(Bio)transformation and Toxicity of Nitroaromatic Compounds: Trace Organic Pollutants in Groundwater and Surface Run-off

**Student:** Christopher Olivares

**Advisers:** Reyes Sierra-Alvarez and Jim A. Field

**Department:** Chemical and Environmental Engineering

**College:** College of Engineering

**Acknowledgements:**

University of Arizona

Technology and Research Initiative Fund 2014/2015

Water Sustainability Graduate Student Fellowship Program
1. Introduction

\( a. \) Nitroaromatic compounds as environmental hazards

Nitroaromatic compounds are one of the most common chemical classes that are used in industries. The main uses include explosives, dyes, pesticides, and synthetic blocks for pharmaceuticals (Ju and Parales, 2010). As this class of chemical compounds are synthesized, used, and disposed, they might represent an important environmental hazard since many of these compounds are xenobiotic in nature. Moreover, several nitroaromatic compounds are toxic (Keith and Telliard, 1979; Roldan, Perez-Reinado et al., 2008; Rylott, Lorenz et al., 2011), mutagenic (Purohit and Basu, 2000), and recalcitrant (Ju and Parales, 2010; Rylott, Lorenz et al., 2011). Given these considerations, natural attenuation processes for these environmental hazard posed by these compounds might be lacking or might require deeper understanding of the environmental fate of these contaminants.

Once a site has been tainted with nitroaromatics, biodegradation by common aerobic pathways, such as oxidative attacks, is generally difficult due to the electron-withdrawing character of the nitro group and the stability of the aromatic ring (Knackmuss, 1996; Lenke, Achtanch et al., 2000). Nonetheless, nitroaromatic do not linger in the environment unchanged, since soil biological and abiotic factors can reduce nitroaromatic compounds and form aromatic amines and other products, such as azo oligomers (Hawari, Halasz et al., 1998; Olivares, Liang et al., 2013), in low-oxygen environments, such as saturated soils and soils with low dissolved oxygen. During these biochemical reactions, hydroxylamine and nitroso compounds may form, which are known to be highly toxic and mutagenic (Roldan, Perez-Reinado et al., 2008). Moreover, aromatic amines are more soluble in water, and are thus more mobile in aquifers near a polluted site.

\( b. \) Motivation and objectives

Because of reasons stated above, nitroaromatics and their transformation products might pose a relevant risk to surrounding ecosystems and public health. In order to better understand the hazard posed by this suite of chemicals, it is important to characterize the products formed and assess the toxicity posed by all of these compounds. In order to study the fate of nitroaromatic compounds, 2,4-dinitroanisole (DNAN) was chosen as a model compound (Fig.1). DNAN was used in dye synthesis and it is currently used as a novel explosive component (Davies, Provatas et al., 2006), that is expected to replace conventional explosives, such as TNT due to its higher resistance to accidental explosions (Boddu, Abburi et al., 2009).

The objectives of this research proposal are: 1) to investigate the (bio)transformation of a model nitroaromatic compound, 2) to assess individual toxicity effects of the parent nitroaromatic compound and identifiable transformation products (that are either commercially available or can be readily synthesized), and 3) to study changes in the toxicity of transformation products formed along the course of biotransformation. With this study,
we hope to elucidate mechanisms of transformation and toxicity of nitroaromatic contaminants in aqueous systems. This knowledge will help minimize hazards posed by these contaminants by gaining insights to develop biological treatment methods for nitroaromatic compounds.

2. Arizona water issue addressed

Since a majority of these compounds are not present naturally, they constitute an important section of xenobiotic compounds once they are released to the environment. In the case of Arizona, agricultural areas, such as Yuma, AZ, could represent a source of nitroaromatic pesticides, such as 2-sec-butyl-4,6-dinitrophenol (commercially known as Dinoseb). Moreover, military sites with legacy firing range activities, such as Florence, AZ, and Camp Navajo Flagstaff, AZ, might represent an additional source of nitroaromatic pollutants into ecosystems in Arizona. These agricultural and defense related nitroaromatic pollutant sources could infiltrate into groundwater or contaminate surface waters during run-off, which could pollute adjacent waters.

3. Methods

a. Task 1 – Characterization of transformation products in aqueous systems.

Anaerobic biotransformation assays will be setup to study biotransformation in aqueous soil solutions. An inoculum consisting of 100 mg of Camp Navajo soil (Flagstaff, AZ) will be added to 10 mL mineral salts medium amended with 20 mM pyruvate. The assays will contain 500 μM DNAN. Test tubes will be incubated at different times so that they have the following incubation times upon sampling: 0.5, 1, 5, 10, 20, 30, and 50 days. Incubations will occur in the dark (30°C, orbital shaker 115 rpm). Upon harvesting, supernatant samples will be retrieved with a syringe. To prevent autoxidation of unstable products, samples will be diluted in 250 ppm ascorbic acid. Samples will be analyzed in ultrahigh pressure liquid chromatography (UHPLC) as described in Olivares, Liang et al. (2013). Samples will also be characterized at the Arizona Laboratory for Emerging Contaminants and analyzed using UHPLC-quadrupole-Time-of-Flight-Mass Spectrometry (UHPLC-Q-ToF-MS) to elucidate transformation products. Proxy calibration curves will be developed for non-commercially available compounds based on similar chemicals to get an approximate abundance of each resolved product.
b. Task 2 – Microtox toxicity studies

i. From the resolved transformation products in Task 1, those that are commercially available (or readily synthesized) will be assessed individually in Microtox, an acute toxicity test that correlates well to aquatic toxicity (Bulich and Isenberg, 1981). The test uses bioluminescence of *Aliivibrio fischeri*, as described in Liang, Olivares et al. (2013). Different concentrations of each compound will be tested to identify concentrations resulting in 50% inhibition of total bioluminescence (IC$_{50}$) for individual compounds and build a library of IC$_{50}$ for the transformation products and the parent nitroaromatic compound, DNAN.

ii. Samples from the incubations developed in Task 1 will be analyzed using Microtox with a modified procedure from Task 2.i. Briefly, absolute toxicity will be tested at a single dilution (tentatively 1:10 or original sample) of the supernatant from each test tube in Task 1 to see if transformation changes will increase or decrease toxicity levels and correlate it to the abundance of metabolites found in UHPLC-Q-ToF-MS in Task 1. This will help test toxic effects from unstable and non-commercially available transformation products, as well as synergistic toxicity effects from mixtures of these products.

4. Key findings

a. Task 1 – Characterization of transformation products in aqueous systems.

DNAN transformation products of anaerobic assays were characterized and semi-quantitated in LC-MS-QToF. Briefly, DNAN was reduced to aromatic amine products (2-methoxy-5-nitroaniline and 2,4-diaminoanisole), which then coupled forming azo dimers and other oligomers. The relative abundances of each of these transformation products were semi-quantitated by the parent ion signal. Overall, as Fig. 2 shows, aromatic amines and other monomers were formed (ion m/z 139-193), which then lead to the formation of oligomers (m/z 228-431). At the end of the experiment, 50 days, the primary products were dimer and oligomer structures.

b. Task 2 – Microtox (*Aliivibrio fischeri*) toxicity studies

i. Microtox toxicity assays for standards and best available surrogates.

DNAN, and its main transformation products, 2-methoxy-5-nitroaniline and 2,4,diaminoanisole, as well as other products detected were characterized for inhibition potential to bioluminescence in the microbial toxicity model *Aliivibrio fischeri*. Table I shows the main chemicals tested as well as their chemical structures and the concentration needed to cause 50% inhibition in bioluminescence (IC$_{50}$). Overall, it can be seen that the full aromatic amine product, 2,4-diaminoanisole resulted in a higher IC$_{50}$, which indicates that they have a lower toxicity potential (2.72 times less toxic on a molar basis) than the
parent compound, DNAN. However, the best available dimer surrogate, 3,3’-dimethoxy-4,4’-diamino-azobenzene, caused 1.9 times more inhibition on a molar basis than DNAN.

**ii. Microtox toxicity assays for complex biotransformation product mixtures.**

Bioluminescence from Microtox assay was recorded for samples taken from different incubation times during the anaerobic biotransformation of DNAN. As Fig. 3 shows, the initial part shaded in green represents that the primary component is DNAN. The yellow shade indicates that the main chemical compound class are reduced aromatic amines. Furthermore, the region shaded in blue shows that the predominant species are dimers and other oligomers. Over these qualitative chemical compound abundance regions, we can see the overlapped changes in bioluminescence. As DNAN is reduced to aromatic amines, the bioluminescence stays constant at ~100% (0-5 days). However, as the aromatic amines start to couple and form dimers and other oligomers (10-20), the bioluminescence sharply decreased from 70 to 35%. This suggest that the formation of azo dimers coincides with an increase period of toxicity. Afterwards, the bioluminescence remained stable for longer periods of times and slightly decreased in the latter portion of the experiment.

Overall, the toxicity changes during the formation of complex mixture of anaerobic DNAN (bio)transformation products suggest that the main change in toxicity occurs when dimers start to form in the mixture. This could be due to nitroso radicals formed during the formation of azo(oxy) and azo dimers. The toxicity remains when the dimers are present.

5. **Key conclusions and future work**

Overall, our results indicate that the main transformation pathway for DNAN in saturated soil and anoxic niche soil environments was reduction of the nitro functional group to yield aromatic amines. These products, in turn, remained reactive to couple and formed azo dimers as well as other oligomers. The initial part of the biotransformation does not change significantly the toxicity potential of DNAN, however, the formation of dimers coincided with a significant increase in toxicity potential as detected by a bioluminescence inhibition assay with *Allivibrio fischeri*.

Even though our results suggest the formation of more toxic species with dimers, future work will explore possible options of diminishing the bioavailability of the dimers formed so that they may be removed from the aqueous phase and overall help find an efficient bioremediation technique for nitroaromatic polluted sites in Arizona and elsewhere.

6. **References**


7. Tables and figures

Table I. Microtox IC$_{50}$ for DNAN and transformation products

<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
<th>Microtox IC$_{50}$ (µM) ($A. fischeri$)</th>
<th>Toxicity potential (highest +++), (lowest +)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4-dinitroanisole (DNAN)</td>
<td><img src="image1.png" alt="Structure" /></td>
<td>56.9</td>
<td>++</td>
</tr>
<tr>
<td>2-methoxy-5-nitroaniline</td>
<td><img src="image2.png" alt="Structure" /></td>
<td>47.7</td>
<td>++</td>
</tr>
<tr>
<td>2,4-diaminoanisole</td>
<td><img src="image3.png" alt="Structure" /></td>
<td>155.6</td>
<td>+</td>
</tr>
<tr>
<td>3,3’dimethoxy-4,4’diamino-azobenzene (best available dimer surrogate)</td>
<td><img src="image4.png" alt="Structure" /></td>
<td>29.8</td>
<td>+++</td>
</tr>
</tbody>
</table>

![Fig. 1.](image5.png) DNAN biotransformation pathways and main products detected.
Fig. 2. Semi-quantitation (in peak area units) of products detected in LC-MS-QToF of DNAN anaerobic biotransformation. DNAN is shown in secondary axis.

Fig. 3. Normalized bioluminescence with respect to a toxicant-free control. Green means primary component is DNAN. Yellow means that primary amines abound. Blue represents that the majority of products are oligomers.