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## Microbial toxicity and characterization of DNAN (bio)transformation product mixtures

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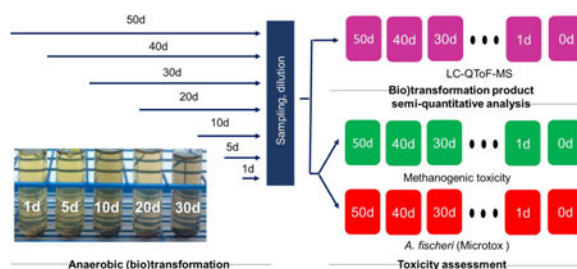
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### Abstract

2,4-Dinitroanisole (DNAN) is an emerging insensitive munitions compound. It undergoes rapid (bio)transformation in soils and anaerobic sludge. The primary transformation pathway catalyzed by a combination of biotic and abiotic factors is nitrogroup reduction 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 monomer products and azo-dimer and trimer surrogates to acetoclastic methanogens and the marine bioluminescent bacterium, *Allivibrio fischeri*, were evaluated. Methanogens were severely inhibited by 3-nitro-4-methoxyaniline (MENA), with a 50%-inhibiting concentration (IC<sub>50</sub>) of 25 μM, which is more toxic than DNAN with the same assay, but posed a lower toxicity to *A. fischeri* (IC<sub>50</sub> = 219 μM). On the other hand, N-(5-amino-2-methoxyphenyl) acetamide (Ac-DAAN) was the least inhibitory test-compound for both microbial targets. Azo-dimer and trimer surrogates were very highly toxic to both microbial systems, with a toxicity similar or stronger than that of DNAN. A semi-quantitative LC-QTOF-MS method was employed to determine product mixture profiles at different stages of biotransformation, and compared with the microbial toxicity of the product-mixtures formed. Methanogenic toxicity increased due to putative reactive nitroso-intermediates as DNAN was reduced. However, the inhibition later attenuated as dimers became the predominant products in the mixtures. In contrast, *A. fischeri* tolerated the initial biotransformation products but were highly inhibited by the predominant azo-dimer products formed at longer incubation times, suggesting these ultimate products are more toxic than DNAN.

### Graphical abstract

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## Keywords

munitions; 2,4-dinitroanisole; biotransformation; microbial toxicity; dimers; liquid chromatography quadrupole time-of-flight mass spectrometry

## 1. 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) (Davies et al., 2006). Once released to the environment, DNAN is readily (bio)transformed via nitro-reduction in natural and engineered systems by a combination of abiotic (Ahn et al., 2011; Niedzwiecka et al., 2013; Hawari et al., 2015; Linker et al., 2015; Olivares et al., 2016) and biotic processes (Platten et al., 2010; Olivares et al., 2013; Olivares et al., 2016). The initial nitro-reduction occurs with a dominant regioselectivity towards the *ortho* nitro group (Platten et al., 2010; Perreault et al., 2012; Hawari et al., 2015; Olivares et al., 2016), yielding 2-methoxy-5-nitroaniline (MENA), but its regioisomer 3-methoxy-4-nitroaniline (iMENA) has been found in few studies (Schroer et al., 2015; Olivares et al., 2016). Continued nitro-reduction leads to the aromatic amine 2,4-diaminoanisole (DAAN) and reactive intermediates that form azo-dimers (Platten et al., 2010; Olivares et al., 2013; Olivares et al., 2016). Parallel anaerobic (bio)transformation pathways have been reported, such as N-acetylation of products of nitrogroup reduction (Perreault et al., 2012; Olivares et al., 2013), as well as N-methylation and step-wise O-demethoxylation of azo-dimers (Olivares et al., 2013; Olivares et al., 2016), creating a large array of possible products.

As DNAN is transformed, its products may pose an environmental hazard yet to be characterized. Most toxicity studies have been focused on the parent compound, DNAN (Dodard et al., 2013; Kennedy et al., 2015), but little attention has been given to its (bio)transformation products. While there has been an initial characterization of the microbial toxicity of the primary DNAN nitrogroup-reduction-products (Liang et al., 2013), the toxicity posed by the majority of transformation products is unknown.

Therefore, the occurrence of (bio)transformation products should be assessed and put in context with their toxicity potential. 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 temporal changes in product mixture composition to toxicity changes during transformation using acetoclastic methanogens and *A. fischeri*, as microbial toxicity targets.

## 2. Materials and Methods

### 2.1 Chemicals and biological materials

Fig. 1 shows the chemical structures of monomer DNAN (bio)transformation products and azo-dimer/trimer surrogates assayed. 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 Butnersoil (water content = 20.7%), described in (Krzmarzick et al., 2015; Olivares et al., 2016), were sieved (2 mm) and stored at 4 °C before use. 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 assays.

### 2.2 Staggered DNAN soil/sludge (bio)transformation assays

DNAN was incubated anaerobically in soil or sludge microcosms to obtain product mixtures at different (bio)transformation stages. DNAN (500 µM final concentration) was added to 10 mL of mineral medium (Olivares et al., 2013) (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 soil (100 mg wet) or sludge (75 mg wet). The headspace was flushed with He/CO<sub>2</sub> (80/20 %). The tubes were sealed with t-butyl caps and aluminum seals, and incubated in the dark at 30°C in an orbital shaker (115 rpm). Tubes were prepared at different times and incubated immediately so that when samples were collected on the same day, the overall (bio)transformation time elapsed would be 0, 1, 5, 10, 20, 30, 40, or 50 days of anaerobic incubation. Sampling was performed in an anaerobic hood to minimize oxidation of 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 the Supplementary Material, Fig. S-1.

### 2.3 Microbial toxicity assays

**2.3.1 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 (Liang et al., 2013), inoculated with 1.5 g VSS L<sup>-1</sup> 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<sub>2</sub> (80/20% v/v) and crimped with aluminum seals and t-butyl rubber septa. The bottles were incubated overnight, and flushed with He/CO<sub>2</sub> (80/20% v/v) before addition of the individual test-compounds: iMENA (10-200 μM), Ac-DAAN (80-8,000 μM), dimer L (4-83 μM), and BBY (0.12-1.92 μM). In addition, mixtures of biotransformation products from a starting DNAN concentration of 500 μM diluted 54-fold in aqueous solutions were utilized in the methanogenic toxicity bioassays. The dilution was chosen based on preliminary experiments to avoid complete methanogenesis inhibition that would prevent toxicity changes to be detected. Separate samples of these aqueous solutions containing mixtures of biotransformation products were harvested at different stages of DNAN (bio)transformation to detect temporal changes in toxicity reflecting the shifting mixture of intermediates and products during (bio)transformation. All experiments were run in duplicate.

CH<sub>4</sub> was monitored for each treatment and the toxicant-free control (Fig. S-2) using a gas chromatograph coupled to a flame ionization detector (GC-FID) (Ochoa-Herrera et al., 2009). The rate of CH<sub>4</sub> production was used to determine acetoclastic methanogen inhibition by comparing the rate of methane production in treatments normalized to a test-compound/mixture-free control.

**2.3.2 A. fischeri (Microtox)**—The Microtox (Bulich and Isenberg, 1981) assay was also used to characterize the toxicity of the (bio)transformation products/surrogates. Bioluminescence after 30 min chemical exposure was monitored. The individual compounds tested included iMENA (2.5-650 μM), Ac-DAAN (9-2,285 μM), dimer L (29-226 μM), and BBY (0.40-103 μM). As described for the acetoclastic methanogenesis assay, aqueous mixtures of biotransformation compounds from a starting DNAN concentration of 500 μM that were recovered from different stages of DNAN (bio)transformation at a single 36-fold dilution were also evaluated in the Microtox toxicity bioassays. In preliminary tests, this dilution allowed for toxicity changes to be detected in Microtox without being completely inhibitory. All experiments were run in duplicate.

## 2.4 Analytical methods to assess (bio)transformation products

**2.4.1 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 × 100 mm, 2.2 μm) (Thermo Fisher Scientific, Waltham, WA, USA) and a methanol/H<sub>2</sub>O eluent (isocratic 40/60, v/v, 0.25 mL min<sup>-1</sup>) 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.

**2.4.2 UHPLC-Q-ToF-MS**—MENA, DAAN, and oligomer products in the liquid phase were semi-quantified without internal standards on an UltiMate 3000 UHPLC (Dionex, Sunnyvale, CA) using the same chromatography parameters and column described above for UHPLC-DAD, that was coupled to a TripleTOF 5600 quadrupole time-of-flight mass

spectrometer (Q-ToF-MS) (AB Sciex, Framingham, MA, USA). The Q-ToF-MS was run with an electrospray ionization source in positive mode at 450°C with a capillary setting of 5.5 kV, and declustering potential of 80 V. N<sub>2</sub> was used as curtain gas, desolvation gas, and nebulizer gas at 30, 35, and 35 psi, respectively. Transformation products were detected and quantified by integrating accurate, selected parent ion (M+H<sup>+</sup>) mass chromatogram peaks from a survey scan (m/z 30-1000) based on newly detected, and previously characterized (Olivares et al., 2013), transformation products. Both of these are shown in Table 1: previously characterized products (above) and *m/z* values for the uncharacterized products (below; [M+H]<sup>+</sup> (retention time, min)). A potential cation [M]<sup>+</sup> with *m/z* 247.0425\* (retention time 1.52 min) was also detected. Semi-quantitative comparisons of transformation product abundances are based on integrated peak areas of parent mass ions extracted from mass chromatograms using a 10 mDa window centered on the calculated masses. AnalystTF 1.6 with PeakView 1.2.0.3 and MultiQuant version 2.1 were used to develop the ion list and to quantify individual ion masses from integrated mass chromatogram peaks.

Other analyses, including pH and volatile suspended solids content in sludge, were performed according to standard methods (APHA, 2005).

### 3. Results and Discussion

#### 3.1 Biotransformation product profiles

An overview of (bio)transformation products and transformation pathways observed in this work are shown in Fig. 1 Panel A. DNAN underwent nitro-reduction primarily to MENA (but also to iMENA), and subsequent nitro-reduction led to DAAN, as reported for anaerobic soil and sludge systems (Platten et al., 2010; Olivares et al., 2013; Olivares et al., 2016). Nitro-group reduction is known to occur biologically via the broad family of nitroreductases (Roldan et al., 2008) or by abiotic factors, such as ferrous iron adsorbed to mineral surfaces (Klausen et al., 1995; Elsner et al., 2004) and sulfide via natural organic matter electron transfer (van Beelen and Burris, 1995). During the course of nitro-group reduction, reactive hydroxylamino and nitroso intermediates are formed (Bryant and DeLuca, 1991; Spain, 1995), which can lead to further reactions. A putative nitroso intermediate coupled with DAAN to form an azo-dimer, 3,3'-diamino-4,4'-dimethoxy-azobenzene (*m/z* 273). In anaerobic environments, azo-dimerization could be explained by direct coupling of nitroso- and amino-derivatives, which has been used in azo-dye syntheses under reducing conditions (Moglie et al., 2008; Zhao et al., 2011). In some cases, *m/z* 273 underwent reductive azo-cleavage to DAAN via a hydrazo intermediate, 3,3'-diamino-4,4'-dimethoxy-hydrazobenzene (*m/z* 275). Reductive azo-cleavage via hydrazo intermediates has been shown to occur in biological reducing environments (Bin et al., 2004). In addition to the main (bio)transformation pathway, there were secondary routes. For instance, the detection of Ac-DAAN indicated DAAN underwent N-acetylation, a reaction reported to occur biologically in anaerobic conditions with aromatic amines (Razo-Flores et al., 1999). Another parallel pathway involved reactions of azo-dimer *m/z* 273, such as N-methylation to yield 5-((3-Amino-4-methoxyphenyl)diazanyl)-2-methoxy-N-

methyleaniline( $m/z$  285) and O-demethoxylation, forming 4' methoxy-3-methylamino-3' methyleneamino-azobenzene( $m/z$  269).

DNAN was (bio)transformed by microbial and abiotic processes in soil (Hawari et al., 2015; Olivares et al., 2016) and sludge (Platten et al., 2010; Olivares et al., 2013). DNAN was readily reduced within 10 days in the anaerobic sludge and in the Camp Navajo soil, while for the Camp Butner soil, DNAN was completely removed by 30 days (Fig. 2A). The specific rate of reduction per unit dwt solids was highest for the sludge and Camp Navajo soil (45.6 and 56.7  $\mu\text{mol DNAN d}^{-1} \text{dwt g}^{-1}$ ), while Camp Butner had the slowest (14.3  $\mu\text{mol DNAN d}^{-1} \text{g dwt soil}^{-1}$ ).

Very limited amounts of MENA and DAAN were detected during (bio)transformation based on HPLC-DAD (Fig. S-3), suggesting that additional transformation products had been formed. Completing a mass balance accounting for DNAN (bio)transformation has been problematic when relying exclusively on HPLC-DAD (Olivares et al., 2013). While additional products can be confidently identified by mass spectrometry techniques, not all of the compounds identified can be quantified (Platten et al., 2010; Perreault et al., 2012; Olivares et al., 2013).

The individual profile of (bio)transformation product mixtures formed at different stages of DNAN (bio)transformation for the two soils, Camp Butner (Fig. 2A) and Navajo (Fig. 3A), as well as for the anaerobic sludge (Fig. 4A), were determined using LC-QToF-MS to detect products that were below detection in the 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 ( $3 \times 10^7$  area units for DAAN). A higher amount of DAAN in the system might result from better reducing conditions. For instance, 2,4,6-triaminotoluene from TNT reduction has only been reported below -200 mV (Esteve-Núñez et al., 2001). A hydrazo compound ( $m/z$  275 in Fig. 1) had a higher occurrence in the sludge (Fig. 4) and is believed to be an intermediate formed during the reductive cleavage of azo-dimers and 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 20 d of (bio)transformation, but overall MENA was at least twice as 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 MENA, iMENA, DAAN, and Ac-DAAN detectable by QToF-MS in all three systems studied, suggesting that dimerization occurs rapidly during nitro-reduction. However, at longer incubation times (9-30 days), the major abundant species were 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. 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 (Thorn and Kennedy, 2002). In anaerobic sludge, the primary dimers detected included  $m/z$  285 and 269, with significant contributions of  $m/z$  273 and 275.

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 (Figs. S-5 through S-7). 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 (Fig. S-6). Camp Navajo has more organic carbon than Camp Butner (5.24 versus 2.07 % dwt soil) (Krzmarzick et al., 2015), 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 (Drzyzga et al., 1999).

Data obtained from the semi-quantitative mass spectrometry analysis allowed a limited interpretation of the cumulative mass chromatogram peak areas. Because the method lacked calibration standards, it mainly provided temporal comparative information for each compound, since different ionization efficiencies likely exist between compound species; although magnitude differences in abundance between separate species may also be interpreted. And although matrix effects may exist, they were restrained by use of the same matrix (a mineral salts basal medium) in all biotransformation mass spectrometry analyses.

### 3.2 Monomeric (bio)transformation products as pure compounds

Based on the (bio)transformation products detected in this work, a chemical library with monomer products and best available surrogates for azo-dimer and trimer compounds was created for microbial toxicity assessment. Most monomeric metabolites from DNAN reduction had decreased microbial toxicity with the exception of the MENA isomers (Table 2, Fig. 5). DAAN and especially Ac-DAAN were many fold less toxic than DNAN, whereas iMENA and MENA were more toxic than DNAN in the case of methanogens and Microtox, respectively. This is consistent with nitroaniline isomers from nitroreduction of 2,4-

dinitrotoluene being more toxic than the parent compound in *A. fischeri* (Dodard et al., 1999). 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 (Donlon et al., 1995). During the course of (bio)transformation, regiospecificity in DNAN (bio)conversion could impact the overall toxicity. For instance, nitro-reduction of DNAN has predominantly been reported in the *ortho* group in biological systems (Perreault et al., 2012; Olivares et al., 2013; Hawari et al., 2015), whereas evidence of *para*-nitro-reduction has been found primarily in abiotic systems (Ahn et al., 2011; Hawari et al., 2015), with one account of biological production (Schroer et al., 2015). Dominance of nitroreduction in the *ortho* group has been attributed to overall molecular stability due to H-bonding with the neighboring methoxy group (Hawari et al., 2015). Given these considerations, the toxicity of the nitroaniline isomers formed from DNAN nitro-reduction is expected to be primarily due to MENA.

On the other hand, there are other reactions during (bio)transformation that could yield less toxic products. Complete nitro-reduction to aromatic amines has been considered as a detoxification mechanism (Gorontzy et al., 1993), and aromatic amines were generally less cytotoxic than nitroaromatic counterparts to methanogens (Donlon et al., 1995; Liang et al., 2013) and *A. fischeri* (Neuwoehner et al., 2007; Liang et al., 2013). In a previous study we reported DAAN as less toxic than DNAN or MENA in both microbial assays (Fig. 5) (Liang et al., 2013). In addition, other transformation pathways could further decrease the inhibition potential of aromatic amines. For instance, DAAN N-acetylation resulted in a considerable decrease in toxicity (Fig. S-3 panels A1,B1 and Fig. 5). 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<sub>50</sub>) of 911 μM 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 (Tweedy et al., 1970; Bruns-Nagel et al., 2000).

### 3.3 Surrogates of dimers and trimers

The azo-oligomers tested, dimer L and BBY, were among the compounds causing the strongest toxicity. Dimer L was a potent toxicant to *A. fischeri* (IC<sub>50</sub>=29.8 μM) and methanogens (IC<sub>50</sub> = 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 (Honeycutt et al., 1996). BBY was found to be the most toxic of all compounds tested with an IC<sub>50</sub> in each of the microbial systems of 0.7 μM. Even though both systems had the same IC<sub>50</sub>, *A. fischeri* were more sensitive than methanogens, since complete inhibition occurred at 1.4 μM (Fig. S-3 Panel A2).

The IC<sub>50</sub> values reported in this work are based on the molarity of the chemical species. However, a comparison of toxicity for micromolar monomer equivalents (two for dimer L, three for BBY), reveals dimer L to be less toxic than DNAN (open symbols, Fig. 5), and



BBY to be the most toxic tested in this work, and still considerably more toxic than DNAN (Fig. 5).

### 3.4 Exposures to DNAN (bio)transformation mixtures

**3.4.1 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 targets were very susceptible to toxicity of the mixture of (bio)transformation products. The sample mixtures were diluted significantly (54-fold for methanogens and 36-fold for *A. fischeri*) before exposure, because at lower dilutions, there was complete inhibition in both assays which did not allow observable differences in inhibition across the (bio)transformation timeline.

At early stages of DNAN bioconversion, methanogenic activity dropped sharply across all systems assayed (Figs. 2B-4B). This period of increased inhibition lasted as long as DNAN was detectable in aqueous solutions. The dip in activity was the shortest in anaerobic sludge (1d) (Fig. 4B), while for Camp Butner (with the slowest rate of DNAN (bio)conversion), it lasted for 30 days (Fig. 3B).

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 (Gorontzy et al., 1993). 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 (Honeycutt et al., 1996). DNAN nitroso-derivatives have been found in abiotic (Hawari et al., 2015) and biotic systems (Olivares et al., 2016), as well as for other nitroaromatic compounds such as 2,4-dinitrotoluene (Liu et al., 1984). Hydroxylamino products have also been reported in DNAN (Perreault et al., 2012; Olivares et al., 2016) and TNT (Knicker et al., 2001) 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 (Smith and March, 2006) or amino derivatives (Moglie et al., 2008; Zhao et al., 2011).

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. In all cases, after the initial inhibition, there was a consistent recovery and detoxification compared to the initial stage of (bio)transformation, eventually achieving restoration of 100% activity compared to the controls. Thus, the complete toxicity profile of DNAN (bio)transformation towards methanogens could be described by a Big Dipper constellation pattern (particularly in Fig. 2B), with initial inhibition and latter activity recovery.

For *A. fischeri*, there was little inhibition during initial (bio)transformation of DNAN in all systems, but inhibition started to increase when more than half of the DNAN had been converted (Figs. 2B-4B). Unlike methanogens, *A. fischeri* may 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 (Wang et al., 2010). 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 was also significant detection of dimer products ( $m/z > 200$ ), which could be directly responsible for affecting bioluminescence due to inhibition in growth. With Camp Navajo soil, 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 dimer products. Based on the oscillation of toxicity from 20-50d in the anaerobic sludge system, dimer abundance might be directly related to *A. fischeri* inhibition.

## 4. Conclusions

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. Increased methanogenic inhibition during nitroreduction of DNAN. This increase is attributed to putative reactive nitroso-intermediates, while mixtures rich with azo-dimers were severely toxic to *A. fischeri*. On the other hand, N-acetylation greatly reduced toxicity of DNAN (bio)transformation products. N-acetylation and azo-dimerization of amines are transformation pathways that have been reported to be in direct competition with each other (Tweedy et al., 1970). Both of these processes have been identified in microbial systems in the presence of DNAN (Perreault et al., 2012; Olivares et al., 2013), 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.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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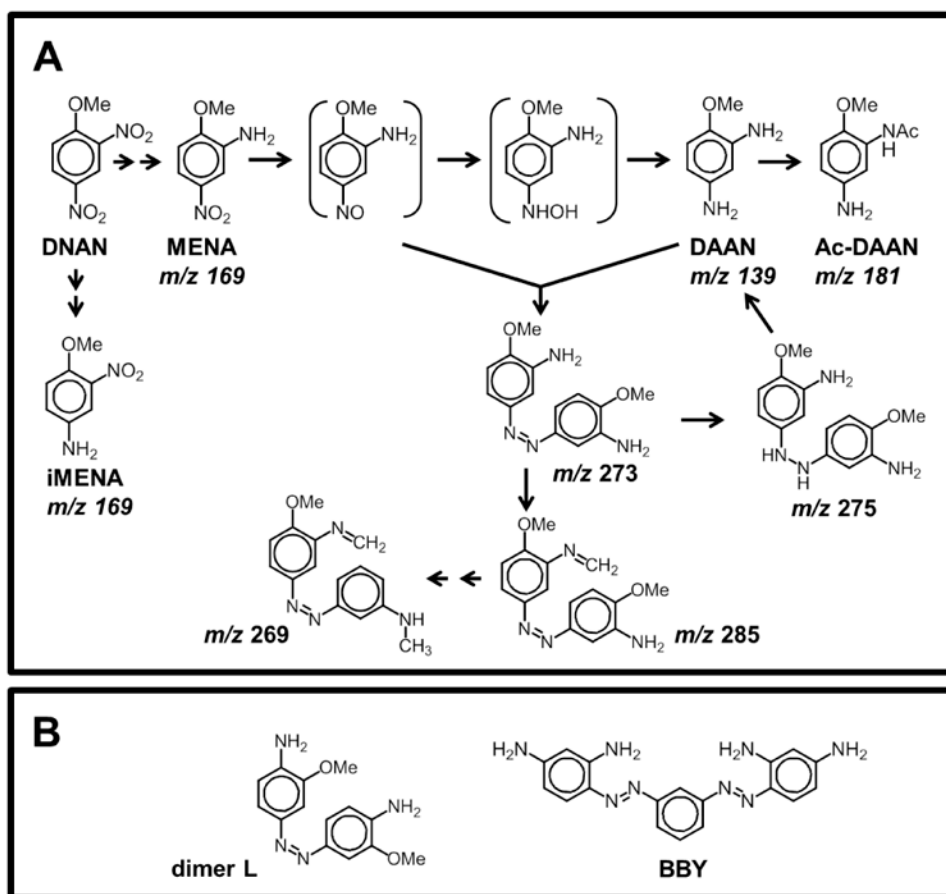
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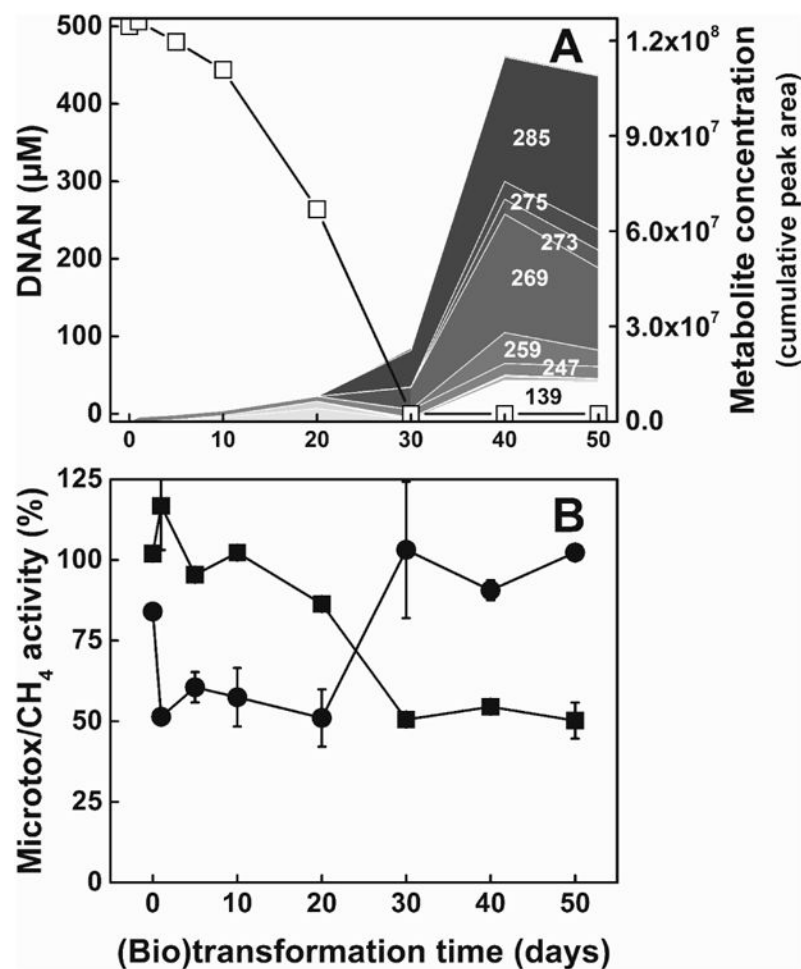
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### Highlights

- DNAN (bio)transformation products caused microbial toxicity.
- 3-nitro-4-methoxyaniline caused 50% inhibition to methanogens at 25  $\mu\text{M}$ .
- N-(5-amino-2-methoxyphenyl) acetamide was the least inhibitory compound.
- Azo-dimer and trimer surrogates were of similar or greater toxicity than DNAN.
- Methanogens were inhibited by putative reactive intermediates during nitro-reduction.

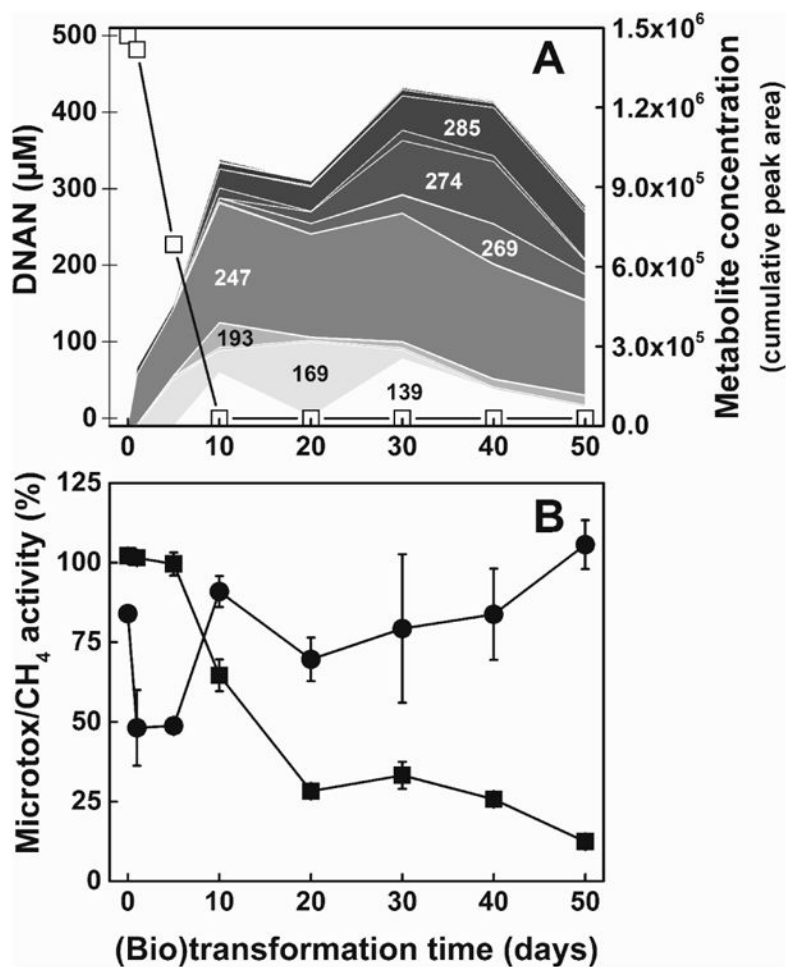


**Fig. 1.** Panel A: (Bio)transformation pathways of DNAN in anaerobic incubations of soils and sludge. Microbial toxicity was evaluated for the monomer compounds shown. Panel B: Azo-oligomer surrogate compounds used for microbial toxicity. Notation: OMe = methoxy; Ac = acetyl. MENA and iMENA are isobaric regioisomers.

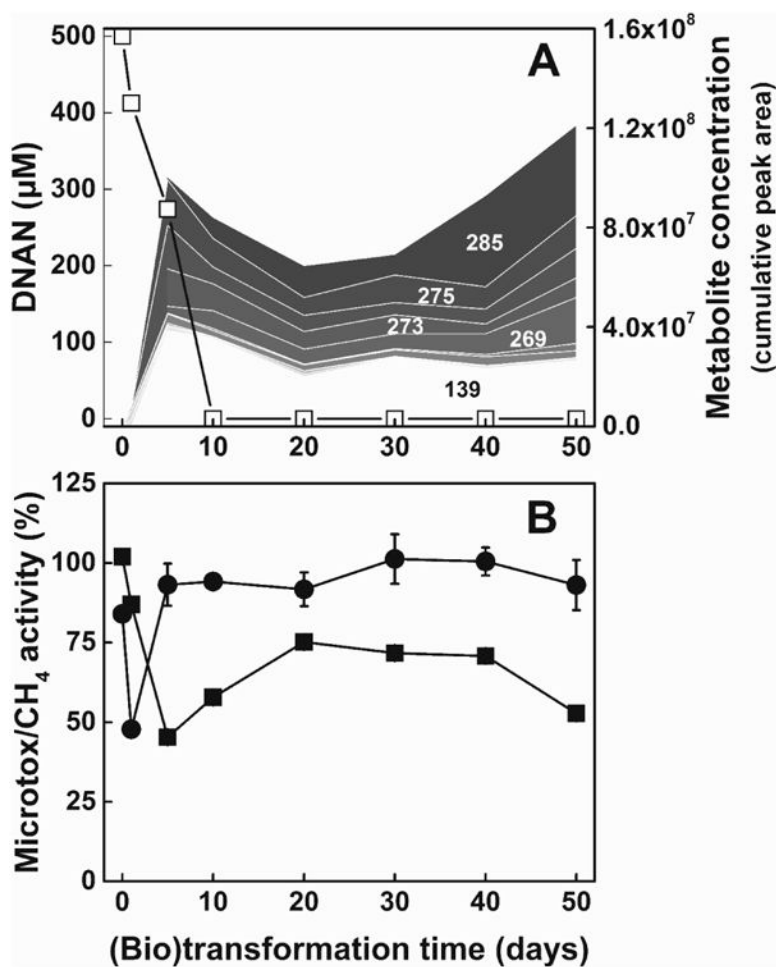


**Fig. 2.** DNAN anaerobic (bio)transformation in Camp Butner soil and collective microbial toxicity of the product mixtures during incubation. Panel A: DNAN (□) formed transformation products (stacked area, from light to dark in ascending molecular mass):139.0866, 165.0659, 169.0608, 181.0972, 185.0652, 193.0607, 228.0768, 243.0877, 243.1241, 245.1300, 247.0425, 259.1190, 267.0975, 269.1397, 273.1347, 274.0715, 275.1503, 285.1347, 299.1179, 301.1289, 313.1289, 325.1659, 327.1452, 431.1569. The predominant [M+H]<sup>+</sup> values are underlined and are also labeled in the plot. Panel B: Impact of mixtures of transformation products formed during (bio)transformation on *A. fischeri* bioluminescence (■) and acetoclastic methanogens (●).

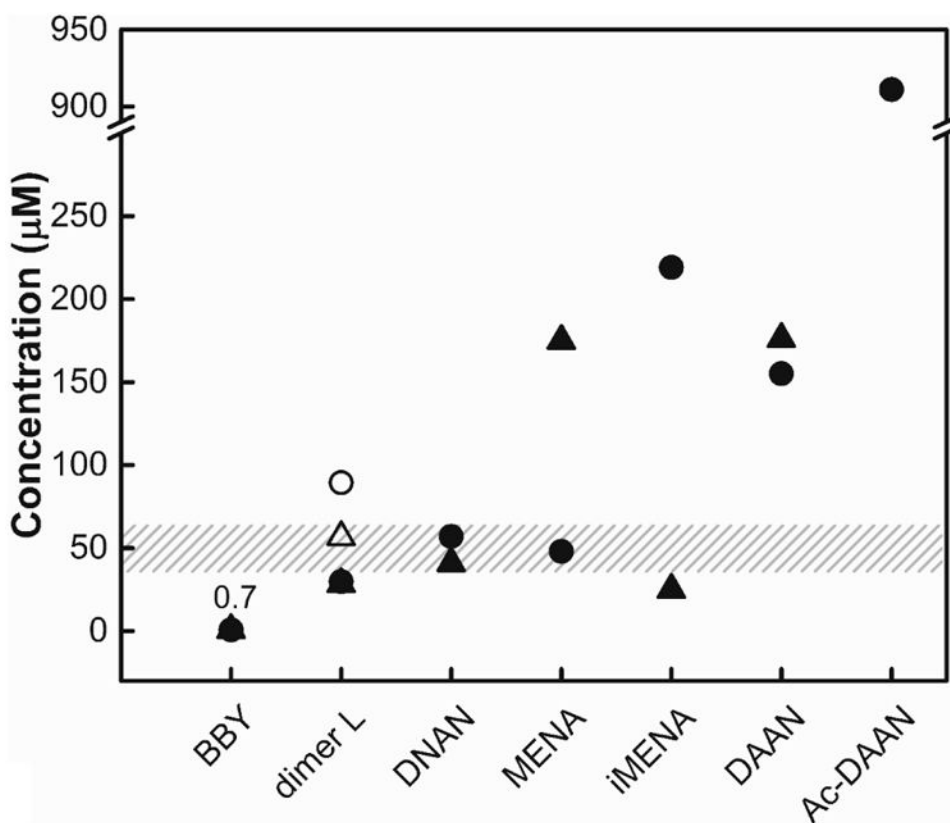




**Fig. 3.** DNAN anaerobic (bio)transformation in Camp Navajo soil and collective microbial toxicity of the product mixtures during incubation. Panel A: DNAN (□) formed transformation products (stacked area, from light to dark in ascending molecular mass): 139.0866, 165.0659, 169.0608, 181.0972, 185.0652, 193.0607, 228.0768, 243.0877, 243.1241, 245.1300, 247.0425, 259.1190, 267.0975, 269.1397, 273.1347, 274.0715, 275.1503, 285.1347, 299.1179, 301.1289, 313.1289, 325.1659, 327.1452, 431.1569. The predominant [M+H]<sup>+</sup> values are underlined and are also labeled in the plot. Panel B: Impact of mixtures of transformation products formed during (bio)transformation on *A. fischeri* bioluminescence (■) and acetoclastic methanogens (●).



**Fig. 4.** DNAN anaerobic (bio)transformation in anaerobic sludge and collective microbial toxicity of the product mixtures during incubation. Panel A: DNAN ( $\square$ ) formed transformation products (stacked area, from light to dark in ascending molecular mass): 139.0866, 165.0659, 169.0608, 181.0972, 185.0652, 193.0607, 228.0768, 243.0877, 243.1241, 245.1300, 247.0425, 259.1190, 267.0975, 269.1397, 273.1347, 274.0715, 275.1503, 285.1347, 299.1179, 301.1289, 313.1289, 325.1659, 327.1452, 431.1569. The predominant  $[\text{M}+\text{H}]^+$  values are underlined and are also labeled in the plot. Panel B: Impact of mixtures of transformation products formed during (bio)transformation on *A. fischeri* bioluminescence ( $\blacksquare$ ) and acetoclastic methanogens ( $\bullet$ ).



**Fig. 5.** Fifty-percent inhibition concentrations (IC<sub>50</sub>) for acetoclastic methanogens (▲) and *A. fischeri* (●) for DNAN and its (bio)transformation products and azo-oligomer surrogates. Shaded area indicates DNAN IC<sub>50</sub> range. Open symbols in dimer L show adjusted concentrations to monomer equivalents. Ac-DAAN did not cause inhibition to methanogens. The BBY IC<sub>50</sub> for both microbial targets was 0.7 µM.

**Table 1**

Selected parent ion list for semi-quantitative LC-MS determination of soluble products formed during anaerobic DNAN (bio)transformation in soil or sludge. A mass range of 0.01 Da was used to select ions  $[M+H]^+$  to measure for peak integration.

Chemical compound (identifier); Molecular formula;	CAS #	Calculated	
		$[M+H]^+$	Retention time (min)
2,4-diaminoanisole (DAAN); C <sub>7</sub> H <sub>10</sub> N <sub>2</sub> O	615-05-4	139.0866	2.51
2-methoxy-5-nitroaniline (MENA); C <sub>7</sub> H <sub>8</sub> N <sub>2</sub> O <sub>3</sub>	99-59-2	169.0608	6.23
3-nitro-4-methoxyaniline (iMENA); C <sub>7</sub> H <sub>8</sub> N <sub>2</sub> O <sub>3</sub>	577-72-0	169.0608	2.99
N-(5-amino-2-methoxyphenyl) acetamide (Ac-DAAN); C <sub>9</sub> H <sub>12</sub> N <sub>2</sub> O <sub>2</sub>	64353-88-4	181.0972	2.14
3-amino-3'-nitro-azobenzene ( <i>m/z</i> 243); C <sub>12</sub> H <sub>10</sub> N <sub>4</sub> O <sub>2</sub>	61390-99-6	243.0877	1.87
4'-methoxy-3-methylamino-3' methyleneamino-azobenzene ( <i>m/z</i> 269); C <sub>15</sub> H <sub>16</sub> N <sub>4</sub> O	N/A	269.1397	5.09
3,3'-diamino-4,4' dimethoxy-azobenzene ( <i>m/z</i> 273); C <sub>14</sub> H <sub>16</sub> N <sub>4</sub> O <sub>2</sub>	N/A	273.1347	13.75
3,3'-diamino-4,4' dimethoxy-hydrazobenzene ( <i>m/z</i> 275); C <sub>14</sub> H <sub>18</sub> N <sub>4</sub> O <sub>2</sub>	N/A	275.1503	3.48
5-((3-Amino-4-methoxyphenyl)diazanyl)-2-methoxy-N-methyleaniline ( <i>m/z</i> 285); C <sub>15</sub> H <sub>16</sub> N <sub>4</sub> O <sub>2</sub>	N/A	285.1347	10.61

Unassigned parent compounds; *m/z* (ret. time in min): 165.0659 (2.84), 185.0652 (4.66), 193.0607 (2.52), 228.0768 (2.86), 243.1241 (13.66), 245.1300 (4.13), 247.0425 (1.52), 259.1190 (9.02), 267.0975 (1.63), 274.0715 (11.24), 299.1179 (7.45), 301.1289 (12.3), 313.1289 (3.21), 325.1659 (12.07), 327.1452 (3.81), 431.1569 (1.32).

**Table 2**  
 Summary of inhibitory concentrations for DNAN, (bio)transformation products and best available surrogates to acetoclastic methanogens and *A. fischeri* (Microtox)

Compound	Methanogens			Microtox			Source
	IC <sub>20</sub>	IC <sub>50</sub>	IC <sub>80</sub>	IC <sub>20</sub>	IC <sub>50</sub>	IC <sub>80</sub>	
DNAN	16	41	70	27	57	110	Liang et al., 2013
MENA	121	175	275	13	48	143	Liang et al., 2013
DAAN	72	176	327	39	155	>302 <sup>a</sup>	Liang et al., 2013
iMENA	8	25	43.4	114	219	>446 <sup>b</sup>	This study
Ac-DAAN	>8000 <sup>c</sup>	>8000 <sup>c</sup>	>8000 <sup>c</sup>	660	911	1266	This study
dimer-L	22	65	76	18.6	29.8	49.7	This study
BBY	0.4	0.71	1.2	0.3	0.7	3.14	This study

<sup>a</sup> 302 µM DAAN caused 75% inhibition to *A. fischeri*.

<sup>b</sup> 446 µM iMENA caused 62% inhibition to *A. fischeri*.

<sup>c</sup> 8000 µM Ac-DAAN caused 2% inhibition to acetoclastic methanogens.