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(Bio)transformation of 2,4-dinitroanisole (DNAN) in soils

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HIGHLIGHTS

- DNAN anaerobic transformation was faster than aerobic conversion.
- Anaerobic DNAN conversion rate correlated well with soil organic carbon (≤2.07%).
- H_2 added as electron donor enhanced DNAN biotransformation.
- DNAN nitroreduction lead to monomer products which coupled to form azo dimers.
- Anaerobic transformation pathway and azo dimer formation mechanism were proposed.

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ABSTRACT

Recent studies have begun to assess the environmental fate and toxicity of 2,4-dinitroanisole (DNAN), an insensitive munition compound of interest to defense agencies. Aerobic and anaerobic DNAN biotransformation in soils was evaluated in this study. Under aerobic conditions, there was little evidence of transformation; most observed removal was attributed to adsorption and subsequent slow chemical reactions. Under anaerobic conditions, DNAN was reductively (bio)transformed and the rate of the transformation was positively correlated with soil organic carbon (OC) up to a threshold of 2.07% OC. H_2 addition enhanced the nitroreduction rate compared to endogenous treatments lacking H_2. Heat-killed treatments provided rates similar to the endogenous treatment, suggesting that abiotic factors play a role in DNAN reduction. Ten (bio)transformation products were detected by high-resolution mass spectrometry. The proposed transformation pathway involves reduction of DNAN to aromatic amines, with putative reactive nitroso-intermediates coupling with the amines to form azo dimers. Secondary reactions include *N*-alkyl substitution, *O*-demethylation (sometimes followed by dehydroxylation), and removal of an *N*-containing group. Globally, our results suggest that the main reaction DNAN undergoes in anaerobic soils is nitroreduction to 2-methoxy-5-nitroaniline (MENA) and 2,4-diaminoanisole (DAAN), followed by anaerobic coupling reactions yielding azo-dimers. The dimers were subsequently subject to further (bio)transformations.

1. Introduction

2,4-Dinitroanisole (DNAN) is an insensitive munitions compound (IMC) being considered by defense agencies and industries to replace conventional explosives, such as 2,4,6-trinitrotoluene (TNT) [1,2]. As the word "insensitive" suggests, IMCs are less prone to accidental explosions. Within the last 15 years, there has been an increased interest in the chemical and physical properties of DNAN [1,3]. More recently, researchers have started to study the hazard that DNAN (either alone or in munitions formulations) may pose once released to the environment, such as residues on firing ranges resulting from incomplete detonation [4–7]. In order to understand the extent of the environmental risk, more data are needed on the environmental fate of DNAN, particularly in natural systems.

(Bio)transformation has been studied as a key component of the environmental fate of DNAN. Investigations in mixed consortia and pure cultures have been carried out in aerobic and anaerobic conditions leading to two major transformation routes. In the first route, nitro groups in DNAN were reduced to aromatic amines biologically [8–11] and abiotically with metallic or ferrous iron

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[4,11,12]. The reduction of DNAN to aromatic amines occurs via nitroso and hydroxylamino intermediates [8,11], which are potentially toxic and mutagenic [13,14] and can react with amines to form azo-dimers [15,16]. These dimers have been observed in aerobic conditions [8], anaerobic conditions [10], and in anaerobic transformation samples exposed to air without an antioxidant agent [9,11]. Additionally, microbial DNAN *O*-demethylation has been reported, yielding 2,4-dinitrophenol (2,4-DNP) [17,18]. In some cases, 2,4-DNP formed a hydride–Meisenheimer complex, releasing NO_2^- [18].

If DNAN is not mineralized, there are two main mechanisms for the loss of DNAN and intermediates from solution: (1) reversible adsorption or (2) irreversible incorporation into humic substances. DNAN is relatively hydrophobic (log Kow = 1.58–1.61) and has been found to sorb reversibly onto soil [3,11]. While the adsorption may be attributed largely to organic components in soil, as DNAN is reported to bind strongly to lignin [19], DNAN has also been found to have strong affinity for K^+-montmorillonite, a secondary clay mineral in soils [20]. Moreover, its reduced transformation products were prone to sorb irreversibly onto soils in oxic conditions, with sorptive affinity increasing with number of amino groups [11].

Previous studies have focused on enriched cultures, whose biodegradation mechanisms might differ from those occurring in natural systems where processes are driven by natural mixed soil microbial populations, as well as abiotic soil components. In order to elucidate key soil parameters and conditions that influence the fate of DNAN in natural systems, we performed (bio)transformation assays with a diversity of soils provided as suspensions under anaerobic and aerobic conditions. Our objectives were (1) to characterize the (bio)transformation potential for a diversity of soil types in aerobic and anaerobic conditions, (2) to assess inherent soil characteristics and culture conditions that enhance (bio)transformation, and (3) to resolve (bio)transformation pathways by identifying intermediates.

2. Materials and methods

2.1. Chemicals

2,4-Dinitroanisole (DNAN) (CAS # 119-27-7, 98% purity) was purchased from Alfa Aesar (Ward Hill, MA, USA). 2-Methoxy-5-nitroaniline (MENA) (CAS # 99-59-2, 98% purity) and 2,4-diaminoanisole (DAAN) (CAS # 615-05-4, analytical standard) were obtained from Sigma–Aldrich (St. Louis, MO, USA). 4-Methoxy-3-nitroaniline (iMENA) (CAS # 577-72-0, 97% purity) was obtained from Accela ChemBio (San Diego, CA, USA). All chemicals were ACS reagent grade.

2.2. Inocula and basal medium

Seven different agricultural or military surface soils, characterized previously, were used as inocula [21] (relevant soil parameters can be found in Supplementary material Table S-1). Prior to use, soils were sieved using a 2 mm mesh, and stored in sealed plastic bags at 4 °C.

Basal medium was prepared with ultrapure water (NANOpure Infinity™, Barnstead International, Dubuque, IA, USA) and was composed of (in mg L^{-1}): K_2HPO_4 (250), $CaCl_2{\cdot}2H_2O$ (10), $MgSO_4{\cdot}7H_2O$ (100), $MgCl_2{\cdot}6H_2O$ (100), NH_4Cl (280), yeast extract (10), and trace element solution [10] (1 mL L^{-1}). Resazurin (200 mg L^{-1}) was used as a redox indicator [22]. A bicarbonate (48 mM) based buffer was used (with 20% CO_2 in head space) for anaerobic assays, while a phosphate buffer (20 mM) was used for aerobic assays. The final pH of the basal medium was adjusted to 7.2 with HCl. All experiment treatments and controls were conducted in duplicate.

2.3. DNAN soil biotransformation survey assays

2.3.1. Aerobic soil biotransformation survey assays

Aerobic biotransformation assays were performed as described previously [10]. Briefly, soil (2.5 g, wet weight) was added to 50 mL of basal medium containing 150 μM DNAN. Water was added to compensate evaporation during the incubation as assessed weekly by weight measurements.

2.3.2. Anaerobic soil biotransformation survey assays

Anaerobic biotransformation assays were conducted as reported before [10]. Soil samples (5.0 g, wet weight) were added to 100 mL of basal medium containing 150 μM DNAN. H_2 was supplied as electron donor using a H_2/CO_2 (80:20, v/v) mixture to an overpressure of 1.5 atm. Three soils with the fastest DNAN conversion (Catlin, Camp Butner, and Camp Navajo) were further studied by including heat-killed soil (3 consecutive daily cycles, autoclaved at 121 °C for 50 min), and a treatment without addition of H_2 (endogenous).

2.3.3. Sample processing

Liquid samples (0.5 mL) were diluted (1:3) in 375 ppm ascorbic acid (anaerobic assays) to prevent autoxidation of aromatic amine products upon air exposure [9,11]. Samples were then centrifuged (9600 *g*, 10 min), and stored at 4 °C. All samples were analyzed within three days. Additional frozen samples from 11 days of incubation from the fast (bio) transforming soils (Catlin, Camp Butner, Camp Navajo, Camp Ripley) and samples from 34 days of incubation from the slow (bio) transforming soils (Roger Rd., Maricopa) were analyzed with ultra-high pressure liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (UHPLC-QToF-MS).

2.4. Analytical methods

2.4.1. UHPLC-DAD

Supernatant samples were analyzed using an Agilent 1290 Infinity Series (Santa Clara, CA, USA) ultra-high pressure liquid chromatograph coupled to a diode array detector (UHPLC-DAD). An Acclaim RSLC Explosives E2 column (2.1 × 100 mm, 2.2 μm) (Thermo Fisher Scientific, Waltham, WA, USA) was used at room temperature. A methanol/H_2O (40/60% *v/v*) mobile phase was run isocratically (0.25 mL min^{-1}, 15 min). Detection of DNAN, MENA and DAAN was performed at 300, 254 and 210 nm, respectively. Retention times were 9 min for DNAN, 5 min for MENA, and 2.4 min for DAAN.

2.4.2. UHPLC-QToF-MS

High resolution full scan mass spectra were obtained from 10 μL injections using liquid chromatography introduction to a TripleTOF® 5600 QToF-MS (AB Sciex, Framingham, MA) equipped with an electrospray ionization (ESI) source kept at 450 °C in the positive mode. UHPLC parameters used were the same as those described for UHPLC-DAD, on an UltiMate 3000 UHPLC (Dionex, Sunnyvale, CA). For identification of parent ion peaks as well as fragmentation patterns, information dependent acquisition (IDA); 0.1 s cycle time, 6 triggered ions per cycle, mass range 35–1000 spectra were also obtained in ESI positive ion mode with a capillary setting of 5.5 kV, a declustering potential of 80 V, and curtain gas, desolvation gas, and nebulizer gas levels at 30, 35, and 35 psi, respectively, with N_2. Analyst TF 1.6 with PeakView 1.2.0.3 and Formula Finder 1.1.0.0 were used to process spectral data and to

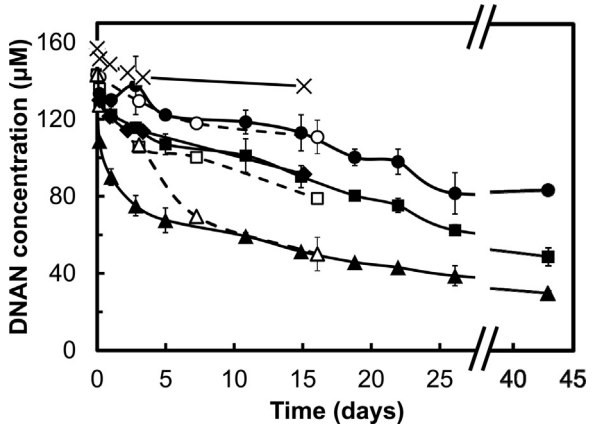

Fig. 1. Aerobic removal of DNAN in soils (50 wet g L^{-1}) in mineral medium. Live treatments (continuous line): Camp Butner (■), Camp Ripley (●), Catlin (▲), Camp Navajo (♦), Florence (**X**). Heat-killed treatments (dashed line): Camp Butner (□), Camp Ripley (○), and Catlin (Δ). No heat-killed treatment available for Florence and Camp Navajo. Averages with error bars are reported.

identify molecular formulae. Instrument calibration was accomplished by automated infusion of a solution periodically, over a mass range of 35–1000. Some metabolites were detected using direct infusion (30 μL min^{-1}) into the QToF-MS.

2.4.3. Bioavailable iron, organic carbon, and water content

Total organic carbon (OC) was calculated from the difference between total carbon and total inorganic carbon. Total carbon was determined by combustion at 900 °C and total inorganic carbon was determined by phosphoric acid addition followed by combustion at 200 °C, in both cases followed by infrared detection of $CO_{2(g)}$, using a Shimadzu 5000 A-SSM TOC Analyzer (Columbia, MD). Sequential extraction of the soils was carried out targeting the following (operationally-defined) solid phases of iron (Fe) following the methods from Richard and Inskeep [23]: (i) water extractable, (ii) exchangeable (using ammonium acetate), (iii) amorphous/poorly crystalline (using acid ammonium oxalate in dark), and (iv) total free oxides (using citrate–dithionate–bicarbonate). Bioavailable Fe was operationally defined as the sum of water, ammonium acetate, and ammonium oxalate extractable pools. The extracted samples were analyzed for total iron in each fraction (done in triplicate) using an Elan DRC-II inductively coupled plasma mass spectrometer (ICP–MS) (PerkinElmer, Waltham, MA, USA). Water content in soil was determined (in duplicate) by oven drying (overnight) at 105 °C.

2.4.4. Statistical analyses

One-way analysis of variance (ANOVA) was conducted for bioavailable Fe and soil texture in OriginPro 9.1 (OriginLab, Northampton, MA, USA). Linear correlations for OC with anaerobic DNAN degradation rate and for DNAN adsorbed in aerobic assays after 4 h were determined by calculating R^2 coefficient and a two-sided *t*-test.

3. Results

3.1. Aerobic soil survey of biotransformation

The bioconversion of DNAN under aerobic conditions was investigated in seven different soils. In some cases, heat-killed soil controls were included. Overall, there was slow conversion of DNAN in all of the soils surveyed (Fig. 1). After rapid initial DNAN decrease (4–24 h), the removal rate was low in the live treatments (0.42–2.28 μM d^{-1}). The rates and extent of DNAN removal achieved were similar to those observed in the heat-killed soil.

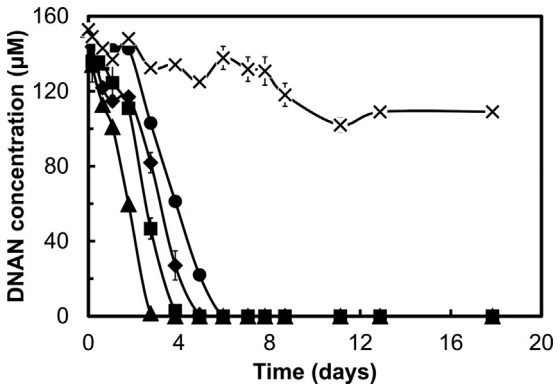

Fig. 2. Anaerobic transformation of DNAN in soils (50 wet g L^{-1}) in mineral medium and amended with H_2: Camp Butner (■), Camp Ripley (●), Catlin (▲), Camp Navajo (♦), and Florence (**X**). Averages with error bars are reported. Please note time scale is compared to Fig. 1.

Initial DNAN removal was not associated with any significant HPLC resolvable transformation products except for minimal amounts of MENA (0–1.4% of DNAN (Fig. S-1 in SI)). However, the removal was linearly correlated with the soil organic carbon (OC) (R^2 = 0.9005, n = 5, two-sided *t*-test p = 0.015) (Fig. S-2 in SI). DNAN initial loss could then be attributed to adsorption.

Generally, initial adsorption was followed by slow DNAN transformation. The transformation rate decreased after 4–8 d, suggesting that abiotic factors responsible for reducing DNAN became exhausted. By 15 days of incubation, Catlin, Camp Navajo, and Camp Butner soils had the most DNAN removed due to adsorption and transformation (Fig. 1); accounting for 64, 42, and 37% of DNAN, respectively. The soils that were incubated for a longer period (43 d) Catlin, Camp Butner, and Camp Ripley; showed an overall removal of 45–80%.

3.2. Anaerobic soil survey of biotransformation

DNAN bioconversion was investigated under anaerobic conditions using H_2 as an electron donor. Two main groups of soils can be distinguished by their respective fast and slow rates of DNAN (bio)transformation (Fig. 2). Zero-order DNAN transformation rates for fast soils ranged between 38.9 73.1 μM DNAN d^{-1} (Fig. 3, Table 1). Complete DNAN removal was achieved within 6 d in the fast soils (Catlin, Camp Butner, Camp Navajo, and Camp Ripley). These soils had lag phases ranging from 0.2 to 2.6 d. Soils with slow (bio)transformation rates (Florence, Maricopa, Roger Rd.) transformed DNAN at 4.51 11.6 μM d^{-1}, providing ≤33% of total DNAN removal after 9 d. In Fig. 2, only Florence soil is shown as example for the slow soils; data for Maricopa and Roger Rd are not shown but Table 1 summarizes DNAN anaerobic (bio) transformation data for all soils.

Anaerobic DNAN (bio)transformation occurred faster and to a greater extent than in aerobic conditions. In the fast degrading soils, 50% of DNAN was removed within 2–4 d under anaerobic conditions (compared to ≥15 days for aerobic conditions). For fast (bio)transforming soils, all DNAN was removed within 6 d, whereas in aerobic conditions, there was residual DNAN by 43 d for the same soils.

Soil parameters (OC content, bioavailable Fe, and soil texture) were tested for correlation with DNAN conversion rate. Fast DNAN-(bio) transforming soils had OC contents above 2% (Fig. 3), while slow soils had a low OC content (<2%). There was a strong relationship between transformation rate and OC (R^2 = 0.9797, n = 5, two-sided *t*-test p = 0.001). Each percent OC means an incre-

Table 1
Features of anaerobic biotransformation of DNAN with H_2 amendment for the seven soils surveyed.

Soil	Lag phase	Conversion rate DNAN[a]	Max. yield products[b]		
	(d)	(μM d^{-1})	(μmol g^{-1} dwt soil d^{-1})	(μM)	(%)[c]
Roger Rd.	7.8	11.6	0.24	10	6.67
Maricopa	3.8	4.51	0.09	28	18.7
Catlin	0.2	69.3	1.43	69	46.1
Camp Ripley	1.1	38.9	0.78	107	71.3
Camp Butner	1.5	68.7	1.66	101	67.7
Camp Navajo	2.6	73.1	1.60	116	77.0
Florence	4.9	9.5	0.20	29	19.3

[a] Conversion rate after lag phase.
[b] MENA + DAAN (resolved with HPLC-DAD).
[c] MENA+DAAN divided by DNAN added (150 μM).

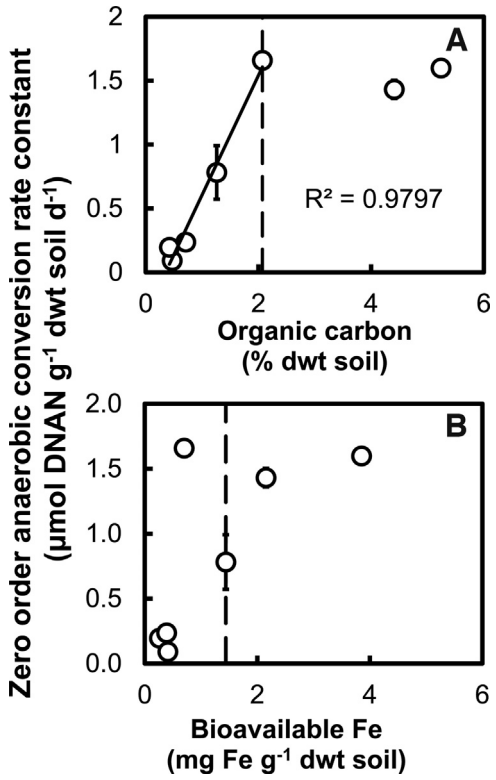

Fig. 3. Correlations of zero-order rate constant with soil organic carbon (OC) content (Panel A) and soil bioavailable Fe (Panel B) during DNAN anaerobic soil biotransformation assays. Rate was calculated from the end of the lag phase (Table 1) until DNAN was no longer detected. The vertical dashed line indicates the threshold of linear correlation of rate constant with OC and Fe. Linear regression for OC in Panel A valid for 0–2.07% OC. Two-sided *t*-test, $n = 5$, $p \leq 0.005$.

ment of 0.593 μmol DNAN g^{-1} dry weight (dwt) soil d^{-1} up to 2.07% OC (Fig. 3). Above this value the rate did not increase. While bioavailable Fe did not show significant linear correlation at the 95% confidence level, one-way ANOVA showed that there was significant difference in the degradation rates based on its concentration (F test = 93.8, $p = 6.87 \times 10^{-5}$). Furthermore, one-way ANOVA also showed significant differences in soil texture (F test = 102, $p = 1.53 \times 10^{-5}$). Based on these observations, 2.07% OC was a threshold range that distinguished fast from slow DNAN-converting soils.

Three of the fast (bio)transforming soils were selected for further study by comparing full live treatment (containing H_2) with endogenous (no H_2 added) and heat-killed controls. DNAN conversion occurred in all cases (Fig. 4). H_2 amendment enhanced DNAN conversion by 2–6 fold compared to endogenous treatments. Heat-killed and endogenous treatments had similar rates, 17.9–44.3 and 11.1–47.3 μM d^{-1}, respectively, which suggest that with no electron donor amendment, abiotic transformation is dominant.

The highest yields of UHPLC-DAD detectable monomer products (MENA, DAAN) for these three fast biotransforming soils in aqueous phase generally occurred in the H_2 amended (46.0–77.0% of DNAN) and the endogenous (56.8–68.2%) treatments. The lowest concentrations generally occurred in heat-killed controls (46.6–58.9%). After 10 d, DNAN recovery was 0%, and no DNAN, MENA, or DAAN were detected. This indicated the formation of transformation products that were either not detectable in UHPLC-DAD or bound to the soil.

3.3. Products identified in UHPLC and infusion QToF-MS

Ten chemical structures were detected in liquid samples using QToF-MS techniques. Table 2 shows the compounds, retention times, mass to charge ratios (m/z values as $[M+H]^+$), as well as fragments detected where available. 2,4′Dimethoxy-4-nitro-3′nitroso–azobenzene was detected by infusion QToF-MS and the rest of the compounds were detected using UHPLC-QToF-MS. The high-resolution masses measured for all compounds detected were within 0.7 ppm of predicted monoisotopic masses. Mass spectral details and fragmentation patterns are shown in Figs. S-3–S-12. MENA and DAAN were confirmed in the samples analyzed, with $[M+H]^+$ m/z values detected 0.7 and 0.1 ppm away from the calculated ones, respectively. An isomer of MENA, 4-methoxy-5-nitroaniline (iMENA) (**J,** Fig. 6) was detected and exhibited a shorter retention time (2.2 min) than MENA (5.3 min), indicative of greater hydrophilicity. The assignment is further supported due by the stronger dipole moment and lower log Kow (0.80 versus 1.47) of iMENA compared to MENA [11]. In addition, the fragmentation pattern was also different between the isomers (Table 2). Since iMENA was only detected by UHPLC-QToF-MS, it could be inferred that it was produced at considerably smaller amounts than MENA. To date, iMENA has only been reported in abiotic reduction of DNAN with zero-valent iron [4] compared to the regioselective formation of MENA in biological systems [11]. Therefore, its presence might indicate that abiotic processes contributed to nitroreduction.

Besides monomeric products, a total of seven dimers were identified. Three of them have been reported during DNAN incubations with anaerobic sludge: 3,3′diamino-4,4′dimethoxy–azobenzene (**E**), 3,3′diamino-4-hydroxy-4′methoxy–azobenzene (**F**), and 4,4′dimethoxy-3-methylamino-3′methyleneamino–azobenzene (**G**) (Table 2, Fig. 6) [10]. The other four were new dimers identified in this study. They included: 2,4′dimethoxy-4-nitro-3′nitroso–azobenzene (**C**), 2,2′dimethoxy-5-hydroxylamino–azobenzene (**D**), 4′methoxy-3-methylamino-3′methyleneamino–azobenzene (**H**), and 3-amino-3′nitro-4,4′dimethoxy–azobenzene (**I**) (see Table 2 and Fig. 6).

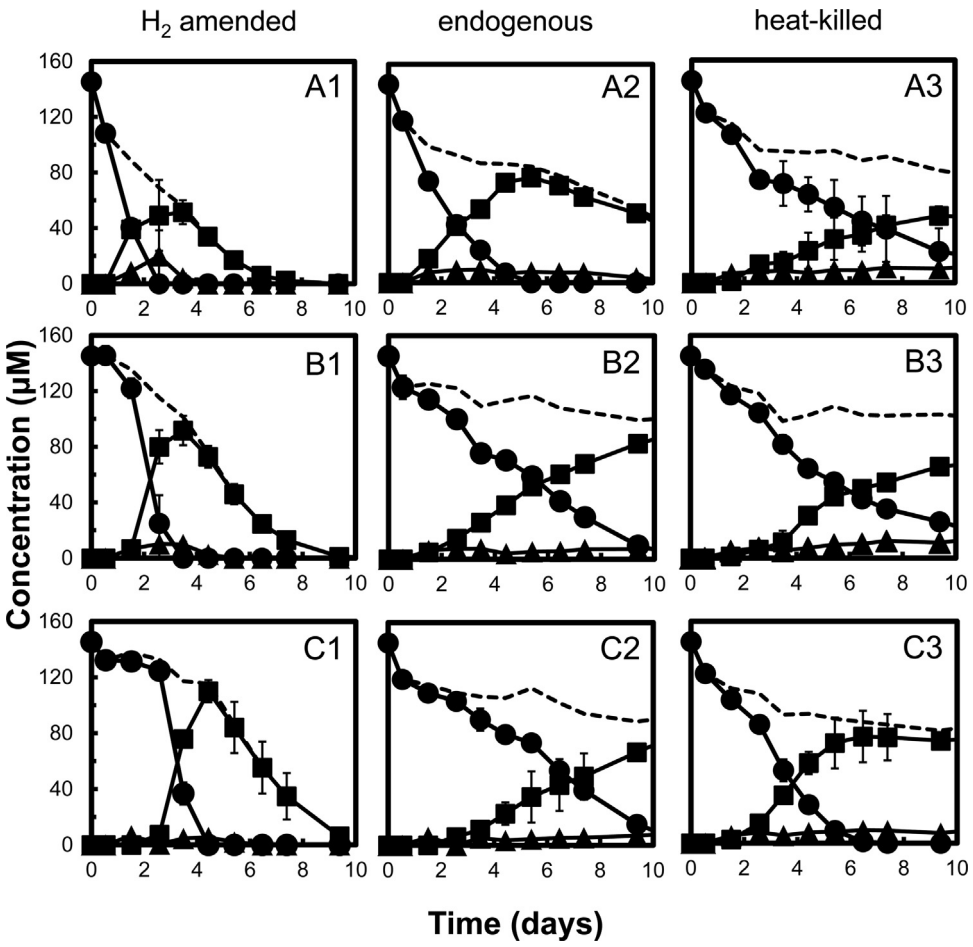

Fig. 4. Concentrations of DNAN (●), MENA (■), and DAAN (▲) and their sum (- - -) during the anaerobic (bio) transformation of DNAN with 50 wet g L^{-1} soil for Catlin (A), Camp Butner (B), and Camp Navajo (C) soils. H_2 added as electron donor (1), live soil (endogenous) (2), and heat-killed soil (3). Averages with error bars are reported.

4. Discussion

Complete DNAN (bio) conversion was restricted to anaerobic conditions. Aerobically, initial partial adsorption and subsequent slow transformation was observed in live and heat-killed soils. Rapid conversion occurred in anaerobic conditions, at a rate that was highly correlated to soil OC content up 2.07%, after which the rates were apparently saturated. An important component of the transformation was due to chemical reactivity of the soil as evidenced by transformation in heat-killed soil. However, rates in live soil were accelerated with the addition of H_2.

4.1. Aerobic transformation

Aerobically, DNAN was removed initially due to possible sorption onto the soil. Hawari et al. [11] calculated soil OC to water partitioning coefficients for DNAN (Koc = 215–364 L kg^{-1}) in two soils containing 2.5–34% total OC, indicative of the strong affinity of the compound to organic matter. The adsorption of nitroaromatics, such as TNT, is known to be correlated with soil organic matter (SOM).

Besides adsorption, a small fraction of DNAN underwent reduction to MENA under aerobic conditions but no further reduction to DAAN was detected. These results indicate that DNAN was also partially reduced under aerobic condition. Aerobic oxidation is problematic for compounds with electron withdrawing moieties, such as multiple nitro groups [13,24,25]. Each additional nitro group shifts the electrons away from the carbon skeleton [26], making the oxidation of the carbon skeleton more difficult and the reduction to amines more likely [27]. While a recent study has reported aerobic DNAN mineralization for a bacterial isolate from a munitions wastewater treatment plant [18], our findings do not show similar reactions in soils, even after extended incubations (43 d). Therefore, reduction is the most likely pathway for DNAN biotransformation in soils, and this was clearly much slower in aerobic conditions.

4.2. Anaerobic transformation

There was rapid formation of aromatic amines, MENA and DAAN, under anaerobic conditions. This is consistent with reports on anaerobic conversion of nitroaromatics with multiple nitro groups [28–30]. Previous research has also demonstrated biological anaerobic conversion of DNAN to MENA and DAAN under anaerobic conditions [9–11].

In our soil assays, the transformation rates were well correlated with soil OC content. Firstly, natural decay of assimilable fractions of SOM can supply electron equivalents for reduction of nitro groups. Assimilable carbon in SOM has been shown for soils [31], waters [32], as well as anaerobic sediments [33]. Secondly, humic material can act as electron shuttles as has been demonstrated by quinones representing redox active moieties in natural SOM that stimulate the reduction of nitroaromatics by ferrous iron and sulfide [34,35]. Lastly, high SOM also correlates with high bacterial counts [36], which may catalyze nitroreduction.

Table 2
Molecular formulae, retention times, calculated and measured *m/z* values, and spectral data determined by UHPLC and infusion Q-ToF-MS for the identified transformation products.

Compound/ structure in Fig. 6 (bold)	Molecular formula [M]	Retention time (min)	Calculated $[M+H]^+$	Measured $[M+H]^+$	Spectral data ([a]Int.)
2,4-Diaminoanisole (DAAN) (B)	$C_7H_{10}N_2O$	1.9	139.0866	139.0865	139.0865 (31), 124.0635 (100), 108.0687 (28), 95.0604 (25), 80.0504 (19)
2-Methoxy-5-nitroaniline (MENA) (A)	$C_7H_8N_2O_3$	5.3	169.0608	169.0615	169.0615 (52), 154.0377 (25), 123.0683 (100), 108.0447 (49), 96.0449 (7), 80.0505 (37)
4-Methoxy-5-nitroaniline (iMENA) (J)	$C_7H_8N_2O_3$	2.2	169.0608	169.0602	169.0602 (72), 154.0366 (29), 123.0676 (16), 122.0597 (100), 108.0444 (21), 94.0649 (23), 77.0395 (18)
3,3′Diamino-4-hydroxy-4′methoxy-azobenzene (F)	$C_{13}H_{14}N_4O_2$	2.6	259.1190	259.1191	259.1191 (87), 242.0919 (27), 228.1005 (34), 227.0923 (100), 199.0977 (70)
4′Methoxy-3-methylamino-3′methyleneamino-azobenzene (H)	$C_{15}H_{16}N_4O$	2.3	269.1397	269.1398	269.1398 (100), 254.1167 (38), 237.1132 (57)
3,3′Diamino-4,4′dimethoxy-azobenzene (E)	$C_{14}H_{16}N_4O_2$	1.6	273.1346	273.1351	273.1351 (54), 256.1079 (35), 242.1161 (60), 227.0924 (100), 199.0978 (11), 151.0860 (14)
2,2′Dimethoxy-5-hydroxylamino-azobenene (D)	$C_{14}H_{15}N_3O_3$	4.9	274.1186	274.1180	274.1180 (24), 243.1010 (100), 228.0764 (69), 200.0806 (34), 172.0863 (10), 143.0591 (10)
4,4′Dimethoxy-3-methylamino-3′methyleneamino-azobenzene (G)	$C_{16}H_{18}N_4O_2$	3.0	299.1503	299.1509	299.1509 (100), 284.1277 (29), 267.1241 (14)
3-Amino-3′nitro-4,4′dimethoxy-azobenzene (I)	$C_{14}H_{14}N_4O_4$	3.4	303.1088	303.1088	303.1088 (100), 271.0810 (19), 257.0651 (19), 225.0888 (6), 227.1042 (16), 151.0856 (24)
2,4′Dimethoxy-4-nitro-3′nitroso-azobenzene (C)	$C_{14}H_{12}N_4O_5$	N/A[b]	317.0880	317.0879	N/A[b]

[a] Int. = % intensity normalized to highest *m/z* for each compound.
[b] Compound detected in infusion-Q-ToF-MS. Fragmentation not available.

Reduction of DNAN also occurred in heat-killed soils, particularly in Camp Navajo soil (which has the highest OC and bioavailable Fe). DNAN abiotic reduction has been reported to result from reaction with Fe(II) alone and with ferrous–ligand complexes [12]. In anaerobic sediments, SOM decay is known to be a major source of electron-donating substrate for Fe(III) reduction [37]. Furthermore, autoclaving soils has been reported to increase 6-fold Fe(II) content due to Fe(III) reduction during heating [38]. Therefore, autoclaving could have reduced Fe(III) to Fe(II) coupled to SOM oxidation, which in turn could promote abiotic reduction of DNAN afterwards.

4.3. Products of anaerobic conversion and biotransformation pathway

The initial reduction of DNAN occurred primarily in the *ortho* position, as reported previously [8,9,11], yielding MENA. In the endogenous and heat-killed treatments there was no further transformation (up to 9 d), except for Catlin soil. However, in the H_2 treatments, the MENA formed was readily consumed and did not result in any accumulation of DAAN. Higher reducing conditions, due to the addition of an electron donor (H_2) favored the conversion of MENA. DAAN was not detected, possibly due to binding with humic substances or coupling reactions with nitroso derivatives to form dimers, and, therefore, it did not accumulate.

QToF-MS indicated azo dimer formation. While these products have been regarded as artifacts formed during sample processing [11], strict anaerobic conditions were maintained in the incubations and ascorbic acid was used as antioxidant to prevent formation of artifacts. Azo dimers were also detected previously during incubations of DNAN with anaerobic sludge [9,10], as well as in similar treatments with TNT [16]. Furthermore, there is a body of literature that uses reductive techniques to synthesize azo dyes from nitroaromatics [39–43]. Therefore, we propose that azo dimers are formed as a product of (bio)transformation of nitroaromatics under anaerobic conditions. Two plausible mechanisms for azo product formation are shown in Fig. 5. The most likely explanation for dimer formation under strict anaerobic conditions is a condensation reaction between a nitroso intermediate and an amino containing compound since aromatic amines were demonstrated to accumulate. This mechanism has been proposed for azo dye synthesis by reduction of nitroaromatics with nano-iron [39] and also by a one-step reaction of aromatic amines with nitroaromatics under basic conditions and high temperature (10 mM, 105 °C). [40]. This proposed reaction would form structures **C, E, and I** in Fig. 6 due to the condensation between a nitroso-bearing compound and an amino group in MENA or DAAN. 2,4′-Dimethoxy-4-nitro-3′nitroso-azobenzene (**C**, Fig. 6), provides evidence of nitroso bearing transformation products. Alternatively, azo dimers can be formed via the reaction of nitroso-intermediates with hydroxylamine intermediates to azoxybenzenes, that can potentially be reduced to azo compounds [44,45]. We detected a tentative azo compound bearing a hydroxylamino group, 2,2′dimethoxy-5-hydroxylamino-azobenzene (**D**, Fig. 6). Although we did not detect any hydroxylamino-bearing monomers, Perreault et al. [8] detected 2-hydroxylamino-4-nitroanisole, during the reduction of DNAN in aerobic conditions. DNAN hydroxylamino intermediates might not accumulate in soil, as hydroxylamino products from TNT reduction have been reported to bind to soil irreversibly [46].

The azo dimers can be subject to further metabolism. An additional plausible reaction is the reduction of the azo dimers to form aromatic amines again. This is a well-known reaction causing cleavage of azo dyes under reducing conditions [47–49].

Besides the formation of dimers, other reactions are proposed based on metabolites observed with QToF-MS, such as

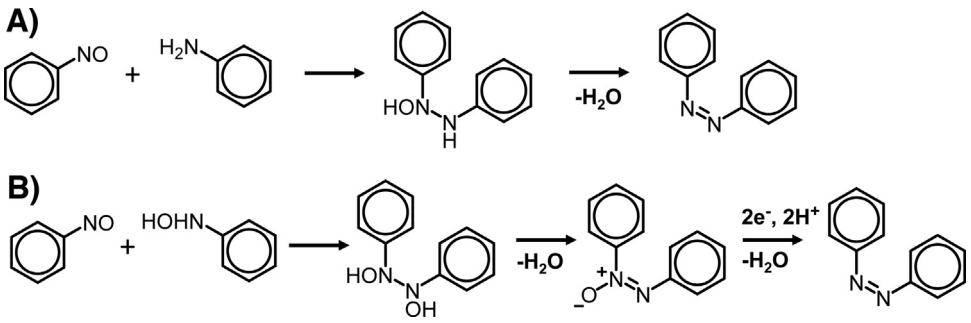

Fig. 5. Possible mechanisms for coupling between reduced intermediates of nitroaromatic compounds: (A) coupling of nitrosobenzenes with aromatic amines [39,40]; (B) coupling of nitrosobenzenes with phenylhydroxylamines [45].

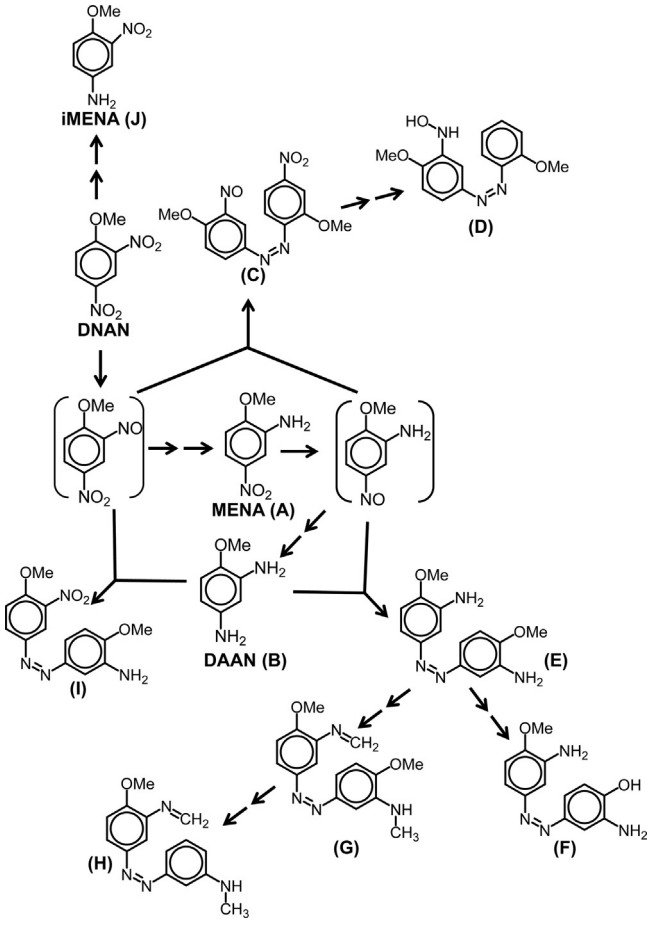

Fig. 6. Metabolites detected with infusion and UHPLC QToF-MS experiments and transformation pathway proposed. Compounds in parentheses were not detected in this work but are known intermediates in the literature. Double arrows indicate that reactions require multiple steps.

O-demethylation, two-step demethoxylation, and *N*-substitution. *O*-demethylation of the methoxy group yields a hydroxyl group in the transformation of 3,3′diamino-4,4′dimethoxy–azobenzene (**E**, Fig. 6) to 3,3′diamino-4-hydroxy-4′methoxy–azobenzene (**F**, Fig. 6). This reaction has been reported during anaerobic incubations of DNAN and 4-nitroanisole [8,10,50]. After *O*-demethylation, dehydroxylation could follow, together comprising a two-step demethoxylation. This series of reactions is suggested between 4,4′dimethoxy-3-methylamino-3′methyleneamino–azobenzene (**G**, Fig. 6) and 4′methoxy-3-methylamino-3′methyleneamino-azobenzene (**H**, Fig. 6). Two-step demethoxylation has been reported previously for the biotransformation of DNAN in anaerobic sludge [10]. Another reaction proposed is *N*-substitution with alkyl groups, leading to observed *N*-methyl and *N*-methylene containing dimers (**G, H**; Fig. 6), which has also been reported previously in anaerobic sludge biotransformation [10]. It is possible that partially degraded labile components could be a source of alkylating amines. Finally, another compound was detected and tentatively assigned the structure 2,2′dimethoxy-5-hydroxylamino-azobenzene (**D**, Fig. 6). This dimer suggests nitrogen removal from the structure (azo dimers from DNAN have four *N*-bearing groups while structure **D** in Fig. 6 has only three). Nitro group removal from an aromatic ring in reductive conditions can occur during nitroreduction to hydroxylamino followed by *N* removal as NH_4^+ yielding a diol [51]. However, the diol product of this reaction scheme was not detected in 2,2′dimethoxy-5-hydroxylamino–azobenene (**D**). Overall, these reactions indicate that azo dimers formed from reduced products of DNAN bioconversion continue to undergo transformations in anaerobic soil environments.

5. Conclusion

DNAN underwent (bio)transformation in soils, particularly in anaerobic conditions, due to biotic and abiotic processes. The major reaction pathway involved nitro-group reduction to MENA, and to a very minor extent, DAAN. Products from DNAN reduction coupled to form azo dimers that continued to be (bio)transformed with *O*-demethylation and *N*-substitution reactions. Taken together, our results indicate that DNAN is readily reductively (bio)transformed in natural soils, and a full suite of transformation products are formed such as aromatic amines and azo dimers, which can impact the fate of DNAN in the environment.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhazmat.2015.10.059.

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