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1           **Complexes of humic acid with cationic surfactants support the**  
2           **supramolecular view of extracted humic matter**

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34 **Abstract**

35 The structural organization of extracted humic matter, polymeric or supramolecular, has been  
36 a long-standing controversial issue. In this paper, we show that the interaction between a  
37 reference humic material, the Suwannee River Humic Acid (SRHA), and a homologous series  
38 of cationic surfactants (n-alkyl trimethyl ammonium chloride) provides key insights to resolve  
39 the controversy. By combining measurements of turbidity, electrophoretic mobility, surface  
40 tension, and cryogenic transmission electron microscopy, we show that (i) the binding of  
41 cationic surfactant to the anionic humic acid is initially triggered by electrostatic interactions;  
42 (ii) the contrasting evolution of turbidity-surfactant concentration curves implies that the  
43 surfactant alkyl chain interacts with the hydrophobic moieties of humic matter from very low  
44 concentrations of surfactant; (iii) a drastic restructuring of humic matter occurs upon surfactant  
45 binding, which brings out supplementary anionic humic sites; (iv) in the presence of C12  
46 cationic surfactant, SRHA spontaneously forms stable nanoscale unilamellar vesicles, which,  
47 in addition to the high surface activity of complexes, could be of interest in the remediation of  
48 oil-contaminated environments; (v) the sequence of molecular structures obtained from SRHA  
49 with increasing amounts of C12-cationic surfactant - humic-rich vesicles, aggregates,  
50 surfactant-rich vesicles – bears striking similarities with the phase behaviour of aqueous  
51 mixtures of anionic and cationic surfactants, thus supporting the supramolecular view of humic  
52 structure.

53

54 **Keywords:** Suwannee River Humic Acid, cationic surfactant, DTAC, CTAC,  
55 Supramolecular organization, Cryo-transmission electron microscopy

56

## 57 **1. Introduction**

58 Humic substances are traditionally extracted by alkali from natural organic matter (NOM)  
59 present in soils, sediments and natural waters, and are then further classified into humic acid  
60 and fulvic acid according to their solubility in water (Thurman and Malcolm 1981). Whether  
61 those operationally defined substances represent a native state of NOM has been a legitimate  
62 and recurrent debate in the literature (Burdon 2001; Kelleher and Simpson 2006; Lehman and  
63 Kleber 2015; Baveye and Wander 2019; Olk et al. 2019). Another controversial issue, also  
64 related to the nature of NOM, concerns the organization of extracted matter. Humic substances  
65 are generally regarded as macromolecular structures resulting either from the polymerisation  
66 of phenolic/peptidic/carbohydrate compounds (Stevenson 1994; Swift 1999; Gerke 2018) or  
67 from the abiotic and/or biotic degradation of original pre-existing biopolymers (Hayes et al.  
68 1989; Burdon 2001). They have also been described as micelle-like or pseudomicelles  
69 aggregates (Wershaw 1999; von Wandruszka 2000), and more recently as supramolecular  
70 assemblies of small heterogeneous molecules essentially associated through hydrogen bonds  
71 and hydrophobic interactions (Piccolo 2001; Simpson et al. 2002, Sutton and Sposito 2005).

72 The argumentation for a macromolecular humic structure is based on the fact that the humic  
73 matter retains its structural integrity through the extensive fractionation procedures that  
74 precede reliable molecular weight assessments (Swift 1999; Cameron et al. 1972). On the other  
75 hand, experimental support for a supramolecular structure essentially relies on the  
76 disaggregation of humic matter detected after the addition of organic acids to disrupt the  
77 hydrogen bonds that hold the humic components within molecular aggregates (Piccolo and  
78 Conte 1999; Piccolo 2001; Simpson et al. 2002; Smejkalova and Piccolo 2008). The enhanced  
79 solubilisation of non-polar organic compounds in the presence of humic substances,  
80 measurements of specific heat capacity, reveal the presence of hydrophobic domains within

81 the humic matter and NOM (Carter and Suffet 1982; Engebretson and von Wandruszka 1997;  
82 von Wandruszka, 2000; Chilom and Rice 2009; Chilom et al. 2013). However, such  
83 hydrophobic domains may be accounted for either by the occurrence of micellar aggregates or  
84 by the formation of hydrophobic cores upon polymer coiling. Likewise, aggregation studies of  
85 extracted humic matter and NOM do not directly identify a macromolecular or supramolecular  
86 structure (Kerner et al. 2003; Chilom et al. 2009; Wells and Stretz 2019).

87 Chaaban et al. (2016) recently proposed to probe the organization of extracted humic matter  
88 through its interaction with varying concentrations of a cationic surfactant. In the case of  
89 macromolecules, the association with the oppositely charged surfactant leads to the formation  
90 of complexes that start to aggregate at a Critical Aggregation Concentration (CAC) in  
91 surfactant (Fig. 1a). Above CAC, further addition of surfactant proceeds through cooperative  
92 binding of supplementary molecules to the complexes that progressively become decorated  
93 with micelle-like structures of surfactant (Jain et al. 2004; Bain et al. 2010; Khan and  
94 Brettmann 2019). Eventually, the polyelectrolyte/surfactant complexes re-solubilize. The CAC  
95 in macromolecule-surfactant suspensions generally occurs several orders of magnitude lower  
96 than the Critical Micellar Concentration (CMC), *i.e.* the concentration at which free surfactant  
97 molecules associate with each other to form micelles (Khan and Brettmann 2019). In the case  
98 of a supramolecular assembly of small heterogeneous molecules, the interaction with the  
99 cationic surfactant may organize new types of self-assemblies such as mixed micelles, vesicles,  
100 or lamellae (Fig. 1d), thus achieving much richer phase behaviour than what would be observed  
101 with macromolecules (Marques et al. 1998; Marques et al. 1999). The occurrence of a given  
102 self-assembly can reasonably be predicted from the geometrical characteristics of individual  
103 components according to the molecular packing concept introduced by Israelachvili et al.  
104 (1976) (Fig. 1b-c-e). In the case of an association between a cationic surfactant and anionic  
105 components such as humic matter, the assembly may contain both complexes and free

106 molecules which leads to more complicated expressions for the packing parameter (Manohar  
107 and Narayanan 2012). Applying the above approach to the Suwannee River fulvic acid  
108 (SRFA), Chaaban et al. (2016) showed that the addition of increasing amounts of Dodecyl-  
109 trimethylammonium chloride (DTAC) successively leads to fulvic acid-rich unilamellar  
110 vesicles, aggregates, DTAC-rich unilamellar vesicles, and mixed micelles. Such a sequence,  
111 consistent with a supramolecular organization of fulvic matter also suggests that a major  
112 component of SRFA has an overall molecular size similar to that of DTAC.

113 This paper extends the previous research by investigating the complexation behaviour of  
114 Suwannee River humic acid (SRHA), a reference aquatic humic matter of the International  
115 Humic Substances Society (IHSS), in the presence of a series of alkyltrimethylammonium  
116 chloride cationic surfactants ( $C_8H_{17}N^+(CH_3)_3Cl^-$  (OTAC),  $C_{12}H_{25}N^+(CH_3)_3Cl^-$  (DTAC),  
117  $C_{16}H_{33}N^+(CH_3)_3Cl^-$  (CTAC)). Turbidity, surface tension, and electrophoretic mobility  
118 measurements are combined with cryogenic transmission electron microscopy observations to  
119 examine the characteristics of complexes formed between SRHA and the homologous series  
120 of surfactants under conditions of concentration relevant to natural aquatic systems. The  
121 formation of oppositely charged humic-rich and surfactant-rich vesicles supports a  
122 supramolecular organization for SRHA.

123

124

125

## 126 **2. Experimental**

127 **2.1 Materials.** Suwannee River Humic acid (SRHA) was purchased from the International  
128 Humic Substances Society (IHSS) (Humic Acid Standard 2S101H). The isolation procedure  
129 of SRHA includes (i) filtration of the river water through a 0.45 $\mu$ m porous membrane filter,  
130 (ii) addition of concentrated HCl to lower the pH of the filtrate to 2.0, (iii) XAD-8 resin  
131 adsorption and then elution with 0.1 M NaOH followed by immediate acidification of eluate  
132 with HCl to avoid oxidation of the humic matter, (iv) centrifugation, washing with water, and  
133 freeze-drying to obtain SRHA in a solid form (Thurman and Malcolm 1981). According to  
134 Thurman and Malcom (1981), the recovery factor is better than 95% for humic substances from  
135 the Suwannee River.

136 The cationic surfactants - Dodecyltrimethyl ammonium chloride ( $C_{12}H_{25}N^+(CH_3)_3Cl^-$  – DTAC  
137  $\geq 99\%$ ), Octyltrimethyl ammonium chloride ( $C_8H_{17}N^+(CH_3)_3Cl^-$  – OTAC  $\geq 97\%$ ) and  
138 Cetyltrimethyl ammonium chloride ( $C_{16}H_{33}N^+(CH_3)_3Cl^-$  – CTAC  $\geq 98\%$ ) - were purchased  
139 from Sigma-Aldrich. The surfactants were used without further purification. Those compounds  
140 are quaternary ammonium salts: they completely dissociate in water and possess a permanent  
141 positive charge. According to literature data, DTAC is readily soluble in water at room  
142 temperature with a CMC of 21.7 mmol/L at 30°C (Perger and Bester-Rogac 2007) and a Krafft  
143 temperature below 0°C (Lachewsky et al. 2005) - the Krafft temperature of a surfactant is the  
144 minimum temperature that ensures the flexibility of the alkyl chain and the formation of  
145 micelles. The CTAC Krafft temperature is about 18°C with a CMC of 1.3 mmol/L at 30°C  
146 (Tan et al. 2010); finally, OTAC possesses a CMC of 250 mmol/L at 28°C with a Krafft  
147 temperature below 0°C (Lindblom and Lindman 1973). As the determination of the Critical  
148 Micelle Concentration is very sensitive to both the presence of impurities and the method of  
149 measurement, the CMCs of commercial surfactants were measured using a Wilhelmy plate  
150 (KRUSS K12 Processor Tensiometer). As shown in Figure S1 of Supporting Information,  
151 CMCs of 20 mmol/L, 1.1 mmol/L, and 200 mmol/L were then obtained for DTAC, CTAC, and

152 OTAC, respectively. The presence of a minimum in the surface tension curve of DTAC  
153 indicates a slight contamination by a non-ionic impurity. In that case, the re-increase in the  
154 surface tension vs concentration curve marks the CMC, the surface-active impurities leaving  
155 the air/water interface as they incorporate the forming DTAC micelles ([Martinez-Balbuena et  
156 al. 2017](#)). Our experimentally determined values of CMC are used throughout the paper.

157 **2.2 Sample preparation.** As this work was initially inspired by the literature on ternary  
158 diagrams of oppositely charged surfactants, which are generally conducted in pure water, all  
159 the solutions were prepared without any supporting electrolyte. The DTAC and CTAC stock  
160 solutions were prepared in deionised water (Millipore, MilliQ, resistivity > 18.2MΩ.cm) at 10  
161 g/L and 200 mg/L, respectively. For OTAC, stock solutions at 10, 35 and 70 g/L were necessary  
162 to minimize the aliquot volume of surfactant solution added to the SRHA suspension. The  
163 concentration of the SRHA suspension was adapted to the technique of investigation used. For  
164 turbidity, surface tension and electrophoretic mobility measurements, a SRHA suspension of  
165 20 mg/L, *i.e.* ~ 12 mg C/L, was prepared by adding the appropriate mass of humic acid into  
166 500 ml of deionised water. For Cryogenic Transmission Electron Microscopy (Cryo-TEM), a  
167 stock solution of 1g/L was used. The SRHA stock solutions were stirred overnight and then  
168 vacuum-filtered through a 0.2 µm cellulose acetate membrane (Macherey-Nagel). Fresh SRHA  
169 suspensions were prepared every day and the dissolved organic carbon of each stock  
170 suspension was checked (Shimadzu TOC-Vcsn). An average value of  $12.28 \pm 1.53$  mg C/L was  
171 obtained for the 20 mg/L suspensions. The suspensions with too much or too little DOC were  
172 discarded.

173 The SRHA/Surfactants complexes were prepared by adding aliquots of the concentrated  
174 surfactant solutions to a total of 10 mL (1 mL for Cryo-TEM) of SRHA suspension. The  
175 SRHA/surfactant suspension was gently shaken (3 times) and then placed in a thermostated  
176 bath at 30°C. The humic acid suspension and the surfactants stock solutions were also



177 maintained at 30°C during the experiments. Such temperature was selected to be above the  
178 Krafft temperature of all surfactants used in the study. The experiments were carried out at pH  
179 4.5 (equilibrium pH of the SRHA stock suspension at a 20 mg/L concentration in pure water)  
180 and at pH 6.7. In the latter case, the pH of the SRHA stock suspension was adjusted by dropwise  
181 addition of NaOH 0.1M (about 250 µL in 0.5 L stock suspension of SRHA). The pH of  
182 SRHA/surfactant mixtures was not readjusted after surfactant addition. The pH was  
183 nevertheless measured in the 10 mL vial at the end of the experiment (Fig. S3).

184 **2.3 Characterization of SRHA/Surfactants complexes.** The turbidity of SRHA/Surfactants  
185 suspensions was measured as a function of time using a HACH LANGE 2100Q turbidimeter.  
186 The conductivity and the pH (WTW Multiline F/SET), the surface tension (KRUSS K12  
187 Processor Tensiometer using a Wihelmy plate), and the electrophoretic mobility of the  
188 complexes (Malvern Zetasizer Nano ZS) were measured after 1h of mixing.

189 The cryo-TEM observations were carried out at the Multiscale Electron Imaging (METI)  
190 platform (University of Toulouse III (Paul Sabatier)) using a *JEOL 2100 Lab6* transmission  
191 electron microscope equipped with a *Gatan 626* electron microscope cryo-holder. The samples  
192 were prepared by depositing 4 µL of SRHA/Surfactant suspension onto a LACEY/CARBON  
193 FILM grid previously ionized using PELCO-easiGlow 91000 glow discharging-cleaning  
194 system. After blotting for 0.2-0.8 seconds (depending on the surfactant concentration) with  
195 filter paper to remove the excess liquid (using a *Leica EM GP* controlled vitrification system,  
196 chamber at 30 °C and 85% relative humidity to avoid evaporation of the liquid), the grid was  
197 immediately plunged into liquid ethane close to its freezing point -183 °C. The vitrified sample  
198 was then transferred to the microscope under liquid nitrogen to prevent any sample perturbation  
199 and the formation of ice crystals. The images were then recorded with a *Gatan ultrascan1000*  
200 *and Gatan ultrascan4000* camera under low dose conditions (~ 10-20 electrons per Å<sup>2</sup>).

201

## 202 **3. RESULTS**

203 **3.1 Turbidity measurements.** Figure 2 compares the turbidity curves obtained 1h after mixing  
204 a suspension of 20 mg/L SRHA with various amounts of surfactants. After 1h, the turbidity  
205 measurements, if not stable, vary less than 10% over the following hour (see Figure S2 in  
206 Supporting Information). The result of the interaction between SRHA and the homologous  
207 series of cationic surfactants is represented as a function of the surfactant concentration  
208 normalized to their respective critical micelle concentration (CMC) to account differences in  
209 water solubility of the surfactants ([Cases and Villieras 1992](#)).

210 Drastic differences are observed in the turbidity curves according to the alkyl chain length of  
211 the surfactant: OTAC addition to SRHA leads to a minor increase in turbidity even at high  
212 surfactant concentration. In contrast, the addition of DTAC to SRHA suspensions is  
213 characterized by a strong initial increase in turbidity that broadly peaks around  $0.28 \text{ CMC}_{\text{DTAC}}$   
214 (5.6 mmol/L), followed by a gradual decrease to reach low turbidity values at high surfactant  
215 concentrations. The addition of DTAC to pure water leads to a negligible increase in turbidity  
216 (fig. 2a). The SRHA/CTAC turbidity curve exhibits two very noisy peaks at roughly 0.09 and  
217  $0.18 \text{ CMC}_{\text{CTAC}}$  (*i.e.* 0.1 and 0.19 mmol/L); the noisy pattern mainly results from the presence  
218 of clearly visible but sparse aggregates in suspension. At pH 6.7 (Figure 2b), the interaction  
219 between SRHA and DTAC leads to two prominent turbidity peaks around 0.15 and  $0.45 \text{ CMC}_{\text{DTAC}}$   
220 (*i.e.* 3 and 9 mmol/L). In the low concentration range, the base of the first turbidity  
221 peak exhibits smaller turbidity values than at pH 4.5. This can be explained by the higher  
222 negative charge of SRHA at pH 6.7 ([Lead et al. 2000](#); [Hosse and Wilkinson 2001](#)), which  
223 requires higher DTAC concentrations to start forming aggregates.

224 The formation of two turbidity peaks for the associations SRHA/CTAC at pH 4.5 and  
225 SRHA/DTAC at pH 6.7, as well as the limited interaction between SRHA and OTAC at natural  
226 pH, reveal complex interactions between the humic acid and the cationic surfactants. Those  
227 interactions certainly involve the charge neutralization of SRHA anionic groups by the positive  
228 head groups of surfactants but also various hydrophobic effects driven by the alkyl chain length  
229 of surfactants. All the surfactants used in this study are 1:1 salts. However, as illustrated in  
230 Figure 2 from additions of various NaCl concentrations to the humic suspension, the change in  
231 solution ionic strength brought by the surfactant does not play any significant role in  
232 determining the turbidity of the suspension in the range of surfactant concentrations  
233 investigated. Moreover, the pH of the SRHA/surfactants suspensions remains close to the  
234 initial pH (Figure S3 in Supporting Information).

235 **3.2 Electrophoretic mobility measurements.** The electrophoretic mobility of SRHA/Surfactant  
236 complexes exhibits two different patterns as a function of surfactant concentration (Figure 3).  
237 For SRHA/DTAC complexes at both pH 4.5 and 6.7, the electrophoretic mobility increases  
238 rapidly at low surfactant additions, and then more slowly at higher DTAC concentrations.  
239 Interestingly, the point of zero mobility (PZM) reached at about 3.2 mmol/L and 5.5 mmol/L  
240 for pHs 4.5 and 6.7, respectively, cannot be related to the turbidity peaks: indeed, the PZM  
241 occurs before the turbidity peak at pH 4.5, and is situated in the valley between the two turbidity  
242 peaks at pH 6.7 (Figure 3b).

243 In contrast, for OTAC and CTAC, the electrophoretic mobility becomes more negative at low  
244 surfactant concentration, reaches a minimum at about 0.01 CMC for both surfactants, and then  
245 increases to reach positive values from 0.06 CMC<sub>CTAC</sub> in the case of CTAC, or decreases to  
246 stabilize at negative values in the case of OTAC. Unlike DTAC, the point of zero mobility of  
247 SRHA/CTAC complexes can be related to the first turbidity peak. It is worth noting that the  
248 initial electrophoretic mobility of SRHA colloids could not be measured with the Nanosizer

249 and that all the data points reported in the plots identify SRHA/surfactant complexes. Previous  
250 capillary electrophoresis measurements carried out by [Hosse and Wilkinson \(2001\)](#) led to  
251 electrophoretic mobilities of SRHA close to -3.8 and to  $-4.9 \cdot 10^8 \text{ m}^2\text{V}^{-1}\text{s}^{-1}$  at pH 4.5 and 6.7,  
252 respectively. This suggests that the electrophoretic mobility of OTAC/SRHA and  
253 CTAC/SRHA complexes comprises an initial maximum not resolved with our measuring  
254 technique.

255 **3.3 Surface tension measurements.** Figure 4 shows the surface tension of SRHA/surfactant  
256 suspensions as a function of surfactant concentration or surfactant concentration normalized to  
257 the corresponding CMC in a semi-log plot. The surface tension of pure surfactant solutions is  
258 also shown for comparison. Two main regimes can be distinguished: at very low surfactant  
259 concentration, *i.e.*  $[\text{surfactant}]/\text{CMC} < 0.1$ , the surface tension of SRHA/surfactant system  
260 decreases more rapidly with concentration than that of pure surfactant. Such synergistic effect  
261 is attributed to the strong electrostatic interaction between the humic anionic groups and the  
262 cationic headgroups of surfactant that leads to humic/surfactant complexes of enhanced  
263 hydrophobicity ([Gamboa and Olea 2006](#)). Following this decrease, the surface tension curve  
264 stabilizes or even exhibits a well-defined breakpoint in the case of CTAC. Upon further  
265 addition of surfactant, the surface tension of SRHA/surfactant system remains fairly constant,  
266 before decreasing again at higher surfactant concentration. Such additional drop in surface  
267 tension suggests the presence of non-associated surfactant in addition to the SRHA/surfactant  
268 complexes. It can also be noted that, in that concentration range ( $[\text{surfactant}]/\text{CMC} \geq 0.1$ ), the  
269 longer the alkyl chain, the higher the surface activity of SRHA/surfactant complexes. At high  
270 surfactant concentration, the surface tension of pure surfactant solutions becomes lower than  
271 that of SRHA/surfactant systems.

272 **3.4 Cryo-TEM observations.** Typical cryo-TEM images at pH 3.1 and at pH 6.7 for the  
273 SRHA/DTAC system, and at pH 3.1 for the SRHA/CTAC system, are shown in Figures 5, 6

274 and 7, respectively. At the concentration of the DTAC stock solution (38 mmol/L), *i.e.* slightly  
275 above the CMC, micelles could not be clearly seen in the micrographs (Fig. 5a). In contrast,  
276 humic structures in the 3-5 nm range and small linear aggregates of those structures, 30 to 140  
277 nm in length, are found for a SRHA concentration of 1 g/L and an equilibrium pH equal to 3.1  
278 (Fig. 5b). Such observations are consistent with previous fluorescence correlation spectroscopy  
279 measurements that suggest a partial aggregation of SRHA at low pH ([Hosse and Wilkinson](#)  
280 [2001](#); [Duval et al. 2005](#)). However, the linear aspect of aggregates likely results from the  
281 intense shearing that occurs during the blotting stage while draining the excess liquid deposited  
282 on the grid ([Lee et al. 2012](#)).

283 Addition of a  $7.6 \cdot 10^{-3}$  mmol/L DTAC concentration to the 1 g/L SRHA suspension leads to the  
284 formation of slightly irregular globules, about 25-50 nm in diameter, marginally aggregated,  
285 and with no internal structure (Fig. 5c2). Such globules may represent the early stages of  
286 interaction between DTAC molecules and SRHA and might be attributed to mixed micelles.  
287 The same amount of surfactant diluted in deionised water leads to featureless micrographs (Fig.  
288 5c1). Further additions of cationic surfactant ( $37.86 \cdot 10^{-3}$  mmol/L and  $188.5 \cdot 10^{-3}$  mmol/L)  
289 reveal a dense packing of unilamellar vesicles often opened and sometimes reminiscent of  
290 faceted self-assemblies (Fig. 5d2 and 5e) ([Vautrin et al. 2004](#)). The vesicular architecture is  
291 identified from the higher electron density at the edges of structures. The diameter of the  
292 vesicles ranges from 15 to 45 nm whereas the thickness of the vesicles walls is in the 1.2-2.8  
293 nm range. Spongy network structures are also found to coexist with the vesicles (*e.g.* Fig. 5d1).  
294 According to [Zheng et al. \(2000\)](#), those structures correspond to entangled threadlike micelles  
295 that form from some severely sheared vesicles during the blotting stage. When the DTAC  
296 concentration is increased to  $375.2 \cdot 10^{-3}$  mmol/L (Fig. 5f), irregular monodisperse structures,  
297 slightly inhomogeneous and about 16 nm in diameter, are observed. Those structures are  
298 partially aggregated and might correspond to almost neutral SRHA/DTAC complexes. Only

299 one DTAC concentration (33 mmol/L) was investigated on the DTAC-rich side of the phase  
300 diagram at natural pH (Fig. 5g). Considering the high dilution of the SRHA suspension  
301 necessary to achieve such DTAC concentration, only a few well-formed vesicles with  
302 diameters ranging 10 to 25 nm were identified (Fig. 5g).

303 At pH 6.7, an addition of  $7.6 \cdot 10^{-3}$  mmol/L of cationic surfactant to the SRHA suspension also  
304 promotes the formation of small irregular vesicular structures more complete than at pH 3.1;  
305 the size of those vesicles ranges from 10 to 50 nm (Fig. 6a1). Increase of the DTAC  
306 concentration to  $226 \cdot 10^{-3}$  mmol/L leads to well-defined spherical vesicles - 16 to 30 nm in  
307 diameter - that form multilamellar structures with ample spacing between entrapped and outer  
308 vesicles (Fig. 6b1). The wall thickness of vesicles remained in the 1.2-2.8 nm range. In both  
309 cases, some deformed vesicles are observed, thus indicating that the vesicles may either merge  
310 or separate from larger assemblies. Branched spongy networks, with budding vesicles at the  
311 higher investigated DTAC concentration, can also be found (Fig. 6a2-b2). The surfactant  
312 concentration added to reach the DTAC-rich side of the phase diagram also implies an  
313 important dilution of the SRHA suspension. At a DTAC concentration of 33 mmol/L, large (~  
314 100 nm in diameter) slightly granulated globules are observed to coexist with infrequent  
315 vesicles. Such globules represent aggregates of neutralized SRHA/DTAC complexes, whereas  
316 an increase of DTAC concentration to 34.9 mmol/L lead to partially aggregated vesicles, about  
317 20 nm in size (Fig. 6d). Therefore, at both investigated pH, a sequence of structures including  
318 humic-rich vesicles, aggregated complexes, and DTAC-rich vesicles, can be observed with  
319 increasing DTAC concentration.

320 As illustrated in Figure 7, when a cationic surfactant with a longer alkyl chain is added to the  
321 SRHA suspension at natural pH, *i.e.* CTAC (C16), aggregated globules are the prevailing  
322 molecular structures. The size of globules increases with the CTAC concentration, from 12-20  
323 nm in size at  $3.12 \cdot 10^{-5}$  mmol/L to 20-32 nm at  $18.7 \cdot 10^{-5}$  mmol/L, reaches 30-60 nm at 93.6

324  $\times 10^{-5}$  mmol/L, and then decreasing to around 40 nm at  $311 \times 10^{-5}$  mmol/L. The aggregates of  
325 globules are ramified at low CTAC concentration, becoming more compact as the surfactant  
326 concentration is increased, and appear to redisperse at the highest investigated concentration  
327 of CTAC.

328 The difference in behaviour between SRHA/DTAC and SRHA/CTAC complexes can be  
329 related to the length of the surfactant alkyl chain. In the case of CTAC, the temperature of the  
330 suspension is only slightly above the Krafft temperature of the surfactant. Upon interaction  
331 between CTAC and SRHA, the neutralization of the negatively charged functional groups of  
332 the humic structure by the positive ammonium headgroups of the surfactant increases the  
333 hydrophobicity of the resulting complex. Near the chain melting temperature of the free  
334 surfactant, the additional hydrophobic interactions between the alkyl chain and the humic  
335 complex might stiffen the CTAC chain, thus facilitating the formation of a precipitate. The  
336 presence of faceted vesicles in the SRHA/DTAC system certainly reflects the coexistence of  
337 ordered alkyl chains in the molecular structures generated with the C12 surfactant ([Vautrin et](#)  
338 [al. 2004](#)).

339

## 340 **4. DISCUSSION**

341 *4.1 Complexation of SRHA with alkyltrimethylammonium cationic surfactants.* Previous  
342 studies on the interactions between humic substances and cationic surfactants were interested  
343 in two main aspects: (i) the fate of the surfactant molecule introduced in the natural  
344 environment either as an organic contaminant through wastewater discharge ([Koopal et al.](#)  
345 [2004](#); [Ishiguro et al. 2007](#); [Hakim and Kobayashi 2019](#)) or as a detergent for the remediation  
346 of aquifers and contaminated soils ([Harwell et al. 1999](#); [Mao et al. 2015](#)); (ii) the chemical  
347 structure of the humic substances, the surfactant being used as a probe of amphiphilic

348 properties of humic matter or as a titrant of the total amount of carboxyl functional groups  
349 (Tombacz et al. 1988; De Nobili et al. 1990; Yee et al. 2006; Shang and Rice 2007; Chaaban  
350 et al. 2016). Overall, the reports concur that the binding of the cationic surfactant to the humic  
351 substance initially proceeds through strong electrostatic interactions between the carboxylic  
352 groups of humic matter and the positively charged head groups of the surfactant. This leads to  
353 the formation of a precipitate when the charge neutralization of the humic matter is achieved  
354 (De Nobili et al. 1989; Koopal et al. 2004; Hakim and Kobayashi 2019). Beyond the point of  
355 charge neutralization, supplementary binding of cationic surfactant to the humic/surfactant  
356 complexes occurs through cooperative hydrophobic effects. A charge reversal and a  
357 restabilization of humic/surfactant complexes can also be observed in this region (De Nobili et  
358 al. 1990; Ishiguro et al. 2007; Hakim and Kobayashi 2019). Highly surface-active complexes  
359 are formed at very low surfactant concentrations, which suggests that structural rearrangements  
360 of the humic substances may occur upon the addition of cationic surfactant (Koopal et al. 2004;  
361 Gambo and Olea 2006).

362

363 Our findings are consistent with the literature, yet they provide further precision about some  
364 key features of the complexation of humic matter with cationic surfactants. The contrasting  
365 evolution of turbidity-surfactant concentration curves, normalized to the respective solubility  
366 of surfactants, suggest that the alkyl chain length plays a significant role in the association  
367 between the cationic surfactant and SRHA for the low surfactant concentrations. Indeed, the  
368 addition of OTAC to the SRHA suspension only produces a minor increase of turbidity in  
369 comparison with that induced by CTAC, even though the electrophoretic mobility curves of  
370 resulting complexes indicate similar neutralization behaviours at low surfactant concentrations  
371 (Fig. 2-3). A likely explanation is that the order-disorder transition of surfactant alkyl chain  
372 occurs at increased temperature after interaction of surfactant with the hydrophobic moieties



373 of humic matter. In the case of DTAC, the presence of vesicles implies that the alkyl chain  
374 maintains a high degree of conformational disorder upon association with SRHA. Likewise,  
375 the shorter alkyl chain of OTAC should also remain disordered. However, the C16-alkyl chain  
376 of CTAC certainly becomes ordered in the SRHA/CTAC complex as suggested by (i) the early  
377 formation of a compact precipitate (Fig.7), and (ii) the surface tension of SRHA/CTAC  
378 suspensions similar to that of pure water in the low surfactant concentration regime unlike the  
379 surface tension of SRHA/DTAC and SRHA/OTAC suspensions (Fig. 4). It is indeed peculiar  
380 that the complexes formed with the longer-chain surfactant are the less surface active at low  
381 surfactant concentrations, thus suggesting that the alkyl chain does not stick out of water under  
382 those conditions.

383 The minimum exhibited in the electrophoretic mobility curves of SRHA/CTAC and  
384 SRHA/DTAC complexes is also an intriguing feature. A similar trend has been reported for  
385 the Suwannee River fulvic acid/DTAC system. In that case, the minimum is interpreted as the  
386 result of a rearrangement of fulvic acid upon neutralization with the cationic surfactant  
387 (Chaaban et al. 2016). Indeed, the initial neutralization of external sites of humic matter by the  
388 cationic surfactant enhances the hydrophobicity of the complex, that then undergoes a  
389 structural rearrangement turning inside-out some previously unattainable negatively charged  
390 sites (Sieliechi et al. 2008). Our results are consistent with such interpretation, although, in the  
391 case of DTAC, the minimum in electrophoretic mobility certainly occurs at surfactant  
392 concentrations slightly lower than those investigated in this study.

393 Only partial neutralization of SRHA is reached with the addition of OTAC, whereas CTAC  
394 and DTAC promote a complete charge compensation of the humic matter. Using the titration  
395 data of SRHA reported by Ritchie and Perdue (2003), it can be verified that the amount of  
396 CTAC added at the point of charge compensation, *i.e.* 0.077 mmol/L, is close to the amount of  
397 negative charges of SRHA at pH 4.5, *i.e.* 0.066 meq/L. This indicates that most (around 85%)

398 CTAC molecules are bound to SRHA. Similar calculations indicate the opposite situation for  
399 DTAC where a minor amount of surfactant molecules seems to be associated with the humic  
400 matter at the point of charge neutralization at either pH 4.5 or 6.7. Such results are in line with  
401 the binding isotherms of dodecyl-pyridinium chloride and cetyl-pyridinium chloride to various  
402 humic acids reported by Ishiguro et al. (2007) and Yee et al. (2006).

403 As the neutralization of SRHA is carried out with monovalent cationic surfactants, further  
404 binding of surfactant above the point of charge compensation is necessarily driven by  
405 cooperative hydrophobic interactions either between interacting alkyl tails of the surfactant or  
406 between alkyl chains and hydrophobic moieties of the humic matter. This change in surfactant  
407 binding mode and the resulting charge reversal of complexes, have been well documented in  
408 previous studies (Ishiguro et al. 2007; Hakim and Kobayashi 2019). However, the break in the  
409 surface tension curve that defines the CAC is also known to signal the onset of cooperative  
410 association of surfactant to the complexes in polyelectrolyte/surfactant systems (Jain et al.  
411 2004; Bain et al. 2010). In our case, CACs of 3.6 mmol/L, 0.4 mmol/L, and 0.022 mmol/L are  
412 obtained at pH 4.5 for OTAC, DTAC, and CTAC, respectively; a CAC of 0.7 mmol/L is found  
413 for DTAC at pH 6.7 (Fig. 4). The CAC values determined for DTAC and CTAC are therefore  
414 much lower than the surfactant concentrations necessary for charge neutralization of SRHA.  
415 Interestingly, The CAC concentrations are in the vicinity of minima of electrophoretic mobility  
416 curves. Furthermore, even though the degree of conformational disorder of surfactant alkyl  
417 chain may vary in the SRHA/surfactant complexes, the logarithm of CACs can be related to  
418 the surfactant chain length just as  $\log(\text{CMC})$  of cationic surfactants (Fig. S4 in Supporting  
419 information). Such dependence implies that micellization is initiated in the process of  
420 SRHA/surfactant interaction and well before charge neutralization. Therefore, after non-  
421 cooperative binding of cationic surfactant to the external carboxyl groups of SRHA, the pooling  
422 of alkyl chains of bound surfactant probably triggers a rearrangement of humic matter to make

423 additional negatively charged sites available for binding. A rapid calculation gives a value of  
424 3.2 kJ/mol for the free energy of binding per CH<sub>2</sub> group which is consistent with previous  
425 studies on humic substances/surfactant binding (Yee et al. 2006).

426

427 **4.2 Clues to a supramolecular organization of extracted humic matter.** The use of a surfactant  
428 as a probe of a given molecular structure, polymeric or self-assembly, primarily relies on the  
429 aggregation behaviour upon addition of increasing concentrations of surfactant. In the  
430 SRHA/n-alkyl trimethylammonium chloride system, both pH and alkyl chain length of the  
431 surfactant, *i.e.* charge of humic matter and strength of hydrophobic interactions, strongly  
432 influence the aggregation. If we only consider DTAC, the turbidity pattern is reminiscent of a  
433 polyelectrolyte/surfactant-like interaction at pH 4.5 (Bakshi and Sachar 2004), whereas the  
434 observation of two distinct turbidity peaks at pH 6.7 is in line with the phase behaviour of a  
435 mixture of oppositely charged surfactants (Marques et al. 1998, 1999), *i.e.* implies a  
436 supramolecular structure of humic matter. Obviously, both molecular structures cannot  
437 simultaneously apply to describe the organization of SRHA.

438 Another key feature in identifying the organization of extracted humic matter is the nature of  
439 turbidity that results from the SRHA/surfactant interaction. At acid pH, direct imaging using  
440 cryo-TEM evidences a sequence of structures comprising globules tentatively attributed to  
441 mixed micelles, small unilamellar humic-rich vesicles, presumably aggregated structures, and  
442 then DTAC-rich vesicles. At pH 6.7, an equivalent sequence of humic-rich vesicles,  
443 SRHA/DTAC aggregated structures, and DTAC-rich vesicles can be observed. Such sequences  
444 bear striking similarities with the phase behaviour of aqueous mixtures of anionic and cationic  
445 surfactants (Herrington et al. 1993; Marques et al. 1998, 1999). Indeed, mixing of oppositely  
446 charged surfactants typically leads to mixed micelles, anionic-rich vesicles, equimolar

447 aggregates, cationic-rich vesicles, and mixed micelles, as a function of mixing ratio. Therefore,  
448 the sequence of molecular structures resulting from the interaction of SRHA with increasing  
449 concentrations of DTAC supports a supramolecular organization of extracted humic matter.  
450 Such conclusion, reached with 1h mixing time, is unlikely to be reversed by using longer  
451 mixing times. The SRHA/DTAC turbidity curve at pH 4.5 may then reflect the close vicinity  
452 of the two regions of vesicles stability in the phase diagram. Moreover, an increased  
453 protonation of SRHA clearly facilitates the formation of a high number of vesicles, thus  
454 suggesting a facilitated restructuring.

455 Synthetic amphiphilic block copolymers have also been shown to assemble into micelles,  
456 lamellae, and a variety of complex vesicular structures including flowerlike vesicles or stacked  
457 bowls (Liu et al. 2019; Wang et al. 2019). However, in that case, the transition process from  
458 one molecular structure to another generally involves shear-induced transformations and the  
459 obtained assemblies are kinetically trapped. In our study, all the vesicles were spontaneously  
460 formed which is also a supplementary feature of aqueous mixtures of anionic and cationic  
461 surfactants (Kaler et al. 1992; Marques et al. 1999). In addition, in the case of amphiphilic  
462 block copolymers the thickness of vesicle wall is about 10 to 30 nm, whereas less than 2.8 nm  
463 thick walls, *i.e.* less than the expected thickness of a bilayer formed from 12-carbon tail  
464 surfactants, was found for the vesicles formed in the SRHA/DTAC system.

465 The thinness of vesicles walls suggests that the building blocks of humic matter are of a size  
466 similar to that of surfactant molecules. Such result is consistent with previous size estimates of  
467 humic matter (Lead et al. 2000). Not knowing the amount of DTAC bound to SRHA, the  
468 geometric packing constraints associated with the formation of vesicles cannot be used to better  
469 define the size of those humic constituents. Nevertheless, estimates for the mean cross-  
470 sectional surface area per SRHA/DTAC complex  $a_{SRHA/DTAC}$  at the air/water interface can be

471 inferred from the surface tension measurements using the Gibbs adsorption isotherm at the  
472 maximum value of  $d\gamma/d\ln(C)$  (Eastoe et al. 1996; Gamboa and Olea 2006):

$$473 \quad a_{SRHA/DTAC} = \frac{1}{\Gamma_{\max} N_a} = - \frac{RT}{N_a} \frac{d\ln(C)}{d\gamma_{eq}}$$

474 in which  $\Gamma_{\max}$  is the surface excess of SRHA/DTAC complexes,  $N_a$  Avogadro's number,  $R$  the  
475 ideal gas constant,  $T$  the absolute temperature,  $\gamma$  the surface tension, and  $C$  the molar  
476 concentration of surfactant. The least-squares fits to the pre-CMC  $d\gamma/d\ln(C)$  data lead to  
477  $a_{SRHA/DTAC}$  values of  $191 \pm 32 \text{ \AA}^2$  and  $69 \pm 25 \text{ \AA}^2$  at pH 4.5 and at pH 6.7, respectively (The  
478 confidence intervals for the  $a_{SRHA/DTAC}$  values are obtained using a Student t-test with a  
479 significance level of 5%). Such values compare favourably with those obtained by Gamboa  
480 and Olea (2006) in the Aldrich humic acid/alkyltrimethyl ammonium Bromide system. In  
481 mixtures of oppositely charged surfactants, previous studies have shown that the mean area per  
482 molecule of complex is slightly less than the sum of individual components cross-sectional  
483 surface areas (Rodakiewicz-Nowak 1981; Eastoe et al. 1996). Using a value of  $40 \text{ \AA}^2$  for  $a_{DTAC}$   
484 (Weiner and Zografis 1965),  $a_{SRHA}$  should then be about  $30 \text{ \AA}^2$  at pH 7 and  $150 \text{ \AA}^2$  at natural  
485 pH. Those cross-sectional surface areas fall in the  $30$  to  $190 \text{ \AA}^2$  range reported in the literature  
486 for humic acids (Chen and Schniter 1978; Kawahigashi and Fujitake 1998; Terashima et al.  
487 2004; Gamboa and Olea 2006; Chaaban et al. 2016). Nevertheless, it should be noted that in  
488 the pre-CMC linear region, SRHA/DRAC complexes remain slightly charged. As a result,  
489 because of electrostatic repulsion, close packing may not be reached and the mean surface area  
490 occupied by the complexes at the air/water interface is certainly slightly overestimated. In any  
491 case, such molecular area is much smaller than those typically found for polyelectrolytes  
492 (Caminati and Gabrielli 1993).

## 493 5. CONCLUSIONS

494 The results presented in this paper shed new light on the interaction mechanisms involved  
495 between humic acid and cationic surfactants under environmentally relevant conditions. In  
496 particular, we show that the hydrophobic interactions occurring between either alkyl chains of  
497 bound surfactants or surfactant alkyl chains and hydrophobic moieties of humic matter, induce  
498 a drastic rearrangement of the humic structure at low surfactant concentration. Those  
499 hydrophobic interactions may stiffen the alkyl tails of surfactants, *i.e.* decrease the order-  
500 disorder transition temperature of alkyl chain, which in the case of C16 chain length promotes  
501 the formation of precipitates. In contrast, shorter alkyl chains remain flexible and trigger the  
502 formation of small nanoscale vesicles in the case of DTAC. Cooperative hydrophobic binding  
503 of surfactant molecules also concurs to the neutralization and the charge reversal of  
504 humic/surfactant complexes except for the short C8-alkyl chains. The formation of vesicular  
505 structures and the high surface activities of complexes formed between humic acid and DTAC,  
506 offer renewed perspectives for the use of cationic surfactants in the remediation of  
507 contaminated ecosystems. Indeed, because of enhanced adsorption onto negatively charged  
508 soil particles and acute toxicity for microorganisms, cationic surfactants are generally not used  
509 for the treatment of oil-contaminated environments (Mao et al. 2015). The possibility of  
510 obtaining negatively charged nanosize vesicles from complexes of humic matter and cationic  
511 surfactant would certainly improve both transport properties and environmental compatibility  
512 of cationic surfactants.

513 The phase behaviour of SRHA/DTAC complexes and the spontaneous formation of nanoscale  
514 unilamellar vesicles are key features of mixtures of oppositely charged surfactants. Such results  
515 support the supramolecular view of humic matter and are completely in line with the  
516 amphiphilic nature of humic substances (Chen and Schnitzer 1978; Guetzloff and Rice 1994;  
517 von Wandruszka 2000; Klavins and Purmalis 2010). As humic substances have been shown to  
518 be mixtures of heterogeneous compounds (Simpson et al. 2002; Sutton and Sposito 2005), it is

519 possible that only part of the humic building blocks participate to the formation of vesicular  
520 structures, the remaining compounds not being detected by our investigation techniques.  
521 Moreover, a self-assembled humic matter suggests that part of NOM might be supramolecular  
522 but does not imply that all NOM is supramolecular. Further investigations should assess how  
523 the extraction process of humic substances could affect their organization structure.

524

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