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Ammonium-assimilating microbiome: A halophilic biosystem rationally optimized by carbon to nitrogen ratios with stable nitrogen conversion and microbial structure

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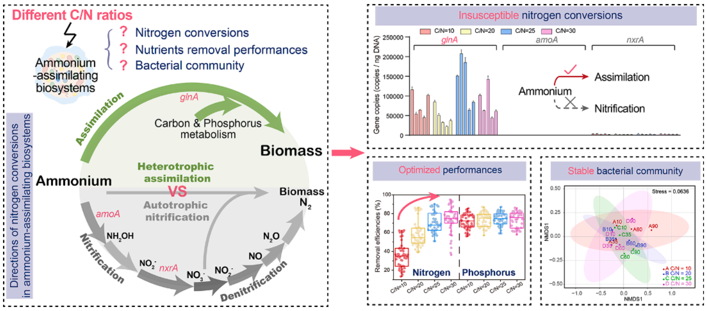
HIGHLIGHTS

- C/N ratios do not change nitrogen conversions in ammonium-assimilating biosystems.
- COD, nitrogen and phosphorus are removed simultaneously at different C/N ratios.
- C/N ratios do not significantly change structure of heterotrophic communities.

GRAPHICAL ABSTRACT



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ABSTRACT

The contradiction between theoretical metabolism of ammonium assimilation and experiential understanding of conventional biosystems makes the rational optimization of the ammonium-assimilating microbiome through carbon to nitrogen (C/N) ratios perplexing. The effect of different C/N ratios on ammonium-assimilating biosystems was investigated in saline wastewater treatment. C/N ratios significantly hindered the nutrient removal efficiency, but ammonium-assimilating biosystems maintained functional stability in nitrogen conversions and microbial communities. With sufficient biomass, higher than 86% ammonium and 73% phosphorus were removed when C/N ratios were higher than 25. Ammonium assimilation dominated the nitrogen metabolism in all biosystems even under relatively low C/N ratios, evidenced by the extremely low abundances of nitrification functional genes. Different C/N ratios did not significantly change the bacterial community structure of ammonium-assimilating biosystems. It is anticipated that the ammonium-assimilating biosystem with advantages of clear metabolic pathway and easy optimization can be applied to nutrient removal and recovery in saline environments.

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1. Introduction

The anthropogenic input of reactive nitrogen into ecosystems through excessive fertilizers application and nitrogen discharge (Yu et al., 2019; Zhang et al., 2015) has perturbed the nitrogen cycle. The process of nitrification and denitrification is the focused strategy for artificial nitrogen management, such as the biological nitrogen removal (BNR) in wastewater treatment plants and the pervasive nitrogen conversion in agricultural soils (Beeckman et al., 2018; Winkler and Straka, 2019). However, these nitrogen conversions are strongly inhibited in saline environments, caused by the detrimental effect of salt on functional microbes (Corsino et al., 2019). The inevitable accumulation of nitrogen intermediates, such as nitrite and nitrate (Carrera et al., 2019), impairs the total nitrogen removal, and the resultant discharge of saline wastewater leads to the eutrophication of water bodies and exacerbates water scarcity (Ma et al., 2020). In agricultural soils, bioavailable nitrogen is converted to unreactive dinitrogen and releases from the biosphere through nitrification–denitrification, along with the nitrate leaching and gaseous nitrous oxide production (Beeckman et al., 2018). High salinity also negatively affected the biodiversity and microorganism activity in soils, leading to the significant inhibition of crop productivity in saline-alkali soils (Singh, 2016). From the perspective of the microbial nitrogen-cycling network, the interconversion of ammonium and organic nitrogen accounts for the largest flux of microbial nitrogen cycling (Kuypers et al., 2018). According to this nature-based ecological nitrogen conversion, an ammonium-assimilating microbiome with effective nutrient removal performance has been firstly built following the bottom-up design as a promising approach for nutrient recovery and recycling for saline wastewater treatment (Zhang et al., 2021). The ammonium-assimilating microbiome has been demonstrated to convert nitrogen to organic nitrogen in biomass, without undesirable intermediate (nitrite and nitrate) accumulation and gaseous nitrogen loss (Zhang et al., 2021). Whether based on conventional nitrification–denitrification or novel ammonium assimilation, biosystems for engineering applications have to face the fluctuation of nutrient and environmental conditions. Thus, it is necessary to promote nitrogen metabolism in saline environments in order to alleviate the pollution caused by nitrogen discharge and to accelerate the improvement of saline-alkali soils.

The critical metabolism driven by functional microbes, such as nitrogen removal in wastewater treatment, is the basis to support the performance optimization in engineering biosystems. Conventionally, nitrification–denitrification has usually been intensified to improve nutrient conversions in saline environments. Yet, this multistep process is generally carried out by autotrophic nitrifiers and heterotrophic denitrifiers, resulting in the inevitable production of intermediates. Specifically, complete nitrification can hardly be achieved in saline environments, mainly due to the different tolerance of ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) to salinity (Pronk et al., 2014). Consequently, other nitrogen metabolism pathways is mandatory to combine with nitrification to eliminate inevitable intermediates, such as denitrification and anammox (Liu et al., 2020; Wang et al., 2022). Changing environmental conditions is usually used to promote nitrogen metabolism, but may easily lead to the unpredictable manipulation of conventional microbiomes. The reason is that different microbial consortia to conduct multistep processes have varying sensitivity to environmental conditions and nutrient affinities for substrates (Lawson et al., 2019). Compared with the multistep process, the process of ammonium assimilation is generally carried out by a group of heterotrophic microbes with a similar function (Zhang et al., 2021), which is postulated to be easily optimized in complex biosystems (Lawson et al., 2019). In this clear pathway, nitrogen is incorporated into central carbon compounds in the TCA cycle via the glutamate dehydrogenase (GDH) or glutamine synthetase/glutamate synthase (GS/GOGAT) pathway. Based on its metabolic characteristics in which ammonium is stored into biomass coupling with the consumption of carbon source, the increase of carbon input could theoretically improve the uptake of nitrogen in ammonium-assimilating biosystems.

The ratio of carbon to nitrogen (C/N) has been considered as one of the critical engineering parameters to improve the nitrogen metabolism of biosystems by changing the microbial community. Different C/N ratios could lead to the ecological niche partition of autotrophic bacteria and heterotrophic bacteria due to their different demand for carbon sources. In practice, the addition of carbon sources has been reported to enhance the nitrogen removal in saline wastewater treatment and nutrient biological turnover in saline soil (Corsino et al., 2019; Liu et al., 2021). This improved BNR performance has been mainly attributed to the enhancement of nitrification in biosystems. Furthermore, it has been reported that heterotrophic nitrification, in replacement of autotrophic nitrification, dominates the BNR when the C/N ratio was higher than 20 (Pan et al., 2020). However, this experiential understanding that extra carbon sources can strengthen the nitrification pathway makes the rational manipulation of the ammonium-assimilating microbiome remain puzzling. The introduction of nitrification in ammonium-assimilating biosystems might cause the invalidation of ammonium-assimilating function in the heterotrophic microbial community, since the ubiquitous nitrifiers are more competitive for nitrogen than ammonium-assimilating microbes (Zhang et al., 2021). This contradiction between the empirical understanding and theoretical mechanism of ammonium assimilation makes it necessary to verify the oriented optimization of the ammonium-assimilating biosystem under the invasion of environmental microorganisms. Therefore, understanding the microbial interactions and their response to different C/N rations is crucial to prove the adaptability and applicability of the ammonium-assimilating biosystem.

For these reasons, this study aimed to (I) uncover the influence of the C/N ratios on treatment performance, functional stability and microbial structure in ammonium-assimilating biosystems, (II) to ascertain the feasibility of applying carbon addition as the optimized parameter in saline wastewater treatment, and (III) to better elucidate the nitrogen metabolism of ammonium-assimilating biosystems.

2. Materials and methods

2.1. Media and culture conditions

The marine bacterium *Psychrobacter aquimaris* has been used as the functional bacteria to build ammonium-assimilating biosystem (Zhang et al., 2021). In previous study, the ammonium-assimilating metabolism was proved that this microbe removed and restored ammonium into biomass without gaseous nitrogen production. Therefore, the effects of C/N ratios on *P. aquimaris* was investigated as a prior verification. *P. aquimaris* was cultured in Luria-Bertani (LB) medium with salinity of 3% (containing 3 g seawater crystal per liter) at 200 rpm and 25 °C for 24 h, and then 10% (v/v) inoculums were inoculated into ammonium medium (AM) under different C/N ratios for subsequent experiments. The dry cell weight was calculated after 24 h (Huang et al., 2018) to reflect the growth of *P. aquimaris.* AM with different C/N ratios contained (per liter): NH_4Cl 200 mg, KH_2PO_4 45 mg; seawater crystal 30 g; sodium acetate 320 mg, 641 mg, 961 mg, 1282 mg, 1602 mg, and 1923 mg, respectively (C/N rations of 5, 10, 15, 20, 25 and 30, respectively). The C/N ratio referred to the chemical oxygen demand (COD) to total nitrogen (TN) ratio in this study.

2.2. Operation of sequencing batch reactors (SBRs)

To investigate the effect of C/N ratios (10, 20, 25 and 30) on ammonium-assimilating biosystems, four identical column-type SBRs were established. The environmental and engineering functions are usually performed by microbiomes instead of pure cultured functional microbes (Lawson et al., 2019). Thus, *P. aquimaris* was used as the functional microbe because of its capability of biofilm formation and ammonium assimilation to build the ammonium-assimilating microbiome with other naturally occurring microbes. The detailed constructing process of ammonium-assimilating biosystems referred to the previous study (Zhang et al., 2021). The cycle time of each SBR was 8 h (5 min feeding, 450 min aeration, 15 min settling and 10 min decanting). The hydraulic retention time (HRT) was 12.8 h, while the dissolved oxygen (DO) was about 2–3 mg/L. Synthetic saline wastewater contained (per liter): NH_4Cl 200 mg, KH_2PO_4 45 mg; seawater crystal 30 g; sodium acetate 641 mg, 1282 mg, 1602 mg, and 1923 mg (C/N ratios of 10, 20, 25 and 30, respectively). The ammonium concentration in synthetic saline referred to the concentration in domestic wastewater (Luo et al., 2021). The salinity of 3% (w/v) was selected to represents the salinity value of saline wastewater, since the average value of salinity was approximately 3% (w/v) in saline wastewater and in seawater contains (Srivastava et al., 2021).

2.3. Batch activity tests of sludge

Batch activity tests were conducted after 90 days of operation to investigate the effect of C/N ratios on nitrogen removal pathways and nutrient performances of sludge. Nitrogen balance was calculated to identify the pathway of ammonium assimilation and nitrogen loss in the system. The total nitrogen (TN) in the biomass were calculated via the subtraction of TN of the system and TN of the supernatant. More detailed calculation was referred to the previous study (Zhang et al., 2021). Specific ammonium utilization rate (SAUR, which represents the amount of ammonia utilization during the first 4 h of the test per sludge mass and time) was used to determine the nitrogen removal capability. The nitrogen utilization tests were also conducted in presence of 30 mg L^{-1} allylthiourea (ATU) (Xu et al., 2018). As the inhibitor of nitrification, ATU was added only in the batch test to distinguish the autotrophic nitrifying capability and heterotrophic ammonium-assimilating capability.

2.4. DNA extraction and community analysis

The genome DNA of sludge samples were extracted to analyse the microbial community and abundances of functional genes. High throughput sequencing was used to reveal the compositions of bacterial community structures. Sludge samples were obtained from four SBRs after 10, 35, 60, and 90 days of operation. Samples were labeled with the name of SBRs (A, B, C and D represented SBRs under the C/N ratios of 10, 20, 25 and 30, respectively.) and sampling time. Genomic DNA of sludge samples was extracted using the FastDNA SPIN Kit for Soil (MP Biomedicals, America). Genomic DNA samples were sent to Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China) for PCR amplification (the V3-V4 region of 16S rDNA) and high-throughput sequencing (Miseq illumina platform).

2.5. Quantitative PCR analysis of functional genes

Nitrogen functional genes were quantified from genomic DNA using qPCR (qTOWER^3 G, Analytic Jena AG, Germany) to identify different nitrogen removal pathways. The primers for nitrogen functional genes and detailed methods were referring to the previous study (Zhang et al., 2021), including glutamine synthetase (*glnA*), ammonium monooxygenase (*amoA*), nitrite oxidoreductase (*nxrA*), nitrate reductase (*napA* and *narG*), and nitrite reductases (*nirS* and *nirK*). Since N_2 and N_2O was not produced in the process of ammonium assimilation (Zhang et al., 2021), functional genes associated with gaseous nitrogen generation were not amplified in this study.

2.6. Co-occurrence network analysis

Network analysis was conducted to explore the co-occurrence of bacterial taxa and to reflect microbial interactions in different SBRs. R v4.0.0 was used to calculate the Spearman's rank correlation coefficient matrix based on the relative abundance of OTUs with the average relative abundance of >0.1% were selected for Spearman's correlation analysis. Connections that were strong ($|r| > 0.60$) and statistically significant (p-value < 0.01) correlations were considered in occurrence network analysis. The occurrence networks were constructed and visualized with Gephi software (v0.9.2) and Cytoscape (v3.8.2). The within-module connectivity (Zi) and among-module connectivity (Pi) were calculated to understand the topological role of OTUs in networks. Based on the value of Zi and Pi, nodes in the network were sorted into four categories: peripherals (interconnected nodes inside a module with only a few connections outside the module, $Zi \leq 2.5$ and $Pi \leq 0.62$), connectors (nodes linking to different modules, $Zi \leq 2.5$, $Pi > 0.62$), module hubs (highly connected nodes inside a module, $Zi > 2.5$, $Pi \leq 0.62$), and network hubs (acting as both module hubs and connectors, $Zi > 2.5$, $Pi > 0.62$) (Olesen et al., 2007).

2.7. Analytical methods

The ammonium, nitrite, nitrate, TN, COD, TP, and mixed liquor suspended solid (MLSS) were calculated based on the Standard Methods. The extracellular polymeric substance (EPS) of the sludge samples was extracted by using a heat extraction method (Zhang et al., 2016). The proteins (PN) and polysaccharides (PS) in EPS were detected using the modified Lowry method (Lowry et al., 1951) and the phenol–sulfuric acid method (DuBois et al., 1956), respectively. The particle size distribution of sludge was measured by Mastersizer (MS2000, Malvern, UK). Elemental analysis was carried out using an elemental analyzer (Vario EL Cube, Elementar, Germany) to analyze the carbon (C), nitrogen (N), hydrogen (H) and Sulphur (S) of dried samples.

The nonmetric multidimensional scaling (NMDS) was performed in R v4.0.0 with the vegan package to show the overall variations of bacterial community composition in SBRs under different C/N ratios. ANOSIM was applied to assess similarities for categorical variables. All data analyses were conducted with SPSS Statistic version 20 (IBM, USA).

3. Results and discussion

3.1. Effect of C/N ratios on wastewater treatment performances

3.1.1. Effect of C/N ratios on nutrient removal performances

The effect of C/N ratios (5, 10, 15, 20, 25 and 30) was firstly investigated in the functional strain *Psychrobacter aquimaris* as a prior verification for ammonium-assimilating biosystems. Results revealed that the efficiency of NH_4^+-N and PO_4^{3-}-P removal increased with the increase of C/N ratios and then remained stable (see Supplementary Material). When the C/N ratios were higher than 20, the effluent concentrations of NH_4^+-N after 48 h were lower than 15.2 mg/L, with the removal efficiencies higher than 76.2%. The nitrogen removal was accompanied by the growth of cells, shown as the increase of biomass with increasing C/N ratios (see Supplementary Material). There was no significant difference in NH_4^+-N removal efficiencies between C/N ratio 25 and 30 ($P > 0.05$). Moreover, a higher than 95% removal efficiency of COD was achieved after 24 h in all systems, indicating the removal of COD was not significantly affected by C/N ratios. Compared with nitrogen removal performance, variations of C/N ratios showed less influence on phosphorus removal performance (see Supplementary Material). The phosphorus removal efficiency remained higher than 50%, and there was no significant difference in phosphorus removal efficiencies when C/N ratios were higher than 15. Therefore, the simultaneous removal of ammonium and phosphorus by *P. aquimaris* revealed that low C/N ratios (5 and 10) hindered nutrient removal, and the C/N ratio of 25 was sufficient for heterotrophic ammonium-assimilating bacteria to achieve efficient nutrient removal.

Results in the heterotrophic bacteria demonstrated that a sufficient carbon source was the prerequisite to achieve efficient nitrogen removal through ammonium assimilation. Considering that heterotrophic microbes consumed COD to remove nitrogen was approximately based on a ratio of 20:1 (Winkler and Straka, 2019), the C/N ratios of 20, 25 and 30 were selected to optimize the nutrient removal performances of ammonium-assimilating biosystems in saline wastewater. Moreover, the C/N ratio of 10 was selected to verify the feasibility of maintaining the functional stability of ammonium-assimilating biosystems at a relatively low C/N ratio, which is also a common C/N ratio in municipal wastewater (Winkler and Straka, 2019).

Four heterotrophic ammonium-assimilating biosystems were established under different C/N ratios referring to the previous study (Zhang et al., 2021), and the nutrient removal performance was investigated during 90 days of operation (Fig. 1). The effluent concentrations of NH_4^+-N decreased with time in all biosystems. The effluent NH_4^+-N was 19.18 mg/L, 7.21 mg/L, 6.29 mg/L and 4.72 mg/L, respectively when C/N ratios were 10, 20, 25 and 30 at the end of operation. With the increasing C/N ratios, the NH_4^+-N removal efficiencies of increased (C/N = 10, 20 and 25) and then remained stable. The average NH_4^+-N removal efficiencies were 36.4 ± 14.3%, 58.7 ± 14.18%, 70.6 ± 11.04% and 73.6 ± 13.9% when C/N ratios were 10, 20, 25 and 30, respectively (Fig. 1e). Notably, NH_4^+-N removal efficiencies at relatively high C/N ratios were significantly higher than that at the C/N ratio of 10, but there was no significant difference in the NH_4^+-N removal efficiencies between the C/N ratios of 25 and 30 (Fig. 1e). Besides, the accumulation of neither nitrite nor nitrate was observed during the whole operation in all biosystems, with average effluent concentrations of NO_2^--N and NO_3^--N lower than 0.4 mg/L (Fig. 1c). The absence of nitrite and nitrate accumulation revealed that ammonium-assimilating biosystems maintained functional stability at different C/N ratios.

The increase of MLSS contributed to the improvement of the NH_4^+-N removal efficiencies (Fig. 1b). The MLSS in all biosystems increased rapidly during the first 30 days, from 0.42 g/L at the start of the operation to 2.6 g/L, 5.8 g/L, 6.4 g/L, and 6.4 g/L when the C/N ratio was 10, 20, 25 and 30 respectively. The low C/N ratio (C/N = 10) significantly hindered the growth of sludge ($P < 0.05$). Moreover, the growth of biomass was impeded by the decreasing temperature, especially when the temperature was lower than 15 °C after 50 days operation (Fig. 1b). The slight increase of MLSS in the last 10 days of operation was probably due to the adaptation of sludge to low temperature contributed by the growth of cold-adapted microbes. Benefiting from the accumulation of

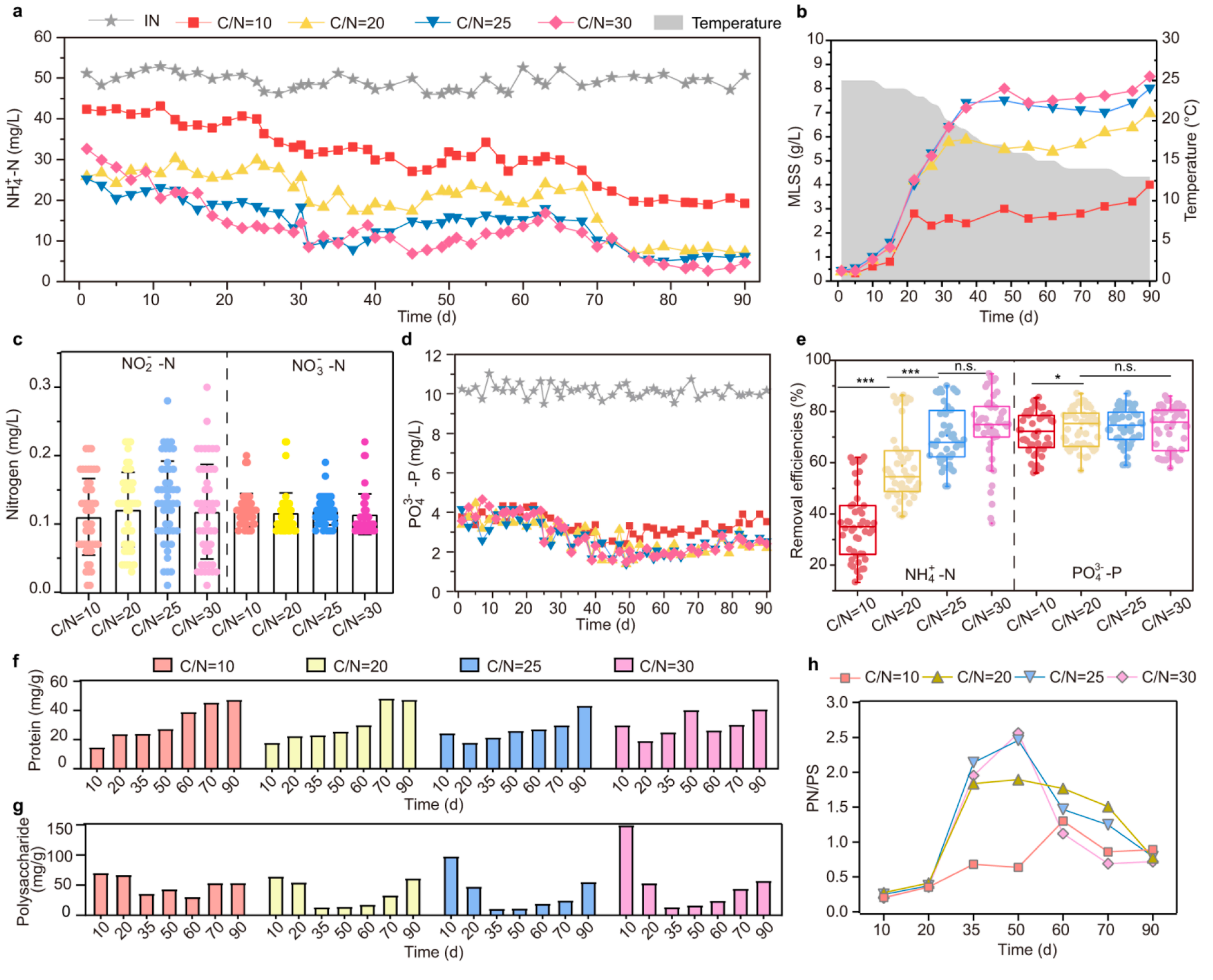


Fig. 1. Effect of C/N ratios on treatment performances of SBRs and EPS of sludge during 120 days of operation. **a,** Variations of NH_4^+-N concentrations in SBRs. **b,** MLSS of SBRs and temperatures. The grey shadow indicates the variation of temperatures. **c,** Concentrations of NO_2^--N and NO_3^--N in SBRs. Box limits, interquartile range; whiskers, minimum to maximum; center line, median; dots, individual data points. **d,** PO_4^{3-}-P removal of SBRs. **e,** Removal efficiencies of NH_4^+-N and PO_4^{3-}-P in SBRs. One-way ANOVA followed by the LSD post hoc test or non-parametric test was used for determining statistical significance between groups (***$P < 0.001$; n.s. not significant). **f,** Protein concentrations in EPS. **g,** Polysaccharide concentrations in EPS. **h,** Protein/polysaccharide ratios (PN/PS).

biomass, the average NH_4^+-N removal efficiencies were stable at levels of 59.14 ± 3.31%, 82.66 ± 5.24%, 86.51 ± 3.66% and 89.35 ± 5.1% respectively in the last 20 days of operation when the C/N ratios were 10, 20, 25 and 30. Hence, results indicated that the high level of biomass ensured efficient nitrogen removal.

The variations of phosphorus concentrations under different C/N ratios were illustrated in Fig. 1d. The removal of phosphorus was significantly hindered when the C/N ratio was as low as 10. Biosystems under different C/N ratios showed very similar performances when the C/N ratio was higher than 20 with average removal efficiencies higher than 73%, and no significant differences were observed. Considering that the consumption of carbon sources is economically costly, the optimal C/N ratio was 25 for the nutrient removal performances that simultaneous ammonium and phosphorus removal could be realized in these ammonium-assimilating biosystems.

3.1.2. Effect of C/N ratios on sludge properties

Concentrations of proteins and polysaccharides in the EPS were monitored. Results in Fig. 1e, f and g revealed that the content of protein increased slowly in all biosystems, from 21.26 ± 6.79 mg/g MLSS at 10 days to 44.26 ± 3.22 mg/g MLSS at 90 days. The polysaccharide concentrations decreased but then increased rapidly to 55.77 ± 3.31 mg/g MLSS. Consequently, the measured ratios of proteins to polysaccharides (PN/PS) decreased after 60 days and then remained lower than 1. No significant difference in proteins and polysaccharide were observed among biosystems under different C/N ratios ($P > 0.05$). The increase of polysaccharide and protein contents was probably due to the drop of temperature (shown in Fig. 1b), since the low temperature stimulated a high amount of EPS to protect cells from cold stress (Reino et al., 2016).

Interestingly, there was no significantly higher sludge yield observed in ammonium-assimilating biosystems than that in the conventional activated sludge (Jimenez et al., 2015; Khursheed and Kazmi, 2011). In this study, the average biomass growth yield of each biosystem was approximately 0.54 g biomass/g COD, 0.49 g biomass/g COD, 0.38 g biomass/g COD, 0.31 g biomass/g COD, when the C/N ratio was 10, 20, 25, and 30, respectively. The similar value of sludge production to the value in the conventional BNR pathway is probably attributed to the similar source of sludge production shared between the ammonium-assimilating biosystem and the conventional activated sludge. In both biosystems, the sludge mainly produced by heterotrophic bacteria. Furthermore, microorganisms in saline environments need more energy to withstand the osmotic stress, generally through accumulating K^+/Cl^- ions or accumulating compatible solutes into cells (Hänelt and Müller, 2013). Both strategies consume energy, and the organic carbon source might be used to withstand the osmotic stress rather than cell proliferation in heterotrophs.

Sludge properties including the particle size distribution and elemental composition were also analyzed. A decrease of particle sizes over time was observed in all biosystems, and the average volume-based sizes were similar in different biosystems (see Supplementary Material). Elemental analysis (see Supplementary Material) revealed that higher C/N ratios resulted in higher content of carbon and nitrogen in the dried sludge, but the atomic C/N ratios in dried sludge samples were similar. This result might be attributed to the similar amount of phosphorus removed and stored in sludge in all biosystems, resulting in relatively higher carbon and nitrogen content in sludge with higher C/N ratios. The above results showed that compared with wastewater treatment performances, C/N ratios had a limited effect on EPS composition, particle size and elemental composition of sludge.

3.2. Batch tests of C/N ratios

To investigate the nitrogen metabolic mechanism of heterotrophic ammonium-assimilating biosystems at different C/N ratios, the NH_4^+-N removal performances were tested in both conditions present and absent of ATU (the ammonia oxidation inhibitor). The NH_4^+-N removal efficiency increased with the increase of organic carbon concentration when C/N ratios were lower than 25, while similar removal efficiencies were observed when C/N ratios were 25 and 30 (Fig. 2a). The highest SAUR was 4.10 mg-N/(g MLSS h) at the C/N of 25. Results in Fig. 2c-f revealed that the ammonium utilization was impervious to the addition of ATU under all C/N ratios. Neither nitrite nor nitrate was detected in all biosystems, indicating the negligible process of nitrification. The removed TN in the supernatant was converted into biomass with the TN in systems stable at 138.99 ± 2.35 mg/L (s.d.), 142.74 ± 3.44 mg/L (s.d.), 146.11 ± 4.39 mg/L (s.d.) and 150.47 ± 2.81 mg/L (s.d.) when the C/N ratios were 10, 20, 25 and 30, respectively. The nitrogen balance between the supernatant and the biomass without gaseous loss revealed that the ammonium-assimilating biosystems maintain functional stability even under relatively low C/N ratio. These results indicated the dominant heterotrophic ammonium assimilation without nitrification and denitrification in these biosystems.

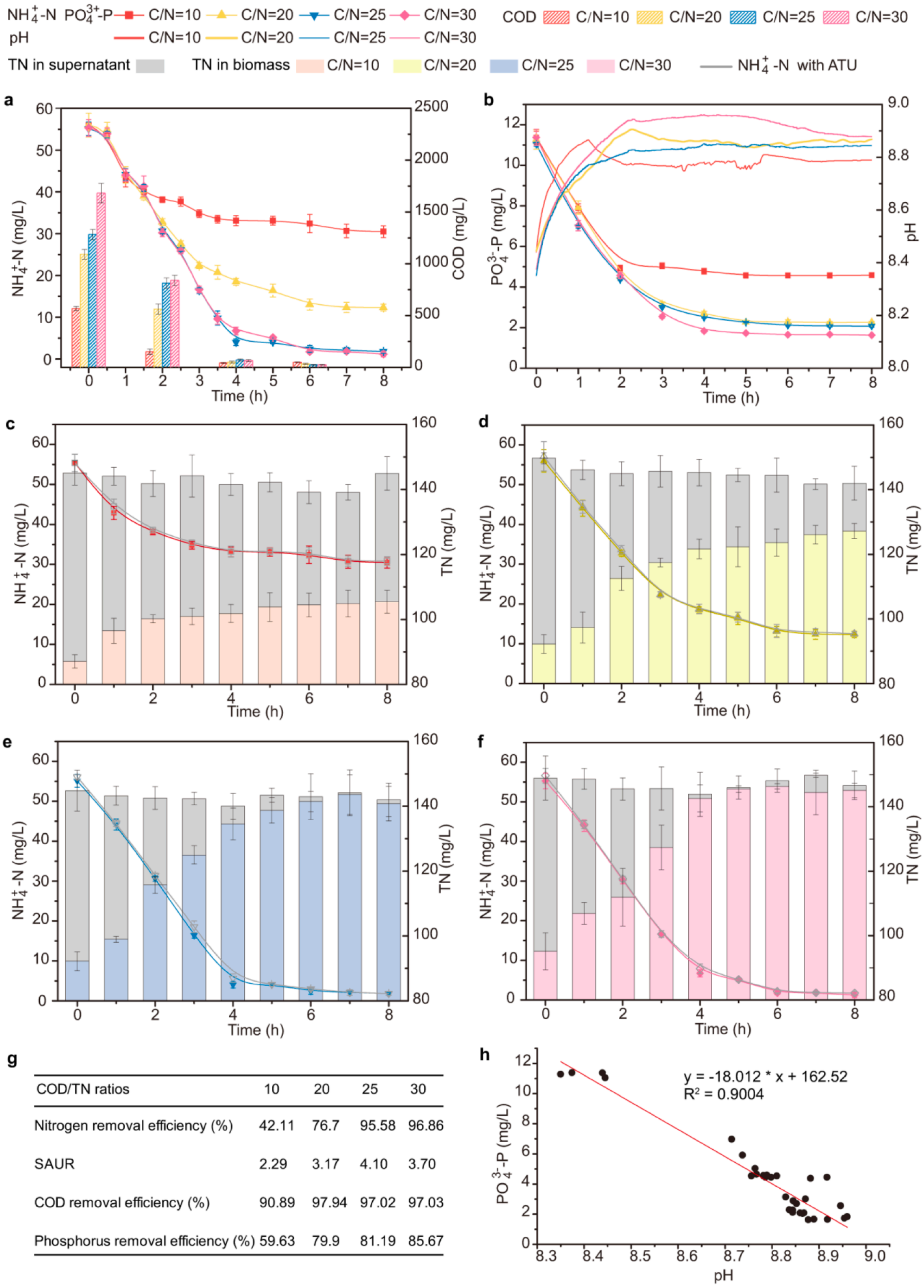
Besides, C/N ratios had less effect on COD (Fig. 2a) and phosphorus removal (Fig. 2b). Fig. 2b showed that pH increased rapidly in all systems, and the pH value reached a similar level when C/N ratios were higher than 20. The correlation analysis between pH and phosphorus concentrations indicated that the bioinduced chemical precipitation of phosphorus caused by the increase of pH value might be the main cause of phosphorus removal (Fig. 2h). This phosphorus removal mechanism seemed to be similar to the previous study (Jiang et al., 2018) but different from the mechanism of PAOs in conventional activated sludge, due to the aerobic removal preocess and absence of PAOs in the microbial community. Hence, the low C/N ratio resulted in a lower final pH value, leading to a lower phosphorus removal efficiency.

3.3. Abundances of nitrogen functional genes and correlation analysis

The abundances of nitrogen functional genes revealed nitrogen removal pathways in these biosystems (Fig. 3). Firstly, the abundances of *glnA* were orders of magnitude higher than the abundances of *amoA* and *nxrA* (Fig. 3a–c). With the extremely low and stable abundances of nitrification functional genes, all four biosystems remained stable ammonium assimilation during the operation with negligible nitrification. Secondly, the abundances of *glnA* in the biosystem under the C/N ratio of 10 were relatively low at an average of 8.18×10^4 ($\pm 2.99 \times 10^3$ s.d) copies per ng DNA. Unsurprisingly, abundances of *glnA* were not significantly different ($P > 0.05$) between the biosystems under C/N ratios of 25 and 30, consistent with similar NH_4^+-N removal performances in these two biosystems. The abundances of *nirK* were significantly higher than that of *nirS*, revealing the selective enrichment of heterotrophic bacteria with *nirK*. This observation was consistent with the explanation that *nirK* gene-harboring microbes preferred a high amount of carbon substrates (Li et al., 2018).

Fig. 3f visually revealed the correlation between various environmental factors. The strongly negative correlation between NH_4^+-N concentrations and C/N ratios suggested that high C/N ratios significantly benefited the NH_4^+-N removal in heterotrophic biosystems. MLSS was also negatively related to NH_4^+-N concentrations, indicating that sufficient biomass was indispensable for efficient heterotrophic nitrogen removal. Moreover, low temperature significantly impeded the growth of biomass, resulting in stagnation in NH_4^+-N removal efficiencies after 50 days. Protein concentrations in EPS were also negatively related to temperature, confirming the speculation that low temperature stimulated protein production.

Briefly, the low C/N ratio significantly hindered the nutrient removal performance but did not change the direction of nitrogen conversions in ammonium-assimilating biosystems. Results of nitrogen removal batch tests and nitrogen functional genes verified that nitrogen metabolism was dominated by assimilation under different C/N ratios in ammonium-assimilating microbial consortia.



COD/TN ratios	10	20	25	30
Nitrogen removal efficiency (%)	42.11	76.7	95.58	96.86
SAUR	2.29	3.17	4.10	3.70
COD removal efficiency (%)	90.89	97.94	97.02	97.03
Phosphorus removal efficiency (%)	59.63	79.9	81.19	85.67

Fig. 2. Batch tests of sludge in SBRs of different C/N ratios. **a,** Effect of C/N ratios on NH_4^+-N and COD removal. **b,** Effect of C/N ratios (10, 20, 25 and 30) on PO_4^{3-}-P removal and changes of pH. **c-f,** Ammonium conversions of ammonium-assimilating biosystems in batch tests under C/N ratios of 10 (c), 20 (d), 25 (e) and 30 (f). **g,** Nutrient removal efficiencies and activity of sludge samples. SAUR (mg-N/(g MLSS h)) represents the specific ammonium utilization rate. **h,** Linear regression analysis for phosphorous concentrations and pH of the four sludge samples under different C/N ratios.

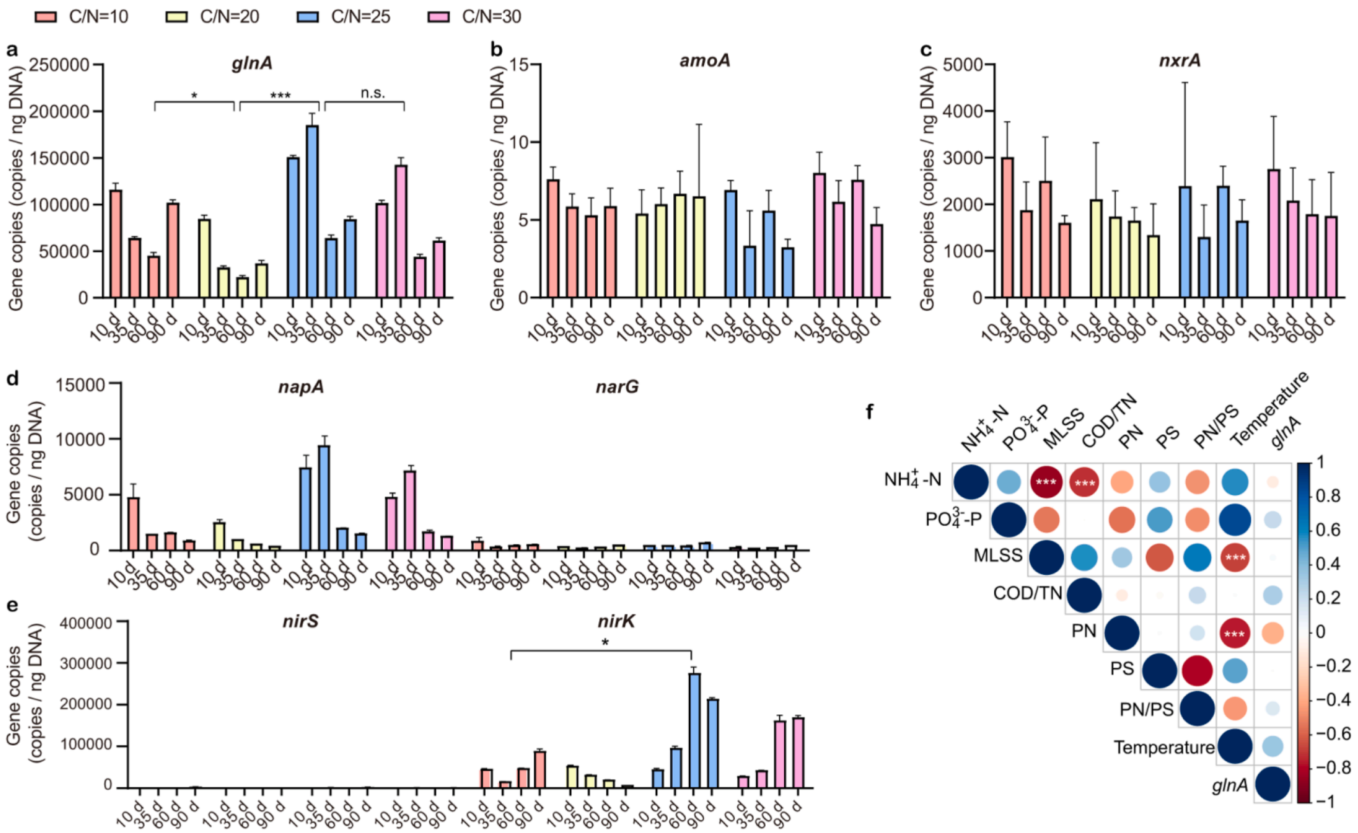


Fig. 3. Abundances of nitrogen functional genes and correlation analysis. **a,** Abundances of glutamine synthetase gene *glnA* (*$P < 0.05$, ***$P < 0.001$, n.s. not significant based on non-parametric Kruskal-Wallis test). **b,** Abundances of ammonia monooxygenase gene *amoA*. **c,** Abundances of nitrite oxidoreductase gene *nxrA*. **d,** Abundances of nitrate reductases genes *napA* and *narG*. **e,** Abundances of nitrite reductases genes *nirS* and *nirK* (*$P < 0.05$ based on non-parametric Kruskal-Wallis test). **f,** Correlation analysis based on Spearman's rank correlation coefficients (***$p < 0.001$).

3.4. The dynamics of bacterial communities and microbial interactions

3.4.1. Compositions of bacterial communities

Results of species richness and bacterial diversity showed that stable and diverse bacterial communities were established in four biosystems, confirmed by the Chao, Shannon and Simpson indexes (Fig. 4a). The bacterial diversity slightly increased with the increase of C/N ratios when C/N ratios were lower than 25. Interestingly, there were no significant differences in both community richness and diversity between samples with C/N ratios based on statistic analysis (Fig. 4a). This result indicated that different C/N ratios did not significantly change the bacterial diversity in heterotrophic systems. On the contrary, the community richness decreased significantly with time (see Supplementary Material). The decrease of species richness after 60 days was probably due to the drop in temperatures, since the low temperature was reported to decrease the richness of the microbial community (Zhou et al., 2018).

Non-metric multidimensional scaling (NMDS) analysis was performed to explore the distributional patterns of the microbial communities in different C/N ratios, following by ANOSIM testing. Consistent with the results of alpha-diversity, bacterial community composition showed no distinct variation among the treatment of different C/N ratios (Fig. 4b). ANOSIM testing was conducted to verify the overall similarity in four biosystems ($R = -0.07726$, $P = 0.762$). Therefore, different C/N ratios did not significantly affect bacterial community compositions.

The dynamics of bacterial communities in four biosystems were shown in Fig. 4c and d. The bacterial phyla were dominated by *Bacteroidetes* (24.3%–69.6%) and *Proteobacteria* (19.4–71.6%), accounting for approximately 90% of the total relative abundance. The domination of *Bacteroidetes* and *Proteobacteria* was consistent with the general fact that *Bacteroidetes* could survive easily in saline environments, and *Proteobacteria* was commonly observed in saline wastewater (Ferrera and Sanchez, 2016). The bacterial communities were further analyzed at the genus level to reveal the more detailed microbial community structure. Samples shared several similar genera, which had the average abundances higher than 1% in four biosystems under different C/N ratios, such as *Xanthomarina* (18.28%), *Saprospiraceae* (17.44%), *Vicingus* (6.99%), *Bradymonadales* (5.90%), *Marinobacterium* (4.70%), *Hyphomonas* (3.79%), *Muricauda* (3.00%), *Ponticoccus* (2.99%), *Stappia* (2.83%), *Pseudoalteromonas* (2.66%), *Halomonas* (2.27%), *Bowmanella* (2.18%), and *Wandonia* (1.60%). These genera were generally reported to be heterotrophic microbes closely related to saline environment metabolism. For example, *Xanthomarina* (Huang et al., 2019; Yao et al., 2021)*, Halomonas* (García-Ruiz et al., 2018), *Marinobacterium* (Pan et al., 2020), *Muricauda* (Jia et al., 2017) and *Pseudoalteromonas* (Jiang et al., 2019) were described as halotolerant heterotrophic bacteria observed in saline wastewater treatment systems; *Saprospiraceae* (Li et al., 2021) and *Hyphomonas* (Yuan et al., 2021) were reported to be vital to utilize organic matters under aerobic conditions. It is worth noting that the representative AOB and NOB, such as *Nitrosomonas* and *Nitrospira*, were not detected in all four biosystems during the whole operation, explaining the absence of nitrification and low abundance of nitrification genes. This observation was similar to a previous report that AOB were almost undetectable when C/N ratios were higher than 5 (Sun et al., 2020). The enrichment of AOB and NOB was completely inhibited in ammonium-assimilating biosystems even under the relatively low C/N ratio of 10. The absence of nitrifiers in biosystems could be attributed to more inhibition on autotrophic microorganisms compared with heterotrophic microorganisms, because of the low growth rate of autotrophic microbes (Ali et al., 2020). Thus, it is difficult for autotrophic nitrifiers to compete with heterotrophic assimilating bacteria to

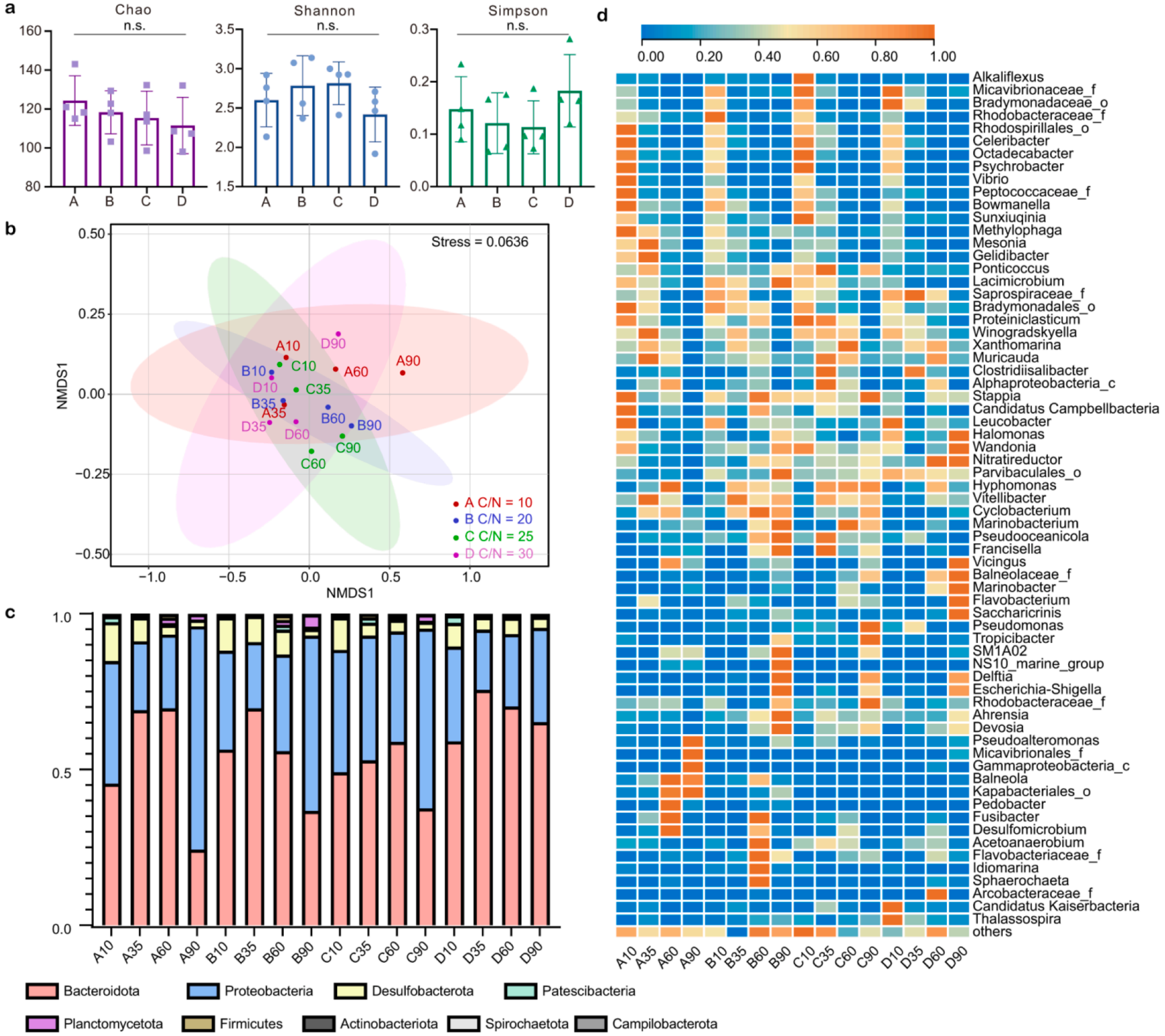


Fig. 4. Structure of bacterial communities in four biosystems. **a,** Bacterial diversity in four SBRs, including Chao, Shannon and Simpson indexes. **b,** Bacterial community compositional structure in the different SBRs as indicated by non-metric multidimensional scaling (NMDS) plots. **c,** Bacterial community structures in four SBRs at phylum level. **d,** The heatmap of major bacterial genera. Genera with an average relative abundance > 0.1% were selected. Those taxa without corresponding taxonomic names at the genus level were indicated by higher level names. "f", "o", "c", "p" or "d" represent family, order, class, phylum or domain respectively.

proliferate under saline conditions in the ammonium-assimilating microbiome in which nitrogen conversion is dominated by assimilation at the beginning (Zhang et al., 2021). Therefore, it was proved that the biosystems under different C/N ratios maintained the stable function of ammonium assimilation from the perspective of microbial community structure.

LEfSe analysis with the linear discriminant analysis (LDA) thoroughly revealed the taxonomic profiling in bacterial communities (Fig. 5a & b). The species with LDA Score > 4 were the biomarker with the statistical difference between groups. Genus *Bowmanella* (3.18%), family *Rhodospirillales* (1.13%), and order *Bradymonadales* (6.24%) were significantly abundant (abundance > 1%) and were biomarkers detected in the biosystem under the C/N ratio of 10. Family *Saprospiraceae* (18.41%) was the biomarker in the biosystem under the C/N ratio of 20. Genus *Xanthomarina* (19.70%), genus *Hyphomonas* (3.94%), family *Flavobacteriaceae* (4.85%) and genus *Muricauda* (2.50%) were biomarkers in the biosystem under the C/N ratio of 25. Genus *Vicingus* (9.03%), genus *Marinobacterium* (1.44%) and order *Micavibrionales* (1.83%) were biomarkers in the biosystem under the C/N ratio of 30. Results of LEfSE analysis revealed the different preferences of heterotrophic bacteria for low or high organic carbon loading. The enrichment of *Rhodospirillales*, *Bowmanella* and *Bradymonadales* in biosystems under the relatively low C/N ratio might be due to the rapid consumption of organic carbon. For instance, *Rhodospirillales* was reported to become predominant when the organic matters were decomposed (Shi et al., 2017). *Bradymonadales* was a recently sequenced order from the marine system and was observed to be enriched in low-strength organic saline wastewater (Cui et al., 2021). On the contrary, biomarkers in the biosystem with the high C/N ratio, such as *Marinobacterium* (Qu et al., 2019), *Micavibrionales* (Crognale et al., 2019), were reported to prefer high organic carbon loading.

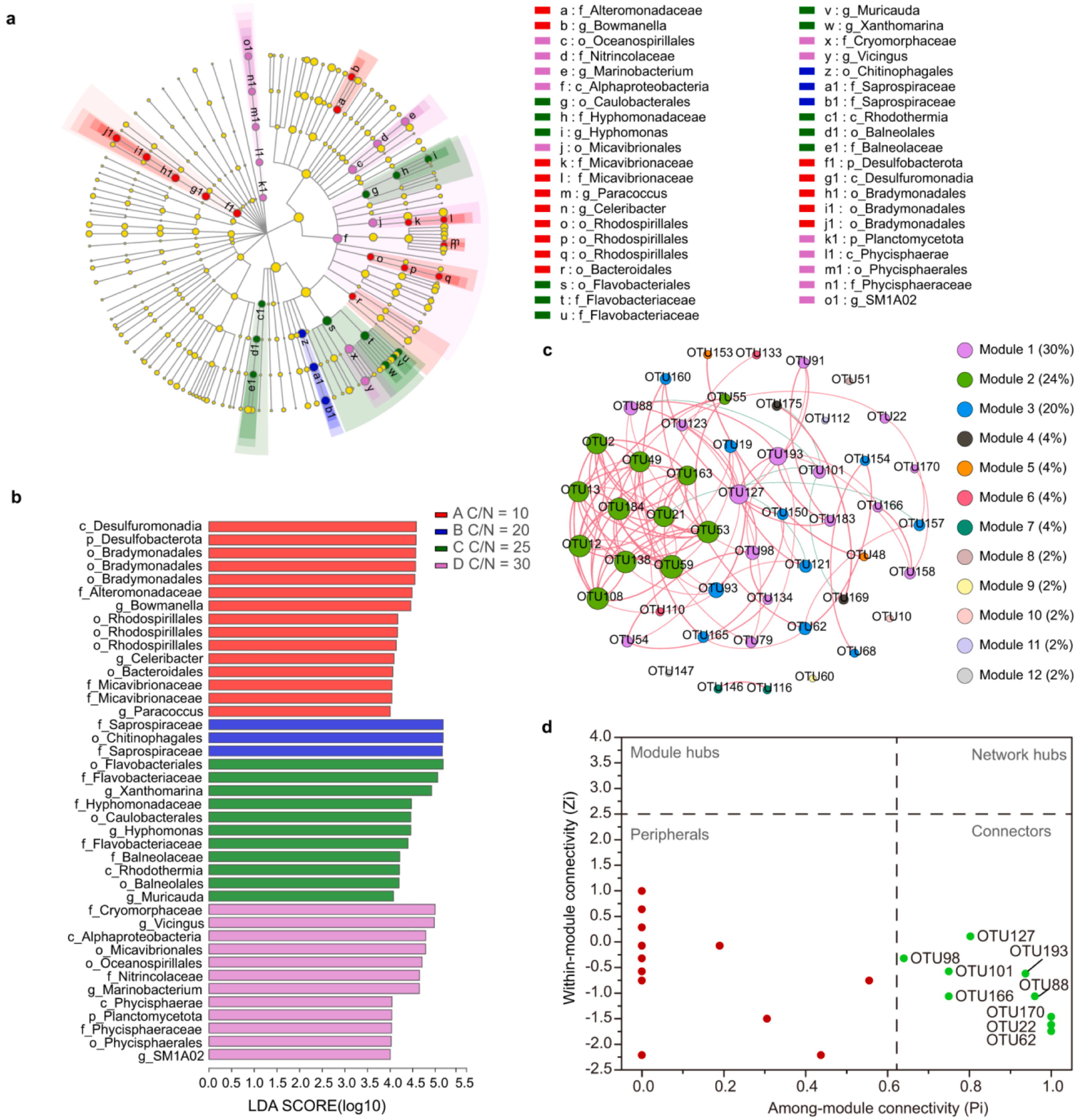


Fig. 5. Analysis of bacterial communities in ammonium-assimilating biosystems. **a,** Cladogram by the linear discriminant analysis (LDA) effect size (LEfSe) statistical results with a threshold value of 4 and phylogenetic levels from phylum to genus. **b,** Histogram of LDA scores computed for features differentially abundant taxa. **c,** Co-occurrence network based on Spearman's rank correlation coefficients between relative abundances of OTUs. OTUs with an average relative abundance > 0.1% were selected for analysis. Connections are shown for strong (Spearman's $\rho > 0.6$) and significant ($p < 0.01$) correlations. Nodes are colored by modularity class. The size of each node is proportional to the number of connections (degree), and the thickness of each connection (edge) is proportional to the value of the Spearman's correlation coefficients. Red and green lines represent significant positive and negative associations, respectively. **d,** Topological roles of nodes (bacterial OTUs) in the network. Threshold values of within-module connectivity (Zi) and among-module connectivity (Pi) for categorizing bacterial OTUs are 2.5 and 0.62, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4.2. Bacterial co-occurrence networks

As there was no significant difference in bacterial community compositions at different C/N ratios, the bacterial co-occurrence network was constructed based on OTUs of all four biosystems samples to comprehensively reflect the interactions between microbes. The network consisted of 50 nodes and 109 edges (Fig. 5c), and the modularity index of 0.511 indicated that the network has a modular structure. The positive associations in the network accounted for 94.5%, suggesting a strong bacterial competition in ammonium-assimilating biosystems.

To identify the distinct topological functions in the network, network nodes can be sorted into four categories (peripherals, module hubs, network hubs and connectors) based on threshold values of Zi = 2.5 and Pi = 0.62. This analysis (Fig. 5d) revealed that the majority of the OTUs were detected at peripherals (Zi < 2.5 and Pi < 0.62), accounting for 58.82%. Of 14 interfered connectors, most of the keystone phylotypes belonged to *Bacteroidetes* (50%) and *Proteobacteria* (42.86%). In more detail, microbes, such as SM1A02, *Devosia*, *Marinobacterium*, and *Tropicibacter*, were identified as connectors (see Supplementary Material). No module hubs or network hubs were found in the network.

To conclude, the ammonium-assimilating bacteria *Psychrobacter* is closely related to several genera, such as *Rhodospirillales* and *Celeribacter*. Several genera, such as SM1A02, *Devosia*, *Marinobacterium*, and *Tropicibacter*, might play a connecting role in the metabolism of the ammonium-assimilating microbiome. As a result, sludge showed similar characteristics, including the EPS compositions, particle sizes and atomic C/N ratios of dried sludge. In addition, the synthetic microbiome built in the laboratory may not be sensitive to environmental disturbance, since it might have a simpler microbial structure than the existing microbiome in the complex environment. Other key environmental parameters, such as temperature (shown in this study) and salinity, might bring more impact on microbial community than the C/N ratios and are deserved to be further studies optimized.

3.5. *The importance of ammonium assimilation in BNR*

The specific function of nitrogen-transforming microbes is usually summarized as the characteristics of nitrogen metabolism pathways in the microbial community, which may lead to unpredictable results in the regulation of biosystems. Although functional microbes are generally classified according to the processes they involving in, such as nitrifiers and denitrifiers (Kuypers et al., 2018), their characteristics can hardly replace or summarize the overall nitrogen metabolic function in complex biosystems because of their metabolic versatility. For instance, recent studies have revealed that approximately half of the nitrogen is removed through the assimilative uptake in pure cultured heterotrophic nitrification-aerobic denitrification (HN-AD) bacteria (Delgadillo-Mirquez et al., 2016; Wang and He, 2020; Yang et al., 2019), and higher proportion of ammonium assimilation was observed under higher C/N ratios in HN-AD bacteria (Zhang et al., 2020). In HN-AD microbial consortia, the highest nitrogen removal efficiency was observed under the C/N of 25 (Pan et al., 2020), similar to results in this study. Unfortunately, BNR is usually attributed to heterotrophic nitrification (Pan et al., 2020), while the pathway of ammonium assimilation is empirically ignored. Therefore, the main metabolism in the microbial community should be given priority in the optimization of biosystems to avoid inefficient regulation.

The ammonium-assimilating biosystem was proved in this study to be a promising approach to the saline wastewater treatment at different C/N ratios. Results of the treatment performance in biosystems indicated that the direction of nitrogen conversion in ammonium-assimilating biosystems remained stable and that the C/N ratio could be used as a clear management strategy to promote the efficiency of nutrient removal. In addition, this approach occupied the advantages of strong resistance to environmental fluctuations without undesirable intermediates. From the perspective of future engineering applications, it has great potential for treating salinity wastewater with high organic carbon sources.

4. Conclusions

Four ammonium-assimilating biosystems constructed under C/N ratios of 10, 20, 25 and 30 showed stable nitrogen removal via ammonium assimilation in simulated saline wastewater. The C/N ratios have a greater effect on the nitrogen removal process than the COD and phosphorus removal process. The optimal nutrient removal performance was achieved when the C/N ratios were higher than 25, with sufficient biomass. Although the different C/N ratios did not significantly change the microbial ecological niche in heterotrophic ammonium-assimilating communities, the differential biomarkers in these systems were identified. Ammonium-assimilating biosystems maintained functional stability even at the relatively low C/N ratio.

CRediT authorship contribution statement

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

Declaration of Competing Interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2022.126911.

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