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Research Paper

Biodegradation of Nonylphenol Ethoxylate Surfactants in Biofilm Reactors

The primary degradation of a technical nonylphenol ethoxylate surfactant with an average chain length of 10 ethoxylate units (NPEO-10) was studied in a flow-through system by means of miniaturized biofilm reactors (mBFR) with bacteria from an activated sludge plant. 5 mg/L of the test compound (total EO concentration) were spiked in synthetic wastewater (SWW) and fed to the reactors continuously for 64 days. Compound removal and the formation of degradation products (DP) were monitored under both oxic and anoxic conditions. Solid-phase extraction and RP-HPLC with fluorescence detection were employed for sample preparation and analysis. Better removal of the parent compound was seen with the oxic reactors (50 to 70 %) than with the anoxic reactors (30 to 50 %). Compared to SWW organic matter, the test compound proved to be of refractory nature. The appearance of degradation products in the effluent was earlier with anoxic reactors despite their lower elimination efficiency. After extraction of biomass only minor amounts of NPEO-10 and metabolites were found, indicating that small amounts were present in adsorbed or intracellular form. Ultimate biodegradation of NPEO-10 and of octylphenol ethoxylates (OPEO-9.5; average chain length of 9.5 EO units) was tested by means of manometric respirometry at a theoretical oxygen demand (ThOD) of 100 mg/L. Whereas NPEO-10 was biodegraded by only 26 %, at best, in 28 days, OPEO-9.5 degradation amounted to (40 ± 5) %.

Biologischer Abbau von Nonylphenolethoxylaten in Biofilmreaktoren

Der Primärabbau eines technischen Nonylphenolethoxylat-Gemisches mit einer durchschnittlichen Kettenlänge von 10 Ethoxygruppen (NPEO-10) wurde unter Verwendung von miniaturisierten Biofilmreaktoren (mBFR) untersucht. Die Reaktoren wurden mit Biomasse aus der Belebungsstufe einer kommunalen Kläranlage inokuliert und als Durchfluss-Testsystem über eine Dauer von 64 Tagen betrieben. Die Konzentration an NPEO-10 betrug 5 mg/L (Gesamtkonzentration aller Ethoxylate) in einer Matrix aus synthetischem Abwasser. Die Elimination der Testsubstanz sowie die Bildung von Abbauprodukten wurde unter oxischen und anoxischen Bedingungen untersucht. Als analytische Methoden wurden Festphasenextraktion und RP-HPLC mit Fluoreszenzdetektion eingesetzt. In den oxischen Reaktoren (50...70 % Elimination) konnte eine bessere NPEO-10-Elimination erreicht werden als in den anoxischen Reaktoren (30...50 % Elimination). Die Testsubstanz erwies sich als refraktärer gegenüber biologischem Abbau als die organische Substanz des synthetischen Abwassers. Trotz der geringeren Eliminationsleistung der anoxischen Reaktoren wurden dort früher Abbauprodukte im Reaktorablauf beobachtet als in den oxischen mBFR. Nach Extraktion der Biomasse der Testreaktoren konnten nur geringe Mengen an NPEO-10 und Abbauprodukten gefunden werden, was zeigte, dass nur ein kleiner Teil in adsorbierter oder intrazellulärer Form vorlag. In einem geschlossenen Respirometer wurde die vollständige biologische Abbaubarkeit von NPEO-10 sowie eines Octylphenolethoxylat-Gemisches (OPEO-9.5; durchschnittliche Kettenlänge von 9.5 Ethoxygruppen) bei einem theoretischen Sauerstoffbedarf (ThOD) von 100 mg/L untersucht. Während NPEO-10 nur zu maximal 26 % des ThOD abgebaut wurde, wurde für OPEO-9.5 ein Abbau von (40 ± 5) % erreicht.

Keywords: Nonionic Surfactant, Nonylphenol, RP-HPLC Fluorescence, Biomass Concentration

Schlagwörter: Nichtionisches Tensid, Nonylphenol, RP-HPLC-Fluoreszenz, Biomassekonzentration

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1 Introduction

Alkylphenol ethoxylates (APEO) are nonionic surfactants which are widely used in the industry as cleaning products and process aids such as emulsifiers or dispersing agents. They are high production volume chemicals. In the year 1995 in Germany alone 23 100 t of nonylphenol (NP) were produced annually, 82% of which were used further for the production of nonylphenol ethoxylates [1]. Of all APEO, nonylphenol ethoxylates (NPEO) form the major share (80...90%). As a result of their widespread use, they are common contaminants in many industrial and domestic wastewaters [2].

Biodegradation (primary degradation) of APEO in sewage treatment plants (STPs) leads to the formation of hydrophilic as well as lipophilic metabolites. It has been estimated that, of all the nonylphenolic compounds that enter the STPs, about 60...65% are released to the environment (as metabolites or parent compounds) via secondary effluents (40%) and sewage sludge (20%) [3]. The major metabolites under aerobic conditions are carboxyethoxylates (APEC) and carboxylated APEC (CAPEC) as well as lower ethoxylates, whereas in the absence of oxygen short-chain APEO with 1 or 2 EO units and alkylphenols (AP) are formed as intermediate products [3, 4].

The short-chain ethoxylates and alkylphenols are more toxic to aquatic life than the parent compounds and have been reported to act as endocrine disrupters in various biological test systems [5, 6]. The lipophilic metabolites have also been reported to accumulate in organisms [5]. Humans are also susceptible to exposure to these compounds via drinking water, and chlorinated derivatives of these compounds have been found in drinking water supplies [7].

Standardized biodegradation test methods, such as closed-bottle test or die-away test, help in assessing the environmental acceptability of compounds as the test results relate to the probable persistence or transience of a compound and its biodegradation intermediates in the environment. These tests give an estimate of the extent of biodegradation, but do not provide any information on the product spectra. The biodegradation of NPEO in STPs has been studied extensively [3, 8], and the process is fairly well understood. One major drawback with studies in STPs is that results are obtainable only about the final concentrations of the metabolites/by-products. Little information is obtained about the transformation pathways.

Due to their versatility biofilm reactors (BFR) are finding increasing use in continuously monitoring the biodegradability of organic compounds. They have been used to characterize the microbial elimination of organic matter [9], to measure biodegradable organic carbon in stream waters [10] as well as to measure the biodegradability of different dissolved organic substances in groundwater [11] and in water [12], or for the study of the biotransformation of organic compounds under oxic and anoxic conditions [7]. BFR are also very suitable to investigate biodegradation pathways of various test compounds under realistic conditions. They were found to be good model systems for STPs and as such have been used for the study of the biodegradability and metabolic transformation of pharmaceuticals [13, 14].

In this work miniaturized biofilm reactors (mBFR) were used to study the biodegradation of a technical NPEO surfactant at a total ethoxylate (EO) concentration of 5 mg/L in a synthetic wastewater (SWW) matrix under both oxic (with aeration) and anoxic (without aeration, presence of nitrate) conditions. Toxicity assessment was performed to ascertain the toxicity of the compound at this concentration. Biodegradation was measured as the decrease in the quantity of the test compound (total ethoxylate concentration) and dissolved organic carbon (DOC) concentration in the reactor effluents as compared to the influents. The total amount of NPEO-10 in the samples was determined by reversed-phase high-performance liquid chromatography (RP-HPLC) with fluorescence detection. To obtain information on the contribution of adsorption and intracellular uptake to overall removal, the reactor biomass was extracted with organic solvents at the end of the study, and the amounts of NPEO and degradation products in the extract were quantified. The method of manometric respirometry was used to determine the extent of the biodegradation of the compound under standard conditions.

2 Material and methods

2.1 Chemicals

Two different technical nonylphenol ethoxylate surfactants were obtained from Sasol Germany GmbH (Marl). The surfactant Marlophen NP10 (NPEO-10; homologues with 1 to 20 EO units, average of 10 EO units) was used for toxicity, mBFR, and manometric respirometry experiments. Marlophen NP3 (NPEO-3; homologues with 0 to 8 EO units; average 3 EO units) was used for toxicity testing only. The octylphenol ethoxylate surfactant Triton X-100 (OPEO-9.5) was purchased from Sigma (Steinheim, Germany). 4n-nonylphenol (4n-NP; purity > 98%) was obtained from Riedel-de-Haen (Seelze, Germany). Methanol (MeOH, HPLC-grade) and *n*-hexane were obtained from Merck, and acetonitrile (AcN, HPLC-grade) and dichloromethane from J. T. Baker (Deventer, Netherlands). All the ingredients for SWW (NaCl, peptone, meat extract, urea, $CaCl_2$, $MgSO_4$), potassium hydrogen phthalate, and sodium acetate were from Merck. Pumice stone with a size range of 0.8 to 3 mm (particle diameter) and a packaging density of ca. 400 kg/m^3 was also obtained from Merck. High-purity Milli-Q water was produced in a MilliQ Plus system (Millipore, USA).

2.2 Toxicity assessment

Acute toxicities of NPEO-10, NPEO-3, and 4n-NP to *Vibrio fischeri* were determined with the luminescence inhibition test according to the German standard method DIN 38412-34/341 [15]. NPEO-3 and 4n-NP were tested to obtain information on the effect of EO chain length on the toxicity. A dilution series consisting of 7 standards was tested for each compound. 2% NaCl solution was used as negative control. All tests were performed in duplicate, except for the control solution which was tested in quadruplicate ($n = 4$). All solutions were maintained at 15 °C and a pH of 7 ± 0.1. The bacteria were exposed to the compound for 30 min. Luminescence measurements were done using a Lumat (Berthold, Bad Wildbad, Germany). EC_{20}, which is usually taken to represent the beginning of the inhibitory effect, was determined.

2.3 Biofilm reactors

Eight mBFR were used for the purpose of this study. Four mBFR were aerated throughout the course of the experiment and are further referred to as oxic mBFR. The second set of 4 reactors was not aerated and are referred to as anoxic mBFR (based on the presence of nitrate in the tap water used for preparation of the synthetic wastewater; annual mean: 3.8 mg/L in 2002 [16]). Of the 4 reactors in one set, one was the control and the other 3 were test reactors. The controls were similar to the test reactors in all respects except that they did not receive input of the test compound. The duration of the study was 64 d after the colonization of the mBFR.

2.3.1 Miniaturized biofilm reactor setup

The mBFR consisted of a stainless steel shell inside which was placed a steel mesh mounted on plastic supports. Within the mesh was placed the media (pumice stone, approximately 2.6 g per reactor) on which the biofilm is formed. Reactor dimensions are 17 mm × 55 mm (diameter x length) with a void volume of 3.2 mL, and a hydraulic retention time of less than 10 min [13, 17]. The mBFR were run at room temperature which was ca. 25 °C. The reactors were placed upright and the liquid flow through them, at the rate of 1 mL/min, was maintained against gravity (upflow) through the use of a peristaltic pump. Aeration for the oxic mBFR was achieved by combining a separate air inlet tube with the liquid tube after the pump. With this setup, dissolved oxygen concentrations above 3 mg/L could be provided in the influent of the aerated (oxic) mBFR, whereas O_2 concentrations in the influent of the non-aerated (anoxic) mBFR remained below 0.5 mg/L [17]. The tubings on the pump were Tygon® with an internal diameter of 1.85 mm (influent) and 0.95 mm (air inlet). All other tubing was teflon. The tubings were cleaned twice a week with a mixture of 35% H_2O_2 and 1 M NaOH to prevent biofilm formation.

2.3.2 Colonization of miniaturized biofilm reactors

The bacterial seed was obtained from the activated sludge (AS) tank of a STP with both industrial and domestic influents. The AS was settled and decanted. 3 L of the decanted solution were diluted with tap water to 10 L. This solution was then fed to the reactors in a cyclic fashion for a week. Everyday concentrates for SWW were added to the solution. After a week the cyclic flow was stopped and thereafter SWW was allowed to flow through the reactors. The mBFR were fed with SWW for approximately 8 weeks, and DOC removal was measured to evaluate reactor performance. During the last couple of weeks the DOC removal averaged 80 to 85% for the oxic, and 70 to 75% for the anoxic reactors.

2.3.3 Influents

Synthetic wastewater (SWW) was prepared by adding 5 mL of concentrate A (16 g peptone from casein, 11 g meat extract, 3 g urea, 3.5 g NaCl, 2 g $CaCl_2 \cdot 2\,H_2O$, 1 g $MgSO_4 \cdot 7\,H_2O$ in 500 mL in demineralized water) and 2 mL of concentrate B (1.675 g K_2HPO_4 in demineralized water) to 10 L of tap water. The SWW had a DOC concentration of approximately 10 mg/L. During the colonization phase only SWW was fed to the reactors. During the course of the experiment the control reactors were fed with SWW, and the test reactors were fed with a SWW solution to which NPEO-10 was spiked to get a concentration of 5 mg/L. At this concentration potentially occurring adsorptive losses of NPEO-10 to container walls were found to be insignificant. New influents were prepared daily and stored in glass containers. The containers were cleaned daily with a mixture of 35% H_2O_2 and 1 M NaOH.

2.3.4 Sampling

The influents as well as the effluents from all 8 reactors were sampled thrice a week on alternate days except for the beginning (where sampling was done on consecutive days) and in the end (where no sampling was done for two weeks prior to sampling on two consecutive days). The samples for DOC measurement (15...25 mL) were taken approximately 2.5 to 3 h after preparation of the influents in the morning. Samples for analysis by HPLC were collected immediately after DOC samples into 50-mL volumetric flasks and extracted within a few hours after collection.

2.3.5 Extraction of biomass

Before extraction the dry weight of the biomass of each reactor was determined after drying at 105 °C for 24 h. The net dry weight of the biomass was calculated from the difference in dry weight before colonization (pumice stone only) and at the

end of the test phase (pumice stone including biofilm). The dried biomass and pumice stone from each reactor were then serially extracted with 20 mL of *n*-hexane and 20 mL of MeOH. During the 2 h extraction period samples were placed on an overhead shaker. After extraction the liquid phase was decanted, the solid phase was washed with another 5 mL of solvent, which was added to the 20 mL, and the liquid was then centrifuged at 2500 1/min for 5 min to remove particles. The clear liquid phase was dried in a N_2 stream at 40 °C, and the dried residue was redissolved in acetonitrile (AcN):water mixture (50:50 v/v). The extracts were analyzed by HPLC.

2.4 Sample preparation and analysis

2.4.1 Solid-phase extraction

Solid-phase extraction (SPE) using Oasis™ HLB extraction cartridges, (V = 6 cm^3, sorbent mass m = 200 mg, Waters, U.S.A.), was performed prior to HPLC analysis. Conditioning of the cartridge was done with 5 mL methanol followed by equilibration with 4 mL water. 50 mL of sample were sucked through the cartridges through teflon tubes at a flow rate of 3 to 4 mL/min. Care was taken to ensure that the same tube was used for influents and each reactor effluent every time. Loaded cartridges were washed with 2 mL of a methanol:H_2O mixture (5:95 v/v) and dried by application of vacuum. Elution was performed with 2 mL dichloromethane (CH_2Cl_2). The eluent was dried in a stream of N_2 at 40 °C. After drying, the residue was dissolved in 1 mL AcN:H_2O mixture (50:50 v/v) to give a concentration factor of 50. As NPEO-10 and 4n-NP concentrations were expected to vary by at least a factor of 10 in the mBFR experiments, different concentration factors were applied for NPEO-10 and 4n-NP analysis. For NPEO-10 analysis, 100 µL of the redissolved residue was again diluted to 1 mL with the AcN:H_2O mixture (50:50 v/v) to get a final concentration factor of 5. To ensure the complete dissolution of the analytes in the organic mixture, the vials containing the solution were kept in an ultrasonic bath for 5 min. In some cases small particles were observed in the final extract (after addition of the organic solvent), and these samples were filtered again using 0.45 µm Minisart RC4 filters (cellulose; Sartorius, Göttingen, Germany). No analyte loss due to filtration was observed. The resultant solution was analyzed with HPLC. The extraction of a SWW sample (blank) served as a method control. SPE recovery was determined for a mixture of NPEO-10 (5 mg/L) and 4n-NP (0.5 mg/L) in SWW, using three replicates each (n = 3).

2.4.2 Sample analysis with HPLC

The analysis of NPEO-10 and metabolites was performed by reversed-phase high-performance liquid chromatography (RP-HPLC) with fluorescence detection. The HPLC system was a HP 1090 with Chemstation software coupled to a HP 1046A fluorescence detector (Agilent Technologies, Waldbronn, Germany). Separations were carried out with a Zorbax RX-C18 column (dimensions 125 mm × 3 mm, 5 µm; Rockland Technologies Inc., USA). The injection volume was 20 µL. The mobile phase was a mixture of A: MilliQ-water and B: HPLC-grade AcN. At a flow rate of 1 mL/min, a gradient elution was performed starting with 70 % B which went up linearly to 90 % B in 15 min and to 100 % B in another 5 min (post time: 5 min). Detection wavelengths were 227 nm (excitation) and 305 nm (emission) at a photomultiplier (pmt) gain of 13 [18]. For quantification, 5 standards for external calibration were used. The standards were a mixture of NPEO-10 (10...50 mg/L) and 4n-NP (1...5 mg/L). 4n-NP was added for the quantification of nonylphenol isomers which were expected as primary degradation metabolites in the mBFR. The stock solutions were made in 100 % AcN and the dilutions therefrom, to get the standards, were made in AcN:H_2O mixture (50:50 v/v). In order to assess the precision of the HPLC method, the lowest and the highest mix standards were analyzed thrice each (n = 3) during each sample analysis cycle, and the average values for the two standards were used for the calibration curve. Limit of detection (LOD) and limit of quantification (LOQ) for the method employed were determined according to the German standard DIN 32645 [19] using 10 standards with concentration ranges of 1.1...11 mg/L for NPEO-10 and 0.11...1.1 mg/L for 4n-NP.

2.5 DOC measurement

The collected samples were filtered through 0.4 µm Isopore™ filters (HTTP; Millipore, U.S.A.) before being analyzed on either a Shimadzu total organic carbon analyzer (Model TOC-5050), or a Sievers total organic carbon analyzer (Model 820) with inorganic carbon remover. Calibration was done with external standards at ρ (C) = 1...10 mg/L with potassium hydrogen phthalate as the standard compound. Standards were prepared in low-organic-carbon water (Milli Q-water). The standards series was measured with every test run, and 2 replicates per sample were measured (n = 2).

2.6 Manometric respirometry

The method of manometric respirometry (as per European standard method, DIN EN 29 408 [20]), which is a 28 day oxygen demand test, was employed to study the ultimate biodegradability of NPEO-10 and OPEO-9.5. The compounds (n = 3) were tested at a theoretical oxygen demand (ThOD) of 100 mg/L. Sodium acetate was used as the positive control, and demineralized water was used as the negative control. A ThOD of 100 mg/L corresponds to 45.0 mg/L of NPEO-10, to 48.8 mg/L of OPEO-9.5, and to 128.7 mg/L of sodium acetate.

The inoculum used in the test was prepared using activated sludge from the same STP as for the mBFR colonization. 5 mL of the activated sludge were centrifuged for 8 min at 2500 1/min. The liquid phase was decanted and the remainder was washed twice with isotonic NaCl, centrifuged and the liquid phase decanted. Finally the residue was resuspended in isotonic NaCl and stored at 4 °C until use. 100 µL of the inoculum were added to 250 mL of sample. The electrolytic respirometer Sapromat (Voith, Heidenheim, Germany) was used to measure the oxygen demand (OD) over 28 d. The biodegradation is reported as % ThOD and calculated according to [12]:

$$\%\ ThOD = [(OD_{sample} - OD_{demin})/ThOD] \cdot 100\% \quad (1)$$

where *OD* is the oxygen demand in mg/L measured with the respirometer.

3 Results and discussion

3.1 Toxicity

The EC_{20} values of the test compounds were 32 mg/L for NPEO-10, 6 mg/L for NPEO-3, and 1 mg/L for 4n-NP. When

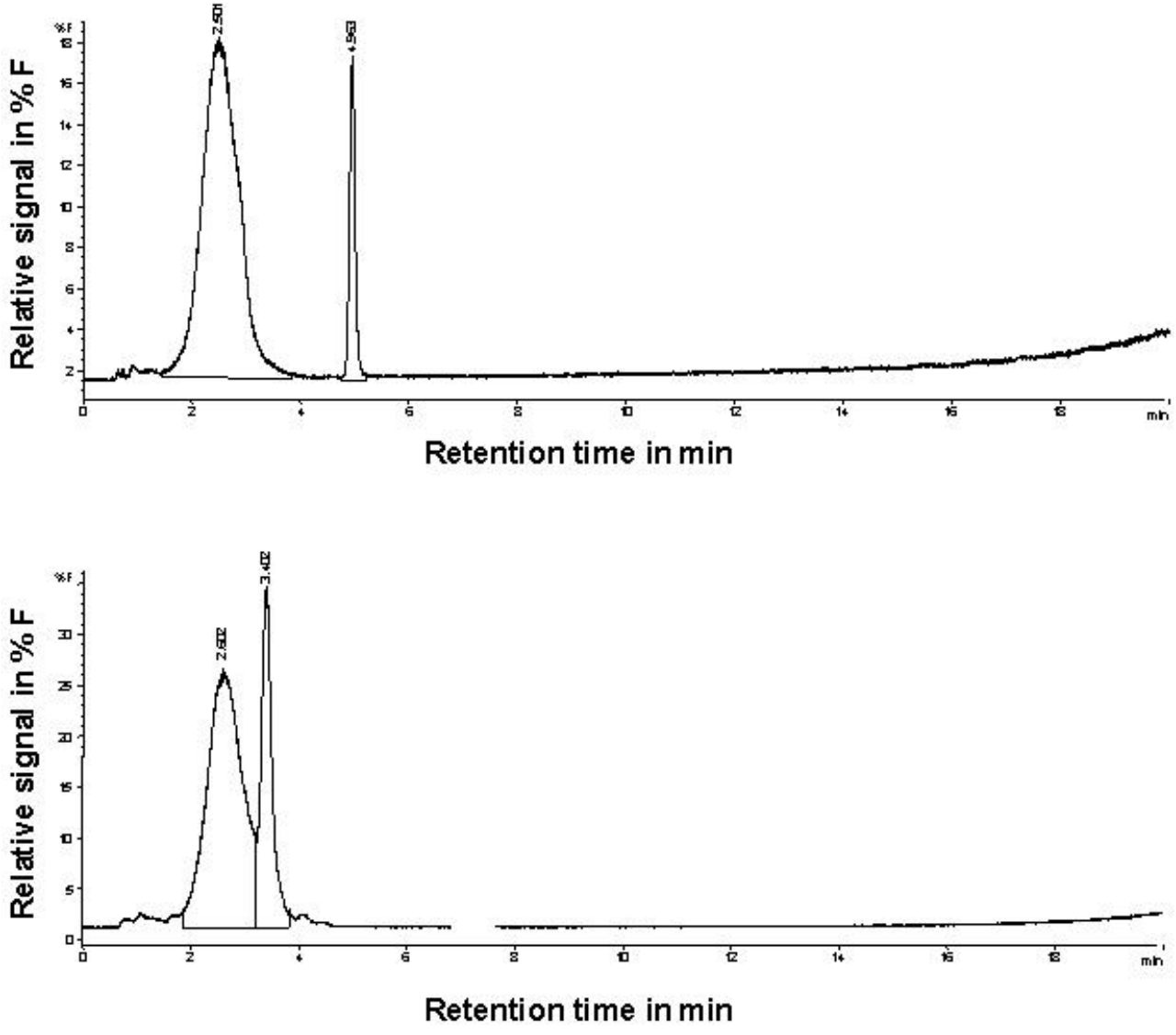

Fig. 1: HPLC-fluorescence chromatograms of NPEO-10 and metabolites. Above: NPEO-10 and 4n-NP standard; below: appearance of degradation products as shoulders in NPEO-10 peak (anoxic reactor).

HPLC-Fluoreszenz-Chromatogramme von NPEO-10 und Abbauprodukten. Oben: NPEO-10- und 4n-NP-Standard; unten: Auftreten von Abbauprodukten als Schulter des NPEO-10-Peaks (anoxischer Reaktor).

comparing the EC_{20} values for the three compounds, a marked decrease is seen as one proceeds from the higher to the lower ethoxylates, and to the completely non-ethoxylated compound. The decrease is only about 5 fold for the reduction in the average length of the EO chain from 10 to 3 units, but for the reduction in average length from 3 to 0 units is about 6 fold. 4n-NP is seen to be more than 30 times more toxic than NPEO-10. This clearly brings out the fact that toxicity increases much more rapidly with decreasing EO chain for lower ethoxylates than for the higher ones. From these tests it is safe to conclude that the NPEO-10 (as Marlophen NP10) would not be toxic to the bacteria at the concentration of 5 mg/L (used in the tests with the mBFR).

3.2 Analytical results

The precision with the SPE method was very good. The recoveries ranged from (96 ± 2)% (mean ± *SD* for $n = 3$) for NPEO-10 and (61 ± 1)% for 4n-NP. The RP-HPLC method was also very reproducible. The coefficient of variation (*CV*) was lower than 0.5% in most cases for the lowest and the highest concentration standards ($n = 3$) and did not exceed 2.5% in any case. The LODs for NPEO-10 (total ethoxylates) and 4n-NP were 0.4 mg/L and 0.02 mg/L, respectively. Likewise the LOQs for the two compounds were 0.8 mg/L and 0.04 mg/L.

NPEO-10 eluted as a broad peak, while the peak for 4n-NP was sharp (Fig. 1, above). Later during the experiment, shoulders on the NPEO-10 peak and eventually separate peaks with retention times midway that of NPEO-10 and 4n-NP appeared, and these were classified as degradation products (DP) (Fig. 1, below). Due to lack of information on the identity of the DP and, hence, also lack of standards, mass concentrations of the metabolites were determined using the NPEO-10 calibration data.

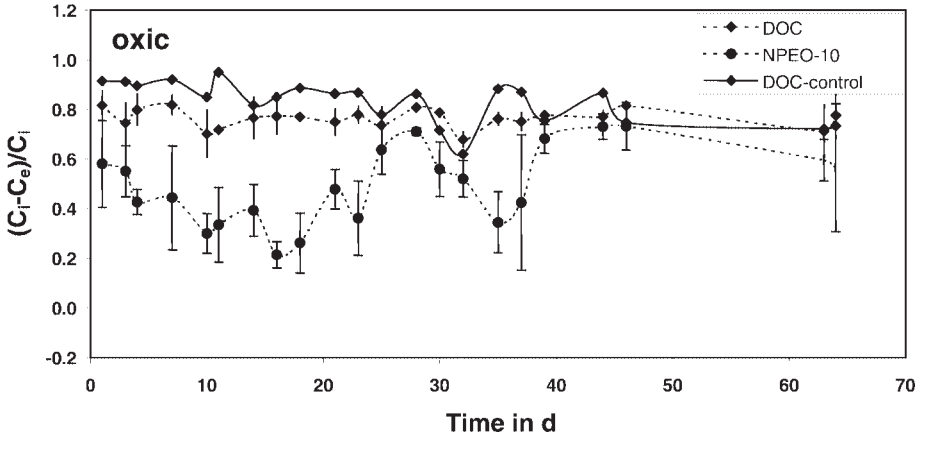

Fig. 2: Removal of NPEO-10 and DOC in oxic mBFR. C_i: concentration of NPEO-10 resp. DOC in influent, C_e: concentration of NPEO-10 resp. DOC in effluent. Error bars: standard deviation for $n = 3$.

Elimination von NPEO-10 und DOC im oxischen miniaturisierten Biofilmreaktor. C_i: Konzentration an NPEO-10 bzw. DOC im Zulauf; C_e: Konzentration an NPEO-10 bzw. DOC im Ablauf. Fehlerbalken: Standardabweichung für $n = 3$.

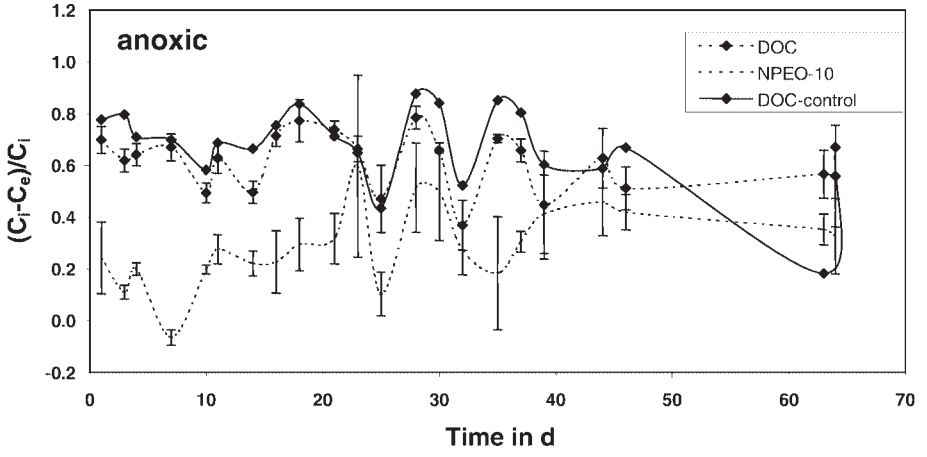

Fig. 3: Removal of NPEO-10 and DOC in anoxic mBFR. C_i: concentration of NPEO-10 resp. DOC in influent, C_e: concentration of NPEO-10 resp. DOC in effluent. Error bars: standard deviation for $n = 3$.

Elimination von NPEO-10 und DOC im anoxischen miniaturisierten Biofilmreaktor. C_i: Konzentration an NPEO-10 bzw. DOC im Zulauf; C_e: Konzentration an NPEO-10 bzw. DOC im Ablauf. Fehlerbalken: Standardabweichung für $n = 3$.

3.3 Primary biodegradation with miniaturized biofilm reactors

3.3.1 Removal of the test compound NPEO-10

Figures 2 and 3 show the removal of NPEO-10 and DOC in the oxic and the anoxic mBFR. Almost instant removal of the test compound is seen in the reactors. A high initial removal of the test compound indicates the presence of an adapted biocenosis [21]. Considering the fact that alkylphenol ethoxylates are widely used compounds which are present in many wastewaters, and hence also in the STPs, it is very likely that the inoculum used for the colonization of the reactors contained microorganisms adapted to the test compound. This conclusion is supported by the observation that a higher initial removal was achieved in the oxic reactors where the adapted species find similar, i. e., oxic, conditions as in the activated sludge tank of the STP. Throughout the whole experiment NPEO-10 removal was better in the oxic than in the anoxic biofilm reactors. The efficiencies for the oxic reactors lie in the range of 40 to 70 % whereas those for the anoxic reactors are mostly around 30 to 50 %. This hints at the greater efficiency of the oxic reactors and underlines the importance of oxygen in the biodegradation process. Throughout the experiment fairly large fluctuations in the removal curves are observed. For the oxic reactors an increase in the removal curve is observed after approximately 25 d whereas it is more like 20 d for anoxic reactors. The precision of the 3 test reactor replicates is poor. As has been stated, SPE and HPLC methods had very good precision; hence, analytical errors do not account for the variations seen among the replicates with regard to removal efficiencies. Instead, the variations are a consequence of the variable nature of biological systems, which generally show lower precision than analytical methods.

3.3.2 Comparison between DOC and NPEO-10 removal

Figures 2 and 3 also show the DOC removal data of the control and test reactors. The efficiency of the oxic control reactors remains at much the same, high level throughout the experiment (ca. 85 %). For the anoxic control reactor, a less efficient DOC removal with larger fluctuations (DOC removals from 40 to 80 %) was found. This suggests that the anoxic biofilm is a more sensitive system which is more difficult to maintain. The DOC removals in the test reactors were similar to the ones in the control reactors and in the range of 75 to 80 % for oxic, and 50 to 75 % for anoxic reactors. The DOC removal curves, including the variations over time, of the test reactors are very similar to the ones of the control reactors for both reactor types. This indicates that the test compound was not toxic to the biofilm microorganisms, and it also shows that the removal of the overall DOC is mainly a function of the degradability of the synthetic wastewater DOC. The overall DOC removal in the test reactors is lower than in the control reactors because a proportion of the DOC (approximately 20 %) comes from NPEO-10 which is removed with a lower efficiency than the SWW DOC. It is evident that the removal efficiency for the DOC is better than the removal efficiency for NPEO-10 in both the oxic and the anoxic reactors. This proves that the test compound is more resistant to biodegradation than the other carbon constituents of the synthetic sewage.

The oxic reactors perform much better than the anoxic reactors both in terms of NPEO-10 as well as DOC removal. Aerobic metabolic systems are generally known for their higher biomass production per mass of substrate than anaerobic systems where a greater proportion of the substrate's energy equivalents are required to support the cell metabolism. This fact is confirmed by the biomass data (Table 1). More biomass was produced in the oxic than in the anoxic reactors even though the substrate concentrations were the same. The greater DOC and NPEO-10 removal in the oxic reactors can thus be a consequence of the differences in biomass. In the oxic reactors more biomass was formed during the colonization phase, and hence a more efficient elimination of NPEO-10 and of DOC is achieved. Better removal in oxic reactors could, on the other hand, also be due to the presence of a more active biofilm. Biofilm activity, however, was not investigated in this work.

Table 1: Biomass in reactors. Values reported for test reactors are mean ± *SD* (1 *s*) in mg.

Biomasse in den Reaktoren. Angaben: Mittelwert ± Standardabweichung (1 *s*) in mg.

Reactor	Biomass dry weight in mg	
	Control reactors (n = 1)	Test reactors (n = 3)
Oxic	240	250 ± 18
Anoxic	140	170 ± 23

3.3.3 Appearance of degradation products

After some time, the occurrence of new peaks was observed in the HPLC chromatograms of both oxic and anoxic mBFR effluents (see Fig. 1, below). It was concluded that these compounds were degradation products of the test compound. Due to lack of analytical methods (LC-MS/MS) at the time of the experiment no identification of the DP was possible. Based on the retention times (between the broad NPEO-10

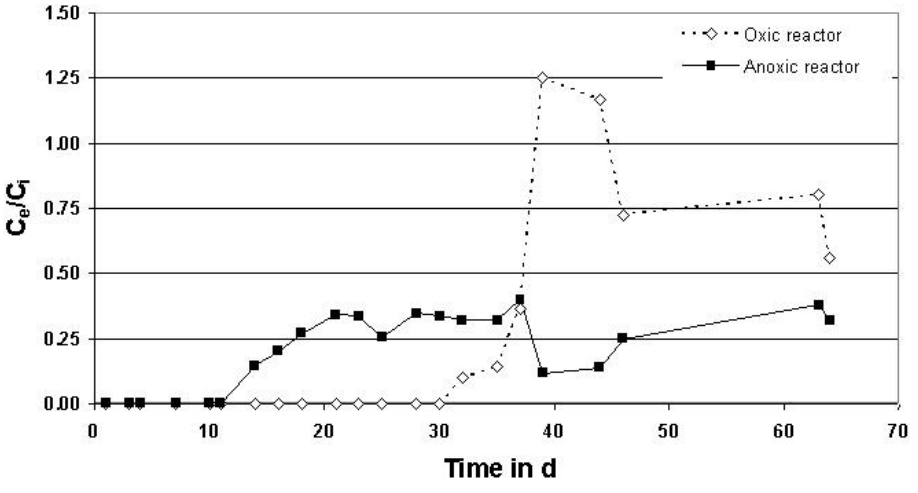

Fig. 4: Formation of degradation products in mBFR. C_i: concentration of NPEO-10 in influent, C_e: concentration of degradation products in effluent. Degradation products were quantified using NPEO-10 calibration data.

Bildung von Abbauprodukten im miniaturisierten Biofilmreaktor. C_i: Konzentration an NPEO-10 im Zulauf; C_e: Konzentration der Abbauprodukte im Ablauf. Quantifizierung der Abbauprodukte unter Verwendung der NPEO-10-Kalibrierdaten.

peak and the 4n-NP peak), and on information in the literature [3, 4], however, it was assumed that the DP should be short-chain ethoxylates, i.e., NPEO with less than 3 EO units. Nonylphenol was not detected as a metabolite in any effluent. Interestingly, even though the removal of the test compound NPEO-10 in the effluent was observed almost instantly, the appearance of the degradation products was not instant. This can be seen in Figure 4.

Degradation products were first observed in the anoxic reactors after about 10 d, and only 20 d later in the oxic mBFR. Whereas DP appear first in the effluents from anoxic mBFR, higher DP concentrations are found in effluents of oxic mBFR where they even exceed influent concentrations ($C_e/C_i > 1$) on two consecutive sampling events. This result demands a modified interpretation of the NPEO-10 removal data. The occurrence of DP in rather large amounts demonstrates that the biodegradation of NPEO-10 is incomplete (i.e., primary degradation only) and that significant amounts of metabolites are released into the mBFR effluent. Based on the NPEO-10 removal (Figs. 2 and 3) and DP formation data, the overall (net) removal of all NPEO compounds can be estimated. With a NPEO-10 removal of 70% and a DP formation of 50% ($C_e/C_i = 0.5$) the net removal is only 20% for oxic reactors, i.e., a large amount of the test compound is released in the form of metabolites instead of being degraded. This effect is, of course, unwanted, especially if similar processes take place in STP as it would lead to the release of NPEO metabolites to the environment.

3.3.4 Extraction of biomass

With the method adopted here (solvent extraction of biomass) no distinction between adsorbed (extracellular) and intracellular compounds was possible. Biomass extraction, however, was expected to yield an estimate of the contribution of adsorption and intracellular uptake to overall NPEO-10 removal, and to provide information on the occurrence of lipophilic metabolites such as nonylphenol in the biofilm. The test compound NPEO-10 was found in extracts of both oxic and anoxic reactors, however, the biomass-specific amounts of NPEO-10 (reported as mg NPEO-10 per kg biomass (dry weight)) were almost 10 times higher in anoxic than in oxic reactors (Fig. 5). Biomass from the anoxic reactors also contained a considerable amount of degradation products (reported as mg DP per kg biomass (dry weight); sum of DP in MeOH and hexane extracts) whereas DP were not quantifiable in oxic biofilms. No nonylphenol could be detected in any of the extracts.

A mass balance was performed to estimate how much of the test compound or its DP had adsorbed onto, or been taken up by the biomass. For this purpose the proportion of the masses of both NPEO-10 and DP in the biofilm relative to the total mass of NPEO-10 fed to the reactor (455 mg per reactor) were calculated. Less than 1% of the total NPEO-10 mass (combined NPEO-10 and DP masses) were found in both oxic and anoxic biofilms. It must be noted that the biomass which was lost due to sloughing was not quantified. Nevertheless, based on this estimate it can be concluded that removal of NPEO-10 from the liquid phase is mainly achieved by biodegradation of the compound.

3.3.5 Discussion of degradation product formation

It is interesting that degradation products were found only after considerable time even though biodegradation of the test compound NPEO-10 started at the very beginning of the ex-

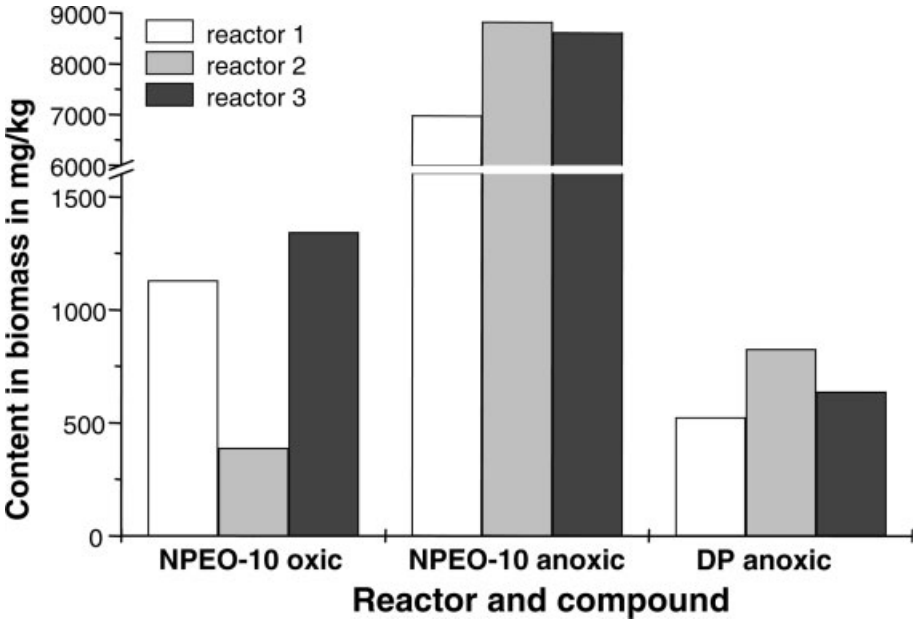

Fig. 5: Content of NPEO-10 and degradation products (DP) in biomass from oxic and anoxic reactors. Contents are given as mg compound per kg biomass (dry weight).

Gehalt an NPEO-10 und Abbauprodukten (DP) in der Biomasse für oxische und anoxische Reaktoren. Angaben in mg Substanz je kg Biomasse (Trockensubstanz).

periment. Furthermore, it is interesting that DP occur first in the effluents from anoxic reactors despite the fact that NPEO-10 elimination was constantly higher in the oxic reactors. The first observation indicates that degradation pathways, or their relative proportions, in the biofilm reactors might have changed over time, resulting in the formation of new compounds. The second observation suggests that this change is related to the presence, or absence of oxygen in the test system, and that the DP observed here are preferably formed under anoxic, or low-oxygen conditions. The occurrence of DP in the effluents from oxic reactors thus indicates that over time areas with low oxygen concentrations are formed in the "oxic" biofilms. This is very typical for a biofilm which grows in a layer-like form and therefore always also contains anoxic or, more generally speaking, anaerobic areas. The detection of DP in the oxic mBFR effluents is thus thought to be related to biofilm growth with increasing duration of the experiment, with DP being formed in the anoxic areas of the otherwise "oxic" biofilm. In the anoxic reactors conditions suitable for the formation of the DP exist from the very beginning, hence DP are detected much earlier here. This explanation is supported by the observation that the biofilms which were removed from the reactors at the end of the experiment were black colored, indicating formation of sulfides under anaerobic conditions. This was the case for both biofilms from oxic as well from anoxic reactors, however, the black color was much more dominant in the anoxic biofilms than in oxic biofilms.

The question remains why no DP are found in any effluent in the beginning of the study. The degradation products observed here are assumed to be short-chain ethoxylates, and the formation of these metabolites has been reported to occur under both aerobic and anaerobic conditions [3, 22]. Under oxic conditions, however, the main metabolites generally are the carboxylated compounds [4]. These compounds were not extracted with our SPE method (no acidification of the samples before extraction). It is therefore possible that initially APEC and CAPEC were formed as main metabolites in the oxic reactors, resulting in measurable removal of the test compound, but no DP detection with our methods. With increasing biofilm growth and the formation of low-oxygen areas a shift in the metabolic pathways could have taken place, resulting in the formation of short-chain EO which are then detected as DP. In the anoxic mBFR primary degradation could have proceeded immediately via shortening of the EO chain, but possibly in the beginning mid-chain EO were dominant which elute within the broad NPEO-10 peak and are therefore also not detected as DP. With increasing time degradation proceeds towards short-chain EO which are again observed as DP in the effluent. Higher DP concentrations in the oxic effluents can again be explained by the presence of more biomass in the those reactors, resulting in a more efficient degradation.

Lower concentrations of NPEO-10 in the oxic biomass agree well with the better elimination efficiency of those reactors. It is assumed that initially the amount of test compound in the biofilms was the same, but that over time more NPEO-10 was degraded in the oxic mBFR, resulting in lower biomass concentrations. The fact that the degradation products were found in anoxic biofilms only is more difficult to explain. A similar result, however, was found in a subsequent study with an identical setup for the mBFR experiment, but different analytical methods. In the anoxic biofilms of the subsequent work

nonylphenol monoethoxylate and nonylphenol were found in the biomass, with the concentrations of both compounds being higher in anoxic than in oxic reactors. Obviously, even for one particular compound, different biomass concentrations can exist in different biofilms, and in our study these differences are seen to be greater than analytical and biological variations (Fig. 5). It is probable that the composition and structure of the biofilms in the oxic and anoxic mBFR was different, resulting in different biomass concentrations of lipophilic metabolites at the end of the study.

3.3.6 Performance of miniaturized biofilm reactors with respect to field studies

In this study, the performance of the mBFR has been evaluated as the elimination of the parent compound from the influent (primary degradation). Here elimination represents all losses from the aqueous phase due to both biotic (primary degradation) and abiotic (adsorption) processes. In the review paper by Thiele et al. [2] it is seen that, in general, the removal efficiencies for the parent NPEO are much better in STPs in the USA (95...99%) than for the STPs in Europe (70...75%). For a study in UK (for APEO) removals as low as 45% have also been reported [2]. This may be due to the different loadings rates, plant operating conditions, wastewater characteristics as well as different microbial populations. In the study with mBFR the variations are much higher – from 13 to 83% – but most of the values lie in the range of 50 to 70%. The wide range of the removal data from the mBFR serves to underline the variability inherent in biological systems. The mBFR data is variable, and an outright comparison with the field data is difficult. Still, mBFR maybe useful if an estimate of the elimination efficiency of the parent APEO in STPs is required.

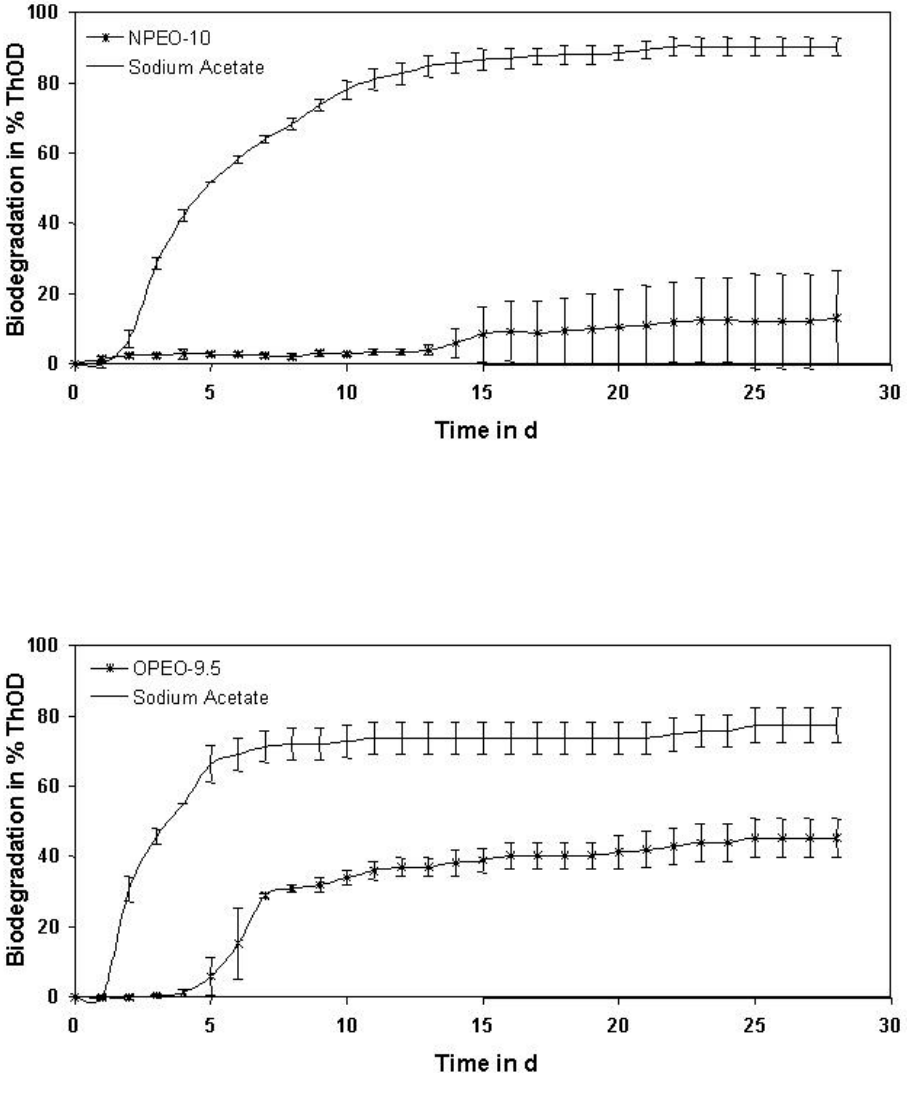

Fig. 6: Ultimate biodegradability of NPEO-10 (above) and OPEO-9.5 (below) with manometric respirometry. Error bars: standard deviations for $n = 2$ (NPEO-10) and $n = 3$ (OPEO-9.5).

Biologischer Sauerstoffbedarf (Respirometer-Test) für NPEO-10 (oben) und OPEO-9.5 (unten). Fehlerbalken: Standardabweichung für $n = 2$ (NPEO-10) und $n = 3$ (OPEO-9.5).

3.4 Ultimate biodegradation: manometric respirometry

Figure 6 shows the degradation of Marlophen NP10 (NPEO-10) and Triton X-100 (OPEO-9.5) with the respirometer. Sodium acetate was degraded very well without any appreciable lag phase in both the cases and attested to the viability of the inoculum. The degradation of NPEO-10 was very low and the lag phase was approximately 13 d, although the end of the lag phase and the beginning of the log phase could not be determined with certainty.

The variations between the NPEO-10 replicates were considerable. Of the three replicates tested for NPEO-10, it was seen that in one of the replicates the oxygen consumption was only 6 mg/L even after 28 d. This replicate was not considered in the data analysis, but this might indicate that at such concentrations (ρ(NPEO-10) = 45.0 mg/L) the compound is toxic to bacteria. After 28 d the biodegradation achieved was 27%, at best. The biodegradability of OPEO-9.5, in contrast, was better and more reproducible. After 28 d a biodegradability of (40 ± 5)% was achieved. The lag phase was only about 7 d. This clearly shows the influence of the length of the alkyl chain on biodegradability. The results for NPEO-10 from this test are basically a logical consequence of the mBFR data which showed that primary degradation already is incomplete (net removal of only 20% for oxic mBFR).

4 Conclusions

The experiments with miniaturized biofilm reactors showed that biodegradation takes place under both oxic and anoxic conditions. The removal of the parent compound (NPEO-10) was more efficient under oxic than under anoxic conditions. Comparison of the DOC removal and the analyte removal indicated that NPEO-10 is a fairly refractory compound. The formation of degradation products, presumably lower ethoxylates, in considerable amounts demonstrated that degradation was incomplete. This effect is unfavorable as it leads to the release of environmentally relevant metabolites into the effluent and, if similar processes occur in sewage treatment plants, also into the environment. In this work the release of the observed DP (short-chain ethoxylates) was concluded to be related to concentrations of dissolved oxygen in the test system. Further work on the identity and relative amounts of different APEO metabolites under various operating conditions of the biodegradation test system is needed because this can provide information on how to control the spectrum of metabolites and on how to achieve ultimate biodegradation. Biofilm reactors in combination with LC-MS/MS analysis would be very suitable for this purpose. The use of bioassays for various effects (e.g., toxicity, estrogenicity) together with analytical methods could help in finding the environmentally most acceptable way of biological APEO treatment.

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List of abbreviations and symbols

AcN	acetonitrile
AP	alkylphenol
APEC	alkylphenol ethoxycarboxylate
APEO	alkylphenol ethoxylate
AS	activated sludge
BFR	biofilm reactor
CAPEC	carboxylated alkylphenol ethoxycarboxylate
C_e	effluent concentration
C_i	influent concentration
CV	coefficient of variation
DOC	dissolved organic carbon
DP	degradation product
EC_{20}	effective concentration; concentration at which 20% of the test organisms show an effect
EO	ethoxylate
LOD	limit of detection
LOQ	limit of quantification
mBFR	miniaturized biofilm reactor
MeOH	methanol
NP	nonylphenol
4n-NP	4n-nonylphenol
NPEO	nonylphenol ethoxylate
NPEO-10	nonylphenol ethoxylate with average of 10 ethoxy units
NPEO-3	nonylphenol ethoxylate with average of 3 ethoxy units
OD	oxygen demand in mg/L
OPEO	octylphenol ethoxylate
OPEO-9.5	octylphenol ethoxylate with average of 9.5 ethoxy units
RP-HPLC	reversed-phase high-performance liquid chromatography
SD	standard deviation
SPE	solid-phase extraction
STP	sewage treatment plant
SWW	synthetic wastewater
ThOD	theoretical oxygen demand in mg/L
ρ	mass concentration

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