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Journal of Chromatography A, 987 (2003) 395–402
JOURNAL OF CHROMATOGRAPHY A
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Process monitoring of anaerobic azo dye degradation by high-performance liquid chromatography–diode array detection continuously coupled to membrane filtration sampling modules

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Abstract

Process integrated microfiltration and ultrafiltration based membrane sampling modules were compared by means of HPLC with diode array detection based monitoring of an anaerobic azo dye biodegradation process. The sampling matrix consisted of anaerobic sludge from a municipal wastewater treatment plant. The hydrolysed azo dye Reactive Black 5 (RB5-H) and three products (ionic and nonionic) released from reductive cleavage under anaerobic conditions were continuously monitored by simultaneously separation by ion-pair chromatography. Microfiltration membrane-based sampling showed no retention for any compound observed. Sampling by ultrafiltration significantly retained the observed ionic compounds between 58 and 83% whereas a nonionic compound was not retained. On-line monitoring of an oxygen-sensitive compound was possible whereas off-line detection failed. Robust long time monitoring could be performed for up to 1 week without cleaning the membrane.

Keywords: Membranes; Sample handling; Ultrafiltration; Microfiltration; Process monitoring; Azo dye

1. Introduction

As compared to process gas chromatography, there is a lack of process liquid chromatography systems to meet the demands of industrial and bioprocess monitoring applications. Bioprocess monitoring means effortless access to continuous real-time information about all variables relevant to a given process which is primarily dependent on a robust sampling technique [1]. On-line and in situ sampling in the area of wastewater screening and biological process monitoring deals with a complex matrix which consists of suspended solids (bacteria cells, mud flakes, particles) and a high number of dissolved molecules characterised by a wide mass range and varying polarity. On-line dialysis, on-line filtration and membrane extraction have been applied for routine target analysis in laboratories and bioanalytical applications [2–4]. Limited life-time and short cleaning intervals are described as the major problems of membrane-based sampling techniques [5]. Membrane-based sampling on-line analysis systems have been established in the area of photometric measurement of parameters like TOC (total organic content), COD, UV–Vis and several anions and cations in industrial and communal sewage treatment plants [6–10]. The primary application of membrane based sampling coupled to chromatography in biotechnology is monitoring and

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0021-9673/02/$ – see front matter
PII: S0021-9673(02)01906-4

controlling of fermentation processes [11–15]. Rehorek et al. [16] first applied ultrafiltration-based sampling coupled to HPLC–diode array detection (DAD)–refractive index (RI) detection for monitoring an anaerobic degradation process of H-acid (1-amino-8-hydroxynaphthalene-3,6-disulfonic acid). Textile wastewater to be treated in a anaerobic bioreactor contains a large number of organic compounds which can be ionic and nonionic of varying polarities. The monitoring of such compounds using continuous process integrated sampling by micro- and ultrafiltration modules was the objective of this study. Other sampling techniques like membrane extraction or on-line solid-phase extraction (SPE) are limited to a number of chemically similar compounds or are not applicable due to a very complex matrix containing suspended bacteria. Aromatic sulphonates with more than one sulphonate group were not extractable by several tested adsorbents [17]. The hydrolysed azo dye Reactive Black 5 (RB5-H) was used as a model substance for textile wastewater as it releases two ionic and one nonionic compound by the anaerobically initiated reductive cleavage of the azo bonds. One ionic compound was oxygen-sensitive and, therefore, not detectable by HPLC–DAD with off-line sampling. Simple and reproducible on-line sampling and sample preparation methods were developed for the monitoring of RB5-H and its metabolites over a time period of up to 1 week without cleaning the membrane of the sampling modules.

2. Experimental

2.1. Chemicals

HPLC-grade LiChrosolv® water and LiChrosolv® gradient grade acetonitrile were obtained from Merck (Darmstadt, Germany). The ion pairing agent tetrabutylammonium hydrogensulphate (TBAHS) was purchased puriss. grade (>99%) from Fluka (Buchs, Switzerland). TBAHS containing eluents were prepared 1 day before use and degassed by ultrasound. Reactive Black 5 and p-Base were obtained from DyStar (Leverkusen, Germany). The conditions for the hydrolysis of the stock solution of the dye were: pH 12 and 80 °C. Hydrolysis products were analysed by HPLC–DAD. Afterwards the stock solution was neutralised to pH 7.

2.2. Membrane sampling and HPLC equipment

A schematic drawing of the monitoring system is shown in Fig. 1. The in situ sampling module ESIP 5441 was purchased from Trace Biotech

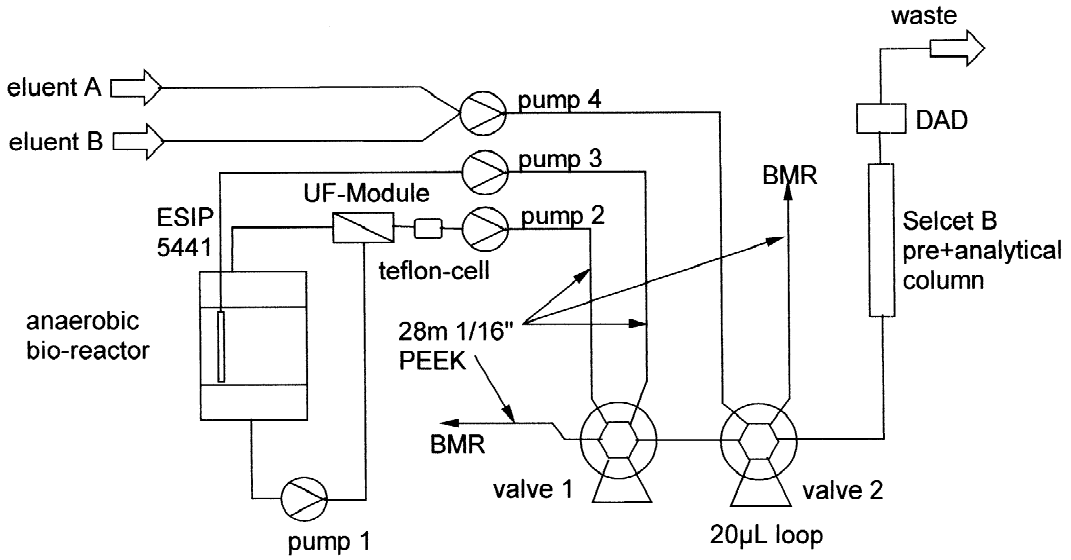

Fig. 1. Schematic figure of membrane sampling modules from bioreactor coupled to HPLC–DAD.

(Braunschweig, Germany). Before use the membrane was preconditioned by pumping 1 ml/min of propanol–water (70:30, v/v) for 2 h. The ultrafiltration membrane cell was described elsewhere by Rehorek et al. [16]. Ultrafiltration membranes were provided by Desal Osmonics (Moers, Germany). Preconditioning was performed with methanol for 5 min. The ultrafiltration permeate was recirculated (Fig. 1 pump 1) into the bioreactor and split into a bypass circuit for the HPLC system far away from the technical laboratory on another floor. The distance between sampling and HPLC was 28 m (56 m circuit). The bypass system (Fig. 1, bypass) consisted exclusively of PEEK tubings (1/16 in. ; 1 in.=2.54 cm). A 58-ml safety PTFE cell (Fig. 1 PTFE cell) with a HPLC filter frit was placed between the ultrafiltration membrane cell (Fig. 1, UF Module) and the bypass circuit pump (Fig. 1, Pump 2) in order to buffer volume stream fluctuations of the ultrafiltration membrane cell and collect gas bubbles before entering the bypass circuit and the HPLC system. A second 56-m bypass circuit connected the ESIP 5441 in situ sampling module (Fig. 1, ESIP 5441) with the HPLC system. The liquid in the bypass circuits was transported by two isocratic pumps (K-120, 0.1–10 ml) from Knauer (Berlin, Germany) and continuously flushed with sample (Fig. 1, Pumps 2 and 3). An electrical six-way switching valve (Fig. 1, Valve 1) was connected with the bypass circuits, the HPLC pump and the HPLC. It was controlled by the HSM software D-7000 which allowed the automatic switching between ultrafiltration based on-line sampling and in situ sampling. LC analyses were performed with a LaChrom® system from Merck (Darmstadt,Germany) with a low pressure gradient pump L-7100, electrical six-way switching valve with 20-μl injection loop (Fig. 1 Valve 2), autosampler L-7250 (off-line injection), column oven L-7450, diode-array detection system L-7450A and an interface L-7000 for system control and data processing with the HSM software D-7000 under Windows NT 4.0.

2.3. Liquid chromatography

The analytical column was a 125×4 mm LiChroCart HPLC-cartridge (LiChrospher 60 RP-select B, 5 μm) from Merck with a select B precolumn which can be used for more than 1000 injections from on-line sampling. Acetonitrile–1 m*M* aqueous TBAHS (10:90, v/v) was used as eluent A against pure acetonitrile as eluent B. From 0 to 5 min the analysis ran isocratically, then a 20-min gradient was run with a two percent slope per minute for B. Within 5 min the gradient decreased by 100% A. Afterwards a 10-min re-equilibration was run. The whole run took 40 min and had to be done under constant 40 °C temperature conditioning.

2.4. Azo dye degradation and calibration

In order to adjust to a concentration of 180 μ*M*, 5.4 m*M* of RB5-H was added to the reactor. The dye was added three times for a step-feed-batch experiment. The monitoring started 1 h before the injection of the prepared dye solution. Analysis with ultrafiltration-based sampling and in situ sampling by ESIP 5441 were run in intervals of 0.75–3.50 h. Due to the fact that dye standards are not commercially available, the dye concentration as well as the concentration of metabolites were calculated according to manufacturers' instructions. Calibration with HPLC–DAD was performed for RB5-H and p-Base. The concentration of p-base ranged between 19 μ*M* and 10 m*M* detected at 270 nm and for RB5-H between 10 μ*M* and 1.25 m*M* detected at 585 nm. The correlation coefficients are $R^2_{\text{p-base }(n=10)}=0.999$ and $R^2_{\text{RB5-H }(n=8)}=0.989$. $\text{RSD}_{\text{p-Base}}=2\%$ and $\text{RSD}_{\text{RB5-H}}=14\%$ was determined by injection ($n=5$) of a standard solution containing 2.5 m*M* p-Base and 1.25 m*M* RB5-H. Concentrations of 7-amino-8-hydroxy-1, 2-naphthaquinone-3, 6-disulphonate-1, 2-diimine ($\text{TAHNDS}_{\text{DP1}}$) and dihydroxynaphthoquinone-3,6-disulfonatediimine ($\text{TAHNDS}_{\text{DP2}}$) were estimated from the stoichiometry of the reduction of RB5-H and correlated to the detection of p-Base. Linear equations for the calculation of the concentration from peak areas are as follows: $c_{\text{RB5-H}}=(\text{peak area}_{585\text{ nm}})/8503$; $c_{\text{p-Base}}=(\text{peak area}_{270\text{ nm}})/2702$; $c_{\text{TAHNDS(DP1/2)}}=(\text{peak area})/2120$ ($c=$ concentration in μ*M*). Retention characteristics of the tested ultrafiltration membranes with respect to the dye and its degradation products were related to the results of in situ sampling based analysis which showed no characteristic retention of any compound as compared to off-line measurements. Off-line

samples were analysed after 10 min centrifugation (8000 *g*) and compared with in situ and on-line sampling based analysis. Off-line data are not shown.

2.5. Bioreactor

Anaerobic bacterial sludge was obtained from a local municipal sewage treatment plant and filled in a 30-l bioreactor. The sludge was immobilised on 1-cm^2 polyurethane foamed carriers coated with activated carbon obtained from Biosys (Leverkusen, Germany) after sieving (2 mm). The sludge was conditioned 14 days under nitrogen atmosphere until an immobilisation rate of >95% could be observed. Redox potential electrode was a PFGR-2 and pH-electrode a HGK-Gel A/AKB, both purchased from Dosatronic (Immenstaad, Germany) and installed in a flow-through-cell flushed by fluid of the bioreactor. The pH was held constant at 6.8 ± 0.2 and temperature was maintained at 39 ± 0.5 °C. The redox-potential (against hydrogen potential) was continuously measured during the experiment: $E_0=-318\pm13$ mV. The anaerobic reactor content was continuously pumped through the ultrafiltration membrane cell in a cross flow mode (180 l/h; 0.5 MPa). The water contained less then 1% suspended bacteria due to the immobilisation. Salt concentrations of the bioreactor fluid were 330 mg_{NaCl}/l and 4 g_{Na2SO_4}/l (other salts concentrations were not determined).

3. Results and discussion

Both process integrated sampling modules continuously coupled to HPLC were suitable for long term on-line monitoring of the biodegradation of an azo dye. Simultaneous separation of both ionic and nonionic solutes sampled from anaerobic sludge was performed by ion-pair chromatography. The chromatograms (see Fig. 2) show the retention order of the four monitored compounds at two wavelengths sampled by in situ and on-line sampling modules. A variation of the retention volume of 10% was observed. p-Base had a retention volume of $V_R=2.9$ ml, $TAHNDS_{DP1}$ one of $V_R=5.5$ ml whereas $TAHNDS_{DP2}$ had $V_R=14.4$ ml and RB5-H $V_R=17$ ml (determined from in situ sampling). The low retention volume of $TAHNDS_{DP1}$ in comparison to $TAHNDS_{DP2}$ is owed to a higher polarity of the ion-paired solute. Both solutes were negatively charged by two sulphonic acid groups in the 3,6-position of naphthalene. An overview of the proposed anaerobic degradation process of RB5-H and formation of metabolites is given in Fig. 3. TAHNDS is rapidly oxidised even in the presence of trace amounts of oxygen and was not detectable by HPLC–DAD–MS [18]. Two autoxidation products were proposed—$TAHNDS_{DP1}$ and $TAHNDS_{DP2}$. $TAHNDS_{DP2}$ is a product of $TAHNDS_{DP1}$ and more stable. Both compounds could be detected and characterised in situ by on-line sampling coupled to HPLC–DAD. $TAHNDS_{DP1}$ was not detectable by off-line sampling. p-Base is another product of the reductive cleavage of RB5-H under anaerobic conditions. The complete reduction of RB5-H was reproducibly monitored (see Fig. 4) for all of the three injections during the step-feed-batch experiment. The anaerobic reductive cleavage of the azo bonds of RB5-H was detected by determination of the metabolites. In the first instant an immediate increase of concentration of p-Base after the injection of RB5-H did not correspond with the decrease of RB5-H and the increase of $TAHNDS_{DP1}$ or $TAHNDS_{DP2}$. The concentration of p-Base in the reactor liquid can be influenced by the injection of high amounts of RB5-H as it effects changes on the adsorption–desorption equilibrium of RB5-H and metabolites especially p-Base adsorbed on polyurethane foamed carriers coated with activated carbon. Chemically initiated displacement and complex reactions contribute to a shift of the equilibrium. Off-line sampled p-Base was detected at approximately two-to-three times higher concentrations which indicates that the sample was influenced from atmospheric oxygen or consecutive reactions (data are not shown). No significant difference in comparison to measurements from off-line samples could be found for RB5-H or $TAHNDS_{DP2}$. Hence, the biological degradation mechanisms and consecutive reactions are not yet fully understood. Oxygen free sampling is elementary for studies of those biodegradation processes of organic compounds in anaerobic sludge as simple off-line sampling is connected with a loss of analytes by oxygen initiated reactions.

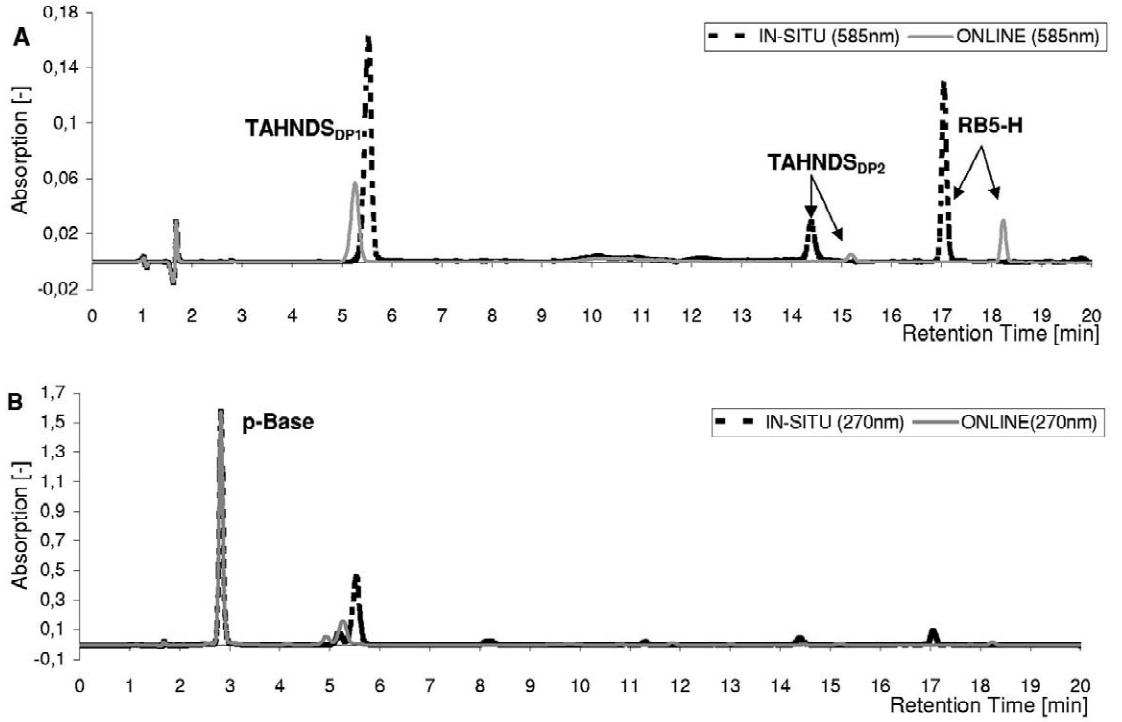

Fig. 2. Comparison of ion-pair chromatography separations of RB5-H and metabolites by online and in situ sampling coupled to HPLC–DAD from bioreactor: (A) 585 nm scanning wavelength, (B) 270 nm scanning wavelength.

Table 1 shows an overview on the characteristics of the membrane based sampling modules. The dead volume of ESIP5441 is 10-fold lower than that of the cross-flow module and retained none of the analytes. Very low sampling dead-times of 4 min can be achieved when a high sampling flow is adjusted. A high sampling flow increases the pressure drop across the inversely working dead-end microfiltration membrane due to an accelerated clogging of the membrane. Previous investigations showed that the cleaning interval time of both systems is very much dependent on the concentration of suspended bacteria and mud. Immobilisation of the bacteria helped to increase the cleaning intervals. Nevertheless, the sampling flow continuously decreased between cleaning intervals. It was recovered by mechanical cleaning of the surface of the membranes. The lifetime of the used ultrafiltration membranes was on average 40 days which is three times shorter than for microfiltration. High shear stress caused by a flux of 180 l/h and a pressure of 0.5 MPa were the reasons for a lower stability of the cross-flow ultrafiltration membranes in comparison to the dead-end microfiltration. In addition, small particles and suspended bacteria in the fluid lead to a continuous abrasive effect on the surface of the membrane.

For the removal of organic compounds, the ultrafiltration membranes are described by their molecular mass cut-off (MWCO) [2]. RB5-H, TAHNDS$_{DP1}$ and TAHNDS$_{DP2}$ were retained by the ultrafiltration membrane with a MWCO of $M_r = 3500$ but not p-Base (see Table 2). TAHNDS$_{DP1}$ ($M_r = 347$) and TAHNDS$_{DP2}$ ($M_r = 348$) were differently retained. This shows that the efficiency of membrane transport for a particular compound is less dependent on its molecular mass. Chemical charge and chemical structure represent the influencing factors on the mass transport for molecules below $M_r < 1000$. Ion-pair chromatography of TAHNDS$_{DP1}$ and TAHNDS$_{DP2}$ confirmed that both analytes signifi-

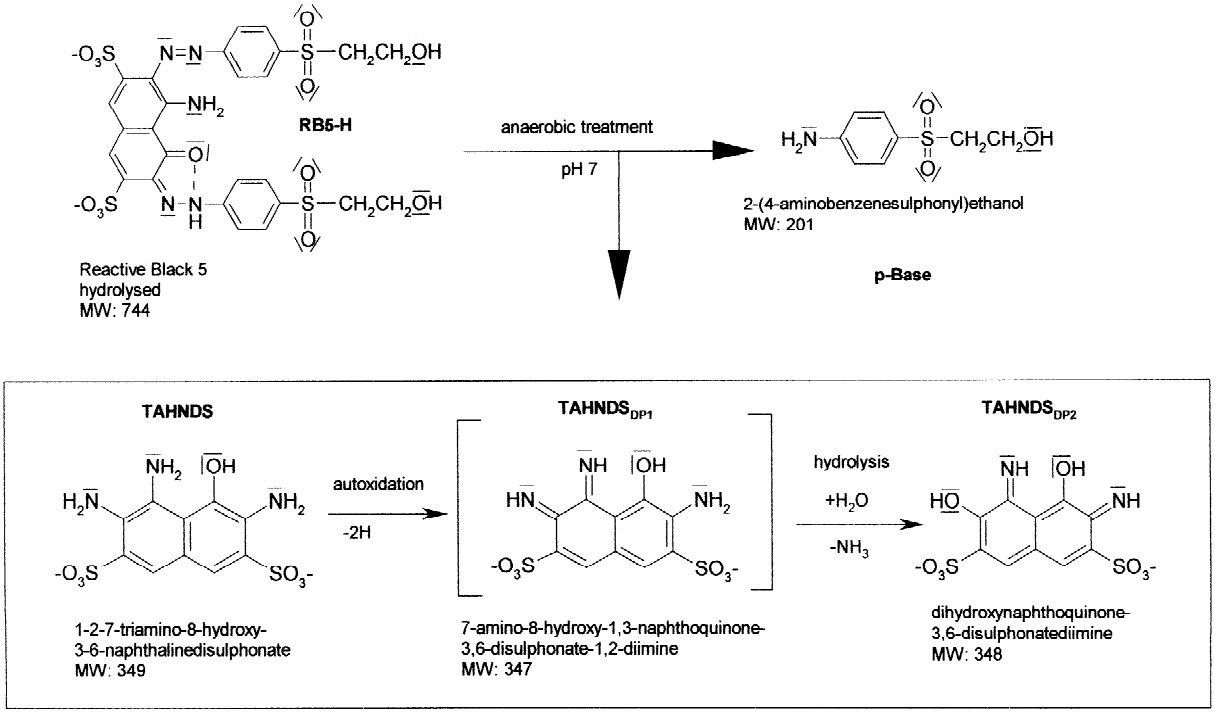

Fig. 3. Anaerobic degradation pathway of RB5-H; proposed reaction of TAHNDS [18].

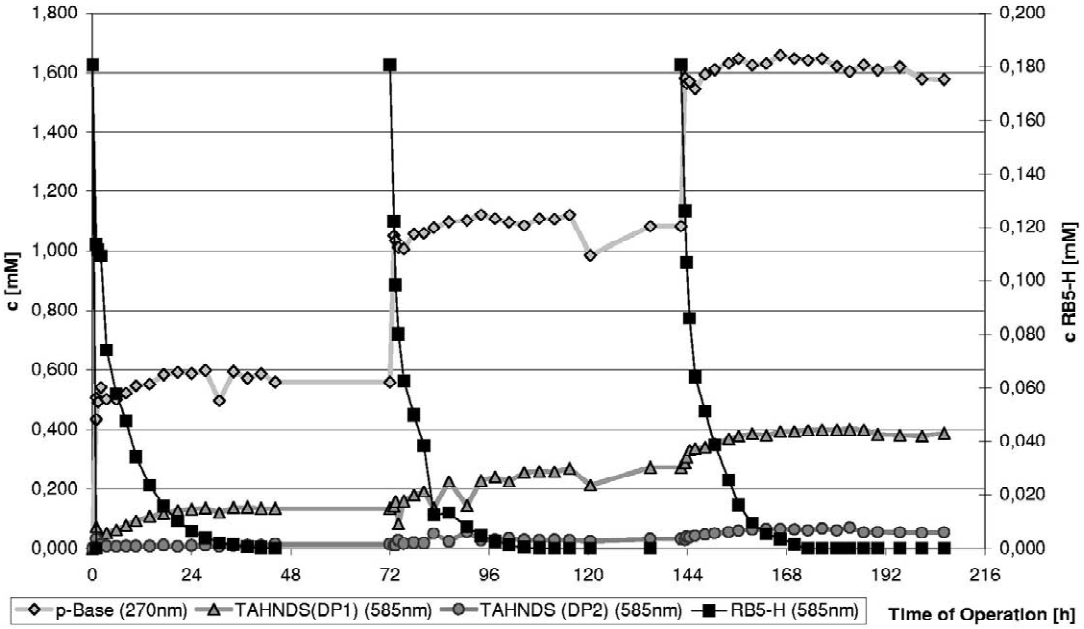

Fig. 4. Monitoring data of step-feed-batch anaerobic degradation of RB5-H calculated from in situ sampling continuously coupled to HPLC–DAD; three times injection of 180 μM RB5-H (at 0, 72 and 144 h).

Table 1
Sampling characteristics of a ultrafiltration (UF) cross-flow membrane module and a inversely operated microfiltration (MF) dead-end in-situ sampling module

	Membrane type	
	GK UF	MF ESIP5441
Manufacturer	Desal Osmonics	Trace Biotech
Membrane surface material	Polyamide	Polypropylene
MWCO (M_r on PPG)	3500	–
Dead volume (module, capillary) (ml)	~63	6
Sampling dead time (min)	20–70	4–30
Membrane area (cm^2)	80	39
Pore size (μm)	–	0.2–0.6
Operating pressure (MPa)	0.5	–
Flow-rate (l/h)	180	–
Permeate flow-rate (ml cm^{-2} h^{-1})	3.2±1.3/3.3±1.7/	2.3–0.3
after 0/1/2/3/4/5/6 (days), $n=3$	1.9±0.4/1.9±0.5/	(SD n.d.)
	1.1±0.1/1.3±(n.d.)/0.9±0.6	
Average cleaning intervals (days)	8±3 ($n=9$)	5±2 ($n=6$)
Life time (days)	39±6 ($n=4$)	117

cantly differ in polarity. The characteristic MWCO is unsuitable as a parameter of ultrafiltration retention characteristics of ionic organic solutes. The applicability of ultrafiltration membrane has to be tested on each specific analyte and this is the objective of further investigations.

4. Conclusions

Membrane-based sampling is a suitable sampling technique for continuous, simultaneous long-time monitoring of ionic and nonionic in anaerobic wastewater treatment processes. It could be demonstrated that, due to the slow kinetics of the reductive cleavage of the azo dye, an analysis time of 40 min and sampling intervals of 2–6 h were sufficient for monitoring of the process even when the sampling dead-time increased to 30 min. Oxygen sensitive metabolites were analysed by both sampling modules without being affected by air. Microfiltration based in situ sampling provides short sampling deadtime and acceptable cleaning intervals for biodegradation processes such as anaerobic azo dye treatment. Operated at very low flow-rates it is an interesting tool for long time monitoring in bioprocesses. The use of ultrafiltration membranes requires testing of the retention characteristics for each compound to be analysed. Mass transport in ultrafiltration membranes is more dependent on chemical structure and charge than one would expect from the MWCO. A reduction of the dead-volume of the ultrafiltration cross-flow module may result in a lower sampling dead time whereby a reduction of flux and pressure can be taken into account. Immobilisation of bacteria is a benefit for longer lifetime of cross-flow membranes but can affect quantitative analysis of solutes if they were adsorbed on the carriers. Further improvement of monitoring by in situ sampling techniques can be achieved by applying more selective detectors to the chromatographic system, e.g. mass selective detectors for HPLC. With a higher selectivity even screening can be applied to wastewater. Structural elucidation of metabolites of biodegradation and

Table 2
Retention characteristics of the tested ultrafiltration and microfiltration membrane

Compound/M_r	Retention (%±SD_{abs}) on membrane type	
	GK UF ($n=49$)[a]	PP MF
		–
p-Base/M_r 201	–	–
$TAHNDS_{DP1}$/M_r 347	58±4	–
$TAHNDS_{DP2}$/M_r 348	83±1	–
RB5-H/M_r 744	68±8	–

[a] MWCO (on PEG) = 3500.
– = no retention.

oxygen initiated consecutive reactions should be the objective of further investigations in order to understand degradation mechanisms and improve biological processes in wastewater treatment.

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