

Project Summary

Title:

Effect of Clean and Polluted Groundwater on Reproduction and Development of *Daphnia*

Project I.D.:

R/UW-BEP-001

Investigator:

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Period of Contract:

1 July 2001 to 30 June 2002

Background/Need:

There is a need for an efficient and effective whole-animal screen for ecological effects of pollutants (see Table 1). Previous work in our lab has focused on herbicides and vertebrate hormones. Several of these chemicals changed normal *Daphnia* development and sex determination, at concentrations found in groundwater. The current proposed research focuses on laboratory assays of low-level concentrations of common-use insecticides that have been reported to be contaminants of groundwater. Information on insecticide effects will contribute toward our evaluation of the *Daphnia* reproduction assay. This assay has been developed and used successfully in the lab for a number of chemical contaminants. Preliminary results suggest that it is crucial to survey insecticides. Our assays provide information on "contamination," (whether due to parent compounds, breakdown products, and chemical mixtures) because we look at the whole-animal response. This is an important feature, because there are so few cost-effective assays that can detect sublethal effects on whole organisms.

Our ultimate goal is to establish the *Daphnia* development and reproduction assays as a surrogate, cost-effective bioassay system for risk assessment. Our results suggest these assays do have value, because *Daphnia* are sensitive to ambient concentrations of contaminant herbicides, and because *Daphnia* respond in characteristic ways to some vertebrate hormones such as thyroxine and some steroid hormones.

Objectives:

The project objective was to characterize effects of common-use insecticides classified as endocrine disruptors on development and sex determination of *Daphnia magna*, using well-established short- (six day) and long-term (30 day) life-table type assays. The endpoints include:

- growth in length, molting frequency, and population growth rate
- fecundity and sex ratio
- deviations from normal morphology in neonates and adults.

Methods:

We exposed *Daphnia* to common-use insecticides in two kinds of assays. These assays have been developed and polished in our lab over the last few years. In the first assay, adult female *Daphnia* are grown under environmental conditions that naturally induce about 50 percent males. After six days (the equivalent of two instars or molting periods), and a renewal of culture medium, the offspring are scored as to gender, survival, and morphology, and the adult females are scored as to

survival, fecundity, morphology, and size. Animals grown in uncontaminated artificial lake water, or in water contaminated with low levels (100 ppb or lower) of a common-use insecticide that occurs in groundwater. In the second assay, neonates are followed with daily observations throughout their lifetime. Animals are measured each day, and we record details of reproduction and development.

Results and Discussion:

Effects of estrogen modulating compounds

Toxaphene (polychlorinated camphenes), an insecticidal mixture of over 670 congeners and widely classified as estrogenic, was the only chemical tested that affected sexual differentiation in *D. magna* (Fig. 1). *Daphnia magna* exposed to 50 and 100 µg/L toxaphene produced 17-44 percent more male clutches compared to the control *D. magna* ($p < 0.01$, Fig.1). In addition to increasing male production, toxaphene exposure (50 µg/L) decreased the average clutch size from 17 to 13 individuals ($p = 0.02$). At the higher concentration of 100 µg/L toxaphene had no effect on fecundity. Toxaphene exposure concentrations below 50 µg/L had no effects on reproduction or growth (Table 2).

Another putative estrogenic insecticide o'p'-DDT, did not alter the natural sex ratio at any of the concentrations tested. However, at 100 µg/L o'p'-DDT decreased survivorship, killing the majority of the *D. magna* by day three of the assay. Di-n-butyl phthalate had no observable effects on the developmental or reproductive endpoints examined in *D. magna* (Table 2).

Effects of thyroid modulating pesticides

Three herbicides reported as disrupting normal thyroid function in vertebrates; acetochlor, metribuzin and alachlor, did not affect sexual differentiation, survivorship, resting egg production, or morphology in *D. magna* (Table 2). The only thyroid modulating herbicide that appeared to have any effect on *D. magna* was acetochlor, and effects were seen below the listed EC₅₀ (48 hours) for *Daphnia* (16 mg/L; Tomlin 1994). An EC₅₀ value represents the concentration at which 50 percent of the organisms show any toxic effect (Effective Concentration). Adult *D. magna* with a six-day exposure to 100 µg/L acetochlor were significantly smaller (4.16 mm) than their respective control (4.28 mm; $p = 0.04$). However, the clutch size of the acetochlor-exposed *D. magna* was not affected by the smaller adult size, hence a smaller brood chamber.

Effects of pesticides with LH, androgenic, or insulin activity

Pesticides with reported activity in vertebrate androgen systems had no effects on *Daphnia*. The o'p'-DDT metabolite p'p'-DDE, which has been shown to function as a hormone (androgen) antagonist in vertebrates, did not impair reproductive or developmental processes in *Daphnia* at sublethal concentrations; however, p'p'-DDE was toxic to *D. magna* at 100 µg/L (Table 1 and 2). Likewise, the androgenic herbicide, linuron, had no toxic effects on *D. magna* at the concentrations tested.

Amitraz an insecticide that has been shown to inhibit insulin secretion in rats (Abu-Basha *et al.* 1999) did not exert toxicity on the daphnid developmental and reproductive processes monitored in this study. The herbicide 2,4-D that has been correlated with elevated LH levels in humans (Garry *et al.* 2001) had no observable effects on the developmental or reproductive endpoints examined in *D. magna*.

Effects of pesticides with no known endocrine activity

Five pesticides were examined that currently have no known impacts on vertebrate endocrine systems: cyanazine, diflufenzuron, chlorsulfuran, diquat and metolachlor. Exposure to 100 µg/L cyanazine significantly reduced the number of *D. magna* that reproduced to 23 percent ($p = 0.01$), while only four percent of the control daphnids failed to produce a clutch in the 12 days monitored. In addition, the average clutch size of the reproducing adult *D. magna* exposed to 100 µg/L cyanazine was significantly smaller ($p = 0.04$) with an average clutch size of 14 individuals vs. 11 individuals in the control. Cyanazine had no effect on sex determination.

Diflubenzuron was highly toxic to *D. magna*, significantly decreasing survivorship at 0.01 µg/L ($p=0.005$; Fig. 2). The LC₅₀ value for diflubenzuron in this six-day assay fell between 0.10 and 0.01 µg/L. Lower diflubenzuron concentrations elicited no adverse effects on growth/molting or reproduction of the daphnids (Table 2).

The remaining pesticides with no known endocrine activity, chlorsulfuran, diquat and metolachlor, did not affect *D. magna* at the concentrations tested (Tables 1 and 2). The herbicide chlorsulfuran has been reported in surface waters at very low concentrations (Table 1). This study indicates that these environmentally relevant concentrations appear to have no apparent effects on *Daphnia* (Tables 1 and 2). Diquat did not affect any of the reproductive or development endpoints monitored in *D. magna* at the concentrations tested. Metolachlor, an herbicide that has been found at concentrations as high as 143 µg/L in Midwestern U.S. streams and rivers, had no effects on *Daphnia* at similar concentrations (Battaglin *et al.* 2000, Table 2).

Conclusions/Implications/Recommendations:

Applicability of Daphnia as a screen for endocrine modulating compounds

Several pesticides affected reproductive and developmental process in *Daphnia*; however, there does not appear to be a pattern between pesticides with particular endocrine classification (reported from vertebrate systems) and effects on specific reproductive and developmental processes in *Daphnia*. Toxaphene a common groundwater contaminant was the only estrogenic compound that affected sexual differentiation in *Daphnia*. Toxaphene exposure (50 and 100 µg/L) increased male production in *Daphnia*, and yet several known estrogenic chemicals (o'p'-DDT, and Di-*n*-butyl Phthalate) had no effect on sexual differentiation in *Daphnia* (Fig. 1, Table 2). This suggests that estrogens may not play a direct role in *Daphnia* sexual differentiation. However, data in other studies imply that weakly estrogenic compounds such as dieldrin and atrazine do affect sex ratio in *Daphnia*. Dodson and colleagues found a decreased proportion of males among young produced by *Daphnia* exposed to dieldrin (1999 a), and an increased proportion of males among young produced by *Daphnia* exposed to atrazine (1999 b).

The question of whether or not pesticides elicit estrogenic activity in *D. magna* is further complicated because there is no universal "gold standard" of estrogen action among vertebrate bioassays (Coldham *et al.* 1997). Toxaphene, although commonly referred to as having estrogenic properties, has also been classified as having thyroid and antiandrogen properties (Waritzet *et al.* 1998, Arcaro *et al.* 2000). Toxaphene has also been reported as not having estrogenic properties (Table 1; Palmer *et al.* 1998). Classification of various chemicals as estrogenic or nonestrogenic is still debated in the scientific community. Therefore, to draw generalizations about all estrogenic compounds is premature.

Developmental and reproductive impairments in *D. magna* by the thyroid modulating compounds (TMC) were inconsistent. Acetochlor was the only chemical with known thyroid activity in vertebrates to have any observable effects on *D. magna*. Acetochlor reduced adult size in the six-day exposure (Table 2). Acetochlor did not decrease daphnid fecundity in this study. Therefore, it is possible that the reduction in growth rate was an endocrine related response; however, the remaining TMC's tested did not have similar affects on growth rates. Although this study did not find consistent evidence of TMC on *D. magna*, it is conceivable that *D. magna* would be affected by TMC. It is unknown if *D. magna* have a thyroid system similar to vertebrates, but other invertebrates do respond to thyroxine. Chino *et al.* (1994) isolated thyroid hormones in the sea urchin (*Hemicentrotus pulcherrimus*) and determined that thyroid hormones function in the formation of the adult rudiment. Thyroxine has also been found to accelerate larval development in the Crown of Thorns Starfish (*Acanthaster planci*; Johnson and Cartwright 1996). Based on results from this study, using *D. magna* to screen for chemicals with thyroid activity may not be effective.

Other vertebrate hormones that have affected invertebrates, such as vertebrate-type steroidal androgens, have disrupted crustacean growth and reproduction. Olmstead and LeBlanc (1998) found that exposure of female daphnids to testosterone significantly inhibited the rate of development of their abdominal process. However, *D. magna* fecundity was not reduced when exposed to the androgenic compounds linuron and DDE at concentrations tested.

Due to the lack of chemicals cited as having effects on insulin or LH activity, only one pesticide was tested from each of these categories. Lutenizing hormone is known to stimulate the crustacean Sand Shrimp (*Crangon crangon*) ovaries resulting in an increase in the number of the generative oocytes, and the number of oogonia (Zukowska-Arendarczyk 1981). Therefore, an herbicide such as 2,4-D, which elevates LH levels in humans, may have a measurable effect on *D. magna* even though the presence of LH in *D. magna* is still unknown (Garry *et al.* 2001); however, based on the reproductive and developmental endpoints examined in this study, I found no effects of 2,4-D on *D. magna*. An insulin-like immunoreactive material was found in the mussel *Mytilus edulis*; therefore, if a chemical can disrupt normal insulin function in vertebrates then it may also be possible to disrupt similar pathways in invertebrates (Fritsch *et al.* 1976). However, amitraz did not disrupt any of the reproductive or development processes examined in *D. magna*.

Many pesticides have never been tested for endocrine effects, and no chemicals have been tested against all hormone systems (Table 1). Therefore, several chemicals with no known endocrine activity were assayed. These chemicals did not elucidate any patterns regarding effects on reproductive and developmental endpoints, therefore, no conclusions can be drawn regarding their effects on the daphnid endocrine system.

Although generalization regarding endocrine classification cannot be drawn from this study, several pesticides did elicit toxic effects on developmental and reproductive processes in *D. magna*. Cyanazine is a triazine herbicide in the same family as atrazine, an herbicide which Dodson *et al.* (1999) found decreased *Daphnia* sex ratio. Although cyanazine had no effect on sex determination in this study, as atrazine did in the Dodson *et al.* (1999) study, cyanazine did lower fecundity and the number of adults that produced offspring. It is possible that *D. magna* reproduction is mediated by endocrine functions, and that 100 µg/L cyanazine disrupts normal endocrine function involved in *D. magna* reproduction.

Based on results from this study, it appears unlikely that *D. magna* would make a good screen for vertebrate endocrine modulating compounds because there was no apparent pattern between pesticides reported with estrogenic, androgenic, thyroid, insulin or LH activity in vertebrates and effects on *D. magna*. However, this *D. magna* assay which monitored sublethal effects related to endocrine-regulated processes such as growth, fecundity and sex determination consistently detected ecologically relevant effects of these pesticides on *D. magna* at environmentally relevant concentrations.

Ecological Implications (daphnid sensitivity)

Any chemical that affects an organism's fitness (i.e. survival, growth rate, fecundity, and/or sexual determination) is likely to have effects that transcend individual responses and affect the entire ecosystem. Several of the pesticides tested in this study appear to disrupt individual developmental and reproductive processes at environmentally relevant concentrations. Toxaphene is a persistent insecticide heavily used in the United States until its use was restricted in 1982. Toxaphene accumulates in ecosystems due to its lipophilic, persistent, volatile nature and appears in regions where it has never been used (DeGeus 1999). Toxaphene has been detected in groundwater at concentrations ranging from 0.1 to 1 mg/L (Bell *et al.* 1996). These concentrations are higher than the concentrations in this study that impaired reproductive and developmental processes in *Daphnia*. Therefore, the effective levels reported in this study are realistic exposures that suggest that *Daphnia* exposed to toxaphene in the wild could be at risk of impaired reproductive development.

Toxaphene not only affected sexual differentiation, but it also decreased the average clutch size (Table 2). Sanders (1980) came to similar conclusions, finding that 0.12 µg/L toxaphene significantly reduced the production of young over a 21-day period in *D. magna*. Toxaphene may affect *Daphnia* population growth rates, since a reduction in mean clutch size (fecundity) is likely to result in a decrease in *Daphnia* population growth rate. This decrease in population growth rate may be further amplified by a reduction in asexual females, which reproduce faster than their sexual counterparts do.

Cyanazine and acetochlor had similar negative impacts on *D. magna*. This study shows reproductive impairment of *D. magna* at a concentration (100 µg/L) substantially lower than the current EC₅₀ listing (42-106 mg/L; Tomlin 1994). Acetochlor disrupted the normal growth patterns of *D. magna*. Acetochlor exposed (100 µg/L) *D. magna* were, on average, 0.12 mm smaller than their respective controls. The acetochlor EC₅₀ (48 hours) for *Daphnia* is currently listed as 16 mg/L (Tomlin 1994) an order of magnitude higher than the concentration that decreased *D. magna* growth in this study.

Diflubenzuron has been regarded as one of the least hazardous insecticides (to vertebrates), primarily due to its specificity to selectively affect chitin synthesis inhibitors (Marx 1977). Chitin, a polysaccharide, is a major component of insect cuticles. Chitin synthesis inhibitors inhibit molting, killing the organism before maturation and preventing reproduction. Nontarget organisms, like crustaceans, also produce chitin. Currently, the listed *Daphnia* EC₅₀ for diflubenzuron (48 hours) is 7.1 µg/L, while this six-day *D. magna* assay found diflubenzuron toxic to *D. magna* at 0.01 µg/L (Table 2). Similarly, Savitz and Wright (1994) found that substantially lower concentrations (0.78 µg/L) than the reported EC₅₀ affected naupliar survival and development in the copepod, *Eurytemora affinis*. Decreased survivorship will have larger ecological ramifications than decreases in fecundity and growth rate.

Daphnia play a key ecological role in lakes and ponds as the dominant herbivores that aid in the transfer of energy from autotrophs to the top of the food web. Determining the vulnerability of *D. magna* to sublethal but environmentally relevant pesticide concentrations is important for the establishment of environmental health standards that will maintain ecological integrity. Pesticides have been widely broadcast and are routinely found in surface and groundwaters at concentrations ranging from 0.001 to 100 µg/l (Table 1). Based on results from this study, *D. magna* are vulnerable to many pesticides found within this range in nature. *Daphnia magna* may be particularly vulnerable to chitin synthesis inhibitors such as diflubenzuron, which reduce survivorship at very low concentrations. Reproductive and developmental processes in *D. magna* are affected by acetochlor, cyanazine, and toxaphene at concentrations found in surface waters. The *D. magna* bioassay appears to be an invaluable tool in determining sublethal but environmentally relevant toxicity of pesticides on aquatic communities and *D. magna* may serve as a useful indicator of water quality.

Related Publications:

Kashian, D.R. and S.I. Dodson. 2002. Evaluation of the use of *Daphnia* for toxicity testing of endocrine disruptors: Effects of vertebrate hormones on development and sex determination in *Daphnia magna*. In Review: submitted to *Journal of Aquatic Ecosystem stress and recovery*.

Kashian D.R., 2002. Reproduction and development in *Daphnia*: The role of hormones, pesticides and detoxification. PhD dissertation. University of Wisconsin-Madison, Wisconsin.

Kashian, D.R. 2002. An investigation of xenobiotic detoxification through P-450 induction in *Daphnia magna*. To be submitted to *Environ. Toxicol. Chem.*

Kashian, D.R. and S.I. Dodson. 2002. Disruption of developmental and sexual determination processes in *Daphnia magna*: A survey of 10 agricultural chemicals. In Review: submitted to *Environ. Toxicol. Chem.*

Key Words:

toxicity, toxaphene, Acetochlor o'p'-DDT, Di-n-butyl phthalate, p'p-DDE, linuron, alachlor, metribuzin, amitraz, 2,4-D chlorosulfuran, cyanazine, diflubenzuron, metolachlor and diquat