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Anaerobic nonylphenol ethoxylate degradation coupled to nitrate reduction in a modified biodegradability batch test

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Abstract

The aim of this work was to elucidate the role of nitrate as a terminal electron acceptor on the biodegradation of NPEO. We have characterized the products of NPEO degradation by mixed microbial communities in anaerobic batch tests by means of HPLC, ^{1}H NMR and GC–MS. Anaerobic degradation of NPEO was strictly dependent on the presence of nitrate. Within seven days of anoxic incubation, NP2EO appeared as the major degradation product. After 21 days, NP was the main species detected, and was not degraded further even after 35 days. Nitrate concentration decreased in parallel with NPEO de-ethoxylation. A transient accumulation of nitrite was observed within the time period in which NP formation reached its maximum production. The observed generation of nonylphenol coupled to nitrate reduction suggests that the microbial consortium possessed an alternate pathway for the degradation of NPEO, which was not accessible under aerobic conditions.

Keywords: Nonylphenol polyethoxylates; Endocrine disruptor; Anoxic biodegradation

1. Introduction

The fate of surfactants in the environment is a matter of concern, considering the substantial release that follows their use in domestic and industrial product formulations and as an adjuvant in bioremediation in the field (Ward et al., 2003). Commercial NPEO are complex mixtures of isomers with differently branched nonyl substituents, containing a range of units in the condensed ethylene oxide (EO) chain, usually between 3 and 20.

A large proportion of this class of nonionic surfactants is ultimately discharged through wastewater treatment plants (WWTP). As a result of the discharge of treated, but also untreated effluents (Hale et al., 2000), NPEO breakdown products consisting mainly of shorter ethoxylated homologues and nonylphenol (NP), accumulate in the aquatic environment (Ying et al., 2002). These metabolites are suspected to have endocrine disrupting activity, in addition to other toxic effects (White et al., 1994). Emissions from WWTPs vary significantly with respect to the content of NPEO and NP (Lee and Peart, 1999; Hale et al., 2000). This variability has been attributed to the load of the surfactants in influent streams, plant design and operating conditions, and other factors such as temperature of treatment (Maguire, 1999).

Aerobic biodegradation of branched nonylphenol ethoxylates by both pure and mixed cultures leads to the formation of shorter ethoxymers ranging from 1 to 4 EOs (John and White, 1998; Manzano et al., 1999; Lozada et al., 2004), nonylphenol ethoxycarboxylates (NPEC)

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(Field and Reed, 1996; Di Corcia et al., 2000) and highly water soluble short ethoxy forms of the carboxylated side alkyl chain (CAPEC) (Di Corcia et al., 2000; Ferguson and Brownawell, 2003), which are more resistant to degradation. The extent of anaerobic degradation appears to be slower (Salanitro and Diaz, 1995; Ferguson and Brownawell, 2003), but proceeds one step further to form NP as the ultimate degradation product (Ejlertsson et al., 1999). Despite the environmental significance of nitrate as electron acceptor, there are only few (and conflicting) reports about APEOs degradation under nitrate-reducing conditions (Jimenez-Gonzalez et al., 2003; Chang et al., 2004; Mohan et al., 2006). Further understanding of the mechanisms of anaerobic transformation of NPEO in the environment has been hampered by the lack of clear evidence of the nitrate-dependent, anaerobic degradation of NPEO. The aim of this study was to assess whether NPEO has any potential for biodegradation under denitrifying conditions. Inherent biodegradability tests attain most favorable conditions for biodegradation by the use of high inoculum to substrate ratio. Specific analytical methods were used to follow the course of biodegradation. High-performance liquid chromatography (HPLC) provided the basis for the analysis of the degradation profile of NPEO. Coupled gas chromatography–mass spectrometry (GC–MS) was used for confirmation of the presence of 4-nonylphenol (NP) (Giger et al., 1981). Positive identification of NP was further supported by proton nuclear magnetic resonance (Flanagan et al., 1963; Giger et al., 1984), which also allowed the quantification of the relative extent of deethoxylation.

2. Materials and methods

2.1. Chemicals and reagents

Cyclohexane, hexanes (95% *n*-hexane/5% branched isomers) and 2-propanol were of HPLC grade (J.T.Baker, NJ, USA). Technical NP (85% 4-NP, Fluka, Buchs, Switzerland), 4-NP1EO and 4-NP2EO (Promochem, Wesel, Germany), were used as analyte standards. Nonylphenol ethoxylate was a commercial preparation with an average number of 10 ethylene oxide units (NP10EO), purchased from Proquimia SA, Chile. All other reagents were analytical grade.

2.2. Experimental set-up

Inocula were obtained from two replicate 3 l semi-continuous activated sludge units, which had been started with sludge obtained from the aeration chamber of a treatment plant receiving mixed industrial and domestic wastewater. A number of 16S rDNA-based analyses revealed high bacterial diversity in the sludge (Lozada et al., 2004). A composite sample taken from both units was added in each bioreactor to yield volatile suspended solids (VSS) concentration of 320 ± 15 mg l^{-1}, measured according to standard methods.

Bioreactors consisted of 250 ml brown-glass serum bottles containing mineral medium, according to the Zahn–Wellens method (ISO:9888, 1999), amended with NPEO, provided as the sole added organic carbon (except for carbon associated with the biomass) to a final concentration of 300 mg l^{-1}. Dissolved oxygen was displaced by bubbling N_2 for 10 min, after which its absence was verified by the method of Winkler. Bottles were capped with butyl rubber stoppers and aluminum caps. Potassium nitrate was added to a final concentration of 10 mM. Endogen activity controls (biomass + mineral medium + NO_3^-), fermentative controls (biomass + mineral medium + NPEO) and alternative conditions (potassium sulfate or potassium carbonate 10 mM) were also performed. Reactors were placed under diffuse light at 24 ± 2 °C and reaction mixtures were homogenized by magnetic stirring.

2.3. Sampling and analytical procedures

Time course experiments were performed by setting up replicate serum bottles. Following incubation, flasks were sacrificed at various times for analysis. Total flask contents were transferred to polypropylene tubes and centrifuged at 15 000 rpm for 15 min. A volume of 150 ml of pooled supernatants were immediately processed by solid phase extraction (SPE) in polypropylene columns packed with 0.5 g of C-18 reverse phase sorbent (ICN, Belgium). Elution was carried out with 1 ml ethyl acetate at a flow of 1 ml min^{-1}. Eluates were collected in 2 ml borosilicate vials and evaporated gently with N_2. Dried extracts were stored at 4 °C. The remaining 50 ml of supernatant was used to follow nitrate depletion by both UV detection and ion chromatography. Colorimetric determination of nitrite was performed with an AquaMerck kit (Merck, Germany).

2.4. HPLC instrumentation and procedures

The liquid chromatography system consisted of a Spectra SERIES P200 binary pump and a SpectraSERIES UV100 UV/visible detector (Thermo Separation Products, USA). Data were acquired and processed with the Konikrom 5.2 software (Konik Instruments, Spain). Separations were performed by using a 5 μm particle aminosilica column, 250×4.6 mm (Pinnacle II, Restek, USA). Elution of individual oligomers was carried out by binary gradient. Mobile phases were A: hexanes (95% *n*-hexane/5% branched hexanes) and B: 2-propanol. Column temperature was controlled to 50 ± 0.1 °C using an Eppendorf CH-30 column heater and an Eppendorf TC-50 controller (Alltech, IL, USA). The program followed was: 10 min isocratic hold with 96% A–4% B, then a linear gradient through 50% A–50% B in 32 min and finally 6 min isocratic hold with 100% B. Flow rate was 1.0 ml min^{-1}. Identification of chromatographic peaks for compounds with less than three ethylene oxide units was made directly with

standard spikes of t-NP, 4-NP1EO and 4-NP2EO from 1.0 g l^{-1} stock solutions of the chemicals in cyclohexane, while the higher ethoxymers were assigned from their HPLC elution sequence.

All assays included replicates ($n \geqslant 2$) and blanks for quality control. Detection limit was around 4 mg l^{-1} for the NPEO surfactant mixture and 0.1 mg l^{-1} for NP, NP1EO and NP2EO. The presence of NP10EO or any of its metabolites in the blank samples was never detected by HPLC analysis. Recovery tests for NPEO were performed by subjecting the samples at time 0 to the same protocol applied to the time course samples. Results showed a recovery of 70% for the surfactant, with a relative standard deviation of 20% ($n = 2$). Recoveries of the lipophilic degradation products (NP, NP1EO and NP2EO) were assessed by spiking nanopure water with known concentrations of each compound. Recoveries ranged from 85% to 90%, with relative standard deviations of 10% ($n = 8$).

2.5. Gas chromatography coupled to mass spectrometry (GC–MS)

GC–MS analyses were performed with a Hewlett Packard HP-5890 Series II split–splitless gas chromatograph, equipped with an HP-Innowax capillary column (30 m long), coupled to a HP 5971A quadrupole mass spectrometry detector (electron impact ionization at 70 eV) operated in scan mode. One μl of sample was injected using an Agilent 6890 Series injector. Instrumental conditions were: injector and detector temperature: 280 °C; oven temperature programming: 160 °C (1 min)–8 °C min^{-1}–290 °C; column flow rate: 1.8 ml min^{-1}.

2.6. Nuclear magnetic resonance (^1H NMR)

NMR experiments were carried out at 25 °C on a Bruker Avance DPX400 MHz spectrometer equipped with

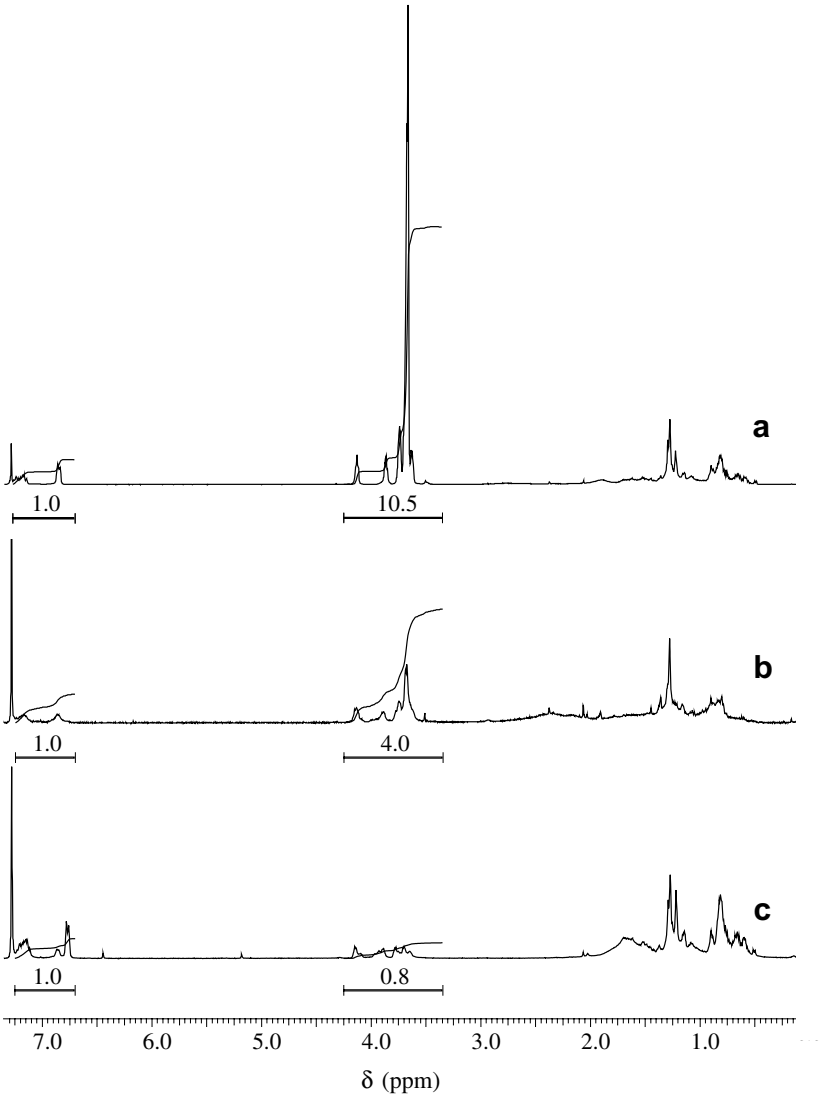

Fig. 1. Proton NMR spectra (400 MHz) of nonylphenol ethoxylate (NPEO) extracts and peak integrals of the protons of the ethoxy (EO) and aromatic (Aro) regions. (a) Stock NPEO, (b) after 35 days of aerobic incubation, (c) after anoxic incubation in the presence of 10 mM potassium nitrate.

a ^{1}H–^{13}C–^{15}N–^{31}P QNP probe. Dried extracts from the SPE procedure were dissolved in 1 ml of deuterated chloroform (99.8%, Merck, Germany) and transferred to 5 mm NMR tubes (Wilmad–Labglass, NJ, USA). Tetramethylsilane was used as an internal standard. Main spectra acquisition characteristic were: pulse program zg30; spectral width of 20 ppm; resolution of 0.12 Hz/point; recycle delay of 1 s; 16 scans and 32 K time domain points.

3. Results

Primary biodegradation of NPEO was initially followed through magnetic resonance analysis. The average ethoxylate units value, calculated from the ratios of the integration of the signals between 3.0 ppm and 4.2 ppm to those between 6.7 ppm and 7.25 ppm had been previously used for categorization of APEOs (Flanagan et al., 1963). The former signals were assigned to the protons in the ethoxy moiety, while the latter were assigned to the protons in the aromatic ring. This ratio (EO/Aro ratio) indicated an average of approximately 10 ethoxy units for the spectrum taken at the start of the experiments (Fig. 1a). As expected, NPEO degradation occurred upon aerobic incubation (Fig. 1b), and the EO/Aro ratio decreased to 4. In the absence of molecular oxygen, a reduction in the EO/Aro ratio to approximately 0.8 also indicated that degradation took place when nitrate was used as an alternative terminal electron acceptor (Fig. 1c). In contrast, the EO/Aro ratio remained unchanged in tests conducted for 35 days under fermentative conditions or when the incubations were performed under anoxic conditions with added sulfate or carbonate (not shown).

HPLC data on samples taken over a time scale of several days showed that degradation of NPEO under anoxic conditions evolved into the formation of NP as the main end product (Fig. 2). Within seven days after the start of the experiment NP2EO appeared as the major degradation product (Fig. 2b). NP1EO became the predominant metabolite within 14 days, though a noticeable amount of NP2EO was still present (Fig. 2c). After 21 days, NP was the main species detected by HPLC (Fig. 2d), and the chromatographic profile remained unaltered after 35 days (Fig. 1e).

Formation of NP was further confirmed by means of GC–MS (Fig. 3). In agreement with HPLC data, detection of NP became clear within 21 days. Ten peaks were resolved by gas chromatography. The mass spectra of these peaks exhibited the same molecular ion of m/z 220, with fragment ions at m/z 107, 121, 135, 149, and 191 (Fig. 3 and Fig. S1). It has been shown previously that nonylphenol isomers could be classified into three groups according to the base peak (Lee and Peart, 1995). Four of the isomers exhibited mass spectra with a base peak at m/z 135 ($[M–COCH_2–C_6H_{13}]^+$) suggesting an α, α-dimethyl structure on the nonyl chain. The second group of NP isomers had a base peak at m/z 149 ($[M–COCH_2–C_5H_{11}]^+$) consistent with structures having α-methyl-ethyl side chains. The

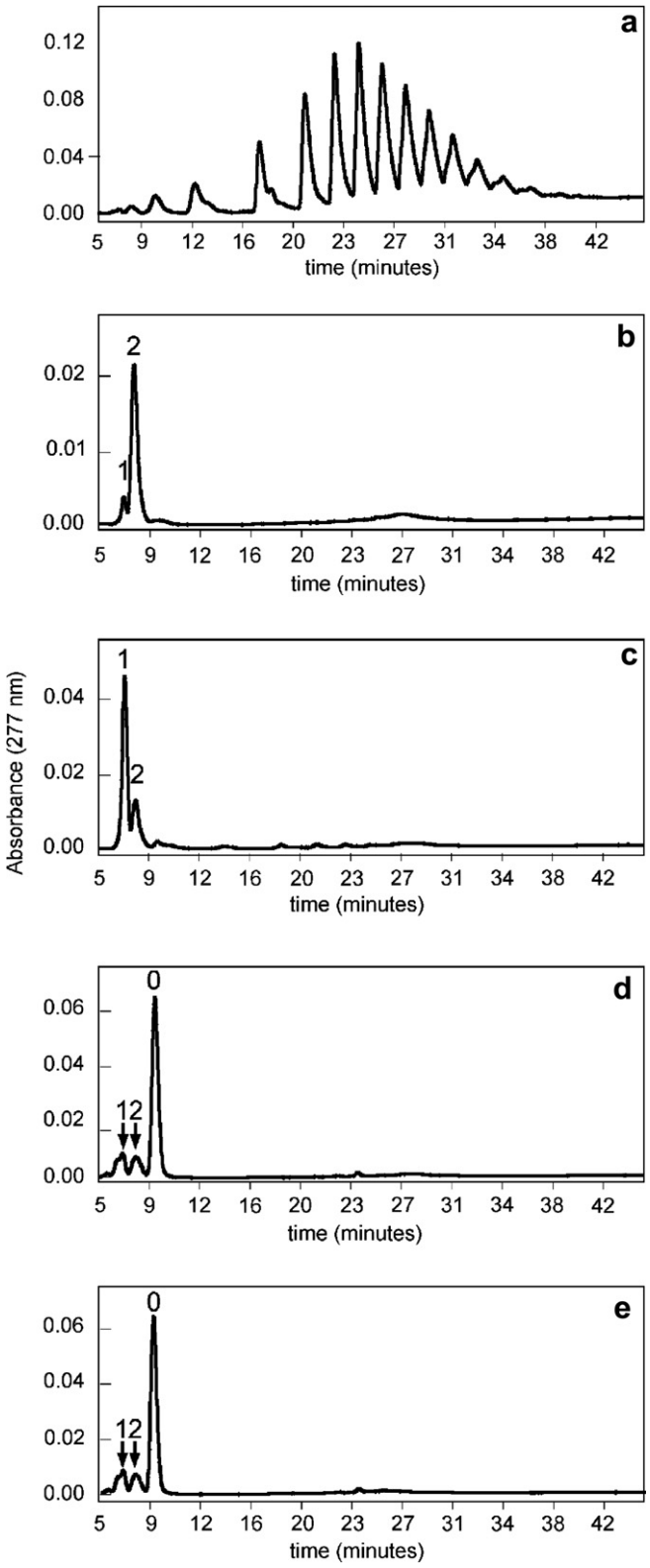

Fig. 2. High-performance liquid chromatography profiles of test samples taken from experiments incubated anaerobically in the presence of 10 mM potassium nitrate at (a) the beginning of the experiments, (b) 7 days, (c) 14 days, (d) 21 days and (e) 35 days. Peak numbers correspond to NPEOs with the indicated chain length. Identification of chromatographic peaks was made directly with the spike of standards of NP, 4-NP1EO and 4-NP2EO.

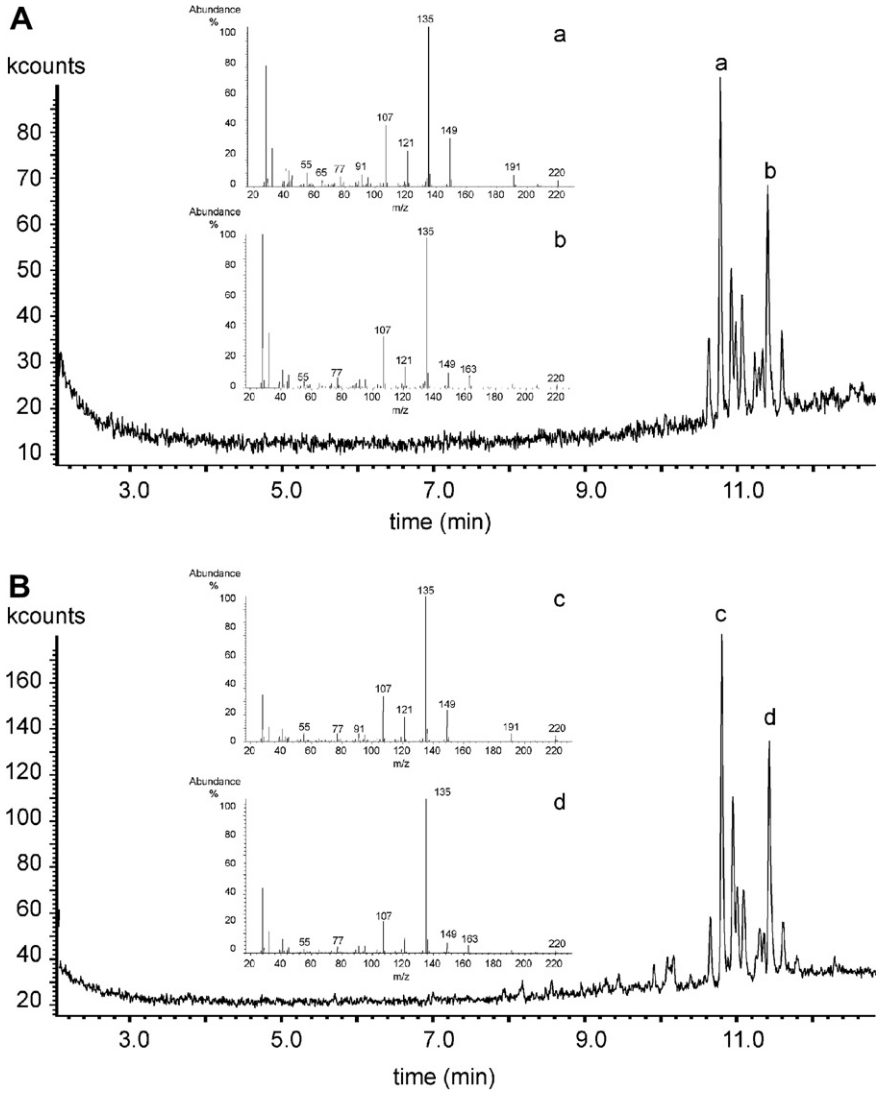

Fig. 3. (A) GC/MS chromatogram of the product of degradation of NPEO by the mixed consortia after 21 days of anoxic incubation in the presence of 10 mM potassium nitrate. (B) GC/MS chromatogram of technical 4-NP. Insets show mass spectra obtained for two selected isomeric components, eluting with retention times of (a) 10.74 min, (b) 11.38 min, (c) 10.77 min and (d) 11.40 min.

third group of two isomers had a base peak at *m*/*z* 121 ($[M–COCH_2–C_7H_{15}]^+$), consistent with a substituted benzyl ion having the α-methyl structure (Lee and Peart, 1995).

The profile of NP isomers detected after NPEO degradation (Fig. 3A) was similar to the pattern observed in the technical grade formulation used as standard (Fig. 3B), suggesting that most of the isomers experienced the same signs of susceptibility to anoxic biodegradation. Two exceptions were the isomers eluting at 11.04 min and 11.20 min, which displayed higher relative intensities in the product of anoxic degradation, compared with those present in technical grade NP. The deviations may be due to differences in isomer composition of the raw material used to synthesize NPEO or to the higher recalcitrance of the two referred isomers. We are not able to distinguish between these two possibilities with the present evidence. Yet the distinction may be important, as estrogenic activity may vary among isomeric forms of nonylphenol (Kim et al., 2004).

Inspection of low field resonances in ^1H NMR spectra obtained from the samples taken at 21, 28 or 35 days after the start of the experiment showed that the chemical shift of aromatic protons in ortho to the phenoxy moiety in NPEO was strongly influenced by the substitution in the phenolic oxygen. As a result, it was possible to address quantitatively the ratio between NP and the mixture of NPEOs in the aqueous phase. Signals between 6.86 ppm and 6.84 ppm (Fig. 4a), assigned to the protons in position 2- and 6-, splitted into two populations at day 35 (Fig. 4b). One of the populations shifted to slightly lower fields, between 6.87 ppm and 6.85 ppm, while the second one was shifted to upper fields (6.76–6.78 ppm), matching the chemical shift of the corresponding protons in NP (Fig. 4c). On the basis of the integration of the respective

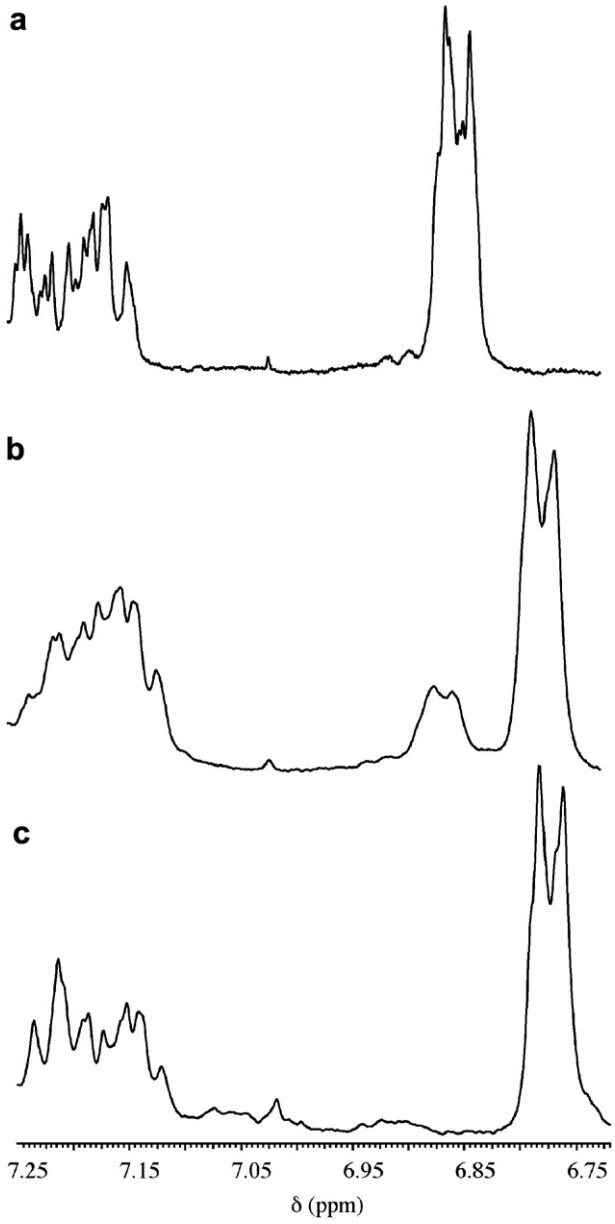

Fig. 4. Aromatic region of ^{1}H NMR spectra of samples taken at days (a) 0 and (b) 35 of anaerobic incubation in the presence of 10 mM potassium nitrate and (c) technical grade NP.

signals, the proportion of aromatic rings containing non-alkylated oxygen. (i.e., NP) at day 35 was estimated as 70% of total NPEO species. The calculation of the average number of ethoxy groups after 35 days of anoxic incubation, obtained from the ^{1}H NMR spectra, was 0.8 when the ratio of the integration of the ethoxy and aromatic peaks (EO/Aro ratio) included the aromatic protons of NP (see Fig. 1a). This ratio was weighted by the higher proton content of the longer chain homologues. A careful examination of Fig. 4 allowed the evaluation of the ratio between the protons of the ethoxy units (EO = 5.85) and the protons assigned to the 2- and 6-protons in the aromatic ring of NPEO molecules, excluding NP ($Aro_{EO} = 1.05$) by integration of the areas under the

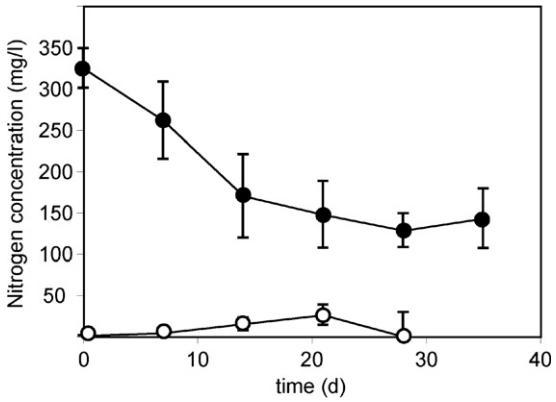

Fig. 5. Time curves of nitrate (solid circles) and nitrite (blank circles) during anoxic incubation, using NPEO as the only source of carbon. Error bars indicate standard deviation ($n = 6$).

appropriate NMR signals. Considering that only half of the protons in the aromatic ring were accounted for, the corrected value for the average ethoxylation degree of NPEO molecules that did not go through complete de-ethoxylation could be estimated as 2.8.

Nitrate concentration decreased in parallel with NPEO degradation while nitrite concentration increased from day 7 up to a maximum of 26.5 mg^{-1} of NO_2^-–N at day 21, in concurrence with NP formation (Fig. 5).

4. Discussion

Since the first report describing the finding of significant concentrations of NP in anaerobic digesters (Giger et al., 1984), a considerable amount of evidence has been collected from a variety of environments documenting the biodegradation of NPEOs and the subsequent accumulation of NPs under anaerobic conditions (Maguire, 1999). Previous work suggested that microbial biotransformation of NPEO proceeds via separate pathways in oxic and anoxic environments (Ferguson and Brownawell, 2003). While both geochemical conditions can lead to the disappearance of higher NPEO homologs through shortening of the ethoxy chain, only anoxic conditions lead to complete de-ethoxylation. Since further anaerobic degradation was never observed, NP represents the ultimate degradation product of NPEO under anaerobic conditions (Giger et al., 1984; Ejlertsson et al., 1999; Maguire, 1999). In contrast, several studies indicated that mineralization of NP is feasible in aerobic environments (Ekelund et al., 1993; Topp and Starratt, 2000). Collectively, these results suggest that alternating redox conditions would be required in order to achieve complete mineralization of NPEO. According to our experimental set-up, anaerobic degradation of NPEO was strictly dependent on the presence of nitrate. There was no indication of biodegradation of NPEO when nitrate was replaced by sulfate, bicarbonate or in electron acceptor-free controls. These results contrast with previous works that achieved biodegradation of

NPEOs under methanogenic conditions in landfilled solid waste (Ejlertsson et al., 1999), sludge (Giger et al., 1984; Ejlertsson et al., 1999) and wastewater (Schroder, 2001), and with the finding of enhanced degradation rates of NP1EO under sulfate-reducing conditions, compared to inoculated controls (Chang et al., 2004). On the other hand, other studies did not find net production of NP during anaerobic NPEO degradation (Ferguson and Brownawell, 2003), and a more recent report found no evidence of biodegradation of octylphenol ethoxylate under nitrate-reducing conditions (Mohan et al., 2006).

A transient accumulation of nitrite was observed within the time period in which NP formation reached its maximum production. Two possible explanations are consistent with this finding. The lack or deficiency of electron sources from organic carbon has been reported to influence the accumulation of nitrite during denitrification, as nitrate respiration out-competes nitrite respiration (Oh and Silverstein, 1999). Alternatively, not all organisms that utilize nitrate can perform the entire denitrification pathway (Philips et al., 2002). A model with enrichment of facultative anaerobes that reduce nitrate only to nitrite on the expense of true denitrifiers has been proposed to account for nitrite accumulation frequently observed during wastewater treatment (Wilderer et al., 1987). Several process conditions, such as the carbon source, C/N ratio and hydraulic retention time (Martienssen and Schöps, 1999), will influence the composition of the microbial community, and ultimately the efficiency of nitrite reduction.

The anoxic degradation of NPEO halted after yielding for the most part NP, even though nitrate was still largely available. Whether this was due in part to the absence of nitrite, the presence of more recalcitrant NPEO isomers or the increased cell death, the reasons why the degradation did not advance further remain to be elucidated.

The above discussion points to the fact that anoxic biodegradation of APEO is a rather complex transformation that necessitates a specialized combination of electron acceptor and microorganisms to occur. Therefore, the fate of these nonionic surfactants in the environment is not only determined by local geochemical conditions, but is also highly dependent on the composition of the autochthonous microbial community.

After the discharge of treated or untreated NPEO, the species that enter the aquatic environment may be the substrate for further degradation. In this work, we focused solely on the degradation processes taking place in the aqueous phase. In addition to enzymatically-mediated transformation, NPEO removal may be affected by sorptive processes, which in turn are influenced by the degree of ethoxylation. High partition coefficients for *n*-octanol/water have been determined for NP (4.48) and for short chain NPEO (EO: 1-3) (4.2) (Ahel and Giger, 1993). Therefore, as the shortening of the ethoxy chain progresses, the breakdown products are more likely adsorbed to the organic phases of the sludge and suspended solids (John et al., 2000). In effect, the rapid disappearance of newly formed OP from the aqueous phase of an upflow anoxic sludge blanket reactor continuously fed with octylphenol polyethoxylates was rationalized in terms of sorption to the sludge components (Jimenez-Gonzalez et al., 2003). However, sorption characteristics may vary with the substrate and with the type of organic matter (Moore and Matos, 1999). Under the experimental conditions used in this work, sorption saturation appeared to have been reached at 21 days. While looking only at the solution phase prevented us from performing a mass balance for each metabolite, the observation that NP is the main species present in the aqueous phase after incubation under nitrate-reducing conditions is strengthened by the fact that the more lipophilic homologues will associate more readily with organic phases of the sludge (Ahel and Giger, 1993; John et al., 2000), and therefore their concentrations (i.e., the extent of biodegradation) would be most likely underestimated. In conclusion, we have shown that NPEO degradation continues to NP under nitrate-reducing conditions in the presence of a suitable microbial consortium. Because nonylphenol is amenable to further degradation by aerobic nonylphenol metabolizing bacteria, the results presented here provide a basis for further development of sequential anoxic–aerobic biodegradation strategies for the treatment of sewage containing nonylphenol ethoxylates.

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Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chemosphere.2007.01.078.

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