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Stable partial nitritation of mature landfill leachate in a continuous flow bioreactor: Long-term performance, microbial community evolution, and mechanisms

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ABSTRACT

A continuous flow bioreactor was operated for 300 days to investigate partial nitritation (PN) of mature landfill leachate, establishing the long-term performance of the system in terms of the microbial community composition, evolution, and interactions. The stable operation phase (31–300 d) began after a 30 days of start-up period, reaching an average nitrite accumulation ratio (NAR) of 94.43% and a ratio of nitrite nitrogen to ammonia nitrogen (NO_2^--N/NH_4^+-N) of 1.16. Some fulvic-like and humic-like compounds and proteins were effectively degraded in anaerobic and anoxic tanks, which was consistent with the corresponding abundance of methanogens and syntrophic bacteria in the anaerobic tank, and organic matter degrading bacteria in the anoxic tank. The ammonia-oxidizing bacteria (AOB) *Nitrosomonas* was found to be the key functional bacteria, exhibiting an increase in abundance from 0.27% to 6.38%, due to its collaborative interactions with organic matter degrading bacteria. *In-situ* inhibition of nitrite-oxidizing bacteria (NOB) was achieved using a combination of free ammonia (FA) and free nitrous acid (FNA), low dissolved oxygen (DO) with fewer bioavailable organics conditions were employed to maintain stable PN and a specific ratio of NO_2^--N/NH_4^+-N, without an adverse impact on AOB. The synergistic relationships between AOB and both denitrifying bacteria and organic matter degrading bacteria, were found to contribute to the enhanced PN performance and microbial community structure stability. These findings provide a theoretical guidance for the effective application of PN-Anammox for mature landfill leachate treatment.

Rapid social and economic development in recent years has significantly increased global consumption and production, leading to the increased generation of municipal solid waste (MSW). It has been estimated that by 2025, over 2.2 billion tons of MSW will be produced worldwide [1]. Sanitary landfill remains a widely used strategy for MSW disposal worldwide, particularly in most developed countries [2], due to its simple operational procedures and low treatment costs. Landfill leachate is released from landfill, which usually contains a large amount of refractory organic compounds, ammonia nitrogen, heavy metals, high salinity, and toxic contaminants [3]. Therefore, developing efficient and economical methods for the treatment of landfill leachate is an increasingly important research focus, with the aim of minimizing its hazardous effect on both human and environmental health.

At present, physicochemical and biological technologies are the primary methods applied for the treatment of landfill leachate [4]. However, the practical application of most physicochemical methods is limited, such as membrane treatment and coagulation/flocculation, as they cannot completely remove pollutants due to the production of secondary pollution during treatment and often have high operational costs [5,6]. As an alternative approach, biological treatment has been widely applied for pollutants removal, providing a simple, efficient, and low-operational cost strategy, that is especially useful for nitrogen elimination. However, with increasing landfill age, biodegradable organics are gradually degraded, resulting in a low chemical oxygen demand (COD) and a low ratio of biological oxygen demand (BOD) to chemical oxygen demand in leachate. Typically, mature landfill leachate is classified

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as containing COD concentrations below 4000 mg/L and a BOD to COD ratio below 0.1 [5]. To achieve efficient nitrogen removal from mature landfill leachate using conventional biotechnology (nitrification and denitrification processes), large amounts of an external carbon source and a high level of aeration are needed, leading to higher levels of sludge production [7]. To overcome these challenges, the Anammox-based process has recently been developed, with this autotrophic biological denitrification technology providing a promising alternative approach, that can reduce operational costs and improve the overall treatment performance.

A crucial prerequisite to the effective application of Anammox-based processes is to achieve stable partial nitritation (PN) and maintain an appropriate ratio of nitrite nitrogen to ammonia nitrogen (NO_2^--N/NH_4^+-N = 1.0–1.3) [8]. To achieve efficient PN, ammonium-oxidizing bacteria (AOB) must be retained within the system, while nitrite-oxidizing bacteria (NOB) must be suppressed or washed out. Previous studies have achieved PN by regulating operation parameters based on different growth characteristics of AOB and NOB, such as temperature, or the concentrations of dissolved oxygen (DO), free ammonia (FA), and free nitrous acid (FNA) [9]. Due to the typically high levels of ammonia nitrogen and alkalinity in landfill leachate, and the differences in inhibitory concentrations of FA and FNA on AOB and NOB, high FA and FNA are the commonly maintained to achieve PN. Chen *et al.* [10] achieved PN of mature landfill leachate using a zeolite biological aerated filter, with the inhibition of NOB maintained by a combination of FA and FNA, while the NO_2^--N/NH_4^+-N ratio was kept at approximately 1.2. Another study used a sequencing batch reactor (SBR) to treat mature landfill leachate with FNA for NOB inhibition, and transformed almost all NH_4^+-N to NO_2^--N, achieving a nitrite accumulation rate (NAR) of >90% [11]. Bruni *et al.* [12] achieved PN in a pilot-scale SBR for landfill leachate treatment, using FA to inhibit NOB, resulting in an ammonia removal efficiency of >80%. Despite the promising results of these studies, either the addition of exogenous carriers was needed to control the NO_2^--N/NH_4^+-N ratio, or NH_4^+-N was provided from another pathway to achieve the Anammox process. Furthermore, the regulation of operational parameters such as FA and FNA to inhibit NOB and achieve PN, will inevitably have a negative impact on AOB activity, reducing the ammonia removal rate (ARR) of the system [13]. Another issue of concern is that NOB were recently found to be capable of adapting to operational conditions causing growth suppression, such as low DO concentrations, side-stream sludge treatment, or inhibition by FNA and FA, therefore disrupting the long-term stability of PN processes [14–17]. Therefore, this study aimed to develop a suitable strategy to achieve long-term stable PN without the use of additional agents, while also simultaneously regulating the ratio of NO_2^--N/NH_4^+-N for Anammox. Most previous research has focused on the operational strategy used to realize PN. However, considering the intrinsic toxicity and varying characteristics of landfill leachate, it is also important to fully explore the microbial community composition, evolution and the interactions between functional microorganisms in the PN system for maintaining long-term stable conditions.

Therefore, a continuous flow bioreactor was established and operated continuously for 300 days to achieve long-term PN of mature landfill leachate, with the bioreactor performance and microbial community comprehensively evaluated. The specific objectives of this study were to: (i) develop a suitable strategy to achieve PN and regulate the ratio of NO_2^--N/NH_4^+-N for Anammox; (ii) systematically evaluate the PN performance during long-term operation and elucidate the PN mechanism; (iii) investigate the microbial community dynamics, identifying key functional microbes and their interactions.

A laboratory-scale continuous flow bioreactor was used for experiments, with a total working volume of 60 L which was equally divided into three tanks, while the sludge was settled through an attached clarifier tank (13.25 L) (Fig. S1 in Supporting information). The raw landfill leachate was fed into the first anaerobic tank by a peristaltic pump, which was packed with braided polypropylene carriers, then flowed through the second anoxic tank, and finally into the oxic tank. Both the anoxic and oxic tanks were aerated, with the DO concentration in the anoxic and oxic tanks maintained at 0.10–0.35 mg/L and 0.20–1.50 mg/L, respectively.

Mechanical stirrers were installed in the anoxic and oxic tanks, maintaining continual agitation at 70 rpm. The sludge retained in the clarifier tank was returned to the anoxic tank using a peristaltic pump, at a reflux ratio of 200%. Throughout the experimental period, the hydraulic retention time (HRT) was kept constant at 5.5 d, while the sludge retention time (SRT) was 15 d and the temperature was maintained at 28 ± 2 °C using an automatic temperature control device. The continuous flow bioreactor operational period was divided into two phases: the start-up phase (1–30 d, phase I) and stable phase (31–300 d, phase II). The mature landfill leachate was diluted with tap water, with the proportion of leachate in the influent of phases I and II being 25% and 50%, respectively.

The seed sludge was collected from a secondary sedimentation tank of a two-stage anoxic-oxic (A/O) treatment system in the Wuhan Chenjiachong Landfill Treatment Engineering Project (Wuhan, China), which has been in operation for 15 years. After inoculation, the mixed liquor suspended solids (MLSS) concentration in the continuous flow bioreactor was approximately 4500 mg/L. Landfill leachate was also collected from the Wuhan Chenjiachong Landfill Treatment Engineering Project (Wuhan, China), and was found to conform to the typical characteristics of mature landfill leachate with a C/N ratio of 1.73 ± 0.52. The specific characteristics of the landfill leachate were as follows: COD = 5127.0 ± 1649.2 mg/L, ammonia nitrogen (NH_4^+-N) = 2926.9 ± 252.2 mg/L, nitrate nitrogen (NO_3^--N) = 32.8 ± 10.4 mg/L, nitrite nitrogen (NO_2^--N) = 1.4 ± 1.1 mg/L, chloride ions (Cl^-) = 5999.6 ± 938.2 mg/L, and pH 8.1–8.5. Furthermore, the five most abundant heavy metals were iron (3.328 ± 0.179 mg/L), arsenic (2.235 ± 0.060 mg/L), barium (0.290 ± 0.004 mg/L), zinc (0.275 ± 0.007 mg/L), and chromium (0.246 ± 0.002 mg/L).

The concentrations of COD, NH_4^+-N, NO_3^--N, NO_2^--N, and TN in samples were measured according to the standard methods, after filtration using a 0.45 µm filter. The pH and DO were measured using a pH meter (pHS-25, Leici, China) and a DO meter (HQ40D, Hach, US), respectively. The water samples were filtered through a 0.45 µm filter prior to three-dimensional excitation and emission matrix (3D-EEM) analysis using a fluorescence spectrophotometer (F-7000, Hitachi, Japan). The emission (Em) spectra were determined from 250 nm to 550 nm at 2 nm increments, while excitation (Ex) wavelengths from 200 nm to 400 nm were determined at 5 nm intervals. The emission and excitation spectra were recorded with slit widths of 5 nm, and at a scanning speed of 2400 nm/min. The final spectra were processed to remove the deionized water background signal.

The NAR and the concentrations of FA and FNA were calculated according to Eqs. 1-3 as follows [18]:

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

$$\mathrm{FA} = \frac{17}{14} \times \frac{\left[NH_4^+ - N\right] \times 10^{pH}}{\exp\left[\frac{6334}{273+T}\right] + 10^{pH}} \tag{2}$$

$$\mathrm{FNA} = \frac{46}{14} \times \frac{\left[NO_2^- - N\right]}{\exp\left[-\frac{2300}{273+T}\right] \times 10^{pH}} \tag{3}$$

where $NO_2^- - N_{eff}$ and $NO_3^- - N_{eff}$ refer to the NO_2^--N and NO_3^--N concentrations in effluent (mg/L); $NO_2^- - N_{inf}$ and $NO_3^- - N_{inf}$ refer to the NO_2^--N and NO_3^--N concentrations in influent (mg/L); and T is the temperature of water within the bioreactor (°C).

DNA was extracted from sludge samples using a PowerSoil DNA Kit (Mobio, US) following the manufacturer's instructions. PCR amplification of the V3-V4 region of bacterial 16S rRNA was performed using the 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) primer set. The V4 region of archaeal 16S rRNA was amplified using the ArBa515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and Arch806R (5′-GGACTACVSGGGTATCTAAT-3′) primer pair. The amplification products were sequenced using the Illumina MiSeq platform (Majorbio Bio–Pharm Technology Co. Ltd., Shanghai, China).

The operational period of the continuous flow bioreactor was divided into two phases. In the start-up stage (phase I), NH_4^+-N concentrations in each tank and in the final effluent were higher than in the influent (Fig. 1a), which was attributed to the liberation of ammonia nitrogen from cell lysis due to the shift in seed sludge from oxygen- and glucose-rich conditions to low DO conditions without the addition of organics [8]. After 14 days of operation, the NH_4^+-N concentration in the anoxic tank began to decrease gradually, and by the 30th day, the NH_4^+-N removal efficiency had reached 49.54%, with a NAR of 100% (Fig. 1b). This suggests that AOB adapted to this environment and effectively became the dominant bacteria in the nitrogen transformation process, signifying the successful start-up of PN within the system.

In phase II, the proportion of landfill leachate in influent was increased to 50%, and the effluent concentrations of NH_4^+-N and NO_2^--N were 527.15 ± 88.10 and 602.70 ± 85.33 mg/L, respectively. The ratio of NO_2^--N/NH_4^+-N was 1.16 ± 0.19 and the NAR was 94.43% ± 1.80%. Therefore, the stable performance of the system

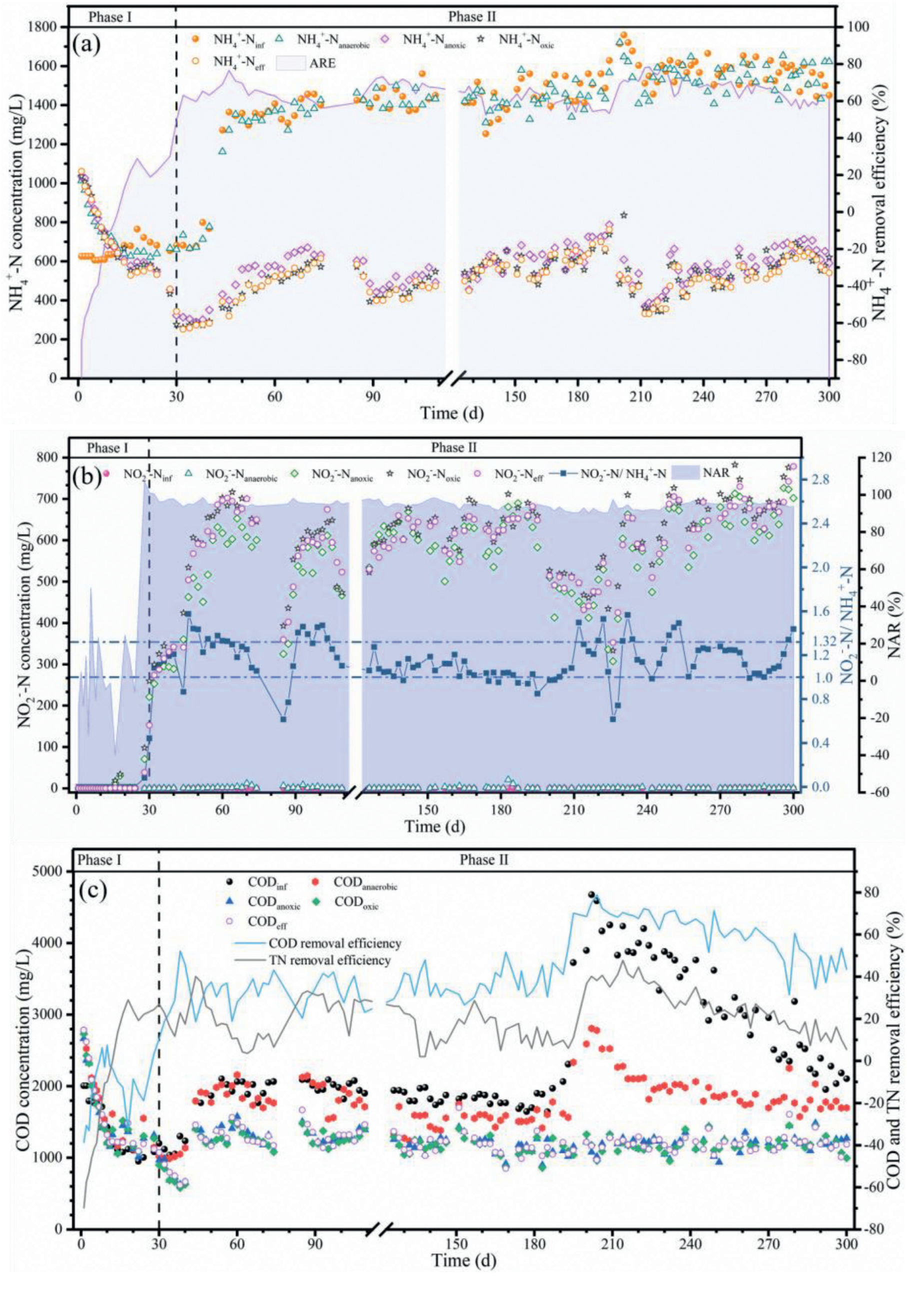

Fig. 1. The long-term performance of the continuous flow bioreactor including (a) NH_4^+-N concentrations in the influent, effluent and each tank and the NH_4^+-N removal efficiency (ARE) of the bioreactor; (b) NO_2^--N concentrations in the influent, effluent and each tank, and variety of NAR and NO_2^--N/NH_4^+-N ratio of the bioreactor; (c) COD concentrations in the influent, effluent and each tank, COD removal efficiency, and TN removal efficiency of the bioreactor.

and the appropriate substrate ratio provided suitable conditions for the subsequent Anammox process. In previous studies, Nhat *et al.* [13] achieved PN in a lab-scale SBR fed with mature municipal landfill leachate after 75 days of operation, while Li *et al.* [19] reported that a 65 days start-up period was required to achieve PN and a satisfactory NO_2^--N/NH_4^+-N ratio (1.20–1.28) in a SBR with intermittent aeration and endpoint pH control. In contrast, Bruni *et al.* [12] failed to establish nitritation due to an excessive nitrogen loading rate. Therefore, the results of the present study show that this system achieved rapid start-up of PN within 30 days in a simple operational procedure, with a comparable treatment performance to previously reported studies. Compared with previous studies, the rapid start-up of the PN system in this study was attributed to the removal of most organics in anaerobic tank and the relatively low DO concentration in oxic tank, which could inhibit the proliferation of heterotrophic bacteria and provide favorable conditions for the growth and activity of AOB.

The concentration of NO_2^--N in the anoxic tank (575.4 ± 88.6 mg/L) was slightly lower than in the oxic tank (623.7 ± 85.6 mg/L) (Fig. 1b), which signified the existence of denitrification in anoxic tank and the further oxidation of NH_4^+-N in oxic tank. The oxic tank was established to further oxidize NH_4^+-N and maintain a suitable NO_2^--N/NH_4^+-N ratio, as denitrification existed in the anoxic tank consumes a portion of NO_2^--N, causing the ratio of NO_2^--N/NH_4^+-N in the anoxic tank to be outside of the range suitable for anammox bacteria (1–1.32). Furthermore, it was found that the removal of TN was closely related to the concentration of organics in leachate (Fig. 1c). During 195–245 days of operation, the relatively higher influent concentration of COD (3927 ± 247 mg/L) resulted in a higher corresponding TN removal efficiency of 37% ± 6%. However, in most cases, the concentration of COD in mature landfill leachate has been low (1910.10 ± 133.51 mg/L), with a corresponding average TN removal efficiency of only 18%.

In phase I, the COD concentration in the effluent was higher overall than in the influent (Fig. 1c), which was attributed to cell lysis. In phase II, from day 42 to 194, the average COD concentrations in the influent and effluent were 1910.10 ± 133.51 mg/L and 1236.29 ± 146.24 mg/L, respectively, corresponding to an average COD removal efficiency of 35.16%. Due to the significant increase in the COD of landfill leachate on day 195, the average proportion of COD degraded in the anaerobic tank increased from 8.26% to 36.35%, and the average COD removal efficiency of the bioreactor increased to 60.55%. However, this increase in COD removal efficiency did not reduce the concentration of COD in the effluent (1198.47 ± 106.07 mg/L), which remained at a similar level to the previous stage. These results show that the residual COD content of effluent was mainly composed of refractory organics, as reported in previous literature [6,20], showing that the large proportion of refractory organics in mature landfill leachate present a major challenge for the efficient removal of COD.

Dissolved organic matter (DOM) accounts for most of the organic matter in leachate and is strongly associated with the COD of the leachate. The compositional distribution of DOM and its transformation, were analyzed based on 3D-EEM spectra (Fig. 2). The fluorescent components of the raw leachate on the 80th day were identified as protein tryptophan-like substances (peaks A and B at the Ex/Em of 230/344 nm and 275/352 nm, respectively) and fulvic-like compounds (peak C at the Ex/Em of 250/450 nm) [21]. On the 280th day, the raw leachate consisted of humic-like substances (peak D at the Ex/Em of 325/410 nm) and fulvic-like compounds (peak C at the Ex/Em of 250/450 nm). It is of note, that fulvic-like and humic-like compounds gradually became dominant during the aging process, indicating the low biodegradability of raw leachate.

Compared to the raw leachate, the fluorescence intensity of the four indicator peaks (A, B, C and D) all decreased slightly in the anaerobic tank, then exhibited a significant decrease in the anoxic tank, with peak A in particular completely degraded. In general, these substances (peak A) were identified as being simple aromatic proteins with Ex < 250 nm and Em < 350 nm, indicating the presence of bioavailable organic substrates [6].

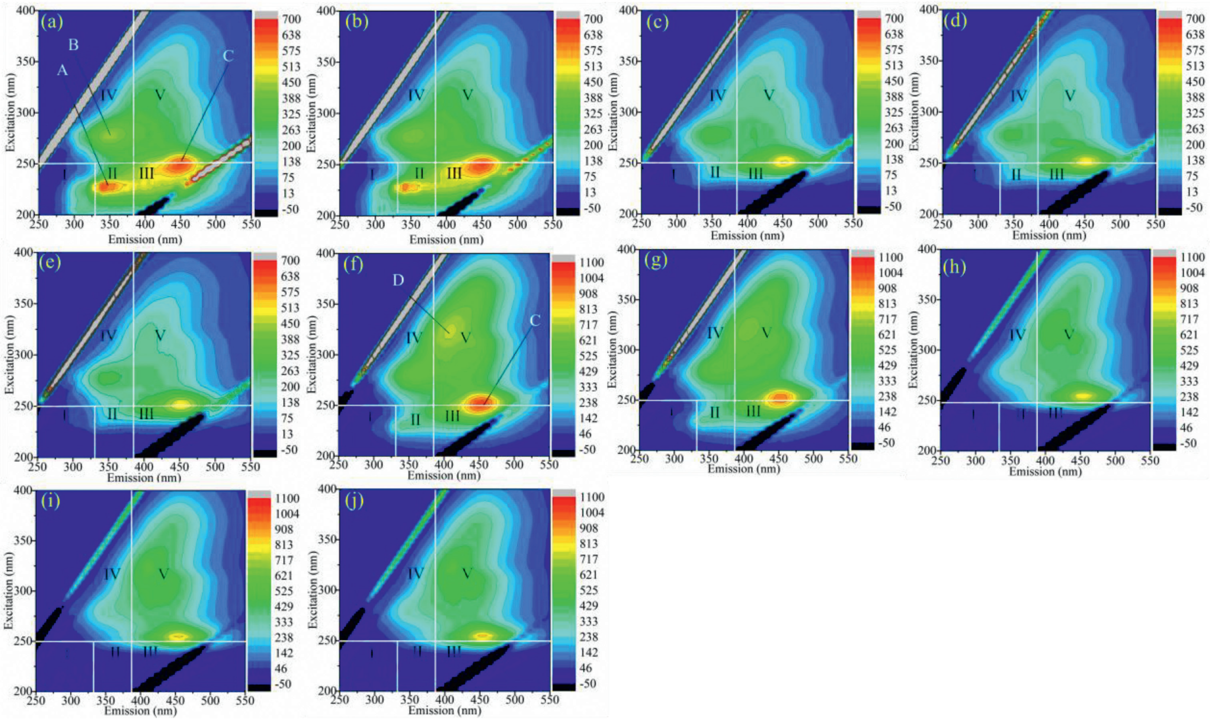

Fig. 2. 3D-EEM spectra of the influent and effluent of the continuous flow bioreactor and the effluent of each tank on the 80th and 280th day: (a) influent, (b) anaerobic, (c) anoxic, (d) oxic and (e) effluent on the 80th day; (f) influent, (g) anaerobic, (h) anoxic, (i) oxic and (j) effluent on the 280th day.

Some organic matter was utilized as a carbon source for denitrification by denitrifying bacteria and the growth of other heterotrophic bacteria and therefore, the fluorescence intensity of tryptophan-like protein, fulvic-like and humic-like substances were all greatly reduced in the anoxic tank. However, the intensity of the peak for these substances in the oxic tank did not change significantly, indicating the low biodegradability of the remaining organics. These results indicate that some proteins, fulvic-like and humic-like compounds were degraded after the treatment using the continuous flow bioreactor. However, the removal of residual organic matter may require some additional chemical methods, such as advanced oxidation processes (AOPs).

The seed sludge sample (S0) was collected on day 0 and sludge samples were collected from the anaerobic, anoxic and oxic tanks on days 30 (A1_30d, A2_30d, O3_30d) and 280 (A1_280d, A2_280d, O3_280d). The Alpha-diversity indices of the seven samples were determined, including diversity indices, richness indices and the coverage index, as shown in Table S1 (Supporting information). The coverage index of all samples was >0.99, indicating a sufficient sequencing depth and that data was representative of the extracted samples. Shannon and Simpson indices showed that microbial diversity increased in phase I compared to the seed sludge, then further increased during phase II in the anaerobic and anoxic tanks, then slightly decreased in the oxic tank. The microbial richness of the anaerobic tank increased initially and then decreased, while the opposite trend was observed in the anoxic and oxic tanks. These results were mainly attributed to the gradual enrichment of microorganisms able to adapt to the system during phase I and the increased abundance of microorganisms able to tolerate low organic matter and DO concentrations and high FA and FNA concentrations. Principal coordinate analysis (PCoA) was performed to investigate changes in microbial community structure within the system (Fig. 3a). The identified sample clusters included A1_30d and A1_280d, A2_30d and O3_30d, A2_280d and O3_280d, all of which were separate from S0, indicating that the microbial community structure altered significantly with treatment time and varying conditions.

To further explore the microbial composition and the evolution of the sludge microbial community, the community structure and relative microbial abundances were analyzed at the phylum

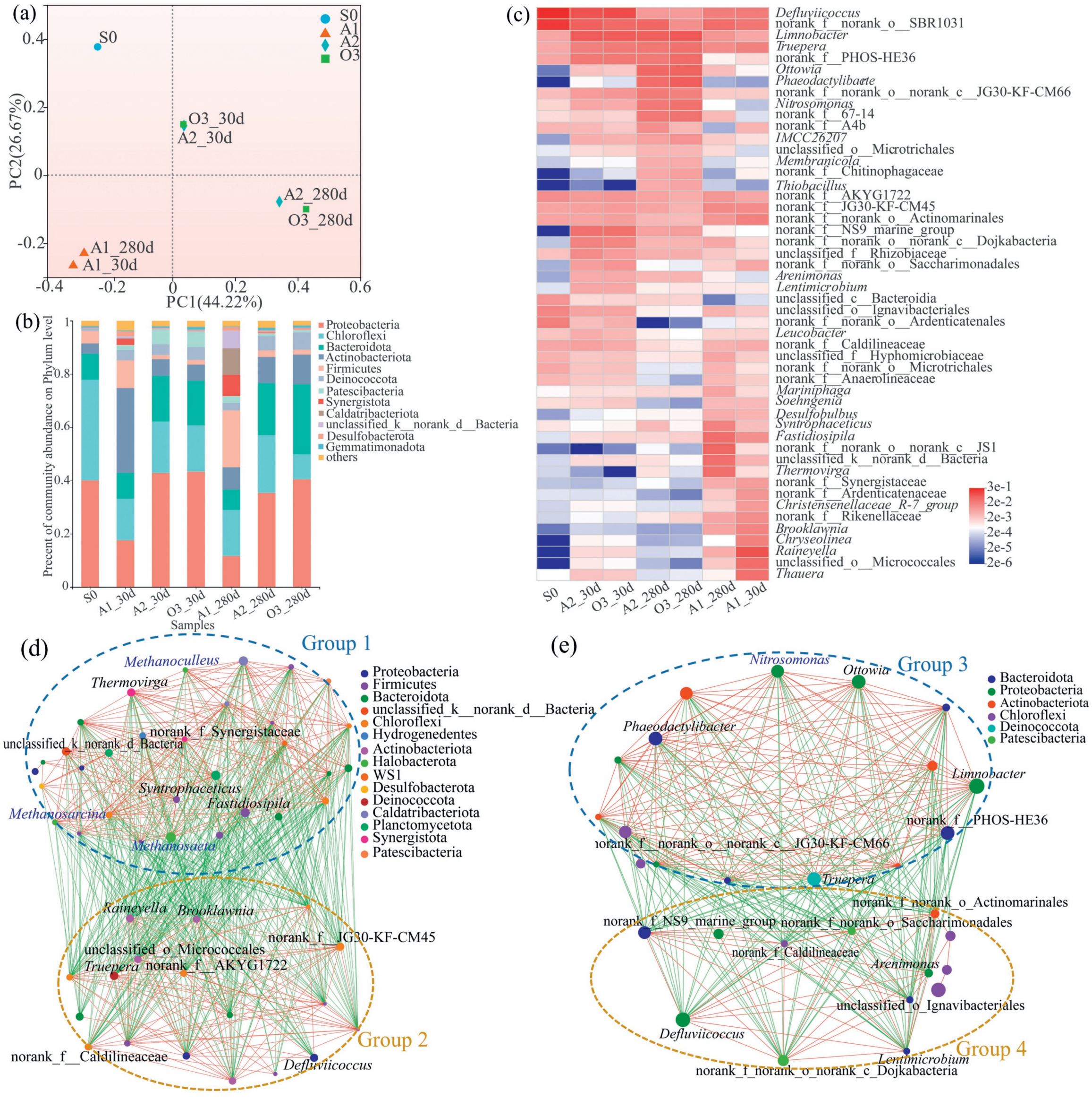

Fig. 3. (a) PCoA analysis based on bacteria community; variations of microbial community structure (b) at phylum level, and (c) at genus level; the genera correlation network analysis of (d) anaerobic tank and (e) oxic tank. The red line represents cooperation relationship and the green line represents competition relationship.

and genus levels. As shown in Fig. 3b, Proteobacteria and Chloroflexi were the predominant phyla, with relative abundances of 11.65%–43.40% and 9.43%–37.80%, respectively. Proteobacteria play an important role in the removal of both organic matter and nitrogen and have previously been reported to be the most abundant phylum in wastewater treatment systems [22]. Chloroflexi is facultative anaerobic bacteria that is capable of aromatic compound degradation and has commonly been identified in wastewater treatment systems [20], which might relate to the degradation of fulvic-like and humic-like compounds in the system. The relative abundance of Bacteroidota in the anoxic and oxic tanks was approximately two-fold higher than in the anaerobic tank, while the abundance of Firmicutes in the anaerobic tank was about 6 to 11-fold higher than in the anoxic and oxic tanks. Bacteroidota has been shown to be capable of hydrolyzing complex macromolecular organic compounds and can decompose carbohydrates into monosaccharides [23], potentially enhancing nitrogen removal from mature landfill leachate by resolving the problem of carbon source limitation. Firmicutes can survive in extremely oligotrophic environments due to its ability to produce endospores, and have been found to be an important acid hydrolyzing microbe that can degrade volatile fatty acids (VFAs) and generate acetic acid [23–25]. Actinobacteriota was also detected within the system, which has been reported to include both anaerobic and autotrophic bacteria [22]. Furthermore, many Firmicutes and Actinobacteriota have been reported to be capable of converting organic compounds (such as proteins and carbohydrates) to short-chain fatty acids (SCFAs) and hydrogen under anaerobic conditions [26]. Synergistota and Caldatribacteriota were the exclusive phyla in the anaerobic tank, with their relative abundances increasing from 2.29% and 0.29% on the 30th day to 8.00% and 9.94% on the 280th day, respectively. Some anaerobic bacteria affiliated with Synergistota have been confirmed to be syntrophic with methanogens, degrading organic acids or alcohols, with Caldatribacteriota also shown to have a syntrophic relationship with methanogens [27].

The microbial community was further investigated at the genus level to determine and compare the abundance of functional bacteria (Fig. 3c). The preponderant genera in the seeding sludge were *Defluviicoccus* and norank_f__norank_o__SBR1031 (an unclassified genus of the order-level lineage *SBR1031*, phylum Chloroflexi), with abundances of 31.90% and 25.83%, respectively. *Defluviicoccus* is a glycogen accumulating organism that is capable of degrading various kinds of organics [28] and its high abundance in the seeding sludge may be due to the addition of glucose as a carbon source in the two-staged A/O landfill treatment process. Nitrogenous compounds could provide satisfactory nutritional conditions for norank_f__norank_o__SBR1031 [29], with the high concentration of ammonia nitrogen in landfill leachate stimulating its extensive enrichment. The relative abundances of *Defluviicoccus* and norank_f__norank_o__SBR1031 in the samples collected on the 30th and 280th day accounted for 1.61%–17.55% and 2.39%–11.00%, respectively, and it was speculated that the degradation of organic matter and ammonia nitrogen in the continuous flow bioreactor system was related to these genera.

As the composition of organic matter in landfill leachate is complex, a large number of microorganisms capable of degrading organic matter were enriched in the anaerobic tank. On the 30th day, *Raineyella*, unclassified_o__Micrococcales (an unclassified genus of the order-level lineage *Micrococcales*, phylum Actinobacteriota), *Thauera*, *Chryseolinea,* and *Brooklawnia* were the main genera associated with the degradation and transformation of organic matter in the anaerobic tank, accounting for 14.77%, 7.58%, 6.76%, 3.87%, and 3.65%, respectively. *Raineyella* was the most abundant microorganism in the anaerobic tank, which has been reported to dominantly coexist with other organic degrading facultative anaerobes in constructed wetlands for mine drainage treatment [30]. It has been proposed that *Raineyella* may play an important role in the transformation and degradation of organic matter in leachate, while the genera *unclassified_o__Micrococcales*, *Thauera* and *Chryseolinea* were found to possess the capacity to degrade refractory compounds, aromatic compounds, polycyclic aromatic hydrocarbons and cellulose [31–34], respectively. *Brooklawnia* are able to produce SCFAs and hydrogen during the anaerobic fermentation of organic matter, degrading glucose to produce propionate [26]. By day 280, a significant shift was observed in the microbial genera in the anaerobic tank, with the genus *norank_f__norank_o__norank_c__JS1* (an unclassified genus of the class-level lineage *JS1*) of the phylum Caldatribacteriota, exhibiting the highest abundance (8.89%). Caldatribacteriota are heterotrophic anaerobes with fermentative potential and have been found to be syntrophic with methanogens by providing substrates for methanogenesis [27]. Thus, it is feasible to assume that the dominance of the genus *norank_f__norank_o__norank_c__JS1* was relevant to, and perhaps vital to the anaerobic fermentation of organic matter for methane production. *Fastidiosipila* and *Syntrophaceticus* belong to Firmicutes and accounted for 7.37% and 3.75% of the community in the anaerobic tank, respectively. *Fastidiosipila* are able to transform carbohydrates and proteins to VFAs [35], while *Syntrophaceticus* are important for acetate metabolism and are syntrophic with methanogenic archaea [36]. Moreover, *Thermovirga* also exhibited a high abundance of 6.71%, which was found to be involved in syntrophic methanogenesis in anaerobic environments and was responsible for the degradation of carbohydrates, proteins, amino acids, and organic acids [37,38].

Methanogenic archaea are strictly anaerobic, with energy metabolism limited to the formation of methane from CO_2 and H_2, methanol, formate, methylamines and/or acetate [39]. The abundance and activity of methanogens are closely related to the transformation and degradation of organic matter in the system. The methanogenic archaeal community in the anaerobic tank could be mainly categorized as acetoclastic, hydrogenotrophic, or methylotrophic methanogens (Table S2 in Supporting information). It is of note, that with ongoing operation of the bioreactor, the abundance of all methanogens increased. *Methanosaeta* were the most abundant methanogens in the system, accounting for 15.58% of all archaea, which is an acetoclastic methanogen that uses acetate for methane production [40]. *Methanosarcina* (1.01%) is an acetoclastic and hydrogenotrophic methanogen, capable of utilizing H_2 or acetate to produce methane [41]. There were also some hydrogenotrophic and methylotrophic methanogens identified with relative low abundances, such as *Methanoculleus*, *Candidatus_Methanofastidiosum*, and *Methanomassiliicoccus*. Overall, these results suggest that the utilization of acetate was the main pathway of methanogenesis in the anaerobic tank. However, no acetate was directly available in the mature landfill leachate, so the enrichment of acetoclastic methanogens mainly depended on their association with syntrophic and acetogenic bacteria (*e.g.*, *norank_f__norank_o__norank_c__JS1*, *Fastidiosipila*, *Syntrophaceticus,* and *Thermovirga*).

Overall, the dominant bacterial population in the anaerobic tank was gradually transformed from bacteria degrading organic matter to bacteria that are syntrophic with methanogens. This cooperation between multiple microbial groups, such as organic degradation bacteria, syntrophic bacteria, acetoclastic methanogens, and hydrogenotrophic methanogens, ensured the degradation or methanogenic utilization of organic matter in the anaerobic tank.

In the anoxic and oxic tanks, the genera *Limnobacter*, *Truepera*, and *norank_f__PHOS-HE36* (an unclassified genus of the family-level lineage *PHOS-HE36*, phylum Bacteroidota) were consistently present at a relatively high abundance, accounting for 10.71%–12.88%, 4.05%–6.46%, and 4.90%–7.43%, respectively. *Limnobacter* have been shown to play an important role in the decomposition of organic matter and denitrification, possibly by decomposing refractory organics into small molecular organics that are usable by other microorganisms [42]. *Truepera* is an important thermophilic denitrifier, which has salt-resistance and has been identified in some extreme environments, including municipal solid waste landfill [38,43,44]. In addition, *norank_f__PHOS-HE36* belongs to the phylum Bacteroidota, which can convert organic matter (such as proteins and polysaccharides) to small organic molecules [45]. It is of note, that the abundances of the genera *Ottowia*, *Phaeodactylibacter*, *Nitrosomonas*, *norank_f__norank_o__norank_c__JG30-KF-CM66* (an unclassified genus of the class-level lineage *JG30-KF-CM66*, phylum Chloroflexi), and *norank_f__67–14* (an unclassified genus of the family-level lineage 67–14, phylum Actinobacteriota) increased significantly after 280 days of operation. *Ottowia* has been widely detected in biological treatment systems for industrial wastewater, due to its capacity to perform hydrolysis and denitrification [46]. *Ottowia* had an abundance of 8.64% in the anoxic tank and 9.59% in the oxic tank, showing that it is capable of both organic matter degradation and denitrification in this continuous flow system under low DO concentration conditions. *Phaeodactylibacter* accounted for 7.80% in the anoxic tank and 11.22% in the oxic tank, with these genera previously proposed to be involved in the heterotrophic nitrosification process [47], suggesting it is credible that it may participate in the NH_4^+-N oxidation process. *Nitrosomonas* were found to have an abundance of 5.14% in the anoxic tank and 6.38% in the oxic tank, with these AOB commonly detected in wastewater treatment systems and responsible for converting NH_4^+-N to NO_2^--N [16]. It was identified that the genus *norank_f__norank_o__norank_c__JG30-KF-CM66* belongs to the phylum Chloroflexi, which are able to assimilate acetate [24], indicating that this genus may significantly contribute to the degradation of organic matter within the system.

Besides, there were also some genera of the phylum Chloroflexi found to be present at low abundance (0.15%–2.97%) which were highly salt-tolerant, such as *norank_f__JG30-KF-CM45, norank_f__Caldilineaceae*, and *norank_f__AKYG1722* (unclassified genera of the family-level lineage JG30-KF-CM45, Caldilineaceae, and AKYG1722, respectively) [48]. These genera may be important to maintaining systematic stability, which was consistent with the high salinity of landfill leachate.

Microbial interaction networks are used to reveal the competitive and cooperative relationships between two different genera. Correlation network analysis results for the anaerobic and oxic tanks are illustrated in Figs. 3d and e, respectively. The genera in these two tanks were divided into two competing groups, which were labelled as group 1 and group 2 in the anaerobic tank, and group 3 and group 4 in the oxic tank. The three major methanogenic archaea (*Methanosaeta*, *Methanosarcina*, and *Methanoculleus*) exhibited obvious cooperative relationships with syntrophic bacteria (*Syntrophaceticus*, *Thermovirga*, and *Fastidiosipila*), while distinct competitive relationships were observed with organic matter degrading bacteria (*Raineyella*, *Defluviicoccus*, and *Brooklawnia*). These results were consistent with the observed succession of microbial communities. The organic matter degrading bacteria that usually appear in nitritation-denitrification systems treating landfill leachate (*e.g.*, *Limnobacter*, *Ottowia*, *norank_f__norank_o__norank_c__JG30-KF-CM66*, and *norank_f__PHOS-HE36*) [11] exhibited a large number of collaborations with *Nitrosomonas* in the oxic tank, which may be crucial to the predominance and activity of *Nitrosomonas* by partly reducing the inhibition of organics [18]. Simultaneously, synergistic relationships were also identified between denitrifying bacteria (*Limnobacter*, *Truepera*, and *Ottowia*) and *Nitrosomonas*. Microorganisms associated with NH_4^+-N oxidization, denitrification and COD degradation can not only coexist while simultaneously serving their respective functions, but they can also be synergistic. Specifically, the NO_2^--N or NO_3^--N produced during NH_4^+-N oxidization could provide metabolic substrates for denitrification. Denitrification lessens the concentration of NO_2^--N and also causes an increase in pH due to the release of alkalinity, resulting in a decrease in FNA, which further reduced the inhibition of microorganisms within the system. Meanwhile, the utilization of organics by organic matter degrading bacteria and denitrifying bacteria reduced the risk of AOB inhibition by more heterotrophic bacteria. These beneficial effects would enhance the PN performance of the system and help form a more stable microbial community structure.

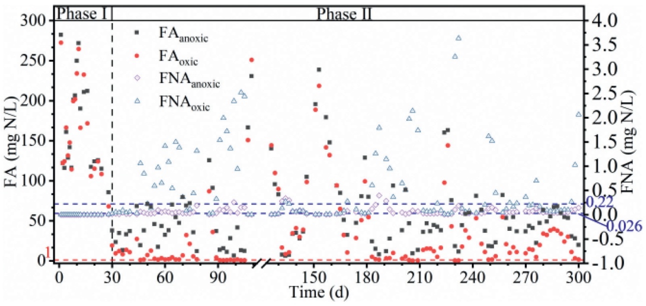

Fig. 4. Variations of FNA and FA concentrations in the anoxic and oxic tanks. The red line represents the inhibitory threshold of FA on NOB and the blue line represents the inhibitory threshold of FNA on NOB.

In this study, a continuous flow bioreactor with anaerobic-anoxic-oxic processes was applied to achieve the partial nitritation of mature landfill leachate. The start-up of this system required only 30 days and then maintained stable operation for 270 days, with the ratio of nitrite nitrogen to ammonia nitrogen remaining stable at 1–1.32, indicating excellent partial nitritation performance. As shown in Fig. 4, during phase II the average FA concentrations in the anoxic and oxic tanks were 55.87 mg N L^{-1} and 31.47 mg N L^{-1}, respectively, while the average FNA concentrations in the anoxic and oxic tanks were 0.068 mg N L^{-1} and 0.548 mg N L^{-1}, respectively. It has previously been reported that the inhibitory concentrations of FA on AOB and NOB were about 10–150 mg N L^{-1} and 0.1–1.0 mg N L^{-1}, respectively [13,18], while the inhibitory concentrations of FNA on AOB and NOB were 0.42–1.72 mg N L^{-1} and 0.026–0.22 mg N L^{-1}, respectively [10,49].

Therefore, the concentrations of FA and FNA in the present system induced a minimal level of AOB growth inhibition, while NOB were effectively restrained by the combination of FA and FNA. Due to the high ammonia nitrogen concentration and alkalinity of the mature landfill leachate, there was a relatively high concentration of FA in the anoxic tank to inhibit NOB. However, the degradation of ammonia nitrogen and the decreased pH in the oxic tank resulted in FA concentrations being below the threshold inhibitory value, while FNA concentration increased accordingly and achieved sufficient NOB suppression. Therefore, stable partial nitritation was achieved in this system *via* the alternate control of FA and FNA in different stages.

DO levels were kept relatively low (0.10–0.35 mg/L in the anoxic tank and 0.20–1.50 mg/L in the oxic tank) in order to prevent excessive ammonia nitrogen from being oxidized and thereby maintaining a specific ratio of nitrite nitrogen to ammonia nitrogen in the effluent. However, DO limitation can also inhibit the activity of AOB due to oxygen competition from heterotrophic organisms, resulting in a low NAR [50]. Therefore, an anaerobic tank was included as the first step, preliminarily removing excess organic matter, reducing the negative impact of organic matter on autotrophic microorganisms. Consequently, long-term stable partial nitritation was achieved under low DO conditions without an adverse impact on AOB.

Long-term stable partial nitritation of mature landfill leachate was achieved using a continuous flow bioreactor, through *in-situ* inhibition by a combination of FA and FNA. The NAR was maintained at around 94.43% for 270 days, with an average NO_2^--N/NH_4^+-N ratio of about 1.16. Microbial analysis showed that the inhibition of organics on AOB (*Nitrosomonas*) was alleviated during ongoing operation, even under relatively low DO conditions, due to the removal of organic matter through the cooperative actions of organic degradation bacteria, syntrophic bacteria, and methanogens. These collaborative interactions between different functional microorganisms further facilitated the predominance and high activity of *Nitrosomonas*, which was vital to the maintenance of an appropriate NO_2^--N/NH_4^+-N ratio and stable partial nitritation within the system.

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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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cclet.2023.108284.

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