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Beth Moore, General Permits Coordinator
Oregon Department of Environmental Quality (DEQ)
Water Quality Permitting and Program Development (WQPPD)
811 SW 6th Ave
Portland, OR 97204-1390

Via email in PDF to: 2300A@deq.state.or.us

Dear Ms. Moore,

These are formal written comments of Beyond Toxics, for the record, in response to the Public Notice of the *Proposed 2300A Water Quality Pesticide General Permit Renewal*, issued July 8, 2016. Please include these comments and all attachments in the record. Documents referenced solely by in-text citation are also intended to be included in this public record and are herein included in this testimony by reference as through set forth in their entirety.

Please contact Lisa Arkin, Executive Director of Beyond Toxics at (541)465-8860 or via email to: larkin@beyondtoxics.org if there are any problems opening or manipulating this document.

SUMMARY OF ARGUMENT

Beyond Toxics, a non-profit organization whose mission is to protect environmental health, writes to comment on the Department of Environmental Quality's ("DEQ") proposed 2300A National Pollution Discharge Elimination System ("NPDES") pesticide general permit renewal action. The proposed general permit is not sufficient to carry out DEQ's management obligations under the Clean Water Act ("CWA") to achieve and maintain Oregon's water quality standards and protect Oregon's designated uses.

These comments address the inadequacies of DEQ's proposed permit to implement the required NPDES water quality standards and management measures under the CWA, its inability and disinterest in evaluating the sufficiency of those management measures to ensure pesticides do not violate Oregon's water quality standards and impair its designated uses, its inability to ensure that those who benefit from degrading the nation's water ways cannot shift the costs of their polluting activities to downstream industries and economic interests, its lack of monitoring and emergency preparedness program to support such an evaluation, and its lack of practices that protect those designated uses.

SPECIFIC WRITTEN COMMENTS

Issue 1: The DEQ's Pest Management Measures are too Ambiguous to Restore and Maintain the Chemical, Physical, and Biological Integrity of Oregon's Waters.

Comment 1: The overarching goal of the CWA is to restore and maintain the chemical, physical, and biological integrity of the nation's waters. 33 U.S.C. § 1251. Many pesticides used for aquatic pest management contain chemical compounds listed as "toxic pollutants" under the CWA. *See* 40 CFR 401.15, listing copper compounds as toxic pollutants. The CWA requires that effluent limitations for toxic and non-conventional pollutants "shall require application of the best available technology economically available...which will result in reasonable further progress toward the national goal of eliminating the discharge of all pollutants." 33 U.S.C. § 1311(b)(2)(A)(i). For toxic pollutants, such as copper-based pesticides, the DEQ must ensure that operator activities are in compliance with the CWA's goal of eliminating the discharge of all [toxic] pollutants. Clear pest management and reporting measures are necessary for achieving this goal.

Under the General Permit, all operators are required to manage their discharge so that it does not "cause or contribute to a violation of water quality standards." 2300A Schedule A, No 1.a, at 12. The permit requires operators to evaluate site-specific pest management measures to minimize the discharge of pollutants from pesticide applications. These general pest management measures are not required to be reported to the DEQ, submitted for public notice or comment, nor require operators to develop an effective or economic plan to manage for the national goal of eliminating the discharge of all toxic pollutants. There is no mechanism in place for the DEQ to provide oversight of an operator's pest management practices to ensure effective regulation of pesticide application that results in point source discharge to surface waters from the use of biological

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pesticides or chemical pesticides that leave a residue. Rather, the DEQ receives annual or- depending on the operator's pesticide application category- monthly reports of pesticide application by operators. Without a pesticide management plan on file, the DEQ cannot ensure that operators are minimizing pesticide application. Thus, the DEQ cannot properly ensure that operator activities under this permit will result in a reasonable further progress toward the national goal of eliminating all toxic pollutants.

Issue 2: Effective Regulation of Discharge from Aquatic Pest Treatments under the CWA is Necessary to Ensure that the Economic Impacts of Pollution of Caused by Aquatic Pest Control are Borne by Those Responsible for the Pollution, Rather than by Other Businesses that Depend on Unpolluted Water Bodies, such as Salmon-Dependent Economies.

Comment 2: (This comment is comprised of and recognizes arguments made by the Pacific Fisherman's Council in a Public Comment for the Coastal Zone Act Reauthorization Amendments) In enacting the comprehensive protections mandated by the CWA, Congress recognized that the degradation of the nation's water ways not only threatened the public's health and recreational uses of rivers, streams, lakes, and other water bodies, but also that other national economic interests – particularly those of fishermen and other downstream businesses not responsible for the pollution – would also be greatly benefitted from enhanced regulation. See, e.g., *A Legislative History of the Water Pollution Control Act Amendments of 1972*, 93d Cong., 1st Sess. (Comm. Print 1973) (“Leg. Hist.”) at 162 (statement of lead Senate Sponsor Muskie) (explaining that urgent action was necessary in view of the “grim realities of lakes, rivers, and bays where all forms of life have been smothered by untreated waste, and oceans which no longer provide us with food”).

Accordingly, Congress established a “national goal that wherever attainable, an interim goal of water quality which provides for the protection and propagation of fish [and] shellfish” be “achieved by July 1, 1983.” 33 U.S.C. §1251(2); see also Leg. Hist. at 189 (statement of Sen. Cooper) (recognizing that protecting fish and shellfish resources “will require a high level of water quality” as well as the “need for a permit system to apply these standards precisely to the sources of discharge of pollutants”); *id.* at 215-16 (statement of Sen. Bayh) (highlighting the protection and restoration of fish and other aquatic resources as a central purpose of the Act); *id.* at 386 (statement of Rep. King) (“There is increasing awareness that the abatement of [water] pollution will . . . enhance supplies of known and potential food products.”); *id.* at 409 (“[I]f [businesses] don't have the quality and quantity of water supply they need to operate and produce their product, they are not going to be in business in any case.”).

Indeed, “externality prevention” – i.e., the notion that those responsible for the economic impacts caused by pollution should bear the burden of preventing or addressing it, rather than shifting that burden to other businesses – “is one of the purposes of many of our modern environmental laws,” including the CWA. Lincoln L. Davies, *Skull Valley Crossroads: Reconciling Native Sovereignty and the Federal Trust*, 68 Md. L. Rev. 290, 359 (2009). This rationale for regulation stems from the “logic of cost-externalization,” which “drives human enterprises to pass on

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potential and actual social costs into the commons of society and the environment.” Zygmunt J.B. Plater, *Environmental Law and Three Economies: Navigating a Sprawling Field of Study, Practice and Societal Governance in Which Everything is Connected to Everything Else*, 23 Harv. Envtl. L. Rev. 359, 365 (1999) (“Humans tend to make decisions on relatively short-term horizons, and in insulated self-referential terms When we are involved in a production activity, we resolutely display an inclination to pass wide the costs, while holding close the benefits and profits. Thus there is a universal tendency of individuals and associations toward cost externalization.”); see also Guido Calabresi & A. Douglas Melamed, *Property Rules, Liability Rules, and Inalienability: One View of the Cathedral*, 85 Harv. L. Rev. 1089 (1972).

Accordingly, a central function of the CWA is to ensure that businesses and local governments will “internalize the cost of pollutant disposal, as opposed to allowing them to discharge pollutants and externalize the cost” to other interests that suffer the effects of pollution they had no responsibility for creating. Jonathan Rosenbloom, *New Day at the Pool: State Preemption, Common Pool Resources, and Non-Place Based Municipal Collaboration*, 36 Harv. Envtl. L. Rev. 445, 463 (2012); see also Noah D. Hall, *Political Externalities, Federalism, and a Proposal for an Interstate Environmental Impact Assessment Policy*, 32 Harv. Envtl. L. Rev. 49, 53-54 (2008) (“Most environmental laws address harms that cross property boundaries and impact the property of another Environmental harms that affect persons and property other than the source of the harm are basic examples of an economic externality.”). The NPDES program is the principal mechanism under the CWA for internalizing costs associated with point source water pollution and degradation that adversely affect downstream economic interests. *Id.* at 73.

Salmon are an important national food resource and the biological basis of a major west coast fishing industry supporting many thousands of jobs. Salmon, however, cannot live in highly polluted waters, and thus their very existence – and the industries that depend upon them – are in turn dependent upon strong enforcement of the CWA.

Salmonids¹ are “anadromous” fish species. This means their eggs are laid far inland in cold, fresh-water mountain streams after full-grown spawning adult salmonids return from the ocean, which they entered two to five years earlier as juveniles. Those eggs then hatch a few weeks later – but can only survive if the water is clear and cold enough to support them. Once they hatch, the emerging juveniles first inhabit their gravel beds until they can grow large enough to gradually migrate downriver to the saltwater estuary as “smolts.” There they biologically adapt to the hostile salt water environment, then migrate out to sea to grow to adulthood – and then return to fresh water to start their amazing lifecycle all over again.

Once these fish enter the ocean, they migrate sometimes thousands of miles north and south along the coastline, eating and growing as they go until reaching maturity and returning to their natal streams to spawn. How they find their way back to the same stream sites where they originally hatched is still a mystery, but is an ability that chemical and sediment pollutants in their natal rivers can easily disrupt.

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Copper is one such chemical that disrupts salmonid olfaction, particularly chemical sensory detection- the behavioral response to chemical signals in the aquatic environment. David H. Baldwin, Christopher P. Tatara, Nathaniel L. Scholz, *Copper-induced olfactory toxicity in salmon and steelhead: Extrapolation across species and rearing environments*, *Aquatic Toxicology*, 101(1):295-297 (2011). Copper compounds are common aquatic pesticide ingredient, and are authorized for use under the general permit. DEQ failed to account for the economic impact of operator pollution to downstream industries in the general permit. DEQ must ensure that the use of copper-based pesticides, and all other compounds found to be harmful to salmonids and their food sources are managed at a level that does not negatively impact the survival of salmonid species, nor Oregon's salmon-dependent industries.

Issue 3: Pesticide Management Measures and Monitoring and Reporting Requirements do not Ensure Adequate Protection to stream and stream bank associated Amphibian, Fish, and Bird populations.

Comment 3: In Oregon and Washington, approximately 53% of general wildlife are riparian associated species. Deanna H. Olson, Paul D. Anderson, Christopher A. Frissell, Hartwell H. Welsh Jr, David F. Bradford, *Biodiversity Management approaches for stream-riparian areas*, *Forest Ecology and Management* 246(1):81-107 (2007). Similarly, all 47 Pacific Northwestern amphibian species are stream-riparian associates. *Id.* These amphibian species include stream- and pond- breeding bank dwellers, such as the endangered Oregon Spotted Frog. *Id.* Stream banks are recognized as “sites of frequent disturbance resulting in relatively heterogeneous and complex microhabitat conditions.” *Id.* at 83. Stream bank microhabitat conditions and associated species are easily disturbed by biological and chemical pest treatments. As described below, amphibians, a stream bank associated species, are highly susceptible to the impacts of environmental toxins due to the high permeability and absorption of toxins by their skin.

In Oregon, pesticide treatment of aquatic systems largely occurs during the agricultural growing season, which coincides with breeding and larval development of many amphibian species. Mark A. Jordan, Abel J. Castañeda, Peter C. Smiley Jr, Robert B. Gillespie, Douglas R. Smith, Kevin W. King, *Influence of instream habitat and water chemistry on amphibians in channelized agricultural headwater streams*, *Agriculture, Ecosystems and Environment*, 230:87-97 (2016). Effects of pesticides on the reproduction, immunity, maturation, and survival of amphibians are widely documented in ecotoxicological studies. *Id.* These studies establish a strong, negative correlation between pesticide pollution to surface waters and the diversity and abundance of amphibians. *Id.* In addition, studies show that only focusing on water chemistry for species abundance and diversity is not effective. *Id.* Rather, an index of water quality *and* physical parameters is positively correlated with species richness. *Id.* at 94, *see also* Alienor Jeliakov, François Chiron, Josette Garnier, Aurélien Besnard, Marie Silvestre, Frédéric Jiguet, *Level-dependence of the relationships between amphibian biodiversity and environment in pond systems within an intensive agricultural landscape*, *Hydrobiologia*, 723(1):7–23 (2014). To ensure that pesticide pollution of surface waters does not decrease the water quality and species richness in Oregon surface waters, DEQ must ensure that operators are managing for all aspects of water quality in pest management measures, such as physical parameters.

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The visual assessment component of the general permit does not adequately manage for species health and physical parameters. Rather than operators conducting on-site visual assessments *during* pesticide applications, DEQ must provide operators with a map or list of potentially affected non-target species consistent with hydrologic basins in Oregon. This resource will aid applicators in pest and water quality management efforts because it will give operators information of the non-target species present in correlative surface waters.

Next, Total Maximum Daily Loads (TMDLs) established by the DEQ and approved by the EPA are listed for OAR 340-041-0324 for the Umpqua River Basin; at OAR 340-041-0304 for the South Coast Basin; at OAR 340-041-0274 for the Rogue River Basin; at OAR 340-041-0234 for the North Coast Basin; and at OAR 340-041-0224 for the MidCoast Basin. Operator activities covered under this permit are required to meet these in-stream TMDL limits. However, limits for stream bank pesticide application are managed under FIFRA label instructions, and are not modified for protecting stream bank health in areas where non-target species are present. All non-target endangered, threatened, and conventional species must be considered as contributors to total ecosystem health and survival interdependency. In assessing the presence of non-target stream bank species at risk for adverse incidents from pesticide pollution, DEQ must establish application limits to protect stream and stream bank associated wildlife species from adverse incidents. In developing the wildlife distribution resource suggested above, DEQ must consider the TMDL limits for each basin to develop a guide, list, or map of areas with stream and stream bank associated wildlife to avoid adverse incidents to non-target species.

Issue 4: Pesticide Management Measures and Monitoring and Reporting Requirements do not Ensure Adequate Protection to Human Exposure to Pest-Treated Surface Waters.

Comment 4: The DEQ requires operators to manage water quality for toxic substances for human health criteria, as defined in OAR 340-041-0033. Pesticides used for aquatic pest treatment under the general permit that contain toxic substances must be managed under human health criteria for Oregon State waters. Therefore, operators using aquatic pesticides containing toxic substances must incorporate human health criteria into pest management measures. These pest management measures for human health must consider exposure limits for at-risk populations, such as children and pregnant women that may come into contact with an operator's applied pesticides, downstream. Young children are particularly vulnerable to exposure of toxic substances in the environment. Jianghong Liu and Erin Schelar, *Pesticide Exposure and Child Neurodevelopment Summary and Implications*, Workplace Health and Safety, 60(5):236-243 (2012). Children's behavior during development can place them at greater risk of exposure to pesticides. *Id.* Similarly, children consume more food and drink per body weight index than adults, increasing dietary exposure to pesticides. *Id.* Exposure to pesticides is compounded in children due to their immature livers and excretory systems, which are not as effective at removing pesticide metabolites as adults. *Id.*, see e.g. Phillip J. Landrigan, Carole A. Kimmel, Adolfo Correa, & Brenda Eskenazi, *Children's Health and the Environment: Public Health Issues and Challenges for Risk Assessment*, Environmental Health Perspectives, 112(2):257-265 (2004). Additionally, studies have demonstrated that pesticide exposure can affect children's reproductive, endocrine, respiratory, neurological, and immune systems. *Pesticide Exposure and Child Neurodevelopment*

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Summary and Implications, 60 Workplace Health and Safety, 5, 236-38. The DEQ must ensure that high-risk populations, such as children are not adversely affected by activities covered under this permit by mandating operators to consider human health criteria of the surface waters they are treating in the development of pest management measures.

For example, glyphosate is an aquatic pesticide authorized for activities under the general permit. Recent studies conducted by the USGS in the Mississippi Basin discovered glyphosate residue from pest treatments in ground water that exceeded permissible levels in drinking water. Jason Vogel, Michael S. Majewski, Paul D. Capel, *Pesticides in Rain in Four Agricultural Watersheds in the United States*, Journal of Environmental Quality, 37:1101-1115 (2008). In these studies, the USGS concluded that glyphosate sprayed in agricultural ditches was the cause of the excessive levels in surface waters used for drinking. *Id.* Glyphosate is linked to birth defects, neurological disorders, fertility issues and cancer. Richard H. Coupe, Stephen J. Kalkhoff, Paul D. Capel, and Caroline Gregoire, *Fate and transport of glyphosate and aminomethylphosphonic acid in surface waters of agricultural basin*, Pesticide Management Science, 68(1):16-30 (2012); M Antoniou, MEM Habib, CV Howard, RC Jennings, C Leifert, RO Nodari, CJ Robinson, and J Fagan, *Teratogenic Effects of Glyphosate-Based Herbicides: Divergence of Regulatory Decisions from Scientific Evidence*, Journal of Environmental and Analytical Toxicology, S4:006 (2012).

Human exposure channels outside of fish, shellfish and surface water consumption must also be analyzed and included in operator monitoring and reporting requirements under the general permit. Such exposure channels include, inhalation from aerial pesticide applications for area-wide pest control treatments covered under this permit. DEQ must ensure operators are considering all impacts to human health in the application methods, frequency, volume, and location of pesticides and their corresponding pest management measures authorized under this permit.

Protections for vulnerable populations such as infants, children and pregnant women must be guaranteed by the general permit requirements, as well as the reporting, tracking and monitoring guidelines. The DEQ must ensure that high-risk populations, such as children are not adversely affected by activities covered under this permit by mandating operators to consider human health criteria of the surface waters they are treating in the development of pest management measures

Issue 5: The DEQ's "Within 3-ft-or-Less" Definition of "Water's Edge" is Arbitrary.

Comment 5: The classification of "Water's Edge" as "within 3 feet of surface waters of the state and conveyances at the time of pesticide application" is an arbitrary classification of the distance for pesticide application and the resulting pollution to impact surface waters. The DEQ did not explain how the 3 feet "Water's Edge" definition was set in the General Permit or the NPDES Waste Discharge Permit Evaluation Report. This flat-rate area is used to evaluate an operator's Annual Treatment Area in determining whether the operator qualifies for mandatory registration of pesticide application operations under the permit.

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The “Water’s Edge” definition disregards the discrete habitat conditions of stream-riparian areas. Pest treatment depends on a number of factors, such as stream bank elevation from the surface water, stream bank soil saturation, and vegetation. For example, a pest treatment for nuisance animal control may begin at 6 feet from the water’s edge, and continue along the stream bank toward the stream. Under the DEQ’s “Water’s Edge” definition, the 3 additional feet of pest treatment is not entered into the Annual area or linear threshold calculation for required registration under the permit.

Issue 6: The General Permit Does Not Provide for Increased Application Protections, Monitoring and Reporting Requirements in Spray Locations Where Vulnerable Species are Present.

Comment 6: DEQ must ensure that, in authorizing operator activities under the pesticide discharge general permit, these activities do not jeopardize or threaten the continued existence or survival of the endangered or threatened species in the state of Oregon. Currently, there are 27 species of fish and 6 species of amphibians that are listed as endangered or threatened species by the State or Oregon or U.S. Fish and Wildlife. *See Oregon Department of Wildlife, Threatened, Endangered, and Candidate Fish and Wildlife Species*, Wildlife Division, Retrieved from (http://www.dfw.state.or.us/wildlife/diversity/species/threatened_endangered_candidate_list.asp), July 17, 2016.

Under the General Permit, DEQ does not require operators to include a consideration of the presence of these species in developing pest management measures, nor are there any increased requirements for site monitoring or water quality evaluation after a spray event. We suggest that, in order to be consistent with the goals of the Oregon State Endangered Species Act, ORS 496.012, and the Federal Endangered Species Act, 15 U.S.C § 1531-1544, that DEQ issue protective measures for pesticide application to surface waters in areas that are designated critical habitat for State or Federally listed endangered or threatened species.

Issue 7: Widespread Sediment Pollution is a Major Factor in Poor River Health in Oregon.

Comment 7: Herbicides can persist in water and bind with soil particulates. For example, Washington State’s Herbicide Risk Assessment (2001) chemical summary of 2,4-dichlorophenoxyacetic acid (2,4-D) lists degradation (half-life) and disappearance time that span weeks to months, depending on the aquatic environment. Washington Department of Ecology, *Herbicide Risk Assessment for the Aquatic Plant Management Final Supplemental Environmental Impact Statement; Appendix C, Volume 3: 2,4-D*, Pub. No 00-10-043, 65-69, (2001). The general permit authorizes registered and unregistered operators to apply 2,4-D for aquatic pest-management. DEQ did not offer pesticide application, daily-load, or water quality limitations for 2,4-D and many other persistent chemical compounds found in pesticides that reflect a chemical’s degradation time in varying aquatic environments. This failure ignores the variability of application of pesticides per volume rate, and its corresponding chemical volatility, degradation rate, and time to disappearance. In fact, DEQ only requires operators that use acrolein-, xylene-, and copper-based pesticides for pet management in irrigation systems

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qualifying under the permit to follow monitoring and sampling requirements for these three compounds. Beyond this, DEQ's guidance for the application of highly persistent chemicals does not extend beyond following the Federal Insecticide, Fungicide, and Rodenticide Act label and to using an optimal amount of pesticide.

The DEQ requires operators to manage water quality for toxic substances and human health criteria under OAR 340-041-0033, but does not require operators to conduct testing or monitoring for toxic substance levels in sediments. By contrast, Washington State's General Permit program for pesticide discharge includes sediment testing standards and monitoring protocols for reporting toxicity and persistence in surface waters affected by aquatic pest control. (WAC 173-204). Monitoring sediment for toxicity to ensure compliance with Oregon State water quality standards should not be overlooked, especially when applicators are authorized to use highly persistent chemicals, such as copper-based pesticides.

Issue 8: Notice of Intent and Public Comment on Permit Coverage for Operators

Comment 8: DEQ does not require any operators authorized for coverage under the General Permit to submit a Notice of Intent to DEQ prior to approval for this permit. The failure to require any operator to file a Notice of Intent for coverage under this permit blocks the public from commenting on DEQ's approval /disapproval of any operator's activities under the permit. Although the general permit does require operators to publish notice to potentially affected water users, and, in some instances, members of the public who are reasonably expected to be affected by the spray, the DEQ does not offer the public an opportunity to comment on the operator's pesticide applications.

The DEQ does not require a Pesticide Discharge Management Program (PDMP) to be submitted at the time of permit application by operators with required registration under this permit. Operators required to develop a PDMP, should be required to file the PDMP at the time of applying for the permit. In requiring this, the DEQ ensures the effectiveness of the operator's proposed PDMP to achieve and maintain Oregon's water quality standards and protect Oregon's designated uses.

We also suggest that, in the approval process of operators required to register to obtain coverage, that the DEQ require applicants to submit a public Notice of Intent (NOI) with a 30-day comment period following the NOI. This is necessary so interested members of the public may provide comment to DEQ and be afforded the opportunity to study the PDMP plan. Further, all registration applications are given the same deadline, and approved in a "bunch" without the public being notified of individual applicant's identity or pesticide application plans. We encourage the DEQ to make the public notice and comment mechanisms of the NPDES permit a priority in the application and approval process of operators that are required to register under the permit.

We suggest that, in the approval process of operators requires to register to obtain coverage, that the DEQ require applicants to submit a public Notice of Intent with a 30-day comment period

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following the NOI. Included in the NOI, we ask that applicants who are required to develop a Pesticide Discharge Management Program (PDMP) under the permit, file the PDMP with the NOI so interested members of the public may comment to DEQ. Currently, the PDMP is not required to be submitted with the registration application to the DEQ. Further, all registration applications are given the same deadline, and approved in a “bunch” without the public being notified of individual applicant’s identity or pesticide application plans. We encourage the DEQ to make the public notice and comment mechanisms of the NPDES permit a priority in the application and approval process of operators that are required to register under the permit.

Issue 10: Operators are not Required to Develop Emergency Spill and Response Plan.

Comment 10: The DEQ requires operators to minimize pesticide product discharge through equipment maintenance, proper mixing, and loading activities in the operator’s pest management measures. An emergency spill and response plan is not included in the pest management measures. Operators are required to report pesticide-related spills and response after a spill incident occurs. The absence of this requirement places an enormous burden on operators to respond, without preparedness, to accidental or negligent spills. We strongly suggest that the DEQ requires operators to include an emergency spill and response plan as a part of the pesticide management measures, with suggested modifications of mandatory reporting as stated above.

CONCLUSION

The further CWA regulation of pesticide discharges to surface water, either marine or freshwater, is entirely consistent with the overarching CWA objective of ensuring the restoration and maintenance of the chemical, physical, and biological integrity of the nation’s water. In addition, further CWA regulation is consistent with the objective of the CWA to ensure that those who benefit from degrading the nation’s waterways cannot shift the costs of their polluting activities to downstream industries and economic interests. The Proposed 2300A General Permit is clearly inadequate to control pesticide discharge from aquatic pest treatments in the state of Oregon. We urge the DEQ to increase the restrictive and protective measures of pesticide applications for aquatic pest control in the general permit to adequately meet these objectives of the CWA.

In short, minimizing the harmful effects of aquatic pest control on Oregon’s surface waters makes excellent environmental and economic sense. Society as a whole benefits through the reduction of externalized environmental damage costs to valuable economic resources and industries, such and salmon-dependent economies, that often occurs far downstream. Additionally, operators covered under this permit otherwise generating pesticide pollution may also benefit by lowering its own expenditures on maintenance of pesticide application hardware, pollution monitoring, and associated chemical and biological control costs for aquatic pest treatment. As in most cases, it is far cheaper to prevent pesticide pollution in the first place than to try to clean it up later.

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Thank you for the opportunity to comment on the *Proposed 2300A Water Quality Pesticide General Permit Renewal*.

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CONSOLIDATED TABLE OF REFERENCES AND LEGAL AUTHORITIES

FEDERAL STATUTES

15 U.S.C § 1531-1544

33 U.S.C. § 1251

33 U.S.C. § 1311

CODE OF FEDERAL REGULATIONS

40 CFR 401.15

LEGISLATIVE HISTORY

A Legislative History of the Water Pollution Control Act Amendments of 1972, 93d Cong., 1st Sess. (1973)

OREGON STATE STATUTES

ORS 496.012

OREGON ADMINISTRATIVE RULE

OAR 340-041-0033

OAR 340-041-0224

OAR 340-041-0234

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OAR 340-041-0304

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WASHINGTON ADMINISTRATIVE RULE

WAC 173-204

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Jianghong Liu and Erin Schelar, *Pesticide Exposure and Child Neurodevelopment Summary and Implications*, Workplace Health and Safety, 60(5):236-243 (2012)

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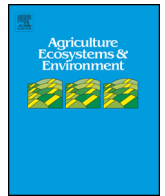
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ATTACHMENTS
In PDF Format

Mark A. Jordan, Abel J. Castañeda, Peter C. Smiley Jr, Robert B. Gillespie, Douglas R. Smith, Kevin W. King, *Influence of instream habitat and water chemistry on amphibians in channelized agricultural headwater streams*, Agriculture, Ecosystems and Environment, 230:87-97 (2016)

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Influence of instream habitat and water chemistry on amphibians in channelized agricultural headwater streams



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ABSTRACT

The widespread use of stream channelization and subsurface tile drainage for removing water from agricultural fields has led to the development of numerous channelized agricultural headwater streams within agricultural watersheds of the Midwestern United States, Canada, and Europe. Channelized agricultural headwater streams have been documented to serve as habitat for amphibians, but information on amphibian habitat relationships within these streams is lacking and needed for developing effective conservation strategies. We quantified instream habitat, water chemistry, and sampled amphibians from seven sites in three channelized streams in Cedar Creek, Indiana in 2008 and 2009 and five sites in five channelized streams in Upper Big Walnut Creek, Ohio in 2009. We conducted an indirect gradient analysis involving the use of principal component analysis and generalized linear mixed effect model analysis to determine which variables had the greatest influence on amphibian community and population structure. Overall, amphibian community and population structure was most strongly correlated with water chemistry rather than instream habitat within channelized agricultural headwater streams in Indiana and Ohio. Eleven of 12 amphibian response variables were most strongly correlated with either a water chemistry gradient of nitrate + nitrite and acetochlor, a gradient of total nitrogen and ammonia, or a gradient of simazine and total phosphorus. Only one amphibian response variable was most strongly correlated with an instream habitat gradient of dissolved oxygen and water depth. Our results suggest that conservation strategies that target reductions of nutrients and herbicides will provide the greatest benefits for amphibians within channelized agricultural headwater streams.

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1. Introduction

Throughout regions dominated by agricultural land use, headwater streams are often channelized or created to maximize the removal of excess water from crop fields with poorly drained soils. The watersheds of these streams are often further modified through the installation of subsurface tile drains. It is estimated that 25% of land within the Midwestern United States would not be available for cultivation without stream channelization and subsurface tile drainage (Skaggs et al., 1994) and it is estimated that these practices impact 80% of the landscape of the region

(Blann et al., 2009). Stream channelization alters instream habitat through increased hydrological dynamics, sediment accumulation, and the simplification of habitat structure (Blann et al., 2009; King et al., 2014). Additionally, water quality within channelized streams is influenced by the application of nutrients and pesticides within the adjacent agricultural fields to promote crop productivity. Surface and subsurface runoff leads to the input of these chemicals within channelized streams at rapid rates, especially following storm events after nutrient and pesticide application (Kladivko et al., 2001; Gaynor et al., 2002; Brown and Van Beinum, 2009; Smiley et al., 2014; Smith et al., 2015; Williams et al., 2015). Channelized streams are also subjected to periodic removal of riparian vegetation and dredging of accumulated sediment to ensure hydraulic capacity is maintained. The management of channelized headwater streams is primarily oriented toward maximizing drainage for agricultural productivity without regard to the potential of these streams to serve as habitat for plants and

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animals (Herzon and Helenius, 2008; Smiley and Gillespie, 2010). Thus, an enhanced understanding of the relationships of the riparian and aquatic biota with the physical and chemical habitat conditions within channelized headwater streams is needed to develop conservation strategies capable of mitigating the effects of agriculture.

Fish and aquatic macroinvertebrate communities have been the subject of most of the ecological research in channelized agricultural headwater streams (Sullivan et al., 2004; Lau et al., 2006; Morris et al., 2006; Smiley and Gillespie, 2010; Leslie et al., 2012; D'Ambrosio et al., 2014). Amphibians are another group that use these degraded streams as habitat but they have received little study (Twisk et al., 2000; Piha et al., 2007b; Maes et al., 2008; Hartel et al., 2011; Smiley et al., 2011). The lack of amphibian research within channelized agricultural headwater streams is likely due in part to a focus on the wetlands and ponds that tend to be preferred habitats for amphibian species found within agricultural landscapes (Kolozsvary and Swihart, 1999; Knutson et al., 2004). Additionally, the presence of predatory fish within channelized agricultural headwater streams may lead to amphibians having a greater preference for isolated wetlands and ponds than channelized agricultural headwater streams. Channelized agricultural headwater streams can serve as dispersal corridors (Reh and Seitz, 1990; Mazerolle, 2004) and may provide much needed refugia for amphibians in agricultural landscapes (Maisonnette and Rioux, 2001; Chester and Robson, 2013) where preferred habitats, in the form of standing surface water, are actively removed and fragmented by drainage practices. The Midwestern United States is characterized by intensified agricultural land use and wetland loss, which makes it highly probable that channelized agricultural headwater streams are serving as amphibian refugia. However, basic information on the occurrence of amphibian species and their habitat relationships within these small degraded streams in this region has not been explored.

Water chemistry may play a prominent role in determining amphibian community and population structure within channelized agricultural headwater streams. The application of agricultural chemicals during the growing season coincides with breeding and larval development in temperate latitudes. Amphibians may be particularly susceptible to the uptake of environmental toxicants due to the high permeability of their skin (Quaranta et al., 2009; Brühl et al., 2011) and the effects of agricultural runoff on the survival, maturation, reproduction, and immunity of amphibians has been widely documented in ecotoxicological studies (Hayes et al., 2003; Relyea, 2005; Rohr et al., 2006; Mann et al., 2009; Smalling et al., 2015). At the landscape level, population declines have been associated with proximity to agricultural land use (Bishop et al., 1999; Houlihan and Findlay, 2003; Davidson, 2004; Piha et al., 2007a). Nutrient and pesticide levels in wetlands have been shown to be negatively correlated with the diversity and abundance of amphibians (Bishop et al., 1999; Houlihan and Findlay, 2003), but whether these relationships also occur in channelized and unchannelized streams in agricultural watersheds in the Midwestern United States remains obscure. Physical habitat variables (*i.e.*, those variables that describe the physico-chemical conditions, the hydrological characteristics, and the substrate and cover types) within streams are also likely to have an important influence on amphibian community and population structure (Twisk et al., 2000; Ficetola et al., 2011). To our knowledge no studies have evaluated the relative influence of water chemistry and instream habitat variables across streams in a homogenous agricultural landscape. Understanding the relative influence of these two habitat factors is important for conservation of amphibians in agricultural watersheds because most conservation efforts in the Midwestern United States only focus on reducing nutrient, pesticide, and sediment

inputs into agricultural watersheds. If amphibians are more strongly correlated with water chemistry then these conservation efforts are likely to benefit amphibians. Conversely, if amphibians are more strongly correlated with instream habitat then conservation efforts focused solely on water chemistry improvements may be ineffective for them.

The objective of this paper is to examine the community and population structure of amphibians, and their relationship to instream habitat and water chemistry in channelized agricultural headwater streams in northeastern Indiana and central Ohio. Following previous field and laboratory research in wetland amphibians, we expected that water chemistry would influence amphibian community and population structure through elevated nutrient and pesticide concentrations. However, water chemistry has been suggested to play a minor role relative to habitat in structuring fish and aquatic macroinvertebrate communities that occupy headwater streams in agricultural regions (Dyer et al., 1997; Fitzpatrick et al., 2001; D'Ambrosio et al., 2009; Leslie et al., 2012). Similar results with fish and aquatic macroinvertebrate communities have also been documented within the watersheds of our study sites (Smiley et al., 2008, 2009; McKinney, 2012; Sanders, 2012), which will enable us to make direct comparisons regarding the observed habitat relationships of amphibians with those of fishes and aquatic macroinvertebrates. Our analysis will provide novel information on the structure of amphibian communities and populations within channelized agricultural headwater streams and will assist with development of conservation strategies for aquatic biota in these streams (Herzon and Helenius, 2008; Smiley and Gillespie, 2010).

2. Materials and methods

2.1. Study area and sampling sites

Cedar Creek (CC) is the largest tributary of the St. Joseph River located in northeast Indiana (latitudes 41°53'78"–41°19'23", longitudes 85°31'88"–84°91'50") and is part of the Maumee River Basin that drains into Lake Erie. The lower 22 km of CC is part of the Indiana State Natural, Scenic, and Recreational River system and this designation provides this section of CC with protection from construction, dam, and drainage projects. This has led to the upper reaches of CC being designated as an Environmental Quality Incentives Program priority area (St. Joseph River Watershed Initiative, 2006). Dominant land use in CC is cropland consisting of soybean and corn and the majority of streams within the CC watershed have been channelized for drainage of agricultural fields. Additionally, increased loadings of nutrients and pesticides from agricultural fields and bacteria from failed septic tanks are nonpoint source pollutants of concern within the watershed (St. Joseph River Watershed Initiative, 2006). Upper Big Walnut Creek (UBWC) is located in central Ohio (latitudes 40°60"–40°32'30", longitudes 82°56'00"–82°42'00") and is part of the Scioto River watershed, which is one of the most biologically diverse watersheds in Ohio that drains into the Ohio River basin (Sanders, 2001). Dominant land use in the UBWC watershed is cropland consisting of soybean and corn. Headwater streams in the watershed are impaired by nutrient enrichment, pathogens, and habitat degradation stemming from current agricultural management practices (Ohio EPA, 2005).

Instream habitat measurements, water samples for nutrients and herbicide measurements, and amphibians were collected from seven sites in three channelized headwater streams within CC in 2008 and 2009 and five sites in five channelized headwater streams in UBWC in 2009. The watershed size of channelized streams in CC ranged from 13 to 43 km² while those in UBWC ranged from 1 to 10 km². Each site was 125 m long and near

automated water samplers or locations where grab sample were collected for the measurement of nutrients and herbicides. These sites possessed riparian habitats consisting mostly of herbaceous riparian vegetation and exhibited the straightened, over-enlarged, trapezoidal channel shape typical of channelized agricultural headwater streams in the Midwestern United States (Smiley et al., 2011).

2.2. Instream habitat measurements

Hydrologic (water depth, velocity, wet width), substrate/cover type, and physicochemical measurements (dissolved oxygen, temperature, pH, and conductivity) were recorded from each site in May and July 2008 and 2009 in CC and May and July 2009 in UBWC. Four measurements of water depth and velocity and one measurement of wet width were obtained from six transects located 25 m apart in each site. One additional transect was established for the calculation of instantaneous discharge and 10 equidistant measurements of water depth and velocity were made along this transect. Water depths were measured with a meter stick or wading rod, water velocity was measured with an electromagnetic velocity meter, and wet widths were measured with a tape measure. The dominant substrate type and cover type was identified at four points along each of the six transects within each site used for the measurement of hydrologic variables. Substrate and cover type categories included clay (particle size <0.004 mm), silt (particle size 0.004–0.06 mm), sand (particle size 0.06–2.0 mm), gravel (particle size 2.0 mm to 64 mm), cobble (particle size 64–256 mm), and boulder (particle size >256 mm), terrestrial vegetation (herbaceous and woody terrestrial plants found within the wetted portion of the stream), aquatic plant, small instream wood, large instream wood, leaf litter, and algae.

We calculated the mean and standard deviation of water depth, water velocity and wet width, and instantaneous discharge from each site during each month of each year. We also calculated five variables (dominant substrate size, substrate type richness, cover type richness, substrate + cover type richness, percent cover types) that described the composition and diversity of substrate and cover types present within each site during each month. Dominant substrate size was calculated by first determining the most frequently occurring substrate type within the site and then assigning a substrate size value. The substrate size values assigned to each substrate type are: (1) clay – 0.002 mm; (2) silt – 0.032 mm; (3) sand – 1.03 mm; (4) gravel – 33.0 mm; (5) cobble – 160 mm; and (6) boulder – 260 mm. In the case of ties the average substrate size value of the two most commonly occurring substrate types was used. Substrate richness is the number of substrate types found in a site and cover type richness is the number of cover types found. Substrate + cover type richness is the number of all substrate and cover types present in a site. Percent cover types is the number of points having at least one cover type divided by the total number of points (24) in a site. *In situ* measurements of dissolved oxygen, temperature, pH, and conductivity were collected from a consistent location (typically a pool or run) within each site during daylight hours (8:00 am EST to 6:00 pm EST) with a multiparameter meter concurrently with hydrologic and substrate/cover types measurements.

2.3. Nutrient and herbicide measurements

Water samples, collected on a weekly basis in CC with automated water samplers for the measurement of nutrients and herbicides, were analyzed by the USDA-ARS National Soil Erosion Research Laboratory. Weekly water samples from the UBWC for the measurement of nutrient and herbicides were analyzed by the USDA-ARS Soil Drainage Research Unit.

Specifically, concentrations of six nutrients (ammonia, nitrate + nitrite, total nitrogen, dissolved reactive phosphorus, total phosphorus, dissolved organic carbon) and four herbicides (acetochlor, atrazine, metolachlor, simazine) were measured from each water sample.

Concentrations of nitrate + nitrite, ammonia, and dissolved reactive phosphorus were determined colorimetrically. Ammonia and nitrate + nitrite were determined by application of the copperized-cadmium or hydrazine-sulfate reduction method and dissolved reactive phosphorus was determined by the ascorbic acid reduction method (Parsons et al., 1984). Total phosphorus analyses were performed on unfiltered samples following alkaline persulfate oxidation (Koroleff, 1983) with subsequent determination of nitrate + nitrite and dissolved reactive phosphorus. Concentrations of total nitrogen were analyzed with alkaline persulfate oxidation (Koroleff, 1983), while concentrations of total nitrogen from CC samples were calculated by as the sum of total Kjeldahl nitrogen and nitrate + nitrite concentrations. Total Kjeldahl nitrogen from CC water samples was measured using U.S. EPA method 351.2 (U.S. EPA, 1983). Dissolved organic carbon was determined by heated-persulfate oxidation using a total organic carbon analyzer with in-line sample acidification and sparging (Menzel and Vaccaro, 1964).

Concentrations of acetochlor, atrazine, metolachlor, and simazine were determined using gas chromatography following standard protocols for pesticide analyses (U.S. EPA, 1995). We selected these herbicides because they are more frequently detected and often occur at greater concentrations than insecticides within agricultural watersheds in the United States (Gilliom, 2007). We calculated the median concentration value of each nutrient and herbicide from water samples collected during a three week period that encompassed the week before amphibian sampling, the week of amphibian sampling, and the week after amphibian sampling. We used a three week time period for our calculation of median concentrations to attain a more representative measure of amphibian exposure to nutrients and herbicides than what would be indicated by a single water sample collected on the day of amphibian sampling.

2.4. Amphibian sampling

Amphibians were collected with unbaited gee minnow traps (43 cm length × 23 cm width, two 2.54 cm openings on each end, 0.32 cm mesh size) in May and July of 2008 and 2009 within CC and in June and August 2009 within UBWC. Fourteen minnow traps were set in each site and were spaced 9 m apart with traps alternating in position from the right bank to the left bank throughout the site. Traps were set for two nights in each month and resulted in 28 trap nights for each site for each month and 56 trap nights in each site per year. At the end of each 48 h period the traps were examined and all captured animals were identified, enumerated, and released. Our sampling design resulted in an equal sampling effort among all sites in each watershed and among all years.

Sixteen amphibian response variables were calculated that described amphibian community and population structure within each site during each month of each year. Community response variables included abundance (number of all amphibians captured), tadpole abundance (number of tadpoles captured), post-metamorph abundance (number of post-metamorph frogs captured), species richness (number of species captured), tadpole species richness (number of species calculated from only tadpole captures), post-metamorph species richness (number of species calculated from only post-metamorph captures), Shannon diversity index (Shannon diversity index calculated based on all amphibians captured; Magurran, 1988), tadpole Shannon diversity

Table 1
Mean (standard deviation) of selected instream habitat variables within channelized agricultural headwater streams from May and July 2008 and 2009 within Cedar Creek, Indiana (CC) and May and July 2009 within the Upper Big Walnut Creek, Ohio (UBWC).

	CC		UBWC	
	May	July	May	July
Water depth (m)	0.25 (0.09)	0.19 (0.08)	0.21 (0.09)	0.14 (0.06)
Water velocity (m/sec)	0.16 (0.16)	0.06 (0.07)	0.04 (0.03)	0.00 (0.01)
Wet width (m)	2.69 (0.86)	2.44 (0.95)	2.63 (0.77)	2.27 (0.78)
Discharge (m ³)	0.12 (0.20)	0.02 (0.04)	0.02 (0.01)	0.00 (0.00)
Dominant substrate	sand	sand	clay	gravel
Substrate + cover type richness	5.36 (1.45)	5.36 (1.15)	7.00 (0.71)	6.40 (0.89)
Percent cover types	61.67 (26.14)	67.86 (28.23)	46.67 (24.01)	87.39 (5.90)
Water temperature (°C)	13.47 (2.70)	19.33 (3.52)	14.19 (1.37)	20.68 (1.86)
Dissolved oxygen (mg/L)	9.60 (3.89)	7.33 (2.66)	10.41 (1.47)	5.38 (2.54)
Conductivity (microsiemens/cm)	579.35 (72.70)	672.62 (52.52)	457.60 (212.56)	758.40 (234.85)
pH	8.31 (0.81)	8.55 (0.83)	7.89 (0.15)	7.74 (0.47)

index (Shannon diversity index calculated based only on tadpoles captured), post-metamorph Shannon diversity index (Shannon diversity index calculated based only on post-metamorphs captured). We also calculated seven population response variables describing the abundance of green frog (*Lithobates clamitans*) tadpoles, bullfrog (*Lithobates catesbeianus*) tadpoles, wood frog (*Lithobates sylvaticus*) tadpoles, green frog post-metamorphs, bullfrog post-metamorphs, northern leopard frog (*Lithobates pipiens*) adults, and wood frog adults. We originally planned on using all 16 amphibian community and population response variables in our generalized linear mixed effect model analyses. However, we eliminated four response variables (bullfrog post-metamorph abundance, wood frog tadpole abundance, wood frog abundance, and northern leopard frog abundance) because these species-life stages had too few occurrences within our dataset (*i.e.*, occurred within <5 sites). Additionally, all three Shannon diversity index response variables were rounded to the nearest whole number to meet the requirements of the generalized linear mixed effect model analyses.

2.5. Statistical analyses

We conducted an indirect gradient analysis involving the combined use of ordination and generalized linear mixed effect model analysis to examine the relationships of instream habitat and water chemistry with the 12 amphibian community and population response variables within channelized agricultural headwater streams. We first conducted two separate principal component analysis (PCA) using PC-ORD (McCune and Mefford, 2011) on the 16 instream habitat variables to obtain the site scores from PCA axis of instream habitat and on 10 water chemistry variables to obtain the site scores from PCA axes of water chemistry. Each PCA axis represents the underlying gradient in combinations of instream habitat and water chemistry variables that occurs among our study sites. We retained the first three PCA axes for use as independent variables within our regression analyses. Our use of PCA also enabled us to objectively reduce the number of independent variables in our generalized linear mixed effect model.

We then performed generalized linear mixed effect model analysis to obtain the standardized coefficients and determine the relationships that occurred between the amphibian community and population response variables with instream habitat and water chemistry. Generalized linear mixed effect models are an extension of the linear modeling process that allows regression models with both fixed and random factors to be fit to data that do not exhibit normal probability distributions (*i.e.*, Poisson, binomial, multinomial) (Zuur et al., 2009). Our initial inspection of our community and population response variables noted the frequent

occurrence of zero values and that the distributions appeared follow the negative binomial distribution. Subsequent Akaike's Information Criteria (AIC) analyses confirmed that generalized linear mixed effect model analyses with either the Poisson or negative binomial distribution would provide greater predictive accuracy than the use of a linear mixed effect model analysis intended for use with normally distributed data. Specifically, we used the site scores from the PCA axes of instream habitat and water chemistry as independent variables and the amphibian response variables as dependent variables in our generalized linear mixed effect model analyses. In all analyses we used the random factors of watersheds and sites nested within watersheds to address the issue of pseudoreplication that results from repeatedly sampling the same sites through time (Zuur et al., 2009). We also used AIC scores to select whether to use the Poisson or the negative binomial distribution within our analysis of each amphibian response variable. Generalized linear mixed effect model analyses with either the Poisson (species richness, tadpole species richness, post-metamorph species richness, abundance, Shannon diversity index, tadpole Shannon diversity index, post-metamorph Shannon diversity index, bullfrog tadpole abundance) or negative binomial distribution (tadpole abundance, post-metamorph abundance, green frog tadpole abundance, green frog post-metamorph abundance) and watersheds and sites as nested random factors were conducted with R (R Core Team, 2014) and the lme4 package [glmer function (Bates et al., 2015)].

3. Results

3.1. Instream habitat summaries and ordination results

Sites within CC exhibited greater water velocity and discharge than sites within UBWC (Table 1). Most sites in CC were dominated by sand substrate, while UBWC sites were dominated by either clay or gravel substrate (Table 1). Mean physicochemical conditions were typically within acceptable levels for aquatic life (Table 1). Sites in CC and UBWC exhibited increases in water temperatures, decreases in dissolved oxygen, and increases in conductivity that would be expected to occur with decreases in water depth, water velocity, wet width, and discharge between the May and July sampling periods (Table 1).

The observed mean and range of nutrient and herbicide concentrations indicated that our sites exhibited wide variations in concentrations expected within agricultural streams (Table 2, Smiley et al., 2014). Mean and maximum total nitrogen and total phosphorus concentrations exceeded levels found to be capable impacting stream fish, macroinvertebrate, and algal community structure (Table 2, Justus et al., 2010). All minimum total phosphorus concentrations and the minimum total nitrogen

Table 2

Mean (range) of water chemistry variables within channelized agricultural headwater streams from May and July 2008 and 2009 within Cedar Creek, Indiana (CC) and May and July 2009 within Upper Big Walnut Creek, Ohio (UBWC).

	CC		UBWC	
	May	July	May	July
Dissolved organic carbon (mg/L)	13.00 (5.78–26.75)	13.62 (4.41–39.49)	13.62 (4.41–39.49)	7.15 (5.13–11.58)
Ammonia (mg/L)	0.21 (0.00–0.67)	0.43 (0.00–7.05)	0.43 (0.00–7.05)	0.05 (0.00–0.25)
Nitrate + nitrite (mg/L)	2.78 (0.09–7.72)	0.50 (0.00–3.07)	0.50 (0.00–3.07)	0.94 (0.01–3.45)
Total nitrogen (mg/L)	5.53 (1.85–13.79)	3.36 (0.07–53.42)	3.36 (0.07–53.42)	1.65 (0.51–4.59)
Dissolved reactive phosphorus (mg/L)	0.02 (0.00–0.29)	0.03 (0.00–0.23)	0.03 (0.00–0.23)	0.07 (0.01–0.25)
Total phosphorus (mg/L)	0.46 (0.02–3.22)	1.14 (0.03–20.28)	1.14 (0.03–20.28)	0.12 (0.02–0.32)
Acetochlor ($\mu\text{g/L}$)	0.60 (0.00–18.35)	0.04 (0.00–0.50)	0.04 (0.00–0.50)	0.00 (0.00–0.00)
Atrazine ($\mu\text{g/L}$)	2.28 (0.00–30.59)	0.12 (0.00–1.53)	0.12 (0.00–1.53)	0.20 (0.00–0.34)
Metolachlor ($\mu\text{g/L}$)	0.99 (0.00–10.90)	0.04 (0.00–0.33)	0.04 (0.00–0.33)	0.14 (0.07–0.65)
Simazine ($\mu\text{g/L}$)	1.20 (0.00–16.71)	0.00 (0.00–0.00)	0.00 (0.00–0.00)	0.10 (0.00–0.23)

concentrations in May within CC and in July within UBWC also exceeded levels found to be capable impacting stream fish, aquatic macroinvertebrate, and algal communities (Table 2, Justus et al., 2010). Additionally, mean and maximum nitrate concentrations did not exceed the minimum level documented to cause amphibian mortality (Table 2, Mann et al., 2009). Mean and maximum concentrations of acetochlor, atrazine, and metolachlor were less than chronic and acute toxicity levels for fish and aquatic macroinvertebrates (Table 2, U.S. EPA, 2015). Mean and maximum concentrations of simazine were less than acute toxicity levels for fish and aquatic macroinvertebrates (Table 2, U.S. EPA, 2015).

Table 3

Loadings for instream habitat variables and percent variance explained for the first three principal component analysis (PCA) axes of instream habitat and for nutrient and herbicide variables on the first three PCA axes of water chemistry from channelized agricultural headwater streams in Cedar Creek, Indiana, and Upper Big Walnut Creek, Ohio, 2008–2009. Underlined loadings are those that were statistically significant (i.e., >0.3 or <-0.3) (Hair et al., 1997) and bolded loadings are those that best characterized the underlying habitat gradients.

	Axis 1	Axis 2	Axis 3
Instream Habitat			
Mean depth	-0.368	-0.055	-0.441
SD depth	<u>-0.156</u>	-0.246	<u>-0.329</u>
Mean velocity	-0.387	0.278	0.118
SD velocity	<u>-0.349</u>	0.263	0.205
Mean wet width	<u>-0.343</u>	-0.062	<u>-0.314</u>
SD wet width	<u>-0.065</u>	-0.196	<u>-0.043</u>
Discharge	-0.388	0.187	-0.077
Dominant substrate score	-0.036	-0.216	0.260
Substrate richness	-0.163	-0.370	0.097
Cover type richness	-0.178	-0.295	0.312
Substrate + cover type richness	-0.223	-0.433	<u>0.267</u>
Percent cover types	-0.178	-0.295	<u>0.312</u>
water temperature	0.213	-0.043	<u>0.267</u>
Dissolved oxygen	-0.230	0.086	0.377
Conductivity	0.199	-0.013	-0.083
pH	0.119	0.402	-0.008
% variance explained by axis	25.7	19.2	10.9
Water Chemistry			
Dissolved organic carbon	0.293	-0.047	<u>-0.356</u>
Ammonia	0.436	<u>-0.392</u>	0.045
Nitrate + nitrite	0.122	<u>0.404</u>	0.503
Total nitrogen	0.460	-0.130	0.290
Dissolved reactive phosphorus	0.147	-0.000	0.320
Total phosphorus	0.396	-0.437	0.089
Acetochlor	0.269	0.185	-0.546
Atrazine	0.345	<u>0.341</u>	-0.291
Metalachlor	0.250	<u>0.331</u>	0.204
Simazine	0.256	0.462	0.015
% variance explained by axis	32.5	20.9	17.1

The first two PCA axes of instream habitat possessed eigenvalues greater than the broken stick eigenvalues (Jackson, 1993) and were used as independent variables in the generalized linear mixed effect model analyses. We also chose to use the third PCA axis of instream habitat as an independent variable in the generalized linear mixed effect model analyses because it represented an interpretable axis that represented a different habitat gradient than the first two PCA axes of instream habitat. These three PCA axes explained 55.8% of the cumulative percent of the variance in the instream habitat data (Table 3). The PCA of instream habitat identified a hydrology gradient (axis 1), a substrate-cover type and pH gradient (axis 2), and a water depth and dissolved oxygen (axis 3) (Table 3). Sites with increasing site scores along the first PCA axis of instream habitat exhibited decreasing discharge, mean velocity, and mean water depth values. Sites with increasing site scores along the second PCA axis of instream habitat had increasing pH values and decreasing diversity of substrate and cover types on the stream bottom. Sites with increasing site scores along the third PCA axis of instream habitat exhibited increasing dissolved oxygen concentrations and decreasing mean water depths.

The first three PCA axes of water chemistry also possessed eigenvalues greater than the broken stick eigenvalues (Jackson, 1993) and were used as independent variables in the generalized linear model analyses. These three PCA axes explained 70.5% of the cumulative percent of the variance in the water chemistry data (Table 3). The PCA of water chemistry identified one nutrient gradient (axis 1) and two nutrient and herbicide gradients (axes 2 and 3) (Table 3). Sites with increasing site scores along the first PCA axis of water chemistry exhibited increasing concentrations of total nitrogen and ammonia. Sites with increasing site scores along the second PCA axis of water chemistry exhibited increasing concentrations of simazine and decreasing total phosphorus concentrations. Sites with increasing site scores along the third PCA axis of water chemistry exhibited increasing nitrate + nitrite concentrations and decreasing acetochlor concentrations.

3.2. Summary of amphibian captures and generalized linear mixed effect model results

We documented four frog species from 153 captures of tadpoles, post-metamorphs, and adult animals during our study (Table 4). No salamanders were captured. Our captures consisted mostly of tadpoles and post-metamorphs (97%), but we also captured northern leopard frog and wood frog adults (Table 4). The three most frequently captured species-life stages captured in both watersheds combined were: (1) green frog tadpoles; (2) bullfrog tadpoles; and (3) green frog post-metamorphs (Table 4). Green

Table 4

Number of captures (#) and relative abundance (%) of frogs captured in channelized agricultural headwater streams within Cedar Creek, Indiana (CC) and Upper Big Walnut Creek, Ohio (UBWC), 2008–2009. Percentages within the table are calculated based on the number of captures from within each watershed.

	CC		UBWC	
	#	%	#	%
All amphibians				
tadpoles	37	53.6	72	85.7
post-metamorphs	28	40.6	12	14.3
adults	4	5.8	0	0
Green Frog (<i>Lithobates clamitans</i>)				
tadpole	22	31.9	37	44.0
post-metamorph	23	33.3	9	10.7
Bullfrog (<i>Lithobates scatesbeianus</i>)				
tadpole	13	18.8	33	39.3
post-metamorph	5	7.2	3	3.6
Wood Frog (<i>Lithobates sylvaticus</i>)				
tadpole	2	2.9	2	2.4
adult	2	2.9	0	0.0
Northern Leopard Frog (<i>Lithobates pipiens</i>)				
adult	2	2.9	0	0.0

frog tadpoles and post-metamorphs, bullfrog tadpoles and post-metamorphs, and wood frog tadpoles were captured in both watersheds, while adult wood frogs and northern leopard frogs were only captured in CC (Table 4).

Overall, the generalized linear mixed effect models indicated that water chemistry influenced amphibian community and population structure more than instream habitat as the greatest standardized coefficient values for all amphibian response variables occurred within the three PCA axes of water chemistry more often than it did for the three PCA axes of instream habitat (Table 5).

Five (species richness, tadpole species richness, tadpole abundance, tadpole Shannon diversity index, bullfrog tadpole abundance) of 12 amphibian response variables were most strongly correlated with PCA axis 3 of water chemistry (i.e., nitrate + nitrite and acetochlor gradient) (Table 5). Four (Shannon diversity index, post-metamorph species richness, post-metamorph abundance, post-metamorph Shannon diversity index) of 12 amphibian response variables were most strongly correlated

with PCA axis 1 of water chemistry (i.e., total nitrogen and ammonia gradient) (Table 5). Only abundance was most strongly correlated with PCA axis 2 of water chemistry (i.e., simazine and total phosphorus gradient) (Table 5).

The generalized linear mixed effect model analysis for green frog tadpole abundance did not converge when all six independent variables were included in the analysis. We reanalyzed this response variable with a reduced generalized linear mixed effect model containing only one instream habitat variable and one water chemistry variable. Specifically, for the reduced model we selected the instream PCA axis (i.e., PCA axis 1) and the water chemistry PCA axis (i.e., PCA axis 3) that exhibited the greatest standardized coefficients in the non-converged full model. Green frog post-metamorph abundance was most strongly correlated with PCA axis 3 of water chemistry (i.e., nitrate + nitrite and acetochlor gradient) (Table 5).

Generalized linear mixed effect model analyses also indicated that species richness, tadpole species richness, tadpole abundance, tadpole Shannon diversity index, green frog tadpole abundance, and bullfrog tadpole abundance were positively correlated with PCA axis 3 of water chemistry, which indicates these response variables increased with increasing nitrate + nitrite concentrations and decreasing acetochlor concentrations (Table 5, Fig. 1). Shannon diversity index, post-metamorph species richness, post-metamorph abundance, and post-metamorph Shannon diversity index were negatively correlated with PCA axis 1 of water chemistry and decreased with increasing total nitrogen and ammonia concentrations (Table 5, Fig. 2). Abundance was positively correlated with PCA axis 2 of water chemistry and increased with increasing simazine concentrations and decreasing total phosphorus concentrations (Table 5, Fig. 3). Green frog post-metamorph abundance was negatively correlated with PCA axis 3 of instream habitat and decreased with increasing dissolved oxygen concentrations and decreasing mean water depths (Table 5, Fig. 3).

4. Discussion

4.1. Community and population level effects of water chemistry

Our results suggest that water chemistry has a greater role in shaping amphibian communities and populations than instream habitat in channelized agricultural headwater streams. Eleven of 12 amphibian response variables exhibited stronger relationships

Table 5

Standardized regression coefficients from generalized linear mixed effect model analyses depicting the relative influence of instream habitat (IH) and water chemistry (WC) on amphibians within channelized agricultural headwater streams in Cedar Creek, Indiana, and Upper Big Walnut Creek, Ohio, 2008–2009. PCA – principal component analysis axis. Bolded standardized coefficients identify the environmental factors with the greatest influence on amphibian community and population response variables. Underlined standardized coefficients are those independent variables that were documented to have significant effect ($P < 0.05$) within the regression model.

	IH PCA 1	IH PCA 2	IH PCA 3	WC PCA 1	WC PCA 2	WC PCA 3
Community						
Species richness	0.054	–0.028	–0.132	–0.486	0.194	0.530
Tadpole species richness	0.018	–0.140	–0.208	–0.360	0.253	0.579
Post-metamorph species richness	0.219	–0.107	0.057	–0.666	0.530	0.376
Abundance	–0.236	–1.159	1.024	–1.006	1.259	–0.098
Tadpole abundance	1.324	–0.583	–2.212	–2.841	2.028	10.541
Post-metamorph abundance	0.350	–0.221	–0.586	–0.609	0.221	0.150
Shannon diversity index	0.048	–0.043	–0.080	–0.344	0.172	0.332
Tadpole Shannon diversity index	0.031	–0.121	–0.221	–0.303	0.219	0.460
Post-metamorph Shannon diversity index	0.217	–0.125	0.092	–0.601	0.517	0.290
Population						
Green Frog tadpole abundance ^a	–	0.896	–	–	–	6.026
Bullfrog tadpole abundance	–0.337	–0.020	–4.047	–6.477	–5.115	12.216
Green Frog post-metamorph abundance	0.186	–0.287	–0.592	–0.530	0.259	–0.132

^a The model for this response variable did not converge and required reanalysis with a reduced number of independent variables. The results of the reduced model are presented above.

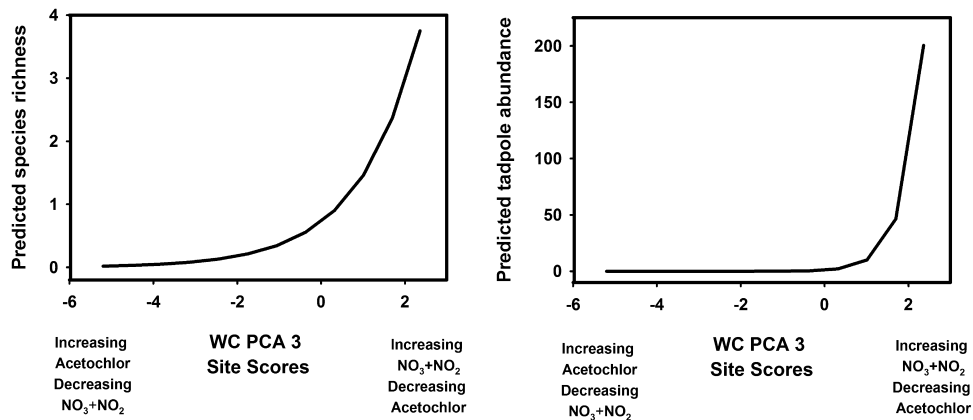


Fig. 1. Predicted relationship of amphibian species richness and tadpole abundance with site scores of the third Principal Component Axis (PCA) axis of water chemistry (WC) within channelized agricultural headwater streams in Cedar Creek, Indiana and Upper Big Walnut Creek, Ohio, 2008–2009. Predicted values were calculated with multiple regression equations using a range of values for the third PCA of WC and the values of the other independent variables held constant.

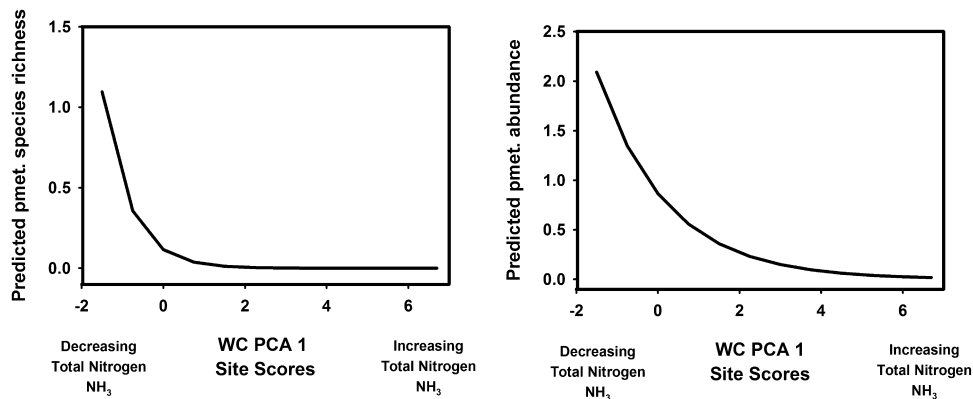


Fig. 2. Predicted relationship of post-metamorph (pmet.) species richness and pmet. abundance with site scores of the first Principal Component Axis (PCA) axis of water chemistry (WC) within channelized agricultural headwater streams in Cedar Creek, Indiana and Upper Big Walnut Creek, Ohio, 2008–2009. Predicted values were calculated with multiple regression equations using a range of values for the first PCA of WC and the values of the other independent variables held constant.

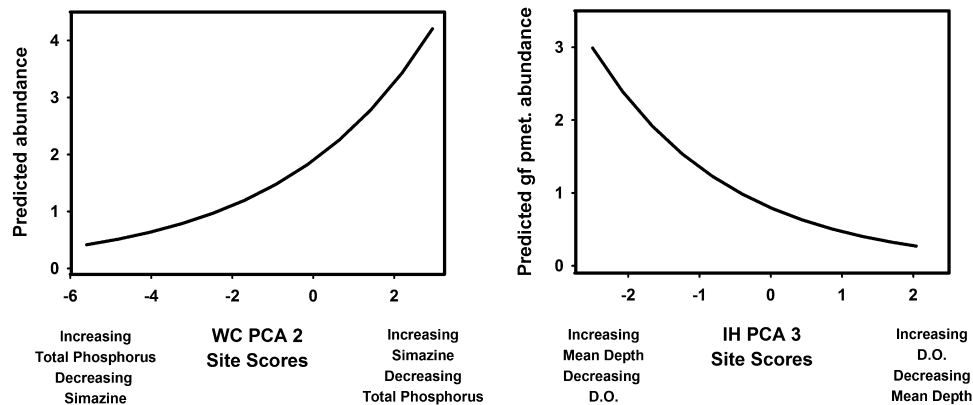


Fig. 3. Predicted relationship of amphibian abundance with site scores of the second Principal Component Axis (PCA) axis of water chemistry (WC) and predicted relationship of green frog post-metamorph (gf pmet.) abundance with third PCA axis of instream habitat (IH) within channelized agricultural headwater streams in Cedar Creek, Indiana and Upper Big Walnut Creek, Ohio, 2008–2009. Predicted values were calculated with multiple regression equations using a range of values for the second PCA of WC (amphibian abundance) or third PCA of IH (gf pmet. abundance) and values of the other independent variables held constant. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

with water chemistry gradients than to those derived from instream habitat variables. In particular, community response variables calculated for all amphibians and tadpoles either increased along a gradient of increasing nitrate + nitrite and decreasing acetochlor concentrations, decreased along a gradient of increasing total nitrogen and ammonia concentrations, or increased along a gradient of increasing simazine and decreasing total phosphorus concentrations. Post-metamorph community response variables decreased along a gradient of increasing total nitrogen and ammonia. At the population level, the abundance of green frog and bullfrog tadpoles increased along a gradient of increasing nitrate + nitrite and decreasing acetochlor concentrations, while the abundance of green frog post-metamorphs decreased as streams became shallower and more oxygenated.

The literature on amphibian habitat relationships within stream ecosystems is sparse and few studies provide information on relationships of amphibians with nutrient and herbicide concentrations in the field. The studies that have been done suggest a minimal role for the effect of water chemistry. In a polder landscape in the Netherlands, it was found that the frequency of dredging for maintenance of hydraulic capacity within channelized streams was correlated with the presence of amphibian larvae, but the amount of nutrients applied on adjacent fields was not (Twisk et al., 2000). Analysis of amphibian communities in streams within a predominantly agricultural landscape in Italy indicated a potential role for nutrient concentrations, but this relationship was likely an indirect effect of adjacent land use (Ficetola et al., 2011). It should be noted that the previous two studies (Twisk et al., 2000; Ficetola et al., 2011) did not quantify herbicide concentrations and both were focused on amphibian occurrence patterns at a landscape scale.

The relationship between water quality and amphibian communities in agricultural wetlands has received more attention (Hecnar and M'Closkey, 1996; Bishop et al., 1999; Brodman et al., 2003; Houlahan and Findlay, 2003; Knutson et al., 2004; Jeliazkov et al., 2014). An index of water quality that included nutrient and physical parameters was positively correlated with species richness in ponds in northern France (Jeliazkov et al., 2014). Nitrogen pollution (total nitrogen, total Kjeldahl nitrogen, and ammonia) has been found to be associated with reduced species richness in wetlands in Wisconsin, USA, (Knutson et al., 2004) and Ontario, Canada (Bishop et al., 1999; Houlahan and Findlay, 2003; but see Hecnar and M'Closkey (1996) for contrasting results). While there is evidence of generally sub-lethal effects of herbicides on individual species (reviewed in Mann et al., 2009), documented effects on community structure are either weak or lacking in natural systems and this has been attributed to low chronic concentrations typically present within agricultural wetlands and other aquatic ecosystems (Knutson et al., 2004). When relationships between water chemistry and community structure have been documented, these relationships occurred as a result of comparing amphibian community structure among wetlands with contrasting land use (Bishop et al., 1999; Houlahan and Findlay, 2003; Knutson et al., 2004), which suggests that land use may be the underlying factor in amphibian response to water quality (Houlahan and Findlay, 2003; Ficetola et al., 2011).

A growing literature on fish and aquatic macroinvertebrate communities in channelized agricultural headwater streams allows the comparison of our results to the habitat relationships exhibited by other taxa found within this stream type. In CC and UBWC, it has been found that instream habitat, specifically hydrology and substrate type, is more important than riparian habitat and water chemistry in explaining variation in the structure of fish (Smiley et al., 2008; Sanders, 2012) and aquatic macroinvertebrate communities (McKinney, 2012). Other studies on fish in channelized headwater streams in Ohio (Dyer et al., 1997;

Miltner and Rankin, 1998; D'Ambrosio et al., 2009) and Wisconsin (Fitzpatrick et al., 2001) have also suggested that water chemistry has less influence relative to instream habitat, geomorphology, riparian habitat, and adjacent land use. Stream size and water velocity has been documented to explain greater variation in aquatic macroinvertebrate community metrics than water quality parameters within channelized agricultural headwater streams in Maryland (Leslie et al., 2012). While water chemistry is considered to be a relatively important habitat factor for fish and aquatic macroinvertebrates in other stream types in North America (Sawyer et al., 2004; Johnson and Ringler, 2014) and Europe (Hering et al., 2006; Leps et al., 2015), these studies were conducted at much larger spatial scales than our study and encompassed greater number of stream types differing in channel modification and stream size.

The relationships of amphibian community and population structure with water chemistry gradients that we have observed are novel. In contrast to much of the literature related to the effects of water chemistry on aquatic biota, a potentially important difference is that our study occurred at a sub-regional scale in two separate watersheds having similar land uses. Although fish and aquatic macroinvertebrates within channelized agricultural headwater streams in CC and UBWC appear to respond to the variation in instream habitat that occurs within and among these sites (Smiley et al., 2008; McKinney, 2012; Sanders, 2012), perhaps the variation in instream habitat is not great enough for amphibians to perceive these streams as complex habitats containing multiple microhabitat types. If so, this would explain why amphibian community structure was more strongly correlated with water chemistry than physical habitat. More research on amphibian use of different microhabitats within channelized agricultural headwater streams is needed to confirm this observation.

While community interactions are complex in water containing nutrient and pesticide mixtures (Boone and James, 2003; Boone et al., 2007; Mann et al., 2009; Relyea, 2009), the specific relationships that we observed are intriguing. Species richness, tadpole species richness, tadpole abundance, and tadpole diversity increased in response to the combined effect of increasing nitrite + nitrate concentrations and decreasing acetochlor concentrations. The nitrite + nitrate concentrations within the streams during amphibian sampling was below 10 mg/L and the lethal effect concentrations compiled by Mann et al. (2009) (>20 mg/L). Such sub-lethal concentrations may benefit tadpoles by increasing productivity which can provide food through increased periphyton biomass (de Wijer et al., 2003; Smith et al., 2006; Boone et al., 2007). Acetochlor effects on amphibians are less well studied, but there is evidence that it can accelerate metamorphosis in northern leopard frogs (Cheek et al., 1999), alter periphyton and phytoplankton dynamics within aquatic food webs, and potentially lead to decreases in pH and dissolved oxygen (Relyea, 2009). We also observed that increasing concentrations of ammonia among sites was correlated with reduced species richness, abundance, and diversity of post-metamorphic amphibians. Ammonia is well known to be highly toxic to tadpoles (Rouse et al., 1999; Mann et al., 2009) and their sensitivity to ammonia can be greater than fish (Diamond et al., 1993). The maximum ammonia concentrations documented in the streams during amphibian sampling exceeded the concentrations capable of causing significant levels of embryo mortality and reductions in embryo and tadpole growth and development in green frogs (0.6 mg/L) and northern leopard frogs (1.5 mg/L) (Jofre and Karasov, 1999) suggesting that such toxicity may be reducing the numbers of individuals that proceed to and complete metamorphosis from channelized agricultural headwater streams. Additionally, we observed increasing amphibian abundance with increasing simazine concentrations and decreasing total phosphorus concentrations. The influence of simazine on

amphibians has not been examined (Smalling et al., 2013), but simazine concentrations in the streams during amphibian sampling were less than acute toxicity levels for fish and aquatic macroinvertebrates (Table 2, U.S. EPA, 2015). In contrast, increasing eutrophication caused by increasing phosphorus concentrations has been linked to increased occurrence of malformations (i.e., missing, extra, or misshapen limbs) in amphibians in North America as a result of increased parasitic infections (Mann et al., 2009). Increasing phosphorus concentrations may also reduce the types and abundance of macroinvertebrate prey preferred by amphibians (Ficetola et al., 2011). Clearly future research needs to quantify the influence of nutrient and pesticide mixtures on amphibians within agricultural streams.

4.2. Channelized agricultural headwater streams as amphibian habitat

Only a handful of studies have documented amphibian use of channelized agricultural headwater streams (Twisk et al., 2000; Piha et al., 2007b; Maes et al., 2008; McDaniel et al., 2008; Hartel et al., 2011), and ours is among the first to do so for the Midwestern United States (Smiley et al., 2011). Our results are consistent with these previous studies in that we documented ranid frogs (Family Ranidae) within channelized agricultural headwater streams in our watersheds. In contrast, previous studies from Europe (Twisk et al., 2000; Maes et al., 2008; Hartel et al., 2011) documented toads (Family Bufonidae, Family Pelobatidae), tree frogs (Family Hylidae), and salamanders (Order Caudata) within channelized agricultural headwater streams in addition to ranid frogs. Additionally, a previous study (McDaniel et al., 2008) in Canada documented toads (Family Bufonidae) in channelized agricultural headwater streams as well as ranid frogs. Among aquatic breeding amphibians that occur in the counties of our study sites, we only captured 4 of 19 species in Indiana (Minton, 2001) and 3 of 24 species in Ohio (Pfungsten, 1998). One of these species (wood frog) is considered to be sensitive to disturbance and prefers forested habitats (Micacchion, 2011), and a second (northern leopard frog) has experienced population declines and is listed as Species of Special Concern in Indiana (Minton, 2001). The reduced amphibian diversity and dominance by tolerant, habitat generalists within our study may be a function of its small spatial scale, the high degree of agricultural land use adjacent to our study areas, and/or the timing of our sampling. Future research in the United States needs to be conducted over the full range of the amphibian breeding season at larger spatial scales to more fully document use of channelized agricultural headwater streams by amphibians.

Green frogs and bullfrogs were the most abundant species within our study sites. Both species favor permanent, open canopy wetlands in forested landscapes but are known to be habitat generalists that will use streams and will readily colonize anthropomorphic bodies of water (Dodd, 2013), and are able to persist in remnant habitats within agricultural landscapes (Kolozsvary and Swihart, 1999). Green frogs and bullfrogs are also tolerant of the presence of fish, a major contributor to tadpole community structure in wetlands (Knutson et al., 2004; Boone et al., 2007; Werner et al., 2007) that has been documented to decrease amphibian diversity and abundance. Bullfrogs are well known to persist in the larval stage for two to three seasons and green frogs in the northern United States and southern Canada have also been documented to remain in larval stage for more than one season (Dodd, 2013). Tadpoles of these two species are exposed to aquatic conditions for a relatively extended period of time, which could increase their susceptibility to agricultural contaminants. While comparative information for bullfrogs is not available, green frogs appear to be more tolerant of reduced water

quality than other amphibian species (Hecnar and M'Closkey, 1996; McDaniel et al., 2008).

Among the species we observed, green frogs have the closest association with stream habitats (Martof, 1953a; Dodd, 2013). In Michigan, green frogs living in streams will migrate to nearby ponds and wetlands to breed (Martof, 1953b), with females spending only one week away from their home stream. Martof (1953a) observed stream-dwelling residents to remain in place to overwinter and it has also been found that green frogs will migrate from ponds and wetlands to overwinter within streams (Lamouroux and Madison, 1999). It is assumed that stream overwintering sites provide higher oxygen availability due to flowing water. While green frogs and bullfrogs may prefer to breed in open canopy ponds and wetlands with long hydroperiods, our captures of tadpoles in channelized agricultural headwater streams confirms that breeding can occur in this stream type. Further study will be required to determine if the use of streams for reproduction is linked to the lack of proximate ponds and wetlands or if there is polymorphism in breeding site choice within populations.

Our results indicate that channelized agricultural headwater streams provide habitat for anurans in anthropogenically disturbed landscapes, but habitat quality within these streams is highly dependent on the use of management practices that maximize their ecological value (Herzon and Helenius, 2008; Chester and Robson, 2013). Our results provide empirical support for previous amphibian management guidelines (BC WLAP, 2004; Kingsbury and Gibson, 2012) for agricultural landscapes in the United States and Canada that recommend reductions in the use of agricultural fertilizers and pesticides for amphibian conservation. Channelized streams with subsurface drainage in the watersheds present a special challenge for managing water quality because subsurface tile drains enable agricultural contaminants to bypass grass filter strips and other types of riparian buffers (Smiley et al., 2011). Thus, effective conservation practices will be those that reduce nutrient and herbicide concentrations within subsurface runoff. Routing subsurface drainage into treatment wetlands created to capture nutrients and pesticides has been promoted to reduce nutrient and pesticide inputs into streams (Smiley et al., 2011). Creation of treatment wetlands within riparian habitats of channelized agricultural headwater streams would also benefit amphibians by providing additional habitat and increasing riparian habitat diversity. Additionally, conservation practices that dissipate subsurface runoff through riparian habitats [i.e., saturated buffers (Jaynes and Isenhardt, 2014)] or practices that filter subsurface runoff [i.e., blind inlets (Smith and Livingston, 2013)] represent additional options for reducing nutrient and pesticide concentrations in channelized agricultural headwater streams. Although we recommend the use of conservation practices targeting water quality improvement at the local scale within channelized agricultural headwater streams as part of amphibian conservation efforts within agricultural landscapes, it is our opinion that amphibian conservation efforts should involve the management of both local and landscape-scale factors. Specifically, management of terrestrial factors (e.g., land use characteristics, riparian corridor width, regional scale density of wetlands, etc.) known to influence amphibian diversity and abundance in agricultural wetlands (Kolozsvary and Swihart, 1999; Houlihan and Findlay, 2003; Semlitsch and Bodie, 2003; Jeliakov et al., 2014) is needed but this has rarely been explored for streams (Maisonneuve and Rioux, 2001; Ficetola et al., 2011).

5. Conclusions

Our results indicate that anuran community and population structure was most strongly correlated with water chemistry (i.e.,

nutrient and herbicide gradients) rather than instream habitat within channelized agricultural headwater streams in Indiana and Ohio. Our results also highlighted the relationships of anuran community and population structure with agricultural contaminants commonly found within agricultural streams. Within these small degraded streams, we documented that anuran community and population response variables either increased along a gradient of increasing nitrate + nitrite and decreasing acetochlor concentrations, decreased along a gradient of increasing total nitrogen and ammonia concentrations, or increased along a gradient of increasing simazine and decreasing total phosphorus concentrations. Our results suggest local scale practices that reduce nutrients and herbicides within channelized agricultural headwater streams will provide greater benefits for amphibians than practices that address physical habitat degradation. Amphibian conservation strategies within agricultural landscapes have focused mostly on wetland conservation. Future research needs to more fully explore the habitat value of channelized agricultural headwater streams and how these streams can be managed to contribute to amphibian conservation efforts.

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Biomonitoring Data for 2,4-Dichlorophenoxyacetic Acid in the United States and Canada: Interpretation in a Public Health Risk Assessment Context Using Biomonitoring Equivalents

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BACKGROUND: Several extensive studies of exposure to 2,4-dichlorophenoxyacetic acid (2,4-D) using urinary concentrations in samples from the general population, farm applicators, and farm family members are now available. Reference doses (RfDs) exist for 2,4-D, and Biomonitoring Equivalents (BEs; concentrations in urine or plasma that are consistent with those RfDs) for 2,4-D have recently been derived and published.

OBJECTIVE: We reviewed the available biomonitoring data for 2,4-D from the United States and Canada and compared them with BE values to draw conclusions regarding the margin of safety for 2,4-D exposures within each population group.

DATA SOURCES: Data on urinary 2,4-D excretion in general and target populations from recent published studies are tabulated and the derivation of BE values for 2,4-D summarized.

DATA SYNTHESIS: The biomonitoring data indicate margins of safety (ratio of BE value to biomarker concentration) of approximately 200 at the central tendency and 50 at the extremes in the general population. Median exposures for applicators and their family members during periods of use appear to be well within acute exposure guidance values.

CONCLUSIONS: Biomonitoring data from these studies indicate that current exposures to 2,4-D are below applicable exposure guidance values. This review demonstrates the value of biomonitoring data in assessing population exposures in the context of existing risk assessments using the BE approach. Risk managers can use this approach to integrate the available biomonitoring data into an overall assessment of current risk management practices for 2,4-D.

KEY WORDS: 2,4-dichlorophenoxyacetic acid, biomonitoring, exposure biomarkers, exposure monitoring, risk assessment. *Environ Health Perspect* 118:177–181 (2010). doi:10.1289/ehp.0900970 available via <http://dx.doi.org/> [Online 12 August 2009]

Biomonitoring data for 2,4-dichlorophenoxyacetic acid (2,4-D) in urine samples are now available from a number of studies of both the general population (including preschool-age children) and farm applicators and their family members [Alexander BH, et al. 2007; Arbuckle and Ritter 2005; Arbuckle et al. 2002, 2004, 2006; Centers for Disease Control and Prevention (CDC) 2005; Morgan et al. 2008]. Such data provide an integrated measure of absorbed dose from all pathways and routes of exposure. The hazards of 2,4-D were recently assessed by the U.S. Environmental Protection Agency (U.S. EPA 2004) and the Canadian Pest Management Regulatory Agency (PMRA 2007). The U.S. EPA–derived reference doses (RfDs) for acute and chronic exposure to 2,4-D are based on external exposure metrics (administered dose), which are not directly useful for evaluating biomonitoring data. However, Biomonitoring Equivalent (BE) values corresponding to RfDs for acute and chronic exposure scenarios are now available (Aylward and Hays 2008) and can be used as a tool for assessing the biomonitoring data directly in a public health risk assessment context, without requiring calculation of

corresponding external dose, as has previously been done (Mage et al. 2004). Here we review urinary biomonitoring data for 2,4-D from several studies in the general population and in farmers and farm family members and evaluates the data in the context of the BE values for 2,4-D presented by Aylward and Hays (2008) to assess the current margin of safety (ratio of exposure guidance value such as an RfD to exposure measures) for population exposures to 2,4-D in the United States and Canada.

Methods

Biomonitoring data. We used urinary biomonitoring data for 2,4-D from several studies of both general population adults and children and from studies of farmers and farm family members, as follows.

The National Center for Environmental Health of the Centers for Disease Control and Prevention (CDC 2005) measured 2,4-D in urine samples collected from a complex, stratified random sample of the civilian, non-institutionalized population of the United States, 6–59 years of age, during 2001–2002, as part of the National Health and Nutrition Examination Survey (NHANES).

Morgan et al. (2004, 2008) recently examined the exposures of 135 preschool children and their adult caregivers to 2,4-D at their homes in North Carolina and Ohio from the Children's Total Exposure to Persistent Pesticides and Other Persistent Organic Pollutants (CTEPP) study. Participants were randomly recruited from homes in six North Carolina and six Ohio counties. Participants were recruited by field staff from homes between February 2000 and February 2001 in North Carolina and January 2001 and November 2001 in Ohio. Monitoring was performed over a 48-hr period at the participants' homes. Spot urine samples and environmental samples including air, soil, dust, hand wipes, and food were collected and analyzed for 2,4-D.

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This research has been subjected to U.S. EPA administrative review and approved for publication. The findings and conclusions in this report are those of the authors and do not necessarily represent the views of Health Canada, the U.S. EPA, or the Centers for Disease Control and Prevention.

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Alexander BH, et al. (2007) reported urinary 2,4-D data from the Farm Family Exposure Study. Participants in the study included 34 farmers in Minnesota and South Carolina who were licensed applicators and their spouses and children ($n = 53$) living on the farm property. Participants collected 24-hr urine samples the day before, the day of, and for 3 days after application of 2,4-D on their farms during the 2000 or 2001 growing season.

Curwin et al. (2005) measured urinary 2,4-D concentrations in 16 farmers 1–5 days after their application of 2,4-D on the farm during the spring and summer of 2001. The evening and the following first morning urine samples were composited.

The Pesticide Exposure Assessment Study measured the extent to which agricultural pesticide applicators and their families in Ontario, Canada, are exposed to pesticides during normal handling practices (Arbuckle and Ritter 2005; Arbuckle et al. 2002, 2004). Farmers from the previously conducted Ontario Farm Family Health Study (Arbuckle et al. 1999) that had reported using phenoxyacetic acid herbicides were telephoned in early 1996 to determine their eligibility for the Pesticide Exposure Assessment Study. To be eligible, the farmer had to *a*) be planning to use 2,4-D or (4-chloro-2-methylphenoxy)acetic acid (MCPA) in the coming growing season, *b*) be the individual who would be handling the herbicides on the farm, *c*) have his or her home on the farm property, and *d*) be currently living with his or her spouse. A total of 126 families provided a spot urine sample before handling either 2,4-D or MCPA and then provided two consecutive 24-hr samples after use of the herbicide. All samples were collected in 1996.

The Agricultural Health Study (AHS)/Pesticide Exposure Study (PES) was designed to evaluate exposure to 2,4-D and chlorpyrifos in a subset of individuals enrolled in the AHS,

which is a large, prospective epidemiologic study of pesticide applicators and their spouses in Iowa and North Carolina designed to assess the relationships between agricultural exposures and disease. Participants in the AHS were contacted randomly and surveyed to ascertain their planned use of the 2,4-D and chlorpyrifos, and then a subset of participants were enrolled in the PES (Thomas et al. 2009). Urinary samples were collected during 2001 and 2002 and included a preapplication first morning void sample, as well as a 24-hr sample starting the day of application (day 1) and, optionally, for days 2–5 as well.

Descriptions of the institutional review board approvals and informed consent information for each of these studies are presented in the cited publications.

RfDs and biomonitoring equivalents.

The U.S. EPA recently conducted a review of 2,4-D and adopted both a chronic oral RfD as well as acute RfDs (applicable to single-day exposures) for this herbicide (U.S. EPA 2004). Table 1 summarizes the derivations of the BE values associated with the RfD values. BEs are defined as the concentration of a chemical or its metabolite in a human biological medium (usually blood or urine) that is consistent with existing exposure guidance values. BE values are screening values corresponding to existing risk assessments and not intended for use as definitive measures of risk for individuals. A full description of the BE approach and application is beyond the scope of this review but is presented elsewhere (Hays and Aylward 2009; Hays et al. 2007, 2008).

The pharmacokinetics of 2,4-D have been studied in two sets of human volunteers (Kohli et al. 1974; Sauerhoff et al. 1977). Both studies found that 2,4-D is eliminated in urine either as the unchanged parent compound (80–95%) or as a conjugate, with urinary half-lives on the order of 1 day. There was no evidence of oxidative metabolism, consistent with data from

other mammalian species (Timchalk 2004). Based on these pharmacokinetic data, continuing exposure for more than 1 week of exposure would result in a steady state in which the amount excreted daily in urine would be approximately equivalent to the amount absorbed each day.

Because 2,4-D is excreted as the parent compound in urine, most biomonitoring evaluations of exposure to 2,4-D have relied on measurements (quantifying both free and conjugated parent compound) in urine samples (CDC 2005; Knopp 1994; Knopp and Glass 1991), although a few kinetic studies have also examined plasma concentrations of 2,4-D in humans and animals (Kohli et al. 1974; Saghir et al. 2006; Sauerhoff et al. 1977; van Ravenzwaay et al. 2003). The relative ease of collection of urine samples compared with blood samples contributes to this choice. From a toxicologic point of view, plasma concentrations of 2,4-D are probably more informative for predicting target tissue concentrations and responses (e.g., neurotoxic responses). This would be particularly true under conditions of episodic, higher-level exposures. However, under conditions of chronic, low-level exposures, urinary excretion rates of 2,4-D should be specific and quantitatively relevant in a framework of a mass-balance assessment. That is, under exposure conditions that approximate steady-state conditions [consistent with the definition of chronic RfDs and related exposure guidance values; see, e.g., the definition of RfD provided under the U.S. EPA Integrated Risk Information System program (U.S. EPA 2009)], daily urinary excretion of 2,4-D should equal daily intake.

The straightforward elimination kinetics of 2,4-D (as parent compound or conjugate in urine with essentially no oxidative metabolism) and the lack of direct relationship between urinary concentration and critical internal dose metrics suggest a simple mass-balance approach

Table 1. RfDs established by the U.S. EPA (2004) for 2,4-D and derivation of corresponding BE values.

Reference value	RfD			
	Chronic	Acute		
		Females of reproductive age	Other general population	Occupational exposure ^a
Underlying study type	Rat chronic dietary bioassay	Rat oral gavage, gestational days 6–15	Rat acute gavage	Rat chronic dietary bioassay
End point	Decreased body weight gain and food consumption, alterations in hematology and clinical chemistry parameters, increased thyroid weights, and decreased testes and ovarian weights	Skeletal variations and malformations	Gait abnormalities	Same as for chronic RfD
POD (NOAEL) (mg/kg-day)	5	25	67	5
Interspecies UF	10	10	10	10
Human equivalent POD (mg/kg-day)	0.5	2.5	6.7	0.5
BE _{POD} (urinary 2,4-D)	20,000 µg/L (30,000 µg/g cr)	40,000 µg/L (70,000 µg/g cr)	100,000 µg/L (200,000 µg/g cr)	20,000 µg/L (30,000 µg/g cr)
Intraspecies UF	10	10	10	10
Database UF ^b	10	10	10	NA
BE _{RfD} (urinary 2,4-D)	200 µg/L (300 µg/g cr)	400 µg/L (700 µg/g cr)	1,000 µg/L (2,000 µg/g cr)	2,000 µg/L (3,000 µg/g cr)

Abbreviations: cr, creatinine; NA, not applicable; NOAEL, no observed adverse effects level; POD, point of departure; UF, uncertainty factor. Details of the derivation are presented by Aylward and Hays (2008).

^aDerivation based on U.S. EPA (2004) memorandum indicating *a*) POD same as for general population chronic RfD, and *b*) desired margin of exposure (ratio between POD and exposure level) of 100, based on UFs of 10 each for inter- and intraspecies variation. ^bUF applied to account for the lack of a developmental neurotoxicity study and the need for a repeated two-generation bioassay with a focus on thyroid and immunotoxicity end points.

for derivation of BE values for urinary 2,4-D consistent with chronic exposure at the chronic RfD. The process of deriving the BE_{POD} and BE_{RfD} values for 2,4-D is detailed by Aylward and Hays (2008) and summarized below and in Table 1.

The point of departure (POD) for the U.S. EPA chronic RfD is a no observed adverse effect level (NOAEL) of 5 mg/kg-day in rats fed 2,4-D chronically in the diet. Applying an uncertainty factor (UF) of 10 for interspecies variation, the human equivalent POD is 0.5 mg/kg-day. Calculating the average concentration of 2,4-D in urine in humans associated with this chronic daily dose (after application of the interspecies UF) yields the BE_{POD} . The daily mass intake at the human equivalent POD was estimated for a variety of child and adult body weights. Estimated distributions of daily creatinine excretion or urinary volume as a function of sex, age, and body size were used in a Monte Carlo analysis to estimate a distribution of creatinine-adjusted urinary 2,4-D concentrations for various age and sex categories [methods are described in detail by Aylward and Hays (2008)]. The average of median estimated creatinine-adjusted 2,4-D concentration consistent with chronic exposure at the human-equivalent POD (BE_{POD}) for 2,4-D for adults (males and females) is approximately 20,000 $\mu\text{g/L}$ or 30,000 $\mu\text{g/g}$ creatinine. These values were consistent with the range of median values identified in the simulations for children of various ages. Concentrations at the 95th percentiles of the estimated distributions were generally within a factor of 2 of the median values.

The BE associated with the chronic RfD was derived by dividing the BE_{POD} , which reflects the interspecies UF of 10, by the UF of 10 for intraspecies variation and the UF of 10 applied by U.S. EPA for database uncertainties (for a total composite UF from the animal POD of 1,000 applied to the animal NOAEL POD). BE values corresponding to the acute RfDs were derived in a similar fashion, except that steady state was not assumed. Based on the urinary elimination half-life of approximately 1 day, an assumption was made that one-half of

the intake dose at the human equivalent POD for the acute RfD values would be eliminated in the first 24 hr after exposures. Average urinary 2,4-D concentrations (both absolute and creatinine adjusted) corresponding to one-half the human equivalent POD doses were estimated, and the intraspecies and database UFs were then applied to obtain the BE_{RfD_acute} values. These BE values are appropriate for use when the exposure is short term and episodic and the timing of the sample collection compared with exposure is known. Table 1 summarizes the derivation and resulting values.

Results

Table 2 summarizes urinary 2,4-D concentrations measured in studies of general population groups (CDC 2005; Morgan et al. 2008). Exposure pathways for persons in the general population may include ingestion of residues in food products, inhalation, and direct contact with dust (Morgan et al. 2004, 2008). Figure 1 presents the measured urinary concentrations in the context of the appropriate BE values based on the U.S. EPA chronic RfD. The urinary levels of 2,4-D observed in the general population samples are far below the BE value corresponding to the U.S. EPA chronic RfD, with median and upper bound measured concentrations more than 100- and 50-fold below the BE_{RfD} .

Table 3 summarizes the corresponding data for farmers and members of their families obtained in the days immediately after application of 2,4-D. Exposure pathways for non-applicators on the farm may include secondary exposure to treated fields, farm machinery, or the applicator, and drift of herbicide during application with resulting inhalation, dermal, and oral exposure after contact with residues on surfaces in the home. Urinary concentrations collected from farm family members in the day or days immediately after application of 2,4-D fell below the applicable acute BE values.

Figure 2 presents measured urinary concentrations in farmers involved in application of 2,4-D in the context of BE values corresponding to the U.S. EPA occupational exposure guidance values. Again, the data suggest an overall margin

of safety, with median or geometric mean levels in farmers involved in application of 2,4-D more than 25-fold below the occupational BE target value. However, some individuals had single spot urinary concentrations that approached the occupational BE target value. The highest urinary level of 2,4-D reported in Thomas et al. (2009) on days 1–5 after application was 2,500 $\mu\text{g/L}$, in excess of the occupational BE value of 2,000 $\mu\text{g/L}$ (data not shown). However, all other reported occupational measurements were below the occupational BE.

Discussion

Available biomonitoring data for 2,4-D in both the general and agricultural populations indicate that current uses and practices suggest exposures that are below the acceptable exposures identified by the U.S. EPA. A “margin of safety” is the ratio between the exposure guidance value and measured exposure. In this analysis, the exposure guidance value (RfD) was converted to a BE_{RfD} value for comparison with the measured biomarker concentrations. General population values indicate a margin of safety compared with the BE_{RfD} of approximately 200 at the central tendency and > 50 at the upper percentiles of exposure. In turn, the BE_{RfD} is 100-fold below the BE_{POD} , which is the biomarker concentration associated with chronic intake in humans at the POD

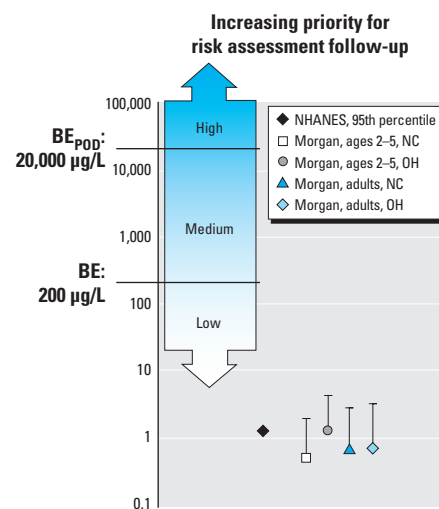


Figure 1. Urinary 2,4-D concentrations ($\mu\text{g/L}$) in general population studies presented in the context of the BE value corresponding to the U.S. EPA RfD for general population chronic exposures. The symbol for data from NHANES (CDC 2005) represents the 95th percentile for all tested participants (median values were below the LOD; see Table 2). The symbols for data from Morgan et al. (2008) (in key, Morgan) represent the median values for the children and adults from two states; bars extend to the 95th percentile for each group. The shaded regions represent concentration ranges associated with low, medium, and high priority for risk assessment follow-up based on the criteria described in the BE communications guidelines (LaKind et al. 2008).

Table 2. Urinary biomonitoring data for samples from the general U.S. population.

Study (n)	Age group (years), population	Sample description	Percentile			
			$\mu\text{g/L}$		$\mu\text{g/g cr}$	
			50th	95th	50th	95th
NHANES, 2001–2002 (CDC 2005)						
546	6–11, USA	Spot	< LOD ^a	1.55	< LOD	1.40
797	12–19, USA	Spot	< LOD	1.24	< LOD	0.662
1,070	20–59, USA	Spot	< LOD	1.27	< LOD	1.04
2,413	All, 6–59, USA	Spot	< LOD	1.27	< LOD	1.08
Morgan et al. (2008)						
66	2–5, NC	48-hr composites	0.5	1.9	1.0 ^b	3.4 ^b
69	2–5, OH	48-hr composites	1.2	4.3	1.5 ^c	5.1 ^c
66	20–44, NC	48-hr composites	0.7	2.8	0.6 ^b	2.3 ^b
69	19–49, OH	48-hr composites	0.7	3.3	0.5 ^c	3.3 ^c

LOD, limit of detection.

^aLOD for NHANES 2001–2002 was 0.2 $\mu\text{g/L}$. ^b $n = 55$. ^c $n = 59$.

extrapolated from animals to humans. The conclusion of a substantial margin of safety holds whether comparisons are made using volume or creatinine-adjusted concentrations. Median or average urinary 2,4-D concentrations for applicators are consistently below the BE values associated with occupational exposure targets set by the U.S. EPA (2004); however, evidence exists for exceptions near the occupational BE target value in a few individuals from the studied occupationally exposed populations. Biomonitoring data for spouses and children of applicators on the day after use of 2,4-D also are less than the BE values associated with general population acute exposure RfDs set by the U.S. EPA (2004).

Other studies have reported related biomonitoring data. Arcury et al. (2007) studied children from North Carolina farm worker families in 2004. Multiple pesticides (or metabolites) were measured in urine samples from these children (1–6 years of age). The median 2,4-D concentration was below the limit of detection (LOD) of 0.2 µg/L (42% of the 60 sampled children had detectable concentrations of 2,4-D, but the range of detected concentrations was not reported). Garry et al. (2001) measured urinary 2,4-D in small numbers of forestry applicators who used a variety of methods to apply the herbicide. Backpack sprayers had the highest measured urinary concentrations during time periods of use, with a median of 160 µg/L and a range up to 1,700 µg/L ($n = 7$). Other modes of application such as use of boom sprayers or aerial applications resulted in lower urinary 2,4-D concentrations, with all measured values < 500 µg/L for boom sprayers and < 100 µg/L for other modes. These values are consistent with the concentrations observed in farm applicators from the Alexander BH, et al. (2007) study and are also below the occupational BE_{RFD} presented in Table 1.

Table 3. Concentrations of 2,4-D measured in urine collected after acute exposure due to agricultural use of 2,4-D.

Group, <i>n</i>	Median (range)		Sample type	Study
	µg/L	µg/g cr		
Applicators				
34	73.1 (1.5–1,856)	45.8 (1.1–533.8)	24 hr	Alexander BH, et al. 2007
43	6.0 (0.5–410.0)	NR	24 hr	Arbuckle et al. 2002
16	13 ^a (NR)	NR	Composite of evening and following morning spot samples	Curwin et al. 2005
28	26 ^b (2.2–1,000)	NR	24 hr	Thomas et al. 2009
Spouses ^c				
34	1.2 (0.5–20)	1.1 (0.2–13.1)	24 hr	Alexander BH, et al. 2007
43	< LOD ^d (< LOD to 61)	NR	24 hr	Arbuckle and Ritter 2005
Children ages 4–17 years				
52	2.9 (0.5–640.4)	2.3 (0.3–660.2)	24 hr	Alexander BH, et al. 2007
Children ages 3–18 years				
91	< LOD ^d (< LOD to 12)	NR	24 hr	Arbuckle et al. 2004

NR, not reported. Concentrations reported are 2,4-D in urine samples collected 1 day after application of 2,4-D on farms in applicators (Alexander BH, et al. 2007; Arbuckle et al. 2002; Thomas et al. 2009) and family members (spouses and children; Alexander BH, et al. 2007; Arbuckle et al. 2004) or in applicators 1–5 days after application (Curwin et al. 2005).

^aGeometric mean for farmers who reported spraying 2,4-D themselves in the previous 1–5 days. ^bGeometric mean. ^cAll spouses were female, and all applicators were male. ^dLOD = 1 µg/L.

The evaluation presented here is based on BE values derived from the U.S. EPA risk assessment of 2,4-D (U.S. EPA 2004). However, the Canadian PMRA has also recently estimated acceptable daily exposures to 2,4-D (PMRA 2007). The derived acute and chronic RfDs are based on the same underlying data as used by the U.S. EPA, with similar or identical choices of POD. However, the PMRA assessment generally applied total UFs approximately 3-fold lower than those applied by the U.S. EPA, resulting in exposure estimates that are approximately 3-fold greater than those set by the U.S. EPA. Thus, the BE_{POD} values associated with the PMRA risk assessment would be essentially identical to those for the corresponding U.S. EPA exposure guidance values. Although BE values were not specifically derived based on the PMRA assessments, corresponding urinary BE values would be approximately 3-fold higher than those derived based on the U.S. EPA RfDs. BE values corresponding to the PMRA acute RfD values for acute exposure in the general population and in females of reproductive age equal to 1,000 and 4,000 µg/L, respectively (2,000 and 7,000 µg/g creatinine). The BE value corresponding to the PMRA acceptable daily intake for chronic exposure would be 700 µg/L (1,000 µg/g creatinine). Thus, reliance on the PMRA risk assessment does not change the overall conclusion of a substantial margin of safety under the various exposure scenarios.

Uncertainties and limitations. BE values are derived based on expected average concentrations (either volume based or creatinine adjusted) in urine under conditions consistent with the underlying exposure guidance value (chronic or acute exposure conditions). Some variability in concentration is expected because of use of spot urine samples, interindividual variability in creatinine excretion rates, and

variability in urinary volume due to hydration status. Morgan et al. (2004, 2008) investigated the variability of 2,4-D concentrations among spot urine samples (i.e., first morning void, after lunch, and before bedtime) collected over the course of 48 hr from 28 adults and 28 children. The maximum measured spot urine value was within a factor of 3 of the mean value in 53 of the 56 individuals, consistent with previous assessments of variability among spot samples (e.g., Scher et al. 2007).

2,4-D is relatively short-lived, with a urinary half-life on the order of 1 day, so for an individual in the general population, a single measurement does not characterize long-term exposure. However, the NHANES urinary data for 2,4-D are representative of the U.S. population, and samples were collected at various times through the year. NHANES data would be expected to capture indications of higher exposures if they were occurring with any frequency, unless such variations were highly seasonal and geographically isolated. Urinary concentration data from Morgan et al. (2004, 2008) collected from two different geographical regions of the United States (North Carolina and Ohio) over the course of a year suggest somewhat higher exposures than reflected in the NHANES data set, but both sets indicate general population exposures far below health-based exposure guidance values.

A notable deficit in the available data for the general population pertains to residential uses of 2,4-D. Unlike exposures to 2,4-D users in agricultural populations, systematic

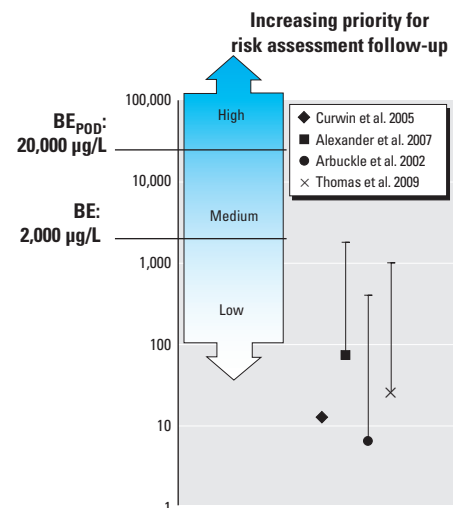


Figure 2. Urinary 2,4-D concentrations (µg/L) in applicators on the day after application of 2,4-D presented in the context of the human-equivalent BE_{POD} and target BE values associated with the occupational risk assessment (U.S. EPA 2004) (see Table 1). Symbols represent the median (or, in the case of Curwin et al. 2005 and Thomas et al. 2009, the geometric mean), and the bars extend to the maximum measured value in each study (not reported for Curwin et al. 2005). For description of shaded regions, see Figure 1 legend.

evaluations of domestic use of the chemical are not available. These episodic exposures would not likely be captured in the NHANES (CDC 2005) or Morgan et al. (2008) data. To the extent that domestic applications do not result in exposures greater than those resulting from agricultural applications, human exposures should be within the margin of safety demonstrated by these existing study data. More research is needed to understand the patterns of domestic use of 2,4-D in residential settings and the resulting potential human exposures to this herbicide in the United States and Canada.

The RfD values derived by the U.S. EPA are based on noncancer end points. 2,4-D has also been assessed for potential carcinogenic effects. Non-Hodgkin lymphoma (NHL) was associated with herbicides and 2,4-D in a series of case-control studies initiated > 20 years ago (Hoar et al. 1986; Zahm et al. 1990). Subsequent case-control and cohort studies have not confirmed these early observations (Burns et al. 2001; De Roos et al. 2003; Hartge et al. 2005; Pearce 1989; Schroeder et al. 2001; Woods et al. 1987). Recent reviews of NHL (Alexander DD, et al. 2007) and 2,4-D (Garabrant and Philbert 2002) have concluded that the epidemiologic evidence remains "scant" and unresponsive for this association.

BE values are screening values and are not intended for use as definitive measures of risk for individuals. They do not represent a bright line between safe and unsafe levels, but rather allow evaluation of biomonitoring data in a public health risk context consistent with the existing risk assessment for 2,4-D (LaKind et al. 2008). Biomarker concentrations below the BE_{RfD} indicate a low priority for risk assessment follow-up, whereas concentrations in excess of the BE_{RfD} but below the BE_{POD} indicate a medium priority for risk assessment follow-up. Values in excess of the BE_{POD} indicate a high priority for risk assessment follow-up. Risk assessment follow-up may include examination of the underlying risk assessment, exposure pathway investigations, or other risk management activities (LaKind et al. 2008). Acute RfDs and the corresponding BE values are targeted at isolated, single-day exposures and are appropriate for use in evaluating biomonitoring data only when there is specific knowledge of a potential acute exposure. The biomonitoring data reviewed here for both members of the general population and applicators generally falls into the range of low priority for risk assessment follow-up, according to the guidelines for BE communication (LaKind et al. 2008).

Conclusions

Considerable population-level and microlevel data are now available regarding domestic and agricultural exposures to 2,4-D as measured by urinary 2,4-D excretion. These data suggest

that current use patterns and risk management efforts by industry and government are likely keeping average exposure to 2,4-D for the general population and in farm family members, and likely other persons potentially exposed from proximity to use of this herbicide, to levels well below current noncancer reference values established both by the U.S. EPA's Office of Pesticide Programs and by Canada's PMRA.

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A Case for Revisiting the Safety of Pesticides: A Closer Look at Neurodevelopment

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A Case for Revisiting the Safety of Pesticides: A Closer Look at Neurodevelopment

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The quality and quantity of the data about the risk posed to humans by individual pesticides vary considerably. Unlike obvious birth defects, most developmental effects cannot be seen at birth or even later in life. Instead, brain and nervous system disturbances are expressed in terms of how an individual behaves and functions, which can vary considerably from birth through adulthood. In this article I challenge the protective value of current pesticide risk assessment strategies in light of the vast numbers of pesticides on the market and the vast number of possible target tissues and end points that often differ depending upon timing of exposure. Using the insecticide chlorpyrifos as a model, I reinforce the need for a new approach to determine the safety of all pesticide classes. Because of the uncertainty that will continue to exist about the safety of pesticides, it is apparent that a new regulatory approach to protect human health is needed. **Key words:** adverse effects, behavior, chlorpyrifos, fetal development, human function, neurodevelopment, pesticides, toxicity. *Environ Health Perspect* 114:10–17 (2006). doi:10.1289/ehp.7940 available via <http://dx.doi.org/> [Online 7 September 2005]

The U.S. Environmental Protection Agency's (EPA) Office of Pesticide Programs (OPP) estimated that 891 pesticide active ingredients were registered in 1997 (Aspelin and Grube 1999) and that 888 million pounds of pesticide active ingredients were used in the United States in 2001 (Kiely et al. 2004). Few of these chemicals are applied alone but rather are applied in formulations using different combinations of several pesticide active ingredients (MeisterPRO 2004). It is not uncommon for many classes of pesticides, such as insecticides, herbicides, and fungicides, to be used on the same crop (National Agricultural Statistics Service 2005). In the case of insecticides, an adjuvant is often added to the formulations to enhance the intensity of the lethal effect. In the case of herbicides, due to the increasing incidence of plant tolerance to a specific pesticide, some formulations now have as many as three active ingredients (MeisterPRO 2004). Each active ingredient has a specific mode of action for controlling a pest, and each active ingredient has its own possible side effects on the wildlife and humans exposed to it. It is impossible to determine the cumulative risk posed to wildlife and humans as the result of releasing vast amounts of pesticide mixtures into the environment.

The quality and quantity of the data about the risk posed to humans by individual pesticides vary considerably. In some instances there are numerous studies about the health effects of a particular pesticide in humans and laboratory animals, and for others there are very few. In general, the longer the active ingredient has been on the market, the greater the number of citations in the peer-reviewed literature. Data are sparse when linking pesticides with neurodevelopmental effects other than for the insecticides chlorpyrifos (CPF),

parathion, and 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT).

Unlike obvious structural defects, most neurodevelopmental effects cannot be seen at birth or even later in life. Instead, adverse effects on the nervous system are expressed in terms of how an individual behaves or functions. Behavior and function vary considerably from birth through adulthood. Functional deficits are not "on" and "off" conditions but instead range from inconsequential through very mild to very severe to totally debilitating. Consequently, it is difficult to quantify neurodevelopmental impairment. Some of the end points used in the laboratory to detect functional impairment of the brain and nervous system are measured at the gene, cell, biochemical, and/or physiologic levels and often require high-tech instrumentation to quantify. At the human level, a battery of tests is continuing to evolve to measure with increasing sensitivity psychomotor, psychologic, clinical, and psychiatric symptoms to better quantify functional impairment.

In this article I have two principal purposes in discussing the inherent risks of using pesticides, the limitations of testing techniques, and the intrinsic incompleteness of all scientific evidence: *a*) to encourage the use of the open literature about the neurodevelopmental effects of all classes of pesticides when setting the criteria for determining their safety and *b*) to encourage a more rigorous regulatory approach to protect human and environmental health in the absence of complete scientific certainty. I begin by presenting unequivocal evidence of pesticide exposure to numerous classes of pesticides during development. This is followed by a section on human epidemiology where only weak data are available linking neurodevelopmental impairment with pesticides.

Next, I present a case study of how CPF cryptically interferes with brain development one stage after another. This is followed with selected laboratory studies demonstrating that other insecticides as well as other pesticide classes target prenatal brain development similar to CPF and share similar and sometimes diverse impacts on the construction and function of the brain. As the data reveal, not only insecticides but other classes of pesticides, such as herbicides and fungicides, can also interfere with neurodevelopment. In the "Discussion" I challenge the protective value of current pesticide risk assessment strategies in light of the vast numbers of pesticide products on the market with untold numbers of targets and mechanisms of action that can cause neurodevelopmental damage.

Evidence of Exposure to Pesticides

Improvements in analytical laboratory equipment and testing procedures have made it easier to detect pesticides and their metabolites at very low concentrations in almost all human tissue. From routinely detecting parts per million (milligrams per kilogram) and more recently to as low as parts per trillion (picograms per kilogram), some laboratories are now able to measure concentrations down to parts per quintillion (femtograms per kilogram). The development of noninvasive sampling methods, such as testing for pesticides and their metabolites in urine, has made it possible to monitor pesticide exposure in infants and children. It is fairly safe to say that every child conceived today in the Northern hemisphere is exposed to pesticides from conception throughout gestation and lactation regardless of where it is born. The herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) was found in approximately 50% of semen samples provided by 97 Ontario, Canada, farmers

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(Arbuckle et al. 1999). The herbicides atrazine, metolachlor, alachlor, and 2,4-D and the insecticides diazinon and the CPF analyte 3,5,6-trichloro-2-pyridinol (TCP) were found in semen of men in central Missouri and in urban Minneapolis, Minnesota (Swan et al. 2003); the insecticides chlordane, dichlorodiphenyldichloroethylene (DDE), heptachlor epoxide, and hexachlorobenzene (HCB) were found in ovarian follicular fluid from women undergoing *in vitro* fertilization in Halifax, Hamilton, and Vancouver, Canada (Jarrell et al. 1993); hexachlorocyclohexane and *p,p'*-DDE were found in amniotic fluid of women undergoing routine amniocentesis in Los Angeles, California (Foster et al. 2000); and nonpersistent pesticides were found in the amniotic fluid of women referred for amniocentesis in the agricultural San Joaquin Valley, California (Bradman et al. 2003). Pesticides were also found in maternal blood, placental, and umbilical cord blood from women experiencing normal births and stillbirths in India (Saxena et al. 1983), from urban and rural mothers during Caesarian section in the Atoya River basin, Nicaragua (Dorea et al. 2001), and from mothers delivering normal and subnormal weight babies (Siddiqui et al. 2003). In addition, pesticides were found in the breast milk of mothers who delivered by Caesarian section in Nicaragua (Dorea et al. 2001), native Alaskan mothers living an indigenous lifestyle (Simonetti et al. 2001), and women living in southwest Greece (Schinas et al. 2000). A median of 8.26 µg/mL CPF (range, 0.40–458.04 µg/mL) was discovered in the meconium of newborns in Manila, Philippines (Ostrea et al. 2002). Six organophosphate (OP) pesticide metabolites were found in the meconium of 20 newborns in New York City (Whyatt and Barr 2001). The babies' first bowel movements held concentrations 10–100 times higher than their cord blood. One metabolite, diethylthiophosphate, was found in all 20 samples; another, diethylphosphate, was found in 19 of 20 samples. Both are metabolites of diazinon, CPF, and several other OP insecticides.

An eastern Washington State research team surveyed OP metabolites in the urine of 210 farmworkers and their children and in dust from their homes and vehicles (Coronado et al. 2004). They segregated farm chores into several classes: harvesting and picking, thinning, loading, transplanting, and pruning. Azinphosmethyl, an OP, was more often found in dust in thinners' homes (92.1% vs. 72.7%) and vehicles (92.6% vs. 76.5%) than in those of workers who did no thinning. Thinners' children had higher concentrations of OP metabolites in their urine, and the metabolites were found more frequently in the children (91.9% detectable in urine), compared to the adults (81.3% detectable; $p = 0.002$).

In Seattle, Washington, investigators measured five OP metabolites in 24-hr urine samples of preschool children (2–5 years of age) who were raised on either a predominantly organic ($n = 18$) or predominantly conventional diet ($n = 21$) (Curl et al. 2003). Pesticide use was also recorded for each home. Median total dimethylphosphate metabolites (0.06 µmol/L) were significantly higher than median total diethyl alkylphosphates (0.02 µmol/L; $p = 0.0001$) in the urine. Those children on a conventional diet had levels of dimethylphosphate metabolites six times higher than those of children on an organic diet (medians = 0.17 and 0.03 µmol/L, respectively; $p = 0.0003$). Median concentrations of both metabolites were almost an order of magnitude higher in the conventionally fed children (0.34 µmol/L vs. 0.04 µmol/L). There were no age differences in the children in the two groups. Home use of pesticides varied, with seven conventional-diet families using OPs versus three organic-diet families using OPs. Although the study group was small and there were difficulties collecting urine samples, this research provides the first empirical data comparing urinary levels of pesticides in youngsters consuming predominantly organic versus conventional diets.

Human Epidemiology

Determining a link between fetal exposure to a specific chemical and long-term expression of a change in health poses a monumental challenge when designing epidemiologic studies. For example, one human epidemiologic study uncovered weak but statistically significant associations between neurodevelopmental impairment as a result of exposure to two pesticides during gestation. In a large study of live births ($n = 1,532$), including 536 children fathered by pesticide applicators, Garry et al. (2002) discovered that “adverse neurologic and neurobehavioral developmental effects clustered among the children born to applicators of the fumigant phosphine [odds ratio (OR) = 2.48; 95% confidence interval (CI), 1.2–5.1].” They also discovered an OR for the herbicide glyphosate (Roundup) of 3.6 (95% CI, 1.3–9.6). Among the children in the phosphine group ($n = 290$), two were diagnosed with autism, which is high compared with the prevalence nationwide, and five were diagnosed with attention deficit disorder/attention deficit hyperactivity disorder (ADD/ADHD). It took years of close interaction with the families in this study to be able to track their pesticide exposure without having to resort to recall and to follow the children's functional development (Garry VF, personal communication). The investigators were cautious about their findings and asked for confirmation.

Another study suggests that CPF might have an effect on head circumference related to

the activity of paraoxonase (PON1), an enzyme that can detoxify CPF before it can inhibit acetylcholinesterase (Berkowitz et al. 2004). Babies with a small reduction in head circumference were from mothers whose TCP concentrations were above the detection limit, and their PON1 activity was in the lowest tertile ($p = 0.014$). Mothers and their infants ($n = 404$) were recruited from East Harlem and other sections of New York City.

In a more recent study, Young et al. (2005) looked at the relationship between maternal OP urine metabolites and infant neurodevelopment. They employed a battery of tests using the Brazelton Neonatal Behavioral Assessment Scale for habituation, orientation, motor performance, range of state, regulation of state, autonomic stability, and reflex in 381 infants younger than 62 days of age. Young et al. (2005) found a significant association between increasing total concentrations of maternal urine OP metabolites representing “approximately 80% of OPs used in the Salinas Valley” and increasing numbers of abnormal reflexes in the infants from days 3 to 62. The median age for testing the infants was day 3. Mothers' urine was tested at 14 and 26 weeks during gestation and at day 7 postpartum. The median urine levels of dialkyl phosphate (DAP), dimethyl phosphate, and diethyl phosphate, respectively, were 132, 97, and 21 mol/L during gestation and 222, 160, and 27 nmol/L after delivery. DAP represents the total of diethyl and dimethyl phosphate metabolites. The dimethyl metabolites could reflect exposure to malathion, oxydemeton-methyl, dimethoate, naled, and methidathion, and the diethyl metabolites could reflect exposure to diazinon, CPF, and disulfoton used in the Salinas Valley. It is important to keep in mind that the OPs are readily metabolized, and exposure can vary considerably and most often is transient and unpredictable. The authors noted that there were large within-person variations in urine levels in this study.

A Case Study: The Cryptic Neurodevelopmental Effects of CPF

The insecticide CPF is an OP pesticide that has been on the market since 1965 to control insects in agriculture, gardens, building construction, and households. In 2002 the use of CPF was restricted to only agricultural applications, and all domestic use was to be completely phased out by 1 January 2005. The metabolites of CPF have been widely reported in human tissue. In a study based on data from the Centers for Disease Control and Prevention's (CDC 2001) first *National Report on Human Exposure to Environmental Chemicals*, Hill et al. (1995) found the CPF analyte TCP in 82% of urine samples ($n = 1,000$) from a broad sample of the U.S.

population between the ages of 20 and 59 years from all regions of the country. The CDC's *Second National Report on Human Exposure to Environmental Chemicals* (CDC 2003) states that the levels of TCP were similar to levels presented in the first *National Report on Human Exposure to Environmental Chemicals* (CDC 2001) but gave no statistics concerning the extent of exposure across the population. Like the other OP insecticides, CPF inhibits the enzyme acetylcholinesterase, which destroys acetylcholine, the neurotransmitter that activates cholinergic neurons. These are an important group of nerve cells that control signals in the peripheral nervous system and in the brain and spinal cord. If acetylcholine is not inactivated immediately by the activity of acetylcholinesterase, it overstimulates the neurons, and tremors, convulsions and death can follow.

As scientists probed deeper into the activity of CPF, a wealth of information surfaced from laboratory studies about its effects on the development and function of the brain and nervous system in embryos, fetuses, and young animals. Although many of the studies were performed on rats and there are differences in the ontogeny of specific parts of the brain between rats and humans, the development of the rat brain through postnatal day (PND) 21 provides a model for the development of the human brain through to birth.

A series of reports starting in 1991 confirmed that CPF is a cholinesterase inhibitor and that neonatal rats were more sensitive than adults when exposed to a single maximum tolerated dose (Pope and Chakraborti 1992; Pope et al. 1991, 1992). These studies also confirmed that the fetus recovers quicker than the adult from cholinesterase inhibition, suggesting that the fetus would be protected from CPF if all the adverse effects were due to cholinesterase inhibition alone. Lassiter et al. (1998), however, wrote that although the fetus could recover faster between repeated doses of CPF, this was only an "illusion that the fetal compartment is less affected than the maternal compartment." Realizing that something other than cholinesterase inhibition was affecting the fetus, a team from Duke University led by Theodore Slotkin gradually began to demonstrate that other mechanisms of action of CPF alter prenatal development of the brain and behavior and that the embryo and fetus are sensitive to cholinesterase inhibition at doses that would not be toxic to an adult (Qiao et al. 2003; Slotkin 2004). These studies provided information about how the brain develops and functions and also provided a chronology of how CPF interferes at successional stages of brain development (Qiao et al. 2002). This team also demonstrated that CPF-oxon, the active metabolite of CPF, is the compound that causes cholinesterase inhibition and that

the actual neuroteratogen is CPF (see Slotkin 2004 for a step-by-step description of how their CPF research progressed).

Slotkin and colleagues demonstrated that as the brain and nervous system are constructed and programmed, there are numerous points in time and at sites where CPF could interfere. CPF attacks the neurons that appear in the earliest stage of brain and central nervous system (CNS) development (Qiao et al. 2004). Neurons process information and are the signaling or transmitting elements in the nervous system. Damage to neurons at this early stage may not be expressed until years later. For example, a brief subtoxic dose of CPF [1 or 5 mg/kg body weight (bw)/day] during neurulation can cause behavioral alterations during adolescence and adulthood (Icenogle et al. 2004). And, although some early symptoms of CPF exposure disappear during certain stages of development, different neurologic symptoms can appear later in life (Qiao et al. 2002, 2003, 2004).

Glial cells that appear later than neurons during early development were shown to be more vulnerable than neurons to CPF (Qiao et al. 2002; Roy et al. 2004). There are more than twice as many glial cells (> 200 billion) in the body than neurons. Glial cells come in many varieties; they are supportive cells critical for normal development and function and serve as a "scaffold" for migration of cells during tissue construction [see Barone et al. (2000) on brain development]. Glial cells also provide nutrition to the neurons and provide a link with the immune system, responding to damage by acting as scavengers of pathogens and neuronal debris. CPF preferentially targets the glial cells among the cells it attacks (Garcia et al. 2002).

Slotkin and colleagues repeatedly demonstrated that CPF toxicity is not limited to cholinesterase inhibition alone but can act by other mechanisms. For example, *in vitro* and *in vivo* studies at three levels of development from DNA to the cell and the whole animal revealed that CPF is far more toxic than previously thought because of this wider range of activity (Crumpton et al. 2000). CPF impairs the binding to DNA of nuclear transcription factors (AP-1 and Sp1) that modulate cell replication and differentiation. When undifferentiated and differentiated neurons were exposed to CPF, the response of some transcription factors varied. Although the activity of one set of cells might not be affected, the activity of another set of cells might be significantly reduced. An independent study at Johns Hopkins University (Schuh et al. 2002) confirmed the ability of CPF to alter the activity of another nuclear transcription factor in cortical neurons, the Ca^{2+} /cAMP response element binding protein (CREB), which is critical for cell survival and differentiation during

development and is critical for memory. CPF increased the activated level of CREB at 0.01 nM, well below the level at which cholinesterase inhibition is expressed and below the typical level of human exposure. Schuh et al. (2002) also demonstrated that CPF-oxon did not cause the alteration, supporting the conclusion of Crumpton et al. (2000) that CPF is more than a cholinesterase inhibitor. Crumpton et al. (2000) also demonstrated that the CPF effects on the development of the forebrain in the rat, which reaches its peak stage of development during gestation, were not as severe as the effects on the cerebellum, which reaches its peak 2 weeks after birth. The cerebellar changes in the later stages of development, however, could not have been the result of cholinesterase inhibition because the cerebellum is not innervated with cholinergic receptors like the forebrain is (Crumpton et al. 2000).

Much of the research undertaken by Slotkin and colleagues demonstrated that models of adult toxicity do not extrapolate to fetuses and would not predict the vulnerability of the embryo to TCP and CPF (Aldridge et al. 2004, 2005a). The ever-changing state of the embryo makes it a more sensitive model for toxicity and a better predictor of long-term, delayed effects. Slotkin and colleagues have demonstrated that the embryo and fetus reveal innumerable mechanisms of action of toxicity that could not be detected in an adult animal. For example, in a series of *in vitro* studies, a 25% increase in reactive oxygen species (ROS) was found 10 min after undifferentiated glial C6 cells were exposed to CPF (Garcia SJ et al. 2001). During some stages of development, selected regions of the brain are vulnerable to CPF by interference with the G-protein in the adenylyl cyclase (AC) cascade by disrupting nuclear transcription DNA binding (Meyer et al. 2003; Slotkin 1999). CPF caused abnormal tissue/cell development in cultured rat embryos through vacuolation of the cytoplasm (Roy et al. 1998). CPF, CPF-oxon, and TCP inhibit DNA synthesis in PC12 cells (typical neuronal cells) and C6 cells (typical glial cells), having a greater effect on the glial cells, with the exception of the TCP (Qiao et al. 2001). Qiao et al. (2001) also showed that CPF is a stronger DNA synthesis inhibitor than CPF-oxon, although it is a weaker cholinesterase inhibitor. Confirming again that certain regions of the developing brain were more susceptible than others, Qiao et al. (2001) found that CPF and TCP suppress DNA synthesis in the epithelium of the forebrain and inhibit neural cell replication. These studies also revealed that serum binding proteins can be protective of DNA antimitotic activity, but because fetuses and newborns have lower concentrations of serum proteins than adults, they could be more vulnerable.

In a series of whole-animal studies looking at damage in rats from the embryo to the adult, Slotkin and colleagues demonstrated again that assays using adult animals cannot predict the long-term delayed effects in the offspring. For example, within hours after 9.5-day-old embryos were exposed to CPF, they showed clear signs of damage that was restricted to the primordial brain (Roy et al. 1998). Upon histologic examination, Roy et al. (1998) found apoptosis and altered mitotic figures, along with gross disruption of the architecture of the developing brain, all in the absence of any gross morphologic defects in the other parts of the embryo. As these animals matured, CPF damage was demonstrable in a wide variety of brain regions. The most vulnerable target was the hippocampus, with the damage expressed both as deficits in nerve activity and as corresponding behavioral abnormalities (Icenogle et al. 2004). Dosing an adult animal similarly would not have provoked these effects of fetal origin.

The complexity of the toxicity of CPF became more apparent as sex-related differences began to appear in *in vivo* assays. The sex-related changes occur when CPF exposure takes place during gestation days (GD) 17–20 (late gestation) and PND1–4 and again at PND11–14. The timing of this exposure in the rat is comparable to human brain development during the perinatal and neonatal period (Aldridge et al. 2004; Meyer et al. 2004a; Slotkin et al. 2001). Late prenatal exposure to CPF has also been shown to cause long-term sex-specific changes in cognitive performance (Levin et al. 2002). Adolescent and adult females were more vulnerable to CPF, based on their number of errors during working- and reference-memory tasks. Levin et al. (2002) also found profound differences between animals exposed to 1 mg/kg and 5 mg/kg CPF, reflecting a U-shaped dose curve. The lowest dose was the most potent in this case, although the highest dose caused the most inhibition of fetal brain cholinesterase. The non-monotonic dose–response curve discovered in the assay, combined with the fact that the results were not dependent on cholinesterase inhibition, raises questions about indirect effects of CPF and its metabolites on the endocrine system via the brain. However, as Slotkin (personal communication) pointed out, hormesis cannot be ruled out until further research proves otherwise. In light of their findings, Levin et al. (2002) noted the need for childhood and adolescent maturation studies and for the development of more sex-selected end points.

At a concentration somewhat higher than human exposure, 50 µg/mL CPF *in vitro* induces the release of norepinephrine from rat brain synaptosomes (Dam et al. 1999). Studies using whole animals confirmed that the release of norepinephrine inhibits synaptogenesis, a

condition that persists to adulthood and is sex specific, long after exposure ceases and cholinesterase activity is restored (Levin et al. 2002). Aldridge et al. (2004) showed that CPF administered during GD9–12 up-regulated serotonin (5-hydroxytryptamine; 5-HT) receptors (5-HT-1 and 5-HT-2) and interfered with the 5-HT protein transporter from the neural tube stage through to adulthood. But during GD17–20, CPF initiated larger effects in regions with greater numbers of 5-HT nerve terminals, which were found more in males. This response continued through PND1–4. In contrast, the 5-HT protein transporter was downregulated in females (Aldridge et al. 2004). Aldridge et al. (2005a,b) performed studies demonstrating abnormalities of 5-HT-related behaviors in developing rats exposed to CPF. The research that preceded this report mapped out the ontogeny of serotonin receptors in the brainstem and forebrain (Aldridge et al. 2003). The authors pointed out that serotonin disruption has been linked to appetitive and affective disorders, and the biologic significance of these findings needs to be clarified. These disorders have been the focus of increasing research attention in recent years as the result of the increasing use of prescription and illicit mind-altering drugs.

Other Pesticide Products That Interfere with Neurodevelopment

There are numerous opportunities during gestation where insecticides and products from several other chemical classes can alter the purpose of a cell, tissue, organ, or system function in the brain or CNS, much like the discoveries presented for CPF.

Herbicides. Over the past 15 years, an Argentinian research team has produced a series of reports on 2,4-D that is comparable to the research on CPF. This team discovered that exposure during lactation to the herbicide 2,4-DBE (the butyl ester of 2,4-D) can alter brain production of 5-HT and its metabolite, 5-hydroxyindoleacetic acid (5-HIAA), in adulthood (Bortolozzi et al. 2001; Evangelista de Duffard et al. 1990; Garcia G et al. 2001). Concentrations of both dopamine and serotonin changed transiently if the animals were exposed only through birth (69 mg/kg bw/day from GD6 to birth; 15 days) and permanently if delivered to the offspring through breastfeeding as well from GD6 to weaning (30 days). Duffard et al. (1996) and Rosso et al. (2000) found that 2,4-D interfered with myelination in the brain as the result of lactational exposure. This caused changes in behavior patterns that included apathy, reduced social interaction, repetitive movements, tremors, and immobility in pups exposed to 2,4-D (Bortolozzi et al. 1999; Evangelista de Duffard et al. 1995). They also discovered that the

serotonergic and dopaminergic effects occurred during postnatal brain development, similar to the effects of CPF. Bortolozzi et al. (1999) and Evangelista de Duffard et al. (1995) also found 2,4-D in breast milk of 2,4-D-fed mothers and in the stomach content, brain, and kidney of 4-day-old pups (Sturtz et al. 2000).

Insecticides. Cassidy et al. (1994) reported that the lowest dose of chlordane used in their studies (100, 500, 5,000 ng/g/day both prenatally and postnatally) caused a dose-dependent reduction in testosterone levels in females in adulthood. The lowest dose they used was 10 times lower than the U.S. EPA's lowest observed adverse effect level (LOAEL) for neurologic effects (1,000 ng/g) and 50 times lower than the U.S. EPA's LOAEL for developmental effects (5,000 ng/g) of chlordane (Cassidy et al. 1994). Females exhibited improved spatial abilities and auditory startle-evoked responses more similar to male responses, and slight increases in body weight. Changes in male mating behavior included shortening of latency to intromission and increased intromissions. The authors speculated that pesticides structurally similar to chlordane cause masculinization of function and behavior in both sexes because the pesticides mimic the sex steroids or change their plasma levels through other enzyme systems. The two lower doses in this study prompted greater change than the highest dose for auditory startle response, mating behavior, and body weight.

Methoxychlor (MXC), an insecticide whose toxicity depends on its conversion to several metabolites, was considered to be an estrogen for many years and only recently was discovered to have antiestrogenic and androgenic properties as well. To measure neurodevelopmental impacts, Palanza et al. (2002) fed pregnant CD-1 mice environmentally relevant doses of MXC (0.02, 0.2, and 2.0 µg/g mother bw/day) from GD11 to GD17 and examined them on postpartum days 2–15. Mothers fed the lowest dose spent less time nursing than the controls, possibly reflecting the inverted U-shaped dose–response curve expressed by endocrine disruptors. At late adolescence the pups exhibited a reduction in novelty seeking (both the environment and objects), with a difference between males and females (Palanza et al. 1999). Male sexual aggression was reduced at puberty but returned to normal in adulthood. The reduction in aggressive behavior in the periadolescent male CD-1 mouse as a result of MXC exposure (20 µg/kg/day) occurred at a dose 100 times lower than the dose at which the Agency for Toxic Substances and Disease Registry (ATSDR 2002) deemed would cause no harm to humans in 1994. The ATSDR recently withdrew this minimum risk level in light of new evidence on MXC.

Dopaminergic neurons in the substantia nigra project to and release dopamine to the corpus striatum of the brain. This section of the brain integrates neuromuscular and behavioral information and is involved in the control of locomotor activity, exploration, and novelty-induced behavior. It also influences social–sexual interactions such as aggression and maternal behavior. The loss of dopamine function in the neurons connecting the corpus striatum with the midbrain of humans is the cause of Parkinson disease. Male offspring of mice exposed to 20 µg/kg/day MXC had fewer dopaminergic receptors in their corpus striatum and were less active than control females (vom Saal et al. 2003). Females exposed to the same concentrations showed a malelike profile in reactivity to novelty. Similar changes in males and females were seen in mice exposed to *o,p'*-DDT in the same study. In an unrelated study, Lamberson et al. (2001) discovered increased locomotor behavior in offspring of Sprague–Dawley rats administered 0.5 mg/kg/day MXC throughout gestation.

Prenatal exposure to aldrin also causes delayed neurologic impairment that extends through to adulthood. Castro et al. (1992) administered 1 mg/kg aldrin subcutaneously to female rats daily from conception to birth and tested their pups on PND1–2 and again on PND90. On PND90, the animals showed loss of locomotor control and behavioral change(s). Aldrin was not measurable in the animals at the time they were tested.

Paraoxon is the oxidized metabolite of parathion and a potent OP cholinesterase inhibitor. Chronic paraoxon exposure (0.1, 0.15, or 0.2 mg/kg subcutaneously) during a stage of rapid cholinergic brain development from PND8 to PND20 in male Wistar rats led to reduced dendritic spine density in the hippocampus without obvious toxic cholinergic signs in any of the animals (Santos et al. 2004). Some animals in the two highest dose groups died in the early days of the study. All doses caused retarded perinatal growth, and brain cholinesterase activity was reduced 60% by PND21.

Johansson et al. (1995) showed that a single exposure to a pesticide before or shortly after birth can sensitize the offspring to low doses of other pesticides later in life, even though there are no immediate changes in the structure and function of the nervous system at the time of exposure. Only as the exposed individual matures do irreversible alterations in structure and function become evident. The researchers exposed mice to one dose of DDT (0.5 mg/kg bw orally) on PND10 and then at 5 months of age exposed them to bioallethrin (0.7 mg/kg bw) (Johansson et al. 1995) or paraoxon (0.7 or 1.4 mg/kg bw) for 7 days (Johansson et al. 1996). When tested 2 months

later, at 7 months of age, the offspring exhibited changes in spontaneous behavior and cholinergic muscarinic receptor density in the cerebral cortex, which led to impairment in learning and memory (Eriksson and Talts 2000). Again, the neurodevelopmental damage was not seen immediately, but instead took 2 months to be expressed. PND10 in the mouse is equivalent to the end of the second trimester in the human. It is during this stage, from the third trimester of pregnancy through 2 years of age in humans, when the neurotransmitter system in the CNS goes through a growth spurt (Eriksson 1997). Throughout these studies the animals showed no clinical signs of toxic symptoms, and the doses used for adult treatment in these studies had no immediate effect on the adult. The dose of DDT used in this study is in the range that human infants might be exposed to during lactation today (Smith 1999). Even though the functional and structural outcomes in the above studies are similar, it should be remembered that they were caused by different mechanisms. For example, bioallethrin causes harm by prolonging sodium channel openings, whereas paraoxon inhibits acetylcholinesterase activity; but they both caused similar neuronal changes, which raises questions about the combined effects of pesticide mixtures on development. These studies support the premise that the differences in susceptibility of adults to pesticides may not be genetic, but rather that susceptibility to pesticides can be acquired by low-dose pesticide exposure earlier in life.

Insecticide and acaricide. Rat pups displayed deficits in learning and retention of memory after exposure to the organochlorine insecticide and acaricide endosulfan (6 mg/kg bw) on PND2–25 (Lakshmana and Raju 1994). The concentrations of the neurotransmitters, noradrenalin, dopamine, and serotonin in the olfactory bulb, hippocampus, visual cortex, brainstem, and cerebellum either increased or decreased depending on the days of examination, PND10 and PND25. The authors ruled out acetylcholinesterase inhibition as the cause of the alterations in the production of the neurotransmitters because they found no differences in acetylcholine activity in any of the regions of the brain used in the study. They suggested that endosulfan directly led to a “re-altering” of the construction of those parts of the brain. By PND25, as the differentiation and organization of the observed tissues proceeded in the presence of endosulfan, the rats’ performance became significantly compromised.

Fungicides. Gray and Ostby (1998) provided an excellent overview of how prenatal exposure to a fungicide can alter sexual behavior and function in adulthood, even though growth and viability are not compromised. The neurobehavioral alterations quantified in

the studies they reviewed include activity level, aggression, mounting frequency, and completed intromissions. In a study using the fungicide vinclozolin, Gray et al. (1994) reported that 100% of the exposed males failed to attain intromission, although there was no reduction in mounting behavior. In subsequent studies, newborn male and female rats were injected on PND2 and PND3 with 200 mg/kg vinclozolin and observed for social behavior on PND36 and PND37 (Hotchkiss et al. 2002). Both males and females exhibited changes in play behavior. Females became involved in increased rough-and-tumble play, a behavior imprinted by male hormones in the brain during early development. Conversely, the males’ rough-and-tumble play was reduced, and they behaved more like unexposed females. Because only one dose was used, this study does not indicate the lowest dose needed to initiate these changes. More recently, on PND34 Colbert et al. (2005) found significantly increased nape contact, pounce, pin, and wrestle play behavior in male offspring of females exposed to 6 and 12 mg/kg bw/day vinclozolin from GD14 to PND3. At a maternal dose of 1.5 mg/kg bw/day vinclozolin, there was a significant increase in penile dysfunction in adulthood. Future studies should include more than one dose, preferably over several orders of magnitude, to take into account the susceptibility and sensitivity of the developing animal.

Discussion

There is a great deal of uncertainty about the neurodevelopmental effects of pesticides among the human studies presented here. Exposure has become too complex because of the hundreds of pesticide active ingredients on the market, confounded by background exposure to industrial chemicals that share similar effects. In addition, functional changes are expressed over a continuum, making it difficult to document the damage which often is expressed as more than one lesion and at different intervals or stages of development. The pesticides discussed here, with the exception of DDT, are still widely used in the United States despite these data. Although this information is available, the U.S. EPA has rarely used the open literature in its risk assessments, generally using only data submitted by manufacturers. Industry continues to use traditional toxicologic protocols that test for cancer, reproductive outcome, mutations, and neurotoxicity, all crude end points in light of what is known today about functional end points. In using manufacturer data, the U.S. EPA misses almost all delayed developmental, morphologic, and functional damage of fetal origin and, in the case of CPF and all OPs, continues to rely primarily on blood cholinesterase inhibition data in risk assessments (Zheng et al. 2000). The

U.S. EPA should accept nonguideline, open literature to determine the toxicity of a chemical. For example, Brucker-Davis (1998) published a comprehensive review of the open literature in which she found 63 pesticides that interfere with the thyroid system—a system known for more than a century to control brain development, intelligence, and behavior. Yet, to date, the U.S. EPA has never taken action on a pesticide because of its interference with the thyroid system.

It would be difficult to find another pesticide in use today that has been as systematically studied as CPF. The amazing litany of diverse mechanisms discovered in the series of CPF studies raises serious questions about the safety of not only CPF and the other OPs but all pesticides in use today. Most astounding is the fact that a large part of CPF's toxicity is not the result of cholinesterase inhibition, but of other newly discovered mechanisms that alter the development and function of a number of regions of the brain and CNS. These findings send a warning that even though an OP pesticide like CPF may have a very high EC_{50} (concentration that produces 50% of the maximum possible effective response) for acute toxicity as a result of cholinesterase inhibition, it may have other toxic strategies that are far more egregious than cholinesterase inhibition. This raises a question about the value of using EC_{50} values if they do not represent the most sensitive end point. Qiao et al. (2003) warn that "developmental neurotoxicity consequent to fetal or childhood CPF exposure may occur in settings in which immediate symptoms of intoxicification are absent." They also point out that in the case of CPF, damage is not always global (referring to the entire brain) but may only interfere in specific regions of the brain during development, which could increase the difficulty of detecting the damage. S.J. Garcia et al. (2001) state that "measurement just of cholinesterase activity is a questionable approach in assigning an appropriate index of safety."

The knowledge gained from a decade of the CPF/brain studies by Slotkin and colleagues and the 2,4-D/brain studies by Evangelista de Duffard and co-workers not only demonstrates the insidious nature of CPF and 2,4-D exposure, but it also demonstrates the weaknesses in current standard practices for determining the safety of a pesticide or any other synthetic chemical. These discoveries demonstrate that a much larger battery of tests must be used when determining the safety of commercial pesticides. Even a U.S. EPA analysis of developmental neurotoxicity studies stated that the U.S. EPA's current developmental neurotoxicologic testing protocol is "not a sensitive indicator of toxicity to the offspring" and urged the U.S. EPA "to further consider if it will use literature data" (Makris

et al. 1998). In this case, "literature data" refers to all of the peer-reviewed reports concerning the pesticide impacts on neurodevelopment that heretofore have not been used for risk assessment by the agency. In the case of CPF and 2,4-D, it appears that those who reviewed the data failed to understand its significance or had other reasons to ignore it. The U.S. EPA needs to convene a panel of independent experts to review these studies for applicability to determine if and how they can be used for registration.

Laboratory studies have clearly revealed neurologic damage after exposure to specific pesticides and in some studies at concentrations equivalent to ambient exposure. Even so, the animal testing for regulatory purposes that takes place today does not attempt to detect adverse health effects at the concentrations at which humans are exposed. Instead, the highest concentrations of chemicals tested are those that can be used without killing the animals or reducing the test mother's weight and her reproductive ability. In most animal studies the pesticides are administered at high oral or subcutaneous doses orally, not reflecting that, for most humans and wildlife, exposure could in many instances be dermal or via inhalation and, in many cases, over a long period of time at low doses. The U.S. EPA currently requires chronic toxicity studies, but it is locked into using high doses to elicit effects and has not overcome the difficulty of detecting effects from chronic or ambient exposure or low doses. In addition, the human pharmacokinetics of pesticide exposure can either enhance or reduce the health impacts depending on individual variations. In some cases the major or minor metabolites are more toxic than the parent compound, which is listed as the active ingredient.

In a recent study, Bowers et al. (2004) found a different profile of developmental neurotoxicity between polychlorinated biphenyls (PCBs; such as Aroclor 1254) alone and with a mixture of organochlorine pesticides. Very low doses of the chemicals together delayed ear opening, affected geotaxis, and reduced grip strength. Ultimately, mortality, growth, thyroid function, and neurobehavioral development were affected. It is safe to say that there are very few people in the developed world today who are not carrying PCBs in their bodies. If animal testing continues to be used for determining the safety of pesticides, at least one group of the test animals should be exposed to PCBs before testing the pesticides for their ability to cause unpredictable interactive effects such as those described above.

It should be pointed out that the same signaling systems (AC cAMP) involved in the sex-selective changes in brain development have also been shown to alter heart and liver function in adulthood (Meyer et al. 2004a, 2004b).

The AC system is ubiquitous throughout the body. In the future, the most efficient, comprehensive assays will take advantage of the fact that most chemicals have more than one effect in one system. Cross-disciplinary teams will be required to design these assays so that every organ system is carefully screened for damage. And most important, this will reduce by thousands the numbers of animals needed for testing. However, improved neurodevelopmental tests with laboratory animals will not fulfill their greatest potential if they are not backed up by better batteries of tests to detect functional disabilities in children. Such new, sophisticated quantitative tests are now available and are being updated regularly. These tests go beyond diagnostic testing to "performance evaluation" and are designed to detect the subtle effects of chronic, low-dose exposure (Davidson et al. 2000).

In conclusion, an entirely new approach to determine the safety of pesticides is needed. It is evident that contemporary acute and chronic toxicity studies are not protective of future generations. The range of doses used in future studies must be more realistic, based on levels found in the environment and human tissue. In this new approach, functional neurologic and behavioral end points should have high priority, as well as the results published in the open literature. In every instance, the impacts of transgenerational exposure on all organ systems must be meticulously inventoried through two generations on all contemporary-use pesticides and new pesticide coming on the market. To protect human health, however, a new regulatory approach is also needed that takes into consideration this vast new knowledge about the neurodevelopmental effects of pesticides, not allowing the uncertainty that accompanies scientific research to serve as an impediment to protective actions.

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