

## Supplementary Materials

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

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## Text S1 Operation condition of the Anammox-EGSB reactor

The anammox-EGSB (working volume = 3 L) has been successfully operated for four years with a loading rate of 4.1 g N/(L·day), and the volatile suspended solids (VSS) is 89% of TSS by mass. The anammox granular sludge, with a size of  $2.4 \pm 0.6$  mm, is dominated by *Brocadia* spp.

## Text S2 Analytical method for DO, $\text{NH}_4^+$ , $\text{NO}_3^-$ , and $\text{NO}_2^-$

$\text{NO}_3^-$  and  $\text{NO}_2^-$  in liquid samples were analyzed in an ion chromatograph (Dionex ICS-1100, Thermo Fisher Scientific, USA) equipped with Dionex IonPac AS22-Fast analytical column (Thermo Fisher Scientific, USA), while  $\text{NH}_4^+$  was measured using an IntelliCAL Ammonia Ion Selective Electrode (ISENH4131, Hach, Loveland, CO, USA). The  $\text{N}_2$  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, Santa Clara, CA, USA) and a thermal conductivity detector. The temperatures of the column, the injection port, and the detector were 40°C, 200°C, and 220°C, respectively. Ultra-high purity helium (purity >99.999%) was used as the carrier gas and the injection volume was 100  $\mu\text{L}$ .

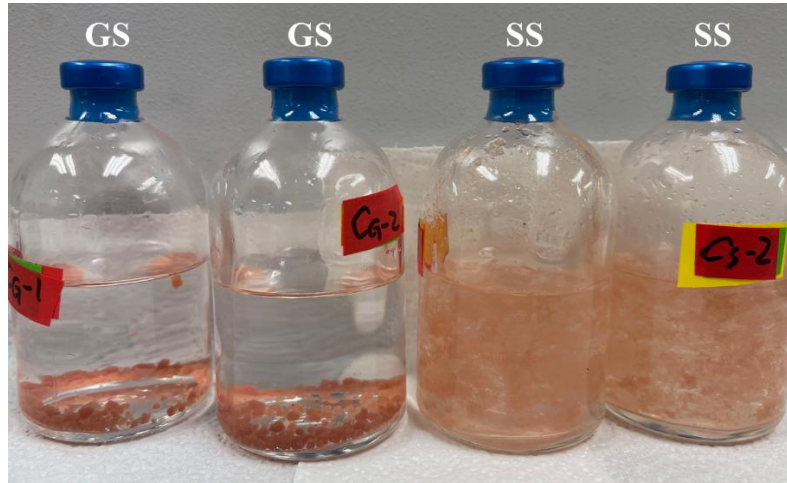


Fig. S1 The picture of granular (GS) and suspended (SS) anammox biomass.

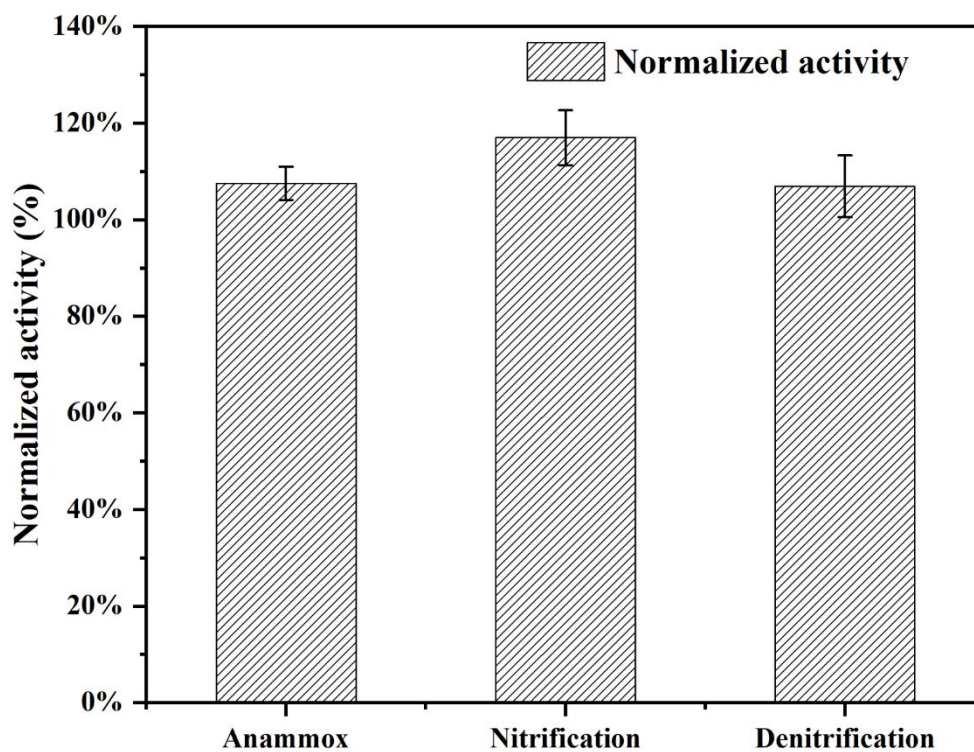
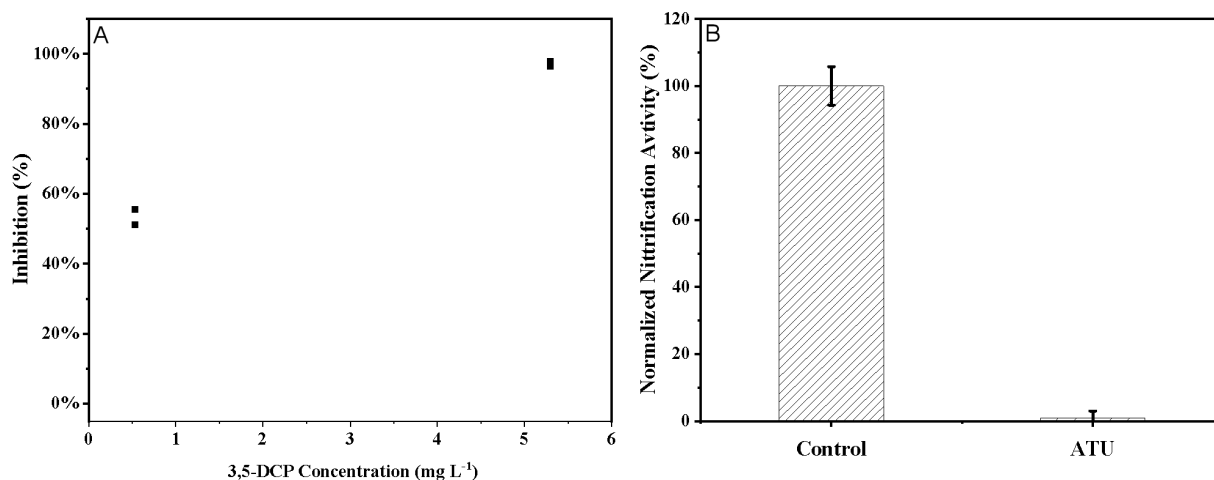
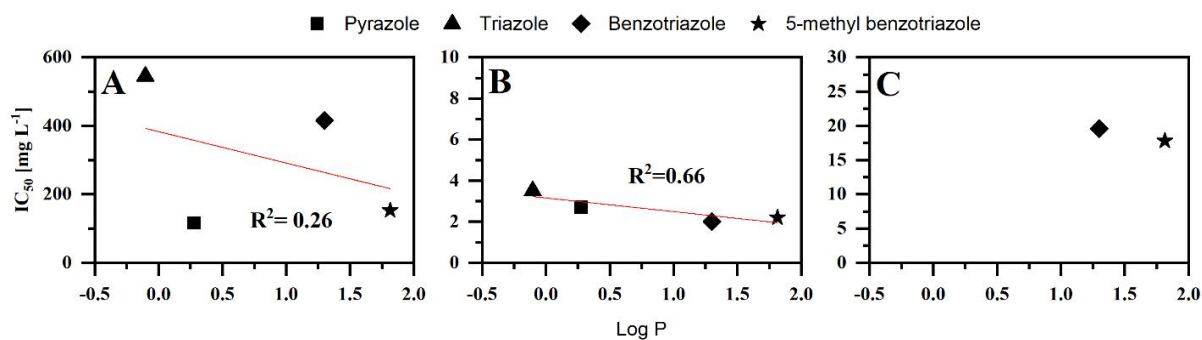


Fig. S2 Normalized BNR activities with 275 mg/L DMSO addition. Error bar represents the standard deviation of triplicate. These results indicated the addition of DMSO for dissolving azoles in the experiments did not cause impacts on the BNR activity. Therefore, the results were reliable for studying the impact of azoles on the activity of anammox, nitrification, and denitrification.



**Fig. S3** (A) Inhibition of reference compound, 3,5-Dichlorophenol, to nitrification activity. (B) Normalized activities of nitrification bioassays with 11.6 mg/L allylthiourea (ATU). Error bar represents the standard deviation of duplicate. The reference substances were tested to assure that the test method and test conditions were reliable, and to check the sensitivity of the activated sludge. The results indicated that 5.5 mg/L of 3,5-DCP and 11.6 mg/L of ATU showed complete inhibition (>99%), confirming the activity of the nitrifying sludge meets the requirements of ISO method 9509 (ISO-9509, 2006).



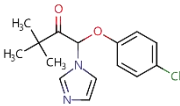
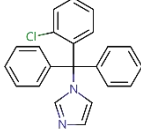
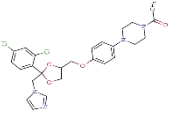
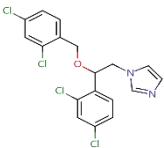
**Fig. S4** Relationship between azoles hydrophobicity (Log P) and their inhibition (IC<sub>50</sub>) on (A) denitrification, (B) nitrification and (C) anammox processes. These results indicated that the toxicity was not correlated to either Log P ( $p > 0.1$ ).

**Table S1** Reported occurrence of azole compounds in different environments.

Azoles	Wastewater ( $\mu\text{g/L}$ )	Surface water ( $\mu\text{g/L}$ )	Ground water ( $\mu\text{g/L}$ )	WWTP sludge ( $\mu\text{g/kg}$ )	Soil ( $\mu\text{g/kg}$ )	Reference
Benzotriazole	12-47	0.02 - 33	<0.001 - 1100	-	0.05 – 4.10	(McNeill and Cancilla 2009; Careghini et al., 2015; Li et al., 2020)
Methyl-benzotriazole	<0.02 - 3.4	<0.01 - 0.05	<17000	-	0.36 – 484.19	(McNeill and Cancilla 2009; Liu et al., 2013; Alotaibi et al., 2015)
Pyrazole	<1020	<0.1	-	-	-	(Li et al., 2020; Li et al., 2021)
Triazole	50 - 75	-	<0.01 – 0.23	-	<0.1 – 2.1	(Rosenbom et al., 2016; Blank et al., 2018; Li et al., 2020)
Clotrimazole	0.26 - 1.8	<0.01 - 0.62	<0.01 - 0.51	<2547	-	(Peng et al., 2012; Chen et al., 2013; Melefa and Nwani 2021; Monapathi et al., 2021)
Climbazole	0.44 - 1.4	<0.53	0.02 –	<1160	-	(Peng et al., 2012;

			0.37			Chen et al., 2013; Zhang et al., 2015)
Miconazole	<0.01 - 1.1	0.01 – 1.45	<0.01 – 0.08	<2069	-	(Peng et al., 2012; Chen et al., 2013) (Patel, 2000; Chen et
Ketoconazole	0.04 – 0.86	<0.01 – 0.14	0.09 – 0.19	60-4500	-	al., 2014; Monopathi et al., 2021)
Fluconazole	0.01 - 27	0.11 - 1.06	0.06 - 13.2	<2547	-	(Patel, 2000; Chen et al., 2014; Monopathi et al., 2021)

**Table S2** Overview of the physical and chemical properties of tested azoles.

Compound	Structure	Solubility in water (mg/L)	Log P	Pk <sub>a1</sub>	Pk <sub>a2</sub>
Climbazole		50	4.34	6.49	-
Clotrimazole		<1	5.84	6.26	-
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<sub>ka1</sub> and P<sub>ka2</sub> represent the P<sub>ka</sub> values of its protonated and neutral species, respectively.*

**Table S3** Inhibition of six other azole compounds on different BNR processes.

Compounds	Test Concentration (mg/L)	Anammox process Inhibition (%)	Observed inhibition	
			Nitrification process Inhibition (%)	Denitrification process Inhibition (%)
Climbazole	20	12 ± 7.0	0 ± 3.4	6 ± 1.2
Clotrimazole	1 (0.8) <sup>a</sup>	0 ± 6.1	7 ± 15.2	0 ± 4.5
Ketoconazole	1	1 ± 3.3	6 ± 6.2	1 ± 3.3
Miconazole	1 (0.8) <sup>a</sup>	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

<sup>a</sup> Tested concentration for Anammox process.

\* Standard deviations are calculated from triplicate groups.

**Table S4** Toxicity parameters of bioassays as a function of concentrations of tested azole compounds.

Process	Compounds	R <sup>2</sup>	Ki		n	
			Value [mg/L]	Stdev	Value [Dimensionless]	Stdev
Nitrification <sup>a</sup>	Benzotriazole	1.00	1.99	0.06	1.24	0.05
	5-Methylbenzotriazole	0.99	2.18	0.08	2.10	0.14
	Pyrazole	0.98	2.69	0.14	1.71	0.14
	1,2,4-Triazole	0.97	3.53	0.22	1.84	0.20
Denitrification	Benzotriazole	0.81	411.9	83.05	1.39	0.38
	5-Methylbenzotriazole	0.96	169.4	13.07	1.35	0.16
	Pyrazole	0.96	115.8	9.06	1.40	0.17
	1,2,4-Triazole	0.88	544.91	151.61	0.69	0.15
Anammox <sup>a</sup>	Benzotriazole	0.98	19.55	1.60	0.84	0.06
	5-Methylbenzotriazole	0.99	17.84	1.14	0.93	0.06
	Pyrazole	–	–	–	–	–
	1,2,4-Triazole	–	–	–	–	–

*a*, Data retrieved from Li et al., 2020 and Lakhey et al., 2020.

\* Standard deviations are calculated from triplicate groups.

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