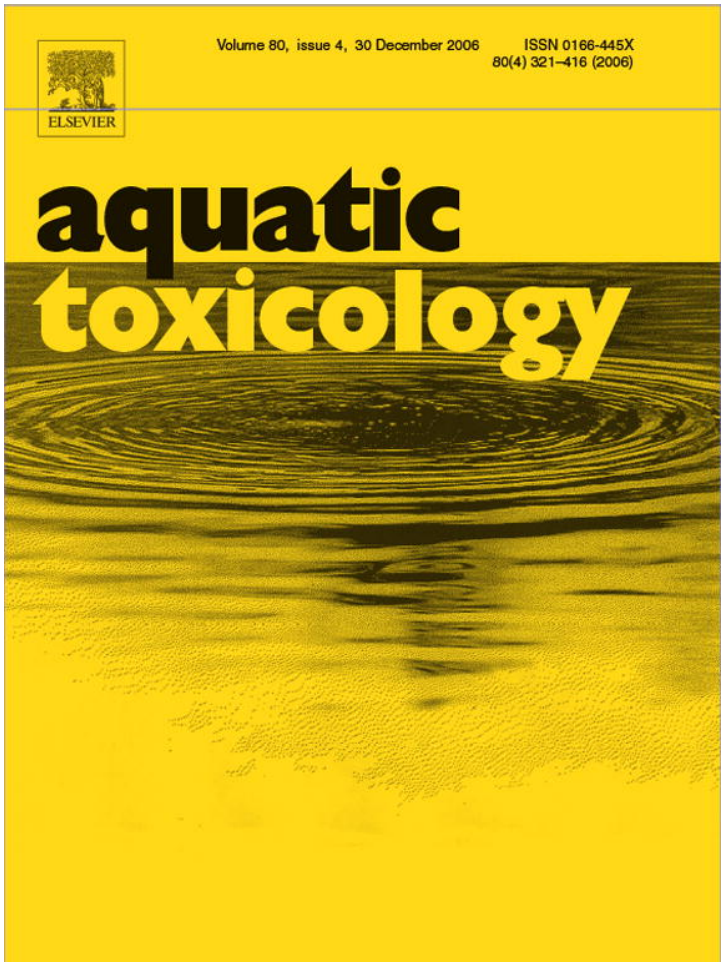

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Bioaccumulation and biotransformation of chiral triazole fungicides in rainbow trout (*Oncorhynchus mykiss*)

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Abstract

There are very little data on the bioaccumulation and biotransformation of current-use pesticides (CUPs) despite the fact that such data are critical in assessing their fate and potential toxic effects in aquatic organisms. To help address this issue, juvenile rainbow trout (*Oncorhynchus mykiss*) were exposed to dietary concentrations of a mixture of chiral triazole fungicides (bromuconazole, cyproconazole, metconazole, myclobutanil, penconazole, propiconazole, tebuconazole, tetraconazole, and triadimefon) and a chiral legacy pesticide [α-hexachlorocyclohexane (α-HCH)] to study the bioaccumulation and biotransformation of these CUPs. Fish accumulated all triazoles rapidly during the 8 day uptake phase, and was followed by rapid elimination, which was estimated by taking accelerated sampling times during the 16 day depuration period. Half-lives ($t_{1/2}$s) and times to 95% elimination (t_{95}s) ranged from 1.0 to 2.5 and 4.5 to 11.0 days, respectively. Chiral analysis suggested no significant selectivity in biotransformation for most of the compounds based on statistically unaltered enantiomer fractions (EFs) in the fish compared to food values; exceptions were a change in EF of myclobutanil and changes in diastereomer fractions (DFs) of propiconazole and cyproconazole. No biotransformation was observed for α-HCH based on consistent EFs in the fish throughout the experiment and a $t_{1/2}$ (15.8 days) that fell within the 95% confidence interval of a log K_{ow}–log $t_{1/2}$ relationship developed for assessing biotransformation of organic contaminants. This relationship did show that biotransformation accounted for the majority (ranging from 59.9 to 90.4%) of the elimination for all triazoles, and that triazole compounds with oxygen and hydroxyl functional groups were more easily biotransformed. This research indicated that chiral analysis may potentially miss biotransformation of CUPs and other potential non-persistent organic contaminants and shows the utility of the log K_{ow}–log $t_{1/2}$ relationship as a mechanistic tool for quantifying biotransformation. Based on the rapid biotransformation of the triazoles, future research should focus on formation of metabolites and their fate and possible effects in the environment.

Keywords: Enantiomers; Diastereomers; Current-use pesticides; Conazoles

1. Introduction

Current-use pesticides (CUPs) (e.g., atrazine, fipronil, diazinon, permethrin, propiconazole) can be defined as those modern pesticides that are currently registered for use, generally developed from chemical synthesis, and are typically used in the agricultural or lawn care sector. They are generally less environmentally persistent having shorter half-lives and lower bioaccumulation than the organochlorine pesticides that they have replaced. There is concern over the wide application of CUPs and their possible detrimental effects on aquatic ecosystem health that may arise from spray drift or surface run-off after rainfall events. Of particular importance are readily formed *in vivo* metabolites of CUPs, which may cause greater harm in aquatic biota than their parent compound (Sinclair and Boxall, 2003). Thus, for accurate risk assessment, there is a need to characterize the persistence and accumulation of CUPs in aquatic biota, including biotransformation rates to metabolites. However, there have been few studies that have addressed this issue for CUPs (Konwick et al., 2006), likely due to their short environmental persistence in biota (i.e., low log K_{ow} values) and the inherent difficulty in identifying the numerous potential biotransformation products. In addition, models based on physical–chemical properties of contaminants to estimate biotransformation rates in fish are limited (Borgå et al., 2004), especially concerning CUPs.

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In 1996, it was estimated that approximately 25% of CUPs were chiral (Williams, 1996), with that number to likely have risen since then because of the increasing complexity of modern pesticides with the greater likelihood of chiral centers. Chiral chemicals exist as two non-superimposable mirror images called enantiomers, often designated as (+) and (−) based on their rotation of plane-polarized light. The manufacture of chiral chemicals results in a racemic (±) mixture containing an equal percentage (50%) of each enantiomer, the form in which they are typically released into the environment. Chemicals can also contain more than one chiral center and thus exist in other stereoisomeric forms. For example, a chemical with two chiral centers would exist as two diastereomers; these are not mirror images of each other, but each would consist of two enantiomers. Enantiomers, unlike diastereomers, have identical physical–chemical (i.e., achiral) properties (Garrison, 2006), and only show differences in selectivity when in a chiral environment. Thus, the relative abundance of enantiomers is subject to change after metabolic processes due to numerous enzymes and receptors having symmetry (i.e., chiral) dependence; this property has been used recently as a tracer for biotransformation (Vetter et al., 2001; Wong et al., 2004). For example, non-racemic enantiomer residues (expressed as enantiomer ratios or fractions) have indicated that fish can biotransform several chiral organochlorines (OCs) as well as CUPs (Wong et al., 2002; Konwick et al., 2006). The use of changes in relative proportions of diastereomers has rarely been used as a biotransformation tracer, but has similar potential to enantiomer ratios. However, this feature may be compromised by the fact that diastereomers can undergo abiotic reactions at different rates.

Another method based on a curve-linear relationship developed between $\log K_{ow}$ and $t_{1/2}$ for a series of recalcitrant PCBs in juvenile rainbow trout (Fisk et al., 1998) can also provide a means of assessing biotransformation, specifically rates, in fish (Fisk et al., 2000; Buckman et al., 2006; Konwick et al., 2006). Based on this method, non-recalcitrant chemicals whose $t_{1/2}$ (determined experimentally using the same protocol) fall below this curve-linear relationship are suggested to be biotransformed, whereas those chemicals that fall on or near this relationship are assumed to not undergo biotransformation. This method has been used to generate biotransformation rates for several polychlorinated alkanes and PCBs, and fipronil in juvenile rainbow trout (Fisk et al., 2000; Buckman et al., 2006; Konwick et al., 2006), with the potential application to other CUPs.

Triazoles as well as the structurally related imidazole fungicides are used as clinical drugs and as agricultural pesticides, including applications for the treatment and protection of cereals, soybeans, and a variety of fruits (Roberts and Hutson, 1999). Their fungicidal effect is a result of the inhibition of cytochrome (CYP) P-450 dependent C14 demethylation of lanosterol, an intermediate in ergosterol biosynthesis (Roberts and Hutson, 1999). Other studies have shown that the inhibition of CYP forms is not limited to sterol biosynthesis (Rodrigues et al., 1988; Ronis et al., 1998). In fish, CYP mediated steroid metabolism (Monod et al., 1993), in addition to xenobiotic metabolism (Levine et al., 1999a; Hegelund et al., 2004), can be altered. In fish exposed to propiconazole, for example, a mixed pattern response in metabolism occurs, whereby CYP1A mRNA levels increase, but EROD activity decreases (Levine et al., 1999a). This alteration in metabolism can lead to potentially higher bioaccumulation and toxicity of contaminants. For example, gizzard shad (*Dorosoma cepedianum*) pre-exposed to clotrimazole had greater bioaccumulation of benzo[*a*]pyrene compared to fish exposed to benzo[*a*]pyrene only (Levine et al., 1997). In addition, fathead minnows (*Pimphales promelas*) that were pretreated with propiconazole showed enhanced acute toxicity after exposure to the pesticide parathion (Levine and Oris, 1999b). Therefore, it is important to understand the fate and toxicokinetics of these fungicides in fish, in part because of their ability to increase the accumulation and toxicity to other contaminants.

We investigated the bioaccumulation and biotransformation of a series of triazole fungicides by dietary exposure to juvenile rainbow trout (*Oncorhynchus mykiss*) to address the lack of such information for CUPs. The ability of this fish to biotransform these chemicals was assessed through chiral analysis and the use of the $\log K_{ow}$–$\log t_{1/2}$ relationship developed for quantifying biotransformation of organic contaminants in rainbow trout (Fisk et al., 1998, 2000). α-HCH was included in this study to expand the $\log K_{ow}$–$\log t_{1/2}$ relationship to lower $\log K_{ow}$ chemicals. In addition, we attempted to explore what role the functional groups attached to the chiral center of the triazoles had on biotransformation. To our knowledge, this is the first experiment to investigate the bioaccumulation and enantioselective biotransformation of triazole fungicides in fish.

2. Materials and methods

2.1. Chemicals and food preparation

All triazoles were obtained from the EPA Repository (EPA National Pesticide Standard Repository, Ft. Meade, MD). α-HCH was obtained from ChemService (West Chester, PA). The purities of all chemical standards were ≥97%. All solvents (Ultra Resi-Analyzed®) were obtained from J.T. Baker (Phillipsburg, NJ).

The spiked food containing a mixture of all the chemicals was made by adding a known quantity of each chemical (dissolved in 300 ml dichloromethane (DCM)) to 150 g of commercial fish food (Zeigler finfish starter, Gardner, PA; 2 mm pellets, 50% protein, 15% lipid, 2% fiber) in a round bottom flask and slowly evaporating the solvent to dryness using a rotary-evaporator. The food was air dried for 24 h and then stored in amber jars at 4 °C. Control food was treated in an identical manner but without the addition of the analytes. The concentrations of each triazole and α-HCH (Table 1) were determined in spiked and control food using the same technique described below for fish tissue. While these concentrations are above those likely to be found in the environment, which have been measured at low part per billion levels (Mortensen et al., 1998), they were required for chemical detection in the fish. Furthermore, the results of this study are applicable regardless of exposure level because first order elimination kinetics (see below) are independent of concentration.

Table 1
Concentrations (μg/g wet wt), enantiomer fractions (EFs), and diastereomer fractions (DFs, if applicable) of triazole fungicides and α-HCH (mean ± S.E.) in control and treated food and standards ($n = 4$)

Treatment	Compound	Concentration in food (μg/g wet wt)	Food EF (DF)[a]	Standard EF (DF)[a]
Control	All[b]	ND[b]	–	–
Exposed	α-HCH	35.82 ± 1.04	0.49 ± 0.01	0.49 ± 0.01
	Myclobutanil	30.15 ± 0.79	0.45 ± 0.01	0.47 ± 0.01
	Triadimefon	29.02 ± 0.47	0.48 ± 0.01	0.48 ± 0.01
	Bromuconazole	33.21 ± 0.88	0.46 ± 0.02, 0.49 ± 0.02, (0.32 ± 0.03)	0.50 ± 0.01, 0.56 ± 0.01, (0.35 ± 0.01)
	Propiconazole	24.96 ± 0.79	0.47 ± 0.03, (0.80 ± 0.01)	0.51 ± 0.01, (0.83 ± 0.01)
	Cyproconazole	23.83 ± 0.87	0.50 ± 0.02, (0.49 ± 0.01)	0.48 ± 0.01, (0.48 ± 0.01)
	Penconazole	31.46 ± 0.94	0.49 ± 0.01	0.50 ± 0.01
	Tebuconazole	26.43 ± 0.71	0.45 ± 0.01	0.45 ± 0.01
	Metconazole	28.14 ± 1.57	(0.49 ± 0.01)[c]	(0.50 ± 0.01)[c]
	Tetraconazole	30.95 ± 0.99	0.50 ± 0.01	0.49 ± 0.01

[a] EFs are given for individual enantiomers first followed by DFs (in parentheses) for diastereomer pairs if the compound had two chiral centers. Unlike for propiconazole and cyproconazole, separation of all four enantiomers was achieved for bromuconazole; therefore EFs are given for each eluting pair of enantiomers followed by the DF for the diastereomer pair (in parentheses).
[b] None of the compounds were detected in control food.
[c] Metconazole has 2 chiral centers, and thus 2 sets of enantiomers. However, these enantiomers were not separated under the GC conditions used, so the 2 peaks observed were diastereomers.

2.2. Exposures

Juvenile rainbow trout (Lake Burton Fish Hatchery, GA) (initial weights 17.6 ± 1.2 g, final weights 25.5 ± 6.0 g, mean ± S.E.) were haphazardly assigned to one of two treatments (exposed or control). Each treatment contained 45 fish distributed among three 50 l fiberglass aquaria (15 each) with re-circulating, dechlorinated tap water chilled to 12 °C, and carbon-filtered to remove any contaminant residues in the water. Fish were maintained on a 12 h light: 12 h dark photoperiod. Fish were exposed to the spiked food mixture (or control food) for 8 days (uptake) followed by 16 days of clean food (depuration) at 1.5% of the mean weight of the fish, corrected for weight gain after each sampling time. Three fish were randomly sampled from both treatments (one per aquaria) on days 1, 2, 4, and 8 of the uptake phase, and 6 h, 12 h, 18 h, 24 h, 36 h, 2 days, 4 days, 8 days, and 16 days of the depuration phase. Sampled fish were separated into liver, gastrointestinal (GI) tract (including stomach and contents, spleen, pyloric caeca, intestines and adipose tissue associated with these organs), and carcass (whole fish minus liver and GI tract to avoid analytes in the undigested food), and were frozen (−4 °C) in aluminum trays until analysis. Only carcass results were used in calculating bioaccumulation parameters and enantiomer/diastereomer fractions.

2.3. Analysis of chemicals in fish tissue

Extraction and clean-up of samples followed similar methods for quantifying organochlorines in fish (Fisk et al., 1998). Prior to extraction, PCB 65 (10 μl of a 65 ppm standard) was added to all samples as a recovery standard. Tissue samples (whole carcass) were freeze-dried and homogenized/extracted in DCM: hexane (1:1 by volume) using a Waring (Torrington, CT) stainless steel blender. Samples were extracted twice; the extracts were then combined, centrifuged, and evaporated to 10 ml, where 10% of the extract was used to determine lipids gravimetrically. Lipids were removed (first 140 ml fraction) from the remaining extract using gel permeation chromatography (GPC) columns packed with 60 g (dry weight) of 200–400 mesh Bio-Beads® S-X3 (Bio-Rad Laboratories, Hercules, CA). The GPC eluate was reduced to 1 ml prior to analysis by gas chromatography.

All analytes were quantified using a Hewlett-Packard 5973 mass spectrometer (MS) linked to a 6890 gas chromatograph (GC) equipped with a BGB 172 (BGB Analytik AG, Switzerland) chiral column, with the exception of tetraconazole, which was quantified by electron capture detection (ECD) using a Chirasel-Dex (Varian-Chrompack, Middelburg, The Netherlands) chiral column. Conditions for the GC–MS were injection temperature, 275 °C; column temperature program, 150–220° at 2°/min, followed by a temperature hold for 60 min; gas flow, 1.5 ml/min; MS inlet temperature, 275°; MS source temperature, 230°; and fragmentation voltage, 70 eV. Conditions for the GC-ECD were: injection temperature, 250 °C; column temperature program, 150–220° at 0.5°/min, followed by a hold of 20 min; column flow was 0.5 ml/min.

GC–MS detection was by selected ion monitoring (SIM). Monitored ions were usually two peaks of the parent ion chlorine isotope cluster with occasional inclusion of another prominent ion as follows: α-HCH, 181, 183; PCB 65, 290, 292; triadimefon, 181, 208, 210; tetraconazole, 256, 336, 338; penconazole, 159, 248, 250; myclobutanil, 179, 181; propiconazole, 173, 259, 261; cyproconazole, 139, 222, 224; tebuconazole, 250, 281; bromuconazole, 281, 295, 297; metconazole, 125, 127, 249, 251. Tetraconazole was observed and its total concentration was measured by the GC–MS system, but its enantiomers could not be separated by the BGB-172 column. They were almost baseline-separated on the Chiralsel-Dex column, so all samples were simultaneously analyzed using this column on the GC with the ECD detector. Myclobutanil was coincidentally separated into its enantiomers on this column, but its enantiomers were better quantitated on the GC–MS system with the BGB column.

Chromatography on the BGB-172 column gave sharp peaks with very little tailing for all compounds studied. Resolution of the enantiomers of α-HCH, penconazole, myclobutanil, and metconazole was baseline or better. Triadimefon and tebuconazole enantiomers were about 75% resolved. Propiconazole, cyproconazole and bromuconazole each have two chiral centers so each exists as two diastereomers, each with two enantiomers. The enantiomers of one of the propiconazole diastereomers were baseline-resolved, while the other diastereomer could not be completely separated from one of the cyproconazole peaks. That peak was apparently one of the enantiomers of cyproconazole, matching the area of one of the other two cyproconazole peaks. The third cyproconazole peak was apparently its other diastereomer, which did not separate into its enantiomers. Bromuconazole produced all four of its expected peaks with several minutes elution time between each peak. Two pairs of enantiomers were observed, the enantiomers of each pair being of equal size, but one pair being larger than the other. As mentioned above, the tetraconazole enantiomers were not separated on the BGB column.

An internal standard was not used, but all extract concentrations were corrected to PCB 65 recovery. Its average recovery, as quantified by reference to its single point external standard, was 73 ± 2% (mean ± S.E.) over all samples. Detection levels (three times signal to noise ratio) for all the triazoles in this study ranged from 0.03 μg/g for penconazole to 0.5 μg/g for each enantiomer of bromuconazole.

Enantiomer fractions (EFs) (Harner et al., 2000) for each chiral analyte were calculated using:

$$\mathrm{EF} = \frac{[\mathrm{E}_1]}{[\mathrm{E}_1] + [\mathrm{E}_2]} \quad (1)$$

where $[E_1]$ and $[E_2]$ are the concentrations of the first and second eluting enantiomers on a given chiral column. Diastereomer fractions (DFs) were also calculated for bromuconazole, propiconazole, and cyproconazole as each of these chemicals has two chiral centers. The DF was calculated in a similar manner as the EF above with:

$$\mathrm{DF} = \frac{[\mathrm{D}_1]}{[\mathrm{D}_1] + [\mathrm{D}_2]} \quad (2)$$

where $[D_1]$ and $[D_2]$ are the concentrations of the first and second eluting diastereomer or enantiomeric pair. Since separation was achieved on all four enantiomers for bromuconazole, unlike for propiconazole and cyproconazole, two EFs are given followed by the DF for each enantiomeric pair, or diastereomer, of bromuconazole (designated commercially as #46 and #47) (Table 1). Elution orders were known for triadimefon and bromuconazole by spiking each racemic standard with one of its pure enantiomers, which were obtained after separation by Chiral Technologies (Exton, PA). The (−) enantiomer of triadimefon, the (+) enantiomer of bromuconazole 46 (the *cis* isomer), and the (−) enantiomer of bromuconazole 47 (the *trans* isomer) eluted first on the BGB-172 column. Mean EF values were all near racemic with both DF and EF standards in agreement with food values (Table 1).

2.4. Data analysis

Growth rate significance was evaluated by fitting all fish weight data to an exponential model ($\ln$ fish weight $= a + bt$; where a is a constant, b the growth rate, and t is time in days from the start of the experiment) (Fisk et al., 1998). Since growth was not found to be significant over the course of the experiment, concentrations were not corrected for growth dilution (see below). Elimination rate (k_d) constants were determined by fitting the mean concentration data obtained during depuration sampling to a first order decay curve ($\ln$ concentration $= a + k_d t$; where a is a constant and t is time in days) (Fisk et al., 1998). Half-life ($t_{1/2}$) values were calculated using $\ln 2/k_d$. Additionally, time to 95% elimination (t_{95}) of each chemical was calculated from $\ln 0.05/k_d$. Steady state biomagnification factors (BMF_{ss}) were calculated from the equation $BMF = C_{fish}/C_{food}$ where C_{fish} is the average concentration assuming steady state in the fish and C_{food} is the average concentration in the food; both concentrations based on lipid content. Steady state was assumed only when concentrations did not continue to increase over three consecutive sampling intervals in the fish (Buckman et al., 2004).

Differences between whole body and liver growth rate constants among treatments were examined by testing the homogeneity of slopes in an analysis of covariance. Tukey's honestly significant difference (HSD) test ($p < 0.05$) was used to compare percent lipid and liver somatic indices (LSI) of the exposed fish to the controls (Systat, Ver 11, SPSS, Chicago, IL).

Biotransformation of each compound was examined using two methods. The first produced quantitative biotransformation rates by comparing the $t_{1/2}$ of each compound in this study with those of 16 known recalcitrant PCBs in juvenile rainbow trout (see Fisk et al., 1998). These 16 recalcitrant PCB congeners are fully chlorinated in the *meta* and *para* positions of the biphenyl rings, and thus have no significant biotransformation, the slowest elimination, and the highest $t_{1/2}$ of PCB congeners in fish for their respective $\log K_{ow}$ value (Niimi and Oliver, 1983). Relative to this $\log K_{ow}$–$\log t_{1/2}$ curve, contaminants of the same $\log K_{ow}$ value that have a depuration rate that is greater (and thus a shorter $t_{1/2}$) are suggested to be biotransformed. Subtracting the regression depuration rate (from the regression curve) at the contaminant's $\log K_{ow}$ value from the experimentally determined depuration rate provides an estimated rate of biotransformation. Compounds with biotransformation rates that approach zero (positive or negative) are assumed to be recalcitrant. α-HCH was included in this study to extrapolate this regression to lower $\log K_{ow}$ values, which allows for the estimation of biotransformation rates for many CUPs, including those from this study. Also, α-HCH has previously been shown to not undergo biotransformation in several studies (Wong et al., 2002; Konwick et al., 2006) and the high spiked food concentration in this study allowed for an accurate estimation of its $t_{1/2}$, including both the rapid initial and slow second phases, upon visual inspection of the raw data. Biotransformation was deemed to be significant and was reported for a contaminant when the mean plus standard error of its $t_{1/2}$ fell below the 95% confidence interval of the $\log K_{ow}$–$\log t_{1/2}$ regression. The second biotransformation method was more qualitative; being based on chiral analysis

by comparing contaminant EFs and DFs in fish to their respective values in food with an analysis of variance by a Tukey's aposteriori test using Systat ($\alpha = 0.05$).

3. Results and discussion

3.1. Effects of pesticide exposure on fish health

Exposure to the triazoles and α-HCH did not appear to influence the health of the rainbow trout. There was no significant difference in lipid percentage or liver somatic index ($p > 0.05$) between treatment and control fish on any sampling day and there were no signs of stress (e.g., coloration change, behavior) or mortality in either treatment. Body weights (two sample *t*-test, $p = 0.14$), and whole fish and liver growth rates ($p > 0.05$) of the exposed fish were not significant over the course of the study, likely due to the variability in fish size and the short duration of the experiment (24 days total) that did not allow for much growth. This is also evident in the low coefficient of determinations (r^2) found for growth rates in both (exposed and control) treatments (≤ 0.23). Concentration data, therefore, were not corrected for growth dilution.

3.2. Bioaccumulation parameters

All compounds were detected in the treated fish on the first collection day, one day after exposure to the spiked food. For a majority of the triazoles, concentrations did not increase or increased only slightly over the uptake period, thus appearing to reach steady state within 1 day (Fig. 1), consistent with their low $t_{1/2}$s (Table 2). Bromuconazole was only detected on two sampling days during the study, thus no bioaccumulation parameters or chiral signatures were calculated for this compound. This was likely due to a combination of bromuconazole's fast elimination and the fact that its detection limit was the highest of all the chemicals analyzed. None of the compounds were detected in control fish on any collection day.

Half-lives of triazoles ranged from 1.0 ± 0.2 days for tebuconazole to 2.5 ± 0.6 days for penconazole in rainbow trout (Table 2). There are very limited data for triazoles in other aquatic species for which to compare these $t_{1/2}$s. However, in mammals, it has been indicated that these compounds are rapidly biotransformed or eliminated ($t_{1/2}$s generally <3 days) in feces and urine with no accumulation in tissues (Roberts and Hutson, 1999), consistent with the short half-lives seen here. There was no increase, or other discernable relationship, of triazole $t_{1/2}$ with $\log K_{ow}$, which is generally found with hydrophobic persistent contaminants in fish (Fisk et al., 1998; Buckman et al., 2004). The time to 95% elimination may be a more appropriate measure for the persistence in biota of the CUPs investigated in this study due to their quick elimination and the need to determine if aquatic species have been exposed to CUPs. Calculated triazole t_{95} values in the fish ranged from 4.5 ± 0.7 days for tebuconazole to 11.0 ± 2.7 days for penconazole, approximately four times longer than their respective $t_{1/2}$s (Table 2). As far as we know, there are no other reported t_{95} values for triazoles or other

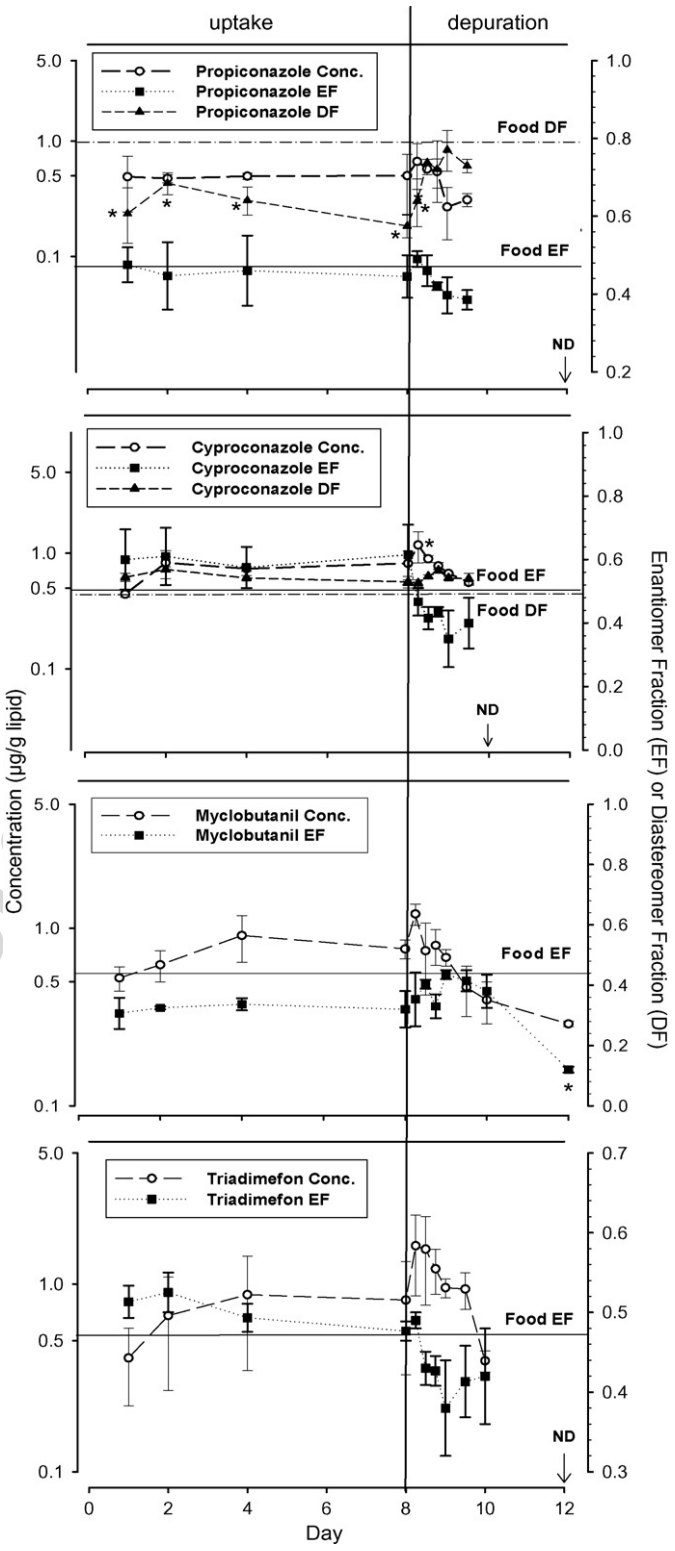
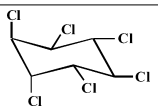
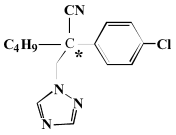
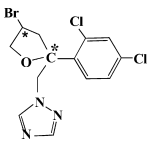
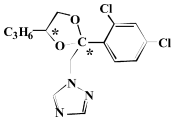
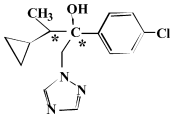
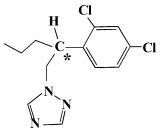
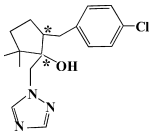
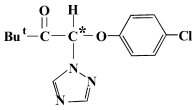
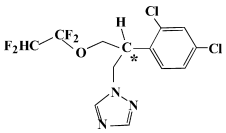
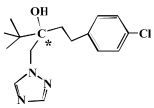


Fig. 1. Concentrations (circles, long dashed lines), enantiomer fractions (EFs) (squares, dotted lines), and diastereomer fractions (DFs) (triangles, small dashed lines, if applicable) of propiconazole, cyproconazole, myclobutanil, and triadimefon in juvenile rainbow trout over time. Each point and error bar represents the mean ± S.E. (if larger than symbol used) of three fish sampled at that time point. An asterisk next to EF or DF symbols indicates a significantly ($p < 0.05$) different value in fish on an individual sampling day compared to food values. All concentrations are the total sum of enantiomer concentrations, with no chemicals detected past day 12.

Table 2
Bioaccumulation parameters (mean ± S.E.) for triazole fungicides and α-HCH via dietary exposure using juvenile rainbow trout carcass data

Compound	Structure[a]	log K_{ow}[b]	Elimination rate, k_d (day^{-1})[c]	Biotransformation rate (day^{-1})[d]	Fraction of elimination due to biotransformation (%)[e]	Elimination $t_{1/2}$ (day)[f]	Time to 95% elimination, t_{95} (day)[g]	BMF_{ss}[h]
α-HCH		3.9	0.047 ± 0.006 (0.90)	NS	–	14.8 ± 1.9	65.0 ± 8.5	0.044
Myclobutanil		2.9	0.334 ± 0.078 (0.77)	0.200	59.9	2.1 ± 0.5	8.9 ± 2.1	0.008
Bromuconazole		3.2						
Propiconazole		3.7	0.633 ± 0.313 (0.58)	0.572	90.4	1.1 ± 0.5	4.7 ± 2.3	0.006
Cyproconazole		2.9	0.503 ± 0.039 (0.89)	0.369	73.4	1.4 ± 0.1	5.9 ± 0.5	0.011
Penconazole		3.7	0.272 ± 0.066 (0.84)	0.211	77.6	2.5 ± 0.6	11.0 ± 2.7	0.010

Metconazole		3.9	0.648 ± 0.122 (0.88)	0.580	89.5	1.1 ± 0.2	4.6 ± 0.9	0.019
Triadimefon		3.1	0.650 ± 0.135 (0.85)	0.541	83.2	1.1 ± 0.2	4.6 ± 1.0	0.012
Tetraconazole		3.6	0.305 ± 0.057 (0.85)	0.237	77.7	2.3 ± 0.4	9.8 ± 1.8	0.009
Tebuconazole		3.7	0.663 ± 0.110 (0.90)	0.552	83.3	1.0 ± 0.2	4.5 ± 0.7	0.012

Values missing for bromoconazole are a result of concentrations in the fish not above detection limits in a large fraction of the samples, therefore they were not calculated. NS = not significant.

[a] Asterisks indicate chiral center(s) for each triazole.

[b] log K_{ow} values for triazoles were taken from Roberts and Hutson (1999), and from Mackay et al. (1997) for α-HCH.

[c] Elimination rate constants (k_ds) were calculated using the model ln concentration = $a + b \times$ time for the 16 day elimination period. Coefficient of determination (r^2) for the model is shown in parentheses.

[d] Biotransformation rate = measured depuration rate − minimum depuration rate. Minimum depuration rates = 0.693/half-life (day), where half-lives were determined from the equation log half-life $= -1.07 + (0.76 \times \log K_{ow}) - (0.05 - \log K_{ow}{}^2)$($r^2 = 0.85$), which assumes no biotransformation.

[e] Percentage of depuration that is estimated to be biotransformed.

[f] Half-life ($t_{1/2}$) was calculated from the equation $t_{1/2} = 0.693/k_d$.

[g] Time to 95% elimination (t_{95}) was calculated from the equation $t_{95} = 2.99/k_d$.

[h] Biomagnification factors at steady state (BMF_{ss}) = C_{fish} (lipid corrected)/C_{food} (lipid corrected).

CUPs in fish, which is needed for addressing exposure for these chemicals.

The sampling scheme employed in this study allowed for an accurate assessment of the triazole bioaccumulation and elimination. Due to the rapid elimination of the triazoles, it was required that fish be collected every 6 h during the initial depuration phase to provide for an estimate of $t_{1/2}$s and t_{95}s. Daily sampling, the common method in bioaccumulation studies with fish, would not have been adequate because many of the triazoles, after cessation of exposure, were not detected past 2 days of depuration (Fig. 1). Furthermore, the sampling method here allowed for an estimate of the assimilation time of the food by the fish. For many of the triazoles, concentrations increased on the first sampling time of depuration (6 h after cessation of exposure to spiked food) (Fig. 1), indicating that assimilation of the food was still occurring 6 h after feeding. Consequently, we used the highest concentration in the depuration phase as our initial value in calculating the elimination rates for the triazoles.

The $t_{1/2}$ of α-HCH (14.8 ± 1.9 days) was longer than that reported in a similar study with rainbow trout ($t_{1/2}$ = 3.9 ± 0.8 days) (Konwick et al., 2006). This is likely due to that fact that α-HCH was detected for a longer proportion of the elimination phase here, perhaps due to a higher spiked food concentration. However, we cannot rule out that cytrochrome (CYP) P450s, or other biotransformation enzymes, were altered as a result of the concomitant exposure to the triazoles, which can have a mixed induction or inhibition response in fish (Levine et al., 1999a; Egaas et al., 1999).

None of the chemicals in this study would biomagnify within aquatic food webs based on the fact that their BMFs are so low (<1) (Table 2). BMF_{ss} values ranged from 0.006 for propiconazole to 0.019 for metconazole (Table 2), driven by their rapid elimination. To our knowledge, there are no other studies in the primary literature reporting BMF values for the triazoles examined in this study. The triazole BMFs were, however, in the range of another CUP, fipronil (BMF = 0.02–0.05), investigated previously using a similar protocol (Konwick et al., 2006). Likewise, the BMF of α-HCH is comparable to previous studies (BMF = 0.013–0.026, Wong et al., 2002). However, field studies have shown α-HCH to biomagnify within Arctic marine food webs (Moisey et al., 2001), which may be due to the larger size of the fish and colder temperatures in this environment, which would decrease metabolism of the chemical, and therefore, its elimination rate (Borgå et al., 2004).

3.3. Chiral biotransformation

The majority of the triazoles in this study did not show significant changes in EFs, which taken alone would indicate little biotransformation for these fungicides (Fig. 1). Although mean EF values did change for several of the chemicals during the elimination phase of the study, including obvious decreasing EF trends for some (see propiconazole, cyproconazole), statistically these values were not different than EFs measured in the food. The exception was myclobutanil, where on the last sampling day that it was detected (day 4 of depuration) the EF value in the fish had decreased significantly compared to the food value, indicating that the first eluting enantiomer E1 was being biotransformed greater. The rapid elimination of these fungicides likely precludes the use of EF measurement to observe biotransformation, especially where there are minimal differences in rates of metabolism between enantiomers, as suggested in this study. On the other hand, the biotransformation of persistent chiral organic chemicals (e.g., PCBs) is slow, and can be more accurately measured through chiral analysis due to the greater time for subtle differences in enantiomer biotransformation to be evident (Buckman et al., 2006). It is possible that with improved detection limits in this study the triazole enantiomers could be quantified over a greater length of time during depuration (see t_{95} values above) allowing for EF trends to be evident, which has been seen in rabbits (Wang et al., 2005).

Significant changes in fish DF values for cyproconazole and propiconazole were seen during this study (Fig. 1), indicating that the diastereomers of these compounds were likely transformed, but not necessarily biotransformed, selectively. For propiconazole, DF values in fish were significantly different from those of the fish food during the uptake phase, as well as during the initial depuration phase (days 1–8.25). The fact that the DF in the fish for propiconazole is lower than that in the food indicates that diastereomer D1 was transformed faster than D2 (Eq. (2)). On the other hand, cyproconazole DF values in fish only became significantly different from those of the food after the cessation of exposure, at which time the DF is higher in the fish than in the food, indicating that diastereomer D2 was transformed faster. The statistically consistent EFs for each of these triazoles would indicate equal rates of biotransformation of the two enantiomers of each diastereomer. Therefore, changes in DFs can possibly be used as an indicator of biotransformation. However, we cannot completely rule out diastereomer-selective abiotic reactions that may have occurred prior to accumulation (i.e., in fish or on food), chemical breakdown by gut flora, or isomerization of diastereomers (Law et al., 2006) as a reason for the observed DF changes.

3.4. Biotransformation using the log K_{ow}–log $t_{1/2}$ relationship

Comparison of triazole $t_{1/2}$s with the curvilinear relationship between log $t_{1/2}$ and log K_{ow} allowed for an estimate of their biotransformation rate (Fig. 2; Table 2). The inclusion of α-HCH in this study with the chemicals studied by Fisk et al. (1998) resulted in a quadratic regression with very similar slopes (b_1 = 0.76, b_2 = −0.05, r^2 = 0.85) to the original regression (b_1 = 1.5, b_2 = −0.1, r^2 = 0.85) reported by Fisk et al. (1998). Based on the position of the triazoles below the log K_{ow}–log $t_{1/2}$ relationship, biotransformation rates ranged from 0.200 to 0.580 day^{-1} and accounted for the major proportion (60–90%) of the triazole elimination (Table 2). There was no attempt to determine the biotransformation rates for the individual enantiomers of the triazoles due to the lack of biotransformation that could be observed in the EF data. In addition, we analyzed for triadimenol, a metabolite of triadimefon, in fish throughout the study to confirm biotransformation of this fungicide. Triadimenol was not found on any sampling day, which may be due

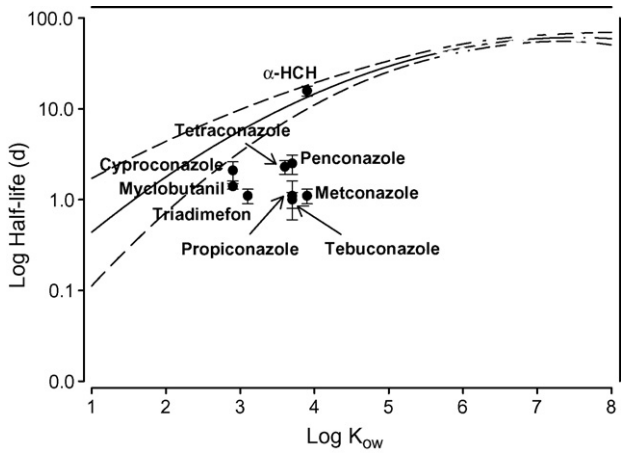


Fig. 2. Log half-life of triazoles in juvenile rainbow trout vs. log K_{ow}. The quadratic regression (solid line) and 95% confidence intervals (dashed lines) shown for the log K_{ow}–log $t_{1/2}$ relationship were derived from 16 recalcitrant PCBs (see Fisk et al., 1998) and α-HCH from this study.

to our analytical procedure, the possibility this metabolite may be in the form of a conjugate through phase II biotransformation, and/or the rapid elimination of this metabolite. As far as we are aware, these are the first reported biotransformation rates for triazoles in fish. It should be noted that further study with additional chemicals of lower log K_{ow} values that are not known to undergo metabolism (besides α-HCH) are needed to clearly establish the log K_{ow}–log $t_{1/2}$ relationship for its further application to less persistent chemicals. Also, since the triazoles in this study were dosed as a mixture, the concomitant exposure to the other triazoles could have potentially affected biotransformation enzymes and therefore the biotransformation rates reported here. Clearly though, risk assessment for these chemicals, and likely many CUPs, should focus on identifying the fate and effects of biotransformation products of CUPs.

An additional objective of the present study was to determine the dependence of biotransformation on the functional groups attached to the chiral center of each triazole. Due to the lack of biotransformation seen in the EF data this objective was not achievable, yet some general observations can be made based on the relationship of functional groups with the biotransformation rates calculated from the log K_{ow}–log $t_{1/2}$ relationship. The triazoles with the highest percentage of elimination attributed to biotransformation had either an oxygen atom or hydroxyl group adjacent to a chiral center (Table 2). On the other hand, two of the triazoles (myclobutanil and penconazole) with the lowest percentage of their elimination through biotransformation did not contain an oxygen atom within the molecule. The oxygen atom has great reaction potential through phase one (oxidation, reduction, hydrolysis) biotransformation reactions, which may result in ring cleavage adjacent to the oxygen atom and/or the formation of hydroxyl groups. Consequently, hydroxyl groups are vulnerable to phase two (synthetic or conjugation) reactions (Landis and Yu, 2004). Both mechanisms of biotransformation ultimately result in more water-soluble and more readily excretable forms. The fact that the triazoles that showed the highest biotransformation percentage have chiral centers located next to oxygen and hydroxyl groups, combined with the small changes in EFs, suggest that biotransformation was rapid for both enantiomers and that there is a low degree of selectivity. Apparent enzymatic degradation without enantiomer selectivity has been seen previously in the phytobiotransformation of chiral *o*,*p*′ DDT in plants (Garrison et al., 2000).

The minor changes in triazole EFs observed in this study are most likely due to biotransformation as opposed to enantioselective uptake or elimination. Enantioselective uptake is unlikely because the transfer of chemicals from the GI tract into the body through mixed micelle vesicles is a passive transport process that is not considered to be stereospecific (Landoni et al., 1997; Drouillard and Norstrom, 2000), although this has yet to be confirmed for non-persistent chemicals. Likewise, elimination of hydrophobic compounds, such as excretion through feces or the gills is considered passive and non enantioselective (Thomann, 1989; Landoni et al., 1997); however, we cannot rule out the breakdown of non-persistent chemicals by gut flora as a possibility for the EF observations.

This study shows the utility of using chiral analysis and the log K_{ow}–log $t_{1/2}$ relationship to provide insights into biotransformation of CUPs. The use of chiral analysis suggested limited enantioselective biotransformation of the triazoles in this study; however, the log K_{ow}–log $t_{1/2}$ relationship did identify biotransformation, likely at the same rate for each enantiomer based on EF data, as the major fraction of the triazole elimination. The results from this study indicate that chiral analysis alone may be an insufficient tool for identifying biotransformation of rapidly metabolized or eliminated chemicals. DFs in this study did provide more information than EFs as well as show the potential for using DFs as an indicator of transformation, likely biotransformation. Half-lives on the order of a day or so would require frequent fish sampling and analysis during the depuration phase, along with improved detection limits, to provide adequate data for more accurate concentration and EF observations. This study did provide enough biotransformation data to show the opportunity for development of a structure-based model to predict bioaccumulation and biotransformation for these chemicals. Our results also highlight the value of the log K_{ow}–log $t_{1/2}$ relationship as a mechanistic tool for quantifying biotransformation for a variety of contaminants, including CUPs, in fish.

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