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Biological transformation pathways of 2,4-dinitro anisole and N-methyl paranitro aniline in anaerobic fluidized-bed bioreactors

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ABSTRACT

The US Army is evaluating new, insensitive explosives to produce safer munitions. Two potential new components are 2,4-dinitro anisole (DNAN) and N-methyl paranitro aniline (MNA), which would eventually make their way to waste streams generated in the production and handling of new munitions. The effectiveness of anaerobic fluidized-bed bioreactors (AFBB) was studied for treatment and transformation of these two new chemical components in munitions. Each compound was fed into a separate reactor and monitored for removal and transformation, using ethanol as the electron donor. The results show that both were degradable using the AFBB system. DNAN was found to transform into diaminoanisole and MNA was found to transform into N-methyl-p-phenylenediamine. Both of these by-products appeared to form azobond polymers after exposure to air. To test the resilience of the reactors, the compounds were removed from the feed streams for 3 week and then reintroduced. DNAN showed that a re-acclimation period was necessary for it to be degraded again, while MNA was removed immediately upon reintroduction. The AFBB technology was shown here to be an effective means of removing the new munitions, but produce secondary compounds that could potentially be just as harmful and require further study.

1. Introduction

The production and handling of explosives and propellants produces wastewater contaminated with energetic compounds. The US Military is currently developing new munitions formulas which are less sensitive. As a result, 2,4-dinitroanisole (DNAN) and N-methyl paranitro aniline (MNA) have been commissioned for production by the US Military and may become major components of this wastewater. DNAN is being used as a replacement for trinitrotoluene (TNT). It is much less sensitive, requiring a higher detonation temperature, but still has similar properties to TNT that make it advantageous for manufacturing explosives (Davies and Provatas, 2006). The addition of DNAN to the munitions formula will reduce the risk of unwanted detonations and any secondary explosions caused by close proximity to initial explosions. MNA is an additive used to lower the melting point of other energetic compounds, such as DNAN, to make manufacturing the explosives easier (Davies and Provatas, 2006). Very little is known about the fate of these compounds in treatment systems or the best conditions for their degradation as they are still under development with no waste streams available. One possible removal method involves an anaerobic fluidized-bed bioreactor (AFBB), which has previously shown promise for other wastewaters from munitions production.

AFBBs have been studied extensively for use with industrial wastes and other more difficult to degrade wastewaters (Grace and Bi, 1997). Advantages of AFBBs over more conventional treatment systems include high solids–liquid interfacial area, low washout, and minimal sludge recycling. Also, Suidan et al. (1988) found that the fluidization decreased the thickness of the concentration boundary layer leading to lower mass transport resistance.

In addition to being studied heavily for general wastes, AFBBs have been used for treating munitions specific waste. Davel (2002) evaluated the treatment of TNT, RDX (royal demolition explosive, or hexahydro-1,3,5-trinitro-1,3,5-triazine), and HMX (high melting explosive, or octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine) using AFBBs and found almost complete transformation of the compounds. Others have shown similar results using dinitrotoluene (Cheng et al., 1996). Atikovic et al. (2008) showed that AFBBs are effective at removing RDX and perchlorate both singularly and when together. A study was also performed on actual manufacturing pinkwater, the term used to describe the water discharged from the manufacture of the munitions containing TNT. In that study Maloney et al. (2002) found that the AFBBs were effective in removing TNT and RDX despite widely varying concentration loads.

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The AFBB is one stage of a two stage process to degrade nitro-based high explosives dissolved in wastewater. The first stage is the anaerobic transformation of the nitro-substituent group to an amino group and the second stage is aerobic treatment of the transformed compound (Berchtold et al., 1995; Vanderloop et al., 1998). Previous studies have shown that some anaerobic transformation products of energetic compounds remain stable in water after the anaerobic stage, such as the 2,4-diaminotoluene resulting from the anaerobic reduction of 2,4-dinitrotoluene, while others become unstable during anaerobic treatment or polymerize upon exposure to oxygen, such as triaminotoluene, which is the reduced product from TNT (Berchtold et al., 1995; Hwang et al., 2000; Davel, 2002).

In many of these studies, ethanol was used as the primary substrate in the system. Ethanol is required as an electron donor to facilitate the degradation or transformation of the compound. Also, it has been shown that more ethanol is required than just the stoichiometric amount needed for the transformation (Atikovic et al., 2008). The additional ethanol is required to remove any oxygen present in the feed stream and that might enter the system through leaks in reactor structure as well as for bacteria synthesis. Determining the lower limit on the ethanol concentration needed is very important to minimizing the cost of running an AFBB system as the ethanol must be added to the waste stream.

The goals of this study were to determine the effectiveness of AFBBs for the treatment of DNAN and MNA and to determine the minimum concentration of ethanol needed to promote full reduction of the two contaminants. To eliminate some of the complexity of the systems, each compound was studied individually in separate reactors. The reactors were charged with silica sand instead of the more customary granular activated carbon (GAC) which was used in many of the prior studies. Sand was selected in order to distinguish between biotransformation and any adsorptive or catalytic interactions that GAC might promote. A recent study showed that GAC is capable of removing over 99% of DNAN (Boddu et al., 2009). The use of sand also eliminates the extended period of time that would be required to load the GAC when changes in the contaminant concentrations are made.

2. Materials and methods

2.1. Chemicals

The DNAN ($C_7H_6N_2O_5$) was first purchased from Sigma Aldrich, St. Louis, Missouri (98% purity). The MNA ($C_7H_8N_2O_2$) was first purchased from Acros Organics, New Jersey (97% purity). The compounds were later supplied directly from the US Army Engineering Research and Development Center – Construction Engineering Research Laboratory (CERL) because new regulations on explosives prohibited the import of the compounds. CERL acquired the explosives from an Army ammunition plant, which manufactured them according to Army specifications anticipated for these munitions. As a result, the purity and other compounds present in the DNAN and MNA were unknown. The ethanol and other solvents used in analysis were purchased from Fisher Scientific, Pittsburgh, Pennsylvania.

2.2. Reactor design

Two 9.1 L AFBB were used to conduct the experiments. Each bioreactor consisted of a jacketed main column and influent and effluent ports (See Supplementary Material (SM), Fig. SM-1). The inner tube (96.5 cm long, 10.2 cm id) was constructed from Plexiglas and is enclosed in an outer Plexiglas tube. Water was circulated through the enclosed space between the two tubes from a constant temperature bath (model 20L-M Fisher Scientific, Pittsburgh, Pennsylvania) to maintain a constant temperature of 37 °C within the column. The recycle lines were made from polyvinyl chloride pipe and the feed and effluent lines were Tygon and neoprene tubing. A gas volume meter (tip meter) and sampling port were placed atop the reactor at the effluent header for measuring gas quantities.

Each AFBB contained 2.0 kg of 16 × 20 US mesh (1.19–0.84 mm) silica sand as the attachment medium. Effluent recycle was used to maintain a bed-expansion of 50%. The influent header of each AFBB was filled with glass marbles to distribute the flow evenly across the column cross section. An effluent port allowed liquid to exit the reactor while still capturing the gas produced during the treatment process. Attachment medium could be withdrawn through a port located at the top of the effluent header. The AFBBs were operated at a medium-empty-bed contact time of 6 h.

Based on work done by Atikovic (2006), the reactors were fed a stream of three different solutions through ports in the recycle line to avoid any precipitation possibilities. A stock solution of the compounds, DNAN for Reactor 1 and MNA for Reactor 2, and ethanol was fed at 5 L d^{-1}. The concentrations in this solution were increased in increments to acclimate the bacteria to the energetic compounds, with the final concentrations being close to solubility, approximately 100 mg L^{-1} for DNAN and 18 mg L^{-1} for MNA. A buffer solution and a nutrient solution were each fed at 0.5 L d^{-1}. The buffer solution contained sodium carbonate, phosphate, and sulfide while the nutrient solution contained salts and vitamins. The complete nutrient composition and concentrations for the buffer and nutrient components are shown in Fig. SM-2. When combined with the buffer and nutrient solutions in the influent, the stock solution concentrations were diluted, producing final influent concentrations of 83.33 mg L^{-1} for DNAN and 15 mg L^{-1} for MNA. The pH was maintained between 6.8 and 7.2.

2.3. Analytical methods

Solution flow rates, pH, and gas production were monitored on a daily basis. Gas composition was measured on a weekly basis for CH_4, CO_2, O_2, and N_2 concentrations. Influent and effluent chemical oxygen demand (COD) and the target compounds were measured on a biweekly basis.

The pH was measured using an Orion Model 720A pH meter (Orion Research, Boston, Massachusetts). COD was measured using Hach Method 8000 on a Hach DR/200 Spectrophotometer (Hach, Loveland, Colorado). Gas analysis was performed on a HP5890 Series II Gas Chromatograph (Hewlett Packard, Wilmington, Delaware) with a thermal conductivity detector using a HP 10 ft (3.05 m) molecular sieve BX-45/60 mesh HP 6 ft (1.83 m) HAYESEP Q 80/100 (SUPELCO, Bellefonte, Pennsylvania) column. Spectrophotometer scans of the effluent were done on a HP 8452 Diode Array Spectrophotometer (Hewlett Packard, Wilmington, Delaware).

A method for HPLC was developed for DNAN and MNA using a modified version of EPA method 8330A. An 1100 series HPLC system (Agilent Technologies, Santa Clara, California) was used with a Zorbax SB-C18 StableBond Analytical 4.6 × 250 mm 5 μm column. The mobile phase was a mixture of 60% water and 40% methanol pumped at 1 mL min^{-1}. The runtime of the method was 25 min. MNA was measured at a wavelength of 220 nm and appeared at approximately 15.2 min. DNAN was measured at a wavelength of 210 nm and appeared at approximately 17.6 min. The samples were filtered through a 0.1 μm filter and diluted with an equivalent volume of methanol before analysis.

Flow injection analysis was used in order to detect products caused by the transformation of MNA and DNAN in the reactors. Effluent samples were introduced on a 1200 series rapid resolution liquid chromatograph that was coupled to a 6410 Triple Quad Mass

Spectrometer (MS) (Agilent Technologies, Santa Clara, California). Analytes were ionized with an orthogonal electrospray ionization interface. The injection volume was 1 μL and the flow rate was 0.4 mL min^{-1}. The binary pump was run under isocratic conditions with a mobile phase of 60% water and 40% methanol with ammonium formate (5 mM) added as a buffer to both solvents. Samples were analyzed in the positive ionization mode with the capillary voltage set at 4 kV. The MS was operated in scan from 80 to 400 *m/z*. Acquisition and peak identification were performed with Agilent MassHunter Workstation Software b.01.00 9 (B48). The samples were filtered through a 0.1 μm filter and injected immediately thereafter.

3. Results and discussion

The AFBBs were seeded with anaerobic digester supernatant and started initially on ethanol as the only organic carbon source. The feed stream at startup contained approximately 500 mg L^{-1} of ethanol. The startup period lasted for 184 d, which is appreciably longer than anticipated, due to leaks that were discovered in the suction side of the recycle line. After these leaks were fixed, startup proceeded rapidly.

After day 184, both DNAN and MNA were introduced into the stock solutions at approximately 25% of the final concentrations. Since these compounds are still under testing for use in munitions, there are no real waste streams available for use in this study. The reactors acclimated to the compounds until stable conditions were achieved, i.e. several consecutive measurements with low and consistent effluent concentrations of the compounds, low COD, and methane gas production near theoretical values. Then, the concentrations were increased to 50% and allowed to stabilize. This process was repeated again for 75% and 100% of the final concentrations of both compounds. After the reactors stabilized again at 100% concentration, the ethanol was lowered to determine the minimum concentration of ethanol required. The ethanol concentration in the feed was lowered in four stages, each stage cutting the concentration in half. Again stability was reached after each change in concentration before it was lowered again. The final ethanol concentration was 31.3 mg L^{-1}. Table 1 gives chronological listing of the feeding conditions as well as other pertinent values for the reactors. The theoretical methane values in this table are for the conversion of ethanol that was not used to reduce DNAN and MNA. The actual methane produced was always less than the theoretical because there was an amount of ethanol required to remove any oxygen that may have entered the system. The exception to this is during the final feeding stage where the amount of gas produced was so low that the tip meter measuring system was not adequate for determining the volume reliably with resulting values slightly higher than expected.

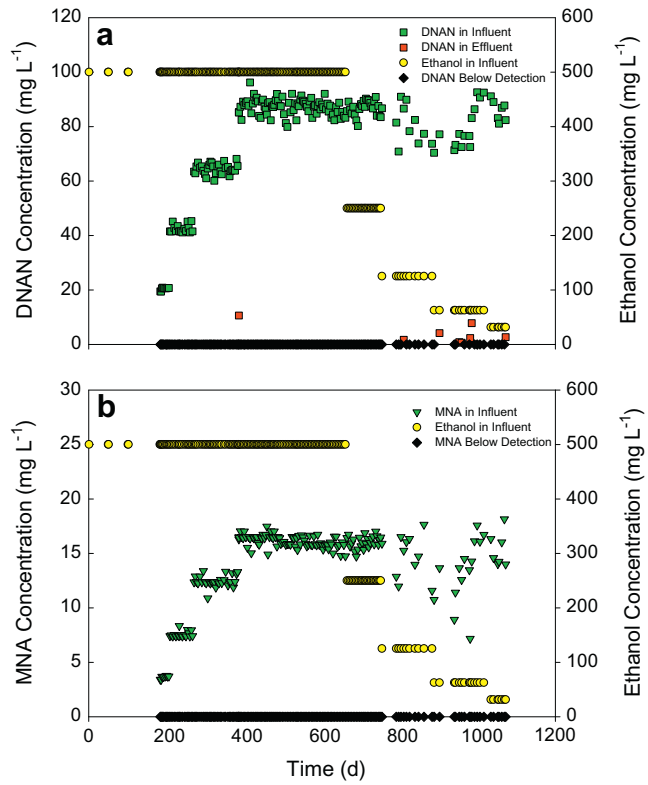

Fig. 1. DNAN (a), Reactor 1, and MNA (b), Reactor 2, influent and effluent concentrations over time along with the ethanol concentration. MNA was never detected in the effluent of Reactor 2.

The reactors transformed the compounds almost completely, as shown in Fig. 1. The analysis done on the HPLC showed the reactors removed the compounds to below the detection limit, 0.5 mg L^{-1} for DNAN and 0.2 mg L^{-1} for MNA, in almost all cases. In a few samplings, DNAN was detected in effluent of Reactor 1 (DNAN only), but these spikes were attributed to pH perturbations that took place after lowering the feed ethanol concentration causing a drop in CO_2 production. The effluent pH in Reactor 1 (DNAN only) became unstable at the lower ethanol concentrations where the influent ethanol concentration was close to the ethanol needed to reduce the DNAN as well as maintain anaerobic conditions in the reactor. Over time, the buffer was adjusted to compensate, but the pH of the reactor fluctuated widely from as low as 6.1 to as high as 7.9 during this time leading to perturbations in performance. Reactor 2 did not exhibit these problems because complete reduction of MNA has an appreciably lower ethanol demand. It is important to note that at around day 750, the compounds for both

Table 1
Reactor values with time.

Days since reactor startup (d)	Compound concentration in feed (% of target)	Ethanol concentration in feed (mg L^{-1})	R1 methane production (actual/theoretical) (mL d^{-1})	R2 methane production (actual/theoretical) (mL d^{-1})	COD value for period for R1 (inf/eff) (mg L^{-1})	COD value for period for R2 (inf/eff) (mg L^{-1})
1	0	500	1940/2310	2070/2390	962/34	963/27
184	25	500	2070/2310	2140/2390	1020/52	998/34
209	50	500	1990/2310	2210/2390	1060/50	1030/22
271	75	500	2110/2310	2150/2390	1090/71	1050/23
386	100	500	2160/2310	2250/2390	1100/80	1060/35
688	100	250	713/1110	929/1190	648/75	566/29
791	100	125	346/506	564/589	430/106	430/35
902	100	62.5	178/207	256/289	283/99	181/31
1034	100	31.3	79/57	141/139	242/75	112/32

reactors were changed from those obtained from specialty manufacturers to those obtained from CERL. The lower purity and inconsistency of the batch obtained from CERL caused the feed concentration to vary greatly.

The removal of DNAN and MNA was achieved through biological transformations into secondary compounds. DNAN was transformed to diaminoanisole ($C_7H_{10}N_2O$). In the electrospray, diaminoanisole experiences an *Ortho*-effect reaction and, therefore, the protonated molecule (139 *m/z*) presented a low intensity peak in the mass spectrum whereas the product of such rearrangement (123 *m/z*) was the most abundant ion (see Fig. 2a) (Schwarz and Levsen, 1982). The transformation was assumed to proceed according to the following chemical equation (see Fig. 4 for chemical structures):

$$C_7H_6O_5N_2 + C_2H_6O \rightarrow C_7H_{10}ON_2 + 2CO_2 + H_2O \quad (1)$$

The transformation of DNAN to diaminoanisole required 19.4 mg L^{-1} of ethanol if a complete conversion occurs. This ethanol demand was below the influent ethanol concentration, leaving approximately 11.9 mg L^{-1} of ethanol for maintaining stable anaerobic conditions.

The effluent from Reactor 1 (DNAN only) exhibited signs of polymerization with resulting precipitation of the polymers upon exposure to oxygen. Sodium sulfite was shown to slow this process

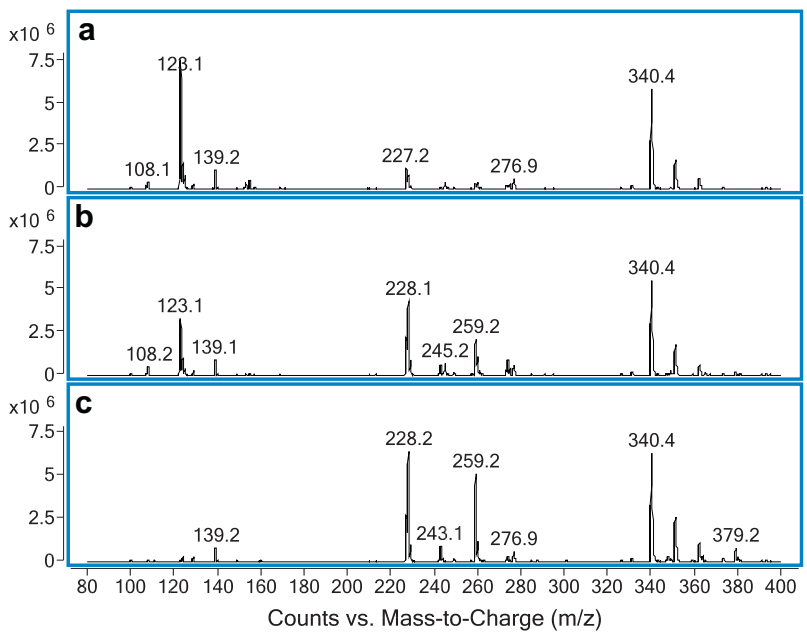

Fig. 2. MS scans of the effluent from the Reactor 1 taken at 0 (a), 2 (b), and 24 h (c) showing the molecular mass ions detected. Note the reduction of the diaminoanisole peaks at 123 *m/z* and 139 *m/z* and the emergence of the dimer peaks at 228 *m/z* and 259 *m/z*.

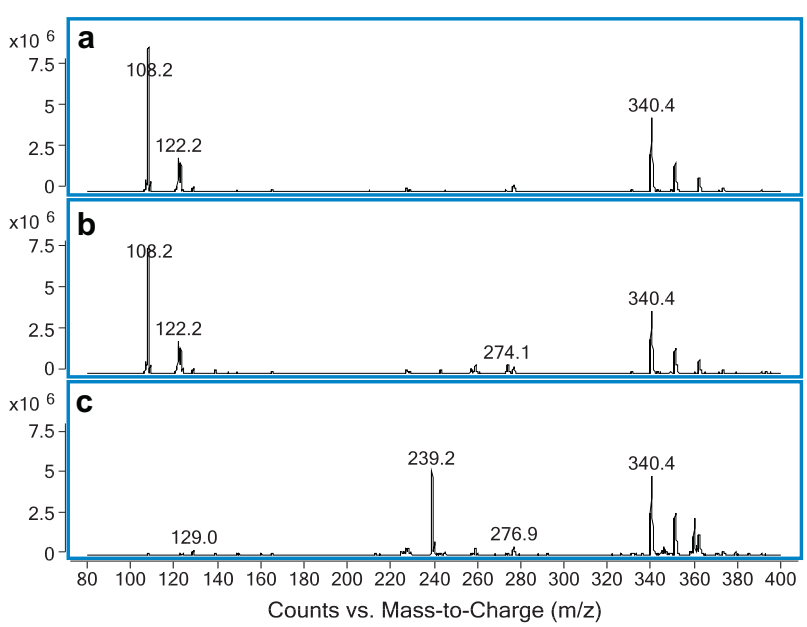

Fig. 3. MS scans of the effluent from the Reactor 2 taken at 0 (a), 2 (b), and 24 h (c) showing the molecular mass ions detected. Note the disappearance of the N-methyl-p-phenylenediamine peaks 108 *m/z* and 123 *m/z* and the emergence of the dimer peak at 239 *m/z*.

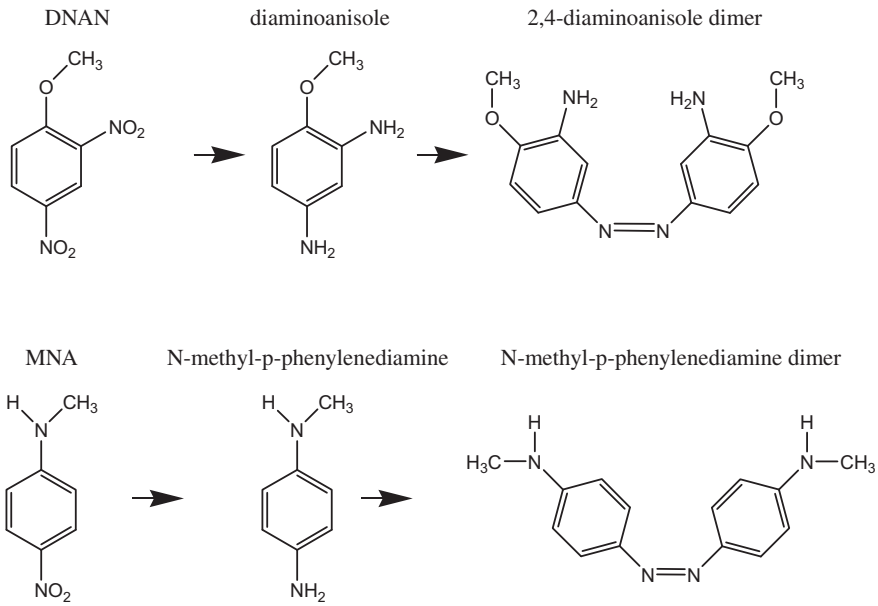

Fig. 4. Chemical structures of 2,4-dinitroanisole (DNAN) and N-methyl paranitro aniline (MNA) and their transformation products, diaminoanisole, N-methyl-p-phenylenediamine, and their dimers.

by removing the oxygen from the sample, but it only delayed it for a short time. The effluent turned from colorless to a dark purple upon exposure, with even filtered effluent samples also turning to dark purple after exposure to oxygen. MS scans, shown in Fig. 2, were repeatedly performed over a 24 h period on an effluent sample that had been exposed to the air. In this figure, scans of the effluent taken at time 0, 2, and 24 h are shown. They show a reduction in the count at 123 *m/z* between 0 and 2 h and the disappearance of the peak entirely after 24 h as well as a reduction in the peak at 139 *m/z* over time. In addition to these decreases, these data also show an increase in peaks at 228 and 259 *m/z*. These peaks increase over time as diaminoanisole decreases suggesting that they are most likely fragmented versions of an azobond dimer ($(C_7H_8ON_2)_2$). The 228 *m/z* fragment would be a diaminoanisole dimer with $[CH_3ON]^+$ cleaved off and the 259 *m/z* fragment would be the same diaminoanisole dimer with $[CH_2]^+$ cleaved off. No evidence could be found to link this behavior directly to a single constituent present in the effluent, although polymerization has been seen with other amines. Yang et al. (2008) showed that diaminotoluene in aerobic sediment can form dimers and trimers. Also, another study showed a precipitate was formed when diaminotoluene was exposed to hydrogen peroxide (Watanabe et al., 1989).

MNA was found to transform into N-methyl-p-phenylenediamine ($C_7H_{10}N_2$) according to the following reaction (see Fig. 4 for chemical structures):

$$2C_7H_8O_2N_2 + C_2H_6O \rightarrow 2C_7H_{10}N_2 + 2CO_2 + H_2O \quad (2)$$

Fig. 3a shows an MS scan of the effluent from Reactor 2 (MNA only). Again, the protonated molecule (123 *m/z*) for N-methyl-p-phenylenediamine had lower intensity than its corresponding fragment $[C_7H_{10}N]^+$ (108 *m/z*). The transformation of MNA required 2.3 mg L^{-1} of ethanol leaving approximately 29 mg L^{-1} for maintaining anaerobic conditions. There was much more available ethanol to support Reactor 2 (MNA only) than was available in Reactor 1 (DNAN only), explaining why the stability problems in Reactor 1 were not seen in Reactor 2 at low ethanol concentrations.

The effluent from Reactor 2 (MNA only) also reacted when exposed to oxygen in a manner similar to Reactor 1 (DNAN only). The color gradually became a purple color, but the color did not become as dark as in Reactor 1 and masses were not visible after an extended period of time. The reaction appeared to be slower than that of Reactor 1 (DNAN only), but the disparity in concentrations could also account for the observed differences. MS scans in Fig. 3 show the disappearance of the byproducts and the formation of a dimer over a 24 h period after the effluent had been exposed to the atmosphere, similar to Reactor 1. The scans show a reduction in the count at 108 *m/z* and 123 *m/z* between 0 and 2 h and the disappearance of the peaks entirely after 24 h. Corresponding to the disappearance of these peaks is the formation of a peak at 239 *m/z*, which could be a dimer of N-methyl-p-phenylenediamine with a cleavage of $2[H]^+$. These polymerization reactions also complicate further treatment since the next step in treatment is commonly aerobic treatment and the macromolecules formed are most probably not amenable to biological treatment. While N-methyl-p-phenylenediamine has not been studied in treatment systems, diaminoanisole has been shown to exhibit removal in activated sludge systems (Ekici et al., 2001). The authors did not provide evidence of mineralization and, based on the above data, removal through polymerization and subsequent capture in the floc is a logical pathway.

Calibration standards for these compounds were not available to permit for quantitative assessment of effluent quality throughout the experiment. To monitor the effluent for changes caused by reduction in the feed ethanol concentration, qualitative analysis was performed on a spectrophotometer over a broad spectrum. The resulting scans show that very little change occurred in the effluent composition over time. Figs. SM-3 and SM-4 show three absorption scans of the effluent from Reactors 1 and 2, respectively, taken during the final three ethanol concentration stages, 125, 62.5, and 31.3 mg L^{-1}. Although the exact amount each of the by-products contributing to the scan is unknown, the consistency across the feeding periods shows that even at very low ethanol concentrations the reactors continue to work effectively.

Arnett et al. (2009) carried out complementary experiments on these systems. They analyzed samples of medium taken at different periods of AFBBs operation for bacterial community structure in order to assess the impact of feed composition on the microbial community within the AFBBs. They reported a significant shift in the dominant bacterial species after introduction of DNAN and MNA into the reactors (Arnett et al., 2009).

After completion of the ethanol reduction phase of the study, a starvation test was performed. Prior to commencement of this test, the concentration of ethanol in the feed was increased back up to 62.5 mg L^{-1} while maintaining the same feed concentrations of DNAN and MNA. The reactors were operated for 34 d during which time reactor performance was stabilized. After reactor stability was achieved, DNAN and MNA were removed from the stock solution for exactly 21 d while maintaining the ethanol feed. The compounds were reintroduced after the 3 week disruption period at their former concentrations. Reactor 2 (MNA only) immediately began removing the MNA while Reactor 1 (DNAN only) removed most, but not all of the DNAN at the beginning with its transformation efficiency improving over time. The lower concentration of MNA explains why it did not require an acclimation period. The acclimation period for DNAN would be significantly reduced or even eliminated if activated carbon was used as the attachment medium instead of sand. Activated carbon would act as a buffer for the compounds, slowly releasing or adsorbing them as the feed concentrations fluctuate.

4. Conclusions

The goal of this research was to look into the effectiveness of AFBBs as a treatment method for a munitions waste containing DNAN and MNA. The general conclusion that can be drawn is that the system will remove them from a waste stream. The reactors showed some resilience by continuing treatment at very low ethanol concentrations and also after a starvation test, which are two situations that would be common in a real plant. If GAC were substituted for the silica sand used in this study, the resilience of these reactors would increase further by smoothing out concentration fluctuations. There is a need to evaluate this technology on real waste streams since such streams are often supersaturated with multiple types of munitions.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chemosphere.2010.08.044.

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