ENVIRONMENTAL FATE, (BIO)TRANSFORMATION, AND TOXICOLOGY OF 2,4-DINITROANISOLE (DNAN) IN SOILS AND WASTEWATER SLUDGE

by

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DEDICATION

A mi mamá (Ruth) y tía Many,

mis hermanas Aranza y Sofía,

a mi sobrina Zuley

y mis abuelitas Gloria y Trini.

que encontremos los electroncitos.
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ABSTRACT

Insensitive munition compounds (IMC) are an emerging class of explosives that are less susceptible to accidental explosions compared to the conventional explosives they will be replacing. An IMC that has been incorporated in several explosives formulations is 2,4-dinitroanisole (DNAN). As the manufacture, storage, and use of these compounds increases, the expected releases in natural and engineered systems might pose an environmental hazard to public health and ecosystems. To date there is little information on the environmental fate and toxicology of DNAN. However, nitroaromatic compounds are known to be toxic, mutagenic and difficult to completely biodegrade. In order to study the fate and (bio)transformation of DNAN, microcosm studies with soils and anaerobic wastewater sludge were performed to determine (bio)transformation pathways and key factors influencing (bio)conversion. Transformation was enhanced in anaerobic conditions, in particular when exogenous electron donor was added. Abiotic transformation (in heat-killed soil) was also significant and dominated transformation reactions in soils that were not amended with exogenous electron donor. The organic carbon content of soils was a key factor that correlated to the anaerobic biotransformation rate. Having identified (bio)transformation products using liquid chromatography coupled to quadrupole time-of-flight mass spectrometry, an overall pathway of (bio)transformation was devised and consistent with nitro-group reduction to form aromatic amines. During the nitro-group reduction, reactive products (e.g. nitroso-intermediates) coupled with amines to form azo-dimers and oligomers. Subsequent transformation pathways included N-alkylation, N-acetylation, and stepwise demethoxylation of these oligomers. The assessment of the toxicity of DNAN and its (bio)transformation
products was performed utilizing microbial toxicity assays and ecotoxicity evaluation with zebrafish (*Danio rerio*) embryos. Overall DNAN severely inhibited methanogens (IC$_{50}$ = 41 µM), the bioluminescent marine bacterium *Aliivibrio fischeri* utilized in the Microtox test (IC$_{50}$ = 57 µM), and nitrifiers (IC$_{50}$ = 49 µM). Reduced aromatic amine products in general were less toxic than DNAN with the exception of 2-methoxy-5-nitroaniline and 3-nitro-4-methoxyaniline, which were similar in toxicity to some of the test organisms as DNAN. Azo-oligomer surrogates were as toxic or more toxic than DNAN, although at trace levels they significantly stimulated activity. N-acetylated amines were found to have by far the lowest toxicity to microorganisms.

In zebrafish embryos, the (bio)transformation product or surrogates 3-nitro-4-methoxyaniline and 2,2'-dimethoxy-4,4'-azodianiline caused developmental abnormalities (each with lowest observable effect level of 6.4 µM). An integrated approach which monitored (bio)transformation product mixture profile in parallel with their toxicity to microbial and zebrafish toxicity was used to characterize toxicity during the time course of the anaerobic (bio)transformation of DNAN. Enhanced inhibition of methanogenic activity and zebrafish mortality were associated with the onset of dimer formation indicating they were being mostly impacted by reactive intermediates formed early in the biotransformation of DNAN. Further accumulation of oligomers was associated with a decrease toxicity. On the other hand, *A. fischeri* bioluminescence became more and more inhibited as the oligomers formed, indicating different responses depending on target organism. Taken globally, the results indicate that DNAN can be readily transformed in soils and wastewater sludge forming both highly toxic (e.g. azo-oligomers) and non-toxic intermediates (e.g. N-acetylated 2,4-diaminoanisole). Depending on target organism, the prolonged formation of oligomer mixtures either resulted in detoxification or recovery of activity.
CHAPTER 1
INTRODUCTION

NITROAROMATIC EXPLOSIVES AS ENVIRONMENTAL TOXICANTS

Nitroaromatic compounds are xenobiotic contaminants that are among the most common industrial chemicals in use today, including pesticides, dyes, explosives and building-blocks for pharmaceuticals [1]. Nitroaromatic explosives are high energy chemical compounds that have a high potential for contaminated sites, and have begun to be characterized—with limited clean-up operations worldwide [2]. Quantifying the extent of pollution due to nitroaromatic compounds has been a challenge. As of 2015, the US Environmental Protection Agency (US EPA) Comprehensive Environmental Response, Compensation, and Liability Information System (CERCLIS) Public Access Database listed 65 sites in the National Priorities List polluted with these chemicals. However, 15 million acres in the US alone have been identified as potentially polluted with energetic compounds [3], which can include nitroaromatic explosives.

INSENSITIVE MUNITIONS (IMs) AND 2,4-DINITROANISOLE

Within the chemical class of nitroaromatic explosives, insensitive munition compounds (IMCs) are emerging highly energetic chemicals that are resistant to shock and have a high detonation temperature, therefore reducing accidental explosions [4]. Due to these properties, which make IMCs safer to handle, defense industries around the world have been replacing conventional munitions with IMC containing formulations during the last decade. One of the first IMCs to be used on a
large scale is 2,4-dinitroanisole (DNAN) [4, 5], which has been included in formulations such as IMX-101, IMX-104, and PAX-21 [6].

There is scarce knowledge on the toxicity and environmental fate of DNAN. As the use of IMC containing formulations increases, releases of these chemicals could occur during manufacture, handling, storage, and at the end of the shelf-life of these munitions. Industrial wastewaters contaminated with DNAN and other IMCs will be produced during manufacture. Likewise, IMCs could impair soils, underlying aquifers, and nearby surface waters, as a consequence of unconsumed ordnance in testing ranges and in the field, or as accidental releases of these IMCs.

The potential release of DNAN to the environment poses a concern since many nitroaromatic compounds are toxic [7-9], mutagenic [10], and resistant to biodegradation [1, 9]. The nitro functional groups are electron withdrawing, and decrease the electronegativity of the aromatic ring. Overall, this makes the molecules less favorable for an oxidative, enzymatic attack conducive to mineralizing the compound [11, 12]. However, this does not necessarily mean that DNAN would be left in the environment without undergoing chemical reactions. A more common pathway for (bio)transformation of poly-nitroaromatic compounds, such as DNAN, is the reduction of nitroaromatic groups to their corresponding aromatic amines [8, 11, 12].

DNAN and the suite of compounds formed during (bio)transformation, may pose an environmental risk that is poorly characterized, particularly in terms of toxicity and environmental fate. Therefore, it is necessary to evaluate the biotransformation of DNAN in natural systems such as soils and water, as well as industrial effluents where residues of this IMC might impact local ecosystems.
(Bio)transformation of DNAN

Nitroreduction

Recent studies have begun to evaluate DNAN (bio)transformation in anaerobic and aerobic conditions in pure and mixed-culture systems [13-16], and some metabolites have been elucidated. Since DNAN is a very oxidized molecule, electrophilic oxygenase attacks, a common pathway in aerobic conditions, are unlikely. Therefore, the primary route of (bio)transformation is reduction, which is favored in anaerobic conditions but can also occur aerobically. DNAN undergoes reduction of the ortho nitro group leading to the formation of 2-methoxy-5-nitroaniline (MENA) [16, 17]. The reduction of the second nitro-group forms 2,4-diaminoanisole (DAAN) [17, 18]. These reactions can be catalyzed biologically with bacterial oxygen-insensitive nitroreductases, via nitroso and hydroxylamino products [8, 19]. Since these enzymes are oxygen-insensitive, nitroreduction could occur aerobically as well as anaerobically. Aerobically, MENA has been detected, but not DAAN [16]. However, in heterogeneous systems, abiotic reactions may also promote nitroreduction, particularly in the presence of Fe(II)-ligand complexes [20], Fe(II)-coated clays [21, 22], and sulfides [23].

Formation of azo-oligomers

Furthermore, during these reduction reactions, there are reactive intermediates that lead to the formation of azo-oligomers [17, 18]. In anaerobic and aerobic conditions, the formation of azo dimers and oligomers have been reported in mixed-
culture incubations of DNAN [13, 16, 17, 24]. There are different mechanisms that might contribute to the formation of azo-oligomers. The first involves a coupling reaction between hydroxylamino and nitroso intermediates, yielding an azoxy-dimer, which leads eventually to the formation of azo-dimers [25]. This mechanism is known to work in aerobic conditions, but azo-dimers have also been reported in anaerobic conditions [17, 18, 24, 26]. Metals present in biological systems in heterogeneous systems, such as sediment microcosms containing metals, could catalyze reoxidation of aromatic amines, yielding azoxy and azo-dimers [26]. An alternative mechanism involves the reaction between amino and nitroso intermediates, which could yield azo-dimers directly. Such a mechanisms is based on azo-dye synthesis from one-step reduction of nitroaromatics with iron [27].

**Parallel pathways of (bio)transformation**

In addition to the primary route of (bio)transformation, there are several secondary (bio)transformation routes that have been identified. O-demethylation–(dehydroxylation), N-acetylation, and N-alkylation reactions have been reported [16, 17]. Recently, denitritation of DNAN was reported for *Nocardioides sp.* strain JS1661 leading to the formation of 2,4-dinitrophenol, followed by a Meisenheimer hydride and release of nitrate [15]; a potential route for mineralization of DNAN when specialized bacteria such as this species are present. As seen, DNAN is far from remaining stagnant in the environment and is transformed by biological and abiotic processes that form (bio)transformation products that alter DNAN’s potential environmental fate and toxicity. A general overview of the primary and secondary DNAN (bio)transformation pathways to date are summarized in Figure 1.1.
**BIO)TRANSFORMATION IMPLICATIONS FROM A SIMILAR COMPOUND, TNT**

The knowledge available for DNAN is relatively recent and limited. However, TNT could be a chemical analog to predict the transformations and fate of DNAN in natural and engineered systems. There is a correspondence between these two compounds due to structural similarities. Biotransformation studies on TNT are widespread and reviews on microbial, fungal and phyto- transformation routes can be found. Aerobically, aerobic bacteria have been reported to partially reduce TNT to hydroxylamino derivatives, which then form azoxy derivatives [28]. In addition, the same review mentions that bacteria classified as *Pseudomonas sp.* had been reported to form Meisenheimer hydride-complexes and release of nitrite had been attributed to the denitritation of TNT. Anaerobically, TNT is known to reduce to aromatic amine derivatives. However, 2,4,6-triaminotoluene (TAT) is known to form only if the redox conditions are strongly reducing: -200 mV or below [28]. Afterwards, TAT leads to formation of azo dimers [26]. Globally, the wealth of knowledge on TNT (bio)transformation could contribute to predict DNAN fate and (bio)conversion potential in natural and engineered systems due to the similarities in the chemical structures of these two compounds.

**Implications of (bio)transformation to the fate of DNAN**

Overall the primary route of reductive (bio)transformation from DNAN yields both more mobile (aromatic amines) and less mobile (azo-oligomer) products, which could affect the distribution and extent of DNAN pollution. The azo oligomers and aromatic amine derivatives could ultimately bind to soil fractions. TNT and its
aromatic amines have been predicted and found to bind tightly to organic matter [9, 29] in soils as well as clays [30]. The mechanism for binding in clays has been predicted to be an electron donor-acceptor pair complex [31]. On the other hand, radiolabeled experiments with $^{14}$C-TNT in soils amended with molasses in anaerobic conditions, have found that 40-70% of the labelled material was bound to humic substances in soils, particularly to the humin fraction [29]. These interactions with soils have generally been classified as chemisorption, or irreversible binding [30, 32].

Figure 1.1 Summary of main biotransformation reactions and transformation products reported for DNAN.
TOXICITY OF NITROAROMATIC COMPOUNDS AND (BIO)TRANSFORMATION PRODUCTS

Many nitroaromatic compounds are toxic and mutagenic to different types of organisms, including bacteria, algae, plants, invertebrates, and mammals [8, 33, 34]. Therefore, adverse outcomes may occur at different trophic levels upon exposure to these chemicals. At the microorganism level, often selected reference assays, including algae [6, 33], bioluminescent bacteria[6, 35-37], and methanogenic archaea [34, 37] have been indicators of impacts of nitroaromatic explosives to microbial populations. For nitroaromatic explosives, benchmarks for different media (sediments, surface waters, etc.) have been developed using ecotoxicology studies to determine concentrations of occurrence of these toxicants that cause concerns about adverse ecological effects [38].

Microbial toxicity of DNAN

Once released to the environmental and to industrial effluents, DNAN could pose a significant environmental hazard to microorganisms. Firstly, in natural environments, key microbial populations in carbon and nitrogen cycling should be monitored for potential toxic effects due to DNAN and other emerging munitions contaminants that may compromise flux of carbon and nitrogen. Secondly, biologically-based wastewater treatment systems are very widespread in municipal and industrial wastewater treatments. Thus characterizing the potential inhibition to key populations in these systems is of high relevance to ensure proper wastewater process functionality.
Data on the inhibitory potential of DNAN towards microorganisms are scarce. To date, there are only two studies that address directly the toxicity of DNAN [6, 37]. With regard to ecotoxicity, DNAN caused growth inhibition in the green algae *Pseudokirchneriella subcapitata* (EC$_{50}$ = 4.0 mg/L), and bioluminescence in the marine bacterium *Allivibrio fischeri* was severely inhibited (IC$_{50}$ = 60.3 mg/L, 30 min exposure) [6, 37]. Acetoclastic methanogens and nitrifiers were also inhibited considerably (IC$_{50}$ = 8.1-9.7 mg/L) [37]. On the other hand, aerobic heterotrophs have been less sensitive to DNAN.

**Ecotoxicity of DNAN**

The last five years have seen considerable development in toxicity evaluation to assess DNAN potency on aquatic, terrestrial, and other relevant ecological toxicity organism models [6, 39]. Fifty-percent lethal concentrations (LC$_{50}$) for different species have been assayed: water fleas (*Ceriodaphnia dubia* 48h-LC$_{50}$ = 42 mg L$^{-1}$ and *Daphnia pulex* 3d-LC$_{50}$ = 18-20.3 mg L$^{-1}$)[39], Northern leopard frog (*Rana pipiens* 96h-LC$_{50}$ = 24.3 mg L$^{-1}$) [40], earthworm (*Einsinia andrei* 7d-LC$_{50}$ = 98 mg kg$^{-1}$) [6], fathead minnow (*Pimephales promelas* 48h-LC$_{50}$ 37 mg L$^{-1}$) [39], zebrafish (*Danio rerio* larval 96h-LC$_{50}$ = 75.3 mg L$^{-1}$, tested as total IMX-101 composition) [41]. In addition to mortality, there are also considerable effects at sublethal concentrations. Moreover, toxicant avoidance test with *E. andrei* (where the worm could choose from a chamber with DNAN vs. one without this toxicant) saw a median effective concentration (EC$_{50}$) that was less than half the lethal values (48h-EC$_{50}$ = 31 mg kg$^{-1}$ soil) [6]. Moreover, phytotoxicity of DNAN has been assays in shoot elongation in ryegrass growth (*Lolium perenne* 19d-EC$_{50}$ = 7 mg kg$^{-1}$) [6]. Moreover,
recently adverse outcome pathways have been developed based on mammalian toxicity literature to describe metabolism of nitroaromatic munitions that cause oxidation of iron in hemoglobin, leading to cyanosis, as well as oxidative stress, resulting in DNA damage and apoptosis [42]. It is possible that DNAN might pose similar toxicity risks. Overall, while the primary endpoint in ecotoxicity studies has been lethality, DNAN sublethal effects should also be evaluated in in vivo models.

**Toxicity of primary (bio)transformation products**

Since sites polluted with DNAN will also have its transformation contaminants, it is important to note the toxicity levels of the transformation products. Nitroreduction has been considered as a detoxification reaction [43] and aromatic amines are much less toxic to methanogens than the nitroaromatic chemical analogs [34]. For instance, the reduction of nitrophenol compounds to aminophenols in an up-flow anaerobic sludge allowed for continuous transformation, even at 25 fold higher levels than those causing inhibition [44]. However, nitroanilines (expected to be formed from single nitrogroup reduction in DNAN) may pose severe inhibition to methanogens [34].

Besides cytotoxicity, other toxicity effects should be considered, such as mutagenicity potential. For instance, TNT and its hydroxylamino biotransformation products were mutagenic to *Salmonella typhimurium* TA98 and TA100[45]. However, an overall decrease in mutagenicity potential has been observed once biotransformation is carried past hydroxylamino derivatives [46]. In regards to DNAN
there are no mutagenicity studies to date, but its reduced metabolite DAAN has shown to be mutagenic and a potential carcinogen in various assays [47-50].

**DNAN and TNT toxicity**

Since insensitive munitions have been formulated as replacements to conventional explosives, it is of interest to consider the relative toxicity with respect to the compounds they seek to replace. In addition, DNAN has been reported to be less toxic in general than TNT in most toxicity model organisms [6, 39, 40]; and depending on the toxicity model, TNT had been reported to be 1.3 to ~100 times more toxic than DNAN [6]. However, since the specific toxicity modes of action between DNAN, TNT, and other poly-nitro toluenes and anisoles are expected to be similar [42], perhaps the increased cytotoxicity potency of TNT compared to DNAN could be due to increased hydrophobicity and partitioning into biological tissue. Octanol-water partitioning coefficients (Log $K_{ow}$) have been positively correlated to cytotoxicity in methanogens [51]. DNAN has a lower reported Log $K_{ow}$ (1.58) compared to TNT (1.86), both at 25 °C [52], which might account for the cytotoxicity difference. Furthermore, the characterization of toxicity emerging munitions compound should also consider specific-toxicity mechanisms unique to DNAN and TNT, as well as those posed by their (bio)transformation products.

**Toxicity implications of (bio)transformation products and mixtures**

Additional toxicity considerations might come from specific mechanisms triggered during (bio)transformation. Some of the products formed during the
(bio)conversion of nitroaromatics might be more toxic than the parent compounds, while others might be less toxic. Of particular concern are hydroxylamino and nitroso intermediates that are formed during nitroreduction, since these unstable products might bind directly to DNA and other biomolecules, leading to toxic effects as well as mutagenesis [46, 53]. In addition, single electron reduction in nitroaromatics caused by oxygen-sensitive nitroreductases and abiotic factors can lead to the production of reactive oxygen species, which in turn can cause oxidative stress into the cell [8, 54]. In addition, azo and azoxy dimers might be equally potent in toxic effects as the parent nitroaromatic compounds [55]. On the other hand, there are some intermediates that could pose a decreased risk compared to the parent nitroaromatic compound, in this case DNAN. For instance, aromatic amines in general have been reported to be about 500-fold less cytotoxic than their nitroaromatic analogs in methanogenic archaea and toxicity was correlated with Log K_{ow} values [34], although this is not the only factor that affect toxicity. In addition, acetylation of aromatic amines has been reported as a potential detoxification reaction in direct competition with formation of azo-oligomers in p-bromoaniline [56]. N-acetyl transferases, which transfer the acetyl group in acetyl Coenzyme A to xenobiotic compounds, are overexpressed in human breast cancer [56, 57].

Assessing toxicity of (bio)transformation mixtures

Characterizing the toxicity implications of individual (bio)transformation products is important to identify transformation routes that lead to environmentally benign products. Mixture profiles of (bio)transformation products will vary depending on the dominant (a)biotic factors in the media and the stage of the (bio)conversion.
Thus, it is important to consider cumulative and synergistic effects from these products as they are formed. This necessitates concerted efforts in characterizing how (bio)transformation mixtures evolve in terms of characterization and quantification of the products as well as evaluating the toxicity of these mixtures in parallel. While the mixture characterization could be achieved by developing analytical methods to detect (bio)transformation products in diverse matrices, the toxicity and bioavailable fraction considerations could be studied using toxicity model microorganisms and multicellular organisms as biological targets [58]. As a relevant example in (bio)transformation of nitroaromatics, *Allivibrio fischeri*, a marine bioluminescent bacterium has been used as a biosensor of water quality during the phytoremediation of TNT with poplar trees to determine if detoxification was achieved [36]. A similar approach could be used in conjunction with (bio)transformation product mixture profile characterization to evaluate environmental hazards, while identifying key events in (bio)transformation that could be linked to drivers of toxicity.
CHAPTER 2

MOTIVATION AND RESEARCH QUESTIONS

As an emerging insensitive munition, DNAN, will be produced in large quantities with potential releases to the environment and engineered systems. Therefore, studies focusing on the environmental fate, (bio)transformation processes and ecotoxicity evaluation are needed. As environmental fate and toxicological knowledge increase, it is paramount to be able to identify key processes and factors influencing transformation of DNAN to environmentally benign end-products in order to guarantee environmental health of ecosystems and surrounding communities where DNAN might be released.

The following research questions are addressed:

- What are the (bio)transformation pathways for DNAN in natural environments and in biological engineered systems (wastewater sludge)?
  - What key biogeochemical factors affect the (bio)conversion?

- What is the inhibitory potential of DNAN and its (bio)transformation products to key microbial populations and \textit{in vivo} aquatic organisms (such as zebrafish \textit{Danio rerio} embryos)?
  - What can we learn about specific toxicity endpoints and modes of action?
• How can (bio)transformation mixtures be characterized analytically concurrent with toxicity studies?
  
  o What key events and (bio)transformation products drive toxicity?
CHAPTER 3
PATHWAYS OF REDUCTIVE 2,4-DINITROANISOLE (DNAN) BIOTRANSFORMATION IN SLUDGE

ABSTRACT: As the use of the insensitive munition compound 2,4-dinitroanisole (DNAN) increases, releases to the environment may pose a threat to local ecosystems. Little is known about the environmental fate of DNAN and the conversions caused by microbial activity. We studied DNAN biotransformation rates in sludge under aerobic, microaerophilic, and anaerobic conditions, detected biotransformation products, and elucidated their chemical structure. The biotransformation of DNAN was most rapid under anaerobic conditions with H\textsubscript{2} as a cosubstrate. The results showed that the ortho nitro group in DNAN is regioselectively reduced to yield 2-methoxy-5-nitroaniline (MENA), and then the para nitro group is reduced to give 2,4-diaminoanisole (DAAN). Both MENA and DAAN were identified as important metabolites in all redox conditions. Azo and hydrazine dimer derivatives formed from the coupling of DNAN reduction products in anaerobic conditions. Secondary pathways included acetylation and methylation of amine moieties, as well as the stepwise O-demethylation and dehydroxylation of methoxy groups. Seven unique metabolites were identified which enabled elucidation of biotransformation pathways. The results taken as a whole suggest that reductive biotransformation is an important fate of DNAN leading to the formation of aromatic amines as well as azo and hydrazine dimeric metabolites.
INTRODUCTION

Insensitive munitions (IMs) are highly energetic compounds that are resistant to shock and have a high detonation temperature, therefore reducing accidental explosions [4]. IMs are replacing conventional explosives, yet little is known about their environmental fate. As the use of IMs increases, industrial wastewaters contaminated with these substances will be produced during manufacture. Likewise, release of IMs into soil, underlying aquifers, and nearby surface waters may occur as a consequence of unconsumed ordnance on firing ranges. One of the first IM ingredients to be used on a large scale in explosive formulations is 2,4-dinitroanisole (DNAN) [4, 5].

The potential release of DNAN to the environment poses a concern since many nitroaromatic compounds are toxic [7-9], mutagenic [10], and resistant to biodegradation [1, 9]. The nitro functional groups are electron withdrawing, and thus are less favorable for an oxidative, enzymatic attack conducive to mineralizing the compound [11, 12]. Instead, a more common pathway for poly-nitroaromatic compounds, like DNAN, is the reduction of nitroaromatic groups to aromatic amines [8, 11, 12]. Intermediates of this reduction, such as hydroxylamine and nitroso aromatics, can bind to DNA and other biomolecules, causing toxic and mutagenic effects [8, 46]. Given the potential toxicity issues of DNAN, there is a need to evaluate the biotransformation of DNAN in natural systems such as soils and water, as well as industrial effluents where residues of this IM might impact local ecosystems. Bioremediation is a low cost and environmentally safe approach that could provide an appropriate solution to treat IM compounds.

Recent studies have begun to evaluate DNAN biotransformation under anaerobic [18] and aerobic [16] conditions, and some biotransformation metabolites
have been elucidated. In the present study, we assessed DNAN biotransformation using mixed microbial cultures (i.e., aerobic and anaerobic sludge) under aerobic, microaerophilic, and anaerobic conditions. Our aims were to compare DNAN biotransformation rates as a function of redox conditions, as well as to determine important biotransformation products. Our results confirmed that DNAN underwent biotransformation under all redox conditions investigated, and that conversion of the nitroaromatic compound was greatly enhanced under reducing conditions. Combination of ultra-high performance liquid chromatography coupled to triple quadrupole mass spectrometry (UHPLC-MS) and high-resolution time of flight mass spectrometry (TOFMS) allowed identification of seven new metabolites which are described here for the first time, and enabled elucidation of new biotransformation pathways.

**MATERIALS AND METHODS**

**Inocula and Basal Medium**

Two different microbial mixed cultures were used in this investigation, anaerobic granular sludge (AGS) and aerobic return activated sludge (RAS). AGS was obtained from a full-scale upward-flow anaerobic sludge blanket reactor treating wastewater at a brewery (Mahou, Guadalajara, Spain). The sludge was washed and sieved to remove fine particles before use in the bioassays. RAS was obtained from a municipal wastewater treatment plant (Ina Road Wastewater Reclamation Facility, Tucson, AZ, USA). The content of volatile suspended solids (VSS) in AGS and RAS
was 7.92% and 0.19% of the wet weight, respectively. The sludge samples were stored in a refrigerator at 4°C.

The basal mineral medium was prepared using ultrapure water (NANOpure Infinity™, Barnstead International, Dubuque, IA, USA) and contained the following (in mg L$^{-1}$): $K_2HPO_4$ (250), $CaCl_2\cdot2H_2O$ (10), $MgSO_4\cdot7H_2O$ (100), $MgCl_2\cdot6H_2O$ (100), $NH_4Cl$ (280), $NaHCO_3$ (4,000), yeast extract (100), and trace element solution (1 mL L$^{-1}$). The solution of trace elements contained (in mg L$^{-1}$): $H_3BO_3$ (50), $FeCl_2\cdot4H_2O$ (2,000), $ZnCl_2$ (50), $MnCl_2\cdot4H_2O$ (50), $(NH_4)_6Mo_7O_24\cdot4H_2O$ (50), $AlCl_3\cdot6H_2O$ (90), $CoCl_2\cdot6H_2O$ (2,000), $NiCl_2\cdot6H_2O$ (50), $CuCl_2\cdot2H_2O$ (30), $NaSeO_3\cdot5H_2O$ (100), EDTA (1,000), resazurin (2,000), 36% HCl (1 mL). Resazurin, a redox sensitive dye that is reduced (turning colorless) at -110 mV (Jacob, 1970), was included in the media to monitor redox potential. The final pH of the basal medium was adjusted to 7.2 with HCl.

**DNAN Biotransformation Assays**

The biotransformation of DNAN was investigated in aerobic, microaerophilic and anaerobic biotransformation assays. The liquid volume in all bioassays was 100 mL. Aerobic biotransformation assays were conducted in glass Erlenmeyer flasks (250 mL) capped with cotton gauze and placed on an orbital shaker at 180 rpm. Microaerophilic biotransformation assays were performed in glass serum flasks (160 mL) capped with cotton gauze and placed on an orbital shaker at 115 rpm. Due to the lower shaking intensity and the higher depth of the liquid medium, the shaking was notably less aggressive compared to the fully aerobic treatment. Anaerobic biotransformation assays were conducted in glass serum flasks (160 mL) sealed with
butyl rubber stoppers and aluminum crimp caps. The culture medium and the headspace were flushed with N₂/CO₂ (80:20, v/v) for 5 min to create anaerobic conditions.

All bioassays included inoculated treatments spiked with DNAN with or without cosubstrate; H₂ or acetate depending on the experiment. Abiotic and heat-killed sludge control experiments were included to account for the potential removal of DNAN by abiotic mechanisms. DNAN was spiked using a stock solution (650 μM DNAN) to a final concentration of 130 μM. Inoculated aerobic and microaerophilic bioassays were supplied with RAS (0.5 g VSS L⁻¹). Prior to addition, RAS was centrifuged (20 min at 2,880 g) and the liquid medium was discarded. Anaerobic bioassays were inoculated with sieved AGS (1.5 g VSS L⁻¹). Heat-killed sludge was prepared by autoclaving the sludge (121°C) for three consecutive days for 50 min the first day and 20 min the next two days. Acetate (1,875 mg L⁻¹) was used as a cosubstrate in both aerobic and microaerophilic biotransformation assays. H₂ was used as a cosubstrate in the anaerobic biotransformation assay, and was supplied by pressurizing the flask headspace to 1.5 atm with a gas mixture (H₂/CO₂, 80:20 v/v) after flushing with N₂/CO₂. The assays were incubated in the dark at 30°C. In the anaerobic biotransformation assays, the flasks were pre-incubated overnight to ensure that the sludge adapted to the medium conditions. DNAN was added to the flasks in the following morning. All assays were performed in duplicate. Evaporation of the liquid in both the aerobic and microaerophilic conditions was monitored during the experimental period to adjust the final results according to the original liquid volume.

Liquid samples were collected periodically for analysis of DNAN and DNAN transformation products. Samples were centrifuged immediately (10 min at 9,600 g) and then analyzed by high-performance liquid chromatography coupled to a diode-
array detector (HPLC-DAD). Anaerobic samples were spiked with 250 mg L\(^{-1}\) ascorbic acid to prevent autoxidation of aromatic amines and other reduced metabolites. Based on sample stability tests performed, ascorbic acid did not chemically reduce DNAN and was therefore chosen as a suitable antioxidant for the anaerobic samples. Dissolved oxygen (DO) and oxidation-reduction potential (ORP) were monitored in experiments replicating the endogenous and cosubstrate conditions for aerobic, microaerophilic, and anaerobic conditions. These experiments were performed in duplicate.

Additional assays were set up to evaluate the anaerobic biotransformation of DNAN with multiple respikes with H\(_2\) as a cosubstrate. The objective was to allow the accumulation of intermediates to help identify intermediates not detected by the routine analyses with HPLC-DAD. DNAN was spiked initially at 40 μM (at 0 h), then at 130 μM (at 23, 47 and 71 h), and finally at 260 μM (periodically between 96 h and 196 d). When the H\(_2\) was consumed, H\(_2\)/CO\(_2\) was resupplied to a pressure of 1.5 atm as described above. After incubation for 84 and 196 d, liquid samples (0.5 mL) were collected. Samples were spiked immediately with ascorbic acid (250 mg L\(^{-1}\)), and filtered through a PTFE membrane (0.45 μm) prior to analysis both by ultra-high performance liquid chromatography coupled to triple quadrupole mass spectrometry (UHPLC-MS) and by time of flight mass spectrometry (TOFMS).

**Analytical Methods**

**HPLC-DAD**

DNAN, 2-methoxy-5-nitroaniline (MENA) and 2,4-diaminoanisole (DAAN) in liquid phase were analyzed using an Agilent 1200 series (Santa Clara, CA, USA) HPLC-
DAD. Samples (20 μL injection) were separated using an E1 Acclaim Explosives column (4.6 x 240 mm, 5 μm) (Dionex, Salt Lake City, UT, USA) at room temperature. The mobile phase (methanol/H2O, 43/57% v/v) was run isocratically at a flow rate of 1 mL min\(^{-1}\) for 30 min. The detector was set to scan for wavelengths of 210, 220, 254, 270, 280, 300, 325, and 360 nm. Detection of DNAN, MENA and DAAN was performed at 300, 254 and 210 nm, respectively. The retention times were 23 min for DNAN, 13 min for MENA, and 7 min for DAAN.

**UHPLC-MS**

UHPLC system consisting of Acquity UPLC\(^{\text{TM}}\) binary solvent manager and Acquity UPLC\(^{\text{TM}}\) BEH C18 column (1.7 μm, 2.1 mm x 50 mm) (Waters, Milford, MA, USA) was used for the separation of DNAN and its biotransformation metabolites. The mobile phase was a mixture of LCMS grade water and acetonitrile (ACN) with a gradient as follows: 0 – 0.5 min, 10% ACN; 0.5 – 5.5 min increasing ACN from 10 to 80%; 5.5 – 6.5 min, hold at 80% ACN; 6.5 – 9.5 min, decreasing ACN from 80 to 10%; 9.5 – 10 min, hold at 10% ACN. A mobile phase flow rate of 0.2 mL min\(^{-1}\) and a column temperature of 30°C produced a pressure of around 4,300 psi. Each injection volume was set to 5 μL. A Quattro Premier XE triple quadrupole MS (Waters, Milford, MA, USA) was used with electrospray ionization (ESI) in positive ionization mode with a capillary voltage of 3.0 kV. The ion source temperature was kept at 120°C and the desolvation gas temperature was set to 300°C. N\(_2\) was used as both cone gas and desolvation gas, and high purity argon was used as the collision gas. Mass Lynx 4.1 software was used to control instruments and to record and process data. Multiple reaction monitoring was used to identify MENA (169 > 154), (169 > 123); and DAAN (139 >107) with dwell time of 100 ms per transition.
**TOF-MS**

High resolution full scan mass spectra were obtained by directly infusion into a TripleTOF™ 5600 quadrupole TOFMS (AB Sciex, Framingham, MA) equipped with ESI source kept at 450 °C. The sample was infused at a flow rate of 10 µL min⁻¹. Spectra were obtained in ESI positive ion mode with a capillary setting of 5.5 kV and declustering potential of 50 V. Curtain gas, desolvation gas, and nebulizer gas levels were kept at 30, 35, and 35 psi, respectively, with nitrogen. High resolution mass spectra were obtained by averaging ca. 100 spectra acquired from a mass range of 35-600 m/z. Analyst TF 1.5.1 and Formula Finder 2.0.2.0 software applications were used to process spectral data and to identify molecular formulae.

DO was monitored with an Orion 081010MD probe (Thermo Fisher Scientific, Beverly, MA, USA). ORP was measured using an Orion 9678BNWP electrode (Thermo Fisher Scientific, Beverly, MA, USA). Other analyses, including pH and VSS content in sludge samples, were performed according to standard methods [59].

**Chemicals**

2,4-Dinitroanisole (DNAN) (CAS # 119-27-7, 98% purity) was purchased from Alfa Aesar (Ward Hill, MA, USA). 2-methoxy-5-nitroaniline (MENA) (CAS # 99-59-2, 98% purity) and 2,4-diaminoanisole (DAAN) (CAS # 615-05-4, analytical standard) were obtained from Sigma-Aldrich (St. Louis, MO, USA).
RESULTS

DNAN Biotransformation under Aerobic, Microaerophilic, and Anaerobic Conditions
The biotransformation of DNAN was evaluated under aerobic, microaerophilic, and anaerobic conditions. In each experiment four treatments were compared. The first treatment was intended to incubate DNAN with sterile medium. This treatment caused no conversion of DNAN irrespective of the redox conditions so the results are not shown. The second treatment was the incubation of DNAN with heat-killed sludge. In the third treatment, which is referred to as ‘endogenous treatment’, DNAN was incubated with live sludge. The fourth treatment had DNAN, live sludge, and a cosubstrate. Under aerobic and microaerophilic conditions the cosubstrate was acetate, whereas under anaerobic conditions it was H₂. DO concentrations and ORP confirmed that aerobic, microaerophilic, or anaerobic conditions were maintained in the various experiments as intended (Figures. A-1 and A-2 in Appendix A).

Aerobic Biotransformation
The DNAN biotransformation rate under fully aerobic conditions was very low (Figure 3.1). Under the best condition (with cosubstrate addition), almost 700 h was required to remove most (87%) of the added DNAN. In the endogenous treatment, only 40% of the nitroaromatic compound was converted in the same time period. In the control with heat-killed sludge, only very minor conversion of DNAN occurred.

Based on HPLC-DAD analysis, MENA and DAAN were identified as products of DNAN biotransformation. In the killed sludge and the endogenous treatments, an initial small decrease in DNAN occurred that may have been due to sludge adsorption. Thereafter, the mass balance indicates that further decreases in
DNAN were accounted for by increases in the products; MENA and DAAN. After approximately 700 h, about 8% and 22% of DNAN was transformed to MENA and DAAN in the heat-killed sludge and endogenous treatments, respectively. In the treatment amended with acetate, conversion of DNAN to MENA and DAAN occurred from the start of the experiment. After 700 h, about 35% and 17% of DNAN was transformed to MENA and DAAN, respectively. The mass balance was not complete in the cosubstrate-amended treatment, since 40% of the DNAN originally added could not be accounted for by the chemical species detected with HPLC-DAD.
Figure 3.1. Aerobic biotransformation of DNAN (●) into MENA (□) and DAAN (▲). Panels: heat-killed sludge (A), live sludge (B), and live sludge supplemented with acetate as co-substrate (C). The dotted dashed line shows the molar sum of the three compounds.
Microaerophilic Biotransformation

Under microaerophilic conditions, the biotransformation of DNAN was much faster in all treatments (Figure 3.2) compared to the fully aerobic condition. In this case, the endogenous and cosubstrate treatments behaved similarly with 80% removal of DNAN in 35 and 52 h, respectively. In the treatments with live sludge, DNAN conversion was notably faster than with heat-killed sludge where only 8% of DNAN was transformed to other compounds during the whole experimental period of 150 h.

MENA and DAAN were also found with HPLC-DAD as the two main metabolites of DNAN biotransformation. MENA was the dominant product occurring in a 3:1 molar ratio compared to DAAN. After incubating for 20 h, the sum of the molar concentrations of DNAN (57 μM), MENA (47 μM), and DAAN (21 μM) was stable and nearly equal to the initial added concentration of DNAN (130 μM). The gap between initial added DNAN and the sum of the final compounds could be due to sludge sorption or to biomineralization. Thus it can be said that DNAN is transformed almost stoichiometrically under microaerophilic conditions to MENA and DAAN. In the treatment with heat-killed sludge, MENA was almost the only product formed.
Figure 3.2. Microaerophilic biotransformation of DNAN (●) into MENA (□) and DAAN (▲). Panels: heat-killed sludge (A), live sludge (B), and live sludge supplemented with acetate as co-substrate (C). The dotted dashed line shows the molar sum of the three compounds.
**Anaerobic Biotransformation**

Under anaerobic conditions, the DNAN biotransformation occurred considerably faster and a higher conversion was observed compared to the aerobic and microaerophilic conditions (Figure 3.3). In the treatment with H$_2$ as cosubstrate, DNAN was nearly fully converted within 12 h. In the endogenous treatment, an almost complete conversion required only 33 h. The heat-killed sludge treatment also retained reducing capacity, converting 88% of the added DNAN by the end of the experimental period of 48 h.

According to the time course of biotransformation products (Figure 3.3), about 75% of DNAN was transformed to MENA in the heat-killed sludge. In the endogenous treatment, about 70% of DNAN was transformed to MENA in 33 h. Afterwards, MENA started to decrease and was transformed into DAAN. In the H$_2$ supplemented treatment, about 28% of DNAN was transformed to MENA during the first 12 h. Thereafter, MENA started to be transformed to DAAN as well. The mass balances in the endogenous and H$_2$-supplemented treatments with live sludge were very poor. At the end of the experiment, the identified products only accounted for approximately 50% and 20% of the DNAN added at the start of the experiment, respectively, suggesting the formation of other biotransformation products that were not detected by HPLC-DAD.
Figure 3.3 Anaerobic biotransformation of DNAN (●) into MENA (□) and DAAN (▲). Panels: heat-killed sludge (A), live sludge (B), and live sludge supplemented with H₂ as co-substrate (C). The dotted dashed line shows the molar sum of the three compounds.
Comparison of DNAN Conversion Rates under Different Redox Conditions
Zeroth-order DNAN conversion rates under the different redox conditions are shown in Figure 3.4 and are representative of initial linear DNAN biotransformation during the first hours. Aerobic and microaerophilic conditions are directly comparable since both used the same inoculum, RAS. However, the anaerobic treatment had an anaerobic inoculum (AGS). The DNAN biotransformation rate increased as the redox conditions shifted from aerobic to anaerobic. The highest initial DNAN biotransformation rate of 16.4 µmol•L⁻¹ h⁻¹ was observed in the anaerobic bioassay with H₂ as cosubstrate. It was approximately 4.2 and 9.4 times higher than those observed in the microaerophilic and aerobic conditions with acetate as cosubstrate. The endogenous, anaerobic biotransformation rate was 8.8 µmol•L⁻¹ h⁻¹, which is 1.9 and 43.2 times those observed in the endogenous microaerophilic and aerobic conditions. Considering specific biotransformation rates per unit of added VSS, the rates in the live sludge had the same order of magnitude for the anaerobic and microaerophilic sludge (see data listed in caption of Figure 3.4).

The DNAN biotransformation data obtained during the course of the different experiments were fitted to a first-order kinetic model. The average rates determined are reported on Table S-I in the Supporting Information Section. The rate constant determined in anaerobic assays amended with cosubstrate (k = 0.306 h⁻¹) was 8.3- and 12.6-fold higher compared to microaerophilic and aerobic assays, respectively.
Figure 3.4. Comparison of the DNAN biotransformation rates in different redox conditions. Treatments: heat-killed sludge (■), endogenous ( ░ ), and cosubstrate (□). Expressed per unit of added biomass, the DNAN biotransformation rates in heat-killed sludge, endogenous and cosubstrate treatments were as follows (in µmol•h⁻¹ g⁻¹ VSS): anaerobic (0.16, 5.86 and 11.0), microaerophilic (0.10, 9.08 and 7.79), and aerobic assays (0.08, 0.41 and 3.49).

Identification of Anaerobic Biotransformation Products from DNAN with H₂ as Cosubstrate

An additional experimental incubation was carried out to identify intermediates in the anaerobic culture using mass spectrometry. The anaerobic sludge supplemented with H₂ was spiked several times with DNAN to generate enough products to facilitate their analysis. The anaerobic sludge amended with H₂ readily
reduced DNAN to MENA and DAAN in a continuous manner after all the DNAN spikes (Figure 3.5). While the transient formation of MENA metabolite disappeared quickly, DAAN seemed to accumulate in the bioassay. Moreover, the concentration of DAAN oscillated and eventually was not detected 220 h after the last DNAN spike.

UHPLC-MS analysis of the products showed a protonated molecular mass \([\text{M+H}]^+\) at \(m/z\) 169 and 139 Da matching formulas of \(C_7H_8N_2O_3\) and \(C_7H_{10}N_2O\). They were also identified with the precursor-transitions: 169>154 and 169>123 for MENA (2; Figure 3.6) and 139>107 for DAAN (5; Figure 3.6). In addition, MENA and DAAN were also confirmed with analytical standards using HPLC-DAD based on retention times. Other compounds detected with UHPLC-MS and TOFMS included azo dimers (7, 10, 11, 12, and 13; Figure 3.6), hydrazine dimers (8 and 14; Figure 3.6), as well as intermediates wherein a primary amine was alkylated with methyl (11; Figure 3.6), methylene (10 and 11; Figure 3.6) and acetyl (9; Figure 3.6) moieties. Dimers were also observed showing evidence of O-demethylation (12; Figure 3.6) and subsequent dehydroxylation (13; Figure 3.6) of the methoxy group. Chemical structures were deduced from molecular formulae determined using Formula Finder 2.0.2.0 wherein monoisotopic masses were measured. The structures were determined based on high resolution molecular formulae in concert with interpretation of possible chemical reactions that nitroaromatics are known to undergo. MENA, DAAN, as well as other compounds found are reported in Table 3.1. The chemical structures and mass spectra recorded for these metabolites can be found in the Supporting Information section (Figs. S-3 to S-17). The retention times determined for the various compounds in the UHPLC-MS analysis are reported on Table 3.1. The dimers did not co-elute with monomeric compound analogues, confirming that dimeric structures were not an artifact of the ionization technique used in this work.
Figure 3.5. Anaerobic biotransformation of DNAN after multiple spikes in an anaerobic bioassay amended with H₂. Legends: DNAN (●) biotransformation into MENA (□) and DAAN (▲). Each respike of DNAN is indicated by a dashed vertical line.
Table 3.1. Molecular formula, retention times determined by UHPLC-MS, calculated m/z values, and m/z values measured by TOFMS for the various identified metabolites.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molecular formula</th>
<th>Structure in Figure 3.6</th>
<th>Retention time (min)</th>
<th>Calculated‡ [M+H]+ measured [M+H]+</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-methoxy-5-nitroaniline (MENA)</td>
<td>C$_7$H$_8$N$_2$O$_3$</td>
<td>2</td>
<td>1.5</td>
<td>169.0866 169.0864</td>
</tr>
<tr>
<td>2,4-diaminoanisole (DAAN)</td>
<td>C$<em>7$H$</em>{10}$N$_2$O</td>
<td>5</td>
<td>0.86</td>
<td>139.0608 139.0579</td>
</tr>
<tr>
<td>3,3’-diamino-4,4’-dimethoxy-azobenzene</td>
<td>C$<em>{14}$H$</em>{16}$N$_4$O$_2$</td>
<td>7</td>
<td>3.7</td>
<td>273.1347 273.1315</td>
</tr>
<tr>
<td>3,3’-diamino-4,4’-dimethoxy-hydrazobenzene</td>
<td>C$<em>{14}$H$</em>{18}$N$_4$O$_2$</td>
<td>8</td>
<td>3.6</td>
<td>275.1503 275.1499</td>
</tr>
<tr>
<td>N-(5-amino-2-methoxyphenyl) acetamide</td>
<td>C$<em>9$H$</em>{12}$N$_2$O$_2$</td>
<td>9</td>
<td>3.2</td>
<td>181.0972 181.0968</td>
</tr>
<tr>
<td>5-((3-amino-4-methoxyphenyl)diazenyl)-2-methoxy-N-methyleneaniline</td>
<td>C$<em>{15}$H$</em>{16}$N$_4$O$_2$</td>
<td>10</td>
<td>4.3</td>
<td>285.1347 285.1346</td>
</tr>
<tr>
<td>2-methoxy-5-((4-methoxy-3-(methylamino)phenyl)diazenyl)-methyleneaniline</td>
<td>C$<em>{16}$H$</em>{18}$N$_4$O$_2$</td>
<td>11</td>
<td>5.3</td>
<td>299.1503 299.1503</td>
</tr>
<tr>
<td>3,3’-diamino-4-hydroxy-4’-methoxy-azobenzene</td>
<td>C$<em>{13}$H$</em>{14}$N$_4$O$_2$</td>
<td>12</td>
<td>4.4</td>
<td>259.1190 259.1159</td>
</tr>
<tr>
<td>3,3’-diamino-4-methoxy-azobenzene</td>
<td>C$<em>{13}$H$</em>{14}$N$_4$O</td>
<td>13</td>
<td>2.5</td>
<td>243.1241 243.1207</td>
</tr>
<tr>
<td>3,3’-diamino-4-methoxy-hydrazobenzene</td>
<td>C$<em>{13}$H$</em>{16}$N$_4$O</td>
<td>14</td>
<td>3.3</td>
<td>245.13 245.0*</td>
</tr>
</tbody>
</table>

* m/z value measured by UHPLC-MS. The compound was not detected by TOFMS. ‡ ChemBioDraw Ultra 12.0.2.1076
**DISCUSSION**

**Reductive Biotransformation**

Although some dinitroaromatic compounds are degraded via oxidative pathways [8], DNAN has a higher tendency for reduction. We observed that the initial biological reduction of DNAN was regioselective favoring the nitro group in the *ortho* position to yield MENA, as other studies have shown for DNAN [16, 18] and other poly-nitroaromatic compounds [43, 60]. MENA was then further reduced to DAAN. Platten, Bailey, Suidan and Maloney [18] studied DNAN transformation in an anaerobic fluidized bed reactor and reported that DNAN was reduced to MENA and DAAN. Perreault, Manno, Halasz, Thiboutot, Ampleman and Hawari [16] studied DNAN transformation in aerobic conditions by soil slurries and *Bacillus* sp strain 13G isolated from the soil slurries and reported that DNAN was reduced to MENA. Moreover, reductive biotransformation has been found in other closely related nitroaromatic compounds, such as 2,4- and 2,6-dinitrotoluene, as well as 2,4,6-trinitrotoluene (TNT) [26, 60, 61].

The multiple nitro functional groups of DNAN cause a strong electron deficiency in the carbon skeleton favoring reductive biotransformation as has been observed in many poly-nitroaromatic compounds in both aerobic and anaerobic conditions [8]. Nitroreductases responsible for nitro group reduction are readily found in bacteria, and they may account for the facile reduction of DNAN in sludge. There are two main types of nitroreductases, oxygen-sensitive and oxygen-insensitive nitroreductases. The latter may account for the reduction of DNAN under aerobic conditions. Oxygen-insensitive nitroreductases use a series of two electron transfer mechanisms and yield nitroso, hydroxylamine, and amino metabolites [8, 19].
oxygen-insensitive reduction may be catalyzed by nitroreductases dependent on NAD(P)H as an electron donor, which may require flavoprotein mononucleotide as a prosthetic group [8, 19, 23].

In this study, we observed reduction of DNAN in the anaerobic and microaerophilic bioassays that contained heat-killed sludge, albeit at a rate that was very much slower than in the bioassays with live sludge. Several authors have reported that reduced components present in sludge can still be active after biological inactivation and cause some reduction of nitroaromatic compounds. Donlon, Razo-Flores, Lettinga and Field [44] detected reduction of 2-nitrophenol by autoclaved methanogenic granular sludge. Beelen and Burris [23] reported reduction of TNT in sediments that had been treated with proteases. In addition, the reduction of nitroaromatic compounds are also attributed to Fe(II) containing minerals [21], as well as to sulfide when electron transfer is mediated by natural organic matter [23, 62].
Figure 3.6. DNAN biotransformation pathways by anaerobic sludge amended with H₂ as a cosubstrate. Dashed lines represent hypothetic routes. Compounds in brackets were not detected but are potential metabolites. Main pathway is shown inside the black box. Secondary routes are presented outside of the box.

**Biotransformation Pathways**

A large array of DNAN metabolites were detected, including seven new products not previously reported. A plausible pathway for DNAN anaerobic biotransformation is proposed in Figure 3.6 which accounts for the detected metabolites. Firstly, the nitro groups in DNAN were reduced leading to MENA and DAAN. Secondly, dimers were formed from reduced intermediates of DNAN. Azo compounds are known to be formed via azoxy dimers [25], such as
those found by Perreault, Manno, Halasz, Thiboutot, Ampleman and Hawari [16]. Afterwards, azo compounds were reduced further to hydrazine metabolites, which in turn were cleaved to form aryl amines. Lastly, secondary methylation, and acetylation reactions of amines occur with the monomeric and dimeric reduced metabolites.

**Reduction of DNAN to Aryl Amines**

The regioselective reduction that we observed from sludge may coincide with oxygen-insensitive nitroreductase activity. These enzymes reduce nitro functional groups to amines in three steps of two electron reductions, yielding the short-lived nitroso (3; Figure 3.6), followed by hydroxylamine (4; Figure 3.6) metabolites [19, 32, 63]. While the nitroso and hydroxylamine metabolites were not measured in this study, Perreault, Manno, Halasz, Thiboutot, Ampleman and Hawari [16] found hydroxylamine metabolites. Moreover, a purified oxygen-insensitive nitroreductase from Klebsiella has been shown to favor the reduction of the ortho nitro group in TNT [64]. Likewise, in our studies we observed that the ortho nitro group in DNAN was reduced first to yield MENA. Afterwards, the para nitro group was reduced to yield DAAN; which had the highest concentration in anaerobic samples measured with HPLC-DAD.

**Dimerization of Reduced DNAN Intermediates**

The concentrations of DAAN fluctuated, disappearing and appearing, in the long term anaerobic experiment with multiple DNAN respikes (Figure 3.5). Indeed, some metabolites can become unstable, such as 2,4-diaminotoluene in aerobic sediment [60] or 2,4,6-triaminotoluene in anaerobic conditions exposed to sludge [26], both of which have been shown to polymerize. Thus, we hypothesized that fluctuating DAAN (Figure 3.5) and poor mass balance observations
(Figure 3.3) in anaerobic conditions may be accounted for by the formation of dimers. This hypothesis was further strengthened by the detection of several dimers in previous evaluations of the biotransformation of DNAN under anaerobic [18], as well as aerobic [16] conditions. Hydroxylamine compounds are known to condense with nitroso intermediates to produce azo compounds (e.g. 7, 10, 11, 12 and 13; Figure 3.6) via azoxy derivatives (e.g. 6; Figure 3.6) [25], and have been found during the anaerobic biological reduction of TNT [26]. Furthermore, the dimers formed could have a regioselectivity favoring the azo(oxy) bond in the para position. We have observed that the first nitro group to be reduced is the ortho one, and other researchers have suggested that the reduction of additional nitro groups is slower [8, 23]. Because of these two reasons, para position nitroso and hydroxylamine derivatives may have a tendency to accumulate more, making the formation of the para-azo(oxy) compounds more likely than ortho-azo(oxy) compounds.

Aromatic amines can be easily oxidized and may polymerize in aerobic conditions [65]. However, dimerization has been shown to occur in the absence of oxygen as well. A mechanism for dimer formation in anaerobic environments has been proposed. Hawari, Halasz, Paquet, Zhou, Spencer, Ampleman and Thiboutot [26] attribute the formation of azo(oxy) compounds from 2,4,6-triaminotoluene to metals present in the sludge, since they can act as electron acceptors and oxidize aryl amines, which will yield nitroso and hydroxylamine products that can form azo(oxy) polymers.

**Reduction of Azo Compounds**

Azo compounds have been reported to be reduced back to their respective aryl amines in AGS by the presence of reduced compounds, such as sulfide [66], or by reduced flavine
compounds [67]. Azo reductases exist as cytoplasmic non-specific enzymes that can reduce azo compounds gratuitously in the metabolic processes of anaerobic bacteria [68]. In addition, there are other enzymes that are commonly found in bacteria, such as quinone reductases, that can reduce azo compounds due to structural similarities between tautomeric forms of quinones and azo compounds [69]. Azo compounds can be reduced first to their hydrazine equivalents before being reductively cleaved back to aromatic amine monomers. We detected hydrazine biotransformation products (8 and 14; Figure 3.6). This finding coincides with hydrazine compounds reported as intermediates during the biological reduction of azo dyes [70-72].

**Methylation and Acetylation Reactions**

Alternative pathways involved addition of an acetyl (9; Figure 3.6) or methylene group (10 and 11; Figure 3.6) to the ortho primary amine of DAAN or related dimers. Acetylation has been reported for 2,4-diamino-6-nitrotoluene by *Pseudomonas fluorescens* [73]. N-arylamine acetyl transferases in bacteria and eukaryotes are responsible for transferring acetyl groups from acetyl coenzyme A to xenobiotic compounds containing amino and hydroxylamine groups [57]. Perreault, Manno, Halasz, Thiboutot, Ampleman and Hawari [16] detected a similar compound to N-(5-amino-2-methoxyphenyl) acetamide (9; Figure 3.6); a DNAN metabolite we found. However, structures with N-methylene groups were unique to our findings.

**O-demethylation and Dehydroxylation Reactions**

Another observed transformation was the O-demethylation of the methoxy group to yield a hydroxyl moiety (12; Figure 3.6). Perreault, Manno, Halasz, Thiboutot, Ampleman and Hawari [16] detected a similar reaction in 2-acetamido-4-nitroanisole and 2-hydroxylamino-4-
nitroanisole, and associated the release of fomaldehyde to this reaction. *Rhodococcus* strains have also been reported to O-demethylate 4-nitroanisole [74]. A set of metabolites unique to our study was detected that lacked the hydroxyl group (13, 14; Figure 3.6), suggesting that the hydroxyl group (formed from O-demethylation) was also removed. Reductive dehydroxylation is a well-known reaction of the intermediate 4-hydroxybenzoyl-coenzyme A yielding benzoyl-coenzyme A during the anaerobic degradation of phenol [75].

**CONCLUSIONS**

DNAN was reduced by sludge to two main metabolites, MENA and DAAN, under all of the different conditions tested. The highest DNAN biotransformation rate was observed in anaerobic conditions enhanced by the addition of H₂, as a cosubstrate. The nitro group in the ortho position was first reduced to an amine to yield MENA, then the para nitro group was reduced to an amine to produce DAAN. During the reductive biotransformation, coupling of DNAN intermediates occurred, yielding azo compounds. These products were further reduced to hydrazine dimers. In addition, a diversity of products was created by parallel pathways of N-methylation, and N-acetylation of primary amines, as well as O-demethylation and dehydroxylation of methoxy groups. These insights about the fate and biotransformation of DNAN will help in understanding environmental health risks from DNAN and provide clues for the bioremediation of DNAN contamination.
CHAPTER 4

(BIO)TRANSFORMATION OF 2,4-DINITROANISOLE (DNAN) IN SOILS

ABSTRACT: Recent studies have begun to assess the environmental fate and toxicity of 2,4-dinitroanisole (DNAN), an insensitive munition compound of interest to defense agencies. Aerobic and anaerobic DNAN biotransformation in soils was evaluated in this study. Under aerobic conditions, there was little evidence of transformation; most observed removal was attributed to adsorption and subsequent slow chemical reactions. Under anaerobic conditions, DNAN was reductively (bio)transformed and the rate of the transformation was positively correlated with soil organic carbon (OC) up to a threshold of 2.07% OC. H2 addition enhanced the nitroreduction rate compared to endogenous treatments lacking H2. Heat-killed treatments provided rates similar to the endogenous treatment, suggesting that abiotic factors play a role in DNAN reduction. Ten (bio)transformation products were detected by high-resolution mass spectrometry. The proposed transformation pathway involves reduction of DNAN to aromatic amines, with putative reactive nitroso-intermediates coupling with the amines to form azo dimers. Secondary reactions include N-alkyl substitution, O-demethylation (sometimes followed by dehydroxylation), and removal of an N-containing group. Globally, our results suggest that the main reaction DNAN undergoes in anaerobic soils is nitroreduction to 2-methoxy-5-nitroaniline (MENA) and 2,4-diaminoanisole (DAAN), followed by anaerobic coupling reactions yielding azo-dimers. The dimers were subsequently subject to further (bio)transformations.
INTRODUCTION

2,4-dinitroanisole (DNAN) is an insensitive munitions compound (IMC) being considered by defense agencies and industries to replace conventional explosives, such as 2,4,6-trinitrotoluene (TNT) [4, 5]. As the word “insensitive” suggests, IMCs are less prone to accidental explosions. Within the last 15 years, there has been an increased interest in the chemical and physical properties of DNAN [5, 76]. More recently, researchers have started to study the hazard that DNAN (either alone or in munitions formulations) may pose once released to the environment, such as residues on firing ranges resulting from incomplete detonation [6, 37, 77, 78]. In order to understand the extent of the environmental risk, more data are needed on the environmental fate of DNAN, particularly in natural systems.

(Bio)transformation has been studied as a key component of the environmental fate of DNAN. Investigations in mixed consortia and pure cultures have been carried out in aerobic and anaerobic conditions leading to two major transformation routes. In the first route, nitro groups in DNAN were reduced to aromatic amines biologically [16-18, 52] and abiotically with metallic or ferrous iron [20, 52, 77]. The reduction of DNAN to aromatic amines occurs via nitroso and hydroxylamino intermediates [16, 52], which are potentially toxic and mutagenic [8, 46] and can react with amines to form azo-dimers [26, 79]. These dimers have been observed in aerobic conditions [16], anaerobic conditions [17], and in anaerobic transformation samples exposed to air without an antioxidant agent [18, 52]. Additionally, microbial DNAN O-demethylation has been reported, yielding 2,4-dinitrophenol (2,4-DNP) [14, 15]. In some cases, 2,4-DNP formed a hydride-Meisenheimer complex, releasing NO$_2^-$ [15].

If DNAN is not mineralized, there are two main mechanisms for the loss of DNAN and intermediates from solution: 1) reversible adsorption or 2) irreversible incorporation into humic
substances. DNAN is relatively hydrophobic (log Kow = 1.58-1.61) and has been found to sorb reversibly onto soil [52, 76]. While the adsorption may be attributed largely to organic components in soil, as DNAN is reported to bind strongly to lignin [80], DNAN has also been found to have strong affinity for K\textsuperscript{+}-montmorillonite, a secondary clay mineral in soils [81]. Moreover, its reduced transformation products were prone to sorb irreversibly onto soils in oxic conditions, with sorptive affinity increasing with number of amino groups [52].

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

**MATERIALS AND METHODS**

**Chemicals**

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

**Inocula and Basal Medium**

Seven different agricultural or military surface soils, characterized previously, were used as inocula [82] (relevant soil parameters can be found in Supplementary Material Table B-1). Prior to use, soils were sieved using a 2 mm mesh, and stored in sealed plastic bags at 4°C.

Basal medium was prepared with ultrapure water (NANOpure Infinity™, Barnstead International, Dubuque, IA, USA) and was composed of (in mg L⁻¹): K₂HPO₄ (250), CaCl₂•2H₂O (10), MgSO₄•7H₂O (100), MgCl₂•6H₂O (100), NH₄Cl (280), yeast extract (10), and trace element solution [17] (1 mL L⁻¹). Resazurin (200 mg L⁻¹) was used as a redox indicator [83]. A bicarbonate (48 mM) based buffer was used (with 20% CO₂ in headspace) for anaerobic assays, while a phosphate buffer (20 mM) was used for aerobic assays. The final pH of the basal medium was adjusted to 7.2 with HCl. All experiment treatments and controls were conducted in duplicate.

**DNAN soil biotransformation survey assays**

**Aerobic soil biotransformation survey assays**

Aerobic biotransformation assays were performed as described previously [17]. Briefly, soil (2.5 g, wet weight) was added to 50 mL of basal medium containing 150 µM DNAN. Water
was added to compensate evaporation during the incubation as assessed weekly by weight measurements.

**Anaerobic soil biotransformation survey assays**

Anaerobic biotransformation assays were conducted as reported before [17]. Soil samples (5.0 g, wet weight) were added to 100 mL of basal medium containing 150 µM DNAN. H\textsubscript{2} was supplied as electron donor using a H\textsubscript{2}/CO\textsubscript{2} (80:20, v/v) mixture to an overpressure of 1.5 atm. Three soils with the fastest DNAN conversion (Catlin, Camp Butner, and Camp Navajo) were further studied by including heat-killed soil (3 consecutive daily cycles, autoclaved at 121 ºC for 50 min), and a treatment without addition of H\textsubscript{2} (endogenous).

**Sample processing**

Liquid samples (0.5 mL) were diluted (1:3) in 375 ppm ascorbic acid (anaerobic assays) to prevent autoxidation of aromatic amine products upon air exposure [18, 52]. Samples were then centrifuged (9,600 g, 10 min), and stored at 4 ºC. All samples were analyzed within three days. Additional frozen samples from 11 days of incubation from the fast (bio)transforming soils (Catlin, Camp Butner, Camp Navajo, Camp Ripley) and samples from 34 days of incubation from the slow (bio)transforming soils (Roger Rd., Maricopa) were analyzed with ultra-high pressure liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (UHPLC-QToF-MS).

**Analytical Methods**

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**UHPLC-DAD**

Supernatant samples were analyzed using an Agilent 1290 Infinity Series (Santa Clara, CA, USA) ultra-high pressure liquid chromatograph coupled to a diode array detector (UHPLC-DAD). An Acclaim RSLC Explosives E2 column (2.1 x 100 mm, 2.2 μm) (Thermo Fisher Scientific, Waltham, WA, USA) was used at room temperature. A methanol/H₂O (40/60% v/v) mobile phase was run isocratically (0.25 mL min⁻¹, 15 min). Detection of DNAN, MENA and DAAN was performed at 300, 254 and 210 nm, respectively. Retention times were 9 min for DNAN, 5 min for MENA, and 2.4 min for DAAN.

**UHPLC-QToF-MS**

High resolution full scan mass spectra were obtained from 10 μL injections using liquid chromatography introduction to a TripleTOF® 5600 quadrupole TOF-MS (AB Sciex, Framingham, MA) equipped with an electrospray ionization (ESI) source kept at 450°C in the positive mode. UHPLC parameters used were the same as those described for UHPLC-DAD, on an UltiMate 3000 UHPLC (Dionex, Sunnyvale, CA). For identification of parent ion peaks as well as fragmentation patterns, information dependent acquisition (IDA); 0.1 sec cycle time, 6 triggered ions per cycle, mass range 35-1000 spectra were also obtained in ESI positive ion mode with a capillary setting of 5.5 kV, a declustering potential of 80 V, and curtain gas, desolvation gas, and nebulizer gas levels at 30, 35, and 35 psi, respectively, with N₂. Analyst TF 1.6 with PeakView 1.2.0.3 and Formula Finder 1.1.0.0 were used to process spectral data and to identify molecular formulae. Instrument calibration was accomplished by automated infusion of a solution periodically, over a mass range of 35-1000. Some metabolites were detected using direct infusion (30 μL min⁻¹) into the Q-ToF-MS.
Bioavailable iron, organic carbon, and water content

Total organic carbon (OC) was calculated from the difference between total carbon and total inorganic carbon. Total carbon was determined by combustion at 900 °C and total inorganic carbon was determined by phosphoric acid addition followed by combustion at 200 °C, in both cases followed by infrared detection of CO$_2$(g), using a Shimadzu 5000A-SSM TOC Analyzer (Columbia, MD). Sequential extraction of the soils was carried out targeting the following (operationally-defined) solid phases of iron (Fe) following the methods from Richard and Inskeep [84]: (i) water extractable, (ii) exchangeable (using ammonium acetate), (iii) amorphous/poorly crystalline (using acid ammonium oxalate in dark), and (iv) total free oxides (using citrate-dithionate-bicarbonate). Bioavailable Fe was operationally defined as the sum of water, ammonium acetate, and ammonium oxalate extractable pools. The extracted samples were analyzed for total iron in each fraction (done in triplicate) using an Elan DRC-II inductively coupled plasma mass spectrometer (ICP-MS) (Perkin Elmer, Waltham, MA, USA). Water content in soil was determined (in duplicate) by oven drying (overnight) at 105 °C.

Statistical Analyses

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

Aerobic soil survey of biotransformation

The bioconversion of DNAN under aerobic conditions was investigated in seven different soils. In some cases, heat-killed soil controls were included. Overall, there was slow conversion of DNAN in all of the soils surveyed (Figure 4.1). After rapid initial DNAN decrease (4-24 h), the removal rate was low in the live treatments (0.42-2.28 µM d⁻¹). The rates and extent of DNAN removal achieved were similar to those observed in the heat-killed soil. Initial DNAN removal was not associated with any significant HPLC resolvable transformation products except for minimal amounts of MENA (0-1.4% of DNAN (Figure B-1 in Appendix B)). However, the removal was linearly correlated with the soil organic carbon (OC) (R² = 0.9005, n=5, two-sided t-test p=0.015) (Figure B-2 in Appendix B). DNAN initial loss could then be attributed to adsorption.

Generally, initial adsorption was followed by slow DNAN transformation. The transformation rate decreased after 4-8 d, suggesting that abiotic factors responsible for reducing DNAN became exhausted. By 15 days of incubation, Catlin, Camp Navajo, and Camp Butner soils had the most DNAN removed due to adsorption and transformation (Figure 4.1); accounting for 64, 42, and 37% of DNAN, respectively. The soils that were incubated for a longer period (43 d) Catlin, Camp Butner, and Camp Ripley; showed an overall removal of 45-80%.
Figure 4.1. Aerobic removal of DNAN in soils (50 wet g L$^{-1}$) in mineral medium. Live treatments (continuous line): Camp Butner (■), Camp Ripley (●), Catlin (▲), Camp Navajo (♦), Florence (X). Heat-killed treatments (dashed line): Camp Butner (□), Camp Ripley (○), and Catlin (Δ). No heat-killed treatment available for Florence and Camp Navajo. Averages with error bars are reported.

Anaerobic soil survey of biotransformation

DNAN bioconversion was investigated under anaerobic conditions using H$_2$ as an electron donor. Two main groups of soils can be distinguished by their respective fast and slow rates of DNAN (bio)transformation (Figure 4.2). Zero-order DNAN transformation rates for fast soils ranged between 38.9-73.1 µM DNAN d$^{-1}$ (Figure 4.3, Table 4.1). Complete DNAN removal
was achieved within 6 d in the fast soils (Catlin, Camp Butner, Camp Navajo, and Camp Ripley). These soils had lag phases ranging from 0.2-2.6 d. Soils with slow (bio)transformation rates (Florence, Maricopa, Roger Rd.) transformed DNAN at 4.51-11.6 µM d\(^{-1}\), providing ≤ 33% of total DNAN removal after 9 d. In Figure 4.2, only Florence soil is shown as example for the slow soils; data for Maricopa and Roger Rd are not shown but Table 4.1 summarizes DNAN anaerobic (bio)transformation data for all soils.

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

Table 4.1. Features of anaerobic biotransformation of DNAN with H\(_2\) amendment for the seven soils surveyed.

<table>
<thead>
<tr>
<th>Soil</th>
<th>Lag phase (d)</th>
<th>Conversion rate DNAN(^\dagger) (µM d(^{-1}))</th>
<th>Max. yield products(^\circ) (µM)</th>
<th>(%(^\ddagger))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Roger Rd.</td>
<td>7.8</td>
<td>11.6</td>
<td>0.24</td>
<td>10</td>
</tr>
<tr>
<td>Maricopa</td>
<td>3.8</td>
<td>4.51</td>
<td>0.09</td>
<td>28</td>
</tr>
<tr>
<td>Catlin</td>
<td>0.2</td>
<td>69.3</td>
<td>1.43</td>
<td>69</td>
</tr>
<tr>
<td>Camp Ripley</td>
<td>1.1</td>
<td>38.9</td>
<td>0.78</td>
<td>107</td>
</tr>
<tr>
<td>Camp Butner</td>
<td>1.5</td>
<td>68.7</td>
<td>1.66</td>
<td>101</td>
</tr>
<tr>
<td>Camp Navajo</td>
<td>2.6</td>
<td>73.1</td>
<td>1.60</td>
<td>116</td>
</tr>
<tr>
<td>Florence</td>
<td>4.9</td>
<td>9.5</td>
<td>0.20</td>
<td>29</td>
</tr>
</tbody>
</table>

\(^\dagger\) conversion rate after lag phase.  
\(^\circ\) MENA + DAAN (resolved with HPLC-DAD)  
\(^\ddagger\) MENA+DAAN divided by DNAN added (150 µM)
Soil parameters (OC content, bioavailable Fe, and soil texture) were tested for correlation with DNAN conversion rate. Fast DNAN-(bio)transforming soils had OC contents above 2% (Figure 4.3), while slow soils had a low OC content (<2%). There was a strong relationship between transformation rate and OC ($R^2 = 0.9797$, n=5, two-sided t-test $p = 0.001$). Each percent OC means an increment of 0.593 µmol DNAN g$^{-1}$ dry weight (dwt) soil d$^{-1}$ up to 2.07% OC (Figure 4.3). Above this value the rate did not increase. While bioavailable Fe did not show significant linear correlation at the 95% confidence level, one-way ANOVA showed that there was significant difference in the degradation rates based on its concentration ($F$ test = 93.8, $p = ...
Furthermore, one-way ANOVA also showed significant differences in soil texture ($F$ test = 102, $p = 1.53 \times 10^{-5}$). Based on these observations, 2.07% OC was a threshold range that distinguished fast from slow DNAN-converting soils.

Three of the fast (bio)transforming soils were selected for further study by comparing full live treatment (containing $H_2$) with endogenous (no $H_2$ added) and heat-killed controls. DNAN conversion occurred in all cases (Figure 4.4). $H_2$ amendment enhanced DNAN conversion by 2-6 fold compared to endogenous treatments. Heat-killed and endogenous treatments had similar rates, 17.9-44.3 and 11.1-47.3 $\mu$M d$^{-1}$, respectively, which suggest that with no electron donor amendment, abiotic transformation is dominant.

The highest yields of UHPLC-DAD detectable monomer products (MENA, DAAN) for these three fast biotransforming soils in aqueous phase generally occurred in the $H_2$ amended (46.0-77.0% of DNAN) and the endogenous (56.8-68.2%) treatments. The lowest concentrations generally occurred in heat-killed controls (46.6-58.9%). After 10 d, DNAN recovery was 0%, and no DNAN, MENA, or DAAN were detected. This indicated the formation of transformation products that were either not detectable in UHPLC-DAD or bound to the soil.
Figure 4.3. Correlations of zero-order rate constant with soil organic carbon (OC) content (Panel A) and soil bioavailable Fe (Panel B) during DNAN anaerobic soil biotransformation assays. Rate was calculated from the end of the lag phase (Table 4.1) until DNAN was no longer detected. Vertical dashed line indicates threshold of linear correlation of rate constant with OC and Fe. Linear regression for OC in Panel A valid for 0-2.07% OC. Two-sided t-test, n = 5, p ≤ 0.005.
Figure 4.4. Concentrations of DNAN (●), MENA (■), and DAAN (▲) and their sum (-----) during the anaerobic (bio)transformation of DNAN with 50 wet g L⁻¹ soil for Catlin (A), Camp Butner (B), and Camp Navajo (C) soils. H₂ added as electron donor (1), live soil (endogenous) (2), and heat-killed soil (3). Averages with error bars are reported.
Products identified in UHPLC and infusion Q-ToF-MS

Ten chemical structures were detected in liquid samples using QToF-MS techniques. Table 4.2 shows the compounds, retention times, mass to charge ratios (m/z values as [M+H]+), as well as fragments detected where available. 2,4’dimethoxy-4-nitro-3’nitroso-azobenzene was detected by infusion Q-ToF-MS and the rest of the compounds were detected using UHPLC-Q-ToF-MS. The high-resolution masses measured for all compounds detected were within 0.7 ppm of predicted monoisotopic masses. Mass spectral details and fragmentation patterns are shown in Figures B-3 to B-12 in Appendix B. MENA and DAAN were confirmed in the samples analyzed, with [M+H]+ m/z values detected 0.7 and 0.1 ppm away from the calculated ones, respectively. An isomer of MENA, 4-methoxy-5-nitroaniline (iMENA) (J; Figure 4.6) was detected and exhibited a shorter retention time (2.2 min) than MENA (5.3 min), indicative of greater hydrophilicity. The assignment is further supported due by the stronger dipole moment and lower log Kow (0.80 versus 1.47) of iMENA compared to MENA [52]. In addition, the fragmentation pattern was also different between the isomers (Table 4.2). Since iMENA was only detected by UHPLC-Q-ToF-MS, it could be inferred that it was produced at considerably smaller amounts than MENA. To date, iMENA has only been reported in abiotic reduction of DNAN with zero-valent iron [77] compared to the regioselective formation of MENA in biological systems [52]. Therefore, its presence might indicate that abiotic processes contributed to nitroreduction.

Besides monomeric products, a total of seven dimers were identified. Three of them have been reported during DNAN incubations with anaerobic sludge: 3,3’diamino-4,4’dimethoxy-azobenzene (E), 3,3’diamino-4-hydroxy-4’methoxy-azobenzene (F), and 4,4’dimethoxy-3-methylamino-3’methyleneamino-azobenzene (G) (Table 4.2, Figure 4.6) [17]. The other four
were new dimers identified in this study. They included: 2,4′-dimethoxy-4-nitro-3′nitroso-azobenzene (C), 2,2′-dimethoxy-5-hydroxylamino-azobenzene (D), 4′methoxy-3-methylamino-3′methyleneamino-azobenzene (H), and 3-amino-3′nitro-4,4′dimethoxy-azobenzene (I) (See Table 4.2 and Figure 4.6).

**DISCUSSION**

Complete DNAN (bio)conversion was restricted to anaerobic conditions. Aerobically, initial partial adsorption and subsequent slow transformation was observed in live and heat-killed soils. Rapid conversion occurred in anaerobic conditions, at a rate that was highly correlated to soil OC content up 2.07%, after which the rates were apparently saturated. An important component of the transformation was due to chemical reactivity of the soil as evidenced by transformation in heat-killed soil. However, rates in live soil were accelerated with the addition of H₂.

**Aerobic transformation**

Aerobically, DNAN was removed initially due to possible sorption onto the soil. Hawari et al. [52] calculated soil OC to water partitioning coefficients for DNAN (Koc = 215-364 L kg⁻¹) in two soils containing 2.5-34% total OC, indicative of the strong affinity of the compound to organic matter. The adsorption of nitroaromatics, such as TNT, is known to be correlated with soil organic matter (SOM).
Besides adsorption, a small fraction of DNAN underwent reduction to MENA under aerobic conditions but no further reduction to DAAN was detected. These results indicate that DNAN was also partially reduced under aerobic condition. Aerobic oxidation is problematic for compounds with electron withdrawing moieties, such as multiple nitro groups [8, 11, 65]. Each additional nitro group shifts the electrons away from the carbon skeleton [54], making the oxidation of the carbon skeleton more difficult and the reduction to amines more likely [85]. While a recent study has reported aerobic DNAN mineralization for a bacterial isolate from a munitions wastewater treatment plant [15], our findings do not show similar reactions in soils, even after extended incubations (43 d). Therefore, reduction is the most likely pathway for DNAN biotransformation in soils, and this was clearly much slower in aerobic conditions.

**Anaerobic transformation**

There was rapid formation of aromatic amines, MENA and DAAN, under anaerobic conditions. This is consistent with reports on anaerobic conversion of nitroaromatics with multiple nitro groups [86-88]. Previous research has also demonstrated biological anaerobic conversion of DNAN to MENA and DAAN under anaerobic conditions [17, 18, 52]. In our soil assays, the transformation rates were well correlated with soil OC content. Firstly, natural decay of assimilable fractions of SOM can supply electron equivalents for reduction of nitro groups. Assimilable carbon in SOM has been shown for soils [89], waters [90], as well as anaerobic sediments [91]. Secondly, humic material can act as electron shuttles as has been demonstrated by quinones representing redox active moieties in natural SOM that stimulate the
reduction of nitroaromatics by ferrous iron and sulfide [92, 93]. Lastly, high SOM also correlates with high bacterial counts [94], which may catalyze nitroreduction.

Reduction of DNAN also occurred in heat-killed soils, particularly in Camp Navajo soil (which has the highest OC and bioavailable Fe). DNAN abiotic reduction has been reported to result from reaction with Fe(II) alone and with ferrous-ligand complexes [20]. In anaerobic sediments, SOM decay is known to be a major source of electron-donating substrate for Fe(III) reduction [95]. Furthermore, autoclaving soils has been reported to increase 6-fold Fe(II) content due to Fe(III) reduction during heating [96]. Therefore, autoclaving could have reduced Fe(III) to Fe(II) coupled to SOM oxidation, which in turn could promote abiotic reduction of DNAN afterwards.

**Products of anaerobic conversion and biotransformation pathway**

The initial reduction of DNAN occurred primarily in the *ortho* position, as reported previously [16, 18, 52], yielding MENA. In the endogenous and heat-killed treatments there was no further transformation (up to 9 d), except for Catlin soil. However, in the H₂ treatments, the MENA formed was readily consumed and did not result in any accumulation of DAAN. Higher reducing conditions, due to the addition of an electron donor (H₂) favored the conversion of MENA. DAAN was not detected, possibly due to binding with humic substances or coupling reactions with nitroso derivatives to form dimers, and, therefore, it did not accumulate.

Q-ToF-MS indicated azo dimer formation. While these products have been regarded as artifacts formed during sample processing [52], strict anaerobic conditions were maintained in the incubations and ascorbic acid was used as antioxidant to prevent formation of artifacts. Azo
dimers were also detected previously during incubations of DNAN with anaerobic sludge [17, 18], as well as in similar treatments with TNT [26]. Furthermore, there is a body of literature that uses reductive techniques to synthesize azo dyes from nitroaromatics [27, 97-100]. Therefore, we propose that azo dimers are formed as a product of (bio)transformation of nitroaromatics under anaerobic conditions. Two plausible mechanisms for azo product formation are shown in Figure 4.5. The most likely explanation for dimer formation under strict anaerobic conditions is a condensation reaction between a nitroso intermediate and an amino containing compound since aromatic amines were demonstrated to accumulate. This mechanism has been proposed for azo dye synthesis by reduction of nitroaromatics with nano-iron [27] and also by a one-step reaction of aromatic amines with nitroaromatics under basic conditions and high temperature (10 mM, 105 °C). [97]. This proposed reaction would form structures C, E, and I in Figure 4.6 due to the condensation between a nitroso-bearing compound and an amino group in MENA or DAAN. 2,4'-Dimethoxy-4-nitro-3’nitroso-azobenzene (C, Figure 4.6), provides evidence of nitroso bearing transformation products. Alternatively, azo dimers can be formed via the reaction of nitroso-intermediates with hydroxylamine intermediates to azoxybenzenes, that can potentially be reduced to azo compounds [25, 101]. We detected a tentative azo compound bearing a hydroxylamino group, 2,2’dimethoxy-5-hydroxylamino-azobenzene (D, Figure 4.6). Although we did not detect any hydroxylamino-bearing monomers, Perreault et al. [16] detected 2-hydroxylamino-4-nitroanisole, during the reduction of DNAN in aerobic conditions. DNAN hydroxylamino intermediates might not accumulate in soil, as hydroxylamino products from TNT reduction have been reported to bind to soil irreversibly [102].
Figure 4.5. Possible mechanisms for coupling between reduced intermediates of nitroaromatic compounds: A) coupling of nitrosobenzenes with aromatic amines [27, 97]; B) coupling of nitrosobenzenes with phenylhydroxylamines [25].

The azo dimers can be subject to further metabolism. An additional plausible reaction is the reduction of the azo dimers to form aromatic amines again. This is a well-known reaction causing cleavage of azo dyes under reducing conditions [66, 69, 103].

Besides the formation of dimers, other reactions are proposed based on metabolites observed with Q-ToF-MS, such as O-demethylation, two-step demethoxylation, and N-substitution. O-demethylation of the methoxy group yields a hydroxyl group in the transformation of 3,3’-diamino-4,4’-dimethoxy-azobenzene (E, Figure 4.6) to 3,3’-diamino-4-hydroxy-4’methoxy-azobenzene (F, Figure 4.6). This reaction has been reported during anaerobic incubations of DNAN and 4-nitroanisole [16, 17, 74]. After O-demethylation, dehydroxylation could follow, together comprising a two-step demethoxylation. This series of reactions is suggested between 4,4’dimethoxy-3-methylamino-3’methyleneamino-azobenzene (G, Figure 4.6) and 4’methoxy-3-methylamino-3’methyleneamino-azobenzene (H, Figure 4.6). Two-step demethoxylation has been reported previously for the biotransformation of DNAN in anaerobic sludge [17]. Another reaction proposed is N-substitution with alkyl groups, leading to
observed N-methyl and N-methylene containing dimers (G, H; Figure 4.6), which has also been reported previously in anaerobic sludge biotransformation [17]. It is possible that partially degraded labile components could be a source of alkylating amines. Finally, another compound was detected and tentatively assigned the structure 2,2’dimethoxy-5-hydroxylamino-azobenzene (D, Figure 4.6). This dimer suggests nitrogen removal from the structure (azo dimers from DNAN have four N-bearing groups while structure D in Figure 4.6 has only three). Nitro group removal from an aromatic ring in reductive conditions can occur during nitroreduction to hydroxylamino followed by N removal as NH$_4^+$ yielding a diol [104]. However, the diol product of this reaction scheme was not detected in 2,2’dimethoxy-5-hydroxylamino-azobenene (D). Overall, these reactions indicate that azo dimers formed from reduced products of DNAN bioconversion continue to undergo transformations in anaerobic soil environments.
Figure 4.6. Metabolites detected with infusion and UHPLC Q-ToF-MS experiments and transformation pathway proposed. Compounds in parentheses were not detected in this work but are known intermediates in the literature. Double arrows indicate that reactions require multiple steps.
CONCLUSION

DNAN underwent (bio)transformation in soils, particularly in anaerobic conditions, due to biotic and abiotic processes. The major reaction pathway involved nitro-group reduction to MENA, and to a very minor extent, DAAN. Products from DNAN reduction coupled to form azo dimers that continued to be (bio)transformed with O-demethylation and N-substitution reactions. Taken together, our results indicate that DNAN is readily reductively (bio)transformed in natural soils, and a full suite of transformation products are formed such as aromatic amines and azo dimers, which can impact the fate of DNAN in the environment.
Table 4.2. Molecular formulae, retention times, calculated and measured m/z values, and spectral data determined by UHPLC and infusion Q-ToF-MS for the identified transformation products. Retention time (RT)

<table>
<thead>
<tr>
<th>Compound/ structure in Figure 4.6 (bold)</th>
<th>Molecular formula [M]</th>
<th>RT (min)</th>
<th>Calculate d [M+H]+</th>
<th>Measured [M+H]+</th>
<th>Spectral data (*Int.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4-diaminooanisole (DAAN) (B)</td>
<td>C₇H₁₀N₂O</td>
<td>1.9</td>
<td>139.0866</td>
<td>139.0865</td>
<td>139.0865 (31), 124.0635 (100), 108.0687 (28), 95.0604 (25), 80.0504 (19)</td>
</tr>
<tr>
<td>2-methoxy-5-nitroaniline (MENA) (A)</td>
<td>C₇H₆N₂O₃</td>
<td>5.3</td>
<td>169.0608</td>
<td>169.0615</td>
<td>169.0615 (52), 154.0377 (25), 123.0683 (100), 108.0447 (49), 96.0449 (7), 80.0505 (37)</td>
</tr>
<tr>
<td>4-methoxy-5-nitroaniline (iMENA) (J)</td>
<td>C₇H₆N₂O₃</td>
<td>2.2</td>
<td>169.0608</td>
<td>169.0602</td>
<td>169.0602 (72), 154.0366 (29), 123.0676 (16), 122.0597 (100), 108.0444 (21), 94.0649 (23), 77.0395 (18)</td>
</tr>
<tr>
<td>3,3’diamino-4-hydroxy-4’methoxy-azobenzene (F)</td>
<td>C₁₃H₁₄N₄O₂</td>
<td>2.6</td>
<td>259.1190</td>
<td>259.1191</td>
<td>259.1191 (87), 242.0919 (27), 228.1005 (34), 227.0923 (100), 199.0977 (70)</td>
</tr>
<tr>
<td>4’methoxy-3-methylamino-3’methyleneamino-azobenzene (H)</td>
<td>C₁₅H₁₆N₄O</td>
<td>2.3</td>
<td>269.1397</td>
<td>269.1398</td>
<td>269.1398 (100), 254.1167 (38), 237.1132 (57)</td>
</tr>
<tr>
<td>3,3’diamino-4,4’dimethoxy-azobenzene (E)</td>
<td>C₁₄H₁₆N₄O₂</td>
<td>1.6</td>
<td>273.1346</td>
<td>273.1351</td>
<td>273.1351 (54), 256.1079 (35), 242.1161 (60), 227.0924 (100), 199.0978 (11), 151.0860 (14)</td>
</tr>
<tr>
<td>2,2’dimethoxy-5-hydroxylamino-azobenene (D)</td>
<td>C₁₄H₁₅N₃O₃</td>
<td>4.9</td>
<td>274.1186</td>
<td>274.1180</td>
<td>274.1180 (24), 243.1010 (100), 228.0764 (69), 200.0806 (34), 172.0863 (10), 143.0591 (10)</td>
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<tr>
<td>4,4’dimethoxy-3-methylamino-3’methyleneamino-azobenzene (G)</td>
<td>C₁₆H₁₈N₄O₄</td>
<td>3.0</td>
<td>299.1503</td>
<td>299.1509</td>
<td>299.1509 (100), 284.1277 (29), 267.1241 (14)</td>
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<td>3-amino-3’nitro-4,4’dimethoxy-azobenzene (I)</td>
<td>C₁₄H₁₄N₄O₄</td>
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<td>303.1088</td>
<td>303.1088</td>
<td>303.1088 (100), 271.0810 (19), 257.0651 (19), 225.0888 (6), 227.1042 (16), 151.0856 (24)</td>
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<tr>
<td>2,4’dimethoxy-4-nitro-3’nitroso-azobenzene (C)</td>
<td>C₁₄H₁₂N₄O₅</td>
<td>N/A</td>
<td>*</td>
<td>317.0880</td>
<td>*</td>
</tr>
</tbody>
</table>

*Int. = % intensity normalized to highest m/z for each compound
** Compound detected in infusion-Q-ToF-MS. Fragmentation not available.
CHAPTER 5

MICROBIAL TOXICITY OF THE INSENSITIVE MUNITIONS COMPOUND, 2,4-DINITROANISOLE (DNAN), AND ITS AROMATIC AMINE METABOLITES

ABSTRACT: 2,4-dinitroanisole (DNAN) is an insensitive munitions compound considered to replace conventional explosives such as 2,4,6-trinitrotoluene (TNT). DNAN undergoes facile microbial reduction to 2-methoxy-5-nitroaniline (MENA) and 2,4-diaminoanisole (DAAN). This study investigated the inhibitory effect of DNAN, MENA, and DAAN towards various microbial targets in anaerobic (acetoclastic methanogens) and aerobic (heterotrophs and nitrifiers) sludge, and the bioluminescent bacterium, *Aliivibrio fischeri*, used in the Microtox assay. Aerobic heterotrophic and nitrifying batch experiments with DAAN could not be performed because the compound underwent extensive autooxidation in these assays. DNAN severely inhibited methanogens, nitrifying bacteria, and *A. fischeri* (50% inhibitory concentrations (IC₅₀) ranging 41-57 µM), but was notably less inhibitory to aerobic heterotrophs (IC₅₀ > 390 µM). Reduction of DNAN to MENA and DAAN lead to a marked decrease in methanogenic inhibition (*i.e.*, DNAN > MENA ≈ DAAN). Reduction of all nitro groups in DNAN also resulted in partial detoxification in assays with *A. fischeri*. In contrast, reduction of a single nitro group did not alter the inhibitory impact of DNAN towards *A. fischeri* and nitrifying bacteria given the similar IC₅₀ values determined for MENA and DNAN in these assays. These results indicate that reductive biotransformation could reduce the inhibitory potential of DNAN.
INTRODUCTION

The defense industry is currently developing insensitive munitions (IM). Utilization of these energetic chemicals is expected to reduce accidental explosions since IMs have a high detonation temperature and enhanced resistance to shocks [76]. 2, 4-dinitroanisole (DNAN) is an IM compound that is being considered as a replacement for the widely used nitroaromatic, 2,4,6-trinitrotoluene (TNT) [5]. While DNAN is less sensitive, other technical properties of this material compare favorably with those of TNT and make it advantageous for the manufacturing explosives formulations [5].

An assessment of the environmental fate and toxicity potential of DNAN is required since DNAN production and usage is expected to increase. Many nitroaromatic compounds are toxic and mutagenic to different types of organisms, including bacteria, algae, plants, invertebrates, and mammals [8, 33]. DAAN has also been shown to be mutagenic and a potential carcinogen in various assays [47-50]. However, data on the inhibitory potential of DNAN towards microorganisms are very scarce. Microbial toxicity could impact biological treatment of effluents containing DNAN and impair the function of natural microbial populations in contaminated soil, which in turn could compromise the effectiveness of soil bioremediation efforts.

DNAN has been reported to undergo microbial transformation to 2-methoxy-5-nitroaniline (MENA) in aerobic conditions [16], and to 2, 4-diaminoanisole (DAAN) in anaerobic conditions [18]. Recently we have also reported that microorganisms present in conventional wastewater treatment systems can reduce the nitro group in DNAN under aerobic, microaerophilic and anaerobic conditions [17]. Both MENA and DAAN were identified as important microbial metabolites in all redox conditions. Initially, the ortho nitro group in DNAN is regioselectively reduced to yield MENA. Subsequently, the para nitro group in MENA is reduced to form the diamino compound, DAAN. Microbial transformation
of DNAN could alter the potential toxic impact of this aromatic compound. Unfortunately, data on the microbial toxicity of the reduced metabolites of DNAN are largely unavailable.

The objective of this study was to evaluate the inhibitory effect of DNAN and its reduced intermediates MENA and DAAN to microorganisms commonly found in the environment under different redox conditions, namely anaerobic methanogens, aerobic heterotrophs and nitrifying bacteria. The inhibitory impact of these compounds was also evaluated using the Microtox assay, a method that relies on bioluminescence measurements in cultures of the bacterium *Aliivibrio fischeri*. The results obtained will contribute to a better understanding of the environmental impact of DNAN and will facilitate the development and optimization of efficient bioremediation technologies for the removal of this nitroaromatic compound.

**MATERIALS AND METHODS**

**Microbial inocula**

Methanogenic sludge, aerobic return activated sludge (RAS), and nitrifying sludge were used as inoculum. The methanogenic sludge was obtained from a full-scale anaerobic bioreactor treating brewery wastewater (Mahou, Guadalajara, Spain). RAS and the nitrifying inoculum were collected from local municipal wastewater treatment plants; Ina Road and Randolph Park Wastewater Reclamation Facilities (Tucson, AZ, USA), respectively. All sludge samples were stored at 4°C. The volatile suspended solid (VSS) content in the methanogenic, RAS, and nitrifying sludge was 7.92, 0.25, and 0.53% (wet wt), respectively.
The aerobic inocula were centrifuged (20 min at 4,000 rpm) and the supernatant was discarded before use in bioassays.

**Culture media**

The basal mineral medium labeled “medium 1” contained (in mg L\(^{-1}\)): K\(_2\)HPO\(_4\) (250), CaCl\(_2\)•2H\(_2\)O (10), MgSO\(_4\)•7H\(_2\)O (100), MgCl\(_2\)•6H\(_2\)O (100), NH\(_4\)Cl (280), NaHCO\(_3\) (4,000), yeast extract (100). The basal medium termed “medium 2” contained (in mg L\(^{-1}\)): NaH\(_2\)PO\(_4\) (1,500), Na\(_2\)HPO\(_4\) (894), NH\(_4\)Cl (164), NaHCO\(_3\) (899). All media were supplemented with 1 mL L\(^{-1}\) of trace element solution [17]. The pH of the basal medium was adjusted to 7.2 with HCl or NaOH, as required.

**Microbial inhibition bioassays**

**Methanogenic toxicity assay**

Assays were conducted in glass flasks (160 mL) with basal medium 1 (25 mL) supplemented with acetate (26 mM) and methanogenic sludge (1.5 g VSS L\(^{-1}\)). All flasks were sealed with butyl rubber stoppers and then flushed with N\(_2\)/CO\(_2\) (80:20, v/v) for 5 min to create anaerobic conditions. The flasks were pre-incubated overnight to ensure that the sludge was adapted to the assay conditions. The following day, DNAN (0-130 µM), MENA (0-500 µM), or DAAN (0-661 µM) were added from concentrated stock solutions. The methane content in the headspace of each flask was measured periodically until the production of methane became constant in the toxicant-free controls. The maximum specific methanogenic activity of the control was 0.16 g CH\(_4\)-COD g VSS\(^{-1}\) d\(^{-1}\).
Aerobic heterotropic inhibition assay

Assays were conducted in serum flasks (160 mL) with medium 1 (25 mL) supplemented with acetate (28 mM) and RAS (0.5 g VSS L\(^{-1}\)). Flasks were spiked with DNAN (0-390 µM) or MENA (0–1,500 µM), sealed with butyl rubber stoppers, and flushed with He/CO\(_2\)/O\(_2\) (60:20:20, v/v) for 5 min. The O\(_2\) content in the headspace of each flask was measured periodically until the O\(_2\) consumption rate resembled that of the endogenous control lacking acetate and toxicant addition. The maximum specific O\(_2\) consumption activity of the uninhibited control was 28 mg COD g VSS\(^{-1}\) d\(^{-1}\).

Nitrification inhibition assays

Assays were conducted in Erlenmeyer flasks (125 mL) containing basal medium 2 (50 mL) and nitrifying sludge (0.5 g VSS L\(^{-1}\)). The flasks were spiked with DNAN (0-520 µM) or MENA (0–480 µM) and then capped with cotton gauzes to facilitate gas exchange. Liquid samples were collected periodically for ammonium analysis. The nitrifying activity of the uninhibited control was 19.3 mg NH\(_4^+\)-N g VSS\(^{-1}\) d\(^{-1}\).

Microtox

Microtox® Model 500 analyzer (Strategic Diagnostics, Inc. SDIX, Newark, DE, USA) was used to measured changes in the bioluminescence produced by the marine bacterium *Aliivibrio fischeri* (lyophilized culture of *A. fischeri* NRRL-B-11177, AZUR Environmental, Carlsbad, CA, USA). DNAN (0-650 µM), MENA (0–1,300µM), DAAN (0-650 µM), and 2,4-dinitrophenol (NDP) (0-650 µM) solutions were tested. Microbial inhibition in Microtox was measured at 25°C in triplicate experiments as previously described [105].
All experiments were conducted in duplicate. The bioassays using sludge were incubated at 30°C in an orbital shaker (115 rpm) in the dark. Flasks without toxicant were included in all the assays and served as uninhibited controls. The maximum specific \( O_2 \) consumption, as well as the nitrifying and methanogenic activities were calculated from the slope of \( O_2 \) consumption, ammonium concentration, and cumulative methane production; respectively. The activities were normalized with respect to the biomass concentration. The initial concentrations of toxicant causing 20, 50 and 80% reduction in activity compared to an uninhibited control were referred to as \( IC_{20}, IC_{50} \) and \( IC_{80} \), respectively.

**Analytical methods and chemicals**

DNAN, MENA, and DAAN were quantified by high-performance liquid chromatograph with diode array detection as previously described [17]. Methane and oxygen in gas samples were determined by gas chromatography with flame ionization- and thermal conductivity detection, respectively [106]. Ammonium was determined using an Orion Thermo combination ion-selective electrode (Mettler Toledo-Seven Multi, Schwerzenbach, Switzerland), and VSS according to standard methods (APHA, 1998).

DNAN (CAS# 119-27-7, 98% purity) was purchased from Alfa Aesar (Ward Hill, MA, USA). MENA (CAS# 99-59-2, 98%), DAAN (CAS# 615-05-4, analytical standard) and 2,4-dinitrophenol (CAS # 51-28-5, 97%) were obtained from Sigma-Aldrich (St. Louis, MO, USA).
RESULTS

Methanogenic inhibition

Figure 5.1A illustrates the time course of methane production in methanogenic activity assays amended with DNAN. The specific methanogenic activities determined in these assays were normalized based on the activity of the DNAN-free control. In each case, the activity was determined during the time period when the control displayed maximum methane production rate (i.e., time 0-31 h). The normalized methanogenic activity as a function of the initial DNAN concentration is shown in Figure 5.2A. The same procedure was utilized to calculate the microbial activities of the different microorganisms evaluated in this study. The IC$_{20}$, IC$_{50}$ and IC$_{80}$ values determined are summarized on Table 5.1.

The rate of methane production in all the assays spiked with DNAN was lower relative to the control indicating methanogenic inhibition. In the treatments with the highest DNAN concentrations, no methane was initially produced. For instance, in the treatments with 78, 104 and 130 µM, methane was not produced during the initial 22, 30, and 40 h of incubation, respectively. The onset of methane production coincided with the complete removal of DNAN due to the reductive transformation of the nitroaromatic compound (Figure 5.1B).
Figure 5.1. Panel A - Methane production by anaerobic granular sludge amended with acetate (26 mM) and exposed to DNAN (in μM): 0 (Δ), 13 (♦), 26 (□), 52 (●), 78 (◊), 104 (■), and 130 (○). Panel B - DNAN degradation, formation of DNAN metabolites, and methane production by anaerobic granular sludge amended with acetate (26 mM) and exposed to DNAN (133 μM). Legends: DNAN (●), and MENA (□), DAAN (▲), and methane (◊).

Figure 5.2A shows the normalized methanogenic activity results determined in assays with DNAN, MENA and DAAN. DNAN was found to be more toxic than MENA and DAAN. The IC$_{50}$ values determined for DNAN, MENA and DAAN were 41, 175, and 176.
μM, respectively (Table 5.1). DNAN, MENA, and DAAN caused complete methanogenic inhibition at 100, 400, and 660 μM, respectively.

Figure 5.2. DNAN, MENA and DAAN inhibition (expressed as percentage of the control activity) towards methanogenic (A), and aerobic microorganisms (B). Panel A- Methanogenic assays: DNAN (●), and MENA (□), and DAAN (▲). Panel B- Aerobic heterotrophic assays: DNAN (○), and MENA (■); Nitrification assays: DNAN (●) and MENA (□).
Inhibition of aerobic heterotrophic bacteria

The inhibitory effect of DNAN on O$_2$ consumption by heterotrophic bacteria in activated sludge is illustrated in Figure 5.3A. All treatments with acetate showed higher O$_2$ consumption rates than the endogenous case during the first 10 h. This period was used to assess O$_2$ consumption. Figure 5.2B compares the normalized activity determined in the aerobic heterotrophic bioassays exposed to various concentrations of DNAN and MENA. Exposure to DNAN (390 µM) for 10 h resulted in moderate inhibition of the O$_2$ consumption rate by the aerobic microorganisms (39% inhibition), whereas MENA was only slightly inhibitory (14% inhibition) at concentrations as high as 1,500 µM. It should be noted that DAAN could not be evaluated in assays utilizing O$_2$ as electron acceptor, i.e., aerobic heterotrophic and nitrifying bioassays, because the compound was not stable under the oxic conditions inherent of these tests. DAAN was observed to undergo rapid spontaneous oxidation in the presence of elemental oxygen.

Inhibition of nitrification

The time course of ammonium consumption by nitrifying bacteria exposed to DNAN is shown in Figure 5.3B. DNAN displayed severe inhibition towards nitrifying microorganisms in aerobic sewage sludge. Nitrification was completely inhibited at DNAN concentrations exceeding 260 µM. At lower concentrations, nitrifying bacteria appeared to become acclimated to the presence of DNAN with time. For example, assays exposed to DNAN concentrations ranging from 65-130 µM were completely inhibited during the first 60
h but their activity increased sharply thereafter, reaching levels close to those observed in the control without DNAN. The observed recovery may be due to transformation of DNAN to less toxic products. In this respect it is interesting to note that chromatographic analysis of the culture samples obtained at the end of the experiment (145 h) indicated considerable removal of DNAN, ranging from 53 to 81% of the initial concentration depending on the assay (Figure C-1, Appendix C). Only a small fraction of the DNAN removed was recovered as MENA and DAAN, suggesting the formation of other unidentified biodegradation products.

Figure 5.2B compares the normalized activity of microbial ammonium oxidation in activated sludge exposed to DNAN and MENA. Both compounds exhibit comparable inhibition towards the nitrifying bacteria as indicated by their similar IC₅₀ values (48-49 µM) (Table 5.1).
Figure 5.3. Panel A- Time course of oxygen consumption by aerobic activated sludge amended with acetate (28 mM) when exposed to DNAN (in μM): 0 (◊), 130 (●), 260 (▲), and 390 (□). Endogenous control lacking acetate and DNAN (♦). Panel B- Time course of ammonium consumption by nitrifying bacteria in aerobic activated sludge in the presence of DNAN (in μM): 0 (●), 65 (○), 130 (▲), 260 (□), and 520 (♦).
**Inhibition towards Aliivibrio fischeri**

Figure 5.4 illustrates the impact of exposure to varying concentrations of DNAN, MENA, and DAAN on the bioluminescence activity of *A. fischeri*, previously classified as *Vibrio fischeri* [107]. The diamino metabolite, DAAN, was the least toxic of the compounds tested and its IC$_{50}$ value (155 μM) was approximately 3-fold higher compared to those of the other nitroaromatic compounds. DNAN and MENA showed very similar inhibitory potential as indicated by their close IC$_{50}$ values, 57 and 48 μM, respectively.

![Figure 5.4. Toxicity of DNAN (●), MENA (□), and DAAN (▲) to Aliivibrio fischeri after 30 min of exposure. Toxicity is expressed as percentage of toxicant-free activity.](image)

Table 5.1 Summary of inhibitory concentrations determined for DNAN and its metabolites MENA and DAAN in various microbial toxicity bioassays.
As previously observed for TNT [63, 108], significant environmental emissions of DNAN could occur during compound manufacture and use in testing and training grounds. Environmental emissions of DNAN are of concern because many nitroaromatic compounds exhibit high toxicity to microorganisms [34, 109-111], and to higher aquatic and terrestrial organisms, including mammals [33, 110, 112-115]. TNT and related nitroaromatic compounds have also been found to be mutagenic and potential carcinogens in studies with several organisms [55, 110, 112, 113, 116]. Only one previous study has attempted to characterize the toxicological profile of DNAN [5] and information on the microbial toxicity of DNAN is largely lacking. We are only aware of a previous study where DNAN was identified as an inhibitor of perchlorate-reducing bacteria [77]. Complete inhibition of the perchlorate-reducing activity of activated sludge was observed in batch assays spiked with 126 µM DNAN. The present study was undertaken to examine the microbial toxicity of DNAN and several of its reduction metabolites to ecologically relevant microbial activity.

**DISCUSSION**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Methanogens</th>
<th>Aerobic heterotrophs</th>
<th>Nitrifiers</th>
<th>Microtox</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC₉₀</td>
<td>IC₅₀</td>
<td>IC₁₀₀</td>
<td>IC₂₀</td>
</tr>
<tr>
<td>DNAN (µM)</td>
<td>16</td>
<td>41</td>
<td>70</td>
<td>158</td>
</tr>
<tr>
<td>MENA (µM)</td>
<td>121</td>
<td>175</td>
<td>27.5</td>
<td>507</td>
</tr>
<tr>
<td>DAAN (µM)</td>
<td>72</td>
<td>176</td>
<td>327</td>
<td>NAᶜ</td>
</tr>
</tbody>
</table>

* NT= Not toxic at the highest concentration tested.
*ᵃ Inhibition at the highest DNAN concentration tested (390 µM) was 39%.
*ᵇ Inhibition at the highest MENA concentration tested (1,500 µM) was 13%.
*ᶜ N.A. = Not available. DAAN was not tested because the compound is very unstable (towards autoxidation) in the presence of O₂.
The results of this research demonstrated that the inhibitory impact microbial of DNAN varied widely depending on the target microbial population. While respiration activity by aerobic heterotrophs was only partly impacted at the highest concentration tested (39% inhibition at 390 µM DNAN), all other microbial targets were severely inhibited at relatively low DNAN concentrations. The IC$_{50}$ values determined in bioassays with methanogens, nitrifying bacteria, and the widely used luminescent bacterium, *A. fischeri*, were relatively close and ranged from 41 to 57 µM (Table 5.1). In agreement with our findings, previous studies have demonstrated that nitroaromatics are highly toxic to methanogenic microorganisms [34, 117-119], and to the bacterium, *A. fischeri* [109, 110, 120]. On the other hand, the sensitivity of aerobic heterotrophic bacteria to these pollutants appears to differ widely. Gram-negative organisms have been found to be more tolerant to nitroaromatics than Gram-positive bacteria [121, 122]. As an example, Fuller and Manning [121] observed that the growth of pure cultures of aerobic Gram-positive bacteria was severely inhibited by concentrations of TNT as low as 44 µM, whereas most Gram-negative organisms were unaffected by concentrations approaching the water solubility of TNT (440 µM). Similarly, cell growth inhibition assays with the Gram-negative bacterium, *Pseudomonas putida*, did not show significant inhibition by TNT and 11 other nitroaromatic compounds, including several TNT metabolites [110].

Comparison of the inhibitory concentrations determined for DNAN with values reported for TNT suggests that DNAN might be somewhat less toxic to microorganisms. Most notably, the 30-min IC$_{50}$ value (57 µM) determined for DNAN in luminescence assays with *A. fischeri* is several times higher compared to those previously reported for TNT (2 to 16 µM) [109, 110, 120]. Unfortunately, although TNT is known to be a potent methanogenic inhibitor [123], detailed studies assessing the impact of varying concentrations of TNT on methanogenesis are lacking. Similarly, information on the inhibitory impact of TNT to
nitrification is very scarce. We are only aware of a study where the IC₅₀ was observed in soil assays exposed to 45 µM TNT [124], a value which is close to the IC₅₀ (49 µM) determined in the current study.

The mechanisms responsible for the inhibitory effects of DNAN have not been investigated to date, but they are likely to overlap to some degree with those of other nitroaromatic compounds. Many nitrophenols are potent uncouplers of oxidative phosphorylation, which are able to abolish the link between substrate oxidation and ATP synthesis without appreciably affecting the electron-transfer mediators [125]. DNAN was also reported recently to be a mitochondrial uncoupler [126]. Furthermore, the inhibitory effect of nitroaromatics has also been related to their hydrophobic character. We have previously demonstrated that the methanogenic toxicity exerted by N-substituted phenols (aminophenols and nitrophenols) was closely correlated with compound apolarity \( (R^2 = 0.95) \) [34], suggesting that partitioning of the nitroaromatics into the lipophilic microbial membranes may have a role in the toxicity. The results of the current work also showed a positive correlation \( (R^2 = 0.75) \) between the methanogenic inhibition (expressed as IC₅₀) exhibited by the various nitroaromatic and aminoaromatic compounds tested and their hydrophobic character (expressed as logarithm of the octanol-water partition coefficient, log \( K_{ow} \)) (Table 5.2). However, the correlation was weak when nitrification and Microtox data were considered. The unique membrane of methanogenic microorganisms appears to be particularly susceptible to disturbance by nitroaromatic compounds. In contrast with sulfate-reducing bacteria and clostridia, methanogenic cells were found to lyse in the presence of nitroaromatics [43]. Interestingly, aromatic amines did not cause cell lysis.
Table 5.2 Properties of tested and structurally-related aromatics compounds. Data for 298.15 K unless otherwise noted.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molecular Formula</th>
<th>MW</th>
<th>Log P</th>
<th>Toxicity- IC\textsubscript{50} (µM)</th>
<th>Methanogenic</th>
<th>Microtox</th>
</tr>
</thead>
<tbody>
<tr>
<td>2, 4-dinitroanisole (DNAN)</td>
<td>C\textsubscript{7}H\textsubscript{6}N\textsubscript{2}O\textsubscript{5}</td>
<td>198</td>
<td>1.61±0.01\textsuperscript{a}</td>
<td>41\textsuperscript{b}</td>
<td>57\textsuperscript{b}</td>
<td></td>
</tr>
<tr>
<td>2-amino-4-nitroanisole (MENA)</td>
<td>C\textsubscript{7}H\textsubscript{8}N\textsubscript{2}O\textsubscript{3}</td>
<td>168</td>
<td>1.71±0.24\textsuperscript{c}</td>
<td>175\textsuperscript{b}</td>
<td>48\textsuperscript{b}</td>
<td></td>
</tr>
<tr>
<td>2, 4-diaminoanisole (DAAN)</td>
<td>C\textsubscript{7}H\textsubscript{10}N\textsubscript{2}O</td>
<td>138</td>
<td>-0.22±0.26\textsuperscript{c}</td>
<td>176\textsuperscript{b}</td>
<td>155\textsuperscript{b}</td>
<td></td>
</tr>
<tr>
<td>2, 4-dinitrophenol (2, 4-DNP)</td>
<td>C\textsubscript{6}H\textsubscript{4}N\textsubscript{2}O\textsubscript{5}</td>
<td>184</td>
<td>1.80\textsuperscript{d}</td>
<td>43\textsuperscript{c}</td>
<td>382\textsuperscript{b}</td>
<td></td>
</tr>
<tr>
<td>2, 4-dinitrotoluene</td>
<td>C\textsubscript{7}H\textsubscript{6}N\textsubscript{2}O\textsubscript{4}</td>
<td>182</td>
<td>2.08±0.18\textsuperscript{e}</td>
<td>27\textsuperscript{c}</td>
<td>248\textsuperscript{f}</td>
<td></td>
</tr>
<tr>
<td>2-amino-4-nitrotoluene</td>
<td>C\textsubscript{7}H\textsubscript{8}NO\textsubscript{2}</td>
<td>152</td>
<td>1.61±0.23\textsuperscript{e}</td>
<td>NA</td>
<td>144\textsuperscript{f}</td>
<td></td>
</tr>
<tr>
<td>2, 4-diaminophenol</td>
<td>C\textsubscript{6}H\textsubscript{8}N\textsubscript{2}O</td>
<td>124</td>
<td>-1.31±0.26\textsuperscript{e}</td>
<td>283\textsuperscript{c}</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>2, 4-diaminotoluene</td>
<td>C\textsubscript{7}H\textsubscript{10}N\textsubscript{2}</td>
<td>122</td>
<td>-0.09±0.25\textsuperscript{e}</td>
<td>NA</td>
<td>826\textsuperscript{f}</td>
<td></td>
</tr>
</tbody>
</table>

NA: Not available. \textsuperscript{a}Boddu, Abburi, Maloney and Damavarapu [76]; \textsuperscript{b} Value calculated in the current study; \textsuperscript{c} Donlon, Razo-Flores, Field and Lettinga [34]; \textsuperscript{d} Schwarzenbach, Stierli, Folsom and Zeyer [127] (294.15 K); \textsuperscript{e} Calculated with Advanced Chemistry Development (ACD/Labs) Software V11.02 (© 1994-2013 ACD/Labs); \textsuperscript{f} Drzyzga, Gorontzy, Schmidt and Blotevogel [109].
Microbial toxicity of DNAN metabolites

Recent studies conducted at our laboratory and elsewhere have shown that DNAN undergoes rapid microbial reduction in anaerobic environments [17, 18]. DNAN is first reduced to MENA, a metabolite that undergoes subsequent reduction to DAAN. While DNAN undergoes rapid biotransformation, the compound does not appear to be mineralized. Therefore, information on the toxicity of DNAN metabolites is important to understand whether microbial degradation processes occurring during natural attenuation or site bioremediation could lead to decreased or enhanced toxicity.

Results obtained for several DNAN metabolites and related N-substituted compounds indicate that the reduction of nitro to amino groups on the aromatic ring was generally associated with a decreased toxicity. The inhibitory impact of DNAN and MENA towards nitrifying bacteria and the marine bacterium, *A. fischeri*, was very similar indicating that reduction of a single nitro group was not sufficient to alleviate the inhibitory effect of the parent compound (Table 5.1). In contrast, MENA was significantly less inhibitory towards methanogenic microorganisms than DNAN. Microbial reduction of the two nitro groups with formation of DAAN led to significant detoxification in bioassays with methanogens and *A. fischeri*. In agreement with these findings, aromatic amines have been reported to be generally less toxic than their corresponding nitroaromatic analogs in studies with several microorganisms, including methanogenic archaea [34, 44], *A. fischeri* [55, 110, 113, 116], and in studies with other aquatic (algae, fish) and terrestrial targets, including mammalian cells [55, 110, 113, 114].

Although the primary metabolites of DNAN reduction, particularly the diamino compound DAAN, were found to be generally less toxic than the parent compound, it is unclear whether microbial reduction of DNAN would lead ultimately to detoxification of the nitroaromatic compound. Anaerobic biotransformation of MENA and DAAN has been
reported to result in an array of secondary metabolites including azo and hydrazine dimer derivatives [17, 18]. Azoxy- and azo-dimers have also been detected during aerobic biotransformation of DNAN [16]. In soil environments, these reduction products are likely to bind to humic compounds, forming humus-bound residue, as reported in soil bioremediation studies with TNT and other nitroaromatic compounds [128-130]. The inhibitory impact of polymeric DNAN derivatives and soil conjugates is yet to be characterized. Previous studies comparing the toxicity of TNT and two of its dimeric azoxy metabolites (4,4’,6,6’-tetranitro-2,2’azoxytoluene and 2,2’,6,6’-tetranitro-4,4’azoxytoluene) have shown that the three contaminants were equally cytotoxic [55]. However, dimeric metabolites detected in microbial transformation studies with DNAN lacked nitro groups. Based on the lower toxicity generally observed when nitro groups are reduced to amine moieties, DNAN dimers are likely to be less toxic than the nitrated TNT dimers.

The impact of microbial transformation on the mutagenicity of DNAN also needs to be investigated. The mutagenic potential of DNAN is well established [5, 47-49, 131]. Intermediates formed during the reduction of DNAN, such as hydroxylamine and nitroso aromatics, may bind to DNA or other biomolecules, causing toxic and mutagenic effects as previously reported for the reduction metabolites of other nitroaromatics [32, 46]. Although the mutagenicity of the microbial products of DNAN reduction is still unknown, extensive mutagenicity investigation of TNT and its microbial metabolites has shown that complete reduction of the nitro group to an amino group seems to decrease the mutagenic effect of the compound [46, 53, 132].
CONCLUSION

Taken as a whole these results indicate that DNAN causes strong acute cytotoxicity in methanogenic and nitrification microbial populations. Preliminary results suggest that microbial reductive transformation may reduce the inhibitory impact of DNAN. Additional toxicity studies with secondary dimeric metabolites of DNAN and their soil conjugates are needed to better characterize the hazard associated with this emerging energetic compound. In addition to cytotoxicity, there is a need to understand the mutagenicity of aromatic amines formed during DNAN metabolism.
CHAPTER 6
MICROBIAL TOXICITY OF DNAN (BIO)TRANSFORMATION PRODUCT MIXTURES CHARACTERIZED BY LC-QTOF-MS AND INDIVIDUAL MODEL COMPOUNDS/SURROGATES OF TRANSFORMATION PRODUCTS

ABSTRACT

2,4-dinitroanisole (DNAN) is an emerging insensitive munitions compound. It undergoes rapid (bio)transformation in soils and anaerobic sludge. The primary transformation pathway is nitroreduction followed by coupling of reactive intermediates to form azo-dimers. Additional pathways include N-acetylation and O-demethoxylation. Toxicity due to (bio)transformation products of DNAN has received little attention. In this study the toxicity of DNAN (bio)transformation products and surrogates to acetoclastic methanogens and the marine bioluminescent bacterium, *Allivibrio fischeri*, were evaluated. The most toxic monomer tested was 3-nitro-4-methoxyaniline, with a 50%-inhibiting concentration (IC_{50}) of 25 µM towards methanogens. On the other hand, N-(5-amino-2-methoxyphenyl) acetamide was the least inhibitory monomer. Azo-oligomer surrogates were found to have a very high toxicity to both microbial systems with a severity that was similar or stronger than DNAN. However, at extremely low concentrations they were stimulatory to methanogenesis. A semi-quantitative method was employed to determine product mixture profiles at different stages of biotransformation with liquid chromatography linked to quadrupole time-of-flight mass spectrometry (LC-QTOF-MS), and compared with the microbial toxicity of the product-mixtures formed (and transferred to toxicity-assays). Methanogen toxicity increased as DNAN was reduced, attributed to putative reactive nitroso-intermediates, but the inhibition later attenuated as dimers became the predominant products in the mixture, due in part to the aforementioned methanogenic stimulation. In contrast *A. fischeri* tolerated the initial biotransformation products but were highly inhibited at longer the incubation times, when the azo-dimers became the dominant products, suggesting these ultimate products are more toxic than DNAN.
INTRODUCTION

2,4-dinitroanisole (DNAN) is an insensitive munitions compound that is less prone to accidental explosions, and has emerged as an important replacement of conventional explosives, such as 2,4,6-trinitrotoluene (TNT)[5]. Once released to the environment, DNAN is readily transformed abiotically[20, 24, 52, 77] and biologically[17, 18, 24] via nitroreduction in natural and engineered systems. The initial nitrogroup-reduction occurs with a dominant regioselectivity towards the ortho nitro group [16, 18, 24, 52], yielding 2-methoxy-5-nitroaniline (MENA), but its regioisomer 3-methoxy-4-nitroaniline (iMENA) has been found in few studies[24, 133]. Continued nitroreduction leads to the aromatic amine product, 2,4-diaminoanisole (DAAN). During nitroreduction, reactive intermediates can also lead to the formation of azo-dimers[17, 18, 24]. In addition to this pathway, alternative anaerobic transformation pathways have been reported, such as N-acetylation of DAAN[16, 17] and O-demethoxylation leading to 2,4-dinitrophenol[24].

As DNAN is transformed, its products may pose an environmental hazard that has yet to be characterized. Most toxicity studies to date have been focused primarily on the parent compound, DNAN [6, 39], with little attention to its transformation products. While there has been an initial characterization of the microbial toxicity of the primary DNAN nitroreduction-products[37], the toxicity posed by oligomer and additional monomer transformation products is unknown. Moreover, the characterization of transformation products past monomer products has been reported only qualitatively based on structure elucidation[16-18, 24], which makes it uncertain if these products are toxic at the levels they are formed.

Therefore, the occurrence of (bio)transformation products should be assessed and put in context with the toxicity risk that DNAN (bio)transformation products might pose. The objectives of this work were: (1) to assay microbial toxicity of individual transformation
products and/or best available surrogate compounds to acetoclastic methanogens and the marine bacterium *Allivibrio fischeri*; and (2) to determine biotransformation product profiles for DNAN incubated anaerobically in soils and anaerobic sludge and link the profile mixture composition to toxicity changes during transformation using acetoclastic methanogens and *A. fischeri*, as microbial toxicity models.

**MATERIALS AND METHODS**

**Chemicals and biological materials**

Figure 6.1 shows the chemical structures of monomer DNAN (bio)transformation products and azo-dimer surrogates assayed for microbial toxicity. 3-nitro-4-methoxyaniline (CAS# 577-72-0, denoted “iMENA”, purity 97%) was procured from Accela ChemBio Inc. (San Diego, CA, USA). N-(5-amino-2-methoxyphenyl) acetamide (CAS# 64353-88-4, denoted “Ac-DAAN”, purity 95%) was purchased from ChemBridge Corporation (San Diego, CA, USA). 2,2’-dimethoxy-4,4’-azodianiline (CAS# 6364-31-4, denoted “dimer L”, purity >90%) was acquired from MolMall Sarl (Lonay, Switzerland). Bismarck Brown Y (*m*-Bis(2,4-diaminophenylazo)-benzene, CAS# 8005-77-4, denoted “BBY”, dye purity 46%) was obtained from Chem-Impex International (Wood Dale, IL, USA). Camp Navajo soil (water content = 9.3%) and Camp Butner soil (water content = 20.7%) soils described in[24, 82], were sieved (2 mm) and stored at 4 °C before use in (bio)transformation assays. The granular sludge (dry weight (dwt) solids =11.2%, volatile suspended solids (VSS) = 7.9% per wet weight) was obtained from a full-scale upflow anaerobic sludge blanket reactor located at an industrial brewery wastewater bioreactor (Mahou, Guadalajara, Spain) and was used for (bio)transformation assays and methanogenic inhibition toxicity. The marine bioluminescent
bacterium *A. fischeri* (lyophilized culture NRRL-B-11177, Modern Water Inc., New Castle, DE, USA) was also used in microbial toxicity.

Figure 6.1 Panel A: (Bio)transformation pathways of DNAN in anaerobic incubations of soils and sludge. Microbial toxicity was evaluated for shown monomer compounds. Panel B: Azo-oligomer surrogate compounds used for microbial toxicity. Notation: OMe = methoxy; Ac = acetyl.

**Staggered DNAN soil/sludge (bio)transformation assays**

DNAN was incubated anaerobically in soil or sludge microcosms to obtain mixtures of products formed at different stages of (bio)transformation. 500 μM DNAN were added to
10 mL of mineral medium[17] (pH =7.2, 18 mM phosphate buffer) amended with 10 mM pyruvate in anaerobic tubes (Bellco Glass Inc., Vineland, NJ, USA). The solutions were inoculated with 100 mg of wet Camp Navajo soil, 100 mg of wet Camp Butner soil or 75 mg of wet anaerobic sludge. The headspace was flushed with He/CO$_2$ (80/20 %). The tubes were sealed with with t-butyl caps and aluminum seals, and subsequently incubated in the dark at 30°C in an orbital shaker at 115 rpm. Tubes were incubated at different times so that when liquid samples were collected at the same day, the overall (bio)transformation time elapsed would be 0, 1, 5, 10, 20, 30, 40, 50 days of anaerobic incubation. Sampling was performed inside and anaerobic hood to minimize autoxidation from reactive products with oxygen. Samples were centrifuged (10 min, 9,600×g), sealed inside the anaerobic chamber, and kept at 4 °C before methanogenic and A. fischeri toxicity assays. All experiments were performed in duplicate. A diagram depicting the staggered (bio)transformation assays and overall workflow is shown in Figure D-1.

**Microbial toxicity assays**

*Acetoclastic methanogens*

The toxicity of (bio)transformation products/surrogates to acetoclastic methanogenesis was assayed in a methanogenic consortia immobilized in anaerobic granular sludge. The assays were performed in 160 mL serum bottles supplemented with 25 mL basal mineral medium at pH 7.2[37], inoculated with 1.5 g VSS L$^{-1}$ of anaerobic granular sludge (7.9% VSS of wet weight), and amended with sodium acetate as substrate (2.6 mM). Serum bottles were flushed with He/CO$_2$ (80/20% v/v) and crimped with aluminum seals and t-butyl rubber septa. The bottles were incubated overnight, and flushed with He/CO$_2$ (80/20% v/v) before addition of the toxicants. iMENA (10-200 µM), Ac-DAAN (80-8,000 µM), dimer L
(4-83 µM), BBY (0.12-1.92 µM) were tested as individual inhibitory compounds. In addition, mixtures of biotransformation products in aqueous solutions at a single dilution of 54-fold (with a starting DNAN concentration of 500 µM) were assayed with the methanogenic sludge. Separate samples of these aqueous solutions containing mixtures of biotransformation products were harvested at different stages of DNAN (bio)transformation in order to detect temporal changes in toxicity due to shifting mixture of intermediates and products during (bio)transformation. All experiments were run in duplicate.

CH₄ was monitored for each treatment and the toxicant-free control (Figure D-2) using a gas chromatograph coupled to a flame ionization detector (GC-FID)[106]. The rate of CH₄ production was used to determine acetoclastic methanogen inhibition by comparing the rate of methane production in treatments normalized to a inhibitor-free control.

**A. fischeri (Microtox)**

The Microtox[105] assay was used to characterize the toxicity of the (bio)transformation products and surrogates. Exposure at 30 min was monitored for individual toxicants. The individual compounds tested included iMENA (2.5-650 µM), Ac-DAAAN (9-2,285 µM), dimer L (0.88-226 µM), BBY (0.40-103 µM). As described for the acetoclastic methanogenesis assay, Microtox was also used to evaluate aqueous mixtures biotransformation compounds recovered from different stages of DNAN (bio)transformation at a single dilution of 36-fold (with a starting DNAN concentration of 500 µM) All experiments were run in duplicate.
Analytical methods to assess (bio)transformation products

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

**UHPLC-Q-ToF-MS**
MENA, DAAN and oligomer transformation products present in the liquid phase were semi-quantitated on an UltiMate 3000 UHPLC (Dionex, Sunnyvale, CA) coupled to a TripleTOF 5600 quadrupole time-of-flight mass spectrometer (Q-ToF-MS) (AB Sciex, Framingham, MA, USA) run with an electrospray ionization source in positive mode at 450°C with a capillary setting of 5.5 kV, a declustering potential of 80 V. N₂ was used as curtain gas, desolvation gas, and nebulizer gas at 30, 35, and 35 psi, respectively. A selected ion list for the transformation products was developed for fragmentation with a collision energy range of 15-45 eV, based products characterized previously [17], as well as other ions detected but that had not been assigned a structure. The m/z values in the list were ([M+H]⁺ (retention time, min)): 139.0866 (2.51), 165.0659 (2.84), 169.0608 (6.23), 181.0972(2.14), 185.0652 (4.66), 193.0607 (2.52), 228.0768 (2.86), 243.0877 (1.87), 243.1241 (13.66), 245.1300 (4.13), 247.0425 (1.52), 259.1190 (9.02), 267.0975 (1.63), 269.1397 (5.09), 273.1347 (13.74), 274.0715 (11.24), 275.1503 (3.48), 285.1347 (10.61), 299.1179 (7.45), 301.1289 (12.3), 313.1289 (3.21), 325.1659 (12.07), 327.1452 (3.81), 431.1569 (1.32) (a shorter version of this list with only characterized products is shown in Table 6.1). The ion
list m/z values had a tolerance of a 10 ppm mass bias, and peak areas from the mass chromatogram were integrated. Analyst TF 1.6 with PeakView 1.2.0.3 and Multiquant version 2.1 were used to develop the ion list and to calculate ion intensity.

Other analyses, including pH, volatile suspended solids content in sludge, were performed according to standard methods [59].
Table 6.1 Selected parent/daughter ion list for semi-quantitative LC-MS determination of soluble products formed during anaerobic MENA soil (bio)transformation. Daughter ions shown indicate they were used to determine the abundance of the products. A total tolerance of 10 ppm for the mass bias was allowed for peak integration of the measured [M+H]$^+$ ions.

<table>
<thead>
<tr>
<th>Chemical compound (identifier); Molecular formula;</th>
<th>CAS #</th>
<th>Measured [M+H]$^+$</th>
<th>Retention time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4-diaminoanisole (DAAN); C$<em>7$H$</em>{10}$N$_2$O</td>
<td>615-05-4</td>
<td>139.0866</td>
<td>2.51</td>
</tr>
<tr>
<td>2-methoxy-5-nitroaniline (MENA); C$_7$H$_8$N$_2$O$_3$</td>
<td>99-59-2</td>
<td>169.0608</td>
<td>6.23</td>
</tr>
<tr>
<td>3-nitro-4-methoxyaniline (iMENA); C$_7$H$_8$N$_2$O$_3$</td>
<td>577-72-0</td>
<td>169.0608</td>
<td>2.99</td>
</tr>
<tr>
<td>N-(5-amino-2-methoxyphenyl) acetamide (Ac-DAAN); C$<em>6$H$</em>{12}$N$_2$O$_2$</td>
<td>64353-88-4</td>
<td>181.0972</td>
<td>2.14</td>
</tr>
<tr>
<td>3-amino-3’-nitro-azobenzene (m/z 243); C$<em>{12}$H$</em>{10}$N$_4$O$_2$</td>
<td>61390-99-6</td>
<td>243.0877</td>
<td>1.87</td>
</tr>
<tr>
<td>4’methoxy-3-methylamino-3’methyleneamino-azobenzene (m/z 269); C$<em>{15}$H$</em>{16}$N$_4$O</td>
<td>N/A</td>
<td>269.1397</td>
<td>5.09</td>
</tr>
<tr>
<td>3,3’-diamino-4,4’dimethoxy-azobenzene (m/z 273); C$<em>{14}$H$</em>{16}$N$_4$O$_2$</td>
<td>N/A</td>
<td>273.1347</td>
<td>13.75</td>
</tr>
<tr>
<td>3,3’-Diamino-4,4’dimethoxy-hydrazobenzene (m/z 275); C$<em>{14}$H$</em>{18}$N$_4$O$_2$</td>
<td>N/A</td>
<td>275.1503</td>
<td>3.48</td>
</tr>
<tr>
<td>5-((3-Amino-4-methoxyphenyl)diazenyl)-2-methoxy-N-methyleneaniline (m/z 285); C$<em>{15}$H$</em>{16}$N$_4$O$_2$</td>
<td>N/A</td>
<td>285.1347</td>
<td>10.61</td>
</tr>
</tbody>
</table>
**RESULTS AND DISCUSSION**

**Monomeric biotransformation products as pure compounds**

Most monomeric metabolites from DNAN reduction had decreased microbial toxicity with the exception of the MENA isomers (Table 6.2, Figure 6.3). DAAN and especially Ac-DAN were many fold less toxic than DNAN, whereas iMENA and MENA were more toxic than DNAN in the case of methanogens and Microtox, respectively. Nitroaniline isomers from nitroreduction of 2,4-dinitrotoluene have been shown to be more toxic than the parent compound in *A. fischeri* [35]. In addition, nitroanilines were also the most toxic compounds in an extensive evaluation of the methanogenic toxicity of 24 different amino- and nitro-substituted benzenes [34]. During the course of (bio)transformation, regiospecificity in DNAN (bio)conversion could impact the overall toxicity. For instance, nitroreduction of DNAN has predominantly been reported in the *ortho* nitro group in biological systems[16, 17, 52], whereas evidence of *para*-NO₂ reduction has been found primarily in abiotic systems[134], with one account of biological production[133]. Dominance of nitroreduction in the *ortho* group has been attributed to overall molecular stability due to H-bonding with the neighboring methoxy group[52]. Given these considerations, the toxicity of the nitroaniline isomers formed from DNAN nitroreduction is expected to be driven by MENA.

On the other hand, there are other reactions during (bio)transformation that could yield to less toxic products. Complete nitroreduction to aromatic amines has been considered as a detoxification mechanism[43], and aromatic amines were generally less cytotoxic than nitroaromatics to methanogens[34, 37] and *A. fischeri* [37, 110]. In a previous study we reported DAAN as less toxic than DNAN or MENA in both microbial assays (Figure 6.3)[37]. In addition, other transformation pathways could further decrease the inhibition.
potential of aromatic amines. For instance, DAAN N-acylation resulted in a considerable decrease in toxicity (Figure 6.2A1, 2B1 and Figure 6.3). Ac-DAAN was the least toxic of all monomers, and did not cause inhibition up to 8 mM to acetoclastic methanogens and had a 50% inhibition concentration (IC$_{50}$) of 911 $\mu$M in Microtox. It had a much lower inhibition than its non-acetylated chemical analog, DAAN. N-acetylation has been reported as a detoxification mechanism for amine moieties in aromatic compounds[56, 128].

Figure 6.2  Inhibition of methanogenic activity (Row A) and $A. fischeri$ bioluminescence (Row B) based on toxicant concentration. Column 1: iMENA (■), Ac-DAAN(▲), dimer L (●). Column 2: BBY (▼).
Surrogates of dimers and trimers

The azo-oligomers tested, dimer L and BBY, were among the compounds causing the strongest toxicity in this work. Dimer L was a potent toxicant to *A. fischeri* (IC$_{50}$ = 29.8 µM) and methanogens (IC$_{50}$ = 65 µM). While there is limited information on toxicity of oligomers formed during the biotransformation of explosives, a study on the cytotoxicity of TNT and its degradation products to H4IIE cells and Chinese hamster ovary-K1 (CHO) cells found that the azoxy-dimers were as toxic as the parent compound[55]. BBY was found to be the most toxic of all compounds tested with an IC$_{50}$ in each of the microbial systems of 0.7 µM. Even though both systems had the same IC$_{50}$, *A. fischeri* were more sensitive than methanogens, since complete inhibition occurred at 1.4 µM (Figure 6.2 Panel A2).

The IC$_{50}$ values reported in this work are based on the molarity of the chemical species. However, a fair comparison of toxicity in the context of DNAN (bio)transformation would consider the micromolar monomer equivalents (2 for dimer L, 3 for BBY). Based on that approach the IC values for dimer L would be less toxic than DNAN (open symbols, Figure 6.3). However, for BBY, even when the IC$_{50}$ values are multiplied by three to adjust for monomer equivalent concentration units, the compound would be the most toxic tested in this work, and still much more toxic than DNAN (Figure 6.3).

Despite the severe inhibition posed by the oligomers, at very low concentrations, dimer L stimulated the methanogenic activity rate. Up to 188% of the test compound-free control activity at 4 µM of the dimer (Figure 6.2 Panel A1). A similar phenomenon was detected for BBY but at a lower concentration range (125% stimulation at 1.23 µM of trimer). Azo-oligomers are highly conjugated molecules that are reminiscent of phenazines, which are electron transport molecules in the respiratory chain of methanogens[135, 136]. Recently, the application of a phenazine, Neutral Red, was claimed in an Australian patent application to increase the rate of methanogenesis[137].
Figure 6.3. Fifty-percent inhibition concentrations (IC$_{50}$) for acetoclastic methanogens (■) and A. fischeri (●) for DNAN and its (bio)transformation products and azo-oligomer surrogates. Shaded area indicates DNAN IC$_{50}$ range. Open symbols in dimer L show adjusted concentrations to monomer equivalents. Ac-DAAN did not cause inhibition to methanogens and the highest concentration tested (8000 µM) is shown instead with an asterisk above the symbol. The BBY IC$_{50}$ for both models was 0.7 µM.
Table 6.2 Summary of inhibitory concentrations for DNAN, (bio)transformation products and best available surrogates to acetoclastic methanogens and \textit{A. fischeri} (Microtox)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Methanogens</th>
<th>Microtox</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC\textsubscript{20}</td>
<td>IC\textsubscript{50}</td>
<td>IC\textsubscript{80}</td>
</tr>
<tr>
<td>DNAN</td>
<td>16</td>
<td>41</td>
<td>70</td>
</tr>
<tr>
<td>MENA</td>
<td>121</td>
<td>175</td>
<td>275</td>
</tr>
<tr>
<td>DAAN</td>
<td>72</td>
<td>176</td>
<td>327</td>
</tr>
<tr>
<td>iMENA</td>
<td>8</td>
<td>25</td>
<td>43.4</td>
</tr>
<tr>
<td>Ac-DAAN</td>
<td>&gt;8000\textsuperscript{c}</td>
<td>&gt;8000\textsuperscript{c}</td>
<td>&gt;8000\textsuperscript{c}</td>
</tr>
<tr>
<td>dimer L</td>
<td>22</td>
<td>65</td>
<td>76</td>
</tr>
<tr>
<td>BBY</td>
<td>0.4</td>
<td>0.71</td>
<td>1.2</td>
</tr>
</tbody>
</table>

\textsuperscript{a}302 \mu M DAAN caused 75\% inhibition to \textit{A. fischeri}.

\textsuperscript{b}446 \mu M iMENA caused 62\% inhibition to \textit{A. fischeri}.

\textsuperscript{c}8000 \mu m Ac-DAAN caused 2\% inhibition to acetoclastic methanogens.

Exposures to DNAN (bio)transformation mixtures

\textit{Biotransformation product profiles}

An overview of (bio)transformation products and transformation pathways observed are shown in Figure 6.1 Panel A. DNAN underwent nitroreduction primarily to MENA, and subsequent nitroreduction led to DAAN. A putative nitroso intermediate coupled with DAAN to form azo-dimer \(m/z\) 273. In some cases, \(m/z\) 273 underwent reductive azo-cleavage to DAAN via a hydrazo intermediate, \(m/z\) 275. In all systems, this dimer underwent N-substitution to yield \(m/z\) 285 and O-demethoxylation, forming \(m/z\) 269.

DNAN was (bio)transformed by microbial and abiotic processes in soil and sludge. DNAN was readily reduced within 10 days in the anaerobic sludge and in the Camp Navajo
soil, while for the Camp Butner, DNAN was completely removed by 30 days (Figure 6.4A).
The specific rate of reduction per unit dwt solids was highest for the sludge and Camp Navajo soil (45.6 and 56.7 μmol DNAN d⁻¹ dwt g⁻¹), while Camp Butner had the slowest (14.3 μmol DNAN d⁻¹ g dwt soil⁻¹).

During the course of DNAN (bio)transformation, very limited amounts of MENA and DAAN were detected in any of the assay system as evidenced by lack of noteworthy accumulation of these intermediates using the HPLC-DAD (Figure D-3), suggesting that additional transformation products had been formed aside from the primary aromatic amine products. Completing mass balances on DNAN (bio)transformation has been problematic relying in HPLC-DAD only[17]. While additional products can be detected and identified by mass spectrometry techniques, not all of the compounds identified can be quantified[16-18].

The individual profile of (bio)transformation product mixtures formed at different stages of DNAN (bio)transformation for the two soils, Camp Butner (Figure 6.4A) and Navajo (Figure 6.5A), as well as for the anaerobic sludge (Figure 6.6A), were determined using LC-QToF-MS to detect products that were below detection in HPLC-DAD. Overall, the rate of DNAN conversion affected the rate of production of monomer and oligomer products. MENA and DAAN were detected primarily between 10-30d of incubation in Camp Navajo soil, but were detected only sporadically in the Camp Butner soil. Unlike the soils, in the anaerobic sludge a consistent amount of DAAN was detected throughout 0-50d of incubation, accounting for slightly less than a third of the total product peak area (3×10⁷ area units for DAAN). A higher amount of DAAN in the system might require more reducing conditions. For instance, 2,4,6-triaminotoluene from TNT reduction has only been reported below -200 mV[32]. In the systems studied in this work, hydrazo compounds had a higher occurrence in the sludge (m/z 275 in Figure 6.1 and 6.6), and as an intermediate formed during the reductive cleavage of azo-dimers, its presence points to a source of DAAN.
Other monomer products, such as iMENA and Ac-DAAN were detected in smaller quantities than MENA or DAAN. For iMENA, the highest amount detected was $2 \times 10^6$ area units in Camp Butner soil at 20d of (bio)transformation, but overall MENA was at least twice as much more abundant than its isomer. For Ac-DAAN, the largest amount detected was in sludge ($8 \times 10^5$ area units), but its signal was on average two orders of magnitude smaller than DAAN.

Azo-dimer products were detected concomitantly with the formation of QToF-MS detectable MENA, iMENA, DAAN, and Ac-DAAN in all three systems studied, suggesting that dimerization occurs very rapidly during the period nitrogroups are being reduced. However, at longer incubation times (9-30 days), the major abundant species were comprised of products with ion $m/z > 200$, attributed to azo-dimers. Based on common dominant (bio)transformation products detected across all systems, dimers underwent other reactions, such as N-substitution and O-demethoxylation.

Besides common dominant products, the relative abundance of each product varied depending on the system. For example in Camp Butner soil incubations, the most abundant ions included $m/z$ 269 and 285, 4’methoxy-3-methylamino-3’methyleneamino-azobenzene and 4,4’-dimethoxy-3-methylamino-3’methyleneamino-azobenzene. In Camp Navajo, the primary products were $m/z$ 247 and 274 (putatively with molecular formulas $C_7H_9N_3O_7^-$ and $C_9H_{11}N_3O_7$, respectively), followed by $m/z$ 269 and 285, like in Camp Butner. The ions $m/z$ 247 and 274 have not been characterized, but could be a product formed during incorporation of DNAN reduced (bio)transformation products with humic substances. Covalent binding between quinone-like and reduced TNT products, a chemical analog to DNAN, have been found in $^{15}$N studies with soil humic substances in aerobic conditions[130]. Moreover, sediment-aromatic amine reactions have been detected and kinetically studied[138]. In the anaerobic sludge, the primary dimers detected included $m/z$ 285 and 269, with significant
contributions of $m/z$ 273 and 275, 4,4’dimethoxy-3,3’diamino-azobenzene and 4,4’dimethoxy-3,3’diamino-hydrazobenzene, respectively.

The largest cumulative amount of products in the aqueous phase, as quantified by peak area, occurred in Camp Butner soil and in the anaerobic sludge ($1.2 \times 10^6$ area units). On the other hand, Camp Navajo soil, had two orders of magnitude less products at longer incubation times (40-50d). A higher abundance in Camp Navajo was also observed with compounds with the highest molecular weight in the list, such as $m/z$ 325 and 327, which were detected in all systems more often after DNAN was depleted (Figures D-4 through D-6). Their concentration was in the range of $\sim 10^5$ area units for Camp Butner and sludge, but $\sim 10^4$ for Camp Navajo. The metabolite $m/z$ 431, a potential trimer based on the molecular mass, was detected only in Camp Navajo soil at a 2.5-fold higher amount than $m/z$ 325 and 327 (Figure D-5). Camp Navajo has more organic carbon than Camp Butner (5.24 versus 2.07 % dwt soil)[82], and the difference in amount of products recovered may be due to irreversible adsorption and covalent incorporation into soil humus. In studies with $^{14}$C radiolabeled TNT incubated with soil, more than half of the label was incorporated into different soil humic fractions[29].

Toxicity profile during (bio)transformation

Acetoclastic methanogenic and $A. fischeri$ bioluminescent inhibition assays were used to assess the overall toxicity of the aqueous extracts of DNAN (bio)transformation product mixtures sampled from the biotransformation assays at different times of anaerobic incubation. Both microbial toxicity models were very susceptible to toxicity of the mixture of (bio)transformation. The samples mixtures had to be diluted significantly (1:54 for methanogens and 1:36 for $A. fischeri$) before exposure, since at lower dilutions, there was
complete inhibition in both assays which did not allow to observe differences in inhibition potential across the (bio)transformation timeline.

At early stages of DNAN (bio)conversion, methanogenic activity dropped sharply as the parent compound disappeared, across all systems assayed (Figures 6.4B-6.6B). This period of increased inhibition lasted as long as DNAN was detectable in aqueous solutions. This period of enhanced toxicity was directly dependent on the rate of DNAN (bio)transformation. The dip in activity was the shortest in anaerobic sludge (1d) (Figure 6.6B), while for Camp Butner (with the slowest rate of DNAN (bio)conversion), lasted for 30 days (Figure 6.4B).

The increase in methanogen inhibition could be attributed to putative reactive intermediates formed during nitroreduction of DNAN, such as nitroso- and hydroxylamino-derivatives. Nitroso and hydroxylamino products have been identified as potentially responsible for methanogen cell lysis (and methanogenic inhibition) during nitroreduction [43]. Cytotoxicity studies on TNT and its reduced (bio)transformation products, showed that 4-hydroxylamino, 2,6-dinitrotoluene could be as toxic or more toxic than TNT [55]. DNAN nitroso- derivatives have been found in abiotic [52] and biotic [24], as well as for other nitroaromatic compounds such as 2,4-dinitrotoluene [45]. Hydroxylamino products have also been reported in DNAN [16, 24] and TNT [139] reductive (bio)transformation studies. Furthermore, azo-dimers detected in this work could be indirect evidence of the occurrence of these intermediates since classic azo formation involves coupling of nitroso-bearing products with either hydroxylamino [25] or amino derivatives [27, 97].

At longer incubation times (30-50d), the diluted mixtures of metabolites present at this stage of (bio)transformation caused very low inhibition to methanogens. Compared to earlier incubation times, when the toxicity was maximum, there was clearly a decrease in
toxicity associated with the formation of azo-dimers. This recovery of activity might be attributed to the disappearance of a steady supply of reactive intermediates from nitroreduction once DNAN is depleted from the system. An additional factor could be the stimulation of methanogenic activity by azo-dimers, such as the effect observed with dimer L exposure up to 17 μM to methanogens in this work. Since the mixtures were diluted 54-fold, this would imply a maximum azo-dimer concentration of 4.6 μM (or 9.2 μM monomer equivalents). The theoretical maximum azo-dimer concentration would be in the stimulatory region. In all cases, after the initial inhibition, there was a consistent recovery and detoxification compared to the initial portion of (bio)transformation, eventually achieving restoration 100% activity compared to the test-compound free controls.

For *A. fischeri*, there was little inhibition during initial conversion DNAN in all systems, but inhibition started to increase when more than half of the DNAN had been converted (Figures 6.3B-6.4B). Unlike methanogens, *A. fischeri* might not be affected by the reactive nitroso and hydroxylamino intermediates if coping mechanisms against radicals and N-reactive species are present. Recently, flamohaemoglobin Hmp in *A. fischeri* has been attributed to protect against NO and reactive oxygen produced by the host squid as an antimicrobial agent during colonization in *A. fischeri*-squid symbiosis[140]. However, at the latter part of DNAN reduction and after DNAN was completely (bio)transformed, there was increased inhibition of bioluminescence. During these stages of (bio)transformation, there were also significant detection of dimer products (*m/z* >200), which could be directly responsible for affecting bioluminescence due to inhibition in growth. In Camp Navajo, the highest inhibition (as evidenced by the lowest normalized activity) occurred at 50 days (corresponding to 88% inhibition) compared to the toxicant-free control. However, in Camp Butner soil and in the anaerobic sludge, the maximum inhibition was 50%. In the anaerobic sludge there was a temporary decrease in inhibition from 20-40d which occurred at the same
time as the amount of detected dimers decreased, and by 50d the inhibition increased again concomitantly with the dimers products. Based on the 1:36 dilution used in Microtox, the maximum theoretical concentration of azo-dimers would be 6.9 μM (or 13.8 μM monomer equivalents), which would be in the inhibitory range based on surrogate L dimer toxicity test.

**Implications of microbial toxicity and (bio)transformation product profiles**

A workflow integrating transformation product semi-quantitation with microbial toxicity provided information about scenarios expected in soils and wastewater sludge systems polluted with DNAN. Two key events during (bio)transformation were identified as potential toxicity drivers. Methanogenic increased inhibition during nitroreduction of DNAN was attributed to reactive hydroxylamino and nitroso intermediates, while azo-dimers rich mixtures were severely toxic to *A. fischeri*. On the other hand, N-acetylation greatly reduced toxicity of DNAN. N-acetylation and azo-dimerization of amines are transformation pathways that have been reported to be in direct competition with each other[56]. Both of these processes have been identified in microbial systems in presence of DNAN[16, 17], although azo-dimerization seems to be the dominant route based on semi-quantitative analysis of this work. However, devising strategies to increase N-acetylation reactions could be exploited in bioremediation efforts to yield non-toxicant products in systems polluted with DNAN.
CHAPTER 7

ZEBRAFISH EMBRYO TOXICITY OF ANAEROBIC BIOTRANSFORMATION PRODUCTS FROM THE INSENSITIVE MUNITION COMPOUND 2,4-DINITROANISOLE (DNAN)

ABSTRACT: 2,4-dinitroanisole (DNAN) is an emerging insensitive munitions compound that readily undergoes anaerobic nitro-group reduction to 2-methoxy-5-nitroaniline (MENA) and 2,4-diaminoanisole (DAAN), followed by subsequent formation of unique azo-dimers. Currently there is scarce knowledge on the ecotoxicity of DNAN (bio)transformation products. In this work, mortality, development, and behavioral effects of DNAN (bio)transformation products were assessed using zebrafish (Danio rerio) embryos. We tested individual products, MENA and DAAN, as well as dimer and trimer surrogates. As pure compounds, 3-nitro-4-methoxyaniline and 2,2’-dimethoxy-4,4’-azodianiline caused significant effects with lowest observable effect levels (LOEL) at 6.4 µM on one or two developmental endpoints, respectively. The latter had six-additional significant developmental endpoints with LOELs of 64 µM. Based on light/dark swimming behavioral tests, DAAN (640 µM) caused reduction in swimming, suggestive of neurotoxicity. No significant mortality occurred (≤ 64 µM) for any of the individual compounds. However, metabolite mixtures formed during different stages of MENA (bio)transformation in soil were characterized using high-resolution mass spectrometry in parallel with zebrafish embryo toxicity assays, which demonstrated significant mortality during the onset of azo-dimer formation. Overall the results indicate that several DNAN (bio)transformation products cause different types of toxicity to zebrafish embryos.
INTRODUCTION

The nitroaromatic compound, 2,4-dinitroanisole (DNAN), is an emerging insensitive munitions compound that has gained the interest of defense industries due to its shock tolerance.[5] As the use of insensitive munitions formulations containing DNAN becomes widespread, discharges to natural environments may threaten diverse organisms vital to ecosystem function. Once released to the environment, nitro groups of DNAN can readily undergo reductive transformation to amino groups due to biological activity of microorganisms[16-18] or abiotic processes.[20]

Rapid (bio)transformation of DNAN to 2-methoxy-5-nitroaniline (MENA) and 2,4-diaminoanisole (DAAN) has been reported in anaerobic environments.[17, 18] Reactive species formed during biotransformation can undergo coupling to form azo dimers (Figure 7.1).[16-18] MENA and DAAN are more hydrophilic than DNAN[52], thus both metabolites might be more mobile in surface and groundwater impacted by DNAN contamination compared to the parent compound. Since DNAN (bio)transformation occurs readily in anaerobic environments, the environmental impact of DNAN pollution could be largely due to its transformation products.

There is a lack of information regarding the ecotoxicity of the products formed during the anaerobic (bio)transformation of DNAN. However, (bio)transformation is expected to alter the toxicity effects of DNAN, as confirmed by early evidence that reductive (bio)transformation during DNAN toxicity assays decreased inhibition towards methanogenic archaea.[37] Similarly, chemical reduction of DNAN with zero-valent iron caused a decrease in the zebrafish (Danio rerio) 48-h mortality.[141] DNAN transformation has been reported in ecotoxicity studies[6, 39, 142, 143], which may complicate toxicological assessment since the observed toxicity effects might be due to either DNAN and/or its transformation products. A few recent studies have characterized the toxic impacts of DNAN to diverse groups of
microorganisms[6, 37], earthworms and plants.[6, 142] Two recent studies have evaluated the
*in vivo* toxicity of DNAN to aquatic vertebrates.[39, 141] However, information on the
developmental and chronic toxic effects associated with DNAN biotransformation products is
lacking.

In the ecotoxicology field, zebrafish embryos have become widely used to monitor
water quality and to aid in environmentally safe product development.[144, 145] The
embryonic zebrafish model is amenable for high-throughput studies and allows evaluation of
developmental and behavioral endpoints in addition to acute toxicity.[146] Early
development is very similar to higher order vertebrates and its transparency allows for non-
invasive and specific developmental assessment endpoints.[147] Moreover, a vast amount of
transcriptomic information along an expanding library of toxicants tested can provide
mechanistic insights of the toxicity effects in zebrafish.[145, 148-150]

Using zebrafish as a toxicology model to test the toxicity of DNAN
(bio)transformation products, the objective of this work was to evaluate developmental
effects of these products (or best commercially-available surrogates). We also tested mixtures
of the products formed at different stages of (bio)transformation during anaerobic incubations
of soils using as a starting point the primary DNAN transformation product, MENA.
Toxicological testing was supported by detailed mass spectrometry studies (using ultra-high
performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry,
UHPLC-Q-ToF-MS) to characterize transformation product mixture profiles.
MATERIALS AND METHODS

Zebrfish embryo assays

A previously reported protocol[149] was used with slight modifications. Embryos with intact chorions were manually placed in 96-well plates containing embryo medium (EM) at 6 hours post fertilization (hpf) and exposed to individual toxicants (10 μL + 90 μL EM) or supernatant solutions of different stages of MENA (bio)transformation (50 μL + 50 μL EM). 32 embryos were exposed per concentration and toxicant, and all controls and treatments included 0.64% dimethylsulfoxide (DMSO) to aid toxicant dissolution. The plates were incubated at 28°C in the dark. At 24 and 120 hpf, development abnormality and mortality assessments were performed (endpoints shown in Table 7.1).[149] Endpoint scoring and statistical analyses were performed in R software as described previously.[149]

In addition, at 120 hpf a larval locomotor behavior assay was performed with alternating light/dark cycles in a Viewpoint Zebrabox (software version 3.0, Life Sciences, Lyon, France).[151] Briefly, at 120 hpf, plates were inserted into the Viewpoint Zebrabox where movement was recorded during the following light/dark cycle: initial light acclimation (10 min), light (10 min), dark (5 min). Long swimming distance (> 2.5 mm min⁻¹) was averaged across all surviving replicates at 120 hpf for a single concentration. Malformed and dead zebrafish were not considered in the locomotor assay. Endpoint scoring and statistical analyses were performed according to the protocols described.[149, 151]
Table 7.1. Zebrafish embryo toxicity endpoints assessed.

<table>
<thead>
<tr>
<th>Time</th>
<th>Endpoints</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 hpf</td>
<td>mortality, developmental delay, spontaneous movement, notochord</td>
</tr>
<tr>
<td>120 hpf</td>
<td>mortality, notochord, yolk sac edema, body axis, eye defect, snout, jaw, otic vesicle, pericardial edema, brain somite, pectoral fin, caudal fin, pigment, circulation, truncated body, touch response</td>
</tr>
</tbody>
</table>

**Individual chemicals tested**

The library of compounds tested included DNAN monomer (bio)transformation products[16-18] and best available surrogates for azo dimers and trimers, since no dimer metabolites were commercially available. 2-methoxy-5-nitroaniline (MENA, CAS# 99-59-2, 98%) and 2-4 diaminoanisole (DAAN, CAS# 615-05-4, analytical standard) were obtained from Sigma-Aldrich (St. Louis, MO, USA). 3-nitro-4-methoxyaniline (iMENA, CAS# 577-72-0, purity 97%) was obtained from Accela ChemBio Inc. (San Diego, CA, USA). N-(5-amino-2-methoxyphenyl) acetamide (Ac-DAAN, CAS# 64353-88-4, purity 95%) was acquired from ChemBridge Corporation (San Diego, CA, USA). 4,4’-azodianiline (denoted “dimer A”, CAS# 538-41-0, purity 95%) was obtained from Alfa Aesar (Ward Hill, MA, USA). 2,2′-dimethoxy-4,4′-azodianiline (denoted “dimer L”, CAS# 6364-31-4, purity >90%) was obtained from MolMall Sarl (Lonay, Switzerland). Bismarck Brown Y (m-Bis(2,4-diaminophenylazo)-benzene, denoted “BBY”, CAS# 8005-77-4, 46%) was purchased from Chem-Impex International (Wood Dale, IL, USA). Figure 7.1 shows chemical structures of these compounds. Solutions were prepared the same day the exposure began and diluted to contain 0.64% DMSO across all wells including controls. This concentration of DMSO is
known to not cause effects in zebrafish embryo assays. Zebrafish embryos were exposed to 0-64 μM concentrations of the test compounds, with the exception of MENA and DAAN which had a range 0-640 μM. In all cases the concentrations tested were chosen to span five orders of magnitude. DNAN was not tested in this work due to the commercialization, transportation, and export control restrictions established by the International Traffic in Arms Regulations (ITAR) of the US Department of State[152].
Figure 7.1. Main anaerobic DNAN (bio)transformation pathway (structures inside box): DNAN undergoes nitroreduction to MENA and DAAN.[17] Reactive intermediates formed during nitro-group reduction enable coupling reactions that form dimers and other oligomers by reacting with aromatic amines [27, 97]. Chemical structures of individual compounds tested based on previously identified DNAN (bio)transformation products (‡) or best available surrogates (§).
Sampling & metabolite analysis from anaerobic (bio)transformation of MENA

Anaerobic bioassays were performed to obtain mixtures of the metabolites formed during different stages of the (bio)transformation of MENA in soil. Anaerobic tubes (Bellco Glass Inc., Vineland, NJ, USA) were filled with 10 mL of mineral medium (described in [17]) containing 18 mM phosphate buffer (pH = 7.2) and 500 μM MENA. The solution was inoculated with 100 mg of fresh Camp Navajo soil (water content 9.3%), previously characterized[82] and amended with 10 mM pyruvate as an exogenous electron donor. The tubes were flushed with He/CO₂ (80/20 %), closed with t-butyl caps and aluminum seals, and subsequently incubated in the dark at 30°C in an orbital shaker at 115 rpm. Samples from the different tubes were all collected on the same day to facilitate simultaneous toxicity testing of all samples and minimize test variability. The tubes were incubated according to a staggered timeline to ensure that samples incubated for 0, 1, 6, 9, 20, and 30 days were available on the sample collection day.

At the end of the incubation period, tubes were opened in an anaerobic hood to avoid autoxidation of unstable products, and the liquid phase was centrifuged (10 min, 9,600×g). Aliquots of the supernatant were collected for immediate analysis by UHPLC-Q-ToF-MS and for toxicity testing. The latter samples were sealed under N₂ gas and then frozen (-20°C) for 2 weeks until the zebrafish embryo toxicity assays were performed.

UHPLC-Q-ToF-MS and UV-VIS analyses

Liquid samples from soil (bio)transformation experiments were analyzed on an UltiMate 3000 UHPLC (Dionex, Sunnyvale, CA) coupled to a TripleTOF® 5600 quadrupole Q-ToF-MS (AB Sciex, Framingham, MA). An Acclaim RSLC Explosives E2 column (2.1 x 100 mm, 2.2 μm) (Thermo Fisher Scientific, Waltham, WA, USA) was used for
chromatographic separation with an isocratic mobile phase consisting of methanol/H₂O (40/60, v/v, 0.25 mL min⁻¹, 15 min) at room temperature. MENA concentration was quantified using UHPLC coupled to a diode-array detector (DAD) at 254 nm (retention time 5 min) before measurement by UHPLC-Q-ToF-MS. The Q-ToF-MS utilized an electrospray ionization (ESI) source operated in positive mode at 450°C with a capillary setting of 5.5 kV, a declustering potential of 80 V, and curtain gas, desolvation gas, and nebulizer gas levels at 30, 35, and 35 psi, respectively, with N₂. A parent compound list was created for fragmentation with a collision energy range of 15-45 eV, based on previously resolved transformation products from DNAN (bio)transformation[17], as well as other detected compounds that had not been assigned a structure. Analyst TF 1.6 with PeakView 1.2.0.3 and Multiquant version 2.1 software were used to develop the parent list as well as integrate peak areas of each analyte detected.

UV-VIS spectra of the supernatant from MENA samples incubated for 1, 6, 9, 20, and 30 days were recorded (200-600 nm) in quartz cuvettes with a UV-1800 Shimadzu spectrophotometer (Columbia, MD, USA). Samples were diluted with a 50 mM phosphate buffer (pH = 7).

RESULTS AND DISCUSSION

Toxicity of model degradation products

Mortality

Freshwater fish species are reported to be as sensitive or more sensitive to nitroaromatics explosives, such as 2,4,6-trinitrotoluene (TNT), than are invertebrates and
amphibians[38, 153-155]; and the lowest 96-h LC$_{50}$ has been reported for rainbow trout (*Oncorhynchus mykiss*) at 3.5 µM TNT[153]. DNAN has been reported to be less toxic than TNT, with a 96-h LC$_{50}$ for DNAN in larval fathead minnows (*Pimephales promelas*) reported at 187 µM[39], whereas for TNT it was 13.6 µM.[156] The 48-h LC$_{50}$ for DNAN in adult zebrafish has recently been reported as 177 µM.[141]

In our work, no statistically significant mortality at 24 and 120 hpf was detected for any of the pure compounds tested (0-640 µM for MENA and DAAN; 0-64 µM for iMENA, Ac-DAAN, dimer A, dimer L, and BBY). Mortality charts for each compound tested can be found in Appendix E (Figures E-1 through E-3). In this study, which used zebrafish embryos with intact chorions, no adverse lethal effect were detected for MENA concentrations up to 640 µM. However, in a previous study with dechorionated zebrafish embryos on compounds from the U.S. EPA ToxCast phase 1 and 2 lists, which included MENA, the 120-hpf mortality lowest observable effect level (LOEL) was reported at 64 µM.[149] Since the chorion is the primary exposure route before hatching[146], this discrepancy suggests that the presence of the chorion could be limiting the diffusion of aromatic amines like MENA. Similar to the monomeric compounds tested in this work, the azo-dimers and trimer (BBY) evaluated did not result in zebrafish mortality at concentrations up to 64 µM. Azo dye dimers and trimers have been tested in aquatic ecotoxicity models, including an extensive dye survey on fathead minnows (*P. promelas*)[157], and more recently on guppy fish (*Poecilia reticulata*)[158], and the Western clawed frog (*Silurana tropicalis*).[159] In contrast with the lack of lethal effects detected in our study, the 96-h LC$_{50}$ value reported in guppy fish for Methyl Red (CAS# 493-52-7), an azo-dimer with N-methyl, amino, and carboxylic acid substituents, was 89 µM[158], and for Basic Brown 4 (CAS# 5421-66-9), an azo trimer with amino and methyl substituents, in fathead minnows it was 12.1 µM.
Overall, the low mortality counts obtained with the pure compounds tested suggest that the individual transformation products/surrogates evaluated in this study pose little acute toxicity risk ($\leq 64 \, \mu$M). This argument is strengthened by the observed decrease in toxicity of TNT on fathead minnows, as TNT was reduced to dinitroaniline products in the assay[153]. While mortality effects were not detected in the concentration ranges tested in this work, other toxicity effects may occur at sublethal concentrations, such as with reproductive[153, 154] and developmental[160] endpoints.
Table 7.2. Developmental Lowest Observable Effect Levels (LOELs) and endpoints with significant morbidity.

<table>
<thead>
<tr>
<th>Chemical compound (identifier)</th>
<th>Surrogate for</th>
<th>Developmental LOEL (μM)</th>
<th>Active Endpoints (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-methoxy-5-nitroaniline (MENA)</td>
<td>N/A</td>
<td>&gt;640</td>
<td>N.D.</td>
</tr>
<tr>
<td>3-nitro-4methoxyaniline (iMENA)</td>
<td>N/A</td>
<td>6.4</td>
<td>yolk sac edema (6.4)</td>
</tr>
<tr>
<td>2,4-diaminoanisole (DAAN)</td>
<td>N/A</td>
<td>&gt;640</td>
<td>N.D.</td>
</tr>
<tr>
<td>N-(5-amino-2-methoxyphenyl) acetamide (Ac-DAAN)</td>
<td>N/A</td>
<td>&gt;64</td>
<td>N.D.</td>
</tr>
<tr>
<td>4,4’-azodianiline (dimer A)</td>
<td>azo dimer</td>
<td>&gt;64</td>
<td>N.D.</td>
</tr>
<tr>
<td>2,2’-dimethoxy-4,4’-azodianiline (dimer L)</td>
<td>azo dimer</td>
<td>6.4</td>
<td>yolk sac and pericardial edemas (6.4); axis, eye, snout, jaw, pectoral fin, touch response (64)</td>
</tr>
<tr>
<td>Bismarck Brown Y (BBY)</td>
<td>azo trimer</td>
<td>&gt;64</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

N/A = Not applicable; N.D. = None detected
Developmental toxicity

Detailed developmental endpoint scoring for all the chemicals tested in this work is shown in the Supplementary Information section, in Figures E-4 through E-10. While most of the compounds tested did not cause developmental abnormalities, dimer L and iMENA caused significant malformations in zebrafish embryos (Table 7.2). iMENA had a developmental LOEL of 6.4 μM based on yolk sac edema formation (Figure E-5), but no other abnormalities were detected. The rest of monomer compounds did not show developmental activity. Developmental toxicity studies with the monomeric compounds tested here have not been previously reported. However, some aromatic amines, such as aniline have been shown to cause developmental toxicity in African clawed frog (*Xenopus laevis*) embryos, albeit at much higher concentrations, 3.9 mM.[161] Of the dimers and trimer tested, only dimer L caused significant developmental abnormalities in the embryos (Figure E-9). Dimer L caused a significant occurrence of yolk sac and pericardial edemas (LOEL = 6.4 μM), and several malformations in six other endpoints at 64 μM (Figure 7.2). A photograph illustrating some of these abnormalities is shown in Figure 7.3. While BBY and dimer A did not show any developmental toxicity in the tested range, in other aquatic species, BBY has been reported to cause malformations in Western clawed frogs at 2.4 mM.[159].
Figure 7.2. Developmental endpoints that caused significant malformations in the zebrafish embryo assay for dimer L. White bars indicate hits above the statistically significant threshold (p ≤ 0.05). The rest of the developmental endpoints did not show significant activity.
Figure 7.3. Representative visual comparison between 120 hpf zebrafish embryos: Control (A), 64 μM dimer L (B). The embryo exposed to dimer L showed developmental abnormalities and visible dimer L uptake (coloration).

**Locomotor behavior assay**

The impact of the various model compounds on zebrafish swimming locomotor behavior was also tested at 120 hpf. Zebrafish have been reported to be more static in presence of light compared to dark periods, and comparing the difference in light/dark cycles can indicate changes in behavior due to exposure to toxicants during development.[151] In this study, long distance swimming motion ranged from 18 to 30 mm in the dark, while it was below 5 mm in light (Figure 7.4). There were no detectable effects in locomotion for any of the chemicals tested, except for DAAN. While there were some minor variations in the distance swum in the dark periods when considering most compounds, the average movement in zebrafish that had been exposed to 640 μM DAAN (4 mm), was eight-fold less than the toxicant–free control (32 mm). In essence 640 μM DAAN caused the motion in the dark period to be reduced to the level normally observed by the embryos in the light periods. This suggests that 640 μM DAAN impaired response to light/dark cycles. Since there were no developmental active endpoints for DAAN (Figure E-6), the lack of response to light/dark periods is not associated with swimming impairment due to malformations. Therefore, this suggests that exposure to 640 μM DAAN could have neurotoxicity effects. To the best of our knowledge, this is the first work to study potential locomotor effects caused by a
nitroaromatic, an aromatic amine, and an azo-dimer exposure in zebrafish during the developmental stage.

Figure 7.4. Average long distance swum recorded in larval locomotor response Viewpoint assay for 120 hpf zebrafish larvae exposed to DAAN (0-640 μM) in dark (Panel A) and light (Panel B) stages.
Toxicity evaluation of MENA (bio)transformation products in anaerobic soil microcosms

Product characterization and semi-quantitation

Based on UHPLC-Q-ToF-MS analyses, six parent ions were selected for semi-quantitation according to their chromatogram peak area abundance (> 1000) (Table 7.3). From these compounds, three had been reported previously as monomer products formed during DNAN (bio)transformation in anaerobic conditions: DAAN and Ac-DAAN.[17] In this work, we report a new transformation product, 3-amino-3’-nitro-azobenzene (m/z 243, Figure E-11), as well as two parent ions that have not been assigned chemical structures [M+H]+ m/z 313.1343 (putative molecular formula C_{16}H_{16}N_{4}O_{3}, Figure E-12) and 393.0836 (putative molecular formula C_{19}H_{12}N_{4}O_{6}, Figure E-13). Based on these formulae, these ions could represent a dimer and a trimer, respectively. Transformation products were quantified using parent ions (m/z [M+H]+), or daughter ion (m/z [M+H-R]+) transitions when the daughter yielded a stronger ionization signal than the precursor (Table 7.3). While standards were available for DAAN and Ac-DAAN, the rest of the analytes were not commercially available, difficult to synthesize, and potentially not stable in air. For consistency, all analytes are reported in peak area units based on UHPLC-Q-ToF-MS, which cannot be unequivocally translated to concentration because of potential variation in ionization efficiencies (Figure 7.5A). MENA was not added to the selected ion list since it ionized several orders of magnitude more strongly than the rest of the analytes. Therefore, its concentration during the (bio)transformation is reported in μM as quantified using UHPLC-DAD (Figure 7.5B).

From initial time to six days of (bio)transformation, trace amounts of all of the analytes were detected, but 23% of MENA had already been removed, indicating more transformation products not shown in Table 7.3 were possibly formed. From day 6 to 9 the
formation of oligomers (mainly \( m/z \) 243, 313, 393) was visible, followed by accumulation of DAAN and Ac-DAAN after day 10. The concentration of all of the analytes increased until day 30, with exception of DAAN, which decreased by 40% from days 20-30.

In order to consider transformation products that might be difficult to separate chromatographically or ionize in the Q-ToF-MS, UV-VIS spectra were also recorded along the course of the (bio)transformation of MENA (Figure E-14). Since oligomers were detected in Q-ToF-MS, the spectral data are summarized as an oligomer index, based on the ratio of absorbance at 400 to 254 nm (Figure 7.5B), the former wavelength being chosen to quantify polymers[162] that have visible absorbance (e.g. azo dyes), and the latter a common wavelength for aromaticity. The 400/254 nm ratio increased from days 1-6, reached a maximum at 9 days of incubation, and then decreased until day 30. The initial increase might be due to the onset of oligomer formation as evidenced by Q-ToF-MS data, whereas the subsequent decrease following day 9 indicates precipitation of larger insoluble oligomers out of solution, even if soluble dimers such as \( m/z \) 243 continue being formed.
Table 7.3. Selected parent/daughter ion list for semi-quantitative LC-MS determination of soluble products formed during anaerobic MENA soil (bio)transformation. Daughter ions shown indicate they were used to determine the abundance of the products.

<table>
<thead>
<tr>
<th>Chemical compound or identifier; Molecular formula</th>
<th>CAS #</th>
<th>Calculated Parent [M+H]^+</th>
<th>Measured Parent [M+H]^+</th>
<th>Daughter [M+H-R]^+</th>
<th>Retention time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4-diaminoanisole (DAAN); C&lt;sub&gt;7&lt;/sub&gt;H&lt;sub&gt;10&lt;/sub&gt;N&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>615-05-4</td>
<td>139.0866</td>
<td>139.0866</td>
<td>N/A</td>
<td>2.86</td>
</tr>
<tr>
<td>N-(5-amino-2-methoxyphenyl) acetamide (Ac-DAAN); C&lt;sub&gt;9&lt;/sub&gt;H&lt;sub&gt;12&lt;/sub&gt;N&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>64353-88-4</td>
<td>181.0972</td>
<td>181.0972</td>
<td>N/A</td>
<td>2.36</td>
</tr>
<tr>
<td>3-amino-3’-nitro-azobenzene (m/z 243)^§; C&lt;sub&gt;12&lt;/sub&gt;H&lt;sub&gt;10&lt;/sub&gt;N&lt;sub&gt;4&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>61390-99-6</td>
<td>243.0877</td>
<td>243.0870</td>
<td>N/A</td>
<td>2.3</td>
</tr>
<tr>
<td>(m/z 313)^*§; C&lt;sub&gt;16&lt;/sub&gt;H&lt;sub&gt;16&lt;/sub&gt;N&lt;sub&gt;4&lt;/sub&gt;O&lt;sub&gt;3&lt;/sub&gt;</td>
<td>N/A</td>
<td>313.1295</td>
<td>313.1312</td>
<td>250.8794</td>
<td>1.60</td>
</tr>
<tr>
<td>(m/z 393)^*§; C&lt;sub&gt;19&lt;/sub&gt;H&lt;sub&gt;12&lt;/sub&gt;N&lt;sub&gt;4&lt;/sub&gt;O&lt;sub&gt;6&lt;/sub&gt;</td>
<td>N/A</td>
<td>393.0830</td>
<td>393.0836</td>
<td>276.0833</td>
<td>1.59</td>
</tr>
</tbody>
</table>

N/A = Not applicable.

* These ions detected have not been assigned a chemical structure.

§ These compounds are reported for the first time as (bio)transformation products.
Zebras in mortality and absence of developmental abnormalities

Zebras in fish embryo mortality assessed at 120 hpf was statistically significant only for
the sample taken at 9 days of (bio)transformation (Figure 7.5A), coinciding with the maxima
recorded for the 400/254 nm absorbance oligomer index (Figure 7.5B) and slightly after the
onset of oligomer formation. This suggests that the early formation of azo dimers is
responsible for an increase in zebrafish embryo mortality. Oligomerization occurs when
reactive nitroso-intermediates react with amines to cause coupling reactions.[27, 97] Thus,
mortality is potentially associated with the reactive intermediates.

There were no active developmental endpoints in any of the samples collected during
MENA biotransformation. The absence of developmental abnormalities in the assay could be
due to several factors, which may contribute synergistically. Firstly, the compound mixtures
were produced at very low concentrations and below any LOEL. Particularly for dimers and
tramer products, for which the maximum molar concentration would be initial parent molarity
(MENA in this case) divided by the number of monomer units (aromatic rings). The most
abundant non-monomer product, according to ionization signal detected in the mixtures, was
an azo-dimer metabolite observed at m/z 243. However, since no developmental
abnormalities in the embryos were observed, either the compound was present below the
LOEL for the surrogate dimer tested, dimer L, or it had an important difference in biological
activity compared to dimer L that made m/z 243 less toxic towards developmental endpoints
in the MENA (bio)transformation product mixtures. Secondly, fewer oligomers may be
bioavailable to the embryos if extensive polymerization occurs that result in oligomers
precipitating from solution or oligomer incorporation into the soil humus.[29] These
mechanisms are supported by the overall decrease of aromaticity of the aqueous phase as
evidenced by the decline in 254 nm absorbance during the course of the (bio)transformation
(Figure E-14). Aromatic amines and azo polymers from similar nitroaromatic compounds,
such as DNAN and TNT, are known to bind irreversibly to soil humic substances.[29, 52] All of these factors could have contributed to an overall lower amount of bioavailable oligomers, and in consequence, no developmental abnormalities in the embryos.

**Toxicity implications of DNAN biotransformation**

The results of this study demonstrate that intermediates of DNAN biotransformation can cause detectable developmental and behavioral toxicity endpoints in zebrafish embryos. Most concerning was the high level of developmental toxicity caused by a surrogate azo-dimer intermediate (dimer L) and iMENA (both at 6.4 μM) as well as evidence of locomotor toxicity caused by DAAN at higher concentrations. Our work approach coupled transformation product identification with toxicological testing to determine the environmental impact of organic pollutants as they undergo transformation in natural systems. The findings from our study will help to advance an understanding of the potential adverse impacts of DNAN biotransformation products in natural systems to aquatic species and vertebrates in general.
Figure 7.5. Characterization of products formed during MENA (bio)transformation coupled to zebrafish toxicity. Panel A: Temporal semi-quantitation of transformation products ([M+H]+ m/z = 139, 181, 243, 313, 393) shown with stacked areas and increasing m/z shown with darker shades and zebrafish mortality assessed at 120 hpf (■). Mortality statistical significance (p < 0.05) is above 4 hits (n = 32). Panel B: MENA concentration (●) and 400 nm/254 nm absorbance index (□) during MENA (bio)transformation.
CHAPTER 8
CONCLUSIONS

(BIO)TRANSFORMATION OF DNAN IN SOILS AND SLUDGE

- DNAN was readily transformed microbially and abiotically in the microcosm studies under aerobic and anaerobic conditions in soils and wastewater sludge.

- Reductive (bio)transformation occurred readily in anaerobic conditions and was stimulated by addition of external electron donor sources, such as H₂.

- The main (bio)transformation pathway was nitroreduction of the ortho nitro group in DNAN to MENA, and the following nitrogroup reduction yielded DAAN. Reactive reduced products during nitroreduction led to azo-dimerization reactions.

- Parallel pathways included acetylation of DAAN and N-substitution and O-demethoxylation in dimers.

- In anaerobic sludge experiments, there was reductive cleavage of azo-dimers to aromatic amines via hydrazo products.
TOXICITY ASSESSMENT OF DNAN AND (BIO)TRANSFORMATION PRODUCTS

- DNAN was severely inhibitory to methanogens, nitrifiers and *A. fischeri*.
- The most toxic (bio)transformation to microorganisms included azo-oligomers, followed by nitroanilines (both with comparable toxicity to the parent compound, DNAN).
- The least toxic compound was Ac-DAAP in all microbial systems.
- Only dimer L and iMENA caused significant developmental abnormalities to Zebrafish embryos.

INTEGRATED (BIO)TRANSFORMATION AND TOXICITY ASSESSMENT

- As DNAN was reduced, increased inhibition of methanogens was recorded. The phenomenon was attributed to reactive hydroxylamino and nitroso intermediates formed.

- *A. fischeri* were not inhibited during nitroreduction of DNAN, but severe inhibition of bioluminescence occurred when azo-oligomers were the most abundant products at the latter stages of (bio)transformation.

- Significant mortality incidence to zebrafish embryos as DNAN was detected at the onset of dimer formation and was attributed to reactive hydroxylamino and nitroso intermediates.
Table A-1. First-order reaction constants for DNAN biotransformation under aerobic, anaerobic and microaerophilic conditions. $R$-squared values were determined for the linearized first-order reaction equation: $\ln \left( \frac{C_0}{C} \right) = kt$

<table>
<thead>
<tr>
<th>Condition</th>
<th>$k$ (h$^{-1}$)</th>
<th>$R^2$</th>
<th>Time period (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aerobic</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endogenous</td>
<td>0.0096</td>
<td>0.885</td>
<td>0-36</td>
</tr>
<tr>
<td>Cosubstrate</td>
<td>0.024</td>
<td>0.969</td>
<td>0-36</td>
</tr>
<tr>
<td><strong>Microaerophilic</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endogenous</td>
<td>0.036</td>
<td>0.962</td>
<td>0-51</td>
</tr>
<tr>
<td>Cosubstrate</td>
<td>0.037</td>
<td>0.965</td>
<td>0-51</td>
</tr>
<tr>
<td><strong>Anaerobic</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endogenous</td>
<td>0.102</td>
<td>0.985</td>
<td>0-34</td>
</tr>
<tr>
<td>Cosubstrate</td>
<td>0.306</td>
<td>0.998</td>
<td>0-12</td>
</tr>
</tbody>
</table>
Figure A-1. Oxidation-reduction potential, ORP (□) and dissolved oxygen concentration (▲) determined in DNAN biotransformation assays conducted under aerobic- (A), microaerophilic- (B), and anaerobic conditions (C) as a function of time. Values reported are averages from endogenous- and cosubstrate treatments.
Figure A-2. Concentration of DNAN determined microbial degradation bioassays conducted under aerobic- (A), microaerophilic- (B), and anaerobic conditions (C) as a function of time. Values reported are averages from endogenous- and cosubstrate treatments.
Figure A-3. Chemical structure of the DNAN metabolites tentatively identified using UHPLC-MS and/or TOFMS (See Table 3.1). The m/z values were determined using ChemDraw.
Figure A-4. 2,4-diaminoanisole (DAAN) (5; Fig. 6) detected in anaerobic bioassays with UHPLC-MS.

Figure A-5. 2-methoxy-5-nitroaniline (MENA) (2; Fig. 6) detected in anaerobic bioassays with UHPLC-MS.
Figure A-6. N-(5-amino-2-methoxyphenyl) acetamide (9; Fig. 6) detected in anaerobic bioassays with UHPLC-MS.

Figure A-7. N-(5-amino-2-methoxyphenyl) acetamide (9; Fig. 6) detected in anaerobic bioassays with TOFMS.
Figure A-8. 3,3’diamino-4,4’dimethoxy-azobenzene (7; Fig. 6) and 3,3’diamino-4,4’dimethoxy-hydrazobenzene (8; Fig. 6) detected in anaerobic bioassays with TOFMS.

Figure A-9. 3,3’diamino-4,4’dimethoxy-hydrazobenzene (8; Fig. 6) detected in anaerobic bioassays with UHPLC-MS.
Figure A-10. 5-((3-amino-4-methoxyphenyl)diazenyl)-2-methoxy-N-methyleneaniline (10; Fig. 6) detected in anaerobic bioassays with UHPLC-MS.

Figure A-11. 2-methoxy-5-((4-methoxy-3-(methylamino)phenyl)diazenyl)-methyleneaniline (11; Fig. 6) detected in anaerobic bioassays with UHPLC-MS.
Figure A-12. 5-((3-amino-4-methoxyphenyl)diazenyl)-2-methoxy -N-methyleneaniline (10; Fig. 6) detected in anaerobic bioassays with TOFMS.

Figure A-13. 2-methoxy-5-((4-methoxy-3-(methylamino) phenyl)diazenyl)-methyleneaniline (11; Fig. 6) detected in anaerobic bioassays with TOFMS.
Figure A-14. 3,3’diamino-4-hydroxy-4’-methoxy-azobenzene (12; Fig 6) detected in anaerobic bioassays with TOFMS.
APPENDIX B
SUPPLEMENTARY DATA FOR CHAPTER 4
Table B-1. Physical and chemical properties of the soils used in this study.

<table>
<thead>
<tr>
<th>Soils</th>
<th>pH(^{1})</th>
<th>Water content</th>
<th>Bioavailable iron</th>
<th>Total organic carbon(^{1})</th>
<th>Sand(^{g})</th>
<th>Silt(^{g})</th>
<th>Clay(^{g})</th>
<th>Textural Class(^{y})</th>
<th>Principal mineralogy: quartz; feldspar; mica; secondary minerals</th>
<th>% dry soil</th>
<th>(g kg(^{-1}) dry soil)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Camp Ripley (MN)</td>
<td>5.96 ± 0.06</td>
<td>0.5 ± 0.06</td>
<td>1.44</td>
<td>12.5 ± 1.50</td>
<td>78.2</td>
<td>13.99</td>
<td>7.82</td>
<td>loamy sand</td>
<td>55 ; 28 ; 16 ; 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Camp Butner (NC)</td>
<td>6.36 ± 0.02</td>
<td>20.7 ± 0.12</td>
<td>0.70</td>
<td>20.69 ± 1.20</td>
<td>68.68</td>
<td>19.83</td>
<td>11.50</td>
<td>sandy loam</td>
<td>67 ; 15 ; 16 ; 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Florence (AZ)</td>
<td>6.96 ± 0.11</td>
<td>2.3 ± 0.06</td>
<td>0.26</td>
<td>4.16 ± 0.20</td>
<td>44.20</td>
<td>28.50</td>
<td>27.30</td>
<td>clay loam</td>
<td>32 ; 27 ; 38 ; 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Camp Navajo (AZ)</td>
<td>6.32 ± 0.01</td>
<td>9.3 ± 0.20</td>
<td>3.85</td>
<td>52.36 ± 3.70</td>
<td>21.48</td>
<td>38.10</td>
<td>40.43</td>
<td>clay</td>
<td>40 ; 13 ; 44.5 ; 2.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maricopa (AZ)</td>
<td>7.75 ± 0.07</td>
<td>1.7 ± 0.09</td>
<td>0.41</td>
<td>4.65 ± 0.40</td>
<td>37.48</td>
<td>21.98</td>
<td>40.55</td>
<td>clay</td>
<td>29 ; 30 ; 33.5 ; 7.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Roger Road (Tucson, AZ)</td>
<td>7.75 ± 0.01</td>
<td>1.6 ± 0.06</td>
<td>0.39</td>
<td>7.07 ± 0.40</td>
<td>23.33</td>
<td>35.10</td>
<td>41.58</td>
<td>clay</td>
<td>24.5 ; 22.5 ; 43 ; 10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Catlin (Champaign, IL)</td>
<td>6.42 ± 0.06</td>
<td>3.3 ± 0.17*</td>
<td>2.15</td>
<td>44.08 ± 1.10</td>
<td>13.50</td>
<td>54.98</td>
<td>31.53</td>
<td>silty clay loam</td>
<td>42.5 ; 13 ; 0.5 ; 44</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Catlin soil was received air dried.

Figure B-1. DNAN (●), MENA (■), and DAAN (▲), sum (---). Aerobic DNAN (bio)transformation. Catlin (A), Camp Ripley (B), Camp Butner (C), Camp Navajo (D), Florence (E). Roger Road and Maricopa soils were not surveyed for aerobic transformation since preliminary experiments showed extremely slow transformation potential.
Figure B-2. Correlation between dry weight (dwt) soil organic carbon content and amount of DNAN removed from liquid phase after 4 hours of incubation attributed to adsorption in aerobic DNAN biotransformation assays. Two-sided t-test, n = 5, p = 0.015. Roger Road and Maricopa soils were not surveyed for aerobic transformation since preliminary experiments showed extremely slow transformation potential.
Fragmentation analysis: parent ion > fragment [loss from parent ion]. Tolerance = 5 ppm:
169.0615 > 154.0377 $[-\text{CH}_3]$ > 123.0683 $[-\text{OCH}_3]$ > 108.0447 $[-\text{CH}_3\text{NO}_2]$ > 96.0449 $[-\text{C}_2\text{H}_3\text{NO}_2]$ > 80.0505

Figure B-3. High-resolution mass spectrum of MENA (Figure 4.6, structure A) and fragmentation pattern as detected by UHPLC-Q-ToFMS.
Fragmentation analysis: parent ion > fragment [loss from parent ion]. Tolerance = 5 ppm:
139.0865 > 124.0635 [-CH₃] > 108.0687 [-OCH₃] > 95.0604 [C₂H₄O] > 80.0504 [-C₂H₅NO]

Figure B-4. High-resolution mass spectrum of DAAN (Figure 4.6, structure B) and fragmentation pattern as detected by UHPLC-Q-ToFMS.
Figure B-5. High-resolution mass spectrum of 2,4’dimethoxy-4-nitro-3’nitrosoazobenzene (Figure 4.6, structure C) as detected by infusion Q-ToFMS.
Fragmentation analysis: parent ion > fragment [loss from parent ion]. Tolerance = 5 ppm:


Figure B-6. High-resolution mass spectrum of 2,2’dimethoxy-5-hydroxylamino-azobenene (Figure 6, structure D) and fragmentation pattern as detected by UHPLC-Q-ToFMS.
Fragmentation analysis: parent ion > fragment [loss from parent ion]. Tolerance = 5 ppm:

Figure B-7. High-resolution mass spectrum of 3,3’-diamino-4,4’-dimethoxy-azobenzene (Figure 6, structure E) and fragmentation pattern as detected by UHPLC-Q-ToFMS.
Fragmentation analysis: parent ion > fragment [loss from parent ion]. Tolerance = 5 ppm:
259.1191 > 242.0919 [-NH₃] > 228.1005 [-OCH₃] > 227.0923 [-CH₃OH] > 199.0977 [-C₂H₄O₂]

Figure B-8. High-resolution mass spectrum of 3,3’diamino-4-hydroxy-4’methoxy-azobenzene (Figure 6, structure F) and fragmentation pattern as detected by UHPLC-Q-ToFMS.
Fragmentation analysis: parent ion > fragment [loss from parent ion]. Tolerance = 5 ppm:
299.1509 > 284.1277 [-CH$_3$] > 267.1241 [-CH$_3$OH]

Figure B-9. High-resolution mass spectrum of 4,4′-dimethoxy-3-methylamino-3′-methyleneamino-azobenzene (Figure 6, structure 7) and fragmentation pattern as detected by UHPLC-Q-ToFMS.
Fragmentation analysis: parent ion > fragment [loss from parent ion]. Tolerance = 5 ppm:
269.1398 > 254.1167 [-CH₃] > 237.1132 [-CH₂OH]

Figure B-10. High-resolution mass spectrum of 4’methoxy-3-methylamino-3’methyleneamino-azobenzene (Figure 6, structure 8) and fragmentation pattern as detected by UHPLC-Q-ToFMS.
Fragmentation analysis: parent ion > fragment [loss from parent ion]. Tolerance = 5 ppm:
303.1088 > 271.081 [-CH₃OH] > 227.1042 [-CH₂NO₃] > 225.0888 [-CH₄NO₂] > 151.0856 [-C₆H₄N₂O₃]

Figure B-11. High-resolution mass spectrum of 3-amino-3’nitro-4,4’dimethoxy-azobenzene (Figure 6, structure 9) and fragmentation pattern as detected by UHPLC-Q-ToFMS.
Fragmentation analysis: parent ion > fragment [loss from parent ion]. Tolerance = 5 ppm:
169.0602 > 154.0366 [-CH₃] > 123.0676 [-NO₂] > 122.0597 [-HNO₂] > 108.0444 [-CH₃NO₂] > 94.0649 > 77.0395

Figure B-12. High-resolution mass spectrum of iMENA (Figure 6, structure 10) and fragmentation pattern as detected by UHPLC-Q-ToFMS.
Figure C-1. Concentration of DNAN, MENA and DAAN determined in the culture medium of nitrification inhibition assays spiked with different initial concentrations of DNAN after 145 h of incubation.
Figure D-1. Workflow pictogram for DNAN staggered (bio)transformation assay, followed by product mixture semi-quantitative analysis and microbial toxicity assessment. Anaerobic incubations were set in duplicate starting at different days so that when all the samples were taken the (bio)transformation product mixture would be a representative mixture for different stages of DNAN (bio)transformation (0, 1, 5, 10, 20, 30, 40, and 50 days of incubation). Samples from the supernatant were taken in an anaerobic hood and diluted for (semi)-quantitation of the products formed, as well as subjected to methanogen and Microtox toxicity assays in order to reconstruct the changes in toxicity of the aqueous phase during DNAN (bio)transformation from 0 to 50 days of anaerobic incubation.
Figure D-2. Toxicity to acetoclastic methanogens. Toxicant-free control (Δ, all panels) Panel A: iMENA 10(♦), 40 (X), 100(●), 150(▲), 200(■) µM. Panel B: Ac-DAAN 400(♦), 1600 (X), 4000(●), 8000 (▲) µM. Panel C: Dimer L: 4 (+), 17(♦), 29(X), 42(●), 63(▲), 83(■) µM. Panel D: BBY 0.12 (+), 0.24(♦), 0.48(X), 0.96(●), 1.44(▲), 1.92(■) µM.
Figure D-3. DNAN (○) (bio)transformation to MENA (□) and DAAN (△) in Camp Butner soil (A), Camp Navajo soil (B), and anaerobic sludge (C), as quantified by HPLC-DAD. Dotted line represents sum of DNAN, MENA, and DAAN.
Figure D-4. Semi-quantitation of metabolites with $m/z$ above 300 during anaerobic DNAN (bio)transformation in Camp Butner soil. $m/z$ 325 (■), 327 (●).
Figure D-5. Semi-quantitation of metabolites with m/z above 300 during anaerobic DNAN (bio)transformation in Camp Navajo soil. m/z 301 (▲), 313 (▼), 325 (■), 327 (●), 431 (♦).
Figure D-6. Semi-quantitation of metabolites with m/z above 300 during anaerobic DNAN (bio)transformation in anaerobic sludge. m/z 325 (■), 327 (●).
Figure E-1. 24 and 120 hpf mortality incidence in zebrafish embryos (out of 32 replicates). Rows: A- MENA, B- iMENA, C- DAAN. No hits above statistical significance.
Figure E-2. 24 and 120 hpf mortality incidence in zebrafish embryos (out of 32 replicates). Rows: D- Ac-DAAN, E- dimer 1, F- Bismarck Brown. No hits above statistical significance.
Figure E-3. 24 and 120 hpf mortality incidence in zebrafish embryos (out of 32 replicates) exposed to dimer L. No hits above statistical significance.
Figure E-4. Activity of developmental endpoints in zebrafish embryos (out of 32 replicates) exposed to MENA. No hits above statistical significance.
Figure E-5. Activity of developmental endpoints in zebrafish embryos (out of 32 replicates) exposed to iMENA. White bars indicate hits above statistical significance ($p \leq 0.05$).
Figure E-6. Activity of developmental endpoints in zebrafish embryos (out of 32 replicates) exposed to DAAN. No hits above statistical significance.
Figure E-7. Activity of developmental endpoints in zebrafish embryos (out of 32 replicates) exposed to Ac-DAAN. No hits above statistical significance.
Figure E-8. Activity of developmental endpoints in zebrafish embryos (out of 32 replicates) exposed to dimer A. No hits above statistical significance.
Figure E-9. Activity of developmental endpoints in zebrafish embryos (out of 32 replicates) exposed to dimer L. White bars indicate hits above statistical significance ($p \leq 0.05$).
Figure E-10. Activity of developmental endpoints in zebrafish embryos (out of 32 replicates) exposed to Bismark Brown. No hits above statistical significance.
Figure E-11. Mass spectrum and fragmentation for 3-amino-3'-nitro-azobenzene (m/z 243), formula: C_{12}H_{10}N_{4}O_{2}

Figure E-12. Mass spectrum and fragmentation for unknown metabolite m/z 313. Putative formula: C_{16}H_{16}N_{4}O_{3}. No structure has been assigned.
Figure E-13. Mass spectrum and fragmentation for unknown metabolite with m/z 393. Putative formula: C_{19}H_{12}N_{4}O_{6}. No structure has been assigned.

Figure E-14. UV-VIS spectra recorded for supernatant samples from 1-30 days of incubation during MENA anaerobic (bio)transformation in 1% Camp Navajo soil.
REFERENCES

[48] T. Aune, E. Dybing, Mutagenic activation of 2,4-diaminoanisole and 2-
aminoanisole in vitro by liver and kidney fractions from aromatic hydrocarbon
[49] E. Dybing, Metabolic activation of 2,4-diaminoanisole, a hair-dye component
Role of cytochrome P-450 metabolism in mutagenicity in vitro, Biochem. Pharmacol.,
26 (1977) 729-734.
[50] Anonymous, Bioassay of 2,4-diaminoanisole sulfate for possible carcinogenicity,
Institutes of Health, Bethesda, MD, USA, 1978.
544-550.
[52] J. Hawari, F. Monteil-Rivera, N.N. Perreault, A. Halasz, L. Paquet, Z. Radovic-
Hrapovic, S. Deschamps, S. Thiboutot, G. Ampleman, Environmental fate of 2,4-
165-175.
[54] B. Stenuit, L. Eyers, S. Fantroussi, S. Agathos, Promising strategies for the
[55] M.E. Honeycutt, A.S. Jarvis, V.A. McFarland, Cytotoxicity and mutagenicity of
[56] B.G. Tweedy, C. Loeppky, J.A. Ross, Metobromuron: Acetylation of the aniline
[57] E. Sim, K. Walters, S. Boukouvala, Arylamine N-acetyltransferases: from
structure to function, Drug metabolism reviews, 40 (2008) 479-510.
Ajaio, C. Eadsforth, M. Galay-Burgos, R. Naidu, R. Oliver, W.J.G.M. Peijnenburg, J.
Römbke, G. Streek, B. Versonnen, From bioavailability science to regulation of
[59] A. American Public Health, A. American Water Works, F. Water Environment,
Standard methods for the examination of water & wastewater, American Public
[60] H. Yang, A. Halasz, J.S. Zhao, F. Monteil-Rivera, J. Hawari, Experimental
evidence for in situ natural attenuation of 2,4- and 2,6-dinitrotoluene in marine
transformation of 14C-labeled 2,4,6-trinitrotoluene in an activated-sludge system,
[63] J.C. Spain, Biodegradation of nitroaromatic compounds, Annual review of
[64] H.-Y. Kim, H.-G. Song, Purification and characterization of NAD(P)H-
dependent nitroreductase I from Klebsiella sp. C1 and enzymatic transformation of
pollutants in cocultures of anaerobic and aerobic bacterial consortia, Antonie van
[120] T. Frische, Screening for soil toxicity and mutagenicity using luminescent bacteria- A case study of the explosive 2,4,6-Trinitrotoluene (TNT), Ecotoxicology and Environmental Safety, 51 (2002) 133-144.
[134] J. Hawari, Biodegradation of RDX and HMX: From basic research to field application, in: J.C. Spain, J.B. Hughes, H.J. Knackmuss (Eds.) Biodegradation of


