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1 **Capture and flocculation of toxic cyanobacteria by amphiphilic peptide dendrimers**
2 **for mitigating harmful blooms**

3
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31

32 **Abstract**

33

34 Harmful and toxic cyanobacterial blooms in freshwater constitute critical environmental

35 problems and are becoming more frequent as a consequence of global climate change.

36 One mitigation strategy is flocculation, which could be achieved with treatment by highly

37 efficient, non-toxic, and biodegradable agents. Here, we report two amphiphilic peptide

38 dendrimers (AmPDs), KK₂ and KK₂K₄, for the removal of the toxic and bloom-forming

39 cyanobacterium *Microcystis aeruginosa* from freshwater. KK₂ and KK₂K₄ are composed

40 of a hydrophobic alkyl chain and a positively charged polylysine dendron of the first and

41 second generation, respectively. Owing to electrostatic interactions with the negatively

42 charged cell surface of *M. aeruginosa*, KK₂ and KK₂K₄ could quickly capture the

43 cyanobacterial cell population via flocculation at very low concentrations. When

44 combined with the natural clay sepiolite, the two AmPDs were even more efficient in

45 promoting cell flocculation. The most performant system, with 3.0 mg/L KK₂K₄ and

46 sepiolite clay, could remove 97.1% of *M. aeruginosa* within 15 min by forming stable

47 flocs. Remarkably, such a system was able to trap toxin molecules released by

48 cyanobacterial cells, thus limiting its destructive impact on ecosystems. This study

49 demonstrates how self-assembling dendrimer materials can resolve cyanobacterial

50 blooms, providing an innovative solution to this environmental challenge.

51

52 **Keywords**

53 Cyanobacterial bloom, Toxin, Amphiphilic dendrimer, Flocculation, Sepiolite clay,

54 Negatively charged microbial surface

55

56 **1. Introduction**

57 Harmful algal blooms are a serious environmental, water quality, and public health issue
58 worldwide. Specifically, cyanobacterial bloom refers to the excessive growth of
59 photoautotrophic cyanobacteria, which form dense biomass and produce toxins,
60 contaminating water and causing adverse effects on human health or even death of
61 domestic and livestock animals [1,2]. In recent years, the frequency, timespan, and
62 intensity of cyanobacterial blooms have increased because of anthropogenic activities [3-
63 5]. This environmental situation is now a major concern for the usage, sustainability, and
64 safety of freshwater reserves with an important economic impact estimated to cost tens of
65 billions of US dollars every year [6]. Industrial, agricultural, and domestic wastewater
66 discharges have led to water body eutrophication, which generates excellent nutritional
67 conditions for cyanobacterial proliferation [1,7]. In addition, many cyanobacteria species
68 prefer warmer temperatures and are benefitting from climate changes, which likely
69 increases the occurrence of harmful blooms [8].

70

71 In particular, cyanobacterial blooms are associated with the production of a large amount
72 of toxins, posing a serious threat to public health. For example, one of the most
73 commonly observed harmful cyanobacteria, *Microcystis aeruginosa*, generates multiple
74 microcystin congeners (MCs) that are hepatic toxins and potentially carcinogenic for
75 humans and other living organisms [9,10]. Among the MCs, the microcystin-LR (MC-
76 LR) is the most abundant and toxic [11]. MC-LR and other MCs are cyclic peptides made
77 by non-ribosomal peptide synthetases, which are relatively stable, and thus difficult to
78 remove from waterbodies affected by cyanobacterial proliferation. In view of the

79 negative impact of cyanobacterial blooms on ecosystems, human health, and the
80 economy, many studies have been undertaken to develop efficient and eco-friendly
81 mitigation strategies.

82

83 Methods to remediate cyanobacterial contaminations include physical, chemical, and
84 biological approaches. Physical strategies such as ultrasonic cracking and air flotation
85 devices are often costly, only applicable to small scale, and can increase the transient
86 release of cyanotoxins [12,13]. Chemical approaches mainly employed oxidants, e.g.
87 copper sulfate, hydrogen peroxide, or diverse herbicides or photocatalysts [14,15]. Large
88 amounts are required to solve algal blooms, augmenting the risk that these molecules,
89 which are also toxic for non-target species, accumulate in the environment. In addition,
90 such treatments often lead to cyanobacterial cell lysis, and thus increase the release of
91 cyanotoxins [16]. Lastly, biological methods exploit predator-prey relationships,
92 nutritional competition between cyanobacteria and harmless organisms, parasitism, or
93 virus infection [17,18]. However, these strategies are often considered too slow for the
94 treatment of sudden cyanobacterial blooms [17,19]. Therefore, an alternative
95 methodology still needs to be developed for the efficient eradication of toxic
96 cyanobacteria.

97

98 One of the most cost-effective and convenient technologies for the removal of harmful
99 cyanobacteria from lakes and/or raw water supply is flocculation [20,21]. The floc-and-
100 sink method aims at displacing cyanobacteria from the water column to the sediment,
101 thus limiting their negative impact [22,23]. However, conventional inorganic flocculants

102 such as metal salts and polyaluminium chloride are generally not suitable for large-scale
103 flocc-and-sink removal of harmful algal blooms due to their toxicity for non-target species
104 [20]. Organic flocculants, including chitosan, tannin, and cationic starch, have also
105 different shortcomings such as low efficiency and high cost [24,25]. Among extensively
106 studied flocculants, natural clays such as sepiolite are some of the few that have been
107 applied in situ for the treatment of algal blooms [26]. Clays are abundant and usually
108 non-toxic, and their flocculation process prevents the release of toxins [27]. Still, their
109 overall efficiency is low, and their surface usually needs modifications with other
110 compounds to increase the electrostatic attraction of negatively charged cyanobacteria
111 [28,29].

112

113 Amphiphilic peptide dendrimers (AmPDs) developed in recent years are tunable organic
114 complex molecules made of hydrophobic alkyl chains and hydrophilic peptides [30].
115 Until now, non-toxic, biocompatible, and biodegradable AmPDs have mainly been
116 designed and explored for biomedical purposes [31]. For instance, they are employed for
117 bioimaging, drug delivery, as transfection vectors, or agents against pathogenic microbes
118 [32-35]. Outside of the biomedical field, applied research on AmPDs has not yet been
119 reported. We report here the use of AmPDs bearing a strong positively charged surface
120 for the removal of negatively charged microbes such as cyanobacteria for mitigating
121 cyanobacterial contaminations. Specifically in this study, two AmPDs, KK₂ and KK₂K₄
122 (Scheme 1), were evaluated for the flocculation and sinking of the toxic cyanobacterium
123 *M. aeruginosa*. KK₂ and KK₂K₄ are composed of a hydrophobic alkyl chain and a
124 positively charged poly(L-lysine) dendron of the first and second generation,

125 respectively. We assessed the cyanobacterial flocculation activity of KK₂ and KK₂K₄
126 alone or in combination with sepiolite clay under different environmental conditions. We
127 also examined the mechanisms involved in the capture by KK₂ and KK₂K₄ of *M.*
128 *aeruginosa* cells. Our results showed that the AmPDs were highly efficient for the
129 flocculation and removal of *M. aeruginosa* populations. In addition, AmPDs removed
130 nitrogen nutrients central to the proliferation of cyanobacteria in freshwater. More
131 importantly, the AmPDs, when combined with sepiolite, became even more active and
132 rapidly captured toxic cyanobacteria without breaking them, thus avoiding a transient
133 increase of MCs in water.

134

135 **2. Experimental section**

136 **2.1. Cultivation of *M. aeruginosa***

137 The model cyanobacterial strain *M. aeruginosa* PCC 7806 was obtained from the
138 Institute of Hydrobiology of the Chinese Academy of Sciences (Wuhan, China). Axenic
139 *M. aeruginosa* was grown at 30 °C with agitation at 150 rpm in BG-11 medium [36]. The
140 microbial cultures were maintained under light at an intensity of 45 μmol photons/m²/s
141 with a 12-h light/dark cycle. For flocculation experiments with the AmPDs and sepiolite,
142 *M. aeruginosa* was first cultivated until reaching the exponential phase. *M. aeruginosa*
143 cell growth and density were monitored by both counting cells with a hemacytometer and
144 an Eclipse Ti2 inverted microscope (Nikon, Tokyo, Japan) and measuring the optical
145 density (OD) at 680 nm with a UV-Vis Evolution 220 spectrophotometer (Thermo Fisher
146 Scientific, Waltham, MA, USA).

147

148

149 **2.2. Synthesis of KK₂ and KK₂K₄ nanoassemblies**

150 For the synthesis of AmPDs, the hydrophilic and hydrophobic segments were prepared
151 separately and then conjugated via click chemistry as previously described [37]. Briefly,
152 the hydrophobic portion consists of a double-tailed alkyl chain with azide groups, while
153 the hydrophilic part constitutes the dendritic peptide segment. The synthesis of the latter
154 began with propargylamine as the starting material and utilized Boc-protected lysine by
155 alternating coupling and deprotection steps. The two segments were joined together
156 through a copper-catalyzed click reaction between the alkyne and azide groups. Copper
157 ions were chelated and subsequently removed with ammonium chloride followed by
158 deprotection to obtain the target products. After washing and further purification by
159 dialysis, the AmPDs were lyophilized. During the synthesis process, the structure of both
160 KK₂ and KK₂K₄ and their intermediates were validated by ¹H nuclear magnetic resonance
161 spectroscopy as previously described [38]. The main difference between KK₂ and KK₂K₄
162 is that the former bears a simpler and smaller peptide dendron exhibiting four terminal
163 amino groups, while the latter is a more complex and larger molecule with eight terminal
164 amino groups.

165

166 In our previous studies [37,38], both AmPDs were shown to readily form, in aqueous
167 environments, nanoassemblies with a positively charged surface (Scheme 1). To achieve
168 that here, lyophilized KK₂ and KK₂K₄ were suspended in a 10 mM sodium phosphate
169 buffer (pH 7.4) at a concentration of 50 mg/L followed by storage for at least 12 h at
170 4 °C. Transmission electron micrographs (TEM) of the AmPD nanoassemblies were

171 taken with a JEM-2100Plus system (JEOL, Tokyo, Japan) at an accelerating voltage of
172 200 kV. To start flocculation experiments, different volumes of these stock solutions were
173 mixed with *M. aeruginosa* cultures.

174

175 **2.3. Flocculation experiments**

176 *M. aeruginosa* flocculation experiments were conducted with concentrations of KK_2 or
177 KK_2K_4 ranging from 0.0 to 7.0 mg/L. Where indicated, 50.0 to 325.0 mg/L of sepiolite
178 (Lingshou County Wancheng Mineral, Shijiazhuang, China) was also added. Sepiolite
179 particles were washed at least 3 times with deionized water before usage.

180

181 Standard flocculation tests were done in triplicate with 10-mL *M. aeruginosa* cultures at a
182 density of ca. 3.0×10^6 cells/mL, a temperature of 30 °C, and under visible light at an
183 intensity of 45 $\mu\text{mol photons/m}^2/\text{s}$. The cell density for this experiment was selected
184 because it is representative of cyanobacterial blooms in lake water [39]. Glass tubes
185 employed for the flocculation tests had a diameter of 16 mm and a height of 50 mm.

186 Where indicated, the salinity of the cell culture was adjusted with 1.0 M NaCl while the
187 pH was adjusted with 0.1 M HCl or NaOH. Agitation was done at different speeds with a
188 MS-M-S10 magnetic stirrer (DLAB, Beijing, China). AmPDs with or without sepiolite
189 were first mixed with the cell culture at 600 rpm for 1 min. Where indicated, the initial
190 agitation speed was slower at 150 or 300 rpm, which had no significant impact on the
191 flocculating performance of KK_2 and KK_2K_4 (Fig. S1). Next, the suspension was
192 maintained at 150 rpm for 14 min. The agitation was then completely stopped and the
193 culture was left on a stand. The beginning of this step was considered as the time 0 of the

194 flocculation process. Subsequently, 1-mL samples were taken 2 cm below the surface of
195 the cell culture at different time points and the OD_{680 nm} was measured with a UV-Vis
196 Evolution 220 spectrophotometer (Thermo Fisher Scientific). In addition, the turbidity of
197 samples was quantified in Formazin Turbidity Unit (FTU) by comparing it
198 spectrophotometrically with formazin standards as previously described [40]. Total
199 nitrogen and phosphorus were measured in the samples with the Chinese national
200 standard method GB/T 11894-1989 and GB/T 11893-1989. Lastly, to assess the flocs'
201 stability, the cell cultures were filtered after the different flocculation treatments with a 60
202 µm nylon membrane. The OD_{680 nm} of the filtrates was then measured. Here, it is
203 expected that flocs with sufficient cohesiveness and size will be retained by the filter
204 while flocs that are not cohesive will be broken by the physical barrier of the filter and
205 pass in the filtrates.

206

207 **2.4. Characterization of the flocs and microcystin-LR quantification**

208 Bright-field light micrographs of the flocs and other samples were taken at a
209 magnification of 100× with an Eclipse Ti2 inverted microscope (Nikon) as described
210 previously [41]. Scanning electron microscopy (SEM) analyses of *M. aeruginosa* flocs
211 were completed with a MIRA system (TESCAN, Brno, Czech Republic) at an
212 accelerating voltage of 15 kV and a sample preparation method reported earlier [42].
213 Briefly, the cyanobacteria flocs were fixed overnight at 4 °C with 2.5% glutaraldehyde.
214 After that, the samples were washed three times with a 10 mM sodium phosphate buffer
215 (pH 7.4). Next, the cells were dehydrated with an ethanol gradient of 30%, 50%, 70%,

216 85%, and 90%. In the final preparation step, samples were freeze-dried for 12 h before
217 being sputter-coated with gold for SEM observation.

218

219 The zeta potentials of the different flocculants and *M. aeruginosa* flocs were measured
220 with a Zetasizer Nano ZS analyzer (Malvern, Malvern, United Kingdom). The
221 concentration of extracellular MC-LR molecules released by *M. aeruginosa* in the growth
222 medium was measured with an ELISA kit (Institute of Hydrobiology of the Chinese
223 Academy of Sciences) as previously described [43] and a Multiskan FC multi-plate
224 reader (Thermo Fisher Scientific).

225

226 **2.5. Cyanobacterial cell viability**

227 The viability of *M. aeruginosa* was evaluated by detecting the red autofluorescence of
228 chlorophyll (Chl) molecules as previously described [44,45]. The Chl content of
229 metabolically active cyanobacteria usually remains constant. Conversely, the death or
230 senescence of cyanobacteria is associated with a massive degradation of Chl molecules
231 [46]. Thus, cyanobacterial cells emitting a red fluorescence during this assay are
232 considered to be metabolically active and viable. Here, an untreated cyanobacterial
233 population as well as flocculation reactions at different time points were first vortexed for
234 30 s. 5 μ L of these preparations were deposited on microscope slides followed by
235 observation with an Eclipse Ti2 inverted microscope (Nikon) at a 540-580 nm excitation
236 wavelength, a 595 nm dichroic beam splitter, and a 600-660 nm emission wavelength
237 [46]. *M. aeruginosa* viability (V) after the different treatments was calculated with Eq. 1:

238

239 $V (\%) = n_{vf} / n_{vi} \times 100$ Eq. 1

240 where n_{vi} is the initial number of cells emitting red fluorescence before the flocculation
241 treatment and n_{vf} is the final number of fluorescent cells after flocculation.

242

243 **2.6. Statistical analysis**

244 Data are reported as means \pm standard deviation ($n \geq 3$). The Student's t-test was
245 employed to determine statistical significance between groups. * indicates p-values \leq
246 0.01.

247

248 **3. Results and discussion**

249 **3.1. Effective capture of *M. aeruginosa* by dendrimers via electrostatic interactions**

250 Because of their positively charged surface, we expect that nanoassemblies of the AmPD
251 KK₂ and KK₂K₄ will form strong electrostatic interactions with negatively charged *M.*
252 *aeruginosa* (Scheme 1) [47]. This, in turn, should lead to cell aggregation and floc
253 formation. To evaluate this hypothesis, a *M. aeruginosa* population was exposed to
254 different concentrations of either KK₂ or KK₂K₄ nanoassemblies exhibiting a diameter of
255 ca. 67-170 nm (Fig. S2). After 30 min at room temperature and circumneutral pH, KK₂
256 at an optimal concentration of 6.0 mg/L removed $96.3 \pm 2.2\%$ of cyanobacterial cells by
257 flocculation (Fig. 1ab). Under the same conditions, a lower KK₂K₄ concentration of 5.0
258 mg/L removed $97.7 \pm 1.2\%$ of cyanobacterial cells. The higher efficiency of KK₂K₄
259 nanoassemblies is likely related to its larger number of branches and surficial amino
260 groups, which increases the electrostatic attraction strength with *M. aeruginosa* cells.
261 Higher concentrations of 7.0 mg/L for KK₂ and 6.0 or 7.0 mg/L for KK₂K₄ resulted in a

262 small decline of flocculation efficiencies compared to when the optimal concentration of
263 either flocculant was added. It is possible that excessive AmPDs compromised the
264 structural cohesiveness of the flocs, leading to a partial release of cyanobacterial cells.
265

266 Time-course studies with either KK_2 or KK_2K_4 showed that the removal process of *M.*
267 *aeruginosa* cells was rapid and mostly happened within 5 min of exposure with $83.2 \pm$
268 1.4% and $90.6 \pm 1.6\%$ of flocculated cells, respectively, before reaching optimal values
269 after 30 min (Fig. 1c). In addition, a visual inspection indicated that the dense flocs
270 formed via cell aggregation using either KK_2 or KK_2K_4 sunk to the bottom of the test
271 tubes (Fig. 1d). As reported in Table S1, when compared with other single inorganic and
272 organic materials developed for the flocculation and sinking of freshwater cyanobacteria,
273 both KK_2 and KK_2K_4 were often more efficient and faster at completing the removal of
274 *M. aeruginosa* from the water column while requiring a lesser amount of reagent. Besides
275 better performance, KK_2 and KK_2K_4 flocculants exhibit additional advantages. Their
276 structures can be customized precisely for the predictable tuning of their properties such
277 as the strength of their interactions with microbes [35,48,49], offering advantages over
278 other materials evaluated for toxic cyanobacteria flocculation which are often ill-defined,
279 complex, or unmodifiable. Thus, AmPDs have a large untapped potential for the future
280 improvement of their capacity to treat cyanobacterial blooms. Furthermore, KK_2 and
281 KK_2K_4 are also biodegradable with good biocompatibility for eukaryotic cells [50,51].
282 This indicates that these flocculants are unlikely to accumulate in the environment and
283 exhibit adverse effects on the flora and fauna.
284

285 Next, light micrographs of *M. aeruginosa* exposed to KK_2 or KK_2K_4 were taken to
286 visualize the formation of aggregates (Fig. 2). Untreated *M. aeruginosa* mostly appeared
287 as individual cells with their characteristic morphology and green color (Fig. 2a) [52].
288 The addition of 2.0 mg/L KK_2 already caused a change in the cell aggregation with the
289 formation of grapes comprising 2-3 microbes (Fig. 2b). When the optimal KK_2
290 concentration of 6.0 mg/L was added, aggregates became more massive and included ca.
291 30 to 120 cells (Fig. 2d). A similar phenomenon was observed with KK_2K_4 where lower
292 concentrations (1.0-3.0 mg/L) of the AmPD resulted in *M. aeruginosa* aggregates of 2 to
293 8 cells (Fig. 2ef). Cyanobacterial cells exposed to the optimal KK_2K_4 concentration of 5.0
294 mg/L assembled in groups of mostly 90-120 cells (Fig. 2g).

295

296 SEM micrographs confirmed the aggregation process. Untreated cells were mainly
297 individual spheres with shape irregularities distinctive of *M. aeruginosa* (Fig. 2h). In
298 comparison, cells exposed to an optimal concentration of KK_2 formed imbricated clusters
299 (Fig. 2i). Similarly, *M. aeruginosa* clumped with KK_2K_4 into flocs made of multiple cells
300 (Fig. 2j). The main reason behind this efficient flocculation process can be ascribed to the
301 positively charged KK_2 and KK_2K_4 nanoassemblies attracting the negatively charged
302 cyanobacterial cells into a cohesive multicellular structure. While the zeta potential of a
303 high-density *M. aeruginosa* preparation was -22.6 ± 0.4 mV, KK_2 and KK_2K_4 exhibited
304 surface charges of 23.9 ± 0.3 mV and 29.1 ± 0.2 mV, respectively (Fig. 2k). Exposing the
305 cyanobacterial cells to KK_2 and KK_2K_4 resulted in flocs with weaker positive surface
306 charges of 2.8 ± 0.3 mV and 4.0 ± 0.2 mV, respectively. These results indicate that
307 AmPDs and *M. aeruginosa* nearly neutralized their respective surface charges by forming

308 strong electrostatic interactions, leading to cell aggregation. They also explain why
309 KK_2K_4 with its higher positive zeta potential is more efficient than KK_2 at removing the
310 toxic cyanobacteria from an aqueous solution.

311

312 **3.2. Synergistic flocculation by dendrimers combined with sepiolite**

313 Widely available clays such as sepiolite are often employed in situ for the flocculation of
314 cyanobacterial bloom because of their abundance as well as low ecotoxicity (Table S2)
315 [53-55]. However, natural clays have a low flocculating efficiency, which means that
316 massive dosages are required, increasing expenses and possible undesired environmental
317 impacts. Here, we assessed both KK_2 and KK_2K_4 in combination with sepiolite for the
318 flocculation of *M. aeruginosa* (Fig. 3). In the first series of experiments, different
319 concentrations of KK_2 and KK_2K_4 were coupled with 200.0 mg/L sepiolite, respectively
320 (Fig. 3a). When compared to AmPDs alone, the addition of sepiolite had two beneficial
321 effects. First, lower concentrations of AmPDs were required to remove most *M.*
322 *aeruginosa* cells. 4.0 mg/L KK_2 and 3.0 mg/L KK_2K_4 with sepiolite were sufficient to
323 flocculate and sink $96.5 \pm 2.4\%$ and $97.1 \pm 2.2\%$ of the cyanobacterial cells, respectively.
324 Second, the removal process was twice faster and reached equilibrium after only 15 min
325 (Fig. 3b). In fact, $88.9 \pm 1.2\%$ and $89.9 \pm 2.0\%$ of *M. aeruginosa* cells have already been
326 removed after 5 min by KK_2 and KK_2K_4 with sepiolite, respectively. Subsequently,
327 optimal KK_2 and KK_2K_4 concentrations were mixed with different quantities of sepiolite
328 (Fig. 3c, Fig. S3). The sepiolite-only control (up to 325.0 mg/L) removed a negligible
329 fraction (below 10%) of the cyanobacterial cells, illustrating the inefficiency of
330 unmodified natural clays in the timeframe investigated. The highest flocculation

331 performance for both AmPDs was observed with sepiolite concentrations of 200.0-250.0
332 mg/L.
333
334 Light micrographs showed the flocculation of *M. aeruginosa* cells by KK₂ or KK₂K₄ with
335 sepiolite (Fig. 4). When compared to 4.0 mg/L KK₂ alone (Fig. 4a), the addition of low
336 concentrations of sepiolite (50.0-100.0 mg/L) already increased the size of the aggregates
337 (Fig. 4bc). On these micrographs, sepiolite appeared as spindle-like black mineral
338 structures. In the presence of 200.0 mg/L sepiolite and KK₂, *M. aeruginosa* aggregates
339 became massive with hundreds of cells (Fig. 4d). A similar effect was observed when 3.0
340 mg/L KK₂K₄ was combined with the same concentration of sepiolite, resulting in the
341 formation of expanded flocs (Fig. 4h).
342
343 The SEM analysis confirmed that *M. aeruginosa* cells aggregated with sepiolite spindles
344 in complex cohesive networks maintained by the AmPDs (Fig. 4i-k). Measurements of
345 the zeta potentials of sepiolite, sepiolite in the presence of KK₂ or KK₂K₄, and sepiolite in
346 the presence of *M. aeruginosa* cells with KK₂ or KK₂K₄ demonstrated the importance of
347 electrostatic interactions for the floc formation (Fig. 4l). While sepiolite alone exhibited a
348 zeta potential of -23.2 ± 6.0 mV, the addition of KK₂ or KK₂K₄ resulted in modified clay
349 with a positive charge of 21.6 ± 0.2 mV and 26.2 ± 0.6 mV, respectively. These results
350 indicate that the AmPD nanoassemblies coated sepiolite particles, changing entirely their
351 surface charge. In the presence of *M. aeruginosa* cells, the positively charged surface of
352 modified sepiolite was neutralized and the flocs had a slightly negative charge,
353 confirming that the cyanobacterial cells closely interacted with the AmPD coat of

354 sepiolite. Several reasons may explain why sepiolite improved the performance of the
355 AmPDs for the capture and removal of *M. aeruginosa* cells from water. Sepiolite with its
356 spindle shape has a significant specific surface area shown to range from 77 to 399 m²/g
357 in the literature [56], and when coated with AmPD molecules, it will form bigger flocs
358 with a greater number of *M. aeruginosa* cells that will settle faster to the bottom. Another
359 possible factor to consider is that multiple coated sepiolite particles may form a matrix
360 encasing toxic cyanobacterial cells and sinking them to the bottom.

361

362 The flocculation systems combining KK₂ or KK₂K₄ and sepiolite described in this proof-
363 of-concept study exhibited excellent performance compared to many other clay-based
364 materials (Table S2), but may still present a challenge for practical applications since a
365 relatively important concentration of sepiolite was required. This could lead to unwanted
366 sediment accumulation altering local ecosystems. Consequently, we aim in future
367 research to develop AmPD flocculating systems requiring less clay while maintaining
368 similar efficiency. This could be achieved, for instance, by coupling KK₂ and KK₂K₄ with
369 clay particles exhibiting different sizes, morphologies, and porosities. These factors,
370 especially a suitable clay particle size, have a major impact on the collision efficiency
371 with microbial cells, and thus on the flocculation process [57].

372

373 **3.3. Dendrimer/sepiolite combination stabilized flocculation and obviated cell death** 374 **and microcystin release**

375 Next, the impact of KK₂ and KK₂K₄ on the viability of *M. aeruginosa* cells was evaluated
376 by monitoring the intrinsic red fluorescence emitted by Chl molecules of intact

377 photosystems from active cells [41]. Among all the tested flocculation systems, KK₂, at a
378 concentration of 6.0 mg/L, did not impact much cell viability nor stimulate the release of
379 MC-LR, the most abundant and potent toxin generated by *M. aeruginosa* (Fig. 5ab) [1].
380 Conversely, KK₂K₄ at 5.0 mg/L considerably compromised the viability of *M.*
381 *aeruginosa* after 24 h (Fig. 5a, Fig. S4). At this time point, KK₂K₄ had killed $74.4 \pm 2.0\%$
382 of cyanobacterial cells from the initial cell suspension. A clear inconvenience of the
383 killing effect of KK₂K₄ was that it increased 1.7 times the extracellular concentration of
384 MC-LR compared to an untreated control (Fig. 5b) [58]. This is probably because
385 damaged cyanobacteria released the cyanotoxin upon the disruption of their cell
386 membranes. It should be mentioned that dendrimers with amino terminals and an overall
387 structure similar to KK₂K₄ were recently reported to have strong antibacterial properties
388 against both Gram-positive and Gram-negative pathogenic bacteria [35,48]. These
389 molecules electrostatically interacted and accumulated around the bacterial cell wall
390 followed by membrane disruption and bacterial killing. At the concentration examined in
391 our study, this is possibly what happened with KK₂K₄.

392

393 Surprisingly, the flocculation system combining 3.0 mg/L KK₂K₄ nanoassemblies and
394 sepiolite behaved differently (Fig. 5ab). It did not induce the death of *M. aeruginosa* cells
395 or the release of MC-LR, which is completely different from the treatment with KK₂K₄
396 alone. This divergence could be due to the lower concentration of KK₂K₄ used in the
397 combination treatment, hence being less potent. It could also be related to strong
398 electrostatic interactions between sepiolite and KK₂K₄, restraining AmPD molecules from
399 penetrating the cell wall and disorganizing lipid bilayers. In addition, with KK₂K₄ and

400 sepiolite in combination, cyanobacterial cells remained settled to the bottom of the water
401 column with only a small increase in viability after 72 h (Fig. S5-S7). This indicates that
402 KK₂K₄ and sepiolite could maintain good control of the cyanobacterial population at least
403 for several days, and not just for a short period.

404

405 More remarkably, the KK₂K₄ with sepiolite system reduced 1.5 times the concentration
406 of MC-LR in the water compared to an untreated control. MC-LR cyclic molecules have
407 two ionizable carboxyl groups not forming peptide bonds and thus are almost exclusively
408 negatively charged at pH higher than 7.0 [59]. Thus, the KK₂K₄ and sepiolite flocculation
409 system, which exhibits a strong positive charge on its surface and does not compromise
410 the integrity of cyanobacterial cells, could also be suitable for the partial elimination of
411 cyanotoxins exhibiting an overall negative charge. Besides MC-LR, *M. aeruginosa*
412 releases other MC congeners that are less toxic and abundant [60]. Further work is
413 required to determine the impact of KK₂ and KK₂K₄ with or without sepiolite on the fate
414 of these uncommon MC congeners.

415

416 It is also of note that KK₂K₄ with sepiolite generated highly stable flocs that were not
417 prone to quick disassembling and the unwanted release of biomass (Fig. 5c). To
418 investigate the floc cohesiveness, *M. aeruginosa* cell suspensions before and after
419 treatment were filtered with a nylon membrane filter. KK₂K₄ with sepiolite was the most
420 performant system with the removal of 96.3% of microbial particles from the aqueous
421 solution by filtration. *M. aeruginosa* flocs formed with KK₂ or KK₂K₄ were slightly less
422 recoverable. The flocculation assay showed that both AmPDs captured and sedimented at

423 least 95% of the cyanobacterial cell population (Fig. 1). Upon filtration, 74.6% and
424 83.7% of the flocculated biomass could be recovered when KK_2 or KK_2K_4 were
425 employed, respectively. This observation denotes that most flocs assembled via KK_2 or
426 KK_2K_4 can be separated from the aqueous solution with only a minority fraction either
427 too small to be filtered out or getting dismantled. KK_2 nanoassemblies with sepiolite was
428 the worst flocculation strategy for subsequent recovery by filtration with the majority of
429 flocculated/sedimented biomass remaining in the aqueous solution.

430

431 **3.4. Removal of total nitrogen by the dendrimers**

432 Cyanobacterial contaminations are directly caused by excessive nitrogen and phosphorus
433 inputs from human activities [1]. While removing cyanobacteria cells, some flocculants
434 can simultaneously adsorb nitrogen and/or phosphorus compounds [23,61]. Our results
435 indicated that KK_2 and KK_2K_4 with or without sepiolite removed large fractions of total
436 nitrogen, but had less impact on phosphorus (Fig. S8). For nitrogen, KK_2 was the most
437 efficient system after a 30-minute reaction with $92.9 \pm 1.9\%$ removed from the aqueous
438 solution. In the case of phosphorus, only KK_2 with sepiolite adsorbed a small fraction of
439 $12.5 \pm 1.8\%$. KK_2 and KK_2K_4 are charged positively, and thus it is not surprising that
440 they adsorbed nitrate anions found in the cyanobacterial suspension. In this study, the
441 cyanobacterial suspensions contained ca. 11 times more nitrate than phosphate ions.
442 Abundant nitrate and cyanobacterial cells may outcompete phosphate for the adsorption
443 sites on the surface of the AmPD molecules, possibly explaining why total phosphorus
444 remained unchanged in most cases. These observations demonstrate that all the AmPD-
445 based systems evaluated can clean concomitantly the biological agent responsible for

446 cyanobacterial contamination as well as key nitrogen pollutants responsible for this
447 harmful phenomenon.

448

449 **3.5. Cyanobacterial flocculation was dependent on salinity and pH**

450 The effect of different environmental conditions such as salinity and pH on the AmPD-
451 driven flocculation process was then investigated (Fig. 6). The salinity of the aqueous
452 solution contaminated with *M. aeruginosa* had an important impact on the efficiency of
453 the aggregation for both KK₂ and KK₂K₄ with or without sepiolite (Fig. 6ab). For
454 instance, *M. aeruginosa* removal was reduced to only $48.3 \pm 2.9\%$ and $31.3 \pm 1.4\%$ with
455 KK₂ and KK₂K₄, respectively, when the NaCl concentration was 0.17 M compared to
456 media with null ionic strength (Fig. 6a). This is likely because ions from the salt interact
457 with the surfaces of AmPDs as well as cyanobacterial cells, changing their electrical
458 charges and interfering with the formation of electrostatic interactions. An increase in
459 salinity is also known to affect the behavior of dendrimeric nanosystems in solution and
460 modify their capacity to form nanoassemblies [62]. This could be another reason why a
461 high salt concentration impeded flocculation. The addition of sepiolite did not mitigate
462 most of the negative effects of augmented salinity. KK₂ and KK₂K₄ nanoassemblies with
463 sepiolite removed only $41.1 \pm 0.7\%$ and $51.5 \pm 1.3\%$ of *M. aeruginosa* cells, respectively,
464 at the highest ionic strength evaluated (Fig. 6b). These observations indicate that the
465 AmPDs studied here are more suitable for treating freshwater contamination such as the
466 ones involving *M. aeruginosa* and may not be as efficient for cyanobacterial proliferation
467 in saltier water.

468

469 The importance of the pH for the activity of both KK_2 and KK_2K_4 combined with or
470 without sepiolite was also investigated (Fig. 6cd). The proliferation of *M. aeruginosa* is
471 favored when the pH becomes basic [63]. In addition, during algal blooms, the
472 photosynthetic activity of cyanobacteria and other microalgae increases the pH between
473 8.0 to 9.5 [64,65]. Thus, the activity of both AmPDs was evaluated at pH values from 6.0
474 to 11.0 (Fig. 6c). Even up to a pH of 10.0, both KK_2 and KK_2K_4 maintained a
475 cyanobacterial removal activity above 85.0%. At pH 11.0, the capture capacity of *M.*
476 *aeruginosa* cells by both KK_2 and KK_2K_4 collapsed. A similar pattern was observed with
477 AmPD-driven flocculation systems comprising sepiolite with cyanobacterial cell removal
478 remaining high until the pH was increased to 11.0 (Fig. 6d). In this condition, amino
479 groups at the surface of AmPDs likely lost their positive charge, which wiped out their
480 capacity to form electrostatic interactions with microbes. Nevertheless, at mild basic pHs
481 observed during cyanobacterial blooms, KK_2 and KK_2K_4 exhibited strong activities for
482 flocculation induction.

483

484 **4. Conclusions**

485 In this proof-of-concept study, we demonstrated that AmPDs harboring positively
486 charged terminals can serve for environment engineering applications to mitigate water
487 bloom via flocculation and sinking of toxic cyanobacteria. Specifically, low
488 concentrations of KK_2 and KK_2K_4 complemented with or without sepiolite removed more
489 than 95% of *M. aeruginosa* cells within 15 to 30 min. The best flocculating system
490 identified was the combination of KK_2K_4 and sepiolite clay, which quickly removed
491 97.1% of cyanobacterial cells and a significant fraction of MC-LR molecules from
492 freshwater by forming highly stable flocs. The AmPDs, which exhibit a strong surficial

493 positive charge, mainly captured the negatively charged cyanobacterial cells through
494 electrostatic interactions. The AmPD molecules were also able to concomitantly remove
495 nitrogen nutrients central to the occurrence of cyanobacterial contaminations. Based on
496 their excellent performance, AmPDs could become a promising new category of
497 materials for two different applications related to cyanobacterial contaminations: in-lake
498 bloom mitigation and water treatment for human utilization. Both purposes have different
499 requirements and additional work is necessary before concluding that AmPDs can be
500 effectively implemented in the real world. This includes subsequent investigations that
501 will establish the long-term effectiveness and the technical, ecological, and economic
502 feasibility of employing AmPD flocculating systems in complex freshwater environments
503 populated by different problematic cyanobacterial species with unique physiologies. We
504 are actively pursuing this research direction.

505

506 **Declaration of competing interest**

507 The authors declare that they have no known competing financial interests or personal
508 relationships that could have appeared to influence the work reported in this paper.

509

510 **Data availability**

511 Data will be made available on request.

512

513

514

515

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520

521 **Appendix A. Supplementary data**

522

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764 **Figure legends**

765 **Scheme 1.** Amphiphilic peptide dendrimers (AmPDs) and their use in the flocculation of
766 *M. aeruginosa*. Both the AmPDs, KK₂ and KK₂K₄, form positively charged
767 nanoassemblies that will interact with the negatively charged surface of *M. aeruginosa*
768 cells, leading to microbial aggregation and the formation of dense flocs that will sink and
769 can be removed from the treated aqueous solution. In the presence of sepiolite clay,
770 AmPD-driven flocculation becomes more efficient. The AmPD nanoassemblies coat
771 sepiolite spindles, which quickly form flocs with cyanobacteria.

772

773 **Fig. 1.** The removal of *M. aeruginosa* by AmPD nanoassemblies. (a) Removal of
774 cyanobacteria and (b) remaining turbidity after 30 min of flocculation treatment with
775 different concentrations of KK₂ or KK₂K₄. (c) Time-course curves of cyanobacterial cell
776 removal with 6.0 mg/L KK₂ or 5.0 mg/L KK₂K₄. (d) Digital images of untreated *M.*
777 *aeruginosa* or cultures treated with either KK₂ or KK₂K₄. FTU: Formazin Turbidity Unit.

778

779 **Fig. 2.** Light microscopy, SEM, and zeta potential analyses of *M. aeruginosa* aggregates
780 with the AmPDs KK₂ and KK₂K₄. Light micrographs of (a) untreated *M. aeruginosa* and
781 *M. aeruginosa* treated with (b) 2.0, (c) 4.0, and (d) 6.0 mg/L KK₂ or (e) 1.0, (f) 3.0, and
782 (g) 5.0 mg/L KK₂K₄. SEM micrographs of (h) untreated *M. aeruginosa* and *M.*
783 *aeruginosa* treated with (i) 6.0 mg/L KK₂ or (j) 5.0 mg/L KK₂K₄ nanoassemblies. (k)
784 Zeta potentials of cyanobacteria, AmPDs, and cyanobacteria with either one of the
785 AmPDs presented in a bar graph. MA: *M. aeruginosa*.

786

787 **Fig. 3.** The flocculation of *M. aeruginosa* by AmPD nanoassemblies combined with
788 sepiolite. (a) Removal of cyanobacteria after 15 min of treatment with 200.0 mg/L
789 sepiolite and different concentrations of KK₂ or KK₂K₄. (b) Time-course curves of
790 cyanobacterial cell removal with 200.0 mg/L sepiolite and either 4.0 mg/L KK₂ or 3.0
791 mg/L KK₂K₄. (c) Removal of cyanobacteria with either 4.0 mg/L KK₂ or 3.0 mg/L
792 KK₂K₄ nanoassemblies and different concentrations of sepiolite clay from 0 to 325.0
793 mg/L. (d-g) Digital images of untreated *M. aeruginosa* or cultures treated with sepiolite
794 alone, and sepiolite with either KK₂ or KK₂K₄. FTU: Formazin Turbidity Unit.

795

796 **Fig. 4.** Light microscopy, SEM, and zeta potential analyses of *M. aeruginosa* aggregates
797 with AmPDs and sepiolite. Light micrographs of the cyanobacteria treated with (a) 4.0
798 mg/L KK₂ only or KK₂ with (b) 50.0, (c) 100.0, and (d) 200.0 mg/L sepiolite.

799 Micrographs of *M. aeruginosa* treated with (e) 3.0 mg/L KK₂K₄ only or KK₂K₄ with (f)
800 50.0, (g) 100.0, and (h) 200.0 mg/L sepiolite. Red arrows indicate sepiolite particles.

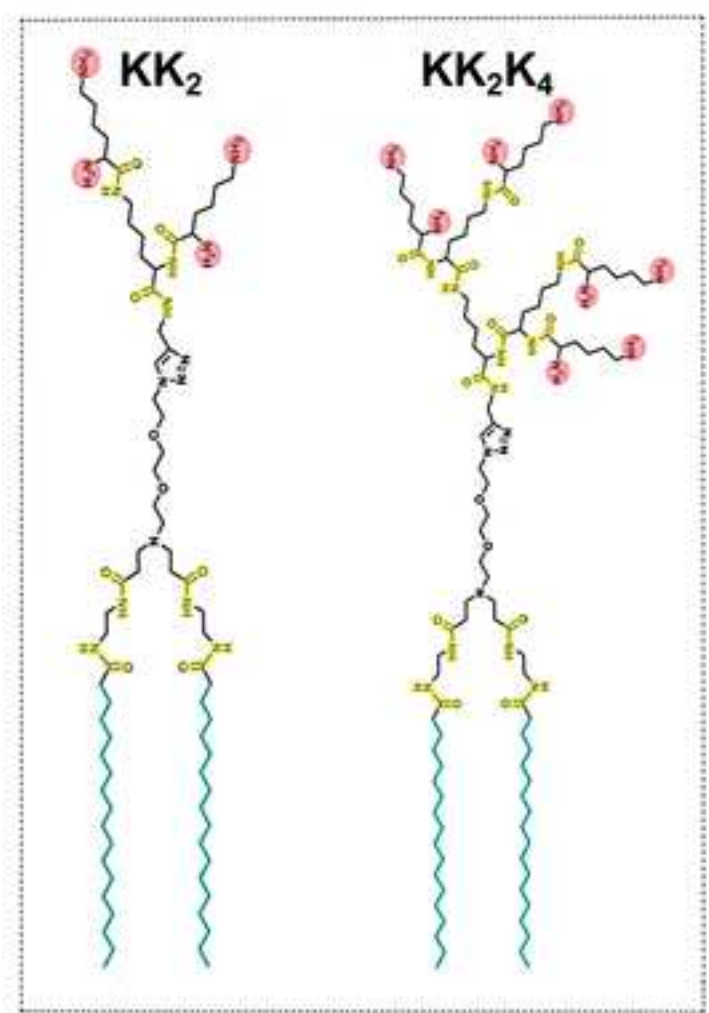
801 SEM micrographs of (i) untreated *M. aeruginosa* and *M. aeruginosa* treated with a
802 combination of (j) KK₂ and sepiolite or (k) KK₂K₄ and sepiolite. (l) Zeta potential bar
803 graph of sepiolite, AmPDs with sepiolite, and cyanobacteria with sepiolite in
804 combination with either one of the AmPDs. MA: *M. aeruginosa*, Sep.: sepiolite.

805

806 **Fig. 5.** The impact of AmPDs and sepiolite on *M. aeruginosa* viability, extracellular MC-
807 LR concentration, and floc stability. (a) *M. aeruginosa* viability 0.5 h and 24 h after
808 different treatments. (b) Extracellular MC-LR concentrations at the end of the
809 flocculation processes with AmPDs and sepiolite. (c) Filtration evaluation of the integrity
810 of the different types of floc. Sep.: sepiolite.

811

812 **Fig. 6.** Effect of salinity and pH on the AmPD-driven flocculation processes. The
813 flocculation of *M aeruginosa* by KK_2 or KK_2K_4 nanoassemblies at different salinity (a)
814 without and (b) with sepiolite, and at different pH (c) without and (d) with sepiolite.
815 FTU: Formazin Turbidity Unit.



AmPD

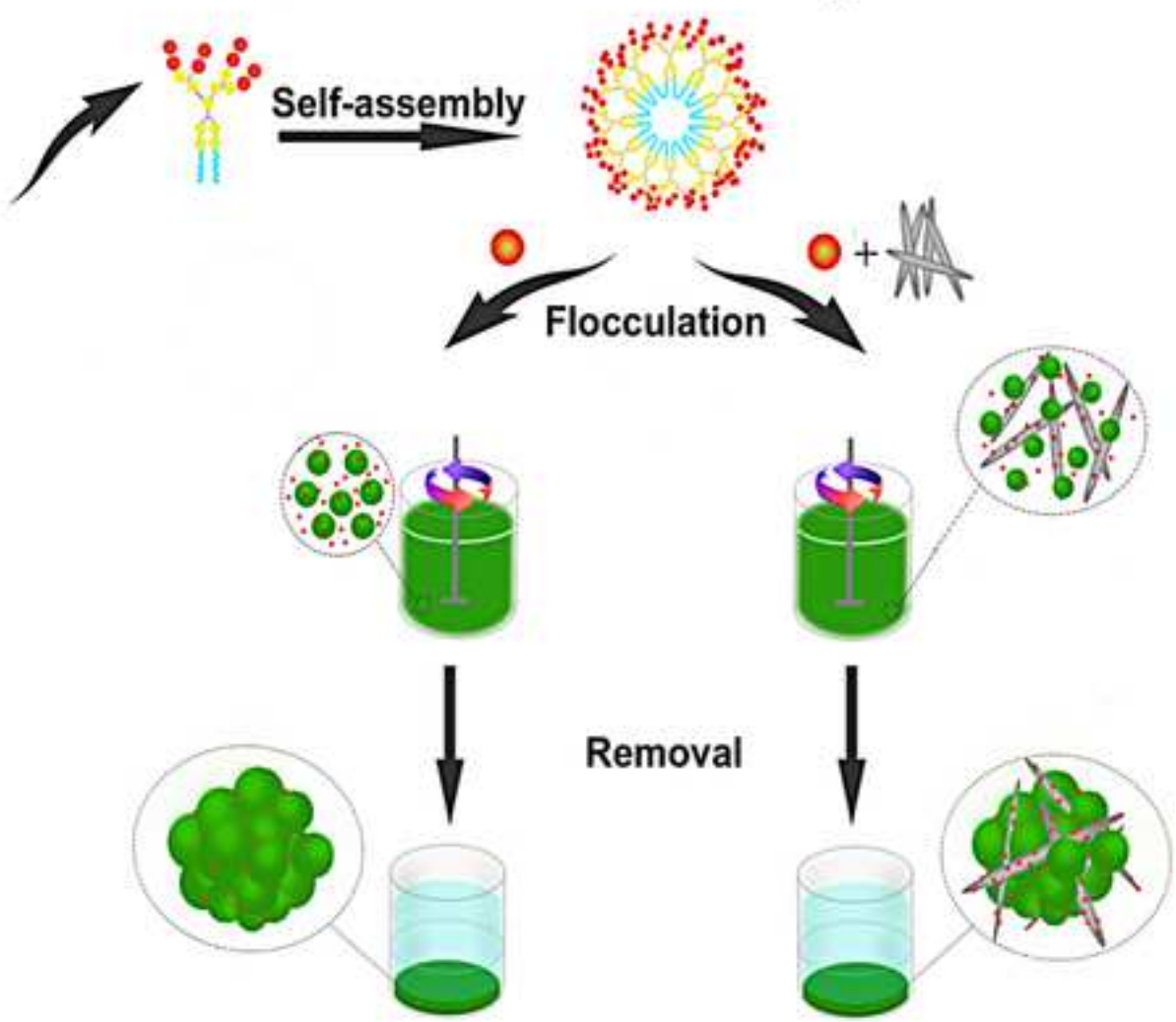
Nanoassembly

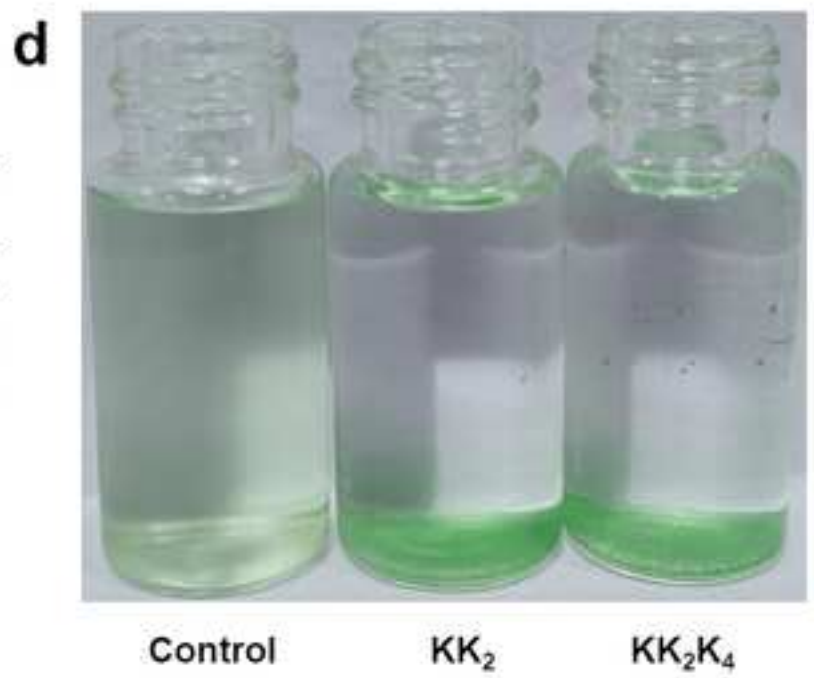
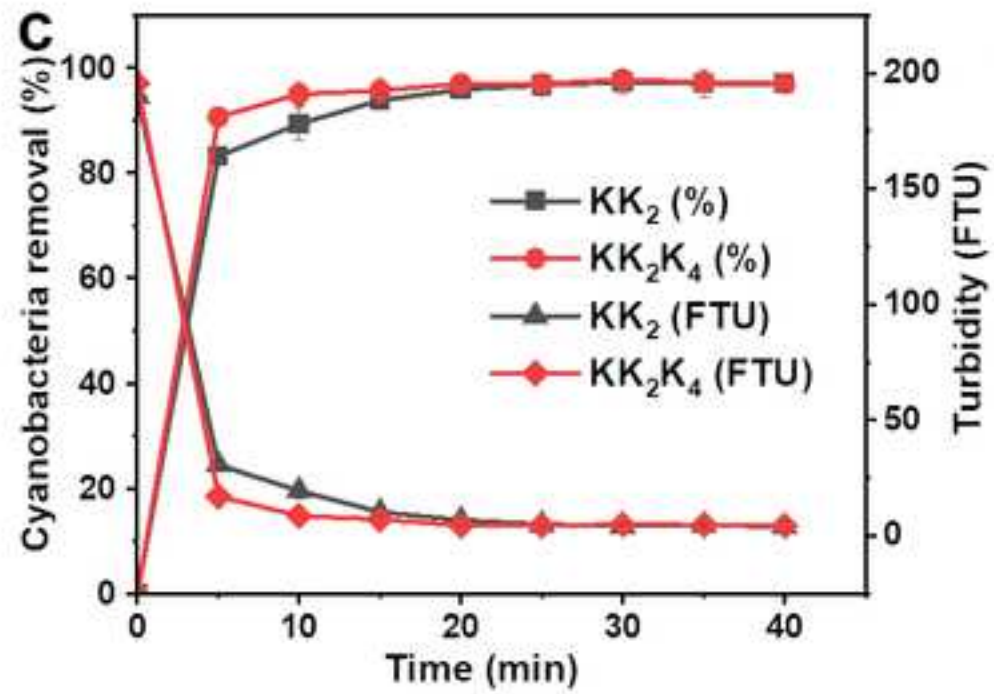
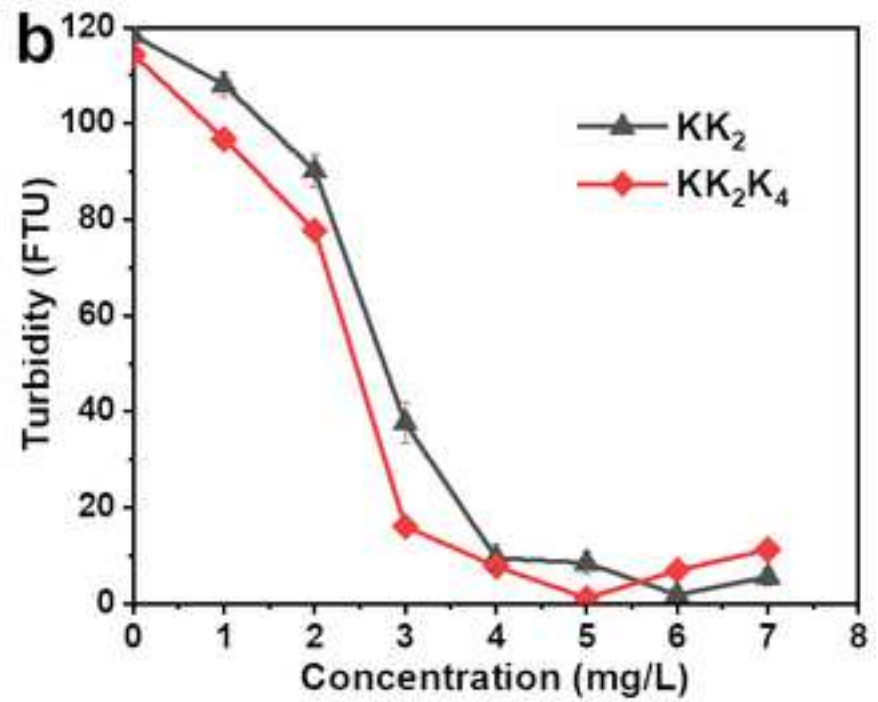
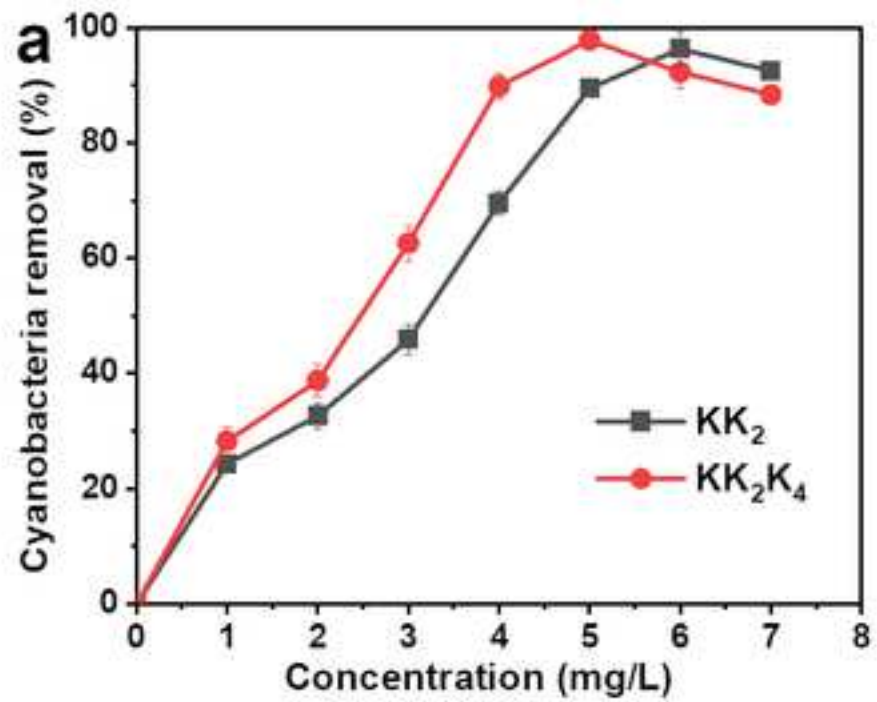
Self-assembly

Flocculation

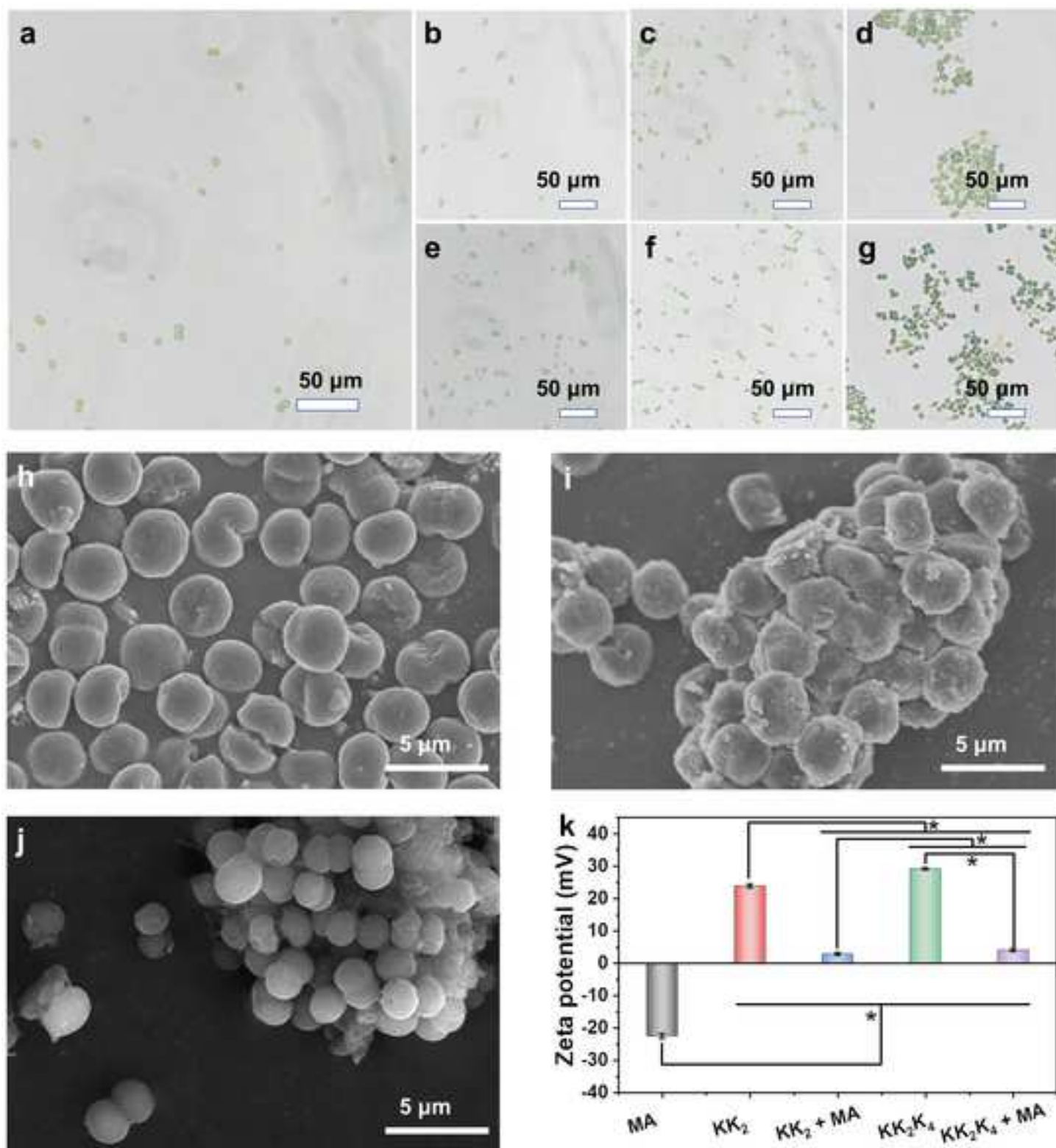
Removal

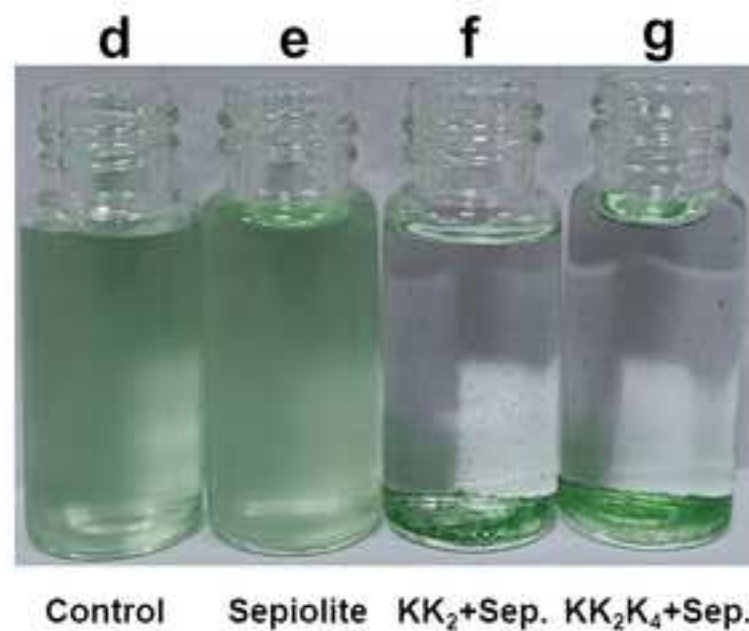
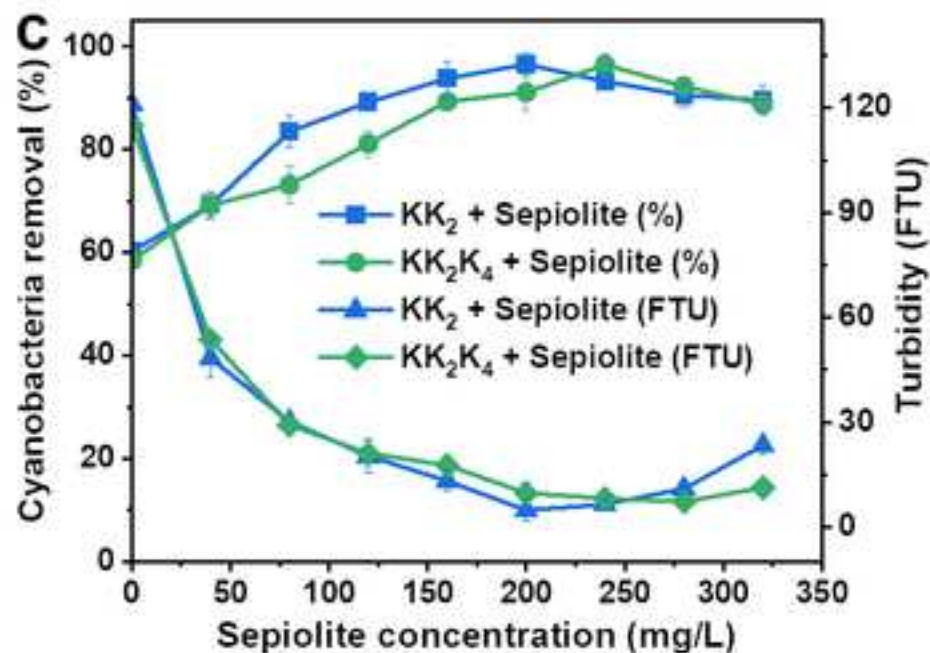
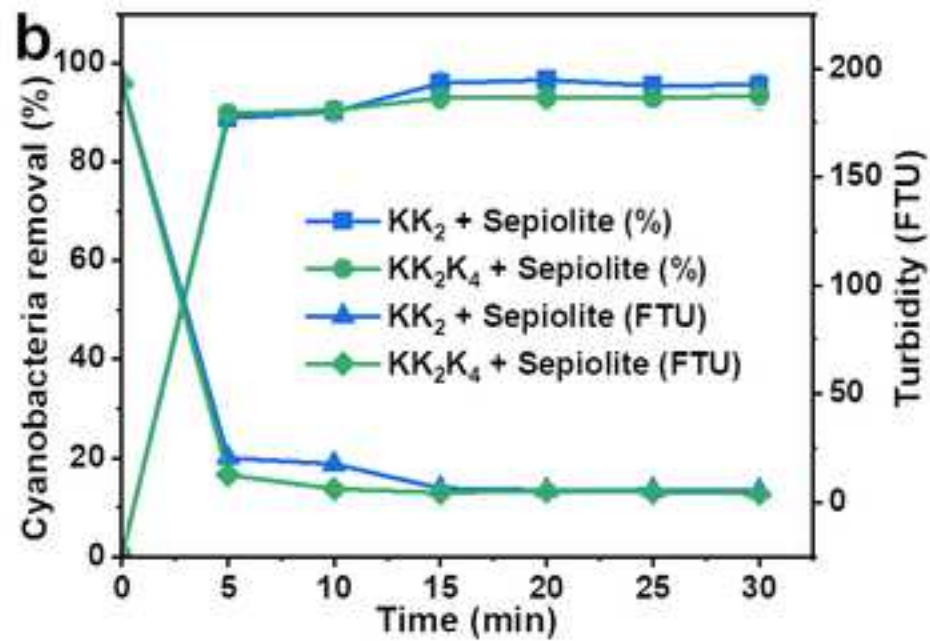
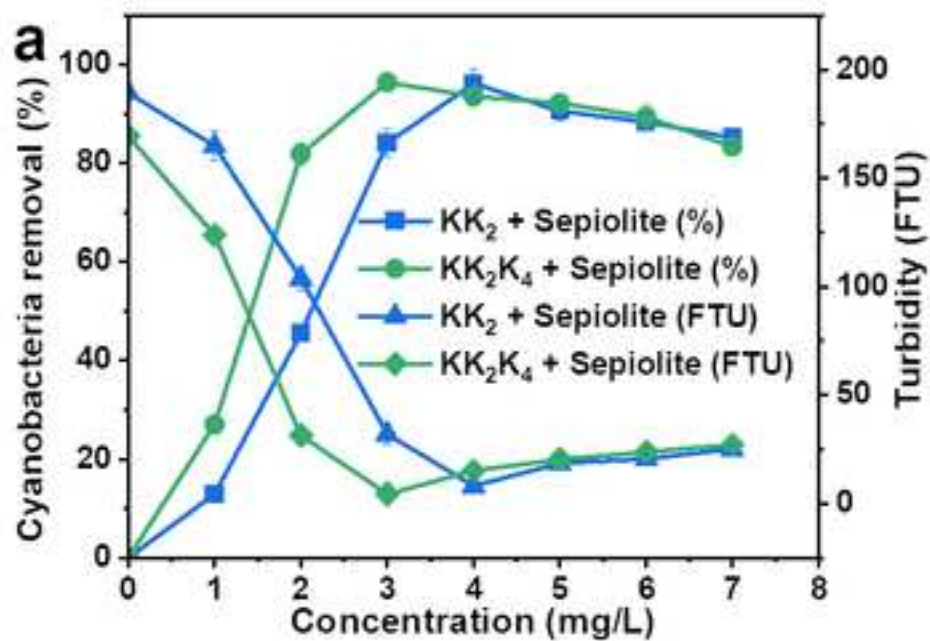
● Nanoassembly  Sepiolite ● *M. aeruginosa*





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