Determination of Chemical Partitioning in *in Vivo* Aquatic High-Throughput Assays

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Introduction

- High-throughput testing (HTT) has been developed as a rapid, low-cost alternative to traditional *in vivo* toxicity testing.
- HTT of aquatic species in 96-well plate formats are under development and results are being compared to traditional toxicity assays.
- Understanding chemical behavior and partitioning in HTT format is critical for comparison of HTT-derived points of departure with those from traditional aquatic toxicity assays.
- The Armitage model within the high-throughput toxicokinetics (httk) R package was used to estimate free chemical concentration of 9 chemicals in *in vivo* fathead minnow *(pimephales promelas*) HTT assays.
- Free chemical concentration (C_{free}) was verified using liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS) in pre- and post-exposure media to compare with model outputs.

Methods

Fathead Minnow Exposures

- Larval fathead minnows (1 day post hatch) were exposed to 9 concentrations of each chemical in 1mL polypropylene 96-well plates (n=5 larvae per concentration) (Figure 1).
- The highest concentration of chemical was set as 2-3x above the known LC_{50}.
- If LC_{50} was not available, the highest concentration was 6 times the MATC (maximal acceptable toxicant concentration; calculated as the geometric mean between the LOEC [lowest observed effect concentration] and NOEC [no observed effect concentration]).
- If neither of these values was available, the known or estimated water solubility was used as the highest concentration.
- Larval fathead minnows were exposed in 700 µL total volume of Lake Superior water spiked with working stock (concentration of DMSO<0.1%).
- The top two concentrations were made in Lake Superior water from a 30 mM and 9.5 mM stock in DMSO. Subsequent concentrations were made in a log dilution series from these two solutions for a total of 9 concentrations at ½ log steps.
- Working stocks were saved in 1:1 acetonitrile for chemical analysis.
- Plates were incubated for 24 hours at 25°C.
- Media was transfered from exposure plates into another well plate, diluted 1:1 with acetonitrile, and stored at -20°C for chemical analysis.

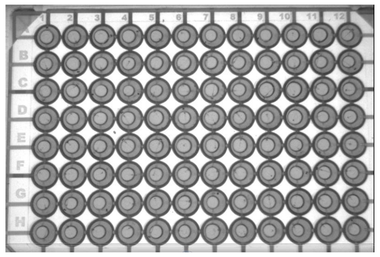

Figure 1. Photograph of larval fathead minnows in exposure plate.

Exposure Distribution Modeling

- The Armitage model[1] within the httk R package[2] was used to model *in vivo* chemical disposition.
- The model is designed for cell-based *in vitro* assays, but parameters were adjusted to estimate chemical disposition in larval fathead minnow (Table 1).
- Input parameters of fathead minnow composition were estimated from 96 h zebrafish[3]. Average fathead minnow mass was estimated from internal US EPA data.

Table 1. Input parameters for the Armitage model.

Well plate parameters	
total volume of well (1.134 mL)	
volume of liquid added (700 uL)	
Fathead minnow parameters	
number of cells (275000)	
mass of cells (8.25mg)	
membrane lipid content of cells (1%)	
structural protein content of cells (6.5%)	
System parameters	
solvent type (Lake Superior water, 0.1% DMSO)	
ionic strength of solvent	
experimental run temperature (25C)	
Chemical parameters	
Concentration	
melting point	
LogKow (octanol-water partitioning coefficient)	
LogKaw (air-water partitioning coefficient)	
water solubility	

Methods

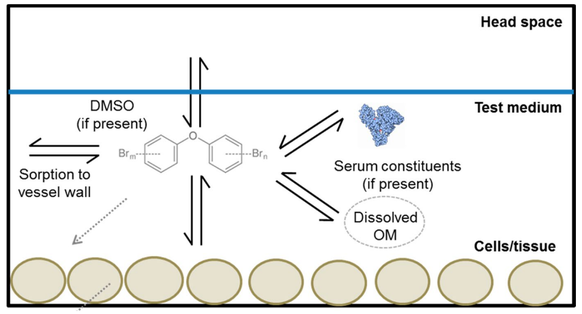

Figure 2. Conceptual representation of an in vitro test system adapted from Armitage et al.[1] DMSO: dimethyl sulfoxide, an example of a cosolvent. OM: organic matter.

Analytical Chemistry

- Chemical stocks used to prepare exposure plates and stored solvent from post-exposure plates were analyzed using LC-ESI-MS using a Vanquish LC system coupled to a TSQ Altis tandem MS.
- Analytical methods were developed for each individual class of compounds. All compounds were run using a reverse phase LC column under gradient separation.
- A stable mass labeled internal standard was used where possible to account for potential matrix effects.

Instrument Parameter Example (used for atrazine, simazine, cyanazine)

- *Thermo Scientific Vanquish LC system*
- Column: StableBond C18, 2.1 x 50mm, 1.8µm
- Column Temp: 40°C
- Injection volume: 1 µL
- Mobile phase: A = H2O+5mM ammonium formate+0.1% formic acid
  B = MeOH+5mM ammonium formate+0.1% formic acid
- Flow rate: 0.4 mL/min
- Gradient: 25% B to 90% B at 3 min held for 1.1 min, return to 25% B.
- Total run time: 6 min
- *Thermo Scientific TSQ Altis triple* quadrupole MS
- Ionization mode: H-ESI, positive
- Gas Temp: 350°C
- Ion Transfer Tube: 350°C
- Sheath Gas : 56.8
- Aux Gas: 1
- Sweep Gas: 1.6
- Capillary voltage: 4500 V

Vanquish LC system coupled to a TSQ Altis tandem MS

Results

Table 2. LogKow inputs and model outputs. C_{plate} = fraction bound to well plate; C_{cell} = fraction bound to cells; C_{free} = fraction free in solvent.

	LogKow	C_{plate}	C_{cell}	C_{free}
Methoxyfenozide	3.7	0.151	0.095	0.754
Estrone	3.13	0.052	0.032	0.916
Nonylphenol	6.06	0.552	0.42	0.028
Atrazine	2.61	0.017	0.011	0.972
Simazine	2.18	0.007	0.005	0.989
Cyanazine	2.22	0.007	0.005	0.988
Parathion	3.83	0.186	0.119	0.694
Fenthion	4.09	0.269	0.175	0.58
Methidathion	2.2	0.007	0.005	0.988

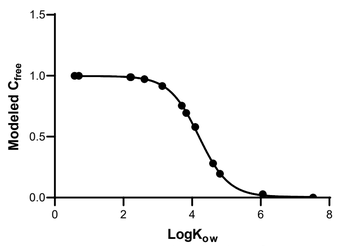

Figure 3. $LogK_{ow}$ inputs and modeled C_{free} for various chemicals.

Results

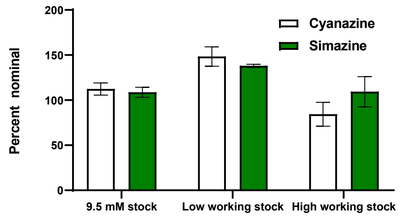

Figure 4. Average percent nominal of chemical stock and working stocks of cyanazine and simazine (Mean±SD, n=3). Working stocks both made from dilution of 9.5 mM stock.

Stock Concentrations (Figure 4)

- Concentrations of 30 mM and 9.5 mM stocks in DMSO used to make exposure plates were measured and were all within 87-118% of nominal.
- Comparing measured concentrations of stocks to working stocks shows that pipetting error may be a large source of error in initial working concentrations.

Model Output vs Measured C_{free} (Figure 5, Figure 6)

- Nominal stock concentrations were used to calculated measured percent recovery of exposure plates for all chemicals except simazine and cyanazine, where measured working stock concentrations were used (Figure 5, 6).
- Top two measured concentrations of atrazine were above or near the measured solubility limit of 28mg/L[4] (highest concentration=52mg/L, second highest=16.4mg/L). These data were excluded because percent recovery was measured at 17% (compared to the median 158%) likely due to solubility issues (Figure 5, 6).
- N values vary depending on the concentrations in the plate and the limit of quantification of the analytical method.
- Measured recovery of parathion, fenthion, and estrone align well with modeled percent recoveries (Figure 5, 6).

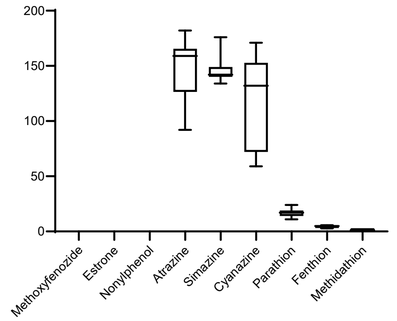

Figure 5. Median percent recovery of chemicals measured in post-24 hour fathead minnow exposure well plates (Median±SD (black box); ±upper and lower limits (error bars)) compared to percent recovery predicted by the Armitage model (red lines). Methoxyfenozide n=18; estrone n=6, nonylphenol n=3, atrazine n=24, simazine n=18, cyanazine n=21, parathion n=12, fenthion n=12, methidathion n=15.

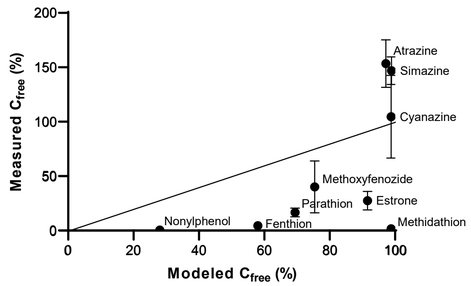

Figure 6. Measured versus modeled percent recovery (Mean±SD). Line: y=x. Methoxyfenozide n=18; estrone n=6, nonylphenol n=3, atrazine n=24, simazine n=18, cyanazine n=21, parathion n=12, fenthion n=12, methidathion n=15.

Discussion and Conclusions

- Dilution of superstocks showed high variability in the two chemicals where working stocks were measured (cyanzine, simazine), indicating the potential for error in the initial preparation of exposure plates.
- C_{free} of triazine herbicides were estimated to be near 100%, but all showed recovery over 100%. This again highlights the potential for dilution-related error and the need to measure final prepared exposure stocks.
- Measured C_{free} of organophosphate compounds (parathion, fenthion, methidathion) were far lower than predicted. Metabolism of fenthion by rainbow trout liver S9 fractions has been previously demonstrated[5], indicating the possibility of *in vivo* metabolism for these compounds.
- Non-volatile chemicals with a $logK_{ow}$ less than 3 are expected to remain near nominal exposure concentrations (Figure 3). As $logK_{ow}$ increases, incorporation of estimated exposure concentrations are important to better estimate true exposure in a HTT scenario.
- Additional model optimization (% protein, % lipid of larval fathead minnows) is underway, and the use of other models estimating in vitro disposition is being investigated.

References

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