Surfactants and Their Aerobic Degradation Products: Formation, Analysis, and Occurrence in the Aquatic Environment

Dissertation
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Meinen Eltern

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LUIS RACIONERO (Die Barbaren des Nordens – Die Zerstörung des mediterranen Lebensgefühls)

The present work was conducted from March 1998 to April 2001 at the ESWE-Institute for Water Research and Water Technology (Wiesbaden, Germany), Johannes Gutenberg-University Mainz.

This Ph.D. thesis is based on the following papers, which have been or will be published as regular contributions to scientific journals or proceedings:

EICHHORN P, KNEPPER TP: α,β-*Unsaturated sulfophenylcarboxylate intermediates detected during aerobic degradation of linear alkylbenzenesulfonate (LAS) surfactant: direct evidence for* ω-*oxygenation followed by* β-*oxidations by liquid chromatography – electrospray mass spectrometry* Environ. Toxicol. Chem. (2001), in press **(Section 4.1)**

DONG W, RADAJEWSKI S, EICHHORN P, DENGER K, KNEPPER TP, MURRELL JC, COOK AM: *Linear alkylbenzenesulfonate (LAS) surfactant: bacterial communities initiate catabolism by various* ω-*oxygenations followed by* β-*oxidation, ring cleavage, and desulfonation* Appl. Environ. Microbiol. (submitted) **(Section 4.1.3)**

KNEPPER TP, EICHHORN P: *Metabolismus von Tensiden: Modell und Realität* In: Weigert B, Steinberg C, Brüggemann R (Eds.): Chemische Streßfaktoren in aquatischen Systemen: Schriftenreihe des interdisziplinären Forschungsverbundes Wasserforschung in Berlin, Wasserforschung e. V. Berlin, (2000), 53-60 **(Section 4.1, 4.2 and 4.3)**

EICHHORN P, KNEPPER TP: *Detaillierte Untersuchungen zum Verhalten von Zuckertensiden in Kläranlagen* In: Reemstma T, Kornmüller A (Eds.) Schriftenreihe Anwendungen der LC-MS in der Wasseranalytik, Biologische Abwasserbehandlung, 11 (1999) 157-168, TU Berlin **(Section 4.2 and 4.3)**

EICHHORN P, KNEPPER TP: *Fate studies of the nonionic surfactant alkylglucamide by liquid chromatography/mass spectrometry* J. Mass. Spectrom. 35 (2000), 468-475 **(Section 4.2)**

EICHHORN P, KNEPPER TP: *Investigations on the metabolism of alkyl polyglucosides and their determination in waste water by means of liquid chromatography-electrospray-mass spectrometry* J. Chrom. A 854 (1999), 221-232 **(Section 4.3)**

EICHHORN P, KNEPPER TP: *Electrospray mass spectrometry studies on the amphoteric surfactant cocamidopropyl betaine* J. Mass Spectrom. (2001), in press **(Section 4.4)**

TRIMPIN S, EICHHORN P, RÄDER HJ, MÜLLEN K, KNEPPER TP: *Recalcitrance of polyvinylpyrrolidone (PVP): Evidence through matrix-assisted laser desorption/ionization time-of-flight mass spectrometry* J. Chrom. A (submitted) **(Section 4.5)**

EICHHORN P, PETROVIC M, BARCELÓ D, KNEPPER TP*: Fate of surfactants and their metabolites in waste water treatment plants* Vom Wasser 95 (2000), 245-268 **(Section 5.1)**

EICHHORN P, RODRIGUES SV, BAUMANN W, KNEPPER TP: *Incomplete degradation of linear alkylbenzene sulfonate surfactants in Brazilian surface waters and pursuit of their polar metabolites in drinking waters* Sci. Total. Environ. (2001), in press **(Section 5.2.1 and 5.3.2)**

EICHHORN P, FLAVIER ME, PAJE ML, KNEPPER TP: *Occurrence and fate of linear and branched alkylbenzenesulfonates and their metabolites in surface waters in the Philippines* Sci. Total Environ. 269 (2001), 75-85 **(Section 5.2.2)**

EICHHORN P, KNEPPER TP, VENTURA F, DIAZ A: *The behavior of polar aromatic sulfonates during drinking water production: A case study on sulfophenyl carboxylates in two European waterworks* Water Res. (accepted for publication) **(Section 5.3.1)**

Selected data from the following publications are presented in the introducing chapters:

RIU J, EICHHORN P, GUERRERO JA, KNEPPER T, BARCELÓ D*: Determination of linear alkylbenzenesulfonates in wastewater treatment works and coastal water by automated solid phase extraction followed by capillary electrophoresis-UV and confirmation by capillary electrophoresis-mass spectrometry* J. Chrom. A 889 (2000), 221-229 **(Section 2.2.3)**

CASTILLO M, RIU J, VENTURA F, BOLEDA R, SCHEDING R, SCHRÖDER H F, NISTOR C, ÉMNEUS J, EICHHORN P, KNEPPER TP, JONKERS CCA, DE VOOGT P, GONZÁLEZ-MAZO E, LEÓN VM, BARCELÓ D: *Inter-laboratory comparison of liquid chromatographic techniques and enzyme-linked immunosorbent assay for the determination of surfactants in wastewaters* J. Chrom. A 889 (2000), 195-209 **(Section 3.11.2)**

ABBREVIATIONS AND ACRONYMS

Chemical substances

ABS	branched alkyl benzenesulfonates
AE	alcohol ethoxylates
AES	alkyl ether sulfates
AG	alkyl glucamides
APEO	alkylphenol ethoxylates
APG	alkyl polyglucosides
AS	alkyl sulfates
CAPB	cocamidopropyl betaines
CDEA	coconut diethanolamide
DATS	dialkyltetralin sulfonates
DTDMAC	ditallowdimethylammonium chloride
LAS	linear alkylbenzene sulfonates
NP	nonylphenol
NPEO	nonylphenol ethoxylates
PEG	poly(ethylene glycol)
PFAC	perfluoroalkane carboxylates
PFAS	perfluoroalkane sulfonates
PVP	poly(vinylpyrrolidone)
SDS	sodium dodecyl sulfate
SPC	sulfophenyl carboxylates
SPC-2H	α,β-unsaturated sulfophenyl carboxylates
SPdC	dicarboxylated sulfophenyl carboxylates
HOAc	acetic acid
IAA	3β-indol acrylic acid
TEA	triethylamine
TEAA	tetraethylammonium acetate

General terms

APCI	atmospheric pressure chemical ionization
API	atmospheric pressure ionization
BOD	biological oxygen demand
CE	capillary electrophoresis
CEC	Council of the European Communities
CI	chemical ionization

CID	collision-induced dissociation
COD	chemical oxygen demand
Da	Dalton
DAD	diode array detector
DOC	dissolved organic carbon
EC	effect concentration
ECETOC	European Centre for Ecotoxicology and Toxicology of Chemicals
EI	electron impact (ionization)
ELSD	evaporative light scattering detector
ESI	electrospray ionization
FAB	fast atom bombardment
FBBR	fixed-bed bioreactor
FIA	flow-injection analysis
FID	flame ionization detector
FL	fluorescence (detection)
GAC	granular activated carbon
GC	gas chromatography
GCB	graphitized carbon black
GPC	gel permeation chromatography
HPLC	high performance liquid chromatography
IC	inhibition concentration
IP	ion-pair
IR	infrared (spectroscopy)
LC	liquid chromatography; lethal concentration
LLE	liquid-liquid extraction
m/z	mass-to-charge ratio
MALDI	matrix-assisted laser desorption/ionization
MBAS	methylene blue active substances
MRM	multiple reaction monitoring
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MW	molecular weight
n. d.	not detected
NCI	negative chemical ionization
NMR	nuclear magnetic resonance
NOEC	no observed effect concentration
NP	normal-phase
OECD	Organization for Economic Co-operation and Development
OR	orifice voltage
PB	particle beam
PIS	product ion scan
PS-DVB	polystyrene-divinylbenzene copolymer

RI	refractive index (detection)
RP	reversed-phase
SAC_{254}	spectral absorption coefficient at 254 nm
SAX	strong anion exchanger
SIM	selected ion monitoring
SIMS	secondary ion mass spectrometry
SPE	solid-phase extraction
TLC	thin layer chromatography
TOC	total organic carbon
ToF	time-of-flight
TSI	thermospray ionization
UV	ultra violet (detection)
WWTP	wastewater treatment plant
XIC	extracted ion chromatogram

SUMMARY

Surfactants are the most important organic chemicals by volume, which are relevant to the environment since of the 10 million tons produced annually the major portion is discharged into sewage after usage. In spite of more than three decades of research, there is still a wide gap to be bridged regarding the knowledge on environmental occurrence, behavior and ecotoxicological effects of surfactants and in particular of their **polar metabolites**. This is due to the fact that sophisticated techniques for sensitive analysis of polar pollutants in challenging environmental matrices have only become available in recent years.

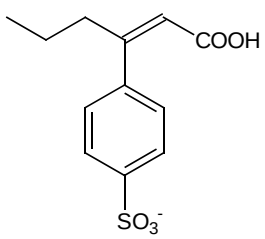
The principal tasks of the present work were

1) to develop and validate sensitive substance-specific methods for the quantitative determination of anionic, non-ionic and amphoteric surfactants and their biochemical degradation intermediates in aqueous environmental samples. These methods were based on advanced hyphenated mass spectrometric techniques.
2) to generate aerobic polar biodegradation products of surfactants in a laboratory-scale fixed-bed bioreactor (FBBR), which was run with a surface water-borne biocoenosis to simulate real environmental conditions.
3) to identify and characterize novel metabolites, generated in 2), by various mass spectrometric methods in order to deduce the surfactant degradation pathway in principle as well as to monitor primary and further biodegradation steps.
4) to gather data on the input and fate of surfactants in the aquatic environment under distinct hydrological and climatic conditions by analyzing the parent compounds and their metabolites in wastewaters and surface waters, in part polluted by untreated waste discharges.
5) to pursue the behavior of persistent surfactant metabolites in waterworks preparing drinking water from surface waters and to determine their residues in tap water.
6) to assess deleterious effects of discovered metabolites employing ecotoxicological bioassays.
7) to compare the results obtained from field studies with those from laboratory experiments in order to confirm the environmental relevance of the latter.

The selection of target analytes was made taking into account output and novelty on the surfactant market. The substances comprised the detergent ingredients anionic linear alkylbenzene sulfonates (LAS) as the single surfactant with the highest production volume known to yield highly polar metabolites, the two non-ionics alkyl glucamides (AG) and alkyl polyglucosides (APG), as well as the amphoteric surfactant cocamidopropyl betaine (CAPB). In addition, the polymeric dye transfer inhibitor poly(vinyl-pyrrolidone) (PVP) was examined.

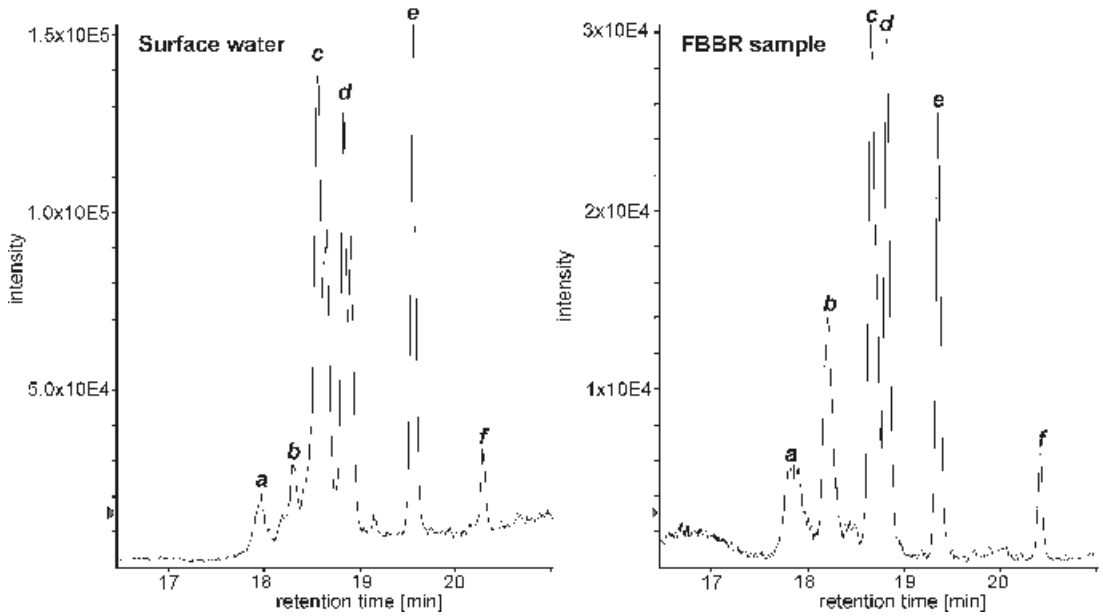
Hitherto no data were available on AG, APG, CAPB, and PVP concerning metabolism and fate in the environment. The degradation pathway of LAS was subject of a great number of studies, but the mechanism was not yet fully understood.

Ultimate evidence for the β-oxidation mechanism of LAS, which was generally accepted but hitherto not unequivocally confirmed, was given through unambigous identification of a suite of C-even α,β-unsaturated sulfophenyl carboxylates (possible structure of C6 homologue see right side) applying liquid chromatography-electrospray ionization-(tandem) mass spectrometry (LC-ESI-MS/MS) under reversed-phase conditions with triethylammonium as volatile ion-pair reagent. The so far unknown intermediates of homologue-pure C12-LAS degradation were released after its initial ω-oxidation within the spirals of β-oxidations of the corresponding sulfophenyl carboxylates (SPC). The latter were well-known for more than three decades as major degradative products of the anionic surfactant.



α,β-unsaturated C6-SPC

It turned out that some SPC isomers proved quite resistant to ultimate degradation and remained as persistent compounds in the test medium. The recalcitrance was attributed to steric hindrance in the aliphatic side chain due to proximity of the carboxylate group to the sulfophenyl ring or a methyl branch, which impeded the progression of degradation via β-oxidations. Significance of these findings to the environment was provided through comparison of the isomer pattern of single SPC homologues persisting in the FBBR with those detected in surface waters receiving residues of surfactant degradation (see figure below for mass trace of C7-SPC).



The metabolism of AG proceeded presumably in analogy to LAS via an ω-oxidation/β-oxidation mechanism. Analysis by LC-ESI-MS enabled unequivocal identification of a transient intermediate, described for the first time, which was postulated to be released from C10-AG after terminal alkyl chain oxidation and threefold shortening by β-oxidations. Ecotoxicity studies on this carboxylated compound were performed after synthesis using a bioluminescence test as well as an amperometric biosensor, both indicating very low toxicity.

Optimizing the ESI-MS detection of APG components, it was observed that in dependence of the constitution of the glucose moiety, sodiated or ammoniated ions were formed preferentially. During rapid

primary degradation of the non-ionic surfactant, no intermediates deriving from destruction of the hydrocarbon chain could be detected. It was inferred that enzymatic cleavage of the glucosidic linkage occurred intracellularly yielding readily degradable substrates.

The amphoteric CAPB showed a pronounced tendency to form cluster ions $[M_n \pm H]^{\pm}$ under both positive and negative ESI ionization. Its primary degradation on the FBBR proceeded without releasing any detectable metabolites.

The experiment on the biodegradability of polymeric PVP with a mean molecular weight of 2.5 kDa, was followed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-ToF-MS), representing the first example of combining the FBBR assay with a substance-specific analytical technique designed for determining high molecular weight compounds. Analysis revealed adsorption losses of higher mass oligomers, detected as singly-charged sodiated ions, but there was no indication of microbially induced alterations of the molecular structure identifiable by an emerging oligomer distribution. Confirmation for strong adsorption behavior was obtained from sorption studies of PVP (5-8 kDa) with sewage sludge.

Quantitative analyses of environmental samples was performed after solid-phase extraction by LC-ESI-MS in negative ion mode. In Spanish and German wastewater treatment plants monitored the two sugar-based surfactants AG and APG, which occurred in influents in the low to mid µg L^{-1} range, were efficiently removed (99.4-100 %). Concentrations of undegraded SPC in raw sewage were always lower than in treated waters, where between 15 and 168 µg L^{-1} were detectable. The alkyl homologue distributions of SPC were dominated by mid-chain C7- and C8-SPC being in accordance with findings on the degradation of commercial LAS in an FBBR where these species were also prominent.

In selected areas in Brazil and the Philippines, where dumping of sewage into rivers is a common practice, the studies focused on pursuing the biotransformation of LAS into SPC. The areas were taken as examples as in these environments surface waters represented a principal source for drinking water production.

In the first study conducted along the course of a river in Brazil the shift in the homologue distribution of LAS and the intermediate formation of SPC indicated the breakdown of the surfactant. The levels of the carboxylated intermediates in the waters were at all stations lower than LAS concentrations and varied between 7.4 to 14 µg L^{-1}.

In the catchment area of the Laguna de Bay, Philippines, LAS were identified together with their alkyl chain-branched homologues ABS in the low to mid µg L^{-1} range. Biodegradation of LAS was obvious by changes in the homologue pattern, while for ABS this indicator was nearly unaffected owing to the poor degradability of ABS. Through MS/MS analysis in multiple ion monitoring mode, linear SPC could be distinguished from their branched congeners because isobaric parent ions of both species produced distinct diagnostic fragments.

The behavior of SPC homologues persisting in surface waters (SPC levels: 2 to 5 µg L^{-1}) was investigated in two waterworks equipped with different treatment technologies. Of the multitude of treatment stages, among them prechlorination, flocculation, activated carbon filtration, ozonation, or subsoil passage, only sand filters with an assumed biological activity were able to diminish substantially the SPC concentrations. This observation was quite unexpected, as those constitutional isomers forming the recalcitrant fraction had not been further metabolized in the surface water.

Insufficient removal of undegraded SPC residues entering waterworks was documented by a monitoring survey of Brazilian drinking water samples. The tap water originated from a waterworks situated at the previously studied river. The levels found ranged from 1.4 and 3.7 µg L^{-1} and are the first reported. The peak pattern of well-resolved isomers of C7-SPC in drinking waters provided a valuable tracer to distinguish between samples from waterworks, which had purified raw waters of different qualities.

In conclusion, the present work illustrates the power of mass spectrometric state-of-the-art techniques for accurate determinations of polar organic pollutants in environmental samples. LC-ESI-MS(MS) is of unparalleled value in identifying and characterizing degradative products and is a highly valuable tool in elucidating metabolic pathways of surfactants. The strategy pursued in this work, i.e. the combination of lab-scale experiments and confirmation of the significance of the findings by analysis of real samples represents a very useful approach to investigating the behavior of surfactants and their metabolites in the environment. The studies conducted demonstrate the necessity for integrating metabolites into analytical protocols in order to obtain a comprehensive picture of the whereabouts of surfactant in the aquatic environment. This is of particular importance in view of strongly polar and recalcitrant degradation products, which are reported to find their ways from wastewaters via surface waters into drinking waters.

ZUSAMMENFASSUNG

Tenside stellen die mengenmäßig bedeutendste Klasse umweltrelevanter, organischer Verbindungen dar, denn der Großteil der jährlich produzierten 10 Millionen Tonnen gelangt nach der Verwendung in das Abwasser. Obwohl Tenside seit mehr als drei Jahrzehnten intensiv untersucht werden, besteht immer noch großer Forschungsbedarf hinsichtlich deren Vorkommen und Verhalten in der Umwelt und ihrer ökotoxikologischen Eigenschaften. Dieses gilt nicht nur für die Tenside selbst, sondern vor allem für deren **polare Abbauprodukte**. Erst in jüngerer Vergangenheit haben moderne Analysenmethoden, die eine empfindliche Bestimmung von polaren Schadstoffen in komplexen Umweltproben ermöglichen, Verbreitung gefunden.

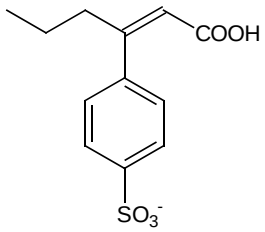
Die Ziele der vorliegenden Arbeit waren

1) die Entwicklung und Validierung von sensitiven und substanz-spezifischen Methoden für die quantitative Bestimmung von anionischen, nichtionischen und amphoteren Tensiden und deren Metaboliten in wässrigen Umweltproben unter Einsatz leistungsfähiger, massenspektrometrischer Analysengeräte,
2) die Gewinnung von aeroben, polaren Abbauprodukten aus Tensiden in einem die realen Umweltbedingungen simulierenden Labor-Festbettbioreaktor (FBBR), dessen Biozönose oberflächenwasserbürtig war,
3) zur Aufklärung des Abbaumechanismus von Tensiden neue, in 2) gewonnene Metabolite zu identifizieren und massenspektrometrisch zu charakterisieren ebenso wie den Primärabbau und den weiteren Abbau zu verfolgen,
4) durch quantitative Untersuchungen von Tensiden und deren Abbauprodukten in Abwasser und Oberflächenwasser Informationen zu ihrem Eintrag und Verhalten bei unterschiedlichen hydrologischen und klimatischen Bedingungen zu erhalten,
5) das Verhalten von persistenten Tensidmetaboliten in Wasserwerken, die belastetes Oberflächenwasser aufbereiten, zu untersuchen und deren Vorkommen im Trinkwasser zu bestimmen,
6) mögliche Schadwirkungen von neu entdeckten Metabolite mittels ökotoxikologischer Biotests abzuschätzen,
7) durch Vergleich der Felddaten mit den Ergebnissen der Laborversuche die Umweltrelevanz der Abbaustudien zu belegen.

Die Auswahl der untersuchten Verbindungen erfolgte unter Berücksichtigung ihres Produktionsvolumens und der Neuheit auf dem Tensidmarkt. Sie umfasste die Waschmittelinhaltsstoffe lineare Alkylbenzolsulfonate (LAS), welches das Tensid mit der höchsten Produktionsmenge darstellte, die beiden nichtionischen Tenside Alkylglucamide (AG) und Alkylpolyglucoside (APG), ebenso wie das amphotere Tensid Cocamidopropylbetain (CAPB). Außerdem wurde der polymere Farbübertragungsinhibitor Polyvinylpyrrolidon (PVP) untersucht.

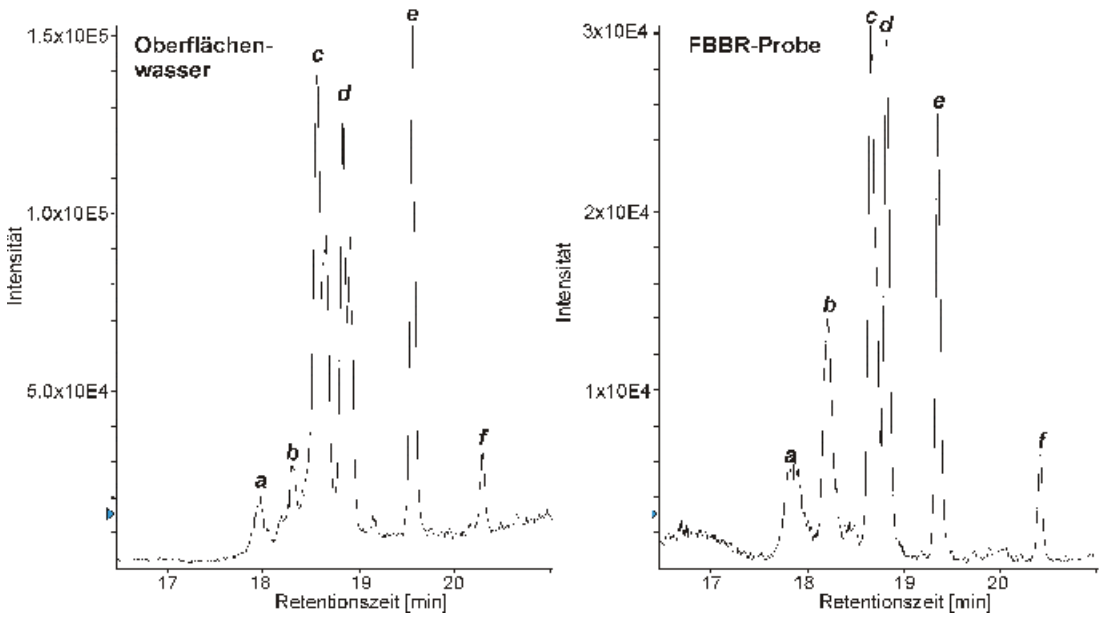
Bisher waren keine Daten zum Metabolismus und Umweltverhalten von AG, APG, CAPB und PVP bekannt. Der Abbaumechanismus von LAS war zwar in zahlreichen Studien untersucht worden, galt aber noch immer als nicht vollständig aufgeklärt.

Mittels Flüssigkeitschromatographie unter Umkehrphasenbedingungen und Kopplung mit der Elektrosprayionisation-(Tandem)Massenspektrometrie (LC-ESI-MS/MS) konnte durch eindeutige Identifizierung einer Reihe α,β-ungesättigter Sulfophenylcarboxylate (SPC-2H; Beispielstruktur siehe rechts) der endgültige Beweis dafür geliefert werden, dass LAS über einen β-Oxidationsmechanismus abgebaut werden. Die bislang unbekannten SPC-2H wurden beim Abbau von C12-LAS nach initialer ω-Oxidation innerhalb der β-Oxidationszyklen der entsprechenden Sulfophenylcarboxylate (SPC) gebildet und freigesetzt. Die SPC waren ihrerseits bereits seit mehr als 30 Jahren als Hauptabbauprodukte der LAS bekannt gewesen.



α,β-ungestättigtes C6-SPC

Von der Vielzahl möglicher SPC-Isomere erwiesen sich einige unter den Laborbedingungen als schwer abbaubar. Dieses wurde durch eine sterische Hinderung in der aliphatischen Seitekette bedingt durch die Nähe der Carboxylatgruppe zum Sulfophenylring oder einer Methylverzweigung zugeschrieben, was den weiteren Abbau über eine β-Oxidation erschwerte. Durch den Vergleich der chromatographischen Trennung der Phenylisomere zwischen einer FBBR-Probe und einem Oberflächenwasser, das SPC-Rückstände erhielt, konnte die Umweltrelevanz der Laborergebnisse bestätigt werden. Beispielhaft sind die Massenspuren des C7-SPC in der Abbildung gegenübergestellt.



Hinsichlich des Abbaus von AG wurde in Analogie zu LAS ein ω-Oxidation/β-Oxidationsmechanismus postuliert. Durch LC-ESI-MS-Analytik gelang es erstmalig, einen transienten Metaboliten zu identifizieren, dessen Bildung aus C10-AG sich nach terminaler Oxidation der Alkylkette und anschließender dreifacher β-Oxidation erklärbar war. Ökotoxikologische Studien des synthetisierten Carboxylats ergaben

sowohl in einem Biolumineszenztest als auch in einem Test mit einem amperometischen Biosensor nur eine sehr geringe Toxizität.

Bei der Optimierung der ESI-MS-Detektion der APG-Komponenten zeigte sich eine Abhängigkeit der Bildung von Natrium- und Ammonium-Addukten von der Konstitution und Konfiguration des Glucoserestes. Während des schnellen Primärabbaus des nichtionischen Tensids konnten keine Intermediate nachgewiesen werden, die aus einem möglichen Abbau der Kohlenwasserstoffkette entstanden waren. Es wurde postulierte, dass der Abbau interzellulär über die Spaltung der glucosidischen Bindung unter Bildung leicht abbaubarer Substrate verlief.

Das amphotere Tensid CAPB zeigte sowohl im positiven als auch im negativen Ionisierungmodus eine starke Tendenz, Clusterionene der Form $[M_n \pm H]^{\pm}$ zu bilden. Bei der Durchführung des FBBR-Experiments konnte kein Primärmetabolit detektiert werden.

Mittels matrix-unterstützter Laserdesorption/-ionzation-Flugzeit-Massenspektrometrie (MALDI-ToF-MS) wurde die Bioabbaubarkeit des polymeres PVP (mittleres Molekulargewicht: 2,5 kDa) verfolgt. Es wurde damit das erste Beispiel vorgestellt, den FBBR-Ansatz mit einer substanz-spezifischen Analysenmethode für hochmolekulare Verbindungen zu kombinieren. Die Messungen der als einfach geladene Natrium-Addukte detektierten Oligomere ergaben keinen Hinweis auf eine mikrobiell bedingte Veränderung der Molekulstruktur sichtbar in Form einer sich neu bildenden Oligomerverteilung. Statt dessen wurde eine Adsorption schwererer PVP-Komponenten beobachtet, was durch ein Zusatzexperiment zur Adsorption von PVP (5-8 kDa) an Klärschlamm bestätigt werden konnte.

Messungen in Umweltmatrices erfolgten mittels (–)-LC-ESI-MS nach Anreicherung der Proben durch Festphasenextraktion. Die im Zulauf der untersuchten spanischen und deutschen Kläranlagen im unteren bis mittleren µg L^{-1}-Bereich vorkommenden Zuckertenside AG und APG wurden während der Abwasserbehandlung effizient entfernt (99,4 bis 100 %). Die Konzentrationen nicht abgebauter SPC waren im Ablauf stets höher als im unbehandelten Abwasser und beliefen sich auf 15 bis 168 µg L^{-1}. Die Alkylhomologenverteilung der SPC wurde von C7- und C8-SPC dominiert, was den Ergebnissen der FBBR-Abbaustudien mit einem technischen LAS-Gemisch entsprach, bei denen ebenfalls diese Spezies vorherrschten.

In ausgewählten Regionen in Brasilien und den Philippinen – in diesen Ländern wurde ein Großteil des Abwassers direkt in Oberflächengewässer eingeleitet – konzentrierten sich die Untersuchungen auf die Biotransformation von LAS zu SPC. Die Auswahl der Gebiete wurde unter dem Aspekt gemacht, dass in jenen Oberflächenwasser eine wichtige Quelle für die Trinkwassergewinnung darstellte.

Bei der ersten Studie entlang eines Flusses in Brasilien wurde der aktive Abbau von LAS durch die Verschiebung des Homologenmusters sowie die intermediäre Bildung der SPC bestätigt. Die Konzentrationen der carboxylierten Abbauprodukte lagen an allen Stationen unter jenen der LAS und variierten im Bereich zwischen 7.4 und 14 µg L^{-1}.

Im Einzugsgebiet der Laguna de Bay, Philippinen, konnten LAS zusammen mit ihren Alkylketten verzweigten Homologen, den sogenannten ABS, im unteren bis mittleren µg L^{-1}-Bereich nachgewiesen werden. Der biologische Abbau der LAS spiegelte sich in der Änderung des Homologenmusters wider, während dieser Indikator für ABS nur Hinweis auf einen sehr begrenzten Abbau ergab. Durch MS/MS-Analysen im multiple reaction monitoring-Modus konnten lineare SPC von ihren isobaren, verzweigten Isomeren unterschieden werden, da sie unterschiedliche, diagnostische Fragmente bildeten.

Das Verhalten von im Oberflächenwasser auftretenden, schwer abbaubaren SPC-Homologen (im Bereich von 2 bis 5 µg L^{-1}) wurde in zwei Wasserwerken verschiedener Bauart untersucht. Von der Vielzahl der Behandlungsschritte wie Vorchlorung, Flockung, Aktivkohlefiltration, Ozonung oder Bodenpassage konnten lediglich Sandfilter mit einer vermeintlich biologischen Aktivität eine deutliche Reduktion der SPC bewirken. Im Hinblick auf die Stabilität der im Oberflächenwasser persistierenden Isomere war dieses Resultat eher unerwartet.

Durch eine Monitoringstudie von Trinkwasserproben in Brasilien konnte die unzureichende Entfernung von nicht abgebauten SPC in einem Oberflächenwasser aufbereitenden Wasserwerk belegt werden. Die Konzentrationen im Trinkwasser bewegten sich zwischen 1,4 und 3,7 µg L^{-1} und stellten damit die ersten dar, die bisher berichtet wurden. Das Peakmuster der gut aufgelösten Isomere von C7-SPC stellte einen nützlichen Marker zur Charakterisierung von Trinkwasserproben unterschiedlicher Provenienz dar.

Zusammenfassend belegt die vorliegende Arbeit die Leistungsfähigkeit massenspektrometrischer State-of-the-art Techniken, die eine exakte Bestimmung polarer, organischer Schadstoffe in Umweltproben ermöglichen. LC-ESI-MS(MS) erweist sich als sehr wertvolles, analytisches Instrumentarium für die Identifizierung und Charakterisierung von Abbauprodukten ebenso wie für die Aufklärung der Abbaumechanismen von Tensiden. Die in dieser Arbeit verfolgte Strategie, basierend auf der Kombination von Laborexperimenten und Bestätigung der Relevanz der gewonnenen Ergebnisse durch Analysen von Realproben, stellt einen sehr nützlichen Ansatz dar, das Verhalten von Tensiden und ihren Metaboliten in der Umwelt zu beschreiben. Die durchgeführten Untersuchungen veranschaulichen die Notwendigkeit, Abbauprodukte in analytische Methoden zu integrieren, um ein möglichst umfassendes Bild des Verbleibs der Ausgangsverbindungen in der aquatischen Umwelt zu erhalten. Dieses ist von besonderer Bedeutung angesichts der Bildung und des Auftretens von stark polaren und schwer abbaubaren Metaboliten, die das Potenzial besitzen, vom Abwasser über Oberflächenwasser in das Trinkwasser zu gelangen.

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1 INTRODUCTION

In industrialized countries wastewaters and surface waters contain a multitude of organic micropollutants originating from various anthropogenic activities in the field of industry, agriculture, and household. With increasing awareness on the deterioration of the quality of the aquatic environment and herewith related possible toxicological effects on organisms living therein, the need urged to enhance the knowledge on the occurrence and behavior of such contaminants.

In the beginnings of environmental analysis of organic pollutants in the 1970s, the research focused on nonpolar hazardous compounds such as the pesticides lindane, dieldrine, aldrine and DDT. Their determination was primarily based on gas chromatographic techniques. Through advancements in the development of sophisticated analytical methodologies, the sensitivity of the methods was steadily improved, which finally allowed the detection of residues occurring at ultra-trace levels (ng L^{-1}) such as e.g. antibiotics. The necessity for evermore increasing sensitivity arose from the fact that very low concentrations of some synthetic pollutants sufficed to exert biological effects on aquatic organisms expressed not only as acute or chronic toxic interactions but also as the potential to act as endocrine disruptor in aquatic life forms.

With the emergence of reliable and robust liquid chromatography-mass spectrometry equipment in the early 1990s, the determination of polar contaminants became increasingly important and is nowadays a major topic in environmental analysis. Next to the possibility for direct sensitive and specific measurements of target analytes, the detection and structure elucidation of metabolites, which frequently exhibit a higher polarity than the precursor compounds, is feasible. The relevance to identify degradation products and to incorporate them into analytical protocols was due to the recognition, that they could potentially be very resistant to ultimate degradation, which might be complemented by an elevated toxicity relative to the parent compound. Such scenario was observed for degradation intermediates of surfactants.

Surfactants themselves are one of the most important organic chemicals relevant to the environment, since after usage they are typically discharged into the sewer and thereby principally enter the environment through release of treated or untreated wastewater. In face of the large quantities of surfactants consumed (about 10 millions tons per annum, worldwide[1]) and emitted into wastewaters and the toxicity towards aquatic organisms (LC_{50} are up to the mg L^{-1} range), contamination of the environment by surface-active agents has generated the need to measure surfactant levels in various compartments. This need will likely increase in the future owing to the continuously growing use of surfactants throughout the world.

[1] A detailed break down into surfactants types and production figures is given in section 1.2., p. 7).

Current needs versus former scopes

Since the mid-1980s a great number of specific analytical methods have been developed for selectively determining trace levels of these pollutants in different matrices (reviewed by Kiewiet and de Voogt, 1996; Marcomini and Zanette, 1996; Matthijs and Hennes, 1991; Morelli and Szajer, 2000 and 2001; Thiele et al., 1997; Vogt and Heinig, 1999). A large part of these examinations focused on the analysis of intact parent compounds of the most widely used anionic surfactant linear alkylbenzene sulfonate (LAS) and the non-ionic nonylphenol ethoxylate (NPEO), which gave rise to environmental concern as it yielded the persistent metabolite nonylphenol (NP) showing a high toxicity.

Conversely, surfactants with comparably low consumption volumes have by date received far less attention. Fulfilling the criteria of high primary degradation (CEC, 1973 and 1982), which should ideally be characterized by loss of surface activity and therewith a substantial reduction in toxicity, and of great extent of mineralization, determined from laboratory experiments, generally sufficed for the acceptance of a surfactant class to be introduced on the market. The example of NPEO degradation, however, has impressively underpinned the necessity for elucidating the holistic metabolic pathway of individual surfactants in order to identify possible recalcitrant intermediate products. Incorporation of such degradation products into a comprehensive risk assessment is indispensable.

To allow a detection and identification of transient or persistent breakdown products, the analytical approaches demand highly sophisticated methods. This holds particularly true in view of the low relative amounts of metabolites to be expected, their complex composition – commercial surfactant mixtures from which they derive can be made up of several tens to hundreds of components –, and their likely polar character. Isolation and preconcentration of the target analytes from environmental matrices must necessarily precede the sensitive analytical determination. The quantitation is an actual problem owing to the lack of authentic standard material. Only with the aid of synthesized reference compounds, which are usually not available from chemical suppliers, will an accurate quantitative analysis be possible.

These obstacles explain why the determination of surfactant degradative products in complex matrices has long been neglected and has only become an issue in environmental analysis over the last years, when the pieces of the puzzle were started to be collected aiming at eventually obtaining a complete picture (Castillo et al., 2000; di Corcia, 1998; di Corcia et al., 1999a; Ding et al., 1999; González-Mazo et al., 1997).

To make a contribution on the aspects outlined and to provide a number of fragments to the imperfect picture were the principle tasks of the present work.

1.1 Objectives and selection of compounds

The objectives of the present work were

1) to develop and validate sensitive substance-specific methods for the quantitative determination of anionic, non-ionic and amphoteric surfactants and their biochemical degradation intermediates in aqueous environmental samples. These methods were based on advanced hyphenated mass spectrometric techniques.

2) to generate aerobic polar biodegradation products of surfactants in a laboratory-scale fixed-bed bioreactor (FBBR), which was run with a surface water-borne biocoenosis to simulate real environmental conditions.
3) to identify and characterize novel metabolites, generated in 2), by various mass spectrometric methods in order to deduce the surfactant degradation pathway in principle as well as to monitor primary and further biodegradation steps.
4) to gather data on the input and fate of surfactants in the aquatic environment under distinct hydrological and climatic conditions by analyzing the parent compounds and their metabolites in wastewaters and surface waters, in part polluted by untreated waste discharges.
5) to pursue the behavior of persistent surfactant metabolites in waterworks preparing drinking water from surface waters and to determine their residues in tap water.
6) to assess deleterious effects of discovered metabolites employing ecotoxicological bioassays.
7) to compare the results obtained from field studies with those from laboratory experiments in order to confirm the environmental relevance of the latter.

The choice of target analytes was made by taking into consideration production and use figures and novelty. Despite a 40-year-history of research, the dominant anionic surfactant LAS was selected since its degradation pathway was hitherto not fully understood. Moreover, there was a strong lack of data on the occurrence of their major degradation intermediates, the sulfophenyl carboxylates, in the aquatic environment and on their whereabouts herein. The two relatively recently introduced non-ionics alkyl glucamide (AG) and alkyl polyglucosides (APG) were studied in order to verify the environmental compatibility of these surfactants entirely manufactured from renewable feedstocks. To that day nothing was reported on their metabolism and fate in WWTP. The increasingly applied surfactant cocamidopropyl betaine (CAPB) was considered due to a general deficit of knowledge on this amphoteric compound.

In addition, the practical worth of the fixed-bed bioreactor and the requirement for alternative analytical approaches was demonstrated by extending the scope to the high molecular synthetic polymer poly(vinylpyrrolidone) (PVP) used as dye transfer inhibitor in detergent formulations. Nothing was reported on the biodegradability and environmental fate of this compounds

1.2 From manufacturing of surfactants to effects on aquatic organisms

In the following sections, detailed knowledge is provided on the characteristics of surfactants, the history of their production, the actual market situation, and the whereabouts and effects of surfactants in the environment after going down the drain.

Function and classification

Immense amounts of chemically very distinct surfactant types are employed in a great variety of fields of application in households, commerce, industry, and agriculture, where their properties of reducing solution surface tension and their tendency to form micelles are exploited. Surfactant solutions exhibit

combinations of cleaning, foaming, wetting, emulsifying, solubilizing, and dispersing properties (Kirk-Othmer, 1999).

All surfactants have in common an asymmetric skeleton with a hydrophobic and a hydrophilic moiety (**Fig. 1-1**). The hydrophobic part generally consists of a linear or a branched alkyl or alkylaryl hydrocarbons chain, which is in turn linked to a hydrophilic group. Due to this bifunctionality, both parts of the surfactant molecule interact differently with water, which is the most commonly used solvent in commercially important surfactant systems.

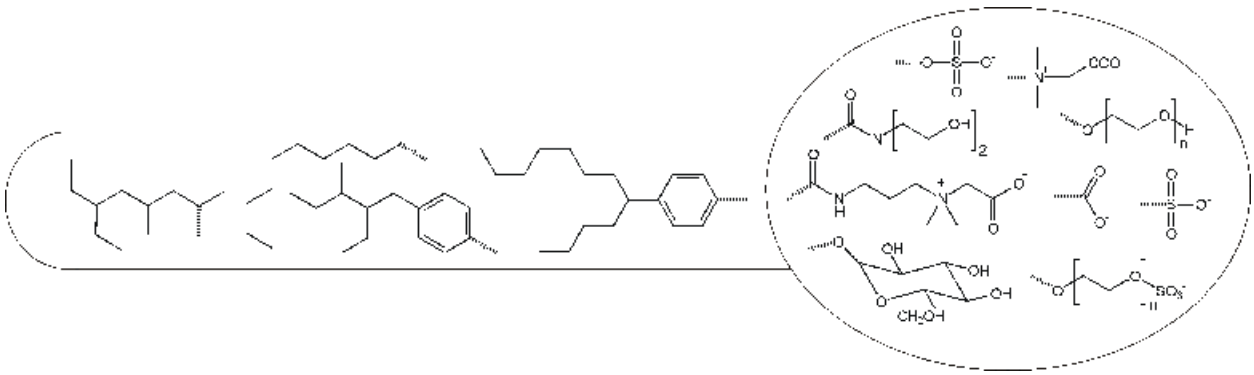


Fig. 1-1: Diagrammatic tail-head model of surfactant molecule showing some important hydrophobic and hydrophilic groups.

Surfactants are classified into four general categories based on their charge **anionics**, **non-ionics**, **cationics** and **amphoterics**. As the chemical homogeneity of a specific surfactant is of little significance regarding its functional properties, commercial surfactant classes are generally complex mixtures.

Surfactant production

Soap was known as the first manmade surfactant. Soaps made from animal fat and wood ashes seem to have been used only for medical purposes in antiquity; not until the 2nd century AD was it recognized as a cleaning agent (Heller, 1984). The first synthetic detergents were developed in the 19th century, when sulfonation of olive and almond oils (1831) and castor oil (1875) yielded appropriate auxiliaries for textile dyeing. At the turn of the century, sodium salts of petroleum sulfonates and cetyl sulfonate were introduced on the market (Chalmers, 1966), but soap remained the only important surface-active agent for many years. Wartime fat shortages during World War I and II stimulated the search for substitutes like, e.g. short-chain alkyl naphthalene sulfonates, made by coupling of alcohols with naphthalene and subsequent sulfonation (Davidsohn and Milwidsky, 1978).

The rapid development of the chemical industry and the increasing importance of surface-active substances in technical and domestic applications led to the introduction of the first synthetic detergent produced entirely on the basis of petrochemical feedstocks. These anionic alkylbenzene sulfonates (ABS; **Fig. 1-2**), which appeared on the market in the early 1930s, rapidly became the major surfactant used in the United States, and later in other countries around the world where advanced chemical technology allowed its production (Jungermann, 1979). The replacement of soap by ABS in most laundry products was essentially driven by two factors: the undesired formation of insoluble calcium and magnesium salts of soap which precipitated in the washing liquor and deposited on the clean surface of the laundry, and the lower production costs of ABS.

The widespread use of ABS mainly in laundry detergents and their subsequent discharge into the sewer, however, led to the unexpected effect of strong foam formation in sewage influent, treated sewage and even in river water (Webster and Halliday, 1959) (**Fig. 1-3**). This observation was directly related to the physical properties of the surfactant, which had originally been responsible for its great success.

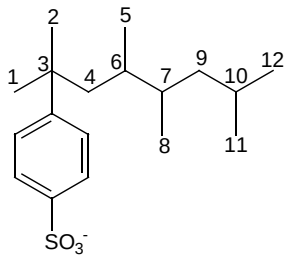


Fig. 1-2: Possible structure of branched C12-alkylbenzene sulfonate (ABS).

Shortly after the appearance of the described environmental problems, it was recognized that ABS derived from tetrapropylene was quite resistant to biodegradation due to the presence of a branched alkyl chain in the hydrophobic moiety (McKinney and Symons, 1959). Legal restrictions in the mid-1960s, in Germany for example, mandating a certain degree of primary degradation for anionic surfactants (Anonymous, 1962), or voluntary industrial bans as in the U.S., led to the development and introduction of an alternative anionic surfactant on the detergent market, the LAS. A modification in the chemical structure consisting of the substitution of the branched alkyl chain by a linear one enhanced the biodegradability profoundly (Swisher, 1987). Quickly after the changeover from ABS to LAS in the mid-1960s, foam-related problems nearly disappeared. In addition, the levels of surfactants in aquatic environments dropped significantly (Sullivan and Evans, 1968).

Fig. 1-3: Foam layers on a river strongly polluted by ABS surfactant.

Since that time an enormous number of surfactants covering a wide range of chemical and physico-chemical properties have been developed for universal as well as specific tasks in domestic and industrial applications (Hager, 1999; Kosswig, 1997; Schwuger and Piorr, 1987). These developments were primarily driven by economic aspects and functional characteristics of the finished products. However, ecological considerations became increasingly important as it became evident that the lesson on ABS was not the last to be learnt.

The cationic surfactant ditallowdimethylammonium chloride (DTDMAC), used since the 1960s as fabric softener, has been found to be substantially enriched onto suspended sludge particles, sediments, and soils, because of an extremely low water solubility and the hydrophobic and electrostatic interactions with negatively charged surfaces (Gerike et al., 1994; Versteeg et al., 1992). For this reason, DTDMAC was almost completely replaced in Europe by more biodegradable cationic compounds (Giolando et al., 1995). Another surfactant, whose use has been severely restricted in several European countries, is non-ionic NPEO, whose partial degradation results in the formation of the nonpolar persistent compound NP, which may exert adverse effects on aquatic organisms and acts as endocrine disruptor (Ahel et al., 1993; Soto et al., 1991).

As for the manufacturing of surfactants, they are produced from both petrochemical resources and/or renewable, mostly oleochemical, feedstocks (Patel et al., 1999; Tsushima, 1996). Crude oil and natural gas make up the first class, while tallow and coconut oil, and increasingly palm kernel oil are the most relevant representatives from the group of renewable resources.

Table 1-1: World production volumes of surfactants, in 1998.

Surfactant type	Thousand tons
Anionics	
Linear alkylbenzene sulfonates (LAS)	3027
Alkyl ethersulfates (AES)	911
Alkyl sulfates (AS)	479
Branched alkylbenzene sulfonates (ABS)	198
Non-ionics	
Alcohol ethoxylates (AE)	849
Alkylphenol ethoxylates (APEO)	701
Alkyl polyglucosides (APG)	80
Alkyl glucamides (AG)	40
Cationics	
Quartenaries	434
Amine oxides	50
Amphoterics	
Betaines	85
Other [a]	3110
TOTAL	9861

[a] does not include soap, Source: CEH, 1998; Karsa, 1998.

Table 1-1 gives an overview on the global production of the most prominent representatives of each surfactant category. By the year 2005, the worldwide demand for surfactants is forecasted to rise 3.6 % annually (Karsa, 1998). Although the market in the industrialized countries, with a very limited number of private surfactant companies, is only slightly growing, the demand is rapidly expanding in other nations particularly in the Asian-Pacific region (CAH, 1999).

Fate and effects of surfactants in the environment

Of the roughly 10 millions tons of surfactants manufactured every year, the major portion enters the wastewater stream after use. Through different routes, residues of surfactants can find their ways into distinct environmental compartments where the inherent properties of surface activity, negligible volatility, and in most instances good water solubility are determining factors for their fate (**Fig. 1-4**).

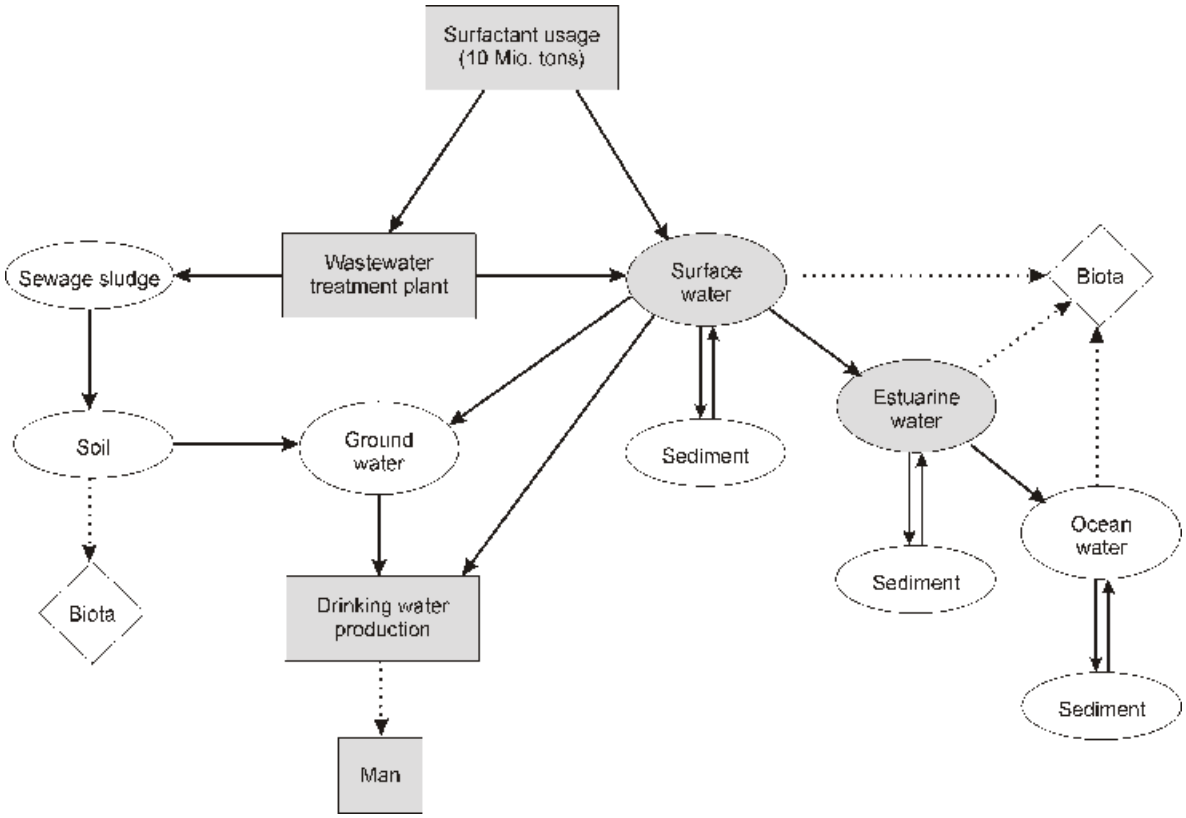


Fig. 1-4: Fate of surfactants and their metabolites in the environment after discharge with sewage.

Regarding the treatment of sewage in wastewater treatment plants (WWTP), the behavior of surfactants depends among other factors on the composition of the wastewater, the operational type of the WWTP, and the physico-chemical properties of the surfactant molecule itself. After removal of solid materials by mechanical means (primary treatment), organic wastewater components are commonly subject to aerobic degradation by a microbial biocoenosis accomplished by either an activated sludge process or a trickling filter (secondary treatment) (NALCO, 1979). The initial step altering the molecular structure of surfactants is referred to as primary degradation (Swisher, 1987). For the majority of surfactants, this is associated with the loss of their surface-active properties. The metabolism ideally results in the complete mineralization of a compound (ultimate degradation) yielding carbon dioxide and water, and inorganic

substances like sulfate, or conversion into bacterial biomass. But this end point is in most cases not attained due to insufficient retention time in the WWTP or very slow degradation kinetics of the parent compound or its intermediates. Hence, surfactants are released together with their biochemical degradation products via treated effluens into surface water bodies serving as receiving waters (Crescenzi et al., 1995; Evans et al., 1994; McAvoy et al., 1998; Popenoe et al., 1994; Rapaport and Eckhoff, 1990).

Depending upon the physical properties of a surfactant, the removal from the mixed liquor may also occur through precipitation of insoluble salts and adsorption onto solids or bacterial flocks, which in turn are subsequently removed with the excess sludge (Field and Reed, 1999; Klotz, 1998; Marcomini and Giger, 1987; McEvoy and Giger, 1986; Petrovic and Barceló, 2000b). Particularly less water soluble and lipophilic surfactants, which can be intact or partly degraded, are eliminated by this route. The removed excess sludge is often decomposed by anaerobic digestion to reduce the content of degradable organic substances (Prats et al., 1997).

The recovered digested sludge is usually further stabilized and is suitable for agricultural purposes as fertilizer and soil conditioner due to its high content of organic material and essential nutrients. This path represents a potential source for surfactants and their metabolites, which have not been degraded during sludge processing, to the terrestrial environment (Gerike et al., 1994; Waters et al., 1989). Surfactants introduced into the soil by sludge application can also interact with soil organisms and crops (Hartmann, 1966; Marschner, 1992). Surfactants were also reported as ground water contaminants (Krueger et al., 1998; Thurman et al., 1987).

On the other hand, residues of the incomplete surfactant degradation entering the aquatic environment via the discharge of WWTP effluent can follow distinct fates. More hydrophobic species with low water solubilities are prone to bind to suspended particles or to sediments (Brownawell, 1997; John et al., 2000; Westall et al., 1999), while polar compounds, predominantly formed during the wastewater treatment by oxidative biotransformation of surfactant molecules, display particularly high water solubility in connection with a high mobility in the aqueous medium. Mobilization over relative long distances is thereby possible unless further degradation occurs. The mobility of persistent surfactants and metabolites in bound or dissolved state in riverine systems enables wide dissemination until these pollutants reach estuaries, coastal regions and at last the marine environment (González-Mazo and Gómez-Parra, 1996; Matthijs and Stalmans, 1993), where they may be removed by scavenging onto particles (Ferguson et al., 2000).

Surfactants emitted into surface waters cause concern – particularly if the wastewater has not experienced any sort of treatment – as some may exhibit relatively high toxicities to aquatic organisms (Guhl and Gode, 1989; Scholz, 1997; Staples et al., 1998) [1]. This is correlated to their inherent surface-active properties primarily contributing to disruption of biomembranes and denaturation of proteins (Swisher, 1987). Notable detoxification may occur if the primary degradation proceeds via the loss of surface-activity leading to more polar compounds (Moreno and Ferrer, 1991), but an enhanced toxicity of e.g. NP as a recalcitrant metabolite of NPEO has likewise been reported (Giger et al., 1984). In addition, this nonpolar compound is known to mimic the effect of the hormone estrogen (Soto et al., 1991).

[1] Toxicity data of LAS: Effective concentration (EC): EC_{50} (*Daphnia*): 8.8 mg L^{-1} and EC_{50} (fish): 4.8 mg L^{-1}; no observed effect concentration (NOEC): NOEC (*Daphnia*): 0.3 mg L^{-1} and NOEC (fish): 0.12 mg L^{-1} (Schöberl, 1997).

Bioaccumulation of surfactants and their degradation intermediates represents a further issue (Ahel et al., 1993; Tolls et al., 1994 and 2000).

Moreover, the mobility of particularly persistent compounds or metabolites may pose a threat to drinking water supplies using contaminated surface water or bank filtration-enriched ground water as a source for the production of potable water. Certain compounds have been reported to persist through the installed technical purification units and are thus ultimately found in drinking waters (Clark et al., 1992; Rivera et al., 1987b; Schröder, 1993a; Ventura et al., 1991).

With the need to monitor the distribution and behavior of surfactants in the environment, a great number of analytical methods based on a multitude of different techniques have been developed. An overview is given in the following chapter.

2 OVERVIEW ON METHODS FOR SURFACTANT ANALYSIS

The analysis of surfactants in environmental matrices has increased the understanding of the removal of these chemicals by WWTP and of their exposure levels to aquatic organisms. Determination at trace levels is usually complicated by the complexity of commercial surfactant blends and by possible interferences from the studied matrix. For this reason, the employment of group-specific sum parameters, like methylene blue active substances (MBAS) for anionics or bismuth active substances for non-ionics (DEV, 1980), is of only limited value since these methods do not generate information on the distribution of individual surfactant components relevant e.g. in environmental risk assessment. Apart from this shortcoming, the sum parameter analysis on spectrophotometric and titrimetric basis is very insensitive and quite unspecific since interferences with natural or synthetic substances can occur, thereby yielding positive or negative bias to the quantification. For example, 1,040 mg L^{-1} of nitrate or 17,900 mg L^{-1} of chloride (pH 1.8[1]) gave the same response as 10 mg L^{-1} AS surfactant in MBAS determination (Evans, 1950).

To surmount the limitations of group-specific sum parameter analysis, sophisticated methods including preconcentration, chromatographic separation and a sensitive and accurate detection are required for the compound-specific analysis of the broad range of existing surfactants. The request for more specific methods is increased when the investigations center not only on the parent compound, but also on qualitative and quantitative analysis of degradation intermediates formed at low concentrations during the wastewater treatment process or in the receiving stream.

2.1 Isolation and enrichment

Prior to the quantitative determination of surfactants from aqueous environmental samples, a preconcentration step is needed to enrich low amounts of target analytes and to remove interfering matrix components. Both aims are attained by employing solid-phase extraction (SPE), which has been shown to be a very powerful and robust alternative to traditional extraction methods of organic compounds, such as e.g. liquid-liquid extraction (LLE) (Bruzzoniti et al., 2000; López-Avila, 1999). It offers the advantages of very low solvent consumption, speed, and ease of handling. Various types of commercially available stationary phases enable the application of distinct separation mechanisms, thereby increasing the selectivity of the adsorbent to the analytes.

To date, typical SPE materials are based on silica gel or highly cross-linked styrene-divinylbenzene (PS-DVB). The former is functionalized with distinct chemical groups to yield various sorbents with non-

[1] Extraction at low pH strongly reduced the co-extraction of inorganic anions.

polar or polar characteristics. Non-polar materials are modified with alkyl groups of different chain length (C18, C8, or C2), while polar sorbents have cyano-, amino-, or diol-bonded groups. Ion-exchange phases have either anionic or cationic functional groups. The main drawback of all silica-based materials arises from their limited pH stability. Reversed-phase (RP) polymeric sorbents such as PS-DVB offer several advantageous: this material can be used under extreme pH conditions, has higher specific surface areas and shows greater retention for polar compounds. Chemical modification of the polymeric resin yields highly selective stationary SPE phases (León-González and Pérez-Arribas, 2000). Another alternative sorbent is non-porous graphitized carbon black (GCB) with positively charged active centers on the surface, which offers the singular feature of behaving as a non-specific RP sorbent and anion exchanger (Creszenzi et al., 1996). By exploiting this feature, the separation of acidic compounds from co-extracted base/neutral ones can be easily achieved by differential elution. In **Table 2-1** (p. 19) a selection of analytical methods is compiled, which are based on SPE for the preconcentration of a range of surfactant classes from wastewater, surface, and drinking water samples.

2.2 Chromatographic separation

2.2.1 Gas chromatography

The application of gas chromatography (GC) offers high resolving capacity, which is advantageous for the separation of complex surfactant mixtures, but the inherently low volatility of most surfactants severely limits a direct analysis. Therefore, a great number of efforts have been mounted to develop derivatization methods designed to transform the analytes into more volatile species.

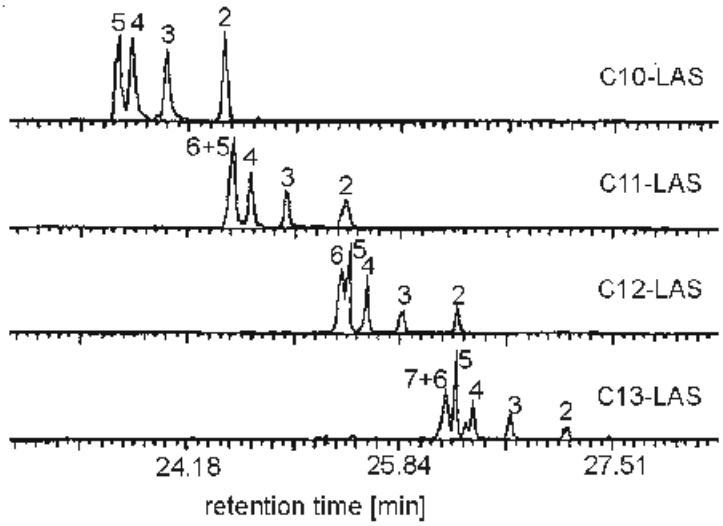


Fig. 2-1: GC-MS chromatograms of butylated LAS. Peak numbers indicate the positional isomer. GC conditions: DB-5MS, 30 m x 0.25 mm i.d., 0.25 µm film, 100 °C for 3 min, 7 °C min^{-1} to 300 °C, hold for 7 min. Adapted from Ding and Fann (2000).

For example, LAS were analyzed as sulfonyl chlorides (Hon-Nami and Hanya, 1978), trifluormethyl esters (Tabor and Barber, 1996), butyl esters (Ding and Fann, 2000; **Fig. 2-1**), or desulfonated products

(Swisher, 1966), NPEO and APG as their silyl derivatives (Billian and Stan, 1998; Rudel et al., 1998) and AE after cleavage with HBr as alkyl bromides (McAvoy et al., 1998). Such sample manipulations, however, are time consuming, and can be susceptible to discriminations. Moreover, for the determination of cationic surfactants GC analysis is not of practical relevance.

2.2.2 Liquid chromatography

Due to the restrictions of GC, high performance liquid chromatography (HPLC) has been recognized as the most appropriate procedure for the analysis of surfactants in the aquatic environment where is has routinely been applied. Although conventional HPLC does not provide the separation power of GC, the chromatographic behavior can be varied within a wide range using different types of stationary phases and compositions of mobile phases.

RP-HPLC working with alkyl-bonded phases is the most common technique since it provides information about the alkyl chain length of surfactant homologues. The optimum resolution of isomeric components, e.g. in LAS mixtures, is attained on long-chain C18-phases (Matthijs and de Hanau, 1987; Nakamura and Morikawa, 1982; Nakae et al., 1981) (**Fig. 2-2**), but the use of short-chain alkyl-bonded reversed phases like C8- or C1-columns is necessary if elution of the isomers as a single peak is desired (Castles et al., 1989; Marcomini et al., 1987). In this instance, the interpretation of the chromatogram becomes easier and lower quantification limits are achieved.

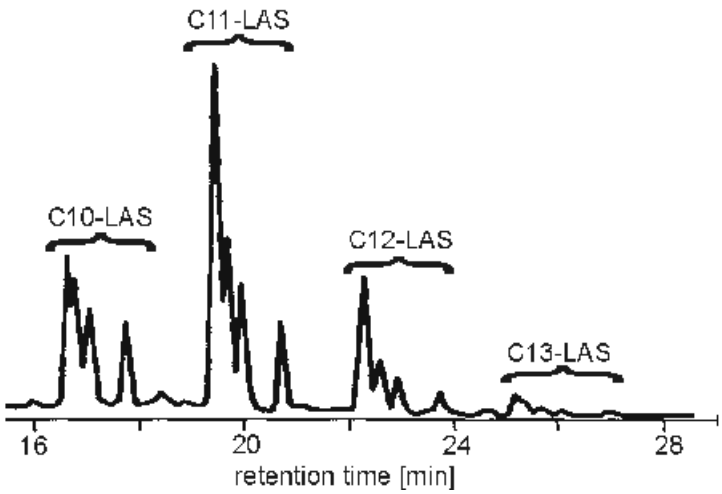


Fig. 2-2: RP-HPLC chromatogram of LAS. HPLC conditions: 250 x 2.1 mm, 5 µm C18 Lichrospher 100 RP, mobile phase A: water-CH_3CN (60:40) + 0.1 M $NaClO_4$, mobile phase B: CH_3CN-water (80:20) +0.1 M $NaClO_4$, from 100 % A to 0 % A within 30 min, hold for 5 min. Adapted from Schröder et al. (1999).

In turn normal-phase (NP) HPLC is often applied to resolve the ethoxylate isomers as present in NPEO or AE mixtures (Kósa et al., 1998; Marcomini and Giger, 1987; Shang et al., 1999). Apart from alumina and silica gel columns, chemically modified silica with aminopropyl-, cyano-, or diol-bonded phases were used to improve the separation of polyethoxylated surfactants (Kiewiet and de Voogt, 1996) (**Fig. 2-3**). Ion exchange chromatography proved to be helpful for the analysis of anionic and cationic surfactant classes (Osburn, 1982; Pan and Pietrzyk, 1995). For the separation of complex surfactant mixtures containing anionics, cationics and amphoterics, two-dimensional HPLC represents a promising approach.

For instance, in the case of anionic surfactants, each surfactant is separated from the matrix according to the type of anionic group by an anion-exchange column, and eluted as a single peak. The eluted surfactant is trapped on a short C8-column and then transferred to a C18-column (Noguchi et al., 1998).

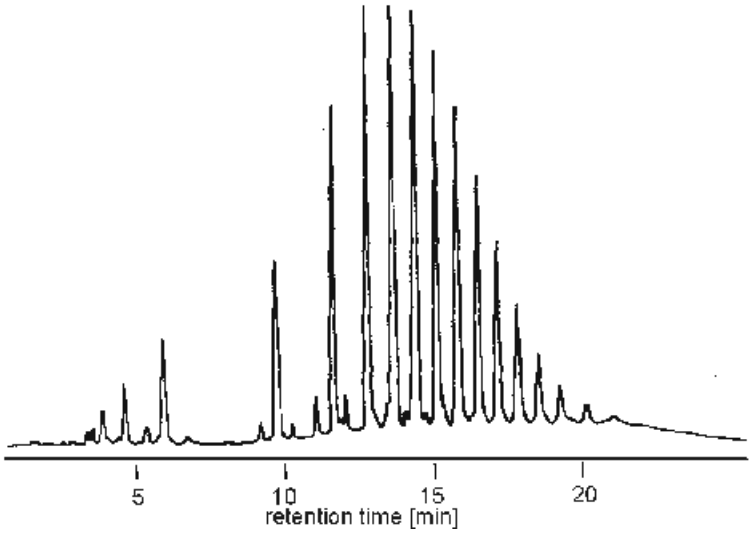


Fig. 2-3: NP-HPLC chromatogram of octylphenol ethoxylate. HPLC conditions: 250 x 4.6 mm, 5 µm Zorbax CN, mobile phase A: heptane, mobile phase B: 2-methoxyethanol–2-propanol (50:50), from 2 % A to 20 % A within 10 min, hold for 20 min. Adapted from Agilent Technologies, HPLC Applications (2001).

Considering detection, optical systems such as ultraviolet (UV) and fluorescence (FL) detectors have been most widely applied for the determination of surfactants with a strong chromophore in the molecule such as the aromatic type LAS and NPEO (Marcomini et al., 1987; Matthijs and de Hanau, 1987; Naylor et al., 1992). These detection methods, however, fail with aliphatic anionic or non-ionic surfactants like AE, AES, or APG lacking suitable chromophoric groups. In these cases, the very low sensitivity refractive index (RI) detector or a conductivity detector, applicable only to charged surfactant classes, can be used. Alternatively, non-UV active compounds can be made amenable to UV or FL detection by pre-injection derivatization with suitable UV-adsorbing or fluorescent agents, or by post-column ion-pair (IP) formation (Fernández et al., 1996; Matthijs and Hennes, 1991).

2.2.3 Capillary electrophoresis

The advantages of capillary electrophoresis (CE), in which the separation is based on differences in the electrophoretic mobilities of the analytes in an electric field, reside in the short analysis time and the very high efficiency when compared with GC and HPLC methods. CE is particularly suited for the analysis of ionic surfactants, where it has been applied to the separation of alkyl homologues of the anionics LAS, AS and alkyl sulfonates (Chen and Pietrzyk, 1993; Heinig et al., 1996; Salimi-Moosavi and Cassidy, 1996; Wheat et al., 1997). The analysis of cationics is problematic because of the strong adsorption of these compounds at the inner surface of the silica capillary. However, by addition of organic modifiers to the buffer, this problem can be overcome (Heinig et al., 1997; Herrero-Martínez et al., 2000; Piera et al., 1997). Non-ionic surfactants have only infrequently been investigated by CE. Their separation can be

achieved using micellar electrokinetic capillary electrophoresis, i.e., through application of a surface-active agent such as sodium dodecyl sulfonate (SDS) a pseudophase is generated. In this case, the distinct migration behavior of e.g. uncharged NPEO ethoxymers results from partition between the organic and the SDS phase (Heinig et al., 1996). Alternatively, ethoxylated surfactants can be derivatized prior to analysis to impart charge on the neutral compound (Barry et al., 1998).

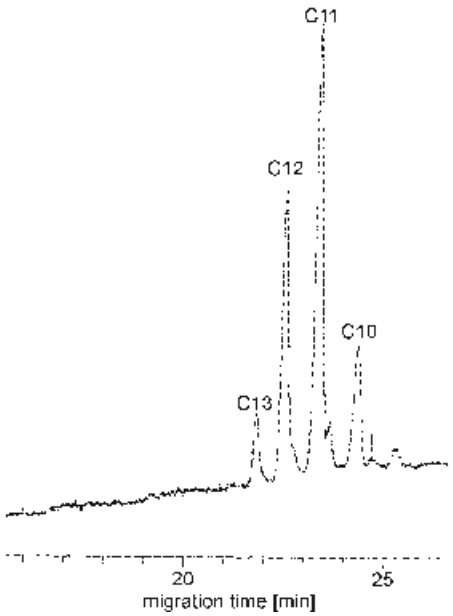


Fig. 2-4: Electropherogram of LAS in extract of WWTP effluent. CE conditions: capillary 40/47 cm, 75 µm i.d., separation voltage: 20 kV, buffer: 50 mM ammonium acetate pH 5.6, 30 % CH_3CN. Adapted from Riu et al. (2000).

Detection systems consist primariyl of optical methods based on UV absorption or indirect UV mode. The application of CE has principally been restricted to the determination of surfactants in raw materials and formulations (Altria et al., 1995; Kelly et al., 1997). The low sensitivity, due to the low injection volumes (1 pL to 100 nL), severely limits extension to environmental trace analysis. Sample preconcentration with high enrichment factors is needed to allow for the detection of the most commonly used surfactants such as LAS (Kanz et al., 1998; Heinig et al., 1998a; Loos et al., 2000; Riu et al., 2000) (**Fig. 2-4**).

2.3 Mass spectrometric detection

In comparison to the above described detection systems, a mass spectrometric (MS) detector, selecting ions in a sample according to their mass-to-charge ratio, offers unequalled selectivity and sensitivity and the opportunity to gather additional information on the molecular weight. The generated mass spectra may provide useful information on the structure of the separated analytes and may therefore assist in structural identification of unknown compounds. These elements are highly valuable in the analysis of traces of surfactants and their degradation products in challenging environmental matrix.

Ionization methods in LC-MS coupling

In combining LC separation with MS detection, three major instrumental difficulties are encountered:

- the flow-rate incompatibility expressed as the need to introduce the mobile phase, flowing at up to 1 mL min^{-1} into the high vacuum of the mass analyzer
- the LC eluent incompatibility due to frequently used non-volatile additives such as buffers or IP reagents
- the difficulty in production of gas phase ions from non-volatile and/or thermally labile compounds.

These problems have largely been solved through the development of a wide variety of powerful LC-MS interfaces (reviewed by Abian, 1999; Niessen, 1998; Niessen and Tinke, 1995). In the following paragraphs the two most amply used types, atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI), are briefly described along with the older techniques particle beam (PB) and thermospray ionization (TSI) whose applications have decreased in recent years. The three atmospheric pressure ionization systems (ESI, APCI, and TSI) combine the liquid inlet system with the ionization source, whereas in PB the ion source is spatially separated from the desolvation chamber.

- *Particle beam.* The liquid stream is nebulized into a spray in a near atmospheric pressure chamber and nearly completely desolvated by various means, leaving a particle beam of solute molecules. This is transferred into the high vacuum ion source where conventional electron impact or chemical ionization takes place, yielding spectra identifiable via standard spectra libraries. Owing to high losses in the interface, the sensitivity of the technique is relatively low.
- *Thermospray ionization.* The liquid is converted into a vapor jet and small droplets are generated with the help of a heated vaporizer tube. The buffer dissolved in the eluent is involved in the ionization process yielding abundant adduct ions produced via statistical charging of individual droplets. Due to the softness of the procedure, no structure-characteristic fragments are formed, which could serve for identification of unknown compounds.
- *Atmospheric pressure chemical ionization.* The column effluent is nebulized into an atmospheric-pressure ion source. Through a corona discharge, electrons initiate the reactant gas-mediated ionization of the analytes. Proton transfers are typical reactions generating $[M+H]^+$ or $[M–H]^-$ ions, although radical ion formation is possible as in high vacuum CI. The ions formed are injected into the high vacuum of the mass spectrometer. APCI typically accepts flow rates of up to 2 mL min^{-1}.
- *Electrospray ionization* [1]. The LC eluent enters the ionization chamber at atmospheric pressure through a narrow capillary tube, the end of which is maintained at a high positive or negative potential (**Fig. 2-5**). The strong electric field creates a mist of small highly charged droplets. Through vaporization of the solvents, the droplets become rapidly smaller until they reach a critical surface charge density. At this point ions begin to desorb from the surface of the droplets into the gas phase and are subsequently extracted into the mass spectrometric analyzer.

[1] Precautions to be taken in quantitative analyis on ESI-MS instruments are discussed in section 3.8.

As with TSI, APCI and ESI are soft ionization techniques usually generating pseudo-molecular ions, adduct ions, and clusters, thereby yielding molecular weight information. The ion source parameters of the two latter modes can, however, be adjusted in such a manner that some in-source fragmentation (also termed as in-source collision-induced dissociation (CID)) is induced (for a discussion of CID relevant to MS/MS see next section). By increasing the voltage applied to the sample cone, which extracts the ions from the atmospheric pressure region and transmits them into the first vacuum region, the ions are accelerated more quickly through the low vacuum region resulting in collisions with abundant solvent or gas molecules. By these means, structurally informative fragmentation is provided. Even if the extent and type of fragmentation are different from and less extensive than electron impact, as is usually the case, in-source CID can be a very valuable tool in identifying unknown compounds.

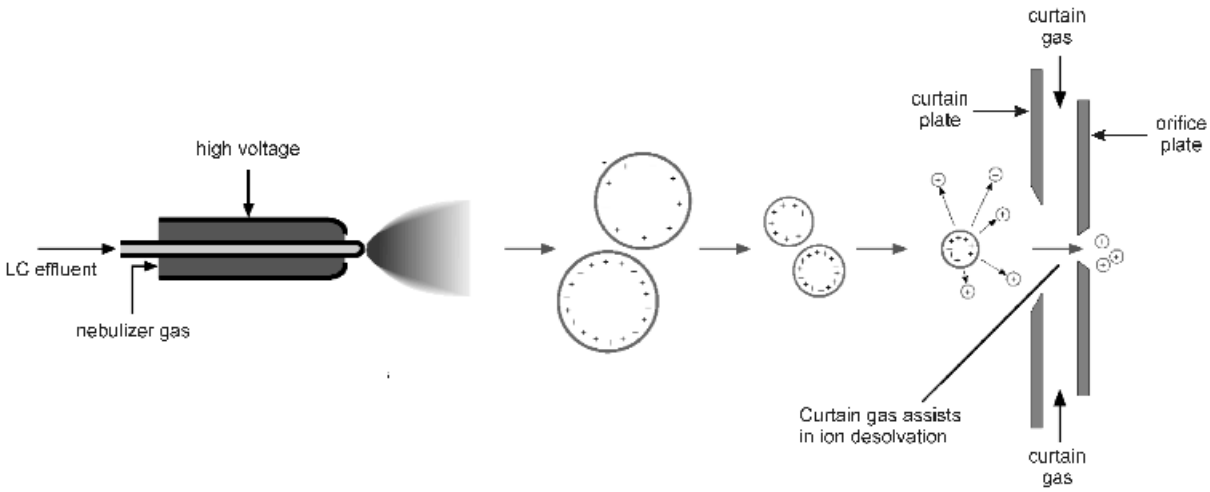


Fig. 2-5: Process of ion generation in electrospray ionization interface. Adapted from Perkin-Elmer Sciex instruction manual of LC-MS API 150.

The suitability of each interface for a particular analysis are determined by the polarity of the analyte and its molecular mass. From the scheme presented in **Fig. 2-6** it can be seen that APCI is advantageous for less to moderately polar compounds, while the most suitable choice for charged analytes, or those for which solution-phase charge can be readily obtained is ESI. A unique feature of ESI is that molecules with high molecular masses appear as multiply charged ions allowing the detection of large biomolecules even on instruments with a rather limited *m/z* range.

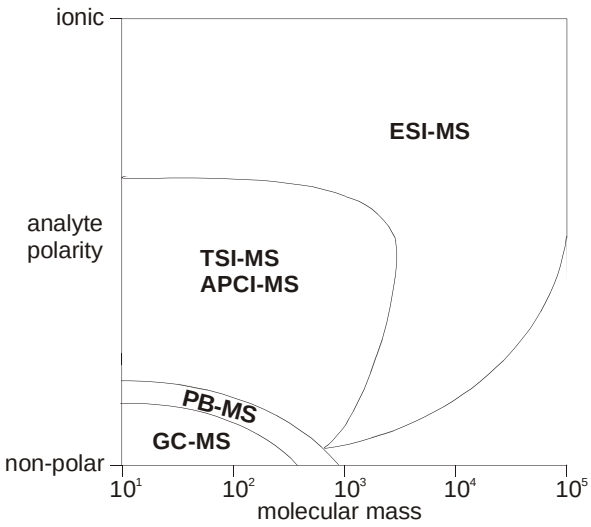


Fig. 2-6: Application ranges of different LC-MS interfaces (adapted from Niessen and Tinke, 1995).

With respect to the analysis of surfactants in environmental samples by LC-MS procedures, TSI-MS approaches were predominantly used until the mid-1990s when robust instruments fitted with soft atmospheric pressure ionization sources (APCI and ESI) became available at reasonable costs.

This trend is reflected by the method collection in **Table 2-1** giving an overview on analytical determinations of surfactants in aqueous matrices. Most methods have focused on LAS, NPEO and AE, while surfactants with lower consumption rates such as CDEA and esterquats or the speciality surfactants PFAS and PFAC, used in fire fighting foams, have received less attention. After preconcentration performed mainly on non-polar alkyl-bonded silica gel or PS-DVB material, the more recent studies were based on MS instruments equipped with ESI and APCI interfaces.

Table 2-1: Determination of surfactants in aqueous environmental matrices applying LC-MS techniques.

Compound	Matrix	Isolation	Ionization/Detection	Reference
LAS	wastewater, surface water	GCB	LC-ESI-MS	di Corcia et al. (1999b)
LAS	coastal water	C18+SAX	LC-ESI-MS	González-Mazo et al. (1997)
LAS	coastal water	C18+SAX	LC-ESI-MS	Riu et al. (1999)
LAS, NPEO	wastewater	GCB	LC-ESI-MS	di Corcia et al. (1994)
LAS, NPEO	surface water	C18 (+SAX)	LC-APCI-MS	Scullion et al. (1996)
LAS, NPEO, AE	wastewater	C18+PS-DVB	LC-APCI/ESI-MS	Castillo et al. (1999)
LAS, NPEO, AE	wastewater	C18+PS-DVB	LC-APCI/ESI-MS	Castillo and Barceló (1999)
LAS, NPEO, AE	wastewater	C18	LC-APCI-MS	Petrovic and Barceló (2000a)
LAS, NPEO, AE	wastewater	C18	LC [a]-APCI/ESI-MS(MS)	Li et al. (2000)
LAS, AES, AE, CDEA	wastewater	C18	TSP-MS-MS	Schröder (1992)
NPEO	surface water	PS-DVB	LC-ESI-MS	Maruyama et al. (2000)
NPEO	surface water	-	LC-ESI-MS	Takino et al. (2000)
NPEO	estuarine water	C18	LC-ESI-MS	Ferguson et al. (2000)
NPEO	estuarine water	C18	LC-ESI-MS	Ferguson et al. (2001)
NPEO	drinking water	LLE	LC-PB-MS	Clark et al. (1992)
NPEO, AE	wastewater	C18	LC [a]-APCI/ESI-MS(MS)	Schröder and Fytianos (1999)
NPEO, AE	wastewater	C18+PS-DVB	LC-APCI-MS	Castillo et al. (2000)
NPEO, AE	wastewater	C18+PS-DVB	LC-APCI-MS	Castillo et al. (2001)
NPEO, AE	wastewater	C18+PS-DVB	LC-APCI-MS	Farré et al. (2001a)
NPEO, AE	wastewater, surface water	GCB	LC-ESI-MS	Creszenzi et al. (1995)
NPEO, AE	wastewater, drinking water	C18	LC [a]-TSP-MS(MS)	Schröder (1993a)
AE	wastewater	C18	LC-TSP-MS(MS)	Schröder (1989)
AE	wastewater	C8	LC-ESI-MS	Evans et al. (1997)
AE	wastewater	C2	LC-ESI-MS	Dunphy et al. (2001)
AE	wastewater, surface water	C8	LC-TSP-MS	Evans et al. (1994)
AE	surface water	C18; PS-DVB	LC [a]-TSP-MS(MS)	Schröder (1995)
AE, AES	wastewater, surface water	C2	LC-ESI-MS	Popenoe et al. (1994)
AE, AES	wastewater	C2	LC-ESI-MS	Matthijs et al. (1999)
PFAS, PFAC	surface water	C18	LC-ESI-MS/MS	Moody et al. (2001)
CDEA	wastewater	C18	LC [a]-TSP-MS(MS)	Schröder (1993b)
Esterquats [b]	wastewater	C2+clean-up	LC-ESI-MS	Waters et al. (2000)
Esterquats [b]	wastewater	IP-LLE	LC-ESI-MS	Radke et al. (1999)

[a] sample introduction into the ion source was done in part by flow-injection analysis bypassing the analytical column; [b] esterquats, cationics.

Mass analyzers and detection modes

In addition to the diversity of ionization techniques available, mass spectrometers offer a selection of mass analyzer configurations. Of note are single and triple quadrupole (MS/MS) instruments, ion trap analyzers, time-of-flight (ToF) analyzers, sector field analyzers, and Fourier transform-ion cyclotron resonance instruments. Mass resolution generally increases for instruments in the listed order. Hybrid variants utilizing various composites of mass analyzers are likewise available and have become commonplace (e. g. quadrupole-ToF).

The instruments may be operated in different detection modes depending upon the objectives of the work. **Table 2-2** gives an overview of the distinct modes of quadrupole mass spectrometers in terms of application, content of information and sensitivity.

Table 2-2: Detection modes of single and triple quadrupole mass spectrometers.

Mode	Detection and application	Information	Sensitivity
Scan	Full scan spectra; identification; structure elucidation	high	low
Selected ion monitoring (SIM)	Detection of selected molecular ions or fragments; quantitation	low	high
Multiple reaction monitoring (MRM) [a]	Detection of selected mass transfers; quantitation	low	high
Product ion scan (PIS) [a]	CID spectra of selected ions	high	medium
Precursor ion scan [a]	Detection of ions with identical fragments	high	medium
Neutral loss scan [a]	Scan with constant loss of neutral group; identification of functional groups	high	medium

[a] modes only available on triple-quad instruments

The potential for generating CID spectra by triple quadrupole instruments has played an important role in the identification of surfactants in complex mixtures analyzed by TSI instruments. Since the softness of this ionization process does not allow inducing in-source fragmentation – this feature is offered solely by ESI and APCI interfaces (see above) – structure-related information could only be gathered by fragmentation of selected precursor ions in the collision cell, which yielded daughter ion spectra (Schröder, 1992, 1993a, 1993b, 1995). The high selectivity of this procedure was exploited for screening purposes of surfactants occurring in wastewaters by performing flow-injection analysis (FIA) of sample extracts, i.e. their direct infusion into the ion source. Time-consuming chromatographic separations could thereby be circumvented. However, the application of FIA-TSI-MS/MS – and FIA-MS/MS methods in general – has its limitations since ambigous results may be obtained when CID spectra of two or more isobaric precursor ions initially present in the extract are recorded. This may lead to misinterpretations in assigning chemical structures and precludes any quantitative assessment.

2.3.1 Matrix-based mass spectrometric methods

In contrast to on-line coupling of LC separation to ESI-MS detection, where the analytes are desorbed from the liquid stream entering the ion source continuously, the desorption of ions from matrix-embedded

samples can be achieved by the impact of particles, as with fast atom bombardment (FAB)-MS, or photons as with matrix-assisted laser desorption/ionization (MALDI)-MS.

In FAB-MS a solid sample or an extract are dissolved in a suitable matrix and bombarded with an atom beam generating molecular or pseudo-molecular ions from the sample. This technique was used for the fast characterization of various surfactants in cosmetic products (Facino et al., 1997) and environmental samples (Simms et al., 1988; Ventura et al., 1988 and 1989).

In MALDI-MS the sample is embedded in a matrix, which enables a very soft transfer of the analytes into the gas phase. Ionization is usually achieved by protonation or cation attachment after irradiation by a laser. The most common MS coupled to MALDI ionization sources is the ToF mass analyzer, which measures the masses of individual ionized molecules as a function of their time-of-flight along a tube of known length. Ions are accelerated by a constant electric field, directed into a flight tube and separated corresponding to their time-of-flight through the tube.

This analytical technique was developed by Karas and Hillenkamp (1988) and has found widespread use in the field of biochemistry (Bakhtiar and Nelson, 2000; Gross and Strupat, 1998) and polymer sciences (Nielen, 1999) as it allows undecomposed transfer of macromolecules into the gas phase. It is ideally suited for high molecular weight compounds (beyond 1 MDa) because of the simplicity of the mass spectra showing mainly singly-charged adduct ions with hardly any fragmentation, and because of the theoretically unlimited mass range of the ToF mass analyzer.

MALDI-ToF-MS has likewise been applied to the rapid mass analyses of surfactants (Ayorinde et al., 1999; Bartsch et al., 1998; Morrow and Ayorinde, 2001; Thomson et al., 1995), but a general drawback of MALDI-ToF-MS is its poor quantitative performance. This arises from inhomogeneities in the sample dispersion within the matrix formed during the crystallization process.

2.3.2 Choice of liquid chromatography-electrospray ionization-mass spectrometry

Of the various techniques existing for surfactant analysis the choice of LC-MS as a cornerstone in the analytical methodologies was made taking into account the following requirements:

- a flexible and efficient chromatographic separation was needed offering the possibility for separating complex mixtures of compounds with a range of polarities. This should have been accomplished without tedious derivatization steps, and should permit direct analysis of aqueous samples.
- high sensitivity was essential for the detection of trace amounts of analytes in combination with high selectivity required for the examination of challenging matrix.
- the detection system should have assisted in the structural elucidation of unknown organic compounds. Moreover, accurate quantitative determinations should be feasible.

In view of the available state-of-the-art ionization interfaces of commercial LC-MS instruments, ESI was preferred over APCI for the present work since it was particularly suitable for mid- to highly polar analytes. The former technique was assumed to be more efficacious in the ionization of highly polar metabolites expected to be formed during surfactant biodegradation. The restriction of LC-ESI-MS to low mass analytes could be compensated by the application of MALDI-ToF-MS for polymeric compounds.

3 EXPERIMENTAL SECTION

3.1 Chemicals

Reference compounds

- Linear alkylbenzene sulfonate, technical blend (13.9 % C10, 37.4% C11, 35.4 % C12, and 23.1 % C13) (courtesy from Petresa S. A., Madrid, Spain)
- Linear alkylbenzene sulfonate, pure C12 (0.01 % C12-LAS-6, 0.1 % C12-LAS-5, 0.6 % C12-LAS-4, 12.1 % C12-LAS-3, 86.8 % C12-LAS-2, 0.1 % <C12-LAS, 0.4 % >C12-LAS; total content of non-linear LAS: 2.9 %) (courtesy from Petresa S. A., Madrid, Spain)
- Sulfophenyl carboxylate, C5-SPC-5 (synthesized by F. Ventura, AGBAR Barcelona, Spain); C11-SPC-2 (synthesized by E. González-Mazo, Cádiz, Spain)
- Alkyl glucamide, technical blend (75 % C12, 25 % C14) (courtesy from H. F. Schröder, Aachen, Germany)
- Alkyl glucamide, C10 (Sigma, Deisenhofen, Germany)
- Alkyl polyglucosides, technical blend Glucopon 210 (courtesy from Henkel AG, Düsseldorf, Germany)
- Alkyl β-monoglucosides, C8, C10 and C12 (Sigma)
- Cocamidopropyl betaine (Witco Co., Steinau a. d. Strasse, Germany)
- Polyvinyl pyrrolidone 2.5 kDa (Polysciences, Eppelheim, Germany), polyvinyl pyrrolidone K17 (7–11 kDa) (courtesy from BASF AG, Ludwigshafen, Germany)

Reagents and solvents

- Acetone, acetonitrile, n-hexane, methanol, all suprasolv (Merck, Darmstadt, Germany)
- Acetic acid (100 %), ammonium chloride, calcium chloride, dipotassium hydrogen phosphate, iron (III) chloride, magnesium sulfate, potassium dihydrogen phosphate, sodium chloride, sodium carbonate, sodium hydrogencarbonate, sodium dihydrogen phosphate, sulfuric acid (Suprapur), tetraethyl ammonium acetate (TEAA) and triethyl amine (TEA) (Merck)
- 3β-hydroxy acrylic acid, sodium trifluoroacetate (Alrich, Steinheim, Germany)
- MilliQ water, MilliQ-Plus 185 (Millipore, Eschborn, Germany)

Due to the ubiquitous occurrence of surfactant in detergents, glassware and sampling apparatus required special treatment prior to use. No detergents were allowed to contact glassware. All glassware was solvent rinsed (acetone) and baked at 250 °C for 8 hours prior to use.

3.2 Instruments

HPLC

- Perkin-Elmer autosampler Serie 200 (Norwalk, USA)
- Perkin-Elmer binary pump Serie 200 (Norwalk, USA)
- Suppressor module 753, micro packed-bed suppressor (Metrohm, Filderstadt, Germany)

MS(-MS)

- Perkin-Elmer Sciex API 150 MCA with turbo ionspray interface (Norwalk, USA)
- Perkin-Elmer Sciex API 365 with turbo ionspray interface (Norwalk, USA)
- Whatman nitrogen generator Typ 75-72 (Whatman GmbH, Göttingen, Germany)
- Syringe pump series, Model 11 (Harvard Apparatus, Holliston, MA, USA)

MALDI-ToF-MS

- Bruker Reflex II equipped with N_2 laser (337 nm) (Bruker, Bremen, Germany)

HPLC columns

- Hypersil ODS, 250x2.1 mm (5 µm) RP C18 (MZ-Analysentechnik, Mainz, Germany).
- Superspher 60 RP-select B, 125x2.1 mm (4 µm) RP C8 (Merck)
- Dual 1 IC (poly(hydroxyethyl-methacrylate) with quaternary ammonium groups) 150x3.0 mm (Metrohm)

Material for sample preparation

- Glass fibre filter, 0.45 µm (Schleicher & Schüll, Dassel, Germany)
- pH-meter with Ag/AgCl-glass electrode (WTW, Weinheim, Germany)
- Glass cartridges 3 mL (Mallinckrodt Baker, Griesheim, Germany)
- PTFE frits (ICT, Bad Homburg, Germany)
- Solid-phase extraction material: LiChrolut RP C18, 40-63 µm; LiChrolut EN, 40-120 µm (Merck)
- Syringe filter, Spartan 13/20, 0.45 µm (Schleicher & Schüll)
- Glass vials, 1 mL, with screw caps (silicon/PTFE seal) (A-Z Analytik-Zubehör, Langen, Germany)
- Kryostat F34 (Julabo, Seelbach, Germany)
- Freeze-drying LYOVAC GT 2-E (Amsco/Finn-Aqua, Hürth, Germany)
- Centrifuge EBA 3 S (Hettich, Tuttlingen, Germany)

Bioassays

- ToxAlert 100® bioluminescence test device using liquid-dried bacterium *Vibrio fischeri* (NRRL B-11177) (Merck)
- Cellsense® biosensor with *Pseudomonas putida* (NCIMB 8277) (Terra Nova Systems, Cambridge, United Kingdom)

3.3 Biodegradation experiments

3.3.1 Literature methods

For the study of the aerobic biodegradability of surfactants in aqueous media, beside the employment of standardized OECD methods for the testing of organic compounds (OECD, 1993), a large variety of assays have been developed differing in instrumental and technical set-up, measured parameters, type and origin of test medium and inoculum used, test duration, and substrate concentration (Swisher, 1987). As for OECD methods their design allows determination of surfactant degradability by measuring DOC removal, CO_2 formation and chemical (COD) or biological oxygen demand (BOD), but they were also used to perform investigations on the metabolic pathway of numerous surfactants (Cavalli et al., 1996; di Corcia et al., 1998a, 1998b, and 1999a; Marcomini et al., 2000a and 2000b; Moreno et al., 1998; Remde and Debus, 1996; Staples et al., 1999).

A rather simple set-up, termed as river die-away test, was introduced by Swisher (1987) to assess the primary biodegradability of surfactants in rivers and was later employed in several studies working with river, estuarine and sea water (Manzano et al, 1999; Perales et al., 1999a; Potter et al., 1999; Sales et al., 1987; Terzic et al., 1992). More sophisticated test systems for simulating the elimination in surface waters were developed by Schöberl et al. (1998) and Boeije et al. (2000) based on a staircase model. The use of trickling filters in turn permitted determination of the degree of bioelimination and mineralization (Baumann et al., 1999; Kölbener et al., 1995b; Mampel et al., 1998). In addition, degradation intermediates could be easily recovered at amounts required for further analytical purposes. Gerike and Jasiak (1985 and 1986) modified the coupled-units test (OECD 303A), normally applied to the simulation of aerobic wastewater treatment, to accumulate recalcitrant metabolites in the test liquor. The reproducibility of this concept was later demonstrated by Guhl and Ranke (1993).

3.3.2 Fixed-bed bioreactor

In this work a biologically active fixed-bed bioreactor (FBBR) operated under aerobic conditions was employed for investigating both the primary degradation of the targeted analytes and their breakdown pathway. The test device, which represented a modification of so-called testfilters widely utilized in German chemical industries and waterworks to study poorly degradable compounds in wastewaters and surface water, respectively, was used benefiting from its simple experimental set-up, ease of operation and the possibility to provide aqueous samples apt for direct injection into the LC-MS. Moreover, wide experience has been gained with the FBBR as model system in terms of assessing the biodegradability of structurally very distinct organic compounds, identifying degradative products and following their formation and decline. Among the tested substances were aliphatic amines, aromatic sulfonates, organic

acids, and phosphates (Karrenbrock et al., 1999; Knepper et al., 1999a), surfactants (Jonkers et al., 2001; Knepper and Kruse, 2000), and a corrosion inhibitor (Knepper et al., 1999b). In these studies the test concentrations of the spiked analytes were chosen depending on the objectives of the studies and the analytical methods applied: low concentrations at environmentally relevant levels (10 µg L^{-1}) were used for examining the primary biodegradability, whereas higher amounts of the parent compounds (10 mg L^{-1}) were selected when aiming at the identification of unknown metabolites, occurring in most instances only at traces in the test liquor, preferably without applying sample enrichment procedures.

Since metabolism studies were a major focus in the present inquiries, the FBBR was amended with the test compounds at concentrations in the mg L^{-1} range. This enabled the detection and mass spectrometric identification of degradation intermediates without requiring high enrichment factors. In order to avoid discrimination of potential breakdown products – presumably exhibiting higher polarity than the spiked surfactant – SPE, frequently applied in previous FBBR studies, was not exploited. Instead in some experiments lyophilization was used.

Set-up of fixed-bed bioreactor

Unfiltered river Rhine water sample (10 L) was continuously pumped in a closed loop from the storage tank upwards into a glass column (Ø: 5 cm, h: 23 cm, V: 310 mL) (**Fig. 3-1**). The pump (Concept b, ProMinent, Heidelberg, Germany) regulated the flow rate to 17 mL min^{-1} and a membrane pump aerated the water in the storage tank with air via a glass frit. The glass column was packed with carrier material up to a height of 18 cm. Teflon tubings (Ø: 6 mm) connected the individual components of the FBBR system. The apparatus was kept in the dark to prevent any photocatalytic degradation and the growth of algae. All experiments took place at 20 ± 3 °C.

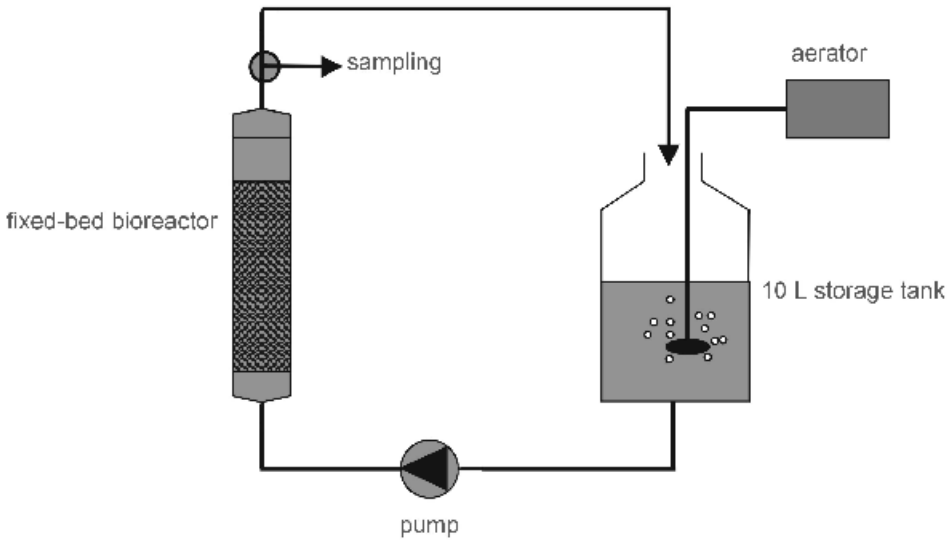


Fig. 3-1: Scheme of fixed-bed bioreactor.

The carrier material was Siran-Carrier (No. 023/02/300, Schott Engineering GmbH, Mainz, Germany), native, porous sintered glass beads used for the immobilization of microorganisms (Ø: 2-3 mm, surface: 80 $m^2 L^{-1}$ or 0.2 $m^2 g^{-1}$, pore Ø: 60-300 µm, ρ: 450 g L^{-1}).

In order to remove the biofilm formed in previous studies a cleaning procedure was carried out: all glass pieces were rinsed with 5 % hydrochloric acid and distilled water. After that, 5 % hydrogen

peroxide (Perhydrol®) (Merck) was pumped over the FBBR for 12 hours. Then it was rinsed twice with distilled water. The Siran-Carrier was cleaned by the same procedure and was finally dried at 110 °C for 12 hours.

Sampling and sample preservation

In all experiments, the samples were stabilized immediately after sampling by addition of 5 % formaldehyde and stored at 4 °C until analysis or further treatment. River Rhine water (km 508) from the day of spiking was analyzed as control to check for background concentration.

3.3.3 Linear alkylbenzene sulfonates

Biodegradation of C12-LAS

10 L river Rhine water were amended with 100 mg L^{-1} C12-LAS (290 µmol L^{-1}) (**Fig. 4-1**, p. 43) without additional nutrient supply. Ten mL-samples were taken daily until day 69. On day 42 a nutrient solution prepared according to OECD testing method 301 (**Table 3-1**) was added to restore the microbial activity. All samples were filtered through a glass fiber filter (0.45 µm, prewashed with methanol and MilliQ-water). Sample aliquots of 1.5 mL were diluted by adding 100 µL of a solution consisting of 500 mM acetic acid (HOAc) and triethyl amine (TEA) each. These solutions were directly injected into the LC-ESI-MS for the determination of degradation intermediates, while for LAS analysis the samples were simply diluted and analyzed.

Table 3-1: Nutrient composition for test concentration of 100 mg DOC L^{-1}, in mg.

KH_2PO_4	K_2HPO_4	Na_2HPO_4	NH_4Cl	$MgSO_4$	$CaCl_2$	$FeCl_3$
85	218	266	25	22.5	27.5	0.25

Biodegradation of C12-LAS under optimized conditions

The same experiment as before was performed with the exception of adding the nutrient solution at the start to assure optimum growing conditions for the microorganisms during the entire assay. FBBR samples were taken daily until day 29.

Adsorption experiment of C12-LAS under optimized conditions

In addition, an analogous trial was run on a clean FBBR adding 5 % formaldehyde to 10 L river Rhine water before fortifying with 1,000 mg C12-LAS. By this, possible adsorption losses of C12-LAS should be assessed. Ten mL-samples were withdrawn daily from the test medium. After filtration and dilution, analyses were performed by LC-ESI-MS.

Adaptation effects on the biodegradation of commercial LAS mixture

A rinsed FBBR was amended with a commercial mixture of LAS at 10 mg L^{-1}. Sampling was performed over 30 days. Hereafter, the water was removed, the FBBR flushed twice with tap water, filled again with

10 L river Rhine water, and supplemented with LAS at the same concentration. Stabilized water samples were filtered and subjected to LC-ESI-MS analyses.

3.3.4 Alkyl glucamides

The degradation experiment was carried out for one week with C10-AG (**Fig. 4-26**, p. 72) as model compound. 10 L of fresh river Rhine water were spiked at a concentration of 1 mg L^{-1}. An aliquot of the 100 mL-samples was analyzed directly to pursue the primary degradation, while 30 mL thereof were concentrated by lyophilization. The residue was re-constituted with 1.5 mL water, containing 3 mM TEAA and filtered prior to analysis anticipating the detection of polar metabolites.

3.3.5 Alkyl polyglucosides

The three β-monoglucosides C8, C10, and C12 (**Fig. 4-36**, p. 81) were spiked to river Rhine water at 1 mg L^{-1} each. Samples (50 mL) were taken in short intervals during a period of 23 hours, stabilized, and filtered prior to injection on the LC-ESI-MS.

3.3.6 Cocamidopropyl betaines

A commercial mixture of the amphoteric surfactants CAPB (**Fig. 4-43**, p. 88) was spiked at 10 mg L^{-1} to the test water. Stabilized samples were directly analyzed for parent compound after filtration and dilution, or concentrated by a factor of 30 by freeze-drying in order to enable detection of low abundance metabolites. For the latter the residue was taken up in 2 mL of water-CH_3CN (95:5, v/v), 5 mM TEA and HOAc and subsequently filtrated before injection.

3.3.7 Poly(vinylpyrrolidone)

Biodegradation experiment

The river Rhine water was supplemented with poly(vinylpyrrolidone) (PVP) (**Fig. 4-49**, p. 96) with a mean molecular weight of 2,5 kDa. Over a period of 30 days, 20 mL-sample were taken every two days except the first day when sampling was done at shorter intervals. For MALDI-ToF-MS measurements, 10 mL of each sample were lyophilized, re-constituted in 1 mL water-CH_3CN (95:5, v/v) and filtered.

Adsorption experiment

Anaerobically digested and dewatered sludge from the municipal WWTP of Wiesbaden was lyophilized and the recovered dry sludge ground with the aid of a mortar. Of this 250 mg were suspended in 5 mL pristine ground water in a 10 mL-centrifuge tube. An aqueous solution of PVP with a molecular weight distribution of 5-8 kDa, obtained from PVP K17 fractionated by gel permeation chromatography (GPC), was used for spiking yielding a final concentration of 100 mg L^{-1}. Two control samples, one containing no PVP spike, the other no sludge, were likewise prepared. Hereafter, the tubes were shaken horizontally during two days. One hundred µL-samples from the supernatant were withdrawn after centrifugation for 15 min at 3000 min^{-1} and submitted to the MALDI sample preparation.

3.4 Solid-phase extraction

For the analysis of the surfactants and degradative products in environmental samples, SPE protocols were developed for the preconcentration of trace amounts of targeted analytes.

General procedure

The SPE cartridges were prepared as follows: Re-usable glass cartridges were filled with the SPE material (see below) and conditioned with 2x2 mL hexane, 2x2 mL acetone, 2x2 mL methanol, and 6x2 mL water. Depending on the origin of the sample, different volumes of 0.45 µm-filtered water were percolated through the cartridge applying a vacuum of 200 mbar generated by a membrane pump. After drying of the adsorbents with an N_2 stream for 1 hour, the enriched analytes were eluted with 3x2 mL methanol. Next, the extract was taken to dryness under a gentle stream of N_2 and reconstituted with 1 mL of the HPLC eluent A of the individual separation (see 3.6).

The volume was chosen depending on the type of examined matrix and hence the expected analyte level: 100-200 mL of wastewater, 500-1000 mL of surface water or 1000 mL of water from waterworks and of drinking water corresponding to enrichment factors of between 100 and 1000.

Recovery studies

Based on established techniques for the simultaneous enrichment of LAS and SPC from water samples, the suitability of a single extraction cartridge filled with RP-C18 was evaluated. In earlier studies, samples were acidified to pH 3 or 1.5 (González-Mazo et al., 1997; Riu et al., 1999 and León et al., 2000; Sarrazin et al., 1997, respectively). Although the adjustment to 1.5 had provided good retention of all SPC homologues including the very polar short-chain SPC (<C6), the higher value of 3.0 yielding poorer recoveries of these species was chosen in this work. This was done taking into consideration that humic acids, occurring in natural freshwater settings, were co-extracted to increasing extent at lowering the pH of the sample (Steen et al., 1999). Subsequent elution of the cartridges with methanol did not only redissolve the compounds of interest but also released the trapped humic substances. Their presence in the extract used for LC analysis without further clean-up should be kept at a minimum level, since the negligible volatility of these interferences resulted in precipitation in the ion source and on the skimmer of the MS thereby gradually reducing the instrumental sensitivity (Steen et al., 1999). Moreover, they might affect the ionization process [1]. Exclusion of those SPC intermediates with chain length <C6 from the protocol was estimated to not substantially affect the quantitative determination since the degradation experiment performed with the commercial LAS mixture showed that the alkyl homologues C7 to C9 were the most prominent metabolites (section 4.1.3.3).

Recovery studies of LAS were performed by spiking secondary effluent of a municipal WWTP, adjusted to pH 3.0, with 100 µg L^{-1} of a commercial mixture of the anionic surfactant. Recoveries of the four alkyl homologues are listed in **Table 3-2**.

With regard to the degradative products of LAS, the spiking experiments were carried out using a mixture containing all investigated alkyl homologues with a representative distribution. This was prepared from the liquor of the FBBR amended with an LAS mixture (see section 4.1.3.3). After finishing the degradation experiment, a volume of the test medium was withdrawn, concentrated by lyophilization,

[1] This issue is discussed in section 3.8.

and reconstituted with methanol. This solution was spiked into groundwater (N=3) corresponding to an SPC concentration of approximately 0.1 µg L^{-1}. SPC were quantified via a calibration made from the methanolic spiking solution (**Table 3-2**).

Table 3-2: Recoveries of SPC and LAS in ground waters and secondary effluents, respectively (in triplicate).

SPC							LAS			
C6	C7	C8	C9	C10	C11	C12	C10	C11	C12	C13
37±6	54±2	50±3	49±3	60±4	54±8	68±7	85±3	85±5	84±4	82±4

The two non-ionic surfactants AG and APG, for which no enrichment techniques for environmental samples were found in the literature, were in a first attempt extracted from spiked WWTP effluent (5 µg L^{-1}) using RP-C18 cartridges. It was found that this material was unsuitable for quantitative enrichment (recovery <30 %). Satisfactory results were obtained using PS-DVB material (Lichrolut EN) (**Table 3-3**).

Table 3-3: Recoveries of AG and APG in secondary effluents (in triplicate).

AG		APG		
C12	C14	C8	C10	C12
89±3	91±3	79±7	82±4	82±5

For the extraction of CAPB a series of attempts were mounted to extract the amphoteric surfactant from a fortified secondary effluent (pH 3 or 7) on cartridges containing RP-C18, Lichrolut EN or anion exchange material or combinations thereof. In all instances, insufficient extraction efficiencies with high standard deviations were obtained. From these findings, it was concluded that CAPB was lost during sample preparation through adsorption onto surfaces of the extraction bottles. Seemingly, the partial cationic character of the charged head group of CAPB led to strong adsorptive forces with the silicate-based glassware. Similar effects were observed at the degradation experiments where in the test device losses between 5 and 70 % were noticed (see **Fig. 4-48**, p. 94). To circumvent the encountered problems, further efforts were made by adding methanol as organic modifier to the sample (5 and 10 %) as well as using polyethylene containers instead of glass vessels. None of these experiments gave acceptable recoveries with satisfactory standard deviations. Due to the lack of a reliable extraction procedure, the occurrence of CAPB in wastewater discharges was not investigated.

3.5 Sampling and sample preparation of environmental samples

Aqueous environmental samples were collected in amber glass bottles containing 3 % formaldehyde for preservation. After filtration, the samples were preconcentrated on SPE cartridges within 24 hours in

order to avoid any degradation and loss of sample integrity. Extracted samples from remote areas were stored at –20 °C prior to air transportation and were finally extracted in our laboratory [1].

Sampling at wastewater treatment plants

Influent and effluent waters were collected in November 1999 in amber glass bottles as 24-h composite samples from two WWTP located in the Catalonian region in Spain (WWTP-2 and WWTP-3). In a third facility (WWTP-1) influent and effluent waters were collected at different times of day (morning, afternoon, night) in order to follow different inputs of domestic and industrial wastewater. Furthermore, a 24-h composite sample of influent and effluent from a German plant (WWTP-4) was taken in February 2001. Technical characteristics of the WWTP are given in
Table 5-1 (p. 103).

Sampling of surface waters (Brazil and Philippines)

At several locations along the Rio Macacu (Rio de Janeiro, Brazil; **Fig. 5-2**, p. 109) grab samples were collected twice within one week during austral winter in August 2000 (dry season). Furthermore, two random water samples from Niterói harbor and the Baía de Guanabara close to Icaraí Beach (Niterói) were taken. In the Philippines near Laguna de Bay (Luzon; **Fig. 5-6**, p. 115) two batches of surface water samples were taken randomly at selected sampling sites in December 1999 and March 2000.

Sampling of tap water and water from waterworks

For monitoring purposes, tap water samples were collected in Niterói (Rio de Janeiro) during the sampling on Rio Macacu, while a more extended campaign was carried out in October 2000 comprising several sites in Niterói and São Gonçalo as well as one location in Rio de Janeiro.

From the waterworks at the Llobregat river (Barcelona, Spain; km 154) and the one at the river Rhine (Wiesbaden, Germany; km 508) water samples were taken on two and three occasions in December 2000/January 2001 and June/July 2000, respectively, from the different stations of the drinking water production plants including raw water from the river.

3.6 High performance liquid chromatography

Solvents were used as supplied without further treatment. Inlet filters in the eluent reservoirs as well as a particle filter placed between the injection valve and the analytical column were employed to protect the column. Conditions of the HPLC separation are given in **Table 3-4**, **Table 3-5**, **Table 3-6**, **Table 3-7**, and **Table 3-8**. Molar concentrations of buffer additives refer to the total volume of the eluents, while pH adjustment was done of the aqueous phase prior to mixing with the organic solvent.

[1] Experiments on the stability of surfactant-loaded SPE cartridges during storage showed that an exposure to room temperature for 7 days or to –20 °C for three month did not lead to notable loss of enriched analytes (Petrovic and Barceló, 2000a).

Table 3-4: HPLC conditions for the separation of LAS, ABS and SPC.

Column	Hypersil MOS, 250x2.1 mm, 5 µm						
Eluent A	water-CH_3CN (95:5, v/v), 5 mM TEA and HOAc						
Eluent B	CH_3CN-water (80:20, v/v), 5 mM TEA and HOAc						
Gradient	t [min]	0	3	18	23	24	44
	% A	100	100	0	0	100	100
Flow	200 µL min^{-1}						
Injection volume	50 µL (10 µL for FBBR analysis on SPC 100 µL for FBBR analysis on SPC-2H)						

Table 3-5: HPLC conditions for the separation of AG.

Column	Superspher 60 RP-select B, 125x2.1 mm, 4 µm						
Eluent A_1	water-CH_3CN (95:5, v/v), pH 7.9 (NH_3)						
Eluent B_1	CH_3CN-water (80:20, v/v), pH 7.9 (NH_3)						
Gradient	t [min]	0	2	17	18	21	22	42
	% A	100	75	50	20	20	100	100
Eluent A_2 [a]	water, 3 mM TEAA						
Eluent B_2 [a]	CH_3CN						
Gradient	t [min]	0	3	23	25	27	47
	% A	95	95	20	20	95	95
Flow	200 µL min^{-1}						
Injection volume	50 µL						

[a] for the analysis of potential metabolites

Table 3-6: Conditions for the separation of C4-glucamide acid on anion exchange column using cation suppressor.

Column	Dual 1 IC, 150x3.0 mm					
Eluent A_2	water, 1.3 mM Na_2CO_3 + 2.0 mM $NaHCO_3$					
Eluent B_2	water, 13 mM Na_2CO_3 + 20 mM $NaHCO_3$					
Gradient	t [min]	0	3	15	20	21	41
	% A	100	100	0	0	100	100
Flow	500 µL min^{-1}, split ratio 1:1						
Injection volume	50 µL						
Regeneration of cation suppressor [a]	water, 50 mM H_2SO_4						
Conditioning of cation suppressor [a]	water						

[a] the suppressor was switched after each run

Table 3-7: HPLC conditions for the separation of APG.

Column	Superspher 60 RP-select B, 125x2.1 mm, 4 µm								
Eluent A	water-CH_3CN (95:5, v/v), pH 7.9 (NH_3)								
Eluent B	CH_3CN-water (80:20, v/v), pH 7.9 (NH_3)								
Gradient	t [min]	0	2	4	19	20	23	24	44
	% A	100	100	75	50	20	20	100	100
Flow	200 µL min^{-1}								
Injection volume	50 µL								

Table 3-8: HPLC conditions for the separation of CAPB.

Column	Superspher 60 RP-select B, 125x2.1 mm, 4 µm						
Eluent A_1	water-CH_3CN (95:5, v/v), pH 7.9 (NH_3)						
Eluent B_1	CH_3CN-water (80:20, v/v), pH 7.9 (NH_3)						
Gradient	t [min]	0	2	24	25	29	30	50
	% A	75	75	20	0	0	75	75
Eluent A_2 [a]	water-CH_3CN (95:5, v/v), 5 mM TEA and HOAc						
Eluent B_2 [a]	CH_3CN-water (80:20, v/v), 5 mM TEA and HOAc						
Gradient	t [min]	0	2	18	22	24	44
	% A	100	100	0	0	100	100
Flow	200 µL min^{-1}						
Injection volume	50 µL						

[a] for the analysis of potential metabolites

3.7 Mass spectrometry

An ionspray voltage of –3000 V was applied to the electrospray emitter tip. A gas flow of 1.53 L min^{-1} was used for the additional pneumatic nebulization (99 % N_2), whereas a drying gas flow (N_2) of 7 L min-^{1} was applied. The ion source temperature was held at 400 °C.

The orifice voltages (OR) were optimized for each analyte by syringe infusion (20 µL min^{-1}) of standard solutions (10 mg L^{-1}) in order to obtain abundant molecular ions and fragments, respectively. In case of ABS, for which no reference compound was available, a highly polluted real sample containing the analyte was injected into the LC-ESI-MS operated in a loop mode, switching continuously between distinct experiments, each applying a different OR. The voltages yielding the most intense signals were selected. The masses of the molecular ions and the fragments are given along with the applied OR in **Table 3-9** and **Table 3-10**.

For LC-ESI-MS/MS analyses on the triple quadrupole MS, the ionization conditions were the same as for the single quadrupole instrument. The source parameters ionspray voltage, nebulizer gas, drying gas and source temperature were identical. For acquisitions in PIS and MRM mode, an OR of -26 V was applied. The N_2-concentration in the collision cell, Q2, was set to 3 (dimensionless) and the accelerator voltage RO2 to 55 V.

Table 3-9: Masses of deprotonated molecular ions and fragment ions (in parenthesis) used for quantitative analysis of LAS and ABS, and applied orifice voltages.

Compound	LAS				ABS			
	C10	C11	C12	C13	C10	C11	C12	C13
m/z	297 (183)	311 (183)	325 (183)	339 (183)	297 (197)	311 (197)	325 (197)	339 (197)
OR [V]	-25 (-90)	-25 (-90)	-25 (-90)	-25 (-90)	-25 (-100)	-25 (-100)	-25 (-100)	-25 (-100)

Table 3-10: Masses of deprotonated molecular ions and fragment ions (in parenthesis) used for quantitative analysis of SPC, AG, APG and CAPB, and applied orifice voltages.

Compound	*m/z*	OR [V]	Compound	*m/z*	OR [V]
C4-SPC	243 (183)	-26 (-90)	C12-AG	376 (212)	-35 (-39)
C5-SPC	257 (183)	-26 (-90)	C14-AG	404 (240)	-35 (-45)
C6-SPC	271 (183)	-26 (-90)			
C7-SPC	285 (183)	-26 (-90)	C8-APG	291 (161)	-28 (-35)
C8-SPC	299 (183)	-26 (-90)	C10-APG	319 (161)	-28 (-35)
C9-SPC	313 (183)	-26 (-90)	C12-APG	347 (161)	-28 (-35)
C10-SPC	327 (183)	-26 (-90)			
C11-SPC	341 (183)	-26 (-90)	C8-CAPB	285 (182)	-20 (-54)
C12-SPC	355 (183)	-26 (-90)	C10-CAPB	313 (210)	-20 (-54)
C13-SPC	369 (183)	-26 (-90)	C12-CAPB	341 (238)	-20 (-54)
			C14-CAPB	369 (266)	-20 (-58)

3.8 Quantitation

For quantitative analysis of organic compounds by means of ESI-MS, one should be aware of two major factors, which may have a strong impact on the outcomes. These are directly associated with the process of ion generation in the interface.

- Formation of distinct adducts with ions originating from the buffer, the sample and/or the introduction system (e.g. H^+, Na^+, K^+, Cl^-, CH_3COO^-) and the formation of cluster and multiply charged ions[1] (Bruins, 1998).
- Ionization suppression caused by co-elution of other analytes or natural matrix components or due to the presence of IP agents or high salt concentrations in the mobile phase buffer used (Ferguson et al., 2000; King et al., 2000; Steen et al., 1999).

The adduct formation can be largely controlled and directed into the formation of a single selected species by adequate choice of the ionization mode (possibly at the expense of sensitivity), the eluent composition (buffer addition, pH adjustment, type of organic modifier) and by optimization of the ion source parameters influencing the stability of individual (adduct) ions. In contrast to the variations in adduct or

[1] These issues are adressed in the individual sections on the method development of the targeted surfactants.

cluster formation, which principally can be diagnosed by recording more than one (adduct)ion in SIM mode, the occurrence of ion suppression requires more careful diagnosis.

A quite reliable method consists in the addition of stable isotope-labelled analytes, which exhibit the same chromatographic and ionization behavior as its unlabeled analogs present in the sample, and which can be distinguished from the latter with the aid of the MS detector separating the different ion masses. However, whereas for many environmental pollutants such as pesticides, pharmaceuticals, or polycyclic aromatic hydrocarbons, a large pool of isotope-labelled compounds is provided by chemical suppliers, the availability of such reference standards for surfactants is very scarce. This is not surprising in light of the difficulties in obtaining even unlabelled defined compositions of commercial surfactants made up of up to several tens to hundreds of homologues and isomers such as e.g. NPEO.

In this instance, the standard addition of a surfactant to the real sample is an appropriate alternative, i.e. along with the analysis of the original sample, a second sample taken is amended with a known concentration of the pure standard material, preferably at a similar level, and prepared for analysis alongside the authentic sample. Comparison of the analytical results provides information on both the extraction efficiencies and possible ion suppressions in the interface.

Quantification of surfactants and metabolites

All quantitative measurements of FBBR and real samples were performed under negative ionization mode in time-scheduled SIM mode recording the $[M–H]^-$ ions together with the corresponding fragment ions (see **Table 3-9** and **Table 3-10**). Identification of the analytes was performed by comparison of retention time and absolute peak area ratio between the deprotonated molecular ion and the fragment ion, relative to the ratio obtained from the authentic standard (±20 %). Retention times of SPC and α,β-unsaturated SPC, for which no standards were available, were determined once by mass spectrometric identification in full scan mode.

The concentration of each analyte in the sample (extract) was calculated by measuring the peak area obtained by selecting ion signals for the parent ion and comparing the peak area with that obtained from standard solutions. These were prepared by dissolving known and appropriate volumes of the working standard solution (10 mg L^{-1} in methanol). Depending on the sensitivity of the analyte, quantitative analyses were done using 5- or 6-point calibrations in the range from 5 to 1000 or 50 to 1000 µg L^{-1} constructing linear calibration graphs (r=0.9991). Calibrations were performed daily for all analytes measured. The set of calibration solutions was run at the beginning of each sample series as well as at the end and if needed within the series. By this, gradual losses of sensitivity due to contamination of the MS instrument by precipitation of inorganic salts or non-volatile matrix components were taken into account. The mean of two calibrations bracketing a sample series was applied for quantitation. Typical graphs of C10- to C13-LAS are shown in **Fig. 3-2**.

Due to the rather narrow linear range of the MS detector and owing to the strongly varying concentrations of the target analytes in environmental samples, dilutions had to be prepared in most instances.

The quantification of individual SPC was performed based on the responses of the individual homologues C5-SPC and C11-SPC. By taking into account that an increasing portion of the organic solvent in the LC eluent enhanced the ionization efficiency (Ikomonou et al., 1991; Molina et al., 1994) (**Fig. 3-2**), the early eluting SPC, ≤C6, were quantified using C5-SPC, while the quantification of those

species eluting closely to C11-SPC, i.e. C7-SPC and higher was performed relating to this longer chain homologue. As no reference substances for α,β-unsaturated SPC were available, these species were quantified in the same manner as was done for the corresponding SPC. The four ABS homologues C10 to C13 were quantified based on the response of corresponding LAS congeners assuming identical molar response factors. Quantification of C14-APG was performed based on the shorter homologue C12-APG.

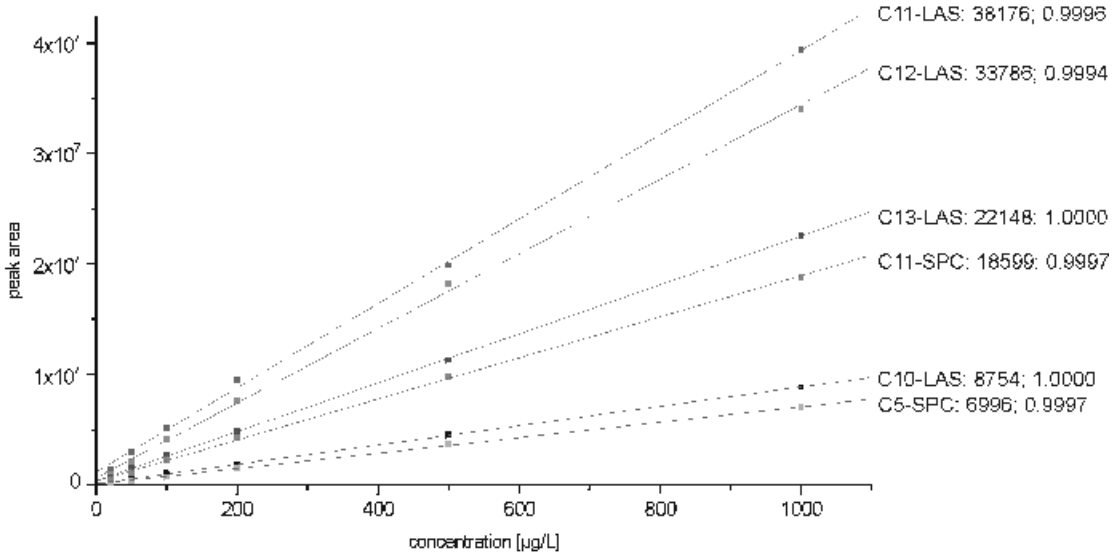


Fig. 3-2: Calibration graphs of LAS and SPC. For each compound the slope of the fitted linear regression and the correlation coefficient are given behind the substance name. The concentrations of the LAS homologues represent the sum concentration of the four components, i.e. for comparison of the ionization efficiencies the value of the slope has to be multiplied with the relative percentage of each homologue in the commercial mixture.

Limits of quantification and detection

The limits of quantification (LOQ) were set as the lowest point of a calibration showing a signal-to-noise ratio =10. Dependent on the enrichment factor applied, different LOQ were obtained. This is shown in **Table 3-11** for influent samples from WWTP preconcentrated by a factor of 100.

Table 3-11: Limits of quantification in WWTP influent samples, in µg L^{-1}.

LAS				SPC	AG		APG
C10	C11	C12	C12	Cn	C12	C14	Cn
0.3	0.1	0.1	0.2	0.5	0.1	0.3	0.1

Owing to the mostly high concentrations found in environmental samples (up to low mg L^{-1} range), requiring in many cases sample dilution prior to injection onto the LC, limits of detection (LOD) were only determined when necessary. Based on a signal-to-noise ratio of 3 the LOD for AG and APG in treated sewage was 0.03 µg L^{-1} with exception of C14-AG amounting to 0.08 µg L^{-1}. In the determination of SPC in waterworks and tap water samples (1000-fold enriched), the LOQ was 0.03 µg L^{-1} for each homologue.

3.9 Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry sample preparation and measurements [1]

Conventional sample preparation

The optimal concentration was found to be 7.5 g L^{-1} (in methanol) of the MALDI matrix 3β-indole acrylic acid (IAA) and 3 g L^{-1} (in methanol) of the cationization agent sodium trifluoroacetate. 10 µL of the IAA solution, 10 µL of the aqueous PVP sample and 1 µL of the salt solution were added together, mixed and applied to the MALDI target (1 µL). After evaporation of the solvent the measurement was obtained readily.

Sample preparation with anchor target

The salt amount was lowered such that 0.5 µL was added to 10 µL of the IAA solution and 10 µL of the aqueous PVP sample. 0.1 µL of the well-mixed MALDI sample mixture was applied to the target spot.

Measurements

The sample target was irradiated with a N_2 laser operated at a pulse rate of 3 Hz. The ions were accelerated by pulsed ion extraction using a voltage of 20 kV. The analyzer was run in reflection mode and the ions were detected using a microchannel plate detector.

3.10 Ecotoxicity assays

3.10.1 ToxAlert 100 bioluminescence test

A standard solution of neutralized C4-glucamide acid (for synthesis see 0) was prepared in 2 % NaCl resulting in a concentration of 2.5 g L^{-1}. From this a dilution series was made in the range from 1.25 to 0.03 g L^{-1}. After addition of the bacteria suspension the reduction of light output in comparison to a reference sample (2 % NaCl) was recorded after 15 and 30 min of exposure.

3.10.2 Cellsense biosensor

Three concentrations in 0.85 % NaCl were prepared from neutralized C4-glucamide acid (3.8, 2.4 and 1.2 g L^{-1}). Following a biosensor stabilization period of 5 min, 100 µL of the redox mediator potassium hexacyano ferrate(III) were added to the samples yielding a final concentration of 5 mg L^{-1}. The response was registered every 4 sec during a period of 15 min. A 0.85 % NaCl solution likewise amended with the redox mediator served as control.

[1] Performed by Max-Planck-Institute for Polymer Research, Mainz.

3.11 Quality assurance

The ability to provide accurate and reliable data is central to the role of analytical chemists not only in areas like e.g. development and manufacture of drugs, food control or drinking water analysis, but also in the field of environmental chemistry, where an increasing need is urging for certified laboratories (ISO 9000 standards). The quality of analytical data is a key factor in the success to identify and monitor contamination of environmental compartments.

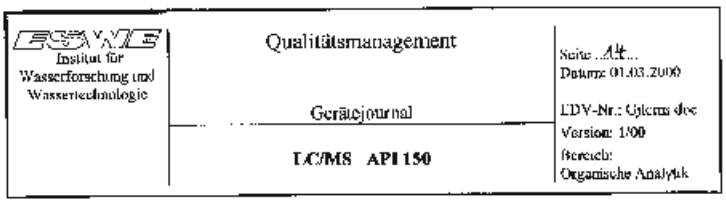
In this light, a large collection of methods applied for routine analysis of prime environmental pollutants have been developed and validated, and are adapted in national or international harmonized protocols (DIN, EPA). Information on the method performance generally provides data on *specificity*, *accuracy*, *precision* (*repeatability* and *reproducibility*), *limit of detection*, *sensitivity*, *applicability* and *practicability*, as appropriate.

In research laboratories, where the objectives are to develop and establish new analytical methods, standardized procedures can commonly not be applied. To prove the reliability of the methods and to guarantee the quality of data generated therewith, characterization of these methods through the afore mentioned performance parameters represents an appropriate mean. Given the possibility to participate in inter-laboratory studies, the own methods should be subjected to such trials.

As a main part of the present work consisted of developing methods and of applying them to the analysis of environmental samples, the following measurements were taken to assure the quality of the data generated:

- Time and date of sampling as well as used reference compounds and chemicals were documented in laboratory journals.
- After elucidation of an adequate material for SPE enrichment, bulk material of the same lot was used throughout the work.
- The LC separations were performed in air-conditioned laboratories to preclude temperature-related shifts in chromatographic retention times. Prior to storage RP-LC columns were rinsed with water-acetonitrile (50:50) and kept filled with this solvent composition. The anion exchange column was stored in pure water.
- Standard operation procedures for the LC-ESI-MS and LC-ESI-MS/MS were followed in the form of instrument journals. These described operation, actual instrument parameters (multiplier voltage, pump vacuum), and maintance work to be performed within fixed intervals (mass calibration, oil change and lubrication). Cleaning of the low and high vacuum region of the MS as well as exchange of original parts by spare parts were documented. Performed analytical measurements were documented in the journals (see **Fig. 3-3** for e.g. sample list; in German).
- Details on MS measurements were additionally documented in chronological order in excel sheets specifying the location of the raw data saved on CD-ROMs and were archived together with the quantitation results.
- The analytical scale and pH meter were calibrated monthly, their function verfied daily (control weight and solution, respectively) and the results reported in control cards (see **Fig. 3-4** for e.g. pH meter; in German).

- The stability of the reference compounds was continuously controlled by comparing previously used calibration series with freshly prepared ones.
- Analytical results obtained were verified for their plausibility, e.g. surfactant concentrations in raw waters were compared with the corresponding values in treated waters. In addition, pattern of alkyl homologues were critically assessed.
- Reproducibility of analytical procedures was verified by standard addition experiments. Repeatability of the MS results and reliability of the autosampler was checked by consecutive injections of identical samples (see section 3.11.1).
- Interlab exercises were performed analyzing waste and surface waters for different surfactant types (see below).



ESWE Institut für Wasserforschung und Wassertechnologie

Qualitätsmanagement

Gerätejournal

LC/MS API 150

Seite: ..14...
Datum: 01.03.2000
LDV-Nr.: Qterms.doc
Version: 1/00
Bereich: Organische Analytik

7 PROBEN - PROTOKOLL

Datum	Name	Matrix / Methode	Anzahl Proben	Vakuum vor Softwarestart	Vakuum nach Erreichen der Betriebstemperatur
18.09.00	elw	LAS Bracken -screening CW	10	$3.4 \cdot 10^{-5}$	$3.2 \cdot 10^{-5}$
19.09.00	elw	LAS Bracken - Quant. CW	21	–	$3.1 \cdot 10^{-5}$
20.09.00	elw	– " –	15	–	$3.2 \cdot 10^{-5}$
21.09.00	elw	– " –	33	–	$3.3 \cdot 10^{-5}$
29.09.00	elw	SPC - Bestimmung - Quantifizierung	5	$3.1 \cdot 10^{-5}$	$2.9 \cdot 10^{-5}$
30.09.00	elw	AB WTP ; HA ein + aus	10	$3.2 \cdot 10^{-5}$	$3.0 \cdot 10^{-5}$
02.10.00	elw	LAS Interlab III HA	15	$3.3 \cdot 10^{-5}$	$3.0 \cdot 10^{-5}$
11.10.00	elw	LAS + SPC ; Code ; Methanol und Stopper	4	$3.5 \cdot 10^{-5}$	$3.2 \cdot 10^{-5}$
16.10.00	elw	SPC Bracken TW	15	$3.5 \cdot 10^{-5}$	$3.1 \cdot 10^{-5}$
18.10.00	elw	Metabolite Code; mit Stopper	5	$3.5 \cdot 10^{-5}$	$3.2 \cdot 10^{-5}$
23.10.00	elw	LPEO Interlab III HA	18	$3.1 \cdot 10^{-5}$	$2.8 \cdot 10^{-5}$
26.10.00	elw	LAS - Adsorption - LSBR	12	$3.3 \cdot 10^{-5}$	$3.0 \cdot 10^{-5}$
12.11.00	elw	AB WTP HA aus	8	$3.3 \cdot 10^{-5}$	$3.0 \cdot 10^{-5}$

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Fig. 3-3: Selected page from LC-ESI-MS instrument journal documenting date, type and number of samples analyzed.

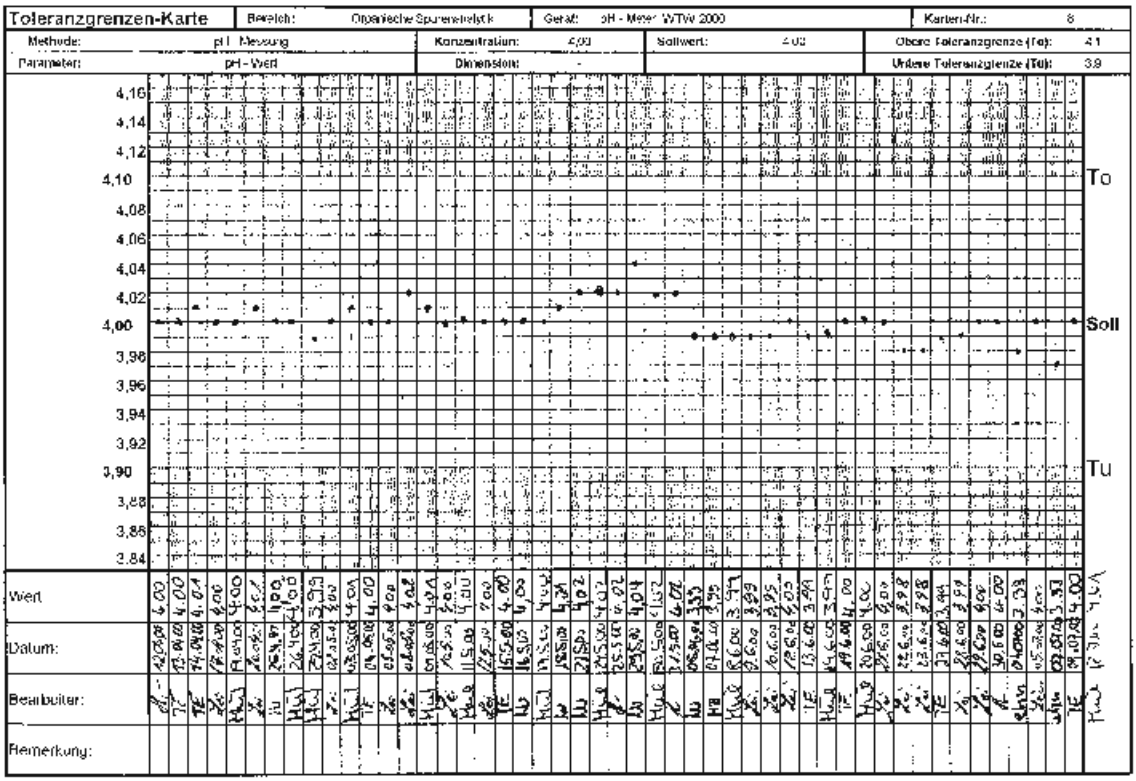

Fig. 3-4: Control card of pH meter from period of April 12, 2000 to July 07, 2000. The tolerance range is 4.00±0.10 (variation from target value corresponds to 2.5 %).

3.11.1 Repeatability study

Repeatability studies were performed on the LC-ESI-MS by consecutive injections of the same sample. An example is shown in **Fig. 3-5** for the analyses of four SPC homologues in a sample from an FBBR degradation experiment.

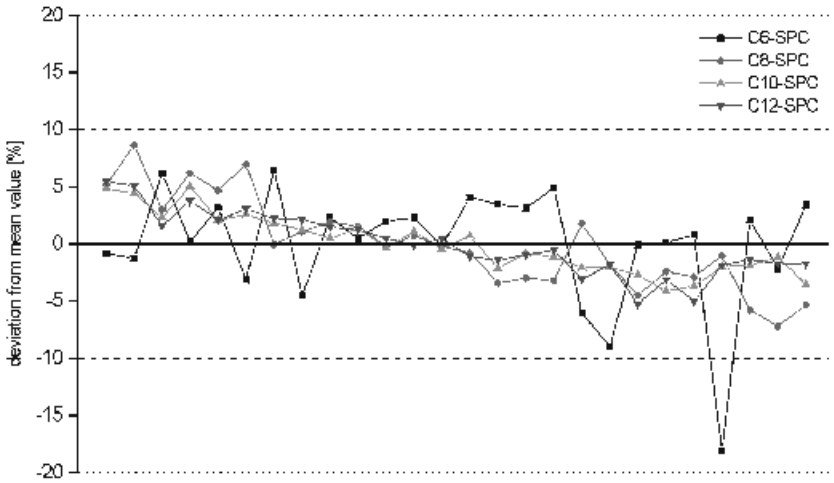

Fig. 3-5: Repeatability of peak area of four SPC homologues, determined from 26 consecutive LC separations of 10 µL-injections of untreated FBBR sample (corresponds to sample from day 14 in **Fig. 4-9**). Total duration: 19 h.

Except for one outlier, the results varied within a range of ±10 % of the mean value. The variability was highest for the short-chain C6-SPC. A positive correlation with slight shifts in retention times could be established for no analyte (C6: 8.1±0.20 min, C8: 19.5±0.16 min; C10: 20.7±0.15 min; C12: 21.9±0.19 min).

Overall, a tendency of decreasing sensitivity was observed during this experiment attributable to the contamination of the mass spectrometer with matrix components (discernible in the crystallization of inorganic salts on the curtain plate). As pointed out in section 3.8 this effect was taken into consideration by regularly placing entire calibration series within a sample sequence.

3.11.2 Interlaboratory exercises

The quality of the analytical methods applied was validated by comparative measurements within interlaboratory exercises. The results of the determination of LAS and NPEO in different spiked and native aqueous matrices are presented as examples in the following paragraphs.

Samples were prepared in the organizing laboratory. After SPE extraction on RP-C18 material, the cartridges were provided to all participants, 4 in number, all performing the quantification by LC-based MS techniques. The results of the LAS analyses are shown in the histogram in **Fig. 3-6**. The values on the first column in each set correspond to the own findings (Lab A)

As can be seen, nearly quantitative recovery of LAS spiked to MilliQ-water at 200 µg L^{-1} is obtained. Comparing the concentrations in primary effluents, one of which fortified with 200 µg L^{-1}, the difference amounts to 180 µg L^{-1} corresponding to 90 %.

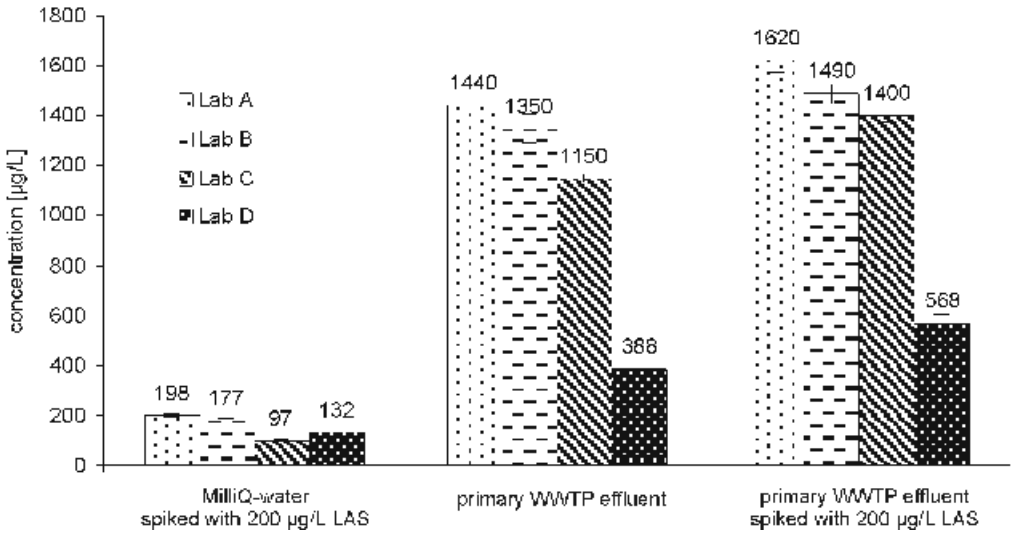

Fig. 3-6: Results from interlab exercise of LAS determination in aqueous matrices. Lab A: (–)-LC-ESI-MS, Lab B: (–)-LC-ESI-MS, Lab C: (–)-LC-ESI-MS, Lab D: (–)-ESI-MS. Error bars correspond to standard deviations of samples prepared in triplicate.

The outcomes of the determination of NPEO in the same samples are shown in **Fig. 3-7**. Recovery of NPEO spiked to MilliQ-water is about 95 %. Of the 200 µg L^{-1} added to the primary effluent prior to extraction 94 % are recovered.

In comparison with the results reported by the other participating laboratories, greater differences are stated for NPEO. This may in part be due to the higher complexity of NPEO, which contrary to LAS has no defined composition. The choice of ethoxymers, on which the quantitation is based, may markedly affect the results, since the ionization efficiencies of individual oligomers are strongly instrument dependent (de Voogt, in preparation). A further observation made in **Fig. 3-6** and **Fig. 3-7** are the generally low values of laboratory D, which may be put down to the fact that this lab carried out the measurements by-passing the analytical column. Under these condition ion suppression during infusion of the complex samples into the MS interface may play a crucial factor.

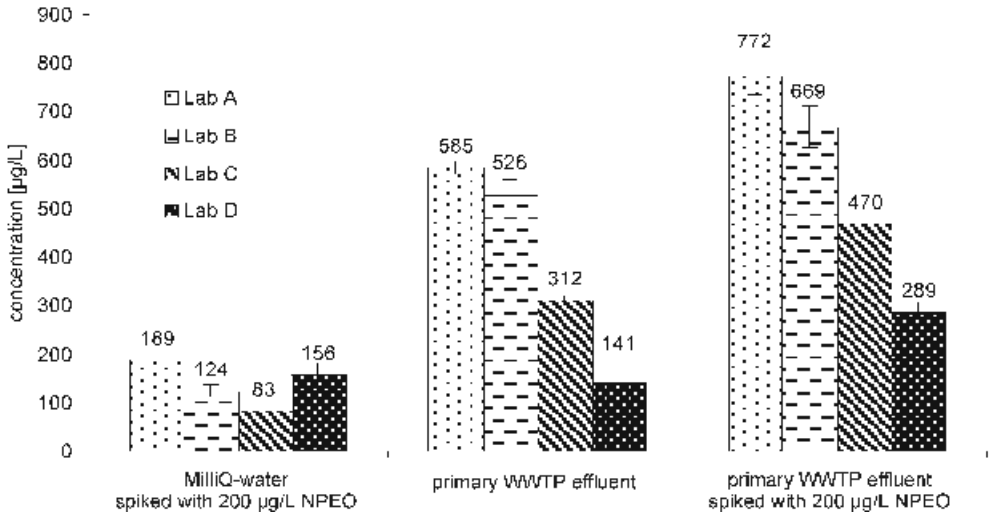

Fig. 3-7: Results from interlab exercise of NPEO determination in aqueous matrices. Lab A: (+)-LC-ESI-MS, Lab B: (+)-LC-ESI-MS, Lab C: (+)-LC-APCI-MS, Lab D: (+)-APCI-MS. Error bars correspond to standard deviations of samples prepared in triplicate.

4 METHOD DEVELOPMENT AND METABOLISM STUDIES

4.1 Alkylbenzene sulfonates and sulfophenyl carboxylates

4.1.1 Introduction

Linear alkylbenzene sulfonate (LAS) is the surfactant with the highest worldwide production volume ($3.0x10^6$ tons per annum) apart from soap. This class of surfactants finds wide application because of its excellent detegent properties, versatility, compatibility with all types of detergent ingredients and best cost/performation ratio among surfactants. Commercial LAS are applied mainly in the formulation of powder and liquid laundry detergents. The calcium salts of LAS are used as emulsifiers in pesticides, and the amine salts are employed in dry cleaning and as degreasing agent in the metal industry (BHK, 1993). LAS generally consist of a mixture of homologues and isomers. Individual components are classified by the length of the alkyl substituent and by the position of attachment of the sufophenyl ring (**Fig. 4-1**). In formulations the alkyl chain length may vary from C10 to C13 with an average distribution of approximately 11.4 to 11.7. Isomer configurations range from the 2-phenyl position to the 7-phenyl position depending on the alkyl chain length.

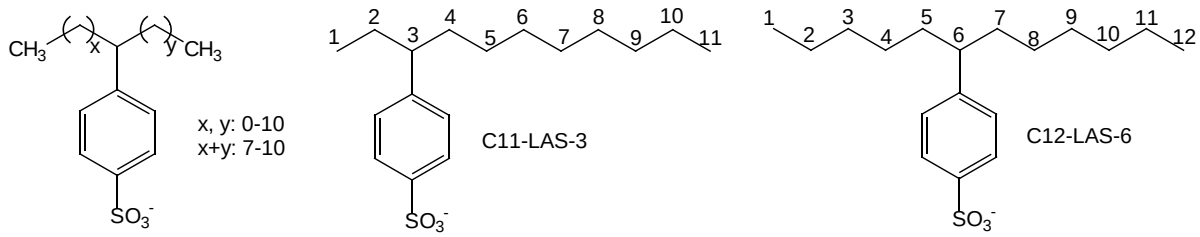

Fig. 4-1: General structure of linear alkylbenzene sulfonates (LAS) (left) and structures of two components (middle and right) illustrating the nomenclature used to identify individual species.

The initial step of LAS synthesis is the Friedel-Crafts alkylation of benzene with n-olefins or alkyl halides catalyzed by either HF or $AlCl_3$. The obtained linear alkyl benzenes are subsequently sulfonated with concentrated sulfuric acid or sulfur trioxide to yield the para-substituted LAS (Kosswig, 1994). Commercial blends usually contain some sulfonated impurities (<1 %), namely dialkyltetralin sulfonates (DATS), branched alkylbenzene sulfonates (ABS), and single methyl-branched LAS (iso-LAS) (**Fig. 4-2**). The level of these impurities depends on the synthetic route used. Alkylation of benzene in the presence of $AlCl_3$ and subsequent sulfonation tends to yield DATS, while ABS are more susceptible to be

formed when HF is used as catalyst in the alkylation step (Drozd and Gorman, 1988; Moreno et al., 1988). Commercial LAS may also contain traces of linear alkylbenzenes resulting from incomplete sulfonation (Kirk-Othmer, 1999).

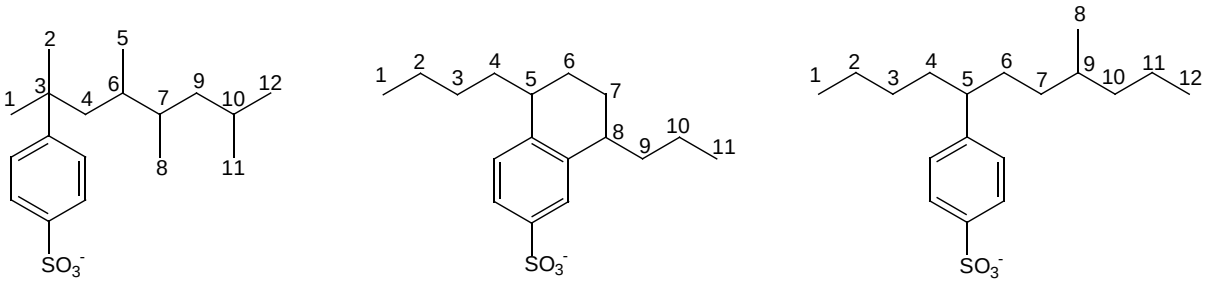

Fig. 4-2: Possible structures of branched C12-alkylbenzene sulfonate (ABS) (left), C11-dialkyltetralin sulfonate (DATS) (middle), and C12-single methyl-branched linear alkylbenzene sulfonate (iso-LAS) (right).

As a consequence of the vast amounts of LAS entering the aquatic environment via wastewater discharge, their environmental fate and distribution has been thoroughly studied for more than three decades. Hence, an enormous body of literature exists describing analytical methods for their determination in distinct environmental compartments (di Corcia et al., 1991; Giger et al.; 1989; González-Mazo et al., 1997; Larson et al., 1989; Matthijs et al., 1999; Trehy et al., 1990), investigations on their biodegradability under both laboratory and field conditions (Berna et al., 1989; Brüschweiler et al., 1991; Knaebel et al., 1990; Larson, 1990; Larson and Payne, 1981; Prats et al., 2000; Sales et al., 1987; Schöberl and Bock, 1980) and assessment of the toxic potential towards aquatic organisms (Aidar et al., 1997; Shcherbakove et al., 1999; Verge and Moreno, 1996 and 2000). In essence, LAS are one of the best-studied organic substances produced at industrial scale with respect to environmental behavior. However, there is still a wide gap to be bridged in understanding the metabolic pathway of LAS and the occurrence and fate of the degradative products in the environment.

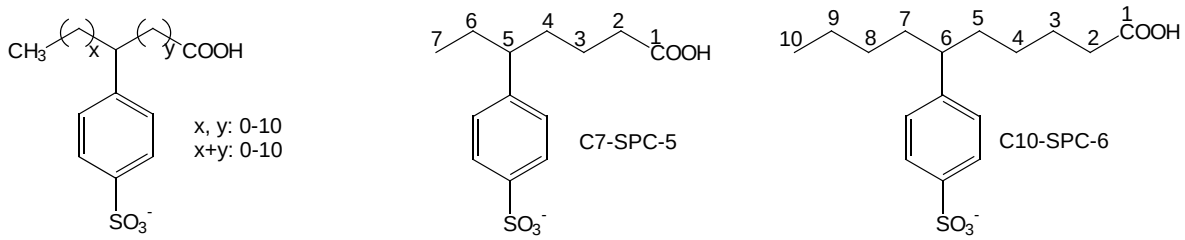

Fig. 4-3: General structure of sulfophenyl carboxylates (SPC) (left) and the structure of two possible components deriving from LAS biodegradation (middle and right) illustrating the nomenclature used to identify individual species.

The major degradation intermediates are sulfophenyl carboxylates (SPC; **Fig. 4-3**), which have been observed in wastewaters and in the environment (di Corcia et al., 1994 and 1999b). SPC originating from commercial LAS constitute even more complex mixtures than the parent compound since the initial attack on the alkyl chain can take place on either side of the molecule and that a wider range of alkyl homologues ranging from C4 to C13 can be produced.

4.1.2 Method development

Review on LAS analysis

Historically, the determination of alkylbenzene sulfonates in aqueous samples was commonly performed by the sum parameter MBAS method, based on the formation of a noncovalent complex of anionic surfactants with methylene blue, which was then extracted into chloroform and detected spectrophotometrically (Larson and Payne, 1981; Olivier, 1961; Swisher, 1966; Webster and Halliday, 1959). This method was rather insensitive and lacked selectivity since it gave positive detects for nearly all anionic surfactants and hence provided no discrimination among various molecularly-distinct surfactant types. Moreover, it suffered from interference of natural and synthetic substances, causing both over- and underestimation of analytical results.

Later, the advent of RP-HPLC allowed more specific determination of anionic surfactants through separation based on the hydrophobic moiety of the surfactant molecule. Through this, the separation, identification, and quantitation of complex surfactant mixtures in terms of their alkyl homologues and phenyl isomers was made possible. The aromatic phenyl ring renders the LAS molecules amenable to UV detection (Matthijs and de Hanau, 1987; Prats et al., 1999; Schröder et al., 1999; Schöberl, 1995; Schöberl et al., 1996; Sigoillot and Nguyen, 1992), while a gain in selectivity is achieved by coupling the HPLC to an FL detector (Castles et al., 1989; Klotz, 1998; Marcomini et al., 1988; Marcomini and Giger, 1987; McAvoy et al., 1993; Prats et al., 1997). Under optimized RP conditions all alkyl homologues are completely separated on the column, whereas positional isomers are only partially resolved.

Higher chromatographic resolution is provided by GC, allowing the baseline separation of all individual components present in LAS mixtures (Brüschweiler et al., 1991; Müller and Noffz, 1965; Swisher, 1966). This technique, however, cannot be applied directly since LAS are too polar and lack volatility. In this instance conversion into more volatile species, e.g. by desulfonation or by esterification, is required prior to injection into the gas chromatograph. This can be combined with MS detection, thereby providing additional information on the analytes (Ding and Chen, 1999; Heywood et al., 1970; McAvoy et al., 1998; Nakae et al., 1981; Reiser et al., 1997). Such sample preparation steps are generally time-consuming and tedious and are susceptible to possible contamination. These shortcomings can be circumvented by employing LC-MS instruments equipped with API interfaces like APCI or ESI.

Methods for the simultaneous detection of LAS and SPC

A common problem the analyst faces when integrating SPC into analytical procedures for the determination of LAS is the scarcity of available reference compounds thereby complicating their identification. In GC, this difficulty can be overcome by exploiting the selectivity of mass spectrometric detection which, however, requires a previous derivatization step of the very polar analytes carrying both a sulfonate and a carboxylate group.

For the application of HPLC the use of a more selective fluorometric or diode array detector instead of a UV detection can be of assistance in identifying SPC. Regarding the simultaneous separation of LAS and their polar metabolites SPC under RP conditions, a gradient elution is necessary in order to provide satisfactory retention of all analytes. While the hydrophobic portion of LAS results in a strong interaction, the oxidized form of this moiety, as present in SPC molecules, greatly alters the retention

behavior. This is especially the case for the most polar SPC having short alkly side chains, which can only be retained on RP columns when IP agents are used.

If an unequivocal identification and quantitation at environmentally realistic trace levels is an objective, then LC-MS is the method of choice since it combines both high sensitivity and selectivity and it offers the possibility to identify unknown compounds through the interpretation of their mass spectra. When transferring established separation methods from HPLC-UV/FL to LC-MS, some modification of buffer systems may be necessary due to concerns over volatility. Therefore, the presence of inorganic salts or IP agents in the mobile phase should be avoided in order to prevent their precipitation in the ion source, which rapidly leads to sensitivity losses.

Table 4-1: Analytical methods for the simultaneous detection of LAS and SPC.

Separation	Detection (Ionization)	Matrix	Reference
GC[a]	MS (NCI)	degradation exp.	Cavalli et al. (1996)
	MS (NCI)	degradation exp.	Kölbener et al. (1996)
	MS (NCI)	wastewater	Trehy et al. (1996)
	MS (NCI)	wastewater, river water	Ding et al. (1998)
	MS (EI, NCI)	river water	Ding et al. (1999)
LC	UV	degradation exp.	Taylor and Nickless (1979)
	UV	degradation exp.	Hrsak (1995)
	UV	surface water	Sarrazin et al. (1997)
	UV	lake sediment	Sarrazin et al. (1999a)
	DAD	degradation exp.	Schulz et al. (2000)
	DAD	degradation exp.	Schleheck et al. (2000)
	FL	degradation exp.	Linder and Allen (1982)
	FL	degradation exp.	Cavalli et al. (1996)
	FL	degradation exp.	Nielsen et al. (1997)
	FL	wastewater	di Corcia et al. (1994)
	FL	coastal water	Marcomini et al. (2000c)
	FL	sea water	González-Mazo et al. (1997)
	FL	sea water, estuarine water	León et al. (2000)
	FL	fish	Tolls et al. (1999)
	FL	fish	Tolls et al. (2000)
	FL	fish, mussel	Sáez et al. (2000)
	MS (ESI)	degradation exp.	di Corcia et al. (1999a)
	MS (ESI)	degradation exp.	Knepper and Kruse (2000)
	MS (ESI)	wastewater, surface water	di Corcia et al. (1999b)
	MS (ESI)	coastal water	Riu et al. (1999)
CE	DAD, FL	wastewater	Kanz et al. (1998)

[a] after previous derivatization

A literature overview describing the analytical methods available for the concurrent determination of LAS and SPC is given in **Table 4-1**. As evident GC has been employed rarely, always in combination with MS, while LC methods with different detection systems have found wider application including

examination of distinct environmental matrices, biota as well as test liquors from laboratory devices studying the degradability of LAS. The usage of LC-ESI-MS has emerged only very recently. Owing to the anionic character of the analytes, an ESI interface is particularly attractive when applying negative ionization.

Optimized separation of alkylbenzene sulfonate and sulfophenyl carboxylate

The analytical method proposed by Riu et al. (1999) for the analysis of coastal water was optimized and adapted to the separation of the SPC homologues C4 to C13 and the LAS congeners C10 to C13. For the gradient elution a binary system made up of water-CH_3CN mixtures was used, modified with equimolar amounts of HOAc and TEA. The protonation of the latter resulted in the formation of the volatile cationic IP agent triethyl ammonium, which allowed sufficient retention of the anionic SPC metabolites. A typical (–)-LC-ESI-MS chromatogram of a sample containing SPC and LAS is displayed in **Fig. 4-4**. Within both analytes´ groups, the retention times increase with increasing alkyl chain length. The four LAS homologues bearing strongly hydrophobic portions elute after their carboxylates intermediates, whose broad range in polarity leads to a wide retention window. In addition, individual phenyl isomers are partially separated (most apparent for C6-SPC in **Fig. 4-4**).

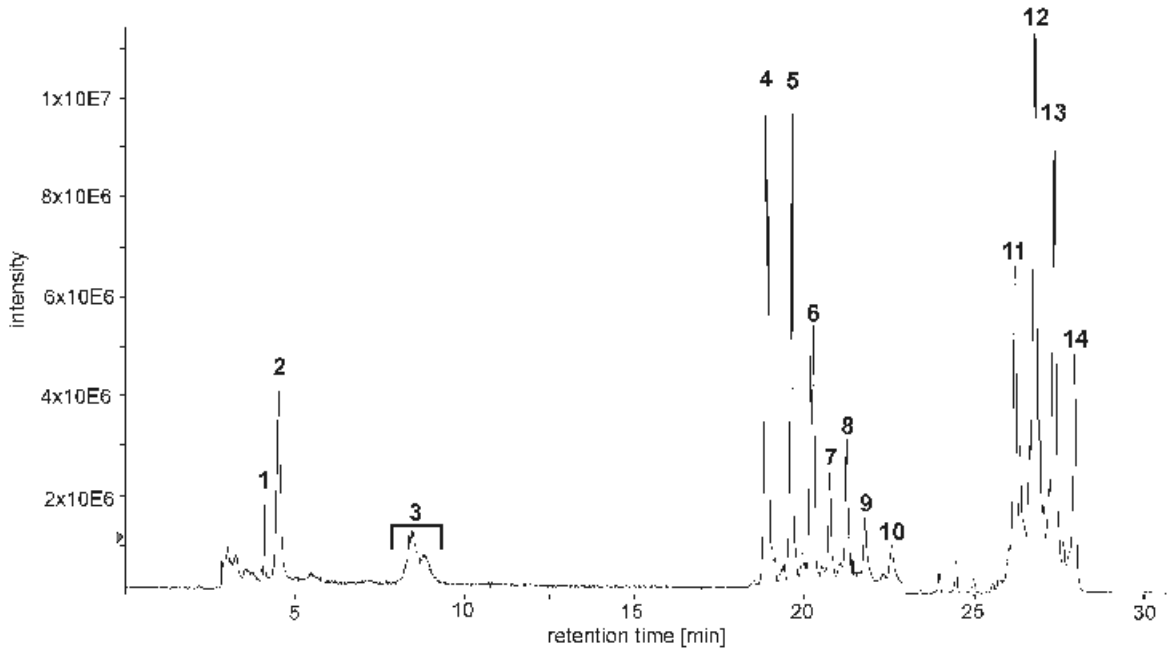

Fig. 4-4: (–)-LC-ESI-MS chromatogram of SPC and LAS separation obtained from FBBR sample of degradation of commercial LAS (see **Fig. 4-23**, day 1). Peak numbering: (1) C4-SPC, (2) C5-SPC, (3) C6-SPC, (4) C7-SPC, (5) C8-SPC, (6) C9-SPC, (7) C10-SPC, (8) C11-SPC, (9) C12-SPC, (10) C13-SPC, (11) C10-LAS, (12) C11-LAS, (13) C12-LAS, (14) C13-LAS. LC conditions: 250 x 2.1 mm, 5 µm C18-bonded silica with a gradient profile: water-CH_3CN, 5 mM HOAc and TEA (95:5 to 20:80, v/v) at 0.2 mL min^{-1}.

The mass spectra of C12-LAS and C6-SPC acquired under negative ion mode are typified by **Fig. 4-5**. The comparatively high orifice voltage of –90 V applied in order to generate abundant fragmentation reflects the stability of the molecules regarding gas-phase CID reactions. Apart from the molecular ions $[M–H]^-$, both compounds yield characteristic fragments occurring at *m/z* 183, corresponding to 4-styrenesulfonate, and *m/z* 119, which can be assigned to 4-styrenephenolate. The ion at *m/z* 80 originates

from the cleavage of the sulfonate group. In addition, a loss of *m/z* 60 is observed for SPC corresponding to the loss of acetic acid liberated from the oxidized alkyl moiety.

With the developed LC-MS method it was possible to analyze the branched analogs of LAS, the ABS. Due to structural dissimilarities in the constitution of the alkyl chain, the retention times differed slightly, hence identification on a mere chromatographic basis was hampered. The MS detector could not distinguish between the two classes of anionic surfactants based solely on molecular ions of the isomeric species, but the assignment of the branched and linear form was permitted due to their different fragmentation behavior under in-source CID. Whereas LAS gave the typical fragment *m/z* 183, ABS could be identified via the diagnostic fragment *m/z* 197 (Borgerding and Hites, 1992; Ogura et al., 1995; Schröder, 1997).

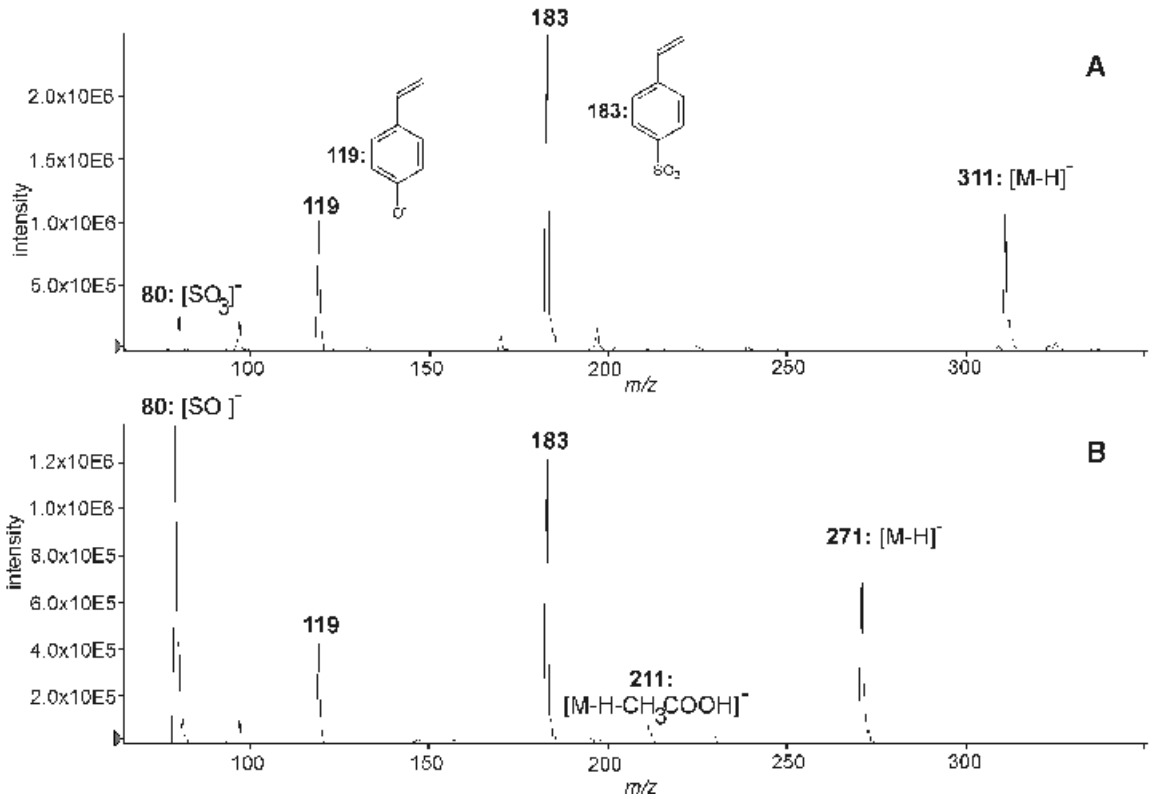

Fig. 4-5: Mass spectra of C12-LAS (**A**) and C6-SPC (**B**) under negative ionization mode (OR: -90 V).

This is illustrated in **Fig. 4-6**, which shows the mass traces of C12-LAS and C12-ABS each with its corresponding fragment. Furthermore, the mass traces also showed that ABS give a broader chromatographic peak than LAS, due to the much higher number of possible isomers of C12-ABS (more than 100) compared to C12-LAS (5 possible isomers).

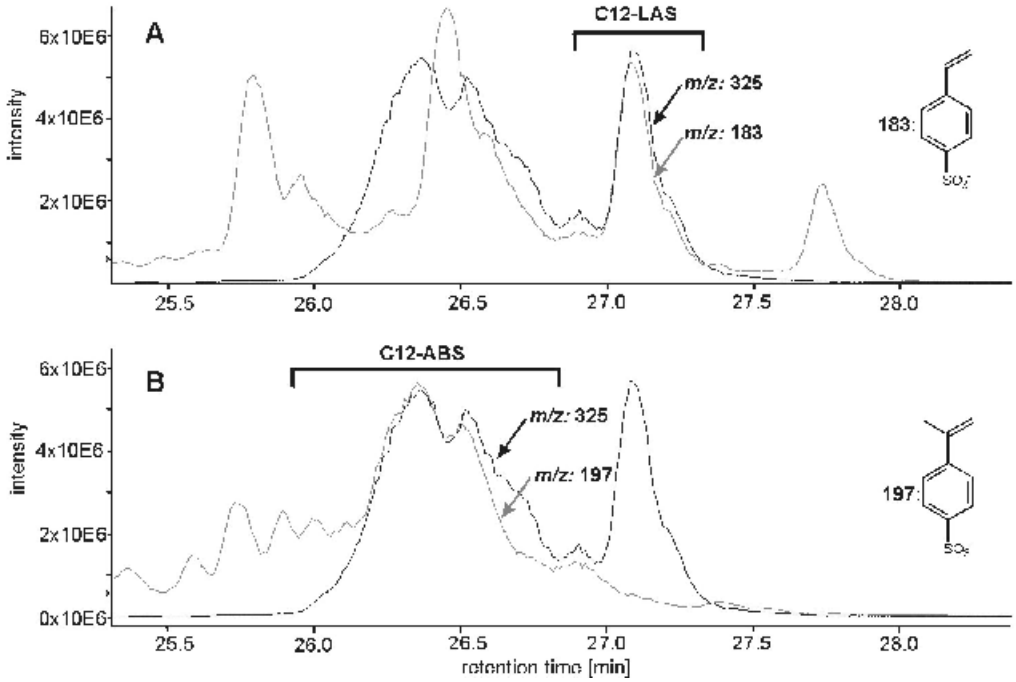

Fig. 4-6: (–)-LC-ESI-MS chromatogram of a surface water sample containing both LAS and ABS; (**A**) ion trace of C12-LAS (*m/z* 325) with fragment ion *m/z* 183, (**B**) ion trace of C12-ABS (*m/z* 325) with fragment ion *m/z* 197. LC conditions as in **Fig. 4-4**.

Regarding the separation, it was pointed out that the IP agent is essential for adequate retention of the very polar SPC on the RP column. The selection of the combination HOAc/TEA is favorable in terms of compatibility with the ESI interface as they are volatile buffer additives. However, IP agents exert an adverse effect on the electrospray ionization process by diminishing the ionization efficiency (Temesi and Law, 1999). The drawback arising from their application can be surmounted by adding a cation exchanger between the analytical column and the MS interface (Gardner et al., 2000; Knepper and Kruse, 2000). Using this technique, all cationic species present in the LC flow are substituted by protons, which in turn do not negatively affect ionization.

The efficacy of the cation suppressor is demonstrated in **Fig. 4-7** comparing the extracted ion chromatograms (XIC) of C7-SPC, constituted of several phenyl isomers, with (B) and without (A) the device. It is apparent that a substantial gain in sensitivity is achieved when operating the system with the suppressor column. The overall peak intensity increases by roughly an order of magnitude. This, however, occurs at the expense of peak resolution. In the first case five peaks can be clearly identified allowing the acquisition of relatively "pure" mass spectra and accurate quantification of single isomers of C7-SPC. On the other hand, the cation suppressor causes appreciable peak broadening.

Since good chromatographic resolution was of more importance than sensitivity, all further LC-ESI-MS measurements were performed without making use of the cation suppressor.

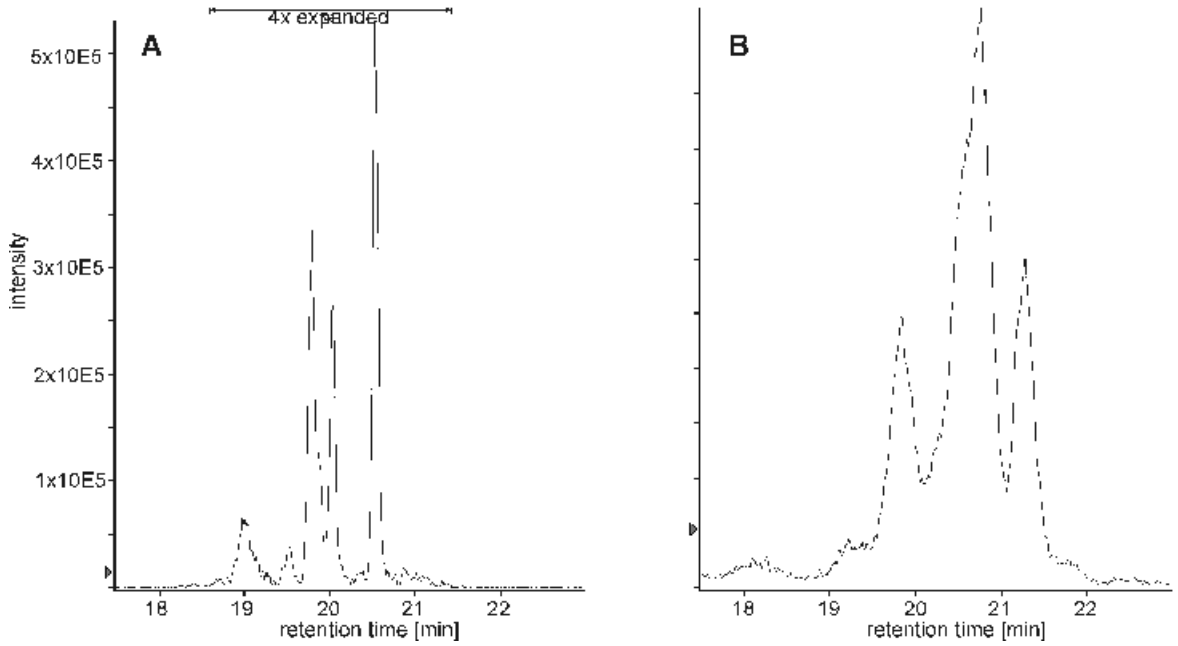

Fig. 4-7: (–)-LC-ESI-MS extracted ion chromatogram of C7-SPC (*m/z* 285) in WWTP effluent (sample from WWTP-1, see **Table 5-2**), (**A**) without cation suppressor, (**B**) with cation suppressor. LC conditions as in **Fig. 4-4**.

4.1.3 Biodegradation tests

Literature review

Since the introduction of LAS more than 30 years ago, an extensive database has been developed supporting their good aerobic biodegradability. A large fraction of these data were gathered from standard OECD laboratory screening tests routinely used in Europe and North America to determine the biodegradation potential of organic substances (OECD, 1993). Evidence for mineralization was provided from non-specific gross biodegradation parameters: O_2 uptake, organic carbon removal, and CO_2 formation (Fischer et al., 1975a and 1975b; Gerike and Fischer, 1979; Gerike et al., 1980; Larson, 1979; Pecenik et al., 1984). Two major factors, however, significantly impact the environmental relevance of screening test results and strongly limit the extrapolation to "real world" settings: (a) standard screening tests do not accurately simulate the physical, chemical, and biological conditions found in environmental systems and (b) the tests ignore several compartments such as sediments, soils, and ground water, estuaries and oceans. These may be important to the biodegradation of surfactants in the environment (see **Fig. 1-4**).

To overcome these limitations a multitude of experiments have been performed to simulate natural environmental settings using both laboratory and field microcosms. These studies were conducted on sediments (Federle and Schwab, 1992; Larson et al., 1993), soils (Branner et al., 1999; Figge and Schöberl, 1989; Litz et al. 1987), ground waters (Krueger et al., 1998; Larson et al., 1989), surface waters (Perales et al., 1999b; Schöberl et al., 1998), estuarine waters (Shimp, 1989; Terzic et al., 1992), and sea water (Quiroga et al., 1999; Quiroga and Sales, 1990; Sales et al. 1987, Vives-Rego et al., 2000). In most

instances a reasonable primary degradation rate of LAS was observed, dependent among other parameters on the history of exposure. In environments with continuous exposure to LAS residues, e.g. by direct discharges of domestic sewage into coastal areas, the microbial communities were rather well adapted to the substrate. The very low kinetics of primary LAS degradation in sediments (Federle and Schwab, 1992) are likely due to oxygen limitation.

A detailed description of the aerobic degradation of LAS was provided by Swisher (1987) and Schöberl (1989). The majority of the published laboratory studies indicated that metabolism starts with oxygenation of one terminal methyl group of the alkyl chain and the conversion of the alcohol to a carboxylic group (ω-oxidation), releasing an SPC with the same number of carbon atoms in the side chain as in the original LAS isomer (**Fig. 4-8**) (Huddleston and Allred, 1963; Payne and Feisal, 1963).

The conversion rates of individual components in a commercial LAS mixture are dependent on the molecular structure: (a) the length of the alkyl chain is positively correlated with the primary degradation rate and (b) phenyl isomers in central positions are degraded more slowly than external isomers (Setzkorn et al., 1964; Swisher, 1963a). Both effects are a direct consequence of the enzymatic attack on the hydrophobic moiety. The relation between the surfactant structure and the biodegradation is termed as "*Swisher´s distance principle"*. In a more general form, it can be formulated as follows: increased distance between the xenobiotic arylsulfonate moiety and the far end of the hydrophobic group increases the speed of primary degradation (Swisher, 1987).

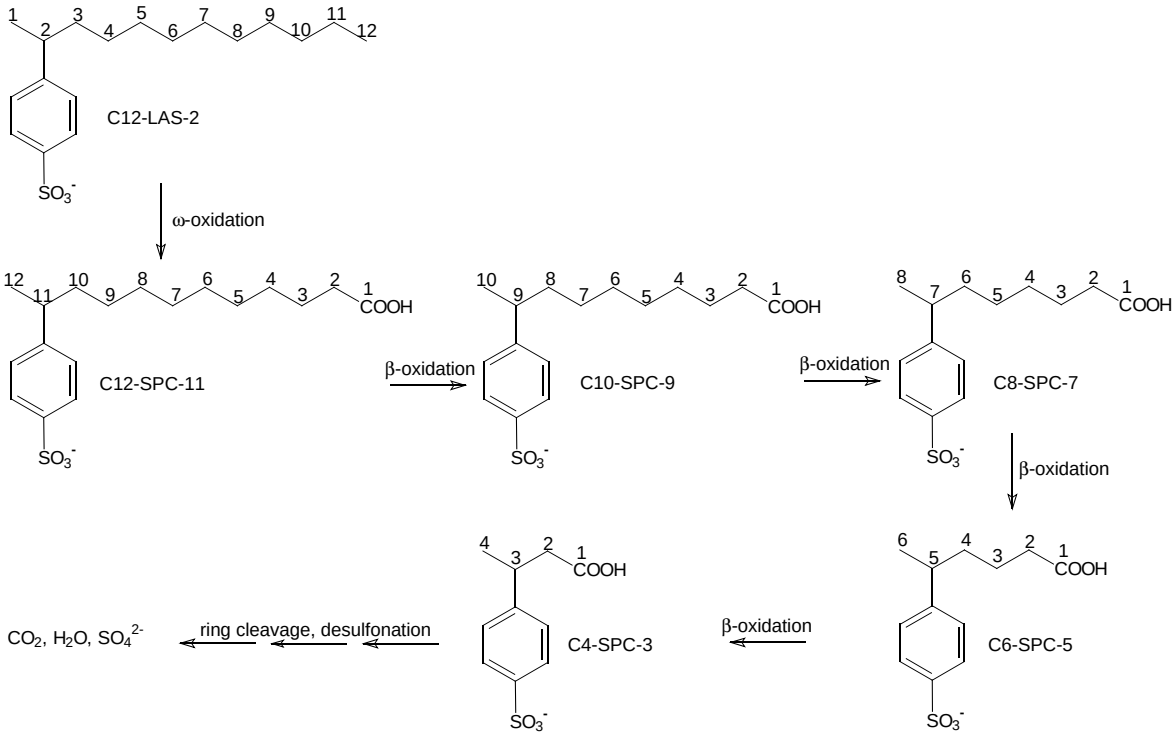

Fig. 4-8: Aerobic degradation pathway of LAS shown for C12-LAS-2.

It is generally accepted that ω-oxidation of LAS is followed by successive oxidative shortening of the alkyl chain by two-carbon units, termed as β-oxidation (**Fig. 4-8**) (Huddleston and Allred, 1963; Swisher, 1963b). The resulting very short-chain SPC are further broken down by ring cleavage, which is

considered the rate-determining step within the total process, and desulfonation resulting ultimately in complete degradation.

Although β-oxidation is certainly the most important mechanism for the destruction of the alkyl chain in LAS, some indication had been given that removal of a single carbon, i.e. α-oxidation, may occur to a minor extent. This alternative route was hypothesized to explain the isolation of C5-SPC as a by-product in the degradation of C12-LAS (Baggi et al., 1974) and the detection of a multitude of both C-even and C-odd SPC intermediates during the degradation of C11-LAS (Eggert et al., 1979).

Following the scheme in **Fig. 4-8**, from a homologue pure C12 starting compound, exclusively C-even SPC should have been formed. Direct support for the scheme was provided by Hrsak and Begonja (1998) and Dong et al. (submitted), who used pure cultures of bacteria, which attacked C12-LAS-2 and C12-LAS-3, respectively. The SPC formed were subject to chain shortening in pure or mixed culture (Hrsak and Begonja, 1998; Dong et al., submitted). The ring cleavage mechanism of one SPC, via 4-sulfocatechol, was established (Schulz et al., 2000) and this is suspected to be common in the degradation of LAS, because mixed cultures which degrade LAS contain high levels of 4-sulfocatechol 1,2-dioxygenase (Dong et al., submitted).

LAS congeners are usually racemic mixtures of optically active compounds (Swisher, 1987), and their transient degradative intermediates are also usually optically active (Kanz et al., 1998). Most of these intermediates are transients, but some can have long half lives (di Corcia et al., 1999a). Some LAS congeners are evidently subject to attack at both methyl groups on the alkyl chain as sulfophenyl dicarboxylates (SPdC) are also detected during the degradation of LAS (di Corcia et al., 1999a).

The observation of SPC and SPdC during the degradation of LAS; however, still does not prove either ω-oxygenation or β-oxidation of SPC as the sole significant mechanism, as Schleheck et al. (2000) realized when they isolated the first heterotrophic bacterium able to attack commercial LAS. They could not distinguish between ω-oxygenation and subterminal oxygenation with subsequent Baeyer-Villiger oxygenation and hydrolysis to yield an SPC. Furthermore, they were working with a pure culture and not with an environmentally realistic microbial consortium.

In order to resolve the ω-oxidation/β-oxidation mechanism of LAS, the following experimental conditions were necessary: (a) the material subjected to the biodegradation study had to be a homologue pure LAS ideally with very low amounts of structurally related by-products from synthesis as these might disturb the chemical analysis of LAS, and their intermediates might complicate the determination of SPC species in the test medium (Kölbener et al., 1996); (b) an experimental set-up based on a mixed microbial community from a natural setting was preferred over isolated cultures as this guaranteed a higher environmental relevance of the outcomes and (c) the applied analytical protocol included a non-discriminating sample preparation to allow an accurate quantification of individual degradation products.

4.1.3.1 C12-Linear alkylbenzene sulfonate – standard conditions

In a first attempt to examine the formation of individual SPC species, homologue pure C12-LAS was added to the FBBR at 100 mg L^{-1} (about 290 µmol L^{-1}). This elevated concentration, found in the aquatic environment only under extreme discharge conditions, was designed to yield sufficient amounts of intermediates for analysis without any sample enrichment that could results in analyte discrimination. In most studies carried out to determine the ultimate biodegradability of LAS (standard OECD tests) lower concentrations – typically in the range between 1 and 20 mg L^{-1} – were chosen to be closer to the levels

found in environmental compartments and also to rule out adverse effects on the biocoenosis caused by the surface-active agent. In comparison to higher organisms, bacterial populations are less sensitive to surfactants: Painter (1986) reported that LAS did not show inhibition at a concentration of 100 mg L^{-1} using the OECD activated sludge respiration inhibition method. Performing the same test procedure Verge and Moreno (1996) determined the toxicity EC_{50} (3 hr) of single LAS homologues to 500 to 1200 mg L^{-1}.

The FBBR was amended with 100 mg L^{-1} LAS, since a similar test concentration proved no problem in other work operating a trickling filter (Kölbener et al., 1995a and 1995b).

The samples collected from the test device were analyzed by LC-ESI-MS for the occurrence of SPC [1]. The C-even SPC were by far the most prominent intermediates, whereas the C-odd SPC (for evolution curve see below) were found at much lower amounts. In **Fig. 4-9** the molar concentrations of C-even SPC are plotted *vs.* time.

In the inset it can be seen that the SPC formation begins in earnest around day 8 or 9. There is a resulting build-up of C-even SPC. The concentrations of the two long-chain SPC, C12 and C10, reach a maximum on day 12. Seemingly, they are rapidly converted into the shorter-chain homologues. In all cases, C6-SPC is the most abundant homologue while its direct precursor, C8-SPC, and its successor, C4-SPC, are detected at substantially lower levels. The increase in C6-SPC is almost linear from the onset of formation until about day 19 when its formation slows down finally reaching a steady level at approximately 80 µmol L^{-1}.

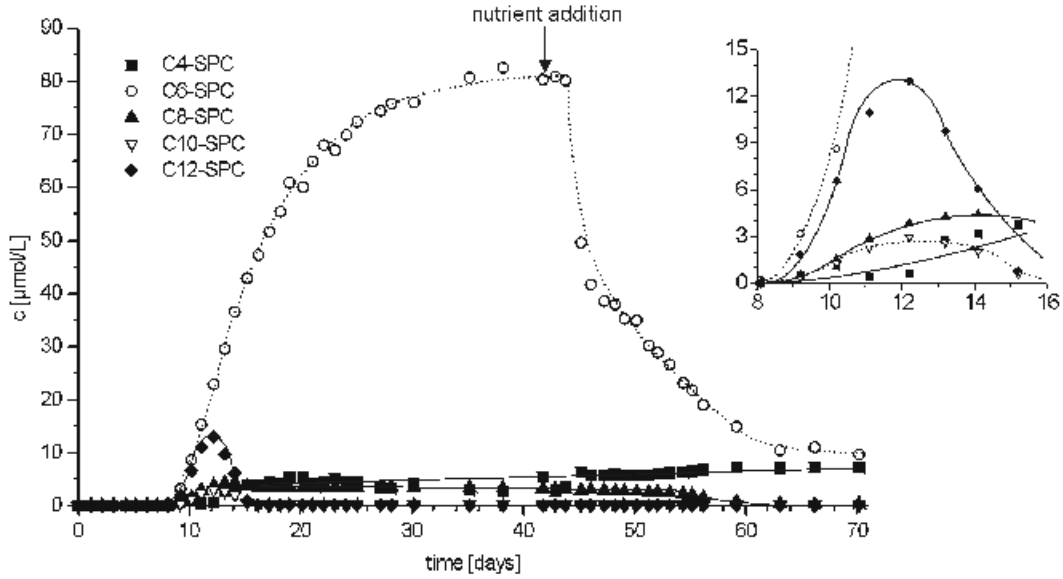

Fig. 4-9: Concentration profile of C-even SPC intermediates formed during biodegradation of C12-LAS on FBBR without initial nutrient supply. The inset shows the evolution between day 8 and 16.

This behavior is explainable by the lack of nutrients in the natural test medium, which were necessary for the metabolism of degrading micro-organisms, since upon addition of a nutrient solution on day 42[2] it

[1] The mass spectrometric identification of SPC is described in section 4.1.3.2.2.

[2] The solution was prepared according to OECD test methods; the composition is given on p. 27.

was observed that the microbial activity was apparently restored. After a short delay of two days, the biodegradation of SPC continued. As can be seen in **Fig. 4-9** the concentration of C6-SPC starts to decrease exponentially on day 44. In parallel, the level of C4-SPC increases slightly. Finally, the concentrations of all species reach relatively constant levels. With respect to C8-SPC, the level remains rather unchanged until the degradation of C6-SPC decreases. This can be attributed to the higher degradation rate of the latter compound.

The proof of the β-oxidation mechanism is visualized in **Fig. 4-10** showing an LC-ESI-MS chromatogram of the FBBR sample taken on day 8. The C-even SPC are identified as the major degradation intermediates. Under the applied chromatographic conditions several isomeric SPC, e.g. four phenyl isomers of C6-SPC, can be separated. The structures of the four theoretically possible C6-SPC are depicted in **Fig. 4-10** [1].

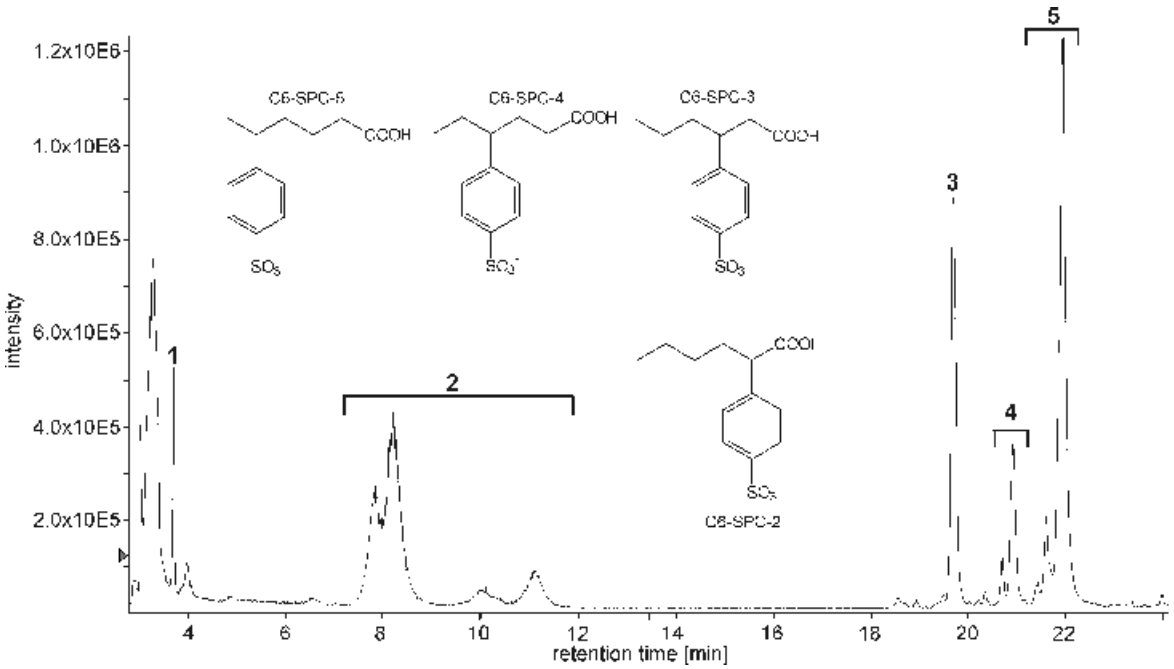

Fig. 4-10: (–)-LC-ESI-MS chromatogram of the FBBR-sample from degradation experiment taken after 8 days. Peak numbering: (1) C4-SPC, (2) C6-SPC, (3) C8-SPC, (4) C10-SPC, (5) C12-SPC. Only the time window where the SPC are eluting is shown; C12-LAS is eluting at 27.5 min under the selected condition. LC conditions as in **Fig. 4-4**.

As mentioned previously some SPC material remained undegraded. Its identity can be further elucidated taking a closer look at the isomer pattern of for instance C6-SPC whose constituents are very well separated.

In **Fig. 4-11** the temporal change in the three most abundant isomers, arbitrarily termed as *a*, *b* and *c*, is plotted semi-quantitatively for the run time between day 35 and the termination of the experiment on day 69 (**Fig. 4-9**). Prior to the addition of the nutrient solution (day 42) they show constant levels, but after reactivation of the micro-organisms it is observed that the single isomers exhibit very distinct degradation rates. Once the micro-organisms start to metabolize C6-SPC, the isomer *a* disappears within two days, whereas the degradation of *b* is somewhat slower. On day 63 this compound is no more

[1] The peak assignment is further discussed in the next section.

detectable. The level of isomer *c*, on the other hand, is unaffected by the nutrient addition. Its level remains constant, indicating a high resistance to further metabolization. At this point an assignment of the species *c* to one of the structures given in **Fig. 4-10** can be made tentatively on a mechanistic basis. C6-SPC-*c* might correspond to C6-SPC-5 since in this isomer the proximity of the sulfophenyl ring would make the attack more difficult than the previous steps, hence the next β-oxidation would be hindered (Swisher, 1987). For an unequivocal elucidation it would be necessary to synthesize all possible C6-SPC isomers.

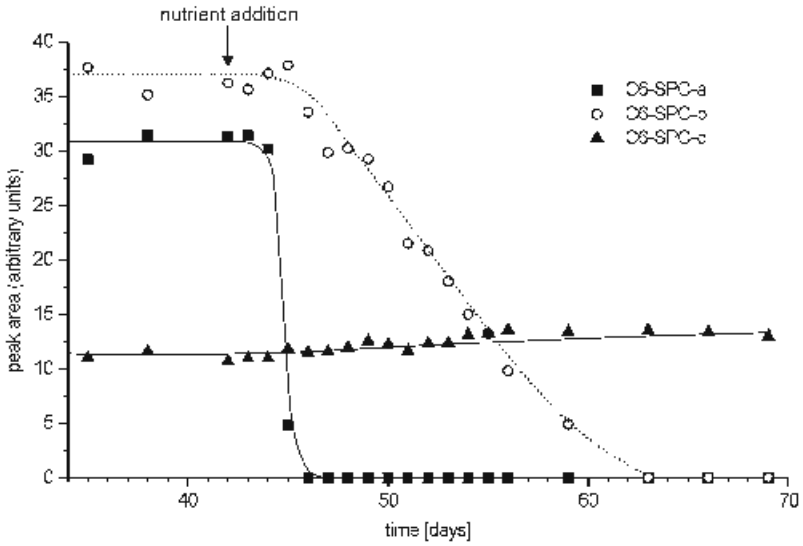

Fig. 4-11: Profile of major C6-SPC isomers, addition of nutrients on day 42. (Of the four isomers separated in **Fig. 4-10**, the first, second and fourth peak were integrated.)

In regard to the detection of C-odd SPC in the present test solution, their occurrence is explainable by the fact that the C12-LAS used contained an impurity of some 0.5 % of C-odd LAS. The concentration profile of the C-odd-SPC is displayed in **Fig. 4-12**.

The long-chain SPC C11 is not detectable, while the levels of C9- and C7-SPC are below 0.3 µmol L^{-1}. The temporal change in C5- and C7-SPC concentrations resemble qualitatively that of C6- and C8-SPC, respectively. C5-SPC is accumulated in the test liquor up to about 5 µmol L^{-1}, obviously because of insufficient nutrient supply. Upon addition of the nutrient solution the concentration drops rapidly until a certain level where a recalcitrant fraction is maintained at a constant level. The degradation of C7-SPC in turn is retarded – as in case of C8-SPC – and a notable decrease begins only at day 58.

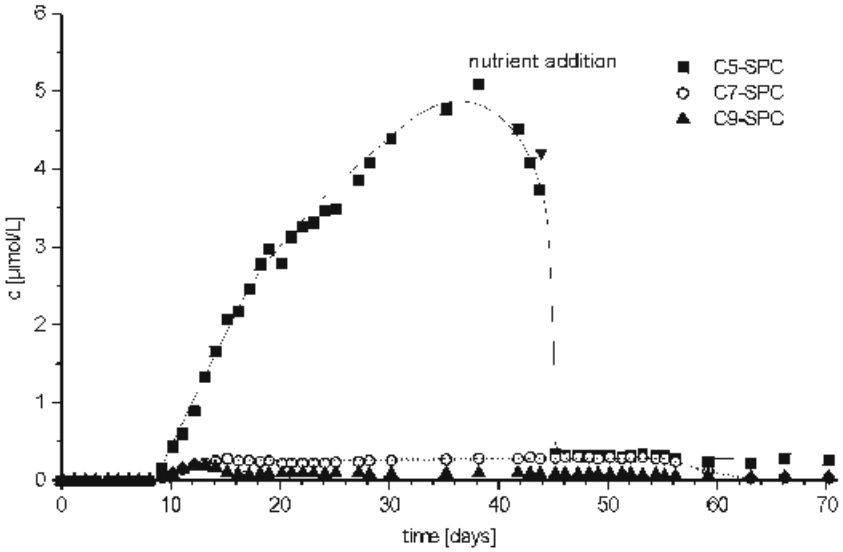

Fig. 4-12: Concentration profile of C-odd SPC intermediates formed during biodegradation of C12-LAS on FBBR without initial nutrient supply.

4.1.3.2 C12-Linear alkylbenzene sulfonate – optimized conditions

The outcomes of the first LAS degradation study, with a high test concentration of 100 mg L^{-1}, proved the necessity for an additional nutrient supply to assure optimum growth conditions of the involved bacteria. Thus, it was one objective in a further experiment to speed up the degradation process by addition of a nutritive solution at the beginning of the assay. A second aspect, which was investigated under these optimum circumstances, was the removal of the parent compound C12-LAS. To this end, it was imperative to distinguish between primary degradation and losses due to adsorption in the system used. Although adsorption had not posed problems in earlier studies using the same equipment – even when assaying relatively nonpolar organic compounds (Knepper et al., 1999a) – the inherent property of surfactants to adhere to surfaces might have been a crucial factor. For this reason two experiments were carried out in parallel in the test devices using identical starting concentrations of C12-LAS (100 mg L^{-1}). To trace the sorption behavior the test medium in one experiment was supplemented with 5 % formaldehyde prior to spiking the surfactant, while the conditions for the other experiment were the same as in the previous section (4.1.3.1).

Sorption of linear alkylbenzene sulfonate

The eluate from the FBBR inhibited with formaldehyde shows an initial rapid loss of LAS followed by a steady loss until day 7, after which the concentration of LAS stays stable at about half the value in the reservoir (**Fig. 4-13**). No degradative products are detected in this experiment, so the loss is attributed to sorption, although precipitation of magnesium and calcium salts might also contribute to the loss of the anionic surfactant (ECOSOL, 1999).

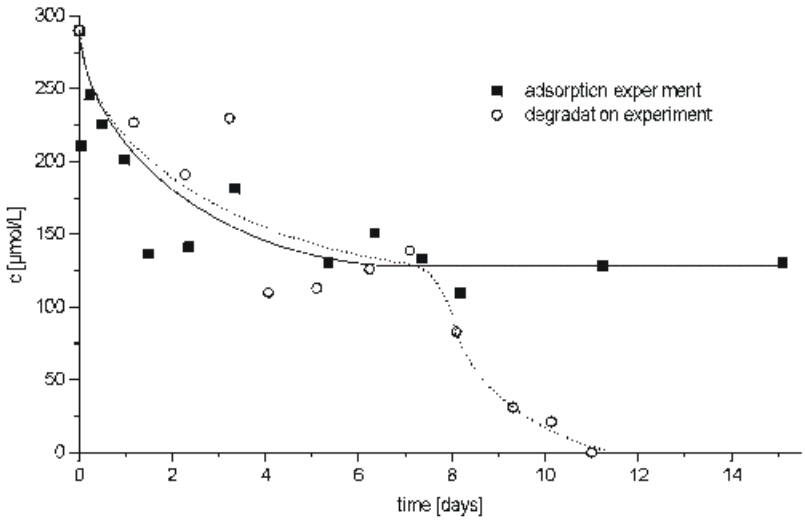

Fig. 4-13: Concentration profile of C12-LAS on FBBR during adsorption and degradation experiment at a surfactant concentration of 100 mg L^{-1}.

Degradation of linear alkylbenzene sulfonate and formation of sulfophenyl carboxylates

The concentration of C12-LAS in the eluate from the FBBR is similar to that in the sorption experiment until day 7, after which the compound disappears within 4 days (**Fig. 4-13**). No degradative intermediates are detected before day 7, which is attributable to acclimation of the micro-organisms to the substrate. Thereafter, degradative intermediates are observed (**Fig. 4-14**), with the C-even C12-, C10-, C8-, C6- and C4-SPC being prominent. The onset of biodegradation is seen to be at day 7, with extensive excretion of all five sets of C-even SPC visible at day 8, concomitant with significant degradation of LAS (**Fig. 4-13**). The formation of C12-SPC can occur only if ω-oxygenation is catalyzed, so the presence of this reaction is proven in **Fig. 4-14** (especially clear in the inset).

Varying concentrations of the C-even SPC are observed during the experiment. The C12- or C10-SPC are never exceed 0.4 µmol L^{-1}. The C8- or C4-SPC reach a maximum concentration about ten-fold higher, whereas the C6-SPC rise to 37 µmol L^{-1} (**Table 4-2**). The concentrations towards the end of the experiment are much lower, usually well below 0.1 µmol L^{-1}. These alterations in the course of an experiment presumably reflect changes in the microbial population and the affinities of different organisms for the surfactants as a carbon source.

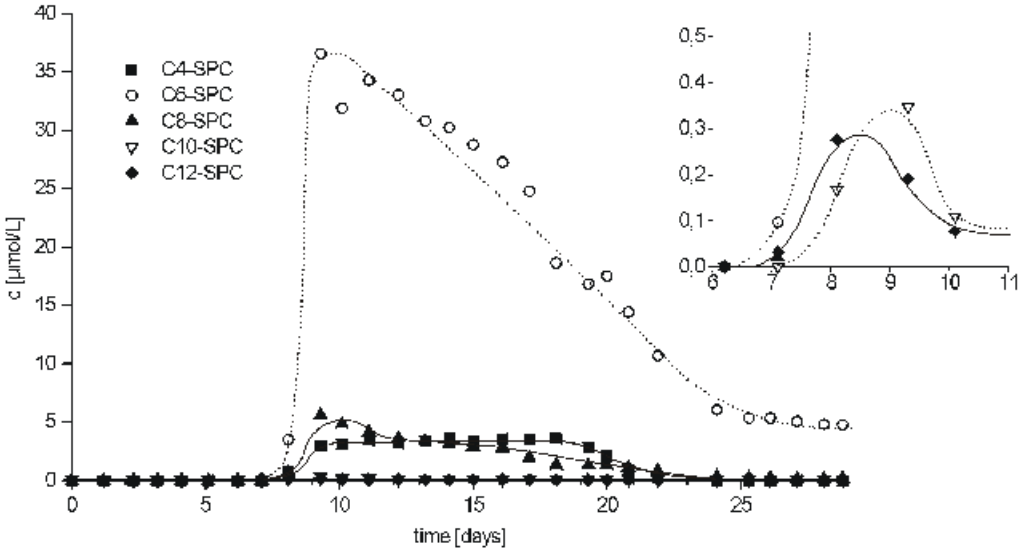

Fig. 4-14: Concentration profile of C-even SPC intermediates formed during biodegradation of C12-LAS on FBBR. The inset shows the evolution of C6-, C10 and C12-SPC between day 6 and 11.

C-odd SPC (C9, C7, and C5) are also observed in the experiment (**Fig. 4-15**; **Table 4-2**). They presumably arise from C11-LAS detected as an impurity in the C12-LAS. With the exception of a transient accumulation of C5-SPC, they are never observed above 1 µmol L^{-1}. The intermediate build-up of about 1.8 µmol L^{-1} C5-SPC on day 9 is due to the excretion of one isomer, which is well separated under the chromatographic conditions (not shown). Seemingly, after release of this isomer into the test solution, it is taken up again into the cells to be further metabolized. By the next day the isomer has disappeared almost quantitatively, while at least one other isomer remains in the test liquor at a relative constant level.

Table 4-2: Molar concentrations of the LAS intermediates, SPC and SPC-2H, in curve maximum and at endpoint of experiment. Spiking concentration: 290 µmol LAS L^{-1}.

	SPC		SPC-2H		SPdC	Desulfon. metabolites
Alkyl chain length (Cn)	c_{max} [µmol L^{-1}]	c_{final} [µmol L^{-1}]	c_{max} [µmol L^{-1}]	c_{final} [µmol L^{-1}]	c [µmol L^{-1}]	c [µmol L^{-1}]
4	3.6	n. d.	n. d.	n. d.	n .d.	n .d.
5	1.7	0.6	n. d.	n. d.	n .d.	n .d.
6	37	4.8	0.67	0.03	n .d.	n .d.
7	0.6	0.04	n. d.	n. d.	n .d.	n .d.
8	5.6	0.1	0.25	0.16	n .d.	n .d.
9	0.1	0.04	n. d.	n. d.	n .d.	n .d.
10	0.4	0.06	0.07	0.03	n .d.	n .d.
11	n. d.	n. d.	n. d.	n. d.	n .d.	n .d.
12	0.3	0.02	0.03	0.01	n .d.	n .d.

n. d.: not detected

In conclusion, it has been observed that (a) the kinetics of the degradation of individual SPC homologues decreases with shortening of the oxidized side chain, i.e. higher concentrations of metastable SPC with shorter alkyl chains are detected and (b) within a suite of phenyl isomers of each SPC homologue some species are quite resistant to further degradation. Hence, an accumulation in the test liquor occurs dominated by shorter chain SPC.

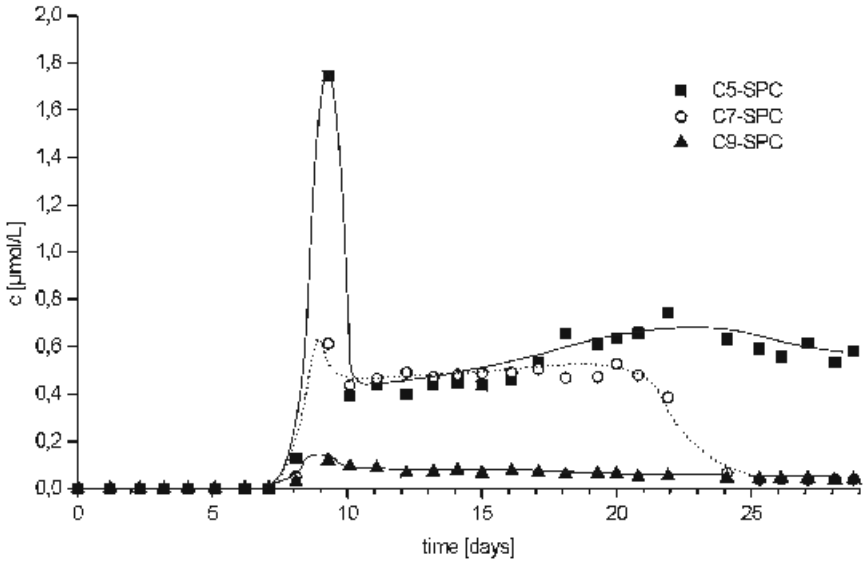

Fig. 4-15: Concentration profile of C-odd SPC intermediates formed during biodegradation of C12-LAS on FBBR.

As for result (a), similar outcomes were reported in other biodegradation assays conducted under controlled conditions regarding the origin of the microbial community and the purity of LAS used. Divo and Cardini (1980) examined the metabolism of single C10- to C13-LAS homologues using an isolated micro-organism belonging to the genus *Pseudomonas*. Depending on the identity of the starting compound, the degradation intermediates had between four and seven carbon atoms in the side chain. Another bacterial strain belonging to the group of α-proteobacteria was isolated from a trickling filter by Schleheck et al. (2000) and investigated for its capacity to degrade C12-LAS-3. Mass spectrometric analysis by MALDI-ToF-MS identified C6-SPC-4 as principal degradative product. Hrsak (1995) made use of a mixed methanotrophic-heterotrophic culture to degrade a C11-LAS mixture consisting of all five possible positional isomers. The major intermediates were C5- and C7-SPC identified by comparison of HPLC retention times with authentic standards. A commercial LAS mixture was submitted to biodegradation in river water used both as a source of organisms and as the medium (Taylor and Nickless, 1979). A series of transient metabolites were formed in the mixture and assigned as C4- to C7-SPC. Cavalli et al. (1996) tested the biodegradability of commercial LAS with a modified OECD 301E procedure over a period of 80 days. The LAS concentration was restored every 4 days with fresh substrate. At the end of the test, four SPC corresponding approximately to 85 % of total SPC were structurally identified by NMR as C6-SPC-3 and C7-SPC-3, while two SPdC (C5 and C7) were likewise recovered.

With respect to (b) the pronounced stability of some degradation intermediates towards further breakdown might be due to positional isomers, in which the vicinity of the sulfophenyl ring is reached and thus the further β-oxidation impeded. This has already been discussed above for the four possible isomers of C6-SPC.

In the literature the persistency of LAS breakdown products was addressed in several studies. The remaining fraction was primarily attributed to metabolites of manufacturing by-products in technical LAS blends used for the assays. Kölbener et al. (1995a) kinetically examined the degradation of commercial LAS in a trickling filter. 15 % of the initially added LAS-carbon remained as DOC in the eluate of which roughly 50 % was specifically identified by HPLC-UV and IR spectroscopy as carboxylated DATS and SPC. In a subsequent study it was shown that the source of the remaining refractory organic carbon (3 to 14 %) was carboxylated DATS and partially degraded ABS (Kölbener et al., 1995b). The same authors identified a series of recalcitrant arylsulfonates originating from biodegradation of pure DATS and ABS (Kölbener et al., 1996). The significance of resistant metabolites of LAS by-products was also claimed by di Corcia et al. (1999a), who identified along with regular SPC, SPdC and mono- and dicarboxylated DATS using mass spectrometry.

Regarding the findings of the own studies, an interference with mono-carboxylated DATS can be ruled out as the molecular ions of these intermediates have an *m/z* of two units lower than that of SPC with the same number of aliphatic carbon atoms [1]. Moreover, they do not produce the fragment *m/z* 183, which was always detected along with the $[M–H]^-$ of SPC [2].

According to the information of the manufacturer of the C12-LAS assayed, it contained 2.9 % of non-linear LAS (Moreno, personal communication). It seems most reasonable that this impurity is made up of iso-LAS, containing a methyl branch located on one of the carbon atoms of the alkyl chain (for structure refer to **Fig. 4-2**). Although β-oxidation is not prevented by the presence of the tertiary carbon, the biodegradation rates are characterized by slow initial phases (Willets, 1974). Hereafter, chain shortening may proceed splitting of one propionate moiety, in place of a acetate moiety. This step is suggested to require alterations in the enzymatic system of the cells (Swisher, 1987).

Support for this hypothesis on iso-LAS is given through the findings of Nielsen et al. (1997) reporting that most of the iso-LAS isomers in a degradation study carried out with radiolabelled substrate underwent ultimate biodegradation (79 to 90 %) but released some (10 to 20 %) of their carbon as water soluble intermediates. Furthermore, Kölbener et al. (1996) could mass spectrometrically identify structures corresponding to iso-C5-SPC, which formed part of the organic residue in LAS biotransformation. In studies on commercial mixtures of LAS, di Corcia et al. (1999a) monitored the formation of different SPC species indicating that iso-SPC exhibited slower degradation rates than the later eluting SPC.

The relevance of the stable compounds originating from LAS breakdown and the elucidation of their possible structures is further discussed in section 4.1.3.3 describing also the environmental significance of SPC intermediates steming from commercial LAS degradation.

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[1] The mass spectrometric identification of LAS metabolites is given in 4.1.3.2.2.

[2] Contrary to LAS and SPC yielding the typical fragment *m/z* 183 corresponding to 4-styrenesulfonate, DATS and their carboxylated intermediates form the diagnostic ion *m/z* 209. This can be assigned as the fragment carrying the intact tetralin structure with its two six-membered rings from which the hydrocarbon chains have been cleaved.

4.1.3.2.1 Novel metabolites from the β-oxidation of sulfophenyl carboxylates

Besides the evidence from the spirals of β-oxidation in C12-LAS biotransformation, further support for β-oxidation cycles would be gathered if other intermediates occurring in this pathway were detectable. β-Oxidation, though known for decades, is still a subject of active research (Eaton et al., 1996; Wanders et al., 1999). And the class of compound that has been identified, albeit in mammalian systems, is the α,β-unsaturated carboxylate.

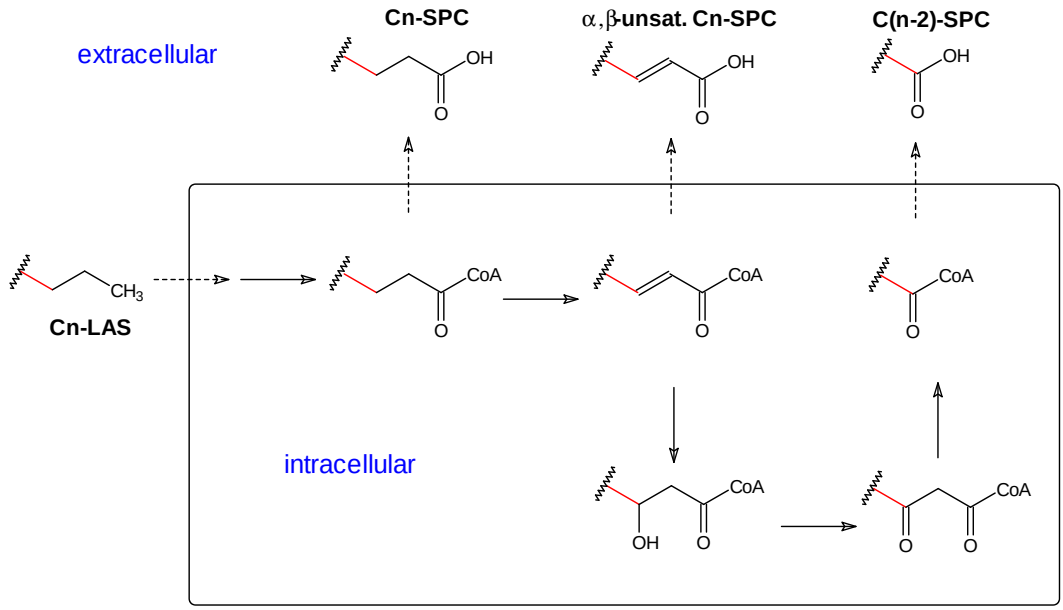

Fig. 4-16: Presumed major pathway for intracellular aerobic biodegradation of LAS (as acetyl-CoA-derivatives) to SPC via ω-oxidation followed by successive oxidative shortening of the alkyl chain by two carbon units (β-oxidation). Intermediates of β-oxidation, such as SPC-2H after enzymatic dehydrogenation are also transported out of the cell after cleavage of the CoA-ester.

Indications for the microbial formation of analogous species in LAS metabolism were found by Kruse (1998) during biotransformation of commercial LAS surfactant on an FBBR, but the low concentrations in the test liquor of the tentative metabolites eluting under the applied RP-HPLC conditions somewhat earlier than the normal SPC did not permit acquisition of full scan mass spectra needed for unequivocal identification. Further evidence for the intermediate formation of a double bond in the alkanoate moiety was reported by Bird (1972). During biodegradation of C11-LAS by a bacterial strain, a new UV adsorption band centered near 260 nm, which was assumed to result from a double bond. A definite confirmation could not be provided in the previous work.

Thus, it was an objective of the current work to attempt detection of α,β-unsaturated SPC (SPC-2H) during the degradation of LAS, since this would be an indicator of β-oxidation (**Fig. 4-16**).

An SPC-2H would have an *m/z* two mass units below that of the corresponding SPC, and compounds of this type were described in the degradation of impurities in LAS, the monocarboxylated DATS (di Corcia et al., 1999a; Field et al., 1992). However, a definite distinction between the known DATS carboxylates (di Corcia et al., 1999a) and the putative SPC-2H could be provided by LC-ESI-MS allowing the structure assignment via the recorded mass spectra.

The postulated SPC-2H (for structure elucidation see below) could be detected in the FBBR samples (**Fig. 4-17**). These C-even compounds were present from about 1 to 10 % of the concentrations of the corresponding SPC (**Table 4-2**, p. 58). No C-odd SPC-2H were detected, presumably due to the low levels of C-odd LAS and C-odd SPC present. Four SPC-2H were detected, C12, C10, C8 and C6. These correspond to the C-even SPC observed which could undergo β-oxidation, so there is direct support for β-oxidation of all relevant SPC. The route of metabolism of LAS observed in the FBBR is thus ω-oxidation followed by β-oxidations.

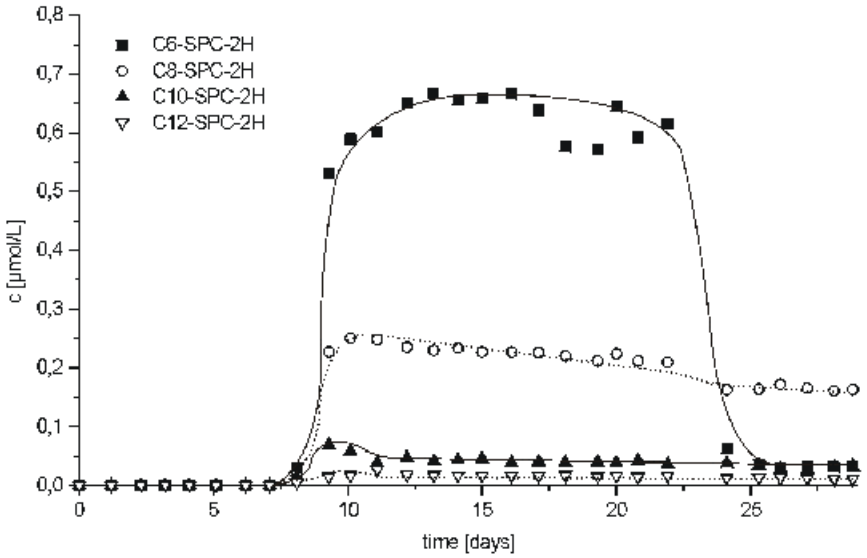

Fig. 4-17: Concentration profile of SPC-2H intermediates formed during biodegradation of C12-LAS on FBBR.

The fact that no C4-SPC-2H is detected, i.e. that the β-oxidation cycle is terminated at C4-SPC, is indicative of a different mechanism in the further degradation process. At this stage desulfonation or ring cleavage begin to dominate (see **Fig. 4-8**): Schulz et al. (2000) grew enrichment cultures on C4-SPC-2 yielding a pure culture of a degradative bacterium capable of metabolizing sequentially the enantiomeric forms of C4-SPC-2. 4-Sulfocatechol was identified as intermediate, which subsequently underwent *ortho*-cleavage. Desulfonation of the same compound was accomplished by another organism, a species of *Pseudomonas putida*, yielding the 4-hydroxyphenyl derivative (Kertesz et al., 1994). A lack of adequate enzymes for the biodegradation of C4-SPC-3 originating from the biotransformation of C12-LAS-2 in fish resulted in an accumulation to about 70 % of the molar concentration of the parent compound (Tolls et al., 2000).

Overall, there is no need to suggest alternative pathways, because the theory is adequate to cover all the observations. There is no need to postulate subterminal oxygenation; if it is present in the FBBR, there is no evidence for it. The better-known attack on both methyl groups of LAS give SPdC is also not observed here.

4.1.3.2.2 Mass spectrometric identification of metabolites

The unambiguous identification of both SPC and SPC-2H was achieved through the interpretation of MS spectra acquired in negative ESI. The mass spectrometric parameters were set so that an OR of –90 V was applied in order to gather maximum information from the fragmentation pattern of SPC and derivatives (**Table 4-3**).

Table 4-3: Observed deprotonated molecular ions $[M–H]^-$ and characteristic fragmentation pattern of SPC, SPC-2H and SPdC.

	SPC		SPC-2H		SPdC	
Alkyl chain length (Cn)	$[M–H]^-$ (*m/z*)	Fragment ions (*m/z*)	$[M–H]^-$ (*m/z*)	Fragment ions (*m/z*)	$[M–H]^-$ (*m/z*)	Fragment ions (*m/z*)
4	243	183				
5	257	183, 197	255 [a]	145, 195, 209, 211		
6	271	183, 211	269	145, 195, 209, 211, 225	301 [a]	183, 239,241
7	285	183, 225	283[a]	145, 183, 195, 209, 211, 239	315 [a]	183, 253, 255
8	299	183, 281	297	145, 183, 195, 209, 211, 253	329 [a]	183, 267, 269
9	313	183, 295	311 [a]	145, 183, 195, 209, 211, 267	343 [a]	183, 281, 283
10	327	183, 309	325	145, 183, 195, 209, 211, 281		
11	341[a]	183, 323	339 [a]	145, 183, 195, 209, 211, 295		
12	355	183, 337	353	145, 183, 195, 209, 211, 309		

[a] only detected in samples from pure culture experiment (Dong et al., submitted)

Regarding the fragmentation pattern observed, there is generally a loss of *m/z* 60 corresponding to the elimination of acetic acid for the short-chain SPC (= C7) or a loss of water, *m/z* 18, for the SPC with a chain length >C7. The most characteristic fragment ions occur at *m/z* 183, corresponding to 4-styrenesulfonate, and *m/z* 119, which can be assigned to 4-styrenephenolate (**Fig. 4-18**). The spectra of individual isomers of a set of SPC homologues differ only slightly making it difficult to assign a definite structure, i.e. the position of the phenyl ring and the location of the carboxylic group.

The SPC-2H eluting in the total ion chromatograms under RP conditions adjacent to the SPC signals with a slightly shorter retention time exhibit a similar fragmentation pattern resulting from a deprotonated molecular ion being *m/z* 2 lower than the ones calculated for the SPC (**Table 4-3**).

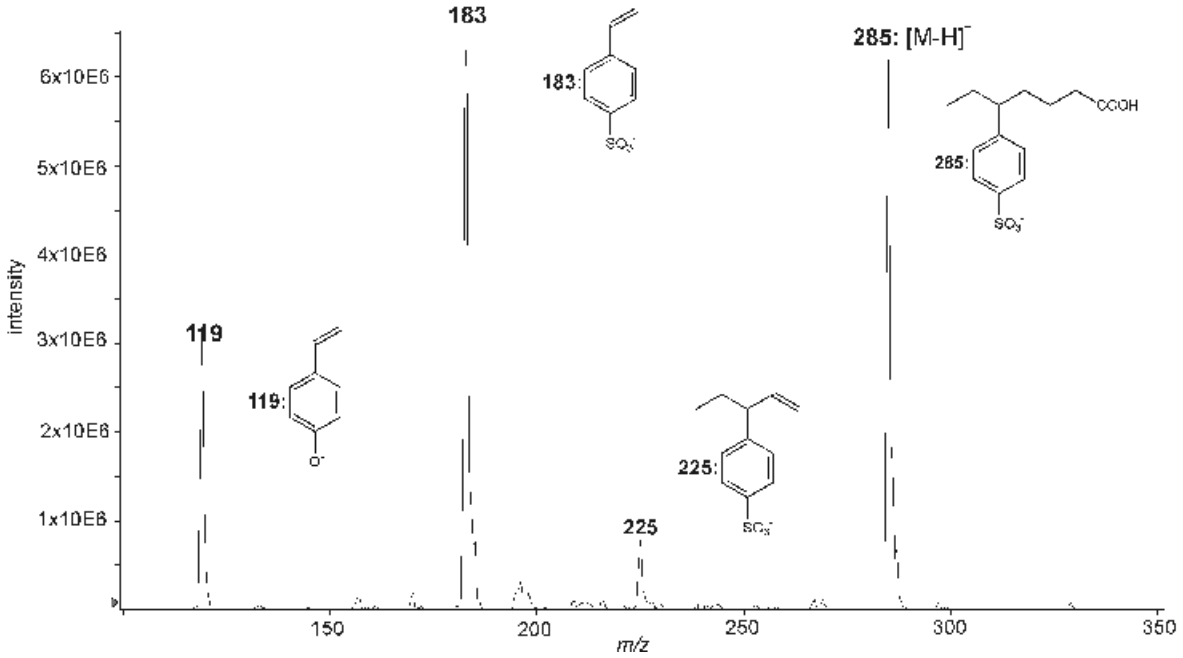

Fig. 4-18: (–)-LC-ESI-MS spectrum of C7-SPC and assignment of the obtained fragments (OR: -90 V). The position of phenyl ring in the ions *m/z* 285 and 225 is arbitrarily chosen.

In **Fig. 4-19** the mass spectrum of C5-SPC-2H is shown as an example of all other SPC-2H-species, which also yield characteristic fragmentation pattern in negative LC-ESI-MS. Besides the deprotonated molecular ion there is always a loss of *m/z* 44 corresponding to the elimination of CO_2 as well as formation of the characteristic fragment ions at *m/z* 211, 209 and 195 corresponding to olefinic 4-benzene sulfonates and *m/z* 145, resulting from the olefinic 4-benzene phenolate. The fragment ion dominating the mass spectrum acquired from the SPC-species, *m/z* 183, which corresponds to the 4-styrenesulfonate, cannot be found in the spectra obtained from the SPC-2H-species <C7-SPC since the double bond already present in the precursor molecule excludes this fragmentation step.

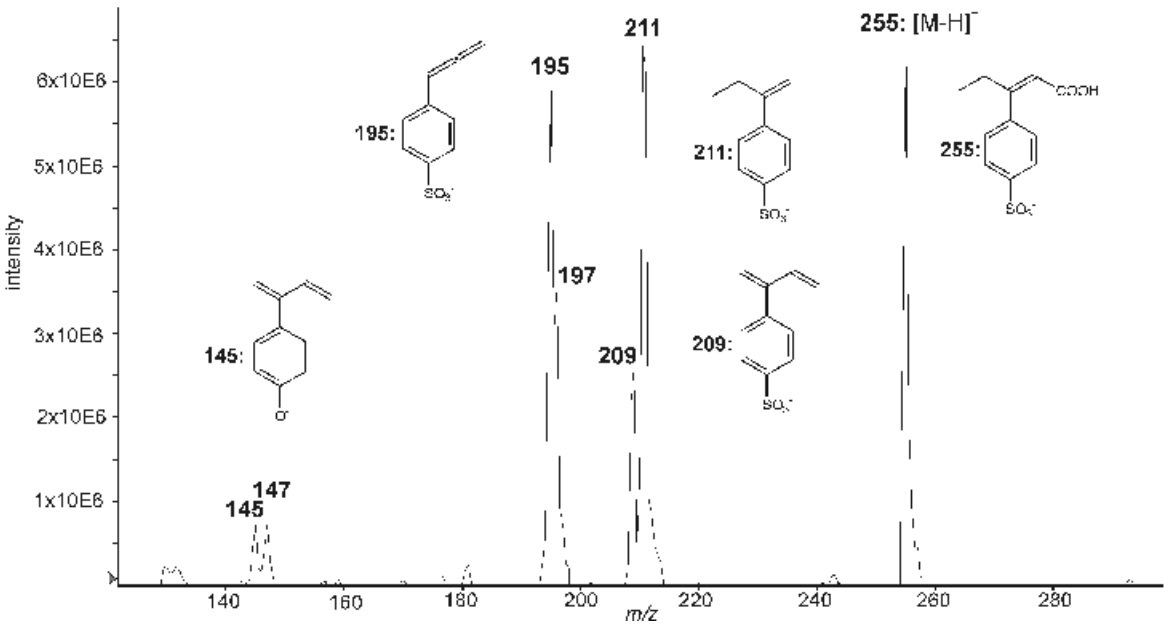

Fig. 4-19: (–)-LC-ESI-MS spectrum of C5-SPC-2H and assignment of the obtained fragments (OR: -90 V). The position of phenyl ring in the ions *m/z* 255 and 211 is arbitrarily chosen.

Apart from SPC and SPC-2H, no other biodegradation products occurring in the degradation pathway as depicted in **Fig. 4-16** nor the reported SPdC were found in this study. This may in part be due to the polarity and/or stability of compounds such as CoA-esters, which makes it almost impossible for them to be excreted out of the cell. Moreover, GC-MS was applied to examine FBBR samples for the presence of desulfonated metabolites [1], which must be formed in the sequence of (ultimate) biodegradation (Swisher, 1987). The lack of detection of such intermediates in the present work (**Table 4-2**) might be explained by the fact that desulfonation is likely to occur at a late stage of biodegradation (Cordon et al., 1970; Taylor and Nickless, 1979).

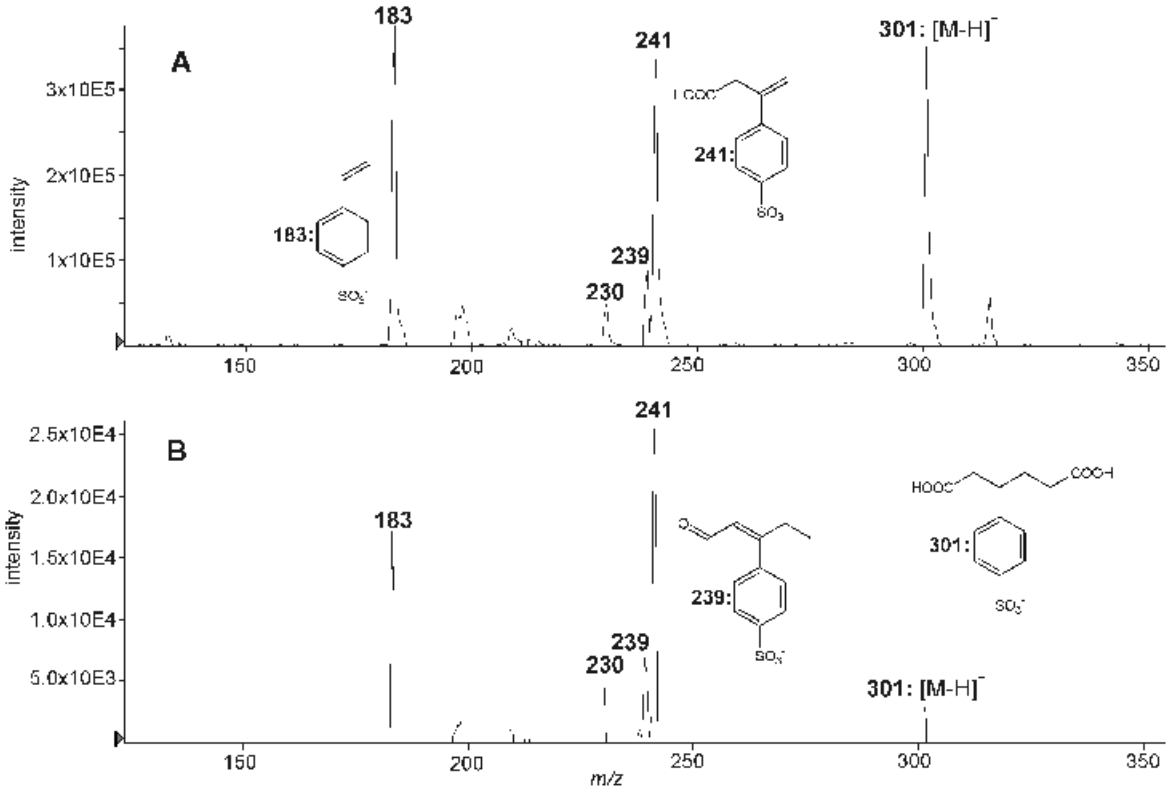

Fig. 4-20: (–)-LC-ESI-MS (**A**) and -MS/MS spectra of C6-SPdC (**B**) and assignment of the obtained fragments (OR: -90 V). The position of phenyl ring in the ions *m/z* 301, 241 and 239 is arbitrarily chosen.

The biological significance of the presented outcomes – particularly the detection of SPC-2H – is emphasized by paralleling studies conducted on the degradation of C12-LAS using a pure culture instead of a consortium of micro-organisms (Dong et al., submitted). In that case, degradation yielded SPdC in addition to SPC and SPC-2H.

Metabolite identification in the study by Dong et al. (submitted) was achieved by LC-ESI-MS operated in negative ionization mode, and the characteristic fragments from that work are compiled for comparative purposes together with the data obtained from the FBBR samples in **Table 4-3**. The mass spectra are quite similar to the ones of the SPC (**Fig. 4-20**): besides the deprotonated molecular ion there are always losses of *m/z* 60, which corresponds to the elimination of acetic acid, as well as *m/z* 62

[1] FBBR samples (5 mL) were extracted with 500 µL hexane and an aliquot of the organic phase injected into the GC-MS acquiring full scan spectra in the mass range from *m/z* 40 to 600.

corresponding to the simultaneous elimination of CO_2 from one carboxylic group together with water from the second carboxylic group located at the opposite end of the alkyl chain. Since the latter fragmentation pattern is only possible for the SPdC, it can be used as a fingerprint for such compounds in the total ion chromatogram. Additionally, the characteristic fragment ion at *m/z* 183 is formed (**Table 4-3**). As for the SPC and SPC-2H, the spectra from the individual SPdC-isomers cannot be distinguished from each other.

In order to confirm the individual metabolite identity assignments and to check for erroneous interpretation due to possible overlapping signals, all fragmentation patterns were proved by acquiring LC-ESI-MS/MS spectra. These were almost identical to the ones obtained by single-MS, as can be seen by comparing exemplary the MS and MS/MS acquired from C6-SPdC (**Fig. 4-20**). Since there was no gain in sensitivity applying tandem-MS, and due to the effect of having sufficiently separated peaks, experiments done for quantification purposes were performed with the single MS. In addition, MRM routinely applied to quantitative measurements by tandem-MS demands for the optimization of relevant parameters of the collision cell. Hence, the analysis on the basis of one or even more fragments is not accurate without having authentic standards, i.e. without having the possibility of prior instrumental tuning.

4.1.3.2.3 Isomer pattern of sulfophenyl carboxylates

To illustrate the different fate of individual SPC isomers in the FBBR experiment – this has been debated above for C6-SPC in the first LAS degradation study after addition of the nutrient solution (see p. 55) – LC-ESI-MS-chromatograms from the samples taken at day 8, 11, 18 and 26 after spiking of C12-LAS were compared with regard to peak pattern. To this end, C6-SPC and C8-SPC were selected since they exhibited the highest sensitivity.

As can be clearly seen, there are at least four different, sufficiently separated C6-SPC-species, occurring in the sample taken at day 8 (**Fig. 4-21**, left side). The individual signals, assigned as *a* to *d* according to the order of elution, all correspond to different isomers of C6-SPC, which behave differently with respect to biodegradation rates and individual final fate. In the sample taken after 11 days, in which the relative amount of the C6-SPC-species is the highest from the samples analyzed, the C6-SPC-isomer *a* is predominant, whereas the isomers *b* and *c* are less abundant compared to the isomer *d*. After 18 days the isomers *a* and *b* are detected at almost equal amounts and after 26 days the apparently recalcitrant isomer *d* is dominates the mixture. In **Fig. 4-21** (right side) the same biodegradation behavior is shown for the different C8-SPC-species. In general, they behave similar to the C6-SPC-isomers, except for the fact, that in the beginning after 8 days almost only the isomer *b* is present. In this case after 26 days isomer *a* dominates the chromatogram with lesser amounts of *b* and *c*.

These observations are in line with earlier observations indicating that those SPC isomers with a greater resistance to further degradation stem from (C12-)LAS in which the phenyl ring is attached to central positions of the alkyl chain or oxidized on the shorter side of the alkyl moiety (Swisher, 1987). In the course of microbial degradation subsequent shortening of the alkyl chain via β-oxidation becomes hindered in close proximity to the aromatic ring.

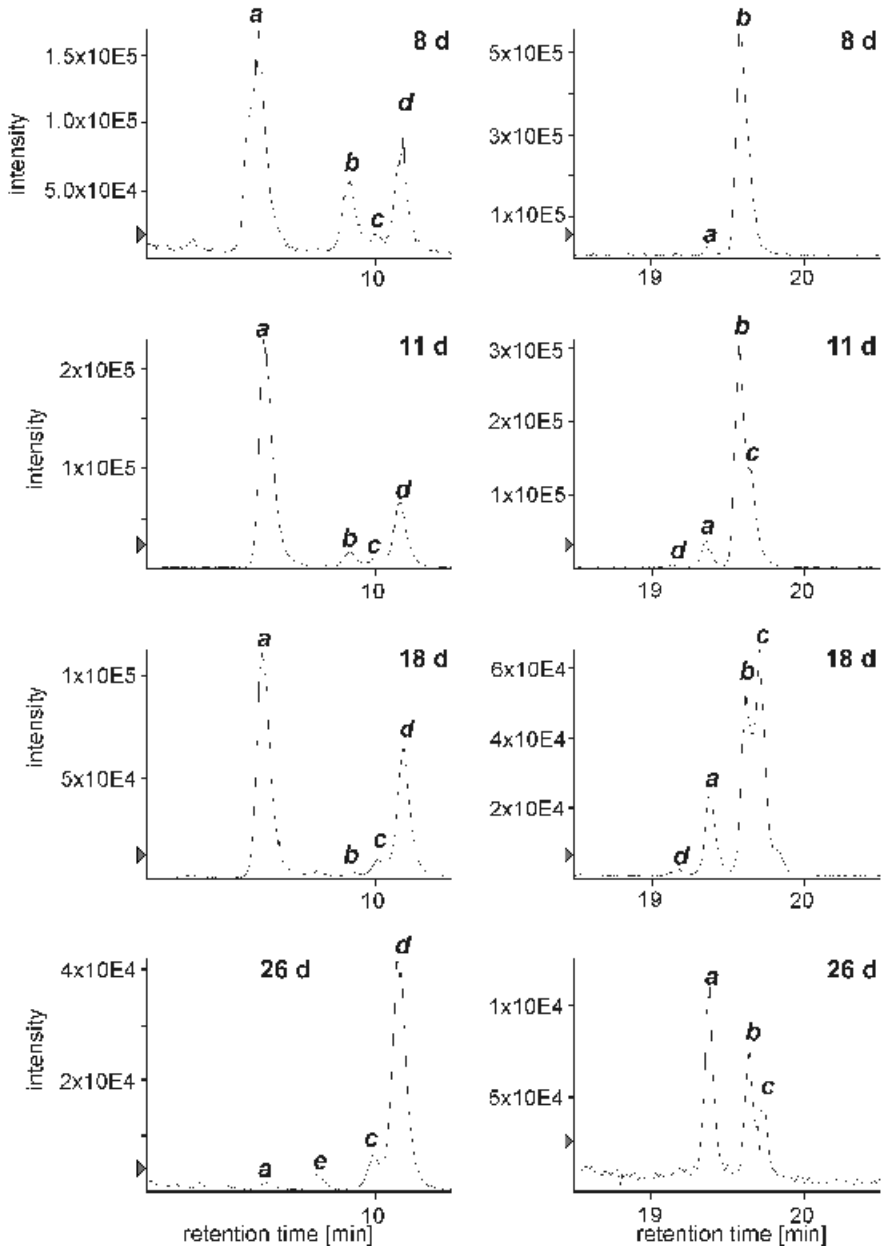

Fig. 4-21: (–)-LC-ESI-MS extracted ion chromatograms of C6-SPC (*m/z* 271; left side) and C8-SPC (*m/z* 299, right side) from samples of FBBR taken after 8, 11, 18 and 26 days after spiking of C12-LAS. Assignment of the individual recalcitrant species according to order of elution and formation (*a* to *e*). LC conditions as in **Fig. 4-4**.

4.1.3.3 Commercial linear alkylbenzene sulfonate– effect of adaptation

A third degradation experiment was performed in order to confirm the build-up of recalcitrant SPC species by assaying a commercial LAS mixture instead of a homologue pure LAS. The choice of an LAS blend would impart higher relevance to the aquatic environment, which is generally exposed to complex mixtures of the anionic surfactant. Furthermore, information may be obtained on the impact of acclimation on the qualitative and quantitative formation of SPC intermediates as well as on the behavior

of recalcitrant phenyl isomers. To this end, the river water in the FBBR was spiked with LAS at 10 mg L^{-1}. Compared to the preceding assays the lower concentration should allow reduction of the test duration prior to reach the final state with constant SPC concentrations. As those degradative products, which were detectable only at high substrate concentration (SPC-2H), were not of interest here, 10 mg L^{-1} were estimated to be sufficient for the specific purpose.

The FBBR samples were directly subjected to LC-ESI-MS analysis without further treatment and SPC were semi-quantitatively determined via absolute peak area. The dependence of LAS primary degradation on the alkyl chain length and position of phenyl attachment was not monitored since this has been thoroughly elucidated since the mid-1960s (Huddleston and Allred, 1963; Larson, 1990; Mann and Reid, 1971; Perales et al., 1999a; Moreno et al., 1998; Terzic et al., 1992).

In **Fig. 4-22** the temporal evolution of C-even (A) and C-odd SPC (B) is plotted. Their formation starts at day 5 with the concomitant release of the entire suite of examined SPC. Then, the curves run through a maximum at day 7 with C7-, C8-, and C9-SPC prevailing. It has to be noted that in quantitative terms the short-chain homologues C4 to C6 are underestimated, as their ionization efficiency is lower than that of the longer chain SPC (see **Fig. 3-2**, p. 36). A relatively constant level of all SPC compounds was achieved on day 15 and maintained until end of the experiment on day 30 (not fully depicted in **Fig. 4-22**). Hereafter, the reservoir tank was emptied, the entire unit was rinsed twice with water, filled again with freshly taken river Rhine water, and finally spiked with 10 mg L^{-1} of the same substrate.

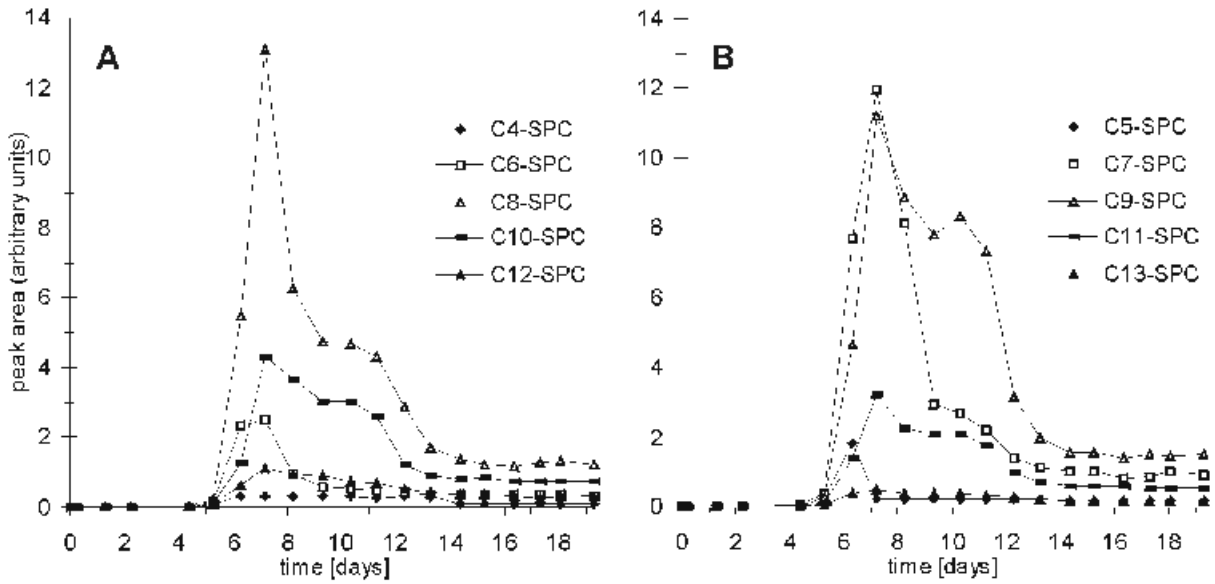

Fig. 4-22: Profile of (**A**) C-even and (**B**) C-odd SPC intermediates formed during biodegradation of commercial LAS on FBBR after first spiking.

It can be seen in **Fig. 4-23** (A and B) that after the first day, SPC are already present in the test liquor. On the apex at day 2 the absolute intensities of single SPC are similar to that observed in the previously described assay, and C7-, C8- and C9-SPC are the most abundant homologues. After day 9, no substantial changes in detected amounts were found. The homologue distribution then greatly resembled that obtained in the first degradation test.

In summary, acclimation of the micro-organisms, settled and immobilized on the glass beads of the fixed-bed during the first run, results in a more rapid degradation of LAS and thus formation of SPC without an acclimation delay. Despite a period of two weeks during which no SPC transformation in the solution is apparent (from about day 15 to day 30), the involved bacteria are instantly capable of producing necessary enzymes for the destruction of freshly added substrate.

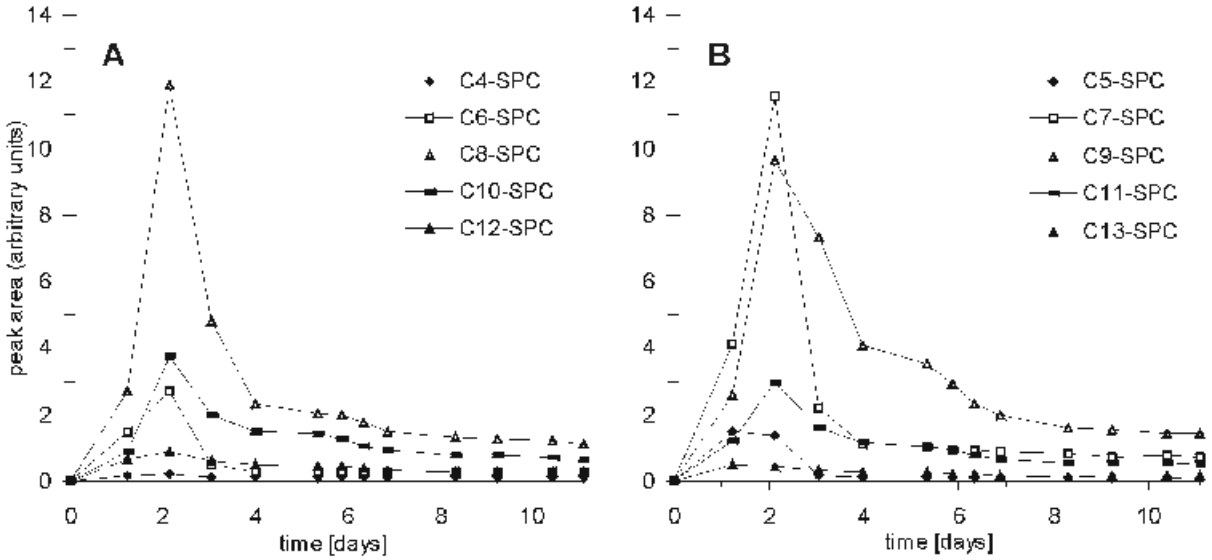

Fig. 4-23: Profile of (**A**) C-even and (**B**) C-odd SPC intermediates formed during biodegradation of commercial LAS on FBBR after second spiking.

This ability is of particular significance in natural waters since it may prevent accumulation of LAS residues in receiving streams: Larson and Payne (1981) studied the biodegradability of LAS in water samples collected from a river at locations above and below the outlet of a WWTP. Although similar extents of ultimate degradation were attained (between 70 and 73 % CO_2), the rate of degradation was about ten-fold lower in the unacclimated water. Moreover, in the latter case a lag phase prior to degradation was noticed, whereas no lag period was obvious in the continuously exposed water. The research of Shimp (1989) demonstrated that exposure of estuarine microbial communities to LAS via municipal effluents enabled the biodegradation of the surfactant. In water taken from a pristine site, less than 10 % of the LAS was converted to CO_2 during the study. In contrast, the surfactant was mineralized to 42 % in water samples from the effluent-exposed site.

Regarding the ultimate fate of SPC in the acclimation experiment above, the observation has been made that it is not affected by the time of exposure to the LAS intermediates. Seemingly, the micro-organisms do not accomplish to complete the degradation of all isomers. The argument could be advanced that the microbial community established on the fixed bed lacks some organisms producing the enzymes needed for a total destruction. It is a generally accepted view that LAS is predominantly biodegraded by a consortium of bacteria (Jiménez et al., 1991; Schleheck et al., 2000; Sigoillot and Nguyen, 1992). A single member of the community may induce the terminal oxidation of the alkyl chain and its shortening by β-oxidations. A second organism may complete the degradation via biologically mediated ring cleavage. Omission of any member of the consortium results in no mineralization of LAS.

Relevance to the environment

To provide evidence that the recalcitrance of some SPC species in the FBBR liquor is not restricted by limitations in the microbial community – it originates from surface water with a known history of LAS exposure – a comparison with the fate of SPC in a natural environment was desired. To this end, the isomer patterns of a selected homologue detected in a steady-state FBBR sample was compared to that found in an extract from the river Rhine [1].

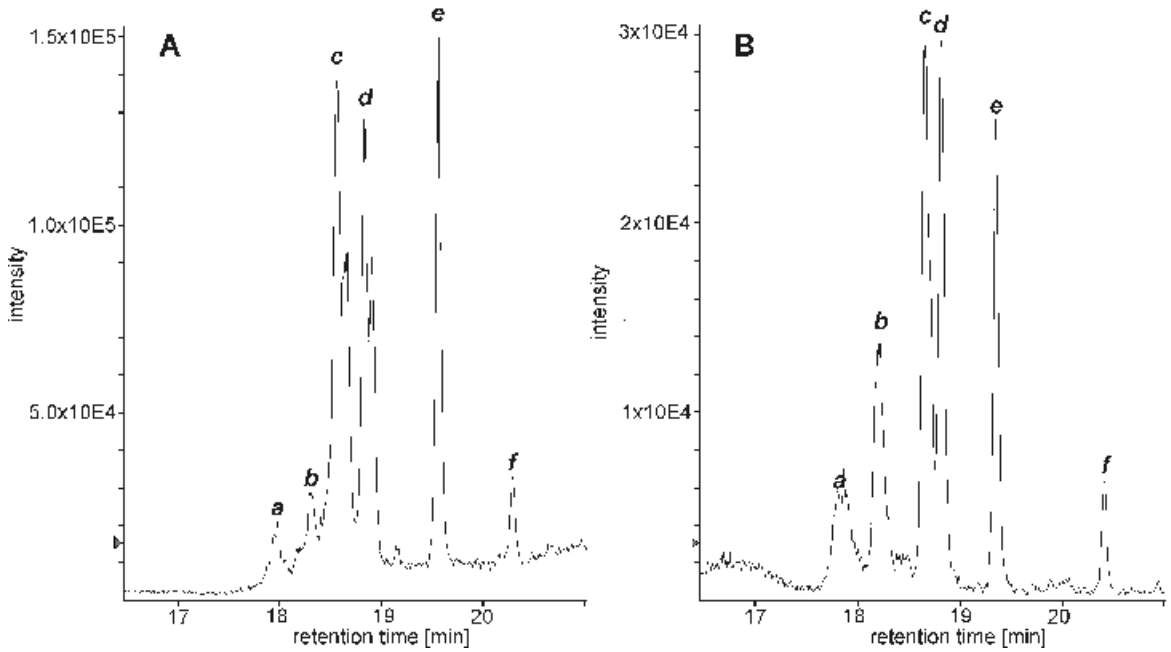

Fig. 4-24: (–)-LC-ESI-MS extracted ion chromatograms of C7-SPC (*m/z* 285) from (**A**) enriched river water sample, and (**B**) FBBR sample taken after 20 days (constant concentrations) where commercial LAS was spiked. Assignment of the individual recalcitrant species according to elution order (*a* to *f*). LC conditions as in **Fig. 4-4**.

In **Fig. 4-24** the XIC of C7-SPC in the enriched river water sample (A) and the FBBR sample (B) are shown. In (A) at least six isomers (*a* to *f*) can be distinguished at retention times between 18 and 21 min. Comparing this chromatogram with one of the enriched river sample, the similarities concerning the different C7-SPC isomers can be clearly discerned. Also, in the river water, six individual isomers can be assigned (*a* to *f*), and these occur at the proper retention time with almost the same ratio of abundance. This proves unambiguously the recalcitrance of the SPC recovered from the FBBR.

Regarding the identity of the C7-SPC constituents persisting in the FBBR liquor and found in the surface water sample, respectively, it might be assumed that they comprise those positional isomers in which the sulfophenyl substituent impedes a subsequent β-oxidation, i.e. namely in C7-SPC-2 and C7-SPC-3. This suppostion, however, does not suffice to explain the high number of signals detected; six are assigned in **Fig. 4-24**.

A possible explanation arises form the fact that iso-LAS, which were also made responsible for part of the SPC residue in the C12-LAS experiment, were present as impurity in the LAS tested. Degradation of iso-LAS-11 or iso-LAS-13 would liberate a set of iso-C7-SPC totaling theoretically 12 constitutional

[1] The extraction procedure is described in section 3.8.

isomers. Their structures are compiled in **Fig. 4-25** and arbitrarily termed as *a* through *l* (in addition, each isomer comprises 4 diastereomers except for *c*, *f* and *i* with only two enantionmers). It can be speculated that those species are most slowly degraded – or being virtually recalcitrant – where both the sulfophenyl ring and the methyl branch are close to the carboxylate group. This holds particularly for the structures *a* and *d* and to a minor extent for *e* and *h*, while the isomers *i* and *l* can be assumed to be most easily converted into iso-C5-SPC.

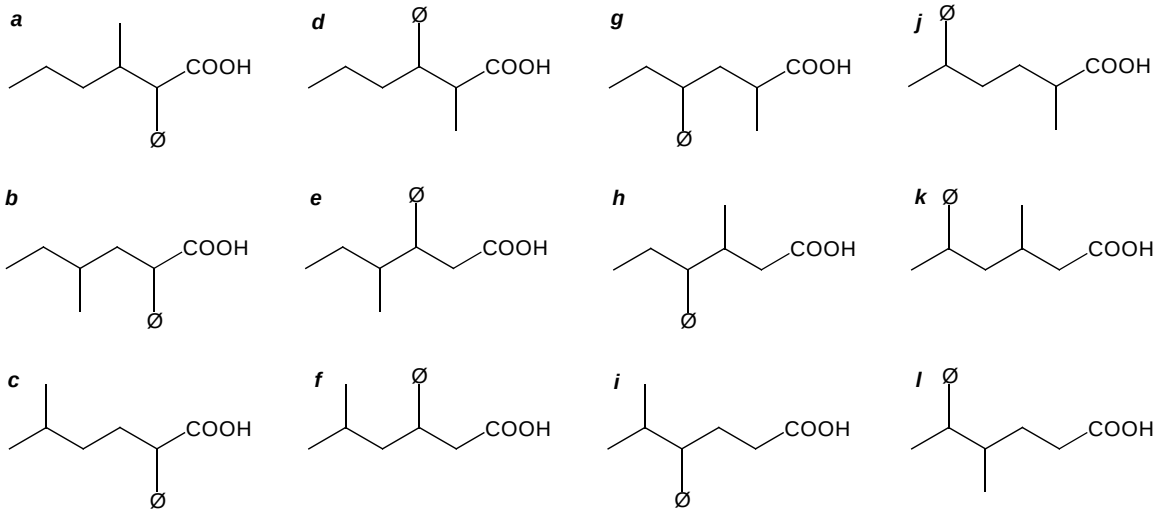

Fig. 4-25: Possible structures of iso-C7-SPC. The "Ø" symbolizes the sulfophenyl group.

Cavalli et al. (1996) claimed that iso-LAS was amenable to biodegradation without producing any evident accumulation of recalcitrant products. The authors suggested that contrasting outcomes of a previous study by Kölbener et al. (1996), who made iso-SPC responsible for the major refractory compounds, arose from the unbalanced microbial community of the particular biodegradation system used. But the own findings demonstrate good accordance between the lab-scale experiment and the conditions in real world settings and indicate that linear SPC cannot exclusively account for the persistent SPC fraction.

Overall, it can be recapitulated that the biodegradation of LAS under laboratory conditions results in the formation of some stable metabolites belonging to the class of SPC. Residues with a very similar composition, defined by peak pattern and retention time, occur in surface waters. Their formation and behavior will thoroughly be discussed in chapter 5. The information gathered from the peak pattern of a single SPC, indicating the progression of LAS/SPC breakdown will allow identification the origin and history of the water.

4.2 Alkyl glucamides

4.2.1 Introduction

During the last years, surfactants on the basis of renewable resources have gained importance due to the demand from the detergent market for more environmentally friendly products. Special interest was focused on raw materials derived from combinations of carbohydrates and vegetable fats and oils. Apart

from APG, in which the sugar moiety and the fatty alkyl part are glucosidically bound (see section 4.3), alkyl glucamides (AG) make up the second most largely produced non-ionic carbohydrate surfactant (40,000 tons per annum) (Karsa, 1998).

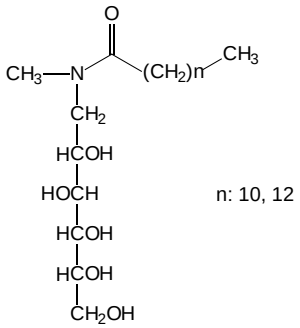

Fig. 4-26: Structure of alkyl glucamides.

These compounds, mainly the two homologues C12- and C14-AG (**Fig. 4-26**), are manufactured by reductive amination of glucose followed by acylation with fatty acid derivatives (Kelkenberg, 1988). AG exhibit very good foaming power and excellent degreasing capacity (Scholz, 1996). Furthermore, they show synergistic effects with anionic surfactants thus reducing the total amount of surfactants applied in formulations and hence emitted into the environment. Currently, AG are predominantly used in cleaning products such as hand dishwashing agents.

4.2.2 Method development

Literature methods

Only a very limited number of methods for the determination of AG have been reported. This is partially due to the relative recent introduction of AG on the surfactant market where is plays the role of a co-surfactant, but also because of the fact of being exclusively manufactured and formulated by the Procter and Gamble Company (Anonymous, 1997; Tsushima, 1997). A method based on HPLC separation with UV detection was established in connection with toxicity studies (Stalmans et al., 1993). The low UV absorbance of the analytes, however, ruled out a sensitive detection of AG at concentrations expected in environmental samples. The same authors presented a more sensitive procedure used for analysis of AG in the liquor from a biodegradation test unit. It comprised enrichment by SPE followed by continuous-flow FAB-MS (Stalmans et al., 1993).

Method development

The separation of a mixture of technical grade AG was achieved by RP-HPLC with subsequent ESI-MS detection. Comparing with other surfactants such as LAS (Nakae et al., 1981) or AE (Kiewiet and de Voogt, 1996), which consist of quite complex mixtures including homologues as well as isomers, the two components of AG, C12- and C14-alkyl, could be well separated and identified (**Fig. 4-27**). Since the LC separation was performed on a C8-RP column, the elution order depends mostly on hydrophobic interactions of the analytes with the column material, i.e. the homologue with the shorter alkyl chain length elute first.

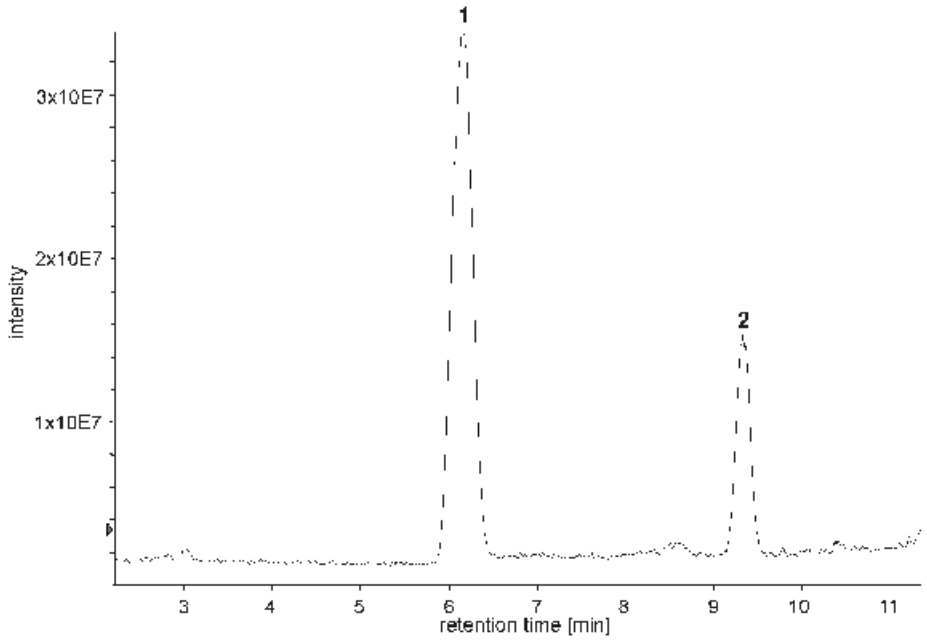

Fig. 4-27: (–)-LC-ESI-MS chromatogram of an AG standard. Peak numbering: (1) C12-glucamide, (2) C14-glucamide. LC conditions: 125 x 2.1 mm, 4 µm, C8-bonded silica with a multistep gradient profile: water-CH_3CN, pH 7.9 (NH_3) at 0.2 mL min^{-1}.

Mass spectrometric detection of AG was achievable in both positive and negative ionization. **Fig. 4-28** displays the spectra of C12-AG, which are characterized by several molecular ions and fragments. The peak assignment with the corresponding masses of all ions is listed for both AG homologues in **Table 4-4**. In (+)-ionization mode apart from the $[M+H]^+$ ion, the sodiated and potassiated ions can be detected. Furthermore, the fragment ion $[M+H–H_2O]^+$ arising from the loss of one water molecule out of the sugar moiety is formed. It is noteworthy that no ammonium adduct ion was observed although ammonia was used for pH adjustment of the eluent. In (+)-ionization mode two fragments were detected both containing the glucose part of the molecule, $[Gluc\text{-}NH_2\text{-}CH_3]^+$ and $[(Gluc–H_2O)\text{-}NH_2\text{-}CH_3]^+$.

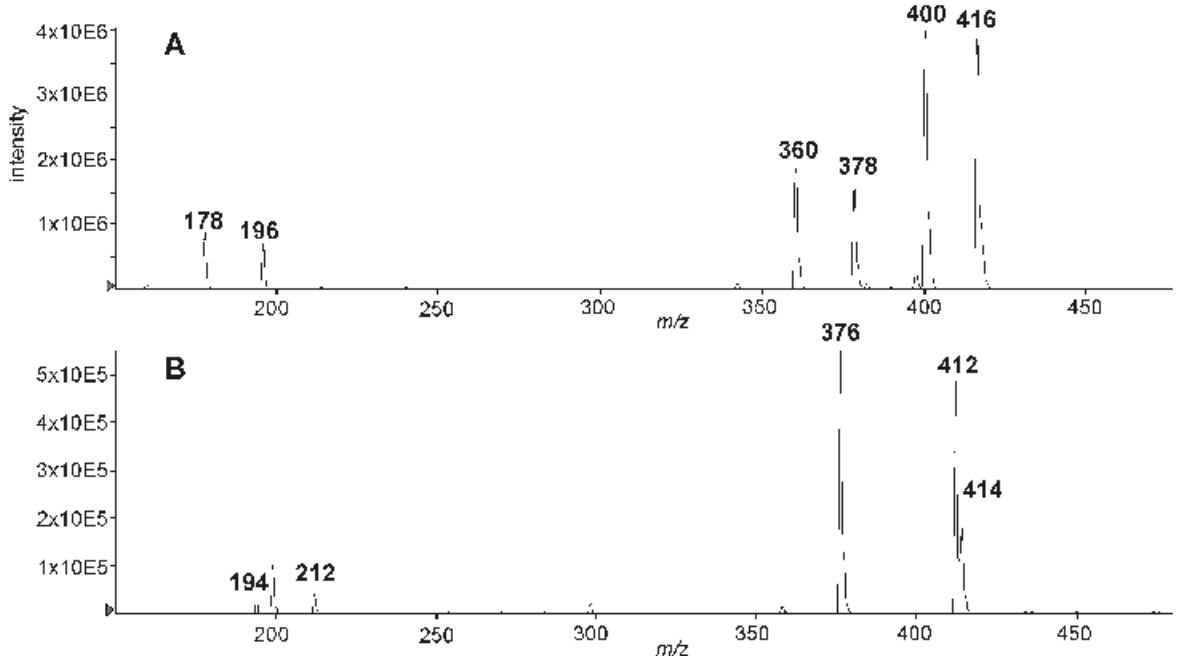

Fig. 4-28: LC-ESI-MS spectra of C12-glucamide under (**A**) positive ionization (OR: –20 V) and (**B**) negative ionization (+35 V). (OR not optimized).

In (–)-ion mode beside the $[M–H]^-$ also the chloride adduct appears in the MS spectra with a similar intensity, but under the MS conditions used for this acquisition the ratio between $[M–H]^-$ and $[M+Cl]^-$ was not optimized in any of these species. However, by optimizing the OR in favor of the highest sensitivity of $[M–H]^-$, the formation of the chloride adduct could be largely suppressed. Furthermore, the elevated pH of the eluent (7.9) assisted in the deprotonation of the parent molecule. The latter forms two fragments, one including the alkyl chain $[CH_3\text{-N-CO-R}]^-$, the other the sugar part $[\text{Gluc-N-CH}_3]^-$.

Table 4-4: Molecular ions and fragments of C12- and C14-AG under positive and negative ionization.

Positive ionization			Negative ionization		
Ion/fragment	C12-AG *m/z*	C14-AG [a] *m/z*	Ion/fragment	C12-AG *m/z*	C14-AG [a] *m/z*
$[M+H]^+$	378	406	$[M–H]^-$	376	404
$[M+Na]^+$	400	428	$[M+^{35}Cl]^-$	412	440
$[M+K]^+$	416	444			
$[M+H–H_2O]^+$	360	388			
$[\text{Gluc-NH}_2\text{-CH}_3]^+$	196	196	$[CH_3\text{-N-CO-R}]^-$ [b]	212	240
$[(\text{Gluc}–H_2O)\text{-NH}_2\text{-CH}_3]^+$	178	178	$[\text{Gluc-N-CH}_3]^-$	194	194

[a] spectra not shown [b] R: alkyl chain

Despite the fact that the sensitivity of (+)-ionization mode was about one order of magnitude higher, the (–)-ion mode was chosen for quantification purposes for two reasons: (a) the intensity ratio of the four possible quantitation ions $[M+H–H_2O]^+$, $[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$ was not reproducible – probably due to varying contents of the cations Na^+ and K^+ in the LC eluent and the sample – and (b) it was

preferable to refer to a qualifier ion including the alkyl chain, since this was more confirmative for identification of the homologues.

4.2.3 Biodegradation test

Literature data

As already pointed out for the analytical methods described so far, results on biodegradation were also very scarce. Stalmans et al. (1993) applied standardized degradation tests using a Batch Activated Sludge system and the modified Sturm test indicating a high mineralization rate. In both tests the DOC removal exceeded 98 % and primary degradation was greater than 99 %.

Metabolism study

In order to investigate the biodegradability and the metabolic pathway of AG, the medium of the FBBR was spiked with a single homologue as model compound, C10-AG, at a concentration of 1 mg L^{-1}. Despite the fact that the higher homologues C12- and C14-AG were predominant in formulations, it was advantageous to work with solitary substances in order to obtain unambiguous results. However, as for the breakdown mechanism, it could be expected that the higher homologues would show an analogous behavior (results are shown below).

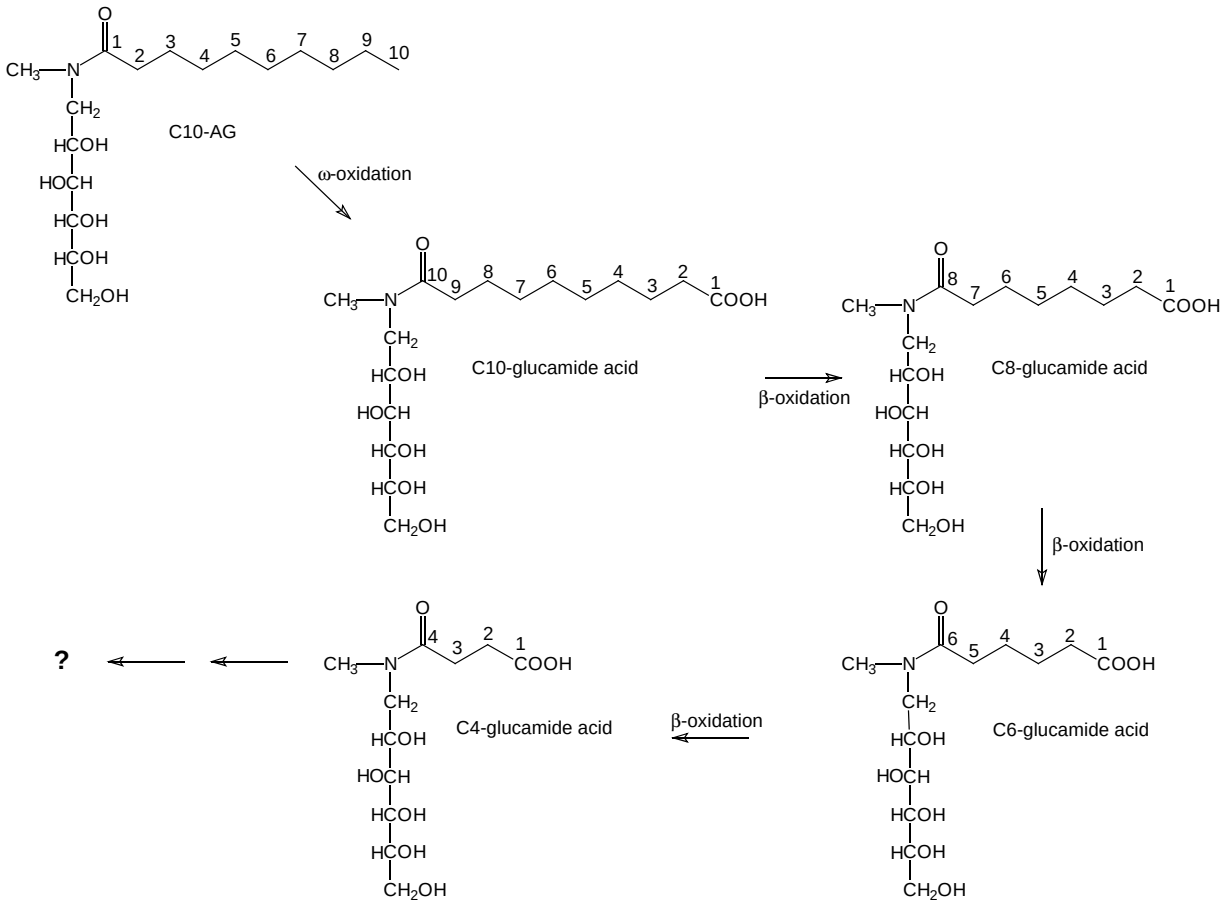

Fig. 4-29: Postulated aerobic breakdown pathway of C10-AG.

After determining the biodegradability of the fortified AG, further interest was focused on breakdown intermediates of this molecule. The strategy pursued to clear up the metabolism of AG was to postulate a mechanism based on the degradation pathway of the structurally related surfactant LAS, which exclusively proceeds via ω-oxidation of the alkyl chain, resulting in the formation of carboxylic acids, which are further broken down through β-oxidations. The corresponding pathway for AG is shown in **Fig. 4-29**.

The mass spectrometric search – lyophilized extracts of FBBR samples were injected on the LC column – for the molecular ions $[M–H]^-$ of the putative carboxylic acids (**Table 4-5**) was unsuccessful regarding the higher homologues C6-, C8-, and C10-glucamide acids, but was successful with the C4-glucamide acid [1].

Table 4-5: Ion masses $[M–H]^-$ of postulated glucamide acids.

Putative metabolite	C10-glucamide acid	C8-glucamide acid	C6-glucamide acid	C4-glucamide acid	C2-glucamide acid
m/z	378	350	322	294	266

Besides the information on the mass of the assumed molecular ion of C4-glucamide acid (*m/z* 294), identification of this metabolite was achieved by assigning a fragment with *m/z* 194. Its formation also occurred in the fragmentation pattern of the parent compound C10-glucamide (**Fig. 4-30**) (ref. to **Table 4-4** for the higher homologues). For both C10-AG and its metabolite, the amide bond was heterolytically cleaved releasing the negatively charged fragment ion *m/z* 194.

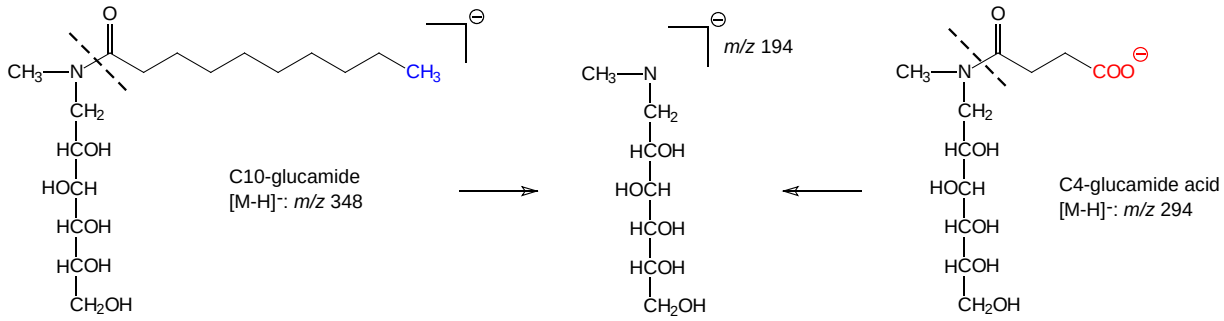

Fig. 4-30: Formation of fragment ion *m/z* 194 out of C10-AG and C4-glucamide acid.

By ramping the OR, a definite assignment of this fragment to the $[M–H]^-$ ion of the breakdown intermediate was accomplished. It can be clearly seen in **Fig. 4-31** that the intensity of the molecular ion $[M–H]^-$ decreases when the voltage is increased from –20 V to –40 V and then finally set to –60 V, whereas the intensity of the fragment (*m/z* 194) reaches a maximum at –40 V.

[1] Due its simplicity the term “glucamide acid” was used in this work to describe the new metabolite.

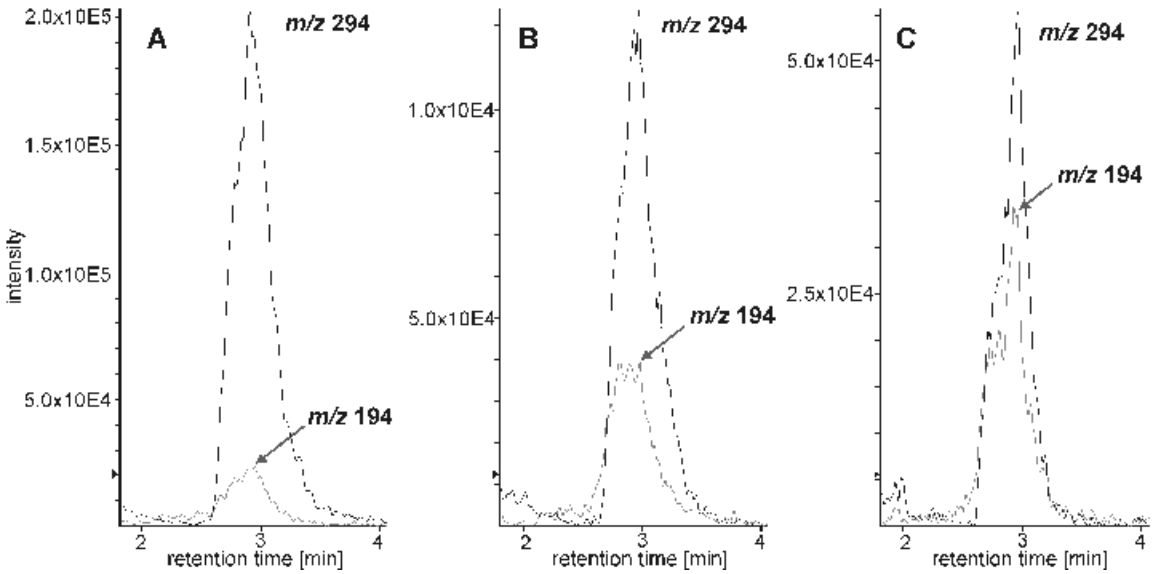

Fig. 4-31: (–)-LC-ESI-MS extracted ion chromatograms of molecular ion (*m/z* 294) and fragment ion (*m/z* 194) of C4-glucamide acid. OR: –20 V (**A**), –40 V (**B**), –60 V (**C**) (intensity of *m/z* 294 is normalized to 100 %). LC conditions: 125 x 2.1 mm, 4 µm C8-bonded silica with a gradient profile: 3 mM TEAA-CH_3CN (3 min isocratic at 95:5, within 20 min to 20:80, v/v) at 0.2 mL min^{-1}.

Detection and unequivocal identification of C4-glucamide acid, occurring as intermediate in the breakdown of C10-AG, confirmed the proposed metabolism as shown in **Fig. 4-29**. Since the alkyl chain was the molecular moiety subject of microbial attack, it might be assumed that the degradation rate increases with increasing alkyl chain length, which holds true for LAS (*Swisher´s distance principle*). Hence, with reference to the metabolization rate of higher AG homologues present in formulated products, it might be expected that the latter undergo faster degradation than C10-AG used in the assay. The measured kinetics of the studied substance can be judged to be rather conservative compared with those of C12- and C14-AG.

Fig. 4-32 displays the separation of C4-glucamide acid and C10-AG under the applied RP condition using the IP agent TEAA. The temporal evolution of both species can be seen in **Fig. 4-33**. No notable adaptation phase of primary degradation is observed. The initial C10-glucamide concentration of 1 mg L^{-1} is halved at about 4 days, while after day 5 the surfactant is no more detectable. The apparently immediate onset of degradation of the parent compound is paralleled by the formation of the metabolite reaching a maximum level after about 4 days. On the following day, the degradation rate of C4-glucamide acid was greater than the rate at which this was being formed, which eventually led to a complete biodegradation of the compound after 6 days.

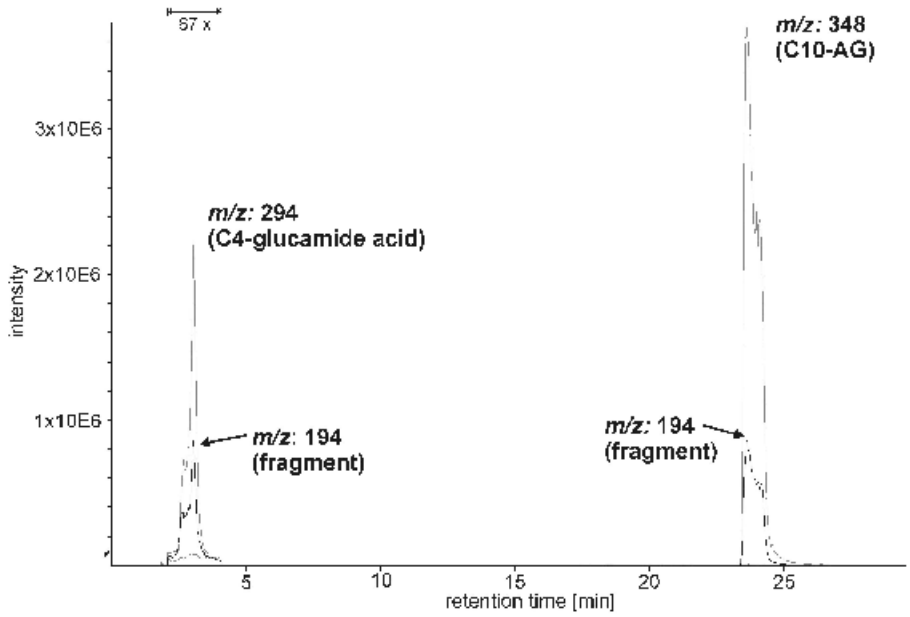

Fig. 4-32: (–)-LC-ESI-MS chromatogram of lyophilized FBBR sample from C10-AG degradation. LC conditions as in Fig. 4-31.

As mentioned before, the higher glucamide acids, C6, C8, and C10, were not detectable in the FBBR samples. The non-detectability of these carboxylic acids can be traced back to their short lifetime in solution or they are even not released from the cells into the bulk. Seemingly, they are rapidly further degraded to the shorter homologues – similar observations were made for long-chain SPC rapidly converted into the shorter-chain species – resulting finally in the formation of C4-glucamide acid. Its relative longevity in turn can be explained by the fact that in contrast to the preceding steps, a subsequent β-oxidation would not lead to a regular carboxylic acid, but to an α-keto acid.

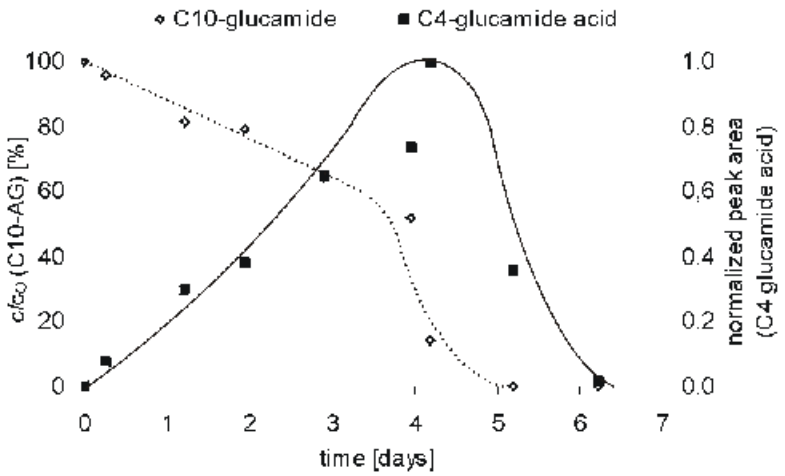

Fig. 4-33: Degradation curve of C10-AG and formation of metabolite C4-glucamide acid.

The final fate of C4-glucamide acid will probably be completed in the course of intracellular processes. This assumption is supported by the findings of Stalmans et al. (1993) measuring high DOC removal and CO_2 formation in standardized biodegradation tests.

Synthesis and isolation of metabolite

For an ecotoxicological characterization of C4-glucamide acid, described in section 6.2, it was indispensable to synthesize this compound as it was not available from chemical suppliers. The organic synthesis was performed by Diaz (unpublished results) reacting N-methyl glucamine with succinic acid dichloride according to the scheme given in **Fig. 4-34**. A series of attempts were mounted to find out optimum reaction conditions in view of preventing formation of by-products arising from the reaction of the acyl halogenide with different functional groups within the N-methyl glucamine molecule. Nonetheless, a subsequent purification step was necessary to remove by-products and unreacted starting material. This turned out to be complicated due to similar solubilities of both the educts and the product as well as of reaction side products. In order to pursue the progress and the quality of the synthetic work, ^{1}H- and ^{13}C-NMR measurements were done by Diaz (unpublished results). Additionally, LC-ESI-MS was applied within this work to check for the purity of the obtained products and to confirm the identity of the components.

As shown in the previous section it was feasible to separate C4-glucamide acid from C10-AG by RP-HPLC owing to very different polarities of both compounds (**Fig. 4-32**). Regarding the chromatography of a mixture containing the very polar species involved in the synthesis of C4-glucamide acid, the use of RP-LC did not offer a reasonable separation. C4-glucamide acid, succinic acid and a by-product, identified as N-methyl glucamide whose secondary amine group and terminal hydroxy group of the sugar moiety were esterified, were nearly co-eluting under the given conditions yielding rather broad peaks (**Fig. 4-35**, A).

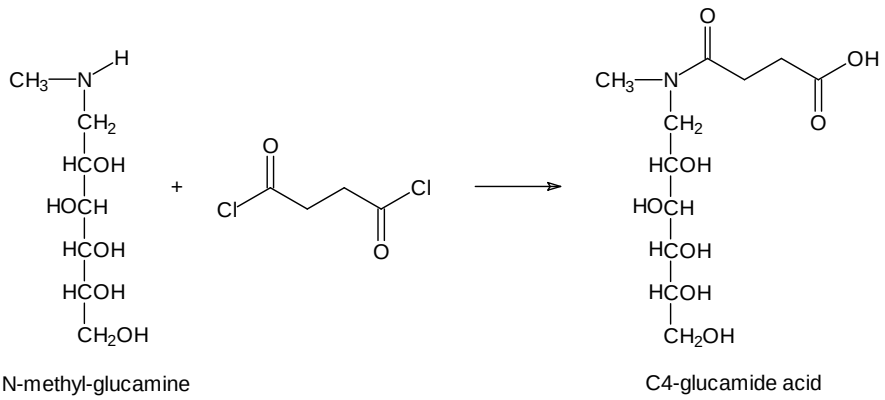

Fig. 4-34: Synthetic route for C4-glucamide acid.

Instead a very good separation was achieved on an anion exchange (AX) column benefiting from the fact that the three compounds are carboxylic acids, i.e. they interact distinctly with the cationic exchanger material. The column used, developed for specific ion chromatography of small inorganic anions (Schäfer et al., 1996), allowed a baseline separation. C4-glucamide acid carrying a single carboxylate group was eluted first, whereas the by-product and the succinic acid, both having two negatively charged groups, were significantly stronger retained (**Fig. 4-35**, B).

The operation of the AX column required aqueous alkaline eluents containing $Na_2CO_3/NaHCO_3$. Such type of eluent was utterly incompatible for a direct coupling of the LC column with the ESI-MS interface since the inorganic salts crystallized instantly upon entering the ion source. The issue was

overcome by placing a cation exchanger before the interface [1]. Substitution of Na^+ ions through protons resulted in the formation of H_2CO_3, i.e. CO_2 in the end, which was compatible with the interface.

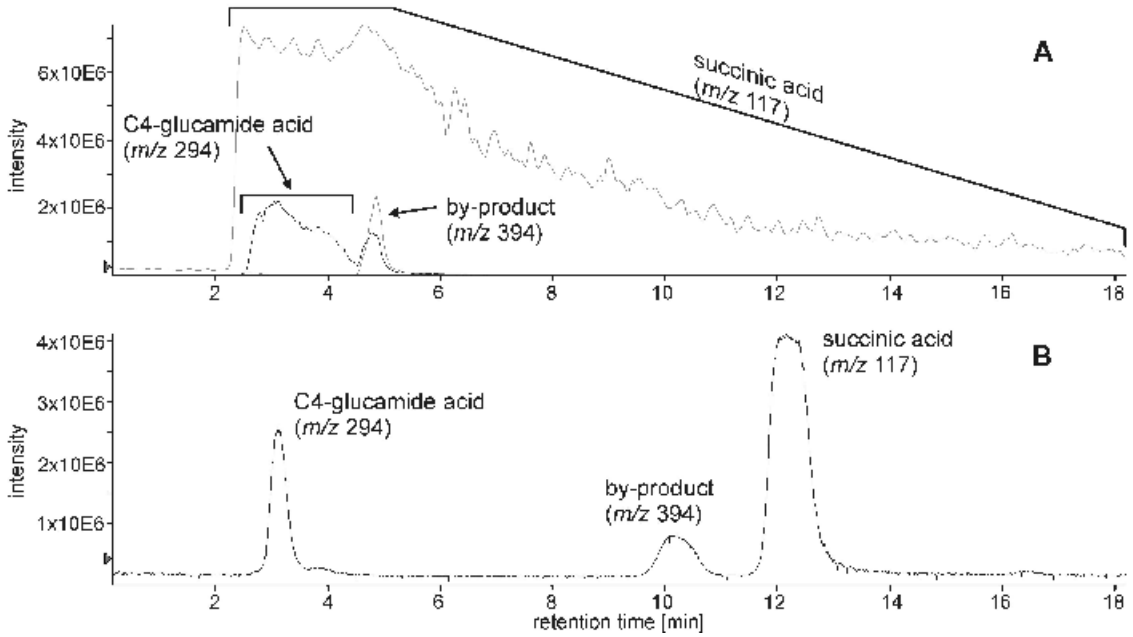

Fig. 4-35: (–)-LC-ESI-MS chromatograms of a reaction mixture of C4-glucamide acid on C8-RP column (**A**) and anion exchanger column (**B**). LC conditions: (A) as in **Fig. 4-27**, (B) 150 x 3.1 mm, anion exchange column with a gradient profile: $Na_2CO_3/NaHCO_3$ (3 min isocratic 1.3/2.0 mM, within 12 min to 13/20 mM) at 0.5 mL min^{-1}, split 1:1.

The usefulness of this instrumental set-up, anion exchange column with cation suppressor, was proven by Bauer et al. (1999) who analyzed several polar and environmentally relevant micropollutants such as the herbicide glyphosate, its metabolite AMPA, and the chelating agent ethylenediamine tetraacetate (EDTA). A similar strategy based on ion-exchange chromatography with a sodium hydroxide eluent, connected with a self-regenerating cation suppressor, was reported by Mohsin (2000) determining anionic compounds in agricultural chemicals.

4.3 Alkyl polyglucosides

4.3.1 Introduction

Alkyl polyglucosides (APG) are non-ionic surfactants produced on the basis of renewable feedstocks such as glucose and fatty alcohols deriving from starch and palm oil, respectively (Hill, 1993; Tsushima, 1996). They were already known since 1893 but it is only since the 1990s that they have been manufactured at industrial scale. These surfactants consist of a complex mixture of a variety of homologues and isomers, including stereoisomers, binding isomers and ring isomers within the glucose moiety (**Fig. 4-36**).

[1] Earlier this device served to eliminating cationic ion-pairing agents, see section 4.1.2.

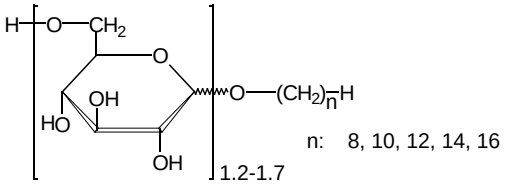

Fig. 4-36: Simplified structure of alkyl polyglucosides.

The global production rate in 1997 amounted to 80,000 t (Karsa, 1998). Even if it is unlikely to become a main surfactant like LAS, AES, or AE, they play an important role as co-surfactant. Due to favorable properties in terms of foaming performance (Förster et al., 1996), synergism with other surfactants (Florescu et al., 1996) and skin compatibility (Hughes and Lew, 1970) they are used with rising extent in cleaning agents, detergents and personal care products (Balzer, 1991). Their usage in pesticide formulations for agricultural applications is profitable due to excellent wetting and penetration characteristics of APG (Garst, 1998).

4.3.2 Method development

Literature methods

Unlike AG, which has only been studied rudimentarily, a comparatively large collection of various analytical methods using chromatographic separation has been found for the characterization of APG (**Table 4-6**).

Table 4-6: Analytical methods for the separation and detection of APG.

Separation	Detection (Ionization)	Matrix	Reference
GC	FID	techn. product	Spilker et al. (1996)
[a]	FID	techn. product	Waldhoff et al. (1998)
	MS (deriv.)	techn. product	Billian and Stan (1998)
TLC	SIMS	techn. product	Buschmann et al. (1996)
LC	RI	techn. product	Klaffke et al. (1998)
	RI	techn. product	Spilker et al. (1996)
	ELSD	techn. product	Elfakir and Lafosse (1997)
	ELSD	techn. product	Heinig et al. (1998b)
	UV	techn. product	Klaffke et al. (1998)
	UV (deriv.)	techn. product	Heinig et al. (1998b)
	MS (ESI)	techn. product	Klaffke et al. (1999)
	MS(MS) (APCI)	techn. product	Billian et al. (2000)
CE	UV (deriv.)	techn. product	Heinig et al. (1998b)

[a] high temperature GC

They all have in common that they focus on bulk chemicals or formulations. This restriction is in part due to difficulties on the availability of appropriate detection systems providing high sensitivity and selectivity needed for the determination of low amounts of APG in complex matrices. Lacking volatility of the majority of APG components disables direct GC analysis. For HPLC rather universal detection devices like RI detector or ELSD have been used since the non-ionic surfactant does not carry a chromophoric group rendering sensitive UV detection impractical. These shortcomings can be circumvented applying LC-MS, which as yet has only been exploited by very few research groups.

Method development

Using RP-HPLC with a C8 column followed by ESI-MS, separation and identification of individual components in a technical blend of APG were accomplished. The detection was possible in both positive and negative ionization mode. **Fig. 4-37** (A) shows a typical LC-ESI-MS chromatogram from a standard mixture of APG registered in negative ion mode scanning a mass range from *m/z* 200 to 600. The complex peak pattern obtained is resolved by extracting the masses of the molecular ions $[M–H]^-$ of the constituents. In **Fig. 4-37**, (B) and (C), the XIC of alkyl monoglucosides (C8, C10 and C12) and alkyl diglucosides (C8, C10 and C12), respectively are depicted. The masses of the corresponding ions are listed in **Table 4-7**.

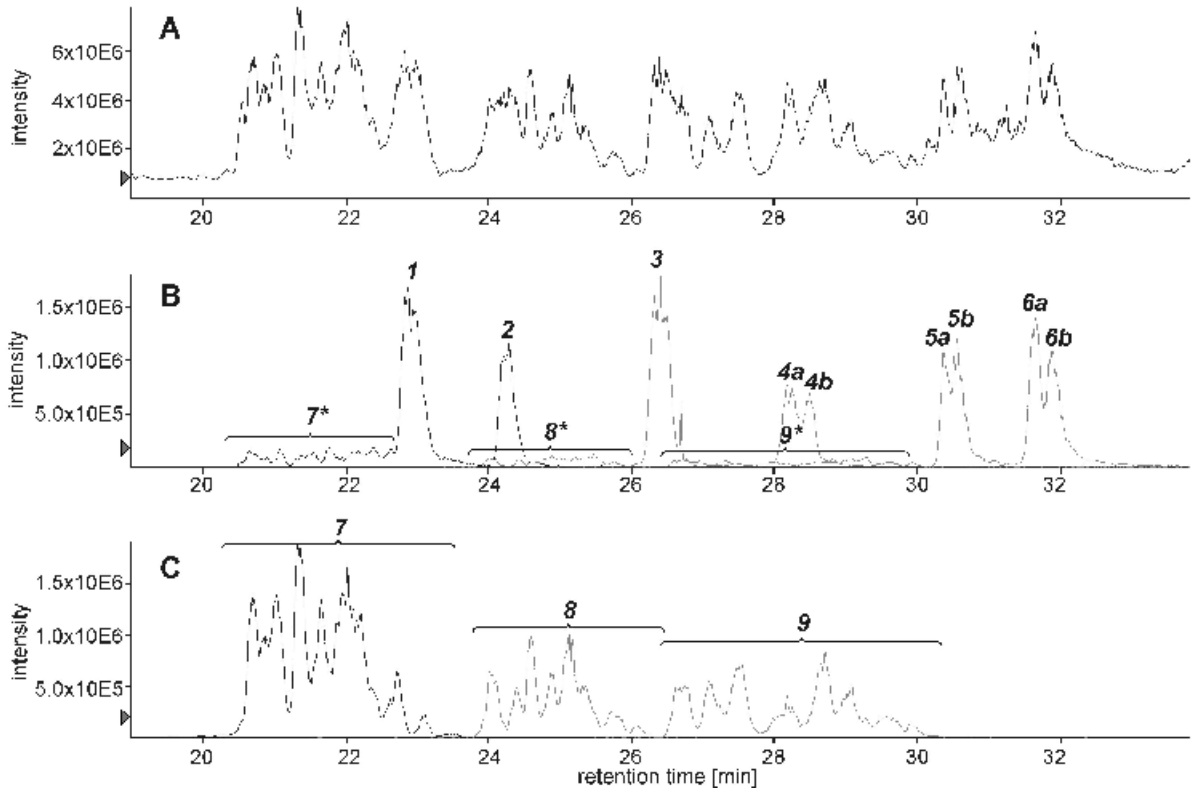

Fig. 4-37: (–)-LC-ESI-MS chromatograms of a standard APG solution. (**A**) total ion current trace from *m/z* 200 to 600 (**B**) XIC of C8-, C10-, and C12-monoglucoside, (**C**) XIC of C8-, C10-, and C12-diglucoside. Peak numbering as in **Table 4-7**; indices *a* and *b* denote different stereoisomeric forms. LC conditions: 125 x 2.1 mm, 4 µm, C8-bonded silica with a multistep gradient profile: water-CH_3CN, pH 7.9 (NH_3) at 0.2 mL min^{-1}.

The order of elution depends on the length of the alkyl chain as well as on the number of glucose units. Increasing chain length results in increased retention time. On the other hand, the introduction of a second glucose moiety leads to weaker retention on the C8-column owing to higher hydrophilicity of the molecule. C8-, C10- and C12-monoglucosides are entirely separated according to the alkyl chain length (B). Moreover, a separation with regard to the ring isomerism of the glucose moiety, i.e. pyranosidic or furanosidic form, is accomplished. Peaks arising from stereoisomeric α- and β-alkyl monoglucosides are partially resolved (indices *a* and *b*). As for alkyl diglucosides (C) the peak pattern of a specific alkyl homologue is much more complex compared to the corresponding monoglucoside. This is due to the higher number of possible isomers including ring isomers, stereoisomers and binding isomers. In the latter case 1,4- or 1,6-glucosidal bonds may link two glucose rings.

Table 4-7: Ion masses (*m/z*) of different molecular and adduct ions of alkyl mono- and diglucosides (as assigned in **Fig. 4-38** and **Fig. 4-40**).

		Ion (*m/z*)		
Compound	Peak no.	$[M–H]^-$	$[M+NH_4]^+$	$[M+Na]^+$
C8-monoglucoside	1, 2	291	310	315
C10-monoglucoside	3, 4	319	338	343
C12-monoglucoside	5, 6	347	366	371
C8-diglucoside	7	453	472	477
C10-diglucoside	8	481	500	505
C12-diglucoside	9	509	528	533

With the selected ion masses beside the assigned monoglucosides also a series of signals with low intensity are occurring, which are denoted as 7^*, 8^* and 9^* in **Fig. 4-37** (B). These signals originate from fragment ions of diglucosides, which are formed by loss of one glucose unit, which results in fragments having the same mass as the corresponding monoglucosides with the identical chain length.

The spectra of C8-monoglucoside registered under positive and negative ionization mode are displayed in **Fig. 4-38**. In (+)-ionization mode the molecule ion peaks of both the sodium and the ammonium adduct are detected, whereas in (–)-ionization mode the base peak is assigned exclusively to $[M–H]^-$. In both instances characteristic fragmentation of the parent compound occurs tracing back to the cleavage of the glucosidal bond. Under positive ionization three glucose fragments are detected, whereas in negative ionization mode a single glucose fragment is observed (**Table 4-8**).

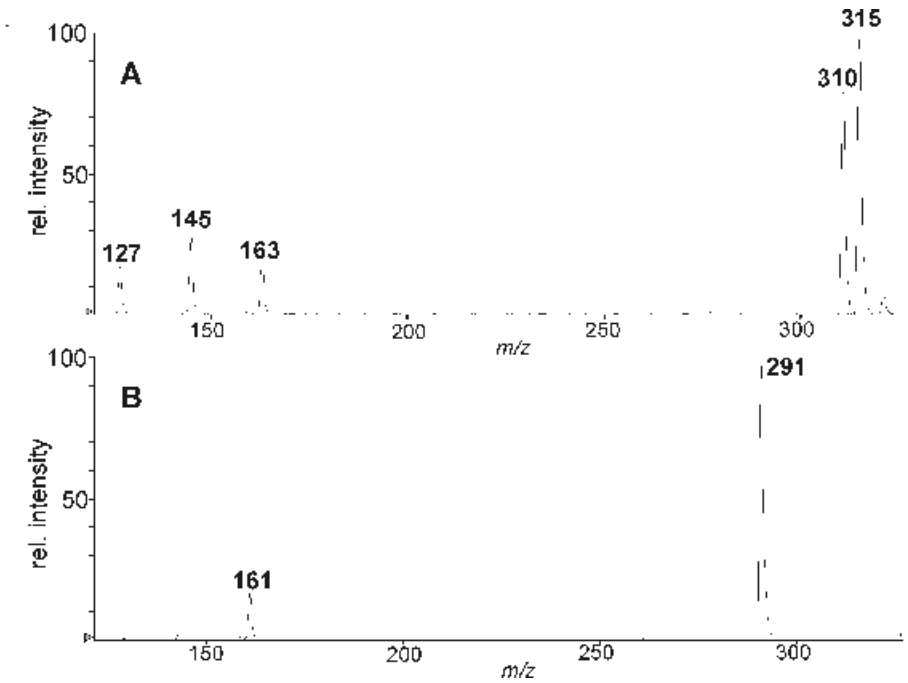

Fig. 4-38: LC-ESI-MS spectra of C8-glucopyranoside under (**A**) positive and (**B**) negative ionization (OR: +32 V and – 40 V).

The impact of the OR on the fragmentation pattern was investigated for C10-β-glucopyranoside, showing that in (–)-ionization mode the intensity of the fragment ion *m/z* 161 relative to the one of the molecular ion rised with increasing voltage (set to –20, –50 and –80 V), whereas the intensity of $[M–H]^-$ (*m/z* 319) run through a maximum (U_{max}: –28 V). When the ESI-MS was set to positive ion mode, formation of sodiated and ammoniated molecular ions was strongly dependent on the OR. Ramping this parameter from 0 to +100 V displayed a rapid decrease of the intensity of $[M+NH_4]^+$, while the intensity of $[M+Na]^+$ was rising until a maximum at +35 V.

Table 4-8: Fragments of alkyl monoglucosides under positive and negative ionization.

Positive ionization		Negative ionization	
Fragment	*m/z*	Fragment	*m/z*
$[Glucose+H–(H_2O)_3]^+$	127	$[Glucose–H_2O–H]^-$	161
$[Glucose+H–(H_2O)_2]^+$	145		
$[Glucose+H–H_2O]^+$	163		

In (+)-ionization mode a distinct selectivity of monoglucosides with respect to the formation of adduct ions was observed. Depending on the ring form (glucopyranoside or glucofuranoside) and the stereoisomerism (α- or β-alkyl monoglucoside), a different affinity towards Na^+ and NH_4^+ became apparent. The molecular structures of the four possible isomers of C8-monoglucoside are shown in **Fig. 4-39**.

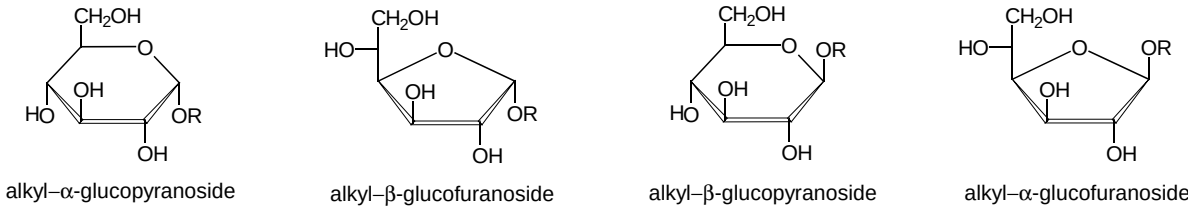

Fig. 4-39: Structures of ring and stereoisomers of alkyl monoglucoside.

The SIM-chromatograms of C8-, C10-, and C12-monoglucoside – each time window corresponds to the sum of $[M+Na]^+$ and $[M+NH_4]^+$ – are displayed in **Fig. 4-40** (A) (masses of the adduct ions as listed in **Table 4-7**). The XIC showing the adduct ions $[M+Na]^+$ and $[M+NH_4]^+$, respectively are given in (B) and (C). While the alkyl glucofuranosides exhibit a higher tendency to form the sodium adduct, the alkyl glucopyranosides are preferably detected as ammonium adduct ion. Also the α- and β-isomers of the glucofuranosides manifest a much higher selectivity for Na^+ and NH_4^+ compared to the two corresponding glucopyranosides.

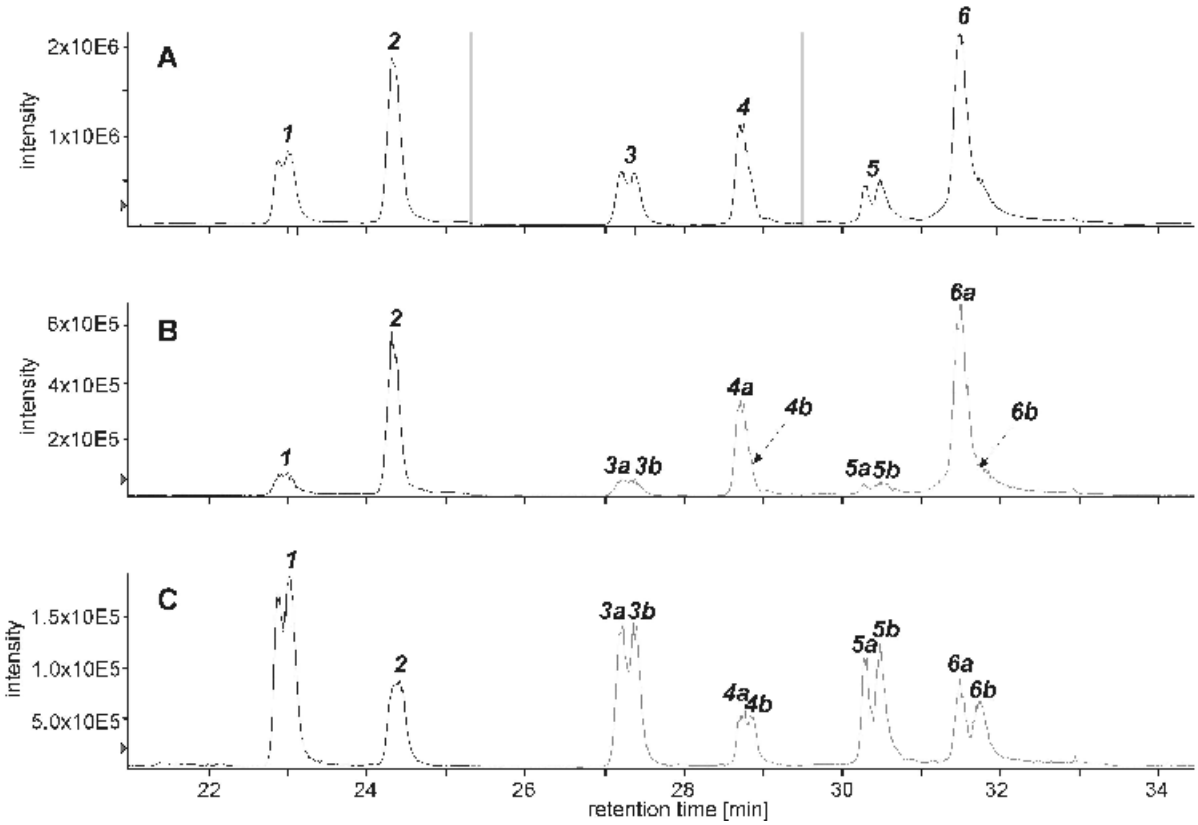

Fig. 4-40: (+)-LC-ESI-MS chromatograms of standard APG solution. (**A**) Sum of XIC of $[M+Na]^+$ and $[M+NH_4]^+$ of C8-, C10-, and C12-monoglucoside, (**B**) XIC of $[M+Na]^+$, (**C**) XIC of $[M+NH_4]^+$. Peak numbering as in **Table 4-7**; *a* denotes α-glucoside, *b* denotes β-glucoside. LC conditions as in **Fig. 4-37**.

The following statements can be made for C12-monoglucoside (*5,6*) as example: (a) α-glucofuranoside (*6a*) has a much higher affinity towards Na^+ compared with β-glucofuranoside (*6b*), (b) the peak of α-glucofuranoside (*6a*) is the most intensive signal among the four $[M+Na]^+$ signals of C12-monoglucoside,

and (c) α-glucopyranoside (*5a*) and β-glucopyranoside (*5b*) show no discrimination of Na^+ or NH_4^+ in terms of formation of the corresponding adducts.

These observations can be explained by specific interactions of the cation with the glucose moiety. Since Na^+ and NH_4^+ have different ion radii (Na^+: 0.95 Å; NH_4^+: 1.43 Å) their coordination with the glucose moiety – presumably via hydroxy groups – depends on the spatial availability, which is determined by the constitution and configuration of the glucose part.

It is noteworthy that sodiated adduct ions are formed and detected even without addition of any sodium salt to the LC eluent. Seemingly the ions originate from impurities in the solvent and standards. Since the concentration of Na^+ is not adjusted to a certain level, the observations in terms of formation of sodium and ammonium adducts may change qualitatively (similar observations were made with AG). Measurements made at a different time revealed that a higher content in sodium resulted in the detection of $[M+NH_4]^+$ only for the glucopyranoside, whereas the sodium adduct is formed from both the glucopyranoside and the glucofuranoside.

Recognizing the variability of the adduct formation under (+)-ionization, which is detrimental to the required reproducibility, negative ion mode was used throughout the remaining work.

4.3.3 Biodegradation test

Literature data

In order to assess the aerobic and anaerobic biodegradability of APG, a series of standard laboratory tests were carried out by Steber et al. (1995) representing either discontinuous screening tests or continuous test systems with activated sludge. Evaluation of the results of sum parameters analysis (DOC removal, TOC removal, COD and BOD) allowed classification of APG as “readily biodegradable” according to OECD definitions. Ultimate biodegradation was proved by the coupled-units test (OECD 303A) giving a DOC removal of 89 % and even the anaerobic biodegradability testing (ECETOC screening test) indicated complete transformation into CO_2 and CH_4 amounting to 84 % theoretical gas production. The findings of Steber et al. (1995) were confirmed in two other studies likewise working with OECD standard tests (Garcia et al., 1997; Madsen et al., 1996). However, all these test methods were exclusively grounded on sum parameters, hence they did not yield any information on the metabolic pathway of APG.

Metabolism studies

River Rhine water was spiked with 1 mg L^{-1} of each C8-, C10-, and C12-β-monoglucoside. In this assay a cocktail of identical concentrations of the three homologues was added to investigate the effect of different alkyl chain lengths. The temporal evolution of the test substance concentrations was followed up by LC-ESI-MS analyzing the FBBR sample without further treatment. In **Fig. 4-41** relative concentrations of each homologue are plotted *vs.* time. The biodegradation curve shows a sigmoidal-shaped course, which can be subdivided into a lag phase, where the acclimation of the micro-organisms to the test substances occurs (0 to 13 h), a degradation phase, in which the compounds are metabolized (13 to 21 h) at increasing speed and a plateau phase, in which degradation is nearly complete (after 1 d). Over the first period a slight dependence of the adaptation rate on the alkyl chain length is observed – increasing chain length goes along with faster degradation – whereas almost no difference is apparent in the final degradation phase.

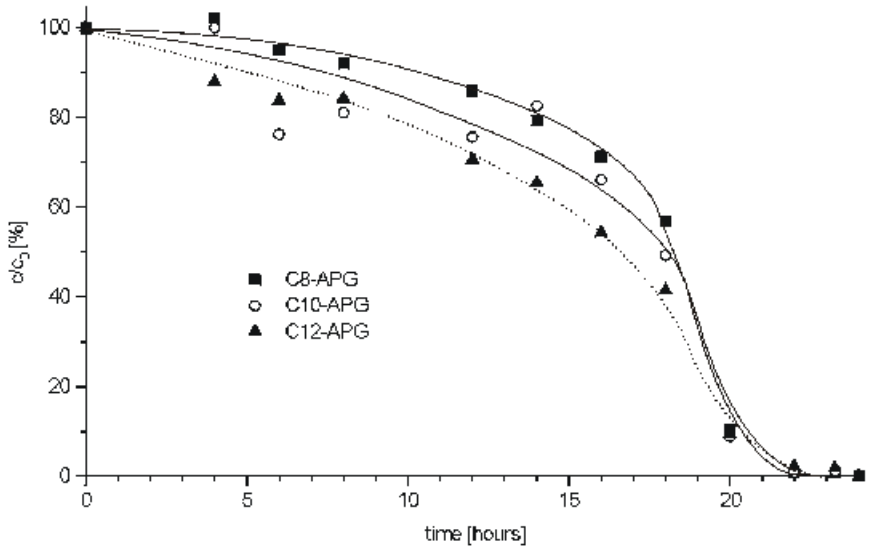

Fig. 4-41: Degradation curve of C8-, C10-, and C12-β-glucopyranoside.

The FBBR samples were also investigated with respect to the occurrence of possible metabolites. A breakdown pathway of the APG was derived from the metabolism of LAS and AG, which are degraded by ω-oxidation of the alkyl chain resulting in carboxylic acids (compare with **Fig. 4-8** and **Fig. 4-29**). These are in turn further degraded by β-oxidation releasing C2-units. The corresponding mechanism for APG is shown in **Fig. 4-42** (Pathway I).

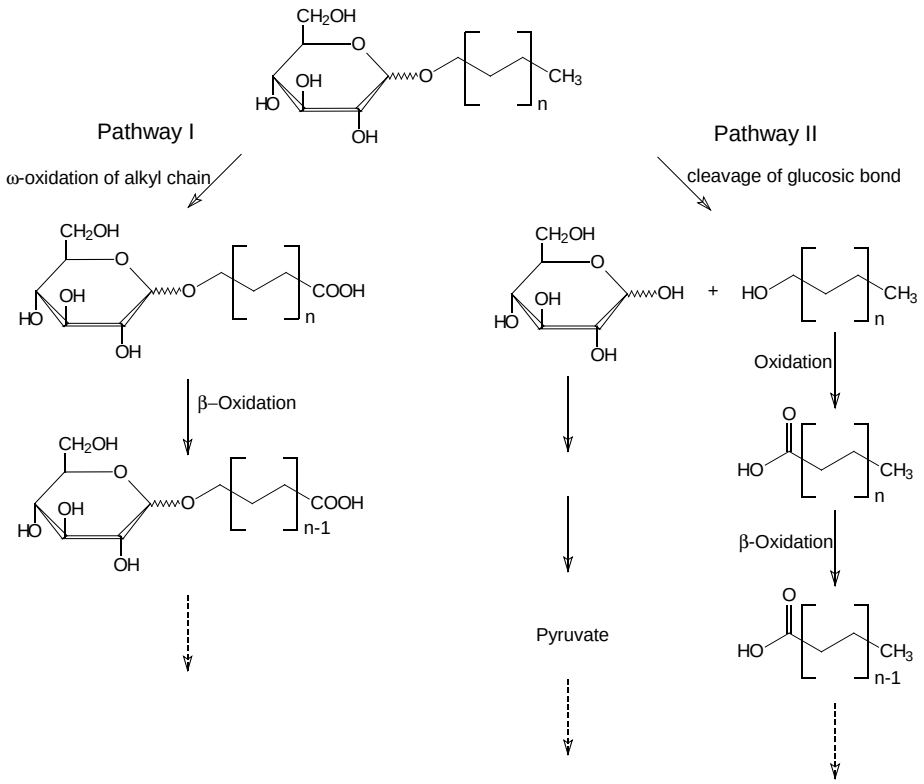

Fig. 4-42: Possible degradation pathways of alkyl glucopyranosides.

The search for the masses of the putative "polyglucoside alcanoic acids" (C8-acid: 322 amu; C10-acid: 350; C12-acid: 378) using LC-ESI-MS was not successful. Neither in (–)-ionization mode where the masses of the ions $[M–H]^-$ and $[M–H–CO_2]^-$ were monitored nor in (+)-ion mode where the supposed acids should have been detected as sodiated adduct ions $[M+Na]^+$ or $[M–H+2Na]^+$, any indication for the presence of the tentative acids was given. An alternative degradation mechanism can give an explanation for the absence of these carboxylic acids in the test medium. This pathway (**Fig. 4-42**, Pathway II) encompasses the cleavage of the glucosidal bond leading to glucose and the fatty alcohol. In this instance glucose is rapidly further metabolized via pyruvate, whereas the fatty alcohol is oxidized to the corresponding acid which subsequently undergoes the classical fatty acid degradation mechanism. The lack of detection of fatty acids, which were also investigated by (–)-LC-ESI-MS, can be traced back to quick intracellular metabolism.

The hypothetic Pathway II is supported by Kroh et al. (1999) investigating the ability of carbohydrolases α-glucosidase, β-glucosidase and isomaltase to break the glucosidal bond between fatty alcohol and carbohydrate. The model compounds C8-α- and C8-β-glucopyranoside were completely hydrolyzed by a mixture of the three enzymes, whereas a technical APG blend, containing also higher oligomers, was not quantitatively cleaved presumptively due to the incapability of the employed enzymes of breaking the linkages between the sugar moieties.

4.4 Cocamidopropyl betaines

4.4.1 Introduction

Next to the most widely used anionic and non-ionic surfactants such as LAS, AES and AE, the share of amphoterics – a major representative is cocamidopropyl betaine (CAPB, **Fig. 4-43**) – has become increasingly important in recent years. These make up about 5 % of the market (Karsa, 1998). Relative high production costs of amphoteric surfactants are warranted by their brilliant synergistic properties in view of foaming and detergency in formulations (Balaguer et al., 1998; Bluestein et al., 1973). On account of the partial anionic and cationic characteristics of the head group, they can be made compatible with anionic and cationic surfactants under specific conditions. The low irritative potential of CAPB on skin and mucous membranes renders it especially beneficial for usage in personal care products like shampoos, conditioners, and cosmetics (Hunter and Fowler, 1998).

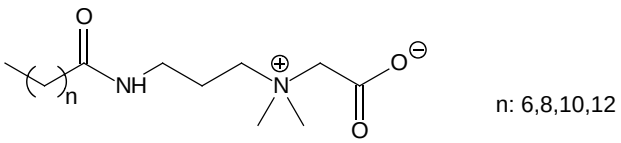

Fig. 4-43: General structure of cocamidopropyl betaine.

4.4.2 Method development

Literature methods

For the determination of amphoteric surfactants in raw materials, commercial mixtures and formulations, a couple of HPLC-based procedures were described. Apart from the separation of alkyl homologues on RP columns (Kondoh and Takano, 1986; Nakamura and Morikawa, 1982), the molecules could also be separated on stationary phases made up of ion-exchanger material owing to the ionic character of the analytes (Matsuzaki et al., 1993). In the work by Wilkes et al. (1994) the separation of CAPB was achieved isocratically with a mixed-mode RP-C8/cation silica column. As for detection methods, the use of an UV or a diode array detector was limited by their low sensitivity due to absence of a strong chromophore in the CAPB molecule (Tegeler et al., 1995). Alternatively, an RI detector or an ELSD were used (Carrer et al., 1999). Another approach determining the total concentration of amphoteric surfactants was based on the photometric analysis of a stable associate formed between the surfactant molecule and the dye Orange II (Boiteux, 1984). Enrichment of the analytes from synthetic wastewater by air stripping into an organic solvent followed by complexation with the dye and photometric analysis allowed to achieve a limit of detection in the lower µg/L range (estimated at 10 µg L^{-1}) (Gerhards and Schulz, 1999). If sensitive detection of individual CAPB components in real environmental samples like sewage water is a prerequisite, LC-MS is indubitably a promising method.

While for the most prominent anionic, non-ionic and cationic surfactants a great number of LC-MS methods has been established, examples for the utilization of MS for the analysis of amphoteric surfactants are very scant, possibly due to difficulties in detecting zwitterionic species. A TSI interface was applied to the qualitative analysis of CAPB in spiked effluent samples from WWTP (Schröder, 1996), but the performance of modern interfaces such as ESI or APCI has not been explored so far.

Method development

The components of a technical CAPB mixture were separated under RP conditions in the order of increasing length of the alkyl chain (**Fig. 4-44**). Since the hydrophobic moiety of the surfactant molecule is derived from coconut oil, the two homologues C12 and C14 form the major constituents according to the distribution in the natural raw product containing approximately 49 % of C12- and 19 % of C14-fatty acid (Reck, 1985).

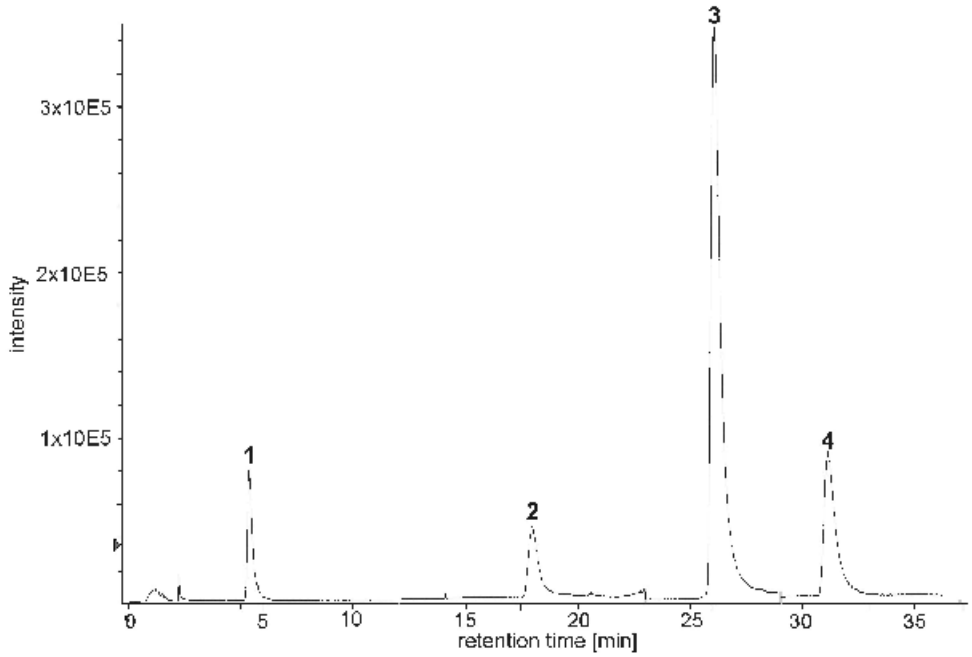

Fig. 4-44: (–)-LC-ESI-MS chromatogram of a CAPB standard. Peak numbering: (1) C8-CAPB, (2) C10-CAPB, (3) C12-CAPB, (4) C14-CAPB. LC conditions: 125 x 2.1 mm, 4 µm, C8-bonded silica with a gradient profile: water-CH_3CN, pH 7.9 (NH_3) at 0.2 mL min^{-1}.

Mass spectrometric detection with ESI was feasible in positive and negative ion modes. In (+)-ionization mode, the mass spectrum is characterized by a series of adduct ions, cluster ions as well as doubly charged monomers. In **Fig. 4-45** the mass spectrum of C12-CAPB is shown and the assigned ions are listed in **Fig. 4-9**. Apart from the protonated molecular ion $[M+H]^+$ at *m/z* 343, the sodiated $[M+Na]^+$ and the potassiated adduct ion $[M+K]^+$ were also detected at *m/z* 365 and *m/z* 381, respectively. Furthermore, dimer and with a minor intensity trimer clusters were observed (not shown in the spectrum) as their protonated, sodiated and potassiated adducts $[2M+H]^+$, $[2M+Na]^+$ and $[2M+K]^+$, and $[3M+H]^+$, $[3M+Na]^+$ and $[3M+K]^+$, respectively (*m/z* values are given in **Table 4-9**). Higher clusters of C12-CAPB could not be investigated due to the limited mass range of the quadrupole MS used (up to *m/z* 1200), but its quadromers can at least be expected since the corresponding species of C8-CAPB were observed (e.g. *m/z* 1145 of $[4M+H]^+$).

Formation of cluster ions under positive ESI was reported for high molecular oligonucleotides (Ding and Anderegg, 1995) and also for low mass compounds such amino acids (Yao et al., 2000; Zhang et al., 1999), tetracycline antibiotics (Kamel et al., 1999) and atrazine (Cai et al., 1996), but this behavior has barely been examined for surfactants. Dimers of the non-ionic APG were only formed at relative high analyte concentrations under flow-injection conditions (Klaffke et al., 1999). In this instance, apart from the ions $[2M+Na]^+$, mixed clusters of two different alkyl homologues were detected. These species, however, disappeared in the LC-MS system working with much lower concentrations. It is likely that bonding between CAPB molecules within the cluster is caused by electrostatic interactions between the zwitterionic head groups resulting in an enhanced stability.

In addition, adducts of the surfactant molecule with two cations leading to doubly charged species were observed (*m/z* 183, 191, 199.5 and 211; **Table 4-9**). While the ammonium ion arises from ammonia added to the eluent for adjusting the pH, the Na^+ and K^+ ion are likely to originate from impurities in the

solvents and the technical surfactant used. Cleavage of the bond between the methylene group and the quaternary nitrogen atom released the stable fragment $[M–H–(N(CH_3)_2\text{-}CH_2\text{-}COO)]^+$, detected at *m/z* 240.

A further ion detected at *m/z* 362 was assigned as the dimer $[2M+H+K]^{2+}$. Since it was the only doubly charged dimer observed, it seems that the combination of two C12-CAPB molecules with one proton and one potassium cation yields an especially stable species.

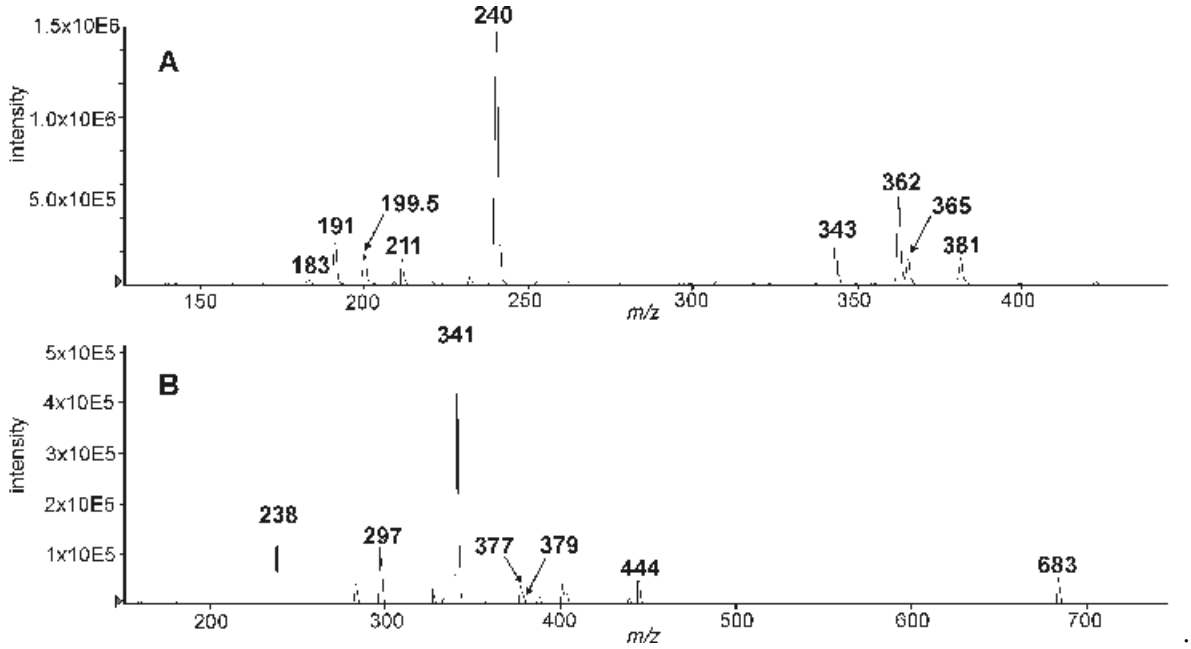

Fig. 4-45: LC-ESI-MS spectra of C12-CAPB: (**A**) positive ionization (OR: +45 V) and (**B**) negative ionization (–20 V).

It is noteworthy that the ratios between the protonated, sodiated, and potassiated forms of the molecular ions, the clusters and the doubly charged ions exhibited relative high day-to-day variations. Obviously, changing contents of competing cations deriving from the sample as well as from impurities in the solvents interfered in the formation of a desired species to be used for instance in quantitative analysis.

The mass spectrum of C12-CAPB acquired in negative ionization is displayed along with the (+)-spectrum in **Fig. 4-45** and the assigned ions are summarized in **Table 4-9**. While the most abundant ion corresponds to the molecular ion $[M–H]^-$ (*m/z* 341), the intensity of the adduct ion $[M+^{35}Cl]^-$ at *m/z* 377 is about 10-fold less. In analogy to the behavior in (+)-ion mode, clusters other than those of monomeric ions of the amphoteric surfactant were also observed; the ion *m/z* 683 was assigned to the dimer $[2M–H]^-$, and the trimer $[3M–H]^-$ was detected at *m/z* 1025. The loss of CO_2 from $[M–H]^-$ yielded the fragment ion *m/z* 297. A further fragment detected at *m/z* 238 corresponded to $[M–H–(N(CH_3)_2\text{-}CH_2\text{-}COO)]^-$. It has to be emphasized that the same fragmentation step occurred under positive ionization (see above). As in this instance the fragment carried two protons more, the ion was detected at two mass units higher (*m/z* 240).

An unusual ion was observed at *m/z* 444. It arose from the dimeric species $[2M–H]^-$, which had lost the fragment $[R\text{-}CO\text{-}NH\text{-}(CH_2)_3]$. Apparently a strong electrostatic interaction between the hydrophilic head groups of the two surfactant molecules – quaternary nitrogen atom of a first molecule interacted with an oxygen atom of the carboxylic group of a second molecule and vice versa – resulted in a pronounced stability of the dimeric form (see also cluster formation under (+)-ion mode), out of which

the fragment [R-CO-NH-$(CH_2)_3$] may be released without destroying the arrangement of the charged moieties.

Table 4-9: Ions and fragments of C12-CAPB detected in positive and negative ionization modes.

Positive ionization		Negative ionization	
Ion/fragment	*m/z*	Ion/fragment	*m/z*
$[M+H]^+$	343	$[M–H]^-$	341
$[M+Na]^+$	365	$[M+^{35}Cl]^-$	377
$[M+K]^+$	381	$[M+^{37}Cl]^-$	379
$[2M+H]^+$	685 [a]	$[2M–H]^-$	683
$[2M+Na]^+$	707 [a]		
$[2M+K]^+$	723 [a]		
$[3M+H]^+$	1027 [a]	$[3M–H]^-$	1025 [a]
$[3M+Na]^+$	1049 [a]		
$[3M+K]^+$	1065 [a]		
$[M+H+Na]^{2+}$	183		
$[M+H+K]^{2+}$	191		
$[M+NH_4+K]^{2+}$	199.5		
$[M+2K]^{2+}$	211		
$[2M+H+K]^{2+}$	362		
$[M+H–(N(CH_3)_2\text{-}CH_2\text{-}COO)]^+$	240 [b]	$[M–H–(N(CH_3)_2\text{-}CH_2\text{-}COO)]^-$	238
		$[M–H–CO_2]^-$	297
		$[2M–(R\text{-}CO\text{-}NH\text{-}(CH_2)_3)]^-$ [c]	444

[a] not shown in selected spectrum window [b] base peak [c] R: alkyl chain

Comparing the overall ion intensity of negative and positive ionization it is marked that the latter is about one order of magnitude more sensitive. A similar behavior was observed for AG and APG also yielding better ionization efficiencies in positive ion mode.

Since less and seemingly more stable molecular and adducts ions were formed under negative ionization, a survey was undertaken on the stability under varying conditions in order to find the most appropriate MS parameters for quantification in the following degradation experiment. To study the effect of different concentrations (0.1, 1, 10, 100 mg L^{-1}) and OR (–20, –50 and –80 V) on the formation of dimer and trimer CAPB clusters and on the extent of fragmentation in negative ion mode, the OR was switched during the same run at intervals of 0.2 sec. This provided the best method for a direct comparison of ion intensities. The MS was operated in SIM mode recording only the ions of interest to obtain the best sensitivity.

The dependence of the area ratio of monomeric to dimeric and to trimeric C12-CAPB on the concentration and the OR is shown in **Fig. 4-46**. At an OR of –20 V the area ratio is most affected by the concentration. A predominance of the monomeric species can be clearly noticed at low analyte concentrations. With increasing concentration of C12-CAPB from 0.1 mg L^{-1} to 100 mg L^{-1}, cluster formation becomes more likely and hence the ratios monomer/dimer (m/d) and monomer/trimer (m/t)

decrease. It should be expected that elevating the voltage from –20 to –50 V would result in a higher ratio since declustering is promoted. However, such a trend cannot be extracted from the diagram representing only area ratios. The absolute intensity of the cluster ions, especially the trimers, is lowered, but the signal intensities of the monomer decrease overproportionally. A further increase of OR to –80 V leads to minor changes of the ratios affecting virtually only the m/t-ratio at 1 mg L^{-1}. It should be mentioned that the $[2M–H]^-$ ion is no more detectable at 0.1 mg L^{-1} when switching from –50 to –80 V. Besides the trimer is not observed at any voltage running the lowest test concentration.

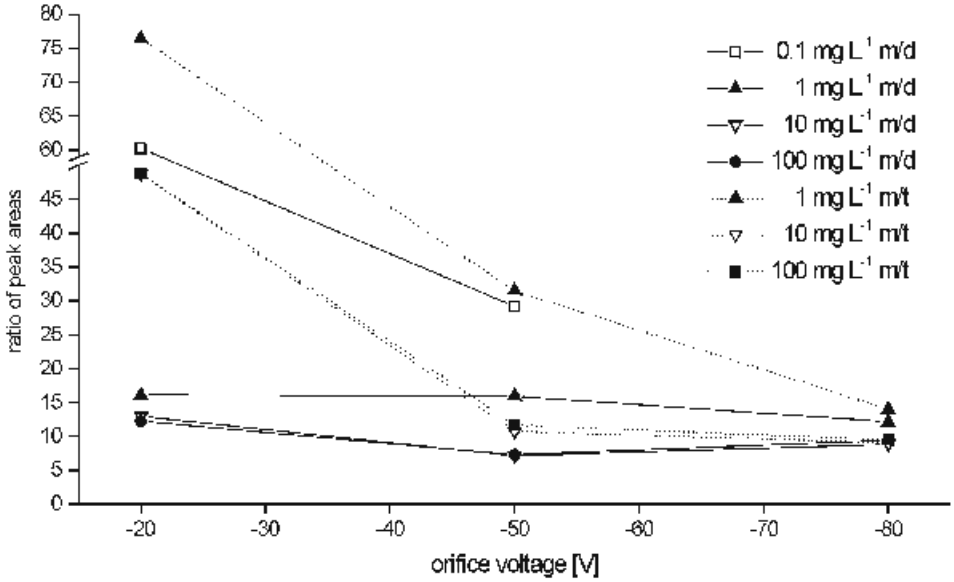

Fig. 4-46: Dependence of peak area ratio of monomer $[M–H]^-$ to dimer $[2M–H]^-$ (m/d) and to trimer $[M–H]^-$ (m/t) on OR and analyte concentration.

In a second study using a concentration of 1 mg L^{-1}, the dependence of absolute signal intensity of $[M–H]^-$, $[2M–H]^-$ and their two fragments $[M–H–(N(CH_3)_2\text{-}CH_2\text{-}COO)]^-$ and $[2M–(R\text{-}CO\text{-}NH\text{-}(CH_2)_3)]^-$, respectively on the OR was investigated (**Fig. 4-47**).

The molecular ion $[M–H]^-$ was by far the most abundant ion at –20 V. While its intensity dropped by more than a factor of four when the OR was set to –50 V, the relative decrease of $[2M–H]^-$ was less pronounced. At the same time, the intensities of both fragments slightly increased. This is a similar behavior to the one described above where the intensity of the monomer decreased overproportionally compared to that of dimer and trimer clusters. The elevation of OR to –80 V finally led to a further reduction of $[M–H]^-$ paralleled by a more intense signal of its fragment. In contrast with this, a loss of peak intensity of the dimer and its corresponding fragment was registered.

Taking into consideration the irreproducible adduct formation observed under positive ion mode and the fact that the molecular ion $[M–H]^-$ is the most prominent ion in negative ionization at low concentrations (=1 mg L^{-1}) prevailing over dimers and trimers, the latter ion mode was considered as better choice for quantitative analysis even if it exhibited a somewhat lower sensitivity. This disadvantage, however, is partly outweighed by the fact that (–)-ion mode is less prone to interferences from co-eluting compounds generally present in real samples.

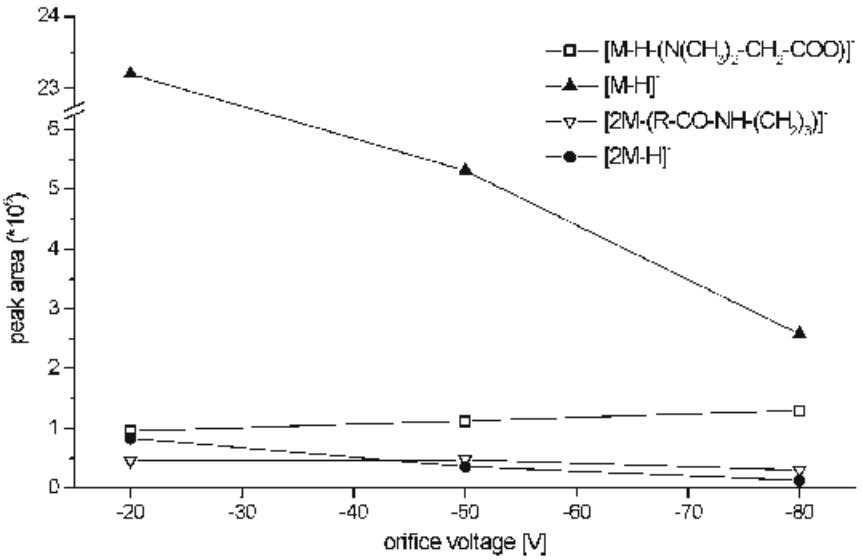

Fig. 4-47: Absolute peak intensities of $[M–H]^-$ and $[2M–H]^-$, and the two fragments (concentration: 1 mg L^{-1}).

4.4.3 Biodegradation test

Literature data

Only few data were published on the degradability of betaines. The information available is contradictory. Swisher (1987) indicated that total degradability is rather poor (45-58 %). Brunner et al. (2000) in turn subjected a series of amphoteric surfactants to biodegradation in an extended OECD 302B test and a laboratory trickling test filter. While the two compounds cocoamphodiacetate and cocoamphodipropionate were mineralized only to a minor extent, CAPB and cocoamphoacetate proved to be nearly totally degradable.

Metabolism study

The degradation study was performed by amending the FBBR with 10 mg L^{-1} of the CAPB mixture. For analysis of CAPB the mass spectrometer was operated in negative ion mode registering $[M–H]^-$ of the four homologues C8- to C12-CAPB and as qualifier ions their corresponding fragments $[M–H–(N(CH_3)_2\text{-}CH_2\text{-}COO)]^-$.

The concentration profile of the analyzed CAPB homologues revealed initial losses attributed to adsorption onto glass surfaces most likely on the porous glass beads used as carrier material of the fixed-bed (**Fig. 4-48**). A correlation between the alkyl chain length and the tendency to adsorption can be established; approximately 70 % of the most hydrophobic component C14-CAPB were lost within the first hours of the experiment. The dissolved concentration of C12-CAPB dropped to about 80 % of the initially present value and the least hydrophobic homologues C10- and C8-CAPB exhibited minor losses in the range of 5 to 10 %. Since after the initial losses of the four homologues relative stable levels were

achieved, it could be assumed that during the first day (for C14 a somewhat shorter time) no significant reduction brought about by microbial degradation occurred, i.e. this period represented the acclimation phase. A substantial decline of C12-CAPB caused by biodegradation was observed after about 26 h, whereas the analogous point of C14-CAPB cannot be clearly defined. After 33 h both compounds have nearly completely disappeared. The concentration decline of the two shorter alkyl chain homologues C8 and C10 required a somewhat longer lag phase, until 33 h after spiking. The rate of biodegradation was still very low, while in the sample taken after 48 h, their concentrations had decreased by more than 95 %. In the 53 h-sample, none of the analytes was detectable anymore.

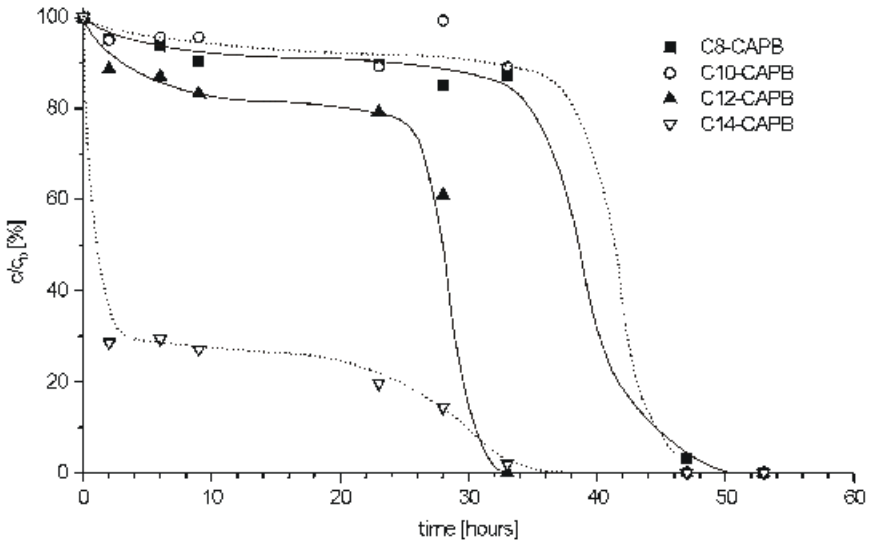

Fig. 4-48: Profile of primary degradation of C8- to C14-CAPB on FBBR at a test concentration of 10 mg L^{-1} (superposed by adsorption).

In conclusion, the degradation rates of the four homologues can be correlated with the length of the hydrocarbon chain. Such kind of relationship was likewise stated for alkyl homologues of LAS (*Swisher´s distance principle*). Higher homologues, i.e. the more lipophilic ones were degraded faster. Initial step in LAS destruction is the ω-oxidation of the terminal methyl group of the alkyl chain resulting in the formation of carboxylic acids. Subsequently, the breakdown proceeds through β-oxidations, i.e. via successive shortening of the alkyl chain by C2-units to form lower carboxylated homologues.

In this respect FBBR samples were investigated for the presence of analogous intermediates of CAPB breakdown. Acquisition of (–)-ionization full-scan chromatograms in the range from *m/z* 120 to 400 as well as the direct search for the theoretical masses in SIM mode did not yield positive results on CAPB with a carboxylated alkyl chain (C14 to C4: *m/z* 391, 363, 335, 307, 279, 251). The investigation on any other compound formed out of the amphoteric surfactant was unsuccessful. Presumably, the intact surfactant molecules were taken up by the cells and rapidly degraded intracellular without releasing any metabolite. This assumption receives support by the results of Brunner et al. (2000) demonstrating high ultimate degradation of CAPB.

4.5 Poly(vinylpyrrolidone)

4.5.1 Introduction

Poly(vinylpyrrolidone) (PVP; **Fig. 4-49**) is a highly water-soluble synthetic polymer with average molecular weights between 2,5 to 1,200 kDa and a broad molecular weight (MW) distribution. These main physiochemical parameters are distinguishing the area of its use. Soluble PVP is obtained by free-radical polymerization of vinylpyrrolidone in water or 2-propanol, yielding the polymeric chain structure (Hallensleben, 1992; Vieweg et al., 1971). The mechanism for terminating the polymerization reaction makes it possible to produce soluble PVP of almost any molecular weight.

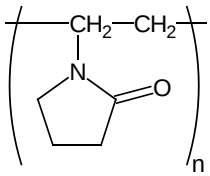

Fig. 4-49: Structure of poly(vinylpyrrolidone). Molecular weight of monomeric unit: 111 Da. End group functionality is dependent on synthetic route.

Soluble PVP was first used during World War II as blood-plasma substitute. Although it had excellent properties for this purpose, it has no longer been used for a number of decades since the organism does not metabolize the polymer, i.e. small quantities of high MW components may remain within the body (Wessel et al., 1971). Due to interaction with low and high molecular weight compounds in aqueous solutions, it is widely used in adhesives, paper manufacturing, food industry, synthetic fibers, and personal care products. Soluble PVP is a very useful and versatile pharmaceutical auxiliary improving the desolvation behavior of drugs (Loftsson et al., 1996; Vélaz et al., 1997). In detergent formulations PVP exhibits building action by promoting soil removal efficiency and preventing redeposition of soil (Tokiwa and Imamura, 1972). Since the early 1990s PVP – and later co-polymeric forms thereof – found a new field of application as dye transfer inhibitor in detergents. Owing to ion-dipole interactions between the sulfo groups of dyes and the flexible polymer, dissolved dyes are stabilized in the washing liquor and hence inhibited to deposit on other laundry (Jäger and Denzinger, 1991; Kistenmacher et al., 1999). The efficiency of the polymer increases with increasing MW.

4.5.2 Method development

Literature methods

No analytical procedures were reported in the literature for the determination of PVP in environmental matrices. However, methods for the identification and characterization of PVP in raw materials and formulations were described. These included IR spectroscopy for qualitative analysis of PVP and photometry of an PVP-iodine complex (Müller, 1968). For MW determinations high osmotic pressure chromatography and gel permeation chromatography (GPC) were used by Xu et al. (1999), while Güner (1997) employed light-scattering photometry. Pyrolysis-GC-MS was applied to ground detergent

products and key fragments formed were used as markers to quickly identify PVP among other dye-transfer inhibitors (Uchiyama et al., 1998).

Method development

All these approaches are not applicable for the detection of individual oligomers of PVP at a low mg L^{-1} level in environmental samples. Due to the limited mass range of the quadrupole MS used, MALDI-ToF-MS was taken into consideration as an extension of a detection method for FBBR investigations. Although signal intensities in MALDI-ToF-MS can vary much stronger than with solvent-based methods in MS, it was the most promising analytical tool for qualitative and semi-quantitative analysis of the polymeric target analyte.

Preliminary tests were conducted by the MPI [1] to evaluate the suitability of different matrices and inorganic salts, providing the cation source. Instrumental parameters were optimized and several PVP distributions with different mean MW were elucidated for their suitability to be used in the FBBR study. Furthermore, the relation between the concentration of PVP, dissolved in MilliQ water or surface water, and the signal intensity in MALDI-ToF-MS was established.

It turned out that PVP with a mean MW of 2.5 kDa [2], mass spectrometrically detected as singly charged sodiated adducts ions with repeating units of *m/z* 111, gave best results in terms of sensitivity, reproducibility and quantitation potential. An additional advantage of the low mass region was a much higher resolution aiding in isotopic resolution and therefore in an increased accuracy. Though non-linearity of the signal intensity in the range from 1 to 1000 mg L^{-1}, corresponding to concentration boundaries yielding very poor mass spectra and saturation of the detector, respectively, was observed, a concentration-dependent intensity was perceived with the highest slope between 10 and 100 mg L^{-1}.

To allow working with the lower concentration of 10 mg L^{-1} in the following FBBR experiment, preconcentration of PVP from spiked surface water by SPE and lyophilization were compared for their appropriateness. It proved that the mass spectra (**Fig. 4-50**) of the SPE enrichment procedure showed an overrepresentation of the lower oligomers (C) in relation to an untreated surface water sample spiked with PVP (A), while lyophilization of the spiked water (B) gave reproducible results without affecting the MW distribution severely. Moreover, MALDI-ToF-MS analysis of the SPE extract revealed the presence of a second oligomer distribution (indicated by ? in spectra (C)) with a repeating unit of *m/z* 111, which is typical of PVP. The identity of this distribution, however, could not be elucidated.

[1] Max Planck-Institute for Polymer Research, Mainz.

[2] End group functionalities of PVP 2.5 kDa were identified through post-source decay MALDI-ToF-MS fragmentation investigations determining the end groups as –OH and $–CH_2$-CHO, respectively.

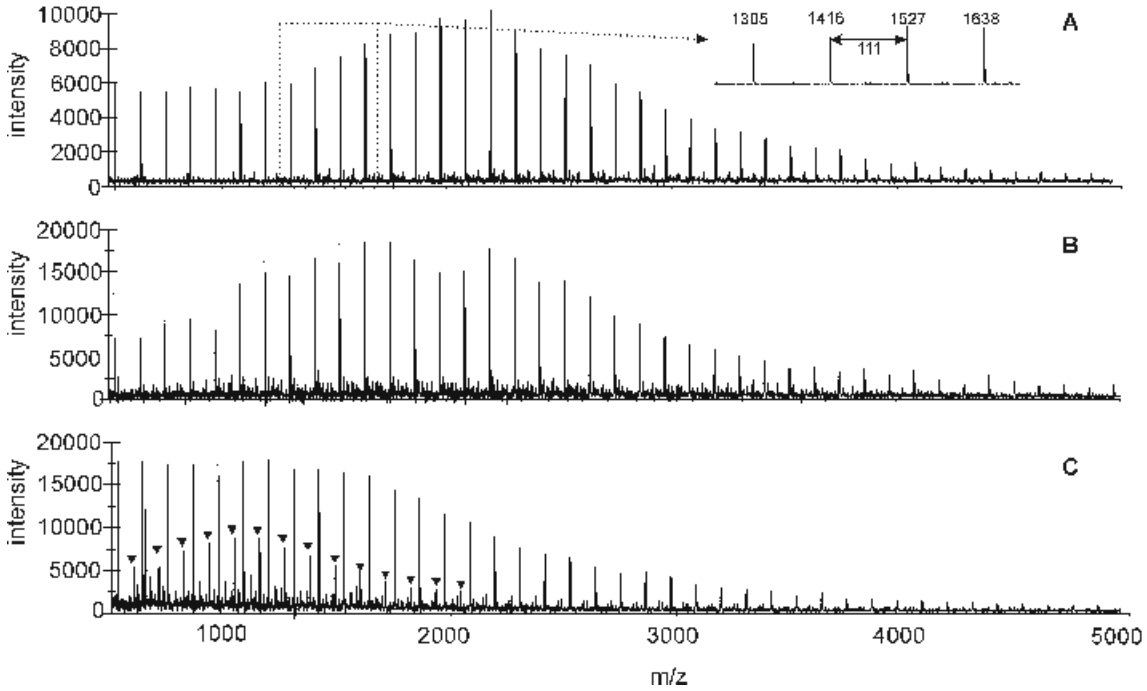

Fig. 4-50: MALDI-ToF-MS spectra of PVP 2.5 kDa of enrichment procedures: (**A**) PVP spiked to surface water, (**B**) lyophilized extract of spiked surface water, and (**C**) SPE extract of PVP spiked surface water. The ion *m/z* 1527 shown in the inset in (**A**) corresponds to $[PVP_{13}+Na]^+$.

4.5.3 Biodegradation test

Literature data

In contrast to many other organic pollutants occurring in wastewaters, such as the surfactants examined in the previous sections, the resistance of synthetic polymers has received far less attention. This is mainly due to the fact that they are not a toxicological issue. Nonetheless, they represent a possible source of contamination to the aquatic environment owing to their mode of application.

Of the most commonly used classes of synthetic polymers, the majority is wholly resistant to biodegradation such as e.g. high MW poly(ethylene), poly(vinyl chloride) or poly(styrene) (Otake et al., 1995). They do not serve as carbon sources for bacteria or fungi, and they are also not subject of co-metabolism. Only a few man-made polymers of commercial relevance are biodegradable including poly(vinyl acetate), poly(vinyl alcohol), and a number of polylactones (Alexander, 1994; Solaro et al., 2000). While studies on crosslinked PVP, synthesized by radical polymerization of vinylpyrrolidone in the presence of divinyl glycol as a crosslinking agent, indicated the possibility of biodegradation (Hong et al., 1996), no information has been found in the literature on homopolymeric PVP.

Metabolism study

For the FBBR biodegradation investigations, the low molecular weight PVP 2.5 kDa was taken and spiked at 10 mg L^{-1} to the test medium. Apart from the above mentioned advantages of this analyte mass region, it appeared to be valuable since it showed a sufficient span to pursue the degradation of the

analyte while not forfeiting a quality decrease in the mass spectra due to interference of background and matrix-adduct-formation commonly observed in the mass region below 1 kDa.

Indication for biodegradation of PVP in the test device should have been received by either detecting a collapse in the analyte distribution after a certain time of acclimation or an emerging oligomeric distribution of a potential metabolite formed. Follow-up of PVP by analyses of freeze-dried samples (enrichment factor of 10) taken over a period of 30 days gave no hints on any microbial degradation of PVP (**Fig. 4-51**). A slow diminishing of higher MW oligomers during the course of the FBBR experiment can be explained by a decrease in concentration, which involves a decrease in signal intensity. This slowly vanishing process very likely does not indicate a biodegradation, since the signal intensities of the smaller oligomers in the polymer distribution were nearly identical after 1 day (not shown) for the whole period of time (C and D) and only varied in the error of reproducibility. It rather could be attributed to an adsorption phenomenon, in particular of higher MW oligomers of PVP to interact and remain to the interior of the FBBR, most likely on the porous glass beads of the fixed bed.

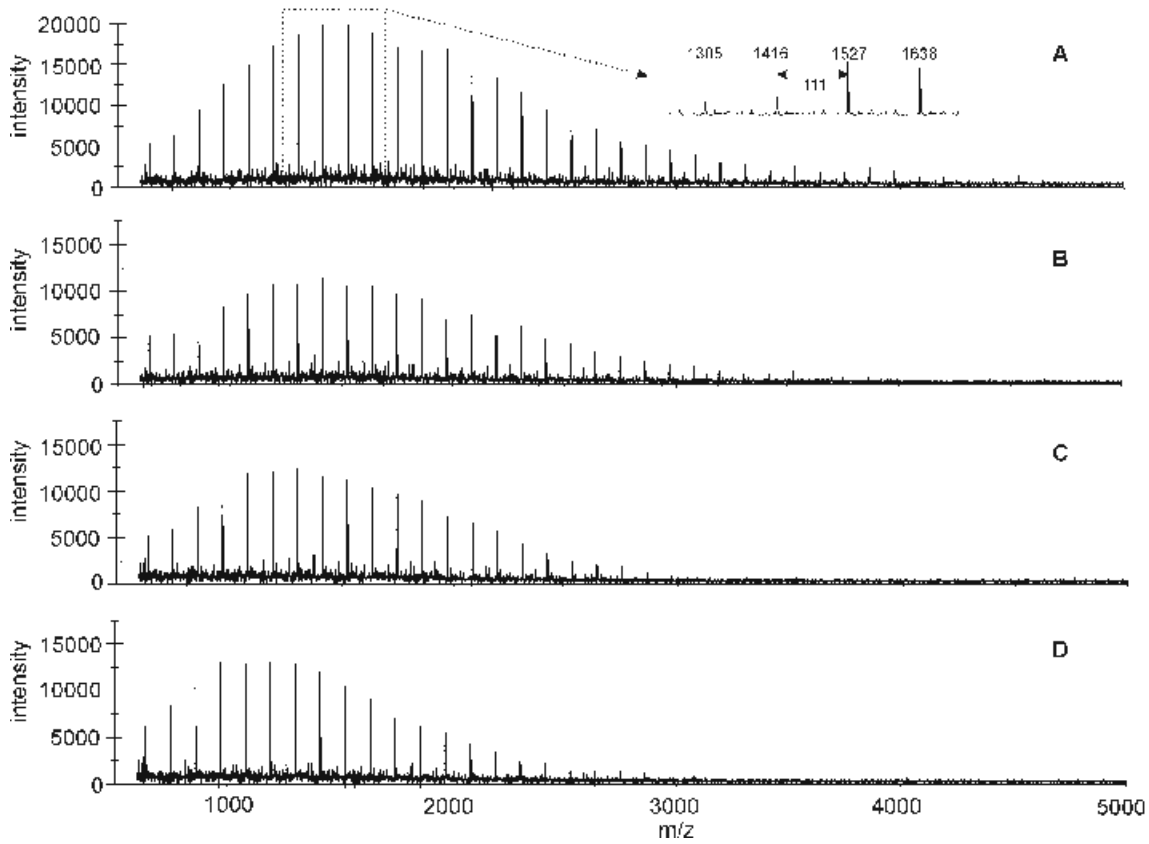

Fig. 4-51: MALDI-ToF-MS spectra of the FBBR degradation course of PVP 2.5 kDa, (**A**) 0 hours, (**B**) 5 hours; (**C**) 8 days, (**D**) 16 days. The ion *m/z* 1527 shown in the inset in (**A**) corresponds to $[PVP_{13}+Na]^+$.

A verification of the recalcitrance of PVP and its strong adsorption tendency was obtained by an experiment performed in sludge suspensions. Dry sewage sludge from a municipal WWTP was mixed with an aqueous PVP solution of 100 mg L^{-1} in a mass ratio of 1:40. The PVP sample was chosen under following aspect: high MW oligomers with a low extent of small oligomeric compounds. The main GPC fraction of PVP-K17 showing a MW distribution with good signal intensities from about 5 to 8 kDa in the mass spectrum was chosen. Adsorption of PVP to the sewage sludge was pursued by analyzing aliquots

of the aqueous supernatant, separated from suspended particles by centrifugation, applying MALDI-ToF-MS.

The sensitivity of the mass spectrometric detection, comprising an identical sample preparation process as used in the biodegradation study, was insufficient to detect PVP already after a short period of incubation with sewage sludge (30 min). For this reason a different sample target (termed as anchor target) was used showing a 10-fold increase in sensitivity compared with the previously applied MALDI target.

MALDI-ToF-MS measurements with this anchor target device of the adsorption experiment were able to detect PVP in the aqueous mother liquor after an incubation period of 30 min, but failed after 2.5 hours (**Fig. 4-52**, A). These observations suggested that adsorption of PVP to the sewage sludge appears to be a very fast process and that the PVP concentration in the mother liquor was below 10 mg L^{-1} after 30 min and below 1 mg L^{-1} after 2.5 hours. The control experiment run in parallel demonstrated that PVP dissolved in water in the absence of sludge did not show any decrease in PVP concentration in the mother liquor (**Fig. 4-52**, B). The results of the adsorption together with the control experiment verify unambiguously that PVP was rapidly absorbed onto the sludge and to a very high extent.

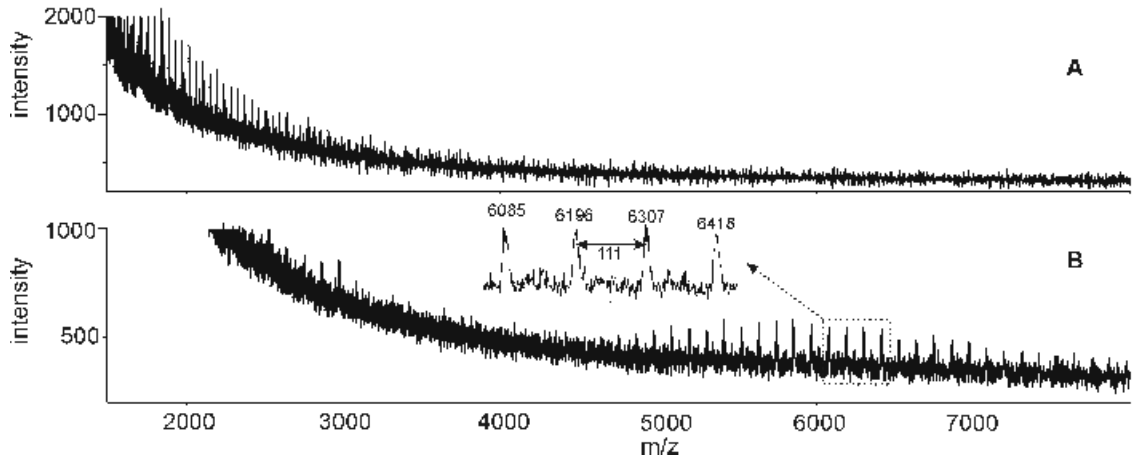

Fig. 4-52: MALDI-ToF MS spectra 2.5 hours samples of the adsorption experiment of PVP (5 to 8 kDa), (**A**) PVP spiked to sludge-water suspension, (**B**) PVP spiked to water.

In the MALDI-ToF-MS measurements of the aqueous phase of the adsorption study there was an additional polymer distribution observed in the mass range below about *m/z* 3,000 with a repeating unit of *m/z* 44, which was attributed to poly(ethylene glycol) (PEG). Verification was easily obtained by a further experiment where the sludge was shaken with water without adding PVP (**Fig. 4-53**).

There is a visible increase observed in the concentration of PEG between 1 hour (A) and 2.5 hours (B), which is then constant during the course of sampling (last sample taken after 32 hours). The experiment showed that the additional polymer distribution, also detected in the samples in **Fig. 4-52**, resulted from PEG, which was already adsorbed onto the sewage sludge and dissolved during the sludge shaking experiment.

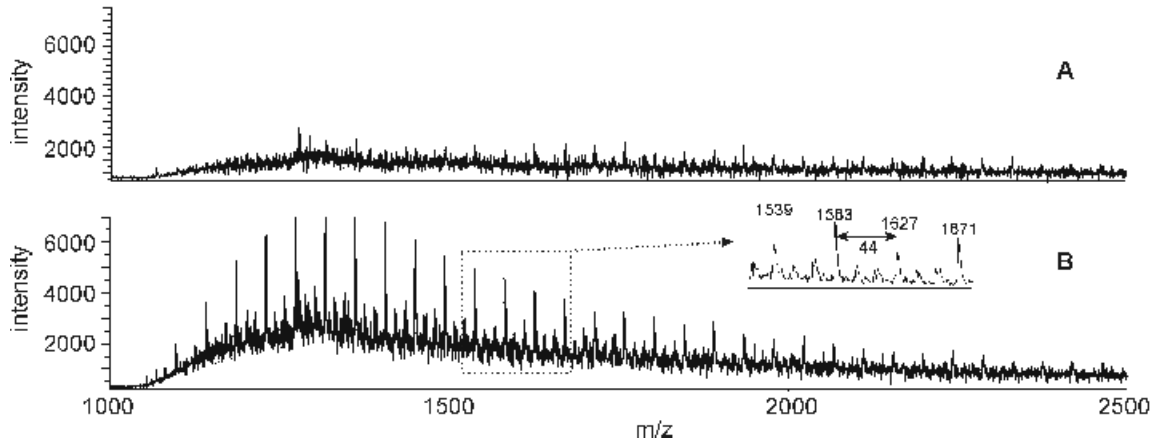

Fig. 4-53: MALDI-ToF MS spectra of samples of the desorption experiment, (**A**) after 1 hour, (**B**) after 2.5 hours.

PEG itself is known to be formed as a metabolite of aerobic biodegradation of polyethoxylated surfactants such as AE and AES (Hoffmann et al., 1999; Marcomini et al., 2000a; Yoshimura and Masuda, 1982). It also originates from commercial formulations where it is present as starting material or by-products from manufacturing processes (Castillo et al., 2000). Despite the high water solubility of PEG, it tends to partition onto sewage sludge during biological wastewater treatment. Residues of PEG in sewage sludge were found at levels between 4 and 31 mg kg^{-1} (Petrovic and Barceló, 2000b), while concentrations in industrial waste effluents were reported to amount to up to 1.0 mg L^{-1} (Castillo et al., 2000).

The findings of the investigations on the biodegradability and the adsorption behavior of PVP allow to assume that this synthetic polymer, discharged into domestic wastes, is eliminated from the dissolved phase in WWTP by adsorption onto sludge owing to its extraordinary complexation property. The question of the final fate and destiny of the adsorbed PVP remains to be answered.

5 SURFACTANTS IN THE AQUATIC ENVIRONMENT

5.1 Monitoring of wastewater treatment plants

A large number of studies conducted on the behavior of surfactants during wastewater treatment have demonstrated the importance of such treatment facilities to eliminate these anthropogenic compounds from the sewage, thereby largely preventing them from reaching the natural aquatic environment. Due to the relevance of LAS, which is the most abundantly used surfactant worldwide, their environmental fate has been the focus of interest since the early 1970s. The majority of monitoring programs concluded – after 1985 most researchers applied substance-specific analytical methods – that LAS is efficiently removed in WWTP by both biological and physical processes. Although SPC were identified as their major degradative products almost four decades ago (Huddleston and Allred, 1963), the knowledge on their occurrence in wastewater discharges is still very scarce primarily because of difficulties in analyzing trace amounts of the polar arylsulfonates in complex matrices. Only a few papers have reported on environmental levels of these breakdown products (di Corcia et al., 1994 and 1999b; Trehy et al., 1996).

On the other hand, the two non-ionics AG and APG, used as co-surfactants in household surfactant formulations, have by date been beyond the scope of research most likely because of the relative low production volumes.

Table 5-1: Technical characteristics of investigated WWTP.

	WWTP-1 Igualada, Spain	WWTP-2 Montornès, Spain	WWTP-3 Abrera, Spain	WWTP-4 Wiesbaden, Germany
Type of treatment	Biological	Physico-chemical	Physico-chemical	Biological
Type of wastewater	Domestic,industrial	Domestic,industrial	Domestic,industrial	Domestic
Input [m^3 day^{-1}]	20,000	30,000	14,000	50,000
Number of habitants in the area	60,000	120,000	43,000	280,000
BOD (influent) [mg L^{-1}]	875	375	370	200
Hydraulic retention time [days]	1.1	-	-	0.9
BOD elimination [%]	> 97	> 60	> 60	99

Keeping in mind this paucity of information, the efficiency of wastewater treatment on the elimination of LAS, AG and APG and the intermediate formation of SPC was investigated by analyzing influent and effluent samples of four selected WWTP differing in type of treatment and sewage composition (**Table 5-1**). Furthermore, these investigations should allow verification of the results of the FBBR experiments in view of the primary surfactants biodegradability and the build-up of persistent metabolites as observed in the LAS laboratory breakdown.

The results from the quantitative analysis of the four target analytes in the raw and treated water samples are shown in **Table 5-2**. As for LAS, the concentrations in the three Spanish WWTP range between 2.0 and 3.2 mg L^{-1}, i.e. levels which are found at the lower end of the range reported in two recent monitoring programs of Dutch and U.S. WWTP with levels ranging between 3.4 and 8.9 mg L^{-1} (Matthijs et al., 1999) and 1.8 and 6.1 mg L^{-1} (McAvoy et al., 1998), respectively. The comparatively low levels detected in the influents may be due to a partial removal of these species during transportation in the sewage system since the three WWTP receive wastewater from remote areas connected via a sewer system of up to 25 km length.

Moreno et al. (1990) reported on the influence of long transportation ways and thus residence times in the drainage system on the removal of LAS. Along a distance of 12 km between the major discharge point and the receiving WWTP, 43 % of the initial LAS were removed through biodegradation. Berna et al. (1989) compared the total levels in raw sewage at nine Spanish WWTP with the theoretical loads calculated based on the per capita consumption. Biodegradation losses in the main sewer (length of up to 40 km) ranged between 28 and 70 % demonstrating the importance of in-sewer removal. The presence of a microbial activity in the entering wastewater of WWTP-1 to 3 is further confirmed by positive detection of SPC (see below).

Table 5-2: Concentrations of surfactants and their metabolites in WWTP (µg L^{-1}).

	WWTP-1		WWTP-2		WWTP-3		WWTP-4	
Compound	Influent [a]	Effluent [a]	Influent	Effluent	Influent	Effluent	Influent	Effluent
LAS	2020	10	2620	91	3170	75	1290	9
SPC	6	53	14	66	22	168	11	15
AG	36	0.2	26	< 0.1	45	< 0.1	60	n. d.
APG	6.5	n.d.	16	n. d.	13	n. d.	8	n. d.

[a] Concentrations are mean of three samples taken on different daytimes; n. d.: not detected

Another indicator for removal in the incoming sewage is the distribution of the four alkyl homologues of LAS with a mean chain length ranging between 11.1 and 11.2 in the influent (laundry detergents: 11.4 to 11.7). This parameter, however, is impacted by biodegradation and adsorption onto suspended solids; both processes discriminate the shorter chain homologues due to their smaller lipophilic moieties. Hence, the contribution of each mechanism is difficult to assess solely based on the determination of dissolved LAS as done here.

In an LAS monitoring exercise conducted by Berna et al. (1989) the proportion of LAS bound to suspended solids was determined by establishing a mass balance for the raw water. At nine WWTP examined the amount of anionic surfactant adsorbed onto solid particles varied between 27 and 67 %.

In comparison to the concentration found in the three Spanish WWTP, the LAS level in the Wiesbaden plant is even lower amounting to only 1.3 mg L^{-1}. It may be suggested that in-sewer removal likewise plays an important role. Despite relative short transport ways in the canalization, the biological activity might be augmented by relative high temperatures of the sewage water. Several hot springs in the underground of the catchment area at the city of Wiesbaden heat the wastewater thus becoming the warmest among all WWTP in Germany (Rudewitt, personal communication).

Based on the LAS concentrations in the treated water from the four WWTP ranging between 9 and 91 µg L^{-1} (**Table 5-2**), total elimination efficiencies were calculated (**Table 5-3**). At all facilities, more than 96 % of LAS present in the influent were removed. These values are in line with the removal averages described in the two studies cited above (99.2 and 99.9 %, respectively).

It is noteworthy that unlike the two activated sludge plants WWTP-1 and 4, the other two treatment works WWTP-2 and 3 yield quite convincing elimination efficiencies only by applying physico-chemical treatment.

Table 5-3: Elimination efficiencies of surfactants in different WWTP (%).

Surfactant	WWTP-1	WWTP-2	WWTP-3	WWTP-4
LAS	99.5	96.5	97.6	99.3
APG	100	100	100	100
AG	99.4	100	100	100

Initiation of biodegradation of LAS during transportation in the sewer is proved by the detection of their degradation intermediates in the untreated raw water in the range between 6 and 22 µg L^{-1} (**Table 5-2**). In the corresponding effluents, the SPC concentrations are always higher spanning a broader range between 15 and 168 µg L^{-1}. The same tendency of increased SPC levels in the treated water was described by Trehy et al. (1996) analyzing wastewater samples by GC-MS after derivatization. In four activated sludge plants treating domestic sewage they found on average 27 µg L^{-1} in the influent and 50 µg L^{-1} in the corresponding effluent. Somewhat higher values were reported by di Corcia et al. (1999b) conducting a long-term study on three WWTP. Mean levels of SPC in untreated waters, quantified by LC-ESI-MS, amounted to 98 µg L^{-1}, while in effluents 153 µg L^{-1} were determined.

The residues of SPC in the treated water of WWTP-1 to 4 entering the receiving stream can be traced back to an unfinished degradation in the plants as a consequence of a limited hydraulic retention time, but it appears that a fraction of the remaining carboxylated LAS intermediates exhibit very slow degradation kinetics. Compared with the XIC of C7-SPC found in the river Rhine and the FBBR experiment, spiked with commercial LAS, (ref. to **Fig. 4-24**) a quite similar peak pattern can be recognized (XIC not shown).

In view of relative distribution of individual SPC species in the four investigated WWTP, different patterns are observed in the effluent samples (**Fig. 5-1**). The most abundant species in WWTP-1 and WWTP-2 are C7-SPC and C8-SPC, while the relative maximum in WWTP-3 centers at C9-SPC possibly because of a slower progression of SPC degradation occurred during sewage treatment. This would explain also the finding that relative high amounts of C10-SPC and C11-SPC compared to those in WWTP-1 and WWTP-2 are found. Regarding the pattern found in WWTP-4 showing the highest

individual contribution of C7-SPC and nearly equal amounts of C6- and C8-SPC, it can be concluded that the SPC degradation has far progressed.

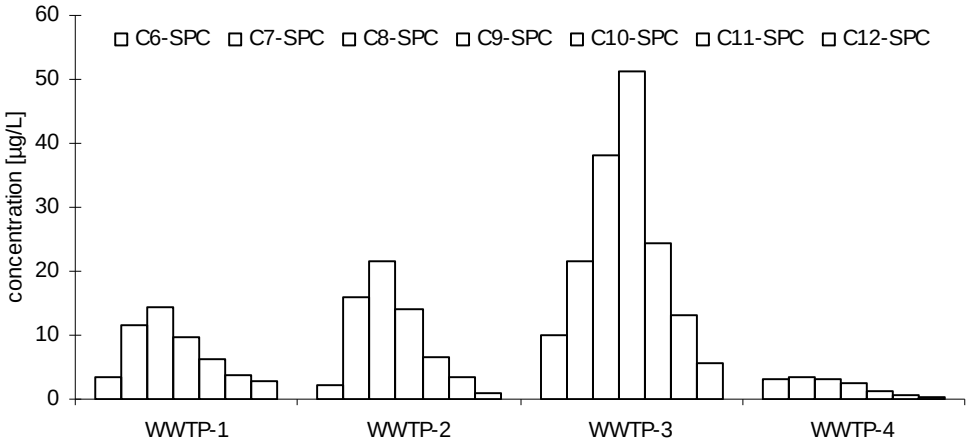

Fig. 5-1: Distribution of SPC homologues in effluents from WWTP.

The measured concentrations of the non-ionics in the raw water samples are between 26 and 60 µg L^{-1} for AG and between 8 and 16 µg L^{-1} for APG (**Table 5-2**). They are therefore less abundant than LAS reflecting their lower application volume due to predominant use as co-surfactant, whereas LAS surfactants usually make up the main surface-active agent in formulations used in household products, as e.g. in laundry detergents. Although only the alkyl monoglucosides C8 to C14 were taken into account for quantification, the values can be considered as rather reliable as the mean degree of polymerization of APG ranges between 1.2 and 1.7 (ref. to **Fig. 4-36**, p. 81). In none of the effluent samples APG are detected and AG only reach a maximum concentration of 0.2 µg L^{-1} in WWTP-1, while in the samples from WWTP-2 and 3 their levels are below the limit of quantification of 0.1 µg L^{-1}. In WWTP-4, no residues of AG are found. Thus, the corresponding removal efficiencies exceed 99.4 % and even reach 100 % in most cases (**Table 5-3**).

These quantitative results on the occurrence of AG and APG, which are reported here for the first time, reveal that the estimations on environmental concentrations presented by Vollebregt and Westra (1998) were fairly unrealistic. Estimations were made for Germany if APG were used as non-ionic surfactant in all-purpose detergents. The calculated concentrations in influent water were predicted to 10 mg L^{-1}, in the corresponding effluent to <100 µg L^{-1}. From the prognosticated level of AG in surface waters (1.5 µg L^{-1}) the authors worked back from the dilution factor of ten and a removal in WWTP of 99.6 % yielding an influent concentration of 3.75 mg L^{-1}.

In conclusion, the survey of the selected WWTP confirms the data gathered from the laboratory degradation experiments with respect to the rapid primary degradability of the studied surfactants. While the two sugar-based non-ionics AG and APG are almost quantitatively eliminated in the course of wastewater treatment with secondary effluent levels generally below 0.1 µg L^{-1}, the LAS concentrations in the treated water are still found in the low to mid µg L^{-1} range. In spite of high removal efficiencies greater than 96 %, the hydraulic retention time of the wastewater in the treatment facility is insufficient to entirely eliminate LAS, which enter the plants at low mg L^{-1} amounts. With regard to their aerobic metabolites, it can be stated that some notable remainders are released into the receiving water bodies. At least a fraction of these species can expected to persist over a longer period of time in the river, in which

the high water solubility of SPC in connection with a negligible bioaccumulation or adsorption onto particulate matter or sediments will enable convective transportation. This is demonstrated in the following sections.

5.2 Surfactant contamination of surface waters by untreated wastes in tropical countries

The preceding monitoring study conducted to assess the removal of LAS, AG, and APG during wastewater treatment has illustrated the capacity of treatment plants to efficaciously reduce the surfactant loads in sewage, thereby avoiding to a great measure that these compounds reach surface waters. In face of the large-scale usage of surfactants consumed daily and subsequently discharged into domestic and industrial sewage water, this is of ample significance for the protection of aquatic life forms in receiving streams. However, the treatment of wastewater in adequate facilities is not available in various regions even in highly industrialized countries. Hence, surfactant residues in wastewaters are continuously emitted into rivers and ocean without previous treatment (Ding et al., 1999; González-Mazo et al., 1997).

To study the impact of direct wastewater discharges to surface waters and to pursue the whereabouts of surfactants in such polluted rivers, areas in two different countries were taken as examples where such situations are widely met. For these purposes the analytical measurements centered on LAS and SPC since higher environmental levels were expected, compared with those of AG and APG, which should allow follow-up of their fate in the examined surface waters. Moreover, the non-ionics were of less interest as they had proved ready biodegradability without leaving behind any recalcitrant degradation intermediate.

5.2.1 Occurrence of linear alkylbenzene sulfonates and sulfophenyl carboxylates in Brazilian surface waters

Wastewater discharge situation in Brazil

The first study was conducted on natural waters in Brazil where only 10 % of the urban population have their sewage treated. In rural areas this value can even be lower than 5 % (Philippi et al., 1999). Hence, surface waters are expected to be substantially affected by domestic wastewater. The objective was to investigate the fate of LAS and SPC in a river located north-eastern from the city of Niterói (state of Rio de Janeiro, Brazil) by monitoring the concentrations at several stations along the river course including their determination in the Baía de Guanabara representing the interface between the freshwater and the marine environment.

Selection and description of area of examination

The selection of the object to be studied was guided by two principal prerequisites: on the one hand, a major point source of wastewater emission should be present in the upper course of the river, prior to which the water was relatively uninfluenced by human activities. On the other hand, the water flow should not be significantly affected by tributaries to the main stream in order to largely rule out concentration changes caused by dilution effects. The section of the Rio Macacu between Cachoeiras de

Macacu and the river's confluence with the Rio Guapi-açu (**Fig. 5-2**) met well the requirements. At Papucaia the river flow varies between ca. 17 $m^3 s^{-1}$ during rainy season and about 6 $m^3 s^{-1}$ during dry periods (SEMADS, 1999).

At the Rio Macacu, receiving discharges of untreated domestic wastewater from several villages located along the riverbank, the towns Cachoeiras de Macacu and Japuíba with populations amounting to 18,000 and 20,000 respectively were identified as the principal discharge points (SEMADS, 1999). Consequently, the river water contained considerable amounts of LAS as well as SPC formed during transportation of the wastewater in the sewerage system and subsequently in the stream.

Furthermore, the Rio Macacu was of particular interest as it was involved in the preparation of drinking water for about 2.5 million people in the region, including the towns of Cachoeiras de Macacu, Niterói and São Gonçalo, all of them located on the east side of the Baía de Guanabara (**Fig. 5-2**). This point is discussed in detail in section 5.3.2.

Alkylbenzene sulfonate and sulfophenyl carboxylate in the Rio Macacu and the Baía de Guanabara

Prior to quantitative determinations of LAS and SPC in the surface waters, it was verified whether the non-linear alkyl chain congeners of LAS, the ABS, could be positively detected. Although these surfactants had been substituted by LAS in the Western World more than 30 years ago in response to their resistance to biodegradation, they are still in extensive usage in some less industrialized countries, which benefit from their favorable production costs and specific application profiles. Analyses of river water proved that ABS were present in no sample examined. This was in line with the non-detectability of this compound in a series of laundry detergent powders purchased on the Brazilian market [1] and analyzed by LC-ESI-MS for their content of surface-active ingredients.

The concentrations of LAS and SPC at the six sampling sites are shown in **Fig. 5-2**. After the passage of Cachoeiras de Macacu, the LAS concentration has increased by about a factor of ten from 14 to 155 µg L^{-1}. Reaching the town entrance of Japuíba, the level has dropped again down to 12 µg L^{-1}, which is likely due to degradation of LAS in the river section between Cachoeiras de Macacu (downstream) and Japuíba. At the three following stations the LAS concentrations average 36, 38 and 31 µg L^{-1}, respectively.

The SPC levels in the Rio Macacu have risen from 1.7 to 12 µg L^{-1} between Cachoeiras de Macacu upstream and downstream site correlating rather well with the concurrent rise of the parent compound LAS. In the next river section up to Japuíba a reduction of SPC is recognized. However, whereas the LAS value has diminished by a factor of 14, the decrease in SPC is much less pronounced changing from 12 to 7.4 µg L^{-1}. A slight increase is observed at the sites Papucaia (town center) and Sambaetiba I and II ranging on average between 12 and 14 µg L^{-1}. This is paralleled by quite steady concentrations of the anionic surfactant at these locations. Although not significant, a certain trend of decreasing LAS and SPC levels might be identified in the river course moving from the sampling station Sambaetiba I to Sambaetiba II (note that the village with 4,000 inhabitants is not directly located at the river bank), which could be attributed to biodegradation of both investigated sulfonates without addition of relevant amounts of urban wastewater in this section of the Rio Macacu.

[1] Brilhante, Campeiro, OMO Progress, (Lever); Minerva Balance, OMO Multiacao (Gessy-Lever); Ariel, ACE (Procter&Gamble); Biju, (Arisco Industrial); SAVON Plus (Industria Brasileira).

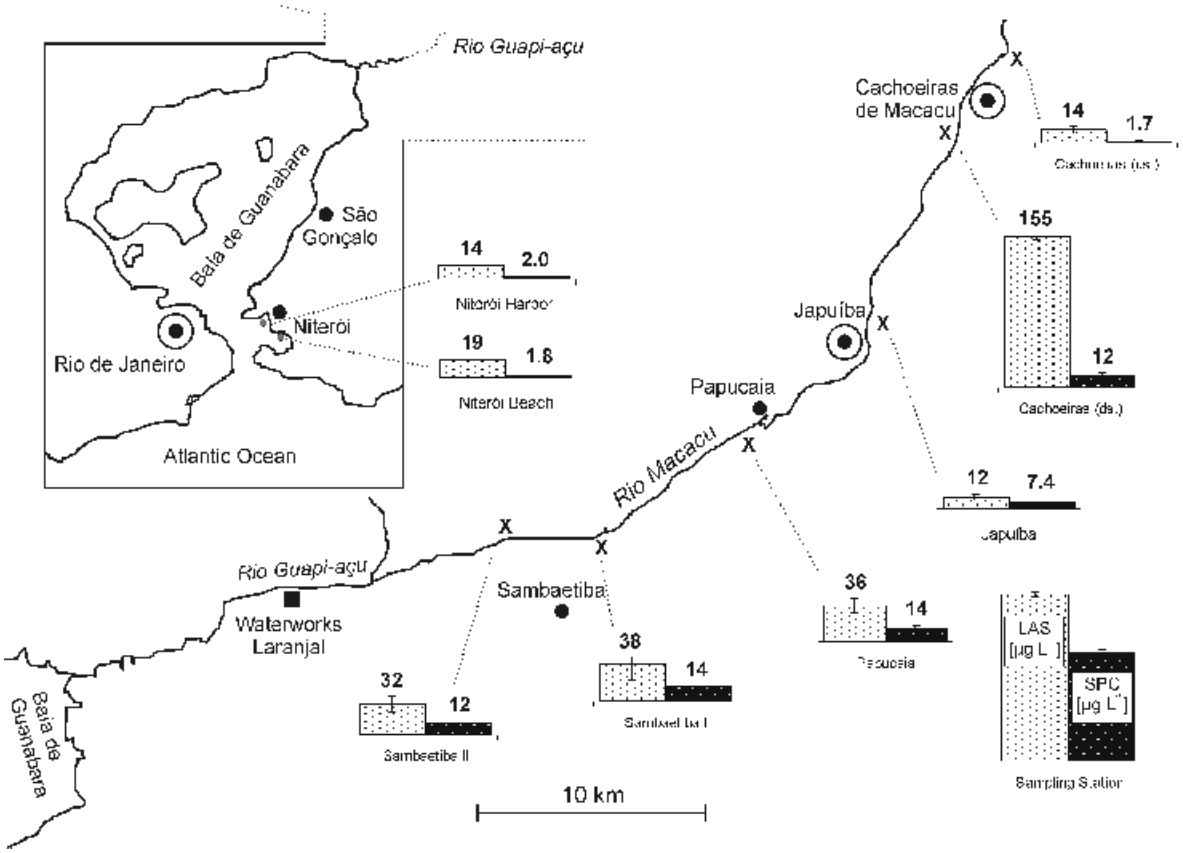

Fig. 5-2: Map of sampling sites at the Rio Macacú (Papucaia: lat. S 22°37´, long. W 42°45´) and the Baía de Guanabara, Brazil and the corresponding concentrations of LAS and SPC.

In the chromatograms in **Fig. 5-3** the change in LAS homologue distribution in samples from an upstream (Cachoeiras de Macacu, downstream) and a downstream station (Sambaetiba II) are shown. The shift towards the shorter alkyl chain LAS is explainable by two factors having the same impact: both biodegradation and adsorption onto suspended particles or sediments (this is further discussed below) preferentially remove the longer chain homologues owing to their higher lipophilicity (Matthijs and de Henau, 1987; Swisher, 1987).

In conclusion, the findings show that microbial communities present in the river are qualified to oxidize LAS yielding long-chain SPC, which are subsequently further broken down to the shorter-chain homologues. Due to the continuous exposure to LAS via municipal wastewater the microbial population is adapted to the surfactant by producing required enzymes for the aerobic degradation (Larson and Payne, 1981). The self-purification capacity of the water is impressively demonstrated between Cachoeiras de Macacu (downstream) and the next sampling site at Japuíba where the surfactant level decreases by more than one order of magnitude. In the lower course of the Rio Macacu near Sambaetiba relative constant concentrations of LAS and SPC suggest that the degradation of both species has slowed down. This might be caused by insufficiently dissolved oxygen being essential for the initial attack of LAS or by the presence of non-degradable and hence accumulated wastewater-borne contaminants, which negatively affect the microbial performance.

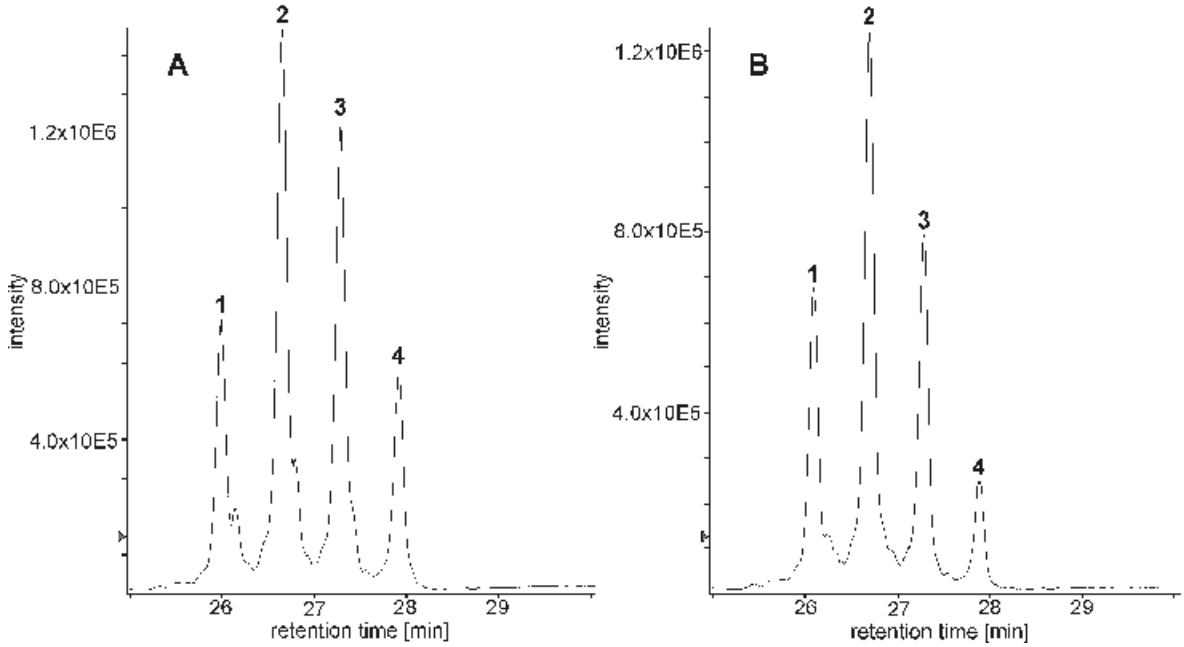

Fig. 5-3: (–)-LC-ESI-MS chromatogram of LAS in Macacu samples collected at (**A**) Cachoeiras de Macacu (downstream) and (**B**) Sambaetiba II. Peak numbering: (1) C10-LAS, (2) C11-LAS, (3) C12-LAS, (4) C13-LAS. (Quantitative conclusions cannot be inferred from the figures as different dilutions were analyzed). LC conditions as in **Fig. 4-4**.

Besides the removal of LAS through biodegradation, the adsorption onto suspended solids and river sediments may likewise contribute to the elimination of LAS from the dissolved phase. Schöberl et al. (1996) carried out several monitoring studies on LAS adsorbed to suspended solids in three small German rivers affected by treated or untreated domestic wastes. On average the amounts of adsorbed LAS were less than 6 % of total LAS in the water body. In freshwater sediments, concentrations of LAS are generally found in the range between zero and 10 mg kg^{-1} (Painter and Zabel, 1988). Such values apply given adequate sewage treatment as demonstrated by the results from Tabor and Barber (1996) who presented data on the spatial distribution of LAS in river water and bottom sediments in the Mississippi River. Dissolved LAS levels averaged 1.7 µg L^{-1}, while the mean sediment concentration was 0.83 mg kg^{-1}. Contrary to this, Rapaport and Eckhoff (1990) found an average concentration in sediments below the outfall of a poorly functioning WWTP of 174 mg kg^{-1} and a corresponding water concentration of 99 µg L^{-1}. These levels appear to be much more realistic in view of the situation found in the Rio Macacu. With respect of the fate of LAS deposited in sediments, most researchers agree that biodegradation is significantly attenuated, due to anoxic or anaerobic conditions at the river bottom (Federle and Schwab, 1992; Tabor and Barber, 1996). Hence, sediment-bound LAS may persist substantially longer than its dissolved form.

The impact of discharges of untreated wastewater of municipal and industrial origin on surface waters was studied in a Taiwanese river by Ding et al. (1999) analyzing LAS and SPC. Concentrations of the anionic surfactant ranged between 11.7 and 135 µg L^{-1}, while the degradative products were found from 0.3 to 3.1 µg L^{-1}. Trehy et al. (1996) reported on levels of both compounds in receiving waters in the U.S. upstream and downstream of domestic WWTP. The values averaged 16 and 35 µg L^{-1} for LAS, while the mean concentration of SPC amounted to 9.3 and 31 µg L^{-1}, respectively. A monitoring study

performed in the Venice Lagoon, Italy, comprised two strongly polluted riverine sites sampled upstream and downstream a WWTP (Marcomini et al., 2000c). The average concentration of LAS was only slightly impacted increasing from 177 to 187 µg L^{-1}, but the SPC level mounted from 368 to 420 µg L^{-1}. From these data describing distinct discharge situations of wastewater it can be seen that the ratio of LAS-to-SPC allows inferring conclusions on the current state of a river and the history of emitted sewage water. A low value as found in the U.S. (0.9) and the Italian work (0.2) is indicative of treated water, whereas elevated values as observed in the Taiwanese (ranging from 270 to 6.7) and in the present study performed on a Brazilian river (between 13 and 1.6) are designative of a high percentage of untreated wastewater.

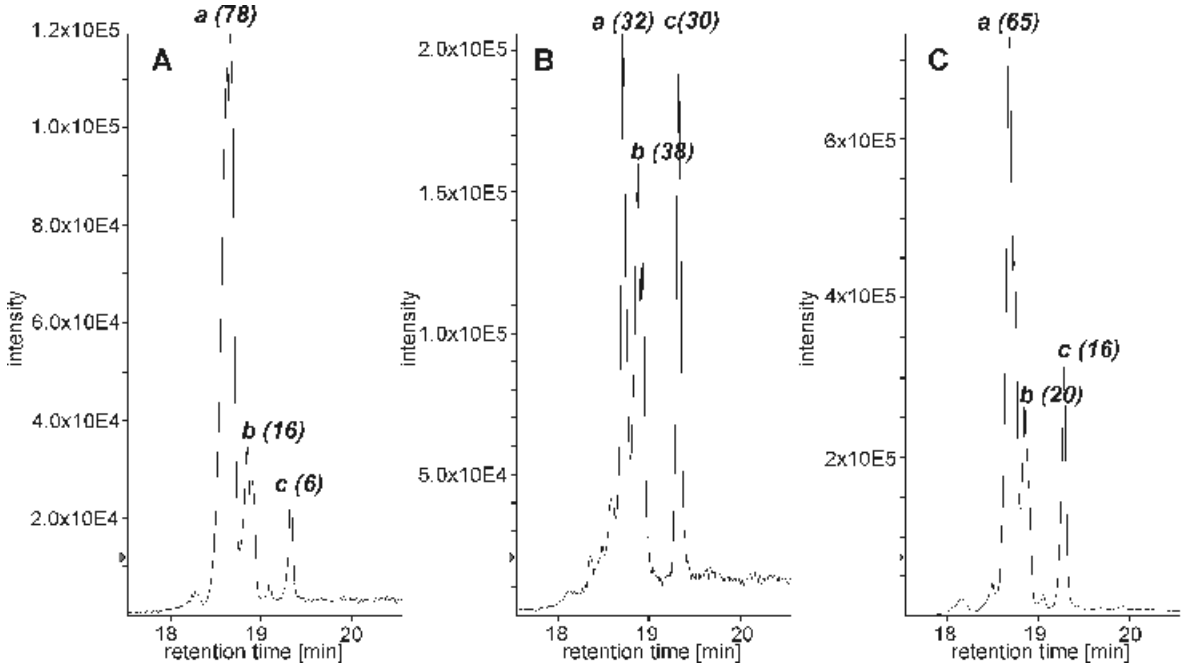

Fig. 5-4: (–)-LC-ESI-MS extracted ion chromatogram of C7-SPC (*m/z* 285) in samples from (**A**) Rio Macacu, (**B**) river Rhine, Germany, and (**C**) Baía de Guanabara. Values in parenthesis indicate relative peak area (*a*+*b*+*c*=100 %). LC conditions as in **Fig. 4-4**.

A further indicator giving hints on the progression of biodegradation of LAS and thereby the build-up of SPC is the peak pattern of phenyl isomers of individual SPC homologues (see **Fig. 4-21**). Since the degradation rate of a particular isomer depends strongly on the position of the attachment of the phenyl ring on the oxidized alkyl chain (Swisher, 1987), the peak distribution observed in the Macacu samples can be used as a marker and be compared to the one recorded from a surface water where a steady state in SPC breakdown was reached (ref. to **Fig. 4-24**).

This is exemplarily illustrated in **Fig. 5-4** for the mass trace of C7-SPC obtained from a Rio Macacu sample (A) and the German river Rhine (B). The relative intensities of the three major peaks, *a*, *b* and *c*, in the latter are representative of a nearly stationary level. On the other hand, the predominance of peak *a* relative to *b* and *c* in **Fig. 5-4** (A) indicates that biodegradation of SPC has not yet been completed. This finding is in accordance with the fact that the concentrations of LAS compared to those of SPC are several factors greater, i.e. SPC may still be liberated by surfactant breakdown. However, the overall rate of conversion of the less rapidly degradable isomers seems to be not especially speedy since very similar

phenyl isomers pattern are observed in all samples taken along the course of the Rio Macacu (data not shown).

With respect to the occurrence of LAS and SPC in the Baía de Guanabara, known to be heavily polluted with oil, land runoff and sewage (Paranhos et al. 1995; Perin et al., 1997), their levels found in the samples taken close to Niterói amounted to 14 and 2.0 µg L^{-1} in the harbor and 19 and 1.8 µg L^{-1} near the beach (**Fig. 5-2**). At both locations the ratios LAS-to-SPC, serving as marker for discharges of untreated sewage waste, is about 10 and therewith lies in a similar range than those observed in the Rio Macacu. It is worth mentioning that C7-SPC in the water from the Baía de Guanabara exhibits a comparable pattern – with a slight shift in favor of *b* and *c* (**Fig. 5-4**, C) – to the one of the Rio Macacu being indicative of an unfinished biodegradation of the target analytes.

Discussing the further fate of both contaminants at the interface to the coastal environment, the change in biochemical and physico-chemical behavior has to be taken into account expressed among other factors by an overall lower metabolic activity of estuarine and marine microbial communities compared with the one of continental waters (Shimp, 1989). Thus, in relation to biodegradative elimination the precipitation of LAS as magnesium and calcium salts is promoted by the higher salinity in these environmental settings and might become the principal elimination route (Madsen and Alexander, 1985). Removal of dissolved LAS from the water phase may also occur by sorption onto particulate matter and sediments (Rubio et al., 1996). In the latter compartment LAS is likely to be accumulated due to low dissolved oxygen content near the bottom of Baía de Guanabara (3.1 mg L^{-1}, Kjerfve et al., 1997), which results in anoxic muds thereby lowering substantially primary degradation rates. Given a sufficient microbial activity the carboxylated intermediates in turn can be assumed to be broken down until reaching the aforediscussed steady state – probably at a slower speed than in fresh waters – whereas elimination by adsorption onto marine sediments is unlikely. The highly polar character and the absence of a hydrophobic moiety, which plays an important role in the interaction with organic matter in sediments (Westall et al., 1999), largely prevent an accumulation. Whereas the anionic surfactant was found at mg kg^{-1} levels in riverine and lake sediments, the corresponding degradation products were not detected along with its parent (Sarrazin et al., 1999b and Trehy et al., 1996, respectively).

5.2.2 Determination of branched and linear alkylbenzene sulfonates and sulfophenyl carboxylates in Philippine surface waters

Wastewater situation in the Philippines

No wastewater facilities exist at municipal level in the Philippines; thus, the water is discharged directly or via drainage systems to surface water bodies contributing to the continued deterioration of water quality. The progressive contamination of potential drinking water resources provokes a general water crisis in the investigated region, which is worsened further by growing population, and problems brought about by urbanization and rapid development.

In the Philippines one encounters the particular situation that LAS is used along with their branched congeners ABS, based on a tetramerized propylene-derived hydrophobic moiety instead of a paraffin-based hydrocarbon chain as in LAS. The continuing usage of ABS, which is not voluntarily

banned or legally restricted as in many other countries[1], is explainable by the availability of cheaper raw materials for ABS production and their favorable physical properties in special formulations like synthetic laundry bars (Wharry et al., 1987). This application form provides better performance and easier handling for manual laundry washing frequently done directly in surface waters.

The resistance of ABS to biochemical degradation in aquatic environments – this had led to strong foam formation in WWTP and sewage-polluted surface water in the 1950s in ABS-applying countries (Fairing and Short, 1956; Webster and Halliday, 1959) – was the reason for their replacement (Heinz and Fischer, 1962). Moreover, ABS had posed a threat to municipal drinking water suppliers, which used surface water as a source for drinking water production (Jente, 1961).

Area description of Laguna de Bay

Laguna de Bay, located in Luzon, Philippines (**Fig. 5-6**, p. 115) has an area of 900 km^2 and a watershed of 2,820 km^2 with a total population estimated at 11.6 million in 1995. The status of Laguna de Bay was described by Barril and Tumlos (1997) based on water quality parameters indicative of the trophic state of the lake, such as the continued flux of organic wastes coming from various sources overloading the capacity of the lake to decompose these wastes and leading to anaerobic condition in certain parts as indicated by increasing production of ammonia.

Water samples were taken from three tributaries: Bucal stream and San Cristobal river in Calamba and San Pedro river, which all drain into Laguna de Bay and three sites representing the outflow from the Napindan Channel to Guadalupe and Pasig river, near the mouth of Manila Bay. These areas are part of metropolitan Manila and are therefore affected by discharges of about 2 millions inhabitants.

Analysis of alkylbenzene sulfonates and sulfophenyl carboxylates

Though LAS and ABS were only partially separated under the applied RP HPLC conditions (**Fig. 5-5**), isobaric homologues could be distinguished with the aid of the fragments *m/z* 183 and 197, respectively (ref. to **Fig. 4-6**, p. 49).

In all investigated water streams LAS as well as ABS are detectable in the low to mid-concentration range in $\mu g\ L^{-1}$. The determined levels in the samples collected in December 1999 and March 2000 are given in **Fig. 5-6**. The calculated ratio of LAS-to-ABS concentration is likewise presented in the same figure. The two series are found to be in good agreement in relation to the total concentration and LAS-to-ABS ratio, indicating that the obtained values are representative of the selected surface waters. In detail, the following interpretations can be made: the sampling site at the Bucal stream located just about 10 m downstream of its spring has an embedded man-made “traditional” basin, which is used for washing clothes. The LAS and ABS burdens are found to be rather low and since the source of surfactants discharge is directly located upstream the sampling point, no significant biodegradation can be expected. Hence, the LAS-to-ABS ratio of about 6 represents a fresh surfactant contamination resulting from washing activities.

[1] Current policies of the Philippine government concerning the use of ABS are described in chapter 7.

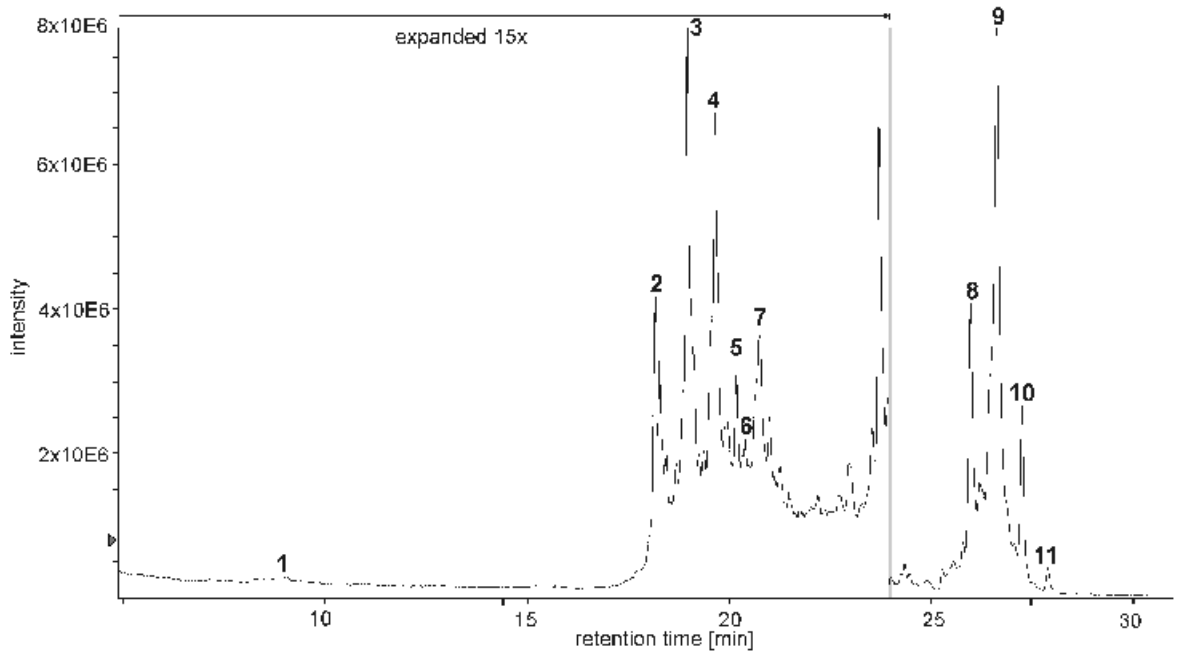

Fig. 5-5: (–)-LC-ESI-MS chromatogram of a sample from San Pedro river containing SPC and alkylbenzene sulfonates. Peak numbering: (1) C6-SPC, (2) C7-SPC, (3) C8-SPC, (4) C9-SPC, (5) C10-SPC, (6) C11-SPC, (7) C12-SPC, (8) C10-benzene sulfonates, (9) C11-benzene sulfonates, (10) C12-benzene sulfonates, (11) C13-benzene sulfonates. LC conditions as in **Fig. 4-4**.

In contrast, the San Pedro river is heavily contaminated by wastes originating from various human activities and industries and therefore it is not surprising that LAS and ABS concentrations of up to 90 µg L^{-1} are found. The mean LAS-to-ABS ratio 1.7 indicates that the surfactant discharges have occurred recently, but a partial degradation of LAS may already have taken place. In the third investigated tributary leading to Laguna de Bay, the San Cristobal river, the concentrations of LAS and ABS amount to about 9 µg L^{-1}, but the LAS-to-ABS ratio is still lower than those found in the Bucal stream and the San Pedro river. This gives an indication that during transportation of the surfactants in the upper course of the San Cristobal river biodegradation of LAS has advanced to a certain extent.

The residues of LAS and ABS detected in the three investigated water streams entering Laguna de Bay can be further reduced by dilution, or biochemical degradation (mainly for LAS). Removal of the surfactants through sorption and precipitation must also be taken into account. Although the tendency to sorption is not particularly strong, an accumulation on the surface of solids is enhanced if substantial amounts of particulate matter are present (Westall et al., 1999), which is the case for Laguna de Bay. This holds particularly true for ABS since many of its derivatives are rather insoluble (Swisher, 1969).

Low values (1 to 2 µg L^{-1}) of LAS and ABS are detected at the entrance of the Napindan Channel, which is the only outlet of Laguna de Bay towards Manila Bay. Two other sampling points discharging into the Manila Bay are located at the Pasig river. An increase in LAS and ABS values is observed at the Guadalupe site (between 4.1 and 6.8 µg L^{-1}) and their concentrations again rise to a high level of up to 75 µg L^{-1} for ABS as it enters Manila Bay.

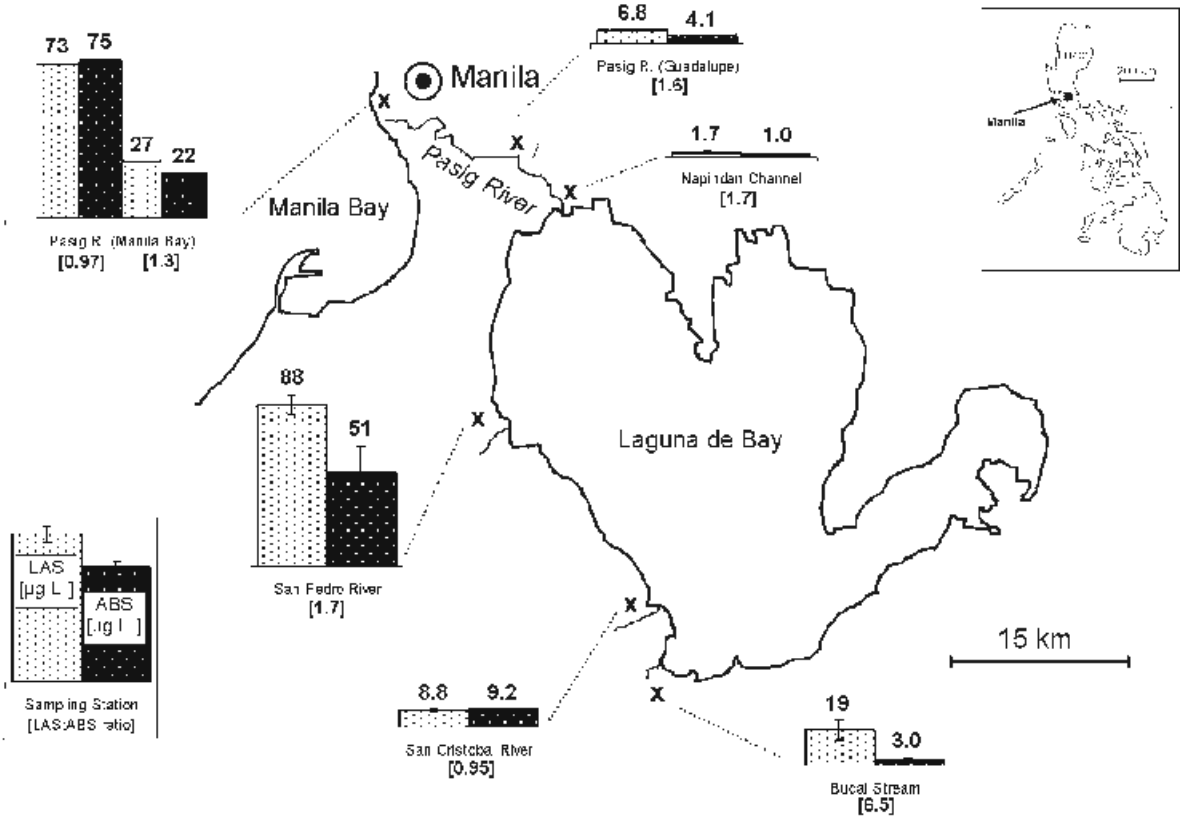

Fig. 5-6: Map of Laguna de Bay, Luzon/Philippines (lat. N 14°02´-14°05´, long. E 121°0`-121°5´) with sampling sites and concentrations. With the exception of the site Pasig River (Manila Bay) where the values of the December 1999 and March 2000 batch are given separately, the values given for the other sampling locations represent mean values.

The significant increase in ABS and LAS concentrations between the outlet of Laguna de Bay at Napindan Channel and the mouth of Manila Bay (Pasig river) is explainable by high emissions of surfactant-loaded wastewaters along the channel course. Approaching the Manila Bay a rising number of industrial effluents together with an increasing number of people in the vicinity of the Pasig river, estimated to be more than 2 million, can be made responsible for the discharge of large amounts of surfactants. Since the average flow in Pasig river is 1.6 $m^3 s^{-1}$ (DENR, 1996) dissolved oxygen is also very low and a rapid aerobic degradation of LAS is limited. On the other hand, the breakdown is likely to be negatively influenced by other industrial pollutants such as petroleum and heavy metals.

Homologue distribution of linear and branched alkylbenzene sulfonates

With continuing degradation of the set of LAS alkyl homologues, the peak pattern of the four components is shifted towards the shorter homologues (*Swisher´s distance principle*). This effect is shown explicitly in **Fig. 5-7** for the samples taken in December 1999; comparable observations were made during the second sampling in March 2000 (data not shown). The LAS homologue pattern in the Bucal stream where washing-related surfactant discharges directly occur upstream the sampling point represents a rather unaffected pattern and corresponds quite well to a technical blend used in detergent formulations (C10- to C13-LAS-ratio of about 1). On the other side, a significant impact of degradation is stated for the

contaminated samples from the San Cristobal river and the Pasig river mouthing to Manila Bay, where an increasing portion of C10-LAS parallels a decrease in C13-LAS (C10-to-C13-LAS-ratio of about 10).

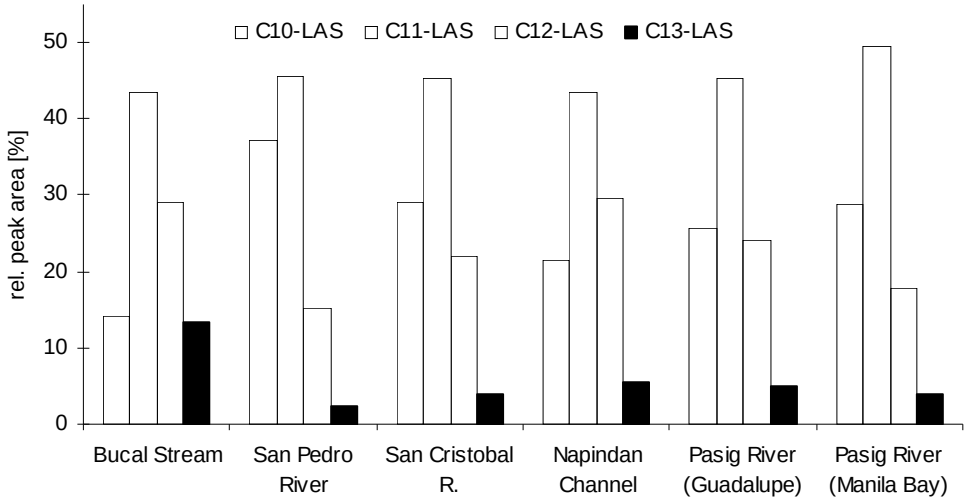

Fig. 5-7: Alkyl homologue distribution of LAS (sample batch from December 1999).

A different situation arises for the poorly degradable ABS (**Fig. 5-8**). In this instance, similar homologue distributions of the branched species are observed for all samples underlining the fact that biodegradation is a negligible way of eliminating this compound. Referring to the above discussed ratio of LAS-to-ABS, this parameter exhibits a good correlation with the degree of degradation described by the sampling site-specific alkyl homologue pattern.

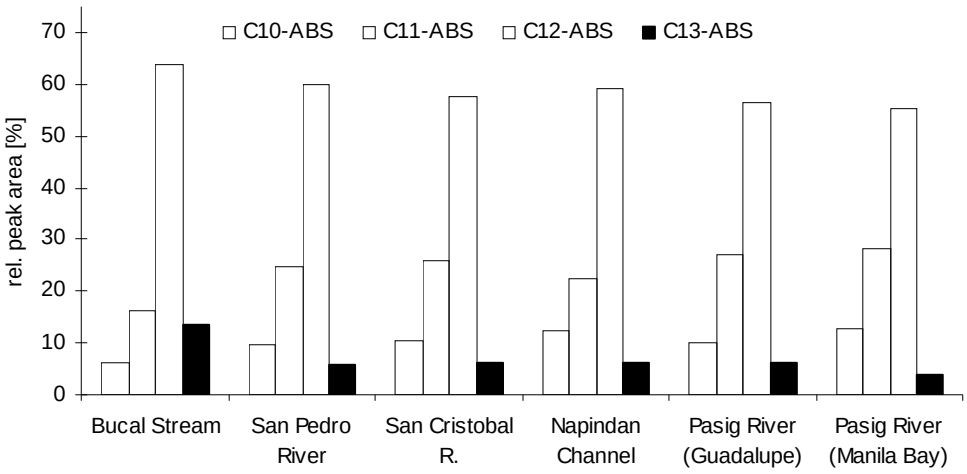

Fig. 5-8: Alkyl homologue distribution of ABS (sample batch from December 1999).

Furthermore, it can be perceived by comparing **Fig. 5-7** (Bucal stream sample) and **Fig. 5-8** that the distinct alkyl homologue distributions are characteristic for LAS and ABS, respectively, which is due to different raw materials used for the synthesis of alkylbenzenes.

Occurrence of sulfophenyl carboxylates

The determination of C6- to C12-SPC in the surface water samples – a typical chromatogram is shown in **Fig. 5-5** – was performed on a semi-quantitative basis by plotting the absolute peak area of each SPC homologue examined. Although the shorter chain SPC are slightly underestimated due to lower recoveries, the interpretation of relative burdens of SPC is possible through comparison of the peaks areas. This is demonstrated in **Fig. 5-9** for the first sample batch taken in December 1999.

A similar homologue distribution is recorded at all sampling points showing a first relative maximum at C9- or C10-SPC and a second maximum at C12-SPC. The detection of the former species stands in concordance with the fact that long-chain SPC are rapidly broken down into lighter SPC, but the rate of conversion decreases with progressive chain shortening resulting in an accumulation of mid-chain SPC. The second maximum at C12-SPC is likely attributable to SPC originating from C12-ABS as the most abundant ABS homologue present in detergents formulations. Support for this assumption is given below.

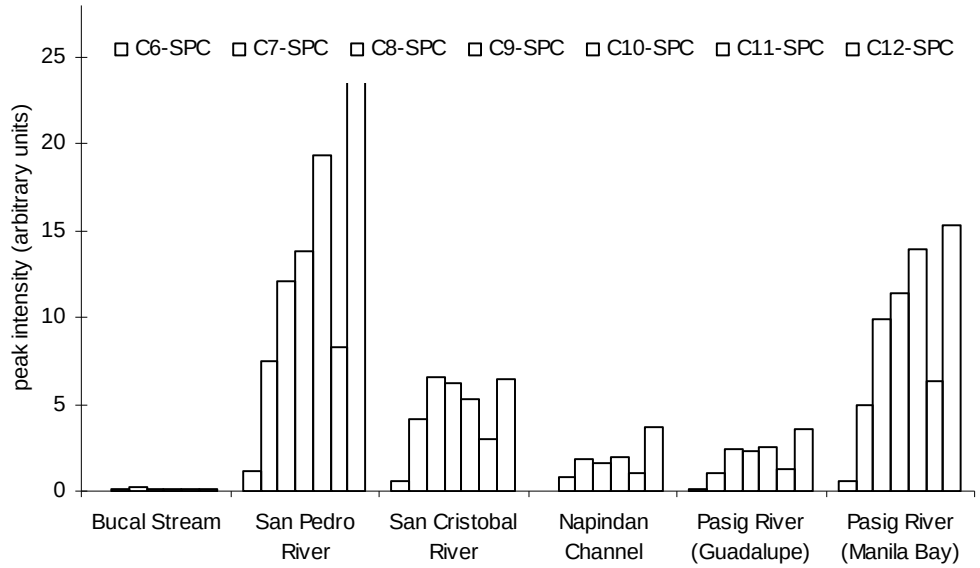

Fig. 5-9: Semi-quantitative analysis of C6- to C12-SPC in investigated surface waters (sample batch from December 1999).

In terms of quantitative measurements, large variations in SPC concentrations are stated. The lowest SPC levels are detected in the Bucal stream, whereas in the San Pedro river and the Pasig river towards Manila Bay, the concentrations are several orders of magnitude higher. The contamination of the San Cristobal river likewise reaches relatively high levels. In addition, taking into account the levels of LAS and ABS, their ratio (**Fig. 5-6**) and the alkyl homologue distribution of LAS (**Fig. 5-7**), the determination of SPC levels provides a complementary tool for the detailed description of the pollution situation in the investigated area. For example, the Bucal stream with rampant washing activities has a high LAS-to-ABS ratio, but contains very few SPC indicating a low level of degradation. On the other hand, high loads of LAS and ABS in the San Pedro river and the Pasig river (Manila Bay) yield large amounts of metabolites presumptively because of active degradation processes.

With respect to the formation of SPC out of ABS it must be remembered that this surfactant is a mixture of hundreds of components, all minor in amount, with distinct constitutions within the alkyl

group. The biological resistance of ABS was attributed to the presence of a quaternary carbon atom in the alkyl chain (McKinney and Symons, 1959; Nelson et al., 1961), which disabled the formation of a double bond as intermediate step of the β-oxidation cycle. A primary degradation, however, i.e. ω-oxidation of the alkyl chain, is not blocked by such a group. Hence, chain carboxylation of ABS is principally feasible and the breakdown might proceed as long as no quaternary carbon atom hinders the metabolism. Or the degradation might be initiated by an open end chain if the other side is blockaded by a terminal quaternary group.

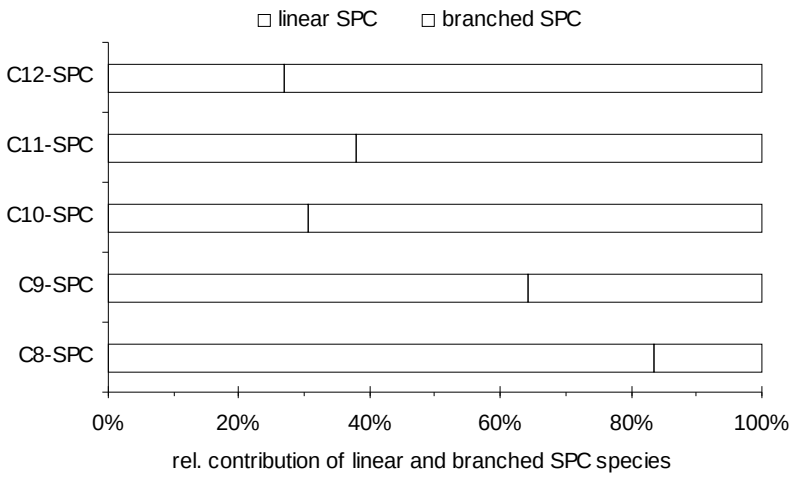

Fig. 5-10: Relative peak intensities of linear and branched SPC homologues determined by LC-ESI-MS/MS operated in MRM mode.

Although no significant shift in the homologue pattern of ABS has been observed in **Fig. 5-8**, indicating no preferential removal of any homologue, the relative contribution of branched SPC homologues to the overall detected amounts of degradative products could be verified by LC-ESI-MS/MS analyses performing MRM measurement of the C8- to C12-SPC parent ions $[M–H]^-$ yielding either the fragment *m/z* 183 for the linear species or *m/z* 197 for the branched congeners. In **Fig. 5-10** the percentages of both species to the total peak area of an alkyl homologue are shown (absolute concentrations could not be determined because no optimization of the Q2 parameters, collision gas concentration and acceleration voltage, influencing the extent of fragmentation was done). Overall, the relative portions of the branched SPC are decreasing for the shorter chain homologues. This is in line with the fact that steric hindrance gets more probable with ongoing destruction ultimately impeding chain shortening via β-oxidation. The exceptional behavior of C10-SPC with its relative high percentage of branched SPC could be assigned to the fact that it is likely to be formed by terminal oxidation of C10-ABS as well as by β-oxidation of C12-ABS, which is the most prominent alkyl homologue in the mixture (**Fig. 5-9**).

The contamination of surface waters by residues of surfactants and their degradative products causes particular concern as these waters are used directly for drinking water production.

5.3 Routes of sulfophenyl carboxylates from surface to drinking waters

Due to the scarcity of ground water supplies in many highly populated regions, the usage of river water for the production of potable water is a common practice. Such water streams usually contain residues of surfactants and their degradation intermediates originating from outfalls of WWTP or direct emissions of wastewater. In the course of drinking water production out of river water, the removal efficiencies of polar pollutants generally depend on their nature and on the kind and arrangement of the natural and/or installed technical purification stages. In a series of investigations carried out in the 1990s the behavior during drinking water treatment of polar pollutants occurring in the raw water was studied comprising e.g. complexing agents (Brauch et al., 2000), aromatic sulfonates (Lange et al., 1995a), intermediates and by-products from industrial syntheses (Haberer and Knepper, 1993; Knepper et al., 1995) as well as metabolites (Knepper et al., 1999b and 2000). Some of these compounds were found to possess the potential of bypassing at least in part all purification steps and were ultimately detected in finished drinking water.

Insufficient removal of highly water soluble surfactants and of their even more polar metabolites during drinking water purification resulted in their positive detection in potable water including APEO (Ghijsen and Hoogenboezem, 2000), nonylphenol carboxylates (Clark et al., 1992), AES and AE (Rivera et al., 1987b) as well as their carboxylated intermediates (Ventura et al., 1991).

Up to now the possible occurrence of very polar LAS intermediates, which are made up of some recalcitrant constituents, has been beyond the scope of interest. However, taking into consideration the high requirements on the quality of drinking water deserving utmost protection (CEC, 1975), there is a need to investigate the behavior of SPC during processing of contaminated raw waters in waterworks.

5.3.1 Behavior of sulfophenyl carboxylates during drinking water production

Two waterworks differing in both raw water quality and treatment technology were chosen to evaluate their capacity to eliminate SPC from the water thereby allowing to draw conclusions on their behavior under distinct processing conditions to be met at other waterworks.

Description of the selected waterworks

A detailed scheme comparing all installed treatment steps of both waterworks is displayed in **Fig. 5-11**. The first waterworks supplying the city of Barcelona, Spain (henceforth denoted waterworks Llobregat) is located at the Llobregat river (km 154). This stream is known to be heavily polluted by discharges from urban and industrial activities, leaching from mining and agriculture (Rivera et al., 1987b; Valero et al., 1998). The second waterworks is placed at the river Rhine at Wiesbaden, Germany, (km 508; denoted waterworks Rhine). A particular characteristic of waterworks Rhine is the subsoil passage performed after granular activated carbon (GAC) filtration. In contrast, the unique features of waterworks Llobregat are breakpoint chlorination of the raw water and ozonation prior to GAC filtration. Furthermore, the daily produced volumes of drinking water are greatly different: 20,000 m^3 at the German waterworks facing 300,000 m^3 at the Spanish waterworks.

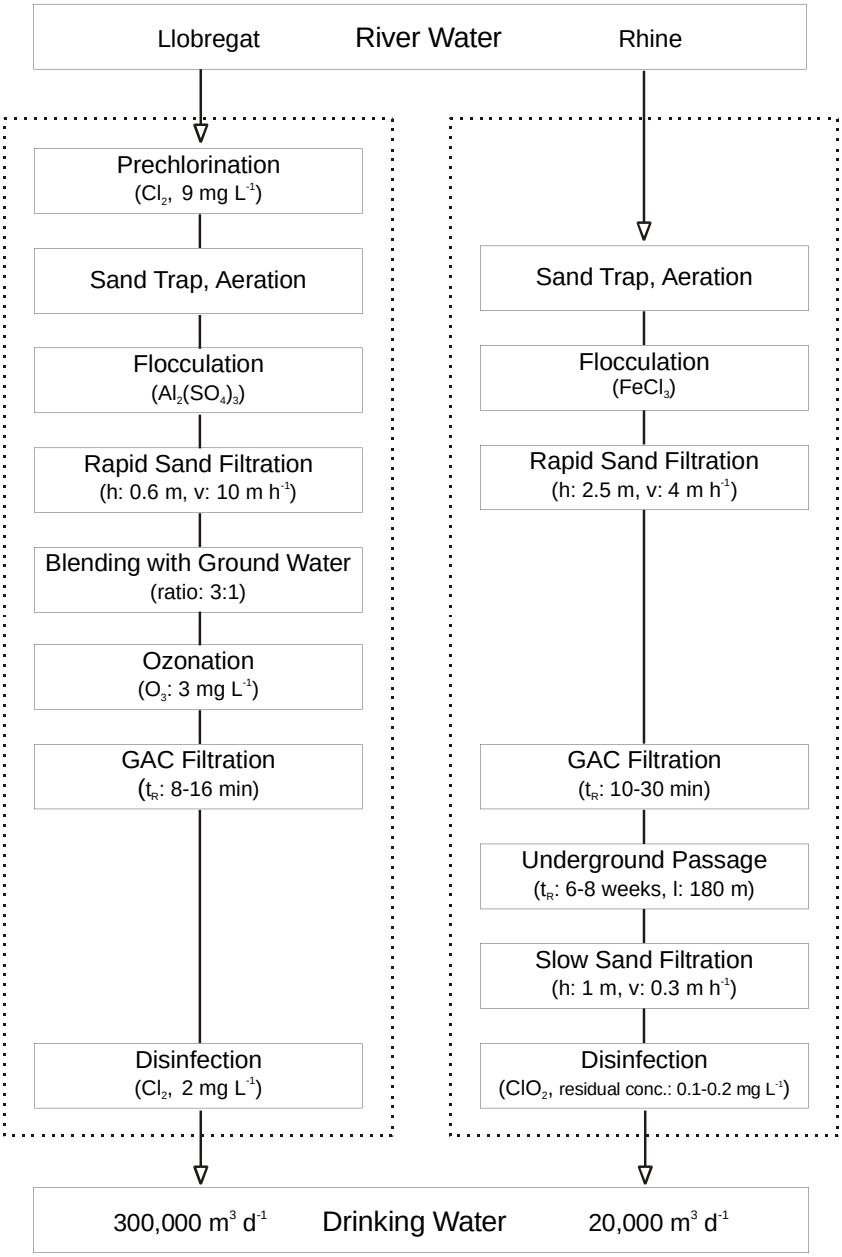

Fig. 5-11: Scheme of drinking water production plants on the Llobregat river, Spain (waterworks Llobregat) and the river Rhine, Germany (waterworks Rhine).

Raw water quality

The river Rhine and the Llobregat river, where the two investigated waterworks withdraw their raw water from, differ substantially in terms of hydrological conditions as well as in physical and chemical parameters of the water. Some selected data characterizing the two rivers are listed in **Table 5-4**. In the Llobregat river the greater percentage of wastewater of both municipal and industrial origin is reflected by higher values of DOC and conductivity. The latter is explainable by emissions from salt mines in the upper courses of the rivers. It has to be noted that seasonal variations of the water quality parameters presented are extremely pronounced in the Llobregat since the regime of this relatively small

Mediterranean river is strongly dependent on meteorological conditions. During dry periods frequently occurring in summer the overall flow of the river may decrease strongly resulting in worsening of the quality due to elevated portion of treated wastewater. Then, foams mainly caused by industrial wastes are present in raw waters representing an obstacle in the later processing. Though it might be expected that higher ambient temperatures in the Spanish river augments the activity of microbial communities, hereby enhancing the self-purification capability of the water, it should be taken into consideration that in many instances biodegradation of e.g. surfactants requires the availability of dissolved oxygen whose solubility, however, decreases with rising water temperature. Concerning intra-annual fluctuations of the water flow in the river Rhine and therewith related pollutant concentrations, these are to a lesser degree seasonally influenced due to a more constant flow of this stream.

Table 5-4: Physico-chemical analysis of raw water.

Parameter	Waterworks Llobregat	Waterworks Rhine	Parameter	Waterworks Llobregat	Waterworks Rhine
River flow [$m^3 s^{-1}$]	3-14	1800	Boron [mg L^{-1}]	0.22-0.24	0.087
pH	7.8-8.2	7.9	NH_4^+ [mg L^{-1}]	0.21-0.96	0.13
DOC [mg C L^{-1}]	5.7-6.0	2.3	NO_3^- [mg L^{-1}]	1.9-8.6	9.8
SAC_{254}	10.5-10.7	5.7	PO_4^{3-} [mg L^{-1}]	0.074-0.16	0.082
Conduct. [µS cm^{-1}]	1290-1735	567	Cl^- [mg L^{-1}]	260-460	61
			Br^- [mg L^{-1}]	0.63-1.18	0.10

Both raw waters taken from the river at the two waterworks contain traces of SPC. The measured concentrations in the raw waters average 5.0 and 1.8 µg L^{-1} in the Llobregat river and the river Rhine, respectively suggesting a higher wastewater percentage in the former. This is corroborated through the LAS levels amounting to 12 and 1.0 µg L^{-1}, respectively. Calculating the ratios LAS-to-SPC as indicator for the progression of LAS breakdown – as done in the interpretation on the pollution extent of the Rio Macacu – values of 2.4 for the Llobregat water and 0.6 for the Rhine water are obtained. Referring to the above remarks on intra-annual variations of the water flow in the Llobregat river, it might be assumed that higher SPC values are present in periods of lower river water levels.

Regarding the distribution of the alkyl homologues analyzed (C6 to C12) in the raw waters, the pattern are shown in the last rows of **Fig. 5-13** (p. 122) and **Fig. 5-16** (p. 125) for the Llobregat river and the river Rhine, respectively. It is noticed that C7-SPC is the most prominent homologue in the Rhine water sample while the relative abundances of the longer-chain homologues C8- and C9-SPC are somewhat greater in the Llobregat water. Since the shorter-chain SPC are formed out of higher homologues, this observation indicates that SPC destruction in the Llobregat river is still continuing.

Fate of sulfophenyl carboxylates in waterworks Llobregat

Total concentrations of SPC during the different stages of water purification are given in **Fig. 5-12**; the fate of single SPC homologues is shown in **Fig. 5-13**. As can be seen neither prechlorination nor flocculation followed by rapid sand filtration have an impact on the elimination. After ozonation and blending with groundwater an SPC reduction by about 50 % is manifested, which is in part attributed to

the mixing. The extent of an oxidative breakdown of the aromatic sulfonates likely to contribute to the decrease cannot be deduced from the analytical results.

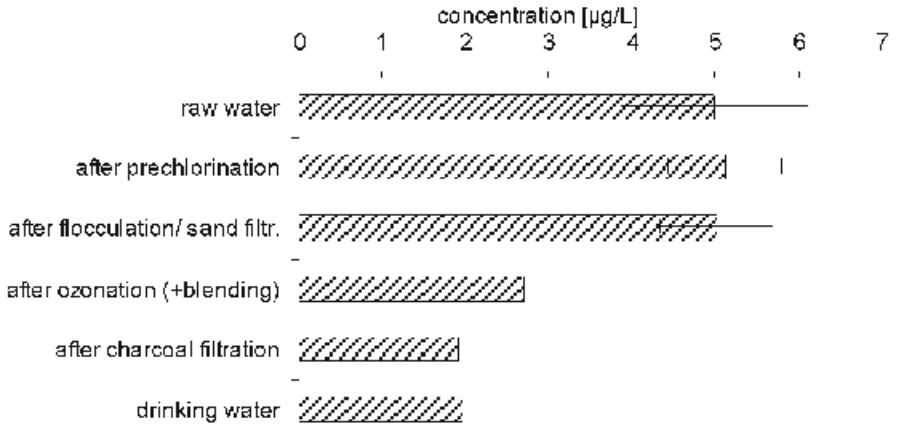

Fig. 5-12: Total concentration of SPC in waters of different treatment steps at waterworks Llobregat (two sample batches).

The capacity of ozone to destroy sulfonated aromatic compounds was assessed by Lange et al. (1995b). They performed field experiments on the removal of naphthalene 1,5-disulfonate in a two-step ozone treatment showing that 40 to 50 % and 10 to 20 % of the compound remained unchanged after the first and the second step, respectively. The same authors reported that *cis*-4-4´-dinitro-2,2´-stilbenedisulfonate was removed by about 80 % by a one-step ozone treatment.

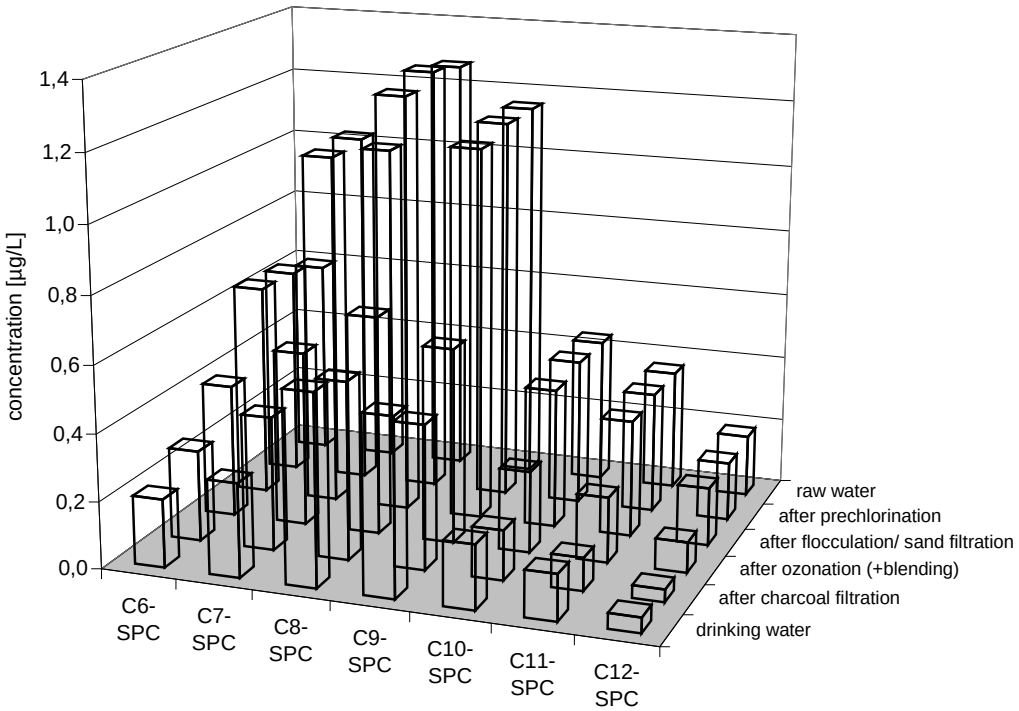

Fig. 5-13: Concentrations of individual SPC homologues in waters of different treatment stages at waterworks Llobregat (two sample batches).

In the waterworks Llobregat no significant removal of SPC is stated during the following stage of GAC filtration. In field investigations it turned out that the elimination efficiency of aromatic sulfonates strongly depended on the process conditions of the individual facility (Lange et al., 1995b). In a long-term study conducted over one year in a waterworks, where the treatment consisted of three main steps (iron removal, manganese removal, and a final filtration step by two parallel GAC adsorbers), the examined naphthalene sulfonates could not always be efficiently removed. Directly after exchange of the activated carbon in the filter, no sulfonate was detectable but with increasing running time, i.e. loading of the filter material, the concentration was steadily increasing due to breakthrough on GAC (Lange et al., 1995b).

Early work by Rivera et al. (1987a) indicated that GAC filters installed at the waterworks Llobregat were capable of retaining different polar pollutants including LAS, NPEO and even PEG but most of them reached – to an unknown portion as the applied FAB-MS method did only yield qualitative results – the drinking water. Hence, further examinations covering at least one GAC cycle should allow gathering more information on the efficacy of GAC filtration to eliminate SPC. When performing lab-scale experiments on the adsorption capability of single compounds, it should be taken into account that the outcomes are strongly influenced by the type of carbon used, e.g. fresh or preloaded carbon (Sacher et al., 2001).

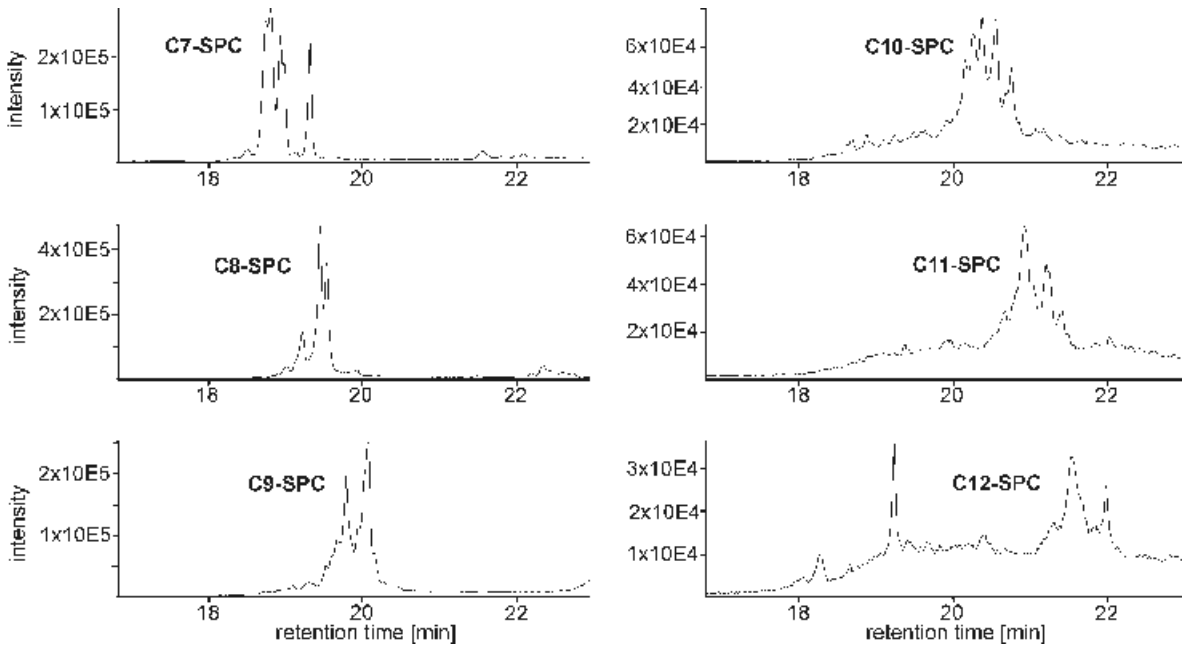

Fig. 5-14: (–)-LC-ESI-MS extracted ion chromatograms of C7- to C12-SPC in treated water from waterworks Llobregat.

Finally, the disinfection of the water with chlorine in the waterworks Llobregat leads to no measurable removal of SPC being around 2 µg L^{-1} in the finished drinking water. The XIC of C7- to C12-SPC in the treated water are shown in **Fig. 5-14**.

Fate of sulfophenyl carboxylates in waterworks Rhine

A quite distinct picture is drawn at this waterworks expressed as a much better reduction of the SPC burden. **Fig. 5-15** and **Fig. 5-16** show that flocculation with iron(III) chloride does not affect the presence

of SPC being in concordance with the behavior noticed in the flocculation process at the waterworks Llobregat. In contrast, a marked reduction is observed during rapid sand filtration; after this stage the SPC level has dropped from initially 1.8 down to 0.25 µg L^{-1}.

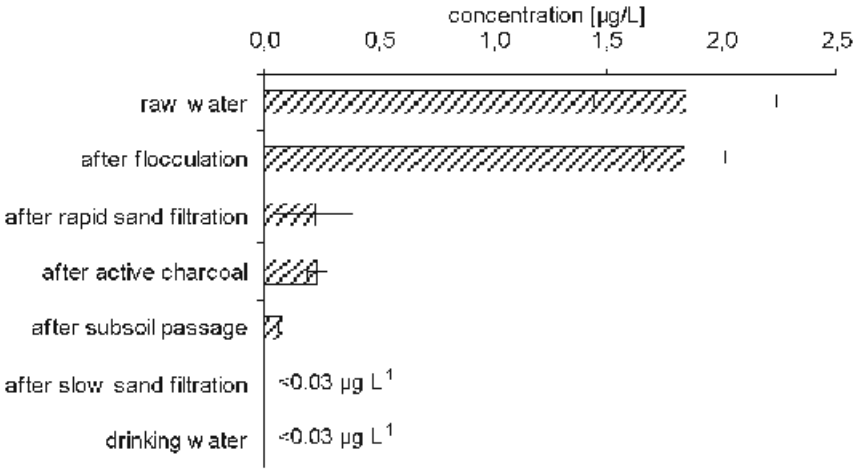

Fig. 5-15: Total concentration of SPC in waters of different treatment steps at waterworks Rhine (three sample batches).

Among all polar substances investigated hitherto in the purification process at waterworks Rhine (Haberer and Knepper, 1993; Knepper et al., 1995; Knepper et al., 2000), a significant removal during this step was never perceived. It could be assumed that the specific elimination of SPC was brought about by micro-organisms residing on the sand grains and being qualified to biodegradation. Such an impact was not observed during the corresponding step in waterworks Llobregat, which might arise from differences in the contact times (about 10-fold less in the latter) and also by the fact that prechlorination of the raw water carried out in the Spanish waterworks most likely slows down biological processes (if present at all) in the subsequent treatment stages.

The next stage in treatment, the GAC filtration, does not result in a measurable decrease of SPC. As already discussed above for the waterworks Llobregat, in this instance the current state of the GAC in terms of covering and breakthrough behavior might be a decisive factor for its performance to adsorb the polar sulfonates.

After the following subsoil passage of about 180 m length their concentration amounts to 0.12 µg L^{-1}. For interpreting this apparent decrease it has to be taken into account that the duration of the underground passage can take up to two months, i.e. the samples before and after this infiltration process, even if they were taken in triplicate over a period of time of six weeks, do not correspond to each other.

In degradation experiments of radio-labelled LAS in soil columns, Branner et al. (1999) observed that microbial transformation products, believed to be SPC, were virtually not retained on the column. Hence, an adsorptive removal of SPC during subsoil passage is rather unlikely to occur. Conversely, the subsequent slow sand filtration leads to a nearly total elimination of the SPC homologues. The residues detected in the water after this step and after the final chlorination with chlorine dioxide are minimal and range below the quantification limit of the applied analytical method (0.03 µg L^{-1}).

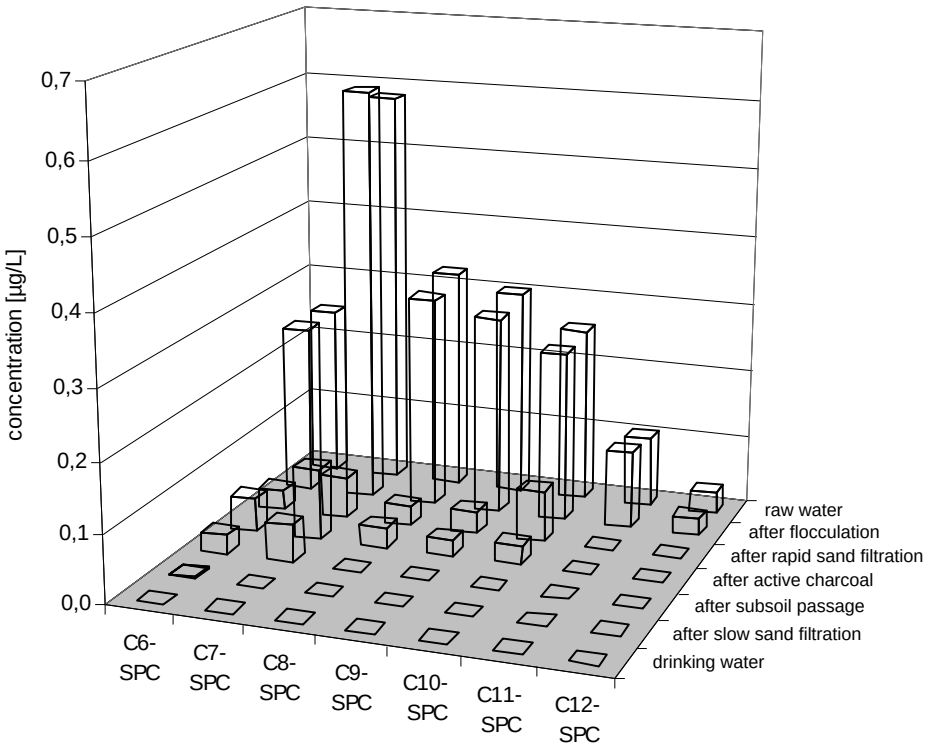

Fig. 5-16: Concentrations of individual SPC homologues in waters of different treatment stages at waterworks Rhine plant (three sample batches).

Comparing the capacities of both waterworks with regard to SPC elimination, it has been marked that the these polar pollutants exhibit a high potential to bypass the purification stages. Only the sand filtration steps with their supposed biological activity contribute significantly to abating the SPC concentrations. This effect is very surprising in view of the resistance of residues of these sulfonates in the river Rhine and in contaminated surface waters in general.

Possible formation of halogenated linear alkylbenzene sulfonates in waterworks Llobregat

It was reported that during breakpoint chlorination of raw water taken from the Llobregat river formation of brominated NP was observed (Ventura et al., 1988 and 1989). This was ascribed to high levels of bromide ions together with the presence in the surface water of NP considered as a recalcitrant metabolite of NPEO. In more recent in-depth investigations on this issue a series of chlorinated and brominated by-products of alkylphenolic surfactants and their degradation intermediates formed during chlorine disinfection were mass spectrometrically identified in chlorinated raw water (Petrovic et al., submitted).

On this background a study was conducted on the potential formation of halogenated by-products of the anionic surfactant LAS likewise present as organic pollutant in the water entering the waterworks (12 µg L^{-1}). Besides LC-ESI-MS analysis of the chlorinated raw water aiming at detection of the putative

single chlorinated and brominated compounds, laboratory experiments were carried out with artificial raw water simulating the prechlorination process [1].

In no instance the hypothetical by-products (e.g. halogenated C12-LAS: ^{35}Cl-C12-LAS: $[M–H]^-$ with *m/z* 403, ^{79}Br-C12-LAS: $[M–H]^-$ with *m/z* 403) could be detected in the water. This might be attributed to sterical hindrance in substituting an aromatic hydrogen atom by a considerably more volume-filling halogen atom. Introduction of a third substituent beside two relatively voluminous ones, the alkyl chain and the sulfonate group, might be disabled. Moreover, the latter acts as electron withdrawing substituent thereby deactivating the aromatic ring in view of an electrophile substitution.

5.3.2 Identification of sulfophenyl carboxylates in Brazilian drinking waters

As indicated by the studies on the two European waterworks, very polar SPC present at trace amounts in surface waters can end up to different degree the treated waters. Therefore, a monitoring exercise was carried out in two towns at the east bank of the Baía de Guanabara, Brazil, which are supplied by the waterworks Laranjal located 3 km downstream the point where the Rio Macacu – investigated previously for its residues of LAS and SPC (see section 5.2.1) – flows into the Rio Guapi-açu (**Fig. 5-2**, p. 109). For comparative purposes samples from the city of Rio de Janeiro receiving its drinking water from another waterworks (Guandu at the Rio Guandu) were also analyzed.

Results of the quantitative determination of SPC in tap water samples from several sites including public buildings and private houses are compiled in **Table 5-5** for two sampling periods. While in August 2000 the concentrations in Niterói averaged 3.3 µg L^{-1}, the mean level was markedly lower in the October samples when 1.6 µg L^{-1} were detected. These differences are likely due to distinct SPC levels in the raw water withdrawn from the Rio Guapi-açu, which in turn vary depending on hydrological settings of the river and status of pollution with domestic wastewaters. The almost identical value found in the October samples from São Gonçalo is in concordance with the fact that both cities receive their drinking water from the same waterworks. Concerning the drinking water from Rio de Janeiro, which is supplied by a waterworks situated in another river catchment area, the SPC concentration was 3.7 µg L^{-1}.

Table 5-5: Concentrations of SPC in drinking water samples.

Sampling site	Sampling period	Concentration [µg L^{-1}]
Niterói	August 2000	3.3 ± 0.4 (3) [a]
Niterói	October 2000	1.6 ± 0.2 (10)
São Gonçalo	October 2000	1.4 ± 0.2 (2)
Rio de Janeiro	October 2000	3.7 ± 0.7 (2)

[a] number of samples analyzed

During sampling it was noticed that most buildings were equipped with reservoir tanks installed on the roofs to safeguard water supply in case of public shortages. These tanks were occasionally subjected to

[1] The experiments were carried out by A. Diaz (AGBAR, Barcelona). Briefly, in the presence of 0.70 mg L^{-1} Br^-, 5 mg L^{-1} LAS were reacted with 9 mg L^{-1} Cl_2. The reaction medium contained 0.05 mg L^{-1} cationic polyelectrolyte and 70 mg L^{-1} $Al_2(SO_4)_3$ or 30 mg L^{-1} aluminium polychloride.

cleaning activities. To exclude that such actions represent a possible contamination source by detergent residues like LAS – out of which SPC might theoretically have been formed – in some instances the water entering the cistern was sampled in addition to that collected directly from the corresponding tap. But no appreciable differences were observed.

At the two supplying waterworks Laranjal and Guandu, evidence for different raw water qualities and presumptively also for distinct treatment stages in water processing is given through comparison of the specific isomer pattern of individual SPC homologues detected in tap water samples from Niterói/São Gonçalo and Rio de Janeiro.

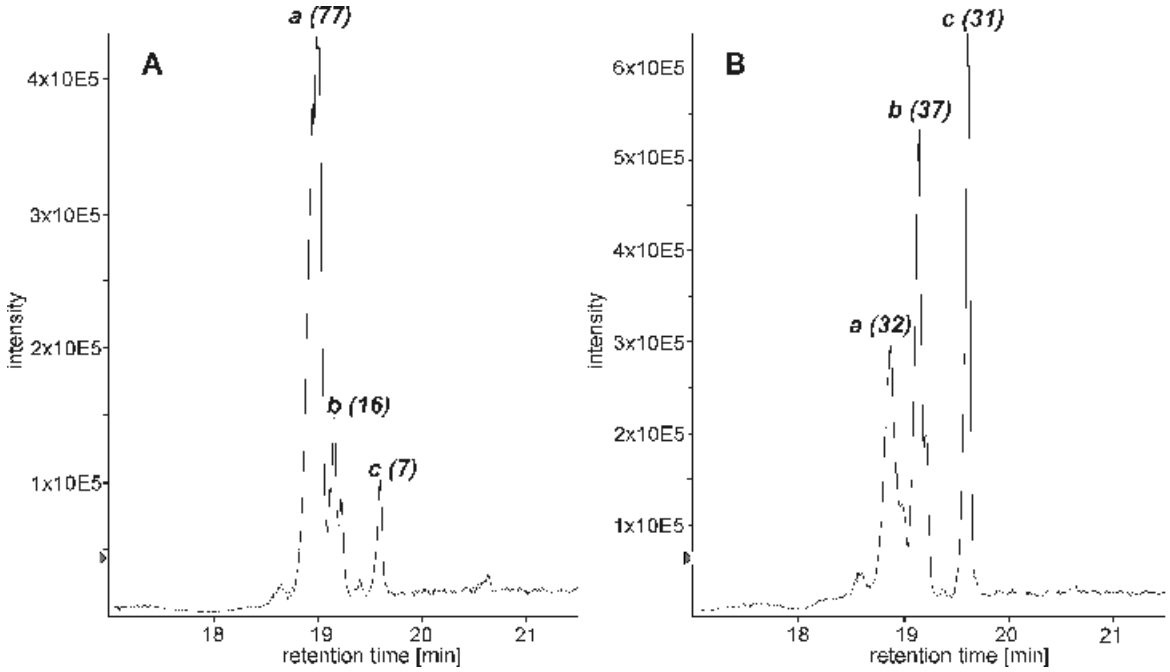

Fig. 5-17: (–)-LC-ESI-MS extracted ion chromatograms of C7-SPC (*m/z* 285) in drinking water samples from (**A**) Niterói and (**B**) Rio de Janeiro. Values in parenthesis indicate relative peak area (*a*+*b*+*c*=100 %). LC conditions as in **Fig. 4-4**.

The XIC of C7-SPC – this homologue also served in section 5.2.1 discussing the progression of SPC formation and breakdown in the Rio Macacu – is displayed in **Fig. 5-17** for samples from Niterói (A), being representative of all samples from Niterói/São Gonçalo, and Rio de Janeiro (B) assigning the three major peaks *a*, *b* and *c* with their relative abundances. While in the Niterói sample peak *a* is prevailing over *b* and *c*, coinciding quite well with the pattern encountered in the Rio Macacu and the Baía de Guanabara, therewith additionally confirming the origin of Niterói drinking water (see **Fig. 5-4**, A and C), the sample from Rio de Janeiro shows a relative homogenous distribution of the three peaks resembling more the pattern found in the River Rhine (**Fig. 5-17**, C) and therewith indicative of a far more progressed biodegradation of SPC in the used raw water.

The isomers pattern of the C7 homologue can thus be employed as a type of fingerprint to elucidate the origin and to assess the quality of raw water used as resource for the production of drinking water.

Overall, the presence of SPC of up to 4 µg L^{-1} in drinking water prompts a discussion on possible adverse effects in connection with to its consumption by humans. This issue is evaluated in chapter 6.

6 RISK ASSESSMENT OF SURFACTANT METABOLITES

In addition to the understanding of biodegradability, ecotoxicological data are necessary for surfactants, which may reach surface waters, in order to assess effects of residues to aquatic organisms. In general, these anthropogenic compounds are relatively high toxic due to inherent surface-active properties primarily contributing to disruption of biomembranes and denaturation of proteins (Swisher, 1987). The aquatic toxicity of single surfactants was thoroughly studied indicating that the response varies strongly between the used test organisms, such as fish, *Daphnia*, algae, or bacteria (Guhl and Gode, 1989; Rosen et al., 2001; Scholz, 1997; Staples et al., 1998). Besides the dependence on the exposed biological species, individual constituents of commercial mixtures, e.g. alkyl homologues of LAS, contribute distinctly to the total toxicity observed (Verge and Moreno, 2000). The presence of unreacted raw materials from manufacturing or by-products formed during synthesis has also to be taken into account measuring the toxicity of technical surfactants blends (Verge et al., 1999).

Usually primary degradation of surfactants goes hand in hand with the loss of surface-activity, leading thereby to more polar compounds. Consequently, the toxicity is notably reduced. For this reason less attention was paid for a long time to degradation intermediates until discovering an enhanced toxicity of some metabolites, relative to the parent compounds, showing a higher lipophilicity than the unbroken substances. The most famous example is NP deriving from breakdown of non-ionic NPEO surfactant. NP exhibits a substantially higher toxicity than the precursor compound (Giger et al., 1984; McLeese et al., 1981). Moreover, this recalcitrant contaminant has caused particular concern since it mimics biological activities of the female hormone estrogen (Soto et al., 1991).

In the following two sections possible effects of SPC, occurring in drinking waters at µg L^{-1} levels, are evaluated regarding the consumption of such water by humans. The toxicity of the transient degradation product of AG, C4-glucamide acid (see section 4.2.3), is evaluated performing two ecotoxicological assays.

6.1 Risks arising from the presence of sulfophenyl carboxylates in drinking waters

No toxicological data on SPC, which is an environmental contaminant deriving from a manmade substance, are available either on mammals or on humans. In contrast, the ecotoxicity to aquatic organisms (fish and *Daphnia*) was explored showing that the LC_{50} was increased by a factor of 200 to 300 by presence of a carboxyl group at the end of the chain and another 10 to 20 times upon shortening the alkanoate chain from 11 to 7 and to 5 (Swisher, 1976). This was attributed to the fact that the toxicity of

LAS is directly related with its surface-active characteristics. Oxidation of the alkyl chain generating carboxylic acids leads to the loss of surface activity. This is in line with the findings of Moreno and Ferrer (1991) monitoring the toxicity towards *Daphnia magna* during biodegradation of various LAS blends. The metabolites formed during this process were far less toxic than the parent molecule regardless of the mean molecular weight the type of LAS assayed. The residual material from trickling filters studies of Kölbener et al. (1995a) showed no detectable impact on the growth of the algae *Selenastrum capricornutum* and the mobility of *Daphnia magna*. Hence, from the viewpoint of mere toxic properties, SPC at µg L^{-1} levels are very unlikely to threaten the health of human beings consuming contaminated drinking water.

A second aspect, which has to be focused on, is the ability of SPC of disturbing the endocrine system of man since a variety of xenobiotic substances with similar chemical properties were shown to exert estrogenic effects on fish and mammals. Unlike several alkylphenol derivatives stemming from the degradation of APEO, a range of SPC and a commercial LAS preparation proved to be not estrogenic in the recombinant estrogen yeast screen utilized by Routlegde and Sumpter (1996). These findings were corroborated using apart from the yeast screen the vitellogenin assay (Navas et al., 1999). Neither C11-LAS nor the two SPC homologues C5 and C11 induced any positive response in the tests.

6.2 Ecotoxicological assessment of the metabolite of alkyl glucamides

Bioluminescence inhibition assay with *Vibrio fischeri*

The short-term bioluminescence test with the marine bacterium *Vibrio fischeri*, originally introduced on the market as Microtox® test, represents a highly standardized bioassay used for the determination of acute toxicity of pure substances, complex effluents and mixtures of chemicals in distinct environmental matrices. The ease of use, speed, and cost effectiveness has made it an attractive tool for toxicity assessment (Castillo et al., 2001; DEV, 1991; Kaiser and Palabrica, 1991; Ribo and Rogers, 1990).

The ToxAlert® bioassay, differing from the original test in its compatibility with liquid-dried bacteria in place of freeze-dried species as demanded in the Microtox® test, was employed to evaluate the acute toxicity of C4-glucamide acid towards *Vibrio fischeri.*

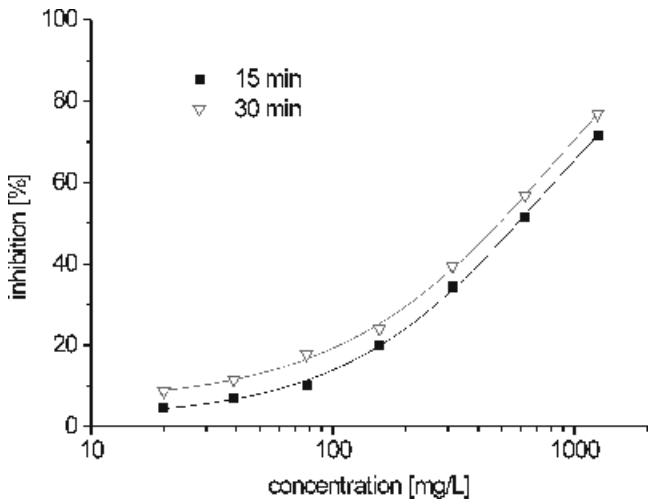

Fig. 6-1: Bioluminescence inhibition curves of C4-glucamide acid.

The dose-response curves shown in **Fig. 6-1** reveal that the luminescence inhibition after 30 min is only slightly different from the value registered after 15 min, which is indicative of a fast interaction of the test compound with the bacteria. The concentrations causing a 50 % reduction of the light emission, termed as IC_{50}, were calculated to be 493 mg L^{-1} and 603 mg L^{-1} after 15 and 30 min, respectively.

Compared with IC_{50} data of other surfactants or their degradation products these values are rather low. The same assay applied by Farré et al. (2001a), as part of wastewater toxicity screening for non-ionic surfactants, yielded IC_{50} for single substances studies of 3.3 mg L^{-1} for C10-AE, 2.7 mg L^{-1} for an NPEO mixture, and 2.6 mg L^{-1} for its persistent metabolite nonylphenol monocarboxylate.

Toxicity analysis with Cellsense biosensor

The Cellsense system was developed as rapid assay of potential toxicity of wastewaters and individual process streams to the active biomass in WWTP (Evans and Rogerson, 1992; Rogerson et al., 1993). Bacterial cells are incorporated into biosensor configurations based on electrochemical transducers, which convert the biochemical signal into a quantifiable electrical signal. This allows the real-time monitoring of metabolic status and the opportunity for fast detection of cellular perturbation due to the presence of toxic compounds. Evans et al. (1998) reported on the use of the biosensor incorporating activated sludge to provide rapid determination of toxicity in wastewaters to activated-sludge based WWTP. In the work of Farré et al. (2001b) the amperometric sensor with immobilized *Escherichia coli* was utilized to assess the toxicity of wastes from textile and tannery industries containing among other pollutants high amounts of diverse surfactants.

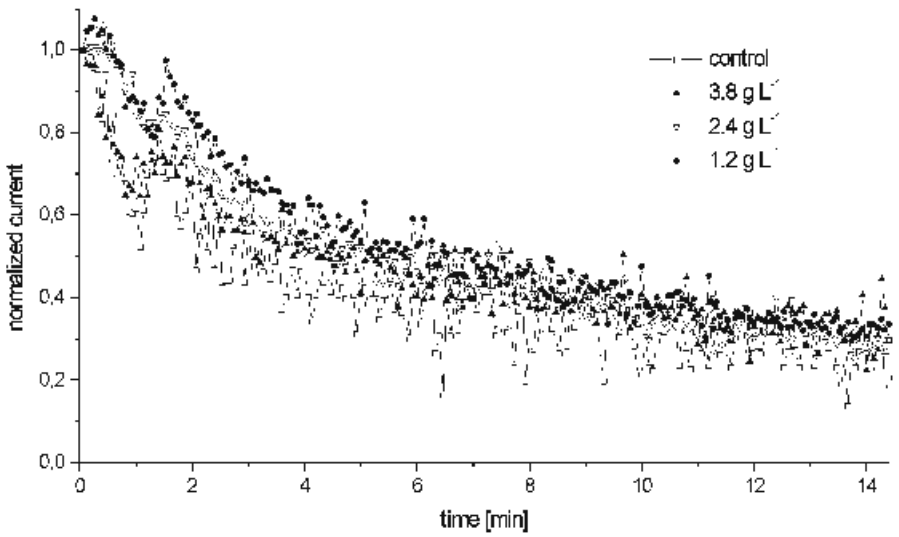

Fig. 6-2: Response curves of Cellsense biosensor to C4-glucamide acid (normalized mean values of duplicates).

In **Fig. 6-2** the effect of three different concentrations of C4-glucamide acid on the metabolic activity of *Pseudomonas putida* used in the Cellsense assay is displayed. While in the presence of a toxic compound the immobilized bacterial population reacts by an immediate and abrupt decline of the measured current, no significant reduction of the signal can be observed for the surfactant metabolite assayed even in the

tested g L^{-1} range. All four curves exhibit a similar exponential decrease, which is consequence of the natural decrease in bacterial activity.

Taking into account the low acute toxicity of C4-glucamide acid established in both bioassays and the expected levels of this transient biodegradation intermediate, which are likely to be 1 or 2 orders of magnitude lower than the parent compounds – occurring in the sewage in the mid µg L^{-1} range – it can be largely ruled out that C4-glucamide acid exerts toxic effects on aquatic organisms. As for its presence in WWTP, it will thus not contribute to the overall toxicity of wastewater. An adverse effect due to endocrine disrupting properties can likewise be excluded as C4-glucamide acid showed no estrogenicity in the MCF-assay (Olea, 2000).

7 CONCLUSIONS AND FUTURE DIRECTIONS

Analytical aspects

The investigations on the fate of surfactants under different discharge conditions demonstrated the importance of wastewater treatment in reducing the amounts of residues entering the natural aquatic environment. Determination of the surfactant proportion eliminated from the dissolved phase through precipitation and adsorption onto particulate matter was beyond the scope of this work, but as concerns LAS a significant percentage is known to be associated to the sludge fraction (15 to 45 %, Berna et al., 1989). Since the xenobiotic arylsulfonates are not degraded during the commonly applied process of anaerobic sludge digestion (Birch et al., 1992; Painter and Mosey, 1992), considerable LAS concentrations of 1,600 to 19,000 mg kg^{-1} were found in anaerobically digested sludge (de Henau et al., 1996; Holt and Bernstein, 1992; McEvoy and Giger, 1986). The use of such sludges as organic fertilizers on arable land represents the most important source of LAS in the terrestrial environments.

Next to the monitoring of LAS, studies are required to investigate the possible formation of SPC in sludge-amended soils. This is of particular interest as these very polar intermediates can be believed to possess high mobility in soil resulting in pronounced leachability (confirmed in laboratory experiments by Branner et al. (1999) and indirectly proved by the lacking removal during underground passage in the waterworks Rhine). The persistency of some SPC isomers is likely to allow their migration into deeper zones until reaching ground water aquifers, thereby endangering the quality of drinking water sources.

In view of the inherent resistance of some SPC isomers to complete mineralization, efforts have to be mounted in order to get more insight into the reasons behind the persistency of these carboxylated arylsulfonates. Therefore, it will be indispensable to fully elucidate the chemical structure of individual components, e.g. after isolation from environmental samples single species could be characterized by LC-ESI-MS/MS in combination with NMR analyses.

The knowledge on the fate of SPC could be further enhanced by taking a closer look on a possibly distinct behavior of SPC stereoisomer in terms of a preferential metabolization of one diasteroimer or enantiomer. Schulz et al. (2000) did a first step in this direction. The authors could isolate an organism sequentially utilizing the two enantiomers of C4-SPC, which were separated on a chiral HPLC column. The general importance of stereospecificity on biodegradability and metabolite formation was also pointed out by Schowanek et al. (1999) investigating the degradation of different stereoisomeric forms of the complexing agent ethylene diamine disuccinic acid. Of the three possible isomers examined only one was rapidly and completely mineralized. Distinct behavior of enantiomers was likewise reported in the

study of Simoni et al. (1996) on 3-phenylbutyrate (this is virtually desulfonated C4-SPC-3). Only the *R* enantiomer was biodegraded, while the *S* enantiomer was co-metabolically transformed.

Since nothing is known on the anaerobic degradability of the amphoteric surfactant CAPB, whose higher alkyl homologues revealed some tendency to adsorption in the laboratory experiments, it cannot be ruled out that these end up in sludge-amended soils. Analytical methods are necessary to follow up their whereabouts in this environmental compartment.

A particularly challenging task, which has so far been almost entirely neglected, will be the analysis of water-soluble synthetic polymers of high MW, like PVP, in both aquatic and terrestrial compartments. For their determination, selective extraction and preconcentration methods are required in order to isolate the analytes from environmental matrices. This needs to be complemented by analytical instrumentation allowing accurate quantitative measurements, which is still a strongly limiting factor in MALDI-ToF-MS analysis. The power of emerging techniques for on-line coupling of MALDI with column separation, such as HPLC and GPC, can potentially become a very promising approach in this field (Gusev, 2000).

Legislative regulations force action

The pollution of the terrestrial ecosystem by high amounts of LAS through sludge application, which may results in detrimental effects on soil organisms and flora and solubilization of hydrophobic sludge contaminants (Jensen, 1999), has given rise to concern and the research on the terrestrial fate and the assessment of the risks caused by high levels of the anionic surfactant in sludge-amended soils has been intensified in recent years.

In the European Community sludge application is currently a vehemently disputed issue between surfactant manufacturers, independent researchers, and policy makers (Berna et al., 2000; CEEP, 2000; CESIO, 2000; CLER, 2000) in face of legislative regulations issued by the Council of the European Communities (CEC). They call for construction and operation of adequate facilities for the biological treatment of wastewater (CEC, 1991), which will ultimately lead to rapidly increasing amounts of sewage sludge produced. Moreover, the CEC released a draft directive setting the cut-off level for LAS to be used in agriculture to 2,600 mg kg^{-1} (CEC, 2000). Hence, by implementation of this directive a large percentage of the sludge from anaerobic treatment will no longer be permitted to be put onto fields.

To mitigate the problem of high LAS concentrations in sludge to be applied on farmland and to fulfill current and projected criteria imposed by EU authorities – and these restrictions are prone to become steadily more severe with the need to dispose of evermore increasing amounts of sewage sludge – composting of sludge might be a promising mechanism for efficiently reducing LAS contents. Prats et al. (1999) reported on a nearly 100 % elimination of LAS after 72-day composting of anaerobically digested sludge. Similar findings were presented by Solbé (1999) stating a quantitative degradation of LAS after composting, which also proved effective in removing NPEO (78-95 %).

In place of trying to find appropriate technical equipment for solving or moderating a man-made problem, the presumptively best choice is to replace a hazardous chemical by suitable alternatives. Despite the repetitive declarations of the surfactant industry classifying LAS as *intrinsically environmentally safe* (Waters and Feijtel, 1995) with *sufficient safety margins* (Cavalli et al., 2000) posing *no significant threat in the long-term* (Jensen, 1999), Sweden has translated the substitution of LAS in detergent products into action. Due to the comparatively high toxicity and the poor anaerobic

biodegradability, this country banned the synthetic compound and replaced it by other surfactants such as AS or AES, which can fulfill the same cleaning function (Hagenfors, 1998). Denmark has likewise focused much on LAS where the Environmental Protection Agency has meanwhile placed this anionic surfactant on the "List of Undesirable Substances" owing to its problemacy in the waste cycle (DEPA, 1998).

A further example for changes on the detergent market are the Philippines. Increasing public awareness of the deterioration of the environment and wasting of water resources solely in exchange of cheaper raw materials used for surfactant production put pressure on manufacturers, which have started to suspend the use of ABS (Phil, 2000a). Recently an act has been approved, which will prohibit manufacture, distribution, and sale of domestic and industrial detergents composed of hard, petrochemically based surfactants (Phil, 2000b). As result new products containing as single surface-active ingredient coconut-based AS were introduced on the market (Phil, 2000c).

Overall, the few examples presented have illustrated the chances for the substitution of a not fully environmentally sound surfactant. The poor anaerobic biodegradation of LAS should not be considered as single pass/fail criterion for its acceptability – the biosphere is predominantly aerobic –, but in face of the enormous amounts of LAS, to which this criterion applies and thus plays a decisive factor, it should be desirable to look out for adequate alternatives taking into consideration economic, functional and above all ecological viewpoints.

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