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Formation of the metabolic intermediate 2,4,4-trimethyl-2-pentanol during incubation of a *Sphingomonas* sp. strain with the xeno-estrogenic octylphenol

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Abstract

Degradation of branched octylphenol was studied in a bacterial culture of a *Sphingomonas* sp. strain. Octylphenol is considered to be the most stable degradation intermediate formed from the corresponding nonionic octylphenol polyethoxylates surfactants during biological wastewater treatment. Since octylphenol can exert estrogenic effects in wildlife, a detailed study of its biodegradation is warranted. The aerobic microbiological transformation of octylphenol was examined with and without the addition of the easily assimilable sodium acetate. In both cases the formation of the metabolite 2,4,4-trimethyl-2-pentanol, representing the intact alkyl chain as a tertiary alcohol, was observed. Since the octylphenol degradation rate was not affected by the presence of acetate, this strain did not show any diauxic metabolic behaviour when incubated with octylphenol and sodium acetate as the sources of carbon and energy. As a result of the biotransformation of octylphenol, its estrogenic potency was removed because it is the phenolic moiety that interacts with the estrogen receptors. This feature opens perspectives for the use of this strain in the framework of an adequate treatment of wastewater with high levels of alkylphenol polyethoxylates.

Abbreviations: NP: nonylphenol; NPnEOs: nonylphenol polyethoxylates; OP: octylphenol

Introduction

In the environmental, ecotoxicological and medical literature the alkylphenols, i.e., nonylphenol (NP) and octylphenol (OP), have received increasing attention during the last decade (Jobling and Sumpter 1993; Nimrod and Benson 1996; TemaNord 1996; Tanghe et al. 1999a; Toppari et al. 1995; White et al. 1994). Their aquatic toxicity and not at least their recently acknowledged endocrine disrupting potential have been the driving force behind a multitude of (eco)toxicological and environmental studies (Ashfield et al. 1998; Gimeno et al. 1997; Liber et al. 1999). Depending on the bio-assay used, NP and OP have an estrogenic potential that is 50 to 100,000 times less compared to a natural estrogen 17β-estradiol (Routledge et al. 1998; Servos 1999; Soto et al. 1995). Because of the use of alkylphenols as alkylphenol polyethoxylates (APnEOs) in the formulation and production of mainly plastics, paints, pesticides and detergents they are omnipresent in the environment (Bennie et al. 1999; Lye et al. 1999; Maguire 1999; Naylor et al. 1992; Tanghe et al. 1999a). Concentrations of NP in surface water reported in literature range from <0.1 to 180 μg/l (Ahel et al. 1994b; Blackburn and Waldock 1995; Naylor et al. 1992; Tanghe et al. 1999a). The presence of OP in the environment is not well documented but concentrations are lower compared to NP due to its lower production volume (Talmage 1994). This represents the potential exposure of animal species and humans with the subsequent disruption of

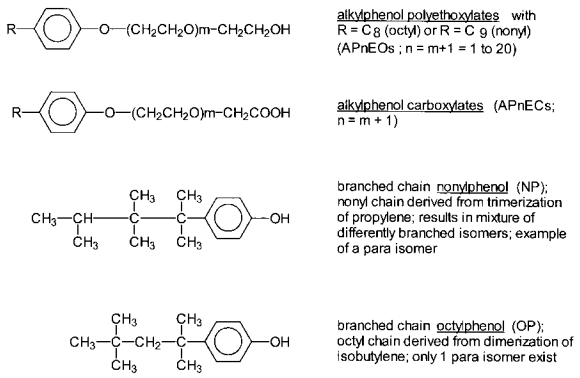

Figure 1. Chemical structures in relation to octylphenol and nonylphenol.

their respective endocrine system. The chemical structures of some alkylphenolic compounds are depicted in Figure 1.

Until recently, branched nonylphenol being a well known representative of the group of xeno-estrogens (mimicking the action of natural female hormones), has been considered to be a biorecalcitrant intermediate of the nonylphenol polyethoxylates (NPnEOs) during biological wastewater treatment (Ahel et al. 1994a; Talmage 1994; TemaNord 1996). However, a bacterial strain (*Sphingomonas* sp.) able to grow on branched NP as the sole carbon and energy source has recently been enriched and isolated from activated sludge (Tanghe et al. 1999b). The degradation kinetics and formation of degradation intermediates from NP have been studied. It was shown that the *Sphingomonas* sp. preferentially degraded the *para*-NP isomers above the *ortho*-NP isomers as could be deduced from the increase of the *ortho*-NP over *para*-NP ratio along the incubation period (HPLC-analysis). Together with the fact that in diethyl ether extracts of the media, no intermediates with an aromatic moiety were detected (GC-MS), it was postulated that the degradation of NP started with a fission of the phenolic ring. However, the search and identification of intermediate metabolites was severely impaired by the fact that the original NP mixture contained a variety of branched alkyl chain isomers. Each mother-isomer could give rise to its own intermediates resulting in a wide scala of products and consequently a complex chromatogram.

To overcome this bottleneck the degradation of a similar alkylphenolic compound, i.e., octylphenol was studied, by the isolated *Sphingomonas* sp. OP, which also contains a branched alkylchain (C8), consists out of only one isomer. This feature should facilitate the search and identification of possible degradation products occurring and/or accumulating in the culture media during incubation.

Materials and methods

Media

Minimum mineral salts medium (MMO) was prepared in sterile MilliQ water (Millipore, Molsheim, France) according to the method of Stanier et al. (1966). The MMO-medium was used as such to incubate the *Sphingomonas* sp. strain after addition of the desired amount of OP (4-(*tert*-octyl)phenol, 97%; Aldrich Chemical Company, Inc., Deisenhofen, Germany). The water solubility of OP at 20.5 °C is about 12.6 mg/l (Ahel & Giger 1993). Luria Bertani (LB) medium was used to culture the *Sphingomonas* sp. strain to obtain high cell densities, to enumerate culture densities and to assess the purity of the cultures. The LB-medium was composed of 10 g of tryptone, 5 g of yeast extract and 10 g of sodium chloride per liter (Oxoid, Ltd., Basingstoke, England; and VEL, Haasrode, Belgium). Fifteen g agar-agar (Merck, Darmstadt, Germany) per liter was added to make plates. A 10-fold dilution series of cultures was made (sterile physiological solution; 0.85% NaCl in MilliQ water), and 100 μl of each dilution was surface plated on LB-agar for enumeration purposes. Liquid cultures and plates were incubated at 28 ± 2 °C.

Determination of OP degradation and formation of intermediates

To monitor OP disappearance and growth of the OP-degrading culture, the following experiment was designed. Two series of Erlenmeyer flasks (250 ml) containing 100 ml of MMO-medium and about 68 to 70 mg OP were simultaneously inoculated with 2 ml of a 12 days old *Sphingomonas* sp. culture, having a OD_{550} of 0.226. The OP dosage corresponded to about 2 g chemical oxygen demand (COD) per liter. In the first series, 8 Erlenmeyer flasks in duplicate contained MMO-medium, OP and 2.6 g sodium acetate per liter corresponding to a COD of 2 g/l from the start of the incubation period (addition of 4 ml of a filter sterilized stock solution of 65 g sodium acetate per liter). For the second series, the sodium acetate was added to the remaining 4 flasks (duplicate) on day 9 of the incubation period. Every 2 to 3 days, two flasks of each series were sacrificed. Both duplicates were sampled

(1 ml) for bacterial growth determination (OD_{550}). Afterwards, the entire contents of one flask (including the plastic tip used to take the 1-ml sample) was used for extraction and OP analysis, and the second for further analysis of COD and degradation intermediates.

Removal of OP in resting cell culture

Resting cells of the *Sphingomonas* sp. strain were cultured in LB-medium (300 ml in a 500 ml Erlenmeyer; rotary shaker at 40 to 50 rpm; 28 ± 2 °C) for 1 week until a plateau in the OD_{610} was obtained. This culture was centrifuged three times (5 min at 5,000 × *g*), decanted and resuspended with MMO-medium. The culture (1.6 10^9 CFU/ml; 1.04 g VSS/l) was divided into 5 aliquots of 50 ml to which ∼680 mg OP/L was added (100 ml Erlenmeyer flasks). At day 2, 4, 7 and 9 a flask was sacrificed for OD_{550} measurements and diethyl ether extraction for intermediates. At day 9 the fifth flask was used for OP analysis.

CODs analysis

The soluble chemical oxygen demand analysis was performed on filtered medium samples (0.22 μm; Millipore). Filters with larger pore size, e.g., 0.45 μm, could not be used because the *Sphingomonas* sp. strain was only retained by 0.22 μm filters. The CODs was analyzed using the Kuvettentest LCK 414 (Dr Lange, Berlin, Germany). Oxidizable compounds react with a sulfuric acid potassium dichromate solution in the presence of silver sulfate as catalyst. The interference of chloride is avoided by the presence of mercury sulfate. The yellow colour of Cr^{6+} is measured using a Multi Diode Array Photometer ISiS 9000 (Dr Lange, Berlin, Germany). The accuracy range of this assay is between 5 and 60 mg O_2/l. If necessary, dilutions of the samples in MilliQ water were performed prior to analysis.

OP analysis

The methodology used for OP analysis was analogous to the one for NP analysis of which an extensive description was given by Tanghe et al. (1998). OP was extracted from samples by an exhaustive steam distillation-extraction technique with cyclohexane as the extraction solvent. The steam distillation method had a detection limit of ∼1 μg of OP per liter. The relative standard deviation on duplicate analysis was smaller than 20%, and the percent recovery on laboratory spiked samples was always more than 80%. The amount of OP present in the collected extracts was determined by normal phase isocratic elution high-performance liquid chromatography (HPLC).

Extraction and determination of degradation products

To 5 ml of culture medium, 1 ml of 1/1 sulfuric acid-water (95 to 96%; VEL), ∼0.8 g of NaCl (VEL), and 2 ml of diethyl ether (VEL) were added. This mixture was vortexed for 15 min and centrifuged for 5 min at 3,000 × *g*. About 1.5 ml of the diethyl ether layer was transferred to a test tube and dried with ∼0.5g of anhydrous sodium sulfate (VEL). These diethyl ether extracts were analyzed with gas chromatography-mass spectrometry (GC-MS) to screen for degradation intermediates of OP. A Varian STAR 3400 capillary gas chromatograph (Varian Associates, Walnut Creek, Calif.), equipped with a J & W Scientific (Folsom, Calif.) (30 m to 0.25 mm) fused silica capillary column (DB-5MS) was directly coupled to an ion-trap detector. Helium N60 was used as a carrier gas with a linear flow velocity of 30 cm/s. Injections were performed with a Varian split-splitless capillary injector equipped with a straight tubular glass insert. A volume of 1 μl was injected in the injector in the split-splitless mode. The splitter was opened 30 s after injection in a split ratio of 40:1. The initial temperature of 50 °C was maintained for 1 min. The temperature was increased with a gradient of 5 °C/min up to 220 °C and consequently up to 280 °C at a rate of 50 °C/min. This maximal temperature was maintained for 1 min. A Varian Saturn type II mass spectrometer was used with a manifold temperature kept at 220 °C. The filament current was 40 μA. The temperatures of the injector and transfer line were kept at 270 and 285 °C, respectively.

Results

OP degradative capacities of the Sphingomonas sp. strain with or without sodium acetate

To impose a stress on the OP degrading capacities of the *Sphingomonas* sp. culture, an easily assimilable source of carbon was added together with OP to the culture medium. Supplementation of an easily assimilable source of carbon (i.e., sodium acetate) to the culture could result in a more prominent accumulation of intermediates of a ‘difficult’ assimilable source of carbon (i.e., OP). In the first series, sodium acetate was added at the beginning of the incubation. In

the second series, this was done halfway the incubation period. OP and sodium acetate were dosed at ∼680 mg/l (∼2 g COD/l) and 2 g COD/l, respectively. During the course of the incubation, OP concentration, CODs concentration, pH and bacterial growth were measured. The diethyl ether extracts were screened for degradation intermediates using GC-MS. In the first series, the removal of OP and CODs followed the same pattern and both coincided with an increase in bacterial growth (Figure 2). After 19 days of incubation, 94% of the added OP was degraded and the CODs concentration leveled off at about 330 to 340 mg O_2/l. In the second series, the OP concentration leveled off at about 220 mg/l at day 9, but decreased further after the addition of sodium acetate resulting in an overall OP removal of 99.5% at day 19. The increase in bacterial growth was influenced by the addition of sodium acetate. At the end of the incubation periods the purity of the *Sphingomonas* sp. strain was verified (LB-agar plating) and proved to have remained axenic.

In both series, the pH of the cultures followed the same pattern (in the range of 6.8 to 9.1; data not shown) as the growth curve, with the exception that the pH reached a plateau value of about 9 from the moment the culture density reached its maximum value of about 1 (OD_{550}) (Figure 2). The pH effect on OP can be considered to be negligible since the pK_{acid} of NP, which has got almost the same physico-chemical characteristics as OP, is in the range of 10.7 (Maguire 1999).

Diethyl ether extracts of aliquots taken from the *Sphingomonas* sp. culture revealed the presence of an intermediate whose concentration profile is shown in Figure 2. The extraction procedure used was evaluated by extraction of water samples spiked with free fatty acids (acetic acid, propionic acid, butyric acid, isobutyric acid, and isocaproic acid [VEL]) and alcohols (*n*-butanol, *n*-pentanol, and *n*-octanol; Sigma-Aldrich S.A., Bornem, Belgium). The levels of recovery for both types of compounds were satisfactory with an extraction efficiency ranging from 70% to 85% depending on the compound, from which it can be deduced that, in case the culture medium contains structurally similar compounds, they will be extracted by this method. The compound was identified as 2,4,4-trimethyl-2-pentanol which is a plausible degradation intermediate of OP. The ratio of the intensities of the peaks in the mass spectrum (Figure 3) corresponded with the identification pattern of the compound. The absence of the molecular ion (M) is typical for a tertiary alcohol. The ion with m/z 115 represents the M-15 = M-CH_3 radical and the ion with m/z 59 ($C_3H_7O^+$) comes from M-71 = M-C_5H_{11} radical. The positive tertiary ion $C_4H_9^+$ gives the m/z 57 peak (fission of the pentanol at the gamma position). The correspondence of the OP consumption and the 2,4,4-trimethyl-2-pentanol appearance suggests a more or less quantitative formation and stability of the alcohol (Figure 2). However, at the end of the incubation period the intermediate seemed to decrease while the OP concentration was more or less stabilized (Figure 2).

Table 1. Evolution of the OD_{550} of the resting cell cultures of the *Sphingomonas* sp. strain in time, OP concentration after 9 days of incubation (∼680 mg OP/l at t = 0) and degradation intermediate formed (2,4,4-trimetyl-2-pentanol)

Time	OD_{550}	OP concn. (mg/l)	Intermediate (peak surface GC/MS)
2 d	1.48	nd	1,998,323
4 d	1.00	nd	17,238,064
7 d	0.89	nd	25,820,731
9 d	0.80	246.5	18,601,277

nd: not determined.

Removal of OP in a resting cell culture of the Sphingomonas sp. strain

In order to induce the formation and/or accumulation of degradation intermediates, resting cell cultures of the *Sphingomonas* sp. strain were incubated with OP. The results of the optical density measurements, the OP concentration at the end of the 9 days incubation period and the intermediates are listed in Table 1. The OD_{550} decreased with time and the amount of OP left after 9 days was in the same range as for the other experiments described above (∼64% degraded). The same metabolic intermediate, i.e., 2,4,4-trimethyl-2-pentanol, was observed in the culture medium and its concentration increased during the incubation run, while at the end a small drop in intermediate concentration could be observed.

Discussion

With the isolation of the *Sphingomonas* sp. strain and the preliminary study of its growth on NP related compounds, it was shown for the first time that an axenic bacterial strain was able to grow on OP as the sole source of carbon and energy (Tanghe et al. 1999b). In

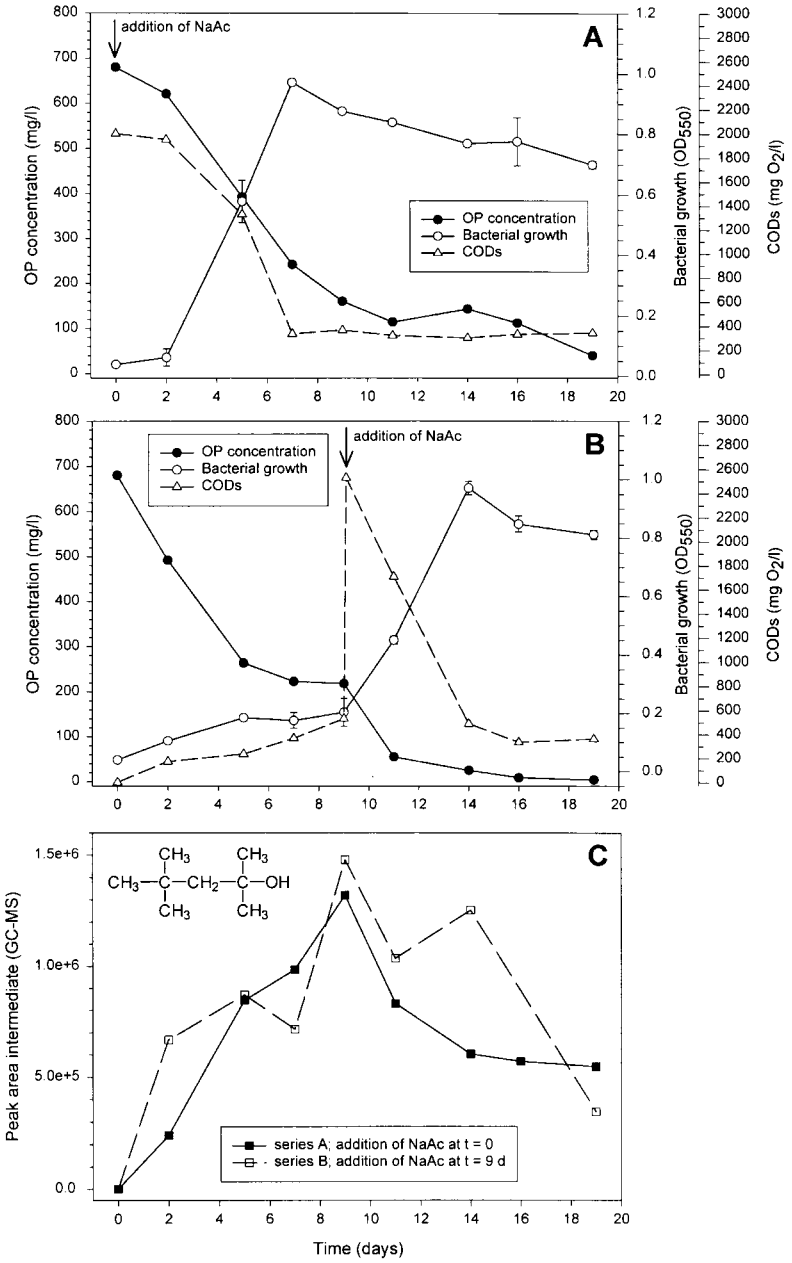

Figure 2. Utilization of OP as a growth substrate for *Sphingomonas* sp. strain together with the addition of sodium acetate (NaAc) (2 g COD/l) at the beginning of the incubation run (**A**), and, halfway the incubation run (**B**). Evolution of the most prominent metabolic intermediate of OP (**C**). The amount of intermediate 2,4,4-trimethyl-2-pentanol is expressed as surface units derived from the peaks in the GC chromatograms. Growth was assessed by OD_{550} (values represent the mean ± standard deviation (n = 2)). OP concentrations were determined by HPLC analysis of steam distillation extracts of the entire culture medium. CODs: water soluble chemical oxygen demand.

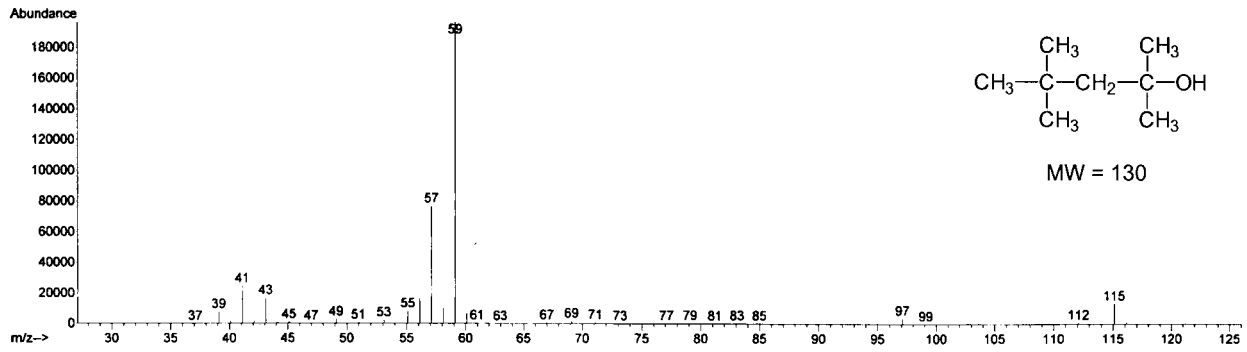

Figure 3. Electron impact mass spectrum of the branched alkyl metabolite detected in the culture medium of a *Sphingomonas* sp. strain incubated with OP as the sole source of carbon and energy. The metabolite was identified as 2,4,4-trimethyl-2-pentanol after comparison with the electron mass spectrum of synthesized 2,4,4-trimethyl-2-pentanol reported by Fujita and Reinhard (1997) (m/z: mass/charge).

the present study the OP degradation and the possible formation of degradation intermediates was further examined.

It has already been shown that the degradation of NP starts with a fission of the phenol ring leaving intermediates of branched alkyl chains with different lengths (Tanghe et al. 1999b). Thus, a similar scenario could be expected for the degradation of OP, but a complete mineralization of the OP molecule induced by the *Spinghomonas* sp. strain, including the branched octyl chain, would be rather unexpected. Indeed ω- or β-oxidation of an alkyl chain only occurs when it is not highly branched (Osburn & Benedict 1966) or when dealing with a linear alkyl chain (Corti et al. 1995). On the other hand, it has been suggested by van Ginkel & Kroon (1993) that the degradation of NP probably proceeds through the breakdown of the alkyl chain, because nonylbenzene, a structurally related compound, is attacked via ω- or β-oxidation of the alkyl chain (Sariaslani et al. 1974). In a study on the ultimate fate of the branched alkyl side chain of APnEOs Di Corcia et al. (1998) showed the presence of relevant amounts of degradation products having both side chains oxidized (alkyl chain and ethoxy chain). According to the latter authors, these species were presumably generated from less extensively branched APnEOs by various oxidative biotransformation mechanisms. All these observations give rise to a panoply of degradation scenarios for OP with their respective intermediates.

The addition of an easily assimilable carbon source, i.e., sodium acetate, to the culture medium of the *Sphingomonas* sp. strain did not slow down the degradation or biotransformation of OP. This is clearly indicated in the experimental series where sodium acetate was added halfway the degradation period. The OP disappearance rate increased again after addition of sodium acetate. The concomitant increase in bacterial density suggests that the increasing level of biomass was responsible for an accelerated OP removal. Yet, comparison of both series (Figure 2) during the first 9 days of incubation does not corroborate these observations, suggesting that solubilization of OP (water solubility: 12.6 mg/l @ 20.5 °C) might be the factor limiting its degradation. Nevertheless, an easy assimilable carbon source did not slow down OP degradation, whether or not dealing with an increased level of biomass due to the addition of sodium acetate ($OD_{550} \approx 0.23$ without, and, $OD_{550} \approx 1.00$ with addition of sodium acetate). Apparently, the *Sphingomonas* sp. strain does not show any diauxic metabolic behaviour when incubated with OP and sodium acetate as the sources of carbon and energy.

It is plausible that higher levels of biomass were responsible for the presence of more relevant concentrations of biotransformation intermediates of OP (Table 1). However, in the *Sphingomonas* sp. resting cell culture the OP degradation proceeded analoguous to the other cultures (Table 1, Figure 2). This observation suggests again that solubilization of the added amount of OP was the limiting factor in its degradation but that the cell age plays a role in the amount of intermediate remaining in the medium. It has already been suggested in literature that dissolution rates of water-insoluble organic compounds (e.g., biphenyls, naphthalene, phenanthrene) can be one of the factors that govern the rates of biodegradation (Bouchez et al. 1995; Stucki & Alexander 1987; Thomas et al. 1986). It is possible that the bacterium utilized dissolved OP and did not obtain OP directly at the surface of the solid crystals suspended in the growth medium. This would imply that once the soluble substrate was depleted the mineralization rate would be limited by the dissolution rate.

The most prominent intermediate that was detected in the *Sphingomonas* sp. culture with the addition of sodium acetate or in the resting cell culture was 2,4,4-trimetyl-2-pentanol. Further work, to confirm the identity of this intermediate by means of NMR or IR is warranted. Yet, the current evidence implies that the alkyl side chain may remain intact as a tertiary alcohol after fission of the aromatic ring of the parent compound. In octylphenol monocarboxylate metabolizing cultures, the metabolite 2,4,4-trimetyl-2-pentanol has been reported to be formed (Fujita & Reinhard 1997). The latter authors identified the compound, following synthesis of the standard of which the retention time and spectrum matched the ones of the unknown. The same compound was observed in our study, as confirmed by the identical mass spectrum reported by Fujita & Reinhard (1997) (no standard product was available in our study). This indicates that branched alkyl chains are not easily mineralized. Apparently the microbial transformation capabilities are somehow limited or hindered. The possibility that the metabolic intermediate 2,4,4-trimetyl-2-pentanol is not completely resistant towards biodegradation is indicated by the fact that at the end of the degradation periods the amount of intermediate tended to decrease (Table 1, Figure 2). A plausible biodegradation pathway of OP is that the *Sphingomonas* sp. strain utilizes all of the carbon of the phenolic ring, includ-

ing the ring point attachment. The latter has also been proposed by Fujita & Reinhard (1997) for the degradation of 4-*tert*-octylphenoxyacetic acid in groundwater enrichment cultures.

The biotransformation of the OP molecule, i.e., degradation of the phenolic moiety, is of utmost importance in relation to its estrogenic potency. Results of Tabira et al. (1999) indicated that the binding of para-alkylphenols to the estrogen receptor is due to the effect of covalent binding of two constituents of the phenol and alkyl groups, which correspond to the A-ring and hydrophobic moiety of the steroid structure, respectively. Thus, through the biodegradative action of the *Sphingomonas* sp. strain the estrogenic potency of the OP should be attenuated.

Monitoring the soluble chemical oxygen demand (CODs) present in the *Sphingomonas* sp. culture medium showed that OP contributed to only a minor fraction of the CODs. Indeed, the latter value does not include the OP because of the low water solubility of OP (12.6 mg/L at 20.5 °C) which makes that the majority of the OP particles is retained on the 0.22 μm pore sized filter. In the series where sodium acetate is added halfway the incubation period, the CODs gradually increased during the first 9 days of incubation. This is probably due to the accumulation of OP degradation intermediates as indicated by the most prominent and identified intermediate 2,4,4-trimetyl-2-pentanol. In both series, with addition of sodium acetate in the beginning respectively halfway the incubation period, the CODs concentration leveled of at about 340 to 360 mg/l. At that time sodium acetate was probably completely converted and OP intermediates and/or cellular excretion products were the only contributors to the remaining CODs level. Together with the decrease observed in the level of the detected OP intermediate, it can be suggested that also the OP degradation intermediates are at least in part susceptible towards further biodegradation. To identify the lower molecular weight intermediates, a derivatization procedure is probably necessary for an adequate detection on the GC configuration used. Further work along this line is warranted in order to make up a mass balance of the biodegradation of octylphenol.

Conclusions

OP could be degraded by a culture of a *Sphingomonas* sp. strain enriched and isolated on a structural analogue NP. In the culture media the metabolic intermediate 2,4,4-trimetyl-2-pentanol could be detected and identified. The results suggest that oxidized branched alkyl chains are rather resistant to biodegradation although further metabolism might be plausible as indicated by the CODs, OP and degradation intermediate measurements. Through this action of biotransformation, the estrogenic activity of OP is most probably decreased due to the ring fission because the phenolic moiety interacts with the estrogen receptor. To the best of our knowledge, this is the first time that the degradation of OP and the formation of a metabolic intermediate in an axenic bacterial culture have been demonstrated.

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