# Removal of nitrogen from secondary effluent of a petrochemical industrial park by a hybrid biofilm-carrier reactor with one-stage ANAMMOX

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## ABSTRACT

A laboratory study was undertaken to explore the capability of one-stage ANAMMOX in a hybrid biofilm-carrier reactor (HBCR) fed with petrochemical wastewater. Under favorable operating conditions in continuous-flow operations (at the dissolved oxygen level of 0.5–1.0 mg $L^{-1}$), the average total nitrogen (TN) removal efficiency reached 62–67% and approximately 90% of TN can be removed by ANAMMOX. In batch operations of the hybrid biofilm-carrier reactor (without adding carbon substrate), the specific TN removal rate of the reactor in which both Kaldnes and nonwoven carriers were kept was two-fold higher than that of the reactor in which only nonwoven carriers were kept. This indicated that the microbial activity of thinner biofilms (Kaldnes carriers) was remarkably higher than that of thicker biofilms (nonwoven carriers). Finally, based on the 16S rRNA clone library, a cluster of ANAMMOX *Candidatus* Kuenenia stuttgartiensis was identified.

**Key words** | ANAMMOX, hybrid biofilm-carrier, petrochemical wastewater, specific total nitrogen removal rate

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## INTRODUCTION

It is known that the conventional nitrification–denitrification process is not very cost-effective for treating wastewater with a high ratio of nitrogen to chemical oxygen demand (COD) because the extra organic carbon (e.g., methanol) required for denitrifying bacteria must be added. Recently, cost-effective ANAMMOX reactors (autotrophic bacteria; no need of organic carbon), especially the one-stage ANAMMOX reactor, have been developed to remove total nitrogen (TN) from wastewater (Kartal *et al.* 2010).

The TN removal rate of a one-stage ANAMMOX biofilm-reactor is governed by complicated bio-kinetics of a mixed culture. Hao *et al.* (2002) reported that, in the one-stage ANAMMOX biofilm-reactor, an ammonium-oxidizing-bacteria biofilm forms the outer layer while an ANAMMOX biofilm forms the inner one. Such a two-layered structure should be beneficial for ammonium oxidizing bacteria to consume oxygen in the outer layer and thereby can prevent the occurrence of inhibition of ANAMMOX (by oxygen) in the inner layer. In the last decade, the competition between ANAMMOX and nitrite oxidizing bacteria for nitrite as well as the competition between ammonium oxidizing bacteria and nitrite oxidizing bacteria for oxygen have been two major issues. Maintaining a very low dissolved oxygen (DO) level of 0.2–1.0 mg $L^{-1}$ in one-stage ANAMMOX biofilm-reactors (or granular sludge reactors) has been postulated to be a key factor favoring ammonium oxidizing bacteria over nitrite oxidizing bacteria (Nielsen *et al.* 2005; Winkler *et al.* 2011). In addition, Winkler *et al.* (2011) reported that the biomass segregation in granular sludge reactors is an important factor in selecting a specific microbial group by varying different sludge retention times.

Biomass aggregation or granulation in granular sludge reactors generally depends on biological, chemical and physical conditions, such as the specific microbial

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ecology, toxic chemicals, shear force, nucleation, initial adhesion, attachment and detachment (Pol *et al.* 2004; Arrojo *et al.* 2006). Thus, the replacement of a granular sludge reactor by a biofilm-carrier reactor can be considered as a fairly good alternative to mitigate the aforesaid disadvantages of a granular sludge reactor. It is noted that many advantages of one-stage ANAMMOX biofilm-reactors have been claimed, including compactness, tolerance to inhibiting substances and the prevention of washout of biomass from the reactor (Gaul *et al.* 2005; Gong *et al.* 2007).

In the present study, a laboratory study was undertaken to explore the capability of one-stage ANAMMOX in removing total nitrogen from petrochemical wastewater. Therefore, a hybrid biofilm-carrier reactor (HBCR) loaded with two different kinds of carriers (Kaldnes and nonwoven carriers) was used to carry out continuous-flow and batch operations. In this article, the microbial activity of biofilms (i.e., expressed in total nitrogen removal rate) and the examination of the microbial community in the hybrid biofilm-carrier reactor are also discussed.

## MATERIALS AND METHODS

### Hybrid biofilm-carrier reactor

A hybrid biofilm-carrier reactor (10 L) loaded with two different kinds of carriers (1,200 pieces of Kaldnes carrier loaded into a 5-L rising compartment; 100 pieces of nonwoven carrier loaded into a 5-L compartment) was used. A schematic diagram of the reactor is presented in Figure 1. A separating plate was inserted into the reactor to divide it into two compartments leading to a rising and a descending flow pattern, respectively. By aerating at the bottom of the rising compartment, a looped-flow regime can be achieved.

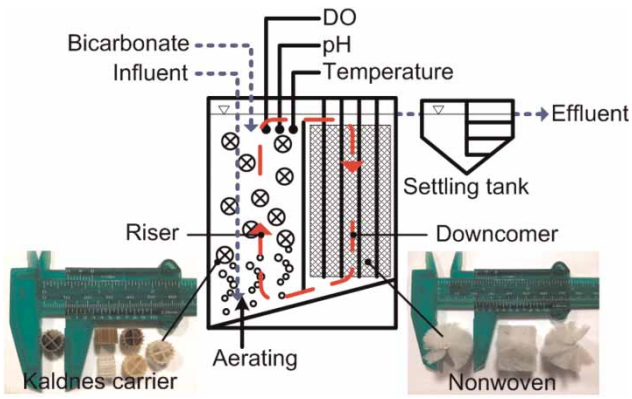


**Figure 1** | Schematic diagram of hybrid biofilm-carrier reactor.

### Bioreactor operation

The hybrid biofilm-carrier reactor was first inoculated with aerobic sludge taken from an activated sludge system located in a local petrochemical industrial park. Then the reactor was fed with petrochemical wastewater (Table 1) to carry out continuous-flow operations (runs 1–5) by maintaining a fixed pH and temperature but altering DO levels. In run 1, the DO, pH and temperature were maintained at 2–4 mg $O_2\,L^{-1}$, $8.0 \pm 0.2$ and $30 \pm 2\,^{\circ}C$, respectively. To ensure that seed sludge can be attached onto both the Kaldnes and the nonwoven carriers, the reactor in run 1 was continuously operated for a longer period of 2 months. In each test run, the $NH_4^+$-N, $NO_2^-$-N and $NO_3^-$-N in the influent and effluent were monitored every 2–3 d. After the aforesaid continuous-flow operations had been completed, the same reactor was also used to conduct batch runs 1 and 2. In batch run 1, both the Kaldnes and nonwoven carriers were kept in the reactor; whereas in batch run 2, the Kaldnes carriers were removed from the reactor but the nonwoven carriers were still kept in the reactor. Except that the petrochemical wastewater was replaced by synthetic wastewater (made by ammonium chloride, necessary nutrients and trace elements, as described in van de Graaf *et al.* (1996)), without the addition of carbon substrate, and the DO level was maintained at 0.5–1.0 mg $L^{-1}$, other control parameters including pH and temperature were kept the same as those in the continuous-flow operations.

**Table 1** | Chemical composition of secondary effluent of petrochemical industrial park

| Constituent | *N* | Average ± SD[a] |
|---|---|---|
| COD (mg $L^{-1}$) | 7 | 310 ± 121 |
| TKN[b] (mg $L^{-1}$) | 7 | 330 ± 89 |
| Organic-N[c] (mg $L^{-1}$) | 7 | 12 ± 5 |
| $NH_4^+$-N (mg $L^{-1}$) | 7 | 325 ± 76 |
| $NO_2^-$-N (mg $L^{-1}$) | 7 | 0.30 ± 0.02 |
| $NO_3^-$-N (mg $L^{-1}$) | 7 | 1.20 ± 0.70 |
| TKN/COD (–) | – | 1.06 |

[a]SD: standard deviation.
[b]TKN: total Kjeldahl nitrogen.
[c]The organic-N was determined by subtracting $NH_4^+$-N from TKN.

## Analytical methods

Total Kjeldahl nitrogen (TKN), $NH_4^+$-N, $NO_2^-$-N $NO_3^-$-N, COD, suspended solids (SS) and volatile suspended solids (VSS) were analyzed according to *Standard Methods* (American Public Health Association (APHA) 1998). The procedures for measurements of the biomass weight (triplicated; expressed in SS and VSS) are briefly described as follows. After batch operations of the hybrid biofilm-carrier reactor were completed in this work, the suspended biomass concentrations in the rising and descending compartments were measured. Also, 20 pieces of the Kaldnes carrier and five pieces of the nonwoven carrier were respectively randomly removed from the rising and descending compartments for the determination of the attached biomass. Then a plastic brush was used to disrupt biofilms from the carriers into a beaker, which was previously filled with 50 mL of de-ionized water. Thereafter, the biomass concentration in the beaker (under a well-agitated condition) was measured, followed by the calculation of the biomass weight.

## Genomic DNA extraction, polymerase chain reaction (PCR) amplification, and phylogenetic analysis

The genomic DNA was extracted from the microbial cells using a modification of the sodium dodecyl sulfate-based extraction method (Miller *et al.* 1999). In the present study, the extracted DNA was used to amplify the 16S rRNA gene to PCR products. For the identification of ANAMMOX, the primers AMX368F (5′-CCT TTC GGG CAT TGC GAA-3′) and 1392R (5′-ACG GGC GGT GTG TAC-3′) were used (Schafer & Muyzer 2001; Schmid *et al.* 2003). For the identification of universal bacteria, 11F (5′-GTT TGA TCC TGG CTC AG-3′) and 1512R (5′-GGY TAC CTT GTT ACG ACT T-3′) were used (Amann *et al.* 1995). Thereafter, the PCR products were purified using the Qiaquick PCR purification kit (Qiagen, Düsseldorf, Germany). According to the manufacturer's protocol (Invitrogen), the purified PCR products were ligated with the pGEM-T Easy Vector System (Promega, Madison, WI, USA) and thereafter transformed into DH5α *Escherichia coli* competent cells. Clones were then sequenced by the Genomic Medicine Center in National Cheng Kung University. Finally, the sequences were aligned using BioEdit and compared to sequences stored in GenBank using BLAST. The obtained 16S rRNA gene sequences were deposited in GenBank under the accession number of KF945664 – KF945679.

# RESULTS AND DISCUSSION

## Performance of continuous-flow operations in hybrid biofilm-carrier reactor

The performance data of continuous-flow operations in the hybrid reactor are presented in Figure 2 and Table 2. Theoretically, the same percentage of $NH_4^+$-N (by neglecting a small amount of $NH_4^+$-N utilized in biosynthesis) should be converted to $NO_2^-$-N plus $NO_3^-$-N if only ammonia

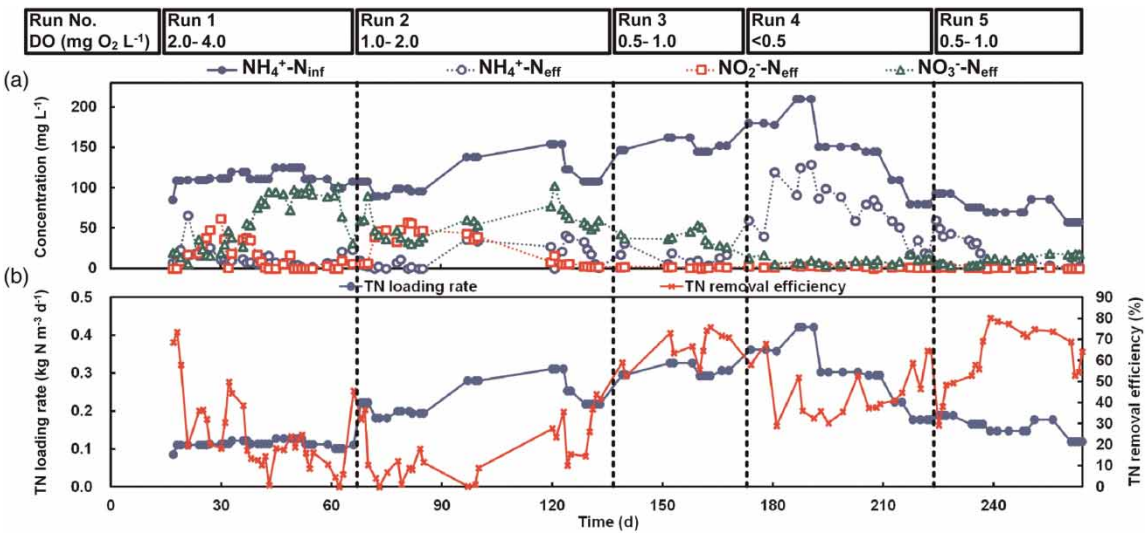


**Figure 2** | (a) $NH_4^+$-N, $NO_2^-$-N and $NO_3^-$-N concentrations; (b) TN loading rate, TN removal rate and TN removal efficiency of continuous-flow hybrid biofilm-carrier reactor.

**Table 2** | Performance data of continuous-flow operations in hybrid biofilm-carrier reactor

| Parameter | Run 1 | Run 2 | Run 3 | Run 4 | Run 5 |
|---|---|---|---|---|---|
| DO (mg $L^{-1}$) | 2.0 – 4.0 | 1.0 – 2.0 | 0.5 – 1.0 | < 0.5 | 0.5 – 1.0 |
| HRT[a] (d) | 1.0 | 0.5 | 0.5 | 0.5 | 0.5 |
| $NH_4^+$-$N_{inf}$ (mg $L^{-1}$) | 112 ± 9 | 115 ± 21 | 152 ± 8 | 145 ± 45 | 75 ± 13 |
| $NH_4^+$-$N_{eff}$ (mg $L^{-1}$) | 12 ± 12 | 15 ± 15 | 11 ± 9 | 70 ± 35 | 20 ± 19 |
| $NO_2^-$-$N_{eff}$ (mg $L^{-1}$) | 13.9 ± 17.1 | 23.6 ± 21.0 | 1.2 ± 0.6 | 1.9 ± 0.9 | 0.5 ± 0.4 |
| $NO_3^-$-$N_{eff}$ (mg $L^{-1}$) | 59.7 ± 33.1 | 55.9 ± 17.4 | 38.4 ± 9.2 | 9.5 ± 3.8 | 10.5 ± 5.4 |
| TN loading rate (kg N $m^{-3}$ $d^{-1}$) | 0.11 ± 0.01 | 0.23 ± 0.04 | 0.31 ± 0.02 | 0.29 ± 0.08 | 0.16 ± 0.02 |
| TN removal efficiency (%) | 25 ± 18 | 19 ± 16 | 67 ± 8 | 45 ± 12 | 62 ± 14 |
| $\Delta NO_3^-$-N/$\Delta NH_4^+$-N[b] (–) | 0.56 ± 0.29 | 0.54 ± 0.14 | 0.27 ± 0.07 | 0.10 ± 0.03 | 0.15 ± 0.09 |

[a]HRT: hydraulic retention time.

[b]The calculated value is based on the stoichiometric biochemical reaction of one-stage ANAMMOX ($NH_3 + 0.85O_2 \rightarrow 0.11NO_3^- + 0.44N_2 + 0.14H^+ + 1.43H_2O$).

oxidizing bacteria and nitrite oxidizing bacteria are present in the reactor (i.e., ANAMMOX is absent). Nonetheless, as shown in Figure 2(a), when the DO levels were maintained at 2–4 (run 1) and 1–2 mg $L^{-1}$ (run 2), 87–89% of $NH_4^+$-N was converted to 49–53% of $NO_3^-$-N and 12–21% of $NO_2^-$-N. This implied that the biochemical reaction of ANAMMOX did occur in the reactor to convert $NH_4^+$-N and $NO_2^-$-N to $N_2$ and $NO_3^-$-N. With a decrease of the DO level to 0.5–1.0 mg $L^{-1}$ (run 3), 92% of $NH_4^+$-N was converted to 25% of $NO_3^-$-N and 1% of $NO_2^-$-N, implying that in the reactor ANAMMOX outcompeted nitrite oxidizing bacteria for nitrite. However, with a further decrease of the DO level to less than 0.5 mg $L^{-1}$ (run 4), the extent of biochemical reactions of ammonia oxidizing bacteria and ANAMMOX markedly decreased because the residual concentration of $NH_4^+$-N (40–100 mg $L^{-1}$) was much higher than that of the previous run 3 (10–20 mg $L^{-1}$). It was repeatedly proven in the following run 5 (i.e., by maintaining the DO level at 0.5–1 mg $L^{-1}$) that a similar result to that of run 3 can also be obtained. Accordingly, if ANAMMOX is expected to prevail in the reactor, it is suggested to maintain the DO level at 0.5–1.0 mg $L^{-1}$. Moreover, Figure 2(b) showed that, at the TN loading rates of 0.11–0.32 kg N $m^{-3}$ $d^{-1}$, the average TN removal efficiencies can reach 67% in run 3 and 62% in run 5 when the DO level was maintained at 0.5–1.0 mg $L^{-1}$. Under such a favorable operating condition for one-stage ANAMMOX, the average TN concentration of 101 mg $L^{-1}$ can be removed from wastewater. It is noted that TN was mainly removed by one-stage ANAMMOX because only approximately 30 mg COD $L^{-1}$ (the average value of the difference between the influent and effluent COD concentrations measured in run 5) was consumed by heterotrophic denitrifying bacteria to reduce approximately 8.1 mg $L^{-1}$ of $NO_3^-$-N to $N_2$ (including the utilization of $NO_3^-$-N in biosynthesis; the calculated value is based on the stoichiometric biochemical reactions of denitrifiers). In other words, approximately 90% of TN was removed by one-stage ANAMMOX. In contrast, under an unfavorable operating condition for one-stage ANAMMOX, the average TN removal efficiencies can only achieve 19–25% in runs 1 and 2 and 45% in run 4 when the DO levels were maintained at 1.0–4.0 mg $L^{-1}$ and less than 0.5 mg $L^{-1}$, respectively.

According to the stoichiometric biochemical reactions of one-stage ANAMMOX, the ratio of $\Delta NO_3^-$-N to $\Delta NH_4^+$-N should be 0.11 if only one-stage ANAMMOX occurs in the reactor (Sliekers *et al.* 2002). As shown in Table 2, even under the favorable operating conditions for one-stage ANAMMOX, the calculated average ratios of $\Delta NO_3^-$-N to $\Delta NH_4^+$-N (0.27 in run 3 and 0.15 in run 5) were relatively higher than 0.11, disclosing that the occurrence of oxidation of nitrite by nitrite oxidizing bacteria cannot be easily avoided.

## Performance of batch operations in hybrid biofilm-carrier reactor

The biomass characteristics and the performance data of batch operations (without the addition of carbon substrate) in the hybrid reactor are presented in Tables 3 and 4, respectively. It is noted that the attached and suspended biomass concentrations were determined right after the batch runs 1 and 2 were completed. As shown in Table 3, the average attached biomass weight in the rising and descending compartments were 5.5 and 12.2 g VSS, respectively; while the average suspended biomass weight in the rising and

**Table 3** | Biomass characteristics determined in hybrid biofilm-carrier reactor

| Parameter | Attached biomass | | Suspended biomass | | Total biomass |
|---|---|---|---|---|---|
| | Kaldnes | Nonwoven | Rising compartment | Descending compartment | |
| VSS (mg piece$^{-1}$) | 4.6 ± 0.4 | 122.0 ± 16.3 | – | – | – |
| Biomass weight (g VSS) | 5.5 | 12.2 | 4.3 | 3.5 | 25.6 |
| Average VSS in reactor (mg L$^{-1}$) | – | – | 960 | 880 | – |
| VSS/SS (–) | 0.27 | 0.34 | 0.46 | 0.48 | – |
| VSS/VSS$_{total}$ (–) | 0.22 | 0.48 | 0.17 | 0.14 | 1.00 |

**Table 4** | Performance data of batch operations in hybrid biofilm-carrier reactor

| Parameter | Run 1 | Run 2[a] |
|---|---|---|
| DO (mg L$^{-1}$) | 0.5–1.0 | 0.5–1.0 |
| Initial $NH_4^+$-N (mg L$^{-1}$) | 170 | 154 |
| Normalized VSS in reactor[b] (mg L$^{-1}$) | 2560 | 2010 |
| TN removal efficiency (%) | 44 | 16 |
| TN removal rate[c] (kg N m$^{-3}$ d$^{-1}$) | 0.10 | 0.04 |
| Specific TN removal rate (kg N kg VSS$^{-1}$ d$^{-1}$) | 0.04 | 0.02 |

[a]Run 2's data were obtained with the Kaldnes carriers removed from the rising compartment, whereas the nonwoven carriers remained in the descending compartment.
[b]The normalized VSS concentration in the reactor was computed by dividing total biomass weight by 10 L of reactor volume.
[c]TN removal rate was determined by linear regression of all data points obtained from the batch operations.

descending compartments were 4.3 and 3.5 g VSS, respectively. In other words, the quantity of biomass retained in the reactor was in the following decreasing order: the biomass attached onto nonwoven carriers, the biomass attached onto Kaldnes reactor, the biomass suspended in the rising compartment, and the biomass suspended in the descending compartment. As expected, a higher TN removal efficiency of 44% was achieved in the reactor in which both Kaldnes and nonwoven carriers were kept; whereas a lower TN removal efficiency of 16% was reached in the reactor in which only nonwoven carriers were kept (Table 4). In order to clarify the capability of biofilms attached onto Kaldnes and nonwoven carriers, in the present study the specific TN removal rate is reasonably used to represent the microbial activity of ANAMMOX. As shown in Table 4, the specific TN removal rate of the reactor in which both Kaldnes and nonwoven carriers were kept (0.04 kg N kg VSS d$^{-1}$) was significantly higher than that of the reactor in which only nonwoven carriers were kept (0.02 kg N kg VSS d$^{-1}$). This indicated that the microbial activity of the biomass attached onto Kaldnes carriers was remarkably higher than that of the biomass attached onto nonwoven carriers. This can be explained as follows: the small size of Kaldnes carriers loaded into the rising compartment resulted in a rigorous mixing effect, leading to a strong shear force to detach biofilms (i.e., formation of thin biofilm). In contrast, the large size of loaded nonwoven carriers (six-fold size of Kaldnes carrier) fixed in the descending compartment resulted in a stagnating effect, leading to a weak shear force to detach biofilms (i.e., formation of thick biofilm). Our observations also comply with the above statements that the biofilm thickness of Kaldnes carriers was much thinner than that of nonwoven carriers. Consequently, the specific TN removal rate in the descending compartment should be diffusion-controlled; whereas the specific TN removal rate in the rising compartment could be reaction-controlled.

## Microbial community in hybrid biofilm-carrier reactor

In the present study, the microbial community of the biofilms attached onto nonwoven carriers (i.e., run 3 of continuous-flow operations) was examined. On the basis of restriction fragment length polymorphism patterns, a bacterial 16S rRNA gene clone library was established. Ninety-eight clones randomly selected from the PCR amplicon of universal primer pair can be categorized into nine phylotypes, whereas 50 clones randomly selected from the PCR amplicon of ANAMMOX-specific primer can be categorized into one phylotype. The sequences of phylotypes of approximately 1,000 bp in length were determined.

The positions of the present study's phylotypes relative to those of the selected GenBank's phylotypes are presented in Figure 3. The phylotype clones HBCRUNI16 and HBCRUNI14 were found to be very closely related to *Nitrosomonas europaea* and *Nitrosomonas* sp. ls, respectively (99% of sequence similarity). The phylotype clone HBCRUNI3

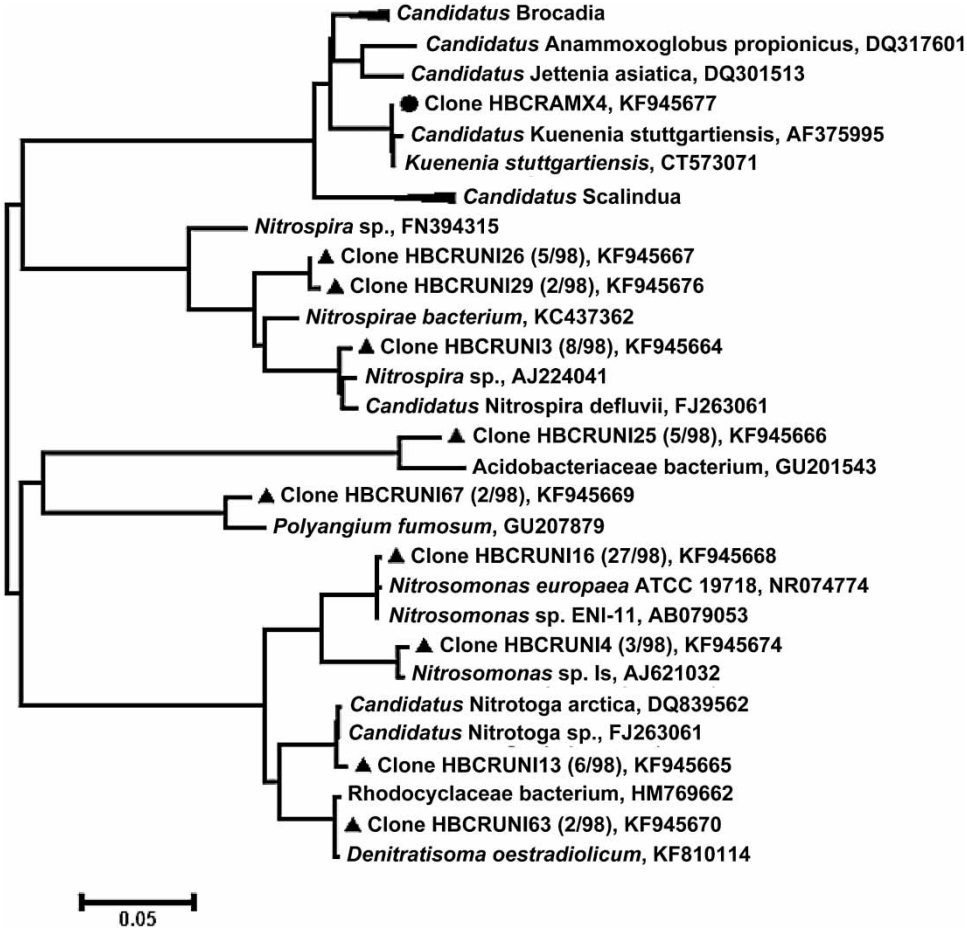


**Figure 3** | A 16s rRNA-gene-based phylogenetic tree of ammonium oxidizing bacteria, nitrite oxidizing bacteria and ANAMMOX in hybrid biofilm-carrier reactor.

was closely related to *Nitrospira* sp. (98% of sequence similarity), while the phylotype clones HBCRUNI26 and HBCRUNI29 were fairly closely related to *Nitrospirae bacterium* (93% of sequence similarity). The phylotype clone HBCRAMX4 was found to be very closely related to *Candidatus* Kuenenia stuttgartiensis (ANAMMOX; 99% of sequence similarity). Accordingly in the present study, ammonium oxidizing bacteria (*Nitrosomonas europaea*; *Nitrosomonas* sp. ls), ANAMMOX (*Candidatus* Kuenenia stuttgartiensis) and nitrite oxidizing bacteria (*Nitrospira* sp.; *Nitrospirae bacterium*) were evidently present in the hybrid biofilm-carrier reactor.

## CONCLUSIONS

According to the continuous-flow operating data of the hybrid biofilm-carrier reactor, at the TN loading rates of 0.11–0.32 kg N $m^{-3}$ $d^{-1}$, the average TN removal efficiency can reach 62–67% when the DO level is maintained at 0.5–1.0 mg $L^{-1}$; whereas the average TN removal efficiencies decreased to 19–25% and 45% when the DO levels were maintained at 1.0–4.0 mg $L^{-1}$ and less than 0.5 mg $L^{-1}$, respectively. Accordingly, if ANAMMOX is expected to prevail in the reactor, it is suggested to maintain the DO level at 0.5–1.0 mg $L^{-1}$.

From the batch operating data of the hybrid biofilm-carrier reactor (without the addition of carbon substrate), the specific TN removal rate of the reactor in which both Kaldnes and nonwoven carriers were kept (0.04 kg N kg VSS $d^{-1}$) was significantly higher than that of the reactor in which only nonwoven carriers were kept (0.02 kg N kg VSS $d^{-1}$). In other words, the microbial activity of the biomass attached onto Kaldnes carriers was remarkably higher than that of the biomass attached onto nonwoven carriers. Based on the 16S rRNA clone library, ANAMMOX (*Candidatus* Kuenenia stuttgartiensis) was evidently present in the hybrid biofilm-carrier reactor.

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