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Determination of anaerobic ammonium oxidation (anammox) bacteria in the domestic wastewater

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ABSTRACT

Wastewater treatment plants (WWTPs) in Malaysia are commonly associated with oxidation tank and activated sludge treatment processes. However, the removal rate of ammoniacal nitrogen from the wastewater still requires alternative technology as compared with the self-purification method. This provides a platform for further investigation of the degradation of nitrogen. Anaerobic ammonium oxidation (anammox) bacteria have been found to provide a shorter pathway for degradation of nitrogen in wastewater with limited presence of oxygen. The temperature, pH, dissolved oxygen (DO), total nitrogen, ammonia, nitrite and nitrate concentrations were analysed for the samples collected from the local WWTP. Influence of the various Malaysia weather conditions upon the treatment process was also observed. The temperature was found to fluctuate between 29.6°C and 32.0°C throughout the experiments at commemorating pH values of between 5.78 and 7.22 and DO concentration of 0.62–1.53 mg/L. Molecular analyses including genomic DNA (gDNA) extraction, polymerase chain reaction amplification and DNA sequencing were conducted to identify the presence of anammox bacteria. The gDNA of the sludge from anoxic and sequencing batch reactor tanks were successfully extracted with the gDNA amount of 24.40 and 31.67 μg, respectively. Anammox bacteria namely *Kuenenia stuttgartiensis* was found in the selected wastewater samples from the local WWTP. Thus, the presence of anammox bacteria enhances the removal of nitrogen from wastewater in a relatively quick and short process with further investigation regarding anammox bacterial growth and development.

Keywords: Anammox; Local wastewater treatment plant; *Kuenenia stuttgartiensis*

1. Introduction

According to Muralikrishna and Manickam [1], wastewater is a mixture of dissolved and dispersed matter, both of organic and inorganic nature. Due to the urbanization and increase in population density, the pollution production has accelerated and a larger amount of wastes are being generated and disposed into the water bodies [2]. According to Mara [3], domestic wastewater indicates the water used by the community, which may contain a variety of materials. Domestic wastewater consists of wastes originated from the human body such as the faeces and urine besides the used water from flushing toilets and sullage.

The International Water Association in 2018 reported that the study conducted at the global level for wastewater

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showed approximately 80% of wastewater is directly discharged, without any type of wastewater treatment application into the nearby waterbodies [4]. Subsequently, this may lead to the enrichment of the nutrients concentration inside waterbodies and cause stimulation of growth for the aquatic plant together with algal bloom, which may result in eutrophication and deterioration of water quality [5]. According to Hu et al. [6], nutrient management is responsible to secure the fresh water source by determining the quality of effluent discharged from WWTPs.

According to studies conducted by local sewerage treatment plant [7], the ammoniacal nitrogen present in the wastewater is being treated using conventional methods including air-stripping (volatilization of gaseous ammonia), breakpoint chlorination (adding chlorine to oxidize ammonia) or ion exchange (type of clay – clinoptilolite for removal of ammonia). However, the removal rate of ammoniacal nitrogen is dependent on the self-purification of the water flows or rivers whereby excessive discharges of pollutants may inhibit the natural waterways. Hence, the discharged effluent containing ammoniacal nitrogen from sewage treatment plants is gradually diluted in the waterways and is reduced to a less toxic compound. However, these processes are known to be time consuming with high cost [8].

Currently, Malaysia is working on to achieve the 6th Sustainable Development Goals, which is Clean Water and Sanitation by implementing alternative models of treatment systems that are less energy intensive, cost-effective and suited to local requirements [12th Malaysia Plan (12 MP) [9]]. Conventionally, most of the sewage treatment companies were using aerobic nitrification and anoxic denitrification to remove the nitrogen in wastewater. However, this process was less economically beneficial due to high energy consumption due to high aeration and external organic carbon demand to degrade the ammoniacal nitrogen [9].

Previous report by Ali and Okabe [10] stated that nations including Taiwan, Germany and Japan are using another alternative process for nitrogen removal called anaerobic ammonium oxidation (anammox) bacteria, in order to reduce energy consumption in WWTP. According to Strous et al. [11], the anammox bacteria will convert the ammonium into N_2 by using nitrite. While for the synthesis of cell biomass ($CH_2O_{0.5}N_{0.15}$), the carbon source is obtained from the bicarbonate, which makes the organisms autotrophs. In the laboratory-scale reactor, the ammonium, nitrite and bicarbonate under a steady-state condition will be converted as in Eq. (1):

$$1NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 0.066CH_2O_{0.5}N_{0.15} + 2.03H_2O \quad (1)$$

The anammox process is more cost-effective and environmental friendly; it also seems to have the capability to reduce the operational costs up to 90% by lowering the aeration to 63% and sludge production to 80%–90% [12–14]. Anammox process in the lab scale has been successfully applied for treatment of various kinds of wastewater such as the landfill leachate, domestic wastewater, municipal wastewater, monosodium glutamate wastewater and pharmaceutical wastewater [15–19].

According to Meng et al. [20], there are five genera of anammox bacteria known to affiliate with the phylum of Planctomycetes, namely *Candidatus Scalindua*, *Candidatus Brocadia*, *Candidatus Kuenenia*, *Candidatus Anammoxglobus* and *Candidatus Jettenia*. Several number of key factors have been discovered as primary drivers affecting the anammox activities during enrichment including operating temperature (25°C–30°C), dissolved oxygen (DO), chemical oxygen demand/total Kjeldahl nitrogen (COD/TKN) ratio, pH (7–8.3), nitrite and phosphate concentration [21,22]. Several anammox bacteria including *Brocadia, Kuenenia, Jettenia, Anammoxoglobus* and *Anammoxomicrobium* are commonly found in WWTPs and freshwater, while *Scalindua* is mostly detected in the marine environments [23–28].

In this study, anammox consortium was determined in locally sourced domestic wastewater, which has potential for the application in wastewater treatment process. The sludge was collected from three different tanks to determine the presence of anammox bacteria. Genomic DNA (gDNA) extraction, polymerase chain reaction (PCR) amplification and DNA sequencing techniques were used for the identification of anammox bacteria in the sludge. PCR amplification method was used as it was found to be effective for detection of anammox bacteria in seeding sludge.

2. Materials and methods

2.1. Sample collection

Selected WWTP for sample collection is located in Kuala Lumpur, Malaysia, with total sewerage catchment of 70 km^2. The WWTP was classified as domestic treatment plant based on previous study by Basri [29] on the raw influent of the plant. According to the database [7], the local WWTP serves for residential areas population equivalents as five per dwelling and is a direct measurement of the population in an area. Fig. 1 illustrates the overall process of WWTP, whilst Fig. 2 illustrates the processes involved in the aeration tank. The plant is designed to operate with a 1 h filling, 1 h reacting, 1 h settling and 1 h drawing and idling phases.

Wastewater samples were collected from two different points, in the anoxic tank and during aeration in the sequencing batch reactor (SBR). According to Wang et al. [30], in the anoxic phase, less oxygen will limit the growth of nitrite oxidizing bacteria (NOB), hence anammox bacteria may obtain excellent advantage over NOB in the case of nitrite consumption. However, the NOB growth may also be inhibited with intermittent aeration operation mode in a reactor; thus, the anammox may have sufficient nitrite for nitrogen removal activity [31]. The samples were collected from the local WWTP thrice a week for 2 months. The wastewater samples were collected in 500-mL HDPE bottles. However, the sample collection from SBR tank during the aeration process started from day-5 onwards.

2.2. Analytical methods

The samples were analysed using Hach-Lange methods. After the samples were collected at each point, within 10 min, the DO was analysed using benchtop EUTECH Instruments DO 2700 (USA). While the temperature and pH were

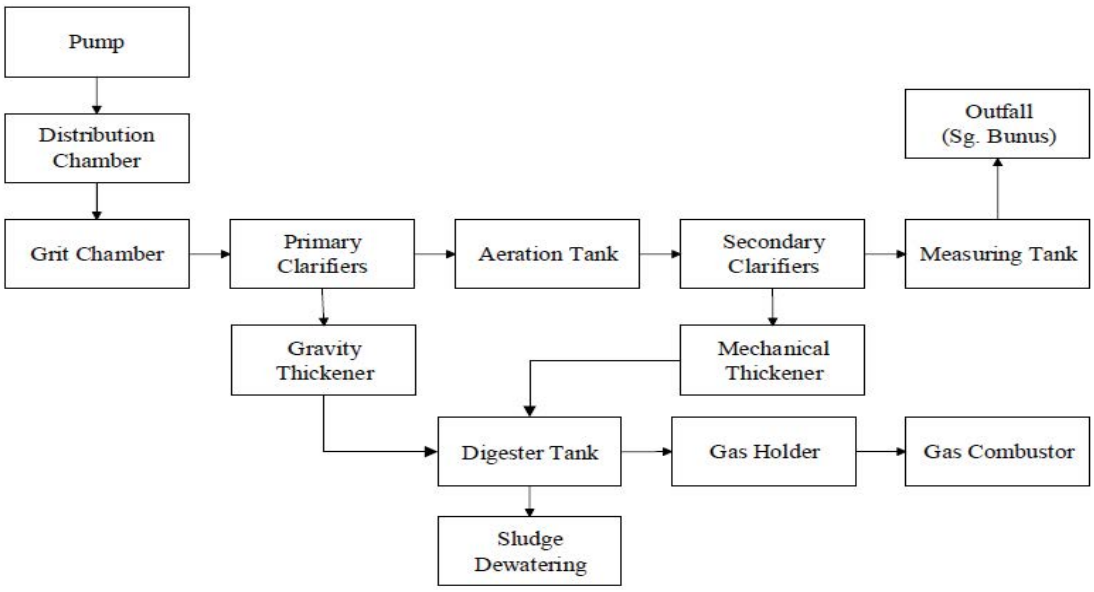

Fig. 1. Overall treatment process of selected WWTP.

Fig. 2. Processes in the aeration tank.

analysed using benchtop EUTECH Instruments pH 2700 (USA). The concentrations of ammoniacal, nitrite and nitrate nitrogen were analysed using salicylate method (Method 10023), diazotization method (Method 10237) and cadmium reduction method (Method 8039), respectively, followed by reading of spectrophotometric values using DR6000 (Hach, USA).

2.3. DNA extraction, PCR amplification cloning and phylogenetic analyses

Sludge from anoxic tank namely, sample A and from SBR tank namely, sample R during aeration process in day-12 was selected for microbial identification as it was freshly sampled on the DNA analysis day. Two sludge samples were transferred into the test tubes and kept in the freezer at –20°C prior to be tested by the commercial laboratory (First BASE Laboratories Sdn Bhd, Malaysia). This is crucial to maintain the integrity of DNA extracted from the samples. For the analyses, CTAB/SDS method was applied for the triplicate extraction of the total genome DNA from the sludge samples. The purification of extracted DNA was conducted using UltraClean Microbial DNA Isolation Kit [32] prior to be used for PCR amplification. Agarose gel electrophoresis analysis of gDNA was conducted by using 1% agarose gel in 1X TAE buffer (10 mM Tris-HCL, 1 mM EDTA, pH 8.0).

For 16S rRNA gene amplification, PCR was conducted according to the manufacturer's instructions. Each sample contained 1 μL of extracted DNA and 10 μL of PCR master mix (0.6 μL of 10 μM forward and reverse primers) [33], respectively. In this study, the partial 16S rRNA fragments were amplified from the extracted genomic DNA for anammox bacteria [34]. The PCR was conducted using the primers Brod541F (5′-GAG CAC GTA GGT GGG TTT GT-3′) and Amx820R (5′-AAA ACC CCT CTA CTT AGT GCC C-3′).

All PCR reaction tests were carried out using 50-ng aliquot of DNA sample with Phusion® High-Fidelity PCR Master Mix (New England Biolabs) [32] in accordance to pre-determined PCR program. The program consisted of an initial 3 min denaturation step at 95°C prior to denaturation at 98°C for 20 s for 35 cycles, annealing at 55°C for 15 s, and extension at 72°C for 1 min followed by a final extension at 72°C for 2 min. However, the result obtained when running in agarose gel electrophoresis was not convincing. Therefore, 20 ng of gDNA was used for annealing temperature as 55°C, 57.6°C, 59.1°C and 61.0°C to identify the perfect temperature to amplify anammox bacteria, respectively. The PCR products appeared at 57.6°C, 59.1°C and 61.0°C with better quality bands than at 55°C due to less smearing effect. Hence, as it was proven that the former temperatures are suitable for annealing anammox DNA, whilst degradation of temperature was conducted for annealing step in the PCR reaction starting from 61°C to 57.6°C.

According to Lee et al. [35], the most effective way to separate DNA fragments of varying sizes particularly ranging from 100 bp to 25 kb is through agarose gel

electrophoresis. Therefore, aliquots of 3 μL PCR products were electrophoresed on 1.7% agarose gels and visualized by using tracking agents such as Orange G and xylene cyanol for this analysis. The reference marker used was 100 bp DNA ladder that have fragment sizes of 1,500; 1,000; 900; 800; 700; 600; 500; 400; 300; 200 and 100 bp, respectively. For PCR products mixing and purification, the PCR products were mixed in equidensity ratios and the mixture was purified using Qiagen Gel Extraction Kit (Qiagen, Germany) [32].

For sequencing, the commercial laboratory (First BASE Laboratories Sdn Bhd, Malaysia) has observed the purified PCR product through electrophoresis. The sequences obtained were compared with the GenBank database through the website [36] to identify the anammox bacteria. The sequence similarity searches were associated with the BLAST (Basic Local Alignment Search Tool) network service and phylogenetic tree, which was constructed accordingly using MEGA X.

3. Result and discussion

3.1. SBR operation performance in WWTP

The performance of the selected WWTP was analysed for 14 alternate days in 2 months. The selection of analysis days was made based on the WWTP recommendations for their peak time for receiving wastewater influent from the surrounding areas without taking into account weather fluctuation that may hamper the samples variations. The performance analyses were conducted for temperature, pH, DO, ammoniacal, nitrite and nitrate nitrogen to determine the suitability of cultivating anammox bacteria in WWTP at industrial scale. The performance of the WWTP was monitored during dry, normal and wet conditions. The values for each performance analyses were tabulated as shown in Table 1, which also describes the samples of effluent from the anoxic and SBR tanks, respectively.

3.1.1. Temperature measurements

Based on the experiment conducted at two different points, the anoxic tank and during the aeration in SBR, the temperature values were different at each point. This was due to the changes in the weather condition during the experimental period of 2 months. Nevertheless, the study period was limited to describe the suitable temperature range for the anammox to survive. Besides, the WWTP is operated in an open tank that is exposed to variations of dry and wet climate conditions throughout the year, which results in fluctuations in the temperature recording of the sampled wastewater at designated sampling points. Moreover, the WWTP also operates as a combined sewer for domestic wastewater and urban runoff. In some instances, the flow rate may exceed the facilities' treatment capacity and can impact treatment performance. Thus, those conditions have resulted in fluctuation temperature readings for the samples at different points every time. For Malaysia scenario, this requires further investigation to identify significant difference in either the loading or performance of the wastewater treatment facilities under wet and dry flow conditions. Fig. 3 describes the variations of temperature in the wastewater samples collected.

The temperatures were fluctuating throughout the sampling time, where during anoxic condition a sudden increase occurred from 30°C to 33.5°C in day-3, decrease on day-5 and fluctuation for the rest of sampling time. The first 4 d of the experimental observations were dried in Kuala Lumpur as the heat wave occurred with maximum temperature of 37°C and minimum temperature of 25°C. Whilst, the rain with thunderstorm occurred on a daily basis from day-8 to day-12 of experimental observations, caused the temperature ranges to be within 23°C to 34°C. However, the other days (day-5 to day-11 and day-13 to day-14) were in normal weather condition with neither rain nor dry condition.

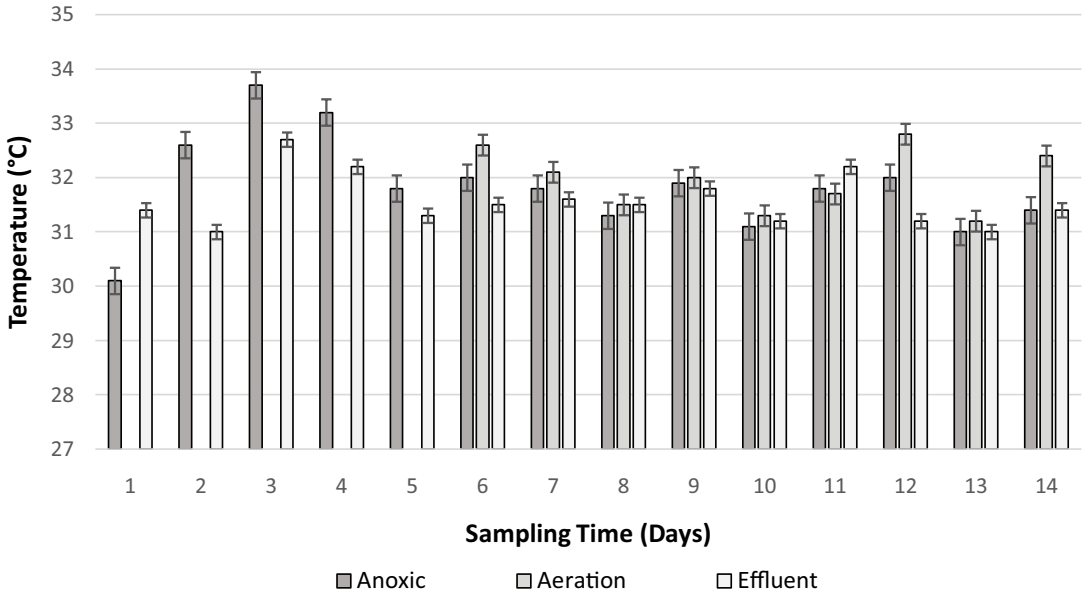

Fig. 3. Variations of temperature in anoxic condition, during aeration and in effluent.

According to Thamdrup and Dalsgaard [37], the anammox bacteria can survive at temperature ranging from 6°C to 43°C with optimal temperature of 26°C to 28°C. Meanwhile, according to Daverey et al. [38], excellent anammox activity results were obtained at optimum temperatures of 30°C–40°C. However at extreme low temperature, the anammox process turns unstable because of nitrite accumulation, which affects the anammox activity [39]. While Ibrahim et al. [22] have summarised the optimal temperature for different type of anammox bacteria species and the species that were compatible with the selected WWTP temperature range were *Brocadia anammoxidans, Brocadia sinica, Kuenenia stuttgartiensis* and *Jettenia caeni.*

3.1.2. pH measurements

The pH value is used to determine the acidity or basicity of an aqueous solution. There pH readings were fluctuating for both samples collected from the SBR anoxic and aeration tanks, respectively. According to Khayan et al. [40], the low pH value in rainwater is influenced by air pollution in the form of carbon dioxide, nitric oxide and sulfur dioxide. Subsequently, the gas reacts with rainwater (H_2O) to form carbonic acid (H_2CO_3), sulfuric acid (H_2SO_4) and nitric acid (H_2NO_3), which further decreases the pH of the rainwater [41,42]. The trend of pH decreasing during rainy days proves the acidity of the rainfall in Kuala Lumpur [43].

While, the average pH readings of the effluent (6.38) in the local WWTP was more acidic as compared with the influent (6.57) in anoxic condition. According to World Health Organization studies, the average pH readings for the rainwater in Malaysia are in the range of 6.5 to 8.5 [44]. The pH values of 6.5 to 8.5 are due to exchangeable base cations such as Ca^{2+}, Mg^{2+}, Na^{+} and K^{+} ions, which dissolved upon the contact of water flowing on the ground with soil and dust lying on the pavement [45].

Based on the experiment conducted at two different points, the range of pH values recorded was in between 5.90 and 7.22. This indicates that the samples were neither neutral nor acidic due to the weather factor. Nevertheless, the samples were found to be more acidic during the rainy days compared with dry and normal days. At the local WWTP, the operations were mainly conducted as an open-pond system whereby these ponds receive rainwater without any cover protection enabling mixing to take place freely (IndahWater, [7]). Therefore, significant influence in the term of pH was observed and described in Fig. 4.

Based on Strous et al. [46], it was proven that the optimum pH within a range of 6.7 to 8.3 able to enhance the growth and activity of anammox bacteria during wastewater treatment. Besides, the pH value in between 6.5 and 9.3 was also reported to be suitable for the anammox activity [47]. According to Van Der Star et al. [48], the different pH values such as 6.3 and 7.3 show that the anammox cell contains two compartments, and subsequently suggests the availability of a proton motive force over an intracytoplasmic membrane. The low pH may cause a decrease in the free ammonia concentrations and lead to increase in the free nitrous acid concentrations. It is vice versa in high pH, where free ammonia concentrations increase and free nitrous acid concentrations drop would be caused [49]. When the pH is lower, the reactor could be operated efficiently with a higher nitrite level [50]. Therefore, the possibility for anammox bacteria to survive according to the pH values of the WWTPs is high.

3.1.3. DO analysis

DO concentration is an important factor to cultivate anammox bacteria. As the bacteria were bound to anaerobes, it was better to keep the DO as low as possible in the process. If the supplied oxygen is higher than the limit for consumption of nitrifiers, consequently the oxygen may reach inhibitory levels for anammox bacteria [51]. Hence, the DO concentrations are usually controlled to prevent reversible or even irreversible inhibition [52].

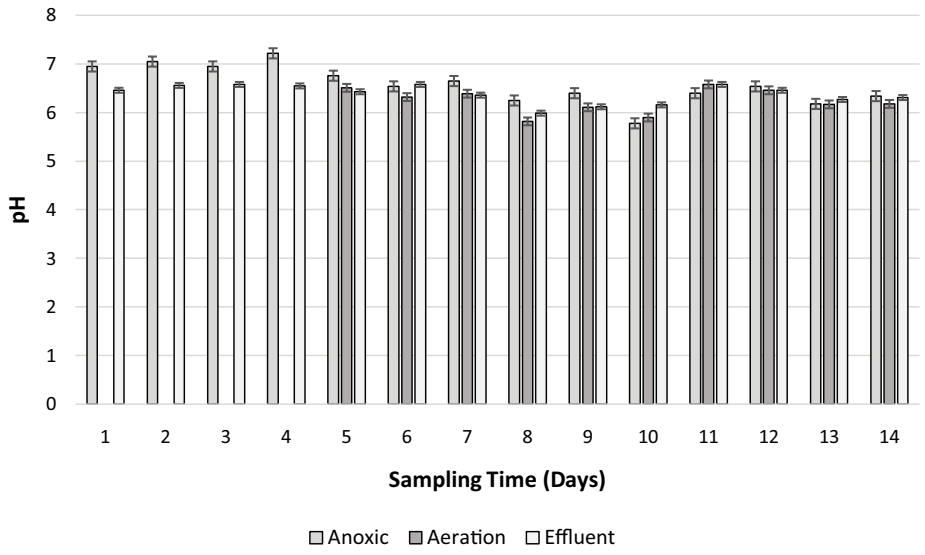

Fig. 4. Variations of pH in anoxic condition, during aeration and in effluent.

The DO concentrations in the anoxic condition were stable for the first 7 d (measured within the range of 0.55 to 0.98 mg/L) and fluctuated afterwards. However, the DO in anoxic tank during the rainy and normal days rose as compared with dry weather, as per tabulated in Table 1. This was due to high MLSS content in the aeration tank of 12,100, 17,800 and 19,800 mg/L, respectively. The rise of high MLSS content was due to the operational issue at the local WWTP. The lack of sludge wasting into the sludge holding tank for dewatering process occurred as limited digester operated due to maintenance. Fig. 5 describes the variations of DO in the wastewater samples collected.

According to Wang et al. [53], when DO is greater than 2 mg/L, the anammox bacteria were still active in aerobic tanks with 0.12–1.13 μmol N g^{-1} h^{-1} in summer and 0.08–0.25 μmol N g^{-1} h^{-1} in winter. Therefore, the possibility for the anammox bacteria to survive in anoxic tank and aeration tank of SBR is high since the DO of both tanks is below 2 mg/L.

3.1.4. Nitrogen removal

The nitrogen removal includes the analysis of total nitrogen (TN), ammonia, nitrite and nitrate concentrations. Currently, there is no standard in Malaysia that limits the discharge of nitrogen (i.e., TN, ammonia, nitrate and nitrite). However, public sewage treatment in Malaysia on average complies to limit ammoniacal nitrogen value below 15 mg/L for Standard A and 25 mg/L for Standard B (IndahWater [7]).

TN in wastewater is the sum of ammonia, nitrite and nitrate. These are the major forms of nitrogen that can be found in wastewater. Ammoniacal nitrogen in the wastewater was the content of ammonium (NH_3–N) that is commonly found in the sewage. Fig. 6 demonstrates the variation of NH_3–N in anoxic tank, aeration tank and effluent of SBR. The ammoniacal nitrogen concentration of SBR effluent was measured to calculate the ammonia removal efficiency in the SBR. The concentrations of NH_3–N in the anoxic tank indicated a sudden decrease at day-2, however increase on day-3 to day-5, whereas on day-6 onwards, the concentrations started to decline. The reason for this phenomenon was probably due to the operation of the plant that inconsistently running the blower during aeration process. Despite that, NH_3–N shows positive removal between 65% and 96% except at day-12 where the removal efficiency was –37.5%.

Nitrite (NO_2–N) in the wastewater indicates the amount of NH_3–N being converted after nitrification process in the anoxic condition. Fig. 7 demonstrates the variations of nitrite concentration in anoxic condition, during aeration and in effluent. Removal efficiency of NO_2–N shows negative values in most days proved the incomplete conversion of nitrite to nitrate and nitrogen gas. This may be due partial nitrification process, in where 50% of the ammonium has

Table 1
Samples of effluent from the anoxic and SBR tanks

	Sample in anoxic tank, Sample A			Sample in SBR tank, Sample R		
Weather conditions	Dry (Hot)	Normal	Wet (Rainy)	Dry (Hot)	Normal	Wet (Rainy)
Temperature (°C)*	32.70 ± 0.75	31.74 ± 0.19	30.88 ± 0.53	32.70 ± 0.14	32.05 ± 0.29	31.33 ± 0.15
pH	6.86 ± 0.31	6.51 ± 0.18	6.29 ± 0.49	6.39 ± 0.10	6.35 ± 0.20	5.96 ± 0.18
DO (mg/L)	0.69 ± 0.13	0.87 ± 0.21	1.67 ± 0.64	1.38 ± 0.35	1.53 ± 0.07	2.47 ± 0.46

DO: dissolved oxygen.
*Temperature describes the temperature of wastewater samples.

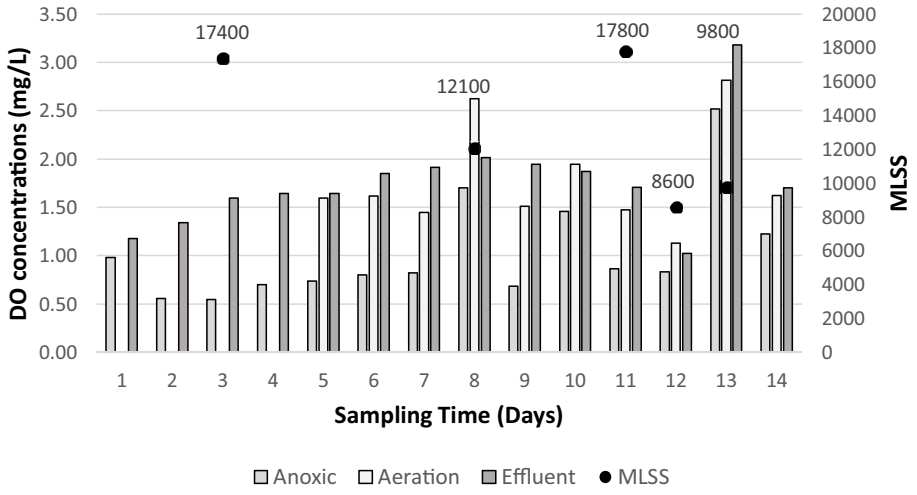

Fig. 5. Variations of dissolved oxygen in anoxic condition, during aeration and in effluent.

been oxidized into nitrite [39]. The factors may be caused by several different processes such as SHARON (single reactor high activity ammonia removal over nitrite), oxygen-limited bioreactors, inhibition of nitrite-oxidizers by free ammonia or free nitrous acid or the recent aerobic granular reactors [54–56]. According to Jin et al. [57], the most difficult aspect in the anammox stability performance is the presence of NO_2^-–N, which is the electron acceptor in the process. Therefore, it can be seen that the local WWTP has NO_2^-–N, which is capable of enhancing the anammox performance.

Nitrate (NO_3–N) in the wastewater indicates the amount of nitrite being converted during denitrification process at the SBR tank. Fig. 8 demonstrates the variations of NO_3–N in anoxic condition, during aeration and in effluent. Concentrations of NO_3–N in all conditions fluctuated due to the inconsistent conversion of nitrite component. At nitrite concentration of 0.014 mg/L on day-14, much higher rate (3.70 mg/L) of nitrate concentration was yielded while at nitrite concentration of 0.019 mg/L lower rate (1.30 mg/L) of nitrate concentration was produced, accordingly. It was observed that the variation of nitrite concentration is within 0.005 mg/L. However, the conversion nitrate is been affected significantly. This may be due to high competition between anammox and NOB for nitrite. In nitratation process, NOB is responsible to convert nitrite to nitrate while in anammox process anammox bacteria converts ammonium

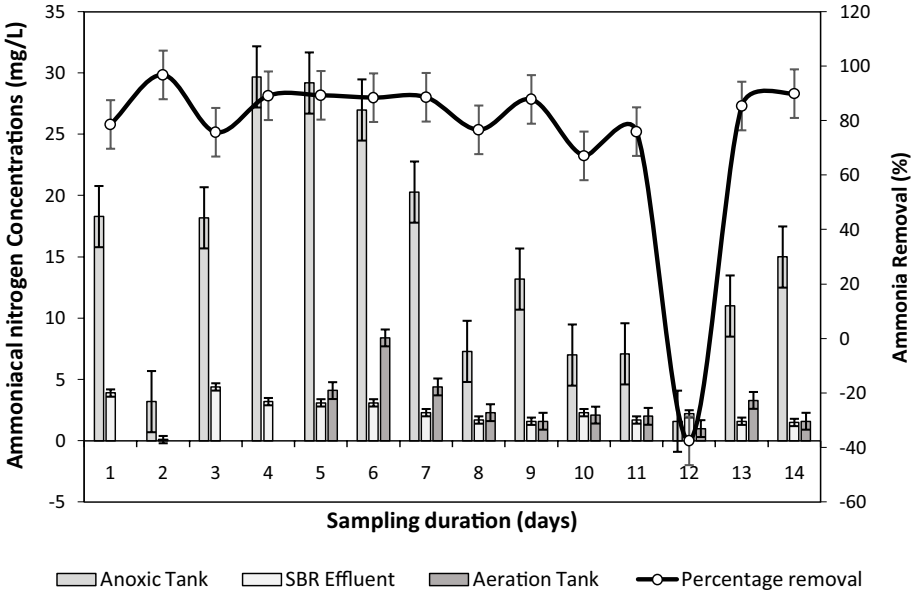

Fig. 6. Ammoniacal nitrogen concentration and removal in anoxic tank, aeration tank and effluent of SBR.

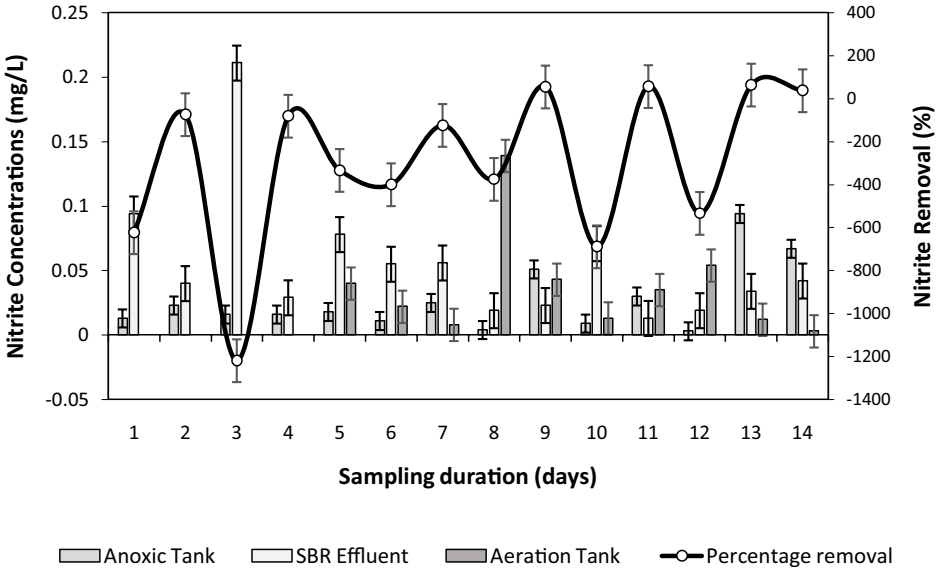

Fig. 7. Nitrite concentration and removal in anoxic tank, aeration tank and effluent of SBR.

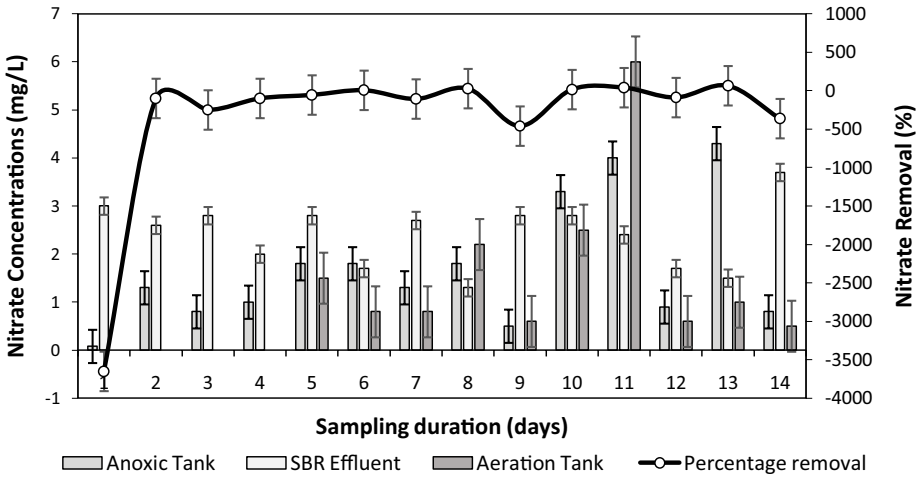

Fig. 8. Nitrate concentration and removal in anoxic tank, aeration tank and effluent of SBR.

to dinitrogen gas using nitrite as electron acceptor [58]. Thus, the nitrate concentration varies due to inconsistent conversion of nitrite component.

The changes of temperature due to weather condition is one of the barrier for steady performance of nitrogen removal performance and anammox species as temperature directly decreased from normal temperature to low temperature during rainy days. This subsequently causes limitation for nitrification and denitrification processes, which led to negative removal efficiency [59]. Another reason of those ammonium, nitrite and nitrate negative removal efficiency was probably due to high sludge content in the SBR tank as it caused inefficient degradation of nitrogen content. High sludge content in the SBR tank was due to the low of sludge washout into sludge holding tank as the dewatering process occurs in one machine instead of two. The percentage of removals was mostly in negative readings due to incomplete reaction of nitrogen cycle, which subsequently caused the rise of nitrogen content in the effluent. However, during aeration process, the concentrations of nitrogen were unstable, which were increasing and decreasing than concentrations of nitrogen in anoxic tank. Furthermore, the timing for collecting the samples was not consistent due to the weather condition. Therefore, the results obtained are different as compared with the previous days.

3.2. Identification of anammox bacteria from domestic wastewater

Based on the analysis conducted, in the wastewater of the selected treatment plant, there were potential for certain anammox bacterial species to survive. According to the physiological characteristics tabulated in Table 1, anammox species that fit into the characteristics of the plant were *Brocadia anammoxidans*, *Brocadia sinica*, *Kuenenia stuttgartiensis* and *Jettenia caeni* [22]. Further analyses on selection of PCR primer were conducted based on the bacteria types.

3.2.1. Microbial community analysis

To determine the presence of anammox bacteria, microbial analyses were conducted in the selected WWTP. Analyses including gDNA extraction, PCR amplification and DNA sequencing were done on the samples. Sludge from anoxic tank, sample A and SBR tank, sample R during aeration process in day-12 was selected for microbial identification as it was freshly sampled on the DNA analysis day.

The extracted gDNA concentrations of the DNA samples measured were 487.95 and 633.36 ng/μL for sample A and R, respectively. From the results, it was calculated that 24.40 and 31.67 μg of gDNA were successfully extracted from anoxic sludge and aeration sludge, respectively. Extracted gDNA from the sample sludge was further tested with 1% agarose gel to identify any contaminant present in the gDNA. 1 kb DNA ladder was used a marker to determine the base pair of the DNA. To ensure ease of data reading, reference band was bold at 3,000 and 1,000 bp. A positive control, M50 was run together the DNAs sample sludge to ensure the analysis compatible with DNA sludge and produce a band. However, a clear band was observed at higher than 10,000 bp marker line. This indicates DNAs obtained from both samples have abundance of bacteria community in the sample. Although smudges happened in the M50 line, the gDNA results were proceeded with PCR amplification as the smudges were not significant.

The 16S rRNA genes of anammox bacteria were successfully amplified using Brod541F and Amx820R. During gel electrophoresis analysis, 1 kb DNA ladder was used a marker to determine the base pair of the DNA. To ensure ease of data reading, reference band were bold at 3,000 and 1,000 bp. A negative control was used in the agarose gel analysis to ensure that the gel is in good condition. As no band appeared on that control lane, the agarose gel was in good condition and the PCR result was reliable.

Then, the preliminary analysis for the PCR products was conducted using different amounts of gDNA. Sample A, gDNA sludge from anoxic tank was used to determine the right amount needed for PCR reaction. Two bands appeared at each sample lane 1, 2 and 3 near the 250 and 1,000 bp marker. According to Li et al. [33], the PCR product size for anammox bacteria was 268 bp. Thus, the band that appeared

at 1,000 bp marker probably was not related to anammox consortium. However, all lanes band have smearing, which indicated that the products were degraded presumably due to the unsuitable annealing temperature. Therefore, another PCR analysis was conducted to determine the suitable annealing temperature. Since the difference of band between all the lanes was insignificant, gDNA sample at lane 1 was used for further analysis as it has the least thickness of band at 250 bp.

The PCR products used different annealing temperatures such as 55°C, 57.8°C, 59.1°C and 61°C for the second PCR analysis [33]. In lane 57.8, 59.1 and 61 only band appeared at slightly higher than 250 bp whereby in lane 55 two bands appeared at 250 bp and 1,000 bp. Therefore, the suitable annealing temperature for anammox reaction would be 57.8°C, 59.1°C and 61°C as the size of PCR products corresponds to the study conducted by Li et al. [33]. Furthermore, lanes 57.6, 59.1 and 61 showed better quality bands than lane 55 due to less smearing effect. As these temperatures suitable for annealing anammox DNA, degradation of temperature was conducted for annealing step in the PCR reaction starting from 61°C to 57.6°C.

The marker of 1 kb DNA ladder was changed to 100 bp DNA ladder due to small band size of anammox bacteria. Based on Fig. 9, the size of PCR products observed was approximately 300 bp in both formulae. Similar band size was obtained in the study by Li et al. [33], when using the same pair of primer which was 268 bp. The study also remarked that 98% of the planctomycetes species in the sample were obtained from marine sediments [33]. Therefore, the PCR products used for sequencing shows the presence of anammox bacteria in large quantity in the local WWTPs.

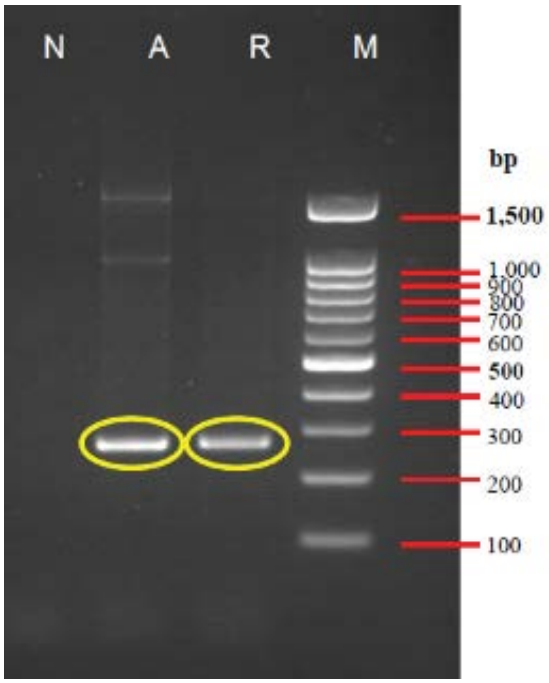

Fig. 9. PCR products of sludge from anoxic tank and during aeration process. Legend: Lane M – 100 bp DNA ladder marker, Lane N – negative control, Lane A – sludge from anoxic tank and Lane R – sludge from SBR tank.

3.2.2. Anammox microbes in the sludges

PCR products undergo cloning before the sequencing analysis. In the sequencing analysis, only forward reaction was involved as the size of the PCR product was smaller than 1,000 bp. The obtained sequences were analysed using BLAST analysis to determine the bacterial community in the sample. In the preliminary sequencing, out of five cloning conducted, two of them showed the presence of *Candidatus Kuenenia stuttgartiensis*.

A total of 40 sequences were BLAST in the NCBI website to compare the presence of anammox bacteria with the database. 20 sequences of DNA were cloned from each of sample A (GAG CAC GTA GGT GGG TT) and R (GGG CAC TAA GTA GAG GGA ATT) and 268 bp of sequences were amplified from each clone. Based on the BLAST results, uncultured bacterium (GenBank accession no.: KU152737.1) has the highest percentage identities of 97% in both samples. This was since the samples were taken from the environmental source. Despite that, *Candidatus Kuenenia stuttgartiensis* (GenBank accession No.: MH581444.1) was detected in both samples with 90.417% percentage identities. The bacteria have 790 bp and the sample matches sequence from 551 to 790 bp.

The fact that DO concentrations play important role in inhibiting anammox bacteria, *Kuenenia stuttgartiensis* managed to be detected in the wastewater [53] and that explains anammox bacteria have the ability to adapt with high DO concentration conditions. According to Kartal et al. [60], *Kuenenia stuttgartiensis* have special characteristics, whereby it can survive in wastewater with high salinity.

Fig. 10 illustrated the phylogenetic tree of both samples with *Candidatus Kuenenia stuttgartiensis*. Two different clones of *Candidatus Kuenenia stuttgartiensis* were chosen to compare with the samples due to their high identities with the sample. Based on the Fig. 10, the samples share the same ancestor with *Candidatus Kuenenia stuttgartiensis* proved that the bacteria present in the samples are descendants of *Candidatus Kuenenia stuttgartiensis*. Furthermore, both sample sequences share same clade with *Candidatus Kuenenia stuttgartiensis* clone S0-S-40 meaning that they have the same characteristics with the clone bacteria.

4. Conclusion

Application of anammox-based system in the treatment of domestic wastewater is uncommon in Malaysia wastewater treatment scenario. This study demonstrated that operational temperature, pH and DO concentrations were highly influenced by the acidity of rains. The temperature was found to fluctuate between 29.6°C and 32.0°C throughout the experiments at commemorating pH values of between 5.78 and 7.22 and DO concentration of 0.62–1.53 mg/L. DO concentrations in both tanks were stable (0.55 to 0.98 mg/L) except during operational issue where maintenance of the digester was carried out. Nitrogen removal of the plant showed negative value for nitrite (–1,218 to –73%) and nitrate (–3,650 to –55%) as the component produce throughout the process. This inefficient degradation was presumably due to the high sludge in the tanks as less amount of sludge was being wasted into the digester.

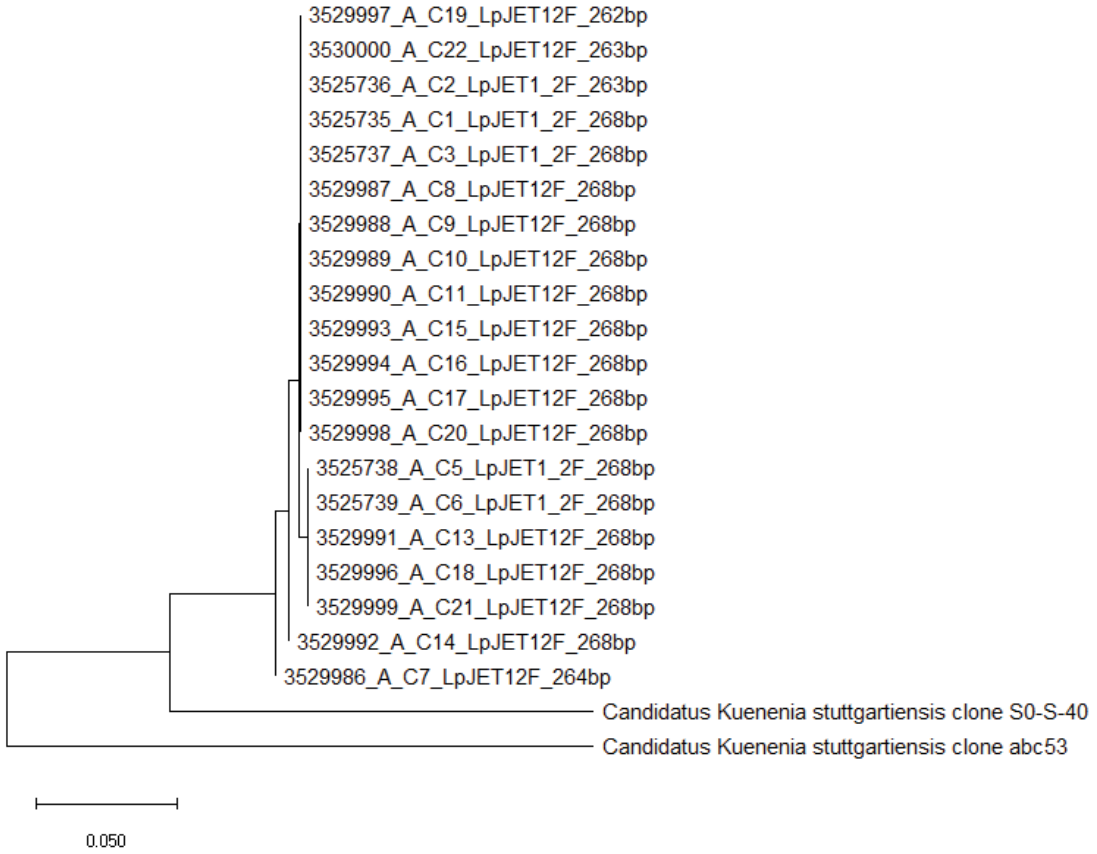

Fig. 10. Phylogenetic tree of both samples with *Candidatus Kuenenia stuttgartiensis*.

The gDNA of the sludge from anoxic tank and SBR tank was successfully extracted with the amount of 24.40 and 31.67 μg, respectively. PCR amplification of the anammox bacteria encountered several problems during the initial stage as the bands appeared smearing in the electrophoresis analysis. Additional test on finding suitable annealing temperature for anammox bacteria was found out to be between 57.6°C and 61°C. It was found that *Candidatus Kuenenia stuttgartiensis* was present in the sampled wastewater, which had the potential to be enhanced to aid domestic wastewater treatment. Thus, the anammox bacteria found in the local WWTP may be able to overcome the challenges faced for nitrogen removal through short and efficient anammox process.

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