Journal of Hazardous Materials 262 (2013) 281–287

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Microbial toxicity of the insensitive munitions compound, 2,4-dinitroanisole (DNAN), and its aromatic amine metabolites

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HIGHLIGHTS

- The microbial toxicity of DNAN and its metabolites was assayed.
- DNAN caused severe inhibition of methanogens, nitrifiers, and *Aliivibrio fischeri*.
- The aromatic amine metabolites were generally less toxic than DNAN.

ARTICLE INFO

Article history:
Received 25 June 2013
Received in revised form 9 August 2013
Accepted 17 August 2013
Available online 28 August 2013

Keywords:
2,4-Dinitroanisole
2-Methoxy-5-nitroaniline
2,4-Diaminoanisole
Microbial toxicity
Microtox

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 toward 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 (IC_{50}) ranging 41–57 μM), but was notably less inhibitory to aerobic heterotrophs (IC_{50} > 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 toward *A. fischeri* and nitrifying bacteria given the similar IC_{50} values determined for MENA and DNAN in these assays. These results indicate that reductive biotransformation could reduce the inhibitory potential of DNAN.

1. 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 [1]. 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) [2]. 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 [2].

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 [3,4]. DAAN has also been shown to be mutagenic and a potential carcinogen in various assays [5–8]. However, data on the inhibitory potential of DNAN toward 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 [9], and to 2,4-diaminoanisole (DAAN) in anaerobic conditions [10]. Recently we have also reported that microorganisms present in conventional

Abbreviations: DNAN, 2,4-dinitroanisole; MENA, 2-methoxy-5-nitroaniline; DAAN, 2,4-diaminoanisole; TNT, 2,4,6-trinitrotoluene; IC50, 50% inhibition concentration; RAS, return activated sludge; VSS, volatile suspended solids.

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http://dx.doi.org/10.1016/j.jhazmat.2013.08.046

wastewater treatment systems can reduce the nitro group in DNAN under aerobic, microaerophilic and anaerobic conditions [11]. 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.

2. Materials and methods

2.1. 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 4000 rpm) and the supernatant was discarded before use in bioassays.

2.2. Culture media

The basal mineral medium labeled "**medium 1**" contained (in mg L^{-1}): K_2HPO_4 (250), $CaCl_2{\cdot}2H_2O$ (10), $MgSO_4{\cdot}7H_2O$ (100), $MgCl_2{\cdot}6H_2O$ (100), NH_4Cl (280), $NaHCO_3$ (4000), yeast extract (100). The basal medium termed "**medium 2**" contained (in mg L^{-1}): NaH_2PO_4 (1500), Na_2HPO_4 (894), NH_4Cl (164), $NaHCO_3$ (899). All media were supplemented with 1 mL L^{-1} of trace element solution [11]. The pH of the basal medium was adjusted to 7.2 with HCl or NaOH, as required.

2.3. Microbial inhibition bioassays

2.3.1. 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}.

2.3.2. 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 (0–1500 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}.

2.3.3. 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 (0–1300 μM), DAAN (0–300 μM), and 2,4-dinitrophenol (0–650 μM) solutions were tested. Microbial inhibition in Microtox was measured at 25 °C in triplicate experiments as previously described [12].

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.

2.4. Analytical methods and chemicals

DNAN, MENA, and DAAN were quantified by high-performance liquid chromatograph with diode array detection as previously described [11]. Methane and oxygen in gas samples were determined by gas chromatography with flame ionization- and thermal conductivity detection, respectively [13]. 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).

3. Results

3.1. Methanogenic inhibition

Fig. 1A illustrates the time course of methane production in methanogenic activity assays amended with DNAN. The specific