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

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Running title:

Mitigation of pentachlorophenol by fungi in forest soils

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Abstract

Pentachlorophenol is globally dispersed and contamination of soil with this biocide adversely affects its functional biodiversity, particularly of fungi - key colonisers. 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 pentachlorophenol but the scientific evidence has previously been lacking. Our data illustrate significant forest contamination through the detection of undefined active sources of pentachlorophenol. By solving the taxonomic diversity and the pentachlorophenol-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 a 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 uncharacterised derivatives, *e.g.* tetrachloroguaiacol isomers. Our study highlights fungi key role in the mineralisation and short lifetime of pentachlorophenol in forest soils and provide novel tools to monitor its degradation in other fungi dominated food-webs.

Key words: pentachlorophenol, tetrachloroguaiacol isomers, fungi, penicillia, biotic degradation, degradation pathway, phase II conjugation reactions

Introduction

Pentachlorophenol (PCP) is recognised as a critical pollutant worldwide, albeit not yet formally classified as a persistent organic pollutant (Crosby et al., 1981; Borysiewicz, 2008). Initially used as wood preservative in the 1930's (Eisler, 1989), its application spread to numerous agricultural, industrial and domestic scenarios (Zheng et al., 2011) and only in the 1980's it became severely restricted due to its high toxicity (*e.g.* probable endocrine disruptor and carcinogen in humans (Zhu and Shan, 2009; Zheng et al., 2013)). By the end of 2008, PCP usage in Europe had virtually ceased (Borysiewicz, 2008), but is still increasing in some countries, *e.g.* in China (Zheng et al., 2012). PCP is also produced as a side product during the degradation of volatile herbicides and pesticides, *e.g.* pentachlorobenzene (Sandau et al., 2000; Phillips et al., 2005). The long history of use of PCP, together with its persistence (can be transported globally, via long-range atmospheric and oceanic transport (Hoferkamp et al., 2010)), has resulted in extensive environmental contamination worldwide (Czaplicka, 2004). Today, PCP is globally detected in human fluids and tissues from exposure in both indoor and outdoor environments (Sandau et al., 2002; Sandanger et al., 2004).

Putative degradation products of PCP have been identified in Mediterranean oak forests/woodlands, particularly in the outer bark of *Quercus suber* L. (cork oak) (Silva Pereira et al., 2000; McLellan et al., 2007). Its incidence and impact in such habitats has never been systematically examined despite the high ecological relevance of these landscapes (Bugalho et al., 2011). 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 (Silva Pereira et al., 2000; Mazzoleni et al., 2005).

PCP has the potential to adversely affect the functional biodiversity in both terrestrial and aquatic niches (Eisler, 1989; Sánchez et al., 2004), with estimated partitioning levels in soil of nearly 95 % (Hattemer-Frey and Travis, 1989). Fungi constitute up to 75 % of the soil microbial biomass and play a key role in preserving the soil functioning and its ecological balance (Harms et al., 2011). They are able to develop strategies to overcome numerous anthropogenic threats due primarily to their broad enzymatic capacities (Gadd, 2001). Numerous studies have demonstrated an increasing abundance and diversity of fungi in chronically stressed and polluted soils (Ramsey et al., 2005; Lladó et al., 2013). 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 (Reddy and Gold, 2000; Gadd, 2001; Field and Sierra-Alvarez, 2008; Carvalho et al., 2011; Bosso and Cristinzio, 2014). 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 (Field and Sierra-Alvarez, 2008; Zhang et al., 2012; Bosso and Cristinzio, 2014). 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 (Zeng et al., 2011).

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 (McLellan et al., 2007; 2013; 2014). 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.

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 categorised through analysis of physicochemical parameters (*e.g.* pH, humidity and carbon/nitrogen ratio), PCP contamination loads, and number of fungal colony forming units (CFUs) (Table S1). The numbers of fungal CFUs were, in general, comparable in all soils, regardless of differences in their PCP levels (Table S1). Through factor analysis (PCA of the quantitative variables), AH_{1-3} soil samples were found to cluster, with PCP concentration exerting the most significant influence (Fig. S1). The PCP levels in AH soils (13.2 - 28.8 $\mu g \cdot kg^{-1}$) were higher than those detected either in FER (4.4 - 14.8 $\mu g \cdot kg^{-1}$) or RR soils (1.7 - 7.0 $\mu g \cdot kg^{-1}$), hence were selected for further study.

AH fungal community comprises 77 cultivable strains

We first characterised the cultivable fungal community at each AH sampling spot. In total, 77 isolates (full details in Table S2) were generated, covering 33 species or species groups, with *Penicillium* species (52 strains) predominating the composition (Table S3). Only six strains remain to be fully characterised (*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 1). The PCP decay in the abiotic controls on the fourteenth day of incubation was *ca.* 9.5%. Only twenty four 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 S2). Out of the 77 strains, 53 could remove PCP at the lowest concentration, whereas 21 could remove PCP at the highest concentration tested (Table 1). Qualitative screening with Remazol Brilliant Blue R (RBBR) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) (Peterson et al., 2009), verified that PCP degradation and oxidoreductase activity (Table 1) were not correlated (Pearson correlation test $p\text{-}value<0.05$).

Each sampling spot showed a unique soil biodiversity of cultivable fungal strains (Table 1 and S3). 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 to either AH_2 or AH_3 (Fig. 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.

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 fourteenth day of incubation, PCP residual concentrations were analysed (Fig. 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 (Figs.

1A and 1B). 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 µM or 38 µM of PCP, over fourteen days, generally increased both richness (*i.e.* number of substrates used) and functional diversity (Shannon index, *H'*) of the communities (Figs. 1C and 1D). 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 $AH_{2\text{-}3}$. 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 sub-products formed either by each strain (Table 2) or community (Table 3) were analysed by UHPLC-ESI-HRMS. The full MS data of the intermediates identified for each culture is available in the Supplementary dataset. Most compounds identifications were confirmed using standards, some of which were synthesised for that purpose (see Experimental section for details).

The full list of PCP degradation intermediates identified (Table 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) (Table 2 and 3). Additional *O*-methylated and sulphated by-products were detected; including the trichloromethoxyphenol-sulphate conjugate (S-TCMP) that involves both conjugation reactions (Table 2). Inspection of the identified compounds revealed several non-chlorinated derivatives (*i.e.* tri- and di-hydroxybenzene isomers) (Table 2 and 3), hence some strains of the fungal soil community were capable of mineralising 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 TeCP, trichlorophenol (TCP) and dichlorotrihydroxybenzene (DCTHB) (Table 4), as well as acetylacrylate (Ac). Traces of pentachlorobenzene (PeCB) were also detected in AH_3 soils.

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

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* (Czaplicka, 2004; Borysiewicz, 2008; Vulykh et al., 2009) impacts on remote and unexpected locations provoking chronic effects (Zheng et al., 2013). 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 (Silva Pereira et al., 2000; McLellan et al., 2007). The bark behaves as an sampler, accumulating both gaseous and particulate pollutants (Zhao et al., 2008) but PCP partition to the soil is likely to be significant (Hattemer-Frey and Travis, 1989). This challenged us to evaluate PCP levels reaching soils by focussing 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 S1). Compared to common practice in other managed forests, this identifies poor forest management, consistent with that reported previously (Boussaidi et al., 2008).

PCP contamination was prevalent at all locations but AH_n soils contained consistently the highest levels (Table S1, Table 4). Levels detected - >10 $\mu g \cdot kg^{-1}$ dry weight - are comparable to those reported for rural areas in China where PCP is currently used to fight the re-emergence of schistosomiasis (Zheng et al., 2012) but much lower than found in the vicinities of wood-mill and storage locations (*e.g.* (Barbeau et al., 1997)). 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 (Borysiewicz, 2008). 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 (Czaplicka, 2004; Borysiewicz, 2008).

To shed light on this topic we evaluated the capacity of soils to degrade the biocide focussing on fungi as its major colonisers. The numbers of fungal CFUs (Table S1) found at each location were comparable to those typically found at similar forest habitats (Ros et al., 2010; Deive et al., 2011), 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 colonising AH_n soils (Table 1, see full details in Table S2-3) reasonably matches previous reports on soils from similar habitats (Orgiazzi et al., 2012; Siles et al., 2014) or with comparable properties (Pasqualetti et al., 2012). Ascomycota, particularly penicillia, typically dominate, as key decomposers, soils with a low abundance of lignin (Ma et al., 2013). 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) (Orgiazzi et al., 2012), 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 (Visagie et al., 2013; Houbraken et al., 2014). Some of the remaining strains need to be fully characterised (6 in total) and hide additional uncharacterised species (*e.g.* isolate DTO 099-G8). The fungal community was dominated by moderate xerophiles, namely penicillia and aspergilla (Vinnere Pettersson and Leong, 2001), accordingly, the number of fungal colonies growing in MEA and DG18 media were comparable, regardless of their divergent water activities (Table S1).

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 1) were not correlated (Pearson correlation test *p-value*<0.05), which is consistent with previous reports (*e.g.* (Carvalho et al., 2013)). 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 1). These data are consistent with the capacity of penicillia to utilise a wide variety of simple aromatic compounds (Field and Sierra-Alvarez, 2008; Leitão, 2009; Bosso and Cristinzio, 2014). Soils are composed of interconnected but distinctive microenvironments holding specific microbial colonisers and concentration/diversity of pollutants (Harms et al., 2011). 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 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. 1). Increasing PCP concentrations led to a continuous decrease in the number (hence biodiversity) of PCP degrading strains (Table 1 and Fig. 1A), while simultaneously increasing both the catabolic richness and diversity of the community (Figs. 1B and 1C). The latter has been often associated with specialisation of microbial communities due to chronic exposure to pollutants (Zhang et al., 2008; Siles et al., 2014). Regardless of this, the persistent strains demonstrated comparable average PCP decay levels in axenic (Fig. 1A) and community (Fig. 1B) cultures.

Overall, data indicated that community interactions hindered the capacity of the strains to remove PCP (Fig. 1B). Ecological interactions are radically altered under *in vitro* conditions, generally favouring competition among strains and reducing the total fungal abundance (Allen, 1965; Engelmoer et al., 2014). 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 2 and Supplementary dataset), deconvoluting the PCP-derived metabolome formed by the cultivable community (Table 3) and defining its PCP degradation pathway (Fig. 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 (Czaplicka, 2006). Regardless of using standards of the three TeCP isomers, their precise identity in cultures (or in the abiotic controls) remains inconclusive due to technical limitations. The degradation intermediates TeCR, TeCHQ and 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) (Czaplicka, 2006) 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 ionisation 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* (Reddy and Gold, 2000) and others (Carvalho et al., 2011). In *Aspergillus nidulans* the catechol branch of the degradation pathway of monochlorophenols ensures its complete mineralisation (Martins et al., 2014b). The identification of TCC implies that biotic transformation of TeCC occurred in some of the axenic cultures. The different branches intersect due to additional hydroxylation of R, HQ and C derivatives, either chlorinated or non-chlorinated, yielding the corresponding trihydroxybenzenes (THB).

The formation of tetrachloroguaiacol (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 (de Jong and Field, 1997; Hiebl et al., 2011). Additional conjugates, namely after *O*-methylation (TCMP), sulphation (S-TCDHB), or both (S-TCMP), were detected in some of the axenic cultures. Similar conjugated compounds have been reported in other eukaryotes, *e.g.* fish and daphnids (Kukkonen and Oikari, 1988; Stehly and Hayton, 1989). In particular, sulphate conjugates and sulphate di-conjugates of PCP degradation intermediates have been identified in other fungi (Pothuluri et al., 1996; Carvalho et al., 2011). These reactions constitute a detoxification mechanism that generally increases the solubility of the toxic compound facilitating its excretion from the cell (Gadd, 2001; Campoy et al., 2009).

Though the PCP-derived metabolome of the AH_n communities retrieved, in general, lower diversity of PCP degradation intermediates and by-products (Table 3), they formed all the tetrachlorinated derivatives previously described, including the previously uncharacterised 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 mineralisation.

The PCP degradation intermediates identified in AH_n soils, namely TeCP, TCP and DCTHB (Table 4), constitute a valuable indicator that the soil microbial community is actively degrading PCP (Field and

Sierra-Alvarez, 2008; Bosso and Cristinzio, 2014). 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 (Yoshida et al., 2007; Field and Sierra-Alvarez, 2008; Bosso and Cristinzio, 2014), 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 4) correlates with the low PCP levels measured in these samples. The origin of Ac, which was previously associated with fungal catabolism of aromatics (Martins et al., 2014b), cannot be certainly attributed to PCP.

Finally, traces of PeCB were also detected in AH_3 soils (Table 4). This compound can yield PCP either biotically or abiotically (Magulova et al., 2011). 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 4) revealed, in addition to TeCP and TCP, dichlorometoxyphenol (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 sub-products is still poorly characterised. This compound cannot be considered as obsolete biocide, particularly since PCP levels in Tunisian *Quercus suber* forests are similar to levels found prevalent where PCP is used (Zheng et al., 2012). 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 (Ben Jamaa et al., 2006)) leading to the so-called "circle of poison" (Karabelas et al., 2009). 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 (Borysiewicz, 2008). 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 (Shamin et al., 2008). 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 specialisation events due to 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 uncharacterised 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 mineralisation and its short lifetime in forest soils, but many questions remain open: *e.g.* are chronic effects of PCP leading to microbial specialisation, 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.

Experimental procedures

Chemicals: If not explicitly stated otherwise, chemicals were of analytical grade and purchased from Sigma Aldrich. *trans*-Acetylacrylate (Alfa Aesar), malt extract agar (MEA) (HiMedia), dichloran-glycerol (DG18) agar (Oxoid) and triton X-100 (GE Healthcare) were also used. All Liquid Chromatography (LC) and Mass Spectrometry (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 (Heasley et al., 1989) and 2,3,5,6-tetrachloro-4-methoxyphenol (drosophilin A) was synthesised as described before (Hiebl et al., 2011).

Collection and physicochemical- characterisation of soil samples: Soil samples were collected in three Tunisian demarked cork oak forests, namely Aîn Hamraia (AH), Fej Errih (FER) and Ras Rajel (RR) in February 2009, as previously described (McLellan et al., 2013). In brief, three locations were chosen within each forest and a composite sample was collected from five sub-samples (0 – 20 cm), sieved to < 2 mm in the field, and immediately conserved (dark, 4 ºC) until analysis. Total organic carbon content, total nitrogen

content, pH, humidity and particle size analysis were performed using standard methodologies (Deive et al., 2011).

To evaluate the diversity of chlorinated compounds, as well as putative sub-products, in the soil samples, a fast-solvent extraction method was applied leading to PCP recovery of > 70 % from a certified reference material containing 2.04 ± 0.18 mg of PCP *per* kg (ERM-CC008, LGC-Promochem, Spain) (McLellan et al., 2013).

Composition of the cultivable fungal communities: Fungi isolation and taxonomic identification were done as previously described (Deive et al., 2011). In brief, aliquots of peptone extracts of each sample (1:10 soil : peptone water, 0.1 % w/v, 1h, 25 ºC, 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 colony forming units (CFUs) was monitored daily (27 ºC, dark), in general, defined after six/seven 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 eight 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 ß-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 (Samson and Varga, 2010; Bensch et al., 2012; Visagie et al., 2013; Samson et al., 2014). 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 ºC, dark, seven days),

were grown in a mineral minimal media (Carvalho et al., 2011; Martins et al., 2014b) containing 1 % w/v of glucose and either 19, 28, 38 or 56 µM of PCP (added after media sterilisation from a 28.2 mM stock in ethanol) under controlled conditions (27 °C, in dark, 90 rpm). After fourteen days of incubation, mycelia were removed by centrifugation (3 minutes, 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, homogenised in 1 mL of methanol and conserved at -20 ºC until further analysis. All assays were executed in triplicates, including controls.

To test the ability of colonising 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. Community level physiological profiles (CLPP) experiments were performed using SF-N2 Biolog plates (Biolog). Soil samples were homogenised in a peptone plus chloramphenicol solution, containing 0, 19 or 38 µM of PCP and incubated for fourteen days (27 ºC, 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 (Liu et al., 2007).

Analysis of PCP-derived metabolome: PCP concentration in the methanolic extracts was quantified using ultra-performance liquid chromatography (UPLC) as previously described (Carvalho et al., 2011). 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 (Martins et al., 2014a; Martins et al., 2014b). MS data was processed by ExactFinder^{TM} 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 principal component analysis (PCA) and multidimensional scaling (MDS) (Fig. S1). Preliminary evaluation of the variance of

the data used Bartlett's and Levene's tests. Pair-wise *t*-tests and Kruskal-Wallis comparisons were used to identify significant differences between the strains PCP degrading capacity, either in axenic or community cultivation, at each PCP concentration tested. All the analyses were performed using the XL-STAT software version 2009.1.02 (Addinsoft).

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Captions to Figures and Tables

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

Table 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). 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.

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

Table 4 – PCP and related compounds identified in the methanolic extracts of Aîn Hamraia (AH), Fej Errih (FER) and Ras Rajel (RR) soils using UHPLC-ESI-HRMS. PCP levels ($\mu g.Kg^{-1}$) are also indicated.

Figure 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 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.

Figure 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.

Table 1

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

	divaricata)						
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			✓	✓

The following strains were unable to germinate in the presence of PCP: *Absidia glauca* (DTO 099-B5); *Absidia pseudocylindrospora* (DTO 099-G6); *Absidia* sp. (DTO 099-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); *Penicillium glabrum* (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 heterogamus* (DTO 099-B2).

Table 2

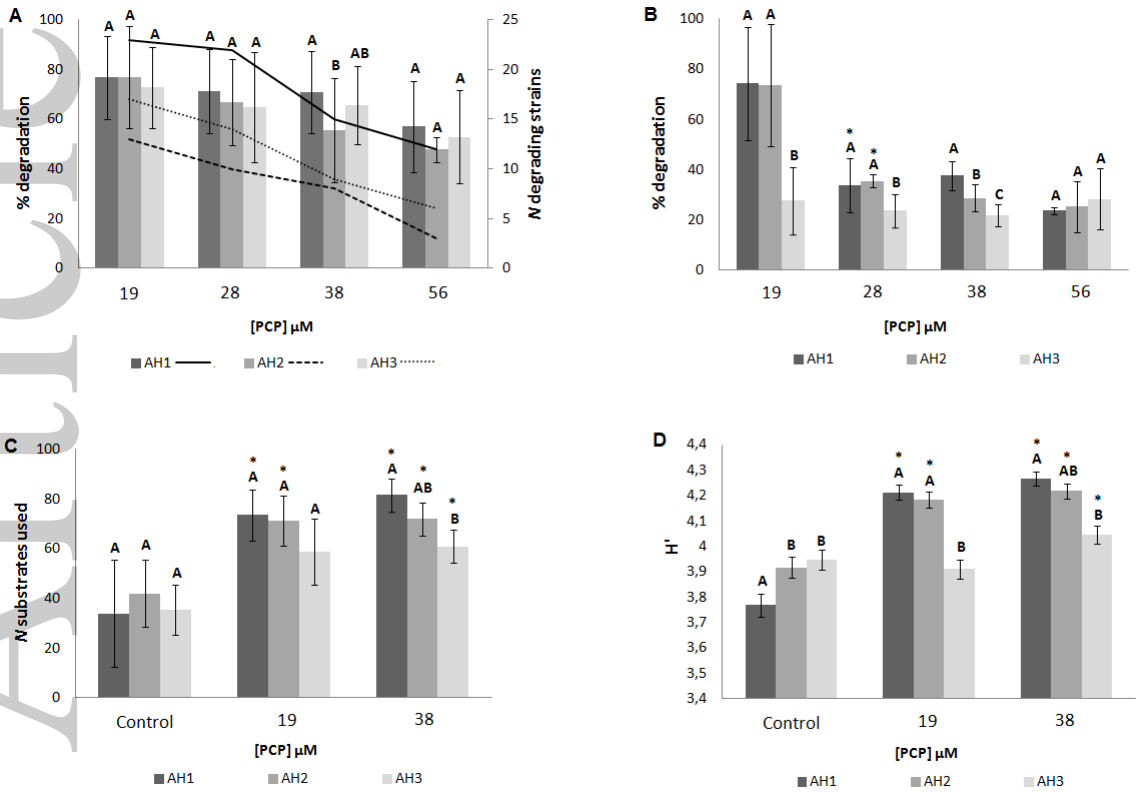
Compound	Abbreviation	Elemental composition	Theoretical mass	Retention time (min)	N_{axenic}	$N_{communities}$	N_{soils}	Abiotic Control
Trihydroxybenzene	THB	$C_6H_6O_3$	125.0245	1.01 - 1.44	18	1	0	
Hydroquinone	HQ	$C_6H_6O_2$	109.0295	1.75 – 1.85	4	0	0	
Acetylacrylate	Ac	$C_5H_5O_3$	113.0246	1.73-1.91	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 - sulphate conjugate*	S-DCDHB	$C_6H_4Cl_2O_5S$	256.9084	4.01 - 4.12	8	0	0	
Trichlorodihydroxybenzene - sulphate 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 - sulphate conjugate*	S-TCMP	$C_7H_5Cl_3O_5S$	304.8854	5.16 - 5.64	13	0	0	
Trichlorocatechol	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	

Table 3

		Community cultures		
Compound	**Abbreviation**	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			

Table 4

Compound	Abbreviation	Soil samples								
		AH_1	AH_2	AH_3	FER 1	FER 2	FER 3	RR 1	RR 2	RR 3
Pentachlorophenol ($\mu g.Kg^{-1}$)	PCP	28.84	13.18	20.72	4.39	14.82	13.20	1.73	6.12	7.03
Acetylacrylate	Ac		✓	✓						
Dichlorotrihydroxybenzene	DCTHB			✓						
Dichlorometoxyphenol	DCMP				✓			✓	✓	
Trichlorophenol	TCP	✓	✓			✓	✓	✓		
Tetrachlorophenol	TeCP	✓					✓			
Drosophilin A	Dro A					✓			✓	✓
Tetrachloro-*o*-guaiacol	TeC-*o*-G				✓					
Pentachlorobenzene	PeCB			✓						



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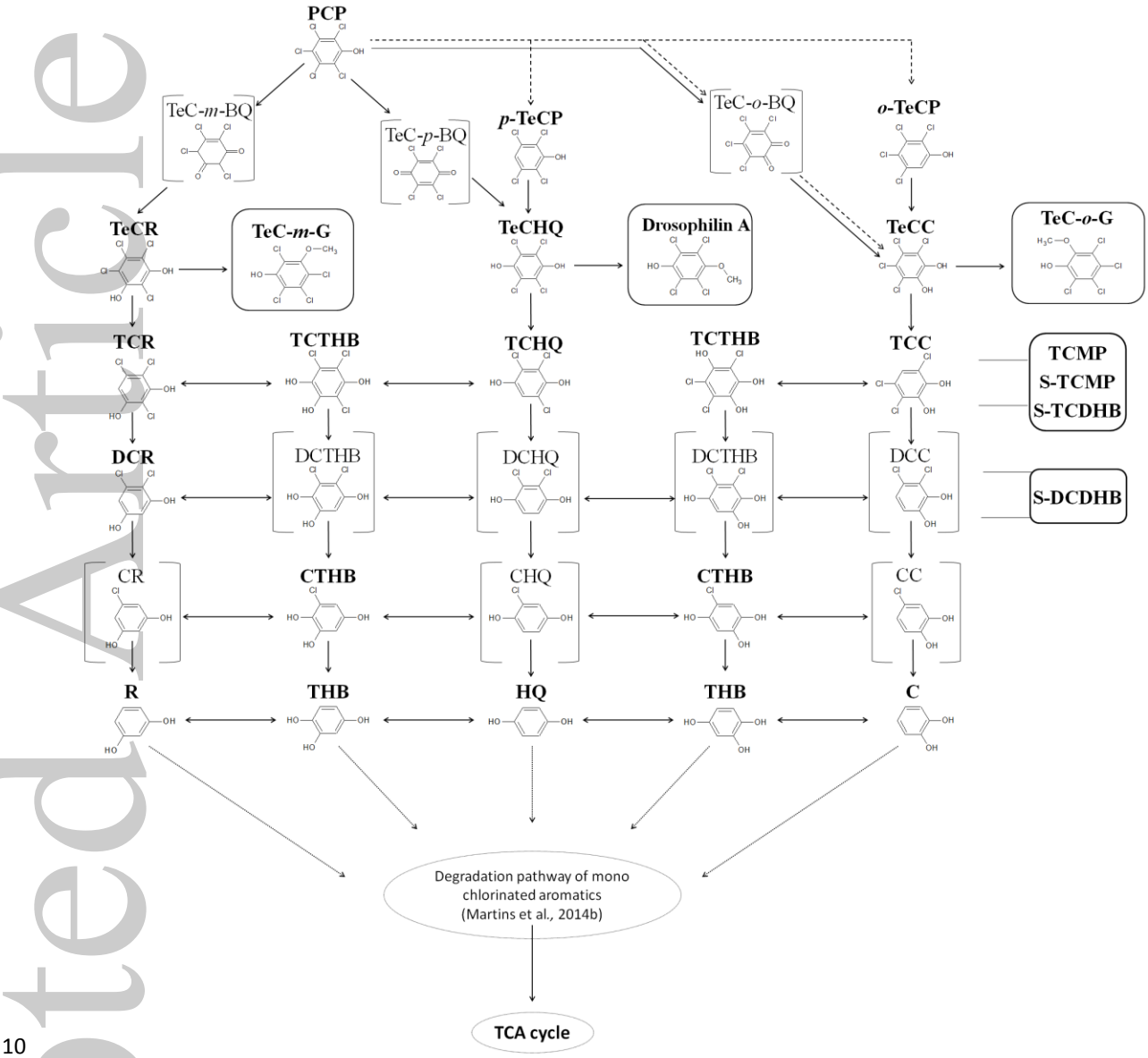

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