Electronic Journal of Biotechnology ISSN: 0717-3458
http://www.ejbiotechnology.info
DOI: 10.2225/vol14-issue4-fulltext-1 *RESEARCH ARTICLE*

Aerobic biodegradation of nonylphenol ethoxylates in shaking-flask test

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Received February 21, 2011 / Accepted April 20, 2011
Published online: July 15, 2011

Abstract Nonylphenol ethoxylates (NPEOs), which are widely used for industrial and domestic purposes, exert adverse effects on wildlife after being used and discharged into the environment. However, their ultimate biodegradability and biodegradation pathway remains unclear. In this study, the aerobic degradability of nonylphenol ethoxylates (NPEOs) by the acclimated microorganisms in active sludge was examined using shaking-flask tests. The degradation of benzene rings in NPEOs was determined using UV spectroscopy and high performance liquid chromatography (HPLC). Results showed that more than 80% of benzene rings were removed after 8-10 days of degradation, and the majority of NPEOs were also removed after 9 days of degradation, indicating NPEOs and the benzene rings could be ultimately degraded by microorganisms in acclimated active sludge. Electrospray ionization-mass spectroscopy (ESI-MS) analysis of biodegradation intermediates indicate that stepwise omega, beta-oxidation of EO chains or fission of EO chains, and further omega, beta-oxidation of alkyl-chain for short-EO-chain NPEOs constitute the main pathway in the early stage, and complete biodegradation occur when the benzene rings in these molecules are opened in the later stage.

Keywords: aerobic biodegradation, degradation pathway, ESI-MS, HPLC, nonylphenol ethoxylates

INTRODUCTION

Nonylphenol ethoxylates (NPEOs) consist of hydrophilic polyethoxy groups and hydrophobic alkyl benzene groups, and belong to a class of nonionic surfactants. Because of their excellent surface activity, NPEOs have been widely used in domestic and industrial product additives, *e.g.* active detergents, textile auxiliaries and pesticide emulsifiers. It is estimated that the annual consumption amount of detergents in China is more than 5 million tons, of which more than 10% are NPEOs (National Bureau of Statistics of China, 2009). NPEOs as well as its degradation intermediates enter into aquatic environment together with treated or untreated wastewater after being used. The residual NPEOs and the degradation intermediates, such as nonylphenol (NP), nonylphenol mono-, di-ethoxylates (NP1EO, NP2EO) and corresponding carboxylic acid (NP1EC, NP2EC), can be detected in contaminated river water and sediments with the reported maximum concentration on the scale of ppm (Kolpin et al. 2002; Loos et al. 2007). The most concern in recent years were the potential endocrine disrupting activities posed by the short-EO-chain NPEOs, as well as NP. Notably, their adverse effects have been reported on aquatic organisms exposed to the contaminated water (Jonkers et al. 2003; Chiu et al. 2010).

Previous studies demonstrated that the primary biodegradation of NPEOs under aerobic conditions is readily achieved by stepwise shorting down the EO chain (Hayashi et al. 2005). But further elimination of these intermediates is difficult, because the benzene rings in these molecules are resistant to degradation (Zhang et al. 2007). These NPEOs and their intermediates with short EO chains can be accumulated in natural environment, especially in surface water and sediments. Meanwhile, the biodegradation pathways of NPEOs are still not clear (Planas et al. 2002; Jahnke et al. 2004). Pathways of stepwise removal of the EO chain and the alkyl chain have been described by Schmitt

(2001). The EO-chain omega, beta-oxidation pathway, alkyl-chain omega, beta-oxidation pathway, and EO-chain fission pathway are the main dissipation modes of EO-chain and alkyl chain.

In this study, the microorganisms in acclimated activity sludge were used to degrade the NPEOs. The biodegradation tests were carried out in shaking flasks on the laboratory scale. And a complete biodegradation pathway including degradation of benzene rings was examined by a series of analysis techniques (Ferguson et al. 2001; Jonkers et al. 2005). According to the above results, a complete biodegradation pathway was proposed. The results can be used for selection of highly efficient strains that can degrade NPEOs, which would benefit the treatment of wastewater containing NPEOs.

MATERIALS AND METHODS

NPEOs (technical grade) with an average of 2 EOs or 10 EOs (purity ≥ 95%) were both obtained from Kasei Kogyo Co. Ltd. (Tokyo, Japan). The stock solutions of NPEOs were prepared in double distilled water with a concentration of 1.0 g/L, which was then ultrasonicated for 30 min to get a stable emulsion solution, and stored in 4ºC for a maximum of four weeks. Other reagents used for culture media and analysis were of analytical grade, and purchased from domestic companies.

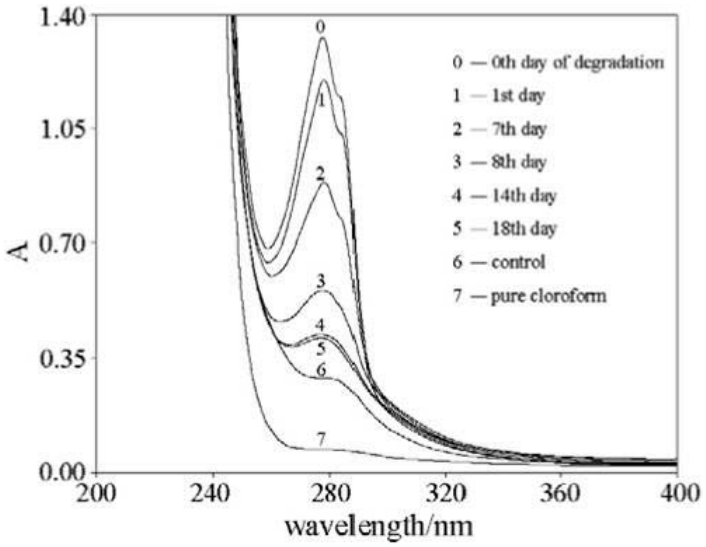


Fig. 1 UV spectra of chloroform solution of benzene rings of degradation intermediates of NP2EO. The maximum absorbance is at 277 nm.

The shaking-flask test for aerobic biodegradation was established according to the International Standards Organization (ISO) method 7827 (International Standards Organization, 1984) and previous study (Zhao et al. 2006). Briefly, 0.5 L of culture medium was prepared in a 1 L shaking flask, containing the following minerals: NH_4Cl, 3.0 g/L; K_2HPO_4, 1.0 g/L; $MgSO_4$, 0.25 g/L; KCl, 0.25 g/L; FeSO4, 0.002 g/L; and yeast extract, 0.3 g/L. NPEOs solution was added to the culture medium to get a concentration of 30 mg/L. Blank control was prepared simultaneously. Three parallel tests for NPEOs and blank were carried out. Before the start of the biodegradation tests, acclimated sludge was need. The flask contained NPEOs of 30 mg/L in culture medium was inoculated with 5 mL of 15 g/L suspended solids content aerobic activated sludge. Then, the flask was placed in a table shaker in a temperature-controlled cabinet at 25ºC and shaken continuously at 200 r/min. After 72 hrs of acclimation, 5 mL of the acclimated solution was added to another 500 mL of freshly prepared culture medium with the concentration of NPEOs kept at 30 mg/L. The acclimation was repeated. Then 5 mL of the twice acclimated solution were inoculated into the NPEOs solutions with an initial concentration of 30 mg/L in culture medium, and biodegradation tests were performed for a maximum of 24 days.

One hundred mL of biodegradation solution was transferred to a 125 mL separatory funnel, and 35.5 g of NaCl was added immediately. The funnel was manually shaken for 1 min for NaCl to dissolve completely. Then the solution was extracted twice with 50 mL of chloroform in total (Tomaszewski et al. 1999; Zhao et al. 2006). Chloroform was collected in a 100 mL vessel through defatted cotton. The combined chloroform phase was dried using a rotary evaporator. The extract was then redissolved in 10 mL of chloroform, and the absorbance at 277 nm was measured with a 1 cm quartz cuvette on a UV-1600 spectrophotometer (Beijing Rui Li, China) to quantify the degradation degree of the benzene ring of the NPEO molecule (Zhao et al. 2006). Then chloroform was removed again by gentle nitrogen gas blowing. The sample was kept in a refrigerator at 4ºC for HPLC (high performance liquid chromatography) and ESI-MS (electrospray ionization mass spectrum) analyses. The extraction recovery efficiencies for NP2EO and NP10EO were 95.3 ± 2.6% and 93.9 ± 8.1%, respectively, at a spiked concentration of 30 mg/L in culture medium.

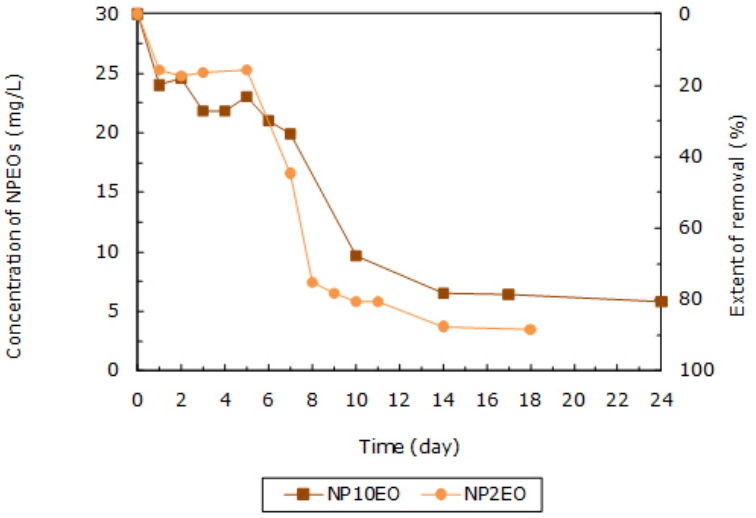


Fig. 2 Time course of removal of NP10EO and NP2EO.

Concentrations of residual NPEOs were also determined using a liquid chromatography technique, which was performed by using a HPLC (Waters, USA) equipped with an automated gradient controller, two Model 510 pumps, an multifunctional sampler injector, a Waters Differential Refractometer R 401, a C18 column (Diamonsil^{TM}, 5 µm, 250 x 4.6 mm, Dikma Corporation, China). The mobile phase was 95% methanol/5% water, and the flow was kept at 1.0 mL/min. The sample extract was dissolved in 0.5 mL of methanol, and filtrated using a 0.22 µm membrane filter. The injection volume was 100 µL.

ESI-MS analysis used for qualitative identification of the potential degradation intermediates was carried out on an API4000 mass spectrometer (Applied Biosystems Corporation, USA). The sample extract was dissolved using 5 mL of Acetonitrile/water (V/V = 1:1) solution, and 0.5 mL of ammonium acetate aqueous solution (w/w = 1:20) was then added into the sample solution. The ionization was carried out in the positive mode, with parameters kept as follows: ESI capillary voltage at 5.3 kV; source block temperature at 100ºC; desolvation temperature at 160ºC; multiplier voltage at 650 V. Nitrogen was used as both desolvation gas with a flow rate of 300 L/h and cone gas with a rate of 50 L/h; the cone voltage was ramped from 30 to 60 V with the full scan mass ranging from 100 to 1000 dalton.

RESULTS AND DISCUSSION

NPEOs (NP2EO and NP10EO) were treated in shaking flasks for over 18-24 days using twice acclimated activated sludge. The extracts of chloroform were examined for the biodegradation of the

benzene rings in NPEOs by measurement of the absorbance at 277 nm. The stepwise decrease of absorbance at 277 nm was found for NP2EO (Figure 1), indicating the benzene ring in the molecule of NP2EO was biodegradable. Because the biodegradation of the benzene ring in NPEO is the key step, the loss of benzene ring in NPEO means that ultimate biodegradation occurs for NPEOs (Zhang et al. 2007). The concentrations of residual NPEOs as well as the extent of removal during the degradation process were illustrated in Figure 2. The concentrations of NP2EO and NP10EO decreased rapidly in the first day from 30 mg/L to 25 mg/L, which was probably due to the adsorption of NPEOs to the bottles wall, or due to the adsorption to the suspended particles of added sludge.

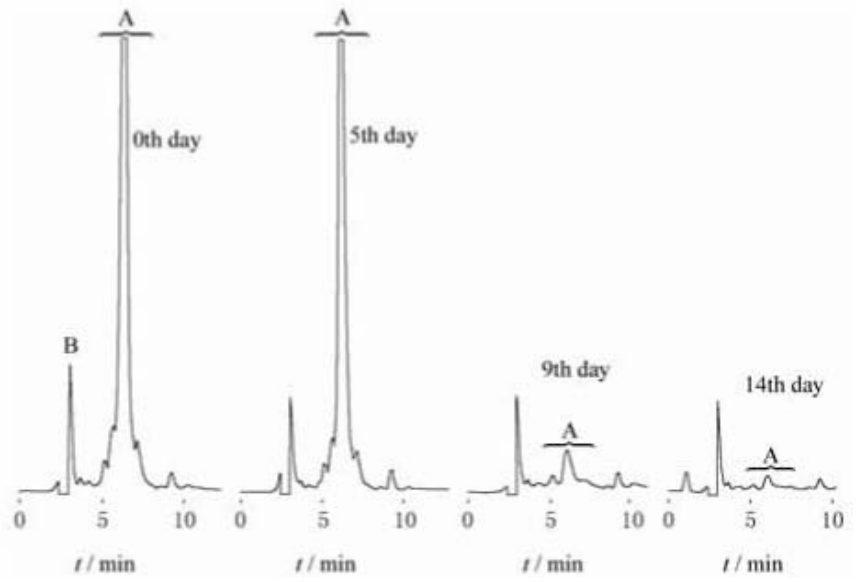


Fig. 3 HPLC chromatograms of biodegradation intermediates of NP2EO in shaking-flask tests.

After the first day, the degradation process was divided into three classic phases: the first phase is the lag phase from 1st day to 5th day with less biodegradation; the second phase is the log phase from 5th day to 10th day; and the last phase is the stationary phase. The concentrations decreased to a stable level after 10 days, with the residual NPEOs at a concentration of about 12-20% of the initial one, and the total removal efficiency of benzene rings was over 80%. The results demonstrated that most of NPEOs could be removed after 8-10 days by microorganisms, except for those adsorbed to bottles wall or suspended particles in degradation liquor.

The biodegradation intermediates of NP2EO for days 0, 5, 9, and 14 were analyzed using Waters HPLC. The chromatograms were shown in Figure 3, where peak B was the solvent, and peaks in group A were the mixtures of NPEOs with different EO units. In Figure 3, the peak intensity reduction between day 5 and day 9 indicates that NP2EO was degraded after the first 5 days, which correlates with the results of UV absorbance at 277 nm. These results indicated that biodegradation of benzene rings was the key step during the entire degradation period, and the intermediates would be degraded immediately after benzene rings in intermediates are opened. The removal of benzene rings indicated that some highly efficient bacterial strains which can degrade NPEOs ultimately were present.

NP10EO and NP2EO samples were treated in shaking-flask tests over 18-24 days. Samples with different degradation period were extracted and analyzed by ESI-MS for identification of potential degradation intermediates. The chromatograms were shown in Figure 4 (corresponding to days 0, 6, 14 of NP10EO) and Figure 5 (corresponding to days 0, 5, 8, 11, 18 of NP2EO). Because ammonium acetate aqueous solution was added to ESI-MS system in order to form ionized adducts to enhance the response of chemicals. NH_4^+ or H^+ were found to combine with NPEOs and the intermediates, and formed ionized adducts. Most of the peaks in the chromatograms were identified according to the molecular masses, which were summarized in Table 1. The peaks in the chromatograms were then marked with corresponding substances and illustrated in Figure 4 and Figure 5.

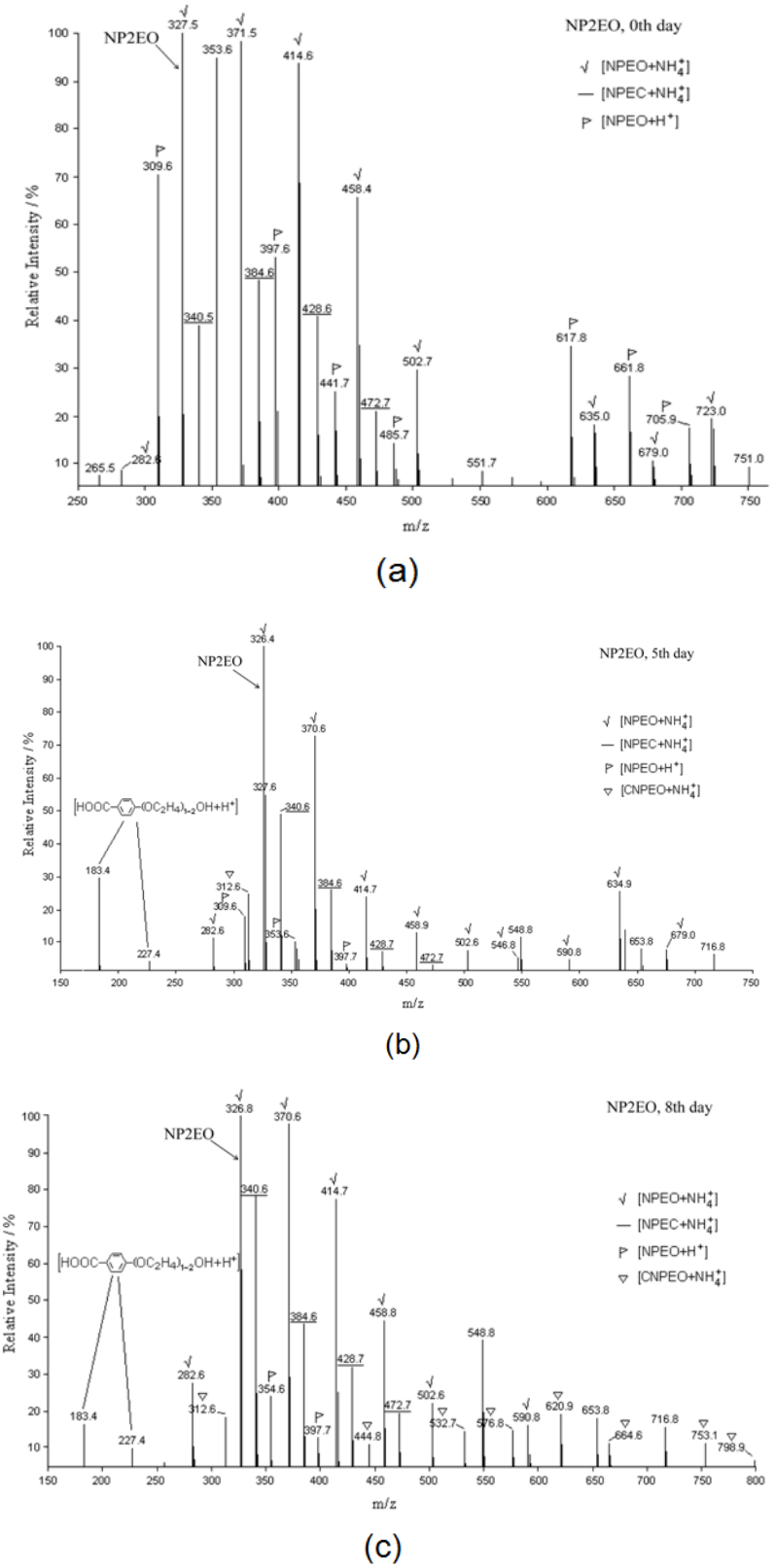
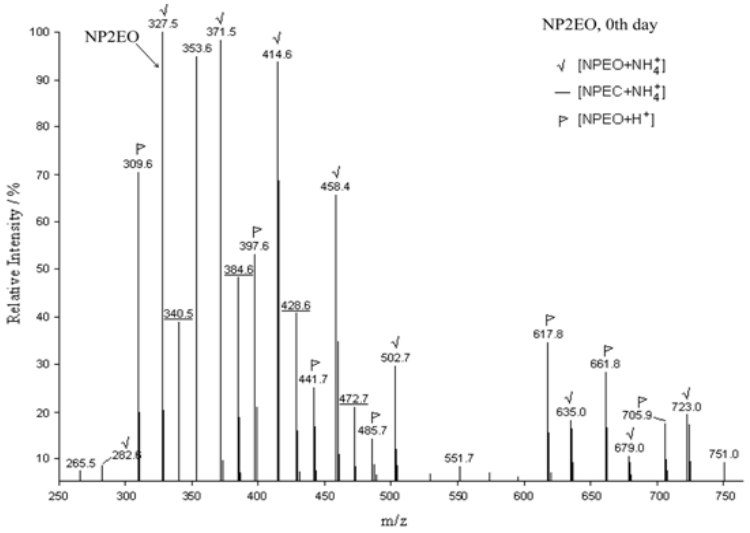
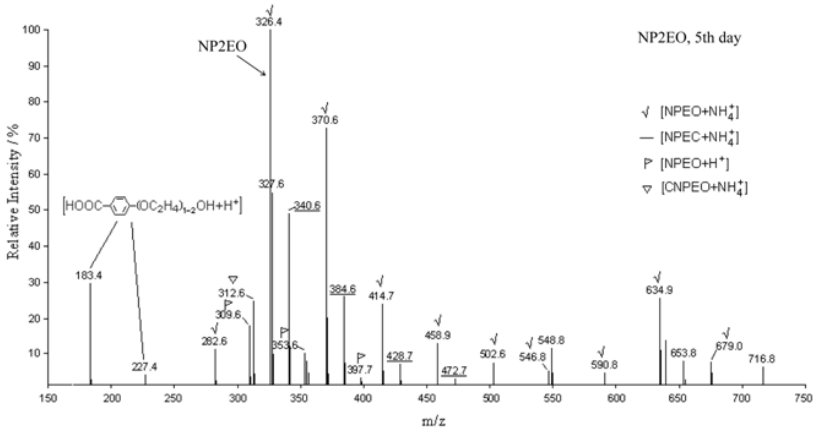


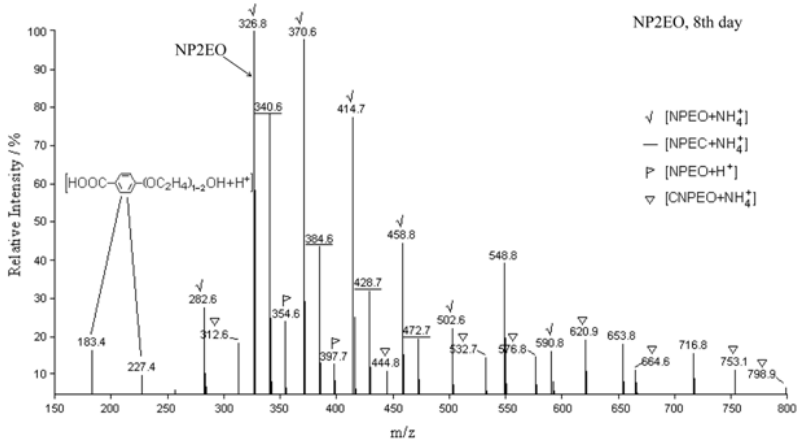
Fig. 4 ESI-MS spectra of NP10EO at degradation time of days 0, 6 and 14.


NP2EO, 0th day
NP2EO
√ [NPEO+NH4+]
— [NPEC+NH4+]
P [NPEO+H+]
Relative Intensity / %
m/z

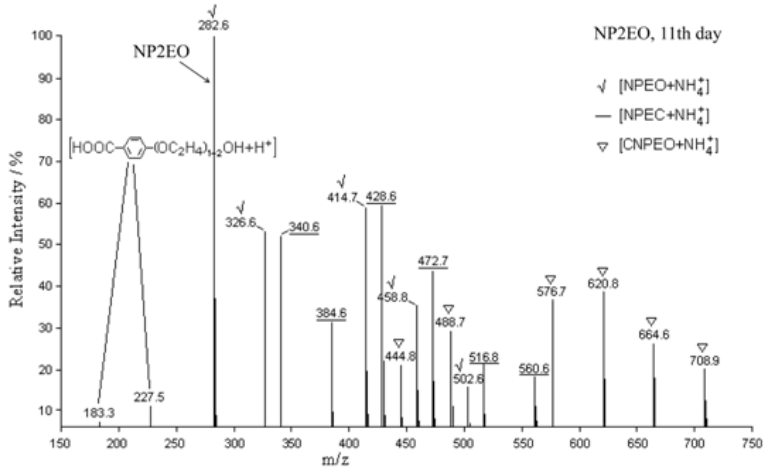
(a)


NP2EO, 5th day
NP2EO
√ [NPEO+NH4+]
— [NPEC+NH4+]
P [NPEO+H+]
▽ [CNPEO+NH4+]
[HOOC-(OC2H4)1-2OH+H+]
Relative Intensity / %
m/z

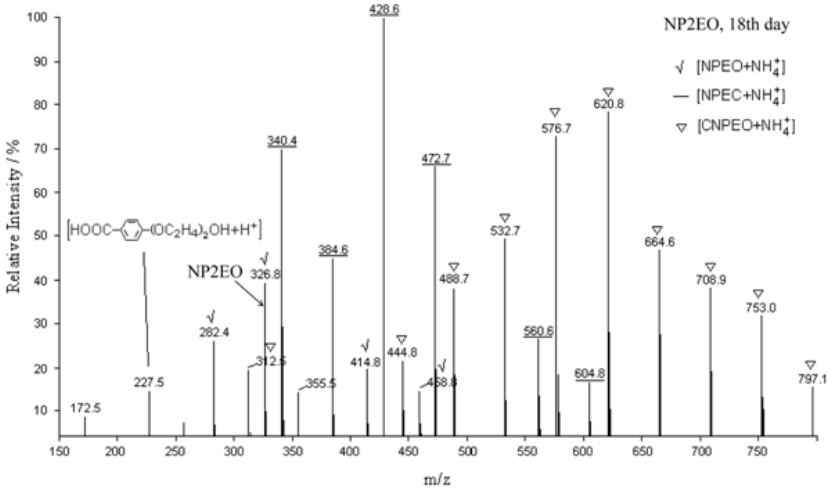
(b)


NP2EO, 8th day
NP2EO
√ [NPEO+NH4+]
— [NPEC+NH4+]
P [NPEO+H+]
▽ [CNPEO+NH4+]
[HOOC-(OC2H4)1-2OH+H+]
Relative Intensity / %
m/z

(c)



(d)



(e)

Fig. 5 ESI-MS spectra of NP2EO at degradation time of days 0, 5, 8, 11 and 18.

Figure 4a and Figure 5a exhibited the chromatograms of initial biodegradation of NP10EO and NP2EO respectively. The peaks of NPEOs + NH_4^+ had mass-to-charge ratios of 282 + 44n, and these ratios differed from each other by multiples of EO units. Because NP10EO sample itself was a mixture of NPEOs with different EO units, and the average EO number was equal to 10. After 6 days, long EO-chain NPEOs were degraded, resulting in short EO-chain NPEOs. Meanwhile, nonylphenol ethoxylates carboxylic acid (NPEC) and alkyl-carboxylated nonylphenol ethoxyaltes (CNPEO) were also detected with the mass-to-charge ratios of 296 + 44n and 312 + 44n respectively, which indicated that EO-chain fission pathway and EO chain omega, beta-oxidation pathway coexisted during the biodegradation process. The results were in agreement with the reported biodegradation pathway of Triton X-100 (4-*tert*-octylphenol ethoxylates with average value of 9 EO number) in literature (Schmitt, 2001; Planas et al. 2002; Franska et al. 2003). In this study, the mass-to-charge ratio of 183 was also detected, which might correspond to the substance of HOOC-C_6H_4-$OC_2H_4OH+H^+$, because some alkyl-chain omega, beta-oxidations of short EO-chain CNPEO could have occurred.

Table. 1 Assignment of main peaks corresponding to mass-to-charge ratios in ESI-MS spectra.

Assignment	Molecular formular	m/z
NPEOs+NH_4^+	$C_9H_{19}C_6H_4(OC_2H_4)_nOH+NH_4^+$	282,326,370,414,458,502,546,590,634,678…
NPEOs+H^+	$C_9H_{19}C_6H_4(OC_2H_4)_nOH+H^+$	265,309,353,397,441,485,529,573,617,661…
NPECs+NH_4^+	$C_9H_{19}C_6H_4(OC_2H_4)_{n-1}OCH_2$ COOH+NH_4^+	296,340,384,428,472,516,560,604,648,692…
CNPEOs+NH_4^+	$HOOCC_8H_{17}C_6H_4(OC_2H_4)_nOH+NH_4^+$	312,356,400,444,488,532,576,620,664,708…
Others	HOOC-C_6H_4-$(OC_2H_4)_{1\sim2}OH+H^+$	183,227

With regard to the NP2EO sample, the biodegradation pattern was similar to that of NP10EO, with EO-chain omega, beta-oxidations on short EO-chain NPEOs taking place and NPEC and CNPEO being produced after 5 days of degradation. The mass-to-charge ratios of 183 and 227 belong to HOOC-C_6H_4-(OC_2H_4)1-2OH+H^+. In the following days, the benzene ring in HOOC-C_6H_4-(OC_2H_4)1–2OH was opened and ultimately degraded to CO_2 and H_2O by microorganisms.

Biodegradation of benzene rings in linear alkylbenzene sulfonate (LAS) has been studied (Hashim et al. 1992; Scott and Jones, 2000), and a similar ring-splitting reaction of benzene rings could probably occur for the substance of HOOC-C_6H_4-$(OC_2H_4)_{1-2}$OH. According to the above analysis, a complete biodegradation pathway of NPEOs was proposed and illustrated in Figure 6.

CONCLUDING REMARKS

The present study demonstrated that benzene rings in NPEOs were degradable by microorganisms in acclimated active sludge. The majority of NPEOs were removed after 8-10 days of degradation. The complete biodegradation pathway of NPEOs was proposed by the identification of degradation intermediates. Stepwise omega, beta-oxidation or fission on EO chains, followed by alkyl-chain omega, beta-oxidation on short EO-chain NPEOs, are the main degradation pathway in the early stage. Then the complete biodegradation occured after benzene rings were opened.

ACKNOWLEDGMENTS

The authors are grateful thanks the supports of Center of Drug Analysis and Test of School of Pharmacy of Shandong University.

Financial support: National Natural Science Foundation of China (No.20872151).

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How to reference this article:

WANG, P.; NONG, X.-H. and GE, J.-H. (2011). Aerobic biodegradation of nonylphenol ethoxylates in shaking-flask test. *Electronic Journal of Biotechnology*, vol. 143, no. 4. http://dx.doi.org/10.2225/vol14-issue4-fulltext-1