Comparison of Nitrogen Removal Efficiencies and Microbial Communities of Full-Scale Anaerobic, Anoxic and Aerobic Processes in Municipal Wastewater Treatment Plants having Low and High COD/TN Ratios

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Abstract

Full-scale anaerobic, anoxic and aerobic (A^2O) process is used worldwide for biological nutrient removal (BNR). However, operation parameters for nitrogen removals and information of microbial communities related to nitrogen removal in full-scale A^2O wastewater treatment plants (WWTPs) having low and high COD/TN ratios are not available. Based on the analysis of four full-scale A^2O WWTPs, it is suggested that maintaining longer SRT of ≥ 30 day and DO of ≥ 0.9±0.2 mg-O_2 L^{-1} is needed to improve nitrogen removal efficiency under low COD/TN ratio (≤ 3.7). On other hand, at high COD/TN ratio (≥ 4.2), DO level of ≥ 2.6 mg-O_2 /L and typical SRT of 19– 25 days would be suggested. It was confirmed that phosphorus removal efficiency significantly improved under BOD/TP ratio of > 20 for A^2O process in these full-scale WWTP.

Microbial distribution analysis showed that ammonia-oxidizing archaea (AOA) was abundant under conditions of low DO level, longer SRT, high temperature and low COD/TN ratio (≤ 3.7). *Nitrosomonas* sp. are mostly found in aerobic tank of full-scale A^2O WWTPs. However, abundances of *Nitrosomonas* sp. are proportional to DO and NH_4^+ concentrations for WWTPs with high COD/TN ratio. *Nitrosospira* sp. are only found under operating condition of longer SRT for WWTPs with low COD/TN ratio. Abundances of *Nitrobacter* sp. are proportional to DO concentration and temperature rather than abundance of *Nitrospira* sp. Predominance of *nosZ*-type denitrifiers were found at low COD/TN ratio. Abundance of denitrifiers by using *nir*S genes was over abundance of denitrifiers by using *nir*K genes at high COD/TN ratios WWTPs.

Introduction

Organic matters and inorganic nutrients are the main contaminants to be treated in municipal wastewaters. Discharge of inorganic nutrients such as nitrogen (N) and phosphorus (P) compounds with to environment is responsible for eutrophication or algal blooms and toxic effects to aquatic life. For this reason, organic matter and inorganic nutrients have to be removed before discharging wastewaters to environment. Biological treatment processes are often recommended because of high removal efficiency and inexpensive operation cost compared to physical and chemical treatment processes. Modified Ludzack-Ettinger (MLE) consisting of anoxic and aerobic processes is specifically designed for N removal. A^2O consists of anaerobic, anoxic and aerobic processes and is used worldwide to remove both N and P biologically in which abundance of microorganisms responsible for various nutrient removal is achieved under designed environmental conditions. With alkalinity provided for nitrification step, A^2O processes can remove both nitrogen and phosphorus efficiently, producing good settling sludge. The energy cost is low and operation is relatively simple. Internal nitrate recycle through proper control of recycling return activated sludge (RAS) from aerobic zone to the anoxic zone is the key to operate A^2O process successfully [1]. When designing A^2O processes, wastewater characteristics and operation parameters including contact time of anaerobic tank, solids retention time (SRT), hydraulic retention time (HRT), DO concentration, etc. must be taken into consideration. Wastewater with proper carbon and total nitrogen ratio, i.e., COD/TN ratio, results in good process performance. Typical COD/TN ratio of ≥ 4 for denitrification process (anoxic tank) and BOD/TP ratio of 20 for biological phosphorus removal process (anaerobic tank) are observed for an A^2O processes with excellent N and P removal [1]. Insufficient carbon source for denitrification process occurs in municipal wastewater with low COD/TN ratio, resulting in low N removal performance [2]. External carbon source addition [3] or maintaining longer SRT [4] are the most effective approaches to improve biological N removal performance for wastewater with low COD/TN ratio. Investigating the effects of SRT on N removal efficiency, Liu et al. [5] reported that system with SRT at 40 day outperformed those with shorter SRTs (5, 10, and 20 day). Phanwilai et al. [4] achieve excellent N removal with a step feed treatment process operated at SRT of 60 day. Biological N removal can be improved by optimizing DO levels. In case of keeping very low DO level of 0–0.5 mg-O_2 L^{-1}, ammonia-oxidizing archaea (AOA) would be the dominant microorganisms group responsible for N removal [6]. Increasing abundance of ammonia-oxidizing bacteria (AOB) was reported with a high DO level of 1.9–3.5 mg-O_2 L^{-1} [6]. The domination of *Nitrospira* was observed at DO below 1.0 mg-O_2 L^{-1} [7].

Temperature and free ammonia (FA) are also important factors affecting the microbial community. Temperature at 10–20ºC was reported to be the optimum range for *Nitrospira* [8] and the temperature at 24–25ºC is favorable for *Nitrobacter* [7]. FA was an inhibitor of NOB activity [9]. Furthermore, *Nitrobacter* is more sensitive to FA than *Nitrospira* [10].

This research work focused on the nitrogen removal performance, identification and quantification of microbes from anaerobic, anoxic, and aerobic tanks of A^2O processes in four full-scale municipal WWTPs having low (≤ 3.7) to high (≥ 4.2) COD/TN ratios. In this work, only nitrogen removal efficiencies were compared and discussed by using results from microbial quantity and communities of bacteria related to nitrogen removal, such as AOA, AOB, nitrite-oxidizing bacteria (NOB), and denitrifying bacteria (DNB) in these four full-scale A^2O WWTPs. In addition, the results from this work could be applied to increase N removal efficiencies of these four full-scale WWTPs.

Materials And Methods

Description of the study sites

A^2O processes from four full-scale WWTPs with wide ranges of COD/TN ratios were selected as the study sites. Two WWTPs were located in Ding Daeng, Bangkok and Samut Prakan Province, Thailand, and are denoted as WWTP TH1 and TH2, respectively. The other two full-scale WWTPs, Dalseocheon and Sincheon WWTPs, were located in Daegu, South Korea, and are denoted as WWTP SK1 and SK2, respectively. A^2O processes in these full-scale WWTPs were mainly designed for BNR, especially for biological both nitrogen and phosphorus removals. The influent COD/TN mass ratios at TH1, TH2, SK1, and SK2 were 3.7, 8.4, 4.2, and 5.6, respectively. TH1 is considered to have low COD/TN ratio (≤ 3.7), while TH2 has relatively high COD/TN ratio (> 4) due to the wastewater being mainly generated from aircrafts, business and commercial buildings, such as hotels and airlines' offices, in the surrounding of Suvarnabhumi airport.

The schematic layout of the full-scale A^2O processes in the TH1 and TH2 is shown in Fig. 1 (A). The schematic layout of full-scale A^2O processes in the SK1 and SK2 (with 1st and 2nd clarifiers) are shown in Fig. 1 (B). No primary clarifier was designed for TH1 and TH2. Two internal recycles are designed in these plants. The first one is from aerobic zone to anoxic zone. The second one is for the return activated sludge (RAS) which was recycled from the 2nd clarifier back to the anaerobic zone.

All wastewater samples from these four full-scale WWTPs were collected from each sampling points (anaerobic, anoxic, and aerobic zones) twice (between 2018 and 2019).

Wastewater quality analysis

All influent and effluent wastewater samples were analyzed for BOD, COD, NH_4^+-N, NO_2^--N, NO_3^--N, organic-N, TKN, TN, TP, TSS, and SS following the Standard Method [11]. Selective effluent samples were measured for *E. coli*.

Microbiological analysis

Molecular analysis of microbes was conducted for selected sludge samples from anaerobic, anoxic, and aerobic zones. Before DNA extraction step, the sludge of each tank was harvested and kept on ice. One mL of sludge was used for DNA extraction according to the procedures of Zhou et al. [12].

Polymerase chain reaction (qPCR)

Using quantitative polymerase chain reaction (qPCR) analysis focused to the microbial abundance. A 20-µL sample was mixed with 1 µL of template DNA and 20 pmol of each primer. All qPCR reactions were performed by using a CFX96 Touch™ Real-Time PCR and CFX Manager version 3.1.1517.0823. (Bio Rad Laboratories, Inc). An efficiency, slope and r^2 values of individual real-time PCR assays are 98.3–106.1%, (−3.1)–(−3.4), 0.993–0.997 and linearity range is 10^1–10^8, see Table S1. Oligonucleotide primers for PCR amplification via qPCR are shown in Table S2.

Denaturing gradient gel electrophoresis (DGGE)

Using denaturing gradient gel electrophoresis (DGGE) analysis focused to the microbial communities responsible for N removal. Total bacteria were identified via 16S-rRNA EUB gene. Ammonium-oxidizing bacteria and archaeal were identified through AOB-and AOA-*amoA* genes. Nitrite-oxidizing bacteria (NOB) were identified *Nitrospira* and *Nitrobacter* via 16S rRNA *NSR* and *Nitro* genes, respectively. Denitrifying bacteria (DNB) were identified via *nirS*, *nirK* and *nosZ* genes. Oligonucleotide primers for DGGE are shown in Table S3. Each 25-µL reaction mixture was added with 1 µ L^{-1} of template DNA with concentration of 10–20 ng µ L^{-1}, 10X *Ex Taq*™ buffer, 5 units/µL *TaKaRa Ex Taq*™, 2.5 mM dNTP Mixture, and 10 pmol of each primer, and the mixture was finally diluted with nuclease-free water. All PCR reactions were performed by using a T100™ Thermal cycler (BioRad Laboratories, CA and USA). (Bio Rad Laboratories, Inc.). The PCR product of 15 µL was loaded into individual lanes on 8% (W/V) acrylamide gel with 35—55% gradient for EUB target and with 35—50% gradient for AOB target. And electrophoresis step was performed in 1x TAE buffer at 58 ºC with a constant voltage at 80 V for 16 h. The shaped DNA band on acrylamide gel was excised by a scalpel. The DNA fragments were eluted by milli-Q water and set aside in a refrigerator for overnight, and then amplified PCR with the same primer without attached CG-camp. Sequencing bases were aligned by using database of the National Center for Biotechnology Information (NCBI).

Loading rate and removal rate calculation

The removal efficiencies (%) of nutrients and contaminants were calculated as Eq. (1).

$$\text{Removal efficiency } (\%) = \frac{C_{inf} - C_{out}}{C_{inf}} \times 100 \quad (1)$$

where C_{inf} and C_{out} are concentrations (mg L^{-1}) of water quality parameters in influent and effluent of a treatment process, respectively.

COD loading rate (kg COD·(m^3·d) $^{-1}$), BOD loading rate (kg BOD·(m^3·d) $^{-1}$), and Ammonia loading rate (ALR) (kg NH_4^+-N·(m^3·d) $^{-1}$) were calculated according to the Eqs. (2), (3) and (4), respectively.

$$\text{COD } (\text{kg-N}/\text{m}^3\bullet\text{d}) = \frac{COD_{inf} \times Q}{V} \quad (2)$$

$$\text{BOD } (\text{kg-N}/\text{m}^3\bullet\text{d}) = \frac{BOD_{inf} \times Q}{V} \quad (3)$$

$$\text{ALR } (\text{kg-N}/\text{m}^3\bullet\text{d}) = \frac{NH_{4\,inf}^+ \times Q}{V} \quad (4)$$

where COD_{inf} and BOD_{inf} are concentration (mg L^{-1}) of COD concentration of influent, (kg COD·(m^3) $^{-1}$) and BOD concentration of influent, (kg BOD·(m^3) $^{-1}$), respectively. $NH_4^+{}_{inf}$ is ammonia concentration of influent, (kg NH_4^+-N·(m^3) $^{-1}$), Q is flow rate, (m^3·d^{-1}), and V is volume of reactor, (m^3).

Free ammonia (FA) was calculated by Eq. (5) according to Anthonisen et al. [13].

$$\text{FA (mg-N/L)} = \frac{17}{14} \times \frac{\left[NH_4^+\right]_{inf} \times 10^{pH}}{\exp\left[\frac{6334}{273+T} + 10^{pH}\right]} \quad (5)$$

where $NH_4^+{}_{inf}$ is ammonium concentration of influent (mg-N L^{-1}) and T is the temperature of effluent (°C).

Statistical analysis for microbial abundances

Two-way multivariate analysis of variance (two-way MANOVA): Tukey's honestly significant difference (HSD, at $p < 0.05$) was performed using Minitab 18.1 for microorganism abundance as copies-DNA. The level of statistical significance and correlations were considered to be statistically significant with the 95%.

Results And Discussion

Major operational parameters of full-scale A^2O processes

Operational parameters of A^2O processes in the four WWTPs are presented in Table 1. The A^2O processes at TH1 were operated at the average flow rate of 218,433 m^3 d^{-1}. The average flow rate was 7,673 m^3 d^{-1} for TH2. The average flow rates at SK1 and SK2 WWTPs were 220,655 and 497,174 m^3 d^{-1}, respectively. Flow rates from high to low are SK2 > SK2 ~ TH1 > TH1. Compared with TH1 (30 mg L^{-1}), TH2 (198 mg L^{-1}), and SK1 (75 mg L^{-1}), high BOD values (260 mg L^{-1}) were found at SK2 because the WWTP received industrial wastewaters as well (approximately 25% of total volume). TH1 received wastewater with very low BOD. It is because that TH1 treated wastewater collected from a combined sewer system with domestic sewage being diluted by stormwater. Infiltration and inflow are able to enter this combined sewer system. Meanwhile, the high temperature inside the sewer lines could promote the degradation of BOD. Finally, the wide practice of septic tanks installation in the residential houses could remove BOD before wastewater entering the sewer lines.

All operational parameters of DO, SRT, and HRT can be found in Table 1. Comparison these operational parameters at TH1, TH2, SK 1, and SK2, low DO level (0.9±0.2 mg-O_2 L^{-1}), longer SRT of 30 day, and HRT (8 hr.) were found at TH1 and high DO level (4±0.5 mg-O_2 L^{-1}), shorter SRT of 17 day and HRT (3.6 hr.) were found at SK1. At TH2, quite high DO level (2.6±0.2 mg-O_2 L^{-1}), SRT of 19 day, and longer HRT (15.4 hr.) were found at TH2 and quite high DO level (3±0.3 mg-O_2 L^{-1}), quite longer SRT of 26 day, and longer HRT (10.1 hr.) were found at SK2. Longer SRT of ≥26 day with DO level (≥ 0.9±0.2 mg-O_2 L^{-1}) could be suggested to use as operation parameters when treating low COD/N ratio wastewaters at TH1 or COD/N ratio of 4.2 at SK2.

Performances of full-scale A^2O processes WWTPs

COD, BOD, and nitrogen removals efficiencies in the full-scale A^2O processes WWTPs are shown in Table 2. COD, BOD, NH_4^+-N, TN, and TP removals efficiencies were 67%, 83%, 95%, 49%, and 35% respectively, at TH1. These values were 98%, 92%, 91%, 86%, and 96% respectively, at TH2. They were 96%, 89%, 99%, 70%, and 95% respectively, at SK1, and were 99%, 96%, 98%, 82%, and 98% respectively, at SK2.

The average nitrogen concentration and removal efficiencies in each month are shown in Figure 2. A^2O processes are designed for biological N and P removals and suitable for municipal WWTP [1]. However, total nitrogen (TN) removal efficiency at TH1 was quite low (only 49%) compared to other plants. The low TN removal performance could be explained by the very low COD/TN ratio (3.7) in the wastewater received at TH1. The low nitrogen removal efficiencies were also reported by Liu et al. [14] for WWTPs treating wastewater of relatively low COD/TN ratios. It was reported that denitrification process could not significantly occur due to the insufficient carbon source for denitrification process with wastewater having relatively low COD/TN ratios of lower than 4. On the contrary, efficient TN removal (86%) was reported for TH2 which received wastewater with COD/TN ratio of 8.4.

For WWTPs treating low COD/TN ratio (≤ 4) wastewater, maintaining longer SRT (≥ 60 day) is recommended to overcome the low TN removal efficiency [4] as the longer SRT would increase nitrifying bacteria abundancy. Meanwhile, long SRT could also enhance NH_4^+ removal by increasing nitrification activity [1]. The effects of SRT on NH_4^+ removal were reported by [5]. The effluent NH_4^+ concentrations in activated sludge process were reported for SRTs at 5 day (2.6±2.3 mg N L^{-1}), 10 day (0.04±0.01 mg N L^{-1}), 20 day (0.03±0.007 mg N L^{-1}), and 40 day (0.02±0.003 mg N L^{-1}), corresponding to NH_4^+-N removal efficiencies of 94.5%, 99.9%, 99.9%, and 99.9%, respectively. The effluent NH_4^+ concentration at TH2 plant was 4.8 mg N L^{-1} and was the highest among the WWTPs studied due to the highest NH_4^+ concentration in the raw water (55.4 mg N L^{-1}). To further enhance the removal of NH_4^+-N, a long SRT of > 19 day should be recommended.

The results of TP removal confirm that the A^2O processes at three WWTPs (BOD/TP ratio of > 20) was able to remove P concentration well (more than 96% of TP removal efficiencies) except TH1 which has lowest BOD/TP ratio of 13 and achieved only 35% of TP removal. To increase phosphorus removal efficiency at TH1 (in case there is low BOD:TP ratio), the chemical treatment by using alum as coagulant would be recommended.

Nitrogen-cycling microbial abundances and predominated existing would be relative with various environmental factors such as dissolved oxygen level, SRT, temperature, pH, ammonium loading rates (ALRs), etc., and will be discussed in the following section.

Nitrogen-cycling microbial abundances and communities

Abundances and communities of ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaeal (AOA)

Autotrophic nitrifying bacteria responsible for ammonia oxidation process are detected at WWTP TH1 and belonged to two orders: *Nitrosomonadales* (affiliated to *Nitrosomonas* sp. *Nitrosospira* sp., *Nitrosococcus* sp., and *Thiobacillus* sp.) and *Rhodocyclales* (affiliated to Azospira sp., *Thauera* sp., and *Zoogloea* sp.) as shown in Table 3. Zhang et al. [15] reported that in full-scale municipal WWTPs, the most important genera of AOB were *Nitrosomonas* and *Nitrosospira*. Furthermore, they mentioned that *Nitrosomonas* were the most dominated ones. Consistently, in the full-scale A^2O WWTPs, *Nitrosomonas* sp. are the most dominant AOB, especially in the WWTPs TH2, SK1, and SK2 which were operated at high DO levels. By contrast, the microbial community of *Nitrosospira* sp. was found at the TH1 because this WWTP was operated under long SRT, a favor condition for the growth of *Nitrosospira* sp. (see Table 3). Although the abundance of *Nitrosospira* sp. is less than that of *Nitrosomonas* sp., the existence of *Nitrosospira* sp. might be suitable a factor for satisfying good nitrification process when the conditions are not optimal for growth of nitrifying bacteria [16].

Figure 3A shows the abundance of AOA-*amoA* genes at WWTP TH1, which is the highest among the WWTPs full-scale A^2O process investigated, and the significance of each tank at TH1 shown the high mean difference of letter grouping in blue clustered column, ($p < 0.05$), see Table S4. The lower DO level, high temperature, and longer SRT (> 30 day) operated in this plant would significantly promote the growth of AOA [17]. Gao et al. [6] studied the effects of DO levels on the growth of AOB-*amoA and* AOA-*amoA*, showing the former is much more abundance under high DO levels of 1.9–3.5 mg-O_2 L^{-1}. Phanwilai et al. [4] analyzed the abundance of microorganisms in the step-feed aerobic tanks of a municipal wastewater treatment plant, reporting that AOA-*amoA* was the most abundance genes in the tank with low DO levels (0.9±0.5 mg-O_2 L^{-1}) while AOB-*amoA* gene was higher than AOA-*amoA* genes in the tank with high DO level (1.8±0.5 mg-O_2 L^{-1}). In this work, the result of AOB and AOA abundance at WWTPs TH2, SK1, and SK2, which are operated at high DO levels of 2.6–4 mg-O_2 L^{-1}, is inline with the results by Gao et al. [6] and Phanwilai et al. [4] (see Figure 3A). Other factors such as the high NH_4^+ loading rate could also increase AOB abundance. The predominated AOB-*amoA* gene over AOA-*amoA* gene at TH2, SK1, and SK2 compared to TH1 could be attributed to the higher NH_4^+ loading rates in these plants (see Table 2), and the significance of the gene ($p < 0.05$) shown difference of letter grouping in orange clustered bar chart (see Table S4). The typical design DO level for a nitrogen-removal process of around 2 mg-O_2 L^{-1} was recommended by [1].

Although abundance of AOA was not found three WWTPs TH2, SK2, and SK3, AOA and AOB would collaborate and offer possible advantage in ammonia oxidation rates at lower ammonia concentration at TH1. It is postulated that in the practical operation, it is desire to maintain low DO level in an aerobic tank to reduce energy and sustaining SRT range base on characteristics of each full-scale WWTP, the abundance of AOA might be possible group of microorganisms to collaborate with AOB for nitrification process. However, in further research on suitable DO level and SRT range would be investigated to find the optimum conditions of growth AOA that could collaborate with AOB.

Abundances and communities of nitrite-oxidizing bacteria (NOB)

Figure 3B shows that *Nitrobacter* sp. was more abundance than *Nitrospira* sp. at WWTP TH1. The DO levels (0.7 to 1.1 mg-O_2 L^{-1}) at TH1 are the lowest among the WWTPs investigated (DO concentration from 2.4 to 4.5 mg-O_2 L^{-1} for the other three WWTPs), and the temperature range at TH1 is moderate high at 27.7–28.1ºC. The low DO condition is favorable for the growth of *Nitrobacter*. Huang et al. [7] reported that DO concentration of > 1.0 mg-O_2 L^{-1} was suitable condition for the growth of *Nitrobacter* while DO concentration of < 1.0 mg-O_2 L^{-1} was optimum condition of *Nitrospira*. Similarly, Park et al. [18] suggested that at the low operational DO concentration of 0.5–0.6 mg-O_2 L^{-1}, *Nitrospira* was selectively enriched over *Nitrobacter* in the activated sludge from a small-scale SBR. Furthermore, Liu and Wang [19] investigated the nitrification performance of activated sludge with the long-term effect of low DO concentration, finding that higher abundance of *Nitrospira* (10^{12}) than abundance of *Nitrobacter* ($10^{10.4}$) under the condition of 0.16 mg-O_2 L^{-1}.

The optimal temperature ranges for *Nitrobacter and Nitrospira* growth are still ambiguous. Huang et al. [7] concluded that *Nitrobacter* was favorable species under the temperature ranges of 24–25ºC while *Nitrospira* dominated at relatively high temperature range of 29–30ºC. On the contrary, Alawi et al. [20] indicated that lower temperature range of 10–20ºC was the optimum condition for *Nitrospira* growth. Roots et al. [21] mentioned that *Nitrospira* increased from 3.1 to 53% under the DO level of 0.2–1.0 mg-O_2 L^{-1} with 99 d of SRT and NH_4^+ 0–14 mg-N/L. Qian et al. [22] found decreasing of *Nitrospira* from 0.44% to 0.04% by DO level of 0.8–1.5 mg-O_2 L^{-1} with SRTs between 33 and 56 d and NH_4^+ 105 mg-N/L. While Sun et al. [23] set a short SRT of 15 d with DO concentration at 1.0 and 2.0 mg-O_2 L^{-1} that increased 1.81 and 2.99%, respectively. Under the longer SRT (30 d) with the DO level (0.7–1.1 mg-O_2 L^{-1}) at TH1 presented low abundance of *Nitrospira* than *Nitrobacter*, while the three plants with the shorter SRT (17 to 26 d) and higher DO level of 2.4 – 4.5 mg-O_2 L^{-1} presented high abundance of *Nitrobacter* than *Nitrospira*. However, the point of SRT could not be one major effect on *Nitrospira* but other factors: DO, temperature, ammonium influent, pH, HRT, FA. ALR could also be the significant factors affecting the competition between *Nitrospira* and *Nitrobacter* [9].

At WWTPs TH2, SK1, and SK2, *Nitrospira* was more abundance than *Nitrobacter*. These plants were operated at relatively DO of 2.6 to 4 mg-O_2 L^{-1}, HRT of 3.6 to 15.4 hr., and SRT of 17 to 26 d. These operation parameters along with ammonium loading rate (ALR, NH_4^+-N /m^3-d) were important factors affecting *Nitrospira* growth but less extent on *Nitrobacter* growth.

Meanwhile, *Nitrobacter* is more sensitive to the free ammonia (FA) concentration compared to *Nitrospira* [10]. Mehrani et al. [9] reported that FA was a major inhibitor on NOB activity. FA concentrations at these three WWTPs (0.25 mg-N L^{-1} for TH2, 0.32 mg-N L^{-1} for SK1, and 0.29 mg-N L^{-1} for SK2) were higher than that at TH1 (0.17 mg-N L^{-1}). It could be postulated that FA concentration was inhibitor to decrease abundance of *Nitrobacter* in these three WWTPs.

In this work, only qPCR technique was used to identify both *Nitrobacter* and *Nitrosipra*. The specific primers to detect nitrifying bacteria population via the DGGE technique were not used. As *Nitrosipra* are able to complete oxidation of NH_4^+ direct to NO_3^- without into NO_2^- (complete ammonia oxidizer, comammox

process), the specific primers to detect nitrifying bacteria population for *Nitrobacter* and *Nitrosipra* are recommended in the further research. In practical, if the information of *Nitrosipra* in full-scale WWTP is reliable, a new approach of comammox process would be applied for increasing BNR in the future.

Abundances and communities of denitrifying bacteria (DNB)

Three coding genes of nitrite (*nirK* or *nirS*) and nitrous oxide (*nosZ*) reductases were evaluated for the abundance of denitrifying bacteria from these four full-scale WWTPs. As indicated in Figure 3C, higher abundance of *nosZ*-type denitrifiers was found at TH1 among WWTPs investigated due to the low COD/TN ratio of ≤ 3.7 in TH1 (see Table S4). The effects of COD/TN ratio on the abundance of *nosZ*-type denitrifiers were consistent with the results reported by Yuan et al. [24] who reported that the *nosZ*-type denitrifiers was two orders of magnitude more at the influent COD/TN ratio of 4.6 (1.29×10^8 copies) compared to that at COD/TN ratio of 8.4 (1.31×10^6 copies) at the Beijing municipal WWTP in China.

The average number of DNB copy presenting at TH1 shows that *nosZ*-type denitrifiers in anoxic and anaerobic tanks were most dominated. Wang et al. [25] found that the abundance of *nosZ* was a good indicator for rechecking anoxic and anaerobic conditions, having more oxygen concentration those conditions. Base on this result, it can be concluded that the DO level in the anoxic and anaerobic tanks of TH1 were quite high, and denitrifying bacteria could not use NO_3^- electron acceptor for denitrification process, resulting in poor denitrification efficiency at TH1 in anoxic condition. As shown in Table 2, DO level in anoxic tank at TH1 was 0.3±0.1 mg-O_2 L^{-1}. It should be noted that the low denitrification efficiency at TH1 could also attributed to the low COD/TN ratio in the receiving water.

Tallec et al. [26] and Jia et al. [27] indicated that low DO concentration in WWTPs favors nitrous oxide (N_2O) production during nitrification/denitrification process. High abundance of *nosZ* gene in denitrifies was also found in aerobic tank of WWTP TH1. Henry et al. [28] indicated that *nosZ*-type denitrifiers could be responsible in N_2O production. It could be postulated that the BNR process at TH1 could produce higher N_2O gas among WWTPs investigated due to the low DO level of this plant (0.9±0.2 mg-O_2 L^{-1}).

On the other hand, the high abundance of *nirS*-type denitrifiers and less abundance of *nosZ*-type denitrifiers were found in anaerobic and anoxic tanks at TH2, SK1, and SK2 due to high DO centration (2.6–4.5 mg-O_2 L^{-1}) operated at the A^2O process. Meanwhile, *nirS*-type denitrifiers were higher than the *nirK*-type denitrifiers at all full-scale WWTPs. Complete denitrification is possible with *nirS*-type denitrifiers [25]. Che et al. [29] found a predominance of *nirS*-type level over *nirK*-type of all eight full-scale municipal WWTPs in different cities of China. Based regression analysis, Zhang et al. [30] suggested that the abundance of *nirK*-type denitrifiers was correlated with temperature and *nirS*-type denitrifiers was linearly correlated with temperature and ammonium concentration.

Both heterotrophic and autotrophic communities of denitrifying bacteria were found as indicated in Table 3. Heterotrophic denitrifying bacteria (*Ilumatobacter* sp., *Comamonas* sp., *Rhodoferax* sp., *Terrimonas* sp., *Niabella* sp., *Sediminibacterium* sp., *Tistrella* sp., *Oryzobacter* sp.) are normally found in WWTPs [31, 32] Autotrophic denitrifying bacteria belonging to Arcobacter (affiliated *Arcobacter suis*) relate to pathogenic bacteria that were found in high abundance in the municipal full-scale biological N and P removals processes [33]. Kristensen et al. [34] reported that pathogenic Arcobacter bacteria was not found in WWTPs with longer SRT (25–35 day) because they could be able to pass through both anoxic and aerobic tanks. In this work, *Arcobacter suis* was only found at SK1. Other filamentous autotrophic denitrifying bacteria were commonly found in wastewater worldwide and were presented. *Chloroflexi* plays a role in sludge flocculation and is more commonly found in WWTPs designed to remove nutrients, and most appearance with a long SRT operation and expose the biomass to anaerobic conditions [35]. *Haliscomenobacter* sp. were filamentous bacteria and satisfied being in phosphorus concentrations [36]. Their filamentous bacteria were found and achieved to remove phosphorus in A^2O processes.

Conclusions

High nitrogen removal performances of full-scale A^2O process (TH2 and SK2 that high COD/TN ratios were 8.4 and 5.6, respectively) are successful with operation parameters, such as DO level (≥ 2.6 mg-O_2 L^{-1}) and SRT (19–26 day). However, to improve nitrogen removal efficiency at WWTPs TH1 (COD/TN ratio of ≤ 3.7) and SK1 (COD/TN ratio of ≤ 4.2) would be possible if longer SRT (> 30 day) is operated. The result from this work is confirm that the A^2O processes are be able to remove P concentration well in case there are BOD/TP ratios of ≥ 19.

Low DO level (0.9 ± 0.2 mg-O_2 L^{-1}) and long SRT under low COD/TN ratio (≤ 3.7) at TH1 is responsible for the high abundances of AOA over AOB. *Nitrosospira* could be an appearance at the long SRT maintaining. In contrary, high COD/TN ratio (> 4.2) contributed the abundance of AOB over AOA. The predominated *Nitrosomonas* were the most existence and others AOB population as *Azospira*, *Nitrosococcus*, *Thiobacillu*, *Thauera*, and *Zoogloea* were found.

The *nirS* outnumbered *nirK*-type denitrifiers. *Nitrospira* sp. are more competitive than *Nitrobacter* sp. at the low operational DO concentration. However, abundance of *Nitrobacter* could be higher than abundance of *Nitrospira* under lower temperature conditions. *Chloroflexi* and *Haliscomenobacter* representative for the autotrophic denitrifying bacterium and *Ilumatobacter*, *Comamonas*, *Rhodoferax*, *Terrimonas*, *Niabella*, *Sediminibacterium*, *Tistrella*, and *Oryzobacter* species played on the heterotrophic denitrifying bacteria. In case of high abundance of gene-type denitrifiers (*nosZ*) could be found in both anaerobic and anoxic tanks. It could be indicated that there were quite high DO concentrations in both tanks. Maintaining low DO level as operation condition in WWTP full-scale A^2O process for saving energy, it could be postulated to produce high amount of N_2O gas rather than operating high DO level. The future research in this area should be recommended in full-scale WWTP.

Declarations

Availability of data and materials

The data used to support the findings of this study are available from the corresponding author upon request.

Competing interests

The authors declare they have no competing interests.

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Authors' contributions

All authors read and approved the final manuscript.

Supaporn Phanwilai: Performing research, analyzing data, and writing on the first draft

Pongsak (Lek) Noophan: Initiative idea of project including funding acquisition, designing research, troubleshooting, analyzing data, and writing- review and editing

Chi-Wang Li: Writing - review and editing

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Tables

Table 1 Operational parameters of the full-scale A2O processes

Parameter	anaerobic, anoxic, and aerobic processes			
	Thailand		Korea	
	TH1	TH2	SK1	SK2
Avg. Flow rate ($m^3\ d^{-1}$)	218,433	7,673	220,655	497,174
SRT (day)	30	19	17	26
HRT (total) (hour)	8	15.4	3.6	10.1
Anaerobic	0.5	1.3	1.0	1.3
Anoxic	1.5	3.1	1.6	2.6
Aerobic	6	11	1.0	6.2
DO ($mg\text{-}O_2\ L^{-1}$)				
Anoxic	0.3 ± 0.1	0.1	Negligible	Negligible
Aerobic	0.9 ± 0.2	2.6 ± 0.2	4 ± 0.5	3 ± 0.3

Table 2 The average physical and chemical characteristics of influent and effluent in full-scale A^2O processes

Parameters		Anaerobic, anoxic, and aerobic (A^2O) processes			
		Thailand		South Korea	
		TH1	TH2	SK1	SK2
pH	Inf.	7.2	6.8	7.2	7.1
	Eff.	7.2	6.9	6.9	6.8
Temp (°C)	Inf.	28.1	27	14-29	11-23
	Eff.	27.7	27	18-30	19-26
SS (mg L^{-1})	Inf.	46.7	178.5	202	407
	Eff.	8.6	4.68	2	1.0
	%	*82*	*97*	*99*	*100*
BOD (mg L^{-1})	Inf.	30.1	197.9	75	260
	Eff.	5.0	3.1	3	1
	%	*83*	*98*	*96*	*99*
COD (mg L^{-1})	Inf.	58	512	88	155
	Eff.	19	40.5	10	6
	%	*67*	*92*	*89*	*96*
NH_4^+ (mg-N L^{-1})	Inf.	11.0	55.4	10.6	4.3
	Eff.	0.6	4.8	0.1	0.1
	%	*95*	*91*	*99*	*98*
NO_3^- (mg-N L^{-1})	Inf.	0.2	NR	0.1	0.1
	Eff.	5.3	NR	6.3	5.8
Alkalinity (mg L^{-1})	Inf.	NR	344.2	NR	NR
	Eff.	NR	154.3	NR	NR
TKN (mg L^{-1})	Inf.	15.4	60.8	NR	NR
	Eff.	2.6	6.1	NR	NR
	%	*83*	*90*	-	-
TN (mg N L^{-1})	Inf.	15.6	61.2	27	49
	Eff.	8.0	8.9	8	9
	%	*49*	*86*	*70*	*82*
TP (mg P L^{-1})	Inf.	2.3	7.1	~~4~~	10
	Eff.	1.5	0.3	0.2	0.2
	%	*35*	*96*	*95*	*98*
E. Coli (MPN)	Inf.	NR	NR	44,845	163,369
	Eff.	NR	NR	22	118
COD/TN ratio		3.7	8.4	4.2	5.6
BOD/TP ratio		13	28	19	26
COD loading rate (kg-COD·(m^3 d) $^{-1}$)		2.83	9.44	2.11	2.86
BOD loading rate (kg-BOD·(m^3 d) $^{-1}$)		1.44	3.65	1.80	4.80
ALR (kg NH_4^+-N·(m^3 d) $^{-1}$)		0.53	1.02	0.25	0.08
FA (mg-N L^{-1})		0.17	0.25	0.32	0.29

Remark: NR = Not record and % = % Removal efficiency

Table 3 Microorganisms' community in four municipal WWTPs of A^2O processes

Sample name		%	Accession No.	A^2/O processes						A^2/O processes					
				TH1			TH2			SK1			SK2		
				Ana	Anx	Aer	Ana	Anx	Aer	Ana	Anx	Aer	Ana	Anx	Aer
Nitrifying bacteria															
Ammonia oxidizing bacteria (AOB)															
Order	Species														
Nitrosomonadales	*Nitrosomonas aestuarii*	90	NR104818.1	+	+	+									
	Nitrosomonas eutropha	93	NR027566.1	+	+	+				+	+	+	+	+	+
	Nitrosomonas communis	97	NR119314.1	+	+	+	+	+	+	+	+	+	+	+	+
	Nitrosomonas halophila	93	NR104817.1	+	+	+				+	+	+			
	Nitrosomonas oligotropha	96	NR104820.1	+	+	+	+	+	+						
	Nitrosomonas ureae	97	NR104814.1	+	+	+	+	+	+						
	Nitrosospira multiformis	96	NR074736.1	+	+	+									
	Nitrosospira tenuis	97	NR114773.1	+	+	+									
	Uncultured Nitrosospira	95	GQ255611.1	+	+	+									
	Thiobacillus thioparus	96	NR117864.1	+	+	+	+	+	+						
Rhodocyclales	*Zoogloea caeni*	91	NR043795.1				+	+	+						
Nitrite oxidizing bacteria (NOB)															
Nitrospirae	*Nitrospira lenta*	99	NR148573.1	+	+	+									
Denitrifying bacteria (DNB)															
Autotrophic denitrifying bacteria															
Campylobacterales	*Arcobacter suis*	81	NR116729.1										+	+	+
Chloroflexi	*Chloroflexi bacterium*	87	KP246879.1												
	Uncultured Chloroflexi	98	GQ366686.1							+	+	+	+	+	+
Rhodocyclales	*Azospira restricta*	97	NR044023.1	+	+	+									
	Thauera aromatica	100	NR026153.1	+	+	+									
	Thauera aminoaromatica	93	NR027211.1												
Saprospirales	*Haliscomenobacter hydrossis*	90	NR074420.1	+	+	+	+	+	+	+	+	+	+	+	+
Heterotrophic denitrifying bacteria															
Acidimicrobiales	*Ilumatobacter fluminis*	86	NR041633.1							+	+	+	+	+	+
Burkholderiales	*Comamonas denitrificans*	99	NR025080.1												
	Comamonas phosphati	96	NR147778.1												
	Rhodoferax ferrireducens	92	NR074760.1				+	+	+	+	+	+	+	+	+
Chitinophagales	*Terrimonas lutea*	96	NR041250.1				+	+	+	+	+	+	+	+	+
	Niabella terrae	92	NR132698.1					+	+						

	Sediminibacterium roseum	82	NR159130.1							+	+	+	+	+	+
Rhodospirillales	*Tistrella mobilis*	91	NR117256.1	+	+	+										
Micrococcales	*Oryzobacter terrae*	98	NR137270.1	+	+	+	+	+	+	+	+	+	+	+	+

Remark: + is DGGE band presenting on acrylamide gel, Ana is an anaerobic tank, Anx is an anoxic tank, and Aer is an aerobic tank.

Figures

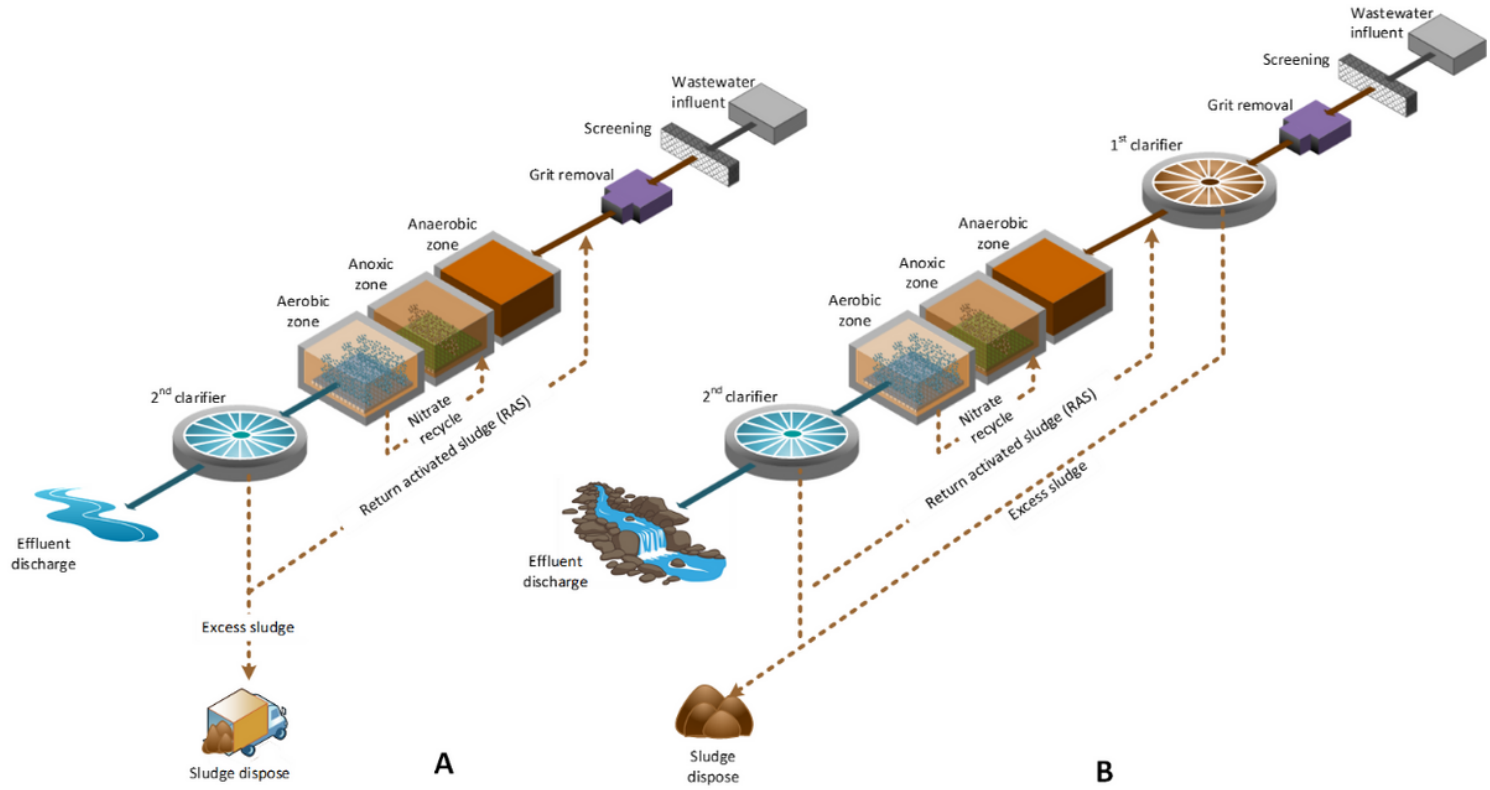

Figure 1

The schematic layout of the full-scale WWTPs at TH1 and TH2 WWTPs (A), and SK1 and SK2 WWTPs (B)

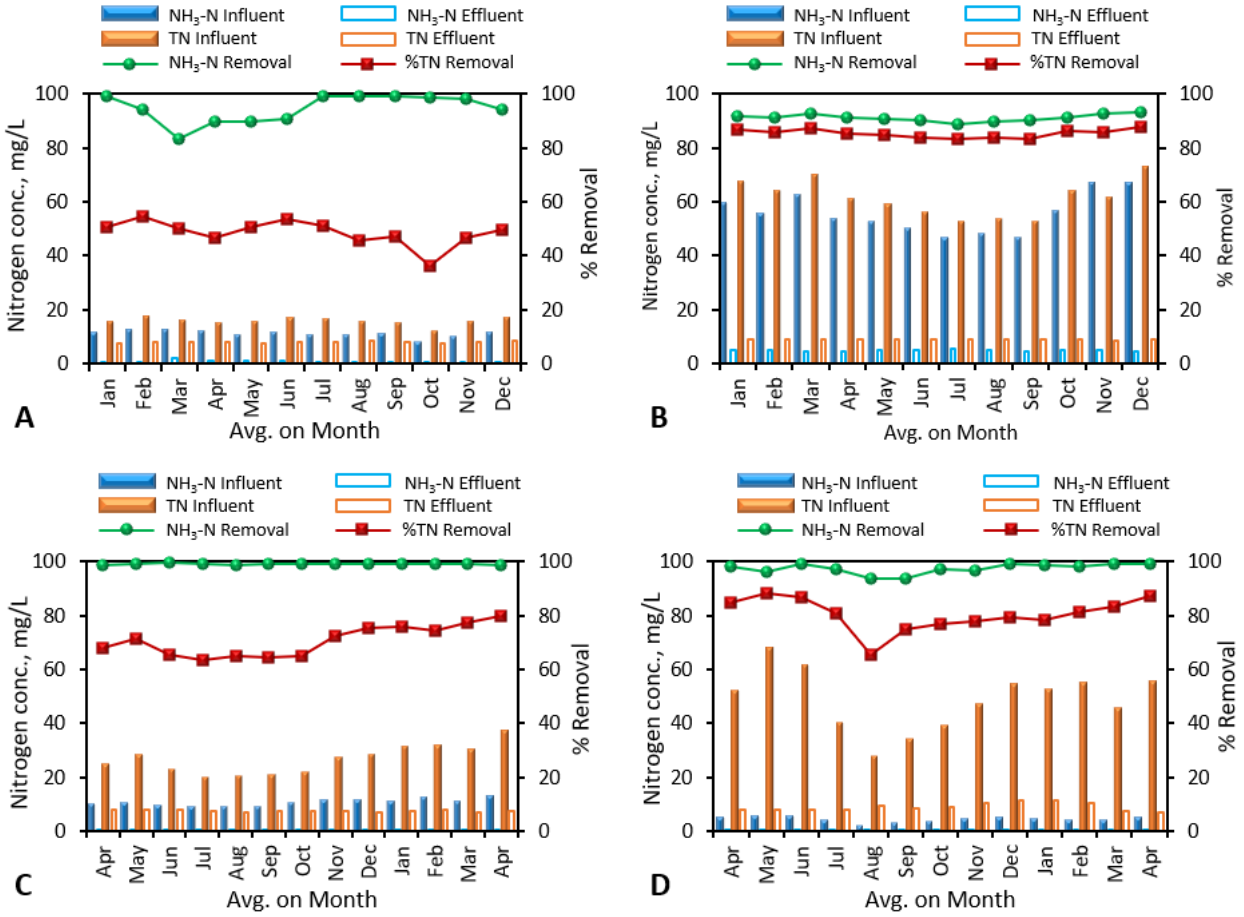

Figure 2

Nitrogen performance A2O processes at (A) TH1, (B) TH2, (C) SK1 and (D) SK2

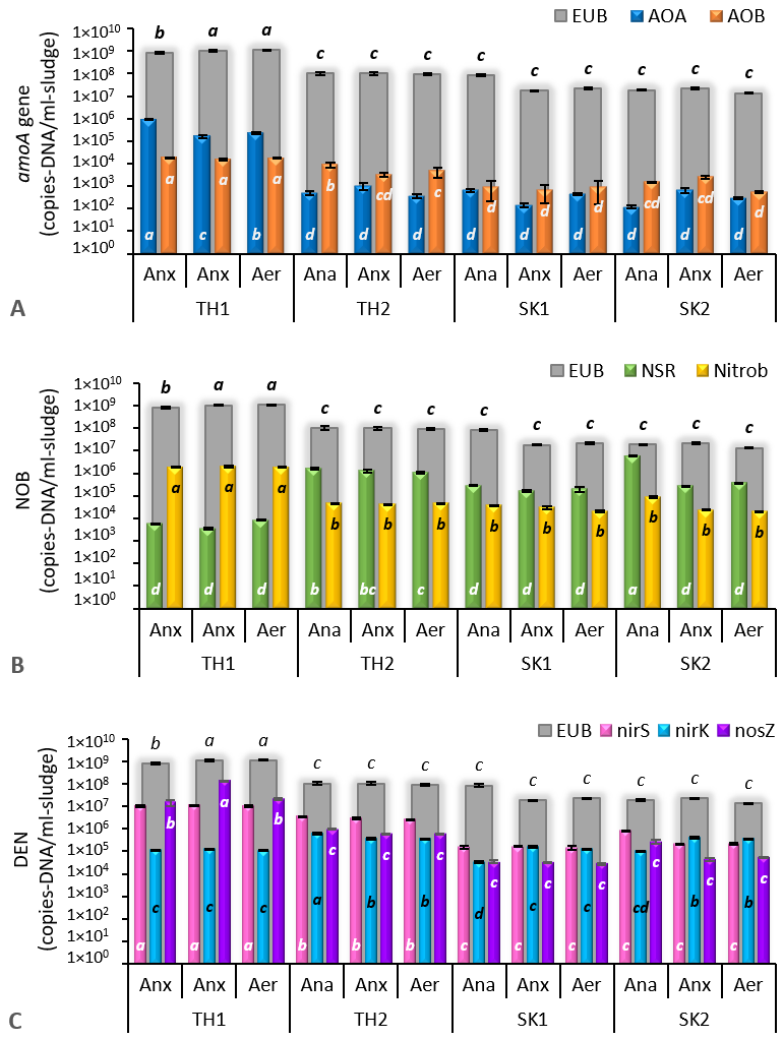

Figure 3

Microbial abundance of (A) AOA and AOB (B) NOB and (C) DEN target at TH1, TH2, SK1, and SK2

Supplementary Files

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