Chemosphere 167 (2017) 478–484

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Sequential anaerobic-aerobic biodegradation of emerging insensitive munitions compound 3-nitro-1,2,4-triazol-5-one (NTO)

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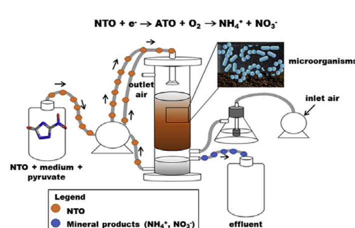
HIGHLIGHTS

- A sequential anaerobic-aerobic approach is proposed for NTO degradation.
- NTO is only biotransformed to ATO under anaerobic conditions.
- ATO is only biodegraded under aerobic conditions.
- Anaerobic microniches in aerobic environments support NTO biodegradation.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 18 August 2016
Received in revised form
7 October 2016
Accepted 10 October 2016
Available online 15 October 2016

Handling Editor: A Adalberto Noyola

Keywords:
3-Nitro-1,2,4-triazol-5-one
3-Amino-1,2,4-triazol-5-one
Biodegradation
Insensitive munitions
Soil

ABSTRACT

Insensitive munitions, such as 3-nitro-1,2,4-triazol-5-one (NTO), are being considered by the U.S. Army as replacements for conventional explosives. Environmental emissions of NTO are expected to increase as its use becomes widespread; but only a few studies have considered the remediation of NTO-contaminated sites. In this study, sequential anaerobic-aerobic biodegradation of NTO was investigated in bioreactors using soil as inoculum. Batch bioassays confirmed microbial reduction of NTO under anaerobic conditions to 3-amino-1,2,4-triazol-5-one (ATO) using pyruvate as electron-donating cosubstrate. However, ATO biodegradation was only observed after the redox condition was switched to aerobic. This study also demonstrated that the high-rate removal of NTO in contaminated water can be attained in a continuous-flow aerated bioreactor. The reactor was first fed ATO as sole energy and nitrogen source prior to NTO addition. After few days, ATO was removed in a sustained fashion by 100%. When NTO was introduced together with electron-donor (pyruvate), NTO degradation increased progressively, reaching a removal efficiency of 93.5%. Mineralization of NTO was evidenced by the partial release of inorganic nitrogen species in the effluent, and lack of ATO accumulation. A plausible hypothesis for these findings is that NTO reduction occurred in anaerobic zones of the biofilm whereas ATO was mineralized in the bulk aerobic zones of the reactor.

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1. Introduction

Traditional explosives, such as 2,4,6-trinitrotoluene (TNT) and hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), require a low amount of heat or pressure to be detonated, leading to accidental

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http://dx.doi.org/10.1016/j.chemosphere.2016.10.032
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explosions. In order to avoid the occurrence of inadvertent detonations, the United States military is replacing existing explosives by insensitive munitions compounds (IMC), which have similar performance, but are more stable than the traditional munitions (Sikder and Sikder, 2004; Smith and Cliff, 1999). The nitroheterocyclic compound 3-nitro-1,2,4-triazol-5-one (NTO) is one of the main ingredients of the insensitive explosives formulations. NTO has shown to have attractive properties as a replacement for traditional explosives such as RDX (Lasota, 2013; Singh and Felix, 2002), and as its use becomes widespread, environmental release of NTO is expected to increase. However, information about the environmental fate and toxicity of NTO is still limited. Concern about the potential environmental impact of NTO is compounded by the high mobility of this chemical. Unlike many other contaminants in groundwater, NTO does not readily sorb to soil surfaces, and it is highly soluble in water (12.8–16.6 g L^{-1} at 19 °C) (Smith and Cliff, 1999; Spear et al., 1989). Measurements of the dissolution of different IMC formulations showed that those containing NTO are likely to reach the groundwater in the absence of biotransformation (Taylor et al., 2015).

Research is being developed in order to assess NTO toxicity. In contrast with TNT, which is well known for its mutagenic potential (Kennel et al., 2000), NTO has been shown to be negative in a variety of genotoxicity and mutagenicity assays (Kirby, 2008; Song, 2008a, 2008b). The subacute and subchronic toxicity of NTO to rats has been evaluated by Crouse et al. (2015). Male adult rats receiving a dose of 1000 mg kg^{-1} body wt d^{-1} NTO or greater during 14 days exhibited decreased food consumption and body mass. Furthermore, NTO affected the reproductive system of the male rats in the high-dose group, causing histological changes and no motile sperm. A few recent studies have reported on the aquatic toxicity of NTO. In a chronic toxicity assay performed with leopard frog tadpoles, the lowest observed effect concentration (LOEC) on the tadpoles swimming distance for NTO was 5 mg L^{-1}, while the LOEC value for TNT was 0.003 mg L^{-1} (Stanley et al., 2015). In contrast, NTO was found to exert very low toxicity in growth inhibition assays with the freshwater organism *Cerodaphnia dubia* (50% inhibitory concentration after 48 h (48-h IC50) = 460 mg L^{-1}) and the unicellular green algae *Selenastrum capricornutum* (LOEC = 2680 mg L^{-1}) (Haley et al., 2009). Although these results suggest that NTO is less toxic than traditional explosives, only a limited number of organisms have been tested to date. In this regard it is worth noting that the explosive compound RDX was found to have very different responses depending on the organism tested (Khan et al., 2012), including RDX-induced convulsion in humans (Woody et al., 1986).

Biotic (Le Campion et al., 1998 ; Le Campion et al., 1999a; Krzmarzick et al., 2015; Richard and Weidhaas, 2014) and abiotic (Le Campion et al., 1999b; Linker et al., 2015) degradation of NTO has been evaluated in a few recent studies. A bacterial strain, *Bacillus licheniformis,* was used to degrade NTO in industrial wastewater (Le Campion et al., 1999a). Firstly, NTO was biotransformed through the reduction of the nitro-group to 3-amino-1,2,4-triazol-5-one (ATO) at pH 6 in the presence of 15 g L^{-1} sucrose as electron donor. Thereafter, ATO was further degraded 40% to mineral products at pH 8, without any additional supply of electron donor. The culture media contained a metabolite identified as urea, and hydroxyurea was a putative assignment for a second metabolite. NTO and ATO biodegradability was also tested using soil microorganisms (Krzmarzick et al., 2015). NTO was found to be reduced to ATO under anaerobic conditions with a wide variety of electron-donating substrates. However, ATO was only further metabolized under aerobic conditions, as evidenced by release of inorganic nitrogen. These results suggest the need of an anaerobic step to activate the molecule for aerobic bioremediation.

The objective of this study is to evaluate NTO and ATO degradation in a sequential anaerobic-aerobic batch reactor and in a continuous-flow reactor using soil microorganisms as inoculum.

2. Material and methods

2.1. Materials

NTO (purity > 95%) was purchased from Interchem (San Pedro, CA, USA) and ATO (purity > 95%) from Princeton BioMolecular Research (Monmouth Junction, NJ, USA). The soil used in this study was collected from the garden of Gortner Laboratory, on the University of Minnesota campus (St. Paul, MN, USA). The soil was stored at 4 °C in a sealed container. The texture of the mineral matter of the soil consisted of 9.8% clay, 38.9% silt and 51.3% sand, determined by a fully automated Beckman Coulter LS 13 320 laser diffraction particle size analyzer (Fullerton, CA, USA) after removal of the organic matter. The pH was 6.92, the water content was 6.2%, and the soil total organic carbon (SOC) determined by loss on ignition was 24.1% (Storer, 1984).

2.2. Biodegradation assays

2.2.1. Batch bioassays

NTO biotransformation was evaluated under only anaerobic, only aerobic, and sequential anaerobic-aerobic conditions. The bioassays were conducted in 160 mL serum bottles with butyl rubber stoppers and aluminum seals containing 400 mg of soil (wet weight) in 40 mL of basal medium. The mineral medium was composed of (in mg L^{-1}) $MgSO_4 \cdot 7H_2O$ (90), $CaCl_2 \cdot 2H_2O$ (15), yeast extract (1), NaCl (20), NTO (195), KH_2PO_4 (796), K_2HPO_4 (1594), and 0.2 mL L^{-1} of a trace elements solution. Sodium pyruvate was also added to the solution as a co-substrate (298 mg L^{-1}). However, the aerobic batch assays did not contain sodium pyruvate and yeast extract. The composition of the trace elements solution (in mg L^{-1}) was: H_3BO_3 (50), $FeCl_2 \cdot 4H_2O$ (2000), $ZnCl_2$ (50), $MnCl_2 \cdot 4H_2O$ (50), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (50), $AlCl_3 \cdot 6H_2O$ (90), $CoCl_2 \cdot 6H_2O$ (2000), $NiCl_2 \cdot 6H_2O$ (50), $CuCl_2 \cdot 2H_2O$ (30), $NaSeO_3 \cdot 5H_2O$ (100), EDTA (1000), and 1 mL L^{-1} of 36% HCl. The pH of the medium was adjusted to 7.0 using NaOH. The headspace of each bottle was flushed according to the treatment. For the anaerobic assays, the bottles were flushed with helium. For the aerobic assays, the bottles were flushed with helium and then 20% of the volume was replaced by pure oxygen at day 0. On day 14, the headspace of these bottles was flushed with a gas mixture containing 61% He, 19% CO_2 and 20% O_2. In the sequential anaerobic–aerobic treatment, the bottles were initially flushed with helium and, after 14 days, the headspace of all bottles was flushed with the same gas mixture of He, CO_2 and O_2. This experiment included two killed control, one in which the soil was autoclaved for 50 min at 121 °C for 3 consecutive days, and one in which the soil was sterilized with 6 mL of a solution of 6% formaldehyde (v/v) for 18 h; a control containing mineral medium and NTO (without soil and co-substrate); an endogenous control, which did not contain NTO; and a control including soil and NTO, but without sodium pyruvate and yeast extract. The bottles were incubated in the dark at 30 °C at 150 rpm. All the assays were performed in duplicate.

Samples were diluted in demineralized water (1:4) and clarified by centrifugation. The supernatant was analyzed for NTO and ATO derivatives, and for inorganic nitrogen species (*i.e*., ammonium, nitrate and nitrite).

2.2.2. Continuous flow reactor

The degradation of ATO and NTO was studied in a continuous flow reactor using 54 g of soil as inoculum. The reactor was made of

acrylic material, and its volume was 350 mL (diameter of 5 cm and column height of 17.8 cm). Approximately 50% of this volume was filled with 1 cm^3 polyurethane sponge blocks (Fig. 1). The reactor was covered with aluminum foil to avoid exposure to light. Humid air was supplied by an air compressor at the bottom of the reactor at a flow rate of approximately 400 cm^3 min^{-1} during all the operation periods. The influent was supplied at the top of the reactor using a peristaltic pump. The influent (pH 7.4) contained the following chemicals (in mg L^{-1}): $MgSO_4 \cdot 7H_2O$ (20), $CaCl_2 \cdot 2H_2O$ (10), $MgCl_2 \cdot 6H_2O$ (20), yeast extract (4), KH_2PO_4 (154), K_2HPO_4 (673), and 0.2 mL L^{-1} of a trace elements solution (previously described). Initially, the medium did not contain ATO. On day 13, the medium was supplemented with ATO (0.6 mM). On day 76, the concentration of ATO was increased to 1.0 mM, and this concentration was maintained during the next 152 days. After day 228, ATO was replaced by NTO (1.0 mM) and sodium pyruvate (1.8 mM, as an electron donor to drive NTO reduction). The reactor was maintained at 22 ± 2 °C and it was operated at an empty bed hydraulic retention time (HRT) of 42 h during most part of the experiment. The reactor contained a recirculation system, and its flow rate was adjusted to 720 mL day^{-1}. The operation of the reactor was divided in four periods (I, IIa, IIb and III), as described in Table 1.

2.2.3. Analytical methods

The concentration of NTO and ATO was analyzed using an Agilent 1200 series high performance liquid chromatograph fitted with a diode array detector (HPLC-DAD, Santa Clara, CA, USA) and a Hypercarb column (50 mm × 4.6 mm, pore size: 5 μm, Thermo Scientific, Waltham, MA, USA). The mobile phase consisted of a gradient of water with 0.1% TFA and acetonitrile according to the following ratio: 0–3 min: 100%/0%, 3–11 min: 85%/15%, 11–17 min: 50%/50%, and 17–20 min: 100%/0%. The retention time of NTO and ATO were 15.0 and 8.9 min, respectively. NTO and ATO peaks were analyzed at 216.5 and 300 nm, respectively.

An ion chromatography (IC) equipped with an ICS-3000 system (Dionex, Sunnyvale, CA, USA) was used to measure the concentration of inorganic nitrogen species. Nitrate and nitrite were analyzed using a Dionex AG18 RFIC column (4 × 250 mm) and KOH (15 mM) as eluent at a flow rate of 1 mL min^{-1}. Ammonium was analyzed using a Dionex IonPac CG16 RFIC column (3 × 250 mm) and methane sulfonic acid (23 mM) as the eluent at flow rate of 0.5 mL min^{-1}. The percentage of nitrogen mineralization was calculated from the difference of the concentrations of total inorganic nitrogen ions in the effluent and influent divided by four times the ATO concentration.

The metabolites formed from ATO degradation were analyzed in a liquid chromatography in tandem with time of flight mass spectrometry (LC-MS/ToF). The samples were injected into a TripleTOFTM 5600 (AB Sciex Instruments, Framingham, MA, USA), and the mobile phase consisted of water with 0.1% TFA and ACN, as previously described for the HPLC analysis. The spectra were obtained using DuoSpray Ion Source (DIS) in both positive and negative mode with a source temperature of 500 °C, and a mass range of 35–1000 *m/z* was acquired. The software Formula Finder was used to identify the molecular formula correspondent to the mass.

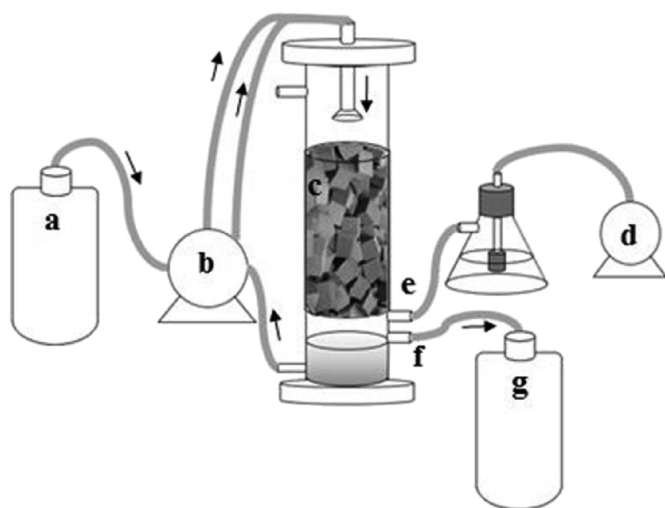

Fig. 1. Scheme of 350 mL aerated bioreactor used for NTO and ATO degradation. Legend: bottle containing influent (**a**), peristaltic pump (**b**), polyurethane foam blocks (**c**), air compressor (**d**), humid air inlet (**e**), effluent sampling port (**f**), and bottle containing effluent (**g**). The arrow indicates the direction of the flow. Influent samples were collected from bottle (**a**) and effluent samples were collected from tube (**f**).

3. Results

3.1. Batch bioassays

NTO biodegradation was tested under different redox regimens. The full removal of NTO was only observed in the sequenced anaerobic-aerobic treatment (Fig. 2, Panel A). Under anaerobic conditions, NTO was completely transformed to ATO within 14 days, and after switching from anaerobic to aerobic conditions, ATO was fully eliminated after a lag phase of approximately 10 days. The complete disappearance of NTO was achieved in 37 days. In the completely anaerobic regimen, all NTO was stoichiometrically converted to ATO. However, ATO was not further metabolized (Fig. 2, Panel B). Under aerobic conditions, the NTO concentration was stable during the entire incubation period (Fig. 2, Panel C). Anaerobic controls without addition of electron donor and yeast extract displayed a slower NTO transformation rate (0.584 mM g^{-1} SOC d^{-1}). After 37 days of incubation, 44% of NTO was biotransformed (Fig. S-4). NTO transformation was also observed in the anaerobic heat killed control, but at a constant and slower rate (0.540 mM g^{-1} SOC d^{-1}) when compared to the live culture (1.543 mM g^{-1} SOC d^{-1}) (Fig. S-1). However, no transformation was observed in the anaerobic control sterilized with formaldehyde (Fig. S-2). In the media only control, the transformation of NTO was not observed in any of the redox regimens (Fig. S-3).

3.2. Continuous flow reactor experiments

3.2.1. Reactor operation with ATO

From day 0 to day 12, the reactor was operated with basal medium. During period IIa, the reactor was fed with 0.6 mM ATO as the only source of carbon and nitrogen. The analysis of the effluent indicates that all the ATO was removed during this period, since the compound was not detected in the effluent (Fig. 3). During period IIb, the concentration of ATO in the influent was increased to 1.0 mM. During this period, ATO was also completely removed from the medium.

The concentration of inorganic nitrogen in the influent and in the effluent was also monitored (Fig. 5, Panel A). Previously to ATO addition in the medium (Period I), very low levels of inorganic nitrogen were measured in the effluent (below 0.125 mM). During period IIa, a progressive increase in the concentration of inorganic nitrogen was observed, reaching values usually above 1.0 mM, indicating that nitrogen was being released from ATO metabolism. For period IIb, the average concentration of inorganic nitrogen was about 1.50 mM. However, these values are below the expected

Table 1
Operational conditions and performance of a laboratory-scale continuous flow reactor treating water containing ATO or NTO during different periods.

Period	Compound added	Days	Flow rate [mL day^{-1}]	ATO influent [μM]	NTO influent [μM]	ATO removal efficiency [%]	NTO removal efficiency [%]	Nitrogen mineralization [%]
I	–	13	154.4 ± 17.2	0 ± 0	0 ± 0	–	–	–
IIa	ATO	63	146.3 ± 11.7	649.1 ± 107.1	0 ± 0	100 ± 0.0	–	36.1 ± 21.5
IIb	ATO	152	213.4 ± 48.2	1024.4 ± 54.5	0 ± 0	99.7 ± 1.1	–	37.8 ± 4.5
III[a]	NTO	79	204.2 ± 18.7	48.1 ± 91.3	930.9 ± 100.8	–	93.5 ± 5.6[b]	41.5 ± 5.1

[a] Addition of 1.8 mM sodium pyruvate as electron donor.
[b] Removal efficiency after the reactor reached steady state (day 298–310).

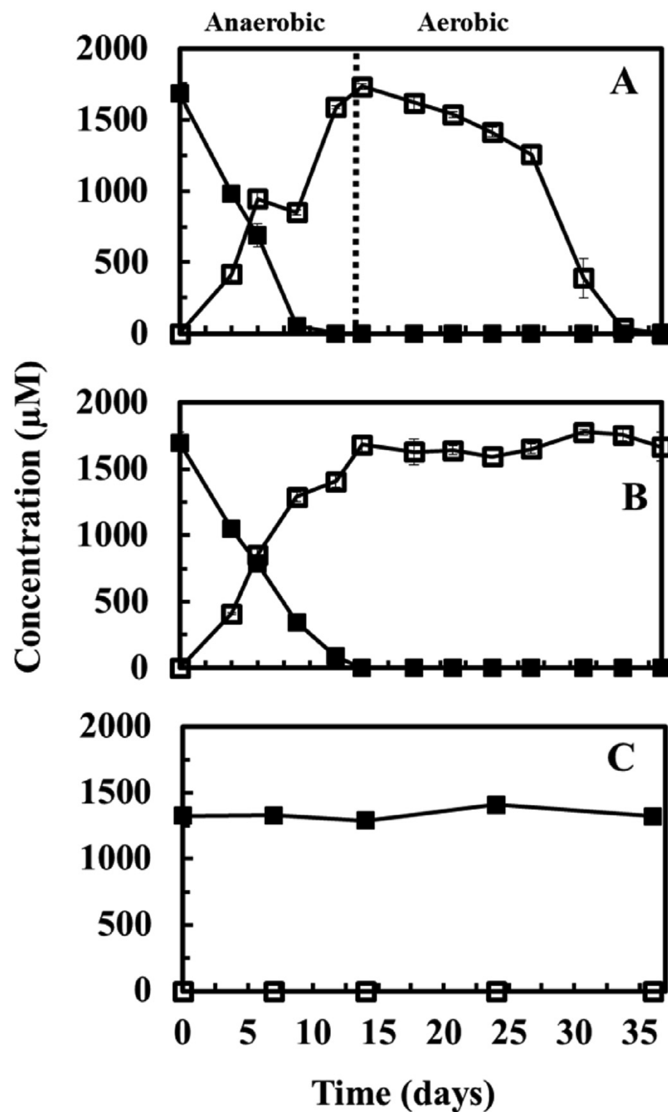

Fig. 2. Bioconversion of NTO under various redox regimens in a soil suspension culture (Gortner soil 10 g L^{-1}) with pyruvate (2.7 mM). The aerobic incubation did not contain pyruvate and yeast extract. **Panel A**: Sequenced anaerobic-aerobic incubation. **Panel B**: Anaerobic incubation. **Panel C**: Aerobic incubation. Legend: NTO live culture (■), ATO live culture (□).

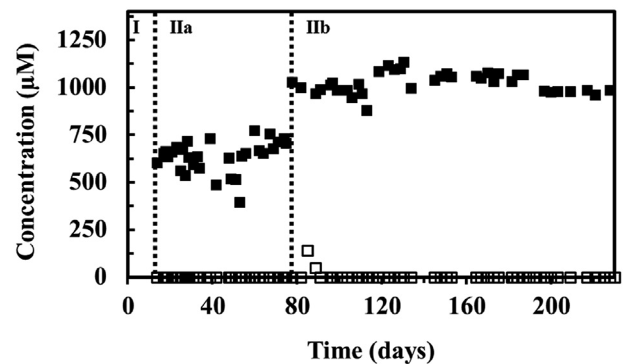

Fig. 3. Disappearance of ATO in aerated continuous flow reactor during periods I (no ATO addition, average flow rate = 154 mL day^{-1}), IIa (600 μM ATO, average flow rate = 146 mL day^{-1}), and IIb (1000 μM ATO, average flow rate = 213 mL day^{-1}). Legend: ATO concentration measured in the influent (■), ATO concentration measured in the effluent (□).

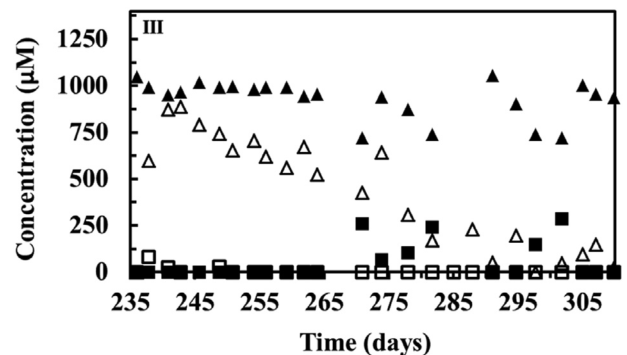

Fig. 4. Disappearance of NTO and ATO in an aerated continuous flow reactor during period III (average flow rate = 204 mL day^{-1}). NTO was added as the target substrate (1000 μM) and sodium pyruvate was added as a cosubstrate (1800 μM). Legend: NTO (▲) and ATO (■) concentration measured in the influent, NTO (Δ) and ATO (□) concentration measured in the effluent.

concentration considering the complete mineralization of ATO, which would release approximately 4 mM inorganic-N. The average percent mineralization of nitrogen was 36.1% and 37.8% for periods IIa and IIb, respectively (Table 1).

3.2.2. Reactor operation with NTO

During period III, the reactor was operated with basal medium containing 1.0 mM NTO and 1.8 mM sodium pyruvate. The concentration of NTO in the influent was relatively lower for certain samples. This variability can be explained by the partial reduction of NTO in the influent container due to the high concentration of pyruvate present in the medium, causing the depletion of oxygen in the bottom of the container. The addition of pyruvate promoted the growth of heterotrophic bacteria, leading to the consumption of oxygen and, as a consequence, creating anaerobic zones in the reactor, while at the same time providing electron donor to drive the reductive transformation of NTO to ATO. The transformation of NTO was not very significant during the first days of period III (Fig. 4). However, the NTO removal efficiency gradually increased with time, and it was close to 94% after 50 days of operation. ATO was not detected in the effluent at significant concentrations

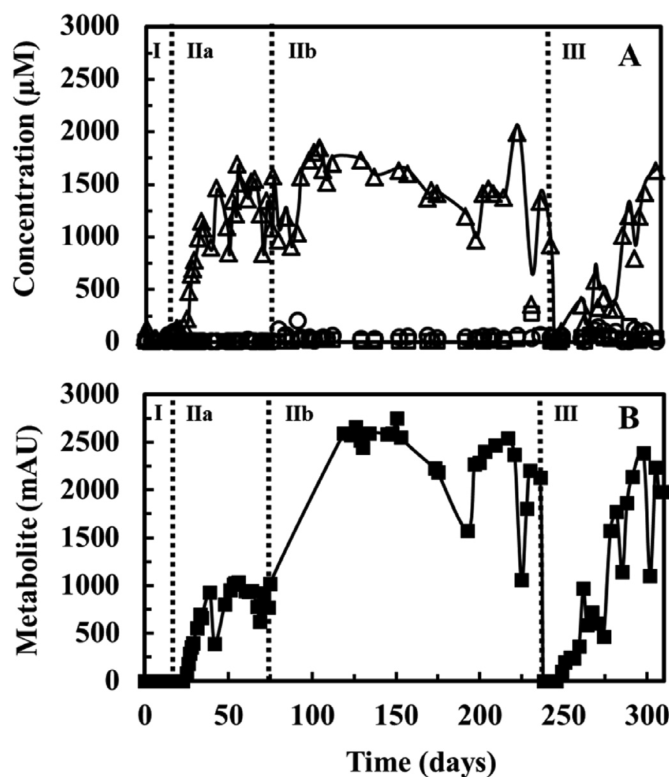

Fig. 5. Mineralization of nitrogen and formation of a metabolite through ATO degradation in an aerated continuous flow reactor. **Panel A**: Concentration of inorganic nitrogen ions in the effluent. **Panel B**: Metabolite peak area measured in the effluent. Legend: Ammonium (○), nitrite (□), and nitrate (Δ) concentration, total nitrogen ions (–) concentration, and metabolite peak area (■) in the effluent.

during the entire period. The lack of ATO in the effluent suggests that ATO yielded from reductive NTO transformation was immediately metabolized (as witnessed in periods IIa and IIb) without any accumulation, which can be viewed as further evidence of ATO metabolism.

The nitrogen mineralization during period III increased with time (Fig. 5, Panel A). When the reactor reached steady state (days 291–310), the average nitrogen mineralization was 41.5%, similarly to the results obtained for the periods in which the reactor was operated with ATO as the sole substrate.

3.2.3. Nitrogen mineralization

As previously stated, only around 40% of the nitrogen was recovered in the effluent, suggesting that while ATO was being removed, a N-containing-metabolite was being formed. HPLC analysis of the effluent showed the presence of a peak at a retention time of 2.9–3.5 min, which was analyzed at the wavelength of 216.5 nm. The metabolite was detected in the effluent consistently during the periods IIa, IIb and III, after NTO started to be metabolized (Fig. 5, Panel B). During the first few weeks after initial ATO addition, ATO disappearance was complete, but N-mineralization was progressively increasing, and this was paralleled with increase in metabolite peak area. In the analysis of samples containing the metabolite in LC-MS/ToF, one compound was identified that correlated with the peak seen in the UV-VIS from the HPLC (Fig. S-5). The unknown metabolite had a charged mass of 107.968 *m/z*, which was correspondent to the molecular formulas HN_3S_2 or C_2H_2NSCl.

A batch experiment using ^{15}N labeled nitrate was performed to evaluate the occurrence of denitrification in basal medium containing Gortner Garden soil and pyruvate. A detailed description of the experiment can be found in the Supplementary Material. The results indicate that denitrification under anaerobic conditions is very significant when compared to the control, especially when pyruvate is present as electron donor (Fig. S-6).

4. Discussion

4.1. NTO reduction

NTO was anaerobically biotransformed to ATO. However, NTO resisted transformation under aerobic conditions. These results suggest that in order to reach full removal, a combination of anaerobic and aerobic conditions must be achieved. This combination can occur as a temporal sequence of anaerobic-aerobic conditions, represented by the batch experiment (Liu et al., 2007; Franca et al., 2015), or simultaneously in a bioreactor which contains an anaerobic microbial population in a bulk aerobic environment (Beunink and Rehm, 1990; Chae et al., 2012; Kato et al., 1993; Mac Conell et al., 2015).

In this study, NTO biotransformation in soil occurred readily under anaerobic conditions. Nonetheless, no biotransformation occurred under aerobic conditions. Similar results were also observed by Krzmarzick et al. (2015). Le Campion et al. (1999a) reported NTO reduction under aerobic conditions in a medium containing fructose, lactate and glucose in high concentrations, accounting for approximately 1000-fold excess of cosubstrate. However, when the growth media was not supplied with other sources of carbon, only 11.8% of NTO was metabolized (Richard and Weidhaas, 2014). NTO was also found to be reduced in the presence of oxygen in a medium containing high amounts of cells suspended in liquid waste with glucose (Le Campion et al., 1999a). In both experiments, the large excess of substrate could cause oxygen limitation in the cultures. The reductive conditions created by microbial O_2 consumption could potentially create anaerobic microniches, accounting for NTO reduction in bulk aerobic environments.

The NTO reduction pathway is driven mainly by biological reactions. As evidence, it was observed in this study that the transformation of NTO did not occur under anaerobic conditions in a control containing soil sterilized with formaldehyde. However, heat killed controls were able to reduce NTO. The autoclaved soil is not necessarily completely comparable to the live soil, since heat treatment could cause changes in the redox active species in the soil (such as conversion Fe^{III} to Fe^{II}). According to Tratnyek and Wolfe (1993), autoclaving the soil may have practical significance in the interpretation of environmental reduction reactions due to changes in soil properties. These changes were found to have an important effect in the reduction rate of nitroaromatic pesticides (Tratnyek and Macalady, 1989). However, NTO reduction was greatly stimulated by the addition of microbial substrate, suggesting a predominantly biological reaction during the reduction of NTO. In batch experiments conducted by Mark et al. (2016), the contribution of biological transformation to the total attenuation of NTO in soils was assessed by comparing autoclaved to untreated soils. A significantly higher NTO mass loss was observed for the untreated soil, indicating that biodegradation plays an important role in NTO attenuation.

4.2. ATO degradation

In this study, ATO was formed stoichiometrically as the main product from NTO reduction. The ATO formed, however, was not further metabolized under anaerobic conditions. ATO elimination under strict anaerobic conditions was not witnessed by Krzmarzick et al. (2015) in incubations lasting 62 days. Nevertheless, evidence

of ATO metabolism under aerobic conditions was observed in two soils, which is consistent with the results of the present study.

In the bioreactor study, we demonstrated that nitrogen in ATO was converted to inorganic nitrogen species (ammonium, nitrate and nitrite), indicating the biodegradation of the compound. The incomplete conversion of ATO-nitrogen to inorganic nitrogen ions indicates that a metabolite may have accumulated. Indeed, an unknown peak with a UV spectrum similar to ATO was detected, and its area followed the concentration of nitrogen mineralized. Le Campion et al. (1999a) detected urea as an ATO degradation product by *B. licheniformis*, which was further degraded to ammonia and carbon dioxide. We did not succeed in detecting urea in our experiments using both biochemical kits and LC-MS, so the possibility of having urea as the unknown metabolite was discarded. However, two chemical structures were found through mass spectrometry as the possible metabolite: HN_3S_2 and C_2H_2NSCl.

Part of the shortfall in the nitrogen mass balance can be attributed to the accumulation of biomass. In order to reduce NTO to ATO, 72 mg L^{-1} of the chemical oxygen demand (COD) of pyruvate was consumed (based on a requirement of 6 e^- equivalents per mole NTO). The remaining COD of pyruvate (144 mg L^{-1}) could be consumed in the aerobic phase, however, assuming a high cell yield of 50% ($COD_{cells}/COD_{pyruvate}$), the generic formula of a bacterial cell as $C_5H_7O_2N$, and the ash content of the biomass as 5%, the nitrogen uptake by the cells would account for approximately 8% and 12% of all the nitrogen in batch experiments and in the bioreactor, respectively. The occurrence of denitrification in Gortner Garden soil amended with pyruvate under anaerobic conditions indicates that a fraction of the nitrogen from ATO mineralization could form nitrogen gas, since anaerobic zones are present in the reactor media.

4.3. Sequential anaerobic-aerobic approach

The oxygen atoms of nitro groups are more electronegative than the nitrogen, leading the nitrogen atom to carry a partial positive charge, therefore serving as an electrophile (Ju and Parales, 2010; Spain, 1995). Due to this characteristic, compounds containing nitro groups are difficult to oxidize, being more prone to reduction in biological systems. In practice, nitroaromatics and other explosives persist for decades in aerobic subsurface environments (Amaral et al., 2009; Bradley et al., 1994). As evidenced by this study, NTO persisted in the soil under aerobic conditions for up to 37 days. Upon reduction of the nitro-groups of explosive compounds to their amines, the molecules become more susceptible to oxidation. Electron donating functional groups, such as the amino group, facilitate the electrophilic attack by pushing electrons into the molecule skeleton, making the atoms in the ring less prone to biotransformation by reduction (Field et al., 1995). Simple aromatic amines, such as aniline, are very recalcitrant under anaerobic conditions. On the other hand, aniline is readily degraded in the presence of oxygen. Wang et al. (2007) observed that the highest degradation levels of aniline by a strain of the *Pseudomonas* family were detected for the highest aeration level tested (6 mg L^{-1} of dissolved oxygen).

The anaerobic step is intended to biotransform the molecule to a compound that is readily oxidizable, and the subsequent oxidation step is expected to allow for extensive biodegradation of the compound. This approach has been demonstrated for a large variety of environmental contaminants with electron withdrawing functional groups. Several nitrogen-containing aromatic compounds, such as nitrobenzene, 4-nitrophenol, aniline and 2,4-dinitrophenol, were reported to be removed simultaneously due to oxic/anoxic cycles in a sequencing batch reactor (Liu et al., 2007). A sequencing anaerobic-aerobic batch reactor was also successful in the treatment of synthetic textile wastewater containing the azo dye Acid Red 14 (Franca et al., 2015). Under anaerobic conditions, the azo bond was reduced, forming two aromatic amines, which were further degraded under aerobic conditions.

4.4. Concurrent aerobic and anaerobic reactions

The simultaneous presence of anaerobic and aerobic zones is possible in a biofilm. The local concentration of oxygen in the biofilm matrix is determined by the balance between consumption and diffusion of dissolved O_2 (Stewart and Franklin, 2008). With increasing depth, the oxygen concentration decreases until it is completely depleted. It was observed by Hooijmans (1990) that in a highly active biofilm, the depth of oxygen penetration may be restricted to only 100–200 μm. This oxygen gradient allows for the presence of a diverse microbial community with distinct metabolisms. This is best illustrated by the degradation of ethanol by anaerobic microorganisms immobilized in anaerobic granular sludge placed in an aerobic bulk environment (Kato et al., 1993). Both oxygen uptake and methanogenesis were reported to occur in parallel in the same biofilm. Furthermore, the presence of facultative substrates, such as ethanol and acetate, was found to enhance the oxygen tolerance. Beunink and Rehm (1990) also observed the coupled reductive and oxidative degradation of 4-chloro-2-nitrophenol (CNP) in an aerobic bioreactor containing an immobilized mixed culture. The culture was able to reduce the nitro group of CNP in the interior of the biofilm and synchronously oxidize the intermediate, 4-chloro-2-aminophenol (CAP), in the aerobic bulk environment. A very diverse bacterial community was found in a sponge-based trickling filter (Mac Conell et al., 2015). Anoxic niches within the sponge were found to provide environmental conditions which supported the growth of denitrifying bacteria, while heterotrophic bacteria with high chemical oxygen demand were more abundant at the outer part of the sponge blocks. The likely explanation for NTO degradation in the bioreactor is similar. Pyruvate added in the medium not only serves as an electron donor, but its degradation led to the depletion of oxygen in the biofilm matrix, forming anaerobic micro zones in the sponge blocks, therefore enabling NTO reduction to ATO, which subsequently was degraded in the aerobic bulk environment of the bioreactor.

5. Conclusion

This study demonstrates that sequencing anaerobic-aerobic biodegradation can efficiently remove NTO from the aqueous phase. The reduction of NTO in an aerobic bulk environment was plausible due to the presence of hypothesized anaerobic microzones, which were formed by addition of cosubstrate. The lack of NTO transformation in a sterilized control indicates that the process is driven mainly by biological reactions. The presence of inorganic nitrogen ions in the reactor effluent suggests partial NTO mineralization. However, the recovery of nitrogen as inorganic aqueous species was only around 40%. Denitrification, accumulation of a N-containing-metabolite formed from ATO degradation, and accumulation of nitrogen in the biomass could account for the missing nitrogen.

Acknowledgments

This study was supported by the Strategic Environmental Research and Development Program (SERDP project ER-2221). C. L. Madeira acknowledges the financial support from CAPES Foundation, Ministry of Education, Brazil, under grant 88888.075208/2013-00, and the training core provided by University of Arizona

NIEHS Superfund Research Program (P42 ES04940). We acknowledge Raju Khatiwada for his help with the soil analysis, Guangbin Li for his help with the denitrification assay, and Stephan M. Cameron for the soil used in this study.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.chemosphere.2016.10.032.

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