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RESEARCH ARTICLE

Enhancing the efficiency of nitrogen removing bacterial population to a wide range of C:N ratio (1.5:1 to 14:1) for simultaneous C & N removal

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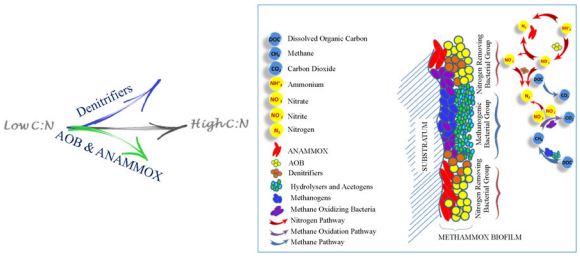
HIGHLIGHTS

- Simultaneous C & N removal in Methammox occurs at wide C:N ratio.
- Biological Nitrogen Removal at wide C:N ratio of 1.5:1 to 14:1 is not reported.
- Ammonia removal shifted from mixotrophy to heterotrophy at high C:N ratio.
- Acetogenic population compensated for ammonia oxidizers at high C:N ratio.
- Methanogens increase the plasticity of nitrogen removers at high C:N ratio.

GRAPHIC ABSTRACT



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ABSTRACT

High C:N ratio in the wastewater limits biological nitrogen removal (BNR), especially in anammox based technologies. The present study attempts to improve the COD tolerance of the BNR process by associating methanogens with nitrogen removing bacterial (NRB) populations. The new microbial system coined as 'Methammox', was investigated for simultaneous removal of COD (C) and ammonia (N) at C:N ratio 1.5:1 to 14:1. The ammonia removal rate (11.5 mg N/g VSS/d) and the COD removal rates (70.6 mg O/g VSS/d) of Methammox was close to that of the NRB (11.1 mg N/g VSS/d) and the methanogenic populations (77.9 mg O/g VSS/d), respectively. The activities established that these two populations existed simultaneously and independently in 'Methammox'. Further studies in biofilm reactor fetched a balanced COD and ammonia removal (55%–60%) at a low C:N ratio ($\leqslant$2:1) and high C:N ratio ($\geqslant$9:1). The population abundance of methanogens was reasonably constant, but the nitrogen removal shifted from mixotrophy to heterotrophy as the C:N ratio shifted from low (C:N$\leqslant$2:1) to high (C:N$\geqslant$9:1). The reduced autotrophic NRB (ammonia- and nitrite-oxidizing bacteria and Anammox) population at a high C:N ratio was compensated by the fermentative group that could carry out denitrification heterotrophically. The functional plasticity of the Methammox system to adjust to a broad C:N ratio opens new frontiers in biological nitrogen removal of high COD containing wastewaters.

1 Introduction

Anammox-based biological nitrogen removal (BNR) processes are preferred over conventional nitrification-denitrification systems for reduced organic carbon cost (electron donor), aeration costs, sludge handling costs and reduced emissions of greenhouse gaseous (GHG) (Joss et al., 2009; Bagchi et al., 2012; Shu et al., 2016). Being autotrophic, anammox based BNR processes need C:N ratio to be 0.5 or less up to ~ 3.0 (Joss et al., 2009; Ni et al., 2012; Chen et al., 2016; Cao et al., 2020). However, since COD is an integral constituent of wastewater, simultaneous nitrogen and COD removal would make the BNR process cost-efficient and energy-efficient.

Sustainable COD removal by autotrophic anammox population is feasible if they team up with heterotrophic microbial populations such as denitrifiers and methanogens. Several studies have been carried out to remove C & N simultaneously, in single-step process, based on anammox-denitrification, short-cut/simultaneous nitrification-denitrification and methanogenesis-denitrification

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processes (Kumar and Lin, 2010; de Almeida et al., 2018; Wang et al., 2019; Peng et al., 2020).

COD can support higher nitrogen removal in an anammox system by supplying electron donors to denitrifiers (Kumar and Lin, 2010). However, a synergistic relationship between the autotrophic and the heterotrophic nitrogen removing bacterial (NRB) groups should exist through the utilization of nitrite/nitrate (Wang et al., 2019). In the processes where denitrifiers team up with autotrophic microorganisms like nitrifiers as in SND (simultaneous nitrification-denitrification), or with anammox as in SAD (simultaneous Anammox and denitrification) and SNAD (simultaneous nitrification, Anammox and denitrification), there are possibilities of the heterotrophic microbial community (mostly the denitrifiers) outcompeting the chemolithotrophic nitrogen removing bacterial (NRB) population in the long run or in the presence of higher C:N ratio (Chamchoi et al., 2008; Tang et al., 2010; Wang et al., 2019). Higher C:N ratio can disbalance the synergy between the denitrifiers and the autotrophs (Wang et al., 2019). Similarly, in anammox based processes like SAD and SNAD, care has to be taken to maintain strict concentrations of either or both dissolved oxygen and organics to prevent cross inhibition between autotrophic and heterotrophic NRB populations (Wang et al., 2019). A threshold C:N ratio of < 1.57 has been essential for maintaining a stable SAD process and increasing C:N ratios from 1.5 to 3.0 could increase denitrification activity in anammox systems (Bi et al., 2015; Shu et al., 2016). In some cases, apart from a low C:N ratio, maintaining a denitrifying bacterium to anammox population ratio between 0.3 and 0.4 has been reported to maintain an efficient nitrogen removal process in the SAD process (Bi et al., 2015). At a high C:N ratio (>3.0), anammox activity is out-competed by denitrifiers for nitrites or may suffer substrate toxicity (Wang et al., 2019; Cao et al., 2020). The studies confirm that the C:N ratio is the bottleneck of anammox-denitrification based simultaneous C & N removal processes that become limited or ineffective in wastewaters with a high C:N ratio.

In methanogenesis-denitrification based processes like ADM (anaerobic denitrification and methanogenesis) and AMODM (aerobic methane oxidation, denitrification coupled to methanogenesis), denitrifiers belonging to group *Firmicutes* and *Bacteroidetes* supply fermentation end products to methanogens and also play a vital role in nitrogen removal (Sundberg et al., 2013; Pitombo et al., 2016; Rachbauer et al., 2017). However, these processes use nitrates instead of ammonia as the nitrogen source. In addition, a few studies have focused on ammonia removal in the methanogenic systems (Saha et al., 2018; Pekyavas and Yangin-Gomec, 2019; Velasco-Garduño et al., 2019) and some have documented enrichment of anammox systems from methanogenic granules (Tang et al., 2010; Pereira et al., 2017; Yangin-Gomec et al., 2017). Pekyavas and Yangin-Gomec (2019) reported a study with anammox and methanogenic population at a C:N ratio of 8.6 in diluted chicken digestate. Similarly, Velasco-Garduño et al. (2019) reported effective biotransformation of ammonia and organic carbon by nitrifying and methanogenic populations, respectively, in a multi-modular hybrid carrousel reactor operated at a C:N ratio of 13–14. The C:N ratio of such methanogenesis based processes are typically on the higher side (>8.0) (Pekyavas and Yangin-Gomec, 2019, Velasco-Garduño et al., 2019). The role of the methanogenic community for organic carbon removal at lower C:N ratios have not yet been investigated and there is a need to evaluate how do methanogens fare in wastewaters with low C:N ratios (up to 1.5).

To the best of our knowledge, simultaneous C & N removal across a wide range of C:N ratios (1.5 to 14) has not been reported. Since methanogens have been shown to exist with both denitrifiers and anammox, the present study aims to explore the feasibility of methanogens and NRB populations to display their independent and functional identities in a single system to simultaneously remove organic carbon and nitrogen across a wide range of C:N ratio (1.5 to 14). The study also determines the microbial interactions between the methanogenic and NRB populations herein referred to as 'Methammox', in a biofilm system through population dynamics and microscopy.

2 Materials and methods

2.1 Biomass

The methanogenic biomass (MB) and NRB biomass were collected from sewage fed, 295-day-old, pilot-scale UASB (Saha et al., 2015) and from a synthetic wastewater-fed, laboratory-scale anaerobic CSTR treating 200 mg/L of NH_4^+-N (Bagchi et al., 2010), respectively. The centrifuged MB and NRB biomass were mixed in a 1:1 ratio on a wet weight basis (77% moisture, w/w) to obtain Methammox biomass.

2.2 Composition of wastewater

The synthetic wastewater was prepared in tap water (van de Graaf et al., 1996).

HPLC grade Methanol (99.9%) and analytical grade ammonium chloride (99.5%) were added to desired concentration as carbon and nitrogen source, respectively (Saha et al., 2018). Analytical grade sodium bicarbonate (99.5%) (0.88 g/L) was used for buffering (Saha et al., 2015). All chemicals were procured from Fischer Scientific, India.

2.3 Batch experiments

The batch experiments were conducted in triplicates, under oxygen-limited conditions, in 125 mL serum bottles. The

total reaction volume in each bottle was 80 mL. For determining the ammonia and COD activity tests of the methanogenic biomass (MB), nitrogen removing biomass (NRB), and Methammox biomass, three different substrates, namely 1) carbon source (C set), 2) nitrogen source (N set) and 3) nitrogen plus carbon source (N + C set) were used. For determining the effect of the C:N ratio for Methammox, methanol and ammonium chloride concentrations were varied to obtain C:N ratio from 3:1 to 20:1. In both the experimental sets, a known weight of biomass, pre-washed with 0.1 M phosphate buffer solution, was incubated in 125 mL serum bottles containing 80 mL synthetic wastewater. The serum bottles were tightly sealed with caps to ensure no entry or escape of gases from the bottles. The average initial dissolved oxygen concentration of synthetic wastewater in each serum bottle was 3.2±0.6 mg/L. The experimental setups were not flushed with inert gas to allow the aerobic population in the biomass to use the available DO and naturally develop anaerobic conditions in the set-ups. The serum bottles were inoculated and incubated under shaking conditions (120 r/min) at 30°C for three days. Headspace pressure was recorded at regular intervals and methane concentration in the headspace was measured at the end of the experiment. The samples withdrawn at regular intervals with syringe, were filtered using syringe filters and were used to measure pH, COD, ammonia, nitrite and nitrate.

2.4 Reactor set-up and operation

The 2 L biofilm reactor (BR), made up of PVC pipe (Fig. S1) was packed with gravels (10–20 mm diameter) supported on a circular Perspex sieve. The effective volume of the reactor was 0.95 L. The BR was fed in an up-flow manner using a Watson Marlow (UK) peristaltic pump. The inlet port was located at the base while the effluent port was provided at the top, 25 cm from the base. A 'U' shaped liquid seal connected to the outlet prevented escape of gases from the effluent. An inverted conical gas collector (14.5 mm ID) was suspended from the lid of the reactor to collect gas in the headspace.

The reactor seeded with 0.5 L Methammox biomass (2 g VSS/L) was fed with synthetic wastewater containing methanol as the carbon source and ammonium chloride as the nitrogen source in an up-flow manner for 187 days. The reactor was operated at 1 d hydraulic retention time (HRT) in 6 phases (Table 1). In these phases, the C:N ratio of the feed was varied between 1.5:1 and 14:1.

2.5 Analytical method

Temperature and pH were measured using a multi-parameter meter (PCD 650 Eutech Instruments, Singapore). TSS, VSS, COD and NH_4^+-N were determined as per the Standard Methods (APHA, 2017), while nitrite and nitrate were estimated by Ion chromatography (830 compact IC Metrohm, Switzerland) (Saha et al., 2018). The dissolved methane was extracted from the effluent (Zhang et al., 2013). The headspace methane was quantified using gas chromatography (Agilent Technologies, USA) (Fotidis et al., 2013). Biofilm samples for scanning electron microscopy were fixed overnight with 2.5% glutaraldehyde in a 0.1-M phosphate buffer. The washed samples were dehydrated sequentially in 30%, 50%, 70%, 90%, and 100% ethanol solutions, respectively (Sinha and Annachhatre, 2007). The pre-fixed and dehydrated samples were loaded onto a stub fixed with a double adhesive tape and viewed under the scanning electron microscope (model No. JEOL JXA-840, Japan). The sludge samples from BR were collected at regular intervals for performing 16S rRNA amplicon sequencing and for quantifying the autotrophic NRB population using real-time PCR. Gravels from the packing medium was randomly selected and the biofilm was scraped using a sterile spatula into a sterile Eppendorf tube for further analysis. The DNA extracts were estimated for quantification of AOB, NOB and Anammox populations using respective primers as per protocols described in Table S1.

2.6 16S rRNA amplicon sequencing

The total genomic DNA of the biomass sample was isolated for amplifying and analyzing the sequences of the V3–V4 region of the 16S rRNA gene. Qiagen Dneasy kit (Qiagen, Germany) was used for DNA extraction, following the manufacturer's protocol. R1 and R2 raw data were obtained by sequencing them on the Miseq

Table 1 Operation of Biofilm Reactor at various C:N ratios

Sr No.	Days	Phases	Mean inlet ammonia (mg NH_4^+-N/L)	Mean inlet COD (mg/L)	C:N ratio
1	0–19	I	100.2±17.7	154.45±23.8	1.5
2	20–40	II	97.0±26.8	227.7±27.7	2.3
3	41–79	III	82.6±13.2	488.8±36.2	6
4	80–100	IV	96.4±21.4	783.6±54.7	8
5	101–145	V	90.5±11.3	804.5±56.8	9
6	146–187	VI	98.8±16.7	1380.2±76.2	14

platform. The primers used to amplify the V3–V4 region were (Yadav et al., 2014);

Forward primer sequence-50CCTACGGGAGGCAG-CAG 30 and

Reverse primer sequence-50ATTACCGCGGCTGCT-GG 30.

FastQC was performed on the raw data and were further processed (Yadav et al., 2014). The data in FastQ format were analyzed using MG-RAST with default parameters using their own QC pipeline. Based on the LCA algorithm, a comparative taxonomic tree with 90% identity and a minimum length of 50 bp for taxonomic classification was obtained.

2.7 Statistical analysis

Statistical calculations like two-way analysis of variance test (ANOVA) and principal component analysis (PCA) was carried using Origin Laboratory 2020. ANOVA and Kruskal–Wallis H-test was also used for comparing the groups of 16S amplicon sequencing profiles in the STAMP tool. The statistically significant features in the amplicon data were further examined with post hoc tests (e.g. Tukey–Kramer and PCA) to determine how groups of the profiles differed from each other.

3 Results and discussion

The study was carried out into three sections. The first section seeks to understand if the methanogens and NRB populations co-exist together in Methammox for independent and simultaneous removal of C & N. The second section evaluates the effect of widely-varying C:N ratio (1.5 to 14) on simultaneous C & N removal by Methammox. And, in the last section, the role of microbial communities in the Methammox system is suggested.

3.1 Can methanogens and NRB be independently functional in Methammox?

To evaluate functional independence of methanogenic and NRB population in Methammox, the removal rates of ammonia (ARR) and COD (CRR) in Methammox were determined using methanol (C), ammonia (N), and ammonia + methanol (N + C) as substrates and compared with those of methanogenic and NRB groups, respectively.

Figure 1 depicts the changes in concentrations of COD, NH_4^+-N, NO_2^--N, and NO_3^--N and headspace methane with time in the different batch set-ups of control (MB and NRB inoculated) and experimental (Methammox inoculated) 'C', 'N' and 'N + C' sets. Figure 2 relates the COD and ammonia removal rates of Methammox to its methanogenic and NRB populations, respectively. The activities with different alphabets are statistically different ($p \leqslant 0.005$) as determined by the two-way analysis of variance (ANOVA) test.

3.1.1 The MB inoculated C, N and N + C sets (Control)

Ammonia removal was observed in both the substrates 'N' and 'N + C' asserting the presence of nitrifiers in methanogenic biomass. The presence of nitrifiers in the anaerobic methanogenic population is well-reported (Díaz et al., 2006; Saha et al., 2018). The versatility of autotrophic nitrifiers to grow and survive in absence of oxygen has also been widely reported (Lam and Kuypers, 2011; Bagchi et al., 2012).

The ammonia removal was quicker in the 'N' sets (Fig. 1(a)). While in 'N + C' sets, ammonia removal was accelerated after more than half of the initial COD was removed (Figs. 1(a) and 1(b), respectively). Zhang et al. (2019) reported delayed and reduced nitrification rate in the presence of organic carbon due to the competition between the fast-growing heterotrophic bacteria and slow-growing autotrophic bacteria. The higher ammonia removal rates (ARR) of 'N + C' sets than those of the 'N' sets (Fig. 2), could be associated with the denitrifiers' activity in the methanogenic biomass (Saha et al., 2018, Cao et al., 2020). Organic carbon supplies electron donors for denitrifiers and increases the overall rate of nitrification (Kumar and Lin, 2010).

COD reduction in the 'C' set progressed more rapidly than in the 'N + C' (Fig. 1(b)), and the COD removal rate (CRR) of the 'C' set was significantly higher than that of the latter (Fig. 2). Both 'C' and 'N + C' sets generated methane in their headspace though methane production reduced in the presence of ammonia in the 'N + C' set (Fig. 1(b)). The results established that the presence of ammonia in MB not only affected methanogenesis but also delayed nitrification. The nitrification is delayed in presence of organics when heterotrophy is the major mode of microbial energy metabolism leading to preferential uptake of organics before ammonia (Steuernagel et al., 2018; Zhang et al., 2019).

3.1.2 The NRB inoculated C, N and N + C sets (Control)

Ammonia removal rate (ARR) in NRB inoculated 'N' set was highest among all experimental sets (Fig. 2) and in contrast to the MB inoculated sets, ammonia removal in the NRB inoculated 'N + C' sets started along with COD removal without any observable lag phase (Figs. 1(c) and 1(d)). The autotrophic nitrifiers are often accompanied by aerobic or anoxic chemoheterotrophs that could carry out organic decomposition along with the former when sufficient organics are present (Gu et al., 2019). Similar microbial conditions must have prevailed in the NRB inoculated 'N + C' set which might have resulted in immediate and simultaneous removal of both C and N

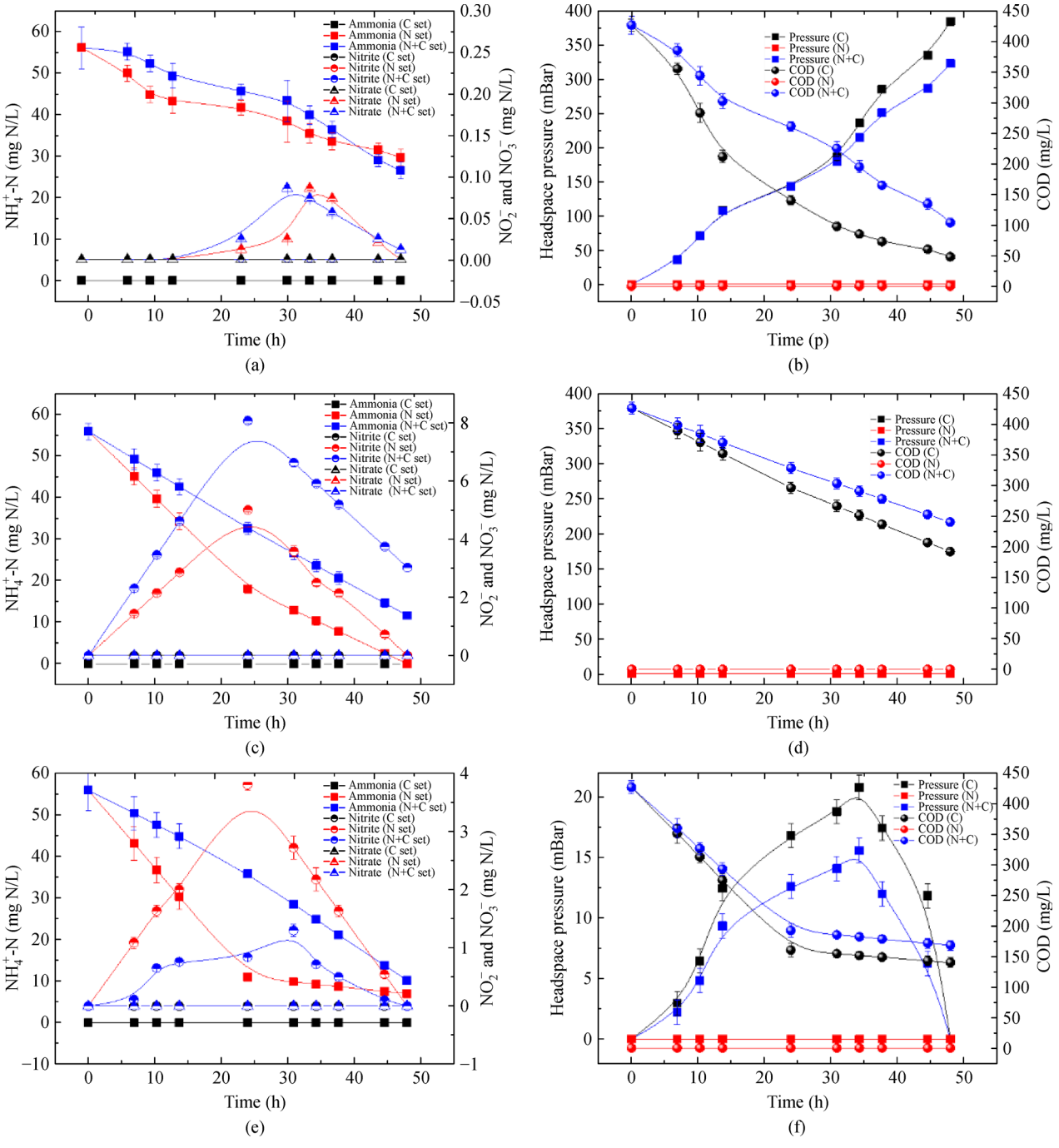


Fig. 1 Line graphs showing the average concentrations of COD (mg/L), ammonia (mg N/L), nitrite (mg N/L), and nitrate (mg N/L) and headspace pressure (mBar) in N, C and N + C substrates inoculated with (a and b) methanogenic biomass (MB), (c and d) Nitrogen removing bacteria (NRB) and (e and f) methammox.

without any lag phase.

Nitrate was not detected in the ammonia containing sets. Although nitrite was detected in low concentrations, it was rapidly consumed after 24 h. Low nitrite concentration and its rapid consumption after 24 h in both the 'N' and 'N + C' sets could be due to the anammox activity or the combined action of anammox and denitrifiers (Ni et al., 2012; Gu et al., 2019). The limiting oxygen in the set-ups could lead to incomplete nitrification explaining the observed absence of nitrates. Certain heterotrophic microbial populations are also capable of consuming nitrate during dissimilatory nitrate reduction to ammonium (DNRA) when organics are available (van den Berg et al., 2017).

Though the CRR of the NRB inoculated set was the least among all the experimental sets, the CRR of the 'C' set was

higher than the 'N + C' set (Fig. 2). Navada et al. (2020) have reported similar observations with higher organic carbon removal by autotrophic nitrifying populations when exposed to acute concentrations of organic carbon than those with sustained organic carbon. Thus, the presence of both the substrates interfered with the chemoheterotrophic activity associated with NRB.

3.1.3 The Methammox inoculated 'C', 'N' and 'N + C' sets (Experimental)

The 'N + C' set of the Methammox inoculated biomass documented simultaneous removal of COD and ammonia (Figs. 1(e) and 1(f)). In contrast to that of NRB, ammonia removal in the 'N + C' set was almost linear and steady but slower than that of the 'N' set. Similar observations were reported by Zhang et al. (2019) where nitrification was delayed and its rate was reduced in the presence of organic carbon. Linear reduction in the ammonia concentration could suggest that ammonia removal was taking place via both the anabolic and catabolic pathways (Velasco-Garduño et al., 2019). Nitrate was not observed but nitrite accumulated briefly in both the ammonia containing sets only to be consumed later (Fig. 1(e)). Velasco-Garduño et al. (2019) reported similar observations in their study.

Thus, from Fig. 2, it can be concluded that ARR of Methammox ('N' set: 13.4 mg N/g VSS/d and 'N + C' set: 11.5 mg N/g VSS/d) was at par with those of the NRB sets ('N' set: 13.9 mg N/g VSS/d, and 'N + C' set: 11.1 mg N/g VSS/d), and the CRR of Methammox ('C' set: 90.1 mg COD/g VSS/d and 'N + C' set: 70.6 mg COD/g VSS/d) was equivalent to those of MB sets ('C' set: 92.6 mg COD/g VSS/d and 'N + C' set: 77.9 mg COD/g VSS/d), respectively. Methane concentration in the headspace was marginally higher in the 'C' sets than those of the N + C sets for both the MB and Methammox biomass, indicating methanogenic activity was not affected by the NRB population in the batch experiments. However, unlike in methanogenic biomass, methane generated in Methammox started reducing after 30 h, indicating the possibility of methane oxidation.

The similarities between ARR and CRR of the Methammox inoculated sets, with those of NRB and MB, demonstrated that ammonia and COD removals were carried out predominately by the NRB and the methanogenic populations, respectively. Simultaneous removals of the ammonia and COD in the Methammox system prove that two different (C utilizing and N utilizing) populations were co-existing simultaneously and functioning independently.

3.2 How do Methammox perform at varied C:N ratio?

The effect of the C:N ratio on the ammonia (N) and COD (C) removal efficacies of the Methammox biomass was studied in both batch and continuously operated biofilm reactor (BR). In the batch experiments, methanogenesis was confirmed by the production of methane in the headspace, while in BR, COD removal rate (CRR) was selected as a measure for carbon removal instead of methane in the biogas since carbon removal potential is not restricted to methanogens alone. In Fig. 3, the methane concentration in the headspace was found to increase with

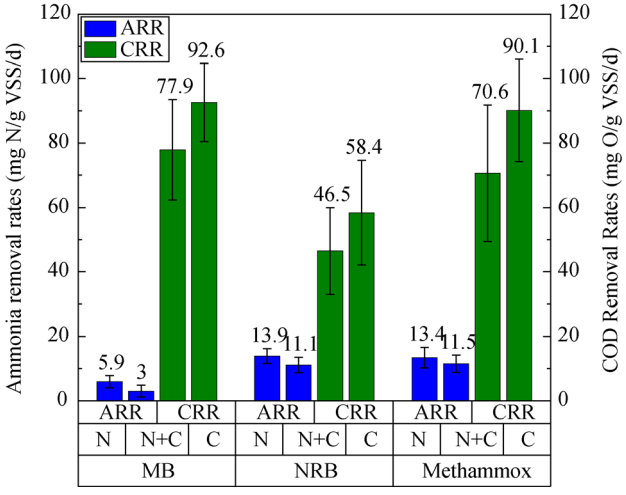


Fig. 2 Bar graphs showing activities of methanogenic biomass (MB), nitrogen removing bacterial (NRB) groups and Methammox in terms of ammonia and COD removal rates (ARR and CRR). Each of the biomasses was inoculated with ammonia (N), ammonia and methanol (N + C) and methanol (C) as substrates. The blue bar represents ARR and the green bars represents CRR. The vertical lines in the graph represent Y-axis error. The rates that do not have statistically ($p \leqslant 0.005$) different values between two different experimental set-ups when determined by a two-way analysis of variance (ANOVA) test are denoted the same alphabets, while those with statistically significant differences are denoted different alphabets above the bar.

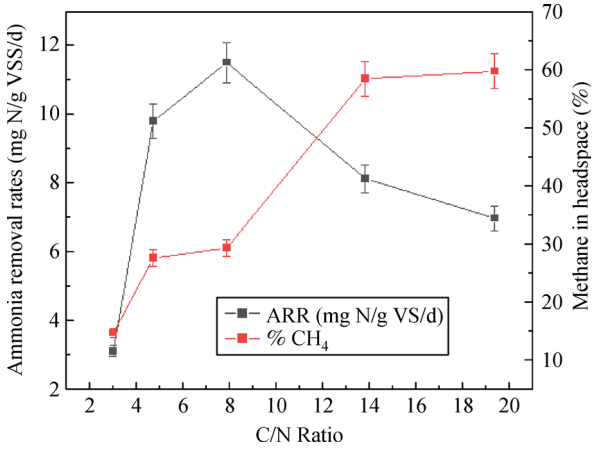


Fig. 3 Effect of various C:N ratios on batch experimental data of Methammox. The black line plot represents ammonia removal rate (ARR) and the red line plot represents per cent methane concentration in the headspace. The vertical lines in the graph represent the Y-axis error.

the rise in C:N ratio, suggesting a high concentration of COD favored methanogenesis. However, the ammonia removal rate showed a peak at C:N ratio of 8:1. Best ammonia removals in anammox based systems are reported at C:N ratio < 3.0 (Bi et al., 2015; Shu et al., 2016). Several researchers reported a significant decrease in the nitrogen removal at C:N ratio >3.0 (Pereira et al., 2017; Wang et al., 2019; Cao et al., 2020). The ability of Methammox to carry out ammonia removal at C:N ratio 8:1 which is higher than the anammox based systems indicated the role of Methammox and its microbial communities in improving tolerance of NRB to a high C:N ratio. Tolerance of anammox based nitrogen removal to a higher C:N ratio in presence of denitrifiers is well reported. In a two-stage nitrification-denitrification system, Pelaz et al. (2018) reported maximum C and N removal in domestic wastewater supplemented with methanol at a C:N ratio of 8.25 with nitrate recycling. Roy et al. (2010) observed the highest nitrogen removal (99%) from shrimp production wastewater at C:N up to 10:1 which decreased at higher C:N ratios.

Subsequent studies on varying C:N ratios were carried out in a continuously operated BR to achieve nitrification, enrich other slow-growing microbial populations and accomplish mutualism between different microbial communities (Navada et al., 2020).

From the performance of the BR at various C:N ratios (Fig. 4(a)), it appears that ARE and CRE are mutually antagonistic with each other at C:N ratios between 3:1 and 9:1. Though the highest ARE and CRE were achieved at C:N ratio of 6:1 and 9:1, respectively, the BR study yielded two C:N optima; one < 2:1 and another >9:1 where both the NRB and methanogenic activities proceed together at equitable capacities (Fig. 4(a)).

Figure 4(b) is a biplot of multivariate Principal Component Analysis (PCA) between various factors. The first principal component extracted in Fig. 4(b) is C:N ratio and the second component includes both the inlet ammonia concentration (mg N/L) and ammonia removal efficiency (ARE %). The gray, orange, blue and purple-colored ovals represent biplot scores of the reactor operated C:N ratio at 14:1, 9:1, 6:1 and 2:1, respectively. Since high variability in data was due to ARE, alignment of the PCA biplot scores for ARE was seen at both higher and lower C:N ratios. At a high C:N ratio, scores were toward high ARE while the lower C:N displayed a balance between the C and N removals (Fig. 4(b)). The long period of operation (187 days) perhaps allowed different microbial communities of biofilm to get polarized into two ideological situations for ammonia removal– one autotrophic and another heterotrophic, at lower and higher C:N ratios, respectively.

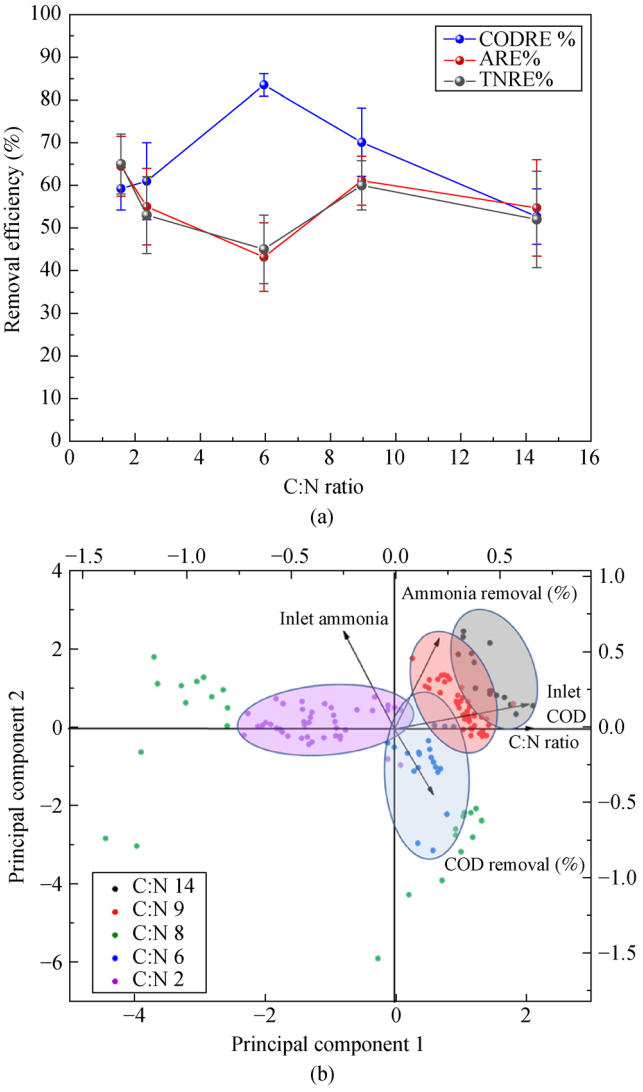


Fig. 4 (a) Effect of various C:N ratios on the performance of Methammox biofilm reactor. The blue, red and the black line plots represent percent COD removal efficiency (% CODRE), ammonia removal efficiency (% ARE), and total nitrogen removal efficiency (% TNRE) respectively. The vertical lines in the graph represent the Y-axis error. (b) Principal component analysis (PCA) performed on biofilm reactor data operated at various C:N ratios (2:1, 6:1, 8:1, 9:1 and 14:1). The first principal component is C:N ratio and the second component is inlet ammonia concentration (mg N/L) and ammonia removal efficiency (ARE %). The gray, orange, blue and purple-colored ovals represent biplot scores of the reactor operated C:N ratio at 14:1, 9:1, 6:1 and 2:1, respectively.

3.3 Interactions between microbial communities of Methammox

C:N ratio plays an important role in determining the stability and functionality of the biological system (Gu et al., 2019). High throughput 16S rRNA Illumina Miseq (V3–V4 region) sequencing of the Methammox biofilm was carried out at four different C:N ratios, namely, 1.5:1, 2:1, 9:1 and 14:1. A total of 517889 classified sequences were obtained, with the majority (92.5%) belonging to domain bacteria and the remaining belonging to archaea. There are 18, 16, 15 and 14 bacterial and archaeal phyla

with abundances >0.1% at C:N ratio 1.5:1, 2:1, 9:1 and 14:1, respectively.

The alpha and beta diversity of the taxonomic profiles were statistically analyzed using the Venn diagram and PCA plot, respectively (Figs. 5(a) and 5(b)). Venn diagram constructed at a distance of 3.0, evaluated the distribution of OTUs among different samples (Fig. 5(a)). Around 34.2% to 44.7% of OTUs were shared among two taxonomic profiles and the number of OTUs specific to each of the samples ranged from 55.3% to 65.8%. The PCA plot loosely clustered the taxonomic data at the lower C:N ratio (1.5:1 and 2:1) while those at higher C:N ratio were distinctly separate against the first and second principal axis, which described 77.7% and 22.1% of the total variation, respectively (Fig. 5(b)).

Figure 6 presents a taxonomic composition of the biofilm samples obtained at four different C:N ratios as per relative abundances of classified reads at phylum, order and class levels. Table 2 lists the abundance of functionally important genera categorised under important groups. Phyla *Proteobacteria* and *Bacteriodetes* dominated the samples at lower C:N ratios while *Firmicutes* dominated at higher C:N ratios (Table 2). *Proteobacteria* mostly comprises nitrifying, denitrifying and methane-oxidizing bacteria while phylum *Bacteriodetes* and *Firmicutes* mostly constitutes fermenters, acetogens and denitrifiers. *Bacteriodetes* and *Firmicutes* play a significant role in supporting methanogenesis and nitrogen removal (Rachbauer et al., 2017).

Nitrosomonas sp. was the most abundant ammonia oxidiser (AOB) (1.3%) detected in BR at low C:N ratios, while the abundance of Nitrite Oxidizing Bacteria (NOB) like *Nitrobacter* and *Nitrospira* were lesser than 0.005% in all the samples implying negligible nitrite-oxidizing activity. The abundance of anammox bacteria, *Candidatus kuenenia* sp. belonging to phylum *Planctomycetes* documented higher abundance (1.6% and 1.7%) at low C:N ratios and decreased to 0.2% and 0.3%, respectively at high C:N ratios. A similar pattern in the population abundance of autotrophic NRB population (AOB, NOB and Anammox) was observed when quantified through real-time PCR (Fig. S2).

Methane Oxidizing Bacterial (MOB) species like *Methylobacillus, Methylocaldum,* were observed in higher abundance at the low C:N ratios. *Methylocaldum* was the most dominating MOB detected in the biomass at C:N ratio 2:1.

The denitrifying bacterial community of the Metham-

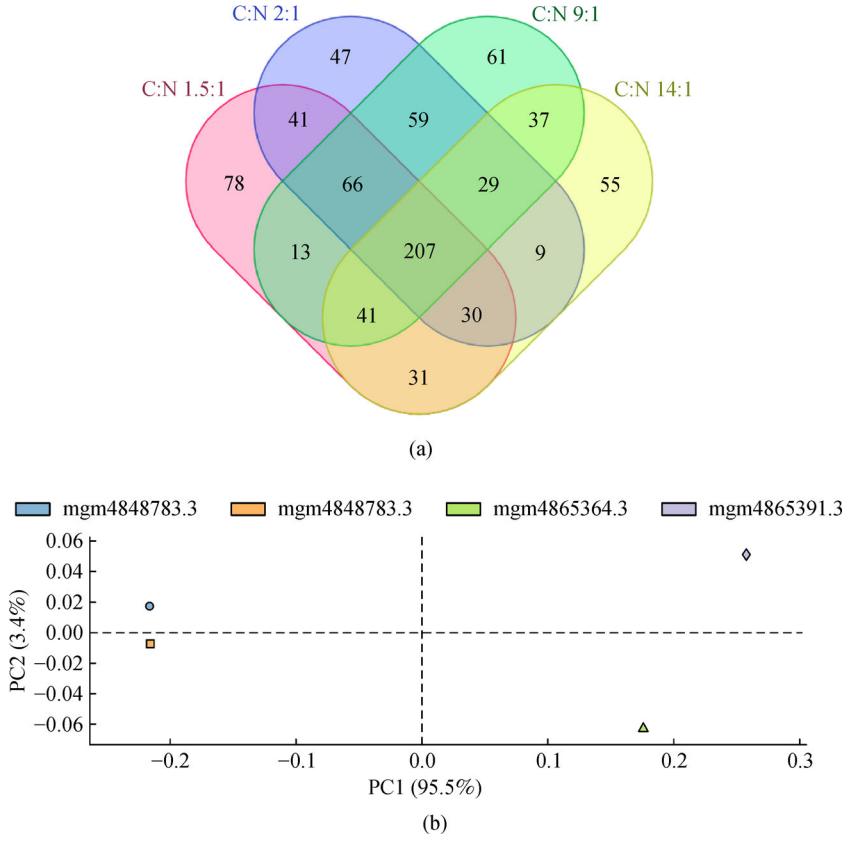


Fig. 5 The alpha and beta diversity of the taxonomic profiles of biofilm reactor at C:N ratios 1.5:1 (mgm 4865364.3), 2:1 (mgm 4865391.3), 9:1 (mgm 4848784.3) and 14:1 (mgm 4848783.3) was statistically analyzed using (a) the Venn diagram and (b) Principal Component Analysis (PCA) plot, respectively.

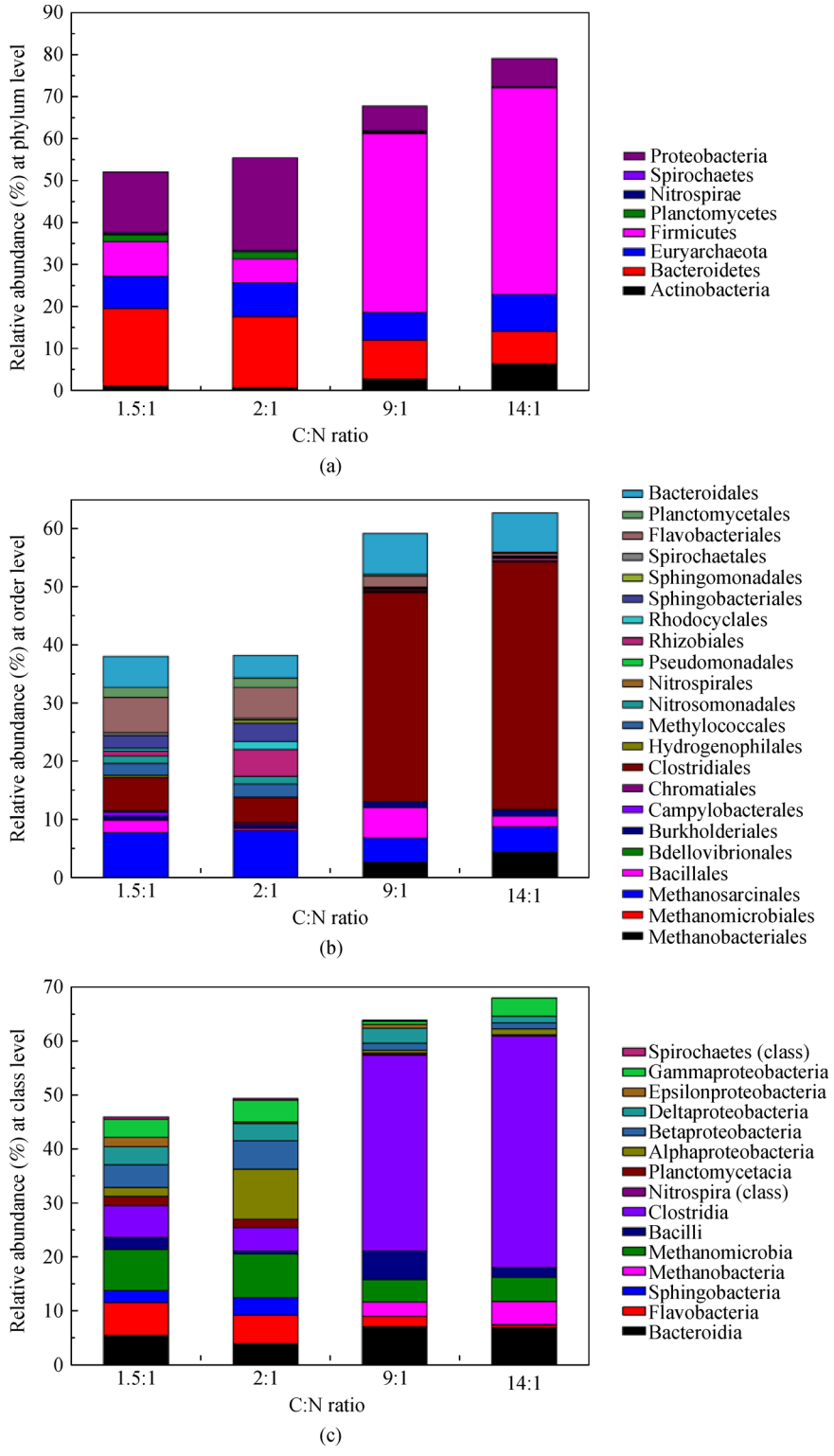


Fig. 6 Stacked bar plots showing per cent relative abundances of populations at C:N ratios 1.5:1, 2:1, 9:1 and 14:1 at (a) phyla, (b) order and (c) class levels.

mox system was comprised majorly by *Proteobacteria, Bacteroidetes* and *Firmicutes* (Sundberg et al., 2013; Pitombo et al., 2016; Pereira et al., 2017; Rachbauer et al., 2017). The denitrifiers constituted 5.7% and 4.8% of the total microbial population at lower C:N ratio and 26.1% and 18.9% at higher C:N ratios, respectively (Table 2).

Table 2 Abundance of various microbial groups in Methammox at various C:N ratios

Functional Group	Phylum	Genera	Abundance (%) at C:N ratio			
			1.5:1	2:1	9:1	14:1
Methanogens	*Archaea*	*Methanobacterium*	0.01	0.01	2.71	3.80
		Methanosaeta	0.07	0.08	4.11	4.18
		Methanomethylovorans	7.51	7.57	< 0.01	< 0.01
		Methanolobus	0.02	0.25	< 0.01	< 0.01
Anammox	*Planctomycetes*	*Candidatus kuenenia*	1.60	1.70	0.30	0.20
Non-Anammox		*Isosphaera*	0.01	0.31	0.00	0.19
		Planctomyces	0.21	1.08	0.01	0.09
		Blastopirellula	0.04	0.01	0.02	0.03
		Pirellula	0.52	0.03	0.04	0.01
Nitrifiers	*Proteobacteria*	*Nitrosomonas*	1.30	1.30	0.06	0.03
		Nitrobacter	< 0.01	0.01	< 0.01	< 0.01
		Nitrospira	< 0.01	< 0.01	< 0.01	< 0.01
Methane Oxidizers	*Proteobacteria*	*Methylobacillus*	0.01	0.21	< 0.01	0.01
		Methylocaldum	0.04	1.87	< 0.01	< 0.01
		Methylobacillus	0.01	0.21	< 0.01	0.01
Denitrifiers	*Proteobacteria*	*Rhodocyclaceae*	0.32	0.23	< 0.01	< 0.01
		*Alcaligens**	< 0.01	< 0.01	0.17	0.08
		Sphingomonas	0.02	0.05	0.01	0.05
		*Comamonas**	0.05	0.08	0.03	0.03
		Comamonadacecae (Unclassified)	0.01	0.06	0.02	0.29
		Dechloromonas	< 0.01	0.05	< 0.01	< 0.01
		Bdellovibrio	0.05	< 0.01	< 0.01	0.05
		*Pseudomonas**	0.03	0.01	< 0.01	0.03
		*Paracoccus**	0.08	0.01	0.02	< 0.01
		Rhodopseudomonas	0.07	0.41	0.12	0.01
		*Thauera**	0.01	0.01	< 0.01	< 0.01
		*Thiothrix**	0.29	0.01	< 0.01	< 0.01
		Hydrogenophaga	0.21	< 0.01	< 0.01	< 0.01
		*Achromobacter**	< 0.01	< 0.01	< 0.01	< 0.01
		*Agrobacterium**	< 0.01	< 0.01	< 0.01	< 0.01
		*Marinomonas**	0.04	< 0.01	< 0.01	< 0.01
	Actinobacteria	*Micrococcus*	< 0.01	< 0.01	< 0.01	< 0.01
	Bacteroidetes	*Terrimonas*	0.58	0.87	0.02	0.02
		Flavobacterium	1.45	0.59	0.13	< 0.01
	Firmicutes	*Clostridium*	1.78	1.83	16.63	11.59
		Clostridiales (Unclassified)	0.08	0.09	1.14	4.49
		*Geobacillus**	0.11	< 0.01	1.82	0.10
		Alicyclobacillus	0.30	0.03	1.76	0.06
		*Bacillus**	0.21	0.36	1.25	0.88
		*Sedimentibacter**	< 0.01	0.07	2.97	1.10

Notes: * also functions as heterotrophic nitrification and aerobic denitrification (HNAD). Total abundances of methanogens, *Planctomycetes*, nitrifiers, methane oxidizers, and denitrifiers, at C:N ratio 1.5:1, 2:1, 9:1 and 14:1 in the same order are [7.61, 7.91, 6.82, 7.97], [1.60, 1.70, 0.30, 0.20], [1.30, 1.31, 0.06, 0.03], [0.07, 2.28, 0.01, 0.01], and [5.69, 4.76, 26.09, 18.78], respectively.

Clostridium (1.8%), *Flavobacterium* (1.5% and 0.6%), *Terrimonas* (0.6% and 0.9%), *Bacillus* (0.2% and 0.36%), and members of family *Rhodocylcaceae* (0.3% and 0.2%) were detected in high abundance at low C:N ratio. On the other hand, *Clostridium* sp., *Bacillus* sp. and unclassified sequences belonging to order Clostridiales was the most dominating denitrifying population common to both the higher C:N ratio (Table 2). Apart from these three populations, *Geobacillus* (1.76%), *Alicyclobacillus* (1.82%), *Flavobacterium* (0.13%), and *Rhodopseudomonas* (0.12%) were abundant in C:N ratio 9:1. Other denitrifying bacteria like *Achromobacter, Agrobacterium*, *Alcaligens*, *Comamonas, Dechloromonas, Hydrogenophaga Marinomonas, Micrococcus, Paracoccus*, *Pseudomonas, Sphingomonas, Thauera, Thiothrix* and others were also detected in lesser abundances in the biomass (Table 2). Most of the denitrifying population detected in the biofilm also carry out heterotrophic nitrification-aerobic denitrification (HNAD). HNAD population can convert ammonium to nitrogen (N_2) via nitrite/nitrate thereby avoiding competition between autotrophic nitrifiers and heterotrophic denitrifiers (Vinothkumar et al., 2021). The genus *Clostridium,* which comes under phylum *Firmicutes,* is not only a denitrifier but also provides fermentation end products for methanogenesis (Sundberg et al., 2013; Pitombo et al., 2016).

The methanogenic population abundance (Phylum *Euryarchaeota*) remained stable across all the C:N ratios and ranged between 6.82% and 7.97% (Table 2) indicating their tolerance to changes in C:N ratio. The archaeal communities in various full-scale anammox reactors reportedly varied from 0.37% to 3.8% of the total microbial population (Pereira et al., 2017) while those in the presence of heterotrophic environment, such as SNAD or in the anammox systems enriched from methanogenic biomass, it varied from around 2% (de Almeida et al., 2018) to 12% of the total microbial population (Pekyavas and Yangin-Gomec, 2019), respectively.

The extended bar plot (Fig. 7) illustrates the significant differences in microbial abundances at the genera level at higher (9:1 and 14:1) and lower (1.5:1 and 2:1) groups of the C:N ratio. Since all the "*p*-values" were less than 0.05, the taxonomic profiles of the biofilm at the lower and higher C:N ratio were significantly different.

At a low C:N ratio, the microbial community structure of Methammox biofilm had all major microbial groups of methanogens (*Euryarchaeota*: 7.6% to 7.9%) and NRB (*Planctomycetes*: ~1.6%, AOB: 1.3%, denitrifiers: ~5%; total NRB: ~8%) co-existing in 1:1 ratio with each other. In addition, the MOB (2.2%) population was observed at a C:N ratio of 2:1. Contrastingly, at a higher C:N ratio, the methanogens (*Euryarchaeota*: 6.8% to 8.0%) and NRB population (denitrifiers: 18%–23%, autotrophic population: $< 0.5\%$) coexisted in a 1:3 ratio with each other. Thus, the microbial abundance and population dynamics of Methammox biofilm demonstrated a shift in the nitrogen metabolism from mixotrophy to heterotrophy with an increase in the C:N ratio.

3.4 Understanding the role of Methammox in simultaneous C & N removal at a wide C:N ratio

The population dynamics of the Methammox biofilm suggests that the community structure must have played an important role at low and high C:N ratios. The image of gravels with attached biofilm is shown in Fig. S3. The Methammox biofilm is likely to have discrete and independent populations of heterotrophic methanogens and autotrophic populations of NRBs interspaced with microbial groups that could connect with both of them. The existence of discreetly separate microcolonies of large coccoid bacterial cells having ~1 μm diameter and those with smaller diameters (0.1–0.2 μm) in the reactor biofilm is evident in the scanning electron micrographs (Fig. 8(a)).

Though the SEM micrographs do not confirm the microbial nature of interspacing groups, it is quite likely that those could be denitrifiers including HNAD and/or MOBs. The most abundant denitrifiers (*Clostridium* and *Bacillus*) detected at high C:N ratio supply acetate for methanogens and undergo fermentation under anoxic conditions with nitrate as electron acceptors using a mechanism commonly described as DNRA process (van den Berg et al., 2017). However, with a low NOB population, limiting nitrates can restrict the DNRA process. The ability of the fermentative population to take up an additional function of denitrifying bacteria compensates for the drop in the abundance of autotrophic NRB population at a high C:N ratio. MOB, under anaerobic conditions, can oxidize methane with nitrate reduction and also can oxidize ammonia (Holmes et al., 1995). The ability of denitrifiers including HNAD and MOBs to associate with both the methanogenic and NRB populations makes them the 'interspacing-connectors'. Analogically, the presence of these interspacing connectors makes the methanogenic and NRB populations functionally independent of each other in Methammox biofilm. The schematic representation of Methammox biofilm depicting two clusters interspaced with methane-oxidizing bacteria and denitrifiers is presented in Fig. 8(b). The interspacing by the MOB and denitrifiers separate the NRB and MB making them functionally independent of each other. The denitrifiers including HNAD that participate in nitrogen metabolism at a high C:N ratio also provide fermentative end products for methanogens and in this way interact with both the microbial populations of Methammox like MB and NRB.

It is worthwhile to mention, while the population of *Euryarchaeota* remained unchanged at both high and low C:N ratios, the major route of methanogenesis shifted from methylotrophy to acetoclastic to fine-tune with the change in the NRB population and ultimately with the C:N ratio. Thus, the methanogens coordinated with NRB through

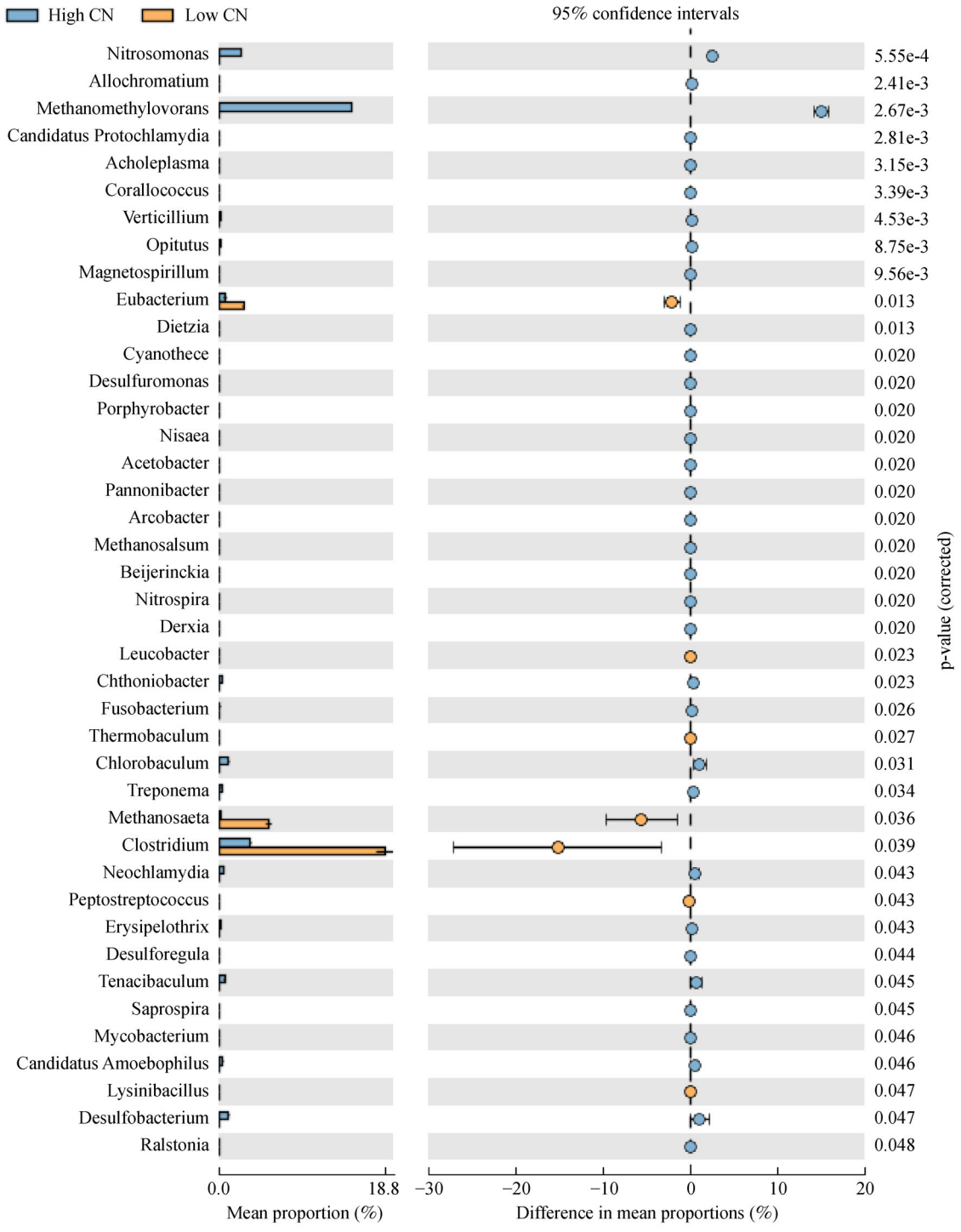
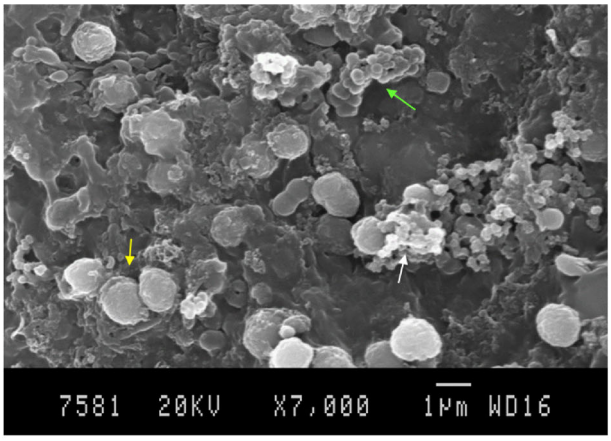


Fig. 7 Extended error bar plots showing significant differences ($p < 0.05$) between mean populations (genera) in low (1.5:1 and 2:1) and high (9:1 and 14:1) C:N ratios shown in orange and blue bars, respectively. Corrected p values are shown at the right.

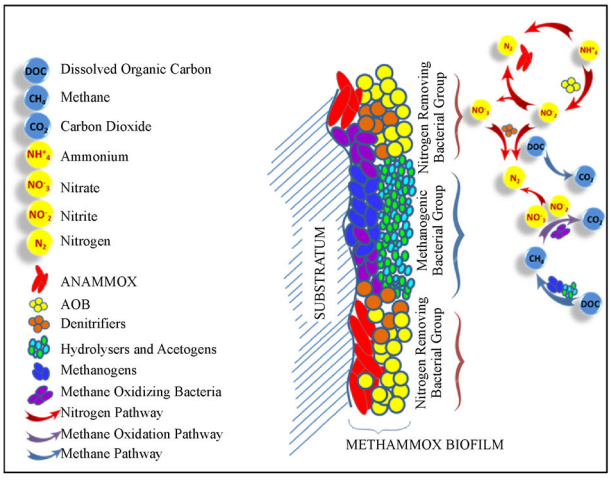
denitrifiers including HNAD and increased resilience of the biological nitrogen removal process to a high C:N ratio. The functional plasticity of the Methammox system to adjust to a broad C:N ratio opens new frontiers in simultaneous C & N removal at both low and high C:N ratio wastewaters.

4 Conclusions

The functional and synergistic association of anaerobic methanogenic population with nitrogen removing bacterial (NRB) groups for simultaneous C and N removal in a single system called ‘Methammox’ was studied at a wide



(a)



(b)

Fig. 8 (a) Scanning electron micrograph of Methammox biofilm taken from the Biofilm Reactor. The discreet clusters of large coccoid bacterial cells having ~1 μm diameter and those with smaller diameter (0.1–0.2 μm) are shown with colored arrows. (b) Schematic representation of Methammox Biofilm consisting of independent clusters of Nitrogen Reducing Bacterial (NRB) group and methanogenic bacterial (MB) group. The two clusters are interspaced with methane-oxidizing bacteria and denitrifiers which take substrates from both groups. The interspacing by the MOB and denitrifiers separate the NRB and MB making them functionally independent of each other. The denitrifiers that participate in nitrogen metabolism at a high C:N ratio also provide fermentative end products for methanogens.

range of C:N ratios. Batch experiments confirmed that both methanogens and NRB exhibited their functional identity in Methammox. The best and balanced simultaneous removals of COD and ammonia were obtained in BR at lower ($\leqslant$2:1) and higher C:N ratio ($\geqslant$9:1). At the lower C:N ratio (1: 5 and 2:1), the microbial population abundance of the NRB and the methanogenic community existed in almost 1:1 ratio. At the higher C:N ratio, the decreased abundance of autotrophic NRB was compensated by denitrifiers that also assisted methanogens through supplying fermentation end products. The methanogenic population showed stable abundance at both lower and higher C:N ratios indicating methanogens can serve as better partners for organic C removal in simultaneous C & N removal systems at both low and high C:N ratio wastewaters.

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