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RESEARCH ARTICLE

Assessing the inhibition potential of azole compounds to biological nitrogen removal processes in wastewater treatment

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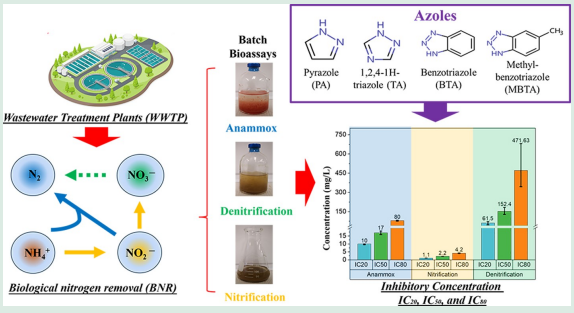
HIGHLIGHTS

- Acute inhibition of azoles in biological nitrogen removal processes was evaluated.
- The granular structure did not protect Anammox bacteria from azole inhibition.
- Azoles mainly inhibited ammonium oxidation process in nitrification.
- The nitrification is more sensitive to azoles than Anammox and denitrification.
- Azole inhibition mechanisms in biological nitrogen removal processes were discussed.



ABSTRACT: As emerging organic contaminants (EOCs), azoles have been detected in various environments. However, comprehensive information on the impact of azoles on biological nitrogen removal (BNR) processes in wastewater treatment is limited, particularly regarding the denitrification process. This study aims to investigate the short-term (< 24 h) inhibitory potentials of ten azole compounds on major BNR processes, including nitrification, denitrification, and anaerobic ammonium oxidation (Anammox). At 6 mg/L, pyrazole (PA), triazole (TA), benzotriazole (BTA), and methyl-benzotriazole (MBTA) caused over 90% inhibition of nitrification activity. In comparison, denitrifiers exhibited greater resistance to these azoles, with calculated half-maximal inhibitory concentrations (IC_{50}) of 126, 520, 412, and 152 mg/L, respectively. Regarding Anammox, the calculated IC_{50} was 20 mg/L for BTA and 18 mg/L for MBTA, while PA and TA showed no significant inhibition (< 20%) at concentrations up to 250 mg/L. The granular structure of Anammox sludge did not exhibit additional protection from the inhibition. The ammonium oxidizing process in nitrification showed the highest sensitivity to tested azoles. The results are expected to aid in evaluating the stability of BNR processes for treating azole-containing wastewater and in developing effective strategies to protect BNR systems from disruptions caused by azoles.

KEYWORDS: Emerging organic contaminants, BNR, Anammox, Nitrification, Denitrification, Toxicity

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1 Introduction

With the rapid growth of human activities, municipal and industrial water consumption has increased, leading to the production of large volumes of wastewater rich in nitrogen (N). The N in wastewater comes from various human activities, including those in the petrochemical, pharmaceutical, fertilizer, and food industries, and is present in the forms of ammonia, nitrite, nitrate, and organic N (Carrera et al., 2003). Municipal wastewater treatment for the removal of excessive N content plays a critical role in the sustainable development of both human society and ecosystems. Wastewater treatment plants (WWTPs) use biological nitrogen removal (BNR) processes, including nitrification, denitrification, and anaerobic ammonium oxidation (Anammox), to eliminate N from wastewater (Ma et al., 2016). The nitrification process, which occurs in the presence of oxygen, involves cooperation between ammonium-oxidizing bacteria/archaea (AOB/AOA) and nitrite-oxidizing bacteria (NOB) (Kolovou et al., 2023). Denitrification is an anaerobic process where a diverse group of microorganisms uses organic carbons as electron donors to biologically convert nitrate into nitrogen gas (N_2) (Ma et al., 2016). In comparison, the Anammox process anaerobically converts ammonium and nitrite into N_2.

Azoles, classified as emerging organic contaminants (EOCs), are synthetic chemicals characterized by N-containing heterocyclic ring structures. They are widely used in aircraft de-icing agents, semiconductor manufacturing, and household dishwashing detergents (Li et al., 2020). For decades, more than 25 azole compounds have been employed as broad-spectrum biocides and fungicides in agriculture, representing approximately 20%–25% of the global biocide/fungicide market share (Jørgensen and Heick, 2021). The European Union and China use up to 1000 and 3800 t of climbazole per annum, respectively (Chen and Ying, 2015). Due to the substantial demand for azoles, the United States Environmental Protection Agency (US EPA) has listed benzotriazole (BTA) and pyrazole (PA) derivatives as high-production volume chemicals, indicating that their total production and import exceed one million pounds per year (US EPA, 2023). Azole compounds are commonly detected in various environmental matrices, including surface water, groundwater, wastewater, biosludge, and soil (Chen and Ying, 2015; Liu et al., 2021). A literature review on the occurrence and concentrations of azole compounds in various environments was conducted, with results presented in Table S1, showing a wide range from ng/L to mg/L. In municipal wastewater, azole concentrations vary significantly, from < 0.02 μg/L (BTA) to 1020 μg/L (PA), depending on the specific compounds and the influent sources received by WWTPs. Additionally, WWTPs receiving substantial industrial wastewater, such as from the semiconductor industry, often exhibit higher azole concentrations, posing greater risks to the stability and performance of biological treatment processes (Struk-Sokołowska et al., 2022).

The presence of azole compounds in wastewater, along with their resistance to biodegradation—particularly in unadapted biological systems—can significantly undermine the effectiveness and reliability of BNR processes in WWTPs (Chen et al., 2018). A previous study found that activated sludge lacked the capacity to degrade azoles within a 24-h timeframe (Jog et al., 2021). Therefore, azoles may accumulate within WWTPs and affect BNR processes. Pagga et al. (2006) reported that PA and BTA inhibited nitrifying bacteria in sewage sludge, with a half-maximal inhibitory concentration (IC_{50}) of less than 0.5 mg/L. Other studies demonstrated that azoles inhibit Anammox and nitrification processes by 50% at concentrations of 18–20 and 2–3 mg/L, respectively (Lakhey et al., 2020; Li et al., 2020). The specific inhibitory mechanisms of azoles remain unclear. As fungicides, azoles typically target the CYP51 enzyme in fungi and disrupt cell membrane integrity (Draskau and Svingen, 2022). Some studies suggest that azoles may similarly inhibit biological nitrification by interfering with key enzymes, such as ammonia monooxygenase (AMO) (Li et al., 2020). Given that different enzymes and membrane compositions are involved in various microorganisms in BNR processes, such as nitrifiers, denitrifiers, and Anammox bacteria, the extent of azole inhibition may depend on both the type of azoles and the specific characteristics of the BNR process. However, limited information is available regarding the impact of azoles on the denitrification process in wastewater, hindering a comprehensive evaluation of the reliability and performance of BNR processes in WWTPs. Since both traditional BNR processes (e.g., nitrification/denitrification) and innovative processes (e.g., partial nitrification-Anammox (PN/A) and partial denitrification-Anammox (PdN/A)) involve combined N-related biological processes (Li et al., 2023a; Zhu et al., 2024), this knowledge gap makes it difficult to accurately assess azoles' inhibition on N removal efficiency in WWTPs and limits the development of effective strategies to protect BNR processes from such inhibition (Fu et al., 2024).

To address this knowledge gap and provide a comprehensive comparison of the impacts of different azole compounds on various BNR processes in WWTPs, batch experiments were conducted in this study to assess the impact of short-term (< 24 h) exposure to ten azoles on nitrification, denitrification, and Anammox processes, with a focus on PA, triazole (TA), BTA, and methyl-benzotriazole (MBTA) due to their widespread use, detection in wastewater, and structural properties. The inhibitory parameter, IC_{50}, was determined, and the results were compared across different BNR processes. Specifically, the study evaluated the extent of azole-induced inhibition on ammonium and nitrite-oxidizing processes in nitrification. Additionally, the potential role of the granular structure of Anammox bacteria in resisting azole inhibition was examined. The findings provide insight into both the potential inhibitory mechanisms of azole compounds and the defense mechanisms of BNR bacteria. This understanding can assist WWTPs in developing strategies to mitigate azole-induced inhibition, ensuring consistent N removal efficiency.

2 Methods

2.1 Chemicals

TA and MBTA were purchased from Acros Organics Chemicals (Geel, Belgium). BTA and miconazole were purchased from Alfa Aesar (Ward Hill, Massachusetts, USA). PA, clotrimazole, climbazole, and fluconazole were purchased from Tokyo Chemical Industry (Tokyo, Japan). Tioconazole was purchased from Combi-Blocks, Inc (San Diego, CA, USA). All purchased azole compounds were ACS reagent grade with purity of ⩾ 98%. Table 1 summarized the primary physicochemical properties of PA, TA, BTA, and MBTA, while the major physicochemical properties of other tested azoles were summarized in Table S2.

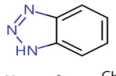
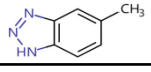
2.2 Sludge

Nitrification: The nitrification sludge used in this work was collected from the aerobic zone of a local municipal WWTP (Laurel, MD, USA), which includes a four-stage Bardenpho BNR. The WWTP influent was analyzed prior to sludge collection, and no azoles were detected. Additionally, there was no evidence of the WWTP receiving azole-containing wastewater in the past (communication with WWTP operators and undetectable azoles in the influent), suggesting that the BNR processes were unlikely to have previously adapted to azoles. After collection, the sludge was transported on ice to the laboratory on the same day as the experiments. A washing-centrifuge-resuspend procedure was conducted to prepare sludge suspension with 3 g total suspended solids (TSS)/L (Li et al., 2020). The nitrification activity was in the range of 2–4 mg NH_4^+-N/(g-TSS·h), meeting the requirement by the International Organization for Standardization (ISO) method 9509 (ISO-9509, 2006).

Denitrification: The denitrification sludge was collected from the anoxic basin of the same WWTP mentioned above. Before conducting the bioassays, the sludge was anaerobically mixed at room temperature to consume the remaining nitrate (NO_3^-). The monitored denitrification rate was in the range of 0.3 ± 0.1 mg N_2-N/(g-TSS·h).

Anammox: Granular Anammox biomass was collected from a lab-scale Anammox expanded granular sludge bed reactor (EGSB), exhibiting an Anammox activity at 0.6 ± 0.1 g N_2-N /(g-VSS·d), with more details provided in Supplementary Information (SI). The EGSB has been successfully operated for five years and has not been previously exposed to azoles. Suspended Anammox biomass was acquired with the

Table 1 Overview of the physical and chemical properties of tested azoles

Compound	Structure	Solubility in water (mg/L)	Log P	Pk_a1	Pk_a2
Pyrazole (PA)		72,850	0.28	2.67	14.76
Triazole (TA)		241,000	−0.10	0.31	9.34
Benzotriazole (BTA)		7,570	1.30	0.22	9.04
Methyl-benzotriazole (MBTA)		2,390	1.82	0.45	9.12

Notes: 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. Pk_a1 and Pk_a2 represent the Pk_a values of its protonated and neutral species, respectively.

syringe needles using a procedure reported in a prior study (Chen et al., 2024). A picture of suspended and granular Anammox biomass is provided in Fig. S1.

2.3 Experimental set up

The inhibition bioassays for nitrification, denitrification, and Anammox were conducted following either the standard methods or well-established methods reported in the literature (Sánchez et al., 2000; ISO-9509, 2006; Lakhey et al., 2020; Chen et al., 2024). Table 2 summarizes the experimental setup and operational conditions. The azole concentrations used in the Anammox and nitrification assays are summarized in Table 3, while azole concentrations of 0, 2.5, 10, 50, 100, and 250 mg/L were selected for the denitrification assays. Given that azole concentrations in municipal wastewater typically range from ng/L to mg/L (Table S1), the concentrations tested in this study encompass this range, representing relevant scenarios for azole contamination in municipal wastewater. Dimethyl sulfoxide (DMSO) from MP Biomedicals, USA (purity > 99%) was utilized at concentrations below 275 mg/L to aid in dissolving some azoles with low solubility (< 1 mg/L, as detailed in Table S2). The concentration of DMSO up to 275 mg/L showed no inhibition to the activity of BNR and the data is depicted in Fig. S2. Azole degradation was not observed in either bioassays or the abiotic controls (used to explore self-degradation) during the experimental period (24 h) (data not shown).

2.3.1 Nitrification inhibition assays

The inhibitory impact of azole compounds on nitrification activity was evaluated following Method ISO 9509 (ISO-9509, 2006). A medium with NH_4Cl (Thermo Scientific Chemicals, USA, purity > 98%) as the substrate was introduced into a 250 mL flask, followed by the addition of the tested azole compounds to achieve the specified concentrations (Table 3). Following the standard requirements, 3,5-Dichlorophenol (3,5-DCP, Thermo Scientific Chemicals, USA, purity >99%) and allylthiourea (ATU, Thermo Scientific Chemicals, USA, purity > 98%) were used as reference inhibitors, confirming the quality of the nitrifying sludge used in this study meet the requirements by Method ISO 9509 (ISO-9509, 2006) (Fig. S3).

2.3.2 Denitrification inhibition assays

Denitrification inhibition assays were conducted in serum bottles with a total volume of 118 mL. The

Table 2 Summary of the experimental setup and operation parameters in inhibition bioassays

Parameters	Nitrification	Denitrification	Anammox
Total Vol. (mL)	250	118	118
Working Vol. (mL)	80	60	60
Sludge	Suspended sludge from aerobic basin at WWTP	Suspended sludge from anoxic basin at WWTP	Granular/crushed sludge from Lab-scale EGSB
TSS (g/L)	1.5±0.0	1.9±0.0	0.8±0.0
VSS (g/L)	0.9±0.0	1.5±0.0	0.8±0.0
Substrate (mg/L)	NH_4^+-N = 56	CH_3COO^--C = 200 NO_3^--N = 50	NH_4^+-N = 75 NO_2^--N = 100
DO (mg/L)	>4	<0.5	<0.5
Temperature (°C)	22±2	37±1	30±1
Shaking (r/min)	120	180	120
pH	7.6±0.1	7.5±0.1	7.2±0.1
Buffer	Bicarbonate	Phosphate	HEPE
Medium (mg/L)	$NaHCO_3$ (504)	$MgSO_4 \cdot 7H_2O$ (90), $CaCl_2 \cdot 2H_2O$ (14), KCl (36) Yeast extract (1) KH_2PO_4 (3400) K_2HPO_4 (4300) Trace element[a)]	$NaH_2PO_4 \cdot H_2O$ (58) $CaCl_2 \cdot 2H_2O$ (100) $MgSO_4 \cdot 7H_2O$ (200) $NaHCO_3$ (50) HEPE (5950) Trace element[b)]

Notes: a), b) following the recipe used by Sánchez et al. (2000) and Lakhey et. al. (2020), respectively. Chemicals, including NH_4Cl, CH_3COONa, $NaNO_2$, KNO_3, $NaHCO_3$, $MgSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2H_2O$, KCl, KH_2PO_4, K_2HPO_4, and $NaH_2PO_4 \cdot H_2O$, were purchased from Thermo Scientific Chemicals (USA) in ACS reagent grade with a purity of ≥ 98%. HEPE was purchased from MilliporeSigma (USA) with purity > 99%.

Table 3 The inhibition of tested azoles in Anammox and nitrification at selected concentrations

Azoles	Anammox		Nitrification	
	Tested concentrations (mg/L)	Inhibition[#] (%)	Tested concentrations (mg/L)	Inhibition[#] (%)
Pyrazole (PA)	250	13 ± 8	6	96 ± 1
Triazole (TA)	250	NI[*]	6	99 ± 1
Benzotriazole (BTA)	250	87 ± 4	7.5	99 ± 1
	20	48 ± 1	7.5	
Methyl-benzotriazole (MBTA)	250	94 ± 3	6	96 ± 1
	18	55 ± 6	6	

Notes: * NI, no inhibition (<5 %) observed. # Standard deviations are calculated from triplicates groups.

bottles were assembled following the setup presented in Table 2 and the order: medium → azole compounds → DI water → sludge →seal and helium gas flush for 5 mins →injection of concentrated KNO_3 and CH_3COONa (Chen et al., 2024). KNO_3 and CH_3COONa were purchased from Thermo Scientific Chemicals (USA) with a purity of ⩾ 98%. Headspace gas samples were collected hourly for N_2 measurement after incubation on a shaker at 180 r/min and 37 ± 1 °C, following established denitrification bioassay from the literature (Sánchez et al., 2000).

2.3.3 Anammox inhibition assays

Similar to the Anammox activity bioassays reported in a prior study (Chen et al., 2024), 118 mL serum bottles were used to test the impact of azole compounds at different concentrations on Anammox (Table 3). The assembly of the bottles followed the procedure described in the denitrification inhibition assays with different substrates and mediums as described in Table 2.

2.3.4 Extended inhibition experiments

Nine sequencing batch reactors (SBR) with a total volume of 1.15 L (350 mL headspace and 900 mL effective volume) were used to examine the impacts of extended azole exposure on BNR processes. The addition of each sludge was performed following the same TSS and volatile suspended solids (VSS) concentrations used in the acute inhibition assays mentioned above. The setup of the SBRs, including DO, temperature, and pH, and the preparation of the influent and substrate concentrations in the influent followed those described in Table 2. The reactors were operated in parallel in a 12-h cycle, consisting 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%, with mixing provided by a stir station set to 120 r/min. Only BTA and PA were selected for testing in the extended inhibition experiments, based on the results from the acute inhibition assays, which indicated that these compounds exhibited the highest inhibition potential for BNR processes (results and discussion are provided in the following sections). The reactors were named A_C, A_{BTA}, A_{PA}, N_C, N_{BTA}, N_{PA}, DN_C, DN_{BTA}, and DN_{PA}, where A, N, and DN represent Anammox, nitrification, and denitrification, respectively. C, BTA, and PA indicate control (without the addition of azoles), treatment with BTA, or treatment with PA, respectively. The concentrations of BTA or PA used in each reactor were selected based on the results from acute inhibition assays, as follows: BTA at 20 mg/L in A_{BTA}, PA at 200 mg/L in A_{PA}, BTA at 2.5 mg/L in N_{BTA}, PA at 2.7 mg/L in N_{PA}, BTA at 2.5 mg/L in DN_{BTA}, PA at 2.7 mg/L in DN_{PA}, and 0 mg/L azoles in A_C, N_C, and DN_C. Higher azole concentrations were selected for the Anammox SBRs due to the IC_{50} of 19.6 mg/L for BTA and no inhibition observed at 250 mg/L for PA in the acute inhibition assays.

2.4 Analytical methods

Different N species (N_2, NH_4^+, NO_3^-, and NO_2^-) in the gas and liquid samples were analyzed using a gas chromatograph (Agilent Technologies, Santa Clara, CA, USA), an ammonium electrode (ISENH4131, Hach, Loveland, CO, USA), and an ion chromatograph (Dionex ICS-1100, Thermo Fisher Scientific, USA), respectively, with details provided in SI. Dissolved oxygen (DO) in liquid samples was determined with an optical DO probe (LBOD101 Sensor, Hach, Loveland, CO, USA), while additional parameters, including TSS, VSS, and pH, were analyzed according to standard methods (APHA, 2017).

2.5 Data processing

The specific activity (SA) in nitrification bioassays was calculated from the time course of NO_x^--N (NO_3^- and NO_2^-) production and expressed as mg NO_x^--N /(g-VSS·d) following the previous work (Li et al., 2020). The SA in denitrification and Anammox bioassays were determined through N_2 gas production rate and expressed as mg N_2-N /(g-VSS·d) (Sánchez et al., 2000; Chen et al., 2024). The normalized activity (NA) was defined as the ratio of the SA in the treatment group to the maximum specific activity (SA_{max}) in the control group, as shown in Eq. (1). The BNR bioassay data were analyzed with an inhibition model (Eq. (2)) that has been successfully used to assess the inhibition behaviors in various BNR processes (He et al., 2019; Li et al., 2020; Chen et al., 2024):

$$NA(\%) = [SA/SA_{max}] \times 100, \quad (1)$$

$$NA(\%) = NA_{max}/[1 + (C/IC_{50})^n], \quad (2)$$

where NA_{max} is the maximum NA value, C is the azole concentration, and n is the dimensionless inhibition order. The IC_{50} is the concentration causing a 50% loss of the treatment group in the BNR bioassays.

Non-linear data fitting and statistical significance analysis were performed using Origin 2021b (OriginLab, Northampton, MA, USA), in accordance with methodology outlined in our previous study (Chen et al., 2024). Variations in the results were evaluated using factorial analysis of variance (ANOVA) combined with Tukey's test and Student's t-test, with a significance threshold of $p < 0.05$. Reported data are presented as means with standard errors.

3 Results and discussion

3.1 Assessment of azoles inhibition in biological nitrogen removal (BNR) processes

3.1.1 Anaerobic ammonium oxidation (Anammox)

The inhibitory potential of four azoles (PA, TA, BTA, and MBTA) was confirmed through preliminary tests at specific concentrations (shown in Table 3). These tests revealed that PA and TA at 250 mg/L each caused less than 20% inhibition of the Anammox process, while 20 mg/L BTA and 18 mg/L MBTA resulted in inhibition of 48% ± 1% and 55% ± 6%, respectively. These findings are consistent with those reported in a previous study (Lakhey et al., 2020). However, previous studies did not investigate the role of the multilayered granular structure of Anammox in protecting the bacteria from azole inhibition (Lakhey et al., 2020; Chen et al., 2021). It remains unclear how the granular structure helps Anammox resist azole compounds. These azoles (250 mg/L PA, 250 mg/L TA, 20 mg/L BTA, and 18 mg/L MBTA) were further tested for the response of both granular and suspended sludge, with the results illustrated in Fig. 1. Both Anammox granular sludge and suspended sludge exhibited specific Anammox activity (SAA) of 0.5 ± 0.0 g N_2-N /(g-VSS·d) in the absence of azoles. 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 slight decrease ($p > 0.05$) in Anammox activity was observed in both Anammox granular and suspended sludge with the addition of PA and TA. Although activity in the Anammox suspended sludge groups appeared lower than in granular sludge, statistical analysis showed that this difference was not significant ($p > 0.05$). Similar results were obtained in the groups with BTA and MBTA. Similarly, previous work observed no difference in the inhibitory impact of crude glycerol and methanol on the activity of Anammox granular and suspended sludge (Chen et al., 2024). Although the granular structure of Anammox sludge provides physical resistance to the diffusion of substrates and inhibitors, azoles were still able to penetrate and affect the Anammox cells, as they were not degraded during the experiment. While the granular structure of Anammox offers advantages in full-scale

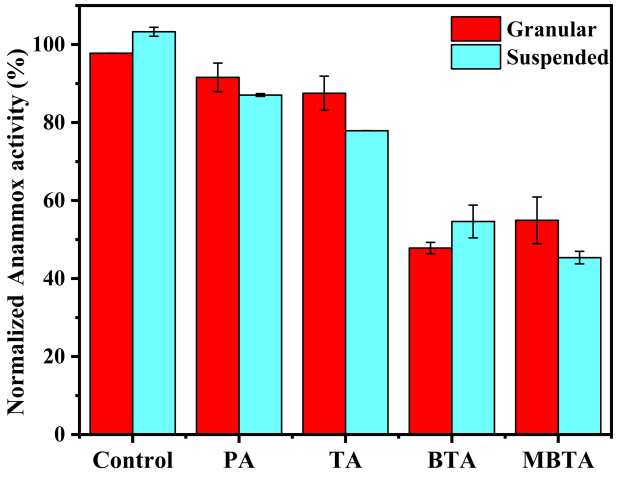


Fig. 1 Normalized Anammox activity of Anammox granular and suspended sludge exposed to different azole compounds. The tested azole concentrations were 250, 250, 20, and 18 mg/L for pyrazole (PA), triazole (TA), benzotriazole (BTA), and methyl benzotriazole (MBTA), respectively. Error bar represents the standard deviation of duplicates.

applications, such as shortening the start-up period in PN/A or PdN/A processes, it may not effectively protect the Anammox process from certain inhibitors (Adams et al., 2022).

3.1.2 Nitrification

A preliminary test was conducted to confirm azole inhibition potential on nitrification by using four azoles (PA, TA, BTA, and MBTA) with the selected concentrations listed in Table 3. The results showed that four azole compounds caused severe inhibition (> 90%) of the nitrification process at concentration levels ranging from 6 to 8 mg/L, which is two orders of magnitude lower than those observed in Anammox bioassays (Table 3). The nitrification process is more sensitive to azole inhibition at low concentrations (mg/L) (Li et al., 2020). A similar study on nitrification inhibition found the calculated IC_{50} for PA and BTA to be below 0.5 mg/L (Pagga et al., 2006). However, it remains unclear which step of the nitrification process, AOB/AOA or NOB, experienced the most inhibition. This lack of clarity impedes the understanding of how azoles might impact the scalability of the PN/A process. To better understand the impact of azoles on the ammonium and nitrite oxidation processes, batch experiments were conducted using ammonium and nitrite as sole substrates. The ammonium and nitrite consumption rates were compared to those of the control groups without azole addition. It should be noted that the tested concentrations of ammonium (56 mg NH_4^+-N /L) and nitrite (56 mg NO_2^--N/L) in this work were below the levels reported to cause substrate inhibition in the nitrification process (Kim et al., 2008). As shown in Fig. 2, the nitrification activity in the control groups, without the addition of azoles, measured 3.7 ± 0.3 mg NH_4^+-N /(g-TSS·h) with ammonium and 2.6 ± 0.2 mg NO_2^--N /(g-TSS·h) with nitrite added as the sole substrate, respectively. These results meet the ISO method 9509 requirements and align with previous work, which reported higher ammonium oxidation rates than nitrite oxidation rates at the temperature used in this study (ISO-9509, 2006; Kim et al., 2008). 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 based on estimates from previous studies that these levels cause 50% inhibition of nitrification activity (Li et al., 2020). As shown in Fig. 2, a more pronounced inhibition was observed in the ammonium groups, except for PA, compared to the nitrite groups where nitrite was the sole substrate ($p < 0.05$). NA calculated in the ammonium groups for TA, BTA, and MBTA significantly decreased ($p < 0.05$) by

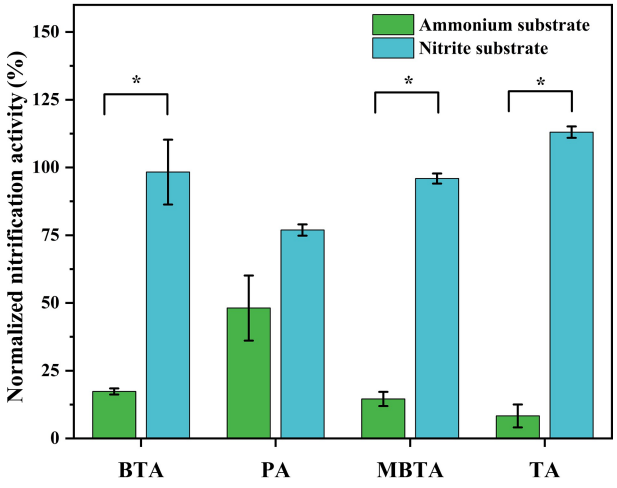


Fig. 2 Normalized nitrification activity of azole toxicity bioassays with ammonium or nitrite added as sole substrate. The tested azole concentrations were 2.7, 3.5, 2.0, and 2.2 mg/L for pyrazole (PA), triazole (TA), benzotriazole (BTA), and methyl benzotriazole (MBTA), respectively. The asterisk (*) indicated the p value of t-test. *, $p \leqslant 0.05$. Error bar represents the standard deviation of duplicates.

83%–92%. For the PA group, although the NA was higher in the nitrite group (77%) compared to the ammonium group (48%), the results were not statistically significant ($p = 0.21$). A review of the literature shows that little information is available for comparing the inhibition of azoles on AOB/AOA and NOB. Kolovou et al. (2023) investigated the inhibition effects of two triazoles (1-butyl-4-propyl-triazole and 1,4-dibutyltriazole) on AOA and AOB at concentrations of 83.5 and 62.6 mg/L, respectively. The results revealed that AOA experienced more significant inhibition ($p < 0.05$) compared to AOB (Kolovou et al., 2023). However, they could not conclude whether the microorganisms involved in the ammonium oxidizing process are more sensitive to the tested azoles than those responsible for nitrite oxidation. In this study, azoles caused greater inhibition during the ammonium oxidation step, indicating the potential for ammonium accumulation when treating wastewater containing azoles.

3.1.3 Denitrification

Inhibition bioassays were performed on denitrification with the four selected compounds over a concentration range of 0–250 mg/L, and the results are presented in Fig. 3. Denitrifiers showed greater sensitivity to PA and MBTA compared to TA and BTA. At a concentration of 100 mg/L, PA and MBTA caused 53% ± 2% and 67% ± 4% inhibition, respectively. In comparison, 25%

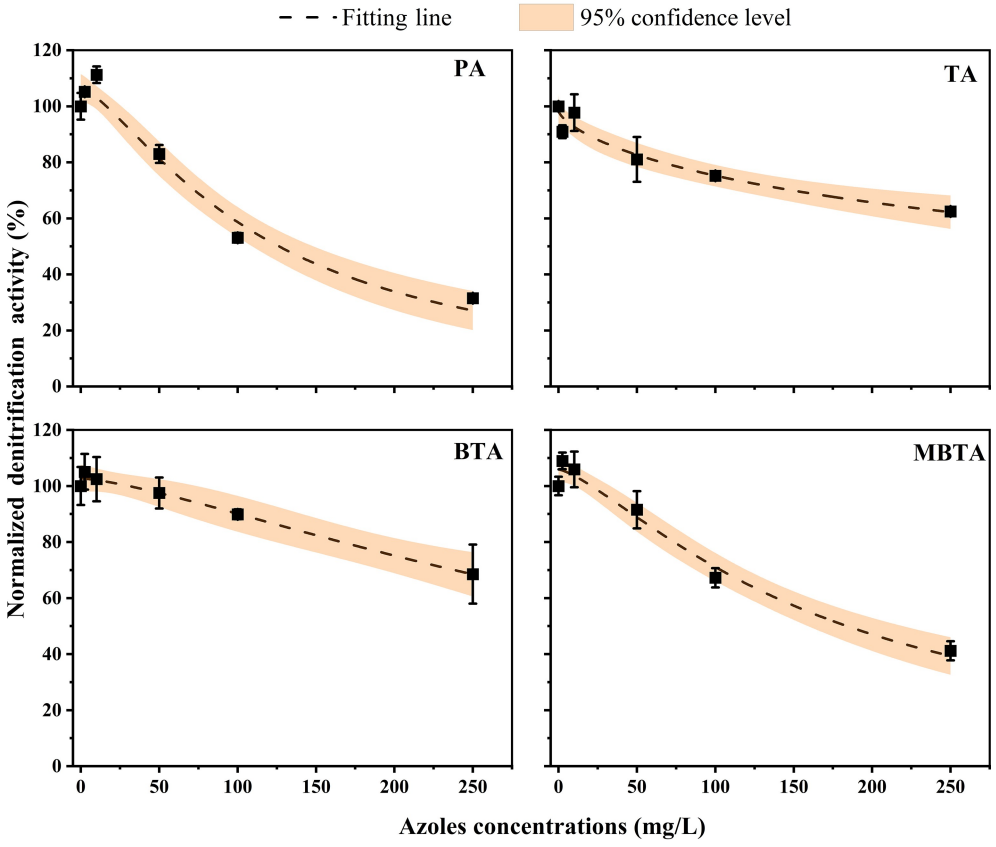


Fig. 3 Inhibitory impact of pyrazole (PA), triazole (TA), benzotriazole (BTA), and methyl benzotriazole (MBTA) on the denitrification activity at various azoles concentrations. The dash lines show the fit of the experimental data to an empirical inhibition model (Eq. (2)), while the shadow area represents the 95% confidence range. Error bar represents the standard deviation of triplicate.

and 10% inhibition of denitrification were observed in the groups with 100 mg/L of TA and BTA. Since the concentration of 100 mg/L azoles is rarely detected in wastewater (Table S1), the results imply a low risk to denitrification processes when exposed to the tested azoles. To the best of our knowledge, limited information is available on the inhibitory effects of azoles on the denitrification process in WWTPs, underscoring the significance of the findings in this study. Previous research has primarily focused on the toxicity of azoles on N-related biological processes, such as nitrification and denitrification, in soil environments. For instance, it was reported that the application of 3,5-dimethylpyrazole at 200 mg/kg completely inhibited nitrification and denitrification in soil (Zebarth et al., 2019). In another study, the application of tebuconazole at 187.5 g per hectare increased nitrate accumulation from 10.3 to 20.3 mg/kg in the soil (Saha et al., 2016). The findings in this study contribute to bridging this knowledge gap, demonstrating that denitrification is more stable compared to Anammox and nitrification processes under azole exposure in wastewater treatment. In both traditional nitrification/denitrification and PdN/A systems, the reduction of nitrate or nitrite and the consumption of organic carbon through denitrification are not significantly affected by azoles.

3.1.4 Other azoles

Various azole derivatives are synthesized from PA, TA, BTA, and MBTA. Six additional azoles including climbazole, clotrimazole, ketoconazole, miconazole, fluconazole, and tioconazole were tested to assess the impact of more structurally complex azoles on BNR processes. As shown in Table S3, varying concentrations were applied for each azole in the bioassays. Different responses were observed across BNR processes exposed to these azoles. Anammox granular sludge showed a reduction in activity (>10% inhibition) upon exposure to climbazole, miconazole, and tioconazole. In comparison, nitrification and

denitrification processes showed < 10% decreases in activity when exposed to these azoles at the tested concentrations (1–100 mg/L due to the solubility). The highest inhibition (22%) was found in Anammox granular sludge when exposed to 0.8 mg/L of miconazole, while nitrification and denitrification only showed < 5% inhibition at 1 mg/L. Azoles with higher hydrophobicity and a greater octanol-water partition coefficient (Log P) may accumulate in WWTP sludge, potentially causing severe adverse impacts on BNR processes (Bhagat et al., 2021). Azoles such as clotrimazole, ketoconazole, and miconazole (Log P > 4), have been reported to accumulate in aquatic organisms, sludge, and sediments (Chen and Ying, 2015). A previous study detected the accumulation of miconazole, clotrimazole, and ketoconazole (up to 76.9 ng/g dry weight) in the bed sediment of the Pearl River Delta, China (Huang et al., 2013). The potential accumulation of azoles within WWTPs raises concerns regarding their impact on BNR processes, especially for biofilm-based technologies, like the ANITA™ Mox (Wang et al., 2018).

3.2 Comparison of the responses of biological nitrogen removal (BNR) processes to azoles: sensitivity and inhibition mechanisms

3.2.1 Sensitivity of BNR processes to azoles

Compared with previous studies that primarily investigated individual BNR processes, such as nitrification and Anammox (Lakhey et al., 2020; Li et al., 2020; Jog et al., 2022), this study offers a broader perspective by comparing the impacts of azole compounds across multiple BNR processes. It connects the calculated inhibition parameters with real-world azole concentrations detected in wastewater, providing practical insights into their potential effects on wastewater treatment systems. The biological activity data obtained from the Anammox granular sludge, nitrification sludge, and denitrification sludge were fitted to an empirical inhibition model (Eq. (2)) to calculate the IC_{20}, IC_{50}, and IC_{80} values, with the key inhibition parameters, *Ki* and *n*, were determined and included in Table S4 (Lakhey et al., 2020; Li et al., 2020; Jog et al., 2021).

As shown in Fig. 4, compared to Anammox and denitrification, the nitrification process exhibited the highest sensitivity to tested azoles. The calculated IC_{20}, IC_{50}, and IC_{80} values for PA, TA, BTA, and MBTA in nitrification bioassays were below 8 mg/L, indicating that exposure of nitrifiers to azoles at environmentally relevant concentrations (a few mg/L) may result in significant (> 20%) inhibition. The toxicity order of the tested azoles for nitrification is as follows (based on IC_{50}): TA < PA < MBTA < BTA (inhibition from low to high). In comparison, the IC_{20} values for BTA and MBTA in Anammox inhibition bioassays were calculated at 10 mg/L, while no IC values could be determined for PA and TA due to non-significant inhibition (< 20%) at the highest tested concentrations (250 mg/L). Based on the IC_{50}, the toxicity of MBTA (17.0 mg/L) is slightly higher than BTA (19.6 mg/L) in the Anammox process. The highest IC_{20}, IC_{50}, and IC_{80}

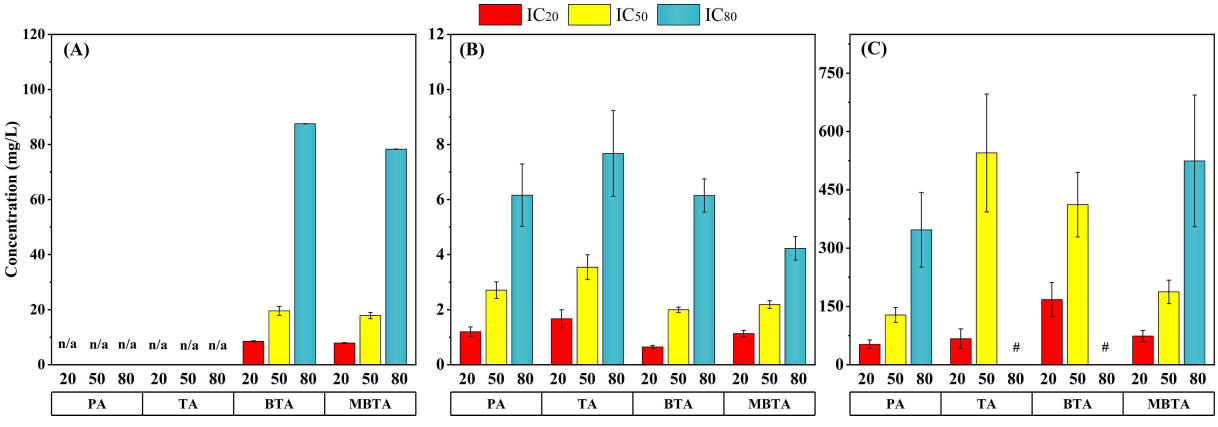


Fig. 4 Comparison of the concentrations of pyrazole (PA), triazole (TA), benzotriazole (BTA), and methyl benzotriazole (MBTA) resulting in 20%, 50%, and 80% (IC_{20}, IC_{50}, and IC_{80}) inhibition in Anammox (A), nitrification (B), and denitrification (C). It should be noted that the tested azole concentration was in the range of 0–250 mg/L. The values of IC_{20}, IC_{50}, and IC_{80} exceeding 250 mg/L were determined using empirical inhibition model Eq. (2), with the error bars representing a 95% confidence interval. n/a: no inhibitory concentration values can be calculated from the inhibition model (Eq. (2)) due to the non-significant inhibition (<20%) observed at the tested azoles concentrations (up to 250 mg/L). #: IC_{80} value of TA and BTA for denitrification is not included due to low confidence from the model calculation.

values were observed in the denitrification inhibition bioassays. As shown in Fig. 4, calculated IC_{20} and IC_{50} values for all tested azoles exceeded 50.0 and 120.0 mg/L, respectively. The estimated toxicity order for denitrification, based on IC_{50}, is TA < BTA < MBTA < PA (from least to most inhibitory).

However, the risk of azole compounds to the performance and stability of different BNR processes should be carefully evaluated by considering the concentrations of azole compounds typically found in real-world wastewater. Comparing the IC_{20} values obtained from the acute inhibition assays in this study (Fig. 4 and Table S4) with the real-world concentrations of azole compounds detected in wastewater (Table S1) suggests that the presence of PA (< 1.02 mg/L) and BTA (0.012–0.047 mg/L) may cause significant (> 20%) negative impact on the nitrification process. In contrast, Anammox and denitrification processes are less likely to be affected by the levels of azoles typically present in municipal WWTPs. Especially, BNR processes are commonly used in combination in wastewater treatment, with the nitrification process serving as a prerequisite step for Anammox in the PN/A process and denitrification in traditional nitrification-denitrification systems. Disruption of the nitrification process by PA and BTA could impair downstream units in the BNR system, potentially leading to system failure. The PdN/A system may have higher azole inhibition resistance when treating azole-containing wastewater. However, it should be noted that WWTPs receiving substantial industrial wastewater containing higher concentrations of azoles, such as from the semiconductor industry, may experience greater risks to the stability and performance of biological treatment processes (Struk-Sokołowska et al., 2022). Meanwhile, increasing municipal water consumption has led to lower hydraulic retention times in WWTPs, making it even more challenging for biomass to adapt to azole inhibition (Hou and Hu, 2022). In contrast, acute azole inhibition of BNR processes can significantly reduce N removal efficiency in under 24 h, leading to the release of non-compliant wastewater effluent, posing risks to both ecosystems and human health (Jing et al., 2022).

3.2.2 Extended exposure of BNR processes to azoles

To further evaluate the potential difference between the short- and long-term impacts of azole exposure on BNR processes, extended inhibition experiments were conducted, with the results presented in Fig. 5. These experiments lasted for 23 and 27 d for the Anammox and denitrification SBRs, respectively, while the nitrification SBRs were terminated after 8 d due to significant disruption of nitrification processes observed in the treatment groups with PA (96% activity reduction) or BTA (45% activity reduction), as shown in Fig. 5. The activity of the control reactors with no azoles addition was monitored at the beginning and end of the experiments. The calculated SA (expressed in mg N/(g-TSS·h)) in the control SBRs at the beginning and end of the experiments were as follows: 30.5 (Day 0) and 23.2 (Day 23) in Anammox SBR (A_C), 3.2 (Day 0) and 5.8 (Day 8) in nitrification SBR (N_C), and 9.0 (Day 0) and 17.9 (Day 27) in denitrification SBR (DN_C). These control measurements were used to calculate the NA by determining the ratio of the SA in the treatment group to the SA in the control group. In addition to monitoring the activity of control groups, the activity of each treatment group was also evaluated on Day 0 before the addition of azole compounds and compared with control SBRs to calculate NA. As shown in Fig. 5,

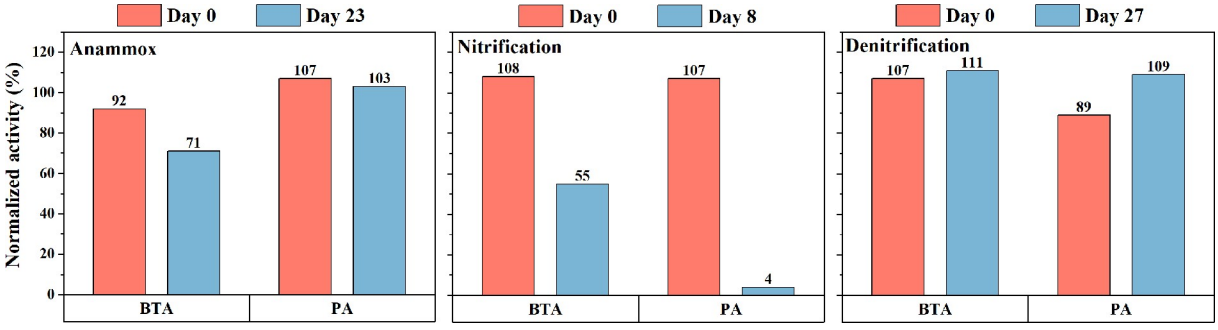


Fig. 5 Normalized activity (NA) of different BNR processes before and after extended azole exposure. Anammox (8-d), nitrification (23-d), and denitrification (27-d) in SBRs. NA was calculated as the ratio of the specific activity (SA) in the treatment group to the SA in the control SBR with no addition of azoles. Tested azoles included benzotriazole (BTA) and pyrazole (PA) with the concentrations as follows: BTA at 20 mg/L and PA at 200 mg/L in Anammox SBRs, BTA at 2.5 mg/L and PA at 2.7 mg/L in nitrification SBRs, and BTA at 2.5 mg/L and PA at 2.7 mg/L in denitrification SBRs.

most of the treatment groups showed an NA within 100% ± 10%, with the exception of DN_{BTA}, which exhibited slightly lower activity (NA of 89%). These results were consistent with or exceeded the SA obtained in the control groups during the acute inhibition assays, indicating the successful operation of the bioreactors throughout the experiments.

In reactor A_{BTA}, Anammox bacteria were exposed to 20 mg/L BTA, a concentration close to the IC_{50} (19.6 mg/L) calculated from the acute inhibition assay (Fig. 4 and Table S4). After 23 d of exposure, a 39% inhibition was observed compared to the control Anammox SBR (A_C). This reduced inhibition in the extended inhibition experiment suggests the potential of adaptation or increased resistance of Anammox bacteria to the presence of BTA over time (Wang et al., 2021). In contrast, the presence of 200 mg/L PA showed no observable impact on Anammox, consistent with findings from the acute inhibition assays. Similarly, no inhibition was observed when denitrifiers were exposed to PA and BTA for 27 d. Compared to the IC_{50} values for denitrification, 412.0 mg/L for BTA and 128.1 mg/L for PA (Fig. 4), the concentrations added to reactors DN_{BTA} (2.5 mg/L) and DN_{PA} (2.7 mg/L) were much lower. Together with the results in acute inhibition assays, these results suggest that Anammox and denitrification process exhibit high resistance to azoles under both acute (< 24 h) and extended exposure (23–27 d) conditions. For the nitrification SBRs, the concentration of BTA (2.5 mg/L) and PA (2.7 mg/L) added in N_{BTA} and N_{PA}, respectively, were similar to their IC_{50} values (2.0 mg/L for BTA and 2.7 mg/L for PA) obtained in acute inhibition assays. The exposure of nitrifiers to 2.5 mg/L BTA caused a 45% activity reduction in N_{BTA}, comparable to the 50% inhibition observed at 2.0 mg/L BTA in the acute inhibition assays. However, N_{PA} exhibited a 96% activity reduction after 8 d of exposure to 2.7 mg/L PA (Fig. 5), a significantly more severe inhibition compared to 50% inhibition seen at the same PA level in the acute inhibition assays (Fig. 4). This result indicated a higher inhibitory potential of long-term PA exposure on nitrifiers compared to short-term exposure, underscoring the importance of further investigating the long-term impacts of azoles on BNR processes. Such studies should consider the potential for microbial adaptation, alterations in community composition, and changes in functional diversity (Pagga et al., 2006; Dvořák et al., 2013; Yang et al., 2024).

It is also important to note that azole degradation has been observed in activated sludge from WWTPs (Liu et al., 2011; Yuan et al., 2014; Jog et al., 2021). However, the incubation time required to observe degradation can take several months for the biomass to adapt. A previous study reported that degradation of BTA and MBTA by 70% and 80%, respectively, took up to 77 d in microcosms containing aquifer sediment materials (Liu et al., 2013). In this study, no significant degradation (< 5%) of added azoles was detected in both acute inhibition assays (lasting less than 24 h) and extended inhibition experiments (lasting between 8–27 d) (data not shown). However, longer exposure of BNR processes to emerging contaminants, such as azoles, has a greater potential to induce the degradation of organic contaminants and influence microbial community dynamics and functional diversity (Li et al., 2023b). These aspects require further investigation in future studies. Results from these extended experiments should be interpreted cautiously when applying them to practical scenarios in wastewater treatment. In addition to measuring NH_4^+, NO_2^-, NO_3, and N_2 conducted in this study, future research should monitor the greenhouse gas (GHG) production, such as nitrous oxide (N_2O), to assess the impacts of azole compounds on GHG emission during BNR processes. GHG emission from BNR processes in wastewater treatment is one of the major anthropogenic sources of GHGs globally (WRF, 2019). Especially, N_2O, which forms during both nitrification and denitrification, has 264 times the global warming potential of CO_2 (Wunderlin et al., 2012; WRF, 2019). The emissions of N_2O have considerably increased due to the expansion of WWTPs and the corresponding rise in wastewater production (Campos et al., 2016; Yang et al., 2024).

3.2.3 Inhibition/resistance mechanisms to azoles

While the exact mechanisms of azole compounds in inhibiting BNR processes remain unclear, several hypotheses have been proposed. The disruption of the integrity of functional structures within cells (e.g., cell membrane) by organic inhibitors can lead to a loss of essential materials and/or disruption of the proton gradient across the membrane, ultimately resulting in reduced activity and potential cell death (Hu et al., 2003). To determine if the hydrophobic character of the azole compounds correlates with their toxicity to BNR processes, the effect of hydrophobicity (expressed in Log P) on the BNR inhibition of four tested azoles (i.e., PA, TA, BTA, and MBTA) was tested. The IC_{50} values of four tested azole compounds were plotted against the Log P (Fig. S4). The results showed a weak correlation (R^2 = 0.26–0.66) between inhibition and Log P in BNR processes. Hydrophobicity alone is insufficient to explain the inhibition of azole compounds on denitrification (as shown in this study), as well as on

nitrification and Anammox processes (Lakhey et al., 2020; Jog et al., 2022). To the best of our knowledge, this study is the first to explore the relationship between Log P and azole inhibition in denitrification within wastewater treatment systems, while also comparing the inhibitory effects of azoles across different BNR processes.

Another suspected inhibition mechanism involves the interaction of azoles with enzymes and other biomolecules within cells, a mode of action commonly associated with their antifungal properties (Elias et al., 2019). Balding et al. (2008) reported that azoles may bind to the iron cores of cytochrome P450 enzymes, which typically act as monooxygenase. Azoles have also been found to bind to metal cores in other oxygenase enzymes, such as nitric oxide dioxygenases (El Hammi et al., 2011). In BNR processes, azoles may inhibit AMO by binding to its copper-containing active site (Jog et al., 2022). In this study, the higher sensitivity of ammonium oxidation compared to nitrite oxidation to tested azoles in nitrification (Fig. 2) supports this inhibition mechanism. However, further analysis is needed to clarify the differences in AOA/AOB inhibition (Kolovou et al., 2023). Similar binding mechanisms may occur in Anammox cells, as many key enzymes in Anammox bacteria contain metal prosthetic groups, such as copper in nitrite reductase, copper or zinc in hydrazine synthase, and iron in cytochrome C (Wang et al., 2022). However, the tested azoles showed less inhibitory impact on the Anammox process compared to nitrification in this study (Table 3 and Fig. 4). One key difference between Anammox bacteria and nitrifying bacteria is the presence of a unique organelle in Anammox cells, called the Anammoxosome. This organelle occupies a significant portion of the cell volume and serves as the site for the major Anammox reactions, a feature not found in other organisms (van Teeseling et al., 2013). The low permeability of the Anammoxosome membrane, attributed to the presence of ether-linked and ester-linked ladderanes and lipids, may decrease the chance of azoles reaching or accumulating in the sensitive region within Anammox cells (van Niftrik and Jetten, 2012). The unique cell structure of Anammox bacteria, rather than the multilayer granular structure, is suspected to provide protection to the Anammox process against azole inhibition. The resistance of the denitrification process to azoles may be attributed to the high diversity of the microbial community involved in denitrification within the denitrifying sludge. Denitrifiers consist of a larger number of genera of facultative anaerobes, contributing to the high microbial diversity of denitrifying sludge (Ma et al., 2016). The complete denitrification process involves multiple key enzymes, including different types of nitrate reductases, nitrite reductases, and nitric oxide reductases found in various bacteria (Feng et al., 2020). The high microbial versatility of denitrifying sludge in adapting to environmental stresses, such as azoles, may explain its resistance to these compounds. While certain denitrification species or enzymes may be adversely impacted by azoles, metabolic interactions among the various species present in denitrifying sludge may still be synergistic, ultimately sustaining denitrification activity in wastewater treatment (Pishgar et al., 2019).

This study compared the impacts of azoles on BNR processes and highlighted the knowledge gaps in potential inhibition and resistance mechanisms. However, beyond the inhibition and resistance mechanisms discussed above, other factors may also influence BNR processes after exposure to azoles, such as extracellular polymeric substances (EPS), reactive oxygen species (ROS), quorum sensing (QS) bacterial communication, and energy metabolism (Zhang et al., 2021; Kannan and Sivaperumal, 2023). Additionally, emerging pollutants in wastewater have been reported to affect microbial community structures (Yang et al., 2024). Previous studies reported that azole compounds (e.g., tetraconazole, tebuconazole, and propiconazole) can induce microbiome shifts in soil environments, affecting the structure, genetic diversity, and functional diversity of bacterial communities (Sułowicz et al., 2016; Reichman et al., 2021). However, limited information is available regarding their effects on microbial communities in BNR process during wastewater treatment. Therefore, further studies are needed to explore the role of these factors in azole-induced inhibition, including their impacts on enzyme activities, microbial cell properties, and microbial community dynamics within BNR process.

4 Conclusions

While previous studies primarily focused on nitrification and Anammox individually, this study assessed the inhibitory potential of azole compounds on the denitrification process and provided comprehensive insights by comparing the impact of azoles across various BNR processes in wastewater treatment. The negative effects of azoles on BNR performance vary depending on the type of azoles and the specific characteristics of the BNR process. In WWTPs that have not previously treated azole-containing wastewater, acute exposure (< 24 h) to azoles,

particularly PA and BTA, may lead to reduced performance of the BNR system due to the inhibition of the nitrification process. As the prerequisite step for Anammox and denitrification in wastewater treatment, nitrification has a higher sensitivity to azoles and may experience substantial inhibition (> 20%) when exposed to low levels of azoles (IC_{20} of 1.2 mg/L for PA and 0.65 mg/L for BTA), thereby endangering the stability of BNR processes in WWTPs. Prolonged exposure (8 d) to PA (2.7 mg/L), however, may result in severe disruption (> 90% activity reduction) or even complete failure of the nitrification process. Results from this study demonstrated a greater inhibition potential of azole compounds on the ammonium oxidation over the nitrite oxidation in nitrification. In the Anammox process, the granular structure did not provide additional protection against azole inhibition. These results underscore the importance for WWTPs to evaluate the inhibitory potential of azoles on BNR processes, especially in nitrification-involved technologies, before treating azole-containing wastewater. They also highlight the need to develop effective strategies to safeguard BNR systems from azole-induced disruption or failure.

CRediT Authorship Contribution Statement

Xiaojue Chen: Investigation, Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing -original draft, Writing-review & editing. **Emily A. Speierman**: Conceptualization, Writing-review & editing. **Liu Jiang**: Conceptualization, Writing-review & editing. **Khashayar Aghilinasrollahabadi**: Investigation, Writing-review & editing. **Camila A. Proano**: Investigation, Writing-review & editing. **Marya O. Anderson**: Conceptualization, Writing-review & editing. **Guangbin Li**: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing-review & editing.

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

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