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Tracing of surfactants in the biological wastewater treatment process and the identification of their metabolites by flow injection–mass spectrometry and liquid chromatography–mass spectrometry and –tandem mass spectrometry

Horst Fr. Schröder*

Institut für Siedlungswasserwirtschaft, Aachen University of Technology, Templergraben 55, D-52056 Aachen, Germany

Abstract

Results of aerobic biodegradation of alkyl ethoxylates (AEOs), of nonylphenol polyethoxylates (NPEOs), and of NPEO derivatives (sulfonates and sulfates), as well as anaerobic NPEO biodegradation monitored by flow injection analysis (FIA) or liquid chromatographic separation (LC) in combination with mass (MS) and tandem mass spectrometry (MS–MS) are presented. The application of visual pattern recognition in the FIA–MS mode showed quite different degradation pathways for C_{13}-AEOs, so that aldehyde compounds as metabolites could be confirmed by collision-induced dissociation for the first time. Methyl ethers of AEO compounds were found to be persistent under aerobic conditions, while NPEO degradation resulted in nonylphenol polyether carboxylates. FIA– and LC–MS proved that NPEO derivatives used as anionic surfactants were either non-biodegradable (nonylphenol diethoxy sulfonate) or were primarily degraded (nonylphenol polyethoxy sulfates) into compounds of the same molar masses yet of different retention behaviour. Anaerobic degradation of NPEOs led to the generation of nonylphenols, which was confirmed by GC–MS.

Keywords: Water analysis; Flow injection analysis; Mass spectrometry; Environmental analysis; Surfactants; Ethoxylates; Nonylphenol ethoxylates

1. Introduction

The production of surfactants — including soaps — had a volume of nearly $30 \cdot 10^9$ kg per-year worldwide in 1996 [1]. Surfactants represent one of the major, most multi-purpose groups of organic compounds. They are among the organic chemicals with the highest production rates, with a prognosticated upward trend of more than 3.5% for 2005 [2]. Their main fields of application, as anionic, nonionic, cationic and amphoteric surface-active compounds, are: industry (cleaning products, food, and industrial processing), household (laundry, dishwashing, etc.), and personal care (soaps, shampoos, cosmetics, etc.).

After their application in aqueous systems, these surfactants are discharged with wastewater. Although legislation stipulates their primary degradability, they can not be completely mineralized because of insufficient hydraulic retention times in wastewater treatment plants (WWTPs). Though biological wastewater treatment eliminates more than 95% of

*Tel.: +49-241-153-252; fax: +49-241-918-2740.

E-mail address: hf.schroeder@post.rwth-aachen.de (H.Fr. Schröder).

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the pollutants surfactants and their metabolites, they are quite often detected in over-proportional concentrations in wastewater effluents.

Compounds of this type have been found in drinking water which had been produced from surface water by bank filtration [3]. In addition, surfactants adsorbed at the biological sewage sludge are discharged in their original, or a partly degraded form, whenever excess sludge is being removed in order to be used agriculturally as fertilizer. Rain then carries the surfactants and their metabolites away from the fields into ground and surface waters.

Not only the vast amounts of surfactants that are discharged into wastewaters and thereby into surface waters, but also the broad variety of their chemical structures (hundreds of homologues, oligomers and isomers) turns them into a group of priority among environmental pollutants [4–7] — all the more so, if we consider their high water solubility and the persistence of their metabolites.

The task of having to detect, identify, and quantify surfactants in aqueous solutions, also in the form of matrix-free standards, may confront an analyst with considerable problems. Substance-class-specific determination methods [8,9], still in use as standard analysis on a spectrophotometric and titrimetric basis, are not only rather insensitive but also quite unspecific. Interferences with other compounds of similar structure lead to wrong (i.e., positive) results [10,11]. At the same time, neither primary degradation products nor strongly modified metabolites as they form during wastewater treatment are detectable.

Our objective was to elucidate the degradation processes of anthropogenic surfactants by selected immobilized biocoenoses of different WWTPs. Primary degradation products containing the basic structure of the surfactant molecules, sometimes persistent and/or toxic against water organisms, have been the compounds of interest. Volatile fatty acids, di-acids, etc., however, which are easily degraded and mineralized in the wasterwater treatment process, were not monitored. For this purpose, procedures under standardized aerobic and anaerobic conditions were applied for the degradation of the surfactants. A flow injection analysis bypassing the analytical column in combination with mass spectrometry (FIA–MS) and LC–MS were applied in order to trace both the precursor surfactants and their metabolites. Soft ionization techniques, such as atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI), were used. In order to identify metabolites, collision-induced dissociation (CID) and high-resolution MS measurements were applied.

2. Experimental

2.1. Materials

Samples from WWTP effluents were taken in order to cultivate the immobilized aerobic biocoenosis. For this purpose, effluent samples from the different WWTPs of Aachen (Germany) were applied and we used the effluent of the sludge stabilization of Aachen-Soers WWTP for our anaerobic degradation experiments. Glass foam beads (SIRAN-Carrier No. 023/02/300) produced by Schott Engineering (Mainz, Germany) were used for immobilization of bacteria. All parts of the reactors were made of glass and the pipes were made of PTFE.

Polyethylene glycols (PEGs), polypropylene glycols (PPGs) and the polyethylene glycol 600 diacid (PEG-diacid; $HOOC–(CH_2–CH_2–O)_x–CH_2–COOH$ ($x=3–15$)) for calibration purposes were of analytical reagent grade (Merck, Darmstadt, Germany) or technical grade (Fluka, Buchs, Switzerland), respectively. The 4-*n*-nonylphenol (Pestanal) for quantification was purchased from Riedel-de Haen (Seelze, Germany). The technical blends of surfactants used for spiking in the biodegradation experiments {alkyl polyethoxylates [AEOs: C_{13}-$(EO)_x$; $x=1–16$, average number of EOs: 8], nonylphenol polyethoxylates [$NP(EO)_x$; $x=4–18$, average number of EOs: 10], and nonylphenol polyethoxylate-sulfates ($NPEO-SO_4$) [$NP(EO)_x-SO_4$; $x=1–15$, average number of EOs: 10]} were products by Hüls (Marl, Germany). The methylated alkyl polyethoxylate blend Dehypon [AEOs: $C_{11–13}$-$(EO)_x$-CH_3; $x=1–16$, average number of EOs: 8], was a product of Henkel (Düsseldorf, Germany). The nonylphenol diethoxysulfonate [$NP(EO)_2-SO_3$, Triton X-200] was donated by N. Paxéus (Göteborg, Sweden).

Methanol, used for desorption of water pollutants

from the solid-phase materials, acetone and methanol for solid-phase extraction (SPE)-conditioning purposes, and cyclohexane applied for nonylphenol extraction were Nanograde solvents by Promochem (Wesel, Germany). Acetonitrile and methanol used as mobile phases were of HPLC grade (Promochem). Both were used in gradient elution in combination with Milli-Q-purified water (Millipore, Milford, MA, USA). Ammonium acetate used in the APCI process and as ion-pairing reagent and anhydrous Na_2SO_4 for drying purposes and sodium acetate used as feed in anaerobic degradation experiments were of analytical-reagent grade from Merck.

Nitrogen for drying of solid-phase cartridges was of 99.999% purity, nitrogen used as sheath gas in APCI ionization was of 5.0 purity, and argon used as collision gas was of technical grade (Linde, Germany).

Commercially available SPE cartridges filled with C_{18} material from Baker (Deventer, Netherlands) were used for concentration of the surfactants and their metabolites. The cartridges were conditioned as prescribed by the manufacturer. The glass-fibre filters which were used for the pre-treatment of the water samples were obtained from Schleicher and Schüll (Dassel, Germany). Before use, the glass-fibre filters were heated to 400°C.

2.2. Biodegradation experiments, sampling and sample preparation

The 10 l aerobic-laboratory-scale reactor and the aerobic treatment procedure were described by Karrenbrock et al. [12]. Devices that were modified for anaerobic degradation allowed a handling under strict exclusion of oxygen. For the immobilization of biocoenosis, all effluents were first pumped into closed-loop systems (2 ml min^{-1}) over glass foam beads located in glass columns. After a period of 5 days, during which the immobilization of the bacteria on the glass foam beads could be achieved, the biodegradation experiments could be started. The anaerobic biocoenosis obtained as effluent of the anaerobic sludge stabilisation tank was separated from the sludge liquor when the mixture was allowed to stand for several h under strict exclusion of oxygen. Then the sludge liquor was tranfered into the anaerobic degradation devices. The suspended bacteria were circled by pumping to allow to immobilize on the glass foam beads contained in the glass columns.

All reactors were stirred by means of magnetic stirrers. The biodegradation experiments were performed in a dark room at a temperature of 20°C. Diffused air aeration using compressed air was adjusted to 5 l h^{-1} dosed into the aerobic laboratory-scale reactor by plate diffusers. Under aerobic and anaerobic conditions the quantities of wastewater pumped through the columns filled with glass foam beads were adjusted to about 1 l h^{-1}.

The different types of surfactants dissolved in 1 ml of methanol were added into the reactors. The absolute quantities of surfactants spiked into the biodegradation devices were chosen to reach an initial concentration of 5 mg l^{-1}.

To obtain a maximum of information, the conditions for sampling, sample preparation, and MS monitoring had at first been adapted to the compounds under research. After an hour of spiking and mixing the reaction media by stirring, we took the first samples, which represented the start concentration. The sampling periods were chosen in accordance with the different degrees of biodegradability. So in order to monitor the AEO surfactant blend, 100 ml samples were taken from the biodegradation reactors every 12 h. For NPEOs and NPEO derivative monitoring, samples were taken once a day at a fixed time. A total of 25 ml of the samples were filtered by glass fibre filter before the surfactants and their metabolites were extracted using SPE cartridges filled with 100 mg of C_{18} material and pretreated as prescribed by the manufacturer. During the weekend, the samples were frozen and stored at −80°C before they were extracted by SPE. A minimum of three C_{18} solid-phase cartridges were loaded with an aliquote of the same sample. After the extraction procedure the columns were rinsed with two column volumes of purified water before they were dried in a gentle stream of nitrogen. One was used for methanol elution (1 ml) and FIA–MS measurement to get an overview. Remaining cartridges were stored below −18°C until the compounds concentrated were desorbed prior to measurement using 2 ml of methanol. Without any further pretreatment these solutions could be applied for FIA– and LC–MS or GC–MS measurements, respectively.

For anaerobic treatment, a quantity of the NPEO blend resulting in a NPEO concentration of 5 mg l^{-1} was added. It had been dissolved in 1 ml of methanol. To ensure anaerobic conditions sodium acetate dissolved in water in a concentration of 50 mg l^{-1} wastewater was added in accordance to the redox potential which was kept <-380 mV. For degradation, monitoring samples for FIA, LC–MS, and GC–MS were first taken 2 h after a spiking with NPEOs. Sampling was repeated every 3 days. For APCI-FIA(+) and LC–MS(+) determinations, C_{18}-SPE was applied as cited above. For GC–MS analysis of nonylphenols (NPs), samples from the anaerobic treatment reactor had been acidified with phosphoric acid before they were steam-destilled. NPs were extracted from the condensate by cyclohexane. The extract was dried by anhydrous Na_2SO_4, collected in a pear shape flask and reduced in a gentle stream of nitrogen to a volume of 1 ml prior to GC–MS analysis.

2.3. Gas chromatography–mass spectrometry

For determination of NPs, nonylphenol monoethoxylate [$NP(EO)_1$], and nonylphenol diethoxylate [$NP(EO)_2$], we used a Varian (Darmstadt, Germany) Model 3400 GC system with a fused-silica capillary column coupled with a TSQ 700 (Finnigan MAT, San Jose, CA, USA). The conditions were as follows: carrier gas, helium; linear gas velocity, 15 cm s^{-1}; injector temperature, 280°C; transfer line temperature, 280°C; column, SE-54 CS Chromatographie Service (Langerwehe, Germany), film thickness 0.25 μm (50 m×0.32 mm I.D.).

Combined with GC, we applied electron impact (EI) ionization with an ionization energy of 70 eV. Under these conditions, the pressure in the ion source was $3 \cdot 10^{-3}$ Torr (1 Torr=133.322 Pa). In the vacuum system of the mass spectrometer, it was $3 \cdot 10^{-6}$ Torr. The electron multiplier operated at 1200 V with a conversion dynode voltage at 15 kV. The temperature in the ion source was 150°C.

GC–MS analysis was performed by scanning at 1 s from 50 to 350 u.

For quantification under MID conditions, the GC–MS conditions the limits of detection (LODs, expressed as injected amount) on the TSQ MS detector were: NPs, 4-*n*-NP: 0.1 ng μl^{-1}; $NP(EO)_1$, $NP(EO)_2$: 1 ng μl^{-1}.

2.4. Flow injection analysis

The conditions in FIA bypassing the analytical column coupled with MS, MS–MS detection on a TSQ 700 were as follows: injection volume: 10 μl; mobile phase methanol–water (30:70) containing 0.05 *M* ammonium acetate. The overall flow-rate was 0.6 ml min^{-1} (Waters Model 510 pump). FIA–MS analysis was performed by scanning at 1 s from 200 to 1200 u.

Under FIA conditions for follow up of surfactants 10 μl were injected. For quantification the LODs on the TSQ 700 were: AEOs [APCI(+)], NPEOs [APCI(+)], nonylphenol polyether carboxylates [NPECs; APCI(±)] and NPEO-SO_4 [APCI(−)]: 2 ng μl^{-1}; Triton X-200 [APCI(−)]: 0.2 ng μl^{-1}. Metabolites were ''quantified'' by using the precursor compounds as surrogate standards under the estimation of comparable response factors to get trend information. For accurate mass measurements, which were to be performed on a MAT 900 ST hybrid sector field-ion trap mass spectrometer or on a MAT 95 XL Trap MS (Finnigan MAT), we used a syringe pump (Harvard, Southnatick, MA, USA) for the transfer of the methanolic eluates into the mass spectrometry. A flow-rate of 10 μl min^{-1} or 25 μl min^{-1} were adjusted, respectively. FIA–MS analyses were performed by scanning at 2 s from 570 to 700 u (MAT 900 ST) or 585 to 635 u (MAT 95 XL Trap).

2.5. Liquid chromatographic conditions

LC separations under non-ion-pairing conditions were carried out with a Multospher 100 RP 8-5 column (C_8, 5 μm, spherical; 250×4.6 mm I.D.) from CS Chromatographie Service. Gradient elution by means of acetonitrile or methanol (A) in combination with Milli-Q-purified water (B) was applied. The gradient was programmed as follows: Starting with A–B (30:70) the concentration was increased linearly to A–B (90:10) within 12 min. Up to 30 min the composition was kept constant. The overall flow-rate was adjusted to 1.0 ml min^{-1}. Ion-pairing separation of the methylated alkyl polyethoxylate blend was performed on a Spherisorb 5 ODS 2

column (C_{18}, 5 μm, spherical; 125×4.6 mm I.D.) from CS applying a gradient mixture of methanol and ammonium acetate solution ($5 \cdot 10^{-3}$ *M*). Starting with 30% methanol the concentration was increased linearily to 90% within 15 min.

LC separations coupled with MS, MS–MS (TSQ 700; Finnigan MAT), and UV detection were achieved with a Waters (Milford, MA, USA) Model 600 MS system or a SpectraSystem P4000 pump [Thermo Separation Products (TSP), San Jose, CA, USA]. A Waters Model 510 pump was used for post-column addition of 0.1 *M* ammonium acetate solution in the APCI mode. A Waters 996 photodiode array detector system (DAD-UV) in combination with a Millenium 2010 data system (Millipore) was connected in-line with the APCI or ESI interfaces (Finnigan MAT).

Applying APCI ionization, 0.5 ml min^{-1} of 0.1 *M* ammonium acetate was added after passing the UV-DAD, which resulted in an overall flow-rate of 1.5 ml min^{-1}. The post-column flow split ratio was 1:2 in favor of the MS in APCI mode or waste, respectively.

2.6. MS and MS–MS systems

A TSQ 700 mass spectrometer combined with a DEC 5000/33 data station, a MAT 900 and a MAT 95 XL Trap mass spectrometer (Finnigan MAT) were used for research work. The APCI interface and the ESI interfaces were obtained from Finnigan MAT.

For coupling the LC system with the TSQ 700 mass spectrometer, the following conditions for APCI ionization using ammonium acetate were chosen: vaporizer temperature, 400°C, capillary temperature, 180°C. Corona voltage was operated at 5 kV. The potential of capillary, tube lens and API octapole were chosen as 50, 50 or −3 V, respectively. Sheath gas pressure was operated at $2.81 \cdot 10^{5}$ Pa. Under the above-mentioned conditions, the ion source pressure was 0.3 Torr, and the pressure in the vacuum system of the mass spectrometer was $2 \cdot 10^{-5}$ Torr.

The electron multiplier operated at 1200 V and the conversion dynode at 15 kV. In the MS–MS mode, too, the ion source pressure was 0.5 Torr. Under CID conditions the pressure in quadrupole 2 (collision cell) was, unless otherwise specified in the captions to the figures, 1.3 m Torr. The collision energy was adjusted from −10 to −50 eV. The electron multiplier voltage in quadrupole 3 varied between 1200 and 1700 V with a conversion dynode voltage at 15 kV.

Low-resolution FIA and LC analyses on the TSQ 700 were performed, recording APCI or ESI mass spectra scanning from 100 to 1200 u at 1 or 3 s, respectively. FIA bypassing the analytical column with MS or MS–MS (daughter- and parent ion-mode) detection was performed while we accumulated a maximum of 50 scans after injection. The mass spectrum averaging the total ion current from the beginning of the signal up to the end in FIA–MS mode was termed ''overview spectrum''.

APCI and ESI ionization on the TSQ 700 or ESI ionization on the MAT 900 and MAT 95 XL Trap were carried out in positive and negative modes.

Multiple MS (MS^n) experiments were performed on the MAT 95 XL Trap, collision energy in the MS^n experiments were chosen to 30% of CID energy.

Under LC–MS conditions for follow up of surfactants 10 μl were injected. For quantification the LODs on the TSQ 700 were: AEOs [APCI(+)], NPEOs [APCI(+)], NPECs [APCI(±)] and NPEO-SO_4 [APCI(−)]: 0.2 ng μl^{-1}; Triton X-200 [APCI(−)]: 0.02 ng μl^{-1}. For metabolite quantifications see Section 2.4.

2.7. High-resolution accurate mass determination

For LC–MS separation the SPE-C_{18} eluates were injected with a Rheodyne injection valve (20 μl loop) into a 25 cm×4.6 mm I.D. Multospher 100 RP 8, column (particle size 5 μm). The initial mobile phase was methanol–water (30:70; v/v) and then a methanol gradient to 90% of methanol at 15 min using a Rheos 4000A pump. The HPLC flow was post-column splitted into the electrospray source of a MAT 900 ST MS spectrometer at a flow-rate of 10 μl min^{-1}. For FIA–ESI-MS applications on a MAT 95 XL Trap MS spectrometry the SPE-C_{18} eluates were transferred continuously (25 μl min^{-1}) via ESI source into the MS using a syringe pump.

Medium-resolution (MR) and high-resolution

(HR) accurate mass measurements were performed on hybrid two sector field-ion trap mass spectrometers (MAT 900 ST with a EBT geometry) or (MAT 95 XL Trap with BET geometry both from Finnigan MAT, respectively.

Measurements were performed with a magnetic scan from m/z 100 to 1000 at 3 s per decade. ESI in the negative ion mode (Finnigan MAT ESI 2, heated metal capillary) was used with 3 kV spray voltage. HR accurate mass was determined at 3500 resolution using an electrical scan from m/z 570 to 700 with a 2-s cycle time. PPG dissolved in methanol was used as a reference standard (conc. 5 nmol ml^{-1}) in the medium-resolution experiments. The solution was added through sheath liquid tube at 10 $\mu l\ min^{-1}$.

HR ESI experiments were operated in the negative ion mode (Finnigan MAT ESI 2, heated fused-silica capillary) with 2.75 kV spray voltage. HR accurate mass was determined at 10 000 resolution using an electrical scan from m/z 585 to 635 with a 1.3-s cycle time. The calibration standard PEG-diacid dissolved in methanol was applied in a concentration of 10 mg l^{-1}.

3. Results and discussion

The biodegradation process of anthropogenic surfactants usually starts with a primary degradation step but then often results in an incomplete ultimate degradation (mineralization). One of the most prominent persistent types of surfactant metabolites are NPs, which originate from non-ionic surfactant mixtures, NPEOs. The dead end metabolites, the nonylphenols, as just one alternative to NPEO degradation, are a mixture of isomers which differ in their C_9-alkyl chain structures. Some of these NP isomers have toxic effects on the biocoenosis in the biological wastewater treatment process [13]. Their estrogenic effects have been verified by in vitro and in vivo experiments [14–16]. Other surfactants, e.g. coconut diethanol amide, however, are easily degradable in the wastewater treatment process, as we have observed in many WWTP effluents in Germany. We wanted to point out these differences in the biodegradability of surfactants, the different ways in which these compounds could degrade and develop a method to monitor surfactants and their metabolites. Therefore we examined industrial blends of AEOs, NPEOs, and some NPEO derivatives under standard degradation conditions by submitting these compounds to an aerobic biodegradation by means of fixed bed biocoenoses of four WWTPs of the town of Aachen (Germany). The bacteria were immobilized on glass foam beads in a test filter device [12]. Additionally, NPEOs were anaerobically degraded by a fixed bed biocoenosis cultivated from the effluent of the sludge stabilization tank of Aachen-Soers WWTP by means of a modified device for biodegradation under strict oxygen exclusion.

Three alternative types of biodegradation may occur under aerobic conditions: (1) Immediate biodegradation, i.e. primary degradation followed by a rapid ultimate degradation resulting in mineralization products water, CO_2 and inorganic salts. This is not a standard procedure in surfactant biodegradation. (2) Biodegradation may result in primary degradation products. Compounds are converted to metabolites — surfactants under these conditions may lose their surface activity — before these metabolites will be finally degraded (mineralized) more or less rapidly. (3) The compounds resist biodegradation and can be found unchanged in structure and not reduced in quantity over a long monitoring period.

To obtain maximum information, the periods for taking samples were chosen according to their prospected degrees of biodegradability in order to avoid any loss of information about intermediates with short half lives in the dynamic degradation processes. The use of the matrix-free technique we applied for biodegradation rendered sequential elution by solvents with different polarities obsolete.

The application of soft atmospheric pressure ionization (API) techniques, such as APCI and ESI, first provided information about the molar masses of compounds which were ionized under the given conditions. A visual pattern by means of these full scan FIA–MS spectra enabled us to obtain a quick overview about the biodegradation result, while we compared the FIA–MS spectra of different sampling dates. The biodegradation can also be pursued semi-quantitatively by selected ion detection in the FIA–MS mode. More precise results, though, can be obtained by the time-consuming LC–MS method

which also permits us to distinguish the isomeric compounds and to characterize them by their retention times. For unequivocally identification the generation of daughter ions by MS–MS was essential.

3.1. Aerobic degradation of alkylethoxylates

Since they form an excellent example for the pattern recognition screening approach of FIA–MS, the biodegradation results of an easily degradable C_{13}-alkyl polyethoxylate surfactant blend should be presented and discussed first. Then the results of biodegradation of NPEOs and of the scarcely degradable NPEO derivatives or the persistent Triton X-200 will be reported on.

In order to monitor the precursor compounds and the generation of their metabolites, we applied the quite efficient and rapid monitoring procedure by FIA–MS. So the comparison of the patterns of the FIA–MS overview spectra from samples taken on different days or from different reactors allowed, on the one side, rapid recognition of alternative biodegradation pathways and, on the other side, made the progress of biodegradation in the reactors apparent. Since intervals between the sampling dates during AEO degradation were kept short enough, it was ensured that even arising intermediates with short half lives could be recognized during the monitoring process.

The quantification of selected mass traces of compounds of interest in the time-saving FIA–MS mode also provided semi-quantitative results about the degradation or generation of surfactants and metabolites, respectively.

The industrial blend of AEOs with their general structural formula $C_nH_{2n+1}O–(CH_2–CH_2–O)_xH$ allows an excellent follow-up by visual comparison of the FIA–MS overview spectra, since all AEO compounds and most of their metabolites contain polyether moieties. These moieties consist of glycol units which can be easily recognized because their equally spaced ions with $\Delta\ m/z$ 44 build up characteristic patterns of signals (cf. Fig. 1). Stable conditions (temperature; aeration intensity and contact time with the biocoenoses immobilized on glass foam beads) were chosen when this mixture of surfactant compounds was used for biodegradation experiments. These continued over periods of several days up to several weeks according to the biodegradability of the surfactants through the different biocoenoses.

So Fig. 1a–f presents the FIA–APCI-MS(+) overview spectra of this AEO mixture along with the primary degradation products (metabolites) as ammonium adduct ions ($[M+NH_4]^+$). We submitted the C_{13}-AEO blend with its average polyethoxylate chain length of 8 to aerobic biodegradation by applying the different biocoenoses of the four WWTPs of Aachen. Under these conditions, the AEO blend $C_nH_{2n+1}O–(CH_2–CH_2–O)_xH$ ($n=13$; $x=1–16$) with equally spaced ions ($\Delta\ m/z$ 44) at m/z 262, 306, 350, . . . 922 (Fig. 1a) in all cases was metabolized completely, and resulting in primary degradation products. Biodegradation occurred on quite different pathways which became recognizable via their resultant products. This becomes apparent by means of pattern recognition of FIA–MS spectra of precursor and metabolite ions that are obtained under positive APCI. The m/z ratios of precursor and metabolite ions are supporting these results. So in Fig. 1b, the ion pattern resembles the ions in Fig. 1a, but compared to Fig. 1a their m/z ratios have changed. Now they contain equally spaced ions (Δ m/z 44) at m/z 304, 348, 392, . . . 832 which differ from the precursor compounds by $\Delta\ m/z\ -2$. The result of aerobic biodegradation was that the terminal polyglycol units of the AEO precursors were oxidized to carbonylic groups [$C_nH_{2n+1}O–(CH_2–CH_2–O)_{x-1}–CH_2–C(H)O$; $x=1–13$], as was reported for alkyl polypropoxylates [17] or for polypropylene glycols [18]. This was discovered for the first time. As will be reported later, these findings could be verified by FIA–MS–MS daughter ion spectra of selected prominent parent ions of the carbonylic compounds.

The FIA–MS spectrum of the primary biodegradation products of the second WWTP (Fig. 1c) contained equally spaced ions with $\Delta\ m/z$ 44, too. But now there were less ions. Also, these ions had m/z ratios of 364, 408, 452, 496 and 540 differing by Δ $m/z\ +14$ from their precursor AEOs. Daughter ion spectra proved that these ions belonged to the metabolites of the AEOs carboxylated in the terminal position [$C_nH_{2n+1}O–(CH_2–CH_2–O)_{x-1}–CH_2–COOH$; $x=2–6$] [19]. The reaction can be described as a ω-glycol oxidation followed by a β-glycol

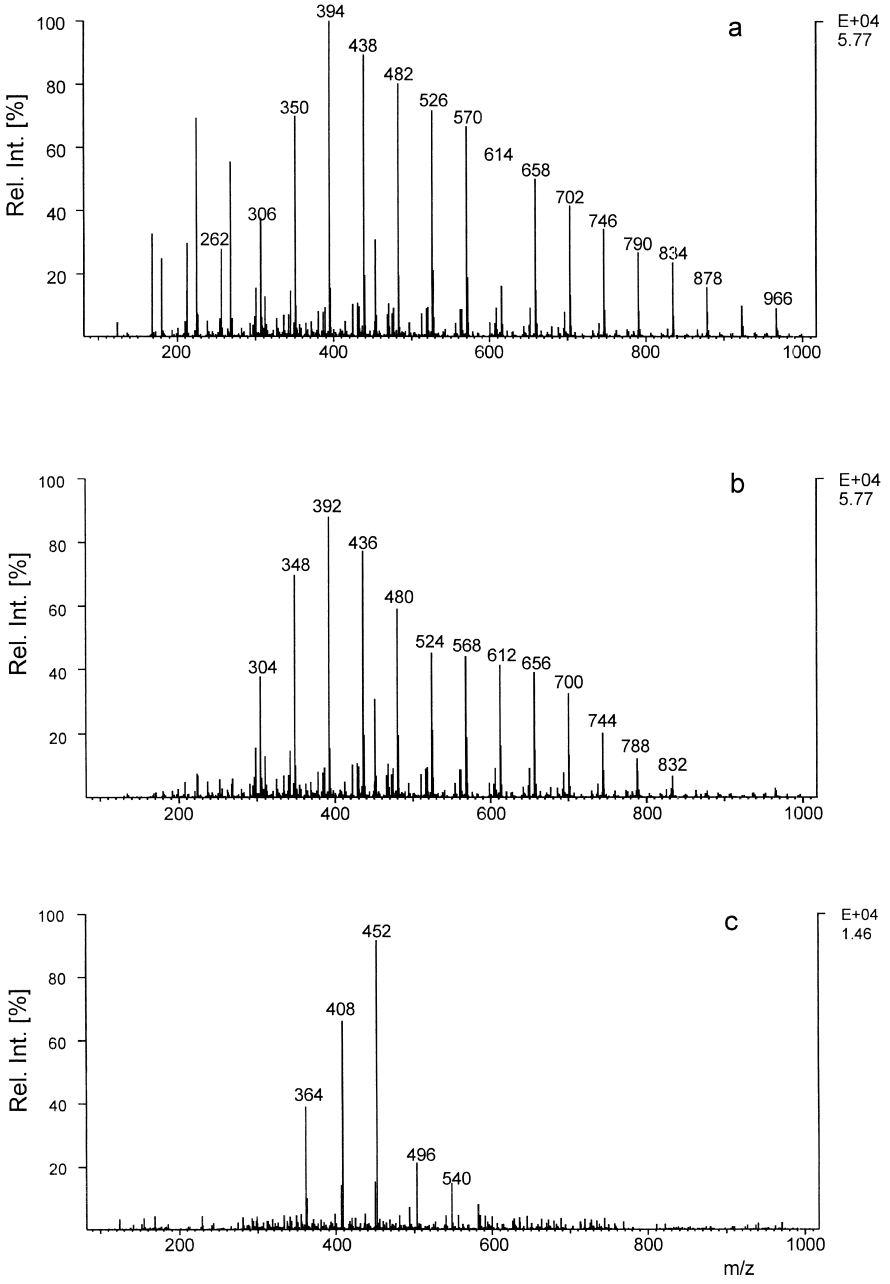

Fig. 1. (a) APCI-MS(+) loop injection spectrum for alkyl polyethoxylate blend (AEO) used for spiking purposes obtained bypassing the analytical column (FIA–MS), subsequently termed ''overview spectrum''. (b) FIA–APCI-MS spectrum for primary degradation products of AEO-spiked wastewater sample after aerobic degradation using biocoenosis of wastewater treatment plant (WWTP) 1 resulting in aldehyde compounds. (c) FIA–MS spectrum as in (b) after application of biocoenosis of WWTP 2 resulting in carboxylated AEO compounds. (d) FIA–MS spectrum as in (b) after application of biocoenosis of WWTP 3 resulting in polyethylene glycols as detectable metabolites (PEGs). (e) FIA–MS spectrum of metabolites (carboxylated PEGs) of primary degradation products (PEGs) of AEOs as in (d). (f) FIA–MS spectrum as in (b) after application of biocoenosis of WWTP 4 resulting in a mixture of primary degradation products of AEOs (carboxylated AEOs and PEGs). C_{18} SPE; eluent, methanol. Positive APCI ionization. For FIA and MS conditions, see Section 2.

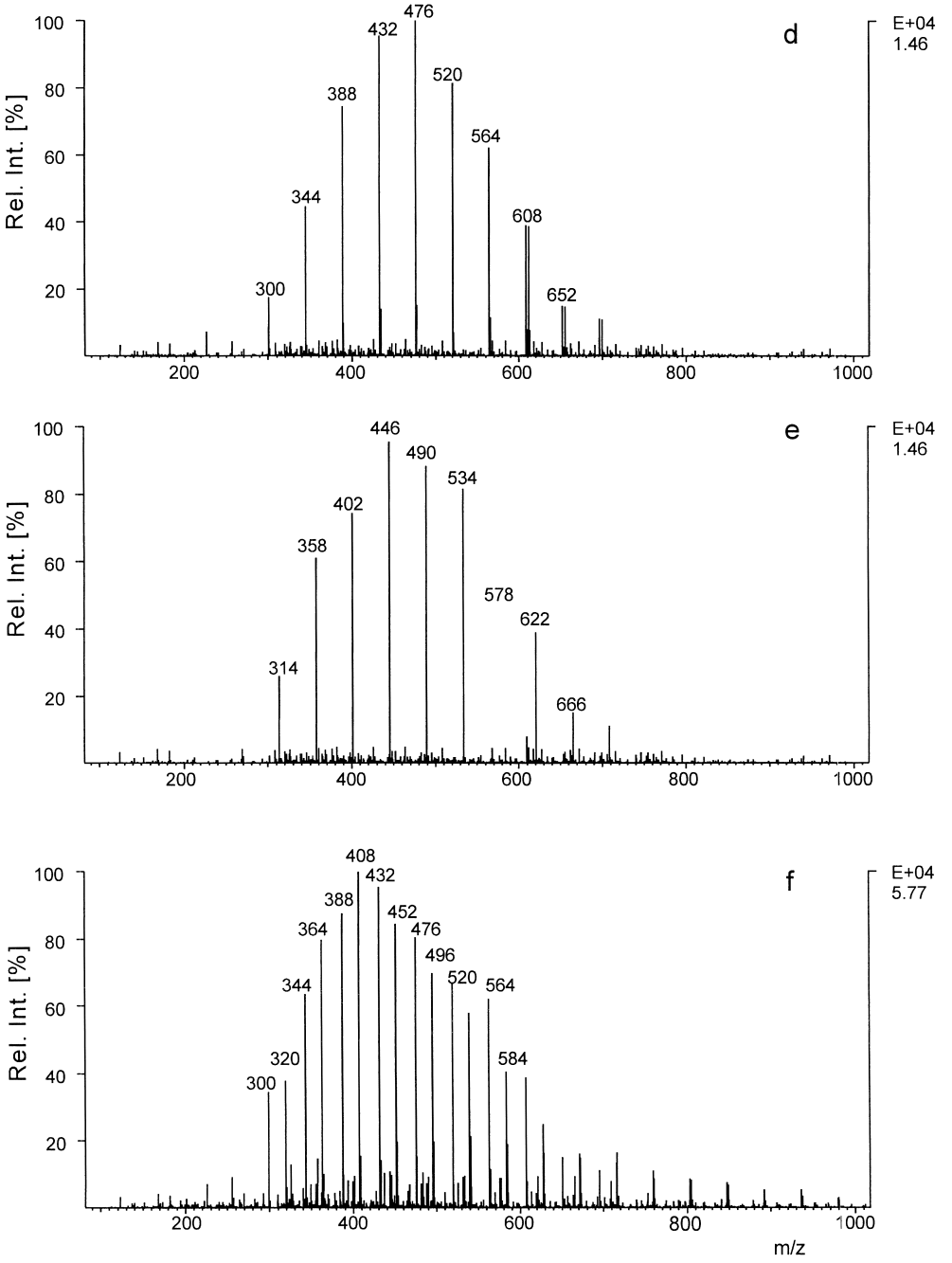

Fig. 1. (*continued*)

oxidation in combination with a shortening of the polyether chains.

The ions shown in Fig. 1d resulted from a biodegradation pathway which leads to the intramolecular scission of the bond between the alcohol moiety and the polyether chain. Parallelly, non-

oxidative cleavages of C_2 units had led to shorter chain polyethers. The polyether moieties, PEGs, can be detected by means of ESI- or APCI-MS in the positive mode because of their equidistant positive ammonium adduct ions at m/z 344, 388, 432, . . . 696 [HO–(CH_2–CH_2–O]$_x$–H; x=7–15], whereas the sensitivity for the detection of alcohol, the other arising metabolite, was too poor. An extended monitoring of this reactor for more than 2 weeks proved that the primary degradation products PEGs themselves were also degraded to carboxylated PEGs with the general formula HO–(CH_2–CH_2–O)$_x$–CH_2–COOH. Also an oxidative cleavage of C_2 units had occurred. The metabolites of this primary degradation product of intermediates could be ionized as $[M+NH_4]^+$ ions at m/z 314, 358, 402, . . . 622 (x= 5–12) (Fig. 1e), again equally spaced with Δ m/z 44.

At a glance, a complex mixture of metabolites resulting from a intramolecular bond scission between the alcohol and the PEG chain and the ω and β-glycol oxidation of AEO compounds could also be observed under aerobic biodegradation conditions (Fig. 1f). This more complex mixture of metabolites, which each consists of a series of Δ m/z 44 ions, and some more ions in the underground, showed a pattern that is quite different from all other FIA–MS spectra of AEO biodegradation (cf. Figs. 1b–e).

In order to confirm all FIA–MS examination results of AEO biodegradation, daughter ion spectra in the FIA–MS–MS mode with CID were performed [20]. Besides characteristic fragments from alkyl chains of AEOs (m/z 71, 85 and 99), i.e. polyether chain fragments which could substantiate preliminary statements about the identity of metabolites, we found the following daughter ions: For carbonylic (m/z 87, 131 and 175) and carboxylic AEO metabolites (m/z 103 and 147) (Fig. 1b or c), PEGs as primary AEO degradation products (Fig. 1d) could be verified via daughters at m/z 45, 89, 133 and 177, whereas the PEG-carboxylates in Fig. 1e show fragments varying by Δ −2 u (m/z 87, 131 and 175) from PEG daughter ions. The mixture of metabolites in Fig. 1f was identified as PEGs and AEO carboxylates by PEG daughters at m/z 45, 89, 133 and 177 and by AEO-carboxylate daughters at m/z 103 and 147.

A slight chemical modification in the easily degradable AEO molecules, however, leads to a persistent surfactant mixture. The compounds shown with their FIA–APCI-MS(+) spectrum (cf. Fig. 2a) ionized as $[M+NH_4]^+$ ions have the general structural formula $C_nH_{2n+1}O$–(CH_2–CH_2–O)$_x$–CH_3 (n=13–16; $x_{average}$=8). These AEO-CH_3 compounds were found to be persistent against aerobic treatment over a period of more than 6 weeks. Here, the protons of the terminal hydroxyl groups of the polyether moieties of the AEO molecules are substituted by methyl groups, closing and protecting this surfactant mixture against any aerobic biochemical degradation. By FIA–MS–MS(+) it was possible to confirm this result, whereas LC separation of this complex mixture of isomers and homologues under ion-pairing conditions failed even under mass trace analytical conditions. LC–APCI-MS(+) resulted in broad, unresolved signals as shown in Fig. 2b presenting the total ion current (TIC) trace and selected mass traces of equally space ions (Δ m/z 14) differing by the number of methylene groups (–CH_2–) in the alkyl chains. Because of a missing chromophore in these molecules UV detection was impossible.

In addition to its persistence in the aerobic degradation process, this mixture has found to be highly toxic against water organisms [11] because of their higher lipophilicity compared to normal AEOs.

The time-saving analytical procedure of FIA–MS for tracing the primary degradation of AEOs and FIA–MS–MS in order to confirm the identity of metabolites was possible because biodegradation pathways of non-alkylated AEOs, as shown in Fig. 3, have been comprehensively examined and are well-known. Different degradation pathways (cf. Fig. 3) were found. Besides the pathway 1 in combination with pathway 6 (intramolecular scission and glycol unit cut-off) ω-glycol oxidation in combination with β-glycol oxidation (pathways 3 and 5) and ω-glycol oxidation resulting in carbonylic AEO metabolites,were reported here for the first time. Compounds of this type had been found as metabolites of alkyl polypropoxylated surfactants [17]. The CID spectrum together with the fragmentation behaviour of one selected carbonylic homologue (m/z 392) is shown in Fig. 4. The ω-alkyl oxidation, however, reported as important primary degradation step [20], was not found. In addition, non-biodegradability of

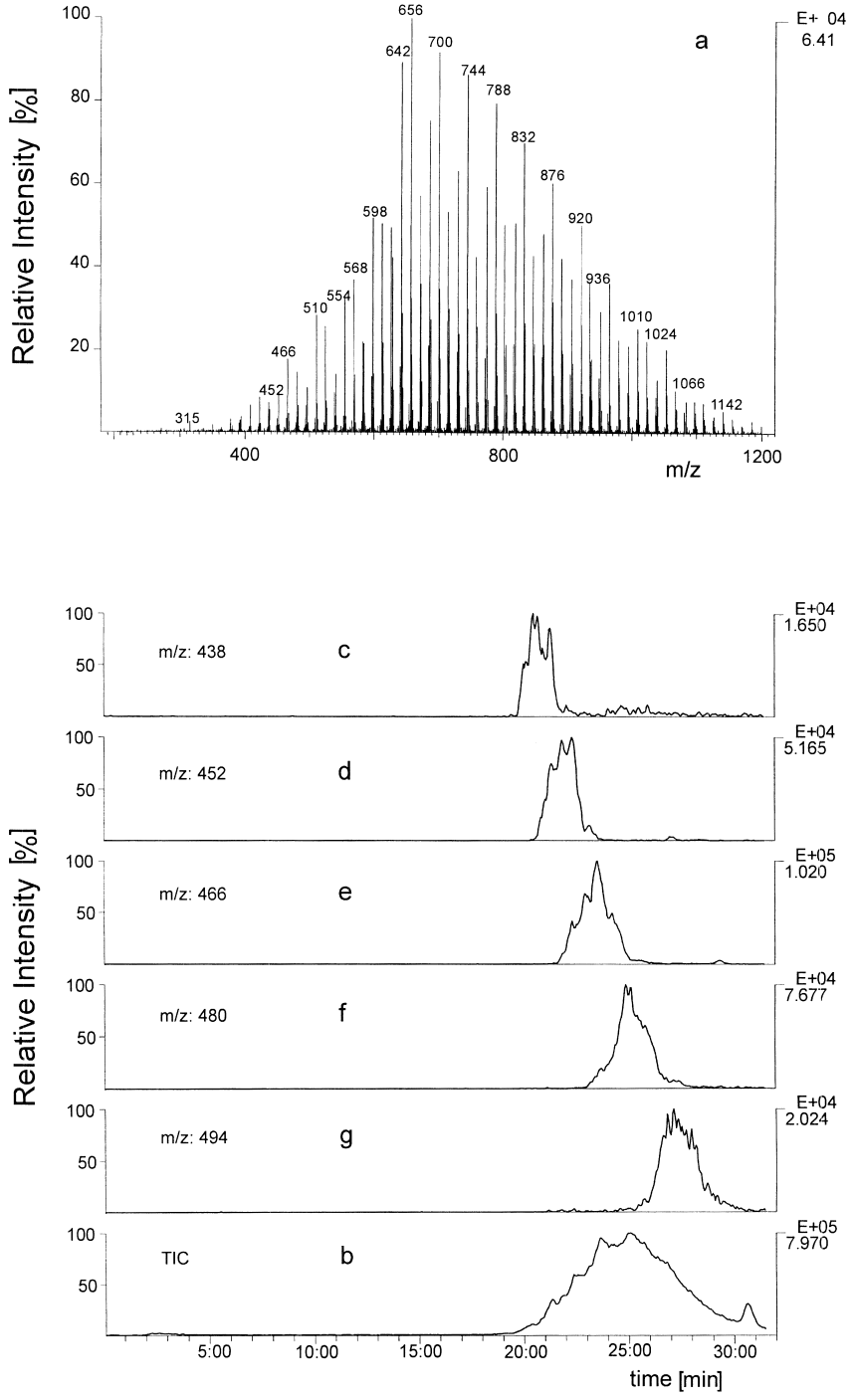

Fig. 2. (a) FIA–APCI-MS(+) overview spectrum of non-biodegradable methylated AEO blend (AEO-CH_3) with general structural formula $C_nH_{2n+1}O–(CH_2–CH_2–O)_x–CH_3$ (n=13–16; $x_{average}$=8). (b) LC–APCI-MS(+) total-ion current tracing (TIC) and (c–g) selected ion current tracings (m/z 438–494; Δ m/z 14) for standard solution of AEO-CH_3 blend. For FIA, LC and MS conditions, see Section 2.

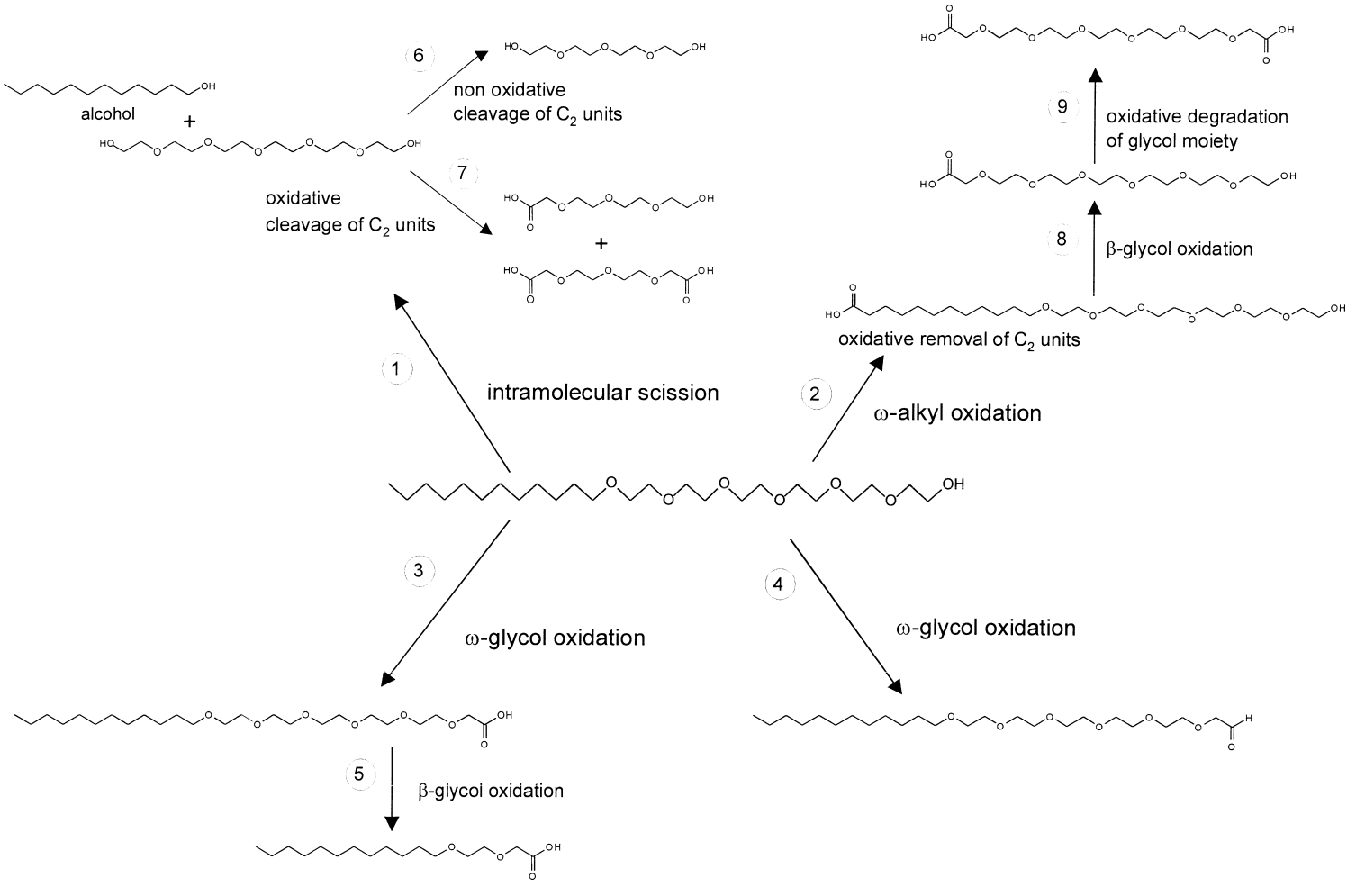

Fig. 3. Scheme of alternative biodegradation pathways of AEO compounds as in Fig. 1a which could be recognized partly in the FIA–MS overview spectra shown in the Fig. 1b–1f.

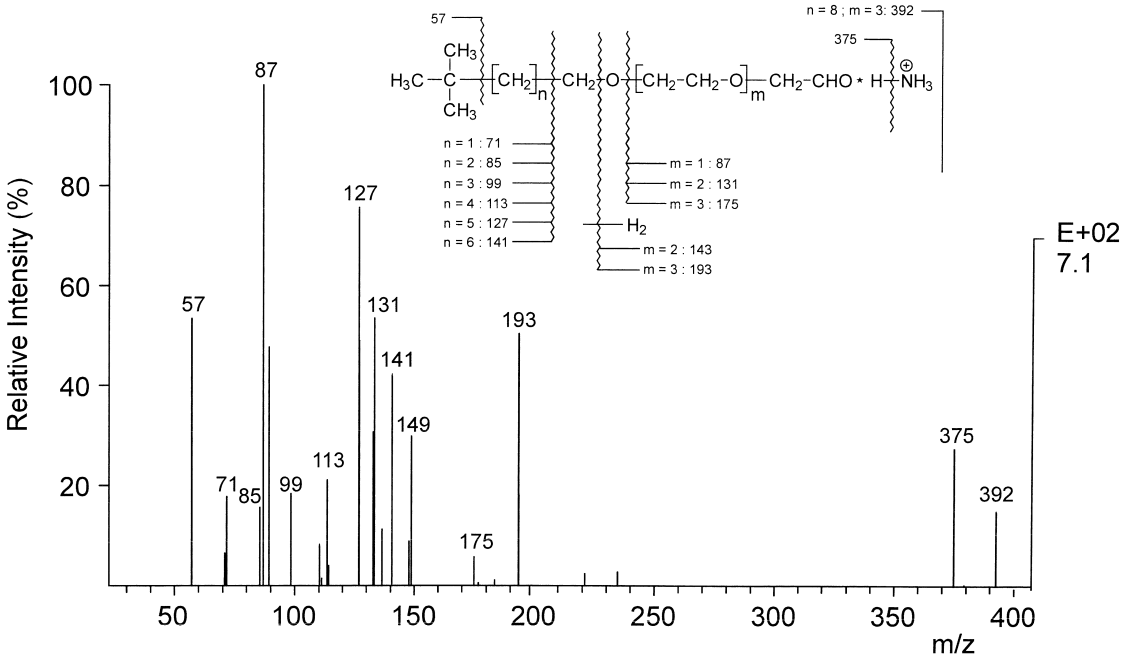

Fig. 4. FIA–APCI-MS–MS(+) daughter-ion mass spectrum (TSQ) and fragmentation behaviour under CID conditions of selected AEO metabolite ion (*m*/*z* 392) as in Fig. 1b, identified as carbonylic homologue. For FIA and MS–MS conditions, see Section 2.

AEOs alkylated in the terminal polyether moiety could be confirmed when we applied the FIA–MS–MS approach.

3.2. Aerobic degradation of nonylphenolethoxylates

The aerobic degradation of NPEOs was also examined comprehensively but reports about anaerobic degradation were quite rare [21,22]. Such a mixture, with a worldwide production of more than $0.5 \cdot 10^9$ kg per year, is applied as an economical non-ionic surfactant with a quite favorable profile of performance. Yet the degradation of NPEOs may lead to NPs, which are toxic to the wastewater biocoenosis and are known as compounds with endocrine disruptor activity. The pathway mainly leading to NPs has been described as a mixture of aerobic and anaerobic conditions [22], but the lack of valid information about it induced us in its further examination.

The aerobic biodegradation of NPEOs was monitored by FIA–APCI-MS(±) and LC–APCI-MS(+).We observed, along with a continuous decrease of NPEOs (C_9H_{19}–C_6H_4–O–$(CH_2$–CH_2–$O)_x$–H; x=3-20; m/z 326-1030), an increase of the primary degradation products of NPEOs, NPECs (m/z 340–824), which differed from NPEOs by +Δ m/z 14. Their structural formula was reported as: C_9H_{19}–C_6H_4–O–$(CH_2$–CH_2–$O)_{x-1}$–CH_2–COOH (x=2–11). In the FIA–MS(+) spectra, both types of compounds could be observed as $[M+NH_4]^+$ ions. The generated NPEC compounds were oxidized and shortened in their polyether chains by ω and in parallel β-glycol oxidation, as has been described for AEO compounds, too. The time-dependent monitoring of the biodegradation of NPEOs vs. NPECs proved that the concentration of short-chain NPEC compounds at first increased because of degradative chain shortening, reaching a maximum for the selected ions m/z 516 and 736 between day 4 and 5. Then the concentrations of these compounds started to decrease. In parallel FIA–MS proved that long-chain NPEOs disappeared from the beginning (Fig. 5). Long-chain NPECs (x>5) had disappeared entirely on the 16th degradation day. Despite the use of different biocoenoses in our NPEO degradation

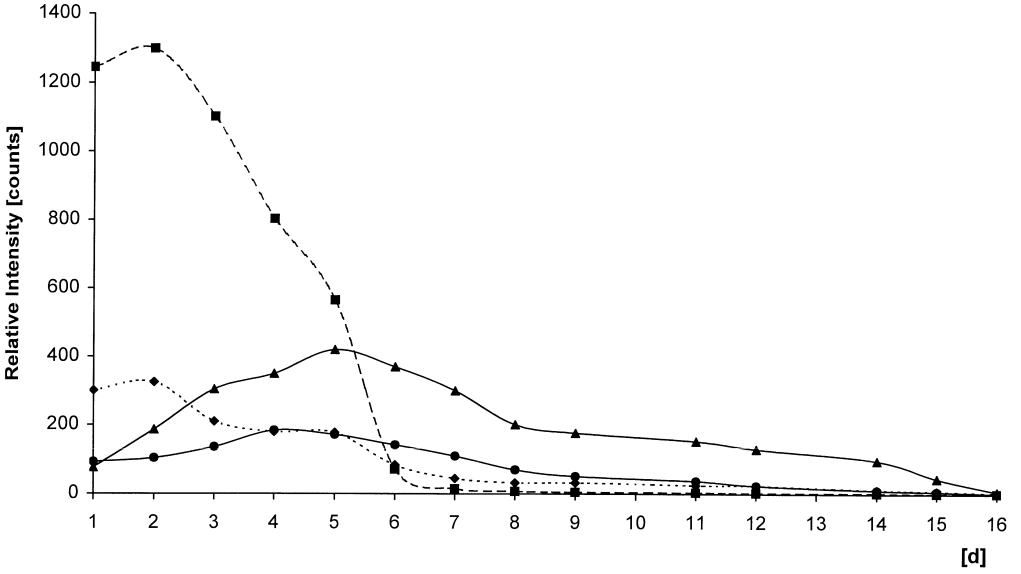

Fig. 5. Results of aerobic biodegradation of nonylphenol polyethoxylates (NPEOs) monitored by FIA–APCI-MS(+) recording selected ions over a period of 16 days. NPEO ions: m/z 502 (♦) and 722 (■); nonylphenol polyethercarboxylate ions (NPEC): m/z 516 (▲) and 736 (●). C_{18} SPE; eluent, methanol. For FIA and MS conditions, see Section 2.

experiments, as in AEO biodegradation, we did not detect NPEO compounds that were carboxylated in the alkyl chains (CNPEO), as has been reported by Di Corcia et al. [23]. GC–MS analysis after steam destillations of precursor compound mixture and wastewater after biodegradation confirmed that only marginal NP concentrations were generated from NPEOs under aerobic treatment conditions. These results corresponded to the literature [21,22].

3.3. Aerobic degradation of anionic nonylphenolethoxylate derivatives

The mixture of NPEOs also functions as a basis for the synthesis of nonylphenolethoxylates derivatives which are used as anionic surfactants. Their data of toxicity against bacteria (*Vibro fisheri*) and *Daphnia magna* for these NPEO derivatives had been promising compared to those of NPEOs, but nothing was known about the aerobic biodegradation behaviour of these compounds [20]. Therefore the biodegradation of selected NPEO derivatives, such as nonylphenol diethoxysulfonate [$NP(EO)_2$-SO_3; Triton X-200, structural formula: C_9H_{19}–C_6H_4–O–$(CH_2$–CH_2–$O)_2$–CH_2–SO_3H] and the mixture of nonylphenol polyethoxysulfates [NPEO-SO_4, structural formula: C_9H_{19}–C_6H_4–O–$(CH_2$–CH_2–$O)_x$–SO_3H] were studied under aerobic conditions in our test filter devices. The aim was to find out whether NPs were generated in the degradation process which should be accompanied by an increase of toxicity against water organisms [11,24,25].

At first, we examined the aerobic biodegradation of Triton X-200, a compound with a moderate toxicity compared to NPs. The samples which were taken daily from the devices were monitored by FIA–MS recorded in positive and negative modes. Under the aerobic conditions we applied, the FIA–MS overview spectra confirmed that $NP(EO)_2$-SO_3 was a persistent, non-biodegradable surfactant. From the start of our monitoring till its final phase, all FIA–MS spectra contained just one prominent ion, along with small amounts of impurities from synthesis. In contrast to the behaviour of nonylphenol polyethoxy sulfates (NPEO–SO_4), which can be negatively ionized only as $[M-H]^-$ ions, positive ionization of $NP(EO)_2$-SO_3 resulted in a $[M+NH_4]^+$ ion with m/z 420. In the negative APCI mode, an $[M-H]^-$ ion at m/z 401 could be observed.

Whilst our examinations of biodegradability were

performed over a period of 3 months, impurities in the FIA–MS spectra disappeared within 2 weeks. The molecular ion of Triton X-200, ionized in the positive or negative mode, however, could be observed during the whole time. FIA–APCI-MS(±) quantification in the single ion mode (SIM, m/z 420 or 401) applied to samples of different sampling dates, proved a reduction of less than 10% for Triton X-200 after 3 months.

The identity of the $NP(EO)_2$-SO_3 compound in all samples that had been used for quantification was verified by retention time (t_R) comparison LC–APCI-MS(±) and by FIA–APCI-MS–MS(±) in the daughter ion mode (Fig. 6a and b) using the precursor compound as standard. In the positive TSQ CID spectrum of $NP(EO)_2$-SO_3 the parent ion 420 disappeared and the daughter ion m/z 291 (cf. inset in Fig. 6a) known as characteristic NPEO daughter ion [20] here also was observed as base peak. Application of CID furthermore led to alkyl fragments (m/z 57, 71, and 113) and ions at m/z 165 and 121 resulting from the abstraction of the alkyl chain of ion 291 or from the abstraction of the alkyl chain and one glycol unit, respectively (Fig. 6a). Fragmentation of the negative parent ion m/z 401 by TSQ CID resulted in negative ions not yet identified with the exception of the ion with m/z 80 ($[SO_3]^-$). This fragment is a characteristic for alkyl and aryl sulfonates [20]. Under CID conditions applied on a TSQ the negative parent ion 401 remained base peak (Fig. 6b). MS–MS experiments with the $NP(EO)_2$-SO_3 blend on the trap coupled to the MAT 95 XL led to comparable results as obtained by CID on the TSQ.

The mixture of the NPEO derivative nonylphenol polyethoxysulfate was also submitted to aerobic biodegradation. The LC–APCI-MS(−) TIC trace (Fig. 7), along with the mass spectrum of the prominent signal, shows that the industrial blend consisted of NPEO-SO_4 homologues [C_9H_{19}–C_6H_4–O–(CH_2–CH_2–O)$_x$–SO_3H] with polyether chains of x=1–15 glycol units. Prior to biodegradation, the SPE eluate of the surfactant mixture dissolved in wastewater was ionized with Δ m/z 44 equally spaced $[M-H]^-$ ions at m/z 343 up to 959. Besides the prominent pattern of ions of the NPEO-SO_4 mixture, there was alkylphenol sulfate (m/z 299) and, to a small extent, ions of other impurities present. The positive ionization of the NPEO-SO_4 mixture resulted in ammonium adduct ions m/z 326–942 equally space with Δ m/z 44. These NPEO ions were generated by cleavage of the EO-SO_3-bond during the destructive ionization process of the precursor NPEO-SO_4 [20]. For homologues at m/z 607 and 651 selected from the series of negative parent ions no structural information could be obtained by CID on the TSQ despite the variation of CID parameters. Daughter ions could neither be generated with a moderate sensitivity nor ions were reproducible and therefore could not be used for identification.

The results of biodegradation of the NPEO-SO_4 mixture were monitored by FIA–MS with daily samples. Changes in the pattern of ions in the overview spectra recorded by FIA–APCI-MS(−) were only marginal. After a treatment and sampling period of 5 days, LC–APCI-MS(−) was performed. The total ion current trace and the UV-trace 220 nm of the surfactant mixture after biodegradation is presented in Fig. 8. This LC–MS of C_{18}-SPE eluate proved that a biodegradation has taken place. The NPEO-SO_4 mixture was reduced in concentration (compare RICs in Figs. 7 and 8; t_R window 13.5–16.0 min) while a mixture of metabolites in parallel was generated (t_R: 5.5–6.0 min). Yet this could not be recognized with FIA–MS recorded under low-resolution conditions on a triple-quad MS, since metabolites of the NPEO-SO_4 mixture show the same molar masses as their precursor compounds.

Structural information about the metabolites of NPEO-SO_4 applying CID on the TSQ was not available as found for NPEO-SO_4, too. MS^n experiments operated on an ion trap coupled to the MAT 95 provided more information about the structure of the metabolites. Negatively charged daughter ions of both, the mixture of metabolite ions and the precursor NPEO-SO_4 mixture ions could be generated. MS–MS spectra of the metabolite ion at m/z 607 resulted in the same daughter ions as the precursor compound ion m/z 607 u: so from both parent ions series of ions at m/z 211, 255, 299, 343 and 387 u, all equally spaced with Δ 44 u could be observed. In addition ions at 521 u were obtained from both parent ions at 607 u. The metabolite ion 607 u, however, resulted in an additional daughter ion at m/z 564 (Fig. 9b) not observed in the CID spectrum

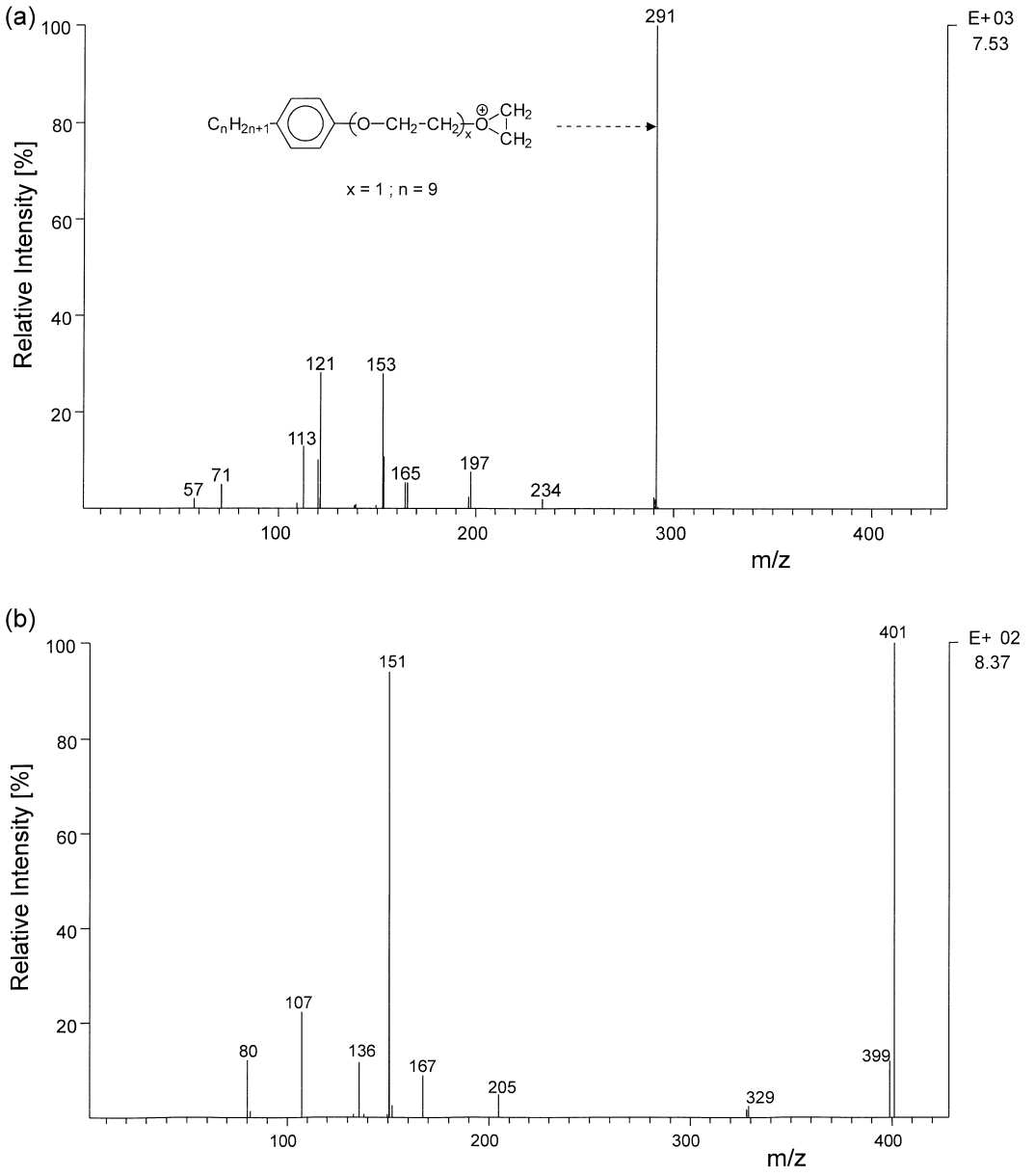

Fig. 6. Confirmation of results of aerobic biodegradation monitoring of Triton X-200 (NPEO-SO_3, structural formula: C_9H_{19}–C_6H_4–O–$(CH_2$–CH_2–$O)_2$–CH_2–SO_3H) by FIA–APCI-MS–MS recorded on TSQ. (a) Positive daughter-ion mass spectrum of NPEO-SO_3 with the structural formula of fragment ion m/z 291 (base peak) in the inset. (b) FIA–Negative APCI-MS–MS spectrum as in (a). C_{18} SPE of wastewater; eluent, methanol. For FIA and MS–MS conditions, see Section 2.

of the NPEO-SO_4 homologue (Fig. 9a). MS–MS–MS experiments performed with the ion trap confirmed that besides the fragment ions m/z 97 ($[HOSO_3]^-$) the Δ 44 u equally spaced fragment ions (m/z 211, 255, 299, 343 and 387) arose from the sequential loss of (CH_2–CH_2–O) moieties of the fragment ions $[CH_2{=}CH$–O–$(CH_2$–CH_2–$O)_6$–$SO_3]^-$ at m/z 387 which can be observed in the

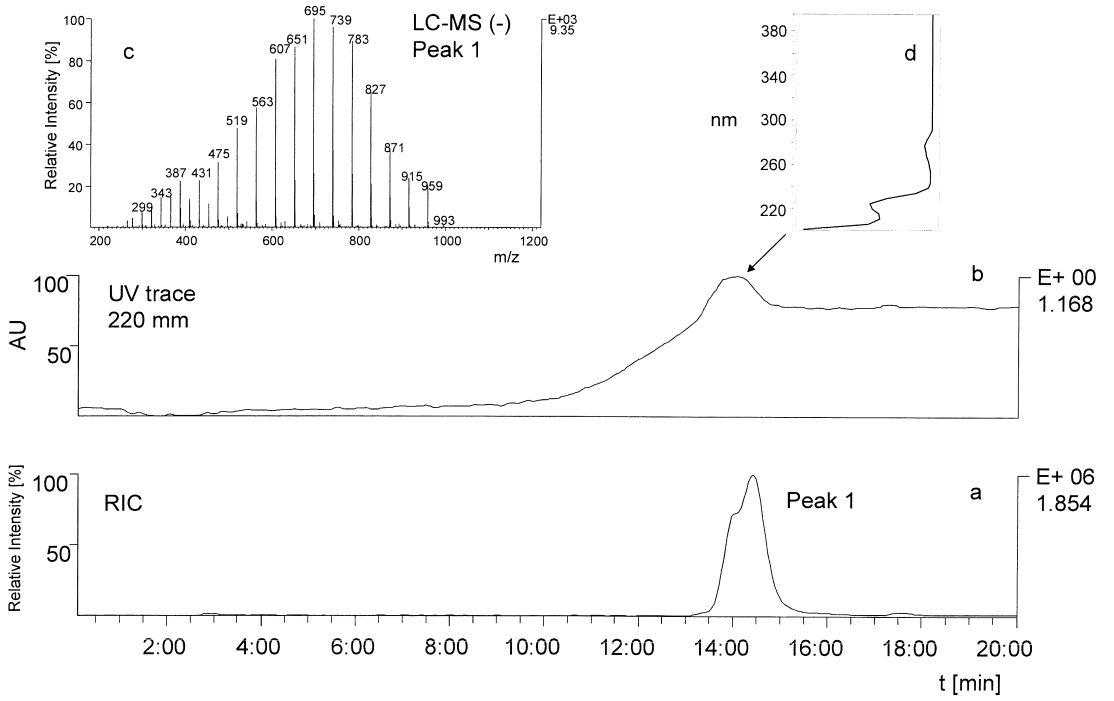

Fig. 7. (a) LC–MS total-ion current tracing for wastewater sample spiked with nonylphenol polyether sulfates (NPEO-SO_4, general structural formula: C_9H_{19}–C_6H_4–O–(CH_2–CH_2–O)$_x$–SO_3H, x=1–15). C_{18} solid-phase extract; negative APCI ionization. (b) UV trace (220 nm) of (a). (c) Averaged mass spectra of peak 1 (range 13.5–15.0 min) (d); UV spectrum as in (c). For UV, FIA–, and LC–MS conditions, see Section 2.

fragmentation process of both compounds the NPEO-SO_4 metabolite and the precursor NPEO-SO_4. Both compounds on the one hand showed quite similar daughter ions (cf. Fig. 9a and b) on the other hand behaved quite different in LC separation (cf. Figs. 7 and 8). Nevertheless the final information about the structure of these two different types of compounds could not yet be obtained.

Therefore medium and high-resolution FIA–MS were applied to acquire a differentiation between the ions of the surfactant mixture and their metabolites.

First medium-resolution accurate mass data (resolution 3500) for the precursor anionic surfactant compound of the industrial blend specified as NPEO-SO_4 (isomer with 7 EO units) were obtained by LC–ESI-MS in the negative mode on the $[M-H]^-$ ion at m/z 607 [reference comp. PPG m/z (calc.) 597.42139, 655.46325]. With 607.3132 u we found an excellent agreement with the calculated mass (607.315210 u) for the elemental composition of the deprotonated NPEO-SO_4 [isomer with 7 EO units, with the structural formula C_9H_{19}–C_6H_4–O–(CH_2–CH_2–O)$_7$–SO_3^-]. Better results were obtained when two selected ions [x=10 and 11; m/z (calc.) 587.291497, 631.317710] of the mixture of homologues of the PEG-diacid [HOOC–(CH_2–CH_2–O)$_x$–CH_2–COOH (x=3–15)] negatively ionized as $[M-H]^-$ ions ([HOOC-PEG-COO]$^-$) were applied as internal standards. No other logical elemental composition than $C_{29}H_{51}S_1O_{11}$ was found by computer search within a ±0.002 mass unit tolerance and the elemental upper limits C 31; H 100; O 20; S 1. This result confirms that the elemental composition of the neutral precursor compound is $C_{29}H_{52}S_1O_{11}$ after a hydrogen will be added to the deprotonated molecule that has been mass-measured.

The unknown metabolite was determined with 607.3011 u applying a resolution of 3.500. With the application of PEG-diacid as standard an increase of concentration was possible resulting in an increased resolution up to 10 000. So the compound was determined with 607.29738 u. Despite increased

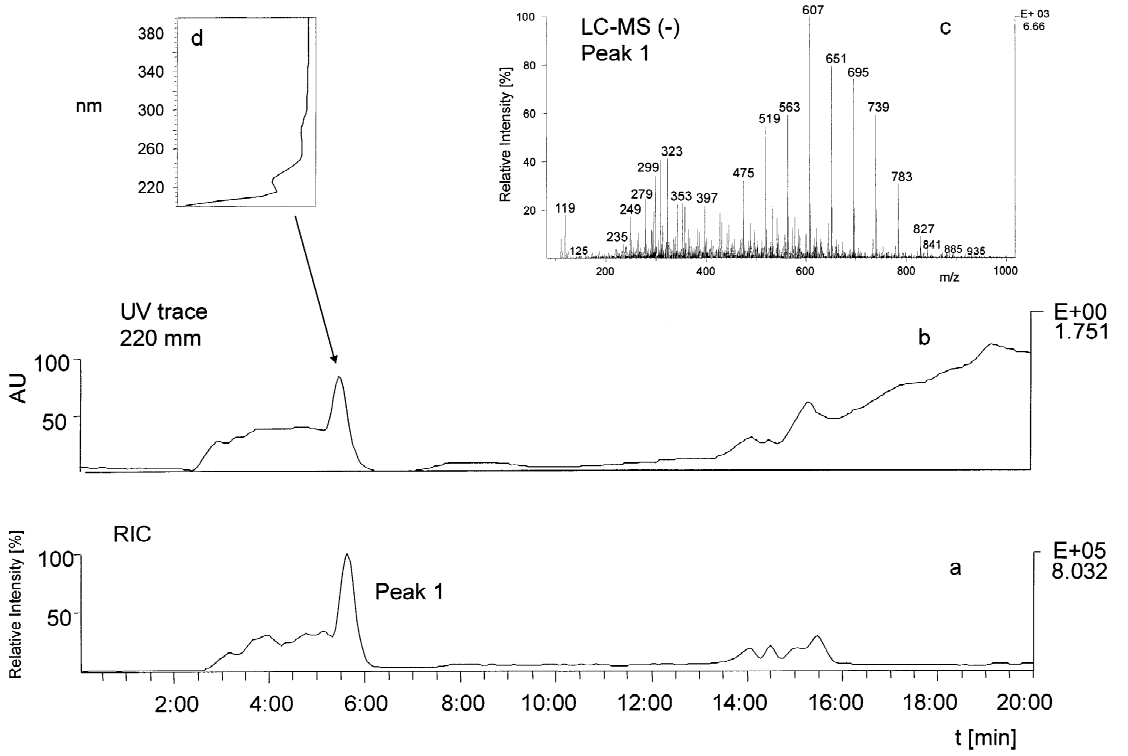

Fig. 8. (a) LC–MS total-ion current tracing for wastewater sample after aerobic biodegradation and spiked with nonylphenol polyether sulfates (NPEO-SO_4) prior to biodegradation. C_{18} solid-phase extract; negative APCI ionization. (b) UV trace (220 nm) of (a). (c) averaged mass spectra of peak 1 (range 5.5–6.0 min) (d); UV spectrum as in (c).). For UV, FIA–, and LC–MS conditions, see Section 2.

resolution we received non-plausible elemental mass compositions. Therefore the metabolite could not yet be identified.

3.4. Anaerobic degradation of nonylphenolethoxylates

The aerobic degradation of NPEOs is quite well examined [21], but information on the anaerobic process of NPEO biodegradation, which finally results in the persistent metabolites NPs, as it can be found in the wastewater sludge of some treatment plants or in sludge amened soils, is quite rare [26]. Therefore we studied the anaerobic biodegradation of NPEOs under matrix-reduced conditions, applying glass foam beads to immobilize biocoenosis. The reactor system was modified to make a strict exclusion of oxygen possible. Then the effluent of a sludge stabilization tank was added and was circled in the reactor passing the glass bead column. This closed circle was equipped with a device which allowed the determination of the oxygen content in the reaction medium wastewater during the whole anaerobic treatment period. Anaerobic conditions (redox potential <-380 mV) were sustained by adding 50 mg l^{-1} per-day of ammonium acetate dissolved in very small amounts of water. After an equilibration period of 2 days the NPEO blend was added. The first samples for FIA, LC–MS and GC–MS were taken 2 h after spiking with NPEOs. FIA–MS(+) of wastewater extract is shown in Fig. 10a. Since anaerobic degradation processes take more time, sampling was repeated every 3 days. First modifications in the NPEO patterns worth mentioning could be observed in the samples of day 9, which were submitted to FIA–APCI-MS(+) and LC–MS(+) determination. Comparing the peak height in the overview spectra here a shortening of the polyether chains could be observed (Fig. 10b). At the beginning of the examination, the NPEO blend had

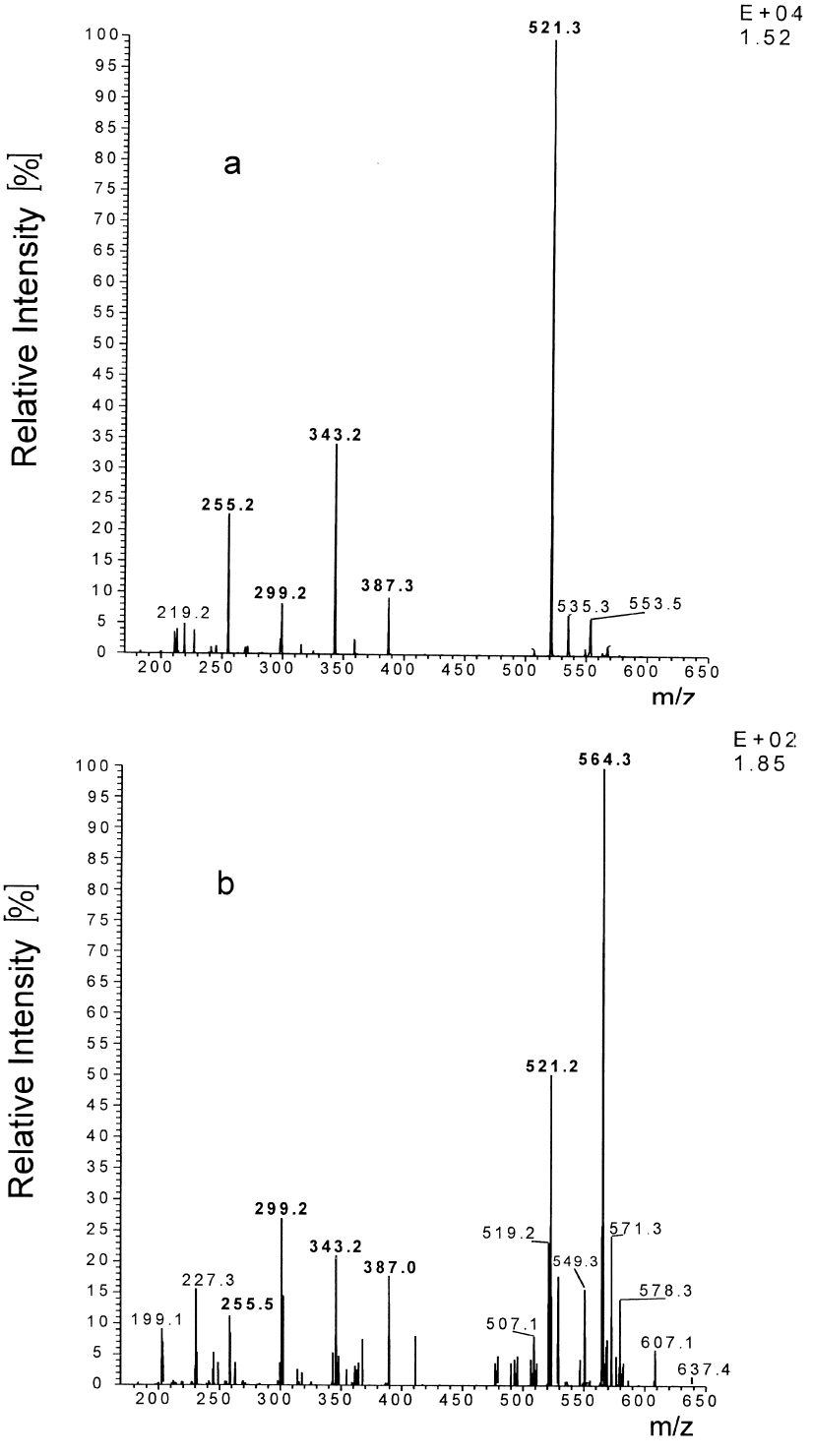

Fig. 9. FIA–APCI-MS–MS(−) daughter-ion mass spectrum recorded by ion trap for (a) selected parent ion at m/z 607 u of NPEO-SO_4 mixture; (b) daughter-ion mass spectrum of metabolite ion at m/z 607 u obtained by biodegradation of NPEO-SO_4 mixture. For FIA and MS–MS conditions, see Section 2.

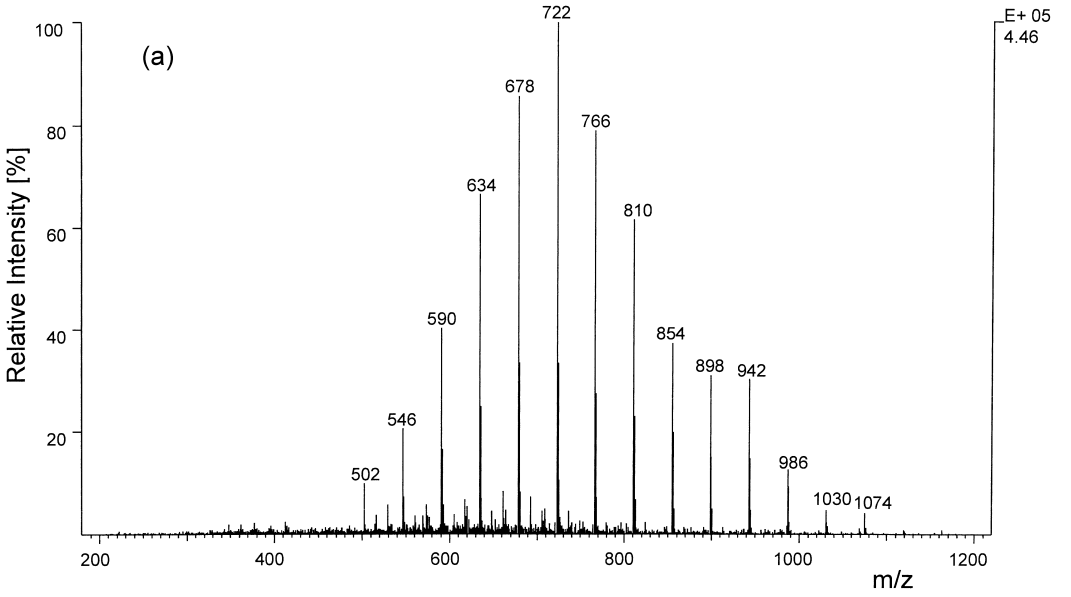

Fig. 10. FIA–APCI-MS(+) overview spectra for (a) wastewater sample spiked with long chain NPEO blend prior to anaerobic biodegradation, (b) wastewater sample as in (a) after an anaerobic biodegradation period of 9 days, and (c) after an anaerobic treatment period of 25 days. C_{18} SPE; eluent, methanol. For FIA and MS conditions, see Section 2.

consisted of a series of homologues with 6 to 19 polyether units ($[M+NH_4]^+$: m/z 502-1074), but now concentration of the long chain NPEO homologues decreased and homologues with only two and three polyether units (m/z 326, 370) were recorded by FIA–MS(+). In parallel to the NPEO surfactants compounds with Δ m/z +14 could be observed. Ions with these m/z ratios were also recorded during aerobic biodegradation. Despite the fact that the reactor was kept strictly anaerobic these compounds with equally spaced ions of Δ m/z+14 at m/z 340, 384, . . . 824 could be confirmed as NPECs by FIA–MS–MS(+), LC–MS(±), and LC–MS–MS(+) (Fig. 10b). The retention time as well as daughter ion spectra of selected ions were found to be identical with compounds isolated from aerobic NPEO biodegradation process and here used as standards. After an anaerobic treatment of more than 4 weeks, short chain NPECs were still present, but long chain NPEOs could not be observed any longer by FIA–MS(+) (Fig. 10c).

We expected that biodegradation of NPEO blend resulted in NPs. The detection of NPs, $NP(EO)_1$ and $NP(EO)_2$ using the APCI or ESI interface of Finnigan was impossible. Despite the variation of ionization conditions sensitivity was too low to acquire reliable data. Therefore the steam-volatile NPs and the short-chain NPEOs were determined by GC–MS in the electron impact EI mode after steam destillation or in the SPE eluates. TICs traces of NPs, $NP(EO)_1$, and $NP(EO)_2$ analyses for all compounds show broad signals because of the number of isomeric compounds (Fig. 11). By means of 4-*n*-NP added as internal standard the compounds could be quantified.

In contrast to aerobic degradation, a mass balance of NPEOs vs. NPs, i.e., monitoring the disappearance of NPEOs with the simultaneous appearance of NPs in the anaerobic biodegration process was not quite easy. While aerobic degradation led to metabolites more polar than their precursor compounds which therefore were dissolved in the water phase, anaerobic degradation of NPEOs, however, led to the more lipophilic NP metabolites. NPs were adsorbed at the sludge which first was suspended in the anaerobic wastewater effluent when the degradation device was filled and later on was concentrated at the bottom of the glass beads column according to the flow inside the reactor system. In contrast to aerobic degradation, the biocoenosis in the sludge stabiliza-

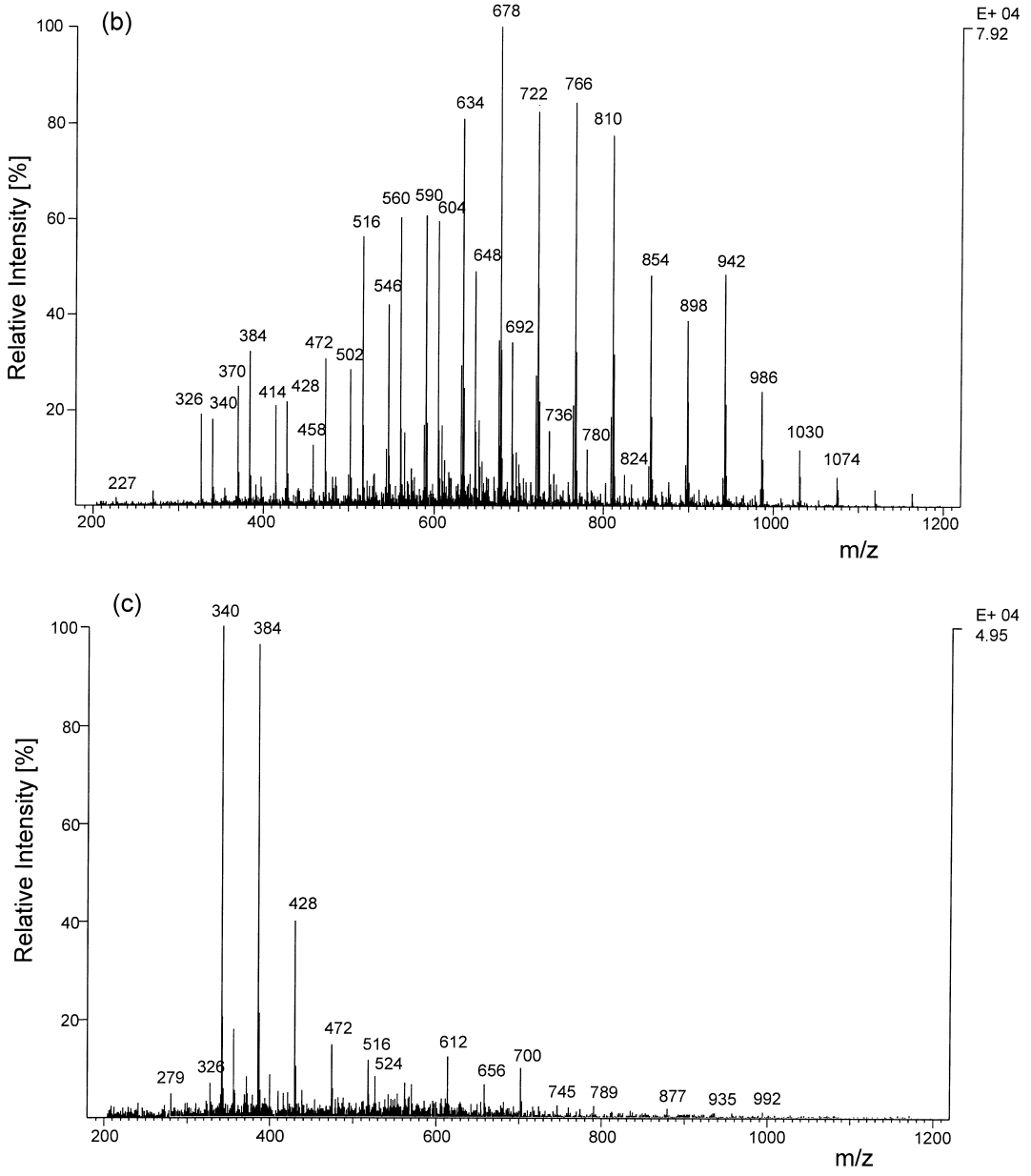

Fig. 10. (*continued*)

tion effluent grew so steadily under anaerobic biodegradation conditions that after 6 weeks there was a surprising manifold of bacterial mass. This biomass concentrated the lipophilic NPs by adsorption, pretending a decrease of the total amount of NPEOs and NPs in the aqueous phase of the reactor system. A semi-quantitative estimation in the whole system after cancellation of the examinations by using the concentration of NPs in the wastewater and in an aliquote of the wastewater/sludge suspension from the anaerobic reactor confirmed this finding of an elevated NP content in the sludge of the reactor.

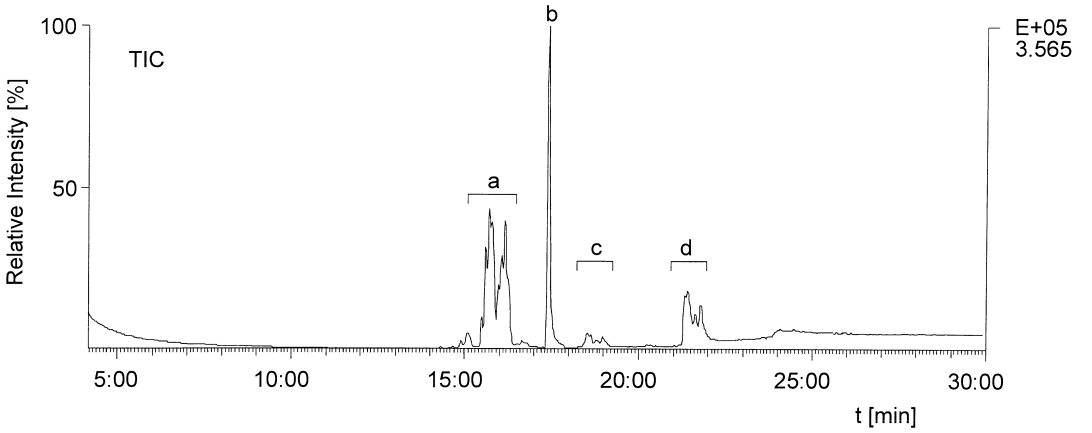

Fig. 11. GC–EI(+)-MS total-ion current tracing for NPEO spiked wastewater sample after an anaerobic biodegradation period of >4 weeks containing the peaks of (a) NPs, (b) 4-*n*-NP (internal standard), (c) $NP(EO)_1$, and (d) $NP(EO)_2$. C_{18} SPE; eluent, methanol. For GC–EI-MS conditions, see Section 2.

Aquatic toxicity in parallel monitored in the water phases by *Daphnia magna* and *vibrio fischeri* for NPEOs and NPEO-derivative did not change worth mentioning during biodegradation process.

4. Conclusions

For biodegradation monitoring of surfactants, the FIA–MS screening method applying pattern recognition has proved to be a fortunate approach which provides a quick overview of results. Especially compounds containing polyether substructures are easy to recognize in their FIA–MS spectra because of their characteristic, equally spaced ion patterns of polyethers. For unequivocal confirmation of results FIA-MS–MS, LC–MS, and –MS–MS is often essential. If standard compounds are not available for comparison of daughter ions or if the generation of daughter ions fail, accurate mass determination applied by HR-MS improves the chance of identification and confirmation.

As FIA–MS screening proved, the non-ionic surfactant mixture, a C_{13} AEO blend, was found to be easily degraded under aerobic conditions. When the biocoenoses of the four existing Aachen WWTPs were applied for biodegradation of the same AEO compound, different degradation pathways of AEOs could be observed by FIA–MS. The differing pattern of ion series obtained made these results apparent and FIA–MS–MS was then used for confirmation.

An explanation of the diversities in the degradation pathways may be hidden in the diversity of the bacteria originating from 4 different WWTPs. Since these WWTPs have to handle different wastewater constituents, the result was a selection of these bacteria and their adaption to different compound mixtures. After immobilization in our reactor devices they did not lose the corresponding properties and therefore degraded the same AEO compound mixture in quite different ways.

FIA–APCI-MS and LC–MS in the positive mode proved that the AEO surfactant mixture methylated in the terminal polyether position was persistent against aerobic biodegradation despite an acclimation time of several weeks.

NPEOs were biochemically degraded under aerobic conditions, resulting in NPECs as primary degradation products, before these compounds themselves were degraded. FIA–MS(+) spectra proved that NPEO degradation took place as a ω and β-glycol oxidation generating NPEO molecules with carboxylated terminal glycol chain links. In parallel the polyether chains were shortened. Quantification of selected ions over the treatment period proved that the concentrations of NPEOs decreased more rapidly than NPEC concentrations increased (cf. Fig. 5). Finally NPEO ions with shorter polyether chains could be observed after aerobic degradation as persistent compounds, whereas no NPs in any concentrations worth mentioning could be detected.

From our knowledge the anaerobic biodegradation

of NPEOs under strict exclusion of oxygen (redox potential <-380 mV) was first monitored by MS. The reaction pathway led from long polyether chain compounds to homologues with shortened polyether chains besides NPEC homologues. The reason why oxygenated compounds were formed under these conditions in repeated experiments can not be explained but the presence of oxidised inorganic sulphur compounds which, theoretically would be able to form carboxylic compounds, could be excluded.

After cancellation of the degradation treatment, NPEOs with 1 or 2 glycol units [$NP(EO)_1$ and $NP(EO)_2$] could be analysed by GC–EI-MS(+) together with NPs in the SPE wastewater extracts in small amounts (cf. Fig. 11). Steam destillation of an anaerobic sludge suspension and GC–MS analysis proved that over-proportional concentrations of the NP metabolites were present in the sludge.

Mass balance calculations on the basis of the quantities of NPEOs spiked and concentrations of NPs, $NP(EO)_1$, and $NP(EO)_2$ observed by GC–MS after biodegradation indicated that besides high concentrations of the NPEO metabolites ''nonylphenols'' small amounts of additional NPEO primary degradation products [$NP(EO)_1$ and $NP(EO)_2$] were still present. I.e., no mineralization of the dominant recalcitrant metabolites, the NP isomers had taken place moreover NP isomers were concentrated in the sludge but were not dissolve in the wastewater phase. Under the conditions applied NP isomers were only eliminable from wastewater but not anaerobically degradable.

The anionic derivatives of NPEOs, the NPEO-sulfonate, and the NPEO-sulfates showed a quite different degradation behaviour under aerobic conditions if compared to the non-ionic NPEOs. Nonylphenol diethoxy sulfonate (Triton X-200) was not degradable under aerobic conditions, while aerobic degradation of NPEO-sulfate led to primary degradation products. But NPEO-sulfates metabolites could only be recognized, when LC–MS(−) was applied, because homologues of precursor compounds and metabolites were detected with ions of the same m/z ratios. Remarkable different retention times were observed, but no differentiation by means of daughter ions in the FIA mode was possible. Under CID conditions operated on the TSQ, neither reproducable daughter ions of the NPEO-sulfates nor those of their metabolites could be generated.

An elucidation of the structures or of the elemental composition of the unknown metabolites via medium-resolution MS was not possible because of a lack of material for analysis. After we obtained the metabolite mixture after a further biodegradation experiment a resolution of 10 000 was obtained under LC and FIA–MS(−) conditions on the MAT 95 XL Trap instrument by using PEG-diacid for calibration purposes in the accurate mass measurements. Experiments using the precursor surfactant as standard to distinguish, under FIA-MS conditions, between the masses determined for surfactant compound (607.3132 u) and metabolite (607.3011 u) that both were present in the mixture, is only possible with a resolution of $>50\,000$. This can only be realised with a sector field mass spectrometer in combination with a highly concentrated sample, containing a compound which exhibited one or two molecular ions but not series of ions like the surfactant blend or the biodegraded surfactants. The propositions of elemental compositions obtained from HR measurements applying PEG-diacid were non-plausible (range ± 20 mmu), too.

To explain the structure of these metabolite homologues because of their fragmentation behavior was not yet possible. The generation of daughter ions of $NPEO\text{-}SO_4$ precursor molecules or their metabolites by negative MS–MS(−) on the TSQ was impossible whereas CID on the ion trap provided a lot of information. The structural differences between both types of molecules — metabolites and precursors — were found absolutely marginal as confirmed by FIA–MS–MS^n results operated on an ion trap coupled to a MAT 95 (cf. Fig. 9a and b). Despite this marginal variation in their structures the large differences in their behavior under RP-C_{18} separation conditions, however, were impressive.

Our results confirmed the observation that the $NPEO\text{-}SO_4$ mixture was degraded, but aerobic biodegradation did not start with a bond scission between polyether chain and sulfate moiety. Moreover the degradation started in the alkyl chain as observed by MS–MS–MS results.

FIA– and LC–MS and –MS–MS in the positive and/or negative mode proved that under aerobic biodegradation conditions NPEOs and the two anionic NPEO derivatives behaved quite differently. These findings were also in good agreement with the toxicity testing data determined in the water phases

before and after biodegradation. These data had proved a marginal increase of toxicity in the water phases during aerobic and anaerobic NPEO degradation because of the generation of small amounts of NPs. As under anaerobic conditions the NP metabolites were adsorbed by the sludge and therefore could not be found in the wastewater toxicity only increased moderately. No changes in toxicity were observed when NPEO derivatives could be observed as persistent compound [$NP(EO)_2$-SO_3] or were biodegraded ($NPEO$-SO_4). The explanation of this behaviour under biodegradation or CID conditions is hidden in the electronic and structural differences of these surfactants, although the same substructure of NPs is present in all compounds.

The differences in the behavior of NPEOs vs. NPEO derivatives as well as AEOs vs. methylated AEO compounds demonstrate that prognosis of biodegradability is not quite easy.

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