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Effects of Caffeine and COD from Coffee Wastewater on Anaerobic Ammonium Oxidation (Anammox) Activities

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Abstract: An anaerobic ammonium oxidation (anammox) process was employed to remove nitrogen from wastewater generated from a coffee brewing facility. The effects of caffeine and chemical oxygen demand (COD) in coffee wastewater on anammox activity were investigated. The anammox activity was inhibited in synthetic wastewater with a caffeine concentration greater than 350 mg/L. Daily additions of caffeine at 2.5 mg/L for 28 days to the same substrate did not inhibit anammox activity. However, daily additions of coffee wastewater with COD of ≥387 mg/L and caffeine at 2.5 mg/L significantly inhibited anammox activity. Because the pH was increased in the system, resulting in an increase in free ammonia (FA) concentration, one could postulate that FA is an inhibitor of anammox activity. Quantitative polymerase chain reaction (qPCR) analysis was employed to determine the populations of anammox and denitrifying bacteria. Coffee wastewater with bacterial COD to total nitrogen (bCOD:TN) ratios of 0.3–0.6:1 did not have any effect on the abundances of anammox and denitrifying bacteria. The results from this work suggest that biodegradable COD (bCOD) rather than total COD (TCOD) should be used for calculating the COD:TN ratio during the study of the effects of nitrogen removal from real wastewaters using the anammox process. A not-competitive model could fit the anammox inhibition with caffeine concentrations at 50–500 mg/L with maximum specific anammox activity (SAA_{max}) of 0.594 mg-N/mg-volatile suspended solids (VSS)/d and inhibitory constant (K_i) of 480.97 mg/L.

Keywords: caffeine; coffee wastewater; inhibition; anammox process

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1. Introduction

Coffee is one of the most popular drinks in the world with about one third of the world's population consuming it. Businesses related to the production and serving of coffee are booming [1]. Wastewaters generated from coffee production contain high organic matter as total chemical oxygen demand (TCOD) as high as 35,600 mg/L, soluble COD (SCOD) of 12,800 mg/L), high nitrogen content (as total Kjeldahl Nitrogen or TKN) of 560 mg N/L, and high caffeine (>1000 mg/L) [1–3]. Due to its high COD, an anaerobic treatment system is strongly recommended to treat this type of wastewater. Although the anaerobic treatment process could significantly remove organic matter in terms of COD, nitrogen removal has been reported to not be very efficient [2,4–6]. Thus, a subsequent nitrogen removal process should be used for treating the anaerobic-treated effluent. Due to a low COD:TN (total nitrogen) ratio of this treated wastewater, conventional biological treatment (nitrification and denitrification processes) is not an ideal choice as several disadvantages are associated with this process, specifically high oxygen concentration demand, requirement for additional carbon source for the denitrification process, high sludge production, and significantly increased nitrous gas (N_2O) emission [7–9].

In this study, the anammox process was suggested as an alternative approach to treat coffee wastewater for biological nitrogen removal (BNR). Unlike conventional nitrification and denitrification processes, the anammox process converts nitrite (NO_2^-) to nitrogen gas (N_2) with ammonium (NH_4^+) as the electron donor with the formation of nitrate (NO_3^-) as indicated in the following reaction.

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

Many advantages, such as low energy cost, no additional organic carbon requirement, lower sludge production, and lower N_2O gas emissions, are frequently cited for anammox process [10,11]. Furthermore, the anammox process is a recommended process to remove nitrogen from wastewaters with high ammonium–nitrogen but low carbon concentrations, that is, low COD:TN ratio.

Many researchers [12–15] have applied the anammox process for treating wastewaters with low carbon and high NH_4^+ concentrations (COD:TN ratio < 1), such as supernatant from the anaerobic treatment of sludge from domestic wastewater, pharmaceutical wastewater, livestock wastewater, and landfill leachate. So far, no study has applied the anammox process to treat coffee wastewater. Several studies [16–18] have reported that caffeine is an antimicrobial agent. Ramanavičienė et al. [16] indicated that caffeine is an effective antimicrobial agent against *E. coli* through the damage of DNA and inhibition of protein synthesis. It is possible that the presence of caffeine with antibiotics (penicillin, amoxicillin, ampicillin, and benzylpenicillin) could enhance the inhibition of microbial activities, producing an even greater inhibition than that reported for caffeine on *Staphylococcus aureus* [19] or furazolidone against Vibrio [20]. Additional work could better define the suitability of the anammox process in the presence of various inhibitors.

The main goal of this study was to investigate the effect of caffeine and COD from coffee wastewater on the anammox process for both acute and long-term periods by observing specific anammox activity (SAA) in a suspended growth system. A simple Monod-based model is used to distinguish the mechanisms for caffeine inhibition on anammox activity. The results from this work could lay a foundation for application of the anammox process for nitrogen removal from coffee wastewater.

2. Material and Methods

2.1. Experimental Setup and Preparation of Wastewaters

Enriched anammox cultures were harvested from anaerobic sequencing batch reactors (ASBRs) maintained in the Environmental Engineering's laboratory of Faculty of Engineering, Kasetsart University, Bangkok. The cultures were immediately transferred to each experimental reactor that was used throughout this study. The initial biomass concentration in each suspended growth reactor was around 0.8–1.2 g/L. To ensure an oxygen free environment, an inert gas mixture (95% Ar and 5% CO_2) was bubbled into the reactors to expel dissolved oxygen (DO). All reactors were shielded from light and operated at constant room temperature (25–30 °C). All samples for acute and long-term experiments were obtained by using a needle joint with a syringe and filtered through a 0.45-µm nylon membrane filter before measurement. Impeller speed by magnetic stirrer is fixed only 40 rpm through experimental work because high share stress could affect the size of anammox granule.

2.1.1. Preparation of Synthetic Wastewater

A synthetic wastewater was prepared to contain ammonium–nitrogen concentration of 15 mM and nitrite-nitrogen of 19.8 mM, replicating the 1:1.32 ammonia–nitrogen to nitrite-nitrogen ratio used by Egli et al. [21] and Van Dongen et al. [22]. These concentrations were achieved by dissolving 990 mg $(NH_4)_2SO_4$ and 1346 mg $NaNO_2$ per liter synthetic wastewater. (210 mg NH_4^+-N/L and 273 mg $NO_{2\_}^-$-N/L). Other minor ingredients in the synthetic wastewater per liter were 937.3 mg $KHCO_3$, 7.5 mg $FeSO_4 \cdot 7H_2O$, 28.77 mg $Na_2EDTA \cdot 2H_2O$, 18.75 mg KH_2PO_4, 150 mg $MgSO_4 \cdot 7H_2O$, 394.68 mg $CaCl_2 \cdot 2H_2O$, 0.06 mg

$Na_2O_3Se{\cdot}5H_2O$, 0.165 mg $MoNa_2O_4{\cdot}2H_2O$, 0.187 g $CuSO_4{\cdot}5H_2O$, 0.322 mg $ZnSO_4{\cdot}7H_2O$, 0.742 mg $MnCl_2{\cdot}4H_2O$, 0.18 mg $CoCl_2{\cdot}6H_2O$ and 0.142 mg $NiCl_2{\cdot}6H_2O$. The characteristics of the synthetic wastewater are shown in Table 1.

Table 1. Characteristics of synthetic wastewater in this study.

Parameter	Value
pH	7.75 ± 0.11
Total Nitrogen (mg N/L)	471.67 ± 3.94
Ammonium (mg N/L)	209.44 ± 6.16
Nitrite (mg N/L)	262.23 ± 5.06

2.1.2. Coffee Wastewater

The coffee sample was collected from an espresso prepared at Café Amazon, Bangkok, Thailand. Prior to its use, the coffee sample was stored in the refrigerator. Just before the experiment, it was allowed to warm to room temperature. A 1 mL portion of the coffee sample was diluted to 1 L with the synthetic wastewater described above to produce the coffee wastewater for the study. The characteristics of the coffee wastewater after mixing with synthetic wastewater are given in Table 2.

Table 2. Characteristics of coffee wastewater (after mixing with synthetic wastewater) in this study.

Parameter	Value
pH	4.21
COD (mg/L)	387.05
bCOD (mg/L)	120.23
Caffeine (mg/L)	3.12

Note: Coffee wastewater was used in this experiment. (1 mL portion of the coffee sample was diluted to 1 L with the synthetic.).

2.1.3. Acute Effects of Caffeine Dosing on Anammox Activity

To investigate the acute effects of caffeine dosing on anammox activity, glass vials with 100 mL maximum volume (working volume 80 mL) were used as reactors. After filling with the mixtures to be tested and sparging with inert gas, the vials were sealed. Constant mixing was carried out with an orbital shaker set at 120 rpm.

2.1.4. Long-Term Effect of Caffeine Dosing on Anammox Activity

To investigate the long-term effect of caffeine dosing on anammox activity, a cylindrical acrylic vessel with 1.8 L maximum volume (working volume 1 L) was used as a reactor and operated in an oxygen free environment. Initially, a 500 mL portion of anammox culture was mixed with 500 mL synthetic wastewater containing 5 mg/L caffeine in the reaction vessel. This test was operated as a sequencing batch reactor (SBR) system as follows: fill (11 min), reaction time with mixing (23.5 h), settle (10 min) and decant (9 min). All reactors were mixed with magnetic bar on a magnetic stirrer. For each cycle, 50% of supernatant was decanted and a new caffeine solution was added.

2.1.5. Effect of Cod from Coffee Wastewater on Anammox Activity. Recovery of Anammox Cultures after Stopping the Addition of Coffee Wastewater

To investigate the effect of COD from coffee wastewater on anammox activity, a SBR was used as described in the previous section. At the start, a 500 mL portion of anammox culture was mixed with 500 mL prepared coffee wastewater in the SBR. After purging DO with inert gas sparging, the SBR followed this sequence: fill (20 min), reaction time

with mixing (23 h), settle (25 min), and decant (15 min). For each sequence, 500 mL of supernatant was decanted and fresh coffee wastewater was added.

2.2. Experimental Tests

2.2.1. Acute Effects of Caffeine Dosing on Anammox Activity

Caffeine (99% purity, Glentham Life Science, Corsham, UK), in quantities of 0, 100, 500, 700 and 1000 mg were diluted to 1 L with the prepared synthetic wastewater. Aliquots of 40 mL of the caffeine solutions were transferred into test reactors (vials). After mixing with 40 mL anammox culture, the caffeine concentrations in each experimental vial were 0, 50, 250, 350 and 500 mg/L, respectively. The NH_4^+ and NO_2^- concentrations were determined from the beginning of the experiment to a reaction time of 7 h. Specific anammox activity (SAA) was calculated based on the removal rate of NH_4^+ and NO_2^-; see Section 2.3.

2.2.2. Long-Term Effect of Caffeine Dosing on Anammox Activity

The long-term effect of caffeine on anammox activity was studied by feeding a caffeine solution daily to the anammox system. The daily additions of 500 mL of caffeine solution (5 mg caffeine/L in the synthetic wastewater) maintained a 2.5 mg/L initial caffeine concentration in the reactor. The reactor was operated for 4 weeks. Each week the anammox activities were determined versus time by taking hourly samples of 20 mL for 7 h in order to measure NH_4^+ and NO_2^- concentrations and biomass concentration after feeding

2.2.3. Effect of Cod from Coffee Wastewater on Anammox Activity and Recovery of Anammox Cultures after Stopping the Addition of Coffee Wastewater

The SBR was operated for 28 days during which daily additions of 500 mL coffee wastewater were fed after decantation. The reactor contents after feeding coffee wastewater were found to contain 154 mg/L COD and 74 mg/L bCOD. After the SBR system was operated in the presence of coffee wastewater for 28 days, the system was fed with synthetic wastewater only for another 28 days to observe the reversibility of anammox inhibition.

2.3. Specific Anammox Activity (SAA)

Specific anammox activity (SAA) was calculated using the removal rate of NH_4^+-N and NO_2^--N by analyzing hourly samples from the reactor contents. The amount of total nitrogen (TN) (NH_4^+-N plus NO_2^--N) removed was plotted against reaction time, and the slope of line was normalized with the biomass of anammox bacteria in terms of volatile suspended solids (VSS). The SAA was expressed in the terms of mg N mg^{-1} VSS d^{-1}, as shown in Equation (2). From this term, the percent of specific anammox activity in the presence of the coffee wastewater was calculated as Equation in (3).

$$\text{SAA (mg-N/mg-VSS/d)} = \frac{\text{slope of } NH_4^+\text{-N} + \text{slope of } NO_2^-\text{-N}}{\text{MLVSS} - \text{day}} \times 24\ \text{h} \quad (2)$$

Equation (3) was used to evaluate the effect of caffeine on the SSA.

$$\text{SAA, \%} = \frac{\text{SAA}}{\text{SAA}_i} \times 100 \quad (3)$$

SAA_i is the maximum specific activity on the control assay (no presence of caffeine) and SAA is the maximum specific activity observed with various caffeine concentrations.

2.4. Free Ammonia Calculation

The concentration of free ammonia (FA) is calculated using Equation (4)

$$\text{FA} = \frac{17 \times NH_4^+ \times 10^{\text{pH}}}{14 \times (e^{\frac{6344}{273+\text{T}}} + 10^{\text{pH}})} \quad (4)$$

where FA is the calculated concentration of free ammonia in mg N/L, NH_4 is the concentration of ammonium in mg N/L determined by analysis, pH is for using of this formula is solution of SBR during reaction time, and T is the temperature of water in °C.

2.5. Analytical Methods

2.5.1. Chemical Analysis

All chemical analysis in this work was conducted according to the Standard Methods of APHA [23]. Ammonium concentration was determined by the titrimetric method (4500-NH_3). Nitrite concentration was determined based on the colorimetric method using a spectrophotometer (U-2800, Hitachi, Tokyo, Japan), at wavelength of 534 nm and a light path of 1 cm (4500-NO_2^-). BOD concentration was determined following the Azide Modification of Iodometric Method (5210B). COD concentration was determined by Titrimetric method (5220C). Suspended and volatile solids concentrations were determined by drying at 103–105 °C and 550 °C, respectively.

2.5.2. Solid-Phase Extraction Procedure for Caffeine Extraction

C18 SPE cartridge (VertiPak^{TM} C18 SPE, Vertical Chromatography, Thailand) was used for caffeine extraction. Before sample loading, the solid-phase adsorbent was preconditioned with 3 mL of methanol and 3 mL of water. 10-mL of sample was loaded into a syringe, connected to the cartridge with PTEE tubes, pushed through the cartridge slowly. The caffeine-loaded solid phase was washed with 3 mL of water and eluted with 3 mL of methanol. Caffeine concentration in the eluted sample was then determined.

2.5.3. Determination of Caffeine Concentration

A High Performance Liquid Chromatography (HPLC, LC-20A, Shimadzu Corporation Tokyo, Japan) was used to measure the concentration of caffeine following the method of Motora and Beyene [24]. A reverse phase column (ODS 250 × 4.6 mm) was used for the separation of caffeine and column temperature was set at 25 °C. Water and methanol at the volume ratio of 65 to 35 were used as the mobile phase and the flow rate of mobile phase was 1 mL/min. The photodiode array detector was set at 272 nm. The injection volume was 10 µL. The limit of detection of caffeine analysis by using HPLC in this work was 1 mg/L.

2.5.4. Statistical Analysis

Statistical analyses (one-way ANOVA) were used to compare the presentation of all effluent samples during experimental works and including performance of the different biomasses (total bacteria, AOB, NOB, and DNB). Statistical significance tested at p-values ≤ 0.05 were applied by using Excel program.

2.5.5. Quantitative Polymerase Chain Reaction (qPCR)

Biomass samples were collected for the molecular analysis. The biomass samples were extracted using phenol/chloroform extraction protocol adapted from Zhou et al. [25]. DNA concentrations were measured using a NanoPhotometer® N60 (Implen, California, USA). The abundance of total bacteria, denitrifying bacteria (DNB), and anammox bacteria were quantified using quantitative polymerase chain reaction (qPCR). Table 3 shows the target genes for total bacteria, DNB, and anammox bacteria and their corresponding primers. The qPCR analysis was carried out using a CFX96 Touch Real-Time PCR Detection System (BioRad Laboratories, California, USA). Each 20 µL of PCR mixture contained 8.2 µL of quantiNova SYBR Green Master MIX (2X) (New England Biolabs, Massachusetts, USA), 0.4 µL of forward and reverse primer (20 µM), 1 µL of template DNA, and 10 µL of dH_2O. Thermal cycling conditions were denaturation at 95 °C for 3 min, followed by 95 °C for 10 s, 53–59 °C for 20 s, and 72 °C for 10 s. Standard curves were generated by 5-fold serial dilutions of plasmid DNA containing specific target gene inserts.

Table 3. List of PCR primers for the amplification.

Target Gene	Primer	Sequence (5′-3′)	References
EUB (Total bacteria)	338F 518R	ACTCCTACGGGAGGCAGCAG ATTACCGCGGCTGCTGG	Muyzer et al. [26]
hzo (Anammox bacteria)	hzocl1F1 hzocl1R2	TGYAAGACYTGYCAYTGG ACTCCAGATRTGCTGACC	Schmid et al. [27]
nirS (Denitrifying bacteria, DNB)	Cd3aF R3cd	GTSAACGTSAAGGARACSGG GASTTCGGRTGSGTCTTGA	Throback et al. [28]

3. Results and Discussion

3.1. Acute Effects of Caffeine Dosing on Anammox Activity

Figure 1 shows the removal efficiencies for total nitrogen (NH_4^+-N plus NO_2^--N) under the caffeine concentrations of 0, 50, 250, 350, and 500 mg/L. The total nitrogen removal efficiencies decreased from 83% to 55% with increased caffeine concentrations from 50 to 500 mg/L. For caffeine concentrations of 0, 50, 250, 350, and 500 mg/L, the specific anammox activities (SAA) were 0.663, 0.527, 0.436, 0.311, and 0.300 mg-N/mg-VSS/d, respectively, as indicated in Figure 2. For the same caffeine concentrations, the values for SAA inhibition were 0%, 0%, 10%, 32%, and 33%, respectively, shown in Figure 2. There was no significant inhibition of anammox activity with caffeine concentrations less than 250 mg/L. The anammox activity was severely compromised (> 32%) with the caffeine concentration >350 mg/L. Ramanavičienė et al. [16] reported that a caffeine concentration of 1% by volume, with nutrient broth medium containing 0.12% glucose could damage DNA of *E. coli* and inhibit protein synthesis. Banerjee and Chatterjee [20] showed that a 500 mg/L caffeine concentration in the presence of an antibiotic (furazolidone concentrations of 0.15, 0.40, 0.50, 0.7, and 0.8 mg/L) would decrease the survival of Vibrio bacteria. Moreover, Esimone et al. [19] reported that caffeine concentrations of 5 and 10 mg/mL, mixed with an antibiotic (amoxicillin concentrations of 7.81, 15, 15.23, 31.25, 125, 250, and 500 mg/mL) could increase the inhibition of the activity of *Staphylococcus aureus*. In this research work, it was found that anammox bacteria are more sensitive to caffeine concentration than other microorganisms (*E. coli*, *Staphylococcus aureus*, and Vibrio).

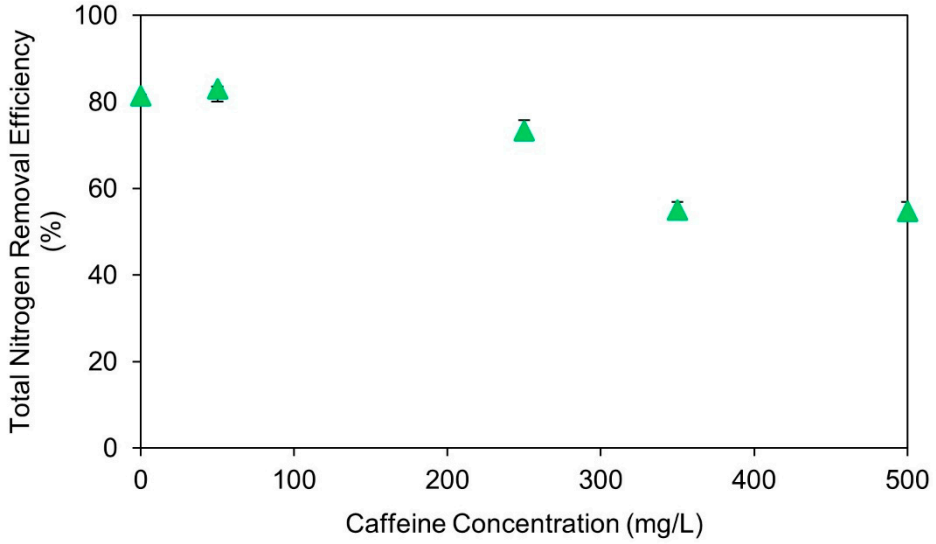

Figure 1. Percentage of total nitrogen removal efficiency at the end of 7 h as a function caffeine dosing concentration (0, 50, 250, 350, and 500 mg/L).

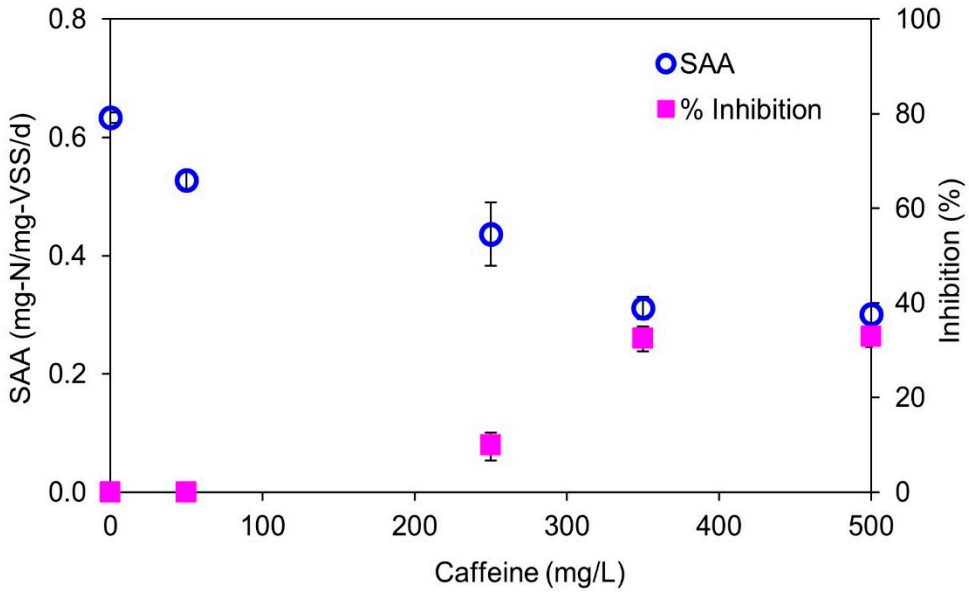

Figure 2. Specific anammox activity (SAA) and percent inhibition versus the acute caffeine dosing concentration.

The Monod-base not-competitive model was used to describe caffeine inhibition. As indicated in Figure 3, the not-competitive model could fit the data with SAA_{max} of 0.594 mg-N/mg-VSS/d and K_i of 480.97 mg/L.

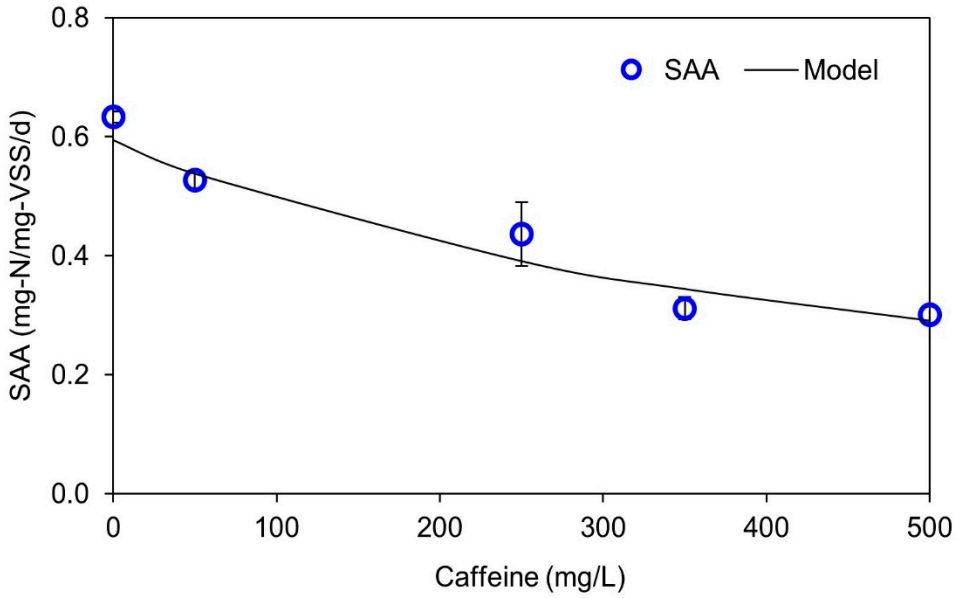

Figure 3. Specific anammox activity vs. caffeine concentration. The line represents the best fit of non- or an un-competitive model.

Chen et al. [29] studied effect of quinoline on anammox activity. Quinoline is one type of nitrogen heterocyclic compound that are found in refractory coking wastewater. They found that only 13.1 mg/L of quinoline concentration would affect anammox activity. A non-competitive model was able to be used in this case. By comparison, quinoline is much more toxic than caffeine on anammox activity. Li et al. [30] investigated the effect of pyridine (that is also found in refractory coking wastewater) on anammox activity. They used a non-competitive model to explain anammox activity and reported the Ki of pyridine at 135.19 mg/L.

Since the not-competitive model could not distinguish between a non-competitive model (expectation is that caffeine is able to bind free enzyme and substrate from the binding site) and an un-competitive model (expectation is that caffeine is able to bind site away from the substrate binding site), the mechanism for caffeine inhibition on anammox activity could not be described clearly. Further investigation on the mechanism for caffeine inhibition is needed.

3.2. Long-Term Effect of Caffeine Dosing on Anammox Activity

To study the long-term effect of caffeine dosing on anammox activity the initial total nitrogen was 252 mg N/L. The reactor was fed a synthetic wastewater containing 2.5 mg/L caffeine. Although caffeine concentrations in treated effluents from anaerobic wastewater treatment plants from a soft drink company are around 7–300 µg/L [1], the higher concentration used in this research is considered reasonable. The higher concentration should expedite the inhibition process. In addition, the HPLC detection limit for caffeine is 1 mg/L, as used in this work.

For the control system, i.e., no caffeine in the feed solution, the nitrogen removal efficiency averaged around 96% and the SAA range was from 1.35 to 0.87 mg-N/mg-VSS/d. See Figures 4 and 5. With the experimental system being fed with caffeine, the nitrogen removal efficiency was decreased slightly to 94% for the first day, slightly decreased to 81% after a week of operation, and then stabilized around 90–94% thereafter as shown in Figure 4. A comparison of SAA inhibition for the control and the experimental systems reveals a slight inhibition of SAA by caffeine, as shown in Figure 5.

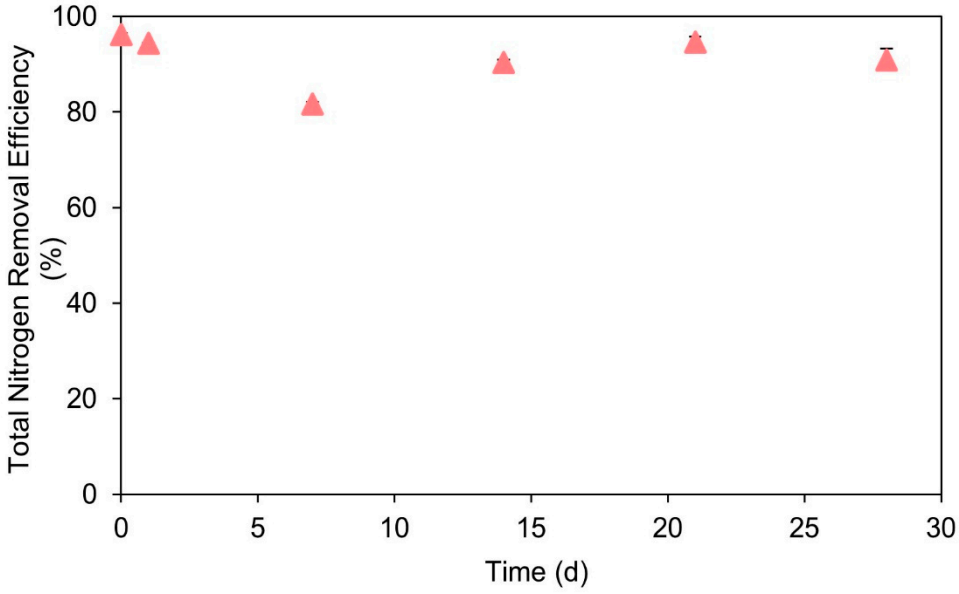

Figure 4. Nitrogen removal efficiency during long-term caffeine dosing.

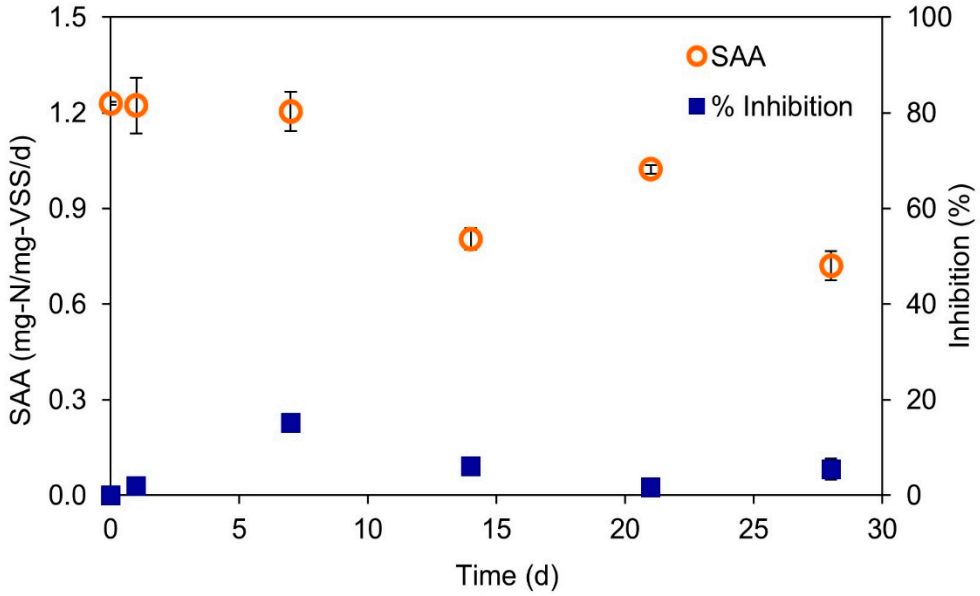

Figure 5. Specific anammox activity (SAA) of control and caffeine and inhibition (%) during the long-term caffeine dosing.

3.3. Treatment of Coffee Wastewater Using Anammox Process and the Recovery of Anammox Cultures after Discontinuance of Coffee Wastewater Additions

The experimental control SBR was started with total nitrogen (TN) concentration (NH_4^+ plus NO_2^-) of 215 mg N/L. The nitrogen removal efficiency was found to be 82% with SAA at 0.867 mg-N/mg-VSS/d. Daily additions of coffee wastewater resulted in the accumulation of COD in the reactor. As indicated in Figure 6, COD in the treated effluent increased from 154 mg/L at day 1 to 508 mg/L at day 28. Meanwhile, the TN concentration increased from 286 mg N/L at day 1 to 391 mg N/L at the end of the 28 day experimental period.

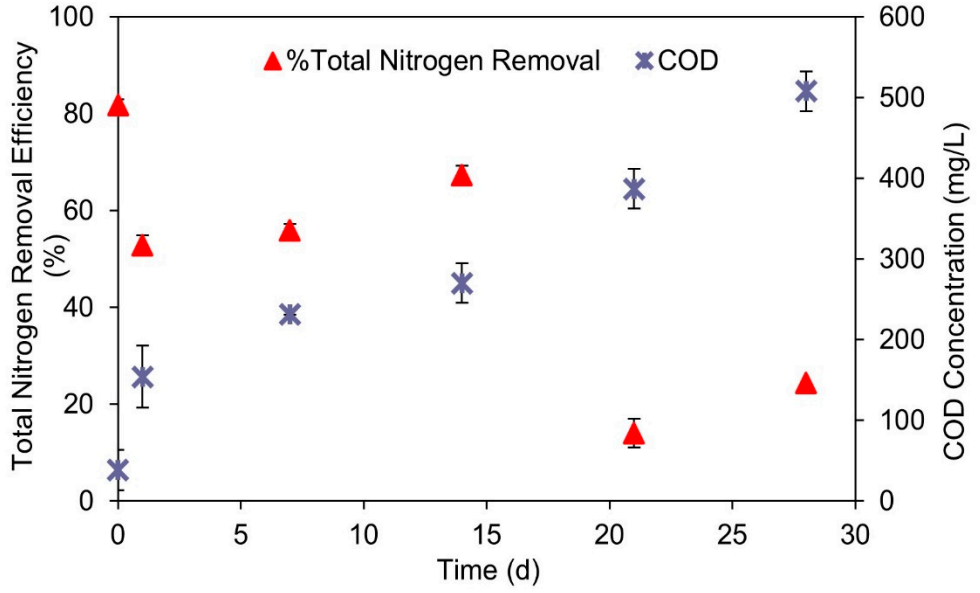

Figure 6. Total nitrogen removal efficiency and COD concentration for the anammox system to treat coffee wastewater.

Anammox activity was inhibited after daily additions of coffee wastewater as indicated in Figure 7. As the caffeine concentration was quite low (2.5 mg/L), caffeine alone

would not have caused the inhibition of SSA. It has been reported that free ammonia (FA) concentrations between 13 and 90 mg N/L could cause significant inhibition of anammox activity [31,32]. In the current study, it was found that FA concentrations increased from 0.06 at hour 1 to 0.79 mg N/L after 7 h of coffee wastewater additions. Meanwhile, FA concentration continually increased to 11.01 mg N/L on day 21. The increasing FA concentration could be the reason for the inhibition of anammox activity. FA concentration would be increased due to the high pH. For this reason, the ammonium–nitrogen removal rate was significantly decreased. Because anammox activity was inhibited, nitrite concentrations increased from 140.8 to 224.3 mg N/L on day 28. Therefore, the nitrogen removal efficiency was decreased. Several studies [21,33,34] have confirmed that high nitrite concentration could be another inhibitor of anammox activity. A previous study [35] reported that nitrite accumulation in an anammox system caused flotation events, which led to biomass washout that affected the removal efficiency.

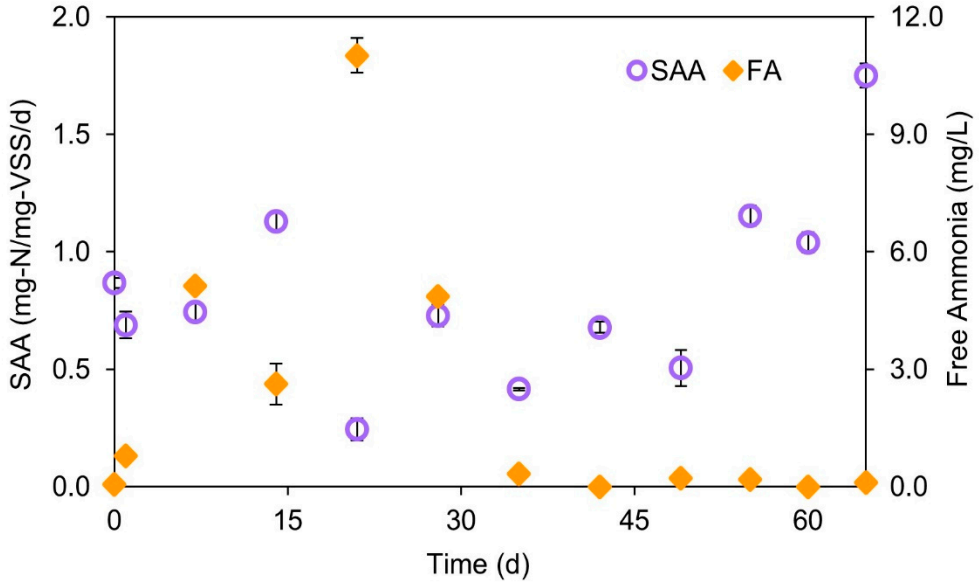

Figure 7. Specific anammox activity (SAA) and free ammonia (FA) concentration during the long-term caffeine dosing.

The accumulation of COD in the reactor contents from 154 mg/L on day 1 to 508 mg/L on day 28 was very similar to the findings of Chamchoi et al. [36]. They reported that when the concentration of COD was >300 mg/L, anammox activity was markedly suppressed.

Figure 8 shows the result of bacteria abundance (total bacteria, anammox bacteria, and denitrifying bacteria (DNB)) in SBR that was analyzed by the qPCR technique. The dominant anammox species in this work were *Candidatus* Kuenenia stuttgartiensis and *Candidatus* Brocadia fulgida. However, the total chemical oxygen demand (TCOD) concentrations from 154 to 508 mg/L from coffee wastewater did not significantly change the abundances of anammox bacteria (ranging from 6.82×10^9 to 1.04×10^{10} copies/g-sludge) and DNB (ranging from 8.4×10^6 to 1.2×10^7 copies/g-sludge) in the system. Leal et al. [37], He et al. [38], and Wang et al. [39] reported that the abundance of DNB was increased when higher COD concentrations were fed to the system. The abundance of DNB found in this work was quite similar to the results from all the cited references [37–39]. The abundance of DNB from the work of the three researchers did not change significantly when COD ranged between 106–251 mg/L.

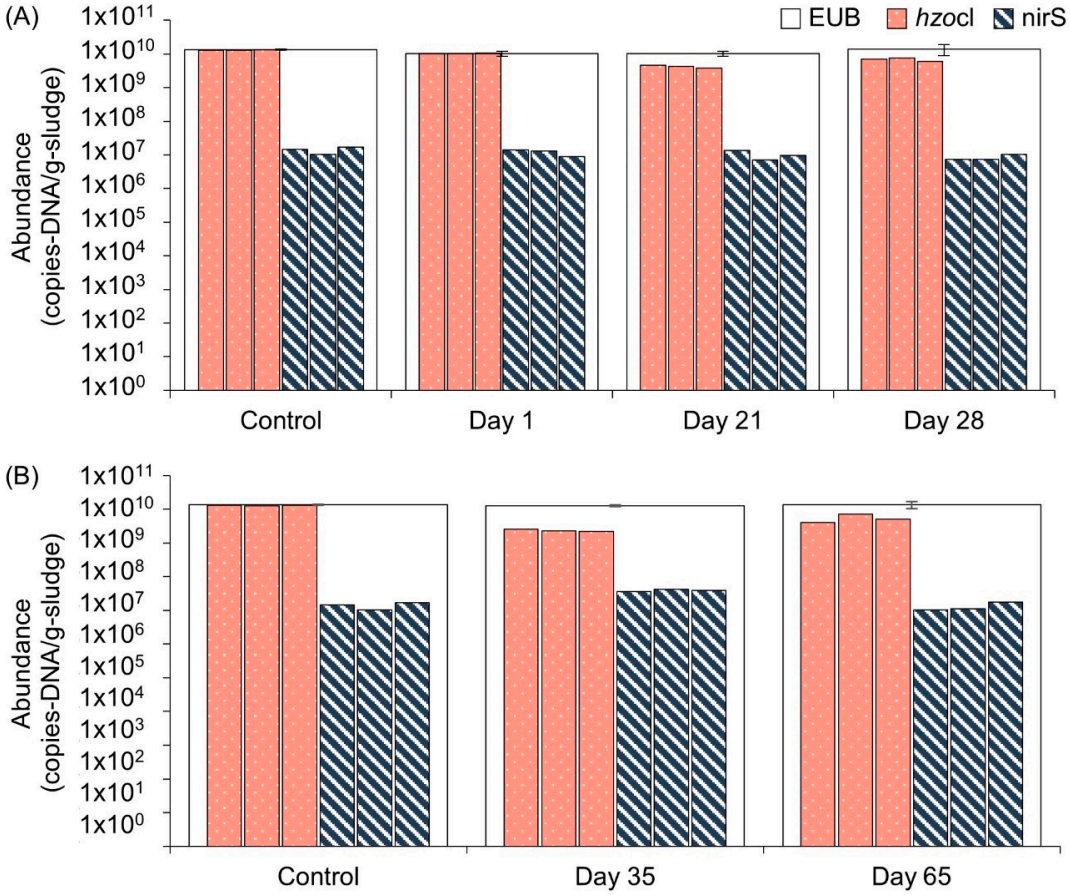

Figure 8. qPCR analysis of microbial abundance of (**A**) anammox and DNB, control, day 1, 21, and 28 during daily addition of COD from coffee wastewater (**B**) anammox and DNB, control day 35 and 65 during recovery.

Although the abundance of DNB from this work was similar to the abundances of DNB from the three referenced studies, one must point out two experimental differences. First, in all of the reference experiments, a synthetic substance (glucose) was used as the COD source. For this reason, TCOD and BOD values are quite similar. However, a real substrate (coffee wastewater) was used in this work as the COD source. Thus, TCOD and BOD values are not the same value. For this reason, when using coffee wastewater, the bCOD should be used instead of TCOD for calculation of the bCOD:TN ratio. The bacterial degradation of total COD (154 mg/L) was equivalent to the bCOD (74 mg/L), or only 48% the value of TCOD. Second, the total nitrogen and organic matter concentrations from the three referenced studies and this work were quite different. For example, in the research of Wang et al. [39], there were three total nitrogen concentrations: 200 mg N/L, 600 mg N/L, and 1000 mg N/L, and the COD:TN ratio was 0.5. In this work, the calculated bCOD:TN ratios were 0.3 at day 1 and 0.4 at day 7, 0.5 at day 14, and 0.6 at days 21 and 28.

Following the 28 days of long-term dosing with caffeine, the system was then fed synthetic wastewater only (no COD from coffee wastewater). The ammonium–nitrogen concentration in the synthetic wastewater was gradually increased from 3 mM to 15 mM. It was shown that the anammox activity slowly recovered over a period of 36 days, at which point the nitrogen removal efficiency has returned to 96%. See Figure 9.

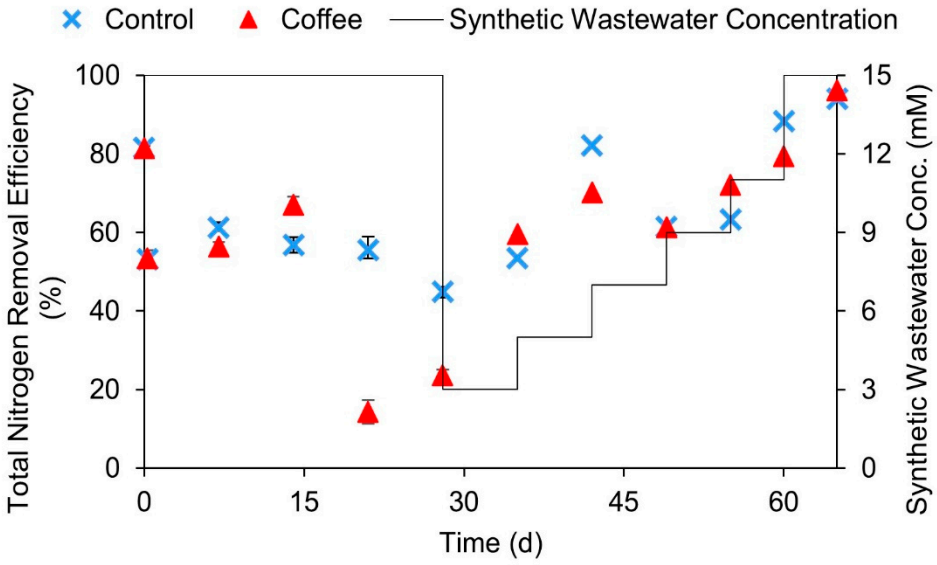

Figure 9. Percentage of total nitrogen removal and synthetic wastewater concentration during long-term coffee dosing.

However, after using the biological treatment process to remove both organic matter and nitrogen from coffee wastewater, the treated effluent of coffee might not be directly discharged to the environment because of color remaining in coffee wastewater. A chemical treatment (adsorption process) using activated carbon might be indicated to complete the process.

4. Conclusions

- The addition of caffeine in concentrations greater than 350 mg/L caffeine significantly inhibits anammox activity.
- There is no long-term effect with low caffeine concentration (2.5 mg/L) on anammox activity.
- The mechanism for caffeine inhibition on anammox bacteria could not be explained clearly because the not-competitive inhibition model could not distinguish between non- and un-competitive inhibition.
- The presence of $\geq$387 mg/L COD from coffee wastewater could significantly inhibit the anammox activity. However, this inhibition effect is reversible. After the discontinuance of coffee wastewater additions to a batch reactor, the anammox activity could recover in 28 days.
- In using an anammox treatment with real wastewater (coffee wastewater) as substrate, bCOD:TN should be used rather than TCOD:TN as the control parameter because bCOD and TCOD are not equal.

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