Desalination and Water Treatment
www.deswater.com
doi: 10.5004/dwt.2020.25065

180 (2020) 317–327
March

Nutritional pattern-driven algal antibiotic treatment: a period-specific and carbon-dependent removal process

Fengyi Zhu[a,§], Xiao Wang[a,§], Xiandi Cui[a], Weili Jiang[b], Jilai Lu[b], Yanhua Liu[a], Yaxin Huang[a], Oscar Lopez Torres[a], Jianqiu Chen[a,*], Ruixin Guo[a,*]

[a]*School of Engineering, China Pharmaceutical University, Nanjing 210009, China, Tel. + 86 25 86185190; Fax: + 86 25 86185190; emails: cjqer@163.com (J. Chen), grxcpu@163.com (R. Guo), 1227708561@qq.com (F. Zhu), 602072284@qq.com (X. Wang), 2603156713@qq.com (X. Cui), 957327801@qq.com (Y. Liu), 1977943170@qq.com (Y. Huang), 1340832@stu.cpu.edu.cn (O.L. Torres)*
[b]*Jiangsu Province Key Laboratory of Environmental Engineering, Nanjing 210036, China, Tel. 025-85588186; Fax: 025-85588186; emails: greenxin@163.com (W. Jiang), 108660712@qq.com (J. Lu)*

Received 7 May 2019; Accepted 23 October 2019

ABSTRACT

Our present study evaluated the potential of algae-mediated antibiotic treatment drove by three nutritional patterns. The relevant lowest removal efficiency of the antibiotic cefradine occurred under the heterotrophic pattern (75.95%–82.35%) and more excellent performance occurred when droved by the mixotrophic pattern (close to 100%). Our results showed out that a process-dependent removal capacity in three steps and the highest removal efficiency was obtained in the second period (24–48 h). Additionally, the effect of N and P was NaAc-dependent. The relatively higher removal rate was obtained when a low concentration of N and P was involved, while a higher concentration of N and P drove higher removal efficiency when NaAc was involved. Additionally, algal photosynthetic pigments and activity of four kinds of enzymes have also been considered. Our result indicated that the enhanced algal photosynthesis drove the removal efficiency up. Finally, PO_4–P was mainly consumed completely during the treatment, regardless of the concentration of P. However, the consumption level of NO_3–N and NaAc were relatively lower than that of P. Our study indicated that the removal efficiency of the algal treatment could be exploited enough which drove by the mixotrophic pattern.

Keywords: Antibiotic; Algae-mediated removal; Nutritional pattern-driven; Period-specific respond; Carbon-dependent

1. Introduction

Due to the considerable eco-toxic impact and associated health issues, emerging contaminant has become an emerging environmental concern. These synthetic chemicals can be transported through the atmosphere and water and, in many cases, find their way into sediment and soil, and can also accumulate in different trophic level organisms including human beings through biomagnification in food chains. Antibiotics, as a widely consumed emerging contaminant, have been used for the treatment and prevention of bacterial infection. However, significant fractions of each human and livestock dose (up to 80%–90%) remain unmetabolized and are released through the excreta [1]. Conventional activated-sludge processes in current wastewater treatment plants (WWTPs) are not designed for the efficient removal of antibiotics. The presence of antibiotics in WWTP effluents is alarming as a selective pressure and is known to stimulate antibiotic resistance in microbial organisms [2].

Although several chemical and physical technologies are used for wastewater treatment in many countries,

* Corresponding authors.

§ These authors contributed equally to this work.

while, large energy and carbon footprint are leading many to explore more sustainable alternatives [3,4]. Microalgae's basic cultivation requirements are light, carbon source, macronutrients such as N and P. N is involved in amino acids, proteins, and chlorophyll production, while P is used for energy transfer, photosynthesis, and nucleic acids formation [5]. Wastewater usually contains organic carbon, nitrogen, phosphorus, and other compounds, making it suitable for the cultivation of algae. Thus, the algae-mediated removal process may be promising in developing advanced and practical technologies for wastewater treatment shortly [6]. Especially, unlike bacteria, eukaryotes are not the target of antibiotics. microalgae-mediated antibiotics treatment has been of growing scientific interest. Previous research has indicated that algae still showed satisfactory growth ability even exposed to the high concentration of antibiotic wastewater [7]. Compared to other antibiotic treatment materials, algae have abundant biomass [8] and the capability to remove contaminants, such as nutrient, organic contaminant, heavy metal, and pathogen from domestic wastewater [9]. Furthermore, the used microalgae can be furnished as a raw material for the production of high-value biofuels and biochemical products, which avoid secondary pollution at the same time [10]. Based on these advantages, algae could, therefore, be considered as an excellent treatment material to remove antibiotics.

The capability of bacteria and fungi cannot overcome some of the major limitations that require carbon and other nutrients in stoichiometric balance for growth and degradation of contaminants. Thus, the traditional biological treatment system has a high capacity for contamination load, while it is less able to adapt to the varied wastewater quality. Microalgae is mainly through the photosynthesis process which uses solar energy to fix atmospheric CO_2. It could also use organic molecules as primary energy and carbon source through heterotrophic nutritional mode. Therefore, microalgae can switch their metabolism between autotrophic and heterotrophic depending on the availability of carbon sources and nutrients, and perform great flexibility and adaptation to survive and thrive in wastewater with varying nutritional conditions. A current review article has summarized the capacity of different microalgae species to remove and uptake of antibiotics [11]. The contribution in the previous study indicated that algae played a dominant role in the removal of the target antibiotics, which compared with the photodegradation and hydrolysis of the antibiotic. The living algae had a better removal capacity, which is about double of that of the dead alga, which indicated that the factors, which are relevant to the algal growth and physiology, could also influence the algal removal performance. Although some studies showed that biochemical factors of considerable relevance to the growth of algae, like light, organic carbon, and inorganic nutrients (e.g., N and P) [12,13], however, the algae in most previous studies, were usually in a given and specific nutritional condition. Whether and how the microalgae tolerate and adapt different nutritional conditions have been still unclear. Due to the impact of antibiotics, the removal efficiency of biological treatment, especially the activated sludge process, is still unsatisfactory. Usually, it is an efficient path to enhance removal efficiency by adjusting nutritive proportion for the antibiotic wastewater. Thus, when the algae-mediated treatment is viewed as an alternative technology, the removal efficiency of the treatment system under different nutritional patterns could be considered.

Therefore, the present study aimed to evaluate the potential of an algae-mediated antibiotic removal process as a cleaner treatment under three nutritional patterns. *Chlorella pyrenoidosa*, a green algae species, was employed. We selected cefradine as the target antibiotic. The compound belongs to the class of β-lactam antibiotics, account for 50%–70% of total antibiotics consumption in the world [14], and, as a widely used cephalosporin, the production output of cefradine increases yearly because of the expanding market demand [15]. The removal rate of the target antibiotic, the algal growth rate, photosynthetic pigments, four kinds key enzymes of carbon metabolism and the consumption of N, P, and NaAc have been considered to test the following hypothesis: (1) the removal efficiency could be exploited enough by an optimum nutritional pattern, (2) the response in algal growth rate, photosynthetic pigments, and activity of four kinds enzymes was period-specific under the nutritional pattern-driving force, and (3) synergic removal of C, N, and P accompanied with the antibiotic removal process.

2. Material and methods

2.1. Chemical and analytical method

The antibiotic cefradine (>98% purity) used in the present study was purchased from Yabang Investment Holding Group Co. Ltd., Changzhou, China. The concentration of antibiotics was determined by high-performance liquid chromatography (HPLC) equipped with an Inertsil ODS column Agilent Technologies Co. Ltd., China, (4.6 mm × 150 mm, 5 μm), and the working curve was $y = 0.0000957x–0.230$ (0.24–60.6 mg/L, $R^2 = 0.9997$). The mobile phase was a mixture of water, acetonitrile, 3.86% sodium acetate and 4% glacial acetic acid (1,364:600:30:6). The injection volume of the samples was 10 μL. The flow rate was 1 mL/min and all detections were performed by a UV detector at the wavelength of 254 nm under 25°C ± 1°C. At the same time, the HPLC method was also developed for the determination of NaAc using water (pH 2.15)-methanol (95:5) as the mobile phase with 0.5 mL/min of flow rate at 30°C ± 1°C, and the detection was implemented at the wavelength of 210 nm [16]. In addition, sodium acetate and cefradine reference substances were precisely weighed and dissolved in the mobile phase to prepare a reference substance solution, which was determined by the same method. Quantification was performed by external standards, which based on peak areas. As a pre-treatment, samples were extracted with disposable filters (NAVIGATOR, MCE, 0.22 μm).

The UV spectrophotometry for determination of nitrate and phosphate content. Nitrate was measured at 220 nm and took reading at a wavelength of 275 nm to remove the influence of dissolved organic matter. The phosphorus concentration in samples after the cells had previously been separated by centrifugation was determined with ascorbic [17].

2.2. Experimental design

C. pyrenoidosa, freshwater alga species which was purchased from the Institute of Hydrobiology of the Chinese

Academy of Sciences. All algae were cultured at intelligence incubator on the photoperiod 12:12 (L:D) under 4,000 lux illumination at 25°C ± 1°C. The experiment was performed in two parts. The green algae *C. pyrenoidosa* in the logarithmic growth phase were centrifuged and resuspended with the target antibiotic. The initial algal density and the concentration of the target antibiotic were set at 10×10^6 cells/mL and 50 mg/L, respectively. The target antibiotic, cefradine was treated by the green algae under three different nutritional patterns (represented autotrophic, heterotrophic and mixotrophic pattern, respectively, shown in Table 1). The concentration of residual antibiotics was determined using HPLC during the treatment process (0, 1, 3, 6, 12, 24, 48, and 72 h, respectively). The part is to evaluate the algal removal efficiency under the three nutritional patterns. Additionally, we also considered assessing the specific response of the green algae which was driven by these nutritional patterns. Algal growth capacity and the concentrations of the target antibiotic were determined at 0, 1, 3, 6, 12, 24, 48, and 72 h, respectively. Meanwhile, the samples were withdrawn at 0, 24, 48 and 72 h to evaluate the consumption of NaAc, NO_3–N, and PO_4–P during the removal process.

Suspension samples of the algae were centrifuged at 4,000 rpm for 10 min, discarded the supernatant. The algal cells in static state were extracted by 95% methanol surrounded by a 60°C water bath for 5 min. The absorbance of extracted supernatant was measured at 470, 645 and 665 nm using a UV/vis spectrophotometer, and the contents of chlorophyll-a, chlorophyll-b and carotenoid in the algal cell were calculated. The activity of these key enzymes was measured by assay kit including ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), Carbonic anhydrase (CA), pyruvate kinase (PK) and hexokinase kinase (HK).

2.3. Statistical analyses

All the data analysis was carried out with Statistic Package for Social Science (SPSS) 19.0. The results were examined by analysis of variance (ANOVA) to identify significant differences ($p < 0.05$). Figures analysis was produced using Originlab 8.5.

3. Results and discussion

3.1. Removal rate of cefradine under three nutritional patterns

C. pyrenoidosa, green algae used in this study, is widely used as nutritional feed additives for humans and animals [18]. A previous study indicated that there was no toxicity on the test organism, which introduced by the antibiotic cefradine itself or the reaction products after the algal treatment [19]. Our previous results indicated that the green alga, *C. pyrenoidosa*, performed high tolerance to the impact of antibiotic cefradine and the algae had a satisfactory growth capacity even if the concentration was at 50 mg/L [20]. Thus, the aim of this part is to evaluate the removal efficiency of the target antibiotic cefradine by the green algae which were driven by three kinds of nutritional patterns (autotrophic, heterotrophic and mixotrophic pattern, respectively). The residual rate of cefradine by the green algae under these nutritional patterns was presented in Fig. 1. In general, relevant lowest removal efficiency occurred when the algae were under the heterotrophic pattern (Group A-1). 75.95% and 82.35% cefradine was residue in Group H-4 and H-2, respectively. However, under the autotrophic pattern (Group A-1), only 10.39% antibiotic remained after a 72 h treatment, and cefradine was non-detected after 72 h treatment when the algae were under these mixotrophic patterns (Group M-1 to M-4). It suggests that the microalgae could be tolerant against the low N and P level and also provide removal capacity of the antibiotic, while more excellent performance occurred when the algae were under the mixotrophic pattern. To exhibit the antibiotic degradation characteristics, linear equations were used to fit the degradation kinetics of the antibiotic by the green algae under these nutritional patterns (in Table 2). Our results showed that strong linear correlations were obtained, regardless of patterns ($R^2 > 0.94$). In our study, the slope would reveal the removal rate of cefradine in a given period (h). The slope under the heterotrophic pattern was always lower than that under the autotrophic pattern, and more excellent performance occurred when the algae were under the mixotrophic pattern. This result provided our first hypothesis.

Table 1
Three nutritional patterns drove the algal removal process

Group	Nutritional patterns				
	NaAc (mg/L)	$NaNO_3$ (mg/L)	K_2HPO_4 (mg/L)	Light	Trophic pattern
H-1	–	–	–	–	Heterotrophic
H-2	–	250	50	–	Heterotrophic
H-3	–	500	100	–	Heterotrophic
H-4	500	250	50	–	Heterotrophic
H-5	500	500	100	–	Heterotrophic
A-1	–	–	–	12: 12 (L:D)	Autotrophic
M-1	–	250	50	12: 12 (L:D)	Mixotrophic
M-2	–	500	100	12: 12 (L:D)	Mixotrophic
M-3	500	250	50	12: 12 (L:D)	Mixotrophic
M-4	500	500	100	12: 12 (L:D)	Mixotrophic

Initial algal density: 10×10^6 cells/mL.
Cefradine concentration: 50 mg/L.

Table 2
Summary of the fitting results of cefradine degradation

Group	Best fitting linear	Equation	R^2	Final removal rate (%)
H-1	Linear	$y = 0.23x + 0.69$	0.9902	17.7
H-2	Linear	$y = 0.23x + 0.96$	0.9791	17.6
H-3	Linear	$y = 0.24x + 0.65$	0.9958	18.2
H-4	Linear	$y = 0.32x–0.02$	0.9913	24.1
H-5	Linear	$y = 0.28x + 1.03$	0.9832	22.2
A-1	Linear	$y = 1.32x–1.97$	0.98365	89.6
M-1	Linear	$y = 1.53x–5.10$	0.96142	100
M-2	Linear	$y = 1.43x–8.87$	0.95826	100
M-3	Linear	$y = 1.56x–5.02$	0.95731	100
M-4	Linear	$y = 1.58x–5.29$	0.94427	100

Performance was undesirable when the algae were under heterotrophic pattern. Although microalgae can switch their metabolism between autotrophic and heterotrophic, light is one of the important growth factors of the algae [12]. Improved capability of microalgae may be explained by enhanced photosynthesis. Light supply is one of the most important variables that influence the first step of photosynthesis and are also correlated with the sequent process. Thus, microalgae-mediated antibiotics treatment was highly dependent on the bio-activity of the algae. Our previous study also indicated that light intensity was related to the algal growth and finally influenced the removal efficiency [20]. Additionally, compared to that in Group A-1, Figs. 1b and c also showed that the superior ability to remove antibiotics occurred when the algae were under the mixotrophic pattern (Group M-1 to M-4). Nutrition regulation is usually viewed as an important and widely used enhancement method. Especially, exogenous organic carbon (EOC) has been applied to influence the metabolism of microorganisms and therefore promotes the removal of difficult-decomposition organic contaminants [21,22]. A previous study indicated that nitrogen and carbon sources act as an electron donor in the pharmaceutical compounds removal process [23]. Our previous work also indicated that glucose and NaAc was consumed as EOC to improve the treatment efficiency of cefradine and amoxicillin, respectively [19]. Thus, in the present study, not only organic carbon but also nitrogen and phosphorus has been considered. Our experimental design in this part was to evaluate whether and how the nutritional pattern (different concentrations of C, N, and P) could drive the algal treatment efficiency improvement. Fig. 1c indicates that there was a significant difference among the four groups (Group M-1, M-2, M-3, and M-4) in the mixotrophic pattern ($p < 0.05$). Our result could reveal that the effect of nitrogen and phosphorus was EOC-dependent. On one hand, under the EOC-free pattern (Group M-1 and M-2), the statistical result showed that the removal rate of cefradine at the relatively high concentration of N and P (Group M-2) was significantly lower than that at low concentration (Group M-1). While when NaAc was involved, a higher concentration of N and P drove higher removal efficiency (Group M-4).

Additionally, although Table 3 indicates that resident antibiotic was not detected in any group under the mixotrophic pattern (final removal rate was about 100%), statistical analysis showed that the removal rate of cefradine varied during the removal process. Compared with Fig. 1b (autotrophic pattern), the C/C_0 value in Fig. 1c (mixotrophic pattern) declined rapidly in the second step (20–40 h), which with a relatively slow decrease in the first step (0–20 h) and the last one (40–70 h), respectively. Significantly accelerated removal efficiency only occurred at the given period, which suggests a process-dependent effect occurred when organic carbon, nitrogen, and phosphorus were involved.

Population growth curves for the green algae during the removal process are presented in Fig. 2a. Generally, the algae exhibited satisfactory growth capacity during every period of the entire removal process. In the first period (0–24 h), the rate of population increased for the green algae under the mixotrophic pattern was significantly higher than that under the autotrophic one (Group A-1, $p < 0.05$). Considering that light was always provided both in the autotrophic and mixotrophic pattern, better growth capacity was might attribute to organic carbon, nitrogen, and phosphorus. Especially, there was no significant difference between that in Group A-1, Group M-1 and Group M-2, which indicated that nitrogen and phosphorus could not influence the algal growth

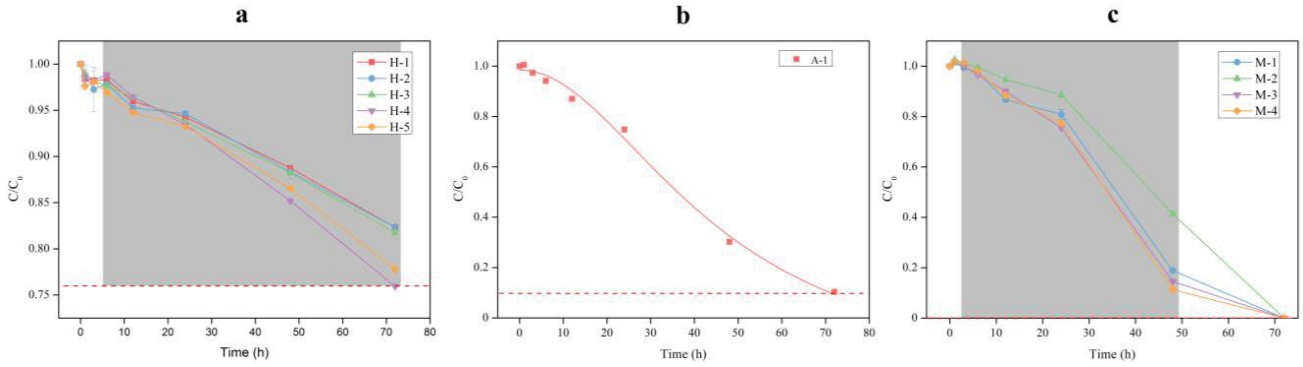

Fig. 1. Removal efficiency of cefradine drove by three nutritional patterns, heterotrophic pattern (a), autotrophic pattern (b) and mixotrophic pattern (c) (The shadow areas in figures represented significant differences ($p < 0.05$) among different groups).

Table 3
Summary of multivariate repeated measurements ANOVA analysis nutritional patterns on various algal responses

Factors	Dependent variables																	
	Removal rate		Density		Chlorophyll-a content		Chlorophyll-b content		Carotenoid content		CA activity		RuBisCO activity		HK activity		PK activity	
	F	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Time	94,477.79	<0.01	4,847.54	<0.01	9,176.70	<0.01	6,190.03	<0.01	2,618.65	<0.01	6,64.93	<0.01	8,45.22	<0.01	2,971.86	<0.01	5,447.78	<0.01
Carbon	NA	1.00	729.51	<0.01	927.94	<0.01	5.94	<0.05	685.91	<0.01	2,188.36	<0.01	2,191.25	<0.01	352.88	<0.01	8.88	<0.01
Nitrogen and phosphorus level	576.10	<0.01	63.60	<0.01	253.93	<0.01	135.21	<0.01	101.34	<0.01	505.92	<0.01	874.46	<0.01	766.55	<0.01	2,061.00	<0.01
Time × Carbon	10,747.81	<0.01	75.39	<0.01	87.56	<0.01	23.27	<0.01	102.78	<0.01	60.94	<0.01	14.53	<0.01	7.50	<0.01	11.87	<0.01
Time × Nitrogen and phosphorus level	3,651.41	<0.01	11.91	<0.01	59.80	<0.01	137.67	<0.01	144.34	<0.01	68.26	<0.01	96.64	<0.01	13.93	<0.01	416.69	<0.01
Carbon × Nitrogen and phosphorus level	NA	1.00	54.17	<0.01	22.89	<0.01	11.82	<0.01	7.90	<0.01	2.03	0.165	66.29	<0.01	2.63	0.11	73.77	<0.01
Time × Carbon × Nitrogen and phosphorus level	6,404.42	<0.01	20.76	<0.01	18.80	<0.01	0.17	0.841	30.41	<0.01	20.61	<0.01	1.39	0.26	0.32	0.73	4.79	<0.05

NA: not available.

independently. When NaAc was involved, the rate of population increased in Group M-3 and M-4 was 1.21 and 1.98 times of that in Group M-1 and Group M-2, respectively.

However, the trends have not occurred in the second period (24–48 h). The algae in Group M-1 and M-2 both performed high growth ability at this stage, and in the final period (48–72 h), the rate of population increased for the green algae in Group M-3 was higher than that in other groups. Our result indicated that, unlike the removal rate of the antibiotic, a higher concentration of N and P has not driven higher algal growth capacity even if NaAc was involved. Comparing the removal rates of the target compounds by the dead and living algal cells, respectively, our previous studies also implied that green algae play a dominant role in the removal of cephalosporin, including cefradine, depending on the population density and vitality [20,24]. Considering that the algal population density also changed during the removal process, the removal rate of the unit algal density per hour, i.e., the "cellular removal rate", should better reflect the removal capacity at the given period. Fig. 2b shows the average removal rates of the antibiotic by the unit algal density when the algae were under the autotrophic and mixotrophic pattern. The highest removal efficiency of the unit algal cells in different patterns was obtained in the second period (24–48 h). Generally, the removal capacity of the unit algal cell displayed a sequence of responses during the algae-mediated treatment in three steps: in step I, the algae received the stimulation of the changing pattern (N and P with/without NaAc) and released a possible compensatory response. In Step II, the algae accelerated the consumption of the antibiotic, and the algae performed a low-level increase in population while no more antibiotic could be consumed in step III. The results provide our hypothesis 2 that the response was period-specific. It's worth noting that compared with the group under the autotrophic pattern, the average removal rate of the unit algal cell not always increased when the algae were under the mixotrophic pattern. Our result provided the hypothesis 2 that a carbon linkage effect occurred when N and P were involved in the algae-mediated removal process. Under NaAc-free pattern (Group M-1 and M-2), the relative higher removal rate was obtained when a low concentration of N and P were involved (Group M-1), while higher concentration of N and P drove higher removal rate when NaAc was involved (Group M-4). Additionally, most antibiotics were removed in Group M-1, M-3 and M-4 after 48 h, while up to 40% antibiotic in Group M-2 was not removed in the first two periods (see in Fig. 1), the residual antibiotic, therefore, caused a relate high removal rate in this group in the last period.

3.2. Response of algal photosynthetic pigment under the nutritional patterns

The removal rate for the target antibiotic by the dead and the living alga was compared before the present study. It indicated that the living alga had a better removal capacity, which was about double that by the dead alga. The analysis of the metabolic product of the target antibiotics underwent the algal treatment was performed for revealing the algal biodegradation of the antibiotic involved (see supporting material). Thus, due to the green algae played a dominant role in the removal of the antibiotic, algal physiological response (such as photosynthetic pigment and carbon metabolism key enzymes) during the removal process should be considered. Our results in Fig. 1 showed that even exogenous C, N and P were added into the algal removal process, the removal efficiency and the final removal rate of the algae under the heterotrophic pattern were much lower than that under the mixotrophic one. It suggests that light also played a non-negligible part in the algal treatment. Photosynthetic pigments usually play a critical role when microalgae can capture light efficiently and convert light energy into chemical energy. Thus, the algal photosynthetic pigments under the mixotrophic pattern were also considered. The contents of three photosynthetic pigments under the autotrophic and mixotrophic patterns are presented in Fig. 3. Generally, the content of these three pigments increased during the algal removal process, regardless of nutritional stress. Our previous study pointed out that although algae could grow after exposure to the target antibiotic chlortetracycline, its photosynthesis function was

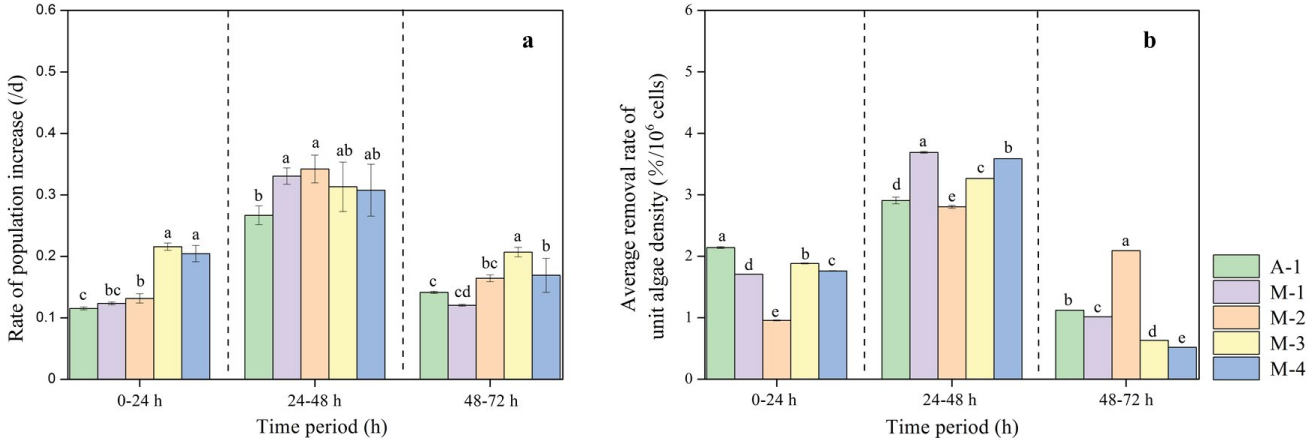

Fig. 2. Algal population growth curves (a) and removal rate of unit algae density (b) at the given period in the algae-mediated removal process.

also disrupted [25]. A decrease of chlorophyll-a content also occurred when *Microcystis aeruginosa* was exposed to the antibiotic cefradine in a relatively shorter-term (24 h), while conversely, for cefradine, the impact on the pigments did not occur in *C. pyrenoidosa* [20].

Fig. 3a shows that the accumulation of chlorophyll-a in Group M-1 and M-2 (low concentration of N and P) were significantly higher than that in Group M-2 and M-4, respectively. A previous study has also provided a similar result. Initial concentrations of both nutrients had a significant effect on the growth and chlorophyll-a content of the green algae *Scenedesmus obliquus* [26]. Additionally, the addition of NaAc significantly improved the level of chlorophyll-a ($p < 0.05$). After 72 h, compared with NaAc-free groups (Group M-1 and M-2), the content of chlorophyll-a in Group M-3 and M-4 increased by 5.73% and 8.45%, respectively. A previous study has also shown that the content of photosynthetic pigments in green algae increased when acetate substrate was added [27].

However, the increased total content of the given photosynthesis pigments was not representative of the algal light capture capacity. It is possible that the algal population growth caused the content of the increased pigment, while these contents in every algal cell might be declined simultaneously. Thus, due to the varied algal population in these groups, the rate of chlorophyll-a content increase of unit algae density in the above-mentioned three periods should be considered. It could better indicate the detail in every algal cell in the given period. The results in Figs. 3a-2, b-2, and c-2 show that the change of these three pigments content varied in these three periods. The content of chlorophyll-a increased during the algae-mediated removal process, regardless of the nutritional patterns. And in most groups, the peak value of the increased rate occurred in the second period (24–48 h). Additionally, the content of carotenoids showed a similar result at the same time period (0–24 h and 24–48 h). While the increased rate of chlorophyll-b decreased first and then increased. chlorophyll-a, the key light-capturing pigment, participates

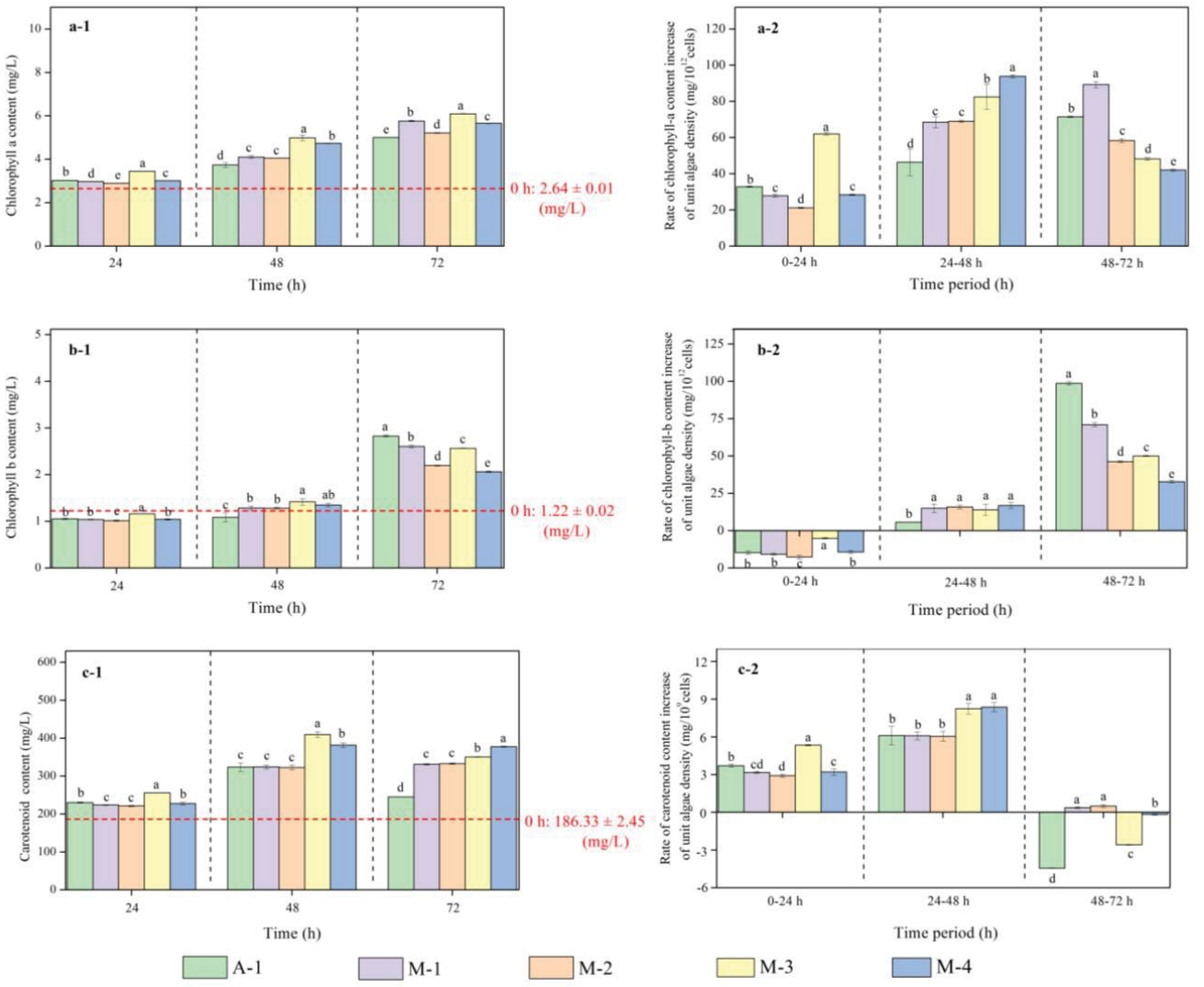

Fig. 3. Contents of the three photosynthetic pigments during 72 h algae-mediated removal process, including chlorophyll-a (a-1 and a-2), chlorophyll-b (b-1 and b-2) and carotenoid contents (c-1 and c-2).

directly in the light reaction of photosynthesis [28]. More chlorophyll-a means that more light could be captured and finally, more adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate could be provided for the subsequent Calvin cycle. The previous study also indicated that chlorophyll-a was improved during the removal process when CO_2 was added and it finally influenced the removal efficiency of the target antibiotic [20]. There was also a positive correlation between the photosynthetic performance and the removal rate. The most increased rate of chlorophyll-a content was obtained in the second period (24–48 h). This event was almost simultaneous with the removal rate (Fig. 2b). A previous study also suggested a positive relationship between leaf photosynthesis and leaf chlorophyll content has been widely observed in rice [29]. Therefore, we suggest that the enhanced algal photosynthesis drove the removal efficiency up. And, more remarkable, the accumulation chlorophyll-a content in Group A-1 (no nutrients addition) was significantly lower than that in other groups. The results showed that the content of chlorophyll is sensitive to the change of inorganic nutrients. For example, the cellular content of chlorophyll-a decreased when the algae under N-limit or P-limit condition [30]. Conversely, N and P in this study could also improve the cellular content of chlorophyll-a in 24–48 h, and there was no significant difference in the increased rate between high and low concentrations of N and P.

3.3. Response of algal carbon metabolism key enzymes under nutritional pattern

There are thousands of enzymes in the organism, which dominate different processes such as metabolism, nutrition, and energy conversion. Therefore, change in key enzyme content was also observed when the green algae underwent different nutritional patterns. Data in Fig. 4 indicate that the activity of CA reduced during the algae-mediated removal process under most nutritional patterns, especially, when EOC was involved (Group M-3 and Group M-4). Generally, there was a positive correlation between the photosynthetic performance and the activity of the enzyme, like CA and RuBisCO [31]. CO_2 could be generated act on the active site of RuBisCO under CA function, thereby improve the fixed efficiency of CO_2 in the presence of O_2 [32,33]. Our previous study also indicated that the activities of CA and RuBisCO of the algae changed when CO_2 at varying concentrations was added [20]. Previously reported results also indicated that the concentration of N and P could influence the level of RuBisCO and CA by restricting CO_2 assimilation [34,35]. In the present study, light and CO_2 were not considered as the nutritional pattern. Thus, a positive response of CA has not occurred, especially, when organic carbon, not the inorganic carbon was added. RuBisCO represents the capacity of the CO_2 fixture. Photosynthesis involves two processes, each with multiple steps. These two stages are known as light-dependent reactions (the photo part of photosynthesis) and light-independent reactions (carbon fixation). In our present study, the content of chlorophyll-a increased during the process (Fig. 2). As the principal photosynthetic pigment, more chlorophyll-a means the algae-mediated removal process could capture more light energy, contributing to faster startup of the light-independent reaction. Thus, a relatively high response values of RuBisCO were obtained in Group M-1 to M-4. Because of that, no more CO_2 was provided into the system, we also found out a process-dependent reduction of the enzymatic activity during the subsequent periods.

The activity of PK and HK under different nutritional patterns was also presented (Fig. 4). Generally, the activity of these two enzymes decreased during the whole algae-mediated removal process, regardless of the nutritional patterns. For PK, the statistical result showed that there was a significant difference ($p < 0.05$) between the group at the low concentration of N and P (Group M-1 and M-3) and a relatively higher one (Group M-2 and M-4). Additionally, at the end of the algae-mediated removal process, the activity of

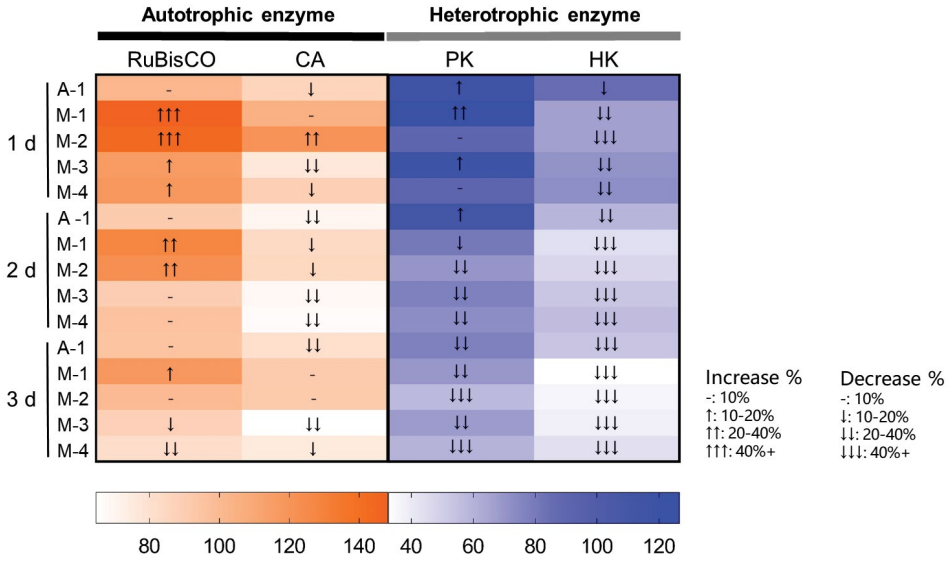

Fig. 4. Response of four key enzymes activities during the algae-mediated removal process, including ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), carbonic anhydrase (CA), pyruvate kinase (PK) and hexokinase kinase (HK).

HK only accounts for 53.82%, 32.59%, 36.02%, 38.13%, and 43.53% of that at the beginning of the process, respectively (Group A-1, M-1 to M-4). Respiratory metabolism is the primary energy supply pathway for algae. Embden–Meyerhof–Parnas (EMP) pathway is the process of transforming glucose into pyruvic acid, which is also an important part of the whole respiratory metabolic activity. HK, PK, and PKF are the three regulatory sites of this pathway, which regulate the respiration of cells [36]. Photophosphorylation and oxidative phosphorylation are two main energy produced processes in the algal cell. NaAc and the antibiotic could not be transferred into the oxidative phosphorylation process directly. Thus, the reduced activity of HK and PK indicated that ATP produced during the algae-mediated removal process was not dependent on the EMP pathway, especially under the mixotrophic pattern. Considering the results in Fig. 3, we noticed that the content of photosynthetic pigments increased during the algae-mediated removal process and the contribution of chlorophyll-a was improved simultaneously. After 48 h, the ratio of chlorophyll-a: chlorophyll-b: carotenoids in Group M-4 was 1.24:0.14:1 × 10^2, which was higher than that in Group A-1 (1.12:0.33:1 × 10^2). Due to that, the initial ratio before the algal treatment was 1.42:0.66:1 × 10^2, we could, therefore, find out that the ratio of chlorophyll-a among the photosynthetic pigment was improved mainly under the mixotrophic pattern. Compared with the activity of enzymes, the content of chlorophyll-a in the algal cell could be viewed as a positive response when C, N, and P were involved in the algae-mediated antibiotic removal process.

Exogenous nutrients have been applied to influence the metabolism of microorganisms and therefore promote the removal of the difficult-decomposition organic contaminants, such as antibiotics, fungicides, and hypnotics [21,22]. The previous study suggested that lincomycin was degraded effectively in a con-metabolic system with glucose as a growth substrate [37]. Co-metabolism is the mechanism that microbial metabolism could consume the external nutrition source, especially carbon source with a simple structure as the growth substrate to enable the body to grow, proliferate and maintain vitality and to induce some certain non-specific enzymes for transforming the organic contaminants (non-growth substrate) [38]. However, NaAc acted as an electron donor to enhance the ciprofloxacin removal by the freshwater microalga *Chlamydomonas mexicana* [39]. In this study, NaAc was not viewed as the substrate in the algal EMP pathway. Similarly, the previous study indicated that when the concentration of the added phosphorus increased from 0.5 to 5 mg/L, the biodegradation of amoxicillin by *Microcystis aeruginosa* increased from 11.5% to 28.2% [40]. It suggests that phosphorus affected photosynthesis and ATP-consumption. We have also observed increased algal population density and content of chlorophyll-a during the algae-mediated removal process which drove the removal efficiency up.

3.4. Consumption of NO_3–N, PO_4–P, and NaAc during the algal removal process

The consumption of NO_3–N, PO_4–P, and NaAc during the algae-mediated removal process is presented in Fig. 5. Generally, after 72 h, PO_4–P was mainly consumed completely, regardless of the concentration of P (>97.0%). Phosphorous is also a key factor in the energy metabolism of microalgae. Inorganic phosphates play an important role in microalgae cell growth and metabolism [41]. A previous study reported that 99% of initial PO_4–P was removed by *Chlorella vulgaris* [42]. In the present study, more than 83% phosphorus consumed in the first period, which also showed a strong ability of *C. pyrenoidosa* to uptake of P [43]. In contrast, the consumption of NO_3–N and NaAc (Figs. 5a-1 and 5c-1) was relatively low, ranged between 9.6%–32.9% and 7.5%–36.2%, respectively. And, more remarkable, the consumption level of NO_3–N, and NaAc was be related to the concentration of N and P. Carbon is the essential component for algal growth. Nitrogen, as the basic element in the synthesis of nucleic acid, protein, and chlorophyll, plays an important role in the growth and metabolism of algae cells. In the present study, more NO_3–N and NaAc could be consumed when the algae were under the relatively low concentration of N and P. The rate of C, N, and P usually influences the consumption. For example, the removal rate of total nitrogen (TN) is reduced to less than 30% when the ratio of N and P is higher than 26 or below 3.7 [44]. In the current study, it indicated that the algae would uptake more organic carbon at low concentration of N and P, which might be attributed to an opportune rate of C, N, and P. On the other hand, the influence of the nutrient consumption taking by the target antibiotic has also been investigated. The consumption of NO_3–N and NaAc in the cefradine treatment groups were significantly lower than those in the antibiotic-free groups ($p < 0.05$) under the low concentration of N and P condition, meanwhile there was no significant difference between different groups under the high concentration ($p > 0.05$). The previous study indicated that significant negative correlations were detected between the tetracycline concentrations and the removal rates of total nitrogen (TN) and total phosphorus (TP) by *Chlamydomonas reinhardtii* [45]. There was a stronger inhibitory effect of tetracycline on the removal of TN than that of TP. Similar results were also observed in our present study.

4. Conclusion

In this study, we evaluated the potential of an algae-mediated antibiotic treatment which drove by three nutritional patterns. Generally, the removal efficiency and the corresponding algal performance varied under different nutritional patterns and the removal efficiency of the algal removal process could be exploited enough which drove by the mixotrophic pattern. The removal capacity of the unit algal cell displayed a sequence of responses during the algae-mediated removal process in three steps. The enhanced algal photosynthesis drove the removal efficiency up. Additionally, the effect of nitrogen and phosphorus was EOC-dependent. When NaAc was involved, a higher concentration of N and P drove higher removal efficiency. PO_4–P was mainly consumed completely, regardless of the concentration of P. However, the total consumption level of NO_3–N and NaAc were relatively lower than that of P but relative more NO_3–N and NaAc in total could be consumed when the algae were under the relatively low concentration of N and P. Thus, the algal antibiotic process, as a cleaner treatment,

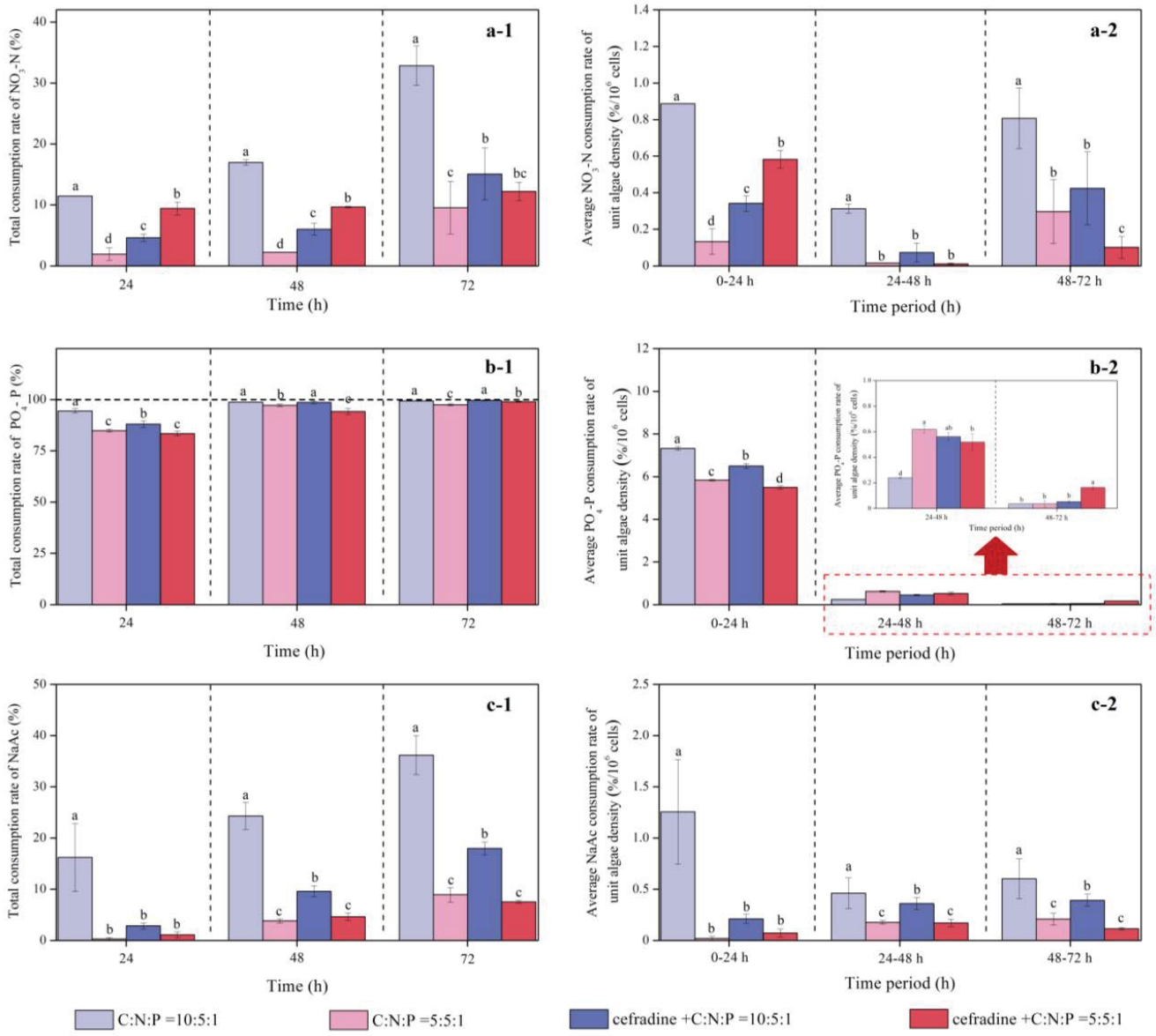

Fig. 5. Consumption of NO_3–N, PO_4–P and NaAc during the algae removal process which was driven by the optimum nutritional pattern.

could perform excellent potential in industrial applications in future.

Acknowledgments

This work was supported by the Jiangsu Key Lab of Environmental Engineering Open Foundation (KF2018005), the National Natural Science Foundation of China (21876207), China Postdoctoral Science Foundation (2018M640540), Double First-Class University project (CPU2018GY21, 24), College Students Innovation Project for the R&D of Novel Drugs (J1310032), College Undergraduate Training Program for Innovation and Entrepreneurship (201810316076G, 2018 10316077G) and National Fund for Fostering Talents of Basic Science (NFFTBS).

References

[1] F. Yin, H. Dong, W. Zhang, Z. Zhu, B. Shang, Antibiotic degradation and microbial community structures during acidification and methanogenesis of swine manure containing chlortetracycline or oxytetracycline, Bioresour. Technol., 250 (2017) 247–255.
[2] A. Gulkowska, H.W. Leung, M.K. So, S. Taniyasu, N. Yamashita, L.W.Y. Yeung, B.J. Richardson, A.P. Lei, J.P. Giesy, P.K.S. Lam, Removal of antibiotics from wastewater by sewage treatment facilities in Hong Kong and Shenzhen, China, Water Res., 42 (2008) 395–403.
[3] W.W. Li, H.Q. Yu, B.E. Rittmann, Chemistry: reuse water pollutants, Nature, 528 (2015) 29–31.
[4] W. Baran, E. Adamek, M. Jajko, A. Sobczak, Removal of veterinary antibiotics from wastewater by electrocoagulation, Chemosphere, 194 (2017) 381–389.

[5] D. Hoh, S. Watson, E. Kan, Algal biofilm reactors for integrated wastewater treatment and biofuel production: a review, Chem. Eng. J., 287 (2016) 466–473.
[6] H. Zou, Y. Wang, Phosphorus removal and recovery from domestic wastewater in a novel process of enhanced biological phosphorus removal coupled with crystallization, Bioresour. Technol., 211 (2016) 87–92.
[7] Y. Liu, S. Chen, J. Zhang, B. Gao, Growth, microcystin-production and proteomic responses of *Microcystis aeruginosa* under long-term exposure to amoxicillin, Water Res., 93 (2016) 141–152.
[8] V. Homem, L. Santos, Degradation and removal methods of antibiotics from aqueous matrices – a review, J. Environ. Manage., 92 (2011) 2304–2347.
[9] R. Muñoz, B. Guieysse, Algal-bacterial processes for the treatment of hazardous contaminants: a review, Water Res., 40 (2006) 2799–2815.
[10] J.K. Kim, B.H. Um, T.H. Kim, Bioethanol production from micro-algae, *Schizocytrium* sp., using hydrothermal treatment and biological conversion, Korean J. Chem. Eng., 29 (2012) 209–214.
[11] J.Q. Xiong, M.B. Kurade, B.H. Jeon, Can microalgae remove pharmaceutical contaminants from water?, Trends Biotechnol., 36 (2018) 30–44.
[12] S.T. Rier, R.J. Stevenson, Effects of light, dissolved organic carbon, and inorganic nutrients [2pt] on the relationship between algae and heterotrophic bacteria in stream periphyton, Hydrobiologia, 489 (2002) 179–184.
[13] J.Y. Joung, H.W. Lee, H. Choi, M.W. Lee, J.M. Park, Influences of organic loading disturbances on the performance of anaerobic filter process to treat purified terephthalic acid wastewater, Bioresour. Technol., 100 (2009) 2457–2461.
[14] A.M. Yehia, H.T. Elbalkiny, S.M. Riad, Y.S. Elsaharty, Chemometrics for resolving spectral data of cephalosporines and tracing their residue in waste water samples, Spectrochim. Acta, Part A, 219 (2019) 436–443.
[15] S. Coenen, M. Ferech, F.M. Haaijerruskamp, C.C. Butler, R.H. Vander Stichele, T.J. Verheij, D.L. Monnet, P. Little, H. Goossens, European surveillance of antimicrobial consumption (ESAC): quality indicators for outpatient antibiotic use in Europe, Qual. Health Care, 16 (2007) 440–445.
[16] S. Zhang, J. Wang, Y. Chen, S. Wang, L. Luo, Y. Luo, H. Liu, Determination of ketoprofen and sodium acetate in high concentration of ketoprofen injection by HPLC, Lat. Am. J. Pharm., 36 (2017) 448–455.
[17] P.S. Chen, T.Y. Toribara, H. Warner, Microdetermination of phosphorus, Anal. Chem., 28 (1956) 215–216.
[18] E.W. Becker, Micro-algae as a source of protein, Biotechnol. Adv., 25 (2007) 207–210.
[19] Y. Du, J. Wang, Z. Wang, O.L. Torres, R. Guo, J. Chen, Exogenous organic carbon as an artificial enhancement method to assist the algal antibiotic treatment system, J. Cleaner Prod., 194 (2018) 624–634.
[20] Y. Du, J. Wang, H. Li, S. Mao, D. Wang, Z. Xiang, R. Guo, J. Chen, The dual function of the algal treatment: antibiotic elimination combined with CO_2 fixation, Chemosphere, 211 (2018) 192–201.
[21] K.M. Onesios, J.T. Yu, E.J. Bouwer, Biodegradation and removal of pharmaceuticals and personal care products in treatment systems: a review, Biodegradation, 20 (2009) 441–466.
[22] A. Dawas-Massalha, S. Gur-Reznik, S. Lerman, I. Sabbah, C.G. Dosoretz, Co-metabolic oxidation of pharmaceutical compounds by a nitrifying bacterial enrichment, Bioresour. Technol., 167 (2014) 336–342.
[23] N.H. Tran, T. Urase, O. Kusakabe, The characteristics of enriched nitrifier culture in the degradation of selected pharmaceutically active compounds, J. Hazard. Mater., 171 (2009) 1051–1057.
[24] R. Guo, J. Chen, Application of alga-activated sludge combined system (AASCS) as a novel treatment to remove cephalosporins, Chem. Eng. J., 260 (2015) 550–556.
[25] R.X. Guo, J.Q. Chen, Phytoplankton toxicity of the antibiotic chlortetracycline and its UV light degradation products, Chemosphere, 87 (2012) 1254–1259.
[26] A. ÇElekli, M. Balcı, The influence of different phosphate and nitrate concentrations on growth, protein and chlorophyll-a content of *Scenedesmus obliquus*, Fresenius Environ. Bull., 18 (2009) 1363–1366.
[27] T.A. Sarma, G. Ahuja, J.I. Khattar, Effect of nutrients and aeration on O_2 evolution and photosynthetic pigments of *Anabaena torulosa* during akinete differentiation, Folia. Microbiol., 45 (2000) 434–438.
[28] A. Patel, S. Tiwari, S.M. Prasad, Toxicity assessment of arsenate and arsenite on growth, chlorophyll-a fluorescence and antioxidant machinery in *Nostoc muscorum*, Ecotoxicol. Environ. Saf., 157 (2018) 369–379.
[29] T. Takai, M. Kondo, M. Yano, T. Yamamoto, A quantitative trait locus for chlorophyll content and its association with leaf photosynthesis in rice, Rice, 3 (2010) 172–180.
[30] Y. Du, Y. Feng, R. Guo, J. Chen, Enhancement by the artificial controlled culture for the algal treatment of antibiotic ceftazidime: a three-step response performance and high-removal efficiency, RSC Adv., 5 (2015) 72755–72763.
[31] I.E. Huertas, B. Colman, G.S. Espie, L.M. Lubian, Active transport of CO_2 by three species of marine microalgae, J. Phycol., 36 (2000) 314–320.
[32] J.R. Xia, K.S. Gao, Impacts of elevated CO_2 concentration on biochemical composition, carbonic anhydrase, and nitrate reductase activity of freshwater green algae, J. Integr. Plant Biol., 47 (2005) 668–675.
[33] P.A. Fernandez, M.Y. Roleda, C.L. Hurd, Effects of ocean acidification on the photosynthetic performance, carbonic anhydrase activity and growth of the giant kelp *Macrocystis pyrifera*, Photosynth. Res., 124 (2015) 293–304.
[34] M.J.D. Rio, Z. Ramazanov, G.G. Reina, Effect of nitrogen supply on photosynthesis and carbonic anhydrase activity in the green seaweed *Ulva rigida* (*Chlorophyta*), Mar. Biol., 123 (1995) 687–691.
[35] J. Beardall, A. Johnston, J. Raven, Environmental regulation of CO_2-concentrating mechanisms in microalgae, Can. J. Bot., 76 (1998) 1010–1017.
[36] G. Tong, M. Yulong, G. Peng, X. Zirong, Antibacterial effects of the Cu(II)-exchanged montmorillonite on *Escherichia coli* K88 and *Salmonella choleraesuis*, Vet. Microbiol., 105 (2005) 113–122.
[37] Y. Li, J. Zhou, B. Gong, Y. Wang, Q. He, Cometabolic degradation of lincomycin in a sequencing batch biofilm reactor (SBBR) and its microbial community, Bioresour. Technol., 214 (2016) 589–595.
[38] K. Fischer, M.J.A.M. Majewsky, Cometabolic degradation of organic wastewater micropollutants by activated sludge and sludge-inherent microorganisms, Appl. Microbiol. Biotechnol., 98 (2014) 6583–6597.
[39] J.Q. Xiong, M.B. Kurade, J.R. Kim, H.S. Roh, B.H. Jeon, Ciprofloxacin toxicity and its co-metabolic removal by a freshwater microalga *Chlamydomonas mexicana*, J. Hazard. Mater., 323 (2016) 212–219.
[40] L. Ying, C. Shi, C. Xiao, Z. Jian, B. Gao, Interactions between *Microcystis aeruginosa* and coexisting amoxicillin contaminant at different phosphorus levels, J. Hazard. Mater., 297 (2015) 83–91.
[41] T. Fazal, A. Mushtaq, F. Rehman, A.U. Khan, N. Rashid, W. Farooq, M.S.U. Rehman, J. Xu, Bioremediation of textile wastewater and successive biodiesel production using microalgae, Renewable Sustainable Energy Rev., 82 (2018) 3107–3126.
[42] G. Markou, D. Iconomou, K. Muylaert, Applying raw poultry litter leachate for the cultivation of *Arthrospira platensis* and *Chlorella vulgaris*, Algal Res., 13 (2016) 79–84.
[43] L. Wang, M. Min, Y. Li, P. Chen, Y. Chen, Y. Liu, Y. Wang, R. Ruan, Cultivation of green algae *Chlorella* sp. in different wastewaters from municipal wastewater treatment plant, Appl. Biochem. Biotechnol., 162 (2010) 1174–1186.
[44] H.J. Choi, S.M. Lee, Effect of the N/P ratio on biomass productivity and nutrient removal from municipal wastewater, Bioprocess. Biosyst. Eng., 38 (2015) 761–766.
[45] J. Li, X. Zheng, K. Liu, S. Sun, X. Li, Effect of tetracycline on the growth and nutrient removal capacity of *Chlamydomonas reinhardtii* in simulated effluent from wastewater treatment plants, Bioresour. Technol., 218 (2016) 1163–1169.