Environ. Chem. **2016**, *13*, 507–516
doi:10.1071/EN15108_AC

Supplementary material

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

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Supplementary introduction for fluorescence measurements

It is well known that surfactants can disrupt cellular membranes[1,2]; 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.[3] 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.[4–6]

Supplementary information on materials and instrumentation used for fluorescence study

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 ($NaH_2PO_4•H_2O$) was purchased from Fisher Scientific and sodium hydrogen phosphate dihydrate ($Na_2HPO_4•2H_2O$) 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). The humic acid standards (Leonardite HA, Florida peat HA, and Suwannee 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 Capital Welders Supply Co. (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 utilised for dynamic light scattering of the liposomes.

Surfactant concentrations

Surfactant concentrations were chosen for the *Artemia* hatching or mortality assays by varying concentration of the surfactants. Concentrations chosen were ones that caused either a hatching or mortality percentage that was <30 % so that any changes caused by HAs would be significant.

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)[7] and CPC (CMC = 40.8 ppm),[8] the concentrations was ~70% of the CMC whereas the SDS (CMC = ~2365 ppm)[9] 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.

Supplementary procedures for the liposome fluorescence study

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 of methanol and 132 μL of chloroform (1 : 2 solution), stirring the solution for 30 min and then evaporating under nitrogen gas for 24 h 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 utilising 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.

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 utilised 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 percentage dye release from the liposomes relative to the lysed membranes:

$$\text{Percentage dye release} = 100\ \% \times (I_H - I_B) \div (I_T - I_B) \quad \text{(S1)}$$

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.

Supplementary information on SRHA-surfactant interactions with model biomembranes from the liposome fluorescence study

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 Fig. S1 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 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 percentage 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.

There is little interaction between the SDS and SRHA in the fresh water environment because of the electrostatic repulsions. However, in the 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.

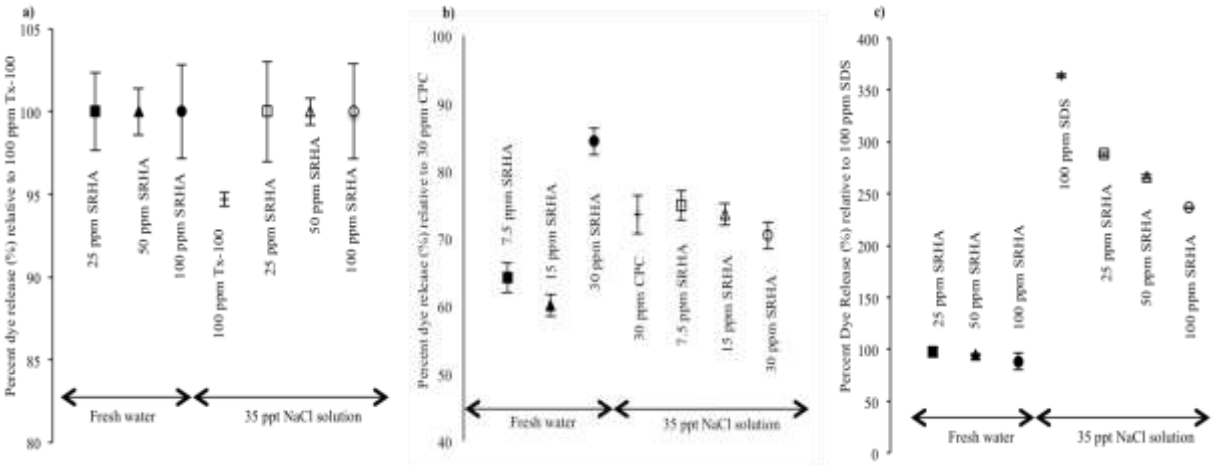

Fig. S1. Percentage liposome SRB dye release induced by (a) Tx-100, (b) CPC and (c) SDS with varying concentrations of SRHA and salinity.

Characterisation data for the HAs used in this study

Non-chemically modified HAs

The following section contains tables summarising the carbon speciation by ^{13}C NMR analysis, metal content, and elemental compositional data (used for calculating the polarity of the different HAs), provided here in support of the discussion presented in the main manuscript.

Table S1. Elemental compositions and stable isotopic ratios of International Humic Substances Society samples (see http://www.humicsubstances.org/, accessed 26 May 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	No data	No data
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

Table S2. ^{13}C NMR estimates of carbon distribution in International Humic Substances Society samples (see http://www.humicsubstances.org/, accessed 26 May 2015)

Electronically integrated peak area percentages

Standard HA	Carbonyl	Carboxyl	Aromatic	Acetal	Heteroapliphatic	Aliphatic	$\Sigma^{15}N$
Suwannee River (SRHA)	6	15	31	7	13	29	no data
Pahokee Peat (FPHA)	5	20	47	4	5	19	1.29
Leonardite (LAHA)	8	15	58	4	1	14	2.13

Table S3. Metal concentrations of HAs as determined by ICP-OES

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

Standard HA	Al (mg kg^{-1})	Ca (mg kg^{-1})	Cr (mg kg^{-1})	Cu (mg kg^{-1})	Fe (mg kg^{-1})	Mn (mg kg^{-1})	Ni (mg kg^{-1})	Si (mg kg^{-1})	Sr (mg kg^{-1})	Zn (mg kg^{-1})
Leonardite HA	2270	3482	48.8	15.4	1535	Below detection	26.6	113	42.3	Below detection
Suwannee River HA	346	381	40.7	20.8	1171	Below detection	Below detection	62.4	1.46	213
Pahokee Peat HA	139	546	Below detection	1.54	1844	Below detection	Below detection	Below detection	12.36	151

Chemically modified HAs

The following section contains tables summarising the carbon speciation by ^{13}C NMR analysis and metal content of the chemically edited HAs, provided here in support of the discussion presented in the main manuscript. It was found that the trends noted in the manuscript for the chemically modified HAs are independent of the differences between them in terms of metal content.

Table S4. Supplementary information for the ^{13}C NMR relative percentage areas of unedited and edited LAHA

Electronically integrated peak area percentages

Standard HA	Carboxyl	Aromatic	Aldyhyde/ Ketone	N- or O- Alkyl	Alkyl
Bleached LAHA	4.03 %	29.86 %	0.0 %	9.43 %	58.44 %
Lipid extracted LAHA	9.99 %	60.71 %	0.47 %	6.28 %	22.56 %
Hydrolysed LAHA	9.08 %	62.38 %	0.71 %	1.78 %	26.05 %
LAHA reference	11.18 %	52.89 %	0.07 %	7.11 %	28.74 %

Table S5. Metal concentrations of chemically modified HAs as determined by ICP-OES

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

Modified HA	Al (mg kg^{-1})	Ca (mg kg^{-1})	Cr (mg kg^{-1})	Cu (mg kg^{-1})	Fe (mg kg^{-1})	Mn (mg kg^{-1})	Ni (mg kg^{-1})	Si (mg kg^{-1})	Sr (mg kg^{-1})	Zn (mg kg^{-1})
Bleached LAHA	393	1856	21.0	62.0	582	12.8	104	362	11.8	38.6
Hydrolysed LAHA	76.5	157	22.9	7.91	168	Below detection	12.7	17.7	1.11	Below detection
Lipid extracted LAHA	3231	5094	62.6	23.7	3134	2.57	37.6	227	59.9	Below detection

Representative images of the *Artemia franciscana* used in this study

Fig. S2 shows images of the *Artemia franciscana* used in this study at two different time points. The images show that the *A. franciscana* are growing as expected and that there are no discernible physiological defects (the same was true in regards to visible motor performance). These images are for *A. franciscana* exposed to 35-ppt NaCl at pH 7.8, in addition to the hatching percentage, further show that under these conditions, the *A.franciscana* are healthy, as was found or all HA solution in the absence of surfactants.

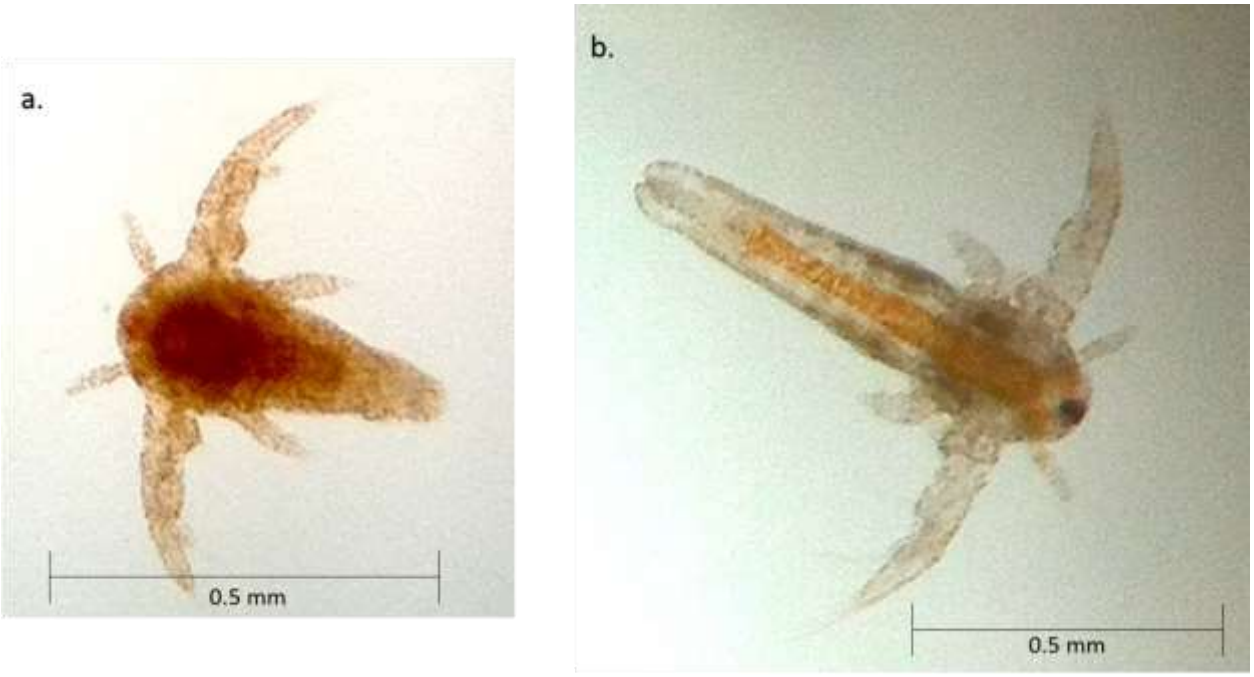

Fig S2. *Artemia franciscana* at (a) 24 h and (b) 48 h in 35-ppt NaCl at pH 7.8.

Hatching and mortality data for *Artemia franciscana* in the presence of the different HAs used in this study

The data in Fig S3 and S4 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[5,6] 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. 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.

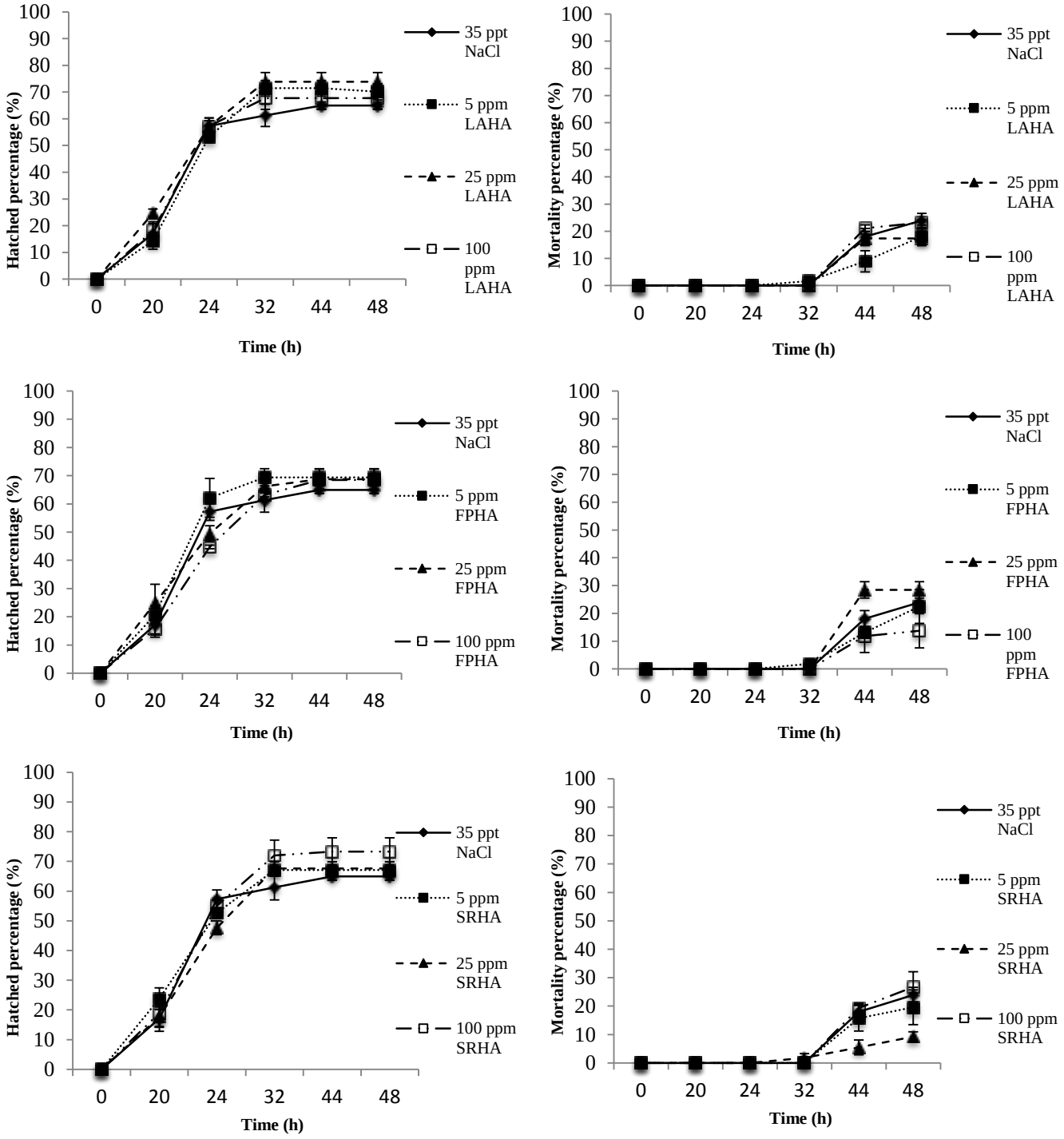

Fig. S3. *Artemia* hatching and mortality assays with (a) LAHA, (b) FPHA and (c) SRHA.

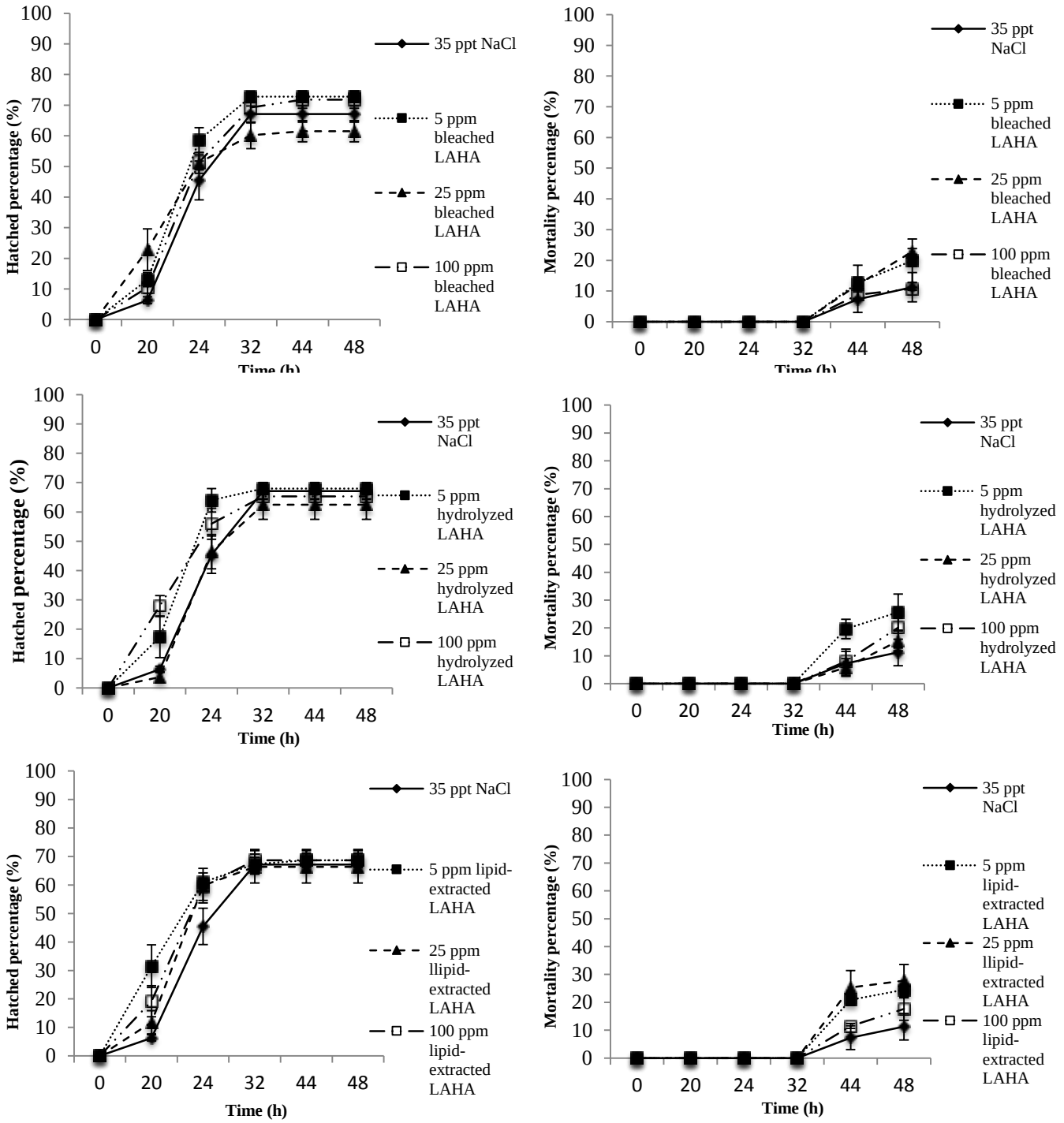

Fig. S4. Chemically modified LAHA *Artemia* hatching and mortality assays.

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