EnvironmentAsia
The international journal by the Thai Society of Higher Education Institutes on Environment

EnvironmentAsia 13 (Special issue) (2020) 38-45
DOI 10.14456/ea.2020.20
ISSN 1906-1714; ONLINE ISSN: 2586-8861

Anammox Process in Thai Wastewater Treatment Systems (Constructed Wetland)

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Abstract

Microbial characterization were identified uncultured *Candidatus* Accumulibacteria phosphatis clade and *Candidatus* Nitrotoga sp. in sediment and root plant biofilm of full scale of constructed wetland site at Laem Phak Bia (located at Phetchaburi Province, Thailand) but not full scale of constructed wetland to treat only storm water at Fukuoka, Japan. This result potentially suggests that constructed wetlands may be able to remove nitrogen under anaerobic conditions without significant organic carbon source. In a pilot-scale constructed wetland, three wetlands cells were inoculated with enriched suspended anammox cultures and fed synthetic wastewater with 50, 70, and 90 mg N/L of both NH_4^+-N and NO_2^--N (1:1.32) at controlled retention time (3 and 5 days).

After 5 months days of operation, at different HRT (3 and 5 days) NH_4^+-N removal efficiencies ranged from 92.3% and 95.5%, respectively and NO_2^--N removal efficiencies from 96.5% to 99.7%, respectively. In the control, NH_4^+-N average removal efficiencies were 82.5% and 82.4%, respectively and NO_2^--N average removal efficiencies were 70.4% and 72.6%, respectively. These results suggest that anammox bacteria can grow and establish naturally in horizontal subsurface-flow constructed wetlands and that such systems may be effective for the treatment of wastewater with low BOD but high nitrogen for water reclamation.

Keywords: Anammox; constructed wetlands; Thailand

1. Introduction

The main source of water pollution in Thailand comes from domestic wastewater (Department of Pollution Control, 2015). For this reason, the Thai Government invested significant funds to build many wastewater treatment plants (WWTPs) to treat domestic wastewater at many big cities like Bangkok, Chiang-Mai, Phetchaburi Provinces, etc. Moreover, the Thai Government tried to promote the use of both reclaimed water and domestic WWTP effluent to solve the water shortages that occur during the summer season. However, the effluent quality from most Thai WWTPs is insufficient for reuse

and water reclamation. A parameter of major concern for reclaimed water and water reuse is nitrogen, but the nitrogen removal efficiency in the nitrification-denitrification process in many WWTPs in Thailand is significantly low because of insufficient carbon content for denitrification (Noophan *et al*. 2007 and 2009). Bangkok has eight WWTPs: Si Phraya, Rattanakosin, Thung Kru, Nongkhaem, Jatujak, Dendaeng, Chongnonsi, and Bang Bue; the average efficiency of nitrogen removal at these seven plants is less than 50%. Adding an external carbon source such as acetate, glucose, ethanol, methanol is a simple way to resolve the issue of insufficient carbon content for denitrification. However, addition of external carbon could be a major operating cost for high volumes of wastewater. Also, skilled plant operation is significantly needed to control addition of the external carbon into these systems.

An alternative process of biological nitrogen removal is anammox (anaerobic ammonium oxidation). The anammox process is capable to provide an efficient method of nitrogen removal from wastewater that contains low BOD but high nitrogen. Although this technology is investigating and developing over two and half decade, the process is still not commonly applied worldwide at full-scale WWTPs. Application of the anammox process in practice faces many main significant obstacles. For example, anammox bacteria require NH_4^+ as an electron donor and NO_2^- as an electron acceptor. Anammox cultures are significantly slow growth or in other words doubling times of anammox bacteria are long. Major thing of anammox process is that as an intermediate the nitrification process, NO_2^- is significantly difficult to maintain at a consistent level for anammox bacteria. Low temperature can significantly affect anammox activity (Tao *et al*. 2012). Furthermore, anammox bacteria are strictly autotrophic, using carbon dioxide as a carbon source. Moreover, the anammox process is more demanding operationally and highly skilled operators are needed. For these reasons, field application of the anammox process at the full scale has been limited. Lackner *et al*. (2014) reported that of nearly 100 full-scale of anammox processes worldwide, 50% were sequencing batch reactors (SBRs), 88% were operated as single-stage systems, and 75% were used for side-stream treatment of municipal wastewater. Based on the literature reviews, there were many researchers such as Kadlec and Knight, 1996; Tao *et al*., 2012, they reported that the potential approach of anammox process might be found in the wetlands because barrier areas between land and waters and could be used to remove nitrogen under condition insufficiency carbon source and maintain water quality.

Many researchers have postulated that the anammox process could be a main of mechanism of nitrogen removal without oxygen and insufficient organic carbon in wetland systems. For these reasons, wetland systems have gained increasing interest in wastewater treatment and as such have been intensively studied around the world. In developed countries such as Europe, Japan, Australia, and United State of American, constructed wetlands are often used in urban and rural areas to treat storm water runoff before discharge to the environment. Moreover, constructed wetlands represent low-cost and appropriate technology for domestic wastewater treatment in developing countries. The aerobic conditions around the plant root biofilm, anaerobic conditions in the subsurface, and high temperature are factors in the treatment performance of constructed wetlands systems. Humbert *et al*. (2012) found anammox bacteria in soil in seven natural wetlands. Wetland systems with anammox should be able to remove nitrogen from influents with low BOD but high nitrogen, despite the lack of carbon source for denitrification. One objective of this research was to identify the dominant ammonia oxidizers (AOB), nitrite oxidizers (NOB) and anammox bacteria in two full-scale constructed wetlands (storm water and domestic wastewater). In addition, nitrogen removal efficiencies in anammox-inoculated pilot-scale constructed wetlands were investigated using synthetic wastewater with low BOD but high nitrogen. The effluent from such constructed wetlands might be used as water reclamation.

2. Materials and Methods

2.1 Full-scale constructed wetlands

Two full-scale constructed wetland were studied. One site located in Fukuoka, Japan (southern Japan) was used to treat only storm water and acted as a control site. In Phetchaburi Province (west direction of Bangkok) a constructed wetland called the Laem Phak Bia Environmental Study and Development Project was used to treat domestic wastewater.

2.2 Pilot-scale constructed wetlands setup

A horizontal surface-flow constructed wetland was used in this pilot-scale nitrogen removal study. Four rectangular stainless steel tanks (50x100x100 cm.) were used to contain the wetland systems. Two types of stone were used: 0.9-1.25 cm stone was added at the bottom of the tank (depth 15 cm) and 2-2.5 cm stone filled the remainder of the tank. A PE pipe outside the tank with a height of 75 cm was used to maintain the water level inside the tank and discharge effluent. In each tank, three PE pipes, 100 cm long, 2.54 cm in diameter, with holes every 5 cm, were used to collect samples from the tank (Figure 1). These three pipes were vertically placed 15, 50, and 85 cm, respectively, across the tank, as shown in Figure 1. The four experimental constructed wetland units, each planted with six umbrella sedge (*Cyperus involucratus* Rottb) plants, were operated at a flow rate of 65 L/day (HLR 13 cm/d). In the pilot-scale wetland systems, three of the four tanks were inoculated, once at the start of the experiment, with enriched anammox bacteria (total 1.5 L, about 500 mL each tank) from the stock culture described below. Both inoculated and uninoculated wetlands were continuously fed with synthetic wastewater (Table 1) containing 50, 70, and 90 mg N/L of both NH_4^+ and NO_2^- (1:1.32 ratio).

2.3 Enriched stock anammox culture used to inoculate pilot-scale of constructed wetlands

An enriched suspended-growth stock anammox culture was maintained in a sequencing batch reactor (SBR). This enriched culture was inoculated with sludge from the anoxic tank of the Nongkhaem WWTP in Bangkok, Thailand. The SBR was a cylindrical vessel with 3 L maximum working volume. The top of the reactor was closed, but a pipeline was used to collect gas. A manually controlled SBR cycle consisted of four periods—fill (5 min), reaction time with mixing with a magnetic stir-bar at 120 rpm on a magnetic stirrer (24 hr.), settle (1 hr.), and decant (5 min.). The decant:recycle ratio of 1:1 was maintained by draining 1.5 L of supernatant. The remaining 1.5 L was retained for the next cycle during which 1.5 L of new medium was added. To limit dissolved oxygen (DO) content, argon gas (95%) and carbon dioxide (5%) were diffused through an air stone into the bottom of the reactor for

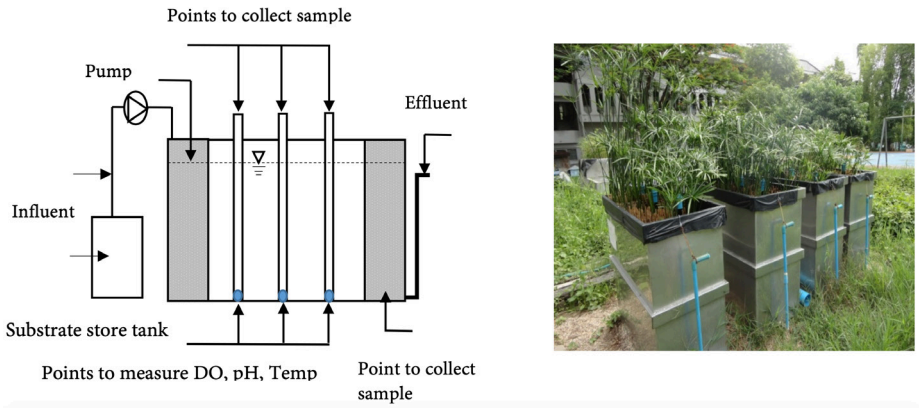

Figure 1. Constructed wetland systems planted with umbrella sedge (*Cyperus involucratus* Rottb)

Table 1. Composition of Synthetic Wastewater

Constituent	Concentration	Unit
$NaNO_2$	50,70, 90 (wetlands) or 273 (anammox stock)	mg N/L
$(NH_4)_2SO_4$	50, 70, 90 (wetlands) or 210 (anammox stock)	mg N/L
$KHCO_3$	1,250	mg/L
KH_2PO_4	18.75	mg P/L
$Na_2EDTA.2H_2O$	26.25	mg/L
$FeSO_4.7H_2O$	7.5	mg/L
$MgSO_4.7H_2O$	150	mg/L
$CaCl_2.2H_2O$	225	mg/L
Trace elements No. 1	1.05	ml/L

Modified from van Dongen *et al.* (2001) and Isaka *et al.* (2006)
Trace element No. 1: 0.06 mg/L Na_2O_3Se $5H_2O$; 0.165 mg/L $MoNa_2O_4$ $2H_2O$; 0.187 mg/L $CuSO_4$ $5H_2O$; 0.322 mg/L $ZnSO_4.7H_2O$; 0.742 mg/L $MnCl_2$ $4H_2O$; 0.18 mg/L $CoCl_2$ $6H_2O$; and 0.142 mg/L $NiCl_2$ $6H_2O$

Table 2. PCR primers for DNA amplification

Specificity group	Primer name	Sequence (5'–3')	Annealing T (°C)	References
All bacteria	Bac338F*	ACTCCTACGGGAGGCAG	52	Yu et al. 2005
	Bac805R	GACTACCAGGGTATCTTATCC	48	
Planctomycetes	PLA46F*	GACTTGCATGCCTAATCC	59	Schmid et al. (2000)

*attached GC-clamp (5′–CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG–3′) (Muyzer *et al.* 1993)

5 min. after filling. The pH of influent and effluent was 7.8±0.4. The concentration ratio of NH4+:NO2- was maintained at 1:1.32. The enriched stock anammox culture was fed with a synthetic wastewater, shown in Table 1.

2.4 Identification of nitrogen-transforming bacteria

Samples for DNA analysis were collected from both constructed wetlands twice at the beginning (January) and end (December) of 2017. The samples were collected from the sediment (around 1-2 cm. from the surface) and biofilm surrounding plant roots. Total DNA was extracted from wetland samples with QIAamp DNA Stool Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. DNA concentrations were determined with a NanoDrop 2000C spectrophotometer (Thermo Scientific).

PCR amplification of bacterial 16S rRNA gene fragments of bacterial group was performed using primers Bac338F-GC/Bac805R (Table 2) as described previously (Yu *et al.* 2005 and Schmid *et al.* 2000). Cycle conditions for the touchdown PCR amplification were denaturation at 94°C for 10 min; 20 cycles consisting of denaturation at 94°C for 30 s, annealing at 65 to 55°C (reducing the temperature by 0.5°C per cycle) and extension at 72°C for 1 min; additional 15 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min; and final extension at 72°C for 7 min. The identity of the dominant ammonia oxidizers (AOB), nitrite oxidizers (NOB) and anammox bacteria was determined by denaturing gradient gel electrophoresis (DGGE). DGGE was performed using a DCode universal mutation detection system (Bio-Rad, USA). The method of DGGE followed Muyzer *et al.* (1993). The gels were prepared in 0.5X TAE buffer (20 mM Tris, 10mM acetic acid, 0.5 mM ethylenediamine tetraacetic acid (EDTA), pH 8.0), which was also used as the electrophoresis buffer. Electrophoresis was run at 58°C, 150 mV at 16 h at a constant voltage. After electrophoresis,

the gels were stained with SYBR Gold nucleic acid gel stain (1:10,000 dilutions) for 40 min, followed by holding in distilled water for 20 min. DGGE images were captured using CUV10 Alphalmager MiNi (Cell Biosciences, Santa Clara, CA). PCR products were purified by a Wizard® SVGel and PCR Clean-up System (Promega, WI, USA). Database searches were conducted using BLAST (Alschul *et al*., 1990) from DNA Data Bank of National Center for Biotechnology Information (NCBI).

3. Results and Discussion

3.1 Full-scale constructed wetland sites

Average water qualities from both full-scale constructed wetland sites are provided in Table 3.

The influent BOD at the constructed wetlands treating domestic wastewater was quite low (Table 3). The main reasons for low BOD in the domestic wastewater at this site are similar to those reported by Noophan *et al.* (2017). Noophan *et al.* (2017) reported that the large WWTP in the Bangkok area (at Bang Sue) was capable of managing larger organic loading but was not designed to significantly remove nitrogen. Reasons for low influent BOD include combined sewage and storm water, infiltration and inflow, and rapid BOD degradation in the sewage pipe because of the warm climate. Bacterial activity increases with increasing temperature; in addition, sewage piping in Bangkok from point source to WWTP is very long, leading to long residence times. In addition, each house in small and large cities in Thailand has a primary treatment system (such as septic tank, grease trap), which removes a significant portion of BOD. These factors are major contributors to the low influent BOD in Thai wastewater. The BOD and nitrogen of influent storm water treated by constructed wetlands was low because the influent did not mix with any domestic wastewater.

Known AOB, NOB and anammox identified in the full-scale constructed wetlands during both first and second survey are summarized in Table 4. AOB *Nitrosospira* sp. but no NOB were found in the sediment of constructed wetlands treating storm water. In constructed wetlands used to treat domestic wastewater, AOB *Nitrosomonas* sp., *Nitrosospira* sp., *Nitrosococcus* sp. and NOB *Nitrospira* sp. were found; nitrification was likely a significant process based on the presence of these microorganisms (Mota *et al*. 2005). Typically, *Nitrospira* sp. is widespread and the key nitrifier in wastewater treatment by wetlands (Pester *et al*. 2014). Daims *et al*. (2001) demonstrated that *Nitrospira* sp. were able to grow in an aerated bioreactor with lower nitrite and oxygen concentrations. Uncultured *Candidatus* Nitrotoga sp. and *Candidatus* Accumulibacteria phosphatis clade were also found in the sediment and root plant biofilm of constructed wetlands treating domestic wastewater; these organisms may be postulated to be related to anaerobic ammonium oxidizers and biological phosphorus removal activities, respectively.

3.2 Pilot-scale constructed wetlands

Literature suggests that wetland systems may have a mechanism to remove nitrogen under conditions with no organic carbon source. Tao *et al.* (2007 and 2012) reported that good conditions to grow anammox bacteria in constructed wetlands are high temperature (25-

Table 3. Water qualities from both of constructed wetlands sites

Parameter	Constructed wetlands to treat storm water		Constructed wetlands to treat domestic wastewater	
	Influent	Effluent	Influent	Effluent
pH	7.0±0.1	7.0±0.1	6.8±0.5	7.1±0.3
Temp (°C) in summer	20±-3	20±-3	25±-3	25±-3
Temp (°C) in winter	17±-3	18±-3	23±-3	22±-3
BOD (mg/L)	2.5±1.5	1±0.5	30±10	2±1
NH_4^+ (mg N/L)	0.5±0.2	0.1±0.1	25±10	0.8±0.5
NO_3^-(mg N/L)	0.2±0.1	0.2±0.1	15±5	2±1

Table 4. Presence of ammonium-oxidizing bacteria, nitrite-oxidizing bacteria, and anaerobic ammonium oxidizers in full-scale constructed wetland sites

Type of microorganism	Constructed Wetland for storm water treatment		Constructed Wetlands for domestic wastewater treatment	
	Sediment	Root plant biofilm	Sediment	Root plant biofilm
AOB				
Nitrosomonas sp.	No	No	Yes	No
Nitrosospira sp..	Yes	No	Yes	Yes
Nitrosococcus sp.	No	No	Yes	Yes
NOB				
Nitrospira sp.	No	No	Yes	Yes
Anaerobic ammonium oxidizers				
Uncultured *Candidatus* Nitrotoga sp.	No	No	Yes	No
Candidatus Accumulibacteria phosphatis clade	No	No	No	Yes

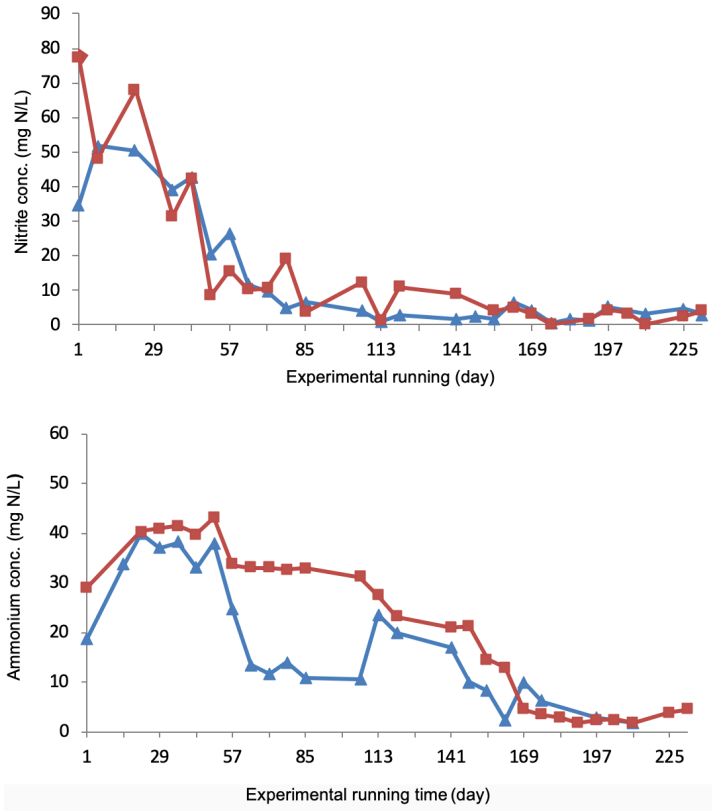

Figure 2. Effluent ammonium and nitrite concentrations *vs.* experimental running time in pilot-scale constructed wetlands fed 50 mg N/L NH_4^+ and NO_2^-. Symbols: ▲ inoculated constructed wetland and ▪ uninoculated constructed wetland (control).

30°C) and high pH (7.6-8.0). More information about how to promote the anammox process in constructed wetlands is important for implementation at the field scale.

After 5 months of operation, ammonium decreased in the effluent from inoculated and uninoculated constructed wetlands receiving 50 mg N/L of NH_4^+ and NO_2^- (Figure 2). At 211 days of operation, ammonia removal efficiencies of inoculated wetlands and uninoculated wetlands were 92.8% and 95.5% respectively, and nitrite removal efficiencies were 96.9% and 96.5% respectively. Also, after 232 days of operation, anammox bacteria could be found in the uninoculated system, showing that anammox bacteria can grow and establish naturally in an uninoculated horizontal subsurface-flow constructed wetland.

In the two pilot-scale wetlands inoculated with enriched anammox bacteria and fed with 70 and 90 mg N/L of NH_4^+ and NO_2^-, NH_4^+ was removed at 93.8% and 92.3% efficiencies, respectively (data not show), and NO_2^- removal efficiencies were 99.7% and 94.1%, respectively (data also not show). The results of this study show that inoculation with anammox and feeding NH_4^+ and NO_2^- concentrations between 50 and 90 mg/L did not affect the startup time for the anammox process in constructed wetlands. However, the startup of these constructed wetlands required several months. Additional conditions that promote startup of the anammox process in wetland systems should be investigated, such as using attached growth as an inoculum.

The anammox inoculum included *Candidatus* Brocadia anammoxidans and *Candidatus* Kuenenia stuttgartiensis. After more than 232 days of the operation, both *Ca*. Brocadia anammoxidans and *Ca*. Kuenenia stuttgartiensis were found in all four constructed wetlands, including the uninoculated wetland. However, the quantities in these four constructed wetland systems have not yet been determined. How to promote the anammox process in constructed wetlands requires further investigation.

4. Conclusions

Uncultured *Candidatus* Nitrotoga sp. and *Candidatus* Accumulibacteria phosphatis clade were found in sediment and root plant biofilm in full-scale constructed wetlands receiving domestic wastewater. These results suggest that constructed wetlands may be effective for treatment of wastewater with low BOD but high nitrogen in subtropical humid-climates countries like Thailand. The effluent from these constructed wetlands is suitable for water reclamation and reuse. Nitrogen removal efficiencies in pilot-scale constructed wetlands with and without inoculated enriched anammox cultures were similar. The results from the pilot-scale constructed wetlands confirm that anammox bacteria could grow under the conditions of 50 to 90 mg N/L NH_4^+ and NO_2^- (1:1.32), temperature 25-30°C and high pH (7.6-8.0).

Acknowledgements

The authors are extremely appreciative for a grant from the Kasetsart University Research and Development Institute (KURDI). The authors also would like to thank Faculty of Engineering, Kasetsart University for good support through this project.

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