

# Aggregation and charge reversal of humic substances in the presence of hydrophobic monovalent counter-ions: Effect of hydrophobicity of humic substances

著者別名	小林 幹佳
journal or publication title	Colloids and surfaces. A, Physicochemical and engineering aspects
volume	540
page range	1-10
year	2018-03
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URL	<a href="http://hdl.handle.net/2241/00151510">http://hdl.handle.net/2241/00151510</a>

doi: 10.1016/j.colsurfa.2017.12.065

1     **Aggregation and charge reversal of humic substances in the presence of**  
2     **hydrophobic monovalent counter-ions: effect of hydrophobicity of humic**  
3                                     **substances**

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## Abstract

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To investigate the effect of hydrophobicity of humic substances (HSs) on their charging and aggregation, we studied the electrophoretic mobility and aggregation-dispersion of HSs in the presence of hydrophobic monovalent cations, namely, tetraphenylphosphonium  $\text{TPP}^+$ . The used HSs were standard Suwannee river fulvic acid (SRFA), Suwannee river humic acid (SRHA), and Leonardite humic acid (LHA) with different contents of aromatic carbons. All of the HSs in the presence of  $\text{TPP}^+$  showed charge reversal. The charge reversal pH or iso-electric point (IEP) of LHA was higher than that of SRFA and SRHA in every concentrations of  $\text{TPP}^+$ , demonstrating the strong hydrophobic interaction between HSs, especially LHA, and  $\text{TPP}^+$ . We also found that the formation of large visible aggregates of all the HSs at lower pH in the presence of  $\text{TPP}^+$ . Large HS aggregates were markedly formed for LHA, manifesting the existence of stronger hydrophobic attraction among LHAs with  $\text{TPP}^+$ . The appearance of aggregates of all the HSs with  $\text{TPP}^+$  was confirmed by microscopic observation and the size determination by dynamic light scattering. The HS aggregates showed fractal structure. The values of fractal dimension  $D_f$  of HS aggregates were 2-2.2 in quiescent conditions, indicating that the HS aggregates with  $\text{TPP}^+$  were formed via cluster-cluster aggregation with restructuring. The  $D_f$  increased to 2.8-2.9 in stirring conditions, implying that the compact aggregates were formed through the continuous aggregate breakage and the regrowth between smaller aggregates and larger aggregates.

Keywords: humic substances, charge reversal, aggregation-dispersion, hydrophobicity

## 32 **Introduction**

33 Humic substances (HSs) are considered as one of the most important sources or sink of  
34 organic carbons in soil and water environments [1] and play a vital role in nutrient cycling, and  
35 the fate and transport of pollutants [2]. The surface active binding sites and highly reactive  
36 nature of HSs [3] influence the binding of organic and inorganic contaminants and also affect  
37 the bioavailability of metal ions and mobility in soil environment [4]. Some previous studies  
38 specially highlight on the structural heterogeneity and the molecular conformation such as  
39 supramolecular and self-assembly [5-8], fractal aggregate [9-13], and spherocolloids, [14].  
40 Some studies on the adsorption of humic substances on minerals surfaces [15] and aggregates  
41 formation [16] were carried out. The charging and aggregation behaviors of minerals particles  
42 and nanoparticles [17-20] with humic substances were also investigated. Nevertheless, there  
43 are scanty data on the structure of humic substances aggregates and the charge reversal of  
44 humic substances itself as natural colloids. A recent study on humic acid co-precipitation with  
45 ferrihydrite discussed about the unlikeliness of the humic acid used as a model colloid, though  
46 the use of humic acid as network of linear macromolecules and large colloids is more consistent  
47 [21].

48 Humic substances with  $\text{Ca}^{2+}$ , lysozyme, surfactants, and polymer can be aggregated to form  
49 large visible and settleable aggregates [4, 22, 23], which are crucially important for the fate  
50 and separation of HSs with chemicals. Generally aggregation and dispersion of colloidal  
51 particles is discussed on the basis of Derjaguin, Landau, Verwey and Overbeek (DLVO) theory,  
52 which assumes that the interparticle interaction is given as the sum of van der Waals and  
53 electrostatic interactions. The latter interaction is influenced by the electric charge of particles.  
54 The aggregation usually occurs at the condition where the net charge becomes zero. Such  
55 condition is called charge neutralization detected as an iso-electric point obtained from

56 electrophoresis. The charge neutralization is often realized by the adsorption of appropriate  
57 amount of oppositely charged ionic substances such as polymers, surfactants, and proteins. The  
58 bindings and adsorption of metal ions depend on different factors such as pH, humic  
59 concentration, types of humic substances, ionic strength, temperature, ternary complexes  
60 formation, etc [24]. The binding of cationic surfactants to humic acid was observed due to the  
61 combined effect of electrostatic and hydrophobic interactions, whereas no notable binding was  
62 observed between anionic surfactants and humic acid [25]. In some cases the overdose of  
63 absorbable ionic substances induces charge reversal. Screening of electrostatic interaction and  
64 charge reversal are observed by the addition of various amounts of polyelectrolyte and  
65 oppositely charged ion as influenced by the amount of adsorption. Several recent researches  
66 report that the hydrophobic interaction plays an important role in charge reversal and/or charge  
67 neutralization for different natural and synthetic colloids and biomaterials [26-31].

68 In addition to the DLVO interaction, some other forces and interactions such as the  
69 hydrophobic interactions [22, 23, 26-31], hydrogen bonding [32, 33], hydration forces [34, 35],  
70 and depletion interactions [36] are recognized to play an important role in aggregation-  
71 dispersion of colloid particles in different environmental systems. Humic substances from  
72 different sources show the aggregation and sedimentation behaviors in the presence of  
73 inorganic salts, organic surfactants, proteins and enzymes [22, 23, 27, 37]. Nevertheless, the  
74 mechanisms behind the aggregation and charging behaviors of humic substances in the  
75 presence of adsorbing ions are still vague. Therefore, the research on the fundamental colloidal  
76 behavior of humic substances is still important in the field of natural colloids.

77 Big hydrophobic ions, such as tetraphenylboron, tetraphenylarsonium, and  
78 tetraphenylphosphonium, are used to modify the interfacial properties of clay colloids [38, 39]  
79 and to monitor permeability and interaction of the lipid membranes and biological cells [40,  
80 41]. Recently, big hydrophobic tetraphenylboron anions and tetraphenylarsonium cations are

81 also selected as tracers to study the extent of hydrophobicity of typical proteins [42]. While it  
82 is inferred that the hydrophobicity of humic substances plays crucial roles in the adsorption  
83 and aggregation, the interaction of humic substances with such big hydrophobic ions have  
84 never been reported.

85 In this context, we came up with performing the study on the influence of hydrophobic  
86 tetraphenylphosphonium (TPP) cations on the behaviors of humic substances. We found that  
87 TPP significantly affects the aggregation and charge reversal of three different humic  
88 substances with different hydrophobicity. In this paper, we report the result of the study on the  
89 electrophoretic mobility and aggregation behaviors of the humic substances with TPP in  
90 different concentration and pH of the solution. To the best of the authors' knowledge, our  
91 results are the first mobility data demonstrating the charge reversal of humic substances itself  
92 and thus are of novelty. We expect that the results obtained in this research can be generalized  
93 to the study of the removal of hydrophobic pollutants and aggregation-dispersion of HSs with  
94 hydrophobic organic pollutants such as dyes and drugs.

## 95 **Experimental**

### 96 **Materials**

97 Three humic substances (Suwannee river fulvic acid, Suwannee river humic acid and  
98 Leonardite humic acid) from International Humic Substances Society (IHSS) were used as  
99 natural organic matters in this study. These are referred to as SRFA, SRHA, and LHA hereafter.  
100 The primary stock solutions of the humic substances were prepared by dissolving solid samples  
101 to KOH solution (Wako Pure Chemical Industries) containing base equivalent or more than the  
102 amount of carboxylic acid groups of each humic substance. Before dissolving the solid sample  
103 to KOH solutions, the supplied samples were oven-dried at 65°C overnight to reduce moisture.  
104 After dissolving solid humic substances to KOH solutions, the suspension (wt. %) were stirred

105 overnight, then the secondary standard solutions were prepared by dilution with deionized  
106 water (Elix, Millipore) to a concentration 500 mg/L. The pH values of the secondary standard  
107 solutions of humic substances were around neutral (6.5-7.2). The concentration of humic  
108 substances (HSs) was maintained at 50 mg/L in every measurement in this study. Some of the  
109 parameters of the HSs from IHSS are listed in Table 1. The HSs used in this research were  
110 chosen depending on their charge groups and hydrophobicity differences based on their carbon  
111 content and aromaticity.

112 The electrolytes of simple KCl (JIS special grade, Wako Pure Chemical Industries) and  
113 hydrophobic tetraphenylphosphonium chloride (TPPCL) (EP grade, Tokyo Chemical Industry  
114 Co.) were used to examine the effect of hydrophobic ions. The salt concentrations were from  
115 10 mM to 100 mM. The pH was controlled by using HCl (Wako Pure Chemical Industries) or  
116 KOH. Carbonate free KOH solutions were prepared by following the method described in ref.  
117 [43]. The salt solutions were filtered (DISMIC 25HP 0.2  $\mu\text{m}$ , ADVANTEC) in every new  
118 preparations. All the solutions of this study were degassed under reduced pressure (GCD-051X,  
119 ULVAC) to avoid the CO<sub>2</sub> contamination prior to our experiments.

## 120 **Methods**

### 121 Electrophoretic mobility measurements

122 The electrophoretic mobilities of the three humic substances (HSs) were measured in the  
123 presence of both KCl and tetraphenylphosphonium chloride (TPPCL) at 20 °C with a Zetasizer  
124 Nano ZS apparatus (Malvern Instruments). The electrophoretic mobility measurements were  
125 carried out as a function of salt concentration of KCl and TPPCL. The KCl of 10 mM and 50  
126 mM and the TPPCL of 10 mM, 50 mM, and 100 mM were used as a function of pH (3-10). For  
127 each condition of salt concentration, the measurements were reproduced and every experiment  
128 was done at least two times and we got similar data. For the measurements of electrophoretic  
129 mobility, all secondary standard solutions of humic substances were sonicated one-time for 20

130 minutes before mixing with water, salts (TPPCL) and KOH/HCl. The concentration of humic  
131 substances (SRFA, SRHA, and LHA) was maintained at 50 mg/L. The pH values of the  
132 suspension of each measurement were checked by a combination electrode (ELP-035, TOA-  
133 DKK).

#### 134 Macroscopic and microscopic observation of aggregation and dispersion

135 Visual observations of aggregates of humic substances (SRHA, SRFA, and LHA) in TPPCL  
136 solutions were performed at 50 mM of TPPCL with 50 mg/L of humic substances of each type  
137 as a function of pH for 24 hours. A series of 5 mL suspensions of 50 mg/L humic substances  
138 in 50 mM of TPPCL at pH 3-10 was prepared in the prewashed and screw-capped polystyrene  
139 bottles and left stand for 24 hours. The pH was controlled by the addition of KOH and HCl. It  
140 was hard to definitely control the sharp pH value at each cases of the experimental  
141 measurement because of the protonation and de-protonation of humic substances at various pH.  
142 The macroscopic pictures and videos (see supporting information) of the suspensions after 24  
143 hours were taken for visual confirmation of the formation of aggregates.

144 After 24 hours later, the aggregated and dispersed suspensions of humic substances in  
145 TPPCL were observed under a microscope for a range of pH from 3 to 9. This microscopic  
146 study was performed to evaluate the tentative size and arrangements of aggregates of humic  
147 substances with TPPCL salts. The microscope used in this study was Shimadzu BA210E,  
148 Moticam 580INT.

#### 149 Dynamic light scattering

150 The size of the particles and/or aggregates of humic substances in TPPCL was determined by  
151 dynamic light scattering (DLS) technique applying backscattering detection (173° detection  
152 optics) at 20 °C with a Zetasizer Nano ZS apparatus (Malvern Instruments). Cleaned disposable  
153 cuvettes containing 1 mL of sample were used in every measurement of DLS. The sample  
154 suspensions of humic substances with TPPCL in the cuvettes were briefly sonicated for 2



155 minutes after mixing, and then the measurements for DLS just started after 5 minutes from the  
156 mixing. After measurements started, it took times for temperature equilibrium at 20°C and for  
157 the optimization of optical condition. Each measurement consisted of 5 runs and the duration  
158 of each run was 10 s.

### 159 Aggregate structure analysis

160 For the aggregates of humic substances (50 mg/L) formed with 50 mM TPPCl, a structural  
161 analysis was performed. For the purpose of aggregates structure analysis, the determination of  
162 fractal dimension of aggregates was carried out by small angle light scattering using SALD  
163 2300 (SHIMDZU), by which the relation of scattered light intensity  $I$  with scattering angle, is  
164 obtained. In the medium of suspension, the magnitude of the incident and scattered wave  
165 vectors difference of light, a scattering vector, is denoted as  $Q$ , and the  $Q$  is expressed as the  
166 following equation (1)

167

$$168 \quad Q = \frac{4\pi n \sin\left(\frac{\theta}{2}\right)}{\lambda} \quad (1)$$

169

170 where  $n$  is the refractive index of the suspending medium,  $\theta$  is the scattering angle, and  $\lambda$  is  
171 the wavelength of the laser light in a vacuum. The fractal dimension  $D_f$  is determined as the  
172 relation of  $I$  and  $Q$  for scattering aggregates. That is, relation among  $I$ ,  $Q$  and  $D_f$  is expressed  
173 as (2)

174

$$175 \quad I \propto Q^{-D_f} \quad (2)$$

176

177 Thus, the log-log scale plot of  $I$  against  $Q$ , with the power law relationships yields a straight  
178 line in a fractal regime. The slope of this straight line is used as fractal dimension  $D_f$  in this  
179 study. Measurements were carried out after mixing of the suspension at pH 3 as a function of

180 different time intervals. The experiment for aggregates structure analysis was carried out no  
181 stirring and upon stirring conditions in a batch cell of SALD 2300 (SHIMDZU) using 12 mL  
182 of solutions in every set-up.

183 Table 1. Some selected composition of the three humic substances reported by IHSS.

IHSS samples	Carbon content % (w/w)	Carboxylic groups (meq/g-C)	Phenolic groups (meq/g-C)	Aromatic carbon (peak area percentages) (165-110 ppm)
SRFA II (Suwannee river fulvic acid)	52.34	11.17	2.84	22
SRHA II (Suwannee river humic acid)	52.63	9.13	3.72	31
LHA (Leonardite humic acid)	63.81	7.46	2.31	58

184

## 185 **Results and Discussion**

### 186 Electrophoretic mobility in KCl

187 The electrophoretic mobility of three HSs (SRFA, SRHA, and LHA) in the presence of KCl  
188 at various pH values are presented in the supporting information. Electrophoretic mobility is  
189 presented with the difference of hydrophobicity (Table 1) of these three humic substances at  
190 10 mM and 50 mM KCl concentrations (see supporting information). All the values of mobility  
191 of HS in KCl solution are negative and thus we observe no charge reversal of HSs in the KCl  
192 solution.

## 193 Electrophoretic mobility of HSs in the presence of TPPCl

194 The electrophoretic mobility of humic substances in the presence of hydrophobic  
195 tetraphenyl phosphonium chloride (TPPCL) at the pH range 3-10 in 10 mM, 50 mM and 100 mM  
196 of TPPCl concentrations is presented in Fig. 1. We find the obvious charge reversal for all three  
197 humic substances (SRFA, SRHA, and LHA) at every concentrations of TPPCl (10 mM, 50  
198 mM, and 100 mM) as demonstrated in Fig. 1. The charge reversal is probably induced by  
199 hydrophobic interactions between  $\text{TPP}^+$  and HSs as inferred from the result in earlier literature  
200 for well-characterized colloidal particles; hydrophobic ions give rise to the charge reversal of  
201 hydrophobic colloids [26, 29, 30, 44, and 46]. The results indicate that all the HSs are  
202 hydrophobic in nature, while FA is well-dissolved in water at all pH. This is because  
203 hydrophobic resin is used to collect FA, meaning that rather hydrophobic fraction of dissolved  
204 natural organic matter at all pH can be collected as fulvic acid.

205 The pH where the charge reversal occurs or the iso-electric point (IEP) of these HSs  
206 increases with the increase in TPPCl concentration (Fig. 1). The IEP also shifts towards higher  
207 pH values with the increase of hydrophobicity or aromaticity (hydrophobicity:  
208 LHA>SRHA>SRFA, see Table 1). At low TPPCl concentration (10 mM), the IEP for SRFA  
209 and SRHA is around pH 3 and it is around pH 4 for LHA. The higher the hydrophobicity is,  
210 the higher the pH of the charge reversal point is. The result indicates more hydrophobic  
211 interaction prevails in the case of LHA with TPPCl than that of SRFA and/or SRHA with  
212 TPPCl.

213 In the presence of TPPCl, the pH range of charge reversal of LHA is more than that of SRFA  
214 and SRHA (Fig. 1 A- Fig. 1 C). The IEPs or charge reversal points for LHA are around pH 4,  
215 pH 6 and pH 7, which are higher than those of SRFA and/or SRHA at pH around 3, 5 and 6 in  
216 10 mM, 50 mM and 100 mM TPPCl, respectively. Hyuang and Kim [45] reported that HSs  
217 with higher content of aromatic groups adsorb more on hydrophobic carbon nanotubes. Thus,

218 Figure 1 provides the evidence of strong hydrophobicity of LHA. Hakim et al. [26] and  
219 Sugimoto et al. [46] suggest that hydrophobic interaction is strong for weakly charged surface.  
220 This suggestion is supported by the data in Figure 1. That is, the charge reversal occurs at low  
221 pH, where HSs are weakly charged. An investigation on the interaction of humate and  
222 cetyltrimethylammonium (CTAB) confirmed the formation of humate-CTAB complex and the  
223 occurrence of charge neutralization and charge reversal by electrostatic and hydrophobic  
224 interactions [47]. These results also support our study.

225 Figure 1 shows that the absolute value of electrophoretic mobility decreases with the  
226 increase of TPPCl concentrations (10 mM-100 mM) at the pH range (7-10). This reduction  
227 could be explained by the compensation of surface charge by the adsorption of TPPCl on humic  
228 substances. The charge reversal points, where the positive electrophoretic mobility of HSs  
229 reverses to negative mobility increases with the increase in TPPCl concentration from 10 mM  
230 to 50 mM. At pH 7-10 at 10 mM and 50 mM of TPPCl, however, the magnitude of mobility  
231 decreases. While this reduction is probably related to the change in conformation of HSs and  
232 ion distribution around HSs, the quantitative description is still difficult. Many studies reported  
233 the zeta potential and electrophoretic mobility of humic substances specifying no inversion of  
234 charges in different mono and divalent electrolytes solutions [4, 33, and 48]. The  
235 electrophoretic mobility of these three humic substances in KCl solutions measured in this  
236 study show no reversal of charges of humic substances (see supporting information),  
237 supporting the previous literature. It should be noted that, to the best of the authors' knowledge,  
238 this paper reports the first data on electrophoretic mobility showing the charge reversal of  
239 humic substances and concurrent relations with aggregation as a factor of hydrophobic  
240 interactions.

241

242

243 Macroscopic and microscopic observations of HSs aggregates formation with TPPCl

244 We performed the visual observation of aggregation and dispersion of humic substances  
245 (SRFA, SRHA, and LHA) in tetraphenylphosphonium chloride (TPPCl) solutions at 50 mM  
246 as a function of pH after 24 hours (Photograph 1). We observed large ramified and interlinked  
247 aggregates of the HSs with TPPCl (Photograph 1 A, B, C) at pH around 3-5 for SRFA and  
248 SRHA and pH around 3-7 for LHA. The stronger network formation of large aggregates of  
249 LHAs at low pH can allow aggregates of LHAs withstand sedimentation. The videos of  
250 macroscopic observation at low pH confirm the strong interconnections and ramification of the  
251 HSs aggregates (see supporting information videos S1 –S5). Smaller size aggregates were also  
252 observed in the pH around 6 in the case of SRFA and SRHA. These smaller and mostly  
253 deposited aggregates, that were not large and ramified like the pH around 3-5, were more  
254 obvious in the case of SRHA. In the case of LHA at higher pH (7-10), smaller settled aggregates  
255 were also observed and those were not large ramified and strongly interconnected. Therefore,  
256 the sediment is clearly seen. We see clear views showing that the interconnection and size of  
257 aggregates decrease with the increase in pH for the HSs-TPPCl suspension. At higher pH  
258 values (7-10), the SRFA and SRHA were more dispersed probably due to weak hydrogen bond  
259 and weak hydrophobic interaction or the domination of electrostatic repulsion over the  
260 attractive interactions with TPPCl and/or humic substances itself. This qualitative observation  
261 of aggregates formation has some limitations due to manual handling and observation by naked  
262 eye.

263 These complex and interlinked aggregates are related to the change of electrophoretic  
264 mobility with pH as described in the previous section. Avena and Wilkinson [49] pointed out  
265 the importance of the hydrophobic interaction and hydrogen bond for the interaction of humic  
266 substances. The probable causes behind the formation of large aggregates are due to the  
267 existence of hydrophobic interactions [50]. This rather strong connection at low pH (3-5)

268 makes it possible that the aggregates withstand the force of gravity in the medium and the  
269 sedimentation is inhibited [51].

270 The microscopic observation was also performed for the confirmation of behavior and  
271 nature of aggregates. In photographs 2, 3, and 4, the structural arrangements and tentative size  
272 of the aggregates can be seen. The photographs 2, 3, and 4 show the aggregates formed at  
273 different pH from 3-9 in 50 mM TPPCl after 24 hours from the mixing. These microscopic  
274 photographs depict the verification of the existence of aggregates with interconnection among  
275 the particles in low pH of SRFA and SRHA. The LHA shows aggregation at all pH with a  
276 decreasing trend of size with pH, although there were some limitations in the microscopic study.  
277 With the increase of hydrophobicity (SRFA<SRHA<LHA), larger and concentrated aggregates  
278 appeared from photographs 2 to 4. The photographs 2, 3, and 4 of optical microscopy show  
279 some smaller units and some large units at pH around 3, and the large aggregates units look to  
280 be formed through the repetition and self-assembly of smaller units. At higher pH value, the  
281 SRFA and SRHA show no larger aggregate units in the photographs 2 and 3 even though at  
282 higher magnification, although LHA shows large aggregates in the higher range of pH. This  
283 result indicates the higher hydrophobicity triggered the aggregation. The repetition and  
284 rearrangements of the aggregates manifest the hierarchical architecture of the aggregates  
285 reported in the previous study [4, 9]. Some authors found similar structural natures and  
286 arrangements of humic acid aggregates with CaCl<sub>2</sub> in different magnification of optical  
287 microscope [52] and transmission electron microscopy studies. During the microscopic  
288 observation of the aggregates in this research, we observed the motion of some aggregates as  
289 a moving unit by Brownian motion of the aggregates. Such motion was also reported in the  
290 earlier study [4]. The motion of the aggregates can also be due to fluid flow and sedimentation  
291 due to gravity in the medium.

292

293 Dynamic light scattering (DLS) and aggregates size

294 To characterize the pH dependence of the aggregation, the DLS measurements of HSs  
295 aggregates in TPPCl solutions were carried out. Figure 2 shows that the hydrodynamic Z-  
296 average diameter of HSs is around 1200-1600 nm at pH 3-4 for SRFA and SRHA with a single  
297 discrete value of Z-average diameter higher than this and at pH 3-5 for LHA, indicating that  
298 the formation of HS aggregates in the presence of TPPCl. The DLS results are consistent with  
299 those by the visual observation described above in the presence of TPPCl. Generally, the  
300 aggregation of colloidal particles occurs around IEP. Our results, however, deviate from this  
301 general rule. Probably, hydrophobic interaction is the driving mechanism of TPP-induced  
302 aggregation. That is, the presence of TPP<sup>+</sup> ions induces the aggregation of HSs by increasing  
303 the hydrophobicity of HSs, and thus enhances the formation of HS aggregates through the  
304 increased tendency of HSs escaping from the water. The largest aggregates size around 2300  
305 nm was found for LHA at pH 3-4. At every pH values, the sizes of aggregates of LHA with  
306 TPPCl are larger than that of SRFA and SRHA with TPPCl. The DLS results manifest the  
307 highest hydrophobicity and the evidence of stronger hydrophobic interaction for LHA. On the  
308 contrary, the aggregates size of HSs with 50 mM TPPCl decreases with increasing pH in every  
309 type of HSs (SRFA, SRHA, and LHA) as expected from the visual observation. At low pH,  
310 less ionization of surface charging groups, the COOH and –OH causes more hydrophobic  
311 interaction between HSs and TPPCl. Furthermore, at low pH, the HSs aggregates are formed  
312 by hydrophobic interaction, hydrogen bonding, and probably some charge-patch attraction. At  
313 high pH, the electrostatic repulsion and weak hydrophobic interaction among HSs predominate  
314 and thus hinder the formation of the larger size of aggregates. In addition we focused on the  
315 formation of aggregates and concentrated the effect of hydrophobicity which provoke us to use  
316 different HSs with varying hydrophobicity regardless the size of primary humic substances. It  
317 was difficult to maintain the same size of primary particles varying with hydrophobicity in this

318 investigation. The decrease of the sizes of aggregates with pH indicating the electrostatic  
319 repulsion at higher negative charge with increasing pH, lower aggregates size and less  
320 hydrophobic interactions. The Z- average diameters of different humic substances with pH  
321 variation in hydrophilic salts were studied in the previous researches conducted by different  
322 groups. They found aggregates of humic acids larger than 1000 nm of Z-average diameter at  
323 low pH [33, 53]. Another group found aggregates of humic acids larger than 1000 nm of  
324 average hydrodynamic radius in NaCl and MgCl<sub>2</sub> at different concentration and time intervals  
325 in pH around 3.6 and 7.1 [54]. The previous study [33] shows the decreasing tendency of size  
326 at high pH due to the hindering of the H-bond and increasing of the electrostatic repulsion. The  
327 larger size at low pH is probably due to the reduction of repulsion and the intermolecular H-  
328 bonding. All our results confirm that the hydrophobic counter-ions TPP<sup>+</sup> induce the  
329 aggregation of humic substances and the degree of hydrophobicity influences the degree of  
330 aggregation of HSs, LHA > SRHA > SRFA. It should be noted here that the Z-average diameter  
331 of aggregates at low pH in this investigation shows higher values in DLS other than the  
332 previous investigation performed in monovalent hydrophilic electrolytes solutions refer to a  
333 hydrophobic interaction for larger aggregation.

#### 334 Fractal dimension and aggregates structure analysis

335 The temporal change in fractal dimension  $D_f$  of the aggregates of HSs in 50 mM TPPCl at  
336 pH 3 is shown in Fig. 3, where the effect of stirring is also demonstrated. In every cases, the  
337 fractal dimension with stirring shows higher values than that of non-stirring condition. Clear  
338 dependence of  $D_f$  on the type of HSs is not found. The values of fractal dimension of HSs are  
339 2-2.31 at no stirring. On the contrary, the values of  $D_f$  of SRFA, SRHA and LHA are raised to  
340 2.8, 2.88, and 2.87 at stirring condition (Fig. 3). The  $D_f$  values without stirring are reasonable  
341 compared to the previous study. The fractal dimension of simulated flocs formed through  
342 diffusion limited cluster-cluster aggregation is around 1.8, while the  $D_f$  around 2.1 can be



343 obtained by reaction limited cluster-cluster aggregation with restructuring [55]. From the  
344 comparison of the simulation results with our data, HSs flocs with TPP are formed through  
345 weaker contact forces allowing restructuring even at most aggregated state at low pH.

346 The values of fractal dimension with stirring are relatively higher than that by previous  
347 studies for aggregates such as kaolin with alum [56], natural organic matters such as humic  
348 acid with chitosan coagulants and Al species [57]. The Fe precipitates flocs showed a  $D_f$  value  
349 2.6 after breakage [58], a modelling of viscosity for coagulated suspension showed  $D_f$  values  
350 around 2.2 - 2.6 [59], and a comparative study of fractal dimension of humic acid flocs and  
351 clay flocs showed higher fractal dimension of humic acid flocs ( $D_f$  around 2.7) than that of  
352 clay flocs [60]. In this study, we found the higher value of  $D_f$ , indicating the more compact  
353 structure of aggregates due to the stirring. Studies revealed that the polydispersity of primary  
354 particles and aggregates cluster of TiO<sub>2</sub> aerosols, polystyrene latex, some polymers, and  
355 hematite particles may have an impact on the compactness and subsequent fractal structure  
356 [61-63]. From this situation in our case we measured the fractals at a fixed pH around 3, though  
357 HSs are not totally monodisperse and this has some effect on fractals structure by influencing  
358 the power law relation in a largely overlapping scattering exponent regimes between scattering  
359 wave vector and scattering intensity. Considering this effect of polydispersity the fractals has  
360 somehow influenced and had a higher value observed due to the presences of small and large  
361 aggregates and particles in stirring condition. Hoekstra et al. [64] reported that  $D_f$  of 2.7 was  
362 obtained for flocs formed in reaction limited regime under shear. Torres et al. [65] showed that  
363  $D_f$  increased to 2.9 through the collision between a single particle and a cluster in a shear flow.  
364 Some recent researches reported high  $D_f$  value around 2.75 of chitosan coagulant for different  
365 aluminium species in humic acid treatment [57], and  $D_f$  value almost 3.0 for polystyrene latex  
366 floc induced by FeCl<sub>3</sub>, NaCl and Al<sub>2</sub>(SO<sub>4</sub>)<sub>3</sub> and found the increase of fractal dimension with  
367 salt and particle concentrations [66]. The comparison of our data with previous studies

368 suggests that the repeated collision between the smaller HS aggregates and larger HS  
369 aggregates during the breakage and re-growth under stirring produces TPP-induced HSs flocs  
370 with higher values of the fractal dimension. This formation of aggregates may be due to the  
371 cluster-cluster aggregation [56] with restructuring and/or penetration of small cluster or broken  
372 aggregates fraction into the large aggregates structure.

373 The aggregation between a small cluster and large cluster, which can often happen in  
374 sheared suspension at high particle concentration, might result in higher  $D_f$ . This is one of the  
375 possible mechanisms of the present high  $D_f$  value. The penetration is also stimulated by soft  
376 permeable structure [48] of humic substances. The aggregation of humic substances in stirring  
377 condition caused by hydrophobic interaction and/or by charge neutralization with TPPCl  
378 undergoes continuous collisions by fluid flow. Also the flow causes frequent collision and  
379 irreversible penetration of some smaller aggregates in the permeable porous structure of HSs  
380 aggregates. These processes make the HSs aggregates more compact in structure, and thus flocs  
381 formed in shear flow have the compact structure [64, 67].

382

## 383 **Conclusion**

384 We found that the obvious charge reversal of all the humic substances (HSs) occurs in the  
385 presence of hydrophobic cation  $TPP^+$  and the iso-electric point (IEP) depends on the  
386 hydrophobicity of HSs and  $TPP^+$  concentration. The HSs form large visible aggregates in  
387 TPPCl solution at low pH for SRFA and SRHA, and in all pH for LHA. The size of LHA  
388 aggregates is also larger than those of SRFA and SRHA. The HS aggregates formed with  $TPP^+$   
389 have large size and fractal structure. This new findings of charge reversal and formation of  
390 large HS aggregates in the presence of hydrophobic counter ion will definitely help us to partly  
391 understand and may unfold a new arena of future research of humic substances. Our findings

392 are available to predict and depict the fate of various hydrophobic ions such as dyes and  
393 medicines in soil and water environment.

#### 394 **Conflict of interest**

395

396 The authors declare that they have no conflict of interest associated with this article.

397

#### 398 **Acknowledgments**

399 The present work was financially supported by JSPS KAKENHI (15H04563 and 16H06382).

400 We would like to thank Mr. Tri Hoa Phan from Department of Chemistry, University of Iowa

401 for his great support during the experimental work.

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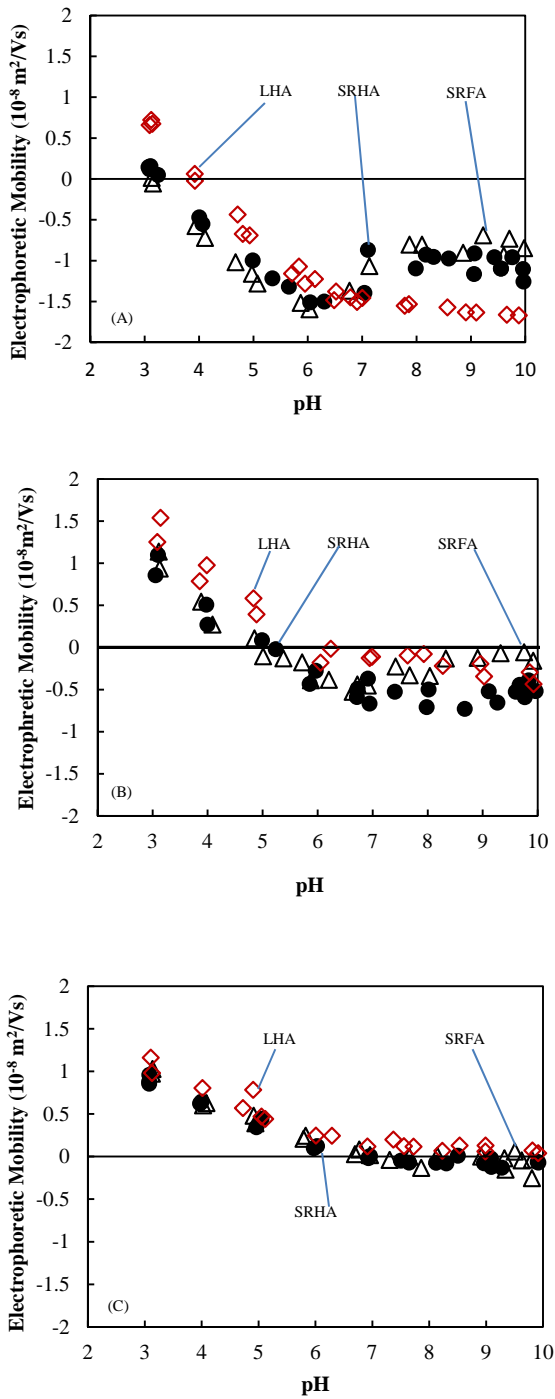
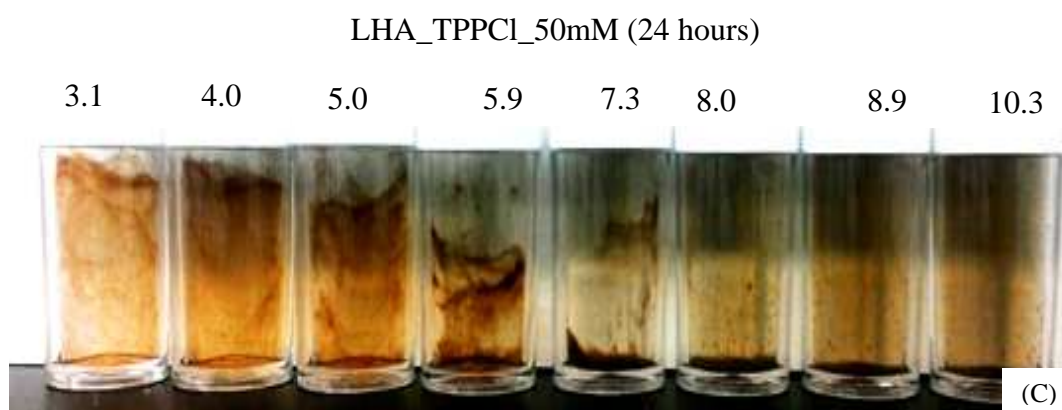
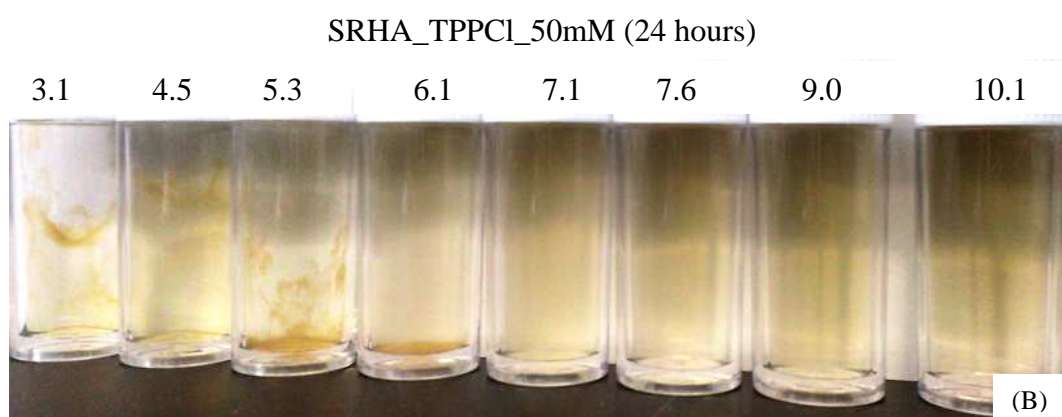
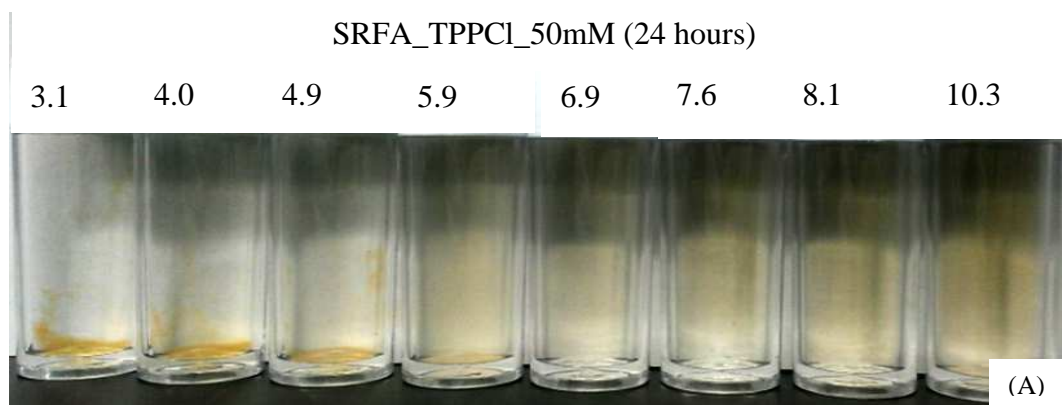
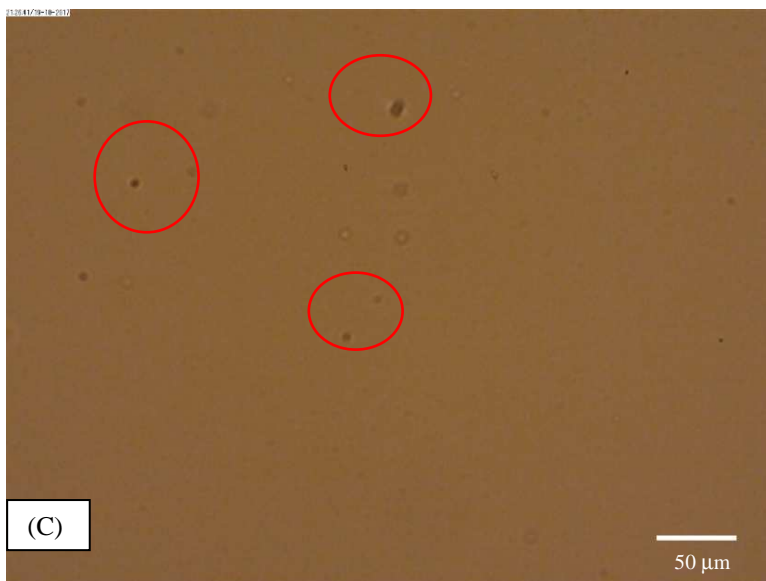
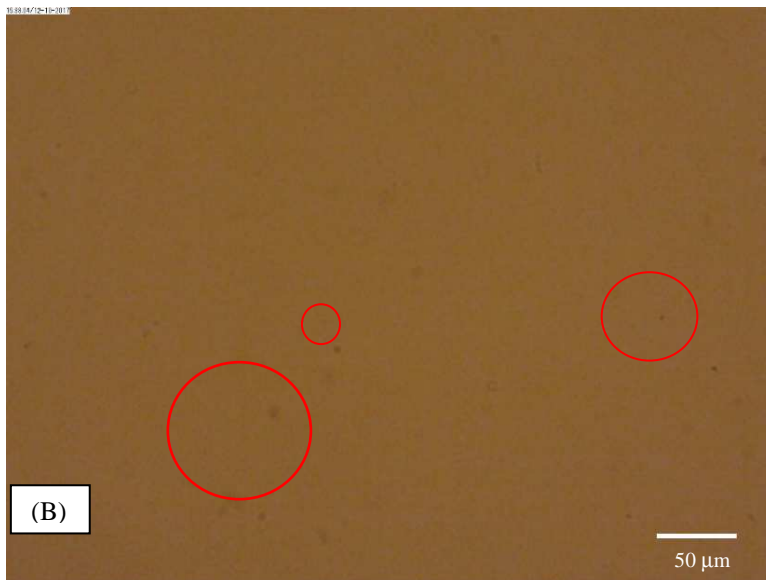
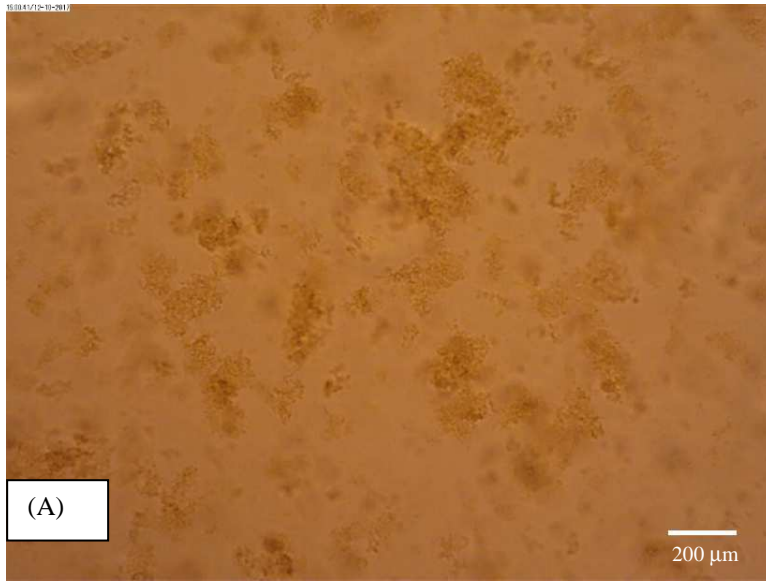


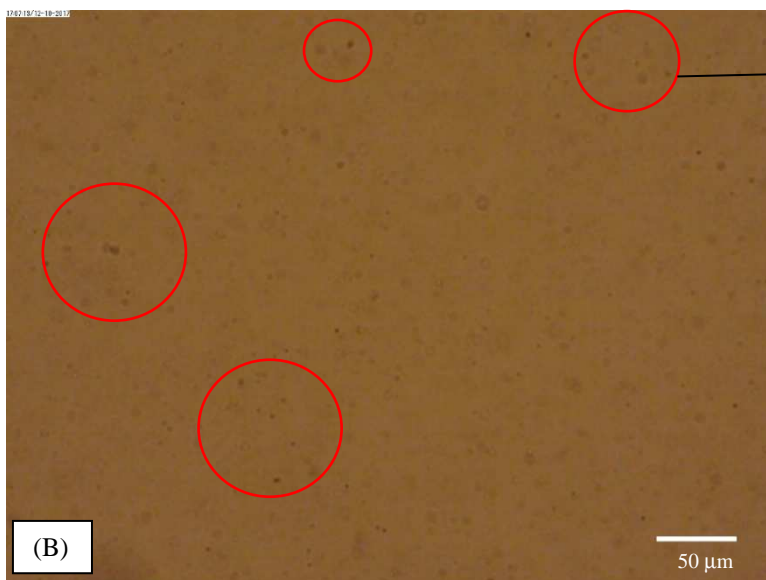
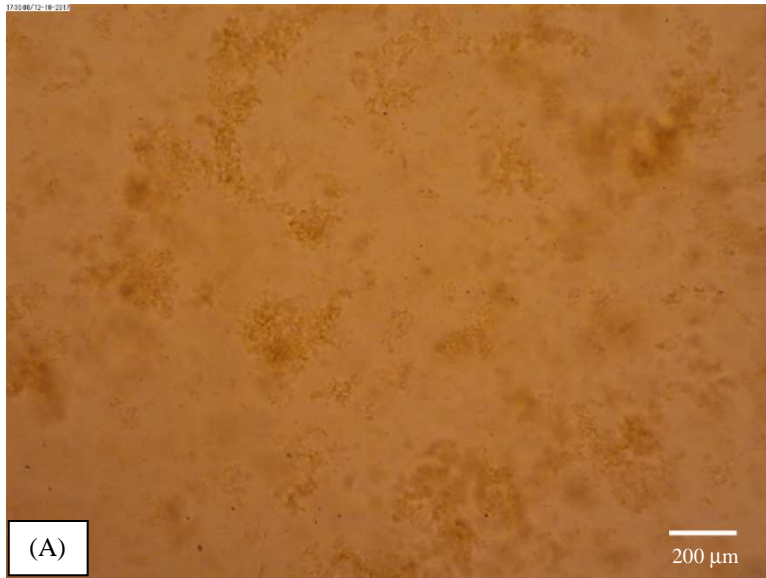
Figure 1. Electrophoretic mobility of Suwanne river fulvic acid (SRFA), Suwannee river humic acid (SRHA) and Leonardite humic acid (LHA) as a function of pH with tetraphenylphosphonium chloride (TPPCL) 10 mM (A), 50 mM (B), and 100 mM (C). Concentration of humic substances (SRFA, SRHA and LHA) is 50 mg/L. Symbols: SRFA ( $\Delta$ ), SRHA ( $\bullet$ ), and LHA ( $\diamond$ ).



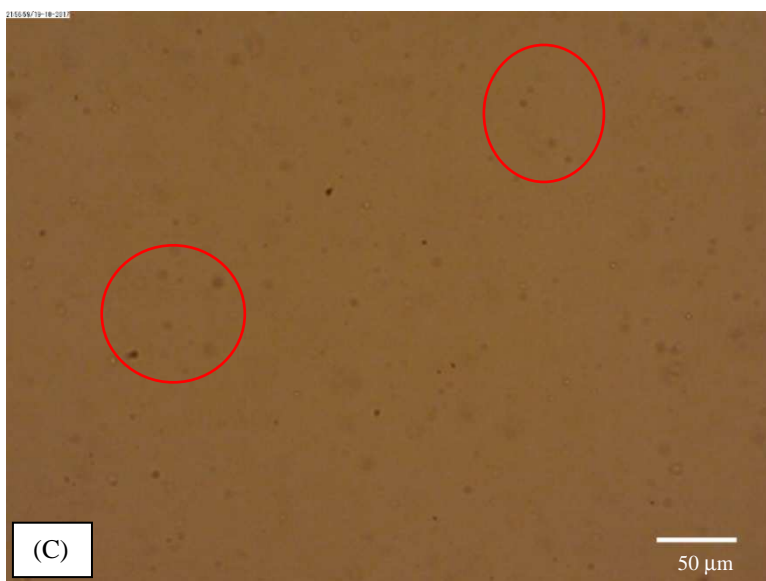
Photograph 1. Macroscopic view of aggregation and re-dispersion for humic substances (SRFA, SRHA, and LHA) in tetraphenylphosphonium chloride (TPPCL) at 50 mM as a function of pH after 24 hours. Photos representing Suwannee river fulvic acid (SRFA) with TPPCl (A), Suwannee river humic acid (SRHA) with TPPCl (B), and Leonardite humic acid (LHA) TPPCl (C). Photo colour was adjusted by using GIMP 2.8.22



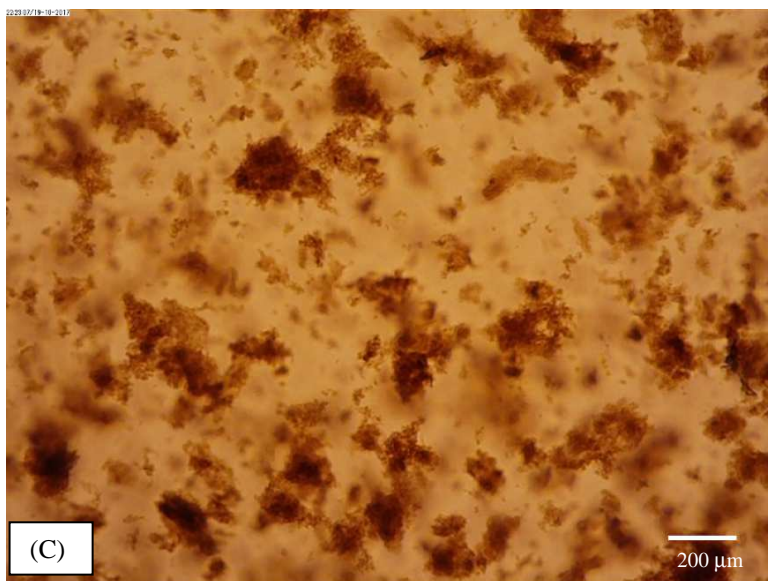
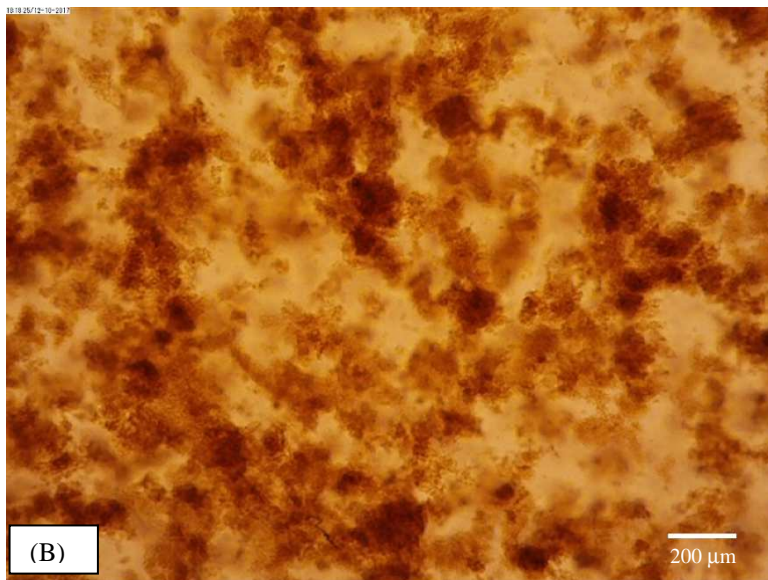
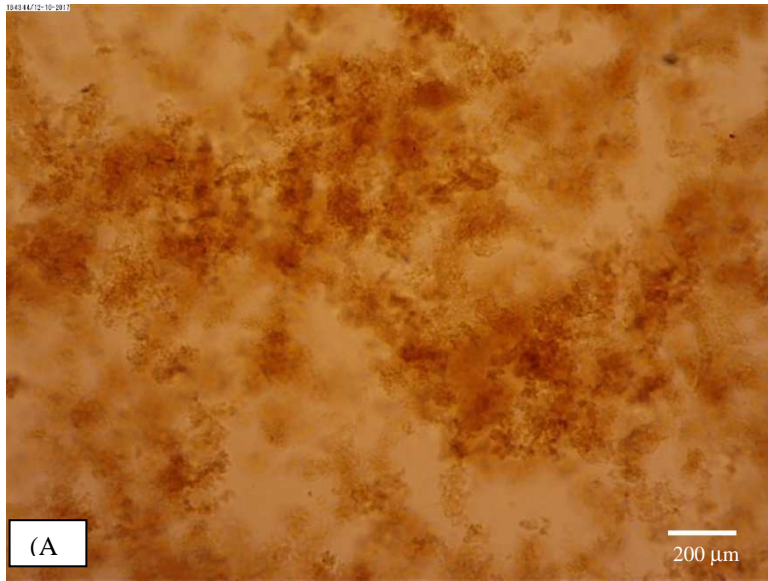
Photograph 2. Microscopic photographs of flocs in Suwannee river fulvic acid (SRFA) with tetraphenylphosphonium chloride (TPPCI) 50 mM at pH 3.1 (A), pH 6.9 (B), and pH 8.9 (C). Scale bar is 200  $\mu\text{m}$  (A), 50  $\mu\text{m}$  (B), and 50  $\mu\text{m}$  (C).



Some small aggregates and particles are dispersed in the suspension. Which indicate the aggregation is induced by tetraphenylphosphonium chloride (TPPCI) at high pH but those are very small in size compared to low pH



Photograph 3. Microscopic photographs of flocs in Suwannee river humic acid (SRHA) with tetraphenylphosphonium chloride (TPPCI) 50 mM at pH 3.2 (A), pH 7.1 (B), and pH 8.8 (C). Scale bar is 200 μm (A), 50 μm (B), and 50 μm (C).



Photograph 4. Microscopic photographs of flocs in Leonardite humic acid (LHA) with tetraphenylphosphonium chloride (TPPCI) 50 mM at pH 3.2 (A), pH 6.8 (B), and pH 8.9 (C). Scale bar is 200  $\mu\text{m}$ .



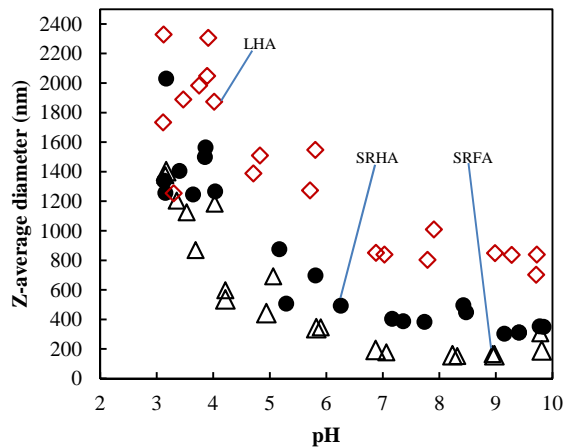


Figure 2. Z average diameter as a function of pH for Suwanne river fulvic acid (SRFA), Suwannee river humic acid (SRHA) and Leonardite humic acid (LHA) in 50 mM tetraphenylphosphonium chloride (TPPCL). Concentration of humic substances (SRFA, SRHA and LHA) is 50 mg/L. Symbols: SRFA ( $\Delta$ ), SRHA ( $\bullet$ ), and LHA ( $\diamond$ ).

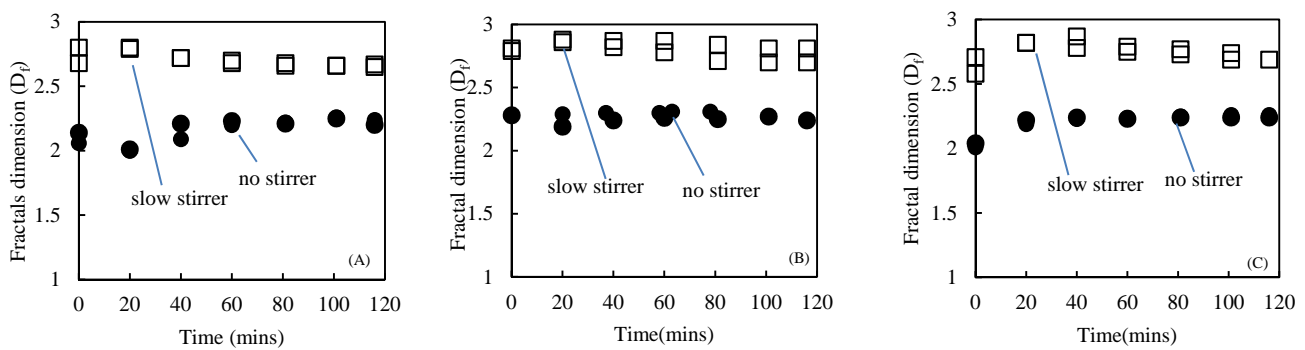


Figure 3. Change in fractal dimension with time in slow stirrer and no stirrer condition of Suwannee river fulvic acid (SRFA) flocs (A), Suwannee river humic acid (SRHA) flocs (B), and Leonardite humic acid (LHA) flocs (C) in 50 mM tetraphenylphosphonium chloride (TPPCL) at pH around 3. Concentration of humic substances (SRFA, SRHA and LHA) is 50 mg/L. Symbols: ( $\square$ ) slow stirrer, and ( $\bullet$ ) no stirrer