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ipso-Substitution – A Novel Pathway for Microbial Metabolism of Endocrine-Disrupting 4-Nonylphenols, 4-Alkoxyphenols, and Bisphenol A

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Abstract: Our studies with *Sphingobium xenophagum* Bayram show that this bacterial strain degrades α-quaternary 4-nonylphenols by an *ipso*-substitution mechanism, whereby the nonylphenol substrates are initially hydroxylated at the *ipso* position to form 4-hydroxy-4-nonylcyclohexa-2,5-dienones (quinols). Subsequently, the α-quaternary side chains are able to detach as short-living cations from these intermediates. Alkyl branches attached to the carbocation help to delocalize and thereby stabilize the positive charge through inductive and hyperconjugative effects, which explains why only alkyl moieties of α-quaternary nonylphenols are released. This view is corroborated by experiments with *S. xenophagum* Bayram, in which the alkyl chains of the non-α-quaternary 4-(1-methyloctyl) phenol (4-NP_2) and 4-*n*-nonylphenol (4-NP_1) were not released, so that the bacterium was unable to utilize these isomers as growth substrates. Analysis of dead end metabolites and experiments with ^{18}O labeled H_2O and O_2 clearly show that in the main degradation pathway the nonyl cation derived from α-quaternary quinols preferentially combines with a molecule of water to yield the corresponding alcohol and hydroquinone. However, the incorporation of significant amounts of O_2-derived oxygen into the nonanol metabolites derived from degradation of certain α,α-dimethyl substituted nonylphenols by strain Bayram strongly indicates the existence of a minor pathway in which the cation undergoes an alternative reaction and attacks the *ipso*-hydroxy group, yielding a 4-alkoxyphenol as an intermediate. Additional growth experiments with strain Bayram revealed that also the two alkoxyphenols 4-*tert*-butoxyphenol and 4-*n*-octyloxyphenol promote growth. Furthermore, strain Bayram's *ipso*-hydroxlating activity is able to transform also bisphenol A.

Keywords: Alkoxyphenols · Bisphenol A · Endocrine disruptors · Nonylphenol · *Sphingobium xenophagum* Bayram · *ipso*-Substitution

Introduction

Nonylphenol (NP), nonylphenol monoethoxylate (NP1EO), and nonylphenol diethoxylate (NP2EO) are known endocrine disruptors.[1,2] They do not appear in the environment because of direct use as chemical products, but they are formed in the course of the biological degradation of nonylphenol polyethoxylates (NPnEO), which are now banned in the EU for water-relevant uses but are still used in the US as commercially important surfactants with industrial, agricultural, and domestic applications.

Degradation of NPnEO proceeds in several phases.[3,4] Scheme 1 shows an overview of biotransformation reactions that have been described to date.[5] It is generally agreed that primary biodegradation of NPnEO in wastewater treatment plants is quite efficient.[4,6,7] However, the overall rate of biodegradation is limited due to the formation of metabolites such as NP2EO, NP1EO, NP, NP1EC, and NP2EC. These metabolites are more recalcitrant than the parent compounds,[6,8] so that they transiently accumulate and can be found in sewage treatment effluents,[6,8,9] sewage sludges, rivers,[9,10] estuaries,[11,12] coastal waters,[13] and sediments.

Maki *et al.*[14] and John and White[15] could isolate bacterial strains that were able to grow on NPnEO as sole carbon source. The strains degrade NPnEO by stepwise exoscission of single glycol units with accumulation of NP2EO as major end product. John and White[15] proposed a mechanism for biotransformation that involves an oxygen-independent hydroxyl shift from the terminal to the penultimate carbon of the terminal ethoxylate unit of NPnEO and dissociation of the resulting hemiacetal to release acetaldehyde and the next-lower homolog, NPn-1EO, which then undergoes further cycles of the same

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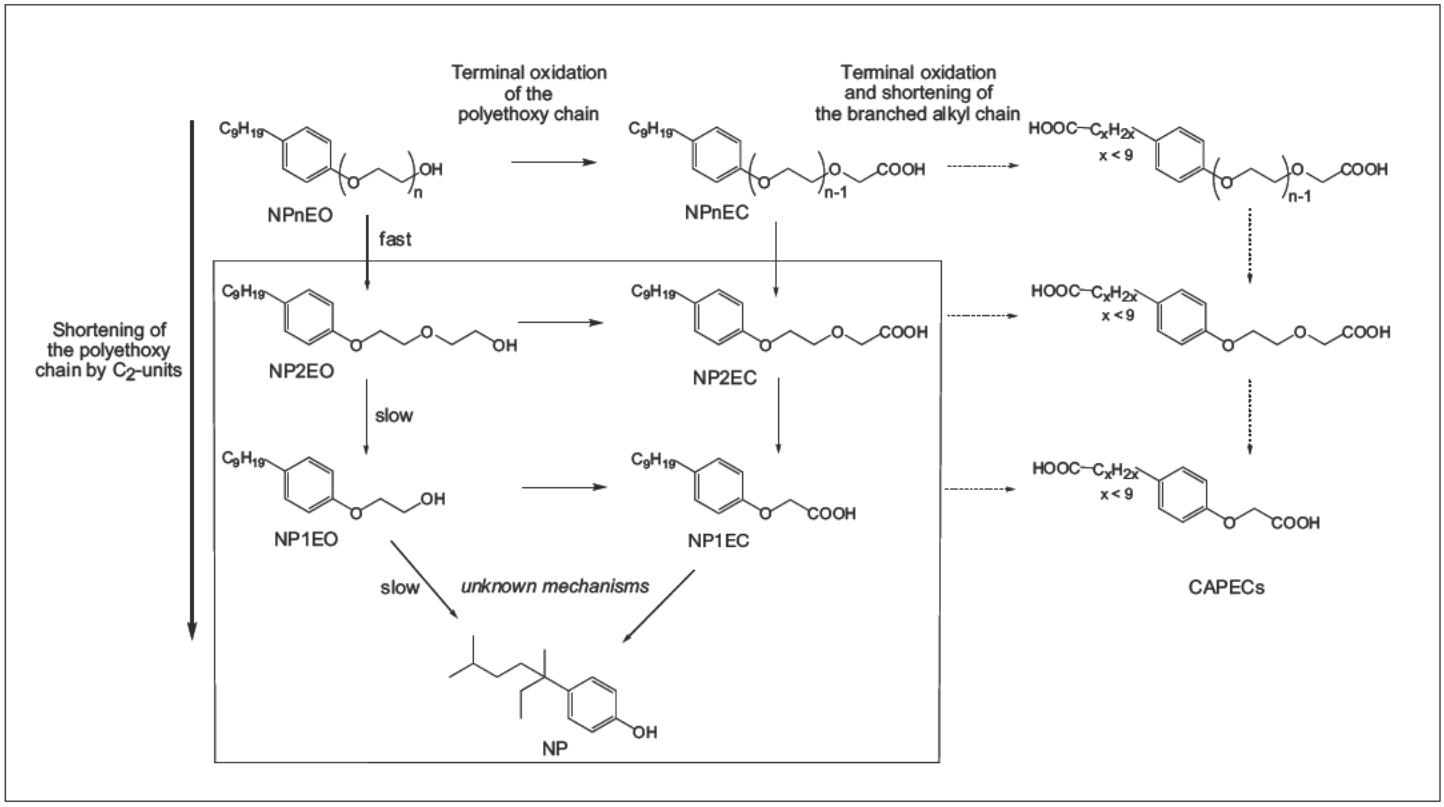

Scheme 1. Transformation of NPnEO to nonylphenol observed in various environmental compartments (adapted from refs. [5,6]). The compounds highlighted by the frame are all weakly estrogenic.

reaction until the ethoxy chain is reduced to two ethoxy units. These sequential reactions are consistent with the results of a study by Kvestak and Ahel,[13] who showed the formation of shortened but non-oxidized NPnEO homologs by mixed estuarine cultures. The fact that NP2EC was not formed during degradation of NPnEO, led the authors to speculate that nonoxidative ether scission may dominate NPnEO biodegradation to shorter homologs and that these longer-lived intermediates may undergo hydroxyl group oxidation as a side reaction, possibly mediated by alcohol dehydrogenases known to occur ubiquitously in bacteria.[15] In a study about biodegradation of NPnEO in river water,[16] the authors could show that, contrary to the situation for sludge, mixed estuarine cultures, and pure strains, the initiating step in Rhine river water is ω-oxidation of the individual ethoxy chains; metabolites with long carboxylated EO chains (NPnEC) were identified. Further degradation gradually yielded short-chain NPnEC with NP2EC as the most abundant species. During this degradation, the nonyl side chain was concomitantly oxidized and metabolites with both a carboxylated ethoxy and a carboxylated alkyl chain (CAPECs) were detected. Interestingly, nonylphenol was not found as a metabolite in this study. These results agree well with earlier studies that showed higher concentrations of NP1EC and NP2EC than of NP1EO and NP2EO in rivers.[6,10]

NPs are toxic and endocrine-disruptive compounds formed during degradation of NPnEO. Because NPnEO are originally synthesized from technical nonylphenol, the nonylphenol released during metabolism of NPnEO is the original technical material. Technical nonylphenol is a complex mixture of more than 100 isomers, which differ in the structure and the position of the alkyl moiety attached to the phenol ring.[17] More than 90% of the mixture consists of *para*-substituted nonylphenols.[18] Given the highly branched alkyl chain substituent, the pronounced recalcitrance of NP did not come as a surprise. As mentioned above, NPs can be found in all environmental compartments that directly or indirectly receive NPnEO. Although NPs are accumulating in such environments, several studies give hints that under aerobic conditions metabolism of NPs is possible.[19–21] Tanghe *et al.*[22] isolated from activated sludge *Sphingomonas* strain TTNP3, which was able to grow at the expense of NP in liquid culture. The authors suggested that degradation of NP by strain TTNP3 starts with a fission of the phenol ring leaving hydroxylated intermediates with branched alkyl chains of different lengths. Strain TTNP degraded the phenol ring of octylphenol and left a branched octylalcohol behind.[23] Another bacterial strain, *Sphingomonas cloacae*, with the ability to degrade NP was isolated from wastewater of a sewage treatment plant in Tokyo.[24] This strain also degrades the ring moiety of NP.[20]

Here we present an overview of our results from the study of the microbial metabolism of nonylphenolic endocrine disruptors, which were achieved within the frame of the NRP50 project. We were able to show that the degradation of NP is initiated by *ipso*-hydroxylation and to elucidate the *ipso*-substitution mechanism. To this end we performed degradation experiments with single pure isomers with defined mixtures of pure isomers as well as with ^{18}O-labeled water and dioxygen. We applied NMR and GC/HPLC-MS techniques to identify the chemical structures of the metabolites.

Bacterial Growth on Single Nonylphenol Isomers

A bacterium utilizing 4-(1-ethyl-1,4-dimethylpentyl)phenol (4-NP_{112}, the numbering system for nonylphenol isomers is described in ref. [25]), one of the main isomers of technical nonylphenol mixtures, as the sole carbon and energy source was isolated from activated sludge of a municipal wastewater treatment plant. To obtain pure cultures, we successively transferred nonylphenol-degrading enrichments. Cultures of stable lineages were plated on agar plates with technical nonylphenol as the substrate. Single colonies were picked and tested for the ability to degrade 4-NP_{112}. However, it appeared that even after several enrichment cycles the majority of the bacteria were not able to degrade

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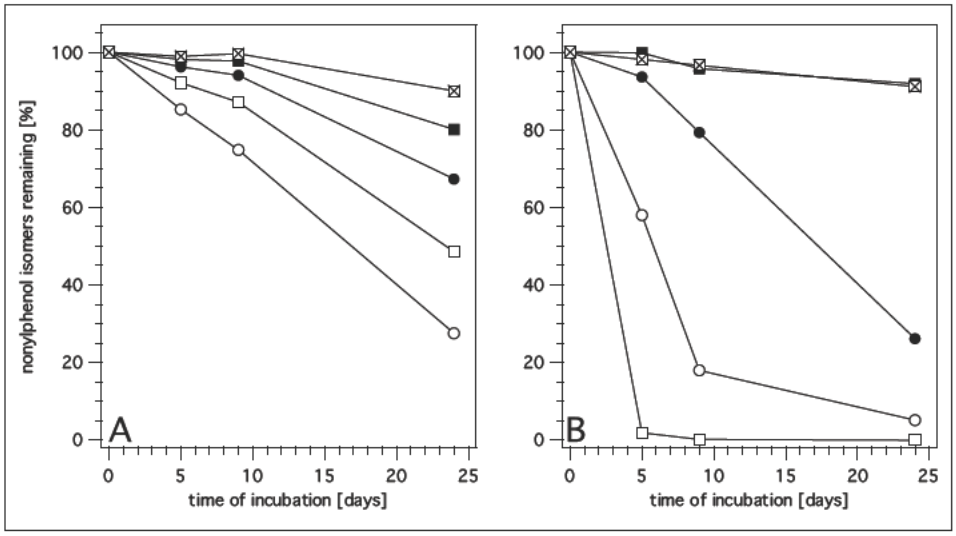


Fig. Degradation of five nonylphenol isomers (4-NP_{93} —○—, 4-NP_{112} —□—, 4-NP_9 —●—, 4-NP_2 —■—, 4-NP_1 —⊠—) by *S. xenophagum* Bayram in minimal medium (the structures of the isomers are shown in Scheme 2). The isomers were added as a mixture (A) and as individual isomers (B) at a concentration of 1 mg/l for each isomer. For details see:[28]

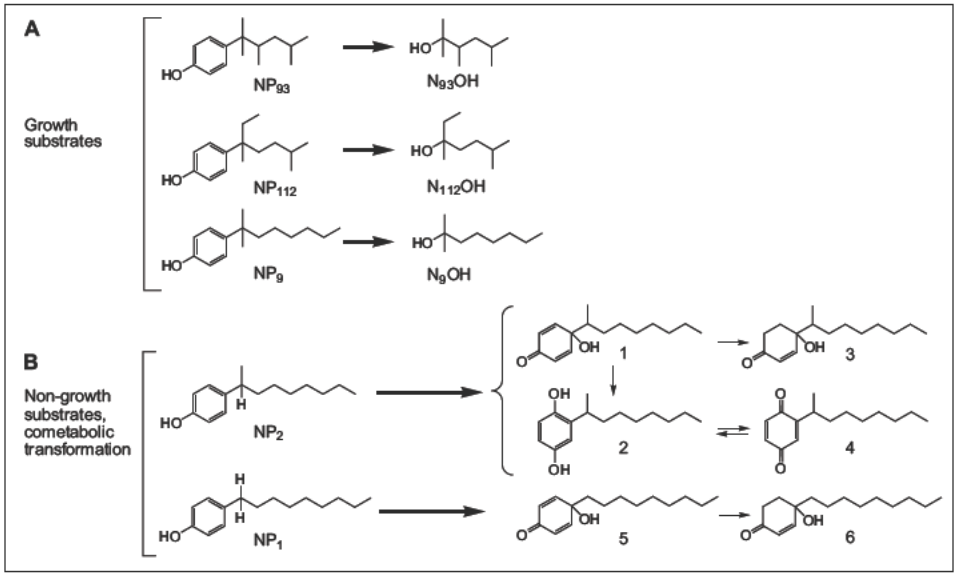
4-nonylphenol. Luckily, active colonies revealed themselves by a transparent halo that formed in the turbid agar after four weeks of incubation. Such colonies were picked and transferred to fresh agar plates and eventually a 4-NP_{112}-degrading bacterium was isolated in pure culture by picking one of the colonies under the view of a binocular microscope. The isolate, designated strain Bayram, was classified as *Sphingomonas xenophaga* and later reclassified as *Sphingobium xenophagum*.[26] Different transfer procedures starting from several enrichment lineages always led to the same type of 4-nonylphenol degrading colony, indicating that Bayram was probably the dominant bacterium in the activated sludge able to grow with 4-NP_{112} as the sole carbon source.

In minimal medium with 1 mg/ml 4-NP_{112}, strain Bayram transformed around 50% of the substrate within three days and after twelve days most of the substrate was metabolized. This degradation rate was similar to the one observed for the transformation of 4-(1-ethyl-1,3-dimethylpentyl)phenol (4-NP_{111}) by *Sphingomonas* sp. strain TTNP3, another nonylphenol-degrading bacterium.[27]

Differential Degradation of Nonylphenol Isomers and Release of the Alkyl Substituent as Nonanol

Our degradation experiments with defined mixtures of nonylphenol isomers clearly showed that *S. xenophagum* strain Bayram differentially transforms these isomers (Fig.). The degradation rate showed a positive correlation with the branching degree of the alkyl substituent – the more highly branched, the faster the degradation.[28] These novel findings contrasted with what had been the prevailing view on the degradation of technical nonylphenol mixtures[22,29,30] and emphasized the importance of taking into consideration isomeric effects when investigating degradation and toxicity of complex mixtures such as technical nonylphenols.

In incubation experiments with a mixture of five isomers, we could identify the nonylalcohols that originated from the isomers with a quaternary carbon atom at the benzylic position. However, no traces of nonan-2-ol and nonan-1-ol, potential metabolites derived from nonylphenol isomers with hydrogen atoms at the benzylic position, could be detected in any experiment, although these nonylphenol isomers were partly degraded. This indicated that nonylphenol isomers with hydrogen atoms at the benzylic position are metabolized in a different way than the more highly branched isomers. We proposed that although such isomers do engage in the main degradation pathway, they are only incompletely degraded, because they are unable to undergo the reaction in which the alkyl group is detached from the aromatic part. Our data further indicated that the ring moiety needs to be cleaved from the alkyl substituent to be degraded and utilized. In our experiments, the alkyl chains of 4-NP_2 and 4-NP_1 were not released. Thus, the bacterium was unable to utilize these isomers as growth substrates. Nevertheless, significant amounts of 4-NP_2 and 4-NP_1 were cometabolically transformed, when supplied together with 4-NP_{93}, 4-NP_{112} and 4-NP_9, which did serve as growth substrates. The low yield coefficient when grown on NP_{112}, indicated that strain Bayram cannot utilize the alkyl part of such compounds as a source of carbon and energy.



Scheme 2. Cometabolic transformation of NP_1 and NP_2 by strain Bayram in the presence of nonylphenol isomers that serve as growth substrates. A) Nonylphenol isomers that serve as substrates. B) Nonylphenol isomers that do not serve as growth substrates and are only cometabolically transformed. For details see:[31,32]

ipso-Hydroxylation of Nonylphenol Isomers

As outlined in the preceding paragraph, we could show that the alkyl moieties of the alkylphenol isomers that serve as growth substrates are transformed to alkyl alcohols with retention of the structures of the original alkyl side chains. Conventional pathways of alkylphenol metabolism cannot explain the formation of such metabolites. As we had available several pure 4-nonylphenol isomers (4-NP_{93}, 4-NP_{112}, and 4-NP_9), we planned to identify intermediates of the degradation pathway when strain Bayram grew on these isomers as the sole carbon and energy sources. Unfortunately,

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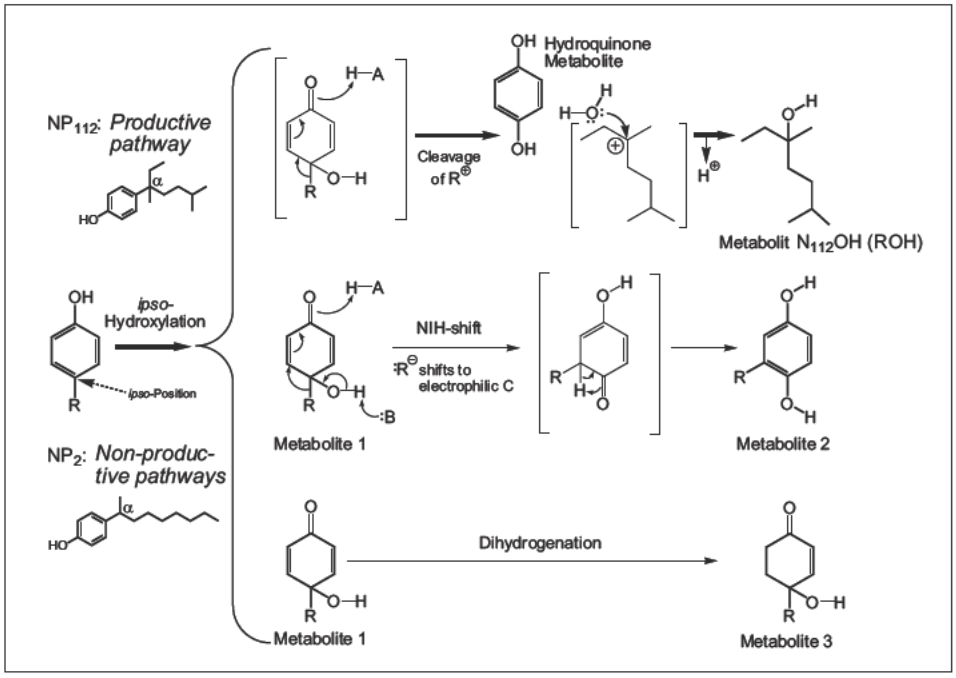

Scheme 3. Pathways proposed for the degradation of nonylphenol isomers in *Sphingobium xenophagum* Bayram. Nonylphenols are transformed by an initial *ipso*-hydroxylation to 4-alkyl-4-hydroxy-cyclohexadienone intermediates (*e.g.* metabolite **1**). As an example for a productive pathway (fat arrow), nonylphenol isomer NP_{112} and as an example for a non-productive pathway, isomer NP_2 is shown. α-Quaternary intermediates dissociate by releasing the alkyl moiety as a cation, which is stabilized by α-alkyl branching. The formed hydroquinone is then further metabolized. Degradation experiments with ^{18}O-labeled oxygen and water clearly showed that the detached cation preferentially reacts with the nucleophilic oxygen atom of a water molecule forming the corresponding nonanol (*e.g.* $N_{112}OH$), which most likely cannot be further metabolized. Intermediates with hydrogen at the α-position are not able to dissociate at all or they undergo a NIH-shift yielding metabolites that cannot be further degraded. A-H and B:, a proton donor and acceptor, respectively.

we could not detect any metabolites in such growth experiments except the C_9 alcohols derived from the nonyl side chains (Scheme 2A). Therefore, we designed experiments, in which the non-growth isomers 4-NP_2 and 4-NP_1 were cometabolically transformed in the presence of the growth substrate 4-NP_{112}. In such experiments, 4-NP_2 and 4-NP_1 were transformed to various metabolites that accumulated in the culture medium. These metabolites were isolated in mg-quantities and their chemical structures were elucidated by NMR spectroscopy and mass spectrometry.[31]

Scheme 2 shows the molecular structures of the metabolites, and Scheme 3 contains a nonylphenol degradation scheme that is based on our experimental data. The structures of metabolites **1** and **5** (Scheme 2, Scheme 3), both with an additional hydroxy substituent at the 4-position of the hexadienone ring, were strong indicators that the initial reaction is a hydroxylation at the anchor carbon atom of the alkyl substituent – an *ipso*-attack – forming 4-alkyl-4-hydroxy-cyclohexadienones (quinols). By analogy, we concluded that the growth substrate isomers undergo the same initial *ipso*-hydroxylation. However, the intermediates derived from the growth substrate isomers do not accumulate, because they are immediately metabolized.

Experiments with ^{18}O-labeled water and dioxygen gave clear indication that the *ipso*-hydroxy group is derived from molecular dioxygen and that in the major pathway for cleavage of the alkyl moiety the resulting nonanol metabolite contains an oxygen atom from water.[32] These results were a clear proof for our view that *ipso*-hydroxylation is the initial reaction in the degradation pathway and showed that an oxygenase was involved. Furthermore, we were able to conclude that the alkyl substituent is released from the quinol intermediate as a carbocation and then preferentially reacts with a water molecule (Scheme 3, productive pathway) to form the corresponding alcohol. The breakdown of the 4-alkyl-4-hydroxy-cyclohexadienone intermediate represents an S_N1 reaction (unimolecular nucleophilic substitution), whereby the carbocation reacts with the solvent (solvolysis) and the dissociation energy is provided by the rearomatization of the leaving carbon ring. However, several α,α-dimethyl-substituted nonanols incorporated a significant amount of O_2-derived oxygen (up to 13%), suggesting the existence of a minor pathway, in which the cation undergoes an alternative (1,2-C,O)-shift and attacks the *ipso*-hydroxy group to form 4-alkoxyphenol intermediates.[32] In strain TTNP3, this alternative reaction most likely leads to dead end metabolites, as this particular strain, in contrast to strain Bayram, seems not able to further degrade 4-alkoxyphenols.[32–34]

In contrast to the quinols derived from α-quaternary nonylphenols, metabolites **1** and **5** do not undergo detachment of the alkyl moiety because the corresponding α-carbocations are not sufficiently stabilized by alkyl substituents in α-position. Alternatively, they serve as substrates for side reactions (Scheme 2 and Scheme 3).

The conversion of metabolite **1** into metabolite **2,** a 2-alkylhydroquinone, involves a (1,2-C,C)-shift of the alkyl substituent, a reaction well-known as the NIH-shift.[31] Small amounts of an NIH-shift product have also been detected in cell extracts of other nonylphenol degrading strains.[27]

In principle, the detachment of the alkyl moiety as a carbocation, the detected (1,2-C,O)-shift, and the (1,2-C,C)-shift (NIH-shift) would be effective in restabilizing the destabilized cyclohexadienone intermediate, as each leads to a rearomatization of the ring moiety. Both detachment of the alkyl chain as carbocation with subsequent addition of water and the (1,2–C,O)-shift, which leads to an intermediate (a 4-alkoxyphenol) that can undergo a second *ipso*-hydroxylation step, are reactions in productive pathways enabling growth on 4-nonylphenols. In contrast, the (1,2–C,C)-shift is disadvantageous in terms of efficiency to break apart the molecule, as it leads to a product, a 2-alkylhydroquinone, in which the alkyl moiety remains connected to the carbon ring by a C–C bond (Scheme 3). We believe that the NIH-shift represents a non-productive side reaction and becomes relevant when other reactions are unfavorable as is the case for 4-nonylphenol isomers with hydrogen atoms at the benzylic α-carbon.

ipso-Substitution is a General Mechanistic Principle for the Removal of a Wide Range of Substituents from *para*-Substituted Phenols

ipso-Substitution is an important mechanism by which cytochrome P450 model systems, liver microsomes, and microorganisms detach various substituents in *ortho*- and *para*-substituted phenols and anilines.[35,36] Depending on its affinity for electrons, the attached group leaves formally either as an anion (type I) or a cation (type II) (Scheme 4A), and several enzymatic systems are known to detach electron-donating as well as electron-withdrawing substituents.[35,37–44] However,

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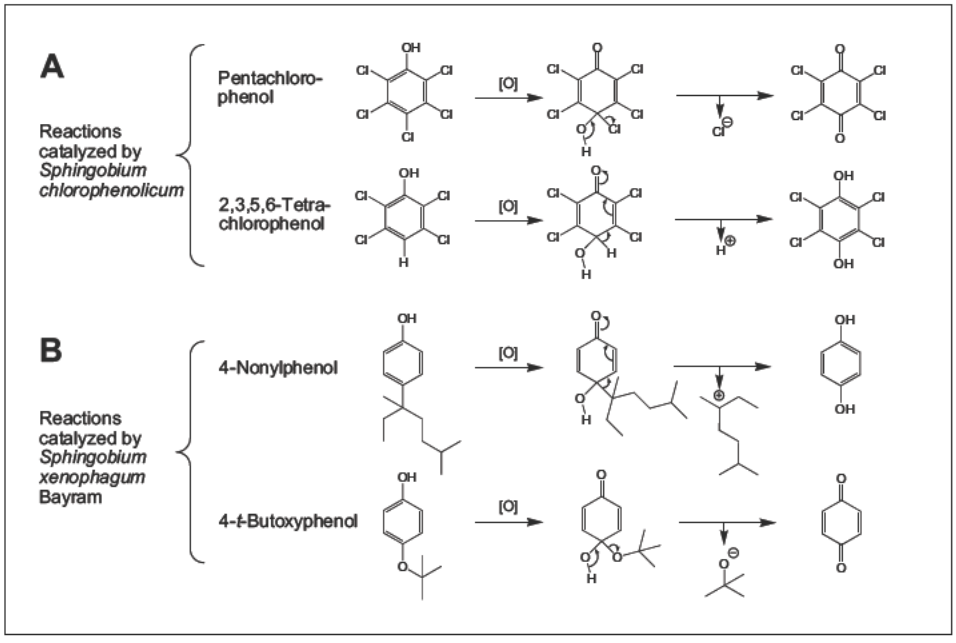
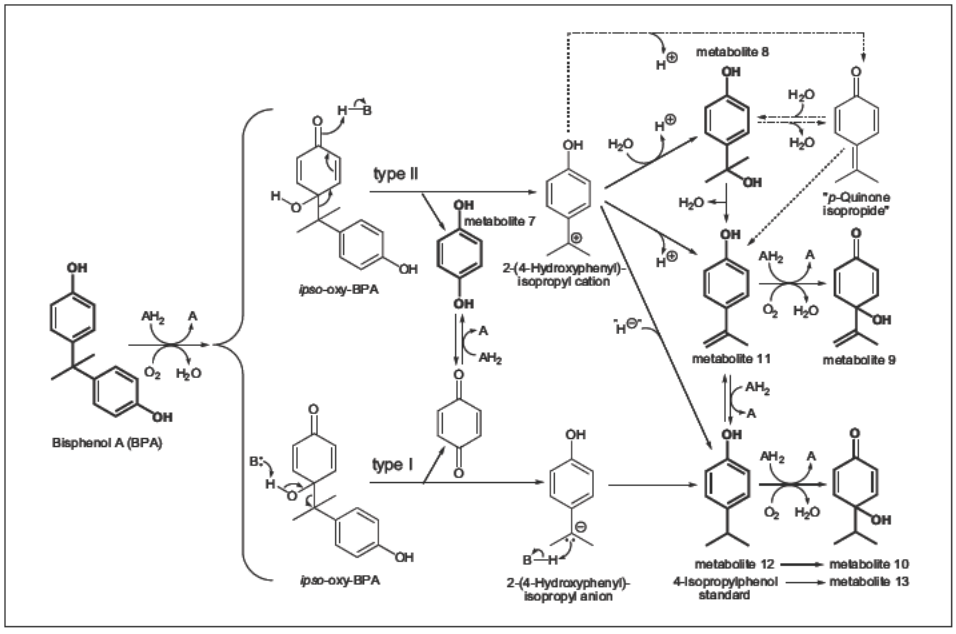


Scheme 4. *ipso*-Substitution of substituents with electron donating (hydrogen and alkyl substituents) and electron withdrawing (chlorine and alkoxy substituents) properties. A) Reactions catalyzed by *S. chlorophenolicum* (hydrogen and chlorine substituents). B) Reactions catalyzed by *S. xenophagum* Bayram (alkyl and alkoxy substituents).



Scheme 5. Pathways proposed for the degradation of bisphenol A and 4-isopropylphenol by strain Bayram leading to the metabolites **7–12** and **13**, respectively. Although *p*-quinone isopropide was not detected in the reaction solution, this reactive compound might play a role in the formation of the metabolites **8** and **11** (dashed arrows). AH_2 and A, a pair of reduced and oxidized cosubstrates, respectively; B–H, a proton donor. The substrate and the detected metabolites are highlighted in bold. For details see:[51]

most studies that considered cationic leaving groups refer to hydrogen.[37,38,40–42,44] As outlined above, we discovered within this project that 4-nonylphenols are metabolized by a type II *ipso*-substitution whereby the α-quaternary alkyl side chains are detached as cations (Scheme 4B). Growth experiments with 4-*tert*-butoxyphenol proved that strain Bayram is able to use this compound as a sole carbon and energy source and we were able to show that 4-*tert*-butoxyphenol is degraded by *ipso*-substitution involving a type I mechanism (Scheme 4B; 4-nonyloxyphenols are most likely transformed analogously). It is tempting to suggest that one and the same *ipso*-hydroxylase is responsible for the transformation of both 4-alkyl- and 4-alkoxyphenols in strain Bayram, since both activities were strongly dependent on the induction of cells with nonylphenol. The putative enzyme would have a remarkably wide substrate range, as activity was observed with α-quaternary 4-alkylphenols, 4-alkoxyphenols with α-primary and -tertiary alkyl moieties, and a 4-aryloxyphenol. However, future work will have to verify whether indeed one and the same enzyme catalyzes the hydroxylation of these different substrates.

Several compounds other than nonylphenols, such as 4-ethylphenol[45] and hexachlorocyclohexane,[46] have been shown to be microbially transformed to hydroquinone that subsequently serves as a ring cleavage substrate. In a reducing environment, the hydroquinone formed is not expected to oxidize to *p*-quinone, a reactive compound that covalently binds to cellular macromolecules and induces oxidative stress.[40] However, *ipso*-substitution of alkoxy moieties and of other electrophilic substituents inevitably results in the formation of this toxic intermediate (Scheme 4).[40,44,47,48] To survive, strains must have developed efficient reducing systems to quickly convert *p*-quinone into hydroquinone.

Many studies have focused on the degradation of the environmental contaminants 4-nonylphenol and bisphenol A, and several microbes are known to degrade both compounds.[49,50] However, the mechanistic principles underlying the observed degradation processes have remained largely unknown so far. Recently, we were able to show that strain Bayram also degrades bisphenol A by an oxidative *ipso*-substitution mechanism, producing several C_3-substituted phenolic derivatives.[51] Among these metabolites, 4-isopropenylphenol and 4-isopropylphenol were transformed by strain Bayram in a further *ipso*-substitution step (Scheme 5). Because of their chemical instability, several of these bisphenol A products could well prove to be quite toxic for various organisms. An *ipso*-mechanism has also been proposed for the degradation of bisphenol A by *Sphingomonas* sp. strain TTNP3, another well-known nonylphenol degrader.[52] Although Bayrams's and TTNP3's modes of degradation of bisphenol A and nonylphenols might appear unique at a first sight, a thorough comparison of literature data strongly indicates that it represents just one example of an *ipso*-substitution mechanism; *ipso*-substitution turns out to be a general mechanistic principle to remove a wide range of substituents from *para*-substituted phenols.

Conclusions

Our results have strong implications for assessments of the environmental fate

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and risk of nonylphenolic compounds. First, differential degradation of nonylphenol mixtures will lead to a change of the relative isomer composition in mixtures upon microbial degradation. As endocrine effects of nonylphenol isomers are dependent on the structure of the nonyl substituent (Gabriel, Routledge, Heidlberger, Rentsch, Guenther, Giger, Sumpter, and Kohler, 'Isomer-specific Degradation and Endocrine Disrupting Activity of Nonylphenols', submitted for publication), proper risk assessments necessitate consideration of such variations in composition when materials from different sources are involved (*e.g.* nonylphenol mixtures extracted from different environmental compartments most likely will have different isomer compositions). Second, 'aging', *i.e.* shifting of the isomer composition due to environmental reactions such as microbial metabolism, photo degradation *etc*., will become very important with regard to the interpretation of analytical data from environmental samples as well as for fate studies. If the importance of isomer-specific effects of nonylphenols and nonylphenolic compounds is confirmed in future studies and also applies to other biological assays, past risk assessment strategies for nonylphenols and nonylphenolic compounds need to be re-evaluated and isomer-specific approaches will have to be implemented.

Ongoing and Future Research

Ongoing and future studies aim at gaining deeper insights into the enzymatic reactions involved in the metabolism of nonylphenolic compounds. We are developing enzymatic assays and plan to purify and characterize the *ipso*-hydroxylating enzyme activity in order to gain an understanding of the substrate spectrum and the catalytic mechanism of the enzyme. This will allow us to better assess the risk of formation of unwanted side products and dead end metabolites during microbial metabolism of these compounds in different environmental matrices.

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