Degradation of the xenoestrogen nonylphenol by aquatic fungi and their laccases

Charles Junghanns,[1] Monika Moeder,[2] Gudrun Krauss,[1] Claudia Martin[1] and Dietmar Schlosser[1]

[1]Department of Groundwater Microbiology, UFZ Centre for Environmental Research Leipzig-Halle, Theodor-Lieser-Strasse 4, D-06120 Halle, Germany

[2]Department of Analytical Chemistry, UFZ Centre for Environmental Research Leipzig-Halle, D-04318 Leipzig, Germany

Correspondence
Dietmar Schlosser
dietmar.schlosser@ufz.de

Received 24 June 2004
Revised 12 October 2004
Accepted 13 October 2004

Degradation of technical nonylphenol (t-NP), known as an endocrine-disrupting compound mixture, was assessed, using the mitosporic fungal strain UHH 1-6-18-4 isolated from nonylphenol-contaminated river water, and a strain of the aquatic hyphomycete *Clavariopsis aquatica*. GC-MS analysis could resolve 12 peaks attributable to nonyl chain-branched t-NP isomers. All were degraded, to individual extents. Analysis of degradation metabolites suggested intracellular hydroxylation of the nonyl moieties of individual t-NP isomers. Further metabolites also indicated shortening of branched nonyl chains, and 4-hydroxybenzoic acid was identified as a t-NP breakdown product in UHH 1-6-18-4. The t-NP degradation efficiency was higher in UHH 1-6-18-4 than in *C. aquatica*, and a lower specificity in degradation of individual t-NP constituents in UHH 1-6-18-4 than in *C. aquatica* was observed. Strain UHH 1-6-18-4 concomitantly produced extracellular laccase under degradation conditions. A mixture of $CuSO_4$ and vanillic acid considerably enhanced laccase production in both fungi. Laccase preparations derived from UHH 1-6-18-4 and *C. aquatica* cultures also converted t-NP. Laccase-catalysed transformation of t-NP led to the formation of products with higher molecular masses than that of the parent compound. These results emphasize a role of fungi occurring in aquatic ecosystems in degradation of water contaminants with endocrine activity, which has not previously been considered. Furthermore, the results are in support of two different mechanisms employed by fungi isolated from aquatic environments to initiate t-NP degradation: hydroxylation of individual t-NP isomers at their branched nonyl chains and further breakdown of the alkyl chains of certain isomers; and attack of t-NP by extracellular laccase, the latter leading to oxidative coupling of primary radical products to compounds with higher molecular masses.

INTRODUCTION

Nonylphenols have increasingly gained attention because of their potential to mimic the action of natural hormones in vertebrates (Ying *et al.*, 2002). They result from incomplete biodegradation of nonylphenol polyethoxylates (NPEOs), which have been widely used as non-ionic surfactants in industrial processes and households (Braun *et al.*, 2003; Ying *et al.*, 2002). Both nonylphenols and NPEOs are discharged into the environment, mainly due to incomplete removal in wastewater treatment facilities (Ying *et al.*, 2002). Nonylphenols are more resistant to biodegradation than their parent compounds and hence are found worldwide in wastewater treatment plant effluents and rivers in concentrations of up to the $\mu g\ l^{-1}$ range (Heemken *et al.*, 2001; Kolpin *et al.*, 2002; Stachel *et al.*, 2003; Ying *et al.*, 2002). Due to their hydrophobicity, they tend to adsorb onto surface water particles and sediments and accumulate in aquatic organisms (Heemken *et al.*, 2001; Ying *et al.*, 2002). Consequently, nonylphenols represent a serious environmental and human health risk. The assessment of biodegradative processes affecting the environmental fate of these pollutants is thus of considerable importance. Nonylphenols are considered as slowly biodegradable under aerobic conditions but persistent under anoxic conditions (Montgomery-Brown *et al.*, 2003; Ying *et al.*, 2002; Yuan *et al.*, 2004), although their anaerobic biodegradability was described recently (Chang *et al.*, 2004). The great variety of NPEO isomers is a consequence of their synthesis via the alkylation of phenol with propylene, which leads to a mixture of different mainly *para*-substituted nonylphenol isomers with

Abbreviations: ABTS, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonate); BSTFA, bis(trimethylsilyl)trifluoroacetamide; 4-n-NP, 4-n-nonylphenol; NPEOs, nonylphenol polyethoxylates; t-NP, technical nonylphenol; TMS, trimethylsilyl.

variously branched side chains (He & Lee; 1996; Kim *et al.*, 2004). Such technical nonylphenol (t-NP) mixtures may consist of more than 30 different isomers (He & Lee, 1996), and certain fractions of the branched side chain isomers exhibited a higher oestrogenic activity than the 4-n-nonylphenol (4-n-NP) with a linear nonyl chain that is often used as a model compound in risk assessment and biodegradation studies (Kim *et al.*, 2004; Vallini *et al.*, 2001). Accordingly, t-NP is of especial environmental relevance. The t-NP isomers with the more branched side chains are assumed to be more resistant to biodegradation than those with the less branched nonyl chains or 4-n-NP (Lalah *et al.*, 2003; Tanghe *et al.*, 1999). As yet, only a few bacterial pure cultures capable of utilizing chain-branched nonylphenols as growth substrates have been described (Corvini *et al.*, 2004; de Vries *et al.*, 2001; Fujii *et al.*, 2000, 2001; Soares *et al.*, 2003; Tanghe *et al.*, 1999), while one yeast has been reported to grow on 4-n-NP (Vallini *et al.*, 2001).

Environmentally ubiquitous mitosporic fungi occur in surface water (de Lope & Sanchez, 2002; Niewolak, 1975; Nikolcheva & Bärlocher, 2004; Tóthová, 1999). Terrestrial isolates of such organisms are known to degrade e.g. polycyclic aromatic hydrocarbons (Cerniglia & Sutherland, 2001), fungicides (Gesell *et al.*, 2001) and chlorophenols (Hofrichter *et al.*, 1994), suggesting that mitosporic fungi may also contribute to the microbial degradation of other organic contaminants present in aquatic environments. Aquatic hyphomycetes are a phylogenetically diverse group of mitosporic fungi specifically adapted to aquatic environments. They initiate the decomposition of organic matter in rivers, streams and lakes by colonilization of plant detritus arising from the riparian vegetation (Bärlocher, 1992; Nikolcheva *et al.*, 2003). Although these fungi are most common in unpolluted, oxygen-rich water, and are sensitive to pollution (Bärlocher, 1992), several strains of aquatic hyphomycetes have also been isolated from contaminated surface and groundwater in recent years (Krauss *et al.*, 2001, 2003a, b; Sridhar & Raviraja, 2001). However, the knowledge about the potential of these organisms to degrade organic xenobiotics is limited to a few examples (Hodkinson, 1976).

Laccases (EC 1.10.3.2) are extracellular multicopper-containing oxidoreductases most prominent in white-rot basidiomycetes, where they are believed to contribute to the biodegradation of lignin. These enzymes are also well known from ascomycetes, imperfect fungi and yeasts (Thurston, 1994). Laccases unspecifically couple the one-electron oxidation of reducing substrates to the reduction of dioxygen to water and oxidize e.g. different phenolic compounds and aromatic amines (Xu, 1996). Their substrate range can be considerably extended in the presence of small, diffusible compounds acting as redox mediators (Bourbonnais & Paice, 1990), particularly in white-rot fungi (Eggert *et al.*, 1996; Johannes & Majcherczyk, 2000). The oxidation of nonylphenol and bisphenol A, another phenolic environmental pollutant with endocrine activity, by laccases from white-rot basidiomycetes (Fukuda *et al.*, 2001; Tsutsumi *et al.*, 2001; Uchida *et al.*, 2001) and soil-derived ascomycetes (Saito *et al.*, 2003) has previously been demonstrated. Both pollutants are also degraded by the extracellular lignin-modifying enzyme manganese peroxidase (EC 1.11.1.13) produced by white-rot fungi (Tsutsumi *et al.*, 2001).

Here, we focus on the degradation of t-NP by a strain of the aquatic hyphomycete *Clavariopsis aquatica*, a species frequently observed in rivers and streams (Baldy *et al.*, 2002; Krauss *et al.*, 2001, 2003a; Nikolcheva *et al.*, 2003), and a strain of a mitosporic fungus isolated from river water containing t-NP as a contaminant. In addition, 4-n-NP was employed in some experiments as a structurally defined reference compound. To our knowledge, the degradation of nonylphenol by fungi isolated from aquatic environments has not previously been described. We also address the potential role of laccases, produced by both of the fungal strains, in the biodegradation of nonylphenol.

METHODS

Chemicals. All chemicals were of analytical grade (gradient grade in the case of chromatography solvents), if not otherwise stated. 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonate) (ABTS), vanillic acid and a penicillin-streptomycin formulation were purchased from Sigma-Aldrich. The t-NP (purity 84 %) was provided by Fluka, and 4-n-NP (purity > 98 %) was obtained from Riedel-de-Haen. All other chemicals were purchased from Merck.

Isolation, identification and maintenance of fungal strains. Strain UHH 1-6-18-4 was isolated in April 2002 from water of the Saale river close to the village of Lettin, which is located at the northern city district boundary of Halle/Saale (central Germany). At the sampling site, t-NP concentrations in river water of about 260, 154 and 206 ng l^{-1} were determined during sampling campaigns in February 2002, March 2003 and September 2003, respectively. Water samples (150 μl) were poured onto agar plates containing 1 % (w/v) malt extract, 1·5 % (w/v) agar, 0·9 % NaCl, 400 000 units penicillin l^{-1} and 0·4 g streptomycin l^{-1} (pH 5·6–5·8) (Krauss *et al.*, 2001), which were incubated at 14 °C for several days. Pure cultures were obtained by transferring single colonies onto fresh agar plates. To avoid undesired sporulation, isolate UHH 1-6-18-4 was routinely maintained in liquid culture, using Erlenmeyer flasks (250 ml) containing 75 ml of a 1 % (w/v) malt extract medium (pH 5·6–5·8). Liquid cultures were incubated at 14 °C and 120 r.p.m. in the dark, and new subcultures were established every 4 weeks. Strain UHH 1-6-18-4 was submitted to the German Collection for Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) for identification. The isolate forms discrete hyaline conidiophores with penicillioid branching, flask-like phialides with a prominent, funnel-shaped collarette and nearly globose apiculate conidia in slimy heads. A brownish orange pigment diffuses into the agar medium. These characteristics point to an affinity with the polyphyletic genus *Phialophora* as described by Gams (2000), but the isolate does not fit any of the species listed there (P. Hoffmann, personal communication).

Clavariopsis aquatica de Wild. strain WD(A)-00-1 was isolated from the brook Steinbach near Waldau (district of Zeitz, Saxony-Anhalt, central Germany), which continuously receives leachates containing tar oil residues arising from the historical disposal of wastes of the former lignite-processing industries in the region. Fallen leaves of alder (*Alnus glutinosa*) were collected in the botanical garden of the

Martin-Luther-University Halle-Wittenberg and air-dried, and autoclaved leaf discs (diameter 1·5 cm) were placed in nylon mesh bags (10 × 10 cm, 1 mm mesh) and exposed in the brook for 4 weeks in June 2001. Leaf discs recovered from the stream were placed in sterile Petri dishes containing distilled water (4–8 leaf discs per Petri dish), and incubated at 10 °C. After 2–6 days, Petri dishes were screened for conidiophores under a stereomicroscope. To isolate pure cultures, individual spores were picked up with capillary pipettes, and aseptically transferred to the penicillin- and streptomycin-containing malt agar plates described above. *C. aquatica* was identified based on its characteristic spore shape by L. Maranová (Czech Collection of Microorganisms, Masaryk University, Brno, Czech Republic). The fungus was maintained at 14 °C on malt agar plates containing 1 % (w/v) malt extract and 1·5 % (w/v) agar.

Nonylphenol-degradation experiments. Liquid culture experiments were conducted in Erlenmeyer flasks (125 ml) containing 37·5 ml of the malt extract medium described above. Flasks were inoculated with either 0·5 ml of a mycelial suspension, prepared by homogenizing 10 agar plugs (7 mm diameter) derived from the margins of well-grown agar plate cultures of *C. aquatica* together with 10 ml sterile distilled water, or 0·5 ml of a mycelial suspension derived from UHH 1-6-18-4 liquid cultures at the onset of the stationary growth phase. Fungal cultures were incubated with agitation (120 r.p.m.) at 14 °C in the dark. t-NP and 4-n-NP were aseptically added from 25 mM stock solutions in methanol to give final concentrations of 250 μM [corresponding to 1 % (v/v) methanol] and 100 μM (corresponding to 0·4 % methanol), respectively, at the time points indicated in the text.

Laccase-containing concentrated crude culture supernatants of both fungi (see below) served as enzyme sources in experiments on the enzymic degradation of t-NP and 4-n-NP. Reaction mixtures consisted of 0·2 U laccase ml^{-1}, t-NP or 4-n-NP at concentrations described in the text (added from 25 mM stock solutions) and 0·1 % (w/v) Tween 80 to improve the solubility of nonylphenols, in 100 mM sodium citrate buffer (pH 4·0). Controls contained laccase preparations that were heat-inactivated by autoclaving at 120 °C for 20 min. Enzyme incubations were carried out with agitation (120 r.p.m.) at 24 °C in the dark.

Analysis of nonylphenols and degradation products. Nonylphenol concentrations in fungal culture supernatants and enzymic degradation experiments were determined by HPLC directly without separation of organic and aqueous phases. Samples (1 ml, cell-free in the case of fungal cultures) were taken at the time points indicated in the text. After addition of an equal volume of methanol and vigorous mixing, samples were centrifuged for 30 min at 20 817 ***g*** and 4 °C. Supernatants were analysed on a Merck-Hitachi HPLC system consisting of an L-7120 pump, an L-7200 autosampler, an L-7420 UV/VIS detector and a LiChrospher 100 RP 18-5 column (Merck-Hitachi). Gradient elution started with 20 % (v/v) acetonitrile in distilled water, which was kept constant for 2 min, followed by a linear increase to 90 % acetonitrile within 10 min. The 90 % acetonitrile concentration was kept constant for 3 min, then linearly decreased back to 20 % within 5 min, and kept constant for another 5 min. The flow rate was 1 ml min^{-1}, and the detection wavelength was 277 nm. The retention time for 4-n-NP was 14·8 min under these conditions. The t-NP mixture eluted as a single peak at 13·9 min. The method was calibrated with external standards.

To resolve t-NP isomers and to detect nonylphenol degradation products by GC-MS, fungal cultures were harvested at the time points indicated in the text and extracted with 62·5 ml ethyl acetate following the simultaneous disintegration of fungal mycelia for 1 min, using a mechanical homogenizer (Ultraturrax; IKA) at 24 000 r.p.m. Ethyl acetate fractions were dried over anhydrous sodium sulfate and evaporated to dryness. Residues were redissolved in 8 ml methanol and subjected to either HPLC or GC-MS analysis. In addition, derivatization of possible H-acidic compounds was carried out with bis(trimethylsilyl)trifluoroacetamide (BSTFA; Merck) after evaporation of the ethyl acetate extracts to dryness (Nakagawa *et al.*, 2001). GC-MS analysis was performed on a 6890 gas chromatograph equipped with a 5973 mass-sensitive detector (Agilent Technologies) and an HP-5 MS fused-silica column (30 m × 0·25 mm i.d., 0·25 μm film thickness; Agilent Technologies) as previously described (Braun *et al.*, 2003). Analysis was conducted in full-scan mode over an *m*/*z* range of 50 to 500 amu. Spectral interpretation was aided by the National Institute of Standards and Technology (NIST) 98 mass spectral library stored in the GC-MS controller unit.

The t-NP concentration in river water was determined by GC-MS after solid-phase microextraction, as previously described (Braun *et al.*, 2003).

Gel-permeation chromatography (GPC) was employed in experiments with concentrated laccases, to detect nonylphenol degradation products with higher molecular masses than that of the parent compound. Samples (1·5 ml) were acidified with 150 μl 1 M HCl and centrifuged for 30 min at 20 817 ***g*** and 4 °C. Supernatants were discarded; pellets were redissolved in 500 μl 0·1 M NaOH and subjected to GPC. For GPC analysis the same HPLC system was used as described above, except that an L-4755 diode-array detector (Merck-Hitachi) was employed and separations were carried out on a HEMA Bio-40 GPC column (8 × 300 mm; PSS GmbH). The solvent system consisted of 20 % (v/v) acetonitrile, 80 % (v/v) distilled water, $NaNO_3$ (5 g l^{-1}), and K_2HPO_4 (2 g l^{-1}). Isocratic elution started at a flow rate of 1 ml min^{-1}, which was kept constant for 3 min. After this, the flow rate was linearly decreased to 0·3 ml min^{-1} within 3 min, and kept at this level for another 20 min. Then it was linearly raised back to 1 ml min^{-1} within 2 min and held for another 5 min. The absorbance was monitored over a wavelength range of 225–600 nm. For estimation of molecular masses, retention times of degradation products were compared with that of polystyrene sulfonate sodium salt molecular mass standards (PSS GmbH).

Production and concentration of laccases. *C. aquatica* was grown in a laboratory fermenter (Biostat MD; Braun Biotech International) in discontinuous culture. The cultivation medium (2 l volume) consisted of the liquid malt extract medium and was inoculated with 100 ml of a homogenized liquid culture of *C. aquatica* at the early phase of stationary growth. The fermenter was operated at 14 °C, an aeration rate of 1 l sterilized air min^{-1}, and a stirring speed of 120 r.p.m. A temperature of 14 °C was chosen because it was more favourable for laccase production than higher temperatures (data not shown). UHH 1-6-18-4 was grown in Erlenmeyer flasks as already described. For both fungi, laccase production was stimulated by addition of a mixture of 1 mM vanillic acid and 50 μM $CuSO_4$ during the exponential growth phase. After reaching maximum laccase activities as monitored by regular enzyme activity measurements, fungal cultures were harvested, and the fungal mycelia were removed by filtration through cellulose nitrate filters (0·45 μm pore size; Sartorius). Cell-free culture supernatants were concentrated with a tangential-flow membrane filter (polysulfone minisette, 10 kDa cut-off; Pall Filtron), and washed several times with 10 mM sodium acetate buffer (pH 5·5).

Enzymic determinations. Malt agar plates containing 2 mM ABTS in addition were used to indicate the production of laccase or peroxidases. Plates were inoculated by placing one agar plug derived from the edge of well-grown fungal cultures on agar plates into the centre of a plate. The development of fungal mycelia and the concomitant colorization of the ABTS plates was followed over several days.

Laccase activities in liquid cultures were determined by following the

oxidation of 3 mM ABTS in 100 mM sodium citrate buffer (pH 4·0) at 420 nm ($\varepsilon_{420}=36$ mM $^{-1}$ cm^{-1}; Johannes & Majcherczyk, 2000), using a microplate reader (SLT Spectra; Tecan). Enzyme activities are expressed as units, where 1 U = 1 μmol product formed min^{-1}.

Gel electrophoresis and staining. SDS-PAGE was performed on 10 % polyacrylamide gels (Laemmli, 1970), using a Mini PROTEAN 3 electrophoresis chamber (Bio-Rad). To provide conditions that allow laccase activity staining (subsequently referred to as SDS-PAGE under non-denaturing and non-reducing conditions), *β*-mercaptoethanol was omitted from the sample buffer, and samples were not boiled prior to loading onto the gel. After the run was complete, the gel was cut to separate lanes containing laccase from those containing molecular mass markers. Laccase-containing lanes were stained for enzyme activity with 3 mM ABTS (Höfer & Schlosser, 1999), and lanes containing molecular mass markers were stained with Coomassie brilliant blue R-250 (Bio-Rad) for protein. A semi-logarithmic plot of molecular masses of standard proteins versus their relative migration distances revealed a linear dependency with a correlation coefficient > 0·99, thus allowing the alignment of apparent molecular masses of laccase activity bands. In addition, proteins on gels containing laccase-active samples from concentrated culture supernatants and molecular mass markers were visualized by silver staining.

Determination of fungal dry weights. Fungal cultures were harvested at the time points indicated in the text; mycelia were removed from fungal cultures by filtration through filter papers (Whatman no. 6), washed with 50 ml distilled water, dried at 80 °C for 24 h, and weighed.

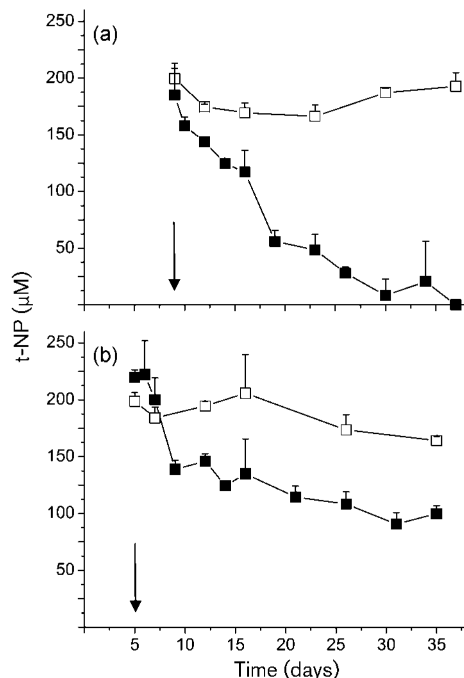

Fig. 1. Time-courses of t-NP sum concentrations in supernatants of active (■) and azide-inactivated (□) cultures of UHH 1-6-18-4 (a) and *C. aquatica* (b), as determined by HPLC. The arrows indicate the respective time point of t-NP addition. Symbols and error bars represent means and standard deviations for triplicate cultures.

RESULTS

Degradation of t-NP and 4-n-NP by fungal cultures

In experiments to follow t-NP degradation by direct HPLC analysis of culture supernatants, the compound mixture was added at 250 μM to fungal cultures pregrown for 9 days (UHH 1-6-18-4, Fig. 1a) and 5 days (*C. aquatica*, Fig. 1b). Cultures additionally contained 0·1 % (w/v) Tween 80, to improve the solubility of t-NP. Inactivated control cultures were prepared by adding sodium azide (500 mg l^{-1}) at the respective time point of t-NP addition, and additional controls contained the uninoculated culture medium and t-NP. The t-NP had completely disappeared in UHH 1-6-18-4 culture supernatants at the end of the experiment and finally decreased to approximately 100 μM in *C. aquatica*. In inactivated controls, t-NP concentrations remained essentially constant for UHH 1-6-18-4 and decreased only slightly for *C. aquatica*. The final recoveries (in relation to the 250 μM initially added) were approximately 77 % (corresponding to 193 μM) and 66 % (corresponding to 164 μM) in inactivated UHH 1-6-18-4 and *C. aquatica* cultures, respectively. In experiments employing uninoculated culture media, t-NP recoveries of 95 % (corresponding to 237·5 μM t-NP) and 105 % (corresponding to 262·5 μM t-NP) with standard deviations of less than 10 % (means from triplicate experiments) were observed after 39 and 35 days of incubation, respectively. This indicates a negligible contribution of processes not attributable to fungal biomass, such as adsorption onto surfaces of the glassware, to the t-NP removal observed in experiments containing fungal mycelia, whereas values for inactivated fungal control cultures suggest some degree of biosorption.

To resolve t-NP isomers and to detect potential degradation products by GC-MS, Tween 80 was omitted from fungal cultures to avoid interference with the GC-MS analysis. The t-NP was added at 250 μM to UHH 1-6-18-4 and *C. aquatica* cultures that were pregrown for 7 days. At this time, control cultures were inactivated as described above. Active cultures and controls were harvested and extracted with ethyl acetate immediately after t-NP addition, after further incubation for 14 days (UHH 1-6-18-4) and 15 days (*C. aquatica*), and after 31 days (UHH 1-6-18-4) and 30 days (*C. aquatica*) following t-NP application (termination of experiments). A final remaining t-NP sum concentration of 25·4 ± 2·9 μM (mean ± standard deviation from triplicate cultures) was detected upon HPLC analysis of extracts from active UHH 1-6-18-4 cultures, whereas 137·3 ± 5·9 μM t-NP was detected in inactivated control cultures. Active *C. aquatica* cultures and corresponding inactivated controls still contained 49·4 ± 7·7 and 133·6 ± 4·9 μM t-NP, respectively, as determined by HPLC after 30 days of t-NP exposure. Thus, the remaining t-NP sum concentrations in active UHH 1-6-18-4 and *C. aquatica* cultures corresponded to 18·5 and 37·0 % of the concentrations in inactivated controls, respectively. The remaining

t-NP concentration detected in ethyl acetate extracts of active UHH 1-6-18-4 cultures, which contrasts with the results from experiments additionally employing Tween 80, where t-NP removal from UHH 1-6-18-4 culture supernatants was finally complete (Fig. 1a), may indicate a limited bioavailability of t-NP in the absence of Tween 80 or binding of a certain amount of t-NP to fungal biomass that was then not susceptible to HPLC analysis of culture supernatants. Upon GC-MS analysis of ethyl acetate extracts, 12 peaks attributable to nonyl chain-branched t-NP isomers could be distinguished by their GC retention times ranging from 14·65 to 15·21 min, and individual MS spectra. The nonylphenol with the linear side chain (4-n-NP) used as a reference compound eluted at a higher retention time of 15·45 min, thus ruling out its contribution to the peak patterns observed with t-NP. Mass spectra of all of the separated t-NP constituents contained the molecular ion (M^+) at *m/z* 220 and the characteristic hydroxyl tropylium ion at *m/z* 107, due to elimination of -C_8H_{17} from the alkyl chain (Wheeler *et al.*, 1997). Individual ion trace patterns of further diagnostic fragments such as those at *m/z* 191 ($[M–C_2H_5]^+$), *m/z* 177 ($[M–C_3H_7]^+$), *m/z* 163 ($[M–C_4H_9]^+$), *m/z* 149 ($[M–C_5H_{11}]^+$), *m/z* 135 ($[M–C_6H_{13}]^+$), and *m/z* 121 ($[M–C_7H_{15}]^+$) indicate several isomer groups with common structural features concerning the branching positions in the nonyl chain (Wheeler *et al.*, 1997). The percentage contents (in relation to the respective concentrations of resolved t-NP constituents in inactivated controls) of individual peaks attributable to t-NP isomers in active UHH 1-6-18-4 and *C. aquatica* cultures are depicted in Fig. 2(a, b); these results indicate that all of the t-NP constituents that could be resolved by the method used had been biodegraded to varying degrees. Generally, all individual t-NP isomer peaks were decreased to a greater extent by UHH 1-6-18-4 than by *C. aquatica.* This result is consistent with the lower remaining t-NP sum concentration in UHH 1-6-18-4 cultures described above. Another marked difference between the two fungal strains was that *C. aquatica* showed a distinct selectivity for the removal of certain individual t-NP constituents, whereas UHH 1-6-18-4 showed this tendency to a much lesser extent (Fig. 2).

GC-MS analysis of ethyl acetate extracts from t-NP-containing fungal cultures that were harvested at incubation times of 14 and 31 days (UHH 1-6-18-4) and 15 and 30 days (*C. aquatica*) after t-NP addition revealed the presence of several metabolites. Such compounds were not detected immediately after t-NP addition or in inactivated controls and were also absent in the untreated parent compound mixture. Most of these compounds showed a molecular ion (M^+) at *m/z* 236 (Table 1, Fig. 3), suggesting hydroxylation of different t-NP isomers. Also, the mass spectra of these t-NP transformation products contained the hydroxyl tropylium ion at *m/z* 107 (Wheeler *et al.*, 1997) known from t-NP isomers (see above). This observation is consistent with the elimination of -$C_8H_{17}O$ from the respective hydroxylated alkyl chains and indicates that an oxidation had occurred at the nonyl chains of individual t-NP isomers

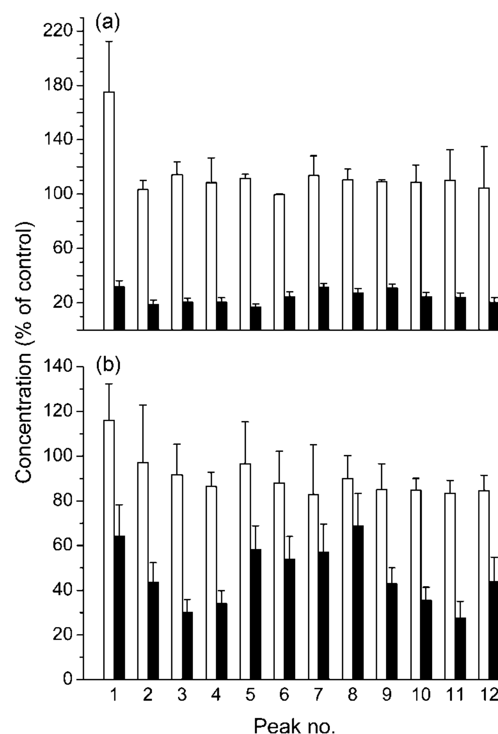

Fig. 2. Percentage concentrations (in relation to the respective concentrations of t-NP constituents in azide-inactivated controls, which represent 100 %) of separated t-NP constituents in active cultures of UHH 1-6-18-4 (a) and *C. aquatica* (b) immediately after t-NP addition (white bars) and after termination of the experiment (black bars; 31 and 30 days after t-NP addition for UHH 1-6-18-4 and *C. aquatica*, respectively). Calculations were based on respective total peak areas of ions corresponding to separated compounds over an *m/z* range of 50–500. Bars and error bars represent means and standard deviations for triplicate cultures.

and not at the aromatic ring. In agreement with such a mechanism, further characteristic ions in mass spectra of t-NP metabolite peaks containing the molecular ion at *m/z* 236 (Table 1) were observed, such as a mostly weak, but structurally significant signal at *m/z* 218, indicating the loss of H_2O primarily present in mass spectra of terminal -OH substituents (Mc Lafferty, 1980). The obviously preferred elimination of an alkyl radical ($M–C_2H_5$) to produce a fragment ion at *m/z* 207 and the loss of -C_5H_{11} to form *m/z* 165 (Table 1) suggests that subterminal oxidation of different nonyl chain-branched t-NP isomers to iso-alcohols had occurred. Furthermore, the mass differences of 73, 87, 101 and 115 amu deduced from corresponding ions at *m/z* 163, 149, 135 and 121, respectively, all indicate losses of hydroxyalkyl radicals (elimination of -C_4H_9O, -$C_5H_{11}O$, -$C_6H_{13}O$ and -$C_7H_{15}O$, respectively). Additionally, ethyl acetate extracts of fungal cultures were treated with BSTFA. The trimethylsilyl (TMS) derivatives thus formed proved the presence of hydroxynonyl phenols (data not shown). A marked difference in the metabolite patterns of the two

Table 1. Mass spectral data and GC retention times of t-NP degradation metabolites detected in fungal cultures after 31 days (UHH 1-6-18-4) and 30 days (*C. aquatica*) of t-NP exposure

Ions with relative intensities below 1·5 % were not listed.

Fungal strain	Peak	GC retention time (min)	Mass spectrum *m/z* (relative intensity)
UHH 1-6-18-4	a	13·07	138 (M^+, 84·4), 121 (100), 93 (25·2), 65 (18·7)
	b	15·32	208 (M^+, 5·1), 191 (2·8), 175 (4·3), 163 (4·7), 149 (13·8), 135 (100), 121 (14·4), 107 (24·7)
	c	15·58	208 (M^+, 10·5), 193 (5·1), 179 (4·2), 165 (8·2), 149 (46·7), 135 (100), 121 (34·3), 107 (35·8)
	d	16·18	236 (M^+, 11·8), 207 (10·0), 189 (30·4), 163 (4·8), 149 (68·8), 135 (100), 121 (13·9), 107 (43·0)
	e	16·34	236 (M^+, 7·0), 207 (10·9), 189 (18·3), 149 (58·6), 135 (100), 121 (15·5), 107 (46·9)
	f	16·44	236 (M^+, 5·7), 149 (7·4), 135 (100), 121 (5·4), 107 (15·0)
	g	16·50	236 (M^+, 9·1), 207 (7·3), 165 (25·1), 149 (8·4), 135 (100), 121 (6·4), 107 (15·7)
	h	16·55	236 (M^+, 8·5), 207 (19·4), 189 (18·3), 163 (9·2), 149 (100), 135 (34·6), 121 (23·5), 107 (49·8)
	i	16·58	236 (M^+, 19·4), 207 (4·0), 165 (13·8), 149 (25·2), 135 (100), 121 (7·9), 107 (19·4)
	k*	16·65	236 (M^+, 9·5), 207 (16·7), 189 (14·7), 163 (10·4), 149 (100), 135 (46·7), 121 (32·2), 107 (56·4)
	l	16·87	236 (M^+, 9·2), 207 (5·1), 163 (10·9), 149 (100), 135 (72·1), 121 (36·5), 107 (53·5)
	m	17·01	236 (M^+, 6·4), 207 (3·5), 165 (8·2), 149 (8·3), 135 (100), 121 (15·7), 107 (18·9)
	n	17·08	236 (M^+, 5·7), 207 (1·5), 165 (3·5), 149 (6·7), 135 (100), 121 (8·3), 107 (15·0)
C. aquatica	o	15·31	208 (M^+, 7·1), 175 (3·0), 149 (2·5), 135 (100), 121 (3·1), 107 (15·8)
	p	15·56	208 (M^+, 5·2), 175 (1·6), 149 (8·2), 135 (100), 121 (9·0), 107 (17·9)
	q	15·85	208 (M^+, 6·7), 179 (4·3), 163 (1·8), 149 (10·6), 135 (100), 121 (6·4), 107 (25·2)
	r*	15·97	236 (M^+, 6·0), 149 (14·7), 135 (100), 121 (8·5), 107 (18·1)
	s*	15·99	236 (M^+, 11·0), 207 (12·9), 189 (44·3), 149 (100), 135 (65·8), 121 (18·5), 107 (54·1)
	t	16·04	208 (M^+, 4·5), 179 (3·8), 161 (5·1), 149 (10·0), 135 (100), 121 (2·7), 107 (23·4)
	u	16·16	236 (M^+, 5·3), 218 (1·7), 149 (6·2), 135 (100), 121 (8·9), 107 (5·4)
	v*	16·18	236 (M^+, 10·8), 207 (11·5), 189 (44·5), 149 (100), 135 (28·8), 121 (15·6), 107 (48·1)
	w*	16·30	236 (M^+, 5·1), 149 (4·3), 135 (100), 121 (5·5), 107 (13·7)
	x	16·58	236 (M^+, 5·2), 154 (5·7), 149 (3·9), 135 (100), 121 (7·3), 107 (13·3)
	k*†	16·65	236 (M^+, 8·6), 207 (16·4), 189 (19·7), 163 (8·4), 149 (100), 135 (40·0), 121 (26·2), 107 (55·4)
	y	16·88	236 (M^+, 9·3), 207 (17·3), 189 (17·7), 149 (100), 135 (63·8), 121 (33·9), 107 (68·4)
	z*	17·06	236 (M^+, 8·2), 207 (17·4), 189 (21·9), 149 (100), 135 (16·8), 121 (18·1), 107 (46·9)

*Mass spectrum also contains an ion at *m/z* 218 with a relative intensity below 1·5 %.

†Most likely identical to metabolite k observed in UHH 1-6-18-4, on the basis of high similarities in GC retention times and mass spectra.

fungi was the much higher concentrations of nonyl chain-hydroxylated t-NP degradation products (molecular ion at *m/z* 236) in *C. aquatica*, where the peak intensities of these metabolites were clearly higher than in UHH 1-6-18-4 (Fig. 3a, b). In both fungi, increasing peak intensities suggest increasing concentrations of the respective hydroxynonyl phenols over time.

Another group of peaks representing t-NP degradation metabolites, which were detected in fungal cultures in lower numbers than compounds attributable to hydroxynonyl phenols, showed a molecular ion at *m/z* 208 in their respective mass spectrum (Table 1, Fig. 4). Like the mass spectra of hydroxynonyl phenols, all mass spectra of this second group of peaks contained the hydroxyl tropylium ion at *m/z* 107 (Wheeler *et al.*, 1997), which together with the observed molecular ion at *m/z* 208 indicates phenolic compounds with alkyl chains shorter than C_9. All of these compounds contained an intense base peak ion at *m/z* 135, which is in agreement with a preferred elimination of $-C_3H_5O_2$ or $-C_4H_9O$ from the alkyl chain and suggests that the respective alkyl chains are not highly branched. Further diagnostic fragment ions were observed at *m/z* 193 (loss of $-CH_3$), 179 (loss of $-C_2H_5$), 165 (loss of $-C_3H_7$), 163 (loss of $-CHO_2$ or $-C_2H_5O$), 149 (loss of $-C_2H_3O_2$ or $-C_3H_7O$) and 121 (loss of $-C_4H_7O_2$ or $-C_5H_{11}O$). The assignment of the exact structures was complicated by the uncertainty of oxygen-containing functional groups and the large number

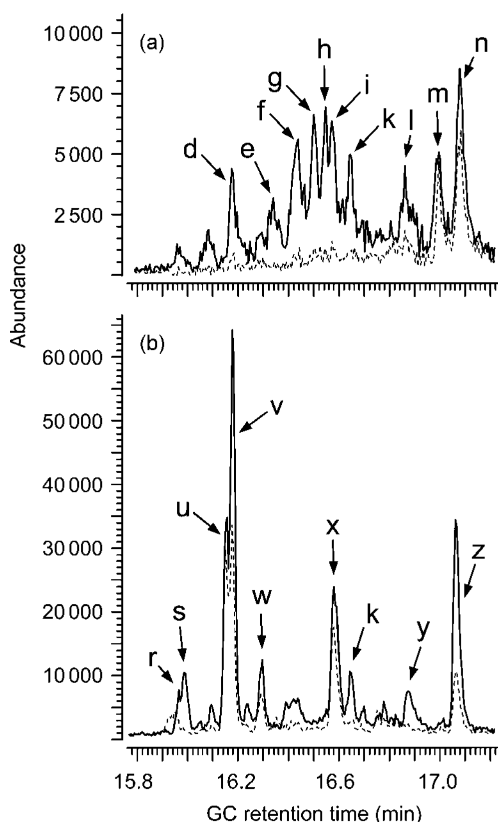

Fig. 3. Representative GC-MS ion trace chromatograms (*m/z* 236) of ethyl acetate extracts from t-NP-containing UHH 1-6-18-4 (a) and *C. aquatica* cultures (b), indicating the formation of nonyl chain-hydroxylated t-NP metabolites from individual t-NP isomers after incubation for 14 days (UHH 1-6-18-4) and 15 days (*C. aquatica*) following t-NP addition (dashed lines) and at the termination of incubation (solid lines; 31 and 30 days of t-NP exposure for UHH 1-6-18-4 and *C. aquatica*, respectively). No peaks were observed immediately after t-NP addition. Labels of prominent peaks refer to those shown in Table 1.

of their possible positions in the respective alkyl chain. Furthermore, coeluting compounds in the GC made the interpretation of mass spectra difficult. Nevertheless, the presence of phenols with shortened alkyl chains was confirmed upon BSTFA derivatization. TMS derivatives with a molecular ion at *m/z* 280 (corresponding to molecular ions at *m/z* 208 observed in underivatized samples) and a prominent fragment ion at *m/z* 265 ($[M-CH_3]^+$), respectively, were detected (data not shown). As observed for the hydroxynonyl phenol metabolites, the concentrations of phenolic t-NP degradation products with shortened alkyl chains were higher in *C. aquatica* than in UHH 1-6-18-4 cultures. Respective metabolite peaks were absent in UHH 1-6-18-4 cultures after 14 days of t-NP exposure. Peak intensities in GC-MS ion-trace chromatograms (*m/z* 208) observed with UHH 1-6-18-4 at the termination of the experiment (31 days after t-NP addition)

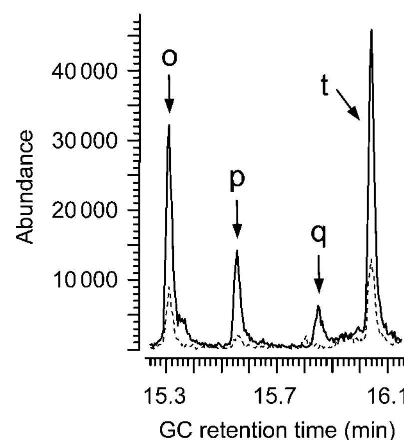

Fig. 4. Representative GC-MS ion trace chromatograms (*m/z* 208) of ethyl acetate extracts from t-NP-containing *C. aquatica* cultures, indicating the formation of phenolic t-NP degradation metabolites with alkyl chains shorter than C_9 after incubation for 15 days following t-NP addition (dashed line) and at the termination of incubation (solid line; 30 days of t-NP exposure). No peaks were observed immediately after t-NP addition. Labels of prominent peaks refer to those shown in Table 1.

were more than 25-fold lower (data not shown) as compared to *C. aquatica* (Fig. 4). In both fungi the intensities of peaks representing phenolic t-NP degradation metabolites with shortened alkyl chains suggest increasing concentrations over time, as also observed for the hydroxynonyl phenol metabolites.

Notably, a t-NP degradation metabolite only observed in UHH 1-6-18-4 in very low concentrations (peak a in Table 1) showed a mass spectrum exactly matching that of 4-hydroxybenzoic acid stored in the NIST 98 database. In ion-trace chromatograms at *m/z* 138, the intensity of the 4-hydroxybenzoic acid peak was maximal after 14 days of t-NP exposure and had decreased by approximately 54 % at the end of incubation (data not shown), indicating further degradation of this metabolite.

Degradation of 4-n-NP was followed by addition of 100 μM of the compound to liquid cultures of UHH 1-6-18-4 and *C. aquatica* pregrown for 5 days. Tween 80 was omitted. Control cultures were inactivated by adding sodium azide (500 mg l^{-1}) at culture day 5. Additional controls consisted of uninoculated culture medium and 4-n-NP. Living fungal cultures and respective controls were incubated for another 42 days, harvested, extracted with ethyl acetate, and extracts were subjected to HPLC. 4-n-NP was completely removed by active UHH 1-6-18-4 cultures and had essentially completely disappeared in *C. aquatica* cultures, where a remaining concentration of 1 ± 0 μM (mean ± standard deviation from triplicate cultures) was detected. The 4-n-NP concentration in uninoculated controls was 113 ± 2 μM (mean ± standard deviation from triplicate experiments), indicating complete recovery. Azide-inactivated cultures

yielded incomplete 4-n-NP recoveries of 49 ± 5 and 79 ± 3 μM (means ± standard deviations from triplicate cultures) for UHH 1-6-18-4 and *C. aquatica*, respectively, possibly due to remaining degradation activities or strong binding to the fungal biomass. GC-MS analysis of ethyl acetate extracts of active cultures could not detect previously described 4-n-NP degradation metabolites such as 9-(4-hydroxyphenyl)nonanoic acid, 3-(4-hydroxyphenyl) propionic acid, 4-hydroxycinnamic acid, 4-hydroxyaceto-phenone, 4-aminoacetophenone or 4-hydroxybenzoic acid (Thibaut *et al.*, 1999; Vallini *et al.*, 2001; Yuan *et al.*, 2004). No peaks potentially representing other degradation products were detected, which may indicate complete degradation of 4-n-NP by both of the fungal strains.

Production of laccases by strain UHH 1-6-18-4 and *C. aquatica*

On ABTS-containing malt agar plates, blue-green zones around the developing fungal mycelia indicated the formation of the ABTS cation radical. The reaction was clearly detectable at essentially comparable intensities with both fungi and covered a radius of approximately 2·5 cm after 5 days, in each case.

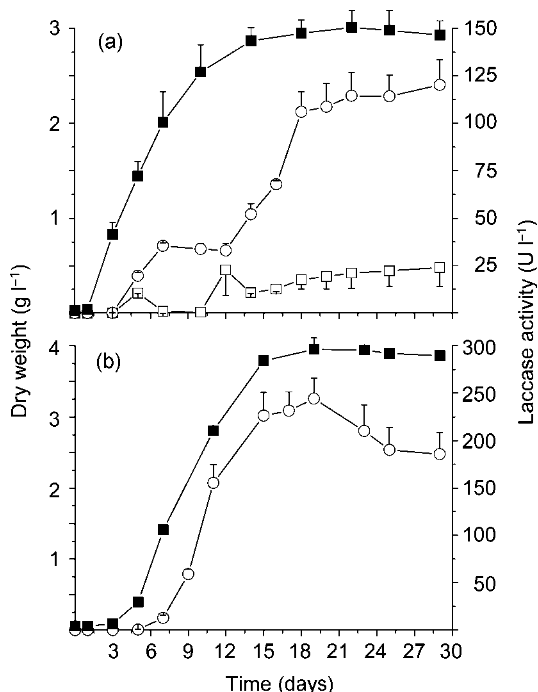

Fig. 5. Time-courses of fungal dry weight (■) and extracellular laccase activities (□, ○) in cultures of UHH 1-6-18-4 (a) and *C. aquatica* (b). ○, Laccase activities in cultures supplemeted with a mixture of 50 μM $CuSO_4$ and 1 mM vanillic acid to stimulate laccase production on culture day 4; □, laccase activities in unsupplemented cultures (not shown for *C. aquatica*, where laccase activities were lower than 1 U l^{-1}. Symbols and error bars represent means and standard deviations for triplicate cultures.

Representative time-courses of laccase activities in culture media of liquid UHH 1-6-18-4 and *C. aquatica* cultures, as well as corresponding fungal dry weights, are shown in Fig. 5(a) and (b), respectively. In a total of four additional experiments conducted under identical conditions, laccase peak titres appeared between 7 and 24 days (UHH 1-6-18-4) and 12 and 17 days (*C. aquatica*) of cultivation, and varied between approximately 11 and 30 U l^{-1} in UHH 1-6-18-4 and about 0·3 and 1 U l^{-1} in *C. aquatica* (data not shown). Thus, *C. aquatica* secretes very little laccase into the culture medium in the absence of inducers whereas UHH 1-6-18-4 produces substantial amounts of extracellular laccase under such conditions. Both t-NP and 4-n-NP were previously shown to enhance laccase activities in the white-rot fungus *Trametes versicolor* (Kollmann *et al.*, 2003). Because of its special environmental relevance, we assessed the effect of t-NP on laccase production and fungal growth of UHH 1-6-18-4 and *C. aquatica* under the degradative culture conditions employed. In both fungi, addition of 250 μM t-NP had no effect on laccase activities or fungal growth (data not shown). In contrast, the addition of a mixture of 50 μM Cu^{2+} (in the form of $CuSO_4$) and 1 mM vanillic acid clearly enhanced laccase titres in UHH 1-6-18-4 up to approximately 125 U l^{-1} (Fig. 5a), and in *C. aquatica* up to about 250 U l^{-1} (Fig. 5b). The fungal dry weights were not affected by the addition of these compounds (data not shown). Manganese peroxidase, manganese-independent peroxidase (peroxidase, EC 1.11.1.7) and lignin peroxidase (EC 1.11.1.14) activities were assessed as previously described (Höfer & Schlosser, 1999; Schlosser *et al.*, 1997) but were not observed during any of the experiments.

Crude culture preparations derived from cultures amended with $CuSO_4$ and vanillic acid were concentrated, and samples from concentrates (corresponding to laccase activities of 150 and 350 mU for UHH 1-6-18-4 and *C. aquatica*, respectively) were subjected to SDS-PAGE under non-denaturing and non-reducing conditions. After activity staining with ABTS, two bands appeared for UHH 1-6-18-4, whereas the sample from *C. aquatica* revealed one major activity band and two less prominent bands appearing at nearly equal intensities (Fig. 6). A comparison with Coomassie brilliant blue R-250-stained molecular mass markers indicated apparent molecular masses of approximately 45 and 51 kDa for the UHH 1-6-18-4 laccase activity bands. The estimated values for the *C. aquatica* enzyme were in the range of 100 kDa for the major activity band and approximately 56 and 43 kDa for the less intense bands, respectively (Fig. 6). Silver staining of additional gels confirmed the apparent molecular masses of the laccases. Corresponding protein bands were detected at molecular masses of approximately 45·5 and 51 kDa for UHH 1-6-18-4, whereas the *C. aquatica* sample revealed proteins with molecular masses of about 100, 56 and 43 kDa (data not shown).

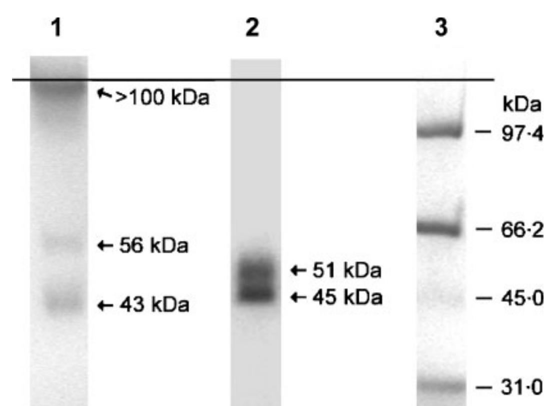

Fig. 6. Gels (SDS-PAGE under non-denaturing and non-reducing conditions) containing concentrated crude culture preparations from $CuSO_4$+vanillic acid-supplemented cultures of *C. aquatica* (1) and UHH 1-6-18-4 (2), and molecular mass markers (3). Bands were stained with ABTS for laccase activity (1, 2) and with Coomassie brilliant blue R-250 for protein (3). The estimated molecular masses of laccase bands are indicated by arrows. The solid line indicates the end of the stacking gel.

Degradation of t-NP and 4-n-NP by laccases from UHH 1-6-18-4 and *C. aquatica*

Laccase-containing concentrated crude culture liquids of both fungi were used as enzyme sources in experiments for enzymic degradation of t-NP and 4-n-NP. Laccase from UHH 1-6-18-4 degraded t-NP and 4-n-NP to a higher extent than laccase from *C. aquatica* (Table 2). Additional experiments addressed the influence of the artificial model redox mediator ABTS on t-NP degradation, because of the special environmental importance of this pollutant. The presence of 1 mM ABTS clearly enhanced degradation of t-NP regardless of the enzyme source, leading to essentially complete degradation within 24 h. Concentrations of t-NP and 4-n-NP remained constant in respective controls containing heat-inactivated enzymes. After 24 h of reaction, the UHH 1-6-18-4 laccase retained approximately 49, 47 and 26 % of its initial activity in experiments containing 4-n-NP, t-NP and t-NP + ABTS, respectively. In experiments with *C. aquatica* laccase, about 15, 5 and 13 % of the initial enzyme activity, respectively, were recovered.

Samples from degradation of t-NP and t-NP + ABTS by laccase from UHH 1-6-18-4 were subjected to gel permeation chromatography (GPC) after 12 days of incubation. The t-NP did not elute under the GPC conditions employed. GPC analysis led to the detection of several distinct peaks absent in heat-inactivated controls (Fig. 7a, b). This indicates the formation of products with higher molecular masses than that of the parent compound, due to oxidative coupling of the primary radical metabolites formed by laccase (Thurston, 1994). A rough estimation of the molecular masses of these products by comparison with retention times of molecular mass markers suggests the formation of di- up to pentamers from t-NP. Laccase-catalysed t-NP degradation in the absence of ABTS led to the detection of three distinct peaks (peaks 1, 2 and 3 in Fig. 7a). In the additional presence of ABTS, two peaks were detected (peaks 4 and 5 in Fig. 7b), which differed from those observed in the absence of ABTS in their retention times, UV/visible spectra, and intensities.

DISCUSSION

Aerobic biodegradation of nonylphenols may be initiated via either oxidative breakdown of the alkyl chain or oxidative attack on the aromatic ring. The proposed mechanism for alkyl chain degradation includes hydroxylation at the terminal carbon position as the first step, oxidation of the resulting alcohol to the corresponding carboxylic acid, and further degradation through β-oxidation (Tanghe *et al.*, 1999; Thibaut *et al.*, 1999; Vallini *et al.*, 2001). Degradation of the 4-n-NP (linear) nonyl moiety was reported to lead to intermediates such as *trans*-4-hydroxycinnamic acid and 4-hydroxyacetophenone in yeast (Vallini *et al.*, 2001), 4-aminoacetophenone in bacteria (Yuan *et al.*, 2004), and 9-(4-hydroxyphenyl)nonanoic acid, 4-hydroxycinnamic acid, 3-(4-hydroxyphenyl)propionic acid and 4-hydroxybenzoic acid in fish (Thibaut *et al.*, 1999). However, nonylphenol degradation via alkyl chain oxidation is thought to be restricted to nonylphenol isomers where the side chain is linear or is at least not highly branched (Tanghe *et al.*, 1999;

Table 2. Degradation of t-NP and 4-n-NP by concentrated laccase-containing crude culture preparations obtained from $CuSO_4$+vanillic acid-supplemented UHH 1-6-18-4 and *C. aquatica* cultures within 24 h of incubation

Values represent means ± SD for triplicate experiments. ABTS was employed at a final concentration of 1 mM. NP, nonylphenol.

Substrate	Laccase source	Initial NP concn (μM)	Final NP concn (μM)	Degradation (%)
4-n-NP	UHH 1-6-18-4	271·3 ± 7·6	146·0 ± 2·3	46·2
4-n-NP	*C. aquatica*	203·8 ± 9·7	158·5 ± 34·7	22·1
t-NP	UHH 1-6-18-4	321·6 ± 22·3	117·3 ± 9·0	63·5
t-NP	*C. aquatica*	235·1 ± 4·4	202·9 ± 11·4	14·0
t-NP + ABTS	UHH 1-6-18-4	196·7 ± 9·3	5·4 ± 0·5	97·2
t-NP + ABTS	*C. aquatica*	224·3 ± 9·3	6·3 ± 0·2	97·2

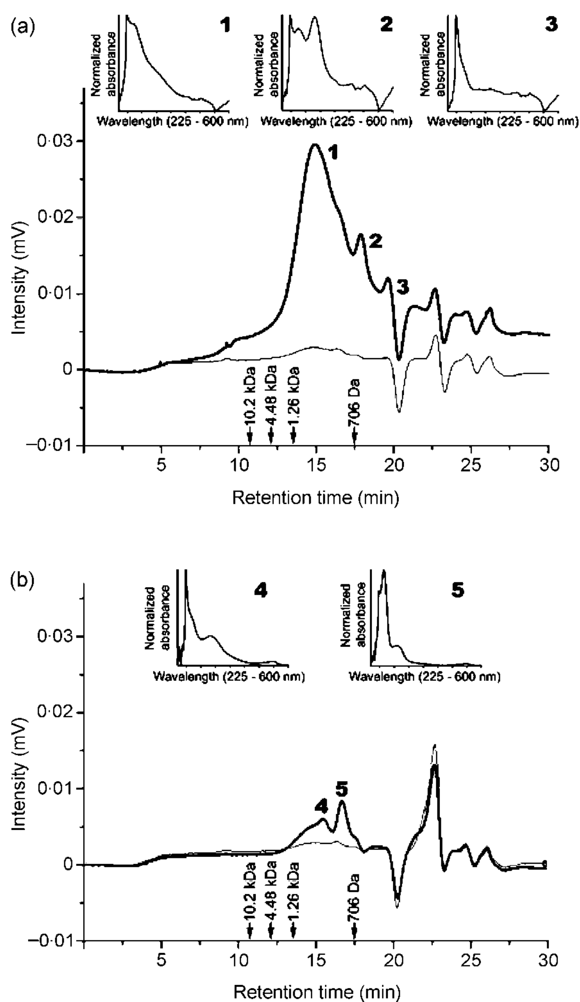

Fig. 7. Representative GPC chromatograms of samples from t-NP degradation by laccase from UHH 1-6-18-4, (a) in the absence and (b) in the presence of ABTS (thick lines). Thin lines represent controls containing heat-inactivated enzyme. Intensity signals (*y*-axes) were obtained from the integration of the respective absorbances over a wavelength range of 225–600 nm. The insets show the normalized UV/visible spectra of labelled peaks. The retention times of molecular mass markers are indicated by arrows.

Vallini *et al.*, 2001). Alternatively, nonylphenol isomers with highly branched alkyl chains initially may be attacked via hydroxylation of the phenolic ring. This has been proposed for bacterial isolates belonging to the genera *Pseudomonas*, *Stenotrophomonas* and *Sphingomonas* (Corvini *et al.*, 2004; de Vries *et al.*, 2001; Fujii *et al.*, 2000, 2001; Soares *et al.*, 2003; Tanghe *et al.*, 1999), and intracellular monooxygenases have been proposed to be responsible for aromatic hydroxylation in *Pseudomonas* and *Sphingomonas* (de Vries *et al.*, 2001; Soares *et al.*, 2003).

Our results show that a mitosporic fungal strain isolated from nonylphenol-contaminated river water, and a strain of the aquatic hyphomycete *C. aquatica*, degraded all of the t-NP constituents analytically resolved under our experimental conditions, to individual extents (Fig. 2). The biodegradative capabilities of *C. aquatica* have already been demonstrated in a previous study, with the fungus being reported to degrade the insecticide 1,1-bis(4-chlorophenyl)-2,2,2-trichloroethane (DDT) to some extent (Dalton & Smith, 1971). Degradation of t-NP was accompanied by the simultaneous formation of several metabolites, and GC-MS analysis of these products implies that either subterminal or terminal hydroxylation had occurred at the respective nonyl moiety of individual t-NP isomers (Fig. 3, Table 1), due to the action of as yet unknown intracellular hydroxylating enzymes. We are not aware of studies describing aromatic metabolites that arise from degradation of complex mixtures of different t-NP isomers with branched side chains by micro-organisms. The detection of phenolic t-NP degradation products with shortened alkyl chains (Fig. 4, Table 1) and the metabolite 4-hydroxybenzoic acid identified in UHH 1-6-18-4 (Table 1) imply subsequent breakdown of hydroxylated, branched nonyl chains of certain t-NP isomers. This may be restricted to t-NP constituents containing less branched nonyl chains, as suggested by the base peak ion at *m*/*z* 135 observed in mass spectra of the t-NP degradation metabolites with shortened alkyl chains (molecular ion at *m*/*z* 208) and their lower numbers as compared to those of hydroxynonyl phenol metabolites (Table 1).

UHH 1-6-18-4 produced extracellular laccase under degradative culture conditions, whereas extracellular laccase titres in the absence of appropriate agents were negligible in *C. aquatica* (Fig. 5). A mixture of Cu^{2+}, which is known to induce differential laccase gene expression in asco- and basidiomycetes (Litvintseva & Henson, 2002; Soden & Dobson, 2001), and the lignin-related compound vanillic acid enhanced laccase production in both fungi investigated (Fig. 5). The concomitant occurrence of different laccase forms as observed (Fig. 6) is a well-known phenomenon in filamentous fungi and may be attributed to multiple laccase genes or post-translational modification (Litvintseva & Henson, 2002; Palmieri *et al.*, 2003; Thurston, 1994). *C. aquatica* has been connected to ascomycetous teleomorphs (Nikolcheva & Bärlocher, 2004; Webster, 1992). In ascomycetes, laccases have frequently been described (Kiiskinen *et al.*, 2002; Litvintseva & Henson, 2002; Saito *et al.*, 2003). Fungi growing on plant material in aquatic environments would be expected to produce laccase under natural conditions; this could also apply to *C. aquatica*, which did not excrete substantial amounts of laccase under the nonylphenol-degradation conditions employed within the present study (Fig. 5).

Cell-free laccase-containing crude culture liquids of both fungi degraded t-NP as well as 4-n-NP (Table 2). The efficiency of t-NP degradation was considerably enhanced in the presence of the model redox mediator ABTS. For nonylphenol and bisphenol A oxidation catalysed by laccase from a soil-derived ascomycete, K_m values of 5 and 10 mM,

respectively, were reported (Saito *et al.*, 2003). Such high K_m values would be rather unfavourable for an efficient removal of these contaminants by laccases in aquatic ecosystems, considering the low pollutant concentrations observed in such environments (Heemken *et al.*, 2001; Kolpin *et al.*, 2002; Stachel *et al.*, 2003; Ying *et al.*, 2002). However, the reported accumulation of nonylphenol and bisphenol A on water and sediment particles (Heemken *et al.*, 2001; Ying *et al.*, 2002), as well as on organic surfaces (Takahashi *et al.*, 2003), may help to overcome kinetic limitations. For nonylphenol, bioaccumulation factors of up to 650 and 990 were reported for river periphytons and benthos, respectively, where bisphenol A was found to be concentrated up to 650- and 170-fold (Takahashi *et al.*, 2003). Also, laccases from aquatic fungi may have kinetic features differing from those of terrestrial species; this needs to be elucidated.

Laccase-catalysed degradation of t-NP led to the formation of products with higher molecular masses than that of the parent compound (Fig. 7), indicating oxidative coupling of primary oxidation metabolites. Similarly, the formation of polymerization products was reported for nonylphenol and bisphenol A degradation by laccases and manganese peroxidases from white-rot fungi (Tsutsumi *et al.*, 2001; Uchida *et al.*, 2001), which led to the removal of the oestrogenic activities of the contaminants (Tsutsumi *et al.*, 2001). In aquatic ecosystems, laccase-catalysed degradation of endocrine disruptors may lead to their removal from these environments by formation of bound residues with organic matter, as already proposed for oxidoreductase-catalysed degradation of chlorophenols in presence of stream fulvic acids (Sarkar *et al.*, 1988).

Together with the results from experiments with whole fungal cultures, the enzymic degradation experiments suggest that an additional extracellular, laccase-based degradation mechanism had contributed to t-NP degradation by UHH 1-6-18-4 under the laboratory conditions employed within the present study. This is supported by the observation that t-NP was more efficiently degraded by UHH 1-6-18-4 than by *C. aquatica* (Figs 1 and 2). A comparison of the fungal dry weights of both strains rules out the possibility that this effect was simply due to a possibly higher biomass of UHH 1-6-18-4. Instead, the opposite was observed (Fig. 5). Also, since laccases are known to unspecifically degrade a broad variety of phenolic compounds, a laccase-catalysed t-NP degradation could be responsible for the lower specificity of degradation of individual t-NP isomers observed in UHH 1-6-18-4 (Fig. 2). Laccase additionally may be involved in further degradation of secreted phenolic t-NP degradation metabolites. This is supported by the peak intensities of these compounds, which were considerably lower in UHH 1-6-18-4 than in *C. aquatica* (Figs 3, 4). Similarly, laccase may contribute to t-NP degradation by *C. aquatica* in its natural aquatic environment.

In conclusion, the results of the present study support a possible role for fungi living in aquatic ecosystems in degradation of water contaminants with endocrine activity. Furthermore, they emphasize two different mechanisms simultaneously employed by aquatic fungi to initiate t-NP degradation: intracellular hydroxylation of individual t-NP isomers at their branched nonyl chains and subsequent side-chain degradation of certain isomers; and extracellular attack of t-NP by laccase, the latter leading to oxidative coupling of primary radical products to compounds with higher molecular masses. Besides the potential role of such degradation processes for natural attenuation processes in freshwater environments, this also offers new perspectives for biotechnological applications such as wastewater treatment.

ACKNOWLEDGEMENTS

We want to thank L. Maranová (Czech Collection of Microorganisms, Masaryk University, Brno, Czech Republic) and P. Hoffmann (German Collection for Micro-organisms and Cell Cultures, Braunschweig, Germany) for their efforts to identify *C. aquatica* and strain UHH 1-6-18-4, respectively. We further greatly acknowledge the support of R. Geyer (UFZ) in helpful suggestions. Also, we are thankful to the German Research Foundation (DFG) for funding of C. Martin.

REFERENCES

Baldy, V., Chauvet, E., Charcosset, J.-Y. & Gessner, M. O. (2002). Microbial dynamics associated with leaves decomposing in the mainstem and floodplain pond of a large river. *Aquat Microb Ecol* **28**, 25–26.

Bärlocher, F. (1992). *The Ecology of Aquatic Hyphomycetes*. Berlin: Springer.

Bourbonnais, R. & Paice, M. G. (1990). Oxidation of non-phenolic substrates. An expanded role for laccase in lignin biodegradation. *FEBS Lett* **267**, 99–102.

Braun, P., Moeder, M., Schrader, St., Popp, P., Kuschk, P. & Engewald, W. (2003). Trace analysis of technical nonylphenol, bisphenol A and 17α-ethinylestradiol in wastewater using solid-phase microextraction and gas chromatography-mass spectrometry. *J Chromatogr A* **988**, 41–51.

Cerniglia, C. E. & Sutherland, J. B. (2001). Bioremediation of polycyclic aromatic hydrocarbons by ligninolytic and non-ligninolytic fungi. In *Fungi in Bioremediation*, pp. 136–187. Edited by G. M. Gadd. Cambridge: Cambridge University Press.

Chang, B. V., Yu, C. H. & Yuan, S. Y. (2004). Degradation of nonylphenol by anaerobic microorganisms from river sediment. *Chemosphere* **55**, 493–500.

Corvini, P. F. X., Vinken, R., Hommes, G., Schmidt, B. & Dohmann, M. (2004). Degradation of the radioactive and non-labelled branched 4(3′,5′-dimethyl-3′-heptyl)-phenol nonylphenol isomer by *Sphingomonas* TTNP3. *Biodegradation* **15**, 9–18.

Dalton, S. A. & Smith, K. A. (1971). DDT and river fungi. *Trans Br Mycol Soc* **58**, suppl., 28.

de Lope, J. L. & Sanchez, J. M. (2002). Eukaryotic diversity in Spain's river of fire. *Nature* **417**, 137.

de Vries, Y. P., Takahara, Y., Ikunaga, Y. & 7 other authors (2001). Organic nutrient-dependent degradation of branched nonylphenol by *Sphingomonas* sp. YT isolated from a river sediment sample. *Microbes Environ* **16**, 240–249.

Eggert, C., Temp, U., Dean, J. F. D. & Eriksson, K.-E. L. (1996). A fungal metabolite mediates degradation of non-phenolic lignin structures and synthetic lignin by laccase. *FEBS Lett* **391**, 144–148.

Fujii, K., Urano, N., Ushio, H., Satomi, M. & Kimura, S. (2000). Profile of a nonylphenol-degrading microflora and its potential for bioremedial applications. *J Biochem* **128**, 909–916.

Fujii, K., Urano, N., Ushio, H., Satomi, M. & Kimura, S. (2001). *Sphingomonas cloacae* sp. nov., a nonylphenol-degrading bacterium isolated from wastewater of a sewage-treatment plant in Tokyo. *Int J Syst Evol Microbiol* **51**, 603–610.

Fukuda, T., Uchida, H., Takashima, Y., Uwajima, T., Kawabata, T. & Suzuki, M. (2001). Degradation of bisphenol A by purified laccase from *Trametes villosa*. *Biochem Biophys Res Commun* **284**, 704–706.

Gams, W. (2000). *Phialophora* and some similar morphologically little-differentiated anamorphs of divergent ascomycetes. *Stud Mycol* **45**, 187–199.

Gesell, M., Hammer, E., Specht, M., Francke, W. & Schauer, F. (2001). Biotransformation of biphenyl by *Paecilomyces lilacinus* and characterization of ring cleavage products. *Appl Environ Microbiol* **67**, 1551–1557.

He, Y. & Lee, H. K. (1996). Separation of structural homologues of alkylphenols and isomers of 4-nonylphenol by cyclodextrin-modified micellar electrokinetic chromatography. *J Chromatogr A* **749**, 227–236.

Heemken, O. P., Reincke, H., Stachel, B. & Theobald, N. (2001). The occurence of xenoestrogens in the Elbe river and the North Sea. *Chemosphere* **45**, 249–259.

Hodkinson, M. (1976). Interactions between aquatic hyphomycetes and DDT. In *Recent Advances in Aquatic Mycology*, pp. 447–467. Edited by E. G. B. Jones. London: Paul Elek.

Höfer, C. & Schlosser, D. (1999). Novel enzymatic oxidation of Mn^{2+} to Mn^{3+} catalyzed by a fungal laccase. *FEBS Lett* **451**, 186–190.

Hofrichter, M., Bublitz, F. & Fritsche, W. (1994). Unspecific degradation of halogenated phenols by the soil fungus *Penicillium frequentans* Bi 7/2. *J Basic Microbiol* **34**, 163–172.

Johannes, C. & Majcherczyk, A. (2000). Natural mediators in the oxidation of polycyclic aromatic hydrocarbons by laccase mediator systems. *Appl Environ Microbiol* **66**, 524–528.

Kiiskinen, L. L., Viikari, L. & Kruus, K. (2002). Purification and characterization of a novel laccase from the ascomycete *Melanocarpus albomyces*. *Appl Microb Biotechnol* **59**, 198–204.

Kim, Y.-S., Katase, T., Sekine, S., Inoue, T., Makino, M., Uchiyama, T., Fujimoto, T. & Yamashita, M. (2004). Variation in estrogenic activity among fractions of a commercial nonylphenol by high performance liquid chromatography. *Chemosphere* **54**, 1127–1134.

Kollmann, A., Brault, A., Touton, I., Dubroca, J., Chaplain, V. & Mougin, C. (2003). Effect of nonylphenol surfactants on fungi following the application of sewage sludge on agricultural soils. *J Environ Qual* **32**, 1269–1276.

Kolpin, A. W., Furlong, E. T., Meyer, M. T., Thurmann, E. M., Zaugg, S. D., Barber, L. B. & Buxton, H. T. (2002). Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999–2000: a national reconnaissance. *Environ Sci Technol* **36**, 1202–1211.

Krauss, G., Bärlocher, F., Wennrich, R., Glässer, W. & Krauss, G.-J. (2001). Aquatic hyphomycetes occur in hyperpolluted waters in Central Germany. *Nova Hedwigia* **72**, 419–428.

Krauss, G., Bärlocher, F. & Krauss, G.-J. (2003a). Effect of pollution on aquatic hyphomycetes. In *Freshwater Mycology* (Fungal Diversity Research Series 10), pp. 211–230. Edited by C. K. M. Tsui & K. D. Hyde. Hong Kong: Fungal Diversity Press.

Krauss, G., Sridhar, K. R., Jung, K., Wennrich, R., Ehrman, J. & Bärlocher, F. (2003b). Aquatic hyphomycetes in polluted groundwater habitats of Central Germany. *Microb Ecol* **45**, 329–339.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Lalah, J. O., Schramm, K.-W., Henkelmann, B., Lenoir, D., Behechti, A., Günther, K. & Kettrup, A. (2003). The dissipation, distribution and fate of a branched ^{14}C-nonylphenol isomer in lake water/sediment systems. *Environ Pollut* **122**, 195–203.

Litvintseva, A. P. & Henson, J. M. (2002). Cloning, characterization, and transcription of three laccase genes from *Gaeumannomyces graminis* var. *tritici*, the take-all fungus. *Appl Environ Microbiol* **68**, 1305–1311.

Mc Lafferty, F. W. (1980). *Interpretation of Mass Spectra*, 3rd edn. Mill Valley, CA: University Science Books.

Montgomery-Brown, J., Drewes, J. E., Fox, P. & Reinhard, M. (2003). Behavior of alkylphenol polyethoxylate metabolites during soil aquifer treatment. *Water Res* **37**, 3672–3681.

Nakagawa, T., Sugaya, N., Sakurai, K., Nakagawa, J., Usukura, K. & Onda, N. (2001). A new finding of trimethylsilyl derivatives of 4-alkyl phenols by positive chemical ionization-GC/MS. *Anal Sci* **17**, supplement, 1597–1600.

Niewolak, S. (1975). The occurrence of microorganisms in the water of some lakes in the district of Węgorzewo. *Acta Hydrobiol* **17**, 371–390.

Nikolcheva, L. G. & Bärlocher, F. (2004). Taxon-specific fungal primers reveal unexpectedly high diversity during leaf decomposition in a stream. *Mycol Prog* **3**, 41–50.

Nikolcheva, L. G., Cockshutt, A. M. & Bärlocher, F. (2003). Determining diversity of freshwater fungi on decaying leaves: comparison of traditional and molecular approaches. *Appl Environ Microbiol* **69**, 2548–2554.

Palmieri, G., Cennamo, G., Faraco, V., Amoresano, A., Sannia, G. & Giardina, P. (2003). Atypical laccase isoenzymes from copper supplemented *Pleurotus ostreatus* cultures. *Enzyme Microb Technol* **33**, 220–230.

Saito, T., Hong, P., Kato, K., Okazaki, M., Inagaki, H., Maeda, S. & Yokogawa, Y. (2003). Purification and characterization of an extracellular laccase of a fungus (family C*haetomiaceae*) isolated from soil. *Enzyme Microb Technol* **33**, 520–526.

Sarkar, J. M., Malcolm, R. L. & Bollag, J.-M. (1988). Enzymatic coupling of 2,4-dichlorophenol to stream fulvic acid in the presence of oxidoreductases. *Soil Sci Soc Am J* **52**, 688–694.

Schlosser, D., Grey, R. & Fritsche, W. (1997). Patterns of ligninolytic enzymes in *Trametes versicolor*. Distribution of extra- and intracellular enzyme activities during cultivation on glucose, wheat straw and beech wood. *Appl Microbiol Biotechnol* **47**, 412–418.

Soares, A., Guieysse, B. & Mattiasson, B. (2003). Aerobic biodegradation of nonylphenol by cold-adapted bacteria. *Biotechnol Lett* **25**, 731–738.

Soden, D. M. & Dobson, A. D. W. (2001). Differential regulation of laccase gene expression in *Pleurotus sajor-caju*. *Microbiology* **147**, 1755–1763.

Sridhar, K. R. & Raviraja, N. S. (2001). Aquatic hyphomycetes and leaf litter processing in polluted and unpolluted habitats. In *Trichomycetes and Other Fungal Groups*, pp. 293–314. Edited by J. K. Misra & B. W. Horn. Enfield, NH: Science Publishers.

Stachel, B., Ehrhorn, U., Heemken, O.-P., Lepom, P., Reincke, H., Sawal, G. & Theobald, N. (2003). Xenoestrogens in the river Elbe and its tributaries. *Environ Pollut* **124**, 497–507.

Takahashi, A., Higashitani, T., Yakou, Y., Saitou, M., Tamamoto, H. & Tanaka, H. (2003). Evaluating bioaccumulation of suspected

endocrine disruptors into periphytons and benthos in the Tama river. *Water Sci Technol* **47**, 71–76.

Tanghe, T., Dhooge, W. & Verstraete, W. (1999). Isolation of a bacterial strain able to degrade branched nonylphenol. *Appl Environ Microbiol* **65**, 746–751.

Thibaut, R., Debrauwer, L., Rao, D. & Cravedi, J. P. (1999). Urinary metabolites of 4-*n*-nonylphenol in rainbow trout (*Oncorhynchus mykiss*). *Sci Total Environ* **233**, 193–200.

Thurston, C. F. (1994). The structure and function of fungal laccases. *Microbiology* **140**, 19–26.

Tóthová, L. (1999). Occurrence of mitosporic fungi in the Slovak section of the Danube river. *Biologia* **54**, 379–385.

Tsutsumi, Y., Haneda, T. & Nishida, T. (2001). Removal of estrogenic activitites of bisphenol A and nonylphenol by oxidative enzymes from lignin-degrading basidiomycetes. *Chemosphere* **42**, 271–276.

Uchida, H., Fukuda, T., Miyamoto, H., Kawabata, T., Suzuki, M. & Uwajima, T. (2001). Polymerization of bisphenol A by purified laccase from *Trametes villosa*. *Biochem Biophys Res Commun* **287**, 355–358.

Vallini, G., Frassinetti, S., D'Andrea, F., Catelani, G. & Agnolucci, M. (2001). Biodegradation of 4-(1-nonyl)phenol by axenic cultures of the yeast *Candida aquaetextoris*: identification of microbial breakdown products and proposal of a possible metabolic pathway. *Int Biodeterior Biodegradation* **47**, 133–140.

Webster, J. (1992). Anamorph–teleomorph relationships. In *The Ecology of Aquatic Hyphomycetes*, pp. 99–117. Edited by F. Bärlocher. Berlin: Springer.

Wheeler, T. F., Heim, J. R., LaTorre, M. R. & Blair Janes, A. (1997). Mass spectral characterization of *p*-nonylphenol isomers using high-resolution capillary GC-MS. *J Chromatogr Sci* **35**, 19–30.

Xu, F. (1996). Oxidation of phenols, anilines and benzenethiols by fungal laccases: correlation between activity and redox potentials as well as halide inhibition. *Biochemistry* **35**, 7608–7614.

Ying, G.-G., Williams, B. & Kookana, R. (2002). Environmental fate of alkylphenols and alkylphenol ethoxylates – a review. *Environ Int* **28**, 215–226.

Yuan, S. Y., Yu, C. H. & Chang, B. V. (2004). Biodegradation of nonylphenol in river sediment. *Environ Int* **127**, 425–430.