

1 **Simultaneous removal of harmful algal blooms and microcystins using**
2 **microorganism and chitosan modified local soil**

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23 **ABSTRACT:** Cyanobacterial harmful algal blooms (cyano-HAB) and
24 microcystins (MCs) can cause potential threat to public health. Here, a method for
25 simultaneous removal of cyano-HAB and MCs was developed using chitosan
26 modified local soil (MLS) flocculation plus microorganism modified soil capping.
27 The experiment was conducted in simulated columns containing algal water
28 collected from Lake Taihu (China). More than 90% algal cells and intracellular
29 MCs were flocculated and removed from water using chitosan-MLS and the sunk
30 flocs were treated by different capping materials including *Pseudomonas sp.* An18
31 modified local soil. During 40 days' incubation, dissolved MC-LR and MC-RR
32 showed 10-fold increase in the flocculation only system. The increase of MC-LR
33 and MC-RR in water was reduced by 30% and 70% in soil capping treatments,
34 however, the total content of MCs in the sediment-water column remained similar
35 with that in the control and flocculation only systems. In contrast, both dissolved
36 MCs and total MCs were reduced by 90% in *Pseudomonas sp.* An18 modified soil
37 capping treatment. The high performance of toxin decomposition was due to the
38 combined effects of flocculation and MCs-degrading bacteria that embedded in
39 the capping material, which prevents dilution of bacteria biomass, concentrates the
40 algal cells, confines the released toxins, and enhances toxin biodegradation.

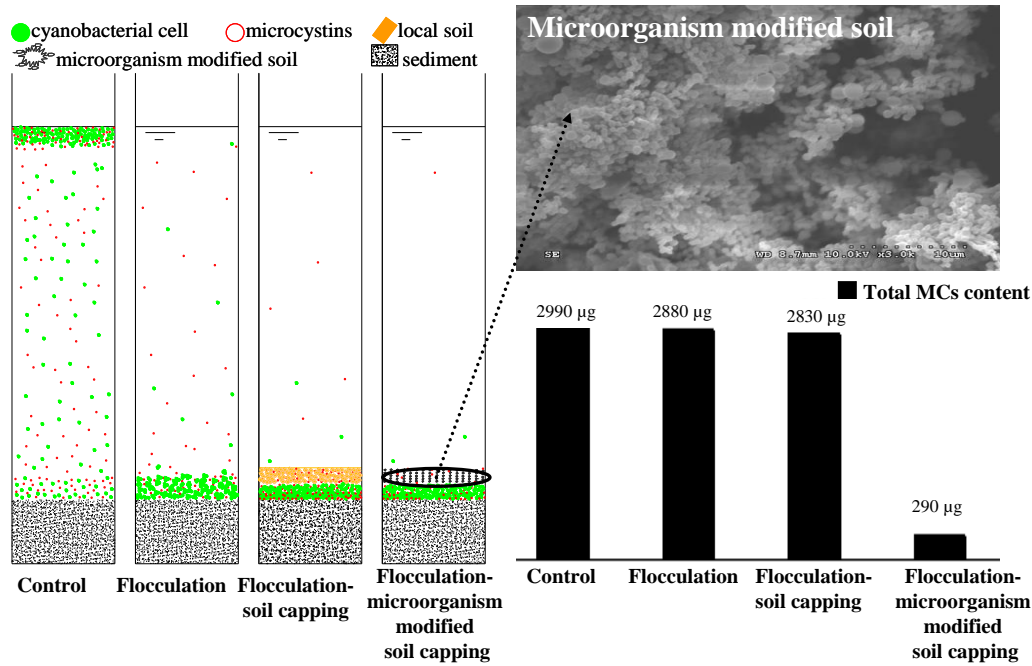
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48 INTRODUCTION

49 Cyanobacteria harmful algal blooms (cyano-HABs) have increasingly
50 occurred worldwide in various water sources,¹ and one of the negative
51 consequences is the production of cyanotoxins.² Microcystins (MCs) are the
52 frequently encountered cyanotoxins that contain over 90 variants.³ MCs are
53 primarily retained in algal cells but released into waters in case of cells lysis,⁴ and
54 can be accumulated in aquatic lives⁵ or transferred to higher trophic levels,⁶ which
55 presents a potential threat to public health as tumor promoters.⁷ Therefore, rapid
56 and sustainable degradation of MCs is imperative during the mitigation of
57 cyano-HABs in natural waters.

58 Several methods including the application of algaecides, chemical and clay
59 flocculation have been tested to remove cyano-HABs in natural waters,⁸⁻¹⁰

60 whereas the methods often result in release of intracellular MCs into water.¹¹
61 Removal of extracellular MCs using activated carbon, plant-mineral composite
62 and phoslock[®] are investigated,¹²⁻¹⁴ however, in addition to the high cost for large
63 scale application, these methods only transfer MCs from water to other media,¹⁵
64 excessive amount of MCs could return back into water column. Only when MCs
65 are degraded to less toxic products can the negative affects be diminished.¹⁶ Pan *et*
66 *al.* propose a chitosan Modified Local Soil Induced Ecological Restoration
67 (MLS-IER) technology to flocculate algal cells and then convert them into
68 submerged vegetation in shallow waters.¹⁷ However, the released toxins could be
69 diffused or mixed back to water column in deep water system where submerged
70 macrophytes cannot be restored. MCs are degradable in natural waters by a wide
71 range of microorganisms,^{5, 18} however, MCs may not be rapidly degraded by
72 indigenous microorganisms if massive intracellular MCs are released due to the
73 intensive lysis of the settled algal cells.¹⁹ Embedding exogenous MCs-degrading
74 bacteria in the capping layer provide a potential alternative to enhance the
75 biodegradation of MCs. However, challenges are presented due to the poor
76 adaptability of MCs-degrading bacteria obtained through conventional artificial
77 culture media (mostly in mineral salts), where the isolated bacteria often cannot
78 form sustainable colonies and continuously degrade MCs in natural waters.^{20, 21}

79 Aiming at improving bacterial adaptability, we have previously proposed a
80 method to isolate MCs-degrading bacteria using concentrated water extract of
81 sediment as natural culture media.^[21] It was proved that the obtained strain

82 *Pseudomonas sp.* An18 displayed enhanced and continuous degradation of MCs
83 in collected Taihu lake water under continuous addition of MCs.²¹ We anticipate
84 that if *Pseudomonas sp.* An18 is embedded into the capping materials, it may
85 greatly enhance the decomposition of MCs following MLS flocculation, since
86 both the toxins and the bacteria can be well confined under the capping layer and
87 the increased availability of MCs as well as the improved bacterial adaptability
88 may work together to enhance the biodegradation of MCs. So far, there are no
89 previous studies of embedding sustainable microorganisms into the capping
90 materials and its combination to MLS flocculation-capping method for
91 simultaneous removal of cyano-HABs and degradation of MCs.

92 In this study, microorganisms modified capping materials were prepared by
93 embedding *Pseudomonas sp.* An18 into Lake Taihu local soil. Simulated column
94 experiment containing Lake Taihu water was conducted to evaluate the effect of
95 chitosan-MLS flocculation followed by *Pseudomonas sp.* An18 modified soil
96 capping on the removal of algal cells and degradation of MC-RR and MC-LR.
97 The objective is to study the effect and the mechanism on cyano-HABs removal
98 and MCs degradation using MLS flocculation and capping method.

99 **MATERIALS AND METHODS**

100 **Toxin Standards and Reagents.** MCs standards (MC-RR and MC-LR) were
101 obtained from Taiwan Algal Science Inc (purity $\geq 95\%$ by HPLC). Enkephalin
102 (internal standard) was purchased from Sigma-Aldrich.

103 **Soil and modifier.** The soil used in flocculation experiment was collected from

104 the bank of Meiliang Bay of Lake Taihu at Wuxi, and was washed with distilled
105 water and dried for 10 h at 90 °C. The soil was sieved through 180 meshes (74
106 µm). Chitosan (solid) was obtained from Qingdao Haisheng Bioengineering
107 Co.Ltd. The molecular weight of chitosan was 500000, and the deacetylation
108 degree was 83.6%. Deionized water was prepared using a Milli-Q filtration
109 system (Millipore, Bedford, MA, USA).

110 **Preparation of Chitosan Modified Soil.** Chitosan was dissolved by adding 100
111 mg chitosan to 10 ml of 1% HCl and mixing until all chitosan was dissolved. This
112 solution was diluted with deionized water to obtain a working solution of 1
113 mg/mL. To modify the soils, a certain volume of chitosan working solution was
114 added to soil suspension. The mixture was well stirred and then ready for use in
115 the flocculation experiment. The SEM picture of chitosan modified soil was
116 presented in our previous study.²²

117 **Preparation of Microorganism Modified Soil.** Local soil was collected from the
118 shore of Lake Taihu (Meiliang Bay, Wuxi, China) and sieved through 40 meshes.
119 *Pseudomonas sp.* An18 was isolated through water extract of sediment followed
120 by concentration treatment according to the method described in our previous
121 study.²¹ The bacteria were incubated in mineral salts medium containing MC-RR
122 (50 µg/L) and MC-LR (6 µg/L) for 3 days, then harvested by centrifugation at
123 10,000 rpm for 5 min and washed twice with 50 mM phosphate buffer (pH 7.0).
124 The local soil were dried in muffle furnace at 250 °C for 3h, then the resultant soil
125 (10g) was mixed with 150 ml *Pseudomonas sp.* An18 suspensions

126 (5×10^{13} CFU/mL) in 250 ml erlenmeyer flask with shaken at 150 rpm for 4h. The
127 suspension solution was centrifuged at 7,000 rpm and the precipitate was washed
128 3 times with sterile water, finally, 1M NaCl was added to liberate the weakly
129 retained bacterial cells.²³ The modified soil was capable of embedding 10^{15}
130 bacterial cells/g soil. The evaluation of *Pseudomonas sp.* An18 proliferation in the
131 modified soil was conducted in mineral salt medium with addition of filter
132 sterilized MCs every 2 days,²⁰ the microorganism modified soil was vortexed to
133 detach the embedded *Pseudomonas sp.* An18 from soil and the bacteria number
134 were counted using plate counting method in mineral salt medium containing
135 MC-RR (50 μ g/L) and MC-LR (6 μ g/L).

136 **Incubation Experiment.** The experiment was conducted in 2000 mL plexiglass
137 columns with diameter of 8.4 cm. The Taihu local soil was autoclaved and used as
138 sediment in the column. A volume of 1500 mL cyano-HABs water were collected
139 from Meiliang Bay, Lake Taihu and added into the columns (the initial algae
140 concentration was 1.38×10^{10} cells/L, concentration of MC-RR and MC-LR were
141 5.14 and 0.84 μ g/L, respectively). Twelve columns containing the same amount of
142 sediment and algal water were prepared. Three of them were used as controls
143 where no flocculants or capping materials were added. Chitosan modified local
144 soil (2 mg/L chitosan and 100 mg/L soil, this optimized dosage was obtained
145 according jar test presented in Figure S1 and S2) was added to all the rest of the
146 columns and then followed by three different capping treatments. No capping
147 materials were added in the flocculation only systems (three columns). Among the

148 rest of the columns, the flocculated algal flocs were capped with 1 cm local soil
149 (three columns) or 1 cm *Pseudomonas sp.* An18 modified soil (three columns).
150 The twelve columns were incubated at 25 °C with an alternating cycle of 12h light
151 and 12h darkness for 40 days.

152 **Sample Collection and Preparation.** Water samples were collected at 1 cm
153 below the water surface every 3 days. After filtration through 0.22 µm membrane,
154 the water was spiked with 100 µl enkephalin internal standard (10 µg/L) to reduce
155 the matrix effects during measurement of MCs.²⁴ The algal cells were enumerated
156 every 3 days. After 40 days' incubation, in order to assess the distribution of
157 MC-RR and MC-LR in different parts of the sediment-water systems, the
158 overlaying water was carefully taken off from each column and filtered through
159 0.45 µm membrane to separate the suspended algal cells. The sediment in the
160 columns were sliced into three sections: 0-1 cm (capping layer), 1-2 cm (flocs
161 layer), and 2-6 cm (sediment layer). For the control system, there was no capping
162 and flocs layer. The capping layer (local soil or *Pseudomonas sp.* An18 modified
163 soil) and sediment layer were freeze-dried and extracted in 0.1 M EDTA-Na₄P₂O₇
164 for three times. After centrifugation at 4000 rpm, the aqueous extractions were
165 passed through SPE cartridge (300 mg, 3 ml, Waters), and the eluted solution was
166 evaporated under nitrogen and dissolved in 1 ml distilled water for UPLC-MS/MS
167 analysis.²⁵ Suspended algal cells and algae flocs were freeze-dried and extracted
168 using 40% (v/v) methanol solution with ultrasonication (300W, 5 mins) three
169 times, followed by the evaporation of the elution containing MCs from SPE

170 cartridge, as was described previously.²⁶ The total content (μg) of MC-LR and
171 MC-RR in the whole systems after 40 days' incubation were calculated according
172 to the toxin concentration in filtered water, suspended algae cells and that in
173 sediment, flocs layer, and capping layer (dry weight).

174 **MCs Determination.** Quantitative analysis of MCs was performed using a
175 LC-MS system equipped with electrospray ionization tandem mass spectrometry
176 (UPLC-ESI-MS/MS) (Acquity UPLC, Quattro Premier XE, Waters, USA).

177 Sample separation was carried out with UPLC system and Acquity UPLC BEH
178 C₁₈ column (i.d. 2.1 mm \times 50 mm, particle size 1.7 μm , pore size 130 \AA , Waters).

179 The column oven was kept at 40 $^{\circ}\text{C}$ and the injection volume was 10 μL . The flow
180 rate used was 0.2 ml/min. Water and acetonitrile were used as mobile phases, the
181 organic phase was linearly increased from 45 % to 60 % in 2.5 min, then increased
182 to 100% in 1.5 min and held for 0.5 min, and finally brought back to 45% and held
183 for 3 min until the next injection. The mass spectrometer was operated in positive
184 mode electrospray ionization in multiple-reaction monitoring mode. Source
185 temperature and desolvation gas temperature were held at 120 and 350 $^{\circ}\text{C}$,
186 respectively. The measured recoveries of the method ranged between
187 91.0-103.2 %. The detection limit was 6.0 and 3.0 ng/L for MC-RR and MC-LR,
188 respectively.

189 **Analysis of MCs Biodegradation Products.** Biodegradation products of the
190 toxins were analyzed using matrix-assisted laser desorption/ionization-time of
191 flight mass spectrometry (MALDI-TOF MS, BIFLEX III, Bruker Inc., USA).

192 After 40 days incubation, MCs from the sediment of local soil capped system and
193 *Pseudomonas sp.* An18 modified soil capped system were extracted. The
194 extracted MCs solution (containing MCs and its degradation products) was dried
195 with nitrogen, then dissolved in distilled water. MALDI-TOF-MS instrument was
196 equipped with a nitrogen laser operating at 337 nm, and a two-state ion source
197 operating in the delayed extraction mode. Briefly, 2 μ L of a saturated solution of
198 a-cyano-4-hydroxycinnamic acid (in 3:2 v/v acetonitrile-0.1% trifluoroacetic acid)
199 were premixed with 2 μ L of the MCs solution. Afterward, 2 μ L of this mixture
200 were applied to the sample plate, and air-dried at 24 °C. Measurements were
201 performed at an acceleration voltage of 20 kV using reflector mode, allowing the
202 determination of monoisotopic mass values. Each spectrum refers to the sum of
203 100-200 individual laser shots.

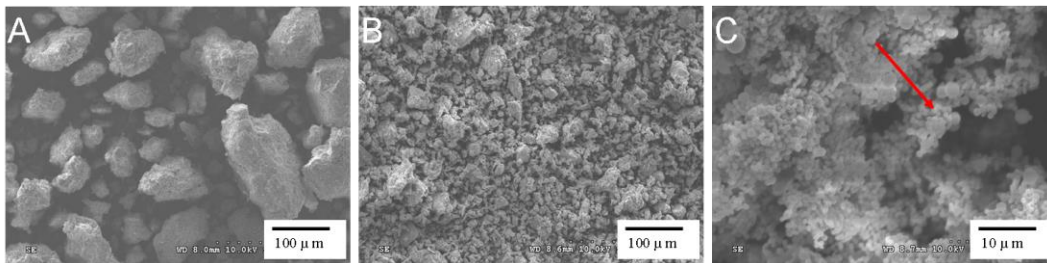
204 **Soil Characterization.** Micrographs of soil and microorganism modified soil
205 were characterized using a XL-30 scanning electron microscope (Philips Corp.,
206 The Netherlands).

207 **Statistical Analysis.** The t-test was used to determine significant differences
208 among different measurements, a significance was assumed when $P < 0.05$.

209 **RESULTS**

210 **Soil Characterization.** Scanning electron microscope (SEM) images illustrated a
211 smooth surface and discrete particles of local soil particles (Figure 1A), while
212 modification by *Pseudomonas sp.* An18 substantially roughened the surface of
213 soil with widely distributed ridges and large density of *Pseudomonas sp.* An18

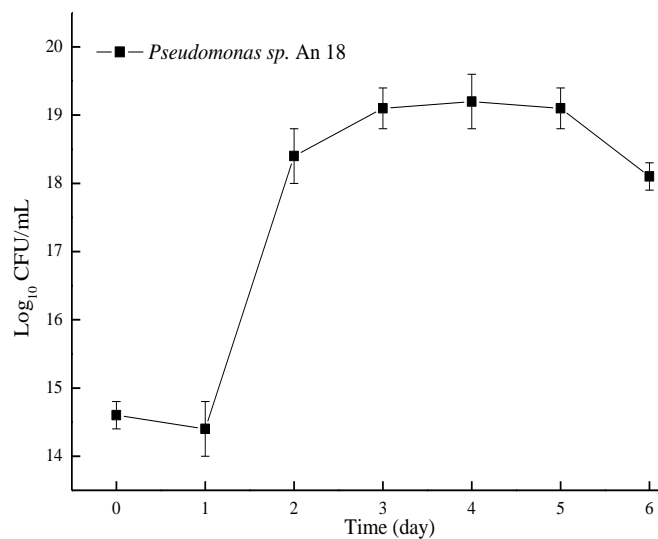
214 (Figure 1B-C). Evaluation of *Pseudomonas sp.* An18 carrying capability revealed
215 the modified soil was capable of embedding 10^{15} bacterial cells/g soil (Figure S3).
216 Proliferation tests showed that the biomass of *Pseudomonas sp.* An18 in the
217 microorganism modified soil dramatically increased after 1 days' lag phase and
218 yielded stable CFU counts (nearly 10^{19} CFU/mL) after 3 days (Figure 2),
219 suggesting the soil was favorable for *Pseudomonas sp.* An18 growth when MCs
220 were supplied.



221

222 **Figure 1.** SEM photographs of local soil and *Pseudomonas sp.* An18 modified
223 soil. (A) local soil (200×magnification), (B) *Pseudomonas sp.* An18 modified soil
224 (200×magnification), (C) *Pseudomonas sp.* An18 modified soil
225 (3000×magnification). The arrow in 1C pointed to *Pseudomonas sp.* An18.

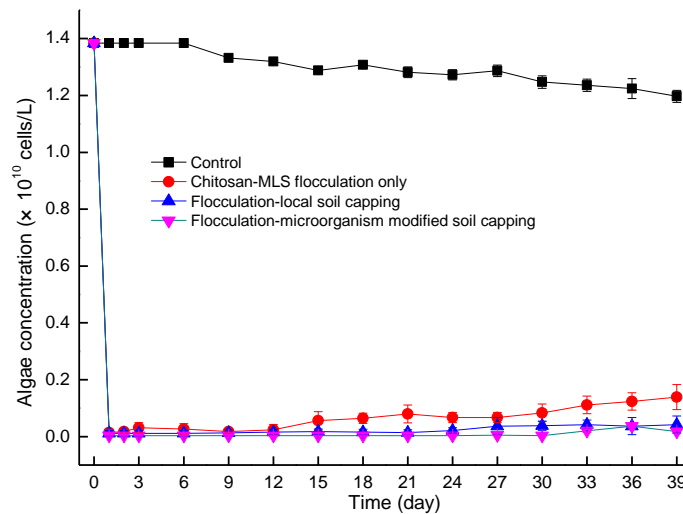
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228 **Figure 2.** Proliferation of *Pseudomonas sp.* An18 on the microorganism modified
229 soil in mineral salts medium containing MCs. Data represent the mean values of
230 triplicate \pm SD.

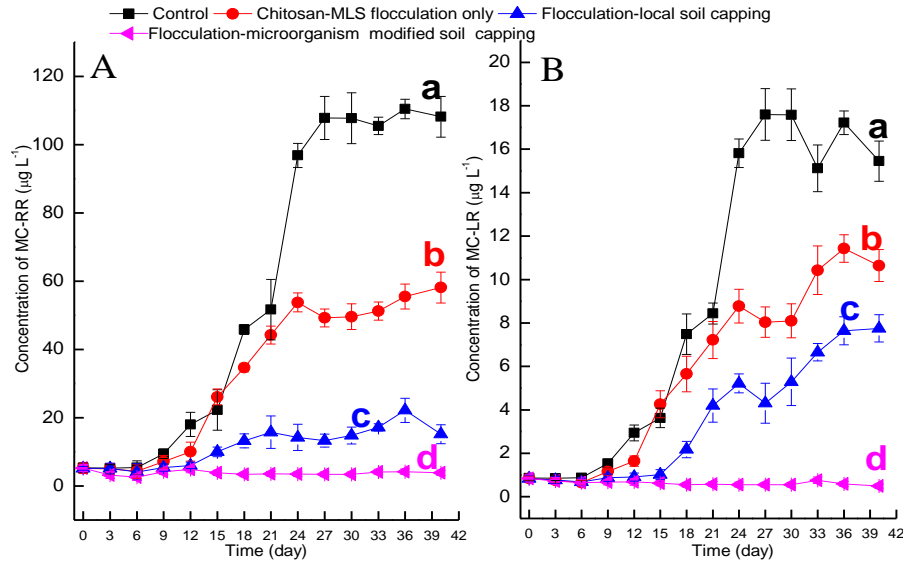
231 **Algae Removal.** In the chitosan-MLS flocculation only systems, the algae
232 removal rate reached 99% within 1 day, however, re-suspension of algal cells
233 occurred in the following days and the removal rate reduced to about 90% at day
234 40 (Figure 3). The re-suspension was effectively avoided when local soil or
235 *Pseudomonas sp.* An18 modified soil was used to cap the sunk algae flocs, where
236 the removal rate remained at 99% throughout the 40 days' period. A small algae
237 removal (maximum of 13% at day 40) was also observed in the control systems
238 (Figure 3).



239 **Figure 3.** Changes in algae concentration in water columns. Data represent the
240 mean values of triplicate \pm SD.
241

242 **MCs in Water Column.** The initial concentration of dissolved MC-RR in the
243 Taihu water sample was 5.14 μ g/L. In the control systems, an obvious increase of
244 dissolved MC-RR was observed from day 9, which reached the maximum level of

245 108.14 $\mu\text{g/L}$ between day 24 to 40 that is 20-fold higher than the initial level
246 (Figure 4A-a). Compared to the control systems, the increase of dissolved MC-RR
247 was reduced by 50% in chitosan-MLS flocculation only systems (Figure 4A-b),
248 70% in flocculation-local soil capping systems (Figure 4A-c), and 96% in
249 flocculation plus *Pseudomonas sp.* An18 modified soil capping systems (Figure
250 4A-d), respectively. The dissolved MC-LR showed a similar trend with that of
251 MC-RR among different treatments. The initial concentration of dissolved
252 MC-LR in Taihu water sample was 0.84 $\mu\text{g/L}$. In the control systems, dissolved
253 MC-LR was quickly increased from day 9 to day 24 and reached 15.45 $\mu\text{g/L}$
254 between day 24 to 40 (Figure 4B-a). The MC-LR release was reduced, at day 40,
255 to 10.64 $\mu\text{g/L}$ by chitosan-MLS flocculation only treatment (Figure 4B-b), 6.65
256 $\mu\text{g/L}$ by flocculation plus local soil capping treatment (Figure 4B-c), and 0.49
257 $\mu\text{g/L}$ by flocculation plus *Pseudomonas sp.* An18 modified soil capping treatment
258 (Figure 4B-d), respectively. The diffusion of released MCs from sediment to water
259 were largely reduced in soil capping treatment (Figure 4A-c, Figure 4B-c) and
260 entirely disappeared in microorganism modified soil treated systems (Figure 4A-d,
261 Figure 4B-d).

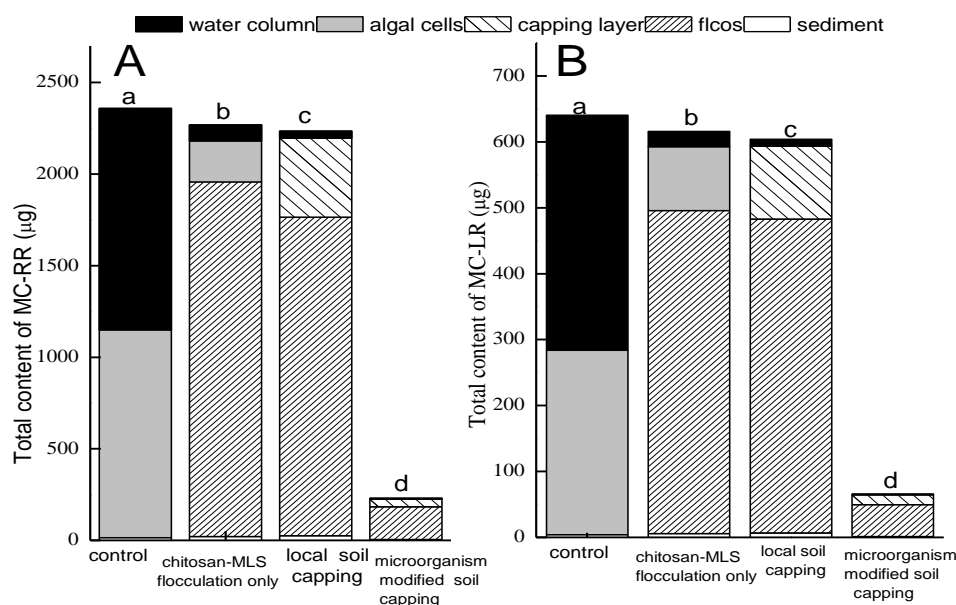


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263 **Figure 4.** Changes of dissolved MC-RR (A) and MC-LR (B) concentration in the
 264 surface water of different systems. a, b, c, d indicate different treatment. Data
 265 represent the mean values of triplicate \pm SD.

266 **Distribution of MCs.** Measurements of MCs in water, algae cells, sediment, flocs,
 267 and capping layer illustrated the distribution of MCs in different parts of the
 268 sediment-water column systems. For the control systems, among the total MC-RR
 269 (2360 μg), 51% of the intracellular MC-RR (1209 μg) was released into the water,
 270 48% was still left in suspended algal cells (1135 μg) and 1% in the sediment at
 271 day 40 (Figure 5A-a). Chitosan-MLS flocculation transferred 85% of the MC-RR
 272 into the sunk algal flocs, and left 10% in suspended algae cells and 4% in water
 273 (Fig. 5A-b). In the flocculation and soil capping treatment, MC-RR was almost
 274 entirely removed from suspended algae cells and water (2% left), 78% and 19%
 275 were transferred into the flocs layer and capping layer (Fig. 5A-c), respectively.
 276 However, the total content of MC-RR in the flocculation only and
 277 flocculation-soil capping systems remained similar to that of the control. In

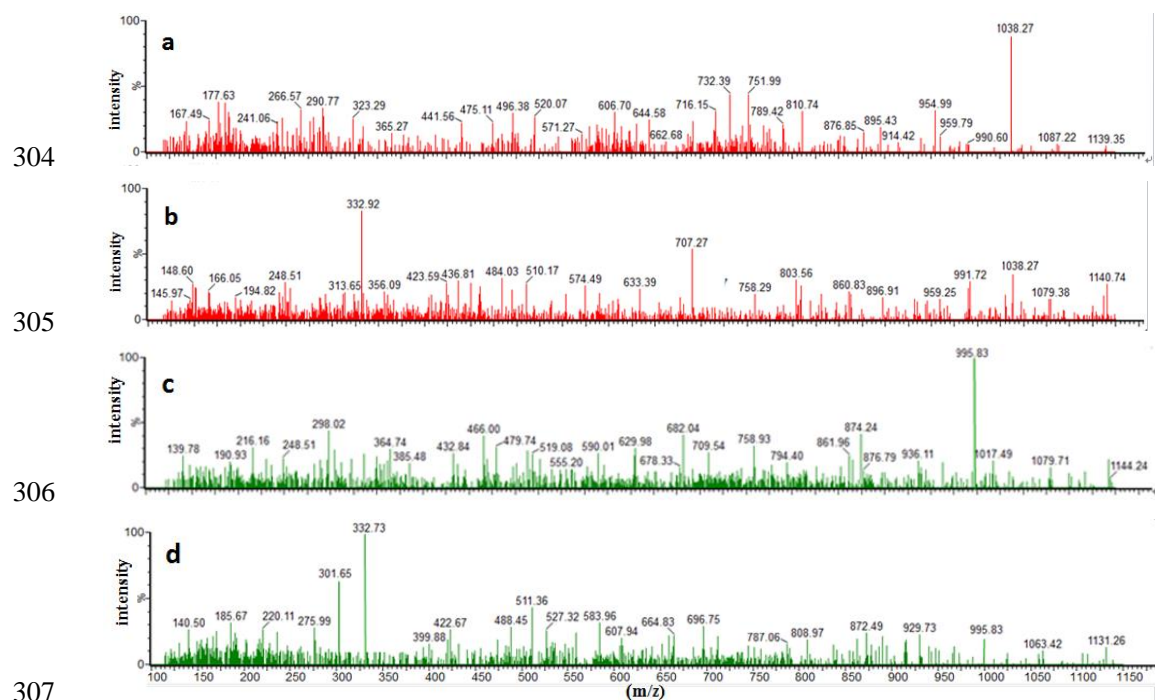
278 contrast, nearly 90% MC-RR in the whole sediment-water system was degraded in
 279 chitosan-MLS flocculation plus *Pseudomonas sp.* An18 modified soil capping
 280 systems (Fig. 5A-d), and the dissolved MC-RR left in the water (6 μg) was
 281 reduced by 201, 13 and 5-fold compared to the control, flocculation only and
 282 flocculation-soil capping systems, where the original total content of toxins were
 283 similar. The distribution of MC-LR followed a similar pattern with that of MC-RR.
 284 The dissolved MC-LR in the control was 356 μg (Figure 5B-a) and was reduced
 285 to 23 and 10 μg in flocculation (Figure 5B-b) and flocculation-soil capping
 286 systems (Figure 5B-c), respectively, but the total MC-LR content (ranged from
 287 620 to 650 μg) remained similar among these three systems (Figure 5B). In
 288 contrast, the total MC-LR content was reduced by 90% and dissolved MC-LR by
 289 99% in flocculation plus *Pseudomonas sp.* An18 modified soil capping treatment
 290 (Figure 5B-d) compared to the control systems.



291
 292 **Figure 5.** Distribution of total content of MC-RR (A) and MC-LR (B) in different
 293 parts of the sediment-water system of each treatment after 40 days' incubation.

294 Data represent the mean values of triplicate.

295 **Biodegradation Products of MCs.** The MS spectrum of MC-RR revealed a
296 major ion at m/z 1038.27 after 40 days incubation in soil capped sediment (Fig.
297 6a), in contrast, two major degradation products of MC-RR (MW=332.92 and
298 707.27) were observed in *Pseudomonas sp.* An18 modified soil capped layer (Fig.
299 6b), corresponding to the reduced intensity of MC-RR (MW=1038.27). Similarly,
300 In comparison to the great intensity of MC-LR in soil capped sediment (Fig. 6c),
301 the predominate ion in *Pseudomonas sp.* An18 modified soil capped layer
302 converted to MW=332.73 and 301.65 (Fig. 6d), of which the MC-LR ion at m/z
303 995.83 almost disappeared.



308 Figure 6. Mass spectra of MC-RR in the sediment of soil capping layer after 40
309 days incubation (a), MC-RR and its biodegradation products after 40 days in the
310 sediment of *Pseudomonas sp.* An18 modified soil capped layer (b), MC-LR in the
311 sediment of soil capping layer after 40 days incubation (c), MC-LR and its

312 biodegradation products after 40 days in the sediment of *Pseudomonas sp.* An18
313 modified soil capped layer (d).

314 **DISCUSSION**

315 **Effect of Flocculation and Capping on Algae and Toxin Removal.** Algal cells
316 tend to suspend and float in water because of the negatively charged cell surface,
317 low specific gravity and specific buoyant structure. Chitosan, a cellulose-like
318 polyelectrolyte biopolymer, is derived from the alkaline deacetylation of
319 crustacean chitin, which possesses several intrinsic characteristics of coagulants
320 and flocculants such as high cationic charge density, long polymer chains,
321 bridging of aggregates and precipitation. The high content of positively charged
322 amine groups in the chitosan structure facilitates electrostatic interaction between
323 polymer chains and negatively charged algal cells,^{27, 28} which caused rapid
324 mitigation of cyano-HABs (Figure 3). For toxin producing cyano-HABs, most
325 toxins are contained inside the cells during the bloom, which will be released and
326 contributed to the dissolved MCs when the cells lysis occur.²⁹ In the
327 chitosan-MLS flocculation only systems, although 99% algal cells were removed
328 from the water column at day 1 (Figure 3), dissolved MC-RR (Figure 4A-b) and
329 MC-LR (Figure 4B-b) began to increase in the water column at day 12 and
330 reached the maximum concentration during day 24 to 40. This result suggests that
331 flocculation alone can only remove algae cells from water column, however,
332 dissolved MCs toxins may be significantly released if the algae flocs are not
333 further treated. The algae flocs may be resuspended back to the water and

334 continue to grow under the field conditions especially in shallow waters.³⁰ By
335 using soil capping after the flocculation, the increase of dissolved MC-RR (Figure
336 4A-c) and MC-LR (Figure 4B-c) from the sediment was postponed about a week
337 and reduced by 74% and 40% compared to the flocculation only treatment at day
338 40. Soil capping not only physically sealed the algal cells in sediment and depress
339 the diffusion of dissolved MCs from the sediment to the water, but also
340 significantly promoted the cells lysis (Figure S4). Algal cell lysis is crucial for the
341 reduction of algae recruitment,³⁰ however, it will result in the release of
342 intracellular MCs.²⁹ If the released toxin is only buried under the capping layer but
343 not degraded, it will be diffused into the water in a postponed period (Figure 4A-c,
344 Figure 4B-c). Previous reports found that once the capping layer was saturated,
345 the excessive toxins could eventually be released into water.³¹ The problems
346 mentioned above can be solved if the capping layer is highly efficient for MCs
347 degradation. Indeed, in the flocculation plus microorganism modified capping
348 systems (1 cm), both MC-RR and MC-LR were almost entirely decomposed in the
349 water (Figure 4A-d, 4B-d) and sediment (Figure 5A-d, 5B-d) throughout the
350 tested period. By increasing the thickness of the *Pseudomonas sp.* An18 modified
351 capping layer, the microorganism may obtain more time to degrade the upcoming
352 MCs molecules under the capping layer.

353 **Adaptability of Incubated Microorganisms in Natural Environment.** In
354 cyano-HABs waters, degradation of MCs by the indigenous MCs-degrading
355 microorganism is one of the main pathways for toxins removal.^{32, 33} In our

356 simulated systems, the maximum dissolved MC-RR and MC-LR reached 108.14
357 $\mu\text{g/L}$ (Figure 4A-a) and 15.45 $\mu\text{g/L}$ (Figure 4B-a) during 40 days' incubation,
358 suggesting long time was required for removal of the toxins by the native
359 microorganism only. This agreed well with the report that MCs can not be rapidly
360 removed relying on the native MCs-degrading microorganisms after abrupt release
361 of MCs under collapse of severe cyano-HABs.¹⁹ Although exogenous
362 MCs-degrading bacteria have been widely studied under laboratory conditions,
363 one of the problems is that most of the incubated bacteria can hardly be
364 sustainable in natural waters due to the poor bacterial adaptability.³⁴

365 The conventional approach of obtaining MCs-degrading bacteria is collection
366 of microorganism communities from MCs contaminated sediment or water,
367 followed by enrichment and isolation in artificial medium (mostly mineral salts
368 medium) where MCs were supplied as the sole carbon and nitrogen source.³⁵ The
369 discrepancy of chemical property between artificial media and natural
370 environment, in addition to the competition with indigenous microorganism
371 communities in natural environment often result in poor bacterial adaptability of
372 the incubated bacteria and limited toxins removal efficiency.³⁶ In order to improve
373 bacterial adaptability in natural waters, an isolation strategy using concentrated
374 natural medium was proposed in our previous study,²¹ by which the obtained
375 *Pseudomonas sp.* An18 showed enhanced adaptability and sustainability for MCs
376 degradation in sampled Taihu Lake water when continuous addition of MCs were
377 degraded.

378 In flocculation only systems, dramatic increase of dissolved MC-RR and
379 MC-LR occurred after nearly 14 days incubation (Figure 4A-b, Figure 4B-b),
380 despite the diffusion of released MCs can be largely reduced in soil capping
381 treatment (Figure 4A-c, Figure 4B-c), the total residual toxins in the experimental
382 systems were not significantly reduced compared to control (Figure 5), in contrast,
383 the increase of dissolved MCs had entirely disappeared (Figure 4A-d, Figure
384 4B-d), the residual MC-RR (Figure 5A-d) and MC-LR (Figure 5B-d) in the whole
385 sediment-water systems displayed 90% reduction in *Pseudomonas sp.* An18
386 microorganism modified soil treated systems, suggesting the adaptability of the
387 incubated *Pseudomonas sp.* An18 strains and biodegradation of MCs in the
388 experimental systems. In this study, *Pseudomonas sp.* An18 was enriched from
389 water extracts of sediment thus experienced selective pressure and subsequent
390 isolation in concentrated water extracts of sediment, which showed similar
391 nutrient condition, component and PH with the real environment and led to
392 adaptation of the re-incubated microbes in the simulated natural water.

393 **Concentrated Microorganisms and MCs by in-situ flocculation-capping.**

394 Biodegradation of MCs by exogenous MCs-degrading bacteria under natural
395 conditions requires not only the adaptation of bacteria, but also the reaction
396 opportunity between the incubated bacteria and toxins.³⁷ Direct addition of
397 isolated bacteria into natural waters may encounter serious dilution problem.³⁴
398 Embedding bacteria into soil can keep relative high bacterial biomass on the soil
399 particles therefore enhance its reactivity with the toxins. Moreover, in natural

400 cyano-HABs waters, the algal cells together with MCs are predominant in the
401 surface of water column,⁵ which may also impair the degradation reaction
402 between toxins and bacteria. In comparison to 51% MC-RR (Figure 5A-a) and
403 56% MC-LR (Figure 5B-a) that were released and diluted in the water column in
404 the control systems, 96% MC-RR (Figure 5A-c) and 98% MC-LR (Figure 5B-c)
405 were confined under the capping layer in flocculation-soil capping systems, hence
406 provided concentrated MCs under the capping layer, suggesting that if
407 MCs-degrading bacteria can be embedded in the capping material, the
408 biodegradation reaction may be enhanced due to the simultaneously raised toxins
409 concentration and bacteria biomass. In this study, the bacteria modified soil loaded
410 nearly 10^{15} *Pseudomonas sp. An18* cells/g modified soil (Figure S3), indicating 1
411 cm thickness capping layer (approximately 140 g) can embed 1.4×10^{17}
412 *Pseudomonas sp. An18* cells, therefore created a micro-environment with higher
413 bacterial biomass. Furthermore, proliferation of *Pseudomonas sp. An18* in the soil
414 led to 10^5 increases of bacteria number during 6 days' incubation (Figure 2),
415 suggesting the soil favored bacteria inhabitation and proliferation.

416 **Degradation of MCs by flocculation-microorganism Modified Soil Capping.**

417 In addition to providing adequate time for bacteria to establish biodegradation
418 ability before they were mixed back to water column, flocculation-microorganism
419 modified soil capping treatment also retained the released MCs within the
420 confined sediment layer. The concentrated toxins and bacteria in-situ hence
421 increased the availability of toxins to *Pseudomonas sp. An18*, as the

422 biodegradation efficiency was positively proportional to the initial toxins
423 concentrations within a certain range,^{10, 19} therefore, 90% degradation of MC-RR
424 (Figure 5A-d) and MC-LR (Figure 5B-d) from the whole sediment-water systems
425 were triggered. This is likely due to that when MCs are released and diffused into
426 the microorganisms modified soil, the toxins are metabolized as carbon and
427 nitrogen source by *Pseudomonas sp.* An 18. This was confirmed by the analysis of
428 MCs biodegradation products by MALDI-TOF mass spectra, which revealed that
429 in *Pseudomonas sp.* An18 modified soil capped systems, the degradation products
430 of MC-RR (MW=332.92 and 707.27, Fig 6b) and MC-LR (MW=332.73 and
431 301.65, Fig 6d) were evident as the concentration of MC-RR and MC-LR
432 decreased in the experimental systems (Figure 5A-d and Figure 5B-d). Moreover,
433 the appearance of MW=332, which was recognized as the Adda residues,^[38] in
434 both the biodegradation products of MC-RR and MC-LR, indicating that the
435 cleavage of Adda-Arg peptide bond, by which the toxicity of MCs can be
436 substantially reduced.³⁹ Zhang et al.(2010) studied the biodegradation pathway of
437 MC-RR bacterium *Sphingopyxis sp.* USTB-05, the results suggested that
438 Adda-Arg peptide bond of MC-RR was cleaved, then a hydrogen and a hydroxyl
439 were combined onto the NH₂ group of Adda and the carboxyl group of arginine,³⁵
440 during which the MW=332 were indicated as the Adda residues.^{35,38} The examined
441 MW=332.92 in the present test implying the similar biodegradation pathway of
442 MCs by *Pseudomonas sp.* An18, which need further investigations.

443 In this column experiments, under the co-effects of prevented bacterial

444 dilution (embedding *Pseudomonas sp.* An18 in the microorganism modified soil),
445 concentrated MCs concentration in-situ (flocculation and microorganism modified
446 soil capping), as well as enhanced bacteria adaptability, the reduction of dissolved
447 MCs (Fig. 4) in water and the decomposition of MCs in the sediment can be
448 achieved (Fig. 5).

449 In order to understand unambiguously the role of incubated *Pseudomonas sp.*
450 An18 and to exclude the biodegradation of MCs by the indigenous microorganism,
451 autoclaved local soil instead of in-situ sediment was used as sediment layer in the
452 column. Under field conditions, natural aquatic sediments have a wide range of
453 MCs-degrading microorganisms, it is possible that the combined effects of
454 indigenous bacteria in the natural sediment and *Pseudomonas sp.* An 18 in the
455 capping layer may influence the efficiency of MCs degradation. In addition, this
456 study was carried out in columns under stationery condition, therefore the
457 re-suspension of sunk algal cells (Figure 3) and diffusion of the released MCs
458 (Figure 4) were depressed through 1cm thickness soil or microorganism modifies
459 soil capping. However, capping as a measure for lake restoration is inevitably
460 influenced by the complicated conditions in the field, such as vertical transport
461 and re-suspension generated by bioturbation⁴⁰ and horizontal transport induced by
462 the wind and current. Although re-suspension of algal flocs has been studied
463 under simulated laboratory conditions,³⁰ the complicated impact of field condition
464 needs further studies at various scales including lab, mesocosm, whole water
465 ponds, and open waters.

466 **Environmental Implications.** The cyano-HABs in eutrophic lakes are usually
467 followed by toxins release due to the decomposition of algal cells. The dissolved
468 MCs in many cyano-HABs lakes can remain a few $\mu\text{g/L}$,^{5, 41} but the toxins can be
469 accumulated in zooplankton, aquatic organisms and eventually bioaccumulated in
470 human bodies through food chain.⁴² Only when MCs are degraded to less toxic
471 products, can the negative affects be diminished.¹⁶ In this study, the
472 flocculation-capping technology converged algal cells coupled with intracellular
473 toxins under the capping layer, accelerated algae decomposition which reduced
474 the recruitment of algae cell, and potentially promote the transfer of released
475 nutrients from algae cells to vegetation if submerged vegetation seeds were
476 included in the capping material in shallow waters. Different methods should be
477 jointly used to achieve both short term and long term effects. For instance,
478 flocculation is important to remove toxic algae from water and create the light
479 conditions for the vegetation restoration in shallow water systems. The problems
480 of floc resuspension and algal toxin release associated with flocculation method
481 can be compromised by using microorganism modified soil/sand capping method.
482 The capping method is also important for maintaining the water clarity for an
483 extended period so that it may create a window period for the restoration of
484 submerged vegetation, where the remediation processes can be relayed into a
485 longer term. Once the excess nutrients are redistributed from water to sediment
486 and from algae to vegetation, the restoration of a healthy food chain and the
487 aquatic ecology may become possible by using the nutrients as a resource. As the

488 released toxins were biodegraded, it is possible to diminish the risk of toxin
489 accumulation in aquatic vegetation, organisms and animals, which may further
490 impact on food web and public health, which needs further multidisciplinary long
491 time studies.

492

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497

498 **Supporting Information Available**

499 Jar test for cyano-HABs removal using chitosan modified soil (Figure S1 and S2)
500 Assessment of *Pseudomonas sp.* An18 carrying capacity of microorganism
501 modified soil. Embedding capability of *Pseudomonas sp.* An18 on the
502 microorganism modified soil (Figure S3). Morphology characterization of algal
503 cells (Figure S4). This information is available free of charge via the Internet at
504 <http://pubs.acs.org>.

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