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Original Research

Advanced wastewater treatment with microalgae-indigenous bacterial interactions

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ABSTRACT

Microalgal-indigenous bacterial wastewater treatment (MBWT) emerges as a promising approach for the concurrent removal of nitrogen (N) and phosphorus (P). Despite its potential, the prevalent use of MBWT in batch systems limits its broader application. Furthermore, the success of MBWT critically depends on the stable self-adaptation and synergistic interactions between microalgae and indigenous bacteria, yet the underlying biological mechanisms are not fully understood. Here we explore the viability and microbial dynamics of a continuous flow microalgae-indigenous bacteria advanced wastewater treatment system (CFMBAWTS) in processing actual secondary effluent, with a focus on varying hydraulic retention times (HRTs). The research highlights a stable, mutually beneficial relationship between indigenous bacteria and microalgae. Microalgae and indigenous bacteria can create an optimal environment for each other by providing essential cofactors (like iron, vitamins, and indole-3-acetic acid), oxygen, dissolved organic matter, and tryptophan. This collaboration leads to effective microbial growth, enhanced N and P removal, and energy generation. The study also uncovers crucial metabolic pathways, functional genes, and patterns of microbial succession. Significantly, the effluent NH_4^+-N and P levels complied with the Chinese national Class-II, Class-V, Class-IA, and Class-IB wastewater discharge standards when the HRT was reduced from 15 to 6 h. Optimal results, including the highest rates of CO_2 fixation (1.23 g L^{-1}), total energy yield (32.35 kJ L^{-1}), and the maximal lipid (33.91%) and carbohydrate (41.91%) content, were observed at an HRT of 15 h. Overall, this study not only confirms the feasibility of CFMBAWTS but also lays a crucial foundation for enhancing our understanding of this technology and propelling its practical application in wastewater treatment plants.

1. Introduction

Currently, nitrogen (N) and phosphorus (P) in secondary effluent (SE) usually fail to reach the stringent wastewater discharge standards due to low carbon (C) source content and imbalanced C:N:P ratio in the secondary wastewater treatment process of the wastewater treatment plants (WWTPs) (Table S1) [1,2]. Therefore, it is necessary to polish the overloaded N (especially nitrate nitrogen (NO_3^--N) and ammonia nitrogen (NH_4^+-N)) and P in SE using an effective advanced treatment [3]. However, the common physio-chemical and membrane advanced treatment technologies, such as advanced oxidation, activated carbon adsorption, and membrane process, face enormous challenges due to high operational costs and considerable N and P resource waste [4–7]. As reported, total phosphorus (TP) and NH_4^+-N recovery efficiencies are 35.7% and 35.8% in China [8]. Moreover, massive carbon emission from conventional biological advanced N and P removal processes is also a major issue for WWTPs, with documented carbon emission intensities exceeding 0.99, 1.17, 0.68, and 0.72 kg CO_2eq kWh^{-1} in South Africa, China, Germany, and the United States, respectively [9]. Based on these, an economical, ecologically friendly, and efficient advanced treatment technology should be developed to overcome these bottlenecks.

Microalgal-bacterial wastewater treatment systems (MBWTS) are a proven sustainable alternative for achieving high CO_2 fixation

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and value-added component accumulation while removing P and N in wastewater without providing additional carbon sources [10,11]. Nevertheless, some demerits must be overcome for large-scale application of MBWTS. Artificial wastewater has been widely used in MBWTS, hindering its practical application. Mixing microalgae with activated sludge is usually an approach to establishing MBWTS, which will enhance biomass harvesting, prolong the culture time, increase the operating cost, and limit further development [12]. Researchers have recently tried exploiting a novel MBWTS (microalgae-based real wastewater treatment system) to promote practical application and sustainability. Microalgae have been shown to cooperate with bacteria in real wastewater (RW) to generate microalgal-bacterial flocs, accumulate carbohydrates and lipids, and improve the effluent quality of RW [13]. Despite this promising breakthrough, the self-adaptation and synergistic interactions between microalgae and indigenous bacteria remain unclear. Undeniably, self-adaptation between microalgae and indigenous bacteria in MBWTS is a prerequisite for sustaining microbial activity, function, and interaction, as well as enhancing the production of energy-rich compounds (carbohydrate or lipid) and the removal of N and P. Microalgae and indigenous bacteria can provide stable self-adaption environments for each other by inhibiting undesirable factors, uptaking N and P, and supplying cofactors and nutrients. The synergistic interaction of these self-adaptation factors can promote microbial growth, nutrient exchange, complete nutrient removal, and energy production. However, the detailed mechanisms, corresponding metabolic pathways, and related functional genes remain unclear. Furthermore, research on microbial succession, the mechanisms of N and P removal between microalgae and indigenous bacteria, CO_2 fixation, and biomass harvesting is still limited, hampering robust support for practical applications.

Besides, microalgal species and batch systems are always major limiting factors for the large-scale expansion of MBWTS. Not all microalgal species can coexist with bacteria and improve biomass harvest in complex wastewater environments [14]. The uncertainty surrounding the optimal microalgal species selection persists due to practical considerations. Batch systems, such as fixed volumes, complex conditions, and long-term operation, pose obstacles to their feasibility on a large scale [15]. In contrast, continuous flow systems (CFS) offer a promising solution by addressing these drawbacks, making them an ideal choice for transitioning MBWTS from lab-scale to practical implementation. Despite this, the feasibility of a continuous flow microalgal-indigenous bacterial advanced wastewater treatment system (CFMBAWTS) for treating real SE is unclear. For CFMBAWTS, hydraulic retention times (HRTs) affect nutrient loading and microbial activity, diversity, and interaction, thereby affecting the overall performance of the wastewater treatment process—an aspect that warrants further exploration [16].

To fill this research gap, this study constructed a CFMBAWTS and determined appropriate microalgal species for treating SE. The study delved into the performance of the CFMBAWTS under different HRTs. Specifically, the variations in N and P, biomass, chlorophyll content, CO_2 fixation, carbohydrates, and lipids were monitored under long-term continuous operation (120 days). HRTs were selected to meet different N and P discharge standards, including Class-II, Class-V, Class-IA, and Class-IB. Finally, the stable self-adaptation mechanisms, synergistic interaction, N and P removal processes, and microbial composition were determined to thoroughly understand the stability and feasibility of CFMBAWTS. This work provides a valuable reference for the applicability of achieving low energy consumption and high-efficiency microalgae-based advanced wastewater treatment, which contributes to promoting the development of microalgae wastewater treatment units under strict wastewater discharge standards.

2. Materials and methods

2.1. Wastewater characteristics

The SE (Raw) used in this study was obtained from a local municipal wastewater treatment plant. The average influent chemical oxygen demand (COD), NO_3^--N, NH_4^+-N, and TP concentrations were 56.34, 4.12, 22.22, and 2.75 mg L^{-1}, respectively. The SE was applied unaltered, without sterilization or additional pretreatment.

2.2. Microalgae species

The microalgal strains *Scenedesmus* sp. F2, *Chlamydomonas* sp. JSC4, and *Chlorella* sp. MA1 were individually isolated from Kaohsiung and Harbin, China. These strains were specifically selected due to their remarkable environmental tolerance, rapid growth rates, and high N and P removal ability. Detailed characteristics of the microalgal cells can be found in Table S2.

2.3. Continuous flow system

A CFMBAWTS was constructed with a specialized continuous flow photobioreactor fed with SE and logarithmic phase microalgae. Throughout the process, influent was introduced from the bottom inlet, while the effluent was discharged through the top outlet. The operating conditions were as follows: temperature 23 ± 2 °C, light intensity 200 μmol m^{-2} s^{-1}, 2.5% CO_2 with a flow rate of 0.04 vvm, pH 7–8, HRTs 15–6 h.

The three microalgal strains (F2, JSC4, and MA1) were established in three CFMBAWTS. Following this, N and P concentrations, biomass, and CO_2 fixation were investigated under the same HRT to determine a preferable microalgal strain for the subsequent experiments.

Following this, tests of CFMBAWTS were divided into four stages: stage I (1–30 days, HRT1 = 15 h), stage II (31–60 days, HRT2 = 12 h), stage III (61–90 days, HRT3 = 8 h), and stage IV (91–120 days, HRT4 = 6 h). In addition, each stage comprised an initial self-adaptation period and a stable self-adaptation period between microalgae and bacteria. The initial self-adaptation period was the foundation for establishing a reliable relationship between microalgae and bacteria, while the stable self-adaptation period was essential to ensure the continuous operation of the CFMBAWTS. Upon reaching the prescribed discharge standards for N and P concentrations for the first time, it signified the initiation of a favorable initial self-adaptation phase. This milestone marked the establishment of an initial and synergistic metabolic bio-system. Subsequently, N and P removal efficiencies remaining constant for over 15 cycles indicated that microalgae and bacteria could continuously adapt, entering a stable self-adaptation period characterized by a stable synergistic interaction. These four stages persisted for over 120 days, each maintaining a stable self-adaptation period lasting 30 days.

2.4. Analytical procedures

The concentrations of COD, TP, NH_4^+-N, and NO_3^--N were measured according to methods as described in prior work [17]. pH was monitored by a pH/Oxi 3420i analyzer (HQ40d, HACH, the United States). Various parameters such as biomass concentration, chlorophyll content, carbohydrate content and composition, fatty acid composition, and lipid content were analyzed using previously reported methods [13,18]. Energy yield and the amounts of CO_2 fixed were calculated following methodologies outlined in the previous report [19].

2.5. *Microbial community and functional genes analysis*

The total DNA from samples in the stable self-adaptation period was extracted using the CTAB/SDS method. The 16S and 18S rRNA genes were amplified with V3—V4 and V4 primer pairs, 341F/805R and 528F/706R, respectively. Detailed PCR reaction conditions were used as previously reported [20]. The purified PCR products of all samples were obtained by Qiagen Gel Extraction Kit (Qiagen, Germany) and then sequenced. The sequencing process was performed on an Illumina NovaSeq platform with paired-end reads of 250 bp. The pollutant removal pathways and functional genes were annotated based on KEGG databases.

3. Results and discussion

3.1. *Selection of the preferable microalgal strain for CFMBAWTS*

As shown in Table S2 and Figs. S1—2, the bacteria in SE did not inhibit microalgal growth in the CFMBAWTS. The biomass concentration of F2 (1.64 g L^{-1}) was higher than that in MA1 (1.57 g L^{-1}, $p < 0.05$) and JSC4 (1.53 g L^{-1}, $p < 0.05$), and no significant differences were observed ($p > 0.05$) between MA1 and JSC4. These results suggest that F2 has a higher tolerance to real SE than MA1 and JSC4. Consistent with previous reports, *Scenedesmus* sp. had higher wastewater tertiary treatment capabilities than *Chlorella* sp., exhibiting higher biomass levels (1.58—1.79 g L^{-1}) and a wider tolerance range for N:P ratio [21]. CO_2 fixation also followed the trend F2 > MA1 > JSC4, with statistically significant differences noted between each pair of strains (F2 vs. MA1, $p < 0.05$; F2 vs. JSC4, $p < 0.05$), except for MA1 vs. JSC4 ($p > 0.05$). This implied that F2 excelled in mitigating CO_2 emissions. This finding aligns with the work of Arbib et al. (2014), who reported a lower CO_2 consumption rate for *Chlorella* sp. compared to *Scenedesmus* sp [21]. Additionally, F2 had superior sedimentation, followed by JSC4 and MA1 (Table S2). This observation could be attributed to the distinct morphology of each strain. Specifically, MA1 with lower dry weight and smaller cell size may prefer suspension in the solution. Conversely, F2 with higher dry weight, larger cells, and unique morphological characteristics, such as flagellum, aggregates of four cells, and large contact surface, tend to form microbial flocs conducive to gravity settling.

The influent's average NH_4^+-N, NO_3^--N, and TP concentrations were 23.22, 4.29, and 2.75 mg L^{-1}, respectively (Figs. S1a—c). At an HRT of 15 h, TP concentrations in the effluents from all reactors were zero, whereas the NH_4^+-N and NO_3^--N concentrations of F2-CFMBAWTS, JSC4-CFMBAWTS, and MA1-CFMBAWTS were below 0.30 and 0.5, 4.5 and 0.7, and 3 and 0.7 mg L^{-1}, respectively. Notably, only the F2-CFMBAWTS upgraded the NH_4^+-N and P concentration in effluent from Class-II of GB18918-2002 to Class-II of GB3838-2002 discharge standard, demonstrating better potential for treating SE. These findings underscore the self-adaption ability of strain F2 in CFMBAWTS due to its superior N and P removal abilities and ease of biomass harvesting. Consequently, F2 was chosen for further experiments.

3.2. *Effect of HRTs on CFMBAWTS performance*

To explore the performance of CFMBAWTS, microalgal biomass, chlorophyll content, CO_2 fixation, and N and P concentrations in the effluents were investigated under different HRTs (Fig. 1). The microalgal biomass and chlorophyll content were consistent with changes in HRTs. High microalgal biomass (1.65 g L^{-1}) and chlorophyll content (14.54 mg L^{-1}) were obtained at 15 h of HRT, while the lowest microalgal biomass (1.38 g L^{-1}) and chlorophyll content (8.45 mg L^{-1}) were obtained when HRT was 6 h. These results indicate that higher influent flow rates can increase nutrient load and enhance microalgal biomass productivity, consistent with previously reported literature [22]. Similarly, the CO_2 fixation rate (0.12 g L^{-1} h^{-1}) was significantly increased under a higher nutrient load. The highest CO_2 fixation (1.23 g L^{-1}) was observed at an HRT of 15 h, indicating that CO_2 fixation ability was closely related to microalgal growth.

HRT influenced the removal of NH_4^+-N, NO_3^--N, and TP during long-term operation. TP could be fully removed when the HRT was higher than 6 h, while NH_4^+-N and NO_3^--N concentrations were 0.07—0.33 and 0.22—0.46, 0.56—1.14 and 0.45—0.77, 2.73—4.12 and 0.70—1.56, and 4.70—6.40 and 1.26—2.64 mg L^{-1} when HRTs were 15, 12, 8, and 6 h, respectively. This indicates that microalgae can assimilate more NH_4^+-N, NO_3^--N, and P and achieve satisfactory water quality for the effluent discharge at longer HRTs. Notably, the NH_4^+-N and P concentrations in effluent complied with Class-II and Class-V (GB3838-2002), as well as Class-IA and Class-IB (GB18918-2002) wastewater discharge standards when HRTs were 15, 12, 8, and 6 h, respectively (Table S1).

3.3. *Effect of HRTs on energy production potential in CFMBAWTS*

Biodiesel and bioethanol are alternative energy sources [23]. Microalgae with rich lipid or carbohydrate contents can be superior precursors for producing biodiesel or bioethanol [24]. The lipid and carbohydrate content and composition, along with the energy yield, were investigated to understand the energy production potential of CFMBAWTS (Fig. 2). The highest total energy yield (32.35 kJ L^{-1}) was achieved when HRT increased from 6 to 15 h, composed of lipids (20.36 kJ L^{-1}) and carbohydrates (11.99 kJ L^{-1}). The highest lipid (33.91%) and carbohydrate (41.91%) contents were obtained when HRT was 15 h. In addition, similar fatty acid and carbohydrate compositions were observed under different HRTs. The glucose content was highest, followed by arabinose and xylose, regardless of HRT changes, indicating that the microalgal biomass could provide a great source material for generating bioethanol (Fig. 2a) [25]. Moreover, microalgae can synthesize considerable amounts of C16—C18 fatty acids suitable for making biodiesel [26]. The percentage of oleic acid (C18:1) is the key factor influencing biodiesel quality as it can largely improve oxidation stability and cold flow properties [27]. Fig. 2b showed that C18:1 was the dominant fatty acid in strain F2-CFMBAWTS, implying that this strain could effectively convert C, N, and P sources from SE to high-quality bioethanol or biodiesel. This characteristic enhances the environmental friendliness of CFMBAWTS and contributes to its economic feasibility.

3.4. *Effect of HRTs on microbial succession in CFMBAWTS*

To reveal the biological self-adaption and synergistic interaction mechanism of CFMBAWTS, the changes of microbial communities under different HRTs were investigated (Fig. 3). As seen in Fig. 3d, Chlorophyta dominated the composition at various HRTs, significantly surpassing its presence in SE. This observation suggests that the N and P levels in SE are favorable nutrients for growing microalgae, and microalgae can adapt to survive in SE due to their excellent self-adaptation abilities [28]. Meanwhile, bacteria supply vitamins, siderophores, growth hormones, and other compounds that promote microalgal growth [29]. Furthermore, the percentage of Chlorophyta increased with increasing HRTs, demonstrating that microalgae could assimilate more N, P, and nutrients from wastewater to achieve higher cell growth, ultimately resulting in lower effluent N and P concentrations.

In Fig. 3a, Proteobacteria, Bacteroidetes, and Firmicutes were the most abundant bacterial phyla in all groups. Compared with the

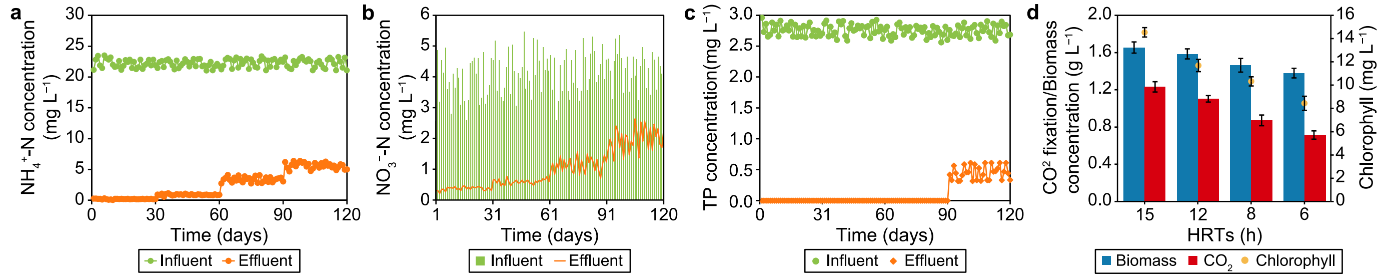

Fig. 1. Variations of NH$_4^+$-N, NO$_3^-$-N, and TP concentrations and characteristics of strain F2 in CFMBAWTS: **a**, Influent and effluent of NH$_4^+$-N; **b**, Influent and effluent of NO$_3^-$-N; **c**, Influent and effluent of TP; **d**, Biomass concentration, chlorophyll content, and carbon fixation ability of strain F2.

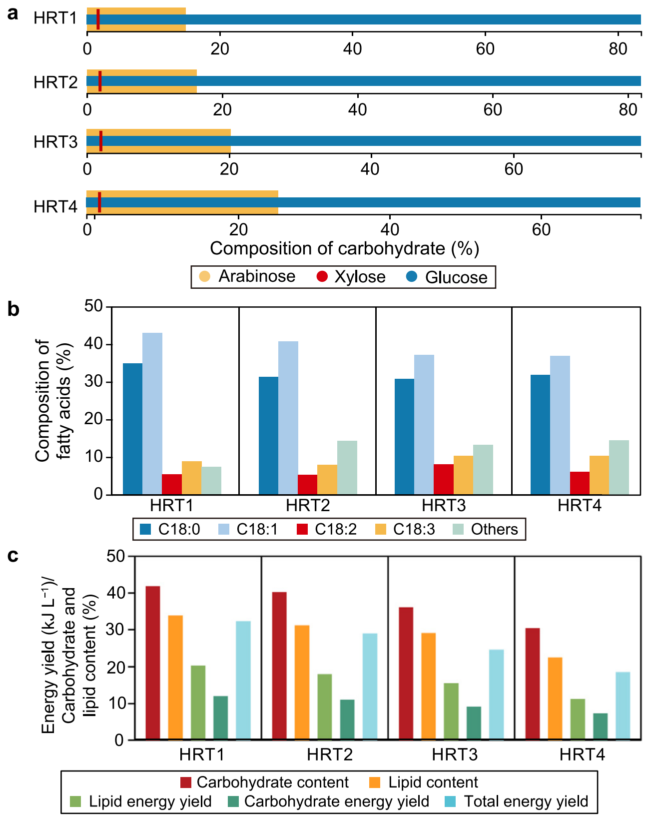

Fig. 2. Energy production of F2-CFMBAWTS: **a**, The compositions of carbohydrates; **b**, The compositions of fatty acids; **c**, The contents of lipids and carbohydrates, and the energy yield.

Raw, the relative abundance of Proteobacteria increased, but the relative abundances of Bacteroidetes and Firmicutes decreased under different HRTs, demonstrating that these three main phyla could establish stable long-term synergistic interaction and self-adaption relationship with microalgae. Proteobacteria promote the formation of microalgal-bacterial flocs, enhancing biomass harvesting and avoiding microorganisms being washed out of the CFMBAWTS [30]. Bacteroidetes and Firmicutes participated in the nitrifying and denitrifying processes, indicating that they could compete with microalgae for N sources, while microalgae produce dissolved oxygen to inhibit their growth [31]. The relative abundance of Cyanobacteria showed a similar tendency under different HRTs and was higher than that in the Raw group, demonstrating that the photosynthesis of Cyanobacteria may also occur with microalgae in CFMBAWTS.

At the class level, the percentages of Alphaproteobacteria and Gammaproteobacteria were varied in all groups (Fig. 3b). The total percentages of Gammaproteobacteria and Alphaproteobacteria in the CFMBAWTS at different HRTs were higher than those in the Raw group. This is likely because Gammaproteobacteria and Alphaproteobacteria could supply vitamins and acyl homoserine lactone signaling molecules to create a favorable self-adaption environment for microalgal growth [32].

In terms of genera level, microalgae changed the domain genera in the Raw group (Fig. 3c). In the CFMBAWTS, the relative abundances of *Sphingopyxis* and *Caulobacter* increased with increasing HRT. *Sphingopyxis* and *Caulobacter* secrete extracellular polymeric substances (EPSs) that could enhance the formation of microalgal-bacterial flocs to strengthen the cross-talk between microalgae and bacteria, achieving lower effluent N and P concentrations and higher microalgal growth at a longer HRT [33]. The relative abundance of *Hyphomicrobium* decreased while the relative abundance of *Brevundimonas* increased when HRT increased from 6 to 15 h, suggesting that microalgae harmed *Hyphomicrobium* by releasing DO [34]. *Brevundimonas* has been shown to boost microalgae growth by a threefold increase [35]. Interestingly, irregular changes in *Thauera* and *Acinetobacter* were observed under different HRTs. The relative abundance of *Thauera* was lower at HRTs of 12 and 15 h. Meanwhile, the relative abundance of *Thauera* was higher under HRTs of 8 and 6 h. *Thauera* is an aerobic denitrifying bacterium that could improve the removal of NO$_3^-$-N and competes with microalgae for NO$_3^-$-N assimilation in an aerobic environment [36]. *Acinetobacter* was enriched at an HRT of 6 h, which could promote microalgal growth by secreting IAA [36]. The relative abundance of *Nitrincola* (nitrifying bacterium) was lower in the CFMBAWTS than in the Raw group, insinuating that microalgae may have a stronger ability to compete for NH$_4^+$-N assimilation than *Nitrincola*. Together, stable self-adaption and synergistic interactions between microalgae and bacteria were obtained, which is conducive to the long-term stability of CFMBAWTS.

3.5. Effect of HRTs on functional genes analysis in CFMBAWTS

To better understand self-adaption and synergistic interaction mechanisms in CFMBAWTS, a series of functional genes was analyzed. In general, the removal pathways of NH$_4^+$-N were mainly nitrification and assimilation [37]. Microalgae could create favorable self-adaption conditions (oxygen-rich) for nitrifying bacterial growth, converting NH$_4^+$-N into NO$_2^-$-N and NO$_3^-$-N. NH$_4^+$-N could be removed by microalgal assimilation. As seen in Fig. 5a, with the increase of HRTs, the abundances of functional genes related to nitrification decreased, demonstrating that microalgae could compete with nitrifying bacteria for NH$_4^+$-N removal and may inhibit them, leading to improve microalgal self-adaption ability and enhance NH$_4^+$-N assimilation. As reported, the intervention of microalgae significantly increased NH$_4^+$-N removal through the

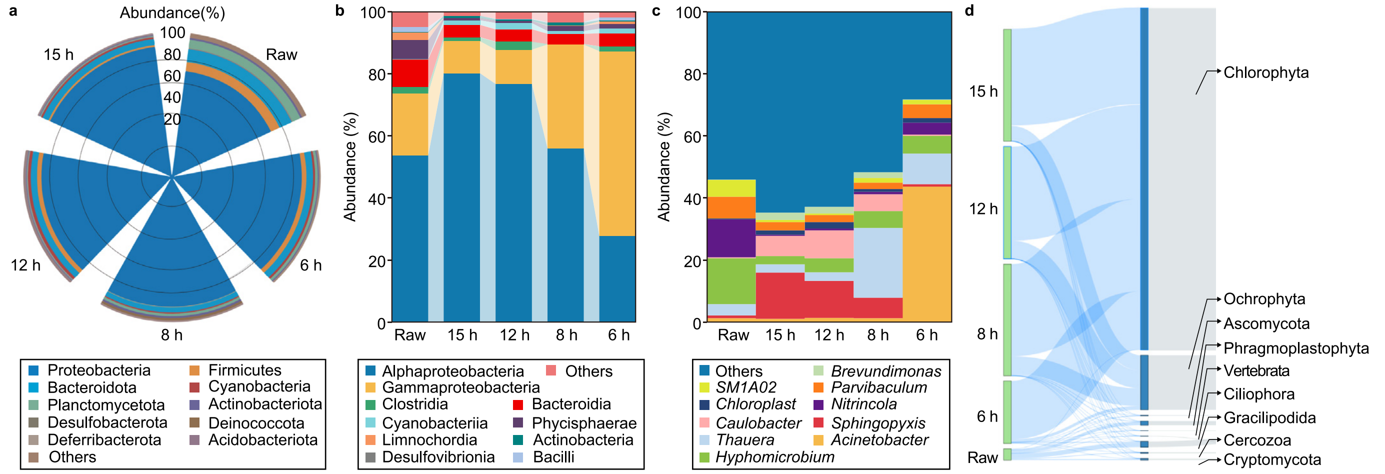

Fig. 3. **a**−**c**, The microbial succession pattern of bacteria at the phylum (**a**), class (**b**), and genus (**c**) levels. **d**, The microbial community compositions of eukaryotes at the phylum level.

assimilation of microalgae, with rates largely higher than the bacteria-alone group [31]. NO_3^--N removal pathways mainly involved assimilatory NO_3^--N reduction (converted to NH_4^+-N and N_2), dissimilatory NO_3^--N reduction, and denitrification [38]. As seen in Fig. 5a, the functional genes related to assimilatory NO_3^--N reduction were found in the lowest abundance compared to dissimilatory NO_3^--N reduction and denitrification, meaning the assimilatory NO_3^--N reduction was not a main pathway to remove NO_3^--N in this system. The initial step in dissimilatory NO_3^--N reduction and denitrification involves the conversion of NO_3^--N to NO_2^--N. The abundance of dissimilation-related genes was higher than those related to denitrification, suggesting that the dissimilatory NO_3^--N reduction was the main route to remove NO_3^--N. Notably, the abundance of dissimilation-related genes in NO_3^--N reduction remained steady under different HRTs, indicating that microalgae could provide a stable self-adaption condition for dissimilatory NO_3^--N reduction. The denitrification-related genes decreased when HRTs increased from 6 to 15 h, indicating that microalgae have a greater effect on influencing denitrification in CFMBAWT than dissimilatory NO_3^--N reduction. This trend may be attributed to the lower oxygen sensitivity of dissimilatory bacteria, which are largely non-strict anaerobic. For the whole NO_3^--N removal pathway, the abundance of functional genes and the effluent NO_3^--N concentrations were decreased when HRT increased from 6 to 15 h, implying that microalgae could make up for the deficiency of bacterial NO_3^--N removal in the presence of DO. P accumulation and assimilation are usually presented as the two main biological pathways in microalgal-bacterial systems (MBS) [38]. As depicted in Fig. 5b, the total abundance of P-related functional genes decreased with rising HRT, implying that P removal occurred mainly through the assimilation of microalgae in CFMBAWTS. This aligns with previous reports highlighting higher P removal efficiency in MBS compared to microalgae or bacteria alone [39,40]. Together, microalgae affected the N and P metabolism of bacteria, effectively compensated for the deficiency of functional bacteria, and enhanced N and P removal by the stable self-adaption and synergistic interactions between microalgae and bacteria. More importantly, this symbiosis improves microalgae biomass accumulation and considerable carbohydrate and lipid production.

However, N and P assimilation are not the only mechanisms for biological self-adaption, synergistic interaction, and carbohydrate/lipid accumulation in microalgae-bacteria systems. As reported, bacteria can supply cofactors (such as vitamins, siderophores, and phytohormones) to promote microalgal growth and carbohydrate and lipid accumulation [41,42]. The metabolic pathways related to the cofactors were investigated in CFMBAWTS (Fig. 4). Cobalamin (vitamin B12), thiamine (vitamin B1), and biotin (vitamin B7) are the main vitamins benefitting microalgal growth [43]. However, most microalgae lack the synthetic pathways to produce vitamins. Therefore, these vitamins needed for microalgae are usually taken from exogenous environmental sources [44], and the vitamin-producing bacteria could exist in MBS to supply the above vitamins to microalgae [45]. This study detected thirty-five, ten, and thirteen genes involved with bacterial vitamin (vitamin B12, B1, and B7, respectively) metabolisms (Fig. 6a,d,c). As a critical step for vitamin B12 synthesis [46], the *cobNST* genes were detected, and the abundance increased with rising HRT. The abundance of functional genes associated with vitamin biosynthesis and transport in bacteria was the highest at an HRT of 15 h, whereas the lowest abundance was discovered when HRT decreased from 15 to 6 h, implying that the exchange of vitamins between bacteria and microalgae had a tight relationship with microalgal growth. More importantly, these vitamins can be directly utilized by microalgal metabolisms; vitamins B7, B1, and B12 would individually participate with the microalgal metabolisms of fatty acid synthesis, primary carbohydrate metabolism, and lipid and carbohydrate metabolism [47] to improve the accumulation of carbohydrate and lipid in microalgae. In addition, riboflavin (vitamin B2) is the essential component of flavin adenine dinucleotide and flavin mononucleotide and could play a vital role in microalgal growth [48]. In this study, six genes (*ribA*, *ribD2*, *ribD*, *ribB*, *ribH*, and *ribE*) related to the metabolic pathway of vitamin B2 were observed (Fig. 6b). The abundance of functional genes was consistent with cell growth trends, implying vitamin B2 may have a beneficial impact on the growth of microalgae.

Additionally, iron is an indispensable element for microalgal metabolism, affecting carbon fixation and cell growth rates. However, the low solubility of iron (in the form of ferric ion, Fe^{3+}) in water may prevent iron uptake by microalgae. Interestingly, siderophores (specific iron chelators) produced by bacteria can be considered vital mediators between bacteria and microalgae and could exhibit high iron binding affinity to increase its solubility [49]. The detailed process is described in Fig. 4. In brief, siderophores bind to iron, converting it to generate Fe^{3+}, and this form is subsequently reduced to ferrous ion (Fe^{2+}) before microalgae

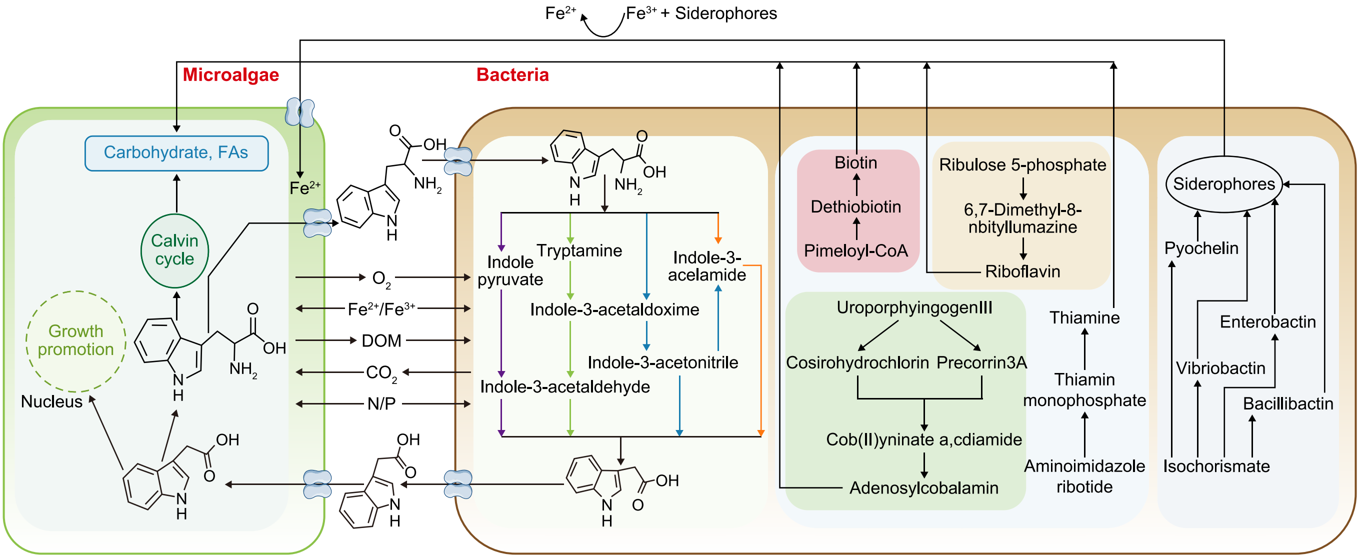

Fig. 4. The key metabolic pathways between microalgae and bacteria.

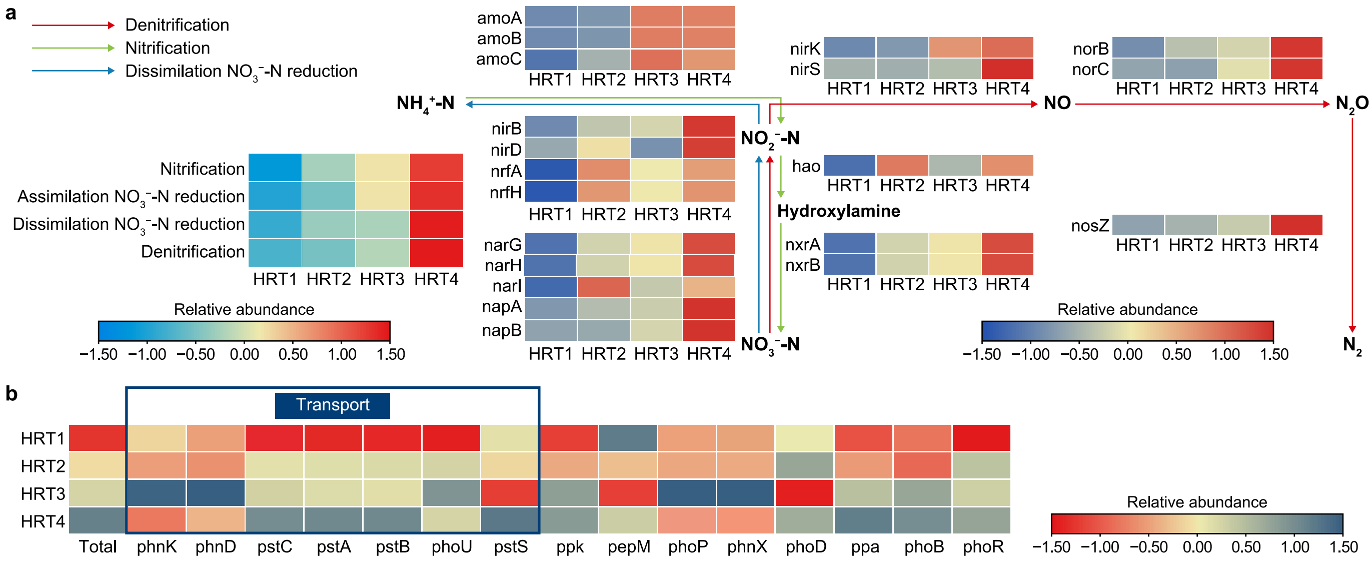

Fig. 5. The metabolic pathways and functional genes related to N (**a**) and P removal (**b**). Total: The total abundance of the functional genes in the metabolic pathways.

uptake through ferrireductases/adjacent Fe^{2+} transporters. Meanwhile, in return, microalgae would provide dissolved organic matter (DOM) to promote the growth of bacteria through “carbon-for-iron mutualism” [50]. As depicted in Fig. 7b, three siderophores (vibriobactin, bacillibactin, and enterobactin) were discovered, known to significantly support iron uptake and growth of microalgae [50,51]. Notably, ten genes (*entF*, *entE*, *entB*, *entA*, *entD*, *vibe*, *vibB*, *DhbF*, *DhbE*, and *DhbB*) related to siderophore synthetase were increased when HRT increased from 6 to 15 h. This indicates that siderophores can enhance microalgae metabolism and accelerate carbohydrate and lipid accumulation [52].

Another key mechanism involved in our study revolved around the synthesis and utilization of indole-3-acetic acid (IAA) (Fig. 4). Specifically, bacteria can use endogenous tryptophan (Trp) as a precursor of IAA biosynthesis as well as secret IAA to promote microalgal growth, Calvin cycle, and fatty acid synthesis. Meanwhile, microalgae secreted Trp to induce the production of more IAA by bacteria, forming a favorable cross-talk between microalgae and bacteria [53]. In CFMBAWTS, bacterial IAA was synthesized from Trp by four pathways: indole-3-pyruvate (IPA), tryptamine (TPM), indole-3-acetonitrile (IAN), and indole-3-acetamide (IAM) (Fig. 7a). Not all genes involved in these pathways have been discovered, such as *IpdC* and *ALDH* (in IPA pathway), *DDC*, *MAO*, and *ALDH* (in TPM pathway), *nthA* (in IAN pathway), and *iAAM* (in IAM pathway). The percentage of these genes increased when HRT increased from 6 h to 15 h, with the abundance of genes in the IPA and IAN pathways notably high. This observation suggests that IPA and IAN are vital pathways for secreting IAA in CFMBAWTS regardless of HRTs. A stable self-adaption process and synergistic interaction always occurred simultaneously, completing continuous pollutant removal and energy production.

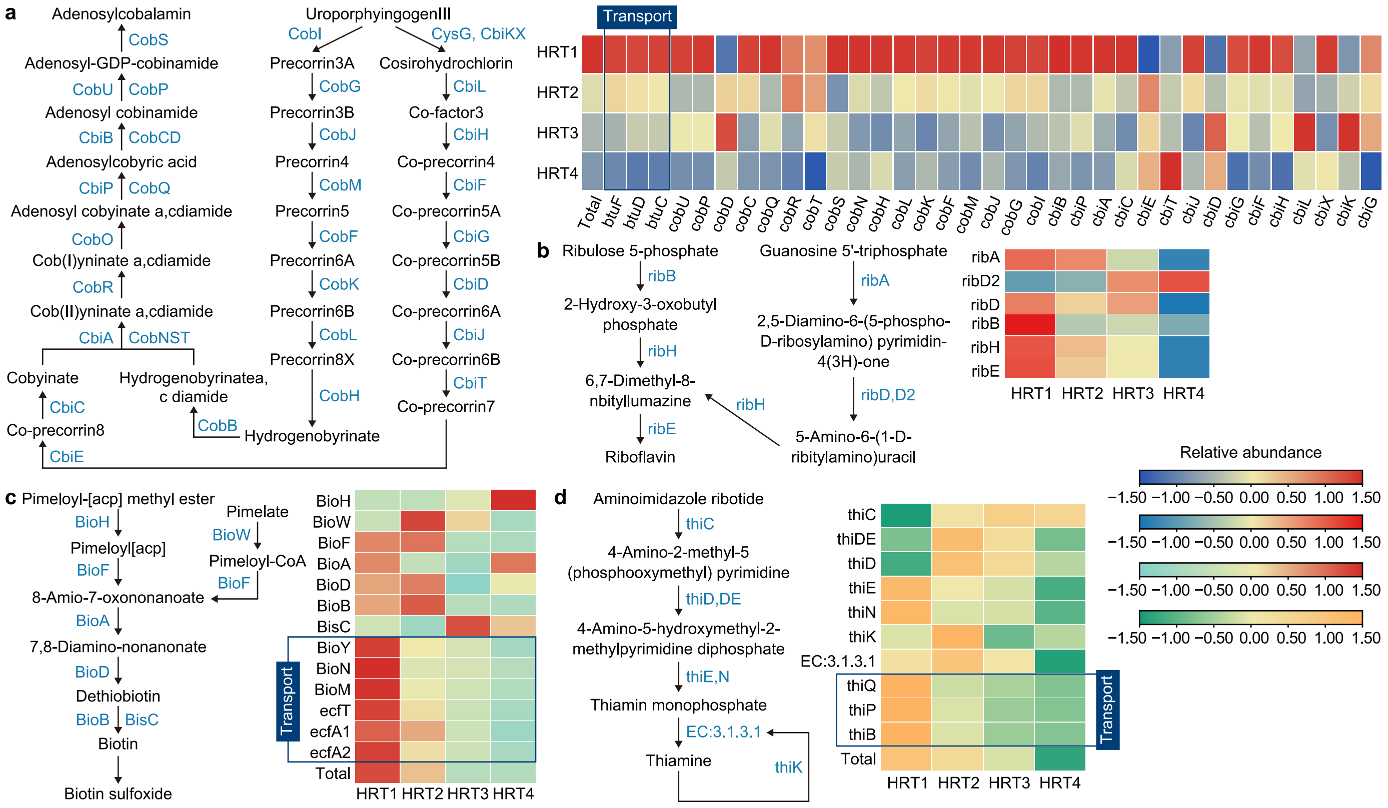

Fig. 6. The biosynthesis and transport of vitamin B12 (**a**), vitamin B2 (**b**), vitamin B7 (**c**), and vitamin B1 (**d**).

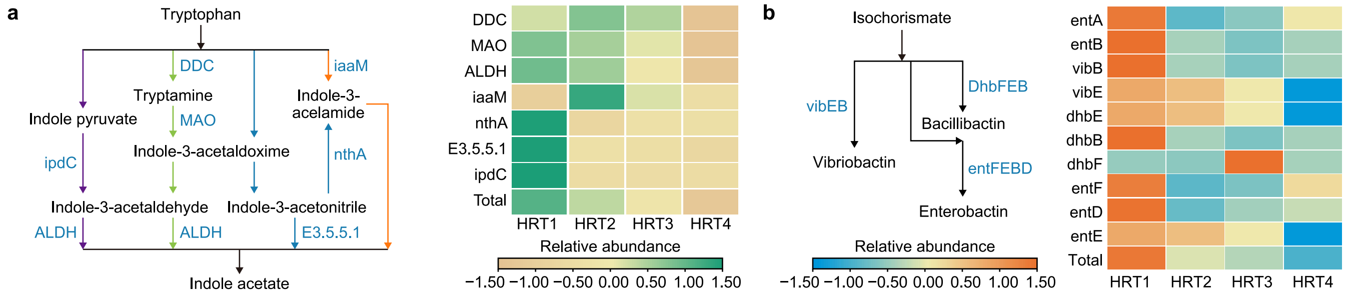

Fig. 7. The biosynthesis and transport of indole-3-acetic acid (**a**) and siderophores (**b**).

3.6. *Perspective and improvement directions for practical application*

While the lab-scale CFMBWT has shown notable advancements in effectively removing N and P from real SE, certain critical hurdles in the practical application of CFMBWT still warrant thorough investigation. Future research should make a more significant effort in the following areas.

(1) How to ensure the stability and high quality of the system's effluent during the natural light-dark cycle?

The impact of the natural light-dark cycle on the stability and effectiveness of microalgae-based wastewater treatment technology has posed a significant challenge, primarily due to the limitations in microalgae's responses to darkness. Fortunately, researchers found that the surplus oxygen produced by microalgae in the daytime could maintain an aerobic atmosphere at nighttime to promote microalgal and bacterial metabolism, thereby achieving simultaneous organics, N, and P removal in MBS [54]. The average removal efficiencies of organics, N, and P in the light-dark cycle were 93.1%, 62.5%, and 80.8%, respectively, when the HRT was 4 h during the nighttime [55]. More importantly, the COD removal efficiency in the daytime was slightly lower than in the nighttime due to the high supersaturation of dissolved oxygen (DO) in the daytime [56]. In summary, strengthening the photosynthesis of microalgae in the daytime and reducing its dependence on light are the keys to ensuring the stability and high quality of the system's effluent during the light-dark cycle. Accordingly, future advancements in CFMBWT should focus on two aspects. Firstly, improving light availability for microalgae during the daytime. Enlarging illumination surface areas and increasing light transmittance are the important factors affecting microbial metabolism [57]. In engineering applications, omnidirectional transparent plexiglass is recommended for CFMBWT. Moreover, the orientation of photobioreactors is crucial, with those oriented north-south receiving more light throughout the year, especially at higher latitudes [58]. Planar waveguides have demonstrated the ability to enhance light availability for microalgal cells in photobioreactors [59]. Given their cost-effectiveness, long lifespan, and superior luminance uniformity, planar waveguides offer a promising industrial application prospect [60]. In conclusion, introducing a planar waveguide into CFMBWT can effectively dilute and homogeneously distribute light within microalgae suspension, presenting a potential solution for industrial-scale applications. Secondly, achieving the appropriate microalgal biomass concentration with a shorter HRT. High microalgal biomass will inhibit its photosynthetic efficiency, while low microalgae biomass is not conducive to removing N and P in wastewater. Moreover, the high influent flow rate can increase the nutrient load and promote microalgae biomass productivity. Therefore, exploring the appropriate microalgae biomass concentration and short HRT is necessary to avoid photoinhibition and improve effluent quality. Collectively, further improvement and validation of CFMBWT on a larger scale are necessary for its practical application.

(2) How to collect and deal with the residual microalgae and bacteria from the system?

Compared to conventional microalgae biomass harvesting techniques, gravity sedimentation is considered an economical solid separation method, accounting for less than 5% of the total cost. It has been widely used for clarifying the treated wastewater [61]. Our study reveals that within the CFMBWT system, microalgal-bacterial flocs can be settled by gravity without additional chemicals. In fact, inside microalgal-bacterial flocs, the interaction of microorganisms provides naturally occurring processes inducing its spontaneous flocculation [62,63]. However, selecting microalgae strains with favorable characteristics (such as cell size and dry weight) is crucial for efficient harvesting. Microalgae with small sizes and low dry weights are prone to suspension in solutions, impeding their collection. Conversely, microalgae with large sizes and high dry weights hinder efficient mixing with the solution. According to our experimental results and extensive experience, we recommend microalgae with a dry weight of about 400–800 mg L^{-1} and a cell size of about 10–20 μm for optimal wastewater treatment. Notably, more excess biomass can be produced during large-scale wastewater treatment, and the harvested biomass can serve as a valuable feedstock for generating biodiesel, bioethanol, biogas, animal feed, fertilizer, protein, pigment, and other value-added products [54]. The effective utilization of biomass resources hinges on factors such as the amount of available biomass, local demand, and economic factors.

4. Conclusions

This study represents the inaugural comprehensive investigation into the performance and mechanisms of CFMBAWTS. Our findings reveal that, with HRTs set at 15, 12, 8, and 6 h, the CFMBAWTS exhibited advanced treatment capabilities for sanitary effluent over a stable operational period of 120 days. As the HRT increased, we observed a corresponding improvement in N and P removal efficiency, elevated microalgae biomass concentration, heightened CO_2 fixation, and excellent energy production. Moreover, the effluent NH_4^+-N and P concertation complied with the strict wastewater discharge standards. Notably, microalgal biomass productivity was decreased with rising HRTs due to low nutrient loading. The primary constituents of microalgal cells in the CFMBAWTS, irrespective of HRTs, included glucose, C18:0, C18:1, C18:2, and C18:3. Crucially, self-adaption and synergistic interaction were major mechanisms for microbial growth, N and P removal, CO_2 fixation, and carbohydrate and lipid accumulation. Overall, this work could provide new insights into the application of CFMBAWTS for future SE treatment and serve as a strong support for the development of MBS.

CRediT authorship contribution statement

Xue Li: Conceptualization, Methodology, Investigation, Writing - Original Draft. **Shengnan Li:** Methodology. **Peng Xie:** Methodology. **Xi Chen:** Methodology, Investigation. **Yuhao Chu:** Conceptualization, Methodology. **Haixing Chang:** Supervision, Conceptualization. **Jian Sun:** Supervision, Funding Acquisition. **Qing Li:** Supervision, Funding Acquisition. **Nanqi Ren:** Supervision, Conceptualization. **Shih-Hsin Ho:** Writing - Review & Editing, Supervision, Project Administration, Funding Acquisition.

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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Appendix A. Supplementary data

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