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# Self-assembly of ammonium assimilation microbiomes regulated by COD/N ratio

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## ABSTRACT

Marine microorganisms have an inherent advantage in the treatment of saline wastewater due to their halophilic properties. Ammonium assimilation is the most important and common nitrogen conversion pathway in the ocean, which means that it may be a suitable nitrogen removal strategy under high salinity conditions. However, the targeted construction of engineering microbiomes with ammonium assimilation function for nitrogen recovery has not been realized. Here, we constructed four halophilic ammonium assimilation microbiomes from marine microbial community under varying chemical oxygen demand (COD) to nitrogen (COD/N) ratios. The regulation of COD/N ratio on microbial self-assembly was explored at the phenotypic, genetic, and microbial levels. The results of nitrogen balance tests, functional genes abundance and microbial community structure confirmed that the microbiomes regulated by different COD/N ratios all performed obligate ammonium assimilation functions. >93% of ammonium, 90% of TN, 98% of COD, and 82% of phosphorus were simultaneously removed by microbial assimilation under the COD/N ratio of 20. COD/N ratios significantly affected the self-assembly of microbiomes by selectively enriching heterotrophic microorganisms with different preference for organic carbon load. Additionally, the increase of COD/N ratio intensified the competition among species within the microbiome (the proportion of negative connections of microbial network increased from 5.0% to 24.4%), which may enhance the stability of community structure. Taken together, these findings can provide theoretical guidance for the construction and optimization of engineering microbiomes for synergistic nitrogen removal and recovery.

## 1. Introduction

For saline wastewater with large organic loads from industrial output, seawater toilet flushing, and leaching of saline-alkali soils, proper treatment is required [1]. However, due to the inhibition of salt on of microbial activities, conventional biotreatment technologies are inappropriate for the saline wastewater treatment [2] and bioremediation of saline-alkali soils [3]. Although freshwater microorganisms can successfully remove nutrients after prolonged domestication (weeks to tens of weeks) [4,5], domesticated biological systems are susceptible because their need to use more energy to maintain the osmotic pressure balance of cells [6]. In contrast, halophilic bacteria are employed to remediate saline wastewater need not take salinity adaptation into account. The ocean is rich in huge numbers of naturally halophilic microorganisms [7], and the microbes have formed stable, cooperative,

and complex microbial communities that can participate in the biogeochemical nitrogen cycle [8,9]. Thus, marine microbial communities appear to have potential for saline wastewater treatment.

The nitrogen conversion pathways and fluxes in marine can give a better reflection of the bias of N conversion in saline environments and may be able to guide the treatment of saline wastewater. In the marine nitrogen cycle, ammonium assimilation is the dominant nitrogen conversion pathway, and its flux (~8800 Tg nitrogen per year) is about four times that of nitrification (~2000 Tg nitrogen per year) and 70 times that of anaerobic ammonia oxidation (anammox) (~125 Tg nitrogen per year) [10]. This means that ammonium assimilation may be a more suitable nitrogen removal pathway than ammonium oxidation under high salinity conditions. Although ammonium assimilation is the most important and common nitrogen conversion pathway in the ocean, even in nature, almost all the regulation of marine microbial communities is

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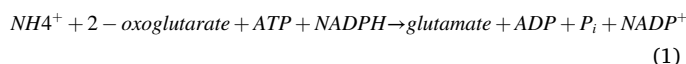
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regulated in the direction of nitrification and denitrification [6,11] and anammox [4] pathways. It is undeniable that these strategies can alleviate the risk of reactive nitrogen (Nr) pollution in saline wastewater. However, these strategies are usually accompanied by accumulation of biotoxic nitrite, emission of greenhouse gases (e.g. nitrous oxide) and loss of active nitrogen [12]. Additionally, these nitrogen removal processes are energy-intensive and result in a waste of nitrogen sources [13]. Although the wasted Nr can be obtained from N<sub>2</sub> in the atmosphere by Haber-Bosch process continuously without limitation, it also aggravates the energy shortage and environmental pollution because Haber-Bosch process is an energy-intensive and carbon emitting process [14,15]. Compared to current biological Nr removal strategies that converting Nr into inert gaseous nitrogen, recovering valuable Nr from waste streams and reusing it is more consistent with the concept and practice of circular economy and sustainable nitrogen management [13,16,17]. Microbial ammonium assimilation may provide a promising and sustainable wastewater treatment strategy that can transform inorganic ammonium in wastewater into biomass organic nitrogen to achieve synergistic nitrogen removal and recovery [14,16]. However, the targeted construction of engineering microbiomes with ammonium assimilation function for nitrogen recovery has not been realized.

Fortunately, the design theory of engineering microbiomes might provide theoretical guidance for the targeted construction of functional microbiomes. The procedures of top-down design include the selection of environmental variables, expected functions and inoculum that may contain microorganisms with desired functions. Following the top-down design, the selected environmental variables (such as nutrient load and redox conditions) can manipulate the self-assembly process of inoculated microbial community to obtain microbiomes with required or expected functions [18,19]. The manipulation of environmental variables on the self-assembly process is usually based on differences in physiological and physicochemical properties (such as differences in growth rates, substrate affinities, environmental suitability, etc.) between functional guilds [19,20]. The inoculum is cultivated under the designed environmental variables or medium composition to obtain or improve the required functions. The inoculated microbial community in this study came from the sediment of the Yellow Sea, in which a halophilic strain *Psychrobacter* A4N01 with obligate heterotrophic ammonium assimilation function was screened, indicating that the inoculum contains microorganisms with desired ammonium assimilation function [12]. In terms of environmental variables, the chemical oxygen demand (COD) to nitrogen (COD/N) ratio of the culture medium was selected as the key culture parameter to achieve or improve the heterotrophic ammonium assimilation function because the assimilation of ammonium into glutamate is the process where the element nitrogen is assimilated by carbon metabolism [21,22]. The assimilation of ammonium to glutamate requires the combination of 2-oxoglutarate, an intermediate product of carbon metabolism under heterotrophic condition (Eq. (1)) [21]. Theoretically, the efficiency of heterotrophic ammonium assimilation can be improved by increasing the COD/N ratio of the culture medium [23]. Additionally, the COD/N ratio can also regulate the characteristics of niche due to the difference in substrate affinity between heterotrophic and autotrophic populations [24–28]. Generally, high COD/N ratio in wastewater is beneficial to the growth and enrichment of heterotrophic microorganisms in the microbial community and inhibiting autotrophic microorganisms [25]. The same phenomenon can also be observed in the nitrogen cycle of soil and wetlands, where increasing COD/N ratio can promote the heterotrophic microbial assimilation [29,30]. Although these evidences indicate that the COD/N ratio plays an important role in regulating microbial population structure and nitrogen metabolism functions, the role of COD/N ratio in the construction of heterotrophic ammonia assimilation bacterial population, especially the regulatory mechanism of self-assembly process and microbial interaction. Revealing the nuances of self-assembly microbiomes under different COD/N ratios is critical for deciphering the response mechanism of microbial communities and guiding the design

of the engineering microbiomes.



This study aimed to construct obligate ammonium assimilation microbiome based on complex marine microbial community for synergistic nitrogen removal and recovery. One focus was on the regulating mechanisms of COD/N ratio on the self-assembly of microbiomes and microbial interactions. Additionally, the phenotypic and genetic characteristics of nitrogen metabolism in the constructed microbiomes were evaluated. These findings can provide theoretical guidance for the construction and optimization of engineering microbiomes for synergistic nitrogen removal and recovery.

## 2. Material and methods

### 2.1. Experimental set-up and inoculum source

Four laboratory-scale sequential batch reactors (SBRs) with a working volume of 3.14 L (diameter of 15.0 cm and height of 20.0 cm) were named A, B, C, and D according to COD/N ratios of 5, 10, 15, and 20, respectively. Four SBRs were operated continuously for 120 days to investigate the regulation of COD/N ratio on the self-assembly process of marine microbial community. The operation cycle of SBR was 8 h, including feeding time of 0.1 h, aeration time of 7.5 h, settling time of 0.3 h, and discharge time of 0.1 h. The volume exchange ratio and hydraulic retention time was 50 % and 16 h. Oxygen was supplied to the reactor by an aeration pump with the aeration flow rate of 1.0–1.2 L min<sup>-1</sup>, resulting in the dissolved oxygen was 4.0–8.0 mg L<sup>-1</sup>. The marine microbial community was inoculated into SBR with different COD/N ratio and continuously cultivated for 120 days. The operating temperature decreased from 26 to 13 °C with the change of ambient temperature (see [Supplementary materials](#)).

Inoculum was collected from surface marine sediment at the station of A4 (123°2'848" E, 36°0'614" N) of the Yellow Sea. The sediment was washed three times before inoculation to remove large inorganic impurities. The initial mixed liquor suspended solids (MLSS) of the four SBRs were approximately 6.0 g L<sup>-1</sup>. It is generally believed that COD/N ratio of 5 and 20 are nutrient limiting thresholds and optimal nutrient ratios of biological treatment processes, respectively [31]. Therefore, COD/N ratio was controlled at 5–20 in this study. In addition, the concentrations of nutrients (e.g. COD and nitrogen) in saline wastewater from different industries are obviously different. According to the conventional concentration of NH<sub>4</sub><sup>+</sup>-N and COD in the tannery wastewater, seawater toilet flushing wastewater and marine-products processing wastewater reported in literature [1,31,32], the concentrations of NH<sub>4</sub><sup>+</sup>-N and COD were set to 100 and 500–2000 mg L<sup>-1</sup> respectively in this study. The synthetic saline wastewater contained: 100 mg L<sup>-1</sup> NH<sub>4</sub><sup>+</sup>-N (NH<sub>4</sub>Cl), 20 mg L<sup>-1</sup> PO<sub>4</sub><sup>3-</sup>-P (K<sub>2</sub>HPO<sub>4</sub>), and 1.0 mg L<sup>-1</sup> of trace element solution. The concentrations of COD (NaAC) were 500, 1000, 1500 and 2000 mg L<sup>-1</sup>, respectively, corresponding to COD/N ratio of 5, 10, 15 and 20. The salinity was adjusted to 3 ‰ by sea salt.

### 2.2. Batch activity tests

To reflect the nitrogen metabolism pathway of different microbiomes, the sludge biomass was collected from four biosystems in the stable period (day 100) for batch experiments outside the biosystems. The obtained biomass was fed for 12 h in nutrient-free wastewater with 3 ‰ salinity under aeration conditions to completely eliminate organic matter and nitrogen. The composition of saline wastewater in batch test was consistent with that of the long-term operating bioreactors. In the Test I and Test II, 100 mL biomass and 400 mL saline wastewater were added in 500 mL beaker, and the MLSS was controlled to 8 g L<sup>-1</sup>. To eliminate the influence of ammonia oxidation in batch test, 30 mg L<sup>-1</sup>



allylthiourea (ATU) was specially added to the batch Test II [33]. The concentration of nutrients was measured hourly during the operation of batch Test I and Test II. Each data is the average of three experiments.

Total nitrogen of biomass (TN-biomass) was calculated by subtracting the total nitrogen of the supernatant (TN-supernatant) after centrifugation from the total nitrogen of the mixture in the system. The concentration of TN-biomass in batch experiments is the net increment of TN-biomass (that is, the concentration of TN-biomass is regarded as  $0 \text{ mg L}^{-1}$  at 0 h).

### 2.3. Analytical methods

The concentrations of  $\text{NH}_4^+\text{-N}$ ,  $\text{NO}_2^-\text{-N}$ ,  $\text{NO}_3^-\text{-N}$ , TN,  $\text{PO}_4^{3-}\text{-P}$ , COD and MLSS were determined according to standard methods [34]. Extracellular polymeric substance (EPS) was extracted according to modified heat method [35] and detailed procedures were shown in [Supplementary materials](#). Proteins (PN) and polysaccharides (PS) content was determined by using the modified Lowry method and anthrone-sulfuric acid method. The procedures of cell surface hydrophobicity (CSH) followed the previous literature [36]. Dissolved oxygen and pH values were monitored by on-line probes (WTW Multi 3630 IDS, Germany).

### 2.4. DNA extraction and high-throughput sequencing

Four microorganism samples were collected from each biosystems on day 5, 35, 75 and 120, and then stored at  $-20^\circ\text{C}$  for high-throughput sequencing. The four samples of each system were named as the system number plus 1, 2, 3 and 4 in chronological order. The detailed procedure of DNA extraction and polymerase chain reaction (PCR) amplification were operated according to the published study [11]. The 16S rRNA gene were amplified with primers 515F and 806R. The amplicon samples were conducted on the Illumina HiSeq platform at Majorbio Bio-pharm Technology Co., Ltd (Shanghai, China). The detailed procedures of microbial community detection and bioinformatics analysis were shown in [Supplementary Material](#).

### 2.5. Quantitative PCR analysis of functional genes

The abundance of ammonium assimilation (glutamine synthetase, *glnA*), nitrifying (*amoA* and *nxrA*), and denitrifying (*napA*, *narG*, *nirK*, *nirS*, and *nosZ*) genes were quantitatively detected on real-time quantitative PCR instrument (Analytik Jena AG, qTOWER 2.2, Germany). The qPCR primers and detailed operation procedures was presented in the [Supplementary materials](#). The abundance of nitrogen functional genes was normalized to number of copies of per ng DNA and plotted with the mean.

### 2.6. Bioinformatics analysis

The top 50 genera of relative abundance were selected to construct microbial networks. Correlations with Spearman correlation coefficients higher than 0.8 ( $p < 0.05$ ) were chosen to represent strong microbial interactions between the two genera. Microbial networks were visualized using Gephi (ver. 0.9.2) [37]. The value of within-module connectivity (Zi) and among-module connectivity (Pi) are calculated using the Cytoscape (ver.2.6.0) [38]. Significance was performed on IBM SPSS V22.0 (IBM, USA). Correlations between the CSH and EPS characteristic were calculated and visualized using “ggplot2” packages (IBM, USA) in R (ver. 4.0.0). Principal coordinates analysis (PCoA) based on Bray-Curtis distance and permutational [multivariate analysis of variance](#) (PERMANOVA) were carried out to visualize the differences of microbiomes under varying COD/N ratios using “vegan” package in R (ver. 4.0.0). Linear discriminant analysis (LDA) effect size (LEfSe) methods was performed on the Galaxy webserver (<https://huttenhower.sph.harvard.edu/galaxy/>) based on the significance threshold of  $\text{LDA} > 4$  [39]. Principle component analysis (PCA) was performed in Canoco

(ver. 5.0) program to reveal the relationships between the system parameters and microbial characteristics. Mantel test analysis was performed using “ggplot2”, “vegan” and “ggcor” packages in R (ver. 4.0.0).

## 3. Results

### 3.1. Treatment performance of microbiomes regulated by different COD/N ratios

The four microbiomes showed significant differences ( $p < 0.01$ ) in nitrogen removal performance under different COD/N ratios. After 15 days, the average removal of  $\text{NH}_4^+\text{-N}$  were  $42.9 \pm 2.8\%$  (s.d.),  $63.4 \pm 1.7\%$  (s.d.),  $78.3 \pm 1.0\%$  (s.d.),  $92.6 \pm 1.2\%$  (s.d.) in the biosystems with COD/N ratio of 5, 10, 15 and 20, respectively ([Fig. 1](#)). The maximum ammonium recovery rate was  $138.9 \text{ mg/L/d}$  under the COD/N ratio of 20. TN removal efficiencies consistent with  $\text{NH}_4^+\text{-N}$ , were  $42.8 \pm 1.1\%$  (s.d.),  $61.8 \pm 0.9\%$  (s.d.),  $76.1 \pm 0.9\%$  (s.d.),  $90.1 \pm 0.8\%$  (s.d.) under COD/N ratio of 5, 10, 15 and 20, respectively. High COD removal efficiency ( $>95\%$ ) was achieved in four biosystems without significant difference. The removal efficiency of phosphorus in the high COD/N biosystem ( $82.5\%$  at COD/N ratio of 20) was significantly higher than that in the low COD/N biosystem ( $77.9\%$  at COD/N ratio of 5).

[Fig. 2](#) showed the dynamics of COD and nitrogen components in a typical cycle of the four biosystems with different COD/N ratio. The removal efficiencies of  $\text{NH}_4^+\text{-N}$  were  $32.9\%$ ,  $54.5\%$ ,  $70.3\%$  and  $89.4\%$  at COD/N ratio of 5, 10, 15 and 20, respectively. No nitrite and nitrate accumulation were detected during the whole process. The decrease in TN-supernatant concentration was accompanied by an increase in TN-biomass. The balanced TN in the mixture (the sum of TN-biomass and TN-supernatant) in the four biosystems indicated that no nitrogen loss in all biosystems. Additionally, there was no significant difference ( $p > 0.05$ ) in the nitrogen removal efficiency in the four biosystems with the addition of  $30 \text{ mg L}^{-1}$  ATU compared with the experiment without the addition of ATU. These evidences confirmed that the four constructed microbiomes remove nitrogen removal by heterotrophic ammonium assimilation rather than nitrification. The effluent concentrations of phosphate and COD remained between 3.0 and 4.3 and  $16.5\text{--}24.5 \text{ mg L}^{-1}$  in the four biosystems, corresponding to the removal efficiencies of phosphorus and COD of  $77.9\text{--}84.5\%$  and  $95.0\text{--}98.0\%$  respectively. There was a correlation ( $R^2 = 0.82501$ ) between the concentration of phosphate and pH value (see [Supplementary Material](#)), indicating that pH value may affect the process of phosphate conversion.

### 3.2. Biomass characteristics

The biomass characteristics of biosystems with different COD/N ratios, including CSH, EPS components and MLSS, were shown in [Fig. 3](#). In the first 20 days, the decreased of MLSS in biosystems were mainly due to the adaptation of microorganisms and the elimination of tiny inorganic impurities in marine sediments. Subsequently, the MLSS of the four biosystems gradually increased, and it was significantly higher in the high COD/N the biosystem than in the low COD/N ratio biosystem. EPS matrix was mainly composed of proteins and polysaccharides. It was found that the contents of EPS, proteins and polysaccharides increased with the increase of COD/N ratio. The contents of EPS increased to the highest of 22.2, 31.6, 33.0, and  $32.9 \text{ mg g}^{-1}$  MLSS on day 75 in the biosystems with COD/N ratio of 5, 10, 15 and 20, respectively. In the stable period (day 20–120), there was no significant difference ( $p > 0.05$ ) in the contents of proteins and polysaccharides under the COD/N ratio of 10–20. However, the content of proteins of biosystem with COD/N ratio of 5 was significantly lower than that of biosystem with COD/N ratio of 15 ( $p = 0.019$ ) and 20 ( $p = 0.024$ ). The PN/PS ratio in biosystems with COD/N ratio of 5, 10, 15 and 20 decreased from 2.0, 2.3, 2.6 and 2.9 on day 75 to approximately 1.0 on day 120 with the temperature decreasing from above  $20^\circ\text{C}$  to below  $15^\circ\text{C}$ . The production of

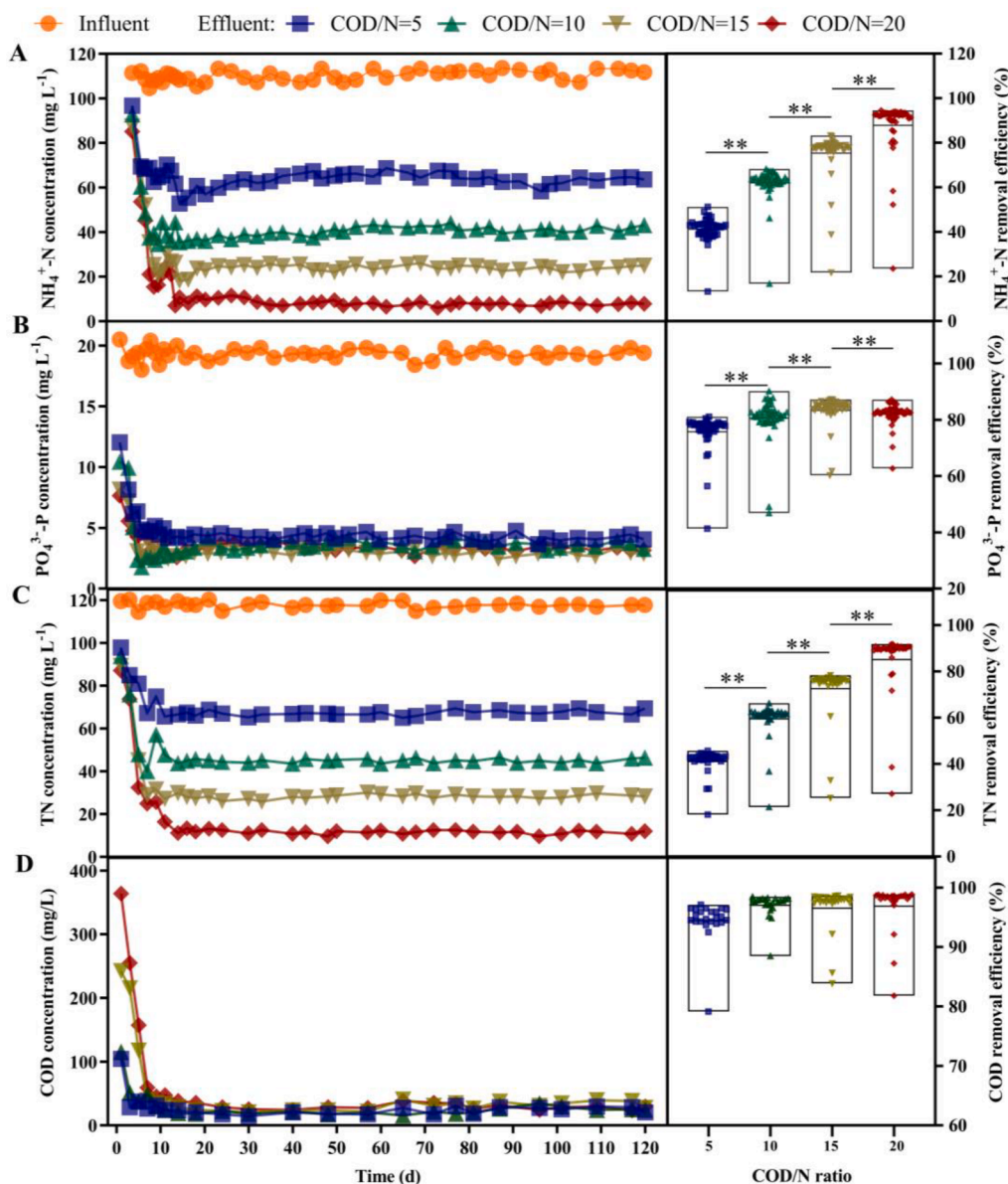


Fig. 1. The long-term treatment performance of microbiomes mediated by different COD/N ratio. Concentrations and efficiencies of ammonium (A), phosphate (B), total nitrogen (C) and COD of the four microbiomes. \* $p < 0.05$ ; \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

polysaccharides may be a protective mechanism against low temperature [40]. Interestingly, the COD/N ratio had no significant effect on the PN/PS ratio of EPS and the CSH ( $p > 0.05$ ). Elemental analysis displayed that the atomic C:N ratio of dried biomass was 4.9, 5.4, 5.7 and 5.9 in the microbiomes with COD/N ratio of 5, 10, 15 and 20, respectively (see Supplementary materials).

### 3.3. Nitrogen metabolism genes regulated by COD/N ratio

Fig. 4 showed the relative abundance of nitrogen metabolism-related genes in different microbiomes. The relative abundance of ammonium assimilation genes *glnA* was as high as  $1.0 \times 10^6$  copies  $\text{ng}^{-1}$  DNA, while the relative abundance of ammonia oxidation gene *amoA* was negligible ( $< 3.0 \times 10^2$  copies  $\text{ng}^{-1}$  DNA). The relative abundance of *glnA* decreased continuously in the biosystems with COD/N ratio of 5 and 10, while it continued to increase in the biosystems with COD/N ratio of 15 and 20 with the operation of the biosystem. Finally, the abundances of *glnA* in biosystem with COD/N of 20 was 5.8 times higher than that in the biosystem with COD/N ratio of 5. The denitrification related genes

*napA*, *narG*, *nirS*, *nirK* and *nosZ* can still be detected in an aerobic environment without nitrate or nitrite accumulation. There was no significant difference in the relative abundance of *narG*, *nirS* and *nirK* under different COD/N ratios. However, the relative abundance of *napA* and *nosZ* decreased by 74.2 %–96.0 % in the four biosystems during operation.

### 3.4. Dynamics of self-assembly microbiomes

#### 3.4.1. Microbial community succession under different COD/N ratio

Alpha diversity analysis exhibited that the species abundance of the microbiome with COD/N ratio of 5 was significantly lower ( $P < 0.05$ ) than that of the other three microbiomes (Fig. 5). In addition, the evenness of high COD/N ratio microbiomes was higher than that of the low COD/N system. Microbial communities with high evenness may respond more calmly to the changing environmental conditions (i.e. pH, temperature, and toxic substances, etc.) [41]. The results of PCoA and PERMANOVA also indicated that there was significant difference (PERMANOVA:  $p < 0.05$ ) between the oligotrophic microbiome (COD/N

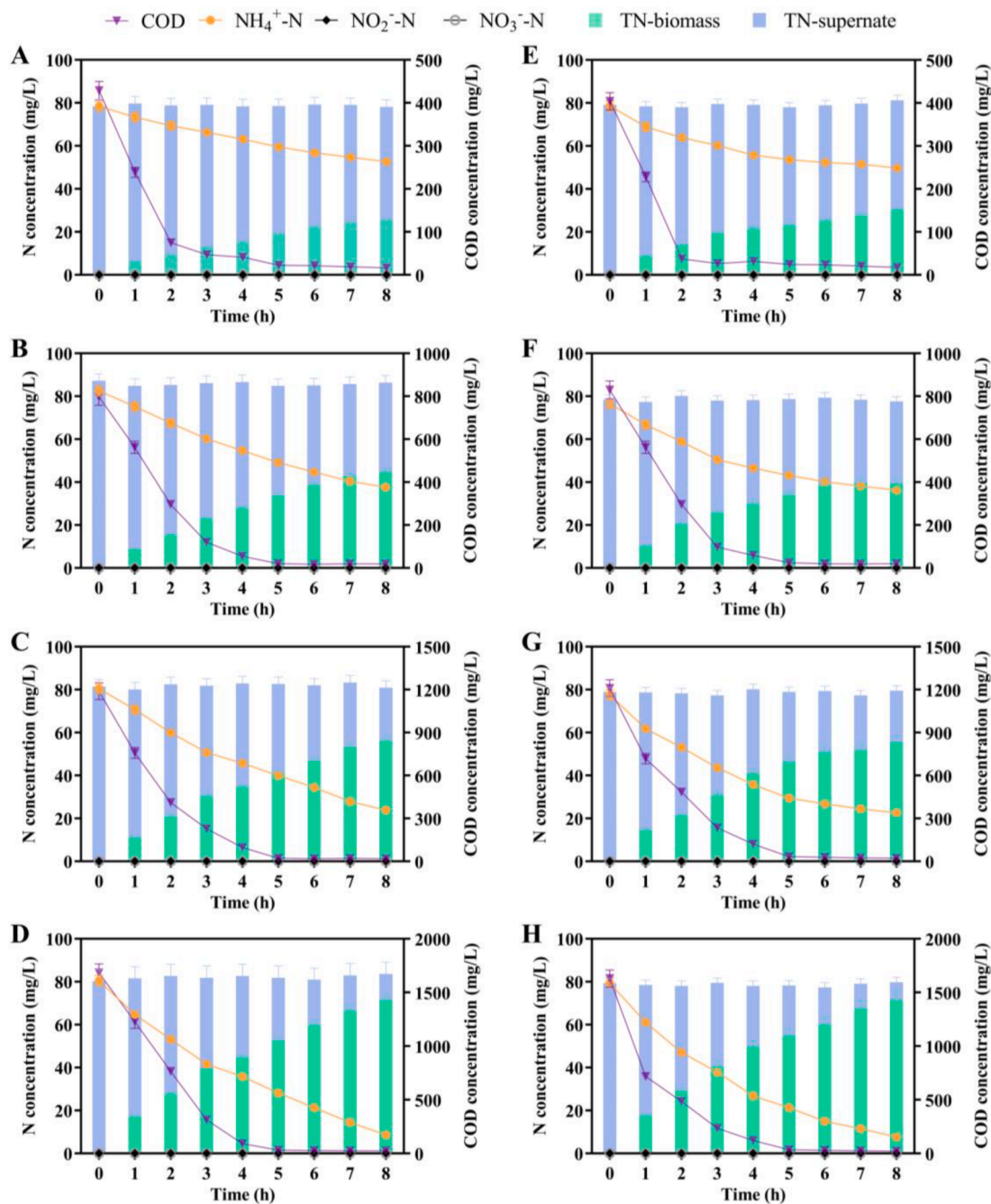


Fig. 2. Dynamics of COD and nitrogen components in a typical cycle of biosystems with COD/N ratio of 5, 10, 15 and 20 without (A, B, C, D) and with 30 mg/L ATU (E, F, G, H).

ratio of 5) and the eutrophic microbiomes (COD/N ratio of 15 and 20).

The community structure of constructed microbiomes under different COD/N ratio was obviously different, revealing that COD/N ratio indeed regulated the self-assembly of ammonium heterotrophic assimilation microbiomes. At phylum level, *Proteobacteria* was the most dominant phylum (23.9 %–67.5 %) in microbiome with the COD/N ratio of 5 and 10, while it was the second dominant phylum in biosystems with the COD/N ratio of 15 and 20 (16.0 %–43.9 %). The relative abundance of *Bacteroidota* was significantly different ( $p < 0.05$ ) among microbiomes under different COD/N ratio. Moreover, the average abundance of phylum *Desulfobacterota* in microbiome with COD/N ratio of 5 (28.7 %) was >10 times that of other microbiomes with COD/N ratio of 10, 15 and 20 (1.4 %–3.0 %). The heatmap of genera with relative abundance >0.01 % was shown in Fig. 5E. *Corynebacterium*, *Halomonas*,

and *Rhodobacteraceae* was the most abundant three genera in indigenous marine sediments. The average abundance of *Xanthomarina* was 3.3 %, 21.1 %, 28.0 % and 35.5 % in microbiomes with COD/N of 5, 10, 15 and 20, respectively. *Xanthomarina* was the dominant genus in microbiomes with COD/N ratio of 15 and 20, and the second dominant genus in microbiome with COD/N ratio of 10. *Marinobacter* and *Thauera* was the most abundant genus the microbiomes with COD/N ratio of 5 and 10, respectively, and the average abundance of *Thauera* ( $43.3 \pm 8.5$  %) was >10 times that of other biosystems. *Vitellibacter* was the second dominant bacterium in the biosystem with COD/N of 15. The average abundance of *Vitellibacter* in microbiomes with COD/N ratio of 15 (14.2 %) and 10 (4.5 %) was higher than that in microbiomes with COD/N ratio of 5 (0.1 %) and 20 (1.0 %). The average abundance of *Fusibacter* increased from <0.1 % to 6.6 % when the COD/N ratio increased from 5



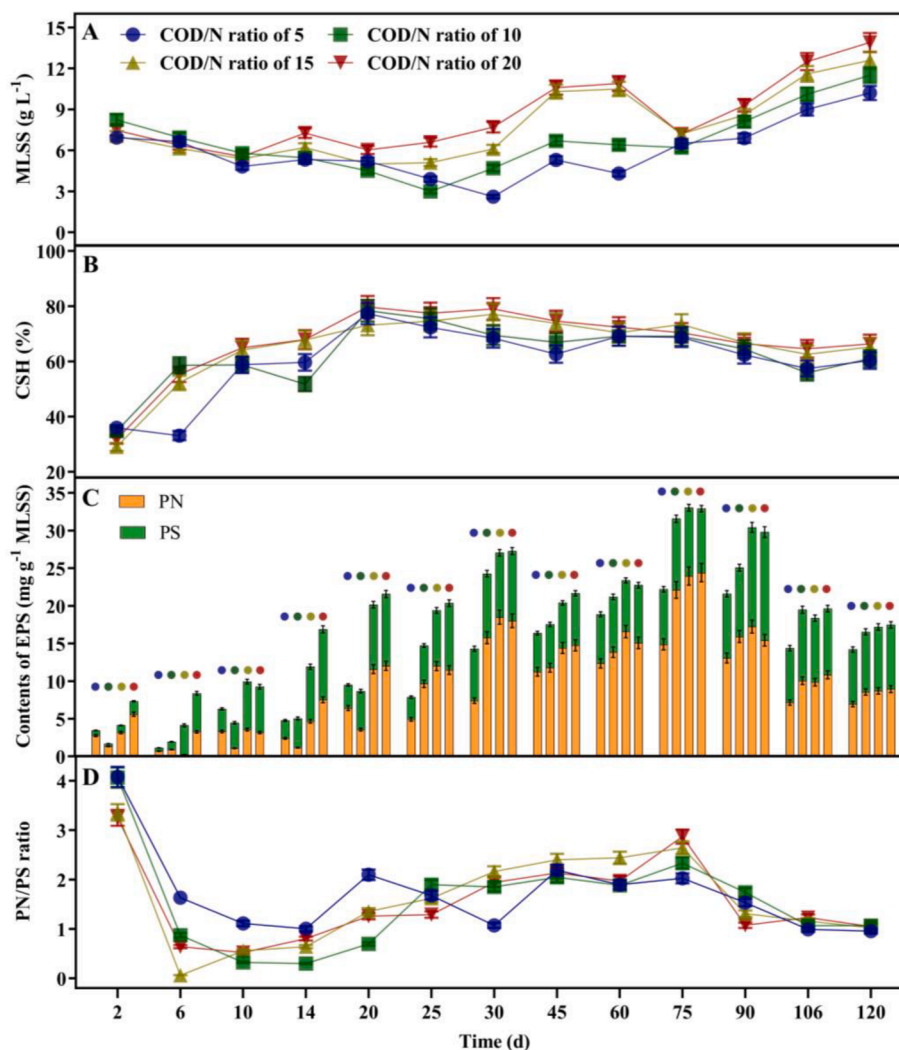


Fig. 3. Sludge properties in microbiomes with different COD/N ratio. (A) Mixed liquor suspended solid (MLSS); (B) Cell surface hydrophobicity (CSH); (C) Contents of PN and PS in EPS; (D) PN/PS ratio in EPS.

to 20. Notably, ammonia-oxidizing bacteria (AOB, e.g. *Nitrosomonas*) and nitrite oxidizing bacteria (NOB, e.g. *Nitrospina* and *Nitrospira*) were completely eliminated within five days, and were not detected in subsequent operations.

The result of LEfSe analysis showed that 5, 7, 6 and 9 biomarker taxa were identified in systems with COD/N ratios of 5, 10, 15 and 20, respectively. Specifically, order *Oceanospirillales* and genus *Acetoanaerobium* was biomarker in the biosystem with COD/N ratio of 5. The abundance of *Acetoanaerobium* in the biosystem with COD/N ratio of 5 is >50 times that in the other three systems. Genus *Thauera* (affiliated with family *Rhodocyclaceae* and phylum *Proteobacteria*), genus *Vitellibacter* and genus *Xanthomarina* were the biomarkers in biosystems with the COD/N ratio of 10, 15 and 20, respectively. The results suggested that the influent COD/N ratio exerted selective pressure on the microbial populations in the self-assembly of the microbiomes.

### 3.4.2. Regulation of COD/N ratio on the co-occurrence pattern of microbiomes

Regulation of COD/N ratio on co-occurrence pattern of microbiomes was further determined by microbial network (Fig. 6). In the constructed networks, there were 221 nodes linked by 4205 edges for COD/N ratio of 5, 189 nodes linked by 1388 edges for COD/N ratio of 10, 209 nodes linked by 2047 edges for COD/N ratio of 15, and 200 nodes linked by 2015 edges for COD/N ratio of 20. A majority of the connections were

positive in the four networks, contributing to 95.0 %, 77.7 %, 75.9 %, and 75.6 % of total interspecific interactions. This result indicates that the cooperative symbiosis was more ubiquitous than competition in heterotrophic ammonium assimilation microbiomes. The proportion of negative correlations in network increased from 5.0 % to 24.4 % with the increase of COD/N ratio from 5 to 20, revealing that the competition between microorganisms in the microbiome under high COD/N ratio may be more intense than that under low COD/N ratio.

The topological role of genera in network were assigned according to within-module connectivity ( $Z_i$ ) and among-module connectivity ( $P_i$ ) (Fig. 6E-H). In these four networks, all nodes were assigned as peripherals or connectors. Connectors are generally considered as key species in the network, maintaining the stability of microbial community [42]. There were 200, 43, 198, and 146 connectors in networks with COD/N ratio of 5, 10, 15, and 20, respectively. More connectors can enhance communication between modules (such as transmitting information or generating intermediate metabolites) to maintain a large and complex module structure.

### 3.5. Relationships between microbial communities and environmental factors

PCA and Mantel test analysis were performed to reveal the relationship between operating parameters, treatment performance,

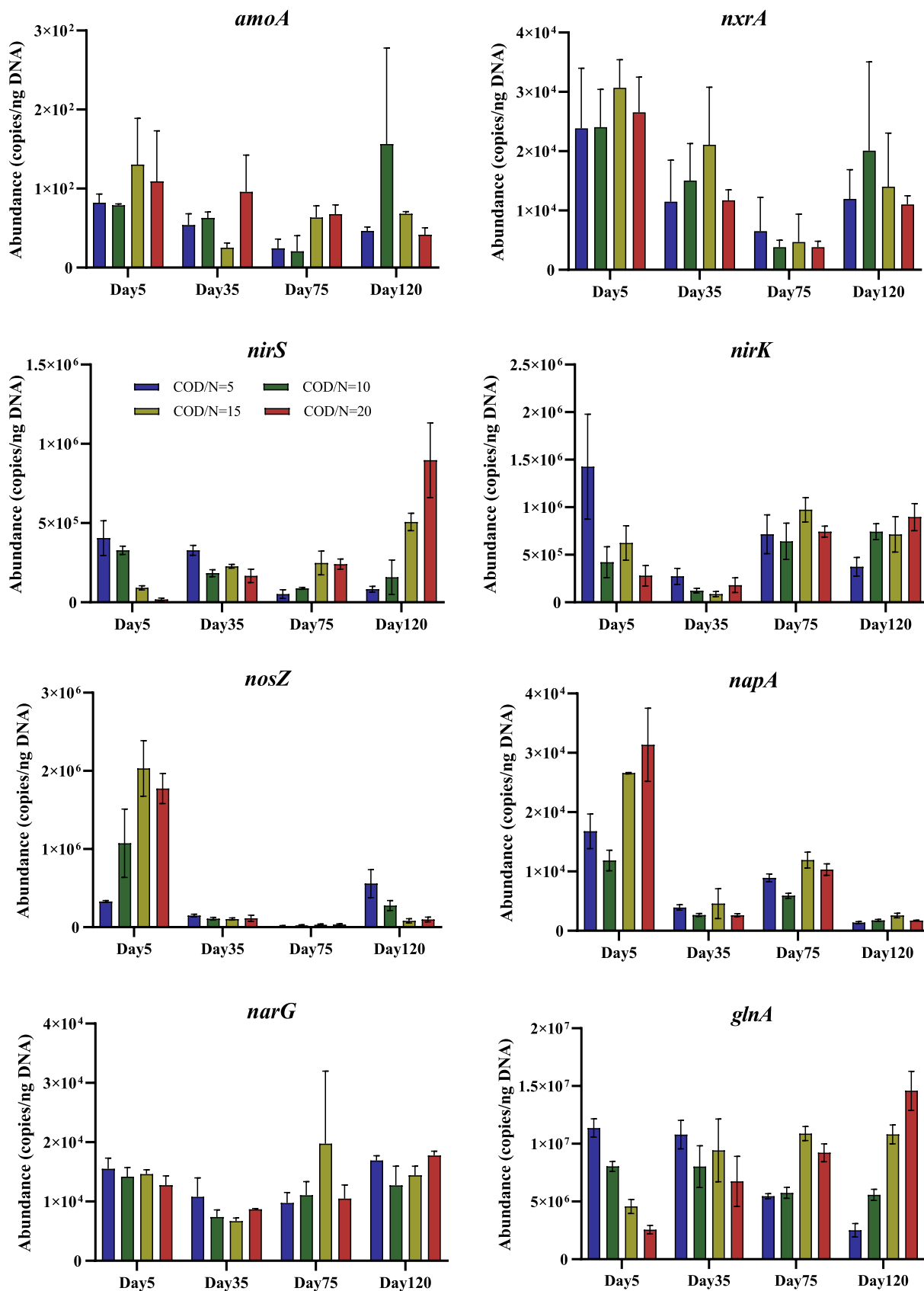
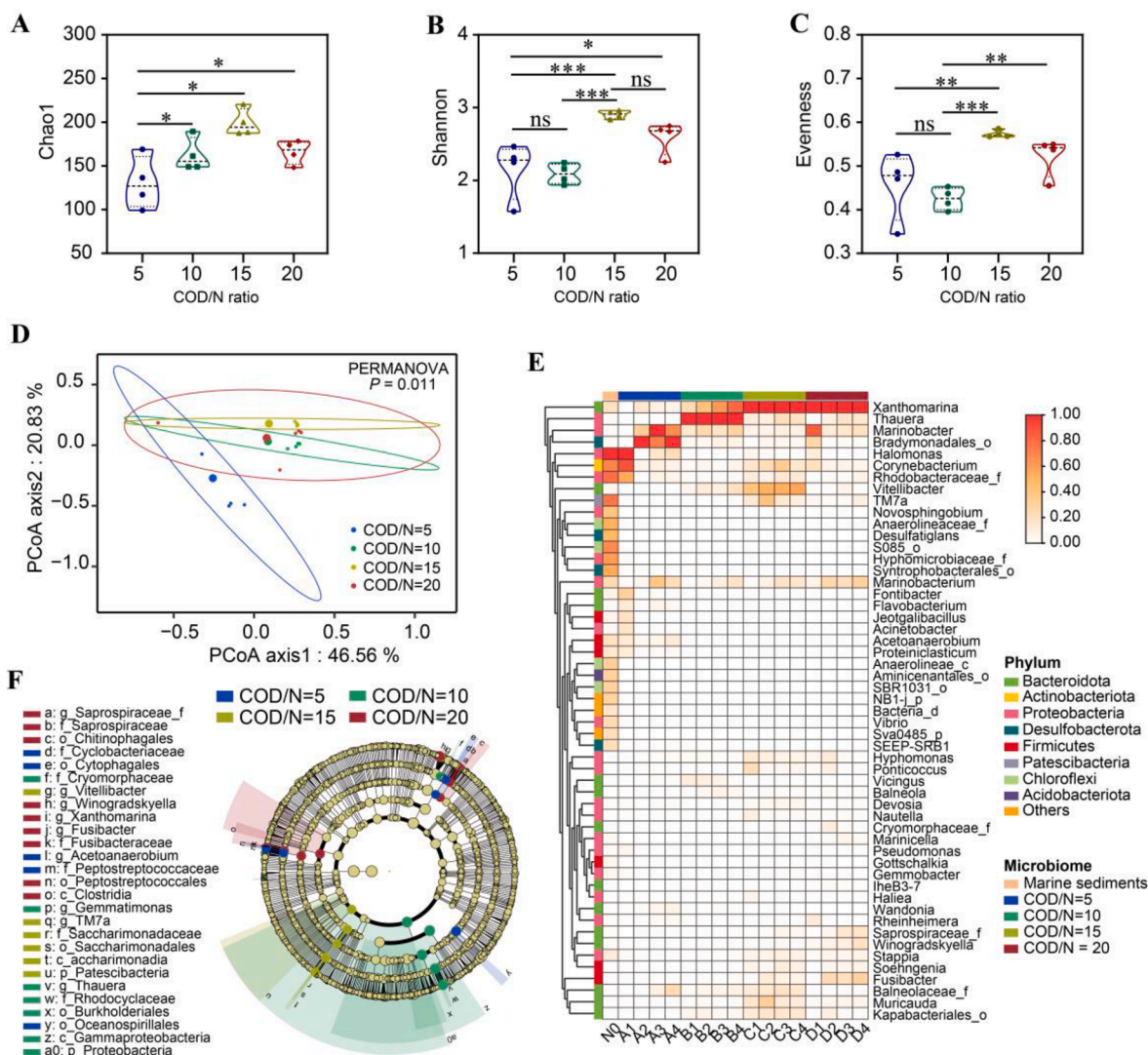


Fig. 4. Relative abundances of nitrogen metabolism-related genes in the four microbiomes.



**Fig. 5.** Microbial diversity indices including Chao1 (A), Shannon (B), Evenness (C) of microbiomes. Statistical significance levels: \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ ; ns = not significant. (D) Principal coordinates analysis (PCoA) based on 16S rRNA data. (E) Heatmap of the microbial community with relative abundance  $> 0.01$  at genus level. (F) Phylogenetic cladogram of microbiomes by using the linear discriminant analysis (LDA) effect size (LEfSe) statistical results of the differentially abundant taxa. Those taxa without corresponding taxonomic names at the genus level were indicated by higher level names. “f”, “o”, “c”, “p” and “d” represent family, order, class, phylum and domain, respectively.

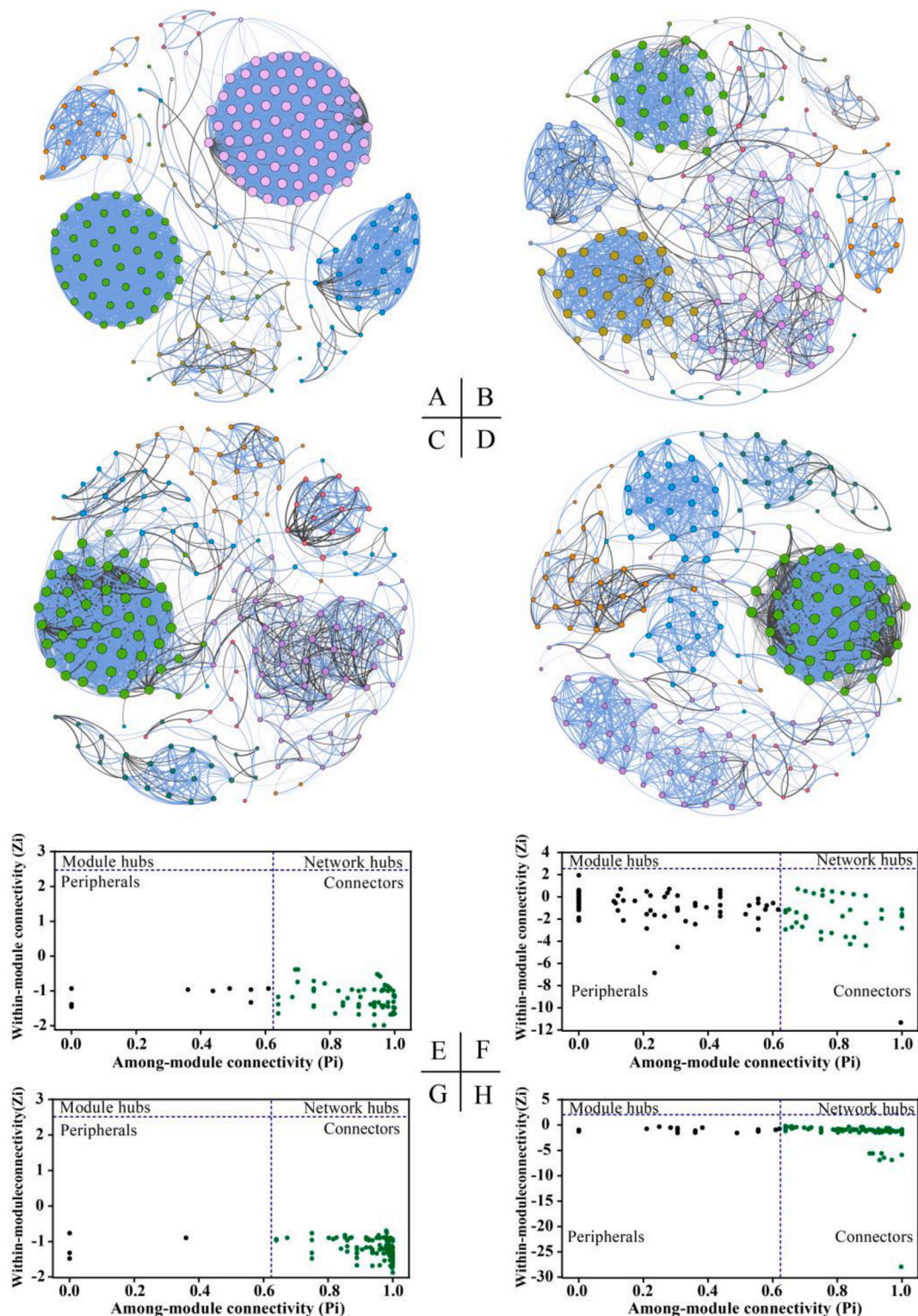
functional genes and microbial populations (Fig. 7). It was obviously observed that the nitrogen removal efficiency and species abundance (represented by Sobs) were positively correlated with the COD/N ratio. Nitrification and denitrification genes have almost no correlation with nitrogen removal efficiency and COD/N ratio (close to right angles). Nitrogen assimilation gene *glnA* has a strong positive correlation with nitrogen removal efficiency and COD/N ratio. The arrow length of temperature is shorter than that of COD/N, indicating that temperature is a secondary influencing factor [43]. Mantel test analysis further evaluated the effects of the operational factors on the microbial community structure and functional genes. Particularly, the COD/N ratios significantly affected the microbial community (Mantel’s  $R > 0.5$ , Mantel’s  $P < 0.05$ ) and nitrogen removal efficiency (Spearman’s  $R > 0.9$ ,  $p < 0.01$ ). Moreover, temperature was significantly negatively correlated with MLSS (Spearman’s  $R > 0.8$ ,  $p < 0.001$ ), but not significantly correlated with nitrogen removal efficiency ( $p > 0.05$ ), indicating that the decrease of temperature inhibited the biomass growth but did not affect the treatment efficiency of ammonium assimilation microbiomes.

## 4. Discussion

### 4.1. COD/N ratio regulated the nutrient metabolism of microbiomes

Phenotypic, genetic and microbial evidences have jointly demonstrated that ammonium in the four constructed halophilic microbiomes was removed by heterotrophic assimilation rather than oxidized by nitrification or anaerobic ammonia oxidation. The ammonium removal efficiency increased linearly with the increase of COD/N ratio ( $R^2 = 0.98139$ ), which was mainly attributed to the proportional assimilation of organic carbon, ammonium and phosphorus for biomass growth [44,45]. The coupled metabolic pathways of carbon, nitrogen and phosphorus in the microbiomes were shown in Fig. 8. During microbial assimilation of nutrients, acetate and phosphate were incorporated into the tricarboxylic acid (TCA) cycle via the acetyl-CoA pathway. Subsequently, the intermediate products of TCA cycle 2-oxoglutarate and ammonium synthesize glutamate into biomass. Sufficient organic carbon in the biosystem with high COD/N provides more substrates (2-oxoglutarate) for the conversion of ammonium to glutamate and glutamine than in the low COD/N ratio condition [45]. The regulation of





**Fig. 6.** Microbial network of ammonium assimilation microbiomes with COD/N ratio of 5 (A), 10 (B), 15 (C) and 20 (D). Genera assigned to the same module have the same color, and the color of the module is random. Modules are randomly colored. A bluish grey line indicated significant positive interactions between two individual nodes, and a gray line indicated significant negative interactions. Z-P plot of OTUs in the microbiomes with COD/N ratio of (E) 5, (F) 10, (G) 15 and (H) 20 based on the basis of topological roles. The nodes were classified into four categories according to the threshold values of  $Z_i = 2.5$  and  $P_i = 0.62$ : module hubs with  $Z_i > 2.5$ ,  $P_i < 0.62$ , network hubs with  $Z_i > 2.5$ ,  $P_i > 0.62$ , connectors with  $Z_i < 2.5$ ,  $P_i > 0.62$ , and peripherals with  $Z_i < 2.5$ ,  $P_i < 0.62$ .

COD/N on ammonium assimilation gene *glnA* is mainly based on energy supply. In general, the synthesis of glutamine from ammonium and glutamate under the action of *glnA* is an energy-consuming process [45]. Therefore, high carbon/energy media is more conducive to the

expression of gene *glnA* than low carbon/energy media. In terms of phosphorus removal, there was no significant correlation between phosphorus removal efficiency and COD/N ratio in the four biosystems, indicating that there are other phosphorus removal pathways in the four



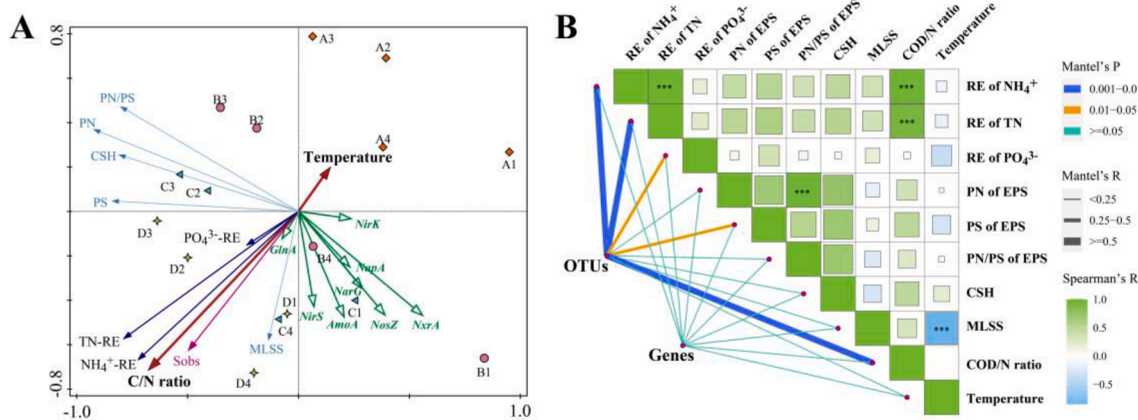


Fig. 7. (A) Principal component analysis (PCA) of the relationship among the system performance, environmental parameters and functional genes. (B) Mantel test analysis with operational conditions effects on the evolution of microbial community and functional genes. (\*p < 0.05; \*p < 0.01, \*\*\*p < 0.001).

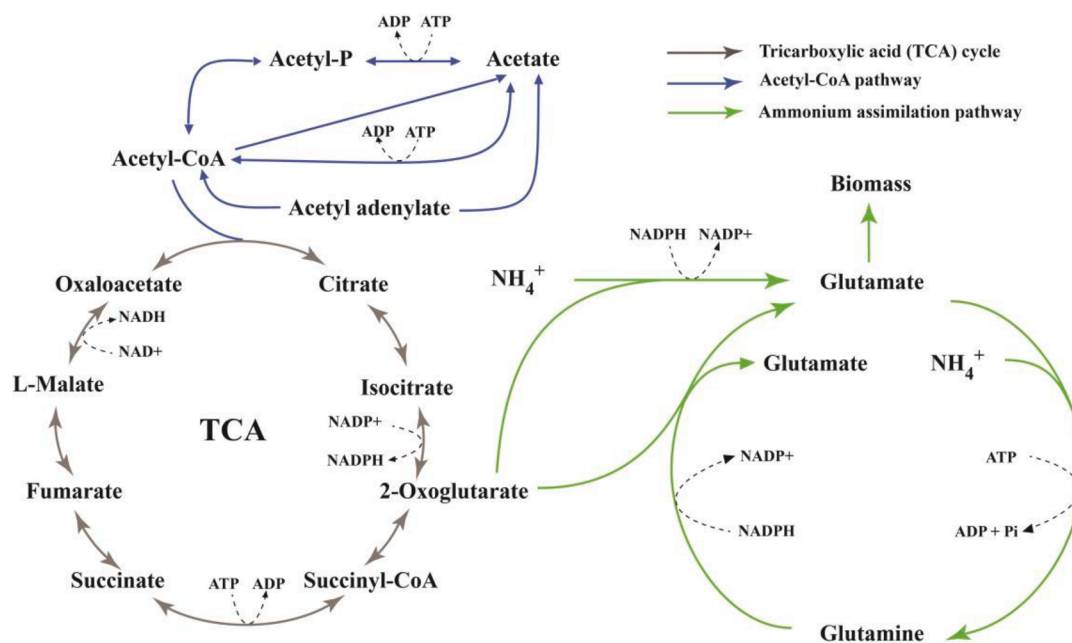


Fig. 8. The coupled metabolic pathways of carbon, nitrogen and phosphorus in the microbiomes.

microbiomes besides assimilation, such as biologically-induced phosphorus precipitation [46]. This was deduced based on the linear relationship between PO<sub>4</sub><sup>3-</sup>-P concentration and pH value in the four biosystems. Stocks of phosphorus, as a key nutrient for the development of life, are decreasing worldwide. However, the mechanism of phosphorus removal and recovery from wastewater will be further explored in the future.

#### 4.2. COD/N ratio regulated the self-assembly of microbiomes

Evidences of microbial community succession verified that COD/N ratio effectively regulated the self-assembly of microbiomes. In terms of microbial diversity, high organic carbon load and COD/N ratio provided sufficient nutrition to support more bacteria and groups. However, deficient available nutrients in biosystems with low COD/N ratio intensified the competition for nutrients among microorganisms, thus eliminating some vulnerable microorganisms [47]. In terms of microbial community succession, the regulation of COD/N ratio on the self-assembly of microbiome was mainly achieved by screening and enriching populations with different preferences for organic carbon

load. For instance, the abundance of *Bacteroidota* [48], a metabolically heterogeneous taxa that prefers organic carbon, increased rapidly with the increase of organic carbon load, which intensified the competition for carbon sources between *Bacteroidota* and *Proteobacteria* [49]. Due to the lack of organic matter, Phylum *Desulfobacterota* with autotrophic preference [50] was significantly enriched in the microbiomes with COD/N of 5. At the genus level, the biomarker order *Oceanospirillales* in the microbiome with COD/N ratio of 5 was considered to be adapted to both autotrophic and mixotrophic conditions [51]. Genus *Acetoanaerobium* has been reported to be able to synthesize acetate from H<sub>2</sub> and CO<sub>2</sub> [52]. In the biosystem with COD/N of 5, homoacetogenic bacteria *Acetoanaerobium* may synthesize organics to feed other microorganisms in the absence of available nutrients, resulting in the actual TN removal efficiency higher than the theoretical ammonium assimilation efficiency. *Thauera* is a common microorganism responsible for heterotrophic nitrogen metabolism in nitrogen removal biosystem, and its adaptation range to COD/N ratio is usually 5–15 [53,54]. Both *Xanthomarina* and *Vitellibacter* are members of the family *Flavobacteriaceae*. They usually perform well in the degradation of high-intensity organic wastewater, and can enhance the degradation of fatty acids,

carbohydrates, proteins and other organic matter [55–58]. During the assembly of microbiome, the selective pressure of COD/N forced microorganisms that can adapt to or prefer a certain nutritional condition to be retained or enriched, while those that cannot adapt were eliminated, thus forming a differentiated microbial community structure.

For native autotrophic nitrifying bacteria, *Nitrosomonas*, *Nitrospina*, and *Nitrospira* in the marine microbial community were completely eliminated after 5 days of culture, and were not detected in subsequent operations. In terms of elimination mechanism of autotrophic nitrifying bacteria, the possibility of salinity inhibition can be ruled out because the salinity of the culture medium prepared with sea salt is consistent with the salinity of the sampling point. In addition, sufficient oxygen content ( $4.0\text{--}8.0\text{ mg L}^{-1}$ ) in the biosystems due to continuous aeration can meet the consumption of autotrophic and heterotrophic bacteria, so the inhibition of oxygen competition on autotrophic bacteria is also eliminated. Autotrophic nitrification usually requires inorganic carbon as carbon sources, while low levels of inorganic carbon limit the growth of nitrifying bacteria [59]. Previous studies have shown that inorganic carbon has a inhibition threshold of  $36\text{--}45\text{ mg L}^{-1}$  for AOB, while there is almost no limit for NOB [60,61]. In this study, the concentration of inorganic carbon (about  $158\text{--}161\text{ mg L}^{-1}$ , mainly carbonate [62] and bicarbonate [63]) in simulated seawater toilet flushing wastewater was consistent with that in seawater because it was prepared with natural sea salt. In addition, organics degradation and  $\text{CO}_2$  supply from aeration can also supplement inorganic carbon in wastewater for autotrophic bacteria growth [60]. Therefore, inorganic carbon supply was also not a factor that inhibited autotrophic bacteria. The elimination of autotrophic nitrifying bacteria was mainly attributed to the selective pressure of organic carbon and the initial inhibition of ammonium assimilation microorganisms. In this study, the minimum COD/N ratio of 5 was still greater than the inhibition threshold of autotrophic bacteria (COD/N of 3) [64,65], resulting in the continuous inhibition of autotrophic bacteria by organic carbon. Interestingly, although there have been previous studies on long-term cultivation of nitrogen removal microbial communities in saline wastewater with COD/N ratio of 5–20, the obligate ammonia assimilation function has not been achieved [25,27,66]. Complete elimination of nitrifying bacteria is difficult process in nitrifying enhanced biological treatment systems even under high salinity conditions [12]. In the construction of engineering microbiome, the selection of original microbial community is also an important factor. The marine microbial community selected in this study contains strain *Psychrobacter aquimaris* with high-efficient ammonium assimilation function [12], which ensured that the original microbial community had ammonium assimilation potential. The initial dominance of ammonium assimilation microbes in indigenous microbial community may suppress autotrophic nitrifying bacteria. Subsequently, the continuous inhibition of organic carbon on autotrophic microorganisms in the culture medium quickly eliminated autotrophic nitrifying bacteria, and achieved complete ammonium assimilation function.

#### 4.3. COD/N ratio regulated the co-occurrence patterns of microbiomes

Members of microbial ecosystems can be assigned to the same or similar ecological niche (modules in the network) based on their nutritional preferences and functional uniqueness [67]. Fewer modules of network with high COD/N ratio than that of low COD/N ratio network indicates that the function of the microbiomes is more specific under high COD/N ratio than that under low COD/N ratio. The proportion of positive correlation in microbial network gradually decreased with an increase of COD/N ratio. In the absence of bioavailable substrates (low COD/N ratio), microorganisms tend to synergistically degrade refractory substrates or closely connect with each other to resist adverse environments (increased cooperation) [47]. For instance, some homo-acetogenic bacteria (e.g. *Acetoanaerobium*) are enriched to synthesize organic matter for other microorganisms. When nutrients are abundant (high COD/N ratio), some species may overgrow and become the

dominant taxa through competition. These dominant genera consume a large amounts of resources, such as oxygen and nutrients, leading to greater competition (negative interactions and more complex networks) among other species [47]. The organic carbon load obviously regulates the interaction of nodes, indicating that the co-occurrence patterns of network may be resource-driven [68].

#### 4.4. Significances and future prospects

Heterotrophic ammonium assimilation is an important pathway in the global nitrogen cycle, while its contribution to nitrogen removal and recovery in wastewater treatment is often ignored [69]. In conventional wastewater treatment plant, ammonia oxidation is still the mainstream strategy. The ultimate destination of nitrogen in ammonia oxidation process is to enter the atmosphere as gaseous nitrogen, which leads to nitrogen loss and accumulation of harmful intermediates. Sustainable economy encourages the recovery of reactive nitrogen from wastewater [16]. Heterotrophic ammonium assimilation may provide a promising sustainable strategy for synergistic nitrogen removal and recovery of wastewater [14]. This process can be completed in a single aerobic heterotrophic unit, avoiding the complex regulation of the intermittent supply of oxygen and organic carbon, the change of redox state and the generation of harmful intermediates [12]. In addition, heterotrophic microorganisms are more robust than autotrophic microorganisms to cope with harsh operating environments (such as high salinity, low temperature, etc.) [38,70]. This study, for the first time, constructed a heterotrophic ammonium assimilation microbiome based on a complex microbial community. The constructed ammonium assimilation microbiomes achieved high ammonium removal and recovery rate ( $138.9\text{ mg/L/d}$ ) at COD/N ratio of 20, which may provide an effective strategy for the efficient recovery of ammonium. Revealing the regulatory mechanism of COD/N ratio on the self-assembly and microbial interaction of the microbiomes can provide theoretical guidance for the construction and optimization of engineering microbiomes for synergistic nitrogen removal and recovery.

Although promising, heterotrophic ammonium assimilation is still in its infancy and some studies need to be prioritized in the future. Firstly, the removal mechanism of phosphorus in the ammonium assimilation microbiomes needs to be clarified, which is important for phosphorus recovery from wastewater. Secondly, the robustness of ammonium assimilation microbiomes in the face of nitrifying bacteria infestation and the mechanism of microbial interaction need to be explored. Thirdly, a comprehensive technoeconomic and environmental evaluations of ammonium assimilation microbiomes need to be developed to guide practical applications. In the future, ammonium assimilation microbiome is expected to be applied in more fields, including but not limited to saline wastewater treatment and alkali-saline soils bioremediation.

## 5. Conclusion

This study constructed halophilic ammonium assimilation microbiomes from complex marine microbial community following a top-down design, and revealed the regulation of COD/N ratio on the self-assembly of microbiome at the phenotypic, genetic and microbial levels. The following conclusions could be drawn:

- The results of nitrogen balance tests, nitrogen metabolism functional genes and microbial community structure confirmed that the four constructed microbiomes under varying COD/N ratio performed obligate ammonium assimilation function.
- >93 % of ammonium, 90 % of TN, 98 % of COD, and 82 % of phosphorus were simultaneously removed under the COD/N ratio of 20.

- The regulation of COD/N ratio on the self-assembly process of microbiomes was through selective enrichment of heterotrophic microorganisms with different preferences for organic carbon load.
- The competition between microorganisms intensified with the increase of COD/N ratio. The dominant genera *Xanthomarina*, *Vitellibacter* and *Fusibacter* consumed a large amount of resources, such as oxygen and nutrients, thus intensifying the competition among other species.

### CRedit authorship contribution statement

**Fei Han:** Conceptualization, Data curation, Methodology, Writing – original draft. **Mengru Zhang:** Data curation. **Zhe Li:** Supervision. **Zhe Liu:** Data curation. **Yufei Han:** Data curation. **Yuke Li:** Supervision. **Weizhi Zhou:** Supervision, Project administration, Writing – review & editing, 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.

### Data availability

Data will be made available on request.

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### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.cej.2022.140782>.

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