

ABSTRACT

Title of Dissertation: CHEMICALS OF EMERGING CONCERNS IN
WASTEWATER TREATMENT: ACUTE AND
CHRONIC IMPACTS OF AZOLES ON
BIOLOGICAL NITROGEN REMOVAL
PROCESSES

Xiaojue Chen

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Dissertation directed by: Dr. Guangbin Li

Assistant Professor

Civil and Environmental Engineering

Nitrogen (N) in wastewater presents in the forms of ammonia, nitrite, nitrate, and/or organic N. Discharging of ineffectively treated N-containing wastewater into water bodies could cause eutrophication, threaten the safety of the ecosystem, and impair the water quality for humans. Various Biological Nitrogen Removal (BNR) processes, including nitrification, denitrification, and anaerobic ammonium oxidation (Anammox), have been successfully applied in wastewater treatment plants (WWTP) for N removal. However, the performance and stability of BNR can be adversely impacted by chemical inhibitors present in wastewater.

Azoles, classified as emerging organic contaminants (EOCs), are a group of man-made chemicals containing N atoms in the heterocyclic ring systems and have been widely applied as aircraft de-icing agents, in semiconductor manufacturing, and household dishwashing detergents. Various azoles have been detected in wastewater streams and their presence within WWTPs may impact the BNR processes. Azole compounds, such as benzotriazole (BTA), have been used as biocides/fungicides in agriculture. However, limited information is available about the potential inhibition of azoles to BNR processes, while guidelines for preventing BNR processes from azole inhibition and methods for system recovery are also unavailable. In this study, we investigated the short- and long-term impacts of azoles on the major BNR processes, including nitrification, denitrification, and Anammox. Besides, potential inhibitory mechanisms of azoles and resistance/adaptation of BNRs were assessed, aiming to develop an effective strategy to prevent BNRs from system interruption and/or efficiency decreasing caused by azoles present in WWTPs.

In short-term impact assessment, both experiment-based lab research and literature review approaches were used to assess the inhibition potential of different azole compounds on major BNR processes. Nine azole compounds (5 diazoles and 4 triazoles) were selected to represent azoles with different structures and physiochemical properties. The concentration (IC_{50}) of azoles needed to inhibit BNR processes by 50% is calculated. Different BNR processes showed various responses to azoles after short-term exposure (<24h). Pyrazole (PA), triazole (TA), BTA, and methyl-benzotriazole (MBTA) at 6 mg /L caused >90% inhibition of nitrification activity, while higher inhibition resistance to these compounds was observed in the denitrification process with the calculated IC_{50} (mg /L) of 126, 520, 412, and 152, respectively. In comparison, 50% inhibition of Anammox activity was observed at the concentration of BTA (20 mg /L) and MBTA (18 mg /L). The differences in azole inhibition were suspected to be related to BNR processes' characteristics including the potential chelation of azoles with enzyme-bound copper (e.g., ammonia monooxygenase, AMO) in nitrifiers, high biodiversity of denitrification sludge, and unique cell structure (e.g., anammoxosome) in Anammox bacteria. In addition, azoles with more functional

groups and/or complicated structures (e.g., climbazole at 20 mg /L & fluconazole at 100 mg /L) exhibited less inhibition on the nitrification (PA and TA at 6 mg/L) and Anammox processes (BTA and MBTA at 20 mg/L). In WWTPs, wastewater contains a variety of different compounds and it is more difficult for azoles with complex structures to chelate with the key BNR enzymes (Kalyani Vikas Jog, 2021). More attention should be paid to azoles with smaller molecule sizes and simpler chemical structures such as PA, BTA, TA, and MBTA.

Compared with short-term experiments (<24 hours), results from long-term exposure (3 to 9 months) of BNR processes to azole provide further insights into understanding the impacts of azole and the system's response under a condition that is closer to practical. Azoles, such as BTA and MBTA, have been found to cause inhibition (>50%) of the Anammox process in short-term experiments at 20 and 18 mg/L levels, separately. However, the long-term influence of azoles, whether inhibitory or not in short-term experiments, on the Anammox process is not well studied. Therefore, in the second part of this work, we aimed to investigate the long-term impact of BTA and PA on the Anammox process and the potential mechanisms by which Anammox bacteria adapt to resist azole inhibition. Through long-term acclimation, the Anammox granular sludge could gain higher resistance to BTA below 30 mg/L. However, further exposure to higher BTA concentrations (40 mg/L) led to a gradual decrease in NAA from 70% to 40%. No inhibition $\geq 10\%$ was observed during short-term exposure at 300 mg/L PA, but long-term exposure to 200 mg/L resulted in more inhibition than short-term exposure. While long-term exposure to 100 mg/L PA did not cause any decrease in Anammox activity, 200 mg/L PA led to 65% reversible inhibition in 40 days. To further investigate the potential mechanisms by which Anammox granular sludge adapts to resist azole inhibition, starvation experiments, ATP content analysis, and microbial composition analysis were conducted. BTA and MBTA were selected as representatives of azoles in the starvation experiment due to their reported inhibitory effects on Anammox granular sludge. Over a 28-day starvation experiment, Anammox activity decreased, and the lag phase increased with starvation time at substrate conditions of 75 mg/L NH_4^+ -N and 100 mg/L NO_2^- -N. However, the presence of BTA and MBTA in the starved

Anammox sludge did not result in a further reduction of Anammox activity and change in Anammox sludge's ATP content levels, suggesting that the short-term energy-required mitigation method may not be the major defense mechanism for Anammox bacteria resisting BTA and MBTA inhibition. Overall, the results indicate that a stepwise acclimation strategy could enhance Anammox resistance to azole inhibition.

As an important pre-step to conventional (nitrification/denitrification) and advanced (Anammox) BNR, the long-term impacts of azoles on the nitrification process were studied using lab-scale sequencing batch reactors (SBR). BTA and PA were selected as two representatives of the azoles due to their wide usage, detection in wastewater, and their structure as benzene-containing triazoles and diazole. Adapted nitrification sludge had better performance in treating wastewater containing azoles. Before acclimation, the addition of 2.5 mg/L BTA and PA decreased 45% and >90% nitrification activity, respectively. After the acclimation with the stepwise increase strategy, no ammonium and nitrite accumulation were observed in the effluent when 2.5 mg/L BTA and 2.7 mg/L PA were added into the system. The normalized nitrification activity (NNA) of the BTA and PA groups were $72.5 \pm 2.5\%$ and $70.2 \pm 2.5\%$, respectively, which were also higher than the non-adapted nitrification sludge in short-term tests. The nitrification sludge developed BTA degradation ability and high degradation rates were achieved during the acclimation process. The removal of N loading didn't impact the BTA degradation process. The addition of an extra 50 mg/L COD increased the BTA removal rate while the extra 200 mg/L COD decreased the BTA removal rate. According to the results, the heterotrophic bacteria that existed in the nitrification sludge may contribute to the BTA degradation. Reported aromatic compound/organic compound degraders such as *Dechloromonas* and *Zoogloea* were identified in the microbial community.

Azoles showed a variable inhibition to denitrifying microorganisms in activated sludge batch tests. In the last part of this work, the long-term inhibition potentials of BTA and PA to the denitrification process were investigated. A stable denitrification process was observed with BTA and PA addition at 2.5 and 2.7 mg/L, respectively, suggesting the low risk of azole inhibiting the denitrification process in WWTPs. No

BTA and PA degradation was observed within the 12-hour operation cycle. However, after increasing the reaction time to 7 days, more than 85% BTA and 45% PA degradation are observed during the process. Furthermore, all the denitrification reactors (Control, BTA, and PA) were found to be able to degrade the BTA and PA at similar degradation rates. The removal of N and organic content (acetate as COD source) loading didn't impact the BTA and PA degradation process. Multiple potential aromatic compound/organic compound degraders were found in the denitrification reactor such as *Thauera*, *Azoarcus*, *Georgfuchsia*, and *Dechloromonas*. Further investigation is needed to examine the contribution of individual species to azole degradation and their synergistic relationship.

The results of this work indicate that the presence of azoles in wastewater has the potential to adversely impact the BNRs in WWTPs, in particular those to which azoles are new. However, proper implementation of the acclimation strategy can enhance the resistance of BNR bacteria to azole inhibition, preventing the system from interruption, even failure, caused by azoles. The synergistic effect of the microbial community may contribute to the attenuation of the azole inhibition to the system. The results provide new insights into understanding how the BNRs respond to chemicals of emerging concerns and are expected to assist WWTP operators in developing effective strategies to recover the system from azole inhibition. This work could provide suggestions for WWTPs to maintain activity and efficiency when treating azole-containing wastewater, identify azole types with high potential risks, and understand which part of the wastewater treatment systems may be impacted.

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CHRONIC IMPACTS OF AZOLES ON BIOLOGICAL NITROGEN REMOVAL PROCESSES

by

Xiaojuan Chen

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Advisory Committee:

Professor Guangbin Li, Chair

Professor Allen P. Davis

Professor Birthe Veno Kjellerup

Professor Heyang Yuan

Professor Stephanie A Yarwood

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Figure 6.3 BTA group N removal performance during operation. (A) Influent and effluent N species; (B) TNRR, NLR, OLR, and ORR (C)NRE, NAR, and ORE: Organic -removal efficiency.

Figure 6.4 PA group N removal performance during operation. A) Influent and effluent N species; (B) TNRR, NLR, OLR, and ORR (C)NRE, NAR, and ORE: Organic -removal efficiency.

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Figure 6.10 Relative abundance of dominant bacteria at genus level in denitrification samples

List of Abbreviations

Anaerobic ammonium oxidation (Anammox)

Ammonium-oxidizing bacteria (AOB)

Ammonia monooxygenase (AMO)

Ammonium loading rate (ALR)

Ammonium removal rate (ARR)

Ammonium removal efficiency (ARE)

Biological N removal (BNR)

Benzotriazole (BTA)

Emerging organic contaminants (EOCs)

Concentration of inhibitor to cause 20% activity decrease (IC₂₀)

Concentration of inhibitor to cause 50% activity decrease (IC₅₀)

Concentration of inhibitor to cause 80% activity decrease (IC₈₀)

Continuing calibration verification (CCV)

High performance liquid chromatography (HPLC)

Methyl-benzotriazole (MBTA)

Nitrogen (N)

Normalized activity (NA)

Normalized Anammox activity (NAA)

Normalized denitrification activity (NDA)

Normalized nitrification activity (NNA)

Nitrate production/ ammonium consumption ratio (R_p)

Nitrate production rate (NPR)

Nitrate removal efficiency (NRE)

Nitrite accumulation ratio (NAR)

Nitrite oxidizing bacteria (NOB)

Nitrite consumption/ammonium consumption ratio (R_s)

Nitrite removal rate (NRR)

Nitrogen loading rate (NLR)

Wastewater treatment plants (WWTP)

Operational taxonomical units (OTUs)

Organic loading rate (OLR)

Organic removal rate (ORR)

Pyrazole (PA)

Partial nitrification/Anammox (PNA)

Partial denitrification Anammox (PdNA)

Specific Anammox activity (SAA)

Specific denitrification activity (SDA)

Specific nitrification activity (SNA)

Sequencing batch reactors (SBR)

Total nitrogen removal rate (TNRR)

Total suspended solids (TSS)

Triazole (TA)

Expanded granular sludge bed reactor (EGSB)

Volatile suspended solids (VSS)

Chapter 1: Introduction

Introduction of BNR processes in wastewater treatment

Nitrogen (N) in wastewater presents in the forms of ammonia, nitrite, nitrate, and/or organic N.

Ineffective treatment of N-containing wastewater could cause eutrophication when wastewater is discharged into water bodies and threaten the safety of the ecosystem and water quality for humans (Hu et al., 2019). Various biological N removal (BNR) processes, including nitrification, denitrification, and Anammox, have been successfully applied in wastewater treatment plants (WWTPs) (B. Ma et al., 2016). Those BNR processes require different substrates, and environments and rely on different reactions for energy. **Table 1.1** synthesizes the common properties and differences of the BNRs applied in WWTPs.

Table 1.1 Common properties and differences of BNRs

	Anammox	Nitrification	Denitrification
Oxygen demand	Anaerobic	Aerobic	Anaerobic
Common metabolism	Autotrophic	Autotrophic	Heterotrophic
Substrates	Electron donor: NH_4^+ Electron acceptor: NO_2^-	Electron donor: NH_4^+ Electron acceptor: O_2	Electron donor: Organic content Electron acceptor: NO_3^-
Intermediates & final product	N_2	NO_2^- , NO_3^-	N_2
Redox condition	-110 to +350 mv	-110 to +110 mv	-50 to +50 mv

*(Environmental, 2008; Gerardi, 2003; Koeve et al., 2010; van Niftrik et al., 2012; Viet et al., 2008)

Nitrification and denitrification are two biological processes that have been used for N removal in WWTPS, which are more effective and cost-effective than physicochemical processes such as ammonia stripping and selective ion exchange (Ge et al., 2015). During the nitrification process, ammonium is firstly oxidized to nitrite and then converted to nitrate under aerobic conditions.

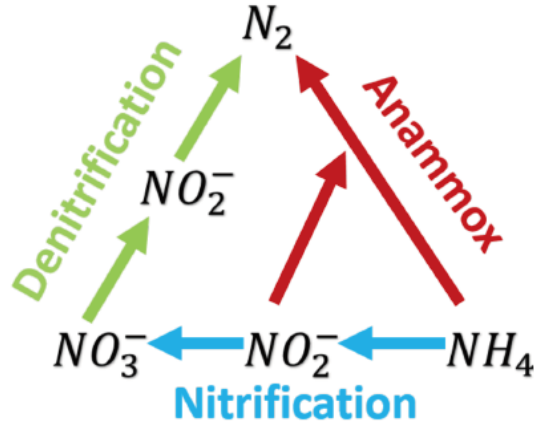
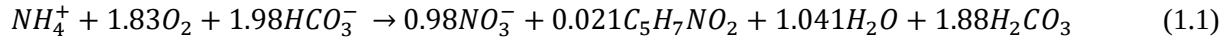


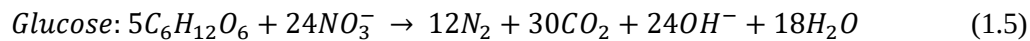
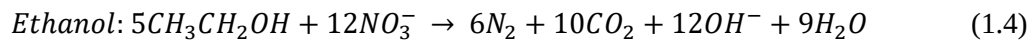
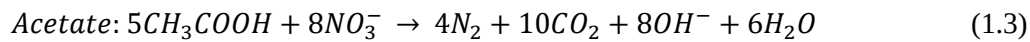
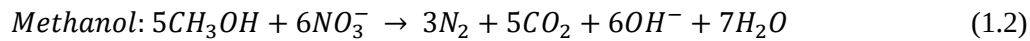
Figure 1.1 Anammox, nitrification and denitrification processes

The overall reaction for the metabolism of *Nitrosomonas* and *Nitrobacter*, which are two typical nitrifiers commonly found in the WWTPs, is presented below (Eq 1.1) (Henze et al., 1997):

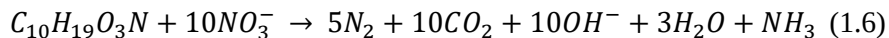


In which the $C_5H_7NO_2$ is used to represent the synthesized bacterial cell.

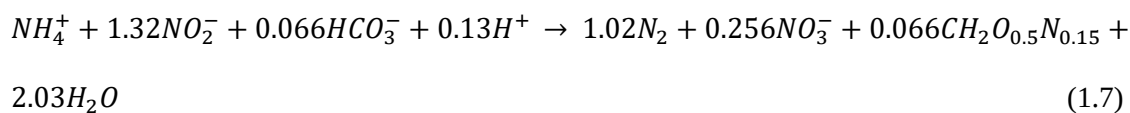
During the denitrification process, nitrate is reduced to molecular dinitrogen gas under anaerobic conditions. Various denitrifiers use different carbon sources for the denitrification process. The correlated stoichiometry equation of the denitrification process also differs from each other. The equations of some common electron donors are shown below (Eq 1.2 to Eq 1.5) (Mokhayeri, 2010; Tchobanoglous et al., 2003):



In the WWTPs, the formula $C_{10}H_{19}O_3N$ is often used to represent the biodegradable organic matter in wastewater and the reaction could also be described as below (Eq 1.6) (EPA, 1993; Mokhayeri, 2010):



The Anammox process is promising biotechnology for N removal in WWTPs. In the Anammox reaction (Eq. 1.7), ammonium and nitrite are converted to dinitrogen gas and nitrate simultaneously (Strous et al., 1998). Compared to traditional nitrification and denitrification, the Anammox process has unique advantages including energy saving (no aeration), less operation cost for chemical addition (no external carbon source requirement) and biosolids management (low biomass yield), and reduction in greenhouse gas emission (Ni et al., 2013). The Anammox process has been successfully applied in treating various N-laden wastewater (e.g., sludge digester liquids, landfill leachate, black wastewater, pharmaceutical wastewater, etc.) (J. Li et al., 2018; Lotti et al., 2015).



Sources and occurrences of azoles in environments

Azoles, classified as emerging organic contaminants (EOCs), are a group of man-made chemicals containing N atoms with heterocyclic ring systems. Azoles have been widely applied in aircraft de-icing agents, semiconductor manufacturing, and household dishwashing detergents (G. Li et al., 2020). Besides, more than 25 azole compounds have been used as biocides/fungicides in agriculture for more than 4 decades (Jørgensen et al., 2021). Azoles have also been used in daily necessities and pharmaceutical products. Climbazole is used at 15 g/L levels as an ingredient of antidandruff shampoo and up to 1000 and 3800 tons of climbazoles were used in Europe and China per annum, respectively (Brienza et al., 2017; Z.-F. Chen et al., 2015). As a result, azoles were reported in different environmental matrices including wastewater, surface water, groundwater, WWTP sludge, and soil. Kahle et al. (2008) reported

that two pharmaceuticals (fluconazole & clotrimazole) and two biocides (propiconazole & tebuconazole) were consistently detected in WWTP influents and lakes. **Table 1.2** synthesized the occurrence of azoles reported in previous literature.

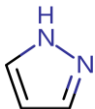
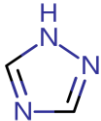
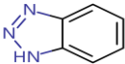
Table 1.2 Reported occurrence of azoles in environmental matrices.

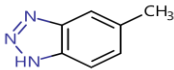
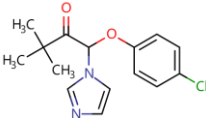
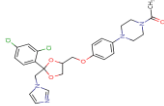
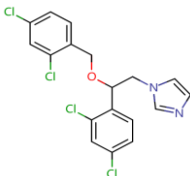
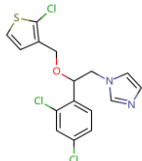
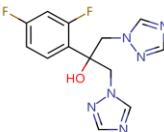
Azoles	Wastewater (µg/L)	Surface water (µg/L)	Ground water (µg/L)	WWTP sludge (µg/kg)	Soil (µg/kg)	Reference
Benzotriazole (BTA)	12-47	0.02 - 33	<0.001 - 1100		0.05 – 4.1	(Careghini et al., 2015; G. Li et al., 2020; McNeill et al., 2009)
Methylbenzotriazole (MBTA)	<0.02 - 3.4	<0.01 - 0.05	<17000		0.36 – 484.19	(Alotaibi et al., 2015; Y.-S. Liu et al., 2013; McNeill et al., 2009)
Pyrazole (PA)	<1020	<0.1	-	-	-	(G. Li et al., 2020; R. Li et al., 2021)
Triazole (TA)	50 - 75	-	<0.01 – 0.23	-	<0.1 – 2.1	(Blank, 2018; G. Li et al., 2020; Rosenbom et al., 2016)
Climbazole	0.44 - 1.4	<0.53	0.02 – 0.37	<1160	-	(Z.-F. Chen et al., 2013; X. Peng et al., 2012; Q.-Q. Zhang et al., 2015)
Miconazole	<0.01 - 1.1	0.01 – 1.45	<0.01 – 0.08	<2069	-	(Z.-F. Chen et al., 2013; X. Peng et al., 2012)
Ketoconazole	0.04 – 0.86	<0.01 – 0.14	0.09 – 0.19	60-4500	-	(Z.-F. Chen et al., 2014; Monapathi et al., 2021; Patel, 2000)
Fluconazole	0.01 - 27	0.11 - 1.06	0.06 - 13.2	<2547	-	(Z.-F. Chen et al., 2014; Monapathi et al., 2021; Patel, 2000)

Physiochemical properties of azoles

Azoles have different structures and physicochemical properties. **Table 1.3** shows the physical-chemical properties of azoles tested in this study. Some azoles such as BTA, MBTA, PA, TA, and fluconazole are soluble in water with low Log P values (partition coefficient of a molecule between octanol and water), which suggests that they prefer to stay in the water phase and have the possibility to reach high concentrations (hundreds mg/L level). For the azoles with higher solubility, they have pK_{a1} (<4.1) and pK_{a2} (>8.5) values, the dominant species of most azoles are the uncharged species at neutral pH. For Climbazole, ketoconazole, miconazole, and tioconazole have low solubilities, their pK_{a1} values are between 6.26 to 6.49. At the operational pH of 7.5 to 7.6 in this study, the percentages of neutral species are more than 90%. Therefore, the results obtained under the tested condition cannot provide information on toxicity impacts due to azole ionization. Previous studies have reported that azoles are resistant to biodegradation, which may result in their presence and/or potential accumulation within WWTPs (Z.-F. Chen et al., 2015). For example, activated sludge showed no degradation for fluconazole and clotrimazole within 24 hours (Kahle et al., 2008).

Table 1.3 Physical chemical properties of azoles

Compound	Structure	Solubility in water (mg/L)	Log P	pK_{a1}	pK_{a2}
PA		72850	0.28	2.67	14.76
TA		241000	-0.10	0.31	9.34
BTA		7570	1.30	0.22	9.04

MBTA		2390	1.82	0.45	9.12
Climbazole		50	4.34	6.49	
Ketoconazole		<1	4.19	6.42	
Miconazole		<1	5.96	6.48	
Tioconazole		<1	5.30	6.48	
Fluconazole		510	0.56	2.3	12.68

*Shown parameters calculated using ChemAxon software. Solubility was calculated at 25 °C. Log P: partition coefficient of a molecule between aqueous and lipophilic phases as octanol and water. P_{ka1} and P_{ka2} represent the P_{ka} values of its protonated and neutral species, respectively.

Current concerns and knowledge gap regarding the impacts of EOCs on the stability of biological processes in WWTPs

The performance and stability of the BNR processes can be impacted by EOCs present in the wastewater. Especially, decreases in performance, even complete failure, of BNR processes have been observed in both lab and full-scale systems when chemical inhibitors enter the system (G. Li et al., 2020; X. Wang et al., 2019). EOCs have raised concerns regarding their inhibition of the BNR processes' efficiency and toxicity to the related microorganisms (D.-Q. Huang et al., 2022). As described in the previous section,

azoles are widely applied in different realms and their potential impact on the efficiency of the WWTPs is not well studied. Azoles such as BTA and MBTA could cause 50% inhibition to the Anammox and nitrification process at 20 and 2.5 mg/L levels, respectively (Lakhey et al., 2020; G. Li et al., 2020). However, the short-term impacts of azoles on denitrification processes and long-term impacts on all the BNR processes are not well investigated. The potential inhibition mechanisms and responses of BNR processes to azole inhibition are not clear. Understanding the short-term and long-term impacts of azoles on BNRs is important and would enhance the stability and efficiency of the N removal system of the WWTPs. At the same time, researchers are seeking methods to prevent or alleviate the inhibition brought by EOCs. However, limited information regarding the methods for alleviating the adverse impact of azoles on BNR processes is available. Adaptation and/or acclimation have been considered a potential method for preventing EOCs inhibition of some biological processes. Development of microorganisms over generations to adapt and/or acclimate to both permanent and transitory changes in environmental conditions has been reported and proved to play a role from the perspective of the survival of species (Biagianti-Risbourg et al., 2013; Morgan-Kiss et al., 2006). Previous studies have reported that adaptation/acclimation could help microbial communities in BNR processes overcome unfavorable conditions such as cold temperatures and chemical inhibitors. When the environmental temperature decreased, Morgan-Kiss et al. (2006) discussed that Anammox bacteria could adapt to the low temperature through the production of antifreeze proteins, kinetic changes in key enzymes, and an increase in the fluidity of cell membranes. The synergistic effect of the BNR microbial community could prevent contact with inhibitors or attenuate the effect of inhibition. Anammox bacteria rely on co-existed nitrifiers such as *N. eutropha* to consume the oxygen transported to the system and maintain an anaerobic environment (S. Liu et al., 2008). Compared to traditional physicochemical pre-treatment, adaptation and acclimation have been considered as a cost-effective method to protect biological processes from adverse impacts (El-Naas et al., 2014; Srivastava et al., 2021). Adapted microorganisms were able to degrade volatile organic compounds such as benzene, toluene, ethyl benzene, and xylene and didn't require any chemical addition or extra facilities construction (El-Naas et al., 2014). Besides, using pre-adapted

microorganisms could improve microorganisms' survival, stability, and performance of biological systems (Megharaj et al., 2011). However, the potential of BNR processes to adapt/acclimate to azole inhibition has not been studied. Therefore, understanding the feasibility of BNR processes in treating azole-containing wastewater is crucial.

Chapter 2: Project Goals

The wide existence of emerging organic contaminants (EOCs) such as azoles in different environment matrices including surface water, groundwater, soil, and especially wastewater are potential risks regarding the stability of biological nitrogen removal (BNR) processes in wastewater treatment. Nevertheless, the understanding of the potential inhibition of BNR processes by EOCs and their underlying mechanisms remains limited. This scarcity of information poses a challenge in formulating comprehensive guidelines to prevent EOC-induced inhibition of BNR processes and lacks sufficient data for devising effective methods for system recovery in such situations. To bridge these knowledge gaps and tackle challenges posed by EOCs in WWTPs, it is important to assess the responses of various BNR processes to both short and long-term exposure to EOCs. Additionally, testing potential strategies to enhance the resistance of BNR processes to inhibition by EOCs is essential for developing effective mitigation measures. In this research, azoles are selected as representative EOCs due to their extensive application, wide detection in wastewater, and potential inhibitory effects on BNR processes. **The overall objective is to improve the stability and performance of BNR systems in WWTPs for treating EOC-containing wastewater.** To achieve these objectives, several sub-tasks were undertaken, as outlined below.

Previous studies examined the impact of azoles on nitrification and anaerobic ammonium oxidation (Anammox), and the results indicated some azole compounds exhibited considerable inhibition on BNR processes. However, the assessment of the variation in the extent of inhibition caused by azoles on different BNR processes remains incomplete (Lakhey et al., 2020; G. Li et al., 2020). **Therefore, the first sub-research question explored the sensitivity of various BNR processes in WWTPs to azole compounds with diverse structures. The objective of Chapter 3 is to assess and compare the effects of acute exposure (short-term, <24 hours) of azoles on various BNR processes. The hypothesis is that the presence of azoles in wastewater may reduce the activity of BNR processes and the adverse impacts of azoles on BNR process performance are contingent upon the specific type of azoles and**

the nature of the BNR process. Understanding the short-term impacts of azoles on BNRs will assist the WWTPs in developing strategies to prevent azole-induced shock loading on BNR processes, thereby ensuring consistent N removal performance.

The integration of the Anammox process has emerged as a viable solution for treating full-scale side streams in combination with traditional nitrogen removal processes, such as the partial nitrification/Anammox process or partial denitrification/Anammox process (Y. Yang et al., 2018). In WWTPs that consistently receive wastewater containing azoles, careful consideration must be given to the potential impact of azoles on the performance of BNR systems operation, as well as on sludge characteristics and microorganisms. Unlike nitrification and denitrification processes, the activity of the Anammox process is highly reliant on substrate conditions. When substrates are limited, the activity and energy status of the Anammox cells may decline. However, the long-term effects of azoles on the Anammox process have not been thoroughly investigated and it remains uncertain whether the Anammox process will rely on energy-intensive methods to mitigate azole inhibition. Consequently, azoles may exert greater inhibition on starved Anammox cells or in environments with lower substrate concentrations. Attention must be directed toward comprehending how azoles affect these critical treatment aspects. **Thus, the second sub-research question studied the impact of azoles on the Anammox integrated BNR system during long-term operation (>3 months). The object of Chapter 4 is to investigate the long-term impact of azoles on the Anammox process under different substrate conditions. The hypotheses are:(1) long-term exposure to azoles would decrease the activity of Anammox granular sludge. (2) The variation of substrate condition will influence the inhibition caused by azoles in the Anammox process.** The anticipated outcomes aim to provide recommendations for sustaining the stability of Anammox-integrated BNR systems when treating azole-containing wastewater over the long-term. Additionally, the analysis will shed light on how the Anammox process may react to azole inhibition across varying nitrogen substrate conditions, including normal, limited, or absent substrate availability.

Nitrification and denitrification processes are the traditional N removal processes and are widely applied in WWTPs. Azoles at low mg levels (<2 mg/L) could cause 50% inhibition of the nitrification process during short-term exposure (G. Li et al., 2020). However, the assessment of long-term inhibition caused by azoles on nitrification was not well investigated. It is important to explore the impact of azoles on the stability of these systems and their microbial composition. Besides, activated sludge systems incorporating nitrification and denitrification processes exhibit higher capability in degrading contaminants. Therefore, the potential of nitrification and denitrification systems to eliminate azoles from wastewater should also be taken into consideration. **The third sub-research question investigated the impact of azoles on the efficiency and stability of nitrification systems in WWTPs. The object of Chapter 5 is to study the long-term impact (>3 months) of azoles on the nitrification processes. The hypothesis is treating azole-containing wastewater will decrease the efficiency and activity of the nitrification system during the long-term exposure process.** Together with the nitrification process or Anammox process, denitrification has been widely applied in WWTPs for water quality improvement (Lu et al., 2014). Similar to the nitrification process, the long-term inhibition caused by azoles on denitrification was not well studied. **The fourth sub-research question discussed the influence of azoles on denitrification performance in WWTPs. The object of Chapter 6 is to study the long-term impact of azoles on the denitrification processes. The hypothesis is Azoles will decrease the denitrification activity during long-term exposure.** The results would further evaluate the influence of azoles on N removal processes and the potential fate of azoles in full-scale systems.

Chapter 3: Assessment of Short-term Impacts of Azoles on Biological Nitrogen Removal Processes in Wastewater

Abstract

The short-term inhibition potential of nine azole compounds (5 diazoles and 4 triazoles) on major biological nitrogen (N) removal (BNR) processes was investigated using both experiment-based lab research and literature review approaches. The half-maximal inhibitory concentrations (IC_{50} s) of azoles are calculated. Different BNR processes showed various responses to azoles after short-term exposure (<24h). Pyrazole (PA), triazole (TA), benzotriazole (BTA), and methyl-benzotriazole (MBTA) at 6 mg /L caused >90% inhibition in nitrification activity, while higher resistance to these compounds was observed in denitrification process with the calculated IC_{50} (mg /L) of 126, 520, 412, and 152, respectively. In comparison, BTA (20 mg /L) and MBTA (18 mg /L). caused 50% inhibition of Anammox activity. The differences in azole inhibition on BNR processes were suspected to be related to inhibition mechanisms and different characteristics of BNR bacteria, including the potential chelation of azoles with enzyme-bound metal cores (e.g., copper in ammonia monooxygenase, AMO), high biodiversity of denitrification sludge, and unique cell structure (e.g., anammoxosome) in Anammox bacteria. In addition, azoles with more functional groups and/or complicated structures (e.g., climbazole at 10 mg /L & fluconazole at 100 mg /L) exhibited less inhibition on the BNR processes when compared to PA, TA, MBTA and BTA.

Introduction

N in wastewater presents itself in the forms of ammonia, nitrite, nitrate, and/or organic N. Ineffective treatment of N-containing wastewater could cause eutrophication when discharged into water bodies and threaten the safety of the ecosystem and water quality for humans (Hu et al., 2019). Various BNR processes, including nitrification, denitrification, and Anammox, have been successfully applied in

wastewater treatment plants (WWTP) (B. Ma et al., 2016). However, the performance and stability of the BNRs could be impacted by a variety of inhibitors that exist within the influence of the wastewater.

Azoles, classified as emerging organic contaminants (EOCs), are a group of man-made chemicals containing N atoms with heterocyclic ring systems and have been widely applied in aircraft de-icing agents, semiconductor manufacturing, and household dishwashing detergents (G. Li et al., 2020). More than 25 Azole compounds have been used as broad-spectrum biocides/fungicides in agriculture for 4 decades, which hold a world market value share of 20-25% (Jørgensen et al., 2021). Azoles have also been used in daily necessities and pharmaceutical products. Climbazole at 15 g/L levels is used as an ingredient in antidandruff shampoo and up to 1000 and 3800 tons of climbazole were used in Europe and China per annum, respectively (Brienza et al., 2017; Z.-F. Chen et al., 2015). Benzotriazoles (BTA) are used as UV stabilizers in plastic products to prevent yellowing (Careghini et al., 2015). As a result of extensive application, azoles were widely detected in different environmental matrices including wastewater, surface water, groundwater, WWTP sludge, and soil. Azoles are resistant to biodegradation during their retention in WWTPs (less than 5% for 2 to 5 days), which may result in their presence and/or potential accumulation within WWTPs (Z.-F. Chen et al., 2015). As fungicides, azoles typically target the CYP51 enzyme in fungi and disrupt cell membrane integrity (Draskau et al., 2022). However, azoles could also cause inhibition in BNR processes. PA and BTA could cause inhibition of nitrifiers in sewage sludge with IC_{50} s of <0.5 mg/L (Pagga et al., 2006). In other studies, azoles were reported to cause 50% inhibition of the Anammox and nitrification process at 18 to 20 mg/L and 2 to 3 mg/L concentration levels, respectively (Lakhey et al., 2020; G. Li et al., 2020). These findings suggest that the presence and accumulation of azole compounds in BNR processes may lead to a decrease in N removal efficiency, potentially resulting in system failure in WWTPs. However, the assessment of the variation in the extent of inhibition caused by azoles of different BNR processes remains incomplete, hindering the development of proper strategies for preventing BNR processes from azole inhibition in WWTPs.

To advance the understanding of the sensitivity of various BNR processes in WWTPs to azole compounds with diverse structures, this work assessed and compared the effects of acute exposure (short term, <24 hours) of different azoles on various BNR processes, including nitrification, denitrification, and anaerobic ammonium oxidation (Anammox). Nine azole compounds were selected for testing to represent azoles based on their widespread application, detection in wastewater, and structural characteristics. Batch bioassays were employed to determine the IC₅₀ values for four selected azoles, including PA, BTA, TA, and MBTA. In addition, the study assessed the extent of inhibition caused by azoles on ammonium and nitrite-oxidizing processes in nitrification, while the potential role of the granular structure of Anammox bacteria in azole inhibition was studied. This allowed researchers to investigate the potential inhibitory mechanisms of azole compounds and the potential defense mechanisms of BNR bacteria. The results are expected to provide insight into the adverse impacts of azoles on BNR process performance. These impacts are anticipated to vary based on the specific type of azoles and the nature of the BNR process. This knowledge will assist the WWTPs in developing effective strategies to prevent azole-induced shock loading on BNR processes, ultimately ensuring consistent N removal performance.

Material and methods

Chemicals and microorganisms

1,2,4-triazole (TA) (CAS # 288-88-0, purity > 98%), N-allylthiourea (ATU) (CAS # 109-57-9, purity > 98%), and 5-methyl benzotriazole (MBTA) (CAS # 136-85-6, purity > 98%) were purchased from Acros Organics B.V.B.A.(Pittsburgh, PA, USA). Benzotriazole (BTA) (CAS # 95-14-7, purity > 99%) and Miconazole (CAS # 22916-47-8, purity > 97%) were purchased from Alfa Aesar (Ward Hill, Massachusetts, USA). Pyrazole (PA) (CAS # 288-13-1, purity > 98%), and fluconazole (CAS # 86386-73-4, purity > 98%) were purchased from Tokyo Chemical Industry (Tokyo, Japan). Tioconazole (CAS # 65899-73-2, purity > 98%) was purchased from Combi-Blocks, Inc (San Diego, CA, USA).

Granular Anammox biomass was obtained from a 3.8 L laboratory-scale Anammox expanded granular sludge bed reactor (EGSB) fed with a synthetic mineral medium at a loading rate of 4.1 g N/L/day. The reactor has been in operation for the last 3 years. The granules contained 0.89 g of volatile suspended solids (VSS) per gram of total suspended solids (TSS) and had a specific Anammox activity (SAA) of 0.55 ± 0.05 g N₂-N/g VSS/day, which is at the same level as other reported work. Q.-Q. Chen et al. (2017) showed that the Anammox activity ranged from 0.47 to 0.65 g N₂-N/g VSS/day.

The suspended sludge used for studying the toxicity of azoles to the nitrification process in this work was activated sludge obtained from an aeration zone of a local municipal WWTP (Laurel, MD, USA), in which the nitrification process was known to happen. All received sludge was immediately stored at 4 °C to maintain the microbial activity. The sludge was collected and stored in a cooler for 1 hour before the bioassay. The sludge was collected and transported to the laboratory on ice within one hour. The specific nitrification activity (SNA) of the collected sludge was in the range of $2.5 - 4.0 \pm 0.5$ mg N/g VSS/hour, which met the required criteria for testing (ISO-8192, 2007; ISO-9509, 2006). The sludge was collected one day before the toxicity bioassays and washed by using chlorine-free tap water. Tap water was collected 24 hours before the experiment and stored in an open container to remove the chlorine. The washing procedure is carried out by centrifuging (1,000 g for 5 min) and discarding the supernatant liquid. Then the sludge was resuspended with chlorine-free tap water and the procedure was repeated two times. The TSS of washed sludge was determined through a standard method (American Public Health Association, 2023). Finally, re-suspend the washed sludge in an appropriate volume of chlorine-free tap water to give the TSS of 3 g/L and aerate until use.

The sludge used for studying the toxicity of azoles to the denitrification process in this work was activated sludge obtained from the anoxic zone of the same WWTP located in (Laurel, MD, USA), in which denitrification was known to be occurring. The procedure of collection, washing, and storage was identical to the nitrification process except the denitrification sludge was flushed with N₂ and stored in anoxic condition after the washing procedure. The denitrification sludge had a specific denitrification

activity (SDA) of 0.26 ± 0.05 g N₂-N/g VSS/day, which was also within the ranges of other reported work. (Cui et al., 2019; J.-J. Wang et al., 2021).

Experiment setup.

BNR toxicity bioassay

Inhibitory effects of selected azole compounds of different BNR processes were tested by following established methods in previous work (Lakhey et al., 2020; G. Li et al., 2020; Sánchez et al., 2000) with minor modifications (shown in **Table 3.1**). The basal medium for the bioassay was prepared using ultrapure water from Hydro AWS 200 (Hydro, Durham, NC, USA). The 118 ml serum bottles were used for Anammox and denitrification experiments, and 250 ml conical flasks were used for the nitrification bioassays. The toxicity bioassays were carried out in triplicates for the treatment groups and control groups. The tested azoles and their concentrations for the Anammox, nitrification, and denitrification processes were selected based on a literature review and preliminary scoping tests as described in **Table S1**. For azoles with lower solubility (≤ 1 mg/L) in water, including climbazole, ketoconazole miconazole, and tioconazole. Dimethyl sulfoxide (DMSO) was used as a co-solvent to prepare azole stock solutions, which was reported to be a suitable co-solvent for azoles such as fluconazole, miconazole, and ketoconazole for improving their solubility in water (Alshehri et al., 2021; Cayman-Chemical, 2022a, 2022b; Hashemzadeh et al., 2015; Vandebosch et al., 2010). A specified volume of the azole stock solution was introduced into the batch experiment to attain the desired test concentrations in the bioassays (shown in **Table S2**). The test concentrations selected were also below the solubilities reported in other studies (Z.-F. Chen et al., 2015; da Silva et al., 2015; Richter et al., 2013). The impact of DMSO on the BNR processes was examined and the results indicated no inhibition ($\geq 10\%$) in the presence of up to 275 mg DMSO/L. This concentration represented the maximum DMSO level used in this study (shown in **Table S1**).

Table 3.1 Operation parameters of BNR batch experiments

Parameters	Anammox	Nitrification	Denitrification
Reactor volume	118 ml serum bottles	250 ml conical flask	118 ml serum bottles
Liquid phase volume	60 ml	80 ml	60 ml
Sludge Addition	Granular sludge at 0.7 g VSS /L	Suspended sludge at 0.7 g VSS /L	Suspended sludge at 1.5g VSS/L
Dissolved oxygen level (DO)	<0.5 mg/L	> 4 mg/L	<0.5 mg/L
Temperature	30 ± 1 °C	22 ± 2 °C	37± 1 °C
Light environment		Dark or in diffused light	
Shaking speed	120 rpm	120 rpm	180 rpm
pH	7.2 ± 0.1	7.6 ± 0.1	7.5 ± 0.1
Substrates	NH ₄ ⁺ -N(75mg/L), NO ₂ ⁻ -N (100 mg/L)	NH ₄ ⁺ -N (56 mg/L)	NO ₃ ⁻ -N (50 mg/L), CH ₃ COO ⁻ -C (200mg/L)
Medium	NaH ₂ PO ₄ ·H ₂ O (58 mg/L), CaCl ₂ ·2H ₂ O (100 mg/L), MgSO ₄ ·7H ₂ O (200 mg/L), NaHCO ₃ (50 mg/L)	NaCl (184 mg/L), NaH ₂ PO ₄ ·H ₂ O (72 mg/L), CaCl ₂ ·2H ₂ O (5 mg/L), MgSO ₄ ·7H ₂ O (34 mg/L), KCl (29 mg/L)	MgSO ₄ ·7H ₂ O (90 mg/L), CaCl ₂ ·2H ₂ O (14 mg/L), KCl (36 mg/L), Yeast extract (1 mg/L)
Trace element solution*	FeSO ₄ ·7H ₂ O (9.15 mg/L), EDTA (19.75 mg/L), ZnSO ₄ ·7H ₂ O (0.43 mg/L), CoCl ₂ ·6H ₂ O (0.24 mg/L), MnCl ₂ (0.63 mg/L), CuSO ₄ ·5H ₂ O (0.25 mg/L), Na ₂ MoO ₄ ·2H ₂ O (0.22 mg/L), NiCl ₂ ·6H ₂ O (0.19 mg/L), Na ₂ SeO ₄ ·10H ₂ O (0.21 mg/L), H ₃ BO ₃ (0.01 mg/L), NaWO ₄ ·2H ₂ O (0.05 mg/L)	FeCl ₃ ·6H ₂ O (0.45 mg/L), EDTA (3 mg/L), ZnSO ₄ ·7H ₂ O (0.04 mg/L), CoCl ₂ ·6H ₂ O (0.05 mg/L), MnCl ₂ (0.04 mg/L), CuSO ₄ ·5H ₂ O (0.01 mg/L), Na ₂ MoO ₄ ·2H ₂ O (0.02 mg/L), H ₃ BO ₃ (0.05 mg/L), KI (0.05 mg/L)	FeSO ₄ ·7H ₂ O (22 mg/L), EDTA (50 mg/L), ZnSO ₄ ·7H ₂ O (22 mg/L), CoCl ₂ ·6H ₂ O (1.61 mg/L), MnCl ₂ (5.06 mg/L), CuSO ₄ ·5H ₂ O (1.57 mg/L), (NH ₄) ₆ Mo ₇ O ₂₄ ·4H ₂ O (1.1 mg/L),
Buffer system	HEPEs buffer	Carbonate buffer	Phosphate buffer

*Prepared according to (van Loosdrecht et al., 2016)

Anammox granular structure disintegration bioassay

To investigate the influence of granular structure on Anammox sludge's resistance to azoles, batch experiments were set up with granular and suspended Anammox sludge. Suspended Anammox sludge was prepared by crushing granular Anammox sludge through syringe needles (20 G, 0.60 mm I.D.) three times. Then the Anammox sludge was suspended in no nitrogen substrate medium as described in **Table**

3.1 for use, while the other portion was left intact. The tested azole concentrations were 20, 250, 250, and 18 mg/L for BTA, PA, TA, and MBTA according to their reported IC_{50} in the Anammox process, respectively (Lakhey et al., 2020). Other parameters used in the batch test are identical to the conditions described in the BNR bioassay.

Nitrification ammonium oxidation bioassay

Nitrification is a two-step process where ammonia is first oxidized to nitrite by ammonia-oxidizing bacteria (AOB) and/or archaea (AOA), and subsequently to nitrate by nitrite-oxidizing bacteria (NOB). To investigate the potential inhibition of azoles on AOB/AOA and NOB, the same amount (56 mg N /L) of ammonium and nitrite was respectively offered in the medium for the nitrification reactors identical to the previous batch bioassays. The tested azole concentrations were 2, 2.7, 3.5, and 2.2 mg/L for BTA, PA, TA, and MBTA according to their reported IC_{50} in the nitrification process, respectively (G. Li et al., 2020). Other parameters used in the batch test are identical to the conditions described in the BNR bioassay.

Analytic methods

The N_2 gas was analyzed using an Agilent 6890 gas chromatograph (GC) (Agilent Technologies, Santa Clara, CA, USA) fitted with an HP-PLOT Molesieve column (30 m $\times$ 0.32 mm, Agilent Technologies) and a thermal conductivity detector. The temperatures of the column, the injection port, and the detector were 40, 200, and 220 °C, respectively. Helium was used as the carrier gas and the injection volume was 100 μ L. Injections were carried out using a Hamilton sample-lock 1710 SL syringe (Hamilton Company, Reno, NV, USA). Nitrate and nitrite in liquid samples were analyzed by Dionex ICS-1100 ion chromatography. Ammonium was measured using an IntelliCAL Ammonia (NH_3) Ion Selective Electrode (ISENH4131, Hach, Loveland, CO, USA). During the measurement, the target compounds with known concentrations were added for continuing calibration verification (CCV) to every 10 samples for quality control. Less than 10% differences between the measured concentrations and the known concentrations were required to accept the measurement results.

DO in liquid samples was measured using an optical O₂ probe (LBOD101 Sensor, Hach, Loveland, CO, USA). Other parameters (TSS, VSS and pH) were determined according to standard methods in triplicate (American Public Health Association, 2023). The limit of detection (LOD) and limit of quantitation (LOQ) of the analytical instruments are listed in **Table S6**.

Data processing

The SNA representing nitrifying rate was calculated by collecting and analyzing the N species at 3.5, 4.25, 5, and 5.75 hours according to previous work (G. Li et al., 2020). The SNA in mg N/g VSS/hour was calculated production of total oxidized nitrite and nitrate as described in the method. Specific Anammox activities (SAA) and specific denitrification activity (SDA) were calculated as the rate of N₂ production per g VSS and were expressed as g N₂-N/g VSS/day. The rate was calculated using linear regression of five or more points. The regression analysis was carried out using Microsoft Excel. The normalized activity (NA) of BNRs was calculated as shown in Eq 3.1:

$$NA(\%) = \frac{SA_t}{SA_c} * 100 \quad (3.1)$$

where SA_t and SA_c (both as g N₂-N/g VSS/day) are the specific activities of the treatment (with azole addition) and control experiment (without azole addition) of the BNRs, respectively.

The NA results were fitted to an empirical inhibition model (Eq 3.2) to determine the IC₅₀ value of the BNR process (G. Li et al., 2020).

$$NA = \frac{NA_{max}}{1 + (I/K_i)^n} \quad (3.2)$$

where NA_{max} is the maximum NA (%), I and K_i are the inhibitor concentration and half inhibition constant, respectively (mg/L), and n is the inhibition order (dimensionless). The value of K_i is equal to IC₅₀. Nonlinear fittings of the data to Eq 3.2 were performed using least-squares minimization of the error using the software OriginPro 9.1 (OriginLab, Northampton, MA, USA).

The t-test was used for statistical analysis of the relationship between azole concentration and the activity of the BNR processes in the batch reactor. A p -value less than 0.05 was required to reject the null hypothesis.

Results and discussion

Assessment of azole inhibition in BNR processes

Anammox

Anammox process anaerobically converts ammonium into N_2 using nitrite as the terminal electron acceptor. It has been widely considered/used as an environmentally sustainable and cost-effective BNR process for treating wastewater, especially ammonia-rich wastewater with low organic matter composition. Previous work examined the acute toxicity of different azole compounds on Anammox granular sludge and concluded high toxicity in MBTA and BTA, and low toxicity in PA and TA. The multilayered structure of Anammox granules has been reported to protect the Anammox bacteria from inhibitors such as nanoparticles and organic matter stress (C. Chen et al., 2021; Z.-Z. Zhang et al., 2017). Prior to the experiments for assessing the performance of Anammox granular and suspended sludge in the presence of azoles, the inhibition potential of four azoles, including PA, TA, BTA, and MBTA, was confirmed in preliminary tests using the selected concentrations (shown in **Table 3.2**). The results indicated that PA and TA at 250 mg/L caused $\leq 25\%$ inhibition of the Anammox process, respectively, while 20 mg/L BTA and 18 mg/L MBTA caused approximately 50% inhibition in Anammox activity. The results align with the previous study (Lakhey et al., 2020).

Table 3.2 Inhibition of tested azoles on NAA

Compounds	Anammox	
	Test concentration (mg/L)	Inhibitory effect (%)
PA	250	13 ± 8
TA	250	NI
BTA	250	87 ± 4
	20	48 ± 1
MBTA	250	94 ± 3
	18	55 ± 6

NI: No $\geq 10\%$ inhibition observed

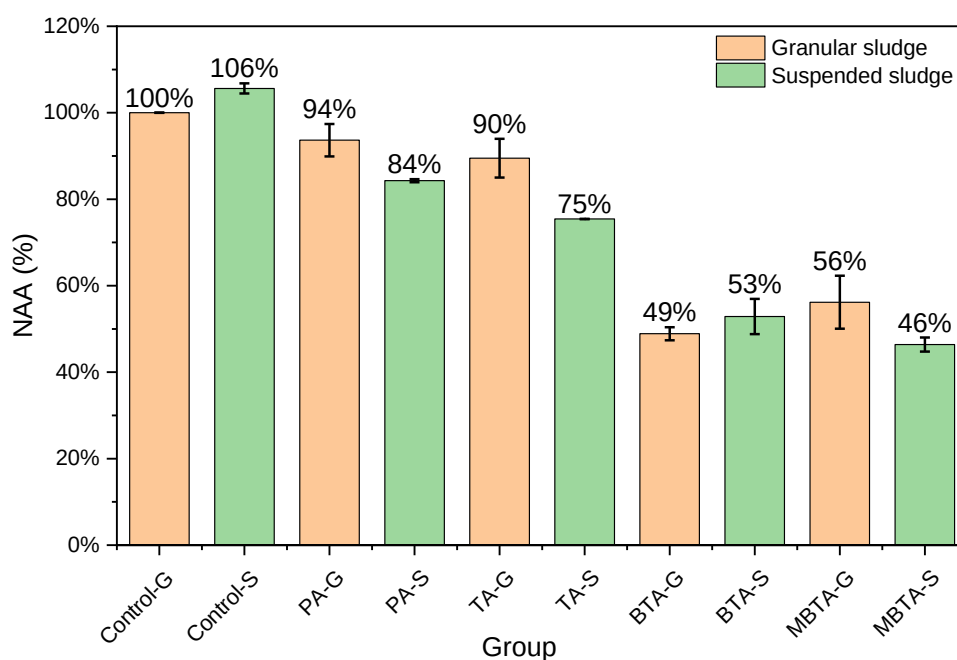


Figure 3.1 Normalized Anammox activity (NAA) of Anammox granular and suspended sludge exposed to different azole compounds. The tested azole concentrations were 250, 250, 20, and 18 mg/L for the PA, TA, BTA, and MBTA, respectively. G represents granular sludge; S represents suspended sludge. Error bar represents the standard deviation of duplicates.

These azoles (i.e., 250 mg/L PA, 250 mg/L TA, 20 mg/L BTA, and 18 mg/L MBTA) were then selected for testing the response of Anammox granular and suspended sludge and the results are illustrated in

Figure 3.1. The Anammox granular sludge exhibited a specific Anammox activity (SAA) of

0.46 ± 0.01 g N₂-N/g VSS/day without the addition of azoles, comparable to the activity of 0.48 ± 0.05 g N₂-N/g VSS/day in Anammox suspended sludge without azole addition. These results indicated a non-significant ($p > 0.05$) impact resulting from the granular crushing procedure used in this work. Compared with the control groups without azole addition, a decrease in Anammox activity was observed in both Anammox granular and suspended sludge with the addition of PA and TA. While the activity in the Anammox suspended sludge groups appeared lower than that in the Anammox granular sludge, statistical analysis revealed that the observed change is not significant ($p > 0.05$). Similar results were obtained in the groups with BTA and MBTA. These results suggested that granular structure did not provide additional protection for Anammox bacteria from the inhibition caused by azoles. Similar results were reported in other work. Our previous work observed a similar inhibitory impact of crude glycerol and methanol on the activity of Anammox granular and suspended sludge (X. Chen et al., 2024).

Nitrification

The nitrification process is carried out by the AOB/AOA together with the NOB, which converts the ammonium to nitrite and nitrite to nitrate, respectively (S. Li et al., 2021). Compared with the Anammox process, the nitrification process was reported to be more sensitive to azole inhibition at low mg/L levels (G. Li et al., 2020). The results showed that four azole compounds caused severe inhibition (>90%) in the nitrification process at concentration levels ranging from 6 to 8 mg/L, which is two orders of magnitude lower than that observed in Anammox bioassays (**Table 3.2**). This is consistent with the previous work (G. Li et al., 2020). However, which step of the nitrification process was the most inhibited was not clear. Thus, in this work, the influence of azoles on AOB/AOA and NOB was investigated, and the results are illustrated in **Figure 3.2**.

Table 3.3 Inhibition of tested azoles on NNA

Compounds	Nitrification	
	Test concentration (mg/L)	Inhibitory effect (%)
PA	6	96 ± 1
TA	6	99 ± 1
BTA	7.5	99 ± 1
MBTA	6	96 ± 1

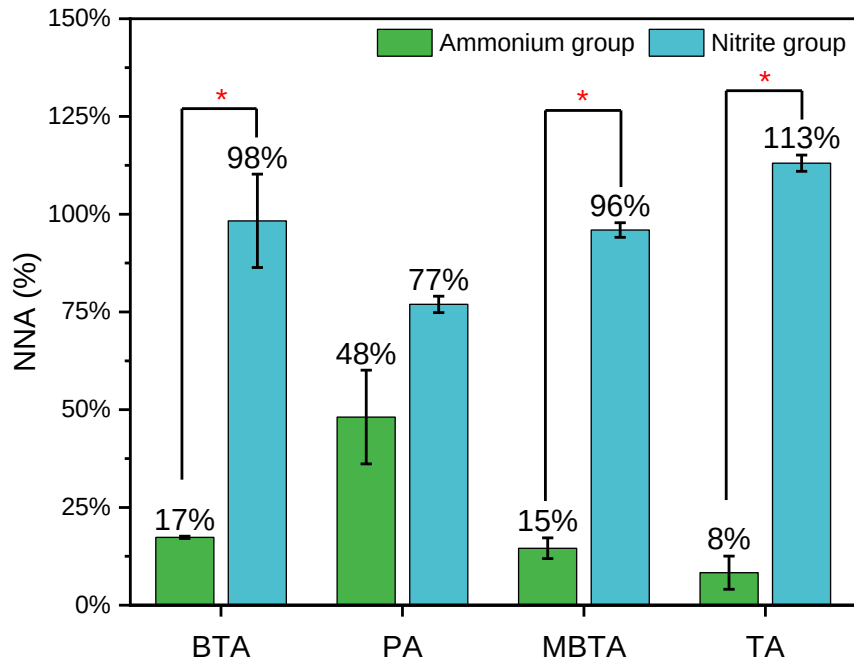


Figure 3.2 Normalized nitrification activity (NNA) of azole toxicity bioassays with ammonium or nitrite added as sole substrate. The tested azole concentrations were 2.7, 3.5, 2.0, and 2.2 mg/L for PA, TA, BTA, and MBTA, respectively. The asterisk (*) indicated the p value of t-test. *: $p \leq 0.05$. Error bar represents the standard deviation of duplicates.

The nitrification activity in the control group, without the addition of azoles, was 3.72 ± 0.3 mg N/g VSS/hour with ammonium and 2.62 ± 0.2 mg N/g VSS/hour with nitrite added as the sole substrate. This indicated the presence of active nitrifiers in the sludge for this test, and there was no observed negative impact on nitrification activity caused by nitrite addition. In the bioassays with the addition of azoles, 2.7, 3.5, 2.0, and 2.2 mg/L for PA, TA, BTA, and MBTA were selected as they were estimated to cause 50% inhibition of nitrification activity by previous work (G. Li et al., 2020). As shown in **Figure 3.2**, a more

pronounced inhibition was observed in the groups except PA with ammonium compared to those with nitrite added as the sole substrate ($p < 0.05$). For the PA group, although higher activities in the nitrite group were observed, the results were not statistically significant ($p = 0.21$). According to **Figure 3.2**, the ammonium oxidation process is more sensitive to azoles compared with the nitrite oxidation process. NAA calculated in the Ammonium groups for TA, BTA, and MBTA significantly decreased ($p < 0.05$) by 83-92%. This finding supported the hypothesis that azoles may impair nitrification by interrupting the enzyme functions (e.g., AMO) related to the ammonium oxidation process (G. Li et al., 2020).

Denitrification

Denitrification plays a significant role in removing N from wastewater through the biological conversion of nitrate to nitrogen gas involving a diverse group of microorganisms. Limiting information is available about the inhibition potential of azoles in the denitrification process in wastewater treatment. Therefore, toxicity bioassays were performed with the four selected compounds over a concentration range of 0-250 mg/L, and the results were presented in **Figure 3.3**. Compared to the anammox and the nitrification process, the denitrification process was less sensitive to azole inhibition. PA and MBTA have higher inhibition effects (70% and 58% inhibition at 250 mg/L, respectively) than the TA (37% inhibition at 250 mg/L) and BTA (31% inhibition at 250 mg/L). Azoles at low concentrations (<50 mg/L) may not cause serious loss in denitrification activity (>20%).

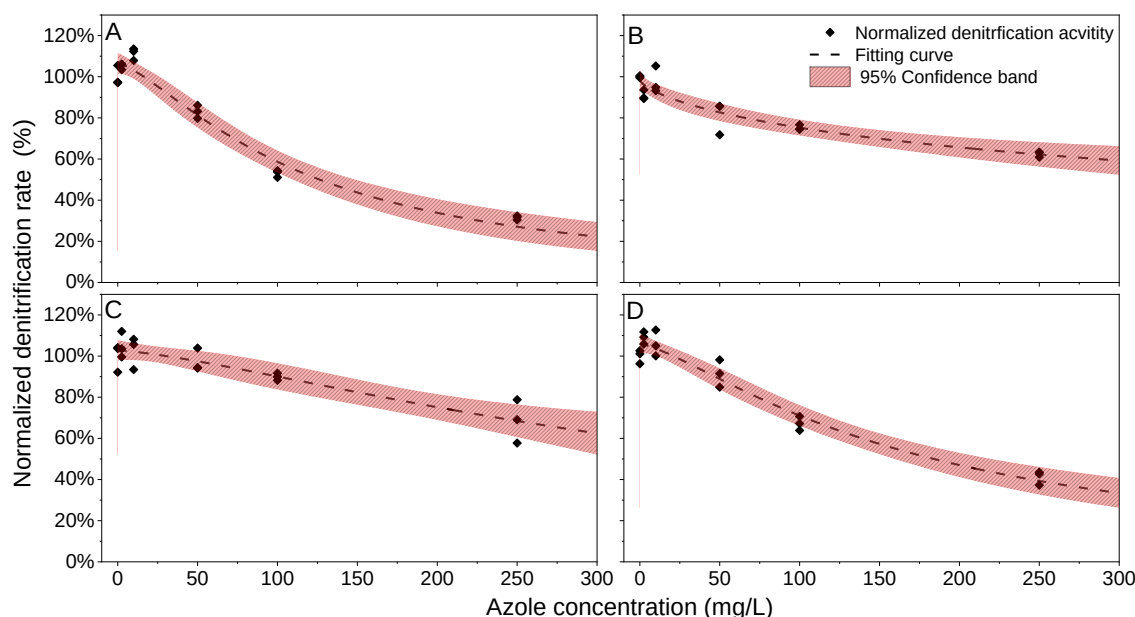


Figure 3.3 Inhibitory impact of PA (A) TA (B) BTA (C) and MBTA (D) on the denitrification activity at various concentrations. Denitrification sludge was obtained from a local municipal WWTP (Laurel, MD, USA). The dash lines show the fit of the experimental data to an empirical inhibition model (Eq.3.2), while shadow area represents the 95% confidence range. All experimental groups were conducted in triplicate. Statistical analysis ($p < 0.05$) revealed that the observed differences between control group and treatment group are significant.

From the previous results, the four azole compounds have different inhibitions on BNR processes. As discussed before, azoles have a lot of derivatives synthesized from the four basic azoles tested in this study. Some of the derivatives have more complex structures and how they would impact the BNR processes is still unknown. The following research question is whether azoles with more complex molecule structures could inhibit the BNR processes. In order to understand the impact of azole structure, six other azole compounds were tested in this study through the screening test. The results are included in **Table 3.4**. Climbazole, miconazole, and tioconazole present less than 25% inhibition to the Anammox process. The nitrification process and denitrification process are nearly not impacted (inhibition < 10%) at the tested concentrations. However, the existence of those azoles in the WWTPs should still raise concerns. The hydrophobic characteristics of those azoles lead to concerns about the potential bioaccumulation in the system and could result in exposure to higher concentrations (Bhagat et al., 2021). Miconazole, clotrimazole, and ketoconazole have been detected in the bed sediment of Pearl River Delta

up to 76.9 ng/g dry weight, which indicates that high Log P azoles are more likely to accumulate in the environment (Q. Huang et al., 2013).

Table 3.4 Inhibition of selected azoles on normalized activities of BNR processes

Compounds	Test Concentration (mg/L)	Observed inhibition		
		Anammox process Inhibition (%)	Nitrification process Inhibition (%)	Denitrification process Inhibition (%)
Climbazole	20	12 ± 7.0%	0 ± 3.4%	6 ± 1.2%
Ketoconazole	1	1 ± 3.3%	6 ± 6.2 %	1 ± 3.3%
Miconazole	1 (0.8) ^a	22 ± 13.5%	3 ± 9.5%	2 ± 2.1%
Tioconazole	10	17 ± 12.9%	4 ± 15.3%	7 ± 1.2%
Fluconazole	100	1 ± 1.7%	0 ± 10.3%	0 ± 3.8%

^a Tested concentration for Anammox process.

Differences of azole inhibition on BNR processes and potential mechanisms

To further explore and answer the research question of how azoles would impact the BNR processes, the inhibition of the tested azole compounds on the BNR process was compared in this section. The potential inhibitory mechanisms of azole compounds were also discussed. The toxicity of the azoles to Anammox, nitrification, and denitrification processes differed from each other. **Figure 3.4** shows the IC₂₀, IC₅₀, and IC₈₀ values of each BNR process derived from literature review and experiment results.

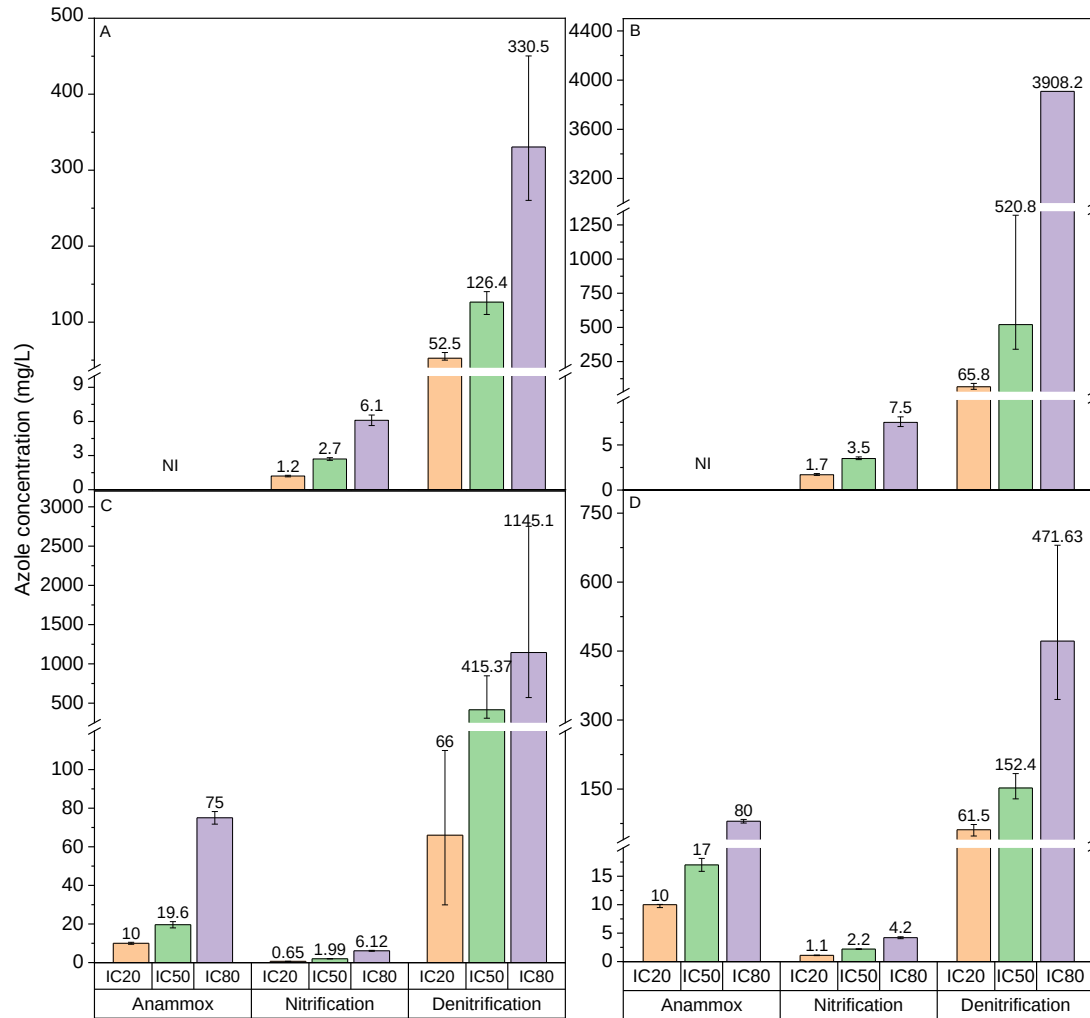


Figure 3.4 Comparison of the concentrations of different azole compounds resulting in 20, 50, and 80 % (IC₂₀, IC₅₀, and IC₈₀) inhibition in Anammox, nitrification, and denitrification. (A) PA, (B) TA, (C) BTA, and (D) MBTA. It should be noted that the tested azoles concentration was in range of 0 - 250 mg/L. The value of IC₂₀, IC₅₀, and IC₈₀ exceeding 250 mg/L were determined using empirical inhibition model (Eq. (3.2)), with the error bars representing a 95% confidence interval. NI: no inhibition ($\geq 10\%$) was observed at the highest tested azole concentration (250 mg/L) (Lakhey et al., 2020; G. Li et al., 2020)

Among all the BNR processes, the denitrification and Anammox processes are more resistant to azole inhibition than the nitrification processes. However, the BNR processes are commonly used in combination in the WWTPs. The nitrification process is a prerequisite step of the Anammox in a partial nitrification-Anammox process and denitrification in traditional nitrification-denitrification systems. The Anammox process has been applied in wastewater treatment as partial nitrification-Anammox or partial denitrification-Anammox systems. If one of the processes was inhibited, the whole BNR system would be impacted. As shown in **Figure 3.4**, 20% inhibition of nitrification activity was observed from 0.65 mg/L

to 2.7 mg/L for the tested four azole compounds. Then the inhibitory effect increased to 80% of the azole concentration to 7 mg/L. The toxicity order of the tested azoles to nitrification is as follows (IC_{50} decreases): in the following order: TA < PA < MBTA < BTA. The Anammox process was resistant to PA inhibition but was inhibited by BTA and MBTA. 20% inhibition of the Anammox activity was observed at 10 mg/L for either BTA or MBTA. Although the denitrification process was less impacted by the BTA and MBTA, the denitrification process was not solely applied in BNR systems. The existence of azoles in the waste may still impact the whole BNR system.

As antifungals, azoles could inhibit the synthesis of ergosterol and damage the cell membrane (Ghannoum et al., 1999). However, how azoles would interact with the BNR microorganisms is not well studied. Azole compounds may be able to pass through the cell membranes and interact with enzymes or other biomolecules to cause inhibition (Lakhey et al., 2020). Several studies hypothesized that the azole compounds may bind to the metal center in key enzymes of the nitrification process such as the copper in ammonium monooxygenase, which results in the inhibition of nitrification activity (Lakhey et al., 2020; McCarty, 1999). Similar in other studies, the N of the azole compound could bind to the central iron in the heme cluster of the P450 enzymes, which are distributed across all domains of life and typically act as monooxygenase (Kunakom et al., 2023; Lakhey et al., 2020; X. Zhang et al., 2020). At the same time, the Anammox bacteria contain very high amounts of cytochrome C, a heme protein that contains iron center, and Fe-involved enzymes (H. Wang et al., 2022). Therefore, the toxicity of some azole compounds towards nitrification and Anammox bacteria may be caused by azole interaction with metal prosthetic groups and the ensuing deactivation of critical enzymes or biomolecules. BNR process with less protected enzyme may be easier to get inhibited by the azole compounds. The nitrification process happens on the cytomembranes of the nitrifiers, which are vulnerable to inhibition (Gerardi, 2003). Similar to this study, the nitrification process was the most sensitive process to azole inhibition, which aligns with the previous hypothesis.

From **Figure 3.4** and **Table 3.2**, the Anammox process was more resistant to BTA and MBTA inhibition than the nitrification process and not influenced by PA and TA. One potential explanation is that Anammox bacteria have unique cell structures that protect the cells from azole inhibition. Anammox bacteria contain an intracytoplasmic compartment called Anammoxosome performing the Anammox process, which occupies most of the cell volume and is bounded by a single bilayer, ladderane-lipid-containing membrane (**Figure S1**) (van Niftrik et al., 2012). The ether-linked and ester-linked ladderanes confer impermeability to Anammoxosome membranes (Fuerst et al., 2006). The unique reaction sites and cell structure of the Anammox bacteria may contribute to their higher resistance to azole inhibition. Unlike the Anammox process, the denitrification process presents higher resistance in all tested compounds compared to the nitrification process and in BTA and MBTA compared to the Anammox process. The denitrification system presented higher resistance compared to the nitrification process. Denitrifiers were composed of a larger number of genera of facultative anaerobes, which contribute to a higher microbial diversity of the denitrification sludge (Gerardi, 2003). The denitrification process is associated with a variety of key enzymes including different types of nitrate reductases, nitrite reductases, and nitric oxide reductases, and thus a complete denitrification process may be carried by different bacteria (Hochstein et al., 1988). One potential explanation for the higher resistance of denitrification is that some of the denitrifiers are resistant to the azole inhibition (Braker et al., 2000). However, the results of this study are not sufficient to support this hypothesis. More investigations will be needed.

Comparison of the azole toxicity and structure

Azoles with different chemical structures and correlated physicochemical properties caused different inhibitions to the BNR processes. Some azole contains only one N-heterocyclic ring such as PA and TA while the other complex azoles have multiple rings, branch structures, and more functional groups. In this section, the relationship between azoles' toxicity to BNR processes and their physicochemical properties is discussed. The log P of the azoles ranges from -0.10 to 5.96, which suggests differences in hydrophobic properties. Some azoles such as PA and TA have solubilities up to 241 g/L. It should be noted that the

tested azoles have $Pk_{a1} < 6.5$ and $Pk_{a2} > 9.0$, which indicates that azole compounds are in uncharged form at the tested pH (see **Table 3.1**) in each BNR process. Data resulting from this work should be carefully used for estimating the toxicity of azoles in the environment where azole compounds predominantly exist in ionized form.

The differences in functional groups of azoles may relate to their toxicity to BNRs. BTA and MBTA have similar IC_{50} values to the Anammox process (20 and 18 mg/L) and nitrification process (1.99 and 2.18 mg/L). At the same time, BTA and MBTA have similar structures and comparable Pk_a and Log P values. The difference between the structure of BTA and MBTA is the methyl group on the benzene ring. The results suggest that the replacement of the H atom by methyl group does not change their toxicity on the Anammox and nitrification process. Comparing the BTA and MBTA to TA, the addition of benzene structure to TA increased the toxicity to the Anammox process. However, benzene itself was found to only cause 26% inhibition on the Anammox process at the concentration of 300 mg/L (Lakhey et al., 2020). This finding indicates that benzene was not the key structure that caused azole inhibition in the Anammox processes. Furthermore, G. Li et al. (2020) suggested the toxicity of the azoles in the nitrification process could be weakened by blocking the N atom with methyl groups. Two methylated PAs were less toxic to nitrification with the calculated IC_{50} of 17.3 mg/L and 49.6 mg/L (G. Li et al., 2020). Thus, the N-containing ring structure of azoles may contribute more to the inhibition of the Anammox and the nitrification process.

The increase in the Cl atom numbers and the addition of benzene caused higher inhibition of the Anammox process. Miconazole and tioconazole have similar structures and physicochemical properties. However, the toxicity of the miconazole and tioconazole to the Anammox process was different. Miconazole and tioconazole caused 22% and 17% inhibition on the Anammox process at 0.8 mg/L and 10 mg/L, respectively. No inhibition ($>10\%$) was observed of either compound in the nitrification and denitrification processes. To further investigate if the physicochemical parameters of azole compounds tested correlate with their toxicity to BNR processes, the IC_{50} values of the PA, TA, BTA, and MBTA

were plotted against the log P, and P_{k_a} values (**Figure S2-S4**). However, no correlation between those parameters of azoles and their inhibition of BNR processes was found.

From the previous sections, the azoles with more complex structures such as branches, higher molecule weight, and higher number of rings present less inhibition effect on BNR processes. According to **Figure 3.4** and **Table 3.4**, PA, TA, BTA, and MBTA presented stronger inhibition to the BNR processes than the other six azole compounds, which contain more functional groups and complex structures. One hypothesis is that the steric effect might occur and prevent the interactions between azoles and enzymes. The azoles may not be able to penetrate the cell membranes or are blocked by those functional groups to interact with enzymes. The existence of functional groups such as extra ring structure, or halogen, S, and O atoms could prevent the azoles from complexing properly with the enzyme-bound metal core (Xiao et al., 2004). A similar phenomenon has been reported in another study. Todoroki et al. (2010) found the acrylate of uniconazole could function as a steric obstacle to prevent the amino acid chelate the P450 enzymes.

Implications

The results in this work indicate that the presence of azoles in wastewater may reduce the activity of BNR processes and the adverse impacts of azoles on BNR process performance are contingent upon the specific type of azoles and the nature of the BNR process. Azoles with larger molecular weight and more branched structures showed lower inhibition potential on BNR processes. Compared with Anammox and denitrification processes, the nitrification process showed a higher sensitivity to azoles. As the prerequisite step of anammox and denitrification, nitrification can experience substantial inhibition (>50%) in the presence of azoles at low concentration levels (<3.5 mg/L), posing a threat to the stability of BNR processes in WWTPs. Within the nitrification process, the inhibition of tested azoles (PA, TA, BTA, and MBTA) was more pronounced in the ammonium oxidation process compared to the nitrite oxidation process. This suggests that azoles may interfere with the function of AMO, a copper-dependent membrane-bound enzyme, through chelation (Lakhey et al., 2020; G. Li et al., 2020), thereby inhibiting

the activity of ABO/AOA. In comparison, the unique cell structure of the Anammox process, anammoxosome bounded by ladderane membranes with low permeability, and the high diversity of denitrification microbial community may contribute to their higher resistance to azole.

Chapter 4: Long-term Effects of Pyrazole and Benzotriazole on Anammox Granular Sludge: Inhibition, Acclimation and Degradation Mechanisms

Abstract

Azoles such as benzotriazole (BTA) and methyl-benzotriazole (MBTA) could cause inhibition (>50%) to the Anaerobic ammonium oxidation (Anammox) process activity in short-term experiments at 20 and 18 mg/L, respectively. At the same time, it was observed that azoles such as pyrazole (PA) and triazole (TA) did not inhibit Anammox activity at concentrations as high as 250 mg/L. However, the long-term influence of azoles, whether inhibitory or non-inhibitory in short-term experiments, on the Anammox process is not well studied. This study aimed to investigate the long-term impact of BTA and PA on the Anammox process and the adaptation of Anammox bacteria to BTA and PA inhibition. Through long-term acclimation, the acclimated Anammox granular sludge showed 25% higher resistance to BTA inhibition below 30 mg/L when compared to unacclimated Anammox sludge. However, further exposure to higher concentrations (40 mg/L) continuously decreased the Anammox activity from 0.213 to 0.120 g N₂-N/g VSS/day within 40 days and no recovery was observed before azoles were removed. On the other hand, 100 mg/L PA did not cause any Anammox activity decrease, but 200 mg/L PA caused 65% reversible inhibition to the Anammox bioreactors in 40 days. Long-term exposure to 200 mg/L caused stronger inhibition than short-term exposure tested in previous work. To investigate the mechanisms by which Anammox granular sludge adapts to resist azole inhibition, starvation experiments under different substrate conditions, ATP content analysis, and microbial composition analysis were implemented. BTA and MBTA were selected as two representatives of azoles in the starvation experiments due to their reported inhibition effect on Anammox granular sludge. Within 28 days, increasing the starvation period decreased Anammox activity and increased the lag phase at high substrate conditions (75 mg/L NH₄⁺-N

and 100 mg/L NO_2^- -N). However, no increase in azole inhibition was observed. Besides, the addition of BTA and MBTA did not cause changes in Anammox ATP content levels, suggesting that the short-term energy-required mitigation methods may not be the major defense mechanisms adapted by Anammox bacteria to resist BTA and MBTA inhibition.

Introduction

The Anammox process has been applied in wastewater treatment through different configurations including partial nitrification/Anammox (PNA) or partial denitrification/Anammox (PdNA) as illustrated in **Figure S5** (M. Zhang et al., 2019). Compared to the traditional nitrification-denitrification systems, PNA or PdNA is a more energy-efficient process for Nitrogen (N) removal from sewage systems (J. Li et al., 2020). However, the efficiency of the Anammox process is not stable and may be impacted by factors such as the Emerging organic contaminants (EOCs) that exist in the wastewater influent (Madeira et al., 2021). Decreases in performance, even complete failure, of the Anammox process, have been observed in both lab and full-scale systems due to the varying quality of receiving wastewater (G. Li et al., 2016; X. Wang et al., 2019)

Azoles have been used in agriculture and personal care products, resulting in the widespread presence of azoles in the environment such as wastewater, surface water, groundwater, sludge, sediment, and bio-solid amended soils (Assress et al., 2019). Azole compounds (e.g., ketoconazole, clotrimazole, and miconazole) are also frequently used as antifungal pharmaceuticals (X. Peng et al., 2012). While applied topically on humans, 90–95% of the remaining ingredients may be directly removed from treated skin by washing and entering the sewage system (X. Peng et al., 2012). The wide application and high-volume usage of azoles raise concerns about their impact on the Anammox processes. A previous study found that 18 mg/L MBTA and 20 mg/L BTA could decrease 50% activity of granular Anammox sludge in short-term batch tests (Lakhay et al., 2020). However, the long-term influence of azoles on the Anammox process is still unknown. How the Anammox community would respond to long-term azole exposure and the potential inhibition defense mechanisms are not well investigated.

Microorganisms possess various mechanisms to counteract the effects of chemical inhibitors. Certain defense strategies, such as certain efflux pumps, necessitate the expenditure of ATP as energy.

Conversely, other methods, such as the cell wall and cell membrane, as well as the formation of unique community structures like biofilms and granular sludge, require less energy investment once established (Zhou et al., 2015). Anammox bacteria would invest energy to mitigate toxicity caused by chemical inhibitors such as nitrite (Carvajal-Arroyo et al., 2014). However, whether Anammox bacteria would invest energy in resisting azole inhibition is unclear. In this study, starvation experiments will be implemented to test if the Anammox bacteria depend on energy to reduce the azole inhibition.

The energy source (available ammonium and nitrite) in wastewater treatment plants (WWTPs) is not always stable and sufficient. In WWTPs, N levels may change in practice and the Anammox cells may suffer from starvation, low energy status, or even an overload of substrates (Muttamara, 1996). For example, Anammox process has been used for treating the side-stream from the dewatering process or anaerobic digester, which contains varied N concentrations (<5 mg N /L to >500 mg N/L) and is accompanied by concentrated contaminants (Izadi et al., 2021; Lackner et al., 2014). At the reported N concentrations in WWTPs, the reaction efficiency of the Anammox sludge could be impacted. Previous studies conducted kinetic analysis on the Anammox process, and the results are fitted with the Michaelis–Menten equation (shown in **Table 4.1**)

Table 4.1 Reported Anammox activity saturation constants.

	$V_{\max}$	K_m	Reference
Ammonia (mg $\text{NH}_4\text{-N/L}$)	32.55	44.86	(Qiao et al., 2017)
	69.59	52.14	
	-	87.1	(Zu et al., 2008)
	-	48.4 – 87.1	(T. Chen et al., 2011)
Nitrite (mg $\text{NO}_2\text{-N/L}$)	39.54	99.18	(Qiao et al., 2017)
	66.23	107.27	

$V_{\max}$: maximum specific growth rate.

K_m : Michaelis–Menten half-saturation constant

According to **Table 4.1**, when the available nitrogen content is low, the Anammox activity could be limited. If energy is needed for the Anammox process to mitigate the azole inhibition, the presence of azoles at a lower concentration may cause more inhibition of the Anammox bacteria. The following consequences may include the loss of activity, a longer recovery phase, and failure of the system. A similar phenomenon has been reported in another study, in which starved Anammox cells are 7 times more sensitive to nitrite inhibition by 50% (Carvajal-Arroyo et al., 2014). The research question of this study is how the Anammox process would respond to long-term azole inhibition and whether different substrate condition would impact their resistance to azole inhibition.

Material and methods

Chemicals and microorganisms

MBTA (CAS # 136-85-6, purity > 98%) was purchased from Acros Organics B.V.B.A. B. BTA (CAS # 95-14-7, purity > 99%) was purchased from Alfa Aesar (Ward Hill, Massachusetts, USA), PA (CAS # 288-13-1, purity > 98%) was purchased from Tokyo Chemical Industry (Tokyo, Japan).

Granular Anammox biomass was obtained from a 3.8 L laboratory-scale Anammox expanded granular sludge bed reactor (EGSB) fed with a synthetic mineral medium at a loading rate of 4.1 g N/L/day. The

reactor has been in operation for the last 3 years. The granules contained 0.89 g of volatile suspended solids (VSS) per gram of total suspended solids (TSS) and had a specific Anammox activity (SAA) of 0.62 ± 0.05 g N₂/g VSS/day, which is at the same level as other reported work. Q.-Q. Chen et al. (2017) showed that the Anammox activity ranged from 0.47 to 0.65 g N₂-N/g VSS/day.

Reactor setup and operation

Three lab-scale batch reactors of 1.15 L sealed bottles including 0.8 L of liquid volume and 0.35 L gas phase were used. pH was controlled at 7.2 to 7.8 by adding 0.5 M HCL or NaOH. The reactors were operated in a dark environment inside a shaker with temperature control (30 ± 1 °C) and shaken at 120 rpm. The reactors were fed with basal medium, and the medium was replaced every 10 to 14 days. During the process, the Anammox sludge was captured by a strainer and washed with fresh basal medium. The Anammox granular sludge was re-added into the reactor with fresh medium for the following operation. Every time after replacing the basal medium, the reactor's liquid phase was flushed with helium for 15 mins and headspace was also flushed with helium gas for 15 mins to form an anaerobic environment. The dissolved oxygen (DO) in the liquid phase and headspace were checked with gas chromatography (GC) and DO probe as described in the following section. An N₂ gas ratio below 3% in the headspace was required. The N₂ gas ratio in the headspace was measured with GC every two days for substrate consumption conditions. Substrates (110 mg NO₂-N/L and 94 mg NH₄-N/L, ratio =1.16) were spiked into the system once the N₂ gas production was stopped for 6 hours. The headspace of the reactors was flushed with helium for at least 15 minutes until the N₂ gas ratio in the headspace was below 3% after substrate addition. During the operation, excessive ammonium (12 ± 0.5 mg NH₄-N/L) was intentionally added at a nitrite consumption/ammonium consumption ratio (R_S) of 1.16, which is lower than the theoretical value of 1.32. The liquid phase samples were analyzed using SEAL AQ300 discrete analyzer (Mequon, WI, USA) for measuring the ammonium, nitrate, and nitrite in liquid samples. The reactor's cap has a rubber area allowing sampling of liquid phase with syringes and collecting gas samples from the headspace. The

cap was replaced every two weeks. It is qualified for maintaining an anaerobic environment for more than a month in the control experiment with identical sampling frequency used in this study.

The basal medium for the bioassay was prepared using ultrapure water. The basal medium contained (mg/L): $\text{NaHPO}_4 \cdot \text{H}_2\text{O}$ (115), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (200), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (400), NaHCO_3 (100), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (5950), which are also the final concentration to the reactor. 1 mL/L of each of the two trace element solutions was added to the medium. The first trace element solution contained 9151 mg/L of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$. The second trace element solution contained (in mg/L): $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (430), $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (240), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (250), $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ (190), $\text{Na}_2\text{SeO}_4 \cdot 10\text{H}_2\text{O}$ (210), H_3BO_3 (14), $\text{NaWO}_4 \cdot 2\text{H}_2\text{O}$ (50), MnCl_2 (629) and $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (220). Azoles were added into each reactor as described in **Table 4.2**.

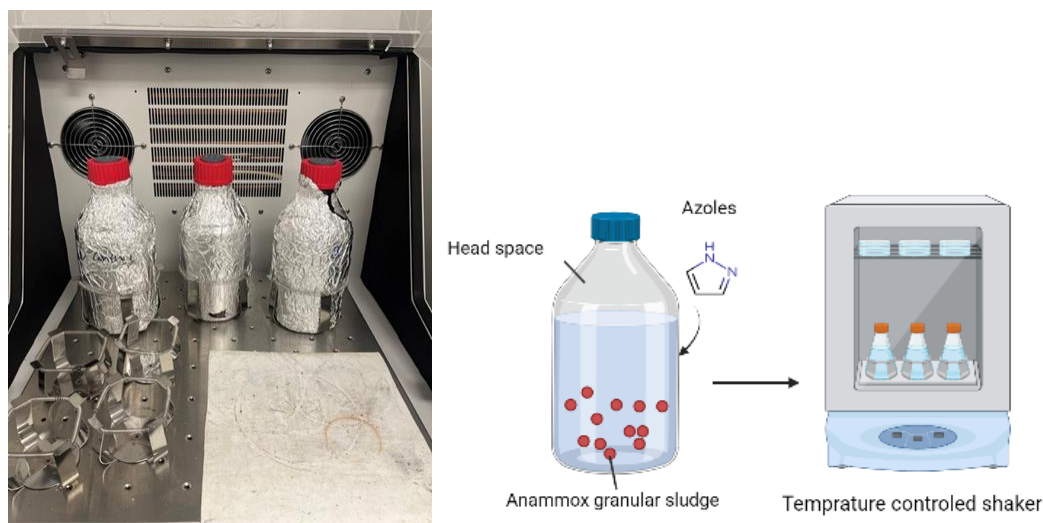


Figure 4.1 Anammox bioreactors(left) and design scheme (right)

Table 4.2 Operation stages of Anammox reactors

Reactor	Stage name	Days	Azole addition (mg/L)
BTA	BTA - A	0 - 10	5
	BTA - B	11 – 21	10
	BTA - C	22 - 85	20
	BTA - D	86 - 139	30
	BTA - E	140 - 180	40
PA	PA - A	0 - 10	50
	PA - B	11 – 21	100
	PA - C	22 - 85	200
	PA – D	86 - 139	100
	PA – E	140 – 180	200
Control	Control	0 - 180	0

Long-term Anammox activity bioassays

The Anammox bioassay was modified from a previous study (Lakhey et al., 2020). During the bioassay, substrates are provided at 110 mg NO₂-N/L and 94 mg NH₄-N/L, respectively. The headspace of the bottles was flushed with helium gas for 15 min each before the bioassay to remove N₂ gas. Gas samples (100 µL) were taken from the headspace at intervals of 1.5 hours to track N₂ production during the incubation period for GC analysis. Typically, at least six gas samples were taken in each bioassay according to the method to calculate the SAA for quality control.

High/low substrate conditions and starvation experiment bioassays

After being taken out of the nursing reactor, the Anammox sludge of the starvation group will be washed with DI water 3 times and 1 time with no substrate medium. Then the sludge will be kept in sealed bottles with basal medium under a dark environment at 30 °C. The liquid phase and headspace of the bottles were flushed with N₂ gas for an anaerobic environment. After a 0, 5, and 14-day starvation period, the Anammox sludge will be taken out for the activity bioassays using a method revised from a previous

study (Lakhey et al., 2020). The bioassays were performed in hermetically sealed 120-mL serum bottles. The liquid volume was 80 ml. Bottles were provided with starved granular anammox biomass. 20 mg/L BTA and 18 mg/L MBTA will be added to the treatment group on the test day and each group will be carried under high (75mg/L NH_4^+ -N and 100 mg/L NO_2^- -N) and low (19 mg/L NH_4^+ -N and 25 mg/L NO_2^- -N) substrate conditions. Gas samples (100 μL) were taken from the headspace at intervals of 1.5 hours to track N_2 production during the incubation period for GC analysis. Each group will be duplicated. For the pre-exposure group, 5 mg/L BTA and MBTA will be added to the system at the beginning of the experiment. After 5, 14, and 28 days, the Anammox activities are tested at high/low substrate conditions using a method identical to the starvation experiment. Gas samples (100 μL) were taken from the headspace at intervals of 1.5 hours to track N_2 production during the incubation period for GC analysis. For comparison, starvation-only groups were also added in this experiment, in which 5 mg/L BTA and 18 mg/L MBTA will be added after the starvation period for the activity test. Each group will be duplicated.

Analytic methods

N_2 gas, DO, TSS, and VSS analysis

The N_2 gas was analyzed using an Agilent 6890 gas chromatograph (GC) (Agilent Technologies, Santa Clara, CA, USA) fitted with an HP-PLOT Molesieve column (30 m $\times$ 0.32 mm, Agilent Technologies) and a thermal conductivity detector. The temperatures of the column, the injection port, and the detector were 40, 200, and 220 $^\circ\text{C}$, respectively. Helium was used as the carrier gas and the injection volume was 100 μL . Injections were carried out using a Hamilton sample-lock 1710 SL syringe (Hamilton Company, Reno, NV, USA). During the measurement, the target compounds with known concentrations were added for continuing calibration verification (CCV) to every 10 samples for quality control. Less than 10% differences between the measured concentrations and the known concentrations were required to accept the measurement results. DO in liquid samples was measured using an optical O_2 probe. (LBOD101 Sensor, Hach, Loveland, CO, USA). Other parameters (TSS, VSS and pH) were determined according to

standard methods (American Public Health Association, 2023). The limit of detection (LOD) and limit of quantitation (LOQ) of the analytical instruments are listed in **Table S6**.

ATP Analysis

The ATP level of whole anammox granular sludge, including anammox bacteria and other bacteria that co-existed in the sludge, was examined. Anammox granular sludge starved for 0, 5, and 14 days is taken for the ATP content analysis. After azole was added to the system, the granular sludge was collected at 0,1,2,4,6,8 and 24 hours for ATP content determination. Negative controls were included using DI water and fresh medium prepared for the reactors. The ATP content was extracted from granular sludge through a published method (Carvajal-Arroyo et al., 2014). The granules (0.10–0.12 g wet weight) were disintegrated through syringe needles (20 G, 0.60 mm I.D.) three times. The disintegrated sludge was suspended in 9 mL of boiling Tris–EDTA (20-2 mM) buffer. The closed vials were incubated in boiling water for 2 min and then submerged in an iced-water bath (Carvajal-Arroyo et al., 2014). The ATP in the extract was quantified using a commercial ATP determination kit (Life Technologies, Grand Island, NY) according to the manufacturer's instructions and measured with a Spark Multimode Microplate Reader (Tecan Group Ltd, Männedorf, canton of Zürich, Switzerland). During the measurement, ATP standards with known concentrations included in the extraction kit were added to every 10 samples for quality control. Less than 10% differences between the measured concentrations and the known concentrations were required to accept the measurement results.

Liquid sample analysis

The liquid sample was collected from the reactor and filtered through 0.45 µm PP filters to measure the concentration of target azole compounds. The concentrations of PA and BTA were measured through a developed method (Lakhey et al., 2021) with minor modifications. The details of the method are described below: Concentrations of PA were analyzed using high-performance liquid chromatography (HPLC) equipped with a diode array detector. Chromatography was performed using the InfinityLab

Poroshell 120 column (2.7 μ m, 4.6 $\times$ 100 mm; C-18 column, Agilent, USA). An isocratic mixture of 10% methanol and 90% water was used as the eluent for a run time of 12 min. The eluent flow rate was 1 mL/minute and the injection volume was 20 μ L. The temperature was maintained at 40°C and the wavelength for PA was 210 nm. Concentrations of azole compounds BTA were analyzed using the same instrument, with an isocratic mixture of 60% methanol and 40% water. The run time was 8 min and the eluent flow rate was 1 mL/min and the injection volume was 20 μ L. The temperature was maintained at 40°C and both chemicals are detected at wavelength of 275 nm. Negative controls were included using DI water and fresh medium prepared for the reactors. Ammonium, nitrate, and nitrite in liquid samples were analyzed by SEAL AQ300 discrete analyzer. (Mequon, WI, USA). During the measurement of nitrogen species and azoles, the target compounds with known concentrations were added for CCV to every 10 samples for quality control. Less than 10% differences between the measured concentrations and the known concentrations were required to accept the measurement results. The LOD and LOQ of the analytical instruments are listed in **Table S6**.

DNA extraction and microbial analysis

The inoculum anammox sludge samples were collected at the beginning of the experiment and the anammox sludge samples of each reactor (Control, BTA, and PA) were collected at the end of the experiment. The DNA was extracted using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc, Carlsbad, California, United States). According to the manufacturer's guidebook, 0.25 g of Anammox sludge sample was used for the DNA extraction. The extracted DNA samples were sent to Molecular Research LLP (Shallowater, TX) for analyzing their bacterial composition with 16S rRNA V4 variable region (PCR primers 515/806) through gene amplicon sequencing. The samples are treated through barcoded amplicon sequencing processes by MR DNA® under the trademark service (bTEFAP®). Negative controls (Blank medium sample without sludge) and positive controls (reference wastewater sludge samples) were used to eliminate background DNA interference. Sequences are joined, sequences <150bp, and sequences with ambiguous base calls are removed. Sequences were quality filtered using a

maximum expected error threshold of 1.0 and dereplicated. The dereplicated or unique sequences are denoised; unique sequences identified with sequencing and/or PCR point errors are removed, followed by chimera removal, thereby providing a denoised sequence of taxonomical units (OTU). Final OTUs were taxonomically classified using BLASTn against a curated database derived from NCBI (MR DNA, Shallowater, TX, USA).

Data processing

The specific anammox activities (SAA) were calculated as the rate of N₂ production per g VSS and were expressed as g N₂ g/ (VSS d). The rate was calculated using linear regression of five or more points. The regression analysis was carried out using Microsoft Excel. The normalized Anammox activity (NAA) was calculated as shown in Eq 4.1:

$$NA(\%) = \frac{SA_t}{SA_c} * 100 \quad (4.1)$$

where SA_t and SA_c (both as g N₂ g/VSS/d) are the specific activities of the treatment (with added azoles) and control experiment (without added azole) of the anammox process, respectively.

In order to quantify the delay between the substrate's injection and the occurrence of the maximum SAA a parameter called lag phase was defined. The lag phase of the Anammox activity bioassay was determined through a method from a previous study (T Lotti et al., 2012). The part of the nitrogen gas production curve at the moment the maximum specific Anammox activity occurred was extrapolated. The lag phase was defined as the time difference between the intercept of the expended trendline to the x-axis and the substrate injection time (**Figure S6**) (T Lotti et al., 2012). T-test was used for statistical analysis of the relationship between azole concentration and the activity of the BNR processes in the batch reactor. A *p*-value less than 0.05 was required to reject the null hypothesis.

Results and discussion

Performance of long-term Anammox reactors with azole addition

The granular sludge was acclimated for 10 days before day 0 in all three reactors without any azole addition, during which steady N₂ gas production and N removal were observed. According to **Figure 4.2**, over the 180 days of operation, the control group achieved stable Anammox performance with a high removal rate (>90%) of ammonium and nitrite. No nitrite accumulation in the system was observed. The remaining ammonium may be attributed to the intentionally added excessive ammonium at an R_s of 1.16, which is lower than the theoretical value of 1.32 to avoid nitrite accumulation. The nitrite loading rate (NLL) was 0.061 ± 0.011 Kg N/m³/day and the ammonium loading rate (ALR) was 0.046 ± 0.007 Kg N/m³/day. The Nitrite removal rate (NRR) was approximately the same as the NLL and nitrite removal efficiency (NRE) >99%. The ammonium removal rate (ARR) was 0.043 ± 0.006 Kg N/m³/day and the ammonium removal efficiency (ARE) was 92.5 ± 3.7 %. The Nitrate production rate (NPR) is 0.006 ± 0.002 Kg N/m³/day. The R_s is 1.43 ± 0.11 and the nitrate production/ ammonium consumption ratio (R_p) is 0.14 ± 0.05, which are within the same level reported in other Anammox studies (Q.-Q. Chen et al., 2017; de Almeida Fernandes et al., 2018). As discussed above, the results from the control group suggest that it could serve as a suitable reference for comparing the impacts of azoles on the nitrification process.

Different concentrations of BTA were loaded to the reactor as described in **Table 4.2**. According to **Figure 4.3**, with the long-term acclimation, the Anammox process continuously happened in the reactor with gradually increasing BTA concentrations. During stage A (days 0 to 10), the shock loading of 5 mg/L BTA didn't cause a decrease in the N removal of the system. No effluent nitrite accumulation was observed, while the ammonium and nitrate effluent concentrations were 6.25 mg NH₄⁺- N/L and 75.2 mg NO₃⁻ N/L, respectively. The ARR and NRR were 0.051 and 0.061 kg N/m³/day with an initial ALR and NLR at 70.052 and 0.061 kg N/m³/day. The ARE and NRE were 98.7% and 100%. When 10 mg/L BTA was loaded in stage B (days 11 to 21), the effluent ammonium and nitrate concentration increased to

67.69 mg NH_4^+ - N/L and 134 mg NO_3^- - N/L, respectively. No impact on nitrite removal was observed. The introduction of low concentration (≤ 10 mg/L) of BTA to the system didn't impact the N removal performance. In stage C (days 22 to 85), 20 mg/L BTA was added to the system. When 20 mg/L BTA was first loaded into the system, the effluent ammonium concentration further increased to 105.32 mg NH_4^+ - N/L and then decreased to below 10 mg NH_4^+ - N/L. The effluent nitrate concentration ranged from 73.05 to 90.59 mg NO_3^- - N/L. The R_s ratio was firstly increased to 1.59 and then gradually decreased to 1.24 in the following 40 days. More than 99% nitrite was removed with NRRs ranging from 0.054 to 0.070 kg N/m³/day.

Similarly, in stages D (days 86 to 139, 30 mg BTA/L) and E (days 140 to 180, 40 mg/L BTA), no nitrite accumulation in the effluent was observed and the effluent ammonium concentration was maintained at below 20 mg NH_4^+ - N/L. The NRR and ARR ranged from 0.044 to 0.067 kg N/m³/day and 0.036 to 0.045 kg N/m³/day, respectively. $\geq 98\%$ nitrite removal and $\geq 95\%$ ammonium removal were observed in the system. The change in nitrite/ammonium consumption ratio was similar to the control group, suggesting that the loading of BTA didn't change the nitrite/ammonium consumption ratio of the Anammox process.

Different concentrations of PA were loaded to the PA reactor as described in **Table 4.2**. According to **Figure 4.4**, within the operation period, the Anammox process continuously occurred in the reactor with gradually increasing PA concentrations. During stage A (days 0 to 10), the shock loading of 50 mg/L PA didn't cause a decrease in the N removal of the system. No effluent nitrite accumulation was observed while the ammonium and nitrate effluent concentrations were 37.2 mg NH_4^+ - N/L and 74.8 mg NO_3^- - N/L, respectively. The ARR and NRR were 0.048 and 0.058 kg N/m³/day with an initial ALR and NLR of 0.052 and 0.061 kg N/m³/day, respectively. The ARE was 94.1 % and NRE was $\geq 99\%$. When 100 mg/L PA was loaded in stage B (days 11 to 21), the effluent ammonium and nitrate concentration increased to 83.89 mg NH_4^+ - N/L and 133.2 mg NO_3^- - N/L, respectively. The ARE and NRE were 88.3 % and $\geq 99\%$. PA below 100 mg/L didn't impact on the N removal during the operation cycle.

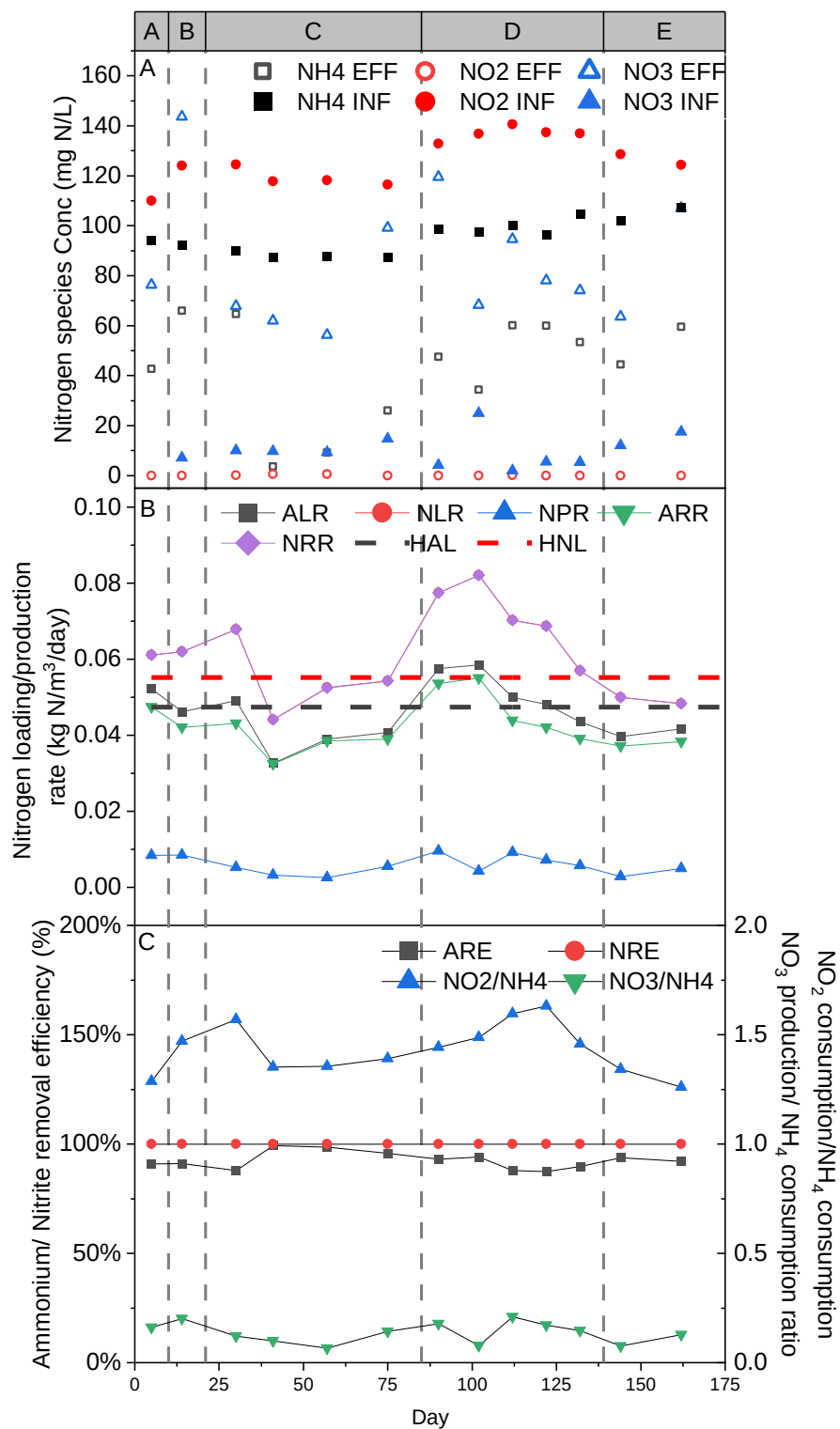


Figure 4.2 N profiles for control Anammox biomass: (A) Influent and effluent N specie; (B) ALR: NH_4^+ -N loading rate; NLR: NO_2^- -N loading rate; NPR: NO_3^- -N production rate; ARR: NH_4^+ -N removal rate; NRR: NO_2^- -N removal rate. ARE: NH_4^+ -N removal efficiency; NRE: NO_2^- -N removal efficiency. Due to the high nitrite removal efficiency, the NLR and NRR overlapped with each other.

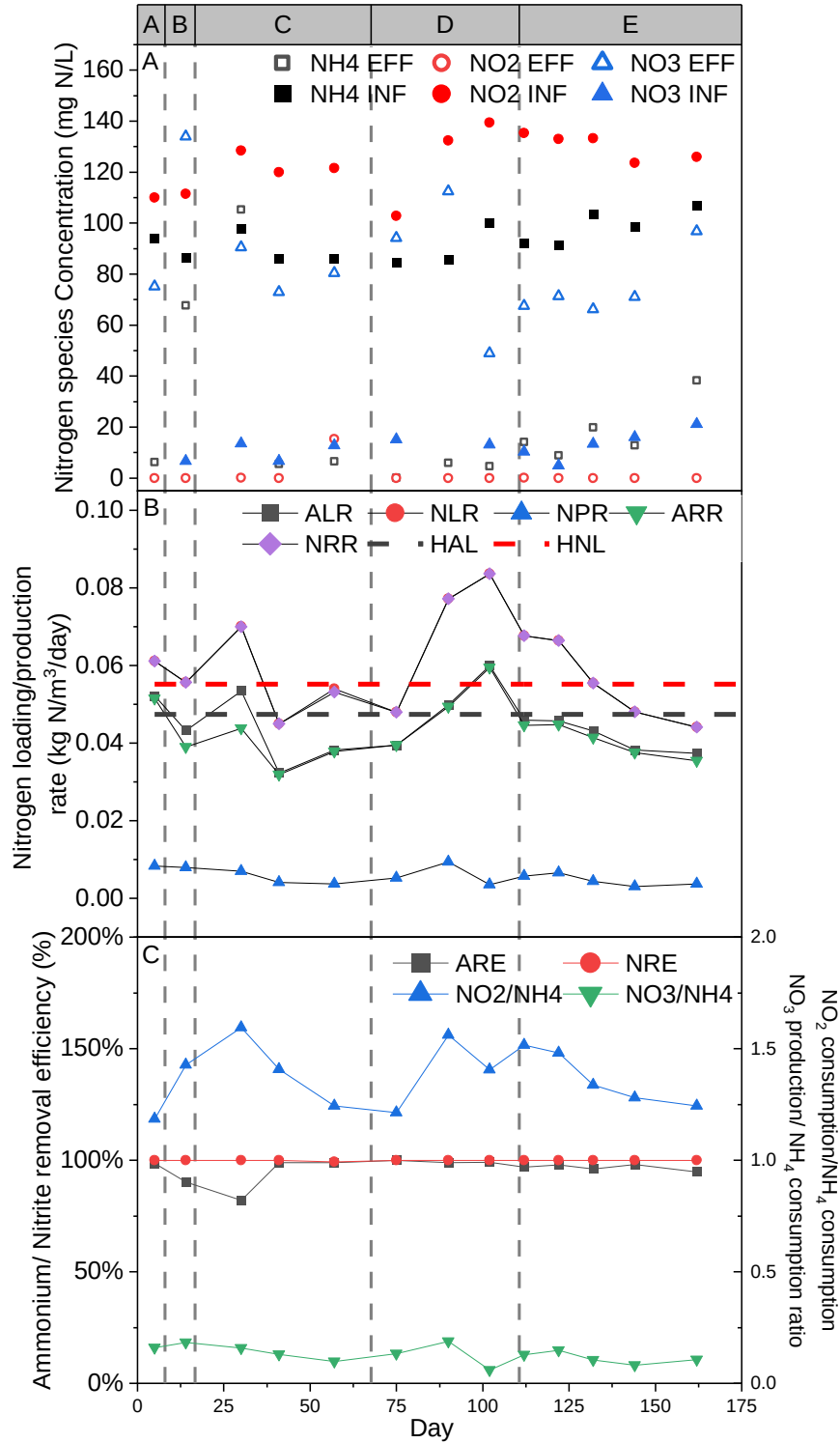


Figure 4.3 N profiles for BTA Anammox biomass: (A) Influent and effluent N specie;(B) ALR, NLR, NPR, ARR, NRR. (C)ARE and NRE. Due to the high nitrite removal efficiency, the NLR and NRR overlapped with each other.

In stage C (days 22 to 85), 200 mg/L PA was added to the system. The effluent ammonium concentrations were ranging from 65.43 to 113.11 mg NH_4^+ - N/L. In comparison to the control group, the PA treatment group exhibited higher levels of ammonium in the effluent. The effluent nitrate concentrations ranged from 88.74 to 147.46 mg NO_3^- - N/L, also surpassing the levels observed in the control and BTA treatment groups. The R_s ratio is 1.52 ± 0.04 , which is higher than the control group (1.42 ± 0.10) and BTA (1.42 ± 0.14) group. More than 99% nitrite was removed while the AREs ranged from 84.6% to 88.4%. At the same time, the ALR was 0.041 ± 0.007 kg N/m³/day. The addition of 200 mg/L PA results in a reduction in ammonium consumption during the Anammox process. Similarly, in stage D (days 86 to 139, 100 mg/L PA) and E (days 140 to 180, 40 mg/L PA), no nitrite accumulation in the effluent was observed. The effluent ammonium concentrations were 119.54 ± 30.28 mg NH_4^+ - N/L. The ALR and ARE ranged from 0.047 ± 0.008 kg N/m³/day and 90.4 ± 3.3 %, respectively. The results were consistent with the previous observation in stage C (days 22 to 85) that high concentrations of PA may cause a decrease in ammonium removal during the Anammox process. A similar phenomenon has been observed in other Anammox inhibition studies, where the addition of excessive sulfur decreased the depletion ratio of ammonium (Jin et al., 2013). The results suggest that 200 mg/L PA impacts the ammonium removal of Anammox bacteria.

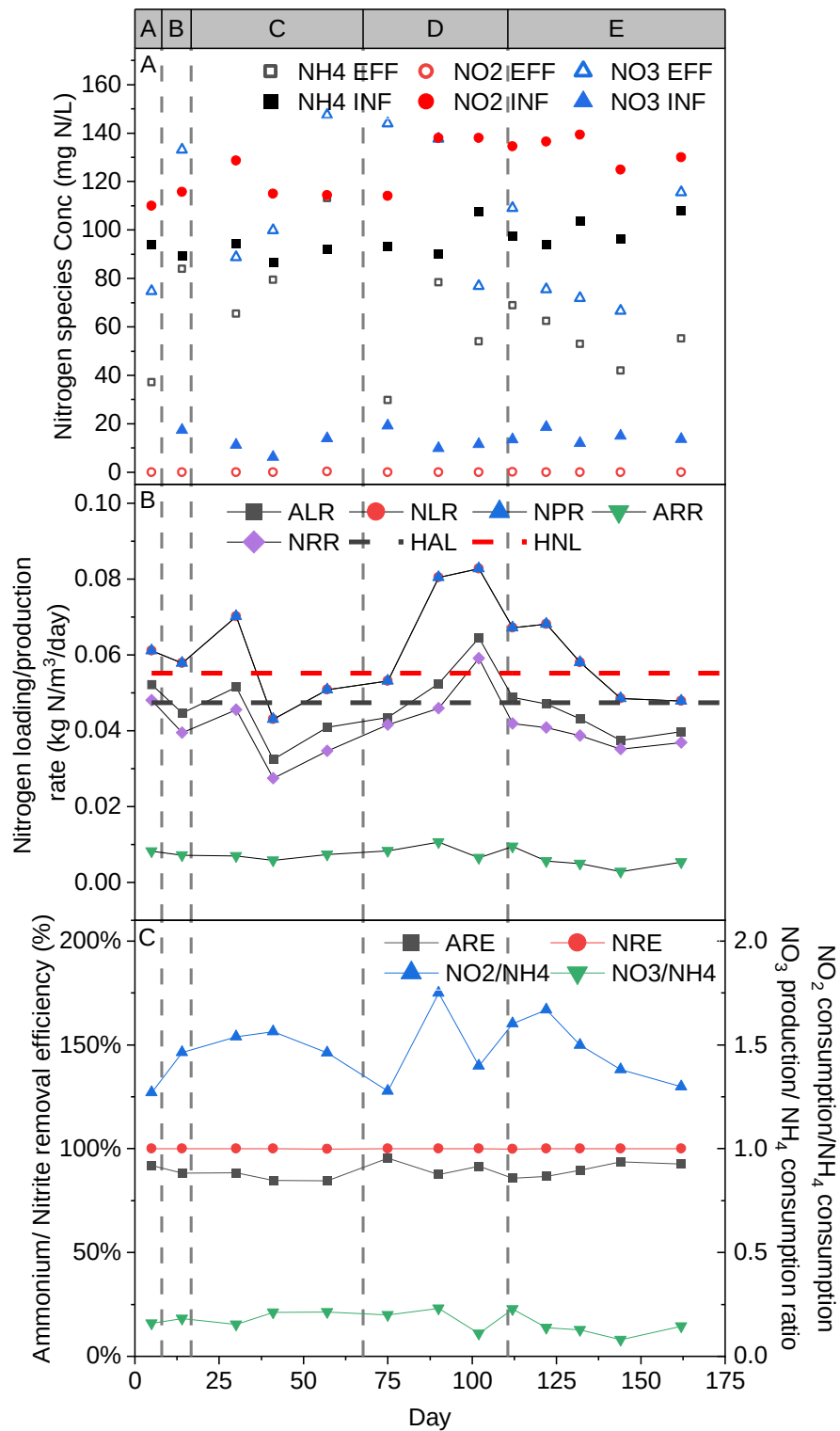


Figure 4.4 N profiles for PA Anammox biomass: (A) Influent and effluent N specie;(B) ALR, NLR, NPR, ARR, NRR. (C)ARE and NRE; Due to the high nitrite removal efficiency, the NLR and NRR are approximately overlapped with each other.

Long-term impacts of azoles on Anammox activity

The NAAs of the BTA treatment group were compared to the theoretical NAAs from the batch tests using unacclimated Anammox sludge. The theoretical NAAs caused by BTA inhibition were aligned with previously reported studies that BTA would cause 10%, 25%, 50%, 60 and 70% inhibition at 5, 10, 20, 30 and 40 mg/L concentration (Lakhey et al., 2020). Over the last 180 days of operation, the average activity of the control group was at 0.52 ± 0.09 g N₂-N/g VSS/day, which is at the same level as other reported work. Q.-Q. Chen et al. (2017) showed that the Anammox activity ranged from 0.47 to 0.65 g N₂-N/g VSS/day.

According to **Figure 4.5**, in stage A (days 0 to 10), the average SAA and NAA in the BTA reactor were 0.558 ± 0.027 g N₂-N/g VSS/day and $90.2 \pm 12.1\%$. When 5 mg/L BTA was loaded into the system, the NAA was lower than the calculated theoretical value. After 5 days of acclimation to the BTA, the NAAs of the treatment group increased to above the theoretical value. When 10 mg/L BTA was loaded into the system, a decrease in SAAs from 0.605 to 0.535 g N₂-N/g VSS/day was observed. However, the NAAs are still higher than the theoretical value. To further evaluate if Anammox bacteria was adapting to the BTA inhibition, 20 mg/L BTA was added in stage C (days 22 to 85). The resistance of the Anammox sludge was maintained during the process. BTA at 20 mg/L could cause inhibition ($\geq 50\%$) to the Anammox process in short-term batch tests (Lakhey et al., 2020). In this study, the NAAs of the BTA group are $72.4 \pm 7.1\%$, which is higher than the theoretical NNA. The results indicated that Anammox bacteria may have developed higher resistance to BTA inhibition during the exposure to low concentrations (mg/L) of BTA. In stage D (days 86 to 139), 30 mg/L BTA was added into the system, however, no changes in the NAAs were observed in the BTA group. The NAAs were maintained at $69.4 \pm 10.0\%$, which was approximately the same level at stage C (days 22 to 85). The increase in the BTA concentration didn't cause further inhibition of the Anammox activity. The results indicate a development of BTA resistance during the acclimation process.

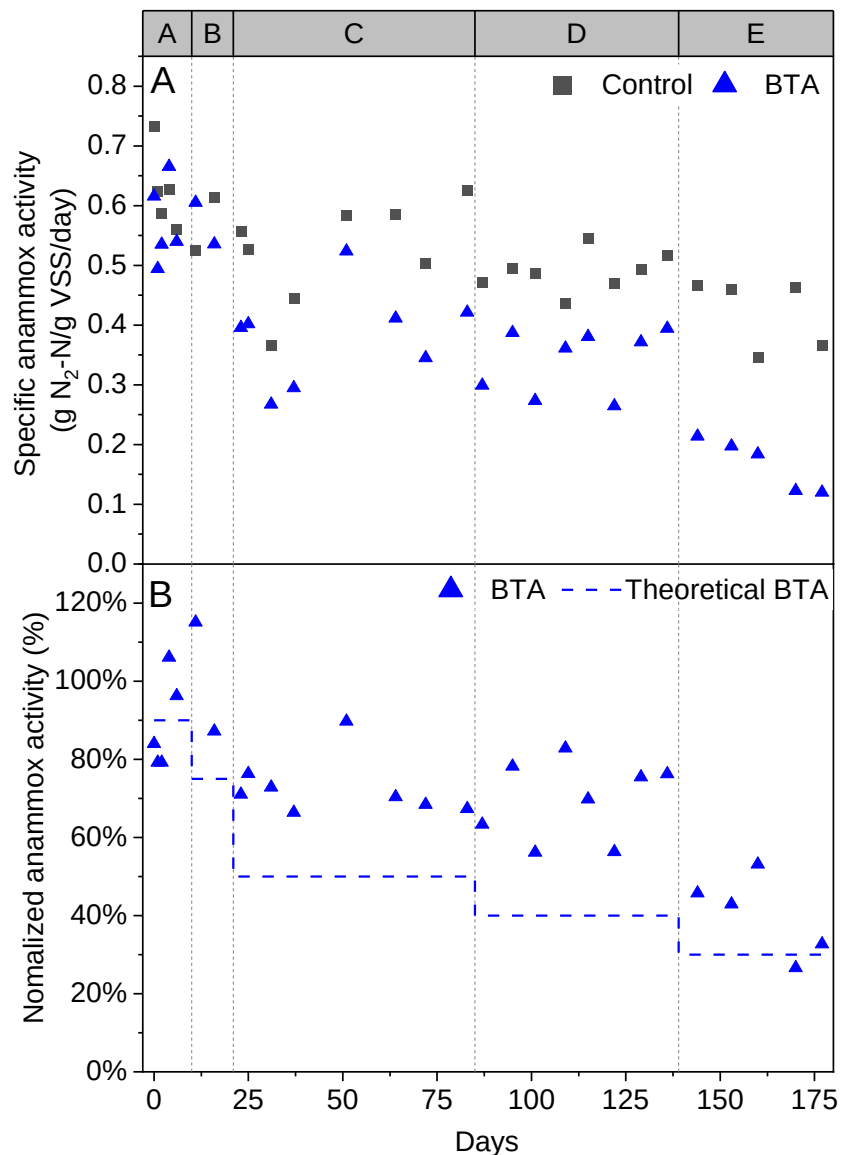


Figure 4.5 (A) SAAs of BTA and control group; (B) NAAs of BTA group and theoretical NAAs

When the BTA concentration was below 30 mg/L, acclimated Anammox cells exhibited higher resistance than unacclimated in short-term batch tests, and stable Anammox activity could be maintained. The results suggest the Anammox bacteria could adapt to the inhibition from azoles. However, no other studies reported the azole adaptation of Anammox bacteria. Similar adaptation processes have been observed in other studies with other compounds. Aromatic organic compounds such as o-cresol, p-nitrophenol, and quinoline are known inhibitors of the Anammox system and are difficult to adapt to (Ramos et al., 2015). However, Toh et al. (2002) found that the Anammox consortium could gradually

acclimate to coke-oven wastewater, which contains phenol up to 330 mg/L, over a 15-month acclimation process. Q.-Q. Chen et al. (2019) reported that Anammox granular sludge lost approximately 80% of its activity in 41 days after exposure to increasing quinoline concentrations (1 to 10 mg/L). However, after the system was recovered, it maintained more than 95% activity in the second exposure to 1-10 mg/L quinoline. Those studies suggest that Anammox bacteria have the potential to adapt to chemical inhibitors through long-term acclimation. In this study, the Anammox system showed the potential to adapt BTA inhibition in lab-scale systems below 30 mg/L BTA. The adapted sludge exhibits higher activity than the non-adapted sludge when exposed to the same BTA concentrations, statistical analysis revealed that the observed change is significant ($p < 0.05$).

In stage E (days 140 to 180), 40 mg/L BTA caused a decrease in the SAAs of the system. The SAAs of the BTA group continuously decreased from 0.213 to 0.120 g N₂-N/g VSS/day. The average NAAs was $40.9 \pm 9.2\%$, which was close to the theoretical value. When the BTA concentration increased to 40 mg/L levels, the NAAs of adapted sludge decreased to the same level as the unadopted sludge at 30%.

However, after BTA was completely removed from the system, the Anammox activity gradually recovered from 0.109 to 0.377 g N₂-N/g VSS/day in 30 days. The inhibition caused by BTA was reversible. Anammox granular sludge may have a limited ability to adapt to 40 mg/L BTA but can recover from the inhibition after BTA was removed by replacing the medium and the concentration was checked by HPLC. Achieving adaptation to the high concentration of inhibitors may take longer than expected and could involve several cycles of exposure and recovery phases (Q.-Q. Chen et al., 2019). This phenomenon has been reported in other N removal processes as well, such as nitrification. In full-scale wastewater treatment processes, adaptation is an important method for maintaining nitrification activity when treating industrial wastewater containing pollutants and the adaptation process could take months to years to achieve stable performance and has the possibility to fail (Dvořák et al., 2014).

According to **Figure 4.6**, during stages A to B (days 0 to 21), no inhibition ($\geq 10\%$) was observed when PA was lower than 100 mg/L. The SAAs of the two stages are 0.664 ± 0.054 and 0.607 ± 0.033 g N₂-N/g

VSS/day, respectively. The NAAs were $113.8 \pm 5.2\%$ and $107.6 \pm 14.1\%$, respectively. The theoretical NAAs were 100%, as PA was found not to be inhibitive to the Anammox process up to 300 mg/L in short-term batch tests (Lakhey et al., 2020). In stage C (days 22 to 85), 200 mg/L PA decreased the Anammox activity from 0.571 to 0.169 g N₂-N/g VSS/day within 47 days. The average decreasing rate was 0.008 g N₂-N/g VSS/day. The results indicate that long-term exposure to PA at 200 mg/L concentration could impact the Anammox activity.

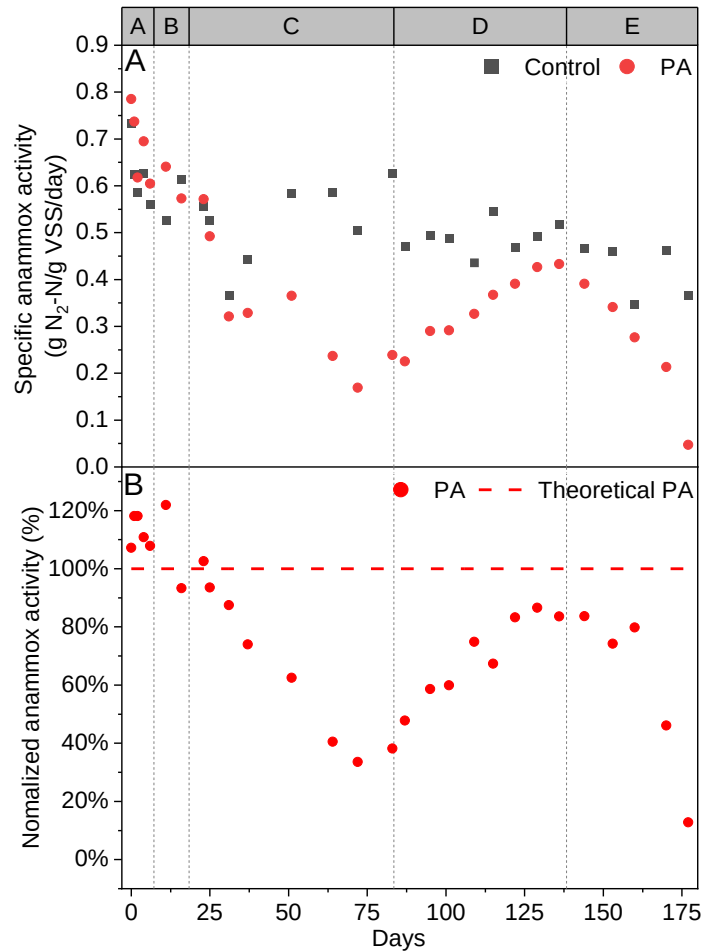


Figure 4.6 (A) SAAs of PA and control group; (B) NAAs of PA group and theoretical NAAs. In stage D (days 86 to 139), when PA concentration returned to 100 mg/L, approximately 80% of Anammox activity was recovered in 50 days, which suggests that the PA inhibition was reversible. In stage E (days 140 to 180), Anammox bacteria were exposed to 200 mg/L PA again. The Anammox

activity decreased from 0.391 to 0.047 g N₂-N/g VSS/day within 25 days. The average decreasing rate was 0.010 g N₂-N/g VSS/day. Anammox granular sludge was not adapted to the 200 mg/L PA. After stage E (days 140 to 180), PA was removed from the system by replacing the medium, and the concentration was checked by HPLC. The SAAs of the PA treatment group did not recover in the following month and the NAAs decreased to below 10% within 14 days. The results suggest that the second exposure to PA caused irreversible inhibition of the Anammox sludge. During the adaptation process, irreversible inhibition could happen if the Anammox bacteria were impaired or even died. In this study, long-term exposure to 200 mg/L PA caused more inhibition than short-term exposure. While limited information about the Anammox process's adaptation to azoles is available, previous studies also suggest that damaged Anammox sludge may not be able to recover from inhibition. Wastewater organic contents, such as toluene (IC₅₀ of 73 - 300 mg/L), sulfathiazole (IC₅₀ of 650 mg/L), and oxytetracycline (IC₅₀ of 250 -1100 mg/L) could have synergic or irreversible inhibition effect on the Anammox process (Hernández et al., 2013; Lakhey et al., 2020; T. Lotti et al., 2012). The Anammox bacteria may die out or lose their activity during the inhibition and are not capable of recovery (Jin et al., 2012).

Compared to the BTA group, PA caused less inhibition of the Anammox process. Anammox bacteria could resist PA inhibition up to 300 mg/L in a short-term batch test (within 48 hours). However, in this study, high concentrations of PA (larger than 200 mg/L) could slowly decrease the Anammox activity statistical analysis revealed that the observed change is significant ($p < 0.05$). This raises the question of how Anammox bacteria respond to the impact of azoles and potential mechanisms are discussed in the following sections.

Biodegradation of azole by acclimated Anammox sludge.

In this study, the concentrations of BTA and PA were monitored to evaluate the potential for degradation within the Anammox system. According to **Figure 4.7**, no significant BTA biodegradation ($p > 0.05$) was observed during the operation. Approximately 10% PA degradation was observed at a 200 mg/L concentration after day 143 ($p < 0.05$). The impact of hydrolysis and sludge adsorption on BTA and PA

could be negligible (Lakhey et al., 2021; Mazioti et al., 2015a). Previous studies have also reported that BTA and PA were not degraded under abiotic conditions (Kalyani V Jog et al., 2021; Lakhey et al., 2021). The degradation ability of Anammox bacteria on PA under inorganic substrate conditions was aligned with previous work (Lakhey et al., 2021). Even though the PA concentrations decreased on day 173, the activity of Anammox kept decreasing during that period. Thus, biodegradation may not be the dominant method for the Anammox process to alleviate the azole inhibition effect.

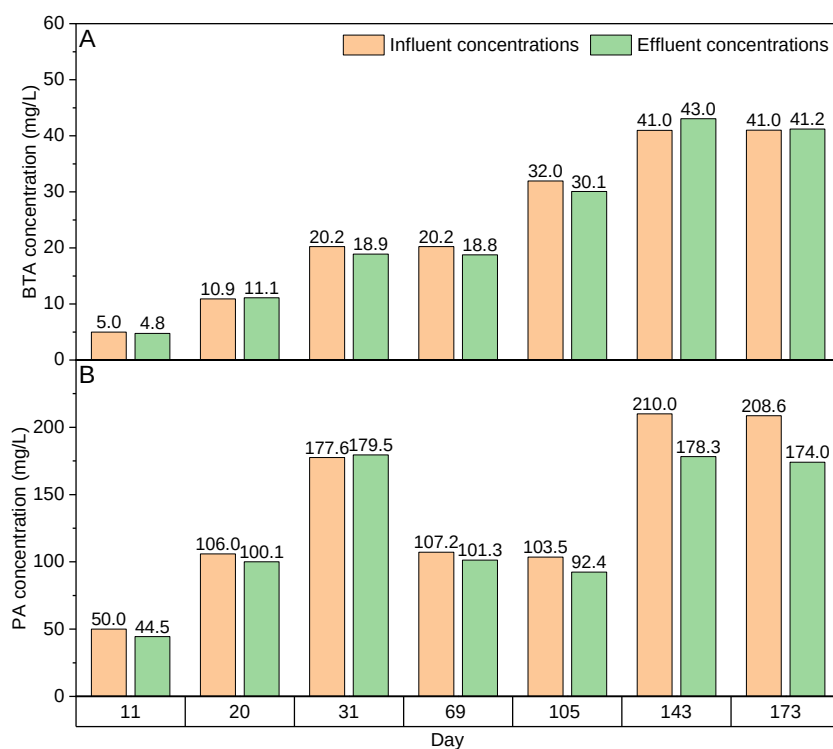


Figure 4.7 Azole concentrations in the anammox reactors over the operation (A) BTA (B) PA.

Long-term effect of azoles on the microbial community

The microbial community in the system was initially seeded using the inoculum sludge obtained from the same source, a 3.8 L laboratory-scale EGSB Anammox reactor. To illustrate the similarities and differences in the microbial composition of each reactor, inoculum samples and sludge samples at the end of the operation in each reactor (control, BTA, and PA) were collected and analyzed. In this study, 22905, 28145, 27192, and 27141 sequences were respectively detected from the Inoculum, Control, BTA, and

PA samples, corresponding to the operational taxonomic units (OTUs) of 99, 108, 106, and 105. As shown in **Figure 4.8**, the OTUs exclusively existed in Inoculum, Control, BTA, and PA were 6, 4, 10, and 5, respectively. A majority of OTUs (63.9%) coexisted in all four samples, while 81.2% of OTUs were shared by two or more samples, suggesting a stable community component in the samples.

The dominant bacteria in phylum levels of Anammox sludge are shown in **Figure 4.9**, with a focus on Anammox bacteria, which are uncultured representatives of the phylum Planctomycetes (Lodha et al., 2021). A higher abundance of *Planctomycetes* was observed in the Inoculum sample (58.5%) compared with Control (6.9%), BTA (1.8%), and PA (6.6%). Meanwhile, *Acidobacteria*, one of the most abundant phylogenetic groups detected in various environments (e.g., WWTP), increased from 2.8% in the Inoculum samples to 32.4%, 32.9%, and 46% in the Control, BTA, and PA samples, respectively (Kristensen et al., 2021; Marti et al., 2014). These results suggested long-term operation (180 days) of the Anammox reactor might result in a shift in dominant bacteria to adapt to a new environment, and such change was more substantial than the impact caused by exposure to azoles. Further analysis of the data was conducted to calculate the Shannon index and Simpson index of all samples. The results showed different values of Shannon and Simpson index in the Control (2.57 and 0.85), BTA (2.73 and 0.86), and PA (2.17 and 0.77) samples (**Table S3**), suggesting a potential change in microbial community composition after azoles exposure.

These results are consistent with a previous report where Anammox sludge was exposed to an aromatic compound, phenol (added stepwise from 0 to 300 mg/L), for 40 days. Their results showed that the Anammox microbial community may alter its composition to adapt to a new environment and/or the addition of inhibitors (Pereira et al., 2014). However, the samples analyzed in this study were collected at the end of the 180-day experiment when the Anammox sludge in BTA and PA reactors were exposed to 40 mg/L BTA and 200 mg/L PA, respectively. These azole concentrations were higher than the typical levels detected in wastewater. Therefore, it is suspected that the microbial community composition may show a higher stability to BTA and PA exposure at lower concentrations (< 10 mg/L).

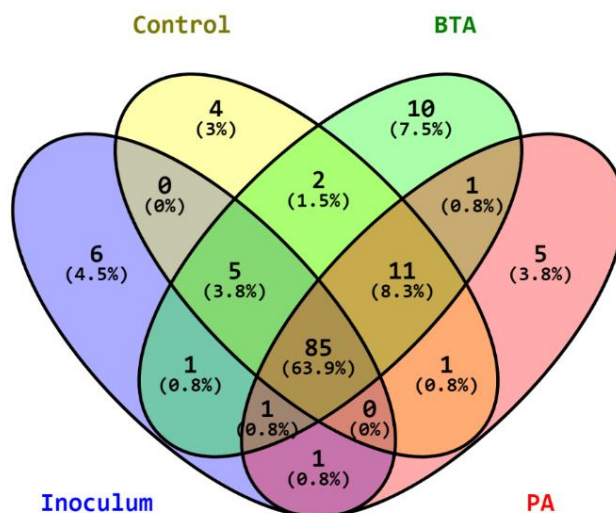


Figure 4.8 OTU numbers in Inoculum, Control, BTA, and PA samples at the end of the experiments.

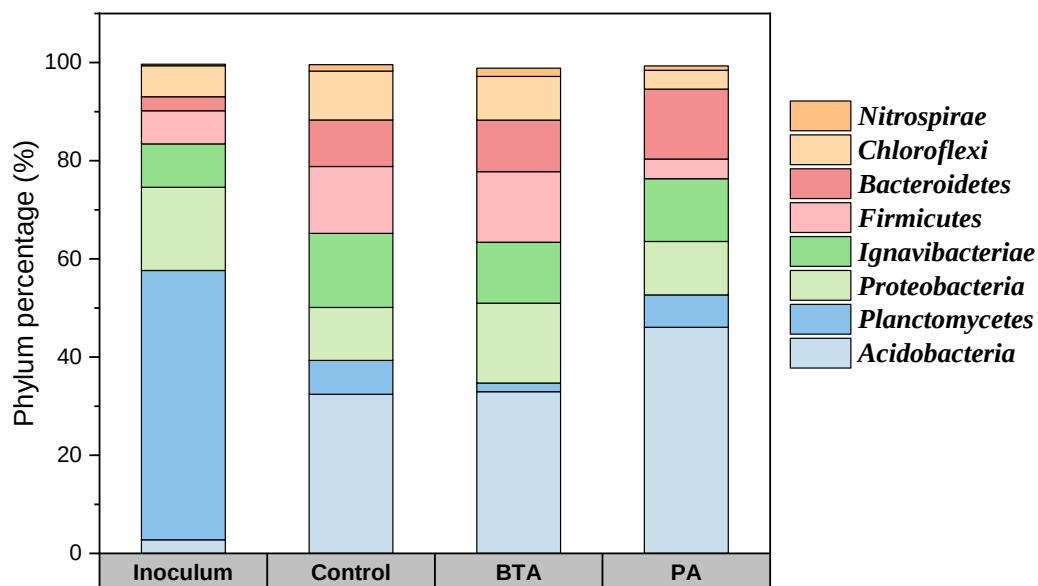


Figure 4.9 Relative abundance of dominant bacteria in phylum level of Anammox sludge in the Inoculum, Control, BTA, and PA samples.

Effect of substrate condition and energy status on Anammox's resistance to azoles

The influence of azoles on fresh/starved Anammox cells was assessed by measuring SAAs and the evolution of ATP content in the biomass over time. According to **Figure 4.10**, at high substrate conditions, with the increase of the starvation period from day 0 to day 14, the Anammox activity of the control group decreased from 0.986 ± 0.058 to 0.692 ± 0.079 g N₂-N/g VSS/day. The activities of the BTA and MBTA treatment group also decreased from 0.613 ± 0.008 to 0.451 ± 0.055 g N₂-N/g VSS/day and from 0.605 ± 0.032 to 0.528 ± 0.0147 g N₂-N/g VSS/day, respectively. These results suggest that an increase in the starvation period leads to a decrease in Anammox activity. The decrease in overall Anammox activity may be caused by the decrease in the amount of viable cells or a decrease in the specific activity of viable bacteria (Qilin Wang et al., 2018). In comparison to the control, both the BTA and MBTA groups continued to induce inhibition of the Anammox process. However, the inhibition effect was not further significantly increased ($p < 0.5$) with the increase of the starvation time (From day 5 to day 14), which suggests that the starved anammox sludge maintained its resistance to azole inhibition during the starvation.

According to **Figure 4.10**, under low substrate conditions, the SAAs of the control group decreased from 0.373 ± 0.021 to 0.232 ± 0.021 g N₂-N/g VSS/day after 5 days of starvation and to 0.307 ± 0.003 g N₂-N/g VSS/day after 14 days starvation. Under low substrate conditions, the Anammox activity was decreased when compared to the high substrate conditions. The results were aligned with the observation reported in the literature, Y. Wang et al. (2019) reported that a decrease in substrate concentration from 45.2 to 7.9 mM led to decreased Anammox activity by about 50% (from 0.4 to 0.21 g N g⁻¹ VSS day⁻¹). Similarly, further increasing starvation period decreased the Anammox activity under low substrate conditions. In comparison to the NAAs under high substrate conditions, both the BTA and MBTA treatment groups experienced further inhibition under low substrate conditions. However, the inhibition effect did not increase with the increase in starvation time.

Table 4.3 summarizes the lag phase of each group during the activity tests. The data are illustrated in **Figure S6**. In the high substrate control group, the lag phase increased from < 0.5 hours to 3.67 hours after 14 days, suggesting that starvation may lead to a delay in Anammox cells utilizing substrates. Comparatively, in the low substrate control group, the Anammox process exhibited a shorter lag phase under low substrate conditions. The difference could be caused by the substrate concentrations. The starved Anammox cells may become more sensitive to substrate inhibition and this results in a longer lag phase. Carvajal-Arroyo et al. (2014) reported that the nitrite IC₅₀ of starved Anammox cells was 7 times lower than in fresh cells, which are 7 mg NO₂-N/L and 52 mg NO₂-N/L, respectively. The lag phase of the BTA and MBTA treatment group increased on day 5 but was approximately the same level as the control on day 14. After 28 days, no gas production was observed for 12 hours for all groups. From the results in **Figure 4.10**, the Anammox process was resistant to starvation and could maintain 70% of the activity content level within 14 days. This observation was consistent with findings from previous studies, suggesting that Anammox bacteria can maintain cell integrity, and most intracellular proteins remain stable during starvation (X. Ma et al., 2018; Xing et al., 2016). In a full-scale system, Anammox sludge was also able to recover within one day after being starved for 2 months (Reeve et al., 2016).

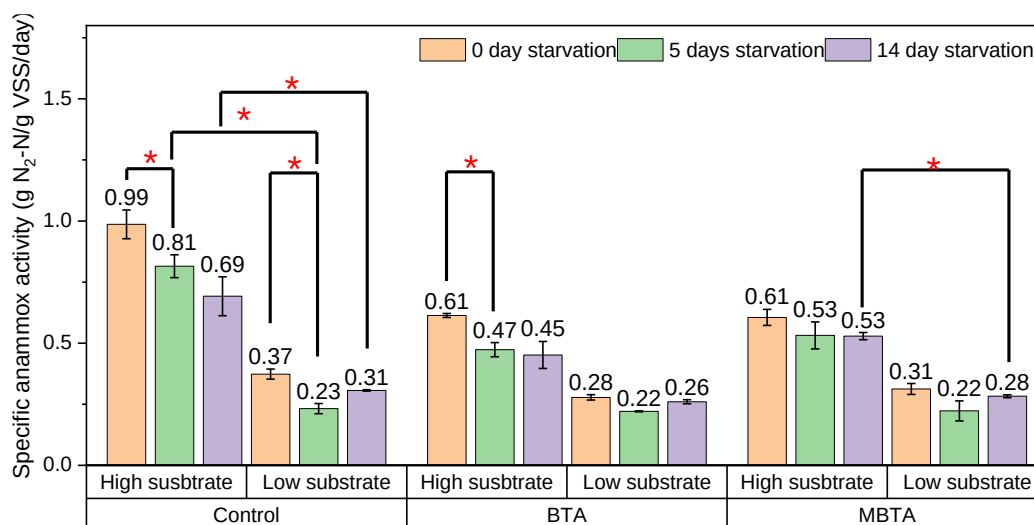


Figure 4.10 Anammox activities during different starvation period. The red color symbols above the columns indicate the p -value between the control group and treatment groups under one category (e.g., 0, 5-, and 14-days starvation experiment data under high substrate condition of control groups. *: $p \leq 0.05$. All experimental groups were conducted in duplicate.

Table 4.3 Lag phase period under different substrate and starvation conditions

Substrate condition	Group	Starvation period			
		Day 0	Day 5	Day 14	Day 28
High substrate condition	Control	<0.5	<0.5	3.68	>12
	BTA	0.96	1.09	2.87	>12
	MBTA	<0.5	1.16	3.12	>12
Low substrate conditions	Control	<0.5	<0.5	<0.5	>12
	BTA	<0.5	<0.5	<0.5	>12
	MBTA	<0.5	<0.5	<0.5	>12

According to **Figure 4.11**, no significant differences ($p > 0.5$) between the BTA and MBTA starvation and pre-exposure group were observed in 14 days starvation. After 5 days starvation period, the SAAs of the BTA starvation group was 0.761 ± 0.010 g N₂-N/g VSS/day while the pre-exposure group was 0.680 ± 0.010 g N₂-N/g VSS/day. At the same time, the MBTA starvation group and pre-exposure group have the same SAAs. After 14 days of starvation, the BTA pre-exposure group is more inhibited than the starvation group. However, the statistic results ($p = 0.23$) suggest the observation is not statistically significant. After 28 days, the Anammox activity dropped below 0.2 g N₂-N/g VSS/day, suggesting that prolonged starvation can affect Anammox activity. After 28 days, the SAAs of all the groups are inhibited. Significant differences ($p < 0.5$) between the high substrate and low substrate groups were observed at 5 days starvation. After 28 days starvation, the BTA and MBTA pre-exposure groups had a higher activities at low substrate conditions when compared to high substrate conditions ($p < 0.5$).

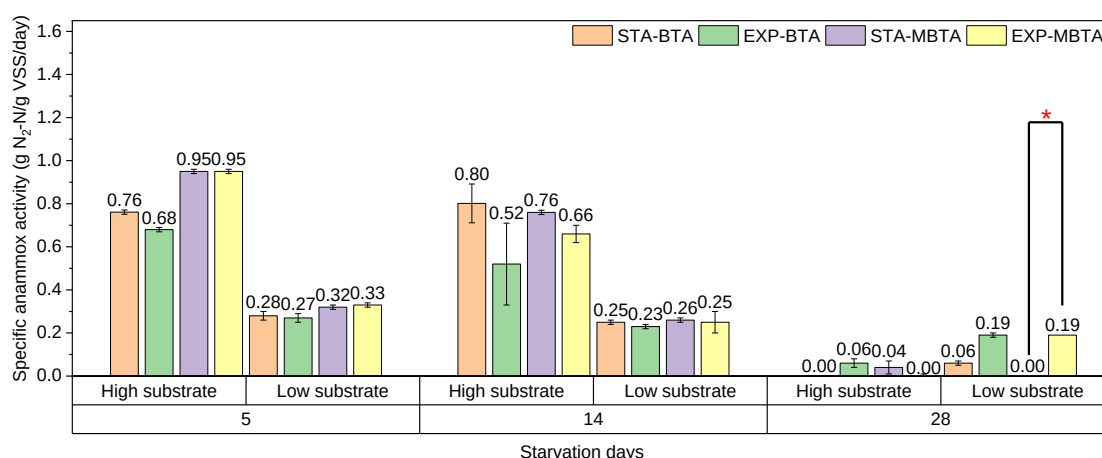


Figure 4.11 SAAs of preexposure experiment at different substrate concentrations. STA: starvation only; EXP: starvation and pre-exposure. The red color symbols above the columns indicate the p -value between the STA group and EXP groups under one category (e.g., 0, 5-, and 14-days starvation experiment data under high substrate condition). *: $p \leq 0.05$. All experimental groups were conducted in duplicate.

Potential defense mechanisms of Anammox granular sludge to azole inhibition

Previous studies have proposed several potential mechanisms by which Anammox bacteria could resist inhibitors. In this study, no degradation of BTA was observed. PA was partially degraded (<10%) after 143 days but the degradation didn't contribute to maintaining Anammox activity. Thus, biodegradation may also not be the major method Anammox bacteria adapted for defense against BTA and PA inhibition.

Previous studies have found the Anammox bacteria invest energy in mitigating the inhibition of nitrite and antibiotic accumulation, which involves the transportation of nitrite out of the Anammox cells (Carvajal-Arroyo et al., 2014; Qian Wang et al., 2023; Yu et al., 2020). To analyze if the Anammox process invests energy in mitigating azole inhibition. The energy status of the Anammox granular sludge was determined through the ATP content analysis. **Figure 4.12** illustrates the ATP content changes within 24 hours at different starvation periods. The ATP content of the Anammox granular sludge decreased from day 0 to day 5 and day 14 kept approximately the same level of ATP from day 5. Maximum ATP levels were observed in fresh cells, the initial ATP content of the control, BTA, and MBTA were 0.172, 0.120, and 0.139 mg ATP/ mg VSS, respectively. After 24 hours, the ATP content decreased to 0.123,

0.094, and 0.0840 mg ATP/ mg VSS. After 5 days of starvation, the ATP content of the three groups was approximately the same level at 0.045 to 0.105 mg ATP/ mg VSS and after 14 days, the ATP content ranged from 0.055 to 0.095 mg ATP/ mg VSS. The statistical data suggest that the results show no significant differences between the control groups and treatment groups' ATP content levels ($p > 0.05$).

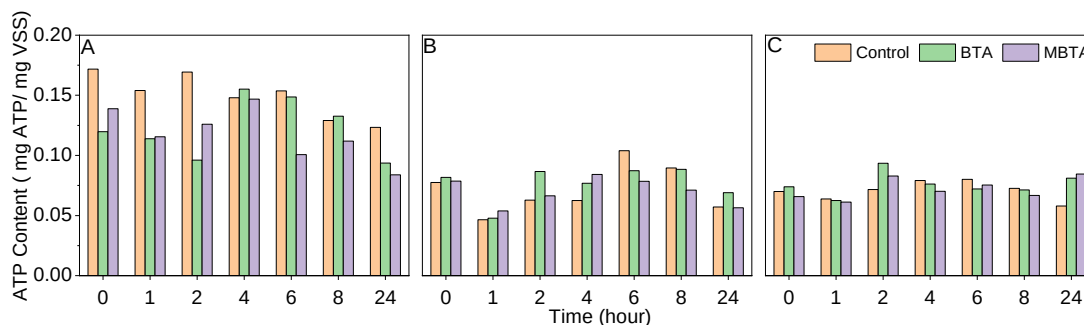


Figure 4.12 ATP content of Anammox granular sludge during starvation time (A) 0 days (B) 5 days (C) 14 days

According to **Figure 4.12**, no increases in ATP were observed after the addition of azoles into the system. This suggests that the Anammox granular sludge didn't regulate its ATP content as a response to azole addition. During short-term exposure, the Anammox granular sludge may not invest extra energy to resist the inhibition caused by BTA and MBTA.

In this study, one hypothesis is that Anammox bacteria may not invest energy immediately to avoid the impact of azole inhibition. During the starvation experiments, BTA and MBTA did not exacerbate inhibition on starved Anammox cells, and no ATP response to BTA and MBTA addition was observed. Therefore, short-term energy-dependent mitigation methods may not be the primary defense mechanisms for the Anammox process against azole inhibition. Several studies hypothesized that the azole compounds may bind to the metal center enzymes and result in the inhibition of the enzyme activities (Lakhey et al., 2020; McCarty, 1999). The unique cell structure of Anammox bacteria, such as membrane ladderane lipids, crateriform structures of the outer membrane, and extracellular polymeric substances (EPS), may serve to prevent contact between inhibitors and Anammox cells or slow down the penetration process (Ozumchelouei et al., 2022). The Anammox microbial communities may regulate their protein or key

enzyme levels, change their membrane properties, and produce more EPS to prevent inhibitor chelation (e.g. Composition and fluid) for adapting to new environments or shocks (Morgan-Kiss et al., 2006; Z.-Z. Zhang et al., 2015). Once the protection structures are built up, abundant energy investment may not be needed to prevent azole inhibition.

Implications

BTA and PA could cause inhibition to the Anammox process in long-term exposure. At the same time, the Anammox process could gain resistance to 0-30 mg/L BTA through long-term acclimation. However, the effect of acclimation also depends on the concentration and inhibitors. Long-term exposure to 200 mg/L caused more inhibition of the Anammox process than short-term exposure and could cause irreversible inhibition. The results indicate that long-term exposure to azoles may cause more inhibition of the Anammox processes. BTA was not degraded in the Anammox system, and less than 20% degradation of PA was observed, which further increased the possibility of long-term exposure to azoles in the Anammox process implemented system. Starvation of the Anammox bacteria didn't increase the inhibition of azoles in the Anammox process, which suggests that Anammox bacteria may not rely on the short-term energy required method in mitigating the azole inhibition. Overall, acclimation to low concentrations of BTA could enhance Anammox resistance to BTA inhibition. At the same time, high concentrations of BTA and PA exposure may cause failure of Anammox acclimation and disrupt the system performance. For the WWTPs going to receive azole-containing wastewater, the concentrations of azoles in the influent should be carefully checked to avoid inhibition. WWTPs could gradually increase the amount of azole-containing wastewater they are going to receive. During the process, the stability of the Anammox process could be enhanced through acclimation and monitoring of the azole concentrations.

Chapter 5: Long-term Impact of Pyrazole and Benzotriazole on Nitrification: System Adaptation and Azoles Biodegradation

Abstract

Azoles have been widely reported in wastewater effluents and receiving waters, raising concerns about their potential toxicity to different biological wastewater treatment processes. In this study, the long-term impacts of azoles on the nitrification process were investigated using lab-scale sequence batch reactors (SBRs). Benzotriazole (BTA) and Pyrazole (PA) were selected as representatives of azoles due to their widespread usage and their structure as basic triazole and diazole. The results showed that nitrifiers could adapt to 2.5 mg/L BTA and 2.7 mg/L PA addition. The adapted nitrification sludge exhibited better performance with the same BTA and PA concentrations than the unadopted sludge.

The first-time addition of 2.5 mg/L BTA and PA led to a decrease of 45% and >90% in nitrification activity, respectively. After acclimation, no ammonium and nitrite accumulation were observed in the effluent when 2.5 mg/L BTA and 2.7 mg/L PA were added into the system. The normalized nitrification activity (NNA) of the BTA and PA group were $72.5 \pm 2.5\%$ and $70.2 \pm 2.5\%$, which were higher than NNAs during the first-time exposure and the theoretical values from short-term tests. The nitrification sludge developed BTA degradation ability and high degradation rates were achieved during the acclimation process. The removal of nitrogen (N) loading didn't impact the BTA degradation process while the addition of extra 200 mg/L COD could decrease the BTA removal rate. The heterotrophic bacteria that existed in the nitrification sludge may contribute to the BTA degradation. The relative abundance of reported aromatic compound/organic compound degraders such as *Dechloromonas* and *Zoogloea* increased in the BTA microbial community.

Introduction

Emerging organic contaminants (EOCs) have been widely applied as pesticides, pharmaceuticals, personal care products, and endocrine-disrupting chemicals, and their occurrences and fates are increasingly gaining attention due to their potential risks to both environmental and human health (Ben et al., 2018; García et al., 2020; J. Li et al., 2021). Azoles, classified as EOCs, are a group of man-made chemicals containing N atoms with heterocyclic ring systems and have been widely used for more than 4 decades detected in wastewater treatment plants (WWTP) effluents and industrial effluents (Z.-F. Chen et al., 2015; Jørgensen et al., 2021; G. Li et al., 2020; Shi et al., 2019). Previous studies show that conventional water treatment processes in WWTPs have limited efficiency in removing EOCs (García et al., 2020; J. Li et al., 2021). BTA and PA are two typical azole compounds that have been found in different water matrices including surface water, groundwater, and WWTPs at concentrations ranging from ng/L to high µg/L (Careghini et al., 2015; G. Li et al., 2020; Shi et al., 2019). In some cases, BTAs have been found up to mg/L level in groundwater below the deicing pad, the regeneration plant, and the snow disposal site (Breedveld et al., 2003). Due to the high demand for azoles, the United States Environmental Protection Agency (EPA) has listed BTA and PA's derivatives as high-production volume chemicals, which means the total production and import of these chemicals exceeds one million pounds per year (U. S. EPA, 2023). The detection of PA in Dutch surface water caused an interruption of drinking water production for 138 days (Piai et al., 2022).

N in wastewater presents in the forms of ammonia, nitrite, nitrate, and/or organic N. Ineffective treatment of N-containing wastewater could cause eutrophication when discharged into water bodies and threaten the safety of the ecosystem and water quality for human beings (Hu et al., 2019). To meet the nutrient discharge requirements of the WWTPs, the nitrification process has been applied widely in favor of the physical-chemical processes (Y. Peng et al., 2006). The nitrification process is carried out by the ammonium-oxidation bacteria (AOB) together with the nitrite oxidation bacteria (NOB), which converts the ammonium to nitrite and nitrite to nitrate, respectively (S. Li et al., 2021). In WWTPs, the nitrification

and denitrification processes are generally carried by the activated sludge, which is composed of a variety of microorganisms including nitrifying bacteria and other autotrophic and heterotrophic microorganisms in aerobic wastewater treatment processes for the removal of nutrients and contaminants. The nitrification process could be impacted by a variety of chemical inhibitors that exist in wastewater such as heavy metals, antibiotics, fertilizer, and other organic compounds (Juliastuti et al., 2003; Xing et al., 2018; L. Yang et al., 2022). Thus, the existence of azoles in WWTPs may become a potential threat to the stability of the biological nitrogen removal (BNR) processes. The short-term impact of azoles on nitrification has been investigated and azoles including BTA and PA could cause inhibition (>50%) to the nitrification process at <1 to 3 mg/L concentrations (G. Li et al., 2020; Pagga et al., 2006). However, limited information is available about the long-term impact of azoles on nitrification processes. The potential inhibition mechanisms of azole in the nitrification process, the guidelines for preventing azole inhibition, and methods for system recovery are also developed. The research question is whether the nitrification process could adapt to long-term azole exposure. Nitrifiers are found able to adapt to known inhibitors such as trimethoprim and benzalkonium chlorides during the long-term exposure experiment, in which gradual adaptation was observed (J. Chen et al., 2021; Schmidt et al., 2012). Understanding the long-term impacts of azoles and the potential adaptation/acclimation of nitrification microorganisms to azole inhibition is important.

The role of activated sludge on EOC degradation has been studied and the biological degradation of BTA and PA under different conditions has been reported. In WWTPs, the removal of BTA and PA depended on the compound's chemical structure and properties, the characteristics of the feeding wastewater, the microbial community composition of the sludge, the operation environment, and the absence or presence of an acclimation period (Mazioti et al., 2015a; Pagga et al., 2006; Shi et al., 2019; Trejo-Castillo et al., 2021). PA is found persistent in the wastewater environment with activated sludge systems without specific treatment while BTA was more easily degraded with activated sludge systems (Kalyani V Jog et al., 2021; Piai et al., 2020; Piai et al., 2022). However, controversial regarding the BTA and PA

biodegradation pathways and mechanisms are reported. Pyrazole was degraded in all batches with/without an additional nitrogen source (235 mg N/L ammonium or 10 mg N/L nitrate) when pyrazole was present at an initial concentration of 6.8 mg/L, which suggest additional nitrogen source is not necessary for PA degradation (Piai et al., 2022) In another study, Kalyani V Jog et al. (2021) reported the addition of external co-substrates (glucose and NH_4^+) was necessary to accelerate the removal of PA. For BTA biodegradation, either heterotrophic bacteria or nitrifying bacteria have been hypothesized as the potential BTA degrader, and different pathways including ammonia monooxygenase (AMO) involved oxidation, utilized as carbon source and co-metabolism were discussed (Y.-S. Liu et al., 2013; Mazioti et al., 2015b; Trejo-Castillo et al., 2021; J. Wang et al., 2022). Therefore, in this study, the impacts of long-term exposure of BTA and PA to the nitrification process are evaluated, and the potential inhibition mechanism and the role of nitrifying bacteria and/or heterotrophic bacteria in BTA removal are investigated.

Materials and methods

Chemicals and microorganisms

Benzotriazole (BTA) (CAS # 95-14-7, purity > 99%) was purchased from Alfa Aesar (Ward Hill, Massachusetts, USA), Pyrazole (PA) (CAS # 288-13-1, purity > 98%) was purchased from Tokyo Chemical Industry (Tokyo, Japan).

The suspended sludge used for studying the toxicity of azoles to the nitrification process in this work was activated sludge obtained from an aeration zone of a WWTP (Laurel, MD, USA), in which the nitrification process was known to happen. All received sludge was immediately stored at 4 °C to maintain the microbial activity. The sludge was collected and stored in a cooler for 1 hour before the bioassay. The nitrifying rate of the collected sludge was in the range of $2.5 - 4.0 \pm 0.5$ mg N per gram of suspended solids per hour (mg N/g VSS/hour), which met the required criteria for testing (ISO-8192, 2007; ISO-9509, 2006). The sludge was collected one day before the toxicity bioassays and washed by

using chlorine-free tap water. Tap water was collected 24 hours before the experiment and stored in an open container to remove the chlorine. The washing procedure is carried out by centrifuging (1,000 g for 5 min) and discarding the supernatant liquid. Then the sludge was resuspended with chlorine-free tap water and the procedure was repeated two times. The TSS of washed sludge was determined through a standard method (American Public Health Association, 2023). Finally, re-suspend the washed sludge in an appropriate volume of chlorine-free tap water to give the TSS of 3 g/L and aerate until use.

Reactor setup and operation

The study was carried out in three laboratory-scale reactors with a working volume of 0.9 L each. The three reactors were operated automatically as SBRs at room temperature (27 ± 2 °C). Mixing was offered by a stir station at 120 rpm. The pH was maintained at $\text{pH } 7.5 \pm 0.5$ using 0.25 M NaOH and 0.25 M HCl. The SBR systems were operated identically in cycles of 12 h including a 10 min fill period, an 11 h reaction period, 40 min for settling, and a 10 min effluent discharge period. The SBR was fed with a synthetic medium containing ammonia and nutrients. 450 ml was pumped out of the reactor during the effluent discharge period. The reactors have a volumetric exchanging ratio of 0.5 and a hydraulic residence time of 24 hours. The total suspended solids (TSS) of the reactor were controlled at 1.2 g TSS/L by the removal of the biomass in the effluent or the addition of extra sludge picked from the WWTP. Aeration was provided during the reaction and DO concentration was maintained at 4 to 7 mg/L. The sludge retention time (SRT) in the three reactors was 100, 70, and 54 days respectively. The three reactors were continuously monitored (online measuring of DO and pH), and the effluent NH_4^+ , NO_2^- , NO_3^- , and azoles were sampled every three days in triplicate. The TSS and volatile suspended solids (VSS) were determined weekly in triplicate.

Nitrifying activated sludge was brought from a domestic wastewater treatment plant and was used as an inoculum to seed all three reactors at the same time. After inoculum for three weeks and all reactors reached steady-state conditions, the three reactors were operated in multiple stages as described in **Table 5.1**. SBR reactor control (Control) was operated as a reference control reactor (no azole added). SBR

reactor BTA (BTA) was the BTA treatment group and BTA was added as described in **Table 5.1**. SBR reactor PA (PA) was the PA treatment group and PA was added as described in **Table 5.1**. The operation of the reactor was divided into two parts. (1) Shock loading of low and high concentrations of azoles. (2) (Stage C to end) Recovery phase and re-loading of azoles with increasing azole concentrations. The synthetic medium was prepared with de-mineralized water and had the following composition: NH_4Cl 305 mg/L as ammonia source, $\text{KH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ 222 mg/L, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 108 mg/L, NaCl 890 mg/L, NaHCO_3 900 mg/L, $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ 5 mg/L, nutrient solution 0.3 mL/L. 10 ml nutrient solution was added to the 1L medium to enhance the microbial growth and contained 1.5 g of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 0.15 g of H_3BO_3 , 0.03 g of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.18 g of KI , 0.12 g of $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.06 g of $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.12 g of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.15 g of $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, and 10 g of EDTA in one liter of de-mineralized water.

Table 5.1 Operation stages of nitrification reactors

Group	Stage name	Days	Azole addition (mg/L)
BTA	BTA - A	0 - 4	0.5
	BTA - B	5 - 7	2.5
	BTA - C	8 - 21	0
	BTA - D	22 - 40	0.5
	BTA - E	41 - 68	0
	BTA - F	69 - 85	0.5
	BTA - G	86 - 250	1
	BTA - H	251 - 280	2.5
PA	PA - A	0 - 4	0.5
	PA - B	5 - 7	2.5
	PA - C	8 - 68	0
	PA - D	69 - 85	0.25
	PA - E	86 - 178	0.5
	PA - F	179 - 212	1
	PA - G	212 - 280	2.7
Control	Control - A	0 - 280	0

Nitrification activity assays

Inhibitory effects of selected azole compounds on nitrification activity were tested by using a modified method following the procedure described in the International Organization for Standardization (ISO) method 9509 (ISO-9509, 2006). The procedure is described as follows: The three reactors were operated automatically at room temperature (27 ± 2 °C). Mixing was offered by a stir station at 120 rpm. The pH was maintained at $\text{pH } 7.5 \pm 0.5$ using 0.25 M NaOH and 0.25 M HCl. The azoles were provided in the influent solution section (as shown in **Table 5.1**) containing 305 mg/L NH_4Cl and the medium mentioned in the previous section. Every batch test was conducted in a new reaction cycle after the filling stage finished, 2 ml of liquid samples were collected at 0, 45, 90, 135, 180, 240, 300 and 360 mins for measuring the NH_4^+ , NO_2^- , NO_3^- and azoles during the monitored cycle. The samples were centrifuged immediately (12000 rpm, 10 min). The supernatants were transferred to clean vials and analyzed for ammonium, nitrite, nitrate, and azoles. For COD measurements, 5 ml liquid samples were collected and filtered with a $0.45 \mu\text{m}$ syringe filter. For mechanism investigation batch tests, extra COD, ammonium, nitrite, and/or ATU were offered by adding stock solution by the end of the filling stage.

Analytical methods

Liquid sample analysis

The liquid sample was collected from the reactor and filtered through $0.45 \mu\text{m}$ PP filters to measure the concentration of target azole compounds. The concentrations of PA and BTA were measured through a developed method (Lakhey et al., 2021) with minor modifications. The details of the method are described below: Concentrations of PA were analyzed using high-performance liquid chromatography (HPLC) equipped with a diode array detector. Chromatography was performed using the InfinityLab Poroshell 120 column ($2.7 \mu\text{m}$, $4.6 \times 100 \text{ mm}$; C-18 column, Agilent, USA). An isocratic mixture of 10% methanol and 90% water was used as the eluent for a run time of 12 min. The eluent flow rate was 1

mL/min and the injection volume was 20 μ L. The temperature was maintained at 40°C and the wavelength for PA was 210 nm, respectively. Concentrations of azole compounds BTA were analyzed using the same instrument, with an isocratic mixture of 60% methanol and 40% water. The run time was 8 min, and the eluent flow rate was 1 mL/min and the injection volume was 20 μ L. The temperature was maintained at 40°C and both chemicals are detected at wavelength of 275 nm. Ammonium, nitrate, and nitrite in liquid samples were analyzed by SEAL AQ300 discrete analyzer in triplicate. (Mequon, WI, USA). During the measurement, the target compounds with known concentrations were added for continuing calibration verification (CCV) to every 10 samples for quality control. Less than 10% differences between the measured concentrations and the known concentrations were required to accept the measurement results. DO in liquid samples was measured using an optical O₂ probe. (LBOD101 Sensor, Hach, Loveland, CO, USA). Other parameters (TSS, pH, etc.) were determined according to standard methods (American Public Health Association, 2023). The limit of detection (LOD) and limit of quantitation (LOQ) of the analytical instruments are listed in **Table S6**.

DNA extraction and microbial analysis

The inoculum sludge samples were collected at the beginning of the experiment and the nitrification sludge samples of each reactor (Control, BTA, and PA) were collected at the end of the experiment. 1 ml of sludge sample with a TSS of 2 g/L was used for the DNA extraction. The DNA was extracted using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc, Carlsbad, California, United States). The extracted DNA samples were sent to Molecular Research LLP (Shallowater, TX) for analyzing their bacterial composition with 16S rRNA V4 variable region (PCR primers 515/806) through gene amplicon sequencing. The samples are treated through barcoded amplicon sequencing processes by MR DNA® under the trademark service (bTEFAP®). Negative controls (Blank medium sample without sludge) and positive controls (reference wastewater sludge samples) were used to eliminate background DNA interference. Sequences are joined, sequences <150bp, and sequences with ambiguous base calls are removed. Sequences were quality filtered using a maximum expected error threshold of 1.0 and

dereplicated. The dereplicated or unique sequences are denoised; unique sequences identified with sequencing and/or PCR point errors are removed, followed by chimera removal, thereby providing a denoised sequence or operational taxonomical units (OTU). Final OTUs were taxonomically classified using BLASTn against a curated database derived from NCBI (MR DNA, Shallowater, TX, USA).

Data processing

The specific nitrification activities (SNA) in different test groups were calculated according to (G. Li et al., 2020). The rate was calculated using linear regression of five or more consecutive points. The regression analysis was carried out using Microsoft Excel. The nitrification normalized activity (NNA) was calculated as shown in Eq 5.1:

$$NNA(\%) = \frac{SA_t}{SA_c} * 100 \quad (5.1)$$

where SA_t and SA_c (both as g N_2 g/VSS/day) are the specific activities of the treatment (with added azoles) and control experiment (without added azole), respectively. The NNA acquired from nitrification activity assays was compared to theoretical NNA from the batch tests using unadopted nitrification sludge from literature and preliminary results from our lab (G. Li et al., 2020).

T-test is used for statistical analysis of the relationship between azole concentration and the activity of the BNR processes. A p -value less than 0.05 is required to reject the null hypothesis.

Results and discussions

Performance of long-term nitrification reactors with azole addition

After a three-week start-up phase preceding day 0, all three reactors reached steady-state conditions. Throughout the 280 days of operation, the control group has maintained stable performance, achieving a high removal rate of ammonium and nitrite. According to **Figure 5.1**, no accumulation of ammonium and nitrite was observed during the process. The ammonium loading rate (ALR) was 0.066 ± 0.007 kg

N/m³/day, and the ammonium removal rate (ARR) was 0.065 ± 0.008 kg N/m³/day. Ammonium removal efficiency (ARE) reached $97.1 \pm 4.1\%$ and the nitrite accumulation rate (NAR) remained below 2% throughout the operational period. The sludge retention time (SRT) for the three reactors is 100, 71, and 54 for the control, BTA, and PA groups, respectively. These results suggest that the control group is being operated effectively, serving as a valuable reference for comparing the impacts of azoles on the nitrification process.

The concentrations of BTA were added into the reactor as described in **Table 5.1**. In Stage A (day 0 to 4), an initial shock loading of 0.5 mg/L BTA resulted in the accumulation of 13.2 mg N/L ammonium and 34.6 mg N/L nitrite in the effluent. The ARR and Nitrite removal rate (NRR) were 0.038 and 0.017 kg N/m³/day, respectively, with an initial ALR of 0.068 kg N/m³/day. The ARE decreased to 79.11%, and the NAR increased from 0% to 44.1%. The introduction of BTA decreased nitrification activity. The results indicate that a shock loading of 0.5 mg/L BTA can cause considerable inhibition of the nitrification process.

In Stage B (days 5 to 7), a shock loading of 2.5 mg/L BTA was introduced into the system. However, less ammonium (7.9 mg NH₄⁺-N/L) and nitrite accumulation (0.3 mg NO₂⁻-N/L) were observed in the effluent. Higher ARR at 0.038 kg N/m³/day and NRR at 0.072 kg N/m³/day were also noted. ARE increased from 79.1% to 90.1%, and NAR decreased from 44.1% to 0.4%. In this stage, the results showed that 2.5 mg/L BTA caused less inhibition on nitrification reactors compared to the first exposure to 0.5 mg/L BTA. The system entered a recovery phase in stage C (days 8 to 21) and then was loaded with 0.5 mg/L BTA again in stage D (days 22 to 40). During the recovery phase, the system fully recovered, with increasing ARE (84% to 91%) and low effluent ammonium and nitrite (0 to 6 mg/L). In Stage D (days 22 to 40), when exposed for the second time to 0.5 mg/L BTA, the nitrification system maintained above 96% ARR and less than 8% NAR. The re-addition of 0.5 mg/L BTA did not affect the nitrification process. Taken together, the nitrification sludge in the BTA treatment group may have developed stronger resistance to BTA inhibition during the acclimation process.

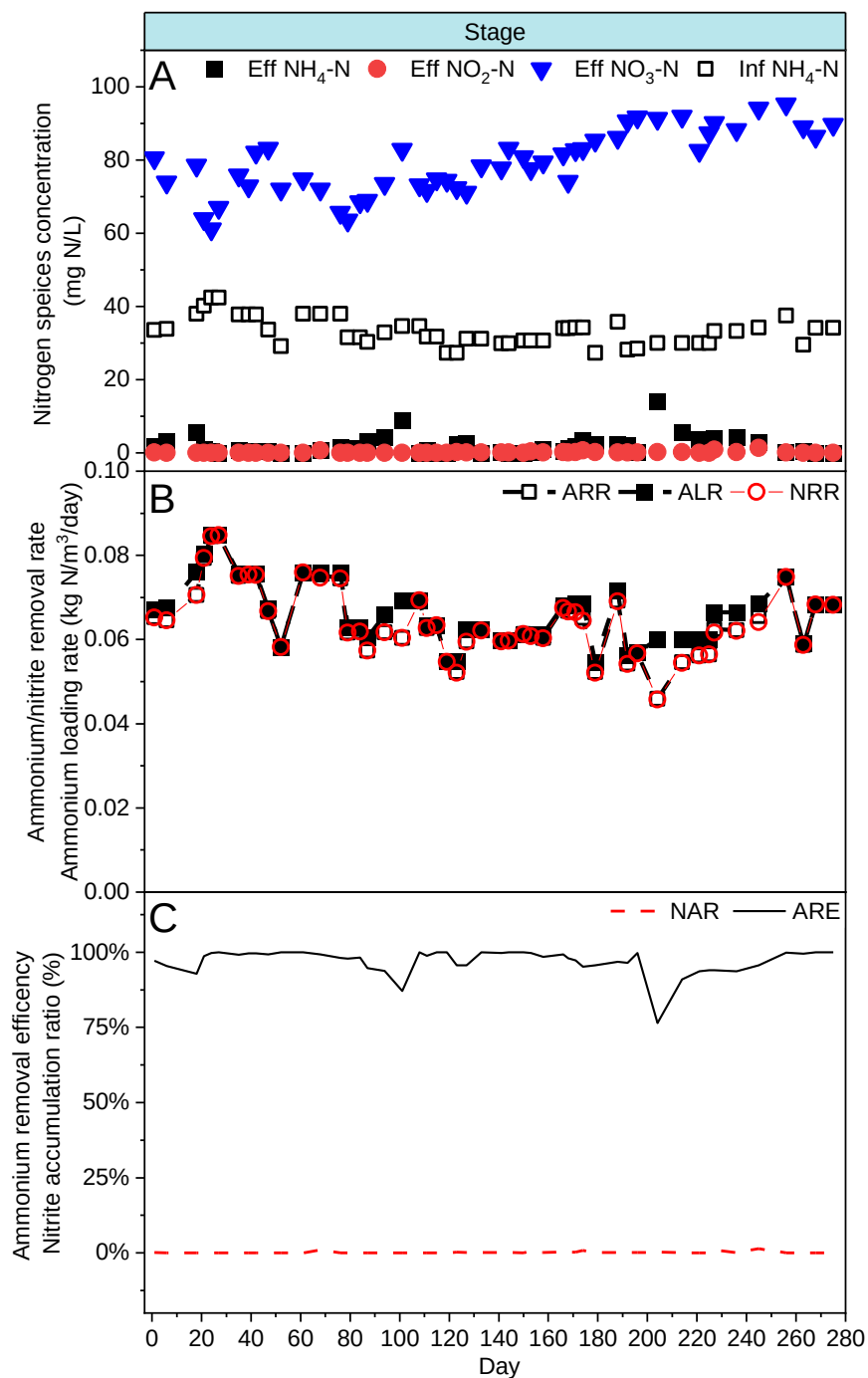


Figure 5.1 Control group N removal performance during operation. A) Influent and effluent N species; (B) ALR: $\text{NH}_4^+\text{-N}$ loading; ARR: $\text{NH}_4^+\text{-N}$ removal rate; NRR: $\text{NO}_2^-\text{-N}$ removal rate. (C) ARE: $\text{NH}_4^+\text{-N}$ removal efficiency; NAR: $\text{NO}_2^-\text{-N}$ Accumulation ratio. A power shut down happened on day 41 and lasted for 3 days. The system was restarted from recovery phase after it.

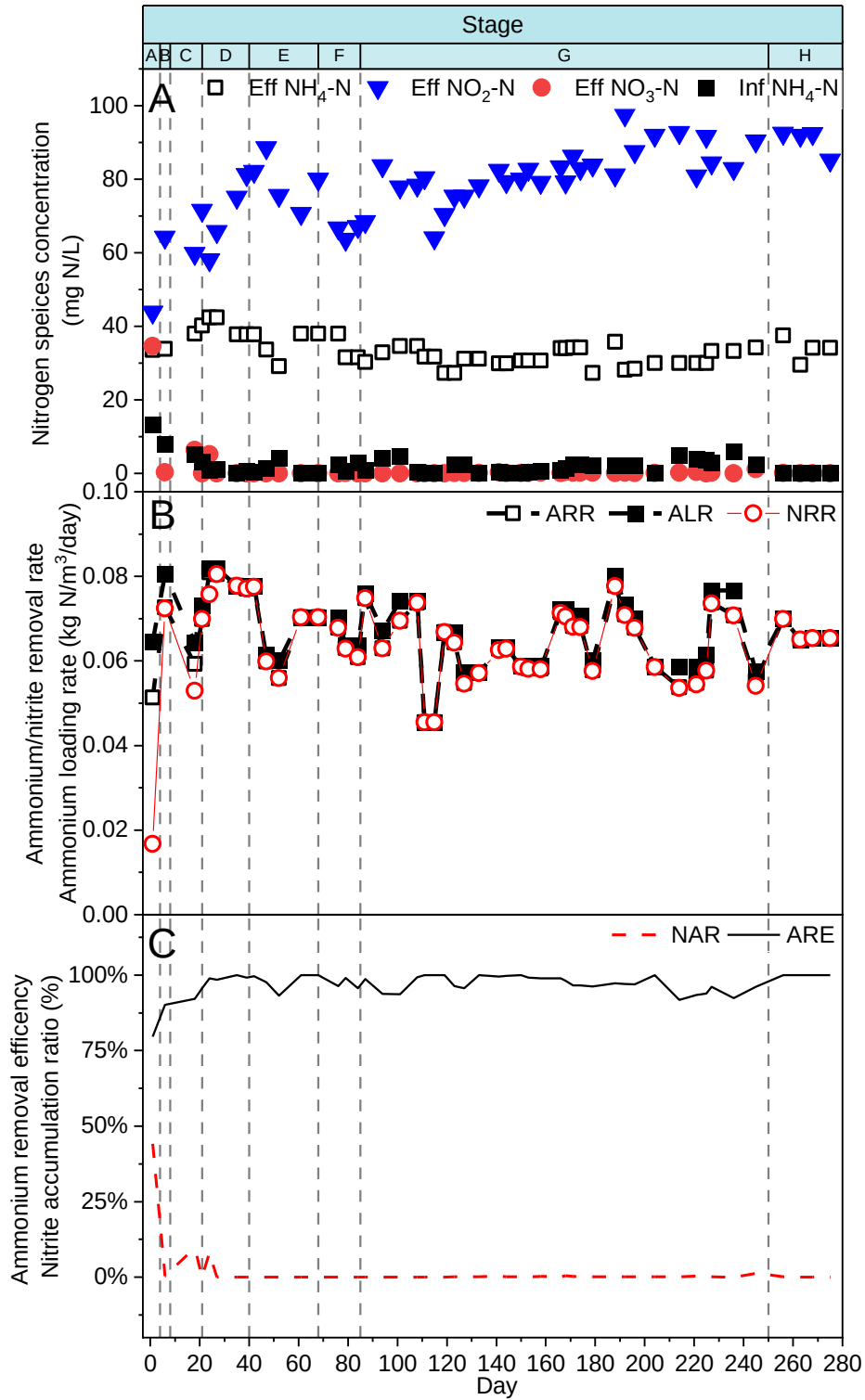


Figure 5.2 BTA group N removal performance during operation. (A) Influent and effluent N species; (B) ALR, ARR, and NRR (C) ARE and NAR. A power shut down happened on day 41 and lasted for 3 days. The system was restarted from recovery phase after it.

From Stage E to F (days 41 to 85), BTA concentrations increased from 0 to 0.5 mg/L. In comparison to stage A (days 0 to 4) and stage D (days 22 to 40), no nitrite accumulation was observed during the process, and effluent ammonium remained below 4 mg N/L. Stable ARR (0.58 to 0.75 N/m³/day) and NRR (0.56 to 0.77 kg N/m³/day) were observed. The AREs were consistently above 86%, demonstrating a higher and more stable performance than the initial phase. The results indicate that the nitrification sludge was not adversely affected by the shock loading of 0.5 mg/L BTA. To further test the nitrification system's ability to resist BTA inhibition, higher BTA concentrations were applied in the subsequent stages.

In Stage G (days 86 to 250), 1 mg/L of BTA was added to the system. Over the 166 days of operation, effluent ammonium, and nitrite concentrations were 1.9 ± 1.6 and 0.2 ± 0.2 mg N/L, respectively. Both ARR and NRR were maintained at 0.63 ± 0.01 N/m³/day, with no observed nitrite accumulation, and ARE remained above 88%. In this stage, the loading of 1 mg/L BTA did not cause shock loading or continuous inhibition of reactor performance. Even with the introduction of 2.5 mg/L BTA in Stage H (days 251 to 280), there was no impact on the reactor's performance. G. Li et al. (2020) reported that 1 mg/L BTA could cause >25% inhibition and 2 mg/L BTA could cause 50% inhibition to the nitrification process. The system demonstrated resistance to azole inhibition and maintained both ammonium and nitrite levels below 1 mg N/L in the system.

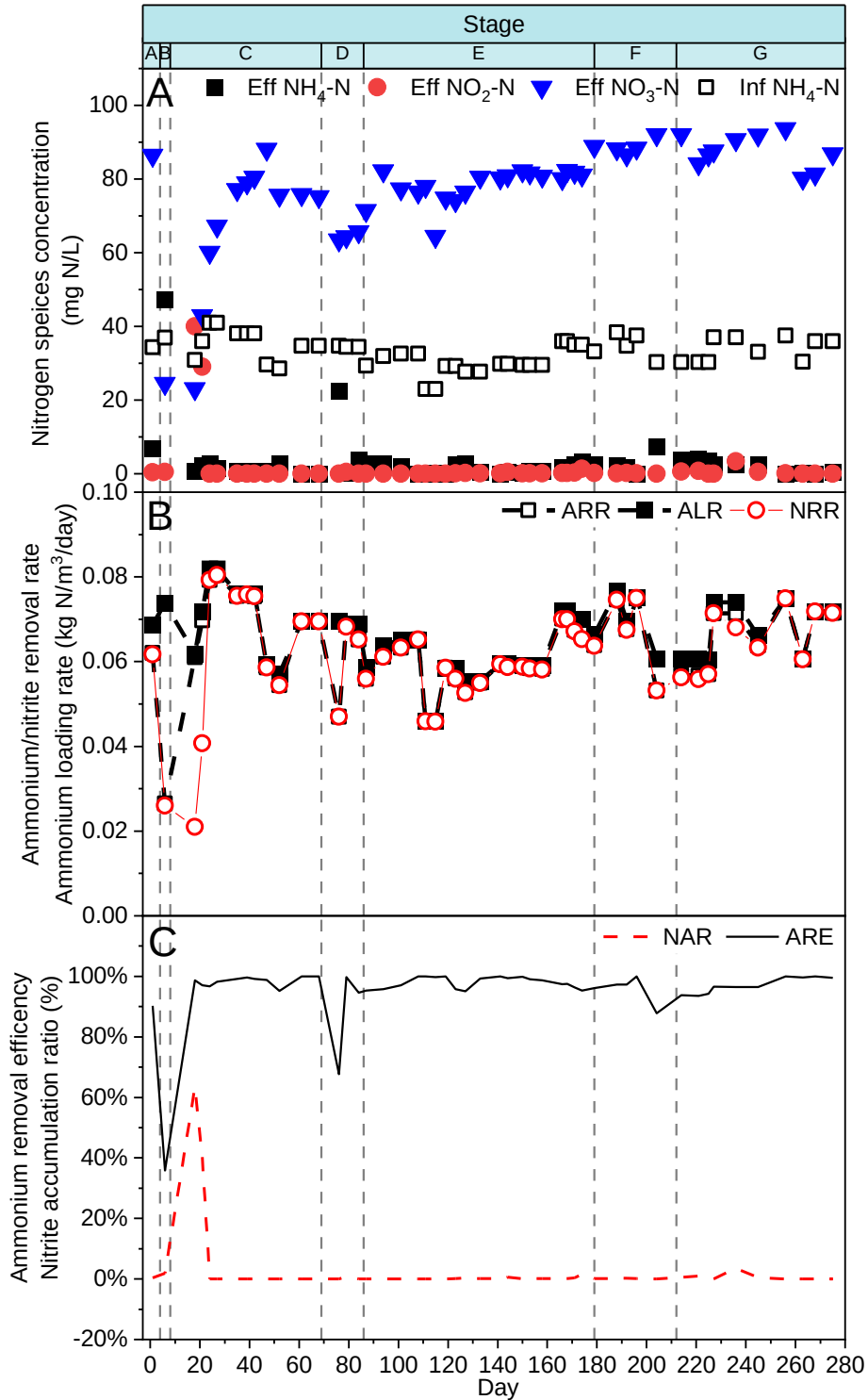


Figure 5.3 PA group N removal performance during operation. (A) Influent and effluent N species; (B) ALR, ARR, and NRR (C) ARE and NAR. A power shut down happened on day 41 and lasted for 3 days. The system was restarted from recovery phase after it.

According to **Figure 5.3**, in Stage A (days 0 to 4), the initial shock loading of 0.5 mg/L PA led to the accumulation of 6.7 mg N/L in the effluent, with no observed nitrite accumulation. Both the ARR and NRR were recorded at 0.061 kg N/m³/day. The introduction of 0.5 mg/L PA caused a decrease in AOB activity, resulting in a 10% decrease in ARR during the initial phase. This finding is different from the BTA treatment group, where inhibition led to a decrease in the removal of both ammonium and nitrite. The difference in structure and physiochemical properties between BTA and PA may influence the way they interact with the microorganisms (Hartman et al., 2013). The ammonium oxidation process is less impacted by 0.5 mg/L shock loading of PA when compared to BTA during long-term exposure.

In Stage B (days 5 to 7), the introduction of 2.5 mg/L of PA inhibited the ammonium oxidation process. The ARE decreased to 35.9% over a 12-hour cycle, resulting in 47.3 mg NH₄⁺-N/L in the effluent. These results align with a previous work, Pagga et al. (2006) reported that shock loading of PA at 2.5 mg/L could inhibit the nitrification process. Combining these findings with the current study, it appears that an unacclimated nitrification system may suffer from sudden PA exposure, causing a decrease in N removal efficiency. After removing PA from the system in Stage C (days 8 to 68), the ammonium removal rate fully recovered to 100%. However, nitrite accumulation was observed, with the NAR increasing from 1.9% to 63.4%. These results suggest that PA inhibited the nitrite oxidation process. Similar results were reported by Pagga et al. (2006), where the addition of 0.75 mg/L of PA for seven days led to inhibition (>75%) of the nitrification process for the following 10 days, gradually recovering after PA removal for 11 days. The study also suggested that the recovery of NOB from PA inhibition takes longer compared to AOB. The difference in recovery rates between AOB and NOB was observed in another study. Nitrite accumulation was observed after 1 mg/L shock load of free cyanide was removed from the system while no ammonium accumulation was observed (Kim et al., 2011).

In Stage D (days 69 to 85), the exposure to 0.25 mg/L PA decreased ARE to 67.7% with no observed inhibition on nitrite removal. The ARR increased to >95% in the following days. Compared to BTA, the nitrification process took a longer time to acclimate to PA. Even if have previously been exposed to PA,

the addition of 0.25 mg/L of PA still caused acute inhibition of the nitrification process. However, the effect of PA inhibition was relieved with the acclimation process proceeding, as observed in Stage E (days 86 to 178). During the second exposure to 0.5 mg/L PA, effluent ammonium and nitrite concentrations remained below 3 and 2 mg N/L over the 92 days of operation, respectively. Simultaneously, ARR and NRR were maintained at 0.06 kg N/m³/day, with ARR above 95% and 98%, respectively. In comparison to Stage A (days 0 to 4), the nitrification system demonstrated enhanced resistance to 0.5 mg/L PA.

In Stages F and G (days 179 to 280), when 1 and 2.7 mg/L of PA were added to the system, no inhibition ($\geq 10\%$) was observed during the process, differing from the results in Stage B (days 5 to 7). Through long-term acclimation to PA, the nitrification system gradually developed resistance to PA inhibition at acclimated and higher concentrations.

Adaptation of nitrifiers activities to azole inhibition

During the start-up phase before day 0, the activities of all three reactors fell within the range of 2-6.5 mg N/g TSS/hour, which is in line with the recommended nitrifying rate for fresh nitrification sludge from WWTPs by the International Organization for Standardization (ISO-9509, 2006). During the 280 days operation, a more pronounced resistance to the BTA inhibition (≤ 30 mg/L) was observed in the groups with the BTA acclimation process ($p < 0.05$). According to **Figure 5.4**, on day 3, the nitrification activity in the BTA reactor was measured at 6.27 mg N/g TSS/hour, while the control reactor was 6.5 mg N/g TSS/hour. The NNA reached 103.6%, surpassing the theoretical SNAs based on short-term batch tests. From the results, the BTA nitrification reactor was no longer inhibited by the 0.5 mg/L BTA after a 3-day acclimation period. During stage B (days 5-7), 2.5 mg/L BTA caused inhibition of the nitrification process. The SNA of the BTA group decreased to 2.88 mg N/g TSS/hour, which is only 55.2% of the control group. However, the NNA is still higher than the theoretical NNA (40%). When compared to the unacclimated sludge, the sludge in the reactor was more resistant to BTA inhibition. The results indicated that previous acclimation to 0.5 mg/L BTA might have increased the azole resistance of the reactor.

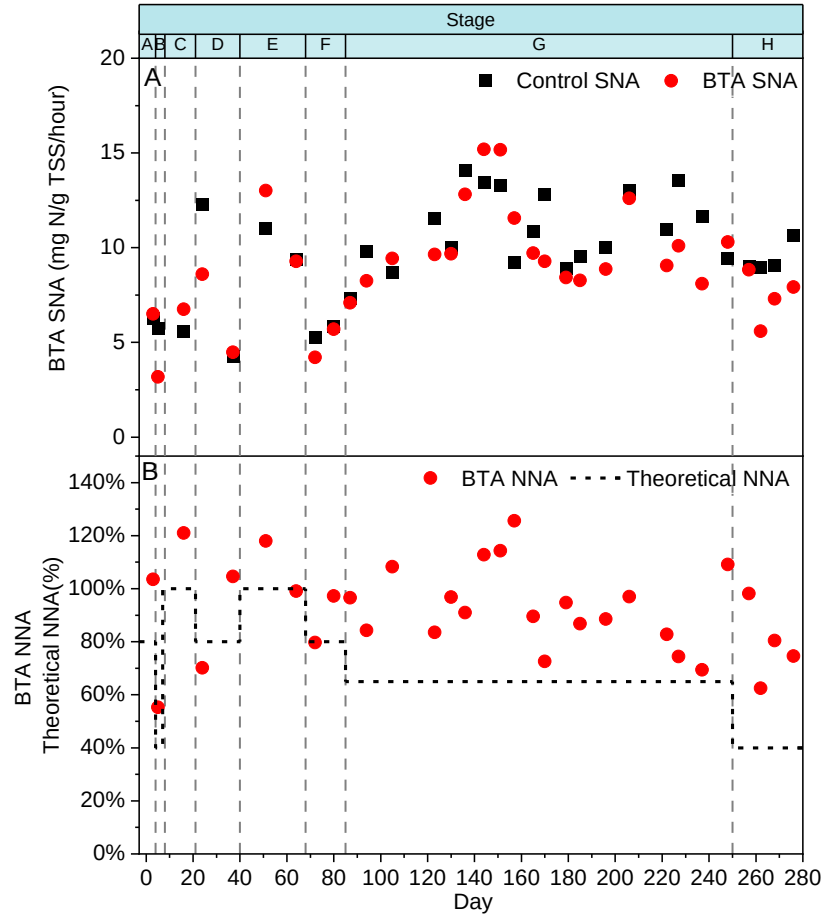


Figure 5.4 (A) SNA of BTA and control group; (B) NNA of BTA group and theoretical SAAs

From Stage C to F (days 22 to 85), 0.5 mg/L BTA was alternately added and removed. Remarkably, the resistance of the nitrification sludge persisted throughout this process. The NNA of the BTA group consistently equaled or surpassed that of the control group and remained higher than the theoretical NNA. In this study, the nitrification process exhibited no inhibition ($\geq 10\%$) even with a BTA concentration of 0.5 mg/L, surpassing the 20% inhibition expected in the short-term batch test. The system's ability to handle incoming perturbations relies on the proper adaptation of the biomass, as highlighted in prior studies (Lefebvre et al., 2006). The results indicate that the system starts to adapt to BTA inhibition. The resistance developed during the acclimation process persisted through the recovery stage. Comparing Stages A (days 0 to 4), D (22 to 40), and F (days 69 to 85) where the same 0.5 mg/L BTA was loaded, the acclimated nitrification sludge demonstrated higher activity in Stages D and F. In Stages G (days 86 to 250), and H (days 251 to 280), 1 and 2.5 mg/L BTA were added to the system and no decrease in SNA

and NNA was observed. The NNAs are higher than the theoretical value. During the acclimation process, the SNAs gradually increased throughout the operation. This phenomenon may be attributed to the enrichment of nitrifiers in the system. With the enrichment of nitrifier microorganisms, laboratory-scale SBR studies have shown that nitrification activity can reach as high as 96 mg-N/g VSS/h (Moussa et al., 2006).

Over the operation, the nitrification sludge developed resistance to BTA during the acclimation process. The average SNA of the control group is 10.94 ± 1.9 mg N/g TSS/hour, while the average SNA of the BTA group is 9.44 ± 2.4 mg N/g TSS/hour. The NNA of stage G (days 86 to 250) is $91 \pm 0.18\%$, and the theoretical NNA was 65%. The performance of the BTA treatment group is 50% higher than the un-acclimated nitrification sludge. When the BTA concentration increased to 2.5 mg/L in stage H (days 251 to 280), the nitrification sludge presented better performance than in stage B (days 5 to 7). The nitrification activity decreased to 2.89 mg N/g TSS/hour when first time exposed to 2.5 mg/L BTA. However, in Stage H (days 251 to 280), the activity level was maintained at 6.94 ± 0.98 mg N/g TSS/hour. The results suggest that the nitrification system was adapting to BTA inhibition in a lab-scale system through acclimation. The nitrification system presented the ability to adapt the azole inhibition. Although this ability was not reported on azole before, other researchers have reported that the nitrification process could adapt to the mixture of municipal wastewater and industrial wastewater (up to 30%) containing a variety of pharmaceuticals and chemicals (Dvořák et al., 2013). Even in a full-scale nitrification process, acclimation is an important method for maintaining nitrification activity when treating industrial wastewater containing pollutants such as aniline and diphenyl guanidine (Dvořák et al., 2014). The adapted nitrification demonstrates higher activity than the non-adapted sludge when exposed to the same BTA concentrations. Adaptation could be considered a potential strategy for treating azole-containing wastewater.

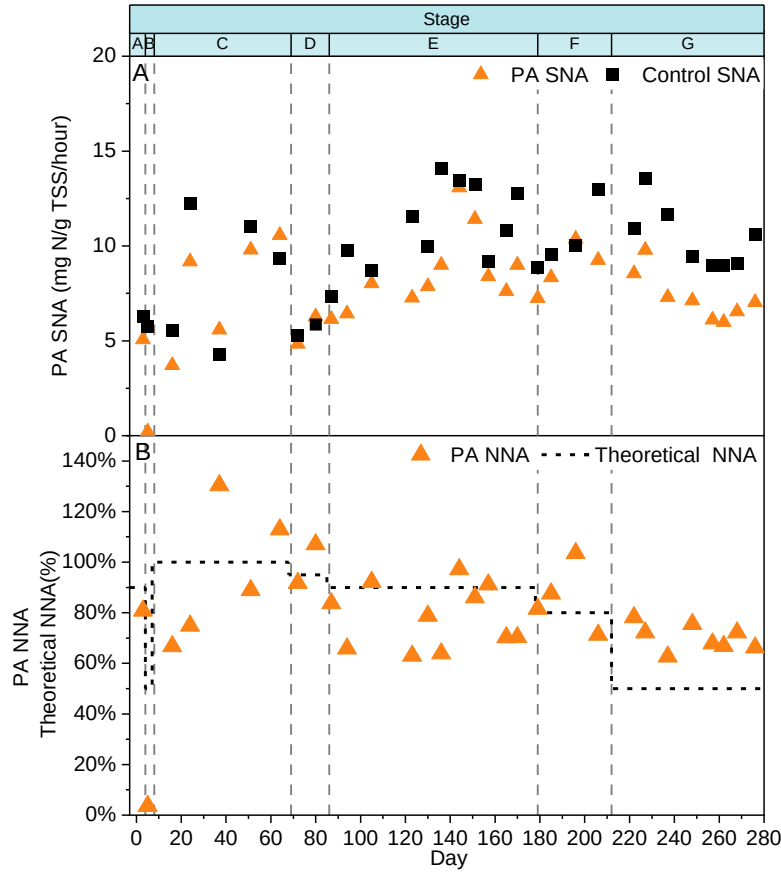


Figure 5.5 (A) SNA of PA and control group; (B) NNA of PA group and theoretical NNAs

For 280 days of operation, a more pronounced resistance to the PA inhibition was observed in the group with the PA acclimation process ($p < 0.05$). According to **Figure 5.5**, in Stage A (days 0-4), the nitrification activities were 6.27, and 5.07 mg N/ g TSS/hour for the control and PA reactor. 0.5 mg/L PA caused 20% inhibition to the treatment group NNA compared to the control group, which is also lower than the theoretical NNA from the batch test. Long-term exposure to PA may have a more severe inhibition impact on the nitrification process compared to short-term exposure. During stage B (days 5-7), 2.5 mg/L PA completely inhibited the nitrification process. The SNA of the PA group decreased to 0.16 mg N/g TSS/hour, which is only 3% of the control group. However, the theoretical NNA is 50%, and the results further support the idea that continuous exposure to PA could cause more serious inhibition of the nitrification process.

Entering the recovery phase in Stage C (days 8 to 68), the SNA of the PA group gradually recovered and maintained at the same level as the control group. After the recovery phase, 0.25 mg/L of PA was added to the system in Stage D (days 69 to 85), and no inhibition ($\geq 10\%$) was observed. However, when 0.5 mg/L of PA was loaded in Stage E (day 86-178), the activity of the PA treatment group was inhibited. The average SNA of the control group was 11.14 ± 2.06 mg N/g TSS/hour, and the PA reactor was 8.40 ± 1.95 mg N/g TSS/hour. The NNA was $78\% \pm 11\%$, while the theoretical NNAS was 90%. After operating for 178 days with intermittent PA addition and recovery, the nitrification process was not well adapted to the PA inhibition at 0.5 mg/L. When compared to the short-term batch test, more inhibition during long-term exposure was observed. However, when 1 mg/L of PA was added to the system in Stage G (days 212 to 280). The NNA of Stage G (days 212 to 280) was $86.1 \pm 11.0\%$, and the theoretical NNA was 80%. Unlike the previous stages, in which the nitrification process was more inhibited compared to the theoretical NNA, the performance of the PA treatment group is higher than the theoretical NNA, suggesting the nitrification sludge started to adapt to PA inhibition.

When the PA concentration increased to 2.7 mg/L, the nitrification sludge presented better performance than in stage B (days 5 to 7) when initially exposed to 2.5 mg/L PA, the nitrification activity was completely inhibited. However, in Stage H (days 212 to 280), the activity level was maintained at 7.3 ± 1.20 mg N/g TSS/hour. The NNA was kept at $70\% \pm 4.8\%$ while the theoretical NNA was 50%. Comparing the performance of the reactor in stages B (days 5 to 7) and H (days 212 to 280), as well as the NNAs in this study to theoretical NNAs, nitrification sludge gradually gained resistance to PA inhibition. Besides, from stages A to H, the NNA of the system was firstly below the theoretical value and then gradually increased to higher than the theoretical value. The pattern further suggested that resistance was developed during the acclimation process. Reduced inhibition effect with the same compound loading could be a sign of inhibition adaptation, Pagga et al. (2006) reported an IC_{50} of N-methyl-aniline was 1.2 mg l/L with adapted sludge but 3.3 mg/L with non-adapted sludge. Compared to the BTA group,

PA caused more inhibition to the nitrification process during the long-term exposure and was harder for the sludge to adapt.

Biodegradation of BTA by acclimated nitrification sludge.

Development of BTA biodegradation ability

In this study, the concentrations of BTA and PA were monitored to evaluate their potential degradation within the nitrification system. In this study, both BTA and PA were found to be stable under sterile conditions in a 5-day control experiment, in which the reactors were operated in identical conditions without sludge. The impact of hydrolysis and sludge adsorption on BTA and PA could be negligible (Lakhey et al., 2021; Mazioti et al., 2015a). Previous studies have also reported that BTA and PA were not degraded under abiotic conditions (Kalyani V Jog et al., 2021; Lakhey et al., 2021). The biodegradation of BTA was not initially observed in this study but gradually developed and enhanced during the acclimation process. Similar phenomena have been documented in other studies focusing on TA biodegradation (Wu et al., 2019). The nitrification sludge could subsequently develop the capacity for azole degradation.

According to **Figure 5.6**, the concentrations of BTA did not decrease within the 12-hour cycle when 0.40 mg/L and 2.56 mg/L BTA were added in Stages A (days 0 to 4) and B (days 5 to 7), respectively. However, in Stage D (days 22 to 40), when 0.43 mg/L BTA was introduced, it was partially degraded by 25% within 4 hours and completely degraded after 12 hours. After 79 days in Stage F (days 69 to 85), 0.44 mg/L BTA was 89% removed within four hours, indicating an increase in the BTA biodegradation ability. With further acclimation to 1 mg/L and 2.5 mg/L BTA, the nitrification sludge demonstrated the capability to degrade 1 mg/L BTA within 4 hours and up to 3.25 mg/L BTA within 12 hours. The statistical data also suggest there is a significant difference ($p < 0.05$) between the BTA concentrations in the effluent (12 hours) and influent (0 hour). The results suggested a strong and fast BTA removal process

continuously happened in the system, which could be a potential strategy for the microbial community to mitigate BTA presence.

PA was found not to be degraded within the nitrification system. According to **Figure 5.6**, the nitrification sludge was unable to degrade PA under aerobic conditions, as the concentration of PA did not change during each cycle. The statistical data also suggest there is not a significant difference ($p > 0.05$) between the PA concentrations in the effluent (12 hours) and influent (0 hour). Poor PA biodegradation (<10%) was observed when tested with the addition of organic carbon with activated sludge (Pagga et al., 2006). On the other side, PA was also reported could be degraded with biological activated carbon filters at 6 mg/L within 20 days with inoculum from a drinking water system (Piai et al., 2022). However, the environment and microorganisms of the drinking water system are different from the wastewater system. The degradation of PA in the wastewater system still needs further investigation. In this study, although the nitrification system developed resistance to PA inhibition, biodegradation was not observed.

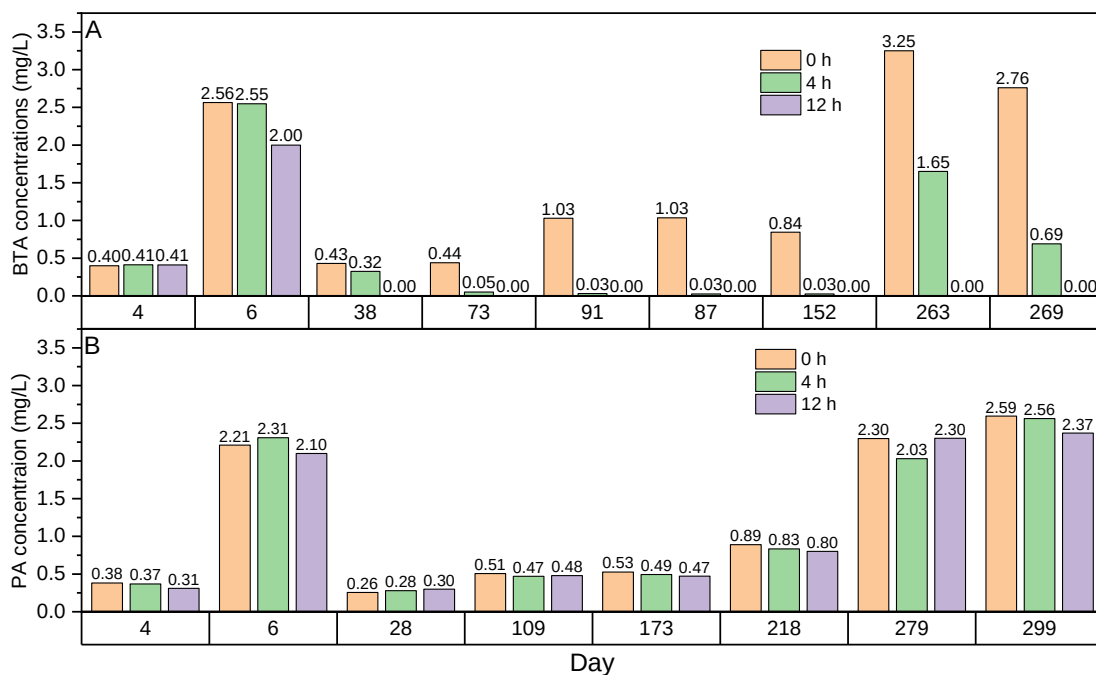


Figure 5.6 Azole concentration in nitrification SBRs at different operation day (A) BTA (B) PA.

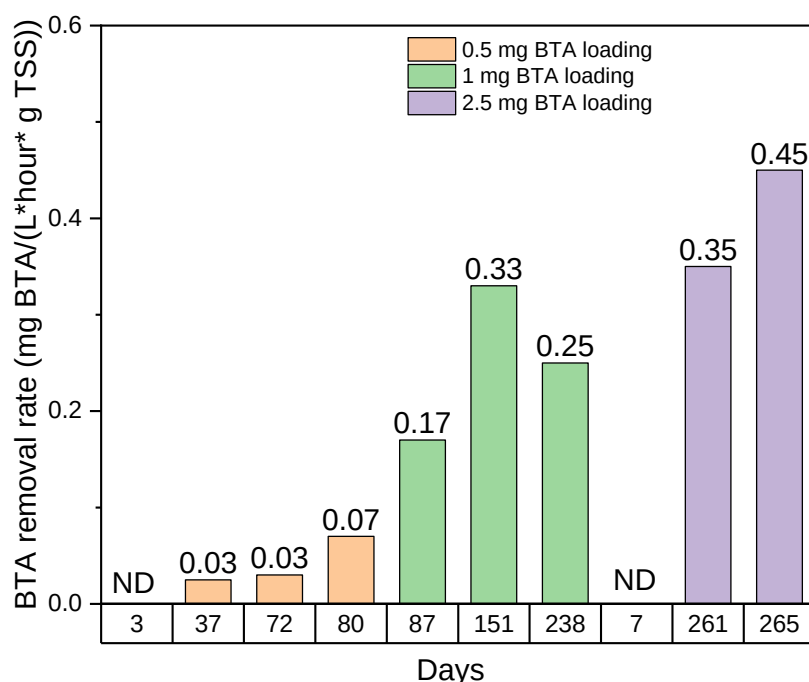


Figure 5.7 BTA biodegradation rate at different operation day

As described in **Table 5.2**, the BTA removal rates reported in this study are higher than those in other BTA biodegradation studies. A highly efficient BTA degradation system was developed through the long-term acclimation of nitrification sludge, achieving a degradation rate of up to 0.47 mg BTA/g TSS/hour. In our work, the BTA biodegradation rates were approximately 10 to 50 times higher than those reported in previous studies.

Table 5.2 BTA removal rates in this study and literature review

System	BTA removal rates	References
Nitrification system with 80 mg/L NH ₄ -N addition	<0.001 to 0.471 mg BTA/g TSS/hour	This study
Nitrification system with 300 mg/L NH ₄ -N addition	<0.001 to 0.008 mg BTA/g TSS/hour	Trejo-Castillo et al. (2021)
Aerated activated sludge system with 200 mg/L COD addition	<0.001 to 0.005 mg BTA/g TSS/hour	(Mazioti et al., 2015a)
Aerated activated sludge system without substrate	< 0.001 mg BTA/g TSS/hour	(Y.-S. Liu et al., 2011)
Aerated activated sludge system with 30 or 270 mg/L COD addition	<0.001 to 0.002 mg BTA/g TSS/hour	(Mazioti et al., 2015b)

According to **Figure 5.7**, the degradation rate increased with the acclimation process within each BTA loading concentration. At 0.5 mg/L BTA, the degradation rate increased from 0 to 0.07 mg BTA/g TSS/hour, and at 1 mg/L BTA, it increased from 0.17 to 0.33 mg BTA/g TSS/hour. For 2.5 mg/L BTA, it was loaded two times in the system in Stages B and H. In Stage B on day 7, no BTA degradation was observed. In Stage H, it increased from 0.35 to 0.47 mg BTA/g TSS/hour. Comparing the degradation rates within the same BTA concentration, the BTA degradation rate was enhanced along with acclimation time. The BTA degradation rates also increased with the increase in BTA concentrations, suggesting the potential of BTA-utilizing mechanisms.

Biodegradation Mechanisms Analysis

There are different hypotheses regarding the potential mechanisms of BTA biodegradation. To further analyze the BTA biodegradation mechanisms, batch tests were applied to analyze the BTA biodegradation under different substrate conditions. According to **Figure 5.8**, the impacts of ammonium and AMO were investigated by removing ammonium from the system and adding 11.6 mg/L allylthiourea (ATU). Ammonium was found not necessary for BTA removal and the AMO enzyme may not play an important role in BTA degradation. The degradation rates of degradation batch experiments are summarized in **Table 5.2**. When ammonium was removed from the system, the BTA removal rate was 0.23 mg BTA/g TSS/hour, falling within the range of 0.17 to 0.33 mg BTA/g TSS/hour when 1 mg/L BTA was added. When 11.6 mg/L ATU was added to the system to inhibit the activity of AMO, the BTA degradation rate was 0.30 mg BTA/g TSS/hour. Previous findings suggested that the addition of ammonium enhanced BTA biodegradation, and AMO was involved in the BTA co-metabolic biotransformation (Trejo-Castillo et al., 2021). However, in this study, BTA was degraded with/without ammonium present. Furthermore, the degradation of BTA was also not impacted by the addition of 11.6 mg/L ATU, which is a known inhibitor of AMO enzymes but has minor effects on heterotrophic bacteria

(Men et al., 2017). In this study, co-metabolism carried by AMO may not be the major pathway for BTA removal.

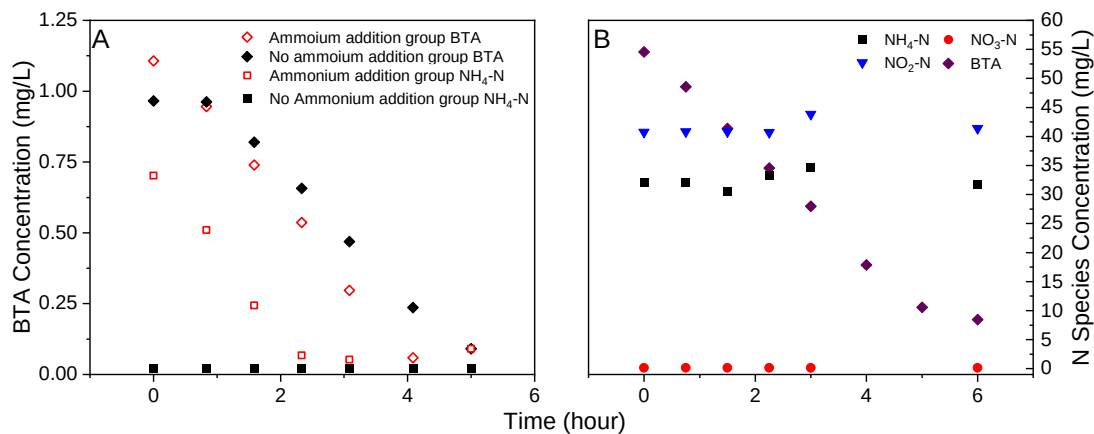


Figure 5.8 BTA degradation batch tests on day 280 (A) With/without ammonium addition (B) With ATU addition

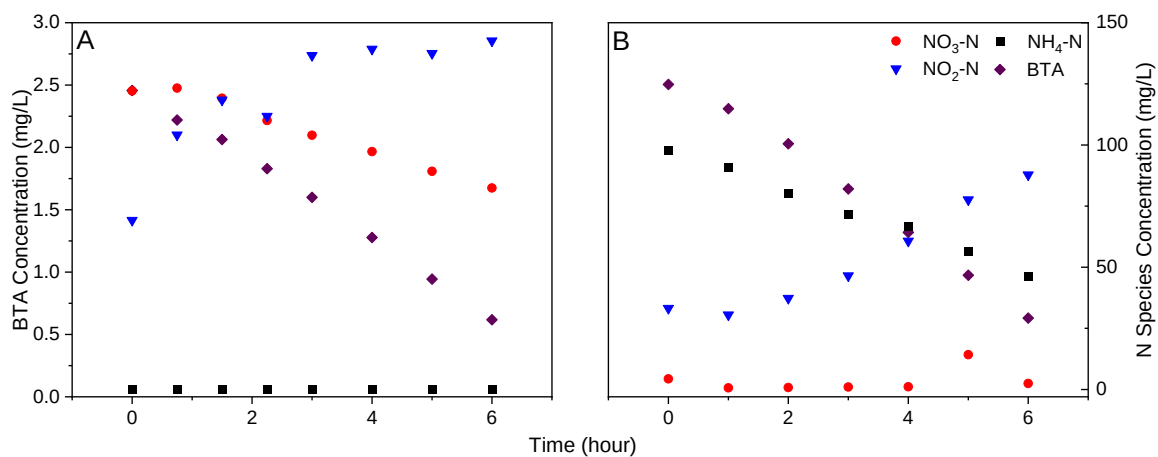


Figure 5.9 BTA degradation batch tests on day 282 (A) With 100 mg/L $\text{NH}_4\text{-N}$ addition (B) 120 mg $\text{NO}_2\text{-N/L}$ addition

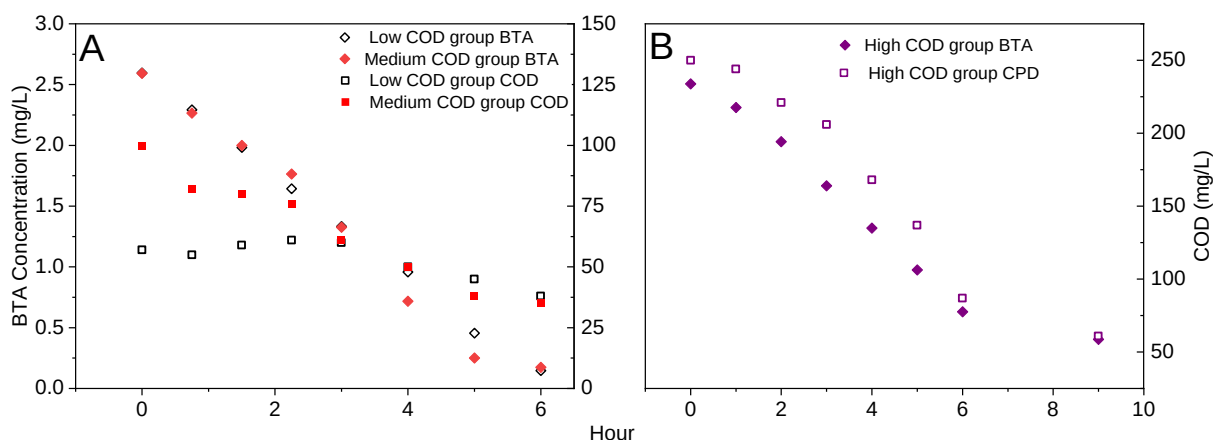


Figure 5.10 BTA degradation batch tests on day 284 (A) With regular and 50 mg/L extra COD addition (B) 200 mg extra COD addition

To further investigate the role of ammonium, nitrite, and COD in BTA biodegradation, additional ammonium, nitrite, and COD were introduced into the system. According to **Figure 5.9** and **Table 5.2**, the addition of 100 mg/L $\text{NH}_4^+\text{-N}$ did not increase the BTA biodegradation rate compared to the group without ammonium addition. The addition of 120 mg/L $\text{NO}_2^-\text{-N}$ resulted in a BTA removal rate of 0.31 mg BTA/g TSS/hour, which is not higher than the group without nitrite addition. These results suggest that the addition of ammonium and/or nitrite does not enhance the BTA degradation process. Other bacteria, rather than nitrifying microorganisms, may play a role in BTA biodegradation.

The addition of an extra 50 mg/L COD did not increase the BTA removal rate at the 2.5 mg/L BTA level (from 0.45 to 0.47 mg BTA/g TSS/hour). However, the addition of 200 mg/L COD decreased the BTA removal rate from 0.45 to 0.26 mg BTA/g TSS/hour. These results suggest that the COD concentration may correspond to the BTA biodegradation ability. The overload of COD decreased the BTA degradation process. J. Wang et al. (2022) reported that BTA may be degraded by degraders and the addition of dissolved organic content may impact the removal of BTA caused by the substrate competition. When multiple substrates are available, the inhibitors may not become the preferred substrates and are more slowly removed from the system (Eiroa et al., 2004).

Table 5.3 Batch tests parameters for BTA degradation mechanisms analysis

Substrate condition	BTA removal rate (mg BTA/g TSS/hour)	Ammonium/nitrite consumption rate (mg N/g TSS/hour)
No ammonium present	0.23	NA
100 mg/L NH ₄ -N	0.22	6.76
11.6 mg/L ATU addition	0.30	<0.01
50 mg COD/L addition	0.47	10.21
200 mg COD/L addition	0.26	8.11
120 mg NO ₂ -N/L addition	0.31	7.28

The biodegradation of BTA may be attributed to the synergistic effect of the microbial community. The heterotrophic microorganisms that co-existed in the nitrification community may utilize BTA as a carbon and/or energy source (Mazioti et al., 2015b). For azole compounds such as benzothiazole-2-sulfonate, it has been reported that biodegradation by heterotrophic bacteria, rather than co-metabolism, is responsible for the contaminant removal in municipal wastewater treatment processes with activated sludge (De Wever et al., 2007). This phenomenon of the nitrification community has also been observed in other inhibitor adaptations such as formaldehyde and phenol (Amor et al., 2005; Eiroa et al., 2004). During the acclimation process, the relative abundance of degraders that co-existed in the microbial community may increase and contribute to the alleviation of BTA inhibition. In this study, PA was not degraded under aerobic conditions with a nitrification system. PA has been reported to be difficult to degrade under aerobic conditions as a sole carbon and N source (Kalyani V Jog et al., 2021). When compared to other azoles such as isoxazoles and isothiazoles, PA's ring structure is more stable in metabolism, which may cause PA to be resistant to degradation in the activated sludge system (Dalvie et al., 2002). The PA ring structure was resistant to both oxidation and reduction (Lumbaque et al., 2021). Unlike other known biodegradable nitrification inhibitors such as phenol, the presence of PA in the system may pose a potential threat to the stability of the nitrification process as it is not easily biodegraded (Kim et al., 2008). The inhibition of nitrification is less with biodegradable substances than with poorly degradable ones

(Pagga et al., 2006). Although the nitrification process could gain resistance to PA inhibition through acclimation, the activity would also be impacted during the initial phase of exposure, resulting in N-rich wastewater being discharged into the environment.

Long-term effect of azoles on the microbial community

The microbial community in the system was initially seeded using the Inoculum sludge obtained from the same source, an aeration zone of a WWTP (Laurel, MD, USA), in which the nitrification process was known to happen. The system may adapt to azole inhibition through the evolution of the microbial community. To illustrate the similarities and differences in the microbial composition of each reactor, inoculum samples and sludge samples at the end of the operation in each reactor (control, BTA, and PA) were collected and analyzed. In this study, 22905, 28146, 27192, and 27141 sequences were respectively detected from the Inoculum, Control, BTA, and PA samples, corresponding to the operational taxonomic units (OTUs) of 358, 338, 430, and 369. As shown in **Figure 5.11**, the OTUs that exclusively existed in Inoculum, Control, BTA, and PA were 89, 3, 5, and 5, respectively. 31.6% OTUs coexisted in all four samples, while 81.5 % of OTUs were shared by two or more samples, suggesting microbial components changed during the operation process.

The microbial community structure and spatial distribution were identified at the genus level and presented in **Figure 5.12**. The right semicircle indicates the relative abundance composition of four samples, and the left semicircle indicates the distribution of bacteria in different samples, where different colors represent different species, and the relative abundance of bacteria is represented by the line width. From **Figure 5.12**, *Nitrospira* remains the dominant genus among all bacteria (40.4%, 20.9%, and 40.2% in control, BTA, and PA, respectively). A higher abundance of *Dechloromonas* was found in the BTA treatment group (11.3%) while the abundance was below 1% in the Control and PA groups and 2.4% in the inoculum. *Dechloromonas* is known as an aromatic compound-degrading bacteria (Terashima et al., 2016). Considering the ring structure of BTA, the presence of the *Dechloromonas* genus may be related to the BTA degradation in the system. Besides, the *Zoogloea* and *Blastochloris* are also found at 7.0% and

5.1% in the BTA treatment group. *Zoogloea* was reported as an aromatic degrader and was important for activated sludge flocs formation (Andrade et al., 2017; P. Li et al., 2005). *Blastochloris* are known as Purple non-sulfur bacteria which are photoheterotrophic and capable of utilizing organic waste (Chitapornpan et al., 2013).

For the PA group, there is a higher abundance of *Ignavibacterium* (6.5%), *Thiohalocapsa*(5.0%), and *Chryseolinea*(5.0%) is observed in the PA group. *Ignavibacterium* was commonly found in the BNR system and as an aromatic degrader(D. Wang et al., 2021). The function of *Thiohalocapsa* and *Chryseolinea* in the wastewater treatment process is not clear while *Chryseolinea* may contribute to better COD removal (Meng et al., 2019). Although the potential aromatic compound degrader was found in the system, no PA degradation was observed in this study. The results suggest that PA is more persistent in the nitrification systems and difficult to remove by the microorganisms.

These results suggested long-term operation (280 days) of the nitrification reactor might result in a shift in dominant bacteria to adapt to a new environment, and such change was more substantial than the impact caused by exposure to azoles. Further analysis of the data was conducted to calculate the Shannon index and Simpson index of all samples. The results showed different values of Shannon and Simpson index in the Control (3.08 and 0.86), BTA (3.51 and 0.92), and PA (2.83 and 0.82) samples (**Table S4**), suggesting a potential change in microbial community composition after azoles exposure.

These results are consistent with previous studies that have reported that nitrification microbial communities changed their composition when adapting to inhibitors such as cyanide and pharmaceuticals (Kim et al., 2011; LaPara et al., 2002). However, the samples analyzed in this study were collected at the end of the 280-day experiment when the nitrification sludge in BTA and PA reactors were exposed to 2.5 mg/L BTA and 2.5 mg/L PA, respectively. The transition of microbial communities at low concentrations is not investigated. Therefore, it is suspected that the microbial community composition may represent the microbial composition at lower concentrations in the previous stages, in which the azole concentrations are from 0.5 to 1 mg/L.

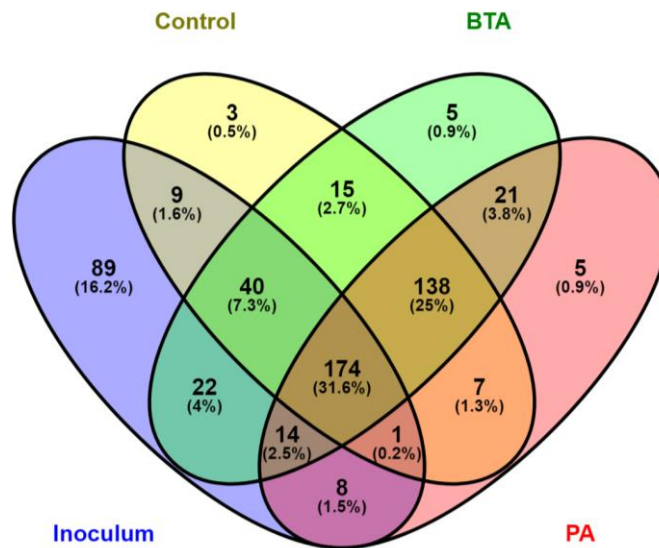


Figure 5.11 OTU numbers in Inoculum, Control, BTA, and PA samples at the end of the experiments.

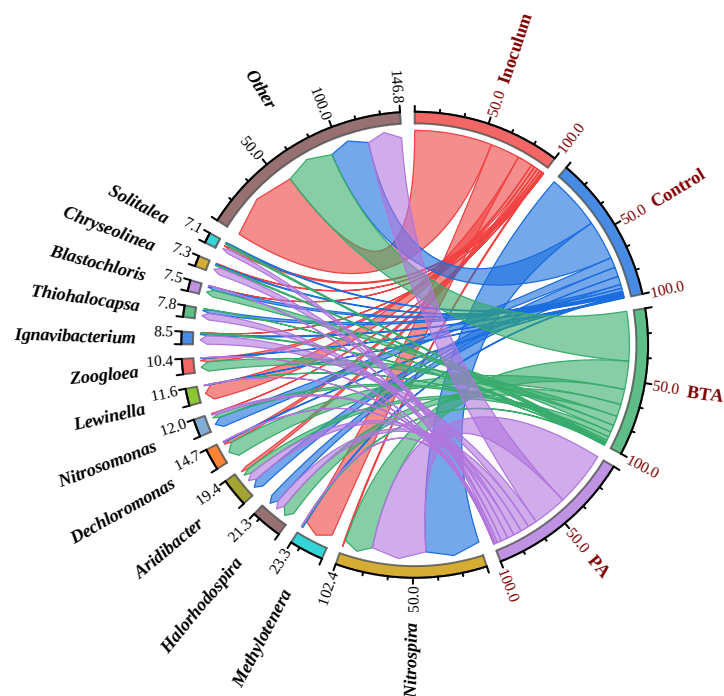


Figure 5.12 Relative abundance of dominant bacteria at genus level of nitrification sludge in the Inoculum, Control, BTA and PA samples.

Implications

The acclimation of the nitrification process to azoles can enhance the resilience of the nitrification process when treating azole-containing wastewater. Compared to unacclimated sludge, acclimated nitrification sludge exhibits better performance with the same concentrations of BTA and PA added to the system. In this study, acclimation to BTA also led to the development of BTA degradation ability in the system. This ability is not only beneficial for the stability of the nitrification process but also for other BNR processes following the nitrification process. The heterotrophic bacteria that existed in the nitrification sludge may contribute to the BTA degradation. The aromatic compound/organic compounds degraders such as *Dechloromonas* and *Zoogloea* are identified in the microbial community while their specific roles need further determination. Overall, more attention still needs to be paid to the presence of azoles in WWTPs. Azoles may pose a potential threat to the stability of the nitrification system. For WWTPs not exposed to azoles before, sudden shock loading of azoles may inhibit the biological nitrogen removal processes. Although the nitrification system may develop higher resistance and/or degradation ability to azoles, the process may take months or even longer. During this period, the quality of N removal may be impacted

Chapter 6: Long-term Impact of Pyrazole and Benzotriazole on Denitrification Process

Abstract

The presence of emerging organic contaminants (EOCs) such as azole compounds in wastewater is a potential threat to the biological nitrogen removal (BNR) process. Azoles showed a variable toxicity to denitrifying microorganisms in activated sludge. In this work, the long-term inhibition potentials of benzotriazole (BTA) and pyrazole (PA) to the denitrification process in wastewater treatment plants (WWTP) were investigated. Stable denitrification processes were observed with BTA and PA inhibition at 2.5 and 2.7 mg/L, respectively. The addition of azoles didn't impact the denitrification activities in the following operation. The results suggested that long-term exposure to BTA and PA at low mg/L will not inhibit the denitrification process in WWTPs. No BTA and PA degradation was observed within the 12-hour operation cycle. However, after increasing the reaction time to 7 days, more than 85% BTA and 45% PA degradation are observed during the process. Furthermore, all the denitrification reactors (Control, BTA, and PA treatment group) were able to degrade BTA and PA at the same time. The removal of N and organic content loading didn't impact the BTA and PA degradation process. The finding indicated the existence of bacteria could utilize BTA and PA or benefit from BTA and PA addition to the system. Multiple potential aromatic compound/organic compound degraders were found in the denitrification community such as *Thauera*, *Azoarcus*, *Georgfuchsia*, and *Dechloromonas*. Although BTA and PA didn't inhibit the denitrification process, the impacts of the potential degradation products were not clear and their impacts on the biological wastewater treatment processes still need investigation.

Introduction

To meet the stringent nutrient discharge requirements of the WWTPs, nitrification, and denitrification have been applied widely in favor of the physical-chemical processes (Y. Peng et al., 2006). EOCs are a

group of chemicals including pharmaceuticals, personal care products, plasticizers, flame retardants, surfactants, and certain pesticides, which may exert ecotoxicological effects at relatively low concentrations (Matamoros et al., 2015). The stability of the system may be impacted by the EOCs presented in the wastewater.

Azoles are used in different realms such as the semiconductor industry, which generates high-strength wastewater containing high concentrations of azoles, amines, hydrogen peroxide, and many other inorganics and organic compounds at the g/L level (R. Li et al., 2021). Azoles are reported EOCs that have been applied in different realms such as antifungal drugs, agriculture, and personal care products, which occurrences have been reported in different matrices (e.g., wastewater, drinking water, and surface water, etc.) and some azoles such as fluconazole represented high risk in human health risk assessment of drinking water (Assress et al., 2020). Some of the azoles have been found persistent in wastewater and their presence may become a potential risk to the stability of BNR processes. Pacholak et al. (2022) reported clotrimazole and epoxiconazole had been reported resistant to biodegradation and showed a biosorption on the activated sludge.

Besides, azoles such as fluconazole are persistent in wastewater treatment even after undergoing activated sludge treatment followed by an anoxic-oxic or by an oxic post-treatment (Madeira et al., 2021); Pacholak et al. (2022) found clotrimazole is resistance EOCs degraders *P. monteilii* LB2 at 70% and *S. maltophilia* AsPCI2.3 at 50% respectively. The short-term effect of azoles on the nitrification process has been investigated and serious inhibition was found at low mg/L concentration levels (G. Li et al., 2020). However, to the best of our knowledge, the long-term impact of azoles and their potential biodegradation derivatives such as imidazole and TA will impact the denitrification processes is still unknown. The inhibition mechanism was not well investigated. Previous studies have different hypotheses of azole inhibition including that the azoles may chelate with enzymes, the hydrocarbons can compete with ammonia for the active site of ammonia monooxygenase (AMO), n-alkynes irreversibly inhibit AMO and

aromatic hydrocarbons deplete cellular reductants that are needed to oxidize ammonia (Bissett et al., 2013; G. Li et al., 2020).

Therefore, an important and at the same time challenging task is to investigate the long-term influence of azoles on the denitrification processes and minimize their adverse impact on the conventional N removal processes. The objective of this study is to investigate the long-term impacts of azoles on denitrification processes.

Materials and Methods

Chemicals and microorganisms

Benzotriazole (BTA) (CAS # 95-14-7, purity > 99%) was purchased from Alfa Aesar (Ward Hill, Massachusetts, USA), Pyrazole (PA) (CAS # 288-13-1, purity > 98%) was purchased from Tokyo Chemical Industry (Tokyo, Japan). COD Digestion Vials (High range, Catalog number 2125915/ low range Catalog number 2125815) were purchased from Hach company (Loveland, Colorado, United States)

The suspended sludge used for studying the toxicity of azoles to the denitrification process in this work was activated sludge obtained from an anoxic zone of a WWTP (Laurel, MD, USA), in which the denitrification process was known to happen. All received sludge was immediately stored at 4 °C to maintain the microbial activity. The sludge was collected and stored in a cooler for 1 hour before the bioassay. The sludge was collected one day before the toxicity bioassays and washed by using chlorine-free tap water. Tap water was collected 24 hours before the experiment and stored in an open container to remove the chlorine. The washing procedure is carried out by centrifuging (1,000 g for 5 min) and discarding the supernatant liquid. Then the sludge was resuspended with chlorine-free tap water and the procedure was repeated two times. The denitrification sludge had a specific denitrification activity (SDA) of 0.26 ± 0.05 g N₂-N/g VSS/day, which was also within the ranges of other reported work. (Cui et al., 2019; J.-J. Wang et al., 2021). The Total suspended solids (TSS) of washed sludge was determined

through a standard method (American Public Health Association, 2023). Finally, re-suspend the washed sludge in an appropriate volume of chlorine-free tap water to give the TSS of 3 g/L.

Reactor setup and operation

Three identical lab-scale sequencing batch reactors (SBR) (Control, BTA group, and PA group), with a volume of 1.15L and liquid phase of 1L. Reactors were operated in parallel in a 12-h cycle, which consisted of the following phases: (1) feeding for 10 min, (2) reaction for 11 h, (3) settling for 40 min, and (4) effluent withdrawal for 10 min. The exchange volume was fixed at 40%. Mixing was offered by a stir station at 120 rpm. All reactors were inoculated with activated sludge described in the previous section and the TSS was maintained at 1.25 g/L through sludge weekly sludge disposed from the system or the addition of extra sludge picked from the WWTP.

Dissolved oxygen (DO) level was kept below 0.5 mg/L through N flushing and the pH was maintained between 6.8 and 7.8 by using either 0.5 M NaOH or 0.5 M HCl. All experiments were conducted at 30 ± 2 °C and controlled with heating pads. The three reactors were continuously monitored (online measuring of DO and pH), and the effluent NH_4^+ , NO_2^- , NO_3^- , and azoles were sampled every three days in duplicate. The TSS and volatile suspended solids (VSS) were determined weekly in triplicate.

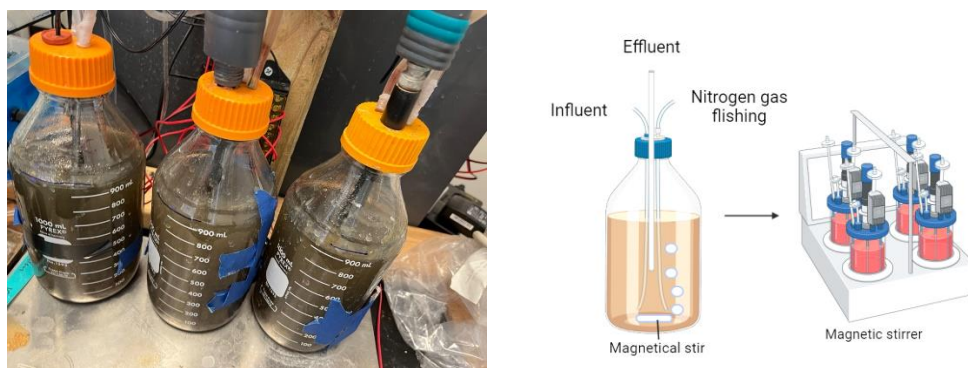


Figure 6.1 Denitrification sequence batch reactors(left) and scheme(right)

After inoculum, the three reactors were operated in two stages as described in **Table 6.1**. From day 0 to day 33 is the startup phase, all three reactors are operated at the same conditions and no azole was added into the system. From day 34 to 80, the control reactor was operated as a reference control reactor (no

azole added). BTA reactor BTA was the treatment group and BTA was added as described in **Table 6.1**.

PA reactor was the PA treatment group and PA was added as described in **Table 6.1**.

Table 6.1 Operation stages of denitrification reactors

Group	Stage name	Days	Azole addition (mg/L)
BTA	BTA - A	0 - 33	0
	BTA - B	34 - 80	2.5
PA	PA - A	0 - 33	0
	PA - B	34 - 80	2.7
Control	Control - A	0 - 80	0

The synthetic medium was prepared with de-mineralized water had the following composition: NaNO_3 460 mg/L as nitrate source, $\text{NaCH}_3\text{COONa} \cdot 3\text{H}_2\text{O}$ 680 mg/L as organic carbon source, $\text{KH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ 18 mg/L, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 90 mg/L, KCl 36 mg/L, NaHCO_3 1000 mg/L, and $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ 14 mg/L. 1 ml nutrient solution was added to the 1L medium. The concentrations of the trace element species in the medium are $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 22 mg/L, EDTA 50 mg/L, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 22 mg/L, $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ 1.61 mg/L, MnCl_2 5.06 mg/L, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 1.57 mg/L, and $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ 1.1 mg/L.

Denitrification bioassay

The inhibitory effect of selected azole compounds on denitrification activity was tested. After the filling stage finished, 4 ml of liquid samples were collected at 0, 20, 45, 90, 150, 210, and 300 mins for measuring the NO_2^- , NO_3^- , and azoles during the monitored cycle. The samples were filtered with 0.45 μm syringe filters. The supernatants were transferred to clean vials and analyzed for COD, nitrite, nitrate, and azoles.

To investigate the azole degradation tests along with the potential mechanisms, batch tests were conducted with the same system and sludge after the long-term experiment ended. The different experiment conditions are described in **Table 6.2**. The liquid samples were collected in the same method as discussed above.

Table 6.2 Denitrification biodegradation bioassay design

Phase/ Groups	I	II	III
Control	2.5 mg/L BTA and 2.5 mg/L PA	2.5 mg/L BTA and 2.5 mg/L PA	2.5 mg/L BTA and 2.5 mg/L PA
BTA	2.5 mg/L BTA	2.5 mg/L BTA and 2.5 mg/L PA	2.5 mg/L BTA and 2.5 mg/L PA
PA	2.5 mg/L PA	2.5 mg/L BTA and 2.5 mg/L PA	2.5 mg/L BTA and 2.5 mg/L PA
Extra modification	-	-	No nitrate and COD addition

Analytical methods

Liquid sample analysis

The liquid sample was collected from the reactor and filtered through 0.45 µm PP filters to measure the concentration of target azole compounds. The concentrations of PA and BTA were measured through a developed method (Lakhey et al., 2021) with minor modifications. The details of the method are described below: Concentrations of PA were analyzed using high-performance liquid chromatography (HPLC) equipped with a diode array detector. Chromatography was performed using the InfinityLab Poroshell 120 column (2.7µm, 4.6 × 100 mm; C-18 column, Agilent, USA). An isocratic mixture of 10% methanol and 90% water was used as the eluent for a run time of 12 min. The eluent flow rate was 1 mL/min and the injection volume was 20µL. The temperature was maintained at 40°C and the wavelength for PA was 210 nm, respectively. Concentrations of azole compounds BTA were analyzed using the same instrument, with an isocratic mixture of 60% methanol and 40% water. The run time was 8 min, and the eluent flow rate was 1 mL/min and the injection volume was 20µL. The temperature was maintained at 40°C and both chemicals are detected at wavelength of 275 nm. Ammonium, nitrate, and nitrite in liquid samples were analyzed by SEAL AQ300 discrete analyzer in triplicate. (Mequon, WI, USA). During the measurement, the target compounds with known concentrations were added for continuing calibration verification (CCV) to every 10 samples for quality control. Less than 10% differences between the

measured concentrations and the known concentrations were required to accept the measurement results. DO in liquid samples was measured using an optical O₂ probe. (LBOD101 Sensor, Hach, Loveland, CO, USA).

DO in liquid samples was measured using an optical O₂ probe (LBOD101 Sensor, Hach, Loveland, CO, USA). COD was determined with Hach DR900 colorimeter with Hach method 8000 (HACH, 2021). The Other parameters (TSS, pH, etc.) were determined according to standard methods ((American Public Health Association, 2023). The limit of detection (LOD) and limit of quantitation (LOQ) of the analytical instruments are listed in **Table S6**. During the measurement, the target compounds with known concentrations were added to every 10 samples for quality control. Less than 10% differences between the measured concentrations and the known concentrations were required to accept the measurement results.

DNA extraction and microbial analysis

The inoculum sludge samples were collected at the beginning of the experiment and the denitrification sludge samples of each reactor (Control, BTA, and PA) were collected at the end of the experiment. 1 ml of sludge sample with a TSS of 2 g/L was used for the DNA extraction. The DNA was extracted using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc, Carlsbad, California, United States). The extracted DNA samples were sent to Molecular Research LLP (Shallowater, TX) for analyzing their bacterial composition with 16S rRNA V4 variable region (PCR primers 515/806) through gene amplicon sequencing. The samples are treated through barcoded amplicon sequencing processes by MR DNA® under the trademark service (bTEFAP®). Negative controls (Blank medium sample without sludge) and positive controls (reference wastewater sludge samples) were used to eliminate background DNA interference. Sequences are joined, sequences <150bp, and sequences with ambiguous base calls are removed. Sequences were quality filtered using a maximum expected error threshold of 1.0 and dereplicated. The dereplicated or unique sequences are denoised; unique sequences identified with sequencing and/or PCR point errors and removed, followed by chimera removal, thereby providing a

denoised sequence or taxonomical units (OTU). Final OTUs were taxonomically classified using BLASTn against a curated database derived from NCBI (MR DNA, Shallowater, TX, USA).

Data analysis

The specific denitrification activities (SDA) in different test groups were calculated from the consumption of total NO₃-N and NO₂-N. The maximum SDA for every test was calculated from the maximum gas production according to Eq 6.1.

$$SDA = \frac{d(NO_3^- - N + NO_2^- - N)}{dt * X_0} \text{ (mg N/g Tss/hour)} \quad (6.1)$$

where SDA is the maximum SDA

$d(NO_3\text{-N} + NO_2\text{-N})/dt$ the maximum N removal rate

X_0 the microbial concentration (g VSS/L)

The rate was calculated using linear regression of five or more consecutive points. The regression analysis was carried out using Microsoft Excel. The normalized denitrification activity (NDA) of BNRs was calculated as Eq 6.2:

$$NDA(\%) = \frac{SDA_t}{SDA_c} * 100 \quad (6.2)$$

where SDA_t and SDA_c (both as g N₂ g/VSS/day) are the specific activities of the treatment (with added azoles) and control experiment (without added azoles) of the BNRs, respectively.

T-test is used for statistical analysis of the relationship between azole concentration and the activity of the BNR processes in batch reactors. A *p*-value less than 0.05 is required to reject the null hypothesis.

Results and discussion

Performance of long-term denitrification reactors with azole addition

During the initial 33 days of the start-up phase, stable removal of nitrate and nitrite was observed, with fluctuation in COD removal. The effluent $\text{NO}_3\text{-N}$, $\text{NO}_2\text{-N}$ and COD were 2.36 ± 3.74 mg N/L, 1.41 ± 2.99 mg N/L and 109.56 ± 60.85 mg COD, respectively. The Nitrite loading rate (NLR) was 0.163 ± 0.019 kg N/m³/day and the total nitrogen removal rate (TNRR) was 0.160 ± 0.018 kg N/m³/day. The organic loading rate (OLR) and organic removal rates (ORR) were 0.535 ± 0.090 and 0.315 ± 0.154 kg COD/m³/day. Nitrate removal efficiency (NRE) was 98.7 ± 2.1 % and NAR was below 1%. The Organic removal efficiency (ORE) was 81.4 ± 7.8 %. The removal rates of nitrate, nitrite, and COD were aligned with the observations in other work (Qian et al., 2019; S. Wang et al., 2019), indicating the successful initiation of the control group reactor during the acclimation phase. As the system entered phase B, effluent nitrate and nitrite levels remained below 2 mg N/L, and COD concentration was 57.17 ± 14.76 mg COD. The NLR was 0.158 ± 0.010 kg N/m³/day and NRE was >98%. The OLR was 0.662 ± 0.069 kg COD/m³/day, and the ORE was 91.3 ± 2.3 %. These results affirm that the control group is operating effectively, serving as a suitable reference for assessing the impacts of azoles on the denitrification process.

According to **Figure 6.3**, the performance of the BTA group is similar to the control group. Stable nitrate and nitrite removal and improving COD removal were observed. The effluent $\text{NO}_3\text{-N}$, $\text{NO}_2\text{-N}$ and COD were 4.39 ± 7.76 mg N/L, 2.72 ± 5.12 mg N/L and 90.63 ± 40.32 mg COD, respectively. The NLR was 0.163 ± 0.019 kg N/m³/day and TNRR was 0.153 ± 0.020 kg N/m³/day. The OLR and ORR were 0.511 ± 0.088 and 0.330 ± 0.116 kg COD/m³/day. NRE was 97.4 ± 4.8 % and NAR was 1.5 ± 2.8 %. The ORE was 83.5 ± 5.6 %. The results suggested that the reactor was successfully started as the control reactor. After entering phase B, 2.5 mg/L BTA was added to the system. The effluent nitrate and nitrite were below 2 mg N/L, and the COD was 55.00 ± 17.37 mg COD. The NLR was 0.167 ± 0.011 kg N/m³/day

and NRE was >98%. The OLR was 0.671 ± 0.078 kg COD/m³/day, and the ORE was 91.84 ± 2.28 %. 2.5 mg/L BTA didn't cause inhibition to the denitrification process. This aligns with our findings in the short-term batch tests.

According to **Figure 6.4**, improving nitrate, nitrite, and COD removal was observed in stage A of the PA treatment group. The effluent NO₃-N, NO₂⁻-N and COD were 3.78 ± 6.42 mg N/L, 2.47 ± 3.69 mg N/L and 96.12 ± 48.19 mg COD, respectively. The NLR was 0.158 ± 0.014 kg N/m³/day and TNRR was 0.152 ± 0.016 kg N/m³/day. The OLR and ORR were 0.485 ± 0.072 and 0.293 ± 0.129 kg COD/m³/day. NRE was 97.78 ± 4.0 % and NAR was below 1%. The ORE was 81.83 ± 7.1 %. The performance of the PA group is at approximately the same level as the control and BTA groups, suggesting that the reactor was successfully started. After entering phase B, 2.5 mg/L PA was added to the system. The effluent nitrate and nitrite were below 2 mg N/L, and the COD was 59.88 ± 16.65 mg COD. The NLR was 0.163 ± 0.012 kg N/m³/day and NRE was >98%. The OLR was 0.691 ± 0.085 kg COD/m³/day, and the ORE was 91.34 ± 2.15 %. Similarly, 2.5 mg/L PA didn't cause inhibition to the denitrification process.

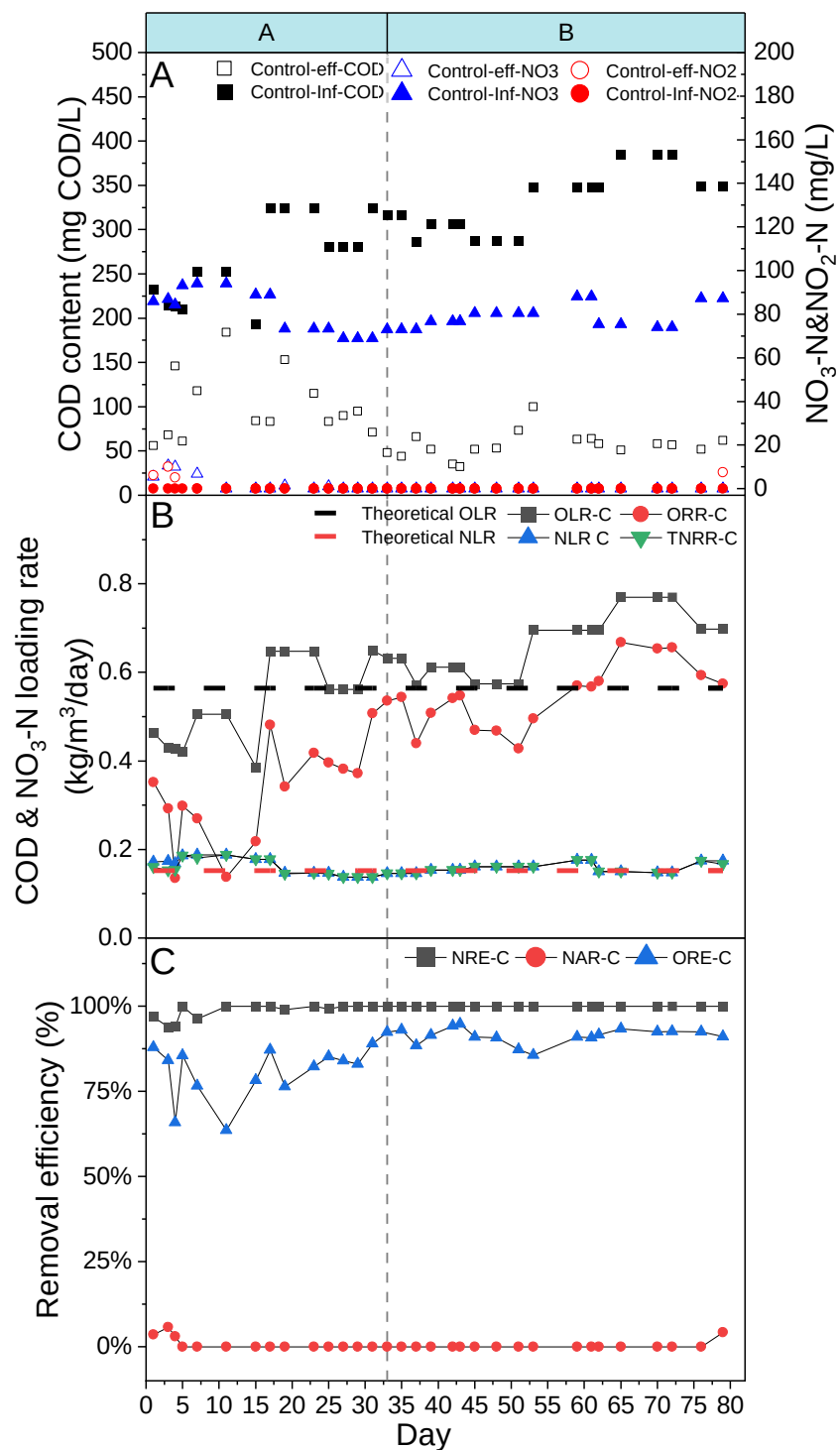


Figure 6.2 Control group N removal performance during operation. A) Influent and effluent N species; (B)NLR: N loading rate; TNRR: Total N removal rate; OLR: Organic loading rate; ORR: Organic removal rate; (C)NRE: Nitrate removal efficiency; NAR: NO_2^- -N accumulation ratio. ORE: Organic - removal efficiency.

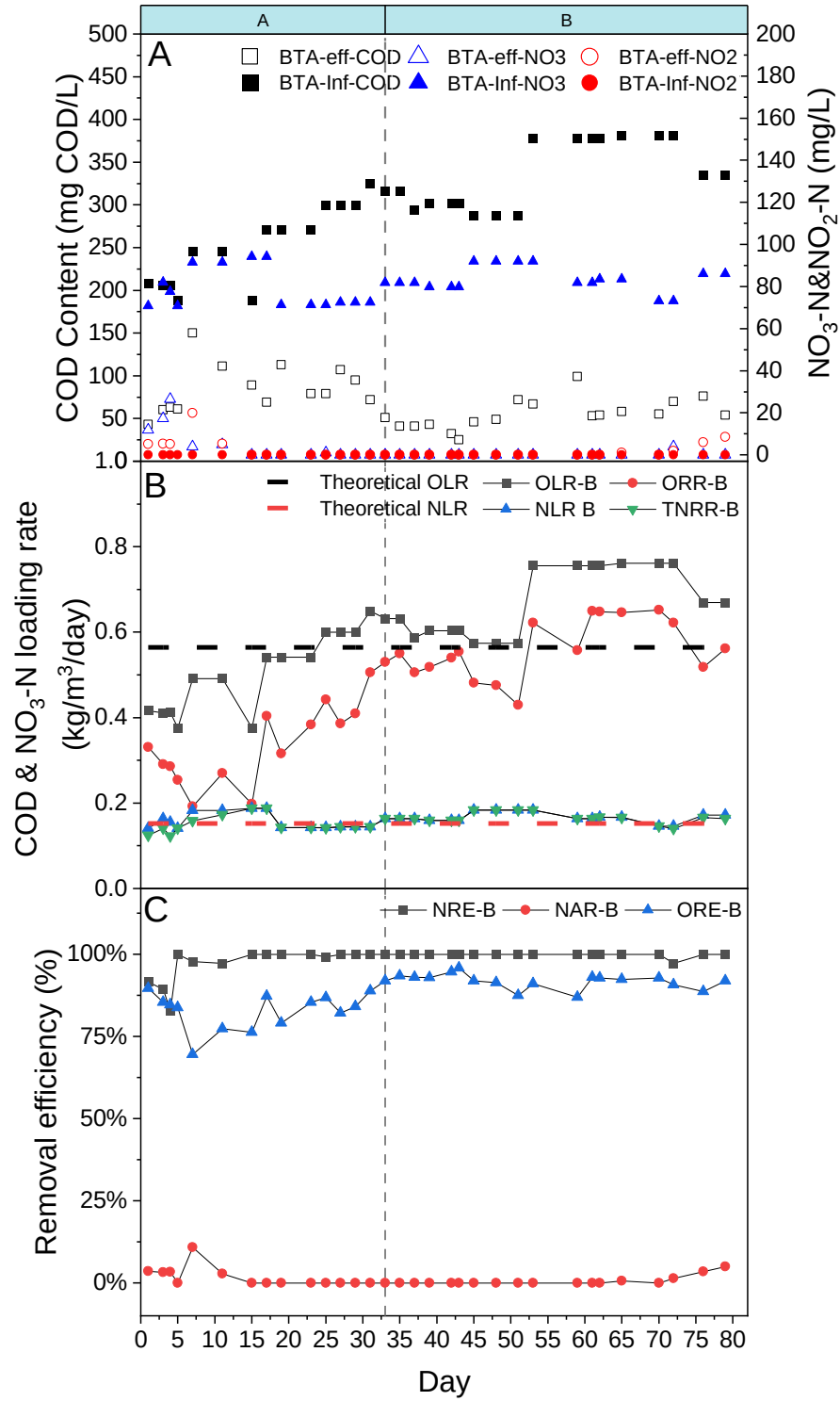


Figure 6.3 BTA group N removal performance during operation. (A) Influent and effluent N species; (B) TNRR, NLR, OLR, and ORR (C) NRE, NAR, and ORE: Organic -removal efficiency.

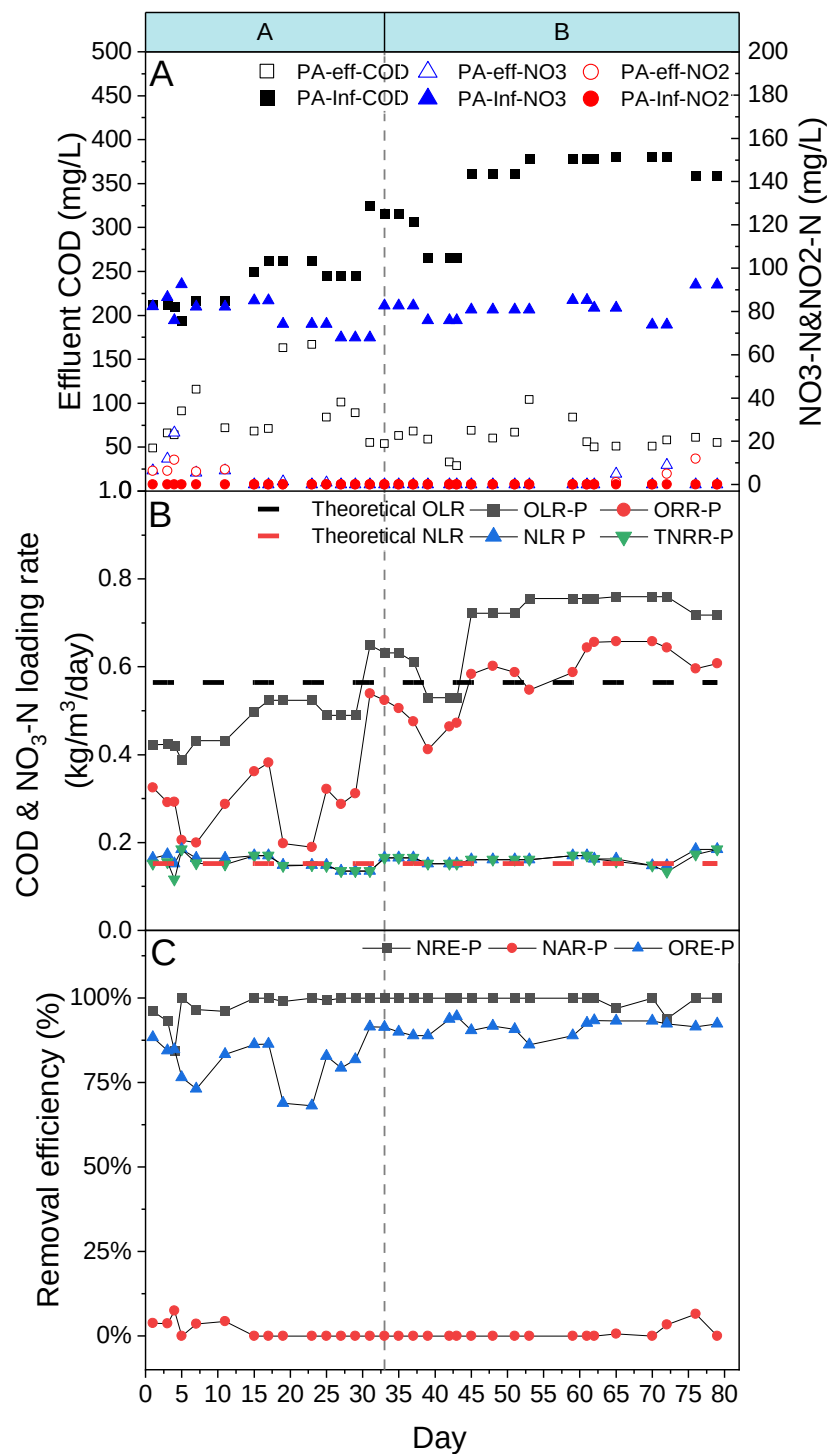


Figure 6.4 PA group N removal performance during operation. A) Influent and effluent N species; (B) TNRR, NLR, OLR, and ORR (C) NRE, NAR, and ORE: Organic -removal efficiency.

Denitrification activities during the operation

Figure 6.5 illustrates the SDA and NDA of the BTA treatment group. During stage A, the SDA of control and BTA groups increased from 3.47 to 25.81 mg N/g TSS/hour and 5.11 to 21.11 mg N/g TSS/hour. The NDA of BTA was 101.60 ± 29.64 %. With the operation proceeding, the activity of the BTA group kept approximately the same level as the control. The increase in the denitrification activities of both groups indicated that both reactors had successfully started. The activities were also within the ranges of other reported work. (Cui et al., 2019; J.-J. Wang et al., 2021). During stage B, 2.5 mg/L BTA was added to the system. The SDA of the control group was 21.50 ± 5.47 mg N/g TSS/hour and the SDA of the BTA group was 21.37 ± 6.16 mg N/g TSS/hour. The activities were maintained at the same level after phase A. The NDA was 100.08 ± 27.12 %, which means the BTA group presented approximately the same nitrogen removal efficiency as the control group. Combined with the results from **Figure 6.3**, long-term exposure to 2.5 mg/L BTA didn't impact the denitrification process. The statistical data also suggest that there are no significant differences between ($p > 0.05$) the treatment group to the control group.

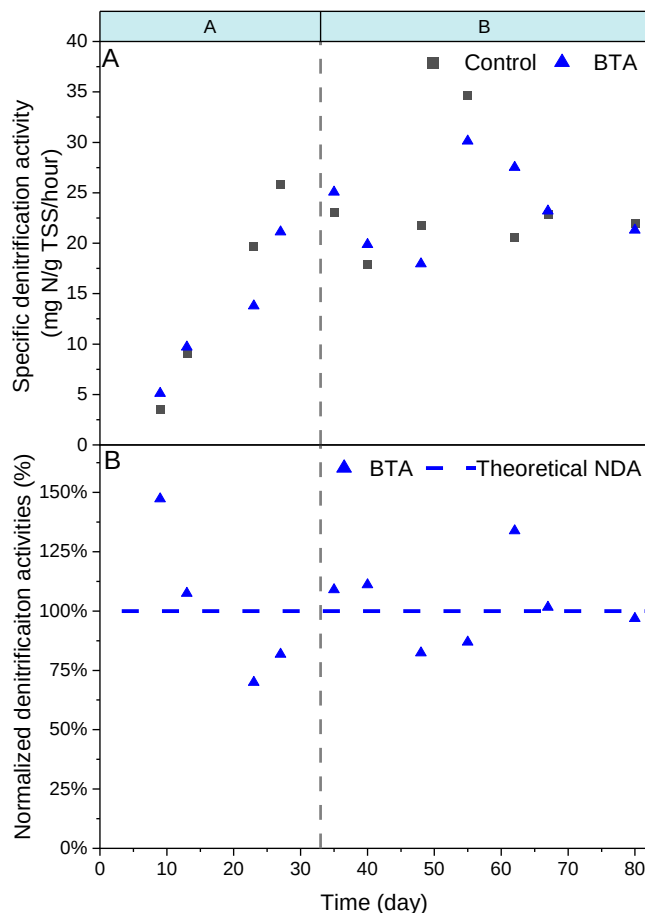


Figure 6.5 Denitrification activities of BTA. (A) SDA of BTA and control group; (B) NDA of BTA group and theoretical NDAS

According to **Figure 6.6**, during stage A, the SDA of PA groups increased from 4.47 to 20.01 mg N/g TSS/hour. The NDA of BTA was $96.69 \pm 19.2\%$. Similar to the BTA group, the activity of the PA group kept approximately the same level as the control. The PA reactor was also successfully started. During stage B, 2.5 mg/L PA was added to the system. The SDA of the PA group was 20.08 ± 8.49 mg N/g TSS/hour. The activities were maintained at the same level as the control and BTA groups. The NDA was $92.43 \pm 36.76\%$. Although the standard deviations are larger than the BTA groups, the PA group also presented approximately the same N removal efficiency as the control group. Combined with the results from **Figure 6.4**, long-term exposure to 2.5 mg/L BTA didn't impact the denitrification process. The statistical data also suggest that there are no significant differences between ($p > 0.05$) the treatment group to the control group.

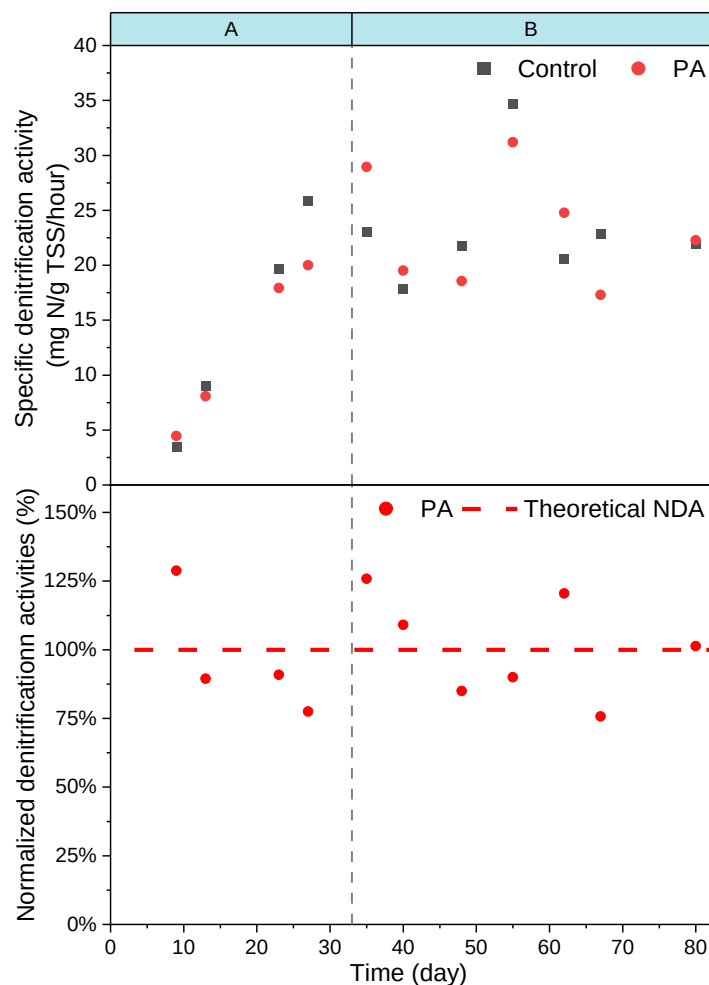


Figure 6.6 Denitrification activities of PA. (A) SDA of PA and control group; (B) NDA of BTA group and theoretical PA

Biodegradation of azole by acclimated denitrification sludge.

In this study, the concentration of BTA and PA was monitored to evaluate the potential of degradation with the nitrification system. In this study, both BTA and PA were found to be stable under sterile conditions as described in previous sections. During the operation, no biodegradation of the BTA and PA was observed within the 12-hour operation cycle. However, previous studies have discussed the potential of azoles and their derivatives degradation under anoxic and/or anaerobic conditions (Q. Huang et al., 2013; Lakhey et al., 2021). Thus, to evaluate the potential degradation of BTA and PA in the

denitrification system, batch experiments with longer operation periods were conducted as described in **Table 6.3** together with the measured BTA degradation rates. The results are illustrated in **Figures 6.7** and **6.8**.

Table 6.3 Azole removal rates in batch tests

Group	BTA removal rate (mg BTA/ g TSS/ hour)		
	Phase I	Phase II	Phase III
Control	0.34	0.42	0.48
BTA	0.28	0.55	0.47
PA	-	0.49	0.41

Group	PA removal rate (mg PA/ g TSS/ hour)		
	Phase I	Phase II	Phase III
Control	0.10	0.20	0.17
BTA	-	0.24	0.14
PA	0.13	0.21	0.18

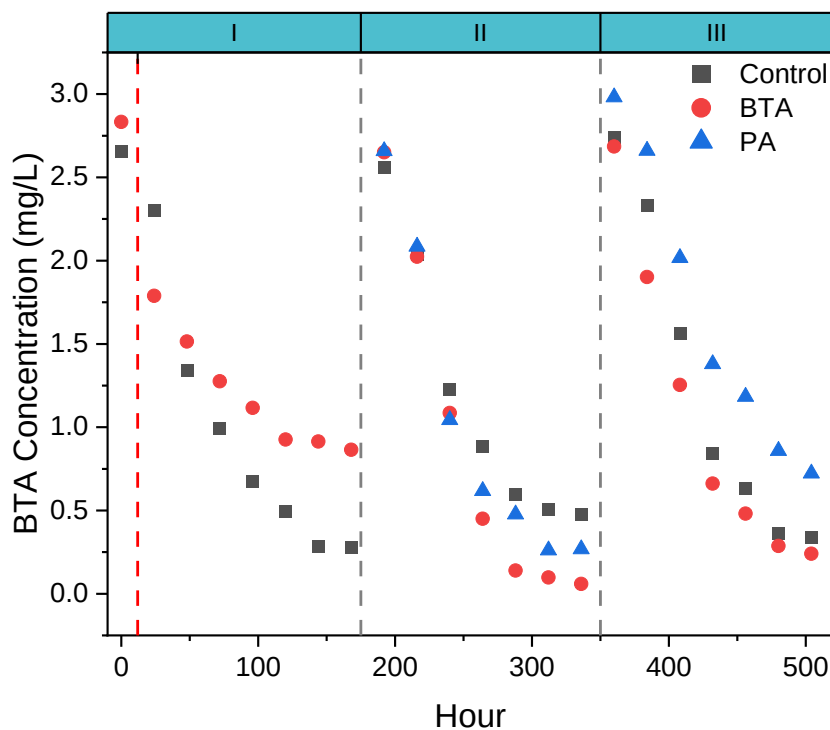


Figure 6.7 BTA degradation experiments. The red line indicated the 12-hour regular cycle time.

According to phase I in **Figure 6.7**, either the control or the BTA group was able to degrade the BTA. The control group BTA decreased from 2.66 to 0.28 mg/L in 7 days. The BTA concentrations of the BTA group decreased from 2.83 to 0.86 mg/L within the same period. The results indicate that the denitrification sludge was able to degrade BTA with/without acclimation. The statistical data also suggest that there are no significant differences in BTA degradation between ($p > 0.05$) the treatment group to the control group. To further verify the finding, the BTA was also added to the PA treatment group in phase II. The BTA concentrations of the control, BTA, and PA groups decreased from 2.56, 2.65, and 2.66 to 0.48, 0.06, and 0.27 mg/L, respectively. The results support the idea that the denitrification microbial community was able to degrade BTA. To investigate the potential degrader, in phase III, nitrate and sodium acetate were not added. The BTA concentrations of the control, BTA, and PA groups decreased from 2.74, 2.69, and 2.98 to 0.34, 0.24, and 0.72 mg/L, respectively. The removal of N and COD addition didn't impact the degradation of BTA. The degradation rates of the batch experiments are summarized in **Table 6.3**. The degradation under anoxic environments has also been reported in other studies. Similar to this study, anoxic sludge from WWTPs has been found able to bio-transform BTA (Trenholm et al., 2020). Besides, Y.-S. Liu et al. (2011) reported that under a nitrate-reducing environment, the degradation of BTA was around 0.0029 mg/L/day. Similarly, Mazioti et al. (2015a) found the 0.03 mg/L BTA was biodegradable under anoxic conditions with a half-life of around 29 hours and suggested potential co-metabolism pathways. In this study, BTA degradation was found in either acclimated or unacclimated sludge, and rates are not impacted after the substrates are removed from the system. The removal of the BTA may have been contributed by some heterotrophic bacteria existed in the denitrification microbial community utilizing the BTA under anoxic conditions (Ghattas et al., 2017). However, the degradation rates reported in this study are longer than some reported HRT of full-scale WWTPs, which suggests the retention of BTA in the anoxic zone may not be enough for complete BTA degradation.

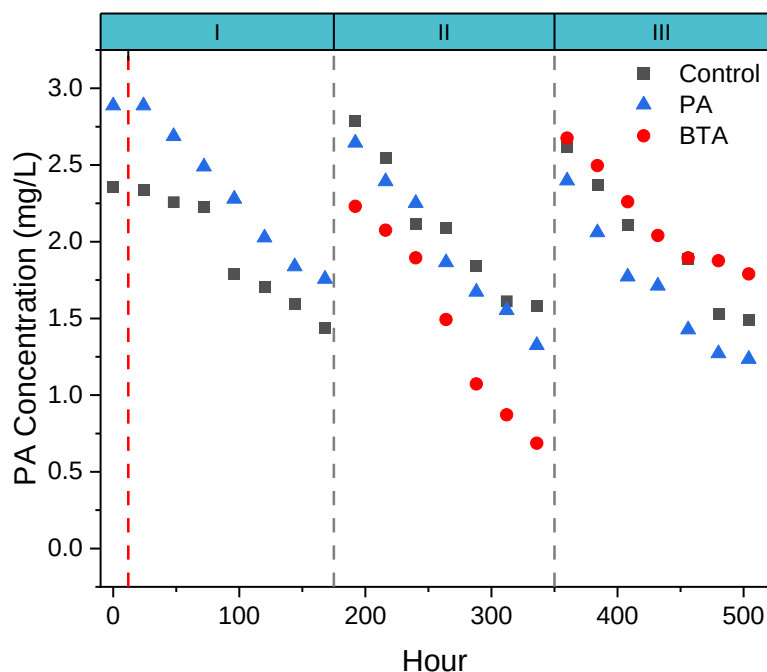


Figure 6.8 PA degradation experiments. The red line indicated the 12-hour regular cycle time. According to phase I in **Figure 6.8**, either the control or the PA group was able to degrade the PA. The control group BTA decreased from 2.36 to 1.44 mg/L in a week. The PA concentrations of the PA group decreased from 2.88 to 1.75 mg/L within the same period. The results indicate that the denitrification sludge was able to degrade the PA. The statistical data also suggest that there are no significant differences in BTA degradation between ($p > 0.05$) the treatment group to the control group. To further verify the finding, the PA was also added to the BTA treatment group in phase II. The PA concentrations of the control, BTA, and PA groups decreased from 2.55, 2.64, and 2.23 to 1.58, 1.32, and 0.69 mg/L, respectively. The results support the idea that the denitrification microbial community was able to degrade PA. To investigate the potential degrader, in phase III, nitrate and sodium acetate were not added. The PA concentrations of the control, BTA, and PA groups decreased from 2.62, 2.40, and 2.68 to 1.49, 1.23, and 1.80 mg/L, respectively. The removal of N and carbon sources didn't impact the degradation of PA. Compared to BTA, the degradation of PA is slower than BTA in this study, which aligns with previous studies that PA is difficult to degrade in wastewater treatment processes (Kalyani V Jog et al., 2021; Pagga et al., 2006). Di Marcantonio et al. (2020) reported that 40% to 60% of 0.1 mg/L PA could

be removed through intermittent switches in aerated/anoxic operation. However, limited information is available regarding the potential anaerobic pathways, degraders, and products. The potential products of oxidative PA metabolism identified in studies with rat liver microcosms and hepatocytes were mono-, di- and tri-hydroxylated PAs (Kalyani V Jog et al., 2021). In this study, PA was degraded either with/without nitrate and acetate addition. Similar to BTA, the potential degrader may co-exist with the denitrification community. A better understanding of the microbial communities may help explain the anoxic biotransformation of BTA and PA.

Long-term effect of azoles on the microbial community

The microbial community in the system was initially seeded using the Inoculum sludge obtained from an anoxic zone of a WWTP (Laurel, MD, USA), in which the denitrification process was known to happen. To illustrate the similarities and differences in the microbial composition of each reactor, inoculum samples and sludge samples in each reactor (control, BTA, and PA) were collected before azole addition (Stage A) and at the end of the operation (Stage B) and analyzed. In this study, different sequences were respectively detected from the Inoculum, Control, BTA, and PA samples, corresponding to the operational taxonomic units (OTUs) were identified as described in **Figure 6.9**. In stage A, the four samples have 90 OTUs (14.4%) shared in common and the exclusive OTUs in Inoculum, Control, BTA, and PA were 164, 15, 11, and 9, respectively. 48.1% of OTUs are shared by Control-A, BTA-A, and PA-A groups, suggesting an adaptation of the inoculum to the new environment. With the addition of azoles, in stage B, 216 OTUs (36%) are shared by Control-B, BTA-B, and PA-B groups, suggesting a stable community component in the samples after azole addition.

According to **Figure 6.10**, an increase in multiple denitrifications involved genus was detected. *Thauera* become the dominant species after inoculum, which is a known denitrifier with metabolic versatility for the remediation of environmental pollutants such as phenol, methylphenol, and indole (Guo et al., 2019; Mao et al., 2010). The *Azoarcus* is also denitrifies reported in wastewater treatment systems and could remove toluene and phenol (Jwa et al., 2020; Song et al., 1999). Besides, *Georgfuchsia* is identified as

nitrate-reducing bacteria and a key player in benzene, toluene, ethylbenzene, and xylenes degradation (Sperfeld et al., 2018). *Dechloromonas* spp. were originally isolated as aromatic, compound-degrading bacteria and facultative anaerobic nitrate-reducing bacteria (Petriglieri et al., 2021). These results suggested that 80 days of operation might result in a shift in dominant bacteria to adapt to a new environment. However, no further changes in the dominant genus were observed after the azole addition. For other genera, *Solitalea* is also a dominant genus that has been found in BNR processes and recognized as hydrolytic bacteria (Jwa et al., 2020; Pang et al., 2022). *Ignavibacterium* sp. is known to grow in anaerobic conditions, producing lactate or alcohol (Jwa et al., 2020). *Flavobacteria* was a common genera found in WWTPs with high antibiotic resistance potential (Zhu et al., 2017). Although a variety of denitrifiers and pollutant degraders have been identified, further analysis should focus on the specific roles of those microorganisms in azole resistance and degradation. For WWTPs going to receive azole-containing wastewater, denitrification systems may be more stable than nitrification and Anammox processes. However, if the concentrations of azoles are higher (>50 mg/L), a decrease (>25%) in denitrification activity may still occur with the azoles tested in this study. Attention should be given to the influent composition and continuous monitoring of the nitrogen removal performance is necessary.

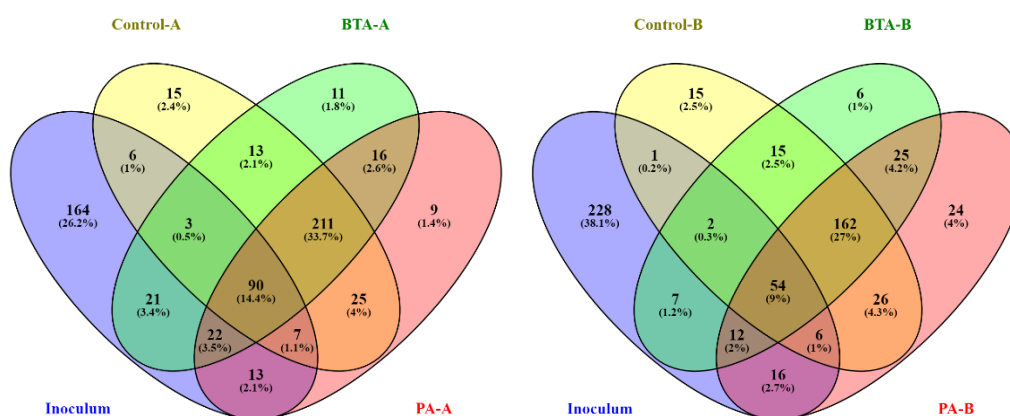


Figure 6.9 OTU numbers in Inoculum, Control, BTA, and PA samples. Inoculum and stage A (Left). Inoculum and stage B (Right)

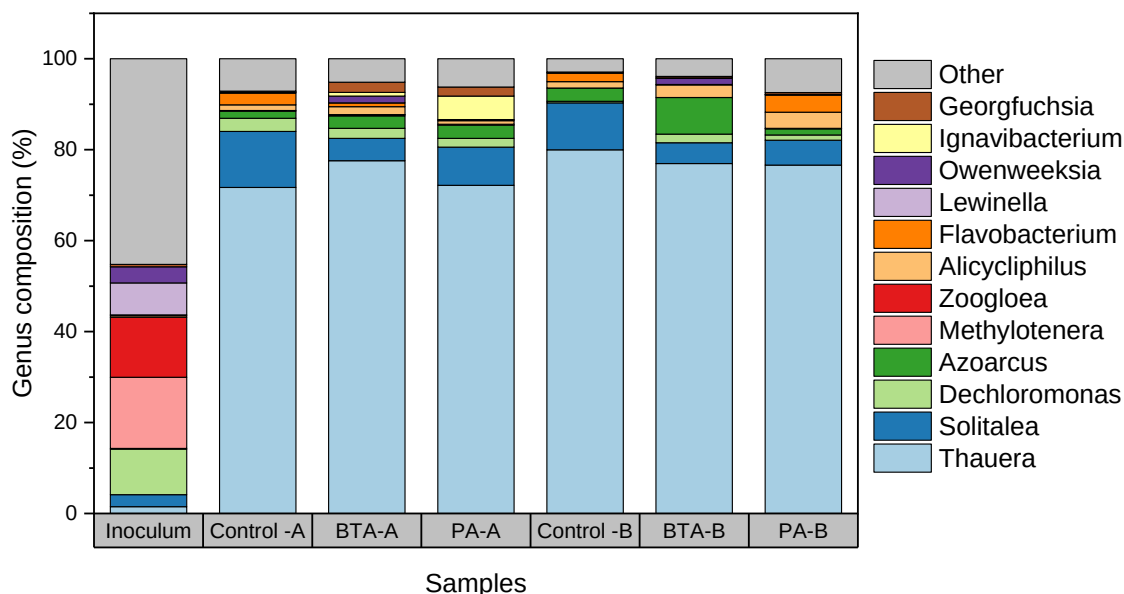


Figure 6.10 Relative abundance of dominant bacteria at genus level of denitrification sludge in the inoculum, control, BTA and PA samples of stage A and B

Implications

The denitrification process is resistant to BTA and PA inhibition at 2.5 mg/L. Following the start-up phase, the addition of azoles did not impact the denitrification activities in the subsequent operation. The existence of multiple aromatic compound/organic compound degraders such as *Thauera*, *Azoarcus*, *Georgfuchsia*, and *Dechloromonas* were identified in the denitrification system. However, the degradation rate of BTA and PA is limited, which may still cause retention of azoles in the BNR systems of WWTPs. Although BTA and PA did not inhibit the denitrification process, the denitrification process also can not eliminate the azoles from the WWTPs. Besides, the impacts of potential degradation products were not clear, and their effects on biological wastewater treatment processes still need investigation.

Chapter 7: Conclusions

The biological nitrogen removal (BNR) processes have been applied in full-scale systems around the world. However, the wide application of azoles in different realms led to concern about its potential risk to BNR processes. To answer the questions of how azoles will impact the BNR processes in the short-term and long-term, what the inhibitory mechanism is, and how to solve the inhibition issue in the system, both batch- and continuous-mode experiments were carried out with representative azoles in this research.

The initial phase of the study involved investigating the short-term effects of benzotriazole (BTA), methyl benzotriazole (MBTA), pyrazole (PA), and triazole (TA) through batch experiments and a literature review. The findings indicated that the inhibitory impact on the nitrification process was more pronounced compared to the Anammox and denitrification processes. As the prerequisite step of Anammox and denitrification, nitrification can be inhibited due to the presence of certain azoles at low mg/L levels (< 5 mg/L) and then interfere with the stability of BNR processes in wastewater treatment plants (WWTPs). The unique cell structure of the Anammox bacteria and the high diversity of the denitrification microbial community may contributed to their higher resistance to azole inhibition. In the nitrification process, the ammonium oxidation process is more inhibited than the nitrite oxidation process. Azoles may interfere with the function of AMO through chelation and thus inhibit the nitrification process. No inhibition ($\geq 10\%$) of azoles with larger molecular and more branched structures on BNR processes was observed.

The second step is investigating the long-term impact of azoles on BNR processes. The study focused on BTA and PA due to their distinct structures and varied inhibition effects on the Anammox process. For the Anammox process, high concentrations of BTA (>40 mg/L) and PA (>200 mg/L) could cause inhibition to the Anammox process in long-term exposure. At the same time, the Anammox process could gain resistance to 0-30 mg/L BTA through long-term acclimation. Long-term exposure to 200 mg/L caused more inhibition of the Anammox process than short-term exposure and could cause irreversible

inhibition. This suggests that extended exposure to azoles may intensify the inhibitory impact on biological processes. Both BTA and PA are persistent in the Anammox system, which further increases the possibility of long-term exposure to azoles in the Anammox process implemented systems. Starvation of the Anammox bacteria didn't increase the inhibition of azoles in the Anammox process, implying Anammox bacteria may not depend on energy-required methods to mitigate the azole inhibition.

The investigation into the long-term effects of BTA and PA on the nitrification process revealed that acclimation to azole toxicity could enhance the resilience of the nitrification process when treating wastewater containing azole. Acclimated nitrification sludge demonstrated better performance when the same concentrations of BTA and PA were added to the system. In this study, acclimation to BTA also led to the development of BTA degradation ability in the system. This ability is not only beneficial for the stability of the nitrification process but also for other BNR processes following the nitrification process. The heterotrophic bacteria that existed in the nitrification sludge may contribute to the BTA degradation. The aromatic compound/organic compounds degraders such as *Dechloromonas* and *Zoogloea* are identified in the microbial community.

The denitrification process is found resistant to BTA and PA inhibition at 2.5 mg/L. After the start-up phase, the addition of azoles did not impact denitrification activities in the subsequent operation. Multiple aromatic compounds and organic compound degraders, including *Thauera*, *Azoarcus*, *Georgfuchsia*, and *Dechloromonas* were identified in the denitrification system. However, the degradation rate of BTA and PA is limited, potentially causing the retention of azoles in the BNR systems of WWTPs. Additionally, the impacts of potential degradation products were unclear, necessitating further investigation into their effects on biological wastewater treatment processes.

This work discussed that BNRs have the potential to acclimate azole inhibition. The azoles selected in this study are azoles that have a variety of derivatives. The concentrations investigated in this study covered the reported azole concentrations in different matrices, the potential higher concentrations based on their physical properties, and the marginal conditions that could offer valuable information. The results

could offer guidance for WWTPs treating azole-containing wastewater such as the maximum wastewater treatment capacity without impacting BNR performance. Future research could focus on azoles with more accessible chemical structures to delve deeper into the inhibition mechanisms. Besides, the adaptation period in this study could take up to months. Potential methods for decreasing the acclimation time could be investigated.

Appendices A

Table S1 Preliminary tests of azoles

Chemicals	Test concentrations and inhibition condition (mg/L)		
	Anammox	Nitrification	Denitrification
PA	NI at 100 mg/L	Not tested	20 ± 1 % inhibition at 50 mg/L
TA	NI at 100 mg/L	Not tested	15 ± 2 % inhibition at 50 mg/L
BTA	52 ± 1 inhibition at 20 mg/L	Not tested	15 ± 2 % inhibition at 50 mg/L
MBTA	53 ± 2 inhibition at 20 mg/L	Not tested	15 ± 1 % inhibition at 50 mg/L
Climbazole	NI at 0.5 mg/L	NI at 0.5 mg/L	NI at 0.5 mg/L
Ketoconazole	NI at 0.5 mg/L	NI at 0.5 mg/L	NI at 0.5 mg/L
Tioconazole	NI at 0.5 mg/L	NI at 0.5 mg/L	NI at 0.5 mg/L
DMSO	NI at 275 mg/L	NI at 275 mg/L	NI at 275 mg/L

*NI: No inhibition ($\geq 10\%$) found.

Table S2 Azole concentrations in bioassays with DMSO added as co-solvent

Chemicals	DMSO stock solution concentrations (g/L)	Target test concentration (mg/L)	DMSO concentration in experiment (mg/L)
Climbazole	40	10	275
Ketoconazole	4	1	
Tioconazole	4	1	

Table S3 Anammox biodiversity evaluation indexes

Samples	Sequence number	OTU	Shannon	Simpson	Evenness
Inoculum	30524	99	1.92	0.68	0.15
Control	29341	108	2.57	0.85	0.18
BTA	29146	106	2.73	0.86	0.18
PA	30354	105	2.17	0.77	0.16

Table S4 Nitrification biodiversity evaluation indexes

Samples	Sequence number	OTU	Shannon	Simpson	Evenness
Inoculum	22905	358	3.97	0.95	0.18
Control	28146	388	3.08	0.86	0.16
BTA	27192	430	3.51	0.92	0.17
PA	27141	369	2.83	0.82	0.15

Table S5 Denitrification biodiversity evaluation indexes

Samples	Sequence number	OTU	Shannon	Simpson	Evenness
Inoculum	21194	326	4.80	0.98	0.17
Control -A	31894	370	2.60	0.67	0.11
BTA -A	32510	387	2.61	0.65	0.11
PA -A	31704	393	2.62	0.68	0.11
Control -B	32915	281	1.82	0.52	0.10
BTA -B	32963	283	2.43	0.69	0.12
PA -B	32465	325	2.30	0.60	0.10

Table S6 Limit of detection and limit of quantitation of analytical instruments

Parameter	Instrument	Limit of detection (mg/L)	Limit of quantitation (mg/L)
NO ₃ -N	Dionex ICS-1100	0.2	0.5

NO ₂ -N	Dionex ICS-1100	0.2	0.5
NH ₄ -N	IntelliCAL Ammonia Ion Selective Electrode	0.2	2
NH ₄ -N	SEAL AQ300 discrete analyzer	0.01	0.05
NO ₃ -N	SEAL AQ300 discrete analyzer	0.01	0.05
NO ₂ -N	SEAL AQ300 discrete analyzer	0.01	0.05
N ₂	Agilent 6890 gas chromatograph	10	10
Chemical oxygen demand	DR900 colorimeter	3	3
BTA	high-performance liquid chromatography	0.05	0.05
PA	high-performance liquid chromatography	0.05	0.05
MBTA	high-performance liquid chromatography	0.05	0.05
TA	high-performance liquid chromatography	0.05	0.05
Dissolved oxygen	LBOD101 Sensor	0.05	0.05
TSS analysis		0.1	0.1
VSS analysis		0.1	0.1

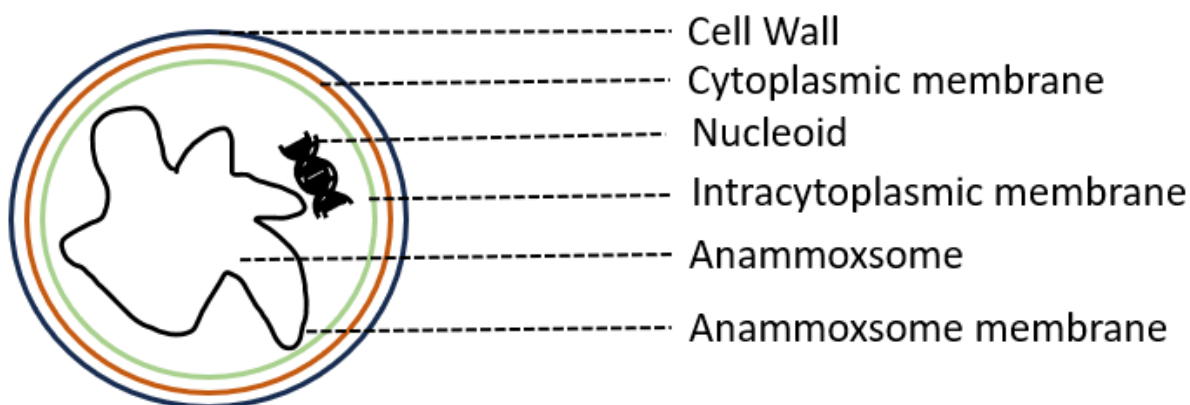


Figure S1 Cellular compartmentalization of Anammox bacteria redrawn from (van Niftrik et al., 2012)

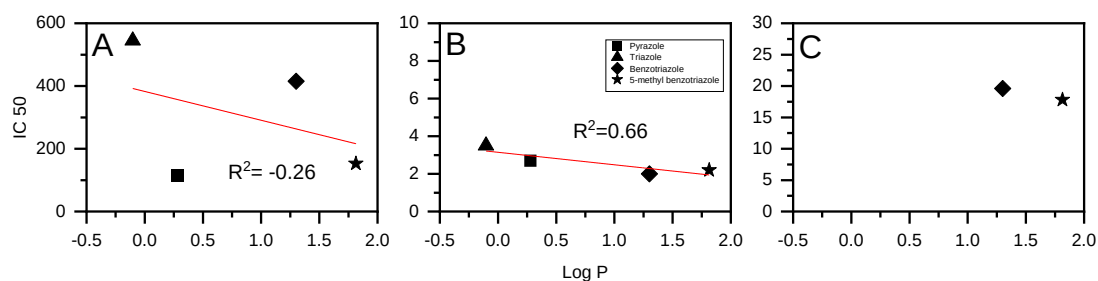


Figure. S2 Effects of hydrophobicity on the (A) Denitrification, (B) Nitrification and (C) Anammox processes toxicity of tested azole compounds

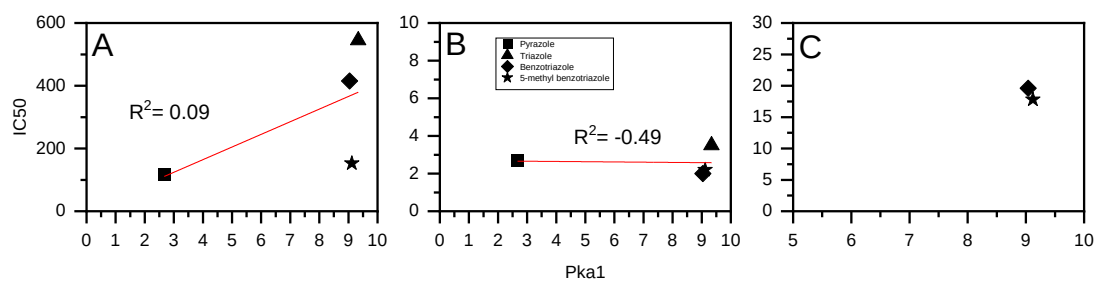


Figure. S3 Effects of dissociation of protonated species on the (A) Denitrification, (B) Nitrification and (C) Anammox processes toxicity of tested azole compounds

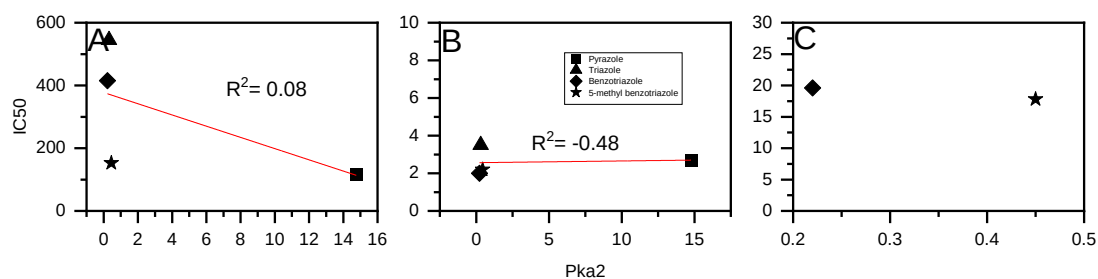


Figure. S3 Effects of dissociation of neutral species on the (A) Denitrification, (B) Nitrification and (C) Anammox processes toxicity of tested azole compounds

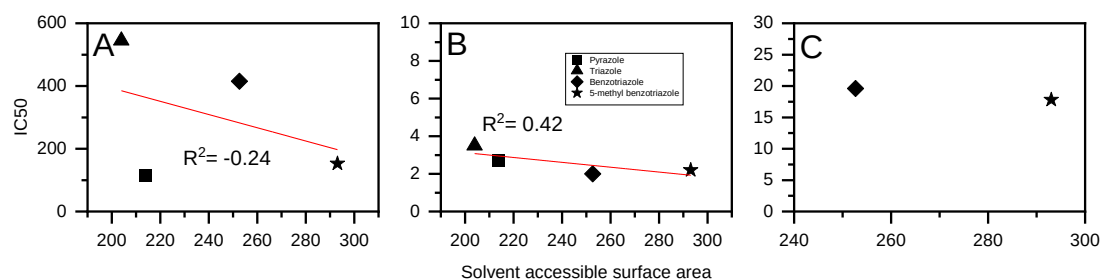


Figure. S4 Effects of Solvent accessible surface area species on the (A) Denitrification, (B) Nitrification and (C) Anammox processes toxicity of tested azole compounds

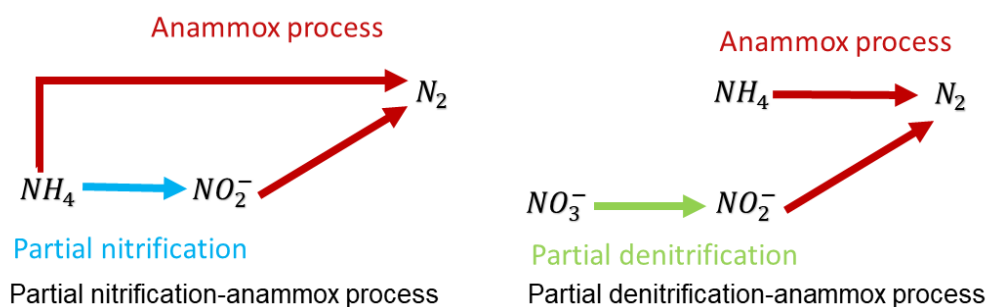


Figure S5 Scheme of partial nitrification-Anammox and partial denitrification-Anammox process

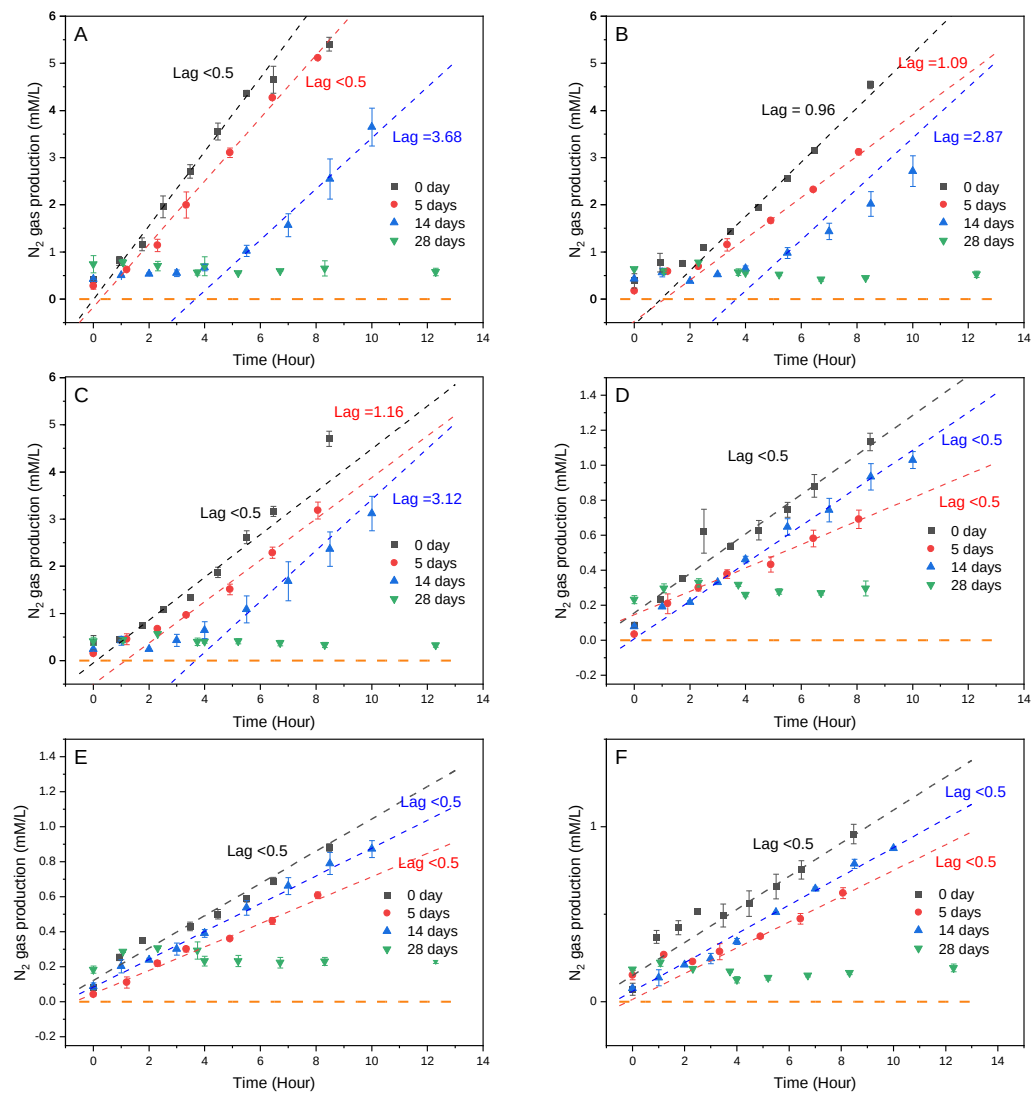


Figure S6 lag phase of Anammox bacteria under different substrate conditions.

Appendices B

Impacts of Crude Glycerol on Anaerobic Ammonium Oxidation (Anammox) Process in Wastewater Treatment

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 (IC_{50}) 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 (TIN) 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 (WRRFs), promoting sustainable development.

Keywords: Waste-Derived Carbon, Anammox, Nutrient Removal, Glycerol, Methanol, Denitrification

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 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 $\text{NH}_4^+\text{-N}$ in the influent into $\text{NO}_2^-\text{-N}$, aiming to attain an ideal stoichiometric substrates relationship of $\text{NH}_4^+\text{-N} : \text{NO}_2^-\text{-N} = 1:1.15 \sim 1:1.32$ for Anammox reaction (Lotti et al., 2014; Strous et al., 1998). To achieve this, two methods have been proposed, including 1) partial oxidation of $\text{NH}_4^+\text{-N}$ by ammonia-oxidizing bacteria (AOB) known as partial nitrification (PN); and 2) $\text{NO}_3^-\text{-N}$ 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 $\text{NO}_2^-\text{-N}$ 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 their application as alternative carbon sources 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 waste management and biological wastewater treatment to develop a sustainable and effective process for utilizing CG for nutrient removal.

Materials and Methods

Waste-derived organic carbons

CG, MicroC 2000, and MicroC 3000 were obtained from a local municipal WRRF in Maryland, USA, which has 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.

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) has been successfully operated for three years with a loading rate of $4.1 \text{ g N L}^{-1} \text{ day}^{-1}$, and the volatile suspended solids (VSS) is 89% of total suspended solids (TSS). The Anammox biomass had an activity of $0.55 \pm 0.05 \text{ g N}_2\text{-N g}^{-1} \text{ VSS day}^{-1}$. 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 (SI, Fig. S1).

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}): $\text{NaHPO}_4 \cdot \text{H}_2\text{O}$ (115), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (200), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (400), NaHCO_3 (100), 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 ($0.70 \text{ g VSS L}^{-1}$), 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 $5.4 \text{ mM NH}_4\text{HCO}_3$) 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 $30 \pm 1^\circ\text{C}$ 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 (shown in Fig. S2).

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 ($30\text{ m} \times 0.32\text{ mm}$, Agilent Technologies) and a thermal conductivity detector. Liquid samples were collected before and after the reaction and filtered ($0.45\text{ }\mu\text{m}$) 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).

Data processing

The specific Anammox activity (SAA, expressed as $\text{N}_2\text{-N g}^{-1}\text{ VSS day}^{-1}$) 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:

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

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).

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

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); K_s 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.

Results and discussion

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 N₂-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 used 700 mg COD L⁻¹ as previous work showed it has a higher inhibitory potential on Anammox bacteria (Güven et al., 2005; Madeira & 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 have been 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 nitrification-denitrification separate centrate treatment process, indicating the growth and enrichment potential of Anammox bacteria in the presence of glycerol (Park et al., 2017). Furthermore, glycerol has been suggested as a protective agent for preserving Anammox bacteria (Chen & 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⁻¹, 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 (IC₅₀) of 3200 mg L⁻¹ 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 ($5.9 \pm 3.6\%$), butyrate ($37.6 \pm 0.7\%$) and formate ($77.3 \pm 2.7\%$) on Anammox activity, the addition of propionate resulted in the most significant reduction, with an inhibition of $97.2 \pm 0.7\%$ (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⁻¹ (equivalent to 467 mg COD L⁻¹) could decrease 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 $\leq 50 \text{ mg L}^{-1}$), Anammox bacteria have 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 $700 \text{ mg COD L}^{-1}$ 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 $600 \text{ mg COD L}^{-1}$, 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 ($< 0.1 \text{ wt. \%}$), while MicroC 3000 is an alcohol blend with a high methanol content, comprising $60 - 80 \text{ wt. \%}$.

% of its composition. The results from the inhibition test for MicroC 2000 and 3000 agreed with our 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.

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 IC₂₀, IC₅₀, IC₈₀, and other parameters summarized in Tables 2 and S1.

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 is consistent with the 96.7 ± 0.5 % inhibition at 2570 mg CG-COD L⁻¹ in a preliminary test (Fig. 1). The results from pure glycerol and methanol groups also aligns 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 R² of 0.98 for both CG and methanol. The calculated IC₅₀ 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 (Table S1). 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 IC₅₀ 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 was reported to display a good potential for promoting PdN in a single-phase PdN-Anammox reactor (Le et al., 2019). However, when PdN is coupled with Anammox in a single-stage reactor, they observed NO_2^- accumulation, suggesting 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 $0.28 \pm 0.01 \text{ g N}_2\text{-N g}^{-1} \text{ VSS day}^{-1}$, respectively, indicating crushing procedure did not have a negative impact on Anammox bacteria. Compared with SAA of $0.79 \pm 0.08 \text{ g N}_2\text{-N g}^{-1} \text{ VSS day}^{-1}$ 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 represent 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 suggests that the

granular structure does not provide additional protection for Anammox bacteria from the inhibition caused by CG and methanol.

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 $\text{NH}_4^+\text{-N}$ but low carbon concentrations (i.e., low COD/TIN ratio, total inorganic nitrogen (TIN) = $\text{NH}_4^+\text{-N}$ + $\text{NO}_2^-\text{-N}$). 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 $\text{NH}_4^+\text{-N}$: $\text{NO}_2^-\text{-N}$ 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 have been reported in the literature, enabling us to make a comparison. It has been reported that when fat milk was added as a COD source, COD/N of 2 may fully inhibit 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 their experiments, TIN concentration was

held constant, and different concentrations 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 compare Anammox activity at 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 $\text{NH}_4^+\text{-N} : \text{NO}_2^-\text{-N}$ 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^- , promotes 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 K_s (mg N L⁻¹) are 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 (Table S2). Various K_s 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 K_s 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 K_s values of 0.1 mg $\text{NH}_4^+\text{-N}$ L⁻¹ and 0.07 mg $\text{NO}_2^-\text{-N}$ L⁻¹ were reported for *Ca. Brocadia* (Jetten et al., 2005). Such varied K_s 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 K_s remained relatively unchanged ($p > 0.1$) at 35.1 ± 9.2 mg N L⁻¹. This result

indicates a non-competitive inhibition mechanism of CG, or more precisely, the inhibitory components within CG, on Anammox bacteria.

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

To supply the substrates at a proper ratio of $\text{NH}_4^+\text{-N}$: $\text{NO}_2^-\text{-N}$ for Anammox, PdN has been 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). To assess if CG can be used for denitrifiers, batch denitrification bioassay was conducted and the experiment setup was provided in the SI. With the addition of KNO_3 (49 mg N L^{-1}), a fast consumption of CG was observed with a calculated consumption rate of $271 \text{ mg COD hr}^{-1}$ in the first hour, corresponding to consumption of 69 % added CG from 570 ± 7.1 to $179.4 \pm 5.9 \text{ mg COD L}^{-1}$ (Fig. S3). At the end of the reaction (7 hrs), 95 % added CG-COD was consumed with the final CG concentration of $28 \pm 1.4 \text{ mg COD L}^{-1}$. This result agrees with previous work indicating 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 $\text{NO}_2^-\text{-N}$ 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.

Conclusion

CG exhibited a good potential to support denitrifier in the two-stage PdN-Anammox process. However, direct exposure of Anammox to CG at $153.7 \text{ mg COD L}^{-1}$, 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.

Appendix A. Supplementary information

E-supplementary data of this work can be found in online version of the paper.

Data availability

Data will be made available on request.

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.

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Figure captions.

Figure 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.

Figure 2. Effects of pure (PG, open square) and crude (CG, closed square) glycerol, and methanol (MeOH, closed 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.

Figure 3. Effects of crude glycerol (CG, closed square) and methanol (MeOH, closed 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.

Figure 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 IC₅₀ 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.

Figure 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 NH₄⁺-N : NO₂⁻-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).

Figure 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 NH₄⁺-N : NO₂⁻-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%. Error bar represents the standard deviation of duplicates.

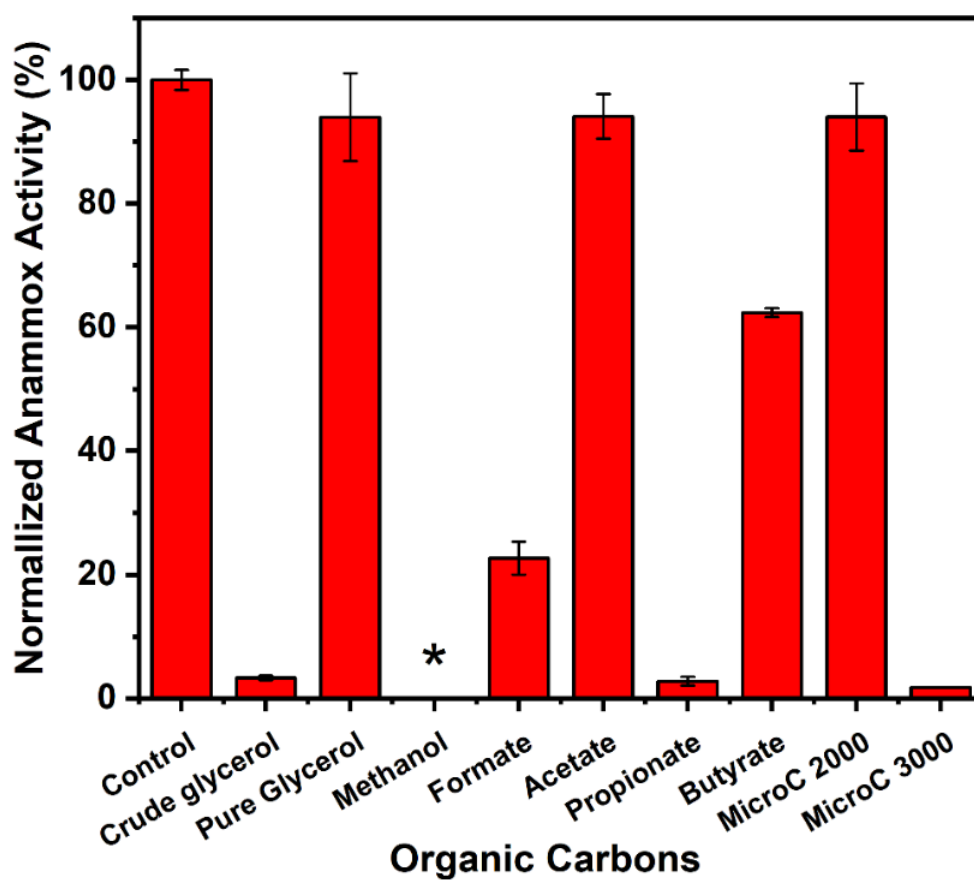


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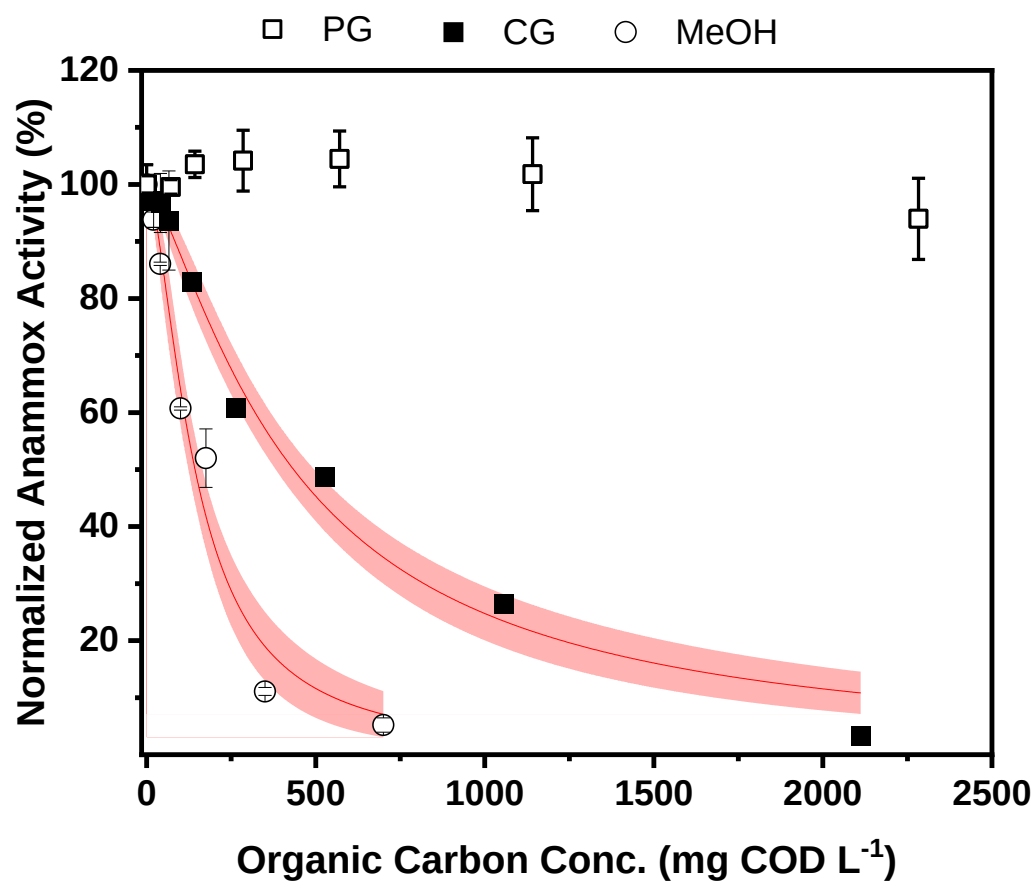


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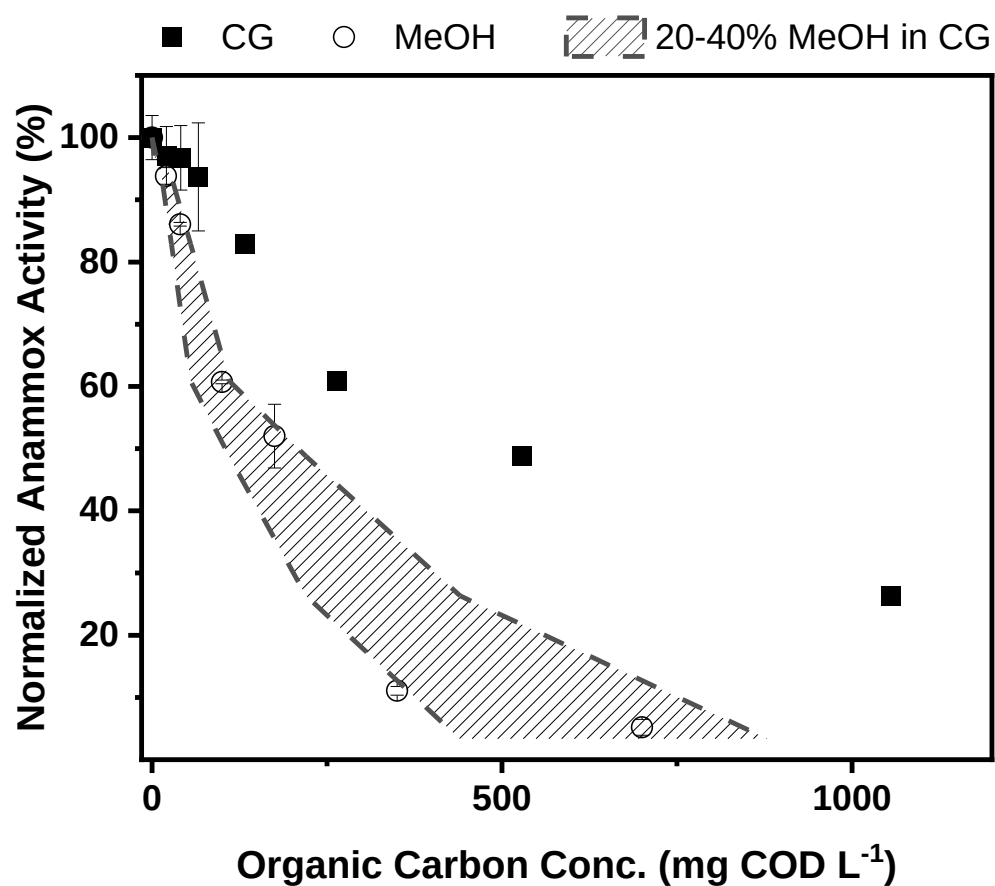


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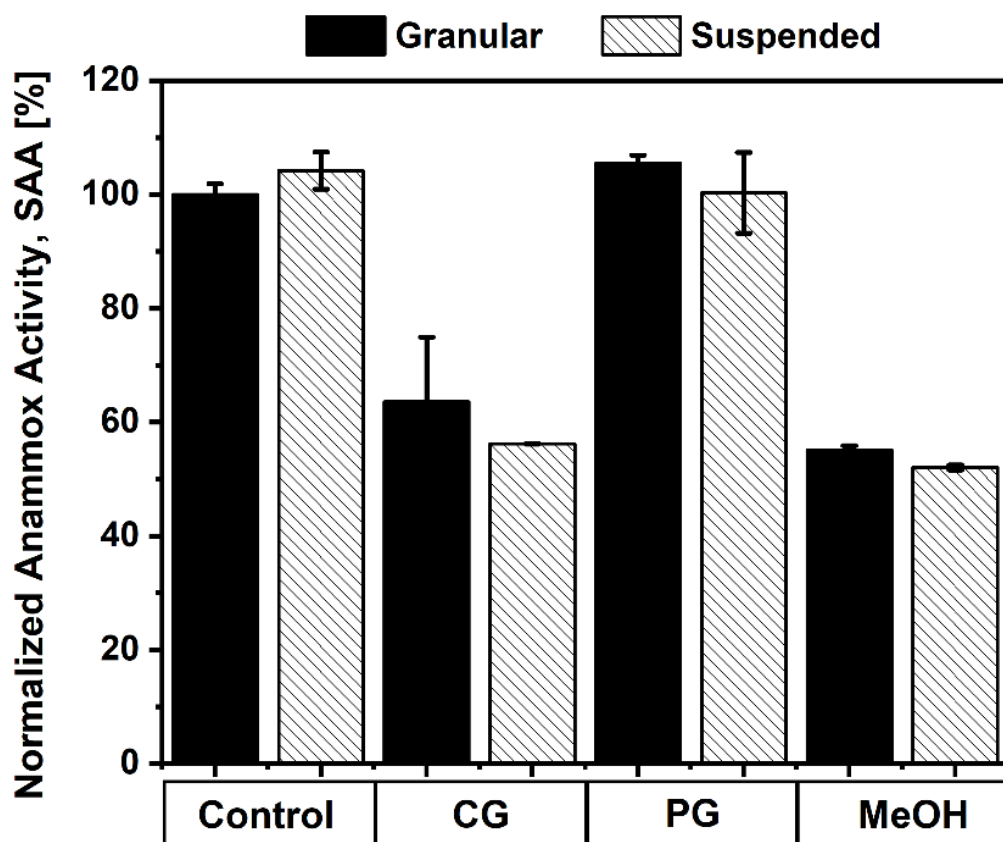


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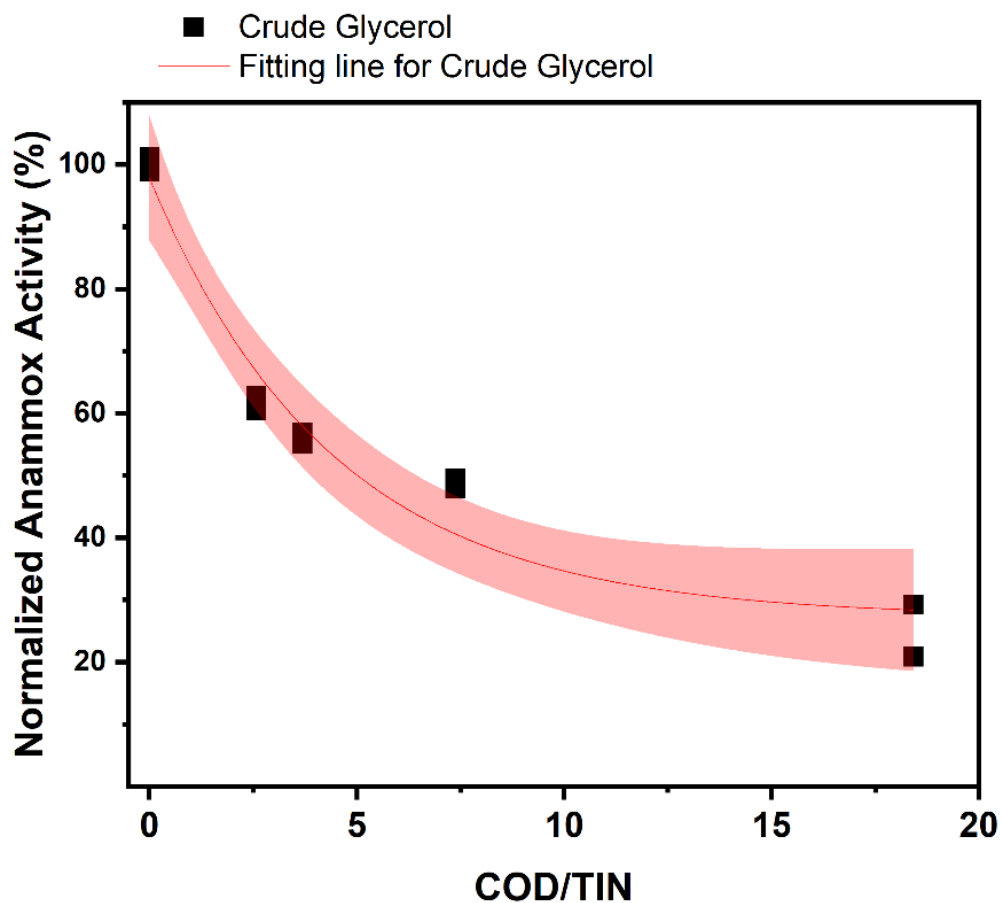


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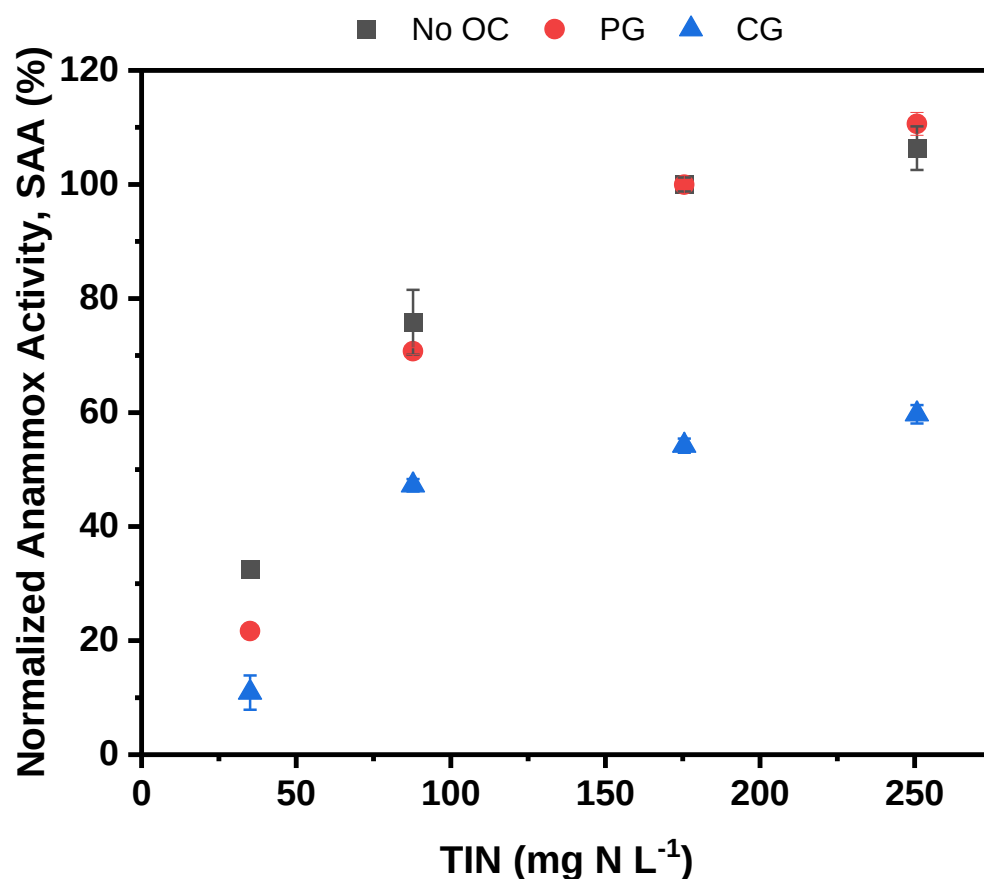


Figure 6. Comparison of the inhibitory impacts of pure glycerol (PG) and crude glycerol (CG) on Anammox activity in the presence of different substrate levels. The concentration of organic carbons (OC) was fixed at 647 mg COD L⁻¹, while various ammonium and nitrite concentration were used as design (mM): 2.5, 6.3, 12.5, and 17.9 mM N at the NH₄⁺-N : NO₂⁻-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 1. The properties of tested waste-derived carbon sources tested.

Source	Description	Color	Density * [g mL ⁻¹]	COD # [g L ⁻¹]	Methanol content * (wt %)
Crude glycerol	Byproduct of biodiesel production 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.

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 = NH₄⁺-N+NO₂⁻-N

The experiments used for obtaining the results were carried out in duplicate.

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