Fungal Unspecific Peroxygentases Oxidize the Majority of Organic EPA Priority Pollutants

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Unspecific peroxygenases (UPOs) are secreted fungal enzymes with promiscuity for oxygen transfer and oxidation reactions. Functionally, they represent hybrids of P450 monoxygenases and heme peroxidases; phylogenetically they belong to the family of heme-thiolate peroxidases. Two UPOs from the basidiomycetous fungi Agrocybe aegerita (AaeUPO) and Marasmius rotula (MroUPO) converted 35 out of 40 compounds listed as EPA priority pollutants, including chlorinated benzenes and their derivatives, halogenated biphenyl ethers, nitroaromatic compounds, polycyclic aromatic hydrocarbons (PAHs) and phthalic acid derivatives. These oxygenations and oxidations resulted in diverse products and—if at all—were limited for three reasons: (i) steric hindrance caused by multiple substitutions or bulkiness of the compound as such (e.g., hexachlorobenzene or large PAHs), (ii) strong inactivation of aromatic rings (e.g., nitrobenzene), and (iii) low water solubility (e.g., complex arenes). The general outcome of our study is that UPOs can be considered as extracellular counterparts of intracellular monoxygenases, both with respect to catalyzed reactions and catalytic versatility. Therefore, they should be taken into consideration as a relevant biocatalytic detoxification and biodegradation tool used by fungi when confronted with toxins, xenobiotics and pollutants in their natural environments.

Keywords: EC 1.11.2.1, peroxidase, xenobiotics, chlorobenzene, nitroaromatics, polycyclic aromatic hydrocarbons, fungi

INTRODUCTION

The most important classes of organic pollutants in the environment are mineral oil constituents as well as halogenated and nitrated products of petrochemicals. Enzymatic transformation and degradation of such recalcitrant compounds, many of them xenobiotics in sensu stricto, generally proceeds via two modes: reductive and oxidative attack (Spain, 1995; Durán and Esposito, 2000; Ye et al., 2004). Oxidoreductases, (e.g., dehydrogenases and oxygenases/peroxidases, respectively) play key roles in both degradative strategies and have been well studied (Durán and Esposito, 2000). Oxidases, oxygenases and peroxidases (POX) can be classified according to their co-substrates; the most important of them—involved in the aerobic degradation of numerous organic pollutants—are shortly discussed below.

Polyphenol oxidases, i.e., laccase (LAC) and tyrosinase (TYR), are copper containing enzymes that catalyze the oxidation of phenolic compounds with dioxygen (O2) as electron acceptor and without the need of additional co-enzymes, such as NAD(P)H. They are found in almost all...
domains of aerobic life and fulfill diverse metabolic functions (Ullrich and Hofrichter, 2007). Whereas, LAC does not directly incorporate oxygen into substrates, TYR can do so along with phenol/catechol oxidation (Jergil et al., 1983; Majcherczyk et al., 1998). Nevertheless, also LAC may indirectly oxygenfunctionalize molecules, for example, polycyclic aromatic hydrocarbons (PAHs) or phenols, via one-electron oxidation followed by disproportionation and water addition (Majcherzyk et al., 1998; Wu et al., 2008).

Oxidases using O2 and reduced coenzymes (e.g., NAD(P)H or FADH2) as oxygen donor (electron acceptor) and electron donor, respectively, are usually referred to as oxygenases (Guengerich, 2001). In dependence on the number of oxygen atoms introduced into the substrate molecule, monooxygenases, and dioxygenases are distinguished. Cytochrome-P450 monoxygenases (P450s) are heme-thiolate proteins where a porphyrin moiety (iron protoporphrin IX; heme) is ligated via a cysteine residue to the polypeptide chain (α-helix) of the apo-enzyme (Munro et al., 2012). The protein superfamily of P450s is highly diverse and comprises versatile intracellular biocatalysts found in all domains of life (Anzenbacher and Anzenbacherová, 2001; Meunier et al., 2004; Munro et al., 2012), and even virally encoded P450s have been described (Lamb et al., 2009). P450s usually utilize NAD(P)H as electron-delivering co-substrate but some of them can also catalyze monoxygenations with peroxides as oxygen donor via the so-called shunt pathway (Munro et al., 2012). While some P450s play specific anabolic roles, e.g., in sterol biosynthesis (Lepesheva and Waterman, 2004), others are rather unspecific and involved in the metabolism of xenobiotics, toxins and drugs (Anzenbacher and Anzenbacherová, 2001; Guengerich, 2001).

Multicomponent monoxygenases (BMMs) represent another family of versatile biocatalysts transferring oxygen to various substrates (Leahy et al., 2003). Thus, toluene 4-monoxygenase and methane monoxygenase are capable of oxygenating—in addition to their eponymous substrates—diverse alkenes and arenes including hardly reactive benzene (Whited and Gibson, 1991; Szazinsky et al., 2004). BMMs have only been found (and characterized) in bacteria and archaea so far (Notomista et al., 2003). Other non-heme monoxygenases contain flavin as prosthetic group (FMOs) (van Berkel et al., 2006; Huijbers et al., 2014). They activate O2 with a reduced flavin cofactor to form a peroxyflavin that attacks the substrate (van Berkel et al., 2006). As P450s, FMOs are found in bacteria and eukaryotes (van Berkel et al., 2006).

Oxygenases that catalyze the incorporation of the entire O2 molecule are called dioxygenases (DIOXs). Most DIOXs are iron containing enzymes, e.g., Rieske-type DIOXs (also referred to as arene DIOXs) (Bugg and Ramaswamy, 2008). Rieske-type DIOXs contain a [2Fe-2S] cluster and preferably catalyze the formation of cis-dihydroxylated metabolites (Ferraro et al., 2005). Arene DIOXs are capable of oxidizing inactivated arenes, such as toluene, benzene and even nitrobenzene (Lessner et al., 2002; Bagnéris et al., 2003).

All types of oxygenases can be involved in the detoxification and biodegradation of organic pollutants and xenobiotics by microorganisms and often they initiate catabolic pathways resulting in the utilization of these compounds as sole carbon and energy sources (Fewson, 1988; Copley, 2000). In this context, the incorporation of oxygen does not only activate the molecules but also increases the compounds’ water solubility and hence their bioavailability. That way, many compounds listed as EPA priority pollutants, such as benzene and its derivatives, become accessible to enzymatic attack by other enzymes (e.g., ring-fission enzymes, POX or LAC) upon hydroxylation.

Secreted peroxidases, such as fungal lignin peroxidase (LIP), manganese peroxidase (MNP), and versatile peroxidase (VP), plant horseradish peroxidase (HRP), animal dehaloperoxidase and lactoperoxidase as well as bacterial and fungal dye-decolorizing peroxidases (DYPs), are typical degradative and detoxifying biocatalysts that utilize hydrogen peroxide as electron acceptor (Camarero et al., 1999; Piontek et al., 2001; Hofrichter, 2002; Osborne et al., 2009; Strittmatter et al., 2011). They all contain heme as prosthetic group that is linked via a proximal histidine to the polypeptide chain (heme-imidazole POX) (Ullrich and Hofrichter, 2007). Heme peroxidases are known from all kingdoms of life (Vlasits et al., 2010). Because of the involvement of some of them in lignin biodegradation (MNP, LIP, and VP), which opens an eco-physiological niche for specialized fungi (basidiomycetous white-rotters and litter-decomposers) and is of general interest for the pulp and paper sector, they have been intensely studied over the last three decades (Kirk and Farrell, 1987; Hofrichter, 2002; Martínez et al., 2009). Interestingly, it has recently been proposed that the appearance of fungal ligninolytic peroxidases led to the end of the carboniferous period (Floudas et al., 2012). In addition to lignin, these enzymes were found to efficiently oxidize diverse organic pollutants as well and hence were proposed to be part of unspecific bioremediation/bioattenuation systems in nature (Poining, 2001; Hammel and Cullen, 2008; Qayyum et al., 2009; Harms et al., 2011).

Unlike the ligninolytic heme peroxidases, the heme iron of chloroperoxidase (CPO) from the ascomycete Caldarriomyces (Leptosphaerium) fumago (Dawson, 1988) is linked to a cysteine (heme-thiolate peroxidase-HTP), as in the case of P450s. In 2004, a new HTP type was discovered, which is presently known as unspecific peroxidase (UPO) (Ullrich et al., 2004; Ullrich and Hofrichter, 2005; Hofrichter and Ullrich, 2006) representing a functional hybrid of peroxidases and P450s (Hofrichter and Ullrich, 2006; Hofrichter et al., 2015). Besides prototypical peroxidase reactions like one electron oxidations, UPO transfers hydrogen peroxide-borne oxygen and catalyze various hydroxylations, e.g., of aromatic and aliphatic hydrocarbons (Kluge et al., 2009, 2012; Aranda et al., 2010; Kinne et al., 2010; Peter et al., 2011; Karich et al., 2013). Moreover, epoxidation, sulfoxidation, heterocyclic N-oxidation, and other

Abbreviations: UPO, unspecific peroxidase; Aae, Agrocybe aegerita; Mro, Marasmius rotula; POX, peroxidases; LAC, laccase; TYR, tyrosinase; PAH, polycyclic aromatic hydrocarbons; P450, cytochrome-P450 monoxygenase; BMM, Multicomponent monoxygenase; TMO, toluene 4-monoxygenase; DIOX, dioxygenase; LIP, lignin peroxidase; MNP, manganese peroxidase; VP, versatile peroxidase; HRP, horseradish peroxidase; DYP, dye-decolorizing peroxidase; CPO, chloroperoxidase; HCB, hexachlorobenzene; PCP, pentachlorophenol; PE, phthalate esters; IP, ionization potential.
cleavage (O-dealkylation) have been reported (Ullrich et al., 2008; Aranda et al., 2009; Kinne et al., 2009b; Kluge et al., 2012), and moreover, UPO has catalase and haloperoxidase activities (Hofrichter et al., 2015). Thus, UPO combines features of LAC/POX (one-electron oxidation), monooxygenases (incorporation of one oxygen atom into the substrate) and POX/catalase (H₂O₂ as co-substrate) and hence represents a multifunctional type of oxidoreductase with almost catalytic promiscuity (Pandya et al., 2014; Hofrichter et al., 2015).

UPO genes are ubiquitous in the fungal kingdom (Eumycota) and beyond that, they have only been found in the (super) phylum of heteroconta (e.g., in fungus-like Peronosporales belonging to the former class of oomycetes and in a few diatoms) (Pecyna, 2016). Horizontal gene transfer from ascomycetes was proposed to be the most probable explanation for the occurrence of UPO genes in the latter organisms, leading to the conclusion that they are an autapomorphic feature in the kingdom of fungi (Pecyna, 2016).

Phylogenetically, Unspecific peroxygenases (UPOs) can be classified into two large groups/families, which differ, among others, in molecular size: (i) group I, the short UPOs with an average mass of 29 kDa and (ii) group II, the long UPOs with an average mass of 44 kDa (compare Figure 1; Hofrichter et al., 2015). The latter are exclusively found in ascomycetes and basidiomycetes, while the former are distributed among all fungal phyla (compare Figure 1; Hofrichter et al., 2015). The herein studied UPOs of Agrocybe aegerita (AaeUPO) and Marasmius rotula (MroUPO) belong to the long and short basidiomycetous UPOs, respectively. Interestingly, the above-mentioned CPO, which had been an “orphan” among the heme peroxidases for decades, can now be classified into group I of UPOs/HTPs as well.

Against the background of widespread occurrence of UPOs in the fungal kingdom and their catalytic versatility, it is worth to study the conversion of a representative number of organic pollutants by these enzymes. So we have tested here

**FIGURE 1** | Neighbor-joining phylogenetic tree of UPO/HTP-sequences using Jukes-Cantor genetic distances; updated according to Hofrichter et al. (2015). Green, Basidiomycota; red, Ascomycota; blue, “Oomycota” (Peronosporales, Saprolegiales); purple, “Zygomycota” (Mucoromycotina); dark blue, Chytridiomycota; and orange, Glomeromycota. The dotted lines separate UPO sequences of groups I and II (short and long UPOs, respectively).
44 substrates, of which 40 are listed as EPA priority pollutants including chlorinated benzenes, halogenated biphenyl ethers, nitroaromatics, PAHs and phthalates (USEPA, 1979).

**MATERIALS AND METHODS**

**Enzyme Preparation and Chemicals**

AaeUPO and MroUPO were prepared as described by Ullrich et al. (2004) and Gröbe et al. (2011), respectively. Final enzyme preparations had specific activities of 98 and 28 U/mg for AaeUPO and MroUPO, respectively. Chemicals used were purchased from Sigma Aldrich-Germany (Munich, Germany) with the highest purity available.

**Enzymatic Assay and Reaction Setup**

Enzymatic activity of UPOs was routinely assayed by following the oxidation of veratryl alcohol to veratraldehyde at 310 nm (ε310 = 9,300 M⁻¹ cm⁻¹) in a buffered reaction mixture (pH 7.0) according to Ullrich et al. (2004). Photometric measurements were performed using a Cary Bio 50 spectrophotometer (Varian Inc., Walnut Creek, CA, USA).

Enzymatic reactions were performed in triplicate in 1.5-mL HPLC vials containing 50 mM potassium phosphate buffer (pH 7) and acetonitrile at concentrations between 5 and 30% vol/vol. The substrate concentration ranged from 0.1 to 1 mM, e.g., in the case of large PAHs and phenolic compounds, respectively. The total reaction volume varied between 0.5 and 1 mL. Reactions were started by addition of H₂O₂ (final concentration 0.5–2 mM). H₂O₂ was added via syringe pumps over 30 min or, in the case of large PAHs, over 2 h. In addition reaction setups, ascorbic acid (4 mM) was added to the reaction mixtures to prevent polymerization starting from intermediate phenoxy radicals formed by one-electron oxidation (Kinne et al., 2009a). The final enzyme activity (AaeUPO and MroUPO) in the reaction mixtures ranged between 0.5 and 1 U/mL (veratryl alcohol units measured at pH 7). Controls for each reaction setup were run without enzyme. Detailed information on the reaction setups, analytical methods and some specific results (e.g., HPLC elution profiles) are given in the Supplementary section.

**Sample Preparation**

Samples from the reaction mixtures were collected 30 min after the reaction had started and stopped by injection into an HPLC system or by addition of 1 mM sodium azide. To ensure dissolving, in a few cases, the reaction mixtures were diluted with acetonitrile (e.g., for the analysis of phthalic acid derivatives or small PAHs, such as acenaphthene) or acetone (e.g., for large PAHs, such as benzo[a]pyrene or benz[a]anthracene) prior to injection.

**HPLC Analyses**

Reaction products were analyzed by HPLC with MS and/or UV-Vis detection using an Agilent Series 1,200 instrument equipped with a diode array detector (Agilent Technologies Deutschland GmbH, Böblingen, Germany). The HPLC system was generally equipped with reversed phase columns; i.e., Luna C18(2), Synergi polar, Kinetex C18 and Kinetex PFP (each supplied by Phenomenex, Aschaffenburg, Germany). An electrospray ionization mass spectrometer (6310 IonTrap, Agilent Technologies Germany GmbH), in negative and positive mode, was used to determine mass-to-charge ratios of substrates tested and metabolites formed in the course of UPO reactions. Oxidation products were identified by comparing their retention times, mass and spectral data with authentic standards (so far available) and with literature data. In the absence of standards or reference data, metabolites were tentatively assigned according to their mass and UV-Vis spectra.

**RESULTS AND DISCUSSION**

The main outcome of the enzymatic oxidation tests with two fungal UPOs and diverse EPA organopollutants is summarized in Table 1. This includes the respective type of substrate functionalization (incorporation of oxygen, release of functional groups, one-electron oxidation, etc.), the relative conversion of the tested compounds in semi-quantitative form (five levels of conversion) and the products formed. Polymerization products were observed in all cases, in which phenolic groups were present in the substrates or emerged in the course of the reaction as intermediates (with the exception of 2,4-dinitrophenol).

**Chlorobenzene and Its Derivatives**

Chlorobenzene (1) was oxygenated by AaeUPO to give 2-chlorophenol (2) and 4-chlorophenol (3) as major products; oxygenation at the meta position of 1 and thus formation of 3-chlorophenol (4) was not observed. Further oxidation of 2 led to 3-chlorocatechol (5) and chlorohydroquinone (6), whereas oxidation of 3 gave 4-chlorocatechol (7); 5, 6, and 7 are direct oxygenation products of 2, 3, or 4, respectively (Figures 2A,B). p-Benzoquinone (8) was detected (Figure 2A) when ascorbic acid was omitted from the reaction mixture of 3; vice versa, 8 was not observed in the presence of ascorbic acid. Hence 8 must be a product deriving from two consecutive or parallel enzymatic one-electron oxidations, which represents a type of oxidative dehalogenation known from LAC and POX (Hammel and Tardone, 1988; Osborne et al., 2007; Kordon et al., 2010). Hydrogen abstraction at the phenolic function of 3 would give a phenoxy radical. Two of the latter can disproportionate to 3 and an arene cation (Ullrich and Hofrichter, 2007). A nucleophile, e.g., water, may add to the aromatic cation and subsequent elimination of hydrochloric acid leaves 8 behind, analogously to the Ritter reaction (Krimen and Cota, 2004). The pathway described resembles the enzymatic dehalogenation of 3 described for dehaloperoxidases (Osborne et al., 2009). Masses of triple hydroxylated products arising from 5 to 7 were detected in low amounts; however, their unambiguous identification was not possible, due to the lacking of authentic standards.

Oxidation of three dichlorobenzenes was indicated by the detection of the corresponding dichlorophenols in the reaction mixture. In the case of 1,3-dichlorophenol, dehalogenation occurred in a second step upon oxygenation giving rise to chlorohydroquinone. The reaction cascade is assumed to proceed analogously to the dehalogenation of 3. Dehalogenation...
| Compound                        | Functional groups introduced and/or released | Relative conversion | Oxidases | References                                                                 |
|--------------------------------|----------------------------------------------|---------------------|----------|-----------------------------------------------------------------------------|
| Chlorobenzene                  | −OH (1x, 2x)                                 | +                   | P450; DIOx | de Bont et al., 1986; Nedelcheva et al., 1998                             |
| 2-Chlorophenol                 | −OH (1x, 2x)                                 | ++ +                | nt       | Wada et al., 1995; Durán and Esposito, 2000; Zhang et al., 2008; Hibi et al., 2012 |
| 1,2-Dichlorobenzene            | −OH                                          | +                   | nt       | Nedelcheva et al., 1998; Jones et al., 2001; Monferran et al., 2007       |
| 1,3-Dichlorobenzene            | −OH; −Cl                                     | ++                  | nt       | de Bont et al., 1986; Jones et al., 2001                                  |
| 1,4-Dichlorobenzene            | −OH                                          | +                   | nt       | de Bont et al., 1986; Spies et al., 1995; Nedelcheva et al., 1998; Jones et al., 2001; Monferran et al., 2007 |
| 2,4-Dichlorophenol             | −OH                                          | +                   | ++ +     | Leontievsky et al., 2001; Hibi et al., 2012                               |
| 1,2,4-Trichlorobenzene         | −OH                                          | ++                  | nt       | van der Meer et al., 1991; Marco-Urrea et al., 2009                      |
| 2,4,6-Trichlorophenol          | −OH; −Cl                                     | ++ +                | nt       | Weser et al., 1997; Reddy et al., 1998; Leontievsky et al., 2001; Fodil et al., 2011; Hibi et al., 2012 |
| Pentachlorophenol              | −                                            | 0                   | 0        | P450; LAC; LIP; MNP; TYR; DYP; VP; FMO                                    |
| Hexachlorobenzene              | −                                            | 0                   | 0        | P450; LAC; LIP; MNP; TYR; DYP; VP; FMO                                    |
| para-Chloro-meta-cresol        | −OH; −Cl                                     | + + +               | nt       | Bollag et al., 1988; Freire et al., 2003                                 |
| 2-Chloronaphthalene            | −OH (1x, 2x)                                 | +                   | nt       | Mori et al., 2003                                                        |
| 3,3-dichlorobenzidine          | −OH                                          | ++                  | nt       | Iba and Thomas, 1988; Imaoka et al., 1997                                |
| 4-Chlorophenyl phenyl ether    | −OH (1x, 2x)                                 | ++                  | nt       | Hundt et al., 1999; Hratsuka et al., 2001                                |
| 4-Bromophenyl phenyl ether     | −OH (1x, 2x)                                 | ++                  | nt       | Hundt et al., 1999                                                        |
| 3-Chlorophenol**               | −OH (1x, 2x)                                 | + + +               | nt       | Wada et al., 1995; Durán and Esposito, 2000; Hibi et al., 2012            |
| 4-Chlorophenol**               | −OH (1x, 2x); −Cl                            | + + +               | nt       | Wada et al., 1995; Durán and Esposito, 2000; Freie et al., 2003; Zhang et al., 2008; Fodil et al., 2011; Hibi et al., 2012; Liers et al., 2014 |
| Nitrobenzene                   | −                                            | 0                   | BMM; DIOx | Ye et al., 2004; Vardar and Wood, 2005; Xiao et al., 2007; Büttner et al., 2015 |
| 2-Nitrophenol                  | −OH                                          | +                   | BMM; FMO; DYP | Spain, 1995; Lessner et al., 2002; Fishman et al., 2004; Ye et al., 2004 |
| 4-Nitrophenol                  | −OH                                          | ++                  | BMM; FMO; DYP | Ye et al., 2004; Vardar and Wood, 2005; Xiao et al., 2007; Büttner et al., 2015 |
| 2,4-Dinitrophenol              | −                                            | 0                   | BMM; FMO; P450; DYP | Spain, 1995; Amato et al., 1998; Kadyala and Spain, 1998; Fishman et al., 2004; Ye et al., 2004; Büttner et al., 2015 |
| 2,4-Dinitrotoluene             | =O                                           | 1                   | DIOx     | Spain, 1995; Johnson et al., 2002; Ye et al., 2004                         |
| 2,6-Dinitrotoluene             | −                                            | 1                   | DIOx     | Nishino et al., 2000; Ye et al., 2004                                    |
| 4,6-Dinitro-o-cresol           | −OH                                          | 1                   | FMO      | Cassidy et al., 1999                                                     |
| Benzidine                      | −OH                                          | 1                   | P450; CPO; HRP; LPO; LAC | Phillips and Leonard, 1976; Yamaezoe et al., 1988; Lakshmi et al., 1996 |

(Continued)
| Compound                     | Functional groups introduced and/or released* | Relative conversion | Oxidases | References                                                                 |
|------------------------------|-----------------------------------------------|--------------------|----------|-----------------------------------------------------------------------------|
|                              |                                               |                    | AaeUPO   | MroUPO                                                                     |
| 1,2-Diphenylhydrazine        | –OH                                           | ++                 | nt       | Wittassek and Angerer, 2008                                                 |
| bis(2-Ethylhexyl) phthalate  | –                                             | 0                  | 0        | P450, Wittassek and Angerer, 2008                                           |
| Butyl benzyl phthalate       | –OH; =O                                       | +                  | 1        | Eaton and Ribbons, 1982                                                    |
| Di-n-butyl phthalate         | –OH; =O                                       | +                  | +        | DIOX                                                                       |
| Di-n-octyl phthalate         | –OH; =O                                       | 1                  | 1        |                                                                             |
| Dethyl phthalate             | –                                             | 0                  | 0        |                                                                             |
| Dimethyl phthalate           | –                                             | 0                  | 0        |                                                                             |
| Acenaphthylene               | –OH (1x, 2x); =O                              | ++                 | ++       | LAC; DIOx; P450, Majcherczyk et al., 1998; Pinyakong et al., 2004; Shimada et al., 2015 |
| Acenaphthene                 | –OH (1x, 2x); =O                              | ++                 | ++       | LIP; LAC; DIOx; P450, Vazquez-Duhalt et al., 1994; Majcherczyk et al., 1998; Pinyakong et al., 2004; Shimada et al., 2015 |
| Benzo(a)pyrene               | –OH (1x, 2x)                                  | +                  | ++       | MNP; LAC; LIP; DIOx; P450, Haemmerli et al., 1986; Warshawsky et al., 1988; Bogan and Lamar, 1995; Sack et al., 1997; Kim et al., 1998; Dobor et al., 2004 |
| Benzo(a)anthracene           | –OH (1x, 2x)                                  | ++                 | +        | MNP; LAC; LIP; P450, Wood et al., 1976; Bogan and Lamar, 1995; Sack et al., 1997; Majcherczyk et al., 1998 |
| Indeno[1,2,3-cd]pyrene        | –OH (1x, 2x)                                  | 1                  | 1        | LAC, Wu et al., 2008                                                       |
| Benzo(b)fluoranthene         | –OH                                           | 0                  | 1        | LIP, Bogan and Lamar, 1995; Majcherczyk et al., 1998                        |
| Benzo(k)fluoranthene         | –OH; (1x, 2x)                                  | 1                  | 1        | LIP, Bogan and Lamar, 1995; Majcherczyk et al., 1998                        |
| Dibenzo[a,j]anthracene       | –OH                                           | 0                  | 0        | LAC, Wu et al., 2008                                                       |
| Benzo(g,h,i)pyrrole          | –OH                                           | 0                  | 0        | LIP, Bogan and Lamar, 1995; Wu et al., 2008                                |
| Perylene**                   | –OH; (1x, 2x)                                  | 1                  | +        | LAC, Majcherczyk et al., 1998                                              |
| 9,10-Dihydrophenanthrene**   | –OH; (1x, 2x)                                  | 1                  | +        |                                                                         |
| 2,4-Dimethylphenol           | –OH; =O                                       | ++                 | nt       | FMO; LAC; HRP, Kilbanov et al., 1980; Aenghi et al., 2001; Ghosh et al., 2008 |
| Benzene                      | +                                             |                    | UPO      | Karich et al., 2013                                                        |
| Naphthalene                  | ++                                            |                    | UPO      | Kluge et al., 2009                                                         |
| Phenol                       | + +                                            |                    | UPO      | Karich et al., 2013                                                        |
| Anisole                      | + +                                            |                    | UPO      | Kinne, 2010                                                                |
| Toluene                      | + +                                            |                    | UPO      | Kinne, 2010                                                                |
| Ethylbenzene                 | + +                                            |                    | UPO      | Kluge et al., 2012                                                         |
| Anthracene                   | +                                              |                    | UPO      | Aranda et al., 2010                                                        |
| Fluorene                     | +                                              |                    | UPO      | Aranda et al., 2010                                                        |
| Phenanthrene                 | +                                              |                    | UPO      | Aranda et al., 2010                                                        |
| Pyrene                       | +                                              |                    | UPO      | Aranda et al., 2010                                                        |

Evaluation of relative conversion was done according to the following pattern: no conversion (“0”), trace amounts of products detected (“t”), little conversion—well detectable products, decrease of substrate < 20% (“+”), moderate conversion—decrease of substrate < 50% (“++”), good conversion—decrease of substrate > 50% (“+++”); *introduced (green) and released functional group (red); **not in the EPA priority pollutants list; “nt” not tested; P450, cytochrome P-450 monooxygenase; DIOx, Rieske-type dioxygenase; LAC, laccase; TYR, tyrosinase; LIP, lignin peroxidase; MNP, manganese peroxidase; VP, versatile peroxidase; HRP, horseradish peroxidase; CPO, chloroperoxidase; DYP, dye-decolorizing peroxidase; LPO, lactoperoxidase; BMM, bacterial multicomponent monooxygenase; FMO, flavin-dependent monooxygenase (flavoprotein monooxygenase).
products were also observed when p-chloro-m-cresol and 2,4,6-trichlorophenol were applied as UPO substrates.

Interestingly, the conversion of chlorinated benzenes did not follow the expected reaction sequence; thus the introduction of chlorine substituents usually decreases the charge density of the aromatic system and hence inactivates the latter. However, all three dichlorobenzenes and 1,2,3-trichlorobenzene where more effectively oxidized by AaeUPO than 1. A possible explanation for that finding could be the “steric fixation” of the substrate molecule inside the heme pocket, positively affected by two or more chlorine substituents, resulting in a closer distance to the enzyme’s reactive compound I and/or less motion within the heme pocket. To our best knowledge, only P450s and DIOX have been reported to oxygenate mono- and dichlorobenzenes (de Bont et al., 1986; Spiess et al., 1995; Nedelcheva et al., 1998; Jones et al., 2001; Monferran et al., 2007).

All three tested chlorophenols were oxygenated by AaeUPO. This was most evident when ascorbic acid was present in the reaction mixtures, which prevented polymerizing side activities. Chlorocatechols (i.e., 5 and 7 and not chlorohydroquinones) were the major products deriving from the oxygenation of 3 and 4. This is an interesting fact, since chlorocatechols are the substrates of ring-cleaving DIOX within intracellular degradation pathways of chlorinated arenes (Kaschabek et al., 1998; Moiseeva et al., 2002). Thus, we can consider UPOs being involved in fungal catabolic routes of chloroaromatics, with the advantage that toxic chlorophenols will not have to be taken up into the hyphae (Mars et al., 1997). Chlorophenols and chlorocatechols can additionally serve as substrates for one-electron oxidations and thus, besides oxygenases (Beadle and Smith, 1982; Xu and Bhandari, 2003), several POX and phenol oxidases (LAC, TYR) were found to oxidize chlorinated phenols and their derivatives to reactive phenoxy radicals (Xu and Bhandari, 2003; Zhang et al., 2008; Hibi et al., 2012).

Neither oxygenation nor one-electron oxidation was observed when hexachlorobenzene (HCB) and pentachlorophenol (PCP) were applied as substrates for AaeUPO and MroUPO. They are the only halogenated compounds tested here that were not converted. Chlorine substituents in higher number may protect the arene C-atoms from attack by UPO’s compound I via steric hindrance and/or the impossibility to find a suitable point of attack. On the other hand, some P450s were found to be able to oxygenate both HCB and PCP (Jones et al., 2001; Crawford et al., 2007), and the phenolic functionality of PCP makes it susceptible to one-electron oxidation catalyzed by phenol oxidases and POX (Reddy and Gold, 2000; Montiel et al., 2004; Jeon et al., 2008; Fodil et al., 2011). All other tested halogenated compounds (compare Table 1) served also as substrates for UPOs, but are not explicitly discussed here; more pieces of information are given in the Supplementary section.

Benzoquinones and polymerization products emerged in all reaction setups where ascorbic acid was omitted. The latter acted as radical scavenger that reduced chlorinated phenoxy radicals formed via one-electron oxidation (peroxidative activity of UPO) and prevented that way radical coupling (Niki, 1991).

**Nitroarenes**

The charge density at the aromatic ring is reduced by nitro substituents; thus, with regard to electrophilic attack, nitroarenes are strongly deactivated compounds (McDaniel and Brown, 1958; Spain, 1995). This property is reflected by the low reactivity of UPOs toward nitroaromatic compounds (compare Table 1) and consequently, oxygenation of nitrobenzene was not observed. On the other hand, 2-nitrophenol and 4-nitrophenol served as substrates and were oxidized into the corresponding dihydroxylated nitrobenzenes, which in turn underwent one-electron oxidation resulting in the formation of coupling products. A second nitro group (e.g., 2,4-dinitrophenol), however, made an enzymatic attack by UPOs impossible. Trace amounts of oxidation products were found when 2,4-dinitrotoluene (9) and 4,6-dinitro-o-cresol were applied as substrates. Since the electron density at the aromatic ring of 9 is lower than in 2,4-dinitrotoluene, it is assumed that hydroxylation took place at toluene’s methyl group, which in case of 2,6-dinitrotoluene is shielded by two flanking nitro groups preventing attack by UPO compound I. A second indication for the oxidation at the benzylic carbon of 9 is the mass shift of “+14” for one of the products detected, which cannot be explained by aromatic ring hydroxylation. In consequence, we conclude that 9 was attacked by two consecutive two-electron oxidations (compare Figure 3) resulting in the formation of 2,4-dinitrobenzaldehyde via the corresponding benzyl alcohol and gem-diol (aldehyde hydrate) intermediates. This finding confirms similar observations previously made for 4-nitrotoluene oxidation by AaeUPO (Kinne et al., 2010).

Overall, these results are not surprising when considering literature data of other enzymes. Only a few oxidoreductases are able to oxidize nitrobenzene, e.g., a few DIOXs and BMMs (Spain, 1995; Fishman et al., 2004). The latter was also found to oxidize nitrophenols (Fishman et al., 2004). Furthermore, oxygenation of nitrophenols was reported for some P450s and FMOs (Cassidy et al., 1999; Ye et al., 2004), whereas one-electron oxidation of nitrophenols can be realized by high-redox potential POX, e.g.,

![Figure 2](image-url)
DYP (Büttner et al., 2015). In contrast, reductive pathways for nitroaromatics are widely distributed in nature and have been well summarized in previous reviews (Spain, 1995; Ye et al., 2004).

**Phthalate Esters**

In the course of a screening, six phthalate esters (PEs) were tested for oxygenation by AaeUPO and MroUPO and three of them were converted: butyl benzyl phthalate, di-n-butyl and di-n-octyl phthalate. In case of the latter, only trace amounts of products could be detected. Analogously to 2,4-dinitrotoluene, the “+14” mass shift of products rules out that the oxygen insertion occurred at the aromatic ring and thus oxygenation at the β-position of the alkyl moieties (i.e., butyl or octyl) is most plausible (Peter et al., 2011). No conversion was observed for bis(2-ethyl-hexyl) phthalate and the short chain PEs, such as dimethyl phthalate and diethyl phthalate.

Most studies dealing with the degradation of PE have used whole cells (bacterial or fungal pure or mixed cultures). Hydrolysis of the ester bond by esterases was the typical reaction observed (Wang et al., 1995, 2003; Staples et al., 1997). The ring of phthalic acid can be oxidized by a specific bacterial DIOX resulting in the formation the corresponding catechol (Eaton and Ribbons, 1982); side chain oxidation of PEs was also reported for human PE metabolism probably realized by liver P450s (Wittassek and Angerer, 2008). Wittassek and Angerer (2008) reported the oxidation of long chain PE, e.g., di(2-ethyl-hexyl) phthalate, by a P450 and emphasized that short chain PEs, e.g., dimethyl phthalate and diethyl phthalate, were not oxidized by this enzyme; an observation that corresponds with our findings here.

**Polycyclic Aromatic Hydrocarbons**

Eleven PAHs were tested for the conversion by AaeUPO and MroUPO. The majority of them was in fact oxygenated and oxidized by both UPOs with the exception of bulky dibenz[a,h]anthracene and benzo[g,h,i]perylene; benzo[b]fluoranthene was a substrate for MroUPO only. In dependence on the particular PAH, the extent of product formation reached from trace amounts (e.g., benzo[k]fluoranthene) to substantial amounts (e.g., acenaphthylene).

From all PAHs tested, acenaphthylene (14) was oxidized to the highest extent by both UPOs. The major product detected was a monohydroxylated metabolite (m/z +16) with a UV-Vis spectrum resembling that of 1-naphthol (Kluge et al., 2009) (data in suplement). Hence, we assume that oxygenation of 14 proceeded via a 4,5-epoxy acenaphthylene intermediate to give 5-hydroxy acenaphthylene, analogously to naphthalene oxygenation catalyzed by UPO (Kluge et al., 2009). Another interesting finding was the detection of acenapthenone (13) in the reaction setup of 14. Oxygenation of 14 led to 1-hydroxy-acenaphthylene (15) that is the enolic form of 13. Again, an epoxide intermediate can be postulated, i.e., 1,2-epoxyacenaphthene (compare Figure 4).

Thirteen (13) was also a product deriving from acenaphthene (11) that was next to 14 the best PAH substrate. Oxygenation of a sp³-carbon (aliphatic carbon) may give 1-acenaphthenol (12); via a second attack at the same carbon (i.e., at C1 position), a geminal diol intermediate (gem-diol, carbonyl hydrate) may be formed that is in equilibrium via spontaneous dehydration with the corresponding ketone (13, Figure 4). Similar to 14, oxygenation at the aromatic system of 11 was observed as well. However, in contrast to the reaction setup of 14, two oxygenation products were detected with UV-Vis spectra resembling those of 1-naphthol and 2-naphthol (Kluge et al., 2009). The reported oxidation pathway of P450s for 11 and 14 is rather similar to the reaction sequences proposed herein, including the proof of 1,2-epoxy-acenaphthene formation (only the formation of 13 was not ascertained for P450s) (Shimada et al., 2015). Conversion of all other (positively) tested PAHs was evident by detection of products with mass shifts of +16 or +32, representing mono- and dihydroxylated products, respectively.

Water solubility and thus bioavailability decreases with increasing size of PAHs and this fact was reflected by their decreasing relative conversion by UPOs the bigger they were (compare Table 2). It has to be noted that the two UPOs tested accomplished the formation of different PAH products and patterns; thus, MroUPO was capable of hydroxylating more bulky PAHs than AaeUPO. This can be explained by the wider heme channel of MroUPOs (11 Å) compared to the relatively narrow channel of AaeUPO (7 Å) (Poraj-Kobielska, 2013; Piontek et al., 2017), which limits AaeUPO to oxidize PAHs that are larger than 6 Å in diameter (e.g., dibenz[a,h]anthracene or benzo[g,h,i]perylene). The results of the UPO-catalyzed conversion of selected PAHs in relation to some physicochemical properties are summarized in Table 2. Aranda and coworkers had already reported about the successful conversion of several PAHs and related compounds by AaeUPO in 2010 (Aranda et al., 2010); four of these PAHs are also listed in Table 2, namely anthracene, fluorene, phenanthrene, and pyrene.

**FIGURE 3** | Proposed oxidation sequence for 2,4-dinitrotoluene (9) leading to 2,4-dinitrobenzaldehyde (10).
are being compared with literature data, it becomes evident that quinones are detectable in decreasing order beginning with benzene > naphthalene > anthracene > 4- and 5-ring PAHs (e.g., benz[a]anthracene and perylene, respectively); hence the formation of quinoid products from arenes, catalyzed by UPOs, is inversely proportional to the size of the aromatic system (Kluge et al., 2007; Aranda et al., 2010; Karich et al., 2013).

Oxidation of PAHs by ligninolytic peroxidases (e.g., MNP, LIP) and LAC is strictly dependent on the substrates' IP and high-molecular mass PAHs, e.g., benzo[g,h,i]perylene or benzo[a]pyrene, were found to be faster oxidized than smaller PAHs, some of which cannot be oxidized at all (e.g., phenanthrene and fluoranthene) (Steffen et al., 2003). In contrast, UPOs favor low-molecular mass PAHs over high-molecular ones as oxygen acceptor and the substrate IP seems to be of minor relevance for the extent of conversion (Table 2).

**Other Substrates**

Benzidine (18), 1,2-diphenylhydrazine (16) and 2,4-dimethylphenol do not really fit into the above classification of potential UPO substrates; therefore, we deal with them herein separately. Treatment of 18 samples with AaeUPO and MroUPO mainly resulted in the formation of coupling products. However, in the case of MroUPO, an oxygenation product of 18 was detectable as well. Again, the larger heme channel of MroUPO (compared to AaeUPO) may explain that fact. We assume that 3-hydroxybenzidine (19) was the product formed, which is most plausible to proceed via a lateral oxidative attack on 18. Azo derivatives based on 18 have been widely used as textile dyes and some of them are known to be carcinogenic (Lowry et al., 1980). Thus, 18 has been subject of various (eco)toxicological studies with focus on its carcinogenicity. In this context, oxygenation/hydroxylation of 18 by P450s as well as its POX-catalyzed oxidation to benzidine diimine ensuing binding to DNA were described (Yamazoe et al., 1988; Lakshmi et al., 1996). To supplement this, we have shown here that UPOs are capable of catalyzing these reactions as well.

Studying 16-conversion turned out to be rather difficult for several reasons. The compound instantaneously autoxidizes in aqueous solution to cis- and trans-azobenzene (17) (Riggin and Howard, 1979) or it rearranges to 18 and diphenylene (Hammond and Shine, 1950; Ghigo et al., 2011); the latter reaction, however, is acid-dependent and was therefore not observed under the conditions applied here. Furthermore, authentic standards of potential 16 products are commercially not available and it was impossible to ionize 16 in a way to get a quality mass spectrum. Nevertheless, one product with a mass spectrum shift of +32 in relation to 16 was detected and might be the result of two hydroxylations at the benzene rings of 16. Two products most probably deriving from the oxygenation of spontaneously formed 17 were additionally detected. The first product's mass spectrum shifted by m/z+16 in relation to 17 (and by m/z+14 compared to 16). The latter mass (+14) could hypothetically stand for a single keto function, which is, however, impossible to emerge at the aromatic rings of 16. Therefore, it may rather represent an oxygenation product of 17 (a hydroxy-azobenzene, 20). Logically, the second product (mass shift m/z+32 compared to
17) presumably resulted from a second hydroxylation of 17 (or it may represent a quinone of a double hydroxylated 16). However, whether cis- or trans-17 served as initial substrate, could not be exactly found out but the decrease of trans-17 in samples containing AaeUPO (compare Supplementary Material) implies the latter. A proposed pathway for spontaneous and UPO-catalyzed hydroxylation of 16 is given in Figure 5. Mammalian metabolism of 17 and derivatives usually proceeds via reductive pathways and 16 typically rearranges to 18 (Walker, 1970; Levine, 1991). Hydroxylation of 17 or 16 by mammalian P450 was not observed (Bray et al., 1951).

In the reaction setup of 2,4-dimethylphenol with AaeUPO, polymerization products, oxygenation products and combinations of both were found. This finding is not surprising, since methylphenols (cresols) are well-known substrates both for one-electron oxidations by POX (or LAC) (Klibanov et al., 1980; Ghosh et al., 2008) and for oxyfunctionalization by P450s (Yan et al., 2005) and other monoxygenases (Arenghi et al., 2001). Thus, our results fit well to these reports and supplement an own previous study dealing with benzene oxidation by AaeUPO, in the course of which phenol emerged as an intermediate that was rapidly further converted (Karich et al., 2013).

Concluding Remarks

The majority of organopollutants tested here (i.e., 35 out of 44 substances, compare Table 1)—including xenobiotics, such as chlorinated benzenes and their derivatives, halogenated biphenyl ethers, nitroaromatic compounds, polycyclic aromatics and phthalates—were oxidatively converted by two fungal model UPOs. UPO-catalyzed oxidations were limited for three main reasons: (i) steric hindrance caused by the number of substituents or general bulkiness of the compound, e.g., hexachlorobenzene or large PAHs, such as benzo[g,h,i]perylene; (ii) strong inactivation of the aromatic ring by electron-withdrawing groups, e.g., nitrobenzene, and (iii) low bioavailability (water solubility) of the potential substrate.

Currently, 41 EPA priority pollutants have been reported to be oxidized by UPOs including the herein tested compounds and several other substances investigated in previous studies. Intensively investigated AaeUPO was found to oxygenate and oxidize numerous substance classes (Aranda et al., 2009, 2010; Kluge et al., 2009, 2012; Kinne et al., 2010; Peter et al., 2011; Poraj-Kobielska et al., 2011; Karich et al., 2013; Poraj-Kobielska, 2013) and at present, as much as 300 aromatic, heterocyclic, aliphatic and alicyclic compounds have been identified to serve as substrates for this enzyme (Hofrichter et al., 2015). This fact points out that UPOs are highly versatile oxidoreductases with an exceptional broad substrate spectrum. Besides UPOs, only P450s realize a comparable catalytic promiscuity for oxyfunctionalization reactions (compare Table 1). In fact, UPOs share with P450s the heme-thiolate as prosthetic group as well as highly reactive compound-I and protonated compound-II intermediates in the catalytic cycle (Yosca et al., 2017).

On the other hand, UPOs and P450s do not share any sequence homology and act in different micro-environments (extracellularly vs. intracellularly). Maybe the catalytic systems of P450s and UPOs complement each other in a suitable way.
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FIGURE 5 | Proposed reaction sequence for the UPO-catalyzed hydroxylation of 1,2-diphenylhydrazine (16) and azobenzene (17). Detection of a double-hydroxylated 1,2-diphenylhydrazine would require a preceding hydroxylation of 16; however, a mono-hydroxylated 1,2-diphenylhydrazine (22) was not detected [1]. (16) spontaneously oxidized to trans- and cis-azobenzene (17); subsequent oxygenation catalyzed by AaeUPO resulted in single and double hydroxylated azobenzenes (20 and 21, respectively). An acid-catalyzed benzidine (18) rearrangement of 16 was not observed. Oxygenation of 18 was catalyzed by MroUPO only.

by eliminating similar, often toxic compounds inside and outside of fungal cells, respectively. Thus, UPOs are secretory enzymes using the rather simple co-substrate (H2O2) that can be generated outside the fungal hyphae, while P450s—as membrane-bound or cytosolic enzymes—use a complex accessory machinery, which allows their precise action in different hyphal compartments. In other words, UPOs can directly interact with the fungus’ micro-environment and do rather “dirty catalytic jobs,” whereas P450s are responsible for the fine-tuning of similar reactions in the cells. UPOs may represent an extracellular equivalent to intracellular P450s, in which they function as a universal fungal detoxification system (“extracellular fungal liver”) that can oxidize plant ingredients, microbial metabolites and xenobiotics. The high number of putative UPO genes distributed among rather different ecological and phylogenetical groups of fungi (compare Figure 1) may strongly support this assumption.

Until now, all substrate conversion studies regarding UPOs have been carried out in cell-free systems with isolated enzyme preparations and it is still unclear, under which circumstances UPOs are induced and expressed in fungi under natural conditions. So it is rather difficult to appraise, which roles (others than detoxification) these enzymes may still play. Whilst the actual physiological functions of UPOs in individual fungi will still have to be elucidated, the wealth of catalyzed reactions is without doubt and in any case, interesting from the environmental and biotechnological points of view. Notably, UPOs do not only complement P450 activities but they may also support the action of extracellular fungal enzyme systems catalyzing one-electron oxidations, as needed for lignin and humus decomposition (i.e., POX and LAC reactions).

Future studies on UPOs will have to focus, amongst others, on the conditions, under which the production and secretion of UPOs are induced and how their activities can be stimulated in different ecological and phylogenetical groups of fungi. Because UPO genes are widely distributed in the whole fungal kingdom and fungi indeed permeate the living scene, a powerful tool may become available to foster bioattenuation processes, such as the self-cleaning function of soils.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: AK, RU, and MH. Performed the experiments: AK. Wrote the manuscript, supervised, and discussed the experiments and data: AK, RU, KS, and MH.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.01463/full#supplementary-material
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