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Short Communication

Anammox enrichment culture has unexpected capabilities to biotransform azole contaminants of emerging concern

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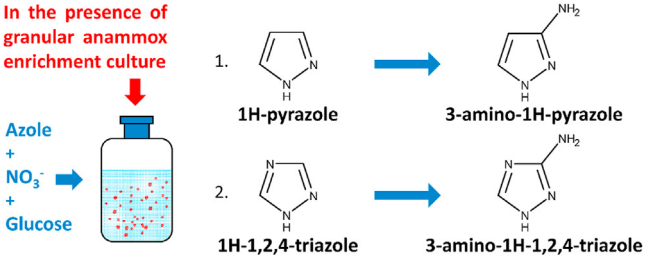
HIGHLIGHTS

- Biotransformation of azoles occurred in an anammox enrichment culture.
- Significant biotransformation of pyrazole and triazole occurred in 6 days.
- Nitrate and glucose promoted the biotransformation process.
- 3-amino-pyrazole and 3-amino-triazole were the biotransformation products.
- High molar product yields were observed for both biotransformation products.

GRAPHICAL ABSTRACT



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ABSTRACT

Azoles are contaminants of emerging concern. They have a ubiquitous presence in the environment due to their wide variety of uses. This study investigated the fate of two commonly occurring azole compounds in an anammox enrichment culture. The results showed that 1H-pyrazole (PA) and 1H-1,2,4-triazole (TA) were biotransformed yielding major biotransformation products, 3-amino-1H-pyrazole and 3-amino-1H-1,2,4-triazole, respectively. Nitrate and glucose greatly stimulated the biotransformation. Under optimized conditions, 80.7% of PA and 16.4% of TA were biotransformed in an incubation period of 6 days. High molar product yield of 84.5% and 83.6% was observed per mole of PA and TA biotransformed, respectively. This novel and selective biotransformation constitutes the first report on the microbial biotransformation of PA and is amongst the very few reports on the biotransformation of TA. This study also provides evidence that anammox enrichments have unexpected capabilities to biotransform organic contaminants of emerging concern.

1. Introduction

1H-Pyrazole (PA) and 1H-1,2,4-triazole (TA) are important azole (nitrogen containing heterocyclic aromatic) compounds. PA and TA have a variety of uses such as corrosion inhibitors and as components of antifungals and other drugs (Dalvie et al., 2002; Karrouchi

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et al., 2018). The presence of these compounds in the environment poses an ecotoxicity and human health risk. Azole-antifungal compounds are known inhibitors of human cytochrome P450 (Dalvie et al., 2002). PA and TA derivatives have been shown to cause degenerative and endocrine disrupting effects in rat and mouse models (Magnusson et al., 1972; Taxvig et al., 2008). PA and TA are known inhibitors of nitrification in soils (McCarty and Bremner, 1989), and wastewater sludge (Li et al., 2020; Pagga et al., 2006).

The treatment of PA, TA and their derivatives has been a challenge. These compounds have high polarity, low volatility and are recalcitrant in nature. Use of physio-chemical methods for the removal of PA and TA derivatives is unfavorable due to the possibility of formation of more toxic intermediates as well as the high cost for treatment (Mandal et al., 2013; Wu et al., 2016). Biological processes can help in overcoming these obstacles. The objective of this study was to investigate the biotransformation of PA and TA in an anammox enrichment culture.

2. Materials and methods

2.1. Anammox enrichment culture (anammox-EC)

Granular anammox biomass was obtained from a 3 L lab-scale anaerobic expanded granular sludge bed (EGSB) reactor. The granules contained 0.81 g of volatile suspended solids (VSS) per gram of total suspended solids, and had a specific anammox activity of 0.85 ± 0.05 g N_2 g^{-1} VSS d^{-1}. A metagenome was constructed from the anammox enrichment culture and is described in the Results and Discussion section below. The data for the metagenomic analysis is provided in the Supporting Information (SI) (SI: Section 2.7).

2.2. Experimental setup

Bioassays were performed in 160 mL serum bottles. The total liquid volume was 100 mL. Bottles were provided with basal medium containing (mg L^{-1}): $NaHPO_4 \cdot H_2O$ (57.5), $CaCl_2 \cdot 2H_2O$ (100), $MgSO_4 \cdot 7H_2O$ (200), $NaHCO_3$ (4000), 1 mL L^{-1} of each of the two trace element solutions (SI: Section 1.1) and the desired concentrations of the test compounds. All assays were inoculated with 0.36 g VSS L^{-1} of the anammox-EC. The liquid and the headspace of the bottles were flushed with the mixture of 20% CO_2 and 80% helium (v/v) for 6 min each. The bottles were sealed using butyl rubber stoppers and aluminum crimp seals. Finally, substrates or other chemicals were added. Following typical values were used throughout the experiments, unless otherwise specified (mM): NH_4^+ (5.41), NO_2^- (7.14), NO_3^- (2), PA (0.37) or TA (0.37), and organic carbon sources (2). All bioassays were incubated in an orbital shaker operating at 115 rpm in a dark environment at 30 ± 2 °C. All experiments were carried out at pH of 7.2 in duplicate. Methods for bioassays for testing anammox activity and killed sludge controls are provided in SI (SI: section 2.3 and 2.4).

2.3. Analytical methods

The concentrations of PA and TA were analyzed using High Performance Liquid Chromatography (HPLC) coupled to a Diode Array Detector (DAD) (1260 Infinity II, Agilent Technologies Inc., Santa Clara, CA, USA) using an Inertsil ODS-SP column (5 μm, 4.6 × 250 mm; C-18 column, GL Sciences, Tokyo, Japan). An isocratic mixture of 10% methanol and 90% water was used as the eluent at a flow rate of 1 mL min^{-1} for a run time of 12 min. The injection volume was 20 μL. The temperature was maintained at 40 °C. PA and TA were detected at 210 and 200 nm, respectively.

The detection of metabolites of the biotransformation process was carried out using Liquid Chromatography - High Resolution Mass Spectrometry (LC-HRMS) using an UltiMate 3000 HPLC (Dionex, Sunnyvale, CA, USA) coupled to an Q Exactive™ Focus Hybrid Quadrupole-Orbitrap™ mass spectrometer (Thermo Scientific, Whaltman, MA, USA). The ionization was carried out using ESI (electrospray ionization) in the positive mode using capillary setting of 2.0 kV. The resolution of the instrument was 70k at a mass range of 50–600 *m/z*. The HPLC method described above was used with slight modifications. An isocratic mixture of 10% methanol and 90% water at a flow rate of 0.3 mL min^{-1} was used as the eluent for a run time of 35 min. The injection volume was 5 μL.

Additional details about the chemicals and analytical methods, including the method for metagenomic analysis, are provided in the SI (SI: Sections 1.1 and 1.2).

3. Results and discussion

3.1. Preliminary experiments leading to the discovery of azole biotransformation

Following our previous study regarding the toxicity of azole compounds towards the anammox bacteria (Lakhey et al., 2020), initial experiments were conducted to test the bioconversion of various azole compounds; 1H-benzotriazole, 5-methyl-1H-benzotriazole, 3,5-dimethyl-1H-pyrazole, PA and TA by the anammox-EC (SI: Section 2.1). In these experiments, azoles were incubated with the anammox-EC in the presence of NH_4^+ and NO_2^- (anammox substrates). Out of all these azoles tested, bioconversion as evidenced by the loss of the parent compound and formation of biotransformation metabolites was only observed with PA and TA. No disappearance of PA and TA occurred in the control experiments without anammox-EC (abiotic controls) (SI: Section 2.2). The disappearance of PA and TA indicated a potential biotransformation process. Killed biomass experiments were conducted to confirm that the disappearance of PA and TA was not due to bio-sorption (SI: Section 2.3). Further experiments were carried out to determine the culture conditions promoting PA and TA biotransformation.

3.2. Identifying essential inorganic and organic substrates

Essential inorganic substrates for the biotransformation process were determined by incubating the anammox-EC individually with the following substrate combinations: no substrates, NH_4^+ only, NO_2^- only, NO_3^- only, and anammox substrates (NH_4^+ and NO_2^-) (Fig. 1-A and 1-D). Only marginal disappearance of PA concentrations occurred in the presence of no substrates, NH_4^+ only or NO_2^- only treatments. Noteworthy disappearance of PA or TA and significant increase in the bioconversion rates (SI: Fig. S3, Section 2.5) occurred in presence of either NO_3^- or the anammox substrates (NH_4^+ and NO_2^-). Since NO_3^- is a byproduct of the anammox reaction (Strous et al., 1998) (Equation (1)); NO_3^- was potentially the essential co-substrate required to drive the bioconversion of PA and TA as opposed to the anammox substrates.

$$NH_4^+ + 1.32\ NO_2^- + 0.066\ HCO_3^- + 0.13\ H^+ \rightarrow 0.066\ CH_2O_{0.5}N_{0.15} + 1.02\ N_2 + 0.26\ NO_3^- + 2.03\ H_2O \quad (1)$$

Experiments were also performed to determine if the addition of an organic carbon source such as glucose, pyruvate, acetate, ethanol and methanol would stimulate PA and TA biotransformation (Fig. 1-B and 1-E). In these experiments, anammox-EC was incubated in basal medium containing NH_4^+, NO_2^-, organic carbon sources (2 mM each) and PA or TA. The results showed that compared to the case with no organic electron donors added, the

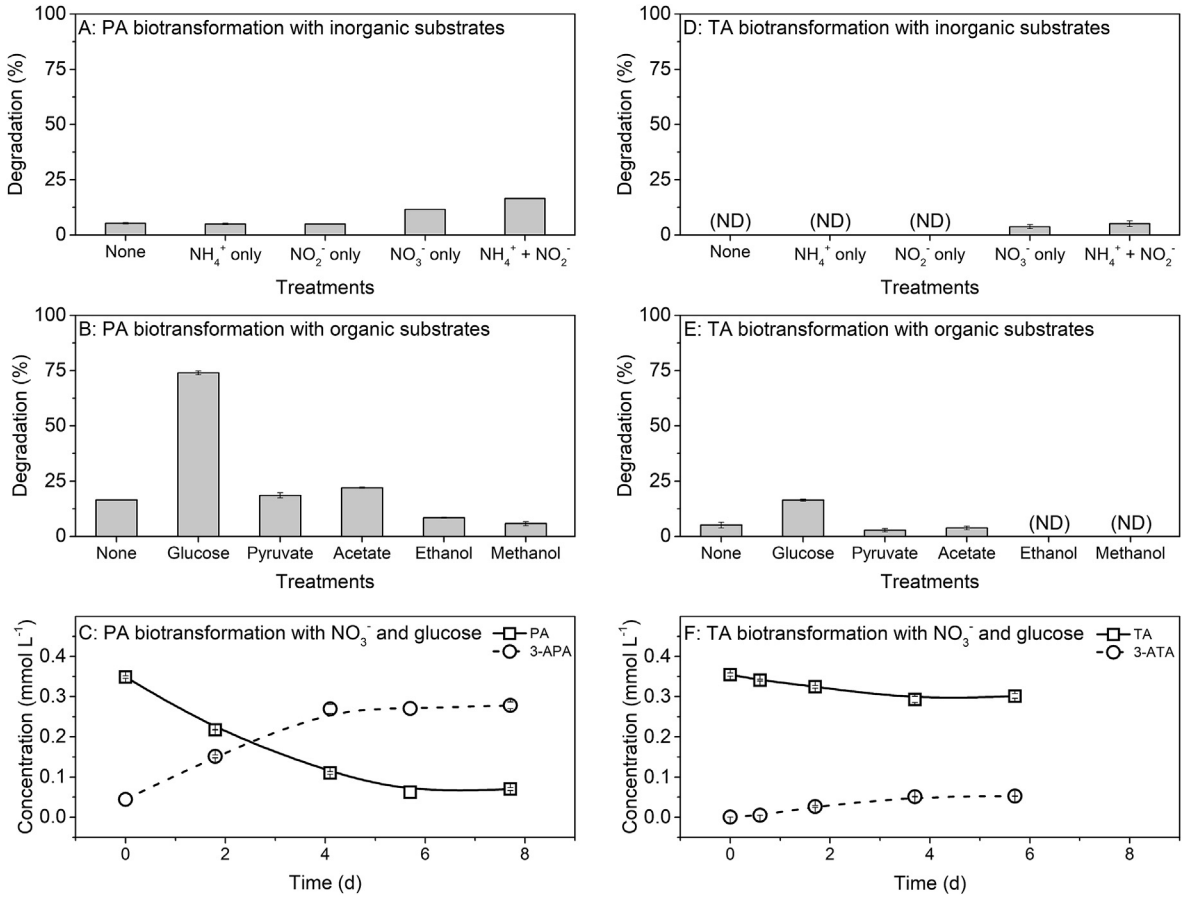

Fig. 1. Substrate scope experiments. All experiments consisted of anammox enrichment culture (0.36 $gVSS^{-1}$ L^{-1}) inoculated in basal medium containing 1H-pyrazole (0.37 mM) or 1H-1,2,4-triazole (0.37 mM), and substrates (as specified). **(1-A, 1-D):** Effect of various inorganic substrate combinations on biotransformation extent (%) of 1H-pyrazole (1-A) and 1H-1,2,4-triazole (1-D). The inorganic substrates were provided as shown in the Fig. 1-A and 1-D at the concentrations of NH_4^+ (5.41 mM), NO_2^- (7.14 mM) and NO_3^- (2 mM). Treatments for which no significant biotransformation was detected are denoted as ND. **(1-B, 1-E):** Effect of various organic substrate combinations on the biotransformation extent (%) of 1H-pyrazole (1-B) and 1H-1,2,4-triazole (1-E). All inoculations were provided with NH_4^+ (5.41 mM) and NO_2^- (7.14 mM). The organic substrates: Glucose, pyruvate, acetate, ethanol and methanol were provided at a concentration of 2 mM each. **(1-C, 1-F):** Time course of 1H-pyrazole (PA) (1-C) and 1H-1,2,4-triazole (TA) (1-F) biotransformation along with the formation of their respective biotransformation products, as 3-amino-1H-pyrazole (3-APA) and 3-amino-1H-1,2,4-triazole (3-ATA), in inoculations containing NO_3^- (2 mM) and glucose (2 mM).

presence of glucose greatly promoted the biotransformation of PA and TA. Acetate also promoted the biotransformation of PA, but the effect was mild. Pyruvate did not promote the biotransformation process whereas ethanol and methanol severely inhibited the PA and TA biotransformation. The results suggested that glucose was the most effective carbon source in promoting PA and TA biotransformation.

3.3. Role of the microbial community in the biotransformation process

The potential role of NO_3^- could point towards a non-conventional anammox metabolic process. The anammox bacteria have been reported to respire using NO_3^- as the terminal electron acceptor when oxidizing simple organic acids (Guven et al., 2005). Alternatively, other bacteria (heterotrophic and denitrifying bacteria) coexisting in the anammox enrichment culture (Zhao et al., 2018) may be responsible for the biotransformation process. Metagenomic analysis was performed to elucidate the microbial community structure in the anammox-EC. During the metagenomic analyses, a total of 17.9 million reads were sequenced and 25 Metagenome Assembled Genomes (MAGs) were found in the constructed metagenome (SI: Tables S1 and S2). One MAG was classified as belonging to the anammox bacterium *Brocadia caroliniensis*. This was the most well-represented in the metagenome, with 26.83% of total reads mapped to it. The remaining 24 MAGS only had from 7.72% to 0.25% reads mapped to each of them, attesting to the fact that the metagenome was enriched with the anammox bacterium. Percent of total reads and GTDBTk classification of MAGs are provided in the SI (SI: Section 2.7).

A possible role of organic carbon sources does not necessarily exclude the anammox bacteria since they are known to metabolize acetate and propionate (Guven et al., 2005). Further analysis was performed to identify the presence of genes involved in glucose metabolism from the MAG recovered in this study, for the anammox bacteria *Brocadia carolinensis*. 12 genes matched to 11 unique clusters of orthologous groups (COGs) that are involved in glucose transport and metabolism, including the ATP binding cassette (ABC)-type transport systems. An additional 18 genes matched to 11 unique COGs associated with cell wall/membrane/envelope biogenesis from glucose. (SI: Tables S3 and S4). These genes provide evidence for the potential ability for uptake and metabolism of glucose by Brocadia. Hence, uptake of glucose by the anammox bacteria is in fact, conceivable. However, to-date, experimental evidence has proved that it is unclear if anammox bacteria are able to metabolize glucose and N-substituted sugars.

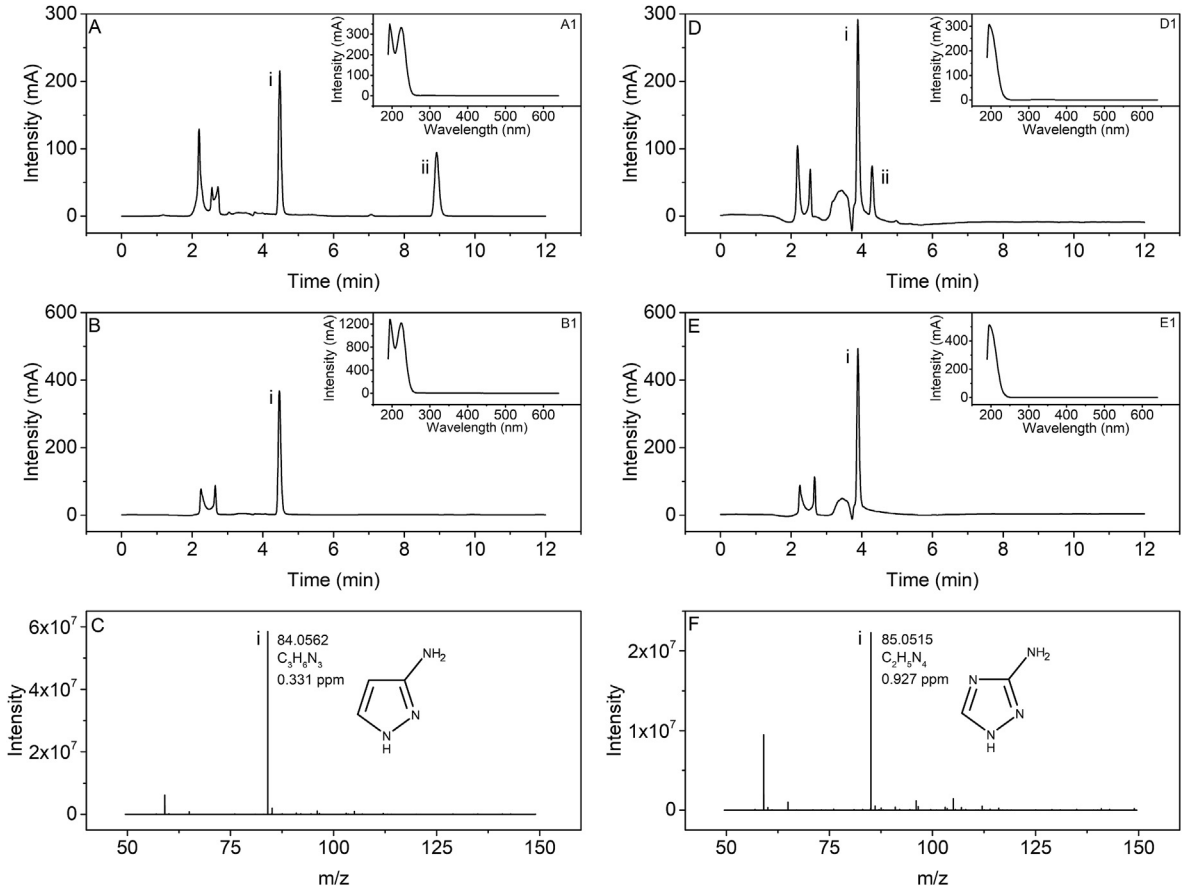

Fig. 2. Characterization of the major biotransformation products of 1H-pyrazole and 1H-1,2,4-triazole biotransformation. Analysis of treatments where anammox enrichment culture (0.36 g VSS L^{-1}) was inoculated in basal medium consisting of 1H-pyrazole (0.37 mM) or 1H-1,2,4-triazole (0.37 mM), NO_3^- (2 mM) and glucose (2 mM). **(2-A, 2-D):** HPLC chromatographs of liquid sampled at the end of 6 days of incubation with 1H-pyrazole (2-A) and 1H-1,2,4-triazole (2-D). In both cases peak (i) is the major biotransformation product whereas peak (ii) is 1H-pyrazole or 1H-1,2,4-triazole. The inset Fig. 2-A1 and 2-D1, are the respective UV–vis spectra for the major biotransformation products. **(2-B, 2-E):** HPLC chromatographs for authentic standards of 3-amino-1H-pyrazole (2-B) and 3-amino-1H-1,2,4-triazole (2-E) and their respective UV–vis spectra shown in inset Fig. 2-B1 and 2-E1. **(2-C, 2-F):** Mass spectra for the major biotransformation product of 1H-pyrazole (2-C) and 1H-1,2,4-triazole (2-F).

Alternatively, bacteria of the phylum Chloroflexota, which commonly coexist in anammox enrichments (Zhao et al., 2018; Kindaichi et al., 2012; Gonzalez-Gil et al., 2015), have been shown to consume glucose and N-acetyl-glucosamine under anoxic conditions (Kindaichi et al., 2012). The occurrence of Chloroflexota in the anammox-EC was also shown by our metagenomic analysis. Six MAGs in the metagenomic assembly were identified as belonging to the class Anaerolineae of the phylum Chloroflexota with a cumulative of 28.5% of the total reads mapped to them. Chloroflexota are hypothesized to coexist in anammox enrichments by consuming cellular byproducts (Kindaichi et al., 2012).

As polysaccharides are an important component of the extracellular polymeric substances (EPS) produced by anammox enrichments (Lotti et al., 2019); it can be hypothesized that the slow biotransformation of PA in the absence of glucose (Fig. 1-A) may be attributable to the sugars present in the polysaccharide-rich EPS.

3.4. Identification of metabolites

During all the experiments, the formation of major unknown biotransformation products was detected by HPLC-DAD analysis for both PA and TA. The formation of unknown metabolites was also evident by identifying unique masses generated over the course of incubation period using LC-HRMS analysis. Only one prominent biotransformation metabolite was detected each, in the PA and TA incubations consisting of anammox-EC, NO_3^- and glucose. The metabolites were detected with both the HPLC-DAD and LC-HRMS. The chemical formula for each of the suspected metabolites was estimated based on their respective accurate masses with the lowest error. The PA biotransformation metabolite had a protonated $[M+H]^+$ mass of 84.0562, corresponding to the molecular formula, $C_3H_6N_3$ with an error of 0.331 ppm (Fig. 2-C). Similarly, the mass $[M+H]^+$ of the TA metabolite was 85.0515, corresponding to the molecular formula, $C_2H_5N_4$ with an error of 0.927 ppm (Fig. 2-F) (calculated using ChemCalc (Patiny and Borel, 2013)). Both chemical formulas suggested the formation of amino derivatives of PA and TA. Hence, to determine the exact structure of the compounds, commercially available authentic standards of several amino derivatives of PA and TA were evaluated based on comparing the retention time (HPLC) and UV–vis spectra (HPLC-DAD) (Fig. 2-A, 2-B, 2-D, and 2-E). The major products for the biotransformation of PA and TA were identified as 3-amino-1H-pyrazole (3-APA) and 3-amino-1H-1,2,4-triazole (3-ATA), respectively.

3.5. Conversion of PA and TA and the fate of the substrates during incubation

The treatment supplemented with 2 mM glucose and 2 mM NO_3^- (Fig. 1-C and 1-F) was found to be ideal for studying the conversion of PA and TA to their respective biotransformation products as well

as evaluating the fate of NO_3^- and glucose during the incubations. Under these optimum conditions, the maximum biotransformation rate (initial rate) for PA and TA was 0.20 and 0.064 mmol g^{-1} VSS d^{-1}, respectively. The maximum biotransformation extent of PA and TA achieved in 6 days was 80.7% and 16.4%, respectively. The product yield (over the first 2 days) of PA to 3-APA was 84.5% and similarly for TA to 3-ATA was 83.6%. Loss in the glucose and NO_3^- concentration was observed (SI: Section 2.6, Fig. S4 and S5). Gas chromatography measurements indicated no significant production of N_2 gas (SI: Fig. S4). As mentioned before, apart from the major metabolites no other metabolites were detected using the HPLC-DAD and LC-HRMS methods.

3.6. Implications

The biotransformation of PA and TA by the anammox-EC is a unique process. To the best of our knowledge, this study constitutes the first report on the microbial biotransformation of PA and is amongst the very few reports on the microbial biotransformation of TA. The biodegradation of TA by bacteria belonging to *Shinella* sp. has been studied previously (Wu et al., 2016) and an array of TA biotransformation products were detected by LC-MS and GC-MS. However, the formation of amino derivative products of PA and TA is a unique type of biotransformation that has not been reported previously. The formation of 3-ATA, otherwise known as the herbicide, 'amitrole', may be a cause for concern. Chronic exposure to 3-ATA is known to cause reproductive defects and carcinogenic effects as shown in animal and human studies (Amitrole: Reregistration, 1996). The US-EPA has categorized 3-ATA as a probable human carcinogen (Amitrole: Reregistration, 1996). The biotransformation of 3-ATA has been studied before. 3-ATA was shown to be degraded by soil microorganisms under aerobic conditions in incubations of several weeks (Oesterreich et al., 1999). Less is known about the ecotoxicity of 3-APA. Further research needs to be done towards finding the microorganism(s) responsible for the biotransformation process. The possibility of amination of PA and TA (like) moieties is an important biotransformation mechanism that needs further investigation. The results demonstrate a novel and highly selective biotransformation of the recalcitrant compounds PA and TA by the anammox-EC. This study also provides evidence that anammox-EC has unexpected capabilities to biotransform organic contaminants of emerging concern.

Credit author statement

Nivrutti Lakhey: Conceptualization, Investigation, Writing - original draft, Reyes Sierra-Alvarez: Conceptualization, Supervision, Matthew Brian Couger: Metagenomic analysis, Mark J. Krzmarzick: Metagenomic analysis, Jim A. Field: Conceptualization, Supervision, 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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2020.128550.

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