2001

Effects of humic acids and salinity on pesticide bioavailability and toxicity as estimated by SPME and toxicity tests

Laurent C. Mézin

College of William and Mary - Virginia Institute of Marine Science

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EFFECTS OF HUMIC ACIDS AND SALINITY ON PESTICIDE BIOAVAILABILITY AND TOXICITY AS ESTIMATED BY SPME AND TOXICITY TESTS.

A Dissertation
Presented to
The Faculty of the School of Marine Science
The College of William and Mary in Virginia

In Partial Fulfillment
Of the Requirements for the Degree of
Doctor of Philosophy

by
Laurent C. Mézin
2001
This dissertation is submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Laurent C. Mézin

Approved, February 2001

Robert C. Hale, Ph.D.
Committee Chairman / Advisor

Mike Unger, Ph.D.

William Reay, Ph.D.

Mike Oesterling, Ph.D.

Peter Van Veld, Ph.D.

Richard Lee, Ph.D.
Skidaway Institute of Oceanography
Savannah, GA
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Abstract:

The interactive effects of humic acids (HAs) and salinity on the bioavailability and toxicity of the organophosphate pesticide chlorpyrifos and the organochlorine pesticide 4,4'-dichlorodiphenyltrichloroethane (DDT) were investigated. The effects of various HAs on the toxicity of chlorpyrifos to the marine bioluminescent bacteria *Vibrio fischeri* were initially assessed with the chronic Microtox® test, conducted at a salinity of 35 parts per thousand (ppt). Environmentally relevant concentrations of Aldrich, Peat, Leonardite and Suwannee River HAs had no significant effect on the toxicity of either chlorpyrifos or copper (Cu), the test’s positive control. As reductions in contaminant toxicity had previously been reported for some contaminants by HAs, it appeared that salinity might be a mitigating factor. Thus salinity effects were further investigated with Aldrich HA only, in freshwater and at lower salinities. HA-pesticide associations were assessed through the pesticides’ relative uptake by solid-phase microextraction (SPME) in the presence of Aldrich HA. Such binding has been hypothesized to reduce contaminant bioavailability. Increasing salinity (0 - 20 ppt) had no effect on the uptake of DDT by SPME, but generally enhanced that of chlorpyrifos. Aldrich HA alone greatly decreased the relative uptake of both pesticides at environmentally relevant DOM concentrations (~ 0-20 mg C/l), and the effect was more pronounced for DDT. Increases in salinity reduced the effects of HA on uptake by SPME, and seemed to have an “effect threshold” between 1 and 5 ppt.

To examine the biological consequences of possible HA-salinity interactions, the
acute toxicities of chlorpyrifos and DDT were assessed using both freshwater 
(*Ceriodaphnia dubia*) and saltwater (*Americamysis bahia*) crustaceans. The DDT 24 hour LC$_{50}$ for *C. dubia* was 1050 ng/l. No definitive value was obtained for *A. bahia*. CPF was more acutely toxic, with LC$_{50}$s of 326 ng/l for *A. bahia* and 78.8 ng/l for *C. dubia*. Results of the acute toxicity experiments were in good agreement with the SPME data; i.e. while pesticide toxicities were reduced by HAs in freshwater, no reductions were seen in saline water (20 ppt). The toxicity reduction in freshwater was proportional to the HA concentration. The difference in toxicity mitigation is believed to be a function of salinity effects on HA-pesticide binding, likely due to conformational changes in the HA molecules, rather than organismal effects.

The high salinity required in the chronic Microtox$^\text{®}$ test and the apparent effect of salt on the HA-pesticide interaction suggest that care must be taken when applying laboratory results to field situations. Results supported the use of nd-SPME to estimate the freely-dissolved fraction and for predicting the bioavailability of compounds to aquatic organisms, under environmentally relevant salinity and HA conditions. As both HA and salinity may affect effluent toxicity, investigators should consider the environmental conditions on-site, as well as downstream, to better assess the toxicity potential and fate of an effluent.
Effects of humic acids and salinity on pesticide bioavailability and toxicity as estimated by SPME and toxicity tests.
**General Introduction:**

Pollutant toxicity is mediated by organism exposure, which is in turn mediated by the contaminant bioavailability. In aquatic environments exposure occurs primarily via the water and through dietary uptake, the relative importance of which are contaminant- and organism-dependant. Hydrophobic contaminants may associate not only with particulates, but also with dissolved organic matter (DOM). A number of studies suggest that waterborne uptake is mostly attributable to the freely-dissolved fraction. Association between contaminants and DOM can reduce the bioavailability of these contaminants and decrease their bioconcentration and toxicity. Other environmental factors have been reported to mediate contaminant toxicity. Water hardness has been identified as a parameter affecting toxicity of metals in freshwater and the ability of DOM to mediate toxicity in these environments. While previous research has determined that increases in ionic strength decrease the magnitude of the DOM-organic pollutant association, little work has examined the effect of salinity on resultant bioavailability or toxicity. As the vast majority of waters in the world are saline and a large percentage of contaminants eventually reach these waters, this is a significant question.

DOM is a naturally occurring constituent of environmental water samples. Dissolved humic materials make up the vast majority of the DOM found in water. They are composed of humic acids (HA), fulvic acids and humins. These classes are separated according to water solubility and molecular weight considerations. HAs are soluble under alkaline, but not acidic conditions [1]. Their molecular weights vary from ~ 1000
to over 300,000 A.M.U. [2, 3]. Typical concentrations in the environment range from 1 to 70 mg/l, with a worldwide average of 5.8 mg/l [4]. HAs result from the partial degradation of organic matter. As a result, their composition is extremely complex and varies not only with its origin, but with the season [5, 6]. Terrestrial, freshwater, marine, and estuarine HAs differ from one another, with estuarine humics being the most complex in nature due to their varied sources. Riverine HAs are more likely to contain lignin residues and be more aromatic than those originating from waters with low or no higher plant input [5]. Bacterial degradation in rivers and estuaries significantly impacts the composition of DOC before it reaches the ocean [7]. Leonardite HA, which is found in conjunction with coal deposits, has a particularly strong aromatic character [8]. Algal and microbially derived humic substances will have a greater aliphatic contribution [9]. HAs may be isolated directly from the environment, but the process is tedious. Moreover, these HAs can vary greatly in composition and structure, even over time at the same location [10]. In contrast, the more constant composition of commercially available HAs (e.g. Aldrich or Fluka HAs), their abundance and low cost make them very attractive as model sorbents [11].

HAs in general are highly aromatic and contain a great number and variety of functional groups which allow them to interact with other compounds in the water. Associations with HAs may occur via H-bonding, ion exchange, Van der Waals forces, protonation, and hydrophobic linkages [2]. These associations may be enhanced by the three-dimensional structure of HAs. Depending on various environmental factors, HAs can form sheet-like or globular structures and associate to form dimers or trimers. This
complex structure allows the formation of intramolecular hydrophobic domains accessible to non-polar molecules [12]. Matthews et al. [13] suggest that HA intermolecular interactions could form a shell within which hydrophobic molecules could be shielded from the polar water molecules. De Paolis and Kukkonen [14] and Engebreston and Wandruszka [8] postulate the existence of intramolecular micelles which act as binding sites for hydrophobic compounds.

The interaction between HAs and compounds have been studied through a range of techniques. Johnson et al. [15], Jiménez et al. [16], and Senseman et al. [17] noted that Aldrich HA in solution associated with various pesticides and decreased their extraction efficiency by solid-phase extraction (SPE). HAs present in natural waters were also shown to associate with pesticides and reduce their recovery by SPE [18]. Laor and Rebhun [19] used a complexation-flocculation technique to determine the binding coefficients of phenanthrene, anthracene, pyrene, and fluoranthene to HAs. The compounds were allowed to associate with the HAs, and the complex was then precipitated by the addition of aluminum sulfate. Binding coefficients were based on the fraction remaining in the supernatant. Rebhun et al. [20], and Yates and von Wandruszka [21] then proposed the method as a way to remove hydrophobic contaminants and metals from water. Schlautman and Morgan [22] and Perminova et al. [23] used fluorescence quenching to measure the association of PAHs with various HAs. Their results indicated that binding to HA increases with the aromaticity of the PAH molecule and the HA, and with the PAH molecule's ability to fit into the intramolecular micelle cavities. Arnold et al. [24] investigated the sorption of triorganotin compounds to HAs by dialysis. They
showed that complexation occurred through association of the compounds’ cations to negatively charged ligands on the HA molecules. Lores and Pennock [25] used ultrafiltration membranes to monitor the association between Suwannee River HA and the metals Cu, Zn, Cd, and Cr. They determined that HA concentrations of 10 mg/l bound at least 40% of each metal in freshwater, but was affected by salinity. Perminova et al. [23] studied the relationships between HA structure and their binding affinities to various PAHs. They found that their $K_{oc}$ is correlated very strongly with the aromaticity of the HA molecules. Aldrich HA has a terrestrial origin. Its molecular characteristics differ somewhat from most other natural HAs (Table 1), and subsequently may have a higher binding affinity for hydrophobic compounds [10, 24, 26, 27]. However, this is not always the case. Sediment HA from Bontoon Reservoir, NJ, showed greater binding capacity for DDT than did Aldrich HA [28]. Landrum et al. [29] found that the partition coefficients of benzo[a]pyrene (BaP) and DDT were an order of magnitude greater with Aldrich HA solutions than with natural waters. However, those of anthracene and tetrachlorobiphenyl did not vary for different HAs, and that of biphenyl was two orders of magnitude smaller with Aldrich HA solutions, under the same conditions. Perminova et al. [23] also described other HAs that had $K_{oc}$s for PAHs that were similar to, or greater than those of Aldrich HA.

The toxicant-HA complex forms rapidly. Beck and Jones [30] found that atrazine and isoproturon sorption to soil was essentially complete within one hour. Alawi et al. [31] noted that the sorption kinetics of organochlorine pesticides to HAs was too fast to be measured by their methods. Saint-Fort and Visser [2] found that the diazinon-HA
Table 1. Molecular composition of various humic acids.
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<tr>
<th>Origin:</th>
<th>Aldrich (a)</th>
<th>Peat (b)</th>
<th>Leonardite (b)</th>
<th>Suwannee River (b)</th>
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<tr>
<td></td>
<td>Terrestrial</td>
<td>Everglades, FL</td>
<td>Oxidized coal seam, ND</td>
<td>Surface water, Suwannee River, GA</td>
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<tr>
<td>% C</td>
<td>69</td>
<td>57</td>
<td>64</td>
<td>53</td>
</tr>
<tr>
<td>% H</td>
<td>5.0</td>
<td>3.6</td>
<td>3.6</td>
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<tr>
<td>% O</td>
<td>39</td>
<td>37</td>
<td>32</td>
<td>43</td>
</tr>
<tr>
<td>% N</td>
<td>0.75</td>
<td>3.7</td>
<td>1.3</td>
<td>1.2</td>
</tr>
<tr>
<td>% S</td>
<td>4.3</td>
<td>0.70</td>
<td>0.77</td>
<td>0.58</td>
</tr>
<tr>
<td>% P</td>
<td>0.15</td>
<td>0.03</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Total (%)</td>
<td>118</td>
<td>98.3</td>
<td>102</td>
<td>102</td>
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(a): [26]; (b): International Humic Substance Society (pers. com. 1997)
interaction was complete within 3 minutes, but that between HA and atrazine or lindane was slower (<1 hour). Schlautman and Morgan [22] described the sorption of three PAHs as complete within three minutes.

The compound-HA interaction is of particular importance because it may affect the compound’s bioavailability, bioaccumulation, activity and degradability, even at naturally occurring HA concentrations.

Caron et al. [32] studied the effect of HA on the sorption of DDT and lindane to sediments. They noted that DDT sorption was reduced, but not lindane’s, and concluded that this could facilitate DDT transport through the aquatic system. Kamiya and Kameyama [33] reported that photo-induced radical generation in HAs increased the degradation of the organophosphate pesticides tested, and that the magnitude of effect varied both with the pesticide’s sensitivity and the HA radical generation potential. Fu et al. [34] also reported increased contaminant degradation in the presence of HAs. They found that Aldrich HA mediated the dechlorination of 1,2,3,4,6,7,9-heptachlorodibenzo-p-dioxin. The association of a contaminant to HAs has also been shown to have effects on its bioavailability and toxicity. HAs of various origins have been shown to decrease BaP uptake and bioaccumulation in the nematode Caenorhabditis elegans [35], the daphnid Daphnia magna [36], and the Atlantic salmon Salmo salar [37]. Muir et al. [38] and Freidig et al. [39] showed reduced bioavailability and uptake of organic pollutants such as DDT and pyrethroid pesticides to the guppy (Poecilia reticulata) and the rainbow trout (Oncorhynchus mykiss) in the presence of Aldrich HA. The uptake and accumulation in fish of metals such as Cu and Cd also decreased in the presence of HAs.
Verhaar et al. [40] were able to model deviations in the relationship between bioconcentration factors (log BCF) and lipophilicity (log K_{ow}) by the presence of low levels of DOC. The relationship between log BCF and log K_{ow} is generally assumed to be linear. In reality, however, it stabilizes at high K_{ow}s. Verhaar et al. reported these deviations could be explained by including in the model low DOC concentrations inherent to any biological system (e.g. through mucus sloughing and feces).

The presence of HAs does not always lead to a decrease in bioavailability. McCarthy and Jimenez [43] showed that while the bioaccumulation of BaP in bluegill sunfish (Lepomis macrochirus) decreased in the presence of HA, that of naphthalene did not, and that this was a reflection of the amount of contaminant bound to the HA. Similarly, Kukkonen and Oikari [44] reported lower bioconcentration in D. magna in the presence of natural humic water for BaP and dehydroabietic acid, but not for pentachlorophenol. DOM has even been shown to enhance bioconcentration up to three times, at low concentration [45].

This change in contaminant bioavailability in the presence of HA is reflected in changes in toxicity. Day [10] studied the effects of Aldrich HA on the accumulation and acute toxicity of fenvalerate, deltamethrin and cyhalothrin to D. magna. Bioaccumulation and toxicity of the pesticides decreased with increased HA concentration. The toxicity of fenvalerate and permethrin was shown to decrease in the presence of Suwannee River and Aldrich HAs, as measured by the acute Microtox® test [3]. Wu et al. [46] and Weinstein and Oris [47] also reported reduced toxicity of the insecticide fenpropathrin and the PAH fluoranthene to fish, with increasing HA concentration. Gensemer et al. [48] noted a
decrease in PAH photoinduced toxicity to plants in the presence of HA.

The effect of HA on toxicity of a contaminant can depend on the species, the contaminant, and the HA. Stuijfzand et al. [49] exposed zebra mussels (Dreissena polymorpha) and midges (Chironomus tentans) to Cu and Cd in the presence of Aldrich HA. Cu toxicity decreased in the presence of HA for the midge, but did not change for the mussel. Cd toxicity increased with HA concentration for the mussel. Bao et al. [50] exposed D. magna to tributyltin chloride and triphenyltin chloride in the presence of various concentrations of peat, sediment, and soil HA. Peat HA effected the greater decrease in toxicity, followed by soil HA. Sediment HA did not significantly affect the toxicity of either contaminant to D. magna.

Salinity may also play a role in pollutant fate, bioavailability, and toxicity. Increasing salinity can affect the solubility of dissolved compounds. This “salting out” effect was first investigated quantitatively by Setchenow in 1889. Buchholtz and Pawliszyn [51] explain that water molecules will preferentially solvate ionic compounds, such as the salt ions, at the expense of neutral compounds. This results in lower solubility of the neutral compounds, and increased sorption to a less polar phase such as C-18 silica. Jiménez et al. [16] and Hela et al. [52] reported increased recovery of hydrophobic pesticides by solid-phase extraction with increasing salinity. This effect will be reversed for polar compounds, where the increased ionic strength of the water may enhance their solubility.

This effect of salinity on the solubility of compounds may be reflected by differences in toxicity. Brecken-Folse et al. [53] reported that the toxicity of 4-
nitrophenol, a methyl-parathion metabolite, decreased with increasing salinity for sheepshead minnow (*Cyprinodon variegatus*) and for grass shrimp (*Paleomonetes* spp.). That of 2,4-dinitrophenol increased for *C. variegatus*, but decreased for *Palaemonetes* spp. in the same study. A study by Linton *et al.* [54] set a counterpoint; they could find no significant effect of salinity for growth or survival of *C. variegatus* exposed to 2,4-dinitrophenol. Fulton [55] reported an increase in acephate toxicity to the mummichog (*Fundulus heteroclitus*) with increasing salinity. The toxicity of guthion to the threespined stickleback (*Gasterosteus aculeatus*) and that of Aroclor 1254 to a panaeid shrimp also increased with salinity [56, 57]. Cadmium toxicity to the blue crab (*Callinectes sapidus*) and the flounder (*Platichthys flesus*) increased with decreasing salinity [58, 59]. This may have been due to the complexation of Cd with the ions in solution. Brown *et al.* [60] found that Cd uptake in the bivalve *Potamocorbula amurensis* decreased with increasing salinity. Wood *et al.* [61] determined that ionic silver complexes with chloride ions in saline water, thereby greatly reducing its toxicity.

Other salinity-dependant mechanisms can affect toxicity. El-Alfy and Schlenk [62] determined that the salinity induced toxicity of aldicarb to the Japanese medaka (*Oryzias latipes*) might be attributed in part to the salinity-dependant upregulation of flavin-containing monooxygenases which increased the transformation of aldicarb to a more toxic metabolite. Pentachlorophenol uptake rate in *O. latipes* decreased, and its clearance rate increased with increasing salinity, resulting in decreased accumulation [63].

Salinity can affect the normal physiology of organisms. For example, the gill
activity of carbonic anhydrase, an enzyme involved in the excretion of CO₂, increased with increasing salinity. The enzyme activity in the mesonephros of the same fish, the tilapia *Oreochromis hornorum*, remained stable [64]. Ion imbalance at a given salinity HAs also been a source of toxicity. Douglas and Horne [65] determined the suite of ions essential to the mysid *Americamysis bahia*. Its survival was significantly impacted when these ions varied from their relative proportions in seawater. An imbalance in ionic composition of an irrigation drain effluent accounted for some of its toxicity to the striped bass (*Morone saxatilis*), and the amphipod *Hyalella azteca* [66, 67]. The toxicity of concentrates from a Florida drinking water plant was significantly reduced when its ionic ratio was manipulated to resemble that of seawater [68].

Salinity may also have an effect on HAs in solution and their associations with contaminants. HAs contain both hydrophilic and hydrophobic moieties, and their sorption to mineral particles has been observed to increase with increased salinity [69]. The rheology and three-dimensional structure of HAs are dictated by intramolecular interactions of the molecule’s functional groups [22]. Increased ionic strength (salinity) of the water will affect these interactions and ultimately the shape of the HA molecules. De Paolis and Kukkonen [14] and Engebreston and Wandruszka [8] postulated the existence of intramolecular micelles which may act as binding sites for hydrophobic compounds. Changes in molecular conformations due to salinity changes could result in the occlusion of these sites to binding. Increasing salinity can result in enhanced molecular coiling, and eventually precipitation of the HA as the electrical double layer surrounding the HA molecule is suppressed [5, 69]. The ‘salting out’ effect can therefore
also alter the affinity of compounds to DOM.

The decreased recovery of some pesticides and metals in the presence of dissolved HA may be mitigated by increasing the salinity of the solution. The addition of 10 % NaCl improved the SPE recovery for 3 of 12 intermediate polarity pesticides in the presence of 10 mg/l Aldrich HA [16]. The association between Suwannee River HA and Zn, Cd, and Cr was also decreased with increased salinity, though that of Cu proved more complex. Its association with Suwannee River HA first decreased at 3 ppt (from ~70 % to 30 % bound), then increased to ~ 60 % bound at 15 ppt [25]. Means [70] and other authors (e.g. [71]) even have reported that pyrene, permethrin, and bis(2-ethylhexyl)phthalate sorbed more to organic matter with increased salinity.

These combined effects of salinity and HAs have been shown to be more pronounced for hydrophobic contaminants and will be more important for those compounds which routinely enter an aqueous environment, such as pesticides. Though most pesticide uses are terrestrial, they can reach and impact riverine and estuarine environments in a variety of ways. Pesticides can enter the water by direct spraying, long-range atmospheric deposition, spills, and runoff from agricultural land [72]. Rain events can wash up to 95 % of applied pesticides from the plants into ground water and streams [73].

Organochlorine pesticides such as DDT (Figure 1a) were synthesized and widely used in the 20th century, but their persistence in the environment and effects on non-target organisms were eventually seen by some to exceed the benefits of their use. Indeed, in a study of chemical residues in US fish, dichlorodiphenyldichloroethylene (DDE), a stable
Figure 1. Molecular structures of CPF (1a) and DDT (1b).
and toxic metabolite of DDT, was the most ubiquitous synthetic organic contaminant reported, detected in 98.6% of the sampled sites [74]. A 1991 study by the National Oceanic and Atmospheric Administration (NOAA) found total DDT (sum of DDT, dichlorodiphenyldichloroethane (DDD) and DDE) to be the most frequently detected organochlorine insecticide in estuarine bedded sediment [75]. DDT’s acute toxicity is due to its ability to slow the repolarization of the nerve membrane following stimulation. Exposure may result in neurons being completely depolarized, even after small stimuli. DDT may also disrupt endocrine function, interfering with normal fertility and reproduction [75].

DDT was first described by Zeigler in 1873 and was marketed in 1942, for crop protection and human hygiene [76]. It was widely used during WWII to control typhus and malaria in allied troops and certain civilian populations. Agricultural use increased greatly after the war, but insects started to develop a resistance to the pesticide [77, 78]. In 1959, use in the USA peaked at 36 million kg of active ingredient. DDT’s persistence and toxicity to non-target biota resulted in its ban in the USA in 1972, except in case of a public health emergency such as an outbreak of malaria [79]. DDT is still in use, mainly in tropical countries, primarily to control mosquito-borne malaria [79]. Illegal uses of DDT also occur and contribute to current environmental concentrations [80]. It is highly stable in the environment, with a calculated half-life of 15 years in soil [75], and reported half-lives in water ranging from 28 to 56 days [79]. DDT will preferentially partition to organic matter ($K_{oc}$: $4.3 \times 10^5$). Its high $K_{ow}$ ($\sim 10^6$) and persistence in organisms mean it may bioaccumulate to toxic levels [75]. DDT degrades and is metabolized to DDD and
DDE, which are also toxic. For example, DDE has been linked to reproductive
dysfunctions in sea lions [81], and in decreases in hatching success in the double-crested
Comoran [82], the bald eagle, and other birds [83, 84, 85]. DDD has been linked to
lesions in winter flounder [86]. The wide use and persistence of DDT has made it and its
metabolites ubiquitous in the environment, even decades after its ban in the area. Total
DDT has been found in Californian otters [81], river dolphins in India [87], wolves in
Spain [88], and porpoises in Sweden [89]. It has been detected in human milk samples
from Italy and the USA [90, 91]. It is likely to remain a significant environmental
concern for decades to come, even if all current uses are stopped.

In response to the persistence and relative lack of specificity of organochlorine
pesticides, other classes of pesticides have been developed such as carbamate, pyrethroid
and organophosphate pesticides. There are 39 currently registered organophosphate
pesticides in the US. They account for 8 % of the total US pesticide use, in terms of kg of
active ingredients, – 50 % of the total insecticide treated acreage, and 65 % of the active
ingredient weight used (27 million kg). Another 8 million kg are applied in non-
aricultural settings [92]. Chlorpyrifos (CPF) (Figure 1b) has been one of the dominant
organophosphate insecticides in the USA and the Chesapeake Bay basin [93, 94, 95, 96].
CPF is classified as “hazardous” by NOAA [95] because of its potential to impact the
coastal aquatic environment.

Organophosphate pesticides such as CPF bind and inactivate acetylcholine
esterase, the enzyme responsible for hydrolyzing the neural transmitter acetylcholine.
Subsequent accumulation of acetylcholine in the nerve synapses results in nerve over-
stimulation and 'jamming'. The autonomous and central nervous systems, as well as the neuromuscular junctions can be adversely affected, resulting in muscle convulsions, paralysis and death [76, 97]. CPF is fairly hydrophobic, albeit less than DDT, with an octanol-water partitioning coefficient of $\sim 10^5$ [98]. It has a $K_{ow}$ of 6070 and a fresh water solubility of 0.4 mg/l [99]. The half-life of CPF in water is greatly affected by pH; at 25 °C, it has a half-life in water of 1.5 days at pH 8.0, and 35-78 days at pH 7.0 [100, 101]. CPF’s half-life varied from 2 weeks to a year [101] in soils, and was estimated at 24 days in sediment from a Floridian salt marsh [102]. Readman et al. [103] determined that CPF had the potential to seriously contaminate and affect marine systems due to its stability.

McConnell et al. [104] described CPF as the most heavily applied insecticide in the Chesapeake Bay basin. Annual usage in the USA was estimated at 4.5 to 9 million kg per year in 1990-1991 [104]. The US Environmental Protection Agency (EPA) estimated an average domestic consumption of $\sim 13$ million kg per year based on available data from 1987 to 1998 [105], split evenly between agricultural and non-agricultural uses. Agricultural uses of CPF included treatment of corn and alfalfa [104, 106]. About 73% of brussel sprouts, 46% of cranberries, and 44% of broccoli were treated with CPF [105]. Other applications included turf applications, indoor pest control, and animal-care products [105, 106].

In a study assessing the health risks of pesticides to humans and wildlife in California, CPF ranked in the top-10 most hazardous current-use pesticides [107]. Smith [108] ranked it “highly toxic” to wildlife species. Odenkirchen and Eisler [109] noted
that CPF concentrations of 35 to 580 ng/l were toxic to several species of freshwater and marine invertebrates and fishes. Representative, low-end 96 hr LC₅₀'s are 40 ng/l for *Hyalella azteca* [110], 80 ng/l for *Paratya australiensis* [111], and 130 ng/l for the fish *Pimephales promelas* [109]. The US EPA found the CPF threat to human and the environment to be so great that its revised risk assessment of June 2000 mandated the phasing out of most home and some agricultural uses by the end of 2004.

CPF has been detected at acutely toxic concentrations in the environment. Gomez-Gomez *et al.* [112] detected CPF concentrations of 200 ng/l in the Guadalete River, Spain. Kimbrough and Litke [113] found concentrations of 300 ng/l in Colorado streams. Thompson and Treble [114] detected 32,000 ng/l of CPF in a Canadian pond. Water samples collected from sewage systems near animal-care facilities have contained from 2,000 to 7,000 ng/l [115]. Over 30% of the samples collected from California urban waterways contained detectable amounts of CPF, with median and maximum concentrations of 50 and 190 ng/l [106]. CPF has even been found in marine ice (170 ng/l) and seawater (up to 70 ng/l) in the Bering and Chukchi seas [116].

While some studies have investigated the effect of salinity or HAs on compounds and their toxicity, few studies have considered their interactive effects on a contaminant's bioavailability, and even fewer on its resultant toxicity. The current study estimated the combined effects of salinity and HAs on the bioavailability of two representative organophosphate and organochlorine pesticides, CPF and DDT, and assessed the resultant effect on their toxicity.
Objectives:

The specific objectives of this study were to:

1) Determine CPF’s and DDT’s freely-dissolved fractions, a measure of their bioavailability, in the presence of HAs at various salinities.

2) Determine any change in toxicity of CPF and DDT in the presence of various concentrations of HAs using acute and chronic toxicity tests in fresh- and saltwater.

3) Correlate the DDT- and chlorpyrifos-HA interactions to the (observed) changes in toxicity.

Null hypotheses

$H_{0,1}$: CPF and DDT will not bind to HAs. Their freely-dissolved fraction will not be modified by the presence of HAs.

$H_{0,2}$: Salinity will not modify HA-pesticide interactions.

$H_{0,3}$: HAs will not impact the pesticides’ toxicities, at any salinity tested.
Chapter 1:

The effects of various humic acids on the toxicity of copper and chlorpyrifos, as measured by the chronic Microtox® test.

Abstract:

The effects of four different HAs (Aldrich, Peat, Leonardite, and Suwannee River) on the toxicity of copper (Cu) and chlorpyrifos (CPF) were assessed by the chronic Microtox® test. No significant effect of any of these HAs (5 mg/l) on Cu or CPF toxicity was observed. The lack of significant effect on toxicity is hypothesized to be largely a result of the relatively high salinity of the test (35 ppt), which may have decreased interactions between the HAs and the contaminants. This suggests that: 1) salinity may be a more important environmental variable than HA type in the control of toxicity; 2) care should be taken when applying results of laboratory bioassays to field scenarios, as not only may organismal sensitivities differ, but ambient environmental conditions may significantly alter the outcome.

Introduction:

Pesticides are intentionally applied to the natural environment and may enter water bodies in a variety of ways, including direct spraying, ground water transport, or
overland runoff after a rain event (e.g. [72]). Hydrophobic pesticides will preferentially associate with particulate or dissolved organic matter such as humic acids (HA), rather than remain freely dissolved. HAs can affect the fate and toxicity of pesticides [10, 33, 50] by altering their transport and decreasing their bioavailability [117]. The effect of HAs will depend on the extent of these interactions, and in turn on the properties of the pesticides and HAs. HAs are derived from the degradation of organic matter, and are differentiated from fulvic acids and humins by their relative water solubility and molecular weight. Their composition is complex, and varies not only with origin, but with season [5, 6]. For example, Leonardite HA, derived from coal seams, has a strong aromatic character [8], whereas HAs derived from lower plant matter are typically more aliphatic [9]. The composition of some representative HAs are presented in Table 1. HA characteristics of importance with respect to pollutant binding include aromaticity (H/C ratio). In a study of 26 different humic materials, Perminova et al. [23] found that the magnitude of their association ($K_{oc}$) with pyrene, fluoranthene, and anthracene was strongly correlated with the humic materials’ aromaticity. A previous study by Chin et al. [27] has yielded similar results for six humic materials and pyrene. De Paolis and Kukkonen [14] reported that binding ($K_{oc}$) of BaP and pentachlorophenol to humic acids was correlated to aromaticity and functional group content. Thomsen et al. [118] cited aromaticity and polarity as important factors in assessing the association of esfenvalerate to seven different dissolved humic materials.

In the past few decades, environmental protection legislation has increased the need to perform toxicity tests on commercial products and environmental samples, and
has led to the development of a wide array of assays. The acute Microtox® toxicity test was introduced in the 1980s. It uses the decrease in light output of bioluminescent bacteria (*Vibrio fischeri*) following exposure to a toxicant as an endpoint (the EC$_{50}$, or concentration that results in a 50% reduction in light emission by the bacteria). The test is rapid (5-30 minute endpoints), and requires a minimal amount of space and manpower. Variants of the test include acute, solid-plate, mutagenic, and chronic tests (22 hour). Advantages to using bacteria in toxicity tests include: 1) their high surface to volume ratio, which maximizes the exposure potential, and minimizes the distance from the uptake site (cell surface) to the site of action of the toxicant [119]; 2) the test bacteria have complex biochemical pathways similar to the higher organisms typically used in toxicity testing, but have shorter life cycles (e.g. 120 minute generation time for *V. fischeri*); 3) they are inexpensive [120]; and 4) their use in acute toxicity testing does not pose an ethical concern. The chronic Microtox® test was introduced around 1994 and presents several advantages over the acute format. Because it spans several generation times, the toxicant can affect both the reproductive cycle and the induction of light production [121, 122]. This increases the sensitivity of the chronic test ~ 20 fold, as compared to the acute Microtox® test, making it more comparable to the *Ceriodaphnia dubia* 4-day survival and reproduction test [122]. Bulich *et al.* [123] compared the lowest observable effect concentrations (LOECs), as determined in the chronic Microtox® test, and the four-day daphnid *Ceriodaphnia dubia* survival and reproduction test, using measured or reported values for nine toxicants. The chronic Microtox® LOECs were lower than the daphnid’s for Cu (0.019 vs 0.04 mg/l), chromium (0.035 vs 3 mg/l) and
the pesticide 2,4-D (0.3 vs 40 mg/l). They were equal to the daphnid’s for zinc (0.1 mg/l), and 10 to 25 times higher for cadmium, nickel, lead, diazinon, and methoxychlor.

A literature search revealed only one study on the effect of natural DOM on compound toxicity in the chronic Microtox® assay. Hsieh et al. [120] assessed the impact of Suwannee River fulvic acid (2 to 25 mg/l) on the chronic Microtox® EC_{50} of Cu. They concluded that fulvic acid itself was non-toxic up to 6.25 mg/l. Cu toxicity was reduced by half by the addition of up to 20 mg/l fulvic acid. No statistical analysis was performed. The chronic Microtox® test uses Cu (as CuSO_4) as a positive control. Its EC_{50} is 20 to 40 µg/l [124].

Acute Microtox® tests have been conducted on organophosphate pesticides in the presence of HAs, and the results have varied. Benson and Long [1] found an EC_{50} increase (toxicity reduction) for CPF in the presence of 0.5 mg/l of Aldrich HA (from 3.86 to 6.65 mg/l; 5 min. test), but a toxicity increase for methyl parathion (from 0.543 to 0.363 mg/l; 5 min. test). Kadlec and Benson [125] found toxicity decreases for methyl parathion (from 0.396 to 0.551 mg/l; 5 min. test) and for guthion (from 0.300 to 0.497 mg/l; 5 min. test), but no significant toxicity change for CPF or the pyrethroid insecticide fenvalerate, using natural water with DOM concentrations of 2.4 to 6.8 mg/l. The 15 min. EC_{50} of permethrin, another pyrethroid insecticide, increased in the presence of 50 mg/l Aldrich HA (from 2.73 to 6.08 µmol), or 0.5 mg/l of the Suwannee River HA (from 2.73 to 3.48 µmol) [3]. These EC_{50} values, though significant, do underline the relative insensitivity of the acute Microtox® test to those contaminants. For example, representative permethrin and CPF acutely toxic concentrations to invertebrates and
fishes are 100 to 1,000,000 times lower than those presented here [109, 126].

Though some data exist on the effect of HAs on the toxicity of CPF as assessed by the acute Microtox® test, the effects of different HAs in the more sensitive chronic Microtox® on the toxicity of CPF have yet to be ascertained.

**Objectives:**

This study’s objectives were to use the chronic Microtox® test to:

1) Determine the Cu (as CuSO₄) 22 hour EC₅₀ to *Vibrio fischeri*.
2) Determine the CPF 22 hour EC₅₀ to *Vibrio fischeri*.
3) Assess the effect of different HAs on CPF toxicity to *Vibrio fischeri*.
4) Assess the effect of different HAs on Cu toxicity to *Vibrio fischeri*.

**Null hypotheses:**

H₀₁: Cu is not toxic to *Vibrio fischeri*.
H₀₂: CPF is not toxic to *Vibrio fischeri*.
H₀₃: HAs are not toxic to *Vibrio fischeri*.
H₀₄: Addition of HAs will not affect the toxicity of Cu to *Vibrio fischeri*.
H₀₅: Addition of HAs will not affect the toxicity of CPF to *Vibrio fischeri*.

**Materials and Methods:**

**Microtox® reagents and glassware:**

The reagent activation solution, reagent, test media, and positive control solution
(copper sulfate) were purchased from Azur Environmental (Carlsbad, CA). The reconstitution solution (high purity water) was Milli-Q water (17.7 MΩ cm), obtained in-house. Test vials (Azur Environmental) were used once and discarded. All pipettes and pipette tips were rinsed in reconstitution solution immediately prior to use.

**Test medium solutions:**

Test media solutions are those to which the bacteria are added immediately prior to the test, and in which the tests are conducted. They are “reconstituted” from a freeze-dried state by the addition of 36 ml of water or sample, and contain nutrients and salts (35 ppt) necessary to sustain *V. fischeri* growth and reproduction. All solutions were filtered to 0.45 µm with pre-rinsed cellulose nitrate membrane filters (Whatman, Clifton, NJ).

**Chlorpyrifos (CPF) and HA solutions:**

CPF (Chem Services, West Chester, PA) test solutions (309 µg/ml) were made in acetone. Stock solutions (100 mg/l) of Aldrich (Aldrich Chemical Company, Inc., Milwaukee, WI), peat, Leonardite, and Suwannee River HAs (International Humic Substances Society, St Paul, MN) were prepared by dissolution in high-purity water (pH ~ 10). The pH of the solution was then reduced to ~ 7.0 (HCl). Working HA solutions of varying concentrations were prepared by diluting stock solution with reconstitution solution.
Water quality:

No DOC values were measured. However, previously determined data suggest that the water to which no HA were added had low DOC levels (<1 mg C/l). Those to which HA were added likely had DOC levels somewhat less than 3 mg C/l. In accordance with the chronic Microtox® protocol, the salinity used in the chronic test was 35 ppt, and higher than those used later in this study (0 to 20 ppt; Chapters 2 and 3).

The chronic Microtox® test:

Chronic Microtox® tests consisted of five replicate control cuvettes containing no toxicant; four replicate serial dilution series consisting of five concentrations of the positive control (CuSO₄); and four additional replicate dilution series also consisting of five concentrations of the toxicant to be tested (Cu, CPF, or peat, Leonardite, or Aldrich HA). When the effect of HAs on Cu or CPF toxicity were assessed, the second set of dilution series contained a constant 5 mg/l concentration of a HA (peat, Leonardite, Suwannee River, or Aldrich), along with decreasing concentrations of either Cu or CPF. This HA concentration had been reported previously to affect the toxicity of compounds with the acute Microtox® test with Aldrich HA, and is environmentally relevant [1, 4]. The cuvettes were incubated ~ 22 hours at 27 °C, and the bioluminescence produced by bacteria in each cuvette measured using a model 500 analyzer (Azur Environmental). Proprietary software (Azur Environmental) was then used to calculate the EC₅₀.
Results:

Chronic toxicity results:

The Cu (positive control) chronic EC$_{50}$ was computed for 46 replicate tests for which a good dose-response relationship was obtained, i.e. where increases in toxicity (decreases in light emission) had a good coefficient of determination ($R^2 > 0.80$) with increases in Cu concentration (Figure 2). Another 10 tests failed to meet that criteria. The mean calculated 22 hour EC$_{50}$ (± standard deviation) from these was 9.51 µg/l (± 2.77). The CPF chronic toxicity was computed for seven tests. All tests had $R^2 > 0.80$ (Figure 3). The mean CPF 22 hour EC$_{50}$ was 466 µg/l (± 191). No definite trend in toxicity could be ascertained for the HAs alone. Assessment of HA toxicity was conducted for three of the four HAs at concentrations ranging from < 1 to 100 mg/l. The fit of toxicity curves was generally poor ($R^2$ from 0.00 to < 0.80) and no definite toxicity could be determined for HAs at a concentration of 10 mg/l. No Microtox® assay was conducted for Suwannee River HA by itself. Sample toxicity response curves for water, Cu, CPF, and Aldrich, peat and Leonardite HA are presented in Figure 4.

HA mitigation of toxicity:

The effect of the four HAs (5 mg/l) on the toxicities of Cu and CPF are presented in Figures 2 and 3, respectively. Six (Aldrich), four (Leonardite and Suwannee River), and three (Peat) tests were conducted with the HAs. For Cu (Figure 2), three tests with Leonardite and peat, four with Suwannee River, and six with Aldrich HA showed a good dose-response ($R^2 > 0.80$). No statistically significant effect of any of the HAs on Cu
Figure 2. Toxicity ($EC_{50}$) of Cu to *Vibrio fischeri*, as measured by the chronic Microtox® test without added HA, or in the presence of 4 different HAs (5 mg/l).
Figure 3. Toxicity (EC₅₀) of CPF to *Vibrio fischeri*, as measured by the chronic Microtox® test without added HA, or in the presence of 4 different HAs (5 mg/l).
Figure 4. Representative chronic Microtox® test response curves, for:
   a) water (non-toxic)
   b) copper
   c) CPF
   d) Aldrich humic acid
   e) peat humic acid
   f) Leonardite humic acid
toxicity could be discerned by ANOVA (p < 0.05). The mean EC$_{50}$s for Cu in the presence of 5 mg/l HA were 8.35 ± 2.62 µg/l (Aldrich), 7.66 ± 1.93 µg/l (Leonardite), 10.84 ± 2.62 µg/l (peat), and 10.42 ± 1.88 µg/l (Suwannee River HA). For CPF (Figure 3), three tests showed a good toxicity response ($R^2 \geq 0.80$) for each of the four HAs. No statistically significant effect of any of the HAs (5 mg/l) on CPF EC$_{50}$ could be discerned (p < 0.05). The mean EC$_{50}$s for CPF in the presence of 5 mg/l HA were 357 ± 159 µg/l (Aldrich), 295 ± 96 µg/l (Leonardite), 306 ± 42 µg/l (peat), and 212 ± 47 µg/l (Suwannee River HA).

**Discussion:**

**Cu EC$_{50}$:**

The mean EC$_{50}$ for Cu determined in this study with no HA added was 9.51 µg/l. This is 2 to 4 times lower than EC$_{50}$s reported by previous investigators [120, 124]. The mean EC$_{20}$ was 6.4 µg/l (± 2.4), and within the range reported by Azur Environmental personnel (≈ 2 - 20 µg/l) (personal communication). Toussaint et al. [127] reported the acute Microtox® test EC$_{50}$s, *Ceriodaphnia dubia's* (48 hour) and *Americamysis bahia's* (96 hour) LC$_{50}$s for Cu to be 1300, 27, and 16 µg/l, respectively. While the acute Microtox® test was by far less sensitive than the 48 and 96 hour tests for the other two organisms, the 22 hour chronic Microtox® test showed comparable sensitivity to Cu. The chronic Microtox® test EC$_{50}$s in the present study had a relative standard deviation (RSD) of 29%. Sources of variance that could have increased the RSD include operator
manipulation, such as faulty pipetting, differences in reagent quality, or glassware contamination.

**CPF EC$_{50}$**

A literature search did not uncover any literature values for the chronic Microtox$^\text{b}$ EC$_{50}$ of CPF. The value obtained in this study was 466 µg/l. The RSD associated with this value was relatively large (41 %). Benson and Long [1] determined acute Microtox$^\text{c}$ EC$_{50}$ of 3860 to 5840 µg/l for CPF, depending on the time endpoint (5 to 30 minutes), 10-fold higher than the chronic value determined here. Huynh et al. [122] noted that the chronic test was in general about 20 times more sensitive than the acute test. According to the measured CPF Microtox$^\text{c}$ EC$_{50}$, *V. fischeri* is not among the most sensitive organisms to CPF. For example, CPF 96 hour LC$_{50}$s of ~9 µg/l for rainbow trout [101], 0.06 µg/l for *Ceriodaphnia dubia* [128], and 0.04 µg/l for *Americamysis bahia* [102] have been reported. Presumably, this lack of sensitivity is a result of CPF’s main mode of action. CPF acts on the nervous system, which is absent in single cell bacteria such as *V. fischeri*.

**HA mitigation of Cu toxicity:**

The HAs tested (Aldrich, Leonardite, peat, and Suwannee River) did not have a significant effect on Cu EC$_{50}$ in the chronic Microtox$^\text{c}$ test, at a nominal HA concentration of 5 mg/l. RSDs for these tests were 31, 25, 24, and 18 %, respectively. The various HA tests were performed concurrently, so this decrease in RSD by HA is not
due to increased operator experience. Hsieh et al. [120] assessed the mitigation of Cu toxicity by Suwannee River fulvic acid (0 to 25 mg/l) using chronic Microtox®, in triplicate experiments. They reported a Cu-only EC$_{50}$ of 20 to 40 µg/l. The RSDs ranged from 5% (no fulvic acid added) to 54% (5 mg/l fulvic acid added). The Cu EC$_{50}$s increased (toxicity decreased) when 2 to 20 mg/l fulvic acid were added (16% increase at 2 mg/l, 21% at 20 mg/l, with a maximum increase of 51% at 10 mg/l), even though fulvic acids alone had shown toxicity at nominal concentrations as low as 6.25 mg/l. Hsieh et al. [120] concluded that fulvic acids significantly decreased Cu toxicity, apparently without the benefit of statistical analysis. Morel et al. [129] used the acute Microtox® assay to assess the complexation of Cu to Aldrich HA through changes in toxicity. They reported no significant effect of Aldrich HA on Cu toxicity until a nominal HA concentration of 50 mg/l or more was reached. These results agree with those of the present study. The potential HA-only toxicity was not assessed by Morel et al..

**HA mitigation of CPF toxicity:**

Aldrich, Leonardite, Peat, and Suwannee River HAs (5 mg/l nominal concentration) had no statistically significant effect on CPF chronic Microtox® EC$_{50}$. The RSDs for the tests with each HA were 45, 33, 14, and 22%, respectively. Available literature revealed two acute Microtox® studies that assessed the effect of DOM on CPF toxicity. Benson and Long [1] examined the impact of Aldrich HA (“final concentrations” of 0 - 100 mg/l) on the toxicity of a variety of organophosphate and carbamate pesticides, including CPF. No replication of the tests themselves was...
apparent. These same authors found significant decreases in the toxicity of CPF, azinphos-methyl, and carbofuran at HA concentrations of 0.5 mg/l and above. In contrast, they reported increases in methyl-parathion toxicity under the same conditions, and in carbaryl toxicity at a HA concentration of 100 mg/l. They did not examine the toxicity of Aldrich HA alone. Kadlec and Benson [125], using natural waters from different streams (total organic carbon: 2.4 to 6.8 mg/l), found some significant decreases in the toxicity of azinphos-methyl and methyl parathion, but none for CPF and fenvalerate.

Because the acute and chronic Microtox® tests are conducted at different salinities (20 and 35 ppt, respectively), and considering the potential effect of salinity on HA binding to pesticides, care must be taken in comparing the results. Salinity notwithstanding, the chronic test is expected to be more sensitive to HA mitigation of toxicity, as it is the more sensitive test overall. However, its salinity probably decreases the interactions between HAs and pesticides that might occur in freshwater. In addition, the osmotic adjustment intrinsic to the Microtox® tests could result in significant complexation of toxic, ionic metals to chloride ions, and artificially reduce toxicity [130]. Hartwell et al. [131] attempted to assess the effect of salinity on the Microtox® toxicity of tire leachates. The toxicity of the leachates seemed to decrease with increasing salinity perhaps as a result of toxicant-salt interactions. As these test were conducted well outside the recommended salinity parameters for the Microtox® tests, the increased toxicity observed at the lower salinities could be explained by osmotic stress. Because of the potential effect of salinity on sample toxicity and contaminant-HA association, neither
Microtox® test can be recommended for the assessment of the effects of HA on contaminant toxicity in freshwater.

The apparent disparity in the observed effects of HAs on contaminant toxicity between the acute (20 ppt) and chronic (35 ppt) Microtox® tests, and the lack of toxicity mitigation of any HA in the present study (35 ppt) indicate that salinity may be a more important parameter in the final determination of toxicity than the specific HA present. Moreover, the chronic Microtox® test is not sensitive enough to detect toxic concentrations of some contaminants. The chronic test’s sensitivity (EC₅₀) to CPF is 1000-fold lower than representative acutely toxic concentrations to invertebrates and fishes [109].

The use of more ‘classical’ organisms such as *Ceriodaphnia dubia* and *Americamysis bahia* would not only allow toxicity testing in both fresh and salt water, but also at contaminant concentrations that are more environmentally relevant.
Chapter 2:

Combined effects of humic acids and salinity on the solid-phase microextraction of DDT and chlorpyrifos.

Abstract:

The unbound portion of dissolved pollutants is generally presumed to be the most bioavailable to aquatic organisms. The effects of humic acids (HAs) and salinity on the freely dissolved fraction of chlorpyrifos (CPF) and 4,4'-DDT in water were assessed through their relative uptake by solid-phase microextraction (SPME) in the presence of Aldrich HA. Increasing salinity (0 - 20 ppt) alone had no effect on the uptake of DDT by the SPME fiber, but generally enhanced that of CPF. HAs decreased the SPME uptake of DDT at 10 mg/l, but 100 mg/l was required to decrease that of CPF. The presence of salt at 5 to 20 ppt greatly reduced the pesticide-HA binding for CPF and DDT. HA and salinity effects appear to be contaminant-dependant and may impact the contaminant’s environmental fate, transport, and bioavailability, especially in estuarine situations.

Introduction:

The freely-dissolved contaminant fraction has been reported to be the most bioavailable fraction [132]. Changes in the freely dissolved fraction of contaminants can
therefore lead to changes in bioavailability, uptake, and resultant toxicity. For example, Freidig et al. [39] measured the association ($K_{DOC}$) of tetra- and hexachlorobenzene with Aldrich humic acid (HA) and found that the $K_{DOC}$ was a good predictor of changes in uptake rates of these contaminants by the guppy (*Poecilia reticulata*). Day [10] reported that Aldrich HA both decreased the freely dissolved concentrations of three pyrethroid pesticides (fenvalerate, deltamethrin, and cyhalothrin), and decreased their bioaccumulation in and toxicity to *Daphnia magna*.

Various methods have been used to assess the interaction between HAs and contaminants. Burgess and Ryba [133] used C-18 solid phase chromatography to separate freely-dissolved and humic-bound PCBs. Johnson et al. [15] used the same method to assess the interaction between Aldrich HA and organophosphate pesticides. Laor and Rebhun [19] used flocculation to remove HA-PAH complexes from solution, and determine their binding coefficients. Perminova et al. [23] used fluorescence quenching to determine the binding affinities of PAHs to 26 different humic materials.

Recently, investigators have turned to negligible-depletion solid-phase microextraction (nd-SPME) to assess partition coefficients of compounds in solution (e.g. [134, 135]). Solid phase microextraction (SPME) is a solventless extraction technique introduced in the late 1980's [136]. A fused-silica fiber coated with a stationary phase is put in contact with a sample, or in the headspace above the sample, and the compounds therein are allowed to partition into the stationary phase. The fiber is then removed and the sorbed analytes are desorbed in the heated injection port of a gas chromatograph [137]. Alternatively, the sample can be analyzed by HPLC [138]. One important
advantage of SPME is that only small sample amounts (1-10 ml) are required [136]. Extractions can be halted during the dynamic phase of partitioning, or continue until steady-state is reached [139]. The latter requires more time, but has the advantages of ease of calculation and greater sensitivity. In the past decade, SPME has been used to analyze for pesticides [139, 140], volatile fatty acids [141], Brazilian nut and green tea aromas [142, 143], phenols [144], and other compounds (e.g. [138, 145, 146]).

It is important to note that SPME is not an exhaustive extraction method. Rather, it is based on the partitioning of the analytes between the sample matrix (or its headspace) and the fiber. As only the freely-dissolved fraction of the analyte in available for uptake by the SPME fiber [117], partition coefficients can then be determined for the analyte in a multi-phase matrix such as HA-containing water. Vaes et al. [147] stated that in order to measure the freely dissolved concentrations using SPME, 1) the percentage of chemical extracted should be minimized (no more than 5 %) to avoid disturbing the equilibrium between the chemical and the matrix; and 2) the sample matrix should not disturb the sorption kinetics of the chemical into the SPME fiber (assuming measurements are made in the kinetic phase of absorption). Low SPME stationary phase and high relative sample volumes are helpful in attaining “negligible depletion” of the analyte. Sampling at steady-state satisfies the second requirement. Artola-Garicano et al. [135] used decreases in nd-SPME uptake to determine tissue-blood partition coefficients for pesticides. Vaes et al. [147] measured the binding of polar compounds to bovine serum albumin. Pörschmann et al. [117] used it to study the sorption of organotin compounds to particulate and dissolved organic matter.
In the last few years, nd-SPME has emerged as a valuable biomimetic tool. Van der Wal et al. [148] used 7 μm polydimethylsiloxane (PDMS) nd-SPME to predict body burdens of some PCBs, PAHs, and other compounds in model terrestrial organisms exposed to contaminated soils. Verbruggen et al. [149] used a polyacrylate fiber similarly, to model body residues and estimate baseline toxicity for a variety of compounds. Bearden et al. [150] investigated the bioavailability of 1-octanol to the freshwater ciliate (*Tetrahymena pyriformis*) in the presence of growth media protein (proteose peptone). Vaes et al. [147] showed that the freely dissolved fraction of some polar compounds decreased as their hydrophobicity increased, in the presence of bovine serum albumin. Vaes et al. [151] assessed the toxicologically relevant concentrations of a variety of compounds by measuring the phospholipid/water partition coefficients, and the freely dissolved concentrations of a range of compounds in in-vitro systems. Urrestarazu Ramos et al. [134] used a 7 μm PDMS fiber to estimate the freely dissolved fraction of pentachlorobenzene, hexachlorobenzene, PCB 77, and DDT in the presence of Aldrich HA. A concurrent estimation of bioavailability of pentachlorobenzene and PCB 77 to *Daphnia magna* showed a decrease in body burdens, as measured by GC analyses of hexane extracts from pooled daphnids.

SPME was initially developed as a rapid and simple analytical technique for measuring organic pollutants in solution. Many investigators determined that increasing the salinity of a sample could increase the analyte uptake by the SPME fiber. For example, Dewulf et al. [152] investigated the effects of salinity and Aldrich HA on the SPME uptake of volatile organic carbons. Analyte uptake by SPME decreased at high
HA concentrations (100 and 500 mg/l), and increased with increased salinity (0 - 100 ppt). Other studies show similar results [51, 144, 153, 154, 155].

This effect of salinity on SPME uptake of compounds is driven by the "salting out" effect. Increasing the ionic strength of a solution decreases the solubility of non-polar compounds, and increases that of polar compounds (e.g. [51]), thereby increasing or decreasing the amount of compound sorbed on the solid phase at steady-state. The effect of salinity on organophosphate pesticide (OPP) uptake by SPME has been investigated, with varying results. Choudhury et al. [156] found significant increases in OPP uptake by a 100 μm PDMS fiber with increases in salinity. Jiménez et al. [157] reported that 200 ppt NaCl increased the uptake of OPPs by 7 μm PDMS fibers by 10-100 %, but that this result did not hold for a thicker or different SPME phase. Jinno et al. [158] showed that diazinon uptake by a polyacrylate fiber reached a maximum at 130 ppt NaCl. Lopez-Avila et al. [137] found no effect of NaCl addition under similar conditions (100 ppt NaCl) for a suite of OPPs. Valor et al. [159] determined that seawater (36 ppt salt) increased the polyacrylate SPME uptake of the OPPs tested by 4 to 22 %. With a few exceptions, Magdic et al. [160] found that the addition of NaCl (0 - 400 ppt) decreased the uptake of OPPs by the same fiber. Beltran et al. [139] and Magdic et al. [160] observed both increases and decreases in organophosphate pesticide uptake by SPME with the addition of NaCl, depending on the polarity of the pesticide.

Though their separate effects have been well examined, a literature search did not reveal any studies examining the interactive effects of salinity and HA on SPME partitioning of contaminants in water. In an earlier study (Chapter 1), the type of HA was
found to be less important than the salinity of the solution in determining the toxicity mitigation of the organophosphate and organochlorine pesticides CPF and 4,4'-DDT. This study therefore focused on the association of these two pesticides to the commercially available Aldrich HA.

**Objectives:**

The objectives of this study were to:

1) Determine SPME time-to-steady-state with and without Aldrich HA at environmentally relevant salinities (0 and 20 ppt) for two pesticides of concern, DDT and CPF.

2) Assess the change in SPME uptake at steady-state of DDT and CPF under various combinations of salinity (0 - 20 ppt) and environmentally relevant HA concentrations (0 - 100 mg/l).

**Null hypotheses:**

$H_{0\,1}$: Addition of HA will affect the time-to-steady-state an aqueous solution of DDT or CPF and the SPME fiber.

$H_{0\,2}$: The presence of HA will not affect amount of DDT or CPF taken up by the SPME fiber in an aqueous solution of the pesticides.

$H_{0\,3}$: Salt content will not affect SPME uptake of DDT or CPF in solution, with or without HA.
Materials and Methods:

Glassware:

Pre-cleaned glassware was ignited at 450°C overnight prior to use. Glassware that did not fit in the furnace was instead cleaned with 4M HCl, followed by multiple rinses in deionized (DI) water. The water was displaced by HPLC-grade acetone (Burdick & Jackson, Muskegon, MI) and the glassware was dried.

Humic acid solutions:

Stock solutions of 100 mg/l HA solutions were made by stirring 200 mg of HA (Aldrich Chemical Company, Inc., Milwaukee, WI) in 1800 ml of DI water at pH=10 (NaOH) overnight in a brown bottle. The pH was then reduced to pH=7.8 (HCl) and the solution diluted to 2000 ml.

Solutions of 0, 10, and 100 mg/l HA at each of five salinities (0, 1, 5, 10, and 20 ppt) were made by first diluting the stock solution with water. Low salinity water (0 and 1 ppt) was amended with 30 mg/l MgSO₄•7H₂O, 30 mg/l CaSO₄•2H₂O, 2 mg/l KCl, 20 mg/l CaCl₂•2H₂O (Fisher Scientific, Fair Lawn, NJ), and 48 mg/l NaHCO₃ (Mallinckrodt, Paris KY) per liter to ‘mimic’ freshwater [50]. These salts increased the salinity by only ~ 0.10 ppt. Saline solutions (1 to 20 ppt) were made by adding the appropriate amounts of Hawaiian Marine Mix (Hawaiian Marine Imports, Houston, TX). At 5 ppt salinity, concentrations of the most abundant ions in the Hawaiian Marine Mix salts added were greater than those added to the “low salinity water”. All solutions were vigorously aerated for at least 24 hours in brown bottles, and their pH controlled to 7.8 (HCl). They
were then filtered to 0.45 µm with pre-rinsed cellulose nitrate membrane filters (Whatman, Clifton, NJ) prior to use. To reduce clogging of the filters, higher HA-concentration solutions were prefilted with ignited (450 °C, 4 hours), pre-rinsed A/E glass fiber filters (Gelman Sciences, Inc. Ann Arbor, MI).

**Pesticides:**

CPF (Chem Services, West Chester, PA) and 4,4'-DDT (EPA Research Laboratories, Research Triangle Park, NC) stock solutions (0.66 and 3.16 µg/ml, respectively) were prepared in acetone.

**SPME fibers:**

SPME fibers with a 7 µm PDMS coating, 1 cm in length, were purchased from Supelco (Bellefonte, PA), and conditioned as per manufacturer’s instructions. Additionally, fibers were conditioned for 10 minutes at 270 °C immediately before each use. A sample desorption from a previous experiment was deemed equivalent.

**SPME extractions:**

All extractions were done at room temperature, using 60 ml vials. Vials were wrapped in aluminum foil to eliminate UV radiation effects on HA or pesticides. Water samples (200 ml) were spiked 12 hours prior to extraction with 200 µl of pesticide stock solution (to nominal concentrations of 660 ng/l CPF and 3160 ng/l DDT), and kept in the dark. The samples were then mixed by pipetting and ~ 60 ml aliquots transferred to
extraction vials, maintaining a ~ 2 ml headspace. Each vial was capped with a pre-drilled teflon-lined septum. The fibers were rinsed briefly in DI water, prior to desorption, to remove any dissolved salts or HA. Preliminary data showed that a ten minute desorption at 270 °C was sufficient to eliminate carry-over between extractions. Each of three fibers extracted a pair of samples. For the effect of HA on SPME uptake, a pair consisted of an HA-free sample and a sample containing HA at the desired concentration analyzed by a single fiber over sequential extractions. Similarly, for the effect of salinity, a pair consisted of a fresh water and a saline sample. Percent pesticide uptake for the HA or saltwater samples were calculated relative to the HA- or salt-free sample from each sample pair. All extractions were conducted at steady-state, except for those used initially for the determination of time to steady-state.

Gas chromatography:

CPF was analyzed on a Varian 3400 GC with a thermionic specific detector (TSD) and a 30 m 1710 column (J&W Scientific, Folsom, CA). Injector and detector temperatures were 270 and 300 °C, respectively. The injections were carried out with the split closed for the first 2 minutes. Oven temperature was held at 75 °C for 2 minutes, then increased to 270 °C at 7° per minute, and held for 5 minutes. DDT was analyzed on a Varian 3400 GC/FID with a 60 m DB5 column (J&W Scientific, Folsom, CA). Injector and detector temperatures were 270 and 320 °C, respectively. Oven temperature was held at 75 °C for 2 minutes, then increased to 320 °C at 10° per minute, and held for 10 minutes.
**Determination of time-to-steady-state:**

As all subsequent extractions were to be done with the samples and fibers at steady-state, time to steady-state was determined for each pesticide solution. Time to steady-state was established in the presence and absence of HA to account for potential effect of HAs.

For each pesticide, the time to steady-state was initially determined by triplicate extractions in a time series (0, 5, 9, 12, and 19 hours), using 0 ppt water with no HA added. Time to steady-state at 20 ppt salinity and/or with 100 mg/l HA were confirmed using the last two time points in the series (12 and 19 hrs).

**Determination of the effects of HA and salinity:**

The effect of Aldrich HA on SPME uptake of each pesticide at different salinities was determined using the described methodology, under HA and salinity conditions relevant to an estuarine setting (Aldrich HA concentrations of 0, 10 and 100 mg/l, and salinities of 0, 1, 5, 10, and 20 ppt).

**Dissolved organic carbon (DOC) measurements:**

Aliquots of water samples were collected just prior to spiking of the pesticides for DOC determinations and frozen for later analysis [161]. A sample of Hawaiian Marine Mix salts was also collected for total organic carbon (TOC) analysis. Samples were analyzed using a TOC analyzer (Shimadzu model TOC-5000) with a non-dispersive infrared detector. The analyses were performed by the VIMS Analytical Service Center.
Statistical design and analysis:

Data were checked for normality by the chi-square test, and for homoscedasticity using Bartlett's test. Differences in uptake as a function of salinity were analyzed by ANOVA and paired sample t-tests, for each triplicate extraction pair. Regression analyses were done when appropriate. SPME uptakes at different salinities in the presence of HA were analyzed by ANCOVA, with salinity as the concomitant variable. When differences were found, Tukey's multiple comparison test was performed to elucidate the differences. Results are presented with ± 1 standard deviation in parentheses.

Results:

The Chi-square tests and Bartlett's tests performed on all SPME data showed that the data were normally distributed and had homogeneous variances. No transformations were made.

DOC data:

Representative DOC concentrations for the SPME water samples are presented in Figure 5. With no Aldrich HA added (0 mg/l), the mean DOC concentration in the water was 1.0 mg C/l (± 0.4), and varied with salinity from a low of 0.64 mg C/l in 1 ppt to 1.5 mg/l in 10 and 20 ppt. At a nominal concentration of 10 mg/l HA, the average DOC concentration was 3.0 mg C/l (± 0.3), and also increased slightly with salinity. At a nominal concentration of 100 mg/l HA, DOC concentration tended to decrease with
Figure 5. Measured dissolved organic carbon content of Aldrich HA solutions (0, 10, and 100 mg/l) at various salinities (0 to 20 ppt).
increasing salinity. DOC concentrations ranged from 21.8 mg C/l in 1 ppt to 16.8 mg C/l in 20 ppt. The Hawaiian Marine Mix salts contained 0.035 % total organic carbon.

**Time-to-steady-state:**

The times to steady state were first determined for CPF and DDT at 0 ppt and 0 mg/l HA. The time series included 5, 9, 12, and 19 hours (Figures 6 and 7). After 5 hours of exposure of the fiber to these solutions, ~ 80 % of the pesticide had been extracted relative to a 12 hour exposure, for both CPF (76.0 % ± 5.8) and DDT (79.7 % ± 7.1). Nineteen hours of exposure did not significantly increase the amounts of pesticide extracted, relative to that of 12 hours. The amounts extracted at 19 hours, relative to 12 hours, were 109 % (± 11.7 %) for CPF and 106 % (± 8.9 %) for DDT. It was therefore concluded that steady state was achieved within 12 hours for both CPF and DDT, at 0 ppt and 0 mg/l HA. Further experiments were conducted to ensure that the time-to-steady-state was not significantly longer in the presence of HA, or at a higher salinity.

Exposures of 12 and 19 hours were conducted, at 0 and 20 ppt, with and without 100 mg/l HA added (Figure 8). No significant differences in amount extracted were detected at the 5 % level between 12 and 19 hours for either pesticide, at any combination of salinity and HA, suggesting that steady state was achieved within 12 hours.

**Extraction efficiency:**

The extraction efficiencies were calculated using 12 hour fiber exposures to pesticide-spiked water samples at 0 ppt salinity and 0 mg/l HA added, followed by
Figure 6. Time to steady state curve for the uptake of CPF by SPME, at 0 ppt salinity and 0 mg/l Aldrich humic acid added. The uptake is relative to that at 12 hours. Error bars represent ± 1 standard deviation.
Figure 7. Time to steady state curve for the uptake of DDT by SPME, at 0 ppt salinity and 0 mg/l Aldrich humic acid added. The uptake is relative to that at 12 hours. Error bars represent ±1 standard deviation.
Figure 8. Percent uptake by SPME of CPF and DDT after 19 hours of equilibration, relative to 12 hours, under varying salinity (0 and 20 ppt) and HA concentration (0 and 100 mg/l). Error bars represent ± 1 standard deviation.
comparison to external standards. The SPME extraction efficiency for CPF was calculated to be ~ 3 %, and satisfied the guidelines for negligible depletion experiments [147]. The SPME extraction efficiency for DDT was ~ 19 %, exceeding the guidelines for negligible depletion. However, the extraction conditions were not modified to lower the extraction efficiency as: 1) significantly larger extraction volumes and times would have been needed to attain steady state under negligible depletion conditions; and 2) preliminary results showed that the existing conditions were sufficient to show differences in SPME uptake in the presence of HA, the primary goal of this study.

Effect of salinity SPME uptake:

The effects of salinity were examined at 1, 5, 10, and 20 ppt, without the addition of Aldrich HA. Extractions were conducted at 12 hours (steady state). Salinity had a significant effect on the amount of CPF extracted by SPME. Extractions were significantly greater in saline than in freshwater conditions, except for 1 ppt (Figure 9). The percent CPF extracted from each salinity tested, relative to freshwater, were 89.9 (± 4.0) at 1 ppt, 105 % (± 1.3 %) at 5 ppt, 123 % (± 4.9 %) at 10 ppt, and 133 % (± 4.2 %) at 20 ppt. The following regression of percent extraction against salinity was computed and was significant (p < 0.05):

\[
\% \text{ CPF extraction} = 92.7 + 2.20 \text{ salinity} \quad (R^2 = 0.87)
\]

In contrast to CPF, salinity had no significant effect on DDT extraction under the
Figure 9. Percent uptake by SPME of CPF at salinities of 1 to 20 ppt, relative to that in freshwater. Uptake measured under steady state conditions. Error bars represent ±1 standard deviation. A star over a bar indicates a significant difference from 0 ppt salinity.
conditions tested (Figure 10). The percent DDT extracted from each salinity tested, relative to freshwater, were 97.8 % (± 10.2 %) at 1 ppt, 100 % (± 5.7 %) at 5 ppt, 100 % (± 2.6 %) at 10 ppt, and 92.6 % (± 4.6 %) at 20 ppt. The slight decrease at 20 ppt was not significant (p = 0.109).

Effect of humic acids:

The effects of 10 and 100 mg/l HA at salinities ranging from 0 to 20 ppt were examined for CPF and DDT at steady state. The relative CPF uptake by the fibers at different salinities and HA concentrations are presented in Figure 11. A HA concentration of 10 mg/l had no effect on extraction efficiencies at any of the five salinities studied. Relative efficiencies ranged from 98.8 % (± 5.1 %) at 0 ppt, to 99.9 % (± 5.3 %) at 20 ppt, with a high of 105 % (± 7.1 %) at 5 ppt. In contrast, CPF uptake by the fibers were significantly decreased (p < 0.01) at 0 and 1 ppt, with 100 mg/l HA, 68.7 % (± 0.4 %) and 68.8 % (± 2.1 %), respectively. A large increase in uptake occurred between 1 and 5 ppt, where the relative efficiency was 95.3 % (± 4.2 %). This was not significantly different from either 100 %, nor any of the higher salinity treatments. Overall, an ANCOVA showed significant effects for salinity (p < 0.01), HA (p < 0.01) and their interaction (p < 0.01).

DDT relative uptake efficiencies in the presence of 10 mg/l HA were similar to those of CPF with 100 mg/l HA (Figure 12). Relative efficiencies at 0 and 1 ppt were similar (56.7 ± 10.7 % and 56.6 ± 9.0 %, respectively) and significantly different from the 0 mg/l HA treatments (p < 0.05). At salinities of 5 ppt and higher, DDT extraction
Figure 10. Percent uptake by SPME of DDT at salinities of 1 to 20 ppt, relative to that in freshwater. Uptake measured under steady state conditions. Error bars represent ± 1 standard deviation.
Figure 11. Percent uptake by SPME of CPF in the presence of 10 and 100 mg/l Aldrich HA, and at salinities of 0 to 20 ppt. Uptakes were measured under steady state conditions, and are relative to those at the same salinity without Aldrich HA added. A star indicates a significant difference from 0 mg/l HA. Error bars represent ± 1 standard deviation.
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Figure 12. Percent uptake by SPME of DDT in the presence of 10 and 100 mg/l Aldrich HA, and at salinities of 0 to 20 ppt. Uptakes were measured under steady state conditions, and are relative to those at the same salinity with 0 mg/l Aldrich HA added. Tukey test results are presented by HA concentration. Treatments overlain with a symbol are significantly different from 0 mg/l HA, and different from treatments overlain with a different symbol (p < 0.05). Error bars represent ± 1 standard deviation.
efficiencies increased significantly to 93.3 ± 7.0 % (5 ppt), 87.9 ± 12.2 % (10 ppt), and 88.4 ± 8.7 % (20 ppt), and were not significantly different (p > 0.05) from the 0 mg/l HA treatments nor from each other. At a HA concentration of 100 mg/l, DDT relative uptake efficiencies were affected over the entire salinity range tested (Figure 12). DDT uptake efficiencies at 0 and 1 ppt (17.8 ± 7.9 % and 20.0 ± 4.6 %, respectively) were significantly lower than at any of the other salinities. They increased at greater salinities to 52.8 ± 3.4 % (5 ppt) and 59.1 ± 6.4 % (10 ppt). At 20 ppt, the fiber uptake efficiency was significantly higher than at any other salinity in the 100 mg/l treatment (84.0 ± 1.7 %), but still lower than in the 0 mg/l HA treatment. An ANCOVA showed significant effects for salinity (p < 0.01), HA (p < 0.01) and their interaction (p < 0.01). The following regression of relative extraction efficiency against salinity was computed and was significant (p < 0.01):

\[
\% \text{ DDT extracted} = 22.59 + 3.31 \text{ salinity} \quad (R^2 = 0.88)
\]

**Discussion:**

**DOC data:**

The DOC values in the samples where no HA was added show low concentrations of organic carbon. While the source of that organic carbon is uncertain, low level contamination of the water supply is suspected. The addition of Hawaiian Marine Mix salts roughly doubled the DOC, from ~ 0.7 to 1.5 mg C/l. This was a lower contribution than might have been expected from the salts’ TOC analysis (0.035 % TOC). An Aldrich
HA concentration of 10 mg/l produced a DOC concentration of ~ 3 mg C/l, independent of salinity. It appeared that HA precipitation between 0 and 20 ppt was minimal for that HA concentration. Work by other authors supports the hypothesis that HA conformation can change with its concentration (e.g. [12, 162]). The independence of Aldrich HA solubility vis-a-vis salinity indicates that any conformational changes resulting from salinity changes are not sufficient to precipitate HAs at 10 mg/l. Salinity seemed to have an effect on HA solubility when the nominal HA concentration reached 100 mg/l. The DOC content of the 100 mg/l HA solutions decreased from ~ 20 mg/l to ~ 17 mg/l, when salinity increased from 0 to 20 ppt. Visible organic matter residues observed on the filters post-filtration were additional evidence of some HA precipitation under these conditions.

The DOC concentrations in the present experiment encompass the range likely to be found in most environments. Rivers and lakes typically have DOC concentrations of 0.5 to 4 mg C/l [45], though higher concentrations are not uncommon. DOC concentrations in the Lena River, Siberian arctic, were reported to vary from 3.6 to 12 mg C/l [163]. Wong and Oatts [164] reported DOC values of 5.7 mg C/l in the James River (0 ppt), and 4.2 mg C/l at the mouth of the Chesapeake Bay (26 ppt). Waters from Finnish lakes had DOC concentrations of 20 to 38 mg C/l [44, 165, 166]. Chin et al. [27] found 35 mg C/l DOC in the surface waters of the Suwannee River, Georgia. In an extreme case, an open pond receiving coal wastewater had a DOC concentration of ~ 180 mg C/l [167].
Time-to-steady-state:

The extraction times and volumes used in this study greatly exceed those in most other SPME studies. For example, Beltran et al. [139] used 60 minute extractions of 3 ml samples for various organophosphate pesticide SPME analyses. Jiménez et al. [157] evaluated SPME as an extraction method for pesticides residues in 3 ml samples of diluted honey with maximum extraction times of three hours. Neither study attempted to minimize depletion of the analytes in the samples, and only a few of the pesticides analyzed reached steady state. The present study attempted to achieve both negligible depletion and steady state, which required larger sample volumes and longer extraction times. The large sample volumes used required increased sampling times to account for decreases in mixing efficiency [168].

Few of the studies that attempted to measure freely-dissolved concentrations, and should have been concerned with negligible depletion, had equilibration times long enough to achieve steady state. A study by Oomen et al. [169] specifically examined non-steady-state nd-SPME to determine the freely dissolved concentrations of PCBs and lindane in various media. Likewise, Verbruggen et al. [149] fitted a curve to SPME uptake data in a simulated bioconcentration study of 28 chemicals. Urrestarazu Ramos et al. [134] used nd-SPME to determine the partition coefficients of hydrophobic chemicals, including DDT, between water and HAs. In that study, only pentachlorobenzene seemed to approach steady state within the 60 minute extraction times used.

In work measuring the binding of aniline, nitrobenzene, 4-chloro-3-methylphenol, and 4-n-pentylphenol to bovine serum albumin, Vaes et al. [147] permitted the
compounds examined to reach steady state. The target compounds were polar, however, and steady state conditions under negligible depletion criteria were achieved in under 30 minutes with 1.5 ml samples. Another study by Van der Wal et al. [148], using headspace nd-SPME to predict body burdens of a suite of hydrophobic chemicals from contaminated soils, conducted extractions as long as 36 days, without achieving steady state for some compounds. Pörschmann et al. [117, 167] determined the binding state (i.e. freely-dissolved vs bound) of organotin compounds, phenols, and PAHs in solutions rich in humic organic matter using 7µm PDMS SPME fibers. Their extractions were conducted at steady state and required equilibration times up to 20 hours for 40 ml samples. These conditions are very similar to those of the present study.

Effect of salinity and HA on time-to-steady-state:

In samples saturated with NaCl, Buchholz and Pawliszyn [144] reported increased equilibration times (40 to 60 min.) relative to freshwater for the phenols regulated by US EPA methods 604 and 625 and Ontario MISA Group 20 regulations. No effect of salinity was seen in the present study on time-to-steady-state. The difference in results can probably be best explained by the fact that different compounds were used, and the conditions of the present study (maximum salinity of 20 ppt) were far removed from NaCl saturation. The lower salinities were chosen to mimic environmentally relevant conditions such as those in an estuary, rather than to maximize the analyte uptake by the fibers.

DOC such as HAs have the potential to slow the uptake of compounds by SPME.
Pörschmann et al. [117] noted this effect with organotin compounds, and suggested it was probably due to an alteration of the stationary water layer around the SPME fiber. Other studies have not directly addressed the issue. No effect of HA on pesticide extraction rate was discerned in this study. It is possible that any HA-related effect might have been masked by a more rate limiting step such as stirring efficiency.

**Absolute extraction efficiency:**

Vaes et al. [147] suggested extraction guidelines for the measurement of the freely dissolved concentration of a compound using SPME. While the extraction parameters for CPF satisfied these guidelines, those of DDT exceeded one of them: DDT absolute extraction efficiency on the fiber was ~ 19 % of the total amount in the sample. Vaes et al. [147] recommended not extracting more than 5 % of the total amount, in order to minimize any disturbance in the compound partitioning between the water and the matrix. A higher percentage extracted, such as for DDT in the present study, might alter the steady state, and produce an overestimate of the freely dissolved fraction of the chemical. However, preliminary results clearly showed a much greater effect of HA on DDT recovery than on CPF recovery. Thus the methodology was not altered to accommodate this recommendation.

Predicting the extraction efficiency of a fiber for a particular compound may be less straightforward than it might appear. Sng et al. [170] compared the extent of extraction of OPPs from water using five commercially available fibers. They determined that fiber polarity was not the main factor affecting the extraction efficiency.
Dugay et al. [171] also found no correlation between the polarity of the fiber solid phase and its affinity for polar compounds. In fact, they determined that the most polar phase used (polyacrylate) did not possess the highest affinity for the more polar pesticides tested. The partitioning of compounds onto the fiber solid phase was found to depend, in part, on the compound’s functional groups. CPF and DDT both have an aromatic character, and for them polarity (e.g. \( K_{ow} \)) appeared to be a good predictor of extraction efficiency: The more polar CPF (\( K_{ow} \sim 10^5 \)), showed less affinity for the non-polar PDMS phase than did DDT (\( K_{ow} \sim 10^6 \)).

**Effect of salinity:**

The effect of salt addition on a fiber’s extraction efficiency depends on the properties of both the fiber and the compound being extracted. As a general rule, an increase in salinity decreases the water solubility of non-polar compounds and increases their uptake by a fiber. For a polar compound, the solubility would be increased, and the uptake decreased [51, 144]. In a study of the US EPA method 507 pesticides, Choudhury et al. [156] found that the addition of NaCl (0 ppt to saturation) significantly increased the uptake of pesticides by a PDMS fiber. Boyd-Boland and Pawliszyn [172] reported that addition of NaCl either increased or decreased the uptake of nitrogen-containing herbicides by a polyacrylate fiber, depending on the herbicide. Bao et al. [173] found no effect of NaCl on uptake by a PDMS fiber for 23 carbonyl compounds.

This last result is similar to the present findings for DDT. Over the range of concentrations tested, salinity had no discernable effect on the amount of DDT extracted.
by PDMS. It should be noted that the salinity range in this study (0 to 20 ppt) was much narrower than those found in the current literature (0 to > 400 ppt) and was obtained by the addition of the major ions in seawater, rather than a single salt. It is possible that greater salt concentrations would have an effect on DDT uptake by that fiber. There is an alternative explanation for the apparent lack of effect on DDT. The DDT concentration used was close to its reported water solubility [75, 76, 174], and salinity-driven decreases in DDT concentration through precipitation might have “canceled out” the increased affinity for the SPME fiber. The high DDT recoveries at 20 ppt seen in Chapter 3 do not lend credence to this hypothesis.

Salinity had a complex effect on the relative extraction efficiency of CPF. The extraction efficiency first decreased to 90% in 1 ppt, then increased to 133% at 20 ppt. The overall increase in efficiency is not unexpected. Jiménez et al. [157] had previously described an increase in pesticide uptake - including CPF - by 7μm PDMS fiber in the presence of 200 ppt NaCl. A reversal in extraction trend, such as seen in the present study, is not unprecedented either. Magdic et al. [160] showed a decrease in CPF uptake by a polyacrylate fiber from 0 to 300 ppt NaCl, but an increase in uptake from 300 to 400 ppt NaCl. No clear explanation for the results was presented. It is possible that the change in uptake in the present study is due to the changing relative concentrations of the different ions in solution, and their effect on the affinity of the solid phase for CPF. Brunk et al. [175], studying the association of phenanthrene to DOC, noted that the effect of salinity depended on a complex interplay between pH, divalent ion concentration, and the specific organic macromolecule (DOC) studied.
Effect of HA:

The magnitude of sorption to a specific organic macromolecule, such as Aldrich HA, also depends on its concentration and the specific sorbate. The present study showed significant differences in SPME extraction efficiencies as a function of pesticide, HA concentration, and salinity.

CPF uptake by the fiber, and presumably its freely dissolved concentration, was less affected by the presence of HA than DDT’s. The freely dissolved CPF concentration did not decrease at any salinity in the presence of 10 mg/l Aldrich HA. However, a decrease in uptake was observed at low salinity (0-1 ppt) in the presence of 100 mg/l HA (~ 20 mg C/l). The relative lack of significant interaction between CPF and HA may be a result of the molecular conformation of the CPF molecule.

The CPF molecule consists of a heteroaromatic group linked to two ethyl chains by a phosphorothioate (O\_3P=S) group. The heteroaromatic group contains a nitrogen atom, and has three chlorine atoms and one oxygen atom bound to it, making it more polar than a simple aromatic ring (Figure 1). This by itself could result in a lower affinity of the CPF molecule to the hydrophobic binding sites within the HA molecule [31]. A three dimensional model of the CPF molecule also shows that one ethyl group is positioned “above” the plane of the aromatic group, resulting in potential steric hindrance to binding with the HA binding sites. Chin et al. [27] measured the binding affinities (K_{DOC}) of pyrene and DDT to Aldrich HA. They noted that the K_{DOC} values were within a factor of two, despite DDT having a K_{ow} value ~ 17 times greater than pyrene. They attributed this difference to the non-planar nature of the DDT molecule, resulting in steric...
hindrance.

The relatively low interaction between CPF and HA was reflected in no observable decrease in fiber uptake at the low HA concentration (10 mg/l). This concentration is equivalent to ~ 3 mg C/l (Figure 5). A study on the recovery of organophosphate and other pesticides by C-18 SPE in the presence of unfiltered Aldrich HA also found no significant decrease in recovery at HA concentrations below ~ 5 mg C/l [52]. In another study, Johnson et al. [15] reported C-18 solid-phase extraction CPF recoveries of 81 to 55 % (relative standard deviations: 25 and 5 %, respectively) in the presence of ~ 2.5 to 10 mg C/l Aldrich HA. These results are comparable to those of the present study.

At the higher HA concentration (100 mg/l; ~ 20 mg C/l), CPF uptake was affected only at 0 and 1 ppt. At salinities of 5 ppt and above, HA addition had no significant effect. While the uptake changes correlate with a slight decrease in measured DOC concentration, from ~ 20 to ~ 17 mg C/l (Figure 5), the main cause behind the increase in CPF uptake with increased salinity is believed to be a result of conformation changes in the HA molecules, rather than a decrease in DOC concentration.

HA molecules are large enough to form intramolecular micelles, e.g. hydrophobic cavities within the molecules, surrounded by more hydrophilic moieties that interact with the surrounding water [14]. It is within these intramolecular micelles that hydrophobic compounds would tend to sorb. The size, shape, and number of these micelles are dependant on HA conformation. The HA conformation in turn is affected by solution chemistry. The three-dimensional structure of the HA molecule may be altered by
protonation, deprotonation, and metal complexation of HA functional groups [71, 176]. The HA molecule may coil up, occluding previously exposed binding sites and lowering its binding capacity [177]. Increases in ionic strength have been shown to reduce the binding of PAHs to humic acids [12, 22]. Hela et al. [52] and Pörschmann et al. [117] observed the same effect on organophosphate pesticide binding to Aldrich HA and on organotin compound SPME recovery in the presence of DOC. Water hardness, has also been shown to affect the association between metals and DOC. Campbell and Evans [178] noted that the sorption of Cd and Pb to Aldrich HA tended to decrease with increasing CaCO$_3$ concentration. Moreover, bioconcentration factors of Cd and Pb in the mussel *Elliptio complanata* were significantly correlated with water hardness, along with HA concentration and pH. Penttinen *et al.* [166] manipulated the hardness of a naturally humic lake water (10.6 mg/l DOC) by adding Mg and Ca ions. Increasing the water hardness (from 0.1 to 2.5 mmol Ca + Mg per liter) decreased the binding coefficient of Cd to DOC. In hard water, Ca ions decreased Cd toxicity to *Daphnia magna*, either through competition of the two ions, or by reducing cell membrane permeability to Cd. Different Ca/Mg ratios may modify the effect of water hardness on trace metal binding and bioavailability. Doig and Liber [179] reported that Ca played a greater role than Mg in protecting *H. azteca* from nickel toxicity.

DDT sorption to HA is greater than that of CPF, and is also affected by increases in salinity. Decreases in DDT uptake were significant at 10 mg/l HA, and resulted in an over 80% decrease in uptake at 100 mg/l HA and 0 and 1 ppt salinity. The DDT molecule consists of two benzene rings connected to a trichloroethyl group (Figure 1).
Each ring has a single Cl atom attached. This makes the DDT molecule much more hydrophobic, and less sterically hindered than the CPF molecule. The greater affinity of HA for DDT is therefore to be expected.

DDT sorption to DOC has been studied previously. Caron et al. [32] noted that addition of DOC to the aqueous phase decreased DDT sorption to sediment. In that study, a natural HA extracted from sediment (~ 7 mg C/l) bound 76 % of the aqueous DDT. Carter and Suffet [28] determined that ~ 75 % of DDT was associated with Bontoon Reservoir, NJ, HA (~ 9 mg/l TOC). The extent of that binding depended on the humic material and its concentration, the pH, and the ionic strength. Urrestarazu-Ramos et al. [134] assessed the freely-dissolved concentration of DDT and other compounds in the presence of Aldrich HA (up to 25 mg/l) in freshwater by SPME using 7 μm PDMS fibers. They reported that ~ 90 % of the DDT was bound to the HA.

There appears to be a threshold on the effect of salinity. Extraction efficiencies of both CPF (at 100 mg/l HA) and DDT (at 10 and 100 mg/l HA) had their greatest increases between 1 and 5 ppt. In fact, there were no significant differences in fiber extraction efficiency for either compound below 5 ppt, and only one set (DDT and 100 mg/l HA) showed significant differences in efficiencies at or above 5 ppt. This change occurred around the cutoff point for the addition of freshwater ions to the water. However, that cutoff point had been chosen with care so that the main ion concentrations would increase with increased salinity. The change in uptake is therefore probably due to that overall increase in salinity.

Environmentally relevant levels of salinity and HA concentration interacted to
alter the freely-dissolved concentrations of the pesticides. This effect was pesticide-dependant, and could impact their fate, transport, and bioavailability. Riverine transport of DDT, and to a lesser extent that of CPF, could be facilitated by their association with DOC. As the salinity of the surrounding water increased, the pesticides would tend to desorb from the DOC back into the freely-dissolved pool and become bioavailable. The dilution of the effluent concurrent to the increase in salinity would likely decrease the freely-dissolved pesticide concentration below toxic levels.
Chapter 3:

Effect of Aldrich HA on toxicity of CPF and DDT to the mysid Americamysis bahia and the daphnid Ceriodaphnia dubia.

Abstract:

The effects of dissolved humic acids (HAs) on the acute toxicities of the organophosphate pesticide chlorpyrifos (CPF) and the organochlorine pesticide 4,4'-DDT were assessed using freshwater (Ceriodaphnia dubia) and saltwater (Americamysis bahia) crustaceans. The effect of filtered Aldrich HAs (10 and 100 mg/l) on organism mortality were determined near the estimated pesticide LC50s. HA had no effect on A. bahia mortality for either pesticide at 20 ppt, but greatly reduced the mortality of C. dubia for both pesticides, at 0 ppt. The effect was proportional to the HA concentration. The difference in toxicity mitigation as a function of salinity is believed to be a function of conformational changes in the humic acid molecules, rather than organismal effects.

Introduction:

HAs can bind to hydrophobic compounds. This compound-HA interaction is of particular importance because it can affect the compound’s bioavailability, bioaccumulation, activity, and degradability [10, 33, 50], even at naturally occurring
concentrations [13]. Kusk [180] found that the bioavailability and toxicity of pirimicarb to *Daphnia magna* decreased in the presence of DOM. Kukkonen *et al.* [36] reported that DOM decreased the bioaccumulation of BaP by *Daphnia magna*. Kukkonen and Oikari [44] found that bioaccumulation of benzo(a)pyrene and dehydroabietic acid in *Daphnia magna* decreased with increased time of interaction between the compounds and HAs (0-8 days). In contrast, they found that pentachlorophenol bioaccumulation was not affected by the presence of HAs. Kim *et al.* [181] and Ma *et al.* [182] examined the effects of HAs on Cu toxicity to *Ceriodaphnia dubia*, and observed decreased Cu toxicity in the presence of HA. The effect was greater with an increased Cu-HA contact time. The Microtox® (an acute bacterial bioassay) 15 minute EC₅₀ of fenvalerate, a pyrethroid insecticide, increased significantly (from 1.23 to 2.06 μmol) in the presence of 50 mg/l of Aldrich HA and 100 mg/l of Suwannee River HA (from 1.23 to 1.71 μmol) [3]. The same study found an increase in the 15 min. EC₅₀ (from 2.73 to 6.08 μmol) of permethrin, another pyrethroid insecticide, at 50 mg/l of Aldrich HA, and at 0.5 mg/l of the Suwannee River HA (from 2.73 to 3.48 μmol). The presence of DOM does not always decrease the bioavailability or toxicity of a contaminant. The uptake of naphthalene by bluegill sunfish was essentially unaffected by 20 mg C/l of dissolved humic material [43]. In a literature review, Haitzer *et al.* [45] even noted that low DOM concentrations (up to 10 mg/l) could enhance bioconcentration of organic chemicals three-fold.

Pesticides are important aquatic contaminants. For example, a study of US fish reported DDT toxic residues in 99% of sampled sites [74], while 30% of Californian
urban waterways samples contained CPF, with a median concentration (50 ng/l) toxic to aquatic organisms [106, 110].

DOM in general, and HAs in particular have previously been shown to interact with both CPF and DDT in solution. Dissolved Aldrich HA (10 mg/l) associated with CPF and reduced its extractability on C-18 SPE columns by 45 % [15]. HAs may also reduce the pesticide load in the environment. Kamiya and Kameyama [33] showed that hydrolysis of organophosphate pesticides, including CPF, can be enhanced by 5 to 45 % in the presence of low concentrations of HAs (5 mg/l). Hydroxyl or carboxyl groups in the HA molecule can undergo photochemically activated radical generation. They hypothesized that these radicals would make pesticides more vulnerable to surface-active hydrolysis. Kamiya and Kameyama [33] reported that radical generation would not occur if those functional groups were in a salt state. Aldrich HA is produced as a Na-salt, and therefore less likely to undergo radical generation, and enhance hydrolysis.

DDT may also associate with HAs. Carter and Suffet [28] equilibrated DDT with various HAs (9 - 16 mg C/l) and found that the majority of DDT was bound to them. Caron et al. [32] also showed that the addition of HA reduced the sorption of DDT to sediment, and increased its partitioning into the dissolved phase, thereby making it more available for transport throughout the aquatic system. Chiou et al. [26, 183] confirmed the apparent increase in DDT water solubility in the presence of HAs. However, they reported that the solubility enhancement depended on the type of HA. They found that commercial HAs examined generally had a greater effect than those isolated from a natural aquatic system. Landrum et al. [29] reported that DDT recovery on C-18 SPE
greatly decreased in the presence of HA, and that Aldrich HA had a larger effect than HA from natural waters.

Toxicity studies also reflect the possible binding of CPF, DDT, and other pesticides to HAs. Acute Microtox® tests have been conducted on organophosphate pesticides in the presence of HAs. Results have varied. Benson and Long [1] found an EC₅₀ increase (toxicity reduction) in the presence of 0.5 mg/l HAs for CPF. Kadlec and Benson [125] found toxicity decreases for methyl parathion and for guthion, but no significant toxicity change for CPF. The results varied with the HA used. Ankley et al. [184] showed that CPF toxicity to the midge *Chironomus tentans* decreased with increasing HA concentration. DDT bioavailability is also affected by the presence of HA. Landrum et al. [132] assessed the bioavailability of DDT and other compounds to the amphipod *Pontoporeia hoyi* and found that the apparent biological uptake rate constant for the compounds decreased with increased HA concentration. DDT uptake rate constants and bioconcentration factors also decreased for rainbow trout in the presence of unfiltered Aldrich HA, through sorption to particulate and DOC [38].

In Chapter 1, the effect of HAs on Cu and CPF toxicity were assessed using the Microtox® chronic test. Aldrich HA was tested along with Leonardite, Peat, and Suwannee River HAs purchased from the International Humic Substances Society. None of the HAs had a significant effect on the EC₅₀s of Cu and CPF at a nominal concentration of 5 mg/l HA, and 35 ppt salinity. Chapter 2 showed that salinity strongly mitigated the association of the pesticides 4,4'-DDT and CPF to Aldrich HA. As the association of contaminants to HAs has been shown to reduce toxicity, the role of varying
salinity in the mitigation of the pesticides toxicities by Aldrich HA was further investigated using the fresh and salt water organisms *Ceriodaphnia dubia* and *Americamysis bahia*.

*Ceriodaphnia dubia:*

The daphnid *Ceriodaphnia dubia* is widely distributed throughout the world, and is an ecologically important species in fresh water plankton communities [185]. *C. dubia* has a sensitivity comparable to that of *Daphnia magna* [186]. It has become a test organism of choice for freshwater acute and chronic toxicity testing in the USA [186], and was first used as such by Mount and Norberg in 1984 [187]. *C. dubia* has yet to gain wide acceptance in Europe [185]. It has been used to monitor the toxicities of effluents [188, 189], freshwater bodies [190, 191, 192, 193], and a variety of compounds (e.g. [194, 195]). *C. dubia* toxicity data for organophosphate pesticides are available in the literature. Bailey *et al.* [106] determined diazinon’s 48 hr LC$_{50}$ (330 ng/l) and its 7-day chronic toxicity (140 ng/l). Parathion, diazinon, malathion, and methyl parathion’s 48 hr LC$_{50}$s were determined to be 230, 500, 2120, and 3500 ng/l, respectively [196]. Roux *et al.* [197] also reported the 48 hr LC$_{50}$ for fenthion (1720 ng/l). Bailey *et al.* [128, 198] found the CPF LC$_{50}$s to *C. dubia* to be 63 - 101 ng/l (24 hours), 58 - 79 ng/l (48 hours), and ~ 55 ng/l (96 hours). Kuivala and Foe [199] determined CPF’s 96 hour LC$_{50}$ to be 80 - 130 ng/l. A search of the literature and the US EPA ACQUIRE database [200] failed to reveal DDT LC$_{50}$ values for *C. dubia*. This may be due in part to the banning of DDT use in the US and most other developed countries before the appearance of the daphnid as a widely used acute test organism.
*Americamysis bahia:*

The mysid *Americamysis bahia* (formerly *Mysidopsis bahia* Molenock), an estuarine crustacean, can be found from the Gulf of Mexico to the temperate waters of the East coast of the United States [201]. Its abundance, ecological importance, and ease of care make it ideal for laboratory toxicity studies [202]. It was first used in toxicity testing in 1977 [203] and has become the standard test organism of the US EPA for marine and estuarine environments [202]. Parkhurst *et al.* [204] found it to be one of three organisms for which acute toxicity data were most extensive. Mysid toxicity tests on various contaminants have been conducted in laboratory exposures varying from a few hours [205] to weeks [206]. It has also been used in field studies [207]. Mysids have often been found to be the most sensitive species tested [208, 209]. Industrial surfactants [210], mycotoxins [211], various effluents [212, 213] and organophosphate pesticides are a few of the contaminants that have been tested using mysids. Cripe *et al.* [214] found that methyl parathion (~ 400 ng/l) and phorate (100 ng/l) decreased the mysid’s swimming stamina in 96 hour tests. The acute and chronic toxicities of azinphos-methyl to mysids were determined by Morton *et al.* [215]. The pesticide’s 96 hr LC$_{50}$ (290 ng/l) and maximum allowable toxicant concentration (24 ng/l) were an order of magnitude lower for the mysid than for the sheepshead minnow, another common test organism. Cripe *et al.* [216] and Cripe [126] assessed the 96 hr LC$_{50}$ of malathion to mysids. Their results ranged from 3300 to 11000 ng/l. The CPF 96 hour LC$_{50}$ to mysids has been estimated to be ~ 35 ng/l [102]. A search of the literature and the US EPA ACQUIRE database [200] also failed to reveal DDT LC$_{50}$ values for the mysid.
Objectives:
The objectives of this study were to determine:

1) the CPF 24 hour LC50 to A. bahia and C. dubia.
2) the DDT 24 hour LC50 to A. bahia and C. dubia.
3) the effect of HA addition on CPF toxicity near its 24 hour LC50 to A. bahia and C. dubia.
4) the effect of HA addition on DDT toxicity near its 24 hour LC50 to A. bahia and C. dubia.

Null hypotheses:
H0 1: DDT is not toxic to either A. bahia or C. dubia.
H0 2: CPF is not toxic to either A. bahia or C. dubia.
H0 3: Addition of HA will not affect the toxicity of DDT to either A. bahia or C. dubia.
H0 4: Addition of HA will not affect the toxicity of CPF to either A. bahia or C. dubia.

Materials and Methods:

Glassware:
Pre-cleaned glassware was ignited at 450°C overnight prior to use. Glassware that did not fit in the furnace was cleaned with 4M HCl, followed by multiple rinses in deionized water. The water was displaced by high purity acetone and the glassware dried.
Chemicals:

Chlorpyrifos (CPF) was purchased from Chem Services (West Chester, PA). 4,4'-DDT was obtained from the EPA Research Laboratories (Research Triangle Park, NC). The pesticide standards were 99.9 and ~99 % pure, respectively. HAs were purchased from Aldrich Chemical Company, Inc., Milwaukee, WI.

Humic acid solutions:

Stock solutions of 100 mg/l HA solutions were made by stirring 200 mg of HA in 1800 ml of deionized water at pH=10 (NaOH) overnight in a brown bottle. The pH was then reduced to pH=7.8 (HCl) and the volume adjusted to 2000 ml.

Solutions of 0, 10, 30, 50, and 100 mg/l HA at salinities of 0 and 20 ppt were made by first diluting the stock solution with water. Fresh water for daphnids was amended with 30 mg/l MgSO₄•7H₂O, 30 mg/l CaSO₄•2H₂O, 2 mg/l KCl, 20 mg/l CaCl₂•2H₂O (Fisher Scientific, Fair Lawn, NJ), and 48 mg/l NaHCO₃ (Mallinckrodt, Paris, KY) per liter [50]. Brackish water for mysids was made by adding 20 g/l of Hawaiian marine mix (Hawaiian Marine Imports, Houston, TX). All solutions were vigorously aerated for at least 24 hours in brown bottles, and their pH controlled (7.8). All solutions were filtered to 0.45 μm with pre-rinsed cellulose nitrate membrane filters (Whatman. Clifton, NJ) prior to use. To reduce clogging of the filters, higher HA-concentration solutions were prefiltered with furnace (450 °C, 4 hours), pre-rinsed type A/E glass fiber filters (Gelman Sciences, Inc. Ann Arbor, MI).
Toxicity test duration:

The toxicity tests for the mysid *Americamysis bahia* and the daphnid *Ceriodaphnia dubia* were terminated after 24 hours. This eliminated the need to feed the organisms. Feeding would have added an additional source of DOM to the test aquaria.

Test water quality:

Water aliquots from the control, HA control, and low, medium, and high concentrations of either pesticide or HA were collected for DOM and pesticide analyses prior to test initiation. Water conductivity, alkalinity and hardness were also measured at that time for the daphnid samples. Immediately before transfer of the test water to the aquaria, dissolved oxygen and temperature were measured in water samples for the control, HA control, and low, medium, and high concentrations of either pesticide or HA. At the end of each experiment, DO was measured in each replicate of the control, HA control, and low, medium, and high concentrations of either pesticide or HA. The water samples from the mysid tests were kept for pesticide analysis. Maximum and minimum temperatures for the duration of the test were recorded from the water bath in which the aquaria were immersed (mysid), or in the test incubator (daphnids).

Mysid and daphnid exposures:

After filtration and vigorous aeration, 800 ml (mysids) or 300 ml (daphnids) of the appropriate aqueous solution were spiked with 800 μl (mysids) or 300 μl (daphnids) of either the acetone carrier or pesticide standard dissolved in acetone, yielding the desired
nominal pesticide concentrations. The aqueous solutions were stored in sealed brown
glass bottles overnight, and brought to temperature prior to test initiation. Solutions
(mysids: 200 ml; daphnids: 18 ml) were transferred to each test aquaria, and the
remainder was analyzed for the pesticide concentration at test initiation. Test aquaria
were “finger bowls” (mysids) or scintillation vials (daphnids).

Four-day old mysids (*Americamysis bahia*) were purchased locally (Chesapeake
Cultures Inc., Hayes, Virginia), and were fed the morning of test initiation, prior to
shipping. Daphnid neonates (<24 hrs old *Ceriodaphnia dubia*) were donated by Coastal
Bioanalysts (Gloucester, Virginia). The organisms were transferred to test aquaria shortly
after arrival. Ten individuals were pipetted carefully into each replicate, the aquaria were
covered loosely with a plastic lid and placed in a water bath at 27 °C (mysids) or in an
incubator at 25 °C (daphnids). All treatments were done in triplicate, and placed in the
water bath or incubator according to random block designs. Light regime was 16/8
light/dark (hours).

Tests to approximate the 24 hr LC₅₀ of either CPF or DDT consisted of a control
(water + solvent carrier), an HA control (100 mg/l Aldrich HA + solvent carrier) and six
pesticide concentrations, with one exception. The mysid DDT LC₅₀ had nine DDT
concentrations. For the mysids, the nominal pesticide concentrations were 80.4, 134,
223, 280, 317 and 397 ng/l CPF and 1610, 2100, 2500, 3160, 4170, 5560, 6950, 8220 and
11100 ng/l DDT. For the daphnids, the nominal pesticide concentrations were 21, 33, 41,
66, 82 and 132 ng/l CPF, and 556, 695, 822, 1110, 1580 and 1900 ng/l DDT. Nominal
concentrations were used to approximate the LC₅₀s. While this approach probably
resulted in an underestimation of toxicity, the goal of this study was not a stringent
determination of LC₅₀s, and the approach was deemed sufficient to determine a pesticide
centrations at which HA effects could be monitored.

Tests to determine the effect of Aldrich HA on pesticide toxicity consisted of a
control, an HA control, and five concentrations of HA spiked with a common
concentration of pesticide. The HA concentrations were 0, 10, 30, 50 and 100 mg/l. The
nominal pesticide concentrations were chosen to approximate the previously estimated
LC₅₀s for the pesticides. They were 322 ng/l (CPF) and 1100 ng/l (DDT) for the mysids,
and 82 ng/l (CPF) and 1100 ng/l (DDT) for the daphnids. After 24 hours, the organisms
were examined under a dissecting scope and enumerated. Mortality was defined as death
or lack of movement or response to gentle prodding (mysid), or swirling of the water
(daphnids).

**Pesticide analysis:**

Water sample volumes were measured and the samples were spiked with known
amounts of surrogate standards to quantify losses occurring during extraction. Surrogate
standards were 1,1'-binaphthyl (BN) for DDT and dichlofenthion (DCF) for CPF. The
samples were extracted twice in separatory funnels with dichloromethane (50 ml x2),
solvent exchanged into hexane and the volume reduced to ~ 200 µl by evaporation under
nitrogen. Internal standards (p-terphenyl for DDT, and ethion for CPF) were added
immediately prior to gas chromatographic analysis. Pesticide concentrations were
determined with the aid of a standard curve, and corrected for surrogate standard
recovery.

CPF was analyzed on a Varian 3400 GC/TSD with a 30 m 1710 column (J&W Scientific, Folsom, CA). Injector and detector temperatures were 270 and 300 °C, respectively. Oven temperature was held at 75 °C for 2 minutes, then increased to 270 °C at 7° per minute, and held for 5 minutes. DDT was analyzed on a Varian 3400 GC/FID with a 60 m DB5 column (J&W Scientific, Folsom, CA). Injector and detector temperatures were 260 and 320 °C, respectively. Oven temperature was held at 75 °C for 2 minutes, then increased to 320 °C at 4° per minute, and held for 10 minutes.

All injections were splitless. The method quantitation limits were ~ 20 ng/l for CPF and ~ 400 ng/l for DDT (200 ml samples).

DOC measurements:

Aliquots of water samples were collected prior to test initiation and frozen for later DOC determination [161]. Samples were analyzed using a total organic carbon analyzer (Shimadzu model TOC-5000) with a non-dispersive infrared detector. The analyses were performed by the VIMS Analytical Service Center.

Statistical design and analysis:

Data were checked for normality of distribution by the Chi-square test and for homoscedasticity using Bartlett's test. 24 hour LC₅₀s were estimated by fitting the data to probit models. The effect of HA on mortality was assessed by ANOVA. Regressions of percent survival against HA concentrations were computed when deemed appropriate.
The Tukey multiple comparison test was used to determine where the differences lay. When data did not satisfy the homoscedasticity requirements, they were analyzed using the Kruskal-Wallis non-parametric test and differences between the means were elucidated by Dunn's multiple comparison test.

Results:

Mysid - 24 hr LC₅₀:

Water quality parameters:

DOC samples were collected post-pesticide spike, and thus contained ~ 0.1% of carrier solvent (acetone), overwhelming the ambient DOC level. Values from a subsequent experiment indicated that the DOC concentration would have been ~ 1 mg C/l.

Water quality data were taken for control, HA control, and low (80.4 ng/l CPF, or 1610 ng/l DDT), medium (223 ng/l CPF, or 4170 ng/l DDT), and high (397 ng/l CPF, or 11100 ng/l DDT) pesticide concentrations. Temperatures in the water bath held steady for the duration of the experiments, between 27.0 and 27.5 °C (CPF), and 27.0 and 28.0 °C (DDT). A single DO value was taken for each treatment at test initiation, as the test water replicates were aliquoted from a single water sample. Triplicate values were taken at test termination. In the CPF LC₅₀ experiment, the lowest DO value measured at any time was in the HA control aquaria at test termination (78.2 ± 1.1 % of saturation). Mean DO values across all treatments in that experiment, at test initiation and termination were 86.0 ± 3.7 %, and 82.2 ± 2.7 % of saturation, respectively. In the experiment for the
estimation of the DDT LC50, mean DO values across all treatments, at test initiation and termination were 92.7 ± 2.0 %, and 88.7 ± 1.2 % of saturation, respectively.

A single initial pesticide concentration was determined for each treatment, for the reason mentioned above. Pesticide concentrations were measured from each triplicate at test termination (Figures 13 and 14).

In the CPF experiment, mean surrogate (DCF) recoveries were 107 % (± 5.6 %) at test initiation and 100 % (± 3.2 %) at test termination. The greater value obtained for the test initiation value is due to a maximum recovery of 115 % for the low CPF concentration (80.4 ng/l). CPF recoveries at test initiation averaged 90.6 ± 6.5 % of nominal, and ranged from 85 % (80.4 ng/l) to 97.3 % (397 ng/l). CPF concentrations at test termination were below the detection limit in the low concentration treatments, and averaged 34.0 ± 4.4 % and 43.3 ± 8.0 % in the medium (223 ng/l) and high concentration (397 ng/l) treatments, respectively. No CPF was detected in the controls at any time.

In the DDT experiment, surrogate (BN) recoveries averaged 107 % (± 7.9 %) at test initiation and 93.2 % (± 5.5 %) at test termination. DDT recoveries at test initiation averaged 110 % (± 9.3 %) of nominal. DDT relative recoveries were similar across all treatments at test termination and averaged 36.6 % (± 5.9 %). Minimum and maximum recoveries were 30.7 ± 5.6 % (1610 ng/l, nominal) and 41.3 ± 1.3 % (11100 ng/l, nominal).
Figure 13. Percent CPF recoveries for the *Americamysis bahia* 24 hour LC$_{50}$ at test initiation (Day 0) and termination (Day 1) from nominal CPF concentrations of 80.4, 223, and 397 ng/l. Error bars represent ± 1 standard deviation.
Figure 14. Percent DDT recoveries for the *Americamysis bahia* 24 hour LC$_{50}$ at test initiation (Day 0) and termination (Day 1) from nominal DDT concentrations of 1610, 4170, and 11,100 ng/l. Error bars represent ± 1 standard deviation.
CPF 24 hr LC50:

The CPF 24 hour LC50 test data for 4-day old mysids are presented in Figure 15. Control and HA control triplicates showed no mortality. One mysid died in one of the triplicates of the lowest CPF concentration (80.4 ng/l nominal) treatment, which resulted in slightly lower survival (97 ± 6 %) than the control and the next higher treatment (134 ng/l), which showed no mortality. Survival decreased monotonically with increased CPF concentration to a minimum of 30 % (± 26 %) at 397 ng/l CPF. The 24 hour LC50 calculated using nominal concentrations was 326 ng/l (95 % fiducial limits 301 - 362 ng/l).

DDT 24 hr LC50:

The DDT 24 hour LC50 data for 4-day old mysids are presented in Figure 16. No mortality was observed in the control replicates. While a preliminary experiment had suggested that the DDT 24 hour LC50 was approximately 3000 ng/l, no clear mortality trend can be seen in these data from 1610 ng/l to 11,100 ng/l. All mortalities exceeded 50 % in the treatments, suggesting that the LC50 is less than 1610 ng/l. Nevertheless, DDT concentrations that produced partial mysid mortality could be identified to assess the effect of HA on DDT toxicity.

Mysid - pesticide - HA:

Water quality parameters:

DOC samples were collected post-CPF spike, and thus contained 0.1 % of carrier solvent. In the DDT experiment, DOC values for the control and 0 mg/l HA treatment
Figure 15. Percent survival of *Americamysis bahia* in its CPF 24 hour LC$_{50}$. Treatments represent the nominal CPF concentrations (ng/l). Error bars represent ± 1 standard deviation.
Figure 16. Percent survival of *Americamysis bahia* in its DDT 24 hour LC$_{50}$. Treatments represent the nominal DDT concentrations (ng/l). Error bars represent ± 1 standard deviation.
% mysid survival

DDT (ng/l)

Ctrl  1610  2100  2500  3160  4170  5560  6950  8220  11100

0  20  40  60  80  100

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were 1.1 and 2.2 mg C/l, respectively (Figure 17). As they were taken from the same water samples, they should have been identical. DOC concentrations increased with HA concentration to ~ 14.8 mg C/l. The HA control and the 100 mg/l treatments, taken from the same water sample, had similar DOC concentrations of 14.7 and 14.9 mg C/l.

Water quality data were taken for control, HA control, and low (0 mg/l), medium (30 mg/l), and high (100 mg/l) HA concentrations. The temperatures in the water bath held steady for the duration of the experiments, between 25.5 and 26.5 °C (CPF) or at 27 °C (DDT). A single DO value was taken for each treatment at test initiation, as the replicates were aliquoted from a single water sample. Triplicate values were taken at test termination. Mean DO values across all treatments in the CPF-HA experiment were 86.9 % (± 0.9 %) of saturation at test initiation, and 82.0 % (± 1.3 %) at test termination. In the DDT-HA experiment, mean DO values at test initiation and termination were 79.5 % (± 2.0 %), and 72.4 % (± 2.7 %) of saturation, respectively.

A single initial pesticide concentration was determined for each treatment. Pesticide concentrations were measured from each triplicate at test termination (Figure 18, 19).

CPF-HA surrogate recoveries averaged 92.9 % (± 6.9 %) at test initiation and 93.2 % (± 7.2 %) at test termination. CPF recoveries at test initiation averaged 88.7 ± 7.1 % of nominal and 35.3 ± 2.5 % at test termination, across all treatments. Minimum and maximum relative CPF recoveries (from a 322 ng/l nominal concentration) at test termination were 33.9 ± 3.9 % (100 mg/l HA) and 36.8 ± 1.7 % (30 mg/l HA).

DDT–HA surrogate (BN) recoveries averaged 81.3 % (± 19.8 %) at test initiation.
Figure 17. DOC content of water samples from the 24 hour Aldrich HA mitigation of DDT toxicity to *Americamysis bahia* experiment. Treatments include control, HA control (100 mg/l HA), and nominal Aldrich HA concentrations of 0, 10, 30, 50, and 100 mg/l prior to DDT spike (1100 ng/l nominal).
Figure 18. Percent CPF recoveries at test initiation (Day 0) and termination (Day 1) for the Aldrich HA mitigation of CPF toxicity to *Americamysis bahia* at nominal Aldrich HA concentrations of 0, 30, and 100 mg/l. Treatments all received a nominal CPF concentration of 322 ng/l. Error bars represent ± 1 standard deviation.
Figure 19. Percent DDT recoveries at test initiation (Day 0) and termination (Day 1) for the Aldrich HA mitigation of DDT toxicity to *Americamysis bahia* at nominal Aldrich HA concentrations of 0, 30, and 100 mg/l. Treatments were at a DDT concentration of 1100 ng/l. Error bars represent ± 1 standard deviation.
The great variation was due to low BN recovery from the HA control sample (54.7 %). Disregarding that datum, surrogate recoveries averaged 87.9 % (± 5.1 %). Surrogate recoveries at test termination averaged 102 % (± 8.5 %). DDT measurements at test initiation averaged 117 % (± 7.2 %) of nominal (1100 ng/l) and 42.2 (± 11.5 %) at test termination, across all treatments. DDT measurements at test termination ranged from 34.6 ± 10.0 % (0 mg/l HA) to 55.2 ± 6.8 % (30 mg/l HA). No pesticide (CPF or DDT) was detected in the controls or HA controls at any time.

**HA mitigation of CPF toxicity:**

The HA mitigation of CPF toxicity data for 4-day old mysids are presented in Figure 20. One mysid died in a single control replicate, which reduced the survival to 96.7 % (± 5.8 %). No mortality was observed in the HA controls. The highest survival occurred in the treatment containing no added HA (63.3 ± 5.8 %). The lowest survival occurred in the 30 mg/l HA treatment. This treatment also had the greatest standard deviation for survival (36.7 ± 20.8 %). Survival data from all treatments containing CPF were normally distributed and had homogeneous distributions, according to the Chi-square and Bartlett’s tests. Mortality between these treatments were not significantly different from each other (p = 0.18).

**HA mitigation of DDT toxicity:**

The HA mitigation of DDT toxicity data for 4-day old mysids are presented in Figure 21. Survival in the HA-control treatments was reduced to 93.3 (± 11.5 %) by the
Figure 20. Percent *Americamysis bahia* survival for the 24 hour Aldrich HA mitigation of CPF toxicity. Treatments include control, HA control (100 mg/l HA), and nominal Aldrich HA concentrations of 0, 30, and 100 mg/l containing a nominal concentration of 322 ng/l CPF. Error bars represent ±1 standard deviation.
Figure 21. Percent survival of *Americamysis bahia* for the 24 hour Aldrich HA mitigation of DDT toxicity. Treatments include control, HA control (100 mg/l HA), and nominal Aldrich HA concentrations of 0, 30, and 100 mg/l containing a nominal concentration of 1100 ng/l DDT. Error bars represent ± 1 standard deviation.
death of two mysids died in a single HA control replicate. No mortality was observed in the controls. Of the HA-exposed treatments, the highest survival occurred in those containing 30 and 50 mg/l HA (76.7 ± 5.8 %). The lowest survival occurred in the 100 mg/l HA treatment. This treatment also had the greatest standard deviation for survival (56.7 ± 20.8 %). Survival data from all treatments containing DDT (1100 ng/l nominal) were normally distributed and had homogeneous distributions, according to the Chi-square and Bartlett’s tests. Mortality between these treatments were not significantly different from each other (p = 0.40).

Daphnid - 24 hr LC50:

Water quality parameters:

Water quality data were taken for control, low (22 ng/l CPF, or 556 ng/l DDT), medium (66 ng/l CPF, or 1110 ng/l DDT), and high (132 ng/l CPF, or 1900 ng/l DDT) pesticide concentrations. The initial incubator temperature was 25.0 °C, and test temperatures ranged from 25.0 to 26.5 °C. Initial DOC concentrations were 0.38 mg C/l (± 0.06).

A single DO value was taken for each treatment at test initiation, as the replicates were aliquotted from a single water sample. Mean % DO saturations at test initiation and termination were 101 % (± 0.64 %), and 93.3 % (± 0.99 %), respectively. Water conductivity, alkalinity, and hardness were measured at test initiation: 140 μMHOS, 33.3 mg/l CaCO₃, and 53.2 mg/l CaCO₃, respectively. Because of low test volumes and the potential for cross-contamination during previous water quality measurements, pesticide
recovery data were determined only at test initiation. In the CPF experiment, mean surrogate (DCF) recoveries were 83.8 % (± 5.9 %). Mean CPF recoveries were 103 % (± 3.3 %) of nominal concentrations (Figure 22). In the DDT experiment, mean surrogate (BN) recoveries were 106 % (± 9.4 %). Mean DDT recoveries were 112 % (± 19.8 %) of nominal concentrations (Figure 22). The greater imprecision for the DDT recovery is due to a high value (134 %) for the lowest DDT concentration (556 ng/l). Initial DOM concentrations were 0.38 mg C/l (± 0.06).

CPF 24 hr LC₅₀:

The CPF 24 hour LC₅₀ data for daphnid neonates are presented in Figure 23. While an effort was made to include 10 individuals per replicate, their small size made counting difficult and some replicates received as few as 9, or as many as 14 individuals. As the DDT and CPF experiments were conducted concurrently, the control replicates were combined and consist of six replicates, rather than three. Mortality was observed in a single control replicate (3 of 12 individuals). Survival decreased monotonically with increased CPF concentration to a minimum of 3.3 % (± 5.8 %) at 132 ng/l CPF. The 24 hour LC₅₀ calculated using nominal concentrations was 78.8 ng/l (95 % fiducial limits 72.4 - 85.9 ng/l).

DDT 24 hr LC₅₀:

The DDT 24 hour LC₅₀ data for daphnid neonates are presented in Figure 24. Survival generally decreased with increasing DDT concentration, and was not different
Figure 22. Percent CPF and DDT recoveries for *Ceriodaphnia dubia* 24 hour LC$_{50}$ at test initiation for control, nominal CPF concentrations of 22, 66, and 132 ng/l, and nominal DDT concentrations of 556, 1110, and 1900 ng/l.
Figure 23. Percent survival of *Ceriodaphnia dubia* in the CPF 24 hour LC$_{50}$ experiment. Treatments represent the nominal CPF concentrations (ng/l). Error bars represent ± 1 standard deviation.
% daphnid survival

CPF (ng/l)

Ctrl  21  33  41  66  82  132
Figure 24. Percent survival of Ceriodaphnia dubia in the DDT 24 hour LC₅₀ experiment. Treatments represent the nominal DDT concentrations (ng/l). Error bars represent ± 1 standard deviation.
from the control treatment until the DDT concentration reached 820 ng/l (survival: 67.8 ± 1.9 %). A single individual survived in one replicate from the highest DDT concentration (1900 ng/l). The 24 hour LC$_{50}$ calculated using nominal concentrations was 1050 ng/l (95 % fiducial limits 851 - 1280 ng/l).

Daphnid - pesticide - HA:

**Water quality parameters:**

Water aliquots were subsampled from each treatment for DOC analysis. The data are presented in Figure 25. Both the control and 0 mg/l HA treatment had low measured DOC concentrations (0.57 and 0.60 mg/l, respectively). The 100 mg/l HA and the HA control samples were taken from the same water sample. Their DOC values were 21.7 and 18.9 mg/l, respectively. The following regression of DOC against HA concentration was computed (mg/l):

$$\text{DOM} = 0.1969 \times \text{HA} - 0.633 \quad (R^2 = 0.99)$$

Water quality data were taken for control, HA control, and 0, 30, and 100 mg/l HA. The initial incubator temperature was 26.0 °C, and ranged from 25.0 to 26.5 °C during the experiment. A single DO value was taken for each treatment at test initiation, as the replicates were aliquoted from a single water sample. The DO data for CPF and DDT were combined because the experiments were combined and run concurrently. The treatment replicates were recombined at test termination to provide sufficient volume for
Figure 25. DOC content of treatments from the 24 hour Aldrich HA mitigation of CPF and DDT toxicity to *Ceriodaphnia dubia* experiment. Treatments include control, HA control (100 mg/l HA), and nominal Aldrich HA concentrations of 0, 10, 30, 50, and 100 mg/l prior to CPF (82 ng/l) or DDT (1100 ng/l) spikes.
a final DO measurement. Mean % DO saturations at test initiation and termination were 91.5 % (± 0.98 %), and 86.9 % (± 0.77 %), respectively. Water conductivity, alkalinity, and hardness were measured at test initiation. Conductivity increased with HA concentration, from 140 µMHOS in the control and 0 mg/l treatments, to 145 µMHOS in the 30 mg/l HA treatment and 165 µMHOS in the 100 mg/l and HA control treatment. Water alkalinity and hardness did not differ between treatments and were 33.3 mg/l CaCO₃ and 53.2 mg/l CaCO₃, respectively.

Because of low test volumes and the potential for cross-contamination during previous water quality measurements, pesticide recovery data were determined only at test initiation. In the CPF-HA experiment, mean surrogate (DCF) recoveries were 105 % (± 14 %). Mean CPF recoveries were 73.3 % (± 2.7 %) of the nominal concentration (82 ng/l) (Figure 26). In the DDT-HA experiment, mean surrogate (BN) recoveries were 98.5 % (± 2.5 %). Mean DDT recoveries were 93.7 % (± 20.6 %) of nominal (1100 ng/l) (Figure 26). DDT recovery increased with HA concentration, from a low of 72.9 % (0 mg/l) to a high of 114 % (100 mg/l HA).

HA mitigation of CPF toxicity:

The HA mitigation of CPF toxicity data for daphnid neonates are presented in Figure 27. No mortality was observed in either the controls or the HA controls. Survival increased monotonously with HA concentration from a low of 19.1 ± 8.7 % (0 mg/l HA) to a high of 91.6 ± 1.1 % (100 mg/l HA).
Figure 26. Percent CPF and DDT recoveries at test initiation for the Aldrich HA mitigation of CPF toxicity to *Ceriodaphnia dubia* at nominal Aldrich HA concentrations of 0, 30, and 100 mg/l. Treatments contained nominal concentrations of either 82 ng/l CPF or 1100 ng/l DDT.

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Figure 27. Percent survival of *Ceriodaphnia dubia* in the 24 hour Aldrich HA mitigation of the CPF toxicity experiment. Treatments include control, HA control (100 mg/l HA), and nominal Aldrich HA concentrations of 0, 10, 30, 50, and 100 mg/l containing a nominal concentration of 82 ng/l CPF. Treatments overlain by a different letter are significantly different from each other (p < 0.05). Error bars represent ± 1 standard deviation.
The following regression of % survival against HA concentration (mg/l) was computed:

\[
\% \text{ survival} = 0.7096 \text{ HA} + 25.044 \quad (R^2 = 0.97)
\]

Survival data from all treatments containing CPF were normally distributed and had homogeneous distributions, according to the Chi-square and Bartlett’s tests. Mortality between these treatments were significantly different from each other (p < 0.001). The results of the Tukey a-posteriori test are presented in Figure 27. Daphnid survival in 100 mg/l HA was higher than in any other concentration. Survival in other concentrations did not differ from those of the next higher or lower HA concentration, but was different from all the others.

HA mitigation of DDT toxicity:

The HA mitigation of DDT toxicity data for daphnid neonates are presented in Figure 28. No mortality was observed in either the controls or the HA controls. Survival increased with HA concentration from a low of 50.6 ± 9.2 % (0 mg/l HA) to ~ 100 % in HA concentrations of 30, 50, and 100 mg/l. No regression was computed, because survival reached 100 % before the highest HA concentration treatment. The lack of variance (no mortality) observed in the 50 mg/l HA treatment resulted in the data not having homogeneous variance. A Kruskal-Wallis non-parametric test found a significant difference between treatments (p < 0.02). Dunn’s multiple comparison test found the 0 mg/l HA treatment to be different from the control (p < 0.05). Conducting analyses only
Figure 28. Percent survival of *Ceriodaphnia dubia* in the 24 hour Aldrich HA mitigation of the DDT toxicity experiment. Treatments include control, HA control (100 mg/l HA), and nominal Aldrich HA concentrations of 0, 10, 30, 50, and 100 mg/l containing a nominal concentration of 1100 ng/l DDT. Treatments overlain by a different letter are significantly different from each other (p < 0.05). Error bars represent ± 1 standard deviation.
% daphnid survival

Ctrl    HA Ctrl
0       10    30    50    100

Aldrich HA (mg/l)

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for data that had non-zero variance (0, 10, 30, and 100 mg/l HA) permitted parametric testing. A Tukey test on these showed that the 0 and 10 mg/l HA treatments were significantly lower than the 30 and 100 mg/l HA treatments.

**Discussion:**

**Water quality:**

The HA concentrations used and resultant DOC levels (0.4 - 21 mg C/l) were commensurate with commonly occurring environmental ranges. For example, Penttinen *et al.* [166] reported DOC ranges in Finnish lakes of 0.2 to 18 mg C/l. Chin *et al.* [27] recorded DOC values reaching 35 mg C/l in the Suwannee River, Georgia. The Parker River (MA) contained ~12 mg/l DOC [7]. DOC values in Chesapeake Bay waters generally ranged from <2.0 to 6.0 mg C/l [164, 188, 217, 218]. York River DOC ranged from 4.7 to 8.4 mg/l [7]. Liu and Dickhut [219] reported high DOC concentrations of 20 mg C/l from Chesapeake Bay surface waters. Sediment pore water DOC in contrasting sites in the Bay ranged from 2.4 to 24 mg C/l [220].

**CPF 24 hr LC$_{50}$ to *A. bahia:***

Mean CPF initial recoveries were 90.6 % of nominal concentrations. The recovery for the concentration (397 ng/l nominal) nearest the estimated LC$_{50}$ (326 ng/l) was 97.3 %. Final (24 hr) relative CPF recoveries were ~40 %. This decrease in recovery was seen in all experiments of this study. No mass balance was conducted, so there is no information on the exact fate of the other ~60 %. Biological uptake,
degradation, sorption to aquaria walls, and volatilization probably all contributed to the decrease in recovery. This underscores the need for periodic renewal of the test solutions, especially under static renewal conditions and for tests longer than 24 hours. This was not done in this case to minimize organism stress and because the test lasted only 24 hours.

A search of the literature and the ACQUIRE database [200] did not reveal any previously collected 24 hr LC₅₀ values for CPF toxicity to *A. bahia*. A preliminary experiment had determined the 96 hr LC₅₀ value to be 28 ng/l (Unpublished results), which was not statistically different from that calculated by Schimmel *et al.* [22]. The present 24 hr LC₅₀ of 326 ng/l is an order of magnitude higher than this 96 hour value. While the former LC₅₀ value exceeds most environmental concentrations detected, higher CPF concentrations have been reported. CPF concentrations as high as 480 ng/l were recorded in irrigation run-off [221]. In the Bohemia River, MD, a tributary of the Chesapeake Bay, CPF was detected in water at concentrations exceeding 900 ng/l [222]. Californian rain and fog samples have contained up to 600 ng/l of CPF [106].

It is important to note that these are acutely toxic concentrations resulting in mortality, and that sub-lethal effects occurring at much lower concentrations can have a significant impact at the population level. For example, the organophosphate pesticide azinphos-methyl significantly reduced mysid brood-size after 26 days of exposure at a concentration (30 ng/l) 15 times lower than its 48 hour LC₅₀ (440 ng/l) [215].
DDT 24 hr LC$_{50}$ to *A. bahia*:

Mean DDT initial recoveries were 110% of nominal concentrations, and final (24 hr) recoveries were 37%. This decrease is very similar to that observed for CPF. The high recovery, especially at the high concentration (11,100 ng/l) is somewhat unexpected, because DDT solubility in water has usually been reported to be lower (1000 to 8000 ng/l) [75, 76, 174]. Higher concentrations were used in this study to encompass the 24 hr LC$_{50}$ of ~ 3000 ng/l suggested in a preliminary experiment. Previous studies have reported even higher DDT LC$_{50}$ values for other aquatic organisms. For example, Hoke *et al.* [223] noted that the 48 hr LC$_{50}$ value for *Gammarus lacustris* had been estimated at 1000 to 9000 ng/l. Anadu *et al.* reported a 96 hr LC$_{50}$ for the mummichog *Fundulus heteroclitus* of ~ 20,000 ng/l [224].

There was no clear trend of increasing mortality with increasing concentration and thus no definite LC$_{20}$ was calculated in the present study. The higher DDT concentrations exceeded the literature values for its solubility in fresh water, and its solubility in saline water is expected to be lower still. It is possible that DDT crystalized out of solution, but remained in suspension. If that were the case, then the mysids might have been exposed to a fairly constant bioavailable DDT concentration, even though the total concentration in the water column exceeded the water solubility. This would account for both the lack of increase in mortality with increasing DDT concentration, and the good initial DDT recoveries. As the primary goal was not to establish LC$_{50}$s for DDT, but rather to provide a reasonable concentration to assess the effect of HA on DDT toxicity, the experiment was not repeated.
CPF 24 hr LC₅₀ to *C. dubia*:

Mean CPF initial recoveries were 103% of nominal concentrations. Due to low test volumes and potential cross-contamination from previous water quality measurements, no CPF recovery data were collected at test termination. A decrease in CPF concentration over time would be expected, as was seen in the mysid experiments. The estimated 24 hr LC₅₀ (78.8 ng/l) was within the range (63 to 101 ng/l) reported by Bailey *et al.* [198]. CPF's 24 hr LC₅₀ for *C. dubia* is one-fourth of that calculated for *A. bahia*. Differences in test conditions non-withstanding, *C. dubia* seems much more sensitive than *A. bahia*. Besides salinity, there were only slight differences in recorded test conditions between the two organisms. Moreover, an increase in salinity may tend to facilitate the partitioning of CPF into biota. It would seem therefore that under identical conditions, *C. dubia* would indeed be the more sensitive of the two species for CPF. The significantly lower LC₅₀ also makes toxicity events due to CPF more likely. Indeed, the median CPF concentration in 90 water samples from urban waterways in northern California was 50 ng/l, and probably sufficient to cause significant mortality.

DDT 24 hr LC₅₀ to *C. dubia*:

The initial concentration determined analytically, whose value was closest to the calculated LC₅₀ (820 ng/l), had a recovery of 96.7% of nominal. The estimated 24 hr LC₅₀ of DDT to *C. dubia* was 1050 ng/l. This concentration is higher than those that can be expected in the environment. Aside from current application sites, DDT effects appear less likely to result from acute exposure than from chronic effects resulting from
bioconcentration of relatively low levels of contamination. Reports of DDT and DDT metabolite bioconcentration or bioaccumulation abound in the literature, from California otters [81] and wolves [88], to shrimp [225], kestrels [226], and humans [91, 227]. Water-only exposures are likely to be a less important route than dietary uptake and close association to contaminated sediment for highly hydrophobic chemicals such as DDT.

**HA mitigation of CPF and DDT toxicity:**

The presence of HA did not affect the toxicity of CPF (82 ng/l nominal) or DDT (1100 ng/l nominal) to mysids. There was a trend suggesting increased CPF toxicity to mysids with increased HA concentration, which mirrored the slight increase in CPF concentration, but it was not statistically significant.

The presence of HA had a great effect on both CPF and DDT toxicity to freshwater daphnids. The toxicity of both compounds decreased with increasing concentrations of HA, with effects visible, albeit not significant, at the lowest HA concentration (10 mg/l). Fewer significant differences were found for DDT. This is mainly because survival in the DDT treatments was greater in the lowest HA concentration (0 mg/l), and reached ~ 100 % in the 30 mg/l HA replicates. The rate of change in survival from 0 to 30 mg/l HA was actually greater in the DDT than in the CPF treatments.

A comparison of these results and the assignment of cause and effect relationships should be done carefully. The two species were tested at different salinities, and no information intrinsic to the experiments is available to allow the unequivocal
determination of the cause of the toxicity changes. There appears to be two main potential agents for the differences observed in this study. Salinity may have interfered with the binding of the pesticides to the HA, and thus modified their bioavailability. Alternatively, the HA-pesticide complex might not have been affected by salinity, and the results could be a function of the ability of the organisms to disrupt the complex and uptake the pesticides.

Both the mysid and the daphnid feed on particulate food items. The mysid is omnivorous [202], while the daphnid feeds mainly on algae such as *Selenastrum capricornutum* [187]. Neither organism is likely to feed on dissolved organic matter such as the HA presented to them in this study. The sensitivity to xenobiotics of both species has been compared to that of *Daphnia magna*, another common freshwater test organism. Versteeg *et al.* [185] found the sensitivity of the two daphnids to be equivalent, over a range of acute tests for 48 different materials, and chronic tests for 20 different materials, including effluents. Robinson [228] also found that the sensitivities of *A. bahia* and *D. magna* were correlated, over a range of 28 compounds tested. These studies do not support the case for the organismal differences as the cause of the changes in toxicity found in the present study.

Salinity has been shown to affect toxicity. Dinitrophenol toxicity decreased with increasing salinity (15 - 30 ppt) for the sheepshead minnow (*Cyprinodon variegatus*), but increased for the grass shrimp (*Palaemonetes* spp.). [53]. Salinity enhanced aldicarb toxicity to Japanese medaka (*Oryzias latipes*) [62]. Fulton [55] assessed the effect of
salinity on the toxicity of pesticides to the mummichog, *Fundulus heteroclitus*. He found an increase in toxicity of acephate with increases in salinity from 5 to 20 ppt. Guthion toxicity was not affected by salinity for either *F. heteroclitus* nor the shrimp *Palaemonetes pugio*, but increased with salinity for the three-spined stickleback (*Gasterosteus aculeatus*). Murphy [229] assessed the effect of salinity on the uptake of DDT, DDD, and DDE in the mosquito fish (*Gambusia affinis*). Bioconcentration of all contaminants decreased with increasing salinity, and the effect was found to be greatest for DDT.

Numerous authors have reported that DOC tends to decrease the toxicity and uptake of compounds by organisms. Natural aquatic humic substances were shown to decrease the uptake and bioconcentration factors of BaP in Atlantic salmon [37]. More recently, Haitzer *et al.* [35] noted a decrease in the BaP uptake by the nematode *Caenorhabditis elegans* as DOC concentrations increased. Aldrich HA also reduced the bioaccumulation and toxicity of anthracene to the fathead minnow (*Pimephales promelas*) and the daphnid (*Daphnia magna*) [230]. Aldrich, Suwannee River and Nordic aquatic HAs reduced the uptake and toxicity of some quaternary ammonium compounds in the fathead minnow [231]. Looser *et al.*, using the midge *Chironomus riparius*, demonstrated that triorganotin compounds (specifically tributyltin and triphenyltin) bound to Aldrich HA were not bioavailable [232]. In a more general report to the EPA Science Advisory Board, Di Toro [233] concluded that the freely dissolved fractions of non-ionic organic chemicals were their bioavailable component. Sediment-associated CPF toxicity to the midge *Chironomus tentans* decreased with increasing
organic carbon content of the sediment [184]. DOC can also affect uptake and toxicity of metals. Cd uptake by the Atlantic salmon (Salmo salar L.) was strongly dependant on humic concentrations [41]. Aldrich HA reduced Ag bioaccumulation in rainbow trout (O. mykiss) and protected the fish against adverse Ag physiological effects (e.g. plasma Na and Cl losses, respiration rates) [234]. These studies all lend weight to the hypothesis that the fraction of a compound that is bound to DOC exhibits reduced bioavailability, and that HA-compound complex is less toxic. However, some studies have reported an opposite effect. One fourth of the studies reviewed by Haitzer et al. [45] showed increased toxicity of compounds in the presence of low concentrations of DOM (< 10 mg/l). Tributyltin chloride toxicity to Daphnia magna significantly decreased in the presence of peat and soil HA, but was not affected by river sediment HA [50]. One may easily hypothesize an increase in solubility of the compounds in the presence of the DOM, resulting in higher concentrations in the water column. The apparent lack of a concomitant decrease in bioavailability due to the presence of DOM might be a result of the conformation of the molecules comprising the DOM, which may result in weak HA-compound interactions in dilute conditions. Most studies have been conducted with freshwater organism, and comparatively little research addresses the effect of DOM in saline waters.

The acute Microtox® bacterial bioluminescence inhibition assay, conducted at 20 ppt, also shows a divergence in the effect of HA on compound toxicity. Fenvalerate, permethrin and Cu toxicity all decreased in the presence of 50 mg/l Aldrich HA [3, 129]. The toxicities of CPF, guthion, and carbofuran decreased with increasing Aldrich HA
concentration, while those of methyl-parathion and carbaryl increased [1]. Kadlec and Benson [125] reported toxicity decreases for methyl parathion and guthion in the presence of natural DOC (2.4 - 6.8 mg C/l), but not for CPF. Chapter 1 here investigating the effect of 5 mg/l of Aldrich, Leonardite, Peat, and Suwannee River HAs on the chronic Microtox® toxicity (35 ppt) of Cu and CPF showed neither mitigation nor enhancement of toxicity by the HAs (Chapter 1).

Though a literature search failed to reveal any studies on the combined effects of salinity and DOC on toxicity, studies on the effect of salinity on the complexation of compounds with DOC do exist. The sorption of phenanthrene to a soil HA was reported to decrease as the ionic strength increased [12]. Schlautman and Morgan [22] had similar results with three other PAHs (anthracene, pyrene, and perylene). The sorption of these compounds to dissolved Suwannee River humic substances also decreased with increased salinity. Organotin compounds followed the same trend [117]. The effect of salinity on metal sorption to HAs appears somewhat less well resolved. Most metals tested by Lores and Pennock [25] also showed decreased sorption to Suwannee River HA with increased salinity. The behavior of Cu was more complex. Its binding to HA decreased with increased salinity up to 3 ppt, then increased to 60 % of the initial value at a salinity of 15 ppt [25]. The sorption of DDT to humic acids under different conditions has also been assessed by Carter and Suffet [28]. They reported a small increase in DDT sorption to Aldrich HA in 0.08 M NaCl, but it was not statistically significant.
General discussion:

Previous studies have assessed the binding of hydrophobic contaminants to HAs [15, 19, 23, 133, 134], and the resultant decrease in their bioavailability and toxicity [10, 13, 132, 180, 181, 182]. A few of these studies have reported that this binding depends on the source and characteristics of the HA (e.g. [50, 165]). The composition of natural DOC and HAs may vary with source and over time, resulting in changes in binding affinities [29, 235]. Terrestrial humics tend to have lower water solubilities, higher aromatic and lower carboxyl contents than fluvial humic substances [176]. Uhle et al. [9] showed that binding of PCBs varied significantly with DOC source. Even HAs from similar sources can have very different properties. For example, Bontoon Reservoir HA had much greater binding affinity for DDT than did HA from Pakim Pond, NJ [28]. It was initially surprising in the present study (Chapter 1) that none of four HAs tested (Aldrich, Leonardite, peat, Suwannee River) had a significant effect on the toxicity of Cu or CPF in the chronic Microtox® test. All the sorption, bioavailability and toxicity studies mentioned above were conducted in freshwater, whereas the chronic Microtox® test is conducted at 35 ppt (equivalent to seawater). Some previous Microtox® toxicity studies have reported HA effects on the toxicities of contaminants such as methyl parathion, guthion, and permethrin. These were conducted using the acute test protocol, at 20 ppt [1, 3, 120, 125]. Furthermore, in some instances these studies contradicted each other, or showed decreases in toxicity with increasing HA concentration. In the present work, the salinity of the chronic Microtox® test appeared to be a possible factor mitigating the effects of HA on contaminant toxicity. Salinity’s effect on bioavailability of a
contaminant in an estuarine setting might therefore override the type of HA present.

A given HA will have varying binding affinities for different compounds, and the extent of association may change with HA concentration and salinity [31, 52]. PAH sorption to HAs has been shown to increase with HA concentration, but decrease with increasing salinity [12, 22]. Similar results have been shown for metals [25] and pesticides [16], including DDT [28]. In the present SPME study (Chapter 2), results suggest that 40 to 50% more DDT than CPF was bound to Aldrich HA, in fresh water (Table 2). The proportion of pesticide bound also varied with HA concentration, increasing by 30% for CPF and 39% for DDT, as the nominal HA concentration increased from 10 mg/l to 100 mg/l. Increasing salinity appeared to decrease the percentage bound for both pesticides (Figures 11 and 12). At 100 mg/l HA, CPF significantly sorbed to HA at the lower salinities (0 and 1 ppt), but not at the higher salinities (5 to 20 ppt). DDT sorption to Aldrich HA at 10 mg/l HA was significant only at lower salinities (0 and 1 ppt), and was greatly reduced (from 88% at 0 ppt to 16% at 20 ppt) at higher HA concentration (i.e. 100 mg/l).

Changes in survival of the crustaceans *A. bahia* and *C. dubia* (Chapter 3), a biological assessor of exposure and bioavailability, paralleled changes in the freely-dissolved concentrations measured by SPME. Four significant decreases in SPME uptake and two significant increases in organism survival were found (Table 2). The two significant increases in survival were matched by decreases in SPME uptake (CPF and DDT at 0 ppt, and 100 mg/l HA). Another significant decrease in SPME recovery (DDT at 0 ppt and 10 mg/l) was matched by a non-significant increase in survival.
Table 2. SPME and biota results synopsis.
<table>
<thead>
<tr>
<th>Salinity (ppt)</th>
<th>Organism</th>
<th>Pesticide</th>
<th>HA Concentration mg/l</th>
<th>Change in % SPME Recovery (✓ = significant)</th>
<th>% Recovery</th>
<th>Change in % Survival from 0 mg/l (✓ = Significant)</th>
<th>% Survival</th>
<th>SPME Estimation of Toxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>C. dubia</td>
<td>CPF</td>
<td>10</td>
<td>-1.2</td>
<td>98.8</td>
<td>12.8</td>
<td>31.9</td>
<td>Good</td>
</tr>
<tr>
<td>0</td>
<td>C. dubia</td>
<td>CPF</td>
<td>100</td>
<td>-31.3 ✓</td>
<td>68.7</td>
<td>72.5 ✓</td>
<td>91.6</td>
<td>Very Good</td>
</tr>
<tr>
<td>0</td>
<td>C. dubia</td>
<td>DDT</td>
<td>10</td>
<td>-43.3 ✓</td>
<td>56.7</td>
<td>12.0</td>
<td>62.6</td>
<td>Fair</td>
</tr>
<tr>
<td>0</td>
<td>C. dubia</td>
<td>DDT</td>
<td>100</td>
<td>-82.2 ✓</td>
<td>17.8</td>
<td>47.2 ✓</td>
<td>97.8</td>
<td>Very Good</td>
</tr>
<tr>
<td>20</td>
<td>A. bahia</td>
<td>CPF</td>
<td>10</td>
<td>-0.1</td>
<td>99.9</td>
<td>-10.0</td>
<td>53.3</td>
<td>Good</td>
</tr>
<tr>
<td>20</td>
<td>A. bahia</td>
<td>CPF</td>
<td>100</td>
<td>-1.8</td>
<td>98.2</td>
<td>-13.3</td>
<td>50.0</td>
<td>Good</td>
</tr>
<tr>
<td>20</td>
<td>A. bahia</td>
<td>DDT</td>
<td>10</td>
<td>-11.6</td>
<td>88.4</td>
<td>-0.6</td>
<td>70.0</td>
<td>Good</td>
</tr>
<tr>
<td>20</td>
<td>A. bahia</td>
<td>DDT</td>
<td>100</td>
<td>-16.0 ✓</td>
<td>84.0</td>
<td>-13.9</td>
<td>56.7</td>
<td>Fair</td>
</tr>
</tbody>
</table>
The experiment that measured the mitigation of DDT toxicity at 0 ppt was most similar to that which estimated the DDT 24 hr \( LC_{50} \) concentration, with a mortality at 0 mg/l HA near 50%. This allowed the use of the probit model’s equation to compare the observed mortality to the predicted mortality computed with the freely dissolved concentration observed in the SPME experiment. At 10 and 100 mg/l HA, DDT recovery decreased to 56.7 and 17.8% of the \( LC_{50} \) (i.e. 590 and 190 ng/l). The model estimates and observed values for survival at 100 mg/l HA were in good agreement (100% and 97.8%, respectively); while those at 10 mg/l HA were 97.5% and 62.6%. In the latter case, the nd-SPME seemed to underestimate the bioavailable fraction. A greater fraction of DDT appeared available to \textit{C. dubia} than to the SPME fiber, as mortalities were greater than expected, even though the SPME extraction efficiency for DDT exceeded the nd-SPME guidelines.

In conclusion, Aldrich HA was shown to bind significantly to both CPF and DDT in fresh water, as measured by SPME. This is in general agreement with previous studies [134, 136, 152, 167]. The association was greater and statistically significant at lower HA concentrations (10 mg/l) for DDT. These physical measures of contaminant-HA binding were reflected in changes in apparent bioavailability, i.e. pesticide toxicity to the freshwater daphnid, \textit{Ceriodaphnia dubia}. Salinity significantly decreased the pesticides’ association to Aldrich HA as determined by SPME. At 20 ppt, CPF was not significantly bound to HA at any concentration (10 and 100 mg/l HA). DDT’s association decreased with increasing salinity. It was still significant, albeit small, at 20 ppt and 100 mg/l HA. These SPME results were also reflected in the toxicity tests with the salt water mysid,
*Americamysis bahia*, at 20 ppt: Aldrich HA did not significantly affect the pesticide toxicities at any HA concentration. The initial toxicity experiments using the chronic Microtox® test were apparently conducted at a salinity high enough (35 ppt) to mitigate any effect of HA on the toxicity of Cu and CPF to *Vibrio fischeri*.

In contrast to the large number of studies done examining the effect of HA characteristics on binding to hydrophobic contaminants and the impact of ionic composition on HA binding of metals and their resultant toxicity to aquatic organisms, virtually no previous research has examined the impact of salinity on the bioavailability (bioaccumulation or toxicity) of hydrophobic organic contaminants in the presence of HAs. The study presented here addressed these issues. Under the conditions of this research, salinity emerged as a more important factor than HA type or concentration. As a consequence, this factor may need to be considered when evaluating possible toxic effects of hydrophobic contaminants in saline waters.
Future Work:

While this research has led to a better understanding of the effects of salinity and HA on the bioavailability and toxicity of contaminants, several questions remain unanswered and merit further mention in the context of this study.

The chronic Microtox® study (Chapter 1) failed to reveal any effect of four different HAs on the toxicity of Cu and CPF. These results seem at odds with previous studies (e.g. [1]) conducted with the acute Microtox® test. This discrepancy might be explained by the different salinities of the tests (20 and 35 ppt), and should be investigated, though the salinity of the acute test may be high enough to mitigate the effects of HA on contaminant toxicity. A related issue involves the ‘type’ of salinity. Previous studies have shown that the ‘water hardness ions’ Ca²⁺ and Mg²⁺ have differing effects on the mitigation of trace metal binding to DOC and resultant bioavailability and toxicity. It may be possible, therefore, that altering the ionic composition of the salts added to control salinity might affect the association and toxicity results. Would using NaCl instead of the complex Hawaiian Marine Mix mixture affect the HA-binding and toxicity results? Are HAs of different composition more or less susceptible to different ionic composition in either fresh or salt water?

A better understanding of the state of DDT in water samples is also needed to clarify the present research. Did DDT crystalize and remain suspended in solution, as was postulated in Chapter 3, or did another process take place, that allowed for both high DDT recovery and a lack of correlation between DDT concentration and daphnid...
mortality?

A direct comparison of estimated (using SPME) and actual (using exposed organisms) bioconcentration, with resultant toxicity would also provide valuable information. The use of larger organisms (e.g. fish or clams) or radioisotopes would facilitate sample analysis.

Finally, the use of a single, euryhaline species to study the effect of HAs would shed additional light on the question of organismal differences as factors in the contaminants' differential toxicities at differing salinities.


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Vita

Laurent C. Mézin