ROLE OF REDOX SHUTTLES IN THE BIOTRANSFORMATION OF INSENSITIVE MUNITION COMPOUNDS BY *GEOBACTER*

by

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As members of the Master's Committee, we certify that we have read the thesis prepared by Stanley Wong, titled *Role of Redox Shuttles in the Biotransformation of Insensitive Munition Compounds by Geobacter* and recommend that it be accepted as fulfilling the thesis requirement for the Master's Degree.

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Dedication

Dedicated to my sister, Jenifer,

who always pushes me to be my best

and inspires all those around her.

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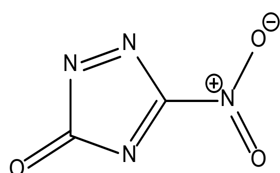
Abstract

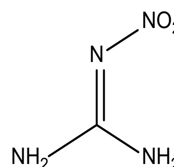
3-Nitro-1,2,4-triazol-5-one (NTO) is a nitroheterocyclic compound that is a component of insensitive explosives. Due to the lower risk of accidental detonation, insensitive munitions are being used more frequently in place of conventional explosives. Environmental contamination with insensitive munition compounds (IMCs) occurs from industrial wastewater discharge and undetonated material in soil. The toxicity of NTO is not fully understood and has created a need to remove it from the environment. Prior research has developed a highly enriched culture for NTO bioreduction. This culture, composed mainly of *Geobacter anodireducens* and *Thauera* species, reduces NTO to 3-amino-1,2,4-triazol-5-one (ATO) and oxidizes acetate to CO_2. This study explores the possibility of using redox mediators to increase the rate of NTO reduction or to expand the range of compounds that could be reduced by the culture. The redox mediator chosen for these experiments is 9,10-anthraquinone-2,6-disulfonate (AQDS), a model compound for other humic substances present in soil. It was found that the addition of AQDS to the enrichment culture with NTO can increase the rate of NTO reduction. The AQDS was directly reduced to 9,10-anthrahydroquinone-2,6-disulfonate (AH2QDS) by the enrichment culture with acetate as an electron donor. The reduced quinone can chemically react with NTO and other nitro group containing compounds to reduce the nitro compounds while oxidizing back into AQDS. This study highlights the potential of increased NTO reduction rates with redox mediators and the potential to expand the substrate spectrum of what can be reduced by this enrichment culture.

Introduction

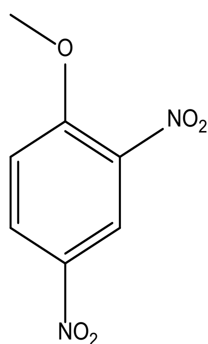
Insensitive munition compounds (IMCs) are compounds designed to replace current explosives (Davies & Provatas, 2006; Defisher et al., 2010). Insensitive munitions are mandated by law in the US due to "accidents, and the subsequent loss of human life" (Defisher et al., 2010). These new compounds have similar performance with improved safety and handling (Indest et al., 2017). Three IMCs are components of insensitive munitions explosive 101 (IMX-101) as a replacement for 2,4,6-trinitrotoluene (TNT) (Picatinny Public Affairs, 2010). These compounds are 2,4-dinitroanisole (DNAN), 3-nitro-1,2,4-triazole-5-one (NTO), and nitroguanidine (NQ) (Figure 1). Another IMX mix, IMX-104, includes DNAN, NTO, and 1,3,5-trinitro-1,3,5-triazine (RDX) (Indest et al., 2017).



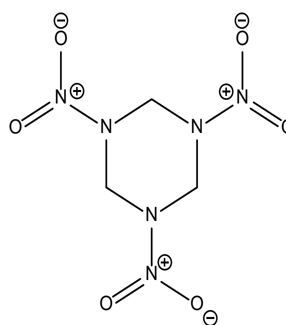
3-Nitro-1,2,4-triazol-5-one (NTO)



Nitroguanidine (NQ)



2,4-Dinitroanisole



1,3,5-Trinitro-1,3,5-triazine

Figure 1. Common compounds associated with insensitive munitions.

Although IMCs have comparable performance, these compounds enter the environment as propellant residues and detonation residues and pose potential problems (Figure 2). NTO is rather acidic and has a high solubility in water (49 g/L at 20˚C) (Becker et al., 1995). This high solubility allows for the compound to spread quickly in the environment through surface and groundwater. In addition, NTO has a low affinity for soil adsorption with an adsorption coefficient less than 1 cm^3/g (Mark et al., 2016). The low affinity implies that the compound can easily reach groundwater. The acidity of NTO also could cause acidification and solubilization of toxic metals in groundwater. The environmental properties of DNAN and NQ are similar to that of NTO. Both DNAN and NQ have low log K_{OW} values at 25 ˚C: 1.58 and 0.148, respectively (Hawari et al., 2015; Haag et al., 1990). These compounds are relatively hydrophilic and are soluble in water albeit not as high in aqueous solubility as NTO; the aqueous solubility of DNAN and NQ at 25˚C are 276 mg/L and 2600 mg/L, respectively (Boddu et al., 2008; Haag et al., 1990). The low Henry's constant of DNAN and NQ (5.51×10^{-4} and $<7 \times 10^{-6}$ at 25 ˚C, respectively) also suggest that the volatilization of these compounds is insignificant. In addition, NQ has a low soil sorption coefficient (less than 0.1) which allows it to readily move through soils (Haag et al., 1990). As IMC production and use increases, it is increasingly important to understand the toxicity of the compounds.

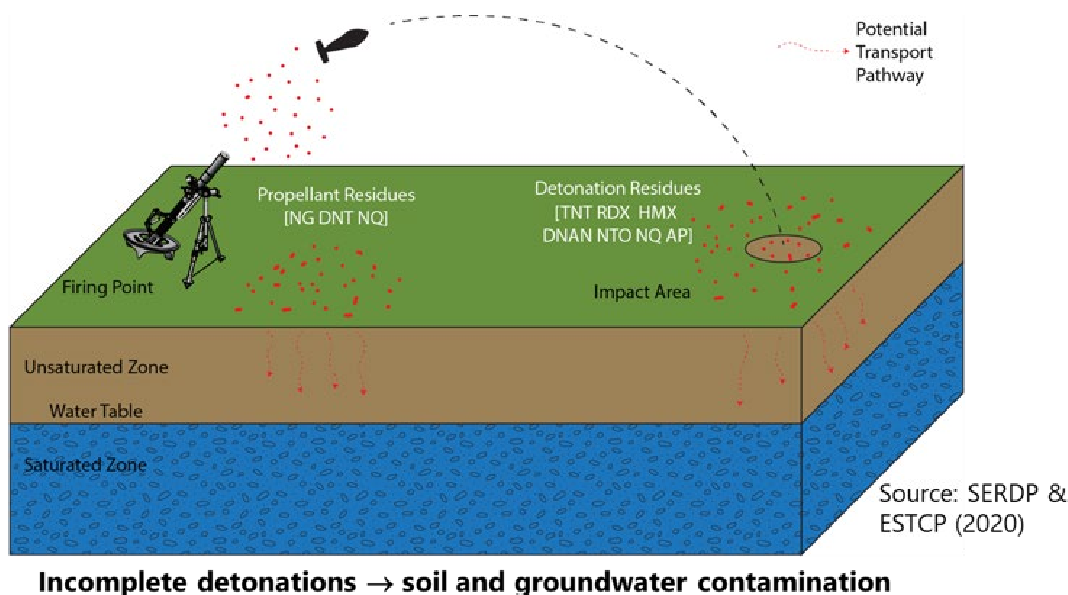


Figure 2. Diagram of energetic compound release on an artillery training range (source: https://www.serdp-estcp.org/energetic_compound/Contaminant-101).

The toxicities of NQ, DNAN, and NTO have been widely studied. NQ toxicity was tested by Burton et al. (1991). It was found that NQ had a lowest observed effect concentration (LOEC) of 440 mg/L in *Cladoceran* species. However, NQ can react in surface water via photolysis (Haag et al., 1990). The photolyzed NQ is much more toxic, with an LOEC of 3.6 mg/L in *Cladoceran* species (Burton et al., 1991). This implies that NQ can be extremely toxic if exposed to light. DNAN was found to be toxic to bacteria, algae, earthworms, and plants (Dodard et al., 2013). This wide range of toxicity implicates DNAN as an ecological hazard. However, the toxicity of DNAN is still less toxic than that of TNT (Hawari et al., 2015). In a 2015 study, Stanley et al. compared the acute and chronic toxicities of DNAN and NTO to that of TNT and RDX. The study found that after 96 hours, the 50% lethal concentrations (LC_{50}) for the species were 4.4 mg/L and 24.3 mg/L for TNT and DNAN, respectively. No significant effects were observed in RDX during a 10-day exposure. The LOEC for TNT, DNAN, and NTO after 28

days were 0.003 mg/L, 2.4 mg/L, and 5.0 mg/L, respectively (Stanley et al., 2015). This data implies that DNAN and NTO are less toxic than TNT, but more toxic than RDX.

Further studies into the toxicity of NTO have been conducted. Haley et al. found that the acute, 24-hour LC_{50} of NTO on freshwater *Cerodaphnia dubia* was 830 mg/L in neutral pH conditions (2009). However, their study found that in non-pH adjusted media, the LC_{50} is much lower at 66 mg/L. This indicates that NTO is much more toxic at lower pHs than at pH near neutral. A different study tested the ecotoxicity of NTO and its reduced compound, 3-amino-1,2,4-trinitro-5-one (ATO) on multiple species (Madeira et al., 2018). The 50% inhibitory concentrations (IC_{50}) on a methanogen species for NTO and ATO are 1.2 mM and over 62.8 mM, respectively (Madeira et al., 2018). In addition, the study found that NTO and ATO had negligible effects on aerobic heterotrophs at concentrations up to 32 mM. High concentrations of both NTO and ATO are required to inhibit the *Aliivibrio fischeri* bacteria used in the microtox assay; NTO and ATO had an IC_{20} of 19.2 mM and 22.4 mM, respectively (Madeira et al., 2018). Madeira also found that *Daphnia magna* was sensitive to ATO, with an LC_{50} of 0.27 mM. Finally, the study also found that 7.5 µM caused abnormal swimming behavior in zebrafish embryos (*Danio rerio*) but concentrations as high as 750 µM did not cause any lethal or developmental effects. Crouse et al. conducted a study to test the chronic oral toxicity of NTO in rats (2015). The team suspended NTO in polyethylene glycol (PEG-200) to minimize problems with acidifying the medium. After a 14-day study, the team found that at a dosage of 2000 mg/kg-d caused a decrease in body mass and food consumption in males. Overall, the toxicity of ATO relative to NTO varies between species. The toxicity of NTO could be lowered or enhanced in the reduction of NTO to ATO based on the target organism. While NTO does seem to be less

toxic than the traditional munitions, only a few species have been tested. The toxicity of IMCs still poses a problem and creates a need to remove these compounds from the environment.

Biodegradation provides a method for removal of toxic compounds from the environment. When biodegradation is high, the concentration of toxic compounds is reduced rapidly (Jørgensen, 2008). In most cases, the biodegradation of compounds is catalyzed by enzymes to convert the compounds into its respective end products (Crawford, 2011). Biodegradation of IMCs have been studied both separately and in a mixture with other IMCs.

The biodegradation of DNAN has been studied extensively. In 2014, a study by Fida et al. isolated a bacterial species that can degrade DNAN aerobically. The *Nocardioides* species strain JS1661 was isolated from activated sludge (Fida et al., 2014). The study found that this process involved the initial hydrolytic release of methanol to form 2,4-dinitrophenol (2,4-DNP). This process was associated with the hydrolase enzyme. Following the initial step, the 2,4-DNP was then further degraded by a hydride-Meisenheimer complex and release of nitrites. Another experiment further explored the application of the *Nocardioides* species. The JS1661 strain was tested in non-sterile soil, aqueous media, and a fluidized bed reactor (Karthikeyan & Spain, 2016). In this study, all three conditions facilitated the complete degradation of DNAN. In addition, there was little or no accumulation of 2,4-DNP, implying that the strain was able to completely degrade DNAN in these conditions. Another strain of bacteria, a *Bacillus* species was also found to biotransform DNAN in soil aerobically (Perreault et al., 2011). Soil microcosms were created for DNAN transformation and carbon and nitrogen supplements were provided to help facilitate the transformation (Perreault et al., 2011). This study found that with carbon and nitrogen supplements, DNAN is completely transformed in 8 days. With only a carbon supplement, complete transformation was achieved in 38 days and with no supplemental carbon

or nitrogen, no transformation was observed. DNAN was cometabolized and formed 2-amino-4-nitroanisole as the primary end product. These studies show that in the presence of oxygen, degradation of DNAN is possible.

The anaerobic degradation of DNAN has also been explored. Platten III et al. studied the anaerobic biotransformation of DNAN in a fluidized bed bioreactor (2010). In the reactor, the team found that the DNAN was transformed into 2,4-diaminoanisole (DAAN), which subsequently formed azobond polymers if exposed to aerobic conditions. In addition, the bioreactor could reacclimate in a short period of time to degrade DNAN if DNAN is removed for three weeks and reintroduced back into the influent (Platten et al., 2010). Olivares et al. characterized another pathway of transformation of DNAN in sludge (2013). This study tested the degradation of DNAN in three different conditions: anaerobic, microaerophilic, and aerobic. The degradation of DNAN was most effective in anaerobic conditions with hydrogen gas as a substrate (Olivares et al., 2013). In the pathway identified in this study, the ortho nitro group on the DNAN is first reduced to an amino group to form 2-methoxy-5-nitroaniline (MENA). The para nitro group is then reduced to an amino group to form DAAN. Azo and hydrazine dimer derivatives also form under the anaerobic conditions (Olivares et al., 2013). The biotransformation of DNAN in soil was also tested (Olivares et al., 2016). In Olivares et al.'s study, there was little evidence of transformation of DNAN in aerobic conditions (2016). However, in anaerobic conditions, biotransformation was possible and was positively correlated with organic carbon (OC) up to 2.07% OC. Moreover, the addition of hydrogen gas enhanced the nitro reduction rate (Olivares et al., 2016). Further, this study found that the rates in sterilized treatments were similar to that of endogenous treatment, suggesting that there may be abiotic factors involved in DNAN biotransformation as well. The reaction of this study matched the

prior one in which DNAN is reduced first to MENA then further to DAAN; this is followed by the formation of azo dimers, which then undergo further biotransformation. All these studies show that DNAN biotransformation is widely studied and is possible in both aerobic and anaerobic conditions.

While DNAN is relatively easier to biodegrade, NQ proves to be a bit more difficult. A 1982 study by Kaplan et al. found that NQ is not susceptible to aerobic degradation in activated sludge. However, NQ was able to be cometabolized in anaerobic conditions to nitrosoguanidine (Kaplan et al., 1982). While no further bioreduction of nitrosoguanidine was observed by Kaplan et al., the compound was found to further decompose nonbiologically to cyanamide, cyanoguanidine, melamine, and guanidine (1982). A later study in 1985 studied the decomposition of NQ in soils (Kaplan & Kaplan). This study found that cometabolic degradation of nitroguanidine at concentrations of up to 150 mg/L is possible with the addition of supplemental carbon in the form of glucose. Kaplan & Kaplan identified ammonia as the primary product with nitrite and nitrate present in very low concentrations (1985). The aerobic biotransformation of NQ was characterized by Haag et al. in 1990. The slow, cometabolic process occurs in natural waters and can be accelerated with the addition of nutrient broth or yeast extract (Haag et al., 1990). The study also found that the anaerobic transformation is also possible with the addition of glucose and yeast extract but much slower than in the aerobic process. In both reactions, Cyanamide is the product (Haag et al., 1990). A *Variovax* strain of bacteria isolated from soil was found to be able to aerobically mineralize NQ (Perreault et al., 2012). This strain could degrade NQ in aerobic microcosms with an added carbon source and was inhibited in the presence of a more favorable nitrogen source (Perreault et al., 2012). Perreault et al.'s study found that NQ turns into nitrourea as an intermediate, where it

decomposes to ammonia, nitrous oxide, and carbon dioxide as the end products (2012). This research suggests a mechanism that starts with the enzymatic hydroxylation of the imine. NQ is a more difficult compound to degrade than DNAN and there is ample research analyzing its decomposition.

Due to the high solubility and toxicity of NTO, it is of particular interest to study the biodegradation of NTO. A 1999 study isolated a species that was able to reduce NTO in aqueous waste (Le Campion et al.). The strain was identified as *Bacillus licheniformis* and was found to reduce NTO to ATO at concentrations as high as 15 g/L; the NTO was completely reduced within 24 hours (Le Campion et al., 1999). Le Campion et al. also found that the ATO ring then got cleaved to urea, carbon dioxide, and a polar compound; this mineralization process took approximately two weeks (1999). A more recent study published in 2015 centered around the degradation of NTO by soil bacteria communities (Krzmarzick et al.). This team tested seven soil inocula and found that all seven were able to reduce NTO to ATO via a hydroxylamine intermediate; the soils were inoculated in anaerobic conditions with hydrogen gas as an electron donor. Krzmarzick et al. then tested the degradation of ATO and found that ATO was only degradable in aerobic conditions while NTO could not be degraded aerobically (2015). This research suggests that a sequential anaerobic-aerobic phase process could effectively reduce NTO and mineralize ATO. Madeira et al. (2017) studied the potential for the sequential anaerobic-aerobic biodegradation of NTO. By inoculating soil in bioreactors with pyruvate as an electron donor, NTO mineralized into inorganic nitrogen species (Madeira et al. 2017). This research confirmed Krzmarzick et al.'s implications. While there is some research in the degradation of NTO and ATO, there is still potential to further understand these mechanisms.

The biodegradation of IMX-101 has also been studied. The simultaneous aerobic biodegradation of IMX-101 constituents was studied by Richard and Weidhaas in 2014. Their study found that the degradation products included nitrourea, 1,2-dihydro-3H-1,2,4-triazol-3one, and 2,4-DNP. Indest et al. explored the biodegradation of IMX-101 in surface soils from two military ranges (2017). This team found that after 30 days, there was complete NTO and DNAN degradation in one soil and complete NTO degradation in another (Indest et al., 2017). This study highlighted the complications of this chemical mixture as some compounds degraded while others did not. Temple et al. studied the fate and transport of DNAN, NTO, and NQ in a mixture (2018). The team found that after 24 hours in soil with a high organic content, DNAN and NTO started degrading. In addition, the two compounds were completely degraded in 60 days (Temple et al., 2018). However, Temple et al. found that there was only a 20% removal of NQ in the mixture after 60 days (2018). The study also suggests that the three compounds do not interact with each other. Overall, the research into IMX-101 indicates that methods able to degrade each constituent individually could be applied to the combinations.

The current enrichment culture in this research group is able to directly reduce NTO to ATO using acetate as an electron donor. This culture acts under anaerobic conditions and is very specific to NTO (Madeira et al., 2021). After analyzing the transgenomics, the culture was found to contain primarily a *Geobacter anodireducens* species (89.3%) and a *Thauera* species (5.5%) most resembling *Thauera humireducens* (Madeira et al., 2021). *Thauera humireducens* was isolated from a microbial fuel cell in 2013 (Yang et al.). This species grows under anaerobic conditions and can oxidize organic compounds to reduce 9,10-anthraquinone-2,6-disulfonate (AQDS) (Yang et al., 2013). AQDS is commonly used as an analog for humic substances generally found in soil. The reduced form of AQDS is 9,10-anthrahydroquinone-2,6-disulfonate

(AH2QDS). In addition, the *Thauera* species can also reduce soluble and insoluble Fe(III) (Ma et al., 2015). Ma et al. found that this species can use acetate, propionate, lactate, and pyruvate as suitable electron donors. The enzymes associated with the reduction of 9,10-anthraquinone-2-sulfonic acid (AQS) are dehydrogenases, quinones, and cytochromes b/c. These studies confirm that *Thauera* species are able to reduce different compounds using aceate as an electron donor.

While *Thauera* is present in the enrichment culture, the bulk of the culture is a *Geobacter* species. A study conducted by Coates et al. suggests that all *Geobacter* species can reduce iron (1996). The research implicates that *Geobacter* is present in environments where Fe(III) reduction is an important process. In addition, *Geobacter* species can grow with Fe(III) as a sole electron acceptor with acetate as an electron donor (Coates et al., 1998). *Geobacter anodireducens* was first discovered in 2014 by Sun et al. This species was found to be a close relative of *Geobacter sulfurreducens* and can reduce soluble and insoluble Fe(III) with acetate as an electron donor (Sun et al., 2014). All strains of *Geobacter* isolated so far are able to reduce Fe(III) (Lovely et al., 2004). In addition to Fe(III) compounds, it was also found that *Geobacter* species are able to reduce AQDS as well. Coates et al. isolated humic reducing *Geobacter* species that used AQDS as an electron acceptor (1998). In addition, these species could also reduce humic soil compounds (Coates et al., 1998). This study suggests that *Geobacter* is an important humic-reducing species. A specific *Geobacter*, *Geobacter metallireducens*, species that used humic substances as electron acceptors for microbial respiration was isolated in 1996 (Lovely et al., 1996). A different specific *Geobacter* species, *Geobacter sulfurreducens*, can transfer electrons to solid phase humic substances (Roden et al., 2010). These experiments suggests that *Geobacter* species can first reduce AQDS to AH2QDS, which can further react with other substances.

The chemical reaction with reduced humic substances with other compounds is of interest. Dunnivant et al. studied the reduction of nitrobenzenes with natural organic matter (NOM) (1992). The abiotic reduction of nitroaromatics was facilitated by NOM constituents (Dunnivant et al., 1992). Dunnivant et al.'s results suggest that these NOM constituents are quinone type moieties (1992). In 1994, Curtis and Reinhard also studied the reductive dehalogenation by AQDS. Their study also found that AH2QDS solutions were able to reduce compounds in a wide range of pHs. These chemical reactions suggest that the presence of AQDS in the enrichment culture could potentially increase the rates of NTO reduction, serving as a redox mediator.

The overall objective of this study is to assess the feasibility of AQDS as a redox mediator in the reduction of NTO by the enrichment culture (Figure 3). The presence of AQDS as a redox mediator could increase the rates of NTO reduction.

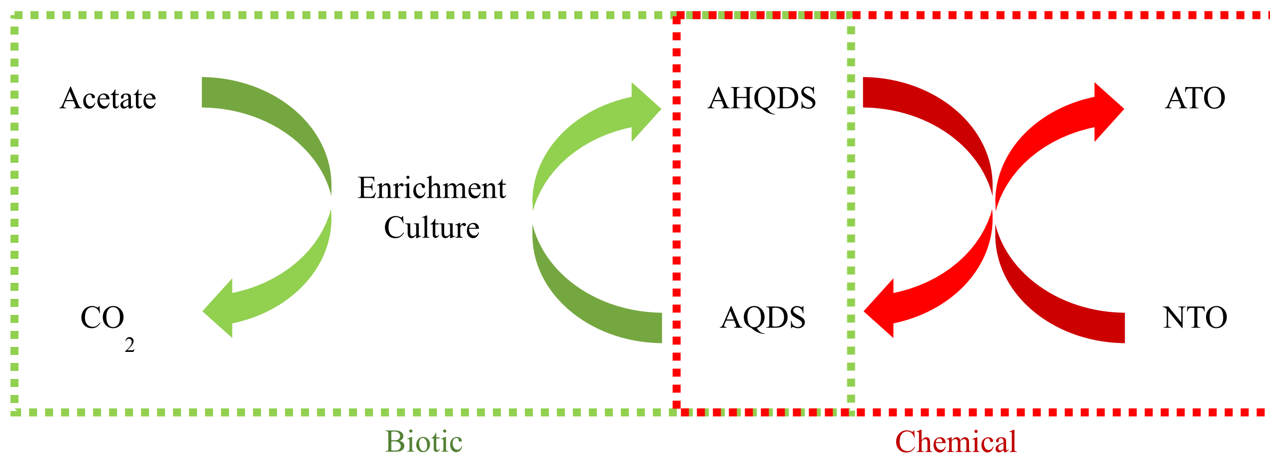


Figure 3. Proposed mechanism for NTO reduction via enrichment culture with AQDS as a redox mediator.

Materials and Methods

Experiment 1: Reduction of NTO with Enrichment Culture and AQDS

The objective of this experiment is to determine if the presence of AQDS in the NTO solution can increase the rate of NTO bioreduction of by the enrichment culture. This whole experiment was done under sterile conditions until sampling. A basal medium was created for the enrichment culture. The basal medium has a final concentration of 10 mg/L NH_4Cl, 10 mg/L $CaCl_2$ dihydrate, 100 mg/L $MgSO_4$ heptahydrate, 250 mg/L KH_2PO_4, a $NaHCO_3$ buffer, excess acetate, and trace elements from a stock solution. In addition, a concentrated AQDS and NTO solution was created with a AQDS disodium salt and NTO, respectively. The basal medium, AQDS solution, and NTO solution was filtered with a 0.22-micron filter into sterile flasks. After combining the medium, NTO, AQDS, and enrichment culture, the liquid and headspace were flushed with 20%/80% CO_2/N_2 to achieve anaerobic conditions. Different AQDS concentrations were tested with 0.5 mM NTO and 2.5% v/v of the enrichment culture. The AQDS concentrations tested were 733 mg/L, 92 mg/L, 1.8 mg/L, 0.4 mg/L, and 0.2 mg/L as AQDS excluding the sodium. A control with no AQDS was also created. All testing conditions were created in duplicate. Samples were collected over the course of 6 days. 1 mL samples were collected and placed in an Eppendorf. The samples were then centrifuged for 10 minutes at 13000 rpm. The supernatant was then transferred to a high-performance liquid chromatograph (HPLC) vial and analyzed in the HPLC for NTO and ATO concentrations. Concentrations were analyzed using an Agilent 1200 series HPLC (HPLC-DAD, Santa Clara, CA, USA) with a diode array detector and a Hypercarb column (Thermo Scientific, Waltham, MA, USA). The column was run with a gradient mobile phase; this phase consisted of water with 0.1% TFA and acetonitrile. The ratio of water with 0.1% TFA to acetonitrile are as follows: minutes 0-3,

100%/0%; minutes 3-11, 85%-15%; minutes 11-17, 50%/50%; minutes 17-18, 100%/0%. The NTO and ATO peaks were analyzed at 300 nm and 216.5 nm, respectively. The retention time of the NTO and ATO peaks were 15 and 8.9 minutes, respectively.

Experiment 2: Biotic Reduction of AQDS by Enrichment Culture with Acetate

The objective of this experiment is to further study the biotic step of the redox mediated reaction. In addition, this assesses whether the direct reduction of AQDS by the enrichment culture with an acetate electron donor is possible. This whole experiment was done under sterile conditions until sampling. The same basal medium as experiment 1 was prepared although without the addition of acetate. A concentrated AQDS solution was created with a AQDS disodium salt. A concentrated acetate solution and was also prepared using sodium acetate. All three solutions were filtered using a 0.22-micron filter. The basal medium, AQDS, and acetate were inoculated with 2.5% v/v enrichment culture. The conditions were created in duplicate, and both the liquid and headspace were flushed with 20%/80% CO_2/N_2 to achieve anaerobic conditions. The final concentration of AQDS was 1 mM and the final concentrations of acetate were 0.5 mM, 0.375 mM, 0.25 mM, and 0.125 mM. Two controls were also created: one with no acetate, and one with 1 mM of acetate but no AQDS. Samples were taken over the course of around 76 hours. At each sampling time, the bottles were brought to the anaerobic chamber to prevent the autooxidation of AH2QDS to AQDS. 1 mL of the sample was centrifuged in an Eppendorf tube for 5 minutes at 10000 rpm. The supernatant was transferred to a cuvette and diluted with a bicarbonate buffer. The samples were analyzed in a colorimeter at 430 nm. The colorimeter was a Thermo Scientific Orion AQUAfast AQ3700 colorimetry meter (Thermo

Scientific, Waltham, MA, USA). The cuvettes used were 1.6 cm in diameter. The extinction coefficient to AH2QDS at 430 nm was found to be 3.19 Au mM^{-1} cm^{-1} (Figure A.1).

Experiment 3: Abiotic Oxidation of AH2QDS via Reduction of Nitro Compounds

The objective of this experiment was to further explore the direct chemical reduction between AH2QDS and NTO. In addition, the feasibility of other nitro compounds to react with AH2QDS was also explored. The nitro compounds tested in addition to NTO were 4-nitroimidazole (4NIm), DNAN, and 4-nitroanisole (4NAn) (Figure 4). Approximately 0.8 mM of AQDS was reduced biotically to AH2QDS with the enrichment culture by inoculating the enrichment culture with 1 mM AQDS and a stoichiometric amount of acetate. 1 mM nitro compound solutions were prepared with a 20 mM phosphate buffer and flushed with 100% nitrogen gas. In an anaerobic chamber, the AH2QDS was filtered through a 0.22-micron syringe filter. AH2QDS and the nitro compound were combined in a 1.6 cm cuvette. The ratio of AH2QDS and nitro compound added was 3:1 to achieve near stoichiometric conditions. Water was also added to dilute the sample to within the measurable range. The final concentrations in the cuvette were ~0.2 mM AH2QDS and 0.083 mM of the nitro compound. The cuvettes were measured in a colorimeter at 430 nm. A reading was taken every 30 s to 1 min. Four replicates were created for each nitro compound. Two controls were also created, each with 4 replicates: one with only AH2QDS and water, and one with only the nitro compound and water. In addition, to understand if the light in the colorimeter was affecting the reaction rates, nitro compound, AH2QDS, and water were combined in a cuvette and wrapped in foil for 5 minutes. This was done in duplicate. After 5 minutes, the cuvette was measured and compared to the cuvettes with a continuous measurement. After running the experiment with NTO and AH2QDS and with

DNAN and AH2QDS, cuvettes were removed from the anaerobic chamber and samples were taken for HPLC analysis. In the HPLC, the Hypercarb column was used to measure NTO and ATO concentrations and the Inertsil ODS-SP column (GL Sciences, Tokyo, Japan) was used to measure DNAN concentrations. The Inertsil column operates with water and methanol as the mobile phase. The ratio of water to methanol is 25%/75%.

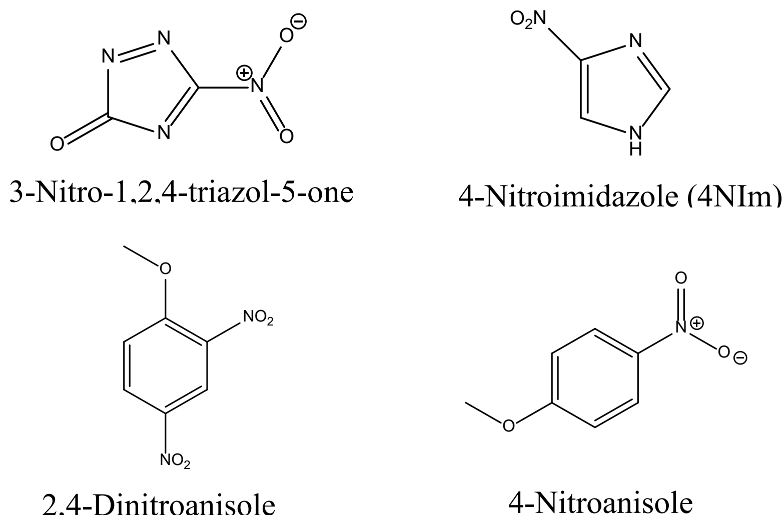


Figure 4. Nitro compounds tested with AH2QDS.

Results

Experiment 1: Reduction of NTO with Enrichment Culture and AQDS

The reduction of NTO to ATO was much faster with higher concentrations of AQDS (Figure 5). Concentrations of AQDS in mg/L refer to concentration as AQDS excluding the sodium ions. At concentrations of 1.8 mg/L of AQDS and higher, the NTO is completely reduced in less than 2.5 days. At an AQDS concentration of 0.4 mg/L, there was complete NTO reduction in about 3 days. The addition of 0.2 mg/L AQDS resulted in complete NTO reduction in less than 3.5 days. Without the addition of AQDS, the reduction of NTO was still possible albeit much slower, with complete NTO reduction in approximately 6 days. The addition of

concentrations of 0.4 mg/L and higher could decrease the time it takes to completely reduce the NTO by half. ATO was produced stoichiometrically to NTO reduction (Figure A.2). The lag time for all conditions was approximately one day.

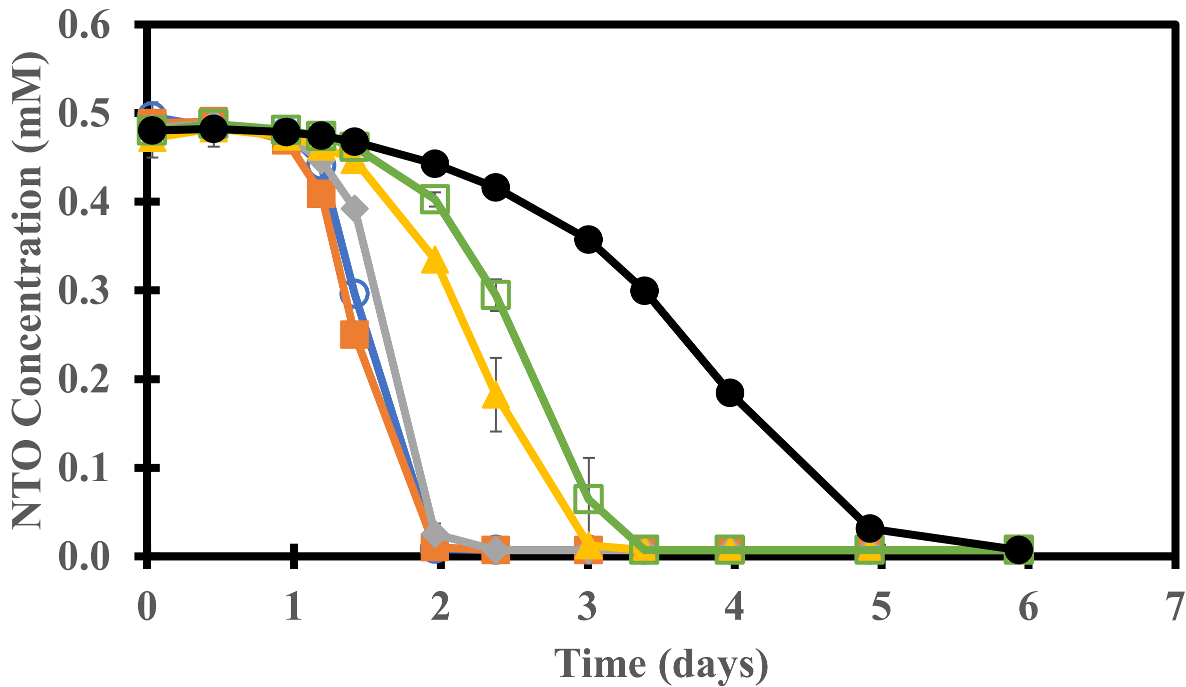


Figure 5. NTO concentration in NTO reduction by anaerobic enrichment culture at different AQDS concentrations. Legend based on the concentration of AQDS: control/no AQDS(●), 0.2 mg/L AQDS (□), 0.4 mg/L AQDS(▲), 1.8 mg/L AQDS (⧫), 92 mg/L AQDS (■), and 733 mg/L (○). Symbols represent the average of two replicates, and error bars represent the standard deviation.

The maximum NTO reduction rate was calculated from the slope at the steepest point on each time course. Overall, the maximum NTO reduction rate increased at with the addition of low concentrations of AQDS. (Figure 6). Increased AQDS concentrations lead to increased maximum NTO reduction rate up to a concentration of 2 mg/L AQDS. The addition of 0.2 mg/L AQDS, resulted in a 68% increase in the maximum NTO reduction rate while the addition of 2

mg/L of AQDS resulted in a 187% increase in the maximum NTO reduction rate. At a concentration of 2.0 mg/L AQDS, the maximum NTO reduction rates level out, and any concentration of AQDS above 2.0 mg/L resulted in a similar NTO reduction rate.

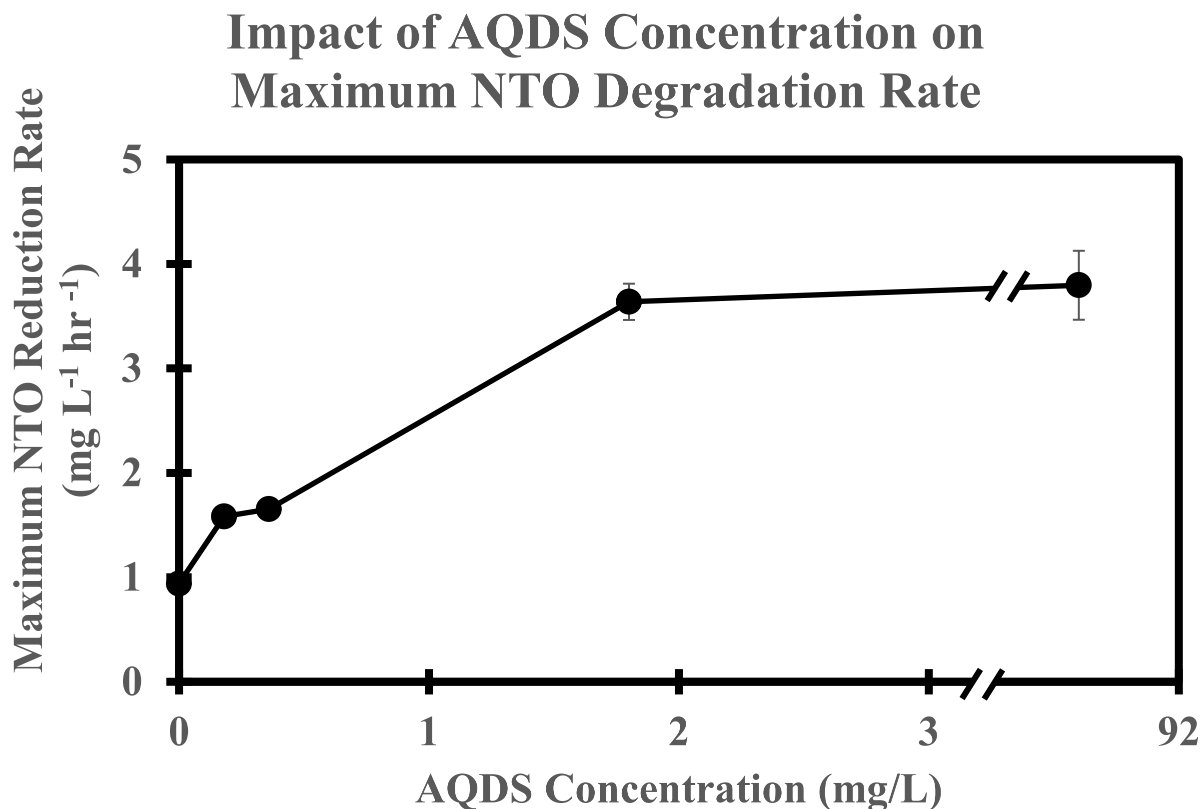


Figure 6. Maximum NTO production rate in NTO reduction by anaerobic enrichment culture at different AQDS concentrations. Symbols represent the maximum slope from an average of two replicates.

Experiment 2: Biotic Reduction of AQDS by Enrichment Culture with Acetate

The presence of acetate led to the production of AH2QDS (Figure 7). The reaction stopped and the AH2QDS concentration remained constant after approximately 72 hours for acetate concentrations of 0.25, 0.375, and 0.5 mM. An acetate concentration of 0.125 mM resulted in the AH2QDS concentration remaining constant after 48 hours. At higher acetate concentrations, a larger amount of AH2QDS was produced. In the bottles that contained a

stochiometric amount of acetate (0.25 mM) and the ones with 50% the stoichiometric amount (0.125 mM), the amount of AH2QDS produced was near stoichiometric to the amount of acetate present. In bottles with excess acetate (0.375 and 0.5 mM), the AQDS was completely reduced to AH2QDS. In the control without acetate, no AH2QDS was produced. In the control without AQDS, there was no significant absorbance in the colorimeter. The lag phase for the bottles with both acetate and AQDS was approximately 10 hours.

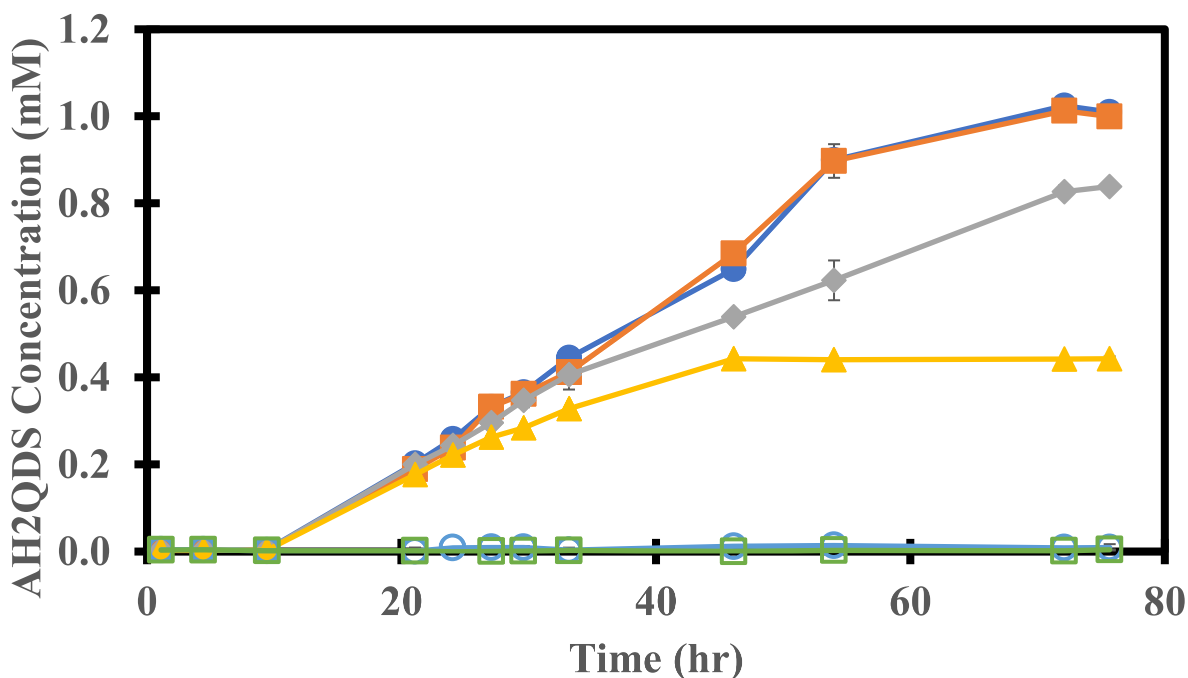


Figure 7. AH2QDS concentration in AQDS reduction by anaerobic enrichment culture at different acetate concentrations. Legend based on the concentration of acetate: 0.5 mM (●), 0.375 mM (■), 0.25 mM (⧫), 0.125 mM (▲), and 0 mM control (○). An additional control with 1 mM acetate and no AQDS was also tested (□). Symbols represent the average of two replicates, and error bars represent the standard deviation.

The maximum AH2QDS production rate was calculated from the slope at the steepest point on each time course. The addition of acetate increases the AH2QDS production rate (Figure 8). As the acetate concentration increases, the maximum AH2QDS production rate increases. There was no observed AH2QDS production when the acetate concentration was zero. While the increase in maximum AH2QDS production rate was large when the acetate concentrations increased at lower concentrations of acetate, the difference was relatively smaller at higher concentrations of acetate, creating a curve that is logarithmic in nature.

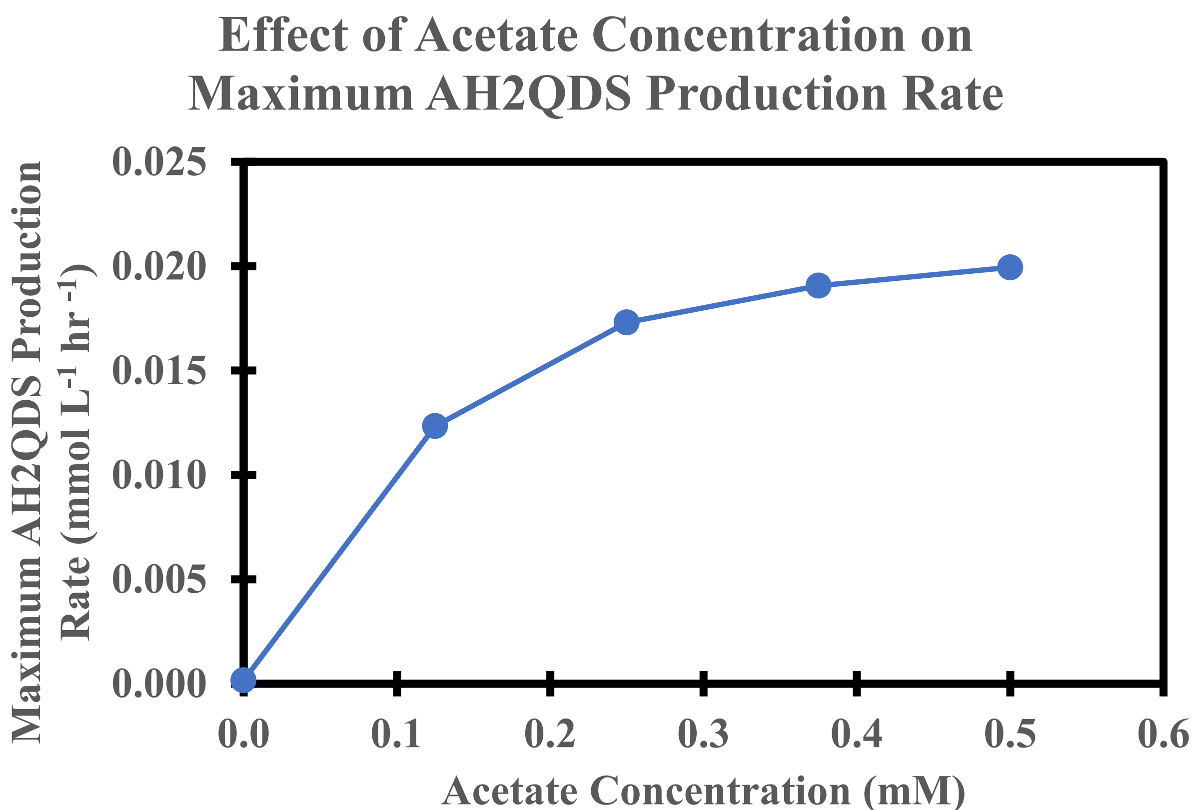


Figure 8. Maximum AH2QDS production rate in AQDS reduction by anaerobic enrichment culture at different acetate concentrations. Symbols represent the maximum slope from an average of three replicates.

Experiment 3: Abiotic Oxidation of AH2QDS via Reduction of Nitro Compounds

The combination of different nitro group containing compounds and AH2QDS resulted in a decrease in AH2QDS concentration over time (Figure 9). When AH2QDS is combined with NTO, the AH2QDS concentration began decreasing immediately. There was a faster decrease in AH2QDS concentration initially. After 30 minutes, there was no detectable concentration of AH2QDS. In the HPLC samples taken after 30 minutes, there was a decrease in the NTO concentration. The NTO concentration removed was near stoichiometric to the AH2QDS concentration removed. A different nitro compound, 4-NIm, was also tested due to its similarity in structure with NTO. The reaction of AH2QDS with 4NIm also resulted in a decrease in AH2QDS concentration albeit at a slower rate. The AH2QDS concentration dropped to around 20 μM after an hour when combined with 4NIm, whereas that same concentration was achieved in 15 minutes when AH2QDS was combined with NTO.

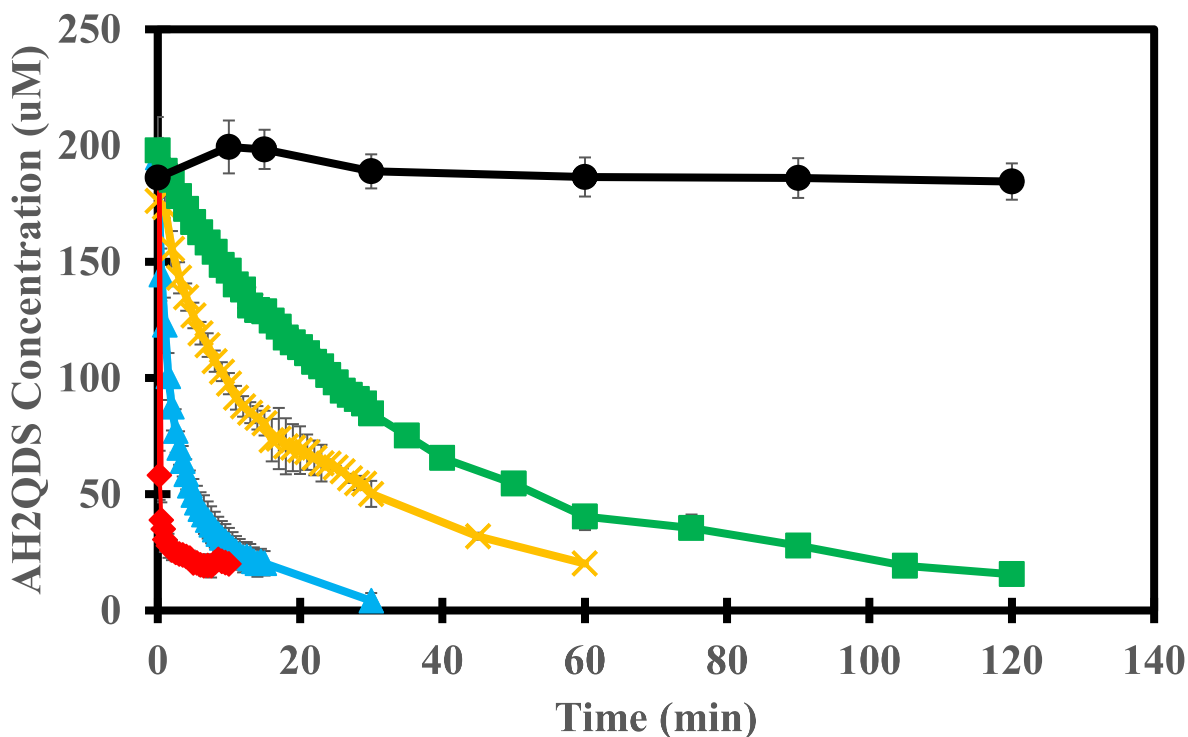


Figure 9. Reaction of 0.2 mM AH2QDS with 0.083 mM of different nitro compounds chemically. AH2QDS measurements were made in a colorimeter at 430 nm. The absorbance of the nitro compounds at 430 were measured to be very low. Legend based on the reaction of AH2QDS with different nitro compounds: control/no nitro compound (●), NTO (▲), 4NIm (✕), DNAN (⧫), and 4NAn (■). Symbols represent average of four replicates, and error bars represent the standard deviation.

DNAN was also tested as a nitro compound. The combination of AH2QDS with DNAN resulted in a rapid decrease in AH2QDS concentration. The AH2QDS concentration had dropped to 20 µM within 6 minutes. After 15 seconds, the concentration of AH2QDS had dropped below 60 µM. The absorbance measured in the cuvette remained constant after 5 minutes. In the HPLC samples taken after 15 minutes, there was no DNAN present in the samples. 4-nitroanisole is a compound that is similar in structure to DNAN, but with only one nitro group on the fourth carbon. The combination of AH2QDS with 4NAn resulted in a decrease in AH2QDS as well,

albeit much slower than in the presence of the other three compounds. The concentration of AH2QDS reached 20 μM after 105 minutes, over 17 times longer than the AH2QDS concentration when combined with DNAN. In an AH2QDS control with no nitro compound present, there was no observed change in the AH2QDS concentration. In the controls with nitro compounds and no AH2QDS, there was no significant absorbance in any of the 4 compounds at 430 nm. The absorbance of the cuvettes kept in the dark after 5 minutes was equivalent to the cuvettes that were being constantly measured in the colorimeter.

Discussion

Experiment 1: Reduction of NTO with Enrichment Culture and AQDS

NTO was able to be reduced to ATO at a faster rate even with the addition of small amounts of AQDS. These results suggest that AQDS can act as a redox mediator in the anaerobic bioreduction of NTO. Similar studies found that enrichment cultures with *Geobacter* spp. could reduce AQDS to AH2QDS, and the AH2QDS could increase the production rate of Fe(II) (Lovely et al., 1996; Chen et al. 2016). The reduced Fe(II) could also further biotransform other organic substances such as pentachlorophenol (PCP) (Chen et al., 2016). These results suggest that the humic reduction could enhance the capacity for the enrichment culture to reduce less accessible electron acceptors. This was observed by Niedźwiecka et al., where *Geobacter metallireducens* was able to use AQDS as a redox mediator to reduce DNAN into MENA and DAAN (2017). In addition, a different study found that the addition of AQDS was able to stimulate the biodegradation of 1,3,5-trinitro-1,3,5-triazine (RDX) (Bhushan et al., 2006). These results implicate the role of AQDS as a redox mediator in the reduction of NTO and other nitro compounds in two steps: a biotic step and an abiotic step.

Experiment 2: Biotic Reduction of AQDS by Enrichment Culture with Acetate

The direct reduction of AQDS to AH2QDS with acetate as an electron donor was observed. The production of AH2QDS was only observed in the presence of acetate. This is similar to Coates et al.'s study that discovered that all species isolated that used AQDS as an electron acceptor and acetate as an electron donor were *Geobacter* spp. (1998). Their study suggested that *geobacter* is an important humic-reducing species. A different *geobacter* species, *Geobacter sulfurreducens*, was also observed to be able to reduce humic substances (Roden et al., 2010). While the amount of AH2QDS reduced when adding a stoichiometric amount of acetate was only observed to be 84% of the AQDS added, it can be assumed that some of the acetate added was not used in the reduction of AQDS, but rather for growth as a carbon source. This is similar to studies where *Geobacter sulfurreducens* is commonly grown with acetate as both an electron donor and as a carbon source and Fe(III) citrate as an electron acceptor (Segura et al., 2018; Yang et al., 2010; Speers and Reguera, 2011). These results support that the culture can reduce AQDS to AH2QDS biotically using acetate as an electron donor. In addition, it suggests that the enrichment culture can not only directly reduce NTO but can directly reduce AQDS as well.

Experiment 3: Abiotic Oxidation of AH2QDS via Reduction of Nitro Compounds

The chemical step of the electron shuttled reaction is supported in the drop in AH2QDS concentration when combined with a nitro compound. In the absence of a nitro compound, there is no reaction. This heavily suggests that the AH2QDS is reacting with the nitro compound. The absence of DNAN and the near stochiometric removal of NTO also supports that the AH2QDS

reacts with the nitro compounds. The direct abiotic reduction of nitrobenzenes by reduced quinones was observed by Dunnivant et al. (1992). In addition, AH2QDS was found to be able to reduce other organic compounds (Curtis & Reinhard, 1994) and could reduce Fe(III) to Fe(II) (Chen et al., 2016). These studies support that AH2QDS can chemically reduce compounds such as NTO and DNAN. In the reaction between AH2QDS with NTO, the presence of light did not increase or decrease the reaction rate between the two compounds. This suggests that there is no photochemical reaction present in this reaction.

The reaction with DNAN was the fastest among the four nitro compounds tested. The additional nitro group makes the compound more reactive. However, in the reaction between DNAN and AH2QDS, the AH2QDS concentration did not drop completely to zero and instead remained constant at around 20 µM after 6 minutes. In addition, there was no DAAN detected in the HPLC while other peaks with small areas were found. This could suggest that DAAN or a reduced intermediate of DNAN could be reacting with each other or with the quinone to dimerize or polymerize (Kadoya et al., 2021; Menezes et al., 2021). Overall, the chemical reaction between DNAN and AH2QDS should be studied further to characterize what products are being formed.

Conclusion and Future Work

These studies demonstrate that the addition of AQDS to the enrichment culture can increase the NTO reduction rate. The culture has shown to be able to biotically reduce the AQDS using acetate as an electron donor. In addition, the reduced form of AQDS, AH2QDS, is able to chemically react with NTO and other nitro compounds; in this reaction, the AH2QDS oxidizes back to AQDS via the reduction of the nitro compound. These results suggest that when AQDS

is added to the NTO-reducing enrichment culture in the presence of NTO, the culture reduces the AQDS to AH2QDS. The AH2QDS reacts with the NTO and the oxidation of AH2QDS by the NTO recycles the quinone as an electron acceptor. The enrichment culture can then make more AH2QDS which can then chemically reduce more NTO to ATO, thus making AQDS a redox mediator in the reduction of NTO via the enrichment culture.

In this study, we had used AQDS as a model quinone for humic compounds found in soils. A future study should test the ability of soil with humic compounds to act as a redox mediator. In addition, the potential for the enrichment culture to reduce other compounds with AQDS that it had not been able to reduce directly should be considered. However, studies should also be considered to determine if other nitro compounds are toxic to the enrichment culture. While AQDS is a great redox mediator, a common trait amongst all *Geobacter* species is the ability to reduce Fe(III). Iron could also serve as a potential redox mediator for this enrichment culture. Finally, the potential for AH2QDS to reduce Fe(III) to Fe(II) which could then further reduce NTO should be explored to see if the presence of two redox mediators could increase the rate more than each redox mediator individually.

Appendix A: Supplementary Data

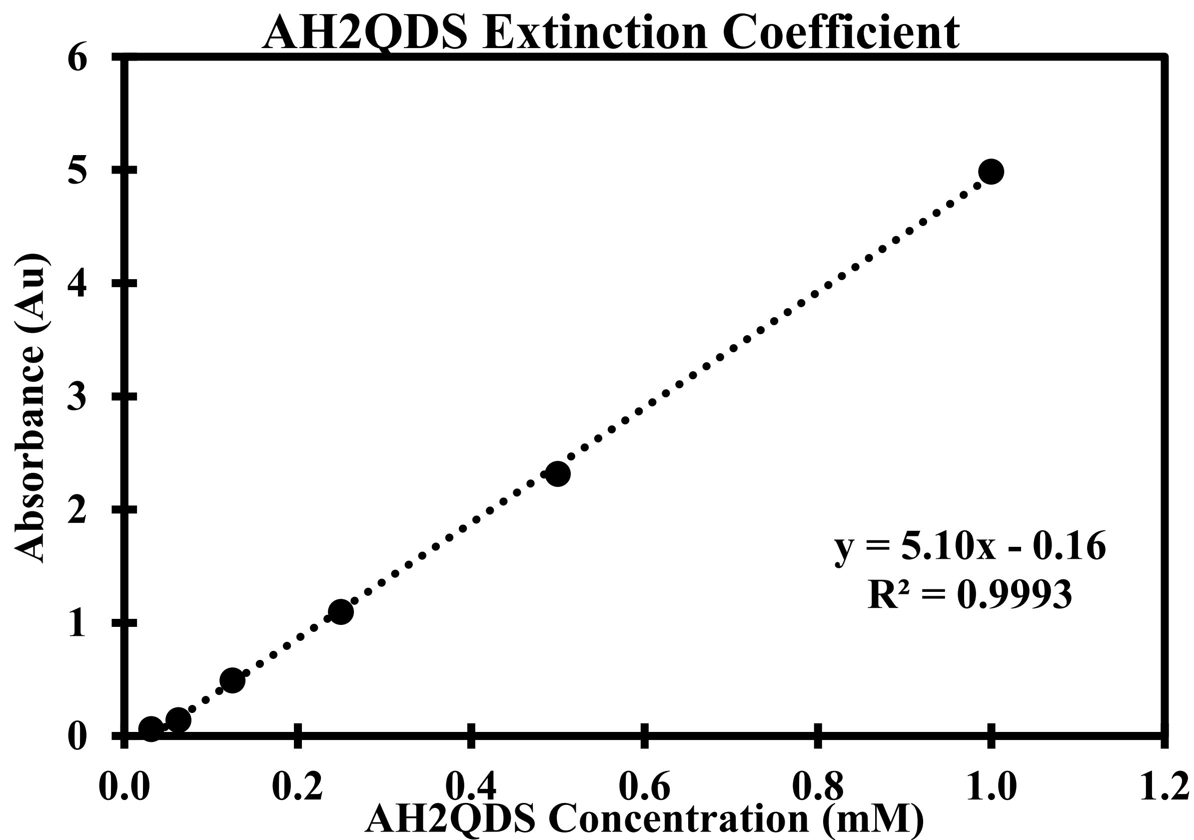


Figure A.1. Determination of extinction coefficient with biotically reduced AH2QDS. The slope of the trendline represents the extinction coefficient in Au/mM for a 1.6 cm cuvette.

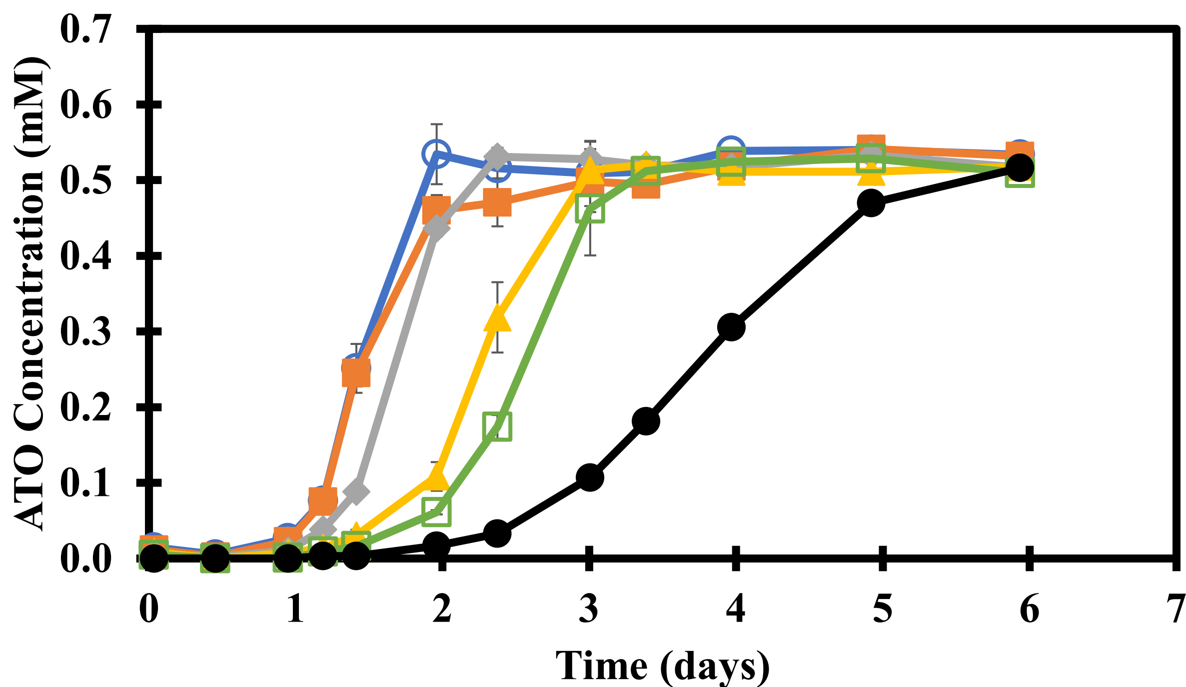


Figure A.2. ATO concentration in NTO reduction by anaerobic enrichment culture at different AQDS concentrations. Legend based on the concentration of AQDS: control/no AQDS(●), 0.2 mg/L AQDS (□), 0.4 mg/L AQDS(▲), 1.8 mg/L AQDS (⧫), 92 mg/L AQDS (■), and 733 mg/L (○). Symbols represent the average of two replicates, and error bars represent the standard deviation.

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