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Characterization of Recalcitrant Intermediates from Biotransformation of the Branched Alkyl Side Chain of Nonylphenol Ethoxylate Surfactants

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Nonylphenol polyethoxylates (A_9PEs) are complex mixtures of isomers and oligomers widely used as surfactants. The weakly estrogenic effects of the A_9PE breakdown products are impelling reformulation of many commercial products and have made "A_9PE free" products an industry trend. Initial A_9PE biotransformation is known to produce metabolites with shortened ethoxy chains, mainly A_9PE_2, and carboxylated ethoxy chains, mainly A_9PE_2C. Very little is known on the ultimate fate of the branched alkyl side chain. We have used liquid chromatography−electrospray−mass spectrometry to elucidate structures of species generated from biotransformation of the branched alkyl chain. According to an OECD protocol, a laboratory A_9PE biodegradation experiment was conducted. After about two weeks from the beginning of the experiment, relevant amounts of species having both side chains oxidized (CAPECs) and 3−8 carbons in the residual alkyl chain appeared in the test solution. These species were presumably generated from less extensively alkyl branched A_9PEC isomers by various oxidative mechanisms. The unreacted most extensively alkyl branched A_9PEC isomers disappeared from the test liquor after more than 3 months by an unknown mechanism. Less abundant, metabolites having only the alkyl chain carboxylated (CAPEs) were also formed. With time, these species were slowly transformed to CAPECs. Apart from a very slow conversion of $CAPE_2$Cs to $CAPE_1$Cs, this metabolite class was extremely recalcitrant to further biotransformation, as they persisted in the test liquor even more than 5 months after their generation. Analysis of a sewage treatment plant effluent showed CAPECs, as a total, were present at concentration of 58 μg/L, accounting for 63% of the total A_9PE metabolites leaving the plant.

Introduction

Alkylphenol ethoxylates (APEs) are one of the most widely used surfactant classes. The annual worldwide production of APE amounts to about 500 ktons (*1*). This surfactant class is applied to a number of domestic, agricultural, and industrial uses. After use, APEs are discharged to sewage treatment plants (STPs) or directly released into the environment. Among APEs, nonylphenol ethoxylates (A_9PEs) are by far the most commonly used, encompassing about 80% of the world market (*2*). General structures of APEs and their well-ascertained initial breakdown products are presented in Figure 1. Commercial A_9PEs are complex mixtures of isomers and oligomers, as the branched alkyl side chain can take many different structural configurations, and the hydrophilic chain may contain from 2 to 20 ethoxy units.

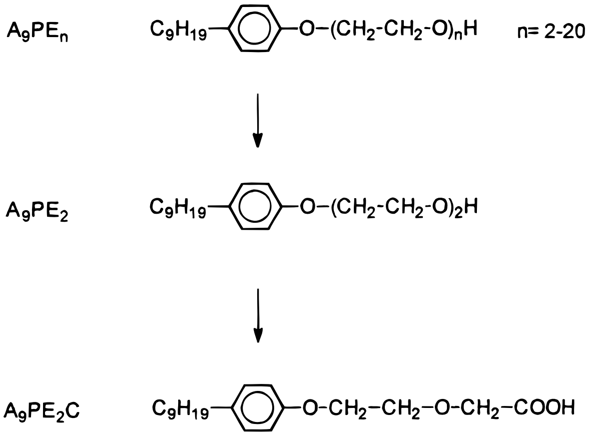

FIGURE 1. Structures and acronyms of nonylphenol polyethoxylate surfactants and their initial most prominent breakdown products.

Initial biodegradation of A_9PEs occurs rather rapidly with loss of surfactant properties. Many studies have shown that A_9PE metabolites are ubiquitous in the environment (*3*−*14*), are more toxic than parent compounds (*7*), and can bioaccumulate in aquatic organisms (*15*, *16*). A recent report has shown that APE intermediates are weakly estrogenic in nature (*17*). In 1996, Jobling et al. (*18*) discovered that a threshold concentration as low as 10 μg/L of APE metabolites can affect fish reproduction. Such concentration levels may be present in surface waters (*5*, *10*). These recent findings on toxicity of A_9PE metabolites have intensified concern over their environmental and human health effects.

Biotransformation of A_9PEs has been the object of many studies, which have been recently reviewed (*19*). It is now generally accepted that the first act of the APE biodegradation process occurs via ether cleavage. This process leads to formation of short-chain APEs, mainly A_9PE_2. Complete deethoxylation with formation of nonylphenol has been observed only under anaerobic conditions (*20*). Under aerobic conditions, short-chain A_9PE biotransformation proceeds via carboxylation of the terminal alcoholic group, with production of persistent A_9PE_nCs. Here, n indicates the number of ethoxy units plus a terminal CH_2COOH unit. Occasionally, the oxidation process of the ethoxy chain can occur simultaneously and compete with that leading to ethoxy chain shortening. Over one year of monitoring, large amounts of A_9PECs with long ethoxy chains were constantly detected in secondary effluents of one of the six major STPs in the area of Rome (*21*).

Further biotransformation of the initial APE metabolites has received very little attention. This oversight may be traced to the general belief that bacterial attack to the branched nonyl side chain is greatly inhibited by steric hindrance. On the basis of analytical data obtained by thin-layer and open air column chromatography with both UV and IR detection, oxidation of the branched alkyl chain was hypothesized by

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Patterson et al. (*22*). Later, Schöberl (*23*) postulated carboxylation of the branched alkyl chain by elemental analysis. In both cases, however, the hypothesis could never be substantiated by sound structural identifications of the produced intermediates. On analyzing tertiary STP effluents by gas chromatography−mass spectrometry (MS), Ding et al. (*24*) detected 10 dicarboxylated A_9PE biotransformation products with the alkyl chain carboxylated (CAPECs). Estimated concentrations of individual CAPECs ranged between 0.9 and 11 μg/L. Because of the complex isomeric structures of the original nonyl side chain, complete structural assignment of the carboxylated alkyl group could not be made.

Very recently (*25*), we have shown that liquid chromatography (LC)−MS with an electrospray (ES) source and a single quadrupole is a powerful technique for unambiguous identification of intermediates originated from biotransformation of alkyl branched alcohol ethoxylate (AE) surfactants. This allowed us to explicate many aspects of the biodegradation pathway of a 2-butyl branched $A_{12}E$ mixture.

The purpose of this work has been that of shedding light upon the fate of the branched alkyl chain of A_9PEs, under laboratory conditions and by using the same instrumentation reported above. Sound evidence for the formation of significant amounts of intermediates having the alkyl chain oxidized has been obtained. To ascertain whether compounds identified in the biodegradation test solution can also be generated in mechanical biological STPs, a short survey was conducted.

Experimental Section

Reagents and Chemicals. The A_9PE surfactant mixture, commercially referred to as Marlophen 810, was supplied by Chemische Werke Hüls AG, Marl, Germany. Marlophen 810 contains A_9PE chain isomers and oligomers with an average of 9 and a range of 1−17 ethoxy units. Imbentin-N/7A, a mixture of mono- and diethoxylated nonylphenol, was received from W. Kolb, AG, Hedingen, Switzerland. A linear alcohol ethoxylate made up of 10 alkyl groups and 6 ethoxy units ($A_{10}E_6$) was purchased from Fluka Bucks, Switzerland. This compound was used as internal standard. Stock solutions of the individual standard and standard mixtures were prepared by dissolving known amounts of them in methanol. Working standard solutions were obtained by further diluting stock solutions with methanol. Trifluoroacetic acid (TFA) was from Aldrich Chemical Co., Milwaukee, WI. For LC analysis, distilled water was further purified by passing it through a Milli-Q Plus apparatus (Millipore, Bedford, MA). Acetonitrile “Plus” of gradient grade was obtained from Carlo Erba, Milan, Italy. To minimize formation of cationized molecules, it was distilled in glass to remove inorganic salt impurities. Other solvents were of analytical grade (Carlo Erba), and they were used as supplied.

Biodegradation Assay. A_9PE biodegradation experiments were conducted under the same previously reported conditions (*25*), that is according to an OECD screening test (*26*). A 5-L test solution containing the necessary organic nutrients and inorganic salts was prepared. Half a milliliter of a filtered fresh effluent of a mechanical-biological STP was added per liter of test solution as the source of microorganisms. The concentration of A_9PEs in the test solution was 5.5 μmol/L. The bioassay was conducted under continuous stirring in a constant-temperature room (21 ± 2 °C) and using a 12-h dark−light cycle. One hundred-milliliter samples were withdrawn at intervals and analyzed in duplicate by the procedure reported below. When not immediately analyzed, samples were stored at 4 °C, after addition of $HgCl_2$ (20 mg/L) to inhibit bacterial activity.

STP Effluent Sample. The 24-h composite sample of a treated sewage was obtained by using flow proportional samplers. This sample was from one mechanical-biological STP (Cobis) that receives mainly domestic wastes and is located in the area of Rome. The sample was collected in a glass bottle, preserved by addition of $HgCl_2$ (20 mg/L), and stored at 4 °C until analysis.

Sample Preparation. Analytes were extracted from the bioassay solution and from the STP effluent by solid-phase extraction (SPE) with a recently introduced form of graphitized carbon black, that is, Carbograph 4 (surface area, 210 m^2/g; 120−400 mesh size, Lara, Rome, Italy). The preparation of the 0.5-g Carbograph 4 SPE cartridge, the extraction apparatus used, and the subsequent analyte extraction procedure have been described elsewhere (*27*).

After passage of 50 mL of the test liquor or 200 mL of the STP effluent sample through the SPE cartridge, this was washed with 50 mL of distilled water. Next, a stepwise desorption procedure was performed to isolate neutral analytes from acidic ones, by suitably modifying a previously reported procedure (*28*). Neutral analytes were eluted by passing 1.5 mL of methanol through the cartridge followed by 12 mL of CH_2Cl_2/CH_3OH (80:20, v/v), at a flow rate of about 4 mL/min. The eluate was collected in a 1.4 cm i.d. glass vial with a conical bottom. The last drops of this solvent mixture were collected by further decreasing the pressure inside the vacuum flask. Acidic metabolites were removed from the sorbent bed and collected in a second vial by elution with 10 mL of CH_2Cl_2/CH_3OH (80:20, v/v) acidified with formic acid, 50 mmol/L.

Both neutral and acidic extracts were taken to dryness in a water bath at 40 °C, under a gentle stream of nitrogen. The neutral residue was reconstituted with 200 μL of a water/methanol solution (40:60, v/v) containing 0.5 μg/mL of the $A_{10}E_6$ internal standard. One-fourth of the final extract was then injected into the LC column.

A_9PE acidic metabolites contained in the other residue were esterified by adding 1 mL of methanol acidified with HCl, 60 mmol/L, and heating this solution at 100 °C for 20 min, after closing the vial with a Teflon lined screw cap. After removing methanol by evaporation, the same procedure as that reported for the neutral residue was followed. Occasionally, the esterification step was omitted and acidic metabolites were analyzed as intact species.

LC−ES−MS Analysis. The LC−ES−MS apparatus and several experimental conditions were the same as reported elsewhere (*28*). The analytes were chromatographed on an “Alltima” 25 cm × 4.6 mm i.d. column filled with 5-μm C-18 reversed-phase packing (Alltech, Sedriano, Italy). For fractionating both neutral and acidic analytes, the phase A was glass distilled acetonitrile and the phase B was water. Both solvents were acidified with TFA, 0.1 mmol/L. The mobile phase composition was 25% A, which was linearly increased to 100% in 50 min. The flow rate of the mobile phase was 1 mL/min, and 50 μL/min of the column effluent was diverted to the ES source, while the rest of the effluent was delivered to a Model 2550 UV detector (Varian) set at 220 nm wavelength. Full-scan LC−MS chromatograms were obtained by scanning the quadrupole from 45 to 600 m/z with a 3-s scan. Unless otherwise specified, the skimmer cone voltage was set at 40 V.

Quantitation. Because of the lack of any standard of acidic A_9PE intermediates, their quantification in the test solution and in STP effluents was rather laborious and could suffer from some inaccuracy. Basically, a procedure analogous to that reported elsewhere (*25*) was followed. A solution (which will be called solution S) containing undetermined quantities of the compounds of interest was obtained by submitting to degradation the A_9PE mixture, following the same procedure described above. The degradation process was stopped after 30 days by $HgCl_2$ addition. Analysis of this solution had demonstrated that the intermediates of interest were extensively formed. Standardization of the analytes in

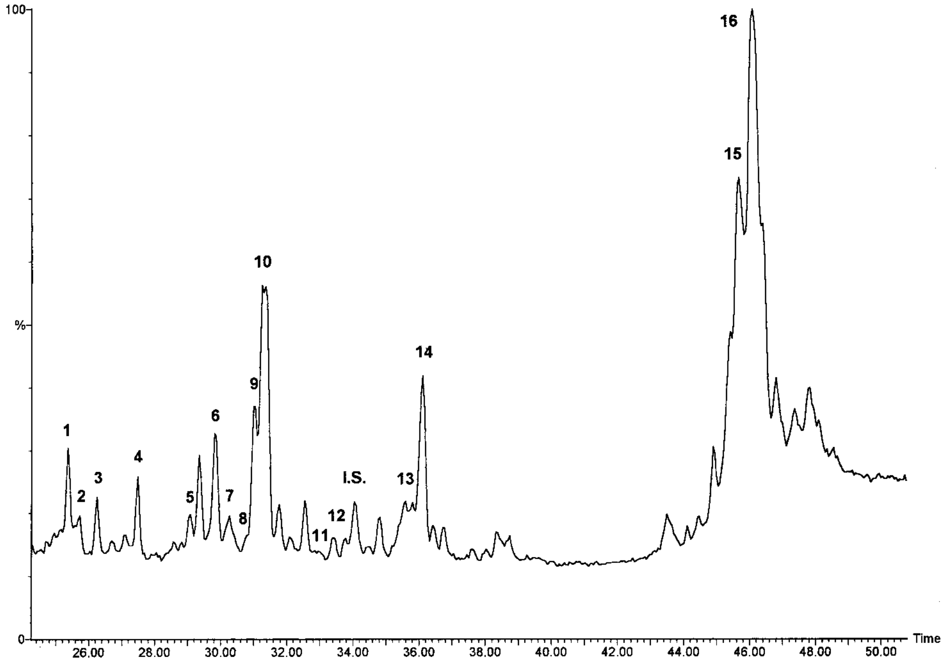

FIGURE 2. Total ion current (TIC) chromatogram obtained by analyzing the acidic extract of a sample of the nonylphenol polyethoxylate (A_9PE) biodegradation test solution taken at day 17. Peak assignment for the most prominent isomer of each homologue: 1, CA_6PE_2; 2, CA_6PE_1; 3, CA_3PE_2C; 4, CA_4PE_2C; 5, CA_5PE_1C; 6, CA_5PE_2C; 7, CA_8PE_2; 8, CA_8PE_1; 9, CA_6PE_1C; 10, CA_6PE_2C; 11, CA_7PE_1C; 12, CA_7PE_2C; 13, CA_8PE_1C; 14, CA_8PE_2C; 15, A_9PE_3C; 16, A_9PE_2C. IS, internal standard. The term "C" just preceding or following the above acronyms indicates carboxylation, respectively, of the alkyl and ethoxylate chains of the A_9PE surfactant mixture.

the solution S relied on the assumption that, when setting the UV detector at 220 nm wavelength, the molar response of the $A_9PE_1 + A_9PE_2$ mixture (Imbentin-N/7A) did not differ significantly from those of species bearing a carboxylic group on the alkyl chain and/or the ethoxylate chain. After determining the composition of the solution S, calibration curves for the analytes of interest were constructed by analyzing with the ES−MS detector five varying volumes of the solution S and by plotting peak areas relative to that of the internal standard versus amounts injected. It is known that the ES−MS detector has a rather restricted range of linear response. When an extract contained analyte amounts sufficient to saturate the detector, the extract was appropriately diluted and reinjected.

Recovery Studies. Recovery of the acidic A_9PE intermediates in the biodegradation test solution was estimated by following a previously reported procedure (*25*). Analyte recovery in STP effluents was assessed by adding known and appropriate volumes of the standardized solution S to 200-mL aliquots of a previously analyzed pooled effluent sample. In both cases, recovery experiments were made in triplicate. For both types of aqueous media, analyte recoveries were better than 90%.

Results and Discussion

General Remarks. By itself, the ES source is capable of producing only adduct ions. This permits assignment of the molecular weight to a certain compound, but structural information is precluded. Yet, structure-significant fragment ions can be obtained by collision between protonated molecules and residual drying gas molecules in the desolvation chamber. So-called in-source collision-induced decomposition (CID) spectra can be obtained by suitably controlling the electrical field between sample and skimmer cones. Providing the analyte is chromatographically separated from any other compound, these in-source CID spectra closely resemble those obtained by the MS−MS technique (*29, 30*). By setting the cone voltage at 40 V, intense signals for diagnostic fragments appeared on spectra, but those for protonated molecules were often scarcely distinguishable. When necessary, therefore, a preliminary chromatographic run was carried out, after setting the cone voltage at 20 V. Under this condition, the CID process was greatly inhibited and spectra displayed intense signals for MH^+ ions.

Acidic intermediates from biotransformation of an alkyl branched AE surfactant mixture were recently detected as intact species by setting the ES−MS system in the positive ion mode (*25*). Although this condition was unfavorable in terms of absolute sensitivity, the structure of the acidic intermediates was fully characterized, as abundant amounts of them were present in the test solution. In a previous work (*28*), acidic ethoxylate species, such as mono- and dicarboxylated poly(oxo)ethylene glycols, were much more efficiently detected by the ES−MS system after their conversion to methyl esters. In the present work, the latter procedure was generally adopted with the purpose of devising a sufficiently sensitive method capable of determining even the relatively low amounts of acidic breakdown products of APEs in an STP effluent.

Intermediate Characterization. Figure 2 shows a selected LC−MS−CID chromatogram obtained by analyzing an acidic sample extract of the biodegradation test solution. This sample was taken at day 17 from the beginning of the experiment. Biotransformation of the initial A_9PE breakdown products, especially A_9PE_2C, gave rise to new metabolite classes. Corresponding low-cone voltage spectra showed that the homologous components of the most prominent class formed a series of protonated molecules at m/z ranging from 381 to 311, regularly spaced by 14 amu. These ion signals were tentatively assigned to intermediates (CA_xPE_2-Cs, $x = 3-8$) generated by various oxidative degradation processes of the alkyl side chain of A_9PE_2Cs. In turn, each homologue produced a series of chromatographic peaks for its various isomers. Due to the large number of isomers (*4*) and the limited efficiency of a LC column, each peak should be regarded not as the product of the elution of a single isomer but rather as the product of the coelution of isomers with similar structural configurations. Definitive confirma-

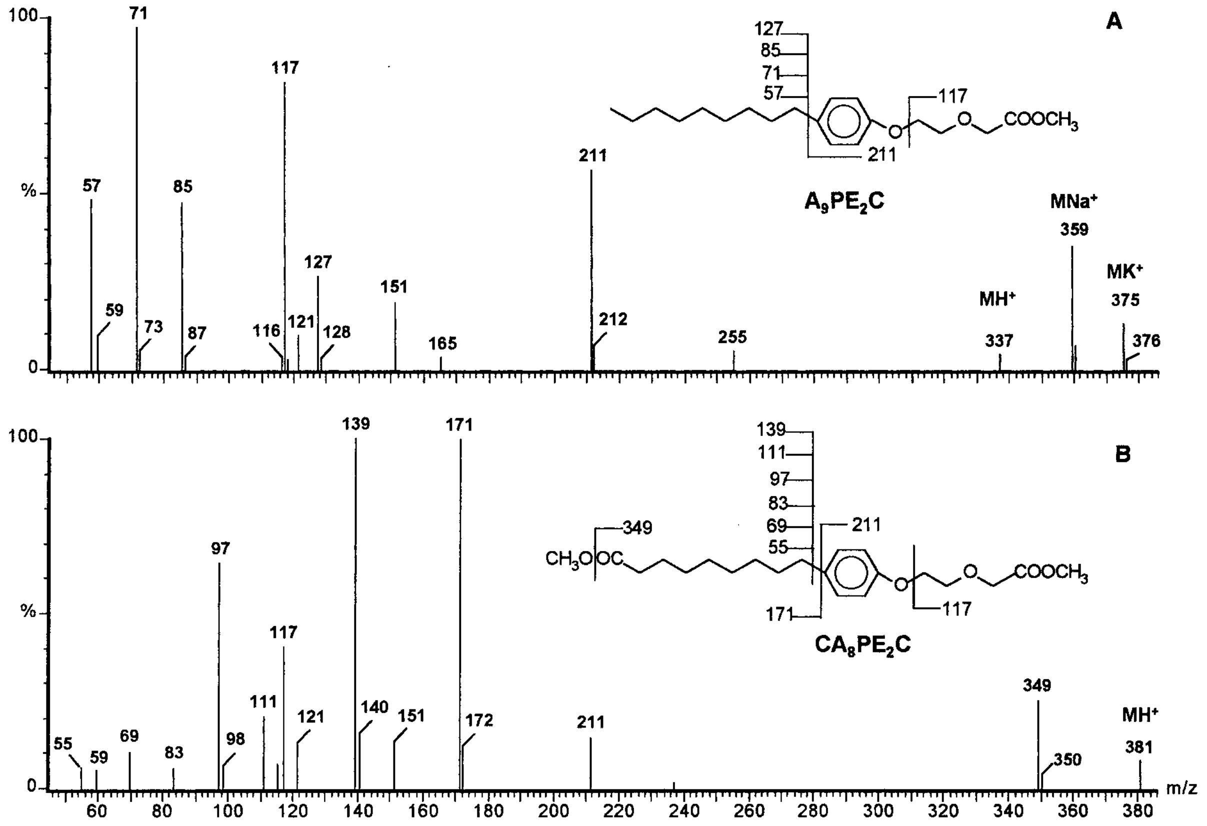

FIGURE 3. CID spectra taken from the apexes of chromatographic peaks (see Figure 2) at (A) 46 min relative to A_9PE_2C and (B) 36 min relative to postulated CA_8PE_2C. The position of the carboxylic group in the alkyl chain was arbitrarily assigned. Moreover, locations of branching points of the alkyl chain have been left undetermined.

tions of the $CAPE_2C$ structures were achieved by the CID process, after raising the cone voltage at 40 V. It has to be pointed out that CID spectra were invariably taken from the apexes of peaks for the most abundant isomer groups.

In Figure 3, the CID spectrum taken from the peak at a retention time of 36.1 min is shown together with that of A_9PE_2C. By comparing these two spectra, it appears they share basically four signals at *m/z* 117, 121, 211, and 151. The first three signals can be assigned respectively to $[CH_2{=}CH{-}O{-}CH_2{-}COOCH_3{+}H]^+$, $[C_6H_5{-}O{-}CH{=}CH_2{+}H]^+$ or $[CH_2{=}CH{-}C_6H_4{-}OH{+}H]^+$, and $[C_6H_5{-}O{-}CH_2{-}CH_2{-}O{-}CH_2{-}COOCH_3{+}H]^+$ fragment ions, while the latter one is presumably formed by removal of methylformiate (−60 amu) from the ion at *m/z* 211. This first examination indicated that the new metabolite was originated from A_9PE_2C biodegradation. Expectedly, the CID spectrum from A_9PE_2C contains characteristic ions at *m/z* 57, 71, 85, and 127, which correspond to the $C_nH_{2n+1}{}^+$ alkyl ion series. The gap of 42 amu between signals at *m/z* 85 and 127 seems to indicate a double methyl or a single ethyl branch point located at the α carbon atom of the alkyl side chain. By observing again the spectrum of the breakdown product of A_9PE_2C, an intense signal at *m/z* 171 is present. This signal could be produced by benzylic bond cleavage, and it is consistent with formation of the $[C_8H_{15}COOCH_3{+}H]^+$ fragment ion. Moreover, it can be noted that the alkyl ion series has been now replaced by ions at 55, 69, 83, 97, 111, and 139, which belong to the acylium ion series $C_nH_{2n-1}{-}C{\equiv}O^+$. These ions could be formed from decomposition of a charged $C_8H_{15}COOCH_3$ side chain, after loss of methanol. This loss also produces the $[MH\text{-}32]^+$ ion at *m/z* 349. Methanol loss followed by formation of acylium ions is a typical fragmentation pattern of fatty acid methyl esters. All these clues led to the conclusion that a group of CA_8PE_2C isomers was produced by the ω-oxidation mechanism of the alkyl side chain of A_9PE_2Cs.

Additional evidence for the formation of A_9PE intermediates bearing a carboxylic group on both opposite side chains was obtained by analyzing them as intact species. CID spectra were then taken and compared with those of the corresponding methylated forms. As an example, in Figure 4 two CID spectra are presented that are supposed to be generated from the same compound, that is, CA_6PE_2C, before and after its methylation. For conciseness, they will be called spectrum A and spectrum M, respectively. Significant correspondences can be recognized by matching these two spectra. First, MH^+ ions differ by 28 amu, as expected in the case of methylation of two carboxylic groups. Second, spectrum A contains the ion at *m/z* 307 ($[MH\text{-}18]^+$) which can be produced by loss of water from the fatty acid side chain of the protonated molecule. Vice versa, the same decomposition mechanism results in CH_3OH removal from the methylated fatty acid chain, generating the signal at *m/z* 321 ($[MH\text{-}32]^+$) on the spectrum M. Third, spectrum A displays signals at *m/z* 103 and 223. These two signals can be assigned to two ions generated by the ether bond cleavage closest to the aromatic ring. Simultaneous appearance of two ions in the spectrum as the result of a single bond rupture is not a rare event in the CID process and arises from alternative arrangement of the proton on the two molecule fragments. Correspondingly, spectrum M shows two signals at *m/z* 117 and 237, each one having 14 amu more than the above two signals. This indicates that both chains attached to the aromatic ring bear a carboxylic group. Finally, benzylic bond cleavage generates either the $[C_6H_{11}{-}COOH{+}H]^+$ ion (*m/z* 129) from the protonated intact molecule (spectrum A) or the $[C_6H_{11}{-}COOCH_3{+}H]^+$ ion (*m/z* 143) from the corresponding protonated methylated form (spectrum M). All these observations led to the conclusion that the ω/β-oxidation mechanism of the alkyl side chains of particular A_9PE_2C isomers led to formation of the species CA_6PE_2Cs.

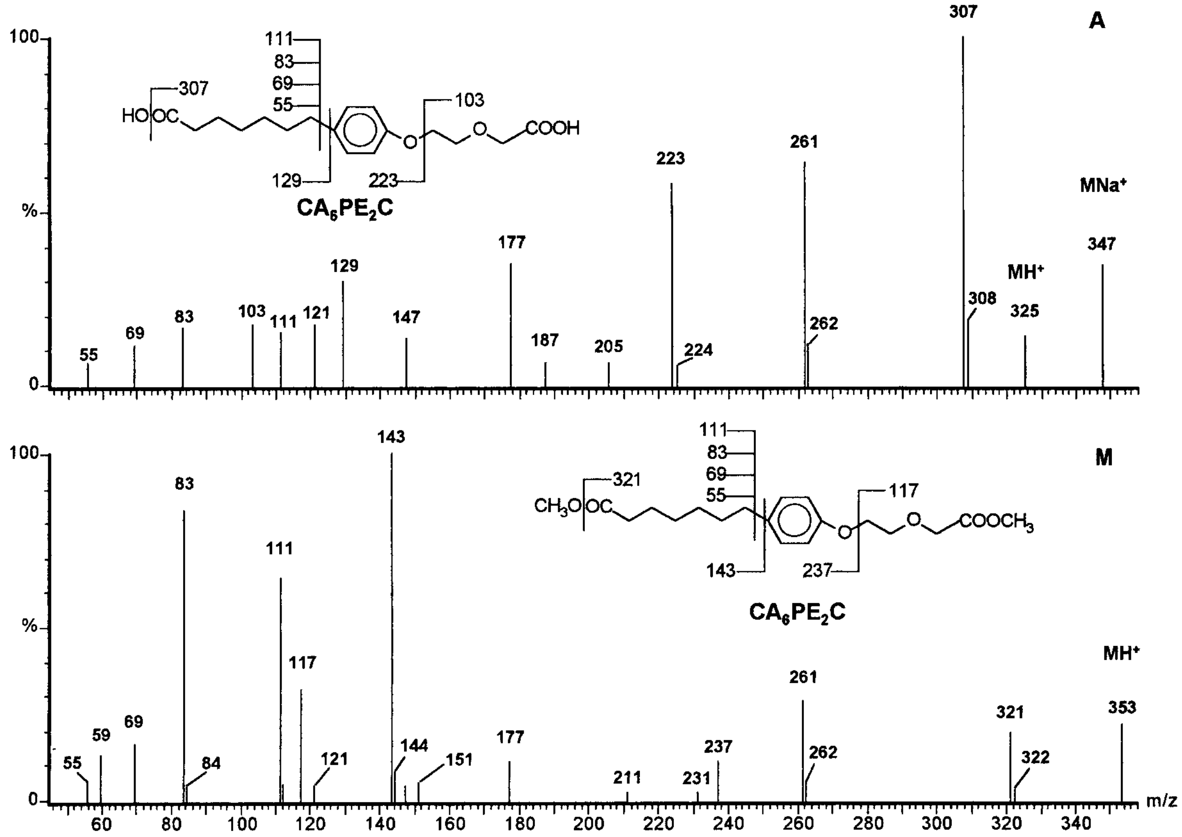

FIGURE 4. CID spectra of (A) intact postulated CA_6PE_2C and (M) its methylated form. The latter spectrum was taken from the apex of the chromatographic peak at 31.2 min (see Figure 2). The position of the carboxylic group in the alkyl chain was arbitrarily assigned. Moreover, locations of branching points of the alkyl chain have been left undetermined.

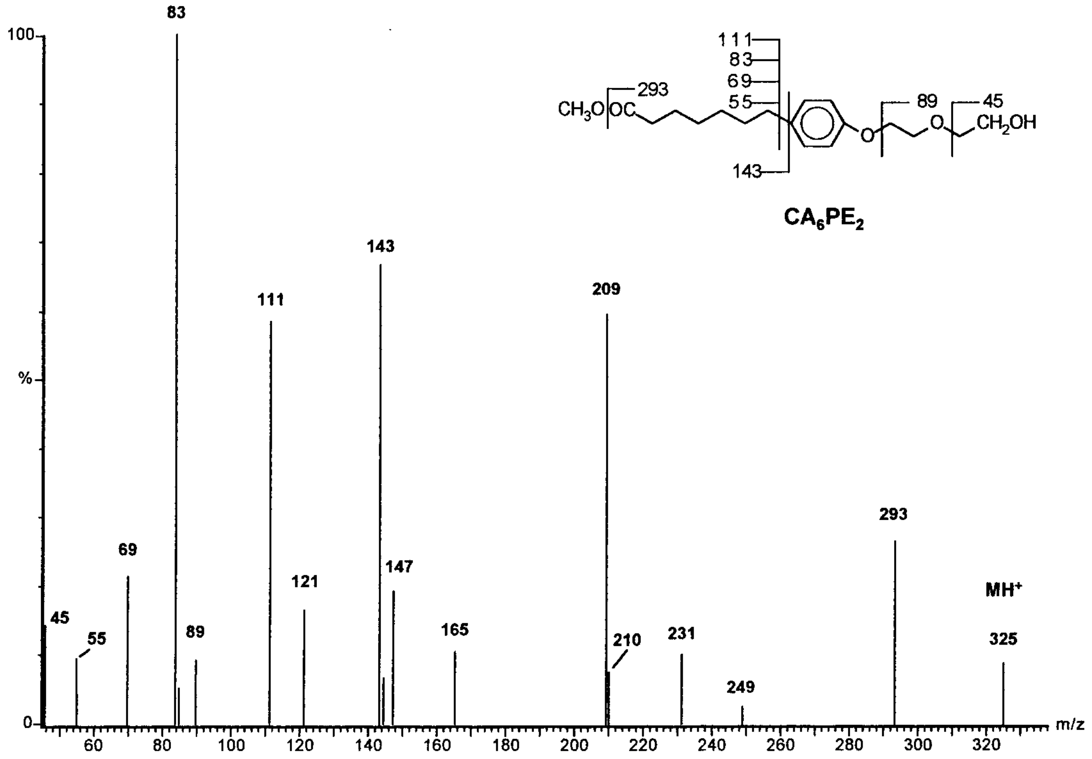

FIGURE 5. CID spectrum taken from the apex of the chromatographic peak at 25.3 min (see Figure 2) and relative to postulated CA_6PE_2. The position of the carboxylic group in the alkyl chain was arbitrarily assigned. Moreover, locations of branching points of the alkyl chain have been left undetermined.

Identities of the other CAPE$_2$Cs were easily obtained by identifying on CID spectra the ion at *m/z* 117 and those signals for fragment ions characterizing the structure of the fatty acid side chain. These ions were those coming from benzylic bond cleavage, that is, $[C_nH_{2n-1}-COOCH_3+H]^+$, and those belonging to the acylium ion series. All these signals were absent in the spectrum of the supposed CA$_3$PE$_2$C species. Therefore, the postulated structure of this metabolite received a minor validation from the CID process compared to those of the other CAPE$_2$Cs.

Besides CAPE$_2$Cs, CAPE$_1$Cs were also formed. Under many aspects, CID spectra of these two groups of intermediates were similar. Structural identification of CA$_x$PE$_1$Cs was readily recognized by the lack on their spectra of the signal at *m/z* 117 (see above) and by the presence of a characterizing signal at *m/z* 193, which was assigned to the $[CH_2{=}CH-C_6H_4-O-CH_2-COOCH_3+H]^+$ fragment ion.

Another class of intermediates, here referred to as CAPEs, was formed as the result of oxidative attack of the alkyl side chain of A$_9$PEs with one and two ethoxy units. The most abundant components of this intermediate family were the CA$_6$PE$_2$ isomers. The CID spectrum of this compound is shown in Figure 5. Evidences for the presence of this compound in the test solution were obtained mainly by comparative examination of this spectrum with that of CA$_6$PE$_2$C (see Figure 4). Both spectra have in common characteristic signals for the ion at *m/z* 143 produced by benzylic bond cleavage as well as for the acylium ion series coming from the fragmentation pattern of the above ion, after methanol loss. Therefore, it was concluded that even the compound under consideration contained a carboxylated alkyl side chain. But, differently from that of CA$_6$PE$_2$C, the spectrum of the supposed CA$_6$PE$_2$ does not contain the signal at *m/z* 117, which indicates ethoxy chain carboxylation (see above). Moreover, the present spectrum contains ions at *m/z* 45 and 89. As shown elsewhere (*25*), these two ions diagnose molecular structures bearing intact ethoxy chains made up of at least two ethoxy units. Final evidence for the formation of the above-mentioned compound was obtained by following one of the fragmentation pathways of its protonated molecule. The ions at *m/z* 209, 165, 147, and 121 could belong to a pattern involving first the loss of C$_4$H$_9$-COOCH$_3$ from the methylated fatty acid side chain to give the $[CH_2{=}CH-C_6H_4-O-(CH_2-CH_2-O)_2H+H]^+$ ion (*m/z* 209), followed then by sequential neutral losses of ethylene oxide, water, and acetylene to give respectively $[CH_2{=}CH-C_6H_4-O-CH_2-CH_2-OH+H]^+$ (*m/z* 165), $[CH_2{=}CH-C_6H_4-O-CH{=}CH_2+H]^+$ (*m/z* 147), and $[CH_2{=}CH-C_6H_4-OH+H]^+$ (*m/z* 121) ions.

In Table 1 signals are reported for structure-significant ions of the most abundantly formed CAPEC and CAPE metabolites. Apart from the anomalous spectrum of CA$_3$PE$_2$C, all other ones contained signals for the acylium ion series with a 28 amu gap close to the signal for the largest acylium ion. This seems to suggest that, among A$_9$PE and A$_9$PEC isomers, only those having a methyl branch point at the α carbon atom of the alkyl side chain were biotransformed respectively to CAPE and CAPEC isomers.

Biodegradation Pathway of A$_9$PEs. A concentration versus time plot of the biotransformation products of A$_9$PEs is shown in Figure 6. For clarity of presentation, changes of the relatively small concentrations of A$_9$PE$_3$C present in the test liquor are not represented. Also, concentrations at any time of both CAPECs and CAPEs are here shown as total concentrations of the two distinct families. Concentrations at selected times of the individual most abundant CAPEC and CAPE homologues and oligomers relative to those of CA$_6$PE$_2$C are reported in Table 2.

As a general consideration, an apparent lack of mass balance was observed in the biodegradation experiments of

TABLE 1. Relative Intensities of Selected Structure Significant Ions in CID Spectra[a] Taken from Chromatographic Peaks[b] for Methyl Esters of Acidic A$_9$PE Intermediates Having the Alkyl Side Chain Carboxylated[c]

RT[e]	MH$^+$	Ions[d], *m/z*																									COMPOUND
		MH$^+$-32	55	69	83	*115*	97	*129*	111	*143*	125	*157*	139	*171*	117	121	151	191[f]	193	211	251[f]	45	89	147	165	209	
36.1	381	25	5	10	5	–	65	–	20	–	–	–	100	95	40	15	15	–	–	15	–	–	–	–	–	–	CA$_8$PE$_2$C
35.1	337	5	10	20	5	–	100	–	20	–	–	–	30	20	–	–	–	–	15	–	–	–	–	–	–	–	CA$_8$PE$_1$C
34.0	367	20	10	5	90	–	50	–	–	–	70	100	–	–	30	10	10	–	–	–	–	–	–	–	–	–	CA$_7$PE$_2$C
31.2	353	20	5	20	85	–	–	–	65	100	–	–	–	–	30	5	5	–	–	5	–	–	–	–	–	–	CA$_6$PE$_2$C
31.0	309	15	10	30	100	–	–	–	45	40	–	–	–	–	–	–	–	–	85	–	–	–	–	–	–	–	CA$_6$PE$_1$C
29.8	339	10	<5	40	–	–	50	30	–	–	–	–	–	–	100	25	40	–	–	15	–	–	–	–	–	–	CA$_5$PE$_2$C
28.4	295	15	5	100	–	–	60	40	–	–	–	–	–	–	–	–	–	–	30	–	–	–	–	–	–	–	CA$_5$PE$_1$C
27.5	325	10	40	–	30	30	–	–	–	–	–	–	–	–	100	20	30	20	–	10	20	–	–	–	–	–	CA$_4$PE$_2$C
26.2	311	–	–	–	–	–	–	–	–	–	–	–	–	–	100	–	–	15	–	–	40	–	–	–	–	–	CA$_3$PE$_2$C
29.7	353	10	10	30	10	–	100	–	25	–	–	–	90	50	–	30	–	–	–	–	–	10	15	10	–	–	CA$_8$PE$_2$
25.4	325	25	10	20	100	–	–	–	60	70	–	–	–	–	–	15	–	–	–	–	–	15	10	20	10	60	CA$_6$PE$_2$

[a] On this Table are reported only ion signals for those species which were isolated from the other ones by the LC column. [b] The spectrum of each homologue was taken from the most intense related isomer chromatographic peak. [c] Skimmer cone voltage = 40 V. [d] Fragment ions generated by rupture of only the benzylic bond are reported in italic. [e] RT = retention time (see Figure 2). [f] Signals at *m/z* 251 and 191 can correspond respectively to $[CH_2{=}CH-CH_2-CH_2-C_6H_4-O-CH_2-CH_2-O-CH_2-COOCH_3+H]^+$ and $[CH_2{=}CH-CH_2-CH_2-C_6H_4-O-CH{=}CH-O-CH_3+H]^+$ fragment ions.

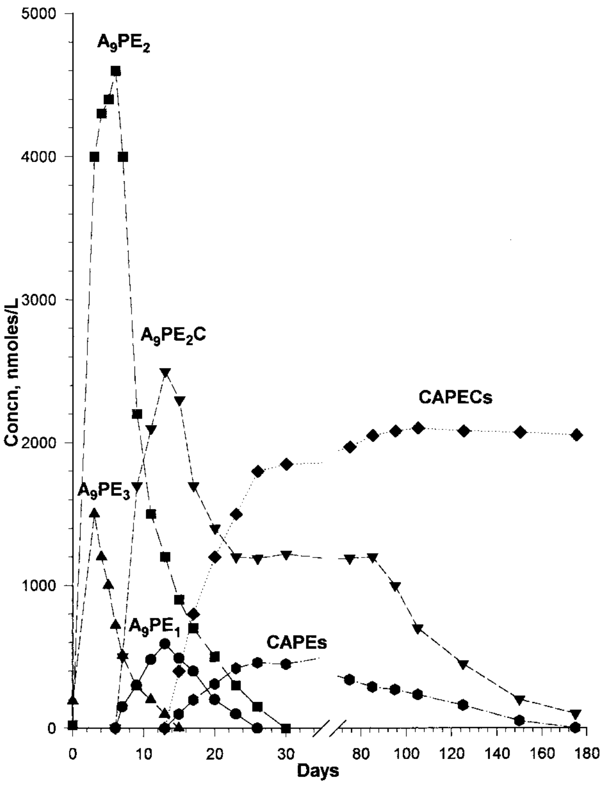

FIGURE 6. Concentration versus time plot of A_9PE metabolites. The acronyms CAPECs and CAPEs indicate two series of metabolite families having, respectively, both side chains carboxylated and the only alkyl side chain carboxylated.

A_9PEs. This effect took place not many days after the initial manifestation of the bacterial activity and became more and more evident in the course of time. The reason for this effect was unclear to us. It could be speculated that a different biodegradation pathway led to formation of intermediates which were not detected by both ES−MS and UV detectors.

According to previous findings (*19*), a rather rapid nonoxidative depolymerization of the ethoxy chain was the first act of the microbial attack to the substrate. This mechanism generated A_9PEs with 1−3 ethoxy units, with A_9PE_2 isomers being by far the most prominent species. Subsequent oxidation of the terminal alcoholic group leading mainly to production of A_9PE_2C was still consistent with previous observations (*19*). Although A_9PE_1 was present in the test liquor, no significant amount of A_9PE_1C was detected. After an acclimation time of about 2 weeks, bacterial attack to the branched alkyl side chain of both A_9PECs and A_9PEs produced respectively CAPECs and CAPEs.

Two noticeable aspects were associated to this event. One was that all CAPE and CAPEC metabolites were produced simultaneously, as though the different biodegradation mechanisms of a branched alkyl chain (*31*) were all at once activated. Maybe, during the two-week acclimation time, various enzymes capable of degrading the less extensively branched alkyl side chains of A_9PEs and A_9PECs were synthesized by the cells. Another unclear aspect was that, although A_9PE_1C was absent in the test liquor, significant amounts of $CAPE_1Cs$ were cogenerated with $CAPE_2Cs$. Then, the former species increased with time presumably by biotransformation of the latter species (see Table 2).

After the event leading to oxidation of the alkyl side chain, the concentration levels of unreacted high-branched A_9PE_2C isomers remained virtually unaltered for more than 2 months. Thereafter, even these recalcitrant species showed a marked

TABLE 2. Relative Concentrations of Individual CAPECs and CAPEs in the Biodegradation Test Solution at Selected Times

	Relative Abundance[a]															
Day	CA_3PE_1C	CA_3PE_2C	CA_4PE_1C	CA_4PE_2C	CA_5PE_1C	CA_5PE_2C	CA_6PE_1C	CA_6PE_2C	CA_7PE_1C	CA_7PE_2C	CA_8PE_1C	CA_8PE_2C	CA_6PE_1	CA_6PE_2	CA_8PE_1	CA_8PE_2
26	nd[b]	5	2	6	6	25	25	100	2	8	16	90	13	30	20	20
51	0.2	4	3	6	9	24	36	100	3	10	24	94	6	26	30	29
75	2	6	5	14	13	34	44	100	5	13	46	96	4	17	18	26
105	3	7	7	15	14	30	45	100	7	17	50	92	3	7	14	23
125	5	6	8	15	16	24	47	100	9	16	53	87	2	3	8	8
175	7	5	11	13	19	22	51	100	13	13	57	86	nd	nd	nd	nd

[a] CA_6PE_2C concn = 100. [b] nd = not detected.

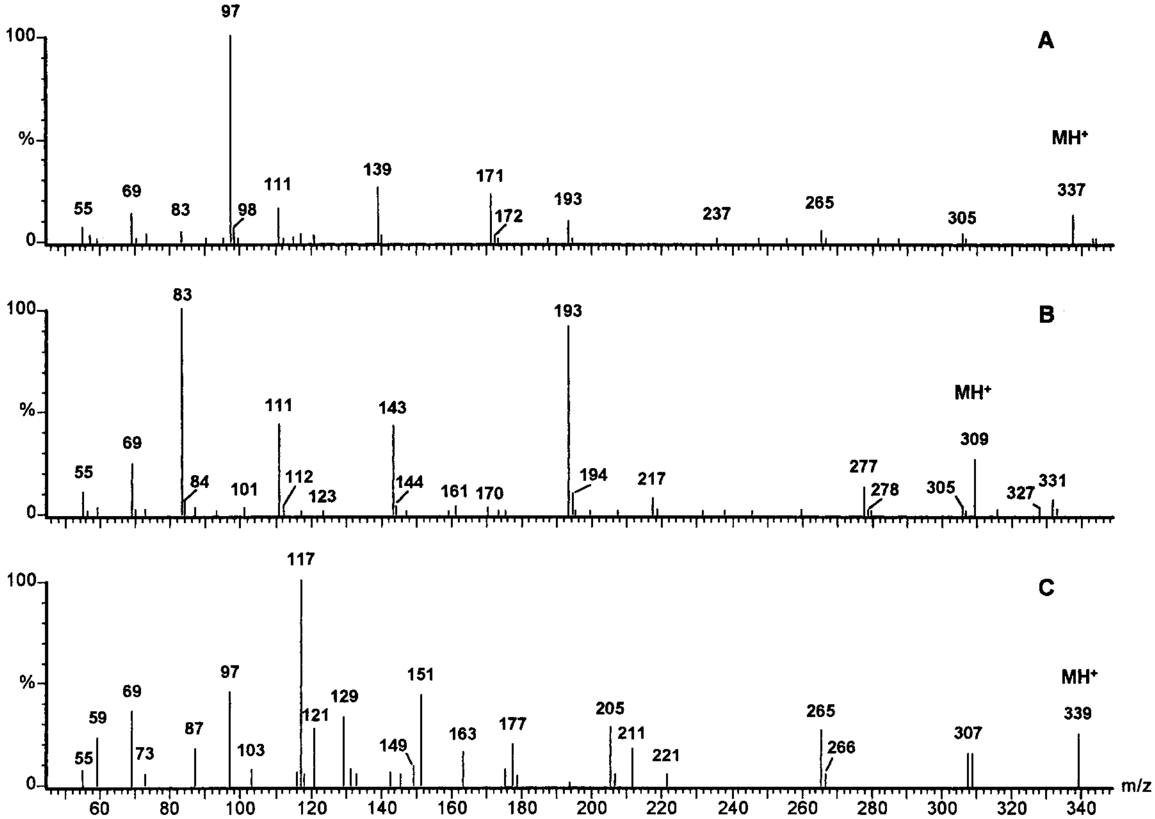

FIGURE 7. CID spectra taken from chromatographic peaks obtained on analyzing the acidic extract of a STP effluent (Cobis, Rome) by the LC−ES−MS apparatus and relative to postulated (A) CA_8PE_1C, (B) CA_6PE_1C, and (C) CA_5PE_2C, respectively.

tendency to disappear from the test solution. After about 170 days from their occurrence, only about 6% of the initial amount still persisted in the biodegradation test solution. By observing the concentration versus time profiles, it appears this group of A_9PE_2C isomers was not converted to CAPECs. We did not investigate the way by which A_9PE_2Cs disappeared from the biodegradation test solution.

After their formation, CAPE concentrations slowly decreased in the course of time. No trace of these intermediates was detected in the test liquor at day 175. Presumably, they were partly converted to CAPECs. This could explain the slight concentration increase of the latter forms occurring at day 75.

Vice versa, CAPECs appeared to be an extremely persistent species. Even after more than 5 months following their generation, no tendency to further biotransformation of these species was observed by us. This was not due to microorganism disappearance in the test liquor, as the biotic mechanism of the conversion of $CAPE_2Cs$ to $CAPE_1Cs$ was still active. The absence of any further interesting event induced us to stop the biodegradation experiment after about six months from its beginning.

Survey of a STP Effluent. The existence and abundance of CAPEC and CAPE species in STP effluents were investigated. An effluent of a STP ("Cobis", Rome) was analyzed according to conditions reported in the Experimental section. From the observation of the resulting mass chromatogram, all the acidic A_9PE biointermediates seemed to be formed in the plant. Definitive identification of the analytes of interest was made by CID spectra. As an example, three of these spectra are visualized in Figure 7. As can be seen, these spectra contain ion signals with relative intensities which fit very well data reported in our personal mass library (see Table 1) for CA_8PE_1C, CA_6PE_1C, and CA_5PE_2C, respectively.

An estimation of the concentration levels of the analytes of interest leaving the STP was made. Results are reported in Table 3. As can be read, CAPECs totaled more than 63% of all A_9PE breakdown products, while only negligible amounts of CAPEs left the plant. Interestingly, CAPEC concentration levels measured by us were similar to those found by Ding et al. (*24*) in a Californian STP effluent, with the difference that this latter effluent contained amounts of $CAPE_1Cs$ much larger than those of $CAPE_2Cs$.

TABLE 3. Concentration Levels of A_9PE Breakdown Products in a STP ("Cobis", Rome, November 1997) Effluent

compound	concentration, µg/L	relative class concentration, %
A_9PE_2[a]	12	13
A_9PE_2C[a]	21	23
CA_8PE_1C	12	
CA_8PE_2C	13	
CA_7PE_1C	2.3	
CA_7PE_2C	1.9	
CA_6PE_1C	7.7	
CA_6PE_2C	10.8	
CA_5PE_1C	2.4	
CA_5PE_2C	2.9	
CA_4PE_1C	1.6	
CA_4PE_2C	1.9	
CA_3PE_1C	0.7	
CA_3PE_2C	0.8	
total CAPECs	58	63
CA_8PE_1	0.18	
CA_8PE_2	0.26	
CA_6PE_1	0.13	
CA_6PE_2	0.11	
total CAPEs	0.68	0.7

[a] Total concentrations of A_9PEs and A_9PECs are reported as concentrations of the most abundant class components.

In conclusion, this study has shown that CAPECs are species extremely recalcitrant to biotransformation. If this property is added to their polar nature and extensive use of parent compounds, one should expect that CAPECs are ubiquitous contaminants of natural waters. With some adjustments, the present analytical method could be adequate to assess the concentration levels of CAPECs in the various aqueous environmental compartments.

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