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Research Paper

Benzimidazole fungicide biotransformation by comammox *Nitrospira* bacteria: Transformation pathways and associated proteomic responses

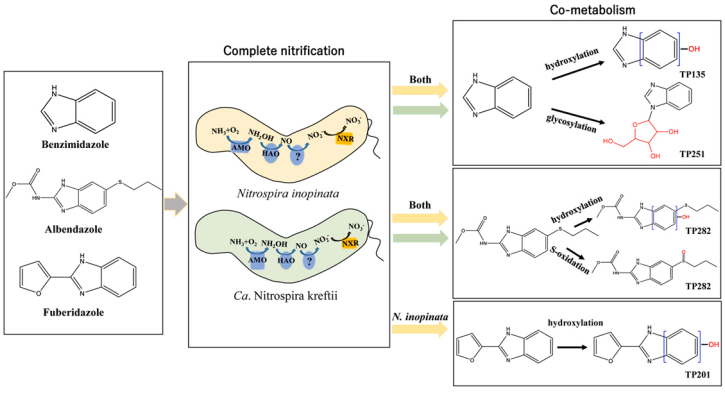
Ping Han [a,b,c,*], Ana B. Rios-Miguel [d], Xiufeng Tang [a], Yaochun Yu [e,f], Li-Jun Zhou [g,**], Lijun Hou [b,c], Min Liu [a,c], Dongyao Sun [a,h], Mike S.M. Jetten [d], Cornelia U. Welte [d], Yujie Men [e,f,***], Sebastian Lücker [d]

[a] Key Laboratory of Geographic Information Science (Ministry of Education), School of Geographic Sciences, East China Normal University, 500 Dongchuan Road, Shanghai 200241, China
[b] State Key Laboratory of Estuarine and Coastal Research, East China Normal University, 500 Dongchuan Road, Shanghai 200241, China
[c] Institute of Eco-Chongming (IEC), 3663 North Zhongshan Road, Shanghai 200062, China
[d] Department of Microbiology, RIBES, Radboud University, Heyendaalseweg 135, 6525 AJ Nijmegen, the Netherlands
[e] Department of Chemical and Environmental Engineering, University of California, Riverside, CA 92521, United States
[f] Department of Civil and Environmental Engineering, University of Illinois at Urbana-Champaign, Urbana, IL 61801, United States
[g] State Key Laboratory of Lake Science and Environment, Nanjing Institute of Geography and Limnology, Chinese Academy of Sciences, Nanjing 210008, China
[h] School of Geography Science and Geomatics Engineering, Suzhou University of Science and Technology, Suzhou 215009, China

HIGHLIGHTS

- Comammox *Nitrospira* distinctively transform benzimidazole fungicides.
- Biotransformation occurs through hydroxylation, S-oxidation, and glycosylation.
- Ammonia monooxygenase likely plays an important role in the biotransformation.
- Benzimidazole exposure induced the expression of the antibiotic resistance gene *acrB*.

GRAPHICAL ABSTRACT



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ABSTRACT

Benzimidazole fungicides are frequently detected in aquatic environments and pose a serious health risk. Here, we investigated the metabolic capacity of the recently discovered complete ammonia-oxidizing (comammox) *Nitrospira inopinata* and *kreftii* to transform a representative set of benzimidazole fungicides (i.e., benzimidazole,

* Corresponding author at: Key Laboratory of Geographic Information Science (Ministry of Education), School of Geographic Sciences, East China Normal University, 500 Dongchuan Road, Shanghai 200241, China.
** Corresponding author.
*** Corresponding author at: Department of Chemical and Environmental Engineering, University of California, Riverside, CA 92521, United States.
E-mail addresses: han@geo.ecnu.edu.cn (P. Han), ljzhou@niglas.ac.cn (L.-J. Zhou), ymen@engr.ucr.edu (Y. Men).

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Comammox *Nitrospira*
Ammonia oxidation
Cometabolism
Biotransformation products

albendazole, carbendazim, fuberidazole, and thiabendazole). Ammonia-oxidizing bacteria and archaea, as well as the canonical nitrite-oxidizing *Nitrospira* exhibited no or minor biotransformation activity towards all the five benzimidazole fungicides. In contrast, the investigated comammox bacteria actively transformed all the five benzimidazole fungicides, except for thiabendazole. The identified transformation products indicated hydroxylation, S-oxidation, and glycosylation as the major biotransformation pathways of benzimidazole fungicides. We speculated that these reactions were catalyzed by comammox-specific ammonia monooxygenase, cytochrome P450 monooxygenases, and glycosylases, respectively. Interestingly, the exposure to albendazole enhanced the expression of the antibiotic resistance gene *acrB* of *Nitrospira inopinata*, suggesting that some benzimidazole fungicides could act as environmental stressors that trigger cellular defense mechanisms. Altogether, this study demonstrated the distinct substrate specificity of comammox bacteria towards benzimidazole fungicides and implies their significant roles in the biotransformation of these fungicides in nitrifying environments.

1. Introduction

Benzimidazoles are aromatic heterocyclic compounds, with a benzene and an imidazole ring, such as benzimidazole, albendazole, carbendazim, fuberidazole and thiabendazole. Benzimidazole fungicides are an important group of biocides, which are mainly used to inhibit the growth of fungi, bacteria, and parasites in crops, animals and humans (Chen et al., 2021; Pan et al., 2012). Additionally, some benzimidazoles have antiviral, anticancer, and antihypertensive properties (Chen et al., 2021). Benzimidazole residues have been frequently detected of μg/L levels in wastewater (Bollmann et al., 2014; Juksu et al., 2019; Liu et al., 2017; Porto et al., 2019), and in various aquatic environments all over the world, including drinking water (ng/L levels) (Belew et al., 2021; Chen et al., 2014; Juksu et al., 2019; Kalogridi et al., 2014; Liu et al., 2015; Montagner et al., 2014; Zamora et al., 2009; Zhou et al., 2020). The excessive use of benzimidazole fungicides has raised considerable concerns due to their severe negative effects on aquatic organisms and human health (Chen et al., 2021; Juksu et al., 2019; Liu et al., 2015).

Biotransformation of specific micropollutants has been observed during ammonia-oxidation in environments with high nitrifying activity (Xu et al., 2016). For example, inhibition experiments and pure culture studies showed that ammonia-oxidizing bacteria (AOB) in nitrifying activated sludge (NAS) were involved in the biotransformation of asulam, clomazone, monuron, trimethoprim, sulfonamide, and fluoroquinolone antibiotics and many other pharmaceuticals (Men et al., 2017; Wu et al., 2021; Zhou et al., 2019, 2021). The complete ammonia-oxidizing (comammox) bacteria within the genus *Nitrospira* have only been discovered recently, but were found to occur in many different nitrifying ecosystems (Daims et al., 2015; Kits et al., 2017; Pjevac et al., 2017; van Kessel et al., 2015). As comammox bacteria have different physiological characteristics compared to their ammonia- and nitrite-oxidizing counterparts, (Daims et al., 2015; Kits et al., 2017; van Kessel et al., 2015), and they were also found in wastewater treatment systems (Spasov et al., 2020; Yang et al., 2020), it is worthwhile to study their biotransformation properties with respect to various micropollutants. We have previously reported the biotransformation of one benzimidazole fungicide, carbendazim by the comammox isolate *Nitrospira inopinata* and this compound was not converted by AOB or ammonia-oxidizing archaea (AOA) (Han et al., 2019). The exclusive biotransformation of carbendazim by *N. inopinata* could be due to the larger substrate promiscuity of its ammonia monooxygenase (AMO) protein complex and/or due to its high substrate affinity (Han et al., 2019). However, it still remains unclear whether (i) *N. inopinata* can also convert other benzimidazole fungicides, (ii) if other comammox strains have similar transformation capabilities, and (iii) which protein complexes of comammox bacteria are involved in the biotransformation of benzimidazole fungicides or induced by them.

Therefore, the objective of this study was to fill those knowledge gaps by examining the biotransformation of a set of benzimidazole fungicides using the two currently available comammox cultures, *N. inopinata* (isolated from a biofilm in a pipe discharging geothermal water (Daims et al., 2015; Kits et al., 2017) and *Ca.* N. kreftii (enriched from a lab-scale nitrifying bioreactor inoculated with biomass from the anaerobic compartment of a trickling filter connected to a recirculation aquaculture system (Sakoula et al., 2021; van Kessel et al., 2015). These two cultures represent comammox bacteria from natural and engineered ecosystems, respectively. We investigated benzimidazole and four derivatives that are commonly used as fungicides, i.e., albendazole, carbendazim, fuberidazole, and thiabendazole. We compared the biotransformation capabilities with those of other nitrifying counterparts, i.e., the AOA and AOB species *Nitrososphaera gargensis* and *Nitrosomonas nitrosa* 18–3D, and the NOB *Nitrospira moscoviensis*. The conversion mechanisms and pathways in the two comammox cultures were further evaluated by analyzing the transformation products when grown in the presence of ammonium, hydroxylamine or nitrite as the primary substrate. Finally, proteomic analyses were performed to determine the protein complexes involved in the biotransformation of benzimidazole fungicides and to evaluate the responses of *N. inopinata* to the exposure of those compounds.

2. Materials and methods

2.1. Selection of benzimidazole fungicides

The five fungicides benzimidazole, albendazole, carbendazim, fuberidazole and thiabendazole were chosen based on their frequent detection in aquatic environments and wastewater, and the diversity in their structures (Belew et al., 2021; Chen et al., 2014; Juksu et al., 2019; Liu et al., 2017, 2015; Montagner et al., 2014; Zhou et al., 2020). All the target compounds were obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Stock solutions of target compounds were prepared in methanol (MeOH) (0.2 g/L) and stored at − 20 °C until use. The physicochemical properties of the selected micropollutants are presented in Table S1.

2.2. Selection of nitrifying microorganisms

Five microorganisms were used to study the biotransformation of the selected fungicides: the comammox bacterium *Nitrospira inopinata* isolated from a hot water well (Daims et al., 2015; Kits et al., 2017); the enrichment culture of *Ca.* Nitrospira kreftii obtained from a lab-scale bioreactor inoculated with the biofilm of the anaerobic compartment of a trickling filter connected to a recirculation aquaculture system (Sakoula et al., 2021; van Kessel et al., 2015), the AOA strain *Nitrososphaera gargensis* (JCM-31473, DSM-103042) isolated from a hot spring (Hatzenpichler et al., 2008; Palatinszky et al., 2015), the AOB strain *Nitrosomonas nitrosa* 18–3D isolated from a biofilm of freshwater mat sediment (Han et al., 2021), and the NOB strain *Nitrospira moscoviensis* isolated from a corroded iron pipe of a heating system (Ehrich et al., 1995).

2.3. Cultivation of nitrifying microorganisms

A modified mineral medium containing (per liter): 50 mg KH_2PO_4, 75 mg KCl, 50 mg $MgSO_4$*$7H_2O$, 584 mg NaCl, 4 g $CaCO_3$, 1 mL of trace element solution (TES), and 1 mL selenium-wolfram solution (SWS)

Table 1
Biodegradation experiments overview. All benzimidazole compounds were added at concentrations of 20 μg/L each, either as mixture of all five compounds or, where indicated, individually. The chemicals added in the control experiments were based on the highest possible concentrations of remained ammonium, nitrite, nitrate and hydroxylamine in the biotic experiments.

Nitrifying cultures	Temperature	Primary substrate (with refeeding upon depletion)			Control experiments (abiotic and dead biomass)	
N. gargensis (AOA)	46 °C	1 mM NH_4Cl			2 mM NH_4Cl, 2 mM $NaNO_2$, and 6 mM $NaNO_3$.	
N. nitrosa 18–3D (AOB)	37 °C	1 mM NH_4Cl			2 mM NH_4Cl, 2 mM $NaNO_2$, and 6 mM $NaNO_3$.	
N. inopinata (comammox)	42 °C	1 mM NH_4Cl 2 mM NH_4Cl (individual-compound experiments)	0.5 mM $NaNO_2$	0.2 mM NH_2OH	2 mM NH_4Cl, 2 mM $NaNO_2$, and 6 mM $NaNO_3$.	
N. moscoviensis (NOB)	37 °C	1 mM $NaNO_2$				
Ca. N. kreftii (comammox enrichment)	20 °C	0.25 mM NH_4Cl	0.5 mM $NaNO_2$	0.1 mM NH_2OH	2 mM NH_4Cl, 0.5 mM $NaNO_2$, and 2 mM $NaNO_3$.	2 mM NH_2OH

(Daims et al., 2015) was used for the cultivation of all nitrifying microorganisms as described elsewhere (Lebedeva et al., 2013). Unless otherwise stated, all nitrifying cultures were grown in 500 mL medium in the dark in 2 L bottles without shaking at the following temperatures and substrate concentrations: 1 mM NH_4Cl at 46 °C (*N. gargensis*); 2 mM NH_4Cl at 37 °C (*N. nitrosa* 18–3D), 1 mM NH_4Cl at 42 °C (*N. inopinata*) or 1 mM $NaNO_2$ at 37 °C (*N. moscoviensis*). The enrichment culture of Ca. *N. kreftii* was kept active in a continuous membrane bioreactor.The bioreactor was run under dark conditions, mechanic stirring, room temperature (20 °C), pH 7.5 and it was fed with NOB mineral salt medium containing 1.25 mM $(NH_4)_2SO_4$ (2.5 mM NH_4^+), 8.56 mM NaCl, 0.42 mM $MgSO_4$ x $7H_2O$, 0.1 mM $CaCl_2$ x $2H_2O$, 1.1 mM KH_2PO_4 and trace elements as described previously (Sakoula et al., 2021).

2.4. Biotransformation experiments

The biotransformation of the benzimidazole fungicides by different nitrifiers was investigated using batch cultures. In general, actively growing nitrifying cultures (including AOA, AOB, comammox and nitrite-oxidizing *Nitrospira*) were harvested by centrifugation at 7000 × *g* at 10 °C for 30 min, followed by washing with fresh medium to remove nitrite/nitrate. Finally, the biomass was 2-fold concentrated by adding half the original volume of fresh medium. In this way we achieved ammonia/nitrite converting rates of approximately 1 mM per week. The initial concentration of each target compound in the experiments was set to 20 μg/L according to their environmental concentrations and our previous experimental setup (Han et al., 2019). Stock solution (0.2 g/L) of target compounds was added into the bottom of empty sterile bottles to let the methanol evaporate. And then washed and concentrated culture was added into 100 mL-Serum bottles to a final volume of 25 mL. All the bottles were incubated at the optimal growth temperatures of the corresponding culture in the dark (see Table 1).

Initially, we tested the AOA *N. gargensis* and AOB *N. nitrosa* 18–3D with a mix of the five compounds (20 μg/L each) and 1 mM NH_4Cl (with refeeding upon depletion) under conditions described in previous work (Han et al., 2019). Abiotic and dead biomass control experiments were set up with medium only or autoclaved cultures (at 121 °C and 103 kPa for 20 min for two cycles), respectively, containing 2 mM NH_4Cl, 2 mM $NaNO_2$ and 6 mM $NaNO_3$ (Table 1). Liquid samples (0.5 mL) were taken after biomass addition at 0 h, 144 h (6 d) and 312 h (13 d) and centrifuged at 10,000 × *g* for 10 min at room temperature. After that, one 200 μL aliquot of each supernatant was stored in 2 mL amber glass vials at 4 °C for LC-MS/MS analysis. The remaining supernatant was kept in 1.5 mL microcentrifuge tubes at 4 °C to determine the concentrations of ammonium and nitrite/nitrate. Cell pellets were stored at − 20 °C for measuring the total protein concentrations.

For the experiments with comammox bacteria, the pure culture *N. inopinata* was incubated with the mixture of the five compounds (20 μg/L each) using 1 mM NH_4Cl, 0.5 mM $NaNO_2$ or 0.2 mM NH_2OH as substrate (with refeeding upon depletion) (Table 1). The NOB strain *N. moscoviensis* was supplied with 1 mM $NaNO_2$ (with refeeding upon depletion). The enriched comammox culture of *Ca.* N. kreftii were incubated at 20 °C and 0.25 mM NH_4Cl, 0.5 mM $NaNO_2$ or 0.1 mM NH_2OH as energy source (with refeeding upon depletion) (Table 1). For *Ca.* N. kreftii, we set up abiotic and dead biomass controls with i) 2 mM NH_4Cl + 0.5 mM $NaNO_2$ + 2 mM $NaNO_3$ and ii) 2 mM NH_2OH to investigate potential abiotic transformation of target compounds by NH_4Cl, $NaNO_2$, $NaNO_3$ or NH_2OH (Table 1). All treatments were carried out in biological triplicates. Liquid samples (0.5 mL) were taken after biomass addition at 0 h, 48 h, 96 h, 144 h, 192 h, 240 h, 288 h, and 336 h for *N. inopinata* and *N. moscoviensis* incubations, and at 0 h, 22 h, 48 h, 95 h, 138.5 h, 191.5 h, 239.5 h, 286.5 h, and 309.5 h for the *Ca.* N. kreftii experiments. Samples were centrifuged and stored as described above.

We also set up experiment with individual compounds (20 μg/L each) and the comammox pure culture *N. inopinata* supplied with 2 mM NH_4Cl with refeeding upon depletion (Table 1). Liquid samples (0.5 mL) were taken after biomass addition at 0 h, 48 h, 96 h, 240 h, 384 h, and 528 h, and centrifuged and stored as described above.

2.5. Analytical detection of micropollutants

All samples were analyzed by Acquity I-class UPLC and Vion IMS QToF MS (Waters, MA, USA) with a C_{18} HSST3 column (particle size 1.7 μm, 2.1 × 100 mm, Waters). The mobile phases were nanopure water (A) and acetonitrile (B) (both containing 0.1% v/v formic acid). The injection volume was 5 μL and the flow rate 0.4 mL/min. Elution was performed using 95% phase A and 5% B for 2 min, followed by linear gradients increasing phase B from 5% to 25% B over 3 min, to 50% B over 2 min, to 100% B over 3 min, kept at 100% B for 2 min, and a final elution step at 95% B for 3 min. Subsequently, the initial conditions were restored for 3 min to equilibrate the column. Spectra were acquired in positive-ion mode, with a scan range from 50 to 1000 *m/z*.

2.6. Transformation product (TP) identification

The screening for transformation products was carried out using the Acquity I-class UPLC and Vion IMS QToF MS (Waters, MA, USA) as described above. For targeted identification, a list of potential TPs for benzimidazole fungicides was used, which consisted of a number of known redox, hydrolysis and conjugation reaction products (Men et al., 2016). Suspected TPs with an intensity above a set threshold > 70% match with the predicted isotopic patterns, and an increasing trend over the time course, were selected for further identification (Men et al., 2016). Furthermore, non-targeted screening was performed to identify potential unknown TPs. TP candidates were chosen by following the criteria: (1) a reasonable peak shape and an intensity above noise; (2) presence in the benzimidazole-added treatments and absence in non-benzimidazole treatments; (3) a reasonable time-series pattern (i.e., continuous increase, or increase and subsequent decrease during the experiment); and (4) a rational molecular formula derived from the exact mass of [M+H], [M+NH_4], [M+Na] or [M-H_2O+H] (with <5 ppm tolerance) and isotopic pattern (isotope ratio difference <10%). Since

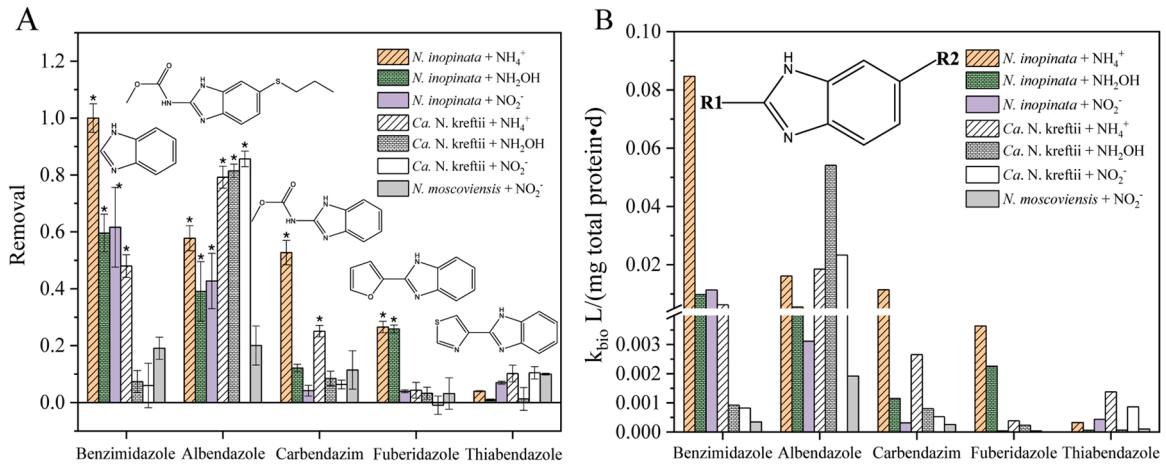

Fig. 1. Biotransformation of benzimidazole fungicides by the comammox species *N. inopinata* and *Ca.* N. kreftii, and the NOB *N. moscoviensis*. (A) Removal of micropollutants by *N. inopinata* (in 336 h), *Ca.* N. kreftii (in 312 h) and *N. moscoviensis* (in 336 h; n = 3; * indicates a statistically significant difference from the abiotic and heat-inactivated biomass controls, $p < 0.05$). (B) First-order biotransformation rate constants (k_{bio}) of benzimidazole, albendazole, carbendazim, fuberidazole and thiabendazole by *N. inopinata*, *Ca.* N. kreftii and *N. moscoviensis*.

reference standards for TP candidates were not commercially available, peak areas of TPs are only used to indicate the formation trend but not for quantification.

2.7. Estimation of kinetic parameters

Calculation of kinetic parameters for benzimidazole fungicide degradation have been previously described using a first-order model (Men et al., 2016). The protein-normalized biotransformation rate constants (k_{bio}) were corrected for sorption and abiotic processes, and used to compare the biotransformation rates among different treatments and nitrifiers. Only time points that showed first-order kinetics according to the biotransformation curves were used for calculation.

$$\frac{dS_c}{dt} = -f_{aq}(k_{bio}X + k_a) \times S_c \quad (1)$$

$$f_{aq} = \frac{S_c}{S_{ct}} \quad (2)$$

$$K_d = \frac{1 - f_{aq}}{f_{aq}X} \quad (3)$$

where S_c is target compound's aqueous concentration, f_{aq} is the dissolved fraction of target compound, k_{bio} is the total protein concentration-normalized biotransformation rate constant, X is the total protein concentration, k_a is the abiotic transformation rate, S_{ct} is the target compound's total concentration, and K_d is the sorption coefficient.

2.8. Proteomic analysis

N. inopinata was inoculated into triplicate bottles after the addition of 100 μg/L benzimidazole or albendazole. Triplicate bottles with no micropollutant addition were used as controls. NH_4Cl (2 mM) was added to each bottle as the growth substrate. Cells were harvested after 96 h incubation by centrifugation at 20,000 × *g* at 4 °C for 15 min. Label-free quantitative proteomic (LFQP) analysis was performed by Novogene Bioinformatics Technology Co Ltd. (Beijing, China). LFQP analysis was used to identify differentially expressed proteins between benzimidazole/albendazole treated and control cells. Analyses included raw data filtering and quality control, protein function annotation including gene ontology (GO), Clusters of Orthologous Group (COG), KEGG and InterPro (IPR), protein expression quantification, differential expression analysis, GO, KEGG and IPR enrichment, and protein-protein interaction. The protein quantitation results were statistically analyzed by performing t-tests. The proteins whose abundance significantly differed between experimental and control groups ($p < 0.05$ and $|\log_2 FC| > 1.5$; corresponding to a ratio <0.67 or >1.5 fold change [FC]), were defined as differentially expressed proteins (DEP).

2.9. Ammonium, nitrite, and nitrate measurements

Ammonium ($NH_4^+ + NH_3$), nitrite (NO_2^-) and nitrate (NO_3^-) were analyzed using colorimetric methods in 96-well microtiter plates as described previously (Kandeler and Gerber, 1988). Briefly, ammonium was measured colorimetrically at 420 nm after reaction with OPA reagent (0.54% (w/v) ortho-phthaldialdehyde, 0.05% (v/v) β-mercaptoethanol and 10% (v/v) ethanol in 400 mM potassium phosphate buffer (pH 7.3)). Nitrite was measured at 540 nm using the Griess reagent method and nitrate concentrations were analyzed by being reduced to nitrite by vanadium chloride first and measured as NO_2^- and NO_3^- (Miranda et al., 2001).

2.10. Total protein measurement

Total protein concentrations were determined using the Pierce BCA Protein Assay Kit (Thermo Scientific, Regensburg, Germany) according to the manufacturer's instructions. Briefly, 20 μL of pelleted sample were thoroughly mixed with 200 μL of working reagent and incubated at 60 °C for 30 min. Afterwards, samples were centrifuged at 20,000 × *g* for 2 min and 100 μL of the supernatant were transferred to a 96 well plate. Protein concentration was determined at 562 nm.

3. Results and discussion

3.1. Specific biotransformation rates of benzimidazole derivatives by comammox bacteria

The five benzimidazoles (i.e., benzimidazole, albendazole, fuberidazole, carbendazim, and thiabendazole) were quite stable and not abiotically transformed in fresh medium or adsorbed to the heat-inactivated biomass controls (Fig. S1-S3). We used two comammox cultures (*N. inopinata* and *Ca.* N. kreftii enrichment) and three canonical nitrifiers (see Table 1) to investigate the transformation of the five benzimidazoles. The two comammox strains showed significant removal (>20%) for four out of the five benzimidazole compounds (except for thiabendazole) when grown on ammonium (Fig. 1 and Fig. 2). The

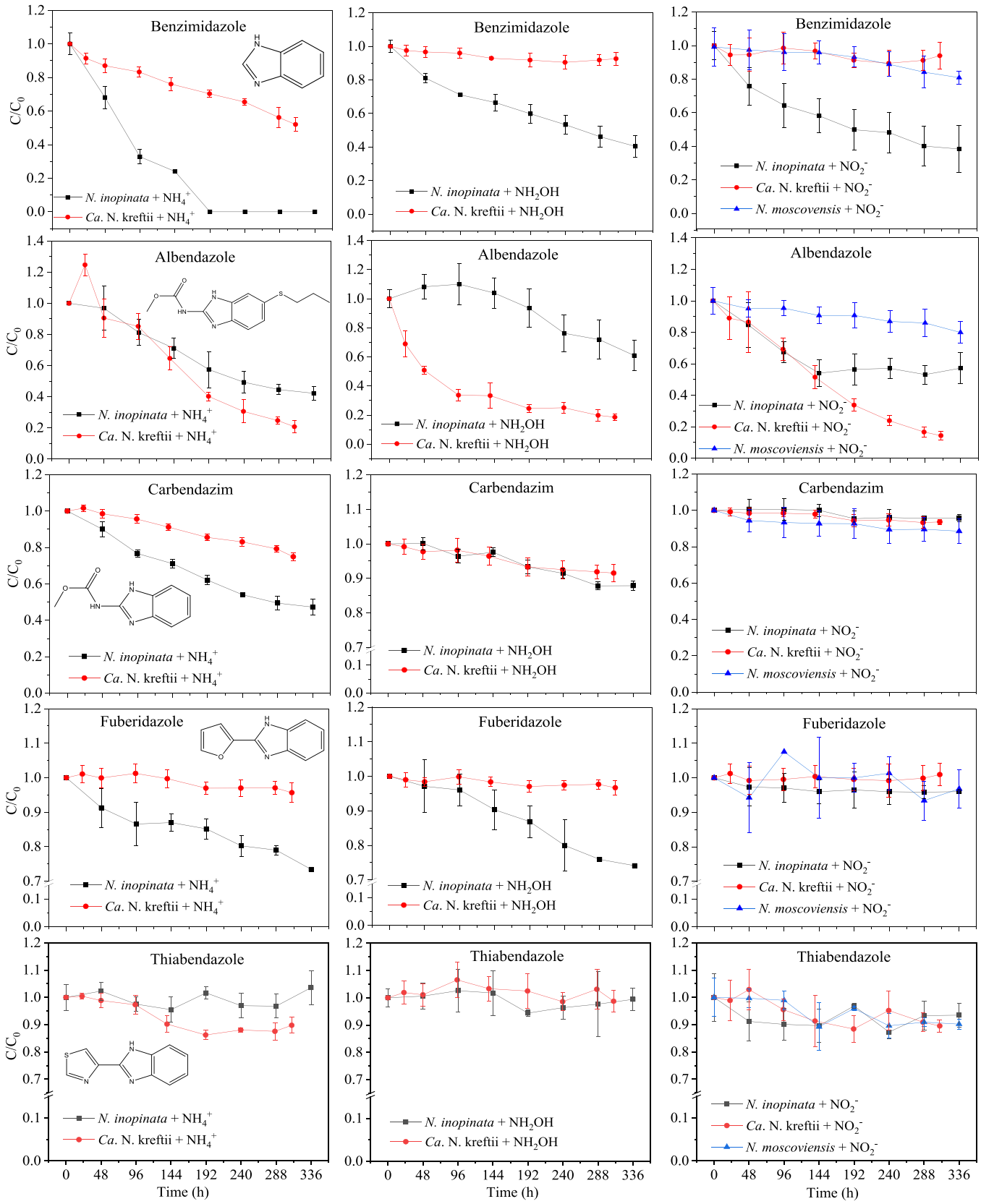

Fig. 2. Removal of benzimidazole, albendazole, carbendazim, fuberidazole and thiabendazole by *N. inopinata*, *Ca.* N. kreftii and *N. moscoviensis*. Left column, incubations with NH_4^+ as substrate; middle, with NH_2OH; and right, with NO_2^-. C/C_0 represents the ratio of micropollutant concentration at T_x vs. T_0. All datapoints represent the mean of biological triplicates ± SD.

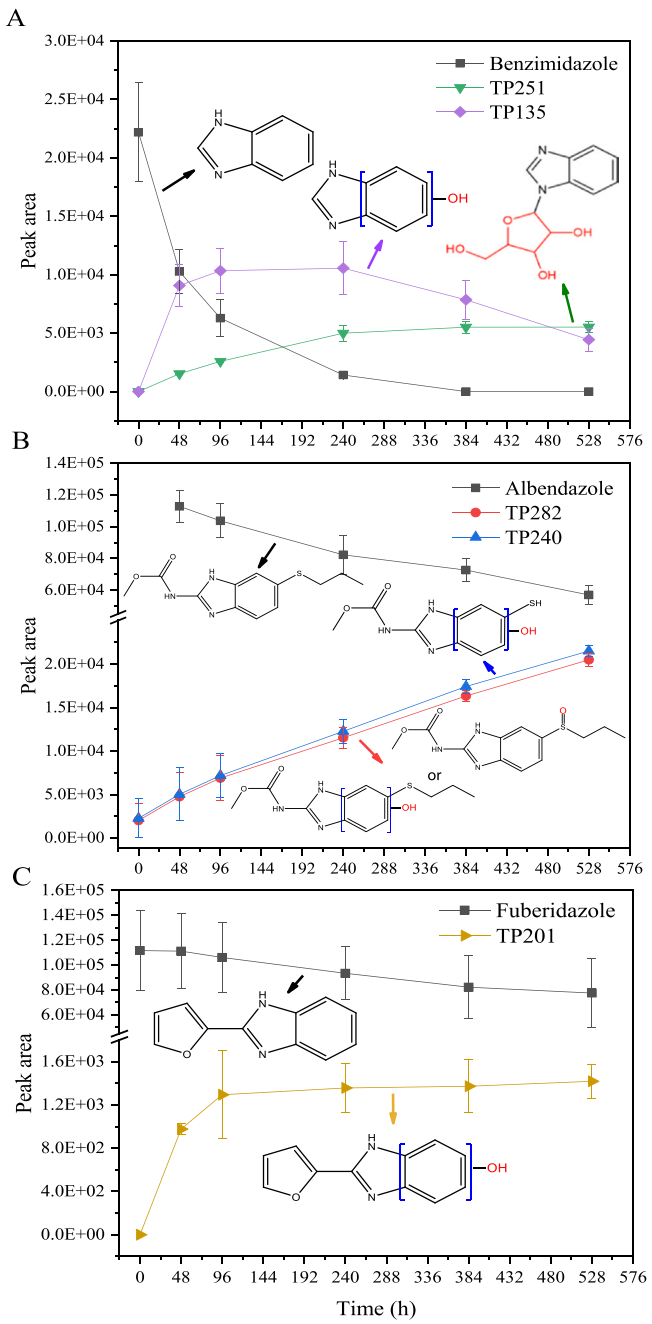

Fig. 3. Biotransformation of selected benzimidazole fungicides by *N. inopinata*. The plots show the degradation of (A) benzimidazole, (B) albendazole and (C) fuberidazole, along with TP formation (n = 3). All incubations were performed in the presence of NH_4^+. Compound concentrations are indicated as peak area.

investigated AOA and AOB species (Fig. S1) did not convert any benzimidazole, while the canonical NOB *N. moscoviensis* only showed slight transformation (<20%) of benzimidazole and albendazole (Fig. 1 and Fig. 2). The results not only corroborated our previous finding of the exclusive biotransformation of carbendazim by *N. inopinata* (Han et al., 2019) but also further demonstrated that the exclusive biotransformation could be extrapolated to a series of benzimidazole fungicides by comammox bacteria in general. It suggests that some distinct properties of comammox bacteria enable them with a more versatile substrate specificity towards micropollutants than other ammonia- and nitrite-oxidizing microorganisms.

Moreover, the biotransformation activity of comammox bacteria was influenced by different substituted R1 groups (Fig. 1B) in the structure of benzimidazole derivatives. Unsubstituted benzimidazole exhibited the highest biotransformation activity (~100% removal in the ammonium treatment) by *N. inopinata* compared to its substituted derivatives (Fig. 1A). With a carbamate group at R1 in carbendazim and albendazole, the biotransformation was about 50–60% for *N. inopinata* in the ammonium treatment. The thioether group at R2 (Fig. 1B) in albendazole did not seem to affect the conversion by *N. inopinata*, but it significantly enhanced the biotransformation by the second comammox strain *Ca.* N. kreftii (Fig. 1). With a heterocyclic group at R1, the biotransformation activity of comammox bacteria further decreased. Fuberidazole, containing an oxole group, was only removed to 30% by *N. inopinata* and was not removed by *Ca.* N. kreftii. With a thiazole group at R1 in thiabendazole, no biotransformation was observed for either comammox strain. The heterocyclic groups at R1 apparently inhibited the binding of the benzimidazole structure to the transforming enzyme, thus significantly decreasing the conversion. This may explain the persistence of thiabendazole in wastewater (Al-Mashaqbeh et al., 2020).

Our previous study suggested that ammonia monooxygenases (AMO) in *N. inopinata* might play a major role in the biotransformation of carbendazim (Han et al., 2019). To further examine the role of nitrifying enzymes, including AMO, in the biotransformation of the four benzimidazole derivatives, we switched the primary substrate from NH_4^+ to NH_2OH, which is the product of ammonia oxidation by AMO, and to NO_2^-, the end product of ammonia oxidation and substrate of the nitrite oxidoreductase (NXR), and compared the biotransformation activities. Feeding comammox with NH_2OH and NO_2^- significantly lowered the biomass-normalized biotransformation rate for nearly all four degradable benzimidazoles (Fig. 1), indicating the important role played by ammonia oxidation and AMO in the biotransformation of benzimidazoles. The only exception was the biotransformation of albendazole by *Ca.* N. kreftii. The biomass-normalized biotransformation rate was substantially enhanced when fed with NH_2OH (Fig. 1B), although similar overall removal rates were observed for all three primary substrates (Fig. 1A). This suggests that the hydroxylamine dehydrogenase (HAO) instead of the AMO of *Ca.* N. kreftii significantly contributed to the biotransformation of albendazole, or its transformation was catalyzed by an enzyme outside of the ammonia oxidation pathway. The latter was corroborated by the stimulated biotransformation of albendazole by *Ca.* N. kreftii grown on nitrite.

3.2. *Biotransformation pathways of benzimidazole fungicides by comammox bacteria*

To further examine the biotransformation mechanisms, we identified the transformation products (TPs) (Table S1) and transformation pathways of the converted benzimidazole fungicides. Hydroxylation of the aromatic ring appeared to be the general transformation pathway for all of the four benzimidazoles (Fig. 3, Fig. S4). Correspondingly, the following hydroxylation products (+O from the parent compound) were detected: TP135 for benzimidazole, TP282 for albendazole, TP208 for carbendazim, and TP201 for fuberidazole (Fig. 3, Table S1). As higher levels of the hydroxylation products were formed when comammox bacteria were grown on ammonia, the hydroxylation was most likely catalyzed by the AMO protein complex, an enzyme with a well-known broad substrate range, including a variety of micropollutants (Han et al., 2019; Men et al., 2016; Wu et al., 2021; Yu et al., 2018; Zhou et al., 2019, 2021).

Additionally, for albendazole with a thioether group at R2 (Fig. 4, Table S1), its +O TP (TP282) was also detected in comammox bacteria grown on hydroxylamine and nitrite (Fig. S4), suggesting that the oxidation could also occur at the thioether position, i.e., S-oxidation. And a different enzyme, such as cytochrome P450, other than AMO might catalyze this reaction. Previous studies demonstrated the ability of cytochrome P450 to oxidize aromatic micropollutants such as diclofenac or carbazole (Durairaj et al., 2016; Ide et al., 2012; Xu et al., 2015). The formation of TP282 in *N. inopinata* grown on hydroxylamine and

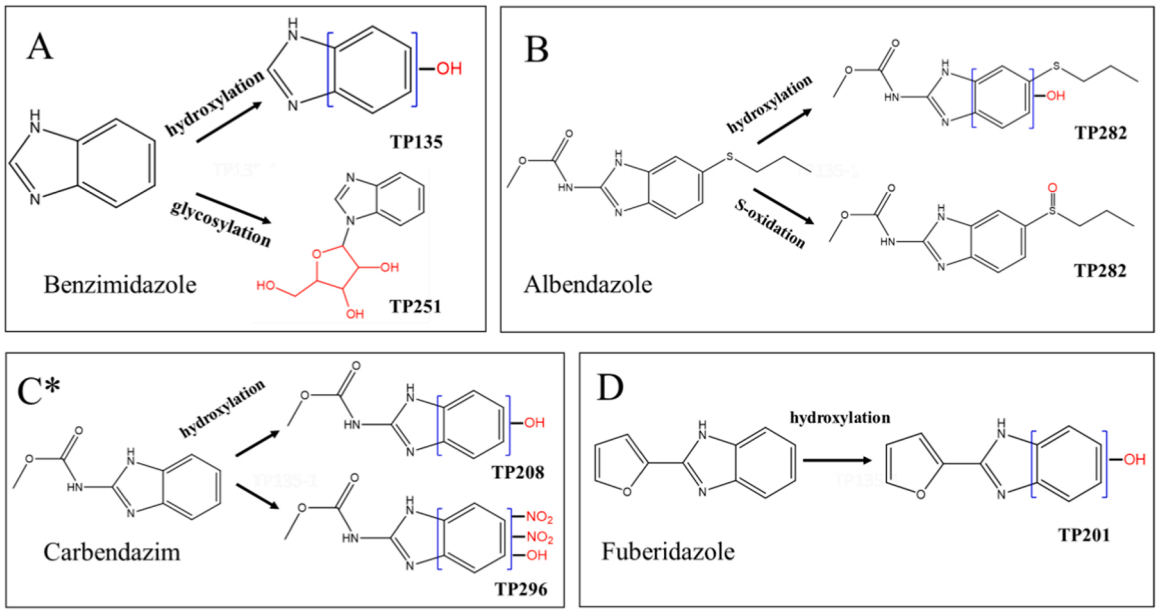

Fig. 4. Proposed transformation pathways of benzimidazole fungicides by comammox *Nitrospira*. (A) benzimidazole, (B) albendazole, (C) carbendazim and (D) fuberidazole. The asterisk indicates information obtained from previous work (Han et al., 2019).

nitrite was much slower than that in *N. inopinata* grown on ammonia (Fig. S4), indicating that the hydroxylation pathway by AMO was the major pathway in the biotransformation of albendazole by *N. inopinata*. In contrast, for *Ca.* N. kreftii, TP282 was formed in both the ammonia- and nitrite-fed cultures at similar levels (Fig. S4), implying that the non-AMO mediated reactions (hydroxylation or S-oxidation) mainly contributed to the formation of TP282 by *Ca.* N. kreftii.

Further removal of the hydroxylation product of benzimidazole (TP135) was observed in *N. inopinata* (Fig. 3A), but not for albendazole or fuberidazole (Fig. 3B and Fig. 3C). The R1 and R2 groups in those two structures (Fig. 4, Table S1) might hinder the removal of these hydroxylation products. In contrast, for carbendazim, which has a carbamate group at R1, *N. inopinata* could further biotransform its hydroxylation product (TP208), likely via a ring cleavage pathway (Fig. 4C) forming the linear product acetylserine (TP148) (Han et al., 2019). Compared to *N. inopinata*, *Ca.* N. kreftii appeared not able to utilize the TP208 hydroxylation product of carbendazim, which accumulated during the incubation (Fig. S4C). This could be due to the much slower biotransformation rate of carbendazim by *Ca.* N. kreftii (Fig. 1B). Similar to carbendazim, the hydroxylation product of benzimidazole (TP135) was further degraded perhaps also through ring-cleavage reactions, forming products that could be utilized in central metabolism (Fig. 3A).

We also observed other reaction types occurring to the R1 or R2 group in the benzimidazole derivatives. For instance, a product from S-dealkylation and hydroxylation of albendazole (TP240, $m/z = 240.0430$, $-C_3H_6 +O$ from albendazole) was identified (Fig. 3B, Fig. 4B and Fig. S4). The cytochrome P450 monooxygenases in the two comammox genomes may carry out the S-dealkylation reaction (Rydberg et al., 2008). Interestingly, *Ca.* N. kreftii exhibited higher removal of albendazole when grown on hydroxylamine, but the formation of TP282 and TP240 did not increase (Fig. S4). It suggests other transformation pathways of albendazole by *Ca.* N. kreftii, which became dominant when grown on hydroxylamine and led to the higher removal of albendazole, although no other transformation products could be identified. Lastly, for benzimidazole we also observed an N-linked glycosylation (ribosylation) forming TP251 (m/z 251.1022, $+C_5H_8O_4$ from benzimidazole) (Fig. 3A and Fig. S4), which could be carried out by glycosylases in the comammox bacteria. Such glycosylation did not occur to the other benzimidazole derivatives, probably due to the presence of R1 or R2 group in those structures.

3.3. Translational regulation in N. inopinata in response to benzimidazole and albendazole

We obtained the proteomes of *N. inopinata* grown on 2 mM ammonium with benzimidazole, albendazole, and in the absence of any micropollutant as the control. Although the nitrate formation of the comammox strains was not affected by the addition of benzimidazole fungicides (Fig. S5) indicating no toxicity to the energy metabolism, we still observed differential expression of a number of proteins in the presence of benzimidazole and albendazole. In the culture with albendazole, a total of 1453 proteins were detected. Compared to the no-addition control, eighteen proteins were significantly differentially expressed with a fold-change greater than 1.5 (t test, $p < 0.05$), with nine proteins up-regulated and nine proteins down-regulated, respectively (Table. S2). In the culture with benzimidazole, 1470 proteins were obtained in total. Compared to the no-addition control, 23 protein abundances were significantly different with a fold-change greater than 1.5 (t test, $p < 0.05$), with thirteen proteins up-regulated and ten proteins down-regulated, respectively (Table. S2).

Among the differentially expressed proteins, a ribosome-inactivating ribosome association toxin (Polyketide_cyc domain-containing protein) was expressed 1.7–2.5 fold lower in the benzimidazole and albendazole treatments, respectively, compared to the control incubation (Fig. 5, Table. S2). Four proteins belonging to the COG class of energy conservation were differentially expressed in the micropollutant treatments compared to the control incubations: ATP synthase subunit b (atpF) and a multiheme cytochrome *c* protein were upregulated in the albendazole treatment, while ferredoxin-thioredoxin reductase subunit B and a cytochrome *c* domain-containing protein were upregulated with benzimidazole (Fig. 5). Within the COG class of intracellular trafficking, secretion, and vesicular transport a TPR_region domain-containing protein was expressed 2.5-fold lower in the albendazole treatment and not expressed in the benzimidazole condition. Similarly, a DctA-YdbH domain-containing protein was expressed 2.5-fold lower in the benzimidazole treatment and not expressed at all with albendazole. Of the COG class of posttranslational modification, protein turnover, and chaperones, a total of five proteins were differentially expressed in the albendazole treatment (one protein with lower expression) and

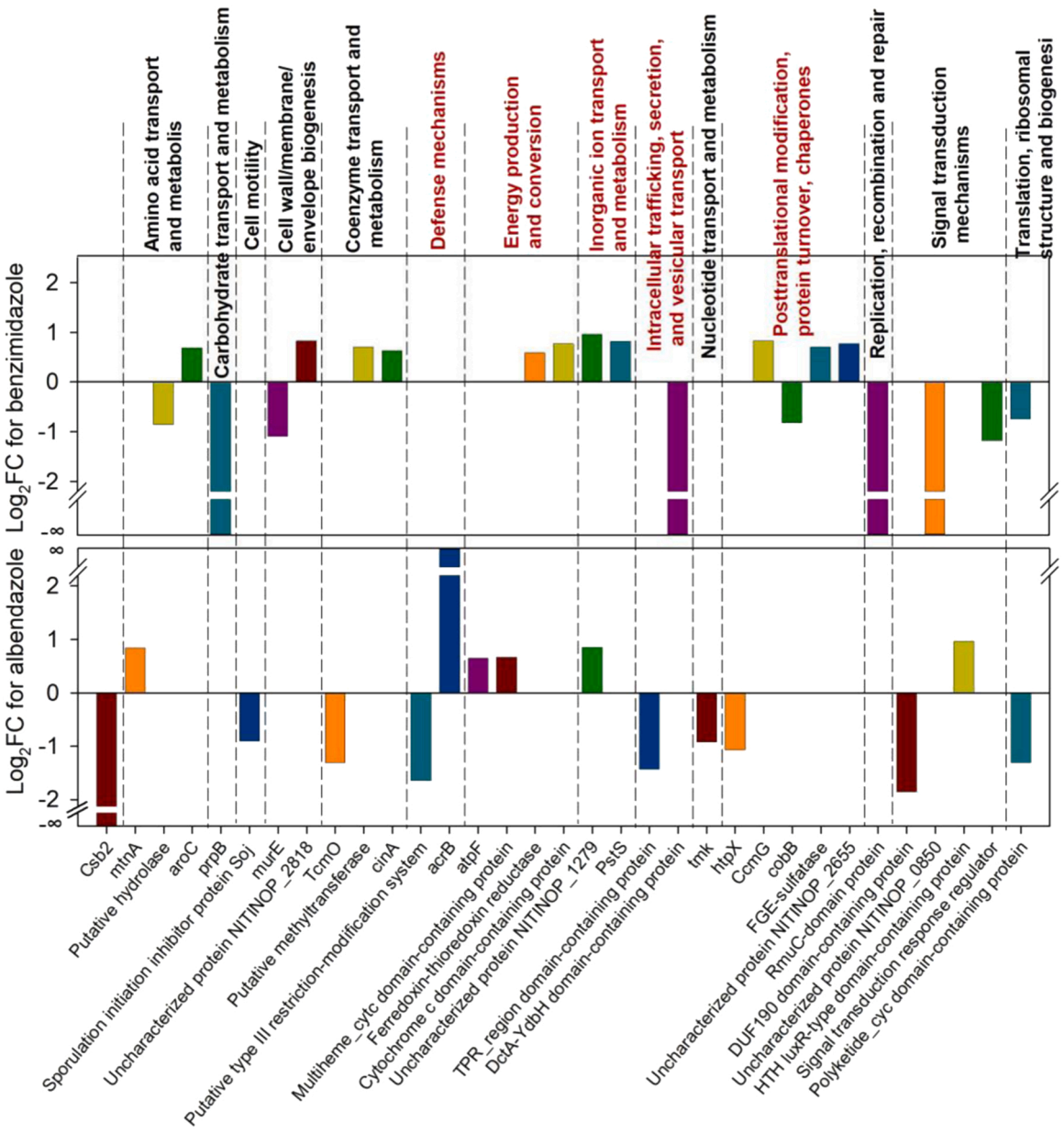

Fig. 5. Proteomic response of *N. inopinata* to benzimidazole fungicide exposure. Significantly changed proteins ($p < 0.05$) in incubations with100 μg/L benzimidazole (top) or albendazole (bottom) compared to control incubations without micropollutant addition.

benzimidazole treatment (one protein lower and three higher expressed; Fig. 5). Two proteins belonging to the COG class of inorganic ion transport and metabolism were more expressed in the benzimidazole treatment, one of which was also upregulated in the albendazole treatment (Fig. 5).

Notably, after exposure to benzimidazole and albendazole, acriflavine resistance protein multidrug efflux pump subunit AcrB was upregulated (Fig. 5). The results indicate that albendazole and benzimidazole exerted toxicity on *N. inopinata*, hence induced the overexpression of an toxin efflux pump to excrete the compounds out of the cell. The overexpression of this efflux pump by benzimidazole fungicides could also promote antibiotic resistance of the comammox bacteria. In a previous study, the exposure to pesticides (including many fungicides, at μg/L level) together with a subinhibitory level of ampicillin synergistically stimulated ampicillin resistance and the cross-resistance to other antibiotics (Xing et al., 2020). Furthermore, the presence of non-antibiotic organic compounds have been previously linked to an enhanced transmission of antibiotic resistance genes (ARGs), including *acrB*, in activated sludge (Jiao et al., 2017; Wang et al., 2020).

Although benzimidazoles affected energy metabolism, translation, transport and antibiotic resistance in *N. inopinata*, no proteins linked to biotransformation mechanisms of such micropollutants like AMO were differentially expressed after fungicide addition, which was consistent with a previous study (Men et al., 2016) and suggested a cometabolic transformation. The concentrations of added fungicides (0.38–0.8 μM) were much lower than the primary substrate ammonium (2 mM), thus they may not be able to alter the expression of the abundant primary pathways. Furthermore, AMO expression might not be regulated by the presence of benzimidazoles since the cell does not get energy out of their hydroxylation. In a previous study with the AOB *N. eutropha* C91, the expression of enzymes of the tricarboxylic acid (TCA) cycle and proteins related to xenobiotic degradation were stimulated by the presence of *p*-cresol, indicating that *N. eutropha* C91 metabolized the formed TPs via the TCA cycle (Kjeldal et al., 2014). The lack of differential expression of xenobiotic degradation pathways in our experiments indicates that

cometabolism could be the biotransformation mechanism of fungicides by comammox, the same as concluded for other micropollutants in several previous studies on ammonia-oxidizers (Han et al., 2019; Men et al., 2016; Zhou et al., 2019, 2021).

3.4. Environmental implications

An important removal route for micropollutants that been frequently found is their biodegradation in nitrifying systems, including natural ecosystems and engineered systems (Daims et al., 2015; Liu et al., 2020; Zhao et al., 2020). Comammox bacteria are occurring in those nitrifying environments as commonly as AOA, AOB, and NOB. However, only few studies on the conversion of micropollutants by comammox pure cultures have been reported so far (Han et al., 2019). Unraveling new ecological roles played by this specific group of autotrophic nitrifying microorganisms is crucial for a better understanding of the environmental fate of micropollutants. This comprehensive study revealed that comammox bacteria were able to convert benzimidazole fungicides, which were not biotransformed by AOB and AOA, although AOB and AOA species showed high biotransformation capabilities for various other micropollutants in previous studies (Men et al., 2016; Yu et al., 2018; Zhou et al., 2019, 2021). This distinct feature of comammox bacteria implies an overlooking removal route of the commonly consumed benzimidazole fungicides in the receiving environments. Moreover, the environmental removal of benzimidazole fungicides could be specifically dependent on and correlated with the environmental activity of comammox bacteria. The specific binding and effective transformation of the benzimidazole structure also warrant further mechanistic investigation in future studies. In addition, the effect of benzimidazoles on comammox bacteria by stimulating the expression of a non-specific antibiotic resistant efflux pump could also be imposed to other bacteria in the environment. More caution would be needed for environments containing benzimidazole fungicides if these fungicides were further proved to be general antibiotic resistance stressors by future studies.

4. Conclusions

In this study we assessed the biotransformation potential for five benzimidazole fungicides by five distinct nitrifying microorganisms (the AOB *Nitrosomonas nitrosa*; the AOA *Nitrososphaera gargensis*; the NOB *Nitrospira moscoviensis*; and the two comammox species *Nitrospira inopinata* and *Ca.* Nitrospira kreftii). We have observed that i) four benzimidazole fungicides (benzimidazole, albendazole, carbendazim, and fuberidazole) were transformed in significant quantities (>20%) by the two comammox species, while AOA and AOB did not transform any benzimidazole fungicide; ii) thiabendazole was not biotransformed by any nitrifier and iii) the group substitutions at R1 and R2 of the benzimidazole rings determine the biotransformation efficiency by both comammox bacteria. Furthermore, benzimidazoles had higher biotransformation efficiencies and rates when ammonium was the growth substrate. The only exception was albendazole transformation by *Ca.* N. kreftii, which had a higher rate when hydroxylamine was added as substrate. The transformation products were mainly formed via hydroxylation on the aromatic ring, which might be catalyzed by the ammonia monooxygenase. Benzimidazole or albendazole induced minor changes in the proteome of *N. inopinata*. However, none of these changes were related to the metabolic degradation of aromatic compounds, so co-metabolism apparently is the primary pathway of biodegradation. An acriflavine resistance protein was upregulated, indicating that benzimidazoles might be expelled from the cell. Further research is required to assess the exact effect biocides exert on ammonia-oxidizing microorganisms and how they might affect the global nitrogen cycle.

CRediT authorship contribution statement

Ping Han: Conceptualization, Investigation, Formal analysis, Data Curation; Writing - Review & Editing; **Ana B. Rios-Migueld:** Sample collection, Formal analysis, Visualization, Writing - Review & Editing; **Xiufeng Tang:** Investigation, Formal analysis, Data Curation; **Yaochun Yu:** Conceptualization, Investigation, Data Curation; **Li-Jun Zhou:** Investigation, Formal analysis, Writing - Review & Editing; **Lijun Hou:** Conceptualization, Investigation; **Min Liu:** Conceptualization, Investigation; **Dongyao Sun:** Investigation, Data Curation; **Mike S.M. Jetten:** Conceptualization, Writing - Review & Editing; **Cornelia U. Welted:** Conceptualization, Writing - Review & Editing; **Yujie Men:** Conceptualization, Investigation, Formal analysis, Writing - Review & Editing; **Sebastian Lückerd:** Conceptualization, Investigation, Writing - Review & Editing.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

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Environmental implication

The recently characterized comammox bacteria are occurring in natural and engineered nitrifying systems where micropollutants removal had been frequently observed. Unraveling new ecological roles played by this specific nitrifying consortium is crucial to better understand the environmental fate of micropollutants. This comprehensive study revealed that comammox bacteria were able to convert benzimidazole fungicides, which in the same time stimulated the expression of a non-specific antibiotic resistant efflux pump and in contrast were not biotransformed by other nitrifiers. This distinct feature of comammox bacteria implies an overlooking removal route of the commonly consumed benzimidazole fungicides in the receiving environments.

Competing interests

The authors declare no competing financial interests.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2022.130558.

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