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Optimizing mixing duration to achieve efficient nitrite accumulation and functional microbiome enrichment in mainstream partial denitrification

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ABSTRACT

Sufficient nitrite production through partial denitrification (PD) is essential for energy-effective nitrogen removal followed by anammox process. However, the underlying impacts of mixing duration of reaction cycle in long-term PD performance is still unclear. This study investigates the impacts of mixing duration on nitrite accumulation in a PD sequencing batch reactor (PD-SBR), by analyzing pH, functional microbiota and metabolic pathways. Results indicated that nitrite production was significantly affected by varying the mixing durations; the maximum nitrate to nitrite transformation ratio (NTR) of 43.30 % was obtained for mixing duration of 1.5 h. However, a deteriorated nitrite production was observed for mixing duration of 2 h, 1 h and 0.5 h with NTR values of 30 %, 36.90 % and 35.60 % respectively. Microbial community analysis revealed that relative abundance of *Azoarcus,* which is known to be capable of denitrification was significantly enhanced in all operational phases. *Thauera*, known for the high nitrite production, was only observed in the samples of 1.5 h and 1-h mixing duration; it showed highest relative abundance of 3.70 % for 1.5 h. Nitrate reduction was more pronounced metabolic pathway under all mixing conditions compared to fresh inoculum and was the most dominant under 1.5 h mixing. Furthermore, the elevated pH (8.30–9.07) played an important role to maintain excellent PD. Overall; this study demonstrated the significance of mixing duration of reaction cycle for robustness and stability of PD by altering the specific functional microbiota and metabolic pathways offering significant insightfulness for integration with anammox to achieve efficient nitrogen removal.

1. Introduction

Conventional biological nitrogen removal (BNR) technologies based on nitrification and denitrification, may face drawbacks for application in wastewater treatment plants (WWTPs) due to their high demand for oxygen supply, carbon sources and excessive sludge disposal which also increase carbon footprint of the plant [1]. Therefore, alternative settings based on anaerobic ammonium oxidation process or anammox have a great potential to reduce energy demand and carbon footprint in WWTP. The anammox process necessitates specific electron acceptors, predominantly nitrite (NO_2^-). Two major pathways which can lead to NO_2^- production in anammox based wastewater treatment bioreactors are partial nitrification (PN) ($NH_4^+ \rightarrow NO_2^-$) and partial denitrification (PD) ($NO_3^- \rightarrow NO_2^-$) [2–6]. Hence, two combinations are plausible: the combination of partial nitrification and anammox (PNA) or combination of partial denitrification and anammox (PDA) for complete nitrogen removal from wastewater. PNA approach is commonly used for ammonia-rich wastewaters but it is less effective in treating mainstream of domestic WWTPs which has lower ammonia concentrations due to struggling to control nitrite-oxidizing bacteria (NOB) [7–9]. Recently, PDA approach emerged as a more promising technology for nitrogen removal from wastewater [1,10–13]. This process has gained much attention for its unique ability to reduce oxygen supply, organic consumption, and activated sludge production as well as potential direct greenhouse gas (GHG) emissions due to N_2O compared to conventional BNR [14,15]. However, a critical factor for the efficiency of the PDA process is maintaining a high and stable nitrate-to-nitrite transformation ratio (NTR), which is essential for effective nitrogen removal [16]. In this process, nitrate (NO_3^-) is reduced to NO_2^- by denitrifying microorganism, followed by ammonium (NH_4^+) oxidation coupled with NO_2^-

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reduction by anammox bacteria. Since autotrophic anammox bacteria have a long doubling time and low sludge yield compared to heterotrophic denitrifiers [17], a stable NO_2^- supply is crucial to support the growth of anammox bacteria, ensuring excellent long-term operational performance.

Similar to PN-based process, achieving stable NO_2^- production with PD is of significant importance when combining with anammox for nitrogen removal. Applying PD for NH_4^+ and NO_3^- rich wastewater is very common however, in mainstream of domestic WWTPs where NH_4^+ is predominant still comes with its challenges since efficient PN is still required to produce sufficient NO_3^-. Besides, many researchers reported that during operation, the PD reactor frequently showed lower activity [18]. This could be due to long-term exposure to low temperatures [19], engineering errors (e.g. miscalculations, design flaws), high substrate loading rates [20,21], and limited mass transfer [22,23]. These issues cause severe sludge flotation and low biomass activity [20,24], resulting in the undesirable occurrence of excess NO_3^- and low NO_2^- production. Finding the right balance in mixing duration of reaction cycle and C/N ratios is the key to make the process work efficiently [25] and overcome lower activity of PD for long-term operations. The C/N ratio was extensively studied previously to evaluate its impacts on PD performance. A high C/N ratio can significantly reduce NO_2^- production during PD, which may suppress the growth of anammox bacteria and further promoting denitrifying microorganisms. Owing to above challenges, efficiency of anammox process could be halted [10,26].

The heterotrophic reduction of NO_3^- is also influenced by various operational conditions and parameters such as mixing duration (refers to the length of time of reaction phase of the SBR cycle) of the reaction cycle for PD reactor [19,27]. For instance, a recent study has evaluated the impacts of influent pH and anoxic reaction time in a single PD reactor inoculated with waste-activated sludge [28]. Briefly, this study concluded that NO_2^- production was decreased, when the anoxic mixing duration exceeded the optimal time. In contrast, Du et al. [29] investigated the influence of extending reaction duration on NO_2^- production in PD process and reported that long reaction time had minor influence on PD performance. These varying results is attributed to their methodological differences, the first study evaluated the effects of mixing duration with relatively long duration along with changing cycle duration, whereas, in the later study, the cycle duration was kept the same but the mixing duration was very short (10–40 min). The contrasting outcomes of above-mentioned studies demands detailed and careful investigation of impacts of mixing duration on PD performance. According to author's best knowledge, there is a critical knowledge-gap in investigation of impacts of mixing durations specifically on the underlying mechanisms of PD. Additionally, analyzing the functional microbiota, metabolic pathways and reactor pH to better understand the long-term PD operations in various mixing durations which is important for a better integration with subsequent anammox process is urgently needed. Moreover, previous studies, evaluated the effects of mixing duration as secondary parameter on relatively small scale and in shorter time-scale, which does not reveal the clear role of mixing duration on NO_2^- accumulation.

Therefore, for the first time, this study aims to achieve long-term PD by assessing the impacts of mixing duration with same cycle time (4 h) on PD performance and investigate the underlying mechanisms by analyzing functional microbiota and essential metabolic pathways. PD in lab-scale SBR reactor under mainstream conditions (low wastewater concentrations of C/N ratio: 1.85) was established to replicate it for long-term performance of NO_2^- production at large scale. Moreover, the impacts and role of different mixing durations (0.5 h, 1 h, 1.5 h and 2 h) on NO_2^- production, microbial community shift, biomass conditioning and activity, pH and COD consumption was studied. Additionally, the suitability of PD effluent for subsequent anammox process was analyzed by evaluating parameters including NO_2^- production rate (NO_2^- production to biomass concentration) and conversion efficiency of NO_3^--N to NO_x^--N. Furthermore, high-throughput sequencing analysis were used to track the evolution of the microbial community dynamics and change in metabolic pathways, aiming to better understand the stability of the PD process for practical applications.

2. Materials and methods

2.1. Experimental equipment and operational condition

The PD process was conducted in a lab-scale SBR with a working volume of 9 L (total volume: 10 L) (height: 36 cm; inner diameter: 19.20 cm), which was connected to two influent tanks (separately providing COD and NO_3^--N) and one effluent tank (Fig. 1). The partial denitrification sequencing batch reactor (PD-SBR) was operated for 160 days at room temperature (22 ± 3 °C), and a magnetic stirrer of 200 rpm (IKA Maxi MR 1, Germany) was used to keep sludge in suspension, while N_2 gas was sparged into the reactor to create anoxic environment to enhance activity of denitrifiers. The reactor was covered with aluminum foil to protect it from sun light. For operating the reactor, the pumps and the stirrer were connected to a Programmable logic controller (PLC) (IKS ComputerSysteme GmbH, Germany). For real-time monitoring of pH, temperature and dissolved oxygen (DO), control system has compatible sensors that were calibrated before starting the operation to measure and monitor each parameter. The PD-SBR reactor was operated with a cycle time of 4 h under an anoxic mode, for NO_2^- production. The details of the operation are given in Table 1.

The PD-SBR was initially run in 4 phases based on different mixing durations (phase 1: 2 h, phase 2: 1.5 h, phase 3: 1 h, phase 4: 0.5 h) keeping the whole cycle duration of 4 h, to investigate the impacts of mixing duration on PD performance. To validate the results of optimum phase (phase 2), internal validation and external validation (phase 5) were simultaneously done in two separate reactors. To eliminate the impacts of infiltrated DO in phase 1, the reactor was operated with repeated mixing duration of 2 h in phase 6. After careful review of literature, the most common anoxic mixing durations were selected for reaction phase of the cycle [1,25,30–32]. The length of every phase was determined by the stable production of NO_2^- or by the regular decrease in NO_2^- production after reaching maximum production in that specific phase. The stable NO_2^- production was defined based on NTR, which indicated that NO_3^- was depleted, and NO_2^- level no longer increased. The whole operation lasted 160 days, with the initial 1–20 days comprised the start-up period.

2.2. Synthetic wastewater and inoculated sludge

Synthetic wastewater containing sodium acetate (CH_3COONa) and sodium nitrate ($NaNO_3$) salts were used as carbon and electron acceptor sources for PD, respectively. Components of the influent synthetic wastewater are shown in Table S1 of Supporting information (SI). Trace elements were added in concentrations as previously reported by Cao et al. [33] and Du et al. [34]. The synthesized NO_3^- containing influent and CH_3COONa solution were prepared every two days to prevent the degradation of substrates and fed to the reactor regularly. A high pH is beneficial for PD process by enhancing NO_2^- production [35], hence the pH was not adjusted, and no buffering chemicals were added to the influent due to the basic and alkaline nature of PD process.

The fresh sludge used for inoculation was obtained from the aerobic reactor at the Kleinsteinbach municipal wastewater treatment plant in Karlsruhe, Germany. The sludge was pre-processed and rinsed three times with deionized water to eliminate large particulate matter and soluble impurities [36,37]. Based on previous studies, to ensure high biological activity and maintain the reaction driving force [38,39], the volatile suspended solids (VSS) concentration after inoculation was adjusted to approximately 2000 ± 200 mg/L. The volume exchange ratio of the reactor was set at 22.22 %.

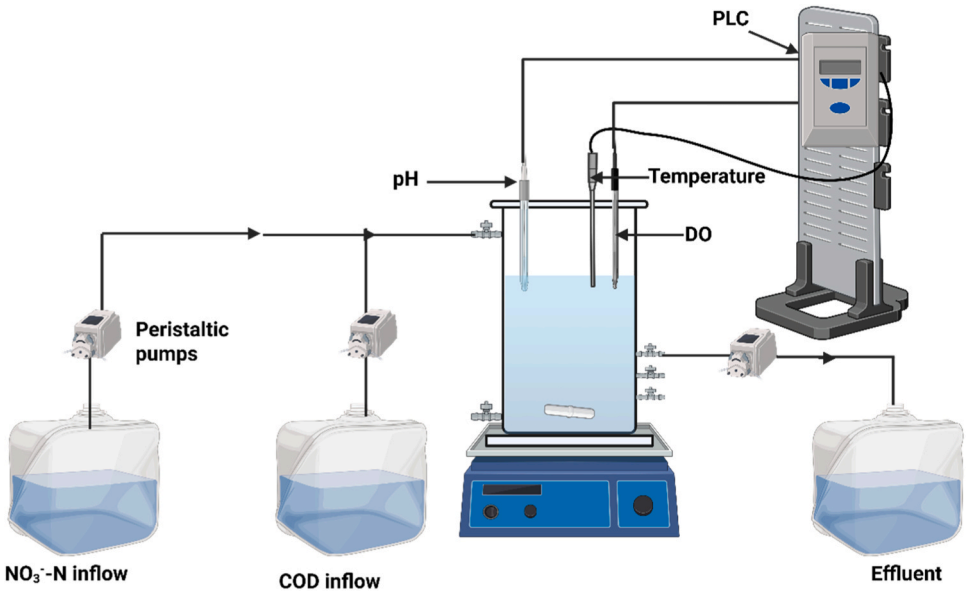

Fig. 1. Sketch of lab-scale sequencing batch reactor (SBR).

Table 1
Operation conditions for the lab-scale PD-SBR over 160 days optimizing operational period.

Items	Phase-1 Day 1–24	Phase-2 Day 24–64	Phase-3 Day 64–104	Phase-4 Day 104–125	Phase-5 Day 125–143	Phase-6 Day 143–160
Cycle time (h)	4	4	4	4	4	4
Feeding time (min)	45	45	45	45	45	45
Mixing duration (h)	2	1.5	1	0.5	1.5	2
Idling time (h)	0.5	1	1.5	2	1	0.5
Effluent time (min)	45	45	45	45	45	45
HRT (h)	18	18	18	18	18	18

2.3. Analytical methods

The samples of PD-SBR were collected three times a week after every 56 h during the effluent phase and analyzed immediately. The collected samples were filtrated through 0.45-μm filter membrane for removing undissolved matter before spectrophotometry. The NO_2^--N, NO_3^--N and COD concentrations were measured using Hach® LCK cuvette tests and spectrophotometer (Hach Lange GmbH, Düsseldorf, Germany). Mixed Liquor Suspended Solids (MLSS) and Mixed Liquor Volatile Suspended Solids (MLVSS) of the PD sludge were measured following the standard methods [40]. Dissolved oxygen (DO) levels and pH were continuously monitored during the SBR operation using PLC (IKS ComputerSysteme GmbH, Germany).

2.4. Microbial diversity analysis

To analyze the microbial community structure, a total of five samples, four sludge samples from the PD-SBR reactor in operation at different mixing durations and one sample from the fresh sludge sample used as inoculum in the reactor (as reference sample) were collected and stored in the freezer for microbial community analysis. The details of sampling are shown in Table 2.

Table 2
Description of the samples used for microbial analysis.

Sample Name	Day of Sample Collection	Mixing Duration
S1	57	1.5 h
S2	76	1 h
S3	118	0.5 h
S4	160	2 h
S5	Starting (Day 1)	Inoculated sludge

The isolation process was done with a suitable kit tailored for NGS applications to extract DNA from the sludge samples according to the standard procedure. PCR amplification (using a Microsynth standard primer set) of the bacterial 16S rRNA genes V4 region was amplified utilizing primers 515F (GTGYCAGCMGCCGCGGTAA) and 806R (GGACTACNVGGGTWTCTAAT). The amplicon metagenomics analysis consisted of clustering reads into OTUs (zOTUs/amplicon sequence variants/ASVs) were carried out at Microsynth AG, Balgach, Switzerland. The taxonomical assignment was provided on each level down to the species level. All raw data were analyzed through Origin and R software. Microsynth AG. (Balgach, Switzerland) undertook the sequencing work.

2.5. Functional profiling and metabolic pathway prediction

To infer the functional potential of the microbial communities based on 16S rRNA gene sequencing data, a predictive metagenomics approach was employed. The amplicon-based taxonomic profiles were analyzed using a pipeline that predicts gene family abundances and reconstructs metabolic pathways from these profiles. Specifically, functional annotation was performed using a combination of curated databases, including KEGG Orthology (KO), Pfam, TIGRFAMs, Clusters of Orthologous Groups (COG), and Enzyme Commission (EC) numbers. Then, metabolic pathway reconstruction and quantification were carried out according to MetaCyc Metabolic Pathway Database (https://metacyc.org) which is a comprehensive, curated resource of experimentally validated metabolic pathways. The resulted profiling allowed to evaluate differences in metabolic potential among microbial communities across various samples (S1–S5). All computational analyses for

functional prediction and pathway inference were conducted by the same sequencing service provider Microsynth AG. (Balgach, Switzerland), who implemented standardized and quality-controlled workflows for this approach.

2.6. Analytical calculations

In the PD process, the nitrate-to-nitrite transformation ratio (NTR), nitrate removal efficiency (NRE) and chemical oxygen demand (COD) removal efficiency (COD-RE) were calculated as follows (Eqs. (1)–(3)) according to the previous researches [9,19,25,28,37,41,42]:

$$\mathrm{NTR}(\%) = \frac{\mathrm{NO_2^-} - \mathrm{N_{eff}} - \quad \mathrm{NO_2^-} - \mathrm{N_{inf}}}{\mathrm{NO_3^-} - \mathrm{N_{inf}} - \quad \mathrm{NO_3^-} - \mathrm{N_{eff}}} \times 100\% \tag{1}$$

$$\mathrm{NRE}(\%) = \quad \frac{\mathrm{NO_3^-} - \mathrm{N_{inf}} - \mathrm{NO_3^-} - \mathrm{N_{eff}}}{\mathrm{NO_3^-} - \mathrm{N_{inf}}} \quad \times 100\% \tag{2}$$

$$\mathrm{COD-RE}(\%) = \frac{\mathrm{COD_{inf}} - \quad \mathrm{COD_{eff}}}{\mathrm{COD_{inf}}} \times 100\% \tag{3}$$

where NO_3^--N_{inf} and NO_2^--N_{inf} were the initial concentrations of NO_3^--N and NO_2^--N respectively in the beginning of reaction time, just after the feeding phase ends, mg/L; NO_2^--N_{eff} and NO_3^--N_{eff} were the corresponding effluent concentrations, respectively, mg/L. COD_{inf} and COD_{eff} were chemical oxygen demand (mg/L) in the influent and effluent.

The nitrite production rate (NPR) and biomass specific nitrite production rate (BSNPR) with units of mg NO_2^--N/L.d and mg NO_2^--N/g.d were calculated in Eqs. (4) and (5):

$$\mathrm{NPR} = \frac{\mathrm{NO_2^-} - \mathrm{N_{eff}} - \quad \mathrm{NO_2^-} - \mathrm{N_{inf}}}{\mathrm{HRT}} \tag{4}$$

$$\mathrm{BSNPR} = \frac{\mathrm{NPR}}{\mathrm{MLVSS}} \tag{5}$$

where NO_2^--N_{inf} was the initial NO_2^- concentration in the beginning of reaction time, mg/L; NO_2^--N_{eff} was the corresponding effluent concentration, mg/L. HRT was the hydraulic retention time (d) of the reactor and MLVSS was the weekly average MLVSS value, g/L.

3. Results and discussion

3.1. PD performance under different mixing durations

To investigate the performance of PD for efficient NO_2^- production under varying mixing durations (2 h, 1.5 h, 1 h, and 0.5 h) keeping the C/N ratio at 1.85 using sodium acetate as carbon source, NO_2^-, NO_3^-, and COD in the effluent of PD-SBR were closely monitored during the operation, as displayed in Fig. 2. The NTR being an important parameter, which reflect the inherent properties of PD [29], was measured regularly during and at the end of each phase. The initial 20 days were considered as start-up period.

In the initial days of PD start-up (Phase 1: day 1–24), the reactor was run with mixing duration of 2 h, there was not enough NO_2^- production (Fig. 2a), the mean effluent NO_2^- concentration was 5.92 mg N/L for the initial phase. This could be due to fresh inoculated sludge and the bacteria could not acclimatize to the new conditions [43,44]. At the 20th day of operation, a NO_2^- production of 16.5 mg N/L was obtained, which showed the adaptation of denitrifiers. The average NTR was 5.01 % and NRE was 28.59 % (Fig. 2b) for phase 1, which indicated that the denitrification was restricted by the presence of DO concentration in the system and NO_3^- can also be consumed aerobically (Fig. S4) [45]. The reason for the infiltration of oxygen in the reactor was usage of a mechanical stirrer through a hole in the lid. Moreover, NO_3^- concentration increased significantly at the 16th day of the operation, which also confirmed the presence of DO in the system and domination of nitrifiers. On day 17 of operation, the mechanical stirrer was replaced by a

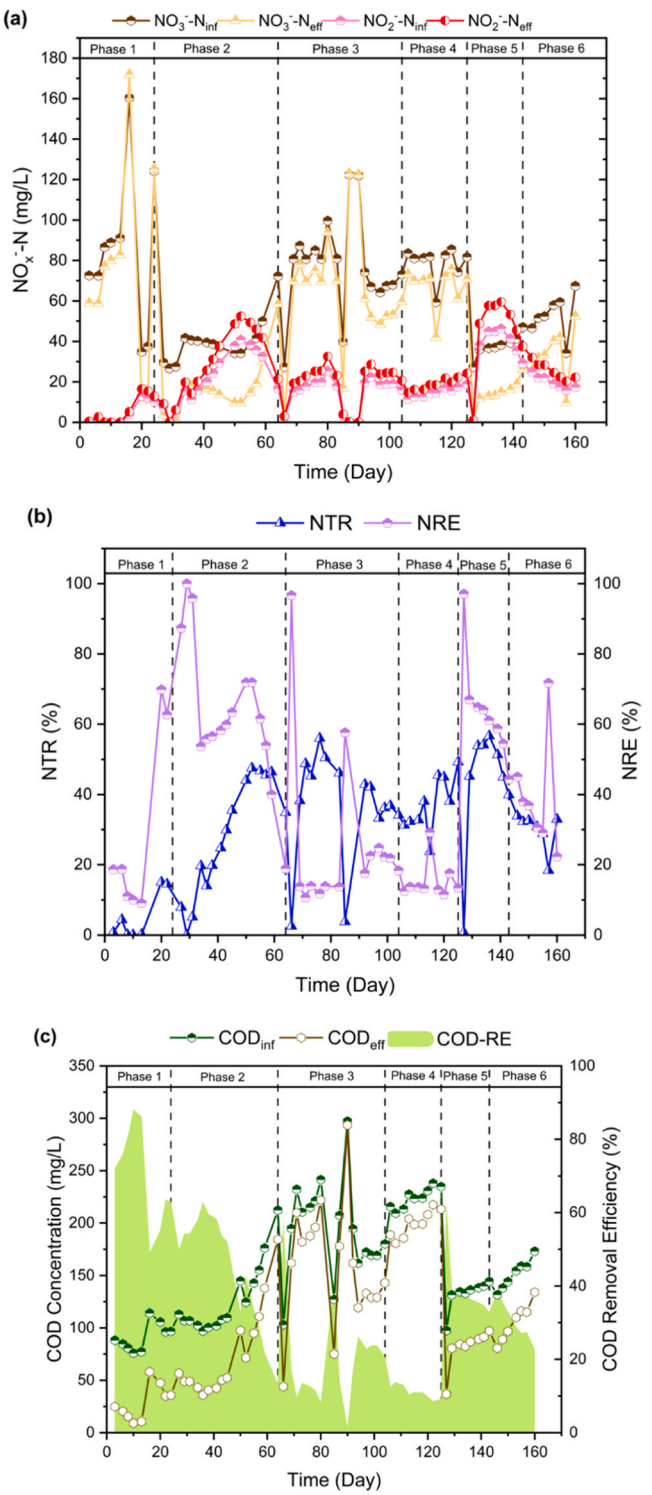

Fig. 2. The performance of lab-scale PD-SBR including (a) NO_3^- reduction and NO_2^- production in anoxic stage (b) NTR & NRE and (c) shifts in COD concentrations and COD-RE.

magnetic stirrer to minimize oxygen infiltration, which was depicted by increase in NTR values afterwards. In phase 1, the COD-RE was the highest of all the phases, with average COD-RE of 70.42 % and effluent average COD concentration of 28.51 mg/L was achieved (Fig. 2c). Whereas, COD-RE was low during the validation (phase 6) of this phase where the infiltration of DO was restricted, this indicated that the infiltration of DO can contribute to COD removal, as oxic process has

better COD-RE than the anoxic process [46].

In phase 2 (day 24–64), the PD reactor was run with mixing duration of 1.5 h. After 5 days of operation, significant and stable NO_2^- accumulation was observed in the reactor (Fig. 2a), which demonstrated the successful start-up of PD. The average effluent NO_2^- was 28.25 mg N/L for this phase, whereas, the average NTR increased from 5.01 % to 28.12 % (Fig. 2b), indicating much higher and more stable NO_2^- production and NO_3^- consumption. Similar results have been reported by recent studies done by Wu et al. [25] and Du et al. [29], which demonstrate that decreasing the anoxic stirring time have improved the NO_2^- production in the PD system. The results of this phase proved that bacterial activities were not inhibited and mixing duration of 1.5 h had a considerable advantage for obtaining high effluent NO_2^- concentration. The average COD-RE was 44.96 %, in the later part of the phase, effluent COD concentration was significantly increased and reached up to maximum of 184.4 mg/L, affected the overall average COD-RE of this phase (Fig. 2c).

In phase 3 (day 64–104), the mixing duration was shortened to 1 h, the NO_2^- production was significantly reduced with average effluent NO_2^- of 19.04 mg N/L (Fig. 2a). The average NTR was 36.90 % and NRE was 25.68 % (Fig. 2b), which are lower than the average values at phase 2 which contradicted with the previous study [10], where NO_2^- production was maximum at lower mixing duration and was decreased from 37.2 mg N/L to 26.8 mg N/L after increasing the mixing duration by 1.5 times. In this phase, it was clear from the Fig. 2a that the NO_2^- production was stable but significantly lower as a substrate for subsequent mainstream anammox process. A significant increase in effluent COD concentration was observed during the 1-hour mixing phase, with a peak value of 293.6 mg/L recorded on day 90 of reactor operation, reflected in the lower average COD removal efficiency of 20 % for this phase (Fig. 2c). This might be due to insufficient electron donors, which causes incomplete denitrification, leading to the accumulation of organic substances. Also, a sudden shift in the microbial community responsible for denitrification can lead to changes in metabolic activity [47,48], which is also evident from Section 3.4.

In phase 4 (day 104–125), the mixing duration was further reduced to 0.5 h. This further decreased NO_2^- production with an average effluent NO_2^- of 18.77 mg N/L achieved during this phase (Fig. 2a). There was no real consumption of NO_3^- in the reactor and higher NO_3^- concentrations were observed in the effluents. The average NTR was 35.63 % and NRE was 15.29 %, which were lower from the previous phases (Fig. 2b). It is also clear from Fig. 2c that a uniform increase in the effluent COD concentrations was noticed during this phase having the average COD-RE of 11.01 %. This high concentration of COD can lead to the effluent NO_2^- decline, that can be due to the sufficient organics providing electrons for complete denitrification [29], as evidenced by the effluent COD, which was increased from 143 mg/L to 213 mg/L. Of all the phases, the 0.5 h mixing duration phase has the lowest NO_2^- production, NTR, NRE and COD-RE values, which indicates that to reach to the desired results, an optimum reaction time is required keeping in mind the energy efficiency of the process.

Given these results of previous phases, the 1.5 h mixing duration performed well in terms of high and stable NO_2^- production in PD, to validate it, a validation of phase 2 results (phase 5: day 125–143) was done. The average effluent NO_2^- was 45.12 mg N/L, the average NTR was 43.34 % and NRE was 63.94 % were quite stable in this phase, which has the shortest startup time due to matured inoculated sludge (Fig. 2a, b). The results of this validation phase justified those of phase 2 and indicated that for PD start-up using matured sludge as inoculum under mixing duration of 1.5 h produced more efficient and stable NO_2^-.

To eliminate the impacts of infiltrated DO and inoculated fresh sludge in phase 1 to get real values for NO_3^-, NO_2^- and COD, in phase 6 (day 143–160), the reactor was operated with repeated mixing duration of 2 h. The average effluent NO_2^- was 25.48 mg N/L (Fig. 2a); the average NTR and NRE were 29.97 % and 39.07 % respectively (Fig. 2b). The average COD-RE was 30.42 %. These values were higher than those of the initial start-up phase, which also had a mixing duration of 2 h confirming the infiltration of DO in the reactor during the start-up days (Fig. S4), but lower than phase 2 and 3, indicated that the low NTR observed in phase 6 can be due to an excessive long mixing duration. Thus, maintaining an optimal anoxic mixing duration was crucial for preventing the reduction of accumulated NO_2^-.

These results indicated that an optimum mixing duration is necessary to achieve efficient NO_2^- accumulation in PD system for subsequent integration with mainstream anammox. Prolonged mixing duration can allow denitrifiers to convert NO_2^- to nitrogen gas, resulting in low NO_2^- accumulation (phase 1 and 6). Whereas, short mixing duration (phase 4) can result in low NO_2^- accumulation due to incomplete reduction of NO_3^-. Therefore, significant NO_2^- accumulation could be easily achieved with mixing duration of 1.5 h and will help in early start-up of PD for mainstream wastewater treatment. Moreover, stable NO_2^- accumulation also depends on infiltrated DO level, which can also negatively influence PD process.

3.2. *Effect of sludge maturation on PD performance*

After running the reactor for 6 phases, it was found that the reactor performed well under the mixing duration of 1.5 h to produce optimum and stable NO_2^- for anammox phase in the next stage. To investigate and highlight the significant variability in PD performance induced by sludge maturation, an external validation was done with mixing duration of 1.5 h in another reactor of same operational conditions with fresh sludge. The results obtained were compared with the results of phase 2 and with internal validation (phase 5) as displayed in Fig. 3. The average effluent NO_2^- obtained in external validation was 34.83 mg N/L, 28.25 mg N/L in phase 2, and 45.12 mg N/L in internal validation. The average NTR, NRE and COD-RE were 46.41 %, 32.14 % and 26.42 % for external validation, 28.12 %, 63.26 % and 44.97 % for phase 2, while it was 43.35 %, 63.95 % and 39.45 % for internal validation respectively. These results clearly indicates that the internal validation done for 1.5 h mixing duration with matured sludge have better and improved results validating the phenomena of sludge fermentation for efficient biological removal of pollutants from wastewater. A study conducted by Li et al. [49], also highlighted that fermented sludge exhibited more activity than that of fresh sludge.

The sludge characteristics largely depend on the surrounding environment, which greatly affects the microbial diversity and richness [50]. Moreover, many microorganisms showed the ability of resilience to the changing environmental conditions, indicated as enhancement in PD activity to prevent the deteriorative reactor performance [51]. It is also clear from the Fig. 3 that it took less time for PD to get to maximum and stable NO_2^- production with matured sludge. The results of phase 2 and external validation were close to each other with slight decrease for phase 2; this might be due to the presence of DO from the initial phase (phase 1) [45]. Overall, the results obtained for phase 2 and external validation were lower than the corresponding internal validation demonstrating the importance of sludge maturation for better PD activity.

Another investigation was conducted to analyze the changes in the properties and behavior of sludge conditioned under different operational phases by determining the factors contributing to increased NO_2^- production rate (NPR). A comparison is shown in Fig. 4, where the normal NO_2^- production rate (BPR) curve shows the rate of NO_2^- production under the influence of different mixing cycle durations at various phases, whereas biomass specific NO_2^- production rate (BSNPR) curve shows the effects of sludge conditioning on NPR during various phases. It is clearly displayed in Fig. 4 that NPR was optimum for phase 2 and phase 5 (1.5 h mixing duration) and was lower for other phases irrespective of the sludge properties substantiating that 1.5 h mixing duration performed well to have maximum and stable NO_2^- production for the anammox phase. It is also clear from the Fig. 4 that the BSNPR was very low at the start of the reactor operations and then increased

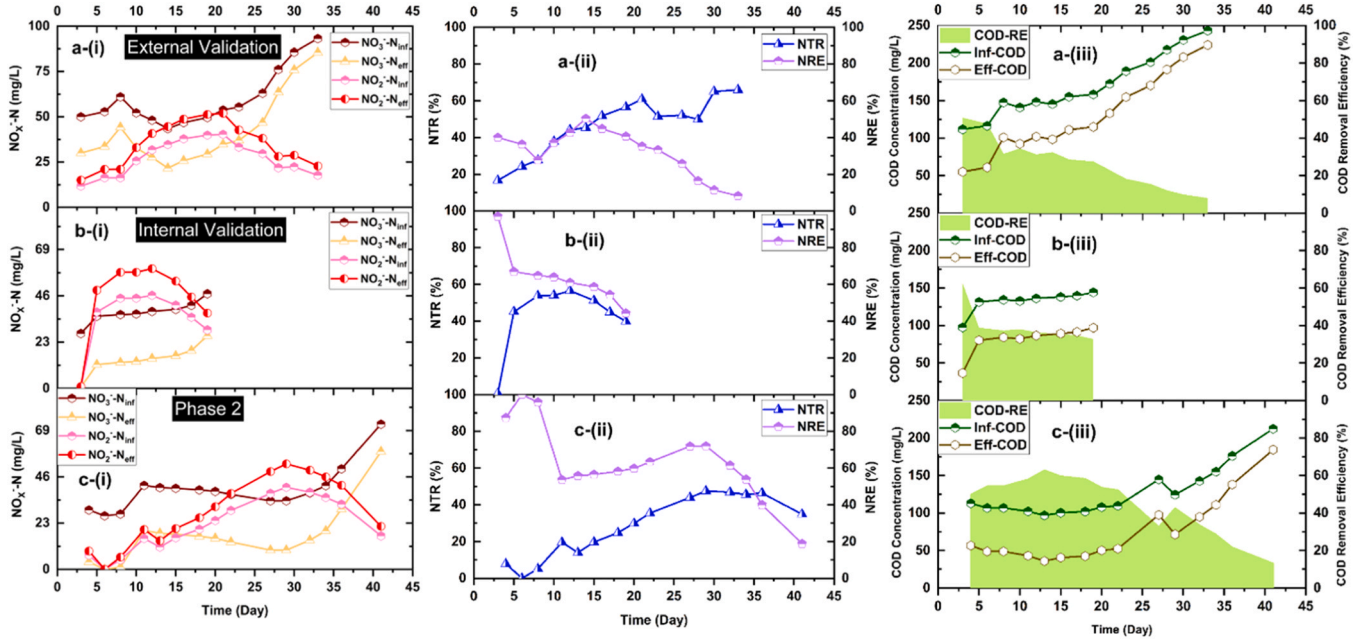

Fig. 3. Comparison of PD-SBR performance for (a) external validation; a-(i) change in nitrate and nitrite concentrations, a-(ii) change in NTR & NRE, a-(iii) change in COD & COD-RE, (b) internal validation; b-(i) change in nitrate and nitrite concentrations, b-(ii) change in NTR & NRE, b-(iii) change in COD & COD-RE, and (c) Phase 2; c-(i) change in nitrate and nitrite concentrations, c-(ii) change in NTR & NRE, c-(iii) change in COD & COD-RE.

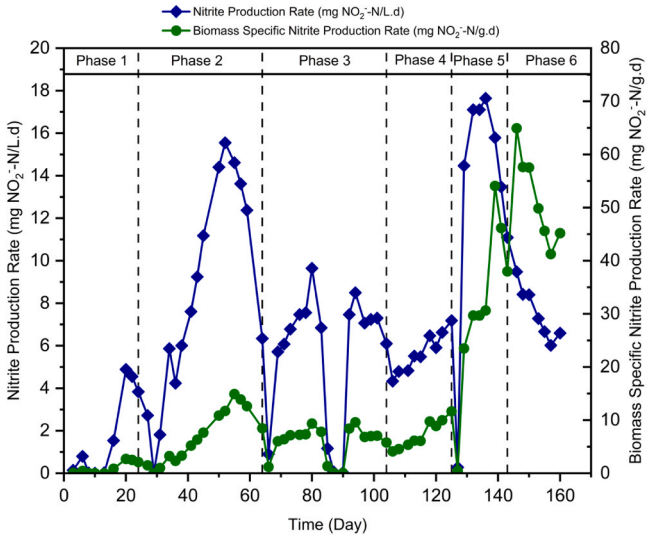

Fig. 4. Comparison of normal nitrite production rate (NPR) and biomass specific nitrite production rate (BSNPR) to evaluate the independence of impacts of mixing duration and sludge conditioning on the performance of PD.

gradually with passage of time and reached to optimum level in the last phase, which demonstrate that the functional microbes took time to acclimatize the reactor condition as reflected by increase in BSNPR in the later phases. To have better results for NTR, NRE, COD-RE, NPR and for BSNPR and to save time, the PD reactor should be operated with matured sludge having suitable anoxic mixing duration.

3.3. Effect of mixing duration on pH in PD system

The accumulation of NO_2^- during denitrification can be attributed to the difference in the reduction rates of NO_3^- and NO_2^- [24,28], a pattern observed throughout all phases of reactor operation for PD (Fig. 2a). This difference and subsequent NO_2^- buildup were linked to the high pH levels monitored during the reactor operation (See Fig. S1 of SI). At high pH (8.30–9.07), protons and electrons competed [52], which inhibited NO_2^- reductase activity [53] and promoted the enrichment of bacterial species with a strong ability to accumulate NO_2^- [20]. Shi et al. [28] found that NO_2^- production was similar regardless of pH control, leading to the decision to operate the PD reactor in this study without pH control.

3.4. Microbial community structure and evaluation responding to change in mixing duration

Comprehensive investigation of functional bacteria is beneficial to demonstrate the underlying mechanisms of NO_2^- accumulation in PD reactors [54]. In this study, the shift of microbial community in long-term operated PD reactor under different anoxic mixing durations revealed the potential relationship with PD functional microorganisms. The microbial community structure in inoculated sludge and four PD-SBR samples were compared and analyzed via Illumina high-throughput sequencing based on bacteria 16S rRNA gene, and the estimated indicators in terms of community richness and microbial diversity are given in Table S2 of SI. The number of retrieved sequence tags are shown as unique reads sequences (2327–7517) and duplicate reads sequences (80,128–94,851) were changed for each sample with change in mixing duration of the reactor. The sequences were further classified into operational taxonomic units (OTUs) and the species richness and diversity related to Shannon and Simpson were compared and assessed. The OTUs of PD-SBR samples were different considerably compared to inoculated sludge showed the lowest species diversity, suggesting that some key specific functional groups might be enhanced and concentrated from the inoculated sludge [55]. Meanwhile, the other Alpha-diversity indicators such as Shannon (4.18 → 3.49 – 2.00) and Simpson (0.0269→ 0.051 – 0.26) for community diversity were all consistent with OTUs (see SI). It could be deduced that the microbial community has changed over the PD-SBR operation under different mixing durations. The observations were also supported by the rank-abundance curves in (See Fig. S2 of SI), proving the shift of PD-SBR sludge and in microbial community compositions influenced by change in mixing duration.

The variation of relative abundance of bacterial community at different mixing duration was investigated at the phylum and genus levels via NGS formation process as displayed in Fig. 5 (The detail data for phylum and genus level are given in Table S3 and Table S4 of SI). The major bacteria at phylum level was *proteobacteria* (Fig. 5a) with a relative abundance range from 74.20 % to 95.20 %, compared with fresh sludge having 31.40 %, as the majority of denitrifying microorganisms belong to this phylum [56] and is commonly found in wastewater treatment bioreactors [57]. Similar results were obtained by Du et al. [29] with *Proteobacteria* being the predominated phylum with a relative abundance as high as 68.50–75.40 %. Collectively, *Proteobacteria, Bacteroides, Firmicutes* and *Planctomycetes* were the main phylum in NGS system, occupying more than 95 % of the total bacterial sequences of phase 2 (1.5 h), phase 3 (1 h), phase 4 (0.5 h), and phase 6 (2 h), where it was 56 % in inoculated sludge (S-5) [30,44].

For mixing duration of 1.5 h, the results of sludge sample analyzed showed that the relative abundance of *Proteobacteria* increased from 31.40 % to 74.20 % of the inoculated sludge, while *Firmicutes* increased from 7.27 % to 15.60 %. In addition, *Planctomycetes* increased from 2.03 % to 2.22 %. The increasing relative abundance of *Proteobacteria* and *Firmicutes* indicated that these bacteria were enriched with NGS formation. Previous studies also reported *Proteobacteria* was the most common shared phylum in sludge samples of PD reactor [30,58]. Meanwhile, the abundance of *Bacteroidetes* decreased from 15.70 % to 4.43 %.

With mixing duration of 1 h, *Proteobacteria* increased from 74.20 % to 84.80 %, while *Bacteroidetes* increased from 4.43 % to 8.44 %. These results indicated that *Proteobacteria* and *Bacteroidetes* were enriched with the decreasing mixing duration. Besides, *Firmicutes* and *Planctomycetes* decreased from 15.60 % to 3.86 % and 2.22 % to 1.01 %, respectively.

Proteobacteria increased from 84.80 % to 95.20 %, while *Bacteroidetes* decreased from 8.44 % to 2.82 % for mixing duration of 0.5 h. Moreover, *Firmicutes* and *Planctomycetes* decreased from 3.86 % to 0.48 % and 1.01 % to 0.761 %, respectively.

While for 2 h mixing duration, *Proteobacteria* abundance was almost the same with slight decreased from 95.20 % to 95.10 %, while that of *Bacteroidetes* increased from 2.82 % to 3.20 %. Abundance of *Firmicutes* decreased from 0.48 % to 0.21 % and *Planctomycetes* decreased from 0.76 % to 0.61 %. These results implied that *Firmicutes* and *Planctomycetes* were constantly eliminated during NGS formation process.

At the genus level (Fig. 5b), top 20 taxa were selected from the data, *Azoarcus* was revealed as the dominant bacteria, which was also identified and previously reported by Liu et al. [59] and Zielińska et al. [60] in wastewater treatment plants, mainly responsible for NO_3^- reduction. It was 0.12 % in the inoculated sludge, its abundance was increased to 6.60 % in sample of 1.5 h mixing duration, and then to 37.50 % in sample of 1 h. The abundance of *Azoarcus* was then decreased from 37.50 % to 1.72 % in 0.5 h, while it was slightly increased to 3.11 % when mixing duration was increased to 2 h, indicating that lower mixing duration would cause a negative influence on the growth of PD functioning bacteria. Abundance of other bacteria such as *Bosea* (0.11 % in inoculated sludge, 0.93 % in 1.5 h, 5.13 % in 1 h, 25.20 % in 0.5 h and 16.90 % in 2 h was increased with decrease in mixing duration, which was also responsible for denitrification [61]. While *Azonexus* which was very low in inoculated sludge, increase to 4.80 % in 1.5 h, 5.63 % in 1 h, 5.04 % in 0.5 h and 7 % in 2 h), abundance was increased with the higher mixing, which belongs to phylum *Pseudomonadota* can mediate NO_3^- reduction [62], clearly demonstrated the shift of denitrifying bacteria community.

It should be noted that *Aquimonas*, which its relative abundance was significantly increased throughout the reactor operation without any impact of mixing duration, is likely to compete with denitrifying bacteria and can affect the NO_2^- production in the reactor [63]. *Thauera*, widely reported as the primary PD functional bacteria responsible for NO_2^- production [64], which was 0.15 % in inoculated sludge, has the highest relative abundance of 3.74 % at mixing duration of 1.5 h, this was likely one of the reason for high NO_2^- production during phase 2. Its abundance was decreased for other phases and was lower than 1 %. These results further demonstrated that change in mixing duration had

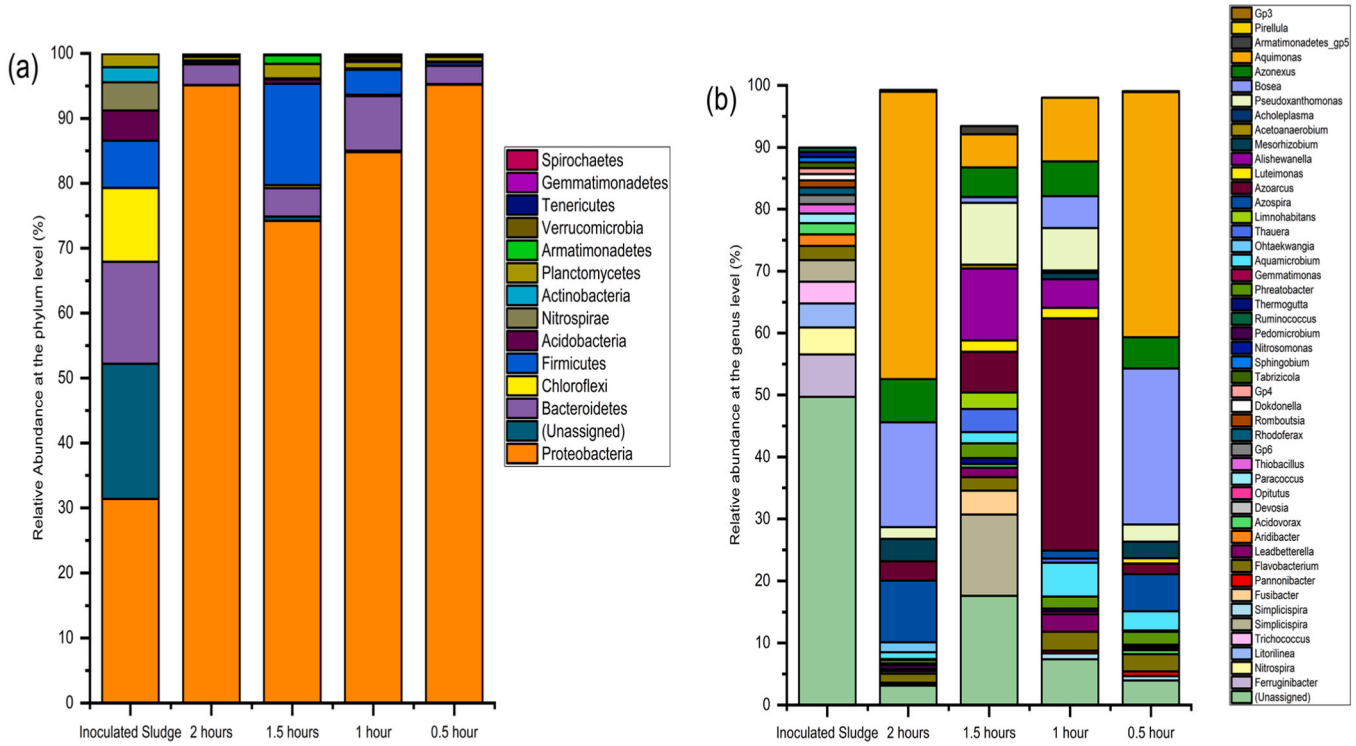

Fig. 5. Relative abundance of microbial community obtained by 16S rRNA amplicon sequencing analysis at (a) phylum and (b) genus level under different mixing durations in comparison to inoculated sludge.

significant impacts on NO_2^- production in the PD process.

3.5. Analysis of functional metabolic pathways

The log2 fold-change analysis was used to compare the major metabolic pathways that changed under different mixing conditions versus inoculate sludge (Fig. 6). The heat map results (Fig. 7) further indicated the relative abundance of pathways related to anaerobic respiration (Fig. 7a) and fermentation pathways (Fig. 7b) as well as facultative biosynthesis and degradation of selected compounds (Fig. 7c), along with other aerobic pathways in Fig. S3 of SI. The results suggested that the system exhibits both anaerobic and aerobic degradation capabilities as major pathways for anaerobic and aerobic respiration were predominated in the microbial communities (Fig. 6). Specifically, NO_3^- reduction was more pronounced under all mixing conditions compared to fresh conditions and being the most dominant pathway under 1.5 h mixing (Fig. 7a). The results also aligned with the NO_3^- reduction efficiency in the bioreactor, where 84.25 % of NO_2^- were accumulated.

The PD reactor facilitated different pyruvate fermentation processes (Fig. 7b), particularly for the formation of isobutane. As the process was more dominant under mixing condition of 1.5 h, we imply fermentation microorganism capable of isobutane production are well adapt to PD process.

However, we observed that the presence of facultative bacteria plays a crucial role in this metabolic flexibility (Fig. 7c). Their ability to switch between electron acceptors depending on oxygen availability further supports the observed trends in NO_3^- reduction and NO_2^- accumulation.

Another key result is that aerobic respiration pathways, including glycolysis, the TCA cycle, and oxidative phosphorylation, were still active across all conditions (see SI). This suggests that even in an anaerobic system, some level of aerobic respiration persists. Importantly, in condition of 1 h mixing duration, aerobic respiration was more abundant than in other conditions, indicating incomplete denitrification due to residual oxygen presence. Occasional aeration events seem to have played a role in this outcome. While the system was not intentionally aerated, minor oxygen exposure might have influenced the microbial activity [65,66]. This occasional oxygen presence appears to have supported incomplete denitrification, preventing full conversion to nitrogen gas and allowing NO_2^- accumulation. Instead of viewing the presence of oxygen as a drawback, it can be reconsidered as a strategic element that enhances PD. Hence, we can hypothesize that controlled occasional oxygen exposure can enhance NO_2^- accumulation while maintaining overall NO_3^- reduction efficiency. This claim also aligns with the need to balance oxygen availability to favor PD over complete denitrification.

In summary, the interplay between mixing strategy, fermentation activity, and occasional aeration influences the efficiency of NO_3^- reduction and NO_2^- accumulation. While the system was not intentionally aerated, minor oxygen exposure might have influenced the microbial activity and appears to have supported incomplete denitrification, preventing full conversion to nitrogen gas and allowing NO_2^- accumulation. These findings highlight the importance of optimizing mixing and oxygen control strategies to achieve the desired metabolic balance in PD systems.

3.6. Importance of efficient PD for synergy with anammox and its practical implications

This study highlights that the change in mixing duration of reaction cycle significantly affect the performance of PD, therefore, optimizing mixing duration can enhance NO_2^- accumulation, reduce energy consumption, enrich the specific functional microbiota and modify metabolic pathways for mainstream conditions. Additionally, NO_2^- accumulation was related to the high pH of the system, which was self-maintained throughout the operational phases with no effects from change in mixing duration, therefore, to yield a high pH and get optimum NO_2^- accumulation, PD should be performed separately so that other processes cannot decreases the pH by consuming the alkalinity [67].These findings are helpful to promote mainstream PD and anammox synergy in WWTPs, as a novel and promising nitrogen removal process having significant economic and environmental benefits.

Moreover, it was found that the carbon source was effectively consumed at optimum mixing duration (1.5 h), which indicate its effectiveness for real mainstream wastewater treatment toward carbon neutrality to cope with climate change issue [68–70]. The insights gained from this research can contribute to design large-scale wastewater treatment systems that operate efficiently under mainstream conditions, thereby significantly reducing energy consumption and operational costs.

Despite the encouraging results, further investigations are necessary to fully realize the potential of PD systems under different mixing durations in treating real mainstream wastewaters, which often contain additional substances [71,72]. These collective inhibitory effects can pose substantial threats to the synergy, stability and efficiency of the PD and anammox process.

4. Conclusions

This research aimed to optimize mixing durations for PD process to enhance NO_2^- production and functional microbiome enrichment in mainstream municipal wastewater treatment and could demonstrate that optimizing mixing duration has significant influence on the NO_2^- production. PD system performed efficiently under mixing duration of 1.5 h having higher NTR of 43.34 %. The pH of the system was not affected by changing the mixing duration and the elevated pH

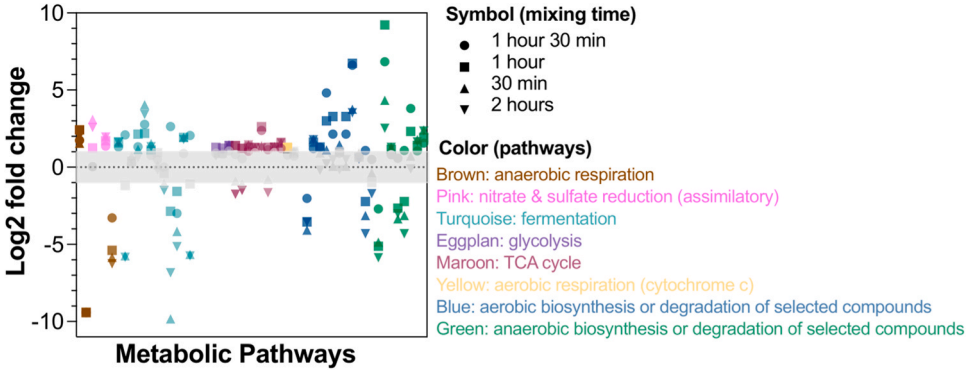

Fig. 6. Log2 fold change analysis of major metabolic pathways affected by different mixing durations compared to inoculated sludge. A manually adjusted threshold of − 1 to 1 indicated no major changes in pathways within this range.

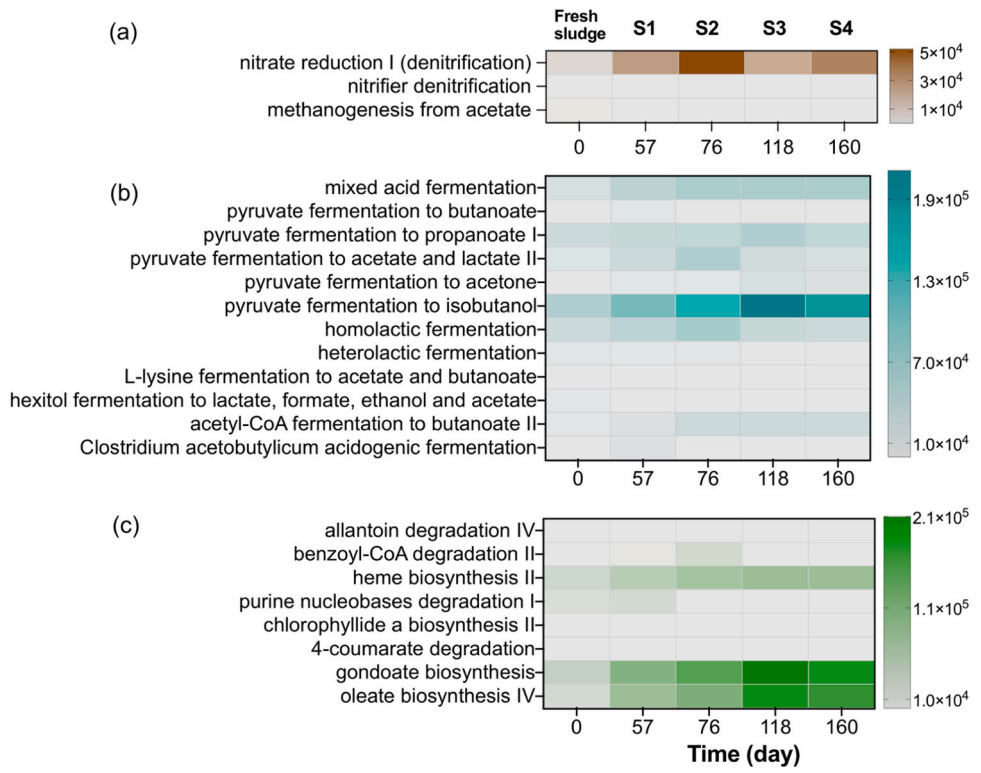

Fig. 7. Selected metabolic pathways abundance within the microbial community under different mixing duration, including of (a) anaerobic respiration (b) fermentation and (c) anaerobic biosynthesis.

(8.30–9.07) played an important role to maintain excellent PD. It was revealed that PD functional bacteria, *Azoarcus* dominated the system in all phases, its relative abundance was significantly decreased to 1.72 % when the mixing duration was reduced to 0.5 h. *Thauera*, responsible for the high NO_2^- production, has the highest relative abundance of 3.74 % at mixing duration of 1.5 h. NO_3^- reduction was more pronounced metabolic pathway under all mixing conditions compared to fresh conditions and was the most dominant under 1.5 h mixing. Consequently, PD was robust in NO_2^- production under 1.5 h mixing duration. These findings offer valuable insights for integrating PD with anammox processes, contributing to energy-efficient nitrogen removal. This technology could be also applied to real mainstream conditions of municipal wastewater treatment in full-scale treatment plants.

CRediT authorship contribution statement

Jafar Ali: Writing – review & editing, Investigation. **Anh Van Le:** Writing – review & editing, Visualization, Data curation. **Mohammad Azari:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Stephan Fuchs:** Writing – review & editing, Formal analysis. **Dara Memarzadeh:** Methodology, Investigation, Data curation. **Naveed Ali:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Data curation, Conceptualization.

Declaration of Competing Interest

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

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jece.2025.118436.

Data availability

Data will be made available on request.

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