Environmental Pollution 210 (2016) 129–136

Contents lists available at ScienceDirect

Environmental Pollution

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

Accumulation and depuration of trinitrotoluene and related extractable and nonextractable (bound) residues in marine fish and mussels

Guilherme R. Lotufo [a, *], Jason B. Belden [b], Jonathon C. Fisher [c], Shou-Feng Chen [d], Richard A. Mowery [d], C. Kevin Chambliss [d], Gunther Rosen [e]

[a] U.S. Army Engineer Research and Development Center, 3909 Halls Ferry Road, Vicksburg, MS 39180, USA
[b] Department of Integrative Biology, Oklahoma State University, Stillwater OK 74078, USA
[c] Department of Natural Sciences, Northwestern State University, Tahlequah, OK 74464, USA
[d] Department of Chemistry, Baylor University, Waco, TX 76798, USA
[e] Space and Naval Warfare (SPAWAR) Systems Center Pacific, 53475 Strothe Rd., San Diego, CA 92152, USA

ARTICLE INFO

Article history:
Received 19 May 2015
Received in revised form
25 November 2015
Accepted 27 November 2015
Available online 18 December 2015

Keywords:
Trinitrotoluene
Bound residue
Nonextractable
Fish
Mussel

ABSTRACT

To determine if trinitrotoluene (TNT) forms nonextractable residues in mussels (*Mytilus galloprovincialis*) and fish (*Cyprinodon variegatus*) and to measure the relative degree of accumulation as compared to extractable TNT and its major metabolites, organisms were exposed to water fortified with ^{14}C-TNT. After 24 h, nonextractable residues made up 75% (mussel) and 83% (fish) while TNT accounted for 2% of total radioactivity. Depuration half-lives for extractable TNT, aminodinitrotoluenes (ADNTs) and diaminonitrotoluenes (DANTs) were fast initially (<0.5 h), but slower for nonextractable residues. Nonextractable residues from organisms were identified as ADNTs and DANTs using 0.1 M HCL for solubilization followed by liquid chromatography-tandem mass spectrometry. Recovered metabolites only accounted for a small fraction of the bound residue quantified using a radiotracer likely because of low extraction or hydrolysis efficiency or alternative pathways of incorporation of radiolabel into tissue.

Published by Elsevier Ltd.

1. Introduction

Manufacturing of explosives and their loading, assembling and packing into munitions for use in testing on training sites, as well as use in the battlefield, has resulted in the contamination of terrestrial and aquatic systems (Monteil-Rivera et al., 2009). Many sites around the world are known to contain explosives and associated compounds in soil, sediment, groundwater or surface water at concentrations that span several orders of magnitude (Talmage et al., 1999; Jenkins et al., 2001). Large amounts of explosives packaged into discarded shells, as well as fragments of explosives formulations remaining following incomplete detonations, may be present in surface soils and in aquatic habitats. Underwater military munitions present in the environment may contaminate the water column as a result of the release of explosives following blow-in-place detonation, corrosion or breaching (Pennington et al., 2008; Lewis et al., 2009; Wang et al., 2011), potentially creating a source compartment for uptake into marine organisms.

The nitroaromatic compound 2,4,6-trinitrotoluene (TNT) is a secondary explosive widely used worldwide mostly for military purposes. TNT has a low potential to accumulate in aquatic invertebrates, fish, and terrestrial invertebrates due to a low degree of lipophilicity and rapid biotransformation (Lotufo et al., 2009). Since TNT is rapidly chemically reduced in most biological systems, the aminated TNT biotransformation products 2- and 4-aminodinitrotoluene (2- and 4-ADNT) are typically found at higher concentrations within tissue than parent TNT in fish and in aquatic and soil invertebrates (Johnson et al., 2000; Conder et al., 2004; Dodard et al., 2004; Belden et al., 2005; Yoo et al., 2006; Rosen and Lotufo, 2007; Belden et al., 2011). Bioconcentration factors ranging from 0.3 to 13.1 L/kg for TNT and its major aminated transformation products 2- and 4-ADNT and 2,4- and 2,6-diaminonitrotoluene (DANTs) have been reported for fish and invertebrates (reviewed in Lotufo et al., 2009 and Lotufo et al., 2013).

Aquatic and terrestrial organisms exposed to TNT bioaccumulate bound, or nonextractable, residues to a larger extent than the sum

* Corresponding author.
E-mail address: guilherme.lotufo@usace.army.mil (G.R. Lotufo).

http://dx.doi.org/10.1016/j.envpol.2015.11.049
0269-7491/Published by Elsevier Ltd.

of extractable TNT and ADNTs (Belden et al., 2005, 2011; Ownby et al., 2005; Lotufo, 2011). In those studies, bioaccumulation of the explosives was measured using a radiolabeled version (^{14}C) of TNT. The formation of bound residues was verified by the inability of acetonitrile (ACN) to extract radiolabeled metabolites from whole-body samples. Acetonitrile efficiently extracts TNT and related compounds and is used in a standard analytical procedure (U.S. Environmental Protection Agency, 2006). The notion of considering these metabolites as nonextractable does not imply that extraction is impossible. Rather it implies that solvents that readily extract the parent compound and primary metabolites did not extract this type of metabolite (Belden et al., 2011). Therefore, nonextractable is experimentally defined in this study as residue that was not extracted by acetonitrile or methanol. Although previous studies demonstrated the prevalence of nonextractable residues compared to TNT, ADNTs, and DANTs in midge (*Chironomus dilutus*), oligochaete (*Lumbriculus variegatus*), channel catfish (*Ictalurus punctuatus*), and sheepshead minnows (*Cyprinodon variegatus*) (Belden et al., 2005; Ownby et al., 2005; Lotufo, 2011), those studies did not investigate the chemical nature or the toxicokinetics of nonextractable residues. Slower rate of depuration for bound residues relative to those for TNT, ADNTs, and DANTs have been speculated (Conder et al., 2004; Belden et al., 2005) and likely contribute to the observed prevalence of nonextractable residues following exposure to TNT.

An investigation of ^{14}C-TNT toxicokinetics in earthworms (Belden et al., 2011) reported depuration half-lives ranging from 61 to 120 h for TNT, ADNTs, and DANTs, but the much slower depuration of nonextractable residues resulted in half-lives ranging from 201 to 240 h. Depuration is defined as the loss of a substance from an organism as a result of any active or passive process (ASTM, 2013). Despite resisting organic solvent extraction, 80% of the nonextractable residue in earthworms was solubilized when residual tissue was treated with a dilute aqueous solution of hydrochloric acid (Belden et al., 2011). Polar conjugates formed between reactive TNT metabolites and proteins (e.g., a sulfinamide conjugate) would not be expected to be solubilized by organic solvents. Previous studies have demonstrated the formation of sulfinimide covalent bonds between reduced metabolites of TNT and nitromusks with hemoglobin (Leung et al., 1995; Bakhtiar and Leung, 1997; and Mottaleb et al., 2012). It is feasible that treatment with dilute acid could potentially break the presumed bond responsible for conjugation and release the TNT-derived portion of the molecule (i.e., reduced metabolites) into aqueous solution as has been reported for similar adducts (Mottaleb et al., 2012). Thus, it is our hypothesis that the nonextractable residues are TNT metabolites present in tissue as conjugates. If nonextractable residues accumulate to a greater extent and have longer half-lives compared to TNT, ADNTs, and DANTs, then insight into the mechanism of TNT toxicity may be gained by elucidating the structure of presumed conjugates. Additionally, nonextractable residues could potentially be useful as a biomarker of exposure for TNT, as proposed for earthworms (Belden et al., 2011) and mammalian hemoglobin (Bakhtiar and Leung, 1997).

The objectives of this study were to 1) assess the toxicokinetics (uptake, depuration and transformation) of TNT in a marine fish (sheepshead minnow) and invertebrate (Mediterranean mussel), including determination of the biological half-life of TNT, its breakdown products and bound residues; and 2) determine whether nonextractable residues can be hydrolyzed, releasing reductive metabolite(s) of TNT that are amenable to analytical identification and measurement in the absence of a radiotracer.

2. Materials and methods

2.1. Experimental organisms

Experimental organisms were laboratory-cultured juvenile (approximately 8-wk old) sheepshead minnows (*C. variegatus*, 85 mg mean individual wet weight) and field-cultured sexually mature Mediterranean mussels (*M. galloprovincialis*, 375 mg mean soft tissue individual wet weight). Fish were purchased from Aquatic BioSystems (Fort Collins, CO) and mussels were purchased from Carlsbad Aquafarm (Carlsbad, CA, USA). Organisms were shipped overnight and acclimatized to exposure conditions for approximately one week prior to experiment initiation. The sheepshead minnow is a common species in southern estuaries of the United States, is cultured in the laboratory and is available from commercial vendors, and widely used in ecotoxicology studies. The Mediterranean mussel is a common species along the west coast of the United States, and is commonly used in biomonitoring studies and in whole effluent toxicity evaluations.

2.2. Chemicals

Trinitrotoluene (uniformly ringed-^{14}C, specific activity 40 ci/mol) was purchased from Perkin Elmer Life Sciences, Boston, MA. Purity was determined to be >96% based on HPLC as described in Belden et al. (2011). The remaining 4% was unidentified. Unlabelled TNT was obtained from Chem Service, Inc. (West Chester, PA, USA). The compounds, 2-amino-4,6-dinitrotoluene (2-ADNT), 4-amino-2,6-dinitrotoluene (4-ADNT), 2,4-diamino-6-nitrotoluene (2,4-DANT), and 2,6-diamino-4-nitrotoluene (2,6-DANT) were obtained from commercial vendors in the highest available purity and used as received. All solvents were high-performance liquid chromatography (HPLC) grade or purer. Liquid scintillation counting (LSC) was performed using Scintisafe Plus 50% (Fisher Scientific, Pittsburgh, PA, USA).

Isotopically-labeled (ring-^{13}C) forms of the hydrolysis products (i.e., ADNTs and DANTs) monitored in this study were prepared in house *via* a simple, one-step reaction. In a typical preparation, 0.25 g of iron powder was added to 2 mL of a 50:50 mixture of aqueous 1% (v/v) acetic acid and methanol. The solution was purged with nitrogen to minimize oxidation of iron. Next, 150 μL of a 1 mg/mL solution of trinitrotoluene (ring-^{13}C) was added, and the reaction was stirred at room temperature. Small volumes of the 50:50 solvent mixture were added, as necessary, to compensate for evaporation and maintain a solution volume of approximately 2 mL. Within min, the stirred solution turned yellow-orange in color. Iron acts as a reducing agent in this reaction, transforming the nitro substituents on TNT into amino groups. The formation of desired transformation products was monitored *via* liquid chromatography-tandem mass spectrometry LC-MS/MS, using the analytical method detailed in Section 2.5 below. Specifically, a peak was observed at the retention time corresponding to the unlabeled analyte (*i.e.*, 2-ADNT; 4-ADNT; 2,4-DANT; or 2,6-DANT) when the mass analyzer was operated in multiple reaction monitoring (MRM) mode. Thus, evidence for formation of each desired reaction product included: (*i*) an LC retention time that matched that observed for the unlabeled analyte, (*ii*) a precursor ion mass (*m/z*) that matched that expected for the protonated (2,4-DANT and 2,6-DANT: $m/z = 174$) or deprotonated (2-ADNT and 4 ADNT: $m/z = 202$) molecular ion, and (*iii*) a product ion mass that corresponded to the same collision induced dissociation reaction(s) that were observed for the unlabeled analyte. A reaction time of 10 min typically produced a light yellow solution that contained ADNTs as the more abundant product (*i.e.*, 2-ADNT and 4-ADNT were the more abundant peaks observed in the LC-MS/MS chromatogram).