Dow AgroSciences Comments:
EPA's Evaluation of the Toxicity Profile of Chlorpyrifos

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Executive Summary

First registered by the US EPA in 1965, chlorpyrifos is a pest control product now registered and used for agricultural applications in more than 100 nations. No pest control product has been studied more extensively, and present applications rest on 4 decades of use, health surveillance of manufacturing workers and applicators, and a scientific database of more than 3600 studies on health and the environment.

In evaluating this database, it is imperative that studies be differentiated based on their relevance to human risk assessment. Not all studies in the database were designed for risk assessment purposes, and many are not appropriate for that use. Due to differing standards, approaches, and methodologies, some studies in the database reach apparently contradictory conclusions, and this can make assimilation and interpretation of the data problematic unless the reviewer actively distinguishes between those studies that provide data appropriate for use in risk assessment and those that do not. When evaluating human risk, global health authorities, regulatory agencies, and professional societies strongly recommend the use of relevant routes of exposure, realistic doses based on estimated human exposure, and study designs with applicability to the human situation.

Chlorpyrifos exerts its primary mechanism of action through inhibition of cholinesterase, an effect that continues to be recognized globally as the most sensitive and appropriate point of departure upon which to assess human risk. Toxicology studies based on regulatory guideline methods demonstrate that with doses that do not cause maternal toxicity chlorpyrifos is not a specific developmental or reproductive toxicant; it is not associated with neurodevelopmental toxicity, nor is it mutagenic or carcinogenic.

While some nonguideline, published studies have reported different findings, these results are less relevant to risk assessment than guideline studies that have been specifically designed to employ a range of doses and use routes of exposure that are most relevant to anticipated human exposures. For example, many nonguideline studies employ a subcutaneous route of administration, often using a vehicle, dimethyl sulfoxide (DMSO) with known systemic and neurotoxic properties at the volumes used. OECD in their draft 2006 Developmental Neurotoxicity Guidelines has recognized the potential problems in data interpretation because of choice of vehicle: "The vehicle should not cause effects that could interfere with the interpretation of the study neither be neurobehaviourally toxic nor have effects on reproduction or development." Many studies also consistently attempt to draw conclusions from effects at doses in excess of the US EPA's recognized no-observed-adverse-effect-level (NOAEL) for cholinesterase inhibition. Effects observed in the presence of cholinesterase inhibition are confounded by cholinergic activity.

Epidemiological studies involving occupational groups with substantially higher exposures than the general population have not reported adverse health impacts. Attention has focused on recent epidemiology studies that collectively present limited and conflicting evidence for effects on neurodevelopment. Only one of these studies has associated chlorpyrifos exposure with neurodevelopmental effects, and that study may be biased by numerous confounding variables and exposure misclassification.

Issues have been raised about neurodevelopmental effects that could be mediated through non-cholinergic mechanism(s) operating at doses below which systemic toxicity occurs. Typically, the doses used in such studies are high enough to inhibit cholinesterase of plasma, RBC, and brain. Such non-cholinergic concerns have been evaluated previously, and review by global authorities has consistently found that cholinesterase inhibition is the most sensitive marker of chlorpyrifos exposure and that, consequently, regulation based on cholinesterase inhibition protects against the potential for noncholinergic effects as well. At this time, information about non-cholinergic effects of chlorpyrifos are deficient in dose-response data, are missing a characterization of the role of dose route and dosing vehicle, and have not identified a sequence of key events, which collectively, preclude the elucidation of non-cholinergic mode(s) of action at this time.

Concomitant with attention on possible neurodevelopmental effects in humans has been the concern – based on the postulate that there may be significant age-related and genetic differences in chlorpyrifos detoxification – that existing regulations may not be sufficiently protective for everyone with the potential to be exposed. Specifically, attention has been directed towards differential expression of the chlorpyrifos-oxon detoxifying enzyme paraoxonase (PON1). Laboratory studies and physiologically-based pharmacokinetic modeling have confirmed that at extreme dose levels PON1 has a modest role in detoxification but no apparent role at environmentally relevant levels of exposure. A recent study indicates that albumin oxonase may play an important complementary role to PON1 oxonase and mitigate against high-dose consequences of low PON1 activity. Moreover, guideline studies have consistently demonstrated that at the established, repeat-dose adult cholinesterase-based NOAEL neonates do not show increased sensitivity. Thus, relative to issues of potential noncholinergic effects or differential sensitivity, there is no compelling scientific basis for reevaluating whether currently established exposure limits are adequately protective, particularly when exposure levels employed in experimental studies are compared to environmental levels of exposure, which are typically orders of magnitude less.

It is essential that critical and objective evaluation of issues in chlorpyrifos toxicity be based on long-standing principles of toxicology and their established application in risk assessment. Given the expanding scientific literature on chlorpyrifos, it is incumbent upon scientists and reviewers to become increasingly discerning as to the potential utility of a study for informing on human health and risk. To this point, many studies continue to utilize dose levels that are well above the current chronic NOAEL used for RfD development, and thus would not, if used as points of departure, impact or improve the risk assessment for humans. Finally, when assessing questions related to sensitivity of various age-groups and exploring new approaches for evaluating risk such as benchmark dose and data-derived extrapolation factors, it is important to maintain the same data quality standard that is required of studies used in current EPA risk assessments for chlorpyrifos. The overwhelming weight of scientific evidence continues to demonstrate - as has been the case for more than forty years - that as registered, used, and regulated, premised upon protection against significant cholinesterase inhibition, chlorpyrifos does not pose an unacceptable health risk to humans.

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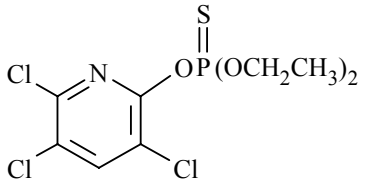
A. Introduction

Chlorpyrifos has been on the market for more than 40 years and was first registered by the EPA in 1965. It is registered in more than 100 nations where it is widely used in agriculture. Today's registered uses of the product rest on a history of over four decades of experience, extensive health surveillance of manufacturing workers and applicators, and an extensive database of 3600 studies on health and the environment. No pest control product has been studied more thoroughly. Studies that support global registrations for chlorpyrifos and which inform on the toxicological profile for chlorpyrifos include guideline compliant toxicity studies and extensive investigation by academic research laboratories. The generation of new data on chlorpyrifos continues and serves as partial basis for the Agency's request for FIFRA SAP guidance.

Chlorpyrifos (CAS No. 2921-88-2) is an organophosphate insecticide commonly used to control insect pests in agriculture. The chemical name is O,O-diethyl O-(3,5,6-trichloro-2-pyridyl) phosphorothioate. Chlorpyrifos is a white to tan crystalline solid at room temperature with a melting point of 41.5 – 42.5° C, boiling point of greater than 300° C, and a very low vapor pressure of 3.35 mPa at 25° C. It has a molecular weight of 350.6. It has a low solubility in water (1 ppm w/v at 25^{o}C) and is soluble in common organic solvents such as acetone, hexane, and toluene. The structure is shown in Text Figure 1.

Text Figure 1. Structure of Chlorpyrifos



The mechanisms of chlorpyrifos-induced toxicity are well described (Timchalk et al. 2000, 2002, 2006, 2007). Chlorpyrifos must be metabolized in the body to chlorpyrifos-oxon (CPF-oxon). Excess CPF-oxon results in an excess of the neurotransmitter, acetylcholine (ACh). An excess of ACh causes overstimulation of the central and peripheral nervous systems and associated toxic responses and adaptive processes. There is no weight-of-evidence that non-cholinergic effects occur at doses that do not inhibit cholinesterase.

The USEPA has convened this SAP for purposes of reviewing relevant literature and studies for chlorpyrifos along with evaluation of several specific areas of interest including (a) recent epidemiological studies involving reported neurodevelopmental effects, (b) potential differential sensitivity in children versus adults, and (c) potential non-cholinergic modes or mechanisms of action.

Dow AgroSciences (DAS) welcomes the opportunity to provide its perspective on each of these areas, but believes there is utility in first describing toxicological principles that have been embraced globally and which have specific relevance when evaluating

chlorpyrifos. Application of these toxicologic principles will clarify confusion that may exist since publication of many chlorpyrifos studies over the past 10-plus years.

B. The Need for Standards When Evaluating and Interpreting the Toxicological Literature for Chlorpyrifos

Key Points

- The chlorpyrifos toxicology literature is diverse and differences in study design, route of administration, dose relative to exposure, dose-related transitions in mechanism, and selection of vehicle are critical when considering relevance and risk to humans.
- Global regulatory agencies have embraced principles of toxicology in risk assessment.
- A number of regulatory agencies and authorities have endorsed pragmatism and applied a weight-of-evidence approach when considering the voluminous literature and diverse number of study designs for use in human risk assessment.
- Risk assessment is one of the primary bases for the design and conduct of regulatory toxicology studies required by law for the registration of chlorpyrifos.
- Consideration of dose-dependent transitions in mechanisms of toxicity is an obligate step in risk assessment and regulatory decision-making.

The scientific literature for chlorpyrifos is voluminous, diverse, and often confusing. Collectively, these data present a challenge to regulators who must analyze that data and judge its relevance for human risk assessment at environmental levels of exposure. There are substantial differences in study design, dose levels, and routes of exposure. These factors are critical to the interpretation and utility of these data for risk assessment. The range in diversity is perhaps the widest between those studies required for registration and those academic investigative studies that explore specific aspects of chlorpyrifos' effects in various test systems under a variety of study designs.

Regulatory Toxicology Testing

The underpinning of scientifically-based, health-protective regulation of human chemical exposure under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) is based on two established procedures: (1) a regulatory toxicology testing program under 40 CFR Part 158 according to OPPTS 870 Series Guidelines and (2) standardized methods of risk

assessment including use of input parameters, mandatory safety factors, and where appropriate, the application of Food Quality Protection Act (FQPA) statutory requirements. The protocols developed for regulatory testing have had extensive input from scientists from academia, government, and industry. The USEPA develops protocols in a lengthy process that is both comprehensive and subjected to peer and public review. The core protocols are designed to identify adverse effects of chemical exposure independent of mechanism of action. The collection of regulatory studies produce an assessment of an extraordinarily long list of morphologic and functional endpoints, at dose levels that range from no-observed-effect levels to clearly toxic doses. Together, these standardized methods and approaches of toxicology and risk assessment provide the foundation for establishing health-based exposure limits protective of all human populations, including potentially sensitive subgroups.

When evaluating the toxicological database and studies on chlorpyrifos, it is imperative to consider route of exposure, selection of vehicle, dose levels and regimens, and other study methodological details that may affect study outcome. To this point, the Society of Toxicology has expressed concern over use in risk assessment of animal studies that are inappropriately designed. Written as an outgrowth of the Society's strategic plan "to promote the use of sound science in risk assessment," Conolly et al note:

> *The relevance of experiments using doses that are many multiples of conceivable human exposures and unrealistic routes of exposure is, at most, quite dubious. Mechanisms of action may be elicited under such conditions that would not occur with relevant routes and exposures levels…*[G]*iven that a major, and possibly the major, application of toxicology data today is protection of the public health via its application to risk assessment, use of routes of exposure and high-dose levels, set primarily for purposes of experimental convenience, should be avoided.*

For chemicals that have been extensively studied, typified by chlorpyrifos, the reviewer is often confronted with two very different types of research or studies: (1) toxicology studies mandated by regulation and conducted under Good Laboratory Practices to conform to prescribed testing methods and that meet time-tested requirements of standardized risk assessment; and (2) studies encompassing a wide variety of approaches and measuring a diverse number of biological endpoints, many of which are not relevant to risk assessment or are of unknown value to risk assessment. While this second category of research can be expected to generate an abundance of hypotheses for future study, scientifically sound regulation of chemicals requires a rigorous application of well-recognized toxicological principles in risk assessment (Neal and Doull, 1995).

Global Efforts Aimed at Clarifying and Embracing Principles of Toxicology for Use in Risk Assessment

All countries that have registered chlorpyrifos have regulated it on the basis of inhibition of plasma, red blood cell (RBC) or brain cholinesterase, in humans and/or animals. The World Health Organization, European Union, and Australia utilize cholinesterase

inhibition from human chlorpyrifos kinetic/biomarker studies to aid in setting exposure standards.

Regulatory conclusions about the safety of chlorpyrifos are dependent on the degree to which principles of toxicology for use in risk assessment are applied. The World Health Organization, European Union, United Kingdom Advisory Committee on Pesticides, and Australian regulatory agencies have been among the more rigorous and transparent in adhering to and applying toxicology principles in hazard and risk assessment of pesticides.

Some of the difficulties that arise as a result of a lack of rigor in the application of basic toxicologic principles when evaluating a body of literature like that for chlorpyrifos were noted in a publication (Neal and Doull, 1995) by two past-presidents of the Society of Toxicology (SOT). The article criticized toxicologists in general, and federal, state and local agencies specifically, for a *"… lack of rigor in application of standard principles for interpretation of toxicology data."* They also noted that *"… toxicology has attracted a number of special interest groups whose concern is not primarily scientific."* Subsequent to this article, the SOT convened an "SOT Task Force to Improve the Scientific Basis of Risk Assessment (Conolly et al., 1999)." Some of the key findings that resulted from this Task Force effort included:

- It is important to use realistic doses and routes of administration of chemicals to avoid generating data that "raise serious questions of relevance."

- Toxicologists too often use dose levels and routes of exposure because of their convenience rather than because of their relevance to risk assessment.

- Oral gavage (in corn oil) was used as an example of a convenient but unrealistic route that can cause unrealistic kinetics in the test animal.

- The use of data generated at unrealistic doses or unrealistic routes of exposure can predict risks that "…have little or no relationship to risk in the real world."

- It is important to acknowledge that dose affects mechanism, and it can be expected that mechanisms will change with dose.

- It is the responsibility of the toxicologist to design studies to be relevant to risk assessment.

The principles, as first clarified by the SOT, have subsequently been endorsed and restated several times by scientific and governmental organizations. Examples follow:

World Health Organization

In 1999, the World Health Organization published "Principles for the Assessment of Risks to Human Health from Exposure to Chemicals" (WHO/IPSC, 1999). They

stated clearly that the studies most relevant to ”hazard identification for risk assessment” are those that use a route of exposure that is similar to that of humans. They also spoke to the need for several dose levels to identify dose-response information "relevant to hazard identification.”

In 2001, the World Health Organization published “Neurotoxicity Risk Assessment for Human Health: Principles and Approaches” (WHO/IPSC, 2001). That report’s recommendations were similar to those stated above, with regard to the importance of using doses, routes, and durations that reasonably approximate human exposure. The report’s recommendations also stressed the importance of using available pharmacokinetic and pharmacodynamic data.

U.S. Environmental Protection Agency:

In 2002, the USEPA published “A Review of the Reference Dose and Reference Concentration Processes” (USEPA, 2002b) which noted the need to characterize databases for possible human effects in the context of “dose, route, duration, and timing of exposure.”

For example, in section 4.3.1.1. *Adequacy of Studies,* page 4-10, Animal studies, one bullet point was “Was an appropriate route and matrix of exposure employed?” Attached to this question was footnote 10: *“The most appropriate route of exposure is the route for which an evaluation is to be made. The toxicity of the chemical may differ with route of exposure because of differences in mechanism of action or toxicokinetics (absorption, distribution, metabolism, and excretion). Development of data to establish dosimetry for the purpose of route-to-route extrapolation is encouraged; however, route-to-route extrapolation is inappropriate when based exclusively upon default assumptions regarding exposure and toxicokinetics. Even within the same route of exposure, responses may differ due to alterations in toxicokinetics, for example, dietary or water exposure versus oral gavage.”*

The USEPA 2002 report emphasized the need for a ”weight-of-evidence” approach that “requires a critical evaluation of the entire body of available data for consistency and biological plausibility.” Importantly, the report noted, the study should be evaluated for possible alterations in metabolism at higher exposure levels.

The Importance of Dose in Affecting Mechanism of Toxicity

Slikker et al. (2004) published the consensus conclusions from two workshops sponsored by the International Life Sciences Institute (ILSI) Health and Environmental Sciences Institute (HESI) on the impact of dose-dependent transitions on the risk-assessment process. Co-sponsors were the Agency on Toxic Substances and Disease Registry (ATSDR), the American Chemistry Council (ACC), the National Institute of Environmental Health Sciences (NIEHS), SOT, and USEPA.

- A ”transition” was described in this publication as a “change with increasing dose in key underlying kinetic and/or dynamic factors that influence the

mechanism responsible for the observed toxicity, resulting in a change in the relationship of the response rate as a function of dose."

- "A transition usually occurs over a range of doses, and reflects a continuum of change, rather than a single point of departure."

- "The demonstration and characterization of a dose-dependent transition should influence the evaluation of data both above and below the transition."

- "… [C]onsideration of dose-dependent transitions in the mechanism of toxicity is an obligate example of integrating the 'best science' into the decision making process."

OECD

In 2006, the Organization for Economic Cooperation and Development (OECD) published a draft for an updated Guideline 426, Developmental Neurotoxicity Study (OECD, 2006). This document addresses many important features in design and interpretation of developmental toxicology studies. For example, there is important guidance information on use of multiple dose levels and understanding how a particular vehicle may affect study outcome. In particular, the vehicle itself should not "be neurobehaviorally toxic" . This OECD document addresses the importance of maternal toxicity, weight-of-evidence, inter-relationships of multiple endpoints and expert judgment, patterns of findings, and the use of statistics as tools and not determinants of effect or no effect:

Dosage
Section 16. *"At least three dose levels and a concurrent control should be used. The dose levels should be spaced to produce a gradation of toxic effects. …"*

Section 18. *"… If a vehicle or other additive is used to facilitate dosing, consideration should be given to the following characteristics: effects on the absorption, distribution, metabolism, or retention of the test substance; effects on the chemical properties of the test substance which may alter its toxic characteristics; and effects on the food or water consumption or the nutritional status of the animals. The vehicle should not cause effects that could interfere with the interpretation of the study neither be neurobehaviourally toxic nor have effects on reproduction or development. For novel vehicle substances, a sham-treated control group should be included in addition to a vehicle control group. Animals in the control group(s) should be handled in an identical manner to test group animals."*

Evaluation and interpretation of results
Section 47. *"A developmental neurotoxicity study will provide information on the effects of repeated exposure to a substance during in utero and early postnatal development. Since emphasis is placed on both general toxicity and*

developmental neurotoxicity endpoints, the results of the study will allow for the discrimination between neurodevelopmental effects occurring in the absence of general maternal toxicity, and those which are only expressed at levels that are also toxic to the maternal animal. Due to the complex interrelationships among study design, statistical analysis, and biological significance of the data, adequate interpretation of developmental neurotoxicity data will involve expert judgment. The interpretation of test results should use a weight-of-evidence-approach (20)(92)(93)(94). Patterns of behavioural or morphological findings, if present, as well as evidence of dose-response should be discussed. Data from all studies relevant to the evaluation of developmental neurotoxicity, including human epidemiological studies or case reports, and experimental animal studies (e.g., toxicokinetic data, structure-activity information, data from other toxicity studies) should be included in this characterization. This includes the relationship between the doses of the test substance and the presence or absence, incidence, and extent of any neurotoxic effect for each sex (20)(95)."

Section 48. *"Evaluation of data should include a discussion of both the biological and statistical significance. Statistical analysis should be viewed as a tool that guides rather than determines the interpretation of data. Lack of statistical significance should not be the sole rationale for concluding a lack of treatment related effect, just as statistical significance should not be the sole justification for concluding a treatment-related effect. To guard against possible false-negative findings and the inherent difficulties in "proving a negative," available positive and historical control data should be discussed, especially when there are no treatment-related effects. The probability of false positives should be discussed in light of the total statistical evaluation of the data (96). The evaluation should include the relationship, if any, between observed neuropathological and behavioural alterations."*

ILSI – Guidance for Interpretation of Developmental Neurotoxicity

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In 2007, toxicologists at workshops convened by the International Life Sciences Institute (ILSI) published guidance to researchers and reviewers of DNT [developmental neurotoxicity] studies on the interpretation of the data generated in these studies (Tyl et al., 2007; Neurotoxicol. Teratol., e-publication). As the report noted:

- *"The overall assessment of biological relevance of treatment-related effects must be carried out in the context of all available data; e.g., historical (untreated or vehicle) controls, positive controls, offspring systemic toxicity data, effects on offspring in relation to maternal toxicity, and any other toxicity data."*

- *"When choosing route, dose levels, vehicle, dosing volume, and concentration, consider pharmacokinetics, mode of action, information from*

other toxicity studies, in particular reproduction/development studies, and data regarding known/expected human exposure."

- *"Historical control data are useful to determine whether the results for the concurrent controls differ from the laboratory's typical results and how consistent the data are over time, from one study to another [15,16]. Such a database is an important factor to consider in determining whether differences from concurrent control values do, in fact, represent compound-related effects. For example, historical control data can be used to determine whether the results for treated animals are well within the range of historical control values [31,46,49,58], which may indicate that the differences from concurrent control values may be unrelated to treatment."*

- *"… In actual practice, however, an evaluation to determine whether there is an indication of a compound-related effect should rely on an integrative evaluation of all the available data in the study (e.g., clinical signs in the offspring and evidence of maternal toxicity), as well as data from other studies (e.g., adult neurotoxicity, reproductive toxicity studies, and other general toxicity studies). Such general (systemic) toxicity information is needed to characterize effects on other organ systems to assist with interpretation of behavioral and other DNT findings."*

- *"Since the offspring are dependent on the mother throughout gestation and lactation until weaning, maternal toxicity can be very detrimental to the pups' growth and survival and can potentially affect nearly every endpoint." "In addition, note that the toxicity endpoints that are measured in the dam in the standard DNT study are few and rather crude (e.g., survival, body weights, feed consumption, clinical observations, optional organ weights) when compared to the large number of sensitive endpoints evaluated in the pups. The standard DNT study protocol requires evidence of maternal toxicity at least at the top dose. Acceptable maternal toxicity is generally defined as at or less than 10% mortality, at or less than 10% reduction in body weight, clinical signs of toxicity [121]. These limits include indicators of rather profound maternal toxicity, and it is likely that these would be associated with offspring toxicity if they occurred during a sensitive time; e.g., during the formation/migration/differentiation of a specific region/layer of the offspring brain. The kind and timing of maternal toxicity and the degree of the effect are important in trying to interpret the role, if any, of the maternal effects on the offspring."*

- *"Though statistical significance is a powerful tool for evaluating toxicological data, it is just a tool that should be used in conjunction with an evaluation of biological relevance and scientific judgment. In this context, a modest difference from control that is not statistically significant may still suggest a relationship to treatment (e.g., if it occurs in a dose-related manner, in both*

sexes, and in conjunction with other DNT effects or with evidence of other types of toxicity). Contrarily, a modest difference from control that is statistically significant but inconsistent with a pattern of effect (i.e., does not occur in a dose-related manner and is not accompanied by any other DNT or toxic effects) may be considered an incidental finding that is unrelated to treatment."

- *"Evaluate the biological relevance of neurodevelopmental findings in the context of other available data, including historical and positive controls, offspring and maternal systemic toxicity, and other toxicity data."*

Chlorpyrifos is a textbook case of the need for such guidance. The toxicology database for chlorpyrifos is large; without guideposts, it is challenging for regulatory risk assessors to judge the utility of individual studies relative to questions involving human health.

C. Chlorpyrifos Toxicology – A Brief Overview with Selected Attention to Guideline Studies Evaluating Developmental Toxicity

Key Points

- Cholinesterase inhibition is the most sensitive endpoint and sentinel mechanism of action for chlorpyrifos.
- Cholinesterase inhibition is recognized globally as the appropriate point of departure for human risk assessment.
- Chlorpyrifos has been evaluated in standard developmental toxicity studies in three species and in a developmental neurotoxicity (DNT) study and has not been associated with selective developmental toxicity at dose levels below those associated with maternal toxicity. Numerous global regulatory agencies have affirmed this interpretation.
- In the DNT study, there were no effects on cognitive function, including a USEPA-validated test of memory and learning in rats exposed to chlorpyrifos in utero and postnatally.

Because of the current attention to numerous studies reporting a variety of effects associated with chlorpyrifos treatment using a wide range of differing test designs and systems, it is relevant to review briefly the toxicological profile for chlorpyrifos as developed over the years through guideline-compliant OPPTS (Office of Prevention, Pesticides and Toxic Substances) testing.

Chlorpyrifos is moderately toxic following acute oral, dermal, and inhalation exposures. It is an organophosphate compound that is an inhibitor of cholinesterase. Cholinesterase activity is returned to control levels by synthesis of new cholinesterase. Inhibition of cholinesterase has long been considered, through extensive and continued evaluation of numerous studies, to be the most sensitive effect in all animal species evaluated and in humans regardless of exposure duration. In subchronic studies in several species, the most sensitive effect following oral exposure is inhibition of plasma and red blood cell (RBC) cholinesterase (ChE). Chlorpyrifos has been evaluated for carcinogenic potential in both rats and mice and was negative in both species. Chlorpyrifos is not mutagenic in bacteria or mammalian cells and was not genotoxic in a number of *in vivo* assays which evaluated multiple endpoints. Chlorpyrifos has been evaluated for chronic toxicity in rats, mice, and dogs, and in all animal species the most sensitive effect was inhibition of plasma, RBC or brain ChE. Clinical signs of neurotoxicity consistent with organophosphate intoxication, in the absence of neuropathology, were observed in rats following an oral dose of 50 mg/kg. Chlorpyrifos is not associated with delayed neuropathy in the hen except for survivors of potentially lethal exposure. Significant inhibition of neurotoxic esterase, an enzyme associated with organophosphate-induced delayed neuropathy, occurs only at doses that are lethal in the absence of medical intervention. Chlorpyrifos is not associated with selective developmental toxicity as evaluated in any of the three species tested. Chlorpyrifos was associated with reproductive toxicity in one generation of rats, but only at dose levels that induced and were associated with maternal toxicity. Chlorpyrifos, as evaluated in the developmental neurotoxicity study, was not associated with effects in the young at dose levels below those associated with maternal toxicity.

Because recent attention on chlorpyrifos has been dominated by reported effects or implications for developmental toxicity, it is relevant to review, in somewhat more detail, results of studies using animal models designed to specifically investigate developmental toxicity potential.

Developmental Toxicity

Chlorpyrifos has been thoroughly evaluated in four standard guideline studies covering three different species and in a guideline developmental neurotoxicity (DNT) study in rats. These study designs were developed through multistakeholder expert input and continual review over the course of many years to explicitly include those parameters required for a thorough and robust design aimed at identification of developmental and reproductive toxicity (DART) effects. Based on the collective results of these four guideline studies, there was no evidence of treatment-related or dose-related effects on malformations, structural abnormalities and variations, altered fetal growth or change in gestational age at delivery.

In the absence of maternal toxicity, there was no evidence of chlorpyrifos-induced postnatal developmental effects, including no evidence of physiological deficits and neurological or neurobehavioral deficits. Chlorpyrifos did not induce transplacental carcinogenesis or somatic or genetic mutations in the conceptus. Effects in one of the developmental studies (Deacon et al, 1979) were limited to embryo/fetal mortality at the

highest dose, accompanied by pronounced maternal toxicity. This was not observed in the other three developmental toxicity studies (Ouelette et al., 1983; Rubin et al., 1987a, 1987b).

While some have interpreted the developmental neurotoxicity study for chlorpyrifos as demonstrative of developmental toxicity (i.e., parietal cortex effects in females at the mid and high dose levels), as detailed in the following section this parietal cortex finding was well within normal variation for developing rats, based on assessments of biological plausibility and exhaustive data analyses undertaken by the study director and study pathologist. In all cases, developmental toxicity NOAELs were at or above those associated with maternal toxicity.

The Chlorpyrifos Developmental Neurotoxicity Study

This section addresses key aspects of the history of the chlorpyrifos DNT study, the study report, and its three supplements. It is critical to understand the history of this study as there were some intervening decisions and perspectives on its outcome and interpretation that did not have the full benefit of additional investigative work that subsequently clarified much of the initial concern over results reported. Hoberman and Garman (2000), Supplement 3, historical control data for morphometrics, was not completed until shortly after the USEPA's June 2000 risk assessment, precluding the Agency's evaluation of this information at that time.

Included in this section is the rationale for the conclusion of both the study director and study pathologist that high-dose effects in pups were a consequence of undernutrition associated with significant maternal toxicity at birth, and that the slightly thinner parietal cortex in mid-dose and high-dose female pups at 2 months of age was not at any time considered to be treatment related. Also included is a discussion of data indicating that the parietal cortex measurements were comfortably within the historical control range. Finally, the conclusions of major regulatory agencies world-wide indicating consistency with the authors' conclusions are reviewed.

Background

The only chlorpyrifos developmental neurotoxicity (DNT) study available today that meets the study-design requirements of regulatory agencies world-wide is the guideline compliant, Good-Laboratory-Practices compliant, chlorpyrifos DNT study conducted by Drs. Alan Hoberman (study director) and Robert Garman (pathologist) at Argus Laboratories in 1998. Not only does this study meet global standards and requirements for study design to evaluate neurotoxicity, sensitivity, and non-cholinergic effects in young animals, it supersedes *in vitro* and other laboratory animal studies that use inappropriate doses and routes of administration - key factors when considering relevance to humans.

Because of the newness of guideline-based developmental neurotoxicity (DNT) studies in 1997, the chlorpyrifos DNT study was conducted under a protocol

developed by Dr. Jacques Maurissen and other toxicologists at The Dow Chemical Company (Dow) in consultation with USEPA toxicologists. Although the study was conducted according to the 1991 DNT guidelines, the 1998 DNT guidelines were under preparation and the purpose of the consultation with USEPA was to design a study that would meet all current expectations for a state-of-the-art DNT study. Although the draft protocol recommended dietary exposure to chlorpyrifos, the USEPA strongly recommended oral gavage. This use of oral gavage was an unfortunate decision that resulted in confounding of pup data from high-dose maternal toxicity. Oral gavage of pregnant rats with chlorpyrifos causes a transient blood Cmax about thirteen times higher than dietary exposure (Marty et al., 2007). Dietary dosing would likely have generated significant inhibition of maternal brain cholinesterase without causing such unrealistic pharmacokinetics and maternal clinical toxicity. The maternal doses were 0, 0.3, 1 or 5 mg/kg/day. The route of exposure to dams was oral gavage in vegetable oil from gestation day 6 to lactation day 10 (birth = lactation day 0).

The USEPA analyzed samples from the chlorpyrifos DNT study for maternal plasma, RBC and brain ChE activity. Because of his experience with chlorpyrifos and cognitive testing, Dr. Mark Stanton of the USEPA was consulted on the design of the cognitive test conducted both just after weaning and again when the pups were about two months old (a T-maze spatial-delayed alternation task to evaluate learning and memory).

The final report was released as Hoberman, 8/19/1998. The pathology report for the pups at two months of age was inadvertently not included in the final report, and was submitted as report Supplement 1, Hoberman, 9/23/1998. The USEPA requested a statistical reanalysis of the morphometric data. The reanalysis was done in consultation with the USEPA and submitted as Supplement 2, Hoberman and Garman, 3/19/1999. The chlorpyrifos DNT study was published in the open literature (Maurissen et al., 2000). No historical DNT morphometric control data were available at the time the chlorpyrifos DNT study was conducted, but Drs. Hoberman and Garman conducted 5 DNT studies soon after the chlorpyrifos study, at the same laboratory and using the same methods, and issued a Supplement 3, Historical control morphometric data (Hoberman and Garman, 10/9/2000). Notably, the morphometric historical control data (Supplement 3) were submitted five months after the USEPA June 8, 2000 risk assessment was published.

Companion Pharmacokinetic Study

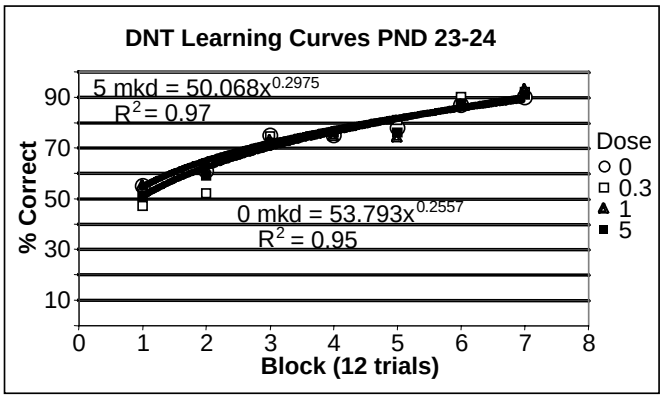
Dow conducted a companion pharmacokinetic study (Mattsson et al., 2000) at nearly the same time as the DNT study. Dams were dosed as in the DNT study; 0, 0.3, 1 or 5 mg/kg/day oral gavage in corn oil from gestation day 6 to lactation day 10. Dams and fetuses were evaluated on Day 21 of pregnancy, and dams and pups on lactation days 1, 5, 11 and 22. Endpoints were cholinesterase inhibition (plasma, RBC, heart, two areas of brain), blood chlorpyrifos levels, blood TCP levels, and milk chlorpyrifos levels. Dams had a high level of inhibition of brain cholinesterase at the

high dose and minor inhibition at the middle dose. Fetuses had inhibition of brain cholinesterase only at the high-dose, and the percent inhibition was less than occurred in their dams. High-dose newborn pups had a very rapid post-natal recovery of cholinesterase activity, and plasma and brain cholinesterase activity was comparable to controls in 5 days. RBC cholinesterase recovery was slightly less, perhaps because RBC cholinesterase recovery is due to replacement of old RBCs with new RBCs, rather than a resynthesis of inhibited cholinesterase. The no-observed-adverse effect level for inhibition of brain cholinesterase was 0.3 mg/kg for dams and 1.0 mg/kg maternal-dose for fetuses and pups. Nursing pups of high-dose dams ingested chlorpyrifos from milk at approximately 0.1 mg/kg/day. Plasma cholinesterase is the most sensitive to inhibition by chlorpyrifos. The recovery of pup plasma cholinesterase to approximate those of controls in 11 days while ingesting 0.1 mg/kg/day of chlorpyrifos indicated this dose level was near or below the threshold for inhibition of adult plasma cholinesterase. If the plasma cholinesterase threshold had been exceeded, then the pup plasma cholinesterase would have attained a new level of inhibition during lactation exposure, and would not have recovered to control values during exposure.

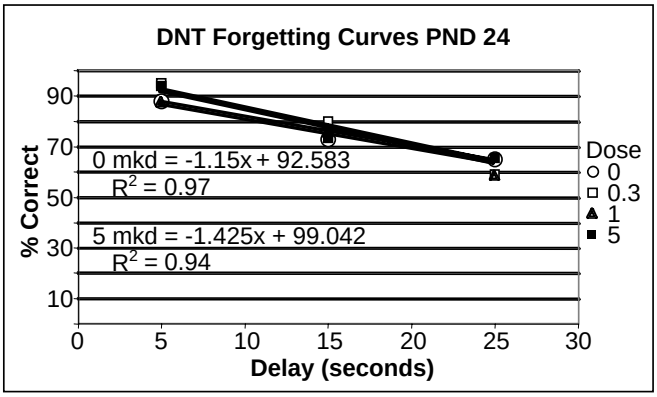
DNT Results

High-dose dams had clinically-evident toxic signs just before and for 4 days subsequent to giving birth (e.g.., muscle fasciculations, hyperpnea, hyperactivity, diminished weight and weight gain). Several pups of high-dose dams died at this time, some in entire litters and some without milk in their stomachs. When maternal clinical signs abated, no more pup deaths occurred. Pups from high-dose dams gained weight more slowly than controls, and several of the developmental measures showed effects consistent with slightly delayed maturation. Despite these signs of delayed maturation, however, pups of high-dose dams performed as well as controls in post-weaning tests of learning and memory (T-maze spatial delayed-alternation task; Text Figures 2 and 3). There was no evidence of maternal toxicity at 1 mg/kg/day, and pups of these dams showed no differences from control that were attributed to treatment.

Text Figure 2. Learning curves/performance for rat pups exposed to chlorpyrifos



Text Figure 3. Forgetting curves/performance for rat pups exposed to chlorpyrifos



For several reasons (discussed below), small but statistically significant differences in the thickness of the parietal cortex of high- and mid-dose female pups at 2 months of age were considered to be random effects and not treatment related. The DNT study concluded that the maternal and developmental NOAEL was 1 mg/kg/day. All adverse effects in offspring of high-dose dams in this study were interpreted by Drs. Hoberman and Garman as secondary to pup undernutrition due to excessive maternal toxicity in high-dose dams.

In its June 8, 2000 risk assessment, the USEPA initially concluded that the chlorpyrifos DNT study showed more severe effects in pups than in dams and that an observed 5% thinner parietal cortex in 2-month old mid- and high-dose female pups was treatment-related (USEPA, 2000a). However, at the time, the USEPA did not have the benefit of the historical morphometric control data (Supplement 3) which

was submitted in October 2000, providing clarifying insight bearing on the Agency's initial conclusion.

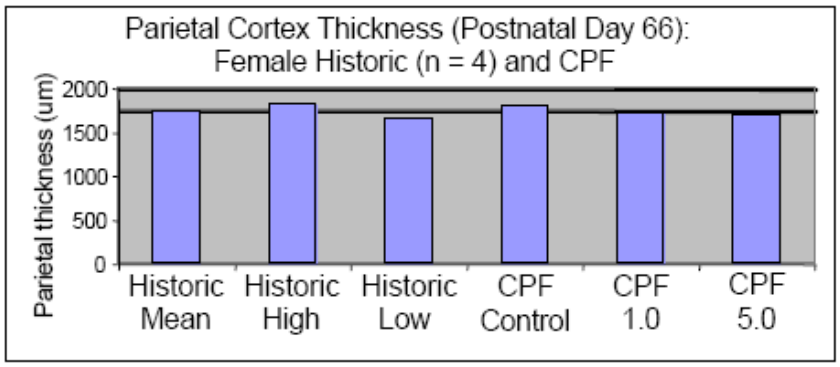
Supplement 3

Supplement 3 was submitted four months after the USEPA issued its June 8, 2000 chlorpyrifos risk assessment. This supplement contained a thorough biological-plausibility evaluation of the parietal cortex findings and reported the results of five subsequent DNT studies for historical reference values. Four of the historical control studies contained data relevant to the parietal cortex.

An examination of the female post-natal day 66 parietal cortex data (Text Figure 4 and accompanying Table) shows how small the differences are between chlorpyrifos controls and low- and high-dose pups. Given a 5X difference in doses, it is difficult to argue that a 5.1% difference from concurrent control (high-dose) is truly different from 4.2% for mid-dose females. Of four historic control studies, the low historic value was 7.6% smaller than the chlorpyrifos control mean. It is readily apparent that the female day-66 parietal cortex measurements from four historic control studies and from the chlorpyrifos DNT study are all within the range of normal variation.

Text Figure 4. DNT Supplement 3 data

Data from Hoberman and Garman, Supplement 3, 10/9/2000.



Female Day 66	Parietal Thickness (um)	% historic mean	% control mean
Hist mean	1738	100.0	97.0
Hist high	1824	104.9	101.8
Hist low	1656	95.3	92.4
CPF Cont	1792	103.1	100.0
1 mg/kg/d	1716	98.7	95.8
5 mg/kg/d	1700	97.8	94.9

The following are quotations from Hoberman, A. and Garman, R. Supplement 3 to the Final Report: Historical Control Morphometric Data, 9 Oct 2000.

In separate papers, the issues of dose, time of exposure, route of exposure and sensitivity of the adult and pup rats to Chlorpyrifos and cholinesterase inhibition have been extensively investigated. All of these investigations have added weight to the original author's conclusions. Specifically, the original report attributes all effects of chlorpyrifos, including those that resulted in morphometric differences in brain areas of high dosage group pups, to slower growth caused by undernutrition of the offspring and not to any type of defective growth or effect on brain development. These effects on brain weight and morphometric measurements were clearly limited to the maternal high dosage group. A statistically significant reduction in the parietal cortex of the F1 generation female adult rats in the middle (1 mg/kg) dosage group was never considered to be related to the test article.

The rationale for concluding that the slightly thinner parietal cortex in the adult females was not related to treatment was as follows:

A. There was a greater than 50% likelihood of a statistical false-positive conclusion (16 ANOVAs on the adult morphometric data).

B. The difference was small (approximately 5%), which was well within the differences that could occur from embedding the brains at different times (one day difference in time between the control and high dose, and one month difference in time between the control and middle dosage group adult females). This would be a "batch" effect.

C. There was a persuasive lack of biological plausibility in the other data. If the slightly thinner parietal cortex were due to a pathological process, then one would expect to find supporting biological data. None were found among the following data sets:

1. When cortical thickness was corrected for body weight, the relative cortical thickness of high-dose females was slightly greater than for middle dosage females. The relative thickness of the parietal cortex in high dose females was not statistically significant as compared to controls.

2. There was no difference in the parietal cortex in high-dose adult males. While differences in dose response often occur between males and females, these differences in sensitivity are seldom large. A 5X difference in dose would be highly likely to affect males if the effect were true.

3. There was no effect on the parietal cortex in the middle dosage group males or females on PD 12. Since the greatest exposure occurred in utero, and neocortical neuronogenesis and migration occur in utero, an effect on the PD 12 pups would be expected.

4. There was no effect on the frontal cortex, even at a dose that was 5X higher. The frontal cortex is adjacent to the parietal cortex, is seen on the same plane of section ,and undergoes development by the same process as the parietal cortex (Bayer et al., Neurotoxicol 14(1): 83-144, 1993). It would be very unusual for pathological processes to alter development of the parietal cortex and not the frontal cortex, given that in utero exposure encompasses the development phases of both cortical areas.

5. No histopathological changes were seen in any brain tissue, including the parietal cortex. Aberrant cortical neuronogenesis and migration would be expected to result in altered cytoarchitecture, especially over a 5X-dose range. The adult female middle and high dosage group parietal cortex had a normal cytoarchitecture when examined by light microscope.

6. There were no changes in complex behaviors. The learning and memory of the delayed spatial alternation task is a set of complex behaviors that were not affected by treatment, in males or in females, even at the high dose. There is a substantial literature concerning the complex role of the parietal cortex on spatial memory. These functions of the parietal cortex increase the likelihood that if the pathological changes had occurred in the parietal cortex in the middle dosage group, then the high dosage group should have demonstrated performance effects on the delayed spatial alternation task. This did not occur.

While one could argue that a particular biological effect might not be detected concurrently with a pathological change in the parietal cortex, it is very difficult to argue that a pathological change in the parietal cortex would not affect any of the above parameters.

The subject of this supplement to the developmental neurotoxicity report is the accumulation of relevant historical control data collected after the original study. The authors have since conducted 5 more studies, and have now demonstrated that these parietal cortical values were well within historical control ranges. The historical average (range) for this value was 1738 micrometers (um) (1656 to 1824 um). The average thickness of the adult female parietal cortex in the 1 mg/kg group was 1716 ± 36.4 um, and the concurrent control value was 1792 ± 36.1 um."

The parietal cortex thickness of middle dosage group females was 1716 um, which was 1.3% smaller than the historical average control and 3.6% thicker than the smallest historical control. Thus, although slightly (about 5%) thinner than concurrent controls (statistically significant), the parietal cortex of the middle dosage group adult females was comfortably within the range of historical control values."

In conclusion, the historical control data have provided an important perspective to the normal variability of morphometric data under the circumstances used in these experiments. In addition, the data have provided additional support to the original conclusions of the authors that the statistically significant differences in adult female parietal cortex were within normal variation and were not treatment related."

The USEPA did not consider this DNT study to be of significant concern relative to developmental neurotoxicity during the revised organophosphate cumulative risk assessment (USEPA, 2002) or in their final cumulative risk assessment (USEPA, 2006). Both of these subsequent EPA reviews considered the published literature on chlorpyrifos developmental toxicity, including Supplement 3, and for those assessments the FQPA factor for chlorpyrifos (repeated exposures) was determined to be 1X.

Conclusions from Regulatory Agencies on the Chlorpyrifos DNT

Various regulatory bodies have evaluated and concluded the following relative to the DNT study for chlorpyrifos:

WHO 1999 toxicology assessment of chlorpyrifos: *"The NOAEL for toxic effects in the pups was 1 mg/kg bw per day on the basis of the decreased viability index, relative brain weight, and delayed sexual maturity, possibly associated with maternal toxicity and subsequent diminished maternal care at the high dose. Cognitive function (learning, memory, and habituation) in the pups were not affected by treatment (Hoberman, 1998)."*

California EPA, DPR, summary of toxicology data (2001): Concluding comments about Supplement 3: *"In the context of the demonstrated high maternal and neonatal toxicity of this dose, the supplemental data reinforce the lack of demonstrated special toxicity of the test article toward the developing nervous system. Supplemental to a previously acceptable study with no adverse effects."* Aldous, 9/26/01.

The UK Advisory Committee on Pesticides (ACP 6(299/03): *"By contrast, the OECD Guideline-compliant developmental neurotoxicity study performed with*

chlorpyrifos covered similar endpoints and established a clear NOAEL (1 mg/kg bw/day) for effects on pups following oral exposure (see Appendix 2, Hoberman, 1998 at section 5.1.7.1 (q), and the evaluation of a supplement to this study at Appendix 3).” p. 3.

APPENDIX 2 - Taken from the UK Advisory Committee on Pesticides (ACP) 264 (277/00) considered by ACP 6 July 2000: *“The NOAEL for effects on pups was 1 mg/kg bw/day, based on decreased viability, lower pup bodyweights and brain weights and delayed sexual maturity at 5 mg/kg bw/day. These effects were consistent with being secondary to maternal toxicity. Cognitive functions in the pups (learning, memory and habituation) were not affected by treatment at any dosage. There were no neuropathology findings in pups at 12 or 66 days of age.”* pp. 92

The UK Advisory Committee on Pesticides (ACP) has evaluated the Hoberman (1998) Supplement 3 on morphometric historical control data:

Pesticide Safety Directorate (PSD) comment: *“The analysis of the morphometric data provided by the company gives a detailed argument as to the ‘lack of biological plausibility’ of the apparent treatment related effects of chlorpyrifos on pup brain sizes. The paper provides some limited historical control data (given the limited length of time such studies have been conducted). Overall there appears to be little consistency to the effects, and since there are no marked differences from control values the overall significance of the findings is unclear.”* In Appendix 3 – Taken from ACP 23 (281/01) considered by ACP 18 January 2001. Supplement 3:a) A supplement to the developmental neurotoxicity study in rats (ACP 264 (277/00) Section 5.1.7.1 (q) Hoberman, A.M. (1998).

Australia 2000a chlorpyrifos toxicology assessment (Supplement 3 not included): *“The morphometric measurements reveal minor variations (approximately 5%) which might be expected for such a small sample (6 animals). The neuropathological microscopical examinations (generally 48 sites/tissues reported) were restricted to the control and high dose animals and no effects of treatment were evident. While data comprising the morphometric measurements were provided for mid-dose days post partum (DPP) 66 females (1 mg/kg/d), no neuropathological examinations were reported for this group. These results suggest that the animals had generally recovered from the delayed development that was evident at DPP 12.”*

Australia 2000b NRA chlorpyrifos summary (based upon analyses in Australia 2000a chlorpyrifos toxicology review): *“There was no evidence that significant developmental or neurological effects were caused by chlorpyrifos in young animals at doses below those that inhibited plasma cholinesterase activity”…”The data on effects of chlorpyrifos in young or developing animals have been reviewed and infants and children are not considered to be at an*

increased risk from chlorpyrifos products that are used according to label instructions."

In summary, from a guideline-compliant perspective involving numerous developmental and developmental neurotoxicity studies designed specifically to capture and detect any untoward effect on developmental processes including cognitive function which may manifest itself in a variety of ways, there are no data that support generalized or specific neurodevelopmental effects resulting from chlorpyrifos exposure.

D. Current Literature on Neurodevelopment – Factors to be Considered when Evaluating Relevance to Human Risk Assessment

Key Points

- Animal studies cited as supportive evidence for biological plausibility in an epidemiology publication have used a route of administration (i.e., subcutaneous in DMSO) and dose levels that are irrelevant for humans.
- The OECD 2006 draft DNT 426 guidelines specifically states: "The vehicle should not cause effects that could interfere with the interpretation of the study, neither be neurobehaviourally toxic…" However, numerous animal studies that are used as the basis for reported neurodevelopmental concerns in humans use a vehicle, subcutaneous DMSO, that possesses neurotoxic properties at the doses used (1 mL/kg). The kinetic and neurobehavioral properties of DMSO present a significant confounding variable when neurotoxicity and neurodevelopment are the key endpoints of evaluation/investigation.
- One of the chief academic investigators that has reported effects on neurodevelopment in animal models and in vitro systems has specifically acknowledged that their research is not designed to have relevance for human risk assessment and regulatory management of chlorpyrifos.
- The majority of studies cited as the basis for potential listing of chlorpyrifos as a California Proposition 65 developmental toxicant use routes (subcutaneous, intraperitoneal) of exposure and doses that are not relevant to humans.
- Even if the studies that employ subcutaneous administration and DMSO as a vehicle are taken at face value, virtually all use a dose (1 mg/kg bw or above) that is above the no-observed-adverse-effect-level (NOAEL) for brain ChEI (relevant target organ) in animals and much higher than the current chronic NOAEL of 0.03 mg/kg bw recognized by the USEPA. These studies would not impact or improve the current exposure limits for humans.

Much of the current interest in chlorpyrifos stems from recent human and animal studies, and it is important to critically assess these with respect to test design, dose, route of exposure, vehicle, and reported effects/outcomes to determine whether there is both

biological plausibility and coherence of findings. Specifically, the epidemiology report of neurodevelopment by Rauh et al (2006) cites, in support of its findings, experimental work in animal models that associates chlorpyrifos exposure with effects on neurocognitive development in rats. Further, the authors suggest that organophosphates may disrupt brain development through noncholinergic mechanisms at doses that cause only minimal acetylcholinesterase inhibition. The specific quotation from Rauh et al (2006) is as follows:

> *"Experimental work showed links between chlorpyrifos exposure during pregnancy and deficits in fetal growth and neurocognitive development in rats.*[10] *Prenatal chlorpyrifos exposure was shown experimentally to inhibit acetylcholinesterase, to downregulate muscarinic receptors, to inhibit the adenylate cyclase signaling cascade, to decrease brain DNA and RNA synthesis, and to suppress neurite outgrowth.*[10–14] *Many organophosphate compounds are lipophilic and cross the placenta.*[15] *Prenatal exposure is a source of concern because acetylcholinesterase seems to act as a neurotropic factor during brain development.*[16] *Organophosphates may also disrupt brain development through noncholinergic mechanisms, at doses that cause only minimal acetylcholinesterase inhibition.*[16–18]*"*

This statement serves as the biological basis for the observation in inner-city children. It is necessary to review the cited studies so as to determine relevance for humans, whether there is consistency in endpoints and reported effects, and how relevant the route of exposure and dosing regimes are for human exposure scenarios (Text Table 1).

Text Table 1. Citation of studies by Rauh et al (2006) as providing basis for neurodevelopmental effects in humans

Citation	Route of Exposure	Vehicle	Dose	Dose-response evaluated	Cholinesterase Inhibition Measured	Effect(s) reported
11: Dam et al., 1998	Subcutaneous (S/C)	DMSO 1 mL/kg	1 mg/kg	No	No	Reductions in DNA synthesis in brainstem, forebrain
12: Johnson et al., 1998	S/C	DMSO 1 mL/kg	1 mg/kg; 5 mg/kg	No	No	Alterations in RNA concentration/content in brainstem/forebrain
13: Slotkin et al., 2005	S/C	DMSO	1 mg/kg	No	No	Elevations in synaptic protein related to 5 HT at 5 months of age
14: Song et al., 1997	S/C	DMSO	1 mg/kg; 5 mg/kg	No	Yes	Deficits in multiple components of the adenylyl cyclase cascade
17: Huff et al., 1994	Cell culture	Acetone	Up to 1 mM	Yes	Yes	Interactions/binding to muscarinic receptors of rat striatum
18: Song et al., 1998	Cell culture	DMSO	1.4-140 uM	Yes	No	Inhibition of DNA synthesis

Several characteristics of these studies have importance when assessing their relevance to humans and when assessing the comparability of these findings to whole animal

guideline studies. First, the route of exposure, subcutaneous administration, is not a typical route employed in guideline studies and is not relevant to humans who may be exposed to chlorpyrifos. Secondly, the vehicle commonly used, dimethylsulfoxide (DMSO), has neurotoxic properties of its own as described in more detail below. Neurobehaviorally-toxic vehicles are specifically prohibited by OECD (2006) draft DNT guidelines. Thirdly, the common dose of chlorpyrifos used in many of the *in vivo* studies (1 mg/kg) is 3333 times higher than the permissible (RfD) human exposure level for chlorpyrifos and more than 16,000 times higher than the actual 95th percentile exposure to children (Barr et al., 2005). In addition, only a few of these studies employed a range of doses, so that even within the limitations of these experimental systems and designs, there is little or no potential for assessing dose-response.

Critical to the question of whether non-cholinergic effects may be operating at dose levels below those at which detectable cholinesterase inhibition (ChEI) occurs, in only a few of the studies was ChEI concurrently measured. When measured, substantial inhibition of ChE was found to exist. The concentrations employed in the *in vitro* studies are also much higher than biologically relevant concentrations, and commonly were conducted without physiologically-relevant levels of albumin (chlorpyrifos binds to proteins, and albumin has chlorpyrifos oxonase activity). For example, incubations of 1 μM chlorpyrifos are 100,000X higher than the mean, circulating blood levels reported from residential use of this pesticide (3.9 pg/mL; Whyatt et al 2005). Finally, a number of different endpoints have been reported making it difficult to determine not only whether the reported effects relate to subsequent downstream events in the neurodevelopment of animals, but also how they might (or might not) fit into a proposed mode of action for chlorpyrifos.

Given the exploratory nature of academic research, it is not surprising to find the myriad number of study designs and accompanying confounding challenges with respect to route, dose, dose-related changes in mechanism, and vehicle. While such research is important in its own right, studies derived from it often have little relevance to regulatory endpoints or the guideline studies conducted to evaluate them. A considerable amount of the academic research on chlorpyrifos has emanated from the laboratory of T.A. Slotkin, who in 2004 described his views on the notable differences between academic toxicology and regulatory toxicology (Slotkin, 2004). In academia, Dr. Slotkin stated a primary emphasis was 'novelty' of findings, publication in top journals, obtaining current funding and opening pathways to funding in the future. In studies such as these, in contrast to regulatory guideline studies, Dr. Slotkin stated:

> "Practical issues that are critical to standardized testing are de-emphasized, such as …"
>
> - "pharmacokinetics/toxicokinetics".
> - "matching of routes of exposure to those of humans".
> - "development of biologically-based dose response models of established hazards".

- "In that sense, the academic approach is entirely deficient in those attributes that are necessary components of the application of research findings to regulatory science."

Another example of where critical analysis of study type, route of exposure, and dosing regime is important for purposes of determining human relevance involves an analysis of the studies (many of which were conducted by the Slotkin laboratory) that California OEHHA included in its listing of animal studies reporting adverse developmental or reproductive effects (OEHHA, 2007). A tabular summary is included below (Text Table 2):

Text Table 2. Categorization of Animal Studies Cited by OEHHA as Reporting Reproductive or Developmental Toxicity

Study	Brief description	Route of exposure	Dose Regimen	Dose Compared to RfD = 0.0003 mg/kg/day*	OEHHA / DARTIC Study Design Compliant**
1. Akhtar et al. 2006	Fetotoxicity and teratogenicity from GD 0-20	Gavage	Dams 9.6-15 mg/kg/day; GD 0-20	32,000 - 50,000 x higher	Yes
2. Venerosi et al. 2006	Behavioral effects after pre- and postnatal exposure	Not reported	3-6 mg/kg/day	10,000 - 20,000 x higher	No
3. Ricceri et al. 2006	Behavioral effects in adult mice after either fetal and/or neonatal exposure	Gavage (dams) and sub-cutaneous (pups; vehicle not specified)	Dams 3-6 mg/kg/day; GD 15-18, Offspring 1-3 mg/kg/day, PND 11-14	10,000 - 20,000 x higher, adults; 3,333 - 10,000 x higher, offspring	No
4. Jeong et al. 2006	Endocrine-disrupting effects	Used chlorpyrifos methyl, not chlorpyrifos	Not applicable	Not applicable	No
5. Aldridge et al. 2005	Adult serotonergic and dopaminergic synaptic activity after pre or neonatal exposure	Subcutaneous in 1 ml/kg DMSO	Dams 1 or 5 mg/kg/day; GD 17-20 Pups 1 mg/kg/day PND 1-4, 5 mg/kg/day PND 11-14	3,333 – 16,666	No
6. Tian et al. 2005	Teratogenicity and developmental toxicity	Intraperitoneal	40-80 mg/kg/day	133,333 - 266,666 x higher	No

Study	Brief description	Route of exposure	Dose Regimen	Dose Compared to RfD = 0.0003 mg/kg/day*	OEHHA / DARTIC Study Design Compliant**
7. Roy et al. 2005	Morphological changes in brain after neonatal exposure	Subcutaneous	Pups 5 mg/kg/day PND 11-14	16,666 x higher	No
8. Icenogle et al. 2004	Behavioral alternations after exposure during neurulation	Subcutaneous in 1 ml/kg DMSO	Dams 1 or 5 mg/kg/day; GD 9-12	3,333 - 16,666 x higher	No
9. Qiao et al. 2004	Cholinergic synaptic activity after exposure during neurulation	Subcutaneous in 1 ml/kg DMSO	Dams 1or5 mg/kg/day; GD 9-12	3,333 – 16,666	No
10. Farag et al. 2003	Developmental toxicity	Gavage	Dams 5-25 mg/kg/day; GD 6-15	16,666 - 83,333 x higher	Yes
11. Tian et al. 2003	Cytogenetic damage in preimplantation embryos	Intraperitoneal	40-80 mg/kg/day	133,333 - 266,666 x higher	No
12. Levin et al. 2002	Behavioral alterations after prenatal exposure	Subcutaneous in 1 ml/kg DMSO	Dams 1 or 5 mg/kg/day; GD 17-20	3,333 - 16,666 x higher	No
13. Breslin et al. 1996	Developmental and reproductive toxicity	Gavage	0.1-15 mg/kg/day	333 - 50,000 x higher	Yes
14. Chanda et al. 1995	Maternal and developmental indicators of toxicity	Subcutaneous	200 mg/kg/day	666,666 x higher	No
15. Muto et al. 1992	Teratogenic and neurotoxic potential	Intraperitoneal	0.03-0.3 mg/kg/day	100 - 1,000 x higher	No
16. Everett 1982	Semen output	Not reported	Not reported	Not available	No
17. Thompson et al. 1971	3-generation reproduction and teratology	Dietary	0.1-1.0 mg/kg/day	333 - 3,333 x higher	Yes

Study	Brief description	Route of exposure	Dose Regimen	Dose Compared to RfD = 0.0003 mg/kg/day*	OEHHA / DARTIC Study Design Compliant**
	study				
18. Qiao et al. 2002	Developmental neurotoxicity	Subcutaneous in 1 ml/kg DMSO	Dams 1,2 or 5 mg/kg/day GD 9-12; 1 to 40 mg/kg/day GD 17-20	3,333 - 16,666 x higher	No
19. Qiao et al. 2003 (online 2002)	Development of acetylcholine systems in forebrain	Subcutaneous in 1 ml/kg DMSO	Dams 1or5 mg/kg/day; GD 17-20	3,333 – 16,666	No
20. Rubin et al. 1987	Pyrinex teratogenicity	Oral (intubation)	0.5-15 mg/kg/day	1,666 - 50,000 x higher	Yes
21. Nimphius 1995	Embryonal and fetal development	Subcutaneous	0.3-10mg/kg/day	1,000 - 33,333 x higher	No

* Current EPA reference dose (RfD) for chronic exposure is 0.0003 mg/kg/day.
** Conformance to guideline study design for evaluation of DARTs under Proposition 65.

From this listing of studies, it is relevant to note route of exposure (developmental toxicity studies typically employ gavage (oral), not subcutaneous exposure) and dosing regimen (i.e., amount), particularly when human risk is being assessed. Many of the studies included in Text Table 2 have no relevance for assessment of risk to humans. Additional comments on route of exposure and vehicle used in test material administration follow.

Inappropriate route of exposure. Many of the studies conducted and reported by Dr. Slotkin and associates (including those listed in Text Table 2) utilize subcutaneous injection (and on one occasion, intracisternal injection, i.e., brain injection to one-day old rat pups) of a bolus dose of chlorpyrifos with a vehicle (DMSO) known to exert neurobehavioral effects of its own (Fossum et al. 1985; Cavaletti et al. 2000; Cavas et al. 2005). The animal studies from Dr. Slotkin and associates combine an inappropriate route of exposure (SQ) with the addition of a confounding toxic compound (DMSO). This type of study design would never be identified as relevant when considering human exposures, and yet it has been used consistently by Dr. Slotkin and associates for many years. It is this experimental study design and accompanying experimental results, in large part, that have served as the basis for the allegations of developmental effects associated with chlorpyrifos (e.g., Rauh et al. 2004, 2006). In spite of the subcutaneous route and use of high doses of DMSO (1 mL/kg/day), no treatment-related effects have been reported by Dr. Slotkin and associates in the absence of inhibition of cholinesterase, with his

laboratory reporting brain ChE inhibition as high as 60%. All doses used by Dr. Slotkin and associates have been above the current EPA NOAEL of 0.03 mg/kg/day.

DMSO alters chlorpyrifos absorption. Dr. Slotkin and associates routinely inject chlorpyrifos into dams and neonates subcutaneously in DMSO to "ensure rapid and complete absorption" (Song et al. 1997, citing Whitney et al. 1995). "Rapid and complete absorption" of chlorpyrifos was an erroneous assumption but had a large impact on selection of endpoints and interpretation of data in subsequent studies. The paper by Whitney et al. (1995) was the first in a long series of chlorpyrifos studies by Dr. Slotkin and associates. In dose-ranging evaluations, Whitney et al. (1995) reported that PND-1 pups given chlorpyrifos subcutaneously in DMSO began to die after 4-hrs post injection. The 4-hr delay for pups to begin to die was comparable to the delay they observed for chlorpyrifos injected subcutaneously in peanut oil vehicle. Peanut oil vehicle was used to prolong chlorpyrifos absorption. The 4-hr delay in onset of deaths in subcutaneous/DMSO pups is not compatible with "rapid and complete absorption" of chlorpyrifos, and should have alerted the investigators that their assumptions on the role of DMSO were incorrect.

The incorrect assumptions (in Whitney et al., 1995) about the rate of chlorpyrifos absorption from subcutaneous DMSO administration invalidate their dose calculations aimed to provide about equal chlorpyrifos exposure to the brain from direct intracisternal injection as from subcutaneous administration in DMSO. The delay in absorption from subcutaneous DMSO would create very different (lower) brain concentrations of chlorpyrifos compared to direct injection. The similar degree of inhibition of brain DNA synthesis from both routes of exposure cannot be explained by similar concentrations of chlorpyrifos in the brain.

The assumption of rapid and complete absorption also led to incorrect timing of tissue samples for evaluation of brain ornithine decarboxylase, a test used to evaluate a generalized decrease in brain metabolism. A lack of effect of chlorpyrifos via subcutaneous DMSO on brain ornithine decarboxylase at 4 hr and 48 hr (the only times sampled) led the authors to conclude a general depression of brain metabolism was not an issue. Whitney et al. (1995) reported, however, that ornithine decarboxylase was inhibited after chlorpyrifos administered subcutaneously in peanut oil at 8, 12, and 24 hrs, but not at 4 or 48 hrs. Had the chlorpyrifos in SQ/peanut oil ornithine decarboxylase data been sampled at only 4 and 48 hrs, it would also appear that a decrease in brain metabolism had occurred. A belief that ornithine decarboxylase was unaffected by chlorpyrifos subcutaneous DMSO, and that brain DNA was affected equally by equal brain concentrations of chlorpyrifos via intracisternal injection or by injection subcutaneously in DMSO, contributed to a conclusion that the parent chlorpyrifos molecule was the active agent in their findings.

The misinterpretation of the time-of-death data in 1995 also prevented the authors from considering the possible systemic and CNS effects of injection of a mild dermal irritant under the skin. Rat pups given 1 mg/lg chlorpyrifos SQ in 1 mL/kg DMSO show writhing behaviors promptly after injection (Marty et al., 2007). Local pain and

irritation do have afferent neural and cytokine effects on CNS biochemistry (discussed more below).

People familiar with the dam-pup interaction literature are usually quite sensitive to how minor alterations in these interactions have effects on pup development. A single 2 mg/kg subcutaneous DMSO dose of chlorpyrifos in PND-1 pups was reported by Whitney et al. (1995) to affect nursing behavior of the pups between the time of injection and 4 hrs post injection. Pups at 4 hrs post injection weighed slightly less than controls ($p = 0.005$), and direct observation indicated less milk in their stomachs. The cause of the altered nursing behavior was not identified, and begs questions about altered pup-maternal behavior. The maternal-pup relationship might have been affected by altered pup behavior, or either dams or pups might respond to sensory factors. Both DMSO and chlorpyrifos are sulfur containing molecules, both have odors, and odors could have been a factor in these studies, although were not considered as such. If a single subcutaneous injection of 2 mg/kg chlorpyrifos in DMSO alters maternal-pup behavior by whatever means, then one must consider similar effects from four daily subcutaneous injections of 1 mg/kg chlorpyrifos in 1 mL/kg DMSO. The net result is that it is impossible to differentiate neurochemical and behavioral effects that may result from chlorpyrifos from those mediated by altered maternal-pup interactions.

Recent data from five-day old rat pups provide evidence that subcutaneous administration in 1 mL/kg DMSO actually delayed absorption of most of the chlorpyrifos for at least two hours (Marty et al., 2007). Chlorpyrifos remained at or near the site of injection instead of being absorbed along with the DMSO. Recent work by Carr et al (2008) also suggests that the chemical properties of DMSO and the volume of dose vehicle employed for these types of studies can significantly impact distribution of chlorpyrifos in the rat (Carr et al., 2008). These findings raise questions critical to risk assessment since it is not possible to characterize chlorpyrifos' behavior or effects in this experimental system in a manner relevant to human exposures or potential health effects.

Central nervous system (CNS) responses may be confounded from cell signaling due to local irritation. The lag in absorption of chlorpyrifos, and the localization of the radiolabel at the site of injection or in the carcass indicate a depot effect. Chlorpyrifos is recognized as a mild dermal irritant by regulatory authorities (WHO 1999). The *in vivo* studies involving subcutaneous administration of chlorpyrifos with DMSO have not evaluated local, systemic, or central nervous system (CNS) consequences of the irritant properties of chlorpyrifos. There is a growing body of literature on circulating cytokines and CNS effects of peripheral pain and inflammation (Swarm et al. 2001; Ruda et al. 2000; Chatterjee et al. 2006).

DMSO itself has recognized CNS effects. As noted above, DMSO, at doses used in Dr. Slotkin's rat pup studies (1 mL/kg), has been reported to have adverse effects on the peripheral nervous system (PNS) and CNS function (Fossum et al. 1985; Cavaletti et al. 2000; Cavas et al. 2005; Authier et al. 2002). There have been no publications by Dr. Slotkin and associates that evaluate the interaction between chlorpyrifos and

DMSO. How chlorpyrifos might act in the absence of DMSO cannot be determined from the data of Dr. Slotkin and associates, and a control group for DMSO does not address the issue of interaction between chlorpyrifos and DMSO. The 2006 OECD draft Developmental Neurotoxicity Guideline is specific that vehicles that affect neurobehavior should not be used.

In summary, when evaluating a study to determine relevance for humans, it is important to clarify the following:

- Route of exposure
- Vehicle employed and whether it represents a confounding variable
- Whether concurrent cholinesterase inhibition was measured or assumed
- Whether doses used inhibited plasma ChE, a critical ”sink” for chlorpyrifos oxon. Oxon availability to the brain increases greatly only after plasma ChE is inhibited (Timchalk et al., 2002)
- Whether there was clear evidence of a dose-response and a reasonable concordance between genders and between logically-related measures
- How the dose levels employed compare to the red blood cell cholinesterase inhibition NOAEL, which serves as a typical point of departure for human risk assessment

To conclude, many of the academic research investigations that have been conducted on chlorpyrifos over the past 10-plus years have inherent limitations and confounding variables that preclude their use for human risk assessment. In particular, (a) the selection of subcutaneous administration, (b) the employment of a vehicle that imparts neurotoxicity at the volumes used, (c) the lack of study replication, (d) a lack of consideration of alternative mechanisms such as pain and local irritation, and impact of odor, and (e) the use of doses that exceed the established NOAEL for regulatory purposes, collectively render these studies of little value when human risk at environmental exposure levels is considered.

E. Epidemiology

Key Points

- An extensive review of epidemiology literature of populations of high exposure concluded there was "no compelling evidence of harm."
- Three birth cohorts dominate the recent epidemiology literature for chlorpyrifos.
- The conclusions on developmental or reproductive endpoints are conflicting and compromised by exposure misclassification and confounding.

Over the 40 year history of chlorpyrifos production and use, numerous epidemiological investigations have been performed and published. These studies have ranged from focused studies of workers at chlorpyrifos manufacturing and processing sites to broad studies of populations in agricultural communities (Albers et al. 2004; Steenland et al. 2000; Yeary et al. 1993; Burns et al. 1998). Although workers in these areas have had exposures above background levels, developmental toxicity has not been reported in these populations. The scientific weight of evidence for the body of epidemiology data indicates that chlorpyrifos is not associated with these adverse developmental endpoints. An expert panel tasked with reviewing the entire chlorpyrifos literature through 1998 concluded that the *"lack of compelling evidence of harm at higher doses of exposure to chlorpyrifos suggests that exposures at dose levels typical in the general population are unlikely to result in adverse health effects"*. (Albers et al. 1999).

The chlorpyrifos-related epidemiology studies of the current decade are dominated by the results from prospective cohort studies of mother and infant pairs, or birth cohort studies. These studies were not designed to specifically test effects from chlorpyrifos, but rather analyzed the available fluid samples for presence of several pesticides and environmental exposures, including chlorpyrifos. Because of the exploratory character of these studies, many potential relationships were investigated, increasing the likelihood of a chance finding. Taken together, the results of these birth cohort studies are conflicting and contradictory and do not implicate chlorpyrifos as a developmental toxicant. Comments relevant to key findings of these studies follow.

Infant health (birth weight, length and head circumference).

Key Points

- The key infant health endpoints are birth weight, birth length and head circumference
- Only 1 of 3 birth cohort studies found a statistically significant negative association. However, all 3 should be considered together in the weight of evidence.
- The use of lipid-unadjusted blood chlorpyrifos levels may result in exposure misclassification.
- In one study, the inverse relationship of head circumference to PON1 activity among births with TCPy above the LOD, was also observed among those with non-detectable TCPy.
- Post cancellation analysis is limited by smaller numbers and lack of data on actual birth weight and length.

Three birth cohort studies have evaluated chlorpyrifos exposure and infant health. These endpoints include birth weight, birth length and head circumference. The three studies and relevant publications (Eskenazi et al. 2004, Berkowitz et al. 2004, and Whyatt et al. 2004) reached conflicting conclusions. The cohorts of Eskenazi (of Salinas Valley, CA) and Berkowitz (of Mount Sinai, NY) used maternal urine to estimate fetal exposure. The cohort of Whyatt (Columbia Center for Children's Environmental Health, CCCEH) used cord blood to estimate in utero exposure to chlorpyrifos. Only Whyatt et al. observed statistically significant correlations between increasing chlorpyrifos levels and decreased birth weight and birth length. In fact, the other studies show increases in birth weight with estimates of rising exposure to chlorpyrifos (Eskenazi et al. 2004, Berkowitz et al. 2004).

As shown in detail in Text Table 3, each study used biological indicators of exposure. Eskenazi et al. also used the dialkyl phosphate metabolites, metabolites for a broader range of organophosphates. In fact, increasing total dialkyl phosphates (DAP) were statistically significantly associated with *increasing* birth length and head circumference. However, the authors have not suggested that organophosphate exposure is somehow beneficial for the developing fetus. Berkowitz et al. also evaluated the infant health with reported use of pesticides by a household member, and any pesticide used. None of the means was significantly different. Lastly, personal air samples were collected in the Columbia cohort study (Whyatt, et al. 2004). Whereas the exposures as measured by the air samples did not correlate with the cord blood results, the air samples were not associated with any measures for infant health. Taken together, all three studies

attempted to evaluate exposure to chlorpyrifos in several ways. The lack of consistency in direction of the association and statistical significance does not support a cause and effect relationship for infant health and chlorpyrifos exposure.

Text Table 3. Summary of infant health epidemiology study results.

Author, year	Exposure indicator	Study area	Birth weight	Birth Length	Head Circumference
Eskenazi, 2004	TCPy in urine	Salinas Valley, CA	+, No	+, No	+, No
	Total DAP in urine		+, No	+, Yes	+, Yes
Berkowitz, 2004	TCPy in urine	Mt Sinai, NYC	+, No	+, No	=, No*
	Reported pesticide use		+, No	+, No	-, No
Whyatt, 2004	Chlorpyrifos in cord blood	Harlem, NYC (CCCEH)	-, Yes	-, Yes	-, No
	Personal air samples		-, No	-, No	-, No

Yes indicates statistically significant at $p < 0.05$;
+ indicates positive association, - indicates negative association, = the groups were equal.
* see additional comments below.

Two reviews of these papers have evaluated possible reasons for the different findings. One review, by Zhao et al (2005), focused on the endpoints of effect. Zhao and co-authors conclude that birth weight, length and head circumference are not adequately sensitive to the exposure levels received by the general population. Instead, they argue that the inhibition of cholinesterase is a more sensitive indicator of effect. The authors estimate internal chlorpyrifos dose (to the fetus) to be about 1/400 of the dose administered to rat fetuses for which cholinesterase inhibition was observed (Mattsson et al., 2000) and conclude that the exposure in the mother-infant pairs was inadequate for a true effect. To more easily put the exposure into context of the birth cohorts, the maximum maternal TCPy values were 32.5 ug/L (Berkowitz et al. 2004) and 56.1 ug/L (Eskenazi et al. 2004) which are approximate to some levels observed among manufacturing workers. However, no depressions of cholinesterase were observed, either for butyl cholinesterase or red blood cell cholinesterase, among workers at this level. (Garabrant et al. 2008). Garabrant et al. estimated that the no effect level of chlorpyrifos dose for butyl cholinesterase is 5 ug/kg/day. This is also several fold higher than the estimated dose for the Columbia infants based on modeling of the cord blood data by Poet et al.. (Poet et al. 2008).

The review by Needham (2005) compared and contrasted the indices of exposure. The studies reported by Berkowitz et al. (2004) and Eskenazi et al. used maternal urine to evaluate levels of the chlorpyrifos metabolite, 3,5,6-trichloro-2-pyridinol (TCPy), while the study reported by Whyatt et al. used cord blood. Needham notes that interpreting exposure using the TCPy metabolite is complicated because the human metabolite and the environmental degradates are the same. Further, the effect of pregnancy on the metabolism of chlorpyrifos is not well understood. Measuring chlorpyrifos in cord blood is problematic because chlorpyrifos is lipophilic. The concentrations may depend upon "the equilibrium between [chlorpyrifos] concentrations in adipose tissue and blood" (Needham 2005). He recommended that the lipid concentrations should be adjusted, as is done in evaluating serum dioxin, since lipid levels may vary widely in the pregnant subjects. Misclassification of exposure may explain the unique and unreplicated observations in the publication by Whyatt et al. (2004) which is the only birth cohort to evaluate exposure with cord blood.

One observation that is often cited with respect to the Berkowitz et al. (2004) study is the reporting of reduced head size among offspring of mothers whose TCPy levels were above the limit of detection (Berkowitz et al. 2004). However, the inverse relationship of head circumference to PON1 activity was also observed among those with non-detectable TCPy (Text Table 4). Low PON1 activity is associated with smaller head circumference independent of TCPy levels. The findings attributing the observed effects to chlorpyrifos exposure are biologically implausible given a 0.3 cm 'beneficial' effect of TCPy for medium PON1 activity and a 0.3 cm 'adverse' effect of TCPy for low PON1 activity.

Text Table 4. (adapted from Berkowitz et al. 2004). Adjusted mean of head circumference by tertiles of maternal paraoxonase activity and TCPy level, Children's Environmental Health Study, Mount Sinai Hospital, 1998-2002.

PON1 Activity (+ SD)	**Head Circumference (cm)**		
	TCPy > LOD	TCPy < LOD	Difference
Low	33.3 ± 1.5	33.6 ± 1.8	-0.3
Medium	34.0 ± 1.5	33.7 ± 1.7	+0.3
High	34.1 ± 1.6	34.1 ± 1.7	0.0

SD: Standard Deviation

A unique comparison was reported by Whyatt et al. (2004) in which the infant health of children born before and after 2001 were analyzed. The residential use of chlorpyrifos was withdrawn in 2001, and exposure to the infants matched with reduced use. Whyatt et al. (2005) reported the mean cord blood levels of chlorpyrifos to be 6.9 pg/g in 1999, 3.5 pg/g in 2000 and 0.9 pg/g in 2001. The regression models indicate a statistically significant decline with each unit log cord blood level for birth weight (beta = -67.3) and birth length (beta = -0.43). This analysis represents 237 infants with the widest range of chlorpyrifos. The post-2001 analysis is limited to only 77 infants and most are at or below the LOD. The authors do not share any power calculations, but it is not

unexpected that statistical significance was not attained. If chlorpyrifos exposure was toxic to the growing fetus, one would expect that removal of the toxic exposure would result in a rebound of the health indicators. Were the babies born after 2001 bigger, longer or healthier than their older counterparts? This is not reported.

Neurodevelopment of the growing child.

Key Points

- Only 2 birth cohorts have published on cognitive development of the children and only 1 reported out to 36 months.
- There are many cofactors associated with neurodevelopment and some may be confounded by chlorpyrifos.
- The study results by Rauh, et al. (2006) are different when four levels of exposure were used compared to two (low and high).
- The majority of measured blood levels used by Rauh et al and Whyatt were below the validated limit of quantitation for the analytical method.
- The test scores do not improve accordingly with marked reduction in chlorpyrifos exposures during the study period.

Two of the three birth cohort studies that reported on infant health also prospectively evaluated the cognitive development of the children. The results are reported by Eskenazi et al. (2007) and Rauh et al. (2006). Both studies evaluated mental and physical development at 12 and 24 months using the Bayley Scales of Infant Development. Neither found a statistically significant relationship with chlorpyrifos exposure as measured in urine or blood (both cord and maternal levels were used).

Because recent attention was made of the statistically significant decline of MDI at 24 months with increasing urinary DAP in the CHAMACOS study, Text Table 5 summarizes the MDI results with DAP and the more specific, and *nonsignificant* TCPy results. Furthermore, when exposure was evaluated using the DAP levels in the child's urine the statistically significant association was positive. In other words, the children with higher organophosphate exposures faired better on their MDI tests. Eskenazi et al (2007) offered anecdotes that are related to exploring behavior and better diet to explain the association. This directional shift supports the hypothesis that at the low level of exposure reported (median 3.3 ug/L of urinary TCPy) of this agricultural population, chlorpyrifos is not a toxic exposure in the causal pathway, but a marker of other behaviors and risk factors.

The publication by Rauh et al. (2006) from the New York City study by the Columbia Center for Children's Environmental Health (CCCEH) further evaluated 228 children at 36 months and found a statistically significant association with chlorpyrifos exposure and the Psychomotor Development Index (PDI). Because the publication by Rauh et al. has not been replicated, it is important to carefully evaluate the results. Critical points for consideration are discussed below.

Text Table 5. Summary of Mental Development index (MDI) results

Age	Berkeley (Log_{10}DAPs) Adj b	Berkeley (≥ median detected TCPy) Adj b	Columbia *High v Low Cpf) Adj b
6 months			
Prenatal	-1.2	0.08	
Child	-0.2		
12 months			
Prenatal	-1.3	-0.65	-0.3
Child	1.4*		
24 Months			
Prenatal	-3.5**	-1.94	-1.5
Child	2.4**		
36 Months			
Prenatal			-3.3*
Child			

* $p < 0.1$, **$p < 0.05$

Sources: Eskenazi et al. 2007, Rauh et al. 2006

(Adapted from Figure 6, page 49, Science Issue Paper,)

Confounding. In a letter to the editor regarding the Rauh et al paper, Cicchetti notes that the high and low exposure groups varied dramatically in characteristics that would also be related to developmental scores (Cicchetti, 2007). Cicchetti suggests that confounding by race, ethnicity, income and other factors related to the home environment is important. Indeed, in another publication on the New York City birth cohort, material hardship, defined as "unmet needs" in food, housing, and clothing were negatively associated with cognitive development (Rauh et al. 2004). This relationship demonstrates that other factors are related to neurodevelopment in this urban population. Since the levels of chlorpyrifos reported in cord blood were highly correlated with the presence of a number of other pesticides such as diazinon, dicloran, 2-isopropoxyphenol, and tetrahydrophthalimide (Whyatt et al., 2003), the conclusions regarding chlorpyrifos may be confounded by complex environmental or maternal factors. Is, for instance, chlorpyrifos an index for pest infestations rather than a toxic exposure?

Further, the cofactors of ethnicity (language), gestational age, maternal education (low), and HOME score were increasingly significant from 12 to 36 months. Given the low R^2 for the regression models (10 – 25%), the variability of the

Mental Development Index (MDI) and Psychomotor Development Index (PDI) score are poorly explained, even with statically significant covariates such chlorpyrifos. These factors suggest that analyses for chlorpyrifos alone are not indicative either of the range of chemical exposures or of other factors potentially influencing development among the study subjects. With a complex dataset, the role of confounding can be more important than the exposure of interest.

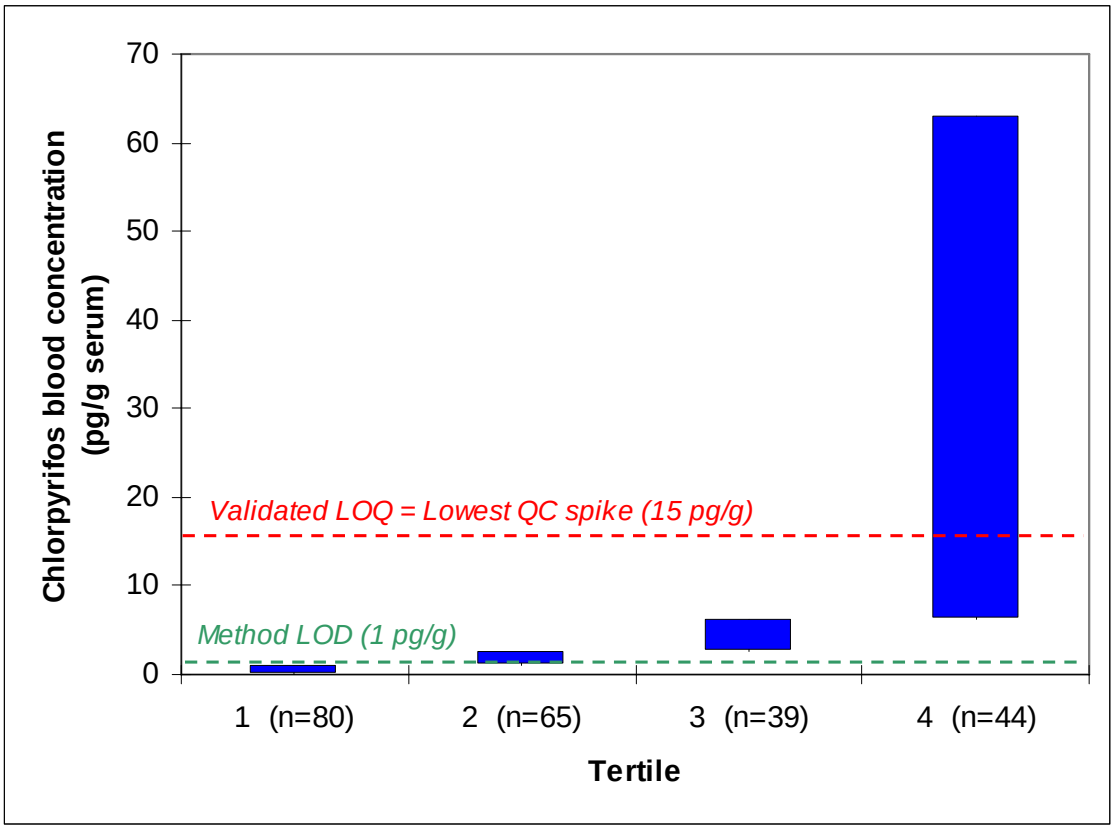
Changing cut-points. The dose response relationship is susceptible to the selection of cut-points. In Rauh et al (2006), the authors describe preliminary statistical analysis that showed no linear dose response in developmental outcomes between non-detectable chlorpyrifos and low, medium or high tertile categories of chlorpyrifos. Accordingly, the low chlorpyrifos tertile had *better* performance than the non-detectable chlorpyrifos group (page 1848). The final analyses in Rauh et al. (2006) were limited to high vs. low exposure levels. Further, there was no attempt to place these exposures in the context of what is known for the broader human population at large. These conflicting results when selecting cut-points arbitrarily reduce the probability of a true cause and effect association.

Chlorpyrifos blood method limit of quantitation. The quantitation of chlorpyrifos in maternal and umbilical cord blood, in support of the human cohort studied by Whyatt et al. (2003, Perera et al. (2003), Whyatt et al. (2004), Whyatt et al. (2005) and Rauh et al. (2006), was conducted at the U.S. CDC laboratory, utilizing the published analytical method of Barr et al. (2002). Concentrations of chlorpyrifos in blood were reported to be extremely low, ranging from ND (1pg/g) to 63 pg/g, with a mean of ~ 5 pg/g. The authors of the analytical method used for these assays defined the limit of detection (LOD) for chlorpyrifos as 1 pg/g serum, via analysis of solvent standards of this compound. Current bioanalytical method validation criteria call for preparation of replicate fortified matrix ("QC spike") samples at the limit of quantitation, to verify accurate determination of analyte concentrations across the range of concentrations expected in a sample set (Braggio et al., 1996; Shah et al. 2000; US FDA 2001; EC 2004). Evaluation of the recovery of analyte fortified into biological matrix is critical, as the endogenous components of the matrix may affect extraction efficiencies, chromatographic separations and/or detector response. However, no QC spikes were reported to be prepared at the stated LOD of 1 pg/g serum during the conduct of these exposure studies. Analyte recoveries were reported in the manuscript for QC spikes at 15 and 50 pg/g serum, showing adequate results down to the concentration of 15 g/g. Based on these criteria requirements and validation results, the validated Limit of Quantitation (LOQ) for measurement of chlorpyrifos in serum by this method should be set at 15 pg/g (Test Figure 5).

As shown in Text Figure 5, the majority of the human cohort study samples were below the fully validated LOQ of 15 pg/g serum (tertiles 1, 2, 3 and a portion of 4). As a result, any statistical analysis between the four exposure subgroups, as conducted by Whyatt et al. (2004, 2005), could be in error, as values below 15 pg/g serum should be reported as non-quantifiable (NQ). The same issue holds true for statistical interpretations by Rauh

et al. (2006). These authors evaluated neurodevelopment effects vs. "high" (> 6.17 pg/g) and "low" (≤ 6.17 pg/g) exposures. Since the fully validated LOQ for this method is above 6.17 pg/g, statistical differences between the "low" and "high" subgroups may not be valid.

Text Figure 5. Comparison of reported blood concentrations from Whyatt/Perera/Rauh cohort by tertile, vs. validated Limit of Quantitation for analytical method.



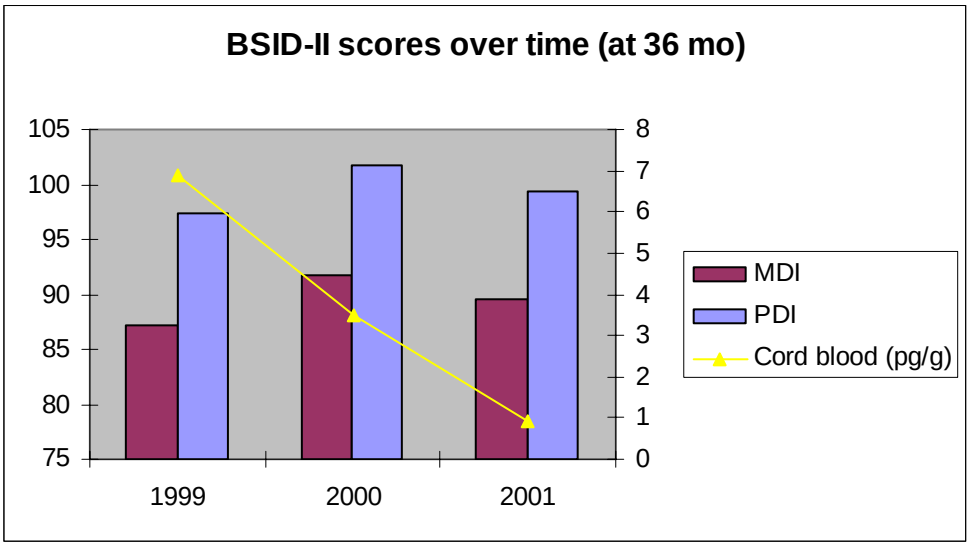
A final quality-control issue with the reported chlorpyrifos blood concentrations from these studies is the lack of evaluation of potential contamination during sample collection and shipment. Current passive dosimetry and biomonitoring methods require preparation of field and/or travel spikes at the time of sample collection (US EPA 1998). These spike samples are shipped and stored with the study samples, to verify no contamination between collection and analysis. Since the blood concentrations of chlorpyrifos in these studies were extremely low, and Whyatt et al. (2002) report measurable environmental concentrations of chlorpyrifos, the lack of field spike results does not rule out sample contamination as a source of the chlorpyrifos detected in the maternal and cord blood samples.

Chlorpyrifos in blood. There were no plasma lipid adjustments to the plasma chlorpyrifos concentrations in the recent human cohort studies. Chlorpyrifos is a lipophilic compound (log Kow 4.96), which is known to partition into lipids (Lowe et

al. 2008; Bartels et al. 2008). Recent studies have shown that the blood:tissue partition coefficients for chlorpyrifos are altered during pregnancy, consistent with documented changes in blood lipid chemistry during gestation (Lowe et al. 2008; McMullin et al. 2008). Since chlorpyrifos is a relatively lipophilic compound, estimates of internal exposure are best made by adjusting plasma concentrations to lipid levels (Lowe et al. 2008; Haddad et al. 2000; Lin et al. 2002). For example, if two women were exposed to the same dose of chlorpyrifos, and one woman had higher levels of plasma lipids, her plasma chlorpyrifos concentration would be higher, even though total body burdens are equivalent, due to a higher blood:adipose partition coefficient. Thus, plasma concentrations of lipophilic chemicals are biomarkers of both exposure and an individual's own lipid chemistry. Blood lipid profiles are known to differ by race and ethnic group and by diet. Different plasma lipid profiles are associated with different birth outcomes (Ophir et al. 2004; Stark et al. 2005; Skidmore et al. 2006; Huter et al. 1997; Koletzko et al. 2007). Without adjusting for lipid relationships, chlorpyrifos exposure may be misclassified.

The natural experiment. In a 2005 publication, Whyatt et al. note that the levels of chlorpyrifos in cord blood decline steadily from 1999 to 2002 (Whyatt et al. 2005), in timely correlation with the withdrawal of chlorpyrifos from residential use and applications. As shown below (Text Figure 6), these mean cord blood levels of chlorpyrifos are 6.9 pg/g in 1999, 3.5 pg/g in 2000 and 0.9 pg/g in 2001. This represents an 87% decline over 3 years. However, the MDI and PDI scores do not improve accordingly. As reported by Rauh, et al. (2006), the MDI and PDI scores did increase from 1999 (pre-cancellation) to 2000 (mid-cancellation) for which only the increase in MDI scores was statistically significant ($p = 0.02$). However, even though the cord blood levels of chlorpyrifos were much lower by 2001, the MDI and PDI performance declined from 2000. If chlorpyrifos exposure at birth was associated with MDI and PDI scores at 36 months, as indicated by the authors, the dramatic reduction in exposure should be correlated with better test scores among the children born later. There is no evidence of a time correlated effect.

Text Figure 6. Changes in Bayley Scores and chlorpyrifos blood levels over time.



BSID: Bayley Scales of Infant Development, MDI: Mental Development Index
PDI: Psychomotor Development Index.
MDI: P= 0.02 (99–00); P> 0.05 (00–01)
PDI: P= 0.06 (99-00); P> 0.05 (00-01)

Other points to consider regarding Rauh et al. (2006) are as follows:

Values within expected variability. The authors report quantitative differences of 7.1 and 3.0 (for Psychomotor and Mental Development Indices, PDI and MDI, respectively) between the high exposure and low exposure groups, yet these differences are well within the expected variability of the Bayley Scales of Infant Development (BSID) (i.e., the average standard error of measurement is 5.21 for the Mental Scale and 6.01 for the Motor Scale) (Black and Matula 2000). These outcomes appear to be of no biological or toxicological (i.e., developmental) significance.

The results may be biased by missing data. IQ measurements were missing for 29 women and cord blood data were missing for 12% of the subjects. Subjectively, the sample mean was substituted for the missing IQ data and maternal levels were used for the missing cord blood data. Since it is well known that maternal IQ can have a profound influence on child development, this is an important factor. The authors describe their rationale for adjusting these omissions as follows: "to maximize sample size." This treatment of data is inappropriate and questionable relative to scientific standards.

Weight of Evidence

Key Points

- The weight of evidence of epidemiology was evaluated quantitatively using the 9 Bradford Hill criteria for causality.
- The probability that chlorpyrifos exposure is associated with adverse infant health (birth weight, length, and head circumference) was 23%.
- The probability that chlorpyrifos exposure is associated with adverse neurodevelopment was 30%.
- The available epidemiology publications on reproductive and development affects in infants and children do NOT support a cause and effect with chlorpyrifos exposure.

A set of criteria formulated by Bradford Hill has been widely used as a guide to determining causality. However, the application of these criteria, while valuable, is rarely systematic. Recently, two epidemiologists conceived of a means by which to estimate weights of each Hill criterion and its impact upon the cause and effect determination (Swaen and van Amelsvoort, 2008). By assigning causal probabilities from the available epidemiology literature, a summary probability score can be determined. This score, from 0 to 100 provides a quantitative determination of the weight of evidence for a body of literature in lieu of vague terms such as strong or weak, and it provides a quantitative estimate of the probability that a given association is causal. The weights were based on an analysis of the weight of evidence for 159 IARC category 1 and 2A carcinogens and the independent evaluation by expert groups who classified these 159 agents as carcinogens.

Using the three publications on birth weight (Berkowitz et al. 2004, Eskenazi et al. 2004, and Whyatt et al. 2004) and the two papers on neurodevelopment (Rauh, 2006; Eskanazi, 2007), we determined the probabilities for the 9 criteria that chlorpyrifos causes lowered birth weight or poor cognitive development, as measured by the Bayley Scales of Infant Development. Since the determination of probabilities for each criterion is a matter of judgment, two epidemiologists independently assigned the probabilities, then selected a compromise value for each. The probabilities are summarized in Text Tables 6 and 7. In so doing, the probability for true cause and effect for infant health and chlorpyrifos exposure (as measured in cord blood) is only 23%. The probability for neurodevelopment is 30%.

The causal inference approach used is both systematic and transparent. The reader can see the criterion for which the epidemiology papers are strong and where they fall short. A qualitative estimate of probability that the association is causal (here, 23% and 30%) helps place the evidence in the context of other epidemiology associations. For example, the probability of cigarette smoking and cancer was determined to be 98.9% (Swaen and van Amelsvoort, 2008).

Epidemiology Summation

A single birth cohort, the CCCEH of inner city children, has recently dominated the epidemiology literature on chlorpyrifos. The results of this study with respect to inverse associations between birth weight and birth length and chlorpyrifos as measured in cord blood, are not confirmed and are diametrically opposed relative to findings reported in two other birth cohort studies. The CCCEH results are also unique in the observation of inverse cognitive development and chlorpyrifos at age 36 months. The CCCEH is the only study to measure exposure in blood, as opposed to chlorpyrifos metabolite in urine. It is unclear if there is exposure misclassification resulting from this unique medium. The determinants of healthy and normal childhood development are complex and may be confounded by exposure to chlorpyrifos. Importantly, when reviewing these studies using the Bradford Hill criteria, the probability of causality is from 23 to 30%. With several key study weaknesses and the lack of another study with similar findings, the weight of the epidemiology evidence does NOT support a cause and effect between chlorpyrifos exposure and developmental effects.

Recent unpublished information from the Columbia study indicates that there is no exposure to the infants born in 2003 and 2004, with 100% of cord blood being below the LOD. Hazard analyses that include these infants are uninformative because there is no exposure. Furthermore, there is no opportunity for risk. Unfortunately, the authors have not reported that any improvement has occurred in the infant health or neurodevelopment compared to the children born earlier with detectable chlorpyrifos in cord blood.

A stated concern of the charge questions is that the cohort members "have been exposed to multiple pesticides, including other OPs." While this is true, all three studies have evaluated either the specific chlorpyrifos metabolite, TCPy, in urine or the parent in blood. Furthermore, equally important to consider are the contributions of other environmental, genetic and cultural factors that may be more strongly associated with the outcomes of interest. A contribution of a mixture or confounder is only critical if there is an elevated risk estimate. In the case of the studies by Berkeley and Mount Sinai, these excesses were not reported. As described above, the statistically significant observations reported by the Columbia investigators may be biased by exposure misclassification or uncontrolled confounding. The weight of evidence of the three birth cohort studies is that chlorpyrifos exposure to humans at environmental levels is not hazardous to the developing child.

Text Table 6. Weight of evidence approach using the Bradford Hill criteria for the association between chlorpyrifos levels and birth weight.

Hill's criterion	Evidence for birth weight	Probability (%)	C1	C2A
Constant			-14.7799	-10.0835
1. Strength	Whyatt reported only betas with a p-value of 0.03. Since not highly significant scored like an OR of 1 - 2.	60	3.7338	1.1538
2. Consistency	Only 1 study of 3 was significant	10	0.4061	0.1803
3. Specificity	Also looked at birth length and head circumference, but for the most part is infant health.	50	-1.3935	-1.9385
4. Temporality	Cord blood chlorpyrifos indicates prenatal exposure. However, with the short half-life we cannot know scope of exposure for entire pregnancy	100	7.657	8.281
5. Dose-response	There was no difference between groups 1 v 2, 2 v 3. The only significant difference is between group 1 and 4 (Table 4)	50	-1.764	-1.767
6. Plausibility	The animal studies show no dose difference unless there is maternal toxicity	5	1.15125	1.08445
7. Coherence	No in utero effects	5	0.004811	-0.0167
8. Experimental evidence	For children born after 2001, chlorpyrifos is no longer associated with birth weight (Table 5). However, the sample size is 2/3 less, and the direction of the change is reversed	50	0.4215	-0.3295
9. Analogy	Whyatt et al found no association with diazinon and birth weight.	20	-0.2588	-0.2022
Sum			9.958161	6.44565
Calculation	$e^{9.958161}/(e^{9.958161}+e^{6.44565})$ =**23.4%**			

C1 is a constant plus the sum of the products of weight1 x probability per criterion
C2A is a constant plus the sum of the products of weight2A x probability

Text Table 7. Weight of evidence approach using the Bradford Hill criteria applied to the association of chlorpyrifos levels and neurodevelopment.

Hill's criterion	Evidence for neurodevelopment	Probability (%)	C1	C2A
Constant			-14.7799	-10.0835
1. Strength	Rauh reported OR of 2.4 (MDI) and 4.5 (PDI). Eskenazi reported only betas (nonsignificant).	80	4.9784	1.5384
2. Consistency	Only 1 study has looked at MDI and PDI at 36 months	10	0.4061	0.1803
3. Specificity	The birth cohorts are not limited to the Bayley scores, but include Attention and ADHD problems and infant health	50	-1.3935	-1.9385
4. Temporality	Cord blood chlorpyrifos predated MDI and PDI testing but this criterion does not distinguish	100	7.657	8.281
5. Dose-response	The analyses are based on low/high. 4 categories were evaluated with category 1 (< LOD) and category 4 (highest tertile) showing similar "lower mean MDI and PDI scores than did the 2 middle levels."	30	-1.0584	-1.0602
6. Plausibility	The animal studies are marked by maternal toxicity only at high dosing.	5	1.15125	1.08445
7. Coherence	No evidence for early effects since no association seen at 12 or 24 months	5	0.004811	-0.0167
8. Experimental evidence	Bayley scores improved from "pre-ban" to "mid-ban" period. However, the scores declined after full implementation of the ban, even though the cord blood levels declined 7 fold.	20	0.1686	-0.1318
9. Analogy	Eskanazi found a significant association with DAP (but not with TCPy	40	-0.5176	-0.4044
Sum			11.39666	7.53255
Calculation	$e^{11.39666}/(e^{11.39666}+e^{7.53255})$ **=30.31%**	0.303161		

C1 is a constant plus the sum of the products of weight1 x probability per criterion

C2A is a constant plus the sum of the products of weight2A x probability

F. Non-Cholinesterase Mechanisms of Chlorpyrifos Neurotoxicity

Key Points

- Chlorpyrifos is regulated globally on inhibition of cholinesterase.
- Potential non-cholinergic mechanisms are not new and have been evaluated historically for regulatory purposes.
- Several independent reviews, including those of regulatory authorities, have concluded that any non-cholinergic effect(s) would not affect regulation of chlorpyrifos based upon inhibition of cholinesterase, still recognized as the most relevant point of departure for risk assessment.
- Any study that reports non-cholinergic effects should also denote whether concurrent levels of cholinesterase were measured and if dose-response comparison was evaluated.
- Non-cholinergic effects that are reported should be evaluated in a mode-of-action framework for determination of relevance to humans.

Chlorpyrifos is regulated world-wide based upon the inhibition of cholinesterase.

Different agencies regulate based upon inhibition of plasma, RBC or brain cholinesterase, depending on the regulatory goals of the agency. A key question in the safety regulation of organophosphate pesticides is "will protection of cholinesterase provide protection against possible non-cholinergic mechanisms of toxicity?" USEPA policy requires that non-cholinergic mechanisms of toxicity be considered in the risk assessment of cholinesterase inhibiting pesticides (USEPA, 2000b).

Because there is significant attention presently directed at putative non-cholinergic effects from chlorpyrifos, particularly in many of the *in vitro* (not whole animal) studies, it is important to evaluate this potential concern and to illustrate that this concern is not new or unique, but rather, has been evaluated and addressed by global regulatory authorities in recent years. While it is generally accepted that the principal mechanism or key event for the toxicity of organophosphate pesticides is the inhibition of acetylcholinesterase (AChE) in muscle and the nervous system (Mileson et al., 1998; USEPA, 2000b; Casida et al., 2004), numerous studies, some of which we have alluded to and discussed previously, have indicated that some mechanisms of toxicity of chlorpyrifos may be mediated by non-cholinergic mechanisms

The following three publications provide useful information for evaluation of organophosphate insecticides for non-cholinergic mechanisms of toxicity. It is important to use all available data and compare dose-response characteristics to see if appreciable

toxicity occurs in the absence of inhibition of cholinesterase. Major reviews in 1998 and 2004 concluded that acetylcholinesterase inhibition is the primary mechanism of toxicity.

Mileson et al. (1998) published the opinions of an expert working group, convened by the ILSI Risk Science Institute, to address whether the anticholinesterase organophosphate pesticides act by a common mechanism of toxicity. In addition, the working group addressed the problem of how to evaluate organophosphate pesticides for a significant level of non-cholinergic toxicity. Regarding mode of action, the workgroup noted that *"Organophosphorus insecticides share a common action of inhibiting acetylcholinesterase; the resulting excess acetylcholine accumulation underlies the principal mechanism of toxicity."*

Mileson et al. (1998) proposed that hypothetical non-cholinergic effects could be evaluated by looking for appreciable toxicity in the absence of significant inhibition of AChE. Mileson et al. discussed using relationships between clinical, pharmacokinetic and *in vitro* data (rate constants against AChE, IC50 versus whole animal ED50, LD50, etc). There was not enough data available to the committee to reach final conclusions about non-cholinergic subgroups, but they did present the potentially useful concept of evaluating correlations between AChE inhibition and other clinical or biological effects.

USEPA (2000b) issued a science policy document on the use of cholinesterase inhibition data in risk assessment identifying the most relevant cholinesterase for risk assessment as brain cholinesterase, followed by RBC and then plasma cholinesterase. This EPA document clearly states that non-cholinergic events must be carefully considered in risk assessment, as follows:

- *"When applying the weight-of-the-evidence approach for selecting critical effect(s) for derivation of a reference dose (RfD) or concentration (RfC), the entire toxicological data base on a pesticide must be evaluated (i.e., there also must be consideration of endpoints not related to the cholinergic consequences of anticholinesterase activity, for instance, liver or developmental toxicity or carcinogenicity)."*

- *"It is possible that, for one or more of the exposure scenarios being evaluated, the non-cholinergic effects will be identified as critical or co-critical, and they may become a more appropriate basis for deriving RfDs or RfCs."*

The relevant point here is that it is implicitly stated by EPA that all toxicities can and should be considered, both non-cholinergic as well as cholinergic, and the Agency has recognized this for a considerable amount of time. It is not as though putative or potential non-cholinergic endpoints or modes of action are only now being considered.

Casida et al. (2004) published an extensive review of cholinergic versus noncholinergic mechanisms of toxicity. This review included 13 *in vitro* and *in vivo* publications from the Slotkin laboratory. Casida et al. concluded:

- *"High-dose laboratory experiments with animal models (e.g., mice, rats, and chickens) are difficult to relate to low-dose, long-term environmental exposure and particularly to actual risks for people."*

- *"The findings reviewed reconfirm the importance of AChE as the primary target and NTE-LysoPLA as the secondary target of greatest interest."*

- *"Chlorpyifos IC_{50} was about 9x lower for AChE than for NTE-LysoPLA, indicating inhibition of NTE-LysoPLA cannot occur without very high levels of inhibition of AChE."*

The USEPA (2002) and UK Advisory Committee on Pesticides (ACP, 2003) Have Determined That Chlorpyrifos Has No Noncholinergic Effects That Affect Regulation.

The USEPA, in the 2002a revised OP Cumulative Risk Assessment, appeared to follow the guidance of the USEPA 2000b policy document on cholinesterase inhibitors and evaluated chlorpyrifos for non-cholinergic developmental effects. Whether intended or not, the USEPA (2002a) evaluation also followed the proposal of Mileson et al. (1998) and looked for evidence of developmental effects at doses below those expected to inhibit cholinesterase. Papers cited in USEPA (2002a) for a variety of possible treatment-related effects include: Johnson *et al.*, 1998; Crumpton *et al.*, 2000; Dam *et al.*, 1999, Dam *et al.*, 2000; Slotkin *et al.*, 2001; Levin *et al.*, 2001; Slotkin *et al.*, 2002). This Agency assessment noted that:

- *In the few prenatal studies where ChE activity was assessed, however, few of these effects occur at dose levels that do not inhibit ChE activity in the fetal brain, and probably none of these effects occur in the absence of ChE inhibition in maternal tissues. In both studies assessing prenatal effects of chlorpyrifos, effects on brain development were noted at dosages (1 mg/kg/day) that did not inhibit fetal brain ChE (Qiao et al., 2002), but would be predicted to show inhibition of maternal blood and brain ChE activity (Maurissen et al., 2000).*

- *In postnatal studies, there are no reports of effects in the absence of ChE inhibition. In some cases, this assertion is made by the authors, but the authors fail to ascertain that the ChE measurements were taken at the time of peak effect. Often the measurements are taken 24 hours after the last dose, rather than assessing ChE activity during the entire dosing period.*

Thus, in 2002a, the USEPA considered the publications from Slotkin's laboratory and noted the apparent lack of developmental effects at doses below those that inhibit cholinesterase. In addition, the USEPA (2002) also evaluated the chlorpyrifos gavage

repeated-dose, adult versus neonate, brain inhibition data of Zheng et al. (2000). USEPA (2002a) concluded there was no meaningful NOAEL difference in sensitivity of pup brain cholinesterase. This is the first time that dose-related mechanisms were a factor in USEPA chlorpyrifos regulation. The chlorpyrifos repeated-dose FQPA factor was reduced to 1X for this assessment.

In 2003, the United Kingdom (UK) Pesticide Safety Directorate (PSD) and the UK Advisory Committee on Pesticides (UK/ACP 2003) reviewed some 25 additional publications, including many from the laboratory of Slotkin and associates for impact on chlorpyrifos reference doses. The review reported the following critical insights:

- *"For this update, key considerations have therefore been: 'Do the studies report effects at dose levels that could impact on the currently proposed regulatory reference dose levels which are based on NOAELs of 1 mg/kg bw/day in humans and dogs (with LOAELs of 2 and 3 mg/kg bw/day, respectively?"* [and]

- *"Do the studies provide evidence of greater sensitivity of fetuses and/or pups than adults to the effects of chlorpyrifos (particularly effects other than cholinesterase inhibition?"*

- Section 3.2 *"In vivo studies (subcutaneous dosing). All of these papers, except for papers by Liu and Pope (1996) and Jett and Navoa (2000), are from the same research group at Duke University, USA."*

- *"The dosing route and the vehicle used (subcutaneous injection in DMSO – designed to maximize exposure) mean that the dose levels used cannot be directly compared with chlorpyrifos reference values (which were derived from oral studies). The pharmacokinetics of chlorpyrifos would also be expected to be completely different following oral ingestion, with first pass metabolism by the liver. Additionally, dermal exposure of operators would not involve such rapid and complete absorption of chlorpyrifos (1% has been proposed based on human data) as occurs following direct subcutaneous injection in DMSO, resulting in a different rate of absorption of chlorpyrifos into the systemic circulation and possible resulting differences in the extent of metabolism. These factors limit the value of the following studies using subcutaneous dosing."*

The PSD review of the additional 25 publications was evaluated by the ACP (Minutes of the 299th Meeting of the ACP on 10 April 2003) with representatives from the following Departments and other organizations present: The Pesticides Safety Directorate (PSD), Department of Health (DH), Health & Safety Executive (HSE), Food Standards Agency (FSA), Scottish Agricultural Science Agency (SASA). … Section 4. Chlorpyrifos Human Health Review. Evaluation of further papers requested by the ACP [ACP 6 (299/2003)]. In this evaluation, the ACP specifically noted that:

- *"As part of this review, members were asked to consider additional papers on developmental neurotoxicity and prenatal exposure in rats."*

- *"The Committee concluded that the papers did not affect their advice on reference doses reached at the meeting in November."*

In summary, both the USEPA (2002a) and the UK/ACP (2003) specifically evaluated publications from the laboratory of Dr. Slotkin and associates for non-cholinergic effects relative to cholinesterase inhibition. Neither agency recognized non-cholinergic effects of a magnitude that caused them to reconsider using cholinesterase inhibition NOAELs for regulation of chlorpyrifos. Furthermore, the USEPA (2002a) determined an FQPA factor of 1X for repeated exposure to chlorpyrifos, bringing the USEPA in agreement with the WHO and EU on the issue of differential sensitivity of the young.

Any study that purports to demonstrate significant non-cholinergic toxicity at dose levels below those where ChEI occurs must be viewed in light of a proposed mode of action for how that effect is related to subsequent downstream effects in whole animals. Additionally, it is critical that concurrent measurement of ChEI be evaluated or included when non-cholinergic effects are investigated as it is not sufficient to infer that dose levels employed are above, below, or equal to where historical reports of ChEI have occurred. Finally, as has been stressed previously, route of administration, vehicle, and dosing regimen in comparison to relevant human exposures should be a sentinel part of the interpretation and determination of study utility for human risk assessment.

G. Age-Related Differences in Cholinesterase Inhibition and Detoxification: Implications for Differential Sensitivity

Key Points

- PON1 is a chlorpyrifos oxon detoxifying enzyme with differential expression in humans. However, PON1 has a modest role in detoxification at very high dose levels and no apparent role at environmentally realistic levels.
- Serum albumin has been shown to have a high capacity to hydrolyze chlorpyrifos oxon at environmentally relevant levels, attenuating any differential sensitivity in PON1 activity
- There are other primary means of chlorpyrifos detoxification at environmentally-relevant levels.
- Differences in PON1 activity have little relevance below 500 ug/kg bw whereas 95% exposure to children is approximately 0.06 ug/kg bw, more than 8000X lower.
- Differential sensitivity of the neonate has not been demonstrated following either gestational exposure or from nursing postnatally (via treated dams).
- Studies conducted for regulatory purposes and according to prescribed guidelines have not demonstrated any differential sensitivity amongst the young.
- Global regulatory authorities have concluded that differential sensitivity of the young or neonate is not apparent following treatment with chlorpyrifos in guideline studies.

There has been an experimental interest in recent years as to potential differential sensitivity between what we will generically describe as 'the young' and adults. This is a legitimate question and one which has downstream ramifications for selection of safety factors in conjunction with FQPA considerations and protection of public health. This paper does not attempt to describe in detail all of the research investigations conducted on this topic. Rather, the intent is to provide some broad perspective on what the weight-of-evidence points to relative to differential sensitivity. Key variables to be considered that bring clarity to questions of relevance to human risk include study design and dose relative to typical human exposures. Understanding dose levels and how they are employed (i.e., administered), in comparison to expected human exposure, is important when evaluating human relevance.

PON1 (Chlorpyrifos-oxonase), a high-dose, dose-related mechanism of detoxification.

Both serum albumin and PON1 have chlorpyrifos-oxonase activity. That albumin has chlorpyrifos oxonase activity in addition to albumin binding was reported by Sultatos et al. (1984). Sultatos et al. (1984) evaluated chlorpyrifos oxonase activity at very high concentrations of oxon (2 mM), and concluded that hydrolysis of the oxon was inefficient. Sogorb et al. (2008) have more extensively evaluated the oxonase activity of human serum albumin and determined that the efficiency of chlorpyrifos oxonase activity is oxon-concentration dependent. The *in vitro* efficiency for protection of AChE increased greatly at oxon concentrations of less than 1 uM, and had about 60% the effectiveness of PON1 at about 0.5 uM chlorpyrifos oxon. For comparison, *in vivo* concentrations of oxon range from low pM in environmentally-exposed humans to nM in high-dose animal studies.

The albumin oxonase data of Sogorb et al. (2008) indicate that even if a person had zero PON1-oxonase activity, at reasonable multiples of environmental exposure, they would still have appreciable levels of albumin-oxonase activity. Albumin-oxonase activity is a plausible explanation for the high degree of tolerance to chlorpyrifos in GD 20 fetal rats (Mattsson et al., 2000) and that which occurred in PON1 knock out mice (PON1 -/-) reported by Dr. Furlong's laboratory (Cole et al., 2005).

Cole et al. (2005) genetically modified mice to express no PON1, or to have normal levels of human PON1 of either $hPON1_{\mathbf{R192}}$ or $hPON1_{\mathbf{Q192}}$. The activity of $hPON1_{\mathbf{R192}}$ is slightly greater against chlorpyrifos-oxon than $hPON1_{\mathbf{Q192}}$. The mice with no PON1 activity (knock-out $PON1^{-/-}$ mice) exposed dermally to high doses of chlorpyrifos had only minor differences in brain ChE inhibition compared to genetically-modified mice that had active expression of normal amounts of human PON1 (Cole et al., 2005, Fig. 4). At a 50 mg/kg dermal dose there was only minimal inhibition of brain ChE in all mice, whether they expressed no PON1 activity ($PON1^{-/-}$) or expressed the more active human $hPON1_{\mathbf{R192}}$ or the less active human $hPON1_{\mathbf{Q192}}$. In Cole et al. (2005) the dermal NOAEL approximated 50 mg/kg regardless of PON1 presence or absence. For comparison, the USEPA short-term dermal NOAEL is 5 mg/kg/day, based on rat dermal exposure data.

The chlorpyrifos dermal dose necessary to inhibit 50% of brain ChE in Cole et al. was about 100 mg/kg. At the brain ChE inhibition ED50, there was less than a 20% difference between those mice with no PON1 activity and those with normal amounts of human PON1 activity.

The data of Cole et al. (2005) are consistent with computer modeling of PON1 position 192 Q/R differences and chlorpyrifos dose-response (Timchalk et al., 2002b). Physiologically-based pharmacokinetic/pharmacodynamic (PBPK/PD) modeled differences in PON1-192 Q/R activity (QQ, QR or RR genetics) showed practically no

effect of PON1 on estimates of brain oxon exposure when oral exposures were below 500 ug/kg/day.

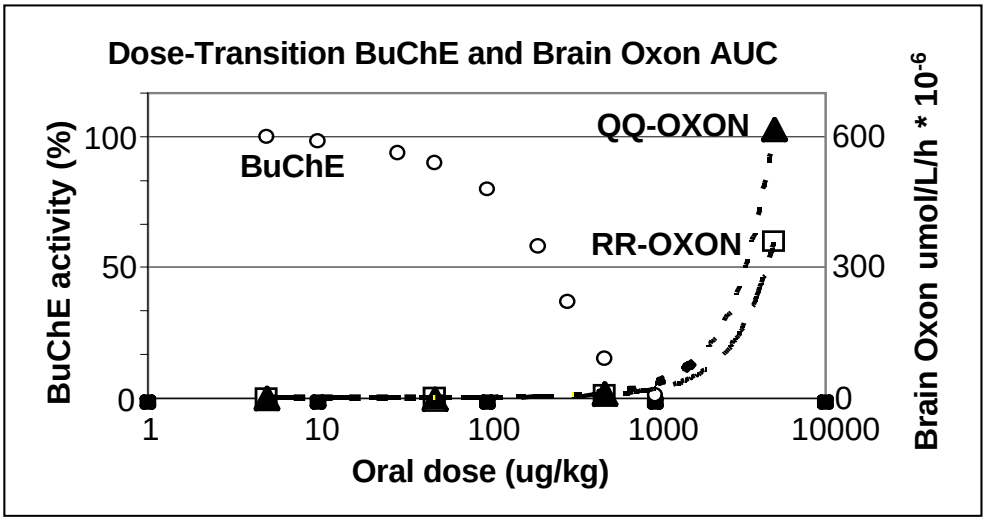
While more precise estimates of the dose-response of albumin-oxonase detoxification of chlorpyrifos are still unavailable, the *in vivo* data of Mattsson et al. (2000) and Cole et al. (2005) indicate that age-related and genetic differences in human PON1 activity and detoxification of chlorpyrifos have little or no practical significance in the real world. PON1 has a modest role in detoxification of chlorpyrifos at very high doses, and no apparent role at environmentally relevant doses. Chlorpyrifos detoxification is layered, and mechanisms independent of PON1 are operational at environmentally relevant doses (Timchalk et al., 2002b; Cole et al., 2005; Sogorb et al., 2008). The importance of factoring dose-related transitions in mechanisms of toxicity into risk assessment has recently been emphasized by two ILSI-sponsored workshops (Slikker et al., 2004), which among other insights reported that:

> *"… consideration of dose-dependent transitions in the mechanism of toxicity is an obligate example of integrating the "best science" into the decision making process."*

The article by Timchalk et al. (2002b) demonstrated through physiologically-based pharmacokinetic (PBPK) modeling that plasma butyrylcholinesterase (BuChE) has as a dose-related mechanism for chlorpyrifos toxicity. Because plasma BuChE is a sink (via stoichiometric binding) for oxon, substantial amounts of plasma BuChE must be inhibited before significant increases in brain oxon exposure occurs. Figure 2 in Timchalk et al (2002b) indicates roughly 60% inhibition of plasma BuChE at 300 ug/kg single oral dose in humans, while nearly all plasma BuChE is inhibited at 500 ug/kg or above. The 500 ug/kg dose appeared to mark the beginning of a dose-related accelerated increase in brain oxon AUC (Table 2).

The Timchalk et al. (2002b) article also demonstrated that PON1, and genetic Q versus R differences in PON1, have little influence at doses below 500 ug/kg on exposure of the brain to oxon. Text Figure 7 combines data on BuChE inhibition (visual BuChE estimate) from Figure 2 and brain oxon area under the curve (AUC) data from Table 2 of Timchalk et al. (2002b). The curves linking the four data points for QQ-oxon AUC or the four data points for the RR-oxon AUC were accomplished by a polynomial fit solely to assist the reader track the AUC data across doses. The polynomial is not intended to describe the dose-response between 500 and 5000 ug/kg doses.

Text Figure 7. Dose relationship between BuChE and brain oxon



The USEPA 2006 Organophosphate Cumulative Risk Assessment applied two principles of toxicology for risk assessment when considering the role of age-related differences in PON1 activity. The USEPA 2006 first considered the dose-dependent role of PON1 as an oxonase (consistent with both Conolly et al. 1999 and Slikker et al., 2004) and then appropriately recognized that the purpose of the risk assessment was the protection of children from environmental levels of OP exposure:

> **USEPA, 2006. b. Intra-species extrapolation (**Section I.B - Page 55 of 522). *"Interpreting the variability in enzyme levels in the context of increased sensitivity to OPs needs to be done cautiously. Timchalk et al. (2002b) used a physiologically-based pharmacokinetic model (PBPK) model for chlorpyrifos to evaluate the impact of variability associated with chlorpyrifos-oxonase polymorphisms on the theoretical concentrations of chlorpyrifos-oxon in the human brain over a range of chlorpyrifos doses. The authors reported that over a range of dose-levels, the response was relatively insensitive to changes in oxonase activity at low doses. However, chlorpyrifos-oxonase status may be an important determinant of sensitivity with increasing dose. The authors further suggest that other esterase detoxification pathways may adequately compensate for lower chlorpyrifos-oxonase activity; hence an increased sensitivity to low chlorpyrifos-oxonase is not observable until other detoxification pathways or esterases have been appreciably depleted or overwhelmed." … "For risk assessment purposes, human responses at low, environmental levels are the most relevant." … "In conclusion, the standard 10x-factor for intra-species extrapolation has been applied to the OP CRA* [cumulative risk assessment]*."*

The dose-related role of PON1 in chlorpyrifos detoxification was confirmed by Cole et al. (2005) in genetically-modified mice. The data in Cole et al. also demonstrate that, even at very high dermal doses of chlorpyrifos in mice (which have very thin skin

compared to human skin), that tolerance to chlorpyrifos exposure was high even in the absence of PON1. The recently described albumin-oxonase activity of human plasma (Sogorb et al., 2008) adds another layer of protection against systemic oxon exposure.

CDC estimates of children's exposure to chlorpyrifos are very low.

For an exposure context, CDC scientists (Barr et al., 2005) estimate the chlorpyrifos 95 %ile exposure of children is 0.06 ug/kg/day. Thus, environmental exposures are several thousands of times less than the dose necessary to begin to discern small differences in PON1 effects on chlorpyrifos detoxification. When PON1 differences in brain oxon did occur at 5000 ug/kg, the brain oxon differences were less than 3X (Timchalk et al., 2002b). Cole et al. (2005) and Timchalk et al. (2002b) stated the PBPK/PD model predicts that lower-level exposures have other esterase detoxification pathways that would compensate for the inter-individual differences in chlorpyrifos-oxonase activity due to the PON1-Q192R polymorphism.

In summation, PON1 oxonase has a modest role in detoxification of chlorpyrifos at very high doses, and no apparent role at environmentally relevant doses. Albumin oxonase activity is also present. Chlorpyrifos detoxification is layered, and mechanisms independent of PON1 are operational at environmentally relevant doses.

Lack of differential sensitivity of the fetus during maternal exposure.

Several studies have evaluated gestational exposure to chlorpyrifos for insight on various outcomes including differential sensitivity, comparative distribution of TCP in the fetus and dam, and dose-response profiles for enzymatic activity (Lassiter et al., 1998; Hunter et al., 1999, and Lassiter et al., 1999). Consistent with the work of Timchalk et al. (2002b) and Cole et al. (2005), a USEPA study by Lassiter et al. (1998) demonstrated that the rat fetus had slightly less inhibition of brain cholinesterase than their dams when their dams were administered chlorpyrifos on gravid-day 18 by single-dose oral gavage at both 7 and 10 mg/kg body weight [These administered dose levels should be compared to 95%-ile exposures to children of 0.06 ug/kg/day]. At these same dose levels, maternal exposure on gravid-days 14 to 18 caused much greater maternal brain cholinesterase inhibition than fetal brain (4.7X less) cholinesterase inhibition. The authors' interpretation was that the fetal brain is able to recover (greater elasticity) more fully (than dams) between sequential exposures to chlorpyrifos. The greater tolerance of fetal than maternal brain cholinesterase to inhibition from maternal gavage exposure was also demonstrated by Mattsson et al., 2000.

A subsequent study to Lassiter et al (1998) which aimed at better understanding the toxicokinetic profile of chlorpyrifos and its metabolites in the fetus and dam reported that only TCP (the low toxicity principal metabolite of chlorpyrifos) was detected in both fetal and maternal liver and brain (administered exposures ranged from 3 to 7 mg/kg; again comparison to anticipated human exposures is critical; Hunter et al., 1999). Neither chlorpyrifos nor chlorpyrifos oxon were detected in either tissue from dams or fetuses. The authors reported that the concentration of TCP in the maternal liver was five-fold

higher than the TCP concentration in fetal liver, but that brain TCP in the fetus was 2-5X that of TCP in the maternal brain. While the authors suggest that the fetus may be differentially exposed to higher levels of chlorpyrifos than the maternal nervous system (following oral exposure at the administered dose levels during late gestation), it is notable that neither chlorpyrifos nor the principal oxon metabolite which is instrumental in ChEI, were detected in either fetal or maternal brain, even following exposures that are thousands of times that of humans. This is not unexpected given the layering of protective mechanisms that exist, a fact that often goes unrecognized and one that is consistent with dose-related transitions in mechanism/protection/detoxification. This layering is noted below:

Tiered layer of human protection

- Portal of entry tissue metabolism →
- Portal of entry tissue A- and B-esterases →
    - Plasma protein binding and albumin oxonase activity →
    - Hepatic metabolism, hepatic A- and B-esterases, albumin oxonase
        - Plasma B-esterases inhibited, then →
        - Plasma A-esterases become involved and →
            - Neural AChE begins to be inhibited

In one additional study in which enzymatic profiles for dams and fetuses were evaluated following GD 14-18 exposure to chlorpyrifos ranging from 3-10 mg/kg/day, the investigators reported significant overt maternal toxicity at 10 mg/kg/day with decreases in ChE activity more notable in the maternal than the fetal brain (Lassiter et al., 1999). There were effects reported for inhibition of carboxylesterase CaE activity in both fetal and maternal liver, but not in either blood or brain CaE, and in no case was fetal activity diminished to a greater extent than maternal activity.

Collectively, these studies do not point to a pattern suggestive of heightened fetal sensitivity to ChEI inhibition or other potentially critical considerations (e.g., oxon in the brain), but rather suggest fetuses are equi- or perhaps more tolerant to the various measurements that these investigators evaluated following maternal chlorpyrifos exposure. What does become apparent from all studies is that protective layering appears to be at work, given that effects were more prominent in first tier-type detoxification tissues (i.e., liver), but not in tissue/organs (i.e., RBC or brain) that are impacted only upon exhaustion/depletion of other (and earlier occurring) protective mechanisms.

Lack of differential sensitivity of cholinesterase of the neonate exposed via nursing of milk from treated dams.

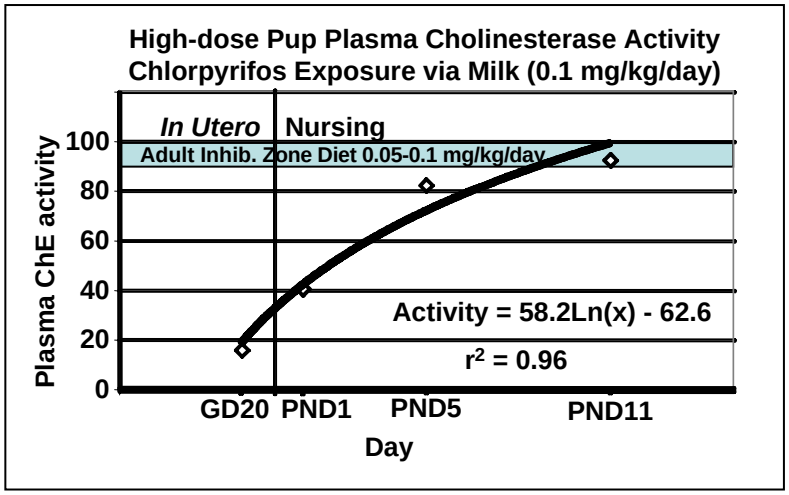
The only data on sensitivity of neonatal rat pups to chlorpyrifos from a natural route of exposure was evaluated in Mattsson et al., 2000. This evaluation of maternal, fetal and neonatal chlorpyrifos kinetics was the 'companion' study to the chlorpyrifos developmental neurotoxicity study (Hoberman, 1998 and supplements 1998, 1999, 2000). Dams were treated from gravid-day 6 to lactation-day 10 by gavage (in oil) at 0, 0.3, 1 and 5 mg/kg/day. Maternal, fetal or pup's cholinesterase activity was evaluated on

gravid-day 20, and postnatal days 1, 5, 11, and 21 (birth = PND 0). Fetal and pup cholinesterase inhibition occurred only at the high maternal dose, and the amount of inhibition was less than in dams.

Chlorpyrifos concentrations in milk were measured, and by integration of blood pharmacokinetic information and published algorithms on milk consumption, an estimate of pup dose from nursing was determined (Mattsson et al. 2000). On Postnatal Days (PND) 1-11, pups of high-dose dams were exposed to approximately 0.1 mg/kg/day of chlorpyrifos via milk. During PND 1-11, brain and plasma cholinesterase activity returned to or very close to control values. RBC cholinesterase recovered more slowly, presumably due to the different mechanism for recovery of RBC cholinesterase activity by replacement of RBC in circulation. At this 0.1 mg/kg/day dose level, differential sensitivity is best addressed by examination of plasma cholinesterase activity as this is the most sensitive to inhibition by chlorpyrifos.

The kinetic principle involved in the following analysis (see Text Figure 8) is that different doses above the threshold for inhibition of cholinesterase will still cause measurable inhibition, but at a different percentage according to dose. The most meaningful adult comparison to pup exposure from milk would be adult exposure to chlorpyrifos by diet. Subchronic and chronic dietary doses of chlorpyrifos to adult rats causes roughly 10% inhibition of plasma cholinesterase at 0.1 mg/kg/day, and no inhibition at 0.05 mg/kg/day (Yano et al., 2000). In Mattsson et al., gravid-day 20 fetal plasma cholinesterase activity was about 15% of control values in the 5 mg/kg/day maternal dosing group. The dose in mg/kg/day to the fetus is unknown, but fetal blood chlorpyrifos concentrations were about half that of their dams 4-hrs post gavage. When born, the estimated dose to the high-dose neonate via nursing was 0.1 mg/kg/day. Plasma cholinesterase activity rapidly rose from 15% activity in the fetus to just above 90% on PND 11 (Text Figure 8). The last day of gavage treatment of dams was postnatal 10. At the lowest dose tested, at maternal gavage 0.3 mg/kg/day, these dams' plasma cholinesterase activity on PND 11 was 84% of control (Mattsson et al, 2000, Figure 2). Thus, PND 11 pup plasma cholinesterase activity increased to 90+ % of control values during exposure to 0.1 mg/kg/day chlorpyrifos via milk, a plasma cholinesterase activity higher than dams administered 0.3 mg/kg/day by gavage and comparable to adult dietary exposures from 0.05 to 0.1 mg/kg/day. The rapid recovery of high-dose pup plasma cholinesterase activity to near control levels during lactation exposure is not consistent with a biologically-meaningful increased sensitivity to chlorpyrifos.

Text Figure 8. Plasma cholinesterase in rat pups following exposure to chlorpyrifos



Lack of differential sensitivity or selective developmental toxicity in the teratogenicity studies for chlorpyrifos

If enhanced sensitivity or selective developmental toxicity did, in fact, occur with chlorpyrifos exposure in the young, then standard teratogenicity studies conducted according to standard design and using prescribed methods would have been expected to yield frank evidence of it. For chlorpyrifos, four such studies in three species have been conducted and in no case have developmental effects or toxicity occurred below doses that are associated with maternal toxicity. Consequently, there is no evidence from standard guideline studies – studies which in some cases employed doses well above those used in the more recently published nonguideline studies of present interest – of advanced sensitivity to chlorpyrifos in the young.

Text Table 8. Guideline developmental studies with Chlorpyrifos

Species	Route	NOAEL (mg/kg bw/day)	LOAEL (mg/kg bw/day)	Reference
Rat	Oral gavage	Dam, 3 Litter, 15	Dam, 15 Litter, none	Ouelette et al., 1983
Rat	Oral gavage	Dam, 2.5 Litter, 15	Dam, 15 Litter, none	Rubin et al., 1987a
Rabbit	Oral gavage	Dam, 81 Litter, 81	Dam, 140 Litter, 140	Rubin et al., 1987b
Mouse	Oral gavage	Dam, 1 Litter, 10	Dam, 10 Litter, 25	Deacon et al., 1979

Lack of meaningful differences in sensitivity of nursing age pups (postnatal days 7 to 21) from repeated oral gavage (in oil) of chlorpyrifos

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> *"Regarding chlorpyrifos, the Agency has not performed a benchmark dose (BMD) analysis but has generated a plot of the data from Zheng et al (2000). Dr. Carey Pope of Oklahoma State University provided the data in Figure I.B-3 to the Agency. The estimated dose to result in 10% brain ChE inhibition (sample obtained @ 4 hrs post-dosing, very close to the time if peak effect cited by EPA) is noted as the dotted line in the graph. At this dose, there is no difference in response between pups and adult rats. Thus, the FQPA factor for chlorpyrifos in the OP CRA for repeated exposures is 1X."*

Of the 33 organophosphates considered in the 2006 USEPA risk assessment, chlorpyrifos was one of only 5 that merited a 1X FQPA factor based upon comparable brain ChE inhibition in pups vs. adult rats to repeated doses. Eleven received FQPA safety factors between 1 and 10, and the others had FQPA factors equal to 10.

The repeated-dose pup and adult gavage data of Zheng et al. (2000) was evaluated by the benchmark dose (BMD) method for a 20% inhibition of brain cholinesterase by Zhao et al (2006). The repeated-dose BMD20 for pups was 1.2 mg/kg/day, and for adults was 1.5 mg/kg/day, indicating a very similar sensitivity at the BMD20. Zhao et al. recommended using inhibition of RBC cholinesterase as the point of departure for risk assessment. There was little difference in either acute or repeated dose BMD20 for RBC ChE between pups and adults.

The need for high quality scientific interpretation of data from gavage studies

Except for the nursing exposure data reported in Mattsson et al. (2000), which is in reality an indirect gavage study, the other data relative to potential pup sensitivity to chlorpyrifos were from oral gavage (in oil) of chlorpyrifos, either to dams or directly to pups. As the Society of Toxicology has made clear (1998 Communiqué, and Conolly et al., 1999), oral gavage is a convenient but unrealistic route of exposure that can cause unrealistic kinetics. The magnitude of the 'gavage distortion' in kinetics has been evaluated.

Marty et al. (2007) reported an approximate thirteen-fold increase in blood chlorpyrifos Cmax in lactating dams administered 5 mg/kg/day chlorpyrifos by oral gavage (in oil), versus the same daily dose via the diet. One would expect a similar distortion in systemic Cmax from oral gavage in pups versus exposure from milk, diet, or contact with the environment. The use of the oral gavage route of exposure in these studies places a special burden on the toxicologist to judge the impact of both dose and route on risk assessment.

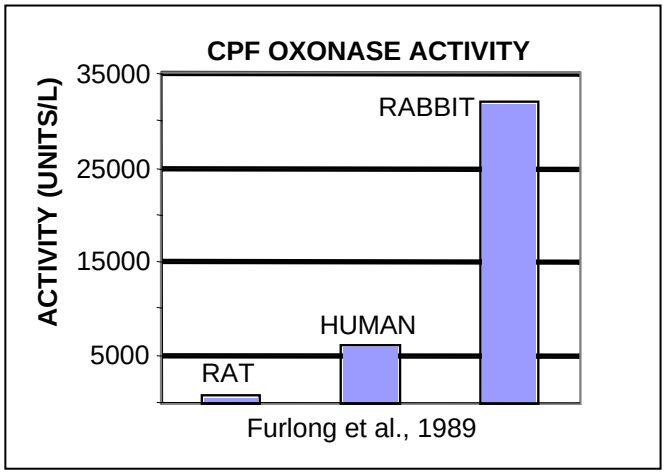
The rat as a model of low PON1 activity

It is also relevant to risk assessment that the test species, the rat, has appreciably lower PON1 chlorpyrifos oxonase activity than humans (Furlong et al., 1989). Thus, the use of rats in the risk assessment process uses an animal model that is deficient in chlorpyrifos oxonase as compared to humans.

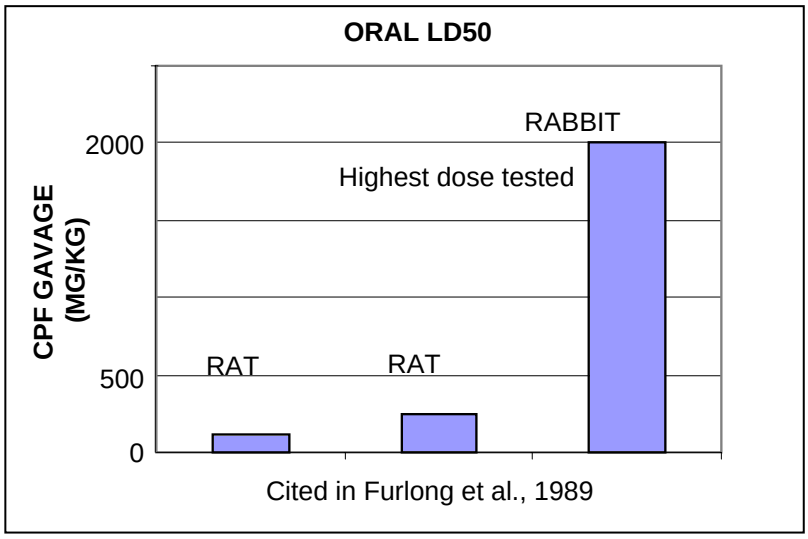
The rabbit teratology data refute a significant role of non-cholinergic mechanisms in developmental toxicity

As shown in Text Figure 9a, rabbits have high levels of chlorpyrifos oxonase. Thus, rabbits tolerate much higher doses of chlorpyrifos than other species (Text Figure 9b). If the parent chlorpyrifos molecule had biologically-relevant toxicity via non-cholinergic mechanisms, then chlorpyrifos doses that cause rabbit toxicity and rabbit developmental toxicity should be similar to other species. In reality, rabbit toxicity and rabbit fetotoxicity occurs at much higher maternal doses than in the rat (Text Figure 9c). Thus, the standard lethality studies and standard developmental toxicity studies on chlorpyrifos provide evidence that non-cholinergic mechanisms are likely to occur only with substantial inhibition of ChE, and that there are no 'non-cholinergic mechanisms' that characterize sensitivity to chlorpyrifos developmental toxicity more accurately than evaluation of cholinesterase inhibition.

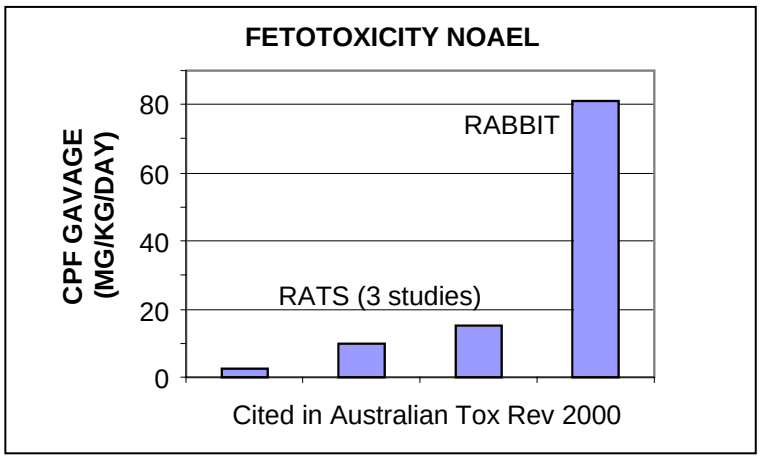
Text Figure 9a. Relative oxonase activity in animals and humans.



Text Figure 9b. Relative tolerance of rabbits to acute lethal doses.



Text Figure 9c. Relative tolerance of rabbits to fetotoxicity.



Evaluations by Regulatory Authorities and Independent Experts on Differential Sensitivity

Numerous regulatory bodies have evaluated chlorpyrifos in the context of potential developmental and/or reproductive toxicity and have sometimes included introspection on differential sensitivity of the young vs. adults. While the dose-response for cholinesterase (ChE) inhibition and corresponding neurotoxicity have long been a focus of regulatory interest and activity regarding chlorpyrifos and other organophosphates, this important endpoint has not been the only focus of regulatory evaluations of chlorpyrifos.

Regulatory agencies assess multiple endpoints for potential health effects for chemicals subject to regulation and evaluation, and scrutiny of DART effects has always been one of the required areas of health effects evaluation. In fact, in recent years, laws such as the 1996 U.S. Food Quality Protection Act have been adopted to ensure that these regulatory programs focus specific attention on the assessment of exposures and mechanisms that may affect children, including DART effects, from regulated pesticides. As a result, chlorpyrifos has been subject to increased scrutiny for evidence of DART by a number of global agencies.

Several expert regulatory bodies, including the EPA, have recently examined the potential DART effects of chlorpyrifos. These thorough evaluations were performed after 1999, using not only the comprehensive developmental and reproductive toxicity studies required by the regulatory agencies, but also some of the published academic literature on potential DART effects that has been reported in recent years.

EPA – Office of Pesticide Programs (2002). The lead agency in the United States for pesticide evaluation and regulation is the EPA's Office of Pesticide Programs. In 2002, the EPA completed its comprehensive evaluation of chlorpyrifos, releasing its

Interim Reregistration Eligibility Decision for Chlorpyrifos (IRED), a document which formed the basis for the Agency's authorization of all currently labeled uses of the product (USEPA/OPPTS 2001). This document was the culmination of more than a decade of data requirements and upgrades, scientific evaluation of all health effects literature and required studies, and the finalization of thousands of pages of "science chapters" leading to EPA's conclusions in the IRED. Though the IRED concludes that neonates are potentially more sensitive to the effects of chlorpyrifos than are adults, this potential sensitivity is related to the ChE inhibition and subsequent cholinergic effects, not due to selective developmental or reproductive effects. The EPA assigned an additional safety factor of 10 to its risk assessment to address the potential increased sensitivity of neonates. These science chapters leading to the IRED conclusions included the EPA's consideration of the recent scientific literature, including some studies published from T. A. Slotkin's laboratory (discussed below).

EPA Human Health Risk Assessment of Chlorpyrifos (2000). The EPA's June 2000 Human Health Risk Assessment of chlorpyrifos is one of several key documents that the Agency developed to contribute to its toxicological conclusions within the IRED (USEPA 2000a). The Human Health Risk Assessment's evaluation of the developmental toxicity data concludes that "*in both mice and rabbits, the developmental effects occurred at maternally toxic doses as indicated by reduced weight gain, and food consumption in both species, and increased mortality in mouse dams*." As to the rat developmental studies, EPA similarly concluded that "*In one rat study, developmental effects (increased post-implantation loss) were noted at 15 mg/kg/day (highest dose tested, HDT), that were also associated with maternal toxicity, while another rat study failed to observe developmental effects at 15 mg/kg/day*."

European Commission – Classification and Labeling (2002). The European Commission recently completed its risk classification process for chlorpyrifos. This process is coordinated by the Commission Working Group on the Classification and Labeling of Dangerous Substances. This Working Group is composed of representatives from several member states with expertise in toxicology and other disciplines involved in the EC Risk Phrases. At its February 2002 meeting, the Working Group considered potential changes to the Risk Phrases for chlorpyrifos. In particular, its debate focused on the possible classification of R64, the Risk Phrase "May cause harm to breastfed babies." The Working Group concluded that this Risk Phrase would be inappropriate for chlorpyrifos. The Working Group also concluded than none of the other potential Risk Phrases involving developmental and reproductive effects (R47, R60, R61, R62, and R63) were appropriate for chlorpyrifos classification.

Australian National Registration Authority (2000). The Australian National Registration Authority ("ANRA") completed its comprehensive evaluation of chlorpyrifos in 2000 (ANRA 2000b). ANRA's over-600-page toxicology evaluation summarized its conclusions regarding animal studies of developmental and reproductive effects by stating that "*exposure to chlorpyrifos had no adverse effects on reproduction. The data on effects of chlorpyrifos in young or developing animals*

have been reviewed and infants and children are not considered to be at an increased risk from chlorpyrifos products that are used according to label instructions" (ANRA 2000b).

California Department of Pesticide Regulation (2001). DPR updated its Summary of Toxicology Data on chlorpyrifos (CalEPA/DPR 2001) and completed a comprehensive draft Risk Characterization Document (RCD) on chlorpyrifos. The conclusions of DPR's evaluation of the toxicology data on chlorpyrifos are summarized in Cochran (2002). DPR's evaluations included all developmental toxicity and reproductive toxicology studies required by DPR and EPA, along with several other documents relating to DART. Furthermore, the studies cited in the OEHHA survey of chlorpyrifos as indicative of DART were assessed by DPR (Cochran 2002), which concluded that "*There is insufficient evidence that human infants are more susceptible to the toxicity of chlorpyrifos than adults and small children and there is no compelling evidence that chlorpyrifos causes any developmental neurotoxicity under physiologically relevant conditions*." In their evaluation of the comprehensive studies required under FIFRA by the EPA and DPR, for the developmental and reproductive toxicity categories, the DPR Summary notes that there is "*no data gap, no adverse effect*". DPR's more detailed conclusions are similar to those of the other agencies discussed previously. For example, in its evaluation of the most recent and comprehensive two-generation dietary reproductive toxicity study, DPR concludes that "*the reproductive findings at 5 mg/kg/day do not warrant a possible adverse effects designation, since brain ChE levels were very markedly depressed at that dose level, and all observed reproductive effects appeared to be due to failure of dams to nurture pups which were otherwise normal*" (CalEPA/DPR 2001).

Independent Reviews: Experts in reproductive and developmental toxicology also reviewed the chlorpyrifos reproductive and developmental literature in 1999, and concluded *"As can be seen, the young in all studies conducted evidenced toxicity at the same or higher dose levels than the adult parent. Chlorpyrifos did not adversely affect reproduction and was not developmentally neurotoxic or teratogenic, and no selective toxicity or sensitivity of the fetus or young animals was apparent in any guideline studies that were scientifically acceptable."* (Schardein and Scialli 1999). CDC similarly reviewed the developmental toxicity data and concluded that chlorpyrifos was not a teratogen (Jackson et al., 1999).

In June 8, 2000, the USEPA (2000a) risk assessment of chlorpyrifos concluded that a 10X FQPA factor was warranted based upon their particular interpretation of the chlorpyrifos DNT study and supported by publications that created uncertainty in their minds about non-cholinergic mechanisms of toxicity. It is notable that WHO (1999) did not feel additional protection was needed for children, even though a senior USEPA toxicologist (Dr. Penny Fenner-Crisp) participated in the WHO evaluation. Nor did the Australian toxicology review of chlorpyrifos recognize a need for additional protection of children. Both the WHO and Australian toxicologists appear attentive to the need to apply principles of toxicology concerning dose, route, maternal toxicity, and relevancy of data for use in risk assessment.

The USEPA position on chlorpyrifos and differential sensitivity for children has shifted since 2000, and the repeated-dose uncertainty factor (i.e., for differential sensitivity) currently embraced by the USEPA is now 1X (from 10X formerly; Organophosphate Cumulative Risk Assessment, USEPA 2002a and 2006). In making this change, the USEPA (2002a) reviewed many studies including several from the laboratory of Slotkin and colleagues, and concluded that there was little evidence of toxicological effects at doses that did not inhibit cholinesterase. USEPA (2002a) also analyzed data from oral gavage of chlorpyrifos to pups and adults and concluded there was no meaningful difference in sensitivity of brain cholinesterase to inhibition from repeated doses of chlorpyrifos. USEPA (2006) reaffirmed these earlier analyses (USEPA, 2002a), and included an analysis of differential sensitivity from differences in PON1 activity, and concluded that PON1 mechanisms of detoxification were dose-related and not a significant factor at environmental levels of exposure. Consequently, USEPA's Cumulative Risk Assessment retained the FQPA factor at 1X for chlorpyrifos.

Conclusion regarding age-related differences

Global thinking on the application of toxicological principles in risk assessment recommends that a weight-of-evidence approach should be used. There are a number of studies and evaluations that have been conducted and analyses undertaken that form a weight-of-evidence perspective that consistent, replicable scientific data demonstrating differential sensitivity to neonates or the young from chlorpyrifos exposure do not exist from relevant doses and routes of exposure. Moreover, this conclusion is apparent even with investigations that have employed dose levels that are thousands of times higher than expected human exposures. Of those toxicology studies most relevant to risk assessment, the weight-of-evidence demonstrates that neonates are not at enhanced risk of harm from chlorpyrifos under realistic exposure scenarios.

Summary

Regulatory decisions regarding chlorpyrifos should remain anchored to the inhibition of cholinesterase. Laboratory animal-derived data along with human evidence continues to support inhibition of cholinesterase as the most sensitive and appropriate endpoint that confers protection to the population based on NOAELs from animal and human studies with the appropriate safety factors applied. While additional animal and human studies have been conducted, some of which report effects presumably below the threshold for cholinesterase inhibition, the weight of scientific evidence, coupled with recognition of the significant differences in exposure between experimental studies and actual environmental exposures, supports the continued reliance on cholinesterase inhibition as a health-protective and conservative point of departure for risk purposes.

In keeping with advancements in risk methodology, if new approaches such as benchmark dose modeling and the generation of data-derived extrapolation factors are to be used in the assessment of chlorpyrifos risk, it is recommended that scientific rigor and

the same standard relative to data quality be applied to insure regulation based on appropriate and relevant scientific data.

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