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Mitigating antibiotic pollution using cyanobacteria: removal efficiency, pathways and metabolism

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ABSTRACT

The occurrence of pharmaceuticals and personal care products (PPCPs) in wastewater poses huge environmental threats, even at trace concentrations, and novel approaches are urged due to the inefficiencies of conventional wastewater treatment plants, especially when processing contaminants at high concentrations. Meanwhile, another widespread problem in the aquatic domain is the occurrence of harmful algal blooms (HABs) which cause serious damage to the ecosystem, but have rarely been investigated for possible valorization. This study investigated the possibilities, mechanisms, and effects of toxin

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release of using a harmful cyanobacterial species, *Microcystis aeruginosa* (*M. aeruginosa*), in order to remove the widely used drug, tetracycline, at high concentration. The results were compared with the performance obtained by the use of the hitherto generally-selected chlorophyte alga *Chlorella pyrenoidosa* (*C. pyrenoidosa*) for tetracycline concentrations of 10-100 mg L^{-1}. *M. aeruginosa* exhibited a much more effective and rapid tetracycline removal (over 98.0% removal in 2 days) than did *C. pyrenoidosa* (36.7%-93.9% in 2 days). A comprehensive kinetic investigation into probable removal pathways indicated that, theoretically, bio-remediation dominated the process by *M. aeruginosa* (71.6%), while only accounting for 20.5% by *C. pyrenoidosa*. Both microalgae promoted the hydrolysis of tetracycline under conditions of increased pH and inhibited abiotic photolytic reactions by the shading effect to the water column, when compared with control experiments. Although identical degradation by-products were identified from treatments by both microalgal species, distinct by-products were also confirmed, unique to each treatment. Moreover, the growth of *M. aeruginosa* biomass exhibited strong tolerance to tetracycline exposure and released significantly lower levels of microcystin-LR, compared with the control systems. This study supports the possibility of reusing HABs species for the effective remediation of antibiotics at high concentrations. We have further suggested possible mechanisms for remediation and demonstrated control of toxin release.

Keywords: Harmful Algal Blooms (HABs), Microalgae, Micropollutants, Microcystin control, PPCPs.

1. Introduction

Pharmaceuticals and personal care products (PPCPs) have been extensively detected in aquatic systems, and pose serious hazards to human health and ecology even at trace levels (Suárez et al., 2008). The global consumption of antibiotics alone has been estimated at 100-200k tons (Qian et al., 2012), however, 25%-75% of the consumed antibiotics are excreted from living systems and contaminate natural waters (Sarmah et al., 2006). Such antibiotics are barely removed in conventional wastewater treatment plants (WWTPs) due to their antibacterial properties (Wang et al., 2013). Tetracycline (Fig. S1.) is the second most widely-used antibiotic, often employed for the treatment of many different bacterial infections as diverse as severe acne, food poisoning, and sexually transmitted diseases. The presence of residual tetracycline at trace levels in the environment as a whole might lead to further development of antibiotic-resistant bacteria, which, in turn, would be a major health problem. Worse still, tetracycline exposure at high concentrations generates the equally serious environmental problem of acute toxicity to the aquatic and edaphic organisms (Daghrir and Drogui, 2013). To date, more attention has been paid to the investigation of tetracycline removal at low concentrations, e.g. μg/L (Kim et al., 2005), however, tetracycline could be present at levels up to 200 mg L^{-1} in pharmaceutical wastewater (Song et al., 2019). The acute toxicity effect from such high concentrations of tetracycline raises significant challenges to conventional wastewater treatment methods. Previous studies have indicated that WWTPs can only achieve up to 30% removal of this drug (Watkinson et al., 2009), which may be attributed to the toxic effects of tetracycline on the bacterial population present in aerobic sludge (Halling-Sørensen, 2001), especially with exposure at high concentration. Thus, development of technology for the effective and economic mitigation of tetracycline at high concentrations is urgently required.

Various approaches, such as flocculation (Fu et al., 2015), advanced oxidation (Hou et al., 2016), and adsorption (Gao et al., 2012), have been proven to effectively remove tetracycline from wastewater at high concentrations. However, such methods require increasing dosage of additional chemicals with rising concentration of tetracycline. Consequently, the large scale application of such techniques is still hindered by their relatively high costs and the potential problem of secondary pollutants (Tang et al., 2016). Microalgal technology has been recognised as an eco-friendly, and cost-effective method for water treatment, including removal of emerging contaminants (Matamoros et al., 2016). A further advantage of this technique is the potential reuse of the algal biomass (Pan et al., 2018). Therefore, microalgal species with high economic value, such as *Chlorella pyrenoidosa* (*C. pyrenoidosa*; Sun et al., 2011), have hitherto been mostly selected for wastewater treatment. Cyanobacteria, although the most well-known species responsible for harmful algal blooms (HABs) in eutrophic waters (Zhang et al., 2018), is often neglected because of the production of toxic microcystins as by-products of metabolism (Hitzfeld et al., 2000). From the perspective of wastewater treatment, its characteristics of rapid growth, high nutrient uptake capability, and low requirement for a favourable environment (e.g. temperature and pH), could make cyanobacteria an ideal microalgae species, if the release of unwanted toxins could be controlled during the process (Rzymski et al., 2014).

Microalgal technology has been demonstrated to effectively remove tetracycline under low initial concentration levels (<2 mg L^{-1}) (Norvill et al., 2017; de Godos et al., 2012). However, studies on microalgal technology for the treatment of wastewater containing high concentrations of tetracycline, have been rarely investigated. Furthermore, some microalgal species exhibit a Gram-negative-bacteria-like prokaryotic structure, in which cases tetracycline could generate biphasic hormetic effects, *i.e.*

inhibition at high doses and stimulation at low doses (Wan et al., 2015). Thus, a high initial concentration of the drug may lead to a dramatically low removal performance, an effect which has rarely been thoroughly investigated. Therefore, research on the removal, by microalgae, of high concentrations of tetracycline from PPCPs-polluted wastewater is needed and is still challenging.

Considering the application of different microalgal species, full-scale microalgae treatment systems, e.g., tubular photo-bioreactor and raceway ponds, have been successfully applied for the treatment of various wastewaters, such as municipal wastewater (Rawat et al., 2011) and livestock effluent (Wang et al., 2016). Nevertheless, the major concern of using cyanobacteria to replace other commonly-used microalgae species, e.g., *C. pyrenoidosa*, is the potential risk of toxin release during algal death and cell lysis (Paerl and Otten, 2013). A previous study has demonstrated the significant decrease of microcystin production and release from cyanobacteria on exposure to tetracycline up to 10 mg L^{-1} (Ye et al., 2017). This effect may have been due to the blocking of peptide synthesis, controlling the production of microcystin-LR (Ye et al., 2017) and a corresponding enhancement in the production of reactive oxygen species (ROS) (Yang et al., 2013) due to the presence of tetracycline. However, release of toxins from cyanobacteria under higher tetracycline concentrations (>10 mg L^{-1}) has rarely been investigated. Release of toxins from cyanobacteria mainly occurs during algal death and cell lysis. Thus, the monitoring of algal cell vitality and the measurement of toxin production during the process is essential in order to evaluate the safety envelope of the treatment technology.

The mechanisms of antibiotics removal, including pathways and metabolism, by different microalgal species, always vary. It is generally agreed that comprehensive pathways, i.e. photolysis, hydrolysis, cation-binding, adsorption, bioaccumulation and

biodegradation, may simultaneously contribute to the removal of antibiotics from the environment (Xiong et al., 2017a). It has been found that photodegradation mainly contributed to tetracycline removal (Norvill et al., 2017), however, de Godos *et al*. (2012) reported that biosorption and photodegradation are both predominant pathways for tetracycline removal during treatment by different microalgae. Thus, quantitative investigation of removal pathways is vitally important for the understanding of cyanobacteria-based treatment of tetracycline. Moreover, Halling-Sørensen *et al*. (2002) have highlighted that anhydrotetracycline (ATC) and 4-epianhydrotetracycline (EATC) could be major by-products of metabolism, and which may be more toxic than the parent compound, during remediation in aqueous conditions (Halling-Sørensen et al., 2002). Thus, identification of the by-products of degradation is also important in order to systematically understand remediation mechanisms (Halling-Sørensen et al., 2002) and potential environmental impacts.

In this study, we investigated the potential use of harmful cyanobacteria to effectively and safely remediate wastewater containing high concentration of tetracycline, and elucidated the probable mechanisms of removal. The removal efficiencies of tetracycline by the cyanobacteria, *Microcystis aeruginosa* (*M. aeruginosa*), were compared with the treatment by representative wastewater treatment microalgal species *C. pyrenoidosa* at relatively high initial concentrations (10-100 mg L^{-1}) of the drug. Different pathways and degradation by-products of tetracycline aided the elucidation of likely removal mechanisms. Algal biomass growth and cell vitality were characterised to estimate possibilities for biomass reuse. Moreover, levels of toxic microcystin-LR, released from *M. aeruginosa*, were monitored, in order to assess water reuse/discharge safety.

2. Materials and methods

2.1 Materials

Two species of microalgae strains, *M. aeruginosa* and *C. pyrenoidosa*, were purchased from the freshwater algae culture collection at the Institute of Hydrobiology (FACHB), Wuhan, China, and cultivated in BG11 media (Table S1) prior use. Tetracycline was obtained from J&K Scientific Ltd., Beijing, China. Microcystin-LR standard (SB05-287-2012) was purchased from the National Standard Material Center of China (Beijing, China). All reagents used were of analytical grade and ultrapure water (18 MΩ·cm) was used in all experiments.

2.2 Experimental operation

The removal performance of tetracycline by the two microalgal species was evaluated under different initial tetracycline concentrations. An appropriate volume of a tetracycline solution in ultra-pure water was added into each BG11 culture medium, resulting in three different concentrations of 10, 50, and 100 mg L^{-1}. Three control groups were set up to elucidate the removal of tetracycline by natural degradation (photolysis and hydrolysis) and binding to divalent cations (calcium, magnesium, cobalt) present in the BG11 culture media. The groups comprised 1) BG11 medium with tetracycline, but without microalgae under irradiation, was set as a *light control*, 2) BG11 medium with tetracycline, but without microalgae and irradiation, was set as a *dark control*, and 3) pure water with tetracycline, but without microalgae and irradiation, was set as a *chemical control*. Media with microalgae addition are hitherto referred to as *treatment groups*.

The treatments were conducted in Erlenmeyer flasks (1L) with 500 mL working volume. Different microalgae species have distinct cell sizes and growth rates, therefore, previous studies usually conducted wastewater treatment experiments under similar initial algal biomass concentrations in order to allow the results to be directly comparable (Arbib et al., 2014). To better compare the tetracycline removal performance, the two

species were separately pre-cultured until the late exponential phase. Afterwards, both microalgal species were inoculated into corresponding flasks to achieve an identical optical density (OD680) of 0.103 ± 0.016, which represented the same biomass concentrations (7.12 x 10^6 cell mL^{-1} *M. aeruginosa* and 7.50 x 10^5 cell mL^{-1} *C. pyrenoidosa*). All flasks were placed in an incubator maintained at 28±1 ^oC and illuminated by white light (full-spectrum white LED; Ledvance GmbH, Garching, Germany; intensity 1600 lux; light:dark ratio of 12:12h). All flasks were swirled manually three times (5 mins each) each day. The experimental setup with the initial tetracycline concentration of 50 mg L^{-1} was selected for comprehensive study into the possible removal pathways. Each group was set up in triplicate and operated for 13 days. In total, 36 treatments were conducted for the investigation.

2.3 Sampling and analysis

2.3.1 Sampling

The pH of each treatment suspension was measured daily directly in the flask using a PHB-600R pH meter (Omega Engineering, Norwalk, USA). In addition, samples (0.2 mL) were taken daily for algal cell counting, while another sample (2 mL) was taken from each flask at 0, 2, 4, 6, 16 and 24 hours, and then daily until day 13, for determination of tetracycline concentration, by ultrahigh-performance liquid chromatography (Waters Acquity UPLC, Waters Corp., Milford, USA) as described later. Samples for investigation into possible tetracycline removal pathways, microcystin-LR production, and detection of degradation by-products, were also taken on the corresponding days.

2.3.2 Tetracycline analysis and removal pathways

Thus, tetracycline removal kinetics by natural hydrolysis, photolysis, and cation-binding were determined by sampling at hour 0, 2, 4, 10, 16, and daily from day 1 to day 11. The chemical control group could be categorised as the abiotic hydrolysis of

tetracycline. The difference between tetracycline removal in the dark and in the chemical control groups was considered to represent tetracycline removal caused by cation-binding. Meanwhile, differences between tetracycline removal in light and dark control groups enabled estimation of abiotic photolysis process. The sampling methods for tetracycline distribution (in water, via bio-adsorption, and via bio-accumulation) are described in Text S1.1. The analysis parameters for UV detection of tetracycline by Acquity UPLC-PDA (Diode Array Detector) are detailed in S1.1.

2.3.3 Detection of degradation by-products

Filtered water samples (supernatant phase, 0.22 μm membrane filter), from treatments spiked with an initial tetracycline concentration of 50 mg L^{-1}, were used to identify tetracycline degradation by-products by analysis with an Acquity UPLC coupled with a Waters Xevo G2 ToF-MS detector (Waters Corp., Milford, USA). Only samples collected at 24 and 312 hours were processed. The working parameters for the Acquity UPLC are described in the supporting information (Text S1. 2) and the working parameters of the ToF-MS were: mass range 50-1200 Da, scan time 1.5 sec, collision energy 6V, collision energy ramp 15 to 30 V, cone voltage 40 V, positive polarity.

2.3.4 Microcystin-LR measurement

The most toxic microcystin compound, microcystin-LR, was detected by UPLC-UV in both intracellular and extracellular fractions in the samples collected at 10, 60 and 240 hours from the treatment groups with an initial tetracycline concentration of 50 mg L^{-1}. Specifically, 50 mL sample was initially centrifuged (4800 rpm for 15 min). The supernatant thus produced was then used for the quantification of released microcystin-LR after pre-concentration by SPE (Oasis R MCX 6cc, 500mg, Waters Corp., Milford, USA). The SPE was initially activated with 100% methanol (10 mL), followed by a wash-out of ultrapure water (20 mL, 3 mL min^{-1}). A gradient concentration of methanol (0-20%,

v/v) was utilized for the wash-out of impurities and methanol (3 ml, 35% v/v) for elution of the microcystin-LR. After collection, the eluent was evaporated under dry nitrogen to 1.2 mL.

The algal pellet was also analysed for intracellular microcystin-LR. Briefly, after washing three times with ultrapure water, the pellet was dissolved with methanol (80% v/v) and freeze-thawing with liquid nitrogen (x3) in order to fragment the algal cells, and to extract the microcystin-LR. Finally, 1 mL sample was concentrated by evaporation under dry nitrogen at room temperature. The working parameters of Acquity UPLC for microcystin-LR detection are described in S.1.3.

2.4 Microalgal growth monitoring

Cell densities of both microalgae species were monitored daily using a hemocytometer. After sampling on day 1, 3, 6, and 10, cell pigments were detected and calculated as described in S1.4.

2.5 Calculation and statistical analysis

The Pseudo-first-order kinetic model (1) was used to simulate the removal, desorption and decline of accumulated tetracycline:

$$\ln(C_0/C) = k \times t \quad (1)$$

Meanwhile, according to a relevant study (Choi et al., 2020), the concentration changes through adsorption and accumulation by microalgae was simulated with modified pseudo-first-order kinetic model (2):

$$\ln(1-C_0/C) = -k \times t \quad (2)$$

where, C_0 is the concentration of tetracycline at time t=0 and C is the tetracycline at time t=t; k represents the pseudo-first-order kinetic rate constant; t is the time.

SPSS 19.0 (IBM Corporation, Armonk, USA) and Origin 8.5 (OriginLab, Northampton, USA) were used to analyse and to plot the data, respectively. One-way ANOVA and Tukey's multiple comparison test were used to compare ($p<0.05$) water quality parameters between different treatment systems at each sampling point.

3. Results and Discussion

3.1 Tetracycline removal efficiency and kinetics

Tetracycline is a chemically reactive compound which can be degraded through photolysis and/or hydrolysis processes (Yi et al., 2016; Jiao et al., 2008). This characteristic supports the observation that the tetracycline degraded in all control groups, even without addition of microalgae. In this study, *C. pyrenoidosa* was selected as a representative, widely-investigated, and safe microalgal species for wastewater treatment (Sun et al., 2011). The comparison of tetracycline removal efficiencies between *M. aeruginosa* and *C. pyrenoidosa* could contribute to a better understanding of the advantages or drawbacks for treatment of wastewater, containing high concentrations of tetracycline, by the newly proposed HABs species over conventional microalgae species. For *C. pyrenoidosa* treatments (Fig. 1a), from an initial concentration of 10 mg L^{-1}, the tetracycline concentration significantly decreased to 0.65 mg L^{-1} (93.9% removal) by day 2 and then reached 0.01 mg L^{-1} (99% removal) on day 11. However, the *M. aeruginosa* treatments showed a much faster removal rate, and achieved 0.01 mg L^{-1} (99% removal) at day 2 which was furthermore maintained until day 11 (Fig. 1a). Previous studies have indicated that higher tetracycline concentration might hinder microalgae growth (Ye et al., 2017) and therefore affect drug removal. This observation is supported in this study by the *C. pyrenoidosa* groups, at the higher initial concentration of 50 mg L^{-1} (Fig. 1b), where tetracycline concentrations decreased faster than those in the control group, until day 4 after which it maintained at 0.7 mg L^{-1} (98.6% removal rate) and performed

similarly to the control. The growth of the algal biomass may act to decrease the light penetration in the water and thus affect photolysis and/or hydrolysis removal of tetracycline. Thus, at the initial concentration of 100 mg L^{-1}, the tetracycline removal in *C. pyrenoidosa* groups became similar, or even less effective, when compared to the control (Fig. 1c).

The *M. aeruginosa* groups consistently exhibited significantly superior performance for tetracycline removal at high initial concentrations of 50 and 100 mg L^{-1}, where the similar tendencies of rapid removal over 98.0% were achieved within 1 and 2 days, respectively (Fig. 1b and c). *M. aeruginosa,* as a typical cyanobacterial alga, demonstrated a rapid reproduction rate, which, however, could lead to the appearance of serious harmful algal blooms within several days in eutrophic lakes (Paerl and Otten, 2013). The existence of *M. aeruginosa* has been observed worldwide, regardless of temperature and pH variance of the water (Paerl and Otten, 2013), which may indicate its high tolerance against any antibiotic effects. The results demonstrated that the harmful cyanobacteria, *M. aeruginosa*, could rapidly and effectively remove tetracycline within limitations of initial concentration. Previous studies indicated that, in algal ponds, only 69±1% tetracycline was removed from an initial concentration of 2 mg L^{-1} (de Godos et al., 2012), and 93% tetracycline was eliminated from an initial concentration of 0.1 mg L^{-1} in 4 days (Norvill et al., 2017). Comparatively, the *M. aeruginosa* demonstrated a much higher and faster removal capacity of tetracycline over a wide range of initial concentrations, up to 100 mg L^{-1}.

Tetracycline removal is widely indicated by studies to follow the pseudo-first-order kinetics in high-rate algal pond (HRAP) systems (de Godos et al., 2012, Norvill et al., 2017). The results of tetracycline removal in this study also exhibited good correspondence with the pseudo-first-order kinetic model (Fig. 1, Table 1, $R^2 > 0.94$).

Generally, elevation of levels of tetracycline significantly decreased the removal rates (k). Except for the *C. pyrenoidosa* with 100 mg L^{-1} tetracycline, in which the growth of microalgae probably inhibited the photolysis of tetracycline and generated lower removal rates than the control, all other groups of both microalgae species achieved greater removal rates than the control. This indicated a significant promotion of the presence of microalgae on tetracycline removal than the natural removal processes contributed by hydrolysis, photolysis, and cation-binding, especially with *M. aeruginosa* (5.1-12.1 times higher than the control). The results further demonstrated that, overall, *M. aeruginosa* exhibited significantly higher removal rates, at all tetracycline levels, than did the *C. pyrenoidosa* ($p<0.05$), indicating a better general tetracycline removal ability of *M. aeruginosa* than the *C. pyrenoidosa*. Compared with previous studies, the pseudo-first-order kinetic rate results also showed statistically better tetracycline removal kinetic achieved by *M.aeruginosa* (0.17-0.077, 10-100 mg L^{-1} tetracycline) than that achieved by the HRAP (0.091-0.038, 0-88 mg TSS L^{-1} biomass, 2 mg L^{-1} tetracycline; (de Godos et al., 2012) and photolysis/hydrolysis processes (0.0014-0.0065, 20-100 mg L^{-1} tetracycline; Yi et al., 2016; Jiao et al., 2008).

3.2 Tetracycline removal pathways

The experiment at the initial tetracycline concentration of 50 mg L^{-1} was further explored in order to understand the contributions from each pathway of removal. Considering the abiotic removal of tetracycline, processes of cation-binding, photolysis, and hydrolysis all contributed to the removal of tetracycline, acting however, at different levels (Fig. 2a-b, and S2a-b). The results from the chemical control group indicated that divalent cations present in the BG11 medium (Table S1), such as calcium, magnesium, and cobalt, could bind with tetracycline to form low solubility complexes (Pala-Ozkok et al., 2019), which contributed to a relatively constant tetracycline removal in the range of

1.42-4.08 mg L^{-1} (2.8-8.0%, Fig. S2a). Meanwhile, abiotic photolysis achieved a significant higher ($p<0.05$) removal rate ($k=0.029$) than did abiotic hydrolysis ($k=0.0069$, Table S2).

Results further indicated that the significantly higher removal rates of tetracycline ($p<0.05$) achieved by microalgae (especially by *M. aeruginosa*) could be contributed through bioadsorption, bioaccumulation and biodegradation processes (Fig. 2c-d and S2c). Simultaneous with the decline in concentration of tetracyclines in water, concentrations adsorbed and accumulated by both microalgae increased to a peak (uptake stage) inside one (*M. aeruginosa*) or two (*C. pyrenoidosa*) days. For the *C. pyrenoidosa* treatment groups, the bioremediation (including bioadsorption, bioaccumulation, and biodegradation) contribution of the algae was always observed to be lower (<10.41 mg L^{-1}, less than 20.5% contribution) than those from abiotic photolysis, hydrolysis, and cation-binding. However, *M. aeruginosa* could be considered more effective for tetracycline bioremediation (39.02 mg L^{-1}, 71.6% contribution) providing a total removal efficiency of 98% after only 24 hours (Fig. 1, 2a and b). Previous studies also illustrated that tetracycline, owing to its hydrophilic nature, could be absorbed by the algal cell (de Godos et al., 2012). The compound has also been found to translocate into an algal cell through water and nutrient uptake pathways (Xiong et al., 2017a). However, the amount of tetracycline existing on cell surfaces (bioadsorption) and intracellular regions (bioaccumulation) in *M. aeruginosa* (1.07 mg L^{-1}, 2%) was much lower than that in *C. pyrenoidosa* (8.02 mg L^{-1}, 16%) on day 1. Afterwards, a gradual decline of concentrations from the peak was followed until day 13 (through either metabolization or release). Both concentrations of bioadsorbed and bioaccumulated tetracycline changed as a consequence of microalgal uptake and translocation, and could be well described by the pseudo-first-order kinetic model ($R2\geq0.90$, Table S2). Generally, in the uptake stage of tetracycline,

the rates of bio-adsorption by both microalgal species were significantly higher than those of bio-accumulation ($p<0.05$) and, overall, *M. aeruginosa* achieved higher rates of bio-adsorption and bio-accumulation (k=0.66 and 0.19, respectively) than did *C. pyrenoidosa* (k=0.37 and 0.12, respectively, Table S2). Nevertheless, *M. aeruginosa* promoted a shorter uptake stage of tetracycline when compared to *C. pyrenoidosa*. In the declining stage of tetracycline, the desorption rates of *M. aeruginosa* (k=0.23) were still significantly higher than that of *C. pyrenoidosa* (k=0.071), however, both species achieved similar decrease rates of accumulated tetracycline (k=0.012 and 0.011, respectively, $p>0.05$). Considering the superior tetracycline removal efficiency in *M. aeruginosa* treatment groups, the species presented significantly higher tetracycline biodegradation either inside the algal cell or by side effects outside the algal cell, when compared with *C. pyrenoidosa.*

Although the contributions of each pathway may vary, the results supported the hypothesis that the possible pathways, such as hydrolysis (tetracycline molecule split by chemically-catalysed addition of water), photolysis (light-activated oxidation), cation-binding (tetracycline binding with divalent cations), biodegradation (decomposition of tetracycline by microbial activity), bioadsorption (tetracycline passively concentrating and binding onto microalgal cells) and bioaccumulation (accumulation of tetracycline in the microalgae cells) (Yi et al., 2016; Jiao et al., 2008; Xiong et al., 2017b), have an effect on tetracycline removal. It should be noted that among the confirmed pathways, more than one mechanism can take place simultaneously and thus can influence each other. For example, the growth of microalgal biomass could reduce the intensity of light inside the water column and thus reduce tetracycline degradation by photolysis (de Godos et al., 2012) (Fig. 1c). Consequently, the realistic photolysis kinetics of tetracycline removal in both microalgal systems should be less than those of the theoretical results (Fig. 2c).

Oppositely, it was observed that the inoculum and growth of both *M. aeruginosa* and *C. pyrenoidosa* led to an increase in solution pH than the controls. Moreover, the *M. aeruginosa* samples generally produced significantly higher pH levels than did the *C. pyrenoidosa* groups ($p<0.05$, Fig. S3). Previous studies have suggested a reduction in tetracycline hydrolysis half-life time by 2.68-3.27 -fold with an increase of pH from 5 to 11 (22-25℃) (Yi et al., 2016). Thus, the realistic hydrolysis of tetracycline under both microalgal treatments should be significantly increased, above those theoretical hydrolysis kinetics (Fig. 2c). The higher levels of pH in the *M. aeruginosa* groups could promote a higher tetracycline hydrolysis compared to that from the *C. pyrenoidosa* groups. Additionally, the elevated photosynthetic processes occurring in microalgal treatments (Fig. S4 and S5) may increase both dissolved oxygen (DO) and pH, increasing the number of reactive oxygen species (ROS) (Norvill et al., 2017), which, in turn, may also lead to the degradation of tetracycline.

3.3 Tetracycline degradation by-products

Water samples from both microalgal treatment groups were analysed by HPLC-ToF-MS in order to determine potential degradation by-products and removal pathways. Based on the results from the LC-ToF-MS analysis (Fig. S6 and S7) and a review of extant literature, we have elucidated that both microalgal systems utilised a common degradation pathway (named PI), but also used additional specific pathways unique to each species.

The results indicated that the tetracycline degradation process proceeded mainly by two reaction patterns, *i.e.* changes to functional groups (gain and loss), and ring-opening reactions (Cao et al., 2016). Relevant structures and ESI-ToFMS spectra are illustrated in Fig. S7. By-product 1 (m/z 415) was detected in the samples (Fig. 3, PI), which indicated the removal of the N-methyl and C3 hydroxyl groups (Shen et al., 2020). Further loss of

the C6 hydroxyl group and the formation of a conjugated double bond at C6-C5a position, resulted in the formation of by-product 2 (m/z 397) (Huang et al., 2019). This molecule could be further degraded via loss of the amide group at position C2 (Huang et al., 2019) and further demethylation of the secondary amine at position C4 (Guo et al., 2018), forming by-product 3 (m/z 340). The ring-opening reaction across C2 and C4a could lead to the formation of by-product 4 (m/z 284) (Huang et al., 2019), while further ring fragmentation across C5a and C11a with concurrent hydration could generate by-product 5 (m/z 183) (Zhou et al., 2020). Notably, most by-products of the PI pathway (m/z 415, 397, 340, 284) were also found in a tetracycline photo-catalysis degradation process mediated by a Bi_2WO_6-based material (Shen et al., 2020; Huang et al., 2019). Meanwhile, the final product from PI (m/z 183) was reported from an activated hydrogen peroxide oxidation process for tetracycline degradation (Zhou et al., 2020). This final product (m/z 183) has been evaluated by Zhou et al (2020) according to the Globally Harmonized System of classification and labelling of chemicals (GHS) method, showing a significantly reduced toxicity relative to the parent tetracycline (Zhou et al., 2020).

In addition to pathway PI, an additional scheme (PII) could be identified for tetracycline degradation by *M. aeruginosa* (Fig. 3a), namely, occurrence of ring-opening across the C1 and C4 positions, and fission of the double bond between C2 and C3 to form the by-product 6 (m/z 163). A further loss of N-methyl resulted in the formation of by-product 7 (m/z 149). Both tetracycline by-products 6 and 7 have been previously reported from a degradation experiment via TiO_2 photo-catalysis (Niu et al., 2013). Therefore, both degradation pathways (PI and PII) may include some processes of photo-catalysis degradation. It has been proved that, under irradiation, the extracellular organic matters (EOMs) released by microalgae could promote the formation of active species, which may lead to indirect photo-degradation of antibiotics (Tian et al., 2019). Moreover,

the ferric ion (in BG11 medium) may further react with the EOMs to generate Fe(III)-carboxylate complexes, resulting in further augmentation of the antibiotic photolysis (Wei et al., 2020). The potential photo-catalysis degradation by-products 6 and 7 have been demonstrated to exhibit a decreased toxicity compared to the parent compound (Niu et al., 2013). However, a different degradation pathway (named PIII, Fig. 3b) was identified in *C. pyrenoidosa* systems, where the double bond at the C11a-C12 position was attacked by OH· to form by-product 8 (m/z 461). This molecule was then further degraded to by-product 9 (m/z 459) via hydrogen abstraction at the C5-C5a position (Ao et al., 2019). The by-products of PIII pathway (m/z 461, 459) were reported from an activated peroxymonosulfate oxidation process (Ao et al., 2019). Moreover, the by-products 8 and 9 from pathway PIII generated by *C. pyrenoidosa*, even reduced its toxicity to algae (based on the GHS evaluation), but still demonstrated the same level of toxicity to fish and daphnid according to previous studies (Pala-Ozkok et al., 2019). Therefore, the defined degradation pathway from *M. aeruginosa* treatment groups may be used to support the rapid rate of tetracycline removal and greater efficiency compared with those from the *C. pyrenoidosa* groups. Further systematic toxicity evolution experiments could help to ensure reductions in toxicity of the by-products.

3.4 Microalgae biomass and vitality response

Microalgal-based wastewater treatment offers the advantage of, in addition to the elimination of the pollutant, potential biomass re-utilization, e.g., as biofuel, biochar, or fertilizer (Kim et al., 2012; Miao et al., 2004). Therefore, during the experiment, we further investigated changes occurring to the biomass, due to tetracycline exposure. Between the two algal species, *M. aeruginosa* demonstrated a better tolerance and growth recovery against tetracycline than did the *C. pyrenoidosa*. Specifically, tetracycline generated an acute inhibition on the growth of *M. aeruginosa*, at all concentrations, after

one day incubation (Fig. 4a). However, the inhibition was temporary, and growth fully recovered afterwards. No significant differences in cell numbers were observed with the 10 and 50 mg L^{-1} tetracycline solutions (4.36 x 10^7 cells mL^{-1} and 3.11 x 10^7 cells mL^{-1}, respectively) which were also similar to the algal cell numbers noted in microalgal control groups (4.19 x 10^7 cells mL^{-1}, $p>0.05$) at day 11 (Fig. 4a). However, 100 mg L^{-1} tetracycline slightly inhibited biomass growth of *M. aeruginosa* ($p<0.05$) until day 11. Comparatively, all three concentrations of tetracycline inhibited the growth of *C. pyrenoidosa* to a greater extent than the microalgal control (1.74 x 10^7 cells mL^{-1}), resulting in a final value of 1.87-9.58 x 10^6 cells mL^{-1} (Fig. 4b). The results agreed well with a previous study, which revealed the inhibition, adaption, and hormesis effects of tetracycline on *M. aeruginosa* at concentrations under 10 mg L^{-1} (Ye et al., 2017). However, our study further extended the tetracycline concentration to 100 mg L^{-1}, and proved a recovery ability of *M. aeruginosa* at tetracycline concentrations under 50 mg L^{-1}.

Additionally, the results of pigment concentration (chlorophyll-a and carotenoid) and fluorescence signal intensity (Fv/Fm) analyses also agreed well with the impacts of cell number, where all tetracycline concentrations generated an acute inhibition on pigment accumulation and photosynthesis intensity (Fv/Fm). However, at tetracycline concentrations under 50 mg L^{-1}, both algal species demonstrated stepwise recoveries in varying degrees (Fig. S5 and S6). We concluded from these results, that *M. aeruginosa* possessed a strong tetracycline removal capacity along with a higher tolerance to tetracycline than did *C. pyrenoidosa*. Meanwhile, the successful recovery ability of *M. aeruginosa* showed the potential for high biomass production, which could benefit re-utilization upon completion of the treatment process.

3.5 Effect of tetracycline on microcystin release

As a species of cyanobacteria, *M. aeruginosa* poses a potential threat for microcystin release, which may cause damage, in humans, to the liver and to the nervous system (Hitzfeld et al., 2000). Consequently, we paid close attention to the investigation of changes in both intercellular and released microcystin during the treatment process. The study of toxin-release of *M. aeruginosa* is significant for broadening the valorization of HABs species. The synthesis of microcystin is induced by a mechanism protective against both abiotic and biotic stress (Babica et al., 2006) and is often coupled with photosynthesis (Walls et al., 2018). Microcystin, however, typically remains intracellularly, unless being released into the surroundings through cell death and lysis, or as extracellular release (Paerl and Otten, 2013). In the present study, at initial tetracycline concentrations of 10 and 50 mg L^{-1}, *M. aeruginosa* cells analysed were in a growing or stable phase, reflected by cell growth curves (Fig. 4a). Thus, intracellular microcystin-LR concentrations were generally higher than those measured in the water (Fig. 5). For intracellular microcystin-LR, tetracycline inhibited photosynthesis (Fig. S5a). It also exerted abiotic stress, which may have resulted in no significant differences in intracellular microcystin-LR production being noted within 60 hours ($p>0.05$, except the systems with 100mg L^{-1} tetracycline), however, being distinctly lowered over 240 hours (Fig. 5a, $p<0.05$). This reduction of induced microcystin-LR may be caused by the long-term damage caused by tetracycline on the synthesis of peptide synthetases, which control microcystin-LR production (Ye et al., 2017).

The microalgal control group simulated the development of *M. aeruginosa* unexposed to tetracycline, where an accelerating growth and metabolism was observed (Fig. 4a). The increasing death and lysis of the cells coupled with *M. aeruginosa* growth could result in an elevated microcystin-LR release into the water (Fig. 5b). Notably, an overall decreasing release of microcystin-LR into the water was detected due to

tetracycline exposure (in the tetracycline treated groups) compared to the microalgal control (except at 10 hours with 10mg L^{-1} tetracycline). This may be mainly due to the reduced growth and metabolic activity of the microalgae, caused by tetracycline. The rapid tetracycline removal ability of *M. aeruginosa*, which produced a removal rate of over 98% in 24 hours (under 50 mg L^{-1} tetracycline) or in 48 hours (up to 100 mg L^{-1} tetracycline), indicated that an optimum, but safe, treatment time for the removal of tetracycline could be set at 48 hours. Within this time, the effluent with initial tetracycline concentrations < 50 mg L^{-1} generated a microcystin-LR concentration lower than 1 μg L^{-1} (Fig.5b). Even for wastewater containing more concentrated tetracycline (up to 100 mg L^{-1}), a low-level microcystin-LR concentration of 1.8 μg L^{-1} was detected in the final effluent, which was much lower than both the concentration in the natural water (Christoffersen and Kaas, 2000) and the guideline concentration for recreation or bathing water of Germany and Australia (< 10 μg L^{-1}) (Burch, 2008).

Summarily, for tetracycline concentrations in the range of 10-100 mg L^{-1}, both intracellular and released microcystin-LR have been found to be reduced by varying degrees. A previous study also confirmed the decrease of synthesized and released microcystin-LR after tetracycline exposure (Ye et al., 2017). Regarding the effective tetracycline removal ability even with low biomass (Fig. 1 and 4a), an appropriate decrease of the initial amount of inoculum, and timely harvest of *M. aeruginosa* cells may further ensure the effluent will conform to safe microcystin-LR levels according to the natural water and recreation or bathing water regulations. In addition, the present study also demonstrated the possibility of the long-term cultivation of low microcystin-LR *M. aeruginosa* biomass by treatment with tetracycline. Nevertheless, real-time monitoring facilities are needed for surveillance during the treatment and cultivation process in order to ensure further water security. Moreover, due to the complexity of real wastewater,

where more complex compounds and other limiting factors may challenge the performance of tetracycline removal as well as release of toxin, further study is needed to investigate the efficacy and safety of the proposed approach with realistic wastewater in scaled-up systems before implementation.

3.6 Insight into future implementation

Along with the rapid development of the microalgal technology, many full-scale systems have been successfully applied for the treatment of various wastewaters, where hydraulic retention times (HRTs) were normally several days (Rawat et al., 2011; Wang et al., 2016). The current study demonstrated that *M. aeruginosa* could reduce high concentrations of tetracycline (up to 100 mg L^{-1}) down to an acceptable level (>98%) in 48 hours, which supported the feasibility of incorporating the process into microalgal treatment systems. Normally, such wastewaters contain both macro nutrients, e.g., N and P (Wang et al., 2016), and trace elements, e.g., metals (Rawat et al., 2011), which could support the growth of microalgae and result in acceptable effluent to meet discharge regulations (Wang et al., 2016). In some circumstances of wastewater with limited available nutrients, such as hospital wastewater, the nutrients could be compensated through the admixture of other high-nutritional wastewaters (Norvill et al., 2017). Moreover, microalgae-based wastewater treatment could also provide additional benefits, such as biodiesel generation (Li et al., 2020), nutrient recovery (Chu et al., 2020), and greenhouse gas emission control (Rawat et al., 2011). The present investigation expanded the potential use of cyanobacteria for antibiotic removal at high concentrations. HABs occur more frequently globally due to the problems of intensive eutrophication, which is detrimental to natural waters (Lyu et al., 2020). Meanwhile, wastewater contaminated with high concentrations of tetracycline, e.g., pharmaceutical wastewater, livestock industry wastes, and emergency releases from hospitals, brings potential resistance-risk

to the ecology. It could further impair the action of the sludge bacteria of WWTPs (Halling-Sørensen, 2001), resulting in low amounts of the tetracycline removed after treatment (Watkinson et al., 2009). By using a typical harmful algal species, *M. aeruginosa,* for the treatment of wastewater contaminated with higher concentrations of tetracycline, the problems of eutrophication and of tetracycline resistance (Fig. 6) could be simultaneously addressed.

In this study, tetracycline was observed to have inhibited the synthesis of microcystin-LR, and the effluent from the process could achieve a safe natural and recreational water quality in respect of microcystin-LR. Notably, other trace organic and inorganic matter released by microalgae may also lead to potential risks, such as the formation of haloacetonitriles during further chlorine disinfection treatment (Pals et al., 2011). However, this has long been a common challenge for the End-of-Pipe technology of chlorine disinfection during the treatment of final wastewater effluents or of drinking water (Pals et al., 2011), which was not yet covered by this study. Therefore, before the application of this treatment process, further investigations, e.g., the treatment performance in real wastewater and comprehensive risk assessment are needed.

4. Conclusion

The present study demonstrated the possibility of reusing harmful algal species for effectively remediating high-concentration antibiotics. The harmful cyanobacterial species, *M. aeruginosa*, provided superior, efficient and rapid capacity for tetracycline removal, compared with *C. pyrenoidosa,* under a wide initial concentration range (10-100 mg L^{-1}). Both microalgal species promoted tetracycline hydrolysis by raising the water pH, however, biodegradation contributed to the major part of the removal by *M. aeruginosa*. Both microalgae species shared a common tetracycline degradation pathway, but also utilised unique species-specific pathways producing different degradation by-

products. The by-products showed no significant increase of toxicity according to published references. The presence of the tetracycline could have significantly inhibited the release of toxic microcystin-LR into the water, thus promoting the relative safety of the final effluent after treatment. Notably, the current study was carried out in the synthetic antibiotic-contaminated water, which provided evidence-based insights into the proposed approach. However, further study is needed in order to evaluate the performance with realistic wastewater in upscaled systems prior to practical implementation.

Declaration of competing interest

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

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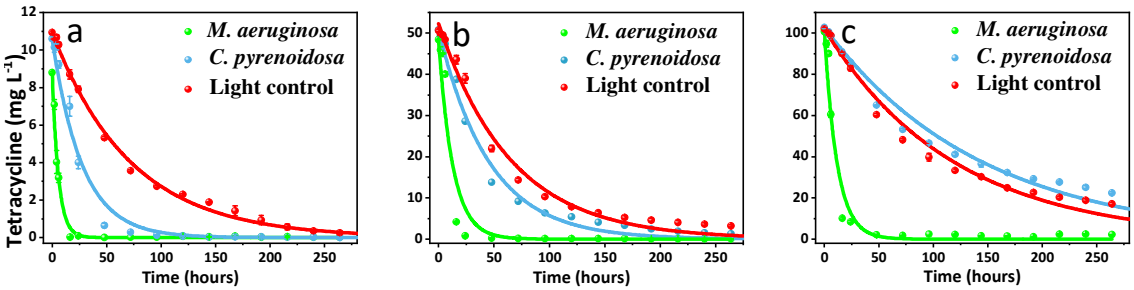


Fig. 1. The dynamics of tetracycline concentration in blank control groups, and treatment groups of *M. aeruginosa* and *C. pyrenoidosa* at initial concentrations of 10 mg L^{-1} (a), 50 mg L^{-1} (b), and 100 mg L^{-1} (c). The solid lines are simulated pseudo-first-order kinetic degradation models.

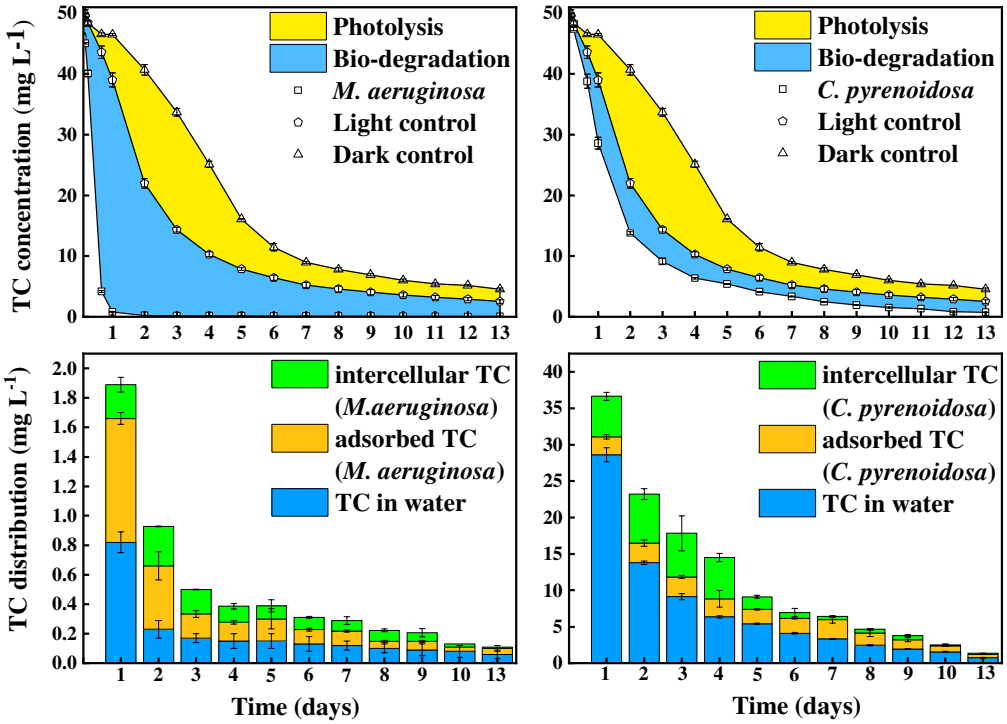
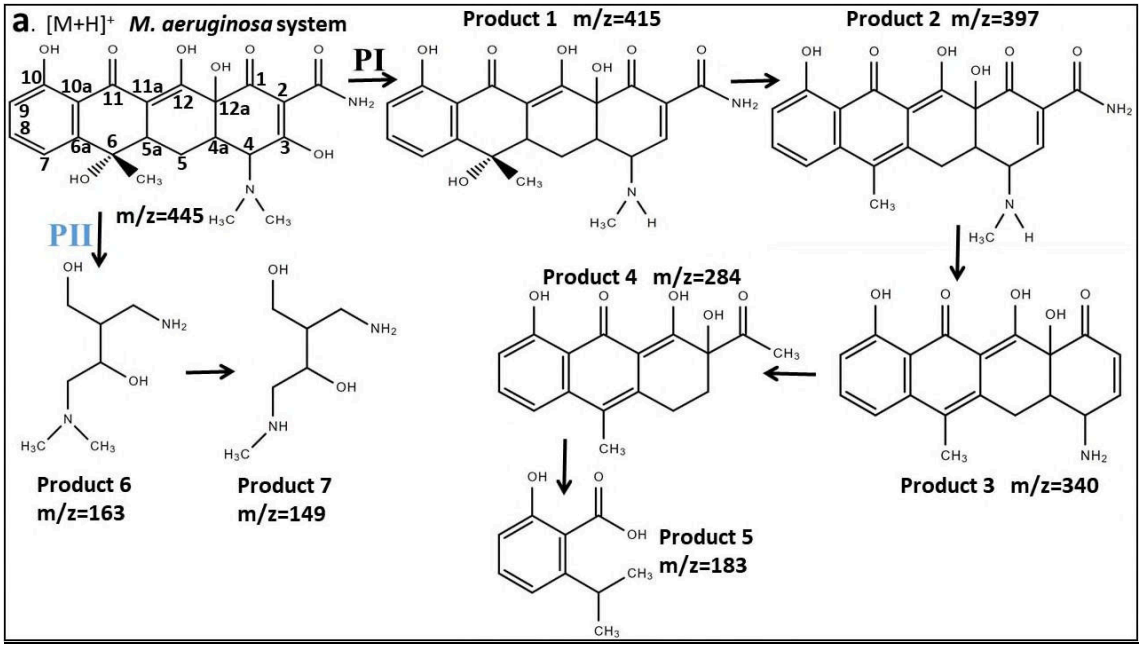


Fig. 2. Theoretically different contributions (photolysis, hydrolysis & cation-binding and bio-remediation) towards tetracycline removal in (a) *M. aeruginosa*, and (b) *C. pyrenoidosa* treatment groups, and distribution (in water, adsorption by microalgae and bio-accumulation into microalgal cells) of residual tetracycline in (c) *M. aeruginosa*, and (d) *C. pyrenoidosa* treatment groups.



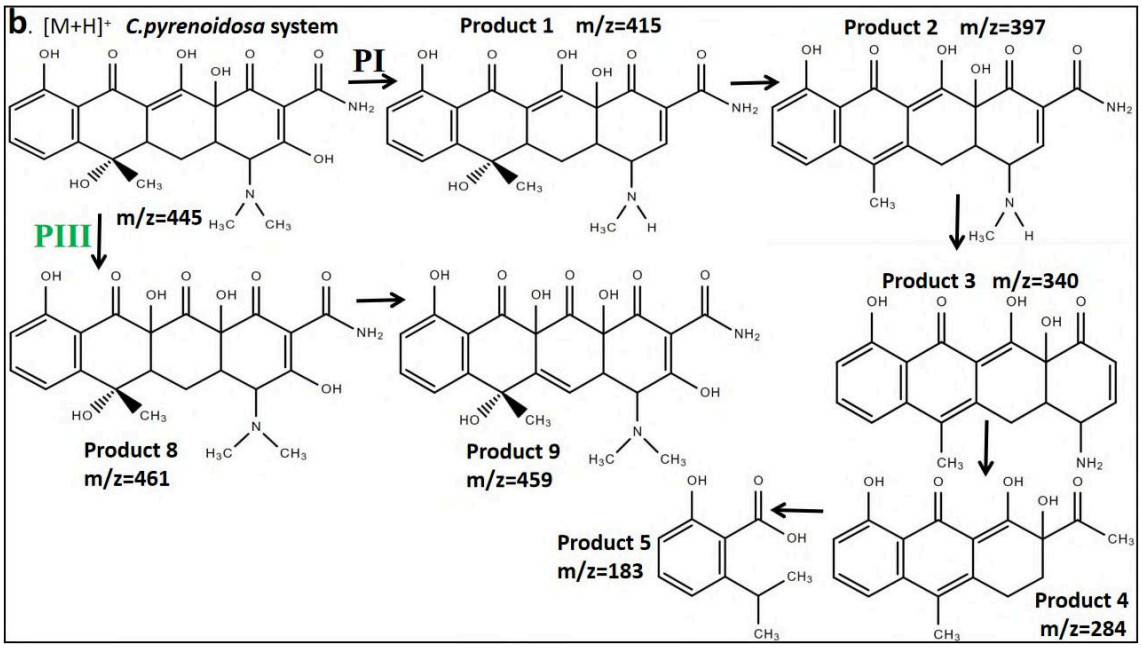


Fig. 3. Tetracycline degradation by-products and potential pathways (PI, PII, and PIII) in (a) *M. aeruginosa*, and (b) *C. pyrenoidosa* treatment groups.

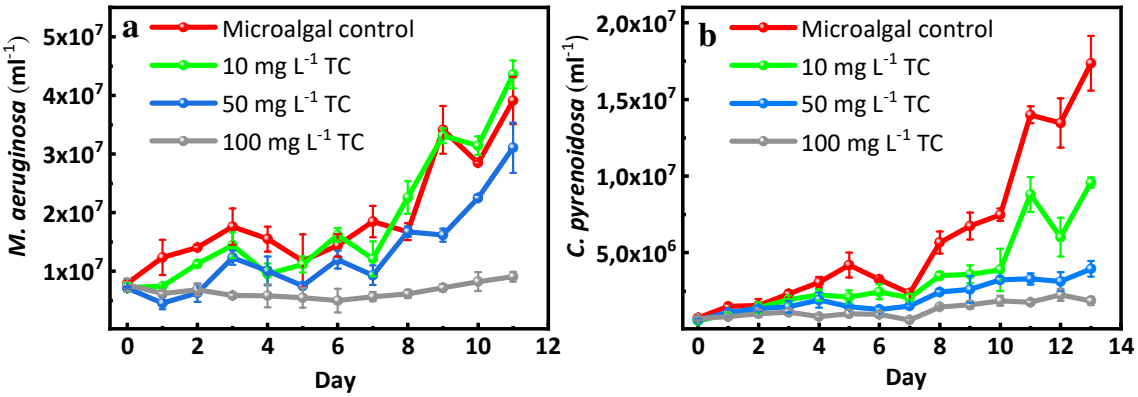


Fig. 4. Cell number growth under varying tetracycline concentrations for (a) *M. aeruginosa*, and (b) *C. pyrenoidosa* treatment groups.

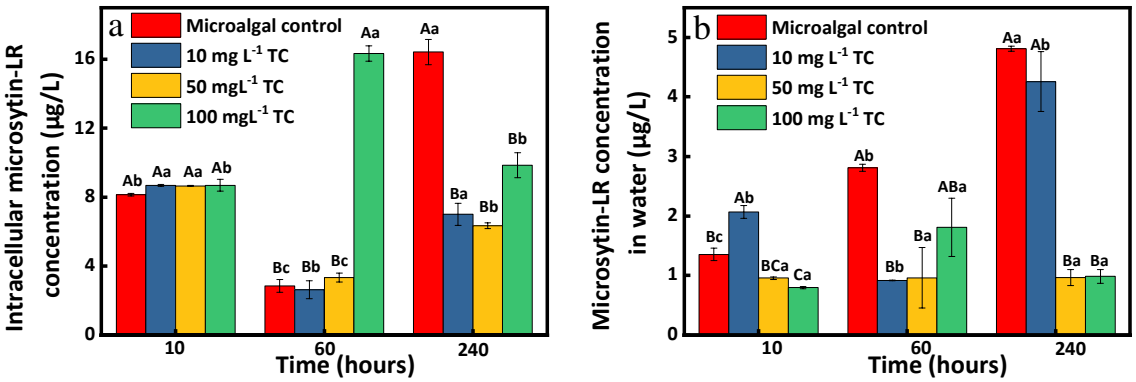


Fig. 5. Microcystin-LR concentrations of (a) intracellular *M. aeruginosa*, and (b) released into water from *M. aeruginosa*. Different uppercase letters above the error bars in each figure represent significant difference ($p < 0.05$) among different treatment groups at the same sampling time. Different lowercase letters above error bars in each figure represent significant difference ($p < 0.05$) of the same treatment group over different sampling times.

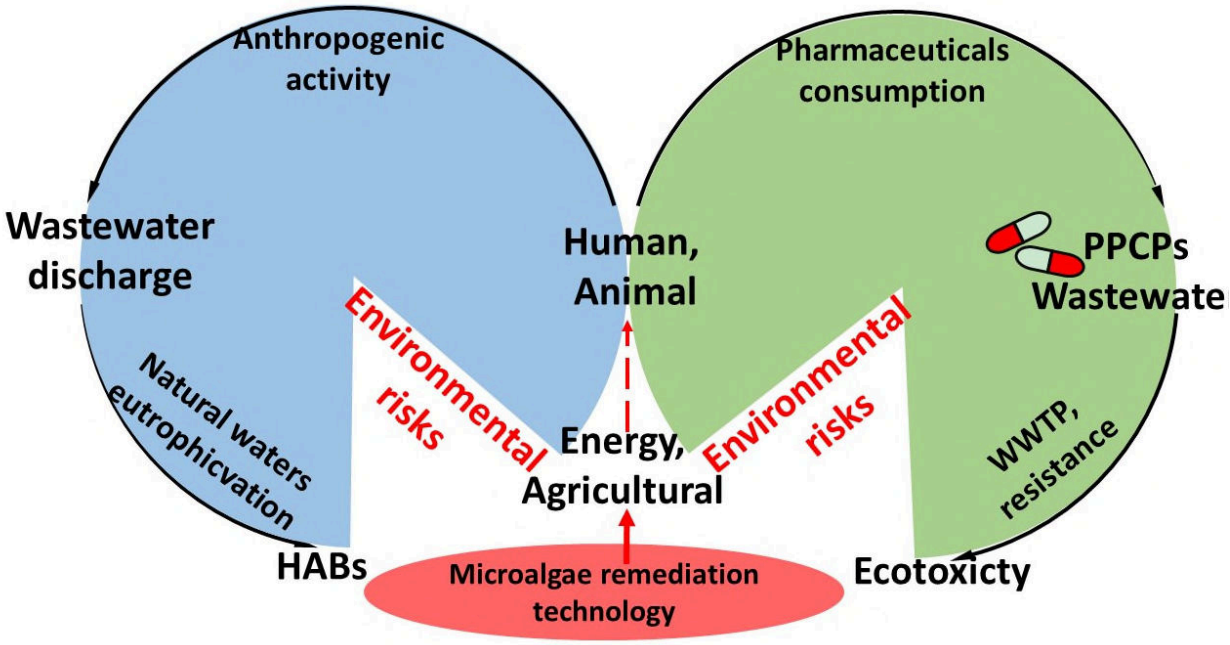


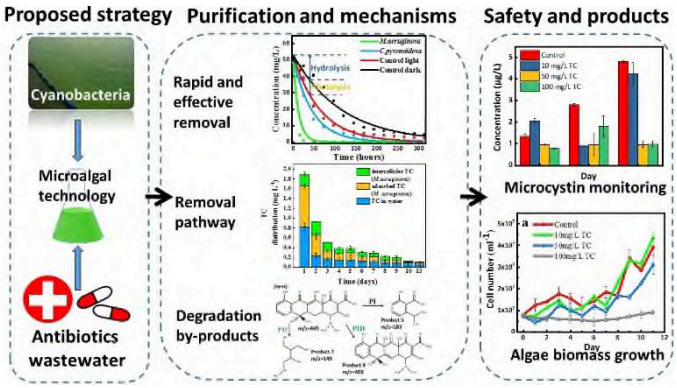
Fig. 6. Sustainable microalgae remediation strategy for addressing the environmental problems caused by pharmaceuticals and personal care products (PPCPs) and Harmful Algae Blooms (HABs).

Table. 1
Pseudo-first-order kinetic model fitting results of tetracycline degradation.

	Initial tetracycline concentration (mg L^{-1})	k value	R^2
M. aeruginosa	10	$1.7 \times 10^{-1} \pm 9.2 \times 10^{-3}$	0.99
	50	$7.7 \times 10^{-2} \pm 1.3 \times 10^{-2}$	0.94

	100	$8.6 \times 10^{-2} \pm 9.7 \times 10^{-3}$	0.97
C. pyrenoidosa	10	$3.6 \times 10^{-2} \pm 2.7 \times 10^{-3}$	0.99
	50	$2.2 \times 10^{-2} \pm 1.1 \times 10^{-3}$	0.99
	100	$7.0 \times 10^{-3} \pm 2.8 \times 10^{-4}$	0.98
Control	10	$1.4 \times 10^{-2} \pm 3.3 \times 10^{-4}$	0.99
	50	$1.5 \times 10^{-2} \pm 8.4 \times 10^{-4}$	0.99
	100	$8.4 \times 10^{-3} \pm 3.6 \times 10^{-4}$	0.98

Graphic abstract



Highlights

- Harmful cyanobacteria *M. aeruginosa* could effectively remediate antibiotic
- Tetracycline removal by *M. aeruginosa* was significantly faster than *C. pyrenoidosa*
- Biometabolism dominated tetracycline removal, led to distinct degradation products
- Biomass growth and cell vitality showed limited inhibition by tetracycline exposure
- Microcystin release was controlled to levels below the recreation water values

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Mitigating antibiotic pollution using cyanobacteria: removal efficiency, pathways and metabolism

Pan, Minmin; Lyu, Tao; Zhan, Lumeng; Matamoros, Victor; Angelidaki, Irini ...

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