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Extraction of extracellular polymeric substances (EPS) from anaerobic granular sludges: comparison of chemical and physical extraction protocols

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Abstract The characteristics of the extracellular polymeric substances (EPS) extracted with nine different extraction protocols from four different types of anaerobic granular sludge were studied. The efficiency of four physical (sonication, heating, cationic exchange resin (CER), and CER associated with sonication) and four chemical (ethylenediaminetetraacetic acid, ethanol, formaldehyde combined with heating, or NaOH) EPS extraction methods was compared to a control extraction protocols (i.e., centrifugation). The nucleic acid content and the protein/polysaccharide ratio of the EPS extracted show that the extraction does not induce abnormal cellular lysis. Chemical extraction protocols give the highest EPS extraction yields (calculated by the mass ratio between sludges and EPS dry weight (DW)). Infrared analyses as well as an extraction yield over 100% or organic carbon content over 1 gg^{-1} of DW revealed, nevertheless, a carry-over of the chemical extractants into the EPS extracts. The EPS of the anaerobic granular sludges investigated are predominantly composed of humic-like substances, proteins, and polysaccharides. The EPS content in each biochemical compound varies depending on the sludge type and extraction technique used. Some extraction techniques lead to a slightly preferential extraction of some EPS compounds, e.g., CER gives a higher protein yield.

Keywords Anaerobic granular sludge · Extracellular polymeric substances (EPS) · Extraction · Biochemical composition

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Introduction

The use of biotechnology to remediate pollution of the environment receives increased attention over the past years. Thus, anaerobic granular sludge has been widely used for over two decades to treat industrial or domestic high strength wastewater (Kassam et al. 2003). This process is based on the degradation of organic pollutants by bacteria agglomerated within granular biofilms. Granules are dense bacterial consortia which enable high biomass retention and withstand shock loadings (Fang 2000). In addition, the high bacterial density of granules allows the treatment of large volumes of wastewater in compact bioreactors.

Extracellular polymeric substances (EPS) are an important part of the biomass (Frølund et al. 1996). These biopolymers are located outside the cells and form a matrix in which microorganisms are immobilized (Liu and Fang

2003). EPS may have a bacterial origin such as bacterial secretions or lysis products, or they are molecules from the wastewater adsorbed on bacterial cells (Wingender et al. 1999). EPS have a crucial role within bacterial consortia and play a key role in the granulation process (Schmidt and Ahring 1996) and the microstructure of the granule (Quarmby and Forster 1995). They also have a protective role in the cell matrix against the adverse influence of the environment (Brown and Lester 1982).

EPS are composed of various organic compounds (Frølund et al. 1996). Their composition depends on the nature of the aggregates. In pure microbial cultures or biofilms, polysaccharides are predominant (Zhang et al. 1998; Sims et al. 2000). In contrast, studies of EPS extracted from activated sludges show a majority of proteins (McSwain et al. 2005; Ge et al. 2007; Park and Novak 2007). In smaller quantities, humic-like substances (Frølund et al. 1995), uronic acids (Tsuneda et al. 2003), and nucleic acids (Zhang et al. 1999) are also EPS constituents. Nucleic acids are released after cell lysis. Large quantities of nucleic acid materials are released when harsh extraction conditions induce cell destruction. Thus, the EPS nucleic acid content is a good marker to estimate the quality of the EPS extraction protocol.

A lot of procedures are available in the literature to extract EPS to study their properties in biofilms. The amount and composition of EPS strictly depend on the extraction method used (Wingender et al. 1999). Physical or chemical techniques can be used, but there is no standard procedure. Centrifugation is commonly considered as a comparative method because it is the less degradative technique and there is no addition of chemical compounds (Comte et al. 2006a). Ultrasonication (Dignac et al. 1998), heating (Zhang et al. 1999), and cation exchange resins (CER; Frølund et al. 1996) are the main physical techniques used. EPS can also be chemically extracted with the addition of ethylenediaminetetraacetic acid (EDTA) (Liu and Fang 2002), alkaline (Frølund et al. 1996), and aldehydic (Fang and Jia 1996) reagents.

The goal of this study is to compare the efficiency of nine EPS extraction protocols from anaerobic granular sludges and obtained EPS composition. The granules were chosen because of the lack of data available on the EPS of this type of biomass in the literature. Four anaerobic granular sludges, sampled in different wastewater treatment plants treating industrial effluent, were selected. Centrifugation was chosen as the "control method". Sonication, CER alone, or CER combined with sonication and heating were chosen as physical extraction techniques. EDTA, ethanol, and formaldehyde treatment associated with heating or sodium hydroxide addition were used as extractants in the chemical extraction protocols. Extraction yield, as well as total organic carbon (TOC), protein, polysaccharide, humic-like substances, uronic acids, nucleic acids, and lipid contents, were determined. An infrared (IR) investigation was also performed to highlight a potential modification of the EPS solution by the extraction procedure.

Materials and methods

Source of biomass

Table 1 summarizes the main characteristics of sludges used in this study and gives references for additional data on the sludges and the bioreactors where they are sampled from. Four different anaerobic granular sludges were selected: three types with a granular shape (Eerbeek, Emmtec and Nedalco) and one more flocculant sludge type containing very small granules (Revico). Eerbeek and Emmtec samples were collected from UASB (Upflow Anaerobic Sludge Blanket) reactors that treat, respectively, paper-mill wastewater and sulfate/ethanol rich wastewater. Nedalco sludge was sampled from an expanded granular sludge bed (EGSB) reactor of a distillery wastewater treatment plant. A fourth granular sludge sample with small granules (diameter <0.5 mm), called Revico, was used to treat, with biogas production, vinasses of Brandy in an anaerobic digester with recirculation. All sludges were stored at 4°C until analysis. The sludges were harvested anaerobically and stored at 4°C until EPS extraction, which were performed within 1 month after sampling. A storage tank with a water seal was used to prevent incoming air.

Composition of the granular sludges

The dry weight (DW) content of the granular sludge was determined by drying a sample at 105°C for 24 h. Then, the volatile dry weight (VDW) content of the granular sludge corresponds to the loss of mass after 2 h at 550°C. The particle size distribution was determined with a laser diffraction particle size analyzer (Beckman Coulter Inc., LS 230, Miami, FL, USA).

Analysis of the mineral composition of sludges was carried out first by a rinsing step with two volumes of deionized water added to one volume of sludge. Then, samples were centrifuged (500 rpm) for 5 min (Jouan KR 22i), and the supernatant was removed. These operations were carried out twice. To pre-oxidize sludge organic matter, 2 mL of hydrogen peroxide (30% Prolabo) was added to about 0.3 g of sludge for 48 h. To digest organic matter, 1 mL of nitric acid (69% Prolabo) and 3 mL of hydrochloric acid (37% Prolabo) were added to the samples for 24 h. Finally, the pretreated sludge was digested in a multiwave 3000 (Anton Paar) according to an adequate 45-min-long microwave program (a 5-min-long ramp to reach

Table 1 Main characteristics of the four anaerobic granular sludge types used in this study

Sludge type	Eerbeek	Emmtec	Nedalco	Revico
Reactor type	UASB	UASB	EGSB	Anaerobic digester
Wastewater type	Paper mill (carbohydrates (mainly starch), acetate, propionate, butyrate, formate and sulfate)	SO_4^{2-}/ethanol	Distillery alcohol distillery wastewater (mainly ethanol)	Brandy vinasse
Metabolic status	Methanogenic and sulfate reducing conditions	Methanogenic and sulfate reducing conditions	Methanogenic conditions	Methanogenic conditions
Sludges retention time	180–200 days	>150 days	>100 days	>400 days
Color of granule	Black	Black	Black	Brown
General characteristics				
pH	7.6±0.1	7.0±0.1	7.8±0.1	7.7±0.1
Organic fraction (% DW)	69.8±0.1	77.9±0.3	87.8±0.2	52.2±0.4
Mineral fraction (% DW)	30.2±0.1	22.1±0.3	12.2±0.2	47.8±0.4
Size range (mm)	0.2–1.6	0.1–1.0	0.6–1.6	<0.5
Mineral element content (μg g^{-1}DW)				
Ca	9,574±89	52,428±6,645	14,840±2,768	124,192±4,779
Mg	1,353±304	1,310±143	530±4	4,949±190
Na	4,641±307	1,327±317	1,896±174	2,737±333
K	1,190±421	2,798±432	2,200±53	18,341±226
P	6,571±1,260	7,767±1,505	5,356±442	34,379±872
S	20,000±1,200	16,110±3,092	12,000±2,000	6,148±749
Si	3,514±3,415	3,456±233	32,831±42,995	2,832±194
Al	3,813±993	7,306±1,046	1,358±228	1,278±98
Mn	214±48	269±58	52±18	355±2
Fe	28,039±10,517	15,078±3,083	18,871±104	2,993±131
Pb	105±56	9±1	91±109	50±2
Zn	5,884±1,662	141±22	503±107	262±9
Ni	2,264±437	44±2	137±39	18±8
Cu	146±28	105±12	863±77	1,962±59
Reference for additional information	Janssen et al. 2009	Roest et al. 2005	Fermoso et al. 2008	Gouzenes 2006

a power of 1,400 W, then a step of 20 min at 1,400 W, and 20 min to cool the sample). Samples were added up to 50 mL with deionized water. Digested samples were sent to ACME Analytical Laboratories ltd. (Vancouver, Canada) to determine their elemental composition using inductively coupled plasma optical emission spectrometer and inductively coupled plasma mass spectrometer. Two replicates were performed for each sample. The sludges were stored at 4°C until physicochemical characterization and EPS extraction.

According to their origin (wastewater treated and bioreactor configuration), the four sludges had a different composition. The pH values of sludge samples are close to neutrality (between 7.0 and 7.8). Sludges are mainly organic; the organic fraction represents 52% (Revico) to 88% (Nedalco) of the total suspended solids in the sludge samples. The amount of inorganic compounds varies depending on the sludge origin. Nevertheless, some similar trends are noticed in Table 1: The main inorganic compounds are calcium which represents 10 to 124 mg g^{-1} DW and iron which represents 3 to 28 mg g^{-1} DW.

EPS extraction

In order to study the effects of the extraction method on the EPS characteristics, nine extraction procedures described in the literature have been selected. Prior to extraction, 150-mL granular sludge was sampled from the stock and washed twice with deionized water.

Five physical extraction protocols were investigated:

- Centrifugation (Liu and Fang 2002): sludge sample centrifuged (20,000×*g*) for 20 min at 4°C (Jouan KR 22i). It is considered as the "control method" because it is the less degradative technique and there is a common step of centrifugation in all other protocols investigated.
- Sonication (Dignac et al. 1998): Sludge samples were sonicated in 50-mL fractions at 37 W for 1 min using a Bandelin Sonopuls GM 70 with a M 73 probe.
- Sonication and CER (Dignac et al. 1998; Liu and Fang 2002): The sludge sample was sonicated as previously described (50-mL fraction, 37 W, 1 min). CER (Dowex 20–50 mesh, Na^+ form, Sigma-Aldrich) was then added (70 gCER g^{-1} sludge VSS), and the mixture was stirred 1 h at 600 rpm with an orbital shaker.
- CER (Liu and Fang 2002): The Dowex resin was mixed with sludge and stirred 1 h at 600 rpm.
- Heating (Zhang et al. 1999): The sludge was heated for 10 min at 80°C.

Four chemical extraction protocols were also tested with as main extractants:

- Formaldehyde and heating (Fang and Jia 1996): 0.06 mL of 36.5% formaldehyde (Prolabo) was added (1 h, 4°C). The mixture was then heated for 10 min at 80°C.
- Formaldehyde and sodium hydroxide (Liu and Fang 2002): Formaldehyde was added as described previously. After 1 h at 4°C, 60 mL of 1 N NaOH (Prolabo) was introduced for 3 h at 4°C.
- Ethanol (Rätto et al. 2006): The sludge sample was first centrifuged (20,000×*g*, 20 min, 4°C). Three volumes of ice-cold ethanol (96%, Elvetec, France) were added to the supernatant. The mixture was incubated overnight at 4°C. The precipitate was collected after a 4,000×*g* centrifugation (15 min).
- EDTA (Liu and Fang 2002): 150 mL of 2% EDTA (99%, Prolabo) was added to sludge for 3 h at 4°C.

Except for the ethanol extraction, the treated samples were centrifuged (20,000×*g*, 20 min, 4°C), and the supernatants were collected as EPS extracts which were stored at −18°C. EPS extractions were performed twice and results displayed are given as "mean value±deviation to the mean value".

EPS characterization

In order to characterize the EPS samples, different parameters were studied. The DW and the VDW content of EPS samples were determined as previously described for the granular sludges. A Phoenix 8000 TOC-meter (Dohrmann) was used to measure the TOC content of the EPS solutions.

The biochemical composition of the EPS extracts was also investigated using colorimetric methods performed with a Spectral Photometer Cadas 50 S, Dr Lange. The main characteristics and references of methods used were summarized in Table 2.

The humic acid content and the protein content of the extracted EPS were determined and corrected using the Frølund et al. (1995) protocols. The uronic acid and polysaccharide contents were corrected using the Blumenkrantz and Asboe Hansen (1973) protocol because of interferences of, respectively, uronic acids and polysaccharides during their measurement. The EPS characteristics are the average values of three measurements of each extracted EPS sample.

Table 2 Main characteristics of the colorimetric methods used for EPS biochemical characterization

EPS content	Calibration curve concentration (mg L^{-1})	Wave length (nm)	Reagent used	Standard	Reference
Proteins	0–200	650	Folin reagent Copper sulfate 0.5% (*w*/*w*)	Bovine albumin serum (96%, Sigma)	Lowry et al. 1951; Frølund et al. 1995
Humic acids like	0–200	650	Folin reagent	Humic acids (Aldrich)	Frølund et al. 1995
Polysaccharides	0–100	492	Phenol 5% (*w*/*w*) Sulfuric acid 95%	Glucose (Rectapur, Prolabo)	Dubois et al. 1956
Uronic acids	0–250	520	Sodium tetraborate 12.5 mM Sulfuric acid 95%	Glucuronic acid (99%, Aldrich)	Blumenkrantz and Asboe Hansen 1973
Nucleic acids	0–50	600	Diphenylamine 0.6% (*w*/*w*) Sulfuric acid 95%	Calf thymus DNA (10 mg mL^{-1}, Aldrich)	Burton 1956
Lipids	0–1,000	540	Vanillin 0.6% (*w*/*w*) Phosphoric acid 85% Sulfuric acid 95%	Commercial olive oil	Frings and Dunn 1970

Infrared spectrometry

EPS extracts were freeze-dried. A pellet of EPS (about 1 mg) and KBr (about 180 mg) was compressed and analyzed using a spectrum 1000 IR spectrometer (Perkin-Elmer).

Results

IR analysis of EPS extracts

The EPS extracted by the control method from the four anaerobic granular sludges investigated (Fig. 1) show similar IR spectra of a qualitative point of view. The same main characteristic bands can be observed with different ratios. Nevertheless, from a semiquantitative point of view, we can notice that the transmittance ratio between characteristic bands is different from one EPS to another one. The large band between 3,200 and 3,400 cm^{-1} is assigned to the stretching vibration of OH into polymeric compounds (Nakanishi and Solomon 1977). The visible weak peak at 2,930 cm^{-1} (asymmetric stretching vibration of CH_2) is related to aliphatic chains of proteins, carbohydrates, lipid, and humic acids (Nakanishi and Solomon 1977). Some regions of the IR spectra can be correlated to biochemical compounds. For instance, proteins are identified by the two bands at 1,640–1,650 cm^{-1} (amide I: stretching vibration of C = O and C = N) and 1,540–1,550 cm^{-1} (amide II: stretching vibration of C–N and deformation vibration of N–H). The bands set at 1,130 cm^{-1} (stretching vibration of C–O–C) and 1,040–1,080 cm^{-1} (stretching vibration of OH) are assigned to polysaccharide structures (Nakanishi and Solomon 1977). Humic-like substances are hardly identifiable because of confused characteristic peaks. Indeed, Chai et al. (2007) considered that humic acids adsorbed intensively at 2,930 and 1,650 cm^{-1} (stretching vibration of C = C). Those bands can also be related to other organic compounds (Nakanishi and Solomon 1977), which implies difficulties to identify them. The IR spectra contain also other peaks with negligible intensity that can be assigned to several organic compounds and thus cannot be assigned to the adsorption bands of specific substances.

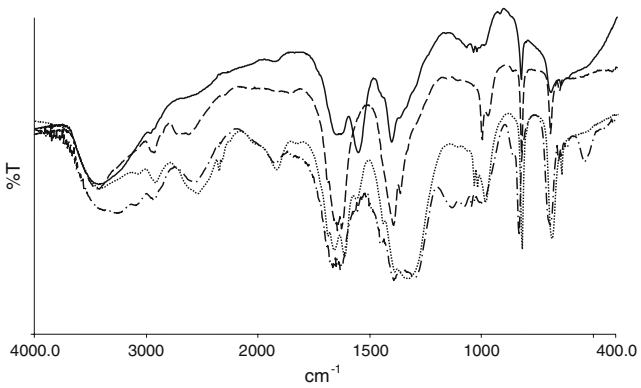

Fig. 1 IR spectra of EPS samples extracted from the four sludges with centrifugation (*solid line* Eerbeek, *dotted line* Emmtec, *dashed–dotted line* Nedalco; *dashed line* Revico)

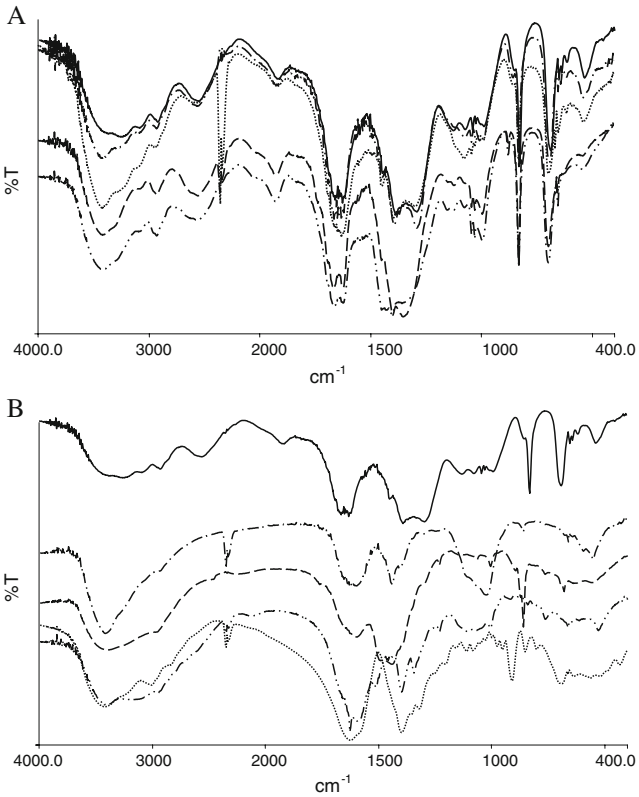

Fig. 2 IR spectra of EPS samples extracted from Nedalco sludge with physical (**a**) and chemical (**b**) extraction techniques In **a**: *solid line* centrifugation, *dashed–dotted line* sonication, *dashed line* CER and sonication, *dashed–double-dotted line* CER, *dotted line* heat. In **b**: *solid line* centrifugation, *dotted line* EDTA, *dashed–dotted line* ethanol, *dashed–double-dotted line* formaldehyde and heat, *dashed line* formaldehyde and NaOH

Figure 2 presents IR spectra of EPS samples extracted by physically (a) and chemically (b) protocols from Nedalco sludge. The same results were recorded for the EPS extracted from the three other anaerobic granular sludges (data not shown). The EPS extracted with physical techniques (Fig. 2a) display very similar IR spectra, and no noticeable differences appear between the IR spectra of the EPS extracted with physical or control techniques. These similarities between IR spectra from EPS extracted with physical methods are in accordance with results of Comte et al. (2006a) obtained with EPS from activated sludges. In contrast, IR spectra of the chemically extracted EPS display different adsorption bands compared to spectra of the control method (Fig. 2b). For the EDTA extraction, the spectra of the EPS of the four sludges investigated are almost similar. Two characteristic bands can be noticed: a

Table 3 Extraction yield for each extraction protocol expressed in % (DW EPS DW^{-1} sludge)

	Extraction yield expressed in % (DW EPSDW^{-1} sludge)			
	Eerbeek	Emmtec	Nedalco	Revico
Centrifugation	17.3±0.2	13.7±0.5	5.3±0.4	5.2±0.4
Sonication	16.4±1.4	ND	5.2±0.2	6.7±0.1
CER + sonication	42.0±0.0	ND	13.4±0.1	14.3±0.1
CER	39.9±1.5	27.4±1.5	12.9±0.6	13.9±0.5
Heating	17.9±0.3	14.8±1.0	6.0±0.2	6.4±0.3
Formaldehyde+heating	27.4±1.1	17.1±0.1	16.1±0.3	6.5±0.1
Formaldehyde + NaOH	112.8±2.3	ND	106.5±0.5	27.0±0.1
Ethanol	19.2±2.2	ND	5.2±0.6	4.0±0.1
EDTA	101.8±5.4	ND	70.1±0.6	21.9±0.0

ND not determined

peak at 1,600–1,550 cm^{-1} related to carboxyl functions and a peak near 1,350 cm^{-1} related to the vibration of C–N. For the ethanol extraction, an intense band around 1,030 cm^{-1} can be noticed which matches to the primary alcohol vibration. For the two formaldehyde extractions, several bands, which were not present on the IR spectrum of the control sample, appeared around 1,500 cm^{-1} (Nakanishi and Solomon 1977).

Extraction yield and TOC content

Table 3 summarizes the extraction yields calculated from the dry weight of the granules used and the dry weight of EPS obtained from the four sludges investigated. These EPS extraction yields differ according to the type of sludge: for the majority of the methods investigated, Eerbeek and Emmtec sludges (except formaldehyde + heating) have nearly the same values as well as the Nedalco and Revico sludges (except for EDTA and formaldehyde + NaOH). Furthermore, the extraction yields differ according to the extraction protocol used, with the same trends for the four sludges: "control" ≈ sonication ≈ ethanol ≤ heating < CER ≤ CER + sonication < formaldehyde + heating ≪ EDTA <<< formaldehyde + NaOH.

Chemical extraction yields are generally higher than the physical extraction ones (Table 3). The combination of formaldehyde and sodium hydroxide gives the highest extraction yields. The combined effects of formaldehyde and NaOH allow an effective extraction. But for EPS from Eerbeek and Nedalco sludges, these extraction yields exceed 100% of DW EPS per DW sludge (Table 3). The EPS extraction yield from Eerbeek sludge with EDTA is also higher than 100%. For the formaldehyde associated with the heating protocol, extraction yields are higher than the ones obtained for the control technique. The ethanol extraction yields are close to those of the control extraction.

Table 4 shows the organic carbon content of each EPS extract. As shown by the extraction yields, two groups appear: Eerbeek and Emmtec with a higher organic carbon content compared to Nedalo and Revico sludges with a low organic carbon content. The differences appear according to the extraction method used. For the four sludges, the

Table 4 Organic carbon content (milligrams of C per gram of DW or milligrams of C per gram of VDW) of the EPS extracted from four different anaerobic granular sludges

	Eerbeek		Emmtec		Nedalco		Revico	
$mgC\,g^{-1}$	DW	VDW	DW	VDW	DW	VDW	DW	VDW
Centrifugation	292±2	493±10	188±5	440±79	52±15	169±8	21±2	80±18
Sonication	303±9	499±4	ND	ND	60±11	133±42	18±1	68±7
CER + sonication	156±2	325±4	ND	ND	51±3	124±20	31±0	135±6
CER	160±3	335±14	103±6	242±6	50±1	144±11	28±2	117±29
Heating	340±15	535±25	198±8	366±5	184±2	343±19	33±2	99±8
Formaldehyde + heating	435±2	606±17	503±4	913±14	463±10	672±150	165±17	557±27
Formaldehyde + NaOH	150±6	331±4	ND	ND	78±9	332±99	54±1	220±15
Ethanol	15,342±975	21,817±5,987	ND	ND	54,422±14,546	68,101±16,482	5,862±1,526	12,061±3,240
EDTA	309±6	557±7	ND	ND	319±11	501±15	221±7	517±19

ND not determined

Table 5 Nucleic acid content (milligrams per gram sludge VSS) and ratio protein content/polysaccharide content of EPS extracts of the four sludge investigated

	Eerbeek		Emmtec		Nedalco		Revico	
	Prot./Polys.	Nucleic acids content (mgg^{-1} sludge VSS)	Prot./Polys.	Nucleic acids content (mgg^{-1} sludge VSS)	Prot./Polys.	Nucleic acids content (mgg^{-1} sludge VSS)	Prot./Polys.	Nucleic acids content (mgg^{-1} sludge VSS)
Centrifugation	1.0±0.1	0.096±0.004	0.2±0.1	0.261±0.013	2.4±0.2	0.065±0.025	3.3±2.7	0.003±0.001
Sonication	1.3±0.1	0.094±0.041	ND	ND	2.9±0.7	0.050±0.003	1.9±0.9	0.004±0.001
CER + sonication	1.7±0.2	0.194±0.019	ND	ND	14.1±2.3	0.055±0.001	4.2±0.2	0.018±0.006
CER	1.3±0.4	0.160±0.007	0.7±0.2	0.065±0.007	13.2±2.5	0.032±0.007	4.6±0.3	0.014±0.001
Heating	2.2±0.9	0.124±0.013	0.6±0.2	0.339±0.030	4.7±1.1	0.079±0.001	3.5±0.1	0.006±0.002
Formaldehyde + heating	1.5±0.1	0.722±0.033	0.4±0.1	0.552±0.195	5.7±0.6	0.184±0.006	1.3±0.5	0.148±0.017
Formaldehyde + NaOH	4.2±0.2	0.097±0.006	ND	ND	7.7±1.0	0.107±0.010	6.1±0.1	0.008±0.002
Ethanol	1.5±0.7	0.169±0.001	ND	ND	2.2±0.2	0.084±0.003	2.6±0.2	0.005±0.002
EDTA	0.4±0.2	0.151±0.006	ND	ND	4.2±1.7	0.092±0.003	2.5±0.4	0.007±0.002

ND not determined, *Prot* proteins, *Polys.* polysaccharides

control and sonication methods give quite similar results. For Eerbeek, Emmtec, and Nedalco, the heating techniques induce the highest organic carbon levels for the physical extraction methods. The lowest organic carbon values are obtained for CER extractions, but these techniques give the highest extraction yields (Table 4).

The amounts of organic carbon for chemically extracted EPS samples are higher than those obtained with the physical extraction protocols. The combination of formaldehyde and heating leads to the highest amount of organic carbon (Table 4). For EPS samples extracted with ethanol, the results exceed 1,000 mg Cg^{-1} DW or VDW which demonstrates, as already shown by IR spectrometry, the carry-over of ethanol into the EPS extract solution. The lowest values of the organic carbon content are obtained with the formaldehyde + NaOH extraction protocol.

Biochemical characterization

Table 5 evaluates the qualitative aspect of the extraction protocols and displays the nucleic acid content and the protein–polysaccharide ratio. These two parameters are often used to estimate the importance of cell lysis during the extraction protocol (McSwain et al. 2005).

Figure 3 presents the main biochemical constituents of the EPS extracts for the four sludges investigated. These EPS are mainly composed of proteins, polysaccharides, and humic-like substances. Uronic acids and nucleic acids are present, but at a lower concentration (Fig. 3). All lipid constituents determined in the EPS samples are below the quantification limit of the colorimetric technique (i.e., 8 mg g^{-1}DW). The content of the biochemical compounds varied in a wide range between the sludge types investigated, but the trends are quite similar whatever the extraction method used. The main constituents of the EPS are humic-like substances for Eerbeek, Emmtec, and Revico anaerobic granular sludges and proteins for Nedalco anaerobic granular sludges. The proportion of each biochemical compound of the EPS varies with the type of extraction protocol used.

The combination of formaldehyde and heating leads to the highest EPS quantity extracted. Humic-like substances are mostly extracted. Nevertheless, the amounts of nucleic acids can be 30 times as high as the control method (Revico control 0.7 mg g^{-1}DW; formaldehyde + heating 29.4 mg g^{-1}DW). EDTA extractions give the lowest EPS content. Ethanol is often used to extract polysaccharides. Results show the highest amounts of polysaccharides, but high concentrations of other compounds are also extracted. For instance, for Nedalco sludge, the extraction with ethanol gives the highest polysaccharide content (57 mg g^{-1}DW), and the protein content is twice higher (125 mg g^{-1}DW). For formaldehyde + NaOH, the EPS contents are close to those obtained with the control method. The main difference is the highest amount of proteins and thus the highest protein to polysaccharide ratio.

For physical techniques, the highest EPS contents are extracted by the heating protocol. As observed for formaldehyde combined to heating, humic-like substances are mostly extracted. Compared to the control method, the CER extractions give a higher protein content and a lower humic-like substance content. Uronic acids and polysaccharides have almost similar concentrations. The protein to polysaccharide ratio can be five times as high as the control

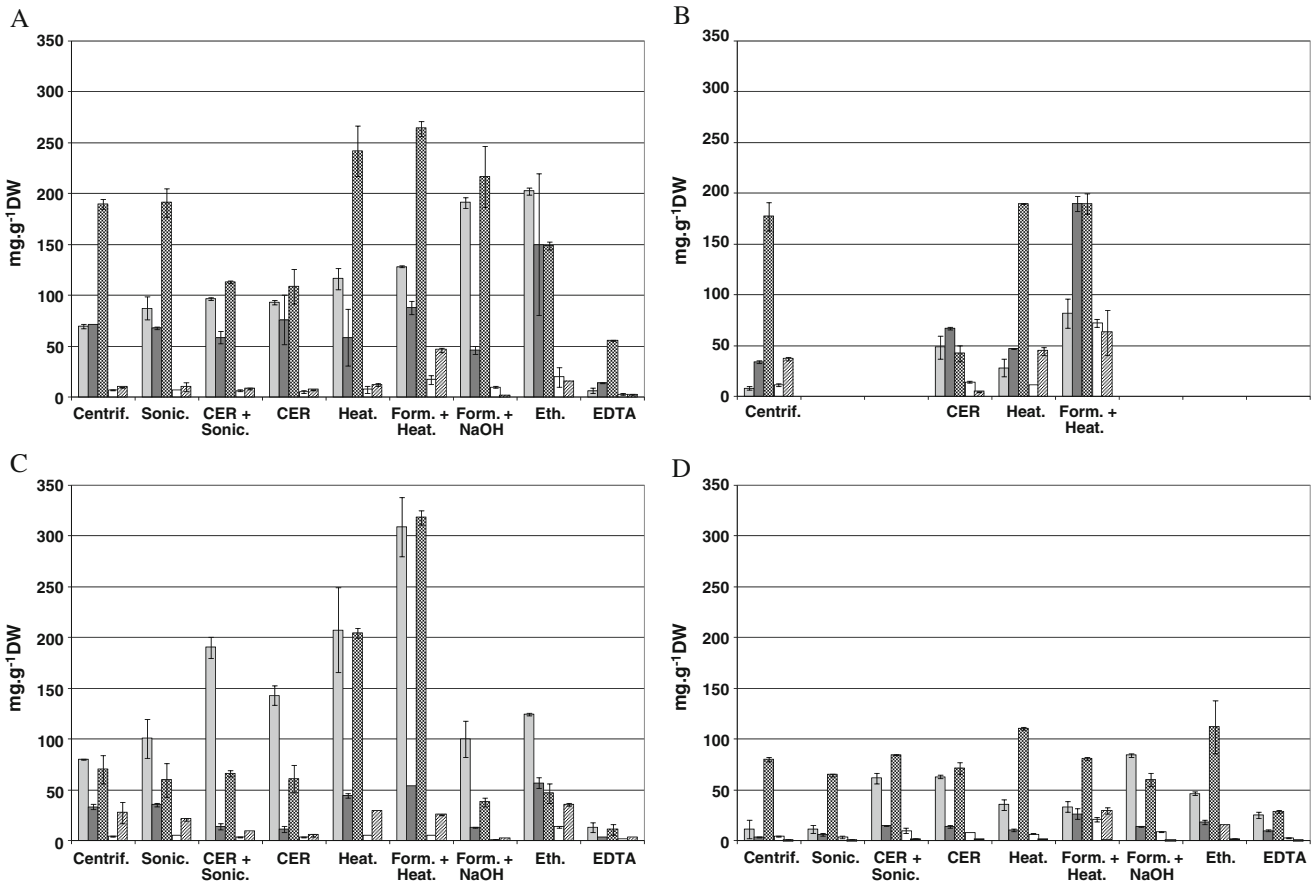

Fig. 3 Biochemical composition of EPS extracts for each sludge type (**a** Eerbeek, **b** Emmtec, **c** Nedalco, **d** Revico). ☐ proteins, ■ polysaccharides, ▨ humic-like substances, ☐ uronic acids, ▨ nucleic acids

protocol (Nedalco control, 2.4; CER, 13.2), but the nucleic acid contents are lower. The biochemical composition of the EPS of samples extracted by centrifugation and sonication does not show significant differences.

Discussion

Organic composition of EPS extracts of anaerobic granular sludges

This study showed that the major EPS constituents are proteins for the Nedalco anaerobic granular sludges and humic like substances for the Eerbeek, Emmtec, and Nedalco sludges. These differences mainly depend on the nature of the wastewater characteristics these granules treat, as shown also for activated sludges by Sponza (2003).

The highest amounts of EPS are found with the chemical extraction protocols. Nevertheless, EDTA extracts yield the lowest quantities of EPS. EDTA can form complexes with EPS (Liu and Fang 2002). These complexes can interfere in the colorimetric analysis. Moreover, Brown and Lester (1982) showed that the protein content by colorimetric determination gave wrong results in the presence of EDTA. The protocol (Frølund et al. 1995) links the content of proteins and humic-like substances. Thus, the amount of proteins and humic-like substances cannot be taken into account for the EDTA extractions. Ras et al. (2008) also have demonstrated that the colorimetric method used to determine the protein content can either overestimate or underestimate the protein concentrations in EPS samples. Thus, three assumptions may be hypothesized: (a) the measured protein content is the real one and differences between the samples really exist, (b) the chemicals used interfere with the protein determination and do not allow to know the real protein content, and (c) the colorimetric method badly estimates the protein content and the protein contents can be determined only by protocols that add no chemicals during the extraction. Moreover, the high organic content, the abnormal extraction yields exceeding 100%, and the small amounts of biochemical compounds confirm a bias in the samples by the chelating agent EDTA.

Ethanol is commonly used to extract specifically polysaccharides. Indeed, polysaccharides are the main constituents, but the contents of proteins and humic-like substances are also significant. The addition of ethanol

takes place after the centrifugation. So only a part of the extracted EPS is trapped by ethanol. The amounts of the extracted organic compounds depend on their solubility in ethanol. Thus, ethanol extraction favors polysaccharide extraction instead of a polysaccharide-specific extraction.

The most important quantities of EPS are extracted with formaldehyde and heating. The disruption of the granule by the heating process facilitates their release. Moreover, this method may allow to extract bound EPS (Comte et al. 2006a). For this extraction method, the nucleic acid content was determined (Fig. 3; Table 5). This can indicate the presence of an abnormal cell lyses during the EPS extraction procedure. Nevertheless, the protein–polysaccharide ratio varied between 0.4 (EPS from Emmtec sludge) and 5.7 (EPS from Nedalco sludge). The values obtained in this work for both the EPS acid nucleic content and the protein/polysaccharide ratio (Table 5) are in accordance with the ones found for dense aerobic granule (Adav and Lee 2008). Indeed, EPS extracted from aerobic dense granules display a protein/polysaccharide ratio in the range of 2.4–6.2 and a nucleic acid content varying in the range of 0.09–0.57 mg g^{-1} of sludge VSS without a significant cell lysis during extraction (Adav and Lee 2008). The apparent high value of the nucleic acid content obtained for the formaldehyde and heating method is in part due to the efficiency of this method to extract organic compounds composing the EPS (Fig. 3) but probably also to a better extraction of the nucleic acid fraction of the EPS. Concerning the other method investigated in this work, the acid nucleic content is lower than the ones obtained with the method using formaldehyde and heating. In all cases, except for extraction using CER, the protein/polysaccharide ratio (Table 5) varied between 0.2 and 7.7. Regarding the nucleic acid content and the protein/polysaccharide ratio, compared to the values obtained on dense aerobic granules (Adav and Lee 2008), the extraction methods selected in this work and applied on anaerobic dense granules do not involve an abnormal cell lysis during the extraction protocols.

The protein extraction fraction of EPS (Fig. 3) is enhanced by the NaOH added combined to formaldehyde. The destabilization of the granule by an alkaline pH may allow the extraction of proteins, such as exoenzymes, which surrounded the cells (Dignac et al. 1998).

Samples extracted by centrifugation and sonication, on the one hand, and CER alone and CER coupled with sonication, on the other hand, display very close compositions. The results of the heating extraction protocol lead to the same conclusion as for the formaldehyde plus heating protocol: a high EPS amount extracted with mainly humic-like substances. The organic carbon content of the EPS (Table 4) of samples extracted with CER is the lowest, except for the Revico sludge which has the highest content of physically extracted EPS extracts. This less dense sludge with the smallest granules (<0.5 mm of diameter) may facilitate the EPS extraction with CER because of stirring. Proteins seem preferentially extracted (Fig. 3; Table 5) by CER, as the resin traps bivalent cations (Ca^{2+} and Mg^{2+}) which are engaged in the linking in the EPS matrix. Dignac et al. (1998) have demonstrated that proteins have more affinity with multivalent cations than polysaccharides in the matrix. Thus, the use of CER to extract EPS facilitates the protein extraction. Moreover, the destabilization of the granule by the resin and the mechanical shear forces applied during the stirring allow the release of the bound EPS. Adav and Lee (2008) have shown that a large fraction of polysaccharides is located in the outer layers of the granules, unlike proteins that are uniformly spread throughout the granule. Thus, when the granular sludge is stirred, the structural disorganization of granules releases more proteins.

Efficiencies of extraction

The efficiency of the extractions is mostly related to the extraction yield (Table 3), but we must also consider the nucleic acid content and the protein/polysaccharide ratio to ensure that an abnormal cell lysis does not occur during the extraction procedure (Table 5). As already demonstrated for dense aerobic granules (Adav and Lee 2008), the nucleic acid content and the proteins/polysaccharide ratio of the EPS extracted from dense anaerobic granule (Table 5) do not display the values that underline an abnormal cell lysis during extraction.

Except for ethanol, the highest extraction yields are obtained with the chemical extraction procedures. The association of formaldehyde with NaOH, which leads to the highest extraction yields, seems to be the most efficient extraction procedure. Formaldehyde itself binds to the cell by reacting with functional groups like carbonyl, amino, or hydroxyl groups (Sutherland et al. 2008). Once fixed to the membrane, it acts as a buffer that prevents cell lysis. The low nucleic acid content confirms this assumption. Furthermore, the addition of NaOH destabilizes the granular structure, the higher pH values promoting the repulsion of negatively charged EPS which facilitates their release from the granule. But the contamination of samples by the chemicals used, as demonstrated by IR spectra (Fig. 2b) and extraction yields over 100% (Table 3), shows that this method cannot be employed to extract EPS in order to study their biochemical properties. The same conclusion can be drawn for extraction protocols based on EDTA and ethanol. For formaldehyde combined with a heating step, extraction yields are higher than the heating extraction alone. Addition of formaldehyde, which is considered to protect the cell membrane during extraction (Sutherland et al. 2008), seems also to increase the EPS extraction yield. Formaldehyde can react with amino groups of proteins or

aminopolysaccharides (Comte et al. 2006a) to alkylate cell wall molecules or EPS and thus destabilizes the structure of granules and increases the release of EPS. But residual formaldehyde can remain in the EPS solution, or the molecules present in the EPS solution can be modified by reaction with the formaldehyde on their amino group which can alter their properties.

The best extraction yields for physical extractions are performed with CER. In contrast to the other physical extractions, CER methods combine two types of mechanisms: the shear force on granules applied by the stirring and the destabilization of the granule structure by removing bivalent cations such as Ca^{2+} (Frølund et al. 1996). Then, the weakened granule structure facilitates the EPS extraction without risks for cell lysis. This mix of both mechanisms can explain the higher yields and lower nucleic acid contents of the CER extractions. The heating protocol leads to higher extraction yields compared to the control protocol. The organic carbon contents are also more important. Thus, the heating disrupts the granular matrix to facilitate EPS extraction and allows to extract higher quantities of EPS than the control protocol. The main drawback which could occur is an abnormal cell lysis induced by the release of intracellular material in the EPS solution if exposure of the granule to heat is badly controlled. A denaturation of a part of the EPS molecules caused by the high temperature (80°C) can also be expected.

The important mineral fraction present in the granular sludges (Table 1) and the compact conformation of granules make it more difficult to extract EPS from anaerobic granules than from activated sludge or aerobic biofilms. The ultrasounds are supposed to break bonds with energy provided by the sonication (Dignac et al. 1998). Nevertheless, compared to the use of CER alone, the use of sonication with CER did not improve the EPS extraction yields (Table 3). It also may be due to the high density of the granules.

Chemical extraction protocols are the most efficient to extract EPS in terms of quantity, but carry-over of chemicals from extraction solution toward the EPS solution has to be taken into account. There are a few differences between the extracts obtained by the physical protocols. As there is no addition of chemicals in these physical protocols, these differences are thus directly linked to the chemical composition of the extracted EPS.

Contamination of EPS samples by chemical extractants

No studies deal with the contamination of EPS samples by the chemical extractants, except Comte et al. (2006b) for activated sludge. The latter authors have shown that the carry-over of chemicals from the extraction solution toward EPS extracts from activated sludges by the chemicals can alter the EPS properties. The IR analyses (Fig. 2a) display no differences between the IR spectra of the physical extraction protocols investigated. But characteristic bands appear for the chemical extraction protocols compared to the control methods (Fig. 2b). The contamination of EPS samples by the chelating reagent EDTA during the extraction was also underlined by Liu and Fang (2002) and Comte et al. (2006a). Specific bands are also noticed for ethanol and formaldehyde. Thus, this contamination may be due to excess of reagent which remains in solution or reactions (adsorption, covalent bonds) between EPS molecules and extractant chemicals.

The organic carbon content and extraction yield confirm this. Aberrant values of extraction yields (over 100%) (Table 3) and huge amounts of organic carbon (over 1,000 mg $C\,g^{-1}DW$; Table 4) for chemical extraction confirm this carry-over of chemicals from the extraction solution toward the EPS extracts. This is also confirmed by the IR spectra (Fig. 2b) and the high organic carbon content of the EPS (Table 4).

Possible consequences of extraction technique on EPS properties

This study shows the effects of the extraction techniques on the quantity and quality of the EPS extracted. The contamination of samples by chemicals used to extract EPS is widely demonstrated with various parameters (IR analysis, organic carbon content, and extraction yield). Chemicals used for extraction can also react with EPS molecules and affect their structure, for example, formaldehyde can react with amine groups of proteins or amino sugars from the EPS, thus modifying the structure and properties of the proteins present in the EPS (Comte et al. 2006a). This contamination of the EPS suspension by chemicals or reactions of chemicals with EPS molecules may impact the physical and the chemical properties of the EPS as previously shown by Comte et al. (2006b) on EPS from activated sludges. Some protocols lead to selective extraction of some EPS compounds, such as the CER extraction which yields large amounts of proteins. These differences of composition may also induce a variation of the EPS properties from one sample to another. For instance, proteins have more affinity with multivalent cations than polysaccharides, and EPS samples with high levels of proteins may therefore complex more metallic cations than those with a majority of polysaccharides. In addition, the binding strength of the different EPS macromolecules may be different, leading to an overall metal binding constant that may differ according to the relative importance of the biochemical compounds present in the extracted EPS. Thus, the efficiency of methods has to take into account a lot of factors such as cell lysis, extraction yield, specificity of the extraction, and the carry-over of

chemicals from the extraction solution toward EPS extracts. The mineral fraction of extracted EPS has not to be ignored as well because it may also have effects during exopolymer analysis.

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