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

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HIGHLIGHTS

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

16

17

ABSTRACT

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

37

38

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

41 1. INTRODUCTION

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

55 Sediments are used by microorganisms as a substrate, and microorganisms play an important
56 role in the biogeochemical processes of sediment, such as the biodegradation of organic
57 matter and cycling of nutrients (Fang et al., 2017). These communities of autotrophic and
58 heterotrophic organisms can attach and grow in the sediment through the production of a
59 biofilm where they are embedded in a matrix of extracellular polymeric substances (EPS)
60 (Romaní et al., 2013). In intact sediment samples, Grant et al. (1986) were able to examine
61 the stabilisation of intertidal sands by diatomaceous mucus films by characterisation of the
62 colloids, polysaccharides, and chlorophyll contents and by scanning electron microscopy.
63 This self-produced three-dimensional structure derived from the metabolism of these
64 microorganisms and environmental inorganic constituents (Mori et al., 2018) affects
65 biostabilisation and deposits of sediment (Fang et al., 2015). The presence of EPS plays an

66 important role in interactions between the sediments and other components of the
67 environment (water, dissolved elements, and microorganisms) (Gerbersdorf and Wieprecht,
68 2015; Shang et al., 2014). This importance is related to the increased chemical reactivity that
69 the presence of this biofilm develops through the biological interface, promoting
70 aggregation/biostabilisation of sediment particles (van de Lageweg et al., 2018) and
71 increasing the chemical reactivity and interaction between the soluble phase and the minerals
72 (Donlan, 2002). This increase in reactivity may be responsible for the change in transport of
73 contaminants such as metals (Comte et al., 2008; Labrenz and Banfield, 2004; Walter, 1997)
74 and organic compounds (Edwards and Kjellerup, 2013). Therefore, understanding the
75 chemical and physical reactivity of the EPS, as well as its composition, is of prime interest.

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

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

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

113 In this study, physical (ultracentrifugation, sonication), chemical (sodium hydroxide,
114 tetraborate, pyrophosphate) and both chemical and physical (cation exchange resin, i.e. CER)

115 extraction methods were performed on three samples of freshwater dam reservoir sediments
116 derived from acidic crystalline bedrock with different contents of organic matter and grain
117 size distributions. Three ratios of sediment mass to extractant were also tested on one
118 sediment to better specify the effect of physicochemical parameters such as the solid:liquid
119 ratio on the composition of EPS extracted from the sediments. The EPS extraction amount
120 was measured by the amount of organic carbon extracted from sediment biofilms, while the
121 effects of the extraction method on the EPS composition were evaluated based on the contents
122 of polysaccharides, proteins, and humic-like substances. The extraction quality regarding the
123 non-extraction of intracellular material was assessed by estimating cellular lysis by measuring
124 the release of ATP in the extract.

125 2. MATERIAL AND METHODS

126 2.1. ORIGIN, SAMPLING AND CHARACTERISTICS OF SEDIMENTS

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

133 The CSG superficial sediment was sampled in November 2016 with the use of an Ekman
134 tipper from 0 to 7 cm depth, and the CPL and VF superficial sediments were sampled in
135 November 2017 with a shovel from 0 to 20 cm in depth. The samples were kept underwater at
136 4°C inside hermetically sealed containers until performing the experiment. The physical and
137 chemical characteristics of the three sediments sampled from dam lakes are presented in Table
138 1.

139 The loss on ignition (LOI) was obtained by weighing previously dried sediment (105°C for 24
140 h) before and after ignition in an oven at 550°C for 2 h. The concentrations of major elements
141 (Si, Al, Fe, K, Mg, and Ca) were measured using an energy dispersive X-ray fluorescence
142 spectrometer (ED-XRF Spectro-XEPOS, Xep05) and were in a similar range between
143 samples. Also, all sediments displayed a neutral pH (Soil Survey Division Staff, 2009). The C
144 content, measured using a Thermo Fisher 2000 elementary analyser, is also presented and was
145 essentially associated with the organic matter in the three samples, since the presence of
146 carbonate minerals was not detected (AFNOR norm NF ISO 10693 (X31-105)). Based on the
147 grain size distribution measured by using a laser diffraction grain size analyser (Malvern-
148 Panalytical Mastersizer 3000) and plotted in a Shepard diagram, the CSG sediment was

149 classified as a silt, while CPL and VF were sandy silts. The CSG and CPL sediments
 150 presented a high organic content (LOI: 18.1% and 19.7%, respectively) whereas the VF
 151 sediment organic content was lower (13.1%). More details on the sampling and sediment
 152 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

153

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

156 2.2. EPS EXTRACTION METHODS

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

161 Nine extraction methods were selected from the literature to investigate their efficiency in
162 extracting the EPS of sediments. Ultracentrifugation (U), which was chosen as the control
163 method, is a physical extraction technique and was the last step in all extraction methods.
164 Sonication (S) was the second physical extraction technique. The chemical extraction
165 techniques were sodium hydroxide (NaOH), sodium pyrophosphate (P), sodium tetraborate
166 (T), and 'mixed extraction techniques', where the previously cited chemical extractants were
167 added after a sonication protocol. The fourth method was the CER method, a mixed method
168 using both chemical and physical means if cationic chemical exchange and physical shearing
169 of resin beads are considered. Centrifugation allows for the separation of water-soluble
170 organic compounds, and is commonly considered as a comparative method as it is the least
171 degradative technique and there is no addition of chemical compounds (Comte et al., 2006).
172 The dispersion induced by sonication is known to enhance EPS extraction. The use of NaOH
173 leads to the ionisation of protonated functional groups in EPS, resulting in strong repulsion
174 forces (Rossi et al., 2018). Sodium tetraborate buffer at pH 9.7 is known to extract organic
175 compounds weakly adsorbed to surface minerals by electrostatic or Van der Waals
176 interactions (Lopez-Sangil and Rovira, 2013). Its extraction mechanism is based on anionic
177 exchange and ionisation of functional groups due to alkaline conditions. Sodium
178 pyrophosphate is a chelating reagent mainly used to extract organic material precipitated by
179 multivalent metallic cations (Lopez-Sangil and Rovira, 2013) without affecting amorphous
180 and/or crystalline oxyhydroxides. Therefore, the involved mechanism is the same as for
181 EDTA extraction, which is well-known for EPS extraction from soils (Rossi et al., 2018) and
182 from sludges (Caudan et al., 2012; Comte et al., 2006; D'Abzac et al., 2010) and was initially
183 applied to sediments (Underwood et al., 1995). Since EDTA extraction is known to be a
184 source of extract contamination that is not removed by dialysis purification and that could
185 lead to the overestimation of EPS metal binding capacity (D'Abzac et al., 2010), sodium

186 pyrophosphate was tested as an alternative to EDTA. Moreover, using mineral salts (i.e.
187 sodium hydroxide, pyrophosphate, or tetraborate) instead of organic extractants has the
188 benefit of not carrying over organic carbon in the solution, which avoids the overestimation of
189 organic carbon in the EPS extract (Comte et al, 2006). The organic carbon content of the
190 extract is often used to measure the quantity of EPS extracted, and the use of an organic
191 extractant, which would increase the C content, forbids this method. By combining physical
192 (shear) and chemical mechanisms (cation exchange) in ‘soft’ pH conditions, CER methods
193 are used for EPS extraction from soil (Redmile-Gordon et al., 2014), sludges (d’Abzac 2010)
194 and sediments (Gerbersdorf et al., 2005). The CER method presents the advantage of
195 maintaining the integrity of cells (preventing contamination by the intracellular material of
196 extraction) and avoiding the co-extraction of humified organic material (Redmile-Gordon et
197 al., 2014). The protocols are briefly described in Table 2.

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

200 In the first step, the effect of the ratio between the sediment and the volume of the extractant
201 solution on the quantity of the EPS was investigated in the CSG sediment. The ratios tested
202 were 1:10, 1:20, and 1:40 (ratio of dry weight of solid at 105°C to liquid, S/L). Note that
203 concerning the extraction with cationic exchange resin, only one solid:liquid ratio was tested,
204 since in the CER method the key parameter is the CER:Volatil Solid ratio, using a 70 g
205 CER/g VS ratio as proposed by Frølund et al. (1996) and Redmile-Gordon et al. (2014). In the
206 second step, the effect of the EPS extraction method was tested on the three sediment samples
207 described above at the optimal solid:liquid ratio among those investigated in the first step
208 (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 pyrophosphate 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 pyrophosphate 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 ₃ PO ₄ + 4 mM NaH ₂ PO 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.

212 *Table 2 : Brief description of extraction protocols.*

213 2.3. CHARACTERISATION OF EPS

214 For each extraction protocol tested, the EPS content was evaluated by measurement of the
215 supernatant total organic carbon (Thermo Scientific Flash 2000/TOC-L TNM-L, Shimadzu).

216 The determination of carbohydrates was performed by the method proposed by Dubois et al.
217 (1956) using the phenol-sulfuric acid reaction and colorimetry (Supplementary material,
218 SM1). Proteins and humic-like compounds of the supernatant were determined using the
219 Lowry procedure described by Frølund et al. (1995). The quantification was carried out by
220 colorimetry with and without the addition of CuSO_4 to omit the colour development due to
221 humic compounds and chromogenic amino acids. For each protocol and ratio, analyses were
222 performed in triplicate.

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

230 ATP quantification was performed using an ATP fluorometric assay kit (Sigma-AldrichTM)
231 with the methodology recommended by the supplier. In brief, 10 μL of sediment extract was
232 added into each well with 0.2 μL of ATP Probe and 2 μL of ATP converter and developer
233 mix. The volume was made up to 100 μL with ATP assay buffer. After development, ATP
234 was measured by fluorometry (FLUOstar Omega, BMG Labtech), with $\lambda_{\text{ex}} = 535 \text{ nm}$ and
235 $\lambda_{\text{em}} = 587 \text{ nm}$.

236 2.4. STATISTICAL ANALYSIS

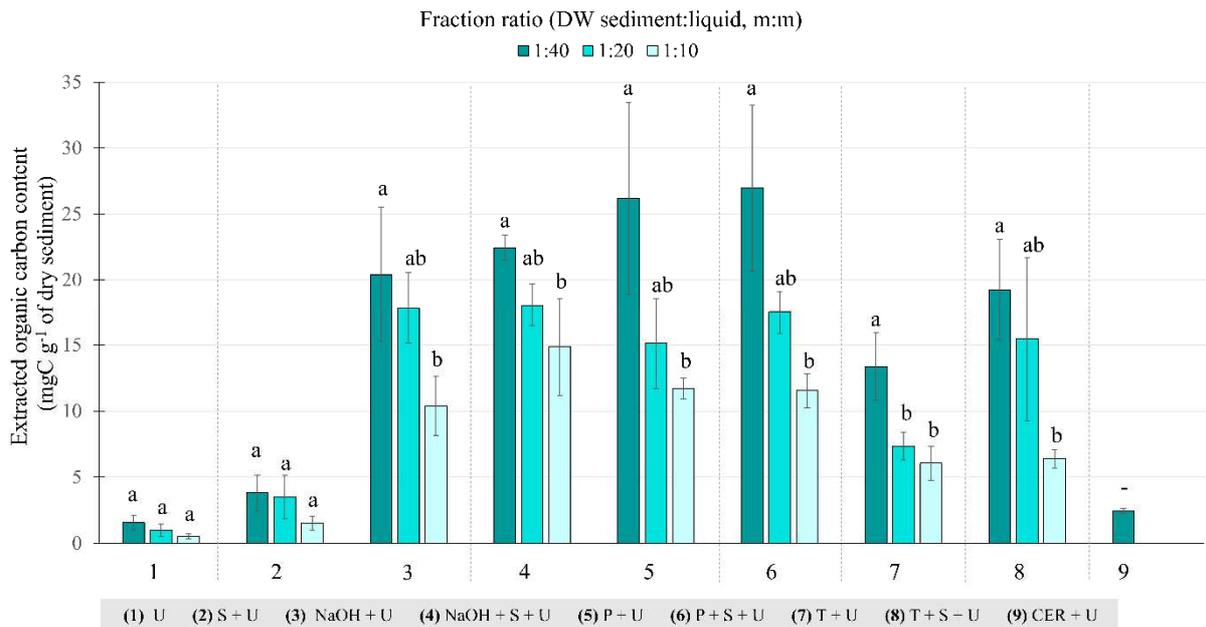
237 The results obtained from the mean of triplicate samples were submitted to analysis of
238 variance and the means were compared by Tukey's test ($p \leq 0.01$). Statistical tests were
239 performed with XLStat software (Addinsoft, France).

240 3. RESULTS AND DISCUSSION

241 3.1. CHOICE OF AN EXTRACTION RATIO (solid/liquid) FOR THE EXTRACTION 242 METHODS

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

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



258

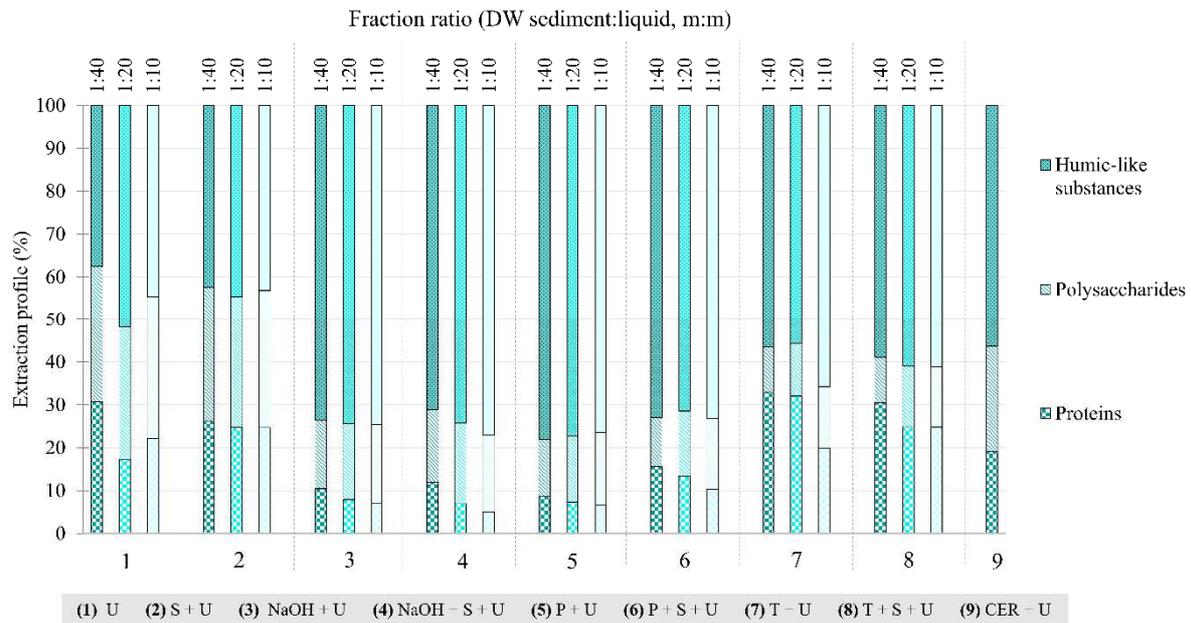
259 *Figure 1: Extracted organic carbon content (mgC g⁻¹ sediment) extracted from CSG sediment*
 260 *with different extraction ratios by the nine extraction methods (means ± standard derivation;*
 261 *n = 3; means followed by the same letter indicate that organic carbon content extracted in*
 262 *different ratios for each method do not differ statistically by Tukey's test (p ≤ 0.01)).*
 263 *Complete data are available in supplementary material SM2.*

264

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

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

275



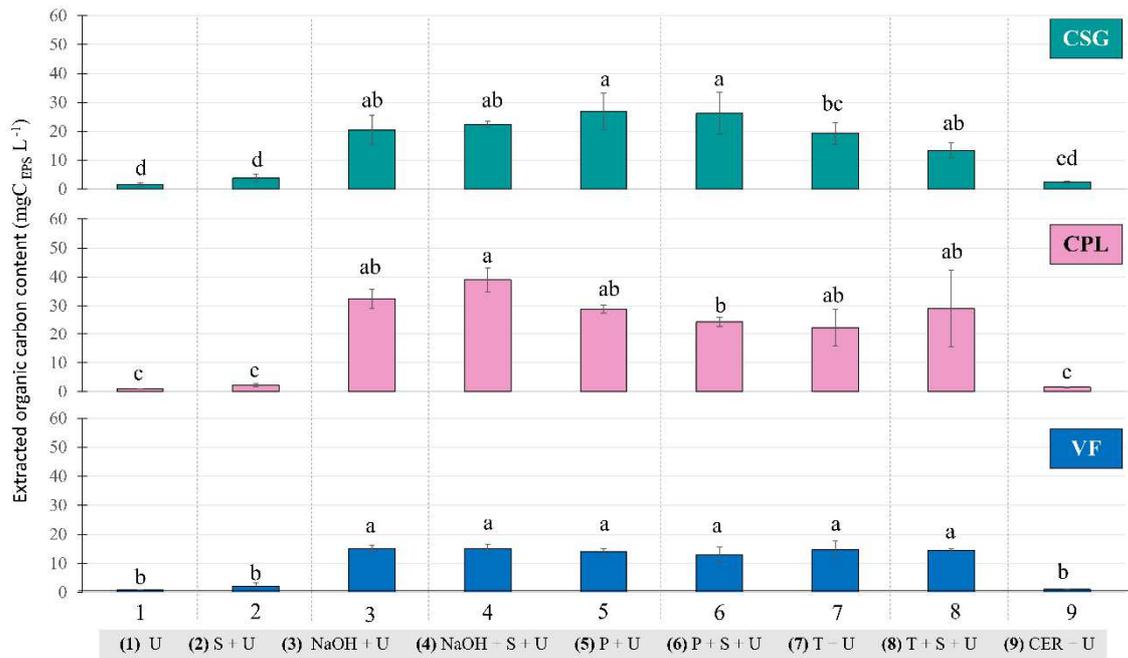
277
 278 *Figure 2: Distribution of proteins, humic-like substances, and polysaccharides from CSG*
 279 *sediment extracts with different extraction ratios for the nine extraction methods tested.*

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

282 3.2. EFFICIENCY OF EXTRACTION METHODS ON THE QUANTITY OF EPS 283 EXTRACTED

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

290



291

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

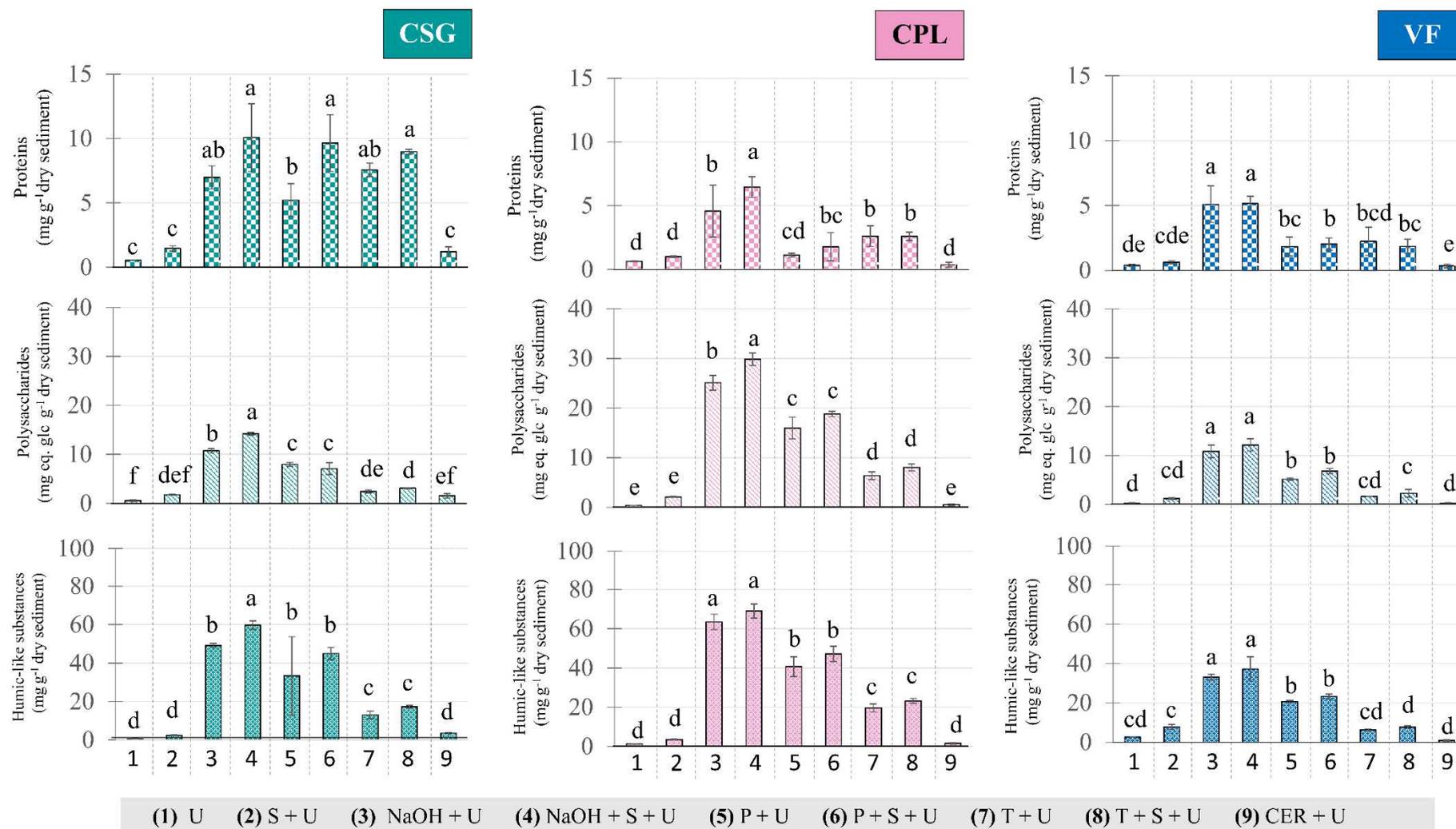
296

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

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

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

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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 $p < 0.01$).

332 3.3. EFFECT OF DIFFERENT EXTRACTION METHODS ON CELL LYSIS

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

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

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

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

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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

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

353

354 3.4. MACROMOLECULE PROFILES OF THE EXTRACTION METHODS

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

362 Indeed, Redmile-Gordon et al., 2014, and Bérard et al. underlined the issue of EPS extract
363 contamination by humic materials from soil. Some reagents used in our work to extract EPS
364 from sediment, such as NaOH, pyrophosphate, or tetraborate, are also communally used to
365 extract organic matter from soil or sediment, but under different experimental conditions
366 (time, concentration, etc), and as such the issue of contamination of the EPS extract with
367 humic materials need to be considered. In contrast to proteins and polysaccharides, humic-like
368 substances are not macromolecules from cells and the reagents of chemicals method can
369 induce the non-specific extraction of humic material from sediment and contaminate EPS
370 extract with humic-like substances not considered to be associated with the EPS. Chemical
371 methods and methods using sodium hydroxide and pyrophosphate show the highest quantity
372 of EPS extracted (Figure 3) with a high content of humic-like substances (Figure 4). By
373 increasing the solution alkalinity, sodium hydroxide extraction permits the breaking of
374 electrostatic bonds, enhanced ionisation of functional groups (phenolic, carboxylic), and
375 repulsive electrostatic forces, leading to higher extraction of humic-like substances (Dick and
376 Burba, 1999; Stevenson, 1994) from EPS, but also from humic materials of the sediment.
377 Indeed, NaOH is widely used for the extraction of humic substances from soil and humic-like
378 substances from EPS are assumed to have the same chemical properties as humic materials
379 from soils. The use of sodium pyrophosphate as a substitute for EDTA leads to a high
380 extraction content, which could be attributed to the dissolution of calcic humates (Pansu and
381 Gautheyrou, 2006). The mechanism of action of pyrophosphate shows that the humic-like
382 substance fraction of the extract originated from humic-like substances associated with the
383 matrix of EPS, but also from humic materials in the sediment. Sodium tetraborate extracted
384 less EPS (Figure 3) than the two other chemicals reagent used, but due to its mechanism of
385 release of organic matter, it can also be assumed to provide non-specific extraction of humic-
386 like substances in EPS. Sodium tetraborate is known to extract organic compounds weakly

387 adsorbed to a mineral matrix by means of electrostatic or Van der Waals interactions, such as
388 hydrogen or cationic bridges with clay particles, through anionic exchange or the ionisation of
389 functional groups (Lopez-Sangil and Rovira, 2013). The specific effects of sodium
390 pyrophosphate and/or sodium tetraborate may be accompanied by more general pH-related
391 effects. This could explain the lower EPS extraction (Figure 3) and lower humic-like
392 substance content (Figure 4) with sodium tetraborate as well as the pH difference (10.2 and
393 9.7, respectively). Indeed, this pH range corresponds with the polyphenol
394 $pK_a[pK_a(\text{PhOH}/\text{PhO}^-) = 9.95]$. The ionisation of these functional groups could vary
395 significantly with pH.

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

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

411 and Figure 2), that centrifugation or centrifugation coupled with ultrasonication methods do
412 not extract humic materials from soil.

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

427 **4. CONCLUSION**

428 The efficiency and characteristics of nine EPS extraction methods were assessed for the first
429 time in three dam reservoir sediments derived from acidic bedrock (magmatic and/or
430 metamorphic). The results highlight the impact of the solid:liquid ratio on the extracted EPS
431 quantities and that increasing the liquid ratio enhanced the extraction of EPS and protein
432 substances through an increase in sediment dispersion. Cell lysis was assessed using ATP
433 measurements and found to be highly dependent on the method used, with a greater
434 prevalence in chemical extractions. Physical extraction methods did not contaminate the EPS

435 extract with extraction reagents, but small amounts of EPS were extracted (< 6 wt% C). In
436 contrast, chemical extraction methods provided a larger amount of EPS (> 16 wt% C) in
437 appearance, but the EPS extract was contaminated by (i) intracellular material due to
438 significant cell lysis, (ii) a high salt content from the chemical reagent used for extraction and
439 (iii) non-specific humic materials from sediment and from EPS.

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

444 **Author contributions**

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

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