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Covalent binding with model quinone compounds unveils the environmental fate of the insensitive munitions reduced product 2,4-diaminoanisole (DAAN) under anoxic conditions

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ABSTRACT

2,4-Dinitroanisole (DNAN) is an insensitive munitions compound expected to replace 2,4,6-trinitrotoluene (TNT). The product of DNAN's reduction in the environment is 2,4-diaminoanisole (DAAN), a toxic and carcinogenic aromatic amine. DAAN is known to become irreversibly incorporated into soil natural organic matter (NOM) after DNAN's reduction. Herein, we investigate the reactions between DAAN and NOM under anoxic conditions, using 1,4-benzoquinone (BQ) and methoxybenzoquinone (MBQ) as model humic moieties of NOM. A new method stopped the fast reactions between DAAN and quinones, capturing the fleeting intermediates. We observed that DAAN incorporation into NOM (represented by BQ and MBQ models) is quinone-dependent and occurs via Michael addition, imine (Schiff-base) formation, and azo bond formation. After dimers are formed, incorporation reactions continue, resulting in trimers and tetramers. After 20 days, 56.4% of dissolved organic carbon from a mixture of DAAN (1 mM) and MBQ (3 mM) had precipitated, indicating an extensive polymerization, with DAAN becoming incorporated into high-molecular-weight humic-like compounds. The present work suggests a new approach for DNAN environmental remediation, in which DNAN anaerobic transformation can be coupled to the formation of non-extractable bound DAAN residues in soil organic matter. This process does not require aerobic conditions nor a specific catalyst.

1. Introduction

The defense industry is replacing conventional explosives with insensitive munitions compounds (IMCs). "Insensitive" means that those compounds are more resistant to unintentional detonation caused by mechanical shocks or high temperatures than conventional explosive constituents (Sikder and Sikder, 2004; Boddu et al., 2008). One of the most important IMCs is 2,4-dinitroanisole (DNAN), which has been replacing 2,4,6-trinitrotoluene (TNT) in munitions formulations (Davies and Provatas, 2006). Unfortunately, DNAN represents an environmental threat, being reported to be toxic to methanogens, nitrifying bacteria, bioluminescent bacteria (Liang et al., 2013), algae, ryegrass, earthworms (Dodard et al., 2013), and zebrafish (Olivares et al., 2016b).

At military firing ranges, chunks of undetonated ordnance can remain in the soil, be dissolved by precipitation, and reach ground and surface water (Taylor et al., 2015). The solubility of DNAN (276 mg L^{-1}) (Boddu et al., 2008) is in the same order of magnitude as that of TNT (100 mg L^{-1}) (Brannon and Pennington, 2002). Such contamination can last for long periods since the decomposition of nitroaromatic compounds under aerobic conditions is not reliable (Amaral et al., 2009). On the other hand, a diversity of microorganisms (Olivares et al., 2016a; Hawari et al., 2015) and some reactive minerals (Khatiwada et al., 2018; Shen et al., 2013) can promote the reduction of nitro groups under anaerobic conditions, producing aromatic amines. This transformation has crucial implications for DNAN's environmental fate.

The reduction of DNAN's ortho nitro group produces 2-methoxy-5-nitroaniline (MENA), while the reduction of the para nitro group produces 4-methoxy-5-nitroaniline (iMENA) (Olivares et al., 2016a; Hawari

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et al., 2015). Further reduction of the remaining nitro group of MENA or iMENA forms 2,4-diaminoanisole (DAAN), an aromatic amine toxic to many microorganisms (Liang et al., 2013; Olivares et al., 2016b) and carcinogenic to mammals (Aune and Dybing, 1979; Ames et al., 1975). Although DNAN reduction to DAAN by biotic and abiotic pathways is well known, DAAN's ultimate environmental fate is not well understood.

In many soil experiments, DAAN does not accumulate after MENA or iMENA reduction. Instead, this compound disappears after a few days (Olivares et al., 2016a, 2013; Hawari et al., 2015). Attempts to recover sorbed DAAN in soil failed, indicating covalent incorporation (Hawari et al., 2015). Furthermore, ^{14}C from ^{14}C radiolabeled DNAN was shown to become irreversibly incorporated into the soil's humin fraction after anaerobic reduction (Olivares et al., 2017). A possible explanation is that the amino groups on DAAN covalently react with quinone moieties of natural organic matter (NOM), as shown for other aromatic amines (Gulkowska et al., 2012; Thorn et al., 1996b). The reactions that govern aromatic amines coupling with quinones are Michael addition and imine formation (Parris, 1980; Kutyrev, 1991). Previous works illustrate these mechanisms by showing that benzidine or 4-methylaniline (aromatic amines) reacted with the quinone compound 2,6-dimethyl-1,4-benzoquinone, producing the corresponding Michael adducts and imines (Ononye et al., 1989; Ononye and Graveel, 1994). Indeed, quinone groups of NOM seem to drive many environmentally relevant redox reactions (Uchimiya and Stone, 2009).

Strong evidence demonstrates that quinones are important moieties of NOM (Uchimiya and Stone, 2009). Firstly, NOM can undergo reversible redox reactions quantified with electron carrier capacity (Uchimiya and Stone, 2009; Ratasuk and Nanny, 2007). Secondly, cyclic voltammetry of NOM has been shown to behave very similarly to cyclic voltammetry of model quinone compounds (Nurmi and Tratnyek, 2002). Semiquinone radicals derived from the one-electron reduction of quinones or the one-electron oxidation of hydroquinones can be detected in NOM by electron paramagnetic resonance (Scott et al., 1998). Such detection is correlated with the reversible redox capacity of the NOM. Additionally, hydroxylamine-treated NOM generates resonances consistent with monoximes attributable to the tautomeric equilibrium between the nitrosophenol and monoxime derivatives of quinones (Thorn et al., 1992), constituting strong evidence for quinones in NOM.

Due to the complexity of NOM, the use of small molecules as models is an efficient way to assess the substitution reactions between aromatic amines, such as DAAN, and quinone moieties present in NOM. Model quinone compounds share similar redox properties, and the results of studies applying one of them can be extrapolated to the others. Methoxybenzoquinone (MBQ) is a model quinone compound worthy of special attention since it is an intermediate of lignin degradation and a potential constituent of NOM (Buswell et al., 1979; Kirk and Lorenz, 1973; Yuan et al., 2016). Also, MBQ was the most susceptible to oxidoreductase enzymes activity among a group of humic model quinones (Buswell et al., 1979).

This study's objective was to investigate the abiotic pathways of DAAN irreversible incorporation into NOM via anoxic reactions with quinones. To this end, we evaluated the formation of oligomers from reactions between DAAN and two model humic constituents, 1,4-benzoquinone (BQ) and methoxybenzoquinone (MBQ). However, those reactions take place in seconds. To work around this problem, we developed a new method to capture fleeting intermediates for examination. This new approach allowed for the first time a comprehensive look at the mechanisms leading to DAAN disappearance in the environment. An additional objective was to assess the formation of high-molecular-weight insoluble polymers from DAAN reactions with quinones.

2. Materials and methods

2.1. Chemicals

We purchased 2,4-diaminoanisole (DAAN, CAS # 615-05-4, 98+% purity) and 1,4-benzoquinone (BQ, CAS # 106-51-4, 98+% purity) from Sigma-Aldrich (Saint Louis, MO, USA). DAAN was furtherly purified as described by Rahaim and Maleczka (2006) to eliminate any possible product of DAAN decomposition before our experiments. Nuclear magnetic resonance (NMR) spectra of the purified DAAN appear in the Supplementary Information (SI, Figs. S-1 and S-2). 1,4-Hydroquinone (HQ, CAS # 123-31-9, 99.5% purity) was purchased from Acros Organics (Morris Plains, NJ, USA). Methoxybenzoquinone (MBQ, CAS # 2880-58-2, 99% purity) and methoxyhydroquinone (MHQ, CAS # 824-46-4, 98% purity) were purchased from TCI America (Portland, OR, USA).

2.2. DAAN incorporation assays

2.2.1. Pairing DAAN and quinones

We performed experiments pairing DAAN with BQ or MBQ using sacrificial test tubes. In all experiments, the pH was held at 5 using a phosphate buffer (13.8 mM of KH_2PO_4 and 16.2 mM of K_2HPO_4). We added 4.5 mL of BQ or MBQ concentrated aqueous solutions (final concentration ranging from 0.3 to 3 mM depending on the experiment) into 25 mL test tubes. We purged the oxygen by flushing the liquid for 4 min with N_2. Then we closed the test tubes with t-butyl caps and aluminum seals and needle flushed the headspace for an additional 4 min. DAAN concentrated solution was prepared and stored in a serum bottle, also flushed with N_2, and sealed to avoid oxygen contamination. The reaction started when we added 0.5 mL of DAAN concentrated solution into the test tubes containing either BQ or MBQ and vortexed immediately. DAAN's added concentration varied from 0.3 to 3.0 mM in our experiments.

To stop the reaction, we added 15 mL of acetonitrile after the appropriate reaction time, vortexed, and froze the sacrificial test tubes at − 80 °C until analysis. Control experiments to verify this method's ability to stop the reactions were performed and can be found in the SI (Fig. S-3). The reaction times varied from 6 s to 15 min. We also performed control experiments with separated DAAN, BQ, HQ, MBQ, and MHQ, and paired DAAN and HQ, or DAAN and MHQ.

2.2.2. Polymerization assay

We performed an experiment pairing DAAN and MBQ to assess the formation of insoluble products over a longer period of time (20 days). We added 26 mL of MBQ concentrated solution (4.6 mM for a final concentration of 3 mM) with the same phosphate buffer described in Section 2.2.1 to 45 mL serum bottles in which oxygen was also purged by flushing with N_2. Then, 4 mL of DAAN concentrated solution (10 mM for a final concentration of 1 mM, previously stored under anoxic conditions) was added to the bottles, which were kept in the dark. Subsamples were removed at appropriate times with syringe and needle and filtered immediately using a 0.22 μm membrane (Millex™ Nonsterile 33 mm Syringe Filters, MilliporeSigma, Burlington, MA, USA) to remove suspended organic carbon. The filter was coupled to the syringe used to collect samples to minimize sample exposure to oxygen before filtration. We diluted the filtered samples four times with ultrapure water and froze them at − 80 °C until analysis. Anoxic control experiments with separated DAAN and MBQ, and paired DAAN and MHQ were also performed. All experiments were performed in duplicate at 23 ± 1 °C.

2.3. Analytical methods

2.3.1. UHPLC – DAD

We analyzed the samples from DAAN-BQ and DAAN-MBQ pairing experiments with UHPLC-DAD (Agilent 1290 Infinity, Santa Clara, CA,

USA). We used a Zorbax SB-C18 column (4.6 × 150 mm, 5 μm; Agilent, Santa Clara, CA, USA). The mobile phase ran isocratically (0.5 mL min^{-1}) at 36 °C for 31.0 min with a gradient of acetonitrile/H_2O (v/v%) in as follows: from 0.0 to 3.0 min held at 5/95, from 3.0 to 18.0 min increasing to 90/10, from 18.0 to 18.5 increasing to 98/2, from 18.5 to 26.0 held at 98/2, from 26.0 to 27.0 decreasing to 5/95, and from 27.0 to 31.0 held at 5/95. The sample injection volume was 20 μL. The compounds were detected at the following retention times and wavelengths: DAAN (10.8 min, 300 nm), BQ (10.6 min, 300 nm), HQ (8.0 min, 290 nm), MBQ (11.0 min, 400 nm), and MHQ (9.3 min, 290 nm). We prepared standards using a 75/25% mixture of acetonitrile/H_2O.

2.3.2. UHPLC-HRAM-MS/MS

We prepared a comprehensive compound library using ChemDraw (PerkinElmer Informatics) including masses of products from different hypothetical reactions between DAAN and quinones to be used in a targeted UHPLC-HRAM-MS/MS analysis of reaction mixtures. Imine formation, Michael addition (Parris, 1980; Kutyrev, 1991), and azo bond formation (Hawari et al., 2015) were the main reactions considered. Derived compounds from DAAN previously found during DNAN biotransformation in soil or sludge were also included (Olivares et al., 2016a, 2013), as well as the possible products of reactions between those compounds and DAAN or quinones. The library included structures ranging from dimers to heptamers (seven monomer units), which totaled 167 different masses.

UHPLC-HRAM-MS/MS (UltiMate 3000 UHPLC, Dionex, Sunnyvale, CA, USA coupled to a Q Exactive Focus Orbitrap mass spectrometer, Thermo Scientific, San Jose, CA, USA) was used to detect targeted compounds in our samples. Chromatographic conditions are described in Section 2.3.1. Positive mode electrospray ionization at 380 °C with a capillary setting of 4.0 kV was used with N_2 at flow rates at 60 for sheath gas and 20 for auxiliary gas in the mass spectrometer ion source. Product ion spectra were acquired using a collision energy of 10 eV. High resolution (35 K) accurate mass measurements for precursors ions $[M + H]^+$ were sought in a survey scan (m/z 90–900 Da). We used Excalibur 4.1.31.9 and TraceFinder 4.1 to process data and identify mass chromatogram peaks.

The instrument was externally calibrated within 1 week of measurements across the mass range of interest (standard deviation < 0.33 ppm) by infusion of Thermo Scientific™ Pierce™ Negative Ion Calibration Solution.

2.3.3. DOC measurements

We measured dissolved organic carbon (DOC) using a Shimadzu Total Carbon Analyzer VCSH (Columbia, MD, USA). We added concentrated hydrochloric acid (HCl) to the samples to adjust the pH to 2. Inorganic carbon was purged by sparging the samples with air using the equipment default configuration. Then, the samples' organic carbon content (filtered immediately after sampling, as explained in Section 2.2.2) was combusted at 680 °C and quantified. Standards were prepared using potassium hydrogen phthalate ($C_8H_5KO_4$) in the appropriate concentration range.

3. Results and discussion

3.1. Transformation of DAAN and quinones

Initially, we tested the reactions between DAAN and BQ (Fig. 1). BQ was partially reduced to HQ. The combination of DAAN and BQ seemed highly reactive since we did not detect DAAN after 6 s nor BQ and HQ after 3 min. We also observed an increase in color and turbidity within the first seconds of reaction. In parallel, we performed controls with DAAN, BQ, and HQ incubated individually, and with DAAN and HQ incubated together (shown in SI, Fig. S-4). The concentrations of DAAN, BQ, and HQ were stable in the controls.

To seek a less reactive system than the combination of DAAN and BQ,

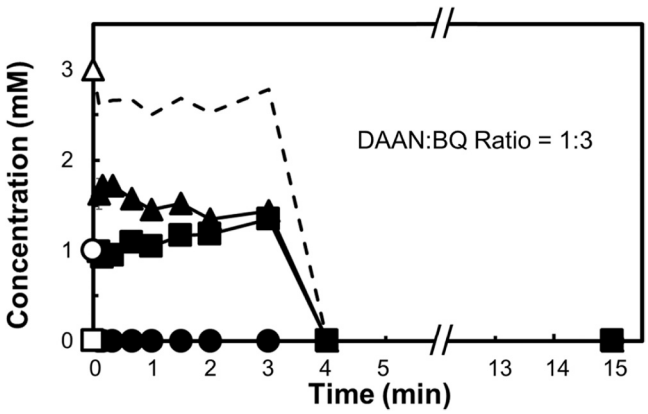

Fig. 1. Concentrations of DAAN (●, ○) BQ (▲, △), and HQ (■, □) incubated in anaerobic conditions. Open shapes refer to the added concentrations of the compounds, while closed shapes refer to measured concentrations. The dashed line represents the sum of BQ and HQ. DAAN was entirely consumed in less than 6 s.

we tested the DAAN reaction with MBQ, a quinone compound with fewer atoms available for nucleophilic attack than BQ. Additionally, there is strong evidence that MBQ is a reliable model for NOM (Buswell et al., 1979; Kirk and Lorenz, 1973; Yuan et al., 2016). We reacted DAAN with MBQ in three different molar ratios, as seen in Fig. 2. When the DAAN:MBQ molar ratio was 1:3 or 1:10, DAAN was again entirely consumed in 6 s or less, while MHQ was formed as a product of MBQ reduction. When the DAAN:MBQ molar ratio was 3:1, the reaction entirely consumed MBQ within 6 s, and MHQ was not detected. In the latter case, DAAN was partially consumed in 6 s, then formed again, possibly due to reversible reactions discussed in Section 3.2. The gradual formation of colored reaction mixtures (shown in SI, Fig. S-5) was observed. Controls with MBQ and MHQ incubated individually and with DAAN and MHQ incubated together did not exhibit a change in the concentration of the analyzed compounds (shown in SI, Figs. S-6 and S-7).

We observed that the DAAN anoxic disappearance was quinone-dependent since pairing DAAN with the reduced forms HQ or MHQ did not lead to DAAN consumption. Moreover, the quinones were not wholly recovered as phenols (Figs. 1 and 2), giving further evidence that they participated in covalent coupling reactions with DAAN. For aerobic conditions, previous studies (Gulkowska et al., 2012, 2013; Thorn et al., 1996b; Thorn and Kennedy, 2002) already indicated that aromatic amines covalently bind to carbonyl groups in NOM. These carbonyl groups are ketone and quinone moieties naturally present in NOM or produced by oxidation of phenolic groups by phenoloxidase enzymes or metal oxides. In this work, however, we studied the DAAN incorporation in anoxic conditions. Furthermore, we used the model quinone compounds directly, without enzymes or other oxidants. Our first results support the findings of Olivares et al. (2017), which suggested DAAN could anoxically bind with NOM in soil. Our next step was to analyze the products of such reactions.

3.2. Identified products and mechanisms of reactions

We analyzed the formed products of the DAAN reactions with BQ and MBQ using a UHPLC-HRAM-MS/MS (Tables 1 and 2). Our approach to stop the reactions at different time points allowed us to detect different products in a range of molecular weights from 229 to 529 g mol^{-1}. Such findings represent the first time that the products of DAAN reaction with quinones were identified. We tentatively assigned the identified products with the most probable structure based on previous studies with DAAN, a strong body of literature about other aromatic amines, and the steric effects expected from the molecules. Figs. 3 and 4 show the

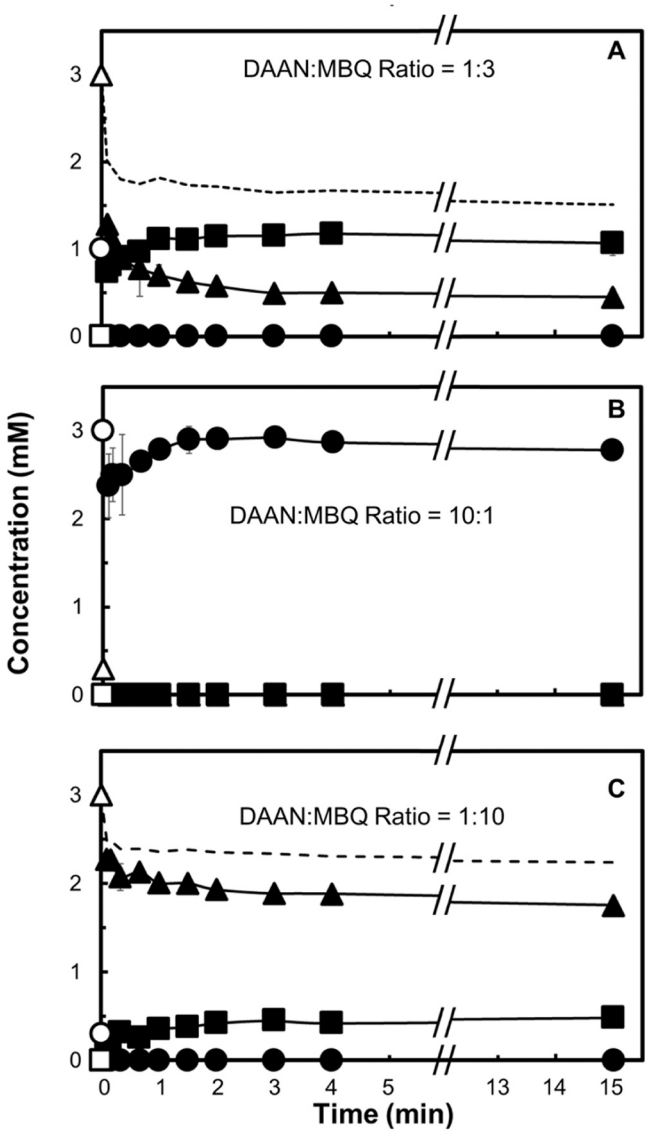

Fig. 2. Concentrations of DAAN (●, ○) MBQ (▲, △) and MHQ (■, □) incubated in anaerobic conditions. Open shapes refer to the added concentrations of the compounds, while closed shapes refer to measured concentrations. The dashed line represents MBQ and MHQ summed. DAAN was entirely consumed in less than 6 s when the DAAN:MBQ molar ratio was 1:3 (panel A) or 1:10 (panel B). MBQ was totally consumed in less than 6 s when the DAAN:MBQ molar ratio was 10:1.

proposed structures. The compounds **F** and **P** had masses that could be assigned to alternative structures (shown in SI, Fig. S-8). However, in the literature, such alternative products have only been attested when nitroso intermediates of DNAN (DAAN's parent compound in the environment) are present in the system (Olivares et al., 2016a; Kadoya et al., 2018), which is not the case in this study.

As shown in Figs. 3 and 4, the identified products originated from a mixture of reactions with multiple combinations. DAAN reacted with both BQ and MBQ resulting in Michael adducts, imines, and azo oligomers. We show semi-quantitative data corresponding to the peak areas in the SI.

A proposed mechanism for the formation of Michael adducts (compounds **B**, **E**, **F**, **G**, **I**, **J**, **L**, **M**, **N**, and **P**) is shown in Fig. 5 (scheme B). Michael addition forms strong bonds that are not easily reversible under environmental conditions (Gulkowska et al., 2012; Parris, 1980; Hsu and Bartha, 1976). After the nucleophilic addition, the remaining quinones probably oxidized the phenol groups in the Michael adducts. This

Table 1
Summary of adducts detected in anaerobic incubation of DAAN with benzoquinone (BQ) via UHPLC-HRAM-MS/MS.

Structure in Fig. 5	Molecular formula [M]	Monoisotopic mass [M + H][a]	Measured mass [M + H][b]	Retention time (min)[c]	[Delta] (ppm)[d]	Spectral data (Int.)	Incubation times in which it was observed
(A)	$C_{13}H_{12}N_2O_2$	229.0972	229.0963–229.0970	14.1–14.2	0.98	213.0656 (24.2), 214.0736 (36.16), 228.0889 (41.92), 229.0969 (100.0)	6 s, 10 s, 20 s, 40 s, 1 min, 1 min 30 s, 2 min, 3 min, 4 min, 15 min
(B)	$C_{13}H_{12}N_2O_3$	245.0921	245.0914–245.0919	15.3–15.4	0.80	213.0655 (28.7), 214.0734 (77.7), 215.0770 (8.5), 231.0762 (7.1), 245.0916 (100.0)	6 s, 10 s, 20 s, 40 s, 1 min, 1 min 30 s, 2 min, 3 min, 4 min, 15 min
(C)	$C_{14}H_{16}N_4O_2$	273.1346	273.1340–273.1342	17.5–16.7	1.40	242.1155 (71.9), 243.1189 (8.6), 256.1076 (11.9), 273.1340 (100.0)	10 s, 20 s, 40 s, 1 min, 1 min 30 s, 2 min, 3 min, 4 min, 15 min
(D)	$C_{20}H_{18}N_4O_3$	363.1452	363.1425–363.1449	18.3–19.7	0.67	363.1440 (100.0)	6 s, 10 s, 40 s, 1 min, 1 min 30 s, 2 min, 3 min, 4 min, 15 min
(E)	$C_{20}H_{20}N_4O_3$	365.1608	365.1594–365.1602	14.1–14.3	1.57	138.0787 (5.1), 227.0810 (100.0), 228.0845 (10.41), 364.1520 (46.41), 365.1595 (61.0)	6 s, 10 s, 20 s, 40 s, 1 min 30 s
(F)	$C_{20}H_{18}N_4O_4$	379.1401	379.1389–379.1409	17.3–17.6	0.31	272.1263 (6.3), 349.1292 (9.7), 350.1366 (32.9), 351.1406 (5.1), 381.1552 (100.0)	6 s, 1 min 30 s, 2 min, 15 min
(G)	$C_{20}H_{20}N_4O_4$	381.1557	381.1543–381.1566	18.0–18.4	1.91	349.1288 (12.5), 350.1367 (42.6), 364.1288 (5.01), 381.1549 (100.0)	20 s, 40 s, 1 min 30 s, 2 min, 3 min, 4 min, 15 min

[a] Calculated in Xcalibur 4.1.31.9.
[b] Range of masses measured in UHPLC-HRAM-MSMS from all time points.
[c] Range of retention times observed in UHPLC-HRAM-MSMS from all time points.
[d] Other observations matched the compound within 4.65 ppm of the calculated monoisotopic mass.

Table 2
Summary of adducts detected in anaerobic incubation of DAAN with methoxybenzoquinone (MBQ) via UHPLC-HRAM-MS/MS.

Structure in Fig. 6	Molecular formula [M]	Monoisotopic mass [M + H][a]	Measured mass [M + H][b]	Retention time (min)[c]	|Delta| (ppm)[d]	Spectral data (Int.)	Incubation times in which it was observed for different molar ratios (DAAN:MBQ)		
							Ratio 1:3	Ratio 1:10	Ratio 10:1
(C)	$C_{14}H_{16}N_4O_2$	273.1346	273.1340–273.1342	17.5–16.7	0.27	242.1155 (71.9), 243.1189 (8.6), 256.1076 (11.9), 273.1340 (100.0)			6 s, 10 s, 20 s, 40 s, 1 min, 1 min 30 s, 3 min, 4 min, 15 min
(H)	$C_{14}H_{14}N_2O_3$	259.1077	259.1072–259.1075	14.25–14.37	0.68	243.0761 (15.5), 244.0840 (21.7), 258.0995 (48.4), 259.1073 (100.0)	6 s, 10 s, 20 s, 40 s, 1 min, 1 min 30 s, 2 min, 3 min, 4 min, 15 min	6 s, 10 s, 20 s, 40 s, 1 min, 1 min 30 s, 2 min, 3 min, 4 min, 15 min	6 s, 10 s, 40 s, 1 min, 1 min 30 s, 2 min, 3 min, 4 min, 15 min
(I)	$C_{14}H_{14}N_2O_4$	275.1026	275.1020–275.1022	14.85–14.92	1.22	244.0838 (29.5), 275.1020 (100.0)	6 s, 10 s, 20 s, 40 s, 1 min, 1 min 30 s, 2 min, 3 min, 4 min, 15 min	6 s, 10 s, 20 s, 40 s, 1 min, 1 min 30 s, 2 min, 3 min, 4 min, 15 min	6 s, 10 s, 20 s, 40 s, 1 min, 1 min 30 s, 2 min, 3 min, 4 min, 15 min
(J)	$C_{14}H_{16}N_2O_4$	277.1183	277.1159–277.1194	10.67–11.23	1.41	n/a	10 s, 20 s, 40 s, 1 min 30 s, 2 min, 3 min, 15 min		
(K)	$C_{21}H_{20}N_4O_4$	383.1557	393.1539–393.1557	15.17–15.97	0.01	n/a	6 s, 10 s, 20 s, 40 s, 1 min, 2 min, 3 min, 4 min, 15 min		
(L)	$C_{21}H_{18}N_2O_6$	395.1238	395.1222–395.1245	15.18–16.91	0.28	363.0968 (28.7), 364.1042 (28.7), 365.1082 (5.7), 394.1150 (25.2), 395.1229 (100.0)	6 s, 10 s, 20 s, 40 s, 1 min, 1 min 30 s, 2 min, 3 min, 4 min, 15 min		
(M)	$C_{21}H_{18}N_2O_7$	411.1187	411.1175–411.1182	15.06–16.50	1.10	411.1176 (100.0)	6 s, 10 s, 20 s, 40 s, 1 min, 1 min 30 s, 2 min, 3 min, 4 min, 15 min	40 s, 1 min, 1 min 30 s, 3 min, 4 min	15 min
(N)	$C_{21}H_{22}N_2O_7$	415.1500	415.1481–415.1497	13.66–13.69	0.70	414.1412 (23.4), 415.1486 (100.0)	1 min, 1 min 30 s, 3 min, 15 min		
(O)	$C_{28}H_{24}N_4O_6$	513.1769	513.1755–513.1766	16.09–16.80	0.44	513.1762 (100.0)	6 s, 1 min 30 s, 3 min, 15 min		
(P)	$C_{28}H_{24}N_4O_7$	529.1718	529.1712–529.1723	16.28–16.34	0.06	501.1765 (21.1), 528.1635 (7.6), 529.1711 (100.0)	6 s, 10 s, 20 s, 40 s, 1 min, 1 min 30 s, 2 min, 3 min, 4 min, 15 min		

[a] Calculated in Xcalibur 4.1.31.9.
[b] Range of masses measured in UHPLC-HRAM-MSMS from all time points.
[c] Range of retention times observed in UHPLC-HRAM-MSMS from all time points.
[d] Other observations matched the compound within 4.65 ppm of the calculated monoisotopic mass.

mechanism finds support from the work of Uchimiya and Stone (Uchimiya and Stone, 2006), which shows that quinones can oxidize hydroquinones. When DAAN reacted with BQ, compound **B** (Fig. 3) was formed by Michael addition. The initially formed Michael Adduct was probably oxidized to compound **B** too fast to be detected. On the other hand, when DAAN reacted with MBQ, we could detect the initial Michael adduct in the system (compound **J** in Fig. 4), as well as its oxidized product (compound **I** in Fig. 4), giving evidence for the mechanism shown in Fig. 5 (scheme B). Another example is compound **N** (Fig. 4), a trimer formed by Michael additions that has the compound **M** as its oxidized product.

We also observed the formation of imines from the reaction of DAAN with MBQ (Fig. 5, scheme C). This process is often seen as a fast and reversible reaction that does not survive long incubations (Parris, 1980). The reversibility is true for anilines reacting with simple quinones since the equilibrium in aqueous systems favors hydrolysis (Thorn et al., 1996b). However, for aromatic amines reacting with substituted quinones, the imines are quite stable products and, in some instances, are the main form of nucleophilic addition (Ononye et al., 1989; Ononye and Graveel, 1994). Our study observed the highest concentration of imine dimers (compounds **A** and **H**) after only 6 s of reaction. After that, the peak areas due to imine products decreased over time, which may indicate that the imine dimers were going through the following processes: (i) being hydrolyzed back to DAAN and quinone, which could have participated in other reactions, (ii) being tautomerized to the Michael adducts, as hypothesized by Gulkowska et al. (2012), (iii) participating in reactions of further oligomerization, generating compounds with higher molecular weight, or (iv) undergoing a combination of these processes. The process (i) seems to be especially important when DAAN is in excess in the system (DAAN:MBQ molar ratio of 10:1), generating an increase in DAAN's concentration after the initial 6 s of reaction. Additionally, compounds **E**, **F**, **D**, **K**, **L**, **O**, and **P** were formed by the oligomerization of the initial dimers and presented imine bonds, which constitute evidence for the process (iii).

A third mechanism, the quinone-dependent formation of azo bonds from DAAN self-coupling in anoxic conditions (Fig. 5, scheme D), is a novel finding. In our study, these nitrogen-nitrogen coupling products presented high peak areas in the UHPLC-HRAM-MS/MS analyses

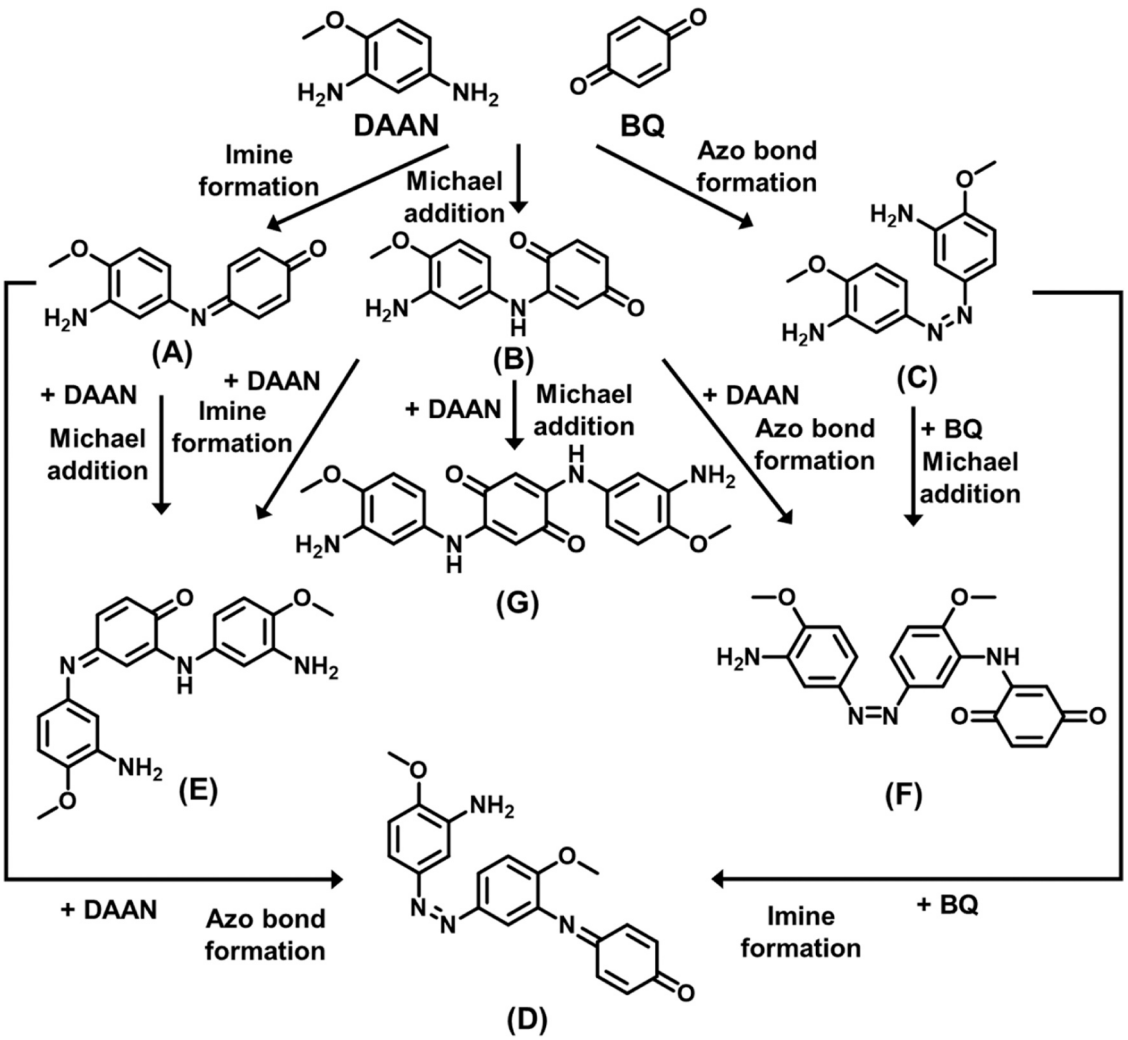

Fig. 3. Proposed formation of oligomers from the incubation of DAAN with BQ.

(plotted in SI, Figs. S-9, S-10, and S-11), comparable to the other main products (imines and Michael adducts). Azo bonds were present in a dimer (compound **C**) and in the trimers and tetramers found (compounds **D**, **F**, **K**, **O**, and **P**), indicating that azo bond formation was important for the polymerization process. Before our work, DAAN was believed to form azo dimers only in three conditions: (i) by auto-oxidation under oxygen exposure in aerobic environments (Hawari et al., 2015; Platten et al., 2010), which is common for other aromatic amines (Konaka et al., 1968), (ii) by coupling reactions with nitroso intermediates of DNAN's reduction in anoxic conditions (Olivares et al., 2016a; Kadoya et al., 2018), or (iii) by one-electron oxidation of the amino group by manganese oxide, as described for other primary aromatic amines (Laha and Luthy, 1990). Thorn et al. (1996) observed azo bonds forming between anilino radicals and free aromatic amino groups in fulvic acids, however with oxygen acting as the oxidant. For anoxic conditions, we propose that the quinone compounds could have acted as oxidants to form DAAN radicals since the oxidation of hydroquinones by quinones can generate semi-quinone radicals (Uchimiya and Stone, 2006). These DAAN radicals could have self-coupled, forming azo dimers. Although many studies described nucleophilic additions as the most important mechanism for the coupling of aromatic amines and quinones (Gulkowska et al., 2012; Hsu and Bartha, 1976; Weber et al., 1996; Bialk et al., 2007; Park et al., 1999; Wang et al., 2002; Colon et al., 2002; Kim et al., 1997), other studies indicate that a combination of nucleophilic and radical reactions drives the process (Thorn and Kennedy, 2002; Thorn et al., 1996a; Camarero et al., 2008, 2005; Carunchio et al., 2001; Dec and Bollag, 2000; Bollag, 1992; Simmons et al., 1989). Similarly to DAAN, compounds **A**, **B**, **H**, and **I**, which are imines or Michael adducts with free amino groups, could have been oxidized by quinones, forming radicals that could have self- or cross-coupled resulting in compounds **D**, **F**, **K**, **O**, and **P**. The presence of azo bonds in the trimers and tetramers formed indicates that azo bonds formation is an important mechanism for the oligomerization process.

The oligomerization process, combining the different reactions, caused the amino groups of DAAN to get locked inside the oligomers, such as in compounds **O** and **P** in Fig. 4. The formation of imines or azo bonds, which are steps involved in the oligomerization, may be reversible. However, it is not the single steps but the progression along the sequence of reactions that determine the strength of the amino group incorporation into the humic-like polymers (Gulkowska et al., 2012; Parris, 1980). Further polymerization is expected to create heterocyclic structures to lock the amino group (Hsu and Bartha, 1976; Thorn et al., 1996a). Nonetheless, we did not detect masses that corresponded to heterocyclic structures in the range of molecular weights investigated in our study. We did observe, though, the precipitation of material during our assays (shown in SI, Fig. S-12), which we further investigated.

3.3. *Formation of insoluble polymers*

We set up an experiment to assess insoluble polymers' formation from DAAN reaction with quinones (Fig. 6). We observed precipitation, expressed by the loss of DOC, only when DAAN and MBQ were

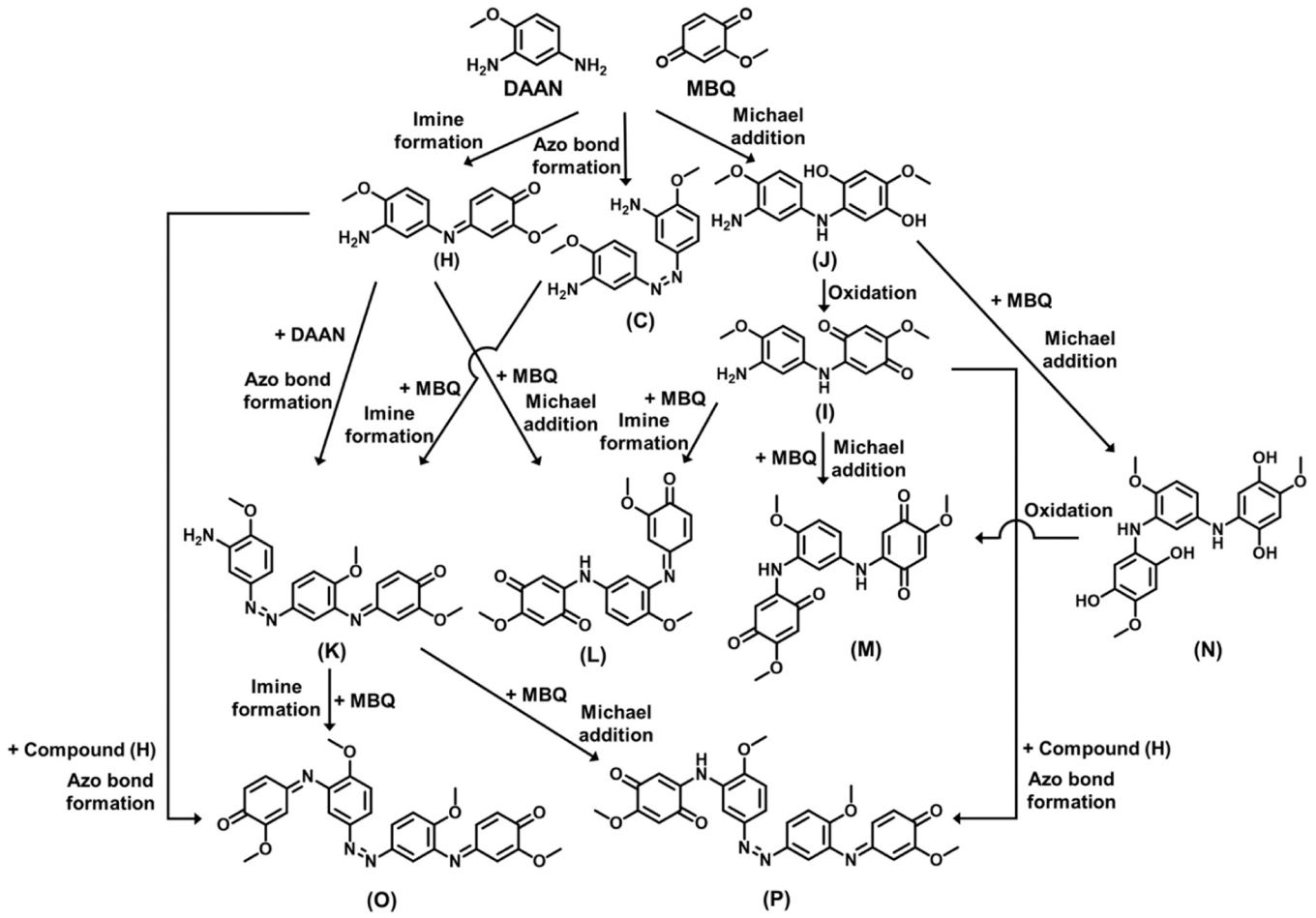

Fig. 4. Proposed formation of oligomers from the incubation of DAAN with MBQ.

incubated together. When DAAN was incubated with MHQ, the DOC remained very close to the theoretical value, evidencing that DAAN incorporation is quinone dependent also for long incubation times. After 20 days of incubation, the precipitation of organic carbon from the reaction of DAAN and MBQ was 58.4% of the theoretical total carbon. Individual DOC contents of DAAN and MBQ incubated separately are shown in SI (Fig. S-13).

A Fourier-transform infrared spectroscopy (FTIR) analysis shown in SI (Fig. S-14) provided a characterization of the precipitated solids, which was consistent with the presence of features and functional groups of the products described in Figs. 3 and 4, such as aromatic C-O and C-N, carbonyl groups, imines, phenyl groups, methoxy groups, aromatic NH or aromatic OH groups. Such results corroborate with the oligomerization process described in Figs. 3 and 4, indicating that the process ultimately led to the formation of insoluble polymers. A polymerization of this extent is expected to incorporate DAAN irreversibly, which reflects DAAN environmental fate.

Our results suggest that DAAN incorporation into quinone moieties of NOM can be applied as a natural attenuation strategy coupled to DNAN anaerobic reduction by microorganisms or reactive minerals in the soil. The literature shows that DNAN biotransformation in many soils is followed by DAAN irreversible disappearance (Olivares et al., 2016a, 2017; Hawari et al., 2015). MENA reduction to DAAN, and not the DAAN disappearance, was the frequent limiting step for DNAN's remediation. Thus, the pool of quinones in most soils seems to be enough to lock DAAN inside NOM. If they are insufficient, phenoloxidase enzymes or other oxidants may be employed to oxidize the phenol moieties in NOM to quinone moieties that promptly initiate a polymerization/humification process in the presence of DAAN. Coupling DNAN anaerobic transformation to the formation of bound DAAN residues in NOM represents a new approach, and a secure end-point, to DNAN remediation.

4. Conclusions

DAAN incorporation into model quinones moieties of NOM was driven by nucleophilic, and possibly radical, coupling reactions. The initial products participated in an oligomerization process involving Michael addition, imine formation, and azo bond formation that ultimately led to the precipitation of insoluble polymers. The self-coupling of DAAN resulting from the oxidation by quinones under anoxic conditions causes an azo dimer formation. Azo bonds were also found in the trimers and tetramers formed, indicating that this is a crucial mechanism for the entire oligomerization process. The incorporation into NOM, demonstrated by DAAN covalent binding with model humic moieties, may represent a safe endpoint for DNAN remediation. Firstly, DNAN can be reduced to DAAN by microorganisms and reactive minerals in the soil. Then, DAAN can be incorporated into NOM, forming non-extractable bound residues. This process does not rely on aerobic conditions or specific catalysts and constitutes a new approach for the cleanup of DNAN-contaminated sites.

CRediT authorship contribution statement

Osmar Menezes: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Visualization. **Warren M. Kadoya**: Conceptualization, Methodology, Investigation. **Savia Gavazza**: Resources, Writing - review & editing,

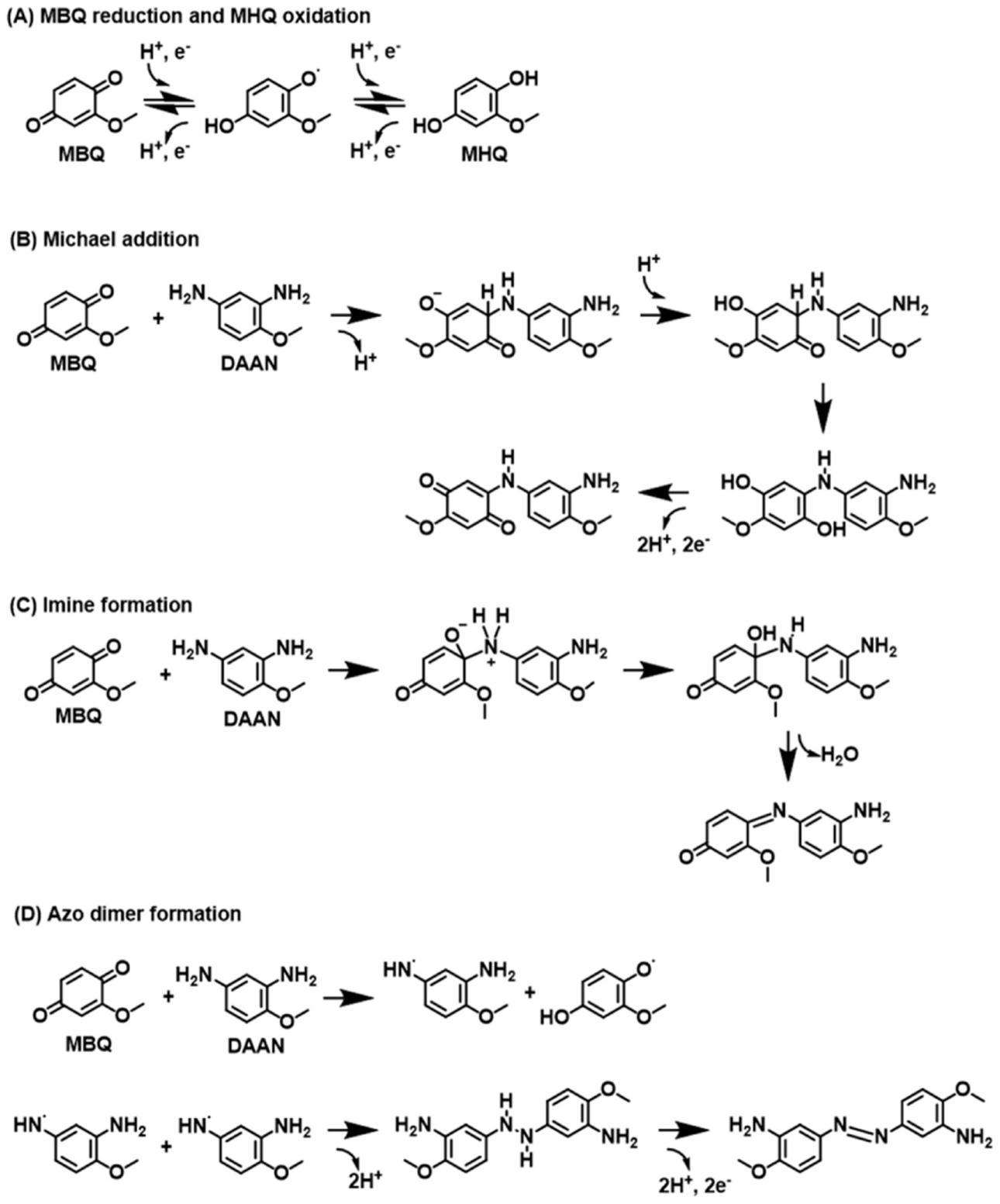

Fig. 5. Proposed mechanisms for the abiotic reactions between DAAN and MBQ under anaerobic conditions. Reversible chemistry of quinones (panel A) is the proposed source or sink of electrons and hydrogen for the other reactions. Imine formation (panel B) and Michael addition (panel C) occurred between DAAN and MBQ. Azo dimer formation (panel D) between two DAAN radicals in anaerobic conditions was quinone-dependent. Reactions between DAAN and BQ followed the same pathways.

Supervision. **Reyes Sierra-Alvarez**: Resources, Supervision, Project administration, Funding aquisition. **Eugene A. Mash**: Conceptualization, Methodology, Resources, Writing - review & editing, Funding acquisition. **Leif Abrell**: Conceptualization, Methodology, Formal analysis, Writing - review & editing, Funding acquisition. **Jim A. Field**: Conceptualization, Methodology, Resources, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition.

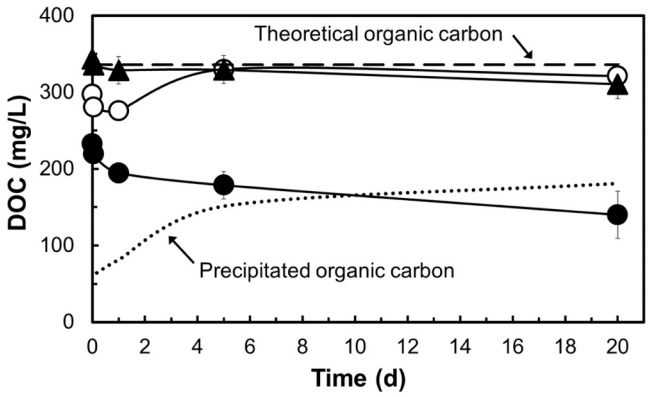

Fig. 6. Dissolved organic carbon (DOC) of anaerobic incubation of paired DAAN + MBQ (●) in comparison to control experiments: separated DAAN + MBQ (○) and paired DAAN + MHQ (▲). Paired means the two compounds were incubated together. Separated means the two compounds were incubated individually, and their DOC contents were measured separately and summed in the graph. Precipitated organic carbon represents the loss of DOC by the polymerization reactions when DAAN and MBQ were paired, and it was calculated by subtracting DOC values of separated DAAN + MBQ from paired DAAN + MBQ.

Declaration of Competing Interest

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

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2021.125459.

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