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Abstract: Nitroaromatic compounds are widely used in agricultural pesticides, pharmaceuticals, military explosives, and other applications. They enter the environment via manufacturing and municipal wastewater discharges and releases from agricultural and military operations. Because of their ubiquity and toxicity, they are considered an important class of environmental contaminants. Nitroaromatics are known to undergo reductive transformation to aromatic amines, and under aerobic conditions they are susceptible to coupling reactions which may lead to their irreversible incorporation into soil organic matter. However, there is also evidence of coupling reactions in the absence of oxygen between reduced intermediates of the insensitive munitions compound 2,4-dinitroanisiole, leading to the formation of azo dimers. The formation of such products is a concern since they may be more toxic than the original nitroaromatic compounds. The objective of this research is to provide evidence of the anaerobic formation of azo coupling products. 4-Nitroanisole was used as a model compound and was spiked into incubations containing anaerobic granular sludge with H2 as the electron donor. Using liquid chromatography, UV-Vis spectroscopy, and mass spectrometry, the formation of the azo dimer 4,4'-dimethoxyazobenzene was confirmed. However, due to the instability of the azo bond under the reducing conditions of our incubations, the azo dimer did not accumulate. Consequently, 4-aminoanisole was the major product formed in our experiment. Other minor suspected coupling products were also detected in our incubations. The results provide clear evidence for the temporal formation of at least one azo dimer in the anaerobic reduction of a model nitroaromatic compound.

Evidence of Anaerobic Coupling Reactions between Reduced Intermediates of 4-Nitroanisole

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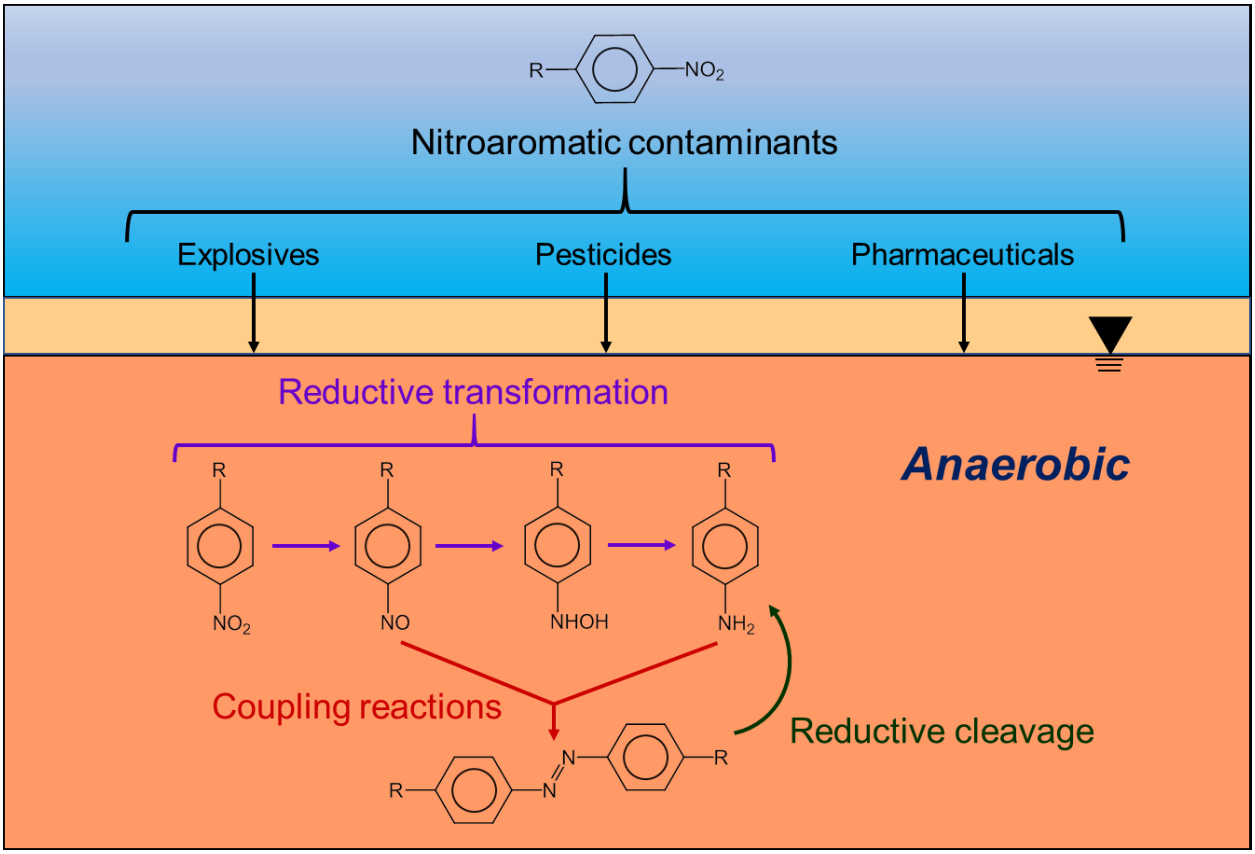
Abstract

Nitroaromatic compounds are widely used in agricultural pesticides, pharmaceuticals, military explosives, and other applications. They enter the environment via manufacturing and municipal wastewater discharges and releases from agricultural and military operations. Because of their ubiquity and toxicity, they are considered an important class of environmental contaminants. Nitroaromatics are known to undergo reductive transformation to aromatic amines, and under aerobic conditions they are susceptible to coupling reactions which may lead to their irreversible incorporation into soil organic matter. However, there is also evidence of coupling reactions in the absence of oxygen between reduced intermediates of the insensitive munitions compound 2,4-dinitroanisiole, leading to the formation of azo dimers. The formation of such products is a concern since they may be more toxic than the original nitroaromatic compounds. The objective of this research is to provide evidence of the anaerobic formation of azo coupling products. 4-Nitroanisole was used as a model compound and was spiked into incubations containing anaerobic granular sludge with H_2 as the electron donor. Using liquid chromatography, UV-Vis spectroscopy, and mass spectrometry, the formation of the azo dimer 4,4'-dimethoxyazobenzene was confirmed. However, due to the instability of the azo bond under the reducing conditions of our incubations, the azo dimer did not accumulate. Consequently, 4-aminoanisole was the major product formed in our experiment. Other minor suspected coupling products were also detected in our incubations. The results provide clear evidence for the temporal formation of at least one azo dimer in the anaerobic reduction of a model nitroaromatic compound.

Keywords: nitroaromatics; anaerobic biotransformation; 2,4-dinitroanisole (DNAN); azobenzenes; insensitive munitions compound.

Graphical Abstract



Highlights

- Study proves adduct formation during reductive transformation of nitroaromatics
- 4,4'-Dimethoxyazobenzene formed during anaerobic transformation of 4-nitroanisole
- Reaction is hypothesized to occur between nitroso and amine intermediates
- 4,4'-Dimethoxyazobenzene is transient and is reductively cleaved to 4-aminoanisole

1. Introduction

Nitroaromatic compounds are a major class of environmental pollutants. They are of significant concern due to their toxicity, production, and use in a wide variety of industries and products. Nitroaromatics are used in the manufacture of explosives (Urbanski, 1984; Davies and Provatas, 2006), pesticides (Zablotowicz et al., 2000; Wang and Arnold, 2003; Keum and Li, 2004), pharmaceuticals (Strauss, 1979; Boelsterli et al., 2006), solid fuels (Powell et al., 1998), and fragrances (Ford, 1998; Gatermann et al., 1998), and enter the environment through various pathways. They are discharged with wastewater effluent from production plants and municipal wastewater treatment plants. Release onto land occurs with the use of explosives on firing ranges and when pesticides are sprayed onto agricultural fields. Some nitroaromatics are known to be mutagenic and/or carcinogenic (Kovacic and Somanathan, 2014). Because of their toxicity and pervasiveness in the environment, these compounds pose an elevated risk to human and environmental health. It is therefore important to develop remediation techniques to clean up sites contaminated with nitroaromatics.

To develop these remediation techniques, a good understanding of the fate of nitroaromatics in the environment is required. Although there are multiple routes of nitroaromatic transformation, including biodegradation (Ju and Parales, 2010), a common fate is their reductive biotransformation to the corresponding aromatic amines (Drzyzga et al., 1998a; Razo-Flores et al., 1999; Hwang et al., 2000; Esteve-Núñez et al., 2001). These reactions are important because many nitroaromatic contaminants encounter anaerobic conditions, such as in saturated soil, groundwater, aquatic sediments, and wastewater (Taylor et al., 2013). Some studies have shown that this transformation may be biologically catalyzed by microorganisms (Rafii et al., 1991; Gorontzy et al., 1993; Boopathy et al., 1998; Ahmad and Hughes, 2000). Enzymes such as nitroreductases, commonly found in both aerobic and anaerobic bacteria, are known to reduce a broad range of nitroaromatic compounds (Nyanhongo et al., 2005; Perez-Reinado et

al., 2008; Gwenin et al., 2011; Pitsawong et al., 2014). Other studies have demonstrated the abiotic reduction of nitroaromatics by surface adsorbed Fe^{2+} and H_2S (Gorontzy et al., 1993). If aromatic amines are exposed to air, they may be oxidized by oxidative enzymes to form radicals that can undergo polymerization reactions (Jensen et al., 1992; Tan et al., 1999; Sierra-Alvarez et al., 2010; Barsing et al., 2011). Furthermore, these aromatic amine radicals are known to form covalent bonds to soil humus (Esteve-Núñez et al., 2001). Evidence of the incorporation of 2,4,6-trinitrotoluene (TNT) into soil humus was demonstrated with both ^{14}C-labeled TNT (Pennington et al., 1995; Drzyzga et al., 1998b; Bruns-Nagel et al., 2000b) and ^{15}N-labeled TNT (Bruns-Nagel et al., 2000a; Knicker et al., 2001). Because ecotoxicological assays have shown that irreversibly bound residues of TNT have reduced toxicity (Lenke et al., 1998; Bruns-Nagel et al., 2000b), soil composting is commonly used to remediate TNT-contaminated soils (Jerger and Woodhull, 2000). The currently held paradigm is that nitroaromatics are reduced in anaerobic environments to aromatic amines, which subsequently undergo aerobic polymerization and incorporation into soil under aerobic conditions (Esteve-Núñez et al., 2001; Snellinx et al., 2002). However, there is also evidence of coupling reactions occurring between reduced nitroaromatic intermediates under anaerobic conditions.

The identity and toxicity of these coupling products need to be determined to develop an effective remediation strategy. Studies have demonstrated that reduced intermediates of nitroaromatics couple with each other under anaerobic conditions to form toxic dimers and oligomers. This coupling is a chemical, not biological, reaction. Azoxy and azo dimers were formed in the bioreduction of TNT (Hawari et al., 1998; Achtnich et al., 1999). Studies of the fate of the insensitive munitions compound 2,4-dinitroanisole (DNAN) in anaerobic sludge and soil revealed the formation of azo dimers (Olivares et al., 2013; Olivares et al., 2016a). Alternative methods of synthesizing azo dyes are currently being explored via the chemical reduction of nitroaromatics using Pd^0, Pt^0, or Fe^0 catalysts and H_2 or isopropanol as the electron donors for

the coupling of the resulting reduced intermediates (Moglie et al., 2008; Hu et al., 2011; Hu et al., 2012; Wang et al., 2013a). Coupling products formed under anaerobic conditions may have higher toxicity than the original nitroaromatics. Dimers and trimers formed in anaerobic incubations of DNAN were found, in most cases, to have higher toxicities to acetoclastic methanogens and marine bioluminescent bacteria *Aliivibirio fischeri* than DNAN (Olivares et al., 2016c). An azo dimer surrogate was shown to cause many more developmental abnormalities in zebrafish (*Danio rerio*) embryos, including bent axis and edema, than monomeric DNAN transformation products (Olivares et al., 2016b). Azo dyes with amino and alkoxy groups were also demonstrated to be mutagenic (Freeman et al., 2013). Although there is compelling evidence that nitroaromatic-derived coupling products form under anaerobic conditions and that such coupling products may have increased toxicity, further research is required to elucidate the nature and mechanisms of formation of these products. The objective of this study is to demonstrate the formation of coupling products, particularly azo dimers, in a simple system of 4-nitroanisole (Figure 1) incubated in anaerobic granular sludge (AGS).

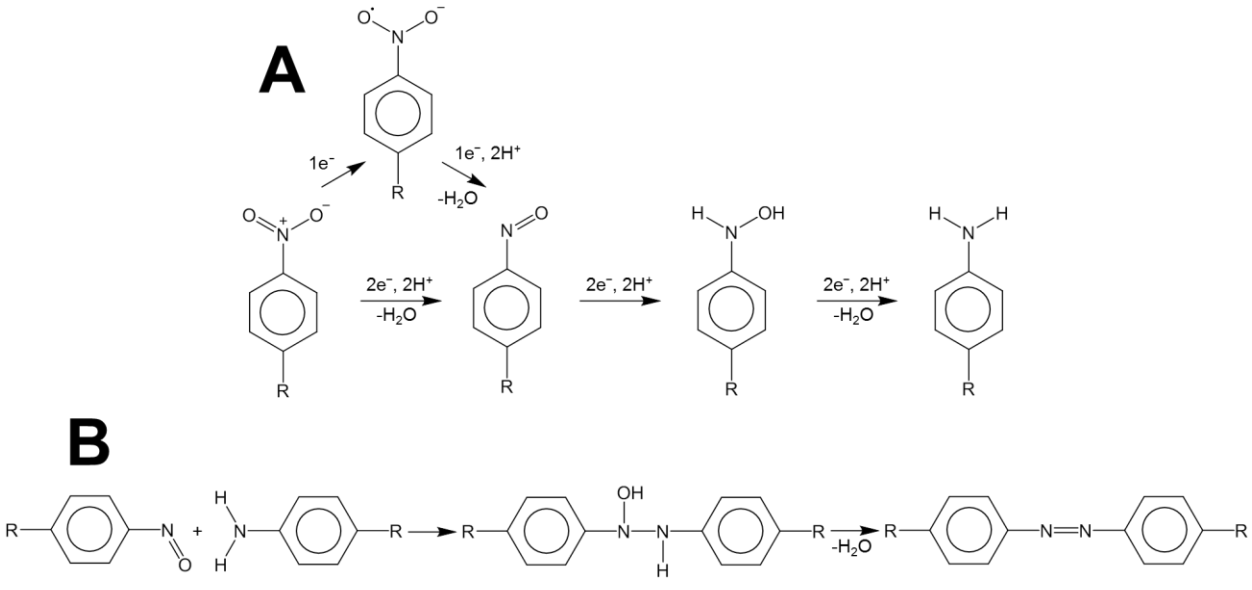


Figure 1. Hypothesized reaction mechanisms of nitroaromatic compounds incubated under anaerobic conditions leading to the formation of an azo dimer. In our experiments, the R group

is a methoxy group. We hypothesize that 4-nitroanisole will first be reduced to intermediates 4-nitrosoanisole, 4-hydroxylaminoanisole, and 4-aminoanisole through biologically catalyzed reactions, as shown in (A). These intermediates, particularly 4-nitrosoanisole and 4-aminoanisole, will then couple abiotically via nucleophilic substitution to form, after loss of H_2O, 4,4'-dimethoxyazobenzene, as shown in (B).

2. Materials and Methods

2.1. Chemicals

The chemicals used in this study are listed in the Supplementary Material.

2.2. Inoculum and mineral medium

The inoculum and mineral medium used in this study are described in the Supplementary Material.

2.3. Anaerobic biotransformation assays

2.3.1. 4-Nitroanisole incubation in anaerobic sludge

To test the formation of azo dimers under anaerobic conditions, we conducted an experiment with three spikes of 4-nitroanisole, a model compound for DNAN, into AGS incubations over a period of 1.6 days. The temporal additions of 4-nitroanisole were intended to provide a steady source of partially reduced nitroaromatic intermediates (i.e. 4-nitrosoanisole) to couple with accumulated 4-aminoanisole, according to our hypothesized mechanism in Figure 1. The objective was to increase the accumulation of coupling products to facilitate analysis. The time interval between 4-nitroanisole spikes was determined by preliminary experiments in which 4-nitroanisole was reduced to 4-aminoanisole in AGS in approximately 24 hours. Four mixtures were tested. In mixture 1, 4-nitroanisole was incubated in AGS and mineral medium with H_2 as the electron donor. In this full experimental treatment, we expected the formation of azo dimers

from the coupling of reduced intermediates of 4-nitroanisole. In mixture 2, 4-aminoanisole was incubated in AGS and mineral medium with H_2 as the electron donor. This control allowed us to confirm that the formation of azo dimers in mixture 1 was not due to the oxidation of aromatic amines. If oxygen was present in these flasks, 4-aminoanisole could be oxidized and couple with itself to form these same azo dimers. In mixture 3, 4-nitroanisole was incubated in mineral medium only. This abiotic control allowed us to attribute the formation of azo dimers in mixture 1 to the reduction of 4-nitroanisole by sludge microbes. We did not anticipate the formation of reduced intermediates of 4-nitroanisole in mineral medium alone. Mixture 4 was an incubation of AGS, mineral medium, and H_2 as the electron donor. This control was used to demonstrate that azo dimers formed in mixture 1 were not artifacts of the sludge matrix.

The experimental setup involved sacrificial, 160 mL glass serum flasks in duplicate. 20 mL mineral medium and 1.5 g VSS/L sieved AGS were added to each flask. The liquid was flushed with N_2/CO_2 (80/20, v/v) and the flasks were sealed with butyl-rubber stoppers and aluminum crimp caps. The headspace was then flushed with N_2/CO_2 and the flask headspace was pressurized to 1.5 atm with H_2/CO_2 (80/20, v/v), with H_2 as the cosubstrate. The flasks were incubated overnight in the dark at 30 °C and 113 rpm to allow for sludge acclimation to the medium conditions. The following day, the headspace was again flushed with N_2/CO_2. For the first spike, 0.2 mL of 400 mM 4-nitroanisole in ethanol was added to flasks containing mixtures 1 and 3 (to a final concentration of 4 mM), 0.2 mL of 400 mM 4-aminoanisole in ethanol was added to flasks containing mixture 2 (to a final concentration of 4 mM), and 0.2 mL of ethanol was added to flasks containing mixture 4. The flasks were again pressurized to 1.5 atm with H_2/CO_2. 0.2 mL of 100 g/L ascorbic acid was added to each flask (to a final concentration of 250 mg/L) to prevent autoxidation of 4-aminoanisole and other reduced compounds. Ascorbic acid was chosen based on previous studies that demonstrated its effectiveness as an antioxidant (Cortinas et al., 2006; Sierra-Alvarez et al., 2010). It also did not chemically reduce 4-

nitroanisole in sample stability tests. For the second and third spikes, only 4-nitroanisole, 4-aminoanisole, and ethanol aliquots were added to the flasks as described above for the first spike. The flasks were incubated as described above between chemical additions and sampling times. Concentrations were adjusted in the calculations to account for the increase in reaction volume due to chemical additions to the flushed flasks.

2.3.2. 4-Methoxyazobenzene incubation in anaerobic sludge

Because we observed the formation and subsequent disappearance of 4,4'-dimethoxyazobenzene in our AGS incubations (Section 3.1), we decided to test the stability of azo compounds in this reducing environment. We incubated 4-methoxyazobenzene, a readily available analog of 4,4'-dimethoxyazobenzene, in flasks containing either mineral medium with AGS and H_2 or only mineral medium. Flasks were prepared exactly as in Section 2.3.1, except only one spike of 0.2 mL of 200 mM 4-methoxyazobenzene in ethanol was delivered into each flask.

2.3.3. Sample processing

Due to the limited aqueous solubility of 4,4'-dimethoxyazobenzene (soluble in 25/75 H_2O/acetonitrile, v/v), 60 mL of acetonitrile was added to each flask at the appropriate time point in the above experiments (Sections 2.3.1 and 2.3.2) to solubilize any products formed. Flasks were incubated for one hour as described in Section 2.3.1, allowing for compounds of interest to become solubilized in the medium, before sampling the liquid. All samples were centrifuged for ten minutes at 13,000 rpm and the supernatant was then analyzed for 4-nitroanisole and transformation products as described in Section 2.4.

2.4. Analytical Methods

2.4.1. UHPLC-DAD

Samples were analyzed using an ultra-high performance liquid chromatograph coupled to a diode-array detector (UHPLC-DAD, Agilent 1290 Infinity, Santa Clara, CA, USA). 5 µL sample injections were separated using an Acclaim RSLC Explosives E2 column (2.1 mm x 100 mm, 2.2 µm; Thermo Fisher Scientific, Waltham, MA) at room temperature. An inline guard column with a filter cartridge was used to remove any particles remaining after centrifugation and supernatant harvest. To analyze samples from the incubation of 4-nitroanisole in AGS (Section 2.3.1), the mobile phase was run at a flow rate of 0.25 mL/min for 20.5 min and consisted of a gradient of methanol/H_2O (v/v) in the following ratios: from 0-5 min held at (50/50), from 5-10 min increasing to (90/10), from 10-15 min held at (90/10), from 15-15.5 min decreasing back to (50/50), and from 15.5-20.5 min held at (50/50). The compounds were analyzed at the following wavelengths and had the following retention times: 4-nitroanisole (300 nm, 7.0 min), 4-aminoanisole (300 nm, 2.1 min), and 4,4'-dimethoxyazobenzene (360 nm, 11.9 min). To analyze samples from the incubation of 4-methoxyazobenzene in AGS (Section 2.3.2), the mobile phase was run at a flow rate of 0.25 mL/min for 25.5 min and consisted of a gradient of methanol/H_2O (v/v) in the following ratios: from 0-5 min held at (15/85), from 5-15 min increasing to (90/10), from 15-20 min held at (90/10), from 20-20.5 min decreasing back to (15/85), and from 20.5-25.5 min held at (15/85). The compounds were analyzed at the following wavelengths and had the following retention times: 4-methoxyazobenzene (360 nm, 16.8 min), aniline (280 nm, 5.6 min), and 4-aminoanisole (300 nm, 7.5 min). All analyzed compounds were compared with prepared standards with respect to retention times and UV-Vis spectra.

2.4.2. UHPLC-Q-ToF-MS

The mass spectrometric analytical methods used to confirm the presence of 4,4'-dimethoxyazobenzene in samples from the incubation of 4-nitroanisole in AGS are described in the Supplementary Material.

3. Results

3.1. 4-Nitroanisole incubation in anaerobic sludge

The results of the anaerobic biotransformation of 4-nitroanisiole to 4-aminoanisole in AGS are shown in Figures 2-6. 4-Nitroanisole was largely converted to 4-aminoanisole, but not entirely. Figure 2 shows the disappearance of 4-nitroanisole after each spike into flasks containing AGS, as measured by UHPLC. 4-Aminoanisole gradually accumulates as 4-nitroanisole is transformed. Figure 3 shows that 76% of the consumed 4-nitroanisole was converted to 4-aminoanisole. The remaining 24% is not accounted for. Although 4-Nitroanisole is reduced in AGS, most likely following the well-known pathway of nitro group reduction (Figure 1A), there is a discrepancy in the mass balance. Consequently, it is essential to study other peaks in the UHPLC chromatograms that formed over the course of the experiment to determine whether coupling reactions like those hypothesized in Figure 1B took place.

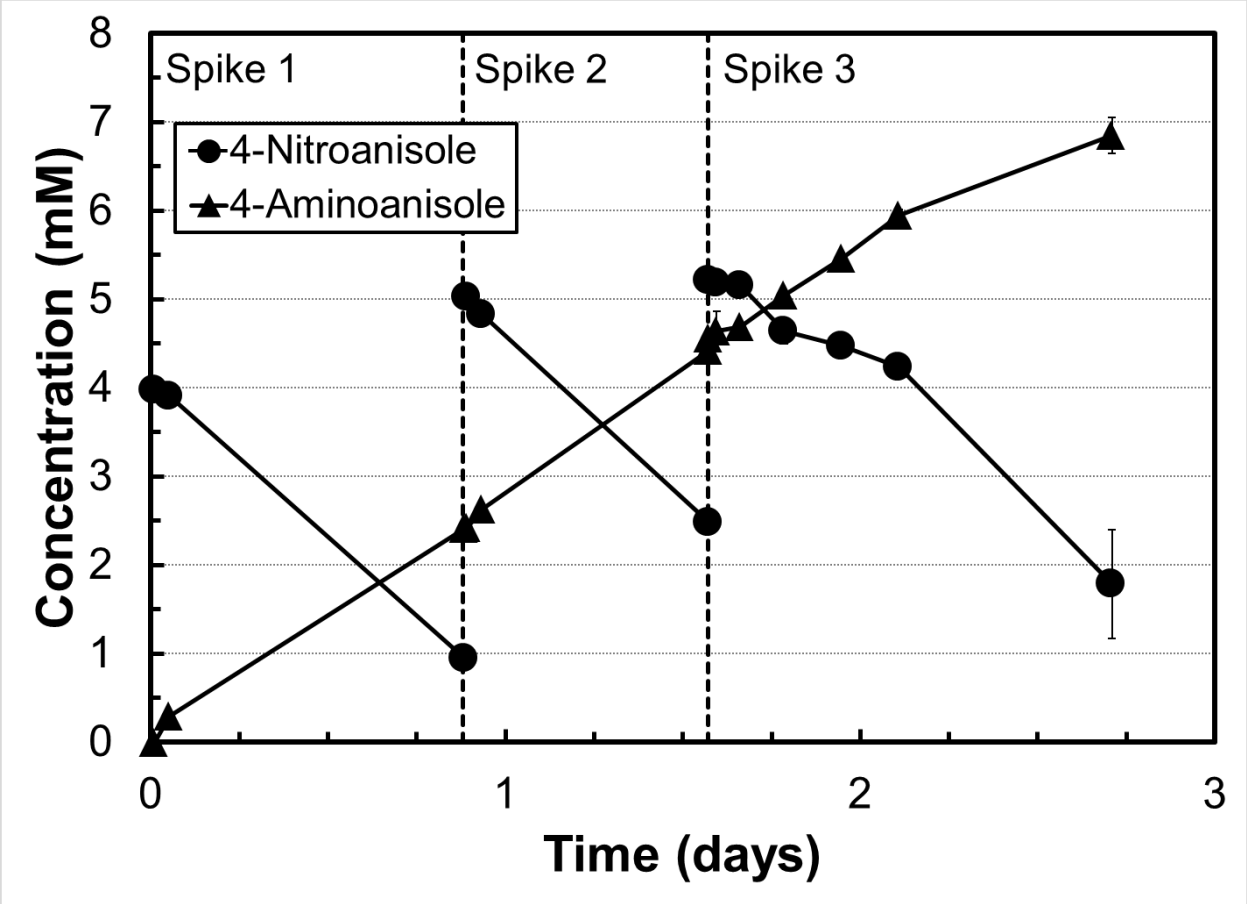


Figure 2. Fate of 4-nitroanisole in anaerobic sludge: nitro group reduction. 4-Nitroanisole transformed into 4-aminoanisole when incubated with anaerobic granular sludge supplied with mineral medium and H_2 as the electron donor (n=2). 4-Nitroanisole was delivered in three 4 mM spikes.

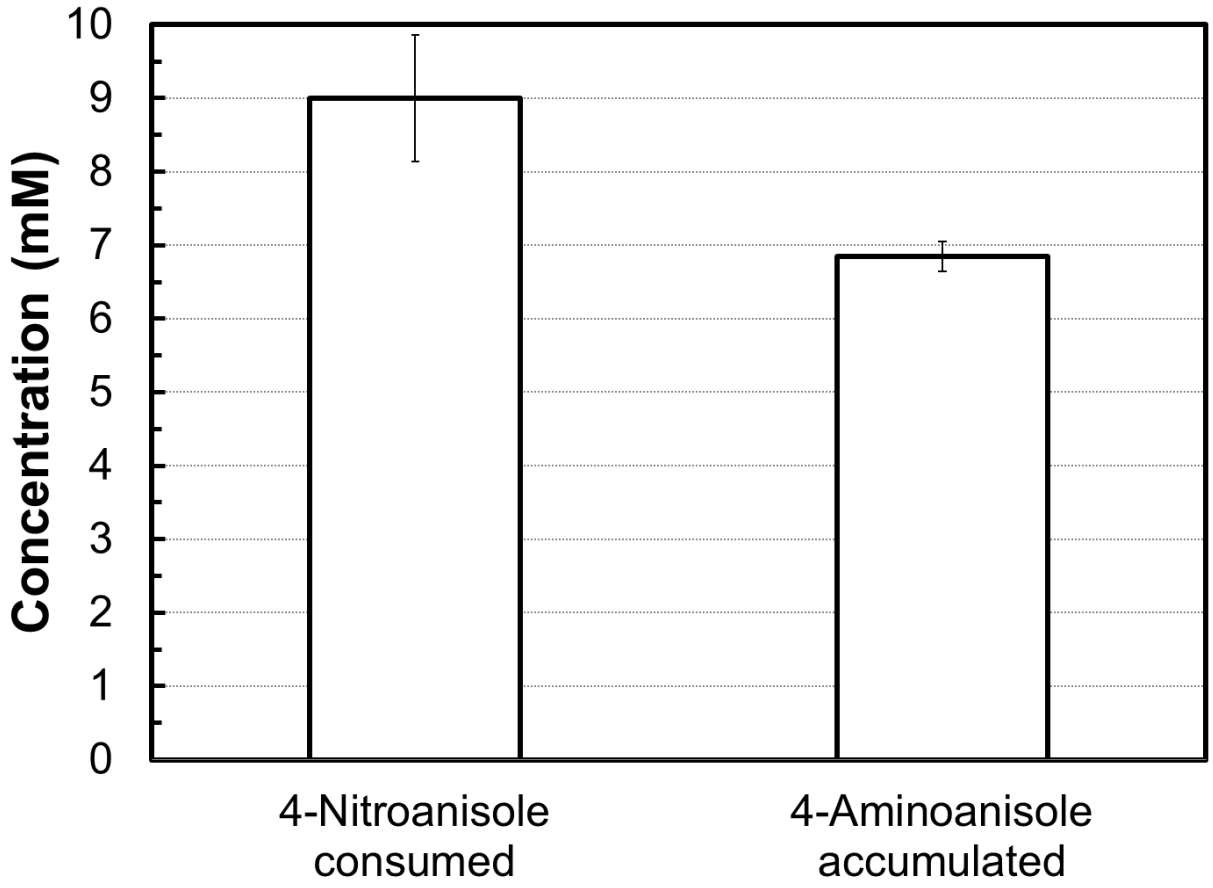
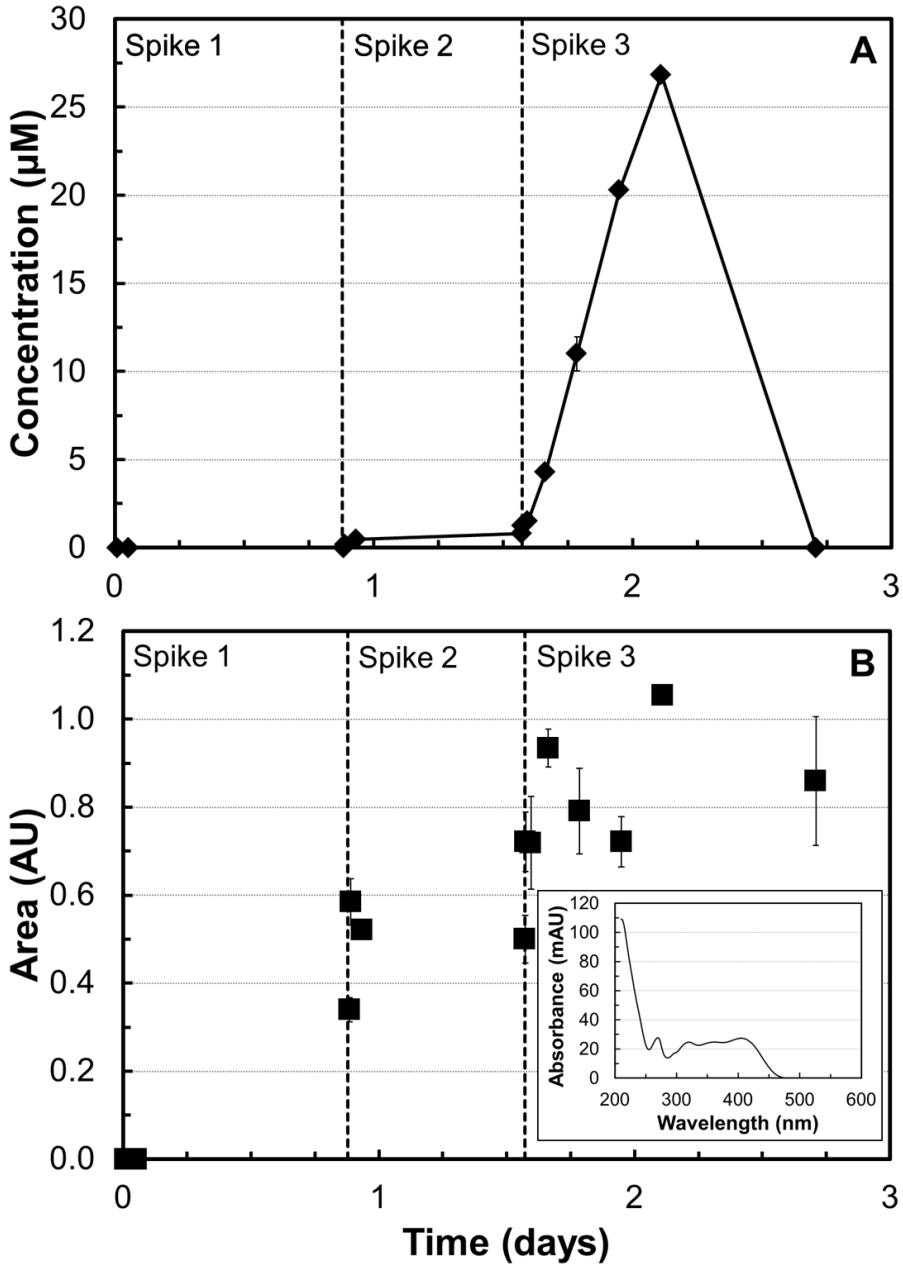


Figure 3. Mass balance of 4-nitroanisole incubated in anaerobic sludge. Overall consumption of 4-nitroanisole and accumulation of 4-aminoanisole at the end of the incubation experiment (4-nitroanisole in anaerobic granular sludge, n=2, shown in Figure 2).

A number of compounds other than 4-aminoanisole were detected in UHPLC after incubating 4-nitroanisole in anaerobic sludge (Figure 4). We observed the transient formation of the coupling product 4,4'-dimethoxyazobenzene (Figure 4A). The peak identified as 4,4'-dimethoxyazobenzene in mixture 1 had the same UHPLC retention time as the standard, as shown in the chromatograms in Figure 5. No peak with this retention time was formed in any of the controls. The UV-Vis spectrum of the peak in mixture 1 closely resembles that of the 4,4'-dimethoxyazobenzene standard, as shown in the inserts in Figure 5. No other peak identified in

any incubation has a similar UV-Vis spectrum, suggesting that this compound is not an artifact or contaminant. Two pieces of mass spectrometric evidence were found that corroborated these UHPLC results. Extracted ion chromatograms for the transition 243.1>107.05 for 4,4'-dimethoxyazobenzene from the incubation sample and chemical standard (shown in Figures 6A and 6B, respectively) are nearly identical. Product ion spectra generated from precursor ion m/z 243.1 from the incubation sample and chemical standard also closely resemble one another (shown in Figures 6C and 6D, respectively). 4,4'-Dimethoxyazobenzene was not detected in any of the controls by LC-MS measurements, confirming the absence of artifacts and contamination. Furthermore, measured accurate parent ion masses were in close agreement. The observed high resolution accurate mass measurement for the commercially obtained dimer standard was 243.1126, 0.8 ppm lower than the calculated mass of 243.1128 for $C_{14}H_{15}N_2O_2^+$, while the observed mass for the experimentally produced dimer was 243.1131, only 1.2 ppm higher than the calculated mass. Through our experimental setup and analytical techniques, we were able to prove that 4,4'-dimethoxyazobenzene is formed when incubating 4-nitroanisole in anaerobic sludge. However, because 4,4'-dimethoxyazobenzene disappeared shortly after its formation (Figure 4A), we looked for additional compounds that may have formed and accumulated.



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290 **Figure 4.** Fate of 4-nitroanisole in anaerobic sludge: coupling product formation. (A) Formation
291 and disappearance of 4,4'-dimethoxyazobenzene (◆) and (B) formation of a possible coupling
292 product (■, peak with 8.2 minute UHPLC retention time) in anaerobic granular sludge
293 incubations supplied with mineral medium, H_2 as the electron donor, and three spikes of 4 mM
294 4-nitroanisole (n=2). The insert in panel B is the UV-Vis spectrum for the possible coupling
295 product.

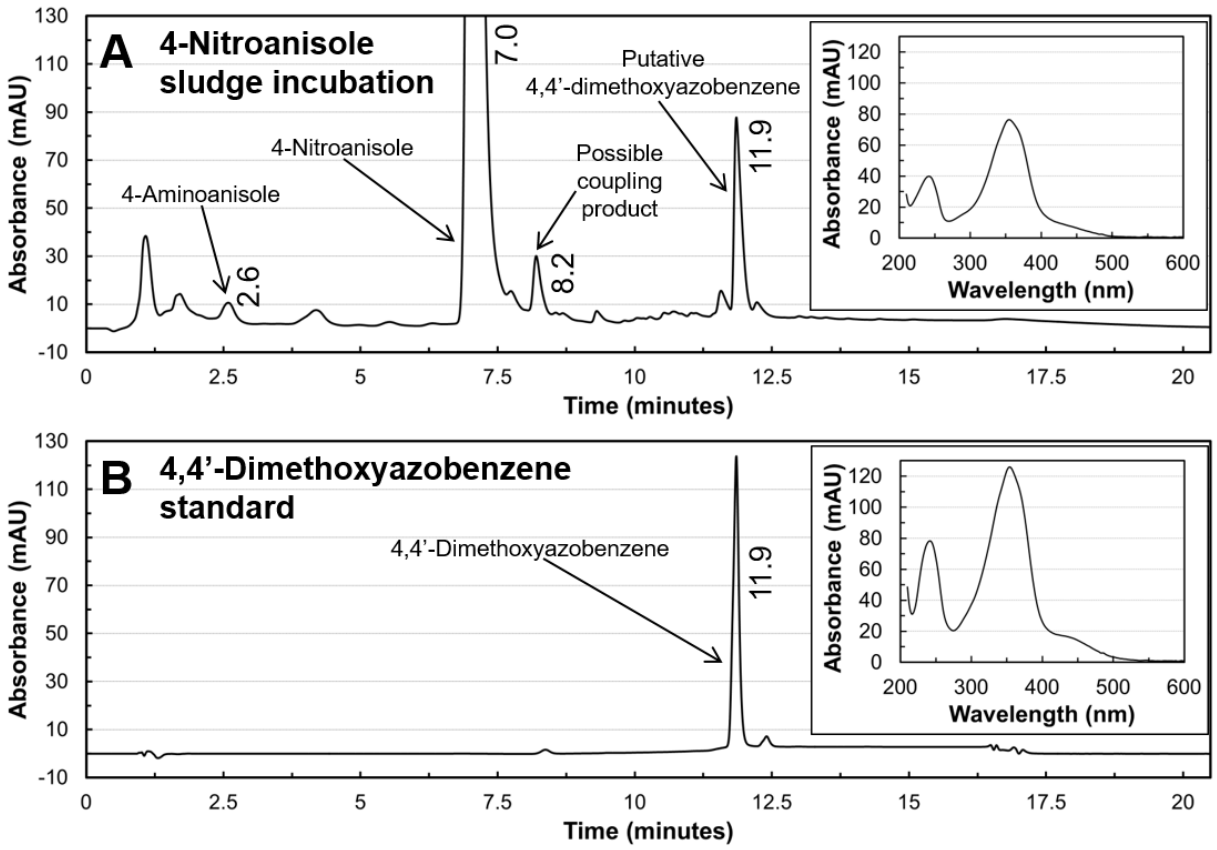

Figure 5. UHPLC and UV-Vis spectral evidence of coupling product formation when incubating 4-nitroanisole in anaerobic sludge. Comparison of UHPLC retention times and UV-Vis spectra of the peaks with retention time of 11.9 minutes, (A) from the 4-nitroanisole incubation sample in anaerobic sludge taken at 2.1 days from Figure 4A and (B) from a 7.8 μM 4,4'-dimethoxyazobenzene standard. The inserts in panels A and B are the UV-Vis spectra corresponding with these peaks with retention times of 11.9 minutes in the sample and the standard, respectively. UHPLC chromatograms were obtained at 360 nm.

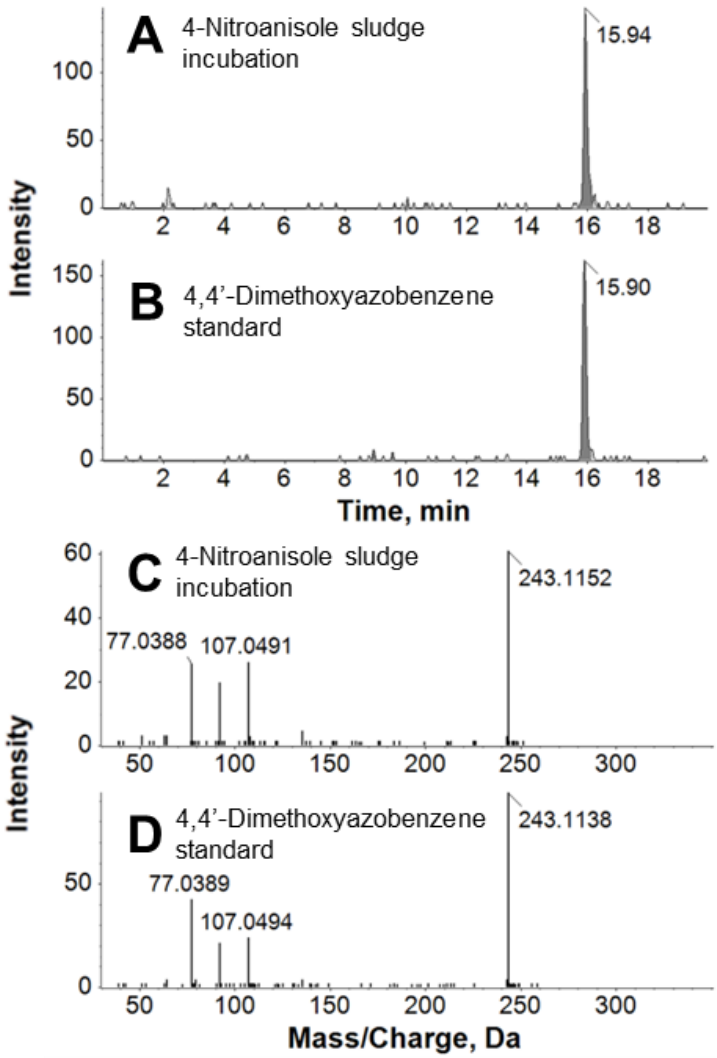

Figure 6. Mass spectrometric evidence of coupling product formation from 4-nitroanisole incubation in anaerobic sludge. Extracted ion chromatograms from selected reaction monitoring transition 243.1>107.05 are shown for (A) acetonitrile extract of incubation products from 4-nitroanisole incubation in anaerobic granular sludge and (B) 1.6 µg/L 4,4'-dimethoxyazobenzene standard. The product ion spectra from the tallest peaks in chromatograms A and B are shown in plots C and D, respectively.

Several small peaks were observed in the UHPLC chromatograms of mixture 1. The largest of these additional peaks, shown in Figure 4B, is a putative coupling product. This peak has

absorbance in the visible light (350-750 nm) range, which is characteristic of azo dimers (Fabian and Hartmann, 1980). Since 4,4'-dimethoxyazobenzene and the putative coupling product were not observed in any of the controls, we concluded that they formed exclusively due to the transformation of 4-nitroanisole incubated in AGS. The concentration of 4-nitroanisole remained stable and no dimers formed when incubated only in mineral medium (Figure S1), which indicates that dimer formation requires the biologically-catalyzed reduction of 4-nitroanisole. Because the concentration of 4-aminoanisole remained stable upon incubation in AGS (Figure S2) and no additional peaks were identified in UHPLC, we assume that our incubation flasks were sufficiently anaerobic and that dimers did not form due to autoxidation of 4-aminoanisole in the presence of oxygen (for example during sample handling). Finally, because no dimers were found in AGS incubations without aromatic compounds, we conclude that the dimers formed in 4-nitroanisole incubations are not artifacts arising from the inoculum. In addition to 4,4'-dimethoxyazobenzene, other coupling products are likely to have formed from the incubation of 4-nitroanisole in anaerobic sludge. However, an explanation is needed for the disappearance of 4,4'-dimethoxyazobenzene after its formation, as shown in Figure 4A after day 2.

3.2. 4-Methoxyazobenzene incubation in anaerobic sludge

To confirm that reductive azo bond cleavage may have caused the disappearance of 4,4'-dimethoxyazobenzene formed in 4-nitroanisole incubations with AGS, we incubated an azo dimer with a closely related structure, 4-methoxyazobenzene, with AGS. 4-Methoxyazobenzene was rapidly converted to the corresponding aromatic amines in AGS, but not in an un-inoculated medium control (Figure S3). The concentration of 4-methoxyazobenzene decreased rapidly when incubated in AGS under the same conditions as the experiment in which 4-nitroanisole was incubated in AGS. As 4-methoxyazobenzene disappeared, aniline and 4-aminoanisole formed and accumulated. These were the expected products from the cleavage of the azo bond under reducing conditions. 4-Methoxyazobenzene was stable when incubated without inoculum,

as shown in Figure S3B, supporting the role of the sludge in catalyzing the azo bond cleavage. The abrupt disappearance of 4,4'-dimethoxyazobenzene after formation in spiking experiments was due to incubation conditions involving AGS and H_2 that are conducive to azo bond cleavage. These observations have important implications for the environmental fate of 4-nitroanisole and other nitroaromatics.

4. Discussion

The present study provides compelling evidence that azo compounds form when nitroaromatics are incubated under anaerobic conditions. The formation of 4,4'-dimethoxyazobenzene in our experiments reflects the findings of other studies in which TNT and DNAN were incubated in sludge and soil. In one study, tetraamino azotoluenes and a putative polyazo precipitate were detected after incubating TNT in anaerobic sludge (Hawari et al., 1998). This is consistent with the formation of 4,4'-dimethoxyazobenzene in our experiments incubating 4-nitroanisole in AGS. However, 2,4,6-triaminotoluene (TAT), an aromatic amine transformation product of TNT, did not accumulate and instead disappeared completely with the formation of azo compounds, unlike the accumulation of 4-aminoanisole in our experiments. The three amino groups on TAT make it much more reactive than 4-aminoanisole. This group also proposed that a one-electron aromatic amine oxidation mechanism may have caused the formation of azo dimers. However, our AGS likely contained fewer trace minerals than the sludge used by this group, since we did not observe any azo dimer formation in our 4-aminoanisole AGS controls (Figure S2). Furthermore, the azo compounds accumulated in that study rather than becoming cleaved back into the corresponding aromatic amines, which could indicate less reducing conditions than in our AGS incubations. In another study, azoxy dimers formed and bound to soil when TNT was incubated under anaerobic conditions (Achtnich et al., 1999). Azoxy compounds may be reduced to azo compounds, as shown in Figure S4 (Smith and March, 2006). Ultimately, the study found that the concentration of azoxy groups disappeared, accompanied by a

corresponding increase in amino group concentration, similar to the accumulation of 4-aminoanisole after 4,4'-dimethoxyazobenzene disappeared from our incubations. When incubating DNAN in the same AGS used in our experiments, the formation of azo and hydrazine dimers was observed (Olivares et al., 2013). Multiple azo and hydrazine compounds were formed because the additional nitro group on DNAN makes it more reactive than 4-nitroanisole. Furthermore, seven azo dimers were detected upon incubating DNAN in soil under anaerobic conditions (Olivares et al., 2016a). 2,4-Diaminoanisole (DAAN), an aromatic amine transformation product of DNAN and analog of 4-aminoanisole, was not detected in solution due to either binding with humic substances or coupling with nitroso intermediates to form dimers. The biotic formation of azo dimers and the subsequent reduction to hydrazine dimers and aromatic amines has been documented in the literature, though differences in nitroaromatic structure and inoculum may have ultimately determined the products that accumulated. Studying the mechanisms of azo dimer formation and transformation will help determine the effects of environmental conditions on the fate of these compounds.

Reduced intermediates of nitroaromatics are thought to couple with each other in the absence of oxygen to form azo dimers and oligomers. Condensation reactions between nitrosoaromatics and aromatic amines or aromatic hydroxylamines are known to produce azo coupling products (Hu et al., 2011; Merino, 2011). Azo coupling may be initiated by nucleophilic attack of the hydroxylamine group (Pizzolatti and Yunes, 1990) or the amine group (Moglie et al., 2008; Zhao et al., 2011) onto the nitroso group. The coupling between nitrosoaromatics and aromatic hydroxylamines is the most widely accepted mechanism (Smith and March, 2006; Hu et al., 2011; Wang et al., 2013a; Wang et al., 2013b). This may explain the formation of azoxy compounds when TNT was incubated in soil (Achtnich et al., 1999), since hydroxylamine intermediates were observed prior to the detection of the azoxy compounds. However, since hydroxylamines are less abundant during nitroaromatic transformation than amines, we

hypothesize that the principal mechanism responsible for dimer formation involves the coupling of nitrosoaromatics and aromatic amines (Figure 1B). The azo dimers in DNAN incubations with soil were hypothesized to have formed from the coupling of nitroso intermediates and aromatic amines (Olivares et al., 2016a). Alternatively, coupling products may form when oxidized soil minerals (e.g. MnO_2) perform a one-electron oxidation of aromatic amines generating a cation radical that initiates the coupling (Laha and Luthy, 1990). Although we did not observe the formation of azo dimers when incubating 4-aminoanisole in AGS, this may be an important pathway in soil or in sludge with high mineral content. Several mechanisms may be responsible for the formation of azo compounds under anaerobic conditions, all of which involve the coupling of reduced intermediates of nitroaromatics. The toxicity of these azo dimers compared with that of the parent nitroaromatics and their reduced intermediates should be considered when designing remediation schemes.

Although azo compounds comprise a minor portion of the products formed in our incubations, their relatively high toxicity may be an issue. Azo compounds have been shown to be more toxic than parent nitroaromatics and corresponding aromatic amines. Therefore, it is desirable to minimize their formation and accumulation in the environment. A surrogate dimer and trimer for DNAN proved to be slightly more toxic or comparable in toxicity to acetoclastic methanogens and *A. fischeri* than DNAN and many fold more toxic than DAAN when comparing IC_{50} values (Liang et al., 2013; Olivares et al., 2016c). When comparing the lowest-observed-effect concentrations (LOECs) on zebrafish embryos, two dimer surrogates and a trimer surrogate for DNAN were found to be ten to 100 times more toxic than DAAN (Olivares et al., 2016b). Therefore, it may be desirable to promote the reduction of azo compounds to the corresponding aromatic amines. Many studies have shown that azo compounds readily undergo reductive biotransformation to the corresponding aromatic amines via azo reductases present in anaerobic bacteria (Walker, 1970; Razo-Flores et al., 1997; Robinson et al., 2001; Stolz, 2001;

Rau et al., 2002; Gavin et al., 2012). Irrigation or addition of an electron donor can help establish and maintain reducing conditions in contaminated soils. Both aromatic amines and azo coupling products with free amine groups may be amenable to humification: removal and detoxification through irreversible binding and incorporation into soil humus. Studies have demonstrated that amine groups on aromatic amines covalently bond to quinone and other carbonyl compounds found in soil humus, forming Schiff bases and Michael adducts (Thorn et al., 1996; Gulkowska et al., 2012). Furthermore, there is evidence that ^{14}C- and ^{15}N-labeled TNT becomes covalently bound to soil under anaerobic incubation (Drzyzga et al., 1998b; Achtnich et al., 1999; Bruns-Nagel et al., 2000a; Knicker et al., 2001). A recent study demonstrated that ^{14}C-labeled DNAN becomes irreversibly incorporated into the nonextractable fraction of soil humus, humin, and that the reduced aromatic amine DAAN forms an insoluble precipitate when paired with 1,4-benzoquinone, a model quinone compound, and humin (Olivares et al., 2017). To lower the risk of nitroaromatic-contaminated sites, reducing conditions should be established to cleave any azo coupling products that may form into the corresponding aromatic amines, which can then become irreversibly bound to soil humus. Soils with low organic carbon content, and therefore low humification capability, can be amended with compost or peat (Olivares et al., 2017). Additional strategies such as containment and oxidative processes may be applied to remove any residual aromatic amines.

5. Conclusions

Nitroaromatics are a complex class of contaminants, and understanding their fate, particularly in anaerobic environments, is crucial in designing remediation methods. This work has demonstrated that azo compounds form via the coupling of intermediates generated when nitroaromatics are reduced in anaerobic sludge incubations. 4,4'-Dimethoxyazobenzene was formed in incubations of 4-nitroanisole in anaerobic granular sludge supplied with mineral medium and H_2 as the electron donor. This evidence is based on matching UHPLC retention

times, UV-Vis spectra, mass spectrometric product ion spectra, and high resolution accurate masses for precursor ions when compared with a commercially obtained dimer standard. This reaction occurs under strict anaerobic conditions and does not require oxygen. The most probable coupling mechanism is the nucleophilic substitution of an aromatic amine onto a nitrosoaromatic compound. The azo dimer that formed in our incubations, however, was not stable and was reductively cleaved to form aromatic amines. This study provides evidence of the formation of azo compounds from the anaerobic incubation of nitroaromatics as well as insight into the risk assessment and remediation of these contaminants. Because aromatic amines are the dominant product, a remediation strategy for nitroaromatic-contaminated soil may involve reduction to aromatic amines followed by incorporation into soil humus, i.e. via covalent bonding between the amines and quinone groups present in the soil organic matter.

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Figure 1

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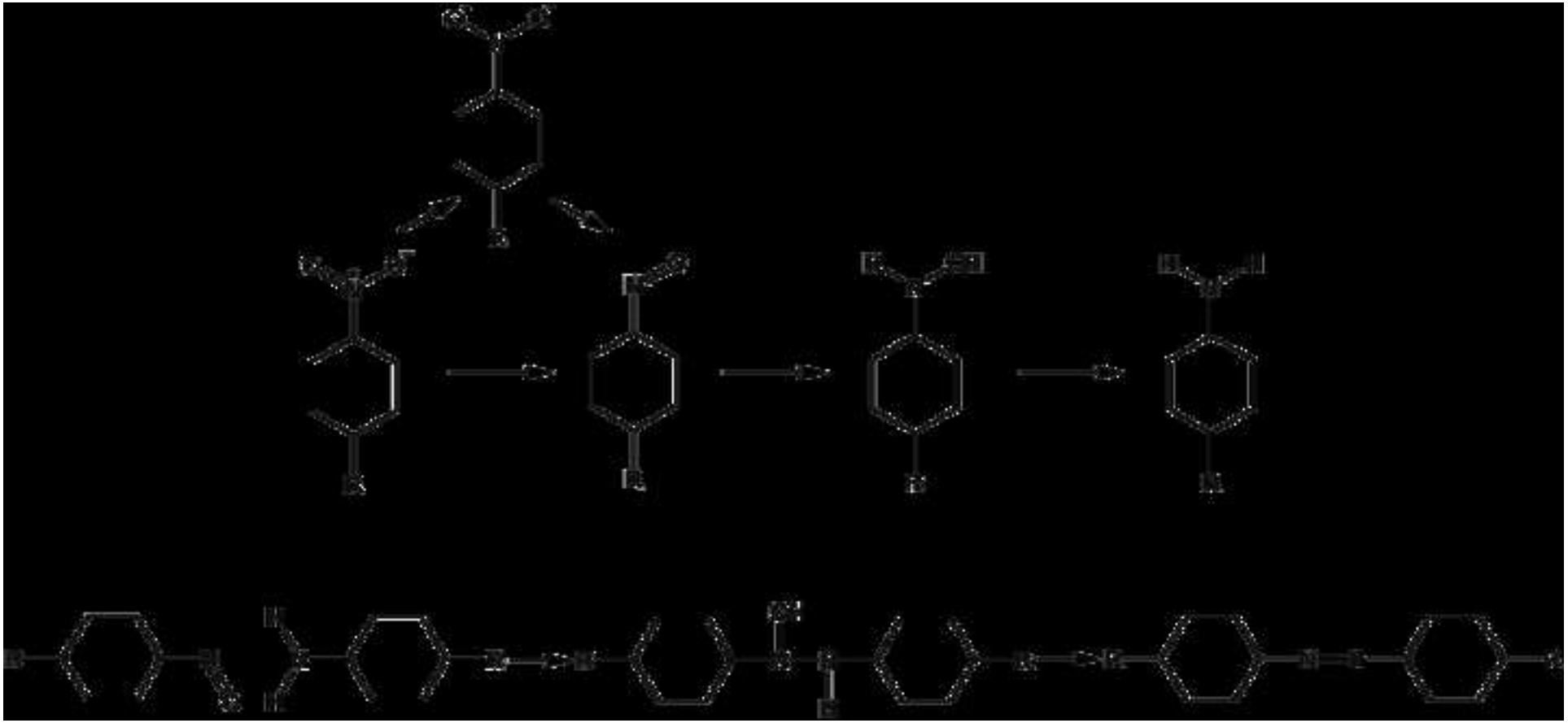

Figure 2

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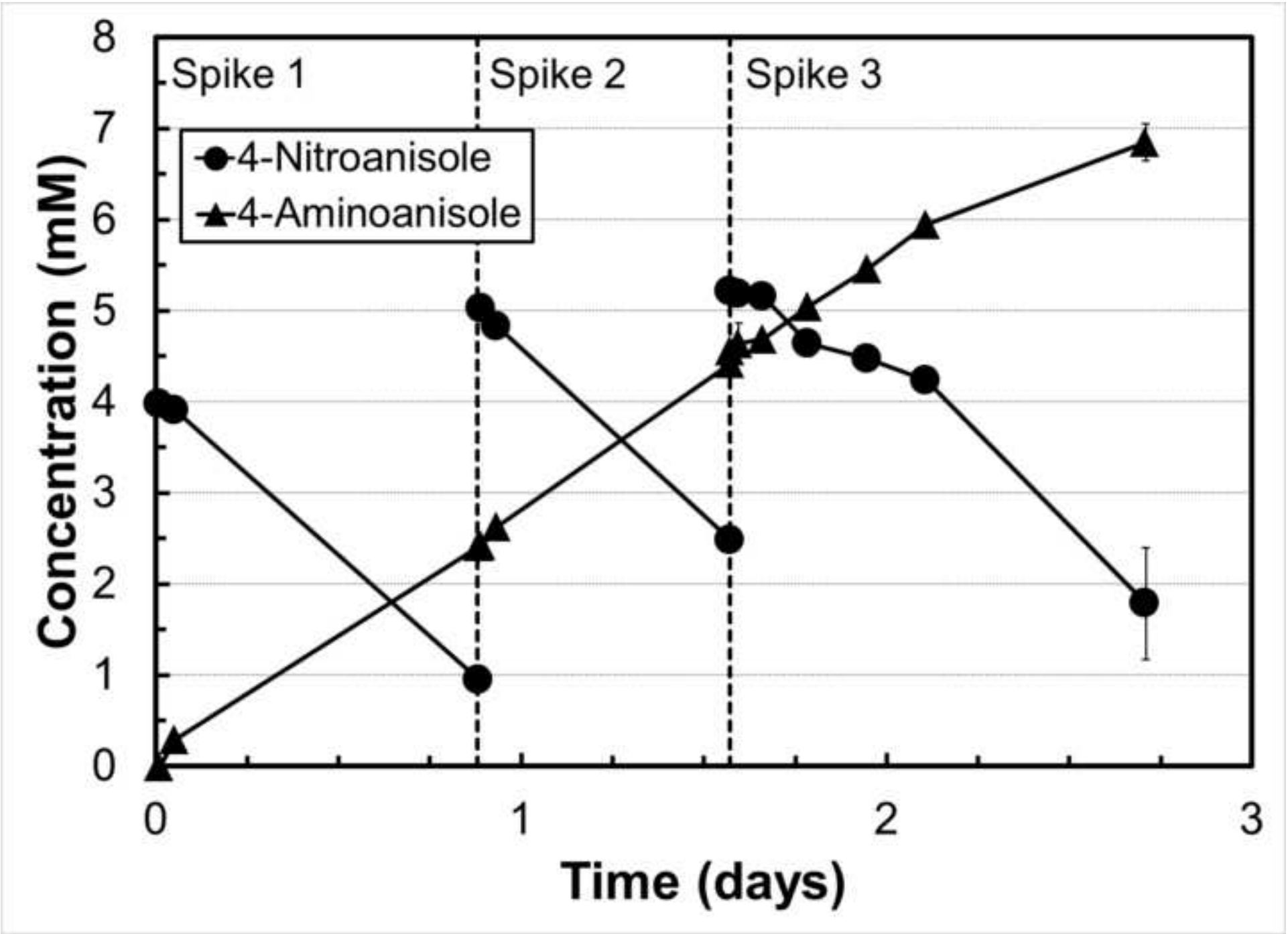

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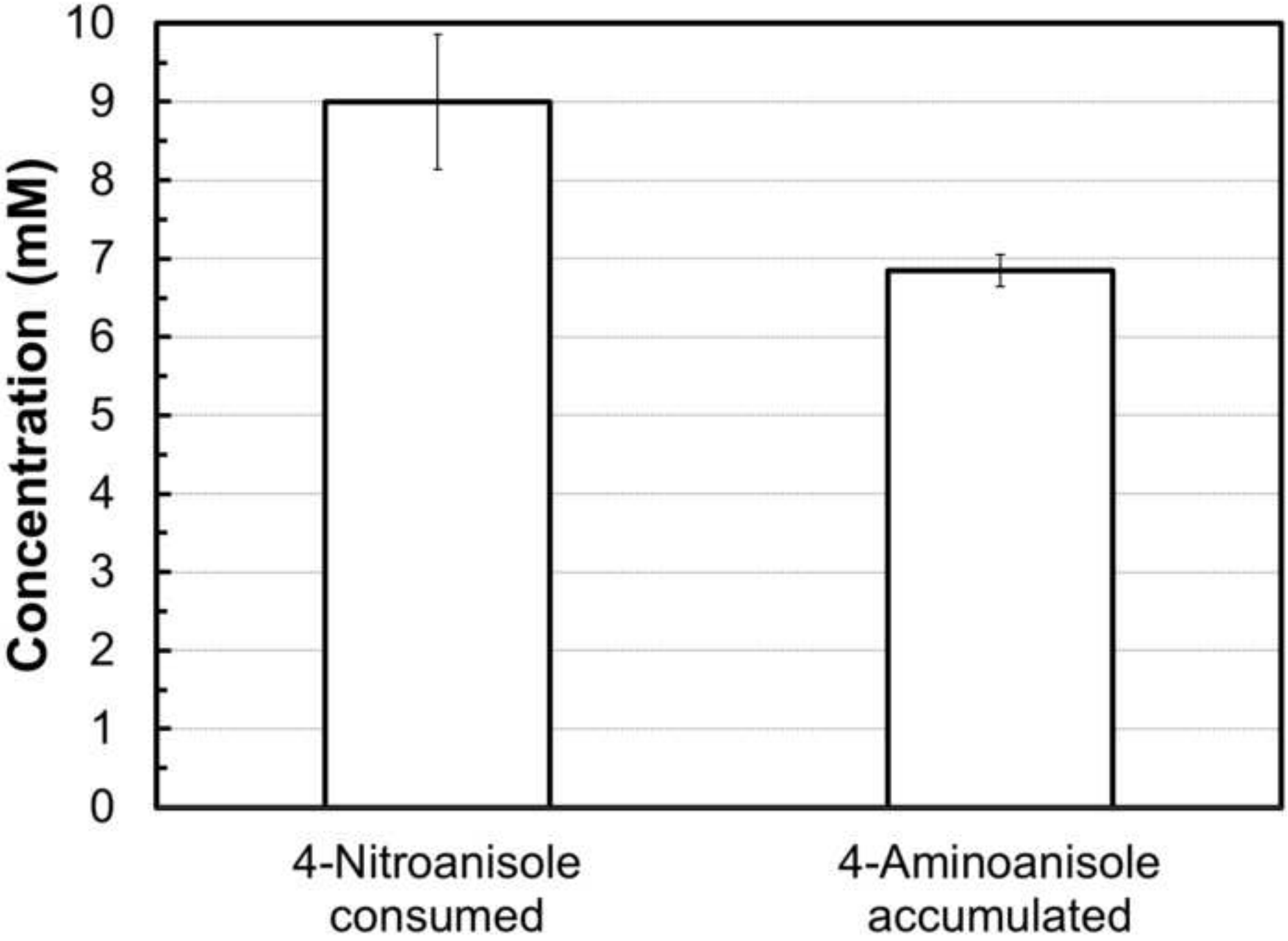

Figure 4

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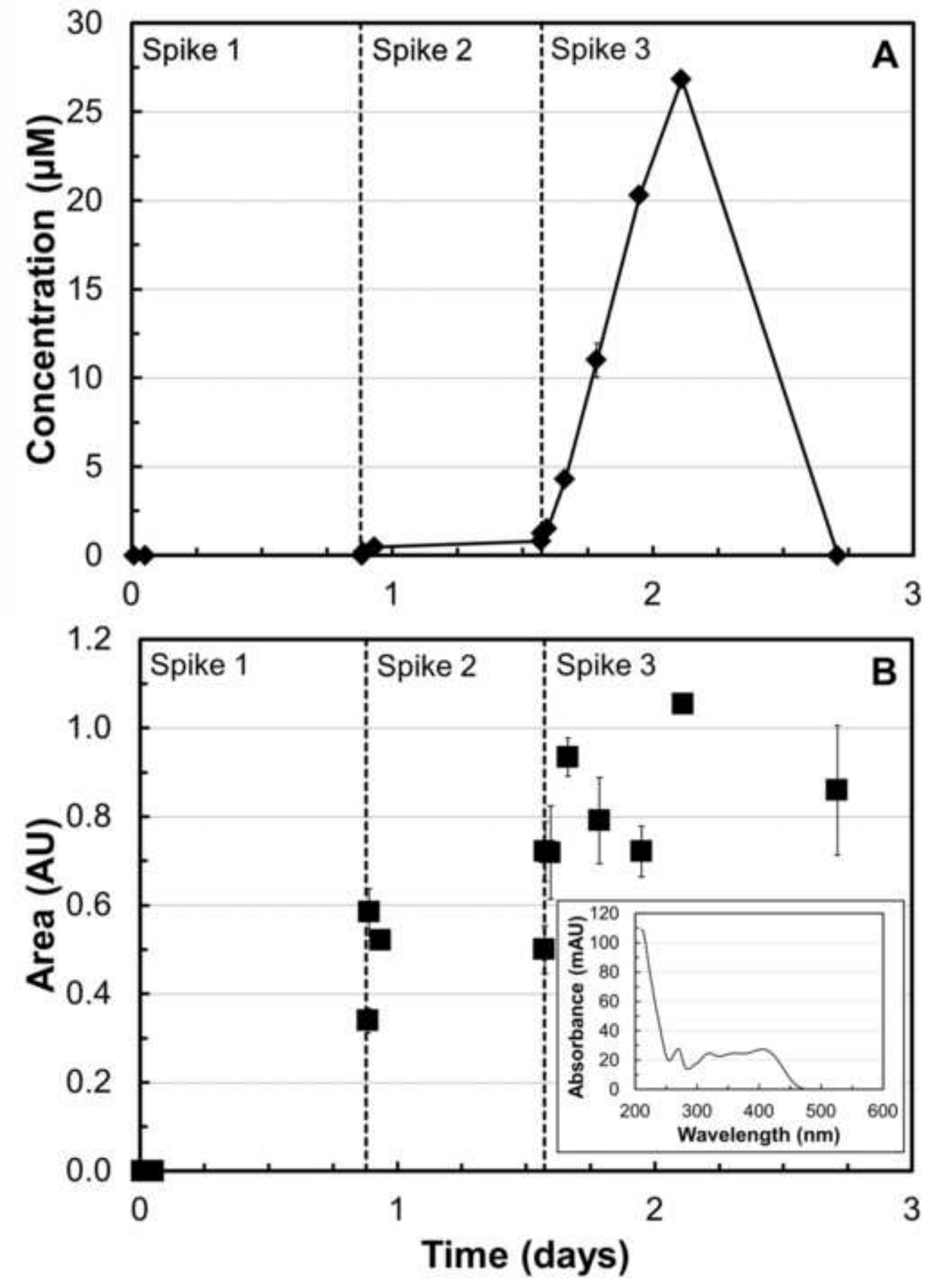

Figure 5

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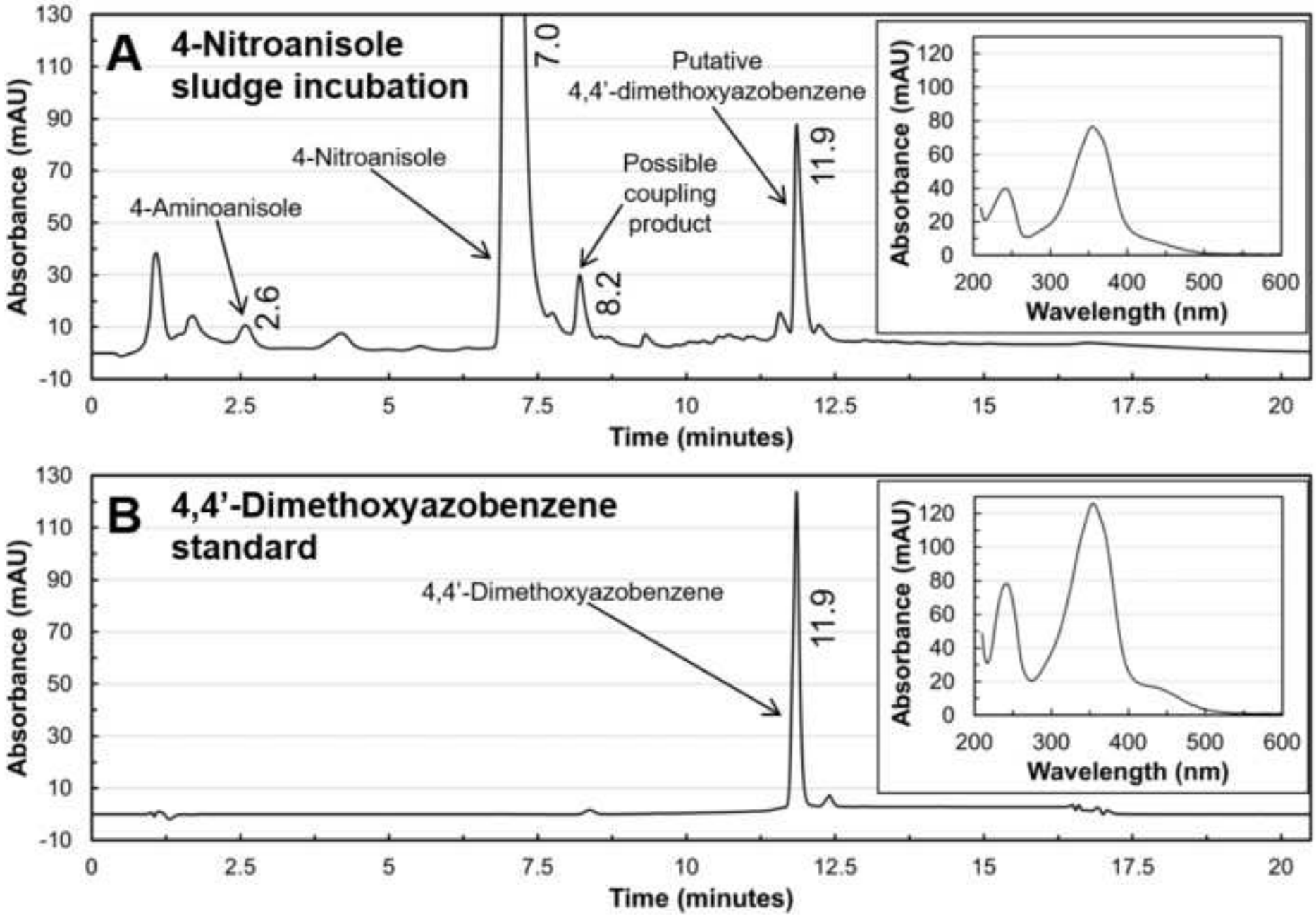

Figure 6

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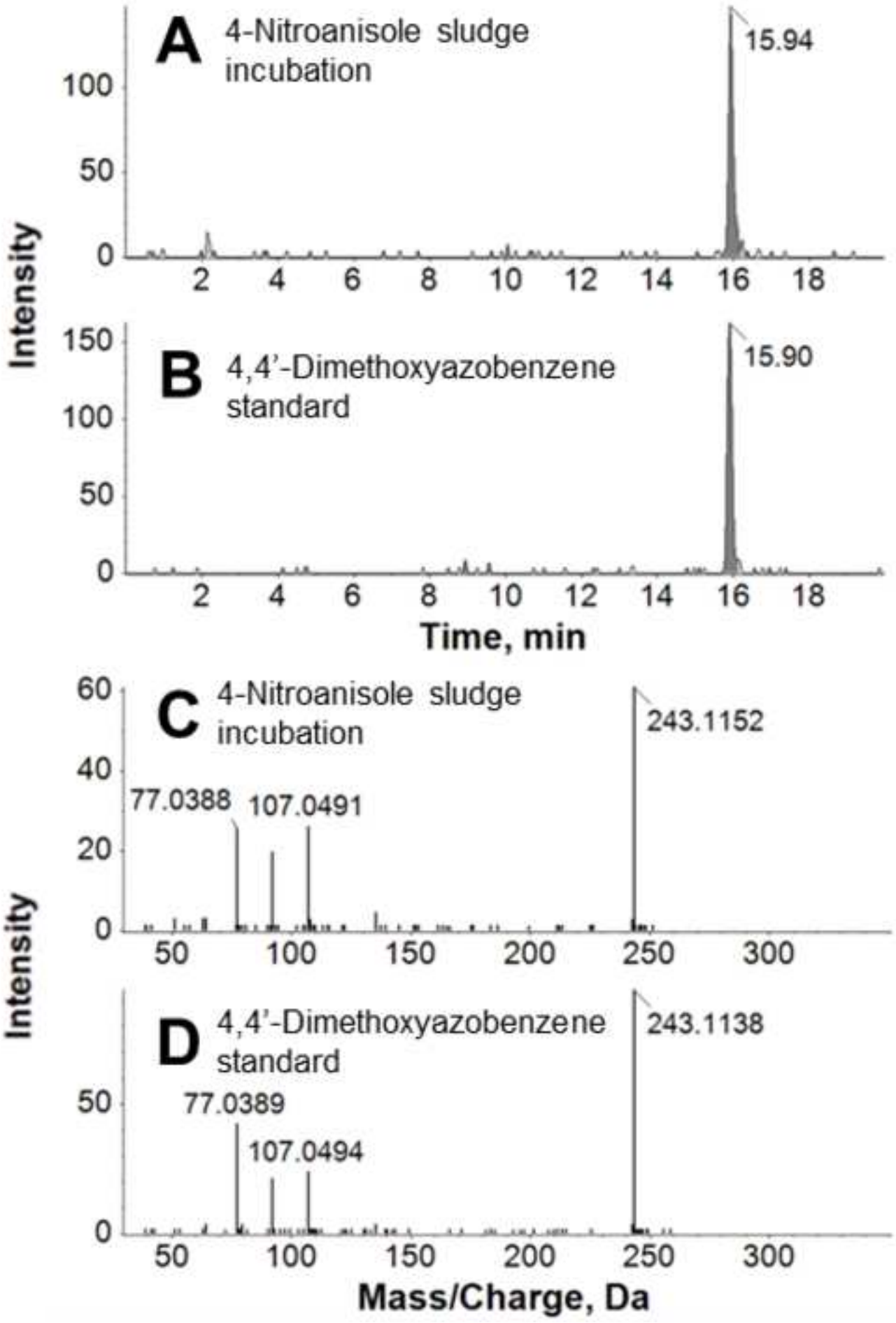

Figure Captions

Evidence of Anaerobic Coupling Reactions between Reduced Intermediates of 4-Nitroanisole

Warren M. Kadoya, Reyes Sierra-Alvarez, Stanley Wong, Leif M. Abrell,

Eugene A. Mash Jr., and Jim A. Field

Figure 1. Hypothesized reaction mechanisms of nitroaromatic compounds incubated under anaerobic conditions leading to the formation of an azo dimer. In our experiments, the R group is a methoxy group. We hypothesize that 4-nitroanisole will first be reduced to intermediates 4-nitrosoanisole, 4-hydroxylaminoanisole, and 4-aminoanisole through biologically catalyzed reactions, as shown in (A). These intermediates, particularly 4-nitrosoanisole and 4-aminoanisole, will then couple via nucleophilic substitution to form, after loss of H_2O, 4,4'-dimethoxyazobenzene, as shown in (B).

Figure 2. Fate of 4-nitroanisole in anaerobic sludge: nitro group reduction. 4-Nitroanisole transformed into 4-aminoanisole when incubated with anaerobic granular sludge supplied with mineral medium and H_2 as the electron donor (n=2). 4-Nitroanisole was delivered in three 4 mM spikes.

Figure 3. Mass balance of 4-nitroanisole incubated in anaerobic sludge. Overall consumption of 4-nitroanisole and accumulation of 4-aminoanisole at the end of the incubation experiment (4-nitroanisole in anaerobic granular sludge, n=2, shown in Figure 2).

Figure 4. Fate of 4-nitroanisole in anaerobic sludge: coupling product formation. (A) Formation and disappearance of 4,4'-dimethoxyazobenzene (◆) and (B) formation of a possible coupling product (■, peak with 8.2 minute UHPLC retention time) in anaerobic granular sludge incubations supplied with mineral medium, H_2 as the electron donor, and three spikes of 4 mM 4-nitroanisole (n=2). The insert in panel B is the UV-Vis spectrum for the possible coupling product.

Figure 5. UHPLC and UV-Vis spectral evidence of coupling product formation when incubating 4-nitroanisole in anaerobic sludge. Comparison of UHPLC retention times and UV-Vis spectra of the peaks with retention time of 11.9 minutes, (A) from the 4-nitroanisole incubation sample in anaerobic sludge taken at 2.1 days from Figure 4A and (B) from a 7.8 μM 4,4'-dimethoxyazobenzene standard. The inserts in panels A and B are the UV-Vis spectra corresponding with these peaks with retention times of 11.9 minutes in the sample and the standard, respectively. UHPLC chromatograms were obtained at 360 nm.

Figure 6. Mass spectral evidence of coupling product formation from 4-nitroanisole incubation in anaerobic sludge. Extracted ion chromatograms from selected reaction monitoring transition 243.1 > 107.05 are shown for (A) acetonitrile extract of incubation products from 4-nitroanisole incubation in anaerobic granular sludge and (B) 1.6 μg/L 4,4'-dimethoxyazobenzene standard. The product ion spectra from the tallest peaks in chromatograms A and B are shown in plots C and D, respectively.

Supplementary Material

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Revised Graphical Abstract