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MECHANISTIC EVALUATION OF THE COMBINED ACTION OF ENVIRONMENTALLY-RELEVANT CHEMICAL MIXTURES ON ENDOCRINE SIGNALING IN CRUSTACEANS

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ABSTRACT

Pharmaceuticals, personal care products, and other chemicals have been shown to accumulate in surface waters of North Carolina and many of these contaminants have the potential to disrupt endocrine signaling processes. The hypothesis was tested that mixtures of pharmaceuticals and other surface water contaminants, at levels individually considered to be safe, can adversely impact population biology of some crustaceans by modulating the endocrine signaling pathway responsible for sex determination. The hormone methyl farnesoate regulates sex determination, hemoglobin production, and other functions in many crustacean species. In the present study, several pharmaceuticals and other major surface water contaminants were evaluated for endocrine-disrupting activity (either as a methyl farnesoate mimic, antagonist, or potentiator). Chemicals were evaluated for their ability to alter sex-determination in offspring of the freshwater crustacean *Daphnia magna.* Combined effects of chemical mixtures of these chemicals were then mathematically modeled to identify mixtures scenarios that might pose risk to crustaceans and perhaps other species. Of the eleven compounds evaluated, 6 mimicked the action of methyl farnesoate and stimulated excess male offspring production. Most potent were the insect growth regulating insecticides pyriproxyfen and fenoxycarb. The insecticide methoprene was moderately active; while, the cyclodiene (dieldrin, chlordane) and cyclohexane (lindane) insecticides were weak methyl farnesoate agonists. Bisphenol A appeared to function as a methyl farnesoate potentiator. Other compounds, including the pharmaceuticals were not active. Mixtures modeling revealed that only the insect growth regulating insecticides were sufficiently potent to contribute to the excess production of male offspring at mixture formulations in which constituents were present at environmentally-relevant concentrations. Mixtures modeling in which constituents were present at equi-potent levels revealed mixture scenarios that elicited significant effects on sex ratios of offspring when constituents were present at levels that, individually, would not elicit effects. This study advanced our understanding of mixtures toxicity, provided significant insight into approaches for assessing the toxicity of chemical mixtures, and contributed to the development of free, interactive website for the evaluation of chemical mixtures toxicity (available at http://wang.tox.ncsu.edu/model5/).

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INTRODUCTION

Numerous studies have demonstrated that pharmaceuticals and personal care products (PPCPs) can escape degradation in sewage treatment facilities and are accumulating in surface waters of the Nation, including North Carolina. Many of these chemicals can interfere with endocrine signaling processes and, in combination with other chemical contaminants, have the potential to elicit adverse effects on populations of ecologically and economically important species. The hypothesis was tested in the present study that *mixtures of environmentally-relevant chemicals, at levels individually considered safe, can adversely impact crustacean population biology by modulating the endocrine signaling pathway responsible for sex determination.* Such studies are critical in order to define environmental concentrations of these chemicals that are truly safe and identify adequate sewage treatment processes that will reduce levels of these contaminants to these established safe levels.

Crustaceans such as Decapod shrimp and crabs are of vital economic importance to North Carolina and the US at large. In addition, other smaller crustaceans such as Branchiopods (water fleas), Ostracods (seed shrimp), Copepods (cyclops), and Cirripedia (barnacles) are key component to fresh water and estuarine ecosystems. These crustaceans serve as a food source for larval and juvenile fish and as major contributors to nutrient cycling and energy transfer through ecosystems. Catch records have shown that many important crustacean populations (e.g., blue crab, brown shrimp) declined or remained suppressed through the 1990's in relation to previous years (Land et al., 1995, Land et al., 1996). These reductions are likely due to various, multiple stressors including storms, over harvesting, and loss of habitat (CBC, 2001, Paerl et al., 2001). Pollution also is often cited as a contributing cause for declining crustacean populations (Reyes et al., 1999, Lund et al., 2000, Wirth et al., 2001).

The status of population stocks of the smaller, keystone crustacean species such as Branchiopods and Copepods is not known. However, with the exception of over harvesting, these populations are subject to the same environmental stressors as the crabs and shrimp, and may be more susceptible to the adverse effects of low levels of environmental contaminants due to their diminutive size and rapid equilibration with environmental levels of contaminants. Indeed, the smaller crustaceans are often viewed as being more susceptible to the toxicity of environmental chemicals as compared to fish and larger crustaceans (Shane, 1994).

Environmental signals regulate a variety of key processes in animal physiology We have shown that some environmental stimuli trigger an endocrine cascade involving the hormone methyl farnesoate in the Branchiopod crustacean *Daphnia magna* (Olmstead and LeBlanc, 2002). This hormone binds to a putative nuclear receptor (as of yet unidentified, but referred to as MfR (Gorr et al., 2006)) to activate gene transcription. Among the processes that are regulated by methyl farnesoate is sex determination (Olmstead and LeBlanc, 2002). In response certain environmental stimuli, such as a decrease in available food, methyl farnesoate signals maturing oocytes in the ovaries of females to develop into males (Olmstead and LeBlanc, 2002). Thus, methyl farnesate

tranduces the signal responsible for environmental sex determination in this species. In addition, the methyl farnesoate signaling pathway regulates hemoglobin production and reproductive maturation in some crustaceans (Laufer and Biggers, 2001, Rider et al., 2004).

The methyl farnesoate signaling pathway regulates critical physiological processes, which if perturbed, can lead to various deleterious conditions. For example, disruption of methyl farnesoate signaling in some crustaceans may alter sex ratios within populations and compromise reproductive capacity (Laufer and Biggers, 2001, Olmstead and LeBlanc, 2002). We have shown that this signaling pathway is susceptible to disruption by environmental chemicals. Some pesticides can mimic the action of methyl farnesoate with a potency 100-times greater than the hormone itself (Olmstead and LeBlanc, 2003). Some ubiquitous environmental chemicals such as bisphenol A and 4-nonphenol appear to potentiate the action of the endogenous hormone resulting in aberrant signaling (Mu et al., 2004). Presumably, some chemicals also can bind the methyl farnesoate receptor and block the action of the hormone (antagonists). Clearly, methyl farnesoate signaling represents an important endocrine pathway that is susceptible to disruption by environmental chemicals.

In the present study, eleven chemicals found in surface waters of the United States and elsewhere, were evaluated for their ability to interfere with normal methyl farnesoate signaling. The combined action of these chemicals was then modeled in an effort to predict environmental scenarios in which mixtures of these chemicals at environmentally relevant concentrations may pose risk.

METHODS

Daphnid culture

The crustacean *Daphnia magna* was used in these experiments. Daphnids were originally acquired from US Environmental Protection Agency, Mid-Continent Ecology Division - Duluth, MN and have been maintained in our laboratory for over ten years. Daphnid media consisted of reconstituted deionized water (192 mg/L $CaSO_4 \cdot H_2O$, 192 mg/L $NaHCO_3$, 120 mg/L $MgSO_4$, 8.0 mg/L KCl, 1.0 μg/L selenium and 1.0 μg/L vitamin B_{12}). All daphnid cultures and experiments were housed in incubators set to 20° C with a 16 h-8 h light-dark cycle. Culture daphnids were maintained at a density of ~50 animals per 1 L of media. Cultures were changed three times per week and adults were discarded and replaced with neonates after three weeks in culture. Culture daphnids were fed twice a day with 2.0 mL (1.4×10^8 cells) of the unicellular green algae *Selenastrum capricornutum* and 1.0 mL (4 mg dry weight) of Tetrafin® fish food suspension (Pet International, Chesterfill, New South Wales, Australia) prepared as described previously (Baldwin and LeBlanc, 1994). The *Selenastrum* was cultured in the laboratory using Bold's basal medium. Daphnids reproduce asexually, via parthenogenesis, under these conditions with the production of only female offspring.

Chemicals

The following chemicals were evaluated in this study.

- Pesticidal insect growth regulators (*pyriproxyfen, fenoxycarb, methoprene*) mimic the action of juvenile hormone III in insects (Dhadialla et al., 1998) and also mimic the action of methyl farnesoate in crustaceans with various potencies (Wang et al., 2005).
- *Dieldrin, cis-chlordane*, and *lindane* are cyclodiene/cyclohexane insecticides. Dieldrin is a weak methyl farnesoate mimic while chlordane has no known effect on methyl farnesoate signaling (Wang et al., 2005). The effects of lindane on methyl farnesoate signaling are unknown.
- *Bisphenol A* and *nonylphenol* are ubiquitous environmental contaminants (Kolpin et al., 2002) with reported methyl farnesoate-like activity (Biggers and Laufer, 2004). In our experience, these compounds may function as methyl farnesoate potentiators rather than true agonists (Mu et al., 2005).
- *Trimethoprim, acetaminophen*, and *ibuprofen* are pharmaceuticals that are frequently detected in surface waters (Kolpin et al., 2002). The ability of these compounds to interfere with methyl farnesoate signaling is not known.

Exposure concentrations of chemicals were used up to the lowest levels that interfered with the production of viable offspring for purposes of establishing whether the compounds elicited effects on methyl farnesoate signaling and when characterizing concentration-response relationships for such effects. Test chemicals were delivered to the exposure solutions in absolute ethanol (0.01% v/v, final concentrations). Control solutions were provided the same concentration of ethanol.

Methyl farnesoate agonist activity

Chemicals were evaluated for methyl farnesoate agonist activity based upon their ability to stimulate the production of male offspring among exposed maternal organisms. These maternal organisms were reared under conditions that promoted the production of only female offspring as described above. The basic experimental design for this assay is outlined in Fig. 1. Adult female daphnids (7-14 days old) carrying embryos in their brood chambers were selected from the cultures and placed individually in 50-ml beakers containing 40 ml media and the desired concentration of test chemical. Test solutions were changed daily and daphnids were observed daily for the release of broods of offspring. Food was provided to each beaker twice daily as 0.05 mL (~0.2 mg dry weight) of Tetrafin® fish food suspension and 0.1 mL (7×10^6 cells) of a suspension of unicellular green algae, *S. capricornutum*. Treatments were replicated 10 times (1 animal per beaker, 10 beakers per treatment). Assays were terminated when all maternal daphnids in the experiment had released their third brood of offspring (typically 7-10 days).

The number of offspring present in the third brood released by each maternal daphnid was determined and sex of individual daphnids within that brood was established. Sex of individual offspring was determined microscopically (10x magnification) with males

being discerned from females by the longer first antennae (Fig. 2) (Olmstead and LeBlanc, 2000). A positive control consisting of either methyl farnesoate or pyriproxyfen at a concentration that stimulates male sex determination among offspring was included to ensure that daphnids were appropriately responding.

Methyl farnesoate antagonist activity Chemicals were screened for methyl farnesoate antagonist activity using the same methods as used for measuring methyl farnesoate agonist activity except that methyl farnesoate also was added to the test solutions. Test chemicals were evaluated in these experiments for their ability to interfere with methyl farnesoate-stimulated male offspring production.

Methyl farnesoate potentiator activity Chemicals were evaluated for methyl farnesoate potentiator activity using the same methods as used for measuring antagonist activity, except that chemicals were evaluated for their ability to enhance male offspring production caused by exogenous methyl farnesoate.

Concentration-response relationships

Chemicals that were found to interfere with normal methyl farnesoate signaling were subjected to definitive characterization of the concentration-response relationship. This characterization was required to define terms that would subsequently be used in the mixtures modeling. The percentage response (male offspring production), was plotted against the log exposure concentrations that elicited the response for a series of chemical exposure concentrations and fit with a sigmoidal line using Origin™ software (Microcal™ Software Inc., Northampton, MA). The logistic equation representing the sigmoidal fit to the data is:

$$R = \frac{1}{1 + \left(\frac{EC50}{C} \right)^{\rho}} \qquad \text{Equation 1}$$

where R is the response, C is the chemical concentration, ρ is the power (Hill slope) of the curve, and $EC50$ is the exposure concentration that would elicit offspring broods with a 50:50 sex ratio. These individual concentration-response curves were subsequently used in mixture modeling as described below.

Mixtures modeling

The Integrated Addition and Interaction (IAI) model, used in this study, consists of four basic components: 1) assignment of chemicals to cassettes with each cassette representing a mechanism of action, 2) incorporating toxicokinetic or toxicodynamic modifying terms into the model to account of chemical interactions, 3) calculating the toxicity associated with each cassette, 4) summing the toxicity of all cassettes (Olmstead and LeBlanc, 2005b, Rider et al., 2005).

Responses associated with individual cassettes Chemicals having the same apparent mechanism of action were assigned to common cassettes. The response (percentage male offspring) associated with each cassette was then calculated using a concentration addition approach (Olmstead and LeBlanc, 2005b):

$$R = \frac{1}{1 + \frac{1}{\left(\sum_{i=1}^{n} \frac{C_i}{EC50_i}\right)^{\rho'}}} \qquad \text{Equation 2}$$

where R is the response to the mixture within the cassette, C_i is the concentration of chemical *i* in the mixture, $EC50_i$ is the concentration of chemical *i* that causes a 50:50 sex ratio among offspring, and ρ' is the average power associated with the chemicals in the cassette.

Joint response associated with the cassettes The joint response of the individual cassettes comprising the mixture was calculated using the response addition equation:

$$R = 1 - \prod_{i=1}^{n} (1 - R_i) \qquad \text{Equation 3}$$

where *R* represents the response to the mixture and R_i is the response to cassette *i*.

Equations 2 and 3 were integrated to establish the response associated with individual cassettes within a mixture and to sum the responses associated with the cassettes (Olmstead and LeBlanc, 2005b):

$$R = 1 - \prod_{I=1}^{N} \left\{ 1 - \frac{1}{1 + \frac{1}{\left(\sum_{i=1}^{n} \frac{C_i}{EC50_i}\right)^{\rho'}}} \right\} \qquad \text{Equation 4}$$

Incorporation of toxicodynamic and toxicokinetic modifiers Coefficients of interaction (*K-functions)* were used to describe the effect of one class of chemical on the response (toxicodynamic interaction) or the effective concentration (toxicokinetic interaction) of the other (Finney, 1942, Mu and LeBlanc, 2004, Rider et al., 2005). *K-functions* were calculated using the following equation:

$$K = \frac{EC50_X}{EC50_{X+Y}} \qquad \text{Equation 5}$$

where $EC50_X$ is the concentration of the target chemical (x) that cause a 50:50 incidence of male sex determination among offspring and $EC50_{X+Y}$ is the EC50 of the target chemical when exposure occurred in the presence of concentration (y) of the interacting chemical (i.e., the chemical responsible for modifying toxicity). A series of *K-functions* were derived over the effective concentration range of chemical y. These *K-functions* were then plotted against the concentration of interacting chemical from which they were derived (chemical y). The logistic equation that defined this relationship was used to calculate *K-functions* for any concentration of chemical y during mixture toxicity modeling. *K-functions* were integrated into the model to describe the toxicodynamic interactions observed. *K-functions* were used to describe toxicodynamic interactions by incorporating them into the equation as modifiers of the response of the target cassette:

$$R = 1 - \prod_{I=1}^{N} \left\{ 1 - \left(k_{a,i}(C_a) \right) \frac{1}{1 + \frac{1}{\left(\sum_{i=1}^{n} \frac{C_i}{EC50_i} \right)^{\rho'}}} \right\} \qquad \text{Equation 7}$$

where $k_{a,i}$ is a function that describes the extent to which effector chemical in the mixture alters the response associated with the affected chemical. Toxicokinetic interactions were similarly incorporated into the equation by multiplying C_i by the $k_{a,i}$ function.

This modeling approach was used to assess the combined action of the chemicals at various concentrations on methyl farnesoate signaling. Modeling was performed using the CATAM program (available at http://wang.tox.ncsu.edu/model5/). Support from this grant was instrumental in establishing this free-access interactive website.

RESULTS AND DISCUSSION

Individual chemical characterization

The eleven chemicals were evaluated for their ability to interfere with normal methyl farnesoate signaling. Results of this evaluation and relevant statistical parameters (EC50 and ρ) generated from the evaluations, that would be used in subsequent mixtures modeling, are summarized in Table 1. The insect growth regulators pyriproxyfen (Fig. 3) and fenoxycarb (Fig. 4) were potent mimics of methyl farnesoate with both compounds causing male sex determination with an EC50 value of 0.1 µg/L. The insect growth regulator methoprene (Fig. 5) also exhibited methyl farnesoate mimicry, but with significantly lower potency (Table 1).

The cyclodiene/cyclohexane insecticides, dieldrin, chlordane, and lindane, exhibited low and variable methyl farnesoate-like activity. Dieldrin, chlordane, and lindane stimulated male sex determination among offspring at levels between 0 to 20%, 0 to 55%, and 0 to 2%, respectively. Co-exposure with low level methyl farnesoate (5.0 μg/L) enhanced male offspring production in a cyclodiene concentration-dependent manner (Fig. 6) suggesting that these compounds may potentiate the activity of the hormone. However, at high concentrations of methyl farnesoate that resulted in significant male sex determination, these compounds tended to block male sex determination (Fig. 7). Taken together, these results suggests that the cyclodiene/cyclohexane insecticides are mixed agonists/anatagonists and not potentiators. We propose that at low levels of methyl farnesoate, these weak agonist bind and activate the methyl farnesoate receptor resulting in low, but measurable, agonistic activity. However, at high levels of methyl farnesoate, these compounds compete with methyl farnesoate for occupancy of the methyl farnesoate receptor. Since the insecticides are intrinsically less potent than the hormone at activating the receptor, successful competition with methyl farnesoate results in a net reduction in activity. Accordingly, these compounds were treated as agonists, since daphnids in these experiments likely had low endogenous methyl farnesoate levels, and not potentiators in the subsequent modeling experiments.

Bisphenol A exhibited no direct methyl farnesoate mimicry, but in the presence of low levels of methyl farnesoate, bisphenol A stimulated male sex determination of offspring (Fig. 8). This scenario suggests potentiation whereby bisphenol A enhanced the activity directly associated with the hormone methyl farnesoate. These effects were observed at high concentrations of bisphenol A relative to methyl farnesoate (i.e., 1000X more bisphenol A) and may be due to bisphenol A successfully competing for esterases that are responsible for inactivating bisphenol A. As a result, methyl farnesoate levels within the organisms increased with increasing bisphenol A exposure concentration. Since this interaction occurs only in the presence of methyl farnesoate and experiments were performed under conditions at which endogenous methyl farnesoate would be minimal (i.e., only females offspring were being produced), this interaction was not incorporated into the mixtures modeling. Nonylphenol ($\leq$100 μg/L), and the three pharmaceuticals ($\leq$1000 μg/L) exhibited no significant effects on the methyl farnesoate signaling pathway.

The statistical parameters used to describe the action of the individual chemicals in the mixtures modeling are summarized in Table 1. EC50 values for pyriproxyfen, fenoxycarb, and methoprene were empirically derived from the concentration-response curves (Figs. 1-3). Dieldrin, chlordane, and lindane were assigned default values that represented the highest concentration that could be tested without adversely affecting the maternal organism (100, 10, and 100 μg/L, respectively). Power functions for the six chemicals varied from 0.9 to 15. All six chemicals were assigned to the same mechanistic cassette for mixtures modeling since they all functioned as methyl farnesoate mimics. The model requires that all chemicals within a cassette share the same power function, and much of the variability in the measured power functions was likely due to deficiencies in the data (i.e., low activity of many of the chemicals). Therefore for modeling purposes, the mean power function of 4.3 was used.

Mixtures modeling: environmental relevant mixtures

A series of 12 mixtures of the six active chemicals was devised where the most concentrated mixture (mixture #12) contained each chemical at its highest estimated level in surface waters (as described in Table 2). Mixtures designed 11 to 1 represent successive 50% dilutions of the original mixture (Table 3). The level of chemicals in between mixtures 8 and 9 represent levels that are more typical of surface water levels in the United States (Kolpin et al., 2002). The combination of chemicals in mixtures 5 through 12 were predicted to cause the production of all-male offspring (Fig. 9) despite the fact that the chemicals were present at concentrations well below levels that are overtly toxic to the maternal organisms (compare LC50 values in Table 2 to chemical concentrations in mixtures in Table 3). Only mixture formulations designated #1 and #2, had no predicted effect on sex determination of offspring (Fig. 9).

Individual mixtures were evaluated to determine which constituents in the mixtures were contributing to the measured effects on sex determination (Table 3). Among the six chemicals, only two – pyriproxyfen and fenoxycarb – contributed to the effects measured with mixtures 6 through 12. Pyriproxyfen, alone, was responsible for the effects measured with mixtures 3 through 5 (Table 3). Thus, while all six chemicals in the mixture had the ability to interfere with sex determination, only the two most potent chemicals contributed to this effect of the mixture.

Previous studies with polycyclic aromatic hydrocarbon mixtures had led us to suggest that the toxicity of a chemical mixture can typically be associated with a few constituents of the mixture (Olmstead and LeBlanc, 2005a). The present results seemed to confirm this suggestion. To further explore this phenomenon, 100 mixtures of 100 chemicals were designed where EC50 values and concentrations of the chemicals in each mixture were randomly determined within defined ranges for typical environmental contaminants. The response to each mixture was then modeled. For 99% of the mixtures, toxicity was due to the combined action of 2 to 12 chemicals in the mix (Fig. 10). Most commonly, toxicity was due to the combined action of 8 or 9 chemicals. These results indicate that, on average, toxicity associated with a chemical mixture is due to $\leq$10% of the chemicals in the mix.

Mixtures modeling: equipotent mixtures constituents

Another series of modeling experiments were performed where the six chemicals that stimulate male sex determination were present in the most concentrated mix (mixture #22) at either their EC50 value for male sex determination or their default EC50 value (Table 4). Subsequent mixtures (designed 21-13) were successive 50% dilutions. This series of mixtures was evaluated to test the hypothesis that when all constituents are present in the mixture at equi-potent levels, some mixtures will elicit effects despite the fact that individual constituents in the mixture are present at levels that do not elicit effects. Mixtures 19 through 22 elicited effects on sex determination (Fig. 11). Among these mixtures, only mixture 22 contained mixture constituents at levels that individually would result in significant male sex determination. Constituents of all other mixtures

were present at concentrations that individually would not significantly impact sex determinations. Results confirm and highlight a major consideration in mixtures toxicity assessment; that, chemicals within a mixture may be individually deemed nontoxic, but in combination can result in significant toxicity.

Mixtures modeling: chemical interactions

A decrease in the requested budget precluded evaluation of potential chemical interactions on the toxicity of the chemical mixtures.

SUMMARY AND CONCLUSIONS

The purpose of this study was to test the hypothesis that *mixtures of environmentally-relevant chemicals, at levels individually considered safe, can adversely impact crustacean population biology by modulating the endocrine signaling pathway responsible for sex determination.* Results of the study support this hypothesis since several mixture formulations in which constituents were present at levels that would have no significant impact of sex ratios of daphnid populations (Table 4, Fig. 11, mixtures 19-21) were predicted to significantly increase the proportion of male offspring produced by the organisms.

Among the 11 chemicals evaluated in this study, the insecticides pyriproxyfen and fenoxycarb were principally responsible for the effects on crustaceans at environmentally-relevant concentrations. The high potency of these compounds with respect to their ability to mimic the action of methyl farnesoate indicates that these compounds should be viewed with concern in surface waters do to their ability to interfere with the endocrine physiology of crustaceans. Estimates of environmental concentrations of these compounds stem largely from fate model predictions. In light of the potential for these compounds to adversely affect crustacean populations, we recommend that direct analytical assessments of level s of these compounds in surface waters be undertaken.

The three pharmaceuticals evaluated in this study trimethoprim, acetaminophen, and ibuprofen were selected because of their common occurrence in surface waters (Kolpin et al., 2002). These compounds did not contribute to altered sex ratios of crustaceans measured in this study. Based upon these results, these pharmaceuticals should not be viewed as chemicals of concern with respect to the endocrine physiology of crustaceans. Future studies should be expanded to include other classes of pharmaceuticals and personal care products know to exist in the environment and well the potential for PPCPs to modulate the activity of active compounds (i.e., pyriproxyfen and fenoxycarb).

Results from this study demonstrated that some compounds (i.e., cyclohexanes, cyclodienes, phenols) can interfere with crustacean endocrine physiology as weak mixed agonists/antagonists or as potentiators of methyl farnesoate . Among the compounds evaluated, potency of the compounds was extremely low or non-detectable. Accordingly,

these compounds would not be viewed as chemicals of concern at levels typically found in the environment, even when present in combination.

Finally, results from this research have provided significant insight into the sources of toxicity of environmentally-relevant chemical mixtures. Specifically, experimental results and modeling exercises performed all support the hypothesis that toxicity of chemical mixtures can typically be ascribed to a few (i.e., <10%) of the constituents of the mixture. The implications of this observation are potentially profound, indicating that toxicity assessments of chemical mixtures can be reduced to the evaluation of a few constituents of the mixture. Complexity is the major hurdle that must be overcome in the hazard assessment of chemical mixtures. The identification of measures that can reduce complexity will significantly enhance progress towards these needed hazard assessments.

RECOMMENTATIONS

1. Assessments of surface water contaminants need to incorporate analyses for insect growth regulating insecticides, including pyriproxyfen and fenoxycarb, since these compounds are used with increasing frequency and are potent mimics of some hormones.

2. Future assessments of mixtures toxicity, as performed in this research, should be expanded to include other pharmaceuticals and personal care products that are commonly measured in surface waters. These would include erythromycin, lincomycin, caffeine, 1,7-dimethylxanthine, and codeine.

3. Research is needed to gain further insight in interactions among chemicals in mixtures that would result in greater-than-additive toxicity. Such data should then be used to validate the IAI model, used in this research, for the assessment of chemical mixtures toxicity when such interactions exist.

4. Current databases of know chemical mixture in surface water should be subjected to rigorous IAI modeling to evaluate potential risks associated with these mixtures.

Table 1 Results of the assessment of the individual chemicals for their ability to mimic methyl farnesoate signaling by acting as an agonist.

Chemical	Agonist	EC50 (μg/L)	ρ
Pyriproxyfen	yes	0.11	5.8
Fenoxycarb	yes	0.10	15
Methoprene	yes	489	2.1
Dieldrin	yes	>100	1
Chlordane	yes	>10	0.9
Lindane	yes	>100	~1
Bisphenol A	no	-	-
Nonylphenol	no	-	-
Trimethoprim	no	-	-
Acetaminophen	no	-	-
Ibuprofen	no	-	-

Table 2 Environmental concentrations of the mixtures constitutents and their toxicity to daphnids. Shaded values under *maximum concentration* represent maximum detected values in a survey of surface waters in the United States (Kolpin et al., 2002) or the Netherlands (Anon., 2006) (fenoxycarb). For these chemicals, *environmental concentrations* represent 1/10 of the maximum value. Shaded values under *environmental concentrations* represent estimated levels in surface water associated with normal use (Ingersoll et al., 1999, USEPA, 2003) and *maximum* values for these are set at 10X the environmental concentration. LC50 concentrations were derived from various literature sources and represent the concentration that is lethal to 50% of the exposed population.

	Concentration (μg/L)		
Chemical	Environmental	Maximum	LC50
Pyriproxyfen	3.8	38	400
Fenoxycarb	0.55	5.5	400
Methoprene	10	100	360
Dieldrin	0.021	0.21	190
Chlordane	0.010	0.10	24
Lindane	0.011	0.11	485

Table 3 Concentrations of mixtures constituents used in the model presented in Fig. 9. Mixture number 12 represents all constituents at estimated maximum concentrations found in surface waters (see Table 2). All other mixtures consist of successive 50% dilutions of the mixture. Constituent concentrations below the dotted line represent levels more typically measured in surface waters. Shaded values represent chemicals and concentrations that contributed to the toxicity of the mixture.

Mixture	Concentration (µg/L)					
	Pyriproxyfen	Fenoxycarb	Methoprene	Dieldrin	Chlordane	Lindane
12	38	5.5	100	0.21	0.10	0.11
11	19	2.8	50	0.10	0.050	0.055
10	9.5	1.4	25	0.052	0.025	0.028
9	4.8	0.69	12	0.026	0.012	0.014
8	2.4	0.34	6.2	0.013	0.0062	0.0069
7	1.2	0.17	3.1	0.0066	0.0031	0.0034
6	0.59	0.086	1.6	0.0033	0.0016	0.0017
5	0.30	0.043	0.78	0.0016	0.00078	0.00086
4	0.15	0.021	0.39	0.00082	0.00039	0.00043
3	0.074	0.011	0.20	0.00041	0.00020	0.00022
2	0.037	0.0054	0.098	0.00020	0.000098	0.00011
1	0.019	0.0027	0.049	0.00010	0.000049	0.000054

Table 4 Concentrations of mixtures constituents used in the model presented in Fig. 11. Mixture number 22 represents all constituents at their respective EC50 values for male offspring production (or the default (<) values in Table 1). All successive mixtures consist of 50% dilutions. Shaded values represent chemicals and concentrations that contributed to the toxicity of the mixture.

Mixture	Concentration (µg/L)					
	Pyriproxyfen	Fenoxycarb	Methoprene	Dieldrin	Chlordane	Lindane
22	0.11	0.10	489	100	10	100
21	0.055	0.050	244	50	5.0	50
20	0.028	0.025	122	25	2.5	25
19	0.014	0.012	61	12	1.2	12
18	0.0069	0.0062	30	6.2	0.62	6.2
17	0.0034	0.0031	15	3.1	0.31	3.1
16	0.0017	0.0016	7.6	1.6	0.16	1.6
15	0.00086	0.00078	3.8	0.78	0.078	0.78
14	0.00043	0.00039	1.9	0.39	0.039	0.39
13	0.00022	0.00020	0.96	0.20	0.020	0.20

Figure 1. Assay design for the assessment of the ability of chemicals to stimulate male offspring production.

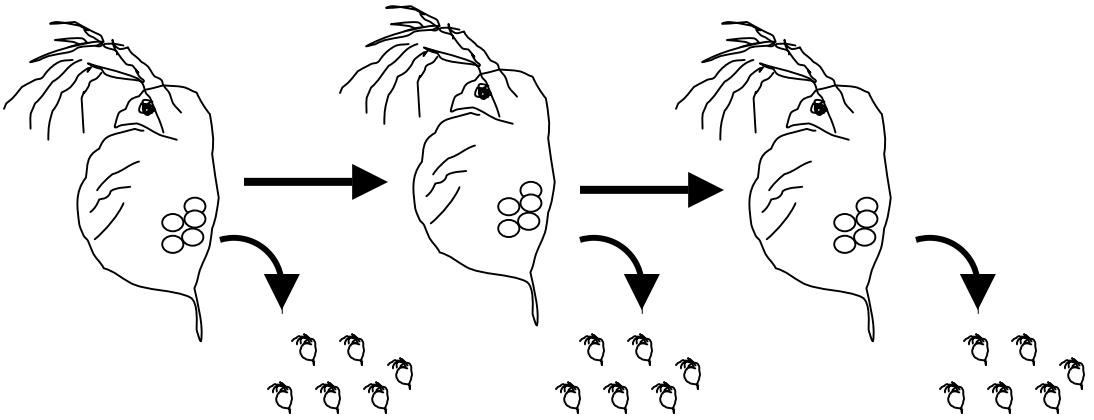
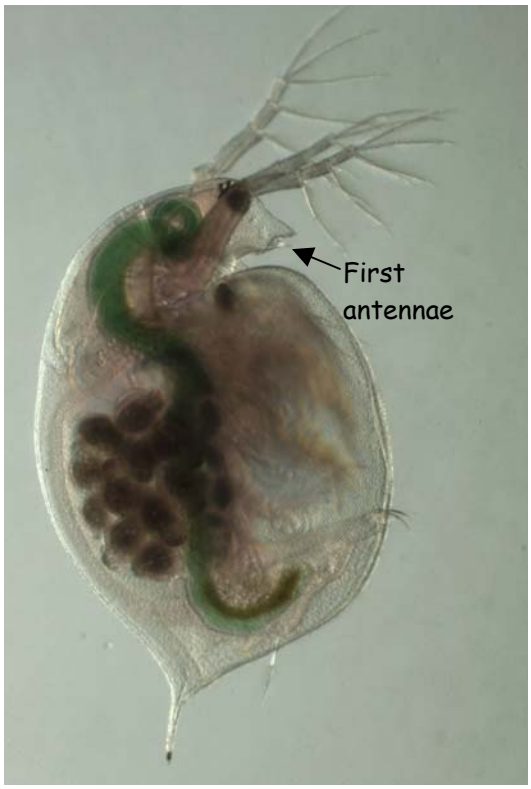
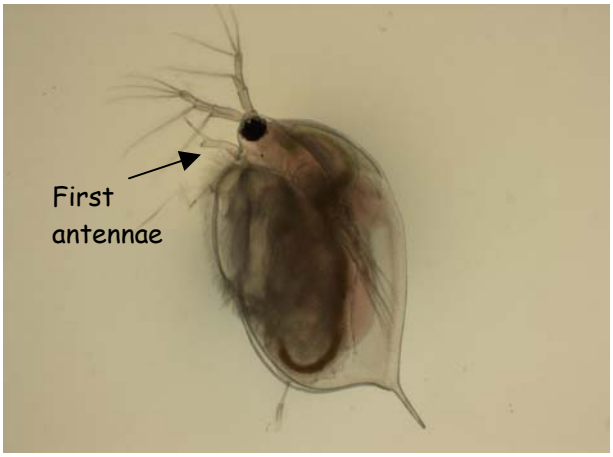


Figure. 2 Anatomy of female and male daphnids. Male daphnids were distinguished from females in this study by the elongated first antennae.



Female



Male

Figure 3. Increase in male offspring production with pyriproxyfen treatment.

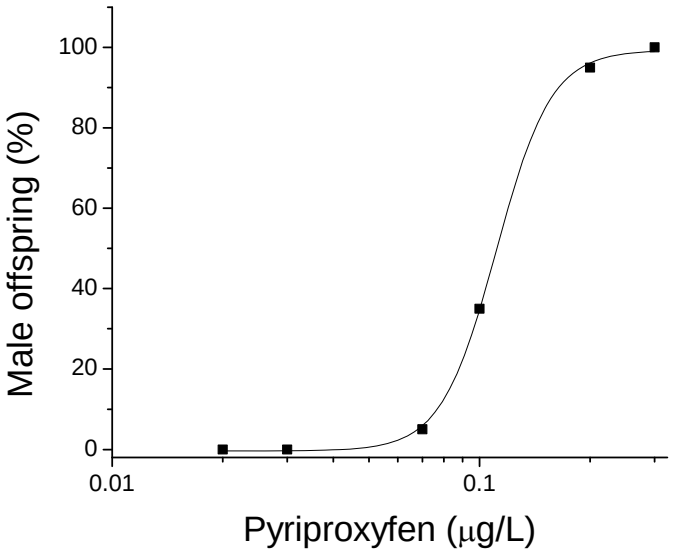


Figure 4. Increase in male offspring production with fenoxycarb treatment.

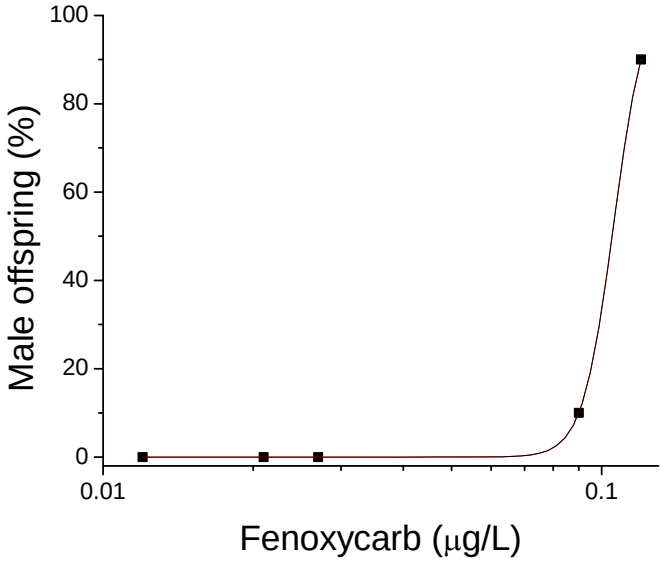


Figure 5. Increase in male offspring production with methoprene treatment.

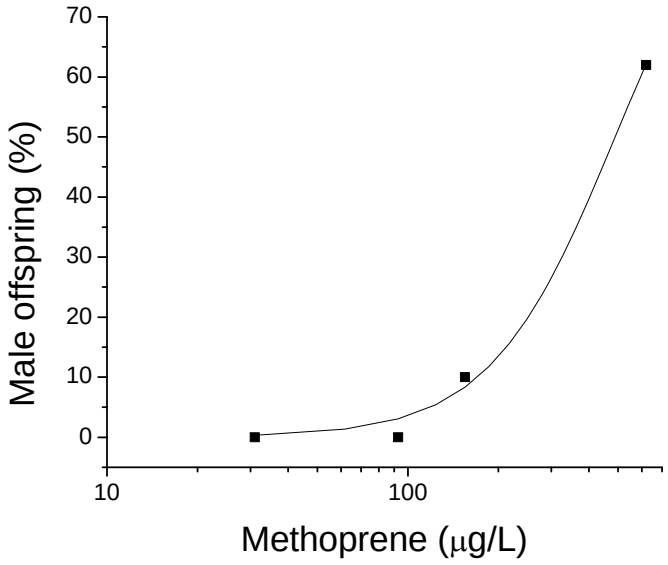


Figure 6. Percentage increase in male offspring production by chlordane in the presence of 5.0 μg/L methyl farnesoate.

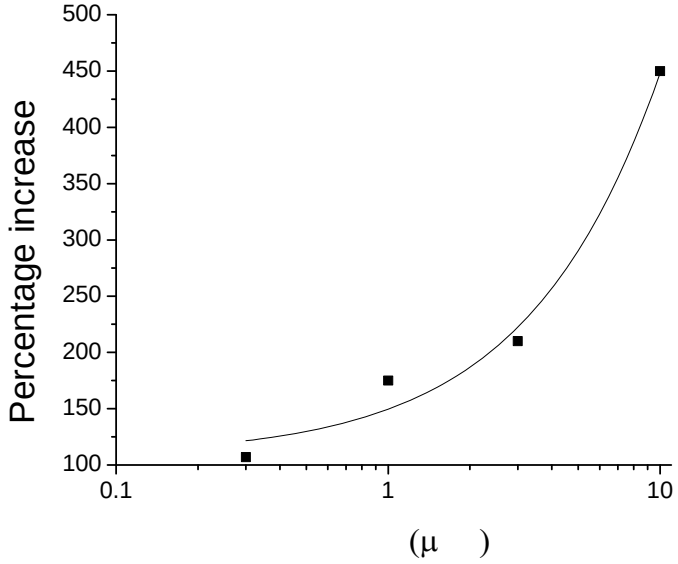


Figure 7. Effect of lindane on male offspring production in the presence of 10 μg/L methyl farnesoate

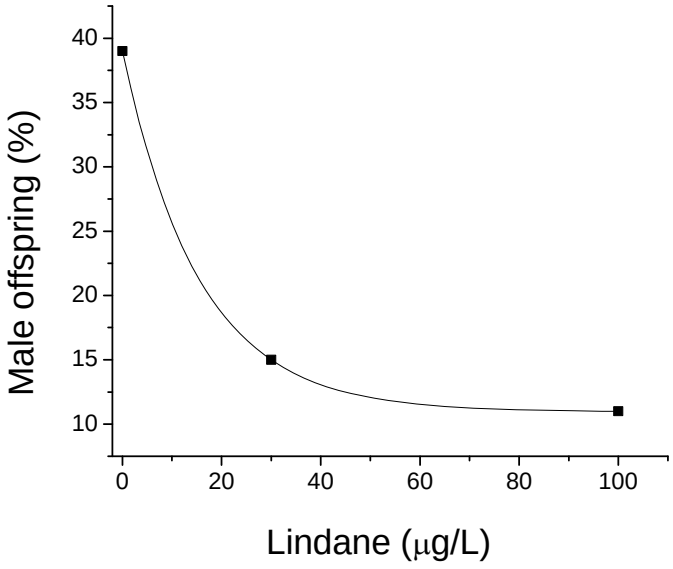


Figure 8 Male sex determination among offspring during exposure to concentrations of bisphenol A in the presence or absence of 10 μg/L methyl farnesoate.

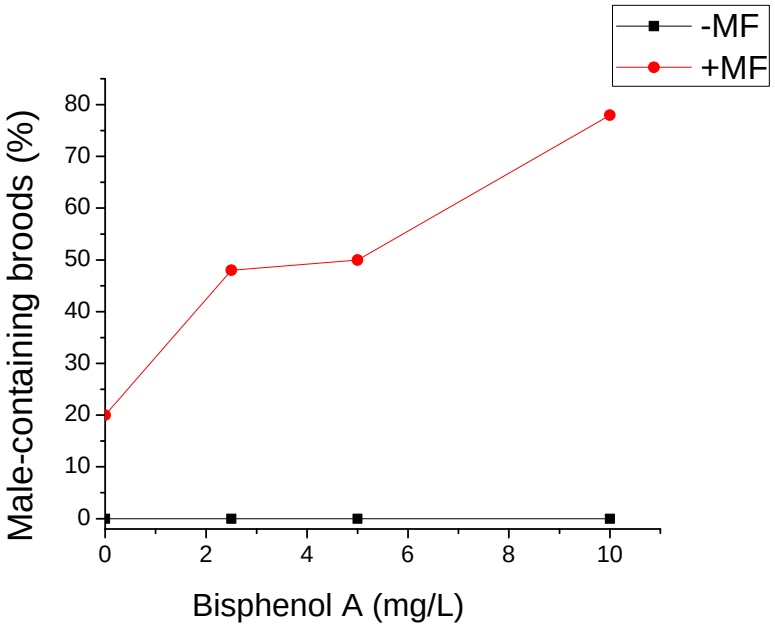


Figure 9. Predicted production of male offspring in response to exposure to chemical mixtures (as described in Table 3).

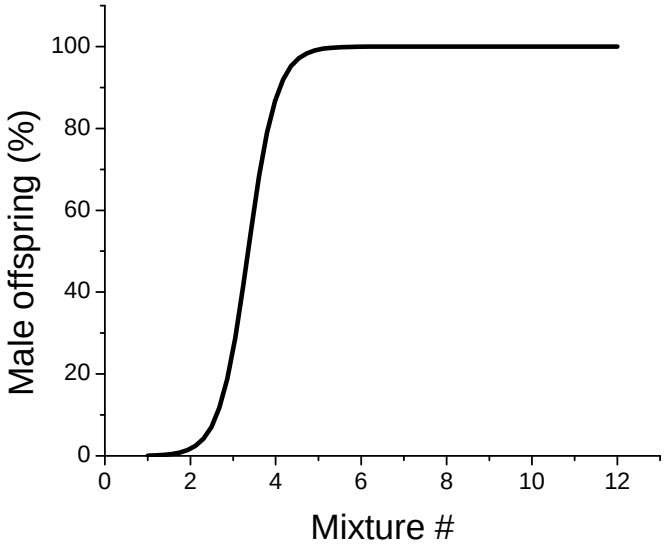


Figure 10 The percentage incidence at which the indicated number of chemicals in 100 mixtures of 100 chemicals contributed to toxicity of the mixture.

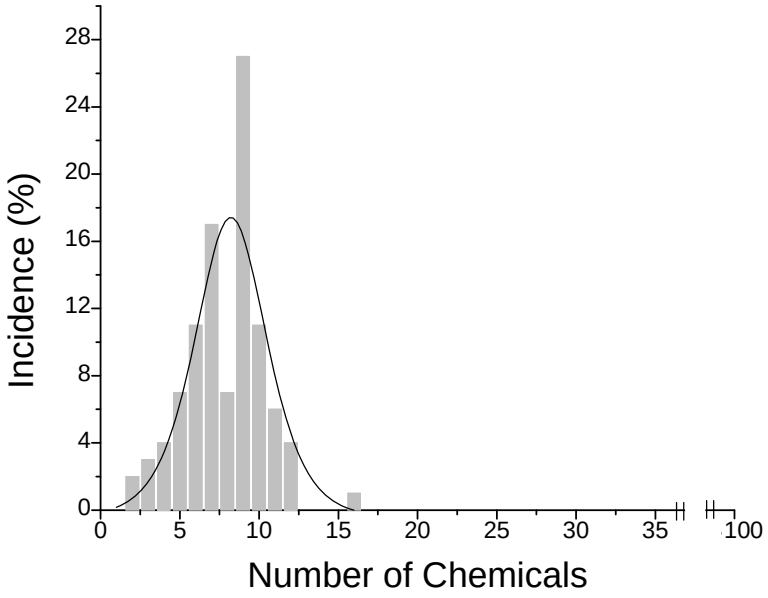
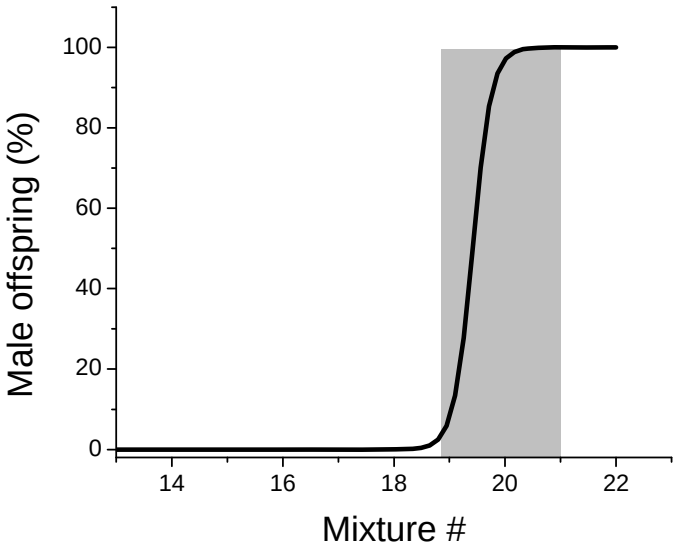


Figure 11. Predicted production of male offspring in response to exposure to chemical mixtures (as described in Table 4). Constituents in mixture 22 are all present at their EC50 for male offspring production (or the default (>) value presented in Table 1). All prior mixtures consist of successive 50% dilutions of the mixture. All constituents in mixtures within the shaded were present at concentrations that, individually, would cause no significant male sex determination (0 to 0.26% incidence), but in combination significantly increased the production of male offspring.



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