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

Available online at www.tshe.org/EA
EnvironmentAsia 8(1) (2015) 34-40

Mixing Intensity Effects of Attached Growth on Enriched Anammox Cultures

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Abstract

Anaerobic ammonium oxidation (anammox) is a promising new technology for the treatment of wastewater with high ammonium and low carbon concentrations. Earlier work suggests that optimal processing would be realized within a sequencing batch reactor (SBR). However, the relatively slow growth of anammox bacteria inhibits the rates of nitrogen removal and biomass yielding. Improved anammox performance has been demonstrated when the bacteria are in granular form or attached to a growth medium. Little has been reported concerning the effect of mixing rate on nitrogen (N) removal with attached anammox bacteria. This work subjected anammox bacteria attached to polystyrene sponge in SBR to various intensities of impeller mixing and studied the effect on NH_4^+ and NO_2^- removal. Nitrogen processing was virtually the same with velocity gradient values between 13.5 and 222 s^{-1}. More vigorous mixing at 407 and 666 s^{-1} values significantly inhibited N removal, likely due to detachment of bacteria from the growth medium. Following the poor N removal at the two higher mixing intensities, agitation was reduced to 24.8 s^{-1} velocity gradient value. Recovery of N removal rates required 2-3 weeks, the slow time attributed to slow reattachment to the growth medium. Denaturing gradient gel electrophoresis (DGGE) analysis identified the prominent anammox species in the experimental study as *Candidatus Brocadia anammoxidans* and *Candidatus Kuenenia stuttgartiensis*.

Keywords: mixing intensity effect; anammox cultures; attached growth; recovery

1. Introduction

Conventional nitrification-denitrification processes would be difficult and costly to apply to wastewaters with high ammonium but low carbon concentrations. A new biological treatment approach, anaerobic ammonium oxidation (anammox) may provide an efficient approach to biological nitrogen removal from these wastewaters. Anammox bacteria are strictly autotrophic using carbon dioxide as a carbon source with ammonium as the electron donor. However, a significant obstacle for field application may occur because the groups of anammox bacteria have very low growth and biomass yielding rates. Theoretically, the growth rate of anammox cultures is extremely slow with doubling times of at least 11 days (Strous *et al*., 1997). Furthermore, the suitable reactor types for anammox cultures are still unclear. On the laboratory scale, Strous *et al*. (1998) strongly recommended that a sequencing batch reactor has been proven to be suitable for anammox cultures. Dapena-Mora *et al*. (2004) studied the growth of anammox bacteria in granular form in SBR and their systems worked well. Fernandez *et al*. (2008) reported that growth of anammox culture in form of granules or having a medium for attached growth could serve to keep anammox culture alive and active in a long-term operation. However, formation of anammox culture granules can be difficult and often takes a long time. For these reasons, most researchers in the wastewater treatment field have postulated that it would be very beneficial to keep as much of the anammox culture as possible in the system such as seen with attached growth or biofilm systems. These systems are able to aggregate anammox bacteria within media with their extracellular polymers attached to a solid surface. Use of such media is simple, reliable, and stable. A natural immobilization of the attached growth system would allow excellent biomass retention and accumulation without the need for separate solids-separation systems. For this reason, an attached growth system would be recommended for use in the anammox process. Furthermore, attached growth reactors could provide operational advantages by keeping all biomass and substrates within a medium rather than being in suspension (Rittmann and McCarty, 2001).

Despite the potential application of the anammox process in wastewater treatment, information about attached growth of anammox cultures has been limited. It was thought that mixing intensity could affect

the performance of N removal by anammox cultures in granular sludge or biofilms. The shear stresses of mixing could cause a partial loss of biomass activity and/or decrease of the size of the biomass granules. At this point, it is unclear how hydrodynamic shear forces influence, the structure and metabolism of anammox biofilms and granular sludge. In addition, still lacking is important information about the recovery of anammox bacteria in attached growth form following upsets due to too vigorous mixing. The aims of this study were to investigate (1) the effect mixing on attached anammox culture by varying velocity gradient values and (2) the recovery time for efficient N removal by anammox culture after too vigorous mixing.

1.1. Velocity Gradient

The velocity gradient, G, equals the square root of energy dissipation or power consumption in a stirred vessel and divided by the absolute viscosity of the fluid and volume according to Eq. (1).

$$G = (P / \mu V)^{0.5} \quad (1)$$

where P is a function of the mixing power input unit volume, (sec^{-1}), μ is the dynamic viscosity, (N sec/m^2), depending on the temperature, and V is the volume of reactor, (m^3). The mixing power consumption in a stirred vessel depends on various geometrical parameters of the impeller, such as diameter, height, and size of the reactor according to Eq. (2)

$$P = N_e \rho N^3 d^5 \quad (2)$$

where the Reynolds number, N_e, is a dimensionless number which characterizes the flow of the fluid around or through some object (pipe, channel, sphere, paddle, particle, etc.), ρ is the fluid density (1000 kg/m^3 for water at ambient temperature), N is the rotating speed (rpm). d is the diameter of the impeller (m). In this work, the Reynolds number (R_e) varied from 5000 to 15,000 during the operational investigation. For dimension analysis can be put into relationship of the Reynolds number as a function of different parameters, depends on the various geometrical parameters of the impeller (diameter (d), height (h) and the diameter of reactor, (D) and the height of reactor, (H), all relationship was below (Eq. 3 and 4).

$$N_e = f(R_e, d/D, H/D, impeller) \quad (3)$$

$$N_e = N\rho D^2 / \mu \quad (4)$$

These formulas were used to calculate velocity gradient (G) from the impeller speed (rpm) in the SBR.

2. Materials and Methods

2.1. Enriched stock anammox culture on attached growth media

An enriched culture of anammox bacteria was first inoculated with sludge from the anoxic tank of the Nongkhaem Wastewater Treatment Plant (WWTP) in Bangkok, Thailand. After inoculation the enriched anammox culture was maintained for 3 months in a SBR to which polystyrene sponge was added as a growth medium. The enriched stock anammox culture on 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 60 rpm on a magnetic stirrer (22 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. Argon gas (95%) and carbon dioxide (5%) were diffused through an air stone at the bottom of the SBR for 5 min to limit dissolved oxygen (DO). The pH of influent and effluent was 7.8± 0.4. The concentration ratio of NH_4^+:NO_2^- was maintained at 1:1.32. The enriched stock anammox culture was fed with a synthetic wastewater (Table 1).

2.2. Mixing intensity effects and recovery on attached growth of anammox culture

Following the above-described attached growth of anammox culture, 15-20 pieces of polystyrene sponge

Table 1. Composition of Synthetic Wastewater

Constituent	Concentration	Unit
$NaNO_2$	273	mg N/L
$(NH_4)_2SO_4$	210	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 $NiCl_2.6H_2O$

were transferred to each experimental SBR as media for attached growth. The working volume of all SBRs in this experimental investigation was 1 L. Before each experiment was conducted in SBR, attached growth cultures in that SBR were allowed to equilibrate a few weeks under the same operating condition as described above. All SBRs were fed with the synthetic wastewater described in Table 1. The effect of mixing intensity on total nitrogen removal was studied by measuring aqueous NH_4^+ and NO_2^- concentrations over time (one time per week over two month period). Impeller mixing speeds were varied from 40-250 rpm, which corresponded to velocity gradient values in the range of 13.5-666 s^{-1}, see Table 2.

After poor nitrogen removal rates were observed with the two maximum mixing intensities (180 and 250 rpm impeller speed or 407 and 666 s^{-1} velocity gradient) impeller speeds were decreased to 60 rpm in order to study the recovery of the anammox culture. The 60 rpm speed was chosen based on earlier observation of satisfactory N removal rates with such mixing.

2.3. Analytical procedures

2.3.1. Nitrogen concentrations

The concentration of ammonium (NH_4^+) in both influent and effluent was measured by using the titration method described in Standard Methods (1995). Nitrite (NO_2^-) concentration was measured by the colorimetric method as described in Standard Methods (1995). The concentrations of nitrate (NO_3^-) were measured by 761 Compact Ion Chromatograph (Methrom, Herisau, Switzerland) equipped with a conductivity detector.

2.3.2. Biomass

The biomass (volatile suspended solids, VSS) of the ammonium oxidizing culture was measured as described in Standard Methods (1995). Biomass was measured at the beginning and at the end of each experiment. Average value was reported in the results.

Table 2. Mixing parameters tested

Impeller Speed (rpm)	Calculated velocity gradient values (s^{-1})
40	13.5
60	24.8
90	144
120	222
180	407
250	666

2.3.3. Specific nitrogen removal rates

The specific nitrogen removal rate is defined as total nitrogen removal divided by biomass at the end of each experiment. Total nitrogen removal was calculated by adding NH_4^+ and NO_2^- loss over time.

2.3.4. Microbial community on attached growth

The identity of the microbial community on attached growth 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). PCR products (18 µL) were run on 10% acrylamide gels with a denaturing gradient of 40-60%. Electrophoresis was performed in 0.5X Tris Acetate EDTA (TAE) buffer, at 150 V and 60ºC for 5 hr. The gels were stained for 40 min with 10% SYBR Green in 0.5X TAE under agitation to observe the bands by UV transillumination. Specific gel bands were excised with a sterilized scalpel and put in Eppendorf tubes with sterilized MilliQ water 25 µL. Samples were re-amplified by PCR with a primer set, VFC and VR and PCR purified with Acc. Prep. PCR&Gel Purification kit (Bioneer). Sequencing of the DNA from the excised bands was carried out by the SolGent Co., Ltd. The isolated sequences were compared with 16S rRNA sequences obtained via BLAST searches of the National Center for Biotechnology Information database (http://blast.ncbi.nlm.nih.gov).

For DNA extraction, the biomass from attached growth stirred by vortex 10 min and DNA extraction was conducted using the UltraClean Soil DNA Isolation Kit (MO BIO Laboratories, Inc.). DNA concentrations were measured with the NanoDrop ND-2000 Spectrophotometer (Thermo scientific) to make sure that there was enough concentration to continue the step. For 16S rRNA analysis, samples from attached growth were amplified by Polymerase Chain Reaction (PCR) using the primers VFC (CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG) and VR (ATT ACC GCG GCT GCT GG). One PCR reaction (20 µL) contained: PCR premix (Bioneer) 2 µL; DNA template, 5 µL; primer VFC (20 pM), 0.5 µL; primer VR (20 pM), 0.5 µL; and sterilized MilliQ water 12 µL. Amplification was performed with touchdown PCR under the following conditions: an initial denaturing step at 95ºC for 5 min; then 20 cycles of denaturing at 94ºC for 1 min, annealing at 65ºC for 1 min and extension at 72ºC for 1 min; followed by 5 cycles of 94ºC for 1 min, 55ºC for 1 min, 72ºC for 1min; final extension at 72ºC for 7 min and then kept at 4ºC.

3. Results and Discussion

3.1. Mixing intensity effect on attached growth of anammox culture

The total nitrogen removal efficiencies over time at 13.5, 24.8, 144, and 222 s^{-1} were quite similar, see Fig. 1. The higher impeller speeds of 407 and 666 s^{-1}, nitrogen removal efficiencies were negatively affected in a significant manner, also shown in Fig. 1.

In other words, at G values between 13.5 and 222 s^{-1} (40, 60, 90, and 120 rpm) specific nitrogen removal rates were not significantly different. However, at G values of 407 and 666 s^{-1} (180 and 250 rpm), the specific nitrogen removal rates were impacted negatively. The results are shown in Fig. 2. The high mixing at G values over 222 s^{-1} could cause decreased anammox bacteria activity by scraping anammox cultures from the polystyrene sponge growth medium. The high stirring speeds could deter biomass attachment to polystyrene sponge because of shear stress. These results were similar to those of Arrojo *et al*. (2006) who investigated the effect of mixing on specific anammox activity and granule diameter. Also, Henzler (2000) reported that the specific input power and the impeller type could affect floc aggregation and breakup of microorganisms and effect of the operating condition of a biological reactor.

3.2. Recovery of anammox culture on attached growth media following vigorous mixing

After the initial study of nitrogen removal at 180 and 250 rpm for 42 days, the impeller speed for each impeller was reduced to 60 rpm, a mixing speed shown to yield good anammox activity. The total nitrogen removal at 60 rpm was then measured for 20 days to determine the time required to achieve adequate removal efficiency. The results are shown in Fig. 3.

It is thought that higher shear with very vigorous mixing breaks up bioflocculation prior to sedimentation. As a result, the removal efficiencies for both ammonium and nitrite are significantly reduced. It was shown that subsequent reduction of mixing to a rate known to be satisfactory (60 rpm) allows anammox activity to improve. However, the process of recovery on mixing intensity effects could require a few weeks. The main reason of this phenomenon could be postulated that after scraping of anammox cultures to polystyrene sponge reattachment to the medium is a low process. Bioflocculation might need some bacterial binding

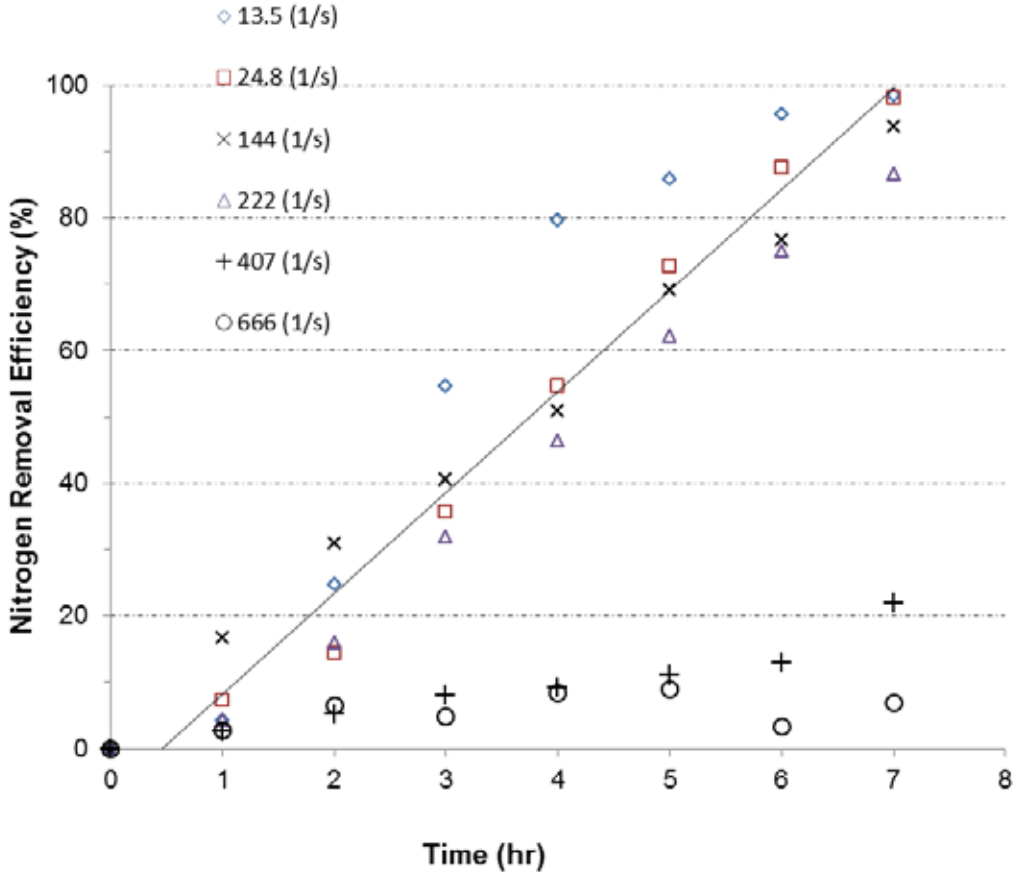

Figure 1. Total nitrogen removal efficiency vs. time with calculated mixing intensity (13.5, 24.8, 144, 407, and 666 s^{-1})

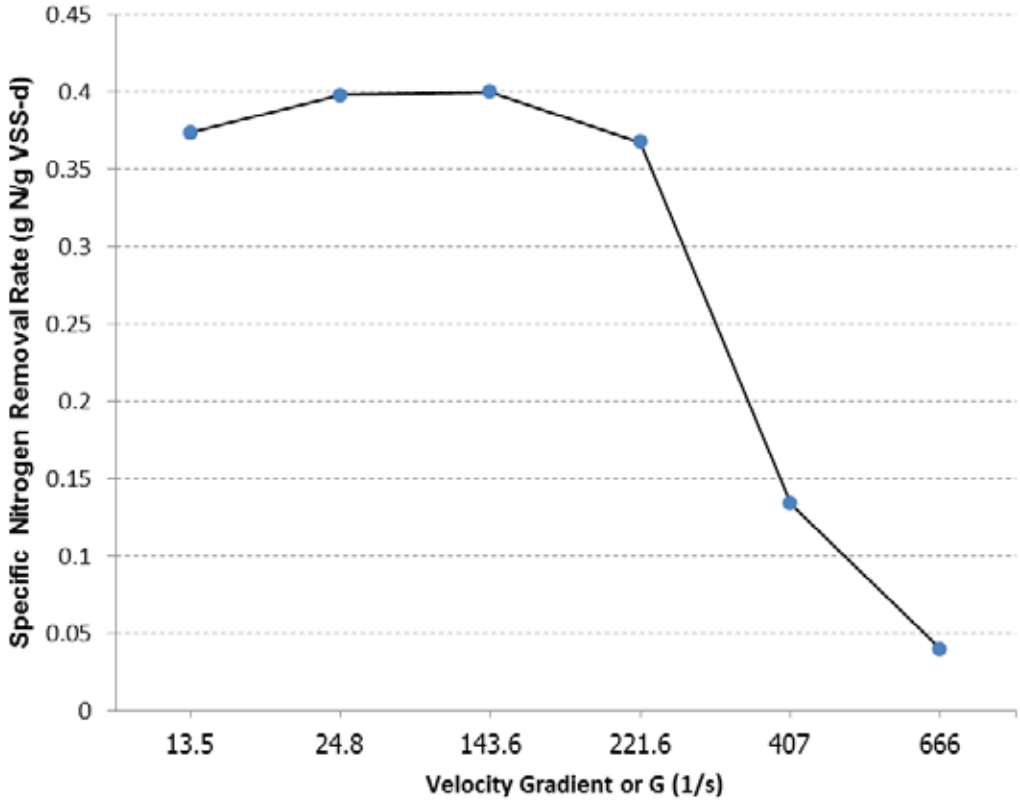

Figure 2. Velocity gradients vs. specific nitrogen removal rate

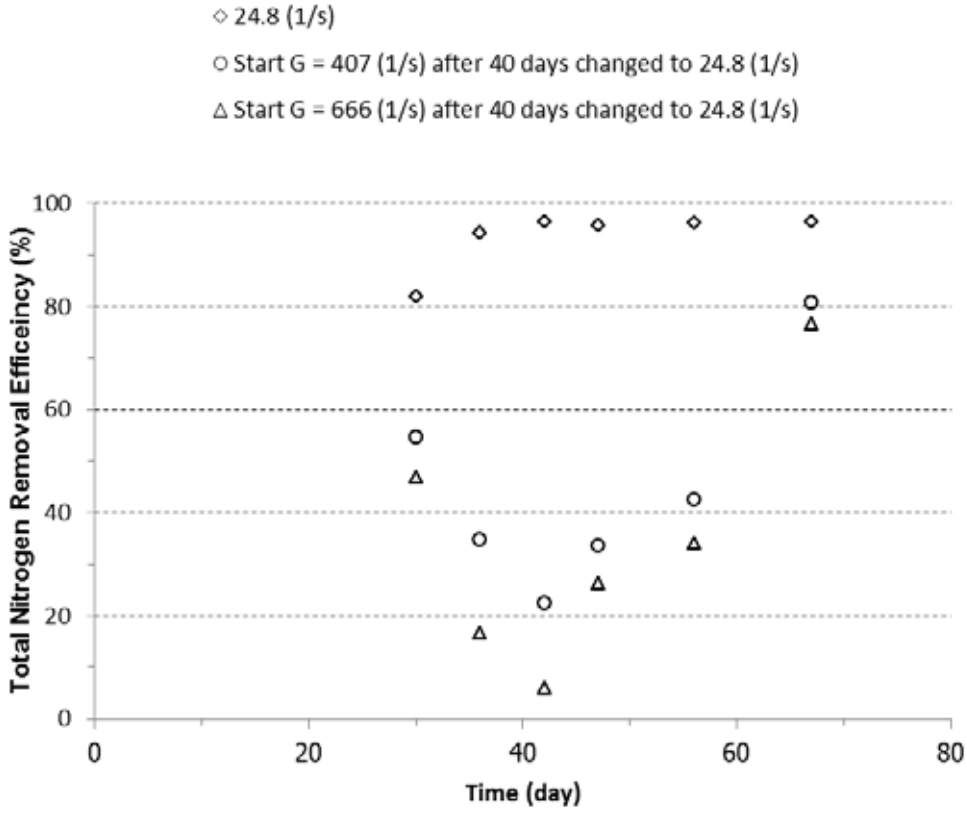

Figure 3. Total nitrogen removals vs. time at the velocity gradients of 24.8, 407 and 666 s^{-1} after 40 days changed to 24.8 s^{-1}

Table 3 Sequence analysis and species identification of selected DGGE bands (the band numbers are shown in the schematic DGGE profiles in Fig. 4)

Band number	Closest relatives	Isolated environment of closest match	Reference accession no.	Identity	Reference journal
1	*Candidatus Brocadia anammoxidans*	Uncultured bacterium, isolate DGGE band Anammox	AM900561	91%	Sanchez-Melsio *et al*. (2009)
2	*Candidatus Kuenenia stuttgartiensis*	Uncultured bacterium clone B70	HQ640561	96%	Phan *et al*. (2012)
3	*Chloroflexi*	Uncultured chloroflexi bacterium clone HUY:A12	AB638620	100%	Kindaichi *et al*. (2012)
4	*Bacillus* spp.	Uncultured bacterium clone HW-1A18	AB376566	100%	Date *et at*. (2009)

chemical from other bacteria. More experiments are still suggested in order to better understand mixing intensity effects of attached growth on anammox cultures.

3.3. DGGE observation of microbial community on attached growth

Two general PCR amplifications followed by denaturing gradient gel electrophoresis (DGGE) were used to reveal the structure of bacteria communities in the sample of enriched anammox culture on attached growth media which were present before the mixing intensity experiments. Analyses on the band patterns by DGGE were duplicated. Some dominant genes bands from the DGGE profile of altered microbial community were sequenced and their Blast results are presented in Table 3.

Fig. 4 shows the image of a DGGE gel of the PCR-amplified products of the sample of enriched anammox culture on attached growth. Comparative analyses with GenBank database using Basic Local Alignment Search Tool (BLAST) program indicated that these sequences were similar to anammox bacteria from different researchers. For example, band number 1 refers to *Candidatus Brocadia anammoxidans* (Sanchez-Melsio *et al*., 2009). Band number 2 refers to *Candidatus Kuenenia stuttgartiensis* (Phan *et al*., 2012). Band number 2 is the brightest, indicating that *Candidatus Kuenenia stuttgartiensis* is the dominant species in the attached growth system in this study. Band number 3 refers to the uncultured *Chloroflexi* coexisting in an anammox reactor (Kindaichi *et al*., 2012). This uncultured sample was found in an anammox reactor which was fed with synthetic nutrient substrates without organic carbon compounds over 2 years in the Kindaichi *et al*. (2012). Band number 4 refers to *Bacillus* spp. which coexists in the anammox culture cultivated from sewage sludge (Yasuhiro *et al*., 2009). Yasuhiro *et al*. found that the different types of seed sludge from wastewater treatment plants could provide different communities of enriched anammox culture.

4. Conclusion

With attached growth of anammox bacteria on polystyrene sponge, neither NH_4^+ nor NO_2^- removal is significantly affected with velocity gradient values from 13-222 s^{-1} (impeller speed 40-120 rpm in this study). However, more vigorous mixing with velocity gradient values of 407 and 666 s^{-1} (impeller at 180 and 250 rpm) greatly reduced both NH_4^+ and NO_2^- removal. Mixing with velocity gradient values > 222 s^{-1} could increase shear stress and detach anammox culture from

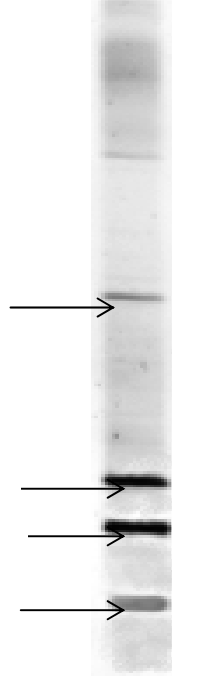

Figure 4. DGGE image of the PCR-amplified products of sample taken from enriched stock culture of anammox culture on attached growth

the growth media. The high mixing rates could affect anammox bacteria dramatically slowing the removal of both NH_4^+ and NO_2^-.

Following too vigorous mixing (>222 s^- velocity gradient) bioflocculation is greatly improved after reducing the mixing intensity to 24.8 s^{-1} or 60 rpm impeller. However, recovery from the effects of intense mixing intensity requires a few weeks. The delayed recovery is attributed to slow reattachment of anammox culture to polystyrene sponge growth medium.

In this work the primary type of anammox bacteria on attached growth as a media of polystyrene sponge was shown to be *Candidatus Kuenenia stuttgartiensis*. *Candidatus Brocadia anammoxidans* was present to a lesser degree.

Acknowledgement

The authors would like to thank the National Research Council of Thailand (NRCT) and the Kasetsart University Research and Development Institute (KURDI) for grants that supported in this research. The authors also would like to thank Faculty of Engineering, Kasetsart University, Bangkok, Thailand and Mr. John F. Elliott, Golden, CO for good supporting through this research.

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Received 10 July 2014
Accepted 26 August 2014

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