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ZEBRAFISH EMBRYO TOXICITY OF ANAEROBIC BIOTRANSFORMATION PRODUCTS FROM THE INSENSITIVE MUNITIONS COMPOUND 2,4-DINITROANISOLE

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Abstract: 2,4-Dinitroanisole (DNAN) is an emerging insensitive munitions compound that readily undergoes anaerobic nitro-group reduction to 2-methoxy-5-nitroaniline (MENA) and 2,4-diaminoanisole (DAAN), followed by formation of unique azo dimers. Currently there is little knowledge on the ecotoxicity of DNAN (bio)transformation products. In the present study, mortality, development, and behavioral effects of DNAN (bio)transformation products were assessed using zebrafish (*Danio rerio*) embryos. The authors tested individual products, MENA and DAAN, as well as dimer and trimer surrogates. As pure compounds, 3-nitro-4-methoxyaniline and 2,2′-dimethoxy-4,4′-azodianiline caused statistically significant effects, with lowest-observable–adverse effect levels (LOAEL) at 6.4 μM on 1 or 2 developmental endpoints, respectively. The latter had 6 additional statistically significant developmental endpoints with LOAELs of 64 μM. Based on light-to-dark swimming behavioral tests, DAAN (640 μM) caused reduction in swimming, suggestive of neurotoxicity. No statistically significant mortality occurred (≤64 μM) for any of the individual compounds. However, metabolite mixtures formed during different stages of MENA (bio)transformation in soil were characterized using high-resolution mass spectrometry in parallel with zebrafish embryo toxicity assays, which demonstrated statistically significant mortality during the onset of azo-dimer formation. Overall the results indicate that several DNAN (bio)transformation products cause different types of toxicity to zebrafish embryos. *Environ Toxicol Chem* 2016;35:2774–2781. © 2016 SETAC

Keywords: 2,4-Dinitroanisole Zebrafish Azo dimer Ecotoxicology Biotransformation Developmental toxicity

INTRODUCTION

The nitroaromatic compound 2,4-dinitroanisole (DNAN) is an emerging insensitive munitions compound that has gained the interest of defense industries because of its shock tolerance [1]. As the use of insensitive munitions formulations containing DNAN becomes widespread, discharges to natural environments may threaten diverse organisms vital to ecosystem function. Once released to the environment by manufacture waste streams or field use [2,3], it is estimated that DNAN may be a continuous source of pollution for several years because of its slow dissolution rate [4]. Moreover, transformation processes in soils might form products with unknown toxic effects. The nitro groups of DNAN can readily undergo reductive transformation to amino groups as a result of direct biological activity of microorganisms [5–7] or in concert with abiotic processes [8].

Rapid (bio)transformation of DNAN to 2-methoxy-5-nitroaniline (MENA) and 2,4-diaminoanisole (DAAN) has been reported in anaerobic environments [6,7]. Reactive species formed during biotransformation can undergo coupling to form azo dimers (Figure 1) [5–7]. The metabolites MENA and DAAN are more hydrophilic than DNAN [9]; thus, both might be more mobile in surface and groundwater impacted by DNAN contamination compared with the parent compound. Because DNAN (bio)transformation occurs readily in anaerobic environments, the environmental impact of DNAN pollution could be largely the result of its transformation products.

There is a lack of information regarding the ecotoxicity of the products formed during the anaerobic (bio)transformation of DNAN. However, (bio)transformation is expected to alter the toxicity effects of DNAN, as confirmed by early evidence that reductive (bio)transformation during DNAN toxicity assays decreased inhibition toward methanogenic archaea [10]. Similarly, chemical reduction of DNAN with 0 valent iron caused a decrease in zebrafish (*Danio rerio*) 48-h mortality [11]. Transformation of DNAN has been reported in ecotoxicity studies [2,3,12,13], which may complicate toxicological assessment because the observed toxicity effects might be the result of DNAN and/or its transformation products. A few recent studies have characterized the toxic impacts of DNAN to diverse groups of microorganisms [3,10], earthworms, and plants [3,12]. Two recent studies have evaluated the in vivo toxicity of DNAN to aquatic vertebrates [2,11]. However, information on the developmental and chronic, toxic effects associated with DNAN biotransformation products is lacking.

In the ecotoxicology field, zebrafish embryos have become widely used to monitor water quality and to aid in environmentally safe product development [14,15]. The embryonic zebrafish model is amenable for high-throughput studies and allows evaluation of developmental and behavioral endpoints in addition to acute toxicity [16]. Early development is very similar to that of higher-order vertebrates, and its transparency allows for noninvasive and specific developmental assessment endpoints [17]. Moreover, a vast amount of

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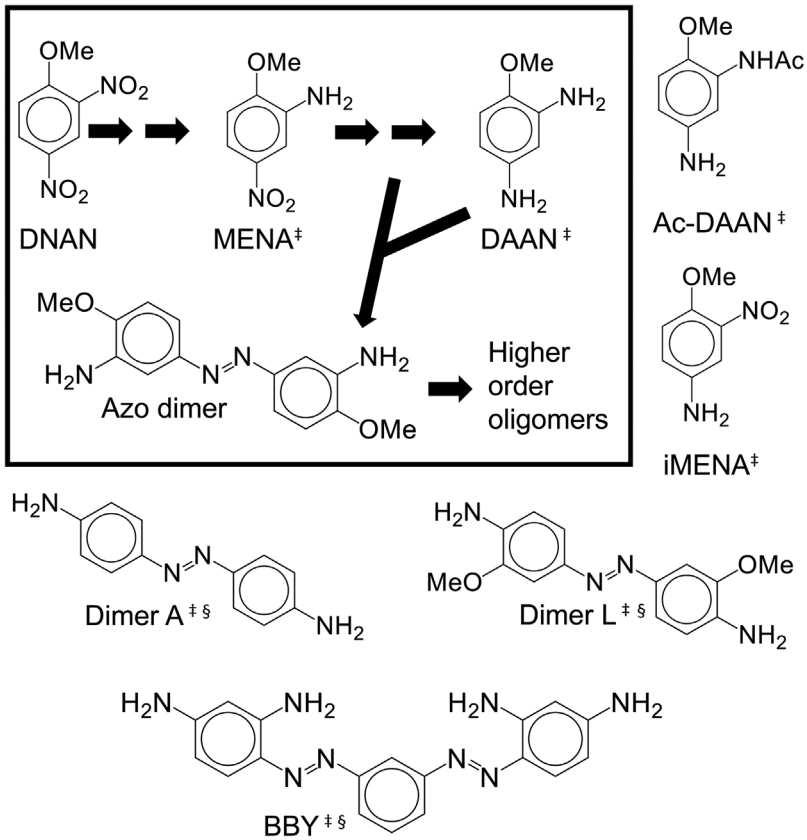

Figure 1. Main anaerobic 2,4-dinitroanisole (DNAN) (bio)transformation pathway (structures inside box): DNAN undergoes nitroreduction to 2-methoxy-5-nitroaniline and 2,4-diaminoanisole [7]. Reactive intermediates formed during nitro-group reduction enable coupling reactions that form dimers and other oligomers by reacting with aromatic amines [37,38]. Chemical structures of individual compounds tested based on ‡previously identified DNAN (bio)transformation products or §best available surrogates. Ac-DAAN = *N*-(5-amino-2-methoxyphenyl) acetamide; BBY = Bismarck brown Y; DAAN = 2,4-diaminoanisole; DNAN = 2,4-dinitroanisole; iMENA = 3-nitro-4-methoxyaniline; MENA = 2-methoxy-5-nitroaniline.

transcriptomic information along an expanding library of toxicants tested can provide mechanistic insights of the toxicity effects in zebrafish [15,18–20].

The objective of the present study was to evaluate developmental toxicity effects of DNAN (bio)transformation products (or best commercially available surrogates) in zebrafish embryos, which were used primarily as a toxicity target representing vertebrate organisms. We also tested mixtures of the products formed at different stages of (bio)transformation and bioavailable in the supernatant during anaerobic incubations of soil solutions using as a starting point the primary DNAN transformation product MENA. Toxicological testing was supported by detailed high-resolution mass spectrometric studies (using ultrahigh-performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry [UHPLC-QToF-MS]) to characterize transformation product mixture profiles.

MATERIALS AND METHODS

Zebrafish embryo assays

A previously reported protocol [19] was used with slight modifications. Embryos with intact chorions were manually placed in 96-well plates containing embryo medium at 6 h postfertilization (hpf) and exposed to individual toxicants (10 μL + 90 μL embryo medium) or supernatant solutions of different stages of MENA (bio)transformation (50 μL + 50 μL embryo medium). Thirty-two embryos were exposed per concentration and toxicant, and all controls and treatments included 0.64% dimethyl sulfoxide (DMSO) to aid toxicant dissolution. Plates were incubated at 28 °C in the dark. At 24 hpf and 120 hpf, development abnormality and mortality assessments were performed (endpoints shown in Table 1) [19]. Endpoint scoring and statistical analyses were performed in R software as described [19].

In addition, at 120 hpf a larval locomotor behavior assay was performed with alternating light-to-dark cycles in a Viewpoint Zebrabox (software Ver 3.0; Life Sciences) [21]. Briefly, at 120 hpf, plates were inserted into the Viewpoint Zebrabox, where movement was recorded during the following light-to-dark cycle: initial light acclimation (10 min), light (10 min), dark (5 min). Long swimming distance (>2.5 mm min^{-1}) was averaged across all surviving replicates at 120 hpf for a single concentration. Malformed and dead zebrafish were not considered in the locomotor assay. Endpoint scoring and statistical analyses were performed according to the protocols described [19,21].

Individual chemicals tested

The library of compounds tested included DNAN monomer (bio)transformation products [5–7] and best available surrogates for azo dimers and trimers because no dimer metabolites were commercially available. Both MENA (Chemical Abstracts Service [CAS] no. 99-59-2, 98%) and DAAN (CAS no. 615-05-4, analytical standard) were obtained from Sigma-Aldrich. Accela ChemBio

Table 1. Zebrafish embryo toxicity endpoints assessed

Time	Endpoints
24 hpf	Mortality, developmental delay, spontaneous movement, notochord
120 hpf	Mortality, notochord, yolk sac edema, body axis, eye defect, snout, jaw, otic vesicle, pericardial edema, brain somite, pectoral fin, caudal fin, pigment, circulation, truncated body, touch response

hpf = hours postfertilization.

provided 3-Nitro-4-methoxyaniline (iMENA; CAS no. 577-72-0, purity 97%). We acquired *N*-(5-Amino-2-methoxyphenyl) acetamide (Ac-DAAN, CAS no. 64353-88-4, purity 95%) from ChemBridge. Alfa Aesar provided 4,4′-Azodianiline (denoted "dimer A"; CAS no. 538-41-0, purity 95%). We obtained the 2,2′-Dimethoxy-4,4′-azodianiline (denoted "dimer L"; CAS no. 6364-31-4, purity >90%) from MolMall Sarl. Bismarck brown Y (*m*-Bis[2,4-diaminophenylazo]-benzene; CAS no. 8005-77-4, 46%) was purchased from Chem-Impex International. Figure 1 shows the chemical structures of these compounds. Solutions were prepared the same day the exposure began and diluted to contain 0.64% DMSO across all wells including controls. This concentration of DMSO is known to not cause effects in zebrafish embryo assays. Zebrafish embryos were exposed to 0 μM to 64 μM concentrations of the test compounds, with the exception of MENA and DAAN which had a range 0 μM to 640 μM. In all cases the concentrations tested were chosen to span 5 orders of magnitude and overlap ranges used in aquatic species in the interspecies toxicology evaluation of DNAN by Kennedy et al. [2]. In the present study DNAN was not tested because of the commercialization, transportation, and export control restrictions established by the International Traffic in Arms Regulations of the US Department of State [22].

Sampling and metabolite analysis from anaerobic (bio) transformation of MENA

Anaerobic bioassays were performed to obtain mixtures of the metabolites formed during different stages of the (bio) transformation of MENA in soil. Anaerobic tubes (Bellco Glass) were filled with 10 mL of mineral medium (described in Olivares et al. [7]) containing 18 mM phosphate buffer (pH 7.2) and 500 μM MENA. The solution was inoculated with 100 mg of fresh Camp Navajo soil (water content 9.3%), previously characterized [23] and amended with 10 mM pyruvate as an exogenous electron donor. The tubes were flushed with He/CO_2 (80/20%), closed with *t*-butyl caps and aluminum seals, and subsequently incubated in the dark at 30 °C in an orbital shaker at 115 rpm. Samples from the different tubes were all collected on the same day to facilitate simultaneous toxicity testing of all samples and minimize test variability. The tubes were incubated according to a staggered timeline to ensure that samples incubated for 0 d, 1 d, 6 d, 9 d, 20 d, and 30 d were available on the sample collection day.

At the end of the incubation period, tubes were opened in an anaerobic hood to avoid autoxidation of unstable products, and the liquid phase was centrifuged (10 min, 9600 *g*). Aliquots of the supernatant were collected for immediate analysis by UHPLC-QToF-MS and for toxicity testing. The latter samples were sealed under N_2 gas and then frozen (–20 °C) for 2 wk until the zebrafish embryo toxicity assays were performed.

UHPLC-QToF-MS and ultraviolet-visible analyses

Liquid samples from soil (bio)transformation experiments were analyzed on an UltiMate 3000 UHPLC (Dionex) coupled to a TripleTOF® 5600 quadrupole QToF-MS (AB Sciex). An Acclaim RSLC Explosives E2 column (2.1 × 100 mm, 2.2 μm; Thermo Fisher Scientific) was used for chromatographic separation with an isocratic mobile phase consisting of methanol/H_2O (40/60, v/v, 0.25 mL min^{-1}, 15 min) at room temperature. The MENA concentration was quantified using UHPLC coupled to a diode-array detector at 254 nm (retention time 5 min) before measurement by UHPLC-QToF-MS. The QToF-MS utilized an electrospray ionization source operated in positive mode at 450 °C with a capillary setting of 5.5 kV, a declustering potential of 80 V, and curtain gas, desolvation gas, and nebulizer gas levels at 30 psi, 35 psi, and 35 psi, respectively, with N_2. A precursor compound list was created for fragmentation with a collision energy range of 15 eV to 45 eV, based on previously resolved transformation products from DNAN (bio)transformation [7], as well as other detected compounds that had not been assigned a structure. Analyst TF 1.6 with PeakView 1.2.0.3 and Multiquant Ver 2.1 software were used to develop the precursor list as well as integrate peak areas of each analyte detected.

The ultraviolet-visible (UV-vis) spectra of the supernatant from MENA samples incubated for 1 d, 6 d, 9 d, 20 d, and 30 d were recorded (200–600 nm) in quartz cuvettes with a UV-1800 Shimadzu spectrophotometer. Samples were diluted with a 50 mM phosphate buffer (pH 7).

RESULTS AND DISCUSSION

Toxicity of model degradation products

Mortality. Freshwater fish species are reported to be as sensitive as or more sensitive than invertebrates and amphibians to nitroaromatic explosives, such as 2,4,6-trinitrotoluene (TNT) [24–27]; and the lowest 96-h 50% lethal concentration (LC50) has been reported for rainbow trout (*Oncorhynchus mykiss*) at 3.5 μM TNT [24]. It has been reported that DNAN is less toxic than TNT, with a 96-h LC50 for DNAN in larval fathead minnows (*Pimephales promelas*) reported at 187 μM [2], whereas for TNT it was 13.6 μM [28]. The 48-h LC50 for DNAN in adult zebrafish has recently been reported as 177 μM [11].

In the present study, no statistically significant mortality at 24 hpf and 120 hpf was detected for any of the pure compounds tested (0–640 μM for MENA and DAAN; 0–64 μM for iMENA, Ac-DAAN, dimer A, dimer L, and Bismarck brown Y). Mortality charts for each compound tested can be found in Supplemental Data, Figures S1 through S3. In the present study, which used zebrafish embryos with intact chorions, no adverse lethal effects were detected for MENA concentrations up to 640 μM. However, in a previous study with dechorionated zebrafish embryos on compounds from the US Environmental Protection Agency's ToxCast phase 1 and 2 lists, which included MENA, the 120-hpf mortality lowest-observable-adverse effect level (LOAEL) was reported at 64 μM [19]. Because the chorion is the primary exposure route before hatching [16], this discrepancy suggests that the presence of the chorion could be limiting the diffusion of aromatic amines like MENA. Similar to the monomeric compounds tested in the present study, the azo dimers and the trimer (Bismarck brown Y) evaluated did not result in zebrafish mortality at

concentrations up to 64 μM. Azo dye dimers and trimers have been tested in aquatic ecotoxicity models, including an extensive dye survey on fathead minnows (*P. promelas*) [29] and, more recently, on guppy fish (*Poecilia reticulata*) [30] and the western clawed frog (*Silurana tropicalis*) [31]. In contrast with the lack of lethal effects detected in the present study, the 96-h LC50 value reported in guppy fish for methyl red (CAS no. 493-52-7), an azo dimer with *N*-methyl, amino, and carboxylic acid substituents, was 89 μM [30] and for basic brown 4 (CAS no. 5421-66-9), an azo trimer with amino and methyl substituents, in fathead minnows it was 12.1 μM.

Overall, the low mortality counts obtained with the pure compounds tested suggest that the individual transformation products and/or surrogates evaluated in the present study pose little acute toxicity risk (≤64 μM). This argument is strengthened by the observed decrease in toxicity of TNT on fathead minnows because TNT was reduced to dinitroaniline products in the assay [24]. Although mortality effects were not detected in the concentration ranges tested in the present study, other toxicity effects may occur at sublethal concentrations, such as with reproductive [24,26] and developmental [32] endpoints.

Developmental toxicity. Detailed developmental endpoint scoring for all the chemicals tested in the present study is shown in Supplemental Data, Figures S4 through S10. Although most of the compounds tested did not cause developmental abnormalities, dimer L and iMENA caused a number of statistically significant malformations in zebrafish embryos (Table 2). Based on yolk sac edema formation, iMENA had a developmental LOAEL of 6.4 μM (Supplemental Data, Figure S5), but no other abnormalities were detected. The rest of the monomer compounds did not show developmental activity. Developmental toxicity studies with the monomeric compounds tested in the present study have not been reported. However, some aromatic amines, such as aniline, have been shown to cause developmental toxicity in African clawed frog (*Xenopus laevis*) embryos, albeit at much higher concentrations, 3.9 mM [33]. Of the dimers and trimer tested, only dimer L caused statistically significant developmental abnormalities in the embryos (Supplemental Data, Figure S9). Dimer L caused a statistically significant occurrence of yolk sac and pericardial edemas (LOAEL = 6.4 μM) and several malformations in 6 other endpoints at 64 μM (Figure 2). Some of these abnormalities are illustrated in Figure 3. Although Bismarck brown Y and dimer A did not show any developmental toxicity in the tested range, in another aquatic species, western clawed frogs, Bismarck brown Y has been reported to cause malformations at 2.4 mM [31].

Locomotor behavior assay. The impact of the various model compounds on zebrafish swimming locomotor behavior was also tested at 120 hpf. Zebrafish have been reported to be more static in the presence of light compared with dark periods, and comparing the difference in light-to-dark cycles can indicate changes in behavior as a result of exposure to toxicants during development [21]. In the present study, long-distance swimming motion ranged from 18 mm to 30 mm in the dark, whereas it was below 5 mm in light (Figure 4). There were no detectable effects on locomotion for any of the chemicals tested, except for DAAN. Although there were some minor variations in the distance swum in the dark periods when considering most compounds, the average movement in zebrafish that had been exposed to 640 μM DAAN (4 mm) was 8-fold less than the toxicant-free control (32 mm). In essence, 640 μM DAAN caused the motion in the dark period to be reduced to the level normally observed by the embryos in the light periods. This suggests that 640 μM DAAN impaired response to light-to-dark cycles. Because there were no active developmental endpoints for DAAN (Supplemental Data, Figure S6), the lack of response to light-to-dark periods is not associated with swimming impairment because of malformations. Therefore, exposure to 640 μM DAAN could have neurotoxicity effects. Energetic compounds, such as hexahydro-1,3,5-trinitro-1,3,5-triazine (also known as RDX) and 2,4,6,8,10,12-Hexanitro-2,4,6,8,10,12-hexaazaisowurtzitane (also known as CL-20), have been reported as neurotoxicants [34]; and prolonged exposure to TNT affected motor and sensory neurons to workers [35].

Toxicity evaluation of MENA (bio)transformation products in anaerobic soil microcosms

Product characterization and semiquantitation. Based on UHPLC-QToF-MS analyses, 6 precursor ions were selected for semiquantitation according to their chromatogram peak area abundance (>1000; Table 3). Of these compounds, 2 had been reported previously as monomer products formed during DNAN (bio)transformation in anaerobic conditions: DAAN and Ac-DAAN [7]. In the present study, we report a new transformation product, 3-amino-3′-nitro-azobenzene (mass-to-charge ratio [*m/z*] 243; Supplemental Data, Figure S11), as well as 2 precursor ions that have not been assigned chemical structures $[M+H]^+$ *m/z* 313.1343 (putative molecular formula $C_{16}H_{16}N_4O_3$; Supplemental Data, Figure S12) and 393.0836 (putative molecular formula $C_{19}H_{12}N_4O_6$; Supplemental Data, Figure S13). Based on these formulae, these ions could represent a dimer and a trimer, respectively. Transformation products were quantified using precursor ions (*m/z* $[M+H]^+$) or product ion (*m/z* $[M+H-R]^+$) transitions when the product

Table 2. Developmental lowest-observed-effect concentrations (LOECs) and endpoints with significant morbidity

Chemical compound (identifier)	Surrogate for	Developmental LOEC (μM)	Active endpoints (μM)
2-Methoxy-5-nitroaniline (MENA)	N/A	>640	ND
3-Nitro-4methoxyaniline (iMENA)	N/A	6.4	Yolk sac edema (6.4)
2,4-Diaminoanisole (DAAN)	N/A	>640	ND
N-(5-Amino-2-methoxyphenyl) acetamide (Ac-DAAN)	N/A	>64	ND
4,4′-Azodianiline (dimer A)	Azo dimer	>64	ND
2,2′-Dimethoxy-4,4′-azodianiline (dimer L)	Azo dimer	6.4	Yolk sac and pericardial edemas (6.4); axis, eye, snout, jaw, pectoral fin, touch response (64)
Bismarck brown Y	Azo trimer	>64	ND

N/A = not applicable; ND = none detected.

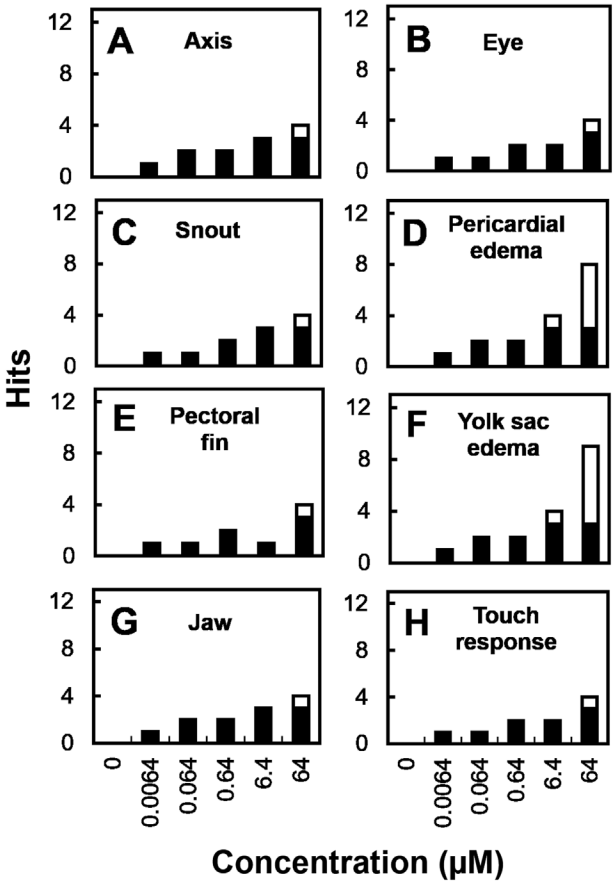

Figure 2. Developmental endpoints that caused malformations in the zebrafish embryo assay for dimer L. White bars indicate hits above the statistically significant threshold ($p \leq 0.05$). The rest of the developmental endpoints did not show statistically significant activity.

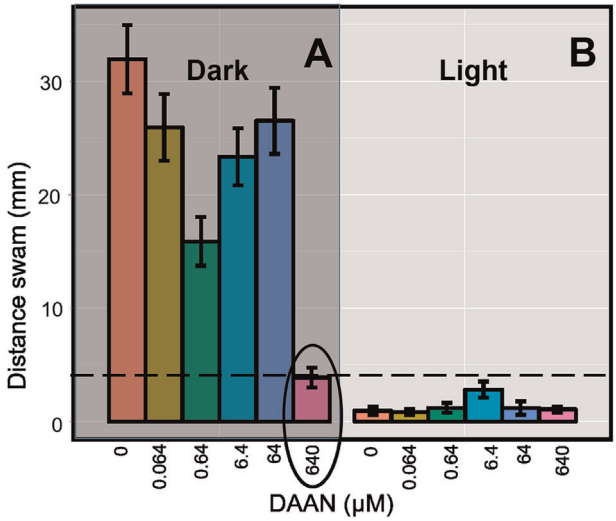

Figure 4. Average long distance swum recorded in larval locomotor response. Viewpoint assay for 120 hpf zebrafish larvae exposed to 2,4-diaminoanisole (0–640 μM) in dark (**A**) and light (**B**) stages. Error bars represent standard deviation of the mean. DAAN = 2,4-diaminoanisole.

yielded a stronger ionization signal than the precursor (Table 3). Although standards were available for DAAN and Ac-DAAN, the rest of the analytes were not commercially available, difficult to synthesize, and potentially not stable in air. For consistency, all analytes are reported in peak area units based on UHPLC-QToF-MS, which cannot be unequivocally translated to concentration because of potential variation in ionization efficiencies (Figure 5A). Because MENA ionized several orders of magnitude more strongly than the rest of the analytes, it was not added to the selected ion list. Therefore, its concentration during the (bio)transformation is reported in micromoles as quantified using the UHPLC diode-array detector (Figure 5B).

From initial time to 6 d of (bio)transformation, trace amounts of all of the analytes were detected, but 23% of MENA had already been removed, indicating that more transformation products not shown in Table 3 were possibly formed. From day 6 to day 9 the formation of oligomers (mainly *m/z* 243, 313, 393) was visible, followed by accumulation of DAAN and Ac-DAAN after day 10. The concentration of all of the analytes increased until day 30, with the exception of DAAN, which decreased by 40% from day 20 to day 30.

To consider transformation products that might be difficult to separate chromatographically or ionize in the QToF-MS, UV-vis spectra were also recorded along the course of the (bio) transformation of MENA (Supplemental Data, Figure S14). Because oligomers were detected in QToF-MS, the spectral data are summarized as an oligomer index, based on the ratio of absorbance at 400 nm to 254 nm (Figure 5B), the former wavelength being chosen to quantify polymers [36] that have visible absorbance (e.g., azo dyes) and the latter being a common wavelength for aromaticity. The 400 nm to 254 nm ratio increased from day 1 to day 6, reached a maximum at 9 d of incubation, and then decreased until day 30. The initial increase might be the result of the onset of oligomer formation as evidenced by QToF-MS data, whereas the subsequent decrease following day 9 indicates precipitation of larger insoluble

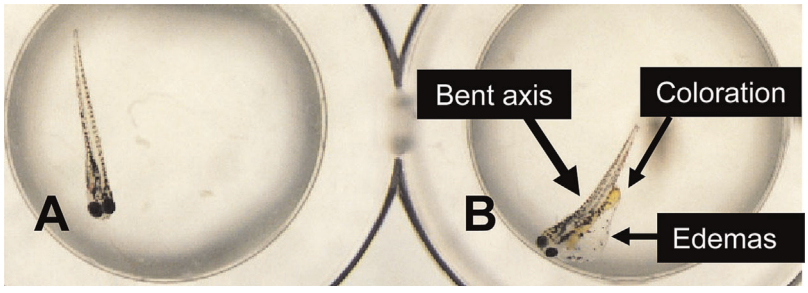

Figure 3. Representative visual comparison between 120 hpf zebrafish embryos: control (**A**), 64 μM dimer L (**B**). The embryo exposed to dimer L showed developmental abnormalities and visible dimer L uptake (coloration).

Table 3. Selected precursor/product ion list for semiquantitative liquid chromatographic–mass spectrometric determination of soluble transformation products formed during anaerobic 2-methoxy-5-nitroaniline soil (bio)transformation[a]

Chemical compound or identifier; molecular formula	CAS no.	Calculated	Measured		
		Precursor $[M+H]^+$	Precursor $[M+H]^+$	Product $[M+H-R]^+$	Retention time (min)
2,4-Diaminoanisole (DAAN); $C_7H_{10}N_2O$	615-05-4	139.0866	139.0866	N/A	2.86
N-(5-Amino-2-methoxyphenyl) acetamide (Ac-DAAN); $C_9H_{12}N_2O_2$	64353-88-4	181.0972	181.0972	N/A	2.36
3-Amino-3'-nitro-azobenzene (*m/z* 243)[b]; $C_{12}H_{10}N_4O_2$	61390-99-6	243.0877	243.0870	N/A	2.3
(*m/z* 313)[b,c]; $C_{16}H_{16}N_4O_3$	N/A	313.1295	313.1312	250.8794	1.60
(*m/z* 393)[b,c]; $C_{19}H_{12}N_4O_6$	N/A	393.0830	393.0836	276.0833	1.59

[a]Product ions shown indicate they were used to determine the abundance of the transformation products
[b]These ions have not been assigned a chemical structure.
[c]These compounds are reported for the first time as (bio)transformation products.
m/z = mass to charge ratio; N/A = not applicable.

oligomers out of solution, even if soluble dimers such as *m/z* 243 continue being formed.

Zebrafish mortality and absence of developmental abnormalities. Zebrafish embryo mortality assessed at 120 hpf was statistically significant only for the sample taken at 9 d of (bio) transformation (Figure 5A), coinciding with the maxima recorded for the 400 nm to 254 nm absorbance oligomer index (Figure 5B) and slightly after the onset of oligomer formation. This suggests that the early formation of azo dimers is responsible for an increase in zebrafish embryo mortality. Oligomerization occurs when reactive nitroso intermediates react with amines to cause coupling reactions [37,38].

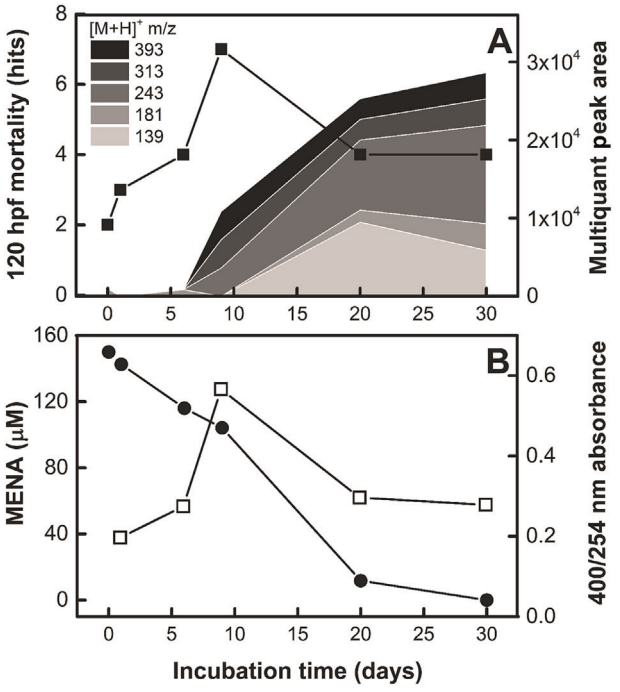

Figure 5. Characterization of transformation products formed during 2-methoxy-5-nitroaniline (MENA) (bio)transformation coupled to zebrafish toxicity. (**A**) Temporal semiquantitation of transformation products ($[M+H]^+$ *m/z* = 139, 181, 243, 313, 393) shown with stacked areas and increasing *m/z* shown with darker shades and zebrafish mortality assessed at 120 hpf (■). Mortality statistical significance ($p < 0.05$) is above 4 hits ($n = 32$). (**B**) Concentration of MENA (●) and 400 nm to 254 nm absorbance index (□) during MENA (bio)transformation.

Thus, mortality is potentially associated with the reactive intermediates.

There were no active developmental endpoints in any of the samples collected during MENA biotransformation. The absence of developmental abnormalities in the assay could be the result of several factors. Firstly, the compound mixtures were produced at very low concentrations and below any LOAEL. Particularly for dimers and trimer products, for which the maximum molar concentration would be initial parent molarity (MENA in this case) divided by the number of monomer units (aromatic rings). The most abundant non-monomer product, according to the ionization signal detected in the mixtures, was an azo-dimer metabolite observed at *m/z* 243. However, because no developmental abnormalities in the embryos were observed, either the compound was present below the LOAEL for the surrogate dimer tested, dimer L, or it had an important difference in biological activity compared with dimer L that made *m/z* 243 less toxic toward developmental endpoints in the MENA (bio)transformation product mixtures. Secondly, fewer oligomers may be bioavailable to the embryos if extensive polymerization occurs that results in oligomers precipitating from solution or oligomer incorporation into the soil humus [39]. These mechanisms are supported by the overall decrease of aromaticity of the aqueous phase as evidenced by the decline in 254 nm absorbance during the course of the (bio) transformation (Supplemental Data, Figure S14). Aromatic amines and azo polymers from similar nitroaromatic compounds, such as DNAN and TNT, are known to bind irreversibly to soil humic substances [9,39]. All of these factors could have contributed to an overall lower amount of bioavailable oligomers and, in consequence, no developmental abnormalities in the embryos.

Toxicity implications of DNAN biotransformation

The present study showed that intermediates of DNAN biotransformation can cause detectable developmental and behavioral toxicity endpoints in zebrafish embryos. Most concerning was the high level of developmental toxicity caused by a surrogate azo-dimer intermediate (dimer L) and iMENA (both at 6.4 μM) as well as evidence of locomotor toxicity caused by DAAN at higher concentrations. Our approach coupled transformation product identification with toxicological testing to determine the environmental impact of organic pollutants as they undergo transformation in natural systems. Although the present study focused on biotransformation, other processes, such as phototransformation [40], could contribute to

additional products that might pose unknown toxicity effects that should be evaluated to complement those in the present study.

To date, environmental fate and toxicology studies on DNAN and other insensitive munitions are currently limited by the lack of information on field-use and manufacture effluent concentrations of this energetic material in different environmental media because of their relatively new use. Although dissolution after detonation trials has been evaluated [4], a comprehensive characterization of the extent of DNAN occurrence in field sites and distribution across different media is needed to understand the range of environmentally relevant concentrations at which DNAN and its biotransformation products are present.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.3446.

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Data Availability—Data are available from the authors (jimfield@email.arizona.edu).

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