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Impacts of crude glycerol on anaerobic ammonium oxidation (Anammox) process in wastewater treatment

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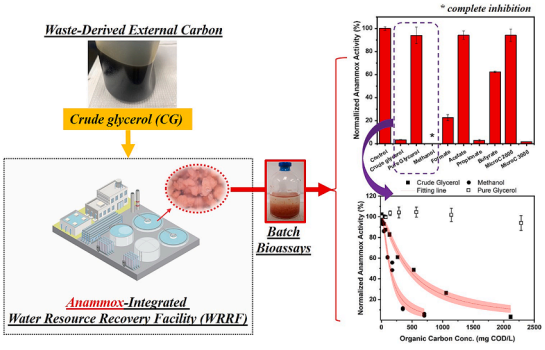
HIGHLIGHTS

- Inhibitory effects of CG and its major components on Anammox were assessed.
- Methanol is the major compound in CG responsible for the Anammox inhibition.
- 153.7 mg CG-COD L^{-1} or COD/TIN ratio of > 1.3 can cause Anammox inhibition by 20 %.
- Increasing the substrate level can promote the Anammox activity inhibited by CG.
- Anammox inhibition by CG is through a non-competitive inhibition mechanism.

GRAPHICAL ABSTRACT



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ABSTRACT

This work investigated the impact of a waste-derived carbon source, crude glycerol (CG), on Anammox. Batch bioassays were conducted to identify inhibitory component(s) in CG, and the relationship between Anammox activity and the concentration of CG, pure glycerol, and methanol were assessed. The results showed that the half-maximal inhibitory concentration of CG and methanol are 434.5 ± 51.8 and 143.0 ± 19.6 mg chemical oxygen demand (COD) L^{-1}, respectively, while pure glycerol at 0–2283 mg COD L^{-1} had no significant adverse effect on Anammox. The results suggested methanol is the major inhibitor in CG via a non-competitive inhibition mechanism. COD/total inorganic nitrogen ratio of > 1.3 was observed to cause a significant Anammox inhibition (>20 %), especially at low substrate level. These results are valuable for evaluating the feasibility of using CG for nitrogen removal in water resource recovery facilities, promoting sustainable development.

1. Introduction

Crude glycerol (CG) is a major byproduct (about 10 wt% of the amount of biodiesel produced) from the biodiesel production process that convert organic wastes (e.g., vegetable oils, grease, used cooking oils, or animal fats) to renewable biofuel (Yang et al., 2012). With the

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rapid expansion of biodiesel plants worldwide, a large amount of CG (around 3.5 billion liters globally in 2022) is produced, making it widely available at a low cost (Anitha et al., 2016; Liu et al., 2022). As CG contains high chemical oxygen demand (COD) due to the presence of glycerol (60–80 wt%), alcohol (6–40 wt%), and other organic compounds, various methods have been proposed to utilize this organic material, such as feedstock for chemicals synthesis, fuel additives, fuel cell application, food for animals, and (co) substrate in fermentation and digestion processes (Kaur et al., 2020). In the wastewater treatment process, CG has the potential to be applied as external carbon source to support the biological nitrogen removal (BNR) process.

A readily available source of biodegradable carbon is often limited in or exhausted prior to denitrification basin in water resource recovery facilities (WRRF). Adding external carbon sources to enhance the BNR has been used in WRRFs to facilitate nutrient removal, such as traditional nitrification–denitrification process (EPA, 2013). In the WRRFs integrated with anaerobic ammonium oxidation (Anammox), an environmentally sustainable biological technology for achieving energy-neutral or positive wastewater treatment (Wang et al., 2022), there is a need for resources inputs, including aeration and/or external carbon sources. These resources are used to partially convert NH_4^+-N in the influent into NO_2^--N, aiming to attain an ideal stoichiometric substrates relationship of NH_4^+-N: NO_2^--N = 1:1.15 ~ 1:1.32 for Anammox reaction (Lotti et al., 2014; Strous et al., 1998). To achieve this, two methods have been proposed, including 1) partial oxidation of NH_4^+-N by ammonia-oxidizing bacteria (AOB) known as partial nitrification (PN); and 2) NO_3^--N reduction by denitrifiers known as partial denitrification (PdN) (Le et al., 2019; Zhang et al., 2019). Compared with PN/Anammox system, PdN/Anammox, a process requires carbon sources, recently has been attracting more attention owing to the more stable NO_2^--N accumulation for Anammox and lower NO_3^--N concentration in the effluent discharge (Du et al., 2015). Compared with methanol, ethanol, and acetate that are derived from fossil fuel-based raw materials, waste-derived carbons, such as CG, have been proposed as more sustainable and safe alternative carbons to supplement the COD for BNR (He et al., 2022; Kim et al., 2017; Ladipo-Obasa et al., 2022; Zubrowska-Sudol et al., 2022). The effectiveness of using glycerol as carbon source for complete denitrification (nitrogen gas as final product) and PdN (aiming to accumulate nitrite) in different environments (i.e., soil, surface water, and wastewater) has been studied and proved (Alessio et al., 2023; De et al., 2022; Schroeder et al., 2020).

When external carbon is added to the heterotrophic PdN/Anammox system to create a favorable COD/N ratio (typically 2.0 – 3.0 reported in previous work) (Zhang et al., 2019), understanding the impact of added carbon on Anammox bacteria is critical to maintain successful nitrogen removal in wastewater treatment. Nevertheless, there is limited information regarding the potential impacts of the components in CG and/or the appropriate dosage of CG, hindering its application as an alternative carbon source in wastewater treatment processes involving Anammox. In this work, CG and its major components, including glycerol, methanol, and volatile fatty acids (VFA, C1-4), were tested to assess their impact to the activity of Anammox bacteria using batch bioassays. As comparison, two commercialized waste-derived carbon, MicroC 2000 and 3000, were also tested. In addition, kinetic parameters were determined to evaluate the inhibitory concentration of CG on Anammox. The study also explored the correlation between Anammox activity, COD/N ratios, and substrate levels in the CG-fed system. The results are expected to benefit researchers and practitioners in both fields of organic wastes management and biological wastewater treatment to develop a sustainable and effective process for utilizing CG for nutrient removal.

2. Materials and methods

2.1. Waste-derived organic carbons

CG, MicroC 2000, and MicroC 3000 were obtained from a local municipal WRRF in Maryland, USA, which used them as supplementary carbon sources for denitrification in the BNR processes. The properties of waste-derived organic carbons are summarized in Table 1. A stock solution for each tested waste-derived organic carbon were prepared by mixing raw carbon solution (obtained from the manufacturers) with MilliQ water. These stock solutions were analyzed for COD, and the obtained results were utilized in designing the experiments to achieve the designated concentrations.

2.2. Anammox sludge

Granular (2.4 ± 0.6 mm) Anammox biomass was obtained from a lab-scale Anammox expanded granular sludge bed reactor (EGSB) dominated by *Ca. Brocadia* spp. The EGSB (working volume = 3L) had been successfully operated for three years with a loading rate of 4.1 g N L^{-1} day^{-1}, and the volatile suspended solids (VSS) was 89 % of total suspended solids (TSS). The Anammox biomass had an activity of 0.55 ± 0.05 g N_2-N g^{-1} VSS day^{-1}. Suspended Anammox sludge was prepared by crushing granular Anammox through syringe needles (23 G, 0.34 mm I.D.) for three times. The pictures of granular and suspended Anammox sludge are provided in Supplementary Information.

2.3. Anammox activity bioassays

Assessment of the impacts of CG, including glycerol and other major components, to Anammox process was conducted in batch bioassays with 118 mL serum bottles (60 mL working volume) following the procedure described in our previous work (Lakhey et al., 2020; Li et al., 2020). The basal medium was prepared using ultrapure water, containing (mg L^{-1}): $NaHPO_4{\cdot}H_2O$ (1 1 5), $CaCl_2{\cdot}2H_2O$ (2 0 0), $MgSO_4{\cdot}7H_2O$ (4 0 0), $NaHCO_3$ (1 0 0), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (5950) and 1 mL L^{-1} of trace element solutions (details in previous work by Lakhey et al. (2020)). After adding basal medium and Anammox biomass (0.70 g VSS L^{-1}), the bottles were sealed with rubber caps and flushed with helium (He) gas for 5 mins to create an anaerobic condition, followed by injection of substrates (7.1 mM $NaNO_2$ and 5.4 mM NH_4HCO_3) to start the reaction. The designated amount of the stock solution of tested organic compounds was injected at final step to prevent pre-exposure of Anammox biomass to these compounds and achieve a custom concentration range (see Table 2). After placed on the shaker at 30 ± 1 °C and 120 rpm, gas samples were collected from headspace at a regular basis for N_2 measurement, while liquid samples were retrieved at the beginning and end of the bioassays for other analysis (i.e., pH, NH_4^+, NO_2^-, and NO_3^-). All bioassays were carried out in duplicates. The potential occurrence of denitrification in the bioassays was tested, and the results indicated a negligible contribution of denitrification to N_2 gas production during the experimental duration (see Supplementary Information).

2.4. Denitrification bioassays

Suspended activated sludge collected from anoxic basin in a local municipal WRRF was used in the denitrification bioassays to examine the bioavailability of CG by denitrifiers. Serum bottles (250 mL working volume) were prepared by adding 3 g TSS L^{-1} sludge, chlorine-free tap water, CG (565 mg COD L^{-1}), and KNO_3 (49 mg N L^{-1}) and the pH was adjusted to 7.2 using 1 M NaOH/HCl. The bottles were then sealed and flushed with He for 5 mins to create the anaerobic condition, followed by incubation at 25 ± 1 °C and 150 rpm. Liquid samples were taken during the experiment for monitoring pH, COD, and concentrations of NH_4^+, NO_3^- and NO_2^-.

Table 1
The properties of tested waste-derived carbon sources.

Source	Description	Color	Density * [g mL^{-1}]	COD # [g L^{-1}]	Methanol content * (wt %)
Crude glycerol	Byproduct of biodiesel produciton fed with soybeans, canola, or waste grease from restaurants	Dark brown	0.96–1.20	1940 ± 65	20–40
MicroC 2000	Waste derived and glycerin-based	Light brown	1.22–1.24	1420 ± 40	<0.1
MicroC 3000	Industrial mixed waste alcohols blend	Dark yellow	0.75–0.90	1270 ± 50	60–80

#, Chemical oxygen demand (COD) was analyzed in triplicate.
*, Data are provided by the manufacturers.

Table 2
Calculated values of 20, 50, and 80 % inhibitory concentration (IC).

Organic Carbons	Tested Concentrations [mg COD L^{-1}]	IC [mg COD L^{-1}] 20 %	50 %	80 %
Crude Glycerol	0–2112	153.7 ± 29.0	434.5 ± 51.8	1228.4 ± 226.3
Pure Glycerol	0–2283	*NA**	*NA*	*NA*
Methanol	0–700	60.8 ± 13.2	143.0 ± 19.6	336.2 ± 65.0

* NA, Not applicable.
COD, Chemical oxygen demand.
The experiments used for obtaining the results were carried out in duplicate.

2.5. Analytical methods

The N_2 in the headspace was analyzed using an Agilent 6890 gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with an HP-PLOT Molesieve column (30 m × 0.32 mm, Agilent Technologies) and a thermal conductivity detector. Liquid samples were collected before and after the reaction and filtered (0.45 μm) prior to analyzing NO_3^- and NO_2^- with an ion chromatograph (Dionex ICS-1100, Thermo Scientific) equipped with Dionex IonPac AS22-Fast analytical column (Li et al., 2016). NH_4^+ was measured using an IntelliCAL Ammonia Ion Selective Electrode (ISENH4131, Hach, Loveland, CO, USA). Other parameters (TSS, VSS, COD, pH, etc.) were determined according to standard methods (APHA, 2017).

2.6. Data processing

The specific Anammox activity (*SAA*, expressed as N_2-N g^{-1} VSS day^{-1}) in each assay was calculated from the time course of N_2 production. The normalized Anammox activity (*NAA*) values for each treatment group were calculated with uninhibited control displayed the maximum specific activity (SAA_{max}) as follows:

$$NAA(\%) = [SAA/SAA_{max}] \times 100 \quad (1)$$

The results obtained in the inhibition bioassays were fitted to an inhibition model:

$$NAA(\%) = NAA_{max} \times [1 + (C/IC_{50})^n]^{-1} \quad (2)$$

where, NAA_{max}, C, and n represent the maximum NAA value, the organic compounds concentration (expressed as mg COD L^{-1}), and the inhibition order (dimensionless), respectively.

Monod model (Eq. (3)) was used to calculate the kinetics parameters of substrate consumption in the presence of CG or PG, and the results were compared with the group without addition of organic carbon (De Prá et al., 2016).

$$q = q_{max} \times S/(Ks + S) \quad (3)$$

where q is the specific substrate conversion rate (g N g^{-1} VSS day^{-1}); q_{max} is the maximum specific substrate conversion rate (g N g^{-1} VSS day^{-1}); S is the substrate concentration (mg N L^{-1}); Ks is the half saturation constant (mg N L^{-1}).

Non-linear fittings of the data to the Eq. (2) and (3) were performed by least-square minimization of the error using Origin 2021b (OriginLab, Northampton, MA, USA), allowing the estimation of the concentration of tested organic compounds causing inhibition at different levels with a 95 % confidence. The significance of differences in the results obtained from the treatment groups were determined by using Origin 2021b for factor analysis of Variance ANOVA with Tukey's test and Student t-text, with the significance levels of $p < 0.05$. The reported values are given as means with standard errors.

3. Results and discussion

3.1. Screening tests for assessing the impact of crude glycerol (CG) and its major components on Anammox activity

Preliminary tests were conducted using batch bioassays to assess the potential inhibitory impacts of CG on Anammox activity. As shown in Fig. 1, compared with the Anammox activity of 0.79 ± 0.08 g N_2-N g^{-1} VSS day^{-1} measured in control group without CG, addition of CG at 2570 mg COD L^{-1} showed a significant reduction in Anammox activity by 96.7 %. To explore the potential inhibitory component(s) in CG, six biodegradable components in CG, including glycerol, methanol, and four VFA (formate, acetate, propionate, and butyrate), were tested using a concentration of 2570 mg COD L^{-1}, except the group of methanol that

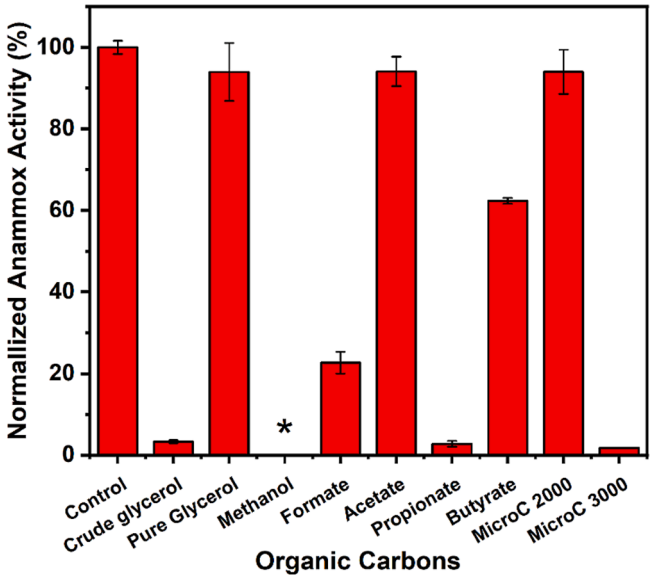

Fig. 1. Screening tests: effects of different organic carbons on Anammox activity. The concentration of organic carbons were 2570 mg COD L^{-1}, except the group of methanol at 700 mg COD L^{-1}. *, no activity was detected. Error bar represents the standard deviation of duplicates.

used 700 mg COD L^{-1} as previous work showed it has a higher inhibitory potential on Anammox bacteria (Güven et al., 2005; Madeira and de Araújo, 2021). The results showed negligible impact on Anammox activity in the groups of glycerol and acetate, while the inhibitory impacts in other groups follow the order (inhibition from low to high): butyrate < formate < propionate < methanol.

These results are consistent with previous work reported in the literature. Both glycerol and acetate were reported to exhibit low/non-toxicity to Anammox activity, indicating their safe use in the wastewater treatment process integrated with Anammox. Anammox bacteria was detected in a glycerol-fed nitritation-denitritation separate centrate treatment process, indicating the growth and enrichment potential of Anammox bacteria in the presence of glycerol (Park et al., 2017). Furthermore, glycerol was suggested as a protective agent for preserving Anammox bacteria (Chen and Jin, 2017). It is noted for its ability to maintain the structural and metabolic integrity of Anammox bacteria when they are exposed to a glycerol-containing environment. In another study, the addition of acetate and glycerol up to 20 mg/L, corresponding to 45 and 80 mg COD L^{-1}, did not show detrimental impact on Anammox activity during both short- and long-term operation (Le et al., 2019). The half-maximal inhibitory concentration (IC_{50}) of 3200 mg/L was reported for sodium acetate, equivalent to 2500 mg COD L^{-1} (Dapena-Mora et al., 2007).

Anammox showed different responses to four tested VFAs at the concentration of 2570 mg COD L^{-1}. When compared to the inhibitory effects of acetate (5.9 ± 3.6 %), butyrate (37.6 ± 0.7 %) and formate (77.3 ± 2.7 %) on Anammox activity, the addition of propionate resulted in the most significant reduction, with an inhibition of 97.2 ± 0.7 % (see Fig. 1). In a previous work that investigated the effects of VFA stress on Anammox process in batch experiments, they found that propionate of 400 mg/L (equivalent to 467 mg COD L^{-1}) reduced ammonium consumption rate of Anammox bacteria by 29 % (Güven et al., 2005). It should be noted that preliminary tests used extremely high concentrations of VFAs for screening the potential inhibitory component in the CG. In wastewater containing low VFA concentration (e.g., typically ⩽ 50 mg/L), Anammox bacteria demonstrated high resistance. Additionally, VFAs can be consumed by heterotrophic organisms that coexist in Anammox process and even by Anammox bacteria due to their metabolic versatility (Yang et al., 2021). These could further reduce the VFA concentration in the system, enabling Anammox bacteria to maintain activity even in environments with higher VFA concentrations.

Compared with other tested organic compounds, the addition of methanol at 700 mg COD L^{-1} caused a complete inhibition of Anammox activity. In comparison, adding methanol at a final concentration of 0.5 mM (equivalent to 24 mg COD L^{-1}) was reported to result in immediate and complete inactivation of Anammox activity (Güven et al., 2005). In another work, the inhibitory effect of methanol on the immobilized Anammox bacteria in a polyethylene glycol gel carrier was studied (Isaka et al., 2008). The results showed that the addition of methanol at concentrations of 5 and 12.5 mM (equivalent to 240 and 600 mg COD L^{-1}, respectively), caused a similar irreversible reduction in Anammox activity (71 %). Therefore, the presence of methanol in CG might play a critical role in contributing to the inhibition of Anammox activity. The inhibition mechanism of methanol on Anammox bacteria is still not clear. Previous work suggested formaldehyde, a chemical converted from methanol within Anammox bacteria, serves as the actual inhibitor causing irreversible inactivation of proteins and enzymes (Güven et al., 2005). Moreover, variations in sensitivity to methanol were reported among different Anammox cultures (Güven et al., 2005; Jensen et al., 2007). To comprehensively understand the inhibitory impact of methanol on Anammox, further studies are necessary, particularly from the genetic level of the microorganisms within the Anammox system.

Two commercialized waste-derived carbon sources (i.e., MicroC 2000 and 3000) were also tested as they have been widely used to supplement carbon in wastewater treatment processes, especially for denitrification. As shown in Table 1, MicroC 2000 is glycerol based and contains minimal methanol (<0.1 wt%), while MicroC 3000 is an alcohol blend with a high methanol content, comprising 60–80 wt% of its composition. The results from the inhibition test for MicroC 2000 and 3000 agreed with the findings about glycerol and methanol (Fig. 1). At a concentration of 2570 mg COD L^{-1}, MicroC 2000 (glycerol-based) showed a minimal inhibition on Anammox activity by 6.0 ± 5.4 %. In contrast, adding MicroC 3000 (methanol-based) resulted in a substantial inhibition of 98.2 ± 0.0 %.

Therefore, considering the high content of methanol (20–40 wt%) and glycerol (60–80 wt%), along with the low content of VFA (<3 wt%) in CG, only CG, glycerol, and methanol were selected for further investigation.

3.2. Inhibitory impact of crude glycerol (CG), glycerol, and methanol on Anammox process

Anammox activity was monitored after exposed to CG, glycerol, and methanol at different concentrations (Table 2). The results are depicted in Fig. 2. The data for CG and methanol groups were also fitted into the inhibition model **Eq. (2)**, with the calculated IC_{20}, IC_{50}, IC_{80}, and other parameters summarized in Tables 2 **and** Supplementary Information.

As shown in Fig. 2, the addition of CG at the concentration of 2112 mg COD L^{-1} caused severe inhibition of Anammox activity by 96.7 ± 0.4 % which was consistent with the 96.7 ± 0.5 % inhibition at 2570 mg CG-COD L^{-1} in the preliminary test (Fig. 1). The results from pure glycerol and methanol groups also aligned with the preliminary test. No noticeable inhibition was detected in the group with pure glycerol of up to 2283 mg COD L^{-1}, while methanol at 700 mg COD L^{-1} caused complete inhibition. The data from this experiment showed a good fitting in the inhibition model with R^2 of 0.98 for both CG and methanol. The calculated IC_{50} of CG and methanol are 434.5 ± 51.8 and 143.0 ± 19.6 mg COD L^{-1}, respectively. These results confirmed the inhibitory impact of CG and methanol on Anammox bacteria, with the inhibition parameters calculated (see Supplementary Information). Previous work reported 3.3 mM methanol (equivalent to 158.4 mg COD L^{-1}) reduced the Anammox activity by 50 % (Isaka et al., 2008), which aligns well with the IC_{50} calculated in Table 2**.** For CG, based on our knowledge, there is limited information available in the literature concerning its inhibition on Anammox activity. Most of the work used pure glycerol and reported no inhibitory impact on Anammox activity. For example, pure glycerol

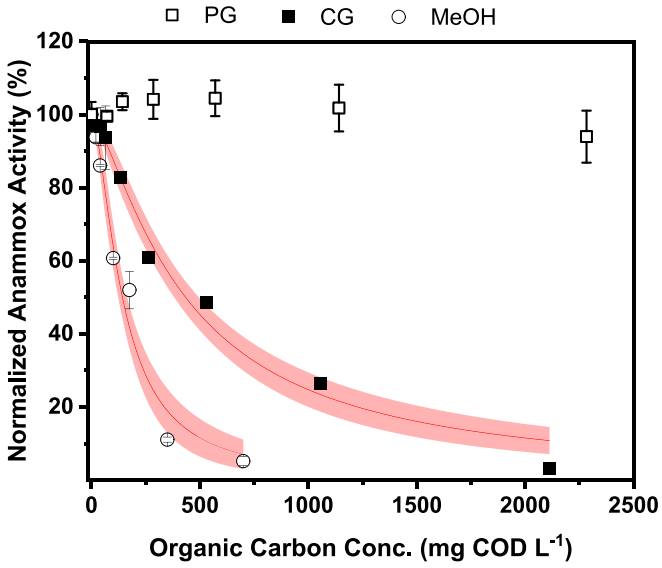

Fig. 2. Effects of pure (PG, open square) and crude (CG, closed square) glycerol, and methanol (MeOH, open circle) on Anammox activity at various glycerol concentrations. Inhibition model Eq. (2) was used to fit the data of CG and MeOH (red line). Error bar represents the standard deviation of duplicates.

was reported to display a good potential for promoting PdN in a single-phase PdN/Anammox reactor (Le et al., 2019). However, when PdN was coupled with Anammox in a single-stage reactor, they observed NO_2^- accumulation, suggested that a pure glycerol-fed system might have difficulty in maintaining a good balance between PdN rates and Anammox rates.

The methanol content in the CG concentrations in Fig. 2 was calculated using 20 and 40 % as the lower and upper weight percentages in CG. The results were plotted in Fig. 3 to depict the relationship between Anammox activity and organic carbon concentration. As shown in Fig. 3, the inhibition curve for the estimated methanol content in CG closely aligns with the methanol inhibition curve, indicating that methanol is the primary component in CG responsible for Anammox inhibition.

Additionally, responses of granular and suspended Anammox sludge to CG, glycerol, and methanol were examined, respectively. The results are presented in Fig. 4. In the treatment groups, IC_{50} of 435 (for CG) and 143 (for methanol) mg COD L^{-1} calculated from the previous experiments (Table 2) were used to add CG and methanol. In the pure glycerol group, a concentration of 435 mg COD L^{-1} was used, which corresponds to the IC_{50} of CG. The results showed that, in the control group without added organic carbons, the measured SAA of granular and suspended Anammox sludge were 0.27 ± 0.00 and 0.28 ± 0.01 g N_2-N g^{-1} VSS day^{-1}, respectively, indicating crushing procedure did not have a negative impact on Anammox bacteria. Compared with SAA of 0.79 ± 0.08 g N_2-N g^{-1} VSS day^{-1} in the control group in the previous experiments, the lower SAA in this instance could be attributed to varying activity levels of the Anammox sludge extracted from the lab-scale Anammox EGSB on different days. Both CG and methanol showed a 50 % inhibition of granular Anammox activity at the IC_{50} concentration of 435 and 143 mg COD L^{-1}, respectively. These results were consistent and reproducible, suggested they accurately represented the actual response. According to the results shown in Fig. 4, a slight, but non-significant ($p > 0.1$), decrease in Anammox activity occurred in the suspended Anammox sludge when exposed to CG and methanol during the batch experiment, compared to granular Anammox bacteria. This result suggested that the granular structure did not provide additional protection for Anammox bacteria from the inhibition caused by CG and methanol.

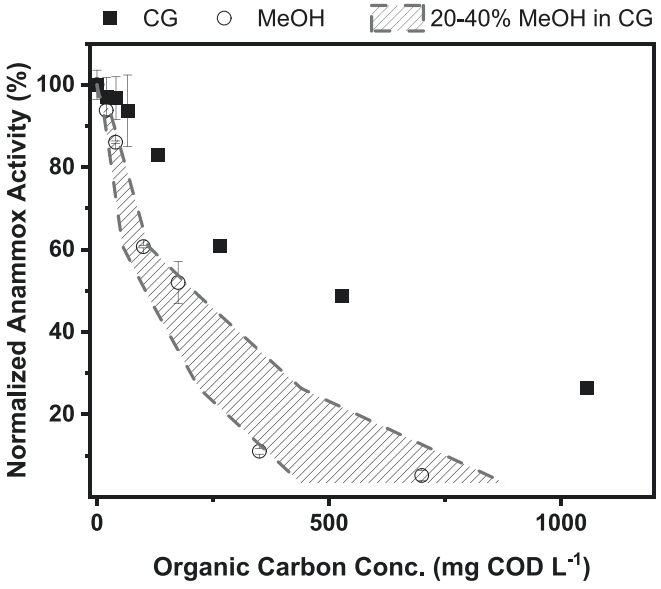

Fig. 3. Effects of crude glycerol (CG, closed square) and methanol (MeOH, open circle) on Anammox activity at various concentrations. The dashed line shaded area indicated the estimated MeOH concentrations in added CG (MeOH content in CG: 20–40 wt%). Error bar represents the standard deviation of duplicates.

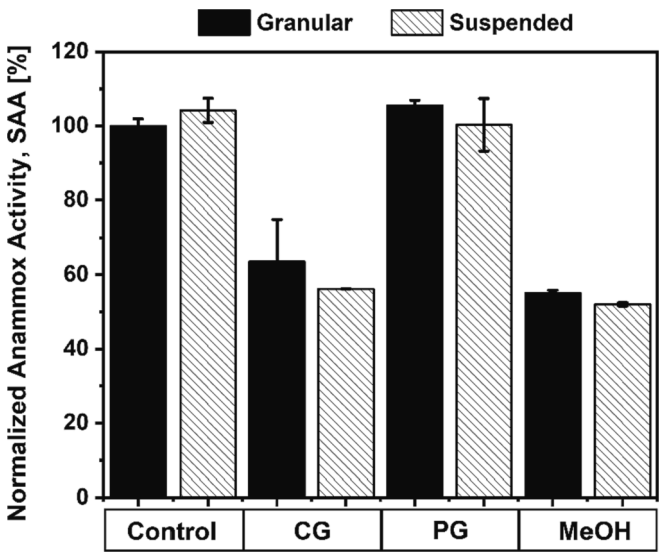

Fig. 4. Comparison of granular and suspended Anammox sludge in the presence of CG (crude glycerol). PG (pure glycerol), and MeOH (methanol). Added concentration of each organic carbons were selected based on the IC_{50} calculated in Table 2. CG: 435 mg COD L^{-1}, MeOH: 143 mg COD L^{-1}. For PG with unnoticeable inhibition in the tested concentration range, 435 mg COD L^{-1} was used for comparing with CG. Error bar represents the standard deviation of duplicates.

3.3. Impact of chemical oxygen demand/total inorganic nitrogen (COD/TIN) ratio and substrate condition on Anammox activity

In addition to the type of organic carbon sources, C/N ratio and substrate level can impact the performance of Anammox process and the microbial community (Xiao et al., 2021). Anammox has been recommended to remove nitrogen from wastewaters with high NH_4^+-N but low carbon concentrations (i.e., low COD/TIN ratio, total inorganic nitrogen (TIN) = NH_4^+-N + NO_2^--N). Four COD/TIN ratios, including 2.6, 3.7, 7.4, and 18.4, were tested by using a fixed CG concentration of 647 mg COD L^{-1} and various TIN concentrations (i.e., 2.5, 6.3, 12.5, and 17.9 mM N at the NH_4^+-N: NO_2^--N ratio of 1:1.3). The results were normalized and compared with a control group containing no CG and 12.5 mM TIN. As shown in Fig. 5, increasing COD/TIN ratio resulted in a lower Anammox activity. The data was fitted in the ExpDecay model ($R^2 = 0.94$), and the calculated COD/TIN ratio that can cause a 50 % reduction in Anammox activity is 5.0 ± 1.1 (Table 3). Although there is still no consensus on what is the C/N ratio that inhibits or affects the Anammox process (Leal et al., 2016), some results were reported in the literature, enabling the researchers to make a comparison. It was reported that when fat milk was added as a COD source, COD/N of 2 may fully inhibited Anammox reaction (Chamchoi et al., 2008). As comparison, glucose was gradually added in the Anammox-sequencing batch reactor (SBR) and no negative impact on nitrogen removal efficiency was found at a COD/N ratio of 5.0. In contrast, other work showed that total nitrogen (TN) removal rates in the batch Anammox reactors increased with increasing COD/N from 0 to 2 (Wang et al., 2019a). They explained that the increasing organic substrate promoted the denitrifier growth and nitrogen removal within the Anammox process. It should also be noted that these varying observations could partially be attributed to differences in the experimental setups for achieving the designated COD/N ratios. Different TIN concentrations were added into the groups with addition of 647 mg COD L^{-1} CG, an inhibitory compound for Anammox. In contrast, their experiments maintained a constant TIN concentration, while varying doses of glucose, a non-inhibitory compound for Anammox, were dosed. This highlights the importance of examining the role of substrate level in the inhibition of Anammox bacteria by organic carbons.

The results depicted in Fig. 6 compared Anammox activity at

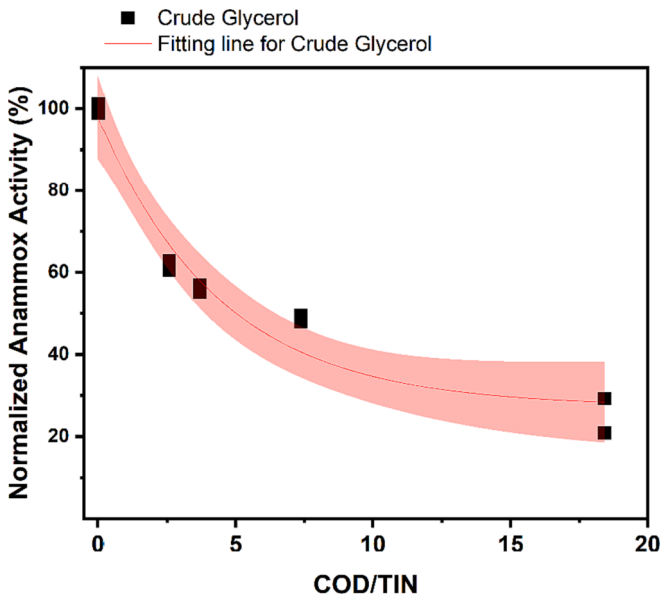

Fig. 5. Sensitivity of Anammox to various COD/TIN ratios. The concentration of crude glycerol was fixed at 647 mg COD L^{-1}, while various ammonium and nitrite concentrations were used (mM): 2.5, 6.3, 12.5, and 17.9 mM N at the NH_4^+-N: NO_2^--N ratio of 1:1.3. Anammox activity obtained in the group without organic carbon addition and 12.5 mM TIN was used for normalizing the activity as 100 %. ExpDecay model was used to fit the data (red line).

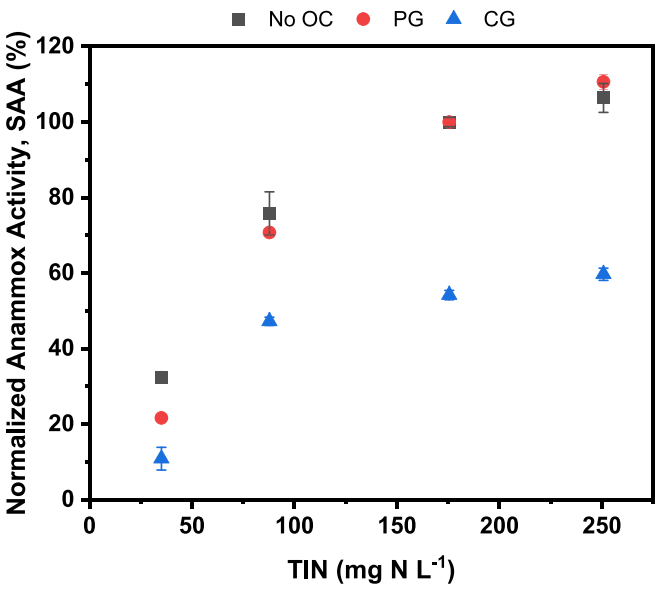

Fig. 6. Comparison of the inhibitory impacts of pure glycerol (PG) and crude glycerol (CG) on anammox activity in the presence of different substrate levels. The concentration of organic carbons was fixed at 647 mg COD L^{-1}, while various ammonium and nitrite concentration were used as design (mM): 2.5, 6.3, 12.5, and 17.9 mM N at the NH_4^+-N: NO_2^--N ratio of 1:1.3. Anammox activity obtained in the group without organic carbon addition (No OC) and 12.5 mM TIN was used for normalizing the activity as 100 %. Error bar represents the standard deviation of duplicates.

Table 3
Calculated values of 20, 50, and 80 % inhibitory COD/TIN# ratio at fixed organic carbon concentration of 647 mg COD L^{-1}.

Organic Carbons	COD/TIN ratio		
	20 %	50 %	80 %
Crude Glycerol	1.3 ± 0.6	5.0 ± 1.1	*NA**

* NA, Not applicable.
#, COD, Chemical oxygen demand. TIN = total inorganic nitrogen = NH_4^+-N + NO_2^--N.
The experiments used for obtaining the results were carried out in duplicate.

different substrate levels with and without organic carbon addition. Four substrate levels (2.5, 6.3, 12.5, and 17.9 mM N at the NH_4^+-N: NO_2^--N ratio of 1:1.3) were selected to represent different wastewater strengths, while pure glycerol and CG were tested to represent organic carbon with no and high inhibition potential to Anammox bacteria. Similar to the observation reported in the literature, increasing substrate level, when it is below the tolerance threshold of Anammox bacteria to NH_4^+ and NO_2^-, promoted the Anammox activity without organic carbon. Previous work reported that a decrease in substrate concentration from 45.2 to 7.9 mM reduced Anammox activity by about 50 % (from 0.4 to 0.21 g N g^{-1} VSS day^{-1}), along with that the relative abundances of Anammox bacteria (*Ca. Kuenenia*) decreased from 43.0 to 27.8 % (Wang et al., 2019b). In this experiment, the addition of pure glycerol at 647 mg COD L^{-1} did not show a negative impact on Anammox under tested substrate conditions. The results from the Monod model **Eq. (3)** showed that q_{max} (g N g^{-1} VSS day^{-1}) and *Ks* (mg N L^{-1}) were 2.0 ± 0.21 and 46.2 ± 9.6, and 2.5 ± 0.4 and 79.0 ± 23.8 for the groups with no organic carbon and addition of pure glycerol, respectively (see Supplementary Information). Various *Ks* and q_{max} values were reported in the literature. For example, q_{max} ranged from 0.2 to 0.3 g N g^{-1} VSS day^{-1}, and substrate *Ks* ranged from 4.9 to 9.7 mg N L^{-1} were reported from the experiments testing both granular and suspended Anammox sludge (Puyol et al., 2013). In other work, lower *Ks* values of 0.1 mg NH_4^+-N L^{-1} and 0.07 mg NO_2^--N L^{-1} were reported for *Ca. Brocadia* (Jetten et al., 2005). Such varied *Ks* and q_{max} reported in different studies may be attributed to the fitting strategy employed, such as the use of simple Monod equation (used in this study) or multiple substrate equations (Puyol et al., 2013). In comparison, the presence of CG at a concentration of 647 mg COD L^{-1} resulted in a significant decrease in Anammox activity of more than 40 % at all tested substrate levels, corresponding to a lower q_{max} of 0.7 ± 0.1 g N g^{-1} VSS day^{-1}, while the *Ks* remained relatively unchanged ($p > 0.1$) at 35.1 ± 9.2 mg N L^{-1}. This result indicated a non-competitive inhibition mechanism of CG, or more precisely, the inhibitory components within CG, on Anammox bacteria.

3.4. Feasibility of using crude glycerol (CG) in partial denitrification (PdN)-Anammox process

To supply the substrates at a proper ratio of NH_4^+-N: NO_2^--N for Anammox, PdN was proposed to produce NO_2^- either in a standalone reactor prior to or within Anammox reactor, corresponding to the two- and single-stage PdN/Anammox process, respectively (You et al., 2020). Batch denitrification bioassay was conducted to assess if CG can be used for denitrifiers. With the addition of KNO_3 (49 mg N/L), a fast consumption of CG was observed with a calculated consumption rate of 271 mg COD hr^{-1} in the first hour, corresponding to consumption of 69 % added CG from 570 ± 7.1 to 179.4 ± 5.9 mg COD L^{-1} (see Supplementary Information). At the end of the reaction (7 hrs), 95 % added CG-COD was consumed with the final CG concentration of 28 ± 1.4 mg COD L^{-1}. This result agreed with previous work indicated that denitrifiers can utilize pure glycerol, methanol, and VFAs (Fu et al., 2022; Le et al., 2019), which are the major components of CG. Together with that glycerol was reported to display an efficient PdN selection (Le et al., 2019), it is expected that CG can be used as a potential carbon source for accumulating NO_2^--N in two-stage PdN/Anammox. However, further efforts are necessary to demonstrate the feasibility of using CG as carbon source for BNR in wastewater treatment by using a PdN/Anammox system.

4. Conclusion

CG exhibited a good potential to support denitrifier in the two-stage PdN/Anammox process. However, direct exposure of Anammox to CG at 153.7 mg COD L^{-1}, such as single-stage PdN/Anammox, may cause a considerable (20 %) inhibition of Anammox activity. Anammox showed higher sensitivity to CG at low substrate levels, like mainstream wastewater. Methanol in CG is the major inhibitor for Anammox via a non-competitive inhibition mechanism. As the composition of CG could vary depending on the feedstock of the biodiesel production and manufacturing processes, the CG quality (e.g., methanol content) should be characterized for accurately estimating its inhibitory effects on Anammox.

5. Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) used the OpenAI ChatGPT in order to improve the language and readability. After using this tool, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

CRediT authorship contribution statement

Xiaojue Chen: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Liu Jiang:** Writing – review & editing, Methodology, Investigation, Formal analysis, Conceptualization. **Khashayar Aghilinasrollahabadi:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Camila A. Proano:** . **Seth Meisler:** Methodology, Investigation, Conceptualization. **Marya O. Anderson:** Resources, Methodology, Conceptualization. **Jinkai Xue:** Writing – review & editing, Resources, Conceptualization. **Guangbin Li:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2023.130271.

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