Solid phase microextraction of aminodinitrotoluenes in tissue

Alethea T. Bowen a, Jason M. Conder b, Thomas W. La Point a,*

a University of North Texas, Department of Biological Sciences, Institute of Applied Sciences, Environmental Science Program, P.O. Box 310559, Denton, TX 76203-0559, USA
b Environ International Corporation, 2010 Main Street, Suite 900, Irvine, CA 92614-7215, USA

Received 5 March 2005; received in revised form 18 July 2005; accepted 21 July 2005
Available online 1 December 2005

Abstract

Tubifex tubifex metabolizes 2,4,6-trinitrotoluene (TNT) to 2-amino-4,6-dinitrotoluene (2ADNT) and 4-amino-2,6-dinitrotoluene (4ADNT). Elimination rates of metabolically-generated ADNTs are low compared to ADNTs absorbed directly from water, suggesting that metabolically-generated ADNTs may be bound or sequestered within tissue and therefore less available for elimination. A solid phase microextraction (SPME) technique was used to extract ADNTs from T. tubifex tissue to investigate the recalcitrance of metabolically-generated ADNTs. As SPME is a gentle, non-depletive, equilibrium sampling technique useful for measuring "available" organic compounds, we hypothesized that metabolically-generated ADNTs would be less extractable than absorbed ADNTs. T. tubifex were exposed to two scenarios to generate tissues containing absorbed ADNTs and metabolically-generated ADNTs. Tissue was then homogenized in a neutral buffer solution. Polyacrylate-coated (PA) SPME fibers were deployed and agitated in tissue homogenates to measure available ADNTs. Extractability of ADNTs from tissue containing metabolically-generated ADNTs was significantly less than expected: 50–60% based on the theoretical fiber–water partition ratio. Extractability of absorbed ADNTs was significantly higher (81–90%), and not significantly different than expected. The lower SPME extractability of metabolically-generated ADNTs may stem from the unavailability of metabolically-generated ADNTs sequestered in tissue or bound to tissue macromolecules during metabolism of TNT to ADNT. Tissue extractions using SPMEs may be able to estimate bound organic residues in tissue and serve to indicate the toxicological bioavailability of tissue-associated organic compounds.

© 2005 Elsevier Ltd. All rights reserved.

Keywords: Nitroaromatic; Tubifex tubifex; Bioavailability; Metabolism

1. Introduction

2,4,6-Trinitrotoluene (TNT) and its reduction products have been found in military bases and army ammunitions plants throughout the United States. Production sites such as Louisiana Army Ammunition and Cornhusker Army Ammunition Plants contain explosives-contaminated soils with concentrations up to 87 000 mg/kg (Steevens et al., 2002). These nitroaromatic (NA) compounds enter into the surface water, groundwater, soils and sediments through runoff (Spain, 2000; Lotufo et al., 2001) and have the potential to affect...
aquatic ecosystems (Renoux et al., 2000). The majority of toxicity research has been performed with TNT parent compound; information on the bioaccumulation and toxicity of its metabolites to aquatic invertebrates is scarce. The most prevalent metabolites, 2-amino-4,6-dinitrotoluene (2ADNT) and 4-amino-2,6-dinitrotoluene (4ADNT), may be even more toxic than the original compound (Lotufo et al., 2001; Lachance et al., 2004; Conder et al., 2004a).

Tiger salamander (Ambystoma tigrinum), earthworm (Eisenia andrei), white potworm (Enchytraeus albidus), and aquatic oligochaete (Tubifex tubifex) (Robidoux et al., 1999; Johnson et al., 2000; Dodard et al., 2004; Conder et al., 2004a) are able to biotransform TNT via detoxification mechanisms or enzymatic and microbial activities. Biotic transformation of TNT to ADNTs follows the general detoxification pathway in that the metabolites are more water-soluble than the parent compound, and thus, should be more-easily eliminated (Boelsterli, 2003). However, Conder et al. (2004a) found that in T. tubifex exposed to TNT, approximately 60% of metabolically-generated ADNTs was not eliminated during the 54 h depuration period in clean water. This phenomenon was specific to metabolically-generated ADNTs, as T. tubifex exposed to ADNTs only in water and then placed in uncontaminated water were able to eliminate their absorbed-ADNT body burdens completely within a 3-h elimination period. The inability of T. tubifex to rapidly and completely eliminate metabolically-generated ADNT was hypothesized to result from incorporating ADNT into tissue macromolecules (Dodard et al., 2004; Conder et al., 2004a). This binding may reduce the ability of metabolically-generated ADNTs to interact with sites of toxic action. This reduction in “toxicologically bioavailability” (Lanno et al., 2004) is crucial to understanding critical body residues for measuring toxicity (McCarty, 1991) and the potential of a compound to bioaccumulate and biomagnify. For example, if metabolically-generated ADNTs are less toxicologically bioavailable due to binding to macromolecules, a higher critical body residue would be expected compared to that associated with absorbed ADNTs.

Solid phase microextraction (SPME) may be a potentially useful, inexpensive, and simple method of evaluating the toxicological bioavailability of organic compound body burdens. SPME is a gentle extraction method which has been used to measure available molecules in environmental compartments such as soil, sediment, and water (Alpendurada, 2000; Mayer et al., 2000; Wells and Lanno, 2000; Leslie et al., 2002a,b; Conder et al., 2004c; Conder and La Point, 2005). This simple and effective technique requires less labor time and solvent (Conder et al., 2003) in the absorption and desorption procedures in environmental sample analyses. Analytes can be desorbed by small amount of solvents followed by high performance liquid chromatography (HPLC) analysis. In contrast to strong solvent extractions, which attempt to extract 100% of organic compound present, SPMEs passively absorb only weakly-bound or dissolved analytes which are able to partition from solution into the SPME’s polymer fiber coating (Wercinski and Pawliszyn, 1999). As SPMEs are capable of predicting the environmental bioavailability of compounds in environmental compartments, it may be possible to use them as indicators of toxicological bioavailability within biological tissues.

The objective of this study was to investigate the use of SPME to measure the toxicological bioavailability of tissue-associated organic compounds in T. tubifex. Our specific hypothesis was that metabolically-generated ADNTs would be less extractable than absorbed ADNTs due to the incorporation of metabolically-generated ADNTs to biological macromolecules. Recalcitrant metabolically-generated ADNTs were expected to favor biological tissues over passive SPME partitioning. This was tested by deploying SPMEs in homogenized T. tubifex tissues containing metabolically-generated or absorbed-ADNTs. As tissues were homogenized, shearing most cellular membrane and organelles, we assumed that if recalcitrance of metabolically-generated ADNTs could be detected by differences in SPME-extractability, that recalcitrance would be evident on a subcellular (homogenized) scale. Tissues were obtained by exposing T. tubifex to TNT followed by a depuration period in water (to generate tissue containing metabolically-generated ADNTs) or by exposing T. tubifex directly to ADNT in water (to generate tissue containing absorbed ADNTs).

2. Materials and methods

2.1. Chemicals used

Pale yellow TNT crystals (molecular mass = 227.1 g/mol, 98% pure) were obtained from Chem Service, Inc., (West Chester, PA, USA). 2ADNT and 4ADNT crystals were obtained from R. Spanggord, SRI International (Menlo Park, CA, USA). Purity was verified via HPLC in our laboratory.

2.2. Experimental setup

Test waters were spiked with an aliquot of 50:50 HPLC grade acetonitrile:ultrapure water (v/v) (Milli-Q® purification system, Millipore, Bedford, MD, USA) containing NAs. Spike solution was added to a volumetric flask and a gentle stream of helium gas was blown along the interior surface of the flask to evaporate acetonitrile. The remaining solvent-free aliquot was re-dissolved with reconstituted hard water (alkalinity = 115 mg CaCO₃,
hardness = 156 mg CaCO₃, and dissolved oxygen = 8.3 mg/l) to make up the final volume. If impurities and undissolved crystals were observed, the water was filtered (1-μm glass-fiber filter).

*T. tubifex* were acquired from in-house cultures (average wet weight is 0.003 ± 0.0003 g/T. tubifex) and rinsed with dechlorinated tap water before exposure to NA-spiked water. Two exposure regimes were used to obtain *T. tubifex* tissues containing metabolically-generated or absorbed ADNTs. To obtain *T. tubifex* containing metabolically-generated ADNTs, *T. tubifex* were exposed to 30 nmol TNT/ml (490 ml/replicate) for 24 h, and then transferred to clean reconstituted water (average wet weight is 0.003 ± 0.0003 g/T. tubifex) filtered (1-m glass–fiber filter). To obtain *T. tubifex* containing absorbed ADNTs, *T. tubifex* were exposed to 10 nmol 2ADNT/ml and 10 nmol 4ADNT/ml (450 ml/replicate) for 24 h. For both exposure regimes, each replicate (four replicates per treatment) contained cm³ 1-mm glass beads and 160 *T. tubifex*. Beakers were covered with perforated aluminum foil to minimize solution loss but allow gas exchange. Water NA concentrations during the TNT-exposures were measured at 0 and 24 h. No mortality was observed during the exposure regimes.

2.3. *SPME fibers*

Eighty-five-μm diameter polyacrylate-coated solid phase microextraction fiber (Supelco, North Harrison Road, Bellefonte, PA, USA) was used to extract ADNTs from tissue homogenates. Bulk fiber was cut into 1-cm pieces with a double-bladed stainless steel razor blade apparatus. Each fiber was secured within the slit of a 1 cm diameter Teflon-coated silicone disc (septa for HPLC vials) so that it could be handled easily. SPME fibers in Teflon discs were rinsed with 50:50 HPLC-grade acetonitrile:ultrapure water solution (v/v) for 10 min (Conder et al., 2003). SPME extract was then analyzed by HPLC (see below). In all experiments, SPMEs extracted approximately 0.3–0.4 nmol NA from the 16-ml homogenate solution, which contained approximately 13–15 nmol NA. Thus, SPMEs extracted less than 5% of the total mass balance, fulfilling the requirement as non-exhaustive, negligible depletion extraction (Vaes et al., 1996; Conder et al., 2003).

Following SPME retrieval, the homogenate was centrifuged at 1000g for 1 min. Five milli litre of homogenate supernatant was combined with 5 ml acetonitrile and filtered through a 0.45-μm glass microfiber filter before HPLC analysis. Water samples and SPME and organism extracts (100-μl injections) were analyzed by HPLC (fixed UV detector at 254 nm) using a Waters Nova-Pak C18 60 Å 4 μm (spherical) 3.9 × 150 mm column with an 84:16 isopropanol:ultrapure water (v/v) isocratic mobile phase. Total running time for the program was approximately 28–30 min with a flow rate at 1.0 ml/min.

2.5. Data analysis

Extractability of 2ADNT and 4ADNT in tissue homogenates was evaluated by normalization by the expected fiber:solution partition coefficient, *Kfs* (Wercinski and Pawliszyn, 1999):

\[
K_{fs} = \frac{C_f}{C_s}
\]

where *Cf* is the concentration of ADNTs (μmol/ml) in the polyacrylate fiber coating and *Cs* is the concentration of ADNTs (μmol/ml) in the homogenate supernatant. Expected *Kfs* values obtained from experiments in ADNT-spiked water were 1043 for 2ADNT and 1393 for 4ADNT at 23 °C (Conder et al., 2003). Extractability of tissue homogenates containing absorbed ADNTs and tissue homogenates containing metabolically-generated
ADNTs was compared via ANOVA and Student–Newman–Keuls (SNK) test a posteriori comparisons ($\alpha \leq 0.05$).

### 2.6. SPME tissue extraction method assumptions

Several crucial assumptions for the SPME tissue extraction for ADNTs were investigated prior to experimentation. First, the ability of Tris–HCl/NaN$_3$ solution to prevent ADNT transformation was evaluated. Tris–HCl/NaN$_3$ was used as a homogenate solution to maintain neutral pH and isotonic conditions and limit the action of bacteria that could transform ADNTs. To evaluate potential loss of ADNTs due to bacterial transformation, ADNTs were added to homogenized $T$. tubifex tissue in the Tris–HCl/NaN$_3$ solution and measured at the end of the 48 h period required for the SPME extraction. Mean percent recovery ($\pm$SD) of spiked 2ADNT and 4ADNT in Tris–HCl/NaN$_3$ solution containing uncontaminated tissue homogenates (at $t = 48$ h) were 104.80% ($\pm$13.06) and 103.26% ($\pm$17.62) respectively. This suggests that transformation of ADNTs in the Tris–HCl/NaN$_3$ solution did not occur.

Although salt solutions as high as 10000 µg/ml do not affect $K_{fs}$ in aqueous solutions (Barschick and Griest, 1998), we examined $K_{fs}$ values tissue-free homogenate solution (Tris–HCl/NaN$_3$). SPMEs were exposed to tissue-free homogenate solution and water spiked at concentrations of 1 nmol 2ADNT/ml and 1 nmol 4ADNT/ml. There were no significant differences in SPME $K_{fs}$ values between Tris–HCl/NaN$_3$ and ultrapure water ($p = 0.44$ and $p = 0.90$, respectively), suggesting that extractability of ADNTs was not affected by Tris–HCl/NaN$_3$.

### 3. Results and discussion

$T$. tubifex tissues used in the experiments contained either metabolically-generated or absorbed ADNTs at tissue concentrations of 30–100 nmol/g w/w ADNT. Mean 2ADNT concentrations in 16 ml tissue homogenate supernatants were 0.93 ($\pm$0.061) nmol/ml and 0.83 nmol/ml ($\pm$0.053) for homogenates containing metabolically-generated and absorbed 2ADNTs, respectively. Mean 4ADNT concentrations of the 16 ml tissue homogenate supernatants were 0.91 ($\pm$0.11) nmol/ml and 0.94 ($\pm$0.07) nmol/ml for homogenates containing metabolically-generated and absorbed 4ADNTs, respectively. TNT was rapidly eliminated from $T$. tubifex during exposure uncontaminated water following TNT exposure and no TNT was detected from tissues containing metabolically-generated ADNTs (Conder et al., 2004a). Mean 2ADNT and 4ADNT concentrations in homogenates containing uncontaminated tissue spiked with known amounts of ADNT were slightly higher than the other homogenates: 1.17 nmol/ml ($\pm$0.12) and 1.4 nmol/ml ($\pm$0.10), respectively.

SPME extractability of 2ADNT and 4ADNT from homogenate containing metabolically-generated ADNTs was significantly less (by a factor of 1.50) than that from homogenates containing absorbed ADNT and spiked ADNT ($p = 0.012$ and 0.003, respectively) (Fig. 1). Extractability of metabolically-generated ADNTs was only 50–60% based on the theoretical fiber-water partition ratio (i.e., mean $K_{fs}$ values in homogenates containing metabolically-generated ADNTs were 625 and 697 compared to expected $K_{fs}$ values in water were 1043 and 1393 (Conder et al., 2003). In contrast, SPME extractability of absorbed and spiked ADNTs was not significantly different ($p > 0.05$), suggesting that absorbed ADNTs easily diffuse from tissue and partition to the SPME. SPME extractability of absorbed and spiked ADNTs did not differ significantly from 100% ($p > 0.05$), suggesting that homogenized tissue did not reduce extractability via absorption of ADNTs.

The lower extractability of metabolically-generated ADNTs is supported by previous research by Conder et al. (2004a), who observed an unexpected recalcitrance of metabolically-generated ADNTs in $T$. tubifex during a 54 h depuration period in uncontaminated water. This recalcitrance was not observed during elimination periods following exposure to ADNT only, as absorbed ADNTs were quickly eliminated with 3 h. Metabolically-generated ADNTs are slowly or not easily eliminated and may be less available within tissues, as reflected by lower SPME extractability. This reduction

![Fig. 1. Mean (±SD) percent extractability (based on amount predicted by SPME-ADNT partition coefficient in water) of SPMEs exposed to 2ADNT (a) and 4ADNT (b) in $T$. tubifex tissue homogenates containing metabolically-generated ADNT, absorbed ADNT, and spiked ADNT. Means with the same letter are not significantly different.](image-url)
in availability may be due to the physiological location in which ADNTs are incorporated into tissue during metabolism of TNT to ADNTs. Absorbed ADNTs may concentrate in the dermis and remain toxicologically available, whereas metabolically-generated ADNTs may be bound within lipid bilayers, where they may be bound to macromolecules. Sequestration of TNT metabolites has been observed by Conder et al. (2004a), who noted that a significant amount of radiolabeled (14C) TNT equivalents (unidentified substances containing the 14C radiolabel originating from 14C-TNT) was not extractable by the strong solvent tissue extraction. It was hypothesized that these unextractable substances were sequestered in tissue and covalently bound to biomolecules. The pool of metabolically-generated ADNTs which are extractable by strong solvent but only partially extractable by SPME (as shown in this paper) may represent an intermediate stage in the partitioning of tissue-associated in T. tubifex. The partial extractability by a gentle, equilibrium-based measurement technique such as the tissue SPME extraction may indicate that metabolically-generated ADNTs are more available than “unextractable” substances, but less available than TNT or absorbed ADNTs that are easily eliminated. Also, it is unknown whether these partially-extractable ADNTs are toxicologically active or bioavailable for dietary uptake by animals which may consume T. tubifex. Dietary bioavailability of TNT in catfish (Ictalurus punctatus) is extremely low (bioaccumulation factor of 2.4 × 10^{-5}, Belden et al., 2005), and it is expected that bioavailability of partially-extracted ADNTs would be as low or lower. The presence of recalcitrant NA residues may complicate the interpretation of tissue-associated NAs in the context of risk assessment.

4. Conclusions

Significantly lower 2ADNT and 4ADNT SPME extractability was observed in T. tubifex tissue homogenates containing metabolically-generated ADNTs compared to homogenates containing absorbed ADNTs or spiked ADNTs. The lower SPME-extractability of metabolically-generated ADNTs corresponds with previous research that notes that metabolically-generated ADNTs may be more recalcitrant in tissue than absorbed ADNTs. This recalcitrance may be due to sequestration and binding of metabolites to tissue macromolecules. Tissue extractions using SPMEs may be able to provide useful information concerning toxicological bioavailability and the partitioning of organic compounds within organisms. Future research should focus on the use of SPMEs in measuring the toxicological bioavailability of compounds not as readily partitioning into lipids. Further, differences between critical body residues and toxicokinetics of metabolically-generated ADNTs deserves further study.

References


Eisenia andrei exposed to amended forest soil. Chemosphere 55, 1339–1348.


