

1 **Hypoxia Remediation and Methane Emission Manipulation Using Surface Oxygen**

2 **Nanobubbles**

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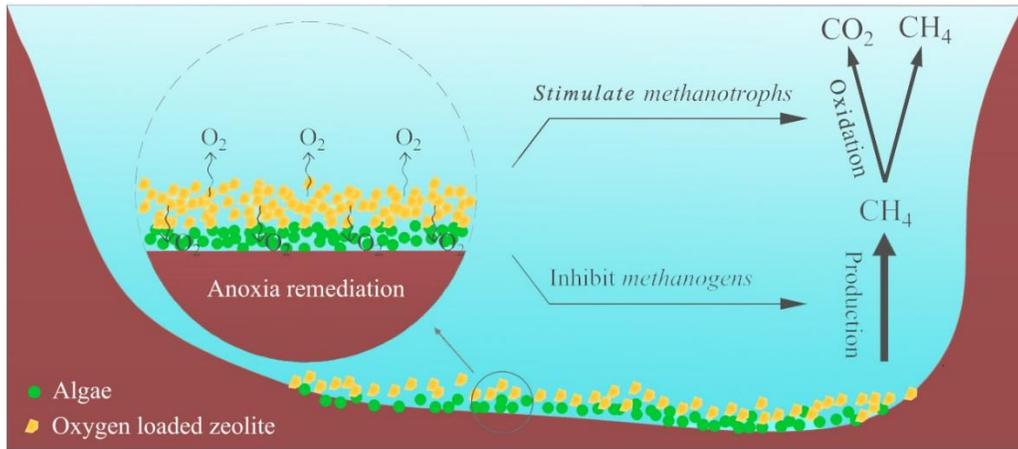
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15

16 **Abstract:** Algal blooms in eutrophic waters often induce anoxia/hypoxia and enhance
17 methane (CH₄) emissions to the atmosphere, which may contribute to global warming. At
18 present, there are very few strategies available to combat this problem. In this study, surface
19 oxygen nanobubbles were tested as a novel approach for anoxia/hypoxia remediation and
20 CH₄ emission control. Incubation column experiments were conducted using sediment and
21 water samples taken from Lake Taihu, China. The results indicated that algae-induced
22 anoxia/hypoxia could be reduced or reversed after oxygen nanobubbles were loaded onto
23 zeolite microporous and delivered to anoxic sediment. Cumulated CH₄ emissions were also
24 reduced by a factor of 3.2 compared to the control. This was mainly attributed to the
25 manipulation of microbial processes using the surface oxygen nanobubbles, which
26 potentially served as oxygen suppliers. The created oxygen enriched environment
27 simultaneously decreased methanogen but increased methanotroph abundances, making a
28 greater fraction of organic carbon recycled as carbon dioxide (CO₂) instead of CH₄. The
29 CH₄/CO₂ emission ratio decreased to 3.4×10^{-3} in the presence of oxygen nanobubbles,
30 compared with 11×10^{-3} in the control, and therefore the global warming potential was
31 reduced. This study proposes a possible strategy for anoxia/hypoxia remediation and CH₄
32 emission control in algal bloom waters, which may benefit global warming mitigation.

33

34 **Table of Contents**



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37 INTRODUCTION

38 In eutrophic waters, algae-induced anoxia/hypoxia is a serious environmental issue that can
39 promote significant methane (CH₄) emission to the atmosphere.¹⁻³ Various strategies for
40 adding oxygen directly into water to combat anoxia/hypoxia have been proposed.⁴⁻⁷
41 However, the challenge is finding a safe and cost-effective method to deliver oxygen to
42 anoxic/hypoxic areas, especially in deep waters. Downward pumping of oxygen-rich
43 midwater was suggested as a solution to alleviate severe hypoxia that occurs annually in
44 the Baltic Sea across an average area of 60×10^3 km².^{8,9} Such an action would consume
45 large amounts of energy and pose ecological risks resulting from disturbance to natural
46 water turnover and circulation patterns.⁸ There is therefore a need for an ecologically safe
47 and cost-effective oxygen delivery technology that can remediate anoxia/hypoxia.

48 Nanobubbles are miniature gas bubbles with the diameters of < 200 nm that exhibit
49 unique characteristics compared to macrobubbles.¹⁰ The high stability and efficient gas
50 solubility of oxygen nanobubbles make them potentially potent oxygen suppliers in water.¹¹
51 There are two types of nanobubbles, known as bulk and surface nanobubbles. Bulk
52 nanobubbles are nanoscopic spherical bubbles dispersed in bulk liquids.¹² However, it still
53 remains a challenge to deliver bulk oxygen nanobubbles to anoxic/hypoxic zones in deep
54 waters as it consumes similar amount of energy as mixing through the use of pumps.
55 Surface nanobubbles are nanoscopic gaseous domains at the solid-liquid interface.^{13,14} Clay
56 particles can act as potential carriers to deliver oxygen nanobubbles to targeted areas,^{15,16}
57 especially deep sediment via natural settling or hydraulic forces, which offers the prospect

58 of anoxia/hypoxia remediation in natural waters. This improvement of the aquatic
59 environment may, in turn, affect microbial processes and achieve CH₄ emission control.
60 However, such a principle has not yet been quantitatively tested.

61 In this study, we used an algal water-sediment simulation system to examine the use of
62 surface oxygen nanobubbles in remediating algal anoxia/hypoxia and reducing CH₄
63 emission. Oxygen nanobubbles were loaded onto natural zeolite microspheres and
64 delivered to bottom water after algae deposition. Zeolites without oxygen nanobubbles
65 were used to identify the capping barrier effect of zeolites on gas emission. Dissolved
66 oxygen (DO) in the water column, oxidation-reduction potential (ORP) at the sediment-
67 water interface, and CH₄ as well as carbon dioxide (CO₂) fluxes across the water-air
68 interface were measured over an incubation period of 20 d. The quantitative polymerase
69 chain reaction (qPCR) method was also employed to analyze the responses of methanogens
70 and methanotrophs in the systems which are responsible for CH₄ production and
71 consumption, respectively.

72 MATERIALS AND METHODS

73 **Oxygen Nanobubble Loaded Zeolites.** Granular natural zeolites (1 ~ 3 mm in diameter)
74 were placed into a pressure-resistant, airtight container and evacuated for 2 h (-0.08 MPa)
75 using a vacuum pump (GM-1.0A, Tianjin Jinteng Experiment Equipment Co. Ltd., China)
76 to remove air from zeolite pores. Oxygen gas was then added to the container (0.12 MPa)
77 and maintained for 4 h. The oxygen nanobubble loaded zeolites were prepared freshly
78 before use. The loaded oxygen was 20.5 mg g⁻¹ zeolites, which was measured using the

79 sodium sulfite adsorption method (See Text S1 in the supporting information). Similarly,
80 zeolites without oxygen nanobubbles were prepared by replacing oxygen gas with nitrogen
81 gas.

82 **Incubation Experiments.** Approximately 35 L of sediment and 190 L of water samples
83 were collected from Lake Taihu (China) using an Ekman grab sampler and a stainless-steel
84 bucket, respectively, and then transported immediately to the laboratory in the dark. Both
85 sediment and water samples were homogenized and then filled into cylinders (8.4 cm in
86 diameter and 150 cm in height) to establish uniform sediment-water cylinders. In this study,
87 twenty seven water-sediment cylinders were established. Each cylinder contained 15 cm
88 of sediment and 120 cm of water column (Figure S2 in the supporting information). These
89 cylinders were allowed to stand for two weeks before treatments. Fresh algal biomass was
90 also collected at the same location as the sediment and water samples, and then freeze-
91 dried (Beijing Boyikang Biological Experiment Equipment Co. Ltd., China) in the
92 laboratory. To simulate algal-derived organic deposition, 0.4 g of dry algal biomass was
93 added to each cylinder (60 mg dry weight/L water) and then settled with modified soil
94 flocculants according to Shi et al. (2016) and Pan et al. (2011).^{17,18} After algal deposition,
95 nine cylinders were treated with 2-cm thick layer (110 ml in volume, 87 g in weight) of
96 oxygen nanobubbles loaded zeolites (O-Ze) by adding them to the bottom water; and nine
97 cylinders were treated with 2-cm thick layer (110 ml in volume, 87 g in weight) of zeolites
98 without oxygen nanobubbles (Ze) to identify the capping barrier effect of zeolites on CH₄
99 emission. The control was run in nine simulation systems without any treatment after algal

100 deposition. Incubation experiments were conducted indoors at room temperature ($22 \pm 2^\circ\text{C}$)
101 over a period of 20 d in the dark.

102 On day 0 (before the prepared cylinder ran), day 1, 3, 5, 7, 10, 13, 16 and 20, the changes
103 of redox conditions and fluxes of CH_4 and CO_2 in these cylinders were analyzed, and 50
104 ml water samples were collected from an outlet of the cylinder (5 cm above the sediment-
105 water interface) for DOC analysis. The DO in the overlying water was measured at 5 cm
106 above the sediment-water interface using a DO meter (JPB-607A, Shanghai INESA
107 Scientific Instrument Co. Ltd., China); and the ORP at the sediment-water interface was
108 measured using an ORP meter (Orion 3-star, Thermo Fisher Scientific, USA). The water
109 DOC was analyzed using a TOC analyzer (Liqui TOC II, Elementar, Germany). On day 0,
110 7 and 20, three cylinders in each treatment were randomly deposed to collect water and
111 sediment samples for microbial analysis. The results were presented as the mean values.

112 Water removed during sampling was replaced with surface water collected from Taihu Lake.

113 **CH_4 and CO_2 Flux Analysis.** Daily fluxes of CH_4 and CO_2 across the water-air interface
114 in the cylinders were analyzed using a static micro-chamber method described by
115 Duchemin et al. (1999).¹⁹ On the sampling date, the micro-chamber was placed onto the
116 water surface in each cylinder. Gas samples (10 ml) were collected before and after 2 h
117 using a 25-ml polypropylene syringe. The samples were injected into a pre-evacuated
118 Exetainer® vial for storage until analysis could be completed by gas chromatograph
119 (7890B, Agilent Technologies, USA). Gas fluxes were calculated based on the changes in
120 gas concentration over 2 h, and then total gas emissions were estimated throughout the 20-

121 day incubation.

122 Since CH₄ has a 25 times higher global warming potential (GWP) than CO₂ on a mass
123 basis over a hundred year horizon,²⁰ carbon emissions with different CH₄/CO₂ ratios often
124 differ in global warming impacts. Thus, the CH₄/CO₂ ratio has been commonly used as an
125 important index for the study of carbon emissions from inland waters.²¹ In this study, the
126 CH₄/CO₂ ratio in each treatment was calculated by dividing CH₄ mass emission by CO₂
127 mass emission. Moreover, the total GWP of each treatment was also estimated and
128 expressed in CO₂ equivalent. The conversion of CH₄ to CO₂ equivalent was conducted by
129 multiplying CH₄ mass emission by a factor of 25.²⁰

130 **DNA Extraction and qPCR.** Approximately 0.4 g of sediment samples were collected for
131 sediment microbial analysis, and 800 mL water was collected from an outlet of the cylinder
132 (5 cm above the sediment-water interface), and filtered through a 0.2 µm pore-size filters
133 (Track-Etched Membranes, Whatman® Nuclepore™) for water microbial analysis. The
134 sediment samples and filters were stored at -80°C before analysis. DNA extraction was
135 undertaken with Powersoil DNA Isolation Kits and Powerwater DNA Isolation Kits
136 (MoBio Laboratories Inc., Carlsbad, CA) according to the manufacturers' instructions,
137 respectively; the DNA subsequently served as templates for qPCR amplification. The
138 methanogens and methanotrophs in the samples were quantified using the qPCR method
139 described below.

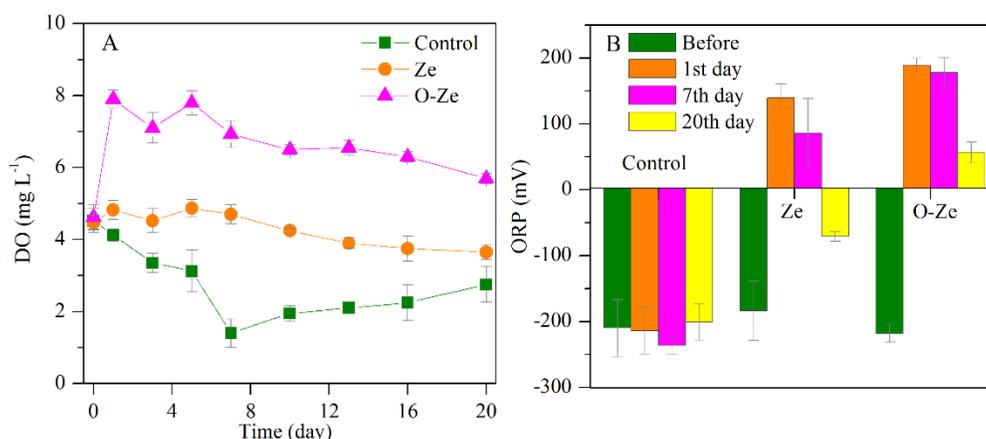
140 The qPCR assay was performed using primers targeting methanogenic archaeal 16S
141 rRNA (primer set, 1106F/1378R) and methanotrophic *pmoA* genes (primer set,

142 A189F/M661R).²²⁻²⁴. Gene copies were amplified and quantified in a Bio-Rad cyclor
143 equipped with the iQ5 real-time fluorescence detection system and software (version 2.0;
144 Bio-Rad). All reactions were completed in a total volume of 20 μL containing 10 μL
145 SYBR[®] *Premix Ex Taq*[™] (TOYOBO, Japan), 0.5 μM of each primer, 0.8 μL bovine serum
146 albumin (BSA, 3 mg/mL, Sigma), double distilled H₂O and template DNA. The qPCR
147 program for archaeal 16S rRNA was as follows: 95°C for 60 s, followed by 40 cycles of
148 95°C for 25 s and 57°C for 30 s and 72°C for 60 s. The qPCR program for *pomA*
149 commenced with 95°C for 60 s, followed by 40 cycles of 95°C for 25 s, 53°C for 30 s and
150 72°C for 60 s. The standard curve was established by a serial dilution ($10^{-2} \sim 10^{-8}$) of known
151 concentration plasmid DNA with the target fragment. All PCRs were run in triplicate on
152 96-well plates (Bio-Rad) sealed with optical-quality sealing tape (Bio-Rad). Three negative
153 controls without DNA template were included for each PCR run.

154 **RESULTS AND DISCUSSION**

155 **Anoxia/hypoxia Remediation.** In the control system, water DO decreased from 4.5 to 1.4
156 mg L⁻¹ within the initial 7 d, and then gradually increased to 2.8 mg L⁻¹ on day 20 (Figure
157 1A). Surface sediment suffered from severe anoxia, and the ORP at the sediment-water
158 interface remained below -200 mV throughout the experiment (Figure 1B). This
159 hypoxia/anoxia frequently occurs in eutrophic waters, which is often driven by algal
160 blooms.²⁵⁻²⁸ When zeolites without oxygen nanobubbles were added (2-cm thick) after
161 algal deposition in the Ze treatment, the DO in the water column maintained a level of 3.6
162 ~ 4.9 mg L⁻¹ throughout the 20-day incubation; and the ORP at the sediment-water interface

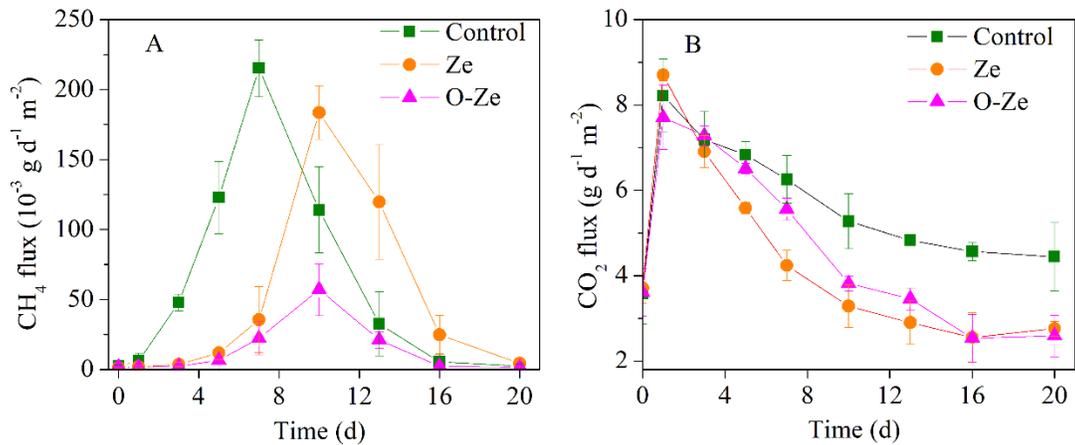
163 was reversed to +139.5 mV on day 1, and then decreased to -70.5 mV on day 20. Unlike
 164 anaerobic sediment, the zeolites that are in constant contact with air and sunshine are
 165 aerobic by nature. Thus, the ORP of the top layer sediment was quickly increased to become
 166 positive during the first few days after the capping (Figure 1B).²⁹ The zeolite capping layer
 167 can prevent oxygen-consuming materials in sediments enter into the overlying water,
 168 resulting in a relatively higher level of water DO in the Ze treatment than in the control.
 169 The remediation effect of zeolite addition was enhanced after the oxygen nanobubbles were
 170 loaded onto zeolites. After oxygen nanobubbles enriched zeolites were added (2-cm thick)
 171 in the O-Ze treatment, the DO in the water column was increased to 7.9 mg L⁻¹ on day 1
 172 and remained above 5.7 mg L⁻¹ throughout the remaining incubation period (Figure 1A);
 173 and the ORP at the sediment-water interface was reversed to +189.0 mV on day 1 and
 174 remained positive throughout the experiment (Figure 1B).



175
 176 **Figure 1.** Water environment changes in the simulated column systems. (A) DO in water
 177 column. (B) ORP at the sediment-water interface. Control was run without any treatment
 178 after algal deposition; O-Ze and Ze represent the addition of zeolites with and without

179 oxygen nanobubbles after algal deposition, respectively. Error bars indicate standard
180 deviations.

181 **CH₄ Emission Mitigation.** A significant CH₄ emission event occurred in the control
182 system. The CH₄ flux across the water-air interface increased and reached a maximum of
183 $215.3 \times 10^{-3} \text{ g d}^{-1} \text{ m}^{-2}$ within the initial 7 d, with a cumulated CH₄ emission of 1.35 g m^{-2}
184 over the 20 days period (Figure 2A). Although zeolite capping yielded a reasonably oxic
185 water environment (Figure 1) in the Ze treatment, a cumulated CH₄ emission of 1.13 g m^{-2}
186 was still detected over the 20-day incubation period. The CH₄ flux reached a maximum
187 of $183.6 \times 10^{-3} \text{ g d}^{-1} \text{ m}^{-2}$ on day 10. In contrast, when oxygen nanobubbles were loaded
188 onto the zeolites and delivered to the bottom water in the O-Ze treatment, the cumulated
189 CH₄ emission was reduced by a factor of 3.2 (0.32 g m^{-2}) compared to the control, and the
190 maximum peak of CH₄ flux was $57.1 \times 10^{-3} \text{ g d}^{-1} \text{ m}^{-2}$ on day 10 (Figure 2A). The CO₂ flux
191 displayed a similar trend in each treatment, initially increasing due to algal organic carbon
192 inputs, and then gradually decreasing to low steady levels as the organic carbon was
193 consumed (Figure 2B); however, the CO₂ fluxes in the Ze and O-Ze were generally lower
194 than in the control, which was potentially due to the capping barrier effect of zeolites
195 carriers. It is important to note that, from day 4 to 16, the CO₂ flux in the O-Ze treatment
196 was higher than that in the Ze treatment, whereas the CH₄ flux in O-Ze was lower than in
197 the Ze (Figure 2A). Over the 20 days period, the cumulated CO₂ emissions were 119.3,
198 88.2 and 95.8 g m^{-2} in the control, Ze, and O-Ze treatments, respectively (Figure. 2B).|

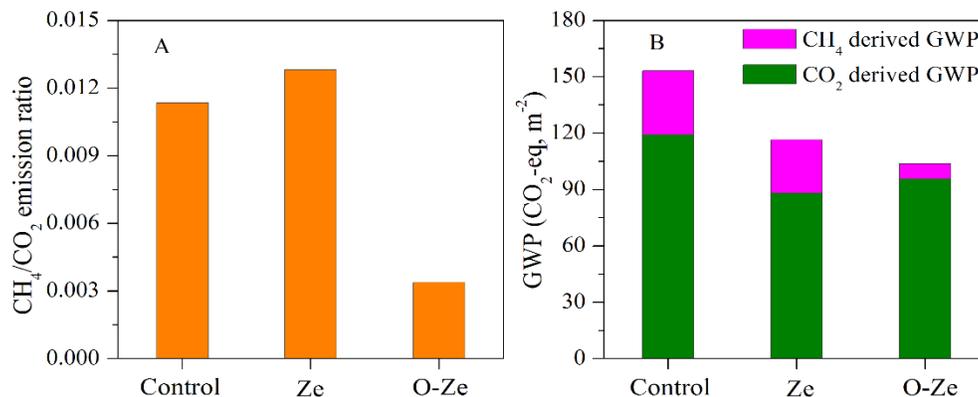


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200 **Figure 2.** CH₄ and CO₂ fluxes across the water-air interface. (A) CH₄ fluxes. (B) CO₂
 201 fluxes. The cumulated gas emissions over the 20-day incubation period were obtained by
 202 the trapezoidal method using MATLAB (MathWorks, USA).

203 In comparison with the control, the addition of zeolites without oxygen nanobubbles
 204 increased the CH₄/CO₂ emission ratio. Over the 20-day incubation period, the CH₄/CO₂
 205 emission ratios reached 11×10^{-3} and 13×10^{-3} in the control and Ze treatments,
 206 respectively. In contrast, the use of oxygen nanobubbles enriched zeolites in the O-Ze
 207 treatment yielded a lower CH₄/CO₂ emission ratio of 3.4×10^{-3} (Figure 3A), suggesting
 208 that a greater fraction of organic carbon was recycled as CO₂ instead of CH₄ in the presence
 209 of the oxygen nanobubbles. This was in line with the fact that the CH₄ flux in the O-Ze
 210 treatment was lower than in the Ze treatment (Figure 2A), whereas the CO₂ flux was higher
 211 in the former than the latter (Figure 2B). As a result of the lower GWP of CO₂ relative to
 212 CH₄, the net global warming effect was reduced as a greater fraction of organic carbon was
 213 recycled as CO₂ instead of CH₄. Total GWP of the carbon emissions was 103.9 g m⁻² (CO₂-
 214 eq) in the O-Ze treatment, as opposed to 153.1 g m⁻² (CO₂-eq) in the control and 116.4 g

215 m⁻² (CO₂-eq) in the Ze treatment (Figure 3B).



216

217 **Figure 3.** CH₄/CO₂ emission ratios and total GWP values over the 20-d incubation period.

218 (A) CH₄/CO₂ emission ratio. (B) GWP values. CH₄ is converted to CO₂ equivalent based

219 on the effect of CH₄ on radiative forcing of the atmosphere relative to the effect of CO₂.

220 The GWP of CH₄ is approximately 25 times higher than CO₂ on a mass basis over a

221 hundred-year horizon. CH₄ emissions were multiplied by a factor of 25, and combined with

222 measured CO₂ emissions to estimate the total GWP of each treatment.

223 **Microbial Process Manipulation.** To elucidate the mechanism of this observation, we

224 quantified methanogens and methanotrophs using the qPCR method based on the

225 methanogenic archaeal 16S rRNA gene and methanotrophic *pmoA* gene, respectively.²²⁻²⁴

226 In the control system, methanogen abundance increased following the algal deposition. The

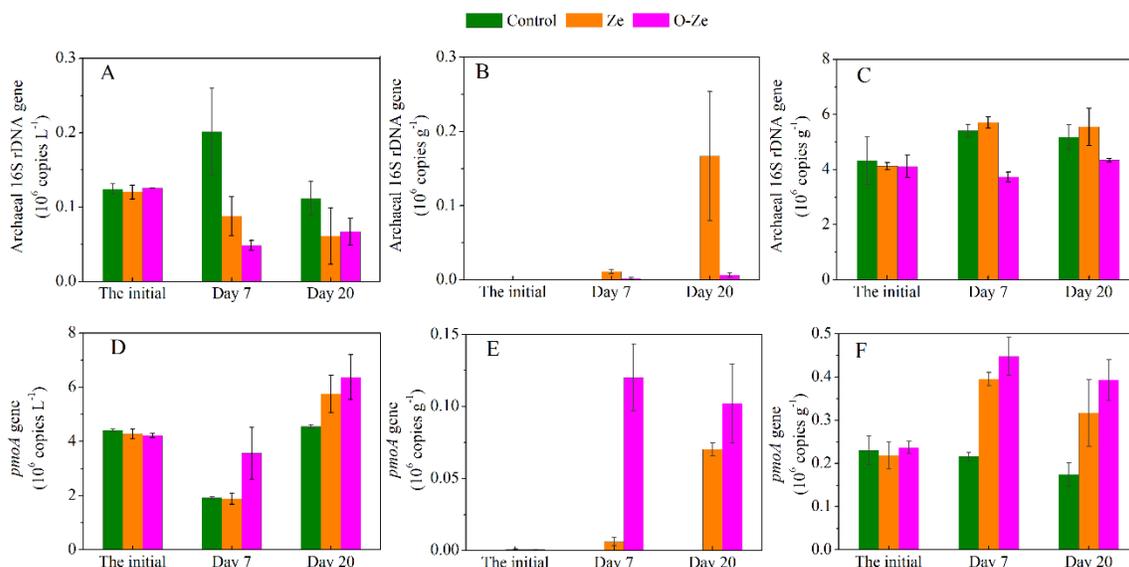
227 archaeal 16S rRNA gene increased from 0.12×10^6 to 0.20×10^6 copies L⁻¹ in the water

228 column (Figure 4A), and from 4.12×10^6 to 5.71×10^6 copies g⁻¹ in the surface sediment

229 (Figure 4C) on day 7, which potentially contributed to the high CH₄ emissions (Figure 2).

230 After algal deposition, the zeolite addition in the Ze system increased methanotroph

231 abundance (Figure 4D,F); however, methanogen blooms occurred in both the zeolite layer
232 and surface sediment, which could be potentially responsible for massive CH₄ production.
233 The archaeal 16S rRNA gene reached 0.17×10^6 and 5.55×10^6 copies g⁻¹ on day 20 in the
234 zeolite layer (Figure 4B) and surface sediment (Figure 4C) in the Ze treatment, respectively.
235 When oxygen nanobubbles enriched zeolites were used in the O-Ze treatment, methanogen
236 abundance declined in both the zeolite layer and surface sediment compared with when
237 zeolites without oxygen nanobubbles were used in the Ze treatment. The archaeal 16S
238 rRNA gene in the O-Ze treatment reached 0.01×10^6 and 4.35×10^6 copies g⁻¹ on day 20
239 in the zeolite layer and surface sediment, respectively (Figure 4B,C). Moreover, the
240 methanotroph abundance in the O-Ze treatment was further increased in the zeolite layer
241 compared with when zeolites without oxygen nanobubbles were used in the Ze treatment.
242 The *pmoA* gene reached 0.12×10^6 copies g⁻¹ on day 7, and 0.10×10^6 copies g⁻¹ on day
243 20 in the O-Ze treatment, while it was only 0.01×10^6 copies g⁻¹ on day 7 and 0.07×10^6
244 copies g⁻¹ on day 20 in the Ze treatment (Figure 4E,F). In natural waters, CH₄ is mainly
245 produced by anaerobic methanogens, and then partially oxidized to CO₂ by aerobic
246 methanotrophs before it is emitted into the atmosphere.³⁰⁻³² Surface oxygen nanobubbles
247 potentially served as oxygen suppliers to the surroundings. The created oxygen enriched
248 environment (Figure 1) favored methanotroph growth and simultaneously inhibited
249 methanogen growth (Figure 4),³³ leading to relatively lower CH₄ emissions and higher CO₂
250 emissions than the zeolites without oxygen nanobubbles (Figure 2).



251

252 **Figure 4.** Abundances of methanogens and methanotrophs in the column simulation

253 systems. Methanogens and methanotrophs were quantified using the q-PCR method based

254 on the archaeal 16S rRNA and *pmoA* genes, respectively. The surface sediment in the Ze

255 and O-Ze referred to the sediment below the zeolite layer. (A) archaeal 16S rRNA gene in

256 the water column; (B) archaeal 16S rRNA gene in the zeolite layer; (C) archaeal 16S rRNA

257 gene in the surface sediment; (D) *pmoA* gene in the water column; (E) *pmoA* gene in the

258 zeolite layer; (F) *pmoA* gene in the surface sediment. Error bars indicate standard deviations.

259 **Implications for Geo-engineering in Hypoxic/anoxic Waters.** When eutrophication

260 occurs, algae often proliferate as dense floating mats.^{34,35} When these blooms die, they sink

261 to the bottom sediment and decay, causing hypoxia/anoxia^{8,36,37} and thereby stimulating

262 CH₄ production.^{38,39} Our results suggest a possible principle for hypoxia/anoxia

263 remediation in natural waters. However, existing technological principles in oxygen

264 delivery are limited to aeration and mixing,^{4,5,40} which fundamentally prevent their large-

265 scale application in hypoxic/anoxic waters that are crucial to the environment. Suspended
266 solids can be flushed through rivers to lakes and coastal waters and gradually settle onto
267 the bottom sediment.^{41,42} If oxygen enriched clay particles can be continuously dispensed
268 through the inflowing rivers, it may provide a geo-engineering method for oxygen delivery
269 especially to the deep waters and sediments. In recent years, geo-engineering methods have
270 proved to be feasible in fixing natural water problems.^{18,43,44} But it still remains a challenge
271 to deliver safe materials (such as clays) cost-effectively into natural water systems.
272 Compared with artificial aeration and mixing, surface oxygen nanobubble delivery should
273 not alter stratification or circulation patterns in the water, but instead follow a natural
274 settling process. Previous studies have indicated that oxygen nanobubbles can remain
275 stable in water for a prolonged period (weeks or months) compared with microbubbles.^{11,45}
276 If they can be dispensed slowly but continuously, they may serve as potential oxygen
277 suppliers for remediating anoxic sediment, which would cause a series of changes in the
278 nitrogen, phosphorus and sulfur cycles in receiving waters.⁴⁶⁻⁴⁸ However, there may be
279 some problems to solve before the large-scale field applications of this method, such as
280 ecological impacts and the dosage of zeolites. It is possible to increase the amount of
281 oxygen absorbed by increasing the porosity of zeolites or screen other effective materials
282 as oxygen carriers (e.g., ecologically safe sediments), however, this requires further studies.
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290

291 **Supporting Information**

292 The system for the analysis of oxygen absorption capacity of zeolites, the algal water-
293 sediment simulation cylinders used in this study.

294

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