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

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Abstract

Surfactants can be extremely toxic to aquatic species and are introduced to the environment in a variety of ways. It is thus important to understand how other environmental constituents, in this case humic acids (HAs), may alter the toxicity of anthropogenic surfactants. Hatching and mortality assays of *Artemia Franciscana* were performed for three different toxic surfactants: Triton X-100 (Tx-100, non-ionic), cetylpyridinium chloride (CPC, cationic), and sodium dodecyl sulfate (SDS, anionic). Humic acids of varying composition and concentrations were added to the assays to determine the toxicity mitigating ability of the HAs. Tx-100 had a significant toxic effect on *Artemia* mortality rates and HAs from terrestrial sources were able to mitigate the toxicity, but an aquatic HA did not. CPC and SDS limited hatching success of the *Artemia* and, as HAs were added, the hatching percentages increased for all HA sources, indicating toxicity mitigation. In order to determine which functional groups within HAs were responsible for the interaction with the surfactants, the HAs were chemically modified by: (i) bleaching to reduce aromatics, (ii) Soxhlet extraction to reduce lipids, and (iii) acid hydrolysis to reduce O- and N-alkyl groups. Although most of the modified HAs had some toxicity mitigating ability for each of the surfactants, there were two notable differences: 1) the lipid-extracted HA did not reduce the toxicity of Tx-100 and 2) the bleached HA had a lower toxicity mitigating ability for CPC than the other modified HAs.

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.[1, 2] The specific chemical composition of HAs varies and is dependent on their biogeochemical origin, but the major chemical constituents are aliphatics, aromatics, and carbohydrates.[1] Because of the high diversity of functional groups and their

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Supplementary Material Details of model membrane leakage studies, investigation passive membrane perturbation cause by different surfactants, and the influence of HAs studied here are presented. Also included are the control hatching assay details of varied concentrations of HAs and chemically modified LAHA with the *Artemia*. Carbon-13 NMR integration data on the three chemically modified LAHA samples are also provided, as well as chemical compositional and metal content data for the HAs used in this study.

amphiphilic nature, HAs can interact with a variety of environmental components, including a wide range of pollutants and biological membranes.[1, 3-7] Such interactions play an important environmental role in the transport and bioavailability of pollutants through the environment.[8-12]

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,[13, 14] 2) a number of remediation practices,[15] 3) as additives in the application of pesticide and herbicide formulations,[16, 17] and 4) urban and industrial run-off.[18] 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,[19] justifying a closer look into their fate in the environment.

A surfactant's toxicity is dependent on its molecular structure, the type of organism,[20-22] and the way the surfactant is ingested or taken-up by the cells.[23] 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[23] and enzyme inhibition have been shown.[24] Toxicity can also be attributed to the disruption of cellular membranes by the surfactant.[23] In general, nonionic and anionic surfactants tend to have similar toxic concentration ranges[23], while cationic surfactants are more toxic to aquatic species.[25, 26] 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[20-22] under a variety of conditions. This association can be relevant to the bioavailability and toxicity of surfactants,[27-30] including the ability of surfactants to perturb cellular biomembranes.[23, 31]

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.[27-29] 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,[32] electrostatic interactions,[27, 28] mixed micelle formation[33] and forced aggregation of the surfactant micelles.[29] 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.[34, 35]

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.

Methodology

Materials

The humic acid standards (Leonardite HA, Florida peat HA, and Suwannee River HA) were obtained from the International Humic Substances Society (Georgia, USA). More details on each of these HAs, including chemical composition, are available in the supplementary material, with further details available on the IHSS website (www.humicsubstances.org, accessed on Aug 8th, 2015). 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 M Ω deionized water was sourced from an apparatus by US filter. *Artemia Franciscana* was purchased from Brine Shrimp Direct (Ogdon, UT). Fisherbrand 100 $\times$ 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*.

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,[36] 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 Fig. 1 for structures) and were chosen for their extensive use.[37-39]

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

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 supplementary material).[40]

Artemia Franciscana have been previously utilized for three different types of toxicology assessments: hatching,[40, 41] short-term mortality (< 48 h),[40, 42] and long-term mortality (> 48 h).[40, 43] 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 are detailed in the supplementary material. These experiments were designed to give further credence to the effects of surfactant-HA associations observed in the *Artemia* hatching assays with a simpler system.

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.

Humic acid chemical modification

Three procedures for chemical modification were performed on LAHA: acid hydrolysis,[44] Soxhlet lipid extraction[45] and bleaching.[46]

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.,[46] 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 AgNO₃ 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.

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 M Ω water. Final dilutions and pH adjustments were made for the final sample solution.

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.

Artemia hatching assay

The hatching and mortality assay procedures used in this study are based on previous *Artemia* toxicity studies.[42, 47, 48] The *Artemia* cysts were first hydrated for two hours in 18 M Ω water kept at 5 °C. Once hydrated, 25 to 28 *Artemia* cysts were placed into individual 100 mm $\times$ 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.[49][50]

$$\text{Hatching percentage} = 100\% \times [\# \text{ of hatched } Artemia] / [total Artemia] \quad (1)$$

$$\text{Mortality percentage} = 100\% \times [\# \text{ of dead } Artemia] / [\# \text{ of hatched } Artemia] \quad (2)$$

Results

Chemically modified humic acid

The ^{13}C NMR spectra for the modified HAs are presented in Fig. 2. 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. 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.

The unmodified and modified HAs used in this study had no toxic effects on the *Artemia*. Both the hatching and mortality percentages were essentially the same for all HA and modified LAHA samples and hence none of the changes in *Artemia* hatching assays performed in subsequent tests can be attributed HAs or their chemical modification (see supplementary material).

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 Fig. 3. The data presented in Fig. 3 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 (Fig. 3A)

Due to the ionic strength and LAHA concentration, it seems logical that this observation is due to LAHA aggregation.[5] 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 (Fig. 3B). SRHA, on the other hand, shows little to no ability to mitigate the toxicity of Tx-100. Fluorescence leakage experiments (see supplementary material) 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.[3]

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 Fig. 4. 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 (Fig. 4A), 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 (Fig. 4B). SRHA mitigates CPC toxicity but not to the same extent as FPHA at higher HA concentration (Fig. 4C).

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 (Fig. 5A). FPHA's SDS toxicity mitigation increased with HA concentration, but not in a linear manner (Fig. 5B). SRHA showed an overall similar mitigating potential to SDS, but again, in a non-linear manner (Fig. 5C). Since HAs are anionic, they are expected to repel the negatively charged sulfate group of the SDS, resulting in limited binding.[28] 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. This phenomenon can be also seen in the fluorescence study with liposome membranes (see supplemental material), 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.

Chemically modified HA 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 Fig. 6A 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 (Fig. 6B). 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 (Fig. 6C). However, in the presence of LAHA (modified or unmodified), the SDS remained still somewhat toxic.

Discussion

Nonionic surfactant – Tx-100

The HA demonstrated the following trend in Tx-100 toxicity mitigation: LAHA > FPFA > SRHA (Fig 3). Conceivably, two possible interactions could be occurring. First, the aromatic component of the HA, by π - π stacking with that of Tx-100,[34, 35] 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, FPFA, and SRHA: 0.51, 0.73, and 0.83, respectively (elemental composition of these HAs are provided in the supplementary material).[51] 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.

Tx-100's toxicity was not mitigated by the lipid-extracted LAHA, which offers strong evidence that the aliphatic lipid-like moieties within HAs that consist of long-chain fatty acids and esters, aliphatic alcohols, and long chain alkanes[52] are responsible for mitigating the toxicity of Tx-100. Coincidentally, an aggregation study found these same components to be largely responsible for the amphiphilic character of HA samples.[45] Thus, the data in Fig. 6A 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 Fig. 3), 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 Fig. 6A 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. 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[53, 54] from an interaction with Tx-100, enhancing their ability to interact with, and reduce the toxicity of, Tx-100.

Cationic surfactant - CPC

For both FPHA (Fig. 4B) and SRHA (Fig. 4C), the CPC toxicity mitigation capacity of the HA does not linearly change with concentration, suggesting partial aggregation of HAs at higher concentrations.[5] 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.[29]

The bleached LAHA had a lower hatching percentage (Fig. 6B), 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 Fig. 4. 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.[36] 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.[34, 35, 55, 56] 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.[53] 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.

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.[28] Unlike FPHA and SRHA, the ability of LAHA to mitigate SDS toxicity was not concentration dependent (Fig 5A). 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.[5] 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 (Fig. 5C). Because of HAs' heterogeneous nature, many different interactions and conformations of moieties within the HA are possible.[57] 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.[53]

Trends observed for the chemically modified LAHA (Fig. 6C) 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.[28]

Summary

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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Stevenson, FJ. Humus Chemistry: Genesis, Composition, Reactions. Second. New York, N.Y: John Wiley & Sons, Inc; 1994.
2. Sutton R, Sposito G. Molecular Structure in Soil Humic Substances: The New View. Environmental Science & Technology. 2005 Dec 01; 39(23):9009–15. 2005. [PubMed: 16382919]
3. Vigneault B, Percot A, Lafleur M, Campbell PGC. Permeability Changes in Model and Phytoplankton Membranes in the Presence of Aquatic Humic Substances. Environ Sci Technol. 2000 Sep 01; 34(18):3907–13.
4. Campbell PGC, Twiss MR, Wilkinson KJ. Accumulation of natural organic matter on the surfaces of living cells: implications for the interaction of toxic solutes with aquatic biota. Can J Fish Aquat Sci. 1997 Apr 8.54:2543–54. Epub 1997.
5. Ojwang' LM, Cook RL. Environmental conditions that influence the ability of humic acids to induce permeability in model biomembranes. Environ Sci Technol. 2013 Jun 27.;47, 8280–7.
6. Tipping, E. Cation binding by humic substances. Cambridge, United Kingdom: Cambridge University Press; 2002. p. 434
7. Elayan NM, Treleaven WD, Cook RL. Monitoring the effect of three humic acids on a model membrane system using 31P NMR. Environ Sci Technol. 2008; 42(5):1531–6. [PubMed: 18441799]

8. Twiss MR, Granier L, Lafrance P, Campbell PGC. Bioaccumulation of 2,2',5,5'-tetrachlorobiphenyl and pyrene by picoplankton (*Synechococcus leopoliensis*, cyanophyceae): Influence of variable humic acid concentrations and pH. *Environ Toxicol Chem.* 1999; 18(9):2063–9.
9. Vigneault B, Campbell PGC. Uptake of cadmium by freshwater green algae: Effects of pH and aquatic humic substances. *J Phycol.* 2005; 41(1):55–61.
10. Parent L, Twiss MR, Campbell PGC. Influences of Natural Dissolved Organic Matter on the Interaction of Aluminum with the Microalga *Chlorella*: A Test of the Free-Ion Model of Trace Metal Toxicity. *Environ Sci Technol.* 1996 Apr 01; 30(5):1713–20.
11. Wilkinson KJ, Bertsch PM, Jagoe CH, Campbell PGC. Surface complexation of aluminum on isolated fish gill cells. *Environ Sci Technol.* 1993; 27(6):1132–8. Epub 1993.
12. McCarthy JF, Jimenez BD, Barbee T. Effect of dissolved humic material on accumulation of polycyclic aromatic hydrocarbons: Structure-activity relationships. *Aquat Toxicol.* 1985; 7(1-2): 15–24.
13. Stalmans M, Matthijs E, De Oude N. Fate and effect of detergent chemicals in the marine and estuarine environment. *Wat Sci Tech.* 1991; 24(10):115–26.
14. Rogers H. Sources, behaviour and fate of organic cotaminants during sewage treatment and in sewage sludges. *Sci Tot Environ.* 1996; 185:3–26.
15. Mulligan CN, Yong RN, Gibbs BF. Surfactant-enhanced remediation of contaminated soil: a review. *Eng Geol.* 2001; 60(1):371–80.
16. Czarnota, M.; Thomas, P. Cooperative Extension. The University of Georgia; 2013. Using surfactants, wetting agents, and adjuvants in the greenhouse.
17. Song H-Y, Kim Y-H, Seok S-J, Gil H-W, Yang J-O, Lee E-Y, et al. Cellular toxicity of surfactants used as herbicide additives. *J Korean Med Sci.* 2012; 27:3–9. [PubMed: 22219606]
18. Zoller, U. Boca. Raton, Florida: CRC Press; 2004. Handbook of detergents: Environmental Impact.
19. Ostroumov, SA. Boca. Raton, Florida: CRC Press; 2006. Biological effects of surfactants.
20. Ying G-G. Fate, behavior and effects of surfactants and their degradation products in the environment. *Environ Int.* 2006; 32:417–31. [PubMed: 16125241]
21. Chen Y, Geurts M, Sjollem SB, Kramer NI, Hermens JL, Droge ST. Acute toxicity of the cationic surfactant C12-Benzalkonium in different bioassays: How test design affects bioavailability and effect concentrations. *Environ Toxicol Chem.* 2014; 33(3):606–15. [PubMed: 24273010]
22. Pavli Ž, Vidakovi -Cifrek Ž, Puntari D. Toxicity of surfactants to green microalgae *Pseudokirchneriella subcapitata* and *Scenedesmus subspicatus* and to marine diatoms *Phaeodactylum tricornutum* and *Skeletonema costatum*. *Chemosphere.* 2005; 61:1061–8. [PubMed: 16263376]
23. Abel P. Toxicity of synthetic detergents to fish and aquatic invertebrates. *J Fish Biol.* 1974; 6:79–198.
24. Cserhati T, Forgacs E, Oros G. Biological activity and environmental impact of anionic surfactants. *Environ Int.* 2002; 28:337–48. [PubMed: 12437283]
25. Lewis M, Wee V. Aquatic safety assessment for cationic surfactants. *Environ Toxicol Chem.* 1983; 2:105–8.
26. Singh R, Gupta N, Singh S, Suman R, Annie K. Toxicity of ionic and non-ionic surfactants to six microbes found in Agra, India. *Bull Environ Contam Toxicol.* 2002; 69:265–70. [PubMed: 12107704]
27. Ishiguro M, Tan W, Koopal LK. Binding of cationic surfactants to humic substances. *Colloid Surface A.* 2007; 306:29–39.
28. Koopal LK, Goloub TP, Davis TA. Binding of ionic surfactants to purified humic acid. *J Colloid Interface Sci.* 2004; 275:360–7. [PubMed: 15178260]
29. Otto WH, Britten DJ, Larive CK. NMR diffusion analysis of surfactant-humic substance interactions. *J Colloid Interface Sci.* 2003; 261:508–13. [PubMed: 16256562]
30. Traina SJ, Mcavoy DC, Versteeg DJ. Association of linear alkylbenzenesulfonates with dissolved humic substances and its effect on bioavailability. *Environ Sci Technology.* 1996; 30(4):1300–9.
31. Luckey, M. Membrane structural biology: with biochemical and biophysical foundations. New York, NY: Cambridge University Press; 2008.

32. Tan W, Koopal LK, Norde W. Interaction between humic acid and lysozyme, studied by dynamic light scattering and isothermal titration calorimetry. *Environ Sci Technol.* 2009; 43(3):591–6. [PubMed: 19244988]
33. Lippold H, Gottschalch U, JKupsch H. Joint influence of surfactants and humic matter on PAH solubility. Are mixed micelles formed? *Chemosphere.* 2008; 70:1979–86. [PubMed: 17980402]
34. Keiluweit M, Kleber M. Molecular-level interactions in soils and sediments: the role of aromatic pi-systems. *Environ Sci Technol.* 2009; 43:3421–9. [PubMed: 19544834]
35. Pignatello, JJ. Interaction of Anthropogenic Organic Chemicals with Organic Matter in Natural Particles. In: Xing, B.; Senesi, N.; Huang, PM., editors. IUPAC series on biophysico-chemical processes in environmental systems, Volume 3 Biophysico-Chemical Processes of Anthropogenic Organic Compounds in Environmental System 3. New York: John Wiley and Sons; p. 2011p. 3-50.
36. Thorn, KA.; Folan, DW.; MacCarthy, P. Characterization of the international humic substances society standard and reference fulvic and humic acids by solution state ³¹C and ¹H NMR. Vol. 1989. Denver, Co: US Department of the Interior; p. 89-4196.
37. Coleman, CW.; Waldroup, AL. Cetylpyridinium Chloride: Claim for Exception. Little Rock, AR: Food and Drug Administration; 1999.
38. TRITON Surfactants: FDA Status of TRITON surfactants. The Dow Chemical Company. 2010
39. SIDS initial assessment profile: sodium dodecyl sulfate, CAS NO: 151-35-3
40. Nunes BS, Carvalho FD, Guilhermino LM, Stappen GV. Use of the genus *Artemia* in ecotoxicity testing. *Environ Pollut.* 2006; 144:453–62. [PubMed: 16677747]
41. MacRae TH, Pandey AS. Effects of metals on early life stages of the brine shrimp, *Artemia*: A developmental toxicity assay. *Arch Environ Contam Toxicol.* 1991 Feb 01; 20(2):247–52. English. [PubMed: 2015000]
42. Arulvasu C, Jennifer SM, Prabhu D, Chandhirasekar D. Toxicity effect of silver nanoparticles in brine shrimp *Artemia*. *Scientific World J.* 2014
43. Manfra L, Savorelli F, Pisapia M, Magaletti E, Cicero AM. Long-term lethal toxicity test with the crustacean *artemia franciscana*. *J Vis Exp.* 2012; (62):3790. [PubMed: 22525984]
44. Almendros G. Effects of different chemical modifications on peat humic acid and their bearing on some agrobiological characteristics of soil. *Commun Soil Sci Plan.* 1994; 25
45. Chilom G, Bruns AS, Rice JA. Aggregation of humic acid in solution: Contributions of different fractions. *Org Geochem.* 2009; 40:455–60.
46. Wise LE, Murphy M, d'Addieco A. Chlorite holocellulose, its fractionation and bearing on summative wood analysis and on studies on the hemicelluloses. *Paper Tr J.* 1946; 122(2):35.
47. Distribution, life cycle, taxonomy, and culture methods: Brine shrimp (*Artemia Salina*). Environmental Protection Agency.
48. Sorgellos P, Wielen CR-VD, Persoone G. The use of *Artemia nauplii* for toxicity tests - a critical analysis. *Ecotox Environ Safe.* 1978; 2:249–55.
49. Matthews RS. *Artemia salina* as a test organism for measuring superoxide-mediated toxicity. *Free Radical Biology and Medicine.* 1995; 18(5):919–22. [PubMed: 7797101]
50. Rodd AL, Creighton MA, Vaslet CA, Rangel-Mendez JR, Hurt RH, Kane AB. Effects of Surface-Engineered Nanoparticle-Based Dispersants for Marine Oil Spills on the Model Organism *Artemia franciscana*. *Environmental Science & Technology.* 2014 Jun 03; 48(11):6419–27. 2014. [PubMed: 24823274]
51. Xing B, McGill WB, Dudas MJ. Cross-correlation of polarity curves to predict partition coefficients of nonionic organic contaminants. *Environ Sci Technol.* 1994; 28:1929–33. [PubMed: 22175935]
52. Chilom G, Rice JA. Structural organization of humic acid in the solid state. *Langmuir.* 2009; 25(16):9012–5. [PubMed: 19408899]
53. Mitchell PJ, Simpson MJ. High affinity sorption domains in soil are blocked by polar soil organic matter components. *Environ Sci Technol.* 2013 Jan 02; 47(1):412–9. [PubMed: 23206246]
54. Lattao C, Birdwell J, Wang JJ, Cook RL. Studying organic matter molecular assemblage within a whole organic soil by nuclear magnetic resonance. *J Environ Qual.* 2008; 37(4):1501–9. English. [PubMed: 18574182]

55. Chin Y-P, Aiken GR, Danielsen KM. Binding of pyrene to aquatic and commercial humic substances: The role of molecular weight and aromaticity. *Environ Sci Technol.* 1997 Jun 01; 31(6):1630–5.
56. Laor Y, Farmer WJ, Aochi Y, Strom PF. Phenanthrene binding and sorption to dissolved and to mineral-associated humic acid. *Water Res.* 1998; 32(6):1923–31.
57. Bonin JL, Simpson MJ. Variation in phenanthrene sorption coefficients with soil organic matter fractionation: The result of structure or conformation? *Environ Sci Technol.* 2007 Jan 01; 41(1): 153–9. [PubMed: 17265941]

Environmental Context

Surfactants, a pollutant class routinely introduced into aquatic environments, can be toxic to a variety of species. It is thus important to understand how surfactants' toxicity is influenced by their interactions with other environmental constituents, including natural organic matter. This article reports the changes in toxicity of three different surfactants to *Artemia Franciscana* (brine shrimp) in the presence of unmodified and chemically modified humic acids.

Fig. 1. Chemical structures of A) Tx-100, B) CPC, and C) SDS

Fig. 2. ^{13}C NMR spectra of the chemically modified Leonardite humic acid (LAHA)

Fig. 3. *Artemia* hatching assays at 100 ppm* Tx-100 and LAHA, FPHA, and SRHA
 *Note: Tx-100 showed significant toxicity levels at 100 ppm and above (3A).

Fig. 4. *Artemia* hatching assays at 3.5 ppm* CPC and LAHA, FPFA, and SRHA

*Note: CPC showed significant toxicity levels at 3.5 ppm and above

Fig. 5. *Artemia* hatching assays at 25 ppm* SDS and LAHA, FPFA, and SRHA

*Note: SDS showed significant toxicity levels at 25 ppm and above

Fig. 6. *Artemia* hatching assays at 100 ppm* Tx-100, 3.5 ppm CPC and 25 ppm SDS in the presence of chemically modified LAHA