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Sequential Nitrification-denitrification Process using Biofilter Reactor for the Removal of Chemical Oxygen Demand and Nitrogen for Tofu Wastewater Treatment

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This study aims to optimize the sequential performance of a nitrification-denitrification (ND) biofilter reactor for nitrogen removal from tofu industry wastewater. The study also supports the Indonesian government's policy to achieve the Sustainable Development Goals through the implementation of sustainable water management in Indonesia. Seeding and acclimatization were conducted at hydraulic retention times (HRTs) of 24, 12, and 6 hours. During nitrification, removal efficiency improved with increasing substrate concentration at a 24-hour HRT. At 100% substrate concentration (915 mg/L), chemical oxygen demand (COD) removal increased until day 19, reaching a maximum of 77.95% at pH 7.3. The maximum ammonia nitrogen (NH_3–N) removal was 86.95% at pH 7.3. However, COD removal efficiency dropped below 70% when the HRT was reduced to 12 and 6 hours at both 50% and 100% substrate concentrations. At a 24-hour HRT, the denitrification reactor achieved 83.25% NH3–N and 67.55% COD removal, while nitrate removal reached 90.75%. Proper HRT was directly related to nitrate removal efficiency, and higher influent nitrate concentrations tended to increase the denitrification rate. The highest nitrate removal efficiency (90.75%) occurred at a 24-hour HRT and pH 7.8.

Keywords: sequential, nitrification-denitrification, reactor, Tofu wastewater.

Introduction

Tofu wastewater contains high protein levels that readily decompose into organic nitrogen. In Medan Deli Serdang, Indonesia, tofu industry effluent typically exhibits chemical oxygen demand (COD) ~ 60,000 mg/L, ammonia nitrogen (NH_3–N) ~ 215 mg/L, and pH 4–5 (Batubara et al., 2023; Yanqoritha et al., 2018; Yanqoritha et al., 2024). Therefore, untreated tofu wastewater poses a significant risk of pollution if discharged directly into water bodies. Ammonia from industrial effluent threatens aquatic ecosystems because it is toxic to aquatic organisms.

Ammonia is particularly harmful as it dissolves in water, forming ammonium (NH_4^+) (Effendi and Sandi, 2018), which contributes to eutrophication, toxic algal blooms, habitat degradation, hypoxia, and increased nitrous oxide emissions (Warneke et al., 2011; Zhou et al., 2011). High nitrate or nitrite exposure has been linked to methemoglobinemia in infants and the formation of carcinogenic nitrosamines (Torrentó et al., 2011; Wiesmann et al., 2006; Rodríguez et al., 2011).

Safe drinking water is critical for public health, and international guidelines set strict limits on nitrogen compounds. The World Health Organization (WHO) and the United States Environmental Protection Agency (EPA) recommend maximum concentrations of 50 mg/L for nitrate and 0.1 mg/L for nitrite. In line with these standards, Indonesia's Government Regulation No. 22/2021 specifies maximum concentrations of 0.5 mg/L for ammonium, 44.3 mg/L for nitrate, and 4.4 mg/L for nitrite (Anh et al., 2006; Kamarehie et al., 2018).

Several other bioreactor configurations for tofu wastewater have been explored beyond the upflow anaerobic sludge blanket (UASB) and hybrid upflow anaerobic sludge blanket (HUASB). Biofilter-assisted anaerobic baffled reactors (ABR) and anaerobic fixed-bed systems (e.g., bamboo carriers) have been reported to achieve high COD removal and methane-rich biogas, supporting energy recovery goals (Ningsih et al., 2024; Dewi et al., 2021). Large-scale multi-stage up-flow anaerobic fixed-bed reactors have also been demonstrated for tofu whey with robust COD removal during extended start-up operation; however, these systems are primarily designed for organic conversion and biogas production and may require long operational periods and additional polishing to meet stringent discharge targets (Sintawardani et al., 2025). Hybrid anaerobic-aerobic attached-growth systems using bioball media can enhance organic reduction; nevertheless, removal efficiency tends to decline as hydraulic retention time (HRT) shortens, and the effluent may still exceed discharge standards, indicating the need for further treatment (Astuti and Ayu, 2019). Furthermore, nitrogen management can be challenging in biofilm systems, where instability and shifts in nitrate reduction pathways (e.g., dissimilatory nitrate reduction to ammonium (DNRA)-related behavior) have been reported during tofu wastewater treatment (Shao et al., 2023). Aerobic biofilter nitrification can reduce ammonia, yet it may lead to nitrate accumulation unless followed by denitrification (Yanqoritha et al., 2024). Collectively, these studies highlight that post-treatment targeting nitrogen compounds is often required to meet stricter effluent criteria.

Nevertheless, effluents from HUASB reactors can still contain high residual COD (~ 915 mg/L) and NH_3–N (~ 215 mg/L), indicating that further treatment is required (Batubara et al., 2023). Anaerobic processes are generally limited in removing nitrogenous compounds, highlighting the need for post-treatment via nitrification and denitrification (Sousa et al., 2008; Tchobanoglous et al., 2014; Henze et al., 2008). Nitrification converts ammonium (NH_4^+) to nitrate (NO_3^-) via two steps: oxidation of ammonium to nitrite (NO_2^-) by *Nitrosomonas* and subsequent oxidation of nitrite to nitrate by *Nitrobacter*. Denitrification then reduces nitrate to gaseous nitrogen under anoxic conditions, using organic matter as an electron donor.

Accordingly, this study evaluates and optimizes a sequential nitrification-denitrification (ND) biofilter reactor using bioballs as attached-growth media as a targeted post-treatment for HUASB effluent, addressing the limited nitrogen removal of anaerobic systems. The effects of hydraulic retention time (HRT) and substrate concentration are examined to assess COD removal and nitrogen transformation/removal (NH_3–N and NO_3^-), aiming to produce an effluent that meets Indonesian discharge standards and supports sustainable water management aligned with the sustainable development goals (SDGs).

Methods

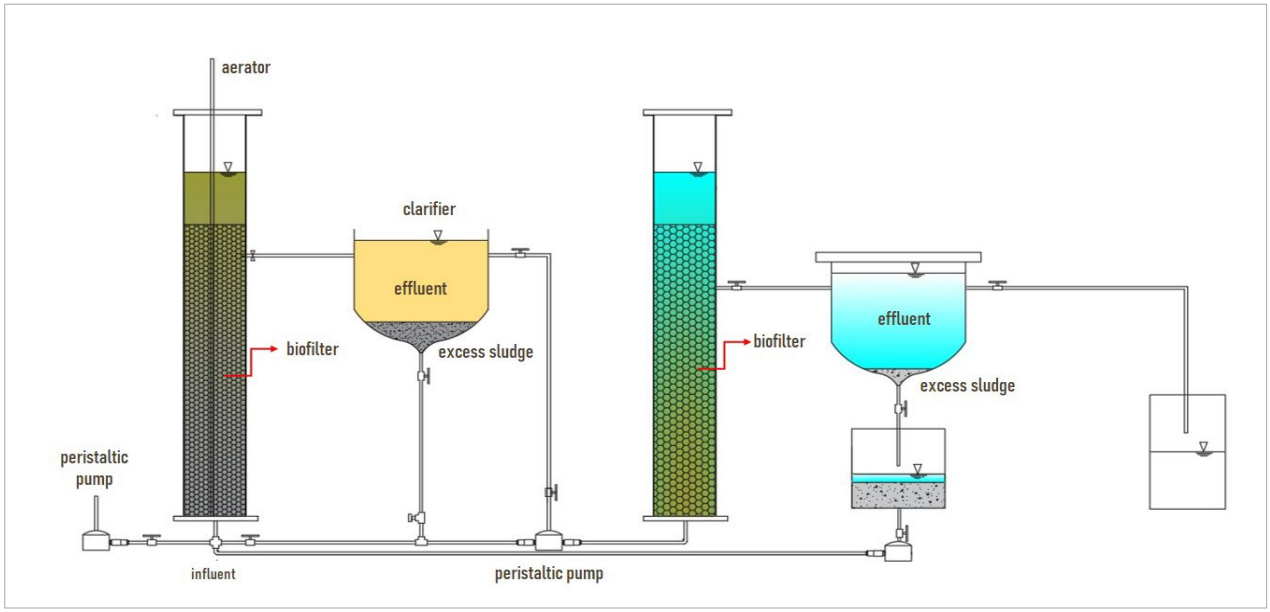
Nitrification-denitrification (ND) reactor

The ND reactor (*Fig.1*) was constructed from acrylic with a diameter of 12 cm. The nitrification unit had a working volume of 10 L and was packed with 169 bioballs as attached-growth biofilter media. The media consisted of black polypropylene (PP) bioballs with a spherical geometry (diameter 3.8 cm) and perforated/structured surfaces; the packed-bed void fraction (porosity) was ε = 0.92 (≈ 92%), providing an open structure favorable for biofilm attachment and stable hydraulics (Yanqoritha et al., 2024; Fatimah et al. 2024).

The nitrification reactor packing corresponded to an estimated media filling ratio of ~ 0.49 (≈ 49% v/v), calculated based on the gross geometric volume of the bioballs. Here, the media filling ratio (~ 49% v/v) refers to the fraction of reactor volume occupied by the gross (geometric) volume of the bioballs, whereas the void fraction (ε = 0.92) describes the openness of the packed bed (i.e., the fraction of bed volume available for water flow). A clarifier was installed downstream of the nitrification unit to serve as an intermediate collection/settling buffer prior to the denitrification stage. The denitrification reactor had a working volume of 8.75 L and was packed with 385 bioballs of the same type and packing characteristics.

Seeding was carried out by mixing sludge and water in a 1:10 ratio, followed by continuous aeration. During seeding and acclimatization, macronutrients were supplemented to prevent nutrient limitation, maintaining an approximate COD:N:P ratio of 100:5:1, to support biomass growth and biofilm development. Biofilm formation occurred within seven days. The acclimatization process started with a 50% substrate concentration and was gradually increased to 100%. The denitrification stage utilizes nitrate produced during the nitrification process as the electron acceptor. Higher influent nitrate concentrations can increase the denitrification rate; however, sufficient dissolved organic carbon must be available to sustain nitrate reduction (Ashok and Hait, 2015; Schipper et al., 2010). Biological denitrification was carried out by denitrifying microorganisms that use nitrate as the terminal electron acceptor and primarily rely on organic carbon (residual COD in the influent and endogenous carbon) as the electron donor; no external inorganic electron donor was added in this study (Lee et al., 2009; You et al., 2020). Anoxic biomass, with a concentration of about 5.5 g volatile suspended solids (VSS) per liter, was taken from the HUASB reactor process system (Wiesmann et al., 2006).

Fig. 1. *Schematic diagram of the nitrification-denitrification reactor*



Seed sludge and the substrate of the ND process

Before inoculation, the seeded sludge was washed with deionized water, and the reactor was confirmed to be oxygen-deficient. The denitrification influent concentrations were approximately NO_3^- = 103.25 mg/L, NH_3–N = 33.48 mg/L, and COD = 226.3 mg/L.

The denitrification reactor was fed directly with the nitrification effluent; therefore, nitrate and COD concentrations (and the resulting nitrate: COD or COD:NO_3^-–N relationship) were not independently adjusted and reflect the carbon availability provided by the upstream process. Residual NH_3–N in the denitrification influent was not intentional, but represents unconverted ammonia due to incomplete nitrification and/or ammonification of organic nitrogen, which is typical in practical post-treatment scenarios.

Reactor operation

The nitrification unit was operated under controlled conditions (pH 6–8.5, DO > 3 mg/L) (Bacta-Pur, 2012; Kutz, 2018; Okabe et al., 2014). After seeding, sequential acclimatization was conducted at different HRTs (24, 12, and 6 h) and substrate concentrations of 50% and 100% until stable operation was achieved. After acclimatization, the mixed liquor suspended solids (MLSS) concentration in the nitrification reactor was approximately 2500 mg/L. To promote anoxic conditions in the denitrification reactor, the nitrification effluent was deoxygenated prior to feeding by sparging pure nitrogen gas (N_2) at 0.5–2.0 L/min for 10–30 min (Di Capua et al., 2022). Pure N_2 sparging was applied as a laboratory-scale method to ensure an anoxic influent to the denitrification unit; large-scale implementation can rely on closed anoxic reactors and process-based DO consumption rather than continuous pure N2 dosing. After nitrification, the effluent was collected in the clarifier and transferred to the denitrification reactor without a dedicated holding period; the clarifier mainly served as an intermediate collection/settling buffer. Subsequently, the reactors were operated at the designated substrate concentrations and HRTs (24, 12, and 6 h) until stable performance was obtained. Denitrification was operated under anoxic conditions at pH 6–9 using the same HRT variations. Routine monitoring was performed daily for pH, COD, NH_3–N, and nitrate (NO_3^-), following the Standard Methods of the American Public Health Association (APHA) (Rice et al., 2012).

Results and Discussion

Process of the nitrification reactor

The results demonstrate that the sequential ND biofilter can function as a targeted post-treatment step for tofu wastewater, particularly under conditions that provide sufficient contact time for attached-growth activity. The reactor was then acclimatized under continuous influent feeding to promote biofilm attachment on the media and gradual adaptation to the tofu wastewater characteristics. Acclimatization was continued for approximately 60 days until stable removal trends were observed. During start-up, nitrification performance was evaluated under different HRTs (24, 12, and 6 h) and stepwise increases in substrate strength (from 50% to 100%). Aeration was supplied to maintain aerobic conditions necessary for ammonia oxidation, and the operating pH was maintained within 6–8.5 (Bacta-Pur, 2012; Kutz, 2018; Okabe et al., 2014). Based on the acclimatization results, the optimal condition was achieved at an HRT of 24 h with 100% substrate concentration; therefore, subsequent operation was conducted at 24 h using the actual substrate concentration.

Fig. 2 shows the COD removal profile under different HRTs and substrate concentrations. At 24 h HRT and 50% substrate concentration, COD removal increased progressively until day 6, reaching 70.15% at pH 6.8. When the substrate concentration was increased to 100% (915 mg/L), COD removal further improved, reaching a maximum of 77.95% on day 19 at pH 7.3. COD is a key parameter for assessing wastewater quality (Alewi et al., 2022; El Abdouni et al., 2021). These results suggest that the longer contact time at 24 h HRT enabled higher and more stable COD removal, consistent with improved organic matter biodegradation.

Similarly, *Fig. 3* presents NH_3–N removal under the same operating conditions. At 24 h HRT and 50% substrate concentration (107.5 mg/L), NH_3–N removal increased steadily to 83.15% by day 7 at pH 6.8. At 100% substrate concentration (215 mg/L), NH_3–N removal increased further and reached 86.95% by day 19 at pH 7.3. Taken together, *Figs. 2* and *3* indicate that the highest COD and NH_3–N removal efficiencies were achieved at 24 h HRT with 100% substrate concentration, highlighting the importance of sufficient HRT for acclimatization and stable nitrification performance.

Fig. 2. *COD removal at varying HRTs and substrate concentrations*

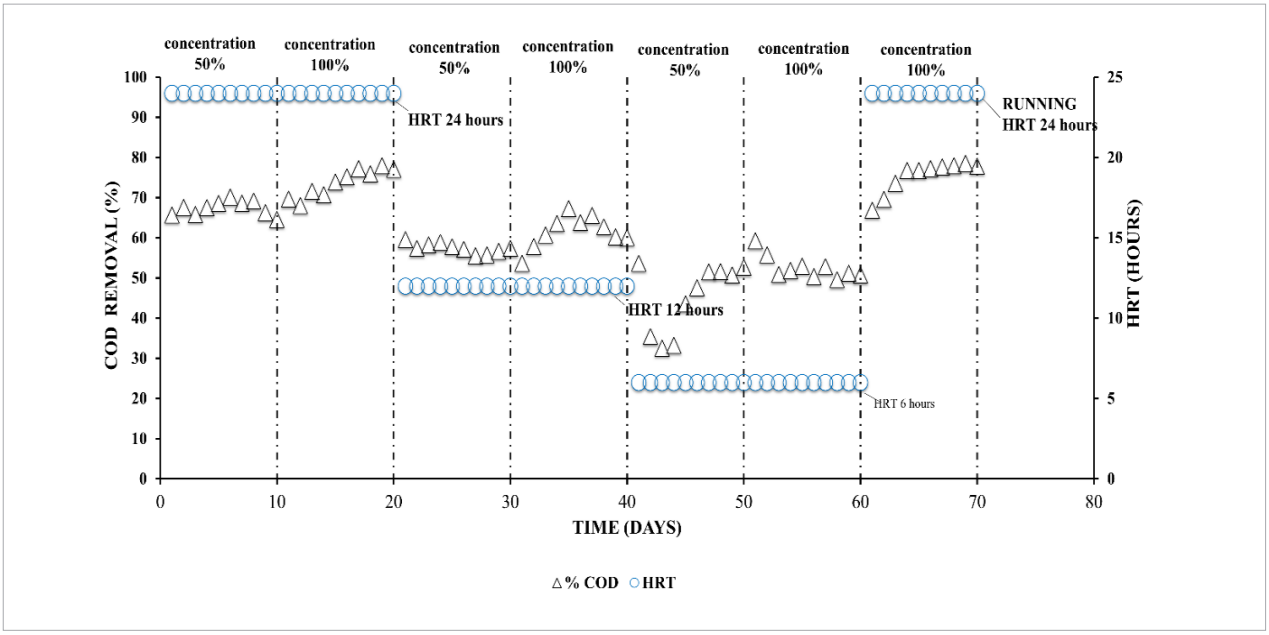
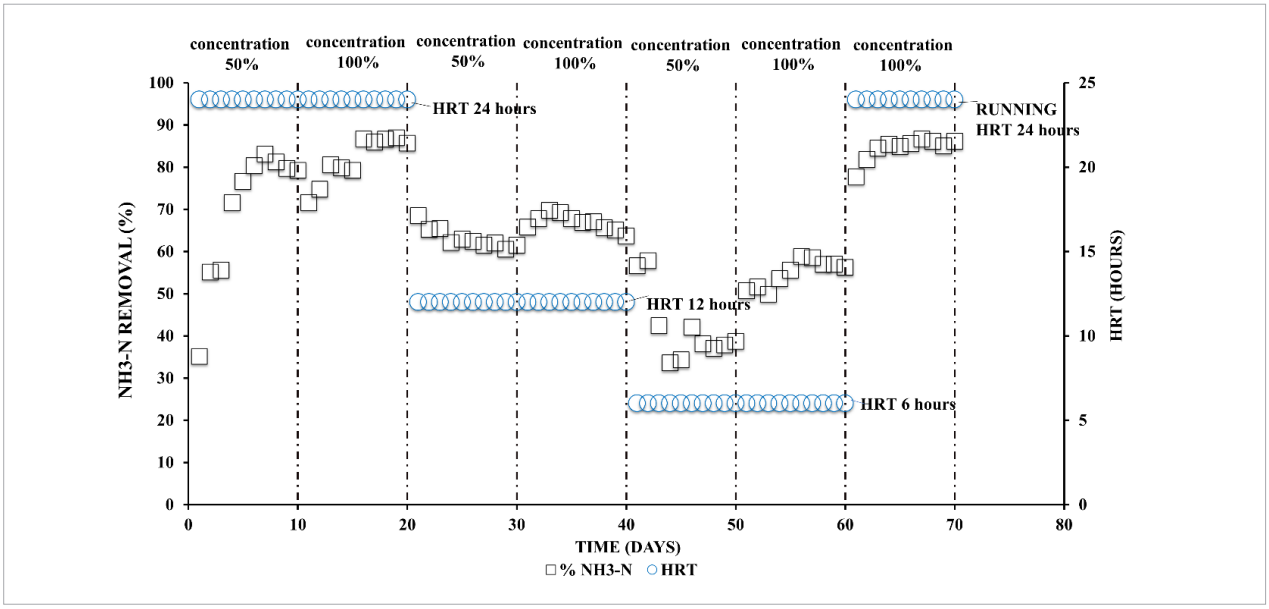


Fig. 3. *NH_3–N removal at varying HRTs and substrate concentrations*



The influence of HRT on removal performance across substrate concentrations is further illustrated in *Figures 4* and *5* for COD and NH_3–N, respectively. At 24 h HRT, removal efficiencies for both parameters were higher than those at 12 h and 6 h under both 50% and 100% substrate concentrations. This pattern indicates that shorter HRTs likely reduced effective contact time and biomass retention/accumulation, thereby limiting overall biological activity. Consequently, a longer HRT promoted biomass accumulation and microbial activity within the reactor, enhancing removal performance (Al-Dhawi et al., 2023; Birniwa et al., 2022; Patel and Kumar, 2016; Rios-Miguel et al., 2021). *Figs. 4* and *5* further show that

shortening the HRT from 24 h to 12 h and 6 h consistently reduced COD and NH_3–N removal across both substrate strengths, indicating insufficient contact time and lower effective biomass retention at higher hydraulic loading. This trend supports the selection of 24 h HRT as the operating condition to maintain stable aerobic oxidation performance during and after acclimatization.

Fig. 4. *The effect of HRT variation on substrate degradation and COD removal efficiency during seeding, acclimatization, and operation*

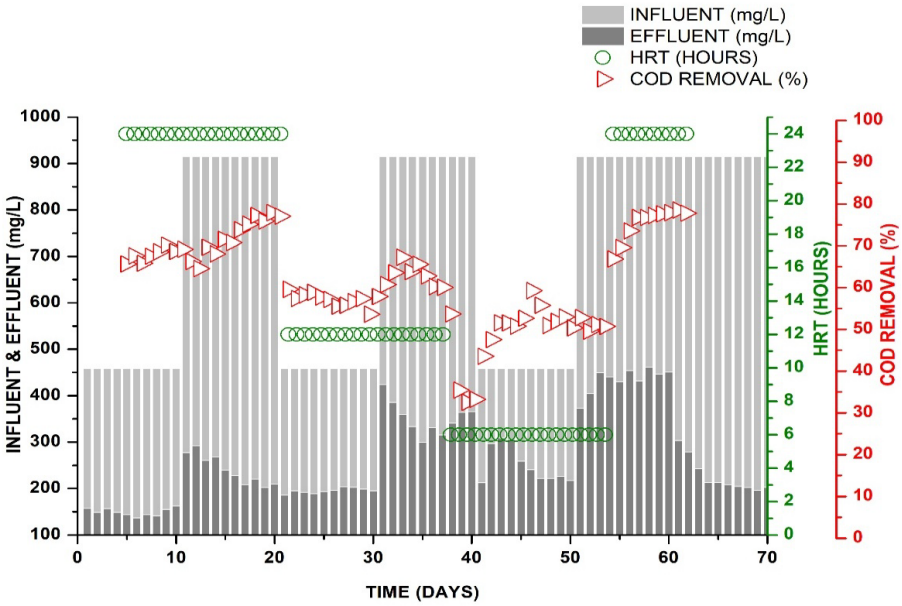
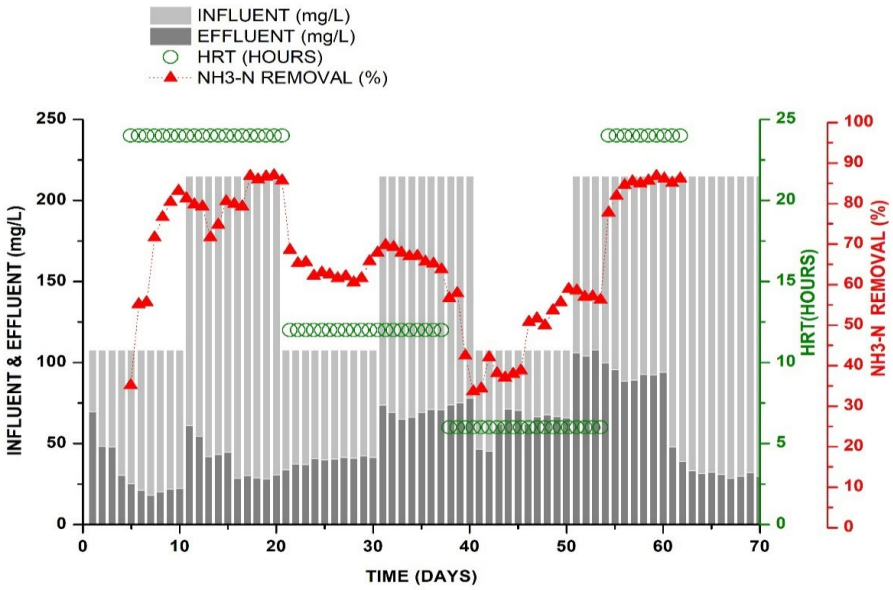


Fig. 5. *The effect of HRT variation on substrate waste removal, NH_3–N removal efficiency during seeding-acclimatization and running process*



The nitrification process employed an aerobic biofilter system with bioballs as a growth medium, which showed an increasing trend with increasing concentration. When treated at a 24-hour HRT, the COD removal rate increased by 77.95% on day 19. However, when HRT was lowered to 12 hours and 6 hours at 50% and 100% substrate concentration treatments, the removal efficiency rate tended to be low, only below 70% against COD removal (*Fig. 6*). This indicates that microorganisms adapted optimally at a 24-h HRT. This also occurs in the rate of NH_3–N removal efficiency (*Fig. 7*). The substrate concentration affects the C/N ratio, where a high C/N ratio inhibits the growth of nitrifiers, especially nitrite-oxidizing bacteria (Ge et al., 2015).

A low C/N ratio is beneficial for nitrite accumulation. Environmental pH has a significant influence on the life activities of microorganisms, so changes in pH have an impact on the nitrification process. The main role of pH is to cause changes in the cell charge of microorganisms, thereby affecting the absorption of nutrients by microorganisms and affecting enzyme activity (Ren et al., 2014). Microbial communities can have acid and alkali inhibition at pH. The highest ammonia removal efficiency was 86.95% at pH 7.3. In general, most heterotrophic nitrification-aerobic bacteria prefer neutral environments or slightly alkaline environments, and the optimal pH range is 6–9 (Zhang et al., 2012).

Fig. 6. *Rate of COD removal efficiency at varying concentrations*

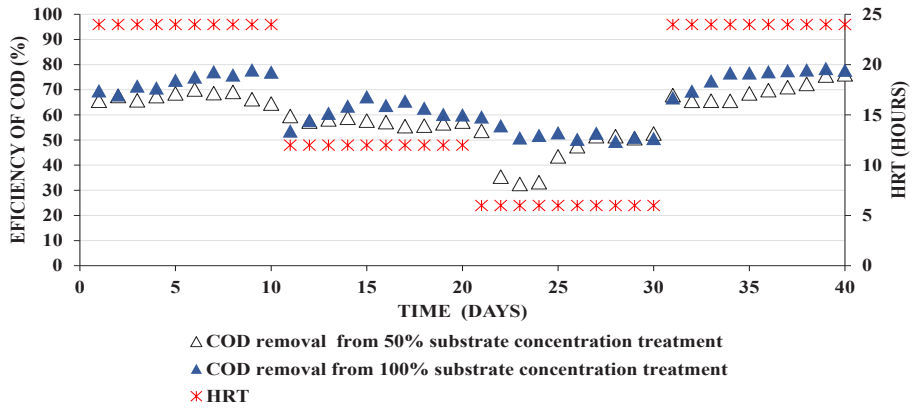
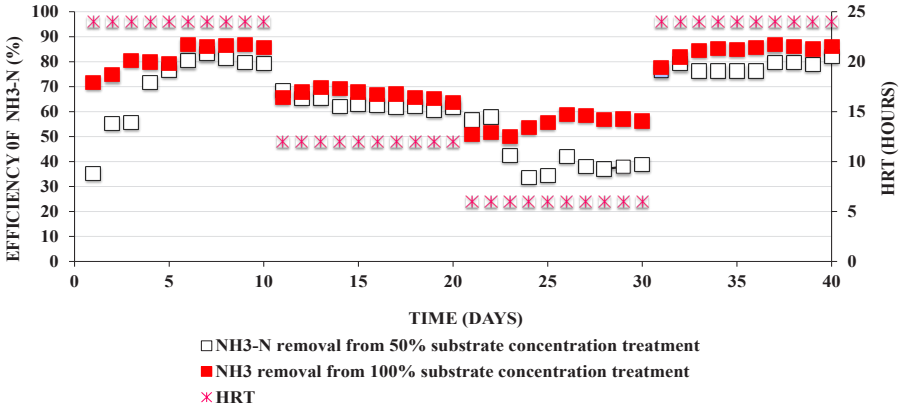


Fig. 7. Rate of NH_3-N removal efficiency at varying concentrations



NO_3–N concentrations increased from 55.06 mg/L in the influent to 103.25 mg/L, 85.55 mg/L, and 67.25 mg/L at HRTs of 24 h, 12 h, and 6 h, respectively. As an oxidative process, nitrification has a high oxygen demand; therefore, dissolved oxygen (DO) is a key factor in maintaining process stability, and pH also contributes to stable nitrification performance (Okabe et al., 2014). Accordingly, sufficiently high DO levels are required in activated sludge systems to sustain DO within flocs and support uniform nitrification activity. At low DO concentrations (< 0.5 mg/L), nitrification is inhibited. Low DO also tends to affect Nitrobacter more than Nitrosomonas, leading to incomplete nitrification and increased NO_2–N in the effluent (Kutz, 2018; Tchobanoglous et al., 2003).

Nitrification is also sensitive to pH (Henze et al., 2008; Kutz, 2018; Okabe et al., 2014; Tchobanoglous et.al, 2003). Removal performance decreases markedly at pH < 6.8, with an optimal pH range of approximately 7.3–8.0. Careful control of operating parameters such as pH and DO strongly influences removal performance and can promote nitrite accumulation within the system. After nitrification, the effluent contained 33.48 mg/L NH_3–N and elevated nitrate concentrations; therefore, a subsequent denitrification step is required.

Process of the denitrification reactor

The nitrification stage generated nitrate, which required further treatment to remove nitrogen from the effluent. Denitrification was therefore conducted in an anoxic attached-growth biofilter reactor. In this study, nitrate served as the terminal electron acceptor, while the reduction process was mainly supported by biodegradable organic carbon (residual COD in the nitrification effluent and endogenous carbon from biomass) as the electron donor; no external inorganic electron donor was applied. Through a series of enzymatic reactions, nitrate is sequentially reduced to gaseous nitrogen (mainly N_2) (Albina et al., 2019; Moloantoa et al., 2022). The denitrification reactor was initiated through seeding on the biofilter media, followed by sequential acclimatization under continuous feeding at HRTs of 24, 12, and 6 h.

COD and NH_3–N removal under the different HRT conditions are presented in *Figs 8.* and *9*, respectively. The highest removal efficiencies were achieved at an HRT of 24 h, with 83.25% NH_3–N removal and 67.55% COD removal. When the HRT was reduced to 12 h and 6 h, COD and NH_3–N removal efficiencies decreased to below 65% on average. HRT is closely associated with nitrate removal efficiency in denitrification reactors (Chu and Wang, 2013; Chu and Wang, 2016; Wang and Chu, 2016). Although higher influent nitrate concentrations can increase the denitrification rate, nitrate removal efficiency may decline when soluble carbon is insufficient relative to nitrate loading; conversely, low influent nitrate can lead to nitrate-limited denitrification (Ashok and Hait, 2015). The decrease in NH_3–N observed in the denitrification reactor likely did not result from denitrification itself, but rather from nitrogen assimilation into biomass and biofilm micro-zones that enabled simultaneous nitrification-denitrification (SND) due to residual DO and/or limited oxygen intrusion and diffusion into the outer biofilm layer.

Fig. 8. *Removal of COD in the denitrification process*

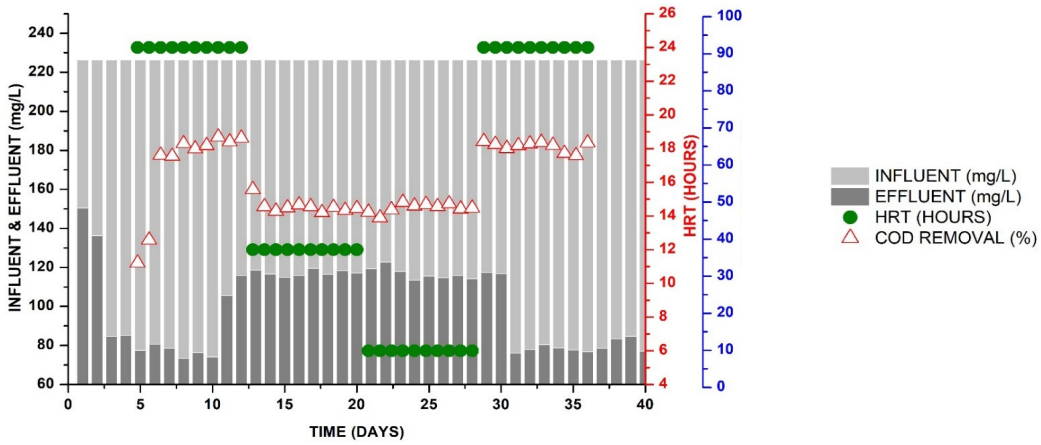


Fig. 9. *Removal of NH_3-N in the denitrification process*

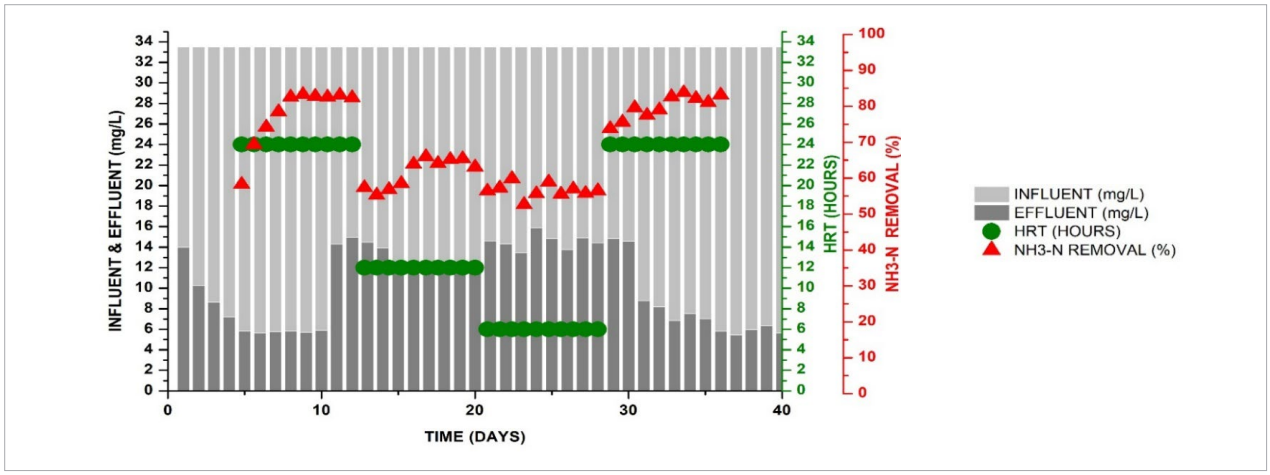
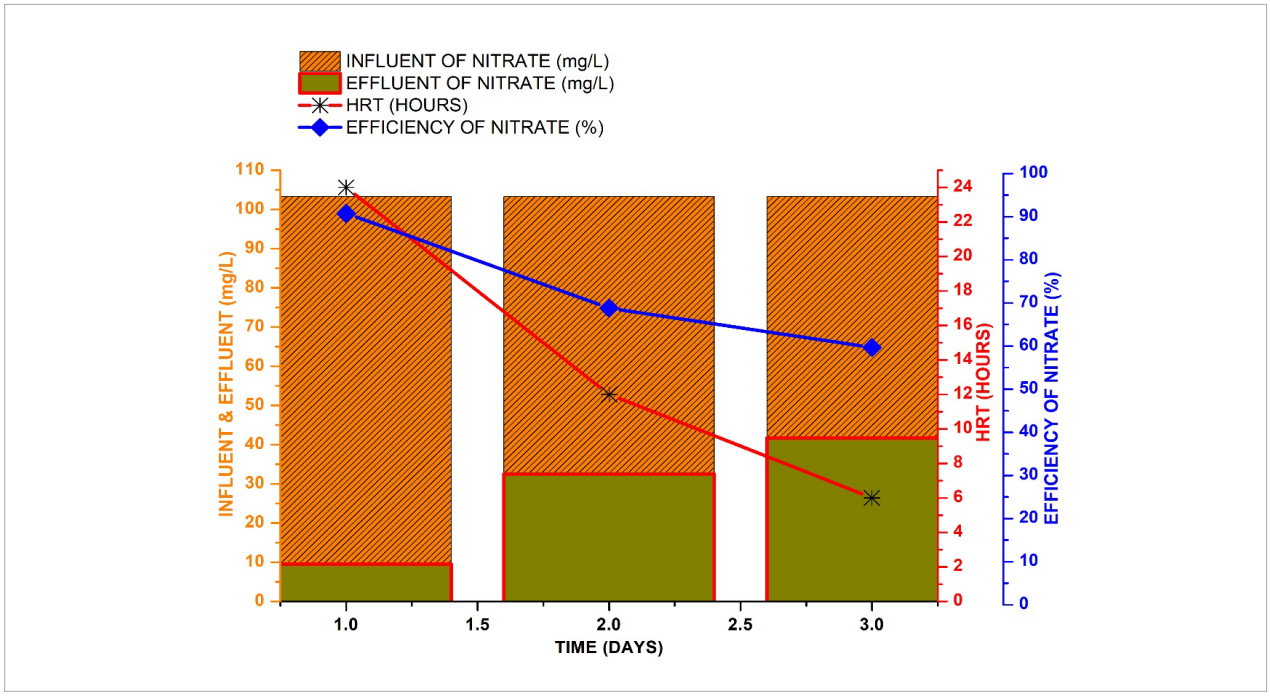


Fig.10 shows that the highest nitrate removal efficiency (90.75%) was achieved at an HRT of 24 h and pH 7.8. When the HRT was reduced to 12 h and 6 h, nitrate removal efficiency decreased to 68.81% and 59.66%, respectively. As HRT decreases, the hydraulic loading rate increases, meaning that more wastewater enters the bioreactor per unit time. This reduces contact time and limits interactions between substrates and microorganisms, which can lead to incomplete biodegradation (Saeed et al., 2021). Similar trends have been reported in the literature, where COD removal decreased as HRT was shortened from 12 h to 6 h (Nor Faekah et al., 2020). In a submerged membrane bioreactor (MBR), COD removal also declined from 97.2% to 91.1% with decreasing HRT (Kankia et al., 2021). In general, shorter HRTs can increase residual COD due to incomplete degradation of organic matter under higher hydraulic loading (APHA, 2012).

Fig. 10. *Rate of nitrate removal*



Although a longer HRT provides more time for bacterial populations to degrade organic substrates and can enhance nitrate removal efficiency, it may also lead to higher residual COD and ammonium in the effluent under certain operating conditions. Therefore, HRT should be optimized to achieve stable and efficient denitrification performance (Chu and Wang, 2016). Higher influent nitrate concentrations may increase the denitrification rate; however, nitrate removal efficiency can decline when soluble carbon is insufficient relative to nitrate loading. Conversely, low influent nitrate concentrations may result in nitrate-limited denitrification(Ashok and Hait, 2015; Schipper et al., 2010). pH is another key parameter influencing denitrification efficiency, as it affects biofilm activity and sludge characteristics (e.g., granulation and flocculation). Denitrifying activity typically occurs within a relatively narrow pH range, with an optimum close to pH 8.0 (Egli et al., 2001; You et al., 2020). Under strongly acidic (pH ≤ 6.5) or alkaline (pH ≥ 8.5) conditions, nitrogen removal performance may deteriorate, as reported for anammox-based systems (Bhuvanesh et al., 2013).

Conclusions

The ND biofilter reactor significantly improved the removal of COD, NH_3–N, and nitrate. The results obtained from this study are as follows:

1 Microorganism cultivation using an attached-growth biofilter system supports microbial growth and development, thereby influencing treatment performance by retaining microorganisms on the filter media. Nitrification was operated under specific conditions, namely pH 6–8.5 and DO > 3 mg/L. The optimal removal depended on the applied HRT and substrate strength; the influent C/N ratio (based on COD as C and nitrogen species as N) was determined by the wastewater composition and varied with substrate concentration, which in turn influenced biological performance. Environmental pH strongly influences microbial activity; therefore, changes in pH can directly affect the nitrification process.

2 The denitrification process used the nitrification effluent as the nitrate-containing influent. Higher influent nitrate concentrations can increase the denitrification rate, provided that sufficient soluble carbon is available. Biological denitrification was performed by denitrifying microorganisms that use nitrate as the terminal electron acceptor and primarily rely on organic carbon (residual COD) as the electron donor to sustain growth. Denitrification was initiated through seeding on the attached-growth biofilter media, followed by sequential acclimatization under continuous feeding at HRTs of 24, 12, and 6 h.

3 Nutrient supplementation (COD: N:P ≈ 100:5:1) was provided during seeding and acclimatization to promote biofilm development. Reactor performance was mainly influenced by HRT and substrate concentration, which together determine carbon availability relative to nitrogen, an important factor for both nitrification and denitrification. Under nitrification conditions, COD and NH_3–N removal reached 77.95% and 86.95%, respectively, at pH 7.3. Heterotrophic nitrifying aerobic bacteria generally perform best under neutral to slightly alkaline conditions (pH 6–9). During denitrification, nitrate removal depended on the influent COD/NO_3^-–N ratio; the relatively low ratio indicated potential carbon limitation, particularly at shorter HRTs.

4 The reactor used was an anoxic denitrification biofilter. COD and NH_3–N removal were evaluated under different HRT conditions. The optimal removal efficiencies were 83.25% for NH_3–N and 67.55% for COD at an HRT of 24 h. When the HRT was reduced to 12 h and 6 h, COD and NH_3–N removal efficiencies decreased, averaging below 65%. The highest nitrate removal efficiency (90.75%) was achieved at 24 h HRT and pH 7.8; when the HRT was lowered to 12 h and 6 h, nitrate removal efficiency decreased to 68.81% and 59.66%, respectively.

5 Despite the promising performance, the sequential ND biofilter has several drawbacks, including strong dependence on HRT (performance decreased at shorter HRTs), the need for strict operational control (aeration/DO for nitrification and anoxic conditions for denitrification), and potential carbon limitation for denitrification; packed-media operation may also require periodic maintenance to avoid biofilm overgrowth or clogging during long-term use.

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