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EXTRACTION OF EXTRACELLULAR POLYMERIC SUBSTANCES FROM DAM LAKE FRESH SEDIMENTS DERIVED FROM CRYSTALLINE BEDROCK

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HIGHLIGHTS

- Nine methods and three S/L ratios tested to achieve sediment EPS extraction
- Extracted organic carbon amount and composition depend on the S/L ratio and method
- Physical methods and CER extract mainly EPS, but in small quantities at S/L=1/40
- Chemical extractions give larger EPS amounts, but are not recommended
- Chemical methods lead to contamination by salts, intracellular, and humic materials

ABSTRACT

Extracellular polymeric substances (EPS) produced by microorganisms have a key role in the sedimentary compartment*, e.g.* promoting aggregation and biostabilisation of sediment particles and increasing chemical reactivity at the water/sediment interface. Therefore, proper extraction methods are needed to study this EPS matrix. In this work, nine extraction methods based on physical (centrifugation, sonication), chemical (sodium hydroxide, sodium pyrophosphate, sodium tetraborate), and both chemical and physical (cation exchange resins, i.e. CER) treatments and their combinations, as well as the solid:liquid ratio used for extraction, were compared based on the quantity and compositions of extracted EPS. The organic carbon extracted was quantified and the nature of biochemical macromolecules (proteins, polysaccharides, and humic-like compounds) was evaluated using colorimetric methods. The amount of ATP was used as an indicator of cell lysis and showed contamination with intracellular materials in EPS extracted with chemical methods. Moreover, chemical extraction presented a large quantity of impurities due to non-removal of reactant salts by ultracentrifugation. For the nine methods tested, humic-like substances represented the main fraction of the extracted EPS, but for chemical extraction, the presence of humic materials from the sediment organic fraction was due to non-specific extraction of the EPS fraction. Therefore, chemicals methods are not recommended to extract EPS from sediment. Despite their low extraction efficiency, physical methods and CER, *i.e.* 'soft' extraction methods, are preferred using a solid:liquid ratio 1:40.

Keywords: EPS, sediment, biofilm, biochemical composition, physical and chemical extraction.

1. INTRODUCTION

Sediments of continental aquatic ecosystems are formed by a wide variety of organic materials of autochthonous and allochthonous origin (Callisto and Esteves, 1996). Their composition and distribution vary with the rock type, vegetation, topography, and meteorology of the site where they are formed. Other factors, such as chemical and biological, limnological, and/or hydrological ones, including the effect of water mass transportation, also influence their formation and distribution (Ward, 1992). In these continental environments, dam reservoirs are areas where sediment accumulation is favoured (up to several centimetres per year) (Arnason and Fletcher, 2003). Dams are artificial constructions that interfere with sediment transport due to the change in flow conditions caused by the strong erosion capacity of the transitory flow and the morphological evolution of the channel bed (Cao et al., 2004). These high sediment accumulations represent an important sedimentary record of current sedimentary processes and events and may have an impact on the environmental quality through interactions with the water column and organisms (Pestana et al., 2019).

Sediments are used by microorganisms as a substrate, and microorganisms play an important role in the biogeochemical processes of sediment, such as the biodegradation of organic matter and cycling of nutrients (Fang et al., 2017). These communities of autotrophic and heterotrophic organisms can attach and grow in the sediment through the production of a biofilm where they are embedded in a matrix of extracellular polymeric substances (EPS) (Romaní et al., 2013). In intact sediment samples, Grant et al. (1986) were able to examine the stabilisation of intertidal sands by diatomaceous mucus films by characterisation of the colloids, polysaccharides, and chlorophyll contents and by scanning electron microscopy. This self-produced three-dimensional structure derived from the metabolism of these microorganisms and environmental inorganic constituents (Mori et al., 2018) affects biostabilisation and deposits of sediment (Fang et al., 2015). The presence of EPS plays an important role in interactions between the sediments and other components of the environment (water, dissolved elements, and microorganisms) (Gerbersdorf and Wieprecht, 2015; Shang et al., 2014). This importance is related to the increased chemical reactivity that the presence of this biofilm develops through the biological interface, promoting aggregation/biostabilisation of sediment particles (van de Lageweg et al., 2018) and increasing the chemical reactivity and interaction between the soluble phase and the minerals (Donlan, 2002). This increase in reactivity may be responsible for the change in transport of contaminants such as metals (Comte et al., 2008; Labrenz and Banfield, 2004; Walter, 1997) and organic compounds (Edwards and Kjellerup, 2013). Therefore, understanding the chemical and physical reactivity of the EPS, as well as its composition, is of prime interest.

EPS consists of different classes of macromolecules that are usually measured after extraction, such as polysaccharides, proteins, and minor traces of nucleic acids and lipids (Flemming and Wingender, 2001), and the relative proportion of each fraction depends on the origin of the biofilm considered (Liu and Fang, 2003; Nouha et al., 2018; Sheng et al., 2010). Moreover, for Decho (2011), as EPS can be defined as relatively ‘large’ molecules contained in the extracellular matrix in the vicinity of cells. A large portion of the EPS fraction does not fit into classical chemical categories and cannot be defined only as proteins, carbohydrates, or lipids. EPS extracted from multispecies natural biofilms contain an important fraction of poorly characterised compounds called humic-like substances due to their similar reactivity to humic materials due to their phenol groups. Bourven et al. (2015) also underlined the presence of glycoproteins and sulphated proteoglycan-like in EPS extracted from anaerobic granular sludge. EPS extract from waste water treatment sludges also contains a mineral fraction bound to the EPS organic matter that structures the EPS matrix, as shown by d’Abzac et al. (2010) in sludges.

To perform detailed analyses of the reactivity and composition of these biofilm components, one approach consisting in the extraction of macromolecules from the EPS matrix can be performed to separate it from other components of the sediments. EPS extraction protocols created for soil and biological sludge are highly documented (Comte et al., 2006; D'Abzac et al., 2010; Liu and Fang, 2002; Redmile-Gordon et al., 2014) and have been applied to sediments in a few cases (Takahashi et al., 2009). However, methods applied to extract EPS from sediment have not deeply investigated the effect of the extraction method on the quantity and composition of the extracted EPS (Battin and Sengschmitt, 1999; Gerbersdorf and Wieprecht, 2015; Taylor et al., 2013). Thus, a better assessment of the impact of several extraction methods (physical and/or chemical) on the nature of EPS extracted from continental surface sediments is needed. Indeed, some properties of the EPS extract, for example, the ability to bind metals, depend on its composition (Comte et al., 2006; Guibaud et al., 2003). Therefore, differences in extracted EPS features such as extracted amount of EPS, composition, or contamination caused by the extractant solution could affect the measurement of EPS reactivity (Bourven et al., 2012).

A first parameter to address the quality of the extracted EPS consists of assessing contamination by the extractants themselves, as suggested by Comte et al. (2006). However, as the extractants used in this study were carbon-free, this point will not be discussed in depth here since it will not impact our measurements based on organic carbon content. Another parameter of assessment of the quality of the extraction method is the amount of cell lysis. The cell lysis rate can be estimated, as presented by Redmile-Gordon et al. (2014) for EPS extracted from soils, by comparing the amount of ATP in the EPS extract to the total ATP content of the sample, thus reflecting the amount of altered cellular biomass.

In this study, physical (ultracentrifugation, sonication), chemical (sodium hydroxide, tetraborate, pyrophosphate) and both chemical and physical (cation exchange resin, i.e. CER) extraction methods were performed on three samples of freshwater dam reservoir sediments derived from acidic crystalline bedrock with different contents of organic matter and grain size distributions. Three ratios of sediment mass to extractant were also tested on one sediment to better specify the effect of physicochemical parameters such as the solid:liquid ratio on the composition of EPS extracted from the sediments. The EPS extraction amount was measured by the amount of organic carbon extracted from sediment biofilms, while the effects of the extraction method on the EPS composition were evaluated based on the contents of polysaccharides, proteins, and humic-like substances. The extraction quality regarding the non-extraction of intracellular material was assessed by estimating cellular lysis by measuring the release of ATP in the extract.

2. MATERIAL AND METHODS

2.1. ORIGIN, SAMPLINGAND CHARACTERISTICS OF SEDIMENTS

Samples were collected from the surface sediments of three dam reservoirs. Sampling sites are located in the eastern (Champsanglard – CSG) and southern (Charpal – CPL; Villefort – VF) edges of the Massif Central (central France). The watershed geology consists essentially of Paleozoic crystalline bedrock (> 85% granitic and/or metamorphic) with minor sandstones or quaternary alluvial deposits derived from the acidic bedrock. The main characteristics of the lakes and their catchments are summarised in Table 1.

The CSG superficial sediment was sampled in November 2016 with the use of an Ekman tipper from 0 to 7 cm depth, and the CPL and VF superficial sediments were sampled in November 2017 with a shovel from 0 to 20 cm in depth. The samples were kept underwater at 4°C inside hermetically sealed containers until performing the experiment. The physical and chemical characteristics of the three sediments sampled from dam lakes are presented in Table 1.

The loss on ignition (LOI) was obtained by weighing previously dried sediment (105°C for 24 h) before and after ignition in an oven at 550°C for 2 h. The concentrations of major elements (Si, Al, Fe, K, Mg, and Ca) were measured using an energy dispersive X-ray fluorescence spectrometer (ED-XRF Spectro-XEPOS, Xep05) and were in a similar range between samples. Also, all sediments displayed a neutral pH (Soil Survey Division Staff, 2009). The C content, measured using a Thermo Fisher 2000 elementary analyser, is also presented and was essentially associated with the organic matter in the three samples, since the presence of carbonate minerals was not detected (AFNOR norm NF ISO 10693 (X31-105)). Based on the grain size distribution measured by using a laser diffraction grain size analyser (Malvern-Panalytical Mastersizer 3000) and plotted in a Shepard diagram, the CSG sediment was classified as a silt, while CPL and VF were sandy silts. The CSG and CPL sediments presented a high organic content (LOI: 18.1% and 19.7%, respectively) whereas the VF sediment organic content was lower (13.1%). More details on the sampling and sediment characteristics can be found in Bascle et al. (2020).

Samples		Champsanglard	Charpal	Villefort
		Dam lake main characteristics		
Abbreviation		CSG	CPL	VF
Latitude		46.2605	44.6225	44.463
Longitude		1.8824	3.5632	3.9251
Dam lake surface km^2		0.6	1.9	1.3
French Region		Nouvelle Aquitaine	Occitanie	Occitanie
Size of watershed km^2		1055	26	128
Watershed surface area with slope >10%		43	22	91
Major land use		Pastureland	Coniferous forest	Copse
		Sediment composition		
Global parameters	pH$_{water}$	6.8	7.0	6.7
	% LOI (loss on ignition)	18.1 ± 0.1	19.7 ± 0.2	13.1 ± 0.1
Granulometry	% Sand (63 µm to 2mm)	9.0 ± 1.0	30.0 ± 1.0	25.0 ± 1.0
	% Silt (2 to 63 µm)	90.0 ± 1.0	67.0 ± 1.0	73.0 ± 1.0
	% Clay (< 2 µm)	1.0 ± 0.1	3.0 ± 0.1	2.0 ± 0.1
Elemental composition	% C	8.0 ± 0.3	9.2 ± 0.3	3.5 ± 0.5
	% Si	27.9 ± 0.1	24.4 ± 0.1	23.3 ± 0.1
	% Al	8.7 ± 0.1	8.7 ± 0.1	6.2 ± 0.1
	% Fe	3.8 ± 0.1	4.5 ± 0.1	6.0 ± 0.1
	% K	4.3 ± 0.1	2.0 ± 0.1	1.8 ± 0.1
	% Mg	0.8 ± 0.1	0.9 ± 0.1	0.5 ± 0.1
	% Ca	0.4 ± 0.1	0.6 ± 0.1	0.1 ± 0.1
	Carbonates (g kg^{-1})	<1	<1	<1

Table 1: Dam lake main and watersheds characteristics and physico-chemical characterisation of sampled sediments

2.2. EPS EXTRACTION METHODS

The samples stored in the containers were sieved at 2 mm (in wet conditions) and the < 2 mm size fractions were immediately pre-treated by centrifugation at 1000 G for 10 min (Heraeus™ Multifuge™ X3, Thermo Scientific™, rotor Fiberlite F13-14x50cy) to remove the excess water from the sample before the application of the different extraction protocols.

Nine extraction methods were selected from the literature to investigate their efficiency in extracting the EPS of sediments. Ultracentrifugation (U), which was chosen as the control method, is a physical extraction technique and was the last step in all extraction methods. Sonication (S) was the second physical extraction technique. The chemical extraction techniques were sodium hydroxide (NaOH), sodium pyrophosphate (P), sodium tetraborate (T), and ‘mixed extraction techniques’, where the previously cited chemical extractants were added after a sonication protocol. The fourth method was the CER method, a mixed method using both chemical and physical means if cationic chemical exchange and physical shearing of resin beads are considered. Centrifugation allows for the separation of water-soluble organic compounds, and is commonly considered as a comparative method as it is the least degradative technique and there is no addition of chemical compounds (Comte et al., 2006). The dispersion induced by sonication is known to enhance EPS extraction. The use of NaOH leads to the ionisation of protonated functional groups in EPS, resulting in strong repulsion forces (Rossi et al., 2018). Sodium tetraborate buffer at pH 9.7 is known to extract organic compounds weakly adsorbed to surface minerals by electrostatic or Van der Waals interactions (Lopez-Sangil and Rovira, 2013). Its extraction mechanism is based on anionic exchange and ionisation of functional groups due to alkaline conditions. Sodium pyrophosphate is a chelating reagent mainly used to extract organic material precipitated by multivalent metallic cations (Lopez-Sangil and Rovira, 2013) without affecting amorphous and/or crystalline oxyhydroxides. Therefore, the involved mechanism is the same as for EDTA extraction, which is well-known for EPS extraction from soils (Rossi et al., 2018) and from sludges (Caudan et al., 2012; Comte et al., 2006; D’Abzac et al., 2010) and was initially applied to sediments (Underwood et al., 1995). Since EDTA extraction is known to be a source of extract contamination that is not removed by dialysis purification and that could lead to the overestimation of EPS metal binding capacity (D’Abzac et al., 2010), sodium pyrophosphate was tested as an alternative to EDTA. Moreover, using mineral salts (i.e. sodium hydroxide, pyrophosphate, or tetraborate) instead of organic extractants has the benefit of not carrying over organic carbon in the solution, which avoids the overestimation of organic carbon in the EPS extract (Comte et al, 2006). The organic carbon content of the extract is often used to measure the quantity of EPS extracted, and the use of an organic extractant, which would increase the C content, forbids this method. By combining physical (shear) and chemical mechanisms (cation exchange) in 'soft' pH conditions, CER methods are used for EPS extraction from soil (Redmile-Gordon et al., 2014), sludges (d'Abzac 2010) and sediments (Gerbersdorf et al., 2005). The CER method presents the advantage of maintaining the integrity of cells (preventing contamination by the intracellular material of extraction) and avoiding the co-extraction of humified organic material (Redmile-Gordon et al., 2014). The protocols are briefly described in Table 2.

Each extraction was performed in triplicate to calculate the average value and standard deviation of each parameter investigated to characterise the extracted EPS.

In the first step, the effect of the ratio between the sediment and the volume of the extractant solution on the quantity of the EPS was investigated in the CSG sediment. The ratios tested were 1:10, 1:20, and 1:40 (ratio of dry weight of solid at 105°C to liquid, S/L). Note that concerning the extraction with cationic exchange resin, only one solid:liquid ratio was tested, since in the CER method the key parameter is the CER:Volatil Solid ratio, using a 70 g CER/g VS ratio as proposed by Frølund et al. (1996) and Redmile-Gordon et al. (2014). In the second step, the effect of the EPS extraction method was tested on the three sediment samples described above at the optimal solid:liquid ratio among those investigated in the first step (i.e., 1:40; see discussion in Section 3.1).

Extraction Type	Method	Abbreviation	Protocol	Protocol adapted from:
Physical	Ultra Pure Water (UPW) + Ultracentrifugation[1]	U	Addition of volume of UPW based on chosen S/L ratio Agitation for 1 hour Ultracentrifugation	Caudan et al. (2012)
	Ultra Pure Water + Sonication + Ultracentrifugation	S + U	Addition of volume of UPW based on chosen S/L ratio Sonication Agitation for 1 hour Ultracentrifugation	Caudan et al. (2012)
Chemical	Sodium Hydroxide + Ultracentrifugation	NaOH + U	Addition of volume of NaOH 0.1M based on chosen S/L ratio Agitation for 16 hours Ultracentrifugation	Comte et al. (2006); Frølund et al. (1996),
	Sodium Hydroxide + Sonication + Ultracentrifugation	NaOH + S + U	Addition of volume of NaOH 0.1M based on chosen S/L ratio Sonication Agitation for 16 hours Ultracentrifugation	Comte et al. (2006); Frølund et al. (1996)
	Sodium Pyrophosphate 0.1 M + Sonication + Ultracentrifugation	P + S	Addition of volume of sodium pyrophophate 0.1M buffer (pH =10.2) based on chosen S/L ratio Agitation for 16 hours Ultracentrifugation	Lopez-Sangil and Rovira (2013); Bascle et al. (2020), D'Abzac et al, 2010
	Sodium Pyrophosphate 0.1 M + Sonication + Ultracentrifugation	P + S + U	Addition of volume of sodium pyrophophate 0.1M buffer (pH =10.2) based on chosen S/L ratio Sonication Agitation for 16 hours Ultracentrifugation	Lopez-Sangil and Rovira (2013); Bascle et al. (2020), D'abzac et al., 2010
	Sodium Tetraborate 0.1 M + Ultracentrifugation	T + U	Addition of volume of sodium tetraborate 0.1M buffer (pH =9.7) based on chosen S/L ratio Agitation for 16 hours Ultracentrifugation	Lopez-Sangil and Rovira (2013); Bascle et al. (2020),
	Sodium Tetraborate 0.1 M + Sonication + Ultracentrifugation	T + S + U	Addition of volume of sodium tetraborate 0.1M buffer (pH =9.7) based on chosen S/L ratio Sonication Agitation for 16 hours Ultracentrifugation	Lopez-Sangil and Rovira (2013); Bascle et al. (2020),
Chemical and physical	Cationic Exchange Resin	CER + U	70 g of CER per gram of volatile solid (Dowex 20–50 mesh, Na+ form, Sigma-Aldrich) added to a volume of buffer solution (2 mM Na_3PO_4 + 4 mM NaH_2PO_4 + 9 mM NaCl + 1mM KCl adjusted to pH 7 with HCl 2M. Agitation for 2 hours Ultracentrifugation	Redmile-Gordon et al. (2014); Frølund et al., 1996,

[1] Ultracentrifugation was carried out for 20 minutes, 10,000 g and at 4°C and sonication was performed at 120 W and 20 kHz for six intervals of 30 seconds, separated by 30 seconds of cooling in ice using a Sonopuls GM 70 (Bandelin) device.

Table 2 : Brief description of extraction protocols.

2.3. CHARACTERISATION OF EPS

For each extraction protocol tested, the EPS content was evaluated by measurement of the supernatant total organic carbon (Thermo Scientific Flash 2000/TOC-L TNM-L, Shimadzu). The determination of carbohydrates was performed by the method proposed by Dubois et al. (1956) using the phenol-sulfuric acid reaction and colorimetry (Supplementary material, SM1). Proteins and humic-like compounds of the supernatant were determined using the Lowry procedure described by Frølund et al. (1995). The quantification was carried out by colorimetry with and without the addition of $CuSO_4$ to omit the colour development due to humic compounds and chromogenic amino acids. For each protocol and ratio, analyses were performed in triplicate.

The quantification of ATP performed on the EPS extract is an indication of the amount of intact cellular biomass (Redmile-Gordon et al., 2014). For the validation of the methods, the total quantities of ATP extractable were measured and compared with the measured ATP contents of each extract for the three sediments. The total ATP extraction was carried out by mixing 100 mg of sediment in 1 mL of cell lysis buffer (assay buffer from ADP/ATP Ratio Assay kit, Sigma-Aldrich^{TM}). After 1 hour of agitation, the supernatant was collected for analysis of the ATP content.

ATP quantification was performed using an ATP fluorometric assay kit (Sigma-Aldrich^{TM}) with the methodology recommended by the supplier. In brief, 10 µL of sediment extract was added into each well with 0.2 µL of ATP Probe and 2 µL of ATP converter and developer mix. The volume was made up to 100 µL with ATP assay buffer. After development, ATP was measured by fluorometry (FLUOstar Omega, BMG Labtech), with λ_{ex}= 535 nm and λ_{em} = 587 nm.

2.4. STATISTICAL ANALYSIS

The results obtained from the mean of triplicate samples were submitted to analysis of variance and the means were compared by Tukey's test ($p \leq 0.01$). Statistical tests were performed with XLStat software (Addinsoft, France).

3. RESULTS AND DISCUSSION

3.1. CHOICE OF AN EXTRACTION RATIO (solid/liquid) FOR THE EXTRACTION METHODS

The choose on a solid liquid ratio to extract EPS was determined according to the quantity of organic carbon extracted and the distribution of three targeted macromolecules, i.e. proteins, polysaccharides, and humic-like substances. It was carried out on CSG sediment due to its relatively high organic content (18.1%), which should ensure that enough matter is extracted for further analysis.

The extracted organic carbon content, obtained by physical methods (ultracentrifugation and sonication + ultracentrifugation) was not statistically different (Tukey test, $p\text{-value} \leq 0.01$) for the three tested liquid:solid ratios. However, for all chemical methods, the ratio used for the extractions were responsible for a statistically significant difference in the extracted organic content (Tukey test, $p\text{-value} \leq 0.01$). The highest contents obtained were extracted by the ratio of 1:40, followed by 1:10, and 1:20 (Figure 1). The greater liquid phase makes it possible to strongly increase the quantity of the organic carbon extracted content, thereby enhancing particle dispersion and improving EPS extraction. The maximum extraction rate was obtained for sodium pyrophosphate coupled with ultracentrifugation (26 mg C g^{-1} dry sediment), which represented *c.a.* 30 wt% of the total C content (80 mg C g^{-1} dry sediment, Table 1).

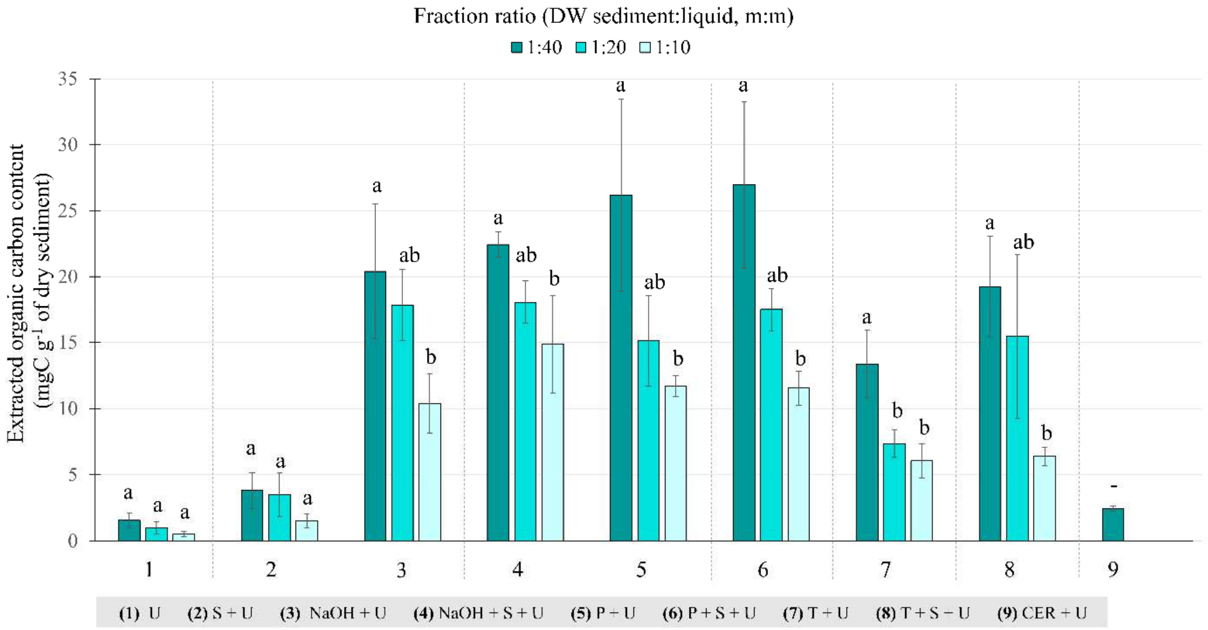

Figure 1: Extracted organic carbon content (mgC g^{-1} sediment) extracted from CSG sediment with different extraction ratios by the nine extraction methods (means ± standard derivation; n = 3; means followed by the same letter indicate that organic carbon content extracted in different ratios for each method do not differ statistically by Tukey's test ($p \leq 0.01$)). Complete data are available in supplementary material SM2.

The profiles of the extracted macromolecules show an impact of the S/L ratio on the distribution of proteins, polysaccharides, and humic-like substances as a function of the solid:liquid ratio. Indeed, polysaccharides are not or slightly affected by the solid:liquid ratio. A small increasing trend of protein ratio was noticed according to the increase in the solid:liquid ratio. In contrast, a small decreasing trend in the relative abundance of humic-like substances was noted (Figure 2) when increasing the solid:liquid ratio.

Due to better picture of the bacterial exo-synthesis activity in link with proteins and polysaccharides contents of the extracts, a greater quantity of organic carbon extracted. Additionally, due to the quantity of sediment required for extraction, the 1:40 ratio was chosen for the extraction of the three sediment EPS by the nine proposed methods.

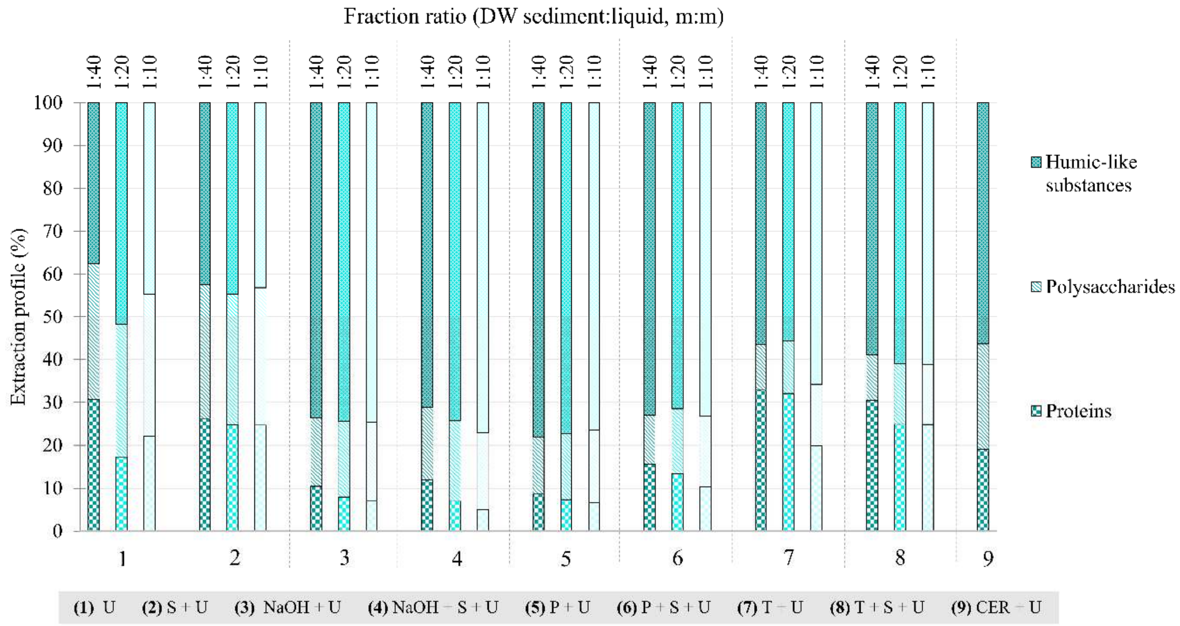

Figure 2: Distribution of proteins, humic-like substances, and polysaccharides from CSG sediment extracts with different extraction ratios for the nine extraction methods tested.

The extracted organic matter, which is more or less enriched in EPS from organic matter, was studied with for the three sediments.

3.2. EFFICIENCY OF EXTRACTION METHODS ON THE QUANTITY OF EPS EXTRACTED

Section 3.1 shows that a solid:liquid ratio of 1:40 is suitable to extract organic carbon from sediment, but it is important to specify if all organic carbon extracted is EPS or contains a portion of humic materials from sediment, as shown in extractions from soils (Redmile-Gordon et al., 2014, Bérard et al., 2020). To specify and discuss their effects on the quality and purity of extracted EPS, the nine selected extraction methods were tested with the three sediments (CSL, CPL, and VF) at a solid:liquid ratio of 1:40.

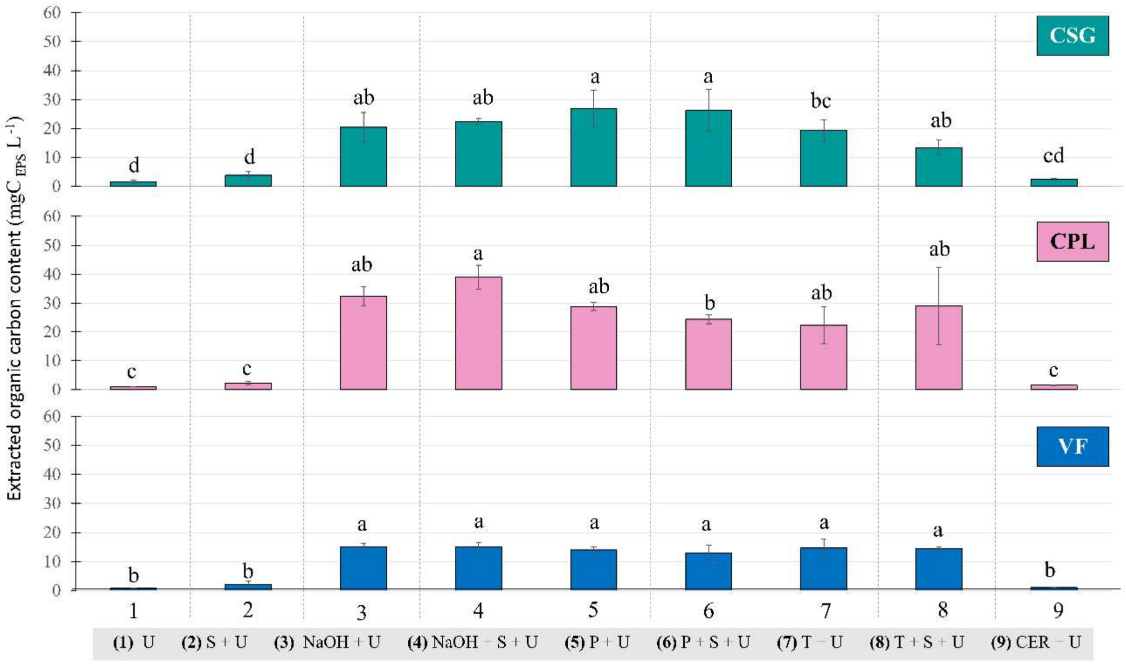

Figure 3: Amount of EPS measured by the organic carbon content (mgC g^{-1} sediment) extracted from the CSG, CPL, and VF sediments by the nine methods tested (means followed by the same letter do not differ statistically among themselves for each treatment by Tukey's test (p-value ≤ 0.01)). Data available in supplementary material SM3).

According to the trend of organic carbon extracted (Figure 3), the extraction yields of EPS vary according to the extraction protocol, which can be divided into two groups: 'soft extraction' based on physical methods (centrifugation and sonication) or mixed extraction, i.e. the CER method, and 'strong extraction' using chemical extractants such as sodium hydroxide, sodium pyrophosphate, or sodium tetraborate. As previously shown (Figure 1), chemical methods extract the more organic carbon from sediment. If sonication coupled with centrifugation enhances the extraction yield for the physical method, coupled with chemical extractant, the benefit of sonication was not consistent and was only present in a few cases (Tukey test, p-value ≤ 0.01) (Figure 3). Nevertheless, the better efficiency of chemicals methods for the extraction of EPS needs to be re-evaluated after considering the following discussion.

Firstly, these tendencies are offset by purity considerations, since the use of chemical extractors leads to the presence of a large amount of salts in the dried extracts, as shown by the predominance of inorganic contents in the extract dry matter (data available in the supplementary material SM2). Thus, the implementation of purification steps may be needed for further characterisation and/or use. Physical and CER methods can avoid these issues, as already discussed by Bérard et al. (2020), but only allow for the extraction of small quantities of EPS (the C content from EPS is less than 4% of the C content from sediment) compared to the others (e.g. up to 30% with tetraborate). Secondly, according to literature on EPS extraction from soils (Redmile-Gordon et al., 2014, in Bérard et al., 2020), chemical reagents can involve non-specific extraction of non-bacterial humic materials from sediment, which can artificially increase the amount of EPS extracted. The extraction specificity needs to be analysed experimentally with other characterisation methods with the comparison of EPS characteristics extracted by soft methods.

For the physical and the CER extraction methods, where EPS extraction specificity is shown in the literature (Redmile-Gordon et al., 2014), we observed that the CSG sediment was the one from which the most EPS has been extracted, while VF and CPL had similar values despite a significant difference in the organic matter content. This suggests that the amount of extractable EPS depends primarily on the nature and/or accessibility of these organic compounds, rather than the amount of organic matter in sediments.

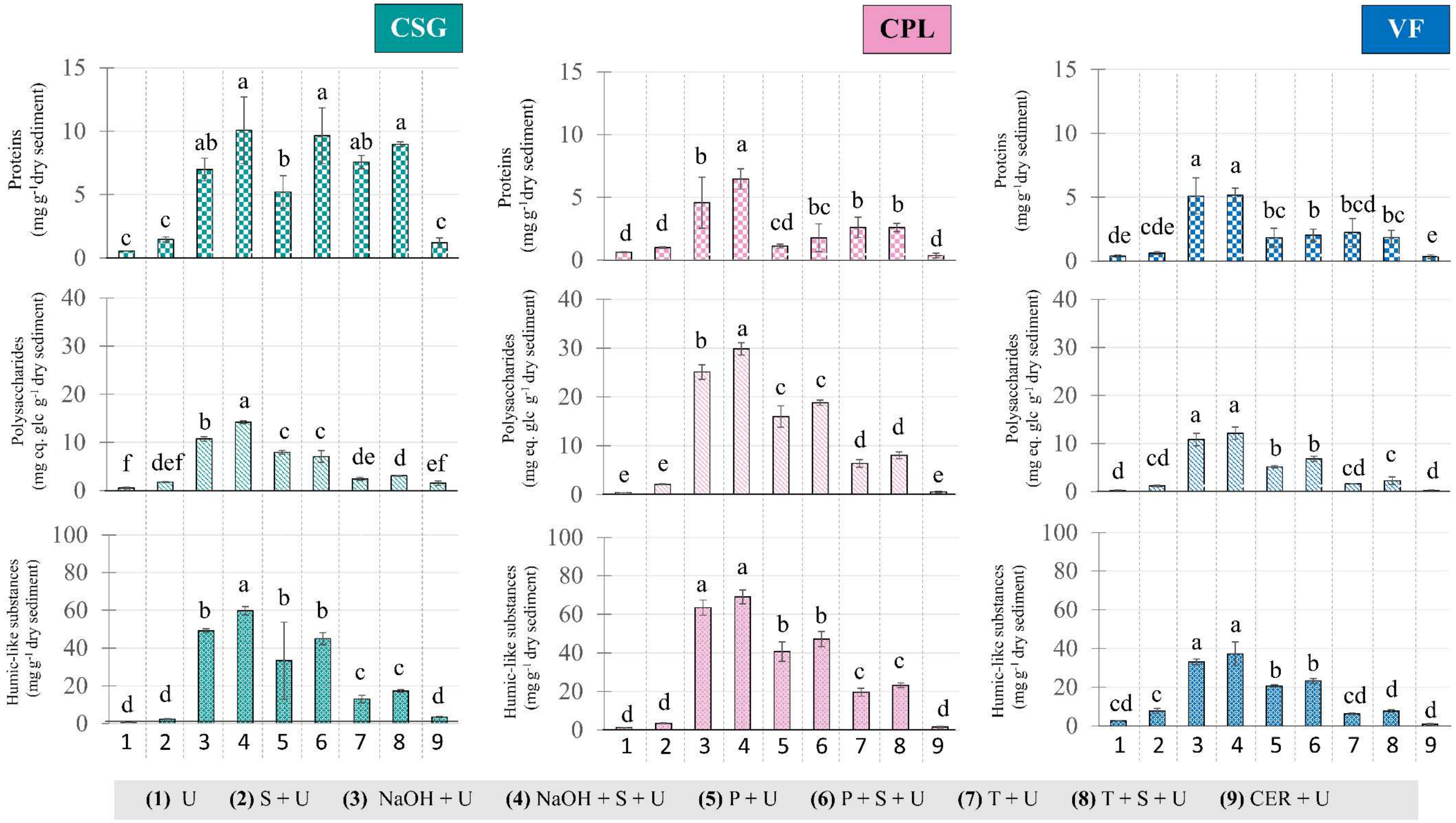

Figure 4: Contents of proteins, polysaccharides and humic-like substances, in the EPS fraction extracted from the CSG, CPL, and VF sediment extracted with different methods at an S/L ratio of 1:40. Means with different letters differ statistically for each data set when comparing the extraction methods (Tukey $p < 0.01$).

3.3. EFFECT OF DIFFERENT EXTRACTION METHODS ON CELL LYSIS

However, before looking at the specificity of each method, their validity was assessed in terms of the 'non-lysis' of microbial cells during the extraction process of EPS from three sediment samples.

The EPS matrix contains small quantities of nucleic acid (Flemming et al, 2001), so an increase in the ATP content in the EPS extracts is used as an indicator of cell lysis and cell membrane permeability (Azeredo et al., 2003). In this study, the amount of ATP in the EPS extract from the three sediments obtained by the nine methods was compared to the total ATP value of each sediment (assumed to represent 100% of the sediment ATP) (Table 3).

The percentage of cells lysed varied with the type of extractor in the following order: U < S+U = CER << P+U < P+S+U < NaOH+U < T+S+U < NaOH+S+U = T+U.

The physical (ultracentrifugation and ultracentrifugation + sonication) and the mixed CER methods presented ATP contents in the EPS extract that were below 5% of the total ATP present in sediments, indicating that a small number of cells was lysed during the extraction process. In contrast, the chemical extraction methods, with or without sonication, led to higher ATP content in the EPS extract, ranging from 7 to 45% of extractable ATP. This suggests the release of intracellular material and that methods using chemical reagents are not satisfactory from this perspective.

Extraction method	Sample site	Average	Standard derivation %
Ultracentrifugation	CSG	0.4	0.1
	CPL	0.9	0.1
	VF	4.0	2.4
Sonication	CSG	0.6	0.1
	CPL	1.2	0.1
	VF	2.1	0.3
NaOH + U	CSG	21.6	1.1
	CPL	11.8	0.1
	VF	26.6	1.6
NaOH + S + U	CSG	24.6	1.4
	CPL	12.9	0.6
	VF	35.9	8.5
P + U	CSG	13.8	1.0
	CPL	8.7	2.3
	VF	16.3	4.1
P + S + U	CSG	12.9	0.1
	CPL	8.4	0.5
	VF	23.9	9.0
T + U	CSG	24.8	0.7
	CPL	17.8	1.7
	VF	14.2	1.2
T + S + U	CSG	24.0	2.4
	CPL	16.0	0.9
	VF	17.9	5.5
CER+U	CSG	0.7	0.2
	CPL	<0.1	0.0
	VF	1.1	0.5

Table 3: ATP percentage of the three sediment extractions obtained with the nine methods compared to the total ATP value of each sediment (representing 100% of the sediment ATP).

3.4. MACROMOLECULE PROFILES OF THE EXTRACTION METHODS

The evolution of the content (in mg g^{-1}dw sediment) of proteins, polysaccharides, and humic-like substances obtained from the CSG, CPL, and VF sediment extracts by different methods for EPS extraction is shown in Figure 4. This presentation compares the ability of methods to extract a greater or lesser amount of a type of macromolecule from a sediment, but we need to keep in mind that chemical methods induce contamination with intracellular macromolecules in the EPS extract (Table 3) and the non-specific extraction of humic materials from sediments, according to the literature.

Indeed, Redmile-Gordon et al., 2014, and Bérard et al. underlined the issue of EPS extract contamination by humic materials from soil. Some reagents used in our work to extract EPS from sediment, such as NaOH, pyrophosphate, or tetraborate, are also communally used to extract organic matter from soil or sediment, but under different experimental conditions (time, concentration, etc), and as such the issue of contamination of the EPS extract with humic materials need to be considered. In contrast to proteins and polysaccharides, humic-like substances are not macromolecules from cells and the reagents of chemicals method can induce the non-specific extraction of humic material from sediment and contaminate EPS extract with humic-like substances not considered to be associated with the EPS. Chemical methods and methods using sodium hydroxide and pyrophosphate show the highest quantity of EPS extracted (Figure 3) with a high content of humic-like substances (Figure 4). By increasing the solution alkalinity, sodium hydroxide extraction permits the breaking of electrostatic bonds, enhanced ionisation of functional groups (phenolic, carboxylic), and repulsive electrostatic forces, leading to higher extraction of humic-like substances (Dick and Burba, 1999; Stevenson, 1994) from EPS, but also from humic materials of the sediment. Indeed, NaOH is widely used for the extraction of humic substances from soil and humic-like substances from EPS are assumed to have the same chemical properties as humic materials from soils. The use of sodium pyrophosphate as a substitute for EDTA leads to a high extraction content, which could be attributed to the dissolution of calcic humates (Pansu and Gautheyrou, 2006). The mechanism of action of pyrophosphate shows that the humic-like substance fraction of the extract originated from humic-like substances associated with the matrix of EPS, but also from humic materials in the sediment. Sodium tetraborate extracted less EPS (Figure 3) than the two other chemicals reagent used, but due to its mechanism of release of organic matter, it can also be assumed to provide non-specific extraction of humic-like substances in EPS. Sodium tetraborate is known to extract organic compounds weakly adsorbed to a mineral matrix by means of electrostatic or Van der Waals interactions, such as hydrogen or cationic bridges with clay particles, through anionic exchange or the ionisation of functional groups (Lopez-Sangil and Rovira, 2013). The specific effects of sodium pyrophosphate and/or sodium tetraborate may be accompanied by more general pH-related effects. This could explain the lower EPS extraction (Figure 3) and lower humic-like substance content (Figure 4) with sodium tetraborate as well as the pH difference (10.2 and 9.7, respectively). Indeed, this pH range corresponds with the polyphenol pKa[pKa($PhOH/PhO^-$) = 9.95]. The ionisation of these functional groups could vary significantly with pH.

The chemical method extracts contained a greater quantity of macromolecules than the physical or CER methods, but from both biofilms and humic materials. Nevertheless, for biological sludges or biofilms, it is well-known that chemical methods are more efficient at extracting EPS than physical or CER methods. In the case of extraction of EPS from sediments, we cannot exclude that more macromolecules from EPS were extracted due to the higher strength of the methods using chemicals. However, it is not currently possible to be confident about this. As highlighted by Seviour et al. (2019), for EPS from a biological matrix, more investigations are required to better understand what is really extracted by EPS extraction methods. This statement may also be applied to EPS extracted from biofilm associated with a mineral and organic matrix such as soils or sediment.

Physical and CER methods extracted a similar quantity of organic carbon regardless of the solid:liquid ratio used (Figure 1), but the relative abundance of humic-like substances for the CER method (Figure 2) was the highest. Redmile-Gordon et al. (2014) considered that the CER method does not significantly extract humic materials from soil. We can assume regarding the extracted organic carbon and the humic-like substance content of EPS (Figure 1 and Figure 2), that centrifugation or centrifugation coupled with ultrasonication methods do not extract humic materials from soil.

If we consider the composition of EPS extracts obtained by physical methods and CER, which did not induce significant contamination of the extract by humic material or the intracellular content of cells (Table 3), we noted that the major compounds in EPS were humic-like substances. The content of proteins and polysaccharides was low (less than 5 mg g^{-1}dw) and showed no or only a slight difference for the three soft methods. Moreover, it can be considered that the humic-like substances and carbohydrate fractions present the same profile while the protein fraction stands out a little more These results differ from the studies of Redmile-Gordon et al. (2014) or Bérard et al. (2020), where EPS was also extracted from soil by the CER method. In these studies, humic-like substances was the main fraction extraction of the extracted EPS, but proteins or polysaccharides were extracted in greater quantities, sometimes with a similar content as humic-like substances. This can be linked to the nature of the biofilm found in the sediment or in the rhizosphere of the soil, but also to the lower content of organic matter in the soils of these studies compared to the high organic matter content of sediment (Table1).

4. CONCLUSION

The efficiency and characteristics of nine EPS extraction methods were assessed for the first time in three dam reservoir sediments derived from acidic bedrock (magmatic and/or metamorphic). The results highlight the impact of the solid:liquid ratio on the extracted EPS quantities and that increasing the liquid ratio enhanced the extraction of EPS and protein substances through an increase in sediment dispersion. Cell lysis was assessed using ATP measurements and found to be highly dependent on the method used, with a greater prevalence in chemical extractions. Physical extraction methods did not contaminate the EPS extract with extraction reagents, but small amounts of EPS were extracted (< 6 wt% C). In contrast, chemical extraction methods provided a larger amount of EPS (> 16 wt% C) in appearance, but the EPS extract was contaminated by (i) intracellular material due to significant cell lysis, (ii) a high salt content from the chemical reagent used for extraction and (iii) non-specific humic materials from sediment and from EPS.

Chemical extractions appear to be weak candidates for the extraction of EPS from sediments. Physical methods and CER seem to guarantee a more specific extraction of EPS from sediments. These results, coupled with the preservation of cells, favour these methods for EPS extraction from sediments.

Author contributions

Thibaut Le Guet: investigation, validation, formal analysis, writing - original draft; **Marilia Camotti Bastos**: investigation, validation, formal analysis, writing - original draft; **Isabelle Bourven**: conceptualisation, methodology, investigation, writing - review editing, **Valentin Robin**: conceptualisation, methodology, writing - review editing; **Gilles Guibaud**: supervision writing- review editing.

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