

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

Han, Fei; Zhang, Mengru; Li, Zhe; Liu, Zhe; Han, Yufei; Li, Yuke; Zhou, Weizhi

DOI

[10.1016/j.cej.2022.140782](https://doi.org/10.1016/j.cej.2022.140782)

Publication date

2023

Document Version

Final published version

Published in

Chemical Engineering Journal

Citation (APA)

Han, F., Zhang, M., Li, Z., Liu, Z., Han, Y., Li, Y., & Zhou, W. (2023). Self-assembly of ammonium assimilation microbiomes regulated by COD/N ratio. *Chemical Engineering Journal*, 455, Article 140782. <https://doi.org/10.1016/j.cej.2022.140782>

Important note

To cite this publication, please use the final published version (if applicable). Please check the document version above.

Copyright

Other than for strictly personal use, it is not permitted to download, forward or distribute the text or part of it, without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license such as Creative Commons.

Takedown policy

Please contact us and provide details if you believe this document breaches copyrights. We will remove access to the work immediately and investigate your claim.

Green Open Access added to TU Delft Institutional Repository

'You share, we take care!' - Taverne project

<https://www.openaccess.nl/en/you-share-we-take-care>

Otherwise as indicated in the copyright section: the publisher is the copyright holder of this work and the author uses the Dutch legislation to make this work public.



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

Fei Han^a, Mengru Zhang^a, Zhe Li^b, Zhe Liu^a, Yufei Han^a, Yuke Li^c, Weizhi Zhou^{b,*}

^a School of Environmental Science and Engineering, Shandong University, Qingdao, Shandong 266000, China

^b School of Civil Engineering, Shandong University, Jinan, Shandong 250002, China

^c Department of Water Management, Faculty of Civil Engineering and Geosciences, Delft University of Technology, Stevinweg 1, 2628 CN Delft, The Netherlands

ARTICLE INFO

Keywords:

Ammonium assimilation microbiomes
Marine microbial community
Self-assembly process
Chemical oxygen demand to nitrogen (COD/N) ratios
Microbial network

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

* Corresponding author.

E-mail address: wzzhou@sdu.edu.cn (W. Zhou).

<https://doi.org/10.1016/j.cej.2022.140782>

Received 7 September 2022; Received in revised form 1 November 2022; Accepted 1 December 2022

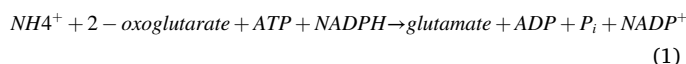
Available online 5 December 2022

1385-8947/© 2022 Elsevier B.V. All rights reserved.

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 N₂ 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⁻¹, resulting in the dissolved oxygen was 4.0–8.0 mg L⁻¹. 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⁻¹. 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 NH₄⁺-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 NH₄⁺-N and COD were set to 100 and 500–2000 mg L⁻¹ respectively in this study. The synthetic saline wastewater contained: 100 mg L⁻¹ NH₄⁺-N (NH₄Cl), 20 mg L⁻¹ PO₄³⁻-P (K₂HPO₄), and 1.0 mg L⁻¹ of trace element solution. The concentrations of COD (NaAC) were 500, 1000, 1500 and 2000 mg L⁻¹, 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⁻¹. To eliminate the influence of ammonia oxidation in batch test, 30 mg L⁻¹

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 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°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 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 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 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°C to below 15°C . The production of

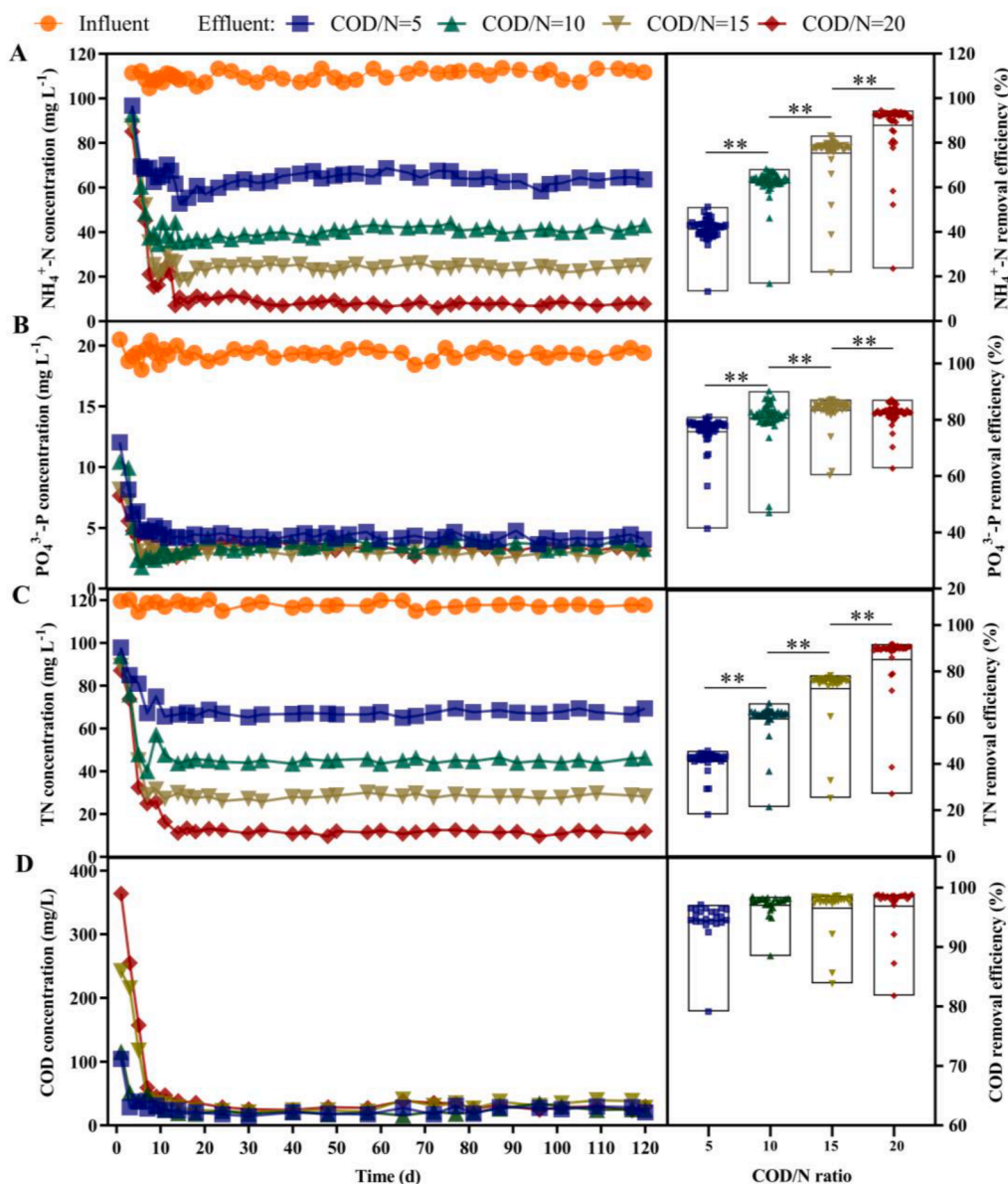


Fig. 1. The long-term treatment performance of microbiomes mediated by different COD/N ratio. Concentrations and efficiencies of ammonium (A), phosphate (B), total nitrogen (C) and COD of the four microbiomes. * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$.

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 1.0×10^6 copies ng^{-1} DNA, while the relative abundance of ammonia oxidation gene *amoA* was negligible ($< 3.0 \times 10^2$ copies 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

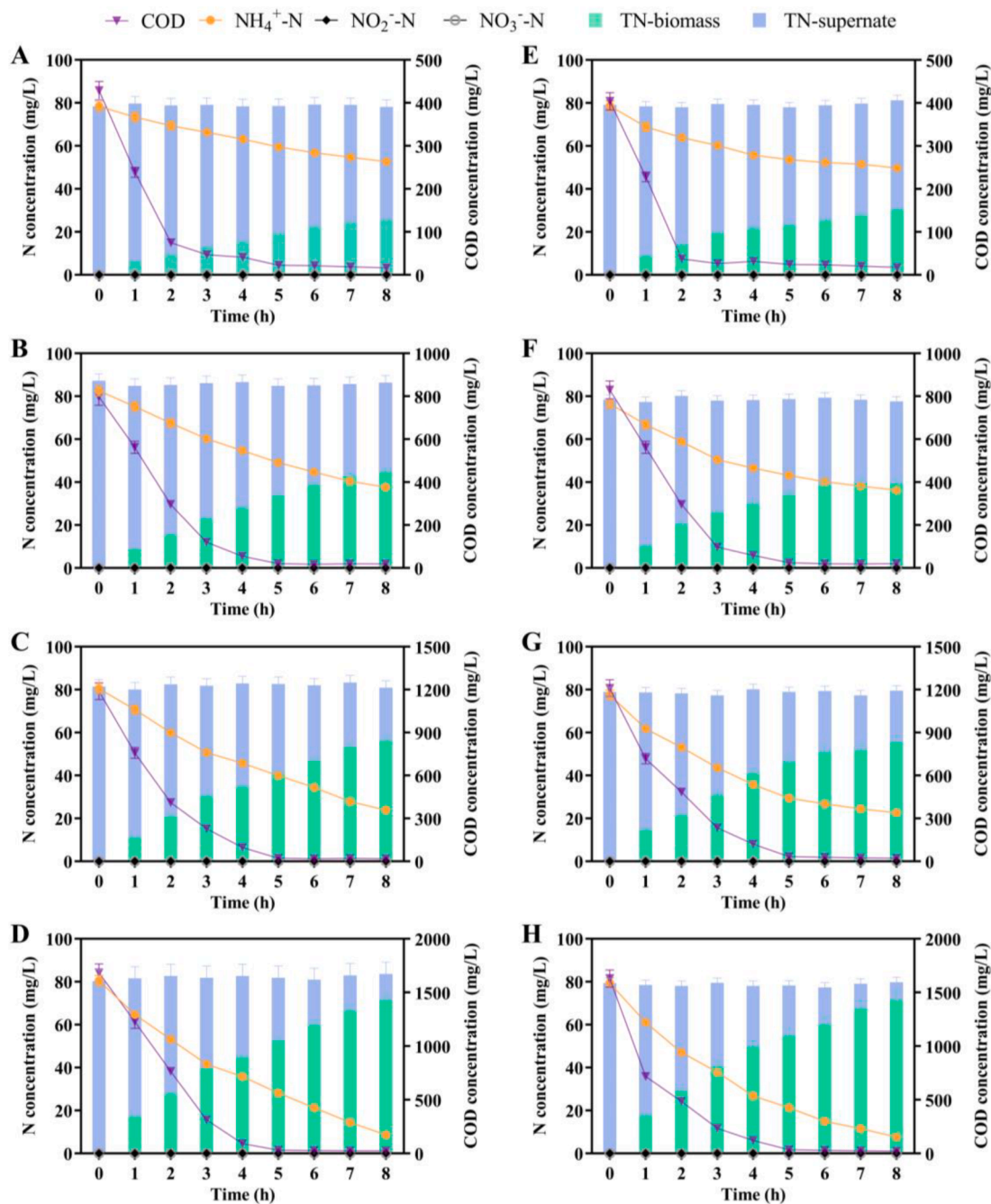


Fig. 2. Dynamics of COD and nitrogen components in a typical cycle of biosystems with COD/N ratio of 5, 10, 15 and 20 without (A, B, C, D) and with 30 mg/L ATU (E, F, G, H).

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 ± 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

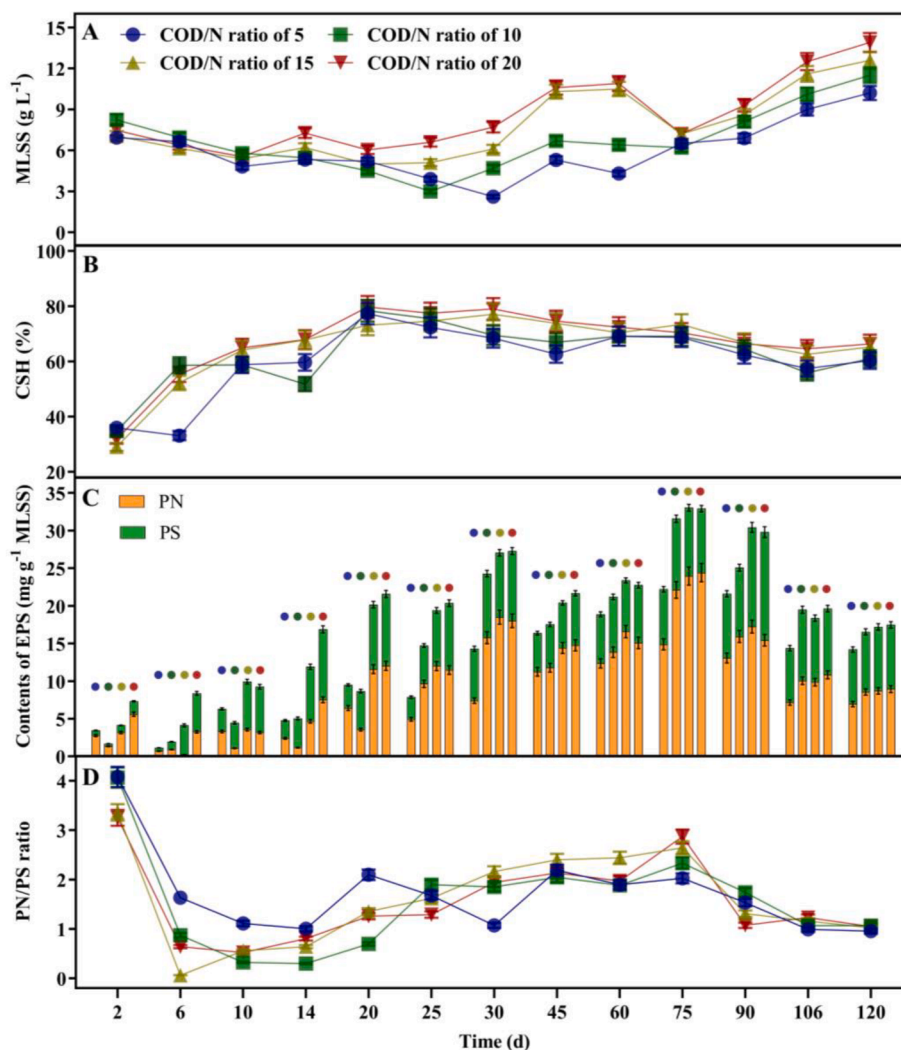


Fig. 3. Sludge properties in microbiomes with different COD/N ratio. (A) Mixed liquor suspended solid (MLSS); (B) Cell surface hydrophobicity (CSH); (C) Contents of PN and PS in EPS; (D) PN/PS ratio in EPS.

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,

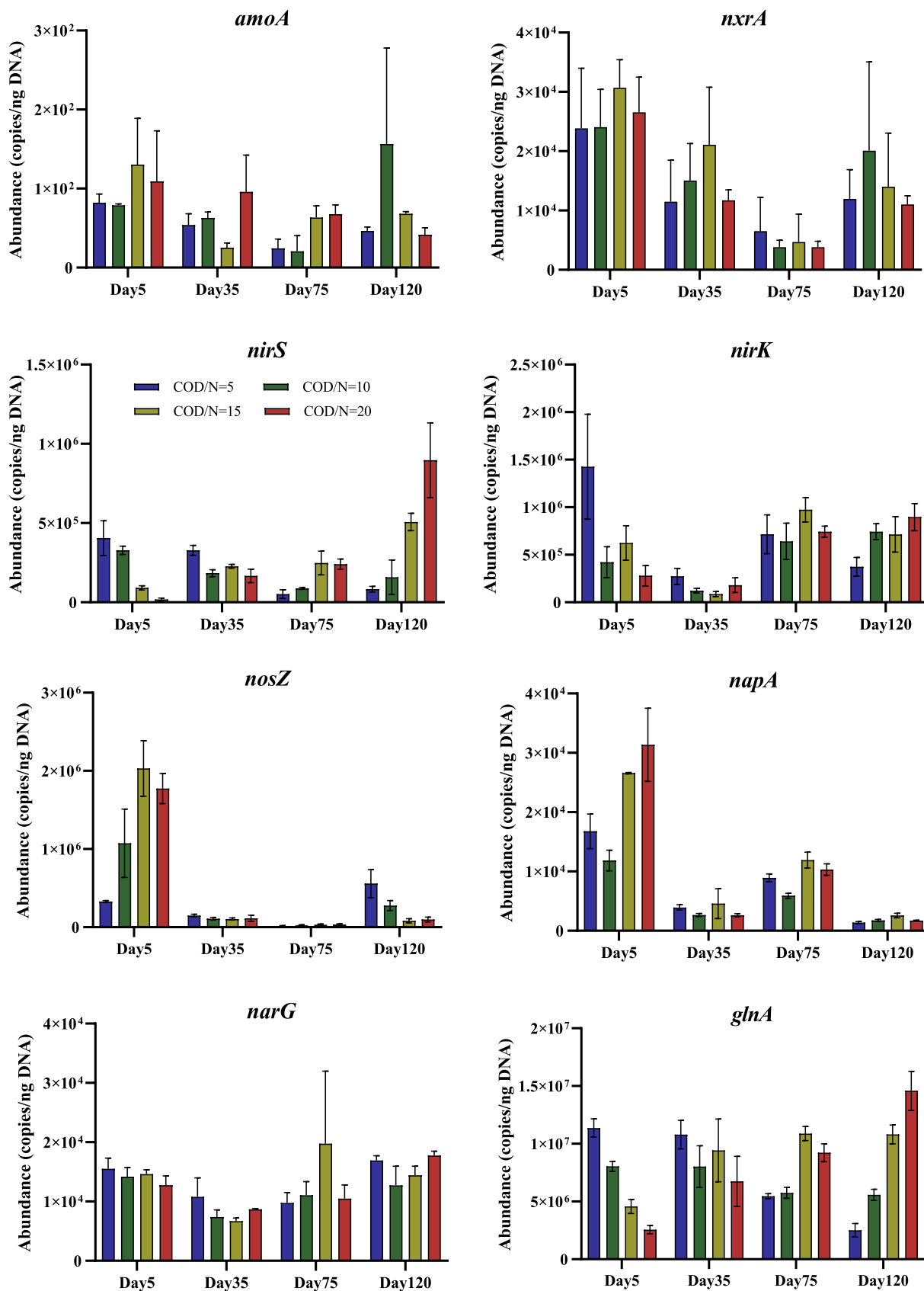


Fig. 4. Relative abundances of nitrogen metabolism-related genes in the four microbiomes.

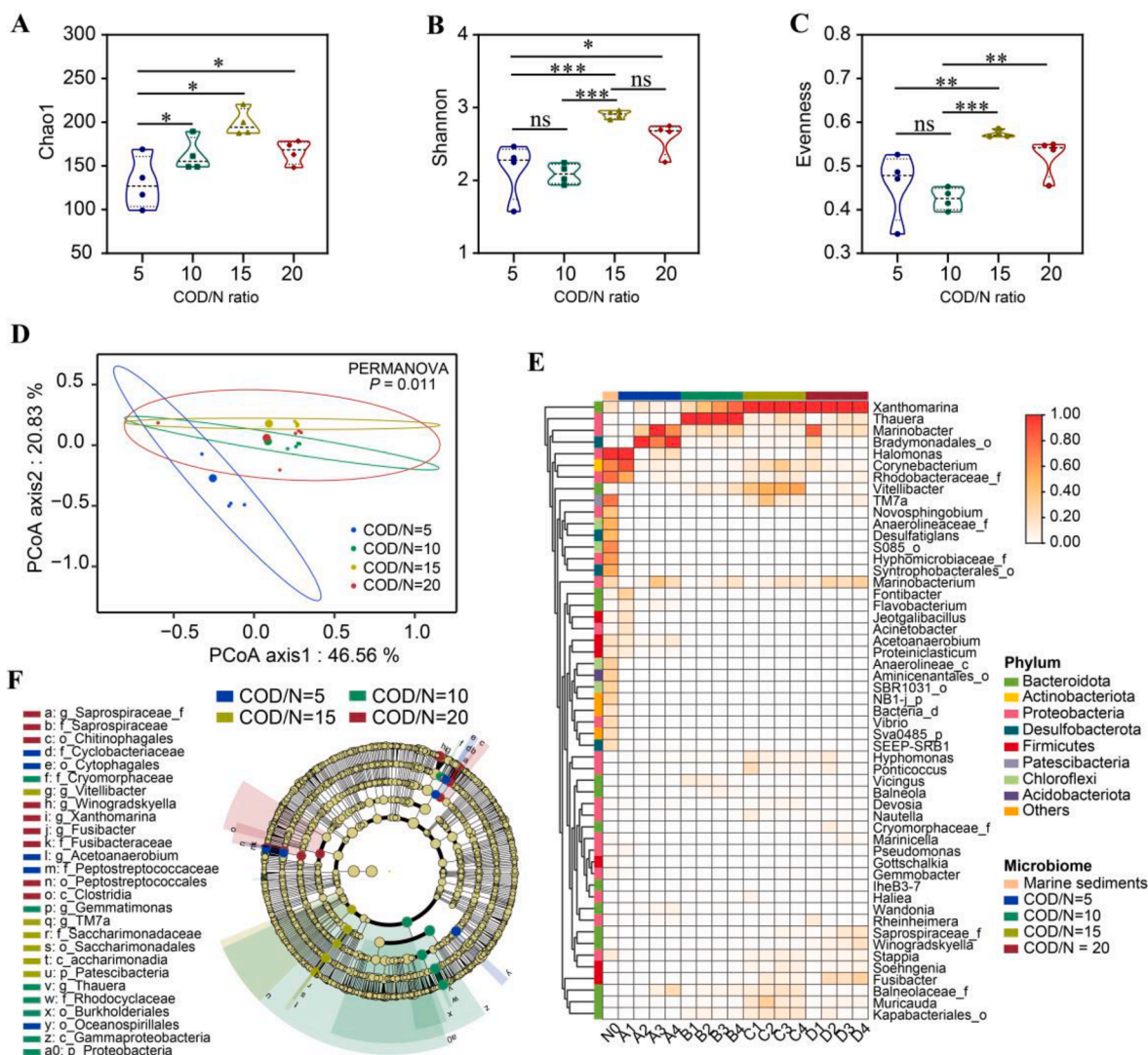


Fig. 5. Microbial diversity indices including Chao1 (A), Shannon (B), Evenness (C) of microbiomes. Statistical significance levels: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns = not significant. (D) Principal coordinates analysis (PCoA) based on 16S rRNA data. (E) Heatmap of the microbial community 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

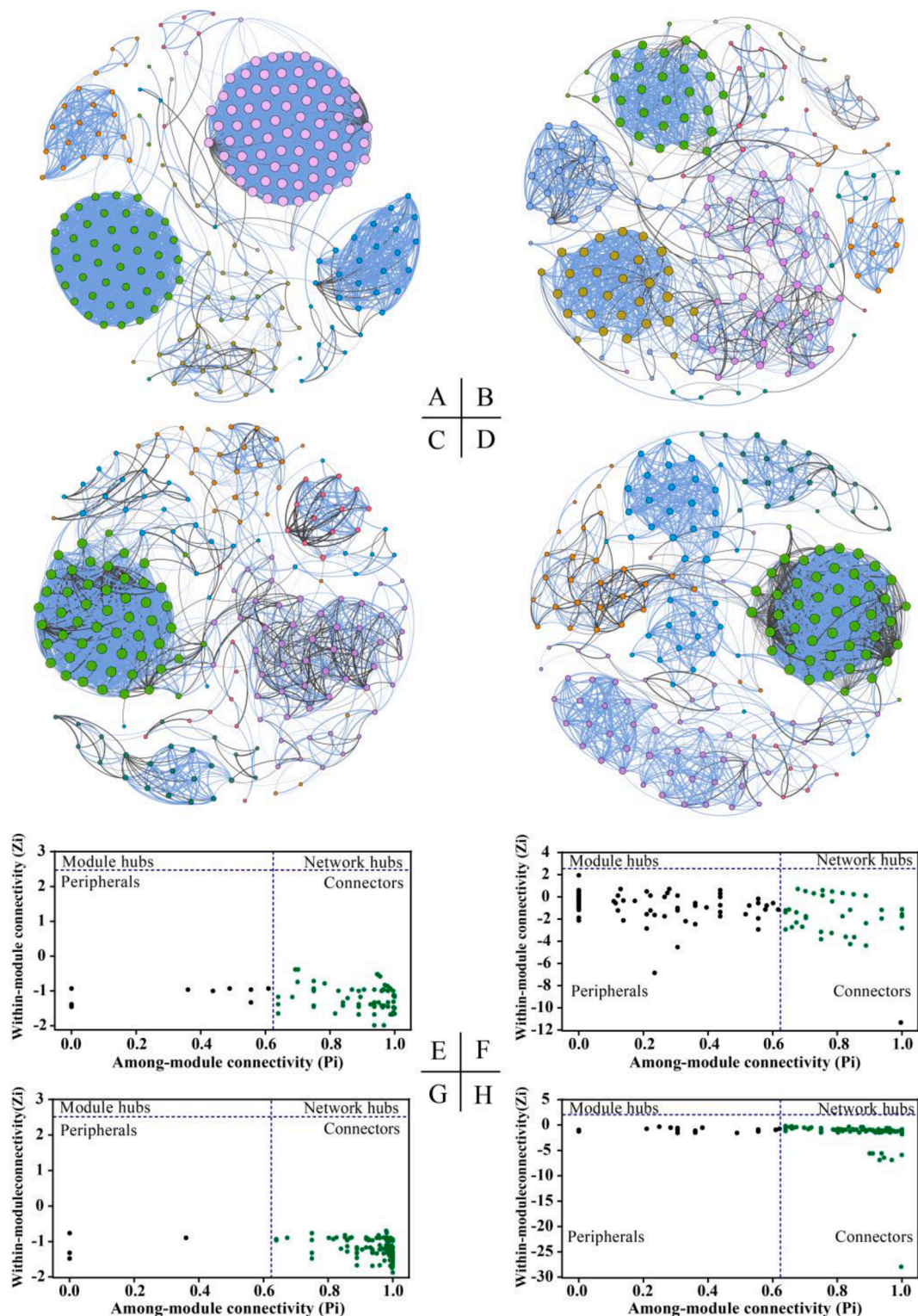


Fig. 6. Microbial network of ammonium assimilation microbiomes with COD/N ratio of 5 (A), 10 (B), 15 (C) and 20 (D). Genera assigned to the same module have the same color, and the color of the module is random. Modules are randomly colored. A bluish grey line indicated significant positive interactions 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

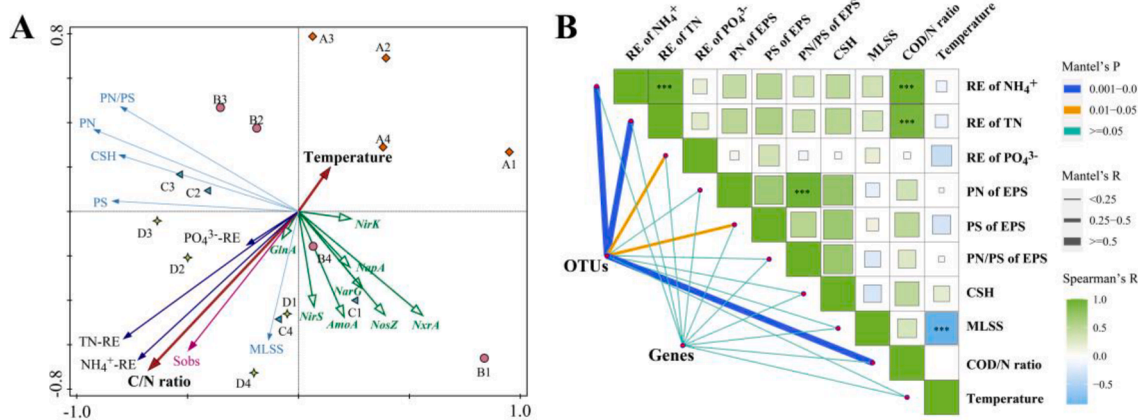


Fig. 7. (A) Principal component analysis (PCA) of the relationship among the system performance, environmental parameters and functional genes. (B) Mental test analysis with operational conditions effects on the evolution of microbial community and functional genes. (*p < 0.05; *p < 0.01, ***p < 0.001).

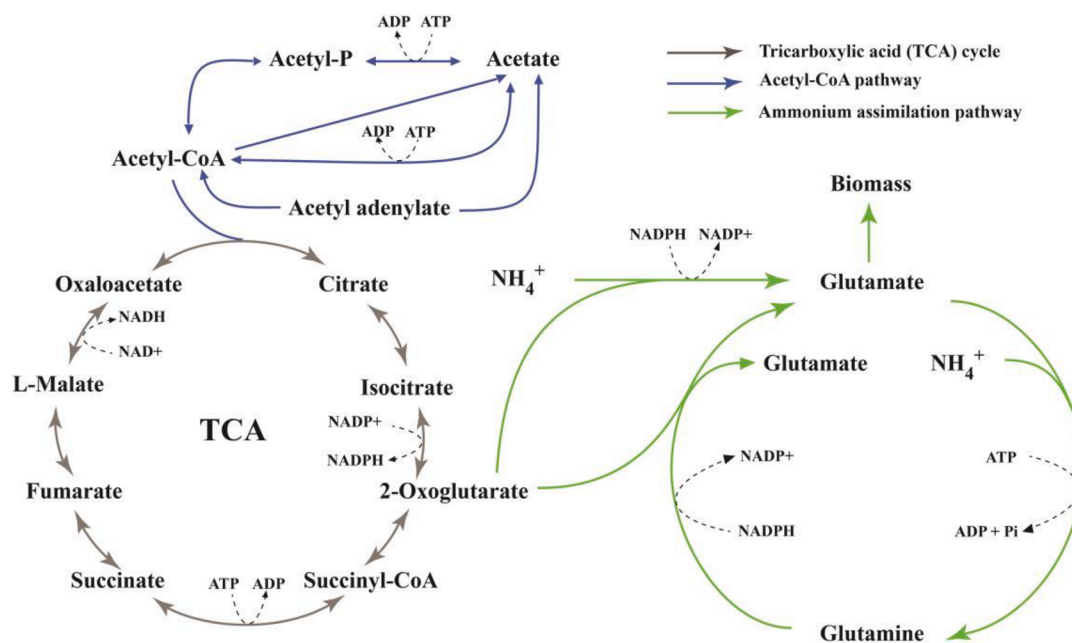


Fig. 8. The coupled metabolic pathways of carbon, nitrogen and phosphorus in the microbiomes.

microbiomes besides assimilation, such as biologically-induced phosphorus precipitation [46]. This was deduced based on the linear relationship between PO_4^{3-} -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_2 and CO_2 [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 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 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.

Acknowledgment

This work was supported by National Natural Science Foundation of China (U1906221); the Major Program of Shandong Province Technological Innovation Project (2020CXGC011403); Open Research Fund Program of Shandong Provincial Key Laboratory of Oilfield Produced Water Treatment and Environmental Pollution Control (SPE-P2021590).

Appendix A. Supplementary data

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

References

- [1] O. Lefebvre, R. Moletta, Treatment of organic pollution in industrial saline wastewater: A literature review, *Water Res.* 40 (2006) 3671–3682.
- [2] M.S. Moussa, D.U. Sumanasekera, S.H. Ibrahim, H.J. Lubberding, C.M. Hooijmans, H.J. Gijzen, M.C.M. van Loosdrecht, Long term effects of salt on activity, population structure and floc characteristics in enriched bacterial cultures of nitrifiers, *Water Res.* 40 (2006) 1377–1388.
- [3] P. Zhang, X. Bing, L. Jiao, H. Xiao, B. Li, H. Sun, Amelioration effects of coastal saline-alkali soil by ball-milled red phosphorus-loaded biochar, *Chem. Eng. J.* 431 (2022), 133904.
- [4] L. Lin, S. Pratt, Z. Li, L. Ye, Adaptation and evolution of freshwater Anammox communities treating saline/brackish wastewater, *Water Res.* 207 (2021), 117815.
- [5] L. Luo, W. Zhou, Y. Yuan, H. Zhong, C. Zhong, Effects of salinity shock on simultaneous nitrification and denitrification by a membrane bioreactor: Performance, sludge activity, and functional microflora, *Sci. Total Environ.* 801 (2021), 149748.
- [6] B.M. Gonzalez-Silva, K.R. Jonassen, I. Bakke, K. Østgaard, O. Vadstein, Nitrification at different salinities: Biofilm community composition and physiological plasticity, *Water Res.* 95 (2016) 48–58.
- [7] J.P. Zehr, J.S. Weitz, I. Joint, How microbes survive in the open ocean, *Science* 357 (2017) 646–647.
- [8] D.A. Hutchins, D.G. Capone, The marine nitrogen cycle: new developments and global change, *Nat. Rev. Microbiol.* (2022).
- [9] A.Z. Worden, M.J. Follows, S.J. Giovannoni, S. Wilken, A.E. Zimmerman, P. J. Keeling, Rethinking the marine carbon cycle: Factoring in the multifarious lifestyles of microbes, *Science* 347 (2015) 1257594.
- [10] M.M.M. Kuypers, H.K. Marchant, B. Kartal, The microbial nitrogen-cycling network, *Nat. Rev. Microbiol.* 16 (2018) 263–276.
- [11] F. Han, M. Zhang, Z. Liu, H. Shang, Q. Li, W. Zhou, Dynamic characteristics of microbial community and soluble microbial products in partial nitrification biofilm system developed from marine sediments treating high salinity wastewater, *J. Environ. Manage.* 290 (2021), 112586.
- [12] M. Zhang, F. Han, Y. Li, Z. Liu, H. Chen, Z. Li, Q. Li, W. Zhou, Nitrogen recovery by a halophilic ammonium-assimilating microbiome: A new strategy for saline wastewater treatment, *Water Res.* 207 (2021), 117832.
- [13] Y. Qin, K. Wang, Q. Xia, S. Yu, M. Zhang, Y. An, X. Zhao, Z. Zhou, Up-concentration of nitrogen from domestic wastewater: A sustainable strategy from removal to recovery, *Chem. Eng. J.* 451 (2023), 138789.
- [14] F. Han, W. Zhou, Nitrogen recovery from wastewater by microbial assimilation – A review, *Bioresour. Technol.* 363 (2022), 127933.
- [15] Y.-J. Lee, B.-L. Lin, M. Xue, K. Tsunemi, Ammonia/ammonium removal/recovery from wastewaters using bioelectrochemical systems (BES): A review, *Bioresour. Technol.* 363 (2022), 127927.
- [16] H. Cruz, Y.Y. Law, J.S. Guest, K. Rabaey, D. Batstone, B. Laycock, W. Verstraete, I. Pikaar, Mainstream Ammonium Recovery to Advance Sustainable Urban Wastewater Management, *Environ. Sci. Technol.* 53 (2019) 11066–11079.
- [17] X. Zhang, Y. Liu, Circular economy-driven ammonium recovery from municipal wastewater: State of the art, challenges and solutions forward, *Bioresour. Technol.* 334 (2021), 125231.
- [18] A. Eng, E. Borenstein, Microbial community design: methods, applications, and opportunities, *Curr. Opin. Biotechnol.* 58 (2019) 117–128.
- [19] C.E. Lawson, W.R. Harcombe, R. Hatzepichler, S.R. Lindemann, F.E. Löffler, M. A. O'Malley, H. García Martín, B.F. Pfeifer, L. Raskin, O.S. Venturelli, D. G. Weissbrodt, D.R. Noguera, K.D. McMahon, Common principles and best practices for engineering microbiomes, *Nat. Rev. Microbiol.* 17 (2019) 725–741.
- [20] U.G. Mueller, J.L. Sachs, Engineering microbiomes to Improve Plant and Animal Health, *Trends Microbiol.* 23 (2015) 606–617.
- [21] W.C. van Heeswijk, H.V. Westerhoff, F.C. Boogerd, Nitrogen assimilation in *Escherichia coli*: putting molecular data into a systems perspective, *Microbiol. Mol. Biol. Rev.* 77 (2013) 628–695.
- [22] A. Gosztolai, J. Schumacher, V. Behrends, J.G. Bundy, F. Heydenreich, M. H. Bennett, M. Buck, M. Barahona, GlnK Facilitates the Dynamic Regulation of Bacterial Nitrogen Assimilation, *Biophys. J.* 112 (2017) 2219–2230.
- [23] T. Hülsen, E.M. Barry, Y. Lu, D. Puyol, J. Keller, D.J. Batstone, Domestic wastewater treatment with purple phototrophic bacteria using a novel continuous photo anaerobic membrane bioreactor, *Water Res.* 100 (2016) 486–495.
- [24] Z. Pan, J. Zhou, Z. Lin, Y. Wang, P. Zhao, J. Zhou, S. Liu, X. He, Effects of COD/TN ratio on nitrogen removal efficiency, microbial community for high saline wastewater treatment based on heterotrophic nitrification-aerobic denitrification process, *Bioresour. Technol.* 301 (2020), 122726.
- [25] Y. Jia, M. Zhou, Y. Chen, Y. Hu, J. Luo, Insight into short-cut of simultaneous nitrification and denitrification process in moving bed biofilm reactor: Effects of carbon to nitrogen ratio, *Chem. Eng. J.* 400 (2020), 125905.
- [26] Y. Zhang, J. Wang, S. Dai, J. Zhao, X. Huang, Y. Sun, J. Chen, Z. Cai, J. Zhang, The effect of C: N ratio on heterotrophic nitrification in acidic soils, *Soil Biol. Biochem.* 137 (2019), 107562.
- [27] R. Pishgar, J.A. Dominic, J.H. Tay, A. Chu, Pilot-scale investigation on nutrient removal characteristics of mineral-rich aerobic granular sludge: Identification of uncommon mechanisms, *Water Res.* 168 (2020), 115151.
- [28] B. Li, S. Irvin, The roles of nitrogen dissimilation and assimilation in biological nitrogen removal treating low, mid, and high strength wastewater, *J. Environ. Eng. Sci.* 6 (2007) 483–490.
- [29] L. Huang, N. Wang, C. Deng, Y. Liang, Q. Wang, M. Liu, Y. Chen, Interactive effect of carbon source with influent COD/N on nitrogen removal and microbial community structure in subsurface flow constructed wetlands, *J. Environ. Manage.* 250 (2019), 109491.
- [30] L. Friedman, H. Mamane, D. Avisar, K. Chandran, The role of influent organic carbon-to-nitrogen (COD/N) ratio in removal rates and shaping microbial ecology in soil aquifer treatment (SAT), *Water Res.* 146 (2018) 197–205.
- [31] D. Todt, A. Heistad, P.D. Jenssen, Load and distribution of organic matter and nutrients in a separated household wastewater stream, *Environ. Technol.* 36 (2015) 1584–1593.
- [32] N. Gharsallah, L. Khannous, N. Souissi, M. Nasri, Biological treatment of saline wastewaters from marine-products processing factories by a fixed-bed reactor, *J. Chem. Technol. Biotechnol.* 77 (2002) 865–870.
- [33] Z.-Z. Zheng, L.-W. Zheng, M.N. Xu, E. Tan, D.A. Hutchins, W. Deng, Y. Zhang, D. Shi, M. Dai, S.-J. Kao, Substrate regulation leads to differential responses of microbial ammonia-oxidizing communities to ocean warming, *Nat. Commun.* 11 (2020) 3511.
- [34] APHA, AWWA, WEF, Standard methods for the examination of water and wastewater (21st ed.), American Public Health Association, Washington, DC, 2005.
- [35] C. Yin, F. Meng, G.H. Chen, Spectroscopic characterization of extracellular polymeric substances from a mixed culture dominated by ammonia-oxidizing bacteria, *Water Res.* 68 (2015) 740–749.
- [36] C. Wan, X. Yang, D.-J. Lee, X.-Y. Wang, Q. Yang, X. Pan, Aerobic granulation of aggregating consortium X9 isolated from aerobic granules and role of cyclic di-GMP, *Bioresour. Technol.* 152 (2014) 557–561.
- [37] F. Han, M. Zhang, H. Shang, Z. Liu, W. Zhou, Microbial community succession, species interactions and metabolic pathways of sulfur-based autotrophic denitrification system in organic-limited nitrate wastewater, *Bioresour. Technol.* 315 (2020), 123826.
- [38] F. Han, M. Zhang, Z. Liu, Y. Han, Q. Li, W. Zhou, Enhancing robustness of halophilic aerobic granule sludge by granular activated carbon at decreasing temperature, *Chemosphere* 292 (2022), 133507.

- [39] Z. Zheng, Y. Cai, Y. Zhang, Y. Zhao, Y. Gao, Z. Cui, Y. Hu, X. Wang, The effects of C/N (10–25) on the relationship of substrates, metabolites, and microorganisms in “inhibited steady-state” of anaerobic digestion, *Water Res.* 188 (2021), 116466.
- [40] Q. He, H. Wang, L. Chen, S. Gao, W. Zhang, J. Song, J. Yu, Robustness of an aerobic granular sludge sequencing batch reactor for low strength and salinity wastewater treatment at ambient to winter temperatures, *J. Hazard. Mater.* 384 (2020), 121454.
- [41] L. Wittebolle, M. Marzorati, L. Clement, A. Balloi, D. Daffonchio, K. Heylen, P. De Vos, W. Verstraete, N. Boon, Initial community evenness favours functionality under selective stress, *Nature* 458 (2009) 623–626.
- [42] J.M. Olesen, J. Bascompte, Y.L. Dupont, P. Jordano, The modularity of pollination networks, *Proceedings of the National Academy of Sciences*, 104 (2007) 19891.
- [43] L. Zhang, J. Zhang, G. Zeng, H. Dong, Y. Chen, C. Huang, Y. Zhu, R. Xu, Y. Cheng, K. Hou, W. Cao, W. Fang, Multivariate relationships between microbial communities and environmental variables during co-composting of sewage sludge and agricultural waste in the presence of PVP-AgNPs, *Bioresour. Technol.* 261 (2018) 10–18.
- [44] G. Capson-Tojo, S. Lin, D.J. Batstone, T. Hülsen, Purple phototrophic bacteria are outcompeted by aerobic heterotrophs in the presence of oxygen, *Water Res.* 194 (2021), 116941.
- [45] L. Reitzer, Nitrogen Assimilation and Global Regulation in *Escherichia coli*, *Annu. Rev. Microbiol.* 57 (2003) 155–176.
- [46] M. Maurer, D. Abramovich, H. Siegrist, W. Gujer, Kinetics of biologically induced phosphorus precipitation in waste-water treatment, *Water Res.* 33 (1999) 484–493.
- [47] B. Zhang, D. Ning, Y. Yang, J.D. Van Nostrand, J. Zhou, X. Wen, Biodegradability of wastewater determines microbial assembly mechanisms in full-scale wastewater treatment plants, *Water Res.* 169 (2020), 115276.
- [48] Q. Zhang, C. Zhang, Y. Zhu, C. Yuan, T. Zhao, Effect of bacteria-to-algae volume ratio on treatment performance and microbial community of a novel heterotrophic nitrification-aerobic denitrification bacteria-Chlorella symbiotic system, *Bioresour. Technol.* 126025 (2021).
- [49] Q. Liu, Y. Sheng, W. Wang, C. Li, G. Zhao, Remediation and its biological responses of Cd contaminated sediments using biochar and minerals with nanoscale zero-valent iron loading, *Sci. Total Environ.* 713 (2020), 136650.
- [50] X. Wang, F. Kong, Y. Li, Q. Li, C. Wang, J. Zhang, M. Xi, Effect of simulated tidal cycle on DOM, nitrogen and phosphorus release from sediment in Dagou River-Jiaozhou Bay estuary, *Sci. Total Environ.* 783 (2021), 147158.
- [51] K. Swan Brandon, M. Martinez-Garcia, M. Preston Christina, A. Sczyrba, T. Woyke, D. Lamy, T. Reinthaler, J. Poulton Nicole, E.D.P. Masland, L. Gomez Monica, E. Sieracki Michael, F. DeLong Edward, J. Herndl Gerhard, R. Stepanauskas, Potential for chemolithoautotrophy among ubiquitous bacteria lineages in the dark ocean, *Science* 333 (2011) 1296–1300.
- [52] C.-Y. Lai, L. Zhou, Z. Yuan, J. Guo, Hydrogen-driven microbial biogas upgrading: Advances, challenges and solutions, *Water Res.* 197 (2021), 117120.
- [53] Z. Xu, X. Chen, H. Li, D. Wan, J. Wan, Combined heterotrophic and autotrophic system for advanced denitrification of municipal secondary effluent in full-scale plant and bacterial community analysis, *Sci. Total Environ.* 717 (2020), 136981.
- [54] Q. Wang, J. He, Complete nitrogen removal via simultaneous nitrification and denitrification by a novel phosphate accumulating *Thauera* sp. strain SND5, *Water Res.* 185 (2020), 116300.
- [55] L. Zhang, J. Wang, G. Fu, Z. Zhang, Simultaneous electricity generation and nitrogen and carbon removal in single-chamber microbial fuel cell for high-salinity wastewater treatment, *J. Clean. Prod.* 276 (2020), 123203.
- [56] Y. Gao, L. Guo, M. Shao, F. Hu, G. Wang, Y. Zhao, M. Gao, C. Jin, Z. She, Heterotrophic denitrification strategy for marine recirculating aquaculture wastewater treatment using mariculture solid wastes fermentation liquid as carbon source: Optimization of COD/NO₃–N ratio and hydraulic retention time, *Bioresour. Technol.* 304 (2020), 122982.
- [57] S. Lu, Q. Wang, M. Gao, C. Zhao, Z. She, Y. Zhao, C. Jin, L. Guo, Effect of aerobic/anoxic duration on the performance, microbial activity and microbial community of sequencing batch biofilm reactor treating synthetic mariculture wastewater, *Bioresour. Technol.* 333 (2021), 125198.
- [58] W. Xiong, L. Wang, N. Zhou, A. Fan, S. Wang, H. Su, High-strength anaerobic digestion wastewater treatment by aerobic granular sludge in a step-by-step strategy, *J. Environ. Manage.* 262 (2020), 110245.
- [59] D. Jiang, W.O. Khunjar, B. Wett, S.N. Murthy, K. Chandran, Characterizing the Metabolic Trade-Off in *Nitrosomonas europaea* in Response to Changes in Inorganic Carbon Supply, *Environ. Sci. Technol.* 49 (2015) 2523–2531.
- [60] A. Guisasola, S. Petzet, J.A. Baeza, J. Carrera, J. Lafuente, Inorganic carbon limitations on nitrification: Experimental assessment and modelling, *Water Res.* 41 (2007) 277–286.
- [61] T. Bressani-Ribeiro, P.G.S. Almeida, C.A.L. Chernicharo, E.I.P. Volcke, Inorganic carbon limitation during nitrogen conversions in sponge-bed trickling filters for mainstream treatment of anaerobic effluent, *Water Res.* 201 (2021), 117337.
- [62] Q. Shangguan, H. Shu, P. Li, K. Lin, R.H. Byrne, Q. Li, D. Yuan, J. Ma, Automated spectrophotometric determination of carbonate ion concentration in seawater using a portable syringe pump based analyzer, *Mar. Chem.* 209 (2019) 120–127.
- [63] C.R. Bryan, A.W. Knight, R.M. Katona, A.C. Sanchez, E.J. Schindelholz, R. F. Schaller, Physical and chemical properties of sea salt deliquescent brines as a function of temperature and relative humidity, *Sci. Total Environ.* 824 (2022), 154462.
- [64] J. Ling, S. Chen, Impact of organic carbon on nitrification performance of different biofilters, *Aquacult. Eng.* 33 (2005) 150–162.
- [65] M. Ali, D.R. Shaw, P.E. Saikaly, Application of an enrichment culture of the marine anammox bacterium “*Ca. Scalindua* sp. AMX11” for nitrogen removal under moderate salinity and in the presence of organic carbon, *Water Res.* 170 (2020), 115345.
- [66] W. Song, D. Xu, X. Bi, H.Y. Ng, X. Shi, Intertidal wetland sediment as a novel inoculation source for developing aerobic granular sludge in membrane bioreactor treating high-salinity antibiotic manufacturing wastewater, *Bioresour. Technol.* 314 (2020), 123715.
- [67] C. Sun, B. Zhang, D. Ning, Y. Zhang, T. Dai, L. Wu, T. Li, W. Liu, J. Zhou, X. Wen, Seasonal dynamics of the microbial community in two full-scale wastewater treatment plants: Diversity, composition, phylogenetic group based assembly and co-occurrence pattern, *Water Res.* 200 (2021), 117295.
- [68] S. Banerjee, C.A. Kirkby, D. Schmutter, A. Bissett, J.A. Kirkegaard, A.E. Richardson, Network analysis reveals functional redundancy and keystone taxa amongst bacterial and fungal communities during organic matter decomposition in an arable soil, *Soil Biol. Biochem.* 97 (2016) 188–198.
- [69] M.K.H. Winkler, L. Straka, New directions in biological nitrogen removal and recovery from wastewater, *Curr. Opin. Biotechnol.* 57 (2019) 50–55.
- [70] F. Han, Z. Li, Q. Li, Z. Liu, Y. Han, Q. Li, W. Zhou, Cooperation of heterotrophic bacteria enables stronger resilience of halophilic assimilation biosystem than nitrification system under long-term stagnation, *Sci. Total Environ.* 848 (2022), 157806.