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Degradation of the radioactive and non-labelled branched 4(3′,5′-dimethyl 3′-heptyl)-phenol nonylphenol isomer by *Sphingomonas* TTNP3

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Abstract

The degradation of the 4(3′,5′-dimethyl-3′-heptyl)-phenol (*p*353NP) nonylphenol isomer in cultures of *Sphingomonas* TTNP3 supplemented with the technical mixture of nonylphenol was first assessed. Then the radioactive and non-labelled form of these diastereomers were both synthesised. The radioactive isomers were synthesised using [ring-U-^{14}C]-labelled phenol and 3,5-dimethyl-3-heptanol by Friedel and Crafts alkylation. The time-course of degradation was performed with and without ^{14}C-*p*353NP; balancing of radioactivity was calculated from different soluble fractions (organic, aqueous), bacterial biomass, and $^{14}CO_2$ evolved as mineralization product. The noticeable portion of ^{14}C bound to biomass showed that at least the aromatic ring of ^{14}C-*p*353NP was degraded and served as energy source and probably as carbon source for bacterial growth. In addition, the appearance of 3,5-dimethyl-3-heptanol, the nonanol corresponding with the side-chain of *p*353NP, was demonstrated in the bacterial media, and its concentration determined during the course of fermentation. Besides the parent ^{14}C-*p*353NP, no other radioactive compounds, i.e. metabolites of ^{14}C-*p*353NP were detected in the media.

Abbreviations: 353NOH – 3,5-dimethyl-3-heptanol (nonanol); LSC – liquid scintillation counting; NP – nonylphenol; *o*NP – *ortho*-nonylphenol; OP – octylphenol; *p*353NP – 4(3′,5′-dimethyl-3′-heptyl)-phenol (nonylphenol); *p*NP – *para*-nonylphenol, *S.* = *Sphingomonas*; t-NP – technical nonylphenol

Introduction

Nonylphenol (NP) is an intensively studied xenobiotic, which has been reported to be involved in undesired endocrine disruption (Soto et al. 1991). In the environment, NP is mainly found as highly persistent microbial degradation product of the world-wide and extensively used nonylphenol polyethoxylates (NP_nEO). High NP-concentration levels are strongly correlated to industrial activity, i.e. downstream their corresponding sewage treatment plants (Giger et al. 1987; Tanghe et al. 1998a, 1999a). In aqueous environments, a rapid diminution of NP concentrations is observed in stream, static water as well as in seawater because of dilution with water flow, surface evaporation, co-distillation and biodegradation. However, as a consequence of its considerable lipophilicity (log K_{OW} = 4.48), NP dissipation in aquatic environments is mainly due to sorption of the xenobiotic to sediments, where it may subsequently accumulate in aquatic fauna and flora or undergo biodegradation (Sundaram & Szeto 1981; Liber et al. 1999; Ekelund et al. 1993).

The *para*-nonylphenol portion of the technical NP (t-NP) product consists of a complex mixture. Commonly, technical NP is synthesised by Friedel and Crafts alkylation of phenol with nonenes resulting predominantly in *para*-substituted derivatives besides

ortho- and di-substituted NP. Commercial nonenes used for alkylation consist of a mixture of propylene trimers and thus, give rise to a number of *p*-NP isomers differing with regard to branching of the alkyl side-chain of *p*-NP. Up to now, 22 isomers could be resolved (Wheeler et al. 1997). Despite the presence of branched alkyl side-chain in NP, which according to van Ginkel (1996) prevents ω- and β-oxidation, NP degradation by microbial consortia was reported in sediments, in soils as well as in sewage treatment plants (Ekelund et al. 1993; Fujii et al. 2000a; Topp & Starratt 2000). The studies suggest that NP degradation by microbial consortia is not only a regional feature of some environments (Topp & Starratt 2000), but rather an ubiquitous potential characteristic of complex microbial communities. Data on the biodegradation of NP in anaerobic environments were not reported; studies with methanogenic treatment revealed that alkylphenols were not degraded under these conditions. Thus, oxygen appears to be a critical parameter for biodegradation of NP. Furthermore, anoxic periods decrease NP elimination in activated sludge, and NP concentrations increase with depth in anaerobic sedimentation layers (Razo-Flores et al. 1996; Tanghe et al. 1998b; Espadaler et al. 1997). Under aerobic conditions, addition of homogenised or non-homogenised sewage sludge containing NP with linear side-chain to soil resulted in a more rapid mineralization of NP, if the way of application was favourable for an increased oxygen diffusion into the sludge particles (Hesselsoe et al. 2001). Among other parameters governing NP-degradation by micro-flora in activated sludge, low temperatures and humic compounds, which may influence bio-availability of dissolved NP, could also have limiting effects. Additionally, adaptation of sludge biocoenosis to NP, bio-augmentation by periodically repeated *inocula* of effluent, as well as higher distribution of NP into sludge improved its biodegradation (Tanghe et al. 1998a, b).

For a long time, it was supposed that a complex micro-flora is a prerequisite for NP-degradation with branched side-chains; only single strains possessing the capacity to degrade NP with linear side-chain, such as *Candida aquaetextoris*, could be isolated (van Ginkel 1996; Vallini et al. 1997). More recently however, two cases of axenic bacterial cultures growing on NP with branched side-chains as sole carbon source have been published. Both strains belong to the gram-negative aerobic *Sphingomonas* genus, also known for the biodegradation of other persistent chemicals (Rieger et al. 2002; Harayama 1997; White et al. 1996). First, Tanghe et al. (1999b) could enrich and isolate a novel *Sphingomonas* strain designated as *S.* TTNP3 from activated sludge. The authors demonstrated preferential degradation of *p*-NP isomers as compared to *o*-NP, and concluded that degradation of *p*-NP started with fission of the aromatic ring, since no intermediates with aromatic moiety could be detected (Tanghe et al. 1999b). Later, Fujii and collaborators characterised the micro-flora profile of waste water samples, in which NP-biodegrading activity had been previously detected. The micro-flora consisted mainly of *Pseudomonas* and *Sphingomonas* bacteria, but only one *Sphingomonas* strain was responsible for NP-degrading activity. The *S.* strain was further examined in a bioreactor in chitosan-encapsulated form; the immobilised bacteria were found to degrade NP as efficiently as the free suspended cells, and could be used several times (Fujii et al. 2000a, b). The isolated micro-organism has been characterised as new *Sphingomonas* species and classified as *S. cloacae* (Fujii et al. 2001). Since optical density or total biomass increased during incubation on minimal medium with a concomitant NP degradation, Tanghe et al. (1999b) and Fujii et al. (2000a, b) assumed that the respective *Sphingomonas* strain utilised NP as sole carbon and energy source. Other similarities were found in NP-degradation pathways, since the corresponding alcohols of the alkyl side-chains of NP appeared to result in dead-end metabolites (Tanghe et al. 2000; Fuji et al. 2000b).

However, these studies were impaired by the fact that *p*-NP is a complex mixture of isomers which gives rise to a complex metabolic pattern. To overcome this difficulty, Tanghe et al. (2000) investigated the biodegradation of octylphenol, a compound closely related to nonylphenol. The use of octylphenol (OP) is advantageous here, because it consists of just one isomer. However, since no propyl- and hexylphenol-degrading activity was detected with *S. cloacae* (Fujii et al. 2000a), it could not be excluded that the degradation of OP by *S.* TTNP3 does not perfectly reflect the degradation of NP. Due to the unavailability of 2,4,4-trimethyl-2-pentanol as authentic reference compound, quantification of this end-metabolite in the assays was impossible. Moreover, they emphasised that a deeper insight into OP and e.g. NP metabolism requires the implementation of mass balance determination (Tanghe et al. 2000).

Recently, an easy method for the chemical synthesis of single NP isomers with branched side- chains has been developed; according to this procedure,

phenol and commercially available defined nonanols were reacted by Friedel-Crafts alkylation (Vinken et al. 2002). Among the isomers synthesised, 4(3′,5′-dimethyl-3′-heptyl)- phenol (*p*353NP), which belongs to group 2 of the *p*-NP isomers (i.e. α-methyl or α-dimethyl substituted with primary carbon in β-position) according to the classification of Wheeler et al. (1997), has been chosen for the present studies. In brief, the present paper reports the degradation of the technical mixture of NP, and the biodegradation of both the radio-labelled and non-labelled NP isomer *p*353NP by *Sphingomonas* TTNP3 isolated by Tanghe et al. (1999b).

Material and methods

Synthesis of 4(3′,5′-dimethyl-3′-heptyl)-phenol (p353NP)

The *p*353NP isomer was synthesised by Friedel-Crafts alkylation from phenol and 3,5-dimethyl-3-heptanol (nonanol), and consisted of 2 diastereomers. Synthesis of [ring-U-^{1}C]-*p*353NP having a specific activity of 298 MBq/mmol and a radiochemical purity of 96.9% was described elsewhere (Vinken et al. 2002).

Additionally, large-scale synthesis of non-labelled *p*353NP was carried out. Experimentally, 1.88 g of phenol, 4.33 g of 3,5-dimethyl-3-heptanol, 35 mL of BF_3-ether complex and 200 mL of petroleum ether were placed in a 500 mL two-necked flask equipped with a reflux condenser and a drying tube filled with $CaCl_2$. The reaction was allowed to run for 15 min at 50 °C with stirring, and then stopped through the addition of 200 mL of water. After intensive stirring for further 15 min, the aqueous phase was removed and the organic phase was washed 5 times with water in order to remove non-reacted phenol, and then dried over Na_2SO_4. After removing the petrolether under vacuum, the product yield was 2.85 g with a purity of 98.0% (GC).

Media and culture

Inocula preparation

Sphingomonas TTNP3 strain was a generous gift from Prof. Verstraete (LabMet, Ghent, Belgium). Precultures were prepared by seeding (0.1%) 100 mL of sterilised standard I medium (Merck, Germany) contained in 500 mL Erlenmeyer flasks with a frozen 20% glycerol stock solution of *S.* TTNP3. The bacteria were incubated for 50 hours on shaker at 28 °C and OD 550 nm was ca. 6.4 (late growth phase).

Preparation of cultures for degradation studies and incubation

For biodegradation experiments, the bacteria were cultivated on MMO synthetic medium (Stanier et al. 1966) with NP added as sole carbon source. Due to the low water solubility of NP, a series of culture vessels had to be set up. Measuring the NP amount at a certain day consisted in working up a whole vessel. To apply the same NP amount of NP (1 g/L) to these vials, it had to be administered dissolved in an organic solvent, which was then evaporated. After this MMO medium was added to the flasks. For radioactive experiments, 83.32 KBq of [ring-U-^{14}C]-*p*353NP and non-labelled *p*353NP were applied to provide the same final concentration. Technical grade NP (t-NP) (Fluka), was prepared as stock solution (100 mg/mL) in CH_2Cl_2. Radio-labelled *p*353NP was dissolved in methanol, whereas non-labelled *p*353NP was prepared at 50 mg/mL in petrolether. Non-radioactive experiments (*p*353NP and t-NP) were performed in 100 mL flasks with 10 mL of MMO (filter sterilised), whereas volumes of radioactive assays were respectively 500 and 50 mL. Moreover, a glass funnel filled with polyurethane foam plugs, glass wool and soda lime pellets was mounted on each flask containing radio-labelled NP in order to trap evaporated NP and $^{14}CO_2$ separately. In order to avoid losses of $^{14}CO_2$, glass funnels were replaced with new ones after 2.5 days.

The biodegradation experiments were started by seeding the culture with 1% (v/v) of the preculture, and flasks were incubated in the dark at 28 °C on a shaker (70 rpm). For each experiment, control flasks without bacteria were prepared similarly, and incubated throughout the entire cultivation interval.

Extraction procedures and sample preparation

Due to NP's high lipophilicity and resulting heterogeneity of the assays, the complete contents of one culture flask was used for each determination of the NP concentration. Prior to extraction, cultures were spiked adequately (100–1000 μg) with 4*n*-NP (linear nonyl chain) dissolved in CH_2Cl_2 (1 mg/mL). By vigorous shaking, culture moulds were extracted successively 3 times with 2 volumes of ethyl acetate. Collected ethyl acetate phases were dried over Na_2SO_4, filtrated (filter paper), and were concentrated

at 190 mbar and 40 °C. Remaining residues were dissolved in 2 and 4 mL ethyl acetate for non-radioactive and radio-labelled experiments, respectively. Occasionally, samples were diluted adequately before final analysis.

After extraction, the remaining aqueous phase of the radioactive biodegradation assays was filtered through pre-weighed 0.2 μm nylon membranes. Filters were dried overnight at 105 °C before being weighed again in order to determine the dry weight. It should be noted that this methodology only allows an approximate measurement of dry-weight since some lipophilic components of *S.* may have been extracted by this procedure.

The membranes were packed in cellulose and combusted in a biological oxidizer OX500 (RJ Harvey Instrument Corporation). Resulting $^{14}CO_2$ was trapped in Carbomax Plus LSC cocktail (Lumac*LSC BV, The Netherlands), and was subjected to liquid scintillation counting.

After filtration, the aqueous phase was frozen and lyophilised. Dry extracts were then resuspended in 4 mL of water and 1 mL acetonitrile and subsequently, filtrated over 0.22 μm (Millipore) filter for further LSC and HPLC analysis.

For HPLC analysis of ethyl acetate extracts, 2 mL of the extracts were gently concentrated under an N_2 stream and re-dissolved in 2 mL methanol. Then, methanolic samples were filtrated (0.22 μm) and adequately diluted prior to HPLC analysis.

Volatile organic compounds and $^{14}CO_2$

Glass fibre and polyurethane foam plugs were removed from glass funnels and were then, submerged in flasks with defined volumes of ethyl acetate. Flasks were stored overnight. Subsequently, ^{14}C contained in these fractions was determined.

Collected soda lime pellets were transferred to a three-necked glass flask connected to a three-step adsorption device (Wendel-apparatus). The $^{14}CO_2$ trapped was released by addition of 25% HCl solution, and flushed by means of an N_2 stream into the adsorption device containing Carbomax Plus LSC cocktail. Radioactivity trapped in the cocktail was then measured by LSC.

LSC measurements

Unless stated otherwise, aliquots of different fractions were added to 10 mL of a Lumasafe scintillation cocktail (Lumac*LSC BV, The Netherlands) for scintillation counting (LSC). Vials were analysed by means of a Beckman LS 5000TD liquid scintillation counter.

Optical density

For non-radioactive biodegradation experiments, optical density was measured at 550 nm in a Beckman DU 7200 spectrophotometer. Optical density was measured individually with three flasks from which the content was in advance filled in a glass vial. From these glass vials, two aliquots were taken and used to measure OD. A mean value was calculated from the two aliquots measurements. Then, mean value and standard deviation was calculated for the three flasks. With taking aliquots, attention was paid to avoid withdraw NP droplets. Blanks were prepared accordingly from a flask containing MMO complemented with NP.

HPLC analysis

Samples derived from aqueous phases
An 100 μL aliquot of the sample (dissolved in acetonitril-water) was injected into a Beckman HPLC-System equipped with a System Gold Programmable Solvent Module 126, a 171 radioisotope detector and a System Gold DAD module 168. UV measurements were performed at 254 nm and 280 nm. The column was a 250/4 Nucleosil 100-5 C18HD (Macherey Nagel), while the mobile phase consisted of water (A) and acetonitrile (B), both acidified with 0.1% acetic acid (flow rate: 1 mL/min). Samples were separated using a gradient program as follows: 20% B in A for 5 min, linear gradient to 100% B during 45 min, and 100% B isocratic for 5 min. Finally, the system returned to its initial conditions (20% B in A) within 5 min, and was kept in this composition for 5 min before the next run was started.

Samples derived from organic extracts
Analysis of samples obtained from ethyl acetate extracts (dissolved in methanol) was performed by means of a HPLC Series 1100 (Hewlett Packard) connected to a Raytest Ramona 2000 radiodetector. UV detection was executed at 220 nm, 254 nm and 280 nm. The chromatographic column was a Zorbax Eclipse XDB Phenyl 4.6 $*$ 150 mm $*$ 5 μm (Agilent, Germany). Aliquots of 100 μL were injected and separated at a flow rate of 1 mL/min. Eluents were as described before. The gradient program was 20% B in A for 2 min, then linear gradient to 100% B during 15 min, and isocratic 100% B for 5 min. Finally, return to 20% B in A was performed within 15 min.

GC analysis

The GC-FID analyses were carried out using an HP 6890 gas chromatograph equipped with a 30 m $*$ 0.32 mm $*$ 0.25 μm phenyl methyl siloxane column (HP-5). The temperature program was 50 °C for 5 min and then 10 °C/min to 280 °C. The injector temperature was 250 °C, the interface temperature 280 °C. Injection volume was 1 μL in splitless mode; carrier gas was N_2 at a nominal flow of 2 mL/min.

For high-resolution GC, a 100 m $*$ 0.25 mm column coated with 0.5 μm 100% dimethylpolysiloxane was used (Restek). The oven temperature program was: initial temperature 100 °C, with 5 °C/min to 200 °C. Injector and detector temperatures were 280 °C and injection volume was 1 μL in splitless mode.

Equipment and method of GC-MS analysis (scan mode) for samples of the ethyl acetate extracts were as described by Vinken et al. (2002).

Results and discussion

Biodegradation of t-NP

Prior to the degradation experiments, t-NP recoveries were verified for the entire procedure including extraction and GC analysis. Recoveries of t-NP in MMO were 95.8% $\pm$ 3.9 (standard deviation for n = 3). Recoveries were also tested in presence of 6 g/L (dry weight) of non-growing bacteria (93.8% $\pm$ 5.2; standard deviation for n = 3). An interval of 72 h for incubation of t-NP with non-growing bacteria was achieved in order to allow for sufficient possible adsorption of t-NP by the bacteria. Furthermore, vessels were gently agitated twice a day in order to maximize adsorption. These findings were considered as sufficient for the subsequent biodegradation studies.

In order to provide a basis for the detection of potential metabolites and to estimate the biodegradability of the *p*353NP diastereomers contained in the isomer mixture, cultures were at first grown on t-NP (Figure 1). During the course of incubation, media were examined by GC. Peak areas corresponding with *p*353NP were determined, and showed that the concentration of the isomer decreased. While in abiotic assays a recovery of 105 $\pm$ 6% of applied t-NP was determined, analysis of extracts of assays inoculated with bacteria showed a rapid decrease of the t-NP concentration. More than 50% of the t-NP added to the

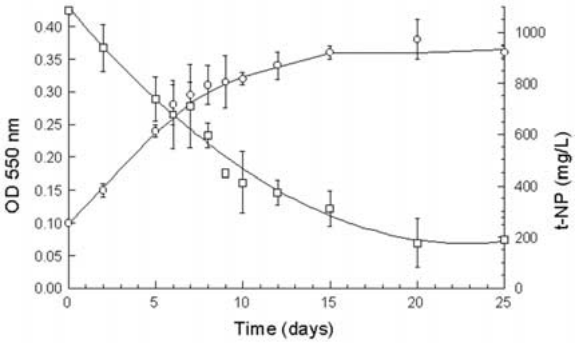

Figure 1. Growth of *S.* TTNP3 on technical nonylphenol (t-NP). OD 550 nm —○—; t-NP concentration —□—. Measurements were performed in three different vessels and error bars represent the standard deviation from these three independent analyses.

cultures were metabolised within 8 days. Concentrations of t-NP then stabilised at ca. 180 mg/L after 20 days of culture. Concomitant to the diminution of t-NP concentration, an increase of the optical density to a maximum of 0.37 after 13–15 days was observed. Thereafter, bacterial growth appeared to slow down stopping after 15 days, when the t-NP concentration had reached its minimal level.

GC-FID analysis of the ethyl acetate extracts revealed a marked diminution of the concentration of the *p*-NP isomers, whereas the concentrations of the *o*-NP isomers seemed to remain constant. In the extracts sampled after 2 days of cultivation, peaks with retention times smaller than those of the *o*-NP and *p*-NP isomers could be detected, and they were presumed originate from the corresponding nonanols of the alkyl-isomers of t-NP. Besides these, no other metabolites could be detected even after derivatisation (trimethylsilylation) with N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) of samples from the organic extracts.

In order to evaluate the degradation pattern of the different isomers and to assess, more particularly, whether *p*353NP was present in the technical mixture and degraded by the bacteria, a t-NP standard solution spiked with *p*353NP, samples derived from the initial as well as final phase of cultivation on t-NP were chromatographed on a 100 m capillary column. The latter renders possible an improved resolution of the different NP isomers. Under the chromatographic conditions utilized, no remarkable differences were detected among the t-NP isomers concerning their consumption by the bacteria (Figure 2).

However, the chromatograms provided first proofs of the presence of the *p*353NP isomer in the t-NP mix-

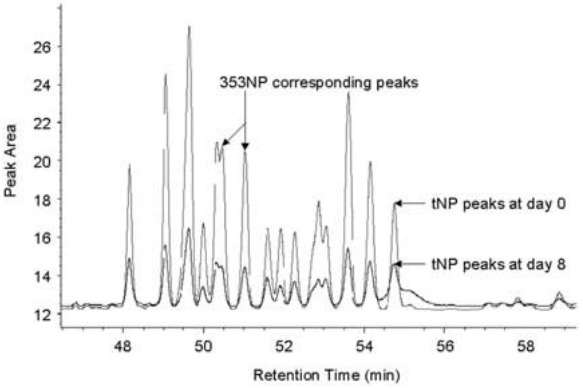

Figure 2. GC-FID gas chromatogram derived after chromatography of tNP on a 100 m column.

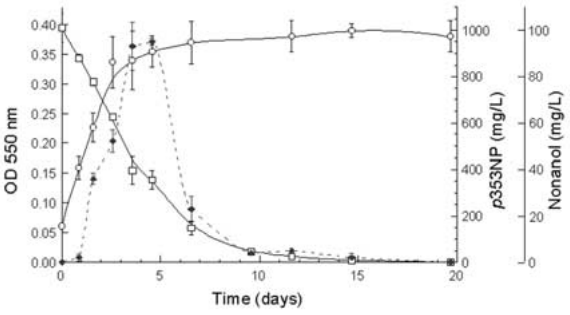

Figure 3. Cultivation of *S.* TTNP3 on non-labelled 4(3′,5′-dimethyl-3′-heptyl)-phenol (*p*353NP). OD 550 nm —○—; *p*353NP —□—; 3,5-dimethyl-3-heptanol (353NOH) --◇--. Measurements were performed in three different vessels and error bars represent the standard deviation from these three independent analyses.

ture, and also of its degradability. This finding was especially crucial for the present study, since *p*353NP was regarded as relevant model isomer of t-NP.

Biodegradation of non labelled p353NP

Whereas 98 ± 1% of applied *p*353NP were recovered after 20 days of incubation in abiotic controls, cultivation of *S.* TTNP3 on *p*353NP revealed that both diastereomers of the compound were completely metabolised after 2 weeks in similar ways (Figure 3). After 3 to 4 days of cultivation, a 50% reduction of the starting concentration of *p*353NP was detected. As already observed for t-NP, the decrease of *p*353NP concentration was correlated to an increase of the optical density of the bacterial culture. Maximal optical density was observed after about 7 days and stabilised when the *p*353NP concentration had reached its minimal level. In contrast to t-NP, *p*353NP appeared to be completely metabolised.

In the case of *p*353NP as substrate, the metabolite pattern was simplified; this result was expected. In addition to the two substrate peaks, GC-MS analysis of the organic extracts revealed the presence of a peak with a R_t = 8.28 min which was thought to originate from the corresponding alcohol of the *p*353NP nonyl chain. The alcohol was unequivocally identified (GC-MS: retention time, MS fragmentation pattern) as 3,5-dimethyl-3-heptanol (353NOH) using the authentic alcohol as reference compound. Characteristic MS fragments of 353NOH were m/z (% abundance) 57 (18.2; M^+-87 = M-C_5H_1O), 73 (100; M^+-71 = M-C_5H_{11}), 115 (16.7; M^+-29 = M–C_2H_5), and 129 (3.8; M^+-15 = M-CH_3), while the molecular ion (m/z 144) was not observed. The concentration of 353NOH determined in the course of the fermentation is displayed in Figure 3. The compound was already detected after one day of cultivation and attained a maximal concentration of 94 mg/L (5 days of cultivation). After 5 days, 353NOH concentration decreased and at the end of the cultivation interval 353NOH could not be detected. As already discussed with t-NP, in derivatised samples (MSTFA) further metabolites (GC-MS) were not found.

Biodegradation of [^{14}C]-p353NP

In order to examine more exactly the fate of *p*353NP during fermentation, cultures of *S.* TTNP3 were grown on [^{14}C]-*p*353NP; corresponding results are shown in Figure 4. A decrease of more than 50% of [^{14}C]-*p*353NP concentrations was observed after 3 days; no [^{14}C]-*p*353NP remained after 18 days of cultivation. Again, biomass (dry-weight) increase was correlated with a decrease of [^{14}C]-*p*353NP concentrations, while a maximum biomass concentration of 298 mg/L was reached after 4 days. As observed for non-labelled *p*353NP, 3,5-dimethyl-3-heptanol was detected in the organic fraction reaching its highest concentration (161 mg/L) between 4 and 9 days of cultivation before decreasing to 0 mg/L.

Complete balancing of radioactivity (organic-, aqueous phase, biomass and $^{14}CO_2$; Table 1) demonstrated that recoveries of radioactivity ranged between 89% and 95% of applied ^{14}C. In the control assays (incubation without bacteria for 18 days), recoveries were 993 mg/L of [^{14}C]-*p*353NP and 94% of the applied radioactivity. The majority of radioactivity was associated with the ethyl acetate phase (92% of the applied ^{14}C), while less than 0.5% were found evaporated. Regarding the flasks inoculated with the bacteria,

Table 1. Balancing of applied ^{14}C radioactivity in the study performed with *S.* TTNP3 on radioactive *p*353NP

Time (days)	Organic phase	Filtrate	Biomass	CO_2	Total recoveries
0	95.0 ± 7.2	0.3 ± 0.2	0	0	95.3 ± 7.4
0.5	89.5 ± 6.7	0.6 ± 0.1	1.3 ± 0.1	0.1 ± 0.1	92.6 ± 7.0
1	76.6 ± 7.4	2.3 ± 0.3	3.2 ± 0.1	6.9 ± 0.8	89.1 ± 8.6
2	60.4 ± 5.7	3.9 ± 0.2	8.4 ± 0.3	20.8 ± 3.3	93.7 ± 9.5
3	49.7 ± 3.2	3.5 ± 0.1	9.2 ± 0.8	28.1 ± 1.5	90.7 ± 5.6
4	31.4 ± 5.9	7.6 ± 0.2	14.2 ± 1.3	35.9 ± 1.9	89.3 ± 9.3
9	14.6 ± 2.8	2.3 ± 0.1	14.1 ± 1.0	60.2 ± 7.2	91.4 ± 11.1
17	5.7 ± 0.9	4.3 ± 0.3	12.2 ± 1.2	69.8 ± 4.8	92.4 ± 7.2

Values of radioactivity for the different fractions are expressed in % of the initial applied radioactivity. Errors represent standard deviation of n = 2 radioactivity measurements of the same culture.

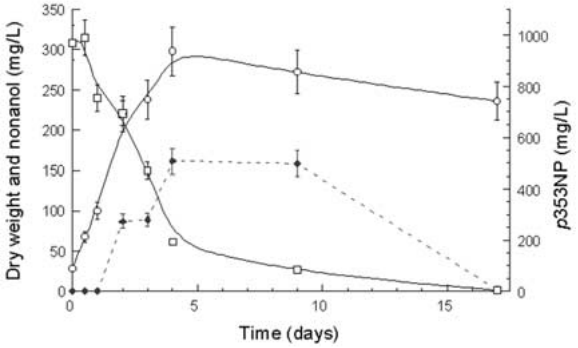

Figure 4. Cultivation of *S.* TTNP3 on radioactive *p*353NP. Dry weight —○—; *p*353NP —□—; *p*353NP 3,5-dimethyl-3-heptanol (353NOH) - -◊- -. The precision of dry weight determination was estimated to be 10%. Other error bars represent standard deviation of n = 2 measurements of the same culture.

the results first showed that the observed decrease of *p*353NP concentration (GC-MS analysis) was correlated with the radioactivity found in the ethyl acetate phase. In addition, the majority of radioactivity (in % of applied ^{14}C) contained in the organic phase at any time during incubation consisted of *p*353NP (data not shown). Thus, non-polar (radioactive) metabolites of *p*353NP did not accumulate to a detectable degree in the medium of the bacterial culture. Concomitant to the increase of biomass, portions of radioactivity associated with the biomass also increased. The only exception was the sample obtained after 12 h of incubation, where biomass increased while no corresponding radioactivity could be observed. This was probably due to remaining nutrients introduced into the incubation vessels with the preculture inoculums. This finding was regarded as evidence that *p*353NP was completely metabolised and used as carbon source for building-up of biomass. A further confirmation for the consumption of *p*353NP (as energy source) was obtained by the data concerning mineralization of *p*353NP, i.e. evolution of $^{14}CO_2$. Whereas with the control, below 1% of applied ^{14}C were found adsorbed as $^{14}CO_2$ onto the soda lime pellets, a large amount (about 70%) of the applied *p*353NP was recovered in form of radioactive CO_2 in the *S.* cultures after 17 days of incubation. A total of 14% of radioactivity was associated with biomass. Assuming that adsorption of NP to growing cells was of the same order as that to the non-growing cells (recovery experiments), adsorption would have been about 6% of applied ^{14}C. This would imply that a part of the 14% of radioactivity associated with biomass was effectively incorporated into biomass. Complementary HPLC analysis of the ethyl acetate extract (resuspended in methanol) revealed the absence of any ^{14}C-substances other than *p*353NP and di-nonylphenol (alkylated in *ortho* and *para* position). The latter was a minor by-product of the synthesis of ^{14}C-*p*353NP (about 2.5% due to GC-MS), and was assumed to be non-degradable due to the reported persistence of *o*-NP isomers. This fact partly explains that radioactivity remained in the organic phase, even after complete absence of *p*353NP (according to GC-MS). These results were confirmed by thin layer chromatography using n-hexane/diethylether (80/20, v/v) as solvent system. Subsequent ^{14}C-examination of the plates did not allow the detection of metabolites in these samples.

After 4 days of incubation, about 7% of applied radioactivity was recovered in the filtrate of the medium (remaining after ethyl acetate extraction and filtration). HPLC analysis of samples derived from aqueous phases proved that the residual radioactivity

contained in these samples was not due to the presence of *p*353NP, *o*- or dialkyl-phenol. This portion of total ^{14}C increased to significant amounts already after 24 h of culture (2.3%). The radio-HPLC chromatograms obtained from corresponding samples showed peaks with noticeably small retention times partly overlapping with the injection peak. In these cases, the radioactivity associated with this considerably polar peak accounted for more of 95% of ^{14}C found in this fraction (liquid scintillation counting).

Conclusion

Neglecting little experimental differences, such as culture conditions, strains, NP source, and analysis, the degradation rates of t-NP and the growth parameters of the bacteria determined in the present investigation were in agreement with those previously reported by Tanghe et al. (1999b) and Fujii et al. (2000b). In the present study, the NP-degrading microorganisms were able to degrade 670 mg/L t-NP within 12 days and 1000 mg/L *p*353NP within 10 days. Tanghe et al. (1999b) reported a residual accumulation of the *o*-NP isomers (ratio *p*-NP/*o*-NP 99/1 to 68/32 after 16 days of incubation) and thus, a relative enrichment of *ortho* isomers. The present work agreed with these reported data. However, due to the lack of a reference substance, concentrations of the *ortho* isomers were not determined. As observed with soil micro-organism communities (Topp & Starratt 2000) by GC-FID analysis (30 m and 100 m columns), our results suggest that the *p*-NP isomers of technical NP were similarly degraded; i.e. different degradation rates due to the chemical structure of the alkyl side-chains were nor observed. GC-MS analysis revealed the presence of nonanol isomers in the extracts. Such compounds have been reported previously (Tanghe et al. 1999; Fujii et al. 2000b), though some ambiguities were left concerning the alcohols identified by Fujii and his collaborators.

Experiments concerning t-NP biodegradation showed the *p*353NP seemed to be degraded like the other isomers of t-NP contained in the mixture. This observation was confirmed by the experiments where *p*353NP degradation alone was investigated. As suggested by the degradation of t-NP, a similar decrease of the concentration of *p*353NP added as sole carbon source was observed. The fact that both diastereomers of the *p*353NP isomer were degraded with equal rates indicates that the stereochemistry of the alkyl side-chain does not play a determinant role for the metabolism of the diastereomers by *S.* TTNP3. This observation agrees with the observation that all t-NP isomers were degraded with similar rates. Nevertheless, *p*353NP consumption rates were higher than those obtained with the t-NP mixture and additionally, the pure isomer was completely exhausted. These differences may partly be explained by the higher precision of the determination of the concentrations of *p*353NP (e.g. separated GC peaks with *p*353NP, 85% purity of t-NP versus 98% purity of *p*353NP). NP tends to be volatile from aqueous solutions (Dachs et al. 1999). However in the present study, the decrease of the *p*353NP concentration in the bacterial assays proved to be only due to its mineralization (evolution of $^{14}CO_2$). After application of radioactive *p*353NP, only 14% of the carbon atoms of the NP aromatic ring were incorporated into bacterial biomass, whereas about 70% are mineralised after 17 days of incubation. Since NP extraction with ethyl acetate prior to radioactivity measurement may lead to extraction of radioactive components incorporated in the biomass, it should be considered that at least 14% of applied radioactivity was associated to biomass. These results are corroborated by those of other investigations on the degradation of radioactive linear NP by different bacterial communities (Hesselsoe et al. 2001; Ekelund et al. 1993), with the exception of results published by Topp & Staratt (2000), who found 40% of NP mineralised and 50% bound to biomass.

In the biodegradation experiment with the *p*353NP diastereomers, a transformation product was formed only in the presence of *S.* TTNP3. The metabolite was unequivocally identified as the detached hydroxylated alkyl chain of *p*353NP, namely 3,5-dimethyl-3-heptanol. Tanghe et al. (2000) reported the formation of 2,4,4-trimethyl-2-pentanol as intermediary degradation product of OP; this metabolite has an alcohol function at the carbon which is attached to the aromatic ring in OP. Interestingly, the present findings on the emergence of 353NOH from *p*353NP metabolism by the bacteria agree with these results. This also holds for similarities concerning mass spectra. Tanghe et al. (2000) postulated that the accumulation of the alcohol in the medium possibly points to the fact that the highly-branched compound is not easily mineralised. However, at the end of incubation, 2,4,4-trimethyl-2-pentanol concentrations decrease while that of OP stabilised (Tanghe et al. 2000). In order to assess the decrease of the 353NOH concentration towards the end of cultivation, incubations of 140 mg/L 353NOH

without *S.* TTNP3 were carried out. After 3 days of incubation, less than 5% of the initially applied nonanol was detected, while 353NOH was absent from the assays after 7 days. The high volatilisation rate of 353NOH in the abiotic control lead us to assume that its disappearance might be explained by mainly volatilisation rather than metabolism. The finding of volatilisation of the alcohol from the aqueous media would agree with literature data on the presence of nonanols in gas phase and media of assays inoculated with bacteria (Fujii et al. 2000b). Thus, conclusions on the possible degradation of the very volatile 353NOH by *S.* TTNP3 was not possible. Ultimately, corresponding studies would require the use of NP ^{14}C-labelled in the alkyl side-chain.

In studies on the microbial degradation of NP_nEO, Di Corcia et al. (1998) detected the formation of large portions of compounds which were oxidised/hydroxylated on both the alkyl and ethoxylate part of the molecules (CAPEC). In the present investigation, such oxidation products could not be detected even after trimethylsilylation (for GC-MS) or LC-MS of corresponding samples (data not shown). In addition to the radiolabelled parent, only highly polar radioactivity was found in the aqueous phase of the media. The origin of this polar compound(s) remained unclear; they may have leaked out from intact or lysed cells as possibly intermediary degradation products of ^{14}C-*p*353NP.

Concerning the degradation pathway of *p*353NP, and, more generally, also those of other NP isomers, some details still remain unclear. Unless the initial reaction of the degradation of *p*353NP is the detachment of the entire alkyl side-chain as corresponding alcohol, the formation of 353NOH via degradation pathways known for a number of aromatic compounds appears quite unlikely. For complete degradation of *p*353NP, an attack of the aromatic ring as first event and a subsequent cleavage of all ring and side-chain carbon atoms (e.g. as C2 units) would imply oxidation of a tetravalent carbon in position 3 of the nonyl chain, which appears to be a considerably stable partial structure of the molecule. Based on the absence of aromatic metabolites of *p*353NP in the extracellular medium, degradation of NP is assumed to start with breakdown of the aromatic ring. However, catabolites with an aromatic moiety may be present only intracellularly, or only in minute intermediary amounts, since they not appear at a bottleneck of the catabolic reaction chain. For a further elucidation of the bacterial catabolism and fate of NP cell-free, enzymatic studies using radioactive methods are required.

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