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Subchronic, chronic, lethal and sublethal toxicity of insensitive munitions mixture formulations relative to individual constituents in *Hyalella azteca*

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HIGHLIGHTS

- Evaluated the toxicity of insensitive munitions (reduced unintended detonation).
- 10 and 35d *Hyalella azteca* water exposures assessing survival, growth, reproduction.
- Relative toxicity (10-d LC50s): DNAN > NTO > NQ; RDX not toxic near solubility limit.
- Sublethal growth and reproduction effects observed for DNAN and IM formulations.
- NTO, RDX or both in IM formulation caused significantly decreased toxicity of DNAN.

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ABSTRACT

Insensitive munitions (IMs) are replacing conventional munitions, improving safety from unintended detonation. IMs are deployed in mixture formulations but little is known about their mixture toxicology. We characterized mixture effects of the IM formulations IMX-101 (mixture of 2,4-dinitroanisole [DNAN], 3-nitro-1,2,4-triazol-5-one [NTO], and nitroguanidine [NQ]) and IMX-104 (DNAN, NTO, and hexahydro-1,3,5-trinitro-1,3,5-triazine [RDX]) in subchronic (10 d) and chronic (35 d) water-only tests in *Hyalella azteca* assessing impacts on survival, growth and reproduction. In 10-d single chemical exposures, DNAN was the most potent constituent, eliciting an LC50 of 16.0 mg/L; the LC50s for NTO and NQ were 891 and 565 mg/L, respectively. RDX did not elicit significant mortality up to 29.5 mg/L, a concentration near its solubility limit. Based on toxic-units (TUs), the toxicity of IMX-101 was driven by the effective concentration of DNAN; however, the presence of NTO, RDX, or both elicited interactive effects causing an approximately 2-fold decrease in lethality for IMX-104. Growth reduction was observed in 10-d exposures to DNAN, IMX-101 and IMX-104, but not for NQ, NTO, or RDX. Longer exposure duration (35 d) to IMX-101, IMX-104, and DNAN resulted in 3–6 times higher sensitivity for lethality and resulted in the most sensitive endpoint for DNAN, RDX, and IMX-101 exposures, decreased reproduction. Slight, but statistically significant, antagonistic responses among IMX-101 constituents were observed for survival and reproduction at 35d. Overall, the results support response-additive summation as a sufficient method to provide conservative hazard assessments of subchronic, chronic, and sublethal IMX-101 and IMX-104 mixture impacts in *H. azteca*.

1. Introduction

Unintended detonation of munitions and munition stockpiles has caused losses of human life, infrastructure, and materiel. The U.S. Department of Defense (DOD), therefore, has a stated goal of replacing traditional munitions with insensitive munitions (IM)

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that are chemically stable enough to withstand mechanical damage, fire, and impact of projectiles during combat operations while still exhibiting similar explosive characteristics to traditional munitions (Isler, 1998; Duncan, 2002). The Army's flagship IM formulations, IMX-101, a mixture of 2,4-dinitroanisole (DNAN), 3-nitro-1,2,4-triazol-5-one (NTO), and nitroguanidine (NQ), and IMX-104, a mixture of DNAN, NTO, and hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), have recently been qualified as replacements for 2,4,6-trinitrotoluene (TNT) and RDX in artillery rounds (Lee et al., 2010; Fung et al., 2010).

Aquatic ecosystems may receive environmental inputs of munitions via contaminated wastewater (Ribeiro et al., 2012; Li et al., 2013; USEPA, 2014). In addition, munitions utilized in live fire can result in the contamination of the soil surface of training ranges that can undergo weathering, dissolution, and transport with the potential of eventually contaminating surface and groundwater and being transported off the training range (Jenkins et al., 2001; Walsh et al., 2008, 2010). Effects characterizations for individual IMs have proliferated in recent time (see references below); however, characterization of the mixture toxicology for IM mixture formulations remains a critical data gap that jeopardizes the accuracy of risk analysis for IMX-101 and IMX-104 formulations that are manufactured and deployed as chemical mixtures.

As foreshadowed above, aquatic toxicity data for individual IMs are becoming increasingly robust, spanning multiple species including both acute and chronic exposure-responses (Table S1). The lethality (LC50) of DNAN in acute exposures ranged from 10.0 to 42.8 mg/L across various aquatic species (Dodard et al., 2013; Liang et al., 2013; Kennedy et al., 2013, 2015; 2017; Stanley et al., 2015; Ou et al., 2015; Gust et al., 2018). Effective concentrations in lethal and sublethal chronic exposures ranged from 1.8 to 13.7 mg/L, with the most sensitive response observed for survival in chronic 28d exposures in leopard frog (*Rana pipiens*) tadpoles (Kennedy et al., 2015; Stanley et al., 2015). The toxicity of NTO varied widely in acute exposures, ranging from a 799 mg/L LC50 in *Ceriodaphnia dubia* to a NOEC of 3954 for bioluminescence in *Aliivibrio fischeri* (Table S1; Kennedy et al., 2017; Madeira et al., 2018; Gust et al., 2018). Chronic NTO exposures also displayed a wide range in effective concentrations, ranging from 5.0 to 3670 mg/L (Pillard et al., 2017; Harvey et al. 2009; Stanley et al., 2015). The potency of NQ tended to be low relative to the other IMs, with acute NOECs for survival ranging from 1550 to 3320 mg/L in fish (Burton et al., 1993; Gust et al., 2018) and acute LC50s ranging from 1174 to 2698 mg/L (Burton et al., 1993; Kennedy et al., 2017; Gust et al., 2017). Effective concentrations of NQ in chronic and sublethal exposures were 440 and 1937 mg/L to *Raphidocelis subcapitata* and *C. dubia* (van der Schalie, 1985; Burton et al., 1993). Although the NQ parent compound is relatively non-toxic, aggressively UV-degraded NQ induced orders of magnitude increases in toxic potency (van der Schalie, 1985; Burton et al., 1993; Kennedy et al., 2017; Gust et al., 2017), and UV-degradation of NTO also showed the potential for increased potency (Kennedy et al., 2017). Finally, RDX had the most robust aquatic toxicity literature-base, where acute NOECs ranged from 7.2 to 75 mg/L and acute LC50s ranged from 3.6 to 28.9 mg/L for various species of fish and invertebrate taxa (Bentley et al., 1977; Burton et al., 1993; Dave et al., 2000; Nipper et al., 2001; Mukhi et al., 2005; Rosen and Lotufo, 2007; Lotufo et al., 2010; Gust et al. 2014, 2018). Chronic and sublethal RDX exposures showed effective exposure levels ranging from 4.8 to 36.7 mg/L (Bentley et al., 1977; Burton et al., 1994; Stanley et al., 2015; Nipper et al., 2001). Overall, DNAN and RDX tended to be more potent toxicants than NTO and NQ as parent compounds (Table S1). Although understanding the toxicity of environmental transformation products of IMs is an important subject (a subject we have investigated in other studies; e.g., Kennedy et al., 2017; Gust et al., 2017), the present study focuses exclusively on parent compounds to elucidate fundamental responses to individual IMs and IM-mixture toxicity characterization.

Toxicity assessment of IM mixture formulations has lagged considerably relative to individual IM constituent evaluations. The toxicity of chemical mixtures can be very complex and deviate from assumptions of an additive response. It is generally hypothesized that chemicals having similar modes of toxic action elicit concentration-additive effects proportional to the summed toxicities of each individual contaminant concentration (Cassee et al., 1998). Concentration-addition has been well demonstrated for chemicals that elicit similar toxicological effects and/or have related quantitative structure–activity relationships (Altenburger et al., 2004; Backhaus and Faust, 2012; Belden et al., 2007; Schmidt et al., 2016). Alternatively, contaminants with dissimilar modes of toxic action are generally hypothesized to act in “independence” of one another, resulting in less than concentration-additive toxicity where effects are characteristic of the mixture constituent present at the highest effective concentration (Walter et al., 2002; Altenburger et al., 2004; Belden et al., 2007). Numerous exceptions to the hypothesis of independence have been observed in the literature, where toxicological interactions occur leading to antagonistic (reduced) or synergistic (increased) toxic effects (Cassee et al., 1998; Ding et al., 2013). The effects of IM mixtures in aquatic exposures have only been investigated for IMX-101 in *P. promelas* (Gust et al., 2018) and *C. dubia* (Kennedy et al., 2017), while IMX-104 mixtures have only been evaluated in *P. promelas* (Gust et al., 2018). In these studies, acute lethal effects indicated that IMX-101 elicited toxicity representative of the component concentration of DNAN within the mixture (Kennedy et al., 2017; Gust et al., 2018). Transcriptomics responses suggested different mechanisms of action among DNAN, NTO and NQ, where expression profiles were indicative of non-interactive responses among these IMX-101 mixture constituents (Gust et al., 2018). Conversely, a slight, yet statistically significant greater than additive effect on acute lethality was observed in the IMX-104 mixture exposure, where transcriptomics revealed probable interactions among DNAN and RDX on oxidative stress mitigation pathways (Gust et al., 2018). To date, IM mixture toxicity assessments have only addressed acute lethal responses. The objective of the present study was to investigate the subchronic (10 d) and chronic (35 d) effects of exposures to IMX-101 and IMX-104 mixture formulations, relative to their respective individual constituents, to assess the potential for interactive toxicity for both lethal and sublethal endpoints, including growth and reproduction in the epibenthic amphipod *Hyalella azteca*.

2. Experimental section

2.1. Chemicals and exposure solutions

Military grade DNAN (>95% pure, Kennedy et al., 2015), NTO (>95% pure), and RDX (approximately 10% octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine [HMX] as impurity) and the IM formulations IMX-101 and IMX-104 were obtained from the Holston Army Ammunition Plant (BAE Systems) and used without further purification. NQ for single chemical exposure was obtained from Sigma-Aldrich (St. Louis, MO, USA). The manufacturer specifications for this NQ indicate that it is 20–25% water for safety purposes. All compounds and formulations were dissolved directly into dechlorinated tap water (City of Vicksburg, MS, municipal source) with no carrier solvent using magnetic stirring for 24–48 h, and diluted with clean dechlorinated tap water to produce the target concentrations. Dilution and control water for all treatments was dechlorinated using activated carbon filtration and fortified