Environmental behaviour of onylphenol ethoxylates in coastal waters
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Aerobic biodegradation studies of nonylphenol ethoxylates in river water using liquid chromatography-electrospray tandem mass spectrometry


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Abstract

The aerobic biodegradation of nonylphenol ethoxylates (A₉PEO) was kinetically investigated in a laboratory scale bioreactor filled with river water, spiked at a concentration of 10 mg L⁻¹ of the nonionic surfactants. Analyses of the samples applying liquid chromatography - electrospray mass spectrometry (LC-ESI-MS) after solid-phase enrichment revealed a relatively fast primary degradation of A₉PEO with >99% degradation observed after 4 days.

Contrary to the generally proposed degradation pathway of ethoxylate (EO) chain shortening, it could be shown that the initiating step of the degradation is ω-carboxylation of the individual ethoxylate chains: metabolites with long carboxylated EO-chains are identified (A₉PEC). Further degradation proceeds gradually into short chain carboxylated EO with the most abundant species being A₉PE₂C. The oxidation of the nonyl chain proceeds concomitantly with this degradation, leading to metabolites having both a carboxylated ethoxylate and alkyl chain of varying lengths (CAPEC). The identity of the CAPEC metabolites was confirmed by the fragmentation pattern obtained with LC-ESI-MS/MS.

Both A₉PEC and CAPEC metabolites are still present in the bioreactor after 31 days.

In the aerobic degradation pathway A₉PEO₂ is formed only to a minor extent and is even further degraded in several days. The endocrine disruptor nonylphenol was not found as a metabolite in this study.

3.1. Introduction

Alkylphenol ethoxylates (APEO) are nonionic surfactants, used in large amounts in industrial and institutional applications. Because of their poor ultimate biodegradability, and the possible environmental hazard of their metabolites, APEO have been replaced in household applications, mainly by alcohol ethoxylates. However, for industrial applications this replacement has not been carried out yet, because of the excellent performance of APEO and their low production costs.

APEO, which are mainly used in aqueous solutions, reach the environment mainly via waste water. Although after secondary waste water treatment often more than 95% of the APEO is removed [1], they are omnipresent in the environment. Concentrations in river waters and marine sediments at ppb levels are reported [2, 3].

The biodegradation of A₉PEO is generally believed to start with a shortening of the ethoxylate chain, leading to short chain A₉PEO containing one or two ethoxylate units.
Further transformation proceeds via oxidation of the ethoxylate chain, producing mainly alkylphenoxy ethoxy acetic acid (A₉PE₂C) and alkylphenoxy acetic acid (A₉PE₁C) (figure 3.1) [4]. In only a few studies A₉PE₃C or A₉PE₄C have been detected in environmental samples [5-8]. To the best of our knowledge, only one paper reports A₉PEC with EO chain lengths longer than four units, which were found in treated waste water effluent [9].

Little has been reported about the further degradation of A₉PEC. In a semi-quantitative biodegradation study by DiCorcia doubly carboxylated metabolites are formed, with both the alkyl and ethoxylate chain oxidized (CAPEC) [10]. Ding detected CAPEC in waste water using TSQ GC-MS [11] and river water using ion-trap GC-MS [8].

It is generally assumed that the endocrine disrupter nonylphenol (NP) is the most persistent metabolite of A₉PEO. However, experimental data on the formation of the metabolite NP from A₉PEO is surprisingly scarce, and mostly under anaerobic conditions has NP been reported to be formed [12, 13]. Only one recent article reports a slight increase in NP concentration from the aerobic degradation of A₉PEO during the composting of wool scour effluent sludge [14].

Studies on the environmental occurrence of A₉PEO degradation products have focused on NP, short chain A₉PEO (1 to 3 EO units), A₉PE₁C and A₉PE₂C. The presence of these metabolites in the environment has been reported in many studies [2, 4, 6, 9, 15-17]. Since several aspects of the fate of A₉PEO and their metabolites remain unclear, detailed studies providing more insight into the biodegradation processes of these surfactants are needed, especially since several of the possible metabolites, notably NP, A₉PEO₁ and A₉PEO₂ have been shown to possess estrogenic activity [18, 19].

The purpose of this study was to elucidate the aerobic biodegradation route of A₉PEO by a microbial community which is ubiquitous in the aquatic environment, using a laboratory scale bioreactor (LSBR) filled with Rhine river water. LC-ESI-MS and LC-ESI-MS/MS were applied to clarify identity and routes of formation of metabolites [20-22].

In this thesis, the acronym A₉PE₂C stands for the oxidized version of A₉PEO₂. Consequently, when the alkyl chain of this compound is also oxidized, the acronym CA₉PE₂C is used.
3.2. Experimental Section

Reagents and standards.
Methanol was HPLC-grade (Merck, Darmstadt, Germany) and Milli-Q water was obtained from a Milli-Q-Plus 185 apparatus. Ammonium acetate (p.a.) was purchased from Merck. Technical mixtures of on average A₉PEO₄ (with an ethoxylate range of 2 to 9) and A₉PEO₁₀ (with an ethoxylate range of 4 to 15) were provided by Shell Amsterdam. The pure A₉PEO₁, A₉PEO₂, A₉PE₁₀ and A₉PE₂₀ standards were synthesized by F. Ventura of AGBAR, Barcelona according to Marcomini et al. [23] and characterized by GC-MS.

Laboratory scale bioreactor setup.
The applied LSBR consists of a glass column filled with SIRAN carrier material (glass beads of 2-3 mm diameter), and a 10 L storage tank aerated with a membrane pump [24]. Water taken from the river Rhine is continuously pumped in a closed loop from the tank upwards through the column at a flow rate of 15 mL min⁻¹. Microorganisms ubiquitously present in river Rhine water settle on the carrier material and form a biofilm [20]. The device has been successfully used in a number of degradation studies of single organic compounds [24, 25], and the degradation pathways of the surfactants alkylpolyglycosides, alkylglucamides and linear alkylbenzene sulfonates was investigated in detail. [20-22]. Two LSBR were spiked with two different technical mixtures of A₉PEO at concentrations of 10 mg L⁻¹ (15 µmol L⁻¹). In both mixtures, with an average ethoxylate chain length of 4 and 10, respectively, also a small amount of octyl- and decylphenol ethoxylates was present. The exact octyl, nonyl and decyl ratio (as determined by LC-MS) was 2.9, 89.9 and 7.2 % respectively for the A₉PEO₁₀ standard, and 2.4, 83.9 and 13.7 % for the A₉PEO₄ standard.

Sample treatment.
Samples of 110 mL were taken every few hours on the first day, twice on the second day, and almost daily after that to a total number of 33 in 31 days. Immediately after sampling, 2 mL of formaldehyde (37% in water/methanol; 90/10) was added to preserve the samples and then they were stored at 5°C. All samples were further processed within three days. Ten mL of the sample was filtered over a 0.45 µm glass fiber filter. The filtrate was analyzed with LC-MS without further treatment to quantify A₉PEO at concentrations higher than 50 µg L⁻¹.
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Solid-Phase Extraction
A SPE method was optimized for the enrichment of A₉PEO, A₉PEC and NP on a single cartridge. Three solid phase materials and a combination of two materials were tested for this purpose. Cartridges were filled either with 500 mg of RP-C₁₈, 500 mg of RP-C₁₈ EC, 300 mg of Lichrolut EN or a combination of 400 mg of RP-C₁₈ and 100 mg of Lichrolut EN (all from Merck, Germany).

Samples were acidified to pH=2 with HCl (3.5 M) before extraction, to ensure that carboxylated A₉PEO metabolites are in their protonated form. Conditioning of the cartridges was done with 10 mL of methanol and then 10 mL of milli-Q water. Subsequently 100 mL of the unfiltered sample was passed through the column. After drying the cartridges for at least 30 min by a stream of nitrogen, the elution was performed with 10 mL of methanol.

The extracts were evaporated with nitrogen until dryness, and then 1 mL of methanol/milli-Q water 1:1 (v:v) was added.

The recovery was determined for a mixture of NP, A₉PE₁C, A₉PE₂C and A₉PEO₁₀ in clean well water, at spiked concentrations of 100, 20, 20 and 5000 μg L⁻¹, respectively. Well water was used because while being free of microcontaminants, it has salt and dissolved organic carbon (DOC) concentrations similar to river Rhine water, and therefore the determined recoveries are more representative for the recoveries of the bioreactor samples.

For all tested compounds, the C₁₈ SPE material provided the best results, with average recoveries (n=5) of 69, 90, 87 and 95% for NP, A₉PE₁C, A₉PE₂C and A₉PEO₁₀, respectively. Therefore this material was used in further experiments.

Liquid Chromatography/ Mass spectrometry
The identification and quantification of A₉PEO and metabolites was performed by liquid chromatography coupled to electrospray mass spectrometry (LC-ESI-MS).

The LC-MS system was a Thermoquest Navigator aQa, equipped with a TSP SCM1000 Vacuum Membrane solvent degasser, a TSP P4000 gradient pump and a TSP AS3000 autosampler.

For separation a Lichrospher RP-C₁₈ column (dimensions 125x2 mm, 3μm) was used. The two mobile phases were pure methanol (A) and water-methanol 3:1 (v:v) containing 2 mM ammonium acetate (B). Ammonium acetate was used to enforce the formation of A₉PEO ammonium adducts over sodium or proton adducts, and at the same time to improve the retention behaviour and peak shape of the A₉PEC.

At a flow rate of 0.25 mL min⁻¹, a gradient elution was performed starting with 10% A. After 2 min, A increased linearly in 8 min to 50%. Then A was further increased to 97% in 21 min, which was kept constant for 12 min. Finally, the composition was brought back to the initial conditions and equilibrated for 9 min.
A9PEO were detected in positive mode, and all metabolites in the negative mode. The probe temperature was 220 °C, and probe and cone voltages were optimized for NP, A9PEC and A9PEO. The optimal values were: probe voltage -3.8 kV and cone voltage -20 V for NP and A9PEC, and probe voltage +4.0 kV and cone voltage +20 V for A9PEO.

For the quantification of A9PEO10, the total abundance of ammonium adducts of A9PEO6 to A9PEO13 [M+NH4]+ were selected, and a quadratic 6-point calibration was used. A9PEO2 could be quantified separately with a pure standard. For this quantification a linear 5-point calibration was used. For the A9PEO10, A9PEO4, A9PEO2 and A9PEO1 standards, the detection limits were 0.10, 0.15, 0.10 and 9.4 μg L⁻¹, respectively.

The quantification of metabolites was performed in SIM mode, with the [M-H] ions selected for NP, A9PEC and CAPEC. Six-point linear calibration curves were prepared for the available standards A9PE1C, A9PE2C and NP. As no standards of the long chain A9PEC and CAPEC were available, their quantification was performed using the A9PE2C calibration curve, assuming long chain A9PEC and CAPEC have the same molar response factor as A9PE2C. Detection limits of NP, A9PE1C and A9PE2C were 0.8, 0.3 and 0.4 μg L⁻¹, respectively.

**LC-ESI-MS/MS conditions.**

For identity confirmation of metabolites, a Perkin Elmer Sciex API 365 tandem mass spectrometer LC-ESI-MS/MS with turbo-ion spray interface was used. Product ion scans were obtained in negative ionization mode using both flow injection analysis and LC separation with a Hypersil MOS column (50x2.1 mm, 5 μm). One mass per analysis was selected to produce a molecular ion using an ionization voltage of -3 kV and an orifice voltage of -15 V at a temperature of 400 °C. In the second mass spectrometer, the molecular ions were fragmented using different fragmentation voltages ranging from -20 to -50 V.

For flow injection analysis, an eluent composition of 70% A was used. The LC separation was performed starting with 10% A for two min, then increasing A to 50% in 3 min, and further increasing to 97% in 7 min. This composition was kept constant for 4 min and finally the mobile phase was returned to the original composition and equilibrated for 6 min.

**3.3. Results and Discussion**

**Biodegradation rates**

The degradation of both A9PEO mixtures in the LSBR started immediately with no observable lag-phase, indicating that no significant acclimation of the micro-organisms was
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necessary. For the A₉PEO₁₀ mixture, the half-life was 10 hours, and a nearly complete (>99%) primary degradation was achieved after 100 hours. When comparing the A₉PEO mass spectra of the samples to the spectrum of the spiked A₉PEO₁₀ standard, it was observed that the oligomer distribution does not change significantly during the first 46 hours of degradation, whereas the total A₉PEO concentration decreases. Only after 76 hours the relative abundance of the shorter ethoxylate chains was found to increase. The concentration of A₉PEO₂ increased during the experiment, and accumulated to 4 mol % of the initially spiked A₉PEO₁₀ concentration. A₉PEO₂ was further degraded rapidly, and had disappeared after 300 hours (see figure 3.2 c). For the A₉PEO₄ experiment, similar results were obtained.

The fact that no gradual change in A₉PEO oligomer distribution towards the shorter ethoxymers was observed during the degradation, is in contradiction with the generally accepted APEO degradation pathway, which states that the degradation starts with a stepwise shortening of the ethoxylate chain [4]. The formation of short chain A₉PEO₂ indicates that ether cleavage of the ethoxylate chain does occur under these well aerated conditions, but only to a minor extent compared to the formation of A₉PEC.

As the ESI/MS is relatively insensitive for A₉PEO₁ compared to A₉PEO₂ (LOD of 9.4 and 0.10 µg L⁻¹, respectively), the possible accumulation of small amounts of A₉PEO₁ cannot be excluded in these experiments. However, in other studies where A₉PEO₁ was found as a metabolite, this was always at concentrations far below those of A₉PEO₂ [10, 26]. It is important to note that no increase in NP concentration was observed during the degradation tests. In the spiked APEO standards, traces of NP are initially present already, and no change in its concentration (slightly above the LOD) was observed during the whole experiment. This is in agreement with previous reports, which state that no NP is formed under aerobic conditions [4, 10]. However, it cannot be ruled out that NP may be formed and removed (by sorption or degradation) at similar rates.

Formation of carboxylated metabolites

Using LC-MS in negative full scan mode, the nonylphenoxy ethoxy acetic acids (A₉PEC) were identified as the first main group of metabolites, which were formed immediately after starting the biodegradation experiment.

The identity of A₉PE₁C and A₉PE₂C was confirmed by standards. For the higher ethoxylates the identification is based on molecular mass [M-H]⁻ and retention time. All A₉PEC are eluting adjacent to each other within a small retention time interval of about 5 min, starting with A₉PE₁C. Only A₉PE₁C and A₉PE₂C are completely separated. The ω-oxidation of the spiked A₉PEO₁₀ mixture led to the formation of A₉PE₁C to A₉PE₆C with an average ethoxycarboxylate chain length of nine (Figure 3.3).
Figure 3.2: Follow-up of $A_9$PEC and $A_9$PE$O_2$ formation during the bioreactor experiment spiked with $A_9$PE$O_{10}$. a) Long-chain $A_9$PEC; b) mid-chain $A_9$PEC; c) $A_9$PE$C_2$ and $A_9$PE$O_2$.

Figure 3.2a shows the accumulation of long-chain $A_9$PEC, with $A_9$PE$C_5$ to $A_9$PE$C_{11}$ as the most abundant species initially. $A_9$PE$C_{15}$ is the highest oligomer observed, present in trace amounts. Concomitant with the biodegradation of the longer chain $A_9$PEC, the concentrations of the mid- to short chain $A_9$PEC ($A_9$PE$C_1$ to $A_9$PE$C_5$) increased (figure 3.2b
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and c). A₉PE₃C and A₉PE₂C were formed as the most abundant species, with A₉PE₂C reaching a maximum concentration of 2.2 µmol L⁻¹ (15% of the originally spiked A₉PEO molar concentration), before being further degraded. The behaviour of A₉PE₁C, which is also commercially produced as a corrosion inhibitor, deviates from the others, as it is further degraded much faster than the other short chain A₉PEC. The exposure and subsequent degradation rate of A₉PE₁C in the environment might be higher than that of A₉PE₂C, for which the actual concentrations are usually higher [2]. The shorter chain A₉PEC (A₉PE₂₋₃C) were still present after 700 hours, amounting to a total concentration of 0.5 mol % of the concentration spiked initially. This relative persistence is in agreement with previous findings, in which short chain A₉PEC were found in wastewater effluents and river water, often in higher concentrations than A₉PEO [10, 17]. Also, the formation of A₉PE₂C as the major metabolite of A₉PEO is consistent with other laboratory biodegradation studies [10, 26] as well as A₉PEO metabolite monitoring studies [2, 4].

![LC-ESI-MS SIM spectrum showing the distribution of A₉PEC from A₉PE₁C to A₉PE₁₅C at t=49 hrs: 1= A₉PE₁C, 2= A₉PE₂C, 3= A₉PE₃C etc.](image)

However, the observed degradation rates of A₉PEO as well as the formation and elimination rates of A₉PEC are higher than those found in other laboratory studies. In a biodegradation study according to OECD 301B, A₉PE₂C started to form after a lag-phase of 6 days, and was still present at half its peak concentration after 80 days [10]. Staples et al. reported half-
lives of A9PEC of 12-22 days using the same protocol, but determining CO2 formation instead of using LC-MS analysis [27]. In a static die-away test of A9PEO in estuarine water samples, A9PE2C started to form after 12 days, and was still increasing after 90 days [26]. Different experimental conditions can account for these differences in degradation rates: in the OECD protocol, a medium with nutrients is inoculated with a small volume of sewage treatment plant effluent or sludge, indicating a small initial microbial population, which requires longer acclimation times. In the estuarine test, the water salinity (18-33‰) can explain a slower degradation rate.

The high degradation rates observed in the present study are an indication that populations of pre-acclimated APEO-degrading bacteria are present in river Rhine water, resulting from a continuous exposure to APEO of the microbial community in this river.

Confirmation of metabolite identity using LC-ESI-MS/MS.

During the degradation of APEO, many other compounds are found to accumulate and later disappear again from the bioreactor. Groups of closely eluting compounds can be identified as ethoxylate homologue series, with mass differences of one ethoxylate unit (m/z=44). In parallel, groups of alkyl homologues can be recognized, eluting some minutes after each other, with mass differences of one methylene group (m/z=14) (figure 3.4).

However, unequivocal identification of these accumulating compounds cannot be performed by LC-MS only, since when solely molecular ion masses are available, these can still be attributed to different groups of homologues. For example, the ethoxylate homologues of A9PEC have the same molecular mass as CA9PEC homologues (having an alkyl chain of nine carbons, and both the alkyl and ethoxylate chain oxidized to an acid), with e.g. CA_nPE_mC corresponding to A_n-1PE_{m+1}C.

In figure 3.5, the mass spectrum of the A9PE2C standard at high cone voltage (-80 V) is shown. The losses of CO2 (m/z 44) and CH2-O-CH2-COO (m/z 88) are characteristic. For both the A9PE1C and A9PE2C standards, the nonylphenoxy fragment (m/z 219) is observed. Further fragmentation of the alkyl chain leads to alkenephenoxy fragments (m/z 203, 189, 175, 161, 147 and 133).

Unfortunately, at high cone voltages, the signal intensity of both the molecular ion and the fragments is very low and therefore many metabolites could not be detected in the samples under these conditions. Moreover, since some metabolites are partially co-eluting and thereby complicating the assignment of the individual fragments to a molecule, LC-ESI-MS/MS was used for an unequivocal assignment.
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Figure 3.4: Extracted Ion chromatograms showing the CAPEC in a bioreactor sample taken after 192 hours.
Figure 3.5: Mass spectrum of A9PE2C at cone voltage -80V.

For 17 compounds that were found to accumulate a product ion scan was performed, using fragmentation voltages of -20, -35 and -50 V in Quadrupole 2 for every compound. The key fragment for identification of these metabolites is the (possibly carboxylated) alkylphenoxy fragment having the highest mass. Nine of these compounds were confirmed to be CAPEC metabolites. In figure 3.6, examples are given of the fragmentation patterns of some of the compounds which were positively identified as CAPEC. In addition to the carboxy-alkylphenoxy fragment, typical fragments are observed where the carboxy-alkylphenoxy fragment has additionally lost a CO2- or an acetic acid group, in the case of CA3PE1.2C leading to fragments of m/z 149 and 133.

Of all the compounds scanned with LC-ESI-MS/MS, only CAPEC with one or two ethoxylate units could be identified positively. Possible metabolites with an oxidated alkyl chain and an intact ethoxylate chain (CAPE), as proposed by DiCorcia et al. [10], were not detected in this experiment.
Figure 3.6: MS-MS fragmentation patterns of some CAPEC (negative ionization mode): a) CA₉PE₂C, b) CA₅PE₂C and c) CA₅PE₁C. The exact branching of the alkyl chain is unknown; the alkyl isomer structures shown are chosen arbitrarily.
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Figure 3.4 shows ion extraction chromatograms of these metabolites. All CAPEC with a phenoxy acetic acid or a phenoxyethoxy acetic acid moiety and alkyl chains of five to nine carbon atoms (CA₅₋₉PE₁₋₂C) were found, with the exception of CA₀PE₁C. A compound with a mass corresponding to CA₉PE₁C is accumulating during the experiment, but in the LC-MS/MS spectrum, the typical carboxy alkylphenoxy fragment (m/z 249) is absent. A fragment of m/z 205 is present in the spectrum, which could either originate from the carboxy alkylphenoxy fragment having lost CO₂, or from the alkylphenoxy fragment of A₈PE₂C. The retention time does not allow to distinguish between CA₀PE₁C or A₈PE₂C, as the compound is eluting between CA₈PE₁C and A₉PE₂C. The presence of both CA₀PE₁C and A₈PE₂C can be expected, with A₈PE₂C originating from the A₈PEO which were present as minor impurities in the spiking standard. Therefore coelution of these compounds may be possible. An attempt to optimize the LC gradient regarding the separation of these compounds was unsuccessful.

The accumulation and further degradation of the positively identified CAPEC during the experiment is shown in figure 3.7. Some uncertainty exists in the quantification of the various CAPEC, as all CAPEC are assumed to have the same molar response as A₉PE₂C. All CAPEC start to form simultaneously at 46 hours, and have disappeared almost completely after 500 hours. CA₀PE₂C is formed in concentrations about one order of magnitude higher than all the other CA₅₋₉PEC. A plausible pathway for the further transformation of these CAPEC would be β-oxidation of the alkyl chain, shortening it by two carbon atoms at a time. Since different alkyl chain lengths were present in the initially spiked APEO standard, this hypothesis cannot be confirmed.

Mass balance

Although there is some uncertainty in the quantitative results for most metabolites because of a lack of standards, it is clear from table 3.1 that a complete mass balance cannot be made. During the first stage of the experiment (i.e. <50 h), the long-chain A₉PEC were prevailing. After several days, the mass balance was dominated by the metabolite A₉PE₂C, as it was present at concentrations one order of magnitude higher than other metabolites.

The reason for the incomplete mass balance is unclear. Possibly other metabolites, not detected by LC-MS were formed, or a large fraction of the AP₉EO₁₀ was completely mineralized, without the accumulation of metabolites.
Figure 3.7: Formation and subsequent degradation of CAPEC in the LSBR: a) CA₉PE₂C and b) CA₅₋₈PE₁C and CA₅₋₈PE₂C (nmol L⁻¹).

<table>
<thead>
<tr>
<th>sampling time (days)</th>
<th>percentage of disappeared APEO detected as metabolites [%]</th>
<th>fraction of total detected metabolite concentration for separate metabolite groups</th>
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<tr>
<td></td>
<td></td>
<td>A₉PEO₂</td>
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<tr>
<td>2</td>
<td>3.9</td>
<td>0.16</td>
</tr>
<tr>
<td>11</td>
<td>19</td>
<td>0.01</td>
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Table 3.1: Mass balance at different sampling times, expressed as the molar percentage of transformed A₉PEO that was found as metabolites in the bioreactor samples of the A₉PEO₁₀ experiment. The relative contribution of different metabolite groups is also shown.
Biodegradation pathway

According to the present results, the aerobic biodegradation of \( \text{A}_9\text{PEO} \) can be divided into three processes (figure 3.8). The first process is a rapid \( \omega \)-oxidation of the ethoxylate chain, which occurs without any observable lag-time. The next process occurs more slowly, in which the long-chain \( \text{A}_9\text{PEC} \) are degraded to short chain \( \text{A}_9\text{PEC} \), mainly \( \text{A}_9\text{PE}_2\text{C} \). The third process is the oxidation of the alkyl chain, which starts as soon as \( \text{A}_9\text{PE}_2\text{C} \) is being formed. The formation of \( \text{A}_9\text{PEO}_2 \) appears to be a minor degradation pathway under the tested aerobic conditions. The reported presence of \( \text{A}_9\text{PEO}_2 \) in the environment is likely to be caused mainly by anaerobic degradation processes.

![Figure 3.8: Newly proposed aerobic biodegradation pathway of alkylphenol ethoxylates. Step (I) is rapid, steps (II) and (III) proceed more slowly. The exact branching of the alkyl chain is unknown; the alkyl isomer shown here is just one of several possibilities.](image)

Taking into account that (i) these experiments were performed using a freshwater microbial population which is omnipresent in the environment, (ii) the above cited abundance of \( \text{A}_9\text{PEO} \) in effluents and sediments, and (iii) the rapid formation of long chain \( \text{A}_9\text{PEC} \), it can be expected that the latter are present in relevant concentrations in many fresh water environments. In addition, short chain metabolites were still present at the end of the experiment, reflecting their relative persistence. Therefore, in future monitoring studies of \( \text{A}_9\text{PEO} \) and their metabolites, both short and long chain metabolites should be included. The present study reveals that formation in aerobic circumstances does not seem a likely source for the occurrence of NP in the environment. However, the formation of NP in 'seemingly aerobic environmental compartments' cannot be ruled out completely, as anaerobic conditions in these compartments may occur locally in for example suspended particles. The \( \text{A}_9\text{PEO} \) transformation processes under anaerobic conditions should be investigated further, and other sources of NP could be considered.
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Acknowledgments

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