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Continuous treatment of N-Methyl-*p*-nitro aniline (MNA) in an Upflow Anaerobic Sludge Blanket (UASB) bioreactor

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Abstract

N-methyl-*p*-nitroaniline (MNA) is an ingredient of insensitive munitions (IM) compounds that serves as a plasticizer and helps reduce unwanted detonations. As its use becomes widespread, MNA waste streams will be generated, necessitating viable treatment options. We studied MNA biodegradation and its inhibition potential to, a representative anaerobic microbial population in wastewater treatment, methanogens. Anaerobic biodegradation and toxicity assays were performed and an up-flow anaerobic sludge blanket reactor (UASB) was operated to test continuous degradation of MNA. MNA was transformed almost stoichiometrically to N-methyl-*p*-phenylenediamine (MPD). MPD was not mineralized, however, it was readily autoxidized and polymerized extensively upon aeration at pH = 9. In the UASB reactor, MNA was fully degraded up to a loading rate of 297.5 $\mu\text{M MNA d}^{-1}$. Regarding toxicity, MNA was very inhibitory to acetoclastic methanogens ($\text{IC}_{50} = 103 \mu\text{M}$) whereas MPD was much less toxic, causing only 13.9% inhibition at the highest concentration tested (1025 μM). The results taken as a whole indicate that anaerobic sludge can transform MNA to MPD continuously, and that the transformation decreases the cytotoxicity of the parent pollutant. MPD can be removed through extensive polymerization. These insights could help define efficient treatment options for waste streams polluted with MNA.

Keywords

N-methyl-*p*-nitroaniline; insensitive munitions; nitroaromatic; anaerobic; biodegradation; microbial toxicity

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1. Introduction

Defense industries are evaluating a new generation of explosive compounds known as insensitive munitions (IMs), which can reduce accidental explosions due to their resistance to shock and their high detonation temperature (Boddu et al., 2009). Examples of these munitions compounds include 2,4-dinitroanisole (DNAN), and 3-nitro-1,2,4-triazole-5-one (NTO). Within explosive formulations, plasticizers such as N-methyl-*p*-nitroaniline (MNA) are added to increase shelf-life of munitions (Boddu et al., 2008a). MNA also decreases the risk of unwanted detonations by decreasing the melting point of insensitive munitions formulations (Boddu et al., 2008b; Platten et al., 2010). MNA is used in Picatinny Explosive Formulation 21 (PAX-21). As IM use becomes widespread, emissions of MNA and other munitions components are expected to increase due to the generation of waste streams by manufacturing facilities, and the use of these materials in fire training grounds (Davies et al., 2006; Boddu et al., 2009).

MNA has low solubility in water; 85 mg L⁻¹ at 25 °C (Boddu et al., 2008a). While the chemical structure of MNA includes both hydrophilic (amino) and hydrophobic (nitro) moieties (Fig. 1), the aqueous solubility of MNA is greatly reduced by the N-methyl substitution (Ahmed and Sandler, 2012). Given its poor solubility in water and its limited mobility in the soil, interaction with biological systems might play an important role in determining the environmental fate and treatment options for this compound. Reduction of MNA to N-methyl-*p*-phenylenediamine (MPD) has been reported in an ethanol-amended anaerobic fluidized bed bioreactor treating MNA and other co-contaminants, such as DNAN and perchlorate (Platten et al., 2013). Regarding the inhibitory potential of MNA, there are no dedicated studies on its cytotoxicity. Nonetheless it is well known that nitroaromatics are toxic to microorganisms (Roldan et al., 2008; Rylott et al., 2011) and many of these compounds are mutagenic (Purohit and Basu, 2000) and recalcitrant (Ju and Parales, 2010; Rylott et al., 2011). MNA has been suggested to be mutagenic based on Ames test results and mutagenicity prediction models (Zeiger et al., 1996). Overall, there is very little knowledge on the impact of MNA in the environment, and studies on environmental fate, biodegradation, and toxicity are needed to identify effective options to treat industrial effluents containing MNA.

The objective of this study was to investigate the continuous treatment of MNA in a laboratory-scale upflow anaerobic sludge bed (UASB) bioreactor and to characterize the inhibitory effects of MNA and its metabolite MPD towards anaerobic methanogenic microorganisms.

2. Materials and Methods

2.1. Chemicals

N-methyl-*p*-nitroaniline (CAS # 100-15-2, 97% purity) was obtained from Alfa-Aesar (Ward Hill, MA, USA) and N-methyl-*p*-phenylenediamine (CAS# 5395-70-0, 98% purity) was acquired from AK Scientific (Union City, CA, USA). All other chemicals used were analytical reagent or HPLC grade.

2.2. Inoculum

Sieved anaerobic granular sludge from an anaerobic wastewater treatment plant treating brewery wastewater (Mahou, Guadalajara, Spain) was used as inoculum. The content of volatile suspended solids (VSS) in the sludge was 7.92% based on wet weight. The acetoclastic methanogenic activity of the sludge was 0.68 g CH₄ (expressed as chemical oxygen demand or COD) per gram VSS per day. The sludge was stored under a N₂ atmosphere at 4°C.

2.3. Basal medium

The basal medium was prepared using Ultrapure water (NANOpure Infinity™, Barnstead International, Dubuque, IA, USA) and contained (in mg L⁻¹): K₂HPO₄ (250), CaCl₂•2H₂O (10), MgSO₄•7H₂O (100), MgCl₂•6H₂O (100), NH₄Cl (280), NaHCO₃ (4,000), yeast extract (100), and trace element solution (1 mL L⁻¹). The trace elements solution contained (in mg L⁻¹): H₃BO₃ (50), FeCl₂•4H₂O (2,000), ZnCl₂ (50), MnCl₂•4H₂O (50), (NH₄)₆Mo₇O₂₄•4H₂O (50), AlCl₃•6H₂O (90), CoCl₂•6H₂O (2,000), NiCl₂•6H₂O (50), CuCl₂•2H₂O (30), NaSeO₃•5H₂O (100), EDTA (1,000), resazurin (200), and 36% HCl (1 mL).

2.4. Anaerobic batch bioassays

Methanogenic toxicity and biodegradation assays were carried out in duplicate using glass serum bottles (160 mL) containing 25 mL of basal culture medium (toxicity) or 100 mL (biodegradation) and inoculated with anaerobic granular sludge (0.5 g VSS L⁻¹). The bottles were sealed with butyl rubber septa and aluminum crimp seals and, subsequently, the headspace was flushed with a gas mixture of N₂/CO₂ (80:20, v/v) to ensure anaerobic conditions and provide buffer capacity (pH = 7.2-7.4). The bottles were incubated in the dark at 30±2°C in an orbital shaker at 115 rpm.

Anaerobic biotransformation assays—The inoculated serum flasks were supplemented with MNA (300 µM). Besides the endogenous treatment, a subset was supplied with both MNA (300 µM) and H₂ as a co-substrate. H₂ was injected as H₂/CO₂ (80/20, v/v) by over-pressurizing the flasks to 1.5 atm after first flushing the headspace with N₂/CO₂ (80/20, v/v). Autoclaved (three consecutive daily 50 min cycles at 121°C) controls and abiotic (no sludge added) controls were run in parallel to account for the potential removal of MNA by abiotic mechanisms. Liquid samples were collected periodically to monitor the concentration of MNA and MNA biotransformation 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).

Methanogenic toxicity assays—Acetate (2.56 g L⁻¹ as CH₃COONa) was added as substrate for acetoclastic methanogens. MNA and MPD were dosed in the range 33-315 µM and 0.13-10.25 mM, respectively. Headspace samples (100 µL) were obtained periodically with a gas-tight glass syringe and analyzed for CH₄ content. The maximum specific methanogenic activity (g CH₄-COD g⁻¹ VSS d⁻¹) in the inhibitor-free control was calculated from the slope of the CH₄ content versus time graph. Methane production was monitored

across all treatment conditions and normalized to the inhibitor-free control, and defined as normalized activity (NA). Inhibition was defined as $100 - \text{NA} (\%)$.

2.5. Continuous-flow bioreactor study

An up-flow anaerobic sludge blanket (UASB) reactor ($L \times ID = 39 \times 14.5$ cm) with liquid volume of 360 mL was operated for 204 days to investigate the continuous treatability of MNA. The reactor was inoculated with anaerobic granular sludge (10 g VSS L^{-1}) and kept in the dark in a climate room at a temperature of $30 \pm 2^\circ\text{C}$. No mechanical mixing was applied in the reactor. The reactor was fed in upflow mode using a peristaltic pump. The feed consisted of the basal mineral medium (pH 7.2) supplemented with a mixture of volatile fatty acids (VFAs) consisting of (in mg COD L^{-1}): acetate (900), propionate (300), and butyrate (300), and a known concentration of MNA. Table 1 summarizes the conditions of operation and the performance of the bioreactor during the various periods of operation, *i.e.*, period I (0 MNA), period II ($39.4 \pm 18.8 \mu\text{M}$ MNA) and IIIa/IIIb ($79.7 \pm 12.0 \mu\text{M}$ MNA). The HRT was maintained at an average of 12.3 ± 0.5 h during period I, II and IIIa (day 0-176), thereafter during period IIIb the HRT was decreased to 6.1 ± 0.3 h. The reactor was incubated in the dark at $30 \pm 2^\circ\text{C}$. The influent was stored in a refrigerator at 4°C to prevent microbial degradation. The methane (CH_4) production was measured by liquid displacement using a 3% (w/v) NaOH solution to scrub out the CO_2 from the biogas. The performance of the reactors was monitored by measuring the CH_4 generation as well as the pH value, and concentration of VFAs, MNA and MPD in the influent and effluent. Additionally, effluent samples were collected on day 128 for mass spectrometry analyses to characterize anaerobic breakdown products from MNA.

2.6. Autoxidation experiments

Autoxidation was performed by placing reactor effluent or an MPD solution in an Erlenmeyer flask open to the atmosphere with a porous stone diffusing air during the exposure time (0–22 h). These experiments were performed at pH 5, 7, and 9, and the pH was adjusted accordingly using HCl or NaOH. Samples were weighed before and after the exposure period, and water loss due to evaporation was reconstituted.

2.7. Analytical methods

Methane was quantified in an HP5890 Series II gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with a flame ionization detector (GC-FID) and a Stabilwax-DA column (Restek Corporation, Bellefonte, PA, USA). The temperatures of the oven, the injector port and the detector were 140, 180, and 250°C , respectively. Helium was used as the carrier gas (5.2 mL min^{-1}), and air and hydrogen as flame source. The injection volume was 100 μL .

UV-VIS spectra of reactor effluent and MPD standards at different oxidation times were recorded from 200–600 nm using quartz cuvettes using a UV-1800 Shimadzu UV spectrophotometer (Columbia, MA, USA). Samples were diluted with a 50 mM phosphate buffer (pH = 7).

MNA in liquid phase was analyzed by an Agilent 1290 series (Santa Clara, CA, USA) high performance liquid chromatograph coupled to a diode array detector (HPLC-DAD). The mobile phase (MeOH/ H₂O, 50/50, v/v) was run isocratically at a flow rate of 1 mL min⁻¹ for 30 min in an E1 Acclaim Explosives column (4.6 × 240 mm, 5 μm) (Dionex, Salt Lake City, UT, USA) at room temperature. Retention time and wavelengths for detection were 10 min and 395 nm for MNA, and 3 min and 254 nm, for MPD.

High resolution mass spectra were acquired from 10 μL injections using UltiMate 3000 UHPLC-DAD (Dionex, Sunnyvale, CA) coupled to a TripleTOF® 5600 quadrupole TOF-MS (AB Sciex, Framingham, MA, USA) equipped with an electrospray ionization (ESI) source kept at 450°C in the positive mode. The HPLC method used was the same as described above. Information dependent acquisition (IDA) experiments were run in ESI positive mode, with 0.1 sec cycle time, 6 triggered ions per cycle, mass range 35-1000. 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 nitrogen. Data was processed with Analyst TF 1.6 with PeakView 1.2.0.3 and Formula Finder 1.1.0.0. Instrument calibration was performed through an atmospheric pressure chemical ionization (APCI) probe spanning a mass range of 35-1000. Some metabolites were detected using direct infusion (30 μL min⁻¹) into the Q-ToF-MS.

Other analyses, such as VSS and pH, were determined according to standard methods (American Public Health et al., 2005).

3. Results & Discussion

3.1 Anaerobic biotransformation of MNA in batch bioassays

MNA was readily biotransformed by the anaerobic culture amended with H₂ as shown in Fig. 2. The compound was completely removed within 72 h of incubation and it was transformed almost stoichiometrically to MPD. There was no significant elimination in the heat-killed and sludge-free controls, indicating that the transformation was mediated by microbial activity. In the heat-killed control, there was an initial decrease of MNA (within 4 h), that corresponded to 8.7% of the total MNA added, and may be attributed to adsorption of MNA onto anaerobic granular sludge. Anaerobic reduction of MNA was also observed in inoculated bioassays lacking H₂ addition (results not shown), but the MNA removal rate was 2-fold higher when H₂ was present (100 vs. 55 μM/day). The results indicate endogenous substrate in the anaerobic biomass can serve as electron donor for the MNA-reducing microorganisms in the sludge as was previously reported with other nitroaromatic compounds (Olivares et al., 2013). In anaerobic conditions, reduction of nitroaromatic compounds to the corresponding amino derivatives is a common pathway that occurs via hydroxylamino and nitroso derivatives (Gorontzy et al., 1993; Boopathy et al., 1998; Razo-Flores et al., 1999). Therefore, reduction of the nitro group in MNA to yield MPD was expected. Platten et al. (2010) also detected MPD during the anaerobic transformation of MNA using mass spectrometry. Although in the current study MNA was readily reduced to MPD, the latter was not degraded further under anaerobic conditions. However, some aromatic amines, namely those with hydroxyl or carboxylic groups, have been reported to be mineralized anaerobically (Razo-Flores et al., 1999). However, as the effluent leaves the

reactor, highly reactive aromatic amines may autoxidize upon exposure to air (Tan et al., 1999; Hassanein et al., 2008; Sierra-Alvarez et al., 2010).

3.2 Continuous anaerobic treatability of MNA-containing synthetic wastewater

The feasibility of treating a synthetic wastewater containing MNA was investigated in an anaerobic UASB bioreactor (Fig. 3). During the duration of this experiment, the pH of the influent and effluent were relatively constant, averaging 7.85 and 7.49, respectively. The bioreactor removed VFA with high efficiencies above 99% throughout the experiment, indicating that at the concentrations tested, MNA did not affect the performance of the bioreactor. The VFA removed were largely recovered as methane as is shown on Table 1. The concentration of MNA supplied to the reactor was increased gradually from 39.4 to 79.7 μM MNA to promote adaptation of the inoculum to the conversion of the aromatic substrate and prevent accumulation of toxic MNA in the effluent. The anaerobic bioreactor completely reduced MNA in the wastewater throughout the experiment even at the highest volumetric loading rate of MNA applied, $297.5 \pm 23.9 \mu\text{M MNA d}^{-1}$. The excellent removal efficiencies maintained in this experiment suggest that the anaerobic process has the potential to treat MNA loading rates exceeding the highest volumetric load tested. MPD was detected in the effluent of the bioreactor from day 63 to 169 often at sub-stoichiometric concentrations. The failure to detect MPD after day 169 was mainly due to the deterioration of the ability of the HPLC column to separate the aromatic amine. The analytical column used is designed for the separation of nitro-aromatic munitions compounds (Liu et al., 2006). Although the column could resolve MPD initially, with increasing usage time the retention time of this compound decreased gradually from 3 min until detection was no longer feasible. The incomplete recovery of MNA as MPD observed before day 169 could also be related to autoxidation of MPD during storage.

The anaerobic transformation products of MNA were not mineralized in the bioreactor as evidenced by the lack of removal of UV absorbance observed at 254 nm (UV_{254}), a wavelength that is strongly correlated with aromaticity (Weishaar et al., 2003). As an example, whereas the average UV_{254} absorbance of the influent between day 166 and 234 was 0.361 ± 0.033 , in the same period the average UV_{254} absorbance of the treated effluent was 0.473 ± 0.027 . Moreover, Platten et al. (2013) reported no mineralization of MNA and DNAN transformation products based on their COD data.

3.3 Autoxidation of MNA degradation products upon exposure to atmospheric oxygen

UV-VIS spectra—Some constituents in the effluent of the anaerobic reactor underwent chemical transformation when exposed to air. The fresh effluent which displayed a light brown color turned dark purple after a few hours of exposure to oxygen. Formation of black particles was also observed when the effluent was allowed to stand in the effluent collector container for several days. To further investigate these oxidative transformations, experiments were conducted where the anaerobic effluent as well as aqueous solutions of MPD – the main product of MNA biotransformation under anaerobic conditions – were subjected to active aeration by passing air through the solution using a fine diffuser. The pH values of the solutions were adjusted to pH 5, 7 and 9 to investigate the impact of pH on the oxidative reaction.

The results obtained indicated that the oxidative transformation of the byproducts from MNA biodegradation increased with aeration time and increasing pH values. Fig. 4 shows the UV absorbance of the solutions at 254 and 400 nm as a function of the aeration time. While 254 nm was chosen as an index of aromaticity, 400 nm has been used as an index for polymers (Koch et al., 2002). At 254 nm and pH 9, there was an increase in the UV absorbance within the first four hours, which then suddenly decreased by 24 hours. This observation was true for both the bioreactor effluent and the MPD standard, (Figs. 4A1-B1), and corresponded to an overall 30-56% decrease of the initial absorbance.. However, at pH 5 and 7, 254 nm absorbance was relatively stable. On the other hand, the absorbance at 400 nm increased with aeration time for all pHs (Figs. 4A2-B2). The effect was most noticeable at pH 7 and 9 in the effluent, and pH 7 for the MPD standard. Increased absorbance in this spectra region (250-500 nm) has been also observed for 2,4-diaminoanisole, the aromatic amine product from DNAN, exposed to air (Hawari et al., 2015). Globally, these observations on changes in absorbance at 254 and 400 nm lead us to hypothesize that as monomers form oligomers (increase at 400 nm) due to autoxidation, some of the oligomers precipitate out of solution (decrease in 254 nm).

Other studies have shown that hydroxylated aromatic amines, such as 4-aminophenol and 5-aminosalicylate, are readily autoxidized in the presence of air (Jensen et al., 1992; Razo-Flores et al., 1997). MPD and related phenylenediamines have also been shown to be unstable in aerobic environments and autoxidize readily when exposed to air (Babich et al., 1992; Platten et al., 2010). The autoxidation of some aromatic amines is catalyzed under alkaline conditions (Joseph et al., 1983; Schüsler-van Hees et al., 1985), which is in agreement with the increased reactivity observed in the present study when the pH of the MDP and effluent samples was increased from 5.0 to 9.0. Moreover, the presence and number of N-methyl groups in *p*-phenylenediamines have been reported to have higher autoxidation rates compared to non-substituted *p*-phenylenediamine (Babich et al., 1992).

LC-QToF-MS—To elucidate the nature of the compounds formed by autoxidation of MPD and other potential MNA degradation products in the effluent of the anaerobic bioreactor, liquid samples from bioreactor effluent and MPD were aerated at alkaline pH and analyzed using LC-QToF-MS. Two oligomers were detected in the oxidized MPD samples, an azo dimer ($m/z = 239$) and a tetramer ($m/z = 479$), corresponding to two and four MPD molecules coupled together (Fig. 1). Detailed information on the LC-QToF-MS spectra of both compounds can be found in the Supplementary Information section (Figs. SI 1-2). There difference between the measured and the theoretical monoisotopic masses for the dimer and tetramer were very small (6.6 and 0.4 ppm, respectively), supporting the proposed chemical structures. Formation of the azo dimer ($[M+H]^+$ $m/z = 239$) by anaerobic biotransformation of MNA has been reported previously (Platten et al., 2010). This structure corresponds to the azo dimer with a double bond between methylene and an amine as seen in Fig. 1. Amino-methylene moieties have been also detected in analogous azo dimers formed from DNAN anaerobic biotransformation (Olivares et al., 2013). It is possible that the double bond is formed during oxidation of the substituted amine. Besides the dimer and tetramer detected, a common feature in fresh and oxidized effluent samples, as well as oxidized MPD samples, was a series of ions ending in 0.9 ($m/z = 158.9, 226.9, 362.9, 430.9, 556.9$). While the

chemical structures of the corresponding compounds could not be elucidated, the repeating m/z difference of 136 suggests that they are formed by increased polymerization (Fig. SI-3). The 0.9 might indicate the presence of another element besides C, H, or N or a potential contamination.

Overall the results obtained indicate that although anaerobic treatment is highly effective in removing MNA, it is unable to mineralize the aromatic derivatives formed. The unstable nature of the degradation products formed upon MNA reduction and their tendency to undergo autoxidation in the presence of atmospheric oxygen has important implications for the treatment of these effluents. Although the oligomeric compounds formed are expected to be poorly biodegradable based on previous studies with azo dyes and other related compounds (Field et al., 1995; Stolz, 2001), these compounds are darkly colored and may cause aesthetic problems if the untreated effluent is discharged to the receiving environment. There is also some concern about the potential toxicity of the azo derivatives. Aromatic azo compounds have been reported to be toxic and mutagenic (Babich et al., 1992; Chung and Cerniglia, 1992; Puvaneswari et al., 2006). Therefore, additional treatment steps may be required if removal of the aromatic compounds is desired. The results obtained in this study confirmed that extended aeration of the anaerobically treated effluent promotes the oxidative transformation of MNA derivatives, particularly under mild alkaline conditions, suggesting the possibility of utilizing such process for effluent treatment. Alkali-catalyzed autoxidation has been shown previously to be an effective method to detoxify tannins, a major class of polyphenolic compounds, by promoting formation of high molecular weight polymers with reduced ability to interact with biological targets due to steric hindrance (Field et al., 1989; Field et al., 1990). Similarly to aromatic amines such as MPD, polyphenolic compounds undergo rapid autoxidation reactions that cause extensive polymerization.

3.4 Methanogenic toxicity of MNA and MPD

Besides understanding the transformations that MNA undergoes, it was necessary to assess the impact of MNA and its anaerobic degradation product, MPD, on the methanogenic activity of anaerobic granular sludge, as it may be critical for the operation of continuous-flow anaerobic bioreactors treating MNA-containing wastewaters. Methanogenesis is the final step in the degradation of organic matter in anaerobic environments and severe inhibition of methanogenic microorganisms can result in treatment failure. Figs. 5A1 and 5B1 show the time course of methane production in acetoclastic methanogenic bioassays exposed to increasing concentrations of MNA and MPD, respectively. The maximum specific methanogenic activities determined in these experiments were normalized based on the activity determined for the inhibitor-free control and the normalized rates as a function of MNA and MPD concentration are shown in Figs. 5A2 and 5B2). The results obtained indicate that exposure to MNA concentrations above 33 μM led to a decrease in the methanogenic activity of the anaerobic mixed culture (Figure 5A1). A nearly complete inhibition was observed at a MNA concentration of 315 μM . Anaerobic reduction of the nitro group in MNA, with formation of the amino derivative MPD, lead to a pronounced decrease in the structure's toxicity towards methanogenesis. The detoxification extent is evident when comparing Figs. 5A2 and 5B2. Whereas a MNA concentration of 103 μM was sufficient to cause 50% methanogenic inhibition (IC_{50}), exposure to the highest

concentration of MPD tested (1025 μM) only caused a small decrease in the methanogenic activity (13.9%).

It is interesting to note that exposure to MNA led to a lag phase in the production of methane, and the length of this lag phase was proportional to the MNA dose (Fig. 5A1). Since it was found that anaerobic granular sludge rapidly reduced MNA in inoculated cultures (Fig. 2), the onset of CH_4 production could be due to the transformation of MNA. A similar observation has been reported for DNAN in toxicity experiments with anaerobic granular sludge, where the reduction of the nitro-aromatic parent compound inhibited methane production, but as it was converted to aromatic amines, CH_4 started to be produced (Liang et al., 2013).

It has been reported that nitroaromatic compounds are more toxic than their aromatic amine analogs and that this difference could be attributed to the higher hydrophobicity of the nitroaromatic compounds. For example, Donlon et al. (1995) have reported a strong linear correlation between the inhibitory potential towards methanogenic microorganisms of structurally related aromatic amines and nitro aromatic compounds and their octanol-water partition coefficient (K_{ow}), widely used as an index of compound hydrophobicity. In spite of the considerably lower inhibitory potential of MPD towards acetoclastic methanogenic microorganisms compared to MNA, this aromatic amine could have adverse effects on other biological systems. Information on the microbial toxicity of N-methylated phenyldiamines is scarce but these compounds have been shown to be cytotoxic in *in vitro* studies with mouse fibroblasts (Babich et al., 1992). In addition, intermediate products from the reduction of nitro groups, such as hydroxylamino and nitroso derivatives, can have toxic and mutagenic effects (Padda et al., 2003; Roldan et al., 2008).

This study focused on anaerobic toxicity due to the microbial populations that may be present in the UASB reactor. However, upon exiting of the reactor, the effluent will likely become exposed to air. Phenylenediamines, such as MPD, are rapidly autoxidize in aerobic conditions. In these cases, MPD will transform, and perhaps the products from such autoxidation could pose toxic effects. Radical cations, superoxide radicals, and hydrogen peroxide can be produced when phenylenediamines autoxidize, and the rate of autoxidation has been correlated to toxicity and muscle damage in rats (Babich et al., 1992). Therefore, study of the impact of oxidative reactions likely to occur under environmental conditions on the toxicity of MNA and MPD degradation products deserves special attention. However, extensive polymerization of amines could be a another environmental fate or designed as post-treatment (section 3.3), leading to settleable polymers, and in turn, and eliminating toxicity risks by dropping out of the aqueous phase. Because of this, more toxicity and fate studies are needed that address autoxidation and polymerization of aromatic amine products from MNA and related compounds.

4. Conclusions

Overall, our findings indicate that MNA is readily reduced by anaerobic sludge in batch and continuous to form MPD, but there was no mineralization detected. A general transformation route is shown in Fig. 1. MNA inhibited methanogenic activity, but MPD was

considerably less inhibitory. MPD was unstable in aerobic environments and autoxidized. Azo oligomer products from MPD were detected in autoxidation experiments, particularly at neutral and higher pH..

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Continuous reduction of N-methyl-p-nitroaniline (MNA) to p-phenylenediamine (MPD) was achieved in an UASB reactor amended with volatile fatty acids.
- MPD is unstable in aerobic conditions and formed polymers upon exposure to air and pH 7.
- MNA severely inhibited acetoclastic methanogens ($IC_{50} = 103 \mu M$), but MPD caused considerably less inhibition.

Fig. 1. Proposed general biotransformation pathway for N-methyl-p-nitroaniline (MNA). Figures in brackets were not detected but are known intermediates of nitroreduction to amino groups.

Fig. 2.
MNA transformed in different treatments: H_2 added (●), heat-killed (■), no sludge added (x). MPD formed (○) in H_2 added treatment.

Fig. 3.
 Panel A - MNA present in the influent (●) and effluent (x) of the UASB reactor. Panel B - MNA loading rate(○).

Fig. 4.

Changes in signal at 254 (row 1) and 400 (row 2) nm at pH 5 (▲), 7 (□), and 9 (●) for reactor effluent (column A) and MPD (column B) exposed to air. The reactor effluent was collected after 204 days of operation.

Fig. 5. MNA (column A) and MPD (column B) toxicity to acetoclastic methanogens. Methane production (row 1) and normalized methanogenic activity curves (row 2). Concentrations tested (1): toxicant-free control (●), MNA – 33 (□), 66 (▲), 131 (○), 230 (■), 315 () μM. MPD – 0.13 (□), 0.64 (▲), 1.28 (○), 2.56 (■), 5.13 (), 10.25 (◆) mM.

Table 1

Conditions of operation and performance of a laboratory-scale reactor treating MNA-containing wastewater during different periods of operation. The feed was supplied with an average concentration of volatile fatty acids of 1.51 ± 0.16 g COD L⁻¹.

	Days	HRT	MNA (influent) (μM)	MPD (effluent) (μM)	MNA loading rate (μM L ⁻¹ d ⁻¹)	MNA removal efficiency ** (%)	VFA removal efficiency ** (%)	VFA conversion to CH ₄ (%)
Period I	0-32	12.3±0.5	0	N/A	N/A	N/A	99.4±0.4	89.7±7.3
Period II	33-67	12.5±0.4	39.4±18.8	11.2±67	73.5±35.4	100	99.8±0.2	96.2±7.2
Period IIIa	68-176	12.2±0.5	80.5±12.9	81.8± 28.1 *	162.9±52.5	100	99.8±0.4	93.8±5.0
Period IIIb	177-204	6.1±0.3	76.5±6.5	*	297.5±23.9	100	99.8±0.1	95.1±6.0

* After day 169, MPD detection became erratic

** Removal efficiency determined solely on remainder MNA or VFA detected at the effluent.