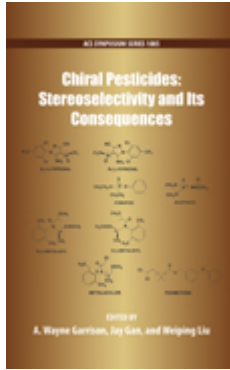
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Chiral Pesticides:
Stereoselectivity and Its
Consequences
EDITED BY
A. Wayne Garrison, Jay Gan, and Weiping Liu

Chiral Pesticides: Stereoselectivity and Its Consequences

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Chiral Pesticides: Stereoselectivity and Its Consequences

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Foreword

The ACS Symposium Series was first published in 1974 to provide a mechanism for publishing symposia quickly in book form. The purpose of the series is to publish timely, comprehensive books developed from the ACS sponsored symposia based on current scientific research. Occasionally, books are developed from symposia sponsored by other organizations when the topic is of keen interest to the chemistry audience.

Before agreeing to publish a book, the proposed table of contents is reviewed for appropriate and comprehensive coverage and for interest to the audience. Some papers may be excluded to better focus the book; others may be added to provide comprehensiveness. When appropriate, overview or introductory chapters are added. Drafts of chapters are peer-reviewed prior to final acceptance or rejection, and manuscripts are prepared in camera-ready format.

As a rule, only original research papers and original review papers are included in the volumes. Verbatim reproductions of previous published papers are not accepted.

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Preface

Chirality as an environmental phenomenon was dealt with in a thorough and interesting manner in a series of three symposia entitled "Modern Chiral Pesticides: Enantioselectivity and Its Consequences", sponsored by the Agrochemical Division of the American Chemical Society and held in Washington, DC (2005), Boston, MA (2007) and San Francisco, CA (2010). All three symposia included speakers from industry, government and academia, representing several European countries, China, and the United States. Corresponding to this broad group of countries, institutions and speakers, the range of topics touched on almost all facets of chirality as it is manifested in environmental and human exposure and toxicity. The 40 oral and 20 poster presentations indeed approached comprehensive coverage: analysis of enantiomers and other stereoisomers; preparative separation of enantiomers; stereoselective occurrences of chiral pesticides in environment soil and water and in wildlife and human tissues and fluids; stereoselective degradation and metabolism of chiral pesticides; and stereoselective toxicity. Symposia oral presentations attracted audiences averaging about 50 interested and critical personnel. All in all, the Agrochemical Division and symposia organizers considered this a successful symposium series.

This book is a result of manuscript contributions by some of the oral and poster presenters to the third symposium in 2010. In addition to symposium participants, invitations were extended to the environmental chiral chemistry community in general, including most of the speakers in the 2005 and 2007 symposia, in an attempt to attain good coverage of this rather broad topic. Thus, we have this ACS Symposium Series book, and trust that it will generate many new ideas from interested readers and inform them of useful techniques for experimental exploration of the somewhat exotic, but important, area of chiral chemistry of pesticides.

From the editors, with much appreciation to all presenters and attendees:

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Chapter 1

An Introduction to Pesticide Chirality and the Consequences of Stereoselectivity

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Thirty percent of known registered pesticides are chiral and usually exhibit some degree of stereoselectivity in their biodegradation rates and/or toxicity. Since the great majority of chiral pesticides are manufactured and applied as their racemic mixtures, there is a need for defining their stereoselectivity to provide for improved risk assessment and establish, in some cases, the rationale for production of single- or enriched-enantiomer products.

Chirality

Chiral chemicals exist as two (or more) species – *enantiomers* – that are non-superimposible mirror images of each other. This *handedness*, so described because our two hands are non-superimposible (hence the root word for "chiral" in Greek is "cheir", meaning hand), requires a chiral center, or center of asymmetry in the molecule. Such asymmetry is usually caused by an sp^3 configured carbon atom to which four different groups are attached. In such cases, bond breakage is required to form the mirror image of an enantiomer. Chiral centers may also originate with a pentavalent phosphorus atom, which also bonds in sp^3 configurations, if it is bonded to 4 different groups counting the double-bonded oxygen or sulfur as one group. Sulfoxide moieties with their non-bonded pair of electrons on sulfur are also not planar, and thus are chiral centers if the three bonded groups are different. There are also chiral centers due to hindered rotation; e.g., the bulky groups in metolachlor (Figure 1) cannot freely rotate about the C-N bond and provide a second center of asymmetry in addition to the asymmetric

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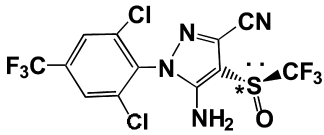
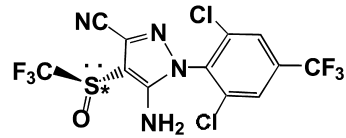
carbon. The major types of asymmetry are illustrated by the examples in Figure 1; most types include several pesticides. In addition, there are other types of asymmetry not discussed here. The reader is directed to the comprehensive book by Eliel, et al (*1*) for more information on chirality of organic molecules.



S-(+)-FIPRONIL



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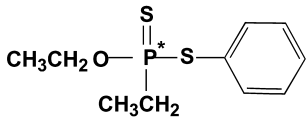

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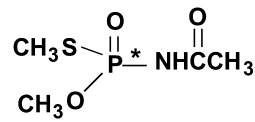

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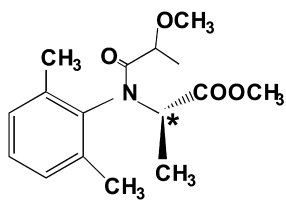

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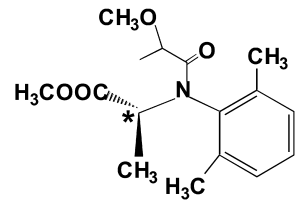

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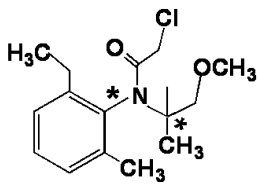
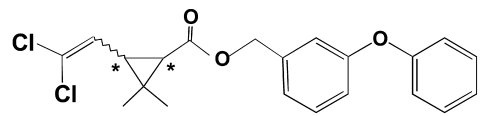


METOLACHLOR



PERMETHRIN

*Figure 1. Examples of chiral pesticides. *Asterisk designates the chiral center(s) of the molecule.*

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The great majority of chiral pesticides are synthetic products, and thus are formed and exist in the pesticide formulation as racemates (*2*) ; i.e., they contain an equal concentration of each enantiomer. A point of clarification: a molecule containing two centers of asymmetry instead of only one, such as permethrin in Figure 1, has 4 enantiomers; however they are not all mirror images of each other. Instead, they exist as *2 pairs* of enantiomers, one pair corresponding to each chiral center. Figure 2 illustrates this for triadimenol (*3*). This concept holds for two or more additional chiral centers, and the number of enantiomers is equal to 2^n where n is the number of chiral centers in the molecule. For example, several pyrethroid pesticides have 3 chiral centers, with a total of 8 enantiomers (3 pairs). Since enantiomers only exist as pairs of mirror image molecules, it is not correct to refer to the enantiomers from different pair sets as "enantiomers". They are, however, all stereoisomers, being isomeric due to differences in spatial arrangement. These definitions are summarized by the axiom that all enantiomers of a particular molecule are stereoisomers of each other, but not all stereoisomers are enantiomers. Stereoisomers that are not enantiomers are called "diastereomers" and they are not mirror images of each other, as with triadimenol in Figure 2.

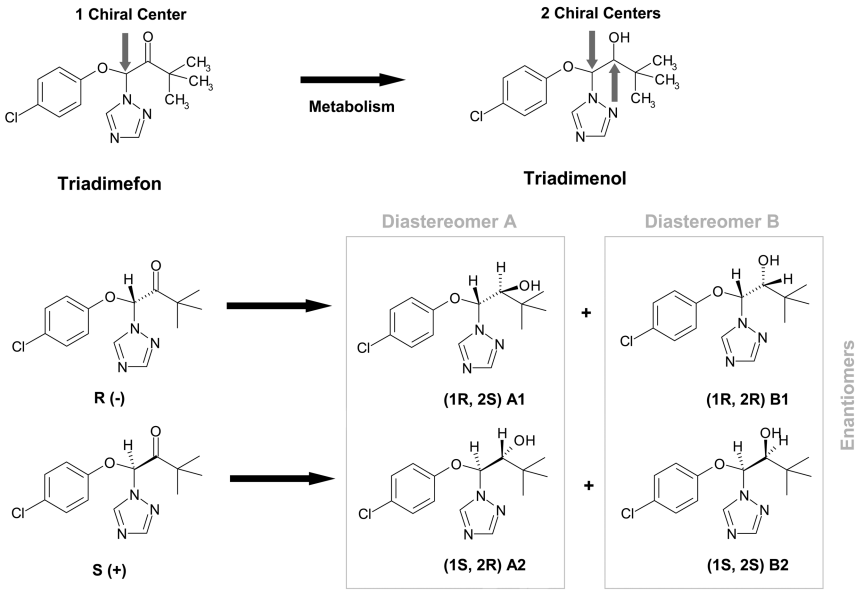

Figure 2. Metabolic transformation of the 2 triadimefon enantiomers to the 4 triadimenol stereoisomers via reduction of the triadimefon prochiral ketone. Reproduced with permission from reference (3). Copyright 2010 John Wiley & Sons.

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The conventions for naming stereoisomers are discussed by Eliel, et al (*1*). Enantiomers differ from each other in their direction of rotation of plane-polarized light, giving rise to (+) or (-) designations, as with fipronil and metalaxyl in Figure 1. But this feature has no bearing on the actual configuration of the molecule; enantiomers also differ in their three dimensional special arrangemnt, or "absolute configuration" . They are thus designated R or S, based on a set of well established rules of substituent order. Although the latter symbols provide a complete description of molecular configuration, the complete name of a chiral molecule usually also includes (+) or (-) to indicate direction of rotation. This is useful as a tag for the enantiomer, since molecular rotation can be easily measured by polarimetry.

Stereoselectivity

The importance of enantiomers is manifested by selectivity in their reactions with chiral systems. For example, reactions with most complex biological molecules such as enzymes, which are generally chiral, as well as with simpler chiral molecules, are all apt to be selective. In other words, one of the enantiomers of the subject molecule reacts preferentially with the chiral host system to which it is exposed. Thus, we have GC, HPLC and CE (capillary electrophoresis) analytical techniques that perform successful enantiomer separations of chiral molecules, as shown with the GC chiral separations of the pair of triadimefon enantiomers and 2 pair of triadimenol enantiomers in Figure 3A (*4*). The analytical system preferentially reacts with one of the enantiomers because the GC (*5*) or HPLC (*6*) immobile phase or the CE chiral selector (*7*) is composed of or contains single enantiomers of chiral molecules or polymers. The resulting products of such reactions are diastereomers; i.e., they are composed of 2 non-paired stereoisomers. This is a very important consequence, since diastereomers differ in their chemical and physical properties and can thus be separated by physical/chemical analytical techniques. This is again shown in Figure 3A; diastereomers A and B could theoretically be separated without the chiral immobile phase. This is not true for actual enantiomers, which differ only in one physical property: the direction of rotation of plane-polarized light.

Selectivity is especially important in enzymatic reaction systems. Most enzymes are enantioselective or stereoselective and react preferentially with one enantiomer or stereoisomer of a chiral pesticide, for example. This results in different rates of biochemical transformation, leading to preferential persistence of the enantiomer or stereoisomer that reacts slowest (*7–9*). This is illustrated in Figure 3B. The microbial transformation of triadimefon, the S-(+)-enantiomer of which reduces faster to triadimenol, leaving more of the R-(-)-enantiomer, produces the 4 stereoisomers of triadimenol in unequal amounts. Stereoselectivity of the enzyme-triadimefon reaction results in isomeric products that vary by an order of magnitude in their relative concentrations (*4*).

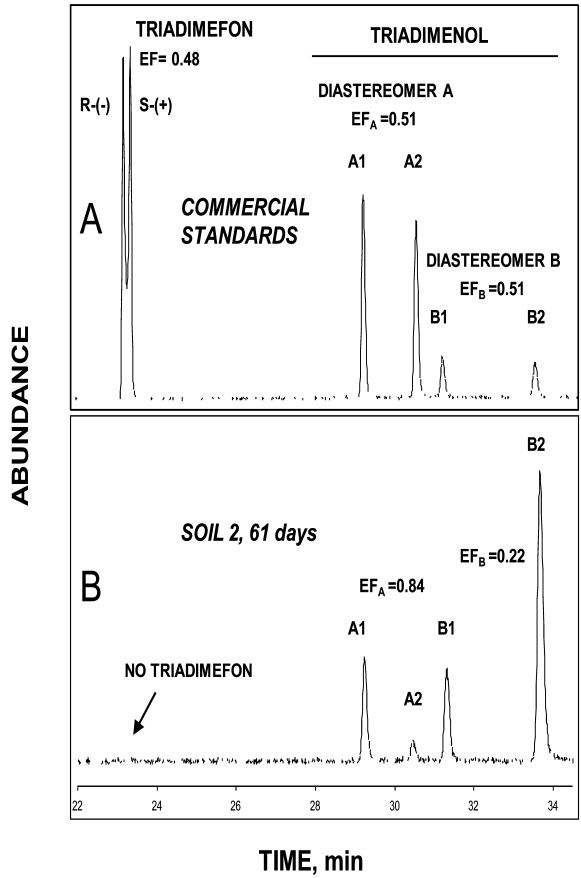

Figure 3. GC-MS (SIM) of (A) triadimefon and triadimenol mixed commercial standards, and (B) triadimefon completely biotransformed to triadimenol after 61 days in soil. EF represents the enantiomeric fraction, EF= [1st eluting enantiomer / [(1st eluting enantiomer + 2nd eluting enantiomer)].

Since toxicity is also the result of an enzymatic reaction, a chiral pesticide often exhibits enantioselective or stereoselective toxicity to plants, animals or humans (*10–12*). These concepts of selectivity in biotransformations and in toxicity are the basis of the concerns about chiral pesticides and their reactivity and thus, as indicated by the title, are the focus of this book.

An Opportunity for Green Chemistry

In 1995, chiral compounds accounted for 25% of all agrochemical compounds used commercially, comprising 26% of the total agrochemical market value (*13*). However, those sold as single isomer products contributed only 7% to the market value. The number of chiral pesticides is increasing along with the general increase in agrochemical products, especially since newer pesticides are often

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more complex molecules that are more likely to contain chiral centers. A 2009 compilation (*14*) reported that about 30%, about 480, of the 1590 known organic chemical pesticides, both legacy and in current use, are chiral molecules.

Key to our interest, however, is the fact that the great majority of chiral pesticides are manufactured as their racemates; i.e., equal mixtures of each enantiomer. These racemic mixtures do not take advantage of the possibility of single- or enriched-enantiomer products, in which the target-active enantiomer is the sole, or at least the enriched, active ingredient. Such products are environmentally *green* in that they minimize adverse impacts of the non-target enantiomer. In fact, the agrochemical industry and government regulators are beginning to take enantioselectivity into account (*2*, *13*). For example, the (R)-enantiomers of the herbicides dichlorprop and mecoprop [as well as the (R)-enantiomers of all phenoxypropionic acid herbicides] are the active enantiomers, killing the weeds, while the (S)-enantiomers are inactive; so to reduce the amount of herbicide used and avoid the possibility of the unnecessary enantiomer causing some adverse impact, several European countries have decreed that only the (R)-enantiomers will be used (*13*).

In addition, the occurrence of chirality should be considered in the risk assessment of pesticides (*15*). In actuality, a chiral pesticide is a mixture of at least two separate molecules (in the case of one chiral center) with their individual fate and effects characteristics, and exhibits the usual anomalous, often non-additive, behavior of chemicals in a mixture. Consideration of chirality in risk assessment can lead to production of safer single- or enriched-enantiomer pesticides, contributing to one of the new research themes of the U.S. Environmental Protection Agency: Chemical Safety for Sustainability.

It is hoped that this book will lead to increased concern with the impact of chirality on pesticide safety by academic researchers, regulators and the agrochemical industry and enhance these green chemistry opportunities and efforts (*16*).

Acknowledgments

Some of this material was used in a previously published article: Garrison, A. W. “Issues on the enantioselectivity of chiral agrochemicals”, CHIMICA OGGI/Chemistry Today, October 2002, pp. 28-32 (www.teknoscienze.com). John Kenneke, U.S. EPA, Athens, GA, designed Figure 2, which is used with permission of John Wiley & Sons and appeared on page 184 of reference (*3*). This paper has been reviewed in accordance with the U.S. Environmental Protection Agency’s peer and administrative review policies and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

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Chapter 2

Chiral Chlordane Components in Environmental Matrices

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Chlordane, a persistent, bioaccumulative and toxic organochlorine pesticide, has been studied for many years. Since the advent of chiral analysis for environmental samples, over 2,400 measurements have been made of various chiral chlordane components. Chlordane enantiomer fractions most often have been reported for air and soil with studies suggesting volatilization from soil is an important source to ambient air, although urban termiticide usage also can influence chiral chlordane measurements. Sediment core studies suggest the small amount of enantioselective degradation of chlordane likely occurs prior to deposition. In general, enantioselective degradation of chlordanes in biota occurs more frequently resulting in more nonracemic values than in other environmental media. There is also more diversity in range and enantiomer preference in biota. Analysis in plants has shown the ability to enantioselectively uptake and transport chlordane compounds from soil to root, from air to leaf and within the plant itself. Observation and measurement of chlordane enantiomers can provide a better understanding of the fate, exposure, toxicity, and risk of chlordanes and other chiral compounds in the environment.

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Introduction

History of Chlordane Use, Regulations, and Environmental Distribution

Chlordane is a persistent, bioaccumulative and toxic organochlorine pesticide used in the United States for forty years (*1*). Due to its extensive usage, both agriculturally and residentially, and its long half-life in the environment, everyone in the US has been exposed to low levels of chlordane, typically from eating contaminated foods or contact with contaminated soil (*2*). The International Agency for Research on Cancer considers chlordane a possible human carcinogen (*3*) and chlordane was included in the Stockholm Convention as one of the original and infamous "Dirty Dozen" persistent organic pollutants (POPs) (*4*).

Technical chlordane consists of more than 140 compounds, including chlordanes, nonachlors and heptachlor (*2*). From its introduction in the 1940s to its eventual cancellation for all uses in the US in 1988, chlordane was used widely for both agricultural and residential pest control (*1*). Use for food crops was banned in 1978 and by 1983, all above-ground use ended. However, chlordane was used for subterranean termite control in the foundation of homes for another five years. Production was voluntarily stopped globally in 1997 by the major manufacturer, Velsicol Chemical Co. (*5*).

Of the multiple compounds found in the technical chlordane mixture, *cis*- and *trans*-chlordane and *trans*-nonachlor are the most abundant species representing ~28% of the mixture (*6–8*). The exact ratio of the *cis*-chlordane isomer (CC, also known as α-chlordane) to the *trans*-chlordane isomer (TC, also called γ-chlordane) in the technical mixture depends on the manufacturing process (*9*), but recent studies have reported this TC/CC ratio to be ~1.0–1.2 (*7*, *8*).

Chlordane can undergo long-range transport due to its semivolatile nature and is considered a persistent organic pollutant (POP). Chlordane compounds and their degradation products have been found throughout the globe from the US (*10–12*) to the Arctic (*13–15*), from Asia (*16*, *17*) to Mexico (*18*) and in all media including air (*16*, *19*, *20*), soil (*21*, *22*), water (*23*), food crops (*24*, *25*) and biota (*26–28*). Chlordane can be taken up into edible plant tissues, both root and aerial, of a number of food crops (*24*, *25*) and has been found to bioaccumulate to the highest trophic levels in food chains, such as the polar bear food chain (*29*), making human exposure by ingestion likely.

Oxychlordane (OXY) is a major metabolite of chlordane that has been found in various media and biota (*3*). Heptachlor (HEPT) is both a component of technical chlordane (~10% by weight) and a breakdown product of it (*3*). Technical heptachlor, a commercially sold pesticide in its own right, contained 20-22% *trans*-chlordane (*30*, *31*) and thus may have increased the TC/CC ratio in the environment. Heptachlor epoxide (HEPX) is an oxidation product of both heptachlor and chlordane (*3*). It has been reported that some metabolites of chlordane, including oxychlordane, heptachlor, and heptachlor epoxide, are more toxic than the parent compounds (*2*).

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Chirality of Chlordane Compounds

Many of the ~140 compounds that make up technical chlordane are chiral. Their chirality comes from having one or more chiral carbon atoms. For illustrative purposes, the carbon atoms of the methanoindene skeleton, common to all chlordane compounds, have been numbered on *cis*-chlordane in Figure 1. While there are chiral centers at both the bridgehead (carbons 3 and 9) and the shared ring carbon atoms (carbons 4 and 8), the strain on such a small ring and across the shared ring bond makes it impossible for both configurations (R or S) to exist at these chiral carbons. Instead, it is typically the carbons of the cyclopentane ring (carbons 5-7) that includes two unconstrained chiral centers (5 and 6) capable of creating stereoisomers. For example, chlordane has a total of six chiral centers, at carbons 3, 4, 5, 6, 8, and 9; however, only the centers at carbons 5 and 6 can exist in all configurations, giving rise to a total of four stereoisomers [(+)- and (–)- *cis*- and *trans*-chlordane]. Nonachlor is achiral because of a mirror plane that runs through carbons 6, 10 and the double bond. Heptachlor, the two heptachlor epoxides, and oxychlordane have a chiral center at carbon 5, creating two stereoisomers for each. Other chiral chlordane compounds have been studied in environmental samples, including MC5 and U82, however, the bulk of research on chlordane stereoisomers has been done with *cis*- and *trans*-chlordane, *exo*- heptachlor epoxide, heptachlor and oxychlordane, and these works will be discussed here. Further references to heptachlor epoxide (HEPX) are for the *exo*-isomer (unless stated explicitly), as *endo*-HEPX is not naturally occurring.

Enantiomer Separations

Many of the chiral compounds in technical chlordane and their degradates have been separated using enantioselective chromatography columns. ChirBase, a repository for stereoisomer separations, has entries for at least eleven chlordane compounds by both gas (GC) and liquid (LC) chromatography, as shown in Table 1 (*32*). Additional separations are found in the literature for *cis*- and *trans*-chlordane, *endo*- and *exo*-heptachlor epoxide, oxychlordane, heptachlor, Compound K, MC4, MC5, MC6, MC7, MC8, photochlordene, photo-heptachlor, photo-heptachlor epoxide, U81, and U82. While a few authors (*33*, *34*) have published LC methods for small-scale preparative separations for chlordane compounds, all environmental measurements have been made using enantioselective gas chromatography with either electron capture detection or mass spectrometry. Reviews of such separations are available (*35–37*), whereas chromatographic conditions for individual experiments are usually published in the methods section of the research papers themselves.

Cyclodextrins (α-, β-, and γ-), and their many derivatives, are the most commonly used chiral stationary phases resulting from their stability, commercial availability, and history of successful separations of chlordane compounds. Due to the complexity of chlorinated contaminants in the environment, similar isotope patterns, and close retention times, caution and quality control measures are necessary to ensure that co-elutions do not occur. For example, endosulfan I coelutes with one of the *cis*-chlordane enantiomers on a Supelco γ-cyclodextrin

column , however this co-elution does not occur on other stationary phases (*38*). It should be noted that single or enriched enantiomers of *cis*- and *trans*-chlordane, oxychlordane, heptachlor, and heptachlor epoxide A and B are commercially available and provide researchers with an easy way to determine the elution order of enantiomers for any chromatographic conditions (*39*).

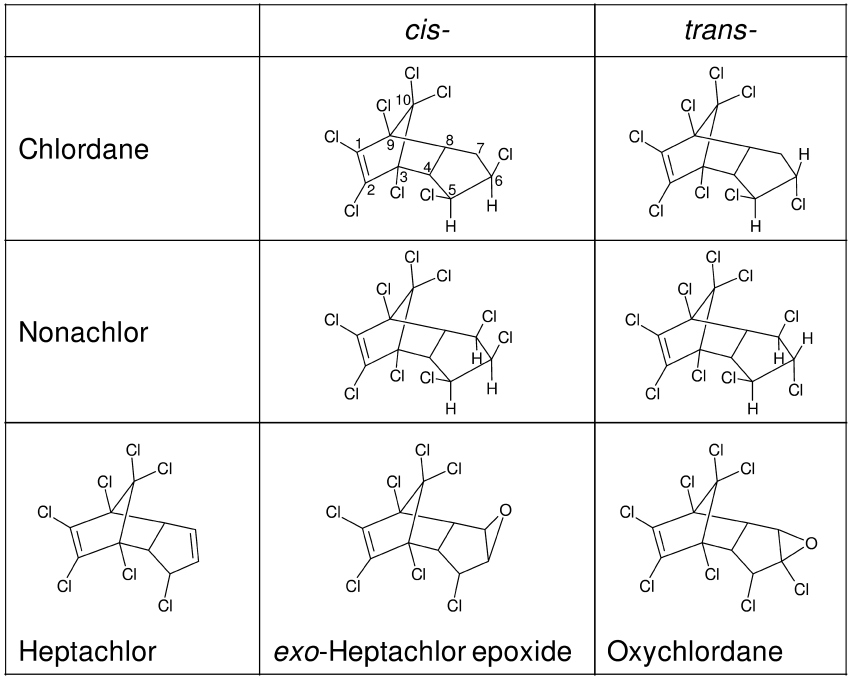

Figure 1. Chemical structures of chlordane compounds.

Chiral Chlordanes in the Environment

While many of the chiral chlordane compounds have been measured in the environment, this review focuses on five: *cis*- and *trans*-chlordane, heptachlor, heptachlor epoxide, and oxychlordane. Over 2,400 enantiomer fraction [EF = (+)/(+) + (–)] or enantiomer ratio [ER = (+)/(–)] measurements for these five compounds have been made in environmental matrices. For this review, all data originally reported as enantiomer ratios were converted to enantiomer fractions to simplify comparison (EF = ER/1+ER). When ER was reported as E1/E2 because the elution order was unknown, other references were consulted to determine the elution order and make the correct conversion; this was impossible for only one reference, so that data was not included. The complete dataset is planned to be available at http://www.epa.gov/heasd/products/products.html or by request from the corresponding author.

Figure 2 shows EF measurements by compound. The vast majority of measurements are for *cis*- and *trans*-chlordane. Heptachlor has very few measurements, likely due to low concentrations (possibly due to lower persistence) and difficulties in separating the heptachlor enantiomers. Figure 3 shows EF measurements by environmental or biological compartments. Air and soil were measured most often and water least. Each matrix will be discussed separately in this review.

Table 1. ChirBase entries for chlordane compounds

Compound	*GC entries*	*LC entries*
1,5 Photo-*cis*-chlordane	3	
2,5 Photo-*cis*-chlordane	3	
Chlordene	3	1
cis-Chlordane	21	5
endo-HEPX	11	1
Heptachlor	17	3
Oxychlordane	16	1
Photo-heptachlor	3	
trans-Chlordane	21	5
exo-HEPX	16	2
U82	4	

Air

Chiral chlordane compounds have been reported in ambient air (both rural and urban), indoor air, and air directly above soils. The most commonly reported are EFs for CC and TC in ambient air, although HEPX ambient air EFs are often reported with these. Both passive and active air samplers have been employed with the vast majority of samples taken from the northern hemisphere, primarily North America and Europe. EF values in published studies varied considerably although most samples, by far, showed CC to be either close to racemic or enriched in the (+)-enantiomer while TC tended to be either close to racemic or enriched in the (–)-enantiomer (Table 2). In general, TC tended to be more enantioselectively degraded than CC for most samples although variations, even within one study, can occur due to temporal trends (*13*, *40*), air-water/air-soil exchange (*41*, *42*), spatial differences (*40*, *43*) and wind direction (*16*).

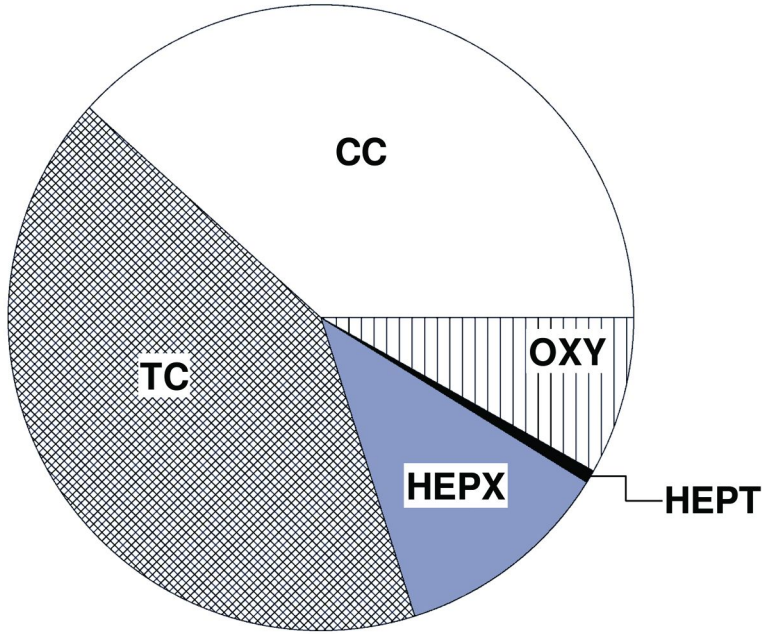

Figure 2. EF measurements by compound.

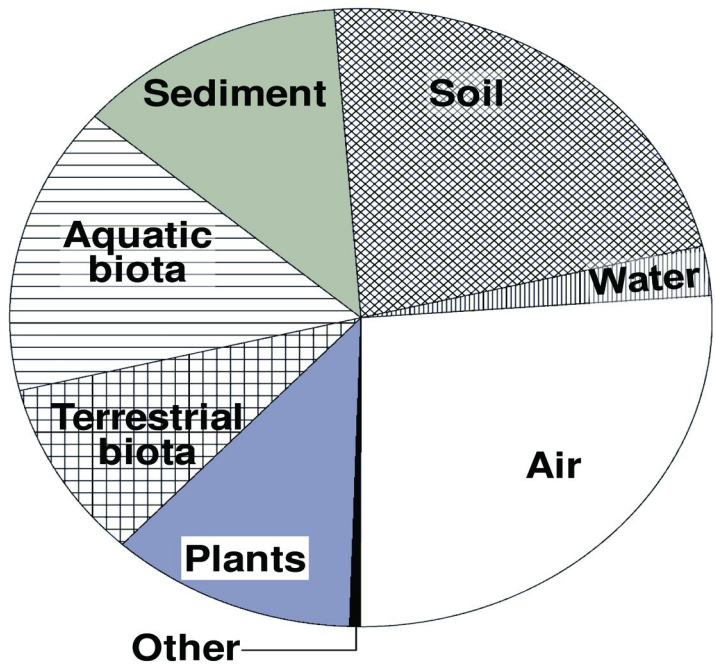

Figure 3. EF measurements by matrix.

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HEPX was reported in ambient air samples about a quarter as often as CC and TC (Table 2). Reported values showed a consistent trend towards higher amounts of the (+)-HEPX enantiomer. It is not known whether this trend is due to selective degradation of the (–)-enantiomer of HEPX or selective formation of the (+)-enantiomer of HEPX from degradation of parent compounds; however, Bidleman et al. (*44*) suggested the most likely source of HEPX in ambient air is that produced by microbial activity in soils rather than HEPT photolysis. Unfortunately, HEPT is only seldom reported, usually due to low concentrations as it is readily degraded in the environment, and the majority of samples were found to be close to racemic. OXY EFs were only reported in one air study with a value of 0.519 found in a sample from Alabama (*7*).

Several studies have shown similar EF signatures [enrichment of (+)-CC and (–)-TC] in ambient air as found in soils from the same area (*10*, *45*, *46*); however, the influence of long range transport and urban termiticide use can affect the EFs in ambient air at any given location (*16*, *20*, *45*). For example, a study in Alabama (*7*) found ambient air EFs for CC and TC as well as MC5 (octachlordane) that were close to racemic. From this data and soil-air exchange models, the authors suggested evaporation from termiticide treated houses in the region might be responsible for the levels in ambient air instead of volatilization from soils. Genauldi et al. (*16*) used enantioselective analysis, quantitative analysis and air mass back trajectories to determine sources of chlordane in the Western US and South Korea. Their results suggested volatilization from urban soils and house foundations were likely the major source but that the Pacific Ocean may also be a source of racemic chlordane to these locations.

To better determine the importance of soil volatilization on EFs in ambient air, several studies measured the EFs of chlordane compounds in air directly above soils known to be contaminated with chlordane (*10*, *25*, *47–49*). Leone et al. (*10*) suggested that although the enantiomeric signatures in the air above the soil generally followed the same patterns as in the soil, both in direction of degradation and relative magnitude, dilution with bulk air caused the air above the soil to be less degraded enantioselectively than the soil. As shown in Figure 4, all studies observed that as the height of the sample collected increases, EFs for both CC and TC became closer to bulk ambient air (i.e., more racemic). Later studies by Eitzer et al. (*47*) and Meijer et al. (*49*) showed that, although air very close to the soil is likely near equilibrium with the soil, the fraction of chlordane contributed to air above the soil decreases quickly with height.

A few studies have looked at the EFs of chlordane compounds in indoor air in personal residences. In all studies, only CC and TC have been reported and values were considered racemic for both when compared to racemic standards (Table 2). In Leone et al. (*50*), it was suggested that a lack of enantioselective degradation of chiral pesticides in indoor environments was likely due to protection from sunlight, moisture, and microbial activity in the homes. High levels of parent relative to metabolite concentrations in the samples further suggest little degradation since application. Differences between the more racemic residues indoors versus non-racemic residues often found in ambient air and other media have been used to differentiate sources of chiral chlordanes (*16*, *44*, *51*).

Due to their relatively high Henry's law constants, chlordane compounds are readily transported across the globe by ambient air. A number of studies have looked at the change of atmospheric chlordane EFs seasonally, spatially, and temporally. Two studies (*40*, *45*) used passive air samplers to look at spatial and seasonal trends in the Great Lakes and throughout North America. Both found strong urban – rural differences and an importance of local sources compared to more distant sources. Bidleman et al. (*13*) measured chlordane compounds in Arctic air samples collected from 1984-1998 and found declining concentrations, likely due to bans in most developed countries in the late 80s, as well as a greater proportion of "recycled" chlordanes from soil emissions (instead of fresher, newer sources). With the withdrawal of chlordane from the world market in 1997, levels are expected to continue to decline and an increase in the importance of recycled sources is likely (*13*). In a separate study, Bidleman et al. (*19*) analyzed archived atmospheric deposition samples, soils, and lake sediment cores from Sweden, Iceland, and the Canadian Arctic representing ~50 years of chlordane accumulation. They found a shift in CC and TC EFs from racemic in historical atmospheric deposition and sediment samples to nonracemic in more recent samples while soil samples were nonracemic throughout, again suggesting a greater influence of soil emissions in current times. More research is needed to further elucidate influences of re-emissions of chlordanes from soils to air.

Water

Data on chlordane enantiomer signatures in natural waters are limited having been reported only in Arctic and North Atlantic marine systems (*23*, *56*, *57*) and three of the Great Lakes (*12*) (Table 2). Hoekstra et al. (*57*) analyzed seawater off the coast of northern Alaska and reported all samples to be approximately racemic for the only two chlordane components quantifiable (CC, HEPX). Two other studies, one in the North Atlantic/Arctic Ocean region (*23*) and another sampling a transect from the Chukchi to the Greenland Sea (*56*) found EFs of TC and CC to be slightly more variable, although averaging around 0.5 for both. Both studies also found HEPX displayed much stronger enantioselective signatures with values typically >0.6. The authors suggested atmospheric transport of residues emitted from soils was the likely source of HEPX to Arctic surface water samples.

Jantunen et al. (*12*) measured EFs for CC, TC and HEPX in water from Lakes Superior, Erie, and Ontario. TC and HEPX were found to be nonracemic in all three lakes; however, CC EFs varied from nonracemic in Lake Ontario (0.480–0.485) to racemic (0.500–0.502) in the other two lakes. The authors suggested different atmospheric sources to the lakes could transport chlordanes with different EF signatures.

Soil

Results of enantioselective analyses of chlordanes in soils have been reported since the 1990s (*46*, *48*, *59*). Nonracemic residues of CC and TC, as well as HEPT, HEPX and OXY, are often found in soils due to enantioselective metabolism. Soils from different regions, with different land uses and histories

can show varied selectivity and EFs can range from racemic (e.g., CC = 0.50 for an agricultural soil in British Columbia, Canada (*59*)) to very nonracemic (e.g., TC = 0.846 for a woodland soil in Switzerland (*43*)). Most soils showed enhancement of the (+)- enantiomer (EF>0.5) for CC, HEPT, and HEPX and the (–)- enantiomer (EF<0.5) for TC and OXY. For the parent compounds, EF values greater than 0.5 and less than 0.5 indicate preferential metabolism of the (–)- and (+)- enantiomers, respectively. However, for the metabolites, HEPX and OXY, nonracemic EFs can occur either from preferential formation of one enantiomer from the parent compound and/or enantioselective degradation of one enantiomer of the metabolite.

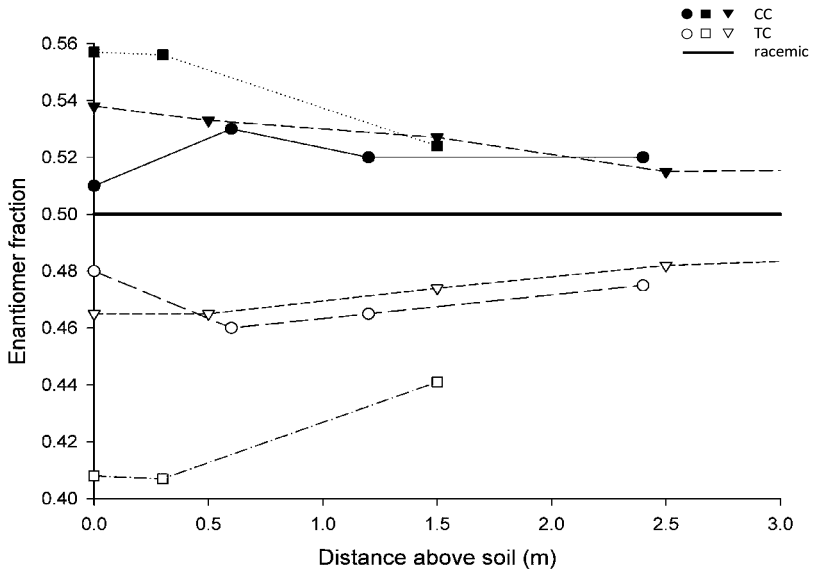

Figure 4. EFs of cis- and trans-chlordane measured in air above soil (□, ○(25), ▼, ▽ (47), ■, □ (49)).

In general, agricultural soils favored degradation of the (–)- CC and (+)- TC enantiomers (Table 2). However, various exceptions exist where racemic or even inverse EFs of CC and TC were found in agricultural soils. Reasons for differences in enantiomer signatures in the various studies were generally not attributable to total organic carbon, pH of soils, concentration of chlordanes or regional differences, although one study showed differences between soils from tilled agricultural fields and those from an ornamental nursery (*60*). Variability is likely due to differences in soil microbial communities, but further research is necessary.

Table 2. Chlordane EF measurements in environmental matrices

Sample type	*CC*	*TC*	*HEPT*	*HEPX*	*OXY*	*Refs*
Air						
Ambient EF range	0.413–0.619	0.372–0.554	0.493–0.521	0.528–0.805	0.591	(*6*, *7*, *12*, *13*, *16*, *19–25*, *38*, *40*, *44*, *45*, *47*, *51–54*)
N[a]	204	219	8	58	1	
% NR (+)[b]	31%	0%	12%	100%		
%NR (-)[b]	4%	67%	0%	0%		
Indoor EF range	0.490–0.505	0.490–0.505	[c]			(*7*, *50*, *51*, *55*)
N	25	25				
% NR (+)	0%	0%				
%NR (-)	0%	0%				
Water						
Water EF range	0.468–0.519	0.452–0.515		0.500–0.980	0.500	(*12*, *23*, *56*, *57*)
N	23	9		17	1	
% NR (+)	5%	0%		94%		
%NR (-)	20%	87%		0%		
Soil						
Agricultural EF range	0.409–0.740	0.237–0.885	0.507–0.526	0.530–0.879	0.367–0.650	(*13*, *16–19*, *22*, *24*, *25*, *39*, *41*, *43*, *46–49*, *51*, *52*, *58–66*)

Sample type	*CC*	*TC*	*HEPT*	*HEPX*	*OXY*	*Refs*
N	98	113	4	16	8	
% NR (+)	81%	2%		100%	29%	
%NR (-)	1%	84%		0%	71%	
Background EF range	0.08–0.846	0.341–0.616				(*16–19*, *21*, *43*, *47*, *54*, *60*, *67*)
N	103	114				
% NR (+)	51%	8%				
%NR (-)	34%	75%				
Sediment						
Suspended EF range	0.503–0.527	0.463–0.530				(*11*)
N	11	11				
% NR (+)	37%	33%				
%NR (-)	0%	44%				
Surficial EF range	0.475–0.528	0.466–0.503		0.603–0.682		(*11*, *67*, *68*)
N	32	32		5		
% NR (+)	53%	0%		100%		
%NR (-)	7%	60%		0%		
Cored EF range	0.488–0.517	0.465–0.518				(*11*, *68*, *69*)

Continued on next page.

Table 2. (Continued). Chlordane EF measurements in environmental matrices

Sample type	*CC*	*TC*	*HEPT*	*HEPX*	*OXY*	*Refs*
N	95	106				
% NR (+)	25%	3%				
%NR (-)	0%	21%				

[a] N = number of measurements included in EF range. [b] % NR (+)= percent of measurements >0.51, thus showing predominance of the (+)- enantiomer. % NR (–)= percent of measurements <0.49, thus showing predominance of the (–)- enantiomer. 0% signifies no measurements were in the selected range. The sum of %NR (+) and %NR (-) may be less than 100% as samples between 0.49-0.51 are considered racemic and not included in these two values. [c] Blank cells indicate no measurements reported.

A number of studies looked at background soils including grassland, forest, woodland, urban and rural but non-agricultural soils. The range of EFs for CC for background soils was greater than in agricultural soils and reversals in preference for the (+)-enantiomer were more common (Table 2). One study by Kurt-Karakus (*43*) looked at chiral pesticide signatures in background soils from around the globe. A significant positive correlation was found between deviation from racemic (DFR= 0.50 – EF) and the percent of soil organic matter (*43*). The authors suggested differences in degradation and reversals in patterns implied that the capability to degrade enantioselectively develops in a localized manner in background soils. They hypothesized that EFs could be used to examine the extent of "mixing" between soil microbial communities.

A few studies looked at more specific soil types. Soils near house foundations, where chlordane was used as a termiticide, were measured in Connecticut (*60*) and found to be racemic. The authors also found very little change in the compositional profile from technical chlordane suggesting minimal degradation of chlordane in these soils since time of application. Two studies looked at amended soils — sludge-treated soils and commercial compost (*62*, *63*). Both types of amended soils were found to follow the same enantioselective pattern for CC and TC as in agricultural soils. In sludge-treated soils, EFs for chlordanes were not statistically different from those measured in untreated soils from the same field. The authors determined the degradation preference of the soil microbial community for chlordanes was not affected by the addition of sludge to the soil (*62*). In the commercial compost, EFs along with compositional profiles for CC and TC were used to determine that the compost matrix was not the same as residential lawn/garden soils and, thus, not simply a soil diluted with compost (*63*).

A few studies measured EFs for HEPT, HEPX, and OXY (Table 2) and for all studies, HEPX was found to be very nonracemic (EFs >>0.5). Residues of OXY showed EFs both greater and less than racemic. It is unclear whether nonracemic HEPX and OXY arise from selective degradation of the metabolite, selective formation from the parent compound, or a combination of both.

Sediment

A few studies have characterized chlordane compounds in sediment and the majority only targeted *cis*- and *trans*-chlordane. When the metabolites HEPX and OXY were studied, they were only found occasionally or not at all (*11*, *27*). These collective studies looked at suspended (*11*), surficial (*11*, *67*, *68*), and cored sediments (*11*, *68*, *69*), as well as a sediment reference material (*27*).

As in other environmental compartments, the EFs were usually >0.5 for CC and <0.5 for TC, although exceptions were reported in each case (Table 2). Surficial sediments showed the largest range but, overall, EFs in sediment did not deviate drastically from racemic with all samples within ± 0.04 of the racemic value. The relatively small deviation from racemic, coupled with a lack of detectable metabolites likely indicates little enantioselective degradation occured in sediments and/or their source materials. The EF for HEPX in surficial sediments ranged between 0.603–0.682 similar to values found in other matrices.

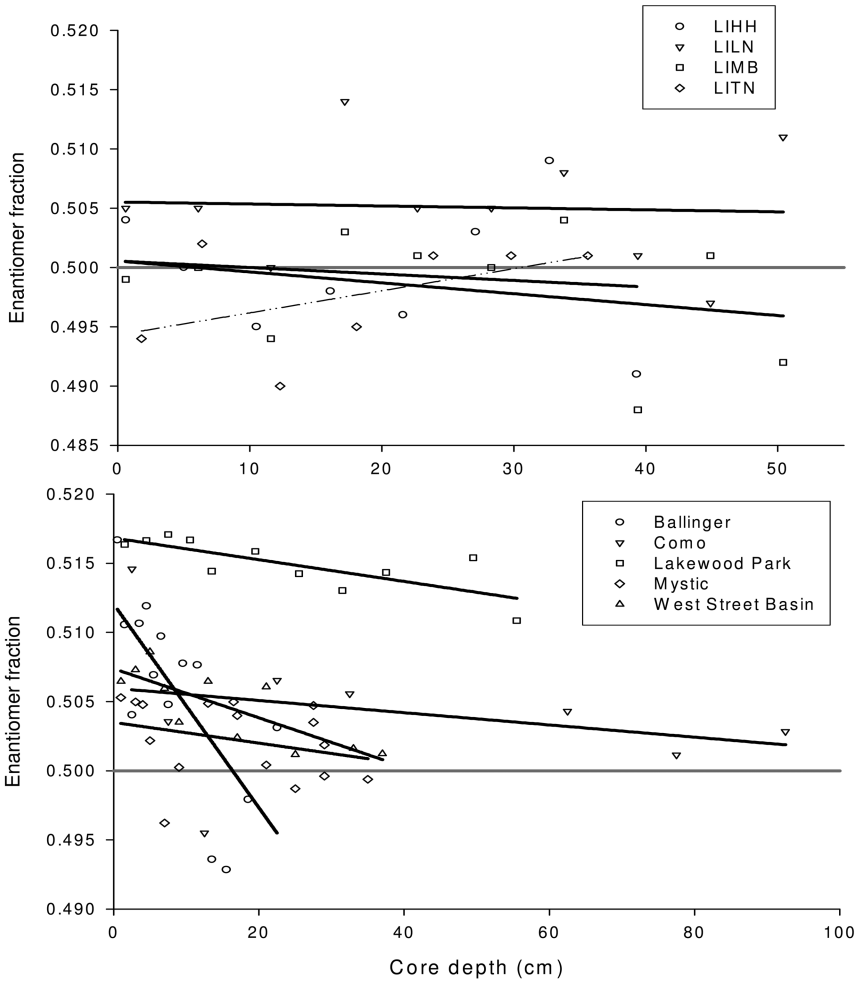

Figure 5. cis-Chlordane trends in sediment cores [LIHH = Hempstead Harbor, LILN = Little Neck Bay, LIMB = Manhasset Bay, LITN = Throgs Neck all in New York; Washington's Lake Ballinger, Texas' Como Lake, Georgia's Lakewood Park Lake, Massachusetts' Upper Mystic Reservoir, and California's West Street Basin (11)]. Grey reference line at EF = 0.50 indicates racemic.

Measurements of chlordane EFs in cored sediments were the most prevalent and allow for temporal comparisons. *cis*-Chlordane EFs were generally closer to racemic in older/deeper core sections compared to EFs in newer core sections where the EF was usually >0.5. This pattern results in a negative slope for trend lines in an EF vs. depth plot (Figure 5). For CC, the trend (though not necessarily statistically significant) was found in eight of nine cores (shown in bold in Figure

5) at Long Island Sound at Hempstead Harbor, Little Neck Bay, and Manhasset Bay (*68*), Washington's Lake Ballinger, Texas' Como Lake, Georgia's Lakewood Park Lake, Massachusetts' Upper Mystic Reservoir, and California's West Street Basin (*11*). *trans*-Chlordane, showed the same trend of more racemic EFs in older/deeper core sections compared to newer core sections but the EF was usually <0.5. This results in an EF vs. time/depth trend line with a positive slope (Figure 6). This trend (though not necessarily statistically significant) was found for six of the ten cores (shown in bold in Figure 6) at Long Island Sound at Little Neck Bay and Throgs Neck (*68*), Washington's Lake Ballinger, Massachusetts' Upper Mystic Reservoir, California's West Street Basin (*11*), and Devon Island's Lake DV09 (*69*). Figure 5 and Figure 6 show that although the slope of the trend line for each location varies, they largely agree in the slope direction for a given compound, negative for CC and positive for TC, possibly indicating similar sources or processes.

The EF trends in sediment cores indicate older inputs of chlordane were from relatively fresh (i.e., racemic) sources, while more recent inputs indicate more weathered (i.e., nonracemic) sources. If enantioselective degradation was occurring within the sediments, the oldest/deepest core sections should have the greatest DFR due to longer times for such reactions (*11*, *68*). Due to the appearance of little or no enantioselective degradation, lack of metabolites, and larger DFRs in more recent sediments, it is more likely that most enantioselective degradation occurs before the sediment is deposited.

One likely source of chlordanes to sediments is nearby soils. Wong et al. (*67*) collected seven soil samples within 100 m of sediment collection sites. They were unable to show a correlation between the CC and TC EFs of these two compartments; however, this may be due to the lakes receiving sediment inputs from several sources. Kurt-Karakus et al. (*43*) reported that some soils show a high spatial variability for EF, especially for different land and chlordane uses (e.g., agricultural, residential). In another study, surficial and suspended sediments collected at or near the same location as cores, though not necessarily at the same time, varied in their agreement with EFs in the top core slices (*11*). These variations indicate a need for additional research to understand interactions and influences of various sources of chlordanes to sediments.

Chlordane Enantiomers in Biota

Enantiomer fractions of chlordane compounds have been measured in a variety of biota, including plants, animals such as fish, birds, seals and even humans (summary in Table 3; examples from specific studies are offered in the appropriate subsections). Several studies targeted metabolic processes by dosing a particular species and measuring the EF over time (*70–74*). Overall, the EFs of chlordane compounds in biota are more frequently nonracemic than EFs measured in other environmental compartments. This is not surprising as the biotic environment is essentially chiral (amino acids, enzymes, proteins, etc.), whereas fate and transport in soil, sediment, air, and water can also be controlled

by achiral physical-chemical processes. There are also more measurements of EFs for the degradates OXY and HEPX in biotic media as these compounds are found more frequently and in higher concentrations than in other environmental compartments. For both parent compounds and metabolites, the range of EFs is broader, likely because a broader collection of enantioselective reactions is possible.

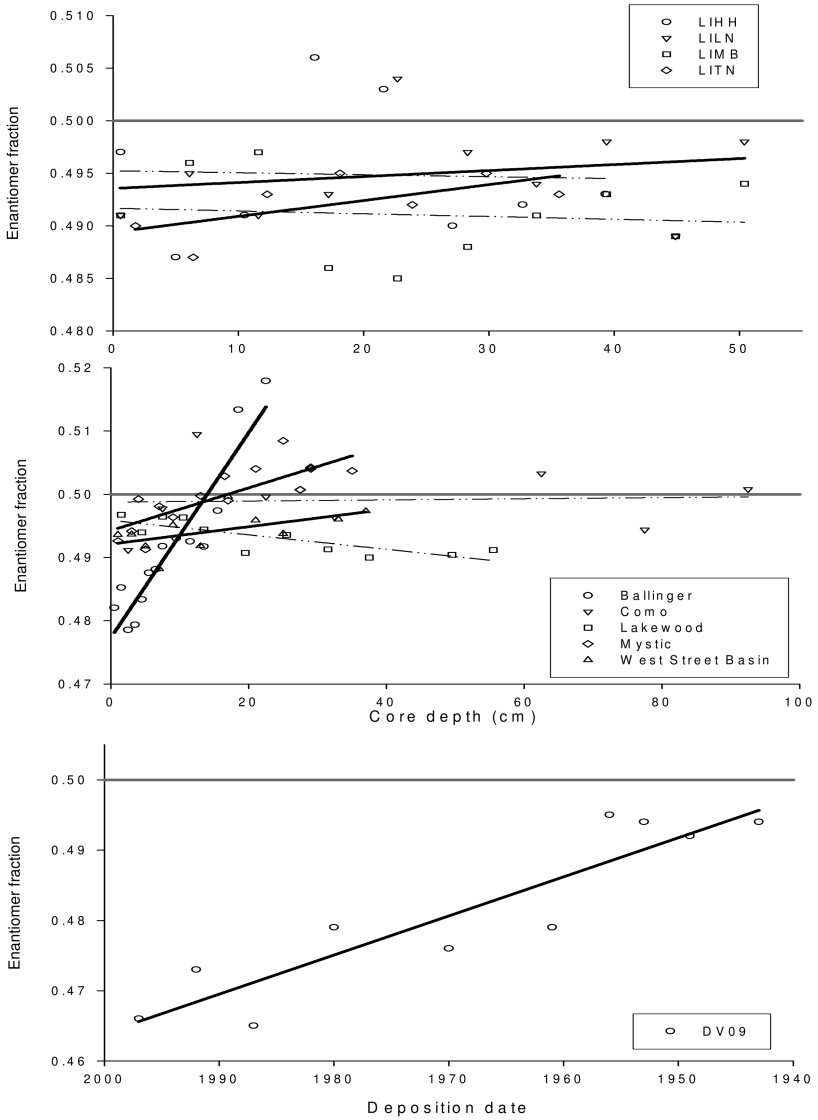

Figure 6. trans-Chlordane trends in sediment cores [Devon Island's Lake DV09 in the Canadian Archipelago (69), see Figure 5 for additional legend abbreviations]. Grey reference line at EF = 0.50 indicates racemic.

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Aquatic Biota

Several studies have addressed biomagnification and aquatic food web relationships for chlordane enantiomers. Generally, the data show the higher the species on the food web, the higher the DFR for chlordane compounds, especially the parent compounds CC and TC.

Mysids (*Mysis relicta)* exposed to TC and *cis*- and *trans*-nonachlor via spiked sediment for 10 days showed an EF of *trans*-chlordane starting at a racemic value of 0.50 and quickly decreasing to 0.00 after 35 days (day 7, EF 0.16; day 10, EF 0.14; day 23, EF 0.02). The EF of OXY also started at racemic and then rose to 0.78–0.89 for all remaining time points. These data suggest that mysids are enantioselectively degrading (+)-TC and enantioselectively producing (+)-OXY (*72*).

A variety of seal species and tissues have been analyzed for chlordane enantiomers. Regardless of where the samples originated, the EFs of CC, TC, HEPX and OXY in liver and brain were <0.50 (enriched in the (–)- enantiomer), while the EFs for OXY in fat or blubber were generally > 0.50 (enriched in the (+)- enantiomer). One study reported average EFs in polar bear fat (f) and liver (l) as CC— 0.78 (f) and 0.56 (l), TC— 0.91 (f) and 0.76 (l), HEPX— 0.68 (f) and 0.77 (l), and OXY— 0.62 (f) and 0.57 (l) (*29*). Two whale species from the wild and a whale blubber reference material were analyzed and showed remarkably similar trends in EFs in all samples for CC (0.07–0.17), TC (0.64–0.89), HEPX (0.62–0.64), and OXY (0.59–0.75) (*27*, *57*). Enantioselective processes, whether enantioselective degradation within the individual or uptake of non-racemic residues through diet, are definitely occurring for chlordane compounds in aquatic biota contributing to nonracemic EFs found in various tissues.

Fish

Three studies measured chlordane EFs in fish oils, including one Standard Reference Material (SRM). The EFs in cod liver oils mostly showed CC <0.50, but TC, HEPX and OXY > 0.50 (*27*, *80*, *84*). In contrast, the EFs in fish oils were closer to racemic for CC and TC, with CC EFs reversed in the enriched enantiomer (*84*).

The enantiomer fraction of chlordane compounds in wild fish (herring, cod, char, salmon, trout) from various water bodies (Resolute Bay, Nunavut, Canada; River Dalälven, Sweden; Arctic Ocean; Baltic, Barents, and Ross Seas), have been reported (Table 3). As expected in biota, nonracemic EFs were most often measured for all compounds, although whole Arctic char had EFs near racemic (0.50–0.52) for CC, TC, HEPX, and OXY, and whole Arctic cod had EFs with a low DRF for CC and TC (0.47–0.53). However, slightly higher DFRs were found in Arctic cod for the metabolites HEPX and OXY (0.52-0.58) (*29*, *57*). The enriched enantiomer in herring is reversed compared to cod and char for both CC and TC (*37*, *75*, *79*, *81*). Two cohorts of Baltic salmon were studied, a control group and a group suffering from M74 syndrome, a disease which affects survival of fry. It has been suggested that negative health effects may cause metabolic changes, but no statistical differences in the concentration or EF of

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several chlordane compounds were found between the two groups, suggesting no relationship between the disease and chlordane body burden (*79*).

Gender and organ differences were noted for chlordane enantiomers in cod. The EF of CC in cod livers averaged 0.54 in males and 0.45 in females; for TC, EF averaged 0.57 in males and 0.39 in females. For both compounds, the difference was statistically significant, and the enriched enantiomer was reversed between genders (*82*). While the EF of CC ranged between 0.415–0.55 for cod muscle, gonad, and liver, the EF of TC ranged between 0.35–0.66 for muscle, 0.38–0.67 for gonad, and 0.29–0.69 for liver. The lowest and highest values for each tissue were always from the same individual, an 11 year old female and a 9 year old male cod, respectively (*81*).

Three studies have investigated enantioselective degradation of chlordane compounds in fish over time. Juvenile rainbow trout were fed food containing HEPX for 32 days, followed by a 96 day depuration period. The EF of HEPX in trout carcasses did not change throughout the experiment, indicating that no enantioselective degradation of HEPX occurred (*73*). Another study with rainbow trout exposed the fish to racemic TC in food for 40 days with a 238 day depuration period. During the experiment, the EF of TC changed from nearly racemic (0.51) at day 13 to nonracemic (0.73) at day 278. The half-life of TC enantiomers was calculated as 231 days for the (+)- enantiomer and 107 days for the (–)- enantiomer (*74*). Another study exposed arctic char to racemic CC and heptachlor by a single intraperitoneal injection, and followed them and their metabolites in muscle and liver for five weeks. Average EFs of CC decreased slightly during the study (to 0.497–0.482 in muscle and 0.492– 0.479 in liver). Enantioselective analysis of heptachlor was not attempted, but the EFs for HEPX in muscle from individual fish ranged between 0.447–0.541. The weekly averages were not statistically different than racemic, and HEPX was below detection in liver. Based on these results, the authors suggest that both CC and HEPT undergo at least some enantioselective degradation in arctic char (*71*).

Terrestrial Biota

Liver in hare, deer, fox, and wolverine have been analyzed for chlordane enantiomers, along with exposure and degradation studies in rats. In liver, the EFs of CC, TC and HEPX were all >0.5 while OXY had a larger range (0.44–0.96) over the range of species (*26*, *39*, *76*, *88*). Factors such as source and route of exposure, time since exposure, general health, and enzyme induction may cause drastically different EF values between individuals.

Sprague-Dawley rats were exposed to either oxychlordane, *trans*-chlordane, or *trans*-nonachlor via daily oral gavage for 28 days and followed for 56 days after dosing concluded. Fat, liver and kidney were analyzed for the enantiomers of oxychlordane and *trans*-chlordane (*70*). The EF of the degradate OXY decreased in fat through the experiment regardless of the exposure chemical indicating more rapid depletion of (+)- OXY or enrichment of the (–)- enantiomer. The EF of OXY in fat was always higher in females than males, regardless of the exposure chemical or time. Similarly, the EF of TC decreased in all tissues over the time course of the experiment, frequently with no (+)- TC detected at later time points.

The results for TC show enrichment of the opposite enantiomer than was found in fish studies (rat TC EF <0.5; fish TC EF >0.5). Females typically had higher TC EFs than males, but the difference was very small in fat. Bondy et al. (*70*) suggest gender differences may have been caused by differences in enzyme induction and/or metabolism.

Birds and Eggs

Bird eggs, liver, fat, and plasma have been analyzed for chlordane enantiomers in a variety of species. The EFs of CC and HEPX in bird eggs were consistent (CC– 0.255 ± 0.009 and between 0.62–0.73 for HEPX) but ranged dramatically for TC (<0.01–0.581). In general, OXY EFs were between 0.60–0.70 in eggs (*28*, *39*, *83*, *87*), except for one study that reported OXY EFs between 0.22–0.38 (*86*). The highest CC EF values in both fat and liver were from the Northern Fulmar (0.53-0.69), whereas all other species had EFs <0.50. For all other samples, there were no significant differences between liver and fat EFs or between species and all samples were shown to have the same enriched enantiomer as in eggs. Small differences in EFs were found between genders but they were not statistically significant. There were also minimal differences between egg yolk and female plasma EFs, indicating that maternal transfer of contaminants in these birds is likely non-stereoselective.

Humans

While chlordane concentrations often have been quantified in humans, to date only one study has been published detailing chlordane enantiomers in humans. The study compared chlordane in adipose tissue of non-Hodgkin's lymphoma patients to a control group. The EFs of CC, HEPX and OXY were all non-racemic, whereas TC was not detected (*89*). Most chlordane compound EFs did not significantly differ between lymphoma patients and the control group, but there was a statistical difference for CC (EF = 0.15 control; 0.23 lymphoma patient) (*89*).

Plants

To date, only one research group, at the Connecticut Agricultural Station, has conducted enantioselective chlordane determinations in plants. Their New Haven campus has an experimental plot that was sprayed with technical chlordane in 1960 (*60*). This soil is well characterized and has been used extensively for research to understand the translocation of chlordane residues from soil to plant compartments.

Table 3. Chlordane EF measurements in aquatic and terrestrial biota

Sample type	*CC*	*TC*	*HEPT*	*HEPX*	*OXY*	*Refs*
Aquatic biota						
Microscopic EF range	0.48–0.62	0.45–0.51	0.52	[a]	0.505–0.889	(*15*, *57*, *72*)
N[b]	8	12	1		6	
Fat/blubber EF range	0.072–0.78	0.16–0.91		0.099–0.68	0.351–0.75	(*27*, *29*, *37*, *57*, *75–79*)
N	23	24		24	24	
Liver/brain EF range	0.194–0.821	0.18–0.91		0.048–0.77	0.306–0.500	(*29*, *37*, *39*, *57*)
N	9	12		13	12	
Fish						
Fish EF range	0.16–0.677	0.167–0.777		0.286–0.644	0.510–0.647	(*27*, *29*, *37*, *57*, *71*, *73–75*, *79–84*)
N	74	84		23	22	
Birds						
Liver EF range	0.16–0.69			0.51–0.77	0.52–0.68	(*14*, *85*)
N	7			13	13	
Fat EF range	0.19–0.53	0.220.40		0.56–0.80	0.55–0.71	(*14*)
N	7	7		7	7	
Plasma EF range	0.267–0.350			0.680–0.693	0.626–0.634	(*28*)
N	2			2	2	

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Sample type	*CC*	*TC*	*HEPT*	*HEPX*	*OXY*	*Refs*
Eggs EF range	0.255	0.01–0.581		0.615–0.730	0.219–0.697	(*28*, *39*, *83*, *86*, *87*)
N	1	7		4	20	
Terrestrial biota						
Terrestrial EF range	0.468–0.519	0.452–0.515		0.500–0.980	0.500	(*26*, *39*, *70*, *76*, *88*)
N	23	9		17	1	
All biota						
%NR(+)[c]	37%	31%		57%	65%	
%NR(-)[c]	52%	65%		39%	30%	

[a] Blank cells indicate no measurements reported. [b] N = number of measurements included in EF range. [c] % NR (+)= percent of measurements >0.51, thus showing predominance of the (+)- enantiomer. % NR (–)= percent of measurements <0.49, thus showing predominance of the (–)- enantiomer. 0% signifies no measurements were in the selected range. The sum of %NR (+) and %NR (-) may be less than 100% as samples between 0.49-0.51 are considered racemic and not included in these two values.

Table 4. Chlordane EF measurements in plants

Sample type	*CC*	*TC*	*HEPX*	*Refs*
Zucchini- Soil exposure				
Root EF range	0.50-0.55	0.42-0.56	[a]	(*25*, *61*, *64*, *66*)
N[b]	13	13		
Stem EF range	0.47-0.58	0.36-0.53		(*25*, *61*, *64*)
N	11	11		
Leaf EF range	0.51-0.58	0.42-0.48		(*25*, *61*, *64*)
N	11	11		
Fruit EF range	0.53-0.65	0.40-0.49		(*25*, *61*, *64*)
N	17	17		
Xylem sap EF range	0.53-0.60	0.45-0.51	0.62-0.65	(*24*, *66*, *90*)
N	5	5	5	
Aerial tissue EF range	0.50-0.55	0.48-0.50	0.65-0.67	(*24*, *66*)
N	3	3	3	
Zucchini- Air exposure				
Root EF range	0.46-0.49	0.50-0.55		(*64*)
N	4	4		
Stem EF range	0.46-0.47	0.47-0.52		(*64*)
N	4	4		
Leaf EF range	0.48-0.54	0.44-0.51		(*64*)
N	4	4		
Fruit EF range	0.53-0.62	0.40-0.49		(*64*)
N	4	4		
Other plants				
Root EF range	0.51-0.55	0.37-0.51	0.58	(*25*, *66*)
N	9	9	2	
Stem EF range	0.46-0.59	0.30-0.54		(*25*)
N	4	4		
Leaf EF range	0.52-0.60	0.30-0.55		(*25*)
N	8	8		
Fruit EF range	0.48-0.56	0.22-0.47		(*25*)
N	6	6		
Xylem sap EF range	0.46-0.53	0.42-0.50	0.43-0.58	(*24*, *66*, *90*)

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Table 4. (Continued). Chlordane EF measurements in plants

Sample type	*CC*	*TC*	*HEPX*	*Refs*
N	6	6	6	
Aerial tissue EF range	0.45-0.48	0.39-0.47	0.44-0.63	(*24*, *66*)
N	4	4	3	
All plants				
%NR(+)[c]	70%	14%	76%	
%NR(–)[c]	20%	70%	24%	

[a] Blank cells indicate no measurements reported. [b] N = number of measurements included in EF range. [c] % NR (+)= percent of measurements >0.51, thus showing predominance of the (+)- enantiomer. % NR (–)= percent of measurements <0.49, thus showing predominance of the (–)- enantiomer. 0% signifies no measurements were in the selected range. The sum of %NR (+) and %NR (-) may be less than 100% as samples between 0.49-0.51 are considered racemic and not included in these two values.

In a series of studies, eight different food crops were exposed to chlordane contaminated soil. Roots, stems, leaves, fruit, peels, and xylem sap were analyzed for chlordane components. Table 4 shows the range of measured EFs in various crops and plant tissues. The EFs were similar in pumpkin, lettuce, spinach, pepper, and tomato for the equivalent compound and tissue (*25*). However, there are notable differences in the CC EFs for xylem sap of zucchini (0.53-0.60) and cucumber (0.46-0.48) (*24*, *66*, *90*), and both chlordanes EFs for aerial tissues of zucchini (0.50-0.55 CC; 0.45-0.46 TC) and cucumber (0.45-0.46 CC; 0.39-0.40 TC) and for fruit of zucchini (0.53-0.65 CC; 0.40-0.49 TC) and cucumber (0.48-0.51 CC; 0.22-0.30 TC) (*24*, *66*). Many of the EFs measured in plants were nonracemic, and EF differences between the plant compartments indicate transport processes in these plants may be enantioselective. The data suggests that the occurrence of enantioselective degradation of CC and TC in plant tissues themselves is unlikely as very little OXY was detected in zucchini plants (*64*), and an increase in DFR for CC and TC did not correspond to an increase in the concentration of OXY (*61*).

In one study, zucchini were exposed to chlordane by both soil and air routes, and chlordane was found to translocate to all plant tissues in both types of exposure. The air in the greenhouse contained racemic CC and TC (EF= 0.50) at high and low concentrations, but all tissues of zucchini, including leaves, which are the most likely tissue to uptake chlordane from air, contained nonracemic chlordane residues (*64*). For the soil route experiment, the EFs of chlordane in high, medium, and low concentration soils were nonracemic and similar in value, with CC = 0.53–0.55 and TC = 0.45–0.48 (*64*). This pattern was similar to that found in other soil studies where CC and TC are on opposite sides of the 0.50 racemic value. The EF pattern in roots, however, did not match that measured in soil, with CC EFs becoming more racemic (0.50–0.53) and TC changing

enantioselectivity (0.51–0.54). This data suggests the soil-to-root transport mechanism was enantioselective (*64*). Additionally, transport within plants was found to also be enantioselective, as best depicted by the higher DFR EFs of CC in zucchini fruit (0.56–0.64) (*64*). The enantiomer and compound distributions into zucchini tissues were different for air and soil routes of exposure (*64*), which may be useful for source apportionment.

Uses of Enantiomer Data

As chlordane compounds are global contaminants that still are found routinely in all environmental media and many species, determining the transport and fate of these chemicals is very important for understanding exposure and effects. To get a better handle on their cycling through the environment and biosphere, more information on the biotic and abiotic degradation of chlordanes is necessary. In general, physical-chemical processes like photolysis, movement between environmental compartments, and non-biological chemical reactions will not change the enantiomer composition (*33*). Biologically mediated processes like adsorption, distribution, metabolism and excretion in an organism very often involve chiral biological molecules and, thus, can change the enantiomer composition of environmental contaminants (*91*). Chlordane was produced as a racemic mixture (*75*), and no anomalous achiral reactions are known that change the enantiomer signature. Generally, racemic enantiomer signatures in the environment indicate it has undergone only achiral reactions whereas nonracemic signatures indicate biologically mediated processing. By examining differences in the enantiomeric signature of various samples, we can learn something of the path/s the chemicals have taken since their application and better determine their future transport and fate.

The first reported uses of chiral chlordane analyses were as tracers of air-surface exchange (*42*, *92*). These first studies, along with more recent ones, used chiral chlordanes to help distinguish the atmospheric transport of freshly applied pesticides from those which are "recycled" from lakes and soil, to determine sources of pesticides, and to investigate biotic vs. abiotic degradation pathways (*52*, *53*, *93*). A few studies used enantioselective analysis to better understand air-water gas exchange and the amount of biotic processing of chlordanes in sediments (*11*, *12*, *23*); however, most work in environmental media has focused on using chiral chlordanes to track air-soil exchange.

The use of enantioselective analysis to follow air-soil exchange has shown the increasing importance of soil emissions of chlordanes to local and regional ambient air signatures, as discussed in an earlier section. Several authors have used differences in chlordane enantiomeric signatures to differentiate urban chlordane use (termiticides) from rural use (agricultural pesticide) (*7*, *45*, *47*, *67*). Bidleman and Falconer derived a relationship to estimate the contribution of chiral compounds from two different sources (*94*). The authors suggested some advantages for using enantiomeric analysis rather than concentration data alone which include lower sample variability for repeat analyses and the fact that EFs

are not affected by analytical recovery factors or abiotic degradation processes during sample storage and recovery.

The use of chiral chlordane analysis to learn about spatial, seasonal and temporal changes in chlordane residues has been discussed already. Several authors have also used enantioselective analysis, along with traditional data (e.g., concentrations, parent-metabolite ratios), to study long range transport of chlordanes (*13*, *16*, *45*). From their studies of chiral pesticides in air from Alabama, Jantunen at al. (*7*) determined that atmospheric transport from the southern US is a continuing source of chlordane to the Great Lakes whereas Genauldi et al. (*16*) combined enantiomer data with other measurements to determine differences in air masses from Asia, the western US, and South Korea.

Other uses include determining differences in metabolism mechanisms and rates between the CC and TC isomers (*17*, *38*), determining patterns in long-term weathering of chlordane in soil (*60*), differentiating surface waters from deeper ocean waters (*23*), and determining that the main source of HEPX in ambient air is metabolism of HEPT in soils, not HEPT photolysis (*44*). Incorvia-Mattina et al. (*25*) determined that many plants grown in contaminated soils alter both concentrations and EFs of chlordanes during uptake and translocation to aerial tissues. The authors suggested that, due to these processes, vegetative matter may contain higher insect toxicities than soil residues. Further work on enantioselective uptake by plants might also help elucidate mechanisms for phytoaccumulation.

Several studies with chlordane compounds have used enantiomer fractions to help determine degradation pathways. As mentioned earlier, the EFs of CC and TC in sediment cores are slightly nonracemic suggesting the enantioselective degradation of chlordane occurred before arriving in sediments (*11*) and that chlordane compounds in this matrix are stable. This evidence can be combined with concentration patterns or other data to make a stronger case for preservation of historic use patterns within sediment cores.

Generally, enantiomer signatures in biota are more nonracemic than in environmental compartments. There are several processes that can contribute to nonracemic EFs in biota including enantioselective uptake from the environment (e.g., air, water), ingestion of nonracemic patterns from food/prey, or enantioselective degradation in the test subject itself. Plant studies have shown that uptake of chlordane from soil via roots and from air via leaves is enantioselective (*64*). A time course study showed that fish tissues have EFs that match the EF of their food at minimal time since exposure (*74*). In species where chlordane degradation is minimal, studies have concluded that accumulation from food is nonstereoselective, thus preserving the enantiomer signature of the lower trophic level (*15*, *57*). Studies in fish, mysids, and rats with chlordane compounds indicate that some species do enantioselectively degrade chlordane compounds (*70*, *72*, *74*). A careful examination of a system can likely pinpoint the enzymes involved and determine enantiomer rate constants and half-lives for both parent and degradate compounds. Nonracemic EFs for degradation products like HEPX and OXY are more complicated to interpret because they may have been enantioselectively formed from a parent compound or enantioselectively degraded themselves.

Conclusions

The studies of chlordane discussed in this review highlight the usefulness of continued monitoring of pesticide enantiomers. Small differences in the environment, be it the exact plant tissue or the gradient of EF with height above soil, can lead to significant differences in enantiomer signature. These differences are important to understanding the mechanisms of pesticide fate and transport. Characterizing enantiomer patterns in biota, plants and environmental media could also point to possible differences in exposure (e.g., plant and animal tissues consumed, respired air, soil ingested during hand-to-mouth activities, etc.). Differences in enantiomer exposure combined with differences in enantiomer toxicity may result in a differential risk for the two enantiomers.

Interpretation of chiral chlordane data is a complex endeavor due to uncertainties such as high variability in EFs over small spatial distances and the very limited knowledge of enantioselective degradation rates and mechanisms. Analytical challenges include a limited number of commercially available chiral columns able to separate chlordanes in complex environmental matrices and variability in stationary phase composition within chiral columns. Future work with chiral chlordane data depends on solving some of these problems. Even so, the use of chlordane EFs, in tandem with traditional measurements, can be a powerful tool for better understanding our environment.

Disclaimer

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Chapter 3

Comparison of the Enantiomer Distribution of Chiral Organochlorine Contaminants in Captive West Greenland Sled Dogs and Polar Bears from Baffin Bay

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Enantioselective analysis is a sensitive means of determining contaminant biotransformation, and is essential for proper assessment of the risk posed by chiral pollutants. Similar enantiomer distributions of chiral organohalogen compounds (OHCs) such as hexachlorocyclohexane (HCH) and chlorinated biphenyls (CBs) were previously reported amongst wild *canoidea* species. Therefore, we investigated the comparative enantioselective biotransformation capabilities for chiral OHCs between captive West Greenland (Baffin Bay) sled dogs (*Canis familiaris*) and free-ranging polar bears (*Ursus maritimus*) from Baffin Island, Canada, to understand bioaccumulation dynamics of individual enantiomers within top-predator

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species better, and to examine the feasibility for cross-species comparisons of enantiomer distributions. Enantiomer fractions (EFs) and enantiomer-specific biomagnification factors (BMFs) of OHCs were determined in West Greenland sled dog adipose, liver, thyroid, adrenal, and brain tissues after exposure to OHC-contaminated minke whale (*Balaenoptera acutorostrata*) blubber (exposed cohort) or pork fat (control cohort) for 20 months. Sled dogs biotransformed chiral OHCs enantioselectively. BMFs of (+)-oxychlordane, (-)-α-HCH, and (-)-CB 149 were 1.8-fold, 3.3-fold and 2.5-fold greater than their antipodes, respectively, leading to an enrichment of these enantiomers in sled dog adipose tissue relative to minke whale blubber. Non-racemic distributions of OHCs in two toxicologically sensitive tissues, thyroid and adrenal glands, are also reported for the first time. Except for CB 91, all OHC EFs were similar between Baffin Island polar bears and captive sled dogs. A comparison of enantiomer-specific BMFs revealed similar bioaccumulation dynamics for oxychlordane and heptachlor epoxide between the two species, but contrasting results for α-HCH, despite similar α-HCH EFs. Enantiomer-specific BMFs provide more reliable information for making cross-species comparisons of enantiomer distributions in biota than EFs alone, particularly for related species (e.g., *canoidea*). However, caution should be exercised in making such comparisons without knowledge of enantiomer distributions in underlying foodwebs.

Introduction

Polar bears (*Ursus maritimus*) are apex predators within arctic ecosystems. They biomagnify high concentrations of organohalogen compounds (OHCs), such as certain polychlorinated biphenyls (PCBs, also CBs), organochlorine pesticides, brominated flame retardants, and persistent metabolites of these compounds in their adipose tissue, internal organs, and blood relative to lower trophic level arctic species (*1*). Concentrations of OHCs in polar bears have been correlated with levels of a variety of biomarkers (e.g., endocrine and immunological) (*1*). For instance, a negative correlation was shown to exist between OHC concentrations and plasma testosterone concentrations in free-ranging male polar bears (*2*). While such correlations suggest possible deleterious effects from exposure, uncertainty in factors such as age, health, genetic variation, reproductive status, and other life history variables of wild polar bears leaves the direct cause and effect relationship between contaminant body-burdens and observed toxicological impairments for polar bears unclear (*3*). It is impractical and ethically problematic to conduct experiments on captive polar bears, and thus studies on a model surrogate species are needed to elucidate such relationships.

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Recently, the sled dog (*Canis familiaris*) has been investigated for use as a surrogate species to study the bioaccumulation, fate and effects of OHCs and other environmental contaminants in wild polar bears, as well as other members of the *canoidea* superfamily, including arctic fox (*Alopex lagopus*), arctic wolf (*Canis lupus arctos*), and wolverine (*Gulo gulo*). The captive West Greenland sled dogs that are the subject of part of the present study were fed wild minke whale (*Balaenoptera acutorostrata*) blubber, which was naturally contaminated with OHCs, for nearly two years to simulate natural exposure conditions. Results have shown that dogs from the exposed cohort exhibited similar biomarker endpoint changes as those reported for wild polar bears for a number of health related parameters, such as vitamin status (*4*), sex hormone homeostasis (*5*), and cellular immune response (*6*). A comprehensive review of the comparative health and toxicological findings in wild polar bear and surrogate model species can be found elsewhere (*3*).

Species-specific metabolic differences, due to dissimilarity in cytochrome P-450 (CYP) isozyme substrate specificity, catalytic activity, or expression levels between species, may lead to a differential accumulation or fate of contaminants, or result in varying degrees of formation of toxic metabolites. Such species-specific differences in biotransformation capacity and bioaccumulation of OHCs may thusly affect the toxicological response, and make cross-species extrapolations of toxicity difficult. Investigations into the comparative fate of OHCs between sled dogs and polar bears found both similarities and differences between species (*7*). Although similarities existed in OHC concentrations between the two species, differences in the inferred biotransformation capability and bioaccumulation of contaminant classes and individual compounds led to variation in contaminant and metabolite patterns between polar bears and sled dogs (*7*).

Enantioselective analysis provides a means of quantifying and observing biological processes that are often difficult to quantify using other techniques (*8–10*), and provides an additional method of comparing the bioaccumulation dynamics and biotransformation capabilities and pathways among species. Chiral compounds exist as pairs of non-superimposable mirror images (enantiomers). Environmentally relevant chiral OHCs include 19 atropisomeric PCB congeners (*11*), many components of technical chlordane and their metabolites, and α-hexachlorocyclohexane (α-HCH). These compounds were released into the environment as racemic mixtures (1:1 mixtures of enantiomers), and because enantiomers possess identical physical and chemical properties, only biological processes (e.g., metabolism, protein binding, active uptake/elimination) will alter the relative proportion of enantiomers in the environment. Within biota, differences in the toxicokinetic behavior between enantiomers will lead to a greater accumulation of one enantiomer over the other (*12*). In addition, biological effects may differ between enantiomers. For instance, (*R*)-(-)-*o,p′*-DDT enantiomer is a weak estrogen mimic, while (*S*)-(+)-*o,p′*-DDT has negligible estrogenic effects (*13*). Likewise, individual PCB enantiomers displayed differing potencies towards hepatic enzyme induction (*14*, *15*) and in the enhancement of cellular Ca^{2+} release (*16*). Thus, enantioselective analysis not only provides an

additional means of investigating the bioaccumulation dynamics of organisms, but is essential for the proper assessment of risk posed by chiral compounds.

The comparison of OHC enantiomer distributions among species is complicated. One of the most notable factors influencing inter-species differences is the enantiomeric substrate selectivity of the enzymes that catalyze metabolism. For instance, complete inversions of enantiomer enrichment have been found between closely related species in some laboratory experiments (*9*, *17*, *18*). Similarities were found in the enantioselective biotransformation of CBs 95 and 149 between arctic char and rainbow trout, although opposite enantiomer selectivity was reported for CB 136 between these two members of the *Salmonidae* family (*9*, *17*, *18*). Likewise, bearded seals (*Erignathus barbatus*) from the coast of Alaska were enriched in (-)-oxychlordane, while ringed seals (*Phoca hispida*) from the same location were enriched in the (+)-enantiomer (*19*). The interpretation of enantiomer distributions in a given predator species is also influenced by consumption of non-racemic quantities of chiral OHCs from prey items. Enantiomer distributions of OHCs were shown to vary in eggs and plasma of glaucous gull from three nearby breeding colonies in the Norwegian Arctic, and were likely a result of differences in enantiomer distributions of the selected OHCs in the preferential food sources of each colony (*20*). However, the degree to which the uptake of food-derived enantiomer distributions affects the measured distribution versus biotransformation within the predator has not been investigated.

The objectives of this study were two-fold. The first was to examine the enantiomer distribution of a suite of chiral OHCs in captive West Greenland (Baffin Bay) sled dog adipose, liver, thyroid, adrenal, and brain tissues after 20-month exposure to naturally OHC-contaminated minke whale blubber (exposed cohort) or pork fat (control cohort), in order to understand bioaccumulation dynamics and fate of individual enantiomers within top-predator species in a controlled experiment. Secondly, the enantiomer distributions of chiral OHCs were determined in adipose tissues of free-ranging polar bears from Canadian Baffin Bay subpopulations, and compared to those in West Greenland sled dog adipose tissues. The goal was to understand species-specific biotransformation capabilities for chiral OHC contaminants better, and to examine the feasibility of using surrogate models for cross-species comparisons of enantiomer distributions.

Materials and Methods

Experimental Design

This research was part of a larger study investigating the overall health effects of persistent environmental contaminants on West Greenland sled dogs. Further details on the experimental design are found elsewhere (*6*, *7*, *21–24*). This animal experiment was conducted under a license granted by the Self-Government of Greenland.

Sixteen 2-month old female sled dogs from the community of Aasiaat, Disco Bay, West Greenland, were divided into control (CON; $n = 8$) and exposed (EXP; $n = 8$) groups. Groups were composed of paired sisters, one in each

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group, to minimize age and genetic variation between groups. EXP dogs were exposed (20 month exposure period) to a daily diet of blubber from an individual minke whale collected off the west coast (Baffin Bay) of Greenland as part of a controlled Greenlandic native subsistence hunt. This blubber was naturally contaminated with a suite of OHCs and other environmental contaminants, including mercury and polybrominated diphenyl ethers (*6*, *7*), simulating real-world exposure to multiple contaminants experienced by wild *canoidea* species. The CON group received relatively non-contaminated pork fat, classified for human consumption, for the same feeding duration. Both cohorts were also fed an equivalent amount of standardized Royal Canin Energy 4300/4800 pellets (https://www.royalcanin.com) to fortify the diet with essential vitamins and nutrients not found in either food source. Daily intake of whale blubber or pork fat was 50-200 g/day, leading to an exposure of 10.4-11.7 μg/kg bodyweight of total OHCs in EXP dogs (*25*).

CON and EXP dogs were subjected to a variety of health-related toxicological tests throughout the experiment, including blood sampling and immune system challenges (*6*, *25*). However, such procedures are not expected to alter the relative biotransformation capacity of the two dogs in either treatment groups. Upon termination of the experiment, dogs (mean age 1.5 ± 0.1 years, range 1.5-2 years) were euthanized. Liver, thyroid, brain, adrenal, and subcutaneous adipose tissues were collected and stored at -20 °C until analysis.

Subcutaneous adipose tissue samples were collected from adult and juvenile polar bears (mean age 6.1 ± 3.0 years) from two subpopulations inhabiting Eastern Baffin Island, Canada: Davis Strait (southern Baffin Island; n =11) and Baffin Bay (northern Baffin Island; n =14). All samples were collected between October 2007 and May 2008 as part of Inuit subsistence hunts. Further details on dates and locations of sample collection, as well as sample handling procedures can be found elsewhere (*26*).

Extraction

Procedures for the extraction and cleanup of sled dog and polar bear tissue samples for PCBs, HCHs, and chlordane-related compounds have been described in detail previously (*7*, *27*, *28*). Briefly, samples were homogenized with Na_2SO_4 and extracted with either 1:1 acetone/*n*-hexanes (brain tissue) or 1:1 dichloromethane:*n*-hexanes (all other tissues). Lipids were removed with the addition of H_2SO_4 and analytes were fractioned into several chemical classes on a Florisil column. Polar bear adipose tissue was homogenized with sodium sulfate and extracted by pressurized liquid extraction, followed by gel permeation and silica gel chromatography prior to analysis (*26*).

Chemical Analysis

Non-enantioselective separation and quantification was performed on an Agilent 6890 (Agilent Technologies, Palo Alto, CA, USA) gas chromatograph-mass spectrometer (GC-MS) with electron impact (EI) ionization, as previously described (*7*, *29*, *30*). Total chlordane (ΣCHL) concentrations

are reported as the sum of six chlordane compounds: Heptachlor epoxide (HEPX), oxychlordane, *trans*-chlordane, *cis*-chlordane, *trans*-nonachlor, and *cis*-nonachlor. Total PCB (ΣPCB) concentrations in sled dogs and minke whale blubber are reported as the sum of 40 congeners (*7*), while ΣPCB concentrations in Baffin Island polar bears represent the sum of 74 congeners (*26*). A complete list of monitored congeners in both species has been published previously (*7*, *26*).

Enantioselective analysis of OHCs was carried out on a Thermo Trace GC Ultra gas chromatograph coupled to a Thermo DSQII mass spectrometer (ThermoFisher Scientific, Waltham, MA, USA). PCBs were detected with electron impact ionization and selected ion-monitoring (*31*). The separation of PCB 95 and 149 enantiomers was achieved on a Chirasil-Dex column (25 m × 0.25 mm i.d. × 0.25 μm d_f, Varian, Walnut Creek, CA, USA) (*32*). A BGB-172 column (30 m × 0.25 mm i.d. × 0.18 μm d_f, Analytik, Adiswil, Switzerland) was used for the separation of PCB 183 enantiomers (*33*). All columns were calibrated with standard solutions containing all 209 PCB congeners to avoid coelutions with other homologous PCB congeners (*32*). OHC pesticides were detected in electron capture negative chemical ionization mode using previously described methods (*34*). Methane was used as the reagent gas at a flow rate of 2.5 mL/min. A BGB-172 was used for the separation of oxychlordane and HEPX. α-HCH, and *cis*-chlordane were separated on a Betadex-120 column (30 m × 0.25 mm i.d. × 0.25 μm d_f, Supelco, Oakville, ON, Canada).

Data Analysis

Model-fitting software (PeakFit v.4.0, Systat, San Jose, CA, USA) was used for deconvolution and integration of partially co-eluting chromatographic peaks (*35–37*). Enantiomer fractions (EFs) were used to quantify enantiomer distributions (*38*). For compounds with unknown enantiomer elution order (CB 95 on Chirasil-Dex and CB 183 on BGB-172) (*31*), the EF is defined as E1/(E1+E2), where E1 and E2 are the peak areas of the first-eluted enantiomer and second-eluted enantiomer, respectively. For all other analytes the EF was determined as the peak area of the (+)-enantiomer divided by the sum of the peak areas of the (+) and (-) enantiomers (*34*, *39*).

Enantiomer fractions and concentration data are presented as mean ± 1 standard deviation unless otherwise noted. Mean measured EFs of all racemic standards ranged from 0.493 to 0.499, depending on the analyte. Non-racemic EFs were determined by statistical comparison to racemic standards. Comparisons between and amongst groups were done using a Student's *t* test or one-way analysis of variance (ANOVA) with Tukey Honestly Significant Difference post-hoc test, respectively, with $\alpha = 0.05$.

Results and Discussion

Organochlorine Contaminant Concentrations

Elevated concentrations of chlorinated OHCs were detected in naturally contaminated minke whale blubber used for the exposed cohort diet, which

had ΣPCB concentrations and ΣCHL concentrations (mean ± standard error) of 1150±60 and 196±5 ng/g wet weight, respectively (*24*). None of the analytes were detected in the control cohort diet or dietary supplements (limit of quantification range: 0.01-1.5 ng/g wet wt., depending on the analyte). Exposure to contaminated minke whale blubber led to increased concentrations of all investigated OHCs in EXP cohort dog tissues. A detailed description of the tissue concentrations and accumulation patterns of OHCs and metabolites in sled dogs arising from exposure to contaminated minke whale blubber was recently published (*7*), and will not be reiterated here. It is important to point out, however, that after two years of exposure, adipose tissue concentrations (mean ± standard error) of ΣPCB and ΣCHL in EXP sled dogs were 2710 ± 500 ng/g wet wt. and 1480 ± 140 ng/g wet wt., respectively (*7*). These concentrations were 56-fold higher in ΣPCB and 43-fold higher in ΣCHL than CON dogs (*7*). Adipose tissue concentrations (mean ± standard error) of ΣPCB and ΣCHL in CON dogs were 48 ± 12 ng/g wet wt. and 34 ± 8 ng/g wet wt., respectively.

No differences in ΣPCB and ΣCHL concentrations were found between the Davis Strait and Baffin Bay polar bear subpopulations of Baffin Island (mean concentrations of 3080 ± 2770 ng/g wet wt. and 1150 ± 690 ng/g wet wt., respectively (*26*). In polar bears and sled dogs, ΣPCB contributed the most to the overall chlorinated OHC body burden. Additionally, oxychlordane was the predominant chlordane compound found in both species. A more detailed description of the congener and compound distributions in sled dogs and Baffin Island polar bears can be found elsewhere (*7*, *26*).

Distributions of OHC Enantiomers in Minke Whale Blubber

Enantiomer distributions of α-HCH, *cis*-chlordane, HEPX, and CB 91 were non-racemic in minke whale blubber (Figures 1 and 2). α-HCH was enriched in the (+)-enantiomer, while both HEPX and *cis*-chlordane were enriched in the (-)-enantiomer (Figure 1). A slight, although not significant, enrichment of (+)-oxychlordane (EF = 0.565 ± 0.022; $p > 0.05$) was also observed (Figure 1). Amongst PCB congeners, only the second-eluting enantiomer of CB 91 was enriched, while CBs 95, 149, 174, and 183 were racemic (Figure 2). This appears to be the first report of enantiomer distributions of chiral OHCs in minke whale blubber. Similar EFs for chlordanes, α-HCH and CB 149, both in the magnitude and the direction of enrichment were reported in bowhead whale from Barrow, AK (*19*). HEPX, on the other hand, was enriched in the (+)-enantiomer in bowhead whales (EF = 0.64) (*19*), in contrast to the enrichment of the antipode in this study.

Accumulation and Disposition of Enantiomers in Sled Dogs

Non-racemic distributions of all analytes except CB 174 were found in the tissues of EXP sled dogs (Figures 1 and 2). CB 95 was below the limits of detection, and therefore EFs could not be quantified. To increase the understanding of the sled dog enantioselective biotransformation and bioaccumulation capacity, biomagnification factors (BMF: concentration of each enantiomer in predator

divided by the concentration in the prey) from minke whale blubber to EXP sled dog adipose tissue were calculated on an enantiomer-specific basis. An inverse relationship exists between the BMF and the elimination rate constant of a compound, based on the equation BMF = $\alpha F/k_{el}$, where α is the assimilation efficiency (%), F is the feeding rate (g_{food} $g_{body\ weight}^{-1}$ day^{-1}), and k_{el} is the elimination rate constant (day^{-1}) (*40*). Feeding rates should be identical for enantiomers, and assimilation efficiencies should also be identical due to the identical physical properties of enantiomers and the passive absorption and uptake of hydrophobic compounds (*41*). The latter assumption is supported by the observation that enantiomer-specific assimilation efficiencies were similar for α-HCH, *trans*-chlordane, CB 95, and CB 136 enantiomers in rainbow trout (*9*). Therefore, the ratio of the enantiomer-specific BMFs provides an approximation of the biotransformation rate constant of the more highly accumulated enantiomer relative to its antipode.

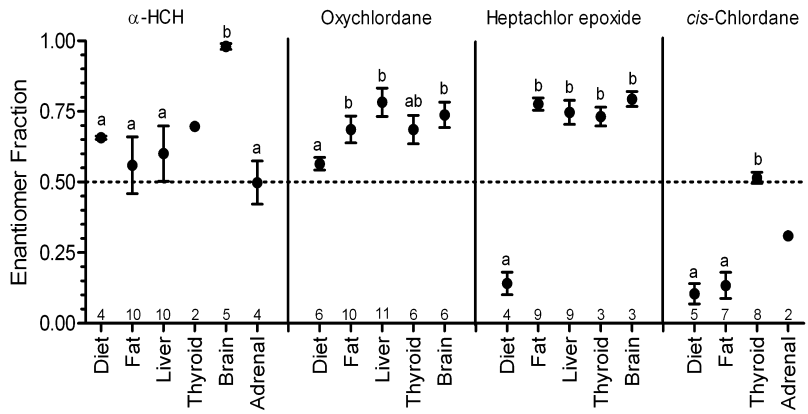

Figure 1. Enantiomer fractions (EFs) of chlorinated organohalogen compounds in exposed cohort sled dog tissues and diet (minke whale blubber). Points indicate mean value, while error bars indicate standard deviation. EF distributions sharing a letter designation are not statistically different. Dotted line represents theoretical racemic EF (EF = 0.5). Numbers above x-axis represent number of samples.

With the exception of (+)-α-HCH, both CB 149 enantiomers, and the first-eluting enantiomer of CB 183, all enantiomers had BMFs greater than 1 in EXP sled dogs (Table I), indicating a propensity for dogs to accumulate these enantiomers from the diet. Conversely, those enantiomers for which the BMF was less than 1 were not being accumulated, and thus were being biotransformed and/or eliminated more rapidly than they were being absorbed from the diet. Moreover, clear differences in enantiomer-specific bioaccumulation and/or biotransformation were evident. The BMF of (+)-oxychlordane was 1.8-fold

higher than that of (-)-oxychlordane, while BMFs of (-)-α-HCH and (-)-CB 149 were 3.3-fold and 2.5-fold greater than their antipodes, respectively. Clear accumulation of (+)-HEPX was also apparent, as the BMFs of (+)- and (-)-HEPX were 19.5 ± 5.9 and 1.1 ± 0.3, respectively. Although minke whale blubber was enriched in (-)-HEPX, the greater accumulation of (+)-HEPX by sled dogs resulted in an inversion in the enantiomer enrichment of HEPX between minke whale blubber and sled dog tissues (Figure 1). Similarly, CB 183 was racemic in minke whale blubber, but was highly enriched in the second-eluting enantiomer in sled dog adipose tissue (EF = 0.139 ± 0.067, Figure 2). The differences in biotransformation between individual OHC enantiomers led to a significant enrichment of the (+)-enantiomer of oxychlordane and HEPX, the (-)-enantiomer of α-HCH and CB 149, and the second eluting enantiomer of CBs 91 and 183 relative to the food. No change in the enantiomer distribution of *cis*-chlordane or CB 174 was found between the food and adipose tissue, resulting in similar BMFs between enantiomers for these two compounds. These changes in OHC EFs between minke whale blubber and sled dog adipose tissues provides strong evidence that sled dogs enantioselectively biotransformed chiral OHCs (Figures 1 and 2). The mechanism responsible for enantiomer enrichment in biota is unknown at the present time, although it has been suggested that CYP-mediated metabolism may be responsible based on the enantioselective metabolism of PCB congeners by isolated CYP isozymes *in vitro* (*42*, *43*). Time-dependent enrichments in the enantiomer distribution of OHCs occur in fish, invertebrates, and mice, further suggesting that metabolic processes may be responsible (*8*, *9*, *12*).

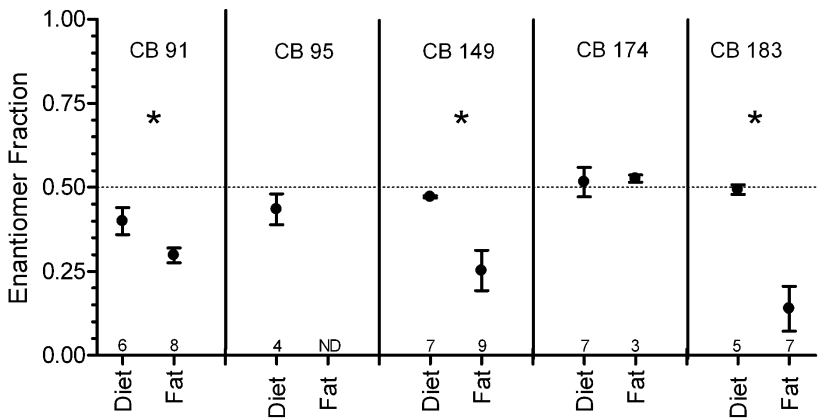

Figure 2. Enantiomer fractions (EFs) of polychlorinated biphenyls in diet (minke whale blubber) and sled dog adipose tissue. Points indicate mean value, while error bars indicate standard deviation. Asterisk indicates significant differences between diet and fat. Dotted line represents theoretical racemic EF (EF = 0.5). Numbers above x-axis represent number of samples. ND = non-detectable.

Table I. Biomagnification Factors (BMFs) and their ratios of individual organohalogen compound enantiomers from minke whales to West Greenland sled dog exposed (EXP) cohort and from ringed seal to polar bears from Resolute Bay, Canada

	Biomagnification Factors[a]			
	EXP Sled Dog		*Resolute Bay (Canada) Polar Bears*[b]	
	BMF	*Ratio*	*BMF*	*Ratio*
(+)-α-HCH	0.60 ± 0.2	3.3	1.4	2.0
(-)-α-HCH	2.0 ± 0.7		0.71	
(+)-HEPX	19.5 ± 5.9	17.7	7.4	3.2
(-)-HEPX	1.1 ± 0.3		2.3	
(+)-Oxychlordane	6.6 ± 4.1	1.8	7.1	1.2
(-)-Oxychlordane	3.6 ± 1.7		5.8	
(+)-*cis*-Chlordane	30.2 ± 13.5	1.2	0.0043	1.1
(-)-*cis*-Chlordane	24.3 ± 9.2		0.0047	
E1-CB 91	na		na	
E2-CB 91	na		na	
(+)-CB 149	0.010 ± 0.005	2.5	na	
(-)-CB 149	0.025 ± 0.008		na	
E1-CB 174	na		na	
E2-CB 174	na		na	
E1-CB 183	0.28 ± 0.15	6.7	na	
E2-CB 183	1.9 ± 0.10		na	

[a] Biomagnification factors (BMF) = concentration of each enantiomer in predator divided by the concentration in the prey. [b] Data from (*54*) E1 = first eluting enantiomer E2 = second eluting enantiomer na = data not available.

The second-eluting enantiomer of CB 183 was eliminated nearly 7 times slower than the first-eluting enantiomer, leading to a significant enrichment of the second eluting enantiomer of CB 183 in EXP sled dog adipose tissue. CB 183 is a metabolic precursor to 4-hydroxylated CB 187 (4-OH-CB187), a major OH-PCB congener in the blood of wildlife species, including polar bears (*27*, *44–46*). Concentrations of 4-OH-CB 187 in the present EXP sled dog plasma were reported to be nearly three-fold higher than the second most abundant OH-PCB congener, and represented approximately 25% of the total burden of OH-PCBs in sled dogs (*7*). Similarly, high concentrations of 4-OH-CB 187 have also been found in polar bears and other wildlife species (*27*, *44–46*). The large ratio of CB 183 enantiomer-specific BMFs in EXP sled dog adipose tissue and the

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enrichment of the second eluting CB 183 enantiomer compared to minke whale blubber suggest substantial biotransformation occurred, which may partially explain the high concentrations of 4-OH-CB 187 in EXP sled dog blood plasma.

Tissue-specific differences in the accumulation of individual enantiomers were also evident. The direction of enantiomer enrichment of chlordane compounds and α-HCH was similar amongst all tissues of the EXP cohort dogs, although some differences existed in the magnitude of enrichment (Figure 1). Extremely non-racemic EFs (EF = 0.960 ± 0.011) of α-HCH were found in the brain tissue, consistent with the highly enriched EFs of α-HCH found in brain tissues of other species, including seals (*47*), rats (*36*), mice and quail (*48*). Enantiomer-specific differences in α-HCH bioaccumulation have been attributed to selective uptake of the (+)-enantiomer across the blood-brain barrier (*36*). An enrichment of (+)-α-HCH was found in all tissues relative to the adrenal gland, which was racemic. Similarly, (+)-oxychlordane in the liver was enriched relative to the fat and thyroid, while the (-)-enantiomer of *cis*-chlordane in fat was enriched relative to the other tissues.

Differences in the accumulation of individual OHC enantiomers among tissues has been reported previously, both in wildlife and laboratory animals. Enantiomer fractions of *trans*-chlordane were different in rats between abdominal fat and the liver (*49*), while differences in the tissue distribution of oxychlordane enantiomers occurred in rats (*49*) and bowhead whales (*19*). In bowhead whales, the liver contained racemic proportions of *cis*-chlordane, while adipose tissue was significantly enriched in the (+)-enantiomer (*19*). Variation in the enantiomer distribution of chiral OHCs between liver and adipose tissue was attributed to metabolism or selective protein binding within the liver (*19*), although the exact mechanism has yet to be elucidated. However, this is the first time that enantiomer distributions of chiral OHCs have been determined in adrenal or thyroid tissue, and therefore the toxicological significance of such findings is unknown. Although no histological changes in either adrenal or thyroid tissue were found in exposed sled dogs (*3*), rats dosed orally with technical chlordane or *trans*-nonachlor (which is metabolized to *trans*-chlordane) developed hypothyroidism and pathological alterations of the thyroid gland (*50*, *51*). Therefore, evaluation of the enantiomer-specific toxicological effects of chiral OHCs and further investigation of the distribution of OHC enantiomers in sensitive tissues are warranted.

Enantiomer distributions of chiral OHCs were similar between CON and EXP cohort dogs for most analytes. This may be expected, due to the age and genetic similarity between CON and EXP groups. However, adipose tissue EFs of oxychlordane in CON dogs were significantly more enriched in the (+)-enantiomer than EXP dogs (Table II). This difference likely resulted from the uptake of more racemic enantiomer distributions of oxychlordane from minke whale blubber by EXP cohort dogs. Oxychlordane was not detectable in pork fat, and the EF of oxychlordane in minke whale blubber (Figure 1) deviated less from racemic than the distributions measured in the tissues of either EXP or CON dogs (Figure 1 and Table II). Enantiomer distributions in sled dogs and wildlife are likely at a steady-state balance between uptake of non-racemic proportions from the food and enantioselective biotransformation (*52*). Changes in the

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distribution of OHC enantiomers in prey items or in the magnitude of exposure to non-racemic quantities will thus alter the enantiomer distribution within the consuming organism.

Table II. Enantiomer Fractions of chlorinated organohalogen contaminants in tissues of *canoidea* species

	CON Sled Dogs[a]	*EXP Sled Dogs*[a]	*Baffin Island Polar Bear*[a]	*Resolute Bay Polar Bear*[b]	*Wolverine*[c]	*Arctic Fox*[c]
α-HCH	0.700 ± 0.196	0.559 ± 0.100	0.639 ± 0.079	0.59	0.423 ± 0.020	0.414 ± 0.036
HEPX	nq	0.776 ± 0.022	0.725 ± 0.089	0.69	0.554 ± 0.019	0.732 ± 0.014
Oxychlor-dane	0.778 ± 0.040	0.686 ± 0.047	0.560 ± 0.067	0.62	0.712 ± 0.020	0.676 ± 0.019
cis-Chlordane	0.131 (n=1)	0.134 ± 0.046	0.299 ± 0.159	0.78	nq	0.607 ± 0.035
CB 91	0.321 (n=1)	0.300 ± 0.022	0.666 ± 0.150	nq	0.497 ± 0.022	0.546 ± 0.060
CB 149	0.315 (n=1)	0.258 ± 0.060	0.403 ± 0.104	nq	0.461 ± 0.030	0.535 ± 0.007
CB 174	0.540 (n=1)	0.526 ± 0.010	nq	nq	nq	nq
CB 183	0.072 (n=1)	0.139 ± 0.067	0.122 ± 0.028	nq	nq	nq

nq = not quantified. [a] Adipose tissue. [b] Adipose tissue data from (*54*). [c] Liver data (mean ± SD) from (*55*).

Comparative Enantiospecific Bioaccumulation

Except for oxychlordane, no differences were found in enantiomer distributions between Baffin Island polar bear sub-populations (data not shown). For oxychlordane, the difference in EF between the Davis Strait subpopulation (EF = 0.512 ± 0.049) and the Baffin Bay subpopulation (EF = 0.597 ± 0.063) may be attributable to differences in prey consumption. Davis Strait and Baffin Bay polar bear subpopulations were shown to feed at different trophic levels and employ different foraging strategies, based on stable isotope and dietary fatty acid analysis (*53*). For the purposes of comparing to sled dogs and other *canoidea* species, both Baffin Island subpopulations were combined into a single data set.

Enantiomer distributions of chiral OHCs have previously been determined in polar bears from Resolute Bay, Canada (*54*), as well in arctic fox and wolverines from the Canadian Arctic (*55*). The overall enantiomer profiles of chiral OHCs in the current study were comparable among Baffin Island polar bears, captive EXP

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sled dogs, and other arctic *canoidea* species (Table II). Similarities in chiral OHC enantiomer distributions amongst *canoidea* species have been noted previously by Hoekstra et al. (*55*) for arctic fox, wolverine, and Resolute Bay polar bears. The results from our study agree well with these previous observations based on visual inspection of the enantiomer distributions. Amongst the OHCs determined in all 4 species, the (+)-enantiomer of both oxychlordane and HEPX was enriched (Table II) in EXP sled dogs, arctic fox (*55*), wolverines (*55*), and polar bears (*54*). Despite similarities in the direction of enrichment, the magnitude of enrichment tended to vary amongst species. For example, the magnitude of enantiomer enrichment of oxychlordane ranged from an EF of 0.560 in Baffin Island polar bears to 0.712 in wolverines, while EFs of HEPX ranged from 0.554 in wolverines to 0.776 in EXP sled dogs (Table II).

The above comparisons, however, are based solely on measured EFs, with no knowledge of the enantiomer distributions within the underlying food web. To account for the dietary uptake of non-racemic distributions of OHCs, the comparative bioaccumulation dynamics and relative rate constants of individual enantiomers were investigated between sled dogs and polar bears from Resolute Bay (*54*) (which is to our knowledge the only data set in which prey species EFs were determined) using enantiomer-specific BMFs (Table II). The average BMF of (+)-oxychlordane between ringed seals and polar bears was 1.2-fold greater than the BMF of (-)-oxychlordane, similar to the 1.8-fold greater BMF of (+)-oxychlordane than (-)-oxychlordane between minke whale blubber and EXP sled dogs (Table I). BMF ratios of greater than unity in both species indicate a preferential elimination of (-)-oxychlordane, although at a rate that is only marginally slower than (+)-oxychlordane. In a similar vein, the BMF of (+)-HEPX in EXP sled dogs was 18 times greater than (-)-HEPX, while in polar bears this ratio was only 3.2 (Table I). In both species, the greater accumulation of (+)-HEPX led to a reversal in the EF between predator and prey, and ultimately to EFs of similar magnitude. Both HEPX and oxychlordane are persistent metabolites, and non-racemic distributions can arise due to the enantioselective metabolism of the parent compounds, or due to the enantioselective biotransformation/elimination of HEPX or oxychlordane itself. The predominant pathway leading to the non-racemic EFs is unclear, but given the comparability in BMF ratios of oxychlordane enantiomers between both species, it is apparent that similarities exist in the enantioselective bioaccumulation dynamics of oxychlordane between polar bears and sled dogs. Likewise, the similarity in enrichment of (+)-HEPX suggests similar mechanisms of enrichment (e.g., enzyme mediated degradation or elimination of HEPX), but differences in rate determining factors, such as catalytic enzyme activity.

Enantiomer distributions of several analytes varied among species. The enrichment of (-)-*cis*-chlordane found in sled dogs and Baffin Island polar bears contrasts the enrichment of the (+)-*cis*-chlordane observed previously in arctic fox (*55*) and Resolute Bay polar bears (*54*) (Table II). However, no alteration in *cis*-chlordane EF was observed between minke whale blubber and EXP sled dog adipose tissues, suggesting that sled dogs were not biotransforming *cis*-chlordane in an enantioselective manner. A similar lack of enantiomer enrichment of *cis*-chlordane was found between ringed seals and polar bears from Resolute

Bay, Canada (*54*). If the other *canoidea* species similarly lack the ability to biotransform *cis*-chlordane enantioselectively, it is possible that the variation amongst species is a result of differences in the enantiomer distribution of *cis*-chlordane within prey items. Thus the observed EF may be a reflection of the enantiomer distribution within prey items rather than *in vivo* metabolism, as observed elsewhere (e.g., for glaucous gulls (*20*)).

Enantiomer fractions of α-HCH in EXP sled dogs were similar in magnitude and direction to those in Baffin Island polar bears and to α-HCH EFs previously reported for polar bears from Resolute Bay, Canada (*54*), although the enrichment of (+)-α-HCH in polar bears and EXP sled dogs contrasts the enrichment of (-)-α-HCH in wolverines and arctic fox (*55*) (Table II). Arctic fox are phylogenetically closer to polar bears than to sled dogs, consistent with our EF observations. The enrichment of (+)-α-HCH in EXP sled dogs and polar bears from both Resolute Bay and Baffin Island suggests similarities in the enantioselective biotransformation capabilities between species. However, as already discussed, (+)-α-HCH was enriched in the tissues of EXP sled dogs due to the consumption of minke whale blubber enriched in the (+)-enantiomer, as the greater BMF of (-)-α-HCH than (+)-α-HCH indicates EXP sled dogs were preferentially eliminating (+)-α-HCH. In contrast, a greater BMF of (+)-α-HCH relative to (-)-α-HCH in Resolute Bay polar bears clearly demonstrates differences in the enantioselective biotransformation and bioaccumulation dynamics between these two species. In addition, the contrasting enantiomer-specific BMFs between the two species, despite similar EFs, further highlights the influences of dietary uptake on the interpretation of EFs in biota.

Enantiomer distributions of CBs 91 and 149 have been measured in arctic fox (*55*), wolverine (*55*), polar bears, and sled dogs, while EFs of CB 183 have only been determined in EXP sled dogs and Baffin Island polar bears. CB 149 was enriched in the (-)-enantiomer in sled dogs, polar bear, and wolverine, but was enriched in the (+)-enantiomer in arctic fox. Similarly, biotransformation of CB 91 in EXP sled dogs resulted in an enrichment of the second eluting enantiomer of CB 91, whereas the antipode was enriched in polar bears and arctic fox (Table II). Enantiomer distributions of PCBs have not been determined in both wild *canoidea* species and their prey, precluding the comparison of enantiomer-specific BMFs between EXP sled dogs and other *canoidea* species. However, racemic distributions of CB 149 and a considerable enrichment of the second eluting enantiomer of CB 91 (EF = 0.063) were found in ringed seals from the Northwater Polynya (NOW) (*56*), a nearby area located on the northern tip of Baffin Bay. Assuming similar enantiomer distributions in the ringed seals from Baffin Island, the enrichment of antipodes of CB 91 between the polar bears and sled dogs suggests differences in the enzyme systems involved in the biotransformation of CB 91. However, without knowledge of the true enantiomer distributions in ringed seals from Baffin Island, this conclusion remains speculative.

No differences were found in the enantiomer distribution of CB 183 between EXP sled dogs and Baffin Island polar bears (Table II). As discussed earlier, metabolism of CB 183 may lead to formation of 4-OH-CB 187, although 4-OH-CB 187 may also be formed metabolically from CB 187. A greater ratio of CB 187 + CB 183 to 4-OH-CB 187, a metric of inferred biotransformation

capacity, was found in East Greenland polar bears compared to EXP sled dogs (*7*). It was postulated that different species-specific biotransformation capacities might be involved in the metabolism of CB 183 between the two species (*7*). While the similarities in both direction and magnitude of the CB 183 enantiomer enrichment between the two species suggests similarities in enantioselective biotransformation of CB 183, other factors (e.g., 4-OH-CB 187 retention or greater metabolic specificity or activity towards CB 187) not investigated here may also play a role in the differences between species in 4-OH-CB 187 accumulation.

Conclusions, Perspectives, and Recommendations

Previous investigations into comparative non-enantioselective bioaccumulation dynamics between EXP sled dogs and polar bears from East Greenland found similarities in the bioaccumulation dynamics of compound classes (i.e. PCBs, chlordanes, etc.) between species, although the accumulation of individual compounds/congeners tended to vary (*7*). Differences were also found between species in OH-PCB metabolite retention and/or formation, with OH-PCB congener patterns in sled dogs being composed primarily of penta- and hexa-chlorinated congeners. Polar bears accumulated greater concentrations of OH-PCBs and the congener profile was dominated by hepta-and octa-chlorinated congeners (*7*). The species-specific congener/compound patterns and differences in metabolite formation were attributed to species-specific differences in biotransformation capacity (CYP enzyme content, activity and specificity), selective retention/excretion of congeners and compounds, or differences in dietary influence between species. Similarly, in this study, both similarities and differences in the enantioselective biotransformation and enantiomer accumulation were found between species. While the biochemical processes mediating the enantioselective biotransformation of OHCs are not well understood, similarities in the direction of enrichment between Resolute Bay polar bears and EXP cohort sled dogs suggests similarity of the substrate specificity responsible for the biotransformation of OHC contaminants between polar bears and sled dogs. However, differences in the relative rates of the biotransformation of individual enantiomers suggest dissimilarity in the rate determining processes (e.g., catalytic enzyme content and/or activity) between species. Due to the lack of data on the enantiomer distributions of chiral OHCs in many Baffin Island prey items of polar bears, arctic fox, and wolverines, it is unclear whether such a conclusion may be extrapolated to these species as well, although the similarities in direction of enrichment suggest that it may. However, differences in the enantiomer preferences in biotransformation of OHCs were also found, most notably in the reversal of enantiomer preference of α-HCH between sled dogs and polar bears. This observation indicates that sled dogs are not a completely accurate surrogate of polar bears, at least for α-HCH.

At present, the toxicological implications of enriched enantiomer distributions are unclear. *In vitro*, exposure of rat hepatocytes to (+)-α-HCH resulted in both higher cell death and greater induction of mitosis than (-)-α-HCH (*57*).

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The almost exclusive presence of (+)-α-HCH in sled dog brain tissues, and the enrichment of (+)-α-HCH in both polar bear and sled dog tissues, suggests the possibility of increased deleterious effects. Enantiomers of several chiral PCB congeners have been shown to induce drug metabolizing enzymes to different extents (*14*, *15*). *In vitro,* individual CB 84 enantiomers differed in potency for increasing translocation of protein kinase C from the cytosol to the cell membrane in rat cerebral granular cells (*58*), and the (-)-enantiomer of CB 136 increased the sensitivity of ryanodine receptors (*16*), which are broadly expressed Ca^{2+} release channels necessary in cellular signaling and muscle contractions. However, none of these PCB congeners were investigated in the current study, and aside from α-HCH, enantiomer-specific toxicological investigations of other analytes is lacking.

In conclusion, this study highlights several challenges in understanding and interpreting enantiomer distributions in biota. Changes in EF between CON and EXP dogs illustrate the role that dietary uptake of non-racemic proportions of chiral contaminants has on resulting EFs in biota, and/or exposure-induced differences in metabolic capacity. Moreover, the reversal in the relative enantiomer-specific biotransformation rate constants between polar bears and sled dogs, despite the similar observed EFs, demonstrates the danger in interpreting EFs in biota or making cross-species comparisons without knowledge of the enantiomer distribution in the underlying foodweb.

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Chapter 4

Enantioselective Separation and Analysis of Chiral Herbicides

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Herbicidal activity and non-target toxicity may be drastically different between enantiomers of a chiral herbicide. The enantiomers of chiral herbicides are usually separated and analyzed by chromatographic methods such as high-performance liquid chromatography (HPLC), gas chromatography (GC), supercritical fluid chromatography (SFC), and capillary electrophoresis (CE). Several chromatographic techniques are used for preparing pure-enantiomer herbicides. Some chiral herbicides have been commercialized with the pure active enantiomer, such as 1′S-metolachlor (aSS, aRS), quizalofop-p-ethyl, haloxyfop-p-methyl, fluazifop-p-butyl, (R)-napropamide, etc.

Introduction

Many of the commonly used herbicides have chiral centers, including amide, phenoxy, imidazolinones, and organophosphorus herbicides. Enantiomers of a chiral compound have identical physical-chemical properties and thus appear as a single compound. However, their biological effects such as toxicity, mutagenicity, carcinogencity, and endocrine disruption activity, are generally different, due to

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the inherent enantioselectivity of biological interactions. This chapter reviews the HPLC, GC, SFC and CE methods in the analysis and preparation of pure enantiomers of chiral herbicides.

Enantioselective Separation and Analysis by HPLC

High-performance liquid chromatography (HPLC) combined with chiral stationary phases (CSPs) is a common approach for enantiomer analysis and preparation. Many types of CSPs have been developed, including Pirkle-type, polysaccharides, cyclodextrins, macrocyclic antibiotics, proteins, crown ethers and ligand exchange, among others (*1*). Profiting from the availability of these CSPs, chiral HPLC methods have seen rapid development and increased applications both for determining optical purity of enantiomers and for preparing enantiopure standards.

Chiral Amide Herbicides

Metolachlor is one chiral amide herbicide, which contains an asymmetrically substituted carbon and a chiral axis, consists of four stereoisomers stable at ambient temperature with a*SS*-, a*RS*-, a*SR*-, and a*RR*-configurations. Two of the four metolachlor isomers were isolated from rac-metolachlor in enantio- (ee>98%) and diastereomerically pure forms by a combination of achiral Hypercarb PH and chiral Chiralcel OD-H HPLC with 98/2 n-hexane/isopropanol as the mobile phase. The enantiomer elution sequence was a*S* prior to a*R* (retention times, a*SS* < a*RS* and a*SR* < a*RR*) and 1'*S* prior to 1'*R* (retention times, a*SS* < a*SR* and a*RS* < a*RR*) (*2*). Baseline separation of four metolachlor stereoisomers was achieved on Chiralcel OD-H using 91/9 hexane/diethyl ether as the mobile phase (*3*). Enantiomers and diastereomers of alachlor, acetochlor, metolachlor, and dimethenamid were separated using achiral and chiral high-resolution GC/MS (HRGC/MS) and chiral HPLC. Chiral HPLC using modified cellulose and phenylglycine columns showed some isomer resolution. A novel thermal equilibration procedure allowed distinction among axial-chiral and C-chiral enantiomers (*4*). Additionally, acetochlor enantiomers were partially resolved with cellulose tris(3,5-dimethylphenylcarbamate) CSP (CDMPC) on HPLC with n-hexane or petroleum ether with different fractions of alcohol (*5*). Dimethenamid-P was completely resolved on a Chiralpak AD-H column (*6*).

Napropamide is another common amide herbicide. It was separated both by normal phase HPLC and by reverse phase HPLC by Liu et al. (*7*, *8*). A method for the chiral separation and micro-determination of napropamide in water was established on a Chiralpak OJ-H column (*10*). The calibration curve for a racemic mixture was linear over the range of 10-100 ng/mL and the correlation coefficient was 0.99 (*7*). The enantiomers of napropamide were further resolved using Chiralcel AD-RH and Chiralcel OD-RH with acetonitrile/H_2O as the mobile phase (*11*). The stereoselectivity of Chiralcel AD-RH was found to be better than Chiralcel OD-RH for napropamide (*8*). In Zhou et al. (*9*), napropamide was

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partially separated (R_s=1.05) using 40/60 acetonitrile/H_2O as the mobile on the amylose tris(3,5-dimethylphenylcarbamate) CSP (ADMPC).

Flamprop, a futher amide herbicide, was resolved on a terguride-based CSP (selectivity factor α 1.09) by using 45% 0.02 M potassium acetate buffer (pH 3.5) and 55% acetonitrile as the elution solvent (*10*).

Chiral Phenoxy Herbicides

The most representative phenoxy herbicides are diclofop, mecopop (MCPP), dichlorprop (DCPP) and their derivartives. They are commonly used to control broad-leaf weeds.

Several phenoxypropionic acid herbicides were separated on two cyclodextin (CD)-derivatived CSPs, Nucleodex α-PM and Nucleodex β-PM. Phenoxypropionic acids may be divided into three different groups. The first has one or two small substituents such as methyl, chlorine or hydroxyl on the aromatic ring (e.g. MCPP, DCPP). The separation of MCPP and DCPP was observed on Nucleodex α-PM, whereas the methyl esters of these compounds were resolved by both Nucleodex α-PM and Nucleodex β-PM. The second has additional small substituents. A further substitution (e.g., fenoprop R1, R2, R3=Cl, R4=H) resulted in the failure of separation on Nucleodex α-PM, but fenoprop was sufficiently resolved on Nucleodex β-PM. The third contains compounds like fenoxaprop or diclofop with large substituents on the aromatic ring. In this case, the methyl or ethyl esters were separated only on Nucleodex β-PM, but not on Nucleodex α-PM (*11*). Resolution of MCPP and DCPP and 2,4-D was achieved on Nucleodex-α-PM CD CSP with 70% methanol and 30% 50 mM NaH_2PO_4 as the mobile in Kohler et al. (*12*) and Bjerg et al. (*13*).

In Padiglioni et al. (*10*), MCPP, DCPP, diclofop, fenoxaprop, fenoprop, fluazifop, haloxyfop, quizalofop-ethyl ester and quizalofop were well resolved on a 150 × 4.6 mm terguride-based CSP using 0.02 M potassium acetate buffer (pH 3.5)-acetonitrile as the mobile phase. Furthermore, a semipreparative-scale separation of fenoprop enantiomers was achieved on a 250 × 7.8 mm (I.D.) column, yielding approximately 1.0 mg of each enantiomer in a single chromatographic run, with a recovery of 88% and optical purity greater than 99%. MCPP, DCPP, and bromacil with a pyrimidinedione ring were better resolved on the native teicoplanin CSPs than the aglycone teicoplanin CSPs with 100% methanol containing 0.1% triethylamine and 0.1% acetic acid (v/v) and 20/80 methanol/H_2O at pH 4.1 with 1% triethylammonium for bromacil (*14*). Furthermore, MCPPM and DCPPM were better resolved on the native teicoplanin CSPs with 20% methanol/80% aqueous buffer (pH 4.1 by triethylammonium, 1%). However, the resolution for bromacil with a pyrimidinedione ring was slightly better on the teicoplanin structurally related A-40926 CSP than on teicoplanin CSP (R_s 2.8 vs. R_s 2.5) (*15*). Rac-diclofop methyl and rac-diclofop acid were baseline separated on a Chiralcel OJ-H column with a mobile phase of hexane/isopropanol/acetic acid (90:10:0.2, v/v) at a flow rate of 0.5 mL/min at 20 °C (*16*). Zhou et al. (*17*) completely resolved these compounds on CDMPC with n-hexane and isopropanol (98:2) containing 0.1% TFA as the mobile phase.

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Enantioseparation of 2-(2,4-dichlorophenoxy)propionic acid (2,4-DP) and MCPP was obtained on a Chirobiotic T column with 5:95 methanol/1% triethylammonium as the mobile phase (*18*).

Fenoxaprop-ethyl was resolved at the baseline on amylose tris(3,5-dimethylphenylcarbamate) CSP (ADMPC) by reversed phase HPLC with methanol/H_2O or acetonitrile/H_2O at a flow rate of 0.5 mL/min, while the enantiomers of quizalofop-ethyl, fluroxypyr-meptyl and 2,4-D-ethylhexyl received partial separation (*9*). Dimethenamid-P, dichlorprop-P, fluazifop-P butyl, mecoprop-P and quizalofop-P ethyl were completely resolved on a normal phase Chiralpak AD-H column (*6*). The optical purity was found to be over 95% ee, while for quizalofop-P ethyl and fluazifop-P butyl, it was in the range 34.1-94.5% ee.

A group of chlorophenoxypropionic acid herbicides 2,2-CPPA, 2,3-CPPA and 2,4-CPPA were separated using capillary LC (*22*). The use of 0.1 mM teicoplanin in the mobile phase was sufficient for baseline enantioresolution of 2,2-CPPA and 2,4-CPPA (*19*).

A group of 2-aryloxypropionic acids (TR-1 to 13) and their esters (TR-19 to 20) were used to evaluate four new brush-type CSP I-IV. The best separation of these herbicides was obtained with CSP I, and the (–)-*S* enantiomers were regularly eluted first. The mechanism of chiral recognition implies a synergistic interaction of the carboxylic acid analyte with the chiral selector and achiral free γ-aminopropyl units on silica (*20*). In Badjah-Hadj-Ahmed et al. (*21*), eleven 2-aryloxypropionic acids and esters were partially separated on a phenylated β-CD CSP when using heptane and either isopropanol or chloroform as the organic mobile phase modifier.

Chiral Imidazolinone Herbicides

Imidazolinones inhibit branched-chain amino acid biosynthesis in plants by targeting acetolactate synthase (ALS).

Five imidazolinone herbicides including imazapyr, imazapic, imazethapyr, imazamox, and imazaquin and their methyl derivatives were separated using reversed phase HPLC with Chiralcel OD-R and normal phase HPLC with Chiralcel OJ (*22*). Enantiomers of imazethapyr, imazaquin, and imazamox were separated on a Chiralcel OD-R column using 50 mM phosphate buffer-acetonitrile as the mobile phase. Enantiomers of imazapyr, imazapic, imazethapyr, imazamox, imazaquin and their five methyl derivatives were resolved on a Chiralcel OJ column using hexane (0.1% trifluoroacetic acid)-alcohol as the mobile phase. The described normal phase method was successfully applied for chiral analysis of imazapyr and imazaquin in spiked soil samples. In a further study (*23*), temperature effects on enantioseparation of these five imidazolinone herbicides and conformation of CSP were evaluated with Chiralcel OJ. The van't Hoff plots of retention factor (k'), distribution constant (K) and separation factor (α) for imazapyr, imazapic, imazethapyr, and imazamox were linear within 15-50 °C. Nonlinear van't Hoff plots of α were observed for imazaquin with mobile phase of n-hexane (0.1% trifluoroacetic acid)-isopropanol at 70/30 or 60/40 (v/v).

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Chiralcel OJ column was found to yield satisfactory results at 15-50 °C but not at ≤ 15 °C.

Liu et al. (*24*) investigated the enantiomeric separation of imazethapyr, imazapyr, and imazaquin on Chiralpak AS, Chiralpak AD, Chiralcel OD, and Chiralcel OJ columns. Chiralcel OJ showed the best chiral resolving capacity among the test columns for the imidazolinones. The optimal chromatographic conditions for complete separation of imidazolinone enantiomers were a mobile phase of hexane/ethanol/acetic acid (77/23/0.1, v/v/v), flow rate of 0.8 mL/min, and a column temperature in the range of 10–30 °C. The researchers also showed that small amounts of enantiopure imidazolinone stereoisomers may be prepared with the developed method.

Enantiomers of imazethapyr were separated on Chiralcel OJ with hexane/ethanol/acetic acid (75/25/0.5 by volume) (*25*, *26*) as shown in Fig. 1, and their absolute configurations were confirmed as S-(+)-IM and R-(–)-IM by the octant rule.

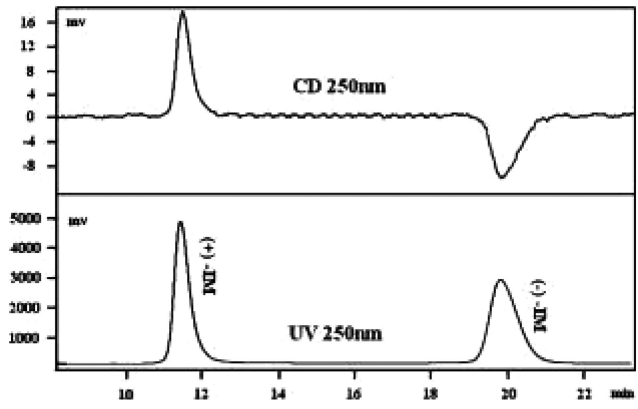

Figure 1. HPLC chromatogram for the enantiomeric separation of imazethapyr on Chiralcel OJ column (25).

Chiral Organophosphorus Herbicides

Five chiral O-aryl O-alkyl N-alkylphosphoramidothioates herbicides were nearly baseline separated on a Pirkle-type column OA-4700 (Chirex(*S*)-LEU and (*R*)-NEA) by HPLC. The chromatographic elution order was *S*>*R* (*27*).

In a report by Li et al. (*28*), enantioselective separation and biological toxicity of a series of 1-(substituted phenoxyacetoxy)alkylphosphonates organophosphorou herbicides were evaluated using Chiralpak AD, Chiralpak AS, Chiralcel OD, and Chiralcel OJ. All the analytes showed baseline resolution (R_s>1.5) on Chiralpak AD which had the best chiral separation capacity.

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Chiral Diphenyl Ether Herbicides

Ethoxyfen-ethyl and lactofen were separated using polysaccharide CSPs by Zhou et al. (*9*, *29–31*). Enantioseparation of ethoxyfen-ethyl was achieved on a self-prepared CDMPC, and with the content of isopropanol in hexane decreased to 1% in the mobile phase, the resolution factor increased to 3.95 (*29*). The two enantiomers of lactofen in soils were baseline separated and semiprepared on CDMPC (hexane/isopropanol 95/5). However, baseline separation was not obtained on a self-prepared ADMPC CSP (*31*). Lactofen was also completely resolved (R_s 2.07) using 80/20 methanol/H_2O as the mobile phase on ADMPC (*9*).

Other Chiral Herbicides

A group of herbicides from different chemical classes, including diclofop-methyl, quizalofop-ethyl, lactofen, fluroxypyr-meptyl, acetochlor, ethofumesate, clethodim, napropamide, fenoxaprop-ethyl and carfentrazone-ethyl, were partially or near-baseline separated on self-prepared amylose tris-(S)-1-phenylethylcarbamate CSP by HPLC with n-hexane/isopropanol as the mobile phase (*31*). Chiral pyrazole phenyl ethers (PPE) are highly active herbicides and their enantiomers were resolved by HPLC using Whelk-O 1. Chromatographic resolution obtained was suitable for determination of enantiomeric purities and, in some cases, for preparative resolution of the enantiomers with enantiomeric excess (ee) > 99% (*32*). (+)- and (–)-enantiomers of thiobencarb sulfoxide were collected with purities greater than 99.0% ee and 99.8% ee on a Chiralcel OB column at 25 °C and with 95/2.5/2.5 hexane/ethanol/methanol as the mobile phase (*33*).

Enantioselective Separation and Analysis by GC

Gas chromatography (GC) is more suitable for trace analysis because of its higher sensitivity, higher precision and smaller injection volume than HPLC. In addition, contaminants and impurities can be separated from the analytes relatively easily.

The most common chiral selectors used for GC are a group of CD and CD-derivatives. Enantiomers and diastereomers of some acetamide pesticides, alachlor, acetochlor, metolachlor, and dimethenamid, were separated using achiral and chiral HRGC/MS. Whereas alachlor is achiral, all other compounds are axial- and/or C-chiral and consist of two or four stereoisomers (enantiomers and diastereomers). Chiral HRGC using a β-CD derivative coated column showed different resolutions of diastereomers and/or enantiomers, while achiral HRGC showed no resolution of diastereomers of metolachlor (and dimethenamid). The resolution of C-chiral enantiomers was easier than the resolution of axial-chiral enantiomers (atropisomers) (*4*). All four metolachlor isomers were identified by HRGC (*2*).

Leachate samples from a waste disposal site in Switzerland and groundwater samples downstream of the landfill were analyzed for residues of MCPP, DCPP, and 2,4-D esterified with 2,3,4,5,6-pentafluorobenzyl (PFB) by means

of enantiomer-specific GC-MS (*12*, *34*). The PFB esters of MCPP and DCPP were nearly baseline separated (R_s=0.9) on a 15 m glass column (0.25 mm i.d.) with an OV-1701 polysiloxane phase containing 35% heptakis(2,3-dimethyl-6-tert-butyldimethylsilyl)-β-CD (TBDM-β-CD) as the chiral selector.

A capillary column BGB-172 (20% tert-butyldimethylsilyl-β-CD dissolved in 15% diphenyl-polysiloxane and 85% dimethylpolysiloxane, GBG Analytik, Adliswil, Switzerland) was used for chiral GC separation of some herbicides by Liu et al. (*35–37*). DCPP was extracted from water, methylated by diazomethane, then separated and determined with a recovery about 90% (*35*). The authors also separated rac-metolachlor and S-metolachlor in soil samples. However, baseline separation was not achieved because of the presence of two chiral elements (asymmetrically substituted carbon and chiral axis nitrogen) (*36*). Furthermore, baseline enantioseparation of DCPPM was achieved both by GC on BGB-172 and by HPLC on Chiralcel OJ-H (*37*).

Enantioselective Separation and Analysis by SFC

As a complementary analytical technique to HPLC, packed-column SFC with sub- and/or supercritical fluid containing organic polar solvents can be used for both analysis and small-scale preparation of optically pure chemicals and enantiomer identification, especially as CSPs are becoming easily available and widely applied. Nearly all conventional HPLC CSPs may be used in the SFC mode except the chiral crown ester CSPs and the protein-based CSPs. Sub- and supercritical carbon dioxide (CO_2) remains as the most commonly used fluid for SFC. Mechanistically, SFC plays a unique role acting as a bridge between GC and LC. Owing to the good diffusibility and low viscosity of supercritical fluids, column equilibration is accomplished more rapidly and enables faster flow rates in SFC than in HPLC. Besides, the higher diffusivity between mobile phase and CSPs yields greater efficiency (smaller plate heights) in resolving analytes.

Generally, SFC shows notable advantages and superior developmental potential for enantiomer separation. The advantages include being environmental friendly with low organic solvent consumption of mobile phase, simple method development, high efficiency on enantioseparation, low column pressure drop, and ease in coupling with chiral columns or MS. However, the high investment of SFC apparatus restricts its widespread application in enantioseparation. To date, the research about chiral herbicides separation by SFC is very limited. In one example is the resolution of the diasteriomeric metolachlor by SFC. It was possible to quickly detect and identify metolachlor and its isomeric ratios in low-concentration samples using the SFC method (*38*).

Enantioselective Separation and Analysis by CE

Capillary electrophoresis (CE) is a simple, efficient, and inexpensive way with unique versatility for chiral separation. CE can be flexibly applied to a wide variety of analytes in different modes. Many separation modes of CE have been

successfully used in chiral separation, including capillary electrochromatography (CEC), micellar electrokinetic capillary chromatography (MECC or MEKC), capillary zone electrophoresis (CZE), capillary gel electrophoresis (CGE), capillary isoelectric focusing (CIEF), and capillary isotachophoresis (CITP) (*1*). CEC and MECC are always considered as the chromatographic techniques in CE. CE offers the advantages of high resolution, high separation efficiency and good reproducibility. In the enantioseparation of chiral herbicides by CE, cyclodextrins (CDs) and its derivatives are often added to the electrophoresis buffer as the chiral selectors.

A mixture of seven chlorophenoxy acid herbicides were successfully separated within 7 min on a 47 cm (40 cm to detector)×50 μm I.D. fused-silica capillary column, where only the enantiomers of 2,4-DP and 2-(2,4,5-trichlorophenoxy)propionic acid (2,4,5-TP) were separated by adding 4 mM α-CD and 1 mM β-CD in the buffer (*39*). Baseline enantiomeric separation of a mixture of six pairs of phenoxypropionic acid herbicides including 2,3-CPPA, 2,2-CPPA, 2,4-CPPA, 2(2,4-DCPPA), 2(2,4,5-TCPPA) and 2-PPA was achieved in less than 30 min by CE with heptakis(6-methoxyethylamine-6-deoxy)-β-CD [β-CD-OMe (VII)] as the chiral selector. The two most substituted herbicides [2(2,4-DCPPA) and 2(2,4,5-TCPPA)] were the best resolved. One of the faster migrating antipodes of 2(2,4,5-TCPPA) co-eluted with one slower antipode of 2(2,4-DCPPA) while baseline separation was obtained for both when they were run separately (*40*).

DCPP was separated using 25 mM sodium tetraborate (Na-TB), pH 8.5, with 25 mM trimethyl-β-CD as the chiral selector, while imazaquin was analyzed with 15 mM dimethyl-β-CD in 50 mM acetate at pH 4.5 (*41*). Enantioseparation of imazaquin enantiomers was conducted with 30 mM hydroxypropyl-β-CD (HP-β-CD) as chiral selector in 50 mM sodium hydrogen phosphate buffer (pH 10.1) by CZE (*42*). In another study (*43*), the two imazethapyr enantiomers were separated using 6% hydroxypropyl-β-CD as the chiral selector in buffer at pH 11.0.

Vancomycin was used as the chiral selector in CZE for the enantiomeric separation of several free acid herbicides including MCPP, fenoprop, DCPP, flamprop, haloxyfop, fluazifop, diclofop and fenoxaprop (*44*). The increase of vancomycin concentration caused a general increase of migration time, resolution and selectivity. Baseline resolution was achieved when 6 mM vancomycin was used. Baseline separation was observed for the enantiomers of fluazifop, halossifop and fenoxaprop, whereas the optical isomers of flamprop could be partially resolved using 100 mM β-alanine-acetate, 50 mM triethylamine in 100% methanol supported with 100 mM allyl-TER by CZE (*45*). Separation times were short compared to similar analyses using HPLC and a terguride CSP.

Separation of Chiral Herbicides by CEC

Capillary electrochromatography (CEC) utilizes a stationary phase rather than a micellar pseudo-stationary one, and therefore is a hybrid technique coupling the selectivity of LC and the separation efficiency of CE. CEC has attracted an increased interest which contains packed CEC (PCEC), open tubular CEC (OTCEC) and emulsion electrokinetric CEC (EECEC).

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The enantiomers of some 2-aryloxypropionic acids together with their ester and amide derivatives were readily separated on the commercially available β-GEM 1 and Whelk-O 1 CSPs. Of the analytes studied, the N,N-diethylamides typically showed the greatest enantioselectivity. Diclofop ethyl, devrinol, and MCPP were separated using the Whelk-O 1 CSP (*46*).

Haloxyfop, fluazifop, fenoxaprop, and flamprop free acids, diclofop, MCPP, DCPP, fenoprop and 2-PPA were separated using a CSP derived from an L-RNA aptamer by CEC after binding to biotin and grafting upon streptavidin-modified porous glass beads (*47*).

A porous monolithic chiral column was prepared by *in situ* copolymerization of glycidyl methacrylate, methyl methacrylate and ethylene glycol dimethacrylate in the presence of formamide and 1-propanol as the porogen solvents for analyzing the DCPP enantiomers. Subsequently, the epoxide groups at the surface of the monolith were reacted with (+)-1-(4-aminobutyl)-(5*R*,8*S*,10*R*)-terguride as the chiral selector. Optimum conditions for the herbicide resolution by CEC were found when using mobile phases consisting of acetic acid/triethylamine mixtures in acetonitrile:methanol (9:1 v/v). Under these conditions, complete separation of DCPP enantiomers in the presence of clofibric acid (internal standard) was achieved in about 5 min (*48*).

A silica based monolithic capillary column derivatized with *O*-9-(tert-butylcarbamoyl)quinidine was prepared for enantiomer separation of DCPP, MCPP and fenoprop. Reasonable baseline separations of enantiomers were accomplished for all analytes after optimization of relevant mobile phase parameters in the anion-exchange CEC system, and the separation was comparable to that obtained on an optimized high density quinidine-carbamate modified organic polymer monolith column (*49*).

Separation of Chiral Herbicides by MEKC

Micellar electrokinetic chromatography (MEKC) separation is based on the differences between interactions of analytes with micelles present in the separation buffer, which can easily separate both charged and neutral solutes with either hydrophobic or hydrophilic properties.

A novel, selective precolumn derivatization reaction was introduced and evaluated in the fluorescence labeling of 2,4-D, (2,4,5-trichlorophenoxy)acetic acid (2,4,5-T), 2-PPA, MCPP, 2,2-CPPA, 2,3-CPPA, 2,4-CPPA, DCPP and silvex with 7-aminonaphthalene-1,3-disulfonic acid (ANDSA) by CE and MEKC mode (*50*). The ANDSA-phenoxy acid herbicide enantiomers exhibited higher chiral resolution than their underivatized analogs in the presence of CD in the running electrolyte. The best enantioselectivity was achieved when 2,3,6-tri-O-methyl-β-CD (TM-β-CD) was used as the chiral selector. Mixed CDs based on β-CD and TM-β-CD proved to be the most effective as far as the enantiomeric resolution of the chiral analytes was concerned. DCPP, MCPP and 2,4-CPPA were separated in the MEKC mode with 600 mM borate, 10 mM sodium phosphate pH 5.0 containing 25 mM TM-α-CD and 50 mM decanoyl-N-methylglucamide (MEGA 10).

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Octyl-β-*D*-maltopyranoside (OM) was evaluated as chiral nonionic surfactant in CE of fluorescece-labeled phenoxy acid herbicides (*51*). The labeling of the analytes with ANDSA permitted a concentration detection limit of 5×10^{-10} M using laser-induced fluorescence detection. The tagging of the phenoxy acid herbicides with ANDSA increased the hydrophobicity of the analytes, thus favoring an enhanced solubilization of the derivatized herbicides in the OM micellar phase. The net results of this effect were a much shorter analysis time and an improved enantiomeric resolution of the derivatives when compared to underivatized phenoxy acid herbicides. Baseline enantiomeric resolution of silvex, DCPP, MCPP, 2,4-CPPA, 2,3-CPPA, 2,2-CPPA and 2-PPA was attained within 30 min using 200 mM sodium phosphate buffer containing 60 mM OM at pH 6.5 (*52*). Except silvex, six phenoxy acid herbicides including DCPP were also baseline separated by performing the separation at 10 °C and using 250 mM sodium phosphate buffer at pH 6.5 that contained 50 mM n-nonyl-β-glucopyranoside (NG) or 70 mM n-octyl-β-glucopyranoside (OG) as surfactant (*53*). In addition, silvex was separated partially with 50 mM N,N-bis-(3-D-gluconamidopropyl)deoxycholamide as the chiral selector, and 400 mM borate treated fused-silica capillaries at pH 10.0, 15 °C, 20.0 kv voltage in the MEKC mode (*54*). Enantiomeric ratios of methyl esters of phenoxy acid herbicides and metolachlor were measured. Each of six CD, including α-CD, β-CD, γ-CD, hydroxypropyl-β-CD, dimethyl-β-CD and trimethyl-β-CD, was then added to the borate-SDS buffer, with and without the organic modifier, to test its effect on the separation of the achiral compounds and the enantiomers of the chiral racemates by CD-MEKC. γ-CD with methanol modifier allowed baseline separation of the three phenoxy acid methyl esters and of the enantiomers of fenoprop methyl ester, but none of the CDs separated the enantiomers of MCPP and DCPPM. Finally, attempts were made to separate the four enantiomers of metolachlor; three of the enantiomers were separated by γ-CD with methanol (*55*).

The enantiomeric resolution of chiral phenoxy acid herbicides was performed by MEKC using several neutral and charged CDs as chiral pseudophase (CD-MEKC). Among the CDs tested, (2-hydroxy)propyl β-CD (HP-β-CD) was found to be the most appropriate for the enantioseparation of phenoxy acids. The use of a 50 mM electrolyte solution in ammonium formate at pH 5.0 containing 15 mM HP-β-CD and a temperature of 40 °C enabled the enantiomeric resolution of four (2-PPA, 2,3-PPA, 2,4-CPPA, and 2-(2,4-DCPPA)) of the six phenoxy acids investigated, with migration times ranging from 9 to 15 min. Mixtures of the two phenoxy acids not enantiomerically resolved (2-(4-chlorophenoxy)-2-methylpropionic acid and 2,4,5-TP) and up to three of the phenoxy acids enantiomerically resolved were separated in about 15 min (*56*).

Thiobencarb sulfoxide, which is produced by S-oxygenation of thiobencarb, was separated using γ-CD together with sodium dodecyl sulfate. The optimum running conditions were found to be 20 mM phosphate-5 mM borate buffer (pH 8.5) containing 60 mM hydroxypropyl-γ-CD and 100 mM sodium dodecyl sulfate with an effective voltage of +20 kV at 20 °C using direct detection at 220 nm (*4*). The resolution (R_s) was approximately 1.7 (*33*).

Enantioselective Separation and Analysis by Other Chromatographic Methods

A preparative enantiomer separation method of DCPP was developed utilizing a purposefully designed, highly enantioselective chiral stationary phase additive (CSPA) cinchona-derived chiral selector derived from bis-1,4-(dihydroquinidinyl)phthalazine in centrifugal partition chromatography (CPC). A solvent system consisting of 10 mM CSPA in methyl tert-butyl ether and 100 mM sodium phosphate buffer (pH 8.0) was identified as a suitable stationary/mobile-phase combination. Complete enantiomer separation of up to 366 mg of racemic DCPP was achieved, corresponding to a sample load equivalent to the molar amount of CSPA employed. Comparison of the preparative performance characteristics of the CPC protocol with that of a HPLC separation using a silica-supported bis-1,4-(dihydroquinidinyl)phthalazine CSP revealed comparable loading capacities for both techniques but a significantly lower solvent consumption for CPC. Given that further progress in instrumental design and engineering of dedicated, highly enantioselective CSPAs can be achieved, CPC may offer a viable alternative to CSP-based HPLC for preparative-scale enantiomer separation (*57*).

Conclusions

Over the last several decades, the enantioseparation of chiral herbicides has been widely studied and has made significant contributions to understanding their stereoselectivity in biological target activity and non-target toxicity. The direct chromatographic separation approaches play a leading role in the separation of chiral herbicides. HPLC combined with CSPs shows its superiority for enantiomer analysis and enantiomer preparation of many common herbicides, especially for the groups including amide herbicides, phenoxy herbicides and imidazolinone herbicides. GC is powerful in trace analysis while CE with diversified modes is also useful because of its maneuverability. The application of herbicides separation by SFC is relatively limited but with high development potential.

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Chapter 5

Enantioselective Separation and Analysis of Synthetic Pyrethroids

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Synthetic pyrethroid (SP) insecticides usually contain 1-3 asymmetric centers, resulting in 1-4 pairs of enantiomers. SPs are generally separated and analyzed by chromatographic resolution, including capillary electrophoresis (CE), supercritical fluid chromatography (SFC), gas chromatography (GC), and high-performance liquid chromatography (HPLC). GC and CE are more suitable for analyzing small quantities and concentrations. HPLC is the preferred preparative method for enantiomeric separation and for analyzing larger, nonvolatile, polar, and thermally labile pesticides.

Introduction

Synthetic pyrethroids (SPs) are analogues of naturally-occurring pyrethrins that occur in pyrethrum, the oleoresin extract of dried chrysanthemum. The insecticidal properties of pyrethrins are derived from ketoalcoholic esters of chrysanthemic acids and pyrethric acids. These acids are strongly lipophilic and rapidly penetrate many insects and paralyze their nervous system (*1*). They were introduced in the early 1980s and are widely used in controlling many insect species in agriculture and in the home (*2*).

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Chirality in a pyrethroid can arise from the acid moiety, the alcohol moiety, or both. Therefore, pyrethroids contain 1-3 asymmetric centers, resulting in a group of chiral insecticides with 1-4 pairs of enantiomers.

SPs are usually applied as single enantiomers or as mixtures enhanced in the more active stereoisomers. Therefore, determination of optical isomers is important for the quality control of SPs. Many modern separation techniques, including crystallization, chemical resolution, biological resolution and chromatographic resolution have appeared in succession. Among them, chromatographic resolution by capillary electrophoresis (CE), supercritical fluid chromatography (SFC), gas chromatography (GC), and high-performance liquid chromatography (HPLC), exhibit dominant advantages (*3*). GC and CE are more suitable for analyzing small quantities and concentrations. In contrast, HPLC has become the preferred method for analyzing larger, nonvolatile, polar, and thermally labile pesticides. Synthetic pyrethroids are generally separated and analyzed by HPLC or GC.

High-Performance Liquid Chromatography

HPLC is quite useful due to its rapid and non-destructive separation property, and there is little enantiomerization during the analysis. Many scientists have devoted considerable efforts to applying HPLC with chiral stationary phases (CSPs) to separate SPs. The CSPs include brush-type (or Pirkle), helical polymers, cavity phases, protein phases and ligand-exchange phases. The results were promising. Previous studies in the resolution of a series of pyrethroids by using enantioselective capillary columns in GC indicated that enantiomers from the cis-stereoisomers were always well separated, while those from trans-stereoisomers remained unresolved even after a very long and gradual elution (*4*).

Four isomers of phenothrin were resolved on a chiral polymer column by Okamoto and co-workers (*5*). Employing a NH2-bound column with (R)-N-(3, 5-dinitrobenzoyl) phenylglycine as the chiral phase, Chapman and co-workers (*6*) obtained incomplete enantiomeric separation for some of the stereoisomers of several SPs, including diastereomers of permethrin (PM), 1S-trans-αR + 1R-trans-αS of cypermethrin (CP), and 1S-cis-αR + 1R-cis-αS and 1S-trans-αR + 1R-trans-αS of cyfluthrin (CF).

Based on Pirkle ionic and covalent columns, Lisseter and Humbling (*7*) separated enantiomers from CP and CF; eight peaks were obtained for both of them, but most peaks were not well separated at the baseline. Six peaks of CP were obtained on a cellulose based chiral column by Edwards and Ford (*8*).

Oi used Sumichiral OA-2500I and OA-4700 columns to resolve enantiomers for a number of pyrethroids. Good resolution was generally obtained for the test compounds, including CP (*9*).

A two-dimensional achiral/chiral HPLC method with circular dichroism (CD) detection was optimized for the stereochemical resolution and determination of the elution order of the eight stereoisomers of synthetic allethrin. A monolithic silica HPLC column was coupled orthogonally to an enantioselective OJ

Daicel column by means of a switching valve. The resolution of cis and trans diastereoisomers on the silica column was obtained by using a mobile phase consisting of n-hexane:tert-butyl methyl ether (96:4) (v/v) at a flow rate of 1mL/min. The cis and trans peaks were then switched to the enantioselective OJ column separately in two subsequent injections. The resolution of the four trans stereoisomers was accomplished by using n-hexane:tert-butyl methyl ether (90:10) (v/v), while the mobile phase composition for the four cis stereoisomers consisted of n-hexane:isopropanol (99.3:0.7) (v/v). The CD-based detection system, together with the injection of pure stereoisomers, allowed the determination of the elution order on the basis of the CD signals of the single stereoisomers. Under the final conditions, the validated method was applied to the determination of stereoisomeric composition and absolute configuration of the prevailing stereoisomers of real samples, i.e. commercial batches of different sources of *d*-allethrin (*10*).

Recently, the enantiomers from several commonly used pyrethroids (i.e. bifenthrin (BF), perrmethrin (PM), cypermethrin (CP) and cyfluthrin (CF)) (Figure 1) were satisfactorily resolved with chiral HPLC and the absolute configurations of each isomer were also identified (*11*, *12*). Table I shows the separation results. Sumichiral OA-2500I served as an effective selector for the enantiomers of cis-bifenthrin (BF), cis-PM and trans-PM using hexane/1,2-dichloroethane (500/1 by volume) as the elution solvent. With respect to the elution of CP and CF, each having three stereocenters, the Sumichiral OA-2500I was initially chosen as the chiral selector, but only six peaks were obtained. The trans-isomers could not be separated, even when 100% hexane was used as the mobile phase with a flow rate of only 0.2 mL/min and at a lower temperature of 0 °C (*13*).

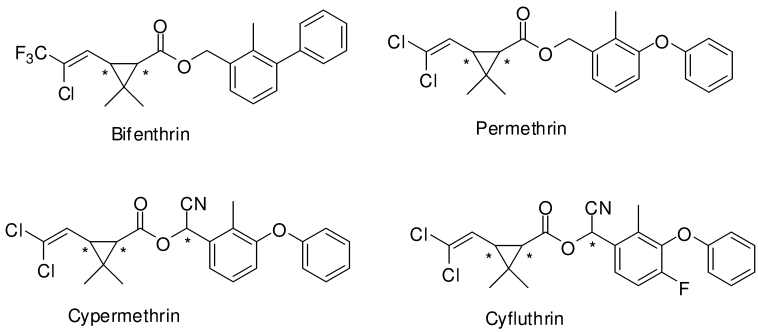

Figure 1. Chemical structures of SPs (I).

However, all eight isomers of both CP and CF were completely separated on two Chirex 00G-3019-OD columns connected in tandem by gradually adjusting the solvent composition of the mobile phase (*14*). When the mobile phase was composed of hexane/1,2-dichloroethane/ethanol at a ratio of 500/1.0/0.005 (by volume), seven peaks of the eight isomers were observed. Fortunately, peak resolution tended to be progressively better when the amounts of 1,2-dichroloethane and ethanol in the mobile phase were enhanced. Eight

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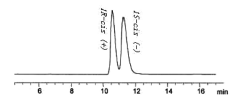
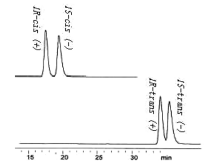
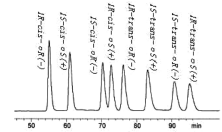
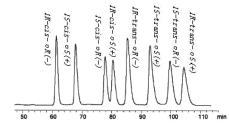
baseline-separated peaks were eventually obtained for both CP and CF at the solvent ratio of 500/30/0.15 (hexane/1,2-dichloroethane/ethanol) (Table I). In addition, the optical rotation of each resolved isomer was indicated by a positive (+) or negative (–) sign on the optical rotatory dispersion spectra. Optical rotations were determined on an in-line laser polarimeter detector (PDR-Chiral, Lake Park, FL, USA) concurrent to the response on the UV detector. Also, by comparing chromatograms from enantiopure and racemic standards (for cis-BF and PM) or peak profiles on chromatograms of various isomer-enriched products (CP and CF), whereby peak retention time could be used as the only evaluation criterion, assignment of absolute configurations for the separated peaks was made.

Table I. Enantioselective separation of SPs by HPLC (I)

Commercial names	*Separation conditions*	*Chromatograms* [a]	*References*
cis-Bifenthrin (BF)	Sumichiral OA-2500I Hexane/1,2-Dichloroethane = 500/1 or 99.5/0.5, room temperature, UV = 230 nm		Ref. 11, 12, 14
cis-Permethrin *trans*-Permethrin (PM)	Sumichiral OA-2500I Hexane/1,2-Dichloroethane = 500/1 or 99.5/0.5, room temperature, UV = 230 nm		Ref. 11, 14
Cypermethrin (CP)	Tandem Chirex 00G-3019-OD Hexane/1,2-Dichloroethane/Ethanol = 500/30/0.15, room temperature, UV = 230 nm		Ref. 14
Cyfluthrin (CF)	Tandem Chirex 00G-3019-OD Hexane/1,2-Dichloroethane/Ethanol = 500/30/0.15, room temperature, UV = 230 nm		Ref. 14

[a] A complete separation was defined as when the Rs exceeded 1.5.

Given that the chirality in both BF and PM originated from 1C and 3C on the cyclopropane ring and that elution occurred under the same chromatographic conditions, peak assignment for cis-PM and trans-PM were made indirectly with inference from cis-BF. But for CP and CF, analytical standards were absolutely not enough and tentative peak assignment was made possible by comparing peak profiles in chromatograms from the different isomer-enriched CP products. Given the great structural similarity between CP and CF, the resolved peaks may be similarly identified for CF. Then the peak assignments were independently

confirmed after enantioselective GC analysis of individual isomers. The isolated isomers were also analyzed by GC-mass spectrometry (MS). In addition, the rotation sign of all resolved isomers was measured using a polarimeter. Finally, the polarities and the absolute configurations of them were identified. The measurements revealed that under these conditions, the 1R isomers in BF or PM appeared to have a (+) rotation sign, whereas the 1S isomers have a (–) rotation, thus it was deduced that in pyrethroids with a chiral αC such as CP or CF, it is likely that the configuration on the αC determines the rotation sign of the isomers.

Other SPs, such as lambda-cyhalothrin (LCT) (*15*), d-trans-prallethrin, d-phenothrin, fenvalerate, tetramethrin and cycloprothrin (*16*) (Figure 2), have also been successfully resolved by HPLC carried out on a Jasco HPLC with various chiral columns (e.g., Chiralpak AD, Chiralpak AS, Chiralcel OD, Chiralcel OJ, Chiralcel OJ-H and Chiralcel OD-H). HPLC-CD (circular dichroism) was used for distinguishing between the enantiomers of LCT according to the CD signal of (–) or (+)-enantiomers (Table II).

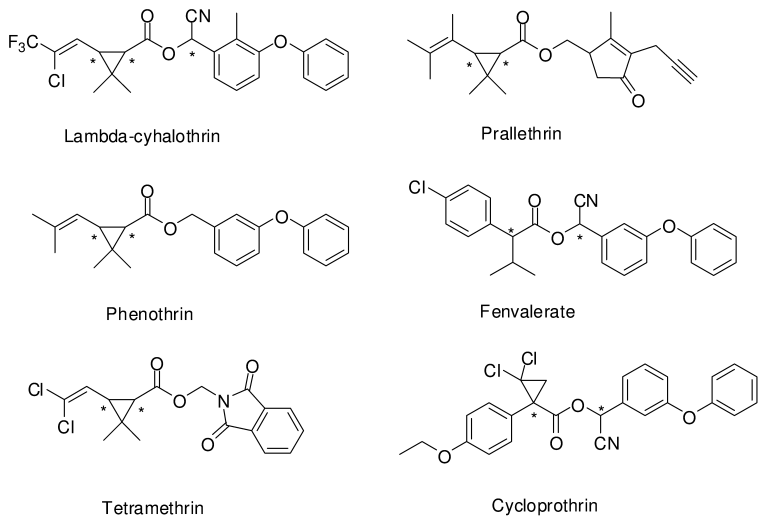
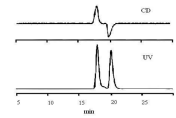
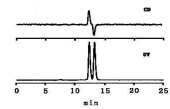
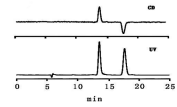
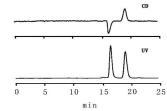
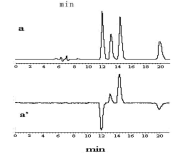
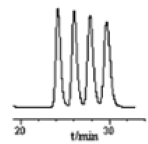
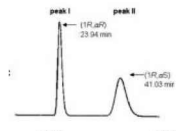
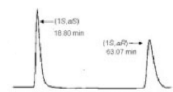
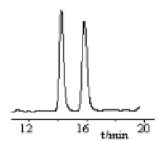


Figure 2. Chemical structures of SPs (II).

A novel CSP was developed recently by bonding the (R)-1-phenyl-2-(4-methylphenyl)ethylamine amide derivative of (S)-valine to aminopropyl silica gel through a 2-amino-3,5-dinitro-1-carboxamido-benzene unit. The new CSP completely separated fenpropathrin and fenvalerate (*17*) using hexane-dichloromethane-ethanol as mobile phase. The results showed that the enantioselectivity of this new CSP for these two compounds was better than that of a Pirkle-type 1-A column.

Table II. Enantioselective separation of SPs by HPLC (II)

Commercial names	*Separation conditions*	*Chromatograms*	*References*
Lambda-cyhalothrin (LCT)	Chiralpak AS Hexane/Ethanol = 95/5 25 °C, 0.4 mL/min, UV = 236 nm		Ref. 15
	Chiralpak AD Hexane/Ethanol = 98/2 25 °C, 0.4 mL/min, UV = 236 nm		Ref. 15
	Chiracel OD Hexane/2-Propanol = 95/5, 25 °C, 0.5 mL/min, UV = 236 nm		Ref. 15
	Chiracel OJ Hexane/Ethanol = 95/5, 25 °C, 0.6 mL/min, UV = 236 nm		Ref. 15
	Chiracel OJ Hexane/2-Propanol = 90/10, 25 °C, 0.4 mL/min, UV = 236 nm		Ref. 15
Fenvalerate	(*R*)-1-phenyl-2-(4-methylphenyl)ethylamine amide derivative of (*S*)-valine to aminopropyl silica gel through a 2-amino-3,5-dinitro-1-carboxamido-benzene unit Hexane/1,2-dichloromethane/ethanol = 98.45/1.2/0.35, Room temperature, 1 mL/min, UV = 230 nm		Ref. 15
Cycloprothrin	Chiralcel OJ-H Hexane/isopropanol = 70/30, 35 °C, 1 mL/min, UV = 254 nm		Ref. 16
	Chiralcel OD-H Hexane/isopropanol = 90/10, 35 °C, 1 mL/min, UV = 254 nm		
Fenpropathrin	(*R*)-1-phenyl-2-(4-methylphenyl)ethylamine amide derivative of (*S*)-valine to aminopropyl silica gel through a 2-amino-3,5-dinitro-1-carboxamido-benzene unit Hexane/1,2-dichloromethane = 94/6, Room temperature, 1 mL/min, UV = 230 nm		Ref. 17

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It was concluded that Chiralcel OD is desirable for the separation of SPs with one chiral center. The phenylcarbamates (Chiralcel OD) have amide groups capable of hydrogen bonding as a hydrogen accepter and donor with polar racemates under normal phase conditions. The substituents on the phenyl groups influence the polarity of the carbamate residues. As for SPs with three chiral centers (e.g., CP and CF), two tandem Chirex 00G-3019-OD columns are the best choice for their HPLC resolution (*18*).

The direct coupling of two columns is currently a common and useful practice. This method can enhance the performance and increase the lifetime of chiral columns.

In addition to analytical separation, chiral HPLC is an effective technique for the preparation of individual isomers of pyrethroids. The resolved isomers can be individually collected at the HPLC outlet. It is a rapid non-destructive tool in which there is little chance of epimerization during the course of analysis.

Gas Chromatography

HPLC has advantages in separation while GC is more suitable for analysis because of its higher sensitivity and lower injection volumes. An attempt to separate individual enantiomers of fenvalerate by GC following derivatization with (1R, 2S, 5R)-(–)-menthol was reported, but epimerization may occur during this procedure (*19*).

An investigation by using GC-ECD with an apolar DB-5 capillary column to separate cypermethrin diastereomers was carried out (*20*). Four enantiomer pairs with the elution order of cis, trans, cis, trans were obtained. Five partially resolved allethrin peaks are observed with the chiral Lipodex C column indicating chiral separation of cis-enantiomers, but it showed no enantioselectivity for the trans-allethrin isomers.

Nie et al. (*21*) utilized several CSPs to separate some ester pyrethroids. Permethylated-β-CD (PM-β-CD), heptakis (2,6-di-O-butyl-3-O-butyryl)-β-CD (DBB-β-CD), heptakis (2,6-di-O-nonyl-3-Otrifluoroacetyl)-β-CD (DNT-β-CD), the mixture of PM-β-CD and DBB-β-CD and the mixture of PM-β-CD and DNT-β-CD. When compared with the single cyclodextrin CSPs derivatives, the enantiomeric separation was improved significantly for some compounds with CSPs containing the mixtures of derivatized cyclodextrins. Synergistic effects were observed for some racemate compounds on the mixed cyclodextrin derivative CSPs. In general, the best resolution was obtained with PM-β-CD + DBB-β-CD stationary phase.

Leicht (*22*) separated diastereomers of cyfluthrin on a HP-1 capillary column and obtained four peaks, and four peaks were also detected by Bolygo and Hadfield (*23*) and Hadfield (*24*) for cypermethrin using capillary columns. Jin and Webster (*25*) also assigned the same order of diastereomers to the four resolved peaks on a DB-5 column.

Based on a HP-5 column, Liu and Gan (*26*) separated the diastereomers and then used a β-cyclodextrin-based enantioselective column (BGB-172) to separate the enantiomers of cypermethrin and cyfluthrin. Resolved peaks were identified

by comparing chromatograms of isomer-enriched cypermethrin products. Diastereomers of both cypermethrin and cyfluthrin were separated on a HP-5 column. On the BGB-172 chiral column, enantiomers of all cis diastereomers were separated, while those of trans diastereomers were not separated (Figure 3). The elution order appears to be regulated by configuration, a finding which may allow peak identification in the absence of isomer standards. The same group (*27*) also combined SPME and GC to analyze enantiomers of chiral contaminants in environmental samples. They determined enantiomers of (Z)-cis-bifenthrin and cis-permethrin in water using coupled SPME and enantioselective gas chromatography. This method has significant advantages when compared to some of the traditional solvent-based procedures. On a β-cyclodextrin-based enantioselective column, enantiomers of cis-bifenthrin or cis-permethrin were baseline separated.

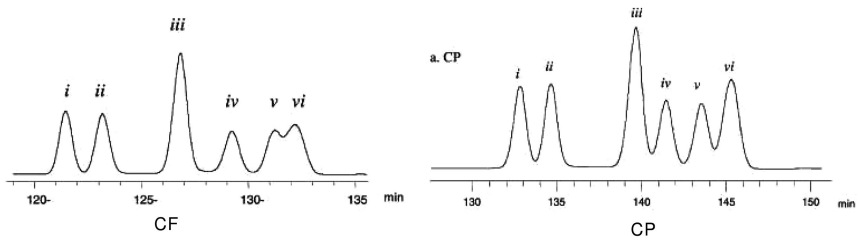

Figure 3. Chiral GC chromatograms of CF and CP. In CF, peak i(1R-3R-αR), ii(1S-3S-αS), iv(1R-3R-αS) and v(1S-3S-αR) are seperated cis enantiomers, while peak iii((1R-3S-αR)+ (1S-3R-αS)) and vi((1R-3S-αS)+ (1S-3R-αR)) are trans enantiomers. In CP, the situation is the same as CF.

This study showed that SPME may be together with enantioselective GC analysis for detecting enantiomers of synthetic pyrethroids in water samples and soil extracts.

The stability of stereoisomers of four commonly used pyrethroids, cis-bifenthrin (cis-BF), permethrin (PM), cypermethrin (CP), and cyfluthrin (CF), was studied during gas chromatography (GC) analysis and sample preparation. Stereoisomers of cis-BF and PM were found to be stable, but those of CP and CF were unstable, under heat or in water. Isomer conversion occurred only at the αC in CP or CF, causing the analyte stereoisomer to convert to an epimer. At a GC inlet temperature of 260 °C, about 9% conversion occurred for CP and CF. In organic solvents and sterile water, stereoisomers of cis-BF and PM were stable, but slow isomer conversion was observed for CP and CF in water at ambient temperature. However, isomer conversion for CP and CF was relatively insignificant (2-3%) when the GC inlet temperature was kept at ≤ 180 °C or when on-column injection was used. Isomer conversion at the αC in water suggests that abiotic processes may also contribute to enantioselectivity observed in the environment for pyrethroids with the asymmetric αC (*28*).

Some pyrethroids show thermal instability and may be degraded during GC analysis resulting in enantiomer conversion. Sometimes pyrethroids can show this effect in organic solvents. Qin and Gan (*29*) demonstrated by using GC analysis that permethrin is stable in some organic solvents (n-hexane, methylene chloride, propan-2-ol, acetone and methanol) but cypermethrin was unstable in acetone and methylene chloride. The extent of enantiomerization was affected by temperature and water as a cosolvent. Results from this study suggested that the exposure to certain solvents and water may cause artefacts in chiral analysis. Also, they showed that for isomer-enriched pyrethroid products such abiotic enantiomerization may render the products less effective because the conversion leads to the formation of inactive stereoisomers.

Subcritical and Supercritical Fluid Chromatography

The separation of enantiomers of SPs by subcritical and supercritical fluid chromatography was investigated using the Pirkle-type chiral stationary phases developed for high-performance liquid chromatography. Although the use of supercritical fluid chromatography is not very extended, it can present some advantages compared to HPLC. For example, when the same columns are used in HPLC and SFC, shorter analysis times can be expected in SFC since the viscosity of supercritical fluid is lower than that for a liquid, and other advantages are easy elimination of the eluent and the use for preparative purposes.

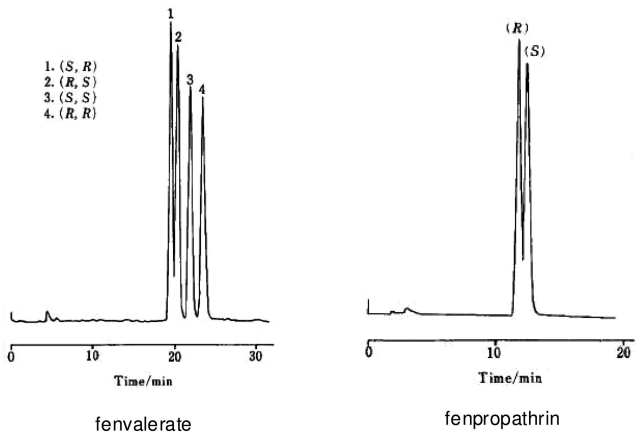

Figure 4. Chiral SFC chromatograms of fenvalerate and fenpropathrin. The conditions for fenvalerate were: mobile phase, carbon dioxide at 300μL/min and 10% ethanol in hexane at 10μL/min; pressure 200kg/cm² (backpressure); column temperature held at 20 °C; detection, UV at 210 nm. The conditions for fenpropathrin were: mobile phase, carbon dioxide at 300μL/min and hexane at 2μL/min; pressure 170kg/cm² (backpressure); column temperature held at 25 °C; detection, UV at 210 nm.

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The enantioselectivities of these stationary phases were found to be sufficient in tests with reference chemicals. Using these stationary phases, enantiomers of SPs with one or two chiral centers in their acid and alcohol moieties were resolved. Significant improvement resulted with the use of lower temperature, and thermodynamic considerations suggested the effectiveness of subcritical fluid chromatography for chiral separation of pyrethroids. The elution order of enantiomers was the same as in high-performance liquid chromatography in the two cases studied. This technique is very promising, as it requires a shorter analysis time (*30*). The separation of fenvalerate was achieved on a Sumichiral OA-2000 chiral column, and fenpropathrin was separated on a Sumichiral OA-4000 chiral column, as presented in Figure 4.

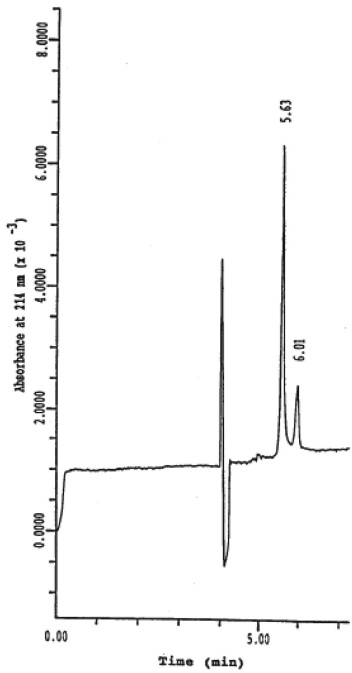

Figure 5. Separation of the geometric isomers of phenothrin (5.63 min, trans, and 6.01 min, cis) by MEKC. Analysis conditions: 50 μm id × 47 cm long (40 cm to detector) capillary column; pressure injection (2 s = 2.0 nL);50mM NaH_2PO_4 + 50mM sodium cholate + 15mM DM-β-CD buffer, pH 7.0; 20 kV (58 μA); 214 nm UV absorbance.

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Micellar Electrokinetic Capillary Chromatography

Micellar electrokinetic capillary chromatography (MECC) and high-performance liquid chromatography (HPLC) were used for the separation of stereoisomers of cypermethrin, alphamethrin, permethrin, and fenpropathrin. Different kinds of cyclodextrins (β-cyclodextrin, hydroxypropyl-β-cyclodextrin, dimethyl-β-cyclodextrin, and γ-cyclodextrin), surfactants (sodium dodecyl sulphate [SDS] and cetyltrimethylammonium bromide [CTAB]), and cations of the background electrolyte (sodium, ammonium, TRIS, and Ammediol) were tested. Optimized conditions (background electrolyte: 50 mmol/L sodium phosphate, pH = 2.5, 150 mmol/L SDS, 150 mg/mL γ-cyclodextrin) allowed the separation of alphamethrin, the eight cypermethrin stereoisomers (eluted in seven peaks) and the separation of two enantiomers of fenpropathrin with resolution Rs = 10 and with n ≈ 500,000 theoretical plates. Different experimental conditions, e.g., mobile phase composition, temperature, injected amount, and flow rate, were also optimized in complementary HPLC experiments. The optimal conditions (stationary phase: ChiralDex, 5 μm; mobile phase: 150 mmol triethylamine/L with H_2SO_4 in water (pH = 3.5) with methanol or acetonitrile; flow rate: 0.8 or 0.6 mL/min; temperature: ambient or 30°C, 20°C, or 10°C; experimental conditions were modified according to the type of analysis) allow chiral discrimination of alphamethrin enantiomers and analysis of permethrin stereoisomers. MECC offers higher efficiency and shorter analysis time than HPLC, but under tested conditions it was shown that the methods complement each other (*31*).

Shea et al. (*32*) separated bioallethrin, fenpropathrin and phenothrin by MEKC. Using an achiral surfactant plus CD, bioallethrin and fenpropathrin enantiomers were baseline resolved by adding DM-β-CD and γ-CD, respectively. While using a chiral surfactant plus CD (sodium cholate), the cis/trans isomers of phenothrin were separated (Figure 5). Also, the enantiomers of fenpropathrin were separated in less than 6 min when the running buffer was supplemented with sodium cholate and DM-β-CD (Figure 6).

A study on enantiomeric separation of cis-bifenthrin by CD-MEKC was carried out by Perez-Fernandez et al. (*33*).The influence of several experimental parameters such as temperature, voltage, type and concentration of surfactant (chiral and achiral) and CD was investigated. The use of the bile salt sodium cholate at a concentration of 100 mM in the presence of 20 mM heptakis (2,3,6-tri-*O*-methyl)-β-CD enabled the separation of cis-BF enantiomers in less than 10 min and with a resolution of 2.8.

Factors Influencing Chromatographic Separation

Chromatographic parameters, including capacity factor (k′), separation factor (α), and resolution factor (Rs) for the resolved enantiomers of chiral pesticides can be calculated and used to evaluate the enantioselectivity of chiral stationary phases (CSPs). The capacity factor k′ for each enantiomer was calculated as

$$k' = (tR - t0) / t0$$

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where tR is the average retention time of duplicate injections of the analyte taken at peak maxima and t0 is the column void time determined by recording the first baseline perturbation. The separation factor α was calculated as

$$\alpha = k2'/k1'$$

where k1′ and k2′ are the capacity factors of first and second eluted enantiomers, respectively. The resolution factor Rs of enantiomers was calculated as

$$Rs = 2\times (t2-t1)/ (w1+w2)$$

where t1 and t2 represent respectively the peak retention time of the first and second eluted enantiomers, and w1 and w2 are the peak widths measured at the peak base of the first and second peaks, respectively. A complete resolution was considered when the Rs exceeded 1.5.

The enantiomers of a chiral compound are usually distinguished by their absolute configuration and/or optical rotation. Recently, CD detectors coupled directly to HPLC/GC have become powerful tools for determining the optical properties of the resolved enantiomers.

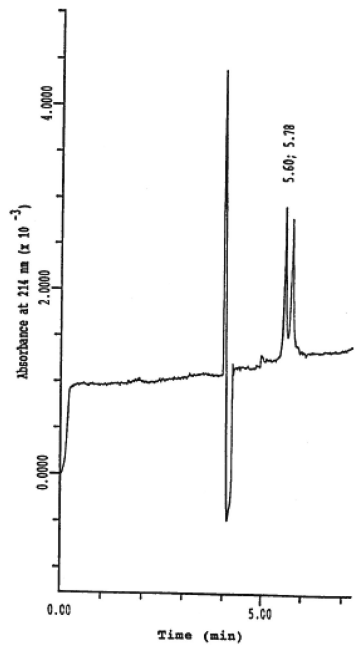

Figure 6. Separation of fenpropathrin enantiomers by MEKC. Analysis conditions: 50 μm id × 47 cm long (40 cm to detector) capillary column; pressure injection (2 s = 2.0 nL); 50 mM NaH_2PO_4 + 50 mM sodium cholate + 15 mM DM-β-CD buffer, pH 7.0; 20 kV (58 μA);214 nm UV absorbance.

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Enantiomeric resolution is highly specific to the CSP (chiral stationary phase) in HPLC/GC analysis. Molecules of chiral pesticides have electronegative atoms (nitrogen, oxygen, or sulfur), C=O groups or phenyl, pyridine or quinoline rings attached directly or indirectly to the stereogenic center, which may lead to different preferences in their interaction with the derivatized polysaccharide-CSPs through hydrogen bonding, π-π, or dipole-dipole interaction. In addition, the degree of steric fit into the chiral cavities of the CSPs may also play a role in chiral recognition. The effects of polar and acidic modifiers, as well as column temperature, are also important. It was suggested that alcohol in the mobile phase not only competes for chiral bonding sites with chiral solutes but can also alter the steric environment of the chiral cavities on the CSP by binding to achiral sites at or near the chiral cavity. Temperature can affect chiral separation in two ways (*34*). One is a kinetic effect that influences the viscosity and the diffusion coefficient of the mobile phase. Another is the thermodynamic effect that changes the separating factor (α).

From the results above, it can be concluded that HPLC is a powerful method for preparing enantiomers-pure samples while GC is more suitable for analysis. Both HPLC and GC chiral separations are based on the nature of the CSP and influenced by parameters such as alcohol content and column temperature.

Thus, chiral HPLC is an effective technique for separation and preparation of individual isomers of pyrethroids. It is a rapid non-destructive technique in which there is little chance of epimerization during the course of analysis, and is suitable for the analysis of technical formulations and terminal residues in biological and environmental matrices.

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Chapter 6

Chiral Pesticides and Environmental Safety

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Enantioselectivity is important in the field of agrochemicals. Although enantiomers may behave differently in their biological activities and in biologically mediated environmental processes, at present the environmental risks of chiral pesticides are mostly understood and regulated as if they were achiral. Studies on environmental enantioselectivity of pesticides first appeared in the early 1990s. Most of the early studies focused on the legacy chiral pesticides, such as α-HCH, chlordane, and *o,p′*-DDT. In this article, the environmental enantioselective consequences, including aquatic toxicity, cell toxicity, endocrine disruption and carcinogenicity of some chiral pesticides, are reviewed. Enantioselectivity in these aspects is expected to result in ecotoxicity that cannot be predicted from our existing knowledge, and must be considered in future environmental risk assessment and regulatory decisions.

Introduction

Chirality is a central concept in structural chemistry. The significance of molecular chirality in the chemical aspects of life sciences has been recognized since the optical isomers of tartrate were separated by Pasteur. The thalidomide tragedy, which happened in the 1960s in Germany, was world-shaking; it was

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caused by the selective teratogenicity of the *S*-enantiomer of this drug. Since then, the recognition of the potential health risks involved in the usage of racemic mixtures of drugs has considered the design and manufacturing of single-enantiomer chiral drugs in the pharmaceutical industry.

Enantioselectivity is also important in the field of agrochemicals. Up to 25% of pesticides sold in 1996 were chiral (*1*), and that proportion is expected to increase as compounds with more complex structures are registered for use (*2*). Although the enantiomers may behave differently in biological activities and in biologically mediated environmental processes (*1*, *3*), the environmental risks of chiral pesticide are mostly understood and regulated as if they were achiral (*4*, *5*). Studies on environmental enantioselectivity of pesticides first appeared in the early 1990s (*6*). Most of the early studies focused on legacy chiral pesticides, such as α-HCH, chlordane, and *o,p'*-DDT (*7*, *8*). Six important chiral pesticides (α-HCH, mecoprop, *cis*-chlordane, *trans*- chlordane, heptachlor *exo*-epoxide and oxychlordane) have also been examined (*8*).

Enantioselective Enrichment and Metabolism of Chiral Pesticides

In the natural environment, hydrophobic persistent organic compounds become increasingly concentration with higher trophic levels in a food web (*9*). The biomagnification of the enantiomers of α-HCH and chlordane was found in the polar bear food chain (*10–12*), where the enantiomeric ratio (ER) (defined as the ratio of the concentrations of the (+)-enantiomer and the (-)-enantiomer) increased from total cod (ER≈1) to blubber and liver samples of ringed seals to liver samples of polar bear (ER=2.3). Many studies found that the ERs in higher trophic animals were influenced by species-specific metabolism and transport processes in the body (*13*, *14*). High deviations from racemic were found for different pesticides in specific organs, for example, in liver, kidney, brain tissue, and spinal marrow. Shen and KatharinaMain (*15*) found that (-)-α-HCH was more easily degraded than the corresponding enantiomer in human placentas, and the EFs decreased with increasing concentrations of α-HCH. A general trend in the deviation from EF=0.5 was found in the following sequence: lower trophic biota < higher trophic biota < liver/kidney <brain. The selective enrichment was believed to be a result of the combined effect of stereoselective degradation/metabolism, complexation, uptake, and excretion within an organism or its organ (*8*).For enantioselective metabolism, one or more enzyme reactions involved in the uptake or in different transformation steps must be enantioselective. This phenomenon is often explained by the three-point model (*16*, *17*). Diastereomeric complexes are formed by combining the enantiomer and biological macromolecules in the process of metabolism, and it leads to stereoselectivity of the substrate. The relative concentrations of enantiomers become different because of the different metabolic rates.

Biotransformation of chiral pesticide may proceed along four different avenues: (A) Two enantioselective enzymes exist, each converting only one substrate enantiomer; (B) Both enantiomers are simultaneously converted by

one enzyme, but at different rates; (C) Sequential conversion of the substrate enantiomers by one enzyme, i.e., the enzyme preferentially transforms one enantiomer, while the other enantiomer is eventually also transformed, only the former is completely transformed; and (D) Enantioselective conversion of one enantiomer by one enzyme and isomerization of the other enantiomer by the isomerase (*18*).

In addition, some minerals exist in the environment that may also affect the enantioselectivity of enzymes. Fang et al. found that the adsorption of lipase on kieselguhr greatly enhanced enantioselectivity of hydrolysis of 2,4-DP with ER increasing from 1.58 to 5.31. The adsorption triggered changes in conformation of the enzyme, thus affecting the microenvironment of the active sites, which may be the major reason for the enhanced enantioselectivity of enzymatic reactions (*19*). The effect of β-cyclodextrins (β-CDs) on enzymatic hydrolysis of chiral dichlorprop methyl ester (DCPPM) was also studied, and the results showed that cyclodextrins affected the enzyme activity and enantioselectivity of chiral pesticide (*20*).

Enantioselective Acute Toxicity of Chiral Pesticides

The biological receptors may include not only enzymes, but also a broad class of biological macromolecules, such as receptors, conduction matter, antibodies, DNA and transporters. Consequently, the enantioselective toxicity of a chiral pesticide may be reflected in many aspects, including lethality, enzyme inhibition, endocrine disturbance, carcinogenicity, teratogenesis, mutagenesis, among others. Due to the enantioselective interactions, enrichment and metabolism of chiral pesticides, the toxicity of chiral pesticides to organisms is also enantioselective.

Pyrethroids

Synthetic pyrethroids (SPs) were introduced in the early 1980s and are widely used in controlling a multitude of agricultural and domestic insect species. They are a family of chiral insecticides with a large number of stereoisomers. The aquatic toxicity of chiral pesticides is often evaluated using *Ceriodaphnia dubia*, *Daphnia magna* and zebrafish as the test animals. The (+)-(Z)-*cis*-BF (bifenthrin) was found to be more active than the (–)-enantiomer by 17 and 22 times for *C. dubia* and *D. magna*, respectively (*21*). The 1*R*-*cis*-BF was the only enantiomer in (Z)-*cis*-BF having toxicity against *C. dubia* (*22*). Two of the most important endpoints, survival and fecundity of *D. magna*, were chosed to evaluate enantioselectivity in chronic aquatic toxicity of *cis*-BF. The results indicated that *cis*-BF possessed obvious enantioselective, long-term, deleterious effects for *D.magna* and 1*R*-*cis*-BF is potentially more toxic than 1*S*-*cis*-BF in the two related endpoints. Enantioselectivity occurred through the 21-d testing period, with significant enantioselectivity observed after 7 and 14d of treatment for different enantiomers of *cis*-BF in a dose-dependent manner. (*23*). For permethrin (PM), the (–)-enantiomer in *cis*- and *trans*-PM was nonlethal for either *C. dubia* or *D. magna*, and the (+)-enantiomer contributed at least 95–97% of the lethal for

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C. dubia and 94–96% of the lethal for *D. magna* in the exposure to the racemate (*21*). The (–)-enantiomer of λ-cyhalothrin (LCT) was >162 times more toxic than its antipode to zebrafish in the acute test, and LCT also induced crooked body, yolk sac edema, and pericardial edema in the embryo test (*24*). Fenvalerate (FV) contains two chiral centers and thus four stereoisomers and enantioselective toxicity was also observed for FV. In the acute toxicity assessment with *D. magna*, the 48-h LC50 values showed that the α*S*-2*S*-FV was 99 times more toxic than α*R*-2*R*-FV (*25*). In the toxicity assay using *D. rerio*, the LC50 value of α*S*-2*S*-FV was 17, 22, 39 and 56 times smaller than α*R*-2*R*-FV at 24, 48, 72 and 96 h after exposure, respectively. 4-day zebrafish embryo-larval bioassays showed that exposure to FV resulted in enantioselectively induced crooked body, yolk sac edema and pericardial edema and that α*S*-2*S*-FV was 3.8 times stronger than the other isomers in 96-h mortality (*25*). In Cypermethrin (CP), only two of the eight enantiomers, 1*R*-*cis*-α*S* and 1*R*-*trans*-α*S*, were found to posses significant toxicity, while the other six isomers were much less active, with LC50 being at least 10 times greater than that for the active enantiomers. The 1*R*-*cis*-α*S* and 1*R*-*trans*-α*S* isomers of cyfluthrin (CF) were 50-100 times more toxic than the other isomers (*26*, *27*).

Organophosphates

The toxicity of chiral organophosphorus pesticides has been analyzed using *Daphnia magna* and cholinesterase enzyme inhibition assay. Enzyme inhibition of leptophos was carried out by using butyryl cholinesterase and acetylcholinesterase (*28*) inhibition tests; (+)- and (−)-leptophos exhibited significant differences in the half maximal (50%) inhibitory concentration (IC50) values. In the toxicity test of *daphnia*, the (−)-leptophos showed a lower toxicity than the (+) form and the racemate (*28*). Toxicity tests of fonofos and profenofos showed that the (–)-enantiomers were more active than the (+)-enantiomers (*21*). The in vitro inhibition toward acetylcholinesterase (AChE) for methamidophos suggested that the (–)-methamidophos was 8.0–12.4 times stronger than the corresponding (+)-form while 48-h acute toxicity test using *D. magna* suggested that the (+)-enantiomer was 7.0 times more toxic than the (–)-enantiomer (*29*). Four stereoisomers of chloramidophos (CP) were separated by Zhou et al. (*30*) and enantioselective toxicities were further measured. Inhibition to AChE (in vitro) and testing of the acute aquatic toxicity to *D. magna* (in vivo) showed that the fourth eluted peak was the most potent inhibitor against BE (Bovine Erythrocytes) - and EE (Electrophorus Electricus)-AChE (in vitro), but it was the least toxic isomer to *D. magna* (in vivo) (*30*). The toxicity assay with isocarbophos using *D. magna* showed a 50-fold difference between the enantiomers (*31*). The butyrylcholinesterase inhibition test showed the (+)-*R*- salithion being about 5.0 times more potent than the (–)-*S*-form, while the test toward *D. magna* indicated that (–)-*S*-salithion was about 3.0 times more toxic than (+)-R-enantiomer (*32*). The acute and delayed toxicities of the four isomers of O,S-dimethyl-N-(2,2,2-trichloro-1-methoxyethyl) phosphoramidothioate (MCP) were evaluated by comparing their acute lethal efficacy against *D. magna*, their inhibitory potentials to AChE, and axon-like outgrowth of the SH-SY5Y cells (*33*). The results implied

that the first eluted peak of MCP had the optimal target selectivity and ecological safety among the four stereoisomers (*33*). Jianmongkol et al. (*34*) examined the relative inhibitory potencies of the four stereoisomers of isomalathion against hen brain AChE and neurotoxic esterase (NTE), and found a 15-fold difference between the strongest(1R, 3R) and weakest (1S, 3S) isomers (*34*). Although the reasons for the discrepancy in in vitro and in vivo toxicity of OPs are not clear, it may be because of a combination of several factors. Firstly, different species of enzymes may have different sensitivities to enantiomers. Secondly, many biological processes, such as metabolism, transfer and accumulation that affect the in vivo toxicity have been found to be enantioselective (*35*).

In summary, the (+)-enantiomers of many OPs, including leptophos, fenamiphos, methamidophos, and isocarbophos, are more active than the (–)-enantiomers in various acute aquatic toxicity tests. However, for fonofos, profenofos, trichloronate and salithion, the (–)-enantiomers are found to be more active. It is generally true that the biotoxicity of most chiral OPs is enantioselective and that only one of the enantiomers contributes significantly to the toxicity of their racemates.

Acetanalides

Acetanalides are an important class of herbicides, the chiral representatives of which are metolachlor and metalaxyl. Since the herbicidal activity of metolachlor resides primarily in its two diastereomeric 1'*S*-isomers (α*SS* and α*RS*), racemic (rac-) metolachlor is now being replaced worldwide by S-metolachlor (*36*). The acute aquatic test for rac- and S-metolachlor with *D. magna* indicated that the toxicity of S-enantiomer was slightly higher than that of the racemate. However, the results of chronic toxicity testing showed that racemate was significantly more toxic than S-metolachlor to *D. magna* in LOEC, no-observed-effect concentration (NOEC), longevity and the number of broods per female (*37*). The effects of rac- and *S*-metolachlor on enzyme (hemolymph lactate dehydrogenase and catalase) activities in the fifth-instar silkworm larvae were also studied, and the results showed that rac-metolachlor was more toxic to silkworms than *S*-metolachlor (*38*). Metalaxyl is used in the control of plant diseases caused by pathogens of the *Oomycota* division. Acute and chronic toxicities of rac- and R-metalaxyl to *D. magna* were compared, and the results showed that R-metalaxyl was more toxic than rac-metalaxyl in long-term effects (*39*).

Imidazolinones

Imidazolinones are widely used because of their broad spectrum of weed control activity and low application rates, as well as low toxicity to animals. All imidazolinone herbicides are chiral and typically consist of two enantiomers. It is known that the imidazolinone enantiomers have different herbicidal activities, with the *R*-enantiomer being 8 to 10 times more inhibitory of the enzyme acetolactate synthase (ALS) than the *S*-enantiomer. However, relatively little information is available on the enantioselectivity of imidazolinones in their

toxicity to nontarget plants. The enantioselective phytotoxicity on the roots of maize (*Zea mays* L.) seedlings was measured for imazethapyr (IM), and the results showed that the *R*-(–)-IM was more effective in damaging maize growth by causing shorter shoot and root lengths, lower dry weights, and more obvious chlorosis than *S*-(+)-IM. Ultrastructural characterization revealed adverse effects of IM on cell organelles in maize root, with *R*-(–)-IM causing the most pronounced damages. Cell organelles such as statocytes, mitochondria, dictyosomes, and endoplasmic reticulum in root were severely damaged by *R*-(–)-IM (*40*). The enantioselective effects on morphology, antioxidant enzyme, oxidant marker and gene transcription in rice seedling (Xiushui 63) of IM were also determined, and the results suggested that *R*-(–)-IM had a stronger effect on the growth of rice than *S*-(+)-IM (*41*).

Phenoxypropanoic Acid Herbicides

Phenoxypropanoic acid herbicides are systemic and post-emergence herbicides that were introduced in the 1940s and 1950s. Representatives of this family include diclofop and dichlorprop. The algal toxicity tests showed that the herbicidal inactive *S*-(–) enantiomers of both diclofop-methyl and diclofop had similar or higher activity than the corresponding *R*-(+) forms. The growth of oat, a target plant, was significantly inhibited by the *R*-(+)-enantiomer (*42*). The enantioselective phytotoxicity of diclofop was determined, and the acute toxicities towards rice (Xiushui 63) seedlings and Hill reaction activities of the chloroplasts indicated significant differences between the two enantiomers (*43*).

Enantioselective Estrogenicity of Chiral Pesticides

Although an increasing number of studies have focused on the occurrence of enantioselectivity of chiral pesticides in environmental fate and acute aquatic toxicity, so far relatively little attention has been given to enantioselectivity in endocrine disruption activity. Among the published studies, most have focused on the enantioselectivity in estrogenic activity of *o,p′*-DDT. McBlain (*44*) and McBlain et al. (*45*, *46*) evaluated the estrogenic activity of *o,p′*-DDT enantiomers in rats and Japanese quail. The results showed that *R*-(−)-*o,p′*-DDT was a more active estrogen than *S*-(+)-*o,p′*-DDT. The alteration of the transcriptional activity of the human estrogen receptor (hER) after administration of *o,p′*-DDT enantiomers was described in yeast reporter gene systems, showing that *R*-(−)-*o,p′*-DDT was an active estrogen mimic, whereas the hER activity induced by *S*-(+)-*o,p′*-DDT was negligible. The estrogen receptor (ER)-binding activity of the enantiomers of a methoxychlor (MXC) metabolite, 1,1,1-trichloro-2-(*p*-hydroxyphenyl)- 2-(*p*-methoxyphenyl) ethane (mono-OH-MXC), was also studied, and the results indicated that the *S*-enantiomer was threefold higher in its binding activity than the *R*-enantiomer (*47*). The enantioselective endocrine disruption potential of dicofol was determined by quantification of β-galactosidase using yeast-based hER gene transcription assay, which showed that the β-galactosidase induction by

(−)-enantiomer was greater than the racemic mixture, while the (+)-enantiomer had negligible estrogenic activity (*48*).

The estrogenic potential of a newer pyrethroid insecticide, bifenthrin, was investigated, and the results showed that the estrogenic potential of 1*S*-*cis*-bifenthrin was much greater than that of the *R*-enantiomer both in the *in vitro* human breast carcinoma MCF-7 cell proliferation assay (i.e., the E-Screen assay) and in the *in vivo* aquatic vertebrate vitellogenin enzyme-linked immunosorbent assay (*49*). Significant differences were observed between the two enantiomers of permethrin (PM) in the induction of hepatic gene transcription by using male adult zebrafish as the test animal. The (−)-trans enantiomer showed the greatest estrogenic activity, with a relative activity 4-fold higher than the 50 ng/L E2 treatment (*50*). A further study using the embryo-larval zebrafish as the test animal showed that the PM racemate and its enantiomers stimulate VTG1, ESRα and cyp19b expression. Significant differences were observed among the four stereoisomers of PM in the induction of estrogen-responsive gene expression, and the (−)-*trans* enantiomer showed the greatest estrogenic activity (*51*).

Enantioselective Cytotoxicity and Genotoxicity of Chiral Pesticides

The assessment of cytotoxicity is an effective method for evaluating environmental and ecological toxicities of pesticides. The relationship of some adverse effects of pesticides and cytotoxicity has been illustrated in several published studies. However, the role of enantioselectivity in toxicity on cell lines is poorly understood for chiral pesticides. The enantioselectivity in cytotoxicity and genotoxicity of bifenthrin (BF) was determined using human amnion epithelial (FL) cell lines. The cell proliferation and cytoflow analysis suggested that 1*S*-*cis*-BF was more toxic than 1*R*-*cis*-BF above the concentration of 7.5 mg/L. FL cells incubated with 1*S*-*cis*-BF exhibited a dose-dependent accumulation of intracellular reactive oxygen species (ROS). In the single cell gel electrophoresis assay (also known as comet assay, is an uncomplicated and sensitive technique for the detection of DNA damage at the level of the individual eukaryotic cell), the number of cells with damaged DNA incubated with 1*S*-*cis*-BF was more than that with 1*R*-*cis*-BF (*52*). In a subsequent study, the enantioselectivity in cytotoxicity and apoptosis mediated by the mitogen-activated protein kinase (MAPK) signalling pathway in the human hepatocellular liver carcinoma (Hep G2) cell line was evaluated. Exposure to 1*S*-*cis*-BF resulted in increased levels of phosphorylated JNK (Jun-N-terminal Kinases)/MAPKs, while exposure to 1*R*-*cis*-BF did not affect the phosphorylated JNK levels (*53*). Pre-treatment with the JNK inhibitor SP600125 blocked 1*S*-*cis*-BF-induced cytotoxicity and apoptosis. Furthermore, 1*S*-*cis*-BF enhanced the production of ROS, while pre-treatment with the antioxidant agent MnTBAP resulted in decreased phosphorylated JNK (*53*). The study demonstrated that *cis*-BF induced apoptosis may occur through the enantioselective activation of JNK/MAPK signalling pathway in Hep G2 cells (*53*).

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Conclusion

Evaluation of chiral pesticides at the enantiomeric level concerning their environmental safety and human health effects is a trend of scientific development. An in-depth study of separation and toxicity of chiral pesticides will provide indispensable technical support and scientific basis for the evaluation of the impact on environmental safety and human health. Although an increasing number of researchers have started relevant investigations, more research results need to be obtained before arriving at a universal theory. The combination of theoretical knowledge of chemistry, biology and other disciplines, and technical means is needed to strengthen research in this area, particularly the research on enantioselective toxicities of chiral pesticides, such as estrogenicity, neurotoxicity and immunotoxicity, which are closely related to human health. Only when the difference of chiral pesticides is fully considered, can we obtain a true and accurate assessment of their risks, therefore providing the basis for the formulation of relevant laws and regulations and the safest use of chiral pesticides.

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Chapter 7

Enantioselective Toxicity of Chiral Pesticides in Aquatic Systems

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Chiral pesticides may differ markedly in their environmental fate, bioavailability and toxicity. A number of reports have shown that chiral pesticides may cause enantioselective toxicities to aquatic organisms. In this chapter, the acute toxic manifestations and long-term effects of chiral pesticides on aquatic organisms, the factors resulting in stereoselective toxicity, and the mechanisms of stereoselective toxicity are reviewed and summarized.

Introduction

The use of pesticides in agriculture is growing at a high speed due to the rise in productivity. In general, pesticides are considered as a class of important pollutants that are widespread throughout the world. More than 40% of the existing agrochemicals in China contain chiral centers, and most are produced and used as racemic mixtures (*1*). However, enantiomers usually differ in their biological properties as a result of their interaction with enzymes or other naturally occurring chiral molecules. Enantioselectivity plays an important role in the environmental fate and ecological risks of a chiral compound because many biologically mediated environmental processes are enantioselective. Many current chiral insecticides have high activity against nontarget organisms

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(*2*). In particular, two classes of insecticides, synthetic pyrethroids (SPs) and organophosphates (OPs), are acutely toxic to a wide range of aquatic organisms at trace levels (*3*, *4*). Contamination of surface aquatic ecosystems by these compounds is a great environmental concern (*5*, *6*). Pesticides may affect estuarine microorganisms via spills, runoff, and drift (*7*). Metolachlor is regularly detected in North American and European surface waters during the crop growing season (*8–10*). In Switzerland, peak exposure concentrations have been shown to surpass the suggested chronic water quality criteria for metolachlor, which is set at 0.3 g L^{-1} (*11*). Mecoprop, dichlorprop and metolachlor concentrations and enantiomer signatures were determined in Ontario streams, with median concentrations of mecoprop is 44 ng L^{-1} (0.5-829 ng L^{-1}), dichlorprop is 3.2 ng L^{-1} (0.5-31 ng L^{-1}) and metolachlor is 94 ng L^{-1} (24-5160 ng L^{-1}), median EFs for mecoprop and dichlorprop were 0.599 and 0.351, and the median SF for metolachlor was 0.863 in 2006-2007 (*12*). Though some chiral compounds such as pyrethroids are known to be strongly adsorbed to soil particles, this is not likely to render them immobile post-application because they can be transported in runoff with the soil particles to which they are attached and end up in sediments (*13*). Once in sediments, they can enter into aquatic ecosystems (*14*) and be bioavailable to the aquatic food web (*15*, *16*). It has also been reported that runoff of agricultural pesticides into estuaries poses significant toxicological risks to resident organisms (*17*). But only one of the two enantiomers may be expected to contribute significantly to the aquatic toxicity. Thus, the study of enantioselective toxicity of chiral pesticides to aquatic organisms is important to evaluate the behavior of chiral pesticides and has gained the interest of many researchers. The acute toxic manifestations and long-term effects of chiral pesticides in aquatic organisms, the factors resulting in stereoselective toxicity, and the mechanism of stereoselective toxicity are reviewed in this chapter.

Acute Toxicity

The EC_{50} (LC_{50} and IC_{50}) is a relatively common index used to evaluate the toxicity of a compound, with a lower value indicating a more toxic potency. Studies have shown that chiral pesticides have a devastating effect on aquatic invertebrates, and dramatic differences between enantiomers have been observed in their acute toxicity to freshwater invertebrates, suggesting that the aquatic toxicity is primarily attributable to a specific enantiomer in the racemate (*2*). The EC_{50} or LC_{50} values of some chiral pesticides reported in the literature for some aquatic species are shown in Table I.

Table I. The toxicity of different chiral pesticides to different kinds of water species

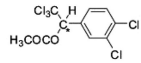
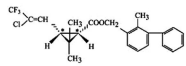
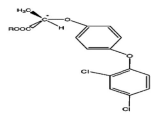
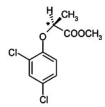
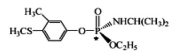
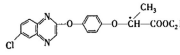
Test chemicals	Structure	Test species	enantiomer	Endpoints	concentration	Reference
Acetofenate		*Danio rerio*	±	$LC_{50,\ 24h}$	1.25 mg/L	(*18*)
			+	$LC_{50,\ 24h}$	0.85 mg/L	
			-	$LC_{50,\ 24h}$	1.16 mg/L	
			±	$LC_{50,\ 48h}$	0.68 mg/L	
			+	$LC_{50,\ 48h}$	0.85 mg/L	
			-	$LC_{50,\ 48h}$	1.16 mg/L	
			±	$LC_{50,\ 72h}$	0.61 mg/L	
			+	$LC_{50,\ 72h}$	0.85 mg/L	
			-	$LC_{50,\ 72h}$	0.67 mg/L	
			±	$LC_{50,\ 96h}$	0.61 mg/L	
			+	$LC_{50,\ 96h}$	0.85 mg/L	
			-	$LC_{50,\ 96h}$	0.52 mg/L	
cis-bifenthrin		*Ceriodaphnia dubia*	±	$LC_{50,\ 96h}$	0.144 μg/L	(*2*)
			+	$LC_{50,\ 96h}$	0.076 μg/L	
			-	$LC_{50,\ 96h}$	1.342 μg/L	
		Daphnia magna	±	$LC_{50,\ 96h}$	0.175 μg/L	
			+	$LC_{50,\ 96h}$	0.081 μg/L	
			-	$LC_{50,\ 96h}$	1.803 μg/L	
Diclofop		*Chlorella pyrenoidosa*	Rac-	$EC_{50,\ 96h}$	8.89 mg/L	(*19*)
			S-	$EC_{50,\ 96h}$	8.74 mg/L	
			R-	$EC_{50,\ 96h}$	9.72 mg/L	
		Chlorella vulgaris	Rac-	$EC_{50,\ 96h}$	4.76 mg/L	
			S-	$EC_{50,\ 96h}$	3.32 mg/L	
			R-	$EC_{50,\ 96h}$	18.74 mg/L	
Dichlorprop-methyl		*Chlorella pyrenoidosa*	R-	$EC_{50,\ 96h}$	7.310 mg/L	(*20*)
			S-	$EC_{50,\ 96h}$	0.783 mg/L	
			Rac-	$EC_{50,\ 96h}$	1.058 mg/L	
		Chlorella vulgaris	R-	$EC_{50,\ 96h}$	8.496 mg/L	
			S-	$EC_{50,\ 96h}$	0.488 mg/L	
			Rac-	$EC_{50,\ 96h}$	0.348 mg/L	
		Scenedesmus obliquus	R-	$EC_{50,\ 96h}$	1.300 mg/L	
			S-	$EC_{50,\ 96h}$	1.377 mg/L	
			Rac-	$EC_{50,\ 96h}$	11.541 mg/L	
fenamiphos		*Daphnia magna*	R-(+)-FAP	$LC_{50,\ 24h}$	5.1 μg/L	(*21*)
			S-(-)-FAP	$LC_{50,\ 24h}$	9.2 μg/L	
			rac-FAP	$LC_{50,\ 24h}$	6.4 μg/L	
			R-(+)-FAP	$LC_{50,\ 48h}$	2.7 μg/L	
			S-(-)-FAP	$LC_{50,\ 48h}$	6.6 μg/L	
			rac-FAP	$LC_{50,\ 48h}$	3.9 μg/L	
Fenoxaprop		*Scenedesmus obliquus*	Rac-	$EC_{50,\ 24h}$	16.1 mg/L	(*22*)
			R-	$EC_{50,\ 24h}$	15.8 mg/L	
			Rac-	$EC_{50,\ 48h}$	8.42 mg/L	
			R-	$EC_{50,\ 48h}$	8.03 mg/L	
			Rac-	$EC_{50,\ 72h}$	7.81 mg/L	
			R-	$EC_{50,\ 72h}$	8.41 mg/L	
			Rac-	$EC_{50,\ 96h}$	9.76 mg/L	
			R-	$EC_{50,\ 96h}$	9.75 mg/L	

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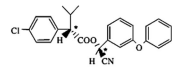
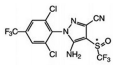
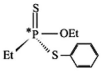
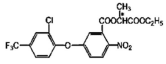
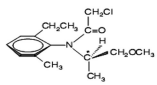
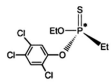
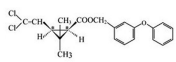
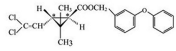
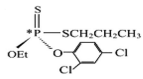
Table I. (Continued). The toxicity of different chiral pesticides to different kinds of water species

Fenvalerate		*Daphnia magna*	FV	$LC_{50,\ 24h}$	0.41 μg/L	(*23*)
			aS-2S- FV	$LC_{50,\ 24h}$	0.86 μg/L	
			aR-2S-FV	$LC_{50,\ 24h}$	24.8 μg/L	
			aS-2R-FV	$LC_{50,\ 24h}$	9.53 μg/L	
			aR-2R-FV	$LC_{50,\ 24h}$	0.49 μg/L	
		Danio rerio	FV	$LC_{50,\ 24h}$	28.8 μg/L	
				$EC_{50,\ 48h}$	17.9 μg/L	
				$EC_{50,\ 72h}$	12.4 μg/L	
				$EC_{50,\ 96h}$	8.29 μg/L	
			aS-2S-FV	$LC_{50,\ 24h}$	29.4 μg/L	
				$EC_{50,\ 48h}$	20.2 μg/L	
				$EC_{50,\ 72h}$	15.8 μg/L	
				$EC_{50,\ 96h}$	10.5 μg/L	
			aR-2S-FV	$LC_{50,\ 24h}$	240 μg/L	
				$EC_{50,\ 48h}$	221 μg/L	
				$EC_{50,\ 72h}$	210 μg/L	
				$EC_{50,\ 96h}$	193 μg/L	
			aS-2R-FV	$LC_{50,\ 24h}$	142 μg/L	
				$EC_{50,\ 48h}$	128 μg/L	
				$EC_{50,\ 72h}$	115 μg/L	
				$EC_{50,\ 96h}$	105 μg/L	
			aR-2R-FV	$LC_{50,\ 24h}$	14.5 μg/L	
				$EC_{50,\ 48h}$	9.98 μg/L	
				$EC_{50,\ 72h}$	5.36 μg/L	
				$EC_{50,\ 96h}$	3.48 '' g/L	
Fipronil		*Ceriodaphnia dubia*	+	$LC_{50,\ 48h}$	10.3 μg/L	(*24*)
			-	$LC_{50,\ 48h}$	31.9 μg/L	
			±	$LC_{50,\ 48h}$	17.7 μg/L	
Fonofos		*Ceriodaphnia dubia*	rac±	$LC_{50,\ 96h}$	0.22 μg/L	(*2*)
			1r +	$LC_{50,\ 96h}$	2.24 μg/L	
			1s-	$LC_{50,\ 96h}$	0.15 μg/L	
		Daphnia magna	±	$LC_{50,\ 96h}$	0.58 μg/L	
			+	$LC_{50,\ 96h}$	3.45 μg/L	
			-	$LC_{50,\ 96h}$	0.23 μg/L	
Lactofen		*Daphnia magna*	rac-	$LC_{50,\ 48h}$	4.30μg/mL	(*25*)
			r-(-)-	$LC_{50,\ 48h}$	0.378μg/mL	
			s-(+)-	$LC_{50,\ 48h}$	17.689μg/mL	
Metolachlor		*Chlorella pyrenoidosa*	Rac-	$EC_{50,\ 24h}$	0.196 mg/L	(*26*)
				$EC_{50,\ 48h}$	0.241 mg/L	
			S-	$EC_{50,\ 24h}$	0.116 mg/L	
				$EC_{50,\ 48h}$	0.106 mg/L	
		Scenedesmus vacuolatus	S-	$EC_{50,\ 18h}$	17 mg/L	(*27*)
				$EC_{50,\ 24h}$	5.5 mg/L	
				$EC_{50,\ 48h}$	2.3 mg/L	
			Rac-	$EC_{50,\ 24h}$	0.232 mg/L	(*28*)
		Daphnia magna	Rac-	$LC_{50,\ 24h}$	69.4 mg/L	(*29*)
			S-	$LC_{50,\ 24h}$	51.2 mg/L	
Trichloronate		*Ceriodaphnia dubia*	±	$LC_{50,\ 96h}$	0.26μg/L	(*30*)
			+	$LC_{50,\ 96h}$	0.68μg/L	
			-	$LC_{50,\ 96h}$	0.06μg/L	
		Daphnia magna	±	$LC_{50,\ 96h}$	0.51μg/L	
			+	$LC_{50,\ 96h}$	1.39μg/L	
			-	$LC_{50,\ 96h}$	0.17μg/L	
Metalaxyl		*Daphnia magna*	Rac-	$LC_{50,\ 48h}$	51.5 mg/L	(*31*)
			R-	$LC_{50,\ 48h}$	41.9 mg/L	

Continued on next page.

Table I. (Continued). The toxicity of different chiral pesticides to different kinds of water species

cis-permethrin		*Ceriodaphnia dubia*	±	$LC_{50,\ 96h}$	0.539 μg/L	(*2*)
			+	$LC_{50,\ 96h}$	0.156 μg/L	
			-	$LC_{50,\ 96h}$	>6.0 μg/L	
		Daphnia magna	±	$LC_{50,\ 96h}$	0.788μg/L	
			+	$LC_{50,\ 96h}$	0.388μg/L	
			-	$LC_{50,\ 96h}$	>6.0 μg/L	
trans-permethrin		*Daphnia magna*	+	$LC_{50,\ 96h}$	0.307 μg/L	
			-	$LC_{50,\ 96h}$	>6.0 μg/L	
			±	$LC_{50,\ 96h}$	0.738 μg/L	
		Ceriodaphnia dubia	+	$LC_{50,\ 96h}$	0.197 μg/L	
			-	$LC_{50,\ 96h}$	>6.0 μg/L	
			±	$LC_{50,\ 96h}$	0.519 μg/L	
Profenofos		*Ceriodaphnia dubia*	+	$LC_{50,\ 96h}$	1.68 μg/L	
			-	$LC_{50,\ 96h}$	0.14 μg/L	
			±	$LC_{50,\ 96h}$	0.17 μg/L	
		Daphnia magna	+	$LC_{50,\ 96h}$	2.32 μg/L	
			-	$LC_{50,\ 96h}$	0.35 μg/L	
			±	$LC_{50,\ 96h}$	0.69 μg/L	

Chiral center is denoted by anasterisk(*)

Long Term Effects

The enantioselective toxicity of chiral pesticides to aquatic organisms can also be shown over time, resulting in long-term effects such as effects on biochemical parameters, structure, and function. Various toxic responses to chiral pesticides have been observed among aquatic organisms. These toxicities can induce biochemical changes in cell components, disturb intracellular signaling pathways, result in blocking mRNA, hamper metabolic protein synthesis, and cause abnormal cell growth/viability.

Enantio-difference of biochemical parameters of treated algaes were observed. The *Chlorophyll a* (*Chla*) and *Chlorophyll b* (*Chlb*) concentrations and the catalase activity of *Chlorella pyrenoidosa* were used to evaluate the enantioselective toxicity of *rac*-metolachlor and *S*-metolachlor (*26*). In another study, a drastic decrease in algal cell permeability occurred when treated with rac-diclofop and the *R*(+) isomer, while for *S*-diclofop, low levels ranging from 1.0 to 5.0 mg L^{-1} increased algal cell permeability to different extents (*19*). *Daphnia magna*, the Japanese medaka *Oryzias latipes*, and the electric eel *Electrophorus electricus* were used in a study by Nillos et al. (*32*) to investigate the enantioselective acetylcholinesterase (AChE) inhibition of the organophosphorus insecticides profenofos, fonofos, and crotoxyphos. The overall results showed variable sensitivity between AChE enzymes from different species as well as variable magnitudes of enantioselectivity in enzyme inhibition. In another study, significantly different endogenous metabolite pattern responses were observed in fish livers when rainbow trout were exposed separately to each of the two enantiomers of triadimefon (*33*).

Chiral pesticides can change cell structure as well. Liu's study on the toxicity of the chiral herbicides *rac*-metolachlor and *S*-metolachlor to *C. pyrenoidosa* showed ultrastructural morphology differences between control cells and cells grown in the presence of the two herbicides. Exposure to *rac*- and *S*-metolachlor resulted in a significant increase in the number and size of starch granules,

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while the size of the pyrenoid was reduced. Although the effects of *rac*- and *S*-metolachlor on the structure of typical cells had no significant difference, the cells treated with *S*-metolachlor were lesser in number than the cells treated with *rac*-metolachlor (*26*). Xu et al. (*34*) investigated the differential toxicities of the pyrethroid insecticide lambda-cyhalothrin to zebrafish (*Danio rerio*). The malformations were induced by the racemate and its (-)-enantiomer at lower concentration (50 μg L^{-1}), whereas the (+)-enantiomer induced malformations at relatively higher concentrations (100 μg L^{-1}). Similarly, the *1R-cis*-αS and *1R-trans-αS* enantiomers of insecticide beta-cypermethrin showed strong developmental toxicities at concentration of 0.1 mg L^{-1}, while the *1S-cis-αR* and *1S-trans-αR* induced no malformations at higher concentration (e.g., 0.3 mg L^{-1}) (*35*).

Chiral pesticides may also lead to physiological parameters changes of aquatic organisms. Survival, longevity and fecundity of the freshwater cladoceran *Moina macrocopa* were reduced at 0.05 mg L^{-1} and higher concentrations of the insecticide *S*-methoprene (*36*). Chiral toxicity of metolachlor in *D. magna* (*29*) showed that longevity and the number of broods per female of *D. magna* were significantly ($P < 0.05$) affected by *rac*- and *S*-metolachlor at different concentrations. Significant inhibitory effects happened when *rac*-metolachlor concentration was higher than 1.0 mg L^{-1}, but there were no significant affects until *S*-metolachlor concentration was higher than 10 mg L^{-1}. Based on the significant difference of reproduction parameters (body length, days-to-first-brood, number of broods/young per female) in different treatments at different concentrations, the *LOEC* and *NOEC* of *Rac*-metalaxyl to *D. magna* were 2 and 1 mg L^{-1}, respectively, whereas those of *R*-metalaxyl were 1 and 0.1 mg L^{-1}, respectively (*31*).

Significant differences were detected between enantiomers of permethrin in the induction of estrogen-responsive gene expression. At the exposure level of 1,000 ng L^{-1}, the vtg1, esr ∂ and cyp 19b of embryo-larval zebrafish responses to the (-)-*trans* enantiomer were about 3.2-, 1.8- and 1.5-fold higher, respectively, than those in the group treated with (+)-*trans* enantiomers ($p < 0.05$). And (+)-*cis* increased the RNA level of the cyp 19b gene about 1.5-fold higher than the (-)-*cis*-enantiomer did (*37*).

Factors That Influence Aquatic Toxicity

The direction and degree of enantioselectivity is not always predictable. Both stereochemistry of a chiral compound and environmental conditions influence the direction and rate of enantioselective degradation, which leads to enantioselective toxicity to aquatic organisms. Enantioselectivity in aquatic toxicity of chiral pesticides may closely depend on bioenvironmental conditions (species and biological and physiological conditions), the nature of the chiral pesticides, time, and even nonbiological environmental factors. As a result of different levels of microbial metabolism, degradation, and biological enrichment in aquatic organisms, chiral pesticides may show different acute and chronic toxicity to aquatic organisms.

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Bioenvironmental Factors

Pesticides can be selectively metabolized by organisms. Enantiomers of chiral pesticides, unlike diastereomers, have identical physical–chemical (i.e., achiral) properties (*38*) 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 (*24*). Aquatic organisms at different physiological stages show sensitivity to chiral pesticides stereoselectively. Days to first brood of *D. magna* was not affected by the presence of either *rac*- and *S*-metolachlor. But after *D. magna* gave birth to the first brood, the difference in toxicity to *D. magna* between *rac*- and *S*-metolachlor became significant (*29*).

Microbially-mediated processes in aquatic environments can include enantioselective degradation and can therefore change the enantiomeric composition over time. In one study, o,p-DDD, a chiral metabolite, was found in fish tissue after exposure to DDT, occurring primarily as the (*S*)-(−)-enantiomer. The enantiomer fraction for o,p-DDD in two-thirds of the fish samples was between 0.29 and 0.44 (*39*), indicating that biotransformation had occurred. In addition, studies about metabolism and dissipation of fipronil (*40*) indicated that it may be converted to desulfinyl, sulfone, and sulfide metabolites, which compared to parent fipronil are reported to have equal or more toxic potential and are more environmentally stable in water and soil. The fipronil was rapidly biotransformed by the rainbow trout with a preference for the biotransformation of the *S*-(+)-enantiomer to fipronil sulfone (*41*). It has also been reported that many ALS-inhibiting herbicides and their derivatives are chiral with one or more enantiomers, such as all the imidazolinone herbicides and some sulfonylurea and sulfonylamino-carbonyltriazolinone derivatives (*42*).

Enantioselective biodegradation of the enantiomers was observed in both field applications and laboratory microcosms (*43*). The isomers of pyrethroids were generally degraded selectively. The *EF* values for *cis*-permethrin obtained ranged from 0.412 to 0.535 in all samples, indicating that there was enantioselective degradation (*44*). Kurt-Karakus et al. compared concentrations and stereoisomer ratios of dichlorprop in Ontario streams in 2006–2007 vs. 2003–2004 (*12*). The results showed that residues were dominated by the *S*(−) enantiomer in both time periods (83–90%). The presence of substantial *S*(−)-mecoprop and *S*(−)dichlorprop residues in all years is likely an indication of enantioselective degradation and/or interconversion of enantiomers within the watershed and / or streams. The enantioselective degradation of α-hexachlorocyclohexane (α-HCH) in the water and snow samples collected at Amituk Lake on Cornwallis Island was found, with *ERs* were 0.77 ± 0.004 (*45*).

Selective degradation of chiral pesticides in the environment lead to difference in their retention time, which may result in different chronic toxicity to the aquatics. Konwick et al. studied bioaccumulation and biotransformation of chiral triazole fungicides in rainbow trout (*Oncorhynchus mykiss*) (*24*). The results showed that half-lives of triazoles ranged from 1.0 ± 0.2 days for tebuconazole to 2.5 ± 0.6 days for penconazole in rainbow trout. The rapid formation of fipronil sulfone, a known metabolite of fipronil, was found to persist

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longer ($t_{1/2}$~2 d) than its parent compound fipronil ($t_{1/2}$~0.6 d) and needs to be considered in fate studies of fipronil (*41*). Pereira and Hostettler (*46*) found no evidence of degradation of deethylatrazine, a major metabolite of atrazine, during its transit time (45–65 d) in the entire navigable reach of the Mississippi River, U.S.A. And a laboratory study found this degradation product to be nearly as toxic as the parent compound to estuarine microbial communities (*47*). Moreover, in some cases, it has been demonstrated that the less active enantiomer persists in the environment for much more time than the enantiomer with more insecticidal activity (*48*). More reports showed that the effects of chiral pesticides on aquatic environments are also due to their enantioselective degradation products, which can be more toxic than the original substances (*40*).

Pesticides can also be bioaccumulated by organisms and then the toxicity of chiral pesticides may change. Zhao et al. (*49*) reported that the enantioselectivity in chronic aquatic toxicity is reasonably consistent with the accumulation of *cis*-BF by organism. As the degradation of chiral pesticides may be enantioselective, resulting in different distribution patterns and bioaccumulation potentials between enantiomers (*50*). A preferential degradation of the (−)-enantiomer of *cis*-bifenthrin resulted in a relative enrichment of the aquatically active (+)-enantiomer (*2*). Some pesticides like endosulfan which was not toxic to algae at levels likely to be found in the environment (< 1 mg L^{-1}), the compound may bioaccumulate in algae and be consumed in higher concentrations by grazers (*7*), such pesticide may pose a threat to higher estuarine organisms. Rao and Lal (*51*) found rapid uptake of the commonly used agricultural pesticide endosulfan in the cyanobacteria *Aulosira* and *Anabaena*, with levels reaching 700 times the exposed dose within 48 h. Pesticides accumulated in prey species may then cause toxicity in the organisms consuming them or continue to bioconcentrate through the food web (*7*). Enrichments in organic nutrients shifted the enantioselectivity for methyl-dichlorprop towards the preferential degradation of the (*S*)-enantiomer (*52*).

Chiral pesticides may be sediment adsorption, and contaminated sediments can be continually resuspended, especially true in estuaries, because of tidal action and dredging. Hence, the degradation of chiral pesticides in sediments can be the potential element leading to enantioselective toxicity to aquatic organisms. *S*-(+)-lactofen or *S*(+)-desethyl lactofen was preferentially degraded in sediments, resulting in relative enrichment of the *R*-(-)-form (*25*). Enantioselective attenuation of *cis*-BF and *cis*-PM was performed in the sediments collected at a site next to a nursery in Southern California (*43*). The results showed that the *S*-enantiomers of BF and PM were preferentially degraded, and then cause relative enrichment of the *R*-enantiomers. Enantioselectivity was also evaluated in the course of degradation of cypermethrin by *Ceriodaphnia dubia* in sediments (*53*).

Nonbiological Environmental Factors

Although nonbiological environmental factors will not change their aquatic toxicity directly, the biological growth and metabolism of the aquatic organisms can be affected by these nonbiological conditions, which may indirectly lead to stereoselectivity in toxicity. Factors such as pH, salinity and nutrient

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concentrations in the system may alter the response of aquatic organisms to pesticides. There is evidence that atrazine becomes more toxic to phytoplankton under nutrient-enriched conditions (*47*). Another study found that natural periphyton communities in low-nutrient environments were less likely to recover from herbicide stress than those in medium- and high-nutrient environments (*54*). Furthermore, the direction of the degradation can be influenced by the sampling location and environmental conditions (*16*). These differences in degradation may due to the variations of the microbial population as a result of pH and soil oxidation state (*55*).

The enantioselective behaviors of chiral compounds in the environment might be shifted when interactions with other chiral receptors. Study showed that chitosan can change the enantioselective bioavailability of dichlorprop (*56*). In the absence of chitosan, the toxicity of (*R*)-enantiomer to *Chlorella pyrenoidosa* was more potent than that of the (*S*)-enantiomer. While (*R*)-enantiomer was less toxic than (*S*)-enantiomer in the presence of chitosan. Results showed that the addition of a certain amount of β-CD and HP-β-CD reduced the toxicity of fenoxaprop (FA) to *Scenedesmus obliquus* and changed the enantioselectivity in toxicity of FA to *S. obliquus* (*22*). Analysis of the joint toxicity of binary mixtures of isocarbophos enantiomers toward *D. magna* clearly showed an additive effect from the individual enantiomers (*1*), implying that the joint acute toxicity for enantiomer mixtures of chiral xenobiotics may show different modes of interactions when present in a mixture.

The enantioselective toxicity to aquatic organisms may vary with different chiral pesticides. Different chiral pesticides (different structure, molecular weight, chiral center, etc.) may show stereoselective toxicity to aquatic organisms. It has been reported that triazoles (myclobutanil and penconazole) with the highest percentage of elimination through biotransformation had either an oxygen atom or hydroxyl group adjacent to a chiral center, while lowest percentage of their elimination through biotransformation did not contain an oxygen atom within the molecule (*24*). The oxygen atom has great reaction potential through phase one (oxidation, reduction, and hydrolysis) biotransformation reactions, which may result in ring cleavage adjacent to the oxygen atom and/or the formation of hydroxyl groups. Some chiral pesticides containing several chiral centers have multiple pairs of enantiomers, although the active ingredients may show the same effects to the targets, the toxicity may be stereoselective. Many other examples showed stereoselective toxicity of chiral pesticides to the same aquatic (Table I).

The toxicity of chiral pesticides to aquatic organisms changes with the concentration. Based on the significant differences in reproduction parameters of *D. magna* exposed to different treatments at different concentrations, the *LOEC* and *NOEC* of *rac*-metolachlor were 0.01 and 0.001 mg L^{-1}, respectively, while those of *S*-metolachlor were 0.5 and 0.1 mg L^{-1}, respectively (*29*). The researches showed that body length, number of broods per female and number of young per female were affected greatly at higher concentration (0.01, 0.1, 1, 2, 5 mg L^{-1}) of *Rac*- and *R*-metalaxyl. And the significant differences between them were showed by *R*-metalaxyl at >1.0mg L^{-1}, by *Rac*-metalaxyl at ≥ 2.0 mg L^{-1} (*31*). Compared to blank control, *rac*-metalaxyl at low level (0.05 and 0.5 mg L^{-1}) stimulated the

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growth of *S. quadricanda*. However, for *R*-metalaxyl it did not influence algae growth at low concentrations (0.05 mg L^{-1} and 0.1 mg L^{-1}) (*57*).

Toxicity of chiral pesticides may vary with the time. Significant differences in the LC_{50} were observed for the two pyrethroid isomers with the (−)-enantiomer 60 times more toxic after 24 h and 162 times more toxic after 96 h than the (+)-λ-cyhalothrin (*18*). The order of EC_{50} (racemate of FV to zebrafish) values were 28.8 g L^{-1}, 17.9 g L^{-1}, 12.4 g L^{-1}, and 8.29 g L^{-1} at 24 h, 48 h, 72 h and 96 h, (Table I), suggesting that the toxicity of *rac*-FV to zebrafish increased with time (*23*). And toxicity of each isomer of FV (*aS-2S*-FV, *aR-2S*-FV, *aS-2R*-FV and *aR-2R*-FV) showed the same trends. Lin et al. (*58*) investigated stereoselective acute aquatic toxicity of fosthiazate to *D. magna*. The differences among isomers varied with the exposure time. The maximum differences observed after exposure of 24, 48, 72 and 96 h were 2.2-fold (pk1/racemate), 3.1-fold (pk1/pk3), 3.1-fold (pk1/pk3), and 2.5-fold (pk1/pk3), respectively.

In addition, testing conditions also play a role in toxicity of chiral pesticides. Study showed that the growth of *S. capricornutum* exposed to the allelochemical ethyl-2-methyl acetoacetate (EMA) under different initial algal densities exhibited a contradictive response. The algal growth was inhibited by EMA at low initial algae densities but stimulated at high initial algae densities (*59*). The sensitivity of *S. vacuolatus* to *S*-metolachlor in synchronous culturing was more than 15 times greater than culturing in continuous light (*27*). Under aerobic or slightly reduced conditions, biodegradation of fipronil in sediments was essentially nonstereoselective, with the enantiomeric fraction (*EF*) similar to racemic (*EF* = 0.5) after 168 d of incubation. However, *EF* decreased to as low as less than 0.1 following short incubations under anaerobic conditions, suggesting preferential degradation of *S*-(+)-fipronil in strongly reduced sediments (*60*). Similar phenomenon of fipronil transformation (to fipronil sulfide) under anaerobic conditions were observed in studies by Jones et al. (*61*).

Mechanisms of Toxicity

Mechanisms of toxicity vary depending on the type of pesticide and the aquatic species exposed. In microorganisms, pesticides have been shown to interfere with respiration, photosynthesis, and biosynthetic reactions as well as cell growth, division, and molecular composition (*7*).

Research showed that paraquat increased the activities of the antioxidant enzymes superoxide dismutase, peroxidase, and catalase compared to the control (*62*). The higher concentration of paraquat, the higher levels of these antioxidant enzymes. Paraquat reduced the transcript abundance of psaB and rbcL to 7.09 and 29.83% of the control, respectively. Another study of ATZ toxicity to zebrafish (*D. rerio*) showed the induction of oxidative stress and the alteration of gene expression in liver and ovary samples from female *D. rerio* (*63*). Yao et al. (*57*) reported that sometimes difference in toxicity for individual forms of chiral pesticides originated from different reaction of individual enantiomer with target enzyme.

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Significant differences were detected between the enantiomers in induction of hepatic gene transcription of male adult zebrafish. At exposure level of 500 ng L^{-1}, the response to the (−)-trans enantiomer was 2.6 and 1.8 times greater than the (+)-*trans* enantiomer based on zebrafish vtg1 and vtg2 mRNA induction ($p < 0.05$), respectively (*64*). It has also been reported that the expressions of estrogen receptor alpha in zebrafish embryo exposed to insecticide acetofenate varied. Data of qRT-PCR showed that there was about 3.2-fold induction in the mRNA levels of *ER∂* between fish exposed to (+)-acetofenate and (-)-acetofenate (*18*). In addition, there was a drastic difference between *1S-cis*-bifenthrin and *1R-cis*- bifenthrin in their relative estrogenic potential for inducing vitellogenin production of the adult male medaka. The average level of vitellogenin in the adult male medaka exposed to *1S-cis*-bifenthrin for 10 d was 1532 ng mg^{-1}, while that with exposure to *1R-cis*-bifenthrin was only 12.45 ng mg^{-1} (*65*).

Cellular systems scavenge the active oxygen species by invoking antioxidative machinery such as superoxide dismutase, catalase, and peroxidase (*66*). It is well known that oxygenation is the first step in the metabolism of pesticides and other organic xenobiotics. In various detoxification reactions, a number of enzymes use glutathione (GSH) and thioredoxins (TRXs1–3) as cofactors, whereas other ROS-removing enzymes, such as superoxide dismutase, glutathione peroxidase, catalase, thioredoxin peroxides and cytochrome c peroxidase, use alternative hydrogen donors like O, -OH, O_2^- , and H_2O_2 (*67*). So, if an antioxidant enzyme in vivo is inhibited by chiral pesticides stereoselectively, it could lead to different stress responses by the organism to each enantiomer of a chiral pesticide.

In chiral notation, *R* and *S* refer to the absolute configuration or the orientation in space of the groups around the chiral center of the enantiomer. This orientation is the important factor in determining the fit with enzymes and other biological molecules (*33*). Thus, the highly stereospecific interaction between an herbicide and an enzyme could be explained by the three-point model proposed by Easson and Stedman in 1993 (*68*) and the four-location model developed by Mesecarin in 2000 (*69*). As the isomers differ in their three-dimensional configurations, exists in two forms which are mirror images of each other, and the enzyme is also chiral with a specific stereostructure (*70*), therefore, there are greater chances that one isomer of a chiral herbicide could block the channel better than the other, causing different enzyme activity. Based on the structural information about the binding site and the inhibitory mechanism, Wang et al. (*21*) used molecular modeling to explain the enantioselective toxicity between *R*-(+)- fenamiphos and *S*-(-)- fenamiphos.

Conclusion

Chiral pesticides can show very different biological activity when exposed to an identical biological environment. The factors resulting in stereoselective toxicity to aquatic organisms involve: biological action (biodegradation, metabolism and bioaccumulation, etc.), abiotic environmental factors (indirect factors), type and dose of chiral pesticides, time and testing conditions as well. Although deleterious effects have been documented in aquatic organisms for

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many chiral pesticides at different levels, the mechanism of toxicity to aquatic organisms remains unclear, especially at the level of stereoselectivity. Aquatic systems may be contaminated with a number of different pesticides, further study is needed to better understand the joint toxicity of chiral pesticide in aquatic.

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Chapter 8

Enantioselectivity in Estrogenic Potential of Chiral Pesticides

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The increasing release of chiral chemicals into the environment dictates attention to a better understanding of enantioselectivity in their human and ecotoxicological effects. Some chiral pesticides are known to have endocrine disruption potential as endocrine disruption chemicals (EDCs). In this review, we outline our endeavor to evaluate the occurrence of enantioselectivity in endocrine disruption of typical chiral pesticides. Given the complexity of endocrine system and the view that the enantioselectivity is expected to result in ecotoxicological effects, more comprehensive research should be focused on discoveries the enantioselective mechanism and cross-disciplinary studies.

Introduction

The endocrine system is one of the most important regulators in human or animal bodies, acting through messengers called hormones. Hormones are transported by the bloodstream to various organs and tissues that recognize and respond to them. Based on the "key and lock" principle, they bind to different kinds of receptors. The endocrine system regulates pivotal functions in the body, including growth, development, reproduction, and metabolism. Nowadays,

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scientists discovered that exogenous substances in the environment may interrupt the normal regulation physiologic function and release excessive or not enough hormones in body. These kinds of substance are called endocrine disrupting chemicals compounds (EDCs). It is well known that prenatal or early postnatal exposure to EDCs could result in permanent and irreversible damage to wildlife and humans (*1*). Several studies on wildlife populations have documented adverse effects that correlated with exposure to one or several EDCs (*2*–*7*). For instance, alligators in Lake Apopka, Florida, which was polluted by a spill of organochlorine pesticides, suffered a host of morphological and hormonally related abnormalities of the male and female reproductive tracts (*2*, *3*). Other hormone-disruption effects include eggshell thinning in avian species (*4*, *5*), sex reversal in Japanese Medaka (*Oryzias latipes*) (*6*), and changes in sexual differentiation and behavior in mice (*7*). Epidemiological studies which were linked with exposure to EDCs reported significant increase in the incidence of breast, prostate and testicular cancer, decreasing sperm counts and semen volume and longer times to conception, all of which injured reproductive fitness to humans (*8*). The situation with environmental exposure to EDCs is grim and it is imperative that we have a comprehensive understanding of this issue.

Pesticide use has increased 50-fold since 1950, and seventy-five percent of all pesticides in the world are used in developed countries (*9*). Many of these compounds are now suspected of being EDCs that can lead to an increase in birth defects, sexual abnormalities and reproductive failure (*10*–*12*). To date, the first important synthetic organic pesticides organochlorine pesticides (OCPs), such as *p,p′*-dichlorodiphenyl trichloroethane (DDT) (*13*), methoxychlor (*14*), endosulfan, toxaphene, and dieldrin, (*15*) have been reported as being estrogenic. Furthermore, the mainstay of modern insecticides– synthetic pyrethroids (SPs) scuh as fenvalerate (Fen) (*16*), and deltamethrin (*17*), and organophosphates (OPs) such as malathion (*18*) and chlorpyrifos (*17*) have been reported as estrogenic pesticides. In 2009, the U. S. Environmental Protection Agency issued the first test orders for pesticides to be screened for their potential effects on the endocrine system (*19*). Testing will eventually be expanded to cover all pesticide chemicals (Table 1). Chiral pesticides account for more than 40% in China (*20*), indicating that one pesticide consists of two or more enantiomers. The more complex structures are being registered for use, in the foreseeable future, is followed by the increasing proportion of chiral pesticides. Enantiomers of chiral pesticides have identical physical and chemical properties except when they interact with enzymes or with other chiral molecules, which lead to different mode of action. Despite this fact, almost all chiral pesticides are manufactured and applied as mixtures of equal amounts of the two enantiomers (racemic mixtures). The uncertainly injecting in the risk assessment of chiral pesticides indicated the necessary to understand the nature of enantioselectivity of single enantiomers.

Recently, many scientific papers have documented non-racemic residues of chiral pesticides in soil, sediment, and water as well as animal tissues (*21*, *22*). In recent years, the importance of stereospecific effect of chiral pesticides has been aroused (*23*, *24*). Endocrine disruption is not merely toxic endpoint as observed in chronic or acute toxicity but an imbalance of body hormone. The safty evaluation of chiral pesticides should involve this property in endocrine disruption since low

level of EDCs can cause the abnormal regulation of hormone when compared to acute toxicity.

Bioassay for Estrogenic Chemicals

There are several methods developed for assessing the ability of potential xenoestrogens, including *in vitro* assays and *in vivo* assays. These *in vitro* methods include, (1) Cell proliferation assay which is referred to as E- SCREEN assay (*15*), which tests the estrogenic potential of xenobiotics by using proliferative effect of estrogenic dependent cell lines and has been proved to be a simple and sensitive *in vitro* test (*25*). (2) Competitive ligand binding, extensively used to investigate ER-ligand interactions (*26*). (3) Induction of protein expression or enzyme activity assays (*27*), which includes the increased expression of secreted proteins such as pS2, cathepsin D, protectin and sexhormone binding globulin. (4) Recombinant receptor/receptor gene assays (*25*) and yeast based assays. The yeast two-hybrid system (YTHS) has been used as a simple screen to prioritize candidates of EDCs (*30*, *31*). It not only provides the opportunity to study the interaction between ligand and ER but also homologous to mammalian cells necessary for controlling transcription (*32*). *In vivo* methods include the end points effect on organ weights, cell differentiation, protein expression and enzyme activities. Among them, the uterine weight assay is considered to be the hallmark of estrogenic activity. The Vitellogenin (VTG) induction in liver cells of oviparous vertebrate species has also been considered as a simple and sensitive method to assess environmental estrogens (*28*, *29*). It cut the labor intensive than *in vivo* assays but still retain its metabolic competence, and can therefore detect in situ.

Except for the robust framework developed for ecological risk assessment for estrogenic disruptors, the solvent using in most of bioassays as a hydrotropy agent requires extra caution in chiral chemical research. A cluster of research showed that several kinds of chemicals, especially SPs, can undergo epimerization or racemization and enantiomerization in solvent such as alcohol (*33–35*). The reason for the racemization could be attributed to the active proton at the chiral center of α-carbon position (*36*). In protic solvent, such as water, alcohol, interconvering enantiomer would be happen through reprotonation regenerates because of the characteristic for easily carbanion creation at chiral center. Chemicals given the structural similarity of α-carbon containing cyano group such as permethrin, cyfluthrin, deltamethrin, Fen, malathrin, triadimefon would occur enantiomerization in solvent, which is dependent the ratio of solvent to water ratio (*33*, *35–38*). Since racemication may contribute to the incorrect interpretation of bioassay data for stereo-specific effects, appropriate solvent should be considered when go through bioassay in solvent-water circumstance.

Enantioselective Estrogenic Activity of Chiral Pesticides

The adverse effects for man-made EDCs on both humans and wildlife have recently become an issue of great concern. Based on the previous researches, some chiral pesticides including OCPs (*39*1), SPs (*40*), atrazine (*41*) are EDCs. More

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and more research indicates that the enantioselectivity is a normal phenomenon of chiral pesticides. Enantioselective endocrine disruption by pesticides is is discussed in this review article.

Table 1. United State Environmental Protection Agency: Endocrine disruptor screening program for pesticides

Chemical names	*CAS Number*
2,4-D	94-75-7
Acephate	30560-19-1
Atrazine	1912-24-9
Benfluralin	1861-40-1
Bifenthrin	82657-04-3
Carbamothioic acid, dipropyl-, *S*-ethyl ester (EPTC)	759-94-4
Carbaryl	63-25-2
Carbofuran	1563-66-2
Chlorothalonil	1897-45-6
Chlorpyrifos	2921-88-2
Chlorthal-Dimethyl (DCPA)	1861-32-1
Cyfluthrin	68359-37-5
Cypermethrin	52315-07-8
Diazinon	333-41-5
Dichlobenil	1194-65-6
Dicofol	115-32-2
Dimethoate	60-51-5
Disulfoton	298-04-4
Endosulfan	115-29-7
Ethoprop	13194-48-4
Esfenvalerate	66230-04-4
Fenbutatin oxide	13356-08-6
Flutolanil	66332-96-5
Linuron	330-55-2
Malathion	121-75-5
Metalaxyl	57837-19-1
Methamidophos	10265-92-6

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Table 1. (Continued). United State Environmental Protection Agency: Endocrine disruptor screening program for pesticides

Chemical names	*CAS Number*
Methidathion	950-37-8
Methomyl	16752-77-5
Methyl parathion	298-00-0
Metolachlor	51218-45-2
Myclobutanil	88671-89-0
Norflurazon	27314-13-2
Oxamyl	23135-22-0
Permethrin	52645-53-1
Phosmet	732-11-6
Piperonyl butoxide	51-03-6
Propachlor	1918-16-7
Propargite	2312-35-8
Propiconazole	60207-90-1
Quintozene (PCNB)	82-68-8
Resmethrin	10453-86-8
Simazine	122-34-9
Tebuconazole	107534-9-63
Tetrachlorvinphos/Gardona	22248-79-9
Triadimefon	43121-43-3

Organochlorine Pesticides

In past few decades, organochlorine pesticides have been widely used around the world. These compounds are particularly persistent in the environment, and are known to accumulate in sediments (*39*), plants and animals. Organochlorines have a wide range of acute and chronic human health effects, including cancer, neurological damage, and birth defects. Many organochlorines are also suspect EDCs, but studies on enantioselective estrogenicity is only available on *o,p′*-DDT [1,1,1-trichloro-2-(o-chlorophenyl)-2-(*p*-chloro- phenyl) ethane], *o,p′*-dicofol [1,1,1-trichloro-2-(o-chlorophenyl)- 2 -(*p*-chloro- phenyl) ethanol] and acetofenate (AF, also known as 7504, plifenate or benzethazet). In Western industrialized countries, the level of *o,p′*-DDT in human blood serum was about $0.3\sim2\times10^{-9}$ mol/L (*42*). Although banned for several decades, it is currently used in some tropical countries for control of malaria vectors, and the spraying of dicofol promotes the releasing of DDT to the environment (*43*). Early in 1968, investigations by Bitman showed the estrogenic activity of *o,p′*-DDT in

mammalian uterus and avian oviduct (*44*). Later in 1974, the compound was found to bind to the rat estrogen receptor (ER) (*45*). After that, researche concerning the estrogenic activity of *o,p′*-DDT was carried on in both *in vitro* and *in vivo* systems (*15*, *46–49*). By the development of chromatograph techniques, the pure enantiomers of *o,p′*-DDT could be obtained to carry on the enantioselectivity studies.

Thus far, about six scientific reports have focused on the enantioselective estrogenic activities of *o,p′*-DDT (*50–55*). All the results suggest that the *R*-(–)-*o,p′*-DDT enantiomer is the more active estrogen mimic (Table 2). *In vivo* test carried out in female rats indicate that the *R*-(–)-*o,p′*-DDT can be more active in increasing the uterine wet weight and uterine glycogen content (*50*). Later the authors found the same phenomenon in thses immature female quail (*51*). These results indicate that the *R*-(–) form of *o,p′*-DDT is more effective than the *S*-(+) form of both mammalian animals and birds. In our recent study, the *R*-(–)-*o,p′*-DDT significantly induced the proliferation in human breast cancer cell MCF-7 when compared with *S*-(+)-*o,p′*-DDT (*56*) , which can be inhibited by the antagonist of ER receptor (*56*). In technical DDT, *o,p′*-DDT is the most important estrogenic chemical among DDT isomers. The enantioselective studies indicated that the estrogenic activity can be mainly attributed to *R*-(-)-*o,p′*-DDT. As a result, considering the estrogenic effect ratio of *R*-(-)-*o,p′*-DDT to DDT and the residue percentile of *R*-(-)-*o,p′*-DDT in DDT would be more accurate in risk assessment of DDT, Meanwhile, this enantioselective estrogen potential of *o,p′*-DDT may due to the enantioselective affinity to estrogen receptors. The technical dicofol is synthesized from technical DDT by chlorination and hydrolysis. The *o,p′*-dicofol contained 20% of technical dicofol, which contains one chiral center as *o,p′*-DDT. The estrogenic activity of (-)-*o,p′*-dicofol is greater than the racemate, while the (+)-enantiomer was found to have no significance effect on yeast-based steroid hormone receptor gene transcription assay (*57*). Other OCPs, for example AF, possess similar chemical structure to DDT. In our study, AF induced not only enantioselective developmental toxicity but also a higher expression level for enantiomer-specific ERα in zebrafish embryo (*58*).

Synthetic Pyrethorids

Synthetic pyrethroids are man-made analogues of pyrethrins which were introduced in the early 1980s and widely utilized in killing a multitude of agricultural and indoor insect species, especially in households. It is estimated that SPs account of 20% of all agricultural insecticides applied to U.S. crop land (*59*), and approximately 70% of total pyrethroid usage occurs in urban areas in California (*60*). Normally, each pyrethroid contains 2-3 chiral centers and thus 2 or 4 pairs of enantiomers , which can arise from the acid moiety, the alcohol moiety, or both (*61*) Many studies showed that pyrethroids possess agonist or antagonist activities in endocrine disruption (*4*, *8*, *17*, *18*, *20*). For example, Fen, sumithrin, cypermethrin and deltamethrin displayed significant estrogenicity in MCF-7 human breast carcinoma cell line and Ishikawa Var-I human endometrial cancer cell line, while Fen and *d-trans* allethrin significantly antagonized the action of progesterone in the T47D human breast carcinoma cell line (*4*, *8*,

17). Bifenthrin (BF), lambda-cyhalothrin (λ-LCT) and Fen also induced thyroid hormone regulation dysfunction in rats (*18*, *20*). Considering the remarkably enantioselective acute toxicity for SPs on daphnids, we also endeavor to study the enantioselectivity in endocrine disruption activity. So far, three kinds of SPs (Table 3) have been investigated for their enantioselectivity in endocrine disruption, including *cis*-BF, permethrin (PM), λ-LCT and Fen.

Table 2. The enantioselective estrogenic activities of (+)-*o,p′*-DDTand (–)-*o,p′*-DDT

Organisms	*Endpoints*	*Estrogenic potency*	*References*
MCF-7	Cell proliferation↑ ERα mRNA expression↓ ERβ mRNA expression↓ Luciferase activity↑	(–)-*o,p′*-DDT > (+)-*o,p′*-DDT	(*43*, *46*)
Female rats	uterine wet weight↑ uterine glycogen content↑ Competition of ER binding↑	(–)-*o,p′*-DDT > (+)-*o,p′*-DDT	(*41*)
Female Japanese quail	oviducal wet weight↑ oviducal glycogen content↑	(–)-*o,p′*-DDT > (+)-*o,p′*-DDT	(*42*)
Two-hybrid yeast	*β*-Galactosidase activity↑	(–)-*o,p′*-DDT > (+)-*o,p′*-DDT	(*45*)
In vitro assay	Competition of ER binding↑	(–)-*o,p′*-DDT > (+)-*o,p′*-DDT	(*44*)

↑: up-regulated; ↓: down-regulated.

To assess the enantioselectivity in estrogenicty of SPs, many *in vivo* and *in vitro* models coupled with molecular docking were used. These data showed that enantioselectivity in estrogenic activity was a normal phenomenon. For example, two isomers of *cis*-BF showed different enantioselective effects on proliferation of the breast cancer cells MCF-7 cells. In the E-SCREEN assay, the relative proliferative effect ratios of 1*S*-*cis*-BF and 1*R*-*cis*-BF were 74.2% and 20.9%, respectively, and the relative proliferative potency ratios were 10% and 1%, respectively. Measurement of increase of vitellogenin level in Japanese medaka (*Oryzias latipes*) showed that 1*S*-*cis*-BF was about 123 times greater than that of the *R* enantiomer. And then, to assess the enantioselective estrogenic activities of

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cis-BF, the yeast two-hybrid assay showed that estrogenic effect of *cis*-BF could be mainly attributed to the 1*S*-*cis*-BF. ERα reporter gene was significantly induced by *rac*-BF and 1*S*-*cis*-BF with a maximal induction at 10^{-7} mol/L. However, the induction change in *β*-galactosidase activity by 1*R*-*cis*-BF was not observed (*62*). The influence on molting to mother daphnid of 1*S*-*cis*-BF in the chronic toxicity to *Daphnia magna* at the last seven days during 21 days exposure could be also attributed to the enantiomer-specific estrogenic activity (*63*). Except for *cis*-BF, three other SPs displayed remarkable enantioselectivity in estrogenic activity in different models. Zhao's report showed that λ- (–)-LCT not only induced the MCF-7 cells proliferation 1.8-fold but also down-regulated the expression of ERα gene about relative 3-fold to λ- (+)-LCT, as did the natural estrogen (*64*). The PM has two chiral centers and thus two pairs of enantiomers. Significant differences were detected among these isomers in the induction of vitellogenin (vtg) gene transcription in both male adult zebrafish and embryo-larval zebrafish (*65*, *66*). The *in vivo* study found that 7 d exposure to 250 ng/L PM racemate and its enantiomers was sufficient to stimulate vtg1, esrα and cyp19b expression, while 1000 ng/L exposure significantly induced gene expression in a pattern similar to that of the control (50 ng/L E_2), except for vtg2. Significant differences were detected between the enantiomers in the induction of estrogen-responsive gene expression. At the exposure level of 1000 ng/L, the vtg1, esrα and cyp19b responses to the (–)-*trans* enantiomer were about 3.2-, 1.8- and 1.5-fold higher, respectively, than those in the group treated with (+)-*trans* enantiomer ($p < 0.05$). In the two *cis*-enantiomer treatment groups, (+)-*cis* increased the mRNA level of the cyp19b gene about 1.5-fold higher than the (−)-*cis*-enantiomer did. Of the four enantiomers, the (−)-*trans* enantiomer showed the greatest estrogenic activity (*65*). For Fen, our recent study with yeast two-hybrid and molecular docking assay (see Fig 1) showed that the *αR*-*2R*-Fen and *αS*-*2S*-Fen can induce the *β*-galactosidase activity, which were also have the stronger developmental toxicity in zebrafish model when compares with the other two isomers (*62*, *67*).

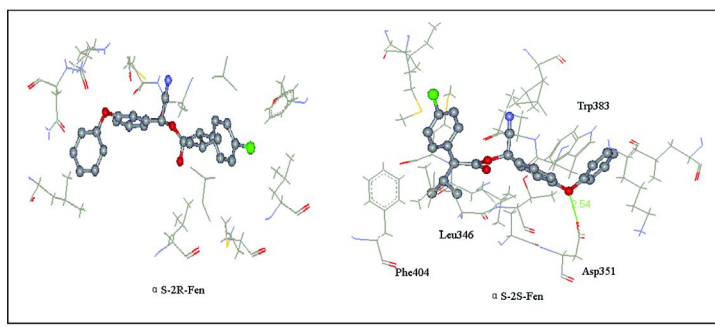

Figure 1. Different binding modes of αS-2R-Fen and αS-2S-Fen with residues in the peripheral 5 Å site of hERα.

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Table 3. The enantioselective estrogenic activities of synthetic pyrethroids (SPs)

Pesticides	*Organisms*	*Endpoints*	*Estrogenic potency*	*References*
Cis-Bifenthrin (*cis*-BF)	MCF-7	Cell proliferation↑	1*S*-*cis*-BF > 1*R*-*cis*-BF ((–)-*cis*-BF > (+)-*cis*-BF)	(*53*, *54*)
	Japanese Madaka	VTG induction↑		
Permethrin (PM)	Male adult zebrafish	Vtg1 mRNA expression↑	(–)-*trans*-PM > (+)-*trans*-PM (+)-*cis*-PM > (–)-*cis*-PM Maximum: (–)-*trans*-PM	(*55*, *56*)
		Vtg2 mRNA expression↑		
	Embryo-larvel zebrafish	Vtg1 mRNA expression↑		
		Esrα mRNA expression↑		
		Cyp19a mRNA expression↑		
		Cyp19b mRNA expression↑		
Lambda-Cyhalothrin (LCT)	MCF-7	Cell proliferation↑	(–)-LCT > (+)-LCT	(*54*)
		pS2 mRNA expression↑		
		ERα mRNA expression↓		

↑: up-regulated; ↓: down-regulated.

Mechanism of Estrogenic Activity and Environmental Implication of Chiral Pesticides

From *in vivo* and *in vitro* studies, researchers have learned much about the mechanisms through which EDCs influence the endocrine system and alter hormonal functions. It is well known that EDCs may mimic or partly mimic naturally occurring hormones in the body like estrogens, androgens and other hormones, potentially producing overstimulation. Sometime the EDCs bind to a receptor within a cell and block the endogenous hormone from binding. The normal signal then fails to occur and the body fails to respond properly. Another mechanism is that EDCs interfere or block the way natural hormones or their receptors such as estrogen receptors are made or controlled, for example, by blocking their metabolism in the liver or other organs. Research on endocrine disruption of chiral pesticides mainly focuses on the enantioselective estrogen

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potential. In the more direct mechanism the endocrine disrupter interacts with the ER producing a series of events to finally mimic and/or inhibit the estrogen effects. By contrast, the indirect mechanism involves several different signaling pathways in which the endocrine disrupters interact with steroidogenic enzymes, binding globulins, growth factors, and/or different receptor systems. When chemicals exert estrogenic activity, the ERα-, ERβ- and the ligand-independent mediated pathway, at the very least, are three pathways that considered as the key point of convergence with ER (*68*).

Based on the research with *o,p′*-DDT using *in vivo* and *in vitro* models, enantioselectivity in the estrogenic activity of *o,p′*-DDT may be correlated with its apparent ability to interact with the ERs. The (-) but not the (+) enantiomer of *o,p′*-DDT inhibits estradiol binding to ERα (*52*, *54*). The cell proliferation and mRNA expression completely blocked by ICI 182,780 also suggested that *o,p′*-DDT acted through the classical estrogen response pathway via ERα (*69*). The two-hybrid yeast assay showed that the *R*-(–)-*o,p′*-DDT induced the mRNA expression of the report gene mediated by hERα, whereas the activity of *S*-(+)-*o,p′*-DDT was negligible (*53*). These data indicate that the two enantiomers have different affiant to ERα. Interference of the ER-mediated signaling pathway may alter the imbalance transcription of mRNA of ERs in testing cells when exposure to EDCs (*41*). Our previous study showed that *R*-(–)-*o,p′*-DDT significantly altered not only ERα but also ERβ mRNA expression in MCF-7 cells. These results suggested that the signaling pathway of ERβ may also play an important role in enantioselectivity in the estrogenic activity of *o,p′*-DDT.

The enantioselective estrogen activity of SPs might be mainly attributed to the stereospecific receptor binding. Molecular docking data shows strong hydrophobic interaction and hydrogen bonding between estrogenic enantiomer of SPs and ERα. Our recent research, for the first time, demonstrated that the enantioselective estrogenic activity of SPs was due to selective binding between their isomers and the ERα receptor. For example, *αS-2S*-Fen embeds in a relatively blocked space comprised of Phe 404, Leu 346 and Trp 383 (Fig 1). There are hydrophobic interactions with the aromatic side chains of Trp 383, and the phenyl rings form a hydrogen bond to the chain of Asp 351, which is a common interaction between ligand and ERα (*45*). However, *αS-2R*-Fen is relatively far from the binding pocket and it can not form orderly interactions. Thus hydrophobic interactions and hydrogen bonding make ER bind to *αS-2S*-Fen but not *αS-2R*-Fen. These models are consistent with the enantioselective estrogenic potencies of *αS-2S*-Fen and *αS-2R*-Fen. In other words, the enantiomer of *αS-2S*-Fen has higher affinity with ERα and shows greater estrogen potential than the other enatiomer. But the molecular mechanisms of enantioselectivity in endocrine disruption by chiral pesticides are far more complicated. So accurate understanding of mechanisms requires further research.

In addition, environment fate of chemical such as biotransformation and biodegradation is also an important mechanism for the estrogenic activity induction. Chemicals like PCBs and BDE-47 increase the endocrine disruption potencies with hundreds of times higher than the parent chemicals by introduction an OH-group during biotransformation (*70*, *71*). The demethylated metabolites of methoxychlor (MXC), an organochlorines pesticide, is 43-time more potent

agonist for estrogenic effects than MXC (*72*). Thus, chemicals metabolize into potentially endocrine disruption active compound seems a common phenomenon. So, what about the chiral chemicals? In 2005, Liu's group speculated that the more estrogenic 1*S*-*cis*-BF might attribute to the preferential uptake the *R* enantiomer or stereoselective metabolism of 1*S*-*cis*-BF to more estrogenic chemical (*56*). A recent research on PM demonstrated this hypothesis. The predominant metabolite formed from the most estrogenic stereoisomer (1S) was the 4′-OH PM which also has higher estrogenic activity in metabolite, while the *trans*-PM is underwent esterase cleavage. The estrogenic activity of the metabolite of PM isomers determined the estrogen potential of the parental PM isomers (*73*). In conclusion, enantioselective estrogenic activity of chiral pesticides may partially result from enantioselective degradation to different potential of estrogenic metabolites.

Conclusions and Areas of Suggested Further Research

In this review article, the enantioselective endocrine disruption of widely distributed chiral pesticides has been discussed. Previous studies imply that the environmental behavior of the active enantiomers, instead of racemate, may bear more relevance to the ecotoxicological effects of chiral pesticides. Combining with other existing documents several important implications may be given.

In this review, most environmental problems associated with chiral pesticides are mainly related to the aquatic environment. This is particularly important for insecticides like SPs, which are widely distributed in aquatic ecosystems and have been shown to have estrogenic activity both for the parent compound and the metabolites. Although the established risk assessment framework for estrogenic evaluation is robust, the enantioselective research on chiral SPs mainly concerning on ESCREEN assay, yeast assay, VTG and its relative gene induction. High tier endocrine disruptor testing such as fish development and fish reproduction tests as well as behavior should be involved in chiral pesticides evaluation to refine the information on the selective estrogen disruption.

As the selective estrogenic potent can be partially attributed the selective biotransformation, the chiral parent chemicals and their metabolites (chiral or achrial) usually co-exist in environment. The estrogenic activity is significant when the metabolites joint together while individual one has no effects. As a result, estrogenic activity should concerning not merely on pure enantiomers but also on the joint effect of enantiomers, metabolites and parent chemicals. Given the high persistent of organochlorine and widespread use of pyrethroid, a more comprehensive understanding of the chronic enantioselective toxicity is imperative for improving risk assessment of chiral pesticides.

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Chapter 9

Phytotoxicity and Environmental Fate of Chiral Herbicides

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Growing concern about the side effects of herbicides on non-target organisms and creatures in the nature has promoted the use of stereo-chemically pure or enantiomerically enriched compounds. The enantioselectivity of the phytotoxicity and environmental effects of herbicides has recently been the focus of much research and is becoming better understood. Because of their different biological properties, enantiomers can selectivity interact with biological systems and behave as very different compounds. It is necessary to study toxicities, metabolic conversions, and migration issues at the chiral level to predict the exact herbicidal effects and pollution load. This review summarizes the preparation technology for optically pure enantiomers, their enantioselective effects on plants, and the biotransformation effects of the main chiral herbicides. This will provide a basis for further study of the chemical and biological behaviors of chiral herbicides and direct research toward the manufacture of optically pure or enantiomerically enriched herbicides with higher efficiency and fewer side effects.

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Introduction

With the development of new classes of pesticides with increasingly complicated structures, and the increase in natural products and their derivatives, the number of chiral compounds has increased tremendously (*1*). Chiral pesticides have been estimated to account for more than 40% of the pesticides currently used in China, among which chiral herbicides occupied the largest proportion (*2*). In recent years, growing concern about the side effects of herbicides on non-target organisms and creatures in the nature has led to research on chiral herbicides. Because of the distinct properties of enantiomers, which have identical physical and chemical properties in an achiral environment (e.g., air-water exchange, sorption, abiotic transformation), but interact with biological systems enantioselectively and may behave as drastically different compounds, enantiomers of a chiral herbicide can be regarded as different substances (*3*, *4*). Thus, it is necessary to study toxicity, metabolic conversion and migration issues at the chiral level and to explore the manufacture of optically pure or enantiomerically enriched herbicides with higher efficiency and fewer side effects (*5*, *6*).

Chiral Herbicides

Since early herbicides had simple structures, optical isomerism was not an issue. With the development of different varieties of pesticides, more and more complicated structures, and increased introduction of natural products and their derivatives as pesticides, the number of chiral compounds has increased tremendously (*1*). In the 1980s, only 19% of agrochemicals used were chiral, but this increased rapidly to 25% in the next decade (*7*). The worldwide market for chiral pesticides grows 10% to 15% per year, and it has been estimated that chiral pesticides account for more than 40% of currently used pesticides in China, among which chiral herbicides occupied the largest proportion (*2*). Among the already commercialized chiral pesticides, optically pure or enantiomerically enriched herbicides account for roughly 50% of total sales (*8*). At present, there are 19 major categories and more than 300 individuals of herbicides on the market. Fourteen categories contain chiral structures, which include phenoxyalkanoic acids, aryloxy phenoxypropanoic acids, imidazolinones and acetanilides and so on. Sixteen varieties of chiral herbicides are marketed and widely used as optically pure or enantiomerically enriched compounds (Table 1).

Preparation Methods of Optically Pure Herbicides

Stereochemically pure or enantiomerically enriched herbicides are highly potent for the target plants, allowing the same herbicidal effects to be achieved at a lower application rate. Therefore, the use of these herbicides could conserve raw materials, reduce the amount of herbicides released into the ecosystem, and avoid some side effects or phytotoxicity. Improvements in methodologies for the preparation of optically pure compounds would provide economic and environmental advantages.

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Table 1. Main Chiral Herbicides

Class of Compound	*Racemic Parent Compound*	*Commercial Single- or Enriched Enantiomer Product*
Phenoxyalkanoic Acids	MCPB, 2,4-DB	Mecoprop-P, Dichlorprop-P
Aryloxy Phenoxypropionic Acids	Fluazifop, Diclofop, Haloxyfop, Quizalofop, Fenoxaprop, Cyhalofop-butyl, Clodinafop-propargyl, Propaquizafop, Quizalofop-P-tefuryl	Diclofop-P, Fluazifop-P, Haloxyfop-P, Fenoxaprop-P-ethyl, Quizalofop-P-ethyl, (R)-Cyhalofop-butyl, Clodinafop-propargyl, Quizalofop-P-tefuryl
Imidazolinones	Imazethapyr, Imazapyr, Imazamox, Imazaquin, Imazamethabenz, Imazapic	
Acetanilides	Metolachlor, Naproanilide, Napropamide, Bromobutide, Dimethenamid, Clomeprop, Flamprop, Carbetamide	S-Metolachlor, S-Dimethenamid, R-Carbetamide
Phenylureas	Cycluron, Daimuron, Clodinafop-propargyl	Daimuron, Clodinafop-propargyl
Cyclonenes	Sethoxydin, Clethodim, Cycloxydim, Tepraloxydim, Cloproxydim	
Pyrimioxyphenyl-propionates	Pyriftalid	
Triazines	Triaziflam	
Diphenyl Ethers	Ethoxyfen-ethyl	R-Ethoxyfen-ethyl
Triazolinones	Carfentrazone-ethyl	
Organophosphorus compounds	Bilanafos, Glufosinate	
Others	Cinmethylin, Flurochloridone, Flurtamone, Indanofan, Tridiphane	

In the past few decades, several single- or enriched-enantiomer herbicide formulations have been developed and promoted in North America and Europe. For example, authorities in the Netherlands and Switzerland have revoked reinstating for racemic mixtures of chiral phenoxy herbicides while approving the registration of single-isomer products (*9*). Sweden has also implemented a tax on agrochemicals based on the weight of the active ingredient (*7*). At present, the biological test reports and preparation techniques for optically pure enantiomers should be provided when registering or reinscribing new herbicides in these countries (*10*). Preparation of optically pure herbicides has become an observable trend.

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Due to the similar physical and chemical properties of the enantiomers, the preparation of optically pure enantiomers is very difficult. As science and technology have progressed, so have the preparation techniques for enantiomers. Optically pure herbicides can be prepared from two ways: isomer separation and asymmetrical synthesis. The first method falls into four main categories: enzymatic resolution, crystallization, achiral derivatives separation and mechanical separation. Asymmetrical synthesis refers to the process of taking an achiral compound and synthetically converting it by one of a number of routes to one isomer of a compound with a chiral center. These methods include asymmetrical hydrogenation (asymmetrical catalysis), asymmetrical dihydroxylation and hydroxamination (*11*); they have been widely applied in the manufacture of chiral herbicides and have achieved outstanding success.

Realization of large-scale industrial production requires feasible routines, high rates of recycling, and excellent optical purity, and relatively low costs (*12*). All are equally important and indispensable for large-scale industrial production. For example, traditional separation techniques have been gradually phased out because of their many faults, including the length of the process, high waste production, low use efficiency of raw materials and high costs due to the consumption of many resolving agents. Separation by chiral column is fast and efficient on a laboratorial scale, but is difficult and expensive on an industrial scale. Highly efficient chirotechnology techniques such as asymmetrical catalysis, enzymatic catalysis and chiral inversion are being used more and more widely, and the industrialization level is continuing to rise (*13*).

Resolution of diastereomers presents an efficient way for the manufacture of optically active herbicides. Herbicides with complicated structures usually have more than one chiral center, resulting in two or more diastereomers. Like enantiomers, diastereomers are expected to have different biological activities, but unlike enantiomers, they also have different physical properties that allow us to separate or manufacture in ratios other than 1:1. For example, chloroacetamide herbicides contain two chiral centers (axial-chiral and C-chiral), producing four stereoisomers. Herbicidal activity is not determined by the chiral axis, but rather by the configuration of the asymmetrically substituted C atom. The low energy required for rotation around the chiral axis results in racemization, two main isomers can be distinguished: α*RS*1'*S* (abbreviated to *S*) and α*RS*1'*R* (or *R*); the racemate is α*RS*1'*RS* (or *RS*). The *S* form has been shown to be much more active than the *R* form to different organisms (*23*, *24*).

Improvements in the methodologies for synthesizing optically pure compounds would result in economic and environmental advantages.

Enantioselective Phytotoxicities of Chiral Herbicides

The chiral nature of living systems has implications for biologically chiral active compounds that interact with them. On a molecular level, chirality represents an intrinsic property of the "building blocks of life", such as amino acids, DNA, RNA, polynucleotides and proteins. As a consequence, metabolic and regulatory processes mediated by biological systems are sensitive to

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stereochemistry (*10*). Enzymes and biological receptors in organisms interact enantioselectively with the individual enantiomers (*14*). When applied, chiral compounds are involved in biochemical interactions, and receptors generally distinguish between enantiomer pairs. The processes of absorption, combination with the target enzyme, and metabolic action for these chiral herbicides in plants are all thought to be enantioselective. Therefore, one enantiomer is target-active or more target-active, and the other enantiomer is inactive or less active and simply adds an extra chemical load to the environment (*5*, *7*).

Phenoxyalkanoic Acid and Aryloxy Phenoxypropanoic Acid Herbicides

Phenoxyalkanoic acids and aryloxy Phenoxypropanoic acids are widely used herbicides that control the growth of grasses by inhibiting plastid acetyl-CoA carboxylase (ACCase), a key enzyme in long-chain fatty acid biosynthesis. Many studies have been devoted to the chirality of these herbicides since their introduction onto the market in the 1940s, 1950s and 1960s. The *R*-enantiomer is believed to be the active (or more active) herbicidal form, while the *S*-enantiomer is thought to be inactive or less active. In the past two to three decades, several single- or enriched-enantiomer formulations have been developed and promoted all over the world; these account for half of the total sales of all commercialized chiral herbicides. Several European countries have strict rules regarding the application of these herbicides. For example, authorities in the Netherlands and Switzerland have revoked registrations for racemic mixtures of chiral phenoxy herbicides while approving registrations of single-isomer products such as mecoprop-P and dichlorprop-P (*9*).

The *R*-(+)-enantiomer of quizalofop-ethyl, one of the typical aryloxy phenoxypropanoic acid herbicides, is 1000 times more toxic than the *S*-(–)-enantiomer to annual grasses. *R*-(+)-quizalofop inhibited the incorporation of [^{14}C]acetate into free fatty acids in excised corn stem-basemeristems, while the *S*-(–)-enantiomer showed no activity (*15*).

R-(+)-diclofop is of high herbicidal activity; plant growth was inhibited significantly by *R*-(+)-diclofop but only slightly by *S*-(–)-diclofop (*16*). However, the enantioselectivity of the aquatic ecotoxicities of *R*-(+)- and *S*-(–)-diclofop on three freshwater algae, Chlorella pyrenoidosa, Chlorella Vulgaris, and Scenedesmus obliquus, was altered. *S*-(–)-diclofop was found to be similarly toxic to or even more toxic than *R*-(+)-diclofop (*17*). The enantioselective toxicity of the *R*- and *S*-enantiomers shifts owing to different objects.

Imidazolinones

Imidazolinones are one of the most important herbicide classes used in many cropping systems because of a broad spectrum of weed control activity, wide crop selectivity, and relatively low usage rates. They inhibit plants by targeting acetolactate synthase (ALS), which catalyzes two different reactions in the biosynthetic pathway of branched-chain amino acids, leucine, isoleucine, and valine (*18*).

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All imidazolinone herbicides are chiral and typically consist of two enantiomers. Imidazolinone enantiomers have been reported to have different herbicidal activities, with the *R*-enantiomer being 8 to 10 times more inhibitory of acetolactate synthase (ALS) than the *S*-enantiomer (*19*, *20*). Optically pure *R*-imidazolinones were protected by a US patent (00108177) in 2000; however, they are often sold commercially as racemates. Little detailed information is available on their enantioselective phytotoxicity and the mode of action of the enantiomers selectively inhibiting the plants. In our previous studies, enantiomers of imidazolinones were obtained by HPLC. We found that the enantiomers of imazethapyr (IM), one of the imidazolinone herbicides, selectively inhibited the plant growth of maize by damaging root morphostructure and ultra-structure. *R*-(–)-IM affected the growth of maize seedlings almost twice as severely as *S*-(+)-IM. The inhibition ability of (±)-IM fell between those of *S*-(+)- and *R*-(–)-IM (*21*, *22*). IM enantiomers enantioselectively suppressed in vitro and in vivo ALS activity. *R*-(–)-IM was two times more toxic than *S*-(+)-IM in controlling in vivo ALS activity, which is in agreement with enantioselective inhibition of plant growth. Computational docking of enantiomers into the ALS enzyme presents a detailed and precise interaction mode of enantiomers with ALS (*23*). The crystal structure of imazaquin (IQ) in complexes with ALS (PDB ID: 1Z8N) was selected to be the receptor for docking. When the IM enantiomers were positioned in the active site of IQ-ALS complex, the isopropyl substituent of R-(–) and S-(+)- IM point to the opposite directions; the former was nearly identical to the corresponding part of IQ, while the latter forms repulsive forces with the side chain of R377 and W574, which may be used as a explanation to elucidate the stereo specificity (Figue 1) (*23*). Additionally, the docked conformations of enantiomers presented the detailed and precise interaction mode of IM with ALS. S-(+)-IM with the distorted dihydroimidazolone ring, although located in the same binding pocket, cannot form orderly interactions with ALS as R-(-)-IM does. One hypothesis was that the dihydroimidazolone ring had to rotate to avoid the steric hindrance between isopropyl and W574(Figue 2) (*23*). *R*-(–)-IM can bind to ALS in its orientation, which is more preferred than that of *S*-(+)-IM (*23*). This study provides important evidence for developing novel and more selective and effective herbicides; that is, the results can aid in choosing the optically pure enantiomer that has the stronger binding ability with the target enzyme, yielding higher herbicidal effects.

Acetanilide Herbicides

Acetanilide herbicides contain two chiral centers (axial-chiral and C-chiral), resulting in four stereoisomers, i.e., α*S*1'*S*, α*R*1'*S*, α*S*1'*R* and α*R*1'*R*. The herbicidal activity of these chloroacetamides are not determined by the chiral axis, but rather by the configuration of the asymmetrically substituted C atom. No major differences in the biological activity of the α*R*- and α*S*-isomers (i.e., axial-chiral) have been found. Furthermore, the low energy required for rotation around the chiral axis results in racemization, so that only two main isomers can be distinguished: α*RS*1'*S* (abbreviated to *S*) and α*RS*1'*R* (or *R*); the racemate is α*RS*1'*RS* (or *RS*). The *S* form has been shown to be much more active than the

R form to different organisms (*24*, *25*). Metolachlor is one famous acetanilide herbicide, first commercialized as a racemate in 1976. Since 1997, it has been gradually substituted by the more active form, the *S*-isomer, which is ten times more inhibitory than the *R*-isomer. Because the *S*-isomer is the active ingredient, metolachlor-P containing 96% *S*-isomer (which was developed by Novartis) is 1.67 times more effective than the 72% *S*-isomer enriched metolachlor. *S*-metolachlor products were registered in New York State as general use products in 2007 (*26*).

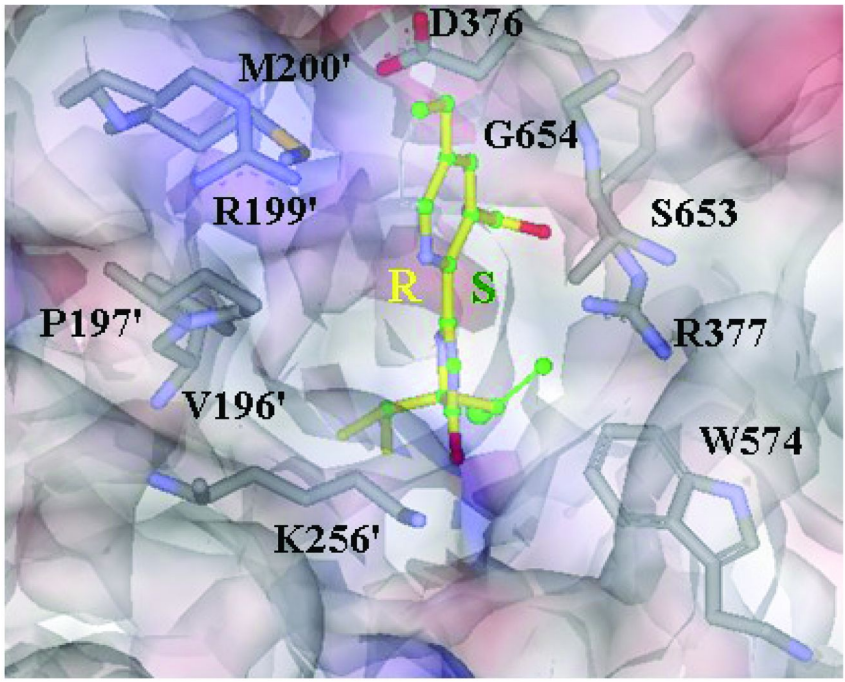

Figure 1. The superposition of R-(–)-IM (carbon atoms shown in yellow color), S-(+)-IM (carbon atoms displayed in green color) and imazaquin (IQ) in the binding pocket of ALS (23).

Another kind of acetanilide herbicide, chloroacetanilides, affects a specific primary target and is probably specific to lipid metabolism or the incorporation of fatty acids into non-lipid compounds. Michel et al. (*27*) investigated the biological activity of the two main stereoisomers of dimethenamid (*R* and *S*) in *Lemna minor* L. and in *S. Acutus* and in greater detail with the green alga *Scenedesmus acutus*. In short-term experiments (up to 5.5 h), the *S*-isomer inhibited [^{14}C]acetate uptake and its incorporation into fatty acids, while the *R*-isomer did not. Incorporation of [^{14}C]acetate into a non-lipid fraction of the algae was strongly inhibited by the *S*-isomer (100 μM), but only slightly inhibited by the *R*-isomer. Fifty percent inhibition of the incorporation of [^{14}C]oleic acid into the same non-lipid fraction was attained with less than 10^{-7} M of the *S*-isomer,

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while10^{-5} M of the *R* form of dimethenamid achieved only a 40% inhibition. Dimethenamid-P with the optically pure isomer was developed by Ehrenstorfer (Augsburg, Germany) and commercialized in 2000. Its application rate is only a half of its racemate.

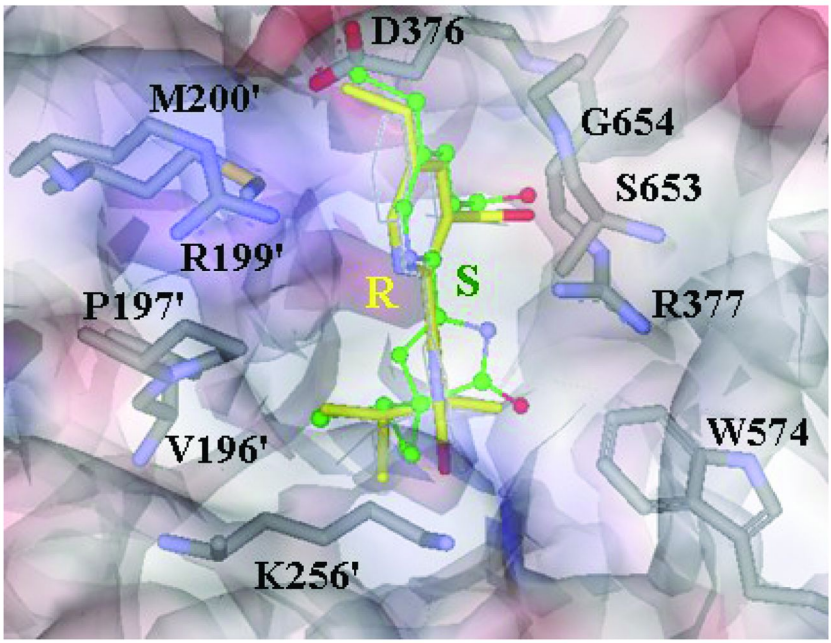

Figure 2. Comparative binding modes of R-(–)-(carbon atoms shown in yellow color) and S-(+)- IM (carbon atoms displayed in green color) with ALS.

Cyclonene Herbicides

Clethodim is a cyclohexanedione herbicide with good herbicidal activity. Optically pure (–)-clethodim development was protected by a US patent (6,300,281 B1) in 2001. (–)-clethodim is more effective in regulating the growth of grass plants than its corresponding racemic mixture or the optically pure (+)-enantiomer. The application can be cut in half by using (–)-clethodim, therefore, the marketing potential will be considerable (*28*).

Phenylureas Herbicides

Ryoo et al. (*29*) synthesized a series of optically active derivatives of desdaimuron *R*-1-(α-methylbenzyl)-3-(substituted-pheny)-urea with various kinds of substituents (alkyl, methoxy, halogen and carbonyl groups) at the aniline moiety and studied the phytotoxic potential of the *R*- and *S*-enantiomers. They found a suitable enantiomer for successful weed control was dependent on the substituent

at the aniline moiety. The *R*-2-iso-Pr and *R*-2-*tert*-Bu derivatives significantly controlled barnyard grass and both annual and perennial *Cyperaceae* paddy weeds. The *R*-2-Et and *R*-2-CF_3 derivatives showed strong herbicidal activity against perennial *Cyperaceae* paddy weeds, while the *S*-enantiomers of the unsubstituted and fluoro derivatives were active against barnyard grass but inactive against other grasses. Furthermore, the enantioselectivity of herbicidal effects was dependent on the type of plant. For example, *R*-1-(α-methylbenzyl)-3-(tolylene)-ureas (*R*-MBTU) efficiently inhibits the root elongation of rice, but not that of wheat. However, S-MBTU strongly inhibits the root elongation of wheat, but barely inhibits that of rice (*30–32*). In addition, *R*-MBTU inhibits the root elongation of a companion weed of wheat, *Beckmannia syzigachne*, and *S*-MBTU inhibits the root elongation of *Echinochloa* spp., which is a companion weed of rice (*30*, *32–35*). These findings indicate that the enantioselectivity of the herbicidal effects of *R*-/*S*-MBTU varies across the plants. This high selectivity is useful for developing novel and more selective and effective herbicides; that is, it can enable us to choose the optically pure enantiomer with the proper substituent that will result in herbicidal effects but no crop injury.

Diphenyl Ethers Herbicides

Diphenyl ethers herbicides are protoporphyrinogenoxidase (Protox) inhibitors. They efficiently control weeds by inhibiting photosynthesis and synthesis of chlorophyll. There are nearly 20 varieties on the market (*36*). phthalide (5-[2-chloro-4-(trifluoromethyl)phenoxy]-3-methylphthalide) is a new diphenyl ether herbicide consisting of a chiral structure. Camilleri et al. (*37*) found that this herbicide enantioselectively causes light-dependent membrane lipid peroxidation in the plants *Vicia faba* and *Hordeum Vulgare*; the *S*-(–)-enantiomer was found to be substantially more active than the *R*-(+)-enantiomer. The enantioselective inhibition arises from the different bindings of the enantiomers with the enzyme, which plays a key role in the biosynthetic path between protoporphyrin IX and protochlorophyllide. The *S*-(–)-enantiomer had a stronger combination ability with the enzyme compared with the *R*-(+)-enantiomer. Another diphenyl ether herbicide, 5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-nitroacetophenone oxime-O-(acetic acid, methyl ester) (DPEI), enantioselectively induced abnormal accumulation of protoporphyrin IX in darkness in the green alga *Chiamydomonas reinhardtii*. The purified *S*-(–)-enantiomer with the greater herbicidal activity induces a 4- to 5-fold greater tetrapyrrole accumulation than the R-(+)-isomer. The site at which DPEs inhibit protoporphyrinogen IX oxidase also shows chiral discrimination (*38*). According to the three-point model proposed by Easson and Stedman in 1993 (*39*) and the four-location model developed by Mesecar in 2000 (*40*), the enantioselective toxicity of the herbicide to the plant may be due to the high stereospecificity interaction between the herbicide and the enzyme. The enantiomers are mirror images of each other that differ in their three-dimensional configurations, and the enzyme is also chiral with a specific stereo structure (*41*); therefore, there is a greater chance with the chiral herbicides that one enantiomer could combine more tightly than the other, resulting in different

enzyme activity. This study provides further evidence for the stereoselective inhibition of enantiomers on enzymes.

Triazine Herbicides

In some herbicides with multi-site inhibition ability, the enantiomers show diverse enantioselectivity when targeting different inhibition sites. The *R*- and *S*-enantiomers may both be highly phytotoxic; however, the mode of action may be totally different. For example, the *R*-enantiomer of Triazine herbicides inhibits root growth in the dark and induces Cytokinin analogue generation. The *S*-enantiomer inhibits photosystem II (*42–45*).

Enantiomers of triaziflam and structurally related Diaminotriazines enantioselectively affect multiple sites of action, including photosystem II electron transport (PET) inhibitory activity, mitotic disruption via inhibition of microtubule formation and inhibition of cellulose synthesis. The *S*-enantiomers of the compounds preferentially inhibit PET and algae growth. In contrast, the *R*-enantiomers of the Diaminotriazines are up to 100 times more potent in inhibiting the growth of cleaver cell suspensions and cress seedlings in the dark than the *S*-enantiomers (*46*). The results suggest that the chiral requirement for strong inhibition of root growth is the *R*-configuration, contrasting with the requirement for the *S*-configuration for inhibition of photosystem II.

The enantioselective phytotoxicities of chiral herbicides on plants are complex. The preferential inhibition shifts when the target objects or some other environmental elements change (*47*). Understanding the different plant biochemical processes in which herbicides are involved can greatly aid in the development of appropriate synthetic chemicals and may help reduce environmental risks.

Enantioselectively Environmental Fates of Chiral Herbicides

Chiral herbicides that enter the environment through point and non-point sources are widely distributed in water, sediment, soil and biota and are ultimately degraded, often enantioselectively by bio-degraded (*48*). For example, the occurrence of chiral Phenoxyalkanoic acids in lakes and rivers in Switzerland has been reported. Buser and Müller found that enantiomerization is biologically mediated and leads to residues of MCPP and DCPP in these waters, which are eventually enriched in their *S* enantiomers (*49*); Imazethapyr was observed to degrade enantioselectively under soil conditions. ER slowly decreased over time from 0.97 (day 1) to 0.58 (day 30) (*50*). The dissipation of Mecoprop under nitrate-reducing conditions was shown to be enantioselective, with the *R*-enantiomers degrading faster than the *S*-enantiomers (*51*).

However, enantioselective degradation of herbicides in the environment is very complicated, as it is related not only to the physical and chemical properties of the herbicide itself, but also to the various environmental factors. Preference shifts may be due to different types of soil, related microbial genotypes and plant species and so on (*3*, *52*). The degradation of the two stereoisomers of

Metalaxyl proved to be enantioselective and dependent on the media: the *S*-(+)-enantiomer showed faster degradation in plants, while the *R*-(–)-enantiomer showed faster degradation in soil (*53*). In some cases, the enantiomer ratio changes when the specificity of the soil microbial populations shifted. For example, soil microorganisms in most forest samples from Brazil preferentially removed (+)-dichlorprop acid, the active form of the herbicide. By comparison, the microbes in nearby pasture samples almost exclusively preferred the (–)-enantiomer (*3*). Significant correlations between the enantioselectivity of lactofen and soil pH were observed under aerobic and anaerobic conditions. In addition, enantioselectivity correlated with soil texture rather than organic carbon (*54*). Romero (*55*) found that the *R*-enantiomer of mecoprop and dichlorprop degraded faster than the *S*-enantiomer, and the enantioselective degradation rates differed across three calcareous soils. However, a study by Garrison (*56*) found that, on the contrary, the S-enantiomer preferentially degraded. These differences may also be due to the different microbial genotypes of the studied soils, as changes in pH, soil texture or organic carbon may result in different population of microbial genotypes that show different degradation preferences.

Enantioselectivity of degradation may differ greatly when targeting different species of plants. Racemic mixtures of 2,4-DP and MCPP were applied to three species of turf grass and four species of broadleaf weeds a period. The preferential degradation of the *S*-(–)-enantiomer of each herbicide was observed in most species of broadleaf weeds that the rates for the *S*-(–)-enantiomers of both herbicides were approximately twice those of the *R*-(+)-enantiomers, while the degradation in all species of grass occurred without enantioselectivity (*51*). The stereoselective degradation of ethofumesate in turf grasses showed that *ER* reached 3.2 at 5 days in Kentucky bluegrass, but 2.9 in tall fescue (*57*).

The fates of enantiomers may thus be very different after application, and the enantiomeric composition of the chiral herbicides may be changed in bio-processes. Therefore, differences in biological activity between the enantiomeric forms of a chiral herbicide may result not from differences in intrinsic activity at the site of action, but rather from differences in rates of metabolic detoxification and other processes that reduce the concentration of the active isomer at the target site. For example, the *R*-(+)-enantiomer of quizalofop-ethyl is 1000 times more toxic to annual grasses than the *S*-(–)-enantiomer. However, they have the same level of toxicity when applied before the weeds germinate because the *S*-(–)-enantiomer converts to the *R*-(+)-enantiomer in the soil within 7 days (*15*). The racemates of Organophosphorus herbicides Bilanafos and Glufosinate can be converted to (-)-enantiomers by microorganisms in the soil (*58*).

Assessments of the risks posed by chemical pollutants to public health and the environment need to take into account the chiral selectivity of microbial and phyto-transformation processes and their alteration as a result of environmental changes.

Conclusion

The phytotoxicity and environmental fate of chiral herbicides are enantioselective. Moreover, enantioselectivity varies according to enzyme properties or environmental conditions.. Further study is needed to better understand the mechanisms of toxicity and the metabolism of individual enantiopure isomers of chiral herbicides in target and non-target organisms. Study of the chirality of herbicides could help us to determine the more active isomer in a stereoisomeric mixture and allow us to remove the less biologically active single stereoisomer or the toxic isomer that has an undesirable toxic response to a non-target organism, thus reducing adverse effects. Using only the stereoisomer that has the desired biological activity will reduce the total amount of chemicals introduced into the environment and therefore merits careful consideration.

The preparation of an enantiomeric pair or chiral synthesis is often a technically difficult and/or laborious and expensive process. However, techniques for asymmetric syntheses and separations of enantiomers have been developed for several herbicides. Further improvements in the methods for preparation of enantiopure isomers can be expected in the future.

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Chapter 10

Enantioselective Cytotoxicity and Molecular Mechanisms of Modern Chiral Pesticides

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The agrochemical industry and government regulators are beginning to take enantioselectivity into account since up to 40% of pesticides are chiral. Cytotoxicity has been developed as an effective method for assessing the eco-safety and health risks of chiral pesticides in diverse *in vitro* models. The application of *in vitro* models may promote understanding of the molecular mechanisms of the enantioselective toxicity of chiral pesticides. In this review, we discuss the enantioselective cytotoxicity of chiral insecticides and herbicides and possible molecular mechanisms. The future role of *in vitro* techniques in the risk assessment of chiral chemicals will expand in light of recent developments in technologies such as genomics and system biology.

Introduction

Up to 40% of modern pesticides in China are chiral molecules, while others contain persistent organic pollutants (POPs) and many other pollutants . With the development of the agrochemical industry, more and more chiral pesticides will be introduced into the market. An inaccurate assessment of risk could result if a potential eco-toxic evaluation and health risk measurement was taken only on the racemic form of chiral pesticides. However, in the past two decades,

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the assessment of the environmental safety of chiral pesticides at enantiomer levels has progressed significantly. Some previous studies also have also suggested that there is an enantioselective toxic effect in development toxicity (*2*), immunotoxicity (*3*), genotoxicity (*4*), endocrine disruption (*5*), and neurotoxicity (*6*, *7*). Many researchers and policymakers ask questions about how to accurately and quickly evaluate the environmental safety of chiral pesticides. In order to improve the risk assessment of chiral pesticides, it is necessary to consider the effects of enantioselectivity.

Conventionally, data resulting from animal (*in vivo*) studies are the main basis for the assessment of toxicological properties of chemicals and pollutants that may be of concern for environmental safety and human health. The same principles apply to the assessment of the enantioselective toxicity of chiral pesticides. As with most chemicals, the animals are also exposed to increasing levels of the chiral pesticides with the intention of eliciting toxicological effects. Unfortunately, these approaches are always slow and expensive. Furthermore, the relevance to human health risk based on *in vivo* assessment is unclear. The importance of accurately and rapidly assessing exposure to chiral pesticides is becoming increasingly evident. The development of *in vitro* methods, including computer-based approaches such as dimensional structure activity relationships, QSARs and mathematical modeling, which are now being used for the risk assessment of pollutants in several fields, have an important role in the hazard and health assessment of chiral pesticides.

A large array of different cell culture systems are being applied in toxicity testing. These systems include cell lines, primary cell cultures, co-culture systems, tissue and cell aggregates and tissue slices. For example, animal cell lines such as macrophages (*3*), natural killer cells (*8*), neuronal cells (*6*, *7*, *9*), lung epithelial cells (*10*); plant cells such as green algae cells (*11*), corn cells (*12*), transgenic plant cells (*12*); primary cells such as hepatocyte cells isolated from the rat or rainbow trout (*13*, *14*); and fungi cells such as yeast cells (*15*) have been widely used to test toxicity for insecticides, herbicides and fungicides. Compared to *in vivo* tests, *in vitro* models are popular because they are quick, relatively inexpensive, and their specific mechanisms of action can be tested. From the public perspective, use of *in vitro* model systems may increase in popularity because their application may allow a reduction in the number of live animals employed in toxicity testing. Among the variety of *in vitro* models, the cellular models have become, at least in some fields, the ideal candidates for toxic investigation. Different types of cell lines have been developed for the toxicological evaluation of pesticides.

Accepted methods for the assessment of acute toxicity, genotoxicity, reproductive toxicity and endocrine disruption of substances are included in the list of OECD guidelines (*16*). *In vitro* cytotoxicity testing has been proposed as an effective method to screen for the environmental safety and eco-toxicity of chemicals. Researchers can either look for toxic compounds if they are interested in developing an agrochemical that targets pests or plants, or they can screen "hits" from the initial screens of highly effective pesticides for unwanted toxic effects after their release into the environment. For chiral pesticides, it is difficult to foresee the changes and side-effects that will occur because the enrichment and accumulation of an enantiomer is not only dependent on environmental

factors, but also on organisms (*17*). As a result, the application of *in vitro* models, especially cytotoxicity tests to assess the ecological risk of different enantiomers of chiral compounds, will highlight the mechanisms by which the chiral pesticides work. Moreover, a pre-validation study on *in vitro* tests would improve the effectiveness of the integration of chiral pesticide management.

Bioassay Using Cytotoxicity

Cytotoxicity is considered as a widely recognized biomarker indicating the harmful effects of chemicals. Pesticide-induced *in vitro* cytotoxicity tests can be categorized basically as follows:

A. *Damage to cell membrane integrity*: Vital dyes, such as Trypan Blue or propidium iodide (PI) are normally excluded from the inside of healthy cells. They have been used for detecting cell viability and the morphology of apoptosis for many pesticide research studies (*3*, *18*). Alternatively, membrane integrity can be assessed by monitoring the passage of substances that are normally sequestered inside cells to the outside. One commonly measured molecule is lactate dehydrogenase (LDH) (*6*, *7*). Live-cell protease biomarkers, which are only active in cells with membrane integrity, have been identified that allow researchers to measure relative numbers of live and dead cells within the same cell population. The proteases from dead cells cannot cross the cell membrane, and can only be measured in culture media after cells have lost their membrane integrity (*19*).
B. *Cell growth:* Cytotoxicity can also be monitored using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) or (methanethiosulfonate)MTS assay, which measures the reducing potential of the cell using a colorimetric reaction. It reflects the cell growth after exposure to pesticides (*3*, *4*, *6*, *7*). A similar redox-based assay has also been developed using fluorescent dye. In addition to using dyes to indicate the redox potential of cells to monitor their viability, researchers have developed assays that use ATP content as a marker of viability (*20*).
C. *Electric cell-substrate impedance sensing (ECIS) technique:* is a label-free approach to follow the cytotoxic response of adherent animal cells in real-time. It provides the kinetics of the cytotoxic response rather than just a snapshot like many colorimetric endpoint assays.
D. *Others:* Cytotoxicity can also be measured by the sulforhodamine B (SRB) assay, WST assay, apoptosis analysis (*21*, *22*) and clonogenic assay (*23*).

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Enantioselective Cytotoxicity of Modern Chiral Pesticides

The chiral molecular building blocks of biology, such as amino acids, DNA, RNA, and proteins are the foundation of life. Almost all biological process occurring in the organisms require chiral enzymes. Therefore, when chiral pollutants such as pesticides enter organisms, the processes of transport, uptake, storage, metabolism, and the mechanisms of toxicity would be structure-specific, even during cytotoxicity.

Organochlorine Pesticides

Organochlorine pesticides (OCPs) are insecticides composed primarily of carbon, hydrogen, and chlorine. They are typically persistent in the environment, accumulate easily in body, and are associated with many chronic diseases such as cognitive function defects (*24*), gynecological disease (*25*) and abnormal immune system function (*26*). Today, several commonly known organochlorines include DDT, aldrin, dieldrin, toxaphene, chlordane and heptachlor have been banned in the U.S., while others are still used.

Acetofenate (AF), an alternative to previously used OCPs, is widely used to control fly populations both indoors and outdoors in Asia (*27*). It is composed of a chiral center and two enantiomers that show enantioselectivity in developmental toxicity in zebra fish (*2*). The differences of cytotoxicity between the two enantiomers also have been investigated in macrophages (*3*) (Table 1). *S*-(+)-AF has stronger effects in the induction of intracellular ROS (reactive oxygen species), DNAdamage and upregulation of p53 gene expression. Also, it induced more apoptosis than *R*-(–)-AF. This suggests that *S*-(+)-AF may have functional effects on the response of macrophages caused in part by oxidative damage.

Synthetic Pyrethroids

Synthetic pyrethroids (SPs) are synthesized derivatives of naturally occurring pyrethrins that are short-lived because of their sensitivity to light, heat and moisture. They are sold as commercial pesticides and globally used against pests in agriculture, homes, communities, hospitals, schools and as a topical lice treatment. The development of SPs, however, heralded a dilemma as the toxic level to non-target aquatic organisms was similar to those for insect larvae (*28*). In addition to aquatic toxicity, mammalian toxicity has also been observed. For example, cytotoxicity has been reported for *cis*-bifenthrin (*cis*-BF) (*29*), permethrin (PM) (*30*), lambda-cyhalothrin (LCT) (*31*), cypermethrin (*32*) and deltamethrin (*33*). However, each synthetic pyrethroid mentioned above contains 2-3 chiral centers, and therefore, 2 or 4 pairs of enantiomers. Reports on enantioselective cytotoxicity have only addressed *cis*-BF, PM and LCT (Table 1).

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Enantioselective cytotoxicity of *cis*-BF conducted with rat pheochromocytoma (PC12), macrophage (RAW264.7), human amnion epithelial (FL) and human hepatocellular liver carcinoma (HepG2) cell lines yielded coherent results (*4*, *6*, *7*, *34*, *35*). *cis*-BF reduces cell viability as follows: 1*S*-*cis*-BF > *rac*-BF > 1*R*-*cis*-BF. The amount of apoptosis induced by 1*S*-cis-BF was approximately 3 times that of 1*R*-*cis*-BF at 0.04 mg/L in RAW264.7 and 50 mg/L in FL cell lines. Treated with two enantiomers of *cis*-BF at 4.2 mg/L and 40 mg/L, 1*S*-*cis*-BF causes a 2-fold increase in apoptosis in PC12s, and 2-fold in Hep G2s compared with 1*R*-*cis*-BF. Moreover, the cytoskeleton was significantly destroyed by the S enantiomer in FL and PC12 cells. These effects can be attributed to the accumulation of ROS and decreased antioxidative enzymes such as superoxide dismutase (SOD) and catalase (CAT), when incubated with 1*S*-*cis*-BF. The cytotoxicity of LCT has only been studied on RAW264.7. The enantiomer of (–)-LCT induced a 2.8-fold increase of apoptosis, along with 3-fold upregulation of p53 compared with (+)-LCT. Among the four enantiomers of permethrin (PM), 1*R*-*trans*-PM is the most toxic enantiomer to PC12s. By remarkably increasing the level of the intracellular ROS, malondialdehyde (MDA), and downregulating the activity of anti-oxidative enzymes by 1*R*-*trans*-PM, PM induced enantioselective oxidative stress and cytotoxicity.

Phenylpyrazole Pesticide

Fipronil is one kind of phenylpyrazole pesticide with a sulfoxide moiety chiral center. It is effective for grass management and against a wide spectrum of pests. Its efficiency in pest control and low rate of application lead to its worldwide use. Consequently, high amounts of detectable residue in the ecosystem pose a risk to non-target species. Enantioselective acute toxicity has been observed in daphnia (*36*) and fish (*37*). Recently, enantioselective cytotoxicity has been explored in primary hepatocytes from rainbow trout (*Oncorhynchus mykiss*) (*37*). (Table 1) In the MTT colorimetric assay, exposure to *S*(+)-fipronil resulted in a 20% reduction in cell viability and a much larger reduction with the *R*(–)-fipronil. Unlike other pesticides, racemic fipronil is strongly more toxic than both of its enantiomers.

Aryloxy Phenoxypropanoic Acid Herbicide

Aryloxy phenoxypropanoic acid herbicides (AOPPs) were introduced in the 1960s by Farbwerke Hoechst AG as a kind of chiral herbicide with stronger effects from *R*-enantiomers than the *S*-form (*38*). Recently, the enantioselective cytotoxicity of diclofop, a representative AOPP, has been studied in different species of algae cells (*11*). A decrease of the final biomass and the maximum growth rate indicated that the *S*-(–)-enantiomer of either diclofop and diclofop-methyl is more toxic to *Chlorella pyrenoidosa* and *Scenedesmus obliquus*. However, enantioselective toxicity is not as significant between *R* and *S* enantiomers in *Chlorella Vulgaris*. This species-specific enantioselectivity might be governed by selective uptake and degradation in algae cells.

Table 1. The enantioselective cytotoxicity of chiral pesticides

Category	*Name*	*Cell lines*	*Endpoint*	*Toxicity order*	*Reference*
Organochlorine pesticides	Acetofenate(AF)	RAW 264.7	Cell viability↓ Apoptosis↑ p53 induction↑ ROS↑	*S*(+)-AF > *R*(–)-AF	(*10*)
Synthetic Pyrethroids	*Cis*-bifenthrin (*cis*-BF)	RAW264.7	Cell viability↓ Apoptosis↑ p53 induction↑	1*S*-*cis*-BF > 1*R*-*cis*-BF	(*11*, *13*, *14*, *38*, *38*, *39*)
		PC12	Cell viability↓ Apoptosis↑ SOD↓,ROS↑,LDH↑ Cytoskeleton damage↑		
		FL	Cell viability↓ Apoptosis↑ ROS↑ Cytoskeleton damage↑		
		Hep G2	Cell viability↓ Apoptosis↑		
	Permethrin (PM)	PC12	Cell viability↓ SOD↓,CAT↓,GSH↓ MDA↑, ROS↑, LDH↑	1*R*-*trans*-PM > 1*S*-*trans*-PM > 1*R*-*cis*-PM > 1*S*-*cis*-PM	
	Lambda-cyhalo thrin(LCT)	RAW 264.7	Cell viability↓ Apoptosis↑ p53 induction↑	(–)-LCT > (+)-LCT	

Category	*Name*	*Cell lines*	*Endpoint*	*Toxicity order*	*Reference*
Phenylpyrazole pesticides	Fipronil	Primary hepatocytes	Cell viability↓	*S*(+)-fipronil > *R*(–)- fipronil	(*40*)
Aryloxy phenoxypropanoic acid herbicides	diclofop (DP) diclofop-methyl (DPM)	*Chlorella pyrenoidosa*	Algae biomass↓ Maxi growth rate↓	*S*(–)-DP/DPM >*R*(+)-DP/DPM	(*18*)
		Scenedes-mus obliquus	Algae biomass↓ Max. growth rate↓	*S*(–)-DP/DPM > *R*(+)-DP/DPM	
		Chlorella Vulgaris	Algae biomass↓ Max. growth rate↓	*S*(–)-DP/DPM = *R*(+)-DP/DPM	

↑: Upregulated; ↓: downregulated; ROS: reactive oxygen species; MDA: malondialdehyde. SOD: super oxide dismutase; LDH: Lactate dehydrogenase; CAT: catalase. PC12: rat pheochromocytoma; FL: human amnion epithelial; HepG2: human hepatocellular liver carcinoma.

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Possible Molecular Mechanisms

The mechanisms of pesticide-induced cytotoxicity are a complex network comprising the alteration of apoptosis-related molecules and proteins, exogenous enzymes, specific organelles and many diverse signaling pathways. Based on previous cytotoxic mechanisms research, the molecular mechanisms of cytotoxicity of pesticides include induction of apoptosis, genotoxicity, oxidative damage and activation of specific cellular signaling pathways. For example, acetofenate (AF) is a widely used insecticide in China and other regions of southeastern Asia. Previous cell viability and apoptosis data showed that AF induced obvious apoptosis in macrophage RAW 264.7 cells. Furthermore, AF induced the generation of intracellular reactive oxygen species (ROS) and DNA damage, and resulted in the alteration of a series of signaling molecules including the upregulation of p53 and cytochrome c protein levels, and a decline in the Bcl-2/Bax protein ratio. These results revealed that the increase of endogenous ROS and DNA damage co-mediated AF-induced cytotoxicity in macrophages may function in the mitochondria and p53 signal pathway (*39*). Primary fish cell apoptosis induced by the herbicide atrazine is involved in mitochondria membrane potential (ψm) disruption, elevation in intracellular Ca^{2+}, generation of ROS and intracellular ATP depletion (*20*). Researchers also reported that HCH (hexachlorocyclohexane) induced ROS production and increased cellular Ca^{2+} influx by influencing the activity of Na^{+}/K^{+}-ATPase and finally caused apoptosis (*38*). However, in most of the mechanisms mentioned above, the chiral pesticide-induced cytotoxic effects in *in vitro* came from the racemic forms. Little is known about the molecular mechanisms of enantioselective cytotoxicity of chiral pesticides. Recently, several groups tried to explain the related mechanisms of selective toxicity caused by pure enantiomers in different cell lines (*3*–*8*).

The enantioselective induction of apoptosis is one of the most common features of enantioselective cytotoxicity. The apoptotic cell undergoes condensation and cleavage of nuclear chromatin, cytoplasmic shrinkage, and change in the refractive index of the cell. For *cis*-BF, relative results showed that 1*S*-*cis*-BF rather than 1*R*-*cis*-BF can selectively induce apoptosis in PC12, macrophage, hepG2 and FL cells (*3*, *4*, *7*, *34*, *35*).

As a result of apoptosis induction, ROS also have been selectively induced in the treated cell lines. Relative toxic enantiomers such as 1*S*-*cis*-BF, 1*R*-*trans*-PM, and *S*-(+)-imazethapyr (IM) significantly elevated the ROS level, followed by the imbalance of antioxidative enzymes, such as SOD, CAT and glutathione (GSH) (*6*, *7*, *40*). The excessive accumulation of ROS has been determined to mediate apoptosis and is regarded as an important apoptotic indicator (*41*, *42*). The production of ROS disrupts the balance of many cellular factors, such as cell membrane integrity. The release of intracellular LDH caused by 1*R*-*trans*-PM and 1*S*-*cis*-BF in PC12 cells indicated that the ROS oxidized the cell lipid layer (*6*). Moreover, cytoskeletal damage is also a biomarker for cytotoxicity. The fragmented cytoskeleton of both FL cells and PC12 cells has been observed after exposure to 1*S*-*cis*-BF (*4*, *7*). Cytotoxic effects might also be induced by DNA damage and genotoxicity. In a comet assay, 1*S*-*cis*-BF and *S*-(+)-AF significantly increased the tail moments of FL cells and macrophages (*3*, *4*).

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Several pesticides have toxic effects on cell growth-mediated cellular signaling pathways in different cell models. Available literature focusing on pesticide-induced cytotoxic signaling pathways has mainly involved the mitogen-activated protein kinase (MAPK), caspase cascade and nuclear factor kappaB (NFκB) signaling pathways. The MAPK family includes the extracellular regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK) and p38 kinase, which are involved in cell survival, proliferation and apoptosis in response to various growth or stress stimuli. The activation of ERK has been implicated in cell proliferation and cell cycle progression (*43*), while JNK and p38 are more commonly activated in response to stress and toxicants which induce cell apoptosis (*44*). Importantly, a number of studies support the concept that a sustained activation of JNK leads to apoptosis.

The previous studies showed that the signaling pathway can be activated by cellular stressors and toxicants such as ROS. Our previous study indicated that the enantioselectivity of *cis*-BF in cytotoxicity was mediated by the MAPK signaling pathway (*35*). In the recent study, (−)-ICP (isocarbophos) enantioselectivity caused a change in the Bax/Bcl-2 ratio triggering the generation of intracellular ROS and sequentially induced sustainable activation of JNK, which in turn results in decrease in cell viability and an increase in cell apoptosis (*45*). Finally, the enantioselective alteration of some mediators of apoptosis such as p53 may cause the enantioselective cytotoxicity of chiral chemicals. *S*-(+)-AF, but not the *R*-form of AF, significantly up-regulated p53 gene expression, which indicated enantioselective toxicity toward macrophages (*3*). These observations provide further insight into enantiomer-selective toxicity pathways which are able to differentiate between enantiomer activities at the molecular level. In phytotoxicity, the chiral herbicide, IM, usually the *S*-(+)-IM form, selectively inhibited plant cell growth by upregulation or downregulation of the transcript abundance of related genes, such as amyotrophic lateral sclerosis (ALS), pyruvate carboxylase (PC) and β–amylase (*40*). In summary, these results illustrate that a single isomer of chiral pesticides may contribute to the activation of cellular signaling pathways leading to enantiomer-specific apoptosis and cytotoxicity. However, the exact molecular mechanisms of cellular signal transduction involved in the enantiomer-specific cytotoxicity and apoptosis of chiral pesticides requires further investigation.

Areas of Suggested Further Research

The development of *in vitro* toxicological assessment of chemicals has made considerable progress over recent decades. The results stated above reveal that the potential for the application of *in vitro* techniques in enantioselective toxicological risk assessment of chiral chemicals is very promising. The use of *in vitro* assays not only provides a valid method for identifying the mechanisms of toxicity, but also provides primary data for risk assessment. However, there are some limitations. The challenges ahead include the following aspects: first, there has been a lack of consensus regarding the predictive value between *in vitro* assay and *in vivo* exposure. The enantioselectivity in cytotoxicity of *cis*-BF in

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different cell lines didn't lead to the same aquatic acute and chronic toxicity in *daphnia magna* (*46*) and zebrafish (*Danio rerio*) (*47*). In other words, in cell lines, 1*S*-*cis*-BF is more toxic than 1*R*-*cis*-BF. But in aquatic organisms, 1*R*-*cis*-BF was more toxic. As a result, *in vitro* bioassays are complementary but not substitutes for *in vivo* tests. Therefore, it is worth emphasizing that *in vitro* assays are the initial step in environmental assessment of chiral pesticides that must include verification of an adverse effect *in vivo*.

Until now, few feasible alternatives have been proposed that are capable of handling the number of chemicals that require timely risk assessment. More recently, the introduction of high-throughput screenings (including transcriptomics, proteomics, and metabonomics) in *in vitro* studies has provided new ways to assess the impact of chemicals on cellular systems (*48*). In our group, we have used microarrays to analyze the enantioselective cytotoxicity of *cis*-BF in monocytes and Hep G2 cells. The data showed that the enantiomers of *cis*-BF induced different gene expression related to cytotoxicity.

The development of structural biology and computer-based approaches such as QSARs, molecular modeling, ligand docking, and molecular dynamics simulation have emerged as a powerful tools to investigate stereoselective chemical-macromolecule (i. e. receptors) interactions (*49*). In our previous study, the molecular docking assay has been determined to be a valid assay to explain the enantioselective estrogen-like activity of some typical chiral pesticides (*50*). It connects the enantioselective toxic results and potentially selective binding between chiral pesticides and macromolecules such as DNA and proteins. Therefore, it will be a major goal for the toxicity assessment of chiral pesticides to develop an integrated testing strategy. The integrated testing strategy is based on the use of two or more approaches including physicochemical, *in vitro*, human (e.g. epidemiological and clinical case reports), *in vivo* and computational methods to assess the toxicity of chiral pesticides.

The emphasis on *in vitro* methods is to provide mechanistic information. The mechanisms of chiral pesticide-induced enantioselective cytotoxicity have proved to be a varied, complex network. For chiral pesticides, the relative complexity of mechanisms lies with chiral enzymes, receptors and other macromolecules. It is clear that further study of the side-effects of chiral pesticides must more extensively focus on mechanisms. In summary, enantioselective toxicity is an important field. It requires more serious attention as the use and development of chiral pesticides increases.

Conclusion

This review suggests that the cytotoxicity of chiral pesticides should be investigated at the enantiomeric level. *In vitro* methods not only play an important role in hazard identification, but also in identifying mechanisms of the enantioselective ecotoxicities of chiral pesticides. It seems that many issues regarding the interactions between chiral pesticides and cytotoxicity remain to be addressed.

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Chapter 11

Evaluating in Vivo Toxicity of Chiral Pesticides Using the Zebrafish (*Danio rerio*) Embryo Model

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Enantioselectivity in toxicology of chiral pesticides has become one of the frontier topics facing toxicology. However, the toxicological data of chiral pesticides is in great scarcity. This is because of the different toxicological profiles between enantiomers and the racemates, and the dearth of enough enantiopure samples for toxicity assay. Zebrafish (*Danio rerio*) embryos provide an attractive model for determining the acute toxicity in relation to environmental risk assessment of chiral pesticides, offering the possibilities to perform small-scale, high-throughput analyses and less sample needs compared with other *in vivo* models. Beyond their application for determining acute toxicity, zebrafish embryos are also excellent models for understanding toxic mechanisms and the indication of possible adverse and long-term effects. Various applications of zebrafish embryos have been used for studying toxicogenomics, such as the effect of chemicals on gene and protein expression patterns. Other major possible applications include studies in metabolism, bioconcentration and biomarkers. These unique properties make them especially suitable for in vivo enantioselective toxicity assay of chiral pesticides.

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Introduction

Enantiomers of chiral pesticides have unique biological properties that make prediction of their toxicity very difficult. Their potential effects on human health and ecosystems are complex (*1*). Therefore, it is important for toxicological evaluation of chiral pesticides to include single enantiomer toxicological and safety evaluations in living bodies, so that actual risks of chiral pesticides are defined and adverse environmental consequences are minimized (*2*). On the other hand, recent advances in separation and analytical techniques have made possible the detection, isolation and preparation of enantiomers, although still on a small scale (*3*). Thus, there is an obvious need for the development of rapid, relevant and efficient testing strategies to evaluate the biological activity and toxic potential of chiral pesticides.

In *vitro* studies such as those using cultured cells are a very important and productive approach to obtain understanding of toxic mechanisms of chiral pesticides, and in fact, they have been widely used. However, *in vitro* systems have the limitation of obtaining appropriate cell lines or primary cells and do not reflect the natural environment of cells in the body. Whole organism test studies can provide the most comprehensive understanding of toxic effects. However, the use of mammals is not only expensive, but also labor, time and dose-consuming. Furthermore, as the ethical concerns increasingly arise, the use of mammals in large scale toxicological screening programs has been limited.

During recent decades, embryos and non-feeding larvae of the vertebrate zebrafish have been developed as cheap, effective alternatives that reduce and refine animal use in *in vivo* research (*4–6*). The zebrafish is a small tropical fish native to the rivers of India and South Asia (*7*). A number of unique features have contributed to its attraction in toxicology, such as its rapid development, easy maintenance in the laboratory, large number of offspring, transparency of embryos and access to experimental manipulation. After 24 hpf (hours postfertilisation), the basic body structure is laid out, and after approximately 2–3 dpf (days post-fertilisation), the embryos can hatch (Figure I). The transparent chorion enables easy observation of development stages (*8*). Zebrafish have a very short reproduction cycle. They reach maturity at the age of about 3 months. One female can spawn more than 100 eggs in each clutch which are fertilised by sperm release from the male into the water. Under good laboratory conditions several thousands embryo can easily be collected daily and used for parallel experimental treatments.

These unique features make zebrafish a complete, developing vertebrate organism, and allow testing at levels of organ and organism toxicity. Also, their anatomic and genomic similarities with humans allow some predictability of mammal and human toxicity (*9*). On the other hand, early developmental life stages are often very sensitive to environmental insult, due to the enormous changes in cellular differentiation, proliferation and migration necessary to form required cell types, tissues and organs (*6*, *10*). Since molecular signaling underlies all of these processes and most toxic responses result from disruption of proper molecular signaling, early developmental life stages are the ideal stage to determine toxicology of chiral pesticides.

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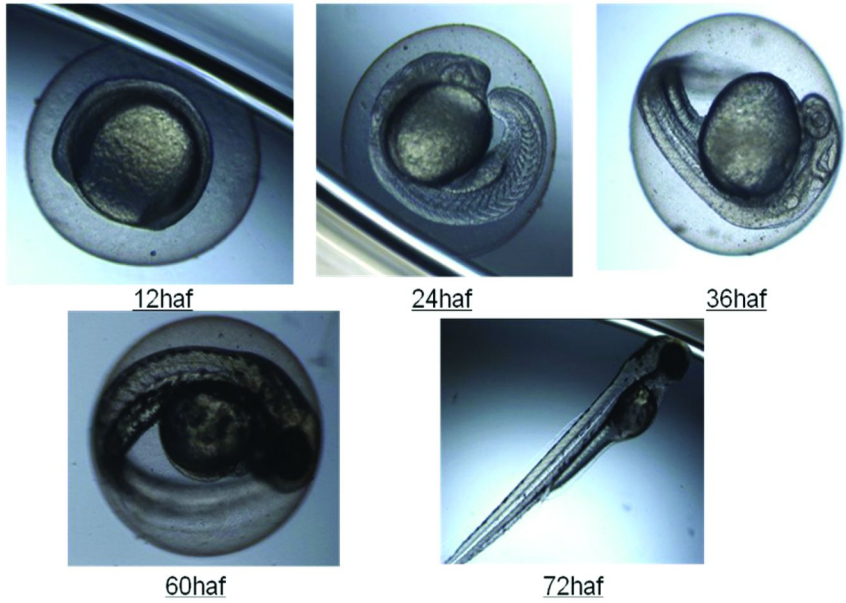

Figure I. Zebrafish embryos at different development stages.

The zebrafish embryo was originally used to study the genetics of development due to its transparency, quick embryonic development, easy collection in high numbers and similarity with human development (*11*). Nowadays, zebrafish offer a model with basic structure not much different from a mouse or a human (*12*). It allows not only phenotypic screens to identify gene function on a large scale, but offers many mutation types for research (*12*, *13*). These mutagenesis protocols are complemented by reverse genetic techniques that allow manipulation of specific gene functions (*14*). In recent years, small chemicals were induced to manipulate the specific developmental pathways of embryos (e.g. inhibitors of Fibroblast Growth Factor, Retinoic acid and Sonic Hedgehog) in a conditional manner (*15–17*). Use of zebrafish embryos has the unique advantages of abundance background knowledge, technology and approaches.

Zebrafish Maintenance and Egg Production

In our studies, zebrafish were maintained in a light/dark cycle of 14:10 h at 26±1 ºC under semi-static conditions with charcoal filtered water. The zebrafish were fed with live brine shrimp (*Artemia nauplii*) twice a day. Since our primary aim was to reproducibly produce large amounts of eggs for exposure studies, we also used commercially available pet shop fish and customized the standard procedures according to our needs. Briefly, embryos were obtained from adult fish free of macroscopically discernable symptoms of infection and disease with a preferable ratio of 1:2 for female to male. Four groups of genitors were placed

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separately in a specific spawning aquarium equipped with a mesh bottom to prevent the eggs from being cannibalized. Spawning was induced in the morning when the light was turned on. Half an hour later, eggs from each aquarium were collected and used immediately for the exposure experiment. For details about the maintenance, feeding and breeding of zebrafish see the Zebrafish Information Network ZFIN (Zebrafish Information Network).

Zebrafish embryos are so small that several of them can even be placed in the well of a 384 well plate. This makes it possible to achieve medium to high throughput toxicity testing. In some recent studies, such systems show the ability to match the chemical entitles with specific biological activity. During recent years, automated screening technologies with zebrafish embryos (i.e. microscopes combined with intelligent image acquisition systems) have been extensively developed, these provide toxicological profiles of toxicants at high spatial and temporal resolution.

Applications of the Zebrafish Model

Acute Toxicity and Teratogenicity

Acute fish toxicity tests are widely required for the testing of pesticides for ecological risk assessment (*18*, *19*). However, as animal welfare legislations becomes more and more important, there is a great public demand for the replacement of animal tests for ethical reasons. Also, less time consuming and expensive replacement of testing methods are required. The zebrafish embryo (up to 3-4 days after hatching) is not considered an animal by current European legislation (*20*), and therefore, its use in research studies is coherent with trying to reduce and replace the use of rodents for toxicity studies. As a matter of fact, the first application of the zebrafish embryo in environmental research was promoted by the aim to develop an alternative to the 96-h acute fish toxicity test (*21*). Acute toxicity in fish embryos correlates very well with acute toxicity in adult fish.And fish embryo test has been submitted to the Organization for Economic Co-operation and Development (OECD) (*22*). In Germany, the zebrafish embryo test was introduced as a standardized ISO assay replacing traditional toxicological tests with adult fish (*21*, *23*).

In our preliminary study with the synthetic pyrethroids of lambda-cyhalothrin, the enantiomers showed the same enantioselective toxicity as in the fish toxicity test, that is, the (−)-enantiomer was more than 100 times toxic than the (+)-enantiomer in 96-h acute toxicity test with zebrafish adults (Table I). The zebrafish embryo acute toxicity test showed the same pattern; the (−)-enantiomer is 7.2 times stronger in 96-h mortality (*24*). In a study with fenvalerate, the *αS*-2*S*-enantiomer was 56 times more toxic than the *αR*-2*R*-enantiomer (*25*). The assay of zebrafish embryo-larval showed that the *αS*-2*S*-enantiomer was 3.8 times stronger than the *αR*-2*R*-enantiomer in 96-h mortality.

Screening for developmental disorders as an indicator of teratogenic effects (such as crooked body) can also be included in analysis of acute toxicity in embryos (*21*, *26*). Other morphological, sublethal endpoints, such as heart beat rates, yolk sac edema and spontaneous movements may also supply useful

information of long-term effects of chemicals. In our study with acetofenate, significant enantioselectivity in developmental toxicities such as yolk sac edema and pericardial edema was observed in zebrafish embryo (*27*). These pattern followed the order (+)-enantiomer > (±) –racemate > (−)-enantiomer. Endpoints include yolk edema, pericardial edema and crooked body. In the study with lambda-cyhalothrin, significant difference was observed between the two enantiomers (*27*) (Table II). The mortality of zebrafish embryo test showed that the (−)-enantiomer was 7.2 times stronger than the (+)-enantiomer in mortality. The teratogenicity also showed the similar patterns. Statistical analysis showed that the (−)-enantiomer was significantly more toxic than the (+)-enantiomer in inducing crooked body and pericardial edema. In other study with fenvalerate, four stereoisomers showed enanioselectively embryonic toxicity in inducing crooked body, yolk sac edema and pericardial edema (*25*). The *αS*-2*S*-isomer was most toxicity in developmental toxicity of zebrafish embryo.

Although most assays using zebrafish embryos rely on morphological endpoints, chiral factors are implicated in the actual teratogenic mechanism of several compounds. In order to further reveal the toxicant-specific mechanisms of action, the gene expression profile should be involved in studies.

Table I. LC_{50} values of enantiomers of lambda-cyhalothrin for aquatic vertebrate zebrafish (μg^{-1}). Ref. (*24*)

Exposure time	*(±)*	*(-)*	*(+)*
24h	2.123±0.062	2.033±0.042	>120
48h	1.105±0.034	1.025±0.034	>120
72h	0.832±0.021	0.794±0.021	>120
96h	0.875±0.032	0.740±0.032	>120

Endocrine Disruption

A wide variety of pesticides have been shown to mimic endogenous hormones. The presence of these compounds in the aquatic environment has been associated with a number of reproductive disorders or disruption of sexual differentiation, particularly in fish (*28*). These chemicals induce endocrine disruption act via estrogen, androgen and thyroid receptors. By measuring the effect of exposure on hormone-responsive genes, the zebrafish embryo model can be an important screening tool for endocrine disrupting compounds. Such a screening assay has been proven to have as much sensitivity as other endpoints and screening systems (*29*, *30*). Furthermore, it is principally possible to monitor of other endpoints like androgenic, glucocorticoid, thyroid or other hormone markers in the embryo model.

Table II. Summary of enantioselective embryo toxicity of lambda-cyhalothrin responsive endpoints (mg L^{-1}))

Developmental defects		*48-h*			*72-h*			*96-h*		
		±	+	-	±	+	-	±	+	-
Mortality (LC_{50})		[a]	[a]	[a]	1.30	[a]	1.30	1.30	[a]	0.18
Malformation EC_{50}	crooked body	0.07	[c]	0.16	0.03	0.46	0.03	0.03	0.09	0.03
	yolk sac edema	0.33	[b]	[b]	[b]	[b]	>0.30	[b]	[b]	[b]
	pericardial edema	0.30	[d]	>0.30	0.07	>0.30	>0.30	0.05	0.34	0.09
NOECs[d]	crooked body	<0.05	[c]	<0.20	<0.05	<0.10	<0.01	<0.05	<0.10	<0.01
	yolk sac edema	<0.05	[b]	[b]	[b]	[b]	<0.30	[b]	[b]	[b]
	pericardial edema	<0.1	[d]	<0.30	<0.05	<0.30	<0.20	<0.05	<0.20	<0.05

[a] Can't be determined, because the highest concentration (0.30ppm) of solvents used in the present study did not result mortality. [b] At all concentrations, no yolk sac edema was observed. [c] At all concentrations, no crooked body was observed. [d] At all concentrations, no pericardial edema was observed. d No observed effect concentrations. Ref. (*27*).

In our previous study, real-time, quantitative polymerase chain reaction was adapted to investigate the induction of estrogen-responsive gene expression in embryo-larval zebrafish after 7 days of exposure to synthetic pyrethroid permethrin (PM) enantiomers. The results indicated significant differences in the induction of estrogen-responsive gene expression between enantiomers. At the exposure level of 1.0 μg L^{-1}, the vtg1, esr*α* and cyp 19b responses to the (−)-*trans* enantiomers were about 3.2-, 1.8- and 1.5-fold higher than those in the group treated with (+)-*trans* enantiomers. In the two cis-enantiomer treatment groups, (+)-*cis*-enantiomer increased the mRNA level of the cyp 19b gene about 1.5-fold higher than the (−)-*cis*-enantiomer (*31*). A similar observation was also obtained for acetofenate. The two enantiomers of acetofenate showed significant differences in inducing estrogen receptor ERα expressions in zebrafish embryos. The data of qRT-PCR showed that there was about 3.2-fold differences in induction of the mRNA levels of ERα between embryo exposed to (+)-or (−)-enantiomer and the racemate (*27*). (Figure II)

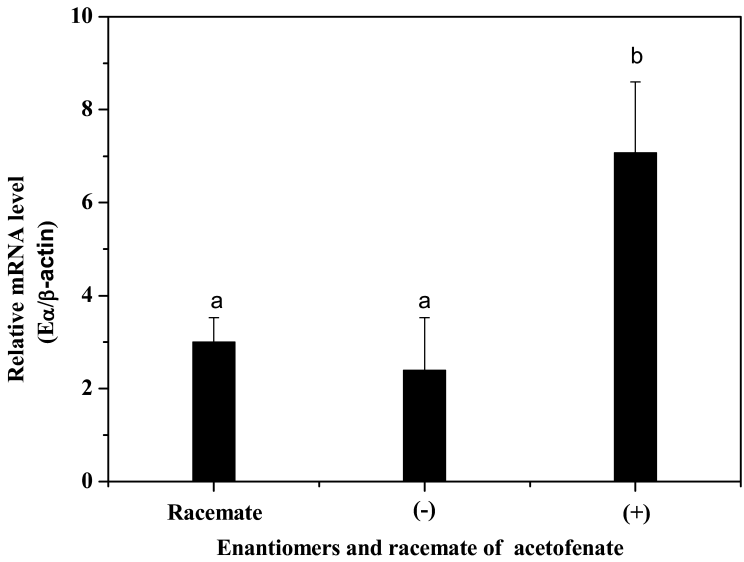

Figure II. Expressions of ERα in zebrafish embryo after 72-h exposed to acetofenate enantiomers and racemate. Values were normalized against β-actin as housekeeping gene, and represent mean mRNA expression value ±S.E.M (n=5) relative to those of male controls. Different letters above adjacent bars indicate a significant difference ($p < 0.05$) between individual enantiomer or racemate, while the same letter indicates no significant difference. Ref. (27).

Recommendations and Perspectives

The rates of uptake and metabolism are important parameters determining the enantioselectivity in bioavailable and effective concentrations of a chiral chemical in an organism. Previous studies have provided evidence that embryos

exhibit a strong bioconcentration potential (*32*). Other studies demonstrated that the differences in the bioaccumulation of different chemicals can be explained by the correlation of bioconcentration and lipophilicity of zebrafish embryo (*33*, *34*). However, the egg membrane could provide a barrier for uptake of chemicals. For instance, chemicals with high lipophilicity, they must overcome the hydrophobic resistance to pass through the chorion and biological membranes to reach the target sites in embryos. On the other hand, the chorion contains pores that might be responsible for size-dependent restrictions (*35*). The bioconcentration might be stage-specific during embryo development. For instance, the yolk could represent an additional reservoir for accumulation of chemicals.

Certain transporter proteins can play a major role in protection of a cell/ organism against toxic compounds. The ATP binding cassette-transporter family is involved in the extrusion of a wide range of chemicals from the cell (*36*). The activities of these transporters could be affected by environmentally relevant chemicals including pesticides (*37*). Interaction of xenobiotics with membranes, or binding to proteins or DNA, can lead to alterations in gene transcription or protein expression. As a result, these changes may affect the metabolomic profile. The application of the zebrafish embryo model in toxicogenomics has showed its capacity to reveal information on the mode of action of potential hazardous compound through their metabolomic pathways (*38*, *39*). Hoyt et al. (*40*) reported a microarray analysis of zebrafish embryos exposed to 4-nonyphenol; the result showed clusters of genes differentially expressed in all of the tested concentrations. After exposure to 2,3,7,8-tetrachlorodibenzo p-dioxin, a cluster of genes down-regulated in the zebrafish embryonic heart was identified by using transcriptome analysis (*41*). The changes of gene expression observed were mostly at concentrations below those that induce morphologic effects.

Enantioselectivity in long term effects of chiral pesticides is especially important in understanding the ecological effects of these chemicals. Although the zebrafish embryo test has been proved a valuable alternative for acute toxicity tests with juvenile/adult fish, there is no replacement available for chronic toxicity test. However, analysis of molecular markers during fish embryo and short-term exposure can indicate the early events for toxic actions of chemicals. The potential of the zebrafish embryo model as a replacement of animal experiments can be used to indicate adverse and long-term effects in fish or fish populations. The molecular markers may respond more sensitively than endpoints of the conventional embryo test and might be used to predict chronic toxicity. Toxicogenomic studies have revealed many biomarkers in zebrafish models, such as biomarkers for immune modulation, endocrine disruption or genotoxicity. Certain gene responses in the zebrafish embryo are exerted in the same range of concentration as toxic responses in a chronic fish early life stage toxicity test (*42*). These gene expressions in fish embryos could identify as specific modes of action and indicate potential sub-lethal, long-term effects not predicted by other methods, such as quantitative structure-activity relationship. Additionally, the effect of gene expression change observed at lower concentrations could be useful to implement gene expression in regulatory tests for chronic toxicity (*43*).

For instance, lots of chiral pesticides can disturb the immune defense system. The innate immune response or components of the underpinning signaling

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pathways have already been established in zebrafish embryos (*42*). Potential immune-modulating effects on fish have not yet been implemented in testing strategies for environmental risk assessment. The zebrafish embryo model would allow establishing an alternative and efficient test system by analyzing the effect of chemicals on components of the innate immune system. The zebrafish embryo model can also be used for analysis of the genotoxic capacity of a toxicant. For instance, detection of single-strand breaks by the comet assay has been developed for zebrafish embryos and applied to demonstrate the genotoxic effects by exposure to river sediments or model compounds as well (*44*). The transgenic approach used in zebrafish can allow analysis of the mutational spectra by DNA sequencing and, hence, on the information on the genotoxicity of chiral pesticides.

With the targeted gene knockout techniques developed for zebrafish embryo, more mutations in genes that have a bearing on the toxic mechanisms will become available in the near future. One example is the acetylcholine esterase gene. The inhibition of acetylcholinesterase (AChE) is the fundamental mechanism of organophophorus insecticides, and these effects can be enantioselective (*1*). However, with the mutant phenotypes of AChE knockout, blueprints of the ideal inhibitor activity can be obtained (*45*).

Numerous other research areas such as developmental toxicity, neurotoxicity, locomotion and behavior of zebrafish embryos were addressed in pilot studies (*46–48*). With the current attention to human toxicity of chiral pesticides, the use of zebrafish in prediction of human health effects of these chemicals is being conducted. For instance, studies have showed that warfarin and aspirin have very similar effects in zebrafish and humans (*49*). However, test results with zebrafish embryo can be more sensitive than with mammals with a few substances (*50*). The zebrafish embryo system offer the complexity of a whole vertebrate organism but could eventually achieve the throughput of traditional toxicological screens, and could help to overcome the bottlenecks of enantioselective toxicity assessment.

Conclusion

Along with the trend of increasing chiral pesticide use, more toxicological information on chiral pesticides at enantiomeric levels is urgently needed (*1*). The zebrafish embryo represents a model with an impressive range of possible applications in toxicology of chiral pesticides. The number of advantages will increase in the near future. The genome is or is mostly known. And there are thousands of mutants available. As a result, the adaptation of molecular and system analyses from biological research is likely to extend to toxicology. Given its experimental advantages, the zebrafish embryo is one of the most promising vertebrate systems for mechanistic toxicology (*51*, *52*). This model can reveal diverse aspects of toxicology and pharmacology, and the additional complexity permits a more complete and precise understanding of toxicological pathophysiology in enantioselectivity. Thus, the environmental safety and human health effects of chiral pesticides can be taken into consideration in order to make sure that they have the least amount of toxic potential.

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Chapter 12

Ecological Risk Assessment Issues for Chiral Pesticides

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The USEPA's ecological risk assessment framework provides a clearly defined process for conducting ecological risk assessments for the registration and re-evaluation of pesticides. The ultimate product of this framework is an ecological risk characterization and assessment, which integrates data on pesticide use, chemistry, and ecotoxicity. Consideration of chiral pesticides in the risk assessment process poses numerous challenges related to the enantioselectivity in environmental fate processes and ecotoxicity. A critical assessment of risk assessment issues for chiral pesticides is discussed in the context of the USEPA risk assessment framework.

Introduction

Before a pesticide can be registered or sold in the United States, the U.S. Environmental Protection Agency (USEPA) evaluates the potential risk of the pesticide to humans, the environment, and non-target species. In its risk assessments, EPA considers the chemical, biological, and toxicological properties of each pesticide. Of the over 900 pesticides registered in the U. S., approximately 30% are chiral compounds (*1*). Because chiral pesticides can exhibit differential toxicity and environmental behavior in soil, water, and air, the chirality of the pesticide needs to be considered in EPA's risk assessments.

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The complexity of a risk assessment depends on a number of factors, including its intended purpose (*3*). Screening-level risk assessments are conservative and generally require only basic data on the environmental chemistry and toxicity of a pesticide. With a screening-level approach, regulators can screen out those pesticides that have a low probability of adverse risk. If risks cannot be dismissed, the screening-level assessment can be refined by considering more detailed information on spatially-dependent variables (e.g., environmental fate data, soil properties, meteorology, agronomic practices) and temporally-dependent variables (e.g., meteorology, pesticide application timing, crop planting dates). The resulting refined assessment results in a more accurate and consequently less conservative estimate of risk.

A risk assessment is comprised of three major components: 1) problem formulation, 2) analysis, and 3) risk characterization (Figure 1) (*4*). The ***problem formulation*** identifies the assessment endpoints and ecological characteristics that are important to protect and describes the mode of action, use characteristics, chemistry, environmental fate, and toxicity of the pesticide and its degradation products. This information is integrated into a conceptual model to identify the routes of dissipation and exposure pathways. More importantly, this integrated information leads to a detailed analysis plan for analyzing data and characterizing risk. In the ***analysis*** phase, the risk assessor develops the exposure and toxicity profiles. The exposure profile provides an estimation of environmental concentrations (EECs) in aquatic and terrestrial environments based on the disposition of the pesticide and the nature of the receiving ecosystem. The toxicity profile summarizes data on the effects of the pesticide and relates them to the assessment endpoints. The final phase, ***risk characterization,*** integrates the analyses from the toxicity and exposure assessments and quantifies risk to ecological entities. This process requires risk analysis and interpretation of the probability for adverse effects.

The objective of this paper is to provide a critical analysis of the USEPA risk assessment framework for chiral pesticides. The paper focuses on an analysis of enantiomers, which have potentially different toxicity and environmental fate properties in terrestrial and aquatic ecosystems.

Problem Formulation

Pesticide Characteristics

Specific tests for determining the environmental behavior, physicochemical properties, and toxicity of pesticides are mandated by the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) and described in the Code of Federal Regulations 40 CFR Part 158 (*2*). In addition, pesticide manufacturers are required to submit data that describe the mixture of chemicals in the end use pesticide product (product chemistry). These data provide information on the identification and concentration of the pesticide product composition, including both active and inert ingredients as well as the physicochemical properties of the pesticide product. Often, a mixture of compounds, which may include enantiomers, diastereomers, and even in some cases, geometric isomers, will be

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considered as a single active ingredient for purposes of registration and labeling. An active ingredient with a mixture of stereoisomers can substantially complicate the conduct of risk assessment since each stereoisomer may have different physical/chemical properties and toxicity characteristics. For this reason, the agrochemical industry is beginning to consider enantioselective toxicity and are isomerically enriching to the more active enantiomer for certain pesticides. For example, the S-enantiomer of metolachlor is the active enantiomer that is registered, while the R-enantiomer is inactive.

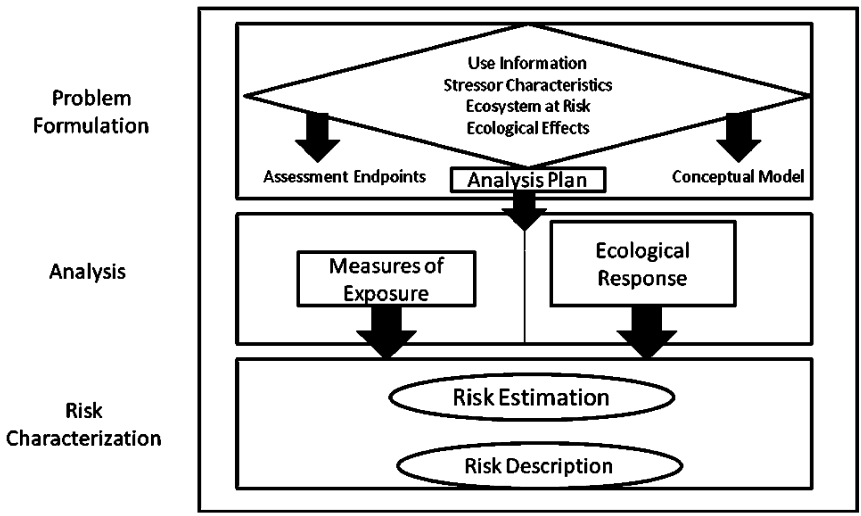

Figure 1. Risk Assessment Framework (4).

Use Area

The use pattern of a pesticide depends on the geographic distribution of crop(s) to which it is applied. Widely used pesticides, such as corn herbicides, can be used anywhere in the United States. In contrast, pesticides applied on regionally grown crops, such as rice insecticides, are only used in rice-growing regions of the United States (e.g., LA, MS, CA, TX, AR). Soil properties, particularly the nature of the organic matter and microbial populations, can vary substantially depending on the parent material, weather, ecology, age of landscape, topography, and agronomic practices of the use area (*5*). Because soil organic matter and microbes are chiral, they can exhibit enantioselective behavior with respect to chiral pesticides. Unfortunately, this variability is not considered in current risk assessments since it is difficult to predict or explain the variability of enantioselectivity among different soils.

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Environmental Fate Characteristics

The environmental fate studies required for pesticide registration are conducted in various media and with different forms of the pesticide, e.g., technical grade, formulated product (Table I) (*2*). Laboratory studies, with the exception of the volatility from soil study, are conducted using the radiolabeled pure active ingredient (abbreviated PAIRA). The use of a radiolabeled test substance in laboratory studies allows for extensive quantification and identification of degradation products. Abiotic degradation studies such as hydrolysis are conducted in sterile buffer solution at pHs 4, 7, and 9 while photodegradation in water studies are conducted in a system buffered at a pH that minimizes the competing hydrolysis rate. Pure enantiomeric pairs are expected to exhibit similar abiotic degradation rates (*6*). To the extent these abiotic reactions dominate the environmental fate of the pesticide, enantioselective behavior is not expected to occur in the environment. However, since microbially mediated degradation processes dominate degradation of most pesticides, enantioselective behavior is expected to occur. For example, microbially mediated hydrolysis of ester pyrethroids is an enantioselective process (*7*).

The USEPA considers microbially mediated degradation in aerobic soil, anaerobic soil, aerobic water/sediment, and anaerobic water/sediment tests. These studies, however, may pose a problem when assessing the extent of enantioselectivity because of the limited representation of soil/sediment/water environments (*8–11*). Aerobic and anaerobic metabolism and field studies require multiple soil/sediment/water systems to represent the universe of use conditions for a pesticide in order to provide an indication of the range of half-lives as well as degradation pathways. The ability of the studies to capture the range of enantioselectivity of metabolism or sorption in different environments is a major uncertainty.

Because there are no guidelines for stipulating enantioselective analytical capabilities, assessing and interpreting environmental fate studies for enantiomers can be particularly challenging. Typically, the extent of enantioselectivity is gauged using comparative analysis of environmental fate data for the racemate and its separated active enantiomer, when these data are available. However, enantioselective analysis is a more appropriate tool to use since it allows calculation of enantiomer ratios or enantiomer fractions (*12*, *13*). Another consideration is the ability to clearly establish the enantioselectivity of metabolism due to the high variation of metabolism rates (typically coefficients of variation = 100%) in laboratory studies. High background variability in metabolism is expected to mask determination of enantioselectivity using enantiomeric ratios or enantiomeric fractions.

Table I. Environmental Fate Data Requirements for Pesticide Registration in the United States

Data	*Media*	*Number of Soil/Water Types*	*Test Substance*
Hydrolysis	Sterile Buffer	None	PAIRA
Photodegradation in Water	Sterile Buffer	None	PAIRA
Photodegradation in Soil	Soil	1	PAIRA
Photodegradation in Air	Air	1	PAIRA
Aerobic Soil Metabolism	Soil	4	PAIRA
Aerobic Aquatic Metabolism	Sediment:Water	2	PAIRA
Anaerobic Soil Metabolism	Soil	2	PAIRA
Anaerobic Aquatic Metabolism	Sediment:Water	2	PAIRA
Batch Equilibrium	Soil	4	PAIRA
Laboratory Volatility	Soil	1	TEP
Field Volatility	Soil	≥1	TEP
Terrestrial Field Dissipation	Soil	≥1	TEP
Aquatic Field Dissipation	Sediment:Water	≥1	TEP
Bioaccumulation in Fish	Laboratory Water	1	TEP

PAIRA-Pure Active Ingredient Radiolabeled TEP- Typical End Use Product

Ecological Effects Characteristics

EPA's ecotoxicity testing strategy is designed to assess the toxicity of the most sensitive species based on studies submitted by the registrant as well as open literature studies (ECOTOX). (Table II) (*3*). Toxicity testing is generally conducted on the technical grade of the active ingredient or technical end use product.

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In general, ecotoxicity studies are designed to assess cumulative residue effects of the pesticide in an exposure media. Because ecotoxicity studies do not require extensive identification of residues, the toxicity endpoint is representative of a mixture of compounds (degradation products, isomers, etc.) rather than an individual compound. Teasing out the relative toxicity of enantiomers is difficult without toxicity data for the individual enantiomers, which are not usually available. Toxicity testing of individual enantiomers can be extremely complex and expensive for pesticides with multiple chiral centers due to the exponential nature of the number of enantiomers per chiral center (2^n, n=number of chiral centers) (*6*).

Table II. Ecotoxicity Data Requirements for Pesticide Registration in the United States

Data	*Typical Test Organisms*	*Test Substance*
Avian Acute Oral Toxicity	Northern bobwhite	TGAI
Avian Acute Dietary Toxicity	Northern bobwhite	TGAI
Avian Reproduction	Northern bobwhite	TGAI
Freshwater fish acute toxicity	Bluegill Sunfish/Rainbow Trout	TGAI and TEP
Freshwater fish early life cycle	Fathead minnow	TGAI
Acute Toxicity freshwater invertebrate	*Daphnia magna*	TGAI and TEP
Acute Toxicity estuarine/ marine invertebrate	Mysid shrimp	TGAI and TEP
Acute Toxicity estuarine/marine fish	Sheepshead minnow	TGAI and TEP
Freshwater invertebrate life cycle	Daphnia magna	TGAI
Honey Bee acute contact toxicity	Honeybee	TGAI
Seedling Emergence	Monocots/Dicots	TEP
Vegetative Vigor	Monocots/Dicots	TEP
Acute Toxicity Algae	Algae	TGAI and TEP
Acute Toxicity Aquatic vascular plant	*Lemma gibba*	TGAI and TEP

TGAI- Technical Grade Active Ingredient TEP- Typical End Use Product

Conceptual Model

A conceptual risk assessment model has many exposure pathways and receptors that may be expected to exhibit enantioselectivity (Figures 2 and 3) (*4*). The dark gray boxes illustrate potential enantioselective environmental fate and transport processes. Receptor organisms, highlighted in light gray, also

have the potential to exhibit enantioselective effects on toxicity endpoints. The models illustrate a high degree of complexity, whereby each chiral compound can theoretically move through the enantioselective exposure pathways at different rates and exhibit different toxicity endpoints in each organism.

Analysis Phase

In the analysis phase, the risk assessor evaluates the exposure of different organisms to pesticides and the relationships between pesticide concentrations and ecological effects. Although there is no unified approach for assessing the risk of chiral pesticides, proposed risk assessment strategies for chiral compounds are based on the relative toxicity of isomers if they are known (*14*, *15*). These risk assessment strategies are based on a complete characterization of the stereochemistry of the pesticide, including chemical nomenclature with appropriate stereochemical descriptors, concentration range of individual isomers, impurities, and availability of an appropriate analytical method for identification and quantification of individual isomers. Critical to these strategies is identification of the *active isomer* as the active ingredient.

Ideally, this *active isomer* is established by the pesticide manufacturer, either by declaration or through toxicity testing of individual isomers. Once the active ingredient is identified, the toxicity of the *active isomer* should be compared with other isomers in the pesticide product. This comparison can be accomplished through toxicity testing of individual isomers or through comparative toxicity testing of the racemic pesticide and isomerically-enriched pesticide. If there is no difference in toxicity among isomers, then the mixture of individual isomers can be considered as a single pesticide. If there is a difference in toxicity among isomers, then toxicity of all isomers should be evaluated. When the *active isomer* is more toxic than the inactive isomers, the inactive isomers can be treated as impurities. When the *active isomer* is less toxic than the inactive isomers, then the development of the pesticide product may require isomeric enrichment. The proposed risk assessment strategies for chiral pesticides, however, lack a comprehensive plan for assessing enantioselectivity of environmental fate processes. Additionally, a risk assessment based on an *active isomer* and inactive *isomers* may be feasible for assessing risk to a single organism, but it may not be practical in assessing toxicity to many different types of organisms (*e.g.* mammal, birds, fish, invertebrates, and plants).

In the past, EPA has attempted to address stereochemistry issues for optical isomers using bridging data to link environment fate and ecological effects of enantiomers in racemic mixtures and enriched enantiomers (*16*). This assessment strategy was developed for a pure enantiomer or partially resolved racemic mixture of a currently registered racemic pesticide. For environmental fate studies, the bridging data have traditionally focused on soil and aquatic laboratory metabolism studies because enantioselective degradation is expected in soil and aquatic environments. The bridging data for ecotoxicity studies have generally considered all available ecotoxicity data on the racemic mixture and enriched enantiomer. A comparison of environmental fate and ecotoxicity data for the

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racemic mixture and enriched enantiomer is used to ensure that relative risk of the enriched isomer is not greater than the racemic pesticide. This testing strategy has been used, for example, to assess risk for S-metolachlor, mefanoxam, and indoxacarb. Although the strategy considers environmental fate and toxicity to different organisms, it is not capable of assessing the impact of individual enantiomers without appropriate enantioselective analytical methods used in the studies. Additionally, the environmental fate studies are conducted in a limited number of environmental media (*e.g.* soils, sediments, waters), which prevents a comprehensive understanding of the extent to which enantioselectivity affects metabolism and sorption in different environmental media.

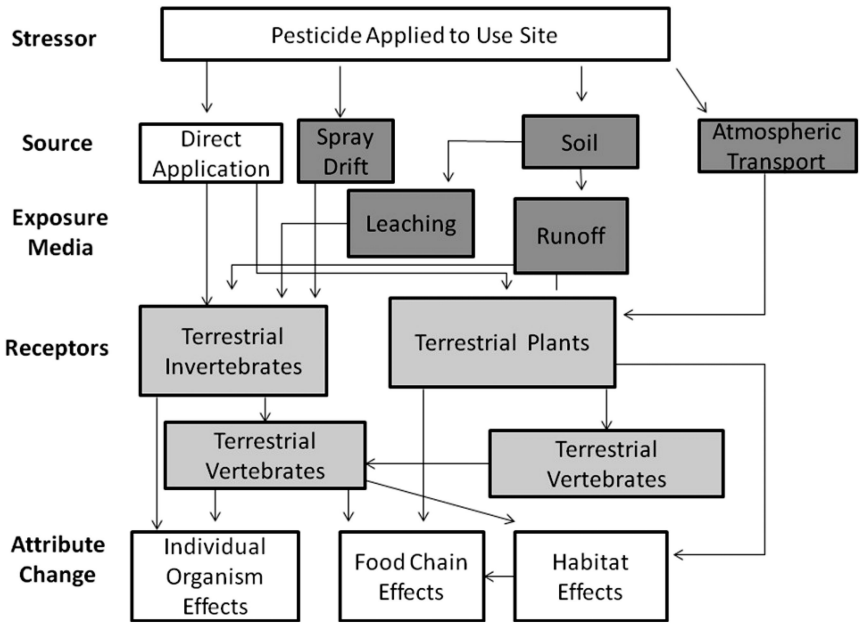

Figure 2. Conceptual Terrestrial Exposure Pathways and Receptor Organisms.

Exposure Assessment

When assessing the exposure of a pesticide, EPA relies on both mathematical models and monitoring data to estimate pesticide exposure. Mathematical models are used to produce estimated environmental concentrations (EECs) in the soil and aquatic environments. Monitoring data are used to characterize the pesticide exposure estimates in the context of mathematical modeling.

Modeling

Exposure assessment is the process of estimating the potential exposure of an organism to a pesticide and its degradation products in aquatic and terrestrial environments. In assessing exposure, EPA typically relies on mathematical models to generate EECs. These estimates are based on laboratory data that describe how fast the pesticide breaks down in the environment and how it moves in the environment. Screening-level models that EPA uses to provide conservative exposure estimates such as GENEEC, PRZM/EXAMS, SCIGROW, and TREX (*17*, *18*). The ability of exposure models to estimate concentrations of chiral pesticides is solely dependent on the availability of enantiospecific environmental fate properties. For some chiral pesticides (*e.g.* azinphos methyl), estimation of the enantiospecific exposure cannot be done because there are no enantioselective environmental fate data.

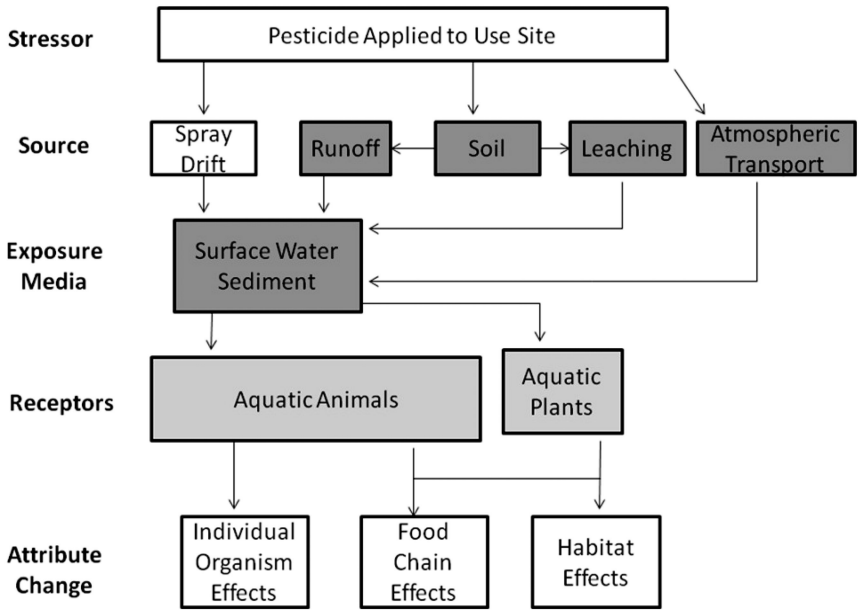

Figure 3. Conceptual Aquatic Exposure Pathways and Receptor Organisms.

Input parameters for EPA's exposure screening models are shown in Table 3. Enantioselective model input parameters include the application rate, soil/water partitioning coefficients, aerobic soil metabolism, aerobic aquatic metabolism, and anaerobic aquatic metabolism.

An important input parameter in exposure modeling is the application rate (lbs a.i./A). If the application rate is reduced, the EECs will be proportionally reduced. The estimated environmental concentration (EEC) is proportionally reduced with a reduction in application rate. This is an important issue because the application rate for a racemic pesticide (50:50 R:S) consists of 50% active enantiomer and 50% inactive enantiomer. Consequently, equivalent application rates for racemic and enantiomerically enriched pesticide will result in up to double the activity (toxicity) for the enantiomerically-enriched pesticide. The application rate of the active isomer for enriched pesticide products (*i.e.*, partially resolved or 100% enriched), however, is generally similar to the application rate of the racemic mixture when normalized for active isomer percentage. Another consideration is the application rate of isomers of unknown toxicity. In these cases, the application rate should reflect the application rate of all isomers to estimate conservative EECs.

A further consideration in exposure modeling is the ability to account for the enantioselectivity in pesticide metabolism and sorption. Variation in enantioselectivity has been attributed to variations in soil microbial populations, redox conditions, and soil pH (*8*, *10*, *11*, *19–22*). As discussed above, a large number of soil and sediment studies may be needed to address relationships of enantioselective degradation rates and soil properties. Aquatic exposure modeling for pesticide registration is conducted using the upper 90th percent confidence bound of the mean half-life. This approach is expected to provide a conservative estimation of persistence in soil and aquatic environments which accounts for the variation in half-lives. However, it is unclear whether this approach correctly addresses enantioselective effects among soils and sediments.

Enantiomerization, which is the inter-conversion of one enantiomer into another, is another consideration in exposure modeling. Enantiomerization has been observed for MCPP and DCPP in soil and surface water with rates ranging from 25 to 65%, respectively, of the degradation rates (*23*). Knowledge of enantiomerization is important in determining the exposure profile for a chiral pesticide because the inactive isomer can form regardless of the application of a single active isomer. This process can only be considered in exposure modeling when enantioselective fate data are available.

Because exposure modeling is sensitive to the sorption coefficient, selection of a representative soil : water partitioning coefficient is important for estimation of environmental concentrations. Enantioselective sorption, though, has not been as intensively studied as enantioselective degradation (*1*). Chiral sorption sites in soil and sediment such as chirally-enriched clays and natural organic matter are expected to enhance enantioselective sorption to chiral compounds (*24*). This ability suggests that enantioselective sorption can be an important consideration in pesticide exposure assessments if data are consistently available to assess the effects. Current guidance for exposure modeling recommends using the mean K_{oc}, or K_d, depending on the relationship of K_d and soil organic-carbon content.

In exposure modeling, EPA considers major degradates (> 10% of the applied parent) and degradation products with known toxicity in addition to the parent compound. For chiral compounds, consideration of degradation products in exposure modeling is further complicated by the enantioselective metabolism, sorption, and toxicity of the chemical. Additionally, enantiomerization needs to be taken into account for chiral degradation products. Degradate modeling in ecological exposure assessment is dependent on the available environmental fate and toxicity data for the degradation products (*25*).

Monitoring

Most monitoring programs generally use multi-residue, non-chiral chromatography methods for analysis, which are generally not capable of separating enantiomers. Therefore, the detection of an isomeric pesticide in water and food monitoring samples cannot preclude potential exposure to a mixture of isomers (enantiomers) or a specific isomer (enantiomer). Exceptions to this statement can be made for detections of registered pesticides with 100% enantiomeric purity with no known enantiomerization.

Although most pesticide monitoring programs are not designed to detect enantiomers, enantioselective environmental fate processes and application of enantiomerically-enriched pesticides have been identified using enantioselective chemical analysis (*20*, *26–28*). Analytical chemistry methods are capable of identifying and quantifying each separate enantiomer and chiral degradation products in soil, water, and fish tissue (*12*, *29*). The ability to differentiate enantiomers in environmental media is the key factor in distinguishing enantioselective sorption, degradation, or bioaccumulation.

Toxicity Assessment

The purpose of the toxicity assessment is to identify the types of effects a chemical can produce in an organism. In toxicity tests, a limited number of species (surrogate species) are used to represent broad taxonomic groups of organisms, and the toxicity endpoint is derived from the most sensitive species tested (Table 4). For pesticide ecological risk assessments, toxicity endpoints include effects on reproduction, growth, or survival (*3*).

The use of a single species indicator organism assumes that enantioselective toxicity is similar for all organisms within a taxa and thus relies on similarity in metabolism across taxa. Because the toxicity testing is generally conducted using the racemic mixture of isomers or a single enriched isomer, the estimation of individual isomer toxicity may be important. For racemic isomer mixtures or partially resolved isomer mixtures, the toxicity endpoint can be adjusted according to the enantiomer fraction (EF) in the exposure media of the toxicity studies (*13*). This approach is scientifically sound as long as there are no synergistic or antagonistic effects among enantiomers.

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Table 3. Enantioselective Data Used in Exposure Modeling for Chiral Pesticides

Input Parameter	*Enantioselective Parameter*	*Exposure Assessment*	*Model Input*
Application Rate	**Yes**	Terrestrial/ Aquatic	lbs ai/A
Application Timing	No	Terrestrial/ Aquatic	Month-Day
Application Interval	No	Terrestrial/ Aquatic	days
Application Technique	No	Terrestrial/ Aquatic	spray method/ incorp. depth
Molecular Weight	No	Terrestrial/ Aquatic	g/mole
Water Solubility	No	Terrestrial/ Aquatic	mg/L
Vapor Pressure	No	Aquatic	torr
Henry's Law Constant	No	Aquatic	Atm-m^3/mol
Octanol-Water Partition Coefficient	No	Aquatic	
Hydrolysis Half-life	No	Aquatic	d^{-1}
Aqueous Photodegradation Half-life	No	Aquatic	d-
Foliar Degradation Half-life	**Possible**	Terrestrial/ Aquatic	d^{-1}
Foliar Washoff Coefficient	**Possible**	Aquatic	cm^{-1}
Soil/Water Partitioning Coefficient	**Yes**	Aquatic	Mean K_{oc} or Lowest non-sand K_d
Aerobic Soil Metabolism Half-life	**Yes**	Aquatic	Upper 90^{th} percent confidence bound on the mean half-life
Aerobic Aquatic Metabolism Half-life	**Yes**	Aquatic	
Anaerobic Aquatic Metabolism Half-life	**Yes**	Aquatic	

Lbs ai/A-pounds active ingredient per acre

Table 4. Toxicity Endpoints Used in USEPA's Ecological Risk Assessments

Organisms	*Toxicity Assessment*	*Toxicity Endpoint*
Aquatic		
Animals	Acute	Lowest EC_{50} or LC_{50}
	Chronic	Lowest NOEC
Terrestrial		
Avian	Acute	Lowest LD_{50} or LC_{50}
	Chronic	Lowest NOEC for 21-week avian reproduction test
Mammalian	Acute	LD_{50} from single oral dose
	Chronic	Lowest NOEC for two-generation reproduction test
Plants		
Terrestrial Non-endangered	Acute	Lowest EC_{25}
Aquatic Vascular and Algae	Acute	Lowest EC_{50}
Terrestrial Endangered	Acute	Lowest EC_{05} or NOEC

Risk Characterization

Risk characterization is the integration of the effects and exposure characterizations to determine the likelihood of risk to aquatic life, wildlife, and plants. For most risk characterizations, EPA uses a deterministic approach or quotient method to compare toxicity to environmental exposure. A risk quotient (RQ=exposure concentration/toxicity endpoint) is a simple screening-level estimate that identifies high- or low-risk situations. The risk quotient can be modified to include the enantiomer fraction and thus account for exposure and toxicity of individual isomers (*13*). Risk quotients for isomers can also be calculated using a toxic equivalence concentration as determined from the toxicity of individual isomers (*30*). Unacceptable risk is assumed when the RQ exceeds a pre-determined level of concern (LOC). Table 5 illustrates EPA's LOCs for assessing ecological effects from pesticides.

Table 5. USEPA/OPP Levels of Concern for Ecological Risk Determination

Organism	*Toxicity Effect*	*USEPA/OPP LOC*
aquatic animals, mammals, birds	Acute	>0.5
aquatic animals	Acute Restricted	>0.1
mammals and Birds		>0.2
aquatic animals	Acute Endangered Species	>0.05
mammals		>0.1
aquatic animals, mammals, bird	Chronic	>1
non-endangered plant	Acute	>1
endangered plant	Acute	>1

Hegemann and Lane, 2002 proposed a model for chiral pesticides based on the enantioselectivity in different environmental media and in different organisms (*9*). The order of enantioselectivity is as follows: enzymes > specific mammalian tissues (kidney, liver, brain) > marine mammals > fish / birds > mollusks > soil > water > air. A compilation of enantioselectivity for different chiral pesticides in various organisms and environmental media is needed for characterizing the potential risk from chiral pesticides.

A critical component of the risk characterization is a description of the uncertainties in a risk assessment. For chiral pesticides, the presence or lack of data on the enantioselective properties of the pesticide can be a significant factor in the confidence level of the resulting risk assessment (*31*). Therefore, a scientifically sound risk characterization considers the possible kinds of enantioselective behavior (as discussed above) and how it could possibly affect the resulting estimated risk. If possible, a description of how the uncertainty in the available source data propagates through the assessment can indicate the sensitivity to potential enantioselective effects. This type of analysis can inform risk managers of the need for additional data if further refinement of the assessment is necessary.

Conclusions

The USEPA risk assessment framework provides a systematic process for evaluating pesticide risk with respect to pesticide use information, environmental fate properties, and ecotoxicity. Although there are several strategies for assessing risk of chiral pesticides, they are dependent on the ability to differentiate toxicity among isomers with little consideration to environmental exposure . These toxicity-based risk assessment strategies for chiral pesticides may not be practical since enantioselective toxicity is expected to be dependent on the organism and taxa (*9*).

Based on a review of the USEPA risk assessment framework, the following issues should be considered in assessing risk for chiral pesticides:

- A clear understanding of the stereochemistry of the pesticide is needed including identification of active and inactive isomers for the target organism;
- Extent of the pesticide use area is an important consideration in assessing the variability in enantioselectivity in pesticide metabolism and sorption;
- Enantioselective analytical methods should be employed in studies used to support pesticide registrations for chiral pesticides;
- The number of test soils, sediments, and aquatic systems required in environmental fate studies for pesticide registrations may not adequately address the variability of enantioselectivity for chiral pesticides;
- The ecotoxicity testing strategy for chiral pesticides should employ methods capable of assessing toxicity of active and inactive isomers;
- Exposure modeling for chiral pesticides is dependent on the availability of enantiospecific environmental fate data;
- Interpretation of monitoring data for chiral pesticides requires consideration of the analytical methods as well as the possibility for enantiomerization;
- Quantification of risk should consider toxicity and exposure to enantiomers of chiral pesticides and the toxic degradation products;
- Addressing uncertainties in risk assessment for chiral pesticides is dependent on understanding variability of enantioselectivity in environmental fate properties and ecotoxicity.

An ecological risk assessment is only as good as the supporting environmental chemistry and ecotoxicity data. For chiral compounds, the most important issue is the availability of enantiospecific environmental fate and ecotoxicity data. Understanding the variability of enantioselectivity for chiral pesticides is critical in formulating reliable risk assessments.

Memoriam

The authors wish to dedicate this book chapter to their late co-author, Dr. Silvia Termes. Her guidance in understanding the role of stereochemistry in pesticide risk assessment was invaluable.

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Chapter 13

Application of Stereoselective Bioassays for Improvement in Pesticide Design: An Example from China Using Methamidophos and Its Derivatives

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Despite the fact that the biological actions of chiral compounds are stereoselective, this concept has been rarely introduced to solve the problems of pesticide design. This chapter offers a typical example involving the original design chain from methamidophos (Me, *O*,*S*-dimethyl phosphoramidothioate) to chloramidophos (CP, *O*,*S*-dimethyl-N-(2,2,2-trichloro-1-hydroxyethyl)phosphoramidothioate), after which *O*,*S*-dimethyl-N-(2,2,2-trichloro-1-methoxyethyl)phosphoramidothioate (MCP) was successfully improved by virtue of stereoisomeric bioassays. Me is so toxic that it was earlier replaced by CP, a newly developed organophosphorus pesticide. However, the subsequent discovery of the storage instability of the commercial formulation of CP greatly inhibited its continuous use. After a great deal of effort, it was confirmed that reactions between CP and the cosolvent methanol primarily resulted in the CP's drop in its formulation and meanwhile, a highly stable new organophosphate−MCP was fortunately gained. Racemic MCP was determined to be highly active to insects and have low acute toxicity towards humans. But MCP has potential to induce delayed neuropathy which makes its

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pesticidal use impossible. Under this background, all of the four stereoisomers (peak 1 to peak 4) of MCP were prepared and collected from a Chiralpak AD column, and their stereospecific profiles for both target and non-target toxicity were measured. An exciting result was was that pair 1, that is the equimolar mixture of peak 1 and peak 3, was about 3.0-fold more active than its racemate but had extremely low potentials to cause acute and delayed neurotoxicities. This product is considered to be worthy of development as a new pesticide both from its biological character and cost effectiveness. The above results imply that possibilities for a new pesticide can be extended when stereoselective biological studies are introduced into the design process.

Introduction

The development of enantiomerically pure or enriched products instead of the original racemic ones has been considered as an effective approach to produce green pesticides (*1–6*). With the emphasis on regulatory, intellectual property, marketing and profitability factors, registration of single- or enriched-enantiomer pesticides has become an important trend in the 21st century (*2*). Our present studies provide a typical example, showing that the concept of stereoselective biological action of chiral compounds can influence the design of a new pesticide. We found that the original design chain from methamidophos (Me, *O*,*S*-dimethyl phosphoramidothioate) to chloramidophos (CP, *O*,*S*-dimethyl-N-(2,2,2- trichloro-1-hydroxyethyl)phosphoramidothioate) and finally to *O*,*S*-dimethyl- N-(2,2,2-trichloro-1-methoxyethyl)phosphoramidothioate (MCP) was successfully improved by use of stereoisomeric bioassays (Fig. 1). Following are the results we obtained as well as some implications for future design efforts and related biological research.

From Me to CP

Me is a broad-spectrum non-fumigant systemic/contact organophosphate insecticide first registered by Miles, Inc. in the United States in 1972 under the trade name Monitor (*7*). It is used mainly on potatoes, tomatoes, and cotton to control insects such as aphids, colorado potato beetles, green peach aphids, and leafhoppers, etc. (*8*). Due to its high efficacy, comparatively low cost and lack of resistance and phytotoxicity (*9*), Me is one of the top ten organophosphorus pesticides (OPs) sold world-wide (*10*). In China, Me was once the most widely used pesticide with an annual production of active ingredient reaching 60,000-70,000 tons in the 1990s (*11*). However, due to its high acute toxicity, the government of China prohibited all production, sale and use of Me as of Dec. 31, 2008 (*12*). Since Me accounts for more than 15 percent of China's total pesticide application (*13*, *14*), a great gap has appeared in agrochemicals in China, and it is very important to develop some safe and cost-effective substitutes.

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Chloramidophos (CP) is a recently developed new organophosphorus pesticide invented in China as an alternative to Me (*15*, *16*). Both the indoor and field tests have indicated that the insecticidal activity of CP is comparable with that of Me. However, CP has low potentials to cause teratogenic, carcinogenic and mutagenic risks, and its acute oral toxicity against rat is only one tenth to one twentieth of that of Me (*17*). CP is synthesized by the aldol condensation reaction of Me with chloral. Since the reaction efficiency of the two raw materials, *i.e.*, the technical Me and chloral are extremely high, the cost of CP is 20% and 60% less than that of Me and acephate, respectively, which iscommonly accepted by farmers (*18*). As a result, CP was provisionally registered in 2005 and has been widely applied in some provinces in China.

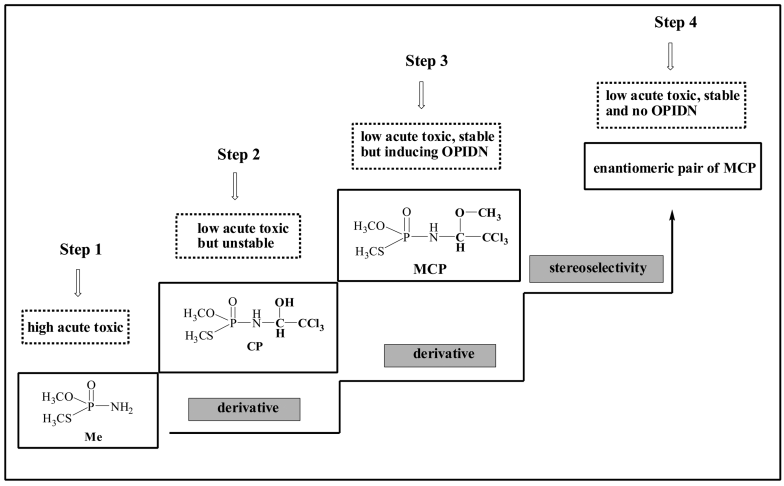

Figure 1. The OP development starting from methamidophos.

From CP to MCP

Recently, the continuous use of CP in agriculture has been questioned due to storage instability of the commercial formulation of CP, which is a 30% emulsifiable concentrate (EC) at room temperature (*19*). It was discovered that the amount of active ingredient in CP EC greatly decreased after storage, whereas the acetylcholinesterase (AChE) inhibitory potential of this formulated CP was unexpectedly increased. For example, 3.5 times increase in toxic effect (IC_{50} from 31.09 mg L^{-1} to 8.82 mg L^{-1}) was obtained in the anti-AChE activity of CP EC after a half-year of room temperature storage, simultaneously with disappearance of 78.6% of the CP. In order to discover the exact reasons for the disappearance of CP in the stored CP EC and further resolve this problem, the products formed during storage needed to be isolated and identified. Moreover, the relationship of CP loss and its toxic enhancement may be important to determine whether CP EC

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can continuously be used in pest control. However, as the components of CP EC were too complex, direct isolation of the reaction products from the formulated CP was very difficult. Fortunately, it was found that the changes of CP EC in both composition and anti-AChE activity might be related to its formulating agents. Therefore when the technical CP was stored at room temperature for half a year, its AChE inhibitory potential decreased (IC_{50} from 16.81 mg L^{-1} to 20.00 mg L^{-1}) rather than increased and the amount of CP only degraded by 8.5 percent. In view of those differences between the technical and formulated versions of CP, it is reasonable to presume that the formulating agents are responsible for the instability of CP. Therefore, as an alternative method to the direct isolation from CP EC, highly purified CP was individually incubated with the formulating constituents and the main products as well as the potentiating materials were isolated and purified.

The formulating agents of CP EC are very complicated, including the solvent benzene, a cosolvent methanol, a skin penetration promoter azone and many polymer emulsifiers. Owing to CP's susceptibility to react with acidic alcohols (*20*), it was initially supposed that the reactions between CP and the cosolvent methanol may play an important role in decomposition and toxic change in CP EC. The following studies were carried out to analyze the spontaneous reactions of CP in the warmed methanol: First, an analytical standard of CP was mixed with methanol and then stored at 50 ± 1 °C for two weeks. At the end of storage, the major products in the heated CP-methanol solution were collected and characterized by semi-preparative HPLC and GC-MS, respectively. Then, the amount of the identified products and their anti-AChE contributions to that of the CP-methanol solution were evaluated for determining the mechanisms based on both composition and toxicological points. In addition, concentrations of the key products, that is, products of the main reaction or those with high anti-AChE activity, were determined in the CP EC by HPLC as previously suggested.

On the basis of the MS fragmentation patterns of the purified products and a comparison of their GC-MS data with those of the standard samples, four compounds other than the original chemical CP were identified. Those were Me, *O*,*O*-dimethyl(2,2,2-trichloro-1-hydroxyethyl) phosphoramidothioate (CSP), O-dimethyl-[(2,2,2)-trichloro-1-methoxyethyl] phosphoramidate (MCPA), and MCP. In the warmed CP-methanol mixture (CM), similar results of CP's loss and toxic enhancement which had been found in the CP EC were observed again. For example, 98.2% of CP was broken down after the 14-day incubation, with a change of the concentration leading to half inhibition of AChE activity (IC_{50}) from 9.19 mg L^{-1} at 1 day to 8.27 mg L^{-1} at 14 days. The contributions of the four products and CP to the total amount of products and the anti-AChE activity of CM were determined and shown in Fig. 2. The results showed two products could be considered as the key products, while the other two did not contribute to the changes in CM from either composition or toxicity aspects. The first key product was MCP, which accounted for more than 80% of the total mass, suggesting that the majority of CP in the CM was reacted with methanol by nucleophilic addition. Moreover, quantitative analysis based on HPLC estimated that about 80% of the lost active ingredient of CP EC had been changed to MCP. Therefore, replacement of hydroxyl group with methoxyl group would also happen in the

CP formulation, primarily resulting in the great decrease of active ingredient (Fig. 3). However, since AChE inhibition of MCP (IC_{50} = 39.65 mg L^{-1}) is only about one fifth of that of CP (IC_{50} = 9.19 mg L^{-1}), MCP must not be a potentiator. On the contrary, Me (IC_{50} = 0.36 mg L^{-1}), the second key product, was about 25.5 times more active against AChE than CP, leading to 3% of the total mass responsible for >75% of the AChE inhibitory potential of CM (Fig. 2). We further estimated that if the anti-AChE activity of CP EC was potentiated to 3.5 times after storage, the mass percent of Me should be increased to 4.1%; while the actual amount of Me in CP EC determined by HPLC was 3.8%. Thus, it can be considered that formation of Me is the probable main factor for the enhancement of AChE inhibitory effect of CP EC during storage. According to a previous study, $(CH_3O)_2P(S)NHCH(OH)CCl_3$ and $(CH_3O)_2P(O)NHCH(OH)CCl_3$, two congeners of CP, could reversibly decompose to $(CH_3O)_2P(S)NH_2$ and chloral or $(CH_3O)_2P(O)NH_2$ and chloral (*21*). Similarly, CP may also decompose to Me and chloral in CP EC (Fig. 3).

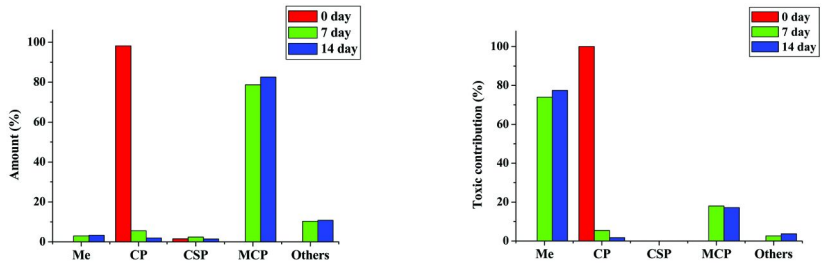

Figure 2. Contributions of the identified products and CP to the total amount and the acetylcholinesterase inhibitory potential of the total CP-methanol reaction mixture.

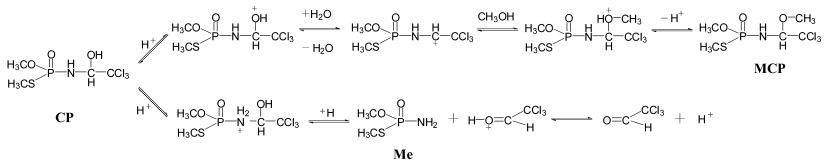

Figure 3. Possible reaction mechanisms of CP to MCP and Me in CP EC.

In conclusion, these results afford two useful pieces of information. First, 30% EC of CP is advised to be limited for sale because the active ingredient, CP will easily decrease and Me, a highly toxic insecticide will form during storage. Moreover, since instability of CP in EC is is responsible for the production of methanol, it will be beneficial to reformulate CP in a less hydrophilic environment. Second, MCP, the newly formed organophosphorus compound, seems stable in

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this type of formulation because its amount was constant during storage. As we mentioned, instability was one of the key factors for the limited use of CP EC. Stability of MCP may make it a good substitute for CP. In the following studies, gram quantities of MCP were synthesized and the target activity and non-target toxicity of MCP were tested for the purpose of assessing the potential of MCP as an insecticide.

Activity and Toxicity of MCP

To testify the insecticidal efficacy of MCP, the lethal activities of a 30% emulsion in water of MCP and a 30% EC of CP against two lepidopterous pests, *Leucania separate* and *Pluteua xylostella*, were compared. The results showed that the concentrations causing 50% mortality (LC_{50}) of the formulated MCP were 329.87 mg L^{-1} and 52.38 mg L^{-1} towards *Leucania separate* and *Pluteua xylostella,* respectively, while those of the formulated CP were 372.21 mg L^{-1} and 53.10 mg L^{-1}, respectively. A subsequent *t* test indicated that these two pairs of values were not significantly different from each other, suggesting that the insecticidal activity of MCP was comparable with that of the original compound CP.

Non-target toxicity to human becomes another decisive factor for examining whether MCP can be developed for agricultural use. Organophosphorus pesticides are responsible for the following two clinical syndromes in humans: one is acute poisoning through inhibition of neural AChE activity causing respiratory failure; the other one is organophosphorus-induced delayed neuropathy (OPIDN), which happens in 1 to 3 weeks after exposure with degeneration of long nerves in the lower limbs and spinal cord (*22*, *23*). Thereupon, both the acute and delayed neurotoxicities were taken into account for hazard identification and risk assessment of MCP. SH-SY5Y human neuroblastoma cells were selected as the biological model for toxicological evaluation, owing to their usefulness in differentiating the acutely toxic OPs (*i.e.*, those highly capable of inhibiting AChE) from neuropathic OPs (*i.e.*, those causing OPIDN). The cholinergic crisis (*i.e.*, acute toxicity) of the OP compound was measured by the IC_{50} values of AChE, the generally acknowledged target enzyme of acute poisoning by OPs. However, the precise molecular mechanisms of OPIDN remain unclear. Literature showed that the OPIDN may be initiated by the inhibition and aging of neuropathy target esterase (NTE) (*22*, *23*), but how the NTE inhibition and aging finally induce OPIDN is still not understood (*24*). Therefore, we chose the resulting morphological biomarker; that is, the concentrations that caused a 50% reduction in the number of axons (ND_{50}) in the differentiated SH-SY5Y cells, to assess the neuropathic toxicity (*i.e.*, delayed toxicity) of OP compounds. Since both the cholinergic and neuropathic risks of Me had been reported (*25*), the acute and delayed neurotoxicities of Me were also measured using the methods above for comparison.

The results showed that IC_{50} of MCP (> 2000 μM) is more than 500 times than that of Me (3.91 μM); *i.e.*, manifesting that MCP is an extremely more friendly structure than Me based on the acute toxicity. In contrast to the

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cholinergic impairment, the morphological observation found a more distinct reduction in the number of axon in MCP-treated cells compared with Me-treated cells (Fig. 4). Further quantitative analysis revealed that the ND_{50}/IC_{50} ratio for racemic Me was > 12.8, meaning that Me might be more potent at inducing acute crisis than at causing OPIDN. These results closely agreed with previous *in vivo* tests indicating that Me caused a mild OPIDN at doses estimated to be greater than eight times the unprotected LD_{50} of adult hens (*26*). In comparison, the ND_{50}/IC_{50} ratio for racemic MCP was smaller than 1, suggesting that the dose of MCP required for producing OPIDN might be lower than that inducing acute poisoning. In other words, MCP seems to more easily induce delayed neurotoxicity than to cause cholinergic crisis. Furthermore, it is suggested that the potential of MCP to inhibit axon outgrowth is mainly attributable to the derivative group at the amino nitrogen. This is analogous to trichlorphon, a pesticide with a similar 2,2,2-trichloro-1-substitiuted-ethyl group, which can also greatly inhibit the axon outgrowth of N2a cells at 1 μg mL^{-1} (*27*).

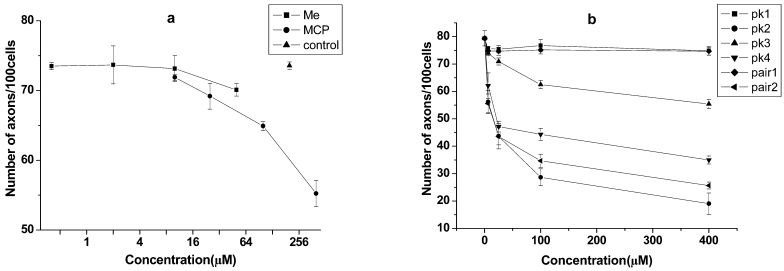

Figure 4. Concentration-response for inhibition of axon outgrowth in SH-SY5Y cells by OPs. a: Me and MCP; b: stereoisomers and equimolar enantiomeric mixture s of MCP.

In conclusion, MCP is a highly active OP but with extremely lower acute toxic to humans. However, MCP has a high potential to induce OPIDN, making its use in agriculture doubtful. Racemic MCP contains two asymmetric centres, one at the phosphorus atom and the other at the carbon atom, yielding four stereoisomers. Considering the selective biological actions among the different optical isomers, the stereoisomeric activity and toxicity of MCP were further measured. We hoped that only specific stereoisomers of MCP would possess the significant toxicity, but the remaining ones may also deserve to be examined.

Stereoselectivity in Activity and Toxicity of MCP

One of the biggest challenges in determining the enantioselective toxicities of chiral pesticide is the preparation of enantiomer standards. After testing various Diacel polysaccharide chiral stationary phases, a satisfactory separation of all four stereoisomers of MCP was only achieved on the Chiralpak AD column using

a mixture of 85% n-hexane and 15% ethanol as mobile phase. The corresponding resolutions (R_s) of every adjacent peaks were all larger than 1.5, suggesting baseline resolution. Under the above conditions, about 5 mg of each single optically pure isomer was collected from the outlet of HPLC. The CD spectra then showed that the first (pk 1) and third (pk 3) eluted isomers are one pair of enantiomers, while the second (pk 2) and fourth (pk 4) isomers are the other pair. We refer to the equimolar mixture of pk 1 and pk 3 as pair 1 and that of pk 2 and pk 4 as pair 2.

Due to the limited amounts of stereoisomer standards of MCP available from the analytical chiral HPLC column, a relatively sensitive arthropod, *Daphnia magna* (*D. magna*), was used as the target organism and the activity of MCP was evaluated by the LC_{50} values of *D. magna*. Another reason for this selection was that the capability of OPs to control insects is definitely due to their inhibition of the type "B" esterases and *D. magna* has been proven to offer an excellent model system to investigate the "B" esterase inhibition patterns (*28*). The values of LC_{50} towards *D. magna* were 1.41 mg L^{-1} and 1.31 mg L^{-1} for racemic MCP and racemic CP, respectively. A *t* test further demonstrated that these two values were not significantly different from each other. As mentioned above, some similar phenomena also can be observed when comparing the insecticidal activities of formulated MCP with those of CP EC against both *Leucania separate* and *PluteUa xylostella*. These comparable results were obtained from *D. magna* and the two target insects support the use of *D. magna* as a target organism for MCP, and therefore it was further used in the activity measurement of the stereoisomers.

Significant differences were observed in the LC_{50} values among the stereoisomers of MCP. The values of LC_{50} of the isomers to *D. magna* were 0.45±0.07, 2.46±0.37, 2.83±0.26, and 1.36±0.32 for pk 1, pk 2, pk 3, and pk 4, respectively, with 1.2–6.3 fold differences among each other. Moreover, among the four optical isomers of MCP, the LC_{50} value of pk1 was about one third that of the racemate, while those of the other isomers were equal to or larger than that of the racemate, indicating pk 1 was predominate in activity. Traditionally, the pesticidal activity of a chiral compound is based on its racemate. If MCP is to consist of only pk 1, only one third of the conventionally used amount would be enough to provide the desired insecticidal efficacy.

Stereoisomeric selectivity of MCP in acute and delayed neurotoxicities were evaluated by the method established above on the basis of SH-SY5Y cells. The results showed that all of the stereoisomers of MCP exhibited low inhibitory potentials in the cell anti-AChE assays, with a 20–30% decrease of AChE activity after exposure at the highest concentration (2000 μM). Considering that none of the stereoisomers or the racemate of MCP proved to be effective inhibitors of an AChE, it should be anticipated that the structure of MCP poses extremely low hazard of causing acute effects to humans. However, inhibitions of the stereoisomers of MCP towards axon growth were markedly stereospecific. As shown in Fig. 4, the order of the potentials for neurodegeneration was pk 2 > pk 4 > pk 3 > pk 1. And the values of ND_{50} indicated that there was at least a 60-fold difference between the strongest and weakest inhibitors. Indeed, the values of ND_{50} of pk 2 and pk 4 were only one-fortieth and one-tenth of that of racemate, respectively, showing that these two isomers were on average 25 times

more potent at inducing OPIDN than the racemate. By contrast, the values of the ND_{50} of pk 1 and pk 3 were both greater than that of racemate, suggesting that their contributions to the delayed neurotoxicity of the racemate were negligible. In other words, the neurite outgrowth inhibitory potency recorded for the racemic MCP was fundamentally due to the effects of pk 2 and pk 4. Furthermore, another exciting result we obtained is that pk 1, the least potent compound at inducing OPIDN, is the most active stereoisomer towards the target organism. Therefore, pk1 must be the optimal substitute for the racemate among the four stereoisomers.

Since the delayed neurotoxicities of stereoisomers of MCP were separated into two groups, the activity and toxicity of equimolar enantiomer mixtures (pair 1 and pair 2) were also measured. The results indicated that the inhibitions of both pair 1 and pair 2 to cell AChE were not significantly different from that of the racemate. Moreover, pair 1, which was 4.4-fold more active than the other, was also more than 45 times less potent at causing OPIDN. Additionally, the insecticidal activity, cholinergic toxicity and neuropathic hazard of pair 1 were all comparable with those of pk 1. Regarding the absence of economically feasible synthetic methods and techniques for production of single optically pure isomers of OPs, pair 1 of MCP shows considerable worth for future applications. And if racemic MCP, is replaced by pair 1, two-thirds of the usual pesticide usage can be eliminated in addition to providing better protection for humans from delayed neurotoxic risks.

Design and Biological Research Implications

In this study, development of the parent insecticide Me was interrupted because of a threat against human health caused by the derivative compound MCP. Under the circumstances, by virtue of stereoselective biological studies, we were fortunate to discover that one of the enantiomeric pairs of MCP exhibited high activity but were extremely safe and worthy to be produced as a new pesticide. These results imply that there is more design flexibility when chiral concepts are introduced into the design step of a pesticide. This approach may be extrapolated to other chemicals, such as compounds created based on the same activity mechanism. These related chemicals produced as racemates may be suitable as active ingredients in some cases. For example, high activities often go with high toxicities. And sometimes, the derivative groups may add new adverse side effects for different toxic endpoints. However, if only specific stereoisomers possess significant toxicity, the remaining ones may become pesticides with low risks both to environment and human health.

Many reports indicate that selective efficacy of optical isomers sometimes can be extrapolated to other pesticides in the same classes. For example, in the phenoxy herbicide class, the R-isomer always has a higher herbicidal activity than the S-isomer (*29*). However, this study emphasizes that research on the asymmetric influence on biological actions of chiral pesticides should still be carried out on a case by case basis at this time. Generally speaking, the biological activity of organophosphorus compounds depends on phosphorus chirality more than on carbon chirality (*30*). But it is unfortunate that economically feasible chemical reactions at a chiral phosphorus centre seem unavailable at present,

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resulting in no enantiopure phosphorus agrochemicals yet marketed (except for bilanafos, which originates from a natural product) (*30*, *31*). That is also the reason that we did not try to change the racemic Me to its single enantiomer, but first changed its structure despite reports of enantiospecific active and toxic profiles of Me (*32*). But when MCP was introduced by adding another chiral centre at a carbon atom, toxicity discrimination unexpectedly materialized between the enantiomeric pairs, making the chiral switch possible because technologies for separation of pair 1 and pair 2 are easier. That is to say, stereoselctivity in biological actions is too complex for prediction of activity from stereoisomer configuration. As a result, more stereoisomeric bioassays and mechanism studies need to be conducted for development of intrinsic rules.

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Editors' Biographies

Dr. A. Wayne Garrison

Dr. A. Wayne Garrison obtained his Ph.D. in organic chemistry from Emory University in Atlanta, Georgia, in 1966. He has been employed as a research chemist with the U.S. Environmental Protection Agency (EPA) and its predecessor organizations for almost 50 years. The objectives of Dr. Garrison's early research were to develop analytical methods, based primarily on GC-MS, for measuring and identifying organic pollutants in drinking water and industrial and municipal effluents. A position as Branch Chief at the Ecosystems Research Division of the EPA in Athens, Georgia, for eight years followed, during which time his research shifted to the environmental fate of organic pollutants. After a research appointment in Germany (1992−93), Dr. Garrison resumed full time research with the EPA, focusing on investigations of the occurrence, fate and exposure of chiral pesticides/pollutants and their stereoisomers in various water, soil and biological matrices. He is the EPA leader in this research, the goal of which is to provide data on the selective fate and effects of the stereoisomers of chiral pesticides so as to encourage the manufacture and use of safer single- or enriched-stereoisomer products, a green chemistry measure. Dr. Garrison has about 60 publications in peer reviewed journals in the above research areas.

Dr. Jay Gan

Dr. Jay Gan is a Professor of Environmental Chemistry at University of California, Riverside. Dr. Gan conducts research on environmental fate and transport of pesticides and emerging organic contaminants, with an emphasis on water quality and risk mitigation. Dr. Gan is the author of over 175 journal articles and 20 book chapters. He is also the senior editor of two ACS books. Dr. Gan teaches environmental chemistry courses to undergraduate and graduate students. Dr. Gan is a Fellow of American Association for the Advancement of Science (AAAS), American Society of Agronomy (ASA), and Soil Science Society of America (SSSA).

Dr. Weiping Liu

Dr. Weiping Liu received his B.Sc. and M.Sc. degrees in Chemistry from Zhejiang University in 1982 and 1989, respectively. He then joined the faculty of the Department of Chemistry at the same university. He spent almost three years as a visiting scholar in University of Sassari, Italy from 1990 to 1992. And then he returned to Zhejiang University in the end of 1992 and became Professor

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and Associate Chairman of the Department of Chemistry in 1995. From 1999 to 2002, he served as Associate Dean of College of Environmental and Resource Sciences, Zhejiang University. He also devoted several years to do environmental chemistry research at U.S. Salinity Laboratory, U.S.A. (1998−1999), and University of California-Riverside, U.S.A. (2002−2005). He got his Ph.D. degree in Applied Biochemistry at Tokyo University of Agriculture, Japan in 2006. He is currently Dean and "Qiushi Scholar" Honor Professor at the College of Environmental and resource Sciences, Zhejiang University, China, and Principal Investigator of "Environmental Chemistry and Toxicology" Innovative Research Team in Chinese University. His research interests are in chiral separation and ecotoxicology of chiral pesticides with modern chromatographic and molecule biological techniques. Dr. Liu has about 180 publications in peer reviewed journals in the above research areas.

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