



Simultaneous partial nitrification, anaerobic ammonium oxidation and denitrification (SNAD) in a full-scale landfill-leachate treatment plant

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ABSTRACT

The occurrence of simultaneous partial nitrification, anaerobic ammonium oxidation and denitrification (SNAD) observed in a single partially aerated full-scale bioreactor treating landfill-leachate is reported in this paper. At present, the full-scale bioreactor is treating an average leachate flow of 304 m³ d⁻¹ with a sludge retention time between 12 and 18 d. The average COD, NH₄⁺-N and NO₃⁻-N concentrations at the upstream end of the bioreactor, i.e., influent, are 554, 634 and 3 mg L⁻¹, respectively; whereas no NO₂⁻-N is detected in the influent. The percentage removals of COD and NH₄⁺-N in the bioreactor were 28% and 80%, respectively. A nitrogen mass balance approach was adopted to analyze the performance of SNAD in the full-scale bioreactor. The total nitrogen (TN) removal by combined partial nitrification and anaerobic ammonium oxidation is 68% and the heterotrophic denitrification contributes to 8% and 23% of TN and COD removals, respectively. The red granule in the bioreactor was analyzed by using fluorescence in situ hybridization and polymerase chain reaction. The results of both analytical methods confirm the presence of anaerobic ammonium oxidizing bacteria as the predominant species along with other Planctomycete-like bacteria. Overall, the SNAD process offers the simultaneous removals of nitrogen and COD in the wastewater.

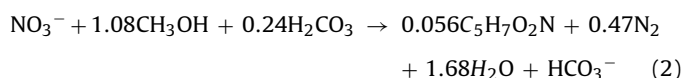
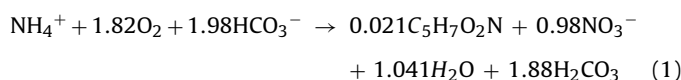
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1. Introduction

Landfilling of municipal waste is still one of the attractive options in the waste management system. However, the generation of leachate, a mixture of high concentration organic and inorganic contaminants including humic acids, ammonia nitrogen, heavy metals, xenobiotics and inorganic salts, remains an inevitable consequence of the existing waste disposal practice. Landfill-leachates need to be pretreated on-site to meet the standards for their discharge into the sewer or their direct disposal into surface water. The hydrosphere has become the main sink for excess nitrogen as a result of human activity [1,2]. Wastewater discharges containing excessive nitrogen can be toxic to aquatic life, deplete dissolved oxygen (DO) levels, cause eutrophication in receiving water bodies and affect the suitability of wastewater for reuse [3]. Therefore, more stringent nutrient discharge regulation has been

implemented to regulate the wastewater treatment plant effluent.

Conventional biological nitrogen removal processes using two reactors for nitrification and denitrification, respectively, for treating municipal and industrial wastewaters have been widely studied. Nitrogen is removed via two biological processes: (i) aerobic nitrification of NH₄⁺ by chemolithoautotrophic bacteria to NO₂⁻ or NO₃⁻ with O₂ as the electron acceptor and (ii) anoxic denitrification of NO₂⁻ or NO₃⁻ to gaseous N₂ by heterotrophic microorganisms using organic matter as carbon and energy source. The overall synthesis and oxidation reaction for nitrification is shown in Eq. (1); the combined dissimilation–synthesis equation for denitrification using methanol as electron donor is shown in Eq. (2) [4].



Generally, the conventional biological nitrogen removal process is used for treating wastewaters with relatively low nitrogen

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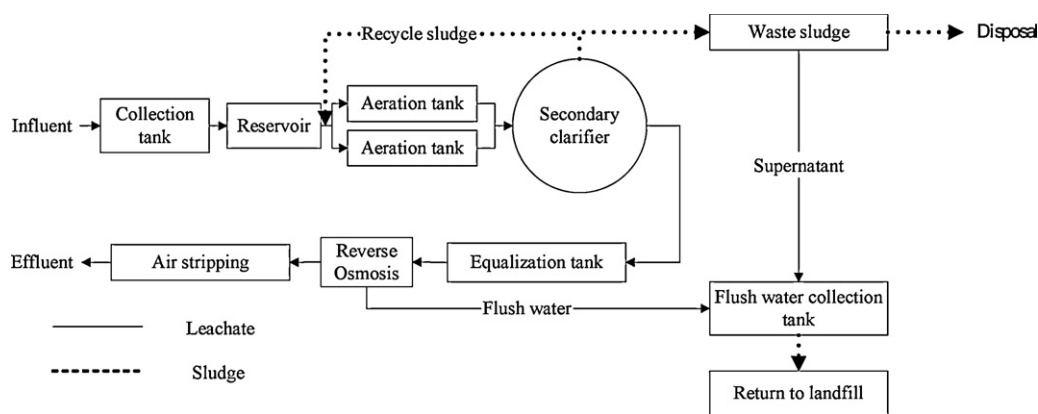
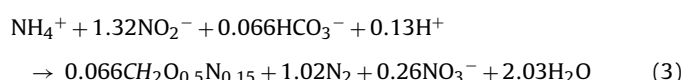


Fig. 1. Schematic diagram of the landfill-leachate treatment plant.

concentrations, i.e., total nitrogen (TN) concentration of approximately less than 100 mg L^{-1} as N. Some wastewaters such as anaerobic digester effluents, landfill-leachate, industrial wastewaters and agricultural surface runoffs contain high concentrations of nitrogen. Anaerobic ammonium oxidation (ANAMMOX) [5] and a single reactor system for high-activity ammonium removal over nitrate (SHARON) [6] are the recent findings for nitrogen removal. Among the nitrogen removal processes, ANAMMOX offers a novel, energy saving and cost-effective biological nitrogen removal method [5]. The process can be implemented using various configurations of reactor, e.g., completely autotrophic nitrogen removal over nitrite (CANON) [7], oxygen limited autotrophic nitrification–denitrification (OLAND) [8], single-stage nitrogen removal using ANAMMOX and partial nitrification (SNAP) [9] and simultaneous partial nitrification, ANAMMOX and denitrification (SNAD) [10], have been developed. ANAMMOX is widely accepted as the most energy saving process for nitrogen removal; it consumes 63% less oxygen and 100% less reducing agent (Eq. (3)) [11–13] than the conventional nitrification–denitrification process.



The biological activity of ANAMMOX has been studied using rotating biological contactor for treating ammonium-rich leachate [14], trickling filter for treating domestic wastewater [15], fixed-and fluidized-bed reactors and sequencing batch reactors for treating a synthetic wastewater [16,17]. However, sequencing batch reactor is considered by many researchers to be a suitable system for cultivating ANAMMOX bacteria [13,17,18].

The ANAMMOX process removes only about 90% of the incoming nitrogen as ammonium/nitrite and leaves about 10% of nitrogen as nitrate in the effluent (Eq. (3)). Moreover, the presence of oxygen and/or organic carbon can completely inhibit the ANAMMOX activity [16,19]. In order to meet stringent TN effluent standards, any improved process using ANAMMOX for treating wastewaters containing nitrogen should address these issues. Alternatively, a combination of ANAMMOX and denitrification in the same reactor would fit the requirements discussed above with facultative heterotrophic denitrifiers being the scavengers to remove residual oxygen, organic carbon and nitrate [20]. The possibility that ANAMMOX may co-exist with nitrification [21,22] or denitrification in a single-batch reactor has also been reported [2,10,23]. In this study, the SNAD process in a full-scale landfill-leachate treatment plant in Taiwan was investigated. The fluorescence in situ hybridization (FISH) and polymerase chain reaction (PCR) techniques were applied to verify the activity of ANAMMOX bacteria in the reactor. In addition, simultaneous nitrogen and chemical oxygen demand

(COD) removals in the treatment plant were evaluated using mass balance approach.

2. Materials and methods

2.1. Description of the full-scale landfill-leachate reactor

The landfill-leachate treatment plant in Taiwan has been in operation since 2006 to treat landfill-leachate with an average flow rate of $304 \text{ m}^3 \text{ d}^{-1}$. The schematic diagram of the treatment plant is shown in Fig. 1 and the wastewater characteristics in each unit process/operation are summarized in Table 1. The phenomena of simultaneous partial nitrification, denitrification and ANAMMOX were observed in two aeration tanks (15.6 m L by 4.1 m W by 3 m D) with 384 m^3 of working volume and 1.26 d of hydraulic residence time. The sludge retention time in the aeration tank was maintained between 12 and 18 d. The concentrations of mixed liquor suspended solids and mixed liquor volatile suspended solids in the aeration tanks were 2110 and 1505 mg L^{-1} , respectively. The aeration tanks were equipped with fine bubble tubular diffusers. The DO concentration in the reactor was maintained at approximately 0.3 mg L^{-1} , which facilitated the co-existence of ANAMMOX microorganisms and denitrifiers. The pH in the aeration tanks was around 7.4 and the temperature was found to be fluctuated under the influence of ambient temperature within $30\text{--}33 \text{ }^\circ\text{C}$ during the course of the study (October–December, 2008). Influent and effluent samples were collected from the aeration tanks on a regular basis for lab analyses. To ensure the data quality, all samples were split and analyzed in both the on-site lab in the landfill-leachate treatment plant and the lab at Institute of Environmental Engineering, National Chiao Tung University, Taiwan. The average COD, $\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$ concentrations at the upstream end of the

Table 1
Main characteristics of leachate.

Parameter ^a	Influent to aeration tank	Effluent from aeration tank
pH	7.9	7.3
TS	2610 ± 22 (3) ^b	1970 ± 31 (3)
VS	554 ± 27 (3)	448 ± 81 (3)
COD	554 ± 97 (15)	399 ± 59 (15)
BOD	57 ± 1 (3)	9 ± 1 (3)
$\text{NO}_2^-\text{-N}$	Not detectable	6 ± 1 (14)
$\text{NO}_3^-\text{-N}$	3 ± 1 (17)	23 ± 12 (17)
$\text{NH}_4^+\text{-N}$	634 ± 143 (18)	126 ± 57 (18)
$\text{PO}_4^{3-}\text{-P}$	4 ± 1 (3)	2 ± 1 (3)

^a All units in table are mg L^{-1} except pH.

^b Average \pm Std. (n).

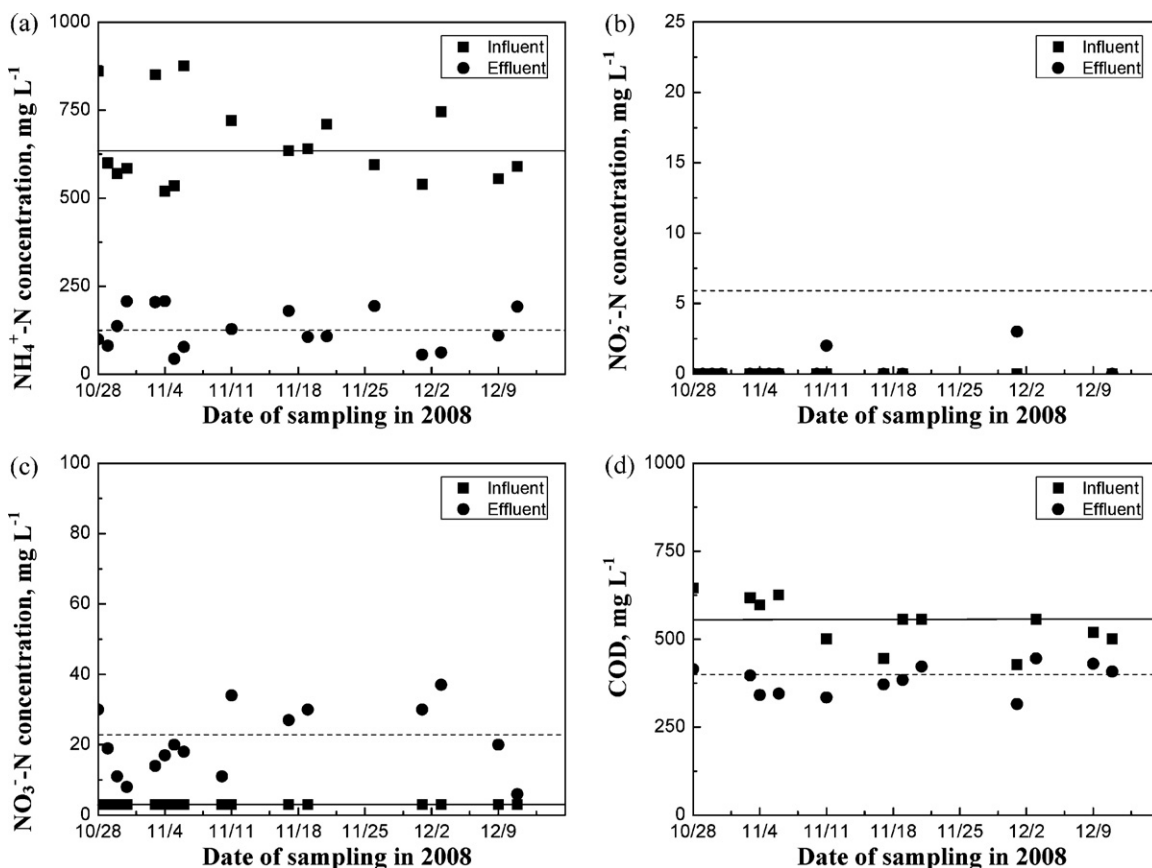


Fig. 2. The influent and effluent profiles of (a) $\text{NH}_4^+\text{-N}$, (b) $\text{NO}_2^-\text{-N}$, (c) $\text{NO}_3^-\text{-N}$ and (d) COD in the aeration tank. (Note: Solid and dash lines indicate the averages of influent and effluent concentrations, respectively.)

bioreactor, i.e., influent, were 554, 634 and 3 mg L⁻¹, respectively; whereas, $\text{NO}_2^-\text{-N}$ concentration was below the detectable limit at all times.

2.2. Chemical analysis

Grab samples of influents and effluents collected from the aeration tank (Fig. 1) were filtered through Whatman GF/F filters (0.45 μm). The filtrate was analyzed for ammonium nitrogen ($\text{NH}_4^+\text{-N}$) using the ammonium-selective electrode method. Nitrite nitrogen ($\text{NO}_2^-\text{-N}$) and nitrate nitrogen ($\text{NO}_3^-\text{-N}$) concentrations were determined spectrophotometrically according to the Standard Methods [24]. The organic matter content of the wastewater was analyzed according to the Standard Methods and expressed as COD [24]. The pH was determined potentiometrically with a digital pH meter (SUNTEX PC-320, Taiwan). The DO was measured with a digital DO meter (SUNTEX DC-5100, Taiwan).

2.3. Polymerase chain reaction (PCR)

The total genomic DNA present in the samples was extracted using the UltraClean Microbial DNA Isolation Kit (MO BIO Laboratories, USA). The 16S rDNA sequences were amplified from the genomic DNA by PCR using 11f (5'-GTTTGATCCTGGCTCAG-3') and 1512r (5'-GGYTACCTGTACGACTT-3') oligonucleotide primers [25] (referred as primer pair I). The thermal cycling consisted of 10 min at 94 °C followed by 35 cycles each of 90 s at 94 °C, 45 s at 52 °C, 120 s at 72 °C and ended by additional 10 min at 72 °C. The nucleotide sequences of PCR products were

determined using the BigDye terminator cycle sequencing kit (Applied Biosystems, USA). The resulting sequences were used to do nucleotide–nucleotide blast search through National Center for Biotechnology Information (NCBI). To amplify 16S rDNA of ANAM-MOX bacterium, PCR was performed using an oligonucleotide primer pair, 16S-1 (5'-AGTGGCGAAAGGTTGAGTAA-3') and 16S-2 (5'-TGTCTCAGTCCAGTGTGGC-3') [26] (referred as primer pair II). The thermal cycling consisted of 10 min at 94 °C followed by 40 cycles each of 15 s at 94 °C, 2 s at 55 °C, 30 s at 68 °C and ended by additional 5 min at 72 °C. In addition, oligonucleotide primer pair specific for amplifying 16S rDNA of ANAMMOX bacterium was used, i.e., 16S-5' (5'-TGGCGCGCTGGTTTAGGC-3') and 16S-3' (5'-GGTTACCTGTACGACT-3') [26] (referred as primer III) with thermal cycling of 10 min at 94 °C followed by 40 cycles each of 15 s at 94 °C, 2 s at 50 °C, 60 s at 68 °C and ended by additional 10 min at 72 °C.

2.4. Fluorescence in situ hybridization (FISH)

The 16S rRNA-targeted oligonucleotide probe used in this study was Amx820 [27] for ANAMMOX bacteria. The probe was synthesized and directly labeled with fluorescein isothiocyanate (FITC) at the 5' end. In situ hybridization was performed according to the procedure described by Amann et al. [28]. A 100 \times objective Olympus BX51 microscope (Olympus Optical Co., Japan) fitted with a mercury bulb and blue, green and red filter sets was used for viewing the slides. The photomicrograph was made using an Olympus U-CMAD 3 camera (Olympus Optical Co., Japan) with exposure times of 0.05 s for DAPI and 0.5 s for Amx820.

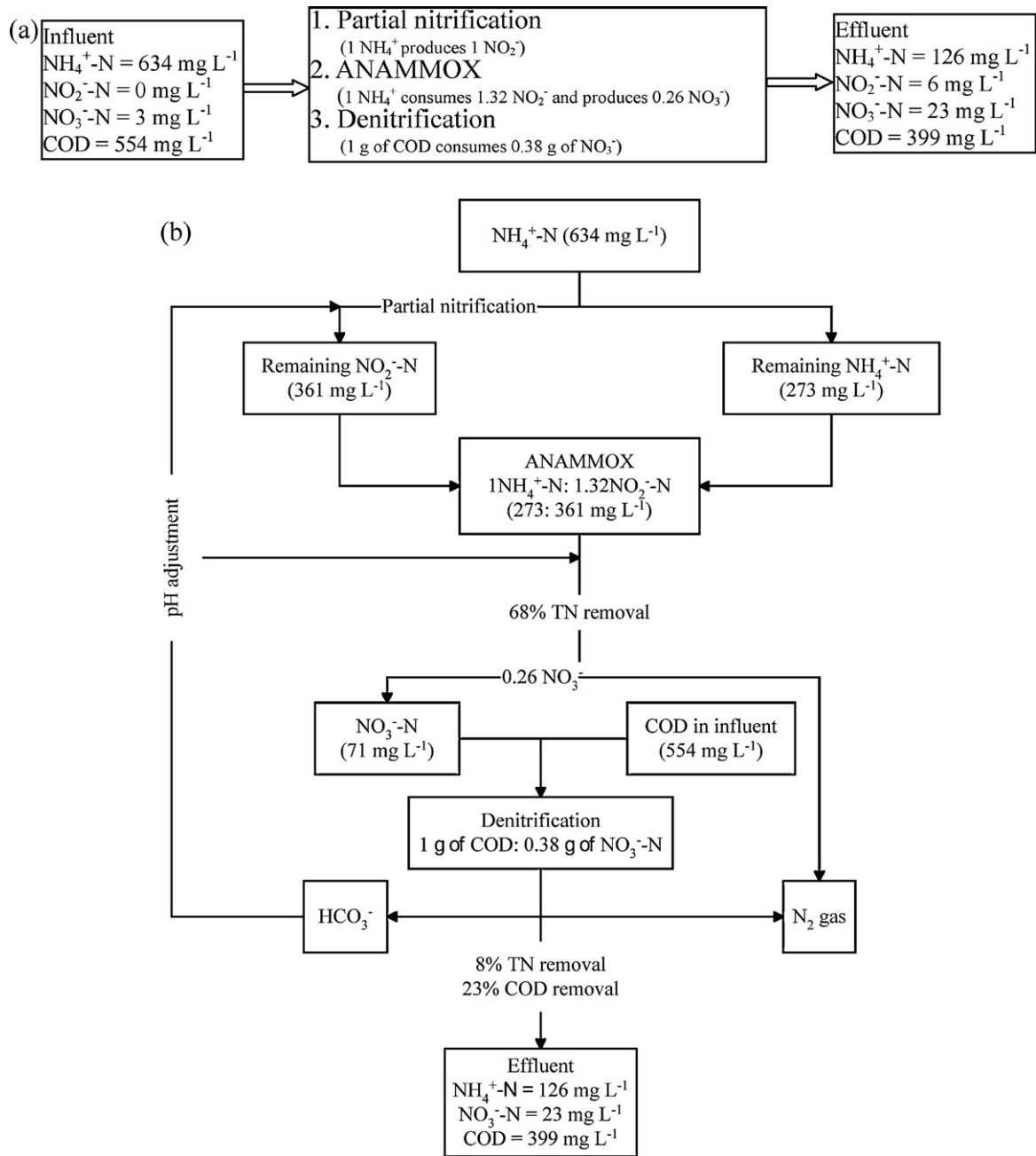


Fig. 3. Schematic diagrams showing (a) leachate characteristics feeding into the aeration tank and (b) the model based evaluation of SNAD process in the aeration tank.

3. Results and discussion

3.1. Nitrogen removal performance

Fig. 2(a–c) show the influent and effluent concentrations of nitrogenous compounds of the aeration tank. The $\text{NO}_2^-\text{-N}$ concentration in the aeration tank influent is below the detectable limit. However, a decrease in $\text{NH}_4^+\text{-N}$ concentration with simultaneous increase in $\text{NO}_3^-\text{-N}$ concentration is observed in the aeration tank. This implies a conventional chemolithoautotrophic oxidation of ammonium to nitrite by ammonium oxidizing bacteria (AOB) and subsequently from nitrite to nitrate by nitrite oxidizing bacteria. The low DO concentration in the aeration tank may inhibit the microbial activity so that a complete nitrification is not observed. Alternatively, $\text{NH}_4^+\text{-N}$ concentration can be consumed by ANAMMOX bacteria. Thermodynamically, ANAMMOX is favor-

able at two molar ratios of ammonium to nitrite, i.e., 1:1 and 1:1.67 [4,29,30]. When the ANAMMOX becomes favorable, two interlinked processes can be hypothesized to occur: (i) partial nitrification, i.e., ammonium to nitrite by the AOB, followed by (ii) ANAMMOX. Oxygen limited conditions can provide an adequate environment for a stable interaction between Nitrosomonas-like aerobic microorganisms and Planctomycete-like anaerobic bacteria [10,31,32]. Moreover, the concept of CANON process is also the combination of partial nitrification and ANAMMOX in a single reactor. Kuai and Verstrete [11] and Hippen et al. [33] used the concepts of CANON process in OLAND and aerobic deammonification processes, respectively [4]. Therefore, AOB is believed to oxidize ammonium to nitrite initially in the aeration tank by consuming DO to create an anaerobic microenvironment. The nitrite produced is utilized along with the remaining ammonium by the ANAMMOX bacteria to be converted into nitrogen gas in the anaerobic

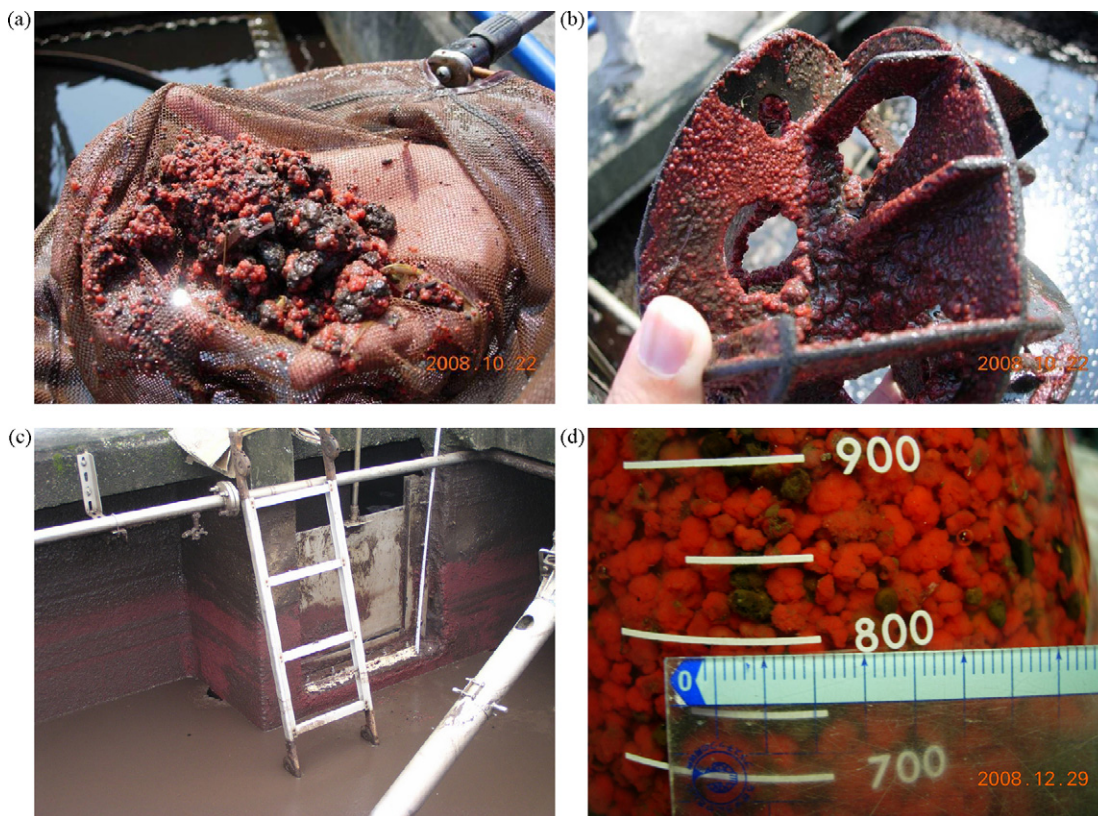


Fig. 4. (a) Granules in the aeration tank, (b) ANAMMOX granules attached on a carrier, (c) attached growth of ANAMMOX on the aeration tank wall and (d) ANAMMOX granules in a flask.

environment [34]. However, quantitative analyses on the ammonium converted to nitrite by AOB and the ammonium utilized in ANAMMOX are highly complicated.

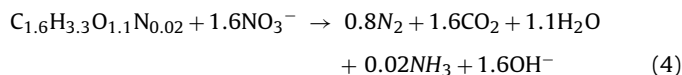
The maximum removal efficiencies of TN and $\text{NH}_4^+\text{-N}$ in the aeration tank are averagely 76% and 80%, respectively. Interestingly, a decrease in COD was also observed (Fig. 2(d)) in the aeration tank with simultaneous reduction of the $\text{NH}_4^+\text{-N}$ concentration. The COD is used by heterotrophic bacteria as carbon and energy sources during denitrification whereas nitrite and/or nitrate are used as electron acceptors. The affinity between nitrite and ANAMMOX bacteria is much higher than that between nitrite and denitrification bacteria [35]. Hence, nitrite produced from the partial nitrification could be consumed by the ANAMMOX bacteria immediately. Thus, the majority of nitrite is consumed by the ANAMMOX bacteria and the nitrate produced from ANAMMOX is consumed by the heterotrophic denitrification with simultaneous COD removal. Subsequently, the nitrite produced from partial denitrification is also consumed by ANAMMOX bacteria. Denitrification and ANAMMOX occur under anoxic condition in the presence of electron donors [3]. Several researchers reported the possibility of denitrification/partial denitrification and ANAMMOX in a single reactor [2,23,36]. Moreover, ANAMMOX was first discovered in a denitrifying-fluidized-bed reactor treating the effluent from a methanogenic reactor [29].

Recently, Chen et al. [10] identified the SNAD process for the simultaneous nitrogen and COD removal using a small scale non-woven rotating biological reactor. The mechanism of simultaneous SNAD is believed to account for the simultaneous removals of nitrogen and COD in the aeration tank of the landfill-leachate treatment plant. The stoichiometric relationships of partial nitrification, ANAMMOX and denitrification are used to estimate the quantities of nitrogen and COD consumed in the treatment plant.

3.2. Model based evaluation of partial nitrification, ANAMMOX and denitrification

If the aeration tank is considered as a black box (Fig. 3(a)), the quantity of nitrogen consumed in partial nitrification, ANAMMOX and denitrification can be modeled based on the stoichiometric equations. The monthly average data are used for modeling and the outcomes are shown in Fig. 3(b).

The modeling assumes stoichiometric relationships for several biological activities in the aeration tanks: (i) partial nitrification occurs in the aeration tank followed by ANAMMOX and denitrification, (ii) in partial nitrification, the molar ratio of $\text{NH}_4^+\text{-N}:\text{NO}_2^-\text{-N}$ is 1:1, (iii) the molar ratio of $\text{NH}_4^+\text{-N}:\text{NO}_2^-\text{-N}$ consumed in ANAMMOX is 1:1.32 and produces 0.26 mol of $\text{NO}_3^-\text{-N}$ (Eq. (3)), subsequently that can be utilized in denitrification, (iv) organic matter composition in wastewater is $\text{C}_{1.6}\text{H}_{3.3}\text{O}_{1.1}\text{N}_{0.02}$ (based on the elemental analysis of wastewater) and therefore, (v) theoretically, denitrification can utilize 1 mol of $\text{NO}_3^-\text{-N}$ per mole of COD consumption as shown in Eq. (4).



It can be seen from Fig. 3(b) that the combined partial nitrification and ANAMMOX remove about 68% of TN in the aeration tank. On the other hand, heterotrophic denitrification is responsible for the removal of 8% TN and 23% COD in the aeration tank. Whereas, the total COD removal in the aeration tank is 28%, i.e., COD reduced from 554 to 399 mgL^{-1} (Fig. 3(b)). The gap between the COD removals (5%) observed in the field samples (28%) and modeling results (23%) could be due to the COD consumption by other heterotrophic organisms in the aeration tank. Denitrification and ANAMMOX produce alkalinity to maintain the solution pH constant

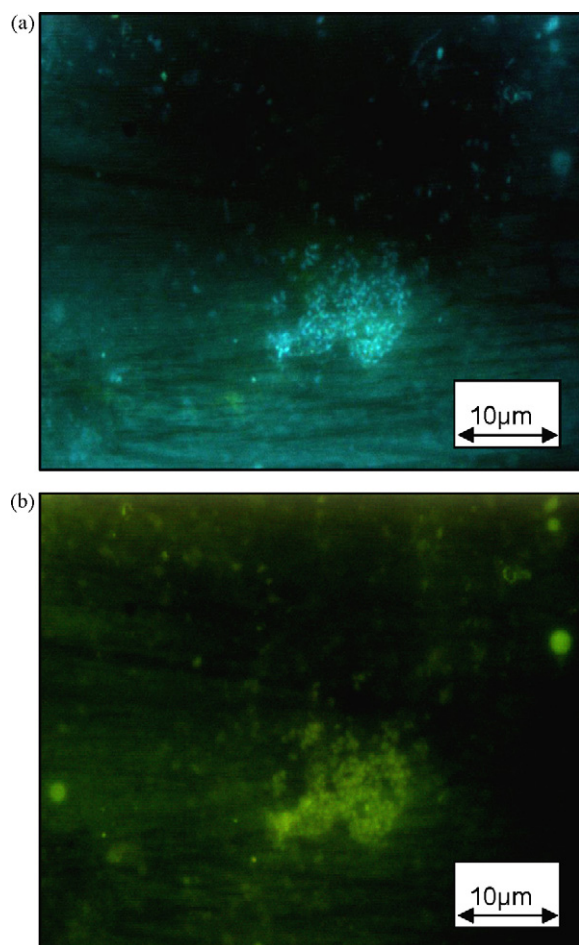


Fig. 5. Epifluorescence micrographs of bacteria granules collected from the aeration tank (a) DAPI staining and (b) Amx820.

while partial nitrification occurs. The stoichiometric calculations of NH_4^+ -N, NO_3^- -N and NO_2^- -N concentrations as shown in Fig. 3(b) are matched well with the corresponding effluent concentrations as shown in Table 1.

3.3. Observation of microbial community

At the time of sample collection for biotechnological analysis, several images of the bacterial granules exist in the aeration tank were captured (Fig. 4). The images show the red granules, which

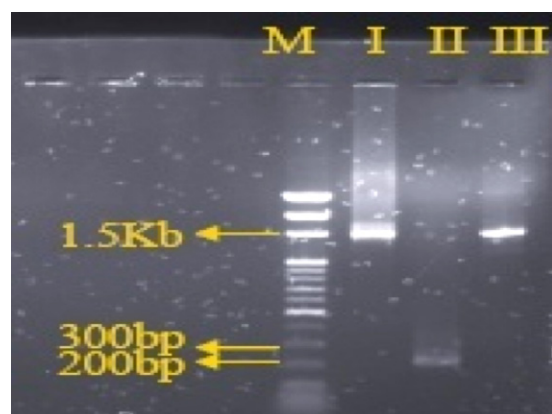


Fig. 6. Electrophoresis profiles of the PCR-amplified DNA fragments (I, II and III represent primer pairs I, II and III, respectively).

Table 2
Outcomes of sequence analysis.

Species identified	Similarity (%)	NCBI no.	Reference
Kuenenia stuttgartiensis	99	CT573071.1	[41]
Candidatus Kuenenia stuttgartiensis	99	AF375995.1	[42]
Anaerobic ammonium oxidizing Planctomycete KOLL2a	99	AJ250882.1	[37]
Candidatus Brocadia fulgida	93	EU478693.1	[43]
Planctomycete KSU-1	93	AB057453.1	[26]
Candidatus Brocadia fulgida	93	DQ459989.1	[44]
Candidatus Jettenia asiatica	92	DQ301513.1	[45]

found to be typical in ANAMMOX reactors [37–39]. The average diameter of ANAMMOX granules found in the field was 5 mm as shown in Fig. 4(d). Comparing the diameter of granules in the first full-scale ANAMMOX reactor, 1.4 mm granules were found [40]. Subsequently, the FISH analysis confirmed the occurrence of ANAMMOX bacteria in the aeration tank (Fig. 5).

Moreover, the results of PCR showed clear bands around 1500 bp marker in lane I (primer pair I), between the 200 and 300 bp markers in lane II (primer pair II) and 1500 bp marker in lane III (primer pair III) (Fig. 6). The results of sequence analysis are shown in Table 2. FISH and PCR results confirm the occurrence of many well-known ANAMMOX species. In addition, further studies are in progress towards the identification of AOB, denitrifiers and other autotrophic denitrifiers in the aeration tank.

4. Conclusions

The nitrogenous and carbonaceous compounds present in the aeration tank are removed by SNAD process. The overall removal efficiencies of nitrogenous and carbonaceous compounds in the SNAD process calculated using stoichiometric equations are 76% and 28%, respectively. The occurrence of the red granules demonstrates the existence of ANAMMOX bacteria; their existence in the granules is confirmed using the FISH and PCR analyses. Future studies are necessary to quantify the nitrifiers, ANAMMOX bacteria and denitrifiers in the aeration tank, using FISH and real-time PCR with more specific probes. Conforming the co-existence of microorganisms that achieve SNAD in a full-scale landfill-leachate treatment plant is a step forward for the treatment of ammonium rich-high strength wastewater.

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