HHS Public Access

Author manuscript

Environ Sci Technol. Author manuscript; available in PMC 2018 January 18.

Published in final edited form as:

Environ Sci Technol. 2017 November 21; 51(22): 13327–13334. doi:10.1021/acs.est.7b03699.

Environmental fate of ^{14}C radiolabeled 2,4-dinitroanisole (DNAN) in soil microcosms

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Abstract

2,4-dinitrosanisole (DNAN) is an insensitive munitions component replacing conventional explosives. While DNAN is known to biotransform in soils to aromatic amines and azo-dimers, it is seldom mineralized by indigenous soil bacteria. Incorporation of DNAN biotransformation products into soil as humus-bound material could serve as a plausible remediation strategy. The present work studied biotransformation of DNAN in soil and sludge microcosms supplemented with uniformly ring-labeled ^{14}C-DNAN to quantify the distribution of label in soil, aqueous, and gaseous phases. Electron donor amendments, different redox conditions (anaerobic, aerobic, sequential anaerobic-aerobic), and the extracellular oxidoreductase enzyme horseradish peroxidase (HRP) were evaluated to maximize incorporation of DNAN biotransformation products into the non-extractable soil humus fraction, humin. Irreversible humin incorporation of ^{14}C-DNAN occurred at higher rates in anaerobic conditions, with a moderate increase when pyruvate was added. Additionally, a single dose of HRP resulted in an instantaneous increased incorporation of ^{14}C-DNAN into the humin fraction. ^{14}C-DNAN incorporation to the humin fraction was strongly correlated (R^2=0.93) by the soil organic carbon (OC) amount present (either intrinsic or amended). Globally, our results suggest that DNAN biotransformation products can be irreversibly bound to humin in soils as a remediation strategy, which can be enhanced by adding soil OC.

Graphical abstract

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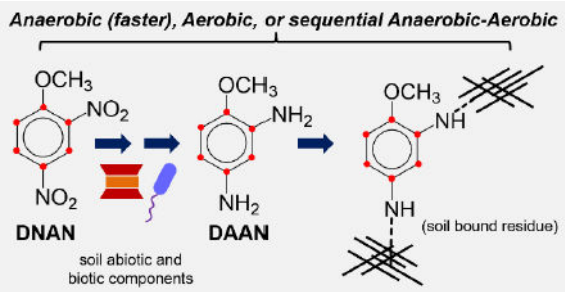
SUPPORTING INFORMATION

Schematic of ^{14}C extraction protocol, HPLC protocol for DAAN, BQ and HQ measurements, and DOC method. DNAN anaerobic biotransformation to MENA and DAAN detected in supernatant in parallel non-labeled assays. Color and loss of DOC upon reaction of DAAN with BQ. Loss of DAAN upon reaction of DAAN with humin.



Keywords

insensitive munitions; 2,4-dinitroanisole (DNAN); 2,4-diaminoanisole (DAAN); biotransformation; bound residue

1. INTRODUCTION

Insensitive munitions (IM) are designed to prevent unintended detonations, greatly improving the safety of soldiers. 2,4-dinitroanisole (DNAN) is widely used in IM formulations (e.g. IMX-101, IMX-104, PAX-48 and PAX-21), to replace the conventional explosive compound, 2,4,6-trinitrotoluene (TNT).[1, 2] There are two primary routes of introduction of DNAN into the environment. Firstly, via unexploded and incompletely detonated ordnance upon field use. Being insensitive, and thus more difficult to detonate, a larger proportion of DNAN might be left unexploded compared to conventional explosives. Remnant portions of DNAN will slowly become dissolved via rainfall events and as such they represent a continuous source of pollution for several years.[3] The second route could be due to wastewater released from munitions manufacturing.

Given the limited solubility of DNAN and moderate octanol-water partition coefficient (Log P), 276 mg L^{-1} and 1.61, respectively at 25° C[4], its transport could be retarded due to adsorption to soil components. DNAN is strongly adsorbed by montmorillonite[5], and there is evidence of strong DNAN sorption to organic matter in soils.[6, 7] Besides adsorption, DNAN is readily (bio)transformed in anaerobic conditions by indigenous soil microorganisms as well as by abiotic processes.[6, 8] The main transformation route is nitro-group reduction to aromatic amines, stimulated by electron donating amendments[9], as well as anaerobic conditions.[6] During nitroreduction, the initially formed nitroso reactive intermediates, can condense with the secondarily formed amino groups to form azo-dimers.[6, 10, 11] Additionally, sulfonation and acetylation of DNAN amino products have also been reported.[9, 12] The bacterium, *Nocardioides* sp. JS1661, [13] was found to mineralize DNAN via *O*-demethylation to 2,4-dinitrophenol and subsequent nitrite release from a Meisenheimer complex intermediate. However, widespread DNAN-mineralizing capability by indigenous soil microorganisms has not been found to date.[6, 9, 14, 15] Instead, aromatic amines are the primary products observed from DNAN in soils[6, 9], which lead to complex oligomer mixtures[6].

Elucidation of DNAN biotransformation products has been accomplished using non-targeted mass spectrometry[6, 9, 12], but a large gap remains to quantify the distribution of these

products across the different matrices (i.e. soil, aqueous medium, gas phase). Quantification has been challenging due to the lack of standards and the potential instability in aerobic environments of metabolites formed anaerobically, as well as irreversibly bound products to soil that might not be extractable. Reduced aromatic amine products of DNAN have been shown to adsorb irreversibly onto soils.[7] We have previously reported condensation reactions leading to azo-dimer structures[6] in soil systems during nitro-group reduction of DNAN.

The objective of the present study is to quantify the fate of DNAN and its (bio)transformation products in soil utilizing uniformly ring radiolabeled ^{14}C-DNAN. One specific goal was to evaluate ^{14}C distribution in soil, liquid, and gas phases during biotransformation in controlled microcosms. A second goal was to evaluate how different strategies of bioremediation impact the fate of ^{14}C-DNAN, with a special focus on its irreversible covalent bonding with soil humus.

2. MATERIALS AND METHODS

2.1 Biological & abiotic materials

2.1.1 Chemicals—2,4-dinitroanisole (DNAN, CAS # 119-27-7, 98% purity) was procured from Alfa-Aesar (Ward Hill, MA, USA). 2,4-diaminoanisole (DAAN) was purchased from Sigma Aldrich, >=98% purity. Uniformly ring labeled ^{14}C-DNAN (77 mCi mmol^{-1}) was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA).

2.2.2 Inocula—Assays were inoculated with granular anaerobic sludge from a brewery wastewater treatment bioreactor (volatile suspended solids=7.9%, Mahou, Guadalajara, Spain), or pristine surface soils from military sites, Camp Butner (CB) and Camp Navajo (CN), described previously.[6, 16] The two soils were selected due to their capability of transforming DNAN to the amino products[6] and their differences in organic carbon (OC) content, 20.7 and 52.4 mg OC g^{-1} soil, respectively, for CB and CN.

2.1.3 Humin purification—A modified protocol was used to purify humin from IHSS Pahokee Peat soil (PPS).[17] PPS was selected due to its high organic carbon content and since it a well-characterized soil. Five grams of PPS were added to 50 mL of 0.5 M NaOH, and placed in a rotating tumbler for four hours. Then, samples were centrifuged (10 min, 4,000 rpm) and the liquid was decanted. The alkaline hydrolysis cycle was repeated ten times. The remaining solids were washed (18 mM phosphate buffer, pH=7.2) until circumneutral pH was achieved. The purified humin was dried overnight in an oven (60 °C) and ground before use. Its OC and water content were 68 and 14 %, both on a dry weight basis.

2.2 ^{14}C-DNAN biotransformation assays

2.2.1 Initial time-course assays—Anaerobic staggered biotransformation assays were run in order to determine changes in ^{14}C distribution during incubation. DNAN (500 μM cold and 1.6 μCi of uniformly ring ^{14}C -labeled) was added to mineral medium[6] (pH =7.2, 18 mM phosphate buffer) amended with 10 mM pyruvate in anaerobic Balch tubes (Bellco Glass Inc., Vineland, NJ, USA). The solutions (10 mL) were inoculated with CN or CB soil (100 mg wet, water content = 9.3 and 20.7 % of dry soil, respectively)[6, 16] or anaerobic

granular sludge[11] (75 mg, volatile suspended solids = 7.9%). The headspace was flushed with He/CO_2 (80/20 %) and the tubes were closed with t-butyl caps and aluminum seals. The tubes were incubated in the dark in an orbital shaker (115 rpm, 30° C). The incubations for each tube set began at different times so that all tubes were extracted for ^{14}C label recovery at the same time. Samples were processed through the extraction protocol within a couple of hours and read in the liquid scintillation counter (LSC) immediately. Upon extraction, tubes had 0, 1, 5, 10, 20, 30, and 50 d of incubation duration. Each incubation duration time was run in duplicate. Parallel non-labeled incubations were run with the same media, inocula, and incubation conditions for HPLC-DAD analysis of DNAN, 2-methoxy-5-nitroaniline (MENA),and DAAN in the supernatant phase.

2.2.2 Anaerobic vs. aerobic treatments—Mineral medium containing DNAN (150 μM cold and 1.6 μCi of uniformly ring ^{14}C –labeled) was inoculated with 250 mg wet weight CN soil in anaerobic tubes. The treatments (10 mL) were run for 40 d and included anaerobic (flushed with He/CO_2) endogenous and 10 mM pyruvate amended assays. No pyruvate amendments to aerobic treatments were included in the experimental design since the consumption of pyruvate would quickly yield anaerobic conditions in the system. The aerobic assays were flushed with air at 0, 10 and 40 d, and the flushed gas was passed through Opti-Fluor and alkali (0.1 M NaOH) traps to capture and detect radiolabeled gaseous species. Triplicate sacrificial assays for ^{14}C extraction were incubated for each several durations (0, 10, 40 d).

2.2.3 Sequential anaerobic-aerobic and peroxidase treatments—In addition to comparing aerobic vs. anaerobic DNAN biotransformation, sequential anaerobic/aerobic treatments were conducted with and without electron donor addition and peroxidase to speed up transformation. To allow sufficient transformation and humus incorporation, these assays were run for longer time, a total of 104 d. Anaerobic conditions were maintained during the first 40 d of incubation, followed by an aerobic phase (40-104 d), produced by flushing air into the headspace of the anaerobic tubes. Three treatments were evaluated: 1) endogenous, 2) 10 mM pyruvate amended at day 0, and 3) 100 mg of Type I Horseradish peroxidase (HRP) and 3% H_2O_2 added at day 40 after switching to aerobic conditions, based on previous protocols.[18, 19] During the aerobic phase, samples were flushed with air at 60, 80, and 104 d and any gaseous species collected with a series of Opti-Fluor and alkali traps. Triplicate sacrificial incubations for ^{14}C extraction were used (0, 10, 40, 60 and 104 d).

2.2.4 Increased soil inoculation and added humin—To investigate if additional endogenous OC from soil and exogenously added humin to a real soil sample would increase significantly the amount of label incorporation into soil humus, DNAN anaerobic (bio)transformation incubations were set up with 250 mg CB soil (wet weight) in 10 mL of basal medium containing DNAN (150 μM cold and 1.6 μCi of ^{14}C–labeled). For the increased soil assays, treatments were inoculated with 5, 10, 25, and 50 g L^{-1} of wet CN soil. For the added humin assays, purified humin was amended (0, 5, 10, and 20 mg) to 250 mg (wet weight) of CB soil. After 35 days of incubation (when azo-dimers are expected to be predominant and no further incorporation of DNAN was expected based on preliminary

experiments), the samples were extracted. A control with only medium and DNAN (hot and cold) were incubated as a control All treatments and controls were run in triplicate.

2.2.5—The pairing of 2,4-diaminoanisole (DAAN) and 1,4-benzoquinone (BQ) was studied in anaerobic batch tube incubation. Stock solutions of DAAN and BQ in a solution of NaH_2PO_4 (pH 4.72, 2 g L^{-1}) were added to the tubes, providing a final concentration of 1.00 mM DAAN and 2.88 mM BQ, and a total volume of 20 mL. The tubes were flushed for 3 minutes with N_2 to remove O_2. Immediately after flushing, samples were taken using a syringe for HPLC and DOC analysis (4 min) and after 5 d. A similar experiment was carried out in which DAAN was incubated with the humin preparation (600 mg in 20 mL tube), controls included humin only and DAAN only.

All samples were centrifuged for 20 min. at 13,000 rpm and the supernatant was then analyzed for DAAN, BQ, and 1,4-hydroquinone (HQ) using UHPLC and DOC methods described in Supplementary Information (SI). Controls included the pairing of DAAN and HQ (2.88 mM), DAAN only, BQ only, and HQ only.

2.3 Analytical methods

2.3.1 UHPLC-DAD—DNAN, MENA, and DAAN were quantified on an ultra-high performance liquid chromatograph coupled to a diode array detector (UHPLC-DAD) with an Acclaim RSLC Explosives E2 column (2.1 × 100 mm, 2.2 μm) (Thermo Fisher Scientific, Waltham, WA, USA) and a methanol/H2O eluent (isocratic 40/60, v/v, 0.25 mL min-1) at room temperature. Detection wavelengths and retention times were (nm:min) 300:9, 254:5, and 210:2.3 for DNAN, MENA, and DAAN, respectively.

2.3.2 ^{14}C extraction—A protocol for ^{14}C recovery from the gas, liquid, and solid phases of the biotransformation soil assays was adapted from Drzyzga et al.[20] (Schematic in Supporting Information (SI) Figure S1) The headspace of the flask was trapped by purging with nitrogen gas through a series of two 20 mL traps: the first consisted of Opti-Fluor scintillation cocktail (Perkin Elmer, Waltham, MA, USA) to trap volatile organic compounds (VOCs), and the second was alkaline solution (0.1 M NaOH) to trap CO_2. For Liquid Scintillation Counting (LSC) the entire contents of the VOC trap were transferred, while only 2 mL of the CO_2 trap were added to 18 mL of Opti-Fluor for the (LSC).

For liquid and solid phase analyses, the anaerobic tubes were opened and the contents transferred to 15 mL centrifuge tubes. The samples were centrifuged (10 min, 4000 rpm). Two mL of the supernatant were mixed with Opti-Fluor for LSC. The remaining of the liquid was decanted before addition of 10 mL MeOH to the pellet, followed by sonication (15 min) and centrifugation. One mL of supernatant was sampled for LSC and mixed with 18 mL of Opti-Fluor for LSC. The procedure was repeated with ethyl-acetate and 0.5 M NaOH (with 2 mL of extract mixed with 18 mL of Opti-Fluor). After these sequential liquid extractions, remaining solids were left to dry at 60 °C overnight. The dry solids (~50 mg) were weighed and combusted in a 400x Harvey Oxidizer (R.J. Harvey Instrument Corp., Tappan, NY, USA). The oven and the catalyst zones were set to 900 and 700 °C, respectively. N_2 and O_2 flows were 340 and 330 cc min^{-1}, respectively. 20 mL of 0.1M NaOH was used to trap CO_2. Two mL of the traps were added to 18 mL of Opti-Fluor for

LSC. Ethyl-acetate and methanol extracts were cumulatively defined as apolar fraction. Soil hydrolyzable products with 0.5 M NaOH were operationally defined as humic acid bound fraction in soils, whereas the remaining OC combusted in the Harvey Oxidizer was operationally defined as humin bound. The Harvey Oxidizer efficiency was 60%, based on unlabeled mannitol combustion using a $BaCl_2$ phenolphtalein titration method to quantify CO_2 trapped in the 0.1 M NaOH solution.[21]

2.3.3 Liquid Scintillation and quenching corrections—^{14}C in samples containing Opti-Fluor scintillation cocktail were measured using a Tricarb 1500 Liquid Scintillation Counter (LSC) (Packard, Downers Grove, IL, USA). Measurements were performed in triplicate for 1 min counting intervals. LSC efficiency was above 85%. Unless otherwise noted, aqueous phase and 0.5 M NaOH samples were corrected for coloration signal quenching.

3. RESULTS AND DISCUSSION

3.1 DNAN reductive biotransformation

The fate of DNAN was studied in CN and CB soils. DNAN biotransformation assays in soil lead to complete biotransformation of DNAN (Figure 1). However, the conversion rate of DNAN in CN soil (56.7 μmol DNAN d^{-1} dwt g^{-1} soil) was four-fold higher than the rate in CB soil (14.3 μmol DNAN d^{-1} dwt g^{-1} soil). CN has a higher OC content (52.4 mg g^{-1} soil) than CB (20.7 mg g^{-1} soil).[6] The higher OC content may increase electron donor supply as natural organic matter (NOM) is degraded and stimulate microbial metabolism capable of driving nitro-group reduction with non-specific flavin containing nitroreductases.[22] The HPLC-resolvable products, MENA and DAAN, were only detected sporadically in the supernatant, with the highest concentrations ranging 80-135 μM (SI, Figure S2). By day 50, no products could be detected any longer with HPLC-DAD. Aromatic amines can react with humic substances becoming irreversibly bound, either through substitution reactions[23, 24] or oxidative radical reactions.[25] In order to obtain a quantitative assessment, experiments utilizing radiolabeled parent munitions compounds were needed.

3.2 ^{14}C label distribution during DNAN biotransformation

The ^{14}C distribution was monitored in aqueous, apolar, humic acid–bound, and humin-bound phases during anaerobic incubations in slurries with two soils (CN and CB) and an anaerobic sludge. During the time course of the incubation, the ^{14}C label was predominantly associated with the aqueous phase. This fraction accounted for 65-80% of the total label for the various soil and sludge microcosms on day 50 (Figure 1). A likely explanation is that the dominant products that remain soluble consist of azo-dimers based on our previous work characterizing the long-term species in DNAN biotransformation assays.[6] Alternatively, the aromatic amines may become substituted in dissolved organic matter.[24] On the other hand, the apolar fractions associated with the methanol extracts ranged from 3-6% of the ^{14}C label in the soil and sludge microcosms. The more hydrophobic ^{14}C label in the ethyl acetate remained below 1%.

The ^{14}C associated with the insoluble humus (humin) of the soil and sludge significantly increased during the incubations. The humin-bound fraction was operationally defined in the present work as the non-extractable fraction combusted by the Harvey oxidizer. On day 50, this fraction accounted for 16-25% of the ^{14}C label in the soil and sludge microcosms. The values were highest in the anaerobic sludge microcosm where ^{14}C incorporation occurred rapidly at the start of the experiment in parallel with a rapid decrease in DNAN. The increase in ^{14}C label in the humin fraction coincided with the decrease in DNAN. Once the DNAN was depleted the ^{14}C in the humin fraction remained relatively constant, suggesting that once the supply of aromatic amines (from DNAN reduction) was depleted, the incorporation of ^{14}C ceased. Based on these observations, most of the ^{14}C associated with DNAN remained associated with the aqueous phase and remained soluble. Nonetheless, a sizeable fraction became irreversibly incorporated as non-soluble bound residue in the soil humin. Similar findings have been reported for TNT biotransformation in soil microcosms[26], where the humin fraction was associated with up to 52.4% of the total label.

No radiolabel above background levels was recovered in the gas phase (i.e. ^{14}C-VOCs or ^{14}C-CO_2), suggesting that ring-cleavage of DNAN was unlikely in the CN and CB soils nor in the anaerobic sludge. This is consistent with findings on ring-labeled ^{14}C TNT in soils[20] and activated sludge.[27] Since the radiolabel in the present work only included the aromatic ring, unlabeled CO_2 might have been released from *O*-demethylation of the methoxy group in DNAN or its reduced products and therefore was not detectable in LSC. *O*-demethylation of DNAN bioconversion products has been detected in soils.[6, 9, 15]

Overall, the recovery of the ^{14}C label was satisfactory, with generally >95 % recovery of the radiolabel added. However, the recovery decreased at longer incubation times, resulting in 76-78 % label recovery in both soils. The aqueous phase had a stronger brown coloration at longer incubation times (>30 d), attributed to fulvic and humic acid release from soils. This coloration decreased the LSC counting efficiency, quenching the scintillation signal. A similar phenomenon had also been observed in TNT soil radiolabeled studies.[20] To address this issue, a quenching correction was performed in subsequent experiments as described in the Methods section.

3.3 Aerobic vs. aerobic conditions

Incorporation of ^{14}C labeled products from DNAN into humin (CN soil) was studied under anaerobic and aerobic conditions. Incubations under anaerobic conditions resulted in greater incorporation of DNAN biotransformation products into the soil humus (Figure 2A) than the aerobic treatment. The pyruvate amended anaerobic treatment had the highest incorporation of ^{14}C (53.4% of label) after 40 d of incubation. This was followed by the endogenous anaerobic treatment (41.2% of label); whereas the aerobic treatment had less than half of the label incorporation compared to the pyruvate amended anaerobic treatment. Moreover, the rate of ^{14}C incorporation was also highest for the pyruvate amended treatment (0.16 nCi mg^{-1} OC_{soil} d^{-1}) (Figure 2B), which was 1.2× the endogenous anaerobic treatment rate, and twice the rate compared to the aerobic treatment. Under anaerobic conditions and added electron donor supply (pyruvate), conversion of nitro-groups to reactive amines occurs readily, which in turn may promote formation of covalently bound products in soil humin.

In addition to redox conditions and the electron donor amendments, the amount of soil also impacted the extent of the bound ^{14}C label. All treatments (25 g L^{-1} soil) had remarkably higher incorporation of ^{14}C compared to the first experiment (Section 3.2) with just 10 g L^{-1} soil. In the initial experiments with 10 g L^{-1}, of soil the ^{14}C-DNAN incorporation corresponded to only 23%; whereas, 25 g L^{-1} of the same soil under the same condition of pyruvate amendment enabled more than twice that level of ^{14}C-DNAN incorporation into soil humus. These results clearly indicate that the quantity of soil (and conceivably its associated OC) in relation to the quantity of DNAN has an important role on the ^{14}C-DNAN incorporation into soil humus.

Smaller quantities of radiolabel were recovered in the remaining extracted phases, apolar and humic acid-bound fractions. The methanol extracted label ranged 2.9-8.1 % of the total label, with the aerobic treatment having the highest amount (0.12 μCi). The 0.5 M NaOH extract, considerable radiolabel was recovered (9.5-11.8%). Finally, no detectable radiolabel was associated with captured gas species above background counts for any of the treatments.

3.4 Anaerobic-aerobic sequential experiments

A sequential combination of anaerobic-aerobic conditions was evaluated as strategy to increase the amount of ^{14}C incorporation into the soil humin utilizing CB soil at a concentration of 25 g L^{-1} (Figure 3). Like in the previous experiments, the majority of the ^{14}C-DNAN incorporation occurred in the humin phase, and the pyruvate amendment increased the initial rate and extent of the ^{14}C incorporation. Upon switching to aerobic conditions, the treatment that was initially amended with pyruvate (Figure 3B) remained relatively stable until the end of the experiment (104d), with no sustained large increase in the fraction of radioactivity incorporated into humin. On the other hand, the endogenous treatment, continued to have ^{14}C incorporated into the humin fraction during the aerobic phase, albeit at a third of the rate of during its anaerobic period. By day 104, both of these treatments had a comparable fraction of the ^{14}C label incorporated into soil humin, ranging between 63-65% of the total label.

Sequential anaerobic-aerobic treatment has been proposed for nitroaromatic compounds either for mineralization or for composting in soils, with the latter being a strategy used for TNT.[28] However, in the findings here for DNAN, most of the incorporation occurred during the anaerobic phase. Despite the continued incorporation during the post aerobic phase, the rate of ^{14}C-incorporation was not improved by switching from anaerobic to aerobic conditions. One of the sequential anaerobic-aerobic treatments included HRP, which was applied to catalyze condensation reactions between aromatic amines[29, 30] and to promote incorporation of TNT metabolites into soil humus.[18, 31] Addition of HRP with H_2O_2, resulted in increasing the incorporation to the humin fraction almost instantaneously from 40 to 49.5% (Figure 3C). After the HRP addition, humin incorporation continued to increase gradually, reaching 58.8% by day 104. Nonetheless, the net benefit (final humin incorporation) compared to the other treatments was not sufficient to pursue HRP addition as a technology.

3.5 Humin and organic carbon addition for higher incorporation

Throughout all the DNAN biotransformation assays performed in the present work under different redox conditions and amendments, ^{14}C was incorporated to a large extent into the humin fraction of the soil humus. Therefore, a range of soil quantities were used to analyze the role of the amount of humin on ^{14}C incorporation, and in another experiment, purified humin was added to the soil. In these experiments, incubations proceeded until ^{14}C incorporation ceased (Figure 4). Increasing the amount of soil used in the biotransformation assays influenced greatly the amount of radiolabeled incorporated. The highest amount of label incorporated into the humin fraction (78.6%) was achieved with 50 g L^{-1} of CN, which was 3.7× higher than the treatment with the lowest amount of CN soil, 5 g L^{-1}. Similarly, addition of purified humin to CB soil, resulted in a higher incorporation of ^{14}C label (Figure 4B). Adding 20 mg of purified humin resulted in the incorporation of 66.8% of the label, while using the unamended soil resulted in only 26.4 % ^{14}C incorporation.

Based on these observations, addition of soil OC (via soil or isolated humin) increased the amount of irreversible binding of DNAN products into the soil. All the treatments in the present work and preliminary experiments with similar redox conditions and incubation time (anaerobic, 40 d) were compiled in Figure 5. As seen, there is a strong positive linear correlation (R^2= 0.95) between the ratio of the OC mass to initial DNAN mass and the percentage of the ^{14}C radiolabel incorporated into the humin fraction (as operationally defined by label recovery from Harvey Oxidizer combustion of exhaustively extracted samples). The relationship holds very well up to 0.8 mg OC / mg DNAN. The plateau suggests saturation of reactive amine groups in reduced DNAN products (e.g. DAAN) by excess OC. Further increments in the OC/DNAN ratio do not increase incorporation of ^{14}C into humin,

3.6 Reaction DAAN with model quinone

To demonstrate the reaction of DAAN with quinone moieties in humus, DAAN was incubated together with 1,4-benzoquinone (BQ). As shown in Figure 6, DAAN was immediately completely removed from solution within 4 min. This reaction coincided with the formation of color, and insoluble materials as can be seen in photographs (SI, Figure S3) and coincided with the loss of dissolved organic carbon (SI, Figure S4). The color change and DAAN removal occurred while tubes were sealed and when BQ was being reduced to HQ, suggesting the reaction of DAAN incorporation into humus was taking place under reducing conditions. No DAAN removal, color change or drop in DOC was observed in controls where DAAN was either incubated alone or together with 1,4-hydroquinone (HQ). HQ was a control since it is not expected to participate in nucleophilic substitution reactions with amines. A similar experiment was conducted by incubating DAAN with the humin preparation. In this experiment, DAAN was also completely removed by humin within 4 min. (SI, Figure S5).

Reduction of all nitro-groups in DNAN and similar compounds might require the lower redox potentials in anaerobic conditions, such as in the case of TNT.[32] The complete reduction of highly nitrated aromatic molecules to amines might be the limiting reaction step to forming azo dimers or bound residue. The current paradigm for recalcitrant

nitroaromatics, such as TNT, has been the assumption that aerobic conditions are needed to polymerize their aromatic amine products to irreversibly bind them into soil organic matter.[33] However, aromatic amines reacted with soil humus when different aromatic amine structures underwent amine nucleophilic substitution with quinones.[23, 24, 34] Abiotic and biotic components in the soil could enable aromatic amine incorporation to soil humin in the absence of oxidizing conditions. Our work on DNAN shows that aerobic reactions are not necessary to irreversibly bind degradation products or to form oligomers.[6] Synthesis of azo dimers under reductive conditions from aromatic amines and reduced nitroaromatic intermediates[35, 36] has been reported, supporting that oxidizing conditions might not be necessary for azo formation.

3.7 Implications for science

Increased incorporation into soil humin of DNAN occurred under anaerobic conditions, with increased rates and extents than those under aerobic conditions. An important prerequisite for bound residue is the formation of aromatic amines that can participate in substitution reactions with each other (forming azo oligomers) [6, 11] or with quinones and quinone moieties in humus yielding anilinohydroquinone adducts[24, 34], and humic substances.[23, 24]

3.8 Implications for DNAN bioremediation

As DNAN-based IMC formulations are used in the environment, affordable and effective remediation strategies are needed. While DNAN is readily reductively biotransformed, complete mineralization is not common in most soils, and irreversible binding to soil OC (intrinsic or amended) could be a promising remediation and pollution control strategy. Our findings show that DNAN biotransformation products are irreversibly bound to soil and OC is a strong predictor of the level of incorporation of DNAN biotransformation products into the insoluble fraction of humus (humin). The OC to DNAN ratio in a soil could potentially be used to estimate a maximum DNAN load that a given soil can irreversibly attenuate. If the intrinsic OC levels are too low, the irreversible attenuation capacity can be improved by adding even more OC in the form of compost or peat to the soil. Our observations show that DNAN could be remediated as the common practice in situ solid-phase treatment (i.e. composting) of TNT via reduction of nitro groups and subsequent humus incorporation.[33, 37, 38] Additional work on the mechanism of soil incorporation of reduced DNAN products could help optimize DNAN remediation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was supported by the Strategic Environmental Research and Development Program (SERDP) project ER-2221 and NSF CBET 1510698. Analyses performed at the Arizona Laboratory for Emerging Contaminants (ALEC) were supported by NSF CBET 0722579, AB Sciex, and additional funding from University of Arizona colleges. CIO was supported by the Mexican National Council for Science and Technology (CONACyT) and the NIEHS Superfund Research Program (P42 ES04940). CLM was supported by 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). The authors thank Stanley Wong for assisting in the research.

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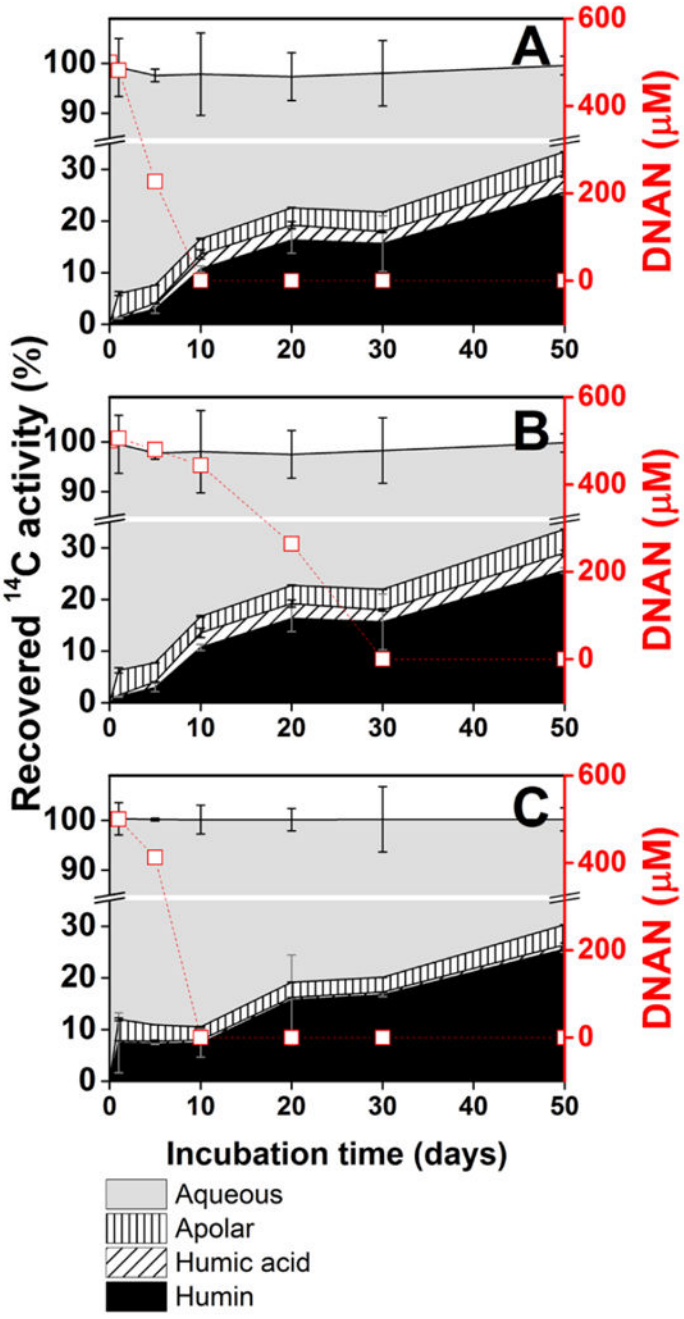

Fig. 1.

Fractionation of ^{14}C-DNAN into different soil components during DNAN (■) anaerobic (bio)transformation in soils and sludge amended with 10 mM pyruvate. Panel A: CN soil (56.7 μmol DNAN dwt soil^{-1} d^{-1}). Panel B: CB soil (14.3 μmol DNAN dwt soil^{-1} d^{-1}). Panel C: anaerobic sludge (45.6 μmol DNAN dwt sludge^{-1} d^{-1}). No detectable ^{14}C label was obtained in the gas phase (volatile organic compounds (VOCs) and CO_2). In this experiment, the aqueous samples were not corrected for scintillation quenching due to coloration.

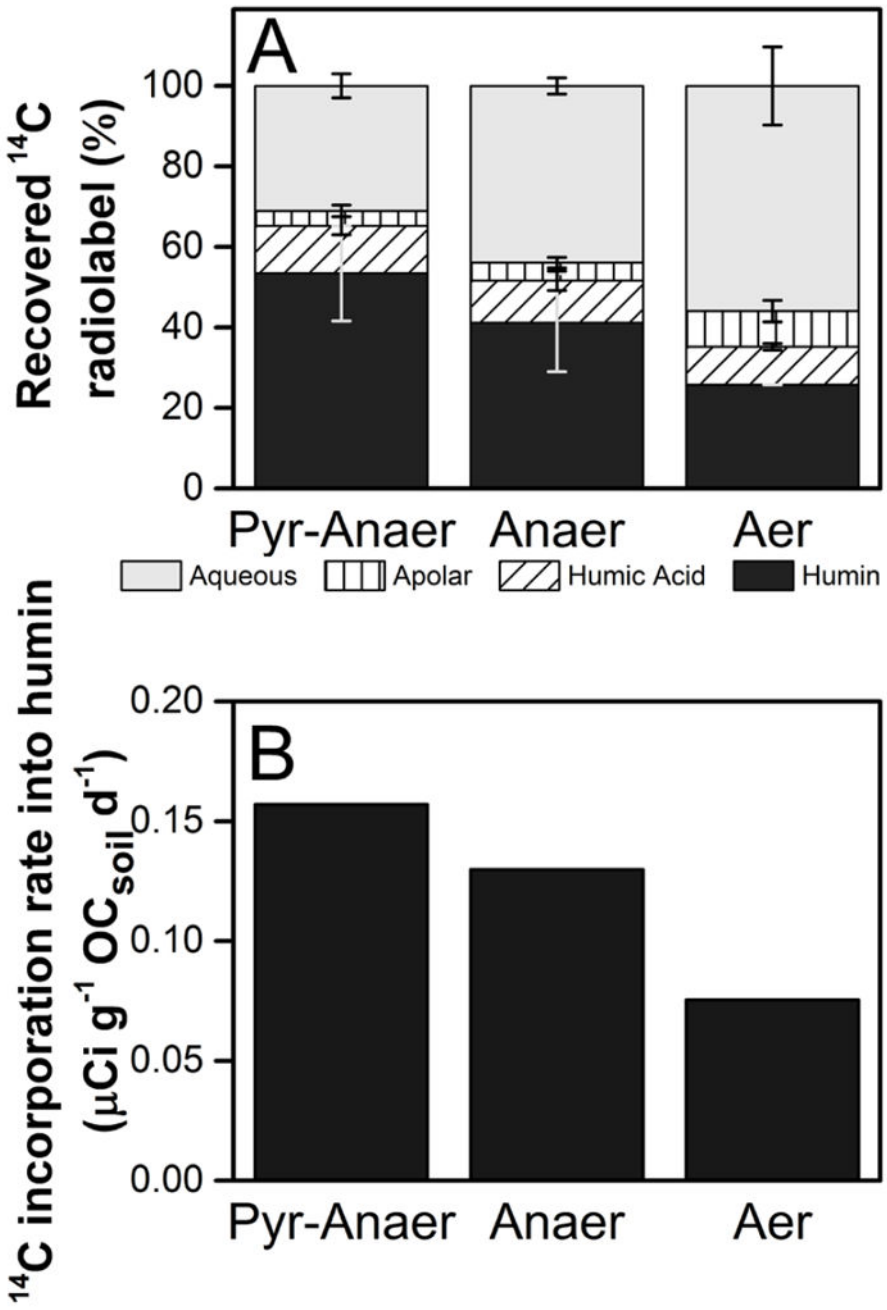

Fig. 2.

Impact of redox conditions on extent and rate of ^{14}C-ring DNAN fractionation into during (bio)transformation in CN soil (25 g L^{-1}). Treatments included: 10 mM pyruvate amended anaerobic (Pyr-Anaer); endogenous anaerobic without pyruvate added (Anaer); and endogenous aerobic without pyruvate added (Aer). Panel A: ^{14}C label distribution at 40 days of incubation. Panel B: ^{14}C-incorporation rate (μCi g^{-1} OC$_{soil}$ d^{-1}) into the humin (0-40d, period of maximum slope). Humin was measured with $^{14}CO_2$ from the combustion of the residual soil in a Harvey Oxidizer after all the sequential extractions.

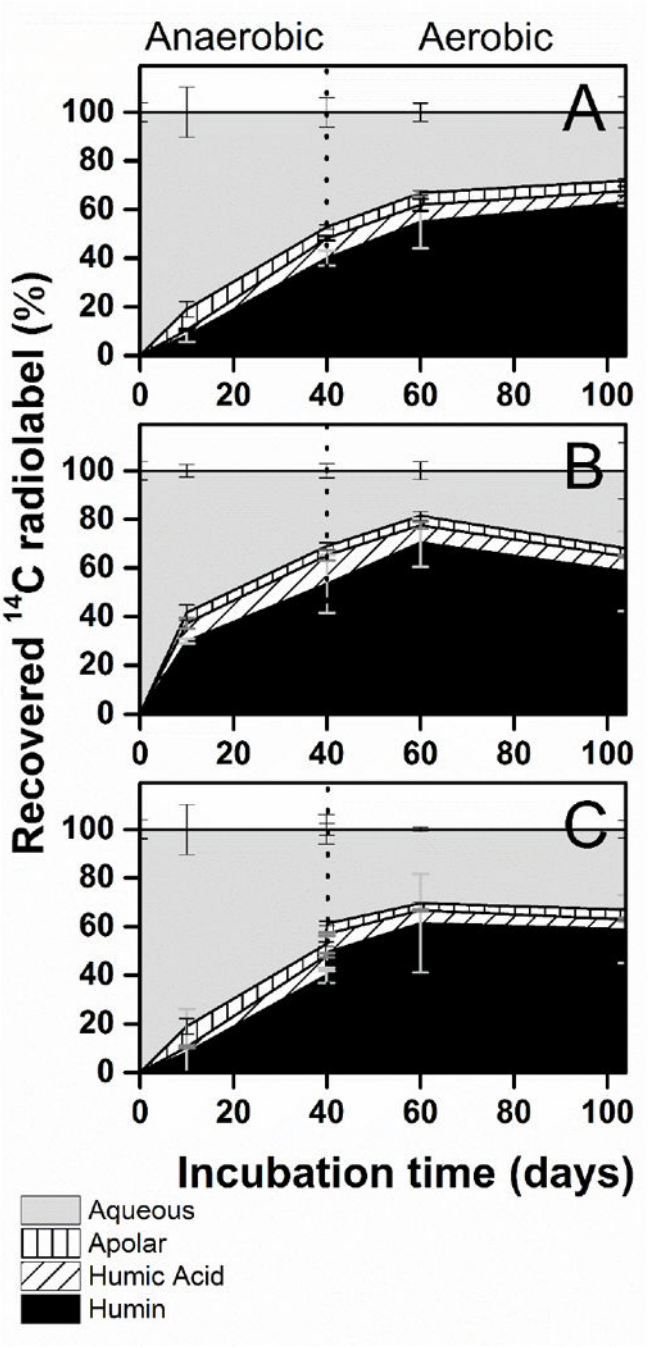

Fig. 3.

Effect of anaerobic/aerobic sequential treatments on ^{14}C-DNAN incorporation into soil fractions during sequential anaerobic(0-40d)-aerobic(40-104d) biotransformation of ^{14}C-ring DNAN in CN soil (25 g L^{-1}). Panel A: endogenous (no pyruvate added). Panel B:amended with 10 mM pyruvate at day 0. Panel C: endogenous with horseradish peroxidase (HRP) and H_2O_2 added at day 40.

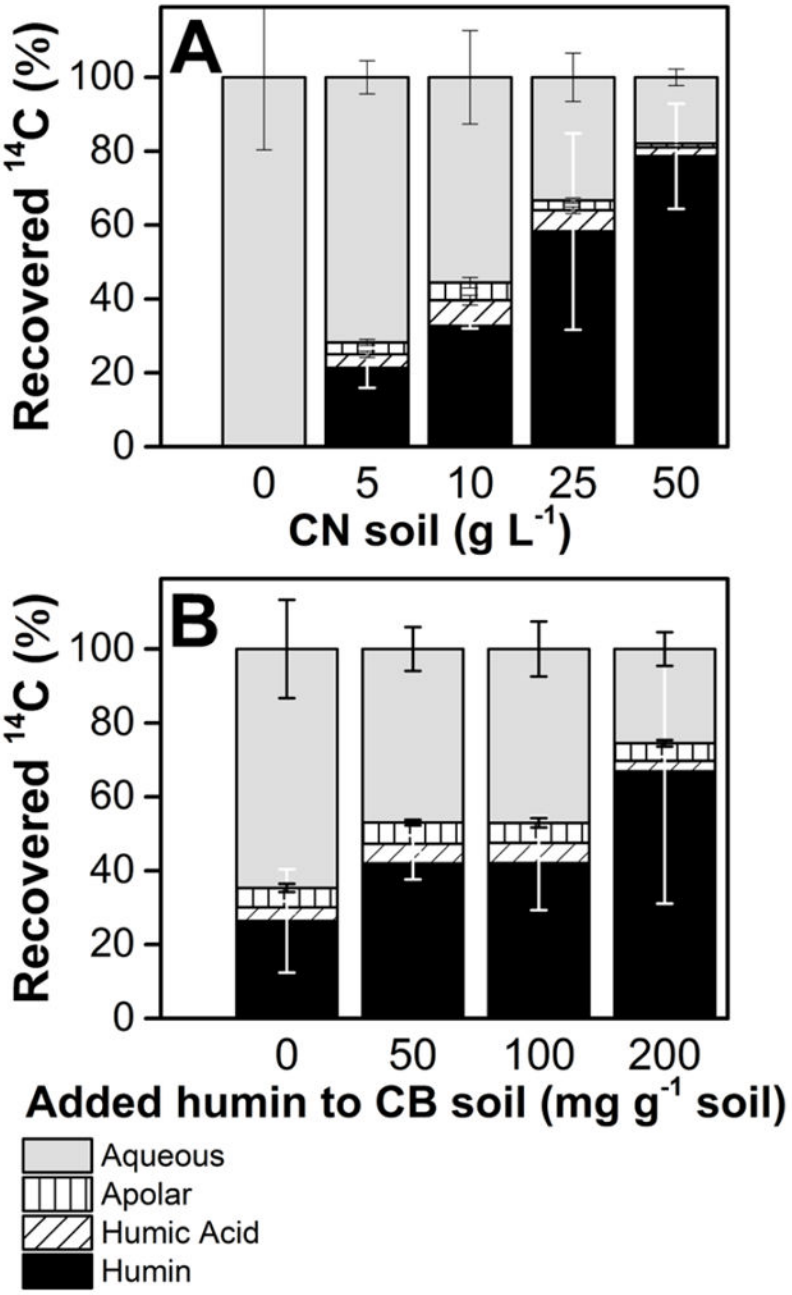

Fig. 4.

Increased incorporation of ^{14}C into soil with increased organic carbon added as natural soil or humin-amended soil during the course of DNAN biotransformation amended with 10 mM pyruvate in anaerobic conditions. Panel A: Incubations with different CN amounts of soil extracted at day 40. Panel B: Incubations with CB soil (25 g L^{-1}) and different amounts of purified humin added.

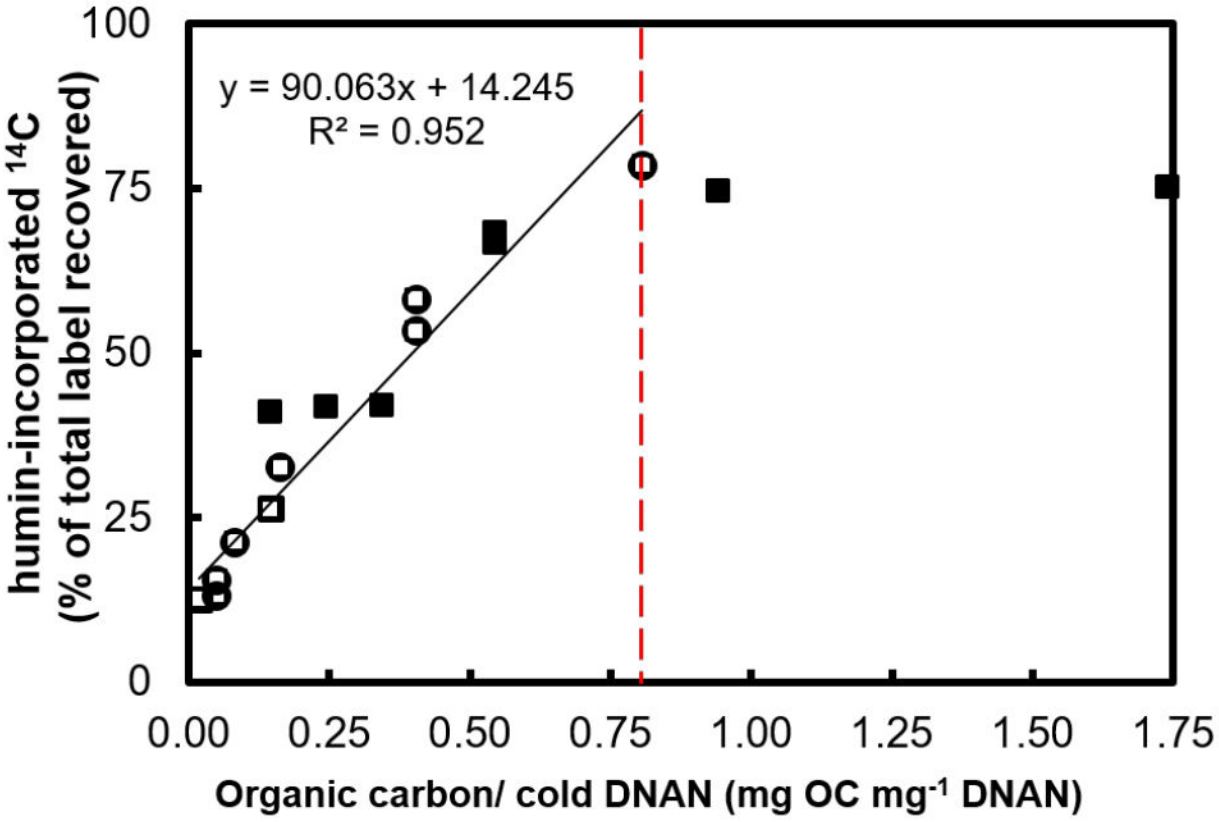

Fig. 5.

Correlation of organic carbon to DNAN ratio with ^{14}C-ring labeled DNAN incorporation into humin extracted at 30-50d of anaerobic incubation amended with 10 mM pyruvate. Incubations with CN soil (○), CB soil (□), CB soil amended with humin: experiment 1 (■) and experiment 2 (▲).

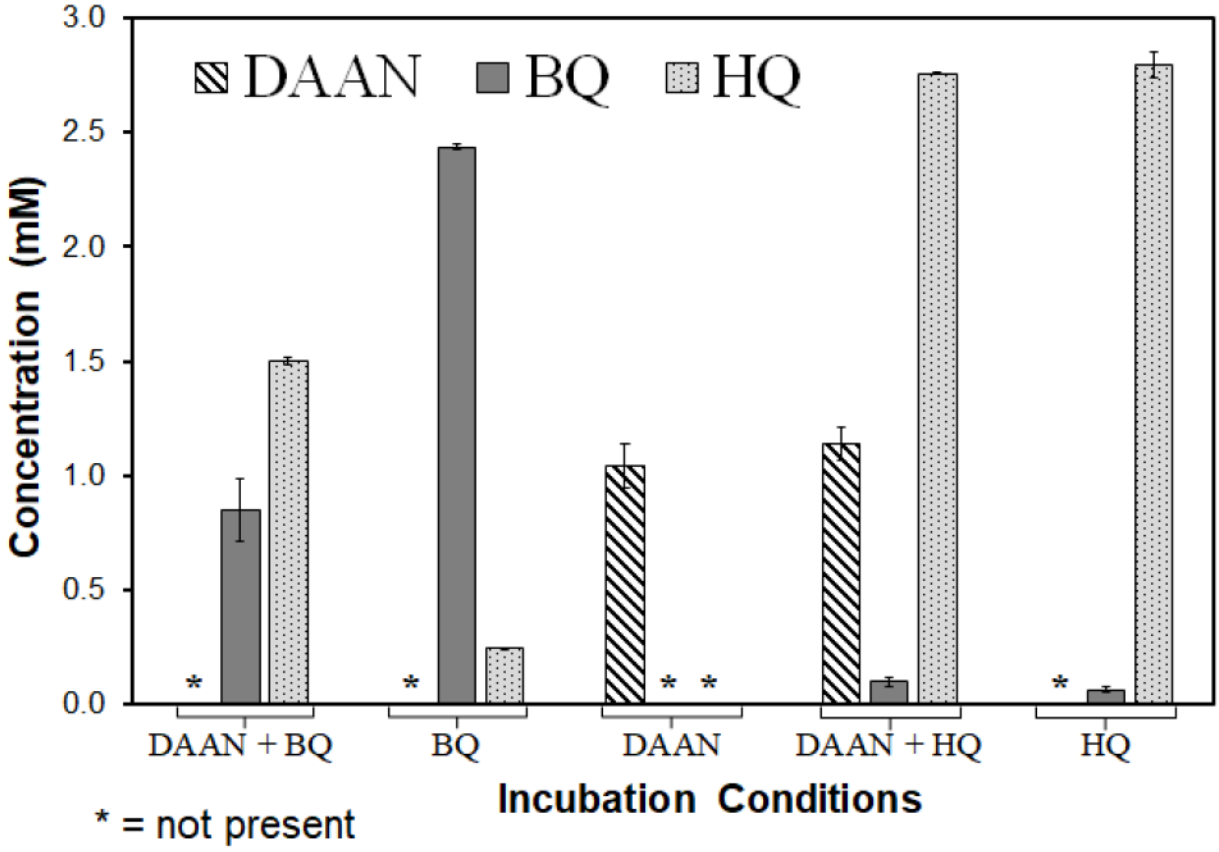

Fig. 6.

Reaction of DAAN (1.0 mM) with BQ (2.9 mM) and lack of reaction with HQ (2.9 mM) at pH 4.7 as measured by UHPLC-DAD. Legend: DAAN+BQ = DAAN and BQ incubated together; and DAAN+HQ = DAAN and HQ incubated together. BQ, HQ and DAAN refer to each of the compounds incubated alone.