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Production and removal of soluble organic nitrogen by nitrifying biofilm

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ABSTRACT

This study investigated soluble organic nitrogen (sON) activity in batch reactors mimicking nitrifying moving bed biofilm reactors (MBBRs). This work was divided into two objectives focusing on the production and degradation of sON, respectively. For the first objective, a synthetic feed that did not contribute any organic nitrogen was used and results indicated that irrespective of the presence of influent organic carbon (0 versus 400 mg COD/L) in the reactors, sON was contributed by the biofilm during nitrification. Although net production of sON was observed, both production and ammonification coexisted which regulated the sON concentration. For the second objective, actual wastewater was fed to the reactors to investigate sON degradation under different carbon to nitrogen (C/N) ratios. A higher concentration of sON was biodegraded in the reactor when fed with influent containing a lower C/N ratio. Overall results suggested that organic carbon bioavailability and/or ammonia concentration influenced the production and ammonification of sON. This study is the first to explore the sON activity by MBBR biofilm and findings from this work could extend the knowledge on the fixed film process with respect to sON activity to regulate and optimize reactor operation in meeting stringent total nitrogen discharge limits.

1. Introduction

Recent guidelines for discharging total nitrogen (TN) are approaching ≤ 5 mg TN/L for several parts of the United States. These guidelines aim to curb the hypoxic conditions and eutrophication issues in vulnerable receiving water bodies. With advancements in science and technology, water resource recovery facilities (WRRFs) are capable of removing $>$ 95% of inorganic nitrogen resulting in soluble organic nitrogen (sON) being a major nitrogen fraction ($>$ 50%) of the effluent TN [1]. Several studies have described that about 60–70% of the total influent sON is removed by activated sludge process (ASP) [2–4] while Simsek et al. [5] found that 37–50% of the influent sON is biodegraded by a trickling filter system. The majority of the research work related to sON degradation has focused on conventional ASP [6–9] while few studies have touched on the fixed film processes, mainly on trickling filter and post-denitrification filters (DNF) [5,10,11].

At WRRFs, moving bed biofilm reactors (MBBRs) are employed usually as a separate stage nitrification process (to nitrify wastewater with a lower carbon to nitrogen (C/N) ratio). Considering the consequences of elevated fraction of sON in the effluent (complication with permit compliance and impairment of receiving water quality), it will be reasonable to identify the available strategies in an MBBR process to control the concentration of sON while avoiding the need for (additional) advanced removal technologies. Simsek et al. [10] investigated the fate of biodegradable sON (bsON) and bioavailable sON (AbsON) in a full-scale WRRF consisting of both ASP and MBBR. The biodegradable fraction of sON or bsON can be biochemically oxidized by bacteria to produce ammonia N [12] whereas the bioavailable fraction of sON or

Abbreviations: AbsON, bioavailable soluble organic nitrogen; ASP, activated sludge process; ATP, adenosine triphosphate; BNR, biological nutrient removal; BOD, biochemical oxygen demand; bsON, biodegradable soluble organic nitrogen; C/N, carbon to nitrogen ratio; COD, chemical oxygen demand; DNF, post-denitrification filters; DO, dissolved oxygen; HPO-ASP, high purity oxygen ASP; MBBR, moving bed biofilm reactors; mg/L, milligram per liter; MWWTP, Moorhead wastewater treatment plant; RLU, relative light units; sCOD, soluble COD; sON, soluble organic nitrogen; SWW, synthetic wastewater; TN, total nitrogen; TSN, total soluble nitrogen; TSS, total suspended solids; VSS, volatile suspended solids; WRRF, water resource recovery facility.

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AbsON can be uptaken by algae or other aquatic plant species for growth [13–15]. Nitrogen cycling can be influenced by the form of sON in the effluent. For instance, dissolved free amino acids can be directly uptaken by (bioavailable to) the algae; however, other forms of sON might have to be first hydrolyzed and/or mineralized (biodegraded) by bacteria making them bioavailable to the algae or other phytoplanktons in the receiving waters [10,16]. Simsek et al. [10] concluded that ASP removed 29% of sON whereas MBBR removed only 4% of sON. The authors suggested that a low C/N ratio, solubilization of particulate organics from the biofilm, and/or release of soluble microbial products (SMPs) might have affected the sON removal in the MBBR process [10].

Hu, Liao, Geng et al. [11] investigated the effect of different C/N ratios (3, 4, 5 and 6) on the removal of sON and AbsON in DNFs. They fed secondary effluent to the filters and noticed the maximum effluent sON at C/N ratio of 3 (1.91 mg sON/L) and no impact on effluent sON for higher C/N ratios i.e., 4 (1.70 mg sON/L), 5 (1.70 mg sON/L) and 6 (1.69 mg sON/L). However, effluent AbsON decreased with increasing C/N ratio suggesting that sON produced by DNFs at higher C/N ratios will be less bioavailable, a scenario favorable for the receiving waters [11]. The studies of Simsek et al. [10] and Hu, Liao, Geng et al. [11] indicated that relatively less sON removal should be expected under a lower C/N ratio. However, no study has explicitly investigated the removal of sON in an MBBR process under different C/N ratios.

Effluent sON from biological treatment processes is primarily from influent- and process-derived sources. The influent-derived sON is the result of recalcitrant organic nitrogen, which is not biodegraded or removed during wastewater treatment [16]. Process-derived sON is released by metabolic activities associated with biological processes (e.g., SMPs and extracellular polymeric substances) [7,14,17,18]. Since process-derived sON is contributed by the growth and decay of microorganisms during the biological treatment processes, process-derived sON is unavoidable and more closely related to operational parameters than influent-derived sON [15]. Approximately 33% of the effluent sON are process-derived while the rest of it is from the influent [19,20]. However, the extent of the biological production of sON varies from one biological system to another [6]. Therefore, reducing the formation of process-derived sON in biological treatment processes will be beneficial in achieving the low TN discharge limits and eventually safeguard the water bodies receiving treated wastewater. Parkin and McCarty [21] investigated the influence of organic loading (glucose, acetate, glucose-acetate mixture) on sON production and found that an increase in organic loading increased sON production in ASP [21]. Although there have been several studies investigating the effect of organic loading (measured as chemical oxygen demand (COD)) on nitrification in an MBBR process [22–25], no study has investigated the effect of organic loading on the production of sON by biofilm particularly those in an MBBR.

Simsek et al. [10] reported sON removal of 29% by ASP and 4% by MBBR (for nitrification) in a full-scale WRRF. Their study suggested that in an MBBR, lower ammonification of sON occurred due to less ability of ammonifying bacteria to compete for oxygen compared to nitrifiers [10]. Ammonification is a major pathway for sON degradation and is considered to be achieved primarily by heterotrophs and phytoplanktons [26]. Ammonia produced from ammonification is transformed via nitrification and/or assimilated by biomass. Since nitrifiers are primarily autotrophs, they are not believed to be directly associated with sON degradation [10,27]. Hence, sON degradation is largely considered to be a heterotrophic bacterial process.

Studies reported that while heterotrophic processes remove higher fraction of sON, reduction in sON concentration was also observed after nitrification stages at full-scale WRRFs highlighting the involvement of nitrifiers in sON biodegradation [5,10]. Wadhawan et al. [28] reported 57% of sON removal through the nitrification process in secondary effluent and lesser removal through the heterotrophic process (38%). The nitrification process biodegraded higher concentration of sON compared to the heterotrophic process. The study also claimed that during the nitrification, ammonia oxidizing bacteria rather than nitrite oxidizing bacteria were responsible for sON degradation and it is the first study that reported the involvement of nitrification in sON degradation. Based on the results from these previous studies [5,10,28], this study aimed at exploring the production of sON and the effect of C/N ratios on sON degradation during nitrification in a MBBR.

The objective of this study was to identify the influence of organic loading and different C/N ratios on sON activity (production and removal) in bench-scale reactors that mimic the nitrification process of MBBRs. Specifically, this study examined the effect of readily biodegradable COD on the production of sON by feeding synthetic wastewater with no organic nitrogen. The study also investigated the effect of different C/N ratios on sON degradation for which the reactors received real wastewater samples representing different C/N ratios. Results from the work could extend our knowledge on the fixed film process with respect to sON activity to regulate and optimize reactor operation in order to achieve low TN discharge limits.

2. Materials and methods

2.1. MBBR carrier and wastewater sample sources and collections

The biofilm carriers shown in Fig. S1 in Supplementary Material (SM) used in this study were collected from a nitrifying MBBR basin of the Moorhead wastewater treatment plant (MWWTP), Moorhead, MN. The biofilm carriers were collected from the basin in bulk using a 5 L bucket and transported within 15 min to a laboratory where experimental work was conducted. The carriers were separated from the liquid phase using a stainless-steel strainer. The separated carriers were weighed, and equal amounts (50% of the reactor volume) were added immediately to four 1 L beakers (batch reactors). To represent varying C/N ratios, grab effluent samples were collected from the equalization basin (C/N = 4.2:1), primary clarifier (C/N = 1.5:1), activated sludge process (C/N = 0.8:1) and MBBR basin (C/N = 0.2:1) as shown in Fig. 1. The C/N ratios were obtained by dividing soluble COD (sCOD) with total soluble nitrogen (TSN) (C/N: 4.2 = 168/39.6, C/N: 1.5 = 74/48.4, C/N: 0.8 = 33/40.1; and C/N: 0.2 = 8/38.8). The collected wastewater samples were used in the experiments immediately after they were brought to the laboratory. Portions of collected wastewater samples were used for the analyses of total suspended solids (TSS), volatile suspended solids (VSS), inorganic nitrogen species and sON. Experimental work (operation of batch reactors as described in subsection 2.4) for each objective (production versus removal) was triplicated. The biofilm carriers as well as wastewater samples were collected three times for each objective corresponding to the triplication.

The MWWTP has a peak pumping capacity of 38,000 m^3/d and an average flow of 15,000 m^3/d. The facility has to be in compliance with the discharge limits for biochemical oxygen demand (BOD) and ammonia but is not regulated for the TN limit. The facility employs high purity oxygen-ASP (HPO-ASP) for removing BOD. A 3024 m^3 MBBR is used to nitrify ammonia in the treated wastewater from HPO-ASP. The hydraulic retention time and sludge retention time of the MBBR are 3.2 h and 32 d, respectively. The reactor is filled approximately 32% with biofilm carriers (21 mm in diameter) that move throughout the reactor with the mixing action caused by the aeration system. More detailed information regarding the MBBR basin and the carriers is tabulated in Table S1 under SM.

2.2. Synthetic wastewater recipe

The synthetic wastewater (SWW) recipe was modified from Nagaoka et. al. [29] to mimic medium-strength domestic wastewater composition. SWW was employed to identify the effect of organic loading (readily biodegradable COD) on sON production in an MBBR process. Therefore, two different solutions of SWW (A and B) were prepared wherein the basic composition remained the same as described in

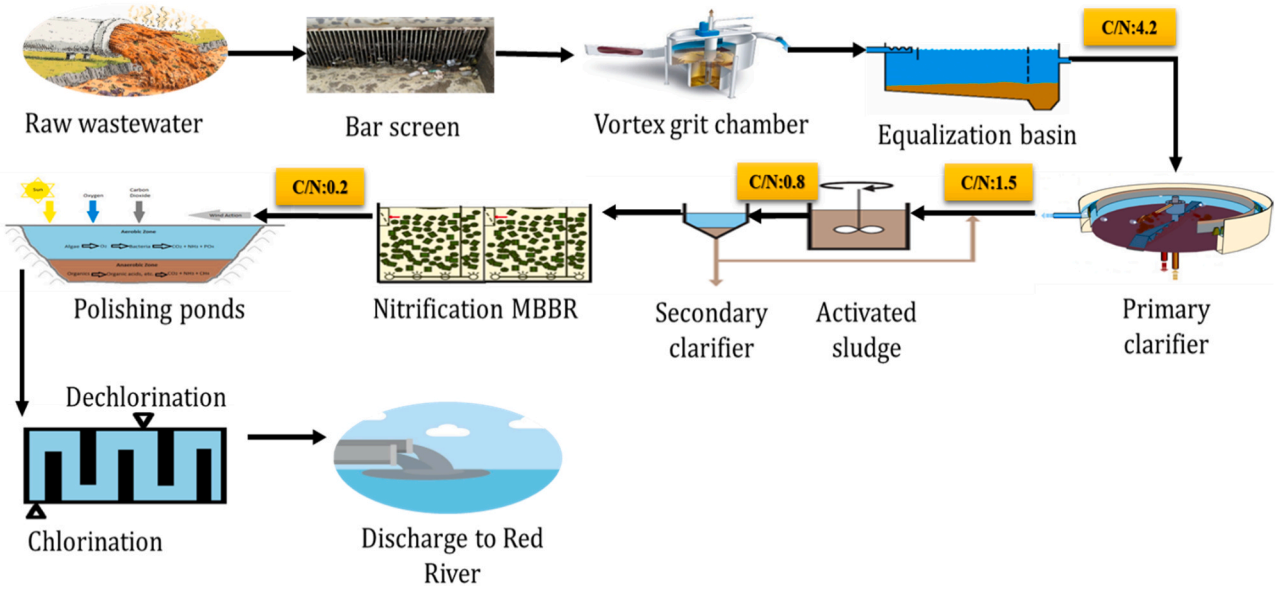

Fig. 1. Graphical representation of the MWWTP. Sampling locations with respective C/N ratios are indicated by yellow boxes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table S2 in SM. SWW A received no-COD, whereas SWW B received 400 mg COD/L using glucose. SWW contributed only inorganic nitrogen (40 mg N/L) via ammonium chloride and no organic nitrogen was in the SWW solution.

2.3. Experimental setup

This study was divided into two parts with two separate focuses: effect of organic loading on sON production and effect of C/N ratio on sON degradation.

2.3.1. Effect of organic loading on sON production

For the first part, bench-scale experiments were conducted to identify the production of sON when the reactors were fed with SWW containing no organic nitrogen (Fig. S2, SM). Glucose is a readily biodegradable source of COD which was added to the SWW that was fed to one of the two reactors. Two 1 L reactors were filled at 50% (reactor volume) with biofilm carriers (~118 g/reactor) collected from the nitrifying MBBR basin of the MWWTP. Each reactor was fed with SWW A (0 mg COD/L) and SWW B (400 mg COD/L) to make up the final volume to 1 L. The resulting organic loading rates were 0 and 2 g sCOD/m^2 of carrier surface whereas ammonia loading rate in each reactor was 0.2 g NH_3-N/m^2 of carrier surface. Both reactors were aerated using air stone-diffusers to maintain a DO concentration at 2–4 mg O_2/L at room temperature (~20 °C). pH in the reactors was maintained at 7.2–7.8 using HCl and $NaHCO_3$. Samples were collected from each reactor every 30 min until ammonia concentration fell below the detection limit (0.015 mg NH_3-N/L).

2.3.2. Effect of C/N ratio on sON degradation

For the second part, batch experiments were conducted to investigate the effect of different C/N ratios on sON degradation (Fig. S3, SM). Fifty percent of the 1 L beakers were filled with biofilm carriers (~115 g/reactor) collected from the MBBR basin. Each reactor was filled to make up a final volume of 1 L with wastewater sample collected from four different locations, i.e., after equalization basin (C/N = 4.2), after primary clarifier (C/N = 1.5), after activated sludge (C/N = 0.8), and after MBBR basin (C/N = 0.2) (Fig. 1). The reactors were operated at room temperature (~20 °C) while maintaining the pH at 7.2–7.8 and DO at 2–4 mg O_2/L. All the reactors were operated continuously until the concentration of ammonia was below the detection limit (0.015 mg NH_3-N/L). Samples were collected from each reactor every 30 min and were analyzed for sON. To examine if different C/N ratios affected the microbial activity in the biofilm attached to the biofilm carriers, an adenosine triphosphate (ATP) assay was used. The ATP assay is an indirect measurement for active cells, including non-culturable cells, based on their metabolic activity. It determined relatively an amount of active biomass in the biofilm attached to the biofilm carrier that was collected immediately before and after the operation of the reactors.

2.4. Analytical techniques

TSS and VSS were analyzed gravimetrically according to Standard Methods [30]. COD was determined using HACH TNT kits (HACH Company, Colorado, USA). COD was measured using the USEPA bioreactor digestion method (HACH method 8000) with low range (3–150 mg COD/L) and high range (20–1500 mg COD/L) testing kits.

Inorganic nitrogen species were measured using the HACH TNT plus kits. Ammonia concentration was measured using the salicylate method (TNT plus method 10205) for ultra-low range (0.015–2.0 mg NH_3-N/L), low range (1.0–12.0 mg NH_3-N/L) and high range (2–47 mg NH_3-N/L). Nitrite concentration was measured using the diazotization method for both low range (0.015–0.6 mg NO_2-N/L) and high range (0.6–6.0 mg NO_2-N/L). The TNT plus method 10207 was used for measuring low range nitrite whereas the TNT plus method 10237 was used for measuring high range nitrite. Nitrate concentration was measured using the dimethylphenol method (TNT plus method 10206) for both low range (0.23–13.5 mg/L NO_3-N) and high range (5–35 mg NO_3-N/L). Total N concentration was measured using the persulfate digestion method (TNT plus method 10208) for low range (1–16 mg TN/L), high range (5–40 mg TN/L), and ultra-high range (20–100 mg TN/L). To determine the concentration, a HACH DR 5000 spectrophotometer was used. The spectrophotometer was calibrated using blank samples and standard solutions as referred in the manual [31]. All the analyses were performed in triplicate on split samples, and the average and standard deviation values are reported.

Since sON concentration is determined indirectly, the reliability of the employed methods should be identified [32]. The accuracy of sON measurement with the HACH kits was verified by preparing standard solutions with known quantities of ammonia (1 mg NH_3-N/L), nitrate (1 mg NO_3^--N/L) and urea (1 mg urea/L) mixed in deionized water. Table S3 in SM displays the error analysis results. The measured sON

concentration (1.04 ± 0.03 mg N/L) was close to the urea concentration (1 mg N/L) added to the standard solution.

Benchtop meters were used to continuously monitor the pH (model 250 A+, Thermo Scientific Orion) and DO (model 850 Thermo Scientific Orion) in the reactors. The pH meter was calibrated daily using the three-point pH calibration method with three different buffer solutions. The DO meter was calibrated daily using the water-saturated air method.

2.5. Measuring soluble fraction and sON

Filtering a wastewater sample through a 0.45 μm pore size membrane filter to obtain soluble fraction can allow colloidal fraction ranging between 0.1 and 0.45 μm diameter to pass through with the filtrate. Hence, the flocculation-filtration technique [33] was employed to remove both particulate and colloidal fractions from the samples. Zinc sulfate ($ZnSO_4$) and sodium hydroxide (NaOH) were added to the sample to flocculate colloids and particulates followed by filtration with a 0.45 μm pore size cellulose acetate membrane filter (PALL Co., Port Washington, NY, USA) to obtain a true soluble fraction. sON concentration was determined as the difference between total soluble nitrogen and total inorganic nitrogen (ammonia + nitrite + nitrate).

2.6. Bacterial growth assessment

The QuenchGone21™ wastewater test kit (Luminultra, New Brunswick, Canada) was used to assess the bacterial activity over a period of time (start and end of reactor operation) in the biofilm attached to the biofilm carriers in each reactor. The assay uses ATP as an indicator of biomass activity. The protocol provided in the QuenchGone21™ wastewater test kit was followed. The ATP assay measures light produced from a luminescent reaction between ATP (from the wastewater sample) and a mixture of luciferin, luciferase (an enzyme which naturally occurs in the tails of fireflies to produce light), and magnesium. Since the oxidation of one molecule of ATP produces one photon, concentration of ATP in a sample is proportional to the emitted light. The light output i.e., relative light units (RLU) was measured with a luminometer. The obtained RLU values were converted to ATP concentrations (μg-ATP/g-biofilm).

3. Results and discussion

3.1. Effect of organic loading on nitrification and sON profile

Fig. 2 displays the nitrification profiles wherein, by 4.5 h of operation, the MBBR carriers added to each reactor successfully nitrified ammonia (> 99%) to below the detection limit. In reactor B (glucose), nitritation and nitratation were achieved relatively earlier than reactor A (control). At 2.5 h of operation, higher removal of influent ammonia was observed under reactor B (94.6%) than reactor A (54.2%). Bassin et al. [23] reported that less time was required to achieve complete removal of ammonia in the presence of organic carbon during the initial operation of a laboratory-scale MBBR. The authors tied the heterotrophic condition (presence of casein peptone, meat extract and urea) to the enrichment of nitrifiers in the biofilm. The presence of higher amounts of organic carbon resulted in greater extracellular polymeric substance production by heterotrophs, which promoted the attachment of nitrifying bacteria to biofilm and reduced loss of nitrifiers through detachment, thereby resulting in an increased nitrification rate [23].

Fig. 3 shows the sON profiles observed in each reactor. Net production of process-derived sON was identified at every time point in each reactor when no organic nitrogen was fed. Residual sON was observed in each reactor at t = 0 h i.e., 0.36 mg sON/L in reactor A (control) and 0.57 mg sON/L in reactor B (glucose). After 4.5 h of operation, reactor A produced 1.63 mg sON/L, whereas reactor B produced 1.58 mg sON/L. Similar to the presence of residual sCOD, residual sON in the aqueous solution was possibly contributed via leaching and or hydrolysis. Although no COD was added to reactor A, sCOD of nearly 70 mg sCOD/L was detected in the reactor at t = 0 min (Fig. S4, SM). The presence of sCOD was attributed to the biofilm carriers added to the reactors from the full-scale MBBR basin. Since the carrier-contributed COD was the residual content from two biological treatment processes (ASP and MBBR), it is assumed that it was biorefractory in nature. The residual sCOD could be released by the following two routes. The residual sCOD leached from the biofilm into the bulk phase and/or readily hydrolyzed particulate matter from the biofilm detached into the bulk phase [34,35]. Both the leaching and detachment were likely promoted by agitation via aeration in the reactors. Therefore, the presence of residual sCOD and sON identified in both reactors were unavoidable.

In reactor A, sON concentration increased during the first 0.5 h of reactor operation, after which it gradually decreased with some minor

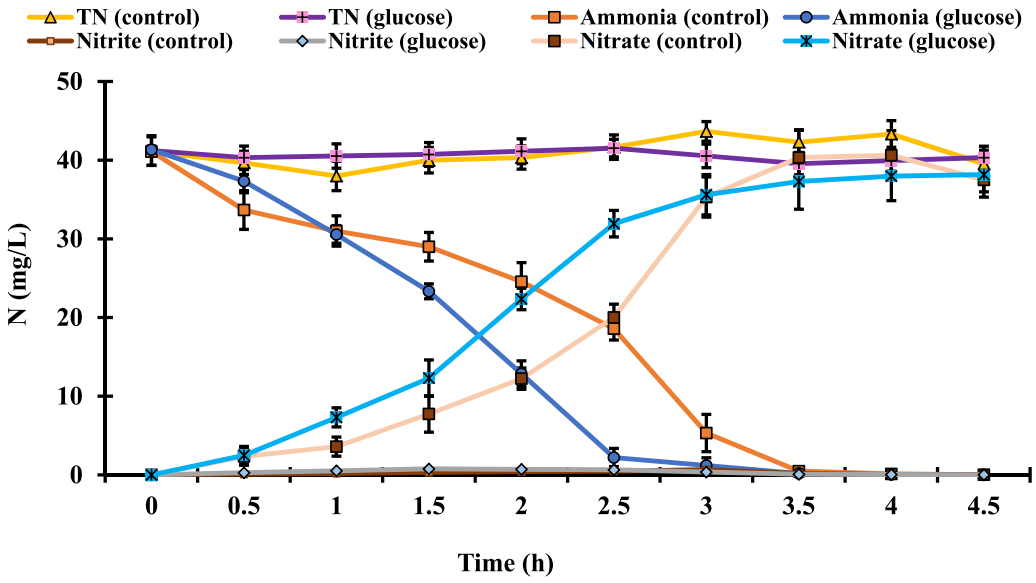

Fig. 2. Nitrification profile of bench-scale MBBRs operated for a duration of 4.5 h. Operation of reactor A (control) started with residual COD, whereas reactor B (glucose) began with a combination of residual + external COD.

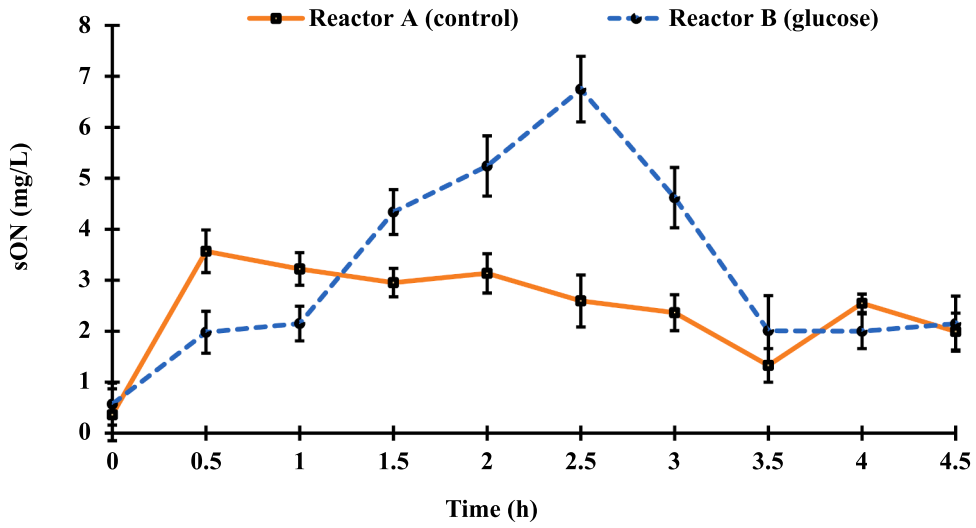

Fig. 3. sON profile in a nitrifying MBBR system in the presence of an residual source of COD (reactor A) and residual + external COD source (reactor B).

fluctuations. sON production in the first 0.5 h of operation was attributed to the prevalence of biomass starvation or decay because organisms in the biofilm possibly got stressed due to change of environmental conditions (real wastewater or feast versus SWW or famine) and low biodegradable organic carbon in the reactor [36,37]. Under stressful periods, excretion of sON to establish a concentration of equilibrium across the cellular membrane increases sON concentration in the reactor [21]. Moreover, starvation can induce bacterial death via programmed cell death or self-destruction of cells under stressful conditions [36], which may as well contribute to sON in the reactor.

In reactor A, lack of organic carbon was expected to significantly affect the heterotrophs, whereas a change in the environment would affect both the heterotrophs and autotrophs. Since ammonia was added to the SWW, autotrophs were assumed to face less stress (i.e., no starvation) than heterotrophs. Considering that biomass decay releases soluble microbial products (specifically biomass associated products that originate from decay), which contribute cellular macromolecules containing organic carbon and nitrogen [38–40]. This organic carbon, which is partially biodegradable, could be oxidized by the heterotrophs as a substrate to support growth or cell maintenance. However, since no increase in COD concentration was observed in reactor A (Fig. S4, SM) and the organic carbon (released from decay) is expected to be available in small amounts, the heterotrophic growth was possibly minimal. Besides, the growth of nitrifiers in the presence of ammonia could have contributed to sON since nitrification was happening in the reactor (Fig. 2). Therefore, a significant fraction of sON release was attributed to heterotrophic activities, particularly the decay than autotrophic growth in the reactor in the first 0.5 h of operation.

The sON concentration in reactor A after t = 0.5 h of operation reduced steadily in the bulk phase, which could be attributed to the dominance of ammonification of sON in the reactor over sON release. Although ammonification dominates after 0.5 h, the heterotrophic decay in the reactor was likely to continue to take place, although submissively beyond 0.5 h of reactor operation. Besides, nitrification was also in progress between 0.5 and 3.5 h of reactor operation. After 3.5 h of operating the reactor, externally added ammonia had significantly depleted (Fig. 2). The sON concentration increased slightly, followed by a small decrease during the last hour of the operation. Since ammonia was essentially gone and sON was present in low concentrations, an increase in sON concentration (t = 3.5 h) is attributed to lack of required nutrients or starvation, leading to endogenous respiration for the autotrophic bacterial cells to obtain energy for maintenance [36]. Since nitrification was reduced after 3.5 h of reactor operation, endogenous respiration of both autotrophs and heterotrophs (pre-existing decay) seems to dominate in the reactor. Thereafter, the excreted sON was possibly ammonified by the surviving bacteria in the next 0.5 h (t = 4.0–4.5 h) to support growth or cell maintenance. Overall, between 0 (0.36 mg sON/L) and 4.5 h (2.0 mg sON/L) for reactor A, a net production of 1.64 mg sON/L was observed. The overall analysis of sON activity highlights the cycling of organic nitrogen under a nutrient-limited environment. This dynamic behavior of sON was also observed by Khan et al. [12], wherein the sON activity of a sample collected from a full-scale WRRF was monitored for 180 d. Heterotrophic growth and decay, and autotrophic growth (nitrification), and ammonification coexist in the nitrifying MBBR, wherein the dominating process determines the fate of sON concentration.

In reactor B, no significant sCOD reduction was observed in the bulk phase of each reactor (Fig. S4, SM). However, for 4.5 h of operation, a net concentration of 26 mg sCOD/L was removed from the bulk phase of reactor B, whereas no reduction was observed for reactor A. Two possibilities have been postulated for the decrease of sCOD concentration observed in the bulk phase of reactor B. Since the addition of glucose was the only difference between the two reactors, the first possibility is that the growth of heterotrophs in the biofilm of reactor B could have enhanced the sCOD removal. The second possibility is that sCOD might have diffused from the liquid phase into the biofilm to equilibrate the concentration gradient. The biofilm composition was not examined; hence, these are only speculations based on bulk phase sCOD analysis.

In reactor B, sON concentration increased consistently until 2.5 h of operation, followed by a sharp decline in the next hour, and then stayed relatively constant until the end of the reactor operation. During the first 2.5 h of operation, the externally added ammonia in reactor B was nitrified (Fig. 2). The drastic increase in sON concentration in 2.5 h of operation highlights that the production overtook the ammonification of sON in the reactor. Unlike reactor A, wherein an increase in sON concentration was primarily attributed to the decay of heterotrophs in the first 0.5 h, the increase in sON concentration in reactor B was attributed to the growth of heterotrophs. Contrary to reactor A, reactor B was fed with readily available organic carbon, which possibly enhanced the growth of heterotrophs, releasing sON because of substrate oxidation [41]. Also, the availability of ammonia supported nitrification further releasing sON from autotrophic growth in the reactor. The higher net production of sON in reactor B compared to reactor A is mainly due to the heterotrophic growth in reactor B, agreeing that heterotrophs are much faster growers than autotrophs.

sON concentration after 2.5 h of operation declined considerably suggesting that ammonification of sON trumped sON production in reactor B. The depletion of readily available ammonia in the reactor

(after 2.5 h) triggered ammonification of the available sON (to generate ammonia for growth). Since the externally added ammonia was the primary source of nitrogen for the bacteria, after depletion, ammonia was generated from the ammonification of the available sON in the reactor. The ammonia generated from ammonification of available sON is assumed to be rapidly nitrified; hence, no increase in the ammonia concentration was observed in the reactor after 2.5 h of operation (Fig. 2). After its decline, the sON concentration did not vary much in the last hour of operating reactor B. Overall, between 0 (0.57 mg sON/L) and 4.5 h (2.15 mg sON/L) for reactor B, a net production of 1.58 mg/L of sON was observed, which was quite close to that observed in reactor A (1.64 mg sON/L).

The net concentration of the sON in each reactor suggests that irrespective of the presence of an organic substrate in the reactor, the MBBR will be contributing sON to the reactor during the nitrification process. However, unlike the batch setup in this objective, in full-scale nitrifying MBBRs, both organic carbon (a small fraction of biodegradable carbon) and ammonia (a significant fraction of TN) are continuously fed. Therefore, there is never really a dearth of those nutrients in the reactor, which suggests that the production of sON may dominate over ammonification of sON, which is less needed when ammonia is always available [7,10]. Besides being low in concentration, the organic carbon in the MBBR influent is mostly recalcitrant in nature. It indicates that heterotrophic growth will not be significantly enhanced under such an environment, resulting in reduced ammonification of sON. Moreover, sON activity is expected to vary with changes in operating and/or substrate conditions. For example, the sON production may increase if COD is not removed well upstream due to organic loading increase and/or inhibition of the biological organic carbon process. Although the final sON concentrations in the two reactors (A and B) were close, they might

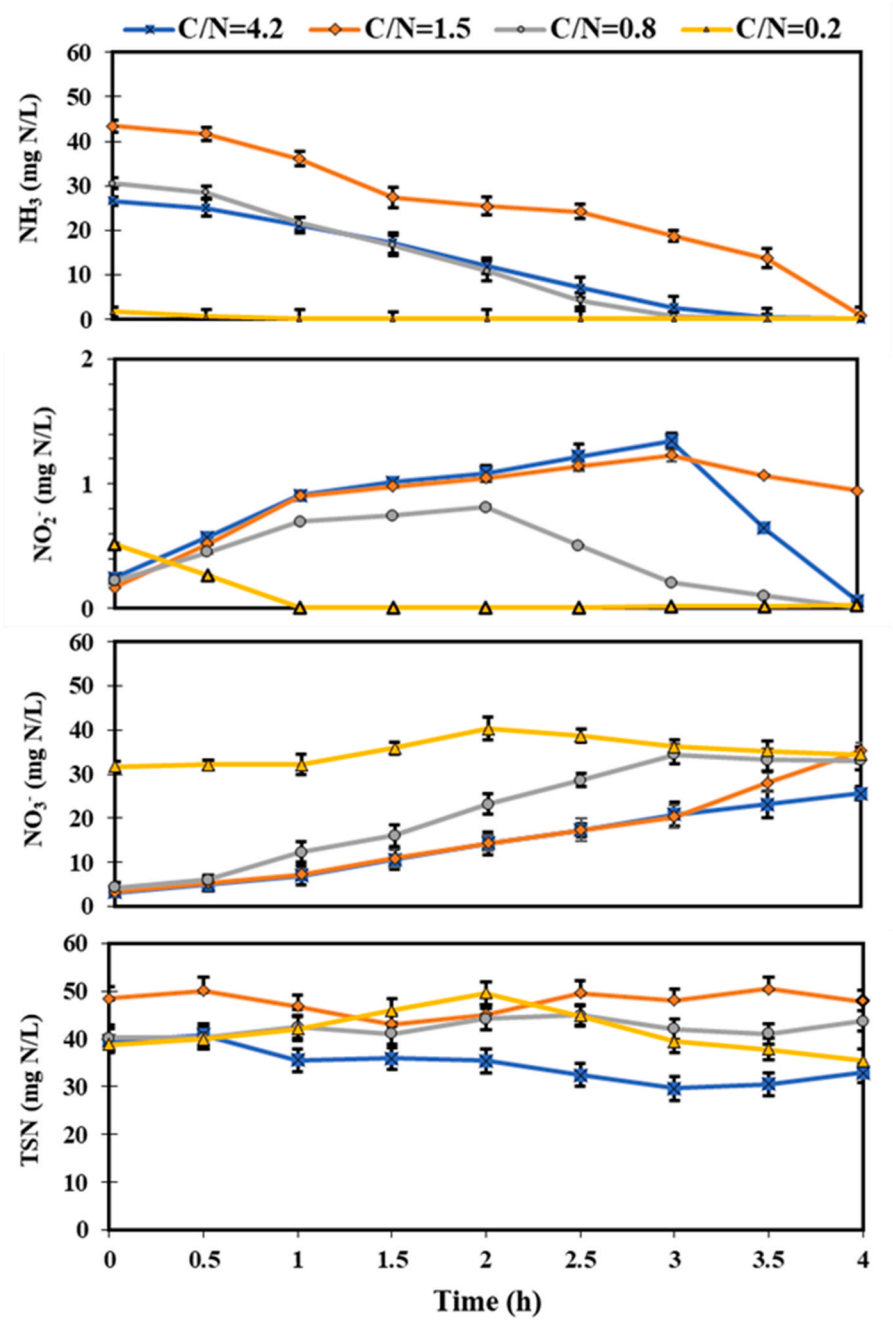

Fig. 4. Nitrogen profile of bench-scale MBBRs when operated under different C/N ratios.

fluctuate if the reactors were further operated due to the difference in the microbial activity occurring within each reactor that governed the organic nitrogen cycling. The findings from this objective display a strong dependence between substrate concentrations (organic carbon and ammonia) and bacterial activity, and these two related factors firmly influence the production or ammonification of sON in a nitrifying MBBR.

3.2. Effect of C/N ratios on sON degradation

3.2.1. Nitrification and sON profile

Fig. 4 displays the nitrogen profiles of the four reactors when fed with real wastewater samples representing C/N ratios of 4.2, 1.5, 0.8 and 0.2. Unlike the previous section, which focused on the production of sON using SWW, this part of the work focused on sON ammonification by using real wastewater samples. After 4 h of operation, ammonia in each reactor was successfully nitrified to below the detection limit. Consistent nitritation and nitratation were observed in the reactors with C/N ratios 4.2, 1.5 and 0.8; however, in the reactor with a C/N ratio of 0.2, after 2 h of operation, ammonia concentration increased from 0.07 to 0.25 mg NH_3-N/L possibly due to ammonification of sON and nitrate concentration decreased from 40.33 to 34.39 mg NO_3-N/L suggesting reduced nitrification. Considering the extremely low initial concentration of ammonia in the reactor (1.66 mg NH_3-N/L), the nitrification rate (0.66 mg NO_3^--N/L/hr) was expected to be lower than those observed in the other reactors. The C/N ratio of 0.2 is based on the final effluent sample in which the majority of ammonia has already been removed. Hence, the low concentration of ammonia likely limited the nitrification rate at the C/N ratio of 0.2.

The nitrification rate at the highest C/N ratio of 4.2 was lower (5.62 mg NO_3^--N/L/hr) than those observed at the C/N ratios of 0.8 (7.18 mg NO_3^--N/L/hr) and 1.5 (7.98 mg NO_3^--N/L/hr). This finding of lower nitrification rate at the highest C/N ratio (Fig. 4c) aligns with the findings from previous studies wherein similar results were reported while using different methods, including biofilters, activated sludge system, MBBRs with different types of carriers [24,42,43]. The C/N ratio of 4.2, besides offering the highest concentrations of organic carbon and ammonia, represents a large pool of bioavailable nutrients, which are expected to enhance the heterotrophic growth substantially. This pool of bioavailable nutrients decreases with decreasing C/N ratios and is expected to affect the bacterial population and activity in each reactor.

The nitrification process is significantly influenced by organic carbon concentration in the reactor [44,45]. Since the outer layers of biofilm are primarily inhabited by heterotrophs, increasing the organic carbon load in the bioreactor further decreases nitrification rate [46]. Whenever influent organic carbon load was increased in an oxic bioreactor, nitrifiers were mostly found in the inner layers of a biofilm wherein only limited oxygen is available for the bacteria to thrive [47]. In this study, the increasing C/N ratio represented an increasing concentration of organic carbon and its bioavailability and hence, enhanced heterotrophic growth in the reactors. The domination of heterotrophs took away the available space and dissolved oxygen for the nitrifiers, thus reducing the nitrification rate in each reactor. Therefore, at the highest C/N ratio (4.2), a reduced nitrification rate was observed. Although nitrification was successfully achieved in each reactor, the rates were highly influenced by the concentration and availability of organic carbon.

The nitrification rate results observed in this part of the work (objective 2) shown in Fig. 4 conflict with those observed in objective 1 (Fig. 2), i.e., a relatively higher nitrification rate was observed in the presence of externally added organic carbon (8.48 mg NO_3^--N/L/hr) than that in the reactor that was not fed with any external carbon source (8.33 mg NO_3^--N/L/hr). Since the reactors in objective 1 were fed with SWW instead of real wastewater, the difference between the characteristics of the two carbon sources may lead to this contrast. The increased nitrification rate in the presence of higher organic loading was also reported by another study that used SWW containing readily available carbon (Casein peptone, meat extract, and urea) [23].

The C/N ratios tested (4.2, 1.5, 0.8, and 0.2) varied because of the sCOD concentrations rather than the TSN concentrations. The decreasing trend reflects the change in sCOD concentration after going through various treatment processes at the MWWTP. The concentration of sCOD changed drastically compared to the concentration of TSN, which was relatively stable but consisted of different nitrogen fractions. Not only the level of COD decreased throughout the treatment train, but also its biodegradability decreased (or its biorecalcitrance increased) after the activated sludge and MBBR (C/N = 0.8 and 0.2). Therefore, at C/N = 0.2, which represents the MBBR effluent, sCOD, besides being lowest in concentration, was the most biorecalcitrant. Regarding the changes in N fractions, organic nitrogen and ammonia dominated the influent (C/N = 4.2) while ammonia concentration dominated after the activated sludge (C/N = 0.8), and nitrate was the major fraction of the MBBR effluent (C/N = 0.2).

Unlike the sCOD/TSN ratio, wherein the variation is primarily attributed to change in sCOD concentrations, the corresponding sCOD/sON ratio reflects the variations contributed by both sCOD and sON concentrations. The sCOD/sON ratios (15.7, 47.3, 6.6, and 1.6), besides being higher than the sCOD/TSN ratios, showcase the influence of sON fraction on the ratio. An overall decreasing trend is observed in the sCOD/sON ratios. However, the significant jump in the ratio (15.7–47.3) indicates the ammonification of biodegradable sON prevailing in the primary clarifier. The decreasing ratios (15.7, 6.6, and 1.6) reflect the production and slower degradation of sON concentrations which is assumed to be less biodegradable after the major ammonification process observed at the sCOD/sON ratio of 47.3. Unlike other biological processes, ammonification is more spontaneous and can exist under both oxic and anoxic conditions [48]. For instance, ammonification takes place faster than nitrification in terms of kinetics because nitrifying bacteria have relatively slow growth rates and a small acceptable pH-range [49,50]. The majority of organic nitrogen before the ASP is urea which is readily oxidized to ammonium to derive metabolically useful energy by the bacteria [14,51]. Therefore, substantial ammonification in the primary clarifier is not uncommon.

This study focuses primarily on four biological processes, i.e., heterotrophic growth, heterotrophic decay, nitrification, and ammonification, in terms of their involvements in regulating the sON activity. The sON profile observed under different C/N ratios is displayed in Fig. 5. Altogether, in reactors with the C/N ratios of 4.2 and 0.2, net removal of 3.9 and 4.1 mg sON/L was observed, respectively, whereas, in the reactors with the C/N ratios of 1.5 and 0.8, net production of 5.6 and 8.7 mg sON/L was observed, respectively.

Overall, the production of sON in the batch reactors with C/N ratios of 1.5 and 0.8 was associated with carbon oxidation and nitrification in the reactors. Although the initial availability of organic carbon, organic nitrogen, and ammonia fluctuated within the two reactors, active metabolic activity (carbon oxidation and nitrification) due to the presence of readily available nutrients results in the net production of sON. Besides, ammonification also coexisted in the reactor but was less dominant. Unlike the reactor with the C/N ratio of 1.5, wherein mainly production dominated the reactor throughout the operation period, in the reactor with the C/N ratio of 0.8, ammonification did overtake the sON production briefly (2.5–3 h), causing a decrease in sON concentration. For the C/N ratio of 0.8, between 2.5 and 3.0 h, ammonification surpassed the production of sON due to the significant depletion of ammonia in the reactor. Thereafter, sON concentration increased in the last hour of operation; however, there was no increase in ammonia concentration (from ammonification). Therefore, nitrification could not have enhanced the sON concentration in the last hour. Lack of both ammonia and organic carbon likely stimulated endogenous respiration of both autotrophs and heterotrophs, thus releasing sON in the reactor [21,52]. Hence, the net production of sON was observed in the reactor with the C/N ratio of 0.8.

The net removal of sON concentration observed in the reactors at C/

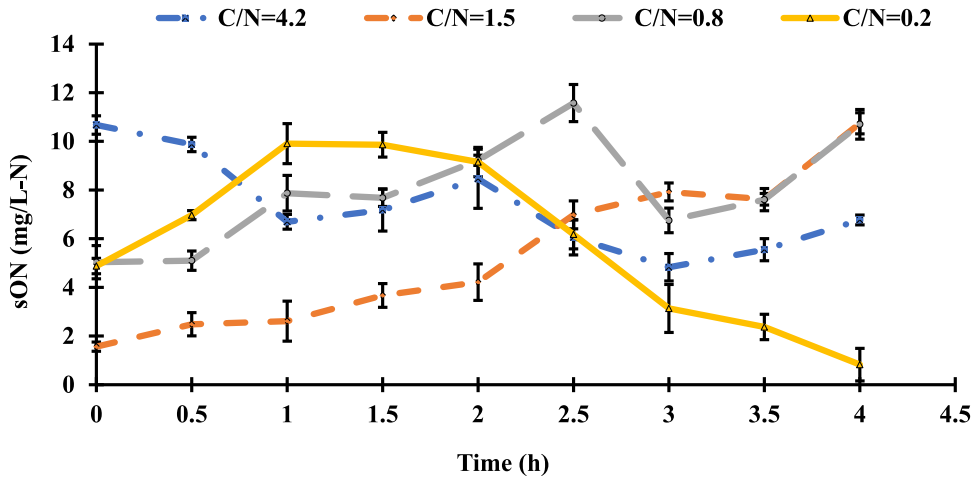

Fig. 5. Soluble organic nitrogen profile of bench-scale MBBRs when operated under different C/N ratios.

N ratios of 4.2 and 0.2 was attributed to the dominance of ammonification over the production of sON overall in each reactor. However, ammonification observed in each reactor was attributed to different reasons. The wastewater sample contributing a C/N ratio of 4.2 provided a large concentration of organic carbon, which must have enhanced the heterotrophic growth in the reactor, consequently resulting in accelerated ammonification of readily available sON (contributed by the sample), predominantly by the heterotrophs. Therefore, with the accelerated growth of the heterotrophs, ammonification of available sON must have dominated over nitrification resulting in higher removal of sON than production. On the contrary, a decline in sON concentration in the reactor with the C/N ratio of 0.2 was attributed to ammonification (of produced sON and sON contributed by the sample) due to ammonia depletion in the reactor. Since biodegradable organic carbon concentration was extremely low, no significant heterotrophic growth was expected, further supporting the dominance of ammonification over the production of sON from heterotrophic growth. The decrease in ATP concentration of the biofilm after 4 h of reactor operation further supports the reduced growth of heterotrophs at C/N ratio of 0.2. More information on the ATP results under each C/N ratio can be found under subsection 3.2.2. Although ammonification dominated the reactor for 3 h (t = 1–4 h), sON production also coexisted in the reactor, which was attributed to endogenous respiration of the biomass due to the lack of readily available nutrients in the reactor, unlike those contributed by the reactor with the C/N ratio of 4.2.

The ammonification of sON in the presence of ammonia in the reactors with the C/N ratios of 4.2 and 0.2 suggests that ammonification was not solely influenced by the absence or depletion of ammonia, as observed under the first objective. The presence of biodegradable organic carbon also governed the ammonification process. Unlike in the first objective wherein sON was biologically produced, in this objective, in the reactor with the C/N ratio of 4.2, sON was influent-derived. Studies showed that influent sON is highly biodegradable (> 80%), whereas biologically produced sON is less biodegradable (up to 60%) [13,15,21,51]. In this study, sON was ammonified irrespective of its source and the presence of ammonia.

For this objective, the variations in sON concentration in each reactor highlight the combined roles of C/N ratio, absolute concentrations of organic carbon and nitrogen, and nutrient biodegradability in influencing the sON activity. The observations suggest that a nitrifying MBBR can degrade more sON when fed with influent containing a low C/N ratio. Biomass in a nitrifying biofilm includes both heterotrophs and nitrifiers [53,54]. Heterotrophs require organic carbon and organic nitrogen for growth, whereas nitrifiers need inorganic nitrogen (ammonia) and inorganic carbon (alkalinity) [27]. Under the low C/N ratio, most of the organic carbon was recalcitrant; hence, heterotrophs targetted sON for ammonification (to obtain nitrogen and carbon via hydrolysis), whereas nitrifiers rapidly nitrified the available ammonia, eventually causing ammonification of available/produced sON (to obtain ammonia). Therefore, ammonification in the reactor was responsible for the higher degradation of sON under the lowest C/N ratio of 0.2.

3.2.2. ATP analysis

To evaluate the effect of batch-fed C/N ratio on the biofilm of a nitrifying MBBR, the ATP assay was used to assess the bacterial activity of the biofilm extracted from the carriers at the start and the end of the MBBR operation (Fig. 6). The ATP concentration measured immediately after starting the operation (t = 0 h) in each reactor was similar because the biofilm carriers were collected from the same basin and at the same time for each run. After 4 h of operation, the ATP concentration increased in the reactors with C/N ratios of 4.2 and 1.5 whereas decreased concentrations were observed in the reactors with C/N ratios of 0.8 and 0.2. As mentioned earlier, the decreasing C/N ratios represent the decreasing concentration and biodegradability of organic carbon along with fluctuations in the inorganic and organic nitrogen fractions depending on the treatment stage in the WRRF from where the wastewater sample was collected. The wastewater samples representing different C/N ratios were exposed to the attached-nitrifying biofilm. The biofilm carriers were collected from the nitrification basin that operates at a low C/N ratio.

Unlike the lower C/N ratios (0.8 and 0.2), as expected, the higher C/N ratios (4.2 and 1.5) seemed to enhance more heterotrophic activity in the biofilm due to the higher bioavailability of organic carbon as a substrate, thus enhancing the microbial activity in these two reactors. The increased ATP concentration in the reactor with the C/N ratio of 4.2 also supports the enhanced ammonification of initial sON as observed in the first hour of reactor operation (Fig. 5). The ammonification in the reactor was attributed to amplified heterotrophic growth in the reactor due to the presence of readily biodegradable organic carbon. Upon comparing the ATP concentrations after 4 h of operation in each reactor, a decreasing trend was observed, suggesting that the influent C/N ratio influenced the microbial activity of the nitrifying MBBRs. Nogueira et al. [55] reported that during the nitrification process, the influent C/N ratio could influence both utilization of oxygen and carbon and the distribution of heterotrophic and nitrifying population within the biofilm layers. The decrease in ATP concentrations with decreasing C/N ratio observed in this study aligns with the results from other studies [23,56,57]. Therefore, the influent C/N ratio, along with independent characteristics (concentration and bioavailability) of carbon and nitrogen, can influence the microbial activity in the biofilms of a nitrifying MBBR, which possibly regulates the sON activity in the bulk phase as well.

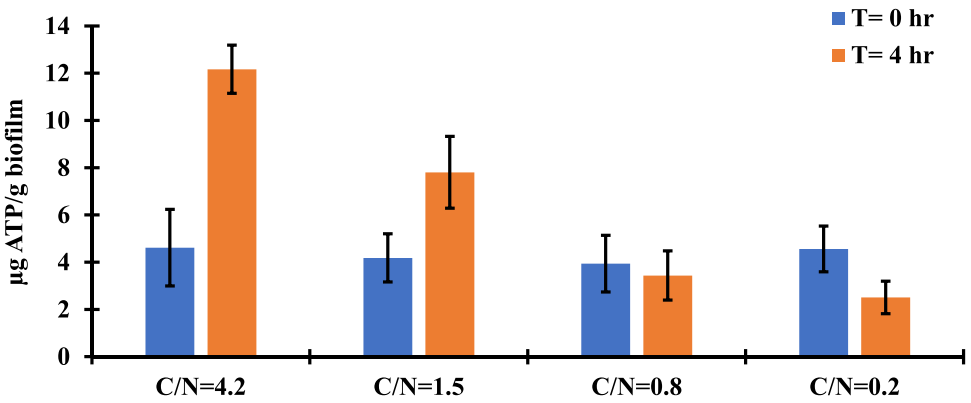

Fig. 6. Change in ATP concentrations measured in the biofilm from reactors operated under different C/N ratios.

3.3. Implications and future perspectives

Based on the findings, the nitrifying biofilms contributed sON regardless of the presence of influent carbon, while the contribution was relatively less when influent carbon was not present. WRRFs with carbon removal and nitrification in the same reactor deal with a high influent C/N ratio. Therefore, removal of sON will require additional air and adequate HRT and SRT for the hydrolysis and ammonification of sON and subsequent nitrification. The additional air requirement will increase the operational cost, which is not economically desirable. The findings also showed higher degradation of sON by the nitrifying biofilms when fed with influent containing a lower C/N ratio. It is therefore beneficial from the sON removal standpoint to separate between organic carbon removal and nitrification stages. A carbon removal reactor prior to nitrification will help lower the C/N ratio, thus enhancing the removal of sON by the nitrifying biofilms. Although the separate-stage configuration is more effective in minimizing effluent sON, it requires additional investments for the construction, and operation and maintenance, which may not be economically feasible, especially for municipal WRRFs operating on limited resources. With increasing eutrophication cases, more WRRFs will likely be regulated for TN, resulting in more wastewater treatment technology upgrades and thus more expenditure.

This study identified that the influent C/N ratio influenced sON degradation by nitrifying biofilm. However, the effect of C/N ratio on the degradation of AbsON by nitrifying biofilms is unknown considering that AbsON stimulates algal growth in the receiving water bodies and affects the nitrogen cycling [58,59]. Previous studies have shown that AbsON concentration decreased with increasing C/N ratio in post-denitrification biofilters [11] and that the formation of AbsON was significantly influenced by microbial activity and microbial community structure [60]. Therefore, the chemical composition and molecular weight distribution of sON should be investigated to better understand the effect of different C/N ratios on effluent AbsON from nitrifying biofilm processes. The chemical composition of sON can be analyzed via advanced analytical techniques such as Fourier-transform ion cyclotron resonance mass spectrometer (FTICR-MS) whereas sON size fractionation can be conducted using membrane filters with different molecular weight cutoffs. Based on the molecular composition, sON molecules can be identified as bioavailable (aliphatic compounds such as proteins or amino acids) and refractory (aromatic compounds such as lignins). Eom et al. [1] reported that sON molecules >1 kDa are potentially less bioavailable unlike smaller sON molecules which are more bioavailable and difficult to remove via advanced treatment processes and hence end up in the receiving water bodies.

This study investigated nitrifying biofilm attached to a specific carrier type (high-density polyethylene (HDPE)-ActiveCell450). A previous study reported that the type of biofilm-carrier affected the quantity and distribution of attached biofilm, which influenced the activity of specific microbial groups in the biofilm [25]. Therefore, the sON activities observed under different C/N ratios in this study may differ when different biofilm-carrier types are used. Future work should examine the biofilms to identify and characterize the microbial community and enzymes involved in sON production and ammonification under different C/N ratios using different types of biofilm carriers. Molecular biology tools such as the next generation sequencing and real-time polymerase chain reaction can be used to identify the presence and expressions of genes involved in the production and removal of sON under different C/N ratios and biofilm carrier-types.

4. Conclusions

This study investigated sON activity in batch nitrifying reactors mimicking MBBRs. The production of sON was examined by feeding SWW (no organic nitrogen), whereas sON degradation was analyzed by feeding actual wastewater samples with different C/N ratios. The study also identified the variation in the activity of the biofilm during nitrification when exposed to different C/N ratios in the influent. This is the first study demonstrating that influent organic carbon concentration influences the production of sON in a nitrifying biofilm reactor. The batch nitrifying biofilm reactors fed with readily biodegradable organic carbon generated a higher concentration of process-derived sON, which may contribute to effluent TN concentration in the absence of an additional treatment process. For the effect of C/N ratio on sON degradation, operating a nitrifying biofilm reactor at a C/N ratio of 0.2 degraded more sON than the other C/N ratios tested. At the higher C/N ratios of 0.8 and 1.5, the production of sON was higher than the degradation, whereas, at the C/N ratio of 4.2, sON degradation was limited. In addition, higher microbial activities of the nitrifying biofilm were observed when fed with influent containing higher C/N ratios. Overall, this study suggests that MBBR, which is known for its ability to provide high nitrification efficiency, can be beneficial in minimizing effluent sON when operated under a lower C/N ratio.

CRediT authorship contribution statement

Ruchi Joshi: Methodology, Formal analysis, Investigation, Data curation, Writing - original draft. **Murthy Kasi:** Conceptualization, Writing - review & editing. **Tanush Wadhawan:** Conceptualization, Writing - review & editing. **Eakalak Khan:** Conceptualization, Project administration, Supervision, Validation, Writing - review & editing.

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. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jece.2021.105440.

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