Degradation pathways of low-ethoxylated nonylphenols by isolated bacteria using an improved method

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Abstract Nonylphenol ethoxylates (NPEOs) with low ethoxylation degree (NP_{av2EO} ; containing two ethoxy units on average) and estrogenic properties are the intermediate products of nonionic surfactant NPEOs. To better understand the environmental fate of low-ethoxylated NPEOs, phylogenetically diverse low-ethoxylated NPEO-degrading bacteria were isolated from activated sludge using gellan gum as the gelling reagent. Four isolates belonging to four genera, i.e., *Pseudomonas* sp. NP522b in γ -*Proteobacteria*, *Variovorax* sp. NP427b and *Ralstonia* sp. NP47a in β -*Proteobacteria*, and *Sphingomonas* sp. NP42a in α -*Proteobacteria* were acquired. *Ralstonia* sp. NP47a or *Sphingomonas* sp. NP42a, have not been reported for the degradation of low-ethoxylated NPEOs previously. The biotransformation pathways of these isolates were investigated. The first three strains (NP522b, NP427b, and NP47a) exhibited high NP_{av2EO} oxidation ability by oxidizing the polyethoxy (EO) chain to form low-ethoxylated nonylphenoxy carboxylates, and then further oxidizing the alkyl chain to form carboxyalkylphenol polyethoxycarboxylates. Furthermore, *Sphingomonas* sp. NP42a degraded NP_{av2EO} through a nonoxidative pathway with nonylphenol monoethoxylate as the dominant product.

Keywords Low-ethoxylated nonylphenol ethoxylates · Degrading bacteria · Biodegradation pathway · Gelling reagent

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Introduction

Nonylphenol ethoxylates (NPEOs), which consist of a polyethoxy chain (EO chain, commonly containing nine ethoxy units) and a branched alkyl chain (C9) connected to a benzene ring (Fig. 1), are a major class of nonionic surfactants used in household, industrial, and agricultural applications (Bergé et al. 2012a,b). Although the production of NPEOs has exhibited a decreasing trend in the USA, Canada, and the EU as a result of highly restrictive regulations (EU 2003; USEPA 2010), worldwide output increased from 300,000 t in 1994 to 500,000 t in 2002 (Berryman et al. 2003; Ying et al. 2002). The increase in consumption by emerging nations such as Brazil, Russia, India, and China has been considered as the main reason (Bergé et al. 2012a).

NPEOs are incompletely degraded to low-ethoxylated NPEOs (containing one to three ethoxy units), nonylphenoxy carboxylates (NPECs), nonylphenol (NP), and carboxyalkylphenol polyethoxycarboxylates (CAPECs) in wastewater treatment plants (WWTPs) and water environments (Ahel et al. 1994; Bergé et al. 2012a,b; Di Corcia et al. 2000; Gonzalez et al. 2007; He et al. 2013; Koh et al. 2008; Maguire 1999; Montgomery-Brown and Reinhard 2003; Nagarnaik et al. 2010; Xu et al. 2011). Bergé et al. (2012a) found that total concentrations of NPEO and their metabolites (mainly low-ethoxylated NPEOs and low-ethoxylated NPECs) were $0.10\text{--}1,179\text{ }\mu\text{g L}^{-1}$ (mean, $4.54\text{ }\mu\text{g L}^{-1}$) in WWTP effluent and $0.03\text{--}215\text{ }\mu\text{g L}^{-1}$ ($0.99\text{ }\mu\text{g L}^{-1}$) in surface water. Some biodegradation intermediates, such as NP and low-ethoxylated NPEOs ($\text{NP}_{1\text{--}3\text{EO}}$), exhibit estrogenic properties, and therefore, the biotransformation pathways of NPEOs have been widely studied using isolated bacteria (John and White 1998; Liu et al. 2006, 2013; Lu et al. 2008; Sato et al. 2001, 2003; Ying et al. 2002). It is generally accepted that at least two EO chain degradation pathways lead to the

Fig. 1 General structures and acronyms of NPEOs and their well-ascertained degradation intermediates

production of metabolites with shortened ethoxy chains (low-ethoxylated NPEOs) and carboxylated ethoxy chains (low-ethoxylated NPECs).

Since low-ethoxylated NPEOs exhibit estrogenic properties (Jobling et al. 1996; Routledge and Sumpter 1996), the final fates of low-ethoxylated NPEOs using isolated bacteria have also been studied. Isolates belonging to the genera *Stenotrophomonas*, *Pseudomonas*, *Ochrobactrum*, *Castellaniella*, *Variovorax*, and *Psychrobacter* degrade NP_{av1.5}EOs (containing 1.5 ethoxy units on average) and NP_{av5.0}EOs (containing 5.0 ethoxy units on average), while their biotransformation pathways and metabolites remain unclear (Di Gioia et al. 2008; Salvadori et al. 2006). Liu et al. (2006) isolated two strains (*Ensifer* sp. and *Pseudomonas* sp.) capable of transforming nonylphenol diethoxylate (NP₂EO) to nonylphenol monoethoxylate (NP₁EO). In comparison with their mother molecules, however, information regarding the fates of low-ethoxylated NPEOs is still very limited. It is not clear, for example, why low-ethoxylated CAPECs, the metabolites with oxidized alkyl and ethoxy side chains, accumulate in the environment as a result of NPEO biotransformation (Ahel et al. 1994; Di Corcia et al. 1998; Jonkers et al. 2001; Montgomery-Brown et al. 2003, 2008).

It is known that isolated bacteria acquired through conventional isolation methods cannot represent the actual distribution of NPEO-degrading bacteria in the environment, and may not be able to reveal the actual biotransformation pathway (Lozada et al. 2004, 2006; Zhang et al. 2008a). Recently, we found two novel high-ethoxylated NPEO (NP_{av9}EO) pathways based on phylogenetically diverse NP_{av9}EOs-degrading isolates acquired using gellan gum as the gelling reagent (Gu et al. 2010). Therefore, it may be possible to acquire phylogenetically diverse low-ethoxylated NPEO-degrading isolates based on the same cultivation strategy. Using this improved method, we isolated novel low-ethoxylated NPEO (NP_{av2}EO)-degrading bacteria from activated sludge and used the isolates to study the biotransformation pathways of NP_{av2}EO. This study will help understand the fates of NPEOs in WWTPs and related environments.

Materials and methods

Reagents and chemicals

Technical grade NPEO surfactant mixtures with an ethoxylate range of 1–4 and an average EO chain length of 2 (NP_{av2}EO) were obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), as a mixture of NP₁EO (32.6 %), NP₂EO (39.0 %), NP₃EO (16.2 %), NP₄EO (5.3 %), and some impurities (6.9 %). Nonylphenol (NP) with a linear alkyl chain (4-n-NP) was used as an internal standard for gas chromatography–mass spectrometry (GC-MS) analysis and was purchased from Fluka (Seinheim, Germany). Mixed NPEC standards with EO chain lengths ranging from 0 to 9 corresponding to NP₁EC up to NP₁₀EC, and NP were obtained from Hayashi Pure Chemicals (Osaka, Japan). We obtained *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1 % trimethyl-chlorosilane, which was used for trimethylsilylation, as well as gellan gum and agar used as gelling reagents from Supelco (Bellefonte, PA, USA). Methanol, dichloromethane, *n*-hexane, and acetonitrile [high-performance liquid chromatography (HPLC) grade] were obtained from the Fisher Company (Muskegon, MI, USA). Ammonium acetate of HPLC grade was purchased from Fluka (Steinheim, Germany). All water (18.2 MΩ) used in this study was purified using PURIC-MXII apparatus (Organo, Japan). General structures and acronyms of NPEOs and their well-ascertained intermediates are presented in Fig. 1.

Screening and identification of low-ethoxylated NPEO-degrading bacteria

A sludge sample was collected from an activated sludge reactor used to treat NPEO-containing wastewater (Zhang et al. 2008b) and inoculated into modified PE03 medium containing NP_{av2}EO (500 mg L^{−1}) as the only carbon source. The NP_{av2}EO were firstly dissolved in hexane, evaporated to dryness, and then redissolved in medium with a final concentration of 500 mg L^{−1}. The modified PE03 medium contained 0.38 g/L of KH₂PO₄, 0.39 g/L of K₂HPO₄, 0.5 g/L of (NH₄)₂SO₄, 5 mL of basal salts solution, and 1 mL of vitamin solution per liter (Hanada et al. 1995). The cultures were then incubated aerobically at 30 °C with shaking at 140 rpm. The enrichment cycles were repeated at 10-day intervals for 6 months (Salvadori et al. 2006). Finally, 100 μL of appropriately diluted culture broth was spread on PE03-NP_{av2}EO (50 mg L^{−1}) gellan gum or agar plates and then incubated at 30 °C in the dark for 2 days. We isolated 130 randomly chosen colonies for each sampling time and then screened them for their ability to grow in the presence of NP_{av2}EO. Next, seven restriction types using *AluI* were selected. Seven morphologically distinct colonies from the seven restriction types, which could grow in the presence of NP_{av2}EO on gellan gum or agar

plates, were further identified by amplification of their 16S rRNA genes using universal bacterial primers 27F and 1492R (Lane 1991). The PCR products were subjected to electrophoresis on 1.2 % agarose gels, stained with ethidium bromide ($0.5 \mu\text{g mL}^{-1}$) and visualized by UV excitation. Furthermore, the PCR products were purified using a QIAquick PCR purification kit (QIAGEN, Japan) according to the manufacturer's protocols, and were then digested for 5 h at 37°C with *AluI*. The seven morphologically distinct colonies from the seven restriction types were selected, purified, and tested for their ability to degrade NP_{av2}EO. The nucleotide sequences of the 16S rRNA genes obtained were then submitted to GenBank and assigned the following accession numbers: NP47a (EU580702), NP42a (EU580703), NP428a (EU580704), NP427b (EU580705), NP522b (EU723211), NP631a (EU723212), and NP526a (EU723213). Strains named with “a” were acquired on the gellan gum-based media, while those named with “b” could be obtained on both media.

Biodegradation of NP_{av2}EO using isolated bacteria

All isolates were previously grown to the log phase in Luria–Bertani liquid medium supplemented with NP_{av2}EO. A 100- μL aliquot of the culture medium, with optical density at 600 nm adjusted to 0.5, was then used to inoculate 250-mL Erlenmeyer flasks containing 100 mL of PE03-NP_{av2}EO medium. The samples were then subjected to constant shaking at 140 rpm and 30°C for 20 days, and growth was evaluated by measuring the optical density at 600 nm using a spectrophotometer (Hitachi, UV3010). All assays were conducted in duplicate, and test blanks were added to measure abiotic losses.

After a designated time, the culture medium (2 mL) was collected from the flasks, stabilized with 20 μL of formaldehyde, and acidified to pH 2 with HCl. The medium was then extracted using an equal volume of ethyl acetate/methanol (7:3, v/v) twice by shaking the mixture vigorously. The collected organic phases were then blown dry under a gentle flow of nitrogen. For liquid chromatography–mass spectrometry (LC-MS) and GC-MS analyses, dried samples were redissolved in 1 mL of methanol/pure water 1:1 (v/v) and 0.4 mL of *n*-hexane hexyl hydride, respectively.

Analytical methods

Low-ethoxylated NPEOs were analyzed using a gas chromatograph (GC) (Agilent Technologies, 6890 GC) interfaced with a DB-5MS capillary column ($30 \text{ m} \times 0.25 \text{ mm} \times 0.25 \mu\text{m}$) and a mass spectrometer (MS) (Agilent Technologies, 5975i MSD) (Isobe et al. 2001). Briefly, the dried samples were redissolved in *n*-hexane, derivatized with BSTFA and then subjected to GC-MS analysis. The GC column temperature was programmed to increase from 50°C (initial equilibrium

time, 2 min) to 200°C via a ramp of $20^\circ\text{C min}^{-1}$, from 200 to 260°C via a ramp of 5°C min^{-1} , and from 260 to 280°C via a ramp of $20^\circ\text{C min}^{-1}$, after which the temperature was maintained at 280°C for 2 min. Helium carrier gas was maintained at a constant flow rate of 1 mL min^{-1} . Compounds were identified by comparison with previously published results (Isobe et al. 2001) and quantified in selective ion monitoring mode.

Analysis of NPECs was conducted using a Waters LC-MS system (Alliance 2690 HPLC system linked to a Micromass ZQ 4000) equipped with a Waters Symmetry ShieldTM RP18 column ($150 \times 2.1 \text{ mm}$, $3.5 \mu\text{m}$) (Zhang et al. 2007). The mobile phase was a mixture of 5 mmol L^{-1} ammonium acetate solution (A) and pure methanol (B). Gradient elution was performed as follows: 30 % methanol held for 5 min, linearly increased to 85 % in 7 min and held for 9 min, and then increased to 97 % and held for 5 min, and finally brought back to 30 % and held for 8 min until the next injection. The flow rate was 0.2 mL min^{-1} . The column oven was maintained at 35°C , and the injection volume was 10 μL . All analyses were performed in the selected ion recording mode using the deprotonated molecular ions of NPECs during negative ionization. The method recovery obtained after stabilization, acidification, and extraction of culture medium spiked with low-ethoxylated NPEOs and NPECs ranged from $93 \pm 10 \%$ to $102 \pm 3.0 \%$ and $75 \pm 4.5 \%$ to $98 \pm 12 \%$, respectively. Relative standard deviation was below 12 %. Instrumental limits of detection and linear range were 25 pg and 2.5–500 $\mu\text{g L}^{-1}$ for NP_{1–10}EC, respectively. Detailed information on the analytical methods is shown in Table S1 (see supplementary data).

Carboxyalkylphenol polyethoxycarboxylates were investigated as per Di Corcia et al. (2000) and Jonkers et al. (2001). Quantification of CAPEC was made more difficult because of the lack of any standard, which may have introduced some inaccuracy. For identity confirmation of CAPECs, LC-ES-MS/MS was used mass spectrometry was carried out on a Waters Quattro Premier XE tandem quadrupole mass spectrometer (Waters Corp., Manchester, UK) using negative electrospray ionization (ESI). The range of m/z , scan period, and scan delay time were 50–500 amu, 0.5 s, and 0.1 s, respectively. The source and desolvation temperatures were held at 120 and 400°C , respectively. Desolvation gas and cone gas were set at flow rates of 600 and 60 L/h, respectively.

Results and discussion

Taxonomic characteristics and NP_{av2}EO degradation capability

Using the improved method, seven isolates grown in the presence of NP_{av2}EO were acquired and labeled as strains NP42a, NP47a, NP428a, NP427b, NP526a, NP631a, and

NP522b, the 16S rRNA gene sequences of which were >99 % homologous with *Sphingomonas chungbukensis* (AF159257), *Ralstonia eutropha* (EU827495), *Alicyclophilus denitrificans* (NR_074585), *Variovorax paradoxus* (AF532868), *Shinella zoogloeoides* (EU346730), *Achromobacter insolitus* (NR_025685), and *Pseudomonas aeruginosa* (GQ926936), respectively. As shown in Fig. 2, these strains were distributed widely in α , β and γ classes of *Proteobacteria* phylum and could be assigned to seven different genera: *Sphingomonas* sp. NP42a, *Ralstonia* sp. NP47a, *Alicyclophilus* sp. NP428a, *Variovorax* sp. NP427b, *Pseudomonas* sp. NP522b, *Shinella* sp. NP526a, and *Achromobacter* sp. NP631a. While all seven strains were acquired on the gellan gum-based media, only two strains (NP522b and NP427b) were obtained on the agar based media, showing gellan gum led to the isolation of phylogenetically diverse strains.

Among the seven isolates, NP522b (*Pseudomonas*), NP427b (*Variovorax*), NP47a (*Ralstonia*), and NP42a (*Sphingomonas*) were found to grow in PE03-NP_{av2}EO medium rapidly within two days and had the ability to degrade NP_{av2}EO. The degradation ratios of NP_{av2}EO and the main products detected in the above four degradation systems are

listed in Table S2 (see supplementary data). No NP_{av2}EO losses were found in the test blanks without strains. Both NP42a (*Sphingomonas*) and NP427b (*Variovorax*) were closely related to previously reported low-ethoxylated NPEOs-degrading bacteria (Di Gioia et al. 2008); however, no members from the latter two genera (NP47a (*Ralstonia*) and NP42a (*Sphingomonas*) have been previously identified as low-ethoxylated NPEO degraders.

Biotransformation pathways of NP_{av2}EO by isolated bacteria

Low-ethoxylated NPEOs are recalcitrant intermediate products of nonionic surfactant NPEOs. In comparison with their mother molecules, information regarding the fates of low-ethoxylated NPEOs is very limited. Liu et al. (2006, 2007) isolated two strains (*Ensifer* sp. and *Pseudomonas* sp.) capable of aerobically degrading low-ethoxylated NPEOs. As shown in Fig. 3a, shorter NPEOs and NPECs were formed through stepwise oxidation of the terminal alcohol group of the EO chain with the final degradation product as NP. We proposed two new pathways as shown in Fig. 3b and c.

Fig. 2 Phylogenetic affiliation of isolated strains NP42a, NP47a, NP428a, NP427b, NP526a, NP631a, and NP522b based on nucleotide sequences of the 16S rRNA genes. The phylogenetic tree was constructed using neighbor-joining algorithm with the Jukes–Cantor distance in the PHYLIP software (<http://evolution.genetics.washington.edu/phylip.html>). The 16S rRNA gene sequence of *Geotoga petraea* (belonging to

the Phylum *Thermotogae*) was selected as the outgroup. Reference sequences and type strain sequences were obtained from the Ribosomal Database Project database (<http://rdp.cme.msu.edu/>) and StrainInfo (<http://www.straininfo.net/>), respectively. GenBank accession numbers are shown in parentheses

Fig. 3 Biodegradation pathways of low-ethoxylated NPEOs proposed by **a** Liu *et al.* (2006, 2007), **b** direct oxidation of NP_{av2}EO, and **c** Hydroxyl shift cleavage of NP_{av2}EO based on the results of this study

Pathway 1: direct oxidation of NP_{av2}EO with NPECs and CAPECs as the major degradation products by *Ralstonia* sp. NP47a, *Variovorax* sp. NP427b, and *Pseudomonas* sp. NP522b

Strains *Ralstonia* sp. NP47a, *Variovorax* sp. NP427b, and *Pseudomonas* sp. NP522b were capable of degrading 53.6, 28.1, and 26.5 % (molar percentage) of NP_{av2}EO on day 10, respectively (Table S2 in supplementary data). The proportion of residual NP₁EO, NP₂EO, and NP₃EO in the mixture did not

change over the duration of the experiment in comparison with the original NP_{av2}EO. At the same time, a considerable amount of NPECs ($n=1-3$) were detected in all culture systems. Figure 4a and b shows that the cell density of strain NP47a (indicated by changes in OD₆₀₀) and the concentrations of three NPEC species increased with decreasing concentration of NP_{av2}EO over a period of 10 days. In our previous study, a strain having the same morphological traits and 16S rRNA gene sequence as NP47a transformed NP₁₁EO to its corresponding carboxylated products (NP₁₁EC) at much

Fig. 4 Utilization of NP_{av2}EO as a growth substrate for strain NP47a. **a** Growth was assessed based on optical density at 600 nm (OD₆₀₀). Residual amounts of NPEOs (EO chain length $n < 3$) were determined by GC-MS analysis of organic extracts of the entire culture medium. **b** Changes in concentration of NPECs ($n = 1-3$) in the culture media during incubation of strain NP47a

lower degradation and bacterial growth rates (Gu et al. 2010). Thus, low-ethoxylated NPEOs appeared more suitable carbon sources for the growth of NP47a. Formation of CAPECs was confirmed on day 10. Mass balances at different sampling times for strain NP47a are summarized in Table 1. The residual NPEOs and the three NPEC and seven CAPEC metabolites accounted for approximately 46.4, 14.6, and 19.0 % of the initial NP_{av2}EO molar concentration on day 10, respectively (Table 1). The incomplete extraction from bacterial cells and the existence of some undetected metabolites might be responsible for the missing mass.

The total ion chromatogram of LC-MS for the degradation sample taken on day 10 showed that besides the co-elution peak of NPECs at 14.2 min, two new peaks were found at 12.8 and 13.1 min (supplementary data Fig. S1). Since they show

Table 1 Mass balances at different sampling times for NP_{av2}EO degradation using strain NP47a

Sampling time (day)	Molar percentage of detected residual NP _{av2} EO to initial NP _{av2} EO (%)	Molar percentage of detected NPECs ($n = 1-3$) to initial NP _{av2} EO (%)				Molar percentage of detected CAPECs to initial NP _{av2} EO (%)
		NP ₁ EC	NP ₂ EC	NP ₃ EC	Total	
0	100	0	0	0	0	—
1	90.1	0.92	1.32	0.72	2.96	—
2	77.7	2.00	3.52	1.28	6.79	—
4	67.4	3.56	4.72	2.36	10.6	—
7	50.9	4.80	6.07	3.60	14.5	—
10	46.4	4.48	6.71	3.40	14.6	19.03

[M-H][−] ions in the negative mode with m/z values of 309 and 351, respectively, the related molecular weights were 310 and 352, which might be assigned to CA₅P₂EC and CA₈P₂EC, respectively (Jonkers et al. 2001). We employed LC-ESI-MS/MS to further confirm this speculation as shown in Fig. S2a (see supplementary data). For m/z 309 at the collision voltage of 20 V, the fragment m/z was 163, which might be attributed to [CH₃–C₄H₈–C₆H₄–O][−] due to the loss of COO[−] group and EO chain cleavage from CA₅P₂EC. Similarly, since there were mass differences in the three methylene groups ($m/z = 42$) between CA₈P₂EC and CA₅P₂EC, the fragment of m/z 351 was m/z 205 (see supplementary data Fig. S2b).

Using the above approach, the other unknown compounds were also assigned as CAPEC metabolites, including CA₇P₂EC ($m/z = 337$), CA₈P₁EC ($m/z = 307$), CA₆P₁EC ($m/z = 279$), CA₅P₁EC ($m/z = 265$), and CA₅P₃EC ($m/z = 353$). As standards of most CAPECs were unavailable, their quantification was performed by assuming that CAPECs had the same molar response factors as the corresponding NPECs (Jonkers et al. 2001). The semiquantitative results of CAPECs formed by strains NP47a, NP427b, and NP522b were calculated as shown in Table 2. The total CAPEC concentrations formed on day 10 were 100.6, 48.7, and 24.3 μM for NP47a, NP427b, and NP522b, respectively. The length of the alkyl chain and EO chain were 5–8 (CA₅EC–CA₈EC) and 1–3 (CA₁EC–CA₃EC), respectively. No products with shorter alkyl chains were found. The highly branched nature of the alkyl chain might have limited the rate and extent of its further oxidation (Montgomery-Brown et al. 2003). Both CA₈PEC and CA₅PEC were the most abundant CAPEC formation species, which was in accordance with previous laboratory-scale bioreactor findings (Jonkers et al. 2001). Researchers have postulated that CA₈PECs and CA₅PECs may result from the ω/β -oxidation of certain NPECs (Montgomery-Brown and Reinhard 2003).

Table 2 Formation of CAPECs (μM) by low-ethoxylated NPEO-degrading isolates on day 10

Strain	CA ₅ P ₁ EC	CA ₅ P ₂ EC	CA ₅ P ₃ EC	CA ₆ P ₁ EC	CA ₇ P ₂ EC	CA ₈ P ₁ EC	CA ₈ P ₂ EC	Total
NP47a	14.12	25.67	6.89	2.60	3.52	5.55	42.27	100.6
NP427b	9.50	19.41	7.94	1.16	0.71	2.71	7.26	48.7
NP522b	4.15	10.09	1.69	0.55	1.02	0.73	5.94	24.2

As shown in Table 2, CAPECs were detected in all three NP_{av2}EO degradation systems on day 10, showing that NP47a, NP427b, and NP522b had high oxidation power to degrade the alkyl group. Since the first detection of CAPECs (CAP₁EC and CAP₂EC) from tertiary treated effluent (Ding et al. 1996), many laboratory and field studies have shown that CAPECs are the main metabolites of NPEOs (Di Corcia et al. 1998; Jonkers et al. 2001; Montgomery-Brown et al. 2003, 2008). Di Corcia et al. (1998) found that CA₃₋₈P₁ECs and CA₃₋₈P₂ECs appeared as the main and extremely persistent intermediates in an OECD biodegradation test of NP_{av9}EO. Their further research showed that CAPECs were the dominant products of NP_{av9}EO biotransformation, accounting for 66 % of all metabolites in the effluents of five WWTPs. Additionally, CA₆PECs and CA₈PECs were the dominant CAPEC species in WWTP effluents, and CAP₂ECs were approximately twice as abundant as CAP₁ECs (Di Corcia et al. 2000). Jonkers et al. (2001) found that CA₉P₂EC, CA₅₋₈P₁ECs, and CA₅₋₈P₂ECs were the main products in a laboratory-scale bioreactor filled with NP_{av9}EO-spiked river water. Thus, both environmental survey and our study with isolated bacteria demonstrated that CAPECs were one of the main products of NPEO biotransformation. However, the estrogenic effect of CAPECs remains poorly understood (Montgomery-Brown and Reinhard 2003).

To date, NPEOs degradation experiments using isolated bacteria only confirmed the formation of low-ethoxylated NPEOs ($n=2, 3$) (John and White 1998; Lu et al. 2008), low-ethoxylated NPEOs and NPECs ($n=2$ or 3) (Liu et al. 2006; Sato et al. 2001, 2003), or NP₁EO, NP₁EC, and NP (Liu et al. 2007; Fig. 3a). Thus, the accumulation of CAPECs observed in many laboratory and field studies (Ahel et al. 1994; Di Corcia et al. 1998; Jonkers et al. 2001; Montgomery-Brown et al. 2003, 2008) could not be explained by the above pathways. Using gellan gum as a gelling reagent, we successfully isolated bacteria capable of transforming low-ethoxylated NPEOs to CAPECs. Thus, Fig. 3b shows the proposed pathway of NP_{av2}EO using *Variovorax* sp. NP427b, *Ralstonia* sp. NP47a, and *Pseudomonas* sp. NP522b, and the oxidation of the EO chain and alkyl group to corresponding NPECs and CAPECs. According to Jonkers et al. (2001) and Di Corcia et al. (1998), CAPECs started to accumulate after NPECs were formed during biotransformation of NPEOs. Montgomery-Brown et al. (2008) has found that alkyl chain oxidation of NP₁EC by

microcosms and the formation of CAP₁ECs is favored under microoxic conditions. Thus, it may be possible that pathway 1 is favored by the microoxic conditions.

Pathway 2: hydroxyl shift cleavage of NP_{av2}EOs with NP₁EO as the major degradation product by strain NP42a (*Sphingomonas*)

Strain NP42a belonging to the *Sphingomonas* genus (α -*Proteobacteria*) degraded NP_{av2}EO rapidly. As shown in Fig. 5, NP₂EO and NP₃EO decreased from 251.7 and 141.7 mol L⁻¹ on day 0 to 75.5 and 8.0 mol L⁻¹ on day 10, respectively, while NP₁EO increased from 156.3 mol L⁻¹ on day 0 to 357.6 mol L⁻¹ on day 10. The total concentration of NP₁₋₃EO decreased from 549.7 to 441.1 mol L⁻¹. NPECs were not formed throughout the whole period, and adsorption by bacterial cells may be responsible for the missing mass. From the above results, it was proposed that biodegradation of the low-ethoxylated NPEOs by NP42a was through the hydroxyl shift cleavage pathway to form NP₁EO, as shown in Fig. 3c. In our previous study, an isolated strain with the same morphological traits and 16S rRNA gene sequence as NP42a degraded NP_{av9}EO with NP₁EO as the major product (Gu et al. 2010). It should be noted, however, that slow

Fig. 5 Changes in concentrations of NPEOs ($n=1-3$) in the culture media during incubation of strain NP42a. Growth was assessed based on optical density at 600 nm (OD₆₀₀)

mineralization of NP₁EO might also be possible, which requires further study.

NP₁EO is regarded as the most toxic and estrogenic NPEO homologue (Metcalf et al. 2001; Servos 1999) and is widely found in many environments as the main metabolite of NPEOs (Ahel et al. 1994; Ferguson et al. 2001; Kolpin 2002). Few pure strains capable of converting NPEOs to NP₁EO have been isolated to date. Liu et al. (2006) isolated two strains (*Ensifer* sp. strain AS08 and *Pseudomonas* sp. strain AS90) capable of transforming NP₂EO to NP₁EO, as shown in Fig. 3a. The present study clearly showed that a member of the genus *Sphingomonas* has the ability to transform low-ethoxylated NPEO to NP₁EO through a nonoxidative pathway. It is known that NP₁EO has been extensively detected in different environments (Ahel et al. 1994; Ferguson et al. 2001; Kolpin 2002), suggesting that pathway 2 may happen under both anaerobic and aerobic conditions (John and White 1998).

In our previous research, we studied the biotransformation of NPEOs during continuous aerobic treatment of sewage spiked with 23 μ M technical NP_{av9}EOs over a period of 90 days (Zhang et al. 2008b), which provided the activated sludge samples for bacterial isolation in the present study. Short-chain NPEOs (NP₁EO–NP₃EO) (1.62–2.03 μ mol/L) and NPECs (2.79–3.30 μ mol/L) accumulated in the treated effluents. However, CAPECs were not found in the effluent throughout the whole treatment experiment (Zhang et al. 2008b).

Conclusions

Four low-ethoxylated NPEO-degrading strains belonging to four genera in three classes (*Pseudomonas* sp. NP522b in γ -*Proteobacteria*, *Variovorax* sp. NP427b and *Ralstonia* sp. NP47a in β -*Proteobacteria*, and *Sphingomonas* sp. NP42a in α -*Proteobacteria*) were isolated from activated sludge by cultivation with gellan gum as the gelling reagent. This was the first time that degradation of low-ethoxylated NPEOs by strains belonging to *Ralstonia* and *Sphingomonas* was observed. A new pathway of the low-ethoxylated NPEOs, i.e., oxidation of low-ethoxylated NPEOs to their corresponding NPECs and CAPECs, was identified using *Pseudomonas* sp. NP522b, *Variovorax* sp. NP427b, and *Ralstonia* sp. NP47a. The nonoxidative pathway with NP₁EO as the dominant product was confirmed by a new low-ethoxylated NPEO-degrading bacteria, *Sphingomonas* strain NP42a.

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