Strain-boosted hyperoxic graphene oxide efficiently loading and improving performances of microcystinase
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SUMMARY
Harmful Microcystis blooms (HMBs) and microcystins (MCs) that are produced by Microcystis seriously threaten water ecosystems and human health. This study demonstrates an eco-friendly strategy for simultaneous removal of MCs and HMBs by adopting unique hyperoxic graphene oxides (HGOs) as carrier and pure microcystinase A (PMlrA) as connecting bridge to form stable HGOs@MlrA composite. After oxidation, HGOs yield inherent structural strain effects for boosting the immobilization of MlrA by material characterization and density functional theory calculations. HGO5 exhibits higher loading capacities for crude MlrA (1,559 mg g⁻¹) and pure MlrA (1,659 mg g⁻¹). Moreover, the performances of HGO5@MlrA composite, including the capability of removing MCs and HMBs, the ecological and human safety compared to MlrA or HGO5 treatment alone, have been studied. These results indicate that HGO5 can be used as a promising candidate material to effectively improve the application potential of MlrA in the simultaneous removal of MCs and HMBs.

INTRODUCTION
The frequency and intensity of harmful Microcystis blooms (HMBs) are increasing globally, with HMBs and microcystins (MCs) produced by Microcystis contributing to the pollution of water environments and seriously threatening the health of animals and human beings. Cyanobacterial blooms frequently occurred in Taihu Lake, Dianchi Lake, and Chaohu Lake, the three largest lakes in China. In 2006, the area of blooms in China lakes was as high as 1363.3 km², decreased to 665.2 km² in 2013, but increased to 775.4 km² in 2018 (Huang et al., 2020; Huisman et al., 2018; Wang et al., 2021). Cyanotoxins were detected 1118 times in 869 freshwater ecosystems in 66 countries around the world, dating from the earliest records until 2018. And the detection frequency of MCs accounted for 63%, ranking the first (Svir/2019). Several physical and chemical treatment strategies have been developed to efficiently alleviate HMBs, but these methods have some problems, including poor selectivity and difficulties in mitigating MCs release after cell lysis (Zhang et al., 2019a, 2019b; Li et al., 2011; Tsai et al., 2019; Feng et al., 2019). Microbial treatments are attractive alternatives but can bring potential risks owing to the secretion of unknown hazardous products (HPs) into water systems or by becoming new ecologically dominant species (Li et al., 2016; Yu et al., 2019). Consequently, the simultaneous and efficient removal of HMBs and MCs has become one of the most important issues in water resource pollution control.

Microcystinase A (MlrA) is the first and key enzyme involved in MCs biodegradation and has attracted extensive research attention owing to the potential for simultaneously removing HMBs and MCs for environmentally industrial application (Dexter et al., 2021; Liu et al., 2020). The cell-free crude enzyme extracts (CMlrA) from recombinant cells are analyzed in most studies (Dexter et al., 2021; Wang et al., 2017). Pure MlrA (PMlrA) was recently purified from CMlrA using commercial amylose-binding resins, as recently reported (Liu et al., 2020). Two types of MlrAs (CMlrA and PMlrA) have been obtained through heterologous expression and have been used for removing HMBs and MCs. However, the low activity yield of PMlrA and the unknown HPs in CMlrA are obstacles to the environmental or industrial application of MlrAs (Liu et al., 2020). Our preliminary studies demonstrated that the activity recovery rate of PMlrA was only 0.33% of that of CMlrA (Table S1). Therefore, the development of an optimized strategy to address both
activity yields and ecological security issues for the actual application of MlrAs to simultaneously remove HMBs and MCs remains a major challenge.

Graphene oxide (GO) nanomaterials have been widely used as promising treatments in many fields because they typically achieve considerably improved performances by tuning morphology, composition, and physicochemical properties (Chaudhary et al., 2021; Sun et al., 2021). However, the structural characteristics of GOs, including their functionalized molecules, microenvironments, and degree of oxidation, have important influences on their performance characteristics including their loading capability, stability, and potential toxicity (Chaudhary et al., 2021; Zhang et al., 2020a; Zolezzi et al., 2018). Our preliminary studies showed that several commercial GOs (CGOs) exhibited different carrying capacities for CMlrA (Table S2). The maximum loading amount of CGO3 (monolayer GO) for CMlrA was notably observed with an approximately 18-fold higher capacity than the GO derivative modified with L-Cys (CysGO) which was the only GO derivative previously used to immobilize MlrA (Wu et al., 2020) (Table S1). Concomitantly, by using the crude maltose-binding protein (CMBP) from recombinant cells without the mlrA gene as control, the CGO3@CMBP composite exhibited better safety than did CGO3 or CMBP (Figure S1). Commercial monolayer GO (CGO3) is expensive to generate, but these results suggest that it is possible to produce an appropriate GO nanomaterial that can improve the loading capacity of GO for CMlrA, while selectively removing HPs from CMlrA.

In this study, several GO derivatives were synthesized using a low-cost graphite powder. A hyperoxic graphene oxide (HGO5) was successfully identified that exhibits impressively high loading capacities for MlrAs that are nearly comparable to those of CGO3. The assembly mechanism of the HGO5@CMlrA composite was further investigated using material characterization and density functional theory calculations. Furthermore, the HGO5@CMlrA composites removed both HMBs and MCs. Lastly, the ecological safety of HGO5@CMlrA composites was also evaluated. This study provides a promising and scalable paradigm whereby HGO5 can be used as a potential nanomaterial to improve the application potential of MlrA in the simultaneous removal of MCs and HMBs.

RESULTS AND DISCUSSION

Characterization of structural strain effects of hyperoxic graphene oxides

Our preliminary experimental results indicated that higher total oxygen content (CTO) in CGOs was associated with higher loading capacities of CGOs for CMlrA (Table S2). Thus, their different immobilization performances could be related to their degree of oxidation (Tan et al., 2017; Zolezzi et al., 2018). To systematically explore the degree of oxidation on the immobilization performance of GO for MlrAs, nine types of HGOs (HGO1-HGO9) were prepared using HGO0 as raw material and by altering the amount of added KMnO4 oxidant and the method of synthesis (M1 or M2) (Hummers and Offeman, 1958; Marcano et al., 2010) (Figures 1A and S2; Table S3). The loading capacities of HGOs for CMlrA were positively correlated with their total oxygen content (CTO) when comparing HGOs prepared with the same raw material and synthesis method. For example, the loading capacity of HGO5 for CMlrA (1,559 mg·g⁻¹) was nearly five times higher than that of HGO3 (303 mg·g⁻¹), while the CTO of HGO5 (46.4%) was nearly nine times higher than that for HGO3 (5.05%) (Table S2). When varying the synthesis method, the loading capability of as-made HGOs for CMlrA significantly differed, even if their CTOs were similar or identical. For example, the CMlrA loading amounts for HGO3 and HGO7 significantly differed (1,101 mg·g⁻¹ and 791 mg·g⁻¹, respectively) although their CTOs were similar (35.4 and 37.5%, respectively). The effects of raw materials on GO loading capacities were also compared when using the same synthesis method. ZGO1 was synthesized using the raw material ZGO0 (Huang et al., 2019) which differed from HGO0 because it used the same synthesis method as used for HGO5. Accordingly, the CTOs and the CMlrA-loading capacities of ZGO1 and HGO5 were significantly different (Table S2).

HGO functions were consequently hypothesized to not only be related to CTO, but also to other structural changes that could have occurred during the oxidation process that then affected the binding of MlrA on HGOs. Strain effect is a commonly used comprehensive factor for evaluating structure-performance relationships in nanomaterial engineering (Li et al., 2020; Luo and Guo, 2017). Scanning electron microscopy (SEM) imaging revealed that the surface roughness and wrinkle degree of HGOs were gradually enhanced with increased KMnO4 addition, indicating that the process of oxidation altered the surface properties of HGOs and clearly generated higher concentrations of lattice defects (Figures 1B and S3). Similar patterns have been observed in CGOs and ZGOs prepared from different sources (Figure S4). The lattice defects of
GOs might be attributable to the insertion of oxygen-containing groups that induce the internal structure and external morphology strain effects in GOs (Reynosa-Martínez et al., 2020; Tan et al., 2017).

X-ray diffraction (XRD) further revealed that a new peak at 2θ = 10.7°, corresponding to the crystal plane of GO (001), began to appear in HGO1 or HGO6, and then gradually increased from HGO1 to HGO5 or from HGO6 to HGO9 (Figures S5B and S6B). Concomitantly, the peak at 2θ = 26° corresponding to the graphite crystal plane (002) became wider and then gradually decreased until it was not detected. These results suggested that HGOs were prepared by destroying C=C bonds and inserting different degrees of oxygen-containing functional groups into the graphite lattice of HGO0, thereby resulting in more tensile strain effects (Li et al., 2020; Reynosa-Martínez et al., 2020; Tan et al., 2017). Similar patterns have been observed in CGOs and ZGOs prepared from different sources (Figure S7B). Furthermore, X-ray photoelectron spectroscopy (XPS) analysis demonstrated that the O1S peak gradually increased, while the C1S peak gradually decreased from HGO1 to HGO5 and from HGO6 to HGO9, indicating that increased KMnO4 addition led to...
gradual increases in the C\textsubscript{TO}S of HGOs (Figures S5D and S6D). The C\textsubscript{TO} value of HGO\textsubscript{5} was up to nine times higher than that of HGO\textsubscript{0} (Table S2). Similar patterns have been observed in CGOs from different sources (Figure S7D). Moreover, high-resolution XPS analysis revealed that variation in several oxygen-containing functional group contents differed when comparing HGO\textsubscript{0} to HGO\textsubscript{5} or HGO\textsubscript{6} to HGO\textsubscript{9} (Figure 1C and Table S2, Figures S8 and S9) (Al-Gaashani et al., 2019). Similar differences were observed in CGOs and ZGOs prepared from different materials (Figure S10).

Raman spectroscopy (RS) imaging revealed that when HGO oxidation degree increased, their intensity ratio (I\textsubscript{D}/I\textsubscript{G}) and full width at half maximum (FWHM) values of the D to G bands also increased, while their C/O ratios decreased (Figures 1D and Table S4, Figures S5A and S6A). These results indicated the presence of increasing strain effects from HGO\textsubscript{0} to HGO\textsubscript{5} and from HGO\textsubscript{6} to HGO\textsubscript{9}, consistent with the SEM characterizations (Reynosa-Martínez et al., 2020; Li et al., 2020). RS variation provides evidence that increasing levels of oxygen-containing functional groups were inserted into the carbon networks of the HGOs, resulting in unique structural strains among different HGOs that could then be modified to achieve desirable HGO properties (Reynosa-Martínez et al., 2020; Li et al., 2020). Similar differences were observed in CGOs prepared from different materials (Figure S7A).

Interestingly, increased HGO interlayer spacings were proportional to the oxidation degree owing to the intercalation of oxygen-containing functional groups in the contiguous aromatic lattices of HGO\textsubscript{0} such as epoxides, ketone carbonyls, and carboxylic groups (Figure 1E). The Bragg law indicated that the interlayer spacing between HGO\textsubscript{5} nanosheets induced a tensile strain rate (\textit{SFGO}5) of 126.1\% compared to HGO\textsubscript{0} (Table S5). Furthermore, the strain effects were positively correlated with the HGO loading levels (Figure 1F). Moreover, Fourier Transform Infrared Spectroscopy (FTIR) analysis demonstrated that increased oxidation degree led to the appearance of different oxygen-containing functional groups in GOs (Figures S5C, S6C, and S7C). A significant band near 3,500 cm\textsuperscript{-1} was identified corresponding to the stretching of the -OH functional group and hydrogen-bonded water (H-O-H), and gradually widened from HGO\textsubscript{0} to HGO\textsubscript{5} and from HGO\textsubscript{6} to HGO\textsubscript{9}, indicating that the hydrophilicity of HGOs increased with increasing oxidation degree (Figures S5C and S6C).

It is worth noting that HGO\textsubscript{1} and HGO\textsubscript{6} were synthesized using the same oxidant (0.3 g KMnO\textsubscript{4}) via M\textsubscript{1} and M\textsubscript{2}, respectively, and the C\textsubscript{TO}S of HGO\textsubscript{1} and HGO\textsubscript{6} were similar. However, their contents of oxygen-containing functional groups, interlayer spacing, and I\textsubscript{D}/I\textsubscript{G} values were significantly different (Tables S2-5S). HGO\textsubscript{3} and HGO\textsubscript{7} also exhibited similar characteristics. Thus, HGOs, that were synthesized by different methods, even when prepared from the same raw material, may have different strain effects, leading to unique functional characteristics. Considering the same amount of oxidant, the HGOs synthesized via M\textsubscript{1} produced more suitable structural strain effects for loading CMlirA than those produced via M\textsubscript{2}, as evaluated by loading capacities (Table S2).

In an aqueous solution (pH 7.4), the zeta potential gradually decreased from HGO\textsubscript{0} to HGO\textsubscript{5} and from HGO\textsubscript{6} to HGO\textsubscript{9}, indicating that higher oxidation degrees led to more stable HGOs (Table S6). In solutions with pH values ranging from 5.3 to 9.3, HGO\textsubscript{5} zeta potentials were lower than those of HGO\textsubscript{0}, indicating that a high degree of oxidation in HGO\textsubscript{5} led to greater stability than HGO\textsubscript{0} over a wider pH range (Table S6). The stabilities of HGOs in aqueous solutions can be attributed to the concentration of oxygenated functional groups and the dissociation of acid functional groups such as carboxylic acid (-COO\textsuperscript{-} + H\textsuperscript{+}) and hydroxyl (OH\textsuperscript{-}) groups (Reynosa-Martínez et al., 2020; Zolezzi et al., 2018).

Combined with the structural characterizations discussed above, the oxidation of HGO\textsubscript{0} resulted in distinct strain effects owing to the introduction of oxygen. The strain effects of HGOs were positively correlated with their C\textsubscript{TO}S, but also included changes in other characteristics such as lattice morphology, oxygen-containing group composition, interlayer spacing, and electronegativity. Consequently, strain effects may optimize the performance of HGOs for effective combination with CMlirA, resulting in greater strain that then leads to higher loading capacities of HGOs for CMlirA (Figure 1F) (Li et al., 2020; Zhang et al., 2014; Luo and Guo, 2017).

**Structural characterization of the HGO\textsubscript{5}@CMlirA composite**

To investigate the role of HGO strain effects in promoting combination with CMlirA, HGO\textsubscript{5} was used as an exemplary material to prepare the HGO\textsubscript{5}@CMlirA composite using physical adsorption and covalent
binding methods (Table S1). Physical adsorption is widely used for enzyme immobilization because it is typically simple to conduct and minimally affects enzyme activity. Covalent binding generally leads to higher loading capacity and stability of nanomaterials than physical adsorption, although it may be a more complex process and considerably affects enzyme activity (Zhuang et al., 2020; Monajati et al., 2018; Chaudhary et al., 2021). The assembly conditions of the two methods for the HGO5@CMlrA composites were also optimized (Figures S11 and S12). Under optimal conditions, the loading capacity of HGO5 for CMlrA increased up to 1,654 mg·g⁻¹ with the covalent cross-linking method, which was slightly higher than that with the adsorption method (1,559 mg·g⁻¹) (Table S1). In contrast, the loading capacity of HGO5 for PMlrA increased by up to 1,659 mg·g⁻¹ when prepared using the adsorption method, representing a slightly higher value than that of CMlrA with the same method (1,559 mg·g⁻¹). These results indicated that HGO5 can efficiently load MlrAs when prepared by both adsorption and covalent methods, representing much higher values than that achieved with L-CysGO (89.77 mg·g⁻¹) (Wu et al., 2020) (Table S1). The adsorption method allowed easy control of high loading performance conditions and thus, the HGO5@CMlrA used in subsequent studies was prepared by the adsorption method unless otherwise indicated.

FTIR spectroscopy revealed a characteristic N-H peak (1,536 cm⁻¹) for HGO5@CMlrA, indicating that CMlrA was successfully loaded onto HGO5 (Figure 2A). C-O-C antisymmetric tensile vibration bands (1,220 cm⁻¹) were observed in both the HGO5 and HGO5@CMlrA, corresponding to the characteristic peaks for C=O (1,616 cm⁻¹), C-O (1,720 cm⁻¹), -OH (3,500 cm⁻¹), as previously reported in other studies (Zhuang et al., 2020; Chaudhary et al., 2021). Thus, most of the functional groups and the skeleton structure of HGO5 were not damaged after CMlrA immobilization.

In addition, XRD analysis revealed that the characteristic diffraction peak of the GO (001) crystal plane at 2θ of 10.7° in HGO5@CMlrA disappeared and an amorphous peak appeared at 2θ of 20-30° that may be caused by crystal structure changes of HGO5 immobilizing CMlrA (Figure 2B). Specifically, the energy-dispersive X-ray spectroscopy (EDS) analysis revealed that the corresponding N element mapping of HGO5@CMlrA was obviously higher than that of HGO5, further confirming that HGO5@CMlrA was successfully prepared (Figure 2D) (Zhuang et al., 2020; Chaudhary et al., 2021). Fluorescent isothiocyanate (FITC)-labeled CMlrA (F-CMlrA) and HGO5@F-CMlrA exhibited higher fluorescence intensities, while HGO5 and CMlrA did not exhibit any measurable fluorescence intensity (Figure S13). Fluorescence confocal scanning electron microscopy (FCSEM) visually confirmed that F-CMlrA was successfully loaded onto HGO5 surfaces (Figure S14).

The above results indicated that the FGO5@MlrA composite was successfully prepared. However, SEM observations indicated that the lamellar thickness of HGO5@CMlrA was clearly greater than that of HGO5 (Figure S15). R5 spectroscopy also revealed that the Ig/I0 and FWHM values of HGO5@CMlrA were higher than those of HGO5, indicating that the loading of CMlrA on HGO5 further increased HGO5 strain (Figures 2C and Table S4) (Chaudhary et al., 2021; Marcana et al., 2010; Krishnamorthy et al., 2013). Moreover, when comparing materials prepared using the same adsorption synthesis method, the loading capacity of HGO5 for CMlrA (up to 1,559 mg·g⁻¹) was 17 times higher than that of CysGO (89.77 mg·g⁻¹) (Wu et al., 2020). The loading capacities were also significantly higher than those of other modified GO derivatives for their respective enzymes (e.g., Glu-r3h-GO for lipase and PEGA-GOMNP for xylanase, 217 mg·g⁻¹ and 273 mg·g⁻¹, respectively) (Zhuang et al., 2020; Mehnati-Najafabadi et al., 2018), and also five times higher than that of HGO5 (303 mg·g⁻¹) (Tables S1 and S2). These results suggest that the HGO5@CMlrA composite may be assembled through a new mechanism differing from that of other modified GO derivatives that simply absorb enzymes on their surfaces (Zhuang et al., 2020; Mehnati-Najafabadi et al., 2018; Wu et al., 2020).

Assembly mechanism of the HGO5@CMlrA composite

GOs have been recently reported to be easily dispersed into monolayer GO nanosheets in aqueous solutions and these dispersed single-layer GO nanosheets can then be reconstituted into multilayer GO nanomaterials by molecular crosslinking. (Zhang et al., 2020a; Chen et al., 2017). Both CGO3 (a monolayer GO) and HGO5 exhibited impressively high carrying capacities for CMlrA (Table S2). Consequently, a new assembly mechanism of the HGO5@CMlrA composite was proposed here that involves HGO5 being dispersed to form monolayer GOs in the aqueous solution, followed by CMlrA acting as a molecular bridge to reassemble the dispersed monolayer GO to form the HGO5@CMlrA composite catalyst (Figure 3A). In contrast, HGO5 maintains a multilayer configuration in the aqueous solution and primarily absorbs CMlrA on its surface (Figure 3B).
The interlayer spacing of HGO₅ was only 0.753 nm, which is not large enough for CMlrA to enter the HGO₅ interlayers (Table S5). AFM imaging was used to validate the proposed assembly mechanism of HGO₅@CMlrA (Figures 3C and 3D). Compared to HGO₀, the HGO₅ lamellae were very irregular and the transparency of graphene layers in HGO₅ was much higher than in HGO₀. HGO₅ thickness (about 1.1 nm) was equivalent to an HGO₅ monolayer, while the thickness of HGO₅@CMlrA increased to around 6-12 nm (equivalent to 8-16 layers of HGO₅) owing to the interlayer spacing of HGO₅ (0.753 nm) (Figure 3C). In contrast, HGO₀ with a thickness of about 20-30 nm is equivalent to 60-90 layers of HGO₀ lamellae, while the thickness of HGO₀@CMlrA decreased to around 10-12 nm owing to the interlayer spacing of HGO₀ (0.333 nm) (Figure 3D). GOs can be dispersed into monolayer GO in the aqueous solution (Zhang et al., 2020a; Klechikov et al., 2015; Sun et al., 2016; Chen et al., 2017). After the addition of CMlrA, dispersed monolayer GO incorporated CMlrA as a connecting bridge, leading to reconstitution into multilayer GO owing to the interactions between CMlrA and monolayer GO. At this stage, the HGO₅@CMlrA composite was successfully and efficiently assembled, resulting in a significantly high loading capacity for CMlrA (Figure 3A). In contrast, HGO₀ was difficult to disperse into monolayers and remained in a multilayer configuration within the aqueous solution. Consequently, CMlrA was only assembled on the surface of multilayer HGO₀, resulting in a low loading capacity for CMlrA, as observed elsewhere (Figure 3B) (Zhuang et al., 2020; Mehnati-Najafabadi et al., 2018; Wu et al., 2020).

The assembly process of the HGO₅@CMlrA composite highly depends on the surface electronegativity of HGO₅. Thus, the density functional theory (DFT) calculation was used to analyze the theoretical rationality of the assembly model of the HGO₅@CMlrA composite (Figures 3E and 3F) (Lu and Chen, 2012; Humphrey et al., 1996). The overall average surface electrostatic potential (ESP) value of HGO₅ (−5.41 eV) was much lower than that of HGO₀ (−0.006 eV), indicating that HGO₅ had stronger electronegativity than HGO₀. Moreover, the penetration distance between HGO₅ and CMlrA (3.97 Å) was much higher than that between HGO₀ and CMlrA (0.90 Å), suggesting that the binding ability of HGO₅ for CMlrA was much stronger than that of HGO₀ in HGO₀@CMlrA.
To confirm the effects of the pH of the reaction solution and the electronegativity of the material on the assembly of HGO5 and CMlrA, HGO5 was further modified with amino groups to obtain HGO5-NH2. The potential value of HGO5-NH2 was higher than that of HGO5, resulting in a 12.8-fold decrease in CMlrA loading on HGO5-NH2 (i.e., only 122 mg/g (C0)) (Table S7). Under assembly conditions of pH 7.4 and 30 min of assembly time, the loading amount of CMlrA by HGO5 reached equilibrium at a loading capacity up to 1,559 mg/g (C0) (Figure S11), while that of HGO5 for PMlrA reached 1,659 mg/g (C0) (Table S1), representing a roughly 20-fold increase over previous reports of Cys-GO for PMlrA (Wu et al., 2020). These results further confirmed that HGO5 combined with CMlrA to assemble the HGO5@CMlrA composite via a novel mechanism different than those established in previous reports (Zhuang et al., 2020; Mehnati-Najafabadi et al., 2018; Wu et al., 2020). In addition, the leakage rates of HGO5@CMlrA obtained owing to adsorption and covalent methods were less than 11% after incubating in different pH buffers for 60 min. Moreover, almost no leakage was observed at pH 7.4, even if the leakage rate was less than 12% at pH 10.5 after 8 h of incubation (Table S8). These results indicated that HGO5 can reconstruct a stable HGO5@CMlrA composite using CMlrA as a molecular bridge by adsorption.

The experiments described above confirmed the novel assembly mechanism of the HGO5@CMlrA composite when using HGO5 combined with CMlrA. All of these factors, including high oxygen and oxygen-containing functional group content, enlargement of interlayer spacing, and enhanced electronegativity
combined to form sufficient strain effects for dispersing HGO₅ into monolayer GO in aqueous solution, are hypothesized to result in increased numbers of free HGO₅ monolayers, while also exposing abundant binding sites, leading to stronger electronegativity that would immobilize CMlrA (Tables S2 and S6). Subsequently, interactions between CMlrA and monolayer GO would promote the formation of the stable HGO₅@CMlrA composite (Zolezzi et al., 2018; Li et al., 2020; Zhang et al., 2014., Wang et al., 2011). This mechanism could explain the high loading capacity of HGO₅ for CMlrA and the assembly of a stable HGO₅@CMlrA composite.

The catalytic performances of the HGO₅@CMlrA composite

HGO₅@CMlrA exhibited efficient degradation activities against the three most common MCs (Figure S16) and had the same degradation product (Figure S17), consistent with the free enzymes CMlrA and PMlrA (Liu et al., 2020), indicating that not only degradation product pathway by HGO₅@CMlrA composite is the same as that of MlrA, but also CMlrA activity was better maintained after cross-linking with HGO₅ nanosheets. The oxygen-containing functional groups on HGO₅ facilitated the adsorption of MC-LR on the surface of HGO₅ through interactions such as electrostatic interactions, hydrophobic interaction, π-π interactions, and hydrogen bonds (Zeng and Kan, 2021). MC-LR is gathering on the surface of HGO₅@CMlrA, which makes MC-LR more accessible to CMlrA and hydrolyzed to chain MC-LR by CMlrA. Notably, the activity recovery rate of HGO₅@CMlrA from CMlrA significantly increased to 46.3%, which was 140 times higher than that of PMlrA from CMlrA (Table S1).

The effect of substrate or enzyme concentration on the degradation activities of HGO₅@CMlrA was investigated (Figure S19). The enzymatic reaction is a first-order reaction when the concentration of MC-LR was low and it was transitioned to zero-order reaction as the concentration of MC-LR increased. Furthermore, the calculation of Michaelis constant (Kₘ) and maximum reaction velocity (Vₘₐₓ) values of HGO₅@CMlrA indicate that the binding capacity of HGO₅@CMlrA to MC-LR was slightly lower than that of free CMlrA to MC-LR, while the specific activity of CMlrA was well-maintained after being immobilized on HGO₅ (Figures S19A and S19B). Degradation activities of HGO₅@CMlrA and free CMlrA toward MC-LR were concentration-dependent (Figure S19C).

The stability of HGO₅@CMlrA catalytic activity is also an important factor requiring evaluation. The results of structure characterization showed that the skeleton of HGO₅@CMlrA did not damage and HGO₅ was slightly reduced in the process of MC-LR degradation (Figure S20). HGO₅@CMlrA outperformed free CMlrA because it maintained more of its initial catalytic activities than free CMlrA under various adverse conditions (Figure S21). Specifically, the alkali tolerance of HGO₅@CMlrA was particularly significantly improved (Figure S21B). This could be attributed to the formation of microenvironments within HGO₅ that hindered the destruction of the conformation and the active site of the enzyme owing to pH stress (Chaudhary et al., 2021). As water pH can range up to about 10.5 during HMBs, the strong alkali resistance of HGO₅@CMlrA renders it more useful than CMlrA for controlling HMB and MCs pollution. The storage stability of enzymes is an important metric to evaluate the potential for their industrial application. The half-life of CMlrA was 10 days, while the half-life of HGO₅@CMlrA was up to 24 days at 4°C, indicating significantly higher storage stability of HGO₅@CMlrA compared to that of CMlrA (Figure S21C). Therefore, the application of HGO₅@CMlrA could effectively be conducted at low cost, while maintaining catalytic activity over time.

Effective inhibition of HGO₅@CMlrA on M. aeruginosa

The effects of HGO₅@CMlrA concentrations and treatment time on the inhibition of Microcystis aeruginosa (M. aeruginosa) were studied (Figure S22). It was shown that the inhibition rates of HGO₅@CMlrA against the growth of M. aeruginosa were time-dependent and concentration-dependent (Figure S22). The inhibition activity of HGO₅@CMlrA against M. aeruginosa was similar to that of free CMlrA and PMlrA, reaching 85.7% after nine days of treatment (Figure S23) (Liu et al., 2020). HGO₅ treatment also exhibited negative effects on M. aeruginosa cell growth, with an inhibitory rate of about 28.7%.
The *M. aeruginosa* was disrupted by HGO5@CMlrA and turned into environmentally friendly organisms that can provide nutrients for the growth of other aquatic organisms (Hadi and Brightwel, 2021). Compared to control group *M. aeruginosa* cells (BC), HGO5@CMlrA and CMlrA group cells all exhibited wrinkling or ruptures, plasmolysis, cell content overflowing, and cell organelle damage, indicating that both HGO5@CMlrA and CMlrA could destroy cellular nucleoid regions. However, the effects of HGO5@CMlrA treatment on cells were more serious (Figure 4A). Cells after HGO5 treatment remained smooth and spherical, although some intracellular changes began to occur, indicating that HGO5 treatment led to adverse effects on *M. aeruginosa* cells. Thus, the destruction of *M. aeruginosa* cells by HGO5@CMlrA may result from the synergistic activities of CMlrA and HGO5, resulting in greater damage to cells than from either component alone.

Chl-a is typically measured to reflect the photosynthetic capacity of *M. aeruginosa* cells. Chl-a content in the HGO5@CMlrA treatment group cells was similar to that observed for the CMlrA treatment, but lower than that in the PMlrA treatment. In contrast, HGO5 exhibited slightly negative effects on Chl-a content, indicating that HGO5@CMlrA and CMlrA treatment led to greater effects on Chl-a than did PMlrA and HGO5 (Figure 4B) (Liu et al., 2020). Fv/Fm and rETRmax are two commonly used parameters for evaluating photosynthetic activity. Fv/Fm represents the maximum quantum conversion efficiency of photosynthetic system II (PSII), while rETRmax represents the maximum electron transfer rates of PSII. Investigation of these changes in photosynthetic systems revealed that HGO5@CMlrA exhibited no significant effect on Fv/Fm, similar to the HGO5 treatment, but had lesser impacts on rETRmax than did the CMlrA treatment (Figures 4C and 4D).
The genetic regulatory mechanisms underlying photosynthesis in *M. aeruginosa* were also studied via their transcriptional responses to *FGO*<sub>5</sub>*@CMlrA* exposure ([Figure S24](#)). *psaB*, *psbD*, and *rbcL* are involved in the photosynthesis pathway. *psaB* encodes the reaction center subunit of PSI. *psbD* encodes the D2 protein of the PSII reaction center. *rbcL* encodes the ribulose-1,5-bisphosphate large subunit that is critical for the Calvin cycle. *FGO*<sub>5</sub>*@CMlrA* had obvious inhibitory effects on the transcription levels of *psaB* and *rbcL* ([Figure S24A](#)). However, *FGO*<sub>5</sub>*@CMlrA* did not significantly affect the transcription levels of *psbD*, while CMlrA significantly inhibited *psbD* ([Liu et al., 2020](#)). Both *FGO*<sub>5</sub>*@CMlrA* and CMlrA treatment effectively degraded total MCs. *FGO*<sub>5</sub>*@CMlrA* treatment was more effective in degrading extracellular MCs (LR<sub>ex</sub>) while CMlrA treatment was more effective in degrading extracellular MCs (LR<sub>ex</sub>) ([Figure 4E](#)). *FGO*<sub>5</sub>*@CMlrA* significantly inhibited the transcriptional level of genes in the MC-LR synthesis pathway including *mcyB*, *mcyD*, and *mcyG* ([Figure S24B](#)), even more so than by PMlrA treatment ([Liu et al., 2020](#)). These results suggest that decreased expression of MC-LR synthesis pathway genes may lead to the reduction of LR<sub>ex</sub> levels under *FGO*<sub>5</sub>*@CMlrA* treatment, consistent with the LR<sub>ex</sub> measured above ([Figure 4E](#)). In addition, *FGO*<sub>5</sub>*@CMlrA* treatment led to better maintenance of a weakly alkaline environment than did CMlrA treatment, while HGO5 treatment alone did not affect culture pH ([Figure 4F](#)). The skeleton of *FGO*<sub>5</sub>*@CMlrA* was not significantly damaged with part of HGO5 reduced after 9 days of co-culture with cyanobacteria, indicating that *FGO*<sub>5</sub>*@CMlrA* was stable during the process of incubation with *M. aeruginosa* ([Figure S20](#)).

Based on the above results, an inhibition mechanism of *FGO*<sub>5</sub>*@CMlrA* against *M. aeruginosa* was proposed ([Figure S5](#)). First, intracellular changes were apparent from *FGO*<sub>5</sub>*@CMlrA* treatment compared to control treatments (MlrA or HGO5 treatment alone). The former also inhibited the expression levels of *rbcL* which is critical for Calvin cycle functioning in addition to MC-LR synthesis genes (*mcyB*, *mcyG*, and *mcyD*) to a much greater extent than did the MlrAs (CMlrA and PMlrA) or HGO5 ([Figure S24C](#)) ([Liu et al., 2020](#)).

The concerted activities of *FGO*<sub>5</sub>*@CMlrA* treatment would, eventually, lead to more serious changes in LR<sub>ex</sub>, cellular nucleoid regions, content overflowing, plasmolysis, and even cell death of *M. aeruginosa* cells ([Figures 4A and 4E](#)) ([Liu et al., 2020](#)). *FGO*<sub>5</sub>*@CMlrA* can also inhibit Chl-a contents and F<sub>etr</sub><sub>max</sub>, resulting in reduced photosynthetic capacity and light energy use efficiency by *M. aeruginosa* ([Figures 4B and 4D](#)). However, *FGO*<sub>5</sub>*@CMlrA* had almost no effect on F<sub>v</sub>/F<sub>m</sub> and *psbD*, indicating that *FGO*<sub>5</sub>*@CMlrA* could not inhibit the light energy conversion efficiency of PSII reaction centers, similar to that observed with HGO5 treatment, but different from that observed in MlrA treatment ([Figures 4C and S24A](#)). The second major type of changes occurred extracellularly. *FGO*<sub>5</sub>*@CMlrA* degraded LR<sub>ex</sub> that was released outside of cells. Furthermore, *FGO*<sub>5</sub>*@CMlrA* maintained a weakly alkaline environment better than CMlrA, while HGO5 did not affect culture pH ([Figure 4F](#)). A highly alkaline environment helps promote HMBs, wherein water pH can be as high as 10.5. Thus, *FGO*<sub>5</sub>*@CMlrA* treatment can better help prevent the formation or maintenance of HMBs than CMlrA or PMlrA treatment alone ([Huang et al., 2020](#); [Huisman et al., 2018](#); [Liu et al., 2020](#)).

**Ecological safety of *FGO*<sub>5</sub>*@CMlrA* composites**

Interestingly, CMlrA and HGO5 treatment promoted the expression levels of MC-LR synthesis genes (*mcyB*, *mcyG*, and *mcyD*) while CMlrA and PMlrA treatments inhibited their expression levels ([Figure S24C](#)). Previous studies have shown that both CMlrA and PMlrA can degrade MC-LR and inhibit *M. aeruginosa* growth, although CMlrA contains unknown HP and carries unknown ecological risks, thereby limiting its application ([Liu et al., 2020](#)). In addition, GOs also exhibit certain toxic characteristics ([Wu et al., 2020](#); [Seabra et al., 2014](#)). Following the above results, an optimization strategy of *FGO*<sub>5</sub>*@CMlrA* treatment combining CMlrA with HGO5 was proposed ([Figure 6A](#)).

In this strategy, the adverse effects of CMlrA from HPs on ecological environments may be reduced by the removal of HPs while HGO5 toxicity may be reduced by beneficial or harmless products (BPs) blocking the free active sites of HGO5 during the synthesis of *FGO*<sub>5</sub>*@CMlrA*. To validate this proposed strategy, a control experiment using *FGO*<sub>5</sub>*@CMBP* was first established that was obtained by the immobilization of CMBP on HGO5 ([Figure 6B](#)) was first established. The *FGO*<sub>5</sub>*@CMBP* composite exhibited a 29.1% inhibition of *M. aeruginosa* after nine days of treatment. In contrast, the inhibition by CMBP was 61.6% and that of the directly mixed experimental group (HGO5 + CMBP) was 59.4%. These results confirmed that *FGO*<sub>5</sub>*@CMBP* reduced the toxicity of CMBP by possibly removing HPs in crude enzyme mixtures, while the toxicity of HGO5 was reduced by BPs and MBP occupying the active sites of HGO5 ([Figure 6A](#)). Similarly, the toxicity of CMlrA may be reduced by removing HPs during the preparation of *FGO*<sub>5</sub>*@CMlrA* while BPs and PMlrA may also reduce the toxicity of HGO5 by occupying the active sites of HGO5. Furthermore,
HGO5@CMlrA treatment slightly inhibited or even promoted the growth of MC-non-producing algal species such as *Synechocystis* PCC6803 and the eukaryotic alga *Chlorella vulgaris* 1227 (Figure S25). These results indicate that HGO5@CMlrA only targets the MC-producing algal species *M. aeruginosa* and exhibits higher application safety than HGO5 and CMlrA.

A laboratory microcosm system was also used to assess the impact of HGO5@CMlrA exposure on the prokaryotic and eukaryotic community structure and diversity in aquatic microcosms by 16 and 18S rRNA sequencing, respectively. Four alpha diversity metrics were evaluated, including the Shannon and Simpson indices that reflect community diversity and evenness, the Chao1 index that reflects species richness based on the estimated number of species, and the Pielou index that reflects species evenness (Figures 7A and S26A) (Zhang et al., 2020b).

The HGO5@CMlrA exposure led to increases in the four alpha diversity indices compared to the CMlrA treatment, in addition to increases in the Simpson and Pielou index values of the prokaryotic community compared with the HGO5 group. Thus, prokaryotic community diversity and evenness in the HGO5@CMlrA group were higher than in the CMlrA group and the HGO5 group (Figure 7A). Among the eukaryotic microbial communities, HGO5@CMlrA exposure led to increases in the four alpha diversity metrics, indicating that eukaryotic community diversity, evenness, and species richness were all higher in the HGO5@CMlrA treatment than in the other three treatments (Figure S26A). Thus, these results indicate that the diversity and evenness of prokaryotic and eukaryotic communities were significantly reduced by CMlrA treatment, but this negative impact could be alleviated after immobilization on FGO5.

Principal coordinates analysis (PCoA) based on Bray-Curtis distances revealed variation in community β-diversity (i.e., species compositional variation) among prokaryotic and eukaryotic communities along with the first principal component (PC1) and the second principal component (PC2) (Figures 7B, 7C, and S26B). The species composition of the HGO5@CMlrA treatment was significantly different from that in the other three treatments, indicating that the prokaryotic and eukaryotic community structures significantly shifted in the HGO5@CMlrA treatment relative to the others. Moreover, some beneficial prokaryotic genera exhibited their highest relative abundances (RAs) in the HGO5@CMlrA treatment compared to the other three treatments (Figure 7D). These taxa included *Acidovorax* which can degrade chlorobenzenes, diazotrophs (*Ideonella and Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*), *Dechloromonas*, and *Polynucleobacter* that can remove...
phosphorus, and Sphaerotilus that can remove heavy metals such as chromium and copper (Figure 7E(top)) (Monferran et al., 2005; Balázs et al., 2021; Petriglieri et al., 2021). In contrast, these beneficial genera exhibited low RAs in the other three treatment communities, indicating that the immobilization of CMlrA on HGO5 can lead to safer aquatic microcosm conditions (Figure 7E(top)). The RAs of some prokaryotes involved in the degradation of MCs such as Aeromonas and Sphingobacterium, significantly increased by 15-fold and 50-fold, respectively, in the HGO5@CMlrA and CMlrA treatments compared to the control and HGO5 treatments (Figure 7E(down)). Notably, Pseudanabaena PCC-7429 within the Cyanobacteria phyla completely disappeared after the HGO5@CMlrA and CMlrA treatments, but was still abundant in the HGO5 and control groups, and thus demonstrates functions of CMlrA involved the removal of MCs and the inhibition of toxic cyanobacterial growth (Figure 7E(down)). Furthermore, some major genera in the eukaryotic communities, including Ciliophora, Peritrichia, and Cyclidium, exhibited significantly increased RAs in the HGO5@CMlrA treatment, indicating that HGO5@CMlrA treatment led to the maintenance of a better trophic structure for eukaryotic organisms compared to the other treatments (Figure S26D). Therefore, the immobilization of CMlrA on HGO5 generally reduced the negative impacts of CMlrA on prokaryotic and eukaryotic communities. These results show that HGO5@CMlrA is a more ecologically safe treatment for aquatic community composition than the use of free CMlrA and HGO5. The effects of CGO3@CMlrA treatment on prokaryotic and eukaryotic communities also exhibited similar trends (Figures S27 and S28), indicating that CMlrA immobilized on GOs can achieve the high ecological safety effects of GOs and CMlrA.

To further evaluate the safety of GOs@CMlrA (HGO5@CMlrA and CGO3@CMlrA) composites toward humans, cytotoxicity and lactate dehydrogenase (LDH) leak rate experiments were conducted with three human cell types (Figures S29–S32). The products of MC-LR after GOs@CMlrA treatment did not exhibit toxicity toward human cells compared to the control group. In contrast, MC-LR exhibited the highest observed toxicity toward the three human cell types. MC-LR was especially toxic toward normal hepatocytes (LO2) relative to the two other types of cancer cells. GOs@CMlrA treatments were also safe to human cells, while HGO5 and CGO3 exhibited certain levels of toxicity toward cells compared to the control. These results are consistent with previous reports showing that MC-LR and GOs are harmful toward human cells (Wang et al., 2021; Seabra et al., 2014).

**Conclusion**

In this study, a unique HGO5 was synthesized and screened with a high loading capacity for proteins that can degrade MCs. A new assembly mechanism was used relative to previously produced other modified GO derivatives, enabling the generation of the HGO5 that can efficiently immobilize CMlrA and maintain the degradation activity of CMlrA against MCs, while simultaneously reducing the toxicity associated with CMlrA and HGO5 alone. The HGO5@CMlrA composite consequently simultaneously removed MCs and inhibited M. aeruginosa growth while exhibiting significantly better ecological and human safety compared to CMlrA or HGO5. The method described here is universal and has potential for widespread application because it is low-cost and incorporates the easily available graphite as the raw material. HGO5@CMlrA exhibited the best effects among the materials produced here. This proof-of-concept study may open new
avenues for GOs and MlrA, while comprehensively controlling HMBs and MCs pollution in water systems via the application of the proposed GOs@CMlrA.

Limitations of the study
This study is fundamental research focused on the improvement of the utilization and security of MlrAs in MCs and M. aeruginosa treatment on the laboratory research scale. Some application technologies need further be developed for the widespread application of HGO5@CMlrA in real water bodies in the future.

STAR METHODS
Detailed methods are provided in the online version of this paper and include the following:

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  - Data and code availability
- **METHOD DETAILS**
  - pMAL-c2X-mlrA-TB1 strains and M. aeruginosa FACHB 905
  - Preparation of HGO nanomaterials
  - Preparation of GO@MlrA composites
  - Characterization of GOs and GO@MlrA composites

Figure 7. Ecological safety of HGO5@CMlrA treatment evaluated with effects on bacterial communities
(A) Alpha diversity index values for bacterial communities.
(B) Principal coordinates analysis (PCoA) ordinations of variation in bacterial community composition across treatments.
(C) Bray-Curtis dissimilarities among treatment communities. Letters indicate statistically significant differences between different groups. Different letters in (C) represent significant differences (p < 0.05) between different treatments.
(D) Major bacterial genera of communities that exhibited relative abundances (RAs) > 1%.
(E) Major genera in the HGO5@CMlrA treatment group that exhibited statistically significant differences relative to other groups (up), in addition to genera in both the HGO5@CMlrA and CMlrA groups that exhibited significantly different abundances among the other two groups (down). Asterisks (*, **, and ***)) indicate statistically significant differences compared to controls (p < 0.05, p < 0.01, and p < 0.001, respectively). Data are represented as mean ± SEM.
SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.104611.

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AUTHOR CONTRIBUTIONS

L.F., H.L, C.C., and H.Q. designed the experiment. L.F. and H.L. wrote the article. H.L., C.C, L.Z, M.Y., Y.H., S.H., M.K., X.G., and Y.F. conducted the experiments. All the authors discussed the article.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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**STAR METHODS**

**KEY RESOURCES TABLE**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Bacterial and virus strains** | | |
| pMAL-c2X vector | New England Biolabs Inc. | Cat#E8000S |
| Microcystis aeruginosa FACHB 905 | Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, China) | FACHB-905 |
| Synechocystis sp. PCC6803 | Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, China) | FACHB-898 |
| Chlorella vulgaris FACHB 1227 | Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, China) | FACHB-1227 |
| pMAL-c2X-miR-TB1 strains | This paper | N/A |
| **Chemicals, peptides, and recombinant proteins** | | |
| Graphite | Maclin Biochemical Technology Co., Ltd. (Shanghai, China) | 7782-42-5 |
| H₂SO₄ | Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China) | 7664-93-9 |
| NaNO₃ | Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China) | 7631-99-4 |
| H₂O₂ | Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China) | 7722-84-1 |
| KMnO₄ | Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China) | 7722-64-7 |
| CGOs | Suzhou Crystal Silicon Electronic Technology Co., Ltd. (Suzhou, China) | 1034343-98-0 |
| ZGO₀ | Professor Heping Zeng of Sun Yat-Sen University | Huang et al. (2019) |
| FITC | Maclin Biochemical Technology Co., Ltd. (Shanghai, China) | 3326-32-7 |
| EDC | Aladdin industrial corporation (Shanghai, China) | 25952-53-8 |
| NHS | Aladdin Biochemical Technology Co., Ltd. (Shanghai, China) | 106627-54-7 |
| MES | Aladdin Biochemical Technology Co., Ltd. (Shanghai, China) | 4432-31-9 |
| MC-LR | Express Technology Co. Ltd (Beijing, China) | 101043-37-2 |
| MC-RR | Express Technology Co. Ltd (Beijing, China) | 111755-37-4 |
| MC-YR | Enzo Life Sciences, Inc. (Beijing, China) | 101064-48-6 |
| **Critical commercial assays** | | |
| CCK-8 Cell Proliferation and Cytotoxicity Assay Kit | Solarbio Co, Ltd | CA1210 |
| LDH activity detection kit | Solarbio Co. Ltd | BC0680 |
| RNeasy Plus Mini Kit | Qiagen, China | Qiagen 74136 |
| Transcriptor First Strand cDNA Synthesis Kit | Roche | 04896866001 |
| LightCycler 480 SYBR Green1 Master | Roche | 04887352001 |

(Continued on next page)
### RESOURCE AVAILABILITY

#### Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Lingling Feng (fll708@mail.ccnu.edu.cn).

#### REAGENT or RESOURCE SOURCE IDENTIFIER

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Experimental models. Cell lines** | | |
| LO2 (normal liver cell line) | ATCC | BJ-0432 |
| Huh7(liver cancer cell line) | ATCC | BJ-0271 |
| ACHN (renal cancer cell line) | ATCC | BJ-0172 |
| **Oligonucleotides** | | |
| Primer for 16s (338F) 5’-ACTCC TACGGGAGGCAGCAG-3’ | Zhang et al., 2020b | N/A |
| Primer for 16s (806R) 5’-GGACT ACVGGGTWTCTA AT-3’ | Zhang et al., 2020b | N/A |
| Primer for 18s (528F) 5’-GCGG TAATTCCAGCTCCAA-3’ | Zhang et al., 2019a, 2019b | N/A |
| Primer for 18s (706R) 5’-AATC CRGAATTTCCACCTCT-3’ | Zhang et al., 2019a, 2019b | N/A |
| Primer for psaB-F CGGTGTA CTGGGGTGTGTATG | Peng et al., 2017 | N/A |
| Primer for psaB-R ACTCGGTTTGGGATGGA | Peng et al., 2017 | N/A |
| Primer for psdD-F TCTTCGGCATCGCTTTTC | Peng et al., 2017 | N/A |
| Primer for psdD-R TCTTCGGCATCGCTTTTC | Peng et al., 2017 | N/A |
| Primer for rbcL-F CGTTTCCCGTGCCTT | Peng et al., 2017 | N/A |
| Primer for rbcL-R CCGAGTT TGGGTTGTAGGTT | Peng et al., 2017 | N/A |
| Primer for mcyB-F CCTACCAGCGCCTTGGA | Peng et al., 2017 | N/A |
| Primer for mcyB-R GAAAAATCC CCAAGATTCTCGAGT | Peng et al., 2017 | N/A |
| Primer for mcyD-F TTAGCTAC ATAACCACCATCCTTC | Gao et al., 2013 | N/A |
| Primer for mcyD-R CTCCCTG GCAGTTTGCAGAT | Gao et al., 2013 | N/A |
| Primer for mcyG AACGACA CGCCGCTCATTAC | Liu et al., 2020 | N/A |
| Primer for mcyG AACATCTG CCGAAGTGACTGC | Liu et al., 2020 | N/A |
| **mlrA gene encoding MlrA from Sphingopyxis sp. C1** | Gene Bank | AB468058.1 |
| **Recombinant DNA** | | |
| Plasmid: pMAM-c2X-mlrA vector | This paper | N/A |
| **Software and algorithms** | | |
| QIIME2 software | Rideout et al., 2019 | https://qiime2.org/ |
| Multiwfn (A Multifunctional Wavefunction Analyzer) | Lu et al., 2012 | http://multiwfn.codeplex.com |
| Visual Molecular Dynamics (VMD) software program | Humphrey et al. (1996) | http://www.ks.uiuc.edu/Research/vmd |
Materials availability
Any materials generated in this study are being made available. The study did not generate new unique reagents or there are restrictions to availability.

Data and code availability
The data are available upon request by contacting lead contact, Lingling Feng (fl708@mail.ccnu.edu.cn). No new code was generated during the course of this study.

METHOD DETAILS

pMAL-c2X-mlrA-TB1 strains and M. aeruginosa FACHB 905
The mlrA gene encoding MlrA from Sphingopyxis sp. C1 (Gene Bank: AB468058.1) was inserted into the pMAL-c2X vector with a maltose-binding protein (MBP) tag to construct the pMAL-c2X-mlrA vector, and the sequenced vector was transformed into E. coli K12 TB1 to obtain the recombinant pMAL-c2X-mlrA-TB1 strains. Briefly, the pMAL-c2X-mlrA-TB1 strains were cultured and then induced with 0.1 mM isopropyl-β-D-thiogalactoside (IPTG). By the way, CMlrA was the cell-free extracts.

M. aeruginosa FACHB 905 purchased from institute of Hydrobiology of the Chinese Academy of Sciences, cultivated in an artificial climate box to simulate natural conditions.

Preparation of HGO nanomaterials
Nine types of graphene oxide (HGO1-HGO9) were synthesized using readily available and low-cost graphite powder (HGO0) as the raw material based on Hummers’ method (Hummers and Offeman, 1958; Marcano et al., 2010). The experimental design is shown in Figures 1A and Table S3. The HGOs exhibited different oxidation degrees and oxygen-containing functional group types (Table S2).

Synthesis of HGO1-HGO5 by Method 1 (M1) based on Hummers’ method (Hummers and Offeman, 1958). Concentrated H2SO4 was first added to a mixture of graphite powder (HGO0) and NaN3 in M1 (Table S3). Different amounts of oxidant KMnO4 were then slowly added in the ice water bath. The reaction mixture was then heated to 98 °C for 15 min, and then maintained at room temperature for 10 min. Then, 30% H2O2 was added, followed by stirring for 1 h. The precipitate was collected by centrifugation and washed once using water and 5% HCl, followed by washing with water until achieving a neutral pH and then drying for later use.

Synthesis of HGO6-HGO9 by Method 2 (M2) based on an improved Hummers’ method (Marcano et al., 2010). For synthesis using M2, a mixture of H2SO4 and H3PO4 was added to a mixture of HGO0 with different dosages of KMnO4 (Table S3). The reaction mixtures were then heated to 50°C, stirred for 12 h, and then cooled to room temperature for 10 min. Then, 30% H2O2 was added, followed by stirring for 2 h. The precipitates were then collected and washed as described above for M1.

Preparation of GO@MlrA composites
MlrAs (CMlrA and PMlrA) and CMBP were prepared as previously described (Liu et al., 2020). Briefly, crude maltose binding protein (CMBP) was used as a control and was prepared from recombinant cells without mlrA. GOs@MlrA preparation was achieved using physical adsorption and covalent binding methods (Zhuang et al., 2020; Monajati et al., 2018).

For the physical adsorption method, 1 mg of GO (HGO5 and other GOs) was dissolved into 1 mL of deionized water and ultra-sonicated for 30 min at room temperature. Then, the precipitate was collected by centrifugation, washed with phosphate buffer solution (PBS, 10 mmol L⁻¹, pH 7.4) three times, and resuspended in PBS to achieve a GO solution with a concentration of 0.625 mg mL⁻¹. MlrAs (5 mg mL⁻¹, 0.4 mL) were then added to the GO solution, followed by gentle shaking for 30 min at room temperature to initiate loading. The precipitate was subsequently collected by centrifugation, washed twice and resuspended with PBS for subsequent characterization and activity determination. The prepared composite was termed GOs@MlrA. The loading amount was calculated as follows:

$$\text{Protein loading amount (mg g}^{-1}\text{)} = \frac{C_0 V_0 - (C_1 V_1 + C_2 V_2)}{m_0}$$
Where \( C_0 \) and \( V_0 \) indicate the initial concentrations and volumes of MlrAs, respectively. \( m_0 \) indicates the initial mass of GO used for enzyme loading. \( V_1 \) and \( V_2 \) indicate the volumes of centrifugated supernatants after loading and the volumes after washing with PBS, respectively. \( C_1 \) and \( C_2 \) indicate the protein concentrations in centrifugated supernatants after loading and after washing with PBS.

The covalent binding method comprised the dissolution of 1 mg GO into 1.6 mL of deionized water, followed by ultrasonication for 30 min at room temperature. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC dissolved in 0.5 mol L\(^{-1}\) MES) and N-hydroxy-sulfosuccinimide sodium salt (NHS, dissolved in 0.5 mol L\(^{-1}\) MES) were added to GO solutions to initiate activation of GO carboxyl groups for 30 min at room temperature. The loading process of MlrA on activated GO was the same as described above for the physical adsorption method.

**Characterization of GOs and GO@MlrA composites**

Several methods were used to characterize the GOs and GO@CMlrA including scanning electron microscopy (SEM), atomic force microscopy (AFM), X-ray diffraction (XRD), X-ray photoelectron spectroscopy (XPS), Fourier transform infrared spectroscopy (FTIR), and Raman spectroscopy (RS).

The SEM instrument (JSM-7900F, JEOL, Japan) was equipped with an energy dispersive spectrometer (EDS, FlatQuad 5060F, Bruker, Germany) and TEM (HT-7700, Hitachi, Japan) to facilitate morphological characterization.

AFM images were obtained using a cypher ES atomic force field microscope (Oxford, England). The samples used for AFM characterization were deposited on a mica substrate. Specifically, HGO\(_0\) and HGO\(_5\) aqueous solutions were dispersed on mica substrates, then air-dried for 30 min prior to AFM characterization. AFM characterization of HGO\(_0@CMlrA\) and HGO\(_5@CMlrA\) was conducted in the aqueous solution.

XRD was performed to evaluate the crystal structures of the materials using a Rigaku D8/ADVANCE X-ray diffractometer (DB/ADVANCE, Bruker, Germany) with nickel-filtered Cu K\(\alpha\) radiation (\(\lambda = 0.15418\) nm). The scan speed was 0.2 s per step with an angle range of 5–70°. The Bragg equation (\(d = n\lambda/2\sin\theta\)) was used to calculate the interlayer space (\(d\)) between nanosheets using an \(n = 1\), an X-ray wavelength (\(\lambda = 0.15418\) nm), and the angle of diffraction as \(\theta\). The average interlayer space was calculated according to the following equation:

\[
\text{Average interlayer space} = \frac{d_1 \times P_1 + d_2 \times P_2}{P_1 + P_2}
\]

Where \(d_1\) indicates the interlayer space that is calculated based on the 2\(\theta\) of the GO crystal plane (001, about 10.7°) and \(d_2\) indicates the interlayer space calculated based on the 2\(\theta\) of the graphite crystal plane (002, about 26°). \(P_1\) and \(P_2\) indicate the peak areas of the characteristic diffraction peaks of the 001 and 002 crystal planes, respectively.

XPS was conducted to analyze the elemental and functional group composition of the materials using a high-resolution X-ray photoelectron spectrometer (EscaLab 250Xi, ThermoFisher, USA).

RS was conducted to analyze the degree of material stacking order using a Raman microscopy system (LabRAM HR Evolution, HORIBA, France) with 532 nm laser excitation.

FTIR spectra were obtained on a Perkin-Elmer spectrometer Nicolet FT-IR 5700 instrument (Thermo, USA) using potassium bromide (KBr) as the background. CMlrA labeled with FITC was prepared to determine the distribution of CMlrA on the surface of HGO\(_0\) using a Leica SP8 fluorescence microscope (Leica Microsystems trading co., Ltd., Shanghai, China). The excitation wavelength was set as 493 nm and the emission wavelength was set as 519 nm.

Material zeta potentials (Malvern Zetasizer Nano ZS90) were measured to evaluate their surface charges. Potentials were measured in samples at pH 7.4 using the same concentration 1 mg mL\(^{-1}\) within water. In addition, the pH-dependence of zeta potentials were measured at pH 5.0, 6.4, 7.4, and 9.3.

All spectra were recorded under the same experimental conditions and all measurements were made in triplicate.
MCs degradation activities of CMlrA and HGO₅@CMlrA

The MCs degradation activities of CMlrA and HGO₅@CMlrA were evaluated over time as we have previously described (Liu et al., 2020). Briefly, reaction mixtures that contained substrates (MCs) and enzymes (CMlrA or HGO₅@CMlrA) were prepared in 50 mmol L⁻¹ PBS, pH 7.0, and incubated at 25°C for 5 min. Then, 20% phosphoric-acid aqueous solution was added to terminate reactions. After centrifugation, residual MCs in the supernatants were detected using HPLC. One unit (U) of MlrA activity was defined as the quantity of enzyme that degrades 1.0 nmol of MC-LR per minute under the above conditions. The stability of the activities including thermal stability, pH tolerance, and storage stability were also evaluated as previously described. The activity recovery rates of enzymes were calculated by: \(\text{Inhibition rate} (%) = \left(\frac{C_t}{C_b}\right) \times 100\%\), where \(C_b\) indicates the Chl-a content of the blank group and \(C_t\) is the Chl-a content of treatment group.

Density functional theory (DFT) simulation calculations

HGO₀ and HGO₅ models were constructed based on XPS analysis. For the HGO₀ model, sp² carbon atoms were contiguous with a hydroxyl group. For the HGO₅ model, part of the sp² carbon atoms were replaced by sp³ carbon atoms, while hydroxyl and epoxides were randomly distributed inside the HGO₅ plane in addition to carbonyl and carboxyl groups that were distributed on the edge of the plane. Several lysines were predicted on the surface of MlrA in the predicted structure of MlrA by the Tencent AI Lab. Consequently, lysine was chosen to construct the models of HGO₀@CMlrA and HGO₅@CMlrA. Density functional theory (DFT) was then used to optimize the conformations of the models. The electrostatic surface potentials were analyzed with Multisfn and drawn with the Visual Molecular Dynamics (VMD) software program to visualize the simulation configurations (Lu et al., 2012; Humphrey et al., 1996).

Effects of GOs@CMlrA on cyanobacterial growth

Cyanobacterial cultures (M. aeruginosa FACHB 905, Synechocystis PCC6803, and Chlorella vulgaris FACHB 1227) were grown to the same cell densities of 3.75 × 10⁶ cells mL⁻¹ that corresponded to Chl-a contents of 0.5 μg mL⁻¹ (Liu et al., 2020). These values correspond to the abundances of Cyanobacteria recorded during HMBs in Taihu Lake. Two milliliters of BG11 or other treatments, in addition to 100 mL of cyanobacterial cultures were placed in sterile 250 mL flasks. The treatment cultures were then investigated based on cellular morphologies, extracellular MC-LR (LRex) contents, intracellular MC-LR (LRin) contents, the mRNA expression levels of genes, the maximum fluorescence quantum yields (Fv/Fm) of cells, the maximum relative electron transfer rates (rETR_max) of cells, Chl-a contents of cells, and media pH. The CMlrA + GOs treatment tested the effects of CMlrA plus GOs (HGO₅ or CGO₃) without immobilization treatment, while the GOs@CMlrA treatment tested the effects of GOs@CMlrA (HGO₅@CMlrA or CGO₃@CMlrA) composites obtained by immobilization treatment.

Chl-a content within cultures and culture media pH were determined at time points of 0, 3, 6, and 9 days using previously described methods (Liu et al., 2020). The inhibition rate (%) was calculated according to the following equation:

Inhibition rate(%) = \left(\frac{C_t - C_b}{C_t}\right) \times 100\%

where \(C_b\) is the Chl-a content of the blank group and \(C_t\) is the Chl-a content of treatment group.

The maximum fluorescence quantum yield (Fv/Fm) and the maximum relative electron transfer rate (rETR_max) were determined at 24, 48 and 72 h using a fast chlorophyll fluorescence measurement on a pulse-amplitude modulation fluorometer (XE-PAM, Walz, Efeltrich, Germany).

Real-time fluorescent quantitative PCR (RT-qPCR) was used to assay the relative mRNA expression levels of genes (psaB, psbD, and rbcL) involved in photosynthetic pathways, in addition to genes (mcyB, mcyD, and mcyG) involved MC synthesis pathways at 24 and 48 h, using a LightCycler 96 PCR instrument (Roche, Switzerland). RT-qPCR methods were the same as previously described (Liu et al., 2020).

SEM, transmission electron microscopy (TEM), and fluorescence confocal scanning microscopy (FCSM) were used to observe cell morphologies after nine days of treatment, as described previously (Liu et al.,...
Samples treated for nine days were used to determine extracellular MC-LR (LRex) and intracellular MC-LR (LRin) contents, as described previously (Liu et al., 2020).

Effects of GOs@CMlrA on ecological microcosms

The effects of GOs@CMlrA on aquatic community compositions were investigated as previously described (Zhang et al., 2020b). Briefly, freshwater samples were collected from a pond (30°17’59”N; 120°10’32”E) at the Zhejiang University of Technology in Hangzhou and then cultured for two days until stable under laboratory conditions before treatment. Then, 150 mL of cultured water samples were dispensed into 250 mL sterilized flasks for different treatment groups. All groups were initialized at the same time and were grown under cool-white fluorescent light (46 μmol m⁻² s⁻¹) with a 12:12 light:dark photoperiod cycle and at 25 ± 0.5°C. Each group comprised four parallel replicates.

To investigate changes in aquatic community composition, 50 mL water samples from different groups were filtered through 0.45 μm pore diameter membranes to collect microbial cells after seven days of exposure. The nucleic acids of aquatic microorganisms were extracted using the cetyltrimethylammonium bromide (CTAB) method. The V3-V4 hypervariable regions of bacterial 16S rRNA genes were then amplified using polymerase chain reaction (PCR) and the primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5’-GGACTACHVGGGTWTCTAAT-3'). In addition, the V4 hypervariable regions of eukaryotic 18S rRNA genes were PCR amplified using the primers 528F (5'-GCGGTAATTCCAGCTCCAAG-3') and 706R (5’-AATCCCRAGATTGTCCCTTCCTCT-3'). The purified amplicons were then paired-end sequenced on an Illumina NovaSeq platform.

Raw sequencing data was quality-filtered to obtain clean data. Specifically, the QIIME2 software program was used to obtain clean data by denoising with Deblur to obtain amplicon sequence variants (ASVs). Taxonomy was assigned to ASVs using the q2-feature-classifier and comparison against the Silva 138 database. The Shannon and Richness diversity indices were calculated using the R (version 4.0.3) vegan and picante packages. The Simpson index reflects the probability that the number of individuals obtained from two consecutive samples of a community belong to different species (Zhang et al., 2020b). PCoA plots were generated based on among-community Gower distances using the R package ggplot 2. Permutational multivariate analysis of variance (PERMANOVA; via ANOSIM tests and based on Gower distances, permutation = 999) tests were used to determine if community compositions differed between treatment and control groups.

GOs@CMlrA effects on human cells

Three human cell lines including normal liver (LO2), liver cancer (Huh7), and renal cancer (ACHN) cell lines were used to evaluate cytotoxicity using a CCK-8 Cell Proliferation and Cytotoxicity Assay Kit (CA1210, Solarbio Co, Ltd). Each cell line was treated with 10 μg mL⁻¹ MC-LR, and sufficient GOs@CMlrA to completely degrade 10 μg mL⁻¹ MC-LR, in addition to an equivalent amount of CMlrA as GOs@CMlrA, an equivalent amount of GOs as GOs@CMlrA, or PMC-LR (a mixture including GOs@CMlrA and MC-LR). A1% methanol aqueous solution was used as the blank control since MC-LR was dissolved in a solution containing a 1% maximum concentration of methanol. In addition, the lactate dehydrogenase (LDH) leakage rate was evaluated using an LDH activity detection kit (BC0680, Solarbio Co. Ltd) after 48 h of treatment, as previously described (Liu et al., 2020).

Quantification and Statistical Analysis

All experiments were performed in at least triplicate and the results shown are the means and standard deviations for three replicates. t-tests were used for statistical analysis of differences, and a p < 0.05 was considered statistically significant. Use origin software and excel software to make diagrams, and use photoshop CC software to combine graphics.

Additional Resources

There are no additional resources need to be declared in this manuscript, additional requests for this can be made by contacting the lead contact.