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Fundamental Studies of Humic Acid's Influence on Pollutant Toxicity to Aquatic Organisms

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FUNDAMENTAL STUDIES OF HUMIC ACID'S INFLUENCE ON POLLUTANT TOXICITY TO AQUATIC ORGANISMS

A Dissertation

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LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
bLAHA	Bleached Leonardite humic acid
CMC	Critical micelle concentration
CNT	Carbon nanotube
CPC	Cetylpyridinium chloride
CP-MAS	Cross polarization magic angle spinning
DLS	Dynamic light scattering
DNA	Deoxyribonucleic acid
EEM	Excitation-emission matrix
FA	Fulvic acid
FPHA	Florida peat humic acid
FTICR	Fourier transform ion cyclotron
GDP	Guanosine diphosphate
GMP	Guanosine monophosphate
GTP	Guanosine triphosphate
HA	Humic acid
hLAHA	Hydrolyzed Leonardite humic acid
HPLC	High-performance liquid chromatography
HS	Humic substance
IHSS	International Humic Substance Society
LAHA	Leonardite humic acid

leLAHA	Lipid-extracted Leonardite humic acid
MWCNT	Multi-walled carbon nanotube
NMR	Nuclear magnetic spectroscopy
NOM	Natural organic matter
OC	Organic carbon
P_i	Inorganic phosphate
POPC	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
ppm	Parts-per-million (mg/L)
ppt	Parts-per-thousand (g/L)
RF	Radio frequency
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulfate
SRB	Sulforhodamine B
SRHA	Suwannee River humic acid
SWCNT	Single-walled carbon nanotube
TC	Total carbon
Tx-100	Triton X-100
UV	Ultraviolet

ABSTRACT

The main purpose of the research presented in this dissertation was to further understand the intricate and convoluted interactions between natural organic material, biological entities, and pollutants. This was achieved by utilizing humic acids (HAs) from differing sources, chemically modified humic acid, two biological entities (model biomembranes and *Artemia Franciscana*), and three types of pollutants (cations, surfactants, and carbon nanotubes).

Fluorescence spectroscopy and model biomembranes were used to measure the change in HA's ability to interact with the biomembranes in the presence of cations. Three differently sourced HAs, chemical modified HAs, and a range of cations were studied to elucidate specific interactions that can occur in the environment. It was determined that the cations limited the ability of humic acids to interact with the biomembranes, which was attributed to humic acid conformation changes in the presence of cations, and the protection capacity increased as the softness of the cation increased.

Artemia Franciscana (*Artemia*) was utilized as an analytic tool to determine the changes in toxicity of surfactants in the presence of humic acid. *Artemia* were exposed to three different surfactants, Triton X-100 (Tx-100), cetylpyridinium chloride (CPC), and sodium dodecyl sulfide (SDS), for both hatching studies and *in vivo* ^{31}P NMR. It was determined by hatching assays that Tx-100 caused mortality after hatching while CPC and SDS inhibited hatching. ^{31}P NMR corroborated these findings by showing an increase in phosphodiester bonds in saline water and in the Tx-100 exposure while there was no increase in the presence of the other two surfactants. HAs from three different sources were added to the surfactant exposures which showed that HAs played a mediation role in terms of toxicity and the extent of mediation was dependent on the type of HA and surfactant.

Artemia was also utilized to measure the toxicity of carbon nanotubes under a variety of conditions. Both single-walled and multi-walled carbon nanotubes that were either in the presence of humic acid or had been sonicated were studied. Overall, there was no significant carbon nanotube toxicity to the *Artemia*.

CHAPTER 1
INTRODUCTION

1.1 Over land and sea – our dependence on the environment

Both land and sea are becoming hot commodities in a world that has an increasing population size. The number of people occupying the globe in 2015 was $\approx$ 7.4 billion, which is more than double that of 1959 ($\approx$ 3 billion) (Nations 2015). The population is expected to continue increasing so that by 2050 it will be $\approx$ 9 billion. The result of this extensive growth in population is a significant decrease in land area and water per person. This decrease is coupled with increasing demands for food, water, materials, energy, and waste deposits. These stressors on land and aqueous environments are a detriment to the quality of soils, water, biological organisms and human life.

A healthy ecosystem successfully provides habitats for all species, food, fresh water, fuel, raw materials, recreational and educational opportunities, cycling of nutrients, and many more important processes (Manahan 1994). Soil and water are necessary as they both play essential roles in keeping the ecosystem healthy. Considering the necessity of soil and water and the increasing demand and inevitable contamination of them, it is imperative to understand their roles in regards to pollutant and biological interactions.

1.2 Aquatic environments

Approximately 71% of the Earth is covered in water and it is essential for living organisms for both biological survival and as habitats (USGS 2016). There are a wide variety of aquatic environments: swamps, rivers, lakes, and oceans. Saline water makes up 96% of the Earth's surface water and can be found in oceans, bays, seas, lakes and estuaries (Shiklomanov 1993, USGS 2016). Humanity relies on these aquatic environments for not only the water itself but also the aquatic organisms they contain. Water is vital to aquatic organisms and the surrounding

environments, including plant life. Healthy aquatic environments rarely contain only pure water as there are a variety of other inputs to the system such as, but not limited to, gases, dissolved natural organic matter, biological waste, minerals and nutrients (Ibanez et al. 2007). These healthy aquatic ecosystems can be easily damaged by contamination either directly or through land drainage.

1.3 Soils and natural organic material

Soils play essential roles in sustaining a healthy ecosystem. It is a medium for plant growth, a regulator of raw materials, it functions as a habitat for soil organisms, it cycle's nutrients, stores organic carbon, and regulates water supplies and purification (Manahan 1994, Krumins et al. 2013). Soils are composed of a complex system of clays, minerals, and organic carbon. In soils, natural organic matter (NOM) is a major source of organic carbon as it is composed of $\approx 50\%$ carbon and it is important in many environmental processes in both land and water (Stevenson 1994). NOMs are created by the degradation of plant, animal, and microbial matter and is thus omnipresent in both terrestrial and aquatic environments (Frimmel 1998). NOM and its interactions with other soil components lend to the quality of the soil as it creates good soil structure, provides pH buffering, and contributes to nutrient uptake and release (Stevenson 1994).

There are two classifications of NOM: non-humic substances and humic substances (HSs) (Stevenson 1994). Non-humic substances consist of things such as carbohydrates while HSs are polydisperse organic molecular assemblies that make up the majority of NOM.

Due to the origin of NOM, HSs' composition is based on its geochemical origin and is incredibly complex. Its intricate chemical composition and polydisperse nature allows for HSs to interact with a variety of components in the environment such as biomembranes and pollutants (Koopal et al. 2004, Lamelas and Slaveykova 2007, Ojwang' and Cook 2013, An et al. 2015).

Pollutant interactions with HSs can influence their bioavailability which, in turn, will influence the fate and bioaccumulation of contaminants. However, these interactions and changes in bioavailability will be dependent on the type of HS, the chemical functionalization of the HSs, and the type of pollutant.

By studying different types of pollutants and different HSs, a greater understanding of the overall health of the environment can be achieved; which is the overall purpose of the studies presented in this dissertation.

1.4 Aquatic organisms

As mentioned previously, NOM is known to interact with components in the environment, including organisms. Studies have shown that NOM can play both a toxic role as it can perturb biomembranes and a protection role as it can decrease toxicity of pollutants, depending on the environment (Lamelas and Slaveykova 2007, Ojwang' and Cook 2013, An, Jho et al. 2015, Deese et al. 2015).

The aquatic environment is home to a large variety of species with a wide range of metabolisms and biochemistries. These organisms include algae, bacteria, small crustaceans, fish, and many more. Biodiversity is a very important aspect of the ecosystem and is an indicator of overall environmental health (Manahan 1994). Pollutants that are introduced into the aquatic environment may affect the biodiversity of the environment because they can affect different organisms in different ways depending on the biochemistry of a specific organism.

Because of the complexity of NOM-pollutant interactions, model biological organisms have the ability to act as an indicator for any changes associated with the interactions such as reduced or increased bioavailability of the toxins.

1.5 Pollutants

With the increase in population, rapid industrialization, new technologies, and the widespread use of xenobiotic chemicals, it is inevitable that an increase in pollution will occur throughout the world. Even if chemicals are well characterized, complete interactions once in the environment will be questionable. The environment is complex and there are numerous components that the xenobiotic pollutants can interact with including soils, water, biological organisms, and other pollutants (Manahan 1994).

There are many concerns about the introduction of pollutants into both soil and aquatic environments as pollutants can be toxic and threaten the health of certain organisms, they can change the environment to become inhospitable to certain organisms, and they can bio-accumulate up the food chain and threaten the health of larger organisms and humans. The pollution of soil can lead to pollution of aquatic environments by leaching of pollutants into groundwater or through runoff (NRC 1997).

It is important to understand as many pollutants in as many environments as possible so we can understand the entire chemistry behind what is occurring in a contamination event. By studying the fundamentals of pollutant interactions in the environment, further studies can be done to either prevent contamination from occurring or to remediate the pollutant if necessary.

1.6 Overview of studies presented

The overall objective of the studies presented in this dissertation is to further understand the complex interactions between humic acids, pollutants, and biological organisms in an aquatic environment.

When investigating environments such as real-life aqueous systems, there is always the problem of complexity due to interactions of the many different components available. Using a

systematic approach and creating a model environmental system with four "simple" components one can start to address the issue of complexity while still maintaining environmental relevancy. The four components are as follows: 1) water, 2) natural organic material, 3) biological entity, and 4) pollutants. This method allows for the variation of the individual components in a systematic manner to better understand specific interactions occurring within the environment.

Since these studies model an aquatic environment, water is the necessary first component. The second component, natural organic material (NOM) is omnipresent in environmental systems and thus it is important to consider the interaction between pollutants and NOM. The NOM chosen for these studies was humic acid (HA). Humic acid is a type of HS that dissolves in water at a pH 2 or above.

The third component is the biological component, which includes model biomembrane systems and an aquatic crustacean *Artemia Franciscana*. These biological systems are considered to be the reporting system of the pollutant and HA interactions under different conditions. These interactions were measured using model biomembranes and fluorescence spectroscopy, *Artemia* hatching and viability assays, and by changes in *Artemia* embryo's phosphometabolite profile as measured by ^{31}P NMR.

The final component is the pollutants. The pollutants studied and presented in this dissertation are a variety of cations, surfactants, and carbon nanotubes.

Because of these four separate components, the experimental approach can be made increasingly complicated depending on which of the components are removed, added or varied.

1.6.1 Biomembrane study with humic acids and cations

Previous studies have shown that HAs perturb biomembranes under certain water conditions and those perturbations can be changed based on pH and temperature (Samson and

Visser 1989, Vigneault et al. 2000, Elayan et al. 2008, Ojwang' and Cook 2013). These studies were performed in "clean" conditions so there is little information about how those HA-biomembrane interactions changes based on any other environmental constituents such as pollutants. The study in chapter 3 was performed to study the changes in the perturbation of biomembranes by HAs under environmental conditions containing metal cations and elucidate the driving forces or interactions behind any changes. The concentrations of metal cations found in aqueous environments are increasing due to water acidification by both natural and human sources so it is necessary to understand how they can affect HA interactions since cations can interact with humic substances by both electrostatic and chelating mechanisms(Schindler et al. 1980, Tipping 2002, Tipping et al. 2002).

It is important to understand how humic acids interact with cellular membranes in the presence of cations. By using this phenomena and fluorescence spectroscopy the passive interaction of humic acid with 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) large unilamellar model biomembranes in the presence of cations can be studied. The chosen cations had a range of charges and affinities for different humic acid components. The cations studied were: K^+, Na^+, Mg^{+2}, Ca^{+2}, Mn^{+2}, Co^{+2}, Cd^{+2}, Fe^{+3}, and Al^{+3}.

Humic acids can be chemically modified in order to determine which functional groups were responsible for the permeation of biomembranes and the binding of the cations (Wise et al. 1946, Almendros 1994, Chilom and Rice 2009). The chemical modifications performed were: (i) bleaching to remove aromatics, (ii) Soxhlet extraction to remove lipids, and (iii) acid hydrolysis to reduce O – and N- alkyl groups.

1.6.2 Surfactants and *Artemia Franciscana*

Surfactants are amphiphilic compounds that are heavily used in both industry and households, inevitably ending up in the environment, and many are known to be toxic to aquatic species (Stalmans et al. 1991, Zoller 2004, Ostroumov 2006). Studies presented in Chapter 4 and 5 were performed not only to better understand the toxicity of different surfactants to *Artemia francsicana* (*Artemia*) but also to better understand how HAs can influence that toxicity.

In this work, *Artemia* was utilized as the biological component for measuring toxicity of three different surfactants, cetylpyridinium chloride (CPC, cationic), Triton X-100 (Tx-100, non-ionic) and sodium dodecyl sulfate (SDS, anionic) in the presence of HAs from three different sources. To further understand which HA moieties interact with the surfactants, chemical modification was done to remove individual components, as listed above.

Artemia embryos were also used with *in vivo* ^{31}P NMR and a peristaltic pump system in order to determine whether the surfactants change the *Artemia's* phosphometabolite profile. By utilizing *in vivo* ^{31}P NMR, near "real time stress" on the embryos and embryo development was measured. The *Artemia* were exposed to the three surfactants. These results were verified with HPLC on *Artemia* embryo phosphometabolite extractions. Humic acid was added to the toxic surfactant solutions and the phosphometabolite embryonic profile of *Artemia* was measured and compared to the profile under toxic conditions to further elucidate any interactions occurring between the surfactants and the HA.

1.6.3 Carbon nanotubes and *Artemia*

Carbon nanotubes are a fairly new technology that is becoming widely produced and used in areas such as medical science, electronics, composites, and even clothing. They are hollow graphite cylinders with high thermal conductivity, high mechanical strength, and low mass density

(Thomsen et al. 2007, Kessler 2011). Since they are a relatively new technology, there are limited and opposing studies on their impact on aquatic environments (Wang et al. 2009, Mwangi et al. 2012, Jackson et al. 2013, Allegri et al. 2016, Kalid et al. 2016). The study presented in chapter 6 is an attempt to understand the toxicity of carbon nanotubes to *Artemia* and how HAs might influence their behavior in the environment.

Hatching assays were performed with *Artemia* under varying CNT conditions including CNTs of different concentrations, diameter and type. HA was added in varying concentrations to determine any affect they might have on the interactions of CNTs with the *Artemia*. Finally, sonication was performed to the CNTs to see if any physically changes could change the chemical properties or toxicity of the CNTs.

To conclude, the work presented in this dissertation is a systematic initial attempt at studying and explaining a multitude of HA-pollutant-biological interactions that are occurring within aquatic environments.

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CHAPTER 2
REVIEW OF RELATED LITERATURE

2.1 Humic substances

Humic substances are molecular assemblies of low molecular weight compounds that are created by the decomposition of natural matter and are highly resistant to further decomposition (Stevenson 1994). Humic substances can be found in soils and waters in both particulate and dissolved forms. They are split into three fractions based on their ability to dissolve in water under certain pH conditions: humin, fulvic acid, and humic acid. Humin is completely insoluble in water, fulvic acid is water soluble at all pHs, and humic acid is water soluble when pH is greater than 2.

The complexity of humic substances should not be underestimated. A detailed study by Hertkorn et al. on a single humic substance (Suwannee river fulvic acid) utilizing Fourier transform ion cyclotron (FTICR) mass spectrometry revealed that the C,H,O-compositional space has 100% coverage of all theoretical space (Hertkorn et al. 2008). Stated differently, all theoretically possible C-H-O combinations were measured by different modes of ionization and there is evidence to suggest that the observed spectra only represent a simplified picture of the complex molecular assemblies. This complexity leads to a polydisperse environmental system that plays a role in distinct interactions with a variety of other environmental components, which lends to the necessity that HSs and their interactions are studied in detail when considering an environmentally relevant system.

2.1.1 Sources, isolation and characterization of humic acids

As a fraction of HSs, humic acids are considered a polydisperse, heterogeneous, and complex mixture of organic molecules that are combined by interactions between the functional groups to create supramolecular structures. It is estimated that the weakly associated molecular

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assemblies range between 200 and 6100 Da (Sutton and Sposito 2005). The hypothetical structure of HAs, seen in Figure 2.1, illustrates just some of the complexity and the variety of functional groups that HAs can have; however, it is now the consensus that HAs are molecular assemblies rather than a single macromolecule.

Aquatic humic acids can be found both in the solid phase (sediment) and dissolved in the liquid phase (water). Degraded microbial matter, plant matter, and animal matter in the aqueous phase can degrade until they become recalcitrant and form humic material (humification). The solubility of HA also allows it to be transported into aquatic environments from terrestrial sources. It is estimated that approximately 2×10^8 tons per year of organic carbon is transported to the ocean (Frimmel 1998).

The concentrations of natural organic material in aqueous environments typical range is 0.5 – 100mg organic carbon/L (OC/L) (Frimmel 1998). The concentration for humic acid is reported by mg OC/L or parts-per-million (ppm) rather than molarity because the complexity of HA does not allow for an exact molecular weight to be known.

The isolation of humic substance fractions is performed on source materials of terrestrial or aquatic origin. Terrestrial sources of humic acid can include soil, peat and lignite while aquatic sources include lakes, rivers, swamps, and marshes. Humic substance fractions are isolated from terrestrial sources via an alkaline extraction with aqueous NaOH.

The extract contains humic and fulvic acid along with dissolved contaminates such as cations. Humic acids are precipitated by lowering the pH of the extract to below 2 and then removing any salts by cation exchange, dialysis or other purification techniques. This results in the three separate fractions that further purified and finally freeze-dried for storage and characterization (IHSS).
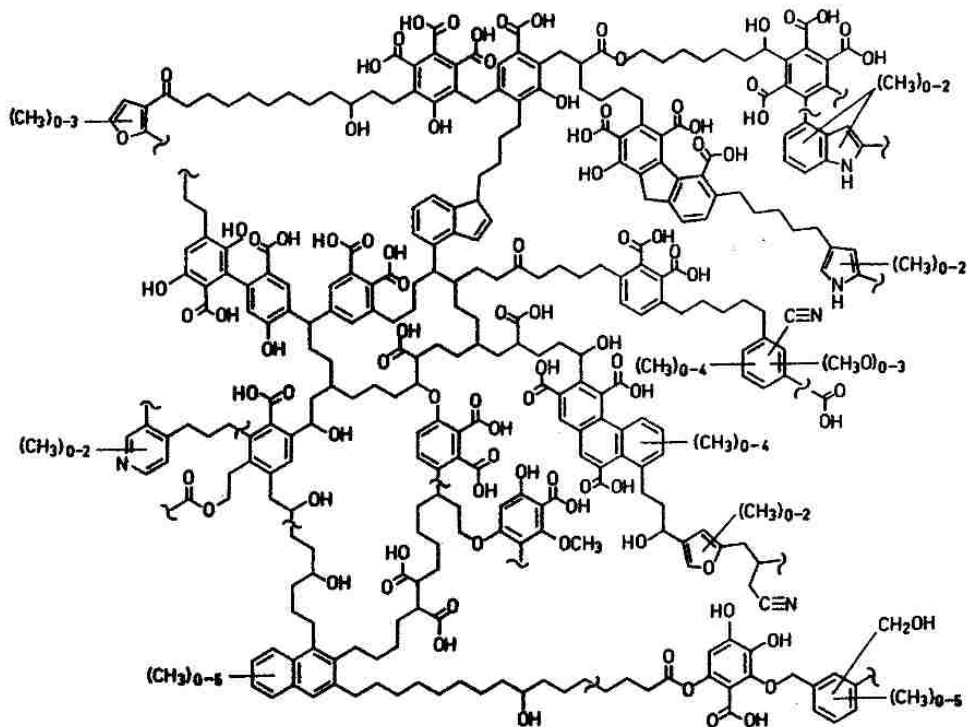
Figure 2.1 Hypothetical structure of humic acid (Schulten and Schnitzer 1993)

There are two methods to isolate humic and fulvic acids from aquatic sources: using an XAD-8 resin or reverse osmosis. The dissolved organic matter in the aqueous phase is fractionated via the XAD-8 resin into hydrophobic and hydrophilic fractions, with the hydrophobic fraction being further fractionated into humic and fulvic acid by pH adjustment as described above. Reverse osmosis has also been used to separate and concentrate dissolved natural organic matter from water. For example, in 2013, a team from the International Humic Substance Society (IHSS) coupled the reverse osmosis method to an electrodialysis method to separate the dissolved NOM and subsequently remove the problematic salts from the Mississippi River in Minneapolis, MN (IHSS 2013).

Because of the complexity of HAs, a variety of characterization techniques are used. One important characteristics of HAs is the elemental composition and, by statistical analysis, certain attributes can be elucidated for different HS fractions (Rice and MacCarthy 1991). Total carbon

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is determined by measuring both the total carbon (TC) and inorganic carbon (IC) and then determining the amount of total organic carbon (TOC) by calculating the difference between TC and IC. Other elements are quantified by elemental analyses such as inductively coupled plasma atomic emission spectroscopy (ICP-AES), ICP-mass spectrometry, or flame atomic absorption spectroscopy (AAS). By comparing the elemental percentages of C, H, N, O, S, and P of ash-free HAs and FAs, Rice and McCarthy determined that there are statistical differences between the composition of different HS fractions and that the elemental composition was dependent on the source of the HS.

Other important characterization techniques for HSs are fluorescence and UV spectroscopy measurements. These types of measurements give information regarding the functional groups of the HAs. UV analysis has been used to estimate aromatic carbon content by measuring absorbance at 254 or 280 nm, which was further validated by other techniques (Chin et al. 1994, Kalbitz et al. 1999, Weishaar et al. 2003). Fluorescence spectroscopy, being that it is more sensitive than UV spectroscopy, is a widely used technique to provide detailed information on the fluorophores of HAs (Chen et al. 2003, Coble 1996, Cook et al. 2009, Stedmon et al. 2003). Fluorescence studies, especially excitation-emission studies, of dissolved organic matter have shown that they contain two major fluorophores that are attributed to moieties that are both protein-like (emissions characteristic to that of tyrosine and tryptophan) and humic-like molecules. Emission-excitation matrices (EEMs) have the ability to characterize HAs as either aquatic or terrestrial sourced based on the wavelength of excitation and emission of their fluorophores(Cook et al. 2009). These differences are attributed to higher heterotrophic activity during degradation of organic matter in aquatic environments versus terrestrial (Coble 1996).

An important characterization technique for HAs is nuclear magnetic resonance spectroscopy (NMR). Specifically, solid state ramp cross polarization magic angle spinning (CP-MAS) ^{13}C NMR. Unlike the other techniques mentioned previously, CP-MAS ^{13}C NMR requires solid and pulverized HA samples, so aquatic samples must be freeze dried before characterization. However, solid state ^{13}C NMR obtains qualitative information on the different carbons present in certain moieties such as aliphatics, carbohydrates (O-alkyl, N-alkyl), aromatic and carboxyl. It also quantifies the percent composition of those functional groups (Cook 2004, Mao et al. 2000).

2.1.2 Composition of humic acids

Since humic acids are created by the degradation of organic matter (e.g. plant materials), they are comprised mostly of carbon (45-64%), oxygen (31-46%), hydrogen (3.2-5.7%), nitrogen (0.6-3.8%) and small amounts of sulfur (0.3-1.3%) and phosphorous (<0.01-0.6%) (Thorn et al. 1989). The compositions of humic acids are dependent on their biogeochemical origin. For example, HAs extracted from a terrestrial source will contain a higher concentration of aromatic moieties (many sourced from lignin) while those from an aquatic source will have higher aliphatic concentrations (Gauthier et al. 1987, Stevenson 1994).

The main functional components in any HA are aliphatics, aromatics, and carbohydrates. It also has hydrophobic and hydrophilic domains. These different characteristics enable HA to have interactions with many different components in the environment.

2.1.3 Chemical modification of humic acids

Chemical modification (or chemical editing) can be utilized to change the composition of HA by removing or reducing certain components by chemical processes. The main techniques can reduce the lipid, carbohydrate or aromatics components of the HA. By reducing one of the

components of the HA, it can be determined what role that component may be playing in the interactions between the HA and its environment.

The lipids found in HA are mainly derived from plant matter. They can come from plant cuticlar materials that make up the waxy protective coating on leaves. The lipid extraction method is a Soxhlet extraction with a benzene:methanol azeotrope. After extraction, the solvent is simply removed by evaporation (Chilom et al. 2009).

The carbohydrate components are primarily found in cellular walls, lignins and stored starches. The method for removing the carbohydrates from the HA is acid hydrolysis by refluxing with 6M HCl (Almendros 1994). The acid hydrolysis breaks the carbon-oxygen bonds of the carbohydrates as well as the carbon-nitrogen bonds of the peptides. The chemical editing process also severs peptide linkages that originate from plant and microbial proteins.

Aromatics make up a large portion of the humic material because of the photosynthetic protein complexes of plants that are mainly aromatic as well as lignin-based materials. The aromatic components are removed by a bleaching procedure in which the HA is bleached in a solution of $NaClO_2$ and acetic acid for three days. The original procedure, by Wise et al, was originally used to isolate wood cellulose; however it was modified by increasing the bleaching time (Gunasekara et al. 2003, Wise et al. 1946).

2.1.4 Types of possible humic acid interactions

Because of the diversity and complexity of HA, it can engage in many different types of interactions with a variety of substances. Interactions occur because HA contains hydrophilic and hydrophobic moieties, strong metal binding sites, a variety of functional groups, and have charged functional groups (Tipping 2002).

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An important intermolecular force that plays a role in interactions between HAs and other environmental components is hydrogen bonding (H-bonding). H-bonding is typically defined as a special type of dipole-dipole interaction that takes place between a hydrogen attached to a highly electronegative atom or "proton donor" (e.g. F, N, O, S, and C) and a proton acceptor (e.g. N, P, O, S, and Se or multiple π-bonds) (Gilli and Gilli 2000). Hydrogen bonding can be classified intro five types: ordinary, double charge assisted, negative or positive charge assisted, resonance assisted, and polarization assisted (Gilli et al. 2009). Ordinary H-bonding is an electrostatic bond and is, thus, relatively weak. It has been postulated that the other types of H-bonds, listed above, have covalent characteristics in addition to the electrostatic interactions (Gilli and Gilli 2000, Gilli et al. 2000). Since HAs contain functional components such as carbohydrates, amino acids and lignin, it also has the ability to H-bond. For xenobiotic pollutants, H-bonding likely involves N, O, and S donors and acceptors (Pignatello 2011). It has also been postulated that H-bonding plays a major role in the interactions between HAs and cellular biomembranes (Ojwang' and Cook 2013).

H-bonding is also an important phenomenon to discuss when considering an aquatic environment because hydrophobic and hydrophilic interactions are dependent on it. Water has unique properties that are attributed to H-bonding. Each water molecule consists of two hydrogen atoms and one oxygen atom with two lone pairs of electrons. This specific molecular structure allows for significant H-bonding between water molecules (Dunnivant and Anders 2006). In an aqueous medium, the H-bonding between water molecules will be thermodynamically favored over other interactions. Hydrophobic interactions are formed when a hydrophobe is introduced into an aqueous medium and the water molecules re-arrange and force hydrophobic entities together so that the energy of the system is still as low as possible. These hydrophobic/hydrophilic interactions are considered to be slightly strongly than Van der Waals forces (Atkins and de Paula

2011). The molecular assemblies of HAs, biomembrane-HA interactions and HA-pollutant interactions can all be affected by the phenomenon of hydrophobic/hydrophilic interactions (Ghabbour and Davies 1999, Ojwang' and Cook 2013, Tan et al. 2009).

Another important type of interaction between HAs and other environmental components is Coulombic, or "electrostatic", interactions. Adsorption of certain compounds, such as metal cations, has been attributed to electrostatic interactions (Vermeer et al. 1998). HAs tend to be anionic at environmentally relevant pH due to moieties such as carboxylic acid groups. The electrostatic interactions of HAs can both attract and repel pollutants or biomembranes depending on the overall charge and the pH of the environment (Ojwang' and Cook 2013, Tipping 2002, Tipping et al. 2002).

Many HAs contain aromatic ring structures that can be polar, nonpolar, or have characteristics of both depending on their substituents (Brown 1975). Because aromatic moieties are present in HA, there is the ability of HAs to undergo π-π interactions between other aromatic-containing environmental constituents. Pi-stacking (or π-π stacking) are noncovalent interactions between aromatic π-bonds. These π-bonds have been modelled and found to take three different conformations: the sandwich, T-shaped, and parallel displaced (see Figure 2.2). These interactions are due to the alignment of the positive electrostatic potential on one ring with the negative electrostatic potential on the other ring. Through computations, it has been determined that the most stable conformations are likely the parallel displaced and the T-shaped (Sinnorkrot and Sherrill 2004).

Pi-stacking or π-π interactions are known to play major roles in the tertiary structure of proteins and other macromolecular structures, the stabilization of the double helical structure of

DNA and complexation in systems containing two compounds with π-containing moieties (Hunter and Sanders 1990).
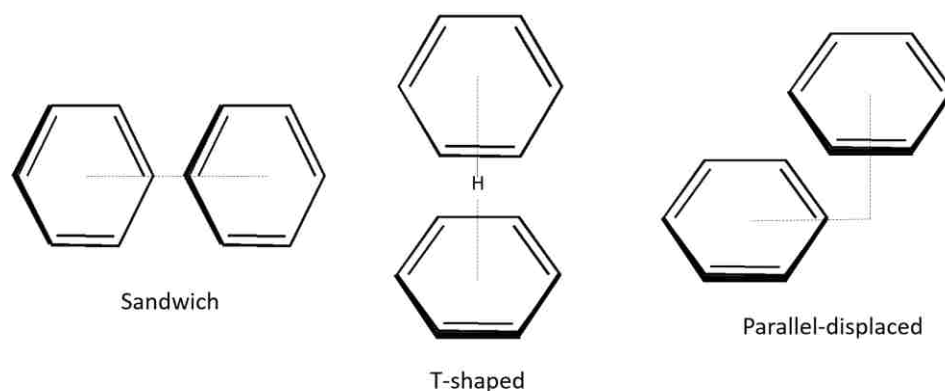
Figure 2.2 Schematic of π-π stacking

It stands to reason that HAs, being molecular assemblies of molecules containing as much as 20-60% total carbon as aromatic moieties (Mao et al. 2000, Schnitzer 1991, Simpson et al. 2001), would also take advantage of the π- π interactions for conformation as well as interactions with other environmental constituents.

2.2 Biological component

2.2.1 Model biomembranes

In every organism, the cellular membrane plays an essential role in biological processes so it is important to understand how other environmental constituents can affect the integrity of the membranes as it can also affect the health of the organism. Cell membranes consist of phospholipids, carbohydrates and proteins (Koster and van Leeuwen 2004). The role of the membrane is to separate the cell's interior from its external environment as well as control transport of selected compounds in and out of the cell. There are two processes by which species can enter or exit the cell: passive and active transport. Passive transport occurs through the phospholipid bilayer while the proteins and carbohydrates in the membrane regulate active

transport. The complexity of real cells can have drawbacks when trying to mechanistically study the passive membrane perturbation by environmental pollutants. Lipid vesicles, or liposomes, have been used extensively in xenobiotic toxicological assessment studies as model biological membranes (Zepik et al. 2008). Membrane perturbation can be studied with a model cell membrane give insight into the fundamental processes of passive transport and reduce the complexity added by a real cell system.

These model biomembrane systems can be created by amphiphilic phospholipids that mimic the natural bi-layered spherical-shaped structures of real cell membranes. The phospholipid bilayer is two layers of the phospholipid arranged such that the hydrophilic phosphate head groups are aligned with the center of the vesicle (containing an aqueous buffer solutions) or towards the external aqueous matrix. The hydrophobic lipid chains are arranged stacked in the center of the bilayer and vesicle formation is driven by hydrophobic and hydrophilic interactions.

The lipid chains of the phospholipid are "packed" within the bilayer and the tightness of that packing is based on the saturation of the lipid as well as the temperature of the environment. Phospholipids have two crystalline phases: liquid and gel (solid). The phase change between the two is at a specific transition temperature (T_m) that is dependent on the degree of lipid saturation. The more saturated the lipid chains, the higher the phase transition temperature. The packing of the lipids is also prone to packing defects. The number of packing defects will increase the closer the temperature is to the transition temperature. Permeability of the membrane will also increase as the temperature nears the transition temperature because of packing defects.

The permeation of the membranes can be investigated by fluorescence spectroscopy by encapsulating a fluorescent dye within the liposome and measuring the changes in fluorescence intensity as the membrane is exposed to different compounds (Vigneault et al. 2000). As

mentioned briefly above, HAs have been shown to interact with biomembranes and have the ability to permeate them. The mechanism of HA-biomembrane interactions was determined through a number of different studies, all leading to the conclusion that the permeation of biomembranes by HA is a two-step process. First, studies by Maurice et al. discovered that fulvic acid (FA) had a preferential adsorption to biomembranes at an acidic pH (Maurice et al. 2004). This work was corroborated by Campbell et al – who also discovered that once the HS was adsorbed to biomembranes, the HSs increased permeability with HAs having the greatest effect out of all the HS fractions (Campbell et al. 1997, Vigneault et al. 2000). Further studies by the Cook research group determined that HAs perturbed biomembranes at acidic pHs, increased temperature increased permeation, and that there was a slow-step and fast-step to the permeation process (determined by kinetics) (Elayan et al. 2008, Ojwang' and Cook 2013). These studies led to the mechanism by which HAs interact with biomembranes which can be seen in Figure 2.3.
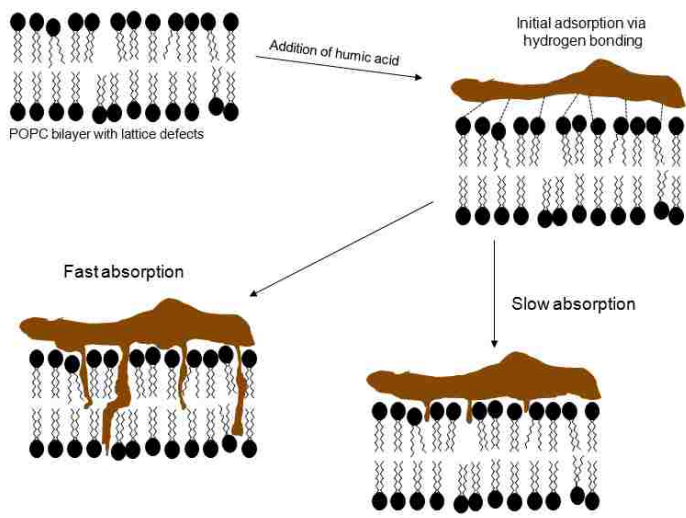
Figure 2.3 Proposed adsorption/absorption HA-biomembrane interactions at acidic pH

The first step is an H-bonding adsorption step between the HAs and the phospholipids; which was determined by the fact that an acidic pH was necessary for the initial adsorption. At an

acidic pH, the HAs carboxylic acid groups are protonated, allowing for H-bonding or bridging. The second step of the interaction is an absorption step, which is the permeation of HA into the biomembrane bilayer. This occurs due to hydrophobic interactions in which the hydrophobic moieties of the HA penetrate to the hydrophobic inner-layer of the lipid biomembranes (see Figure 2.3). Kinetics determined that the second step had both fast and slow aspects to the absorption mechanism. This was determined to be caused by different types of hydrophobic moieties within the HA.

2.2.2 *Artemia Franciscana*

Aquatic toxicology is a large field of study in which the toxicity of a compound is tested with aquatic organisms from a variety of aquatic environments such as fresh water, saline water, and sediment environments. Usually these studies are performed to determine specific toxicity levels of a compound for a particular organism - usually ones that are ecologically relevant - for risk assessments and greater understanding of toxicity mechanisms. However, in the studies presented here, the aquatic organism, *Artemia franciscana* (*Artemia*), and the aquatic toxicology methods were used as indicators to measure interactions between HAs and pollutants known to be toxic to the *Artemia*.

Artemia (commonly known as brine shrimp) are aquatic bisexual micro-crustaceans that have been used extensively in toxicity studies. *Artemia* have been widely used in laboratories because they are commercially available, the cysts (eggs) can stay dormant for long periods of time, they are easy to hatch, they have a short life span and their embryogenesis is well known. In addition to the simplicity of the procedures, lower volumes of toxicants and solutions are needed because of their small size. An unexhausted list of contaminants studied with *Artemia* includes metals, trace elements, toxic cyanobacteria, pharmaceuticals, organic solvents, oil dispersants, and

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silver nanoparticles (Arulvasu et al. 2014, Barahona and Sánchez-Fortún 1999, Cotou et al. 2001, Kokkali et al. 2011, MacRae and Pandey 1991, Matthews 1995, Rodd et al. 2014). An interesting study by Parra el al. determined that there were significant correlations between *Artemia* and mice toxicity of autochthonous plant extracts (Parra et al. 2001). By being able to correlate *Artemia* toxicity with mammals, it reduces the need for expensive and time-consuming bioassays with mammals. *Artemia* have even been sent to space to study their development in a microgravity environment (there were no measurable alterations in development during spaceflight) (Spooner et al. 1994).

Artemia belong to the class *Branchiopoda* that also includes another common test organism, *Daphnia*. They are found in hyper-saline environments such as coastal lagoons, man-made saltpans and permanent salt lakes. There are two main bioassays using Artemia: hatchability assays and mortality of nauplii. The viability of cysts after exposure and the mortality of the nauplii can assess differences in sensitivity of the Artemia for a certain pollutant at different stages of their life cycle. Hatchability assays measure the ability of *Artemia* to hatch from their cysts under certain pollutant or environmental conditions as a decrease in hatching can indicate a toxic response. Mortality assays measure the viability of the *Artemia* after they have hatched.

The stages of growth are well defined for *Artemia* and is illustrated in the Figure 2.4. The "zero" time point is the point in which the *Artemia* are introduced into their saline hatching medium. The first stage (A in Figure 2.4) is when the *Artemia* embryo is encapsulated in a hard shell or "cyst". After approximately 18 hours within the hatching medium, the cyst begins to break open and the embryo begins to emerge. The embryo will completely remove its self from the cysts but still be encased in an inner, flexible, membrane – this is known as the "umbrella stage" based on the shape of the *Artemia* embryos at this time. At approximately 24 hours, they will completely

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hatch into the juvenile stage (instar I) and then, between 36 and 48 hours, they will molt into their first adult stage (instar II).

The embryonic and juvenile *Artemia* get their energy (food) from their yolk but after they molt into the adult stage (instar II) they begin to feed on particulate matter such as algae. Under good conditions, the *Artemia* will continue to molt several more times to a breeding stage where the process begins again with a new generation (Neumeyer et al. 2015, Stappen).
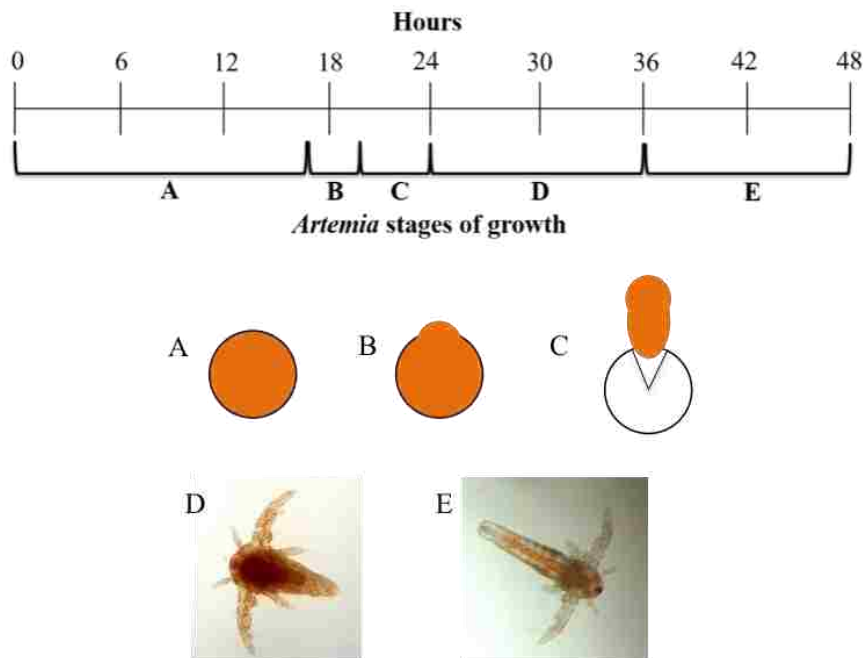
Figure 2.4 Artemia stages of growth (Neumeyer et al. 2015): A: Encapsulated cyst; B: emergence; C: "umbrella stage"; D: juvenile (instar I); E: adult (instar II)

As mentioned previously, not only is the stages of growth of *Artemia* well characterized, but so is their embryogenesis; which is the formation and development of the embryos. A multitude of studies have been performed on *Artemia* embryos. These embryogenesis studies include, but are not limited to, enzymatic processes during development (many different enzymes), gene expression, ribosomal RNA structure and expression, histone roles and structures, trehalose and its role in resumption of metabolic processes, intracellular pH changes, protease inhibitors, and respiratory and osmotic pressure changes (Busa et al. 1982, Chen et al. 2009, Clegg 1997, Covi et

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al. 2005, Kwast et al. 1995, Spooner et al. 1994, Stappen , Wang et al. 2007, Warner and Clegg 2001, Warner et al. 1989, Warner et al. 1995, Zhu et al. 2009).

Of particular interest for the studies in this dissertation is the ATP cycle (Figure 2.5), guanosine triphosphate usage and the gene expression of *Artemia* embryos during development.
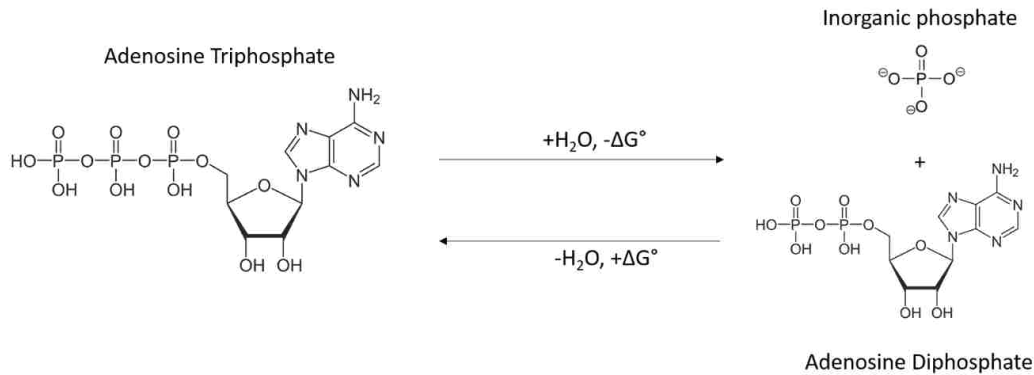
Figure 2.5 Adenosine triphosphate (ATP) cycle

The ATP/ADP cycle is an important biochemical phenomenon that provides short-term energy storage and use for cells. Adenosine triphosphate (ATP) consists of an adenosine (a purine nucleoside) and three phosphates. ATP is converted to adenosine diphosphate (ADP) by removal of a phosphate group, during which energy is released to the cells for use in processes such as development, synthesis, and transport. The major bi-product of this conversion is inorganic phosphate (P_i). During times of stress, cells require energy in order to combat the stress on the organism. This results in a depletion of ATP in the cells as the demand of energy increases, while also increasing the concentrations of both ADP and P_i. Since ATP/ADP concentrations can change during stress, monitoring them can indicate how a cell is handling certain stressors. Because they contain phosphorous, ATP, ADP, and P_i can all be monitored by ^{31}P NMR (discussed later). P_i is also an indicator of intracellular pH within cells as the ^{31}P NMR chemical shift of the P_i peak is dependent on pH (Moon and Richards 1973).

Guanosine triphosphate (GTP) is known to also play a role in providing energy to an organism as well as acting as a substrate in the synthesis of RNA and DNA. It has been established that [GTP] increases over time during the embryogenesis of *Artemia*; which indicates a growth in the system (Warner and Finamore 1967). *Artemia* embryos have a high concentration of guanosine diphosphate before any development occurs and this concentration decreases over time. Warner and Finamore postulated the following mechanism (GMP = guanosine monophosphate):

GMP + GDP $\longleftrightarrow$ [guanosine – 5' – P-P-P-5'-guanosine] $\longleftrightarrow$ guanosine + GTP   (1)
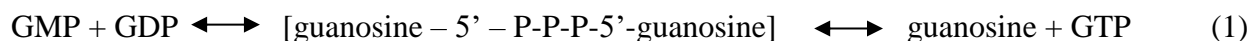
The opposite was also found to be true: when there is stress on the system, [GTP] decreases. Warner and Clegg performed a study on *Artemia* embryos during diapause that measured the concentration changes of some nucleotides, metabolites, and proteins over long-term stress (years) (Warner and Clegg 2001). Their results indicated that [ATP] stayed fairly steady during the first stage of stress while guanosine triphosphate [GTP] declined slowly throughout the process. Although the total processes are still unclear, they came to the conclusion that GTP plays a role in supplying energy as the embryos undergo stress which led them to the theoretical mechanism shown in Figure 2.6.

2.3 Pollutants

2.3.1 Cations

Metal cations are abundant in aquatic environments due to both natural and unnatural sources. Common cations such as Na^+, K^+, and Ca^{2+} are found in large quantity in aquatic environments. Sodium and calcium can be found in areas where fresh water and salt water meet or in areas where there is run-off from mineral deposits or industrial sites. Other cations like less abundant metals: Al^{3+}, Fe^{3+}, Mn^{2+}, Mg^{2+}, Co^{2+} and Cd^{2+} can also be found in aquatic environments. These cations are becoming more abundant because of fresh water acidification. As water becomes

more acidic, these cations can be released from bedrock and sediment. This acidification is especially concern with Al^{3+} because of its high toxicity, as the Al^{3+} species is more readily available in acidic waters.

It is well established that cations and HAs undergo binding interactions and Tipping characterized the binding sites of HAs for cations in three categories: 1) non-specific binding, 2) abundant weak sites, and 3) less-abundant strong sites (Tipping 2002). The non-specific binding sites are defined as sites that likely engage in electrostatic interactions but are not specific to a certain type of cation. Abundant weak sites are those that can engage in binding or chelation with a large number of cations – such as carboxyl acid functional groups. Finally, the less-abundant strong binding sites are those sites that contain atoms such as N or S that will only interact with some cations. A variety of both sorption studies and toxicity studies have shown that cations interact with HAs (Van Dijk 1971). The toxicity studies have shown that some toxic cations, in the presence of HA, become less bioavailable to the organisms in question (Kalis et al. 2006, Lamelas and Slaveykova 2007). The study by Kalis showed that although some the bioavailability of cations could be reduced by HA, in the presence of multiple cations, other cations were more bioavailable. They determined this to be due to competition of the cations to the binding sites of the HAs and those with higher affinities to HA (Cu, Pb, and Fe) bound to the organic matter while cations with lower affinities (e.g. Cd, Zn, Mn) were more available for organism uptake. This further illustrates that HA has different binding sites and affinities for different cations.

Unlike the cation toxicity studies, the study presented in Chapter 3 of this dissertation reports the changes in the ability of HAs to permeate biomembranes (as mentioned above) when different cations are in the environment. This allows for the determination of not only how cations

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bind to HAs but also how binding can change the conformation of the HAs in a way that may affect other environmental interactions.

2.3.2 Surfactants

Many surfactants are commercially available and used widely in every-day life. They are found in household cleaning products, personal care products (soaps), paints, pesticide solutions, polymers, oil recovery, paper industries, and more. Because of this wide usage, they are commonly introduced into the environment. These compounds are amphiphilic and fall into four main categories: nonionic, zwitterionic, cationic and anionic. Nonionic surfactants have no net charge while zwitterionic have both cationic and anionic moieties. Cationic surfactants have a cationic head-group and anionic surfactants have an anionic head-group (Kosswig 2012).

Surfactants can make their way into the environment through a variety of pathways as illustrated in Figure 2.6. One is through household and industry because although wastewater treatment centers may remove some of the surfactants that enter the water system, it has been shown that detectable levels still persist (Rogers 1996, Stalmans et al. 1991, Waters and Feijtel 1995). In addition, some surfactants are deliberately introduced into the environment to remediate contamination by other pollutants or they simply run-off from industry sources. Surfactants are also added into pesticide and herbicide solutions to increase their solubility. All of these sources eventually lead the surfactants into the aquatic environment.

Most surfactants have a hydrophilic (polar) head group and hydrophobic (nonpolar) tail. Because of this amphiphilic nature, surfactants form micelles (spherical assemblies) at a defined concentration known as the critical micellar concentration (CMC). When the concentration of the surfactant is below the CMC, the surfactant exists as its monomers (Luckey 2008).
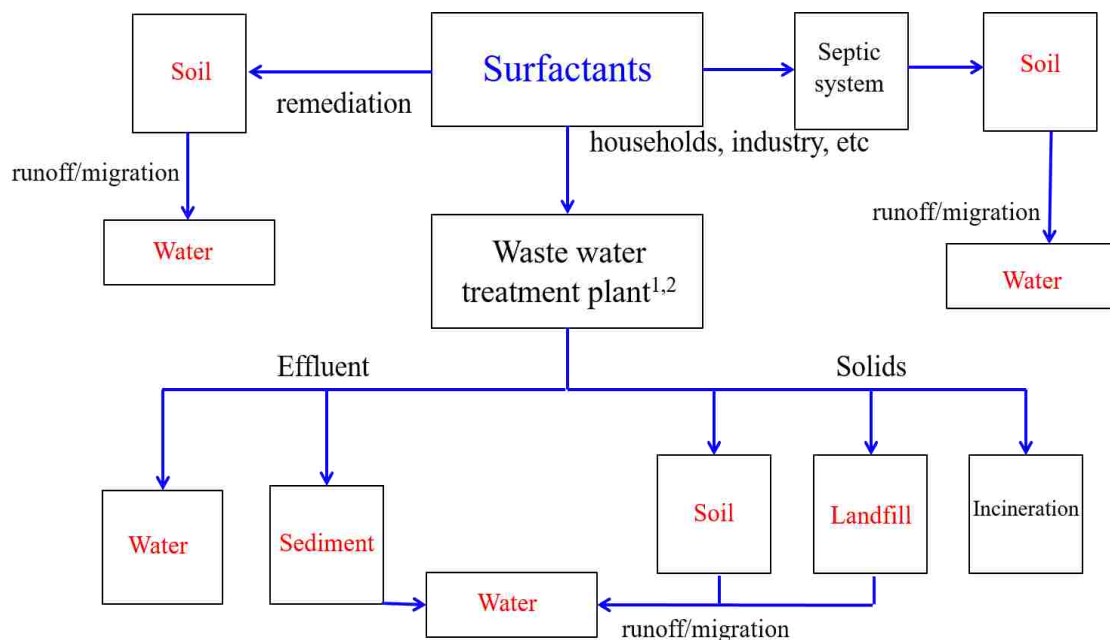
Figure 2.6 Environmental pathways of surfactants into the aquatic environment (Rogers 1996, Stalmans et al. 1991)

Triton-X 100 (TX-100; $C_{14}H_{22}O(C_2H_4O)_n$ where n = 9 or 10) is a common nonionic surfactant used extensively in laboratories and it contains a hydrophilic polyoxyethylene head-group (with approximately 9 – 10 repeats) and octylphenol, seen in Figure 2.7. It tends to be mild, but it can be toxic and will disrupt model biomembranes (liposomes). The CMC for Tx-100 is 0.22 – 0.24 mM or approximately 142.34 – 155.02 ppm (Triton surfactants: FDA status of triton surfactants 2010).
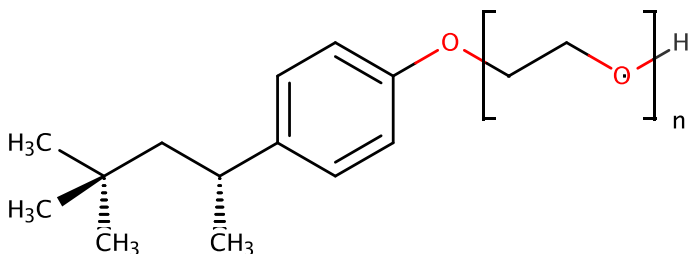
Figure 2.7 Molecular structure of Triton X-100

Cetylpyridinium chloride (CPC; $C_{21}H_{38}NCl$) is a cationic surfactant that is used as an antiseptic in household items such as mouthwashes and navel sprays (Witt et al. 2005), its structure can be seen in Figure 2.8. It is, however, known to be toxic to animals and aquatic organisms. The CMC for CPC is approximately 0.12mM or 40.8 ppm (Safety data sheet: Cetylpyridinium chloride 2015).
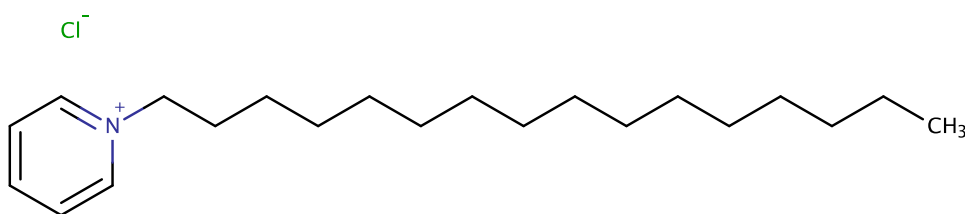
Figure 2.8 Molecular structure of cetylpyridinium chloride

An example of a common anionic surfactant is sodium dodecyl sulfate (SDS; $NaC_{12}H_{25}SO_4$), Figure 2.9. It is found in many domestic cleaning products because of its low-cost synthesis from coconut and palm oils and its effectiveness at dissolving oils and lipids. It is also used commonly to lyse cells in laboratories. The CMC for SDS is approximately 8.2 mM or 23.6 ppm (Moroi et al. 1974).
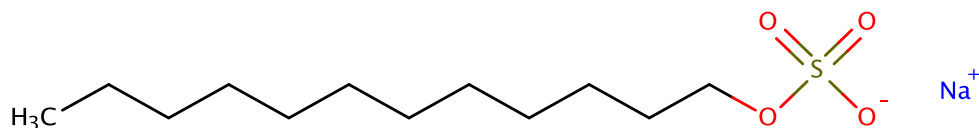
Figure 2.9 Molecular structure of sodium dodecyl sulfate

There have been a variety of studies to determine the interactions and binding of surfactants by HA. Ionic surfactants are expected to interact with HAs through both electrostatic interactions and hydrophobic interactions. Surfactants and HA interactions have been determined in previous studies by monitoring the free concentration of surfactants using surfactant-selective electrodes

and precipitation of the surfactant-HA complex. Because of the anionic nature of surfactants at environmental pHs, it is generally believed that cationic surfactants will interact much more with HA than anionic surfactants – especially in fresh water environments. A study by Koopal et al. between several ionic surfactants showed that there is little to no interaction between a humic acid (purified Aldrich HA; PAHA) and SDS (Koopal et al. 2004); however, there were strong binding interactions between the cationic surfactants CPC and dodecylpyridinium chloride (DPC) (Koopal et al. 2004). They determined the binding to be by both electrostatic and hydrophobic interactions. The affinity for cationic surfactants and HA increased as pH increased (resulting in an increasing negative charge on the HA) which indicated strong electrostatic binding. This study also illustrated that HA can precipitate when bound with a cationic surfactant to the point that the HAs charge is neutralized. This could have implications for other HA interactions in the environment. A further study into cationic surfactants and humic acid binding using iso-electric-points (IEP) showed that surfactants could change the physicochemical characteristics of humic acid (charge density, hydrophobicity and internal structure) as they bound (Ishiguro et al. 2007). A study by Klocking et al. that included cation, anionic and nonionic surfactants, studied the changes in cytotoxicity of the surfactants to cells in the presence of HAs. They found that although HA could reduce the cytotoxicity of cation surfactants, there was no change with the nonionic and anionic surfactants (Klocking et al. 2008). It is believed that anionic surfactants do not interact strongly with HAs because of electrostatic repulsions.

Although the previously mentioned studies did not show any significant interactions between a nonionic surfactant and HA, it has been shown that nonionic surfactants do, in fact, interact with HA. A study by Guangzhi et al. measured the binding of Triton X-100 to HA, soil, humin, and base-extracted soil (Guangzhi et al. 2009). Their results indicated that Triton X-100

not only interacted with all the constituents but that HA had the highest equilibrium binding capacity for the surfactant.

Surfactants are also known to interact with biomembranes. Surfactants are commonly used with biomembranes to extract membrane lipids and proteins or to completely disrupt the membranes. When the concentration of the surfactant is much lower than it's CMC (critical micelle concentration), it can intercalate into the biomembrane's bilayer. As the concentration is increased, the surfactant will completely disrupt the biomembranes bilayer and form mixed micelles (Luckey 2008).

The toxicity of surfactants are dependent on the molecular structure, the type of organism and the way the surfactant is ingested or taken-up by the cell(Abel 1974). The mechanism(s) of toxicity is not well understood and likely has many different pathways. In aquatic species, there has been shown a change in liver and kidney function, gill damage, and enzyme inhibition (Cserháti et al. 2002, Ivanković and Hrenović 2010, Lechuga et al. 2016, Lewis and Wee 1983). Toxicity is also contributed to the disruption of cellular membranes by the surfactant (Abel 1974, Partearroyo et al. 1990). Studies have shown that, in general, nonionic and anionic surfactants tend to have similar toxic concentration ranges (Abel 1974) while cationic surfactants are more toxic to aquatic species (Lewis and Wee 1983, Singh et al. 2002). However; there is no clear relationship between type of surfactant and relative toxicity because the toxicity can also depend on the type of organism (Ivanković and Hrenović 2010, Lechuga et al. 2016, Lewis and Wee 1983).

2.3.3 Carbon nanotubes

Since their discovery in 1991, the use of carbon nanotubes (CNTs) in everyday life has been increasing (Jackson 2013). CNTs are graphene nano-cylinders that can be either single-walled (SW) or multi-walled (MW) where SWCNTs consist of only one cylinder while MWCNTs

consist of two or more cylinders (see Figure 2.10). They are synthesized using chemical vapor

deposition, carbon arc discharge, laser ablation or electrolysis methods (Tasis et al. 2006). They

have a variety of uses in medical science, electronics, and material sciences because of their unique

properties including high mechanical strength, low mass density, and high thermal conductivity

(Eklund et al. 2007). Another appeal of CNTs is their ability to be functionalized to change their

physical and chemical properties based on the desired use of the CNTs (Kuzmany et al. 2004).

The increasing and extensive use of CNTs in everyday products such as clothing, sporting

goods, and batteries, means that they are increasingly likely to enter aquatic environments.
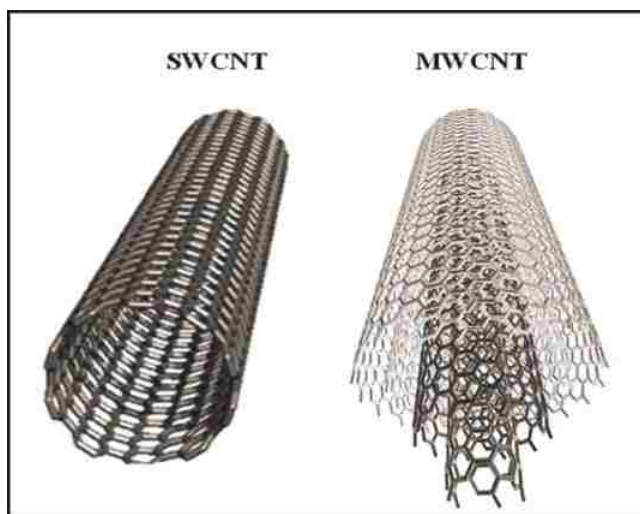
Figure 2.10 Schematic of single walled carbon nanotubes and multi-walled carbon nanotube (Choudhary and Gupta 2011)

CNTs are both hydrophobic and non-biodegradable which creates the concern that they

will accumulate in soils, sediments, or organisms when they enter the aquatic environment

(Edgington et al. 2010, Jackson et al. 2013).

Carbon nanotubes have been studied extensively and they have been shown to have

interactions with both natural organic material and biological organisms (Edgington et al. 2010,

Nowack and Bucheli 2007, Ferguson et al 2008, Kwok et al. 2010). Although there are many

studies about how CNTs interact with environmental components, there are many conflicting

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results and inconclusive data. NOM has been shown to sorb CNTs by hydrophobic interactions (Hyung and Kim 2008). This sorption was shown to allow CNTs to disperse stably in water. Greater dispersion of CNTs have been related to higher toxicity to aquatic organisms. Studies by Edgington et al. and Nowack and Bucheli have shown that the addition of NOM increases the toxicity of CNTs to aquatic organisms (Edgington et al. 2010, Nowack and Bucheli 2007).

There is inconclusive data on the toxicity of CNTs to a variety of organisms. There is not only conflicting data on if they are toxic or not, but there is also conflicting data on how their physical attributes effect toxicity (Allegri et al. 2016, Cheng and Cheng 2012, Du et al. 2013, Edgington et al. 2010, Jackson et al. 2013, Kalid et al. 2016, Lukhele et al. 2015, Mwangi et al. 2012). These physical attributes include whether they are single walled or multi walled, their diameter, and their functionalities. Toxic effects can also vary for different organisms. For example, studies on two different crustaceans showed that one had the ability to intake the CNTs and subsequently eliminate them which caused no toxicity while another crustacean could not eliminate them, causing death (Ferguson et al. 2008, Kwok et al. 2010). Because of this variability, it is necessary to further investigate CNTs, their toxicity, and the role of other environmental constituents such as NOM on their bioavailability.

2.4 Techniques

2.4.1 Fluorescence spectroscopy

Fluorescence spectroscopy is a non-destructive, relatively inexpensive, and sensitive technique that can be used to study HAs and model biomembrane perturbation (Ojwang' and Cook 2013, Vigneault et al. 2000). The fluorescence phenomenon occurs when a fluorophore's (a species that can re-emit light upon light excitation) electron is excited to a higher energy level (excited state) with a photon of appropriate wavelength. After internal conversion to the lowest

excited vibrational state, the electron relaxes back to the excited state by emitting a photon. The emitted photon from fluorescence occurs at a longer wavelength that the excitation wavelength because it loses energy during the internal conversion. Internal conversions can consist of molecular rearrangement to minimize the energy of the excited and ground states as well as vibrational losses.

A simplified illustration of the electronic and vibrational states involved in the process of absorption and fluorescence can be seen in the Jablonski diagram (Figure 2.11) (Lakowicz 2006).
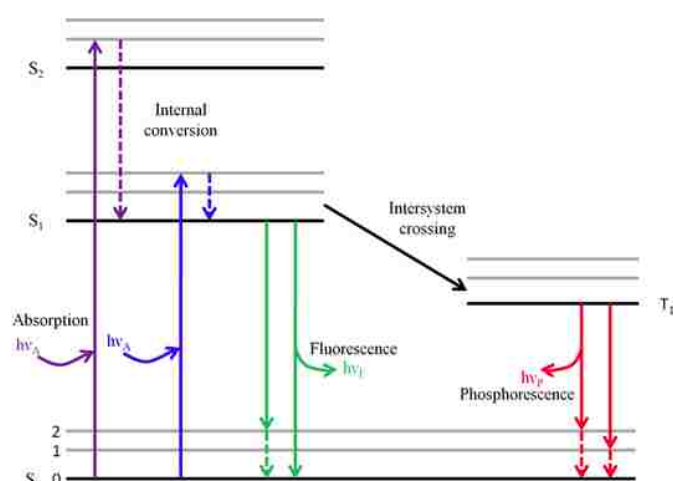
Figure 2.11 Jablonski diagram

By encapsulating a fluorophore in the form of a fluorescent dye within a lipid vesicle (model biomembrane), fluorescence spectroscopy can be utilized to study the leakage of dye from the membrane as induced by humic acids or other environmental factors by taking advantage of the inner filter effect. The inner filter effect is the phenomenon where the fluorescence intensity is not proportional to the concentration of fluorophores because there is an absorbing component in the matrix that absorbs the emitted radiation before reaching the detector. The absorbing component can be the fluorophore itself, which is known as being "self-quenching". Self-quenching dyes, such as sulforhodamine B (Figure 2.12), have low fluorescence intensity at high dye concentrations while having high fluorescence intensity at low concentrations. By

encapsulating high concentrations of dye in lipid vesicles, there is a low fluorescence intensity until the vesicle is perturbed and dye released, thus diluting the dye, and the fluorescence intensity increases. For example, Campbell et al. used fluorescence spectroscopy as a method to measure biomembrane perturbation caused by HAs and determine the ability of toxins to change these interactions (Campbell et al. 1997).
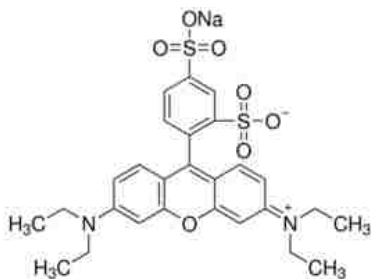
Figure 2.12 Structure of sulforhodamine B fluorescent dye

Fluorescence spectroscopy was used in the studies presented in this dissertation as a way to measure the changes in membrane perturbation by HAs and surfactants in a variety of conditions to elucidate different interactions of HA and its chemical components.

2.4.2 Dynamic light scattering

When light is directed towards a small particle, it is scattered in all directions by the phenomena known as Rayleigh scattering. The DLS instrumentation measures fluctuations in the intensity of scattered light at a particular angle and utilizes an autocorrelation function to determine "time of decay" (Γ) – or the time it takes for the same particle to move to a new point in space. From this, a diffusion coefficient (D) can be calculated:

$$\Gamma = q^2 D \tag{2}$$

Where q is the scattering vector magnitude that is based on experimental conditions.

From this, the size of the particles can be measured through the following calculation:

$$R_h = KT/6\pi\eta D \tag{3}$$

Where R_h is the hydrodynamic ratio, K is is the Boltzmann constant, T is the temperature, and η is the viscosity. Dynamic light scattering is a conventional technique that uses these parameters to determine the average size distribution of particles. In the model biomembrane studies presented in this dissertation, DLS was utilized to verify that the model biomembranes were 100 nm in diameter and monodisperse so to have consistent results.

2.4.3 Nuclear magnetic resonance spectroscopy

When a certain type of atomic nuclei (i.e. magnetizable) is placed into a magnetic field, it can absorb energy in the range of 10 to 900 MHz (and higher) that are considered radio frequency (RF) waves (Keeler 2010). As the RF photon is absorbed, the magnetic moment of the nuclei is tilted away from the external magnetic field (B_o) and it precesses around at the specific resonance frequency (the Larmor frequency). It then relaxes back to the B_o direction, emitting radiation. The Larmor frequency is dependent on the type of magnetic nuclei as well as the strength of the external magnetic field.

$$\text{Larmor frequency } (\omega) = -\gamma B_o \tag{4}$$

Where γ is the gyromagnetic ratio. NMR spectroscopy takes advantage of this physical phenomenon to obtain a spectrum of absorption vs. frequency. The positions of the peaks in an NMR spectrum, or "chemical shifts", are caused by nuclear shielding by surrounding electrons, which causes a change in the precession frequency of the nuclei. Because this shift is dependent on the electron density distribution of corresponding molecular orbitals, the environment of the nuclei can be determined.

Solid state ^{13}C NMR does not typically yield as high of resolution spectra as liquid ^{1}H NMR because the chemical shift anisotropy and dipole-dipole interactions. Anisotropy problems

occur in molecules (e.g. aromatic rings and carbonyls) that contain non-spherical electron densities that, in liquid, are allowed to freely rotate so the signals average while in solid, they are not allowed to rotate. Dipole-dipole interactions in solids cause line broadening as well because of molecules effecting local fields of neighboring nuclei. In order to resolve the two main issues in solid-state ^{13}C NMR, samples are spun at high rates and at the "magic angle" ($\beta = 54.7°$) which simulates liquid-like conditions.

Another major issue with solid state ^{13}C NMR is that ^{13}C is a nucleus with low sensitivity because of its low isotopic abundance and γ compared to ^{1}H. In order to obtain a spectrum with an appropriate signal-to-noise ratio, many measurements must be taken in order to get enough signal. This problem is diminished by use of a ramped amplitude cross polarization technique (Ramp-CP) which occurs when a pulse is applied simultaneously on an I and S spin (Schaefer et al. 1977). This is done by targeting an abundant nucleus such as ^{1}H and magnetizing its spins by applying a $\pi/2$ pulse followed by transferring the magnetization to the ^{13}C nuclei during cross polarization. The cross polarization program is summarized in Figure 2.13.

To successfully obtain efficient cross polarization, the Hartmann-Hahn match must be set properly where the RF fields of the low sensitive spin (^{13}C) is set equal to that of the abundant spin (^{1}H) which is achieved by adjusting the power of both channels:

$$\gamma_{c\text{-}13}\omega_{C\text{-}13} = \gamma_{H\text{-}1}\omega_{H\text{-}1} \tag{5}$$

The amplitude must be ramped because each unique carbon has a different Larmor frequency (ω) (Cook 2004). The ^{1}H protons are decoupled from the ^{13}C signals by a strong RF field after the magnetization is transferred.

In vivo phosphorous nuclear magnetic resonance (^{31}P NMR) spectroscopy has the ability to determine an organism's cellular metabolic processes in real time by monitoring phosphorous

metabolites such as adenosine triphosphate (ATP), adenosine diphosphate (ADP) and inorganic phosphate (P_i). ^{31}P is a highly receptive nucleus and is also more selective than ^1H since ^{31}P is found in far fewer molecular entities, and even fewer that are highly mobile, and thus yield easily discernible NMR signals.
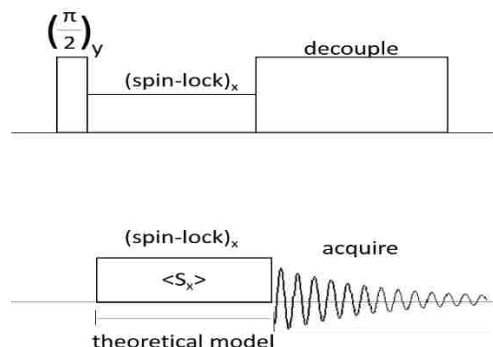
Figure 2.13 Schematic of the basic ramp cross-polarization program

Because it is noninvasive and can give information on the energy cycle of the organisms, *in vivo* ^{31}P NMR can be a valuable tool to gain insight into how living organisms respond to a range of environmental factors, such as pollutants and changes in ionic strength, pH, temperature, etc. These *in vivo* ^{31}P NMR studies are a type of metabolic profiling.

Metabolic profiling is a powerful method in determining biological responses to toxins, stressors and disease in living systems. Because of the complexity of an organism's metabolism, multiple metabolites and biomarkers must be monitored simultaneously. A change in metabolites or biomarkers can signal that a living system is under stress and the organism is trying to adjust for that stress.

The use of the term *metabolite profiling* and *metabolomics* should be addressed. The term *metabolomics* refers to the study of small molecule *metabolite profiles* that cellular processes leave behind. This should not be confused with the *genome* which is constant for an individual organism throughout its life - the *metabolic profile* of an organism changes depending on its environment

and stressors therein. In particular, the use of *in vivo* ^{31}P NMR allows for the study of the phosphometabolite profile; the metabolite profile of only metabolites containing phosphorus.

Environmental metabolite profiling applications fall into two main categories: ecophysiological and ecotoxicogenomics. Ecophysiological studies include natural stressors (e.g. temperature, salinity, pH changes) while ecotoxicogenomics is the study of an organism's metabolic response to xenobiotic pollutants. The NMR work presented in this dissertation would fall under the category of "ecotoxicogenomics".

Tjeerdema and coworkers have done extensive metabolomics studies with *in vivo* 31P NMR and have a series of publications dedicated to this type of study with different organisms under a variety of environmental conditions (Tjeerdema et al. 1993, Viant et al. 2006, Viant et al. 2002). The organisms studied were red and black abalones as well as medaka (*Oryzias latipes*) embryos. They studied changes in metabolomics when varying conditions such as pH, oxygen levels, and temperature. They also studied organisms under the pollutant stressors pentachlorophenol, copper, and dinoseb.

Decapsulated *Artemia* embryos have also been used in previous ^{31}P NMR studies to determine the metabolism changes under anoxia and to show the recovery of the organisms once the anoxic conditions were reversed. A study by Covi, Treleaven, and Hand studied the dissipation of proton gradients of Artemia under anoxic conditions and with the antibiotic bafilomycin using ^{31}P NMR (Covi et al. 2005). They determined that the data could be used to detail the proton gradients within the Artemia embryos to elucidate the metabolic processes when the organism was under stress (Covi et al. 2005).

In vivo ^{13}P NMR for metabolomics has advantages over ^{1}H NMR because of its simplicity. The ^{1}H NMR studies for metabolomics are complex and require multivariate analysis while ^{31}P

NMR is more straightforward because it limits the number of metabolites measured while still providing important information about the organism. Often ^{1}H NMR metabolomics studies, as well as other metabolomics methods (e.g. HPLC, mass spectrometry) requires extraction and purification of the metabolites before analysis. *In vivo* ^{31}P NMR allows for the measuring the organism phospho-metabolites without extraction and while they are still viable; which allows for further studies on "real-time" stressors.

2.4.4 High performance liquid chromatography

High performance liquid chromatography (HPLC) is a separation technique that can be used to separate and quantify multiple components in a mixture. It uses a separation column filled with an adsorbent material (the stationary phase) and a pump that passes pressurized liquid sample though the column. The solvent that carries the sample through the column is known as the mobile phase. Depending on the type of column and the composition of the mobile phase, the analytes will absorb onto the stationary phase for a period of time based on the physical nature of the different sample compounds. The time that it takes for a specific compound, or analyte, to pass through the pressurized column is known as the retention time. The retention time is characteristic for certain compounds so the analyte can be identified. Common detectors for HPLC include UV/Visible spectrometers and mass spectrometers. These detectors can further help to identify and quantify the analyte.

Metabolomic studies require parallel analytical techniques such as mass spectrometry, HPLC, and NMR spectroscopy (Sumner et al. 2007). HPLC can be used to separate and quantify different metabolite after they have been extracted from the organism in question. Extraction of the metabolites generally consists of death of the organism, lyophilization, homogenization, lysis, and purification in order to accurately measure multiple metabolites. It is labor intensive and has

a high degree of error associated with it. However, it can provide verification for other metabolomics techniques such as NMR.

2.5 Overview

The techniques and theories presented above laid the groundwork for the studies presented in this dissertation. The overall goal of this work is to study the HA-pollutant interactions and how those interactions influence biological organisms. These techniques allow for a systematic study of the incredibly complex aquatic environments to lead to a better understanding the role of different constituents in the environment.

2.6 References

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CHAPTER 3
INTERACTIONS OF HUMIC ACIDS AND CATIONS AND THE INFLUENCE OF BIOMEMBRANE PERTURBATION

3.1 Introduction

Decomposition of organic matter in the environment creates assemblies of organic molecules that are also referred to as humic substances (Stevenson 1994). These substances are further classified into three fractions: humin (completely insoluble in water), fulvic acid (FA – water soluble at all pH levels), and humic acid (HA – water soluble at pH >2). The organic matter discussed in this chapter is HA because of its omnipresence in water and terrestrial environments, because standards are commercially available, and it has been shown to have greater membrane permeation than FA (Elayan et al. 2008, Vigneault et al. 2000).

HAs have different physical and chemical properties depending on their biogeochemical origin. They are best viewed as complex, heterogeneous, amphiphilic and polydisperse mixture of organic molecules that create supramolecular structures attributable to the weak interactions between functional groups (Lattao et al. 2008). The main functional groups are aliphatics, aromatics, and carbohydrates (Schulten and Schnitzer 1993, Stevenson 1994). These characteristics enable HA to have many types of interactions in aquatic systems with both pollutants and biomembranes (Elayan et al. 2008, Ojwang' and Cook 2013). There are three types of binding sites in HA: 1) non-specific sites, 2) abundant weak sites and 3) stronger, less abundant sites (Tipping 2002). The non-specific sites are the functional groups with a net negative charge and can attract any positively charged group. This electrostatic interaction can change the conformation of the humic materials as well as its ability to interact with other components. The abundant weak sites are typically made up of carboxyl groups and they can play a chelation role. The stronger, less abundant, sites have differing affinities for different environmental components.

These sites contain the soft donor atoms such as nitrogen, sulfur and phosphorous. It is important to understand how the varying characteristics of HAs can influence the ability of HAs to bind and transport pollutants as well as interact with cellular membranes. By studying both HAs of different biogeochemical sources as well as chemically modified the HAs certain types of interactions can be elucidated.

In every organism, the cellular membrane plays an essential role in biological processes. The membrane separates the cell's interior from the external environment while controlling the transport of selected species in an out of the cell. These biomembranes usually consist of phospholipids, carbohydrates, and proteins (Koster and van Leeuwen 2004). In many cases, the complexity of real cells can have major drawbacks when mechanistically studying interactions in the environment. Model biomembranes created from amphiphilic phospholipids can be studied to reduce this complexity (Lasic 1997, Zepik et al. 2008).

Previous studies have shown that HAs can interact with biomembranes by adsorption and perturbation (Ladokhin et al. 1995, Parent et al. 1996, Samson and Visser 1989, Zhou and Banks 1993). Vigneault et al. investigated model biomembrane and phytoplankton cell permeation by both humic and fulvic acids and found that humic acids will have a higher amount of permeation into the cell than fulvic acid (Vigneault et al. 2000). They determined that pH has a strong influence on the ability of HA and FA to perturb biomembranes, which led them to propose that electrostatic interactions play a role in permeation. Studies by Elayan et al. showed that HA decreased the structural integrity of model biomembranes at low pH using ^{31}P NMR (Elayan et al. 2008). Further studies by the Cook group used fluorescence spectroscopy to investigate the permeation of model biomembranes in the presence of HA at different pH, different HA concentrations, and the kinetics of permeation (Ojwang' 2012, Ojwang' and Cook 2013).

These studies all led to the proposal that there is a two-step process by which the HA permeates biomembranes: Step 1) adsorption and Step 2) absorption. The adsorption step is one in which the HA adsorbs onto the surface of the biomembrane by hydrogen bonding between the negatively charged carboxyl groups of the HA and the phosphate head groups on the membrane. The absorption step is attributed to the ability of hydrophobic moieties in HA to permeate into the hydrophobic layer of the membrane at the packing defect sites. Kinetics studies determined that the absorption step has both a fast and slow component. This is attributed to the different hydrophobic functional groups of the HA interacting with the biomembranes and their different times of diffusion throughout the bilayer.

The interactions between HAs and biomembranes are dependent on two factors: the *composition* and the *conformation* of the HA. The *composition* of the HA is determined by the geochemical source of the HA and any chemical modifications that occur. The *conformation* of the HA changes depending on the environment, changes in composition, or interactions with different compounds such as cations.

Cations such as Na^+, K^+, and Ca^{2+} are naturally abundant in aquatic environments while other less abundant metals such as Al^{3+}, Fe^{3+}, Mn^{2+}, Co^{2+}, and Cd^{2+} can be naturally occurring as well as introduced via urban runoff (Urban runoff quality control guidelines for the province of British Columbia 1992). Because of freshwater acidification, there is concern over an abundance of these cations being released from the bedrock and sediments (Schindler et al. 1980).

Cation and humic acid interactions are well established in the literature in terms of binding sites and conformation changes (Tipping 2002). For example, an early study by Gamble examined electrostatic interactions of natural organic matter (NOM) with Na^+ and K^+ and determined that Na^+ bound to the NOM stronger than K^+(Gamble 1973). Bonn and Fish continued this research by

investigating the possible binding sites of the +1 cations (Bonn and Fish 1993). Di-cations such as Ca^{2+} were shown to have the ability to complex with HA and cause the molecular assemblies to become more compact.

Also, there are a multitude of studies that illustrate the ability of HAs to change the bioavailability of metal ions in the environment (Alstad et al. 2005, An et al. 2015, Guo et al. 2001, Lamelas and Slaveykova 2007, Lamelas et al. 2005, Matsuo et al. 2004, Sanchez-Marin and Beiras 2011). Elkins and Nelson as well as Parent, Twiss, and Campbell investigated the effect of the binding Al^{3+} with HA on the overall toxicity (Elkins and Nelson 2001, Elkins and Nelson 2002, Parent et al. 1996). Tipping el al. also studied Fe^{3+} as well as Al^{3+} and the binding ability of HA (Tipping 2002, Tipping et al. 2002). These studies agreed that Al^{3+} toxicity decreased when HA is added to the system, which suggests that HA's metal binding protects the organisms from up-taking the pollutant.

Cations can interact with humic acid by electrostatic interactions or with covalent interactions at HA binding sites. The diagrams in Figure 3.1 illustrates some of the possible scenarios that could occur when cations and HAs interact. Figure 3.1A demonstrates possible outcome of electrostatic interactions between the non-specific sites of the HA and the positively charged cations and how that affects the HA-biomembrane interactions.

Figure 3.1B illustrates how the electrostatic repulsion of HA groups can be mitigated by cations and thus altering the conformation of the HA. Humic acid has a net negative charge at pH 4.8 and the negative groups will repulse one another and "stretch out" the HA assemblies. As the cations are electrostatically attracted or chemically bound to these negative groups, the electrostatic repulsion will decrease and the HA will become more compact.

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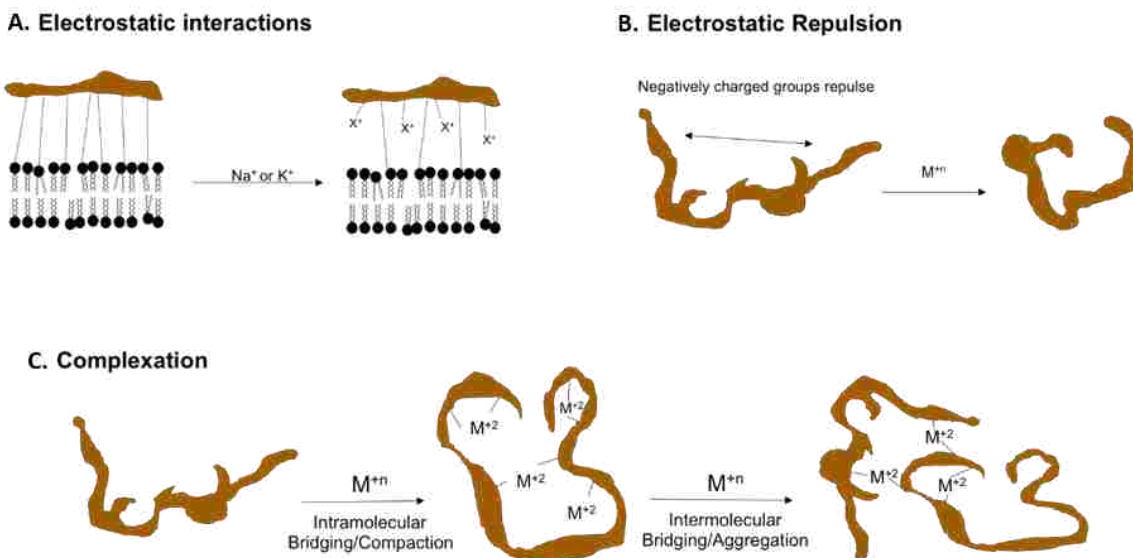
Figure 3.1 Possible humic acid conformation changes in the presence of cations

The final illustration in Figure 3.1C shows how cations can complex with HA functional groups which causes further conformational changes of the HA. This type of complexation can have two different mechanisms: 1) intra-molecular bridging and compaction and 2) inter-molecular bridging and aggregation. Since cations and HAs interact through both electrostatic and complexation, these interactions may cause conformation changes in the HA. These conformation changes can further change how HAs interact with other entities in the environment such as biomembranes. This study aims to investigate the result of conformation changes by cation-HA interactions and how it effects biomembrane-HA perturbation. This will allow for a better understanding of HA interactions in the environment.

3.2 Materials and Methods

3.2.1 Materials

The metal salts were purchased from Sigma Aldrich (Piscataway, NJ) with the exception of cadmium chloride, which was purchased from Fischer Scientific (Somerville, NJ). Sulforhodamine-B dye (SRB), t-octyl-phenoxy polyethoxy ethanol (Triton TX-100), sodium

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acetate, and sodium chlorite were purchased from Sigma Aldrich. The 1-palmitoyal-2-oleoyl-sn-glycero-3-phosphocoline (POPC) was purchased from Avanti Polar Lipids (Alabaster, AL) and the Sephadex G-50 gel for the size exclusion columns was from Healthcare Biosciences (Piscataway, NJ). The humic acid standards (Leonardite HA, Florida peat HA, and Suwanne River HA) were obtained from the International Humic Substances Society (Georgia, USA). Benzene, methanol, and hydrochloric acid were purchased from Fischer Scientific. The nitrogen gas was supplied by the LSU chemistry department, originally sourced from Capital Welders Supply Company (Baton Rouge, LA). Sterile 18 MΩ deionized water was sourced from an apparatus by US filter. All fluorescence measurements were made on a Horiba Jobin Yvon Fluorolog 3 spectrofluorimeter with a FL1073 detector, Spectra Acq computer and a model LF13751 temperature control. A Malvern Zetasizer nano (Worchester, UK) was utilized for dynamic light scattering of the liposomes. Solid state ramp cross-polarization magic angle spinning (CP-MAS) ^{13}C NMR by a Bruker Advance 400 MHz NMR spectrometer with a ramped-amplitude cross-polarization pulse program and magic angle spinning was used to characterize the chemically modified HA.

3.2.2 Experimental design

For this study, 1-palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphocoline (POPC) was chosen for the model biomembranes because phospholipids make up 50 – 60% of eukaryotic cell membranes (Cooper 2000). Also, the transition phase temperature of $T_m = -2.5 \pm 2.4°C$ is well below room temperature so the lipids are completely in the liquid crystalline phase and has minimum packing defects.

Three different humic acids were investigated with solutions of Na^+ and Ca^{2+}: Leonardite humic acid (LAHA), Florida peat humic acid (FPHA) and Suwannee river humic acid (SRHA).

LAHA is a coal source, SRHA is an aquatic source, and FPHA is a peat source. These humic acids have different components based on their sources and the different components have a different propensity to perturb the biomembranes, as shown in previous studies (Ojwang' and Cook 2013).

The cations studied were: K^+, Na^+, Ca^{+2}, Mn^{+2}, Mg^{+2}, Co^{+2}, Cd^{+2}, Al^{+3}, and Fe^{+3}. These cations were chosen for their range of charges, chemical "hardness", and affinities for different functional groups.

3.2.3 Model biomembrane preparation

All biomembranes, humic acid solutions, and cation solutions are in a 0.01 M acetate buffer at pH 4.8. The model biomembranes with encapsulated sulforhodamine B dye were prepared in a similar method to Ladokhin et al. (Ladokhin and Holloway 1995) To create the model biomembranes (or bilayer liposomes), first, a lipid film was created in the bottom of a round bottom flask by dissolving POPC in 66 μL methanol and 132 μL chloroform (1:2 solution). The mixture was stirred for 30 minutes using a rotary evaporator without any pressure applied. The solvent was then evaporated under nitrogen gas for 24 hours resulting in a thin lipid film.

The lipid film was hydrated with 5 mL of 50 mM SRB dye in acetate buffer. sulforhodamine B dye was chosen as the fluorophore because humic acids do not quench it. The dye and lipids were vortexed (Vortex Genie series G560) until the lipid had completely suspended into the dye solution. The common freeze-thaw procedure for creating large multilamellar liposomes was then applied: heat in 80ºC water bath, vortex, and freeze in dry ice and acetone bath. This cycle was repeated three times. Once the cycles were completed, the frozen liposome solution was then thawed in the water bath and extruded under pressure using a Lipex Lipid Extruder (North Lipid, Vancouver, BC, Canada) through a 100 nm pore Whatman Nuclepore polycarbonate track-etched membrane to create large unilamellar liposomes (model

biomembranes) of 100 nm in diameter. After the extrusion, the liposomes were put through two consecutive Sephadex G-50 columns to remove any non-encapsulated (free) dye from the solution. For fluorescence measurements, the liposomes were diluted 2.144 mL per 100mL. Dynamic light scattering (DLS) was used to verify the size and monodispersity of the model biomembranes. The DLS measurements were made with a scattering angle of 90° and a wavelength of 6471 Å.

3.2.4 Stock solution preparation

The humic acid (HA) stock solutions were prepared by dissolving 7 mg of reference HA in NaOH. The HA solution's pH was adjusted with HCl and NaOH until it reached pH 4.8. Then, 15 mL of prepared acetate buffer was added, the vial covered with foil, and the solution was allowed to stir overnight. Once equilibrated, the solution was diluted with acetate buffer to the desired concentration and the pH re-adjusted as needed.

Cation solutions were prepared using chlorine salts of each cation and then serial diluted with acetate buffer to the desired ionic concentrations.

3.2.5 Chemical modification of humic acids

Three procedures for chemical modification were performed on the humic acids (Leonardite HA, Suwannee River HA and Florida Peat HA): acid hydrolysis (Almendros 1994), Soxhlet lipid extraction (Chilom and Rice 2009) and bleaching (Wise et al. 1946). The HAs were characterized by solid state CP-MAS ^{13}C NMR after chemical modification.

The acid hydrolysis reduced the carbohydrate components of the humic acid. To achieve this, 300 mL of 6 M HCl per gram of HA was mixed together and maintaining under reflux for 6 hours. The acid was removed from the HA by dialysis. The modified HA was freeze dried for 24 hours (until completely dry) and then stored in the freezer.

The Soxhlet extraction was used to reduce the lipid components. The HA was placed into the thimble of the assembly and inserted into a Soxhlet extractor fitted with a condenser. Approximately 200 mL of benzene: methanol (3:1) azeotrope was put in a round bottom flask fitted onto the Soxhlet extractor. It was then heated in a sand bath and refluxed for at least 72 hours. Once complete, the thimble was removed from the extractor and the solvent allowed to evaporate in the hood. The modified HA was stored in the freezer.

Bleaching was performed to reduce aromatic components. The original procedure, by Wise et al., was used to isolate wood holocellulose; however, it was modified by increasing the bleaching time. The bleach solution for one gram of HA was as follows: 10 g sodium chlorite, 10 mL glacial acetic acid, and 100 mL deionized water. The HA and the bleach solution was stirred overnight in the hood. It was then centrifuged at 3500 g for 15 min and the bleach solution decanted from the HA. This was repeated 3 times with fresh bleaching solution. The final HA residue was separated by centrifugation followed by dialysis. It was then freeze dried for 24 h or until dry. The modified HA was stored in the freezer.

All chemically modified HA sample solutions were prepared the same as the un-modified HA (previously stated).

3.2.6 ^{13}C NMR Ramp CP-MAS

The LAHA and the freeze-dried chemically modified LAHA samples were ground with a mortar and pestle to ensure a homogeneous sample before the ^{13}C NMR experiments. The chemically modified HA sample was tightly packed into a 2.5 mm high-resolution magic angle spinning zirconium rotor (Bruker). Spectra were acquired at 100 MHz with a spinning rate of 5 kHz and a ramp cross-polarization contact time of 2 ms. The recycle delay time was 1 s and a total of 4096 scans were collected per experiment. The ^{13}C NMR spectra are shown in Figure 3.6.

3.2.7 Fluorescence leakage measurements

The blank for all fluorescence measurements was a 1:3 solution of liposomes and the acetate buffer. Because of inner filter effects, the dye encapsulated in the model biomembrane did not give a strong fluorescence intensity. A control of liposome, buffer, and detergent (Triton X – 100) solution was used as 100% dye release. Triton X-100 is a non-fluorescent detergent that completely lyses the liposomes, causing all the encapsulated dye to be released (Luckey 2008). Once diluted (inner filter effects removed), the dye fluoresces strongly. All measurements from the humic acid and cation samples was compared to the 100% dye release. The fresh water reference was a 1:2:1 solution of liposomes, buffer, and the humic acid solution. The humic acids had a final concentration of 20 ppm in all samples. The cation samples were a 1:2:1 solution of liposomes, cation solution (in buffer) and humic acid.

Fluorescence spectroscopy was utilized to determine the amount of dye released from the liposomes after 10 min. All samples were done in triplicate. The excitation wavelength was 565 nm, and the emission was measured between 575 nm and 700 nm. The maximum intensity used for the percent dye release calculations was the emission at 585 nm. The following equation was used to calculate the percent dye release from the liposomes relative to the lysed membranes:

$$\text{Percent dye release} = 100\ \% \ \text{x}\ (I_H - I_B)/(I_T - I_B) \tag{1}$$

Where I_H is the fluorescence intensity of liposomes in the presence of HA sample, I_B is the fluorescence intensity of the blank (liposomes only), and I_T is the fluorescence intensity of the dye after the liposomes are ruptured.

3.3 Results and Discussion

Dynamic light scattering (DLS) was performed on the unperturbed liposomes in order to verify the size. The DLS data seen in Figure 3.2 illustrates the z-average mean of the liposomes as 100 ± 2 nm and that the size distribution is homogeneous.

3.3.1 Interaction of Na^+ and Ca^{2+} with different humic acids

The change in membrane perturbation by LAHA, FPHA and SRHA were studied with increasing ionic strength of Na^+ and Ca^{2+} in order to determine any binding trends based on changes in dye released from the model biomembranes.
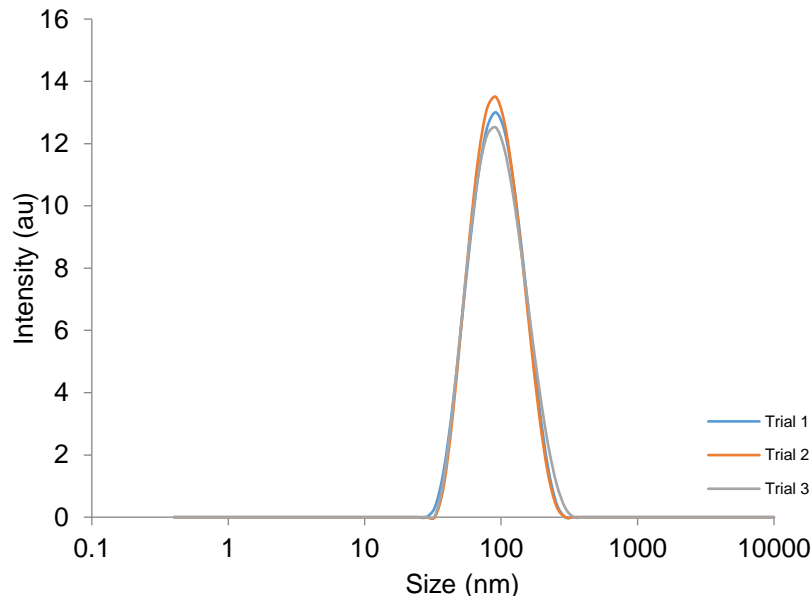
Figure 3.2 Dynamic light scattering of the POPC liposomes. Size: 100 ± 2 nm

Three HAs were utilized to determine if any changes in membrane perturbation were similar for HAs of different sources. Previous studies with these three HAs showed that all of them perturbed the biomembranes via the previously described two-step mechanism. At 20 ppm HA, all three HAs had similar perturbation of the model biomembranes (within 10% dye release of each other) (Ojwang' 2012).

Figure 3.3 and Table 3.1 illustrates the normalized percent dye release from the biomembranes with LAHA, FPHA, and SRHA with increasing ionic strength of Na^+ and Ca^{2+}.

The decrease in percent dye release indicates that the ability of HAs to perturb the model biomembranes has decreased. For all three HAs, both Na^+ and Ca^{2+} reduced their membrane perturbing ability, suggesting that the cations bind with the HAs and limit the availability of the HAs to the biomembranes. It can be seen that Ca^{2+} hindered the HAs perturbation more so than Na^+. It is likely that Na^+ is inhibiting some of the initial adsorption of the negatively charged moieties with the surface of the biomembrane while Ca^{2+} is involved in more complicated binding and complexation (see Figure 3.3).

This is consistent with previous studies that showed that Na^+ generally associates only with humic acids via electrostatic interactions with ionized functional groups (Bonn and Fish 1993, Gamble 1973). Unlike Na^+, Ca^{2+} can create inter- and intra- molecular bridges with the negatively charged carboxylic and phenolic groups with the humic acids – causing the HA conformation to change (see Figure 3.3). The HA molecular assemblies can become more tightly packed and aggregated because of this bridging (Tipping 2002, Wang et al. 2001). The hydrophobic moieties that are responsible for permeation of the model biomembranes become less accessible as the HA molecular assemblies become more compacted – thus, less leakage of the encapsulated dye. The more bridging and compaction that occurs because of the Ca^{2+} binding, the less permeation of the biomembrane.

When comparing the differently sourced HAs, it can be seen that the difference in dye release with Na^+ compared to the fresh water is less with LAHA than FPHA and SRHA – which can be attributed to the composition of the HAs.
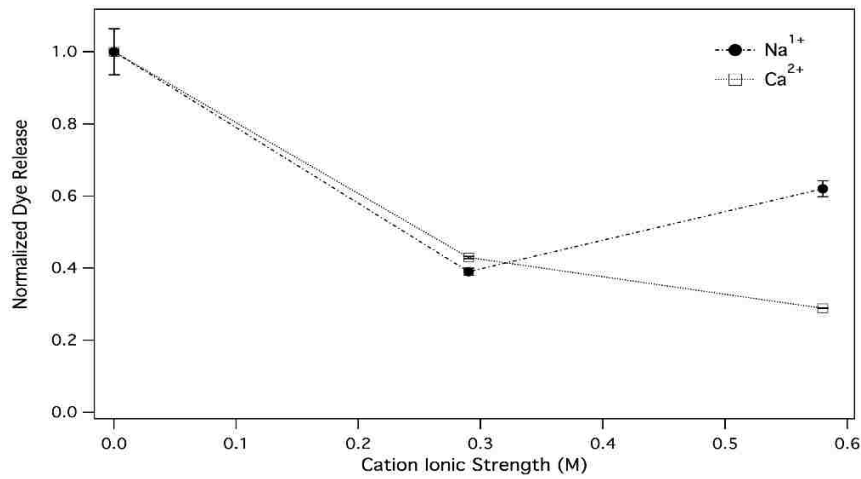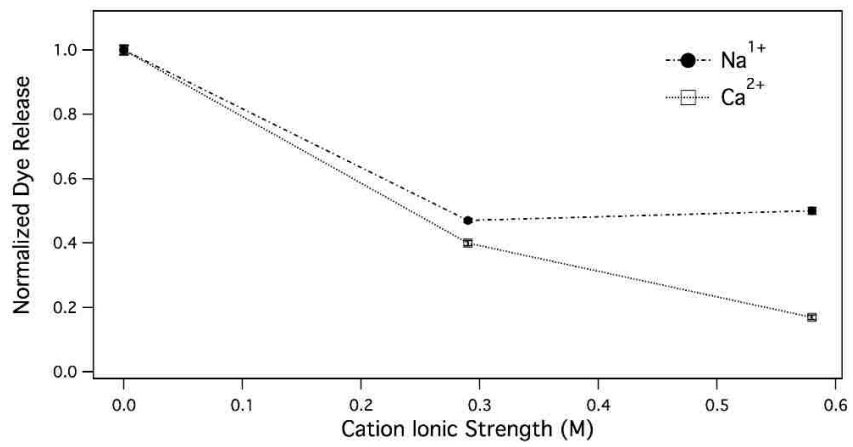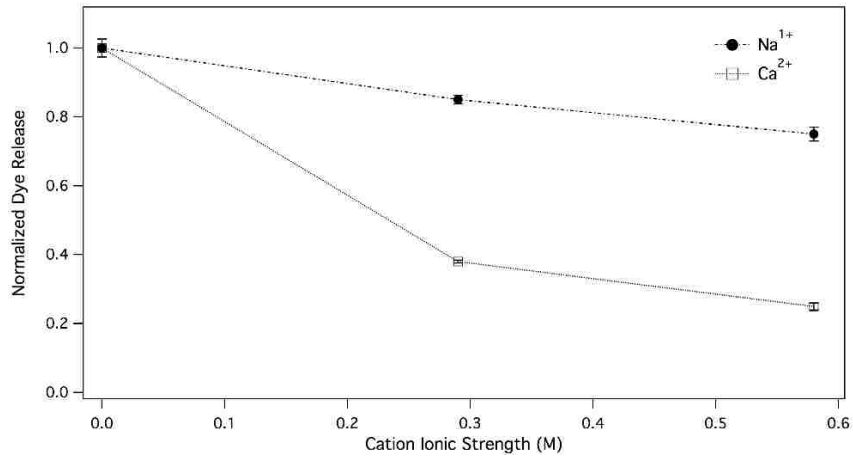
Figure 3.3 Percent leakage of SRB dye as induced by 20 ppm a) LAHA, b) FPHA, and c) SRHA

Table 3.1 Percent dye release as induced by 20 ppm LAHA, FPHA, and SRHA in the presence of Na^+ and Ca^{2+}

		Na^+		Ca^{2+}	
Ionic strength	0 M	0.29 M	0.58 M	0.29 M	0.58 M
20 ppm LAHA	45.93 ± 0.05%	39.10 ± 1.2%	34.59 ± 1.9%	17.48 ± 0.35%	11.33 ± 0.91%
20 ppm FPHA	30.09 ± 0.88%	14.17 ± 0.32%	15.09 ± 0.64%	10.90 ± 0.47%	5.24 ± 0.39%
20 ppm SRHA	22.13 ± 1.41%	8.71 ± 0.43%	13.69 ± 0.98%	9.52± 0.15%	6.39 ± 0.03%

LAHA has less carboxyl and carbohydrate groups than FPHA and SRHA; moieties that attracts Na^+ ("International humic substances society" 2015, Thorn et al. 1989). The addition of Na^+ to those sites limits the adsorption step by hydrogen bonding and thus limits the dye release.

3.3.2 Interaction of mixed solutions of Na and Ca with LAHA

To further corroborate the proposed interactions between Na^+, Ca^{2+} and HAs, mixed solutions of Na^+ and Ca^{2+} with a total ionic strength of 0.29 M were tested with 20 ppm LAHA and the model biomembranes. Figure 3.4 shows that as the percentage of Ca^{2+} in the solution increased, the perturbation of the membranes decreased. This trend gives further support to the model proposed in regards to Ca^{2+}'s ability to reduce HA's biomembrane perturbing potential by inducing inter- and intra-molecular bridging and thus, conformational changes. Tables 3.2, 3.3, and 3.4 provide percent dye release and standard deviations for all dye leakage studies. There was a 2.5% dye release increase when only Ca^{2+} is present compared to the 75% Ca^{2+} solution. It can be assumed that in the mixed solution (75% Ca^{2+} and 25% Na^+), the Na^+ is still electrostatically attracted to some moieties of the humic acid where Ca^{2+} may not be able to bind efficiently.
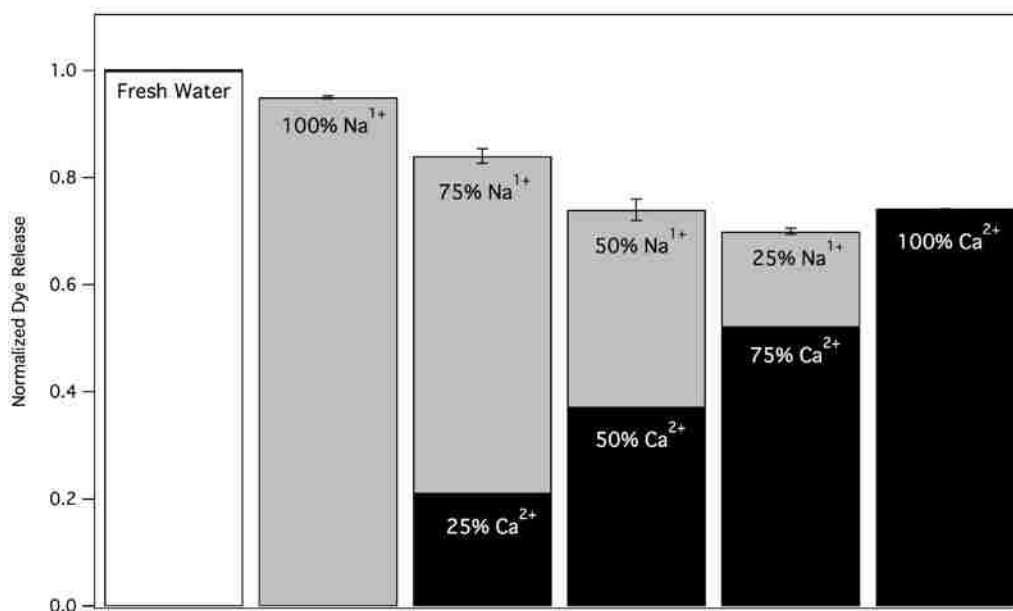
Figure 3.4 Percent leakage of SRB dye as induced by 20 ppm LAHA combined with mixtures of sodium and calcium with a total ionic strength of 0.29 M at pH 4.8

Table 3.2 Percent dye release of SRB dye as induced by 20 ppm LAHA in the presence of Na^+ and Ca^{2+} mixtures

Fresh water	100 % Na^+, 0% Ca^{2+}	75 % Na^+, 25% Ca^{2+}	50% Na^+, 50% Ca^{2+}	25 % Na^+, 75 % Ca^{2+}	0% Na^+, 100% Ca^{2+}
63.58 ± 0.14%	60.63 ± 0.33%	53.42 ± 1.71%	47.2 ± 2.51%	44.53 ± 0.74%	47.03 ± 0.25%

Thus, the Na^+ is giving some protection to the membrane in addition to the Ca^{2+} bridging and complexation. Since HA is complex, different binding affinities may allow different cations to interact with it at different areas; therefore, the HA permeation of the model biomembranes may be decreased even more when two or more cations are involved in binding.

3.3.3 Interaction of cations with LAHA

To further investigate the interaction of LAHA with cations, an expanded set of cations was used: K^+, Na^+, Ca^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+}, Cd^{2+}, Al^{3+}, and Fe^{3+}. The ionic strength of each

solution (2.03 and 4.11 mM) was less than previously explored so to be more environmentally relevant for fresh water.

Table 3.3 Percent dye release as induced by 20 ppm LAHA in the presence of cations

	Ionic strength of cation		
	0	2.03	4.11
K^+	$60.61 \pm 2.70\%$	$59.13 \pm 3.41\%$	$60.53 \pm 1.27\%$
Na^+	$60.61 \pm 2.70\%$	$59.60 \pm 1.96\%$	$55.31 \pm 3.27\%$
Ca^{2+}	$60.61 \pm 2.70\%$	$53.91 \pm 1.50\%$	$52.69 \pm 1.69\%$
Mg^{2+}	$60.61 \pm 2.70\%$	$58.45 \pm 0.78\%$	$55.57 \pm 0.13\%$
Mn^{2+}	$60.61 \pm 2.70\%$	$54.10 \pm 2.08\%$	$51.24 \pm 1.64\%$
Co^{2+}	$60.61 \pm 2.70\%$	$53.71 \pm 1.78\%$	$51.23 \pm 4.07\%$
Cd^{2+}	$60.61 \pm 2.70\%$	$53.63 \pm 2.50\%$	$29.19 \pm 1.39\%$
Al^{3+}	$60.61 \pm 2.70\%$	$7.14 \pm 2.90\%$	$4.75 \pm 2.24\%$
Fe^{3+}	$60.61 \pm 2.70\%$	$14.98 \pm 5.16\%$	$21.20 \pm 6.37\%$

It can be seen in Figure 3.5 that K^+ does not reduce dye release caused by the LAHA to the extent of Na^+, which suggests limited interactions between K^+ and LAHA. Na^+- favored binding with HAs has been previously reported and attributed to the size difference of the ions (Gamble 1973). The smaller Na^+ atoms can more easily bind to the negatively charged moieties within the complex structure of the HA than the larger K^+ ion.

For the +2 cations, the order of leakage reduction is as follows: $Mg^{2+} < Ca^{2+} < Mn^{2+} \approx Co^{2+} \ll Cd^{2+}$. The binding and resulting hindrance of the HA's ability to perturb the biomembranes follows the scheme of hard and soft acids and bases (HSAB) with reduced leakage in the order of hard < intermediate < soft cations. The cations that are classified as "hard" are Mg^{2+}, Ca^{2+}, and Mn^{2+}; Co^{2+} is considered intermediate and Cd^{2+} is considered a soft cation (Huheey 1978).

Since the softer cations limit the permeation of the biomembranes by the HA, it suggests that the less abundant binding sites of the HA must be playing a strong role in chelation with the cations or that the strong binding sites are a main source of membrane permeation.
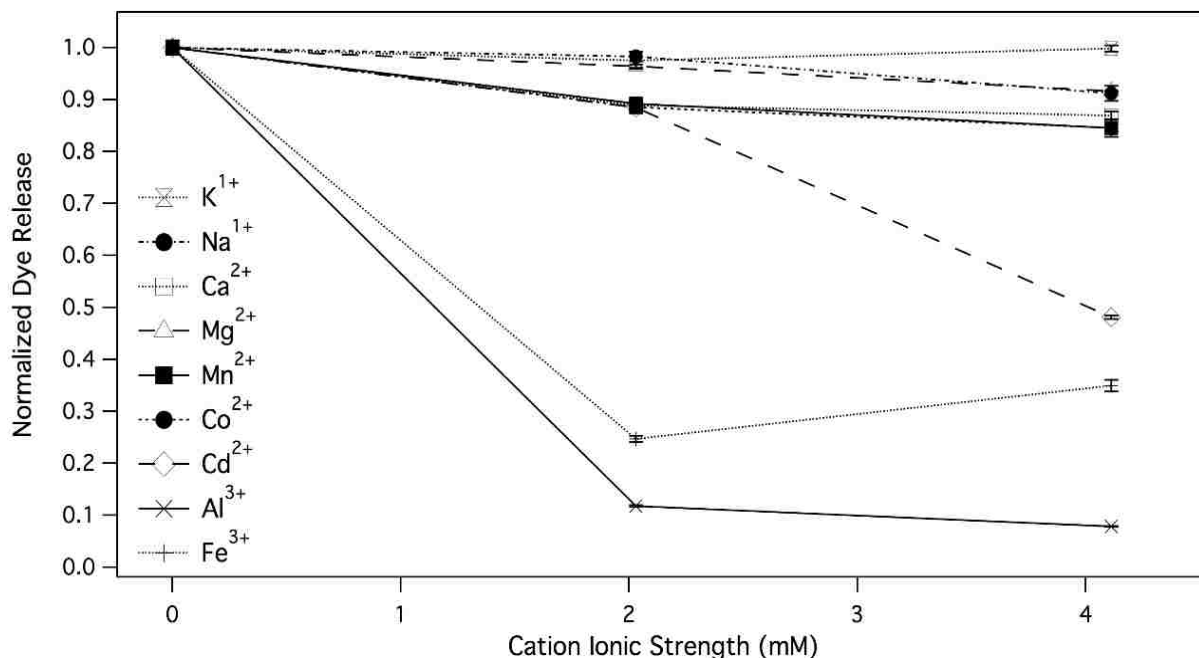
Figure 3.5 Percent leakage of SRB dye as induced by 20 ppm LAHA combined with different cations at pH 4.8

Both Fe^{3+} and Al^{3+} are considered to be hard acids, but there is evidence that they bind much more than the other hard cations studied (Tipping et al. 2002). Both of these +3 cations have been previously shown to complex with HAs with Al^{3+} having a higher affinity with HA than Fe^{3+} (Elkins and Nelson 2002). In this study, the +3 cations followed this trend, with Fe^{3+} reducing the leakage less than Al^{3+}. It can be seen that there is a slight increase in dye release as the concentration of Fe^{3+} increases, which is different than the trend of the other cations studied. This is due to the iron forming $FeOH^{2+}$ in the aqueous solutions and $FeOH^{2+}$ molecules tend to coagulate with each other, thus limiting the iron available to bind with the HA (Elayan et al. 2008).

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3.3.4 Interaction of cations with chemically modified LAHA

The composition of HAs plays a role in both biomembrane perturbation and how the HAs interact with various cations. In order to determine how composition changes could influence HA interactions within the environment, chemical modification was performed on LAHA.
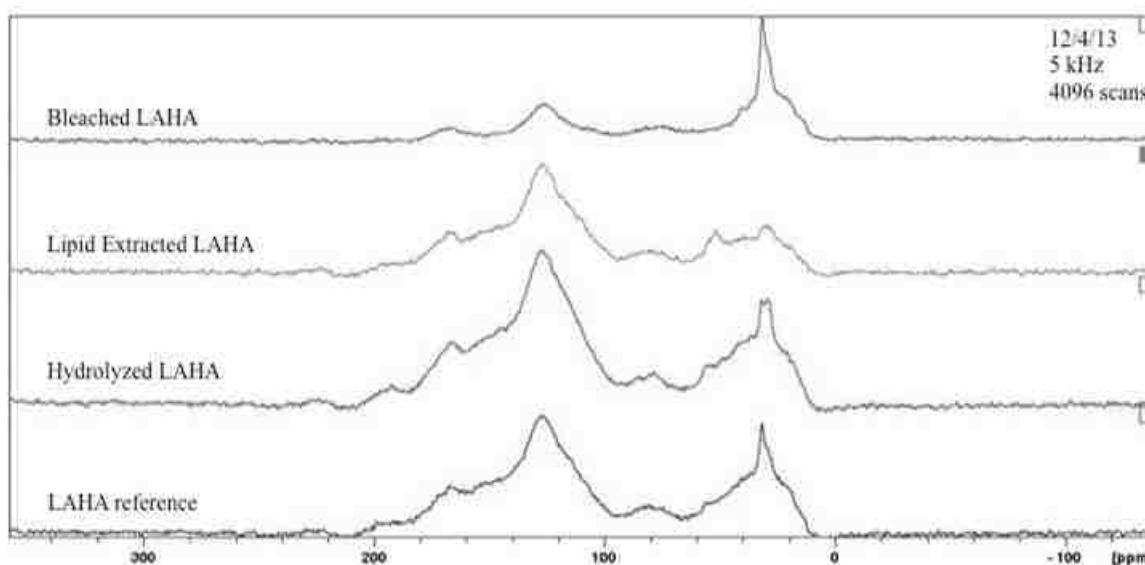
Figure 3.6 Solid-state ^{13}C NMR spectra of chemically modified Leonardite humic acid (LAHA)

Figure 3.6 shows the ^{13}C NMR spectra of the chemically modified HAs. The spectra verify that the chemical modifications designed to reduce certain moieties were successful. The peak percentages of the modified HAs can be visualized in Figure 3.7 and are listed in Table 3.1. These chemical modifications allowed for the study of cation interactions based on different moiety composition; specifically aromatics, carbohydrates, and aromatics.

Bleaching of the LAHA was done to reduce aromatic moieties. The ^{13}C NMR spectrum illustrates the significant reduction in aromatic moieties by the reduction of the relative peak area in the 112-145 ppm chemical shift regions. The low aromatic signal that was measured (30%) can be attributed to aromatic groups from charcoal-like compounds in the HAs that are resistive to oxidative cleavage by the chemical modification (Chefetz et al. 2002).

The acid hydrolysis chemical modification was performed to reduce carbohydrate components. The chemical shift range of 50 – 112 ppm includes the different types of carbohydrate (O-alkyl) carbons. The hydrolyzed LAHA had a peak percentage decrease in that region, indicating successful reduction of carbohydrate moieties. Percent dye releases by these HAs are seen in Figure 3.8.
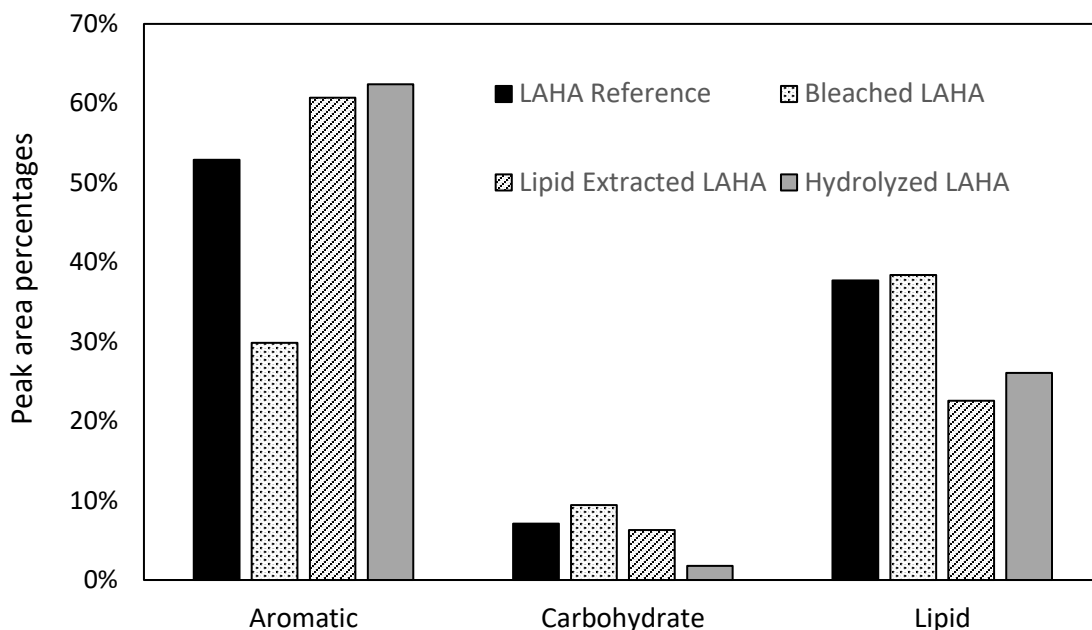
Figure 3.7 ^{13}C NMR relative peak area percentages of unmodified and modified LAHA for aromatic, carbohydrate, and lipid moieties

Table 3.4 ^{13}C NMR relative percent areas of unmodified and modified LAHA

HA	Carboxyl	Aromatic	Aldyhyde /Ketone	N- or O- Alkyl	Alkyl
Bleached LAHA	4.03%	29.86%	0.0%	9.43%	38.44%
Lipid Extracted LAHA	9.99%	60.71%	0.47%	6.28%	22.56%
Hydrolyzed LAHA	9.08%	62.38%	0.71%	1.78%	26.05%
LAHA Reference	11.18%	52.89%	0.07%	7.11%	37.74%
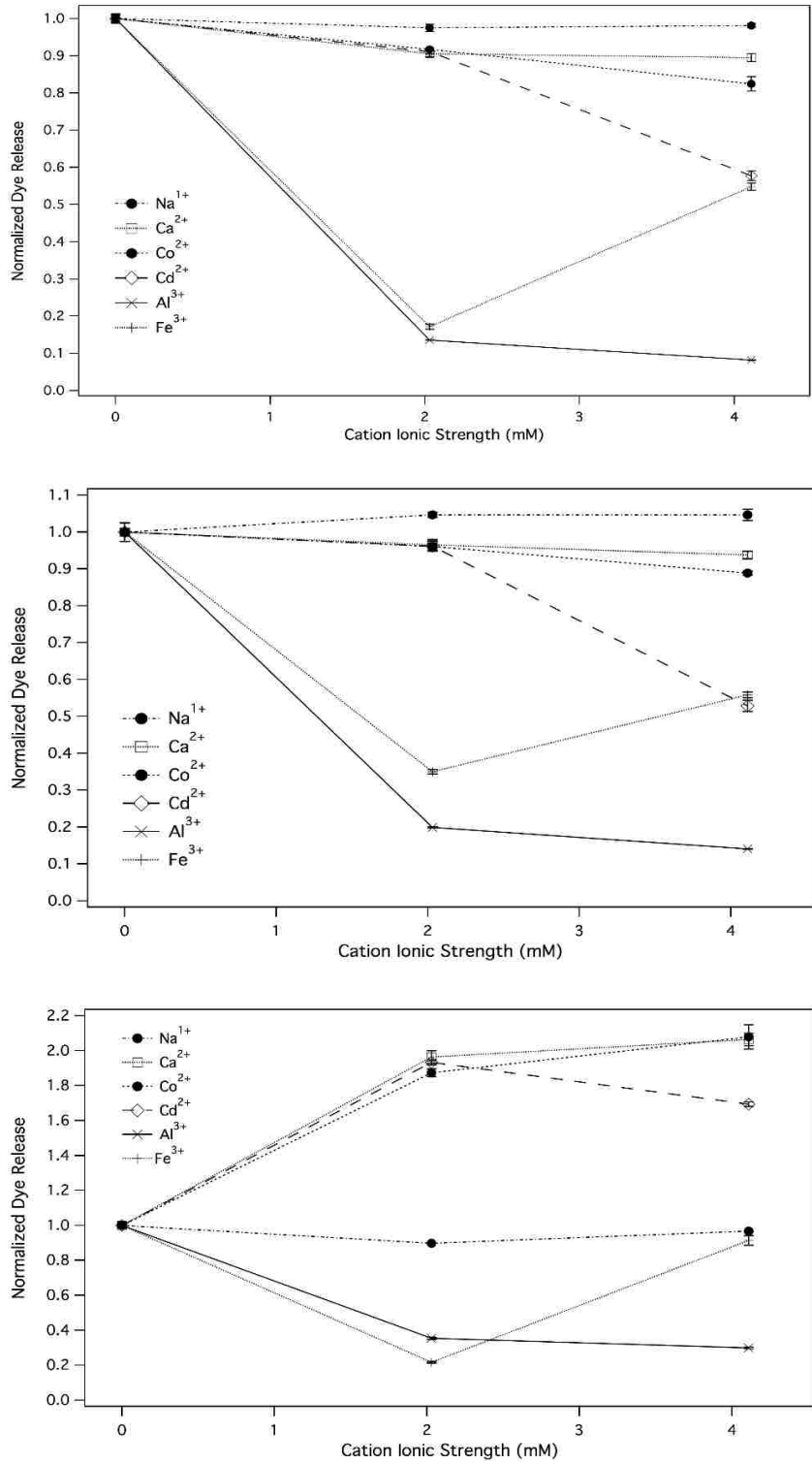
Figure 3.8 Percent leakage of SRB dye as induced by 20 ppm a) hydrolyzed, b) lipid-extracted and c) bleached LAHA combined with different cations at pH 4.8

The lipid extraction was performed to reduce any lipid moieties of the LAHA. The signal within the region 22 – 33 ppm is indicative of lipids, or polymethylene chains. Because of the nature of HA, the majority of lipids they contain are aliphatic. The ^{13}C NMR spectra verifies that the lipid moieties in the lipid-extracted LAHA are reduced relative to the reference LAHA.

Previous studies have been performed to determine the changes in membrane perturbation when the HA is modified (Ojwang' 2012). For LAHA, it was determined that the hydrolyzed and lipid-extracted LAHA had a slightly greater ability to perturb the biomembranes relative to the reference LAHA. This was believed to be caused by the relative increase and availability of membrane-perturbing moieties such as aromatics. It was concluded that the aromatic moieties play a major role in the absorption step of the proposed model for HA-biomembrane interactions. The hydrophobic nature of the aromatics is what allows for membrane perturbation via hydrophobic interactions.

This mechanism was further verified by the fact that the bleached LAHA had a 29% lower percent dye release than the un-modified LAHA. The percent dye release and standard deviations are presented in Table 3.5.

Six cations were investigated with the chemically modified LAHA: a +1 cation (Na^+), a hard +2 cation (Ca^{2+}), a soft +2 cation (Cd^{2+}) and the two +3 metal cations of interest (Al^{3+} and Fe^{3+}).

The bleached LAHA showed a very different trend than the other modified HAs. As can be seen in Table 3.5, when the aromatics were reduced, there was a significant loss in membrane permeation in fresh water as seen by the decreased dye release. However, with the addition of cations, the ability of the bleached LAHA to permeate the membranes was significantly increased.

The percentage of dye release due to the bleached LAHA with Ca^{2+} and Co^{2+} binding is more than that of the unmodified LAHA.

Table 3.5 Percent dye release as induced by chemically modified LAHA in the presence of cations

| | Ionic strength of cation | | |
	0	2.03	4.11
	Hydrolyzed LAHA		
Na^+	$65.26 \pm 2.42\%$	$63.68 \pm 2.11\%$	$64.06 \pm 0.92\%$
Ca^{2+}	$65.26 \pm 2.42\%$	$59.09 \pm 1.77\%$	$58.41 \pm 2.34\%$
Co^{2+}	$65.26 \pm 2.42\%$	$59.44 \pm 1.32\%$	$37.70 \pm 4.55\%$
Cd^{2+}	$65.26 \pm 2.42\%$	$59.87 \pm 0.76\%$	$53.78 \pm 4.76\%$
Al^{3+}	$65.26 \pm 2.42\%$	$7.94 \pm 0.92\%$	$4.80 \pm 2.08\%$
Fe^{3+}	$65.26 \pm 2.42\%$	$10.05 \pm 7.48\%$	$32.10 \pm 3.37\%$
	Lipid extracted LAHA		
Na^+	$59.00 \pm 5.10\%$	$61.70 \pm 1.32\%$	$61.74 \pm 3.00\%$
Ca^{2+}	$59.00 \pm 5.10\%$	$56.93 \pm 3.04\%$	$55.33 \pm 2.14\%$
Co^{2+}	$59.00 \pm 5.10\%$	$56.69 \pm 2.03\%$	$52.41 \pm 1.15\%$
Cd^{2+}	$59.00 \pm 5.10\%$	$56.74 \pm 2.91\%$	$31.20 \pm 5.90\%$
Al^{3+}	$59.00 \pm 5.10\%$	$11.61 \pm 1.25\%$	$8.21 \pm 1.04\%$
Fe^{3+}	$59.00 \pm 5.10\%$	$20.40 \pm 3.69\%$	$32.52 \pm 2.72\%$
	Bleached LAHA		
Na^+	$36.07 \pm 1.22\%$	$32.36 \pm 0.06\%$	$34.90 \pm 1.11\%$
Ca^{2+}	$36.07 \pm 1.22\%$	$70.85 \pm 3.65\%$	$74.48 \pm 3.44\%$
Co^{2+}	$36.07 \pm 1.22\%$	$67.58 \pm 2.36\%$	$75.00 \pm 6.77\%$
Cd^{2+}	$36.07 \pm 1.22\%$	$69.73 \pm 1.55\%$	$61.12 \pm 1.54\%$
Al^{3+}	$36.07 \pm 1.22\%$	$17.06 \pm 3.66\%$	$14.39 \pm 2.75\%$
Fe^{3+}	$36.07 \pm 1.22\%$	$10.47 \pm 4.73\%$	$44.01 \pm 6.17\%$

The Na^+ cation has the same trend as the other modified and unmodified LAHA, which suggests that the complexation of the HAs with the cations is the reason for the increased permeation rather than any electrostatic interactions.

The two main components of the bleached LAHA (aromatics removed) are carbohydrate and aliphatic moieties, which may compete for access to the surface of the biomembrane. The hydrophilic carbohydrates will not absorb into the biomembrane because they are repulsed by the hydrophobic bilayer – this gives the biomembrane protection from permeation. The cations will

bind to the carbohydrate (O- and N- alkyl groups) (Gyurcsik and Nagy 2000), which may remove the competition of the carbohydrates. The hydrophobic aliphatic moieties will then have an increased ability to absorb into the biomembrane, thus having increased permeation.

3.4 Conclusions

This study illustrated that the ability of HAs to passively permeate biomembranes can be mitigated by cation interactions. Changes in the conformation of HAs are caused by cation binding and interactions which limit the availability of certain moieties to the biomembranes. The soft acid was shown to decrease membrane perturbation more successfully than the hard +2 acids, which suggests that the interaction of HAs by bridging for the less abundant binding sites limits the ability of HA to interact with the model biomembrane.

Chemically modified HAs in the presence of cations showed that the carbohydrates and lipids did not play a major role in HA-cation interactions. The aromatic-reduced (bleached) HA with bound cations showed an increase in the ability to permeate the model biomembrane, which may be possible because the +2 cations bind the carbohydrates and allow the aliphatic components to absorb into the bilayer of the membrane.

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CHAPTER 4
SURFACTANT TOXICIY TO *ARTEMIA FRANCISCANA* AND THE INFLUENCE OF HUMIC ACID AND CHEMICAL COMPOSITION*

4.1 Introduction

Humic acids (HAs) are polydisperse, heterogeneous, amphiphilic and complex mixtures of organic molecules. They are created by the decomposition of mainly dead plant matter and combined by a range of interactions, mainly between the functional groups, to create supramolecular structures (Stevenson 1994, Sutton and Sposito 2005). The specific chemical composition of HAs varies and is dependent on their biogeochemical origin, but the major chemical constituents are aliphatics, aromatics, and carbohydrates (Stevenson 1994). Because of the high diversity of functional groups and their amphiphilic nature, HAs can interact with a variety of environmental components, including a wide range of pollutants and biological membranes (Campbell et al. 1997, Elayan et al. 2008, Ojwang' and Cook 2013, Stevenson 1994, Tipping 2002, Vigneault et al. 2000). Such interactions play an important environmental role in the transport and bioavailability of pollutants through the environment (McCarthy et al. 1985, Parent et al. 1996, Twiss et al. 1999, Vigneault and Campbell 2005, Wilkinson et al. 1993).

One class of pollutants with which HAs commonly interact are surfactants. Surfactants can enter the environment by a number of pathways including 1) waste water treatment (Rogers 1996, Stalmans et al. 1991), 2) a number of remediation practices (Mulligan et al. 2001), 3) as additives in the application of pesticide and herbicide formulations (Czarnota and Thomas , Song et al. 2012), and 4) urban and industrial run- off (Zoller 2004).

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Surfactants are amphiphilic compounds and may be nonionic, zwitterionic, cationic and anionic. Surfactants are designed to reduce the surface tension of water and, as a consequence, can affect biological processes, (Ostroumov 2006) justifying a closer look into their fate in the environment.

A surfactant's toxicity is dependent on its molecular structure, the type of organism, (Chen et al. 2014, Pavlić et al. 2005, Ying 2006) and the way the surfactant is ingested or taken-up by the cells (Abel 1974). The mechanism of toxicity is not well understood and likely adopts many different pathways. In aquatic species, a change in liver and kidney function, gill damage (Abel 1974) and enzyme inhibition have been shown (Cserhati et al. 2002). Toxicity can also be attributed to the disruption of cellular membranes by the surfactant (Abel 1974). In general, nonionic and anionic surfactants tend to have similar toxic concentration ranges (Abel 1974), while cationic surfactants are more toxic to aquatic species (Lewis and Wee 1983, Singh et al. 2002). However, there is no clear relationship between the type of a surfactant and relative toxicity. This situation becomes even more complex when surfactants associate with HAs (Chen et al. 2014, Pavlić et al. 2005, Ying 2006) under a variety of conditions. This association can be relevant to the bioavailability and toxicity of surfactants (Ishiguro et al. 2007, Koopal et al. 2004, Otto et al. 2003, Traina et al. 1996), including the ability of surfactants to perturb cellular biomembranes (Abel 1974, Luckey 2008).

The association between HAs and surfactants has been previously studied in terms of binding isotherms. The amphiphilic nature of HAs and surfactants may cause an attraction that both decreases the free concentration of the surfactants and alter the properties of the HAs in solution (Ishiguro et al. 2007, Koopal et al. 2004, Otto et al. 2003). This association between HAs and surfactants could cause the toxicity of the surfactants to be mitigated significantly. The

association of HA with surfactants has been attributed to hydrophobic interactions (Tan et al. 2009), electrostatic interactions (Ishiguro et al. 2007, Koopal et al. 2004), mixed micelle formation (Lippold et al. 2008) and forced aggregation of the surfactant micelles (Otto et al. 2003). Because HAs are complex and often contain aromatic systems, there is also the possibility for other more specific interactions, such as π-π interactions between π-donor and π-acceptor moieties of both the surfactant and the HA (Keiluweit and Kleber 2009, Pignatello 2011).

It is well known that surfactants can disrupt cellular membranes (Abel 1974, Luckey 2008); however, the complexity of real cellular membranes limits the scope of mechanistic studies into how such a disruption changes when humic acid is added to the system. Lipid vesicles, or liposomes, have been used extensively as model biological membranes in xenobiotic toxicological assessment studies (Zepik et al. 2008). Membrane perturbation can be studied with a model cell membrane to give insight into the fundamental processes of passive transport while removing the inherent complexity of a real cell system. The permeation of the membranes by the surfactants can be investigated by fluorescence spectroscopy by encapsulating a fluorescent dye within the liposome and measuring the changes in fluorescence intensity as the membrane is exposed to different environments (Elayan et al. 2008, Ojwang' and Cook 2013, Vigneault et al. 2000).

Consequently, two main questions emerge: 1) how do HAs affect the biomembrane perturbing potential and toxicity of different surfactants and 2) what is the role of different chemical components within HAs? This study is an initial step in addressing these questions by combining model biomembrane fluorescence leakage studies, *Artemia* hatching and mortality assays, and HAs with a range of chemical compositions.

4.2 Materials and Methods

4.2.1 Materials

     The humic acid standards (Leonardite HA, Florida peat HA, and Suwannee River HA) were obtained from the International Humic Substances Society (IHSS, Georgia, USA). More details on each of these HAs, including chemical composition, are available in Tables 4.1-4.3, with further details available on the IHSS website (www.humicsubstances.org, accessed on Aug 8th, 2015).

Table 4.1 Elemental compositions and stable isotopic ratios of IHSS samples ("International humic substances society" 2015)

Standard HA	H_2O	Ash	C	H	O	N	S	P	$\Sigma^{13}C$	$\Sigma^{15}N$
Suwannee River (SRHA)	20.4	1.04	52.63	4.28	42.04	1.17	0.54	0.013	nd	nd
Pahokee Peat (FPHA)	11.1	1.12	56.37	3.82	37.34	3.69	0.71	0.03	-26.0	1.29
Leonardite (LAHA)	7.2	2.58	63.81	3.70	31.27	1.23	0.76	<0.01	-23.8	2.13

(% weight)

Table 4.2. ^{13}C NMR estimates of carbon distribution in IHSS samples ("International humic substances society" 2015)

Standard HA	Carbonyl	Carboxyl	Aromatic	Acetal	Heteroapliphatic	Aliphatic	$\Sigma^{15}N$
Suwannee River (SRHA)	6	15	31	7	13	29	nd
Pahokee Peat (FPHA)	5	20	47	4	5	19	1.29
Leonardite (LAHA)	8	15	58	4	1	14	2.13

(Electronically integrated peak area percentages)

Table 4.3. Metal concentrations of HAs as determined by ICP-OES

Standard HA	Al mg/kg	Ca mg/kg	Cr mg/kg	Cu mg/kg	Fe mg/kg	Mn mg/kg	Ni mg/kg	Si mg/kg	Sr mg/kg	Zn mg/kg
Leonardite HA	2270	3482	48.8	15.4	1535	Below det.	26.6	113	42.3	Below det.
Suwannee River HA	346	381	40.7	20.8	1171	Below det.	Below det.	62.4	1.46	213
Pahokee Peat HA	139	546	Below det.	1.54	1844	Below det.	Below det.	Below det.	12.36	151

(Samples were digested in nitric acid for 16 h at 110° C in pyrex digestion tubes)

The surfactants Triton X-100, cetylpyridinium chloride and sodium dodecyl sulfate were all purchased from Sigma Aldrich (Piscataway, NJ). Sodium chloride and sodium hydrogen carbonate for the saline solution were purchased from Sigma Aldrich. Sterile 18 MΩ deionized water was sourced from an apparatus by US filter. *Artemia Franciscana* was purchased from Brine Shrimp Direct (Ogdon, UT). Fisherbrand 100×15 mm petri dishes were purchased from Fisher Scientific (Somerville, NJ). A VWR mini shaker was used during the hatching assays. An AmScope SE305R-PZ stereoscopic microscope was utilized for observing and counting the Artemia.

Sulforhodamine-B dye (SRB), t-octyl-phenoxy polyethoxy ethanol (Triton TX-100), sodium dodecyl sulfate (SDS), and cetylpyridinium chloride (CPC) were purchased from Sigma Aldrich. Sodium dihydrogen phosphate ($NaH_2PO_4 \bullet H_2O$) was purchased from Fisher Scientific while sodium hydrogen phosphate dihydrate ($Na_2HPO_4 \bullet 2H_2O$) was purchased from Sigma-Aldrich. The 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocoline (POPC) was purchased from Avanti Polar Lipids (Alabaster, AL) and the Sephadex G-50 gel for the size exclusion columns was obtained from Healthcare Biosciences (Piscataway, NJ). Benzene, methanol, and hydrochloric acid were purchased from Fischer Scientific. The nitrogen gas was supplied by Capital Welders

Supply Company (Baton Rouge, LA). All fluorescence measurements were made on a Horiba Jobin Yvon Fluorolog 3 spectrofluorimeter with a FL1073 detector, Spectra Acq computer and a model LF13751 temperature control. A Malvern Zetasizer nano (Worchester, UK) was utilized for dynamic light scattering of the liposomes.

4.2.2 Experimental design

Humic acid (HA) was chosen because it is a major portion of humic substances, is highly amphiphilic, i.e., contains both hydrophilic and hydrophobic functional groups (Thorn et al. 1989), and there are several well-characterized HA standards commercially available. Three humic acids of different sources were chosen to sample a range of HA chemical compositions: Suwanne River HA (SRHA; aquatic source), Pahokee peat HA (FPHA; peat source) and Leonardite HA (LAHA; lignite coal source). Comparing three HAs of different origins can provide only a limited amount of information about the components of HAs that are involved with toxicity mitigation of surfactants. Therefore, to gain deeper insight into the roles played by each specific HA component in the binding of surfactants, three chemical modifications were performed on LAHA: bleaching (reduced aromatics), Soxhlet lipid extraction (reduced lipids), and hydrolysis (reduced carbohydrates). LAHA was chosen as it gives the same trends as the other two HAs and is economically viable.

Triton X-100 (Tx-100), cetylpyridinium chloride (CPC) and sodium dodecyl sulfate (SDS), represent three different classes of surfactants: the non-ionic, cationic, and anionic, respectively (see Figure 4.1 for structures) and were chosen for their extensive use (Coleman and Waldroup 1999, SIDS initial assessment profile: Sodium dodecyl sulfate, Triton surfactants: FDA status of triton surfactants 2010). The structures of these surfactants can be seen in Figure 4.1.

The concentration chosen was one that showed a dramatic difference in either the hatching or mortality percentages compared to the saline water control ($> LC_{50}$). The Tx-100, CPC, and SDS were found to have significant toxicity levels at 100, 3.5, and 25 ppm, respectively. Therefore, these concentrations were also used when testing the toxicity of each surfactant when associated with the HAs.
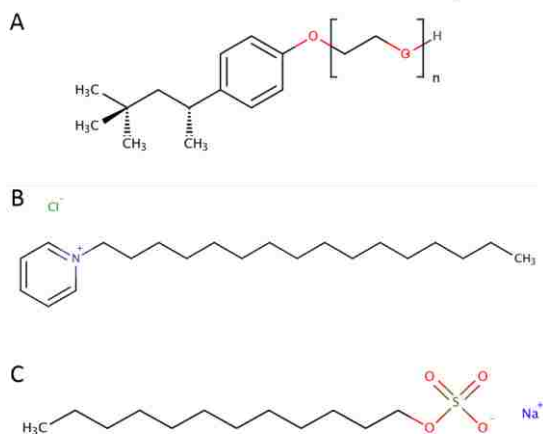
Figure 4.1 Chemical structures of A) Tx-100, B) CPC, and C) SDS

All hatching assays were performed in triplicate and repeated at least three different times to verify reproducibility.

The surfactant concentrations chosen for the model biomembrane were below the critical micelle concentration (CMC) but also high enough to cause significant perturbation of the biomembranes. For Tx-100 (CMC ≈ 150 ppm) (Triton surfactants: Fda status of triton surfactants 2010) and CPC (CMC = 40.8 ppm) (Safety data sheet: Cetylpyridinium chloride 2015), the concentrations was ≈ 70% of the CMC while the SDS (CMC ≈ 2365 ppm) (Moroi et al. 1974) was ≈ 5% of the CMC due to the high percentage dye release in the presence of NaCl. SDS concentration was also limited by humic acid concentrations because a 1:1 ratio of HA to SDS was

desired. *Artemia Franciscana*, or brine shrimp, were chosen as model living organisms as they are commercially available, the cysts (eggs) can stay dormant for long periods of time, they are easy to hatch, and have a short life span.

In addition to the simplicity of the procedures, lower volumes of toxins and solutions are needed relative to other species because of their small size (0.4 – 10 mm in length, depending on age – see Figure 4.2) (Nunes et al. 2006).
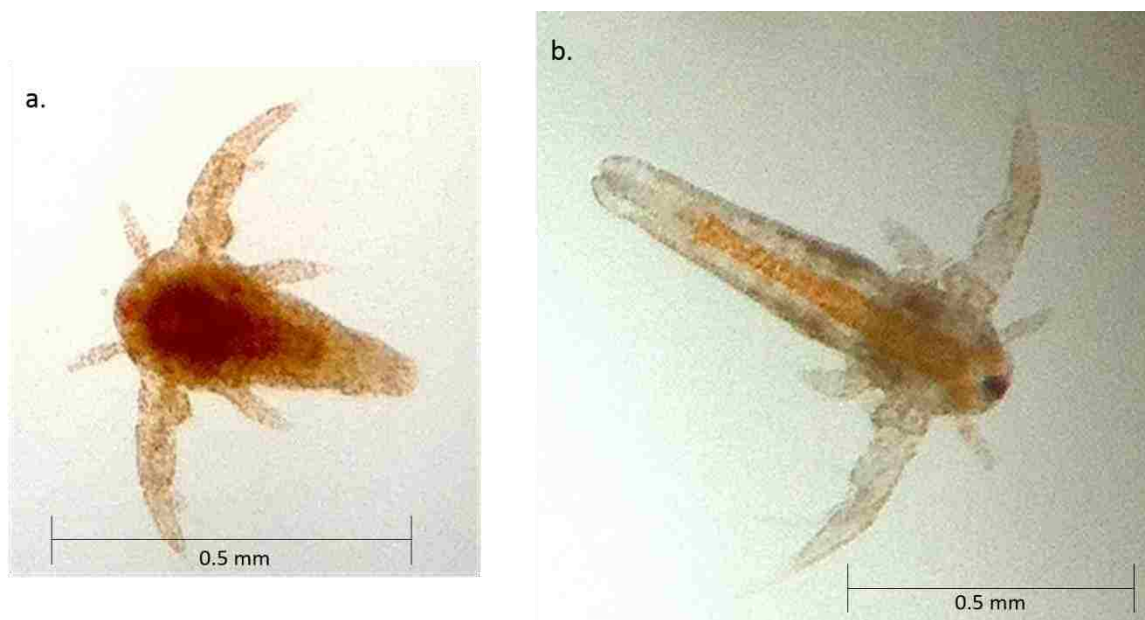
Figure 4.2 *Artemia Franciscana* at a) 24 h and b) 48 h in 35 ppt NaCl at pH 7.8

Artemia Franciscana have been previously utilized for three different types of toxicology assessments: hatching (MacRae and Pandey 1991, Nunes et al. 2006), short-term mortality (≤ 48 h) (Arulvasu et al. 2014, Nunes et al. 2006), and long-term mortality (> 48 h) (Manfra et al. 2012, Nunes et al. 2006). Various pollutant toxicity mechanisms can inhibit hatching or be lethal to the hatched *Artemia*. For this study, only hatching and short-term mortality assays were used because long-term mortality assays would require feeding the *Artemia* algae, which would add another level of complexity when determining toxic effects of surfactants in the presence of humic acids.

Fluorescence experiments with model biomembranes experiments were designed to give further credence to the effects of surfactant-HA associations observed in the *Artemia* hatching assays with a simpler system.

4.2.3 Sample preparation

Humic acid stock solutions were prepared by dissolving approximately 20 mg of HA, including chemically modified HAs, in 18 MΩ deionized water (if HA solubility issues arose at low pH, a small amount of NaOH was added). The pH was adjusted to the desired value by HCl and NaOH. The solutions were diluted with sterile 18 MΩ deionized water and stirred overnight. When necessary, the pH was re-adjusted after the equilibration period.

4.2.4 Humic acid chemical modification

Three procedures for chemical modification were performed on LAHA: acid hydrolysis (Almendros 1994), Soxhlet lipid extraction (Chilom et al. 2009) and bleaching (Wise et al. 1946).

Acid hydrolysis: 300 mL of 6 M HCl per gram of HA were mixed together and maintained under reflux for 6 h. The acid was removed from the HA by dialysis. The modified HA was freeze-dried for 24 h or until completely dry.

Soxhlet extraction: The HA was placed into the thimble of the assembly and inserted into a Soxhlet extractor fitted with a condenser. Approximately 200 mL of benzene: methanol (3:1) azeotrope was placed in a round bottom flask fitted onto the Soxhlet extractor. It was then heated in a sand bath and refluxed for at least 72 hours. Subsequently, the thimble was removed from the extractor and the solvent was allowed to evaporate in the hood.

Bleaching: The original procedure by Wise et al., (Wise et al. 1946) was used to isolate wood holocellulose; however, it was modified in this work by increasing the bleaching time. The bleach solution for one gram of HA contained 10 g sodium chlorite, 10 mL glacial acetic acid, and

100 mL deionized water. The HA and the bleach solution was stirred overnight in the hood. It was then centrifuged at 3500 g for 15 min and the bleach solution was decanted from the HA. This was repeated 3 times with fresh bleaching solution. The final HA residue was separated by centrifugation and followed by dialysis.

The dialysis waste was tested with $AgNO_3$ to verify that all chlorine had been removed before freeze-drying the modified HA. It was then freeze-dried for 24 h or until dry.

The chemically modified and unmodified HAs were characterized by solid state CP-MAS ^{13}C NMR. Homogenized HAs were tightly packed into a 2.5 mm high-resolution magic angle spinning zirconium rotor (Bruker). Spectra were acquired at 100 MHz with a spinning rate of 5 kHz and a ramp polarization contact time of 2 ms. The recycle delay time was 1 s and a total of 4,096 scans were collected per experiment.

4.2.5 Surfactant stock solutions

Stock solutions of 10,000 ppm (1%) of Tx-100, CPC, and SDS were prepared by dissolving 1 g of the surfactant into 100 mL of 18 MΩ water. Final dilutions and pH adjustments were made for the final sample solution.

4.2.6 Humic acid and surfactant mixture solutions

Humic acid and surfactant solutions were prepared by adding appropriate amounts of a 70 parts-per-thousand (ppt) NaCl solution for a final concentration of 35 ppt NaCl (to mimic saline environments), humic acid stock solution and surfactant stock solution into 50 mL volumetric flask. The samples were diluted to 50 mL and the pH was adjusted to 7.8 with sodium hydrogen carbonate. The sample solutions were allowed to equilibrate overnight. The *control solution* was 35 ppt NaCl adjusted to pH 7.8 with sodium hydrogen carbonate for all sample series.

4.2.7 Model biomembrane preparation

For liposome fluorescence measurements, all model biomembranes, humic acid solutions, and surfactant solutions were in a 0.01 M phosphate buffer at pH 7.0.

The sulforhodamine-B (SRB) vesicles were prepared as previously described.[5] In short, a lipid film was created in the bottom of a round bottom flask by dissolving POPC in 66 µL methanol and 132 µL chloroform (1:2 solution), stirring the solution for 30 minutes and then evaporating under nitrogen gas for 24 hours resulting in a thin lipid film. The lipid film was hydrated with 5 mL of 50 mM SRB dye in phosphate buffer and the solution was vortexed until the lipid was fully suspended in solution. The solution then underwent three freeze/thaw cycles (placed in dry ice and acetone until the mixture was completely frozen and then heated to 80°C) to yield large multilamellar vesicles. The thawed vesicles were then extruded utilizing a Lipex Lipid Extruder (North Lipid, Vancouver, BC, Canada) through a 100 nm-pore Whatman Nuclepore polycarbonate track-etched membrane to create large unilamellar liposomes (model biomembranes). Size exclusion chromatography was used to remove the non-encapsulated dye from the dye-loaded liposome solution by passing the liposome solution through three consecutive columns packed with Sephadex-G 50 resin with phosphate buffer as the elution buffer. Dynamic light scattering (DLS) was used to verify the size and monodispersity of the model biomembranes. The DLS measurements were made with a scattering angle of 90° and a wavelength of 6471 Å. The DLS results confirmed the formation of vesicles with a diameter of 100 nm.

4.2.8 Fluorescence measurements

The blank for all fluorescence measurements was a solution of liposomes and the phosphate buffer. Excitation and emissions wavelengths of 565 nm and 585 nm, respectively (the excitation and emission maximum for SRB), were used for all fluorescence measurements. Ten minutes after

introducing the surfactants and humic acids to the liposomes, fluorescence spectroscopy was utilized to determine the amount of dye released from the liposomes due to membrane perturbation. Triplicates were made of each sample and measured by fluorescence to verify reproducibility. The following equation was used to calculate the percent dye release from the liposomes relative to the lysed membranes:

Percent dye release = 100 % x $(I_H - I_B)/(I_T - I_B)$ (1)

where I_H is the fluorescence intensity of liposomes in the presence of HA sample, I_B is the fluorescence intensity of the blank, and I_T is the fluorescence intensity of the dye after the liposomes are ruptured with the surfactant alone.

4.2.9 Artemia hatching assay

The hatching and mortality assay procedures used in this study are based on previous *Artemia* toxicity studies (Arulvasu et al. 2014, Distribution, life cycle, taxonomy, and culture methods: Brine shrimp (artemia salina), Sorgellos et al. 1978). The *Artemia* cysts were first hydrated for two hours in 18 MΩ water kept at 5 °C. Once hydrated, 25 to 28 *Artemia* cysts were placed into individual 100 mm × 15 mm petri dishes and the total amount of cysts was recorded. To each sample, 10 mL of the saline/pollutant solution was added. Three replicates were used for the control and all samples. The petri dishes were placed on a shaker at 100 rpm. The shaker was used to keep the samples aerated to limit anoxia. The *Artemia* were not fed during the 48 h hatching assays. The number of *Artemia* hatched and the number of dead-hatched were counted at 20, 24, 32, 44, and 48 h using a stereomicroscope. Healthy *Artemia* are highly active so individual *Artemia*

were considered dead if there was no movement within five seconds (Matthews 1995, Rodd et al. 2014).

Hatching percentage = 100 % × [# of hatched *Artemia*]/[total *Artemia*]   (2)

Mortality percentage = 100 % × [# of dead *Artemia*]/[# of hatched *Artemia*]   (3)

4.3 Results

4.3.1 Fluorescence results and discussion

Fluorescence spectroscopy was used to determine the changes in surfactant permeation of the model biomembranes in the presence of aquatic Suwannee River humic acid (SRHA, as it was found that SRHA did not quench the release fluorescent dye, under conditions used in this study) by measuring the intensity of fluorescent dye released relative to the surfactant alone. The SRHA concentration was varied while maintaining a constant concentration of surfactants. Since the *Artemia* hatching assays required a salt-water environment, the leakage studies were performed in both fresh water and saline water (35 ppt NaCl). The data presented in Figure 4.3 are the percentage of the dye released from the liposomes relative to the surfactant and liposomes alone in fresh water. SRHA has no effect on the Tx-100's ability to perturb the liposomes in either the fresh water or in 35 ppt NaCl solution. The SRHA did not interact enough with the non-ionic surfactant to cause any changes in the perturbation. The cationic surfactant, CPC, showed a decrease in liposome dye release as SRHA was added except for the 30 ppm SRHA, which had an increase in perturbation relative to the two lower concentrations of SRHA. This was unexpected but may be caused by aggregation of the SRHA at the higher concentration. In 35 ppt NaCl, the CPC alone caused a decreased perturbation of the liposomes relative to the fresh water environment. The salt likely

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plays a protective role by surrounding the negatively charged liposomes with positively charged sodium ions and thus either repelling the positively charged CPC or limiting CPC's access to the liposome. As SRHA was added to the CPC and saline water solution, there was a slight decrease in membrane perturbation, which suggests some interaction between the SRHA and CPC, but the percent dye release was still greater (meaning more perturbation) than that at the low concentrations of SRHA and CPC in fresh water. The sodium ions were likely interacting with the negatively charged moieties of the SRHA and not allowing CPC as much access to the binding sites as in fresh water. This evidence suggests that much of the CPC-SRHA interactions are electrostatic. The anionic surfactant, SDS, had less of an interaction with the liposomes than the other two surfactants, which was exhibited by lower fluorescence intensity (not shown).

The SDS has a much greater dye release in the saline water solution than in the fresh water solution. This constitutes further evidence that the sodium ions surround the negatively charged liposomes, decreasing the repulsion between the liposomes and the SDS, which allows the SDS to permeate the liposome.
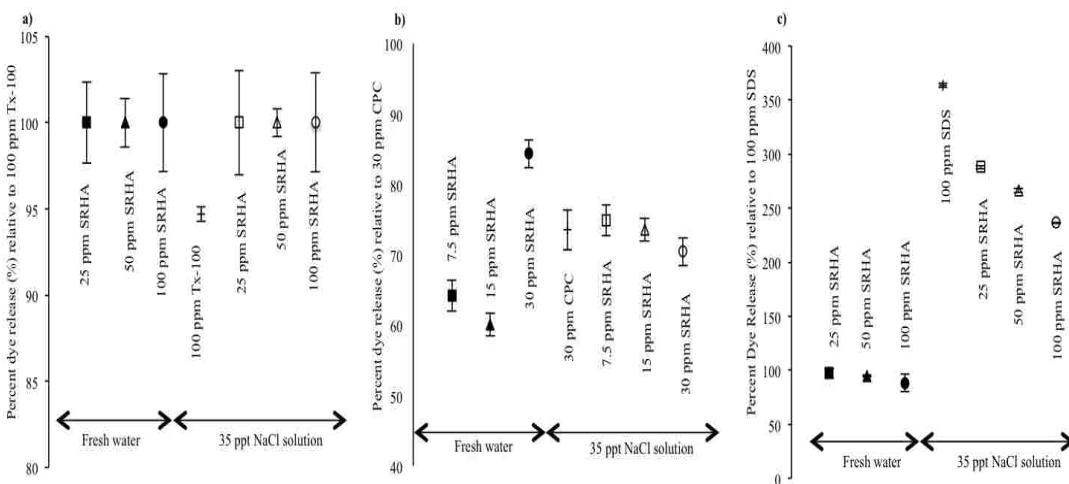
Figure 4.3 Percent liposome SRB dye release induced by a) Tx-100, b) CPC and c) SDS with varying concentrations of SRHA and salinity.

There is little interaction between the SDS and SRHA in the fresh water environment because of the electrostatic repulsions. However, in 35 ppt NaCl solution, there is a significant decrease in liposome perturbation when SRHA is added to the system.

Again, the sodium ions must be playing a role in limiting the electrostatic repulsion and allowing the SRHA to interact with the SDS.

4.3.2 Chemically modified humic acid

The ^{13}C NMR spectra for the modified HAs are presented in Figure 4.4. For the hydrolyzed LAHA, there is a decrease in the O- and N- alkyl region (90-65 ppm) and an increase in relative percentage area of the aromatic region.
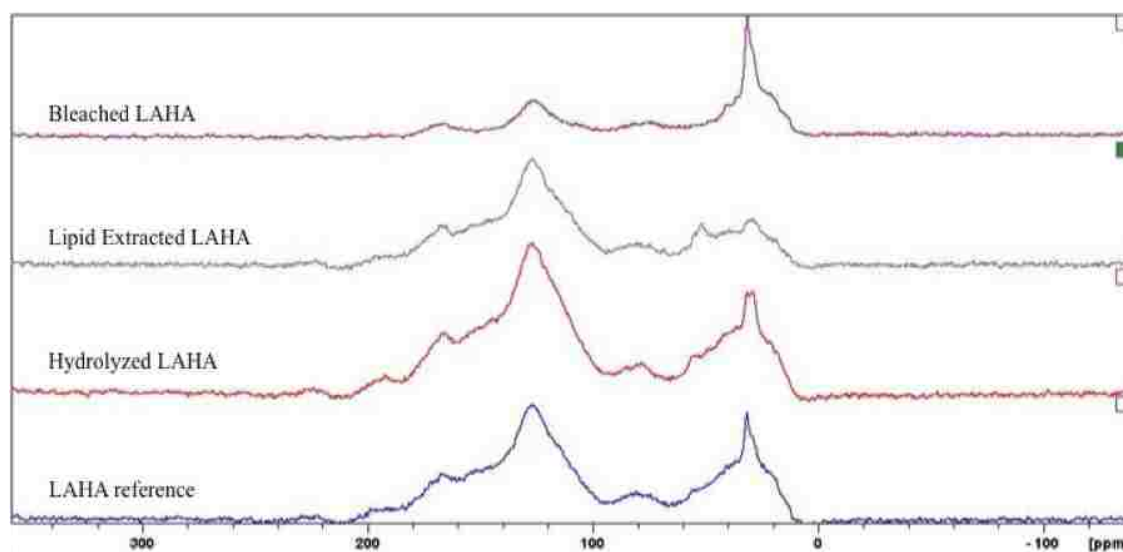
Figure 4.4 ^{13}C NMR spectra of the chemically modified Leonardite humic acid (LAHA)

This indicates a reduction of the carbohydrate moieties versus the other chemical moieties within the sample. In the lipid-extracted LAHA, there is a decrease in the region corresponding to the polymethylene chains and an increase in the relative percent area of the aromatic region, which is consistent with the reduction of lipid moieties versus the other chemical moieties within this sample.

Finally, the bleached LAHA spectrum shows a significant decrease in the relative percent area of in the aromatic region (165 – 90 ppm), and hence, a reduction in aromatic moieties versus the other chemical moieties within this sample. Tables 4.4 and 4.5 provide detailed information on the chemically modified humic acids.

Table 4.4 ^{13}C NMR relative percent areas of unedited and edited LAHA

Standard HA	Carboxyl	Aromatic	Aldyhyde/K etone	N- or O- Alkyl	Alkyl
Bleached LAHA	4.03%	29.86%	0.0%	9.43%	38.44%
Lipid Extracted LAHA	9.99%	60.71%	0.47%	6.28%	22.56%
Hydrolyzed LAHA	9.08%	62.38%	0.71%	1.78%	26.05%
LAHA Reference	11.18%	52.89%	0.07%	7.11%	37.74%

(Electronically integrated peak area percentages)

Table 4.5 Metal concentrations of chemically modified HAs as determined by ICP-MS

Modified HA	Al mg/ kg	Ca mg/ kg	Cr mg/ kg	Cu mg/ kg	Fe mg /kg	Mn mg/kg	Ni mg/ kg	Si mg/ kg	Sr mg/ kg	Zn mg/kg
Bleached LAHA	393	1856	21.0	62.0	582	12.8	104	362	11.8	38.6
Hydrolyzed LAHA	76.5	157	22.9	7.91	168	Below det.	12.7	17.7	1.11	Below det.
Lipid extracted LAHA	3231	5094	62.6	23.7	3134	2.57	37.6	227	59.9	Below det.

(Samples were digested in nitric acid for 16 h at 110° C in pyrex digestion tubes)

The data in Figures 4.5 and 4.6 clearly show that none of the HAs studied were toxic to the *Artemia Franciscana* for the conditions used. In regards to HAs being toxic, we have previously studied (Elayan et al. 2008, Ojwang' and Cook 2013) this phenomenon extensively with model systems by a range of techniques including ^{31}P NMR and fluorescence leakage assays and have

found that HAs can induced passive membrane perturbation at acidic pH, but induce little to no perturbation at pHs of 7 or higher, as used in this study.
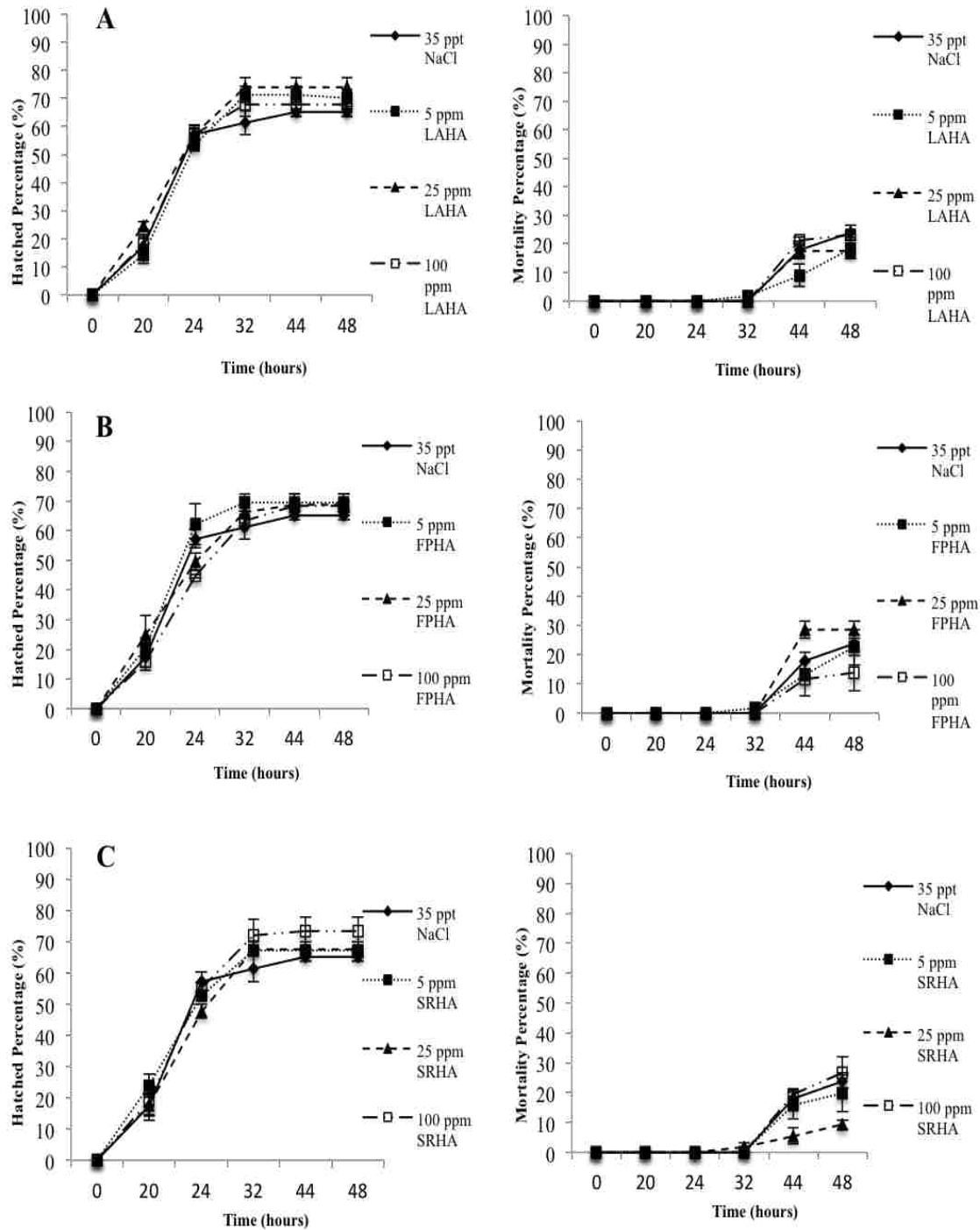
Figure 4.5 *Artemia* hatching and mortality assays with A) LAHA, B) FPHA and C) SRHA
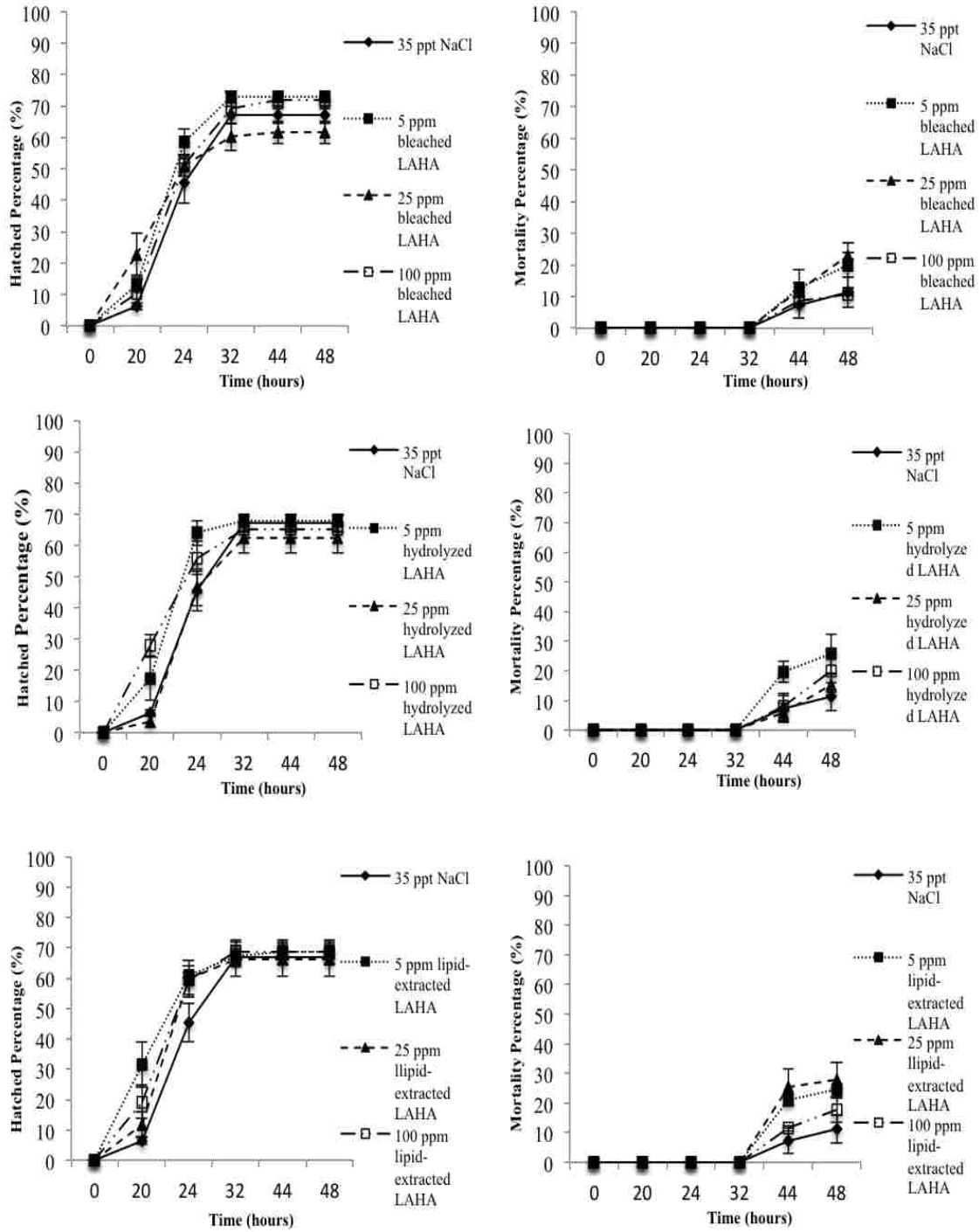
Figure 4.6 Chemically modified LAHA *Artemia* hatching and mortality assays

Thus, the finding here in terms of HAs toxicity is consistent with our previous study on model membrane systems. This is comforting and illustrates the usefulness of model systems as well as living organisms in toxicity studies.

4.3.3 Hatching assays with a non-ionic surfactant – Tx-100

Triton-X 100 (Tx-100), a non-ionic surfactant, showed no effects on the *Artemia's* hatching percentage but it did have a significant effect on mortality at a concentration of 100 ppm and above. The influence of the different HAs at concentrations of 25, 50, and 100 ppm is shown in Figure 4.7. The data presented in Figure 4.7 clearly show that LAHA reduces the toxicity of Tx-100, even at concentrations as low as 25 ppm (versus the 100 ppm Tx-100) and that LAHA's ability to mitigate this toxicity increases at 50 ppm. However, there is no difference in the ability of LAHA to mitigate Tx-100 toxicity between LAHA concentrations of 50 and 100 ppm (Figure 4.7A). Due to the ionic strength and LAHA concentration, it seems logical that this observation is due to LAHA aggregation (Ojwang' and Cook 2013) FPHA also shows an ability to mitigate the toxicity of Tx-100, however to a lesser degree than LAHA and only at concentrations of 50 ppm and higher (Figure 4.7B). SRHA, on the other hand, shows little to no ability to mitigate the toxicity of Tx-100. Fluorescence leakage experiments (Figure 4.3) show a consistent view that SRHA does not reduce the ability of Tx-100 to induce biomembrane permeability. This toxicity enhancement can be caused by SRHA's ability to permeate cellular membranes due to membrane defects caused by the Tx-100 surfactant (Vigneault et al. 2000).

4.3.4 Hatching assays with a cationic surfactant – CPC

Unlike Tx-100, the cationic surfactant cetylpyridinium chloride (CPC) had significant impact on the hatching percentage of the *Artemia* at 3.5 ppm and above but did not affect the mortality percentage. The changes in hatching percentages in the presence of HAs can be seen in

Figure 4.8. The ability to mitigate CPC's toxicity increased with HA concentration, with LAHA being the most effective. In fact, even at 5 ppm LAHA, the hatching percentage was very similar to the control sample (Figure 4.8A), suggesting that the toxicity of CPC is completely mitigated by the LAHA.

FPHA and SRHA have similar CPC toxicity mitigation trends. Unlike the LAHA, even the lowest concentration of 1 ppm has a significant effect on the levels of toxicity. At the highest concentration of 5 ppm, FPHA nearly completely mitigates the toxicity (Figure 4.8B). SRHA mitigates CPC toxicity but not to the same extent as FPHA at higher HA concentration (Figure 4.8C).

4.3.5 Hatching assays with an anionic surfactant – SDS

Similar to CPC, the anionic surfactant SDS also had a significant effect on the *Artemia* hatching percentage but not the mortality percentage. All HAs at concentrations of 5, 10, and 25 ppm showed the ability to mitigate the toxicity of SDS to some extent. Unlike FPHA and SRHA, the ability of LAHA to mitigate SDS toxicity was concentration independent, with all three tested concentrations reducing the toxicity of the SDS surfactant by about half or yielding toxicity midway between the control solution and the SDS solution in the absence of HA (Figure 4.9A). FPHA's SDS toxicity mitigation increased with HA concentration, but not in a linear manner (Figure 4.9B). SRHA showed an overall similar mitigating potential to SDS, but again, in a non-linear manner (Figure 4.9C).

Since HAs are anionic, they are expected to repel the negatively charged sulfate group of the SDS, resulting in limited binding (Koopal et al. 2004). However, the *Artemia* are in a saline environment containing positively charged Na^+ ions. These cations are expected to electrostatically interact with the SDS and the HA to reduce electrostatic repulsion.

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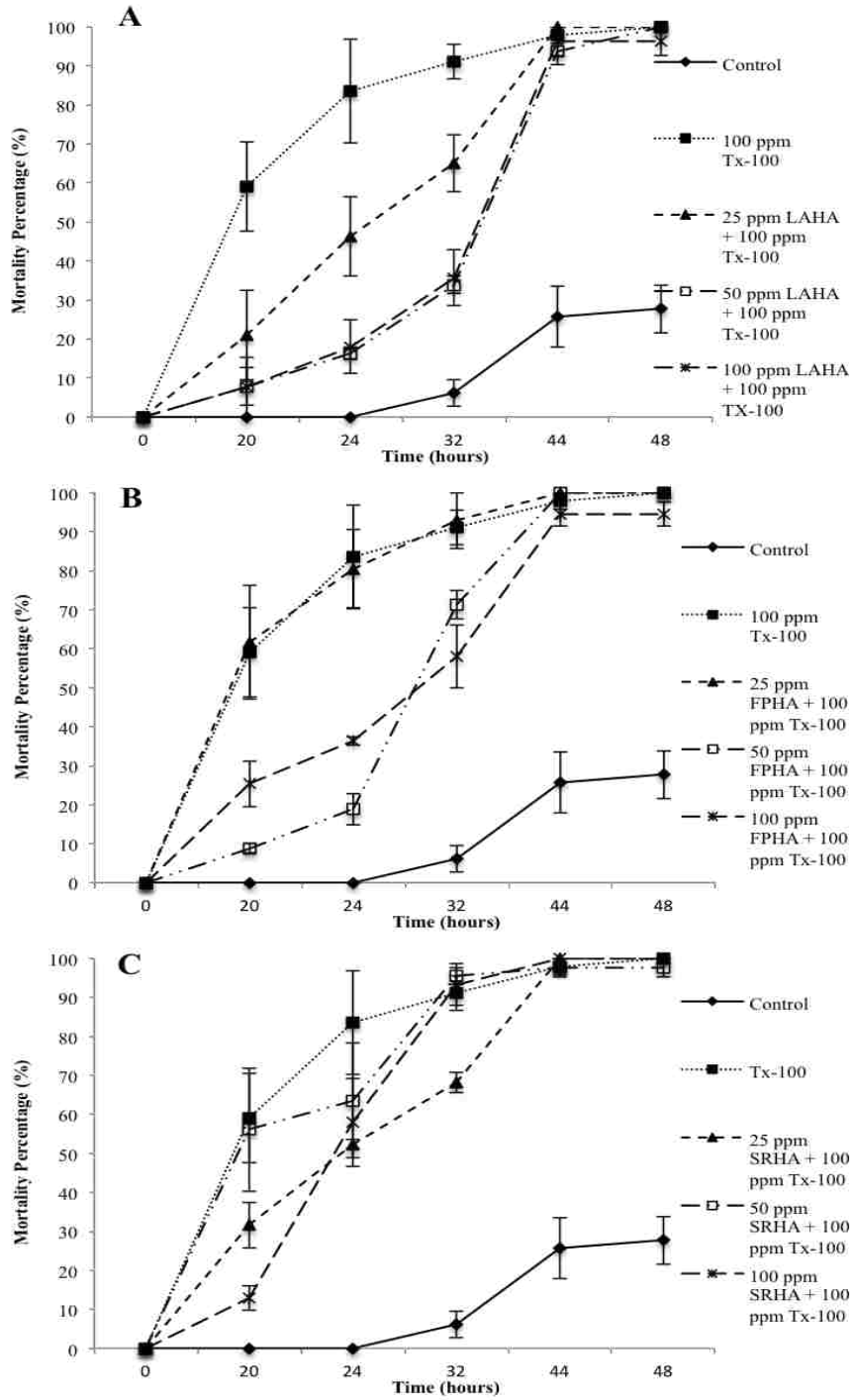
Figure 4.7 *Artemia* hatching assays at 100 ppm Tx-100 and LAHA, FPHA, and SRHA
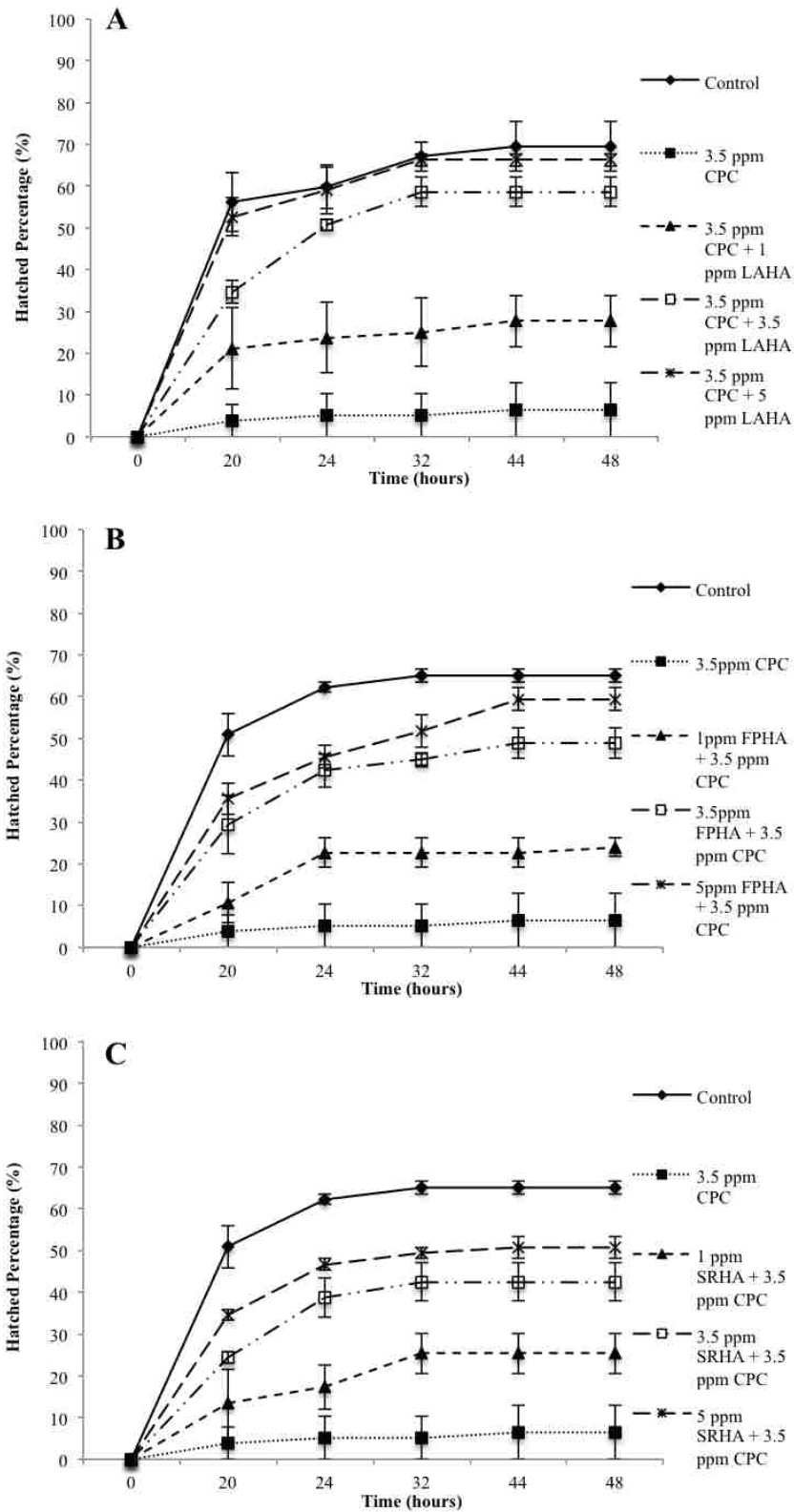
Figure 4.8 *Artemia* hatching assay at 3.5 ppm CPC and LAHA, FPHA, and SRHA.
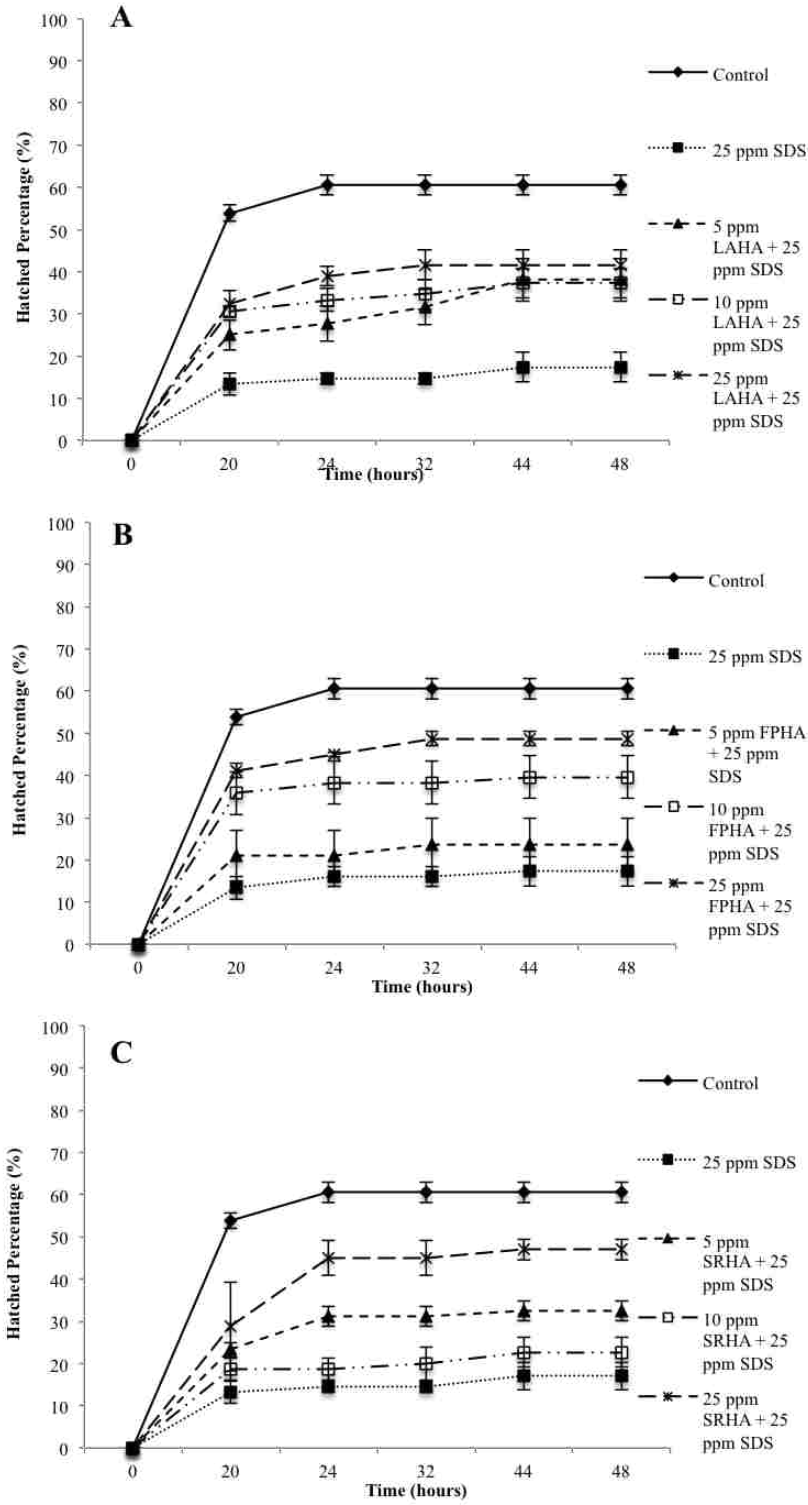
Figure 4.9 *Artemia* hatching assays at 25 ppm SDS and LAHA, FPHA, and SRHA.

This phenomenon can be also seen in the fluorescence study with liposome membranes (see Figure 4.3), whereby the presence of the SDS alone increased membrane permeability in both the saline and fresh water environments, but upon introduction of SRHA, there was a more significant decrease in membrane perturbation in the saline environment relative to that in the fresh water environment.

4.3.6 Chemically modified humic acid with surfactants

While studying HA-surfactant interactions alone provides relatively limited information in terms of the role of HA composition, chemically modified LAHA was utilized to determine specifically which components of the HA may be involved in the interactions. The data in Figure 4.10A show that when the lipids are extracted, the ability of LAHA to mitigate Tx-100's toxicity is almost completely removed. At the same time, samples with enhanced polymethylene chains demonstrate a slight increase in their ability to mitigate Tx-100's toxicity.

The hydrolyzed, lipid-extracted, and the unmodified LAHA all mitigate the CPC toxicity (Figure 4.10B). The bleached LAHA has a lower hatching percentage, indicating that it does not have the ability to mitigate the toxicity of CPC to the same extent as the unmodified, lipid-extracted, and the hydrolyzed LAHA.

All of the chemically modified LAHA somewhat mitigated the toxicity of SDS relative to the SDS alone (Figure 4.10C). However, in the presence of LAHA (modified or unmodified), the SDS remained still somewhat toxic.

4.4 Discussion

4.4.1 Non-ionic surfactant – Tx-100

The HA demonstrated the following trend in Tx-100 toxicity mitgation: LAHA > FPHA > SRHA (Figure 4.7). Conceivably, two possible interactions could be occuring. First, the aromatic

component of the HA, by π-π stacking with that of Tx-100 (Keiluweit and Kleber 2009, Pignatello 2011), could make Tx-100 unavailable to the *Artemia* and essentially mitigate Tx-100's toxicity.

The trend in the ability to mitigate TX-100 toxicity parallels the aromatic content of the different HAs, with LAHA demonstrating the highest, and SRHA having the lowest, such ability. Secondly, the observed trend in Tx-100 toxicity mitigation of the different HAs may be due to their polarity.

The polarity indices obtained by elemental analysis ((O + N) / C) for LAHA, FPHA, and SRHA: 0.51, 0.73, and 0.83, respectively (elemental composition of these HAs are provided in Table 4.1)(Xing et al. 1994). Based on the chemical composition of the HAs, it can be postulated that the less polar or the more hydrophobic the HA, the better it can mitigate Tx-100 toxicity, exploiting interactions with the hydrophobic end of the Tx-100 molecule. The chemical modification of the HA can elucidate which of the proposed interactions is the primary interaction.

Coincidentally, an aggregation study found these same components to be largely responsible for the amphiphilic character of HA samples (Chilom et al. 2009). Thus, the data in Figure 4.10A suggest the amphiphilic character of the lipid component play a large role in HA's ability to interact with the non-ionic Tx-100 surfactant. When this component is removed, that interaction is significantly weakened and the ability of a humic acid to mitigate Tx-100's toxicity is either removed or greatly reduced.

This proposal is also consistent with the more polar HA - such as SRHA - being less able to mitigate Tx-100's toxicity (see Figure 4.7), but it also offers strong evidence against aromatic moieties playing a role in HA's ability to mitigate Tx-100's toxicity. Additionally, the data in Figure 4.10 show that the bleached LAHA, depleted in aromatic content, was still able to mitigate Tx-100 toxicity to almost the same level as the unmodified LAHA.
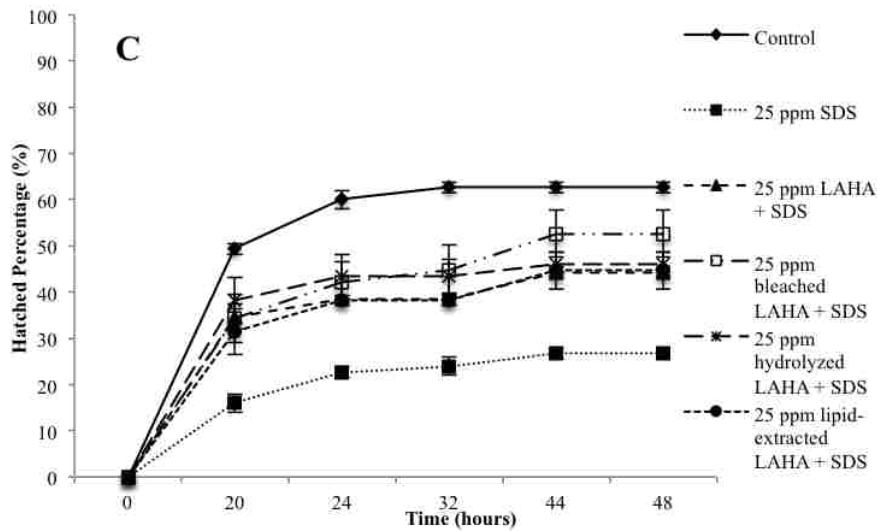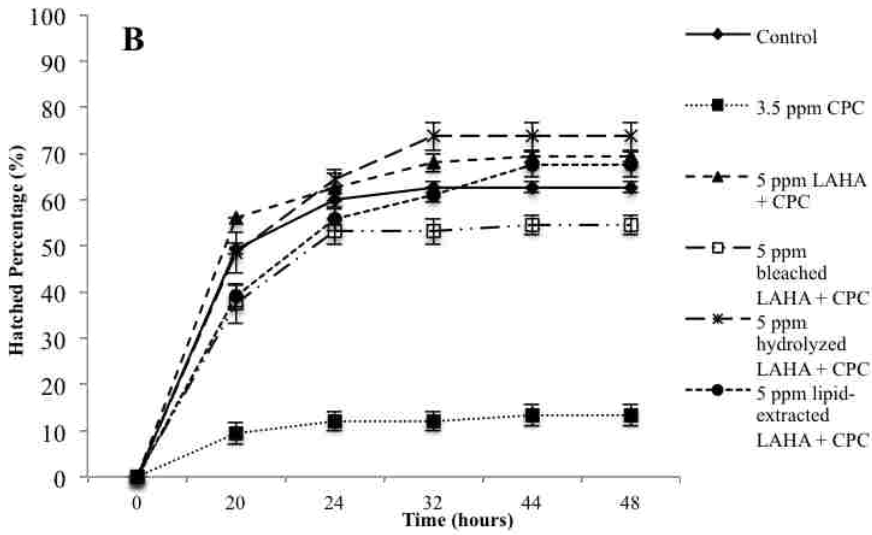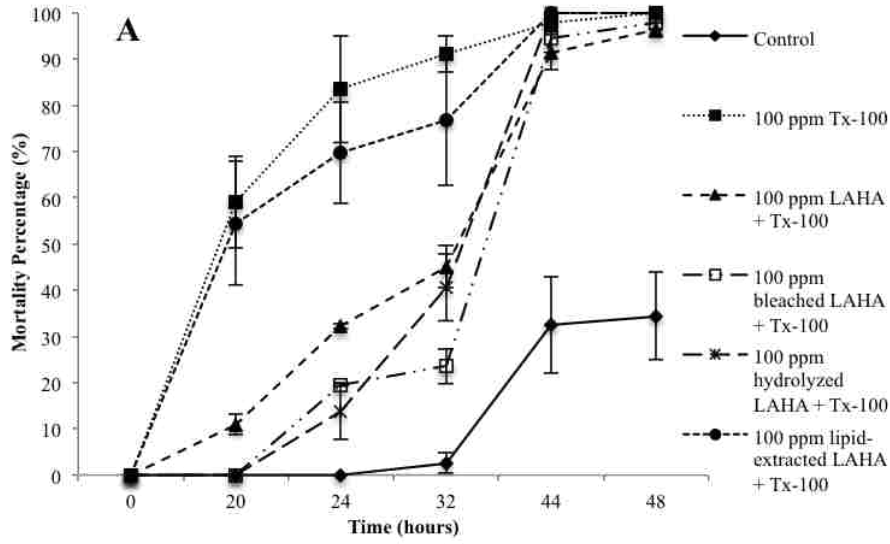
Figure 4.10 *Artemia* hatching assays at 100 ppm Tx-100, 3.5 ppm CPC and 25 ppm SDS in the presence of chemically modified LAHA

This implies the increased importance of "hydrophobic" interactions in mitigating the toxicity of Tx-100 and downplays the possibility of π-π interactions in the interaction of Tx-100 with HAs.

The bleached and hydrolyzed LAHA samples reduced the early stages of Artemia mortality induced by Tx-100. There is a two-fold explanation for this observation. First, the reduction of the aromatic (in the bleached sample) and carbohydrate moieties (in the hydrolyzed sample) concentrates the aliphatic and lipid-like moieties, increasing their toxicity mitigating capacity. In addition, reducing the amount of aromatic and carbohydrate moieties limits the HAs' potential to block lipid-like moieties (Lattao et al. 2008, Mitchell and Simpson 2013) from an interaction with Tx-100, enhancing their ability to interact with, and reduce the toxicity of, Tx-100.

4.4.2 Cationic surfactant - CPC

For both FPHA (Figure 4.8B) and SRHA (Figure 4.8C), the CPC toxicity mitigation capacity of the HA does not linearly change with concentration, suggesting partial aggregation of HAs at higher concentrations (Ojwang' and Cook 2013). This partial aggregation involves HA's hydrophobic moieties (possibly aromatic groups) reducing their availability to interact with the CPC hydrophobic domain.

The trend in the CPC toxicity mitigation by HAs is the same as that seen for Tx-100, and so the same two mechanisms may be proposed, namely the CPC π-π stacking with the aromatic component of the HAs as well as the hydrophobic interactions. In addition, because CPC is cationic and HAs have an overall anionic nature at pH 7.8, it can be assumed that at least some of the CPC-HA interactions are caused by electrostatic attractions and the formation of ion pairs (Otto et al. 2003).

The bleached LAHA had a lower hatching percentage (Figure 4.10B), suggesting that the aromatic moieties play a role in binding CPC. Hence, when the aromatics are removed by bleaching, there are fewer interactions between CPC and the HA. This is consistent with the results obtained for CPC and HAs from different sources, as shown in Figure 4.8. SRHA has a low percentage of aromatic groups and did not demonstrate the CPC toxicity mitigating capacity of LAHA or FPHA, both of which have higher percentages of aromatic groups (Thorn et al. 1989). Since CPC contains a positively charged aromatic, and hence, hydrophobic head group, HAs have the ability to engage in aromatic π-π stacking interactions and/or form ion pairs with the surfactant to mitigate CPC toxicity (Chin et al. 1997, Keiluweit and Kleber 2009, Laor et al. 1998, Pignatello 2011). This explanation is further strengthened by the fact that the hydrolyzed sample has a better toxicity mitigating capacity than the unmodified LAHA, as it has been found that carbohydrate moieties can block aromatic interaction sites (Mitchell and Simpson 2013). In other words, the removal of the carbohydrate moieties frees up aromatic moieties to associate with CPC, and thus, reduce CPC's toxicity. In addition, it appears that the removal of carbohydrate moieties is capable of enhancing the ability of the *Artemia* to hatch in the presence of CPC. Although interesting, this finding is beyond the scope of the presented work, but it will be the subject of future investigations.

4.4.3 Anionic surfactant - SDS

All HAs at concentrations 5, 10 and 25 ppm had the ability to mitigate the toxicity of SDS, likely through the electrostatic and hydrophobic/hydrophilic interactions (Koopal et al. 2004). Unlike FPHA and SRHA, the ability of LAHA to mitigate SDS toxicity was not concentration dependent (Figure 4.9A). This could suggest that either LAHA and SDS bind in a limited and non-specific manner or the possible HA sorption sites for the SDS's sulfur head group (such as nitrogen) are saturated, due to LAHA aggregation and conformational changes. Another possibility

is that LAHA is associating with, and hence, protecting the membrane from SDS's toxic effect, and membrane association sites are saturated even at the lowest LAHA concentration (Ojwang' and Cook 2013). The hatching percentage for the SDS with 5 ppm SRHA was higher than that for SDS with 10 ppm SRHA, which constitutes a discrepancy compared to the mitigation trends of the previous surfactants (Figure 4.9C). Because of HAs' heterogeneous nature, many different interactions and conformations of moieties within the HA are possible (Bonin and Simpson 2007). Accordingly, interactions of SRHA with SDS at a low SRHA concentration may have become limited when the concentration was increased to 10 ppm due to changes in HAs' conformation or an altering of the interaction patterns. As SRHA concentration continued to increase to 25 ppm, those conformational changes may have been overcome. It has been previously proposed that structure, conformation, and accessibility of HA moieties can play a role in HAs' interactions with pollutants (Mitchell and Simpson 2013).

Trends observed for the chemically modified LAHA (Figure 4.10C) are consistent with those reported for HA from different sources and with the proposal that SDS engages in non-specific binding with the HA, which may be due to a combination of electrostatic (repulsions counteracted by the Na^+ ions in the saline solutions) and hydrophobic/hydrophilic interactions (Koopal et al. 2004).

4.5 Conclusions

Overall, the results presented above show that HAs reduce the toxicity of surfactants, and need to be considered in studies of surfactant toxicity. The results of this work show that the chemical composition of the HA is an important factor in determining their effectiveness in mitigating surfactant toxicity and that HAs mitigate the toxicity of the various surfactants

differently. While there is no universal mechanism by which HAs mitigate the toxicity of

surfactants, there is a range of possible mechanisms due to the complex nature of HAs.

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CHAPTER 5
SURFACTANT'S INFLUENCE ON *ARTEMIA FRANCISCANA'S* EMBRYONIC PHOSPHO-METABOLITE PROFILE AND THE INFLUENCE OF HUMIC ACID AS MEASURED BY ^{31}P NMR

5.1 Introduction

Surfactants are a class of amphiphilic water soluble compounds and their toxicity is dependent on the organism being studied, the class of the surfactant, and the structure of the surfactant (Ivanković and Hrenović 2010). There are four classes of surfactants: zwitterionic, nonionic, cationic, and anionic. Surfactants used for household and industrial applications are typically classified as either nonionic, cationic, or anionic (Ivanković and Hrenović 2010). Surfactant toxicity has been a focus of many studies (Ivanković and Hrenović 2010). Due to their heavy use, surfactants inevitably end up in the environment via run-off, waste water treatment plants, remediation treatments, and pesticide formulations (Rogers 1996, Stalmans et al. 1991, Mulligan et al. 2001, Czarnota and Thomas, Song get al. 2012, Zoller 2014). This is of major concern because of their possible toxicity to aquatic organisms (Ostroumov 2006).

Furthermore, humic acids (HAs), which are omnipresent in the environment, have been shown to mitigate the toxicity of a range of pollutants, including surfactants (Deese et al. 2015, Koopal et al. 2004). HAs are complex heterogeneous organic molecular assemblies created by the degradation of organic matter. They are amphiphilic and are made up of a variety of functional groups, mainly carbohydrates, aromatics, and lipids. HA structure and functionality varies depending on its biogeochemical origin. HA-pollutant interactions can be caused by electrostatic or hydrophobic/hydrophilic interactions, as well as by chemical binding (Stevenson 1994). These interactions are dependent on both the type of HA and the pollutant. Previous *Artemia Franciscana* (*Artemia*) hatching assays have shown that surfactant toxicity can be mitigated by HAs and that

mitigation is based on electrostatic interactions, π-π interactions, and the amphiphilic functionality of the HA (Deese et al. 2015).

Artemia, commonly known as brine shrimp, are aquatic crustaceans that are often used in toxicological studies. Pollutant toxicity to *Artemia* has been studied for a range of toxicants, including (but not limited to) surfactants (Deese et al. 2015), oil dispersants (Rodd et al. 2014), pharmaceuticals (Nunes et al. 2005), pesticides (Venkateswara Rao et al. 2007), metals (Kokkali et al. 2011), and nanoparticles (Arulvasu et al. 2014, Rajabi et al. 2015). Depending on the type of pollutant or stressor, the embryonic development and the overall health of *Artemia* can be negatively impacted. Adverse conditions, such as a polluted environment, can cause significant changes and development inhibitions of an embryo as well as retard development after hatching. The changes in development of the *Artemia* are based on the toxicity mechanism of the pollutant. The commonly used toxicological monitoring methods include hatching ability, short-term mortality (≤ 48 h), and long-term mortality (> 48 h). Hatching ability assays with decreased hatching success under toxic conditions suggest that there is either death to the embryos or a delay of the processes required to hatch, while mortality assays measure the toxic response to *Artemia* after they have hatched. Although these methods can provide information on the toxicity of pollutants, they are limited in that they cannot provide any mechanistic insight on the causes of toxicity.

Because of the limited information obtained by *Artemia* hatching assays and the extensive sample preparation required for metabolite extraction, an *in vivo* method for analyzing embryo development under a variety of conditions is desired. Nuclear magnetic resonance spectroscopy (NMR) is an almost ideal technique for *in vivo* experiments because of its non-invasive nature. Phosphorous-31 (^{31}P) NMR is of particular interest because ^{31}P has a high gyromagnetic ratio (γ)

and is a biologically selective isotope. Notably, important metabolites in organisms contain phosphorus, such as adenosine triphosphate (ATP), adenosine diphosphate (ADP), inorganic phosphate (P_i), sugar phosphates, such as glucose-1-phosphate and NAD(P)H, phosphodiester bonds (PDE), and phosphocreatine (PCr). By tracking the changes in concentration of these important phospho-metabolites, the health of the organism in question can be monitored by *in vivo* ^{31}P NMR.

Previous studies found that, while Tx-100 cause mortality after hatching, while cetylpyridinium chloride (CPC) and sodium dodecyl sulfate (SDS) inhibited hatching, and HAs can mitigate these toxic effects (Deese et al. 2015). However, questions remained in regards to the toxicity mechanisms of CPC and SDS as well as to whether real-time measurements could be taken to study this toxicity. These questions have been addressed in this study by utilizing ^{31}P NMR. The specific objectives are to determine if 1) surfactants can measurably change the phospho-metabolite profile of the *Artemia* embryos, 2) changes can be measured in real time, 3) the toxicity mechanisms are based on membrane disruption or a growth inhibition, and 4) the toxicity mitigation by HA phenomenon could be measured by ^{31}P NMR.

5.2 Materials and Methods

The surfactants Tx-100, CPC and SDS were all purchased from Sigma Aldrich (Piscataway, NJ, USA). Sodium chloride and sodium hydrogen carbonate were also purchased from Sigma Aldrich. Sterile 18 MΩ deionized water was sourced from a US filter water purification system. *Artemia franciscana* was purchased from Brine Shrimp Direct (Ogdon, UT, USA). Guanosine 5'-triphosphate sodium standard was purchased from Sigma Aldrich. The Perista Pump SJ 1220 peristaltic pump was manufactured by the ATTO Corporation (Tokyo, Japan). Silicone tubing for the peristaltic pump (0.64 mm inner diameter, 1.27 mm outer diameter,

15.3 m length, and 1.47 mm inner diameter, 1.97 mm outer diameter, 15.3 m length) was purchased from Fisher Scientific (Pittsburg, PA, USA).

5.2.1 Experimental design

In addition to *Artemia Franciscana* being a well-studied model organism for toxicity studies, it was chosen for the *in vivo* ^{31}P NMR studies because its phospho-metabolite is readily measured by ^{31}P NMR, its preparation for NMR studies is simple, and its embryogenesis is well characterized. The development of *Artemia* has been extensively studied and the processes that occur during its embryonic development are well known and thus, any changes due to stressors can be observed (Distribution, life cycle, taxonomy, and culture methods: Brine shrimp (artemia salina), Stappen , Wang et al. 2007, Warner and Clegg 2001, Warner et al. 1995). Commercially available *Artemia* are stored in a diapause (suspended development) state and the *Artemia* will come out of their diapause state when environmental conditions are favorable. The ability of *Artemia* to go into a diapause state makes them ideal for laboratory studies as they can be stored for long periods of time. Optimal conditions for *Artemia* involve a saline environment (20 – 40 ppt NaCl), pH 7.5 - 9, and temperatures between 25 and 30°C (Nunes et al. 2006, Warner et al. 1989). There are several normal stages of development for *Artemia* (Neumeyer et al. 2015). Immediately post-diapause, the *Artemia* are in a cyst stage, in which a spherical embryo is encased within a translucent membrane and a dense shell. The next stages include the emergence, in which the embryo ruptures its outer shell and begins to protrude, the "umbrella" stage, in which the embryo emerges from the shell but has not yet broken the translucent inner membrane, the first instar larva, in which they are free swimming, and finally the second instar, or the "adult stage" (Neumeyer et al. 2015).

The surfactants chosen for the ^{31}P NMR studies were Triton X-100 (Tx-100, non-ionic), cetylpyridinium chloride (CPC, cationic) and sodium dodecyl sulfate (SDS, anionic) as they cover the three common classes of surfactants, are extensively used in households and industries, and the toxic responses of *Artemia* to these surfactants have been previously studied.

Tx-100 is commonly used in laboratories for cellular lysing procedures and in heavy-duty industrial cleaners, CPC is found in mouthwash and is known to be toxic to aquatic organisms, and SDS is found in many household detergents(Safety data sheet: Cetylpyridinium chloride 2015, Sids initial assessment profile: Sodium dodecyl sulfate, Triton surfactants: Fda status of triton surfactants 2010). *Artemia* hatching assays with these surfactants revealed that Tx-100 caused mortality after hatching, while SDS and CPC caused hatching inhibition (Deese et al. 2015).

Humic acids have been shown to remediate the toxic effects of the surfactants to the *Artemia* (See Chapter 4, Deese et al. 2015). Three different HAs and chemically modified HAs have previously been studied with the surfactants to study changes in toxicity to the *Artemia* based on the HA-surfactant interactions (Deese et al. 2015). It was determined that the amphiphilic character of the HAs played a role in Tx-100 toxicity mitigation, aromatic content played a role in CPC toxicity mitigation, and SDS-HA interactions were nonspecific. This initial study provided important but limited information about the toxicity of these surfactants to *Artemia* and the role of HAs in toxicity mitigation. Hence, the objective of this study is to measure the "real time" toxicity of the surfactants and the ability of the HAs to reduce the bioavailability of the surfactants using ^{31}P NMR. Leonardite humic acid (LAHA) was chosen for these studies because it shows similar trends in regards to surfactant toxicity reduction as other HAs, is more economically viable for the experiments discussed below (each individual *in vivo* ^{31}P NMR requires between 5 and 35 mg of LAHA), and has been well characterized as well as extensively studied.

5.2.2 Surfactant stock solution preparation

Stock solutions of the surfactants Tx-100, CPC, and SDS were prepared by dissolving 1 g of the surfactant into 100 mL of 18 MΩ water for a concentration of 10,000 ppm (1%). Any dilutions and pH adjustments were made as necessary for the exposure solutions.

5.2.3 Humic acid stock solution preparation

The stock solution of LAHA was prepared fresh for each experiment by dissolving approximately 150 mg of LAHA in 18 MΩ water. NaOH was added as necessary to dissolve the LAHA in water and the pH was adjusted to pH 7.8 using HCl and NaOH. The solution was diluted to 250 mL using 18 MΩ water, protected from light, and stirred overnight. The pH was checked and, if necessary, adjusted after the equilibration period.

5.2.4 Exposure solutions

Artemia exposures took place in a 35 parts-per-thousand (ppt) sodium chloride (NaCl) solution at pH 7.8. The 35 ppt NaCl solution was prepared by dissolving 35 g of NaCl in 1 L of 18 MΩ water. Sodium hydrogen carbonate ($NAHCO_3$) was added to the solution until a pH of 7.8 was reached. The 35 ppt NaCl solution without any pollutants or HA added was used for the control experiments. For surfactant exposure solutions, appropriate amounts of Tx-100, CPC, or SDS stock solution were added to the 35 ppt NaCl solution for a final surfactant concentration of 100 ppm, 5 ppm, or 35 ppm, respectively. Humic acid exposure solutions were prepared by adding the appropriate amount of LAHA stock solution to the 35 ppt NaCl solution along with any desired surfactant. The final LAHA concentrations were 35 ppm for the LAHA control and SDS solution and 5 ppm for the CPC solution. In order to ascertain toxicity, 35 ppm LAHA in the 35 ppt NaCl solution was used and changes occurring to the *Artemia*'s phospho-metabolite profile in the presence of LAHA were monitored using ^{31}P NMR. Since this was the highest concentration of

LAHA used for the experiments, it was determined that a 5 ppm LAHA control was not needed (especially in light of the large amount of LAHA required). In addition, it has previously been shown that LAHA had no toxic effects on *Artemia* (See Chapter 4, Deese et al. 2015).

5.2.5 Decapsulation of *Artemia Franciscana* embryos

Prior to the ^{31}P NMR analysis and perchloric acid extraction for HPLC analysis, the *Artemia* were decapsulated using a bleaching method. This decapsulation (removal of the cysts encapsulating the embryos, Figure 5.1) method is commonly used when *Artemia* are used as feed for other organisms (Stottrup and McEvoy 2003). Before decapsulation, approximately 5 g of *Artemia* cysts were hydrated with 18 MΩ water in a 250 mL Erlenmeyer flask equipped with a bubbler for 1.5 h in an ice bath. The ice bath was used to keep the *Artemia* cysts below 5°C to prevent any premature development. After complete hydration, the cysts were filtered utilizing a nylon mesh fabric, placed back into the Erlenmeyer flask, and a hypochlorite solution (pure Clorox® bleach) was added with continued aeration.

Figure 5.1 Decapsulation of *Artemia Franciscana* embryos

After approximately 35 s, when the *Artemia* embryos began to turn orange (see Figure 5.1), they were immediately filtered, using the nylon mesh fabric, and rinsed with copious amounts of deionized (D.I.) water. For the NMR experiments, the *Artemia* embryos were packed into a 10 mm NMR tube. For the perchloric extraction step required for the HPLC analysis, the *Artemia* were

directly transferred to their exposure solutions containing 35 ppt NaCl and the appropriate surfactant concentrations.

5.2.6 In vivo ^{31}P NMR

A perfusion system was designed based on previous *in vivo* ^{31}P NMR studies, see Figure 5.3 (Covi et al. 2005, Tjeerdema et al. 1993, Viant et al. 2006). The total length of each tube used for the NMR experiments was approximately 4 m. Decapsulated *Artemia* embryos were packed by gravity into a 10 mm NMR tube along with the pump's tube system (Figure 5.2).

Figure 5.2 Multi-step gravity packing of *Artemia* embryos after packing into a 10 mm NMR tube with glass wool "cap"

The 10 mm NMR tube was filled with D.I. water and the "in" tube of the pump system placed so that the opening was at the bottom of the NMR tube. Small aliquots of the *Artemia* embryos (< 0.5 mL) were added, allowing for settling between each aliquot (Figure 5.2). This multi-step gravity packing method assured that the *Artemia* embryos packed tightly in the NMR tube, limiting any movement once liquid flow begins. Once the *Artemia* filled approximately 4 - 5 cm of the NMR tube, in order to increase packing efficiency, glass wool was placed on top of the *Artemia* and gently pressed to remove any air bubbles.

It was important not to over-fill the *Artemia* embryos as too many embryos would consume too much oxygen, leading to anoxia problems. The glass wool also acted as a "cap" to keep the embryos from being removed from the NMR tube via the "out" tube. A ATTO Perista Pump SJ 1220 peristaltic pump was utilized to pump in fresh, oxygen rich, 35 ppt NaCl exposure solution at pH 7.8 (adjusted with $NaHCO_3$) to the *Artemia,* with a flow rate of 2 mL/min.

A bottom-to-top flow through the system was achieved by pumping fresh solution into the bottom of the NMR tube and then removing the solution from the top of the *Artemia* population. The solution pumped into the NMR tube was aerated so as to saturate the solution with oxygen to limit negative effects of anoxia on *Artemia*. The pump system was constantly monitored to verify that the "in" and "out" tubes were working properly throughout each 5-h long NMR experiment.

All ^{31}P NMR experiments were performed on a Bruker AVIII HD 400 MHz NMR at a controlled temperature of 298 K and equipped with a 10 mm broad band probe operating at the 202.43 MHz. All spectra were baseline and phase corrected. The NMR spectral shift was calibrated using a 80% H_3PO_4/20% D_2O solution, with the phosphate peak serving as the 0 ppm reference. A deuterium lock was not required. The *Artemia* embryos were measured over a period of 12 minutes with 3072 transients, 2048 data points, a spectral width of 82 ppm, a delay time of 0.1 s, a pulse power of – 6 dB, and a pulse angle of 30°. Data were processed with a 25 Hz line-broadening prior to Fourier transformation. All experiments were repeated in triplicate.

After ^{31}P NMR control experiments solely utilizing the 35 ppt NaCl solution, normal hatching success was observed for the *Artemia.* To determine hatching success, embryos within the spectrometer detection window were gently removed from the NMR tube and placed into a 250 mL Erlenmeyer flask equipped with a bubbler that contained 35 ppt NaCl hatching solution. The solution was aerated and hatching was observed after 24 hours.
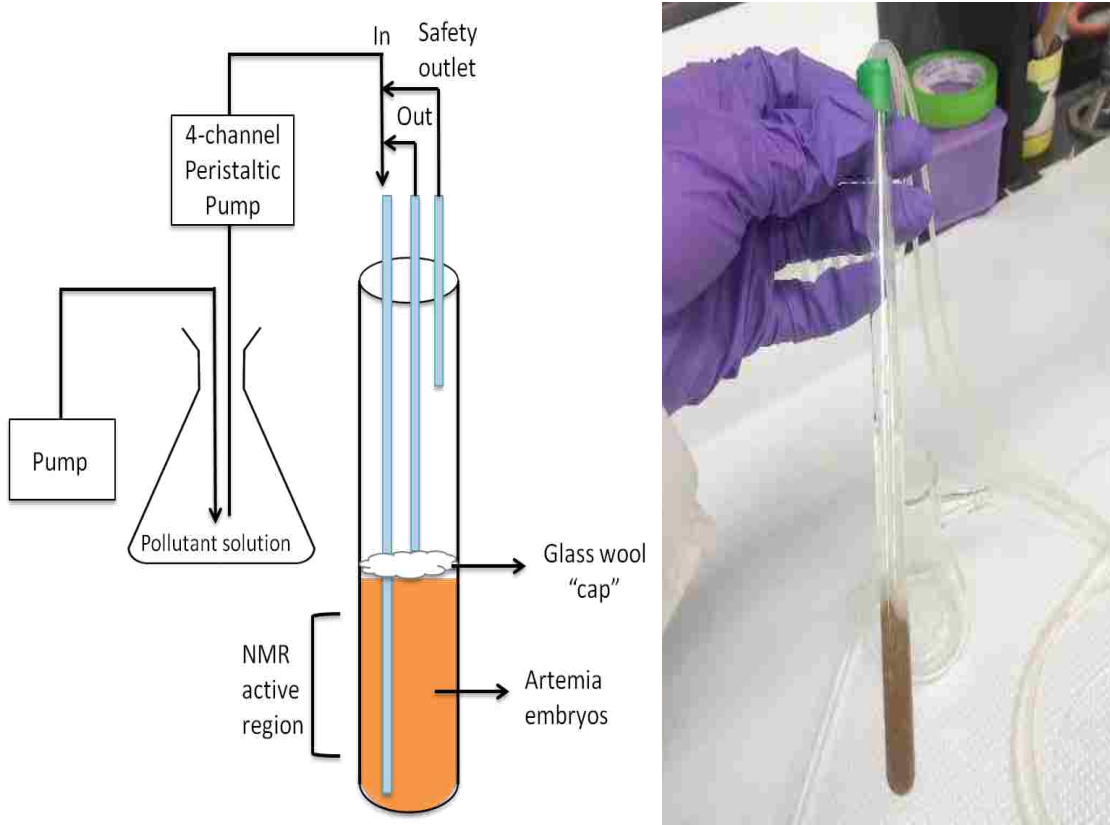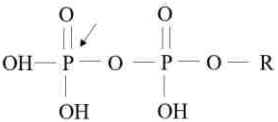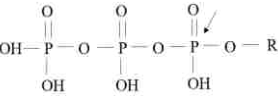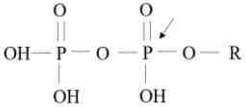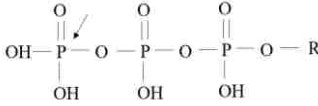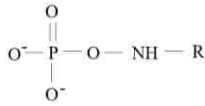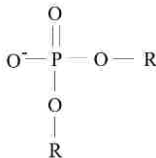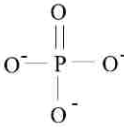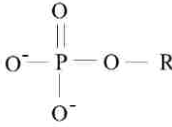
Figure 5.3 Schematic and actual photograph of *in vivo* NMR perfusion system with 10 mm NMR tube

5.2.7 In vivo ^{31}P NMR peaks and trends

This section briefly introduces the chemical meaning of the peaks in the representative *in vivo* ^{31}P NMR spectrum presented in Figure 5.4, as well as a brief review of their use in previous toxicological studies. The main indicator of stress that has been established by numerous studies is measurable changes in the ATP cycle (Covi et al. 2005, Viant et al. 2002, Tjeerdema et al. 1993). It has been found that when an organism is stressed, cells consume a large amount of ATP to generate ADP and the by product P_i. Table 5.1 summarizes the assigned ^{31}P NMR peaks.

Table 5.1 Assigned ^{31}P NMR peaks

Chemical Shift (ppm)	Chemical linkage	Compound Type
-19.0 – -18.6	OH—P(=O)(OH)—O—P(=O)(OH)—O—R	β - adenosine triphosphate
-11.8 – -9.8	OH—P(=O)(OH)—O—P(=O)(OH)—O—P(=O)(OH)—O—R OH—P(=O)(OH)—O—P(=O)(OH)—O—R	α-adenosine triphosphate/ α-adendosine diphosphate
-5.5 – -4.5	OH—P(=O)(OH)—O—P(=O)(OH)—O—P(=O)(OH)—O—R	γ-adenosine triphosphate/ β-adendosine diphosphate
0 - 1	O$^-$—P(=O)(O$^-$)—O—NH—R	Phosphocreatine
1.2 – 1.8	O$^-$—P(=O)(O—R)—O—R	Phosphodiester
2.5 – 3.5	O$^-$—P(=O)(O$^-$)—O$^-$	Inorganic phosphorus
3.7 – 3.9	O$^-$—P(=O)(O$^-$)—O—R	Phosphomonoester

The organism uses the energy from the ATP bonds for necessary biological functions that would be stressed in a toxic environment. The P_i can also be utilized to monitor the intracellular pH of an organism, which can also serve as an indicator of stress. Cells are alkaline under normal conditions and acidification of the cells is considered to be a response to stress.

A variety of biological species, conditions, and toxins have been previously measured by *in vivo* ^{31}P NMR. Tjeerdema, Viant, and co-workers have extensively studied *in vivo* metabolomics using ^{31}P NMR on organisms such as red abalone (Martello et al. 1998, Tjeerdema et al. 1993, Viant et al. 2002) and medaka (*Oryzias latipes*) fish embryos (Pincetich et al. 2005, Viant et al. 2006). These studies illustrated the ability of *in vivo* ^{31}P NMR to characterize the effects of both chemical stressors, such as pollutants, and physical stressors such as anoxia (lack of oxygen); all of which can be monitored in real time. Two studies by Viant and co-workers measured the toxic response to copper by the red abalone (Viant et al. 2002) and medaka fish embryos (Viant et al. 2002). Both organisms showed measurable changes in [ATP], [phosphoarginine], and [P_i] indicating changes in the ATP cycle caused by Cu-induced stress. A study of the effects of dinoseb (a herbicide) on medaka fish embryos which also indicted changes in the ATP cycle by an increase in [P_i], and declines in [ATP] and [PCr] under stressful conditions (Viant et al. 2006). The ^{31}P NMR results of that study were verified with HPLC and ^{1}H NMR measurments of the metabolite extracts of the embryos.

The phospho-metabolite profile of *Artemia franciscana* has been studied with *in vivo* ^{31}P NMR by several groups. Busa et al. measured the metabolite profile of *Artemia* embryos as they developed from a diapause state to a post-diapause state (Busa et al. 1982). Kwast et al. measured the changes in the ^{31}P NMR spectra as the *Artemia* embryos when stressed by low oxygen (anoxic) conditions and measured their ability to recover (Kwast et al. 1995). A study by Covi et al. utilized

Artemia Franciscana and ^{31}P NMR to study *in vivo* changes of the intracellular pH and the ATP cycle during anoxia and under incubation with bafilomycin A$_1$ (a V-ATPase inhibitor) (Covi et al. 2005). The ^{31}P NMR results for these studies showed that, under stress, intracellular acidification occurred, as measured by the upfield shift of the P$_i$ peak as well as a decrease in [ATP]. These studies also found that *Artemia* could recover from anoxia under normal conditions and that the V-ATPase inhibitor bafilomycin limited the intracellular alkalization aspect of the recovery but did not interfere with the [ATP].

The P$_i$ peak is generally used to calculate intracellular pH, as acidification of *Artemia* embryonic cells has been linked to stress on the organism in low-oxygen conditions (Covi et al. 2005, Warner et al. 1989). Because the P$_i$ peak shifts upfield during intracellular acidification, which indicates stress, the shift itself can determine the pH, and the P$_i$ concentration can be correlated with the peak area. As mentioned before, P$_i$ is a by-product of the ATP consumption when an organism is under stress. Hence, the P$_i$ peak shift and the peak area increase serve as good stress indicators. Recent studies with *Artemia* have failed to identify the ^{31}P NMR peak at approximately 2 ppm (the peak labelled PDE in Figure 5.4) (Covi et al. 2005, Kwast et al. 1995). However, studies of organisms with a similar phospho-metabolite profile as the *Artemia* have provided convincing evidence for that peak to be due to a phosphodiester (PDE) peak and corroborated their assertions by such methods as HPLC and ^{1}H NMR (Martello et al. 1998, Pincetich et al. 2005, Tjeerdema et al. 1993, Viant et al. 2006, Viant et al. 2002). For this study, this peak was assigned to PDE with further verification by HPLC.

The ^{31}P NMR profile for the *Artemia* in this study is similar to previous *Artemia* studies as well as having a similar phospholipid profile to that of medaka embryos (Busa et al. 1982, Covi et al. 2005, Kwast et al. 1995, Viant et al. 2006). The detectable peaks (see Figure 5.4.A) are

identified here as a phosphomonoester (PME, sugar phosphate; 3.7 – 3.9 ppm) peak, an inorganic phosphate (P_i; 2.5-3.5 ppm) peak, a phosphodiester (PDE, DNA backbone; 1.2-1.8 ppm) peak, a phosphocreatine (PCr; 0-1 ppm) peak, adenosine triphosphate (ATP; -19.0 - -18.6 ppm) peak, and two peaks attributed to both ATP and adenosine diphosphate (ADP; -11.8 - -9.8 ppm and -5.5 - -4.5 ppm) that are indistinguishable from each other. Figure 5.4B illustrates an example of the ^{31}P NMR of *Artemia* embryos that are "dead" at the time of measurement. This typically occurs when the decapsulation process is either done improperly (e.g. by leaving the embryos in the hypochlorite solution for too long) or when the decapsulation hypochlorite (bleach) solution has degraded over time.

5.2.8 Extraction of phosphorylated metabolites

The phosphorylated metabolites were extracted from decapsulated embryos by a perchloric acid extraction for HPLC analysis, following Viant et al. (Viant et al. 2006). After the *Artemia* were exposed in 35 ppt NaCl and pollutant solutions for 1 h and 5 h time periods (n = 6), aliquots of the *Artemia* embryos were removed and flash frozen with liquid nitrogen. The frozen embryo samples were lyophilized overnight to remove all water. The dry tissue was then homogenized with a mortar and pestle and weighted. All solutions during the extraction procedure were kept at T ≤ 5°C by keeping them in an ice bath throughout the entire experiment. The dry tissue was extracted with 0°C 1.0 M perchloric acid, vortexed for 30 s, and then put into an ice bath for 10 min. The samples were centrifuged at $10,000 \times g$ for 10 min. The supernatant was then removed and neutralized to pH 7.0 with ice-cold 1 M Na_2CO_3, kept on ice for 30 min, and then centrifuged again under the same conditions. The samples were diluted to 10 mL with 18 MΩ water and filtered with 0.45 μm polyvinylidene fluoride filters prior to HPLC analysis.

5.2.9 High performance liquid chromatography

High performance liquid chromatography (HPLC) was performed on the extracted *Artemia* phosphorylated metabolites for verification of the *in vivo* ^{31}P NMR results.
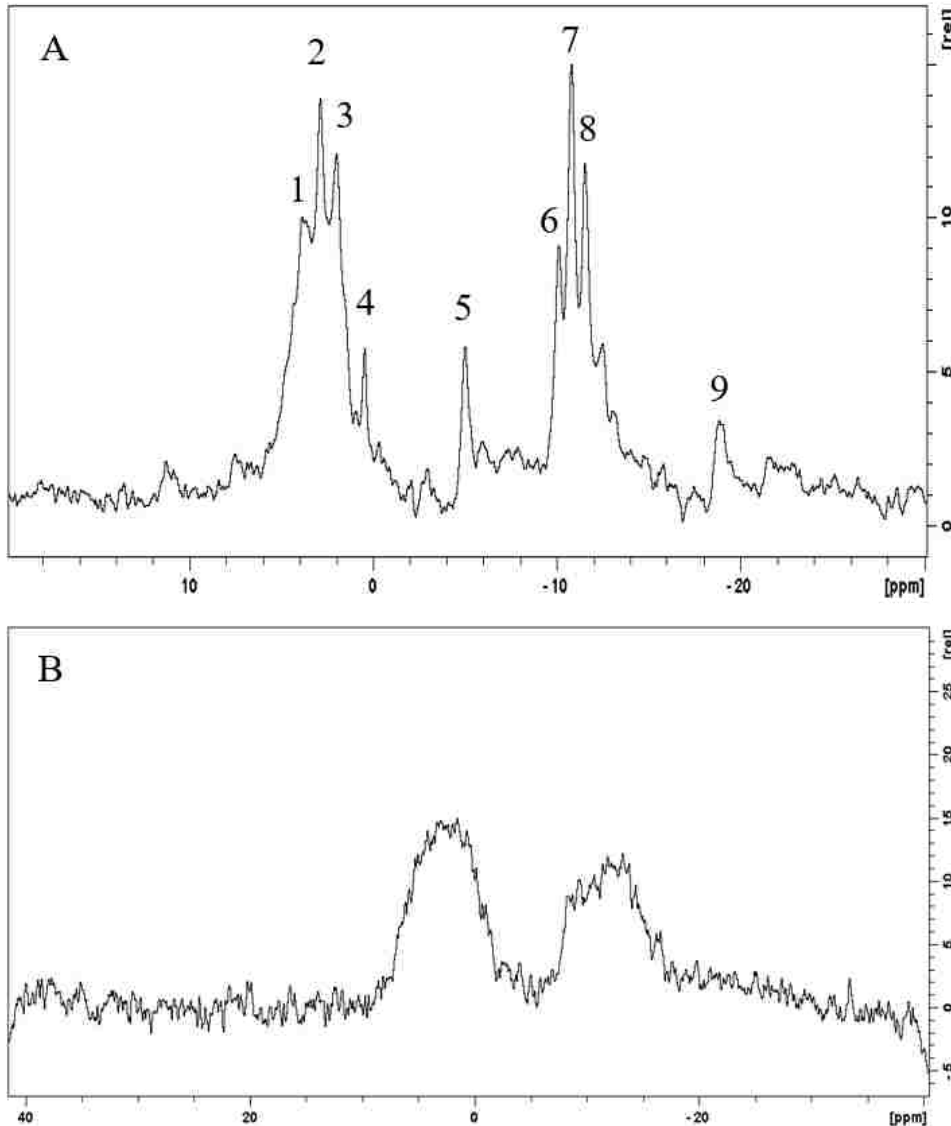
Figure 5.4 A) Representative ^{31}P NMR spectrum of live *Artemia Franciscana*. Peak identities are 1) phosphomonoesters (PME; 3.7 – 3.9 ppm), 2) inorganic phosphate (P_i; 2.5 – 3.5 ppm), 3) phosphodiesters (PDE; 1.2 – 1.8 ppm), 4) phosphocreatine (PCr; 0 – 1 ppm), 6, 7, 8) α-adenosine triphosphate (ATP)/α-adenosine diphosphate (ADP), β-ATP (-5.5 - -4.5 ppm, -11.8 - -9.8 ppm) 9) γ-ATP/β-ATP (-19.0 – 18.6) and B)^{31}P NMR example spectrum of dead *Artemia* embryos

All measurements were obtained utilizing an Agilent 1100 series HPLC with a C_{18} reverse-phase column. The HPLC method used to detect guanosine triphosphate (GTP) from the perchloric

acid extraction was developed by Veciana-Nogues et al. (Veciana-Nogues et al. 1997) with UV detection at 254 nm. A 24-min gradient was used for each sample. The mobile phase A was 0.05 M phosphate buffer and mobile phase B was HPLC-grade methanol. The flow rate was 1 mL/min. The gradient was as follows: 0-9 min 100% A, 0% B; 9 - 14 min 70% A, 30% B 14 - 24 min 100% A, 0% B. Peak assignment was confirmed with the use of standards. A calibration curve for GTP was created with 1, 5, 10, 20, and 30 ppm GTP standard and a $R^2 = 0.99$ was obtained.

5.3 Results

5.3.1 ^{31}P NMR of Artemia in 35 ppt NaCl solution – Control conditions

^{31}P NMR spectra were acquired over 5 h with 35 ppt NaCl at pH 7.8 and under continuous aeration in order to obtain a set of control spectra with which to compare the polluted systems. Representative spectra of the control can be seen in Figure 5.7A. The PME, PCr, and ATP/ADP peaks showed insignificant changes over the course of 5 h. These slight changes are attributed to the constant change in the PME, PCr, and ATP/ADP concentrations as the embryos begin to develop. The PDE peak for the controls had a significant increase after approximately 150 min and continued over the course of the experiment.

5.3.2 Tx-100

Although a significant and steady decrease in [ATP] is indicative of a stressed system, in the case of Tx-100, it is attributable to anoxia rather than stress by the surfactant, as it was not possible to fully aerate the Tx-100 solution due to a "foaming" issue, causing the solution to overflow with bubbles (Figure 5.5). This issue did not occur with the other surfactant solutions, which were aerated successfully and thus, did not exhibit anoxia-related problems.

Figure 5.5 Aeration of Tx-100 solution resulting in excessive "foaming"

^{31}P NMR spectra were collected for the *Artemia* with 100 ppm Tx-100 in 35 ppt NaCl. As can be seen in Figure 5.7B, there was a slight decrease in the [ATP] (specifically peaks at approximately -10.4 ppm and -19 ppm) over the total 5 hr-course of the experiment. This decrease in [ATP] is greater than seen for the other surfactants as can be seen in the data presented in Figure 5.6 (see Appendix 2 for more detailed [13]P NMR spectra representations). The PCr and PME peaks did not exhibit any significant changes over the course of time. Similar to the control *Artemia* spectra, the PDE peak increased significantly over time after $\approx$ 150 min.

5.3.3 CPC and SDS

The signals of the ATP peaks in the CPC spectra were similar to those of the control ^{31}P NMR spectra. Some fluctuation occurred in the [ATP] over time, which was to be expected. However, the increase in [PDE] was significantly lower for the CPC- and SDS-exposed *Artemia* compared to the control and the Tx-100-exposed *Artemia*. These solutions were constantly aerated, to eliminate, or minimize, the effects of anoxia. The *in vivo* ^{31}P NMR intensities were used to calculate a PDE/P_i ratio for each time point obtained.
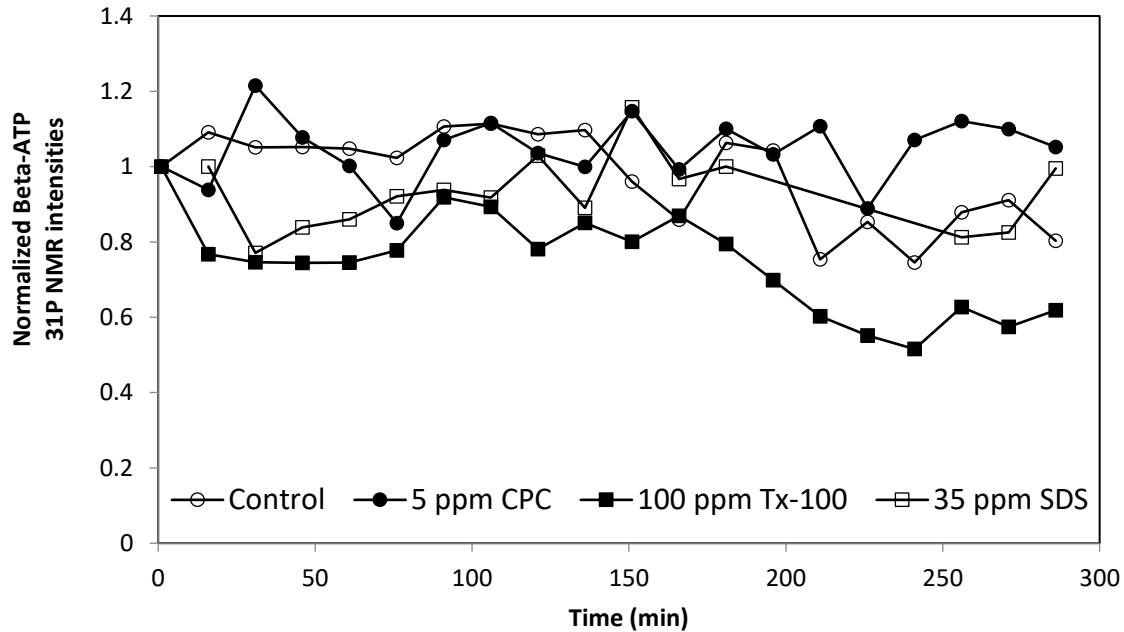
Figure 5.6 Normalized β-ATP (-19 ppm) ^{31}P NMR intensities of *Artemia* under varying conditions

The moving average (n = 3, interval = 2) of the PDE/P_i ratios are shown, with error bars, in Figure 5.8. The PDE/P_i increased consistently over time after ≈ 150 min for both the control and Tx-100 solutions.

This increase was not observed for the CPC and SDS and the PDE/P_i ratio was significantly lower for CPC and SDS solutions compared to the control and Tx-100 solutions after 150 min, as illustrated by the fact that the PDE/P_i ratio did not increase above 1 for the CPC and SDS solutions while for the control and Tx-100 solutions, the PDE/P_i ratio easily exceeded 1.4, especially after 200 min.

5.3.4 HPLC results

High performance liquid chromatography (HPLC) with UV detection was utilized to measure the concentration of guanosine triphosphate (GTP).
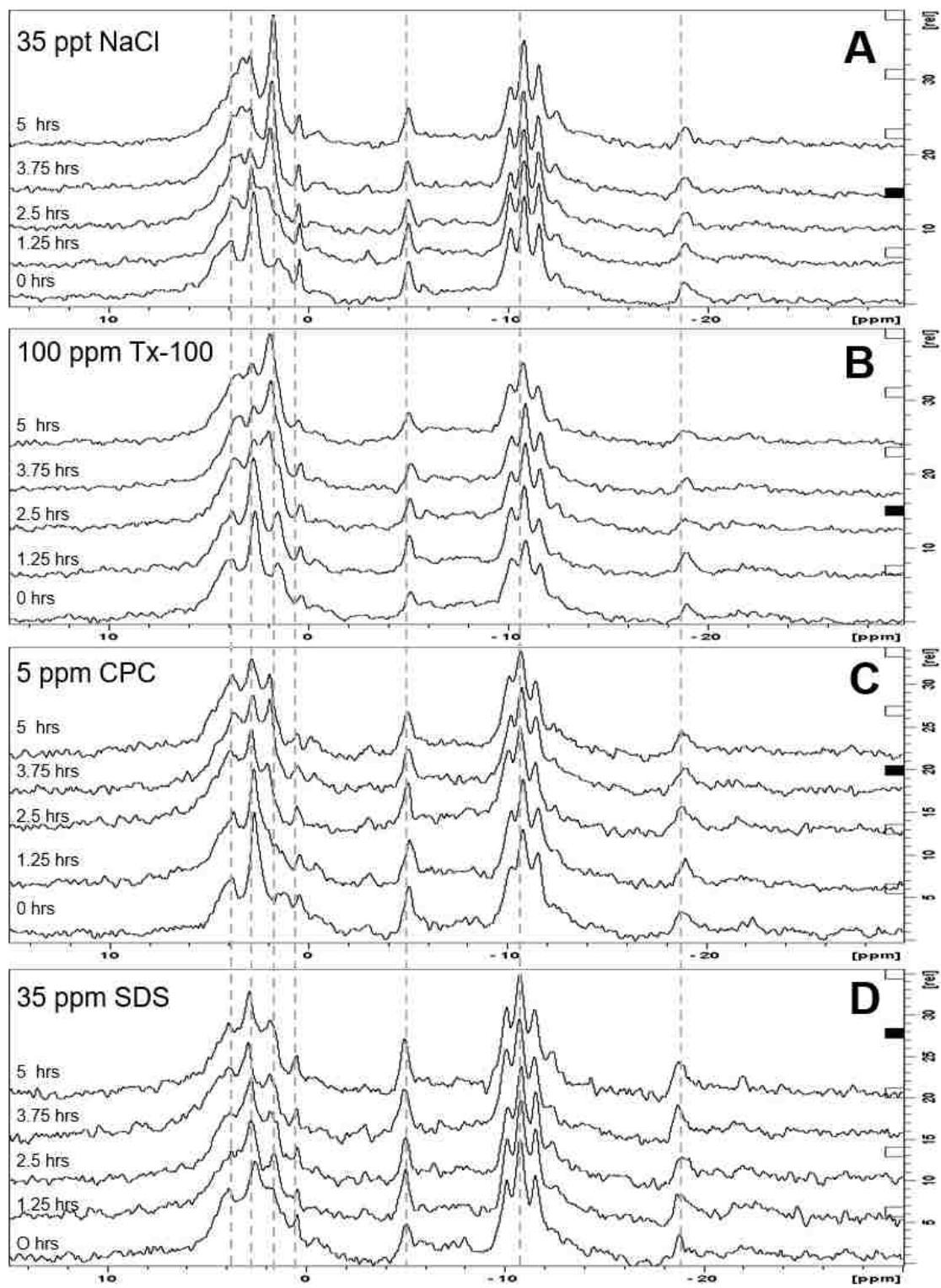
Figure 5.7 Stacked representative ^{31}P NMR spectra for *Artemia* embryos in A) 35 ppt NaCl, B)100 ppm Tx-100, C) 5 ppm CPC and D) 35 ppm SDS

Figure 5.8 ^{31}P NMR intensities of PDE/P$_i$ of *Artemia Franciscana* embryos with 35 ppt NaCl, 5 ppm CPC in 35 ppt NaCl, 35 ppm SDS in 35 ppt NaCl, and 100 ppm Tx-100 in 35 ppt NaCl.

Figure 5.9 illustrates the HPLC results obtained on the *Artemia* embryo extracts as the change in μmoles/mg of dry *Artemia* tissue over 5 h. For the control and 100 ppm Tx-100 solutions, the GTP concentration increases, while for the SDS and CPC solutions, the GTP concentration decreases. The CPC and SDS changes in [GTP] were significantly different ($p < 0.05$) compared to the control sample.

5.3.5 ^{31}P NMR of *Artemia* with addition of LAHA

Humic acid (HA) has been shown to mitigate the toxic effects of surfactants to *Artemia* (Deese et al. 2015). Specifically, Leonardite humic acid (LAHA) was able to interact and reduce the bioavailability of CPC, SDS, and Tx-100, resulting in changes in the hatching or mortality rates of *Artemia*.
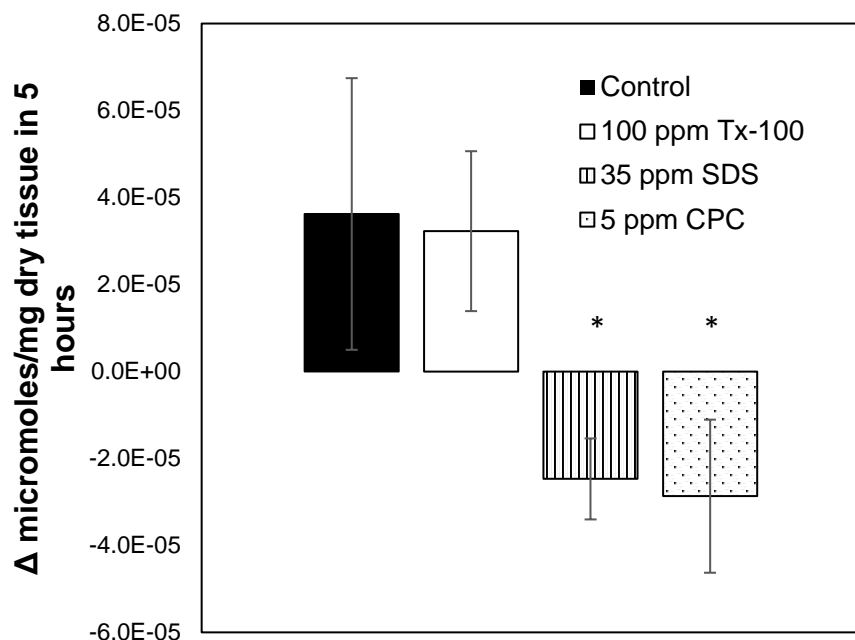
Figure 5.9 The changes in micromoles of guanosine triphosphate (GTP) per milligram of dry *Artemia franciscana* tissue extracts for 5 h exposure as measured by HPLC (* $p < 0.05$ versus the control)

As CPC and SDS induced changes in the *Artemia's* phospho-metabolite profile, LAHA was added to the CPC and SDS toxic solutions in order to determine if LAHA's ability to mitigate the toxicity of these surfactants could be measured and further understood by *in vivo* ^{31}P NMR.

The PDE/P_i ratios of the control, LAHA alone, CPC alone, and CPC plus LAHA were calculated from the ^{31}P NMR data and plotted in Figure 5.10. Both the control and the 35 ppm LAHA in 35 ppt NaCl had PDE/P_i ratios were greater than 1.4 and, as described before, the 5 ppm CPC PDE/P_i ratios were less than 1. Since LAHA was previously shown in the hatching assays to have no effect on the *Artemia* hatching ability, similar trends in the phospho-metabolite profile of the control and LAHA solutions were expected.
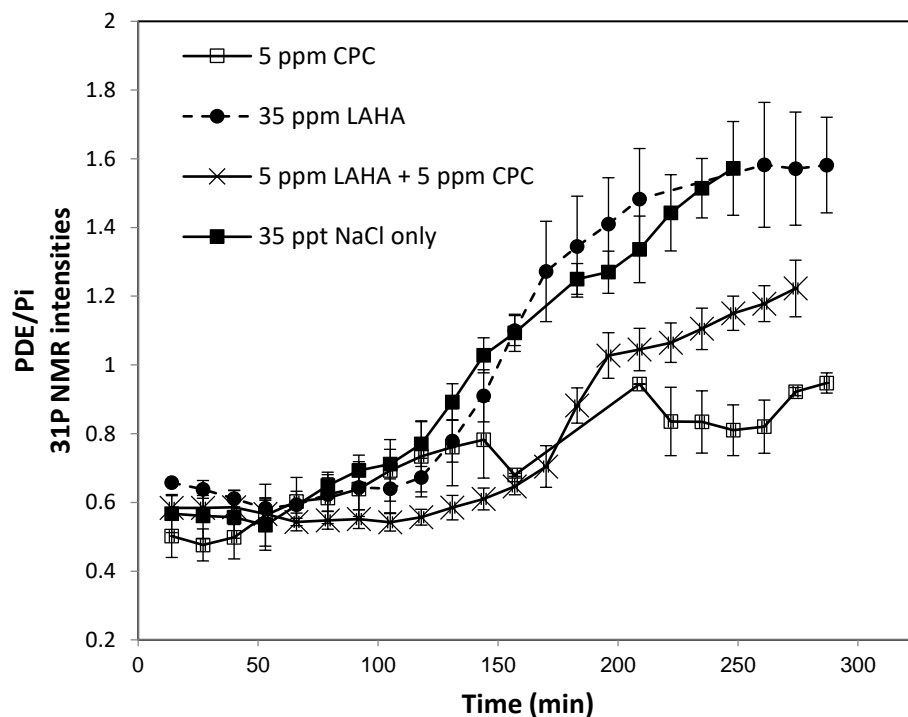
Figure 5.10 ^{31}P NMR intensities of PDE/P$_i$ of *Artemia Franciscana* embryos under 35 ppt NaCl, 35 ppm LAHA in 35 ppt NaCl, 5 ppm CPC in 35 ppt NaCl, and 5 ppm LAHA and 5 ppm CPC in 35 ppt NaCl

When LAHA was added to the CPC solutions, there was an increase in the ratio of PDE/P$_i$ relative to that obtained for the CPC-only solutions.

The PDE/P$_i$ of *Artemia* with CPC and LAHA was above 1 but less than 1.4, falling directly in between the highest ratio obtained the CPC-only solutions and the lowest ratio for the control solutions. Very similar results were obtained with SDS, as shown in Figure 5.11. Based on the PDE/P$_i$ ratio, LAHA mitigates the toxicity of SDS better than the toxicity of CPC to *Artemia*. The data in Table 5.2 further illustrates these points and shows that LAHA significantly ($p < 0.05$) mitigates both CPC and SDS toxicity
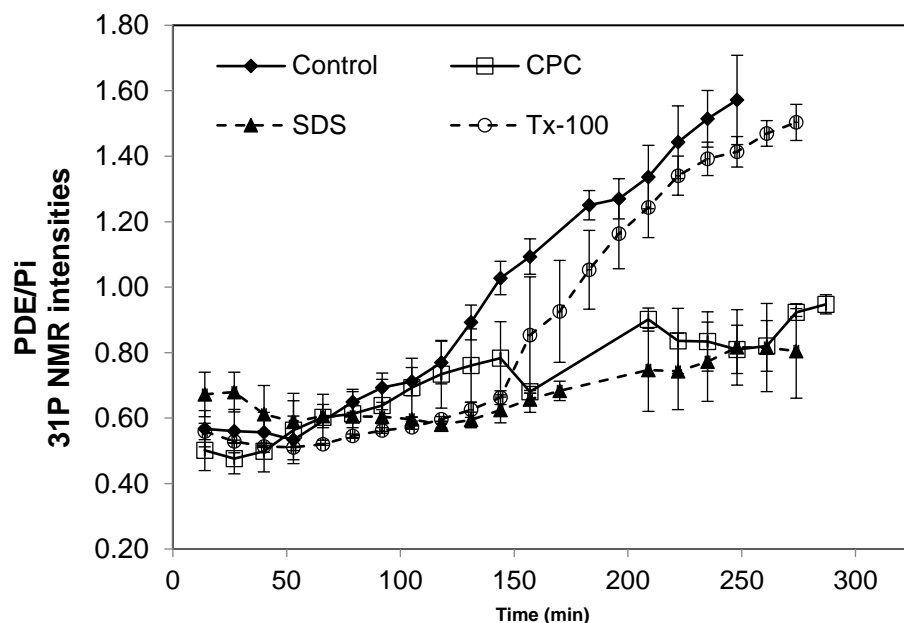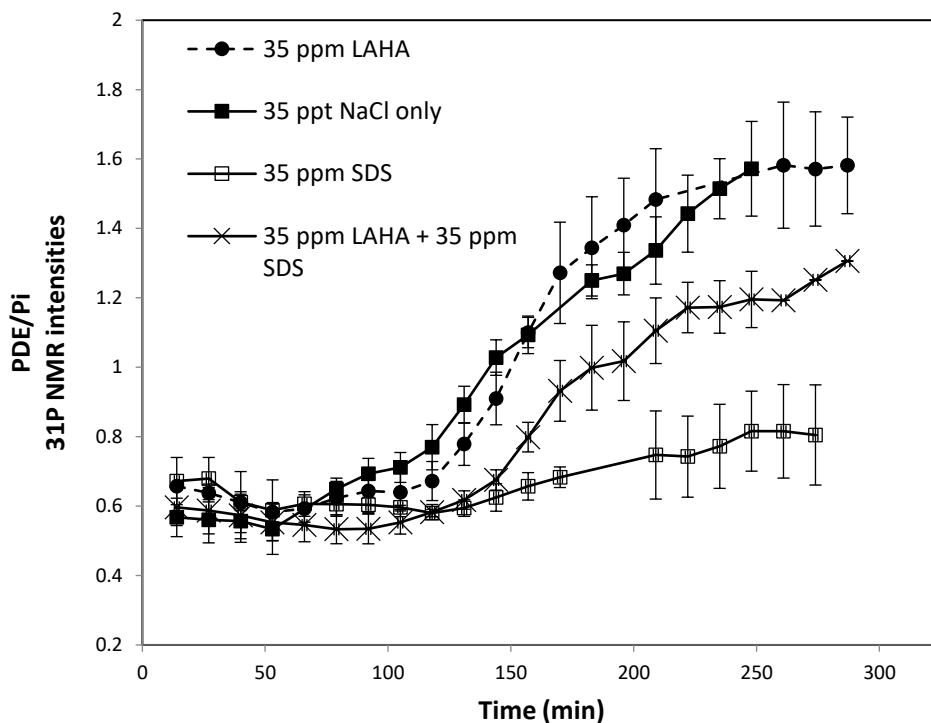
Figure 5.11 ^{31}P NMR intensities of PDE/P$_i$ of *Artemia Franciscana* embryos under 35 ppt NaCl, 35 ppm LAHA in 35 ppt NaCl, 35 ppm SDS in 35 ppt NaCl, and 35 ppm LAHA and 35 ppm SDS in 35 ppt NaCl (*last three time points only repeated in duplicate).

Table 5.2 PDE/P$_i$ ratios of *Artemia Franciscana* embryos measured by in vivo ^{31}P NMR

Time (min)	Exposure Solution						
	35 ppt NaCl	5 ppm CPC	35 ppm SDS	100 ppm Tx100	35 ppm LAHA	5 ppm LAHA + 5 ppm CPC	35 ppm LAHA + 35 ppm SDS
15	0.57± 0.06	0.50±0.06	0.67±0.07	0.56± 0.03	0.66± 0.01	0.58±0.04	0.60±0.05
150	1.03± 0.05	0.78±0.11	0.62±0.04*	0.66± 0.02	0.91±0.08	0.61±0.03*	0.68±0.03
200	1.34± 0.10	0.90±0.04*	0.75±0.13*	1.24± 0.09	1.48± 0.15	1.04±0.06	1.11±0.09
250	1.57±0.14	0.81±0.07*	0.82±0.12*	1.41± 0.05	1.58± 0.18	1.15±0.05†	1.20±0.08†

Values represent mean ± S.E.M. of three replicates. (*$p < 0.05$ at the same time point where null hypothesis = same as control. $^†p < 0.05$ at the same time point where null hypothesis = same as respective surfactant-only exposure)

5.4 Discussion

CPC, SDS, and Tx-100 are known to have toxic effects on *Artemia* and HAs have the ability to mitigate the toxicity of these surfactants. This study addresses the question as to whether these trends could be observed using *in vivo* methods, namely ^{31}P NMR, and if a better understanding of the toxicity can be obtained. The major advantages of ^{31}P NMR is that clean, simple, and directly interpretable data can be obtained and that changes in phospho-metabolites can be measured in real time.

The control ^{31}P NMR spectra indicated some minor variation in levels of ATP throughout the experiment. Although changes in [ATP] can be used to indicate stress on an organism, [ATP] has also been shown to fluctuate greatly during embryogenesis in *Artemia* (Warner and Finamore 1967, Zhu et al. 2009) and other organisms (Moroz and Luzhin 1976) with a significant increase in [ATP] immediately before and during hatching. Post-diapause development of *Artemia* is complex and most of the development events cause changes in energy and thus, changes in ATP demands by the embryo (Zhu et al. 2009). These energy-consuming events consist of protein synthesis, gene transcription, degradation of the yolk, and more. Since the degradation and synthesis of ATP is a cyclic process, the [ATP] can depend on the *Artemia* embryo's point in the cycle at a particular time. Thus, the observed small fluctuations in the ^{31}P NMR ATP/ADP peaks can be attributed to normal fluctuations in energy demands**.**

The major change in the phospho-metabolite profile of *Artemia* in the control over time is the increase in [PDE] after ≈ 150 min. *Artemia* embryos are known to stay in a diapause state until introduced to the proper hatching conditions, such as the right salinity, pH, and temperature (Stappen). Thus, the time between 0 and 150 min is likely the delay time between the time the *Artemia* are introduced to the appropriate hatching conditions and the time when the embryos

begin to develop. Increases in [PDE] have been shown to occur during tissue growth, maturation, and cell replication (Styles 1993) and decreases in [PDE] have been attributed to declining rates of cellular replication (Viant et al. 2006). The increase in [PDE] observed in the control spectra of *Artemia* can be attributed to the significant cell replication occurring during embryonic development.

Triton X-100 is a nonionic surfactant that was used previously in *Artemia* hatching assays to determine the toxicity of Tx-100 and the changes of that toxicity in the presence of HAs (Deese et al. 2015). The hatching assays in this work show that Tx-100 does not have any effect on the hatching ability of the *Artemia*, instead, the Tx-100 causes mortality after the *Artemia* had hatched at a Tx-100 concentration of 100 ppm or above. The spectra of Tx-100-exposed *Artemia* had a decrease in [ATP], which tends to indicate stress. However, this decrease of [ATP] is credited to the slight anoxic conditions caused by the inability to fully aerate the Tx-100 solution due to foaming issues rather than the stress from the surfactant itself.

The [PDE] increase observed in the Tx-100-exposed *Artemia* spectra indicates that there is still cell replication occurring under Tx-100 solutions. Because Tx-100 does not affect the hatching ability of the *Artemia* and since normal processes still need to occur within the cell before hatching, it stands to reason that there should not be any significant differences in the phospho-metabolite profile of the *Artemia* embryos with Tx-100 versus the control. This notion is confirmed by the data in Figures 5.6, 5.7, and 5.8 as well as Table 5.2. It should be noted that data does, however, show lower values across the board for the Tx-100 condition versus the control due to anoxia, as discussed above.

The previous study of *Artemia franciscana* under varying toxic surfactant conditions determined that the cationic surfactant, cetylpyridinium chloride (CPC), affected the hatching

ability of the *Artemia* but not the mortality after hatching (Deese et al. 2015). Only 10% or less of the *Artemia* cysts hatched under CPC conditions at 3.5 ppm or above. Hatching assays allow for the determination of whether a surfactant is toxic and at what concentration; however, they offer very limited information on the toxicity mechanisms. Because the CPC only affects hatching rates and not mortality rates, it can be assumed that CPC either 1) disrupts the cellular membranes of the embryos and causes them to die before hatching (Partearroyo et al. 1990) or 2) inhibits growth of the embryos by inhibiting their metabolism in some way(Roberts and Costello 2003). Surfactants have the ability to lyse cellular membranes and, although lysing tends to occur at surfactant concentrations close to the critical micelle concentration, this ability has been considered to play a role in surfactant toxicity to cells (Partearroyo et al. 1990). It has also been shown that cationic surfactants cause narcosis in aquatic organisms, which is a depression is biological activity typically caused by narcotics (Roberts and Costello 2003).

The presence of clear and stable ATP peaks detected indicates that CPC does not cause initial mortality of the embryos. If the CPC disrupted the cell membranes and caused embryonic death, the expected NMR spectrum would be expected to resemble that presented in Figure 5.4. This suggests that the second toxicity mechanism presented above is more likely.

For the *Artemia* under CPC conditions, the PDE peak increased but not to the extent of the control conditions, as illustrated by the ^{31}P NMR data presented in Figure 5.8. Because the increase in [PDE] is indicative of cell replication and thus, growth of the system, it is suggested that CPC inhibits cell replication. This is consistent with the cationic surfactant studies of aquatic organisms that indicate that cationic surfactants cause a decrease in biological activity (Roberts and Costello 2003).

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The anionic surfactant utilized in the hatching assays, sodium dodecyl sulfate (SDS), was similar to CPC in that it affected the hatching ability of the *Artemia* and did not affect mortality rates. Anionic surfactants have been shown to have a similar influence to cationic surfactants ability to depress biological functions by means of binding to bioactive macromolecules, such as peptides, enzymes, and DNA, causing conformational changes and dissociation (Cserháti et al. 2002). The same toxicity mechanisms as with the CPC can be suggested for SDS – either a perturbation of the cell membrane or some inhibition of growth.

Because the ^{31}P NMR spectra with SDS-exposed *Artemia* shows normal ATP signals, once again the perturbation of the cell membrane does not seem to be a major contributor to the toxicity of SDS. However, the lack of increase in [PDE] indicates that SDS inhibits cell replication or growth, as shown in Figure 5.8. Cationic and anionic surfactants have been shown to attack different components of cells; anionic surfactants bind to peptides and DNA, while cationic surfactants attack cytoplasmic membranes (Ivanković and Hrenović 2010, Ostroumov 2006). Although these two classes of surfactants may have different mechanisms of toxicity, it has been shown that both cause a decrease in biological activity or narcosis (Cserháti et al. 2002, Roberts and Costello 2003).

To verify the ^{31}P NMR finding in regards to reduced [PDE], as induced by CPC and SDS during the course of the *in vivo* ^{31}P NMR exposure studies and its linkage to cell growth and replication, HPLC was utilized to measure [GTP] changes over time. GTP is a metabolite that can act as a source of energy similar to that of ATP, an activator for substrates in metabolic reactions, and as a substrate for DNA replication. Embryogenesis studies of *Artemia* have indicated that an increase in the [GTP] is a major indicator of embryo growth(Warner and Finamore 1967), and a decrease in the [GTP] is indicative of stress on the system and lower hatching rates(Warner and

Clegg 2001). When the [GTP] increases, the growth in the system is assumed; however, under CPC and SDS conditions, the [GTP] significantly ($p < 0.05$) decreased, as shown in Figure 5.9, indicating that there inhibition in the embryo growth. This decrease in the [GTP] corroborates the inhibition of growth as seen in the *in vivo* ^{31}P NMR spectra.

In previous hatching assays, LAHA and, to a lesser extent, Florida Peak humic acid (FPHA) and Suwannee River humic acid (SRHA), were shown to have the ability to reduce the toxicity of Tx-100, CPC and SDS to *Artemia* (see Chapter 4, Deese et al. 2015). Although there was significant toxicity mitigation when these humic acids were present, some toxicity was still evident though slightly lower hatching rates. This phenomenon is clearly evident in the NMR data presented in Figures 5.10 and 5.11 for CPC and SDS, respectively, by the increase in the PDE/P_i ratio. However, the PDE/P_i remains lower than in the controls in the presence of the surfactant and LAHA, indicating that LAHA does not fully mitigate the toxicity of either surfactant. The toxicity mitigation ability of LAHA for these surfactants is attributed to interactions between LAHA and CPC or SDS, which in turn, reduces the bioavailability of these surfactants.

5.5 Conclusions

Surfactants pose a threat to the overall health of the environment as they can be toxic to a variety of organisms. It is also of great importance to measure organisms' responses to surfactants in the presence of other environmental constituents that are known to interact with pollutants, such as HA.

Artemia hatching and mortality assays are commonly used for toxicity measurements; however they offer limited information in terms of toxicity mechanisms and toxic responses before hatching. For example, while hatching assays demonstrated hatching inhibition caused by CPC and SDS surfactants the question of whether this inhibition was caused by membrane disruption

or inhibition of development remained unanswered. In this study, an *in vivo* ^{31}P NMR method was utilized to measure toxicity trends of surfactants and was able to illustrate growth inhibition of the *Artemia* embryos in the presence of CPC and SDS rather than membrane disruption. Over the course of time, the phosphometabolite profile of the 35 ppt NaCl control and Tx-100 solutions, but not the CPC or SDS solutions, show a significant increase in the PDE/P_i ratio for the *Artemia* embryos. These results demonstrate that CPC and SDS inhibit cell replication, and *in vivo* ^{31}P NMR is a powerful, yet direct, tool that provides a noninvasive measure of the growth inhibition, and thus, toxicity, in real time.

The toxicity mitigated effects on *Artemia* as a result of adding LAHA to the CPC and SDS solutions were investigated with the use of ^{31}P NMR and a greater PDE/P_i ratio than that for the CPC or SDS alone. This example illustrates (i) the importance of accounting for all the effects of all environmental matrix components, in particular HAs and other natural organic matter, and (ii) the ability of *in vivo* ^{31}P NMR spectroscopy to monitor the influence of these components.

This study also illustrates a significant advantage of *in vivo* ^{31}P NMR over other methods, such as hatching assays, which offer limited information on the toxicity processes, metabolomics, which utilizes ^{1}H NMR requiring extensive multivariate analysis, and metabolite extractions, which require extensive sample preparation and time for analysis, rendering them unsuitable for real time studies of environmental systems.

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CHAPTER 6
TOXICITY OF CARBON NANOTUBES TO *AREMIA FRANCISCANA* UNDER A VARIETY OF CONDITIONS

6.1 Introduction

Carbon nanotubes (CNTs) are hollow cylinders constructed of graphite sheets that can vary widely in length and diameter. There are two classes of carbon nanotubes: single-walled (SW) and multi-walled (MW). Single-walled CNTs consist of a single cylinder while MWCNTs consist of multiple cylinders that are placed concentrically within each other (Thomsen et al. 2007).

The main appeal of carbon nanotubes is that they have unique physical properties. CNTs have high thermal conductivity, high mechanical strength with flexibility, high electron/hole mobility, and low density. They also have the ability to be functionalized to increase their solubility or their reactivity (Kuzmany et al. 2004). Because of these properties, CNTs have many potential uses and are becoming widely used. CNTs are of particular interest in the fields of medical research, electronics, composites, and material sciences (D'Alessandro et al. 2016, Gerasimenko et al. 2015, Thomsen et al. 2007). CNTs may also be found in consumer products such as sporting goods, batteries, electronics, and clothing items because of their ability to add strength and better electrical conductivity (Kessler 2011).

Because of the increasing demand for CNTs in everyday products and research applications, this nanomaterial will inevitably enter aquatic environments. It is expected that CNTs will enter the environment via general weathering, accidental spillage, and from consumer waste of CNT-containing products (Nowack and Bucheli 2007, Petersen et al. 2011). CNTs are also generally hydrophobic and non-biodegradable so they can accumulate in the environment (Donaldson et al. 2006). It is important to understand the toxicity of CNTs to a variety of organisms

as well as the interactions they may have in the environment with environmental constituents such as natural organic matter (NOM). There have been many studies and reviews that have attempted to assess the sources, behavior, fate and toxicity of CNTs once they enter the environment (Crane et al. 2008, Donaldson et al. 2006, Du et al. 2013, Jackson et al. 2013, Klaine et al. 2012). A wide variety of organisms have been studied with CNTs such as the *Chydorus sphaericus* (water flea) (Velzeboer et al. 2008), *Ambystoma mexicanum* (salamander) (Mouchet et al. 2007), and *Daphina Magna* (water flea)(Kim et al. 2009); however, there has not been consistent or conclusive evidence on CNT toxicity. There have also been several toxicity mechanisms proposed for CNTs. One is biomembrane perturbation of the CNTs by physical penetration, oxidation of the biomembrane, and/or electrostatic interactions (Donaldson et al. 2006, Mwangi et al. 2012). Another proposed mechanism is that the CNTs create reactive oxygen species that are toxic to organelles or cause DNA damage. Some other suggestions are that any toxic effects are cause by metal impurities of the CNTs or that CNTs are inhibiting uptake of nutrients by the organism. It is a general consensus that CNT risk assessment studies must be carried out in a case-by-case basis because of the variability of results depending on physical characteristics of CNTs, the environments, and the organism being studied (Aitken et al. 2010, Jackson et al. 2013).

Another difficult challenge in environmental CNT studies is predicting the concentration of CNTs in soils, sediments, and water (Klaine et al. 2012, Mueller and Nowack 2008, Sun et al. 2014). Models predict that concentrations in soil range from 0.1 – 32 ng/kg and the range in surface water is 0.1 – 16 ng/L. These concentrations are believed to increase as CNT usage increases so they are likely on the low-end in regards to future CNT concentrations.

Because CNTs are hydrophobic, they tend to stay suspended in aqueous solutions and form large aggregates due to van der Waals forces. It is generally believed that by increasing the

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dispersion of CNTs, the bioavailability, and possibly toxicity, will increase (Jackson et al. 2013, Kennedy et al. 2009, Mwangi et al. 2012). Dispersion can be enhanced by functionalization with polar functional groups such as carboxyl groups (-COOH), sonication, and the addition of surfactants or NOM.

A variety of organic functional groups can be added to the surface of CNTs to functionalize them for different uses. CNTs functionalized with carboxyl groups (-COOH) is one of the more commonly studied types. The addition of -COOH reduces the van der Waals forces between the non-polar CNTs thus allowing water molecules to surround them and reduces aggregation (Kennedy et al. 2008). Carboxyl functionalization is achieved by either ultra-sonication in concentrated nitric and sulfuric acid or refluxing in nitric acid. These methods create oxidative defects on the surface of the CNTs resulting in –COOH functionalization (Balasubramanian and Burghard 2004).

Sonication not only helps to disperse CNTs into aquatic solutions, it can also physically alter CNTs by shortening them. These changes, in some cases, were found to increase the toxicity of CNTs. Three aquatic organisms (*Tigriopus japonicas, Oryzias melastigma,* and *Thalassiosira pseudonana*) were studied by Kwok et al. and it was determined that for all three, the EC50s (mean effective concentration) of the double-walled CNTs decreased by a minimum of one magnitude after 1 hour of sonication (Kwok et al. 2010). Another study conducted with zebrafish embryos suggested that the length of MWCNTs play an important role in the toxicity of functionalized CNTs. The CNTs were shortened by sonicating the MWCNTs for 24 and 48 h (Cheng and Cheng 2012).

CNT dispersion can be enhanced in the environment by NOM. NOM is a degradation product of biological matter and is thus omnipresent in the environment. Humic acids (HAs) are a

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type of NOM that are soluble in water at any pH above 2. By nature, NOMs (and HAs) are complex and heterogeneous organic molecular assemblies. This complexity allows for NOM to interact with a variety of xenobiotic pollutants, including CNTs. A NOM sorption study was performed by Hyung and Kim with MWCNTs with varying types of NOM and water quality parameters such as pH and ionic strength (Hyung and Kim 2008). This study determined that the type of NOM, especially the aromatic content, played a significant role in their sorption to MWCNTs. It was also found that as the pH or the ionic strength of the aqueous solution increased, as did the interactions between NOM and CNTs. Some of the interactions between NOM and CNTs are also attributed to the lipophilic groups of the NOM and it has been found that functionalized CNTs interact with NOM more so than non-functionalized (Lu and Su 2007). Previous studies have illustrated that the addition of NOM to CNTs can increase the toxicity of the CNTs to *D. Magna* (Edgington et al. 2010), *Chlorella vulgaris* and *P. subcapita* (Nowack and Bucheli 2007).

Both vertebrates and invertebrates have been studied with CNTs and it has been found that invertebrates are generally more sensitive to CNT toxicity than vertebrates (Jackson et al. 2013). Crustaceans, a type of invertebrate, have been widely studied in terms of CNT toxicity. *Daphnia Magna*, a crustacean commonly known as a water flea, is widely used in aquatic toxicity studies. Both SWCNTs and MWCNTs of different lengths, functionalities, and environmental conditions have been tested with *Daphnia Magna* in acute and chronic toxicity studies where: acute toxicity describes the toxicity effects caused by a toxin in the short term while chronic toxicity are long term effects such as decreased growth, inhibition of nutrition uptake and reproduction problems. A study by Edgington et al. studied *Daphnia Magna* with MWCNTs in the presence of NOM and found that growth was inhibited by the CNTs with NOM increasing that inhibition (Edgington et al. 2010). It was demonstrated that it was not acute CNT toxicity that had negative effects on the

organism but instead it was CNTs aggregating and clogging the gut. Several other studies have been performed with *Daphnia Magna* that seem to agree that toxicity for that organism is based on aggregation of CNTs in the gut (Petersen et al. 2009, Roberts et al. 2007, Zhu et al. 2009).

Other crustaceans have been studied with CNTs. *Ceriodaphnia dubia* and *Tigriopus* j*aponicas* were studied with MWCNTs and it was determined that the inability of them to eliminate CNTs from their gut was the cause for toxicity (Kennedy et al. 2008, Kwok et al. 2010, Li and Huang 2011). This is of concern because of the possibility of bioaccumulation up the food chain as many crustaceans are a source of food for larger organisms. Studies of another crustacean, *A. tenuiremis* with SWCNTs, showed that the organism was able to eliminate the SWCNTs from its gut and did not show significant toxicity (Ferguson et al. 2008).

All toxic CNT effects observed for these organisms were at higher than the predicted environmental concentrations. But, as previously mentioned, those concentrations are expected to increase as CNTs become more widely used.

Artemia Franciscana, or brine shrimp, is a saltwater crustacean that has been extensively used in aquatic toxicology studies, including with nanomaterials (Arulvasu et al. 2014, Nunes et al. 2006, Rajabi et al. 2015). The allure of utilizing *Artemia* for toxicology stems from their commercial availability, the ease of hatching, and the ability to store the cysts (eggs) for long periods of time. This particular organism has not, to current knowledge, been utilized for CNT toxicity studies. The other organisms mentioned are fresh-water organisms while *Artemia* requires a saline environment for survival. Since toxicity can be organism-based, it is ideal to perform toxicity tests on a large variety of organisms, including *Artemia.* It should be noted that the *Artemia* hatching assays performed for this study only indicates any possible acute toxicity at < 48

hours. Toxicity of CNTs to the *Artemia* after more than 48 hours or chronic toxicity cannot be determined with these assays.

The purpose of this study was to determine if there is any measurable toxicity of CNTs to *Artemia franciscana.* If so, does this toxicity change when the CNT's physical characteristics or the environment changed?

6.2 Materials and Methods

6.2.1 Materials

Leonardite HA standard, LAHA, was obtained from the International Humic Substances Society (Saint Paul, MN, USA). Sodium chloride and sodium hydrogen carbonate for the saline solution were purchased from Sigma–Aldrich. All the carbon nanotubes were purchased from Nano Lab (Waltham, MA, USA). Sterile 18 MΩ deionized water was sourced from an apparatus by US Filter (Snellville, GA, USA). *Artemia franciscana* were purchased from Brine Shrimp Direct (Ogden, UT, USA). Fisherbrand 80 x 15-mm Petri dishes were purchased from Fisher Scientific (Somerville, NJ, USA). A VWR (Radnor, PA, USA) mini shaker was used during the hatching assays. An AmScope SE305R-PZ stereoscopic microscope (Irvine, CA, USA) was used for observing and counting the *Artemia*.

6.2.2 Experimental design

Both SWCNTs and MWCNTs were studied in order to determine any difference in toxicity between the two classes of CNTs as previous studies have shown that they have differences in toxicity to a variety of organisms, with SWCNTs generally showing more toxic effects than MWCNTs. Two diameters of MWCNTs were studied, 15 nm (PD15) and 50 nm (PD50), to determine if the diameter of CNTs could play a role in any toxicity. All the CNTs studied were

functionalized with –COOH because the functionality lends to better dispersion in aqueous solutions.

LAHA, a lignite coal sourced HA, was chosen as the natural organic material. HAs are a major portion of NOMs, are soluble in water, and have been shown to interact strongly with non-polar pollutants. In particular, LAHA was chosen because it has previously shown similar binding trends as other HAs from varying sources and it is economically viable. Previous studies have shown that LAHA is non-toxic to *Artemia Franciscana* as it does not affect their hatching abilities or mortality rates (see Chapter 3 for hatching assay results with LAHA).

6.2.3 Stock solution preparation

The saline stock solution was prepared by measuring 70 g of NaCl and diluting it with 2 L of 18 MΩ deionized water for a final concentration of 35 ppt NaCl (to mimic saline environments). It was adjusted to a pH of 7.8 with sodium hydrogen carbonate.

The stock solutions of –COOH functionalized SWCNTs and MWCNTs were prepared by suspending approximately 50 mg of the CNTs in 250 mL of the saline solution. Adjustments to the pH were made if needed.

6.2.4 Sample preparation

LAHA stock solution was made by dissolving approximately 15 mg of LAHA in the saline solution. NaOH was added until the LAHA solid dissolved. The pH was brought back to 7.8 by using HCl and NaOH. The solution was diluted with the saline solution and stirred overnight (wrapped in foil so light doesn't cause it to oxidize) to equilibrate the solution. If necessary the pH was readjusted after the equilibration period.

6.2.5 LAHA and carbon nanotube solutions

LAHA and carbon nanotube solutions were prepared by adding appropriate amounts of the LAHA stock solution and carbon nanotube stock solution into a 50 mL volumetric flask. Some of the CNTs were not well dispersed so the CNT stock solutions were mixed well before dilution. The samples were diluted to 50 mL with the saline solution and the pH was adjusted to 7.8 with sodium hydrogen carbonate. The sample solutions were allowed to equilibrate overnight. The control solution was the saline solution for all sample series.

6.2.6 Sonication of carbon nanotubes

Sonication was performed to disperse the CNTs in the solution. If the CNTs are aggregated, sonication will break up this aggregation. Aggregation happens when the CNTs interact with themselves so they can't interact with their surroundings as much. The CNT solutions were put into 250 mL glass bottles and placed in a water sonication bath. The CNT solutions were sonicated for 30 minutes, 1 hour, and 2 hours at room temperature.

6.2.7 *Artemia* hatching assay

The *Artemia* hatching assay used in this study was based on previous experiments as is described in detail in chapter 3. Briefly, 25 – 28 hydrated *Artemia* eggs are placed into individual 80 x 15 mm petri dishes and the total number recorded. To each dish, 10 mL of a saline-pollutant solution was added. Three replicate samples were made per hatching assay and the hatching assays were performed in duplicate. The petri dishes were placed on a shaker at 100 rpm to keep the *Artemia* solutions oxygenated. The *Artemia* were not fed during the 48 h hatching assays. The number of hatched and dead *Artemia* were counted at 24 and 48 h by using a stereomicroscope. The count for each sample was repeated 4 – 5 times for accuracy. The hatching percentage and mortality percentage was calculated for each sample using the following equations:

$$Hatching\ Percentage = \frac{Hatched\ Artemia}{Number\ of\ initial\ eggs} * 100\% \qquad (1)$$

$$Mortality\ Percentage = \frac{Dead\ Artemia}{Hatched\ Artemia} * 100\% \qquad (2)$$

6.3 Results and Discussion

6.3.1 Varying concentrations of carbon nanotubes

The concentrations of PD15 MWCNTs, PD50 MWCNTs, and SWCNTs were varied from 10 ppm to 100 ppm in order to determine any toxicity of the CNTs alone. These results are measured as both hatching ability and mortality.

As can be seen in Figure 6.1, neither the 100 ppm PD50 or PD15 MWCNTs completely dispersed in the aqueous matrix; however, visually it can be noted that the PD50 MWCNTs seemed to disperse more than PD15.
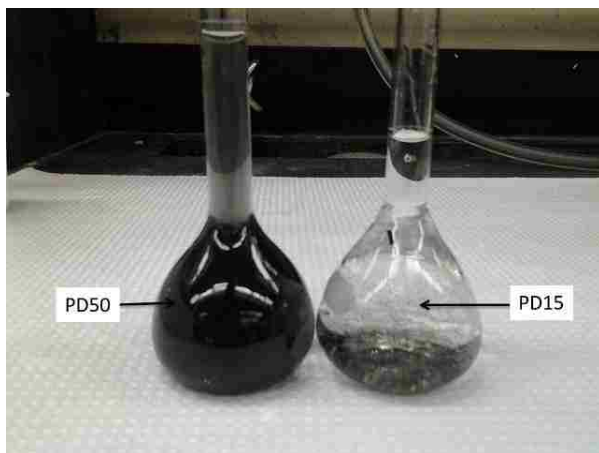

Figure 6.1 100 ppm of PD50 and PD15 MWCNTs

There are no 24 hour mortality percentages shown because there was no death of the *Artemia* in any of the assays at that time. As seen in Figure 6.2, there is a significant decrease (p < 0.05) in hatching percentage at 100 ppm PD15 MWCNTs, suggesting that there is some effect on

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the hatching ability of the *Artemia* once high concentrations of CNTs are reached. However, the mortality percentages for the *Artemia* do not show any significant changes as the concentration of PD15 CNTs increase.

MWCNTs with a diameter of 50 nm were also studied with *Artemia* with varying concentrations (Figure 6.3). The PD50 MWCNTs did not have any significant effects on the hatching percentages or the mortality percentages of the *Artemia* which indicates that there are no toxicity effects under these concentrations. Lowered hatching rates indicate acute toxicity to the embryos before they have the ability to hatch. This is caused by either permeation of the embryonic membrane and then death or an inhibition of growth processes.

The PD50 MWCNTs were more disperse than the PD15 MWCNTs but that did not play a role into the toxicity of the PD50 MWCNTS since the PD15 MWCNTs showed some toxicity at high concentrations while the PD50 MWCNTs did not. Single-walled CNTs were also studied with the *Artemia Franciscana* with varying concentration (Figure 6.4). For the *Artemia*, SWCNTs did not cause a significant decrease in hatching percentage or increase in mortality percentage versus the control group of the *Artemia*.

The effects of diameter on the toxicity of CNTs have had contrasting results in previous studies. The results of some studies showed that the increased diameter of MWCNTs also caused an increase in toxicity (Hamilton Jr et al. 2013, Wang et al. 2009).

The offered mechanism is that as the diameter increases, the rigidity of the MWCNT increase and thus, the increase of a physical/mechanical interaction with cellular membranes. However, other studies demonstrated higher toxicity for CNTs of smaller diameters than those with larger diameters (Allegri et al. 2016, Eom et al. 2015). The offered theory for these studies is either the thinner MWCNTs are able to perturb the cell wall or they are easier to uptake by the

biological organism. Comparing hatching results from the PD15 and PD50 MWCNTs with the *Artemia*, it can be seen that the smaller diameter MWCNTs were more toxic to the embryos. The thinner MWCNTs may have been able to penetrate the embryonic membrane and cause toxicity at very high CNT concentrations, possibly by disrupting the membranes or effecting mechanisms within the cells.

A multitude of studies have been performed to compare SWCNT toxicity with that of MWCNTs. It is the general consensus that SWCNTs are more toxic than MWCNTs (Jackson et al. 2013, Zhu et al. 2009). *Daphnia Magna* is an organism that has been studied with both SWCNTs and MWCNTs where MWCNTs have been shown to be less toxic. It should be noted that the *Daphnia Magna* toxicity assays were performed for mortality, not hatching ability (Thomsen et al. 2007, Zhu et al. 2009). It should also be noted that the mortality studies of *Daphnia Magna* generally conclude that toxicity is not caused by cell perturbation but instead either a clogged gut or by metal toxicity from CNT impurities (Mwangi et al. 2012).

The toxicity indicators in these Artemia studies are both hatching ability and short-term mortality. The SWCNTs may not have the ability or rigidity to penetrate the embryonic membrane while PD15 MWCNTs has increased rigidity because of the multi-walls, but still small enough to penetrate the membrane. The *Artemia* at 48 h did show an accumulation of CNTs in their gut for all assays; however because these are short-term mortality assays (up to 48 h), it is not known if the CNTs clog the gut and cause problems with long-term mortality rates (> 48 h) similar to the *D. Magna* (see Figure 6.5).

6.3.2 Sonication of carbon nanotubes

The CNTs were sonicated in an attempt to better disperse them throughout the aqueous media and possibly make them more bioavailable to the *Artemia*. There have been previous toxicological studies that measured changes in toxicity before and after CNT sonication.
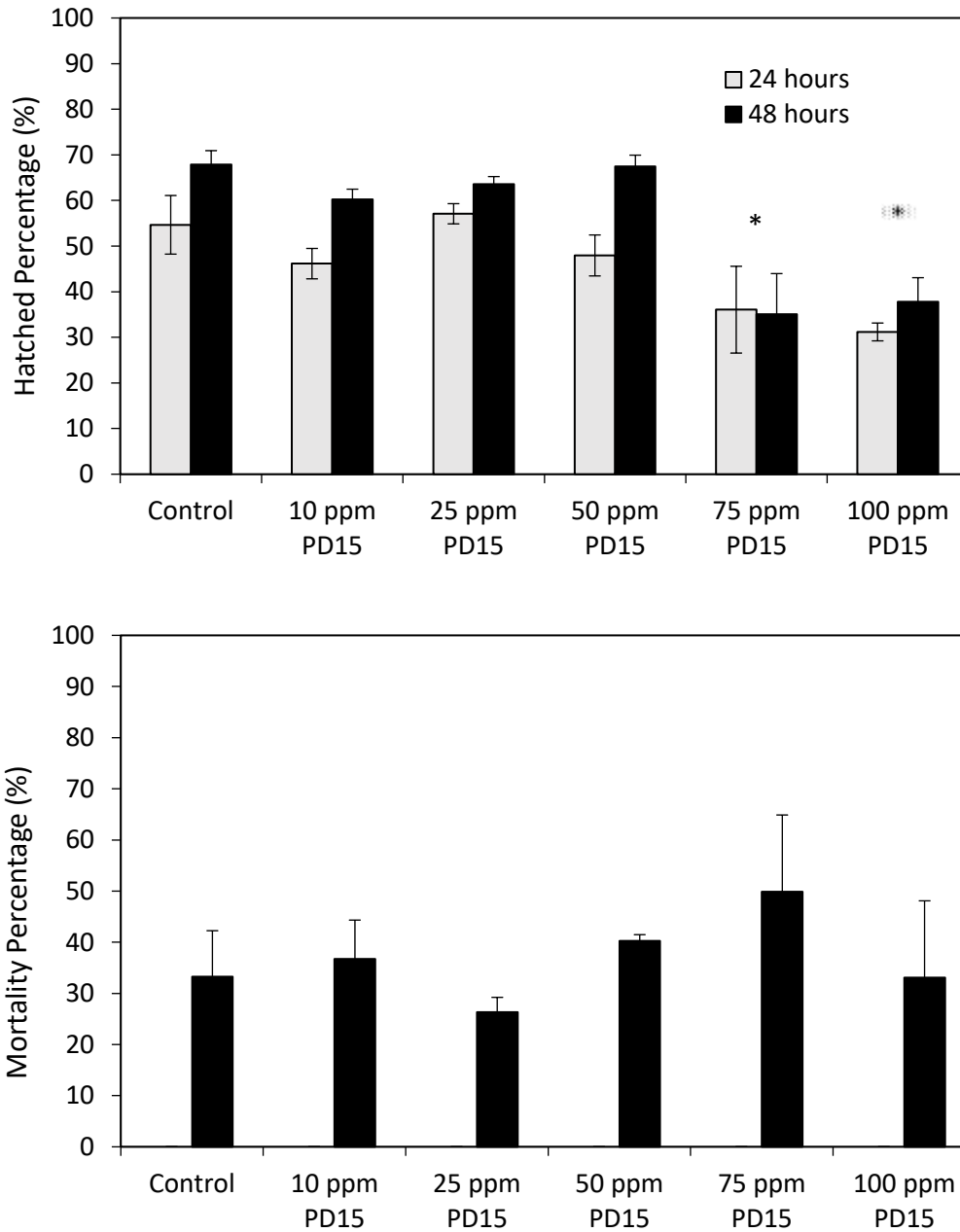

Figure 6.2 Hatching at 24 and 48 hours and mortality percentages at 48 hours of *Artemia* with varying concentrations of PD15 CNTs (*p < 0.05)
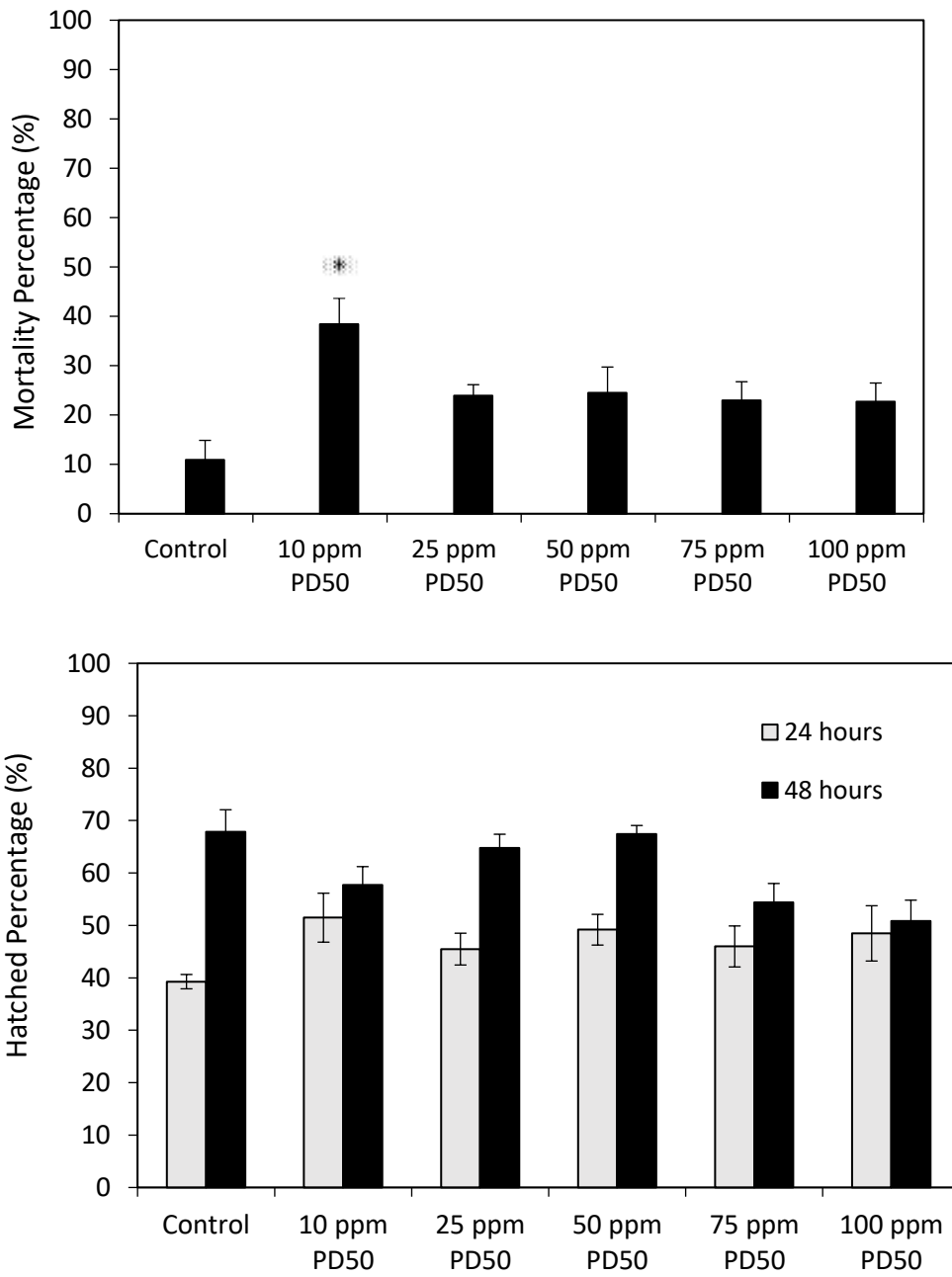
Figure 6.3 Hatching at 24 and 48 hours and mortality percentages at 48 hours of *Artemia* with varying concentrations of PD50 CNTs (*p < 0.05)
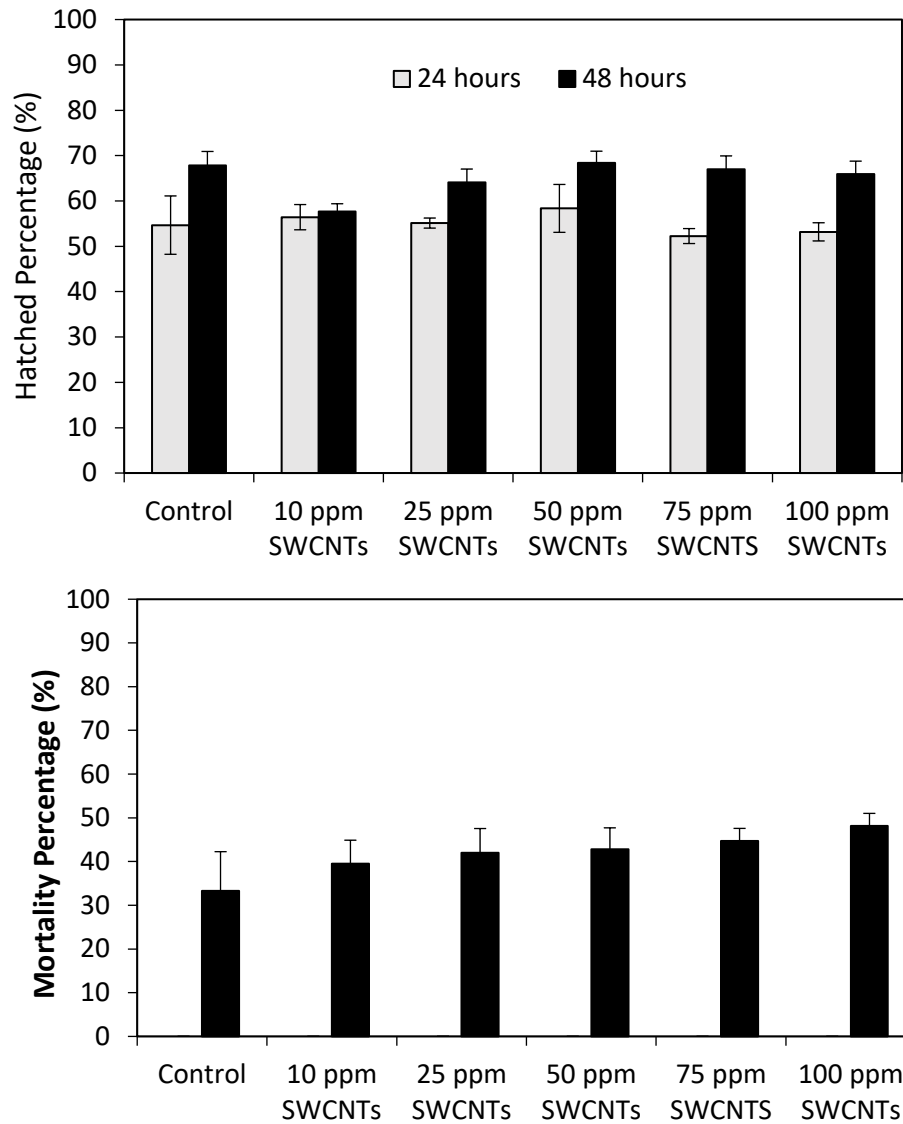
Figure 6.4 Hatching at 24 and 48 hours and mortality percentages at 48 hours of *Artemia* with varying concentrations of SWCNTs
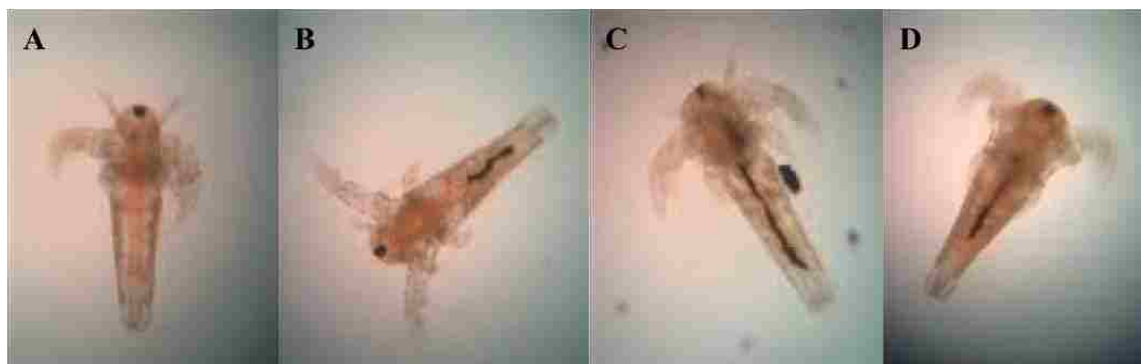

Figure 6.5 *Artemia* at 48 h: A) 35 ppt NaCl only, B) 10 ppm PD15, C) 10 ppm PD50, and D) 10 ppm SWCNT

However, sonication is not a naturally occurring phenomenon and so studying the effects of sonication may not be environmentally relevant. However, it can still help to illustrate if changes in the physical characteristics of CNTs play a major role in toxicity since sonication is also known to shorten CNTs, which can change their interactions with their environment. In Figure 6.6, it can be seen that as sonication time increased, as did the dispersion of the MWCNTS. The effect of sonication is visually more dramatic with the PD15 MWCNTs than the PD50 MWCNTs because the PD50 MWCNTS were already fairly disperse in the solution.
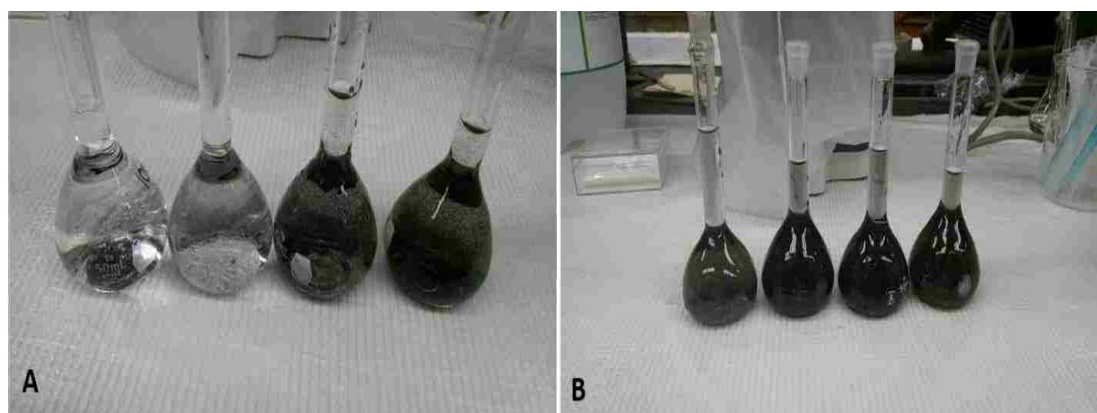

Figure 6.6 Left to right: non-sonicated, 30 min, 1 h, 2 h; A) 10 ppm PD15 MWCNTs B) 25 ppm PD50 MWCTs

In Figure 6.7, it can be seen that although the hatching percentages with the sonicated PD15 MWCNTs are similar to the control, the mortality percentages increase with soniciation time. This suggests that sonication has either allowed the PD15 MWCNTs to disperse and become more bioavailable, or the MWCNTs were shortened and possibly become more toxic as a result. However, the mortality percentage does not exceed 50%.

Unlike the PD515 MWCNTs, the PD50 MWCNTs did not show any toxicity changes after sonication which suggests that if there was further dispersion or shortening of the PD50 MWCNTs, it did not increase toxicity. The diameter, and possibly the membrane-perturbing potential, of the

PD50 MWCNTs would not be changed by sonication. Sonication hatching assays can be seen in
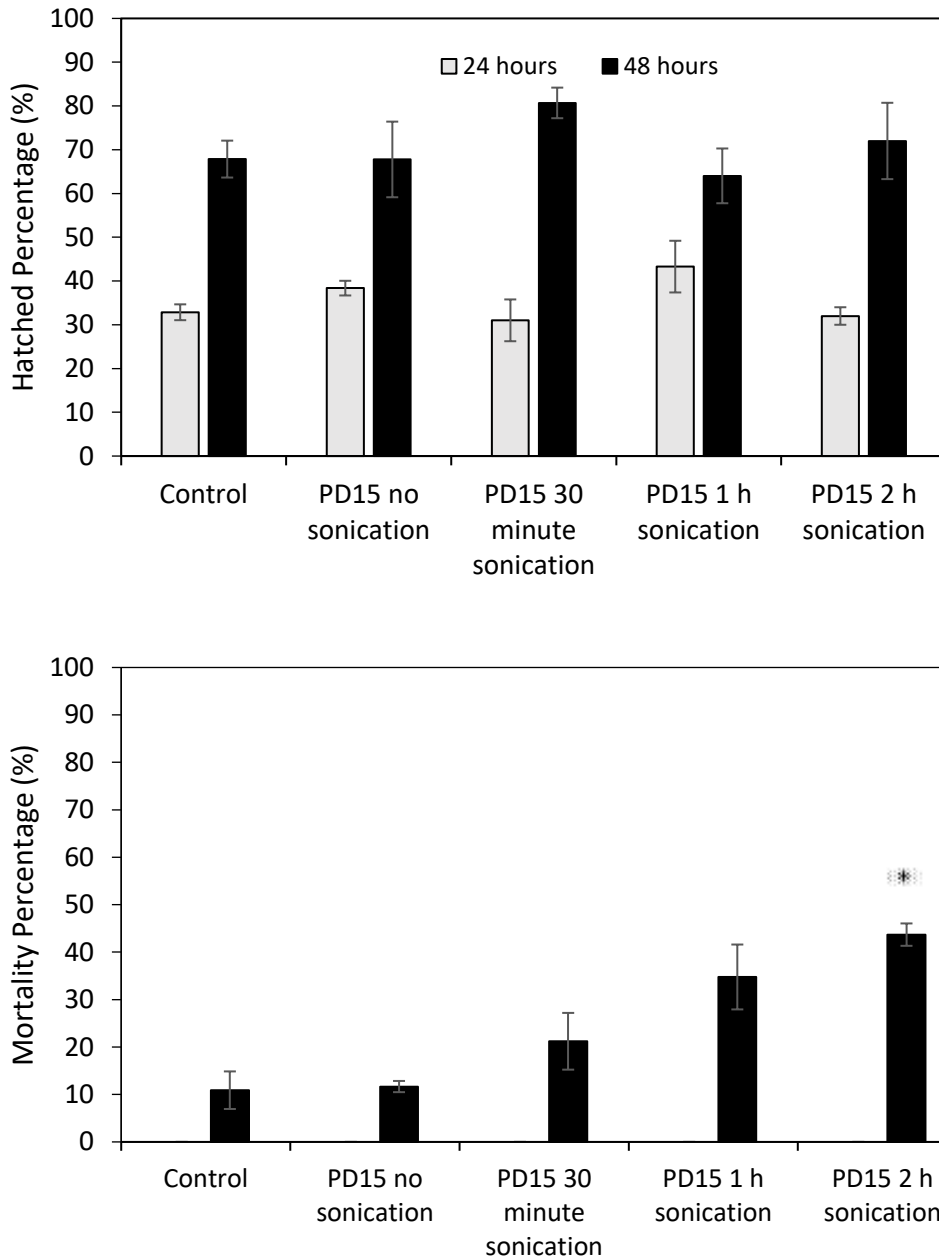
Figures 6.7, 6.8 and 6.9.

Figure 6.7 Hatching at 24 h and 48 h and mortality percentages of *Artemia* at 48 h with 10 ppm PD15 CNTs with varying sonication times (*p < 0.05)
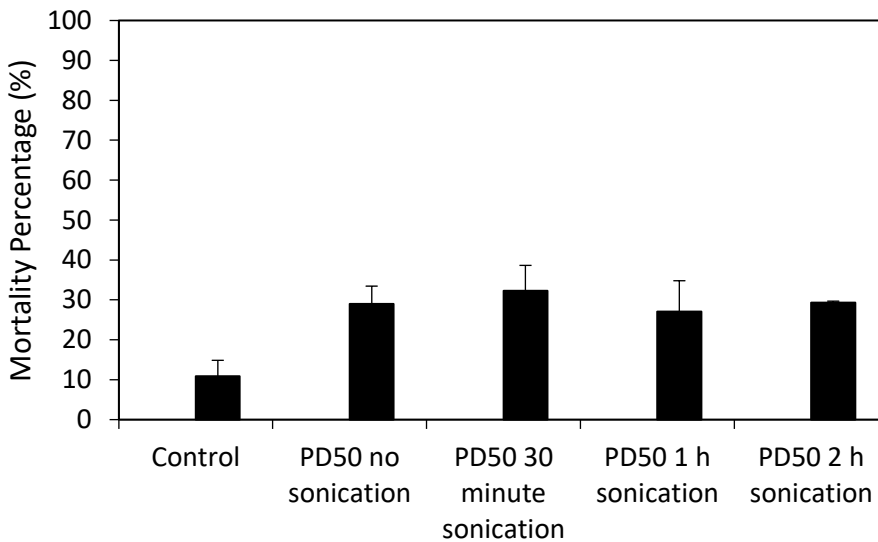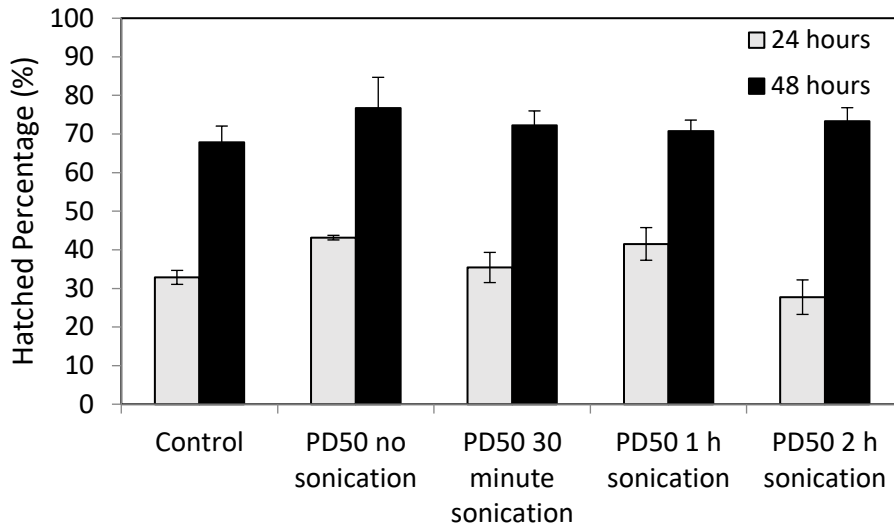
Figure 6.8 Hatching at 24 and 48 h and mortality percentages of *Artemia* at 48 h with 25 ppm PD50 MWCNTs with varying sonication times

Sonication of SWCNTs showed a slight decrease in hatching ability with initial sonication but further sonication did not show a decreased hatching percentage. After 1 hour of sonication, the SWCNT did show a significant increase in the mortality percentage and it is the only hatching assay that was higher than 50% mortality. If sonication had shortened the SWCNTs, the aspect ratio of the diameter would increase and would have possibly allowed for perturbation of the embryonic membrane.
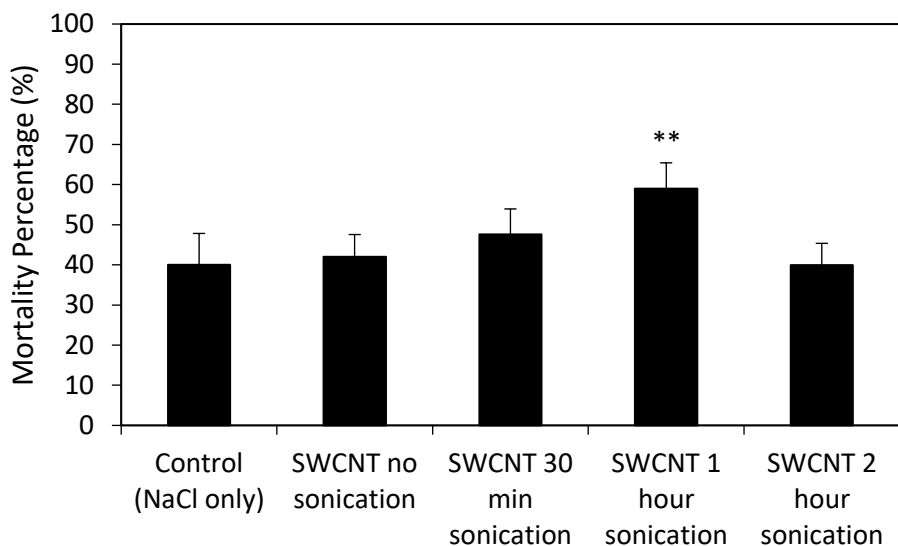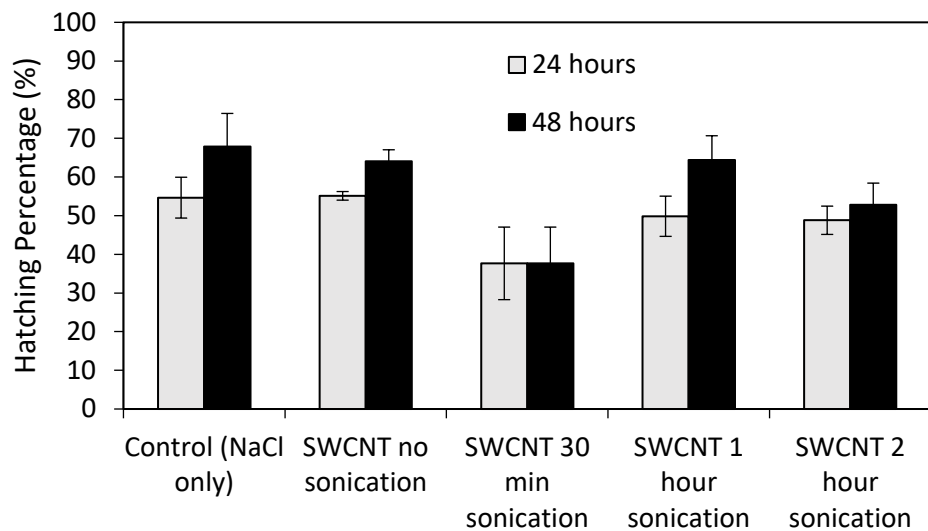
Figure 6.9 Hatching at 24 and 48 h and mortality percentages of *Artemia* at 48 h with 25 ppm SWCNTs with varying sonication times (** $p = 0.05000$)

However, this shortening of the SWCNTs do not seem to play a major role in toxicity since longer sonication times did not continue to show a decreased hatching ability. If shortening the SWCNTs caused toxicity, the longer sonication times should still show lower hatching percentages. This could possible indicate that there is a certain length or range of lengths of CNTs

that are toxic to the *Artemia* and further sonication or continued shortening, removes the CNTs from that range.

6.3.3 Carbon nanotubes with LAHA

For the PD15 MWCNTs, LAHA was added to solutions at two different PD15 MWCNT concentrations: 10 ppm PD15 (Figure 6.10) and 25 ppm PD15 (Figure 6.11). For both cases, LAHA did not have any effect on the toxicity of the PD15 MWCNTs. For 25 ppm PD50 MWCNTS (Figure 6.12) and 50 ppm SWCNTs (Figure 6.13), the addition of LAHA also did not show any toxic effects to the *Artemia*.

Although the LAHA enhances CNT dispersion, this did not result in CNT toxicity. These results indicate that HA did not cause any changes to the bioavailability of the CNTs. As with comparing the toxicity of CNTs with different diameter, so too do studies with NOM and CNTs illustrate contrasting results depending on the organism. A study that measured the toxicity of CNTs in the presence of NOM with *D. Magna* and *C. Dubia* showed that there was no increase in CNT toxicity with NOM (Edgington et al. 2010). These results are in contradiction with a different study utilizing the organism's *C. vulgaris* and *P. subcapita.* In that case, NOM enhanced the CNT toxicity and it was determined that the enhancement was due to NOM dispersing the CNTs and increasing their bioavailability to the organisms (Schwab et al. 2011). The difference in the toxicity changes compared with these two cases is likely because of the difference in organisms and thus the difference in toxicity mechanisms by the CNT. *D. Magna* and *C. Dubia* are crustaceans while *C. vulgaris* and *P. subcapita* are algae. The toxicity mechanism suggested for crustaceans are clogging of the gut and they show no evidence of CNT perturbation of biomembranes of the crustaceans. For algae, the toxicity mechanism suggested is shading of the algae (causing a lack of photosynthesis) and agglomeration of the algae cells.

Figure 6.10 Hatching at 24 and 48 h and mortality percentages of *Artemia* at 48 h with 10 ppm PD15 CNTs with varying LAHA concentrations

The addition NOM may enhance shading and agglomeration effects of the CNTs to the algae, which causes an increase in toxic effects. However, NOM may not increase the ability of CNTs to clog the gut of crustaceans and thus, does not change the levels of toxicity. Because *Artemia* are crustaceans and, in general, did not show acute toxicity to CNTs alone in solution, it is not surprising that NOM does not change the toxicity.
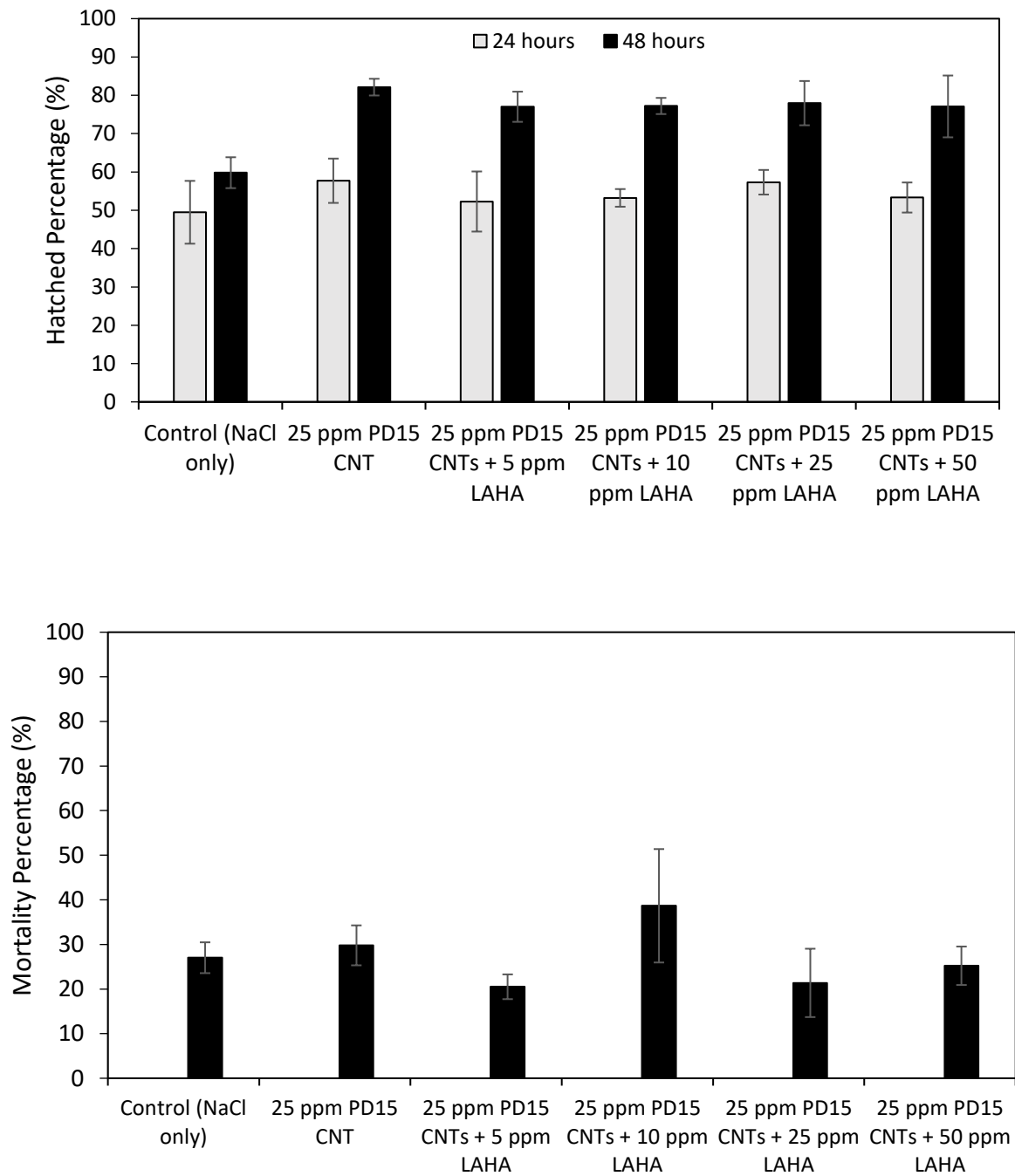
Figure 6.11 Hatching at 24 and 48 h and mortality percentages of *Artemia* at 48 h with 25 ppm PD15 MWCNTs with varying LAHA concentrations

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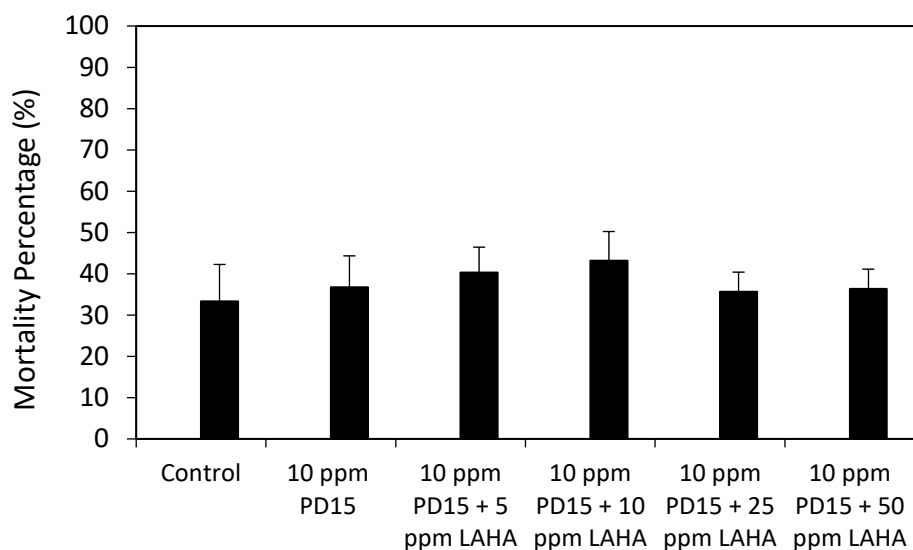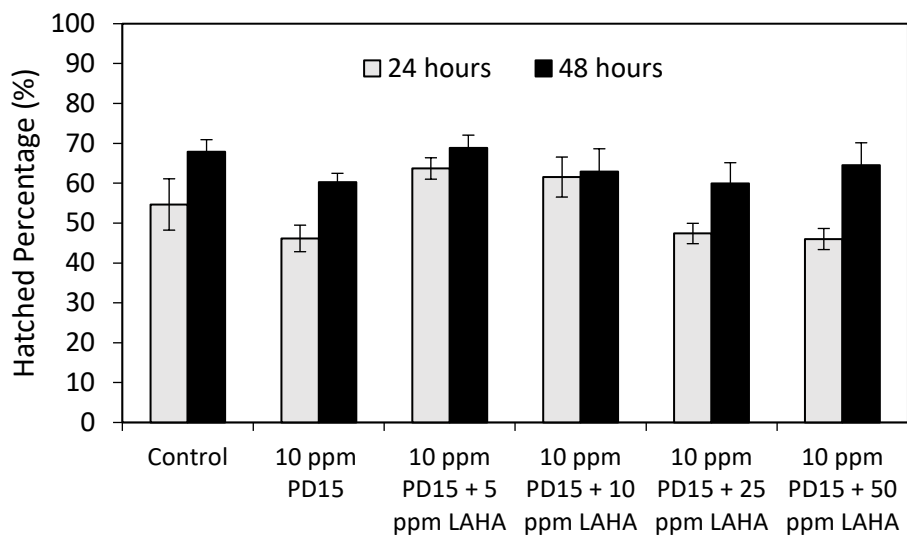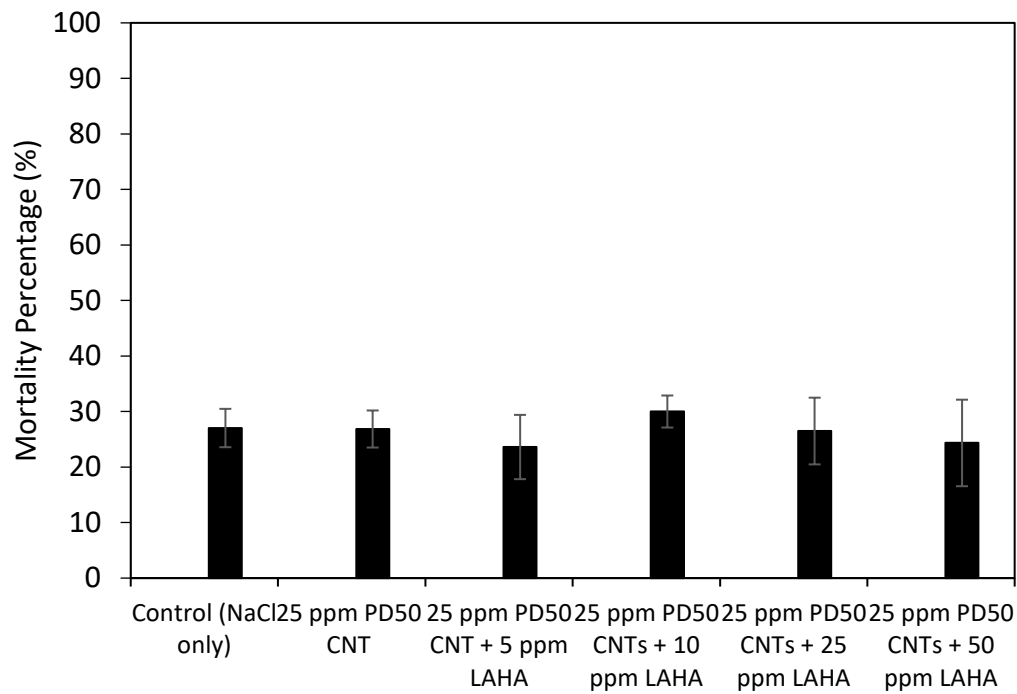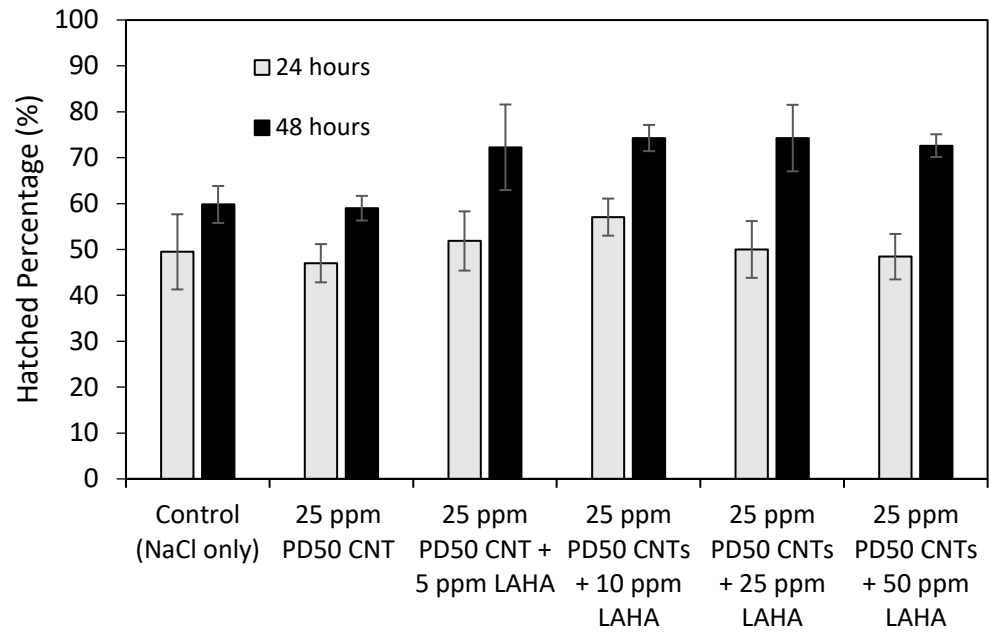Figure 6.12 Hatching at 24 and 48 h and mortality percentages of *Artemia* at 48 h with 25 ppm PD50 MWCNTs with varying LAHA concentrations
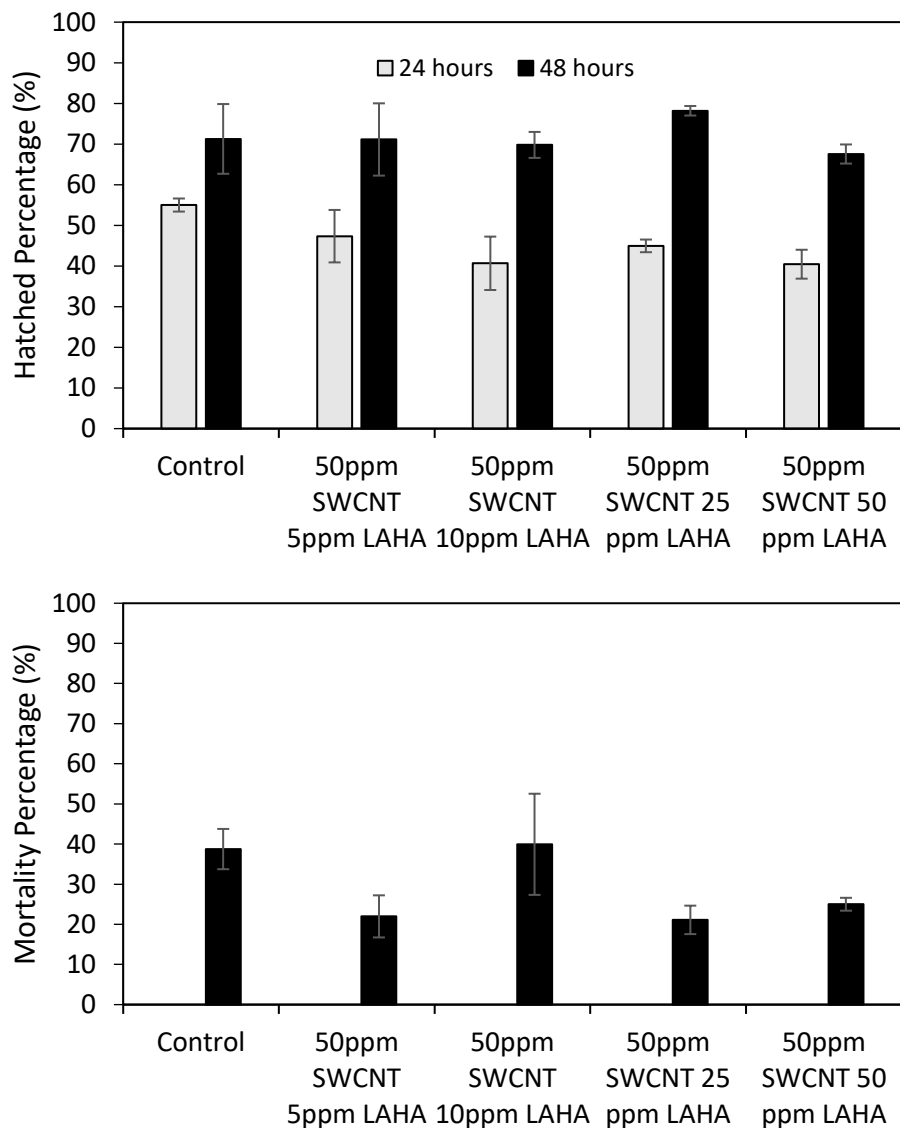
Figure 6.13 Hatching at 24 and 48 h and mortality percentages of *Artemia* at 48 h with 50 ppm SWCNTs with varying LAHA concentrations

6.4 Conclusions

In general, CNTs had little impact on the hatching ability and mortality of *Artemia*. Only under two conditions were CNTs shown to be marginally toxic. As sonication time increased, the mortality percentages of *Artemia* increased with 10 ppm PD15 MWCNTs and SWCNTs up to 1 hour of sonication. Sonication was performed on the CNTs because sonication can both disperse and change the CNTs' length to possible increase bioavailability to the Artemia.

Previous studies have suggested that the addition of a natural organic matter (NOM), such as HA, can cause an increase in toxicity of CNTs because it is a dispersion aid. This phenomena was not observed for any of these CNTs with *Artemia*.

Overall, the CNTs were not significantly toxic to the aquatic species *Artemia Franciscana* under a variety of conditions. Better dispersion of the CNTs naturally, by sonication or by NOM does not play a major role in the toxicity of CNTs to *Artemia Franciscana*. The diameter and rigidity of the CNTs may play a role in the ability of CNTs to perturb the embryonic membrane of the *Artemia.* This was suggested by some hatching inhibition by PD15 MWCNTs and not the PD50 MWCNTs and SWCNTs.

The type of toxicity that can be determined by these assays are acute toxicity. No chronic toxicity could be observed with < 48 h toxicity assays. Any mortality cause by gut clogging or bioaccumulation could not be determined in this study. These results suggest that there needs to be further thorough study on the toxicity of SWCNTs and MWCNTs under a variety of conditions for a more comprehensive understanding.

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CHAPTER 7
CONCLUSIONS AND FUTURE WORK

Although pollutant toxicity to organisms alone is important to understand, for environmental relevancy, other environmental constituents must be taken into account since they may affect toxicity and other environmental behaviors of pollutants. The overarching purpose of this dissertation work was to study and further understand the role of humic acid (HA) interactions with pollutants and biological organisms. The complexity of aquatic environments required the use of a systematic method designed to maintain relevancy while reducing some complexity. This was done by defining four environmental components that could be varied in these experiments: water, natural organic material, biological organisms, and pollutants.

7.1 Interactions of humic acids and cations and the influence on biomembrane perturbation

Previous studies have shown that humic acids can perturb cellular membranes but it was unknown how adding other chemical entities into the environment could effect that perturbation. The fluorescence studies presented in Chapter 3 of this dissertation discusses the influence of cations on the humic acid and its biomembrane interactions. Metal cations are also known to interact with humic substances by both electrostatic and chelating mechanisms and they are of concern due to increasing concentrations in the aquatic environments.

The passive interaction of humic acid with 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) large unilamellar model biomembranes in the presence of cations was measured using fluorescence spectroscopy. The following metal cations studied were chosen because they had a range of affinities to the functional groups found in HAs and charges: Na^+, Ca^{2+}, K^+, Mg^{2+}, Mn^{2+}, Co^{2+}, Cd^{2+}, Fe^{3+}, Al^{3+}.

Three different humic acids (Leonardite, Florida peat and Suwannee River) were studied with the biomembranes in the presence of Na^+ and Ca^{2+}. All three HAs were previously shown to

perturb biomembranes and both cations reduced that ability. Ca^{2+} had a greater ability to reduce the perturbation ability of the HAs by complexation and binding of the HA relative to the weak electrostatic interactions with Na^+. Comparing the three HAs, Leonardite humic acids's (LAHA) perturbing potential was not decreased in the presence of Na^+ to the extent of the other HAs which was attributed to LAHA having lower percentages of carboxyl and carbohydrate groups.

Mixed solutions of Na^+ and Ca^{2+} were studied to verify the difference of interactions between the cations and LAHA. It was found that as the percentage of Ca^{2+} in the mixture increased, the fluorescence intensity (and thus the perturbation of the membranes) decreased. There was more perturbation in the 100% Ca^{2+} solution than the 75% Ca^{2+}/25% Na^+ - illustrating the different binding affinities of different HA moieties for Na^+ and Ca^{2+}.

With the expanded set of cations, the impact of the cations on the LAHA's ability to perturb the biomembranes was measured. The cations protected the biomembranes in the following order: $K^+ < Na^+ < Mg^{+2} < Ca^{+2} < Mn^{+2} \approx Co^{+2} << Cd^{+2} << Fe^{+3} < Al^{+3}$. This order also followed the trend of charge and chemical softness. The greater the charge or the softer the cation, the stronger the binding to LAHA and hence a reduced LAHA-biomembrane interaction. This is attributed to the cations binding to LAHA sites that would have otherwise been involved in biomembrane interactions and that "less abundant" or strong binding sites of LAHA play a large role in chelation.

Finally, chemically modified LAHA was studied with Na^+, Ca^{+2}, Co^{+2}, Cd^{+2}, Fe^{+3} and Al^{+3} in order to determine which functional groups were responsible for the permeation of the biomembranes and the binding of the cations. Three chemical modifications were performed: bleaching to reduce aromatic moieties, Soxhlet extraction to reduce lipid moieties, and acid hydrolysis to reduce carbohydrate moieties. The LAHA perturbation of biomembranes is caused by hydrogen bonding followed by LAHA's hydrophobic moieties absorbing into the

biomembrane's lipid bilayer. Similar trends were seen for the lipid extracted and the acid hydrolyzed LAHA while the bleached humic acid showed a significant increase in membrane perturbation in the presence of the cations. The reduction of aromatics causes a reduction of membrane perturbation and an increased overall percentage of carbohydrate moieties that "protect" the biomembrane. In the presence of cations, the carbohydrates play a role in binding with them and thus, allowing the aliphatic components to have greater hydrophobic interactions with the biomembrane.

This study contributes to the overall goal of this dissertation by demonstrating that the interactions of HAs with biological membranes can be altered depending on other components in the environment, specifically cations.

7.2 Surfactant toxicity to *Artemia Franciscana* and the influence of humic acid and chemical composition

Chapter 4 details the studies of a more complex biological organism and pollutant than the previous chapter in the presence of HA. The aquatic crustacean, *Artemia Franciscana,* was utilized as an indicator for humic acid and pollutant interactions. The pollutants studied were surfactants, Triton X-100 (Tx-100), cetylpyridinium chloride (CPC), and sodium dodecyl sulfide (SDS), which are commonly used in everyday life and are often released into the environment. *Artemia* hatching and mortality assays were performed with the surfactants, three humic acids (LAHA, FPHA, and SRHA), chemically modified LAHA, and combinations in order to determine any surfactant toxicity changes in the presence of humic acids and elucidate any specific interactions that could be occurring. It was found that although all three surfactants were toxic to the *Artemia*, Tx-100 was the only one that affected mortality rates while CPC and SDS affected the hatching rates.

For Tx-100, the toxicity mitigation by HA followed the trend LAHA > FPHA > SRHA. Two possible interactions were proposed: π-π stacking of the aromatic groups of the HA and Tx-

100 and polarity interactions. Chemically modified LAHAs were studied to provide further insight, and it was discoved that the lipid-extracted LAHA did not have the same toxicity mitigation ability as the other HAs. This led to the conclusion that the aliphatic lipid-like moities are responsible for mitigating the toxicity of Tx-100. The trend with CPC toxicity mitigation was the same as that for Tx-100 with the unmodified HAs. However, the lipid-extracted LAHA had toxicity mitigation similar to that as the other HAs while the bleached LAHA had a lower hatching results, suggesting that it has less ability to mitigate toxicity. This observation can be explained by π-π stacking interactions playing a role in toxicity mitigation. It is also likely that there are electrostatic interactions between the cationic CPC and anionic HA moities.

For SDS, all the HAs and chemically modified LAHAs had the ability to mitigate the SDS toxicity. Because there were no trends based on the type of HA or the chemical modification, it suggests that SDS and HA undergo non-specfic binding interactions such as electrostatic or hydrophobic/hydrophilic interactions.

Overall, these studies show that HAs can interact with surfactants, reduce their bioavailability and, thus, their toxicity to *Artemia Francisicana*. This relates to the overall goal of the dissertation by elucidating some specific interactions that occur and demonstrate that while there is no universal mechanism of interaction, the complexity of HA lends to a range of possible mechanisms that can be studied systematically.

7.3 Use of *in vivo* ^{31}P NMR to measure phosphometabolite profile changes of *Artemia Franciscana* under toxic surfactant conditions

The studies presented in Chapter 5 also utilized *Artemia Franciscana* as a toxicity indicator for surfactants; however, rather than measuring the toxicity by hatching and mortality changes, the phospho-metabolite profile was measured by ^{31}P NMR in an attempt to study the toxicity *in vivo*. The embryonic development of *Artemia* is well known and, thus, changes in this development can

be monitored in order to determine toxicity responses before hatching. Since two of the surfactants in the previous study, CPC and SDS, effected hatching ability – it was desired to see if there were significant changes in the phospho-metabolite profile due to toxicity and how HA could affect any changes.

The resulting ^{31}P NMR profile for *Artemia* under normal conditions was similar to that of previous studies by Covi et al., Busaet al., Kwast et al., and also that of medaka embryos studied by Viant et al (Busa et al. 1982, Covi et al. 2005, Kwast et al. 1995, Viant et al. 2006). The peaks were identified as phosphomonoesters (PME), inorganic phosphate (P_i), phosphodiester (PDE), phosphocreatine (PCr), adenosine triphosphate (ATP), and adenosine diphosphate (ADP).

It was discovered that under healthy hatching conditions, the PDE peak of the *Artemia's* phospho-metabolite profile began to increase at approximately 150 min and continue to increase to approximately 300 min (5 h). This was attributed to significant cell replication occurring during early *Artemia* development. Interestingly, under Tx-100 conditions, the trend was the same as the controls while under CPC and SDS conditions, the PDE peak did not increase to the same extent. This suggests that CPC and SDS do not kill the embryos before they hatch, but instead inhibits growth in the embryos so they do not develop to the point of hatching. A perchloric acid extraction of metabolites and high performance liquid chromatography was utilized to measure guanosine triphosphate (GTP) levels of the embryos in order to verify the inhibited growth trend. An increase in GTP has been shown to indicate growth in *Artemia* embryos and the results of the HPLC studies determined that while there was an increase in GTP for both the control and Tx-100 conditions, there was a decrease in GTP concentration under CPC and SDS conditions. Thus, verifying that CPC and SDS causes an inhibition of growth.

Since the addition of LAHA had previously been shown to mitigate the toxicity of these surfactants to the *Artemia*, LAHA was added to CPC and SDS conditions to determine if the addition would mitigate the growth inhibition in a way that could be measured by the in vivo ^{31}P NMR method. The resulting spectra for both LAHA-CPC and LAHA-SDS conditions showed an increase in the PDE peak; however the PDE/P_i ratios were less than that of the controls alone. This may indicate that the LAHA does mitigate toxicity, but only partially, as observed in the previous hatching assays.

This study further verified HA-surfactant interactions mitigate toxicity and it lends to the overall goal of the work described in this dissertation by developing a method to dynamically study these toxic responses that allows for more information about the toxicity mechanisms to be obtained.

7.4 Toxicity of carbon nanotubes (CNTs) to *Artemia Franciscana* under a variety of conditions

Carbon nanotubes are a relatively new pollutant and the use of them in everyday products is increasing. Although there have been many studies performed to determine the toxicity of CNTs and how the physical properties and environment can affect toxicity, there is inconsistency in the results. The variation of results are caused by the different types of organisms, the physical properties of the CNTs, and the different environments, so more work is needed in order to better understand the problems that could occur by these nanomaterials. Chapter 6 of this dissertation details *Artemia* toxicity studies to both single-walled (SW) and double-walled (DW) CNTs, the effects of sonication, and the effects of LAHA. This study contributes to the overall theme of the dissertation work by studying the interactions, or lack thereof, of pollutants (CNTs), HAs, and biological organisms.

It was determined that sonicating 10 ppm of PD15 (diameter = 15 nm) MWCNTs and SWCNTs increased the mortality of the *Artemia* up to a sonication time of 1 h. However, all other conditions studied showed that the CNTs were not measurable toxic to the *Artemia*. It is likely that the CNTs could not penetrate the chorion (outer membrane) of the *Artemia* embryos to cause any toxic effects before hatching and mortality assays of < 48 h do not allow for chronic toxicity or starvation due to gut clogging to be measured.

7.5 Considerations for further research

The studies in this dissertation lead to the opportunity to continue this line of study in which the interactions between pollutants, natural organic material, and biological organisms are systematically varied. The following are some future directions that should be considered in the future.

In Chapter 3, the study of cations and humic acid interactions offered some interesting results in regards to changes in the ability of humic acids to interact with model biomembranes. Some metal cations have been shown to be toxic to *Artemia Franciscana* (Gajbhiye and Hirota 1990, Kokkali et al. 2011, MacRae and Pandey 1991); however, there seem to be no current studies measuring the toxicity of those cations under conditions containing natural organic matter. There was a study noting that there was a synergistic effect of toxicity of some metal cations to *Artemia* and, as Chapter 3 noted, multiple cations in solution (e.g. Na^+ and Ca^{2+}) can lead to greater binding of the HA (Gajbhiye and Hirota 1990). Studies should be continued with a variety of cations and mixtures of cations, HAs, and chemically modified HAs, to further understand pollutant-HA interactions and how it affects the threat to biological organisms.

Similarly to studies utilizing multiple metal cations to study synergistic (additive) toxicity effects - cations and surfactants could be measured as a two-pollutant system with *Artemia* to

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determine if toxicity increases when there is more than one pollutant in the system. Furthermore, the addition of HA and chemically modified HAs could lead to understanding of any possible competitive HA binding of the cations and surfactants.

The *Artemia* studies suggested here could be performed both as hatching and mortality assays as well as ^{31}P NMR studies. While surfactants have been shown to affect embryonic development by inhibiting growth, cations may have a different toxicity mechanism that could be elucidated via the phospho-metabolite profile.

For the ^{31}P NMR studies of *Artemia*, it is important to note that the phospho-metabolite profile measured in this dissertation was slightly different than that of the study previously performed at Louisiana State University (Covi et al. 2005). It was discovered during this dissertation work that the differences were due to differences in the preparation of the embryos before performing ^{31}P NMR. The "Covi spectrum" was accidently replicated when an error occurred during one experiment - the initial *Artemia* preparation failed and *Artemia* that had been sitting in fresh water (not a healthy medium for *Artemia* development) for an hour was used instead. Covi et al. prepared their *Artemia* embryos by hydrating the cysts for 24 hours, while the studies presented here only hydrated them for 1.5 h. This leads to questions that are beyond the scope of this dissertation but would be interesting to look into in the future.

Finally, it is believed that carbon nanotubes can penetrate biomembranes (Kang et al. 2007, Lelimousin and Sansom 2013). As shown in Chapter 6, the CNTs are overall non-toxic to *Artemia Franciscana* but it is not an indicator on the ability of CNTs to permeate biomembranes. An attempt was made to measure perturbation of CNTs with the POPC model biomembranes that were utilized in Chapter 3; however, it was found that the fluorescence of the dye was quenched

by the CNTs leading to inaccurate results. Future work could investigate other methodologies to study passive CNT perturbation of biomembranes by fluorescence spectroscopy.

7.6 References

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APPENDIX A
HATCHING ASSAY DATA TABLES AND P-VALUES

Supplemental to Chapter 4 and Chapter 6

Table A.1 *Artemia* hatching and mortality assays with LAHA, FPHA, and SRHA

Sample Name	20 hours	24 hours	32 hours	44 hours	48 hours
		Average Hatched Percentage			
35 ppt NaCl	17.14 ± 4.33%	57.28 ± 3.09%	61.28 ± 4.30%	64.90 ± 1.33%	64.99 ± 1.33%
5 ppm LAHA	14.36 ± 3.11%	53.23 ± 0.53%	71.44 ± 2.89%	71.44 ± 2.89%	71.44 ± 2.89%
25 ppm LAHA	24.74 ± 1.48%	57.09 ± 2.21%	73.83 ± 3.46%	73.83 ± 3.46%	73.83 ± 3.46%
100 ppm LAHA	18.07 ± 2.69%	57.19 ± 3.08%	67.75 ± 4.19%	67.75 ± 4.19%	67.75 ± 4.19%
5 ppm FPHA	20.62 ± 3.23%	62.14 ± 6.89%	69.43 ± 2.99%	69.43 ± 2.99%	69.43 ± 2.99%
25 ppm FPHA	24.87 ± 6.62%	49.44 ± 2.84%	66.21 ± 4.90%	68.77 ± 3.55%	68.77 ± 3.55%
100 ppm FPHA	15.74 ± 1.81%	44.72 ± 0.62%	63.18 ± 0.71%	68.46 ± 1.67%	68.46 ± 1.67%
5 ppm SRHA	23.79 ± 3.64%	52.67 ± 1.53%	67.13 ± 0.75%	67.13 ± 0.75%	67.13 ± 0.75%
25 ppm SRHA	17.74 ±3.33%	47.67 ± 2.30%	67.61 ± 2.26%	67.61 ± 2.26%	67.61 ± 2.26%
100 ppm SRHA	19.00 ± 4.82%	55.04 ± 1.89%	72.02 ± 5.20%	73.30 ± 1.39%	73.30 ± 1.39%
		Average Mortality Percentage			
35 ppt NaCl	0%	0%	0%	18.01 ± 2.96%	23.90 ± 2.67%
5 ppm LAHA	0%	0%	1.67 ±1.44%	8.92 ± 3.95%	18.14 ± 3.32%
25 ppm LAHA	0%	0%	0%	17.39 ± 2.64%	17.39 ± 2.64%
100 ppm LAHA	0%	0%	0%	21.11 ± 1.20%	23.19 ± 1.0%
5 ppm FPHA	0%	0%	0%	13.09 ± 1.98%	22.49 ± 6.08%
25 ppm FPHA	0%	0%	3.21 ± 1.60%	28.43 ± 2.97%	28.43 ± 2.97%
100 ppm FPHA	0%	0%	0%	11.76 ± 5.88%	13.73 ± 6.12%
5 ppm SRHA	0%	0%	0%	15.69 ± 4.49%	19.61 ± 6.12%
25 ppm SRHA	0%	0%	3.03 ± 1.52%	5.47 ±2.64%	9.28 ± 1.64%
100 ppm SRHA	0%	0%	0%	19.31 ± 1.39%	26.72 ± 5.34%

Table A.2 *Artemia* hatching and mortality assays with bLAHA, hLAHA, and leLAHA

Sample Name	20 hours	24 hours	32 hours	44 hours	48 hours
		Average Hatched Percentage			
35 ppt NaCl	6.00 ± 1.10%	45.45 ± 6.37%	67.13 ± 2.6%)	67.13 ± 2.63%	67.13 ± 2.63%
5 ppm bLAHA	13.03 ± 2.31%	58.56 ± 4.09%	72.77 ± 1.70%	72.77 ± 1.70%	72.77 ± 1.70%
25 ppm bLAHA	22.85 ± 6.79%	51.18 ± 3.44%	60.16 ± 4.33%	61.50 ± 3.45%	61.50 ± 3.45%
100 ppm bLAHA	10.47 ± 4.25%	51.34 ± 2.47%	69.26 ± 5.03%	71.82 ± 2.83%	71.82 ± 2.83%
5 ppm hLAHA	17.33 ± 7.02%	64.00 ± 5.87%	68.00 ± 2.00%	68.00 ± 2.00%	68.00 ± 2.00%
25 ppm hLAHA	3.66 ± 1.85%	46.44 ± 4.00%	62.43 ± 4.94%	62.43 ± 4.94%	62.43 ± 4.94%
100 ppm hLAHA	28.00 ± 3.46%	56.00 ± 5.29%	65.33 ± 1.15%	65.33 ± 1.15%	65.33 ± 1.15%
5 ppm leLAHA	31.51 ± 7.53%	60.99 ± 0.86%	67.36 ± 3.53%	68.69 ± 1.88%	68.69 ± 2.55%
25 ppm leLAHA	11.79 ± 4.04%	59.79 ± 6.05%	66.36 ± 5.68%	66.36 ± 5.68%	66.36 ± 5.68%
100 ppm leLAHA	19.21 ± 5.42%	59.46 ± 4.83%	68.69 ± 3.80%	68.69 ± 3.80%	68.69 ± 3.80%
		Average Mortality Percentage			
35 ppt NaCl	0%	0%	0%	7.31 ±4.26%	11.25 ± 4.75%
5 ppm bLAHA	0%	0%	0%	12.67 ± 5.72%	19.88 ± 7.04%
25 ppm bLAHA	0%	0%	0%	12.10 ± 2.39%	22.88 ± 0.94%
100 ppm bLAHA	0%	0%	0%	8.80 ± 1.27%	10.76 ± 0.45%
5 ppm hLAHA	0%	0%	0%	19.66 ± 3.49%	25.68 ± 6.50%
25 ppm hLAHA	0%	0%	0%	5.83 ± 3.15%	15.30 ± 3.54%
100 ppm hLAHA	0%	0%	0%	7.97 ± 4.47%	20.10 ± 7.28%
5 ppm leLAHA	0%	0%	0%	20.88 ± 1.88%	24.51 ± 0.42%
25 ppm leLAHA	0%	0%	0%	25.37 ± 6.02%	27.75 ± 5.79%
100 ppm leLAHA	0%	0%	0%	11.48 ± 0.85%	17.59 ± 4.05%

Table A.3 *Artemia* mortality percentages with 100 ppm Tx-100 and LAHA, FPHA, SRHA, bLAHA, hLAHA, and leLAHA

Sample name	20 hours	24 hours	32 hours	44 hours	48 hours
			Average Mortality Percentages		
35 ppt NaCl	0%	0%	6.14 ± 3.41%	25.77 ± 7.84%	27. ± 6.16%
100 ppm Tx-100	59.09 ± 11.45%	83.5 ± 13.31%	91.07 ± 4.49%	97.92 ± 2.08%	100%
25 ppm LAHA + 100 ppm Tx-100	21.03 ± 11.59%	46.29 ± 10.12%	65.02 ± 7.34%	100%	100%
50 ppm LAHA + 100 ppm Tx-100	7.78 ± 4.84%	16.35 ± 1.66%	33.83 ± 2.22%	93.70 ± 3.42%	100%
100 ppm LAHA + 100 ppm TX-100	7.69 ± 7.69%	18.01 ± 6.80%	35.73 ± 7.15%	96.30 ± 3.70%	96.30 ± 3.70%
25 ppm FPHA + 100 ppm Tx-100	61.72 ± 14.59%	80.56 ± 10.02%	92.86 ± 7.14%	100%	100%
50 ppm FPHA + 100 ppm Tx-100	8.83 ± 1.14%	18.89 ± 4.01%	71.33 ± 3.67%	100%	100%
100 ppm FPHA + 100 ppm Tx-100	25.37 ± 5.87%	36.35 ± 1.06%	58.03 ± 8.04%	94.53 ± 3.05%	94.53 ± 3.05%
25 ppm SRHA + 100 ppm Tx-100	31.61 ± 5.84%	52.39 ± 1.21%	68.24 ± 2.61%	100%	100%
50 ppm SRHA + 100 ppm Tx-100	56.10 ± 15.87%	63.61 ± 14.68%	95.54 ± 2.25%	97.62 ± 2.38%	97.62 ± 2.38%
100 ppm SRHA + 100 ppm Tx-100	12.89 ± 3.19%	57.95 ± 11.28%	93.33 ± 5.44%	100%	100%
100 ppm LAHA + 100 ppm Tx-100	10.89 ± 2.21%	32.16 ± 0.51%	45.10 ± 4.59%	91.49 ± 3.85%	96.37 ± 1.57%
100 ppm bLAHA + 100 ppm Tx-100	0%	19.58 ± 0.36%	23.58 ± 3.79%	94.41 ± 2.95%	98.04 ± 1.70%
100 ppm hLAHA + 100 ppm Tx-100	0%	13.66 ± 5.98%	40.58 ± 7.19%	100%	100%
100 ppm leLAHA + 100 ppm Tx-100	54.00 ± 13.34%	69.69 ± 10.99%	92.82 ± 0.36%	100%	100%

bLAHA = bleached LAHA, hLAHA = hydrolyzed LAHA, leLAHA = lipid-extracted LAHA

Table A.4 p values for *Artemia* mortality percentages with 100 ppm Tx-100 at 48 hours

	Control	0 ppm LAHA	25 ppm LAHA	50 ppm LAHA	100 ppm LAHA
Control	n/a	0.00	0.01	0.00	0.04
0 ppm LAHA	n/a	n/a	0.02	0.00	0.01
25 ppm LAHA	n/a	n/a	n/a	0.04	0.05
50 ppm LAHA	n/a	n/a	n/a	n/a	0.82
100 ppm LAHA	n/a	n/a	n/a	n/a	n/a

	Control	0 ppm SRHA	25 ppm SRHA	50 ppm SRHA	100 ppm SRHA
Control	n/a	0.00	0.00	0.00	0.10
0 ppm SRHA	n/a	n/a	0.02	0.44	0.47
25 ppm SRHA	n/a	n/a	n/a	0.00	0.91
50 ppm SRHA	n/a	n/a	n/a	n/a	0.39
100 ppm SRHA	n/a	n/a	n/a	n/a	n/a

	Control	0 ppm FPHA	25 ppm FPHA	50 ppm FPHA	100 ppm FPHA
Control	n/a	0.00	0.00	0.00	0.01
0 ppm FPHA	n/a	n/a	0.84	0.03	0.03
25 ppm FPHA	n/a	n/a	n/a	0.08	0.02
50 ppm FPHA	n/a	n/a	n/a	n/a	0.24
100 ppm FPHA	n/a	n/a	n/a	n/a	n/a

	Control	0 ppmHA	LAHA	bLAHA	hLAHA	leLAHA
Control	n/a	0.00	0.01	0.02	0.04	0.00
0 ppm HA	n/a	n/a	0.00	0.00	0.01	0.74
LAHA	n/a	n/a	n/a	0.04	0.67	0.01
bLAHA	n/a	n/a	n/a	n/a	0.17	0.00
hLAHA	n/a	n/a	n/a	n/a	n/a	0.02
leLAHA	n/a	n/a	n/a	n/a	n/a	n/a

Table A.5 *Artemia* hatching percentages with 3.5 ppm CPC and LAHA, FPHA, SRHA, bLAHA, hLAHA, and leLAHA

Sample name	20 hours	24 hours	32 hours	44 hours	48 hours
		Average Hatching Percentages			
35 ppt NaCl	56 ± 7.1%	59.83 ± 5.30%	67.11 ± 3.50%	69.58 ± 5.96%	69.58 ± 5.96%
3.5 ppm CPC	3.85 ± 3.85%	5.13 ± 5.13%	5.13 ± 5.13%	6.41 ± 6.41%	6.41 ± 6.41%
1 ppm LAHA + 3.5 ppm CPC	21.13 ± 9.67%	23.74 ± 8.39%	25.03 ± 8.14%	27.69 ± 6.22%	27.69 ± 6.22%
3.5 ppm LAHA + 3.5 ppm CPC	34.67 ± 2.67%	50.67 ± 1.33%	58.67 ± 3.53%	58.67 ± 3.53%	58.67 ± 3.53%
5 ppm LAHA + 3.5 ppm CPC	52.66 ± 4.56%	58.97 ± 5.59%	66.30 ± 1.50%	66.30 ± 1.50%	66.30 ± 1.50%
1 ppm FPHA + 3.5 ppm CPC	10.67 ± 4.81%	22.67 ± 3.53%	22.67 ± 3.53%	22.67 ± 3.53%	24.00 ± 2.31%
3.5 ppm FPHA + 3.5 ppm CPC	29.47 ± 7.05%	42.35 ± 4.00%	44.92 ± 1.66%	48.87 ± 3.60%	48.47 ± 3.60%
5 ppm FPHA + 3.5 ppm CPC	35.52 ± 3.69%	45.63 ± 2.69%	51.80 ± 3.89%	59.45 ± 2.83%	59.45 ± 2.83%
1 ppm SRHA + 3.5 ppm CPC	13.33 ± 8.11%	17.33 ± 5.33%	25.33 ± 4.81%	25.33 ± 4.81%	25.33 ± 4.81%
3.5 ppm SRHA + 3.5 ppm CPC	24.38 ± 1.37%	38.65 ± 4.69%	42.49 ± 4.61%	42.49 ± 4.61%	42.49 ± 4.61%
5 ppm SRHA + 3.5 ppm Tx-100	34.67 ± 1.33%	46.67 ± 1.33%	49.33 ± 1.33%	50.67 ± 2.67%	50.67 ± 2.67%
5 ppm bLAHA + 3.5 ppm CPC	37.48 ± 4.25%	53.09 ± 2.85%	53.09 ± 2.85%	54.42 ± 2.10%	54.42 ± 2.10%
5 ppm hLAHA + 3.5 ppm CPC	48.56 ± 4.43%	64.46 ± 2.04%	73.69 ± 3.01%	73.69 ± 3.01%	73.69 ± 3.01%
5 ppm leLAHA + 3.5 ppm CPC	39.00 ± 2.36%	55.90 ± 2.62%	61.08 ± 1.59%	67.59 ± 2.71%	67.59 ± 2.71%

bLAHA = bleached LAHA, hLAHA = hydrolyzed LAHA, leLAHA = lipid-extracted LAHA

Table A.6 p values for *Artemia* hatching percentages with 3.5 ppm CPC at 32 hours

	Control	0 ppm LAHA	1 ppm LAHA	3.5 ppm LAHA	5 ppm LAHA
Control	n/a	0.00	0.01	0.21	0.64
0 ppm LAHA	n/a	n/a	0.08	0.01	0.01
1 ppm LAHA	n/a	n/a	n/a	0.02	0.02
3.5 ppm LAHA	n/a	n/a	n/a	n/a	0.15
5 ppm LAHA	n/a	n/a	n/a	n/a	n/a
	Control	0 ppm FPHA	1 ppm FPHA	3.5 ppm FPHA	5 ppm FPHA
Control	n/a	0.01	0.00	0.03	0.18
0 ppm FPHA	n/a	n/a	0.10	0.01	0.01
1 ppm FPHA	n/a	n/a	n/a	0.01	0.00
3.5 ppm FPHA	n/a	n/a	n/a	n/a	0.09
5 ppm FPHA	n/a	n/a	n/a	n/a	n/a
	Control	0 ppm SRHA	1 ppm SRHA	3.5 ppm SRHA	5 ppm SRHA
Control	n/a	0.01	0.01	0.03	0.02
0 ppm SRHA	n/a	n/a	0.08	0.01	0.01
1 ppm SRHA	n/a	n/a	n/a	0.06	0.02
3.5 ppm SRHA	n/a	n/a	n/a	n/a	0.22
5 ppm SRHA	n/a	n/a	n/a	n/a	n/a

	Control	0 ppm HA	LAHA	bLAHA	hLAHA	leLAHA
Control	n/a	0.00	0.02	0.06	0.07	0.25
0 ppm HA	n/a	n/a	0.00	0.00	0.00	0.00
LAHA	n/a	n/a	n/a	0.01	0.34	0.65
bLAHA	n/a	n/a	n/a	n/a	0.10	0.15
hLAHA	n/a	n/a	n/a	n/a	n/a	0.27
leLAHA	n/a	n/a	n/a	n/a	n/a	n/a

Table A.7 *Artemia* hatching percentages with 25 ppm SDS and LAHA, FPHA, SRHA, bLAHA, hLAHA, and leLAHA

Sample name	20 hours	24 hours	32 hours	44 hours	48 hours
	Average Hatching Percentages				
35 ppt NaCl	54 ± 1.90%	60.51 ± 2.37%	60.51 ± 2.37%	60.51 ± 2.37%	60.51 ± 2.37%
25 ppm SDS	13.33 ± 2.67%	14.66 ± 1.33%	14.66 ± 1.33%	17.33 ± 3.53%	17.33 ± 3.53%
5 ppm LAHA + 25 ppm SDS	25.03 ± 3.60%	27.69 ± 4.16%	31.64 ± 4.19%	38.10 ± 5.07%	38.10 ± 5.07%
10 ppm LAHA + 25 ppm SDS	30.66 ± 1.33%	33.33 ± 2.66%	34.67 ± 3.53%	37.33 ± 3.53%	37.33 ± 3.53%
25 ppm LAHA + 25 ppm SDS	32.42 ± 5.42%	38.97 ± 2.28%	41.59 ± 3.54%	41.59 ± 3.54%	41.59 ± 3.54%
5 ppm FPHA + 25 ppm SDS	20.92 ± 5.97%	20.92 ± 5.97%	23.54 ± 6.33%	23.54 ± 6.33%	23.54 ± 6.33%
10 ppm FPHA + 25 ppm SDS	35.79 ± 5.06%	38.31 ± 5.15%	38.31 ± 5.15%	39.64 ± 5.02%	39.64 ± 5.02%
25 ppm FPHA + 25 ppm SDS	41.22 ± 1.74%	45.01 ± 0.57%	48.72 ± 1.67%	48.72 ± 1.66%	48.72 ± 1.66%
5 ppm SRHA + 25 ppm SDS	23.31 ± 1.75%	31.21 ± 2.44%	31.21 ± 2.44%	32.44 ± 2.35%	32.44 ± 2.35%
10 ppm SRHA + 25 ppm SDS	18.67 ± 2.67%	18.67 ± 2.67%	20.00 ± 4.00%	22.67 ± 3.52%	22.67 ± 3.52%
25 ppm SRHA + 25 ppm SDS	28.77 ± 10.54%	45.00 ± 4.08%	45.00 ± 4.08%	47.00 ± 2.45%	47.00 ± 2.45%
25 ppm bLAHA + 25 ppm SDS	34.36 ± 5.28%	42.21 ± 4.45%	44.87 ± 5.36%	52.67 ± 5.13%	52.67 ± 5.13%
25 ppm hLAHA + 25 ppm SDS	38.15 ± 5.00%	43.33 ± 4.93%	43.49 ± 3.71%	46.10 ± 2.59%	46.10 ± 2.59%
25 ppm leLAHA + 25 ppm SDS	31.00 ± 5.01%	38.15 ± 1.01%	38.15 ± 1.01%	44.72 ± 4.05%	44.72 ± 4.05%

Table A.8 p values for *Artemia* hatching percentages with 25 ppm SDS at 32 hours

	Control	0 ppm LAHA	5 ppm LAHA	10 ppm LAHA	25 ppm LAHA
Control	n/a	0.00	0.03	0.01	0.02
0 ppm LAHA	n/a	n/a	0.03	0.02	0.01
5 ppm LAHA	n/a	n/a	n/a	0.91	0.15
10 ppm LAHA	n/a	n/a	n/a	n/a	0.24
25 ppm LAHA	n/a	n/a	n/a	n/a	n/a
	Control	0 ppm FPHA	5 ppm FPHA	10 ppm FPHA	25 ppm FPHA
Control	n/a	0.00	0.02	0.04	0.02
0 ppm FPHA	n/a	n/a	0.45	0.03	0.00
5 ppm FPHA	n/a	n/a	n/a	0.12	0.05
10 ppm FPHA	n/a	n/a	n/a	n/a	0.21
25 ppm FPHA	n/a	n/a	n/a	n/a	n/a
	Control	0 ppm SRHA	5 ppm SRHA	10 ppm SRHA	25 ppm SRHA
Control	n/a	0.00	0.00	0.00	0.15
0 ppm SRHA	n/a	n/a	0.65	0.30	0.01
5 ppm SRHA	n/a	n/a	n/a	0.09	0.05
10 ppm SRHA	n/a	n/a	n/a	n/a	0.01
25 ppm SRHA	n/a	n/a	n/a	n/a	n/a
	Control	0 ppmHA	LAHA	bLAHA	hLAHA	leLAHA
Control	n/a	0.00	0.95	0.23	0.02	0.05
0 ppm HA	n/a	n/a	0.18	0.04	0.01	0.05
LAHA	n/a	n/a	n/a	0.67	0.47	0.44
bLAHA	n/a	n/a	n/a	n/a	0.40	0.35
hLAHA	n/a	n/a	n/a	n/a	n/a	0.82
leLAHA	n/a	n/a	n/a	n/a	n/a	n/a

Table A.9 Hatching and mortality percentages for hatching assays of PD15 CNTs, PD50 CNTs, and SWCNTs with varying concentration and sonication time

	Hatching		Mortality	
	24 h	48 h	24 h	48 h
Control	54.67 ± 6.43%	67.86 ± 3.06%	0%	17.94 ± 8.97%
10 ppm PD15	46.15 ± 3.33%	60.26 ± 7.59%	0%	36.72 ± 7.59%
25 ppm PD15	57.08 ± 2.21%	63.56% ± 2.22%	0%	26.34 ± 2.88%
50 ppm PD15	47.95 ± 4.49%	67.49 ± 1.67%	0%	40.26 ± 1.22%
75 ppm PD15	36.06 ± 9.51%	29.83 ± 13.23%	0%	49.88 ± 15.00%
100 ppm PD15	31.18 ± 1.96%	37.79 ± 5.28%	0%	33.10 ± 15.00%
10 ppm PD50	51.48 ± 4.67%	57.75 ± 5.27%	0%	10.55 ± 5.27%
25 ppm D50	45.48 ± 3.03%	64.79 ± 2.24%	0%	4.47 ± 2.24%
50 ppm PD50	49.19 ± 2.93%	67.46 ± 5.22%	0%	10.45 ± 5.22
75 ppm PD50	45.98 ± 3.91%	54.40 ± 3.79%	0%	22.95 ± 3.79%
100 ppm PD50	48.48 ± 5.28%	50.86 ± 3.79%	0%	22.69 ± 1.26%
10 ppm SWCNTs	55.42 ± 2.78%	57.70 ± 1.68%	0%	39.52 ± 5.36%
25 ppm SWCNTs	55.13 ± 1.11%	64.10 ± 5.53%	0%	42.03 ± 5.53%
50 ppm SWCNTs	58.36 ± 5.28%	68.42 ± 2.55%	0%	42.79 ± 4.91%
75 ppm SWCNTs	52.26 ± 1.65%	67.01 ±2.91%	0%	44.71 ± 2.87%
100 ppm SWCNTs	53.18 ± 2.01%	65.91 ± 2.88%	0%	48.15 ± 2.87%
10 ppm PD15 30 min sonication	31.01 ± 4.78%	80.69 ± 3.49 %	0%	21.21 ± 5.98%
10 ppm PD15 1 h sonication	43.29 ± 5.90%	64.02 ± 6.26%	0%	34.77 ± 6.84%
10 ppm PD15 2 h sonication	32.00 ± 2.00%	72.00 ± 8.72%	0%	43.69 ± 2.36%
25 ppm SWCNTs 30 min sonication	37.67 ± 9.39&	37.67 ± 9.39%	0%	47.62 ± 6.29%
25 ppm SWCNTs 1 h sonication	49.85 ± 5.20%	64.36 ± 6.30%	0%	58.99 ± 9.50%
25 ppm SWCNTs 2 h sonication	49.81 ± 3.66%	52.81 ± 5.61%	0%	39.90 ± 5.46 %

Table A.10 Hatching and mortality percentages for hatching assays of PD15 CNTs, PD50 CNTs, and SWCNTs with LAHA

	Hatching		Mortality	
	24 h	48 h	24 h	48 h
Control	49.48 ± 8.19%	68.67 ± 1.67%	0%	27.03 ± 3.45%
25 ppm PD15	57.71 ± 5.78%	77.46 ± 4.22%	0%	29.80 ± 4.47%
25 ppm PD15 + 5 ppm LAHA	52.29 ± 7.84%	77.01 ± 3.94%	0%	20.51 ± 2.77%
25 ppm PD15 + 10 ppm LAHA	53.23 ± 2.30%	73.88 ± 2.51%	0%	38.67 ± 12.71%
25 ppm PD15 + 25 ppm LAHA	57.32 ± 3.21%	77.95 ± 5.77%	0%	21.38 ± 7.66%
25 ppm PD15 + 50 ppm LAHA	53.33 ± 3.92%	69.76 ± 3.10%	0%	25.22 ± 4.31%
25 ppm PD50	47.00 ± 4.16%	58.99 ± 2.68%	0%	26.85 ± 3.34%
25 ppm PD50 + 5 ppm LAHA	51.85 ± 6.46%	65.84 ± 4.35%	0%	23.61 ± 5.79%
25 ppm PD50 + 10 ppm LAHA	57.05 ± 4.04%	74.29 ± 2.86%	0%	30.00 ± 2.89%
25 ppm PD50 + 25 ppm LAHA	50.00 ± 6.18%	72.62 ± 7.24%	0%	26.49 ± 6.01%
25 ppm PD50 + 50 ppm LAHA	48.43 ± 4.95%	63.71 ± 2.49%	0%	24.34 ± 7.79%
50 ppm SWCNT + 5 ppm LAHA	47.34 ± 6.45%	71.13 ± 8.89%	0%	21.98 ± 5.25%
50 ppm SWCNT + 10 ppm LAHA	40.67 ± 6.57%	69.79 ± 3.19%	0%	39.94 ± 12.60%
50 ppm SWCNT + 25 ppm LAHA	44.35 ± 1.56%	78.19 ± 1.17%	0%	21.11 ± 3.54%
50 ppm SWCNT + 50 ppm LAHA	40.44 ± 3.56%	67.56 ± 2.35%	0%	25.00 ± 1.60%

Table A.11 Hatching and mortality p values for hatching assays of PD15 CNTs, PD50 CNTs, and SWCNTs with varying concentration and sonication time at 48 h versus controls

	Hatching	Mortality
10 ppm PD15	0.31	0.81
25 ppm PD15	0.69	0.58
50 ppm PD15	0.66	0.57
75 ppm PD15	0.01	0.18
100 ppm PD15	0.03	0.99
10 ppm SWCNTs	0.15	0.64
25 ppm SWCNTs	0.81	0.52
50 ppm SWCNTs	0.54	0.48
75 ppm SWCNTS	0.63	0.53
100 ppm SWCNTs	0.91	0.29
10 ppm PD50	0.79	0.09
25 ppm PD50	0.42	0.09
50 ppm PD50	0.24	0.15
75 ppm PD50	0.80	0.11
100 ppm PD50	0.26	0.11
PD15 no sonication	0.88	0.89
PD15 30 minute sonication	0.05	0.29
PD15 1 h sonication	0.88	0.07
PD15 2 h sonication	0.58	0.01
PD50 no sonication	0.10	0.09
PD50 30 minute sonication	0.12	0.08
PD50 1 h sonication	0.14	0.20
PD50 2 h sonication	0.10	0.06
SWCNT 30 min sonication	0.38	0.49
SWCNT 1 hour sonication	0.26	0.49
SWCNT 2 hour sonication	0.81	0.99

Table A.12 Hatching and mortality p values for hatching assays of PD15 CNTs, PD50 CNTs, and SWCNTs with LAHA at 48 h versus controls

	Hatching	Mortality
25 ppm PD15 CNTs + 5 ppm LAHA	0.16	0.78
25 ppm PD15 CNTs + 10 ppm LAHA	0.17	0.31
25 ppm PD15 CNTs + 25 ppm LAHA	0.24	0.83
25 ppm PD15 CNTs + 50 ppm LAHA	0.78	0.99
25 ppm PD50 CNT	0.40	0.99
25 ppm PD50 CNT + 5 ppm LAHA	0.59	0.92
25 ppm PD50 CNTs + 10 ppm LAHA	0.18	0.97
25 ppm PD50 CNTs + 25 ppm LAHA	0.64	0.84
25 ppm PD50 CNTs + 50 ppm LAHA	0.18	0.75
50ppm SWCNT 5ppm LAHA	0.59	0.40
50ppm SWCNT 10ppm LAHA	0.40	0.71
50ppm SWCNT 25 ppm LAHA	0.06	0.36
50ppm SWCNT 50 ppm LAHA	0.63	0.51

COMPLETE REPRESENTATIVE ^{31}P NMR SPECTRA AND HPLC DATA
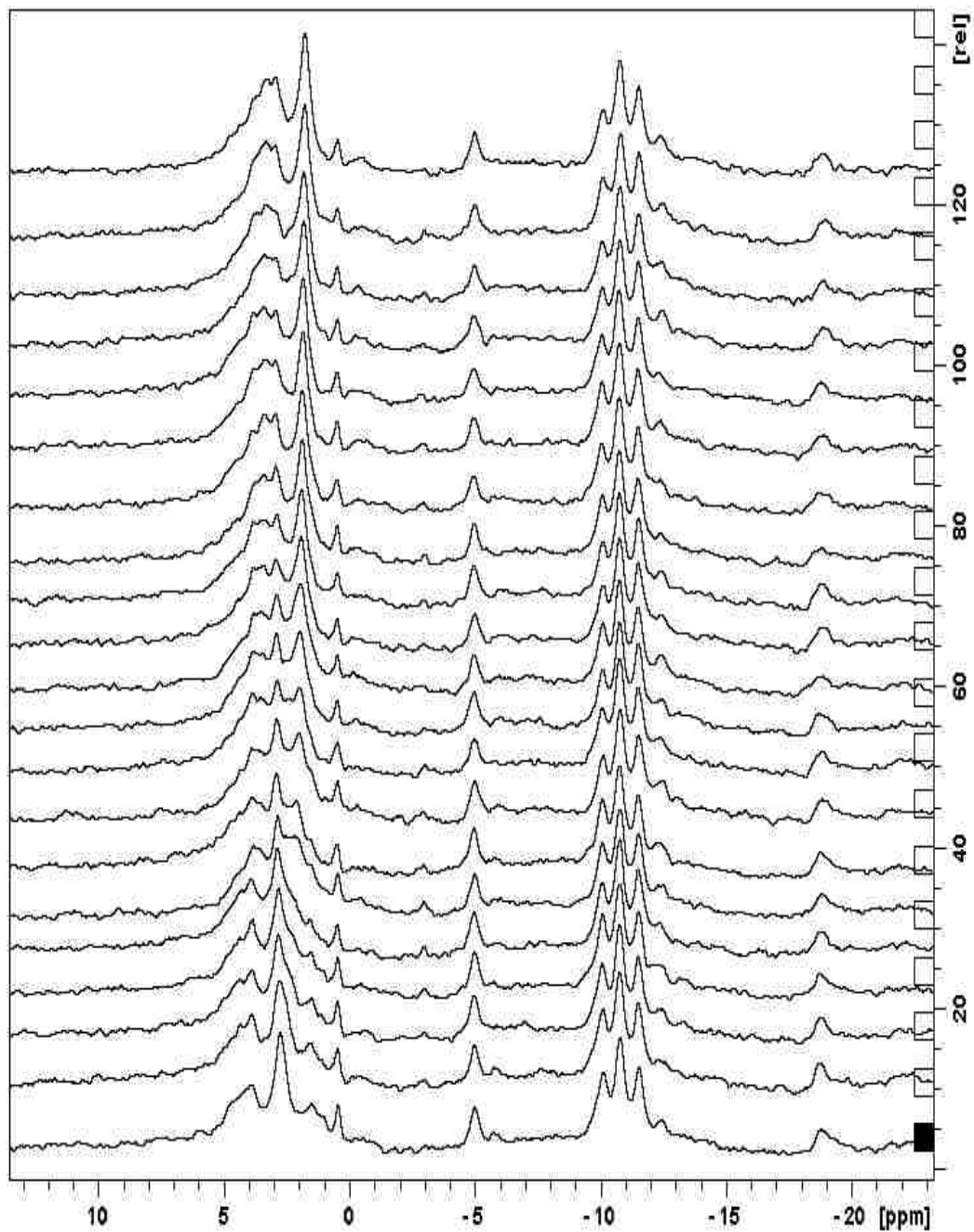
Figure A2.1. Complete representative ^{31}P NMR experiment with 35 ppt NaCl at pH 7.8 (time increasing from bottom-to-top)
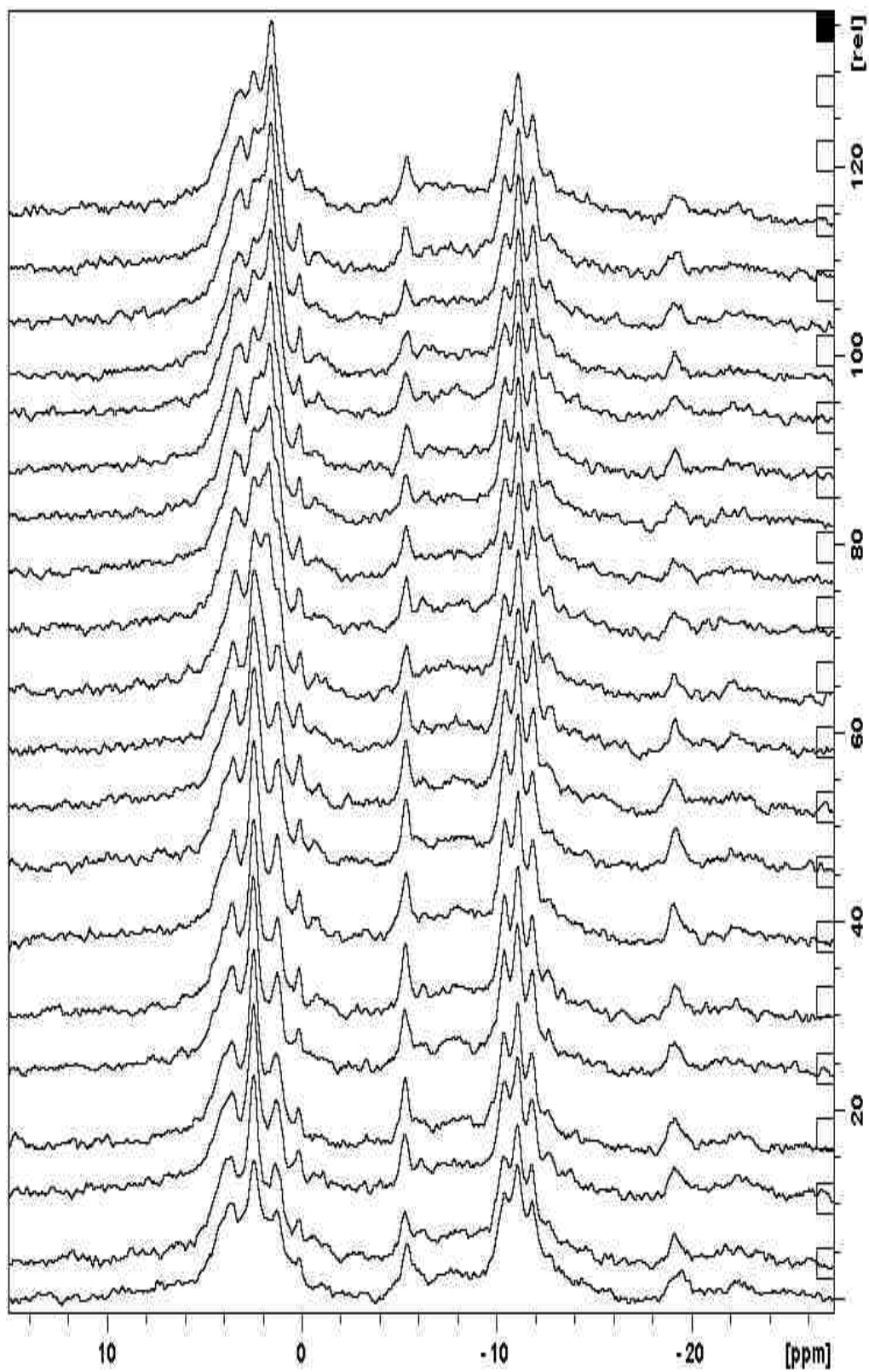
Figure A2.2. Complete representative ^{31}P NMR experiment with 100 ppm Tx100 at pH 7.8 (time increasing from bottom-to-top)
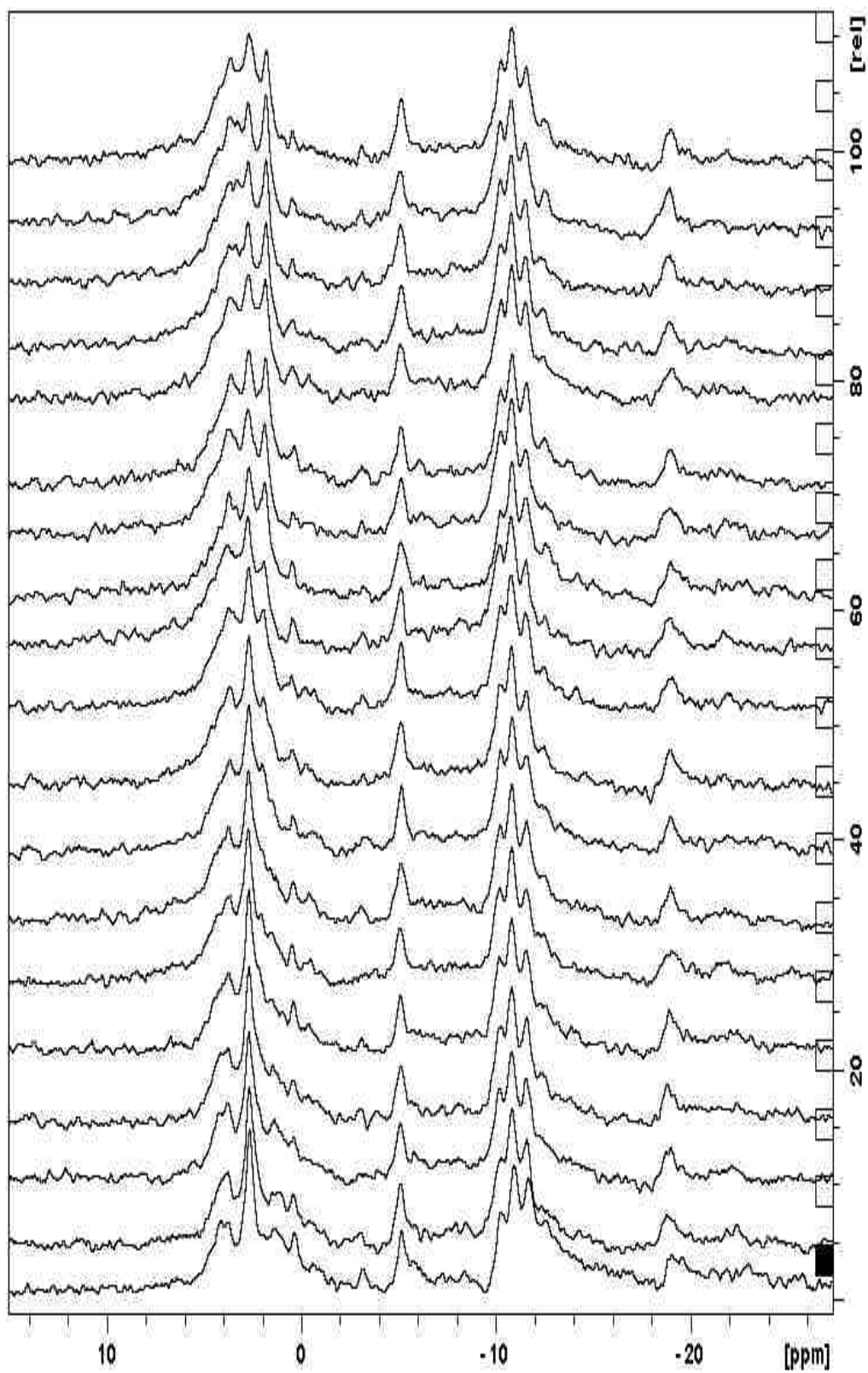
Figure A2.3. Complete representative ^{31}P NMR experiment with 5 ppm CPC at pH 7.8 (time increasing from bottom-to-top)

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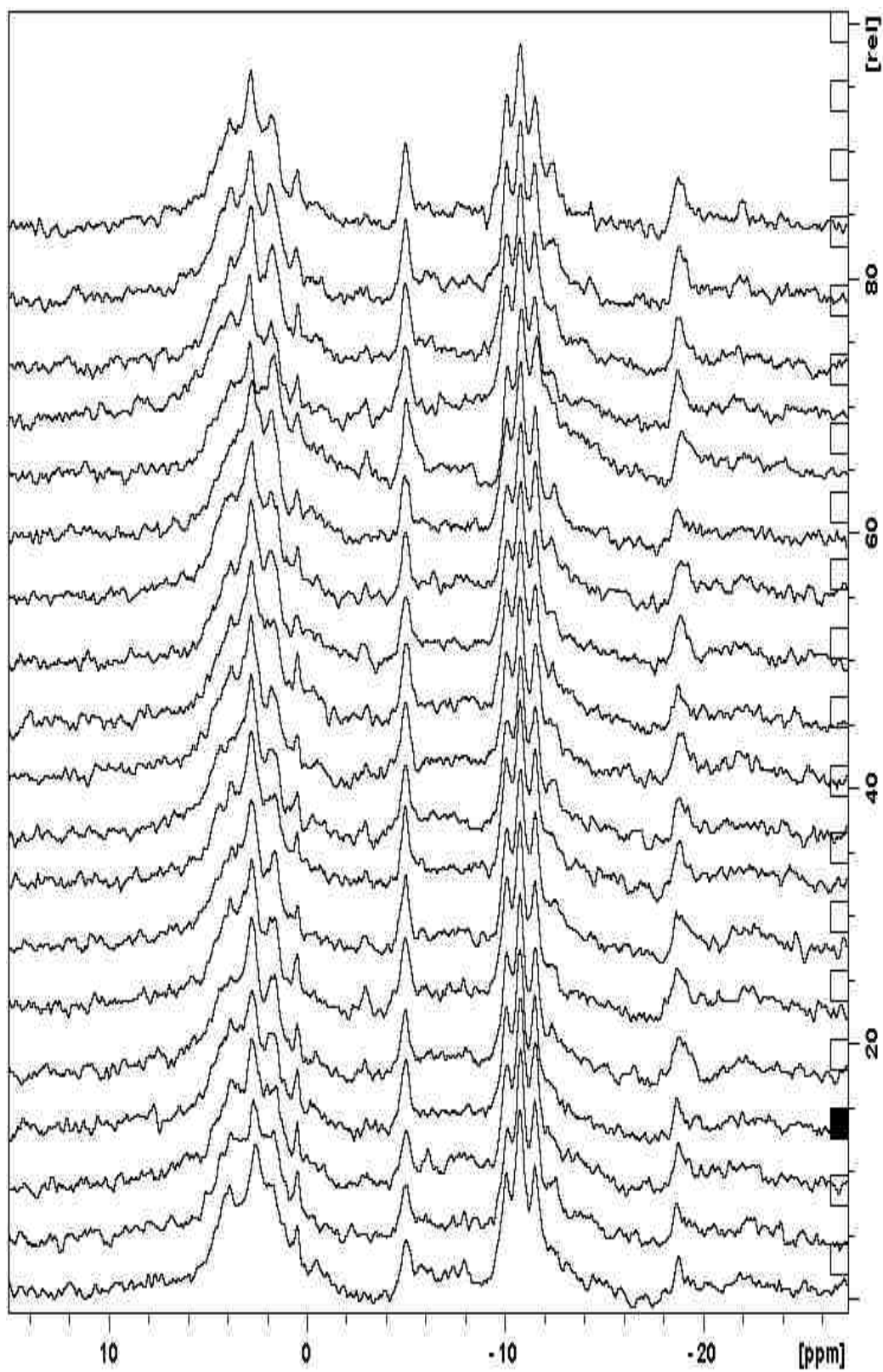
Figure A2.4. Complete representative ^{31}P NMR experiment with 35 ppm SDS at pH 7.8 (time increasing from bottom-to-top)
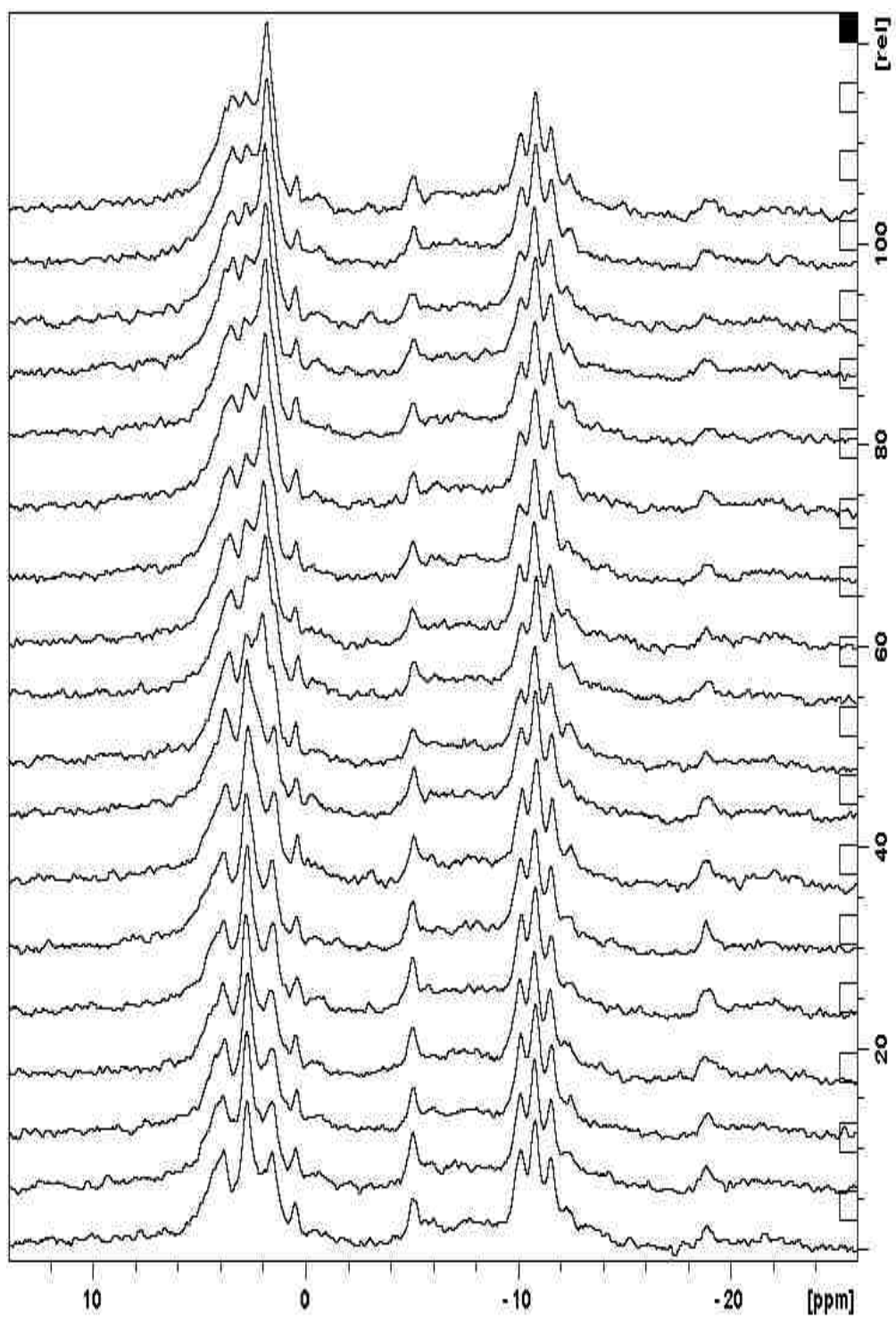
Figure A2.5. Complete representative ^{31}P NMR experiment with 35 ppm LAHA at pH 7.8 (time increasing from bottom-to-top)

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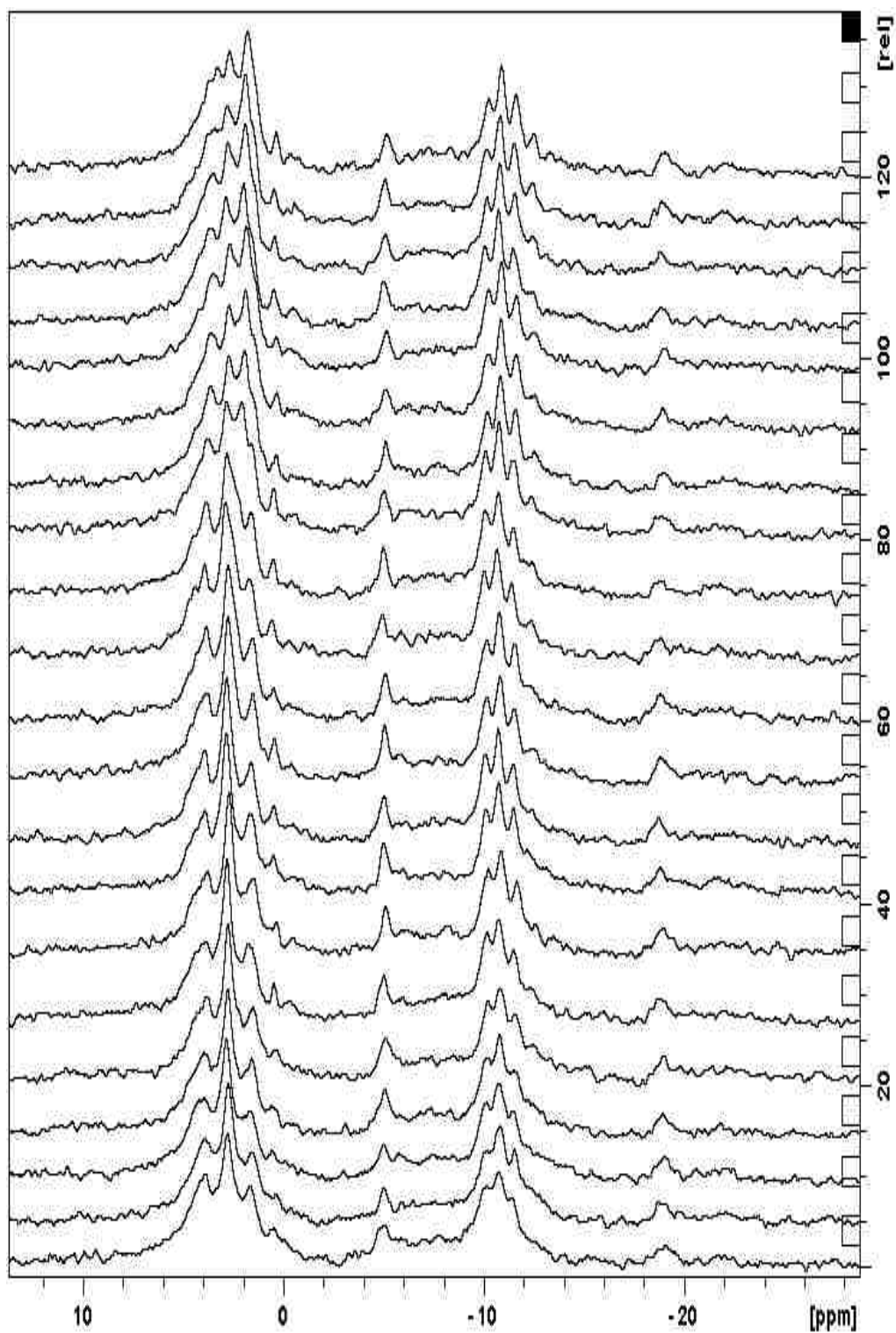
Figure A2.6. Complete representative ^{31}P NMR experiment with 5 ppm CPC and 5 ppm LAHA at pH 7.8 (time increasing from bottom-to-top)
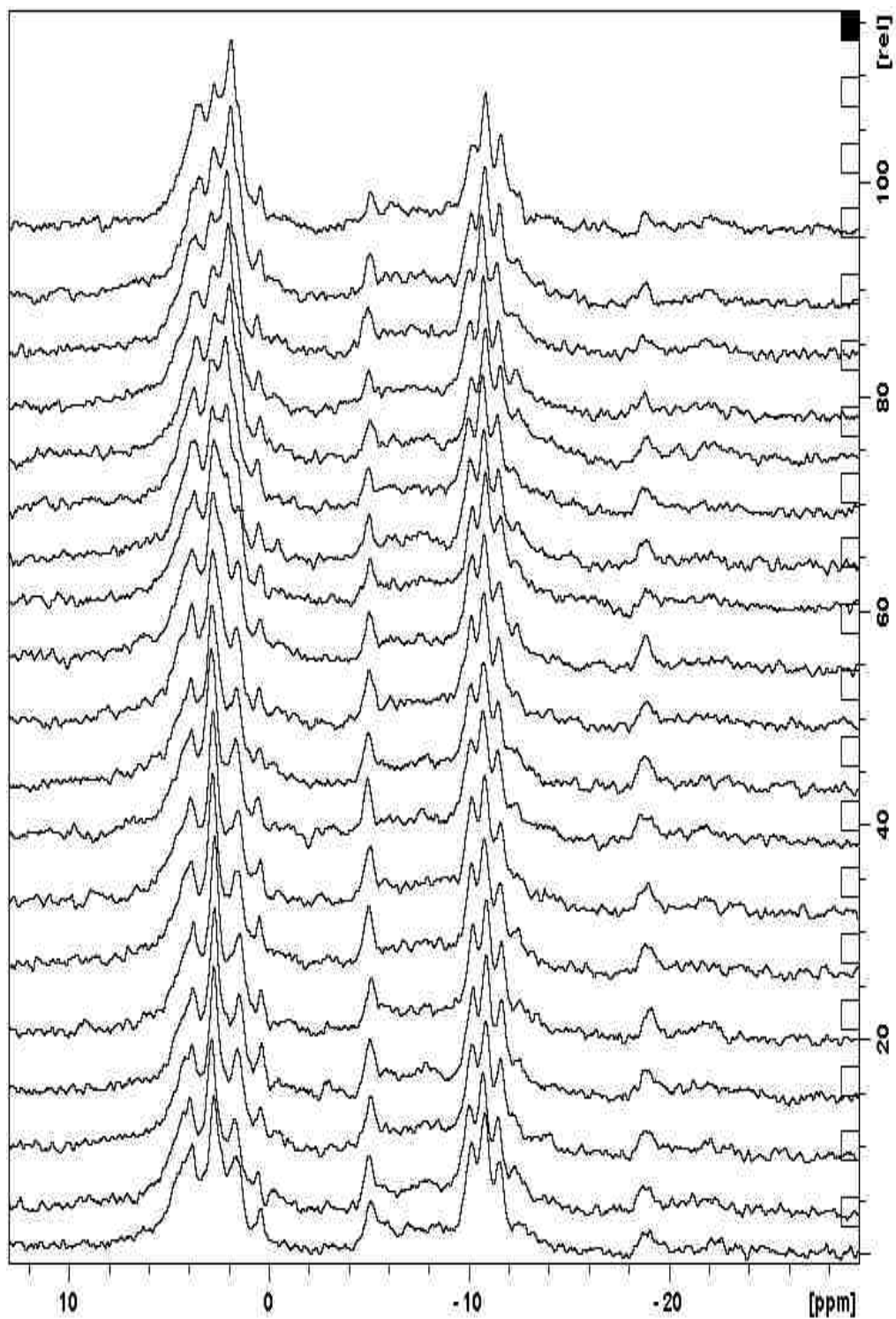
Figure A2.7. Complete representative ^{31}P NMR experiment with 35 ppm SDS and 35 ppm LAHA at pH 7.8 (time increasing from bottom-to-top)

APPENDIX C
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(1) Author signature _[signature]_ Print name Rachel D. Williams Date 05/28/15

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VITA

Rachel Deese (*nee* Williams) was raised and homeschooled in Vicksburg, Mississippi. She received her Associates degree from Hinds Community College in 2009 followed by earning her B.S. degree in Chemistry from the University of Mississippi in 2012. During her junior and senior year at the University of Mississippi, Rachel had the opportunity to conduct research in Dr. James Cizdziel's laboratory, which focused on elemental and mercury analysis in an environmental context. This work led to her interest in analytical chemistry with a focus on environmental studies. With encouragement from her mentor, she decided to further pursue her interests by attending graduate school. Rachel was referred to Dr. Robert Cook at Louisiana State University, where she decided to attend after a brief visit.

For her doctoral studies, Rachel studied the effects of soil components on the toxicity of pollutants to biological entities, with a focus on *Artemia Franciscana* (brine shrimp). Her research demonstrates the ability to utilize *Artemia Franciscana* as an indicator of toxicity and the mediation of toxicity in the presence of humic acid.

Rachel is a candidate to receive her doctorate in December 2016. Immediately after her defense, she will be starting her position as a chemist at the Savannah River National Laboratory.