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Characterization of phenol and cresol biodegradation by compound-specific stable isotope analysis

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ABSTRACT

Microbial degradation of phenol and cresols can occur under oxic and anoxic conditions by different degradation pathways. One recent technique to take insight into reaction mechanisms is compound-specific isotope analysis (CSIA). While enzymes and reaction mechanisms of several degradation pathways have been characterized in (bio)chemical studies, associated isotope fractionation patterns have been rarely reported, possibly due to constraints in current analytical methods. In this study, carbon enrichment factors and apparent kinetic isotope effects ($AKIE_c$) of the initial steps of different aerobic and anaerobic phenol and cresols degradation pathways were analyzed by isotope ratio mass spectrometry connected with liquid chromatography (LC-IRMS). Significant isotope fractionation was detected for aerobic ring hydroxylation, anoxic side chain hydroxylation, and anoxic fumarate addition, while anoxic carboxylation reactions produced small and inconsistent fractionation. The results suggest that several microbial degradation pathways of phenol and cresols are detectable in the environment by CSIA.

1. Introduction

Phenol and cresols are simple-structured phenols of natural or anthropogenic origin. These compounds are usually obtained from petroleum or coal tar and are widely used in chemical, petrochemical as well as pharmaceutical industries. Their presence as pollutants in air and water is therefore also related with these industrial processes (Marrot et al., 2006). The increasing presence of phenols in the environment creates health concerns due to their toxicity; e.g., phenol and cresols are acting corrosively in contact with skin and mucosal membranes. In addition, *p*-cresol is classified as probably carcinogenic to humans (Kahru et al., 2002; Michałowicz and Duda, 2007). Phenol and cresols are highly water-soluble (phenol, 83 g/L; cresol isomers, 22–26 g/L) (Yalkowsky et al., 1987) leading to increased environmental risks as these compounds can be transported in aqueous systems over long distances at high concentrations. For these reasons, it is vital to investigate natural attenuation processes of phenols in the environment. Previous studies have shown that microbial degradation of phenol and cresols play a significant role in the removal of these compounds in polluted environments (Krastanov et al., 2013). Phenol and cresols are biodegradable under both oxic and anoxic conditions by different degradation pathways. Under oxic conditions, phenol can be initially hydroxylated to catechol; the reaction is catalyzed by phenol hydroxylase using molecular oxygen as a co-substrate (Leahy et al., 2003). The aerobic degradation of cresols is initiated as well by hydroxylation via phenol hydroxylases, either occurring at the ring or at the methyl moiety (Hopper and Taylor, 1975; Paller et al., 1995; Powlowski and Shingler, 1994; Solyanikova and Golovleva, 1999). Under anoxic conditions, phenol is firstly carboxylated to 4-hydroxybenzoate prior to reduction of the aromatic ring (Lack and Fuchs, 1994; Schmeling et al., 2004; Schuhle and Fuchs, 2004). Different initial reactions were described for the degradation of cresols under anoxic condition: (i) molecular oxygen-independent hydroxylation (Hopper et al., 1991; McIntire et al., 1985; Rudolphi et al., 1991), (ii) carboxylation (Bisaillon et al., 1991) and (iii) fumarate addition

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(Müller et al., 1999, 2001). The initial reactions of the different degradation pathways and the used strains in this study employing these pathways are summarized in Fig. 1.

CSIA, by which specific pollutants are chromatographically separated prior to isotopic analysis, is a promising new method for environmental monitoring of pollutant biodegradation that proved to be successful for rather non-polar compounds such as hydrocarbons (e.g., BTEX) or chlorinated organics (Schmidt and Jochmann, 2012). CSIA can be used for (i) identifying pollution sources by detecting small variations in the isotopic composition of compounds, and for (ii) assessing the biodegradation of pollutants in the environment by detecting the associated isotope fractionation (Elsner et al., 2005; Schmidt and Jochmann, 2012). The latter approach takes advantage of different reaction rates of isotopologues in rate-limiting steps of (bio)chemical reactions. Due to energetic constraints, the lighter isotopologue reacts usually slightly faster resulting in a residual substrate pool enriched in heavy isotopes at the reactive position in the course of the reaction; correspondingly, the product becomes isotopically lighter. This is termed kinetic isotope effect (KIE). KIEs can be calculated by forming the ratio of the reaction rates of the heavy and light isotopologues, or by applying the Rayleigh equation which relates changing isotope signatures and concentrations of the substrate. By the latter procedure, apparent kinetic isotope effects (AKIEs) are usually derived in studies dealing with the (bio)transformation of environmental relevant pollutants (Elsner et al., 2005). KIEs can be further divided into primary and secondary isotope effects. Isotope effects related to the breakage or formation of chemical bonds to the atom of interest in the reacting substrate are termed primary isotope effects. Isotope effects occurring in the absence of bond breakage or formation in the rate-determining step of a reaction are termed secondary isotope effects. Secondary isotope effects are usually by at least one magnitude lower than primary effects (Elsner et al., 2005). Different types of reactions may lead to isotope fractionation with different extent due to the energy level differences during the bonds dissociation and formation processes in the transition state. Thus, analysis of stable isotope fractionation during biodegradation of a certain compound may indicate the biochemical mechanism by which the compound is initially transformed, hence indicating the biodegradation pathway (Elsner et al., 2005; Meckenstock et al., 2004; Schmidt and Jochmann, 2012).

For quantifying *in situ* biodegradation by CSIA and for identifying distinct reaction mechanisms, it is essential to determine the magnitude of isotope fractionation of the first step of known pollutant degradation pathways in laboratory experiments using model cultures. In recent years, isotope fractionation factors for the biodegradation of various pollutants and degradation pathways have been published, using stable isotopes of several elements (e.g., D/H, $^{13}C/^{12}C$) depending on the compounds structure (Morasch and Hunkeler, 2009). Many of those pollutants belong to the dense non-aqueous phase lipids (DNAPLs), e.g. chlorinated ethenes, or light non-aqueous phase lipids (LNAPLs), e.g. mineral oil components. The isotope signatures of those non-polar compounds are usually analyzed by methods based on gas chromatography–isotope ratio mass spectrometry (GC-IRMS) (Schmidt and Jochmann, 2012).

Sensitive analysis of polar compounds like phenols by GC-IRMS is virtually impossible due to their physical-chemical characteristics: they are hydrophilic and thermo-labile compounds. Therefore,

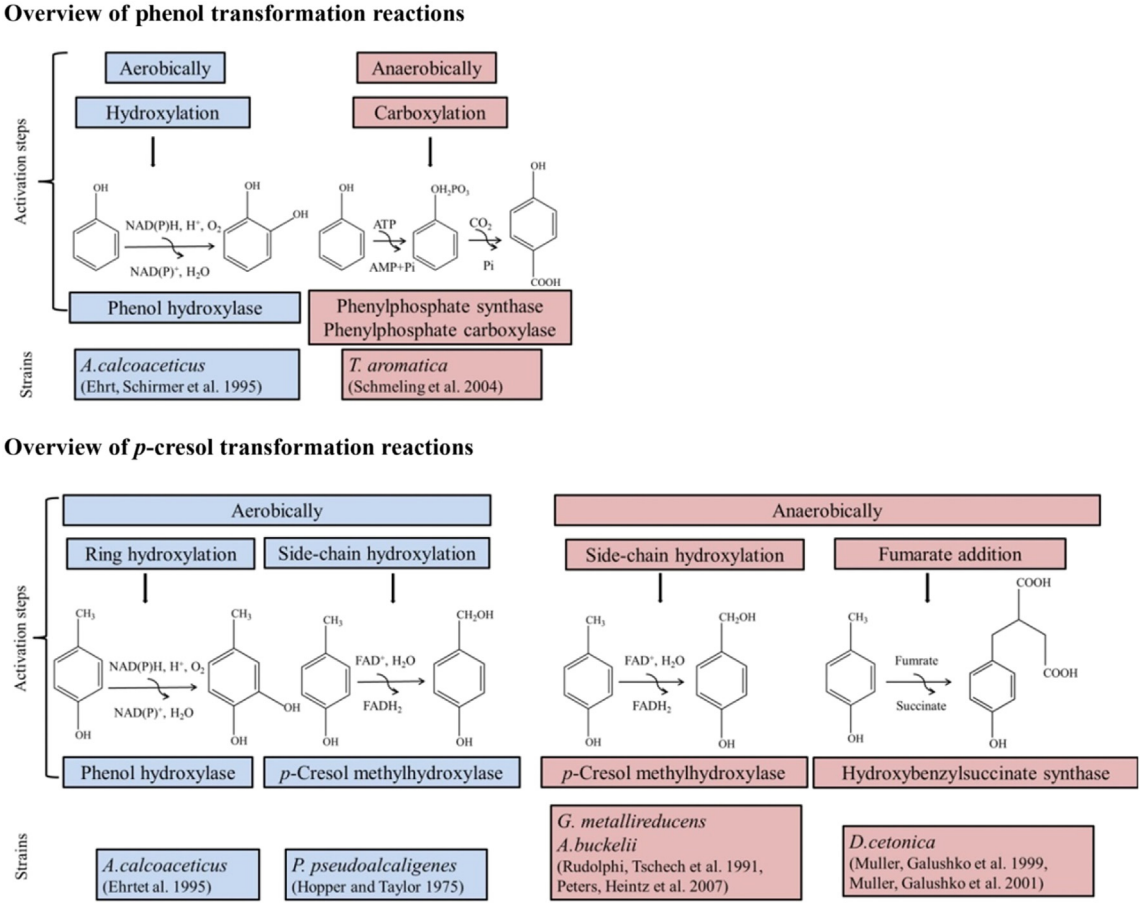

Fig. 1. Initial steps of microbial phenol and p-cresol degradation pathways investigated in this study.