Letter

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Tissue Distribution of Substituted Diphenylamine Antioxidants and Benzotriazole UV Stabilizers in White Sucker (*Catostomus commersonii*) from an Urban Creek in Canada

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ABSTRACT

Substituted diphenylamine antioxidants (SDPAs) and benzotriazole UV stabilizers (BZT-UVs) have been detected in aquatic organisms, but little is known about their tissue distribution and elimination in wildlife. The present study investigates the distribution of SDPAs and BZT-UVs in blood plasma, bile, liver and carcass (without gill and internal organs) of white sucker (*Catostomus commersonii*) and evaluates the extent of biliary excretion in fish. Fish were collected from a creek upstream and downstream of an urban area in Ontario, Canada. Downstream fish showed higher contamination of many target compounds (e.g., 4,4'-bis(α,α-dimethylbenzyl)diphenylamine and monononyl diphenylamine) than those from upstream, indicating the input of these contaminants from the urban area. The concentration (wet weight) of target compounds generally followed the order of liver > carcass homogenate ≥ bile > plasma, indicating that liver is a major tissue for accumulation of these contaminants in fish. Tissue-specific partition coefficients suggest that SDPAs tend to partition from plasma to liver and that the biliary excretion of these contaminants is limited relatively minor pathway of elimination. Only monobutyl diphenylamine was effectively excreted via bile. Our results suggest that future studies should focus on the liver toxicities and biotransformation of these contaminants to better understand their environmental risks.
INTRODUCTION

Substituted diphenylamine antioxidants (SDPAs) and benzotriazole UV stabilizers (BZT-UVs) are organic contaminants of emerging environmental concern. SDPAs are common industrial antioxidants added to engine oil, lubricant, plastic, polyurethane foam and rubber to prevent oxidative degradation. BZT-UVs are a class of additives used in daily commodities (e.g., cosmetics and plastics) and industrial products to minimize color change and degradation of materials caused by solar radiation. SDPAs and BZT-UVs may be released into environments during manufacture, application, and waste disposal processes.

SDPAs and BZT-UVs have been detected in environments such as surface water, wastewater and sewage, sediments, beach sand, dust, and soil. Aquatic organisms may accumulate these contaminants via ingestion, dermal exposure and respiration. The bioaccumulation of BZT-UVs has been reported for organisms including invertebrates, fish, birds, marine mammals and human breast milk. In our previous research, we reported SDPAs and BZT-UVs in fish and invertebrates from freshwater environments in Canada as well as marine mammals in the southern USA. Analysis of European eels (Anguilla anguilla) by non-targeted high resolution mass spectrometry identified µg g⁻¹ levels of SDPAs on a wet weight (ww) basis. The occurrence of SDPAs and BZT-UVs in different environmental compartments indicates that these contaminants are of emerging concern and with an environmental presence on a global scale.

Accumulation of SDPAs and BZT-UVs in wildlife raises the concern of their potential toxicities in aquatic environments. However, the toxicology information for these contaminants is very limited. BZT-UVs may have endocrine disruption potential in humans and fish, and sex-specific chronic
toxicity in rats. A recent risk assessment summarized the modeled and measured toxicities of SDPAs, but many of the references cited in this assessment were not peer-reviewed.

Once accumulated from the aquatic environment, SDPAs and BZT-UVs may be subjected to lymphatic, blood and/or enterohepatic circulation systems and therein be distributed to various tissues in fish. Biliary excretion is an important process for the elimination of xenobiotics from fish. The rate of contaminant excretion into bile has implications for toxicity and detoxification processes.

Endogenous partitioning and elimination processes are affected by many factors such as lipid content and metabolic potency of target tissues, as well as the exposure route and physical-chemical properties of the target compounds. Our previous research demonstrated a range of bioaccumulation factors for a suite of SDPAs and BZT-UVs, suggesting that distribution of these substances in tissues/organs might be congener-specific. Thus, tissue distribution analysis would provide a basis for relevant toxicity assessment of these contaminants. However, much remains unknown about their circulation and tissue distribution. To date, tissue distribution of SDPAs has only been qualitatively reported in the muscle, gonad and eggs of European eels, while the distribution of BZT-UVs has only been studied in the liver and carcass homogenate for few marine species.

In this study, we report the distribution of SDPAs and BZT-UVs in the plasma, liver, bile and carcass of white sucker (Catostomus commersonii) from an urban creek. White sucker is a benthic fish widely distributed across North America including in the Great Lakes Basin. These fish are opportunistic bottom feeders that live in close contact with sediments and thus sediment-borne exposure is a particularly significant vector for contaminants to this species. This is the first quantitative report of tissue-specific partitioning of SDPAs and BZT-UVs in wildlife.
MATERIALS AND METHODS

Chemicals. Details of standards (Table 1 and Figure S1) and other materials are described in Supporting Information (SI).

Sample Collection and Analysis. Details of fish collection, extraction and analysis have been previously described\(^1\) and are summarized with biometric data (Table S1) and QA/QC (Table S2) in SI.

Data Analysis. Data was analyzed using Rstudio V 0.99.903 (Boston, MA, USA) and GraphPad Prism 7.0 (La Jolla, CA, USA). Statistics for data with censored values (< 50% censoring) were conducted using the robust regression on order statistics (ROS) in Rstudio by the Nondetects and Data Analysis (NADA) package (V1.5-6).\(^36\) Concentration is reported as arithmetic mean ± standard error (SE) (ww). Liver/tissue partition coefficients were calculated as the ratio of contaminant concentration (ww) in liver to that in plasma or bile.\(^37,38\) Non-normally distributed data (Shapiro-Wilk test) were logarithmically transformed to approximate a normal distribution before being subjected to statistical \(t\) test or one-way ANOVA analysis. The significance level was set to \(p < 0.05\).

RESULTS AND DISCUSSION

Tissue Distribution. Since SDPAs and BZT-UVs are hydrophobic, lipid content in tissues may affect the distribution of these contaminants in fish. The lipid content was 16 ± 1, 20 ± 5, 25 ± 1 and 22 ± 3 mg g\(^{-1}\)(ww) (mean±SE) in plasma, bile, liver and carcass, respectively. No statistically significant correlation exists between the concentration (ww) of target contaminants and lipid content (g g\(^{-1}\), ww) (\(r\), correlation coefficient: between -0.46 and 0.37; \(\rho\), probability of correlation to be caused by random sampling: between 0.06 and 0.81), indicating that other processes (e.g., biotransformation and
enterohepatic circulation) may be involved in regulating the accumulation of these contaminants in tissues.\(^\text{39}\) Thus, the data was not lipid normalized for comparison in the present study.\(^\text{39}\) A larger sample size may be necessary to confirm the extent of the trend observed using 18 fish in this study and also the lack of lipid correlation to contaminant concentration in our previous whole body homogenate analysis of 20 forage fish (common shiner \((Luxilus cornutus)\) and hornyhead chub \((Nocomis biguttatus)\)) and 35 crayfish \((Orconectes spp.)\).\(^\text{1}\)

All target SDPAs were detected in white sucker tissues. For the six monitored BZT-UVs, only UV234 and UV328 were higher than their corresponding method limit of quantification (MLOQ) (Tables S2 & S3). Downstream fish showed higher detection frequency and concentrations of many target contaminants than those from upstream (Figure 1 & S2), indicating the input of these contaminants from the town (e.g., industry and WWTP), consistent with our previous spatial trend comparison for sediment, water and organisms from the same sampling location.\(^\text{1}\) The concentration of \(\Sigma\)SDPAs \((\text{ww})\) generally followed the order of liver \(>\) carcass \(\geq\) bile \(>\) blood plasma (Figures 1 & S3).

The highest mean concentration of \(\Sigma\)SDPAs was observed in fish liver (upstream: \(5.6 \pm 3.3 \text{ ng g}^{-1} \text{ ww}\); downstream: \(19.8 \pm 2.9 \text{ ng g}^{-1}, \text{ ww}\)) and was 3.7 – 40 times greater than in other tissues (Figure 1). \(\Sigma\)SDPAs \((\text{ww})\) in the liver was significantly higher than in plasma and bile for both upstream and downstream fish (Figures 1 & S3). For C4C8, C8C8, C9C9, diAMS, UV234 and UV328, significantly higher concentrations of these contaminants were detected in downstream liver compared with other tissues (Figure S3). These results indicate that liver may be a major tissue for accumulation of these contaminants in fish and suggest that liver-specific SDPA and BZT-UV toxicities should be addressed in future studies. For C4, the highest concentration was found in downstream bile exceeding other
tissues (Figure S3), indicating compound-specific partitioning and distribution of SDPAs and BZT-UVs in fish tissues.

Liver. In liver, C9C9 was the dominant congener for both upstream and downstream fish (upstream 35%; downstream: 39% of ΣSDPAs) (Figures S3 & S4). C9C9 in liver was present at much greater concentration and percent proportions compared to C9C9 in other selected tissues (< 21%) (Figures S3 & S4). Other major SDPAs in liver included diAMS (27%), C8C8 (19%) and C4C8 (10%) (Figure S4). However, diAMS was the dominant (44-59%) congener in other tissues (Figure S4). Higher levels of UV234 and UV328 in the liver (e.g., downstream UV234: 13 ± 5 ng g\(^{-1}\) ww; UV328: 4.8 ± 1.5 ng g\(^{-1}\) ww) compared with other tissues (downstream UV234: 1.0 ± 0.4 ng g\(^{-1}\) ww in bile, 0.4 ± 0.2 ng g\(^{-1}\) ww in carcass; UV328: 1.4 ± 0.4 ng g\(^{-1}\) ww in carcass) (Figure S3) are consistent with findings by Nakata et al. who reported 3-4 times higher concentrations of UV327 and UV328 in the liver compared to carcass homogenate of shallow water fish from Ariake Sea in Japan.\(^6\)

The mean concentration of UV328 in white sucker liver (4.8 ± 1.5 ng g\(^{-1}\), ww) from our downstream site was comparable with the concentration determined in the livers of sea bass (Lateolabrax japonicas; 2.4 ng g\(^{-1}\), ww) and eagle ray (Aetobatus flagellum; 8.1 ng g\(^{-1}\), ww), but lower than that of mullet (Chelon hematocheilus; 19 ng g\(^{-1}\), ww) and hammerhead shark (Sphnna lewini; 55 ng g\(^{-1}\), ww) all from the Ariake Sea (Japan).\(^6\) UV328 was also detected in the liver of Atlantic cod (Gadus morhua) and the concentration was in the range of <MLOQ-19.5 ng g\(^{-1}\) ww (median < 10 ng g\(^{-1}\) ww).\(^{40}\) In bream (Abramis brama) from German rivers, UV327, UV328 and UV350 were detected in liver and the highest concentration was found for UV327 (155 ng g\(^{-1}\), lipid weight (lw)).\(^{41}\) Taken together, variability in measured liver concentrations of BZT-UVs indicates different usage and
contamination pattern of BZT-UVs in global geographic regions but with a commonality of accumulation in liver.

Carcass. The mean concentrations of different SDPA congeners in upstream white sucker were in the range of 0.03 – 0.21 ng g\(^{-1}\) (ww) in carcass with highest concentration corresponding to C4C8; however C4C8 was not statistically different from downstream fish. In contrast, diAMS (2.20 ± 0.44 ng g\(^{-1}\) ww) was the dominant congener for downstream white sucker. Both diAMS and C9 (0.21 ± 0.05 ng g\(^{-1}\) ww) in downstream fish carcass were significantly higher than in upstream fish (diAMS: 0.12 ± 0.04; C9: 0.03 ± 0.006 ng g\(^{-1}\) ww). Akin to sediment (median concentration: 15.3 ng g\(^{-1}\) downstream vs. 0.34 ng g\(^{-1}\) upstream for C9, 87.7 ng g\(^{-1}\) downstream vs. 1.75 ng g\(^{-1}\) upstream for diAMS), elevated diAMS and C9 in white sucker from the downstream location suggest there are sources of specific SDPAs in the town resulting in elevated concentrations in the aquatic environment including downstream organisms.\(^1\) In addition, UV234 (0.02 – 1.98 ng g\(^{-1}\) ww, median 0.22 ng g\(^{-1}\) ww) and UV328 (<MLOQ – 3.90 ng g\(^{-1}\) ww, median 1.30 ng g\(^{-1}\) ww) were detected frequently (75%) in downstream white sucker carcass but not upstream (33%), also suggesting sources of these substances in the town.

ΣSDPAs in downstream white sucker carcass were higher than the concentrations previously reported in common shiner and hornyhead chub homogenate, but lower than the concentrations in crayfish.\(^1\) UV328 was frequently detected in white sucker carcass and crayfish homogenate, but was not detected in most smaller pelagic fish samples previously analyzed from the same creek.\(^1\) Such variations among species may be correlated with their contact frequency with the contaminated sediment,\(^1\) lifespan and metabolic capacity for these contaminants.
Bile. After liver biotransformation (if any), contaminants and any metabolites are subsequently transferred to bile in the gall bladder for excretion.\textsuperscript{42} In the present study, all target SDPAs were detected in white sucker bile samples. For upstream bile samples, C4C8 (38%) was the dominant congener and followed by C9C9 (22%) and C8C8 (18%) (Figure S4). In contrast, diAMS (49%) was the dominant congener in downstream samples, likely due to the large input of this contaminant from the urban area.\textsuperscript{1} For BZT-UVs, only UV234 was frequently detected in the downstream bile samples with mean concentration of 0.96 ± 0.43 ng g\(^{-1}\) (ww), indicating that other BZT-UVs may undergo biotransformation in the liver or are poor candidates for biliary excretion in fish. The U.S. EPA’s Estimation Program Interface (EPI) Suite (V4.11) estimates the biotransformation rate constants are in the range of 0.0008-0.18 day\(^{-1}\) for target SDPAs and 0.03-0.14 day\(^{-1}\) for target BZT-UVs in a 100 g fish. Of the BZT-UVs monitored in this research, UV234 is unique due to the presence of alkylphenyl substituents whereas the other compounds possess alkyl substituents. Sundt et al.\textsuperscript{43} demonstrated that alkylphenols tend to distribute in fish bile for excretion after chronic exposure via water or food, which is consistent with our data for UV234.

Plasma. Concentrations of SDPAs in the fish plasma were lower than other tissues (Figure 1 & S3), whereas BZT-UVs were not detected in any fish plasma samples. In upstream fish plasma, C4C8 was the dominant congener accounting for 61% of total SDPAs and followed by C8C8 (19%), C9C9 (15%) and C4C4 (4%) (Figure S4). The composition of C4C8 decreased to 30% for the downstream fish plasma samples, while the proportion of diAMS increased to 44% (Figure S4). Increase in the contribution of C9 was observed for downstream fish plasma compared to those fish from upstream (mean: 0% vs. 4%) (Figure S4). Therefore, plasma, liver, and carcass homogenate were all consistently...
elevated in diAMS and C9 in fish sampled downstream of the urban area relative to the upstream location.

**Tissue-specific partition coefficients.** Tissue-specific partition coefficients were calculated for SDPAs to examine whether the extent of liver accumulation was congener-specific. The logarithmic partition coefficients for liver/plasma and liver/bile are presented in Figure 2. Those congeners with log partition coefficients approximating 0 are indicative of an equal distribution between liver and the other tissue, whereas the coefficients >1 represent enrichment in liver over the other tissues. The log (liver/plasma) for SDPAs was all significantly higher than 0.5 suggesting a propensity for magnification in liver except for C4 which had a log liver/plasma partition coefficient of 0.18 ± 0.17. Liver-plasma partition coefficients were positively correlated with log $K_{ow}$ (Figure 2), demonstrating that hydrophobicity is one factor influencing the enhanced concentration of SDPAs in liver relative to plasma.

The log (liver/bile) partition coefficient (Figure 2) of C4 (-1.36 ± 0.27) was significantly lower than 0, indicating the effective biliary excretion of C4 in fish. C4C4 and C9 was equally distributed between liver and bile as evidenced by log (liver-bile) partition coefficients near 0 (-0.08 ± 0.11 for C4C4 and 0.02 ± 0.13 for C9). In contrast, other SDPAs tended to accumulate in liver compared to bile, with log (liver/bile) partition coefficients ranging from 0.36 ± 0.14 (diAMS) to 1.65 ± 0.60 (C9C9). A positive correlation between log (liver/bile) and log $K_{ow}$ was noted (Figure 2), suggesting that less hydrophobic SDPAs are more likely to undergo biliary excretion (Figure 2). For BZT-UVs, the log (liver/bile) was not calculated due to the low detection frequency in the paired liver and bile samples. A limitation of the derived tissue distributions is that all of the fish were fairly uniform - one species,
caught on the same day along a 2.5 km stretch of an urban creek, and with similar length, weight, and age characteristics. Typically tissue distributions of contaminants are achieved using controlled lab-based water-borne or dietary-exposures for which our field-derived results will be a useful comparison.

ASSOCIATED CONTENT

Supporting Information Available

Supporting Information for this manuscript provides details on experimental methods, fish biometric parameters, structure of target compounds, QA/QC results, raw data on detection frequency and concentrations of SDPAs and BZT-UVs in white sucker tissues, concentration and distribution of SDPAs and BZT-UVs in plasma, bile, liver and carcass.

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REFERENCES


Table 1. Chemical names, formula, CAS numbers, acronyms and physical-chemical properties of target compounds. The SDPAs of CAS No. 184378-08-3 and 68608-79-7 are seven substances considered as UVCBs (Unknown or Variable Composition, Complex Reaction Products, or Biological Materials). The representative chemical structures for these SDPAs are shown in Figure S1. All representative chemical structures were identified from Environment and Climate Change Canada’s chemical analysis of standards. For SDPAs, the terms “butyl”, “octyl” and “nonyl” are used to refer to the number of carbon atoms and represent branched alkyl chains.

<table>
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<th>No.</th>
<th>Chemical Name and Formula</th>
<th>CAS No.</th>
<th>Acronyms</th>
<th>Molecular weight (g mol(^{-1}))</th>
<th>Water solubility (mg L(^{-1}))</th>
<th>Log (K_{ow})</th>
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<td>68608-79-7</td>
<td>C8</td>
<td>337.5</td>
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**Figure Captions**

Figure 1. Boxplot comparison of \( \sum \text{SDPAs} \) in white sucker tissues from the upstream (blue) and downstream (red) locations of an urban creek in southern Ontario, Canada. Boxplots are defined as follows: center line, median; boxplot edges, 25\(^{th}\) and 75\(^{th}\) percentile; whiskers, 5\(^{th}\) and 95\(^{th}\) percentile of distribution. Differences between upstream and downstream were tested by \( t \) test with Welch’s correction (* \( p<0.05 \), ** \( p<0.01 \), *** \( p<0.001 \)).

Figure 2. Pearson correlation and linear regression between log \( K_{ow} \) of SDPAs and (A) log (liver/plasma) ratio or (B) log (liver/bile) ratio. Tissue-specific partitioning coefficients are calculated based on wet weight concentrations. Data is presented as mean ± standard error. In the plots, \( r \) represents the Pearson correlation coefficient and \( \rho \) represents the probability for the correlation to be caused by random sampling. \( R^2 \) is linear regression coefficient and \( p \) is the probability for the observed linear regression to be caused by random sampling.
Figure 1.
Figure 2.