

1 **Influence of zeta potential on the flocculation of cyanobacteria cells using**
2 **chitosan modified soil**

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6
7 **Abstract:** Using chitosan modified soil to flocculate and sediment algal cells has been
8 considered as a promising strategy to combat cyanobacteria blooms in natural waters.
9 However, the flocculation efficiency often varies with algal cells with different zeta
10 potential (ZP) attributed to different growth phase or water conditions. This paper
11 investigated the relationship between ZP of *microcystis aeruginosa* and its influence
12 to the flocculation efficiency using chitosan modified soil. Results suggested that the
13 optimal removal efficiency was obtained when the ZP was between -20.7 mV and -6.7
14 mV with a removal efficiency of more than 80% in 30 min and large floc size of >
15 350 μm . When the algal cells were more negatively charged than -20.7 mV, the effect
16 of chitosan modified soil was depressed (< 60%) due to the insufficient charge density
17 of chitosan to neutralize and destabilize the algae suspension. When the algal cells
18 were less negative than -6.7 mV or even positively charged, small floc size (< 120 μm)
19 were formed, which may be difficult to sink under natural water conditions. Therefore,
20 manipulation of ZP provided a viable tool to improve the flocculation efficiency of
21 chitosan modified soil and an important guidance for practical engineering of
22 cyanobacteria blooms control.

23 **Key words:** cyanobacteria blooms, flocculation, chitosan, modified soil, zeta
24 potential

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27 **Introduction**

28 Excess quantities of nutrients have been discharged into fresh waters, inducing a
29 global environmental epidemic of cyanobacteria blooms (Paerl and Huisman, 2008).
30 Such blooms pose serious threats to aquatic life, fish industry, local tourism, and

31 water quality in lakes, rivers and reservoirs (Beaulieu et al., 2005). They also threaten
32 drinking water safety, such as the drinking water crisis in Wuxi City, China in 2007
33 (Guo, 2007).

34 Over the past several decades, many efforts have been done to combat the
35 cyanobacteria blooms (HABs). Among the technologies of mechanical, biological,
36 chemical, genetic and environmental control (Anderson, 2009), significant attention
37 has been focused on the use of clay to flocculate and settle the cyanobacteria cells in
38 natural waters (Anderson, 1997; Sengco et al., 2001; Yu et al., 1994). However, the
39 efficiency of clay alone was low and high loads of caly (0.25--2.5 g/L) (Pan et al.,
40 2006; Sengco et al., 2001; Sun et al., 2004) often lead to various ecological concerns
41 (Lee et al., 2008). Pan et al (2006) found that local soil/sand collected from lake shore
42 after modified by chitosan could be turned into effective flocculants to remove
43 cyanobacteria blooms and improve water quality, which greatly reduced the dosage to
44 11 mg/L and hence minimized the costs and the use of exogenous materials to the
45 aquatic environments. Chitosan, a commercially available product of edible food
46 additives, is derived from the alkaline deacetylation of crustacean chitin and known to
47 be a biodegradable and non-toxic natural polymer. A field application of chitosan
48 modified soil in Lake Taihu and the study of ecological response in time scale of
49 months to year proved its efficiency and ecological safety, 0.1 km² of the HAB layer
50 was disappeared in 10 hours after the dispersion of the chitosan modified soil and the
51 submerged vegetation was successfully restored after 4 months due to the improved
52 water quality (Pan et al., 2011b).

53 The key mechanism of chitosan modified soil/sand to remove cyanobacteria
54 blooms was that the chitosan with long polymer chain and positively charged groups
55 (-NH₃⁺) captured and linked the negatively charged algal cells and other particles, the
56 soils then provided the mass or ballast to carry the flocs to the water sediment (Zou et
57 al., 2006). Therefore, the charge interaction between chitosan and algal cells was
58 critical important for the flocculation process. However, the zeta potential, which
59 gives a measurement of the apparent surface charge of algal cells, often changed
60 because of different growth phase (Henderson et al., 2008a) or water conditions (Zou

61 et al., 2005), which caused the flocculation efficiency of chitosan modified soil
62 variable. For example, when the *microcystis aeruginosa* (M.A.), the main species
63 forming cyanobacteria blooms in Lake Taihu was firstly harvested from the culture
64 medium by centrifuge and then re-dispersed into 0.5% NaCl solution, Zou et al (2006)
65 reported that 80% of the algal cells was removed by 1 mg/L chitosan modified 10
66 mg/L soil in 30 min. However, if directly flocculated in the culture medium,
67 maximally 60% was achieved in 4 hours with the same dosage (Li and Pan, 2013).
68 Further studies proved that after the pretreatment, the magnitude of zeta potential (ZP),
69 which gives a measurement of the apparent surface charge, was significantly reduced
70 from -67.9 mV to -30 mV, which greatly increased the flocculation potential of M.A.
71 cells and hence achieved higher removal efficiency (Li and Pan, 2013).

72 Reducing the magnitude of negative zeta potential means charge neutralization
73 and destabilization, which established the polymer flocculation mechanism (Hjorth
74 and Jorgensen, 2012). Although the ZP as an influence factor affecting the
75 flocculation ability of chitosan has been proposed (Renault et al., 2009), little progress
76 has been done to quantify the effects and study the mechanism how it affected the
77 flocculation efficiency. The use of ZP for monitoring and controlling the coagulation
78 of algal cells using aluminium sulphate has been well researched and found to be of
79 great benefit (Henderson et al., 2008b), it was reported that the optimum removal was
80 measured when the ZP of algal cells was controlled to between -8 mV and +2 mV.
81 However, the main mechanism of chitosan to remove particles in water was the long
82 polymer chain with netting and bridging function (Huang and Chen, 1996; Zou et al.,
83 2006), which was significantly different from the aluminium sulphate functioned
84 mainly as charge neutralizer, the results and mechanism how the ZP affect the
85 removal efficiency of chitosan may be also different. Therefore, if the influence of ZP
86 of cyanobacteria cells on the flocculation ability of chitosan modified soil can be
87 quantified, for example, in which ZP range ideal removal efficiency can be achieved
88 or in which range the algal cells failed to be flocculated, it will give an insight for
89 understanding the flocculation mechanism and provide a useful guidance for practical
90 engineering of cyanobacteria blooms control.

91 Here, the *microcystis aeruginosa* (M.A.), main species forming cyanobacteria
92 blooms was selected in different growth phase and adjusted to possess different ZP by
93 a positively charged protein, *moringa oleifera* seed extract (MO). The relationship
94 between ZP of algal cells and flocculation ability of chitosan was studied. The main
95 objective of this research was to find how the ZP of particles affects the flocculation
96 behavior of chitosan, including removal efficiency, sedimentation kinetics, floc
97 structure and floc size growth. According to these results, an optimized ZP range for
98 algae flocculation using chitosan modified soil was proposed.

99 **1 Materials and methods**

100 **1.1 Algae culture**

101 The M.A cells were obtained from Freshwater Algae Culture Collection at the
102 Institute of Hydrobiology (FACHB), Chinese Academy of Sciences. The culture
103 medium, BG11, was adjusted to pH = 8.0 by adding either 0.1 mol/L HCl solution or
104 0.1 mol/L NaOH solution before autoclaving. The sterilized 500 mL glass flasks
105 containing 300 mL aqueous *M. aeruginosa* medium were maintained at 25 ± 1 °C
106 under cool white fluorescent light of 2000–3000 lx on a 12 h light and 12 h darkness
107 regimen in the illuminating incubator (LRH-250-G, Guangdong Medical Apparatus
108 Co.Ltd., China).

109 **1.2 MO, chitosan, soil and modification**

110 *Moringa oleifera* seed extract (MO) was cationic proteins with molecular mass of
111 6.5–13 kDa and isoelectric points in the range of pH 9.6–11 (Ghebremichael et al.,
112 2005). It was chosen as the ZP adjuster for M.A. cells since as reported, it can
113 significantly reduce ZP of particles (Ndabigengesere et al., 1995). MO seeds were
114 purchased from Shaoguan city (South China) in dry form, having already been
115 removed from the pod. The healthy seeds (about 1.0 cm) were selected and deshelled.
116 The kernels were grounded in a coffee grinder to become particles of ~ 300 μm and
117 stored at room temperature in an airtight container and used in one month (Katayon et
118 al., 2006). To extract the active proteins, 5 g of the seed powder was suspended in 100
119 mL of 1.0 mol/L NaCl solution and the suspension was stirred using a magnetic stirrer
120 for 30 min (Okuda et al., 2001). The solution was then filtered through a glass

121 microfibre filter of 0.45 μm pore size (Whatman GF/C) and the filtrate was used as
122 the ZP adjuster.

123 Chitosan was purchased from Qingdao Yunzhou Bioengineering Co. Ltd. The
124 chitosan flakes were dissolved by adding 500 mg chitosan to 100 mL of 0.5% HAc
125 and stirred until all the chitosan was dissolved. This solution was then diluted with
126 deionized water to obtain a final concentration of 1 g/L before use. The MO and
127 chitosan was prepared freshly for each experiment.

128 The soil was collected from lakeshore of Meiliang Bay, Lake Taihu, washed with
129 deionized water, dried at 100 $^{\circ}\text{C}$ for 10 h, and then grounded and sieved through 180
130 mesh ($< 90 \mu\text{m}$).

131 To modify the soil, a certain volume of chitosan solution (1 mg/L) was added to a
132 clay suspension (10 mg/L). The mixture was well stirred and then ready for use in the
133 flocculation experiment. As the Al_{13} -modified montmorillonite reported by Zhao et al
134 (2012), the chitosan modification also changed the physic-chemical characteristics of
135 soil and hence affected the flocculation behavior, more detailed information can be
136 obtained from our previous publications (i.e., Pan et al (2006) and Zou et al (2006)).

137 **1.3 Algae suspension preparation**

138 M.A. cells with different ZP were obtained by two ways: (1) Algal cells in
139 different growth phase (mid- to late-exponential growth phase and mid- to
140 late-stationary phase) possess different ZP were selected, and then diluted to optical
141 density of 0.150 ± 0.002 at the wavelength of 680 nm ($\text{OD}_{680 \text{ nm}}$) (Pan et al., 2006)
142 using BG11 culture medium; (2) M.A. cells in the mid- to late-stationary phase were
143 chosen and diluted to $\text{OD}_{680 \text{ nm}} = 0.150 \pm 0.002$ using BG11 culture medium, then 1
144 mL, 2 mL, 3 mL, 4 mL and 5 mL of MO was added to 200 mL algae suspension,
145 respectively. The pH of all the solutions were adjusted to 8.0 by adding either 0.1
146 mol/L NaOH or 0.1 mol/L HCl solutions and the ZP of M.A. cells was then
147 determined by Zetasizer 2000 (Malvern Co. Ltd., United Kingdom).

148 **1.4 Algae flocculation**

149 200 mL of each prepared algae suspension was transferred into 300 mL beaker,
150 respectively. According to the pre-experiment (**Fig. 1**), the optimal dosage of 2 mg/L

151 chitosan modified 10 mg/L soil was added to the algae solution and stirred (six-head
152 stirrer ZR3-6, made in Shenzhen, China) at 300 r/min for 1 min, then 120 r/min for 2
153 min, followed by 40 r/min for another 10 min. The solution running without adding
154 any MO, chitosan or soil was set as blank control and only the addition of ZP adjuster
155 (MO) was set as MO control. The solutions were kept standing when the stirrer
156 stopped. Samples (1 mL) from 2 cm below the water surface were collected after
157 sedimentation for 0, 2, 5, 10, 15, 20, 30, 60, 90, 120, 180, 240, 300, 360 and 420 min.
158 The cells were enumerated in a counting chamber of an electromotive microscope
159 (Axioskop 2 mot plus, Carl ZEISS, Germany) after being fixed by Lugol solution. All
160 the flocculation experiments were conducted in triplicate and the results were
161 presented as the mean values. The removal rate of M. A. cells was calculated as
162 (initial cell concentration – sample cell concentration)/initial cell concentration
163 $\times 100\%$.

164 To study the flocs formation and floc size growth of M.A. cells with different ZP
165 after the addition of chitosan modified soil, the floc size during flocculation process
166 was quantitatively monitored with a laser particle size analyzer (Mastersizer 2000,
167 Malvern Co. Ltd., United Kingdom). Samples were drawn into the analyzer and back
168 to the jar by a peristaltic pump (BT00–300M, Baoding Longer Precision Pump Co.
169 Ltd., China) at a flow rate of 35 mL/min (Jarvis et al., 2005). The floc size of samples
170 was determined firstly before going through the pump head, which avoid the flocs
171 breakage. The size was denoted by the measured mean diameter ($D_{0.5}$). After
172 flocculation and sedimentation, the flocs formed by M.A. cells with different ZP were
173 carefully transferred on a glass slide and then photographed by the electromotive
174 microscope (Axioskop 2 mot plus, Carl ZEISS, Germany) for floc structure study.

175 **2 Results and discussion**

176 **2.1 Algae removal in different growth phase**

177 The chitosan modified soil showed different flocculation ability to the M.A. cells
178 in different growth phase (**Fig. 2**). Maximally about 70% of algal cells in the mid- to
179 late-exponential growth phase and 50% in the mid- to late-stationary phase were
180 removed at the optimized dosage of 2 mg/L chitosan modified 10 mg/L soil,

181 respectively. The change of ZP associated with algae proliferation process probably
182 caused the different removal efficiency. When in the mid- to late-exponential growth
183 phase, the ZP of M.A. cells was -26.3 mV, whereas in the mid- to late-stationary phase,
184 it was more negatively charged (-67.9 mV) (**Fig. 2**). This was probably because of the
185 more generation of extracellular organic matter (EOM) in the stationary phase which
186 attached to the algal cells and changed the surface properties (Wang et al., 2013).

187 EOM in appropriate concentration acted as flocculation aid to increase the
188 removal efficiency (Henderson et al., 2008a), however, the increased EOM might
189 increase the magnitude of ZP and thus inhibited the flocculation process (Wang et al.,
190 2013). Compared to some coagulants with strong charge density (e.g. aluminium and
191 ferric), chitosan is a relatively weaker surface charge modifier, which was less
192 effective in reducing the magnitude of ZP and destabilizing algae suspension (Huang
193 and Chen, 1996). Therefore, more negatively charged algal cells often led to lower
194 removal efficiency when using chitosan modified soil.

195 **2.2 Removal efficiency of M.A. cells with different ZP**

196 To study the flocculation of M.A. cells less negatively charged when using
197 chitosan modified soil, the cationic protein, MO was used to adjust the ZP of algal
198 cells in the mid- to late-stationary growth phase. The initial ZP of M.A. cells in the
199 BG11 culture medium was highly negatively charged (-67.9 mV). After the addition
200 of MO of 1 mL, 2 mL, 3 mL, 4 mL and 5 mL, the magnitude of ZP was reduced to
201 -20.7 mV, -6.7 mV, -3.7 mV, +0.4 mV and +2.7 mV, respectively (**Fig. 3A**). Due to the
202 reduction of repulsive force, MO itself can remove some algal cells (**Fig. 3B**). As the
203 reduction of the magnitude of ZP, the removal efficiency was increased and achieved
204 the maximum removal efficiency of 58.7% at the ZP of -6.7 mV, then slightly
205 decreased to 54.5% when the ZP was +2.7 mV after sedimentation for 30 min. The
206 chitosan modified soil showed different flocculation ability to M.A. cells with
207 different ZP (**Fig. 3B**). When directly flocculated in the culture medium without ZP
208 adjusting, only 40% of algal cells were removed in 30 min, whereas the removal
209 efficiency of 80% and 93% was achieved at the ZP of -20.7 mV and -6.7 mV,
210 respectively.

211 Unlike the coagulants, the magnitude of ZP should be reduced to a certain level to
212 help the algal cells overcome the repulsive force and make them self-aggregation and
213 sedimentation, such as the optimal removal rate was obtained at the ZP range between
214 -8 mV and +2 mV when aluminium sulfate was used (Henderson et al., 2008b).
215 Reduction of the magnitude of ZP to -20.7 mV was sufficient for the chitosan to
216 flocculate the M.A. cells (**Fig. 3B**). However, if over-reduced the magnitude of ZP, i.e.
217 less negatively charged than -6.7 mV, the attractive force between positive charged
218 groups of chitosan ($-\text{NH}_3^+$) and the algal cells was weakened, which inhibited the
219 netting and bridging process of chitosan. Although the removal efficiency was still
220 higher than 90% due to reduced repulsive force between algal cells at the ZP range
221 between -3.7 and +2.7 mV (**Fig 3B**), the floc size became small (as discussed below)
222 and hence the overall removal efficiency was decreased.

223 **2.3 Algal flocs formation, size growth and sedimentation**

224 The flocs formation and floc size growth of M.A. cells with different ZP after the
225 addition of chitosan modified soil was directly monitored during flocculation process
226 (**Fig. 4**). The size of algal flocs was limited improved from 2 μm to 30 μm at the ZP of
227 -67.9 mV, which suggested that chitosan cannot capture and link these highly
228 negatively charged cells effectively. As the reduction of the magnitude of the ZP, the
229 floc size was significantly increased to about 360 μm at the ZP of -20.7 mV. The
230 netting and bridging function of chitosan played best when the ZP of M.A. cells was
231 -6.7 mV, the maximum floc size of about 700 μm was achieved. However, if the ZP
232 was less negatively charged than -6.7 mV, as explained above, the weakened attractive
233 force between chitosan and cells led to the decrease of floc size. When the ZP was
234 turned to positive, the floc size was almost the same as MO control (120 μm), which
235 suggested that the netting and bridging function of chitosan was lost under this
236 condition. According to the images of floc structure of M.A cells at the ZP of -6.7 mV
237 and +2.7 mV (**Fig. 5**), the latter was much more fragile and smaller than the former,
238 which directly proved the effect of ZP to the netting and bridging process of chitosan.

239 Algal floc size directly affected the sedimentation kinetics of M.A. cells after
240 flocculation (**Fig. 6**). The maximum removal efficiency of 90% was achieved in 30

241 min for the M.A. cells at the ZP of -6.7 mV due to the rapid formation and size
242 growth of algal flocs (**Fig. 4**), whereas 240 min was needed to obtain the same
243 removal efficiency when the ZP was +2.7 mV and only about 50% of alga cells were
244 removed as long as 480 min when the ZP was -67.9 mV. After adjusting ZP to -6.7 mV,
245 the MO alone can remove algal cells due to the reduction of repulsive force between
246 particles, however, if without the netting and bridging function of chitosan, 120 min
247 was needed to sediment the small MO-flocs (**Fig. 6**).

248 Sedimentation was regarded as a major challenge for chemical coagulation and
249 flocculation treatment of cyanobacteria cells because of the buoyant properties
250 (Gheraout et al., 2010) and low density of algal flocs (Pieterse and Cloot, 1997).
251 Unlike in the constructed water treatment systems, sufficient sedimentation time can
252 be provided to allow the small flocs to settle by creating static water condition and/or
253 appropriate water retention time, or dissolved air bubbles can be generated to float the
254 flocs with low density (Edzwald, 1993). For flocculating cyanobacteria blooms in
255 natural waters, since the small and fluffy flocs were often hard to settle or easy to
256 re-suspend into the water column with the disturbance of water flow and
257 wind-induced waves (Beaulieu et al., 2005). Improving algal floc size and combining
258 with soil particles to increase the floc density was a key process for algae removal
259 when using the chitosan modified soil technology. To this end, manipulating the ZP of
260 M.A. cells to create an optimized condition for chitosan linking and bridging the algal
261 cells and soil particles (**Fig. 4**) is crucial for flocculating cyanobacteria blooms
262 successfully in natural waters. According to the removal efficiency (**Fig. 2** and **Fig. 3**),
263 floc size growth process (**Fig. 4** and **Fig. 5**) and sedimentation kinetics (**Fig. 6**), the
264 optimized ZP range for chitosan modified soil to flocculate M.A. cells was suggested
265 to be between -20.7 mV and -6.7 mV, in which the removal efficiency of more than
266 80% in 30 min and floc size larger than 350 μm can be achieved.

267 **2.4 Environmental implications**

268 Cyanobacteria blooms pose a serious threat to aquatic eco-systems and public
269 health (Guo, 2007; Paerl and Huisman, 2008), flocculating them using chitosan
270 modified soil not only removed the harmful algal cells and reduced the risk level, but

271 more importantly, the water quality including water transparency was also improved
272 and excess nutrients in the algal cells were transferred to the sediment, which created
273 a better condition for subsequently submerged macrophytes restoration in shallow
274 lakes (Pan et al., 2011b). Besides the biodegradable, nontoxic and natural properties
275 (Pan et al., 2011a), chitosan was also reported to be beneficial for the submerged
276 macrophytes growth in aquatic environment (Xu et al., 2005).

277 However, both exciting and unsatisfactory results of the algae removal using
278 chitosan modified soil has been reported (Li and Pan, 2013; Zou et al., 2006). The
279 changes of algal surface charge attributed to the different algae growth phase (Chen et
280 al., 2004) or water condition (Pan et al., 2011a) often led to the flocculation
281 efficiency variable. Our results demonstrated the relationship between ZP of algal
282 cells and its influence on the flocculation efficiency using chitosan modified soil. The
283 optimized ZP range between -20.7 mV and -6.7 mV thus provided a manipulating
284 strategy for practical engineering of cyanobacteria blooms control. For example, since
285 algal cells in different growth phase possess different ZP, the optimized range can
286 guide the researchers or engineers to choose the right time to carry out the
287 cyanobacteria blooms removal, or for the highly negatively charged algal cells, some
288 charge modifiers (e.g. the coagulants) can be dosed first to create a better condition
289 for chitosan to capture and link the particles and increase floc size. Therefore,
290 manipulating the ZP of algal cells as a tool to improve the flocculation efficiency and
291 floc size greatly increased the probability of successful control of cyanobacteria
292 blooms in natural waters when using this chitosan modified soil technology.

293 Since chitosan alone cannot destabilize and capture highly negatively charged
294 algal cells, pre-charge modification was hence important for the successful algae
295 removal. Besides MO, there are some other widely existed proteins with high
296 isoelectric point which possess net positive charge in natural waters shows promise to
297 achieve this goal. Ghebremichael et al (2005) has proved that there are many other
298 small, basic peptides from plants and animals can be used to reduce the surface charge
299 of particles. Therefore, a new bi-component modification method using locally
300 available, biodegradable and nontoxic materials with high charge density and chitosan

301 could be developed, which combines multi mechanism of charge neutralization to
302 manipulate the ZP and netting and bridging function to improve the floc size. If so,
303 the uncertain impact of ZP attributed to different algal growth phase could be
304 overcome and high removal efficiency could be achieved under different water
305 conditions.

306 Finally, it is important to note that besides ZP or some factors directly affected ZP
307 and thus further affected the flocculation ability of chitosan, some other factors
308 irrelevant to ZP also show the control of the performance in flocculation process, such
309 as the origin and the nature of the chitosan (i.e., its intrinsic characteristics such as
310 degree of deacetylation and molecular weight, and the activation conditions of the raw
311 biopolymer), the type of acid used to dissolve the chitosan, the reaction time and
312 temperature, etc (Renault et al., 2009), the quantitative relationship between algae
313 removal efficiency and these factors needs to be further studied.

314 **3 Conclusions**

315 The ZP of algal cells directly affected the flocculation ability of chitosan
316 modified soil. According the removal efficiency, floc size and sedimentation kinetics,
317 an optimized ZP range between -20.7 mV and -6.7 mV was proposed, in which the
318 removal efficiency of more than 80% in 30 min and floc size of larger than 350 μm
319 can be achieved. The quantification of the ZP effect to chitosan flocculation behavior
320 provided a viable tool to increase the probability of successful control of
321 cyanobacteria blooms in natural waters when using this technology. With some
322 additional research, a bi-component modification method combing ZP manipulation
323 and netting and bridging function can be developed.

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