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## Effects of Surface-Engineered Nanoparticle-Based Dispersants for Marine Oil Spills on the Model Organism *Artemia franciscana*

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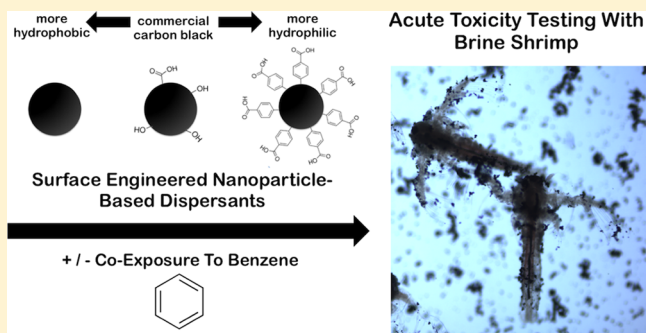
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### S Supporting Information

**ABSTRACT:** Fine particles are under active consideration as alternatives to chemical dispersants for large-scale petroleum spills. Fine carbon particles with engineered surface chemistry have been shown to stabilize oil-in-water emulsions, but the environmental impacts of large-scale particle introduction to the marine environment are unknown. Here we study the impact of surface-engineered carbon-black materials on brine shrimp (*Artemia franciscana*) as a model marine microcrustacean. Mortality was characterized at 50–1000 mg/L, and levels of heat shock protein 70 (hsp70) were characterized at sublethal particle concentrations (25–50 mg/L). Functionalized carbon black (CB) nanoparticles were found to be nontoxic at all concentrations, while hydrophobic (annealed) and as-produced CB induced adverse effects at high concentrations. CB was also shown to adsorb benzene, a model hydrocarbon representing the more soluble and toxic low-molecular weight aromatic fraction of petroleum, but the extent of adsorption was insufficient to mitigate benzene toxicity to *Artemia* in coexposure experiments. At lower benzene concentrations (25–75 mg/L), coexposure with annealed and as-produced CB increased hsp70 protein levels. This study suggests that surface functionalization for increased hydrophilicity can not only improve the performance of CB-based dispersants but also reduce their adverse environmental impacts on marine organisms.



## 1. INTRODUCTION

Synthetic chemical dispersants are used for marine oil spills to limit petroleum accumulation at the surface of the ocean<sup>1</sup> and to accelerate biodegradation processes by increasing the oil–water interfacial area.<sup>2,3</sup> Oil released from an underwater offshore source as in the Deepwater Horizon spill can generate surface oil slicks containing large quantities of hydrocarbons and introduce soluble organics into the water column.<sup>4</sup> Dispersants enhance the emulsification and dissolution of oil into the water, which augments degradation and prevents slick formation, reducing the amount of oil that reaches shorelines.<sup>5,6</sup> However, chemical dispersants can increase the concentration of petroleum hydrocarbons in the water column, enhancing their bioavailability for marine organisms.<sup>3,7,8</sup> Corexit 9500 and 9527, two dispersants used in the Deepwater Horizon oil spill response, have low to moderate toxicity for most marine organisms<sup>6,9</sup> but can enhance the toxicity of oil in others, including rotifers,<sup>10</sup> coral larvae,<sup>11</sup> and copepods.<sup>12</sup> The bioavailability of hydrocarbons to marine organisms is variable and depends on the chemistry, structure, sedimentation, and binding to natural particulates.<sup>13,14</sup> Polycyclic aromatic hydro-

carbons (PAHs) are known to be more toxic than the more abundant, nonaromatic compounds in petroleum.<sup>15,16</sup> Low molecular weight (low-MW) aromatic compounds, like the human carcinogen benzene, have higher water solubility than other component petroleum hydrocarbons and are of particular concern.<sup>17,18</sup> These toxic compounds can volatilize into the atmosphere after a surface spill or partition into deep water following subsurface release, as in the Deepwater Horizon spill.<sup>19</sup> Additionally, offshore oil drilling releases low-MW hydrocarbons (namely benzene, toluene, ethylbenzene, xylene) at levels ~8 mg/L in produced water.<sup>17</sup>

Nanoparticles and fine microparticles can assemble at water–oil interfaces to stabilize fine oil droplets, forming a Pickering emulsion. These particles are a potential alternative to chemical dispersants such as Corexit. Over the past decade, the field of nanotechnology has developed methods to precisely engineer

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the surface properties and behaviors of fine particles, and this ability may be exploited to fabricate a new class of high-efficiency, low-toxicity, particle-based dispersants. These dispersants may act similarly to naturally occurring oil-mineral aggregates (OMAs) formed from suspended mineral fines in seawater agglomerating with spilled oil to form entities that are stable for weeks in seawater without re-coalescing.<sup>20</sup> This is considered a key process in the natural biodegradation of oil in coastal environments.<sup>21</sup>

This article focuses on evaluating the behavior and environmental implications of novel surface-engineered carbon nanoparticle dispersants under current development. Studies have shown that surface-modified carbon black (CB) particles are effective dispersants for stabilizing oil-in-seawater emulsions.<sup>22</sup> Functionalized CB particles are less likely than Corexit 9500A to interact with crude oil to form water-in-oil emulsions, which reduce bioremediation efficiency<sup>22</sup> and make oil removal more difficult by increasing volume and viscosity<sup>23</sup> and reducing the ability to burn.<sup>22</sup> Carbon surfaces are well-known to adsorb organic compounds from aqueous phases and have a particularly high affinity for aromatics. We therefore explored the potential for these engineered particle dispersants, as a secondary feature, to serve as adsorbents for dissolved aromatic compounds from the water column and reduce their bioavailability and toxicity to marine organisms.

We evaluate the impact of engineered particle-based dispersants on biota in the water column using *Artemia franciscana* (brine shrimp), a filter-feeding microcrustacean previously used as a laboratory model for marine zooplankton toxicity testing of petroleum hydrocarbons and dispersants.<sup>24–28</sup> Surfactant-based dispersants like Finasol, Corexit 9500, and Corexit 9527 can cause changes in ATPase activity, respiration, and death in *Artemia*, with the 48 h LC<sub>50</sub> for Corexit 9500 at 21 mg/L and 52–104 mg/L for Corexit 9527.<sup>6,9,26,28,29</sup> Here we assess acute toxicity of novel carbon nanoparticle dispersants using larval mortality<sup>25</sup> and a sublethal stress response characterized by a measurable increase of heat shock protein 70 (hsp70) levels.<sup>30</sup>

A key variable in this study is hydrophilicity or hydrophobicity of the carbon particle surfaces. Successful Pickering dispersants must have surfaces of intermediate hydrophilicity/hydrophobicity,<sup>31</sup> which some particles possess in the native state, but usually requires postsynthesis chemical surface modification. For example, Saha et al. show that CB decorated with hydrophilic benzoic acid functional groups was an effective emulsion stabilizer.<sup>22</sup> Surface functionalization to increase hydrophilicity can also influence physical adsorption on carbon surfaces,<sup>32,33</sup> potentially influencing the bioavailability and toxicity of the particles.<sup>34</sup>

The surface engineering of particle-based dispersants is thus a complex design problem, which requires a type and degree of functionalization that co-optimizes dispersant and adsorbent performance but minimizes potential adverse effects of the particles on biological systems. Here we create three types of surface-engineered CB particles and study their interactions with *Artemia franciscana* and examine the three-way interactions between the particles, benzene (used as a model low-MW aromatic), and the brine shrimp through coexposure experiments.

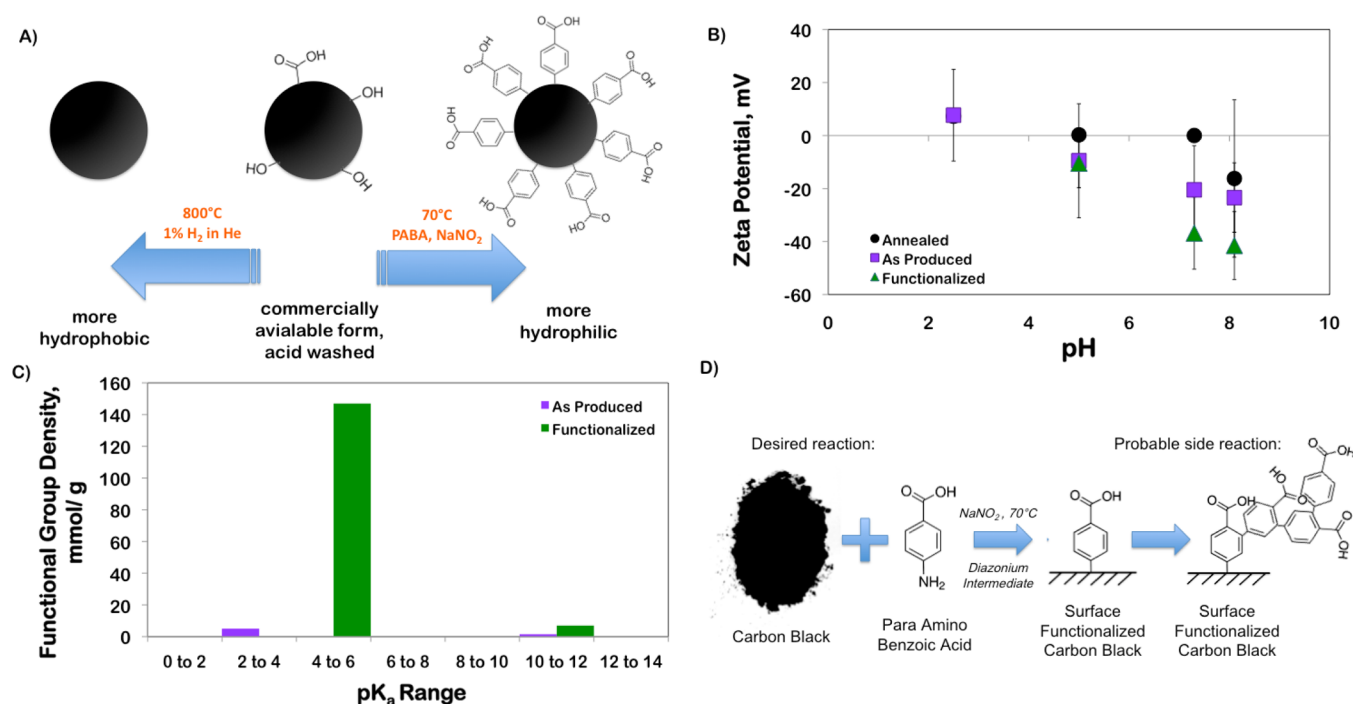
## 2. MATERIALS AND METHODS

### 2.1. Surface Modification of Carbon Particles and Characterization.

Norit SX Ultra powdered activated carbon

(PAC) (Norit, TX, USA) was used as a reference sample in the commercially available form without further modification. Particle size distributions were determined with SEM micrographs using ImageJ. Commercially available CB (Regal 330 from Cabot Corporation) was used as a baseline material and then chemically modified to produce more-hydrophilic and less-hydrophilic versions. This sample, called as-produced CB, was washed with 0.1 M HCl to remove potential metal impurities and then rinsed with deionized water until reaching neutral pH. Particles with increased hydrophobicity were produced by annealing the acid-washed Regal 330 in an environment of 1% hydrogen in helium for 2 h at 800 °C and are referred to as annealed CB. Para-amino benzoic acid (PABA) was used to introduce aryl-carboxylate groups using diazonium chemistry,<sup>35</sup> producing particles with increased hydrophilicity that are designated as functionalized CB. Briefly, 250 mM PABA in nanopure water was added to CB precursor particles in a ratio of 80 mL of solution per 100 mg of CB. This mixture was sonicated for half an hour to ensure good dispersion. Then, 3.3 mL of 600 mM sodium nitrite per 100 mg of carbon were added to the mixture, which was then moved to a water bath at 70 °C. The reaction was mixed with a magnetic stir bar and allowed to proceed for 30 min before being moved to a refrigerator at 4 °C to stop the reaction. The resulting particles were then cleaned extensively using 3.5K molecular weight cutoff dialysis tubing. The differences between the precursor and the modified sample were then characterized. Acid–base titrations were performed with a 907 Titrand automatic titrator (Metrohm, Switzerland). A sample of 0.1 g were dispersed in 50 mL of 0.1 M NaCl as background electrolyte. Titration was carried out by dynamic addition of 1 N NaOH or 1 N HCl to the flask while the solution was stirred continuously. After each addition of titrant, the system was allowed to equilibrate until a stable pH value was obtained. Thermogravimetric analyses were performed with a Thermo Cahn Versa analyzer (Thermo Scientific, MA, USA). Each material was heated from 25 to 800 °C at a rate of 10 °C/min, under N<sub>2</sub> atmosphere. Results can be seen in Figure S1 of the Supporting Information. Particle size distributions for all three versions of CB particles were measured by dynamic light scattering using a Zetasizer Nano S90 (Malvern Instruments, Worcestershire, UK).

**2.2. Benzene Adsorption Experiments.** Known concentrations of benzene (puriss p.a. ACS reagent, Sigma-Aldrich, MO, USA) were dissolved in simulated seawater (Instant Ocean, VA, USA) with a known mass of carbon particles in 50 mL glass vials. The vials were filled completely with no headspace and sealed tightly with PTFE-lined caps. The vials were kept on lab rollers for 48 h to ensure adsorption equilibrium was reached (kinetics data shown in Figure S2 of the Supporting Information). The absorbance of at least 3 samples from each vial was measured at 254 nm in a low-UV quartz cuvette (Thermo Scientific, MA, USA) using a Jasco V-630 Spectrophotometer (Jasco, OK, USA), and replicates were used to calculate standard deviations. Prior to spectrophotometric analysis, the CB was removed using a Millex 0.2 μm PTFE syringe filter (Millipore, MA, USA) to avoid interference with the absorption spectra of benzene. The absorbance was converted for a final concentration of benzene using a previously constructed calibration curve. The adsorbed amount,  $q$ , was determined from the following equation



**Figure 1.** Surface engineering of particle-based dispersants. A) Schematic representation of surface modifications performed. Commercially available (as-produced) CB is washed with 0.1 M HCl to remove impurities and then either annealed to a more hydrophobic version (left) or functionalized using diazonium chemistry to a more hydrophilic form (right). B) Change in zeta potential as a function of pH, at an ionic strength of approximately 165 mM. C) Determination of the pK<sub>a</sub> value distribution by acid–base titration of the functionalized CB as compared to the precursor particles. D) Probable side reaction is the self-polymerization of the diazonium intermediate leading to greater than monolayer coverage of benzoic acid groups.

$$q = \frac{V(C_0 - C_f)}{m_{\text{carbon}}}$$

where  $V$  is the volume of liquid in the vial,  $C_0$  is the initial concentration of benzene in the simulated seawater,  $C_f$  is the concentration of benzene after adsorption equilibrium, and  $m_{\text{carbon}}$  is the total mass of carbon in the vial. Data were then fit with various models (Freundlich, Langmuir, linear) and the best fit was determined by the coefficients of determination. The mathematical model of best fit was then used to determine three-way partitioning between air, simulated seawater, and carbon particles. A literature value for the partition coefficient for benzene between ocean water and air<sup>36</sup> was used after experimental verification. All calculations were performed in MATLAB R2012a.

**2.3. Brine Shrimp Culture and Toxicity Assays.** *Artemia franciscana* cysts from Great Salt Lake, Utah (INVE brand) were stored at  $-20^\circ\text{C}$ . Cysts were hatched under continuous light for 48 h at  $28^\circ\text{C}$  in aerated simulated seawater (Instant Ocean, VA, USA) at a ratio of 1 g cysts/1 L seawater. Based on anatomy and Schrehardt staging system, they have developed into metanauplius II by the end of exposure.<sup>37</sup> Exposure media prepared with seawater and particle dispersants or mixtures of particles and benzene were placed in vials filled completely with no headspace and sealed tightly with PTFE-lined caps and then mixed on lab rollers for 24 h. Benzene treatments were mixed immediately before exposure. As-produced CB, functionalized CB, and PAC were each sonicated for at least 20 min in a water bath (Branson 2510) to facilitate dispersion in simulated seawater. Annealed CB was dispersed in simulated seawater using a tight-fitting glass Dounce homogenizer.

For mortality experiments, 25 mL glass scintillation vials received 15 mL of exposure medium and a minimum of 250 A.

*franciscana* larvae. Larvae were exposed for 24 h at  $19^\circ\text{C}$  and then counted for total mortality. Death was determined by motility after brief (2–3 s) observation under a dissecting microscope. For observation of particle agglomeration on surfaces, brine shrimp were fixed with 4% formaldehyde and visualized using a Nikon Eclipse E800 microscope and brightfield microscopy. Images were obtained using commercially available SPOT software.

To assay hsp70 protein levels, 25 mL glass scintillation vials received 15 mL of exposure medium and a minimum of 500 larvae. Larvae were exposed for 24 h at  $19^\circ\text{C}$ , then collected into lysis buffer, and homogenized to isolate protein for Western blot analysis (protocol available in the Supporting Information). Samples were probed with primary antibodies for hsp70 (abcam 136874) and alpha-tubulin (abcam 52866) for the Western blot analysis, and the final chemiluminescent films were used for band density quantification with NIH ImageJ software.

For brine shrimp mortality and hsp70 protein levels, statistical significance of each treatment was determined using one-way ANOVA followed by Tukey's multiple comparisons test. Data were compared to untreated for individual particle exposures and compared to benzene alone at the given concentration for coexposure studies. To compare trends in sublethal protein levels, the linear regression of the particle coexposures was compared to benzene exposure alone using the extra sum of squares F test. All hsp70 protein data were normalized to total protein using alpha-tubulin protein expression and expressed relative to the hsp70 protein levels induced by menadione sodium bisulfite (MSB) in each experiment. MSB, a strong inducer of oxidative stress in brine shrimp, is used here as a positive control in all toxicity studies.<sup>38</sup> All statistical analysis were performed by GraphPad Prism



version 6 for Mac (GraphPad Software, La Jolla, California USA, www.graphpad.com), and data were considered significant at  $p < 0.05$ . For the low concentration coexposure experiments, particles were considered to have a significant impact on hsp70 levels with a  $p < 0.01$  for a given particle concentration.

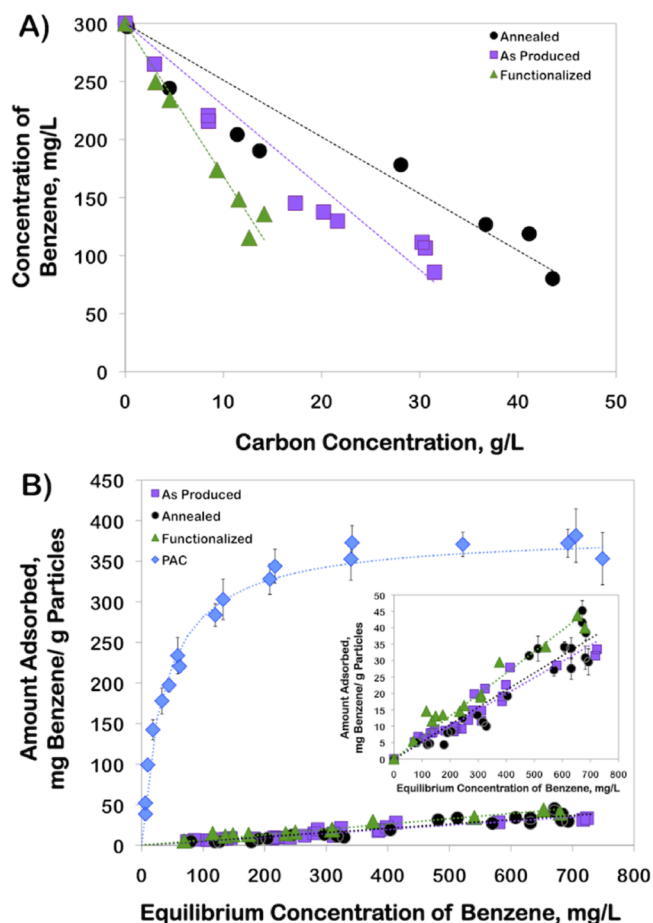
### 3. RESULTS

**3.1. Effect of Surface Properties on Carbon Particle Sorbent Performance.** Three different surface-engineered CB nanoparticles were characterized for this study. Commercial CB was used as a baseline sorbent and is denoted by the name as-produced CB nanoparticles. Particles with increased hydrophobicity were produced by heating in 1% H<sub>2</sub> in He and are called throughout the paper annealed CB nanoparticles (Figure 1A). This process drives off many of the oxygen-containing hydrophilic functional groups and caps the nascent carbon active sites with hydrogen to minimize surface reoxidation in ambient air. Particles with increased hydrophilicity were produced by covalently grafting benzoic acid functional groups through diazonium chemistry and are referred to as functionalized CB nanoparticles (Figure 1A). The increase in acidic surface functional groups was confirmed by measuring the zeta potential (Figure 1B). The number and nature of acidic and basic sites on the particle surface determine the zeta potential vs pH curve shape. The values of the zeta potentials shown in Figure 1B indicate that the number of acidic functional groups increase in the order annealed < as-produced < functionalized, as expected.

Acid–base titrations (Figure 1C) show a clear increase in the number of protonable functional groups on the surface of the CB particle after the functionalization process. There are approximately 150 mmol/g of functional groups added at  $4 < \text{p}K_a < 6$ , which is characteristic of a carboxylic acid moiety. Using an estimated surface area of benzoic acid, it is possible to show the total surface area of these molecules is over 400% that of the original carbon particle surface. This greater-than-monolayer coverage was initially surprising, but a known side reaction in the diazonium functionalization process is grafting onto the aryl ring of a PABA molecule, which produces polymeric coatings on the carbon surfaces (Figure 1D).<sup>39</sup> These polymeric films can have thicknesses from the nanometer<sup>40</sup> to micrometer range.<sup>41</sup> It is likely that many of the carboxylic acid moieties seen in Figure 1C are actually part of the polymer network and are not directly attached to the carbon surface.

Figure 2 compares the ability of the different engineered particle formulations to adsorb dissolved benzene as a model low-MW aromatic hydrocarbon. The raw data are shown in Figure 2A, while 2B is the fundamental equilibrium adsorption isotherm. Thermal annealing slightly increases benzene adsorption, which is consistent with the removal of native oxygen-containing hydrophilic groups in the as-produced material. Planar conjugated adsorbates, such as benzene, often adsorb through hydrophobic and  $\pi$ – $\pi$  interactions with the carbon surface.<sup>42</sup> The oxygen-containing functional groups cover portions of the pristine hydrophobic surface and can also create H-bonded water clusters that propagate from the groups to partially restrict access to the remaining adjacent hydrophobic domains.<sup>32,43</sup>

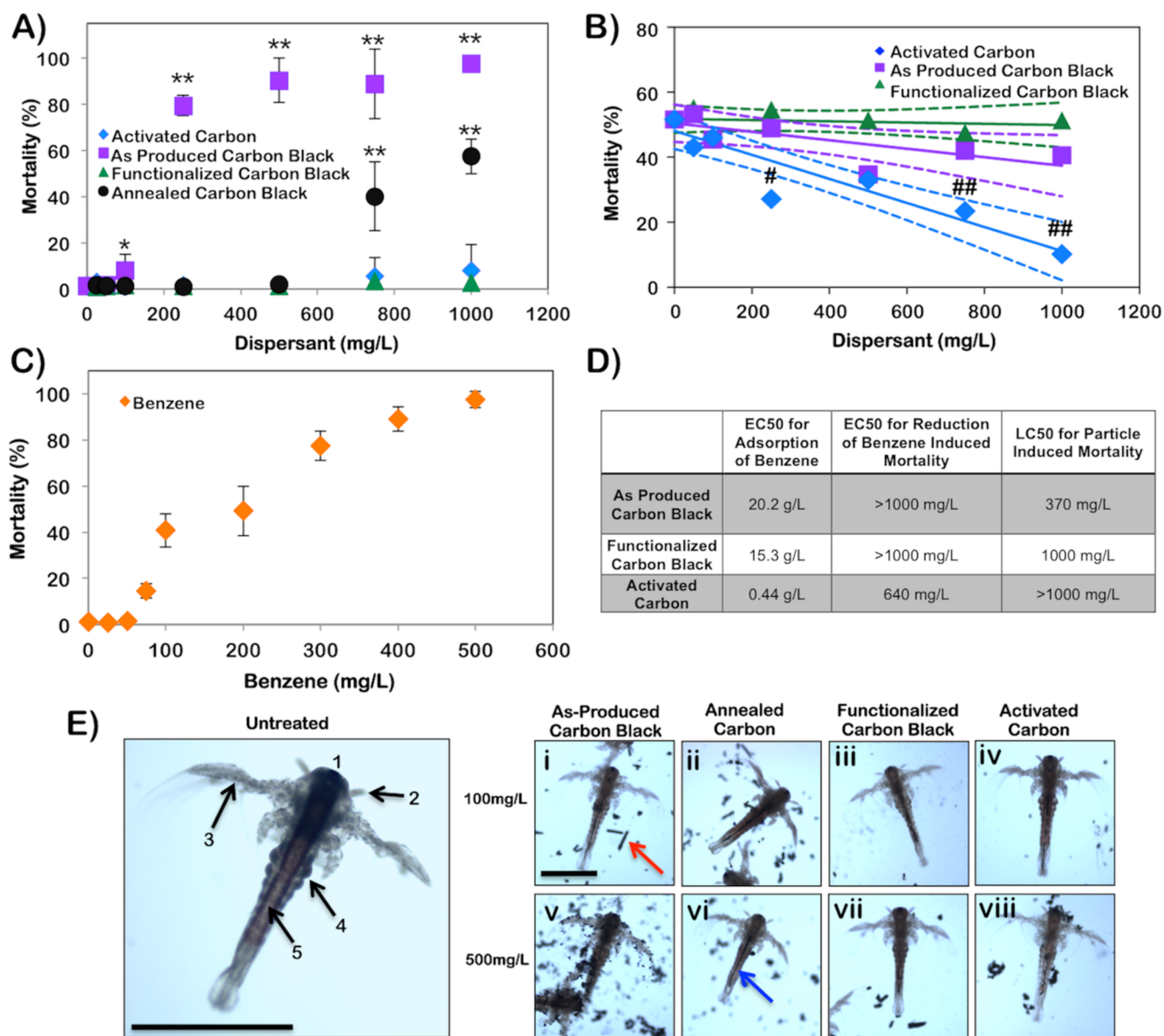
The hydrophilic formulation showed the highest benzene adsorption, which initially seemed inconsistent with the hydrophobic driving forces cited above. We suggest that this



**Figure 2.** Adsorption of benzene as a model low-MW aromatic by various engineered carbon materials. A) Raw adsorption data for three formulations of carbon-black-based dispersants in simulated seawater. B) Adsorption isotherm comparing the particle-based dispersants to powdered activated carbon (PAC) as an engineered sorbent reference material. Inset shows a detailed view of the adsorption isotherm for the particle-based dispersants only.

is additional evidence for the side reaction shown in Figure 1D. The presence of the aromatic polymer film creates an interfacial zone of finite thickness that contains carboxylate groups for surface charge and hydrophilicity (Figure 1A–B) but also numerous aryl groups that provide sites for benzene uptake. This enhanced uptake of benzene is likely caused by the favorable partitioning of benzene into that organic polymer layer from the aqueous phase, rather than simple adsorption onto a carbon surface.

Finally, PAC is much more active as a benzene adsorbent than any of the engineered CB samples (Figure 2B), consistent with its much higher surface area. The activated carbon adsorption data are sufficient to define the full isotherm allowing extraction of Langmuir adsorption parameters, which are  $K = 2.7 \times 10^2 \text{ L/mg}$  ( $2.1 \times 10^3 \text{ L/mol}$ ), maximum adsorption capacity ( $q_{\text{max}} = 385 \text{ mg/g}$ ), and the free energy of adsorption,  $-RT \ln K$ , of  $-19 \text{ kJ/mol}$ . The ratio between the highest observed amount of benzene adsorbed by the as-produced CB ( $\sim 32 \text{ mg/g}$  at an equilibrium concentration of about  $750 \text{ mg/L}$ ), and the  $q_{\text{max}}$  predicted by the Langmuir adsorption model for the PAC ( $\sim 385 \text{ mg/g}$ ) is roughly equivalent to the ratio of their surface areas ( $92 \text{ m}^2/\text{g}$  vs  $1200 \text{ m}^2/\text{g}$ , respectively).

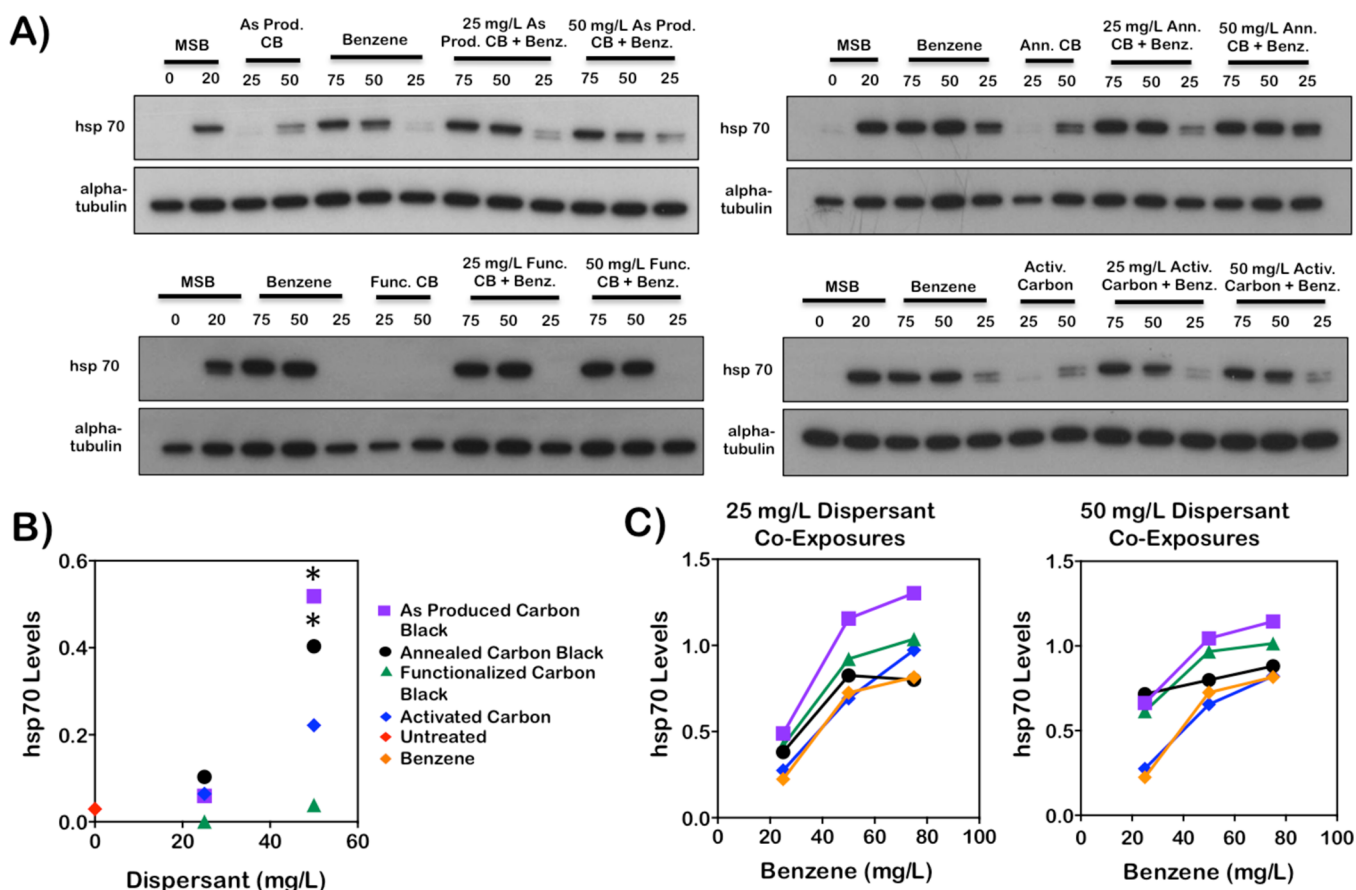


**Figure 3.** Brine shrimp mortality and particle accumulation after 24 h. Error bars indicate standard deviation. Solid and dashed lines represent linear regression and 95% confidence bands, respectively. \* $p < 0.05$ , \*\* $p < 0.01$  compared to untreated, # $p < 0.05$ , ### $p < 0.01$  compared to 200 mg/L benzene. A) Mortality after exposure to CB dispersants and activated carbon. B) Mortality after coexposure to 200 mg/L benzene and particle dispersants. C) Mortality after benzene exposure. D) Calculated values for particle: effective concentration for adsorption of 50% of 200 mg/L benzene onto the carbon surface; reduction of brine shrimp mortality following coexposure to benzene at 200 mg/L by 50%; and  $LC_{50}$  for particles alone. E) The untreated brine shrimp image to the left of the panel demonstrates anatomical features at time of exposure. 1: mouth (underneath and not visible), 2: sensory antennule, 3: locomotory antenna, 4: nonfunctional limb buds, 5: gut lumen. The panel of images at right shows uptake and accumulation of particles on the exoskeleton of whole brine shrimp after 24 h at 100 mg/L (i-iv) and 500 mg/L (v-viii) for all three CB forms and PAC. Red arrow indicates rod-like bundles of excreted particles; blue arrow indicates gut uptake of particles. Scale bar indicates 500 μm.

**3.2. Brine Shrimp Mortality Assays.** To assess acute toxicity, brine shrimp larvae were exposed to the surface-engineered CB nanoparticles and PAC for 24 h and observed for mortality and particle accumulation (Figure 3A). As-produced CB induces significant mortality at concentrations of 100 mg/L and above. Annealed CB shows lower toxicity, with significant mortality only over 500 mg/L. Using a linear regression model, the calculated median lethal concentration ( $LC_{50}$ ) for as-produced CB is ~370 mg/L and for annealed is ~1000 mg/L. Functionalized CB and PAC are less toxic, with insignificant mortality above 750 mg/L. The  $LC_{50}$  for PAC and

functionalized CB are greater than 1000 mg/L and thus outside the range of tested concentrations included in this study.

Light microscopic images of brine shrimp exposed to 100 and 500 mg/L of carbon particles or PAC for 24 h reveal gut uptake of all particle types and particle agglomerates adhering to external surfaces (Figure 3E). Agglomerates of as-produced CB nanoparticles adhered to brine shrimp after exposure to 500–1000 mg/L. High concentrations (1000 mg/L) of annealed CB or PAC formed larger surface agglomerates, while functionalized CB particles remained well-dispersed with minimal surface adherence at all concentrations. Particles can be seen in the gut after all particle exposures, indicating uptake



**Figure 4.** Hsp70 protein levels after 24 h exposure. \* $p < 0.05$ , compared to untreated. A) Western blot analysis of hsp70 and alpha-tubulin protein levels induced by as-produced, annealed, and functionalized CB, activated carbon or benzene, alone or in combination with carbon particles. B) Relative quantitation of hsp70 protein levels induced by particle dispersants, normalized to hsp70 induced by 20 mg/L MSB. C) Relative quantitation of hsp70 levels induced by coexposure to particle dispersants and benzene, normalized to hsp70 levels induced by 20 mg/L MSB.

by filter-feeding. Also visible in the seawater around the brine shrimp are rod-like bundles of particles, which most likely reflect gut uptake and excretion as described by Handy et al.<sup>44</sup> These can be seen after exposure to 100 mg/L as-produced and annealed CB and at all concentrations of functionalized CB.

The mean mortality of untreated brine shrimp in this study was 1.2%. After 24 h of benzene exposure, brine shrimp mortality is evident at an initial concentration of 75 mg/L. Mortality increases in an approximately linear fashion, with an  $LC_{50}$  of ~200 mg/L, until reaching ~100% mortality at a concentration of 500 mg/L benzene (Figure 3C).

**3.3. Impact of Particle Dispersants on Benzene-Induced Mortality.** To test the ability of as-produced or functionalized CB nanoparticles and PAC to mitigate the toxicity of benzene, brine shrimp were coexposed to 200 mg/L benzene (the  $LC_{50}$ ) and a range of particle concentrations. Linear regression plots indicate that coexposure to as-produced CB nanoparticles or PAC induced a dose-dependent decrease in mortality, but only PAC caused a statistically significant mitigation when compared to 200 mg/L benzene (Figure 3B). Coexposure to 1000 mg/L as-produced CB reduced mortality to ~40% as compared to ~10% at the same concentration of PAC. Brine shrimp coexposed to benzene and functionalized nanoparticles showed no attenuation of mortality, even at the highest concentration (~51% at 1000 mg/L). Coexposure to benzene reduced toxicity of as-produced CB particles ~50% over the concentration range of 250–1000 mg/L. Imaging

shows less particle aggregation and accumulation on the surface of brine shrimp coexposed to benzene and as-produced CB or PAC when compared to particles alone (Figure S3).

**3.4. Impact of Particle Dispersants on Sublethal Benzene Toxicity.** To determine the impact of particle dispersants on exposure of brine shrimp to low mortality (75 mg/L) and sublethal (25–50 mg/L) concentrations of benzene, hsp70 protein levels were assessed using Western blot analysis (Figure 4). Hsp70 is a heat shock protein induced by a wide range of physical and chemical stressors including heat, metals, and chemical pollutants.<sup>30</sup> Menadione sodium bisulfite (MSB) was used as a positive reference toxicant to induce high levels of hsp70. Brine shrimp were exposed to particle concentrations of 25 and 50 mg/L alone (Figure 4A) or in combination with benzene (25–75 mg/L) (Figure 4C).

At 50 mg/L, as-produced and annealed CB alone significantly increased hsp70 levels, while exposure to functionalized CB or PAC did not induce this stress protein response (Figure 4B). Using linear regression to analyze protein expression, coexposure with as-produced CB significantly increased hsp70 levels at 25–50 mg/L compared to benzene alone, while annealed CB increased levels at 50 mg/L (Figure 4C). Coexposure to functionalized CB or to PAC did not significantly affect hsp70 protein levels induced by low concentrations of benzene.



## 4. DISCUSSION

This study provides a preliminary safety assessment for particle-based alternative dispersants currently under development for large-scale petroleum spills. Engineered hydrophilic CB particles that show good dispersant performance<sup>22</sup> demonstrate no toxicity. At low concentrations, the functionalized hydrophilic samples did not induce the stress response indicated by increased hsp70 protein levels, and no significant mortality or particle agglomeration was observed at high concentrations. The hydrophobic varieties induced toxicity at concentrations  $\geq 100$  mg/L and were associated with the accumulation of particle agglomerates on the brine shrimp exoskeleton. Other studies have reported that hydrophilic surface treatments can reduce the toxicity of carbon nanomaterials in mammalian cell cultures<sup>34</sup> and aquatic organisms.<sup>45–47</sup> At sublethal concentrations, hydrophobic carbon nanoparticles induced significantly increased hsp70 protein levels.

A mechanism proposed for brine shrimp mortality at high concentrations of hydrophobic carbon nanoparticles is physical adhesion to the exoskeleton (Figure 3) and locomotory appendages that decrease motility. Locomotor impairment has been previously associated with external nanomaterial CB exposure in adult fruit flies, while dietary exposure to the same material had no significant effect.<sup>48</sup> For invertebrates, these engineered carbon nanomaterials act as external physical or mechanical stressors. Other studies using *Daphnia* or *Artemia* exposed to different natural and engineered nanomaterials emphasized the contribution of particle loading of the gut resulting in impaired food uptake and increased mortality after prolonged time periods.<sup>49,50</sup> In this study, both gut uptake and excretion of all tested carbon particles were observed over 24 h similar to observations following exposure to well-dispersed single wall carbon nanotubes.<sup>51</sup> Only the more hydrophobic carbon nanoparticles caused mortality and only at high concentrations. It is noteworthy that the hydrophilic surface treatment required to create effective dispersants<sup>22</sup> also reduces or eliminates adverse effects of the nanoparticles on *Artemia*. Our study, along with previous work by others,<sup>22</sup> demonstrates that functionalized CB particles are both functional as dispersants and safe for marine organisms.

We also examined the potential for particle-based dispersants to adsorb low-MW aromatics from the water column as a secondary useful feature. Using benzene as a model solute, we measure significant physical adsorption but only at particle concentrations  $> 10$  g/L. We would also like to note that these experiments were conducted in artificial seawater and did not include any competitive effects from natural organic matter or other compounds naturally present in seawater. An interesting finding is that the functionalized, hydrophilic CB showed a higher affinity for physical adsorption of benzene than the hydrophobic varieties. This is the opposite of the commonly observed trend,<sup>32,33</sup> and we attribute it to benzene partitioning into an organic layer formed by self-polymerization of the diazonium intermediate on the CB surfaces.

Additionally, this study provides information on the implications of dissolved aromatic hydrocarbons in petroleum/water systems. At high concentrations, inhalation of volatile or low-MW hydrocarbons can cause irritation of the respiratory tract and central nervous system depression.<sup>52</sup> Marine organisms including *Artemia* develop nonpolar narcosis that correlates with the octanol–water partition coefficient of the hydrocarbon.<sup>53–55</sup> In this study, the  $LC_{50}$  of benzene for

*Artemia* was found to be 200 mg/L, and increased levels of the generalized stress protein, hsp70, were induced by sublethal concentrations (25–50 mg/L). Increases in hsp70 protein levels may serve as a generalized stress biomarker that is responsive to both physical and chemical stressors, either alone or in combination.

Also of concern are potential long-term human health consequences of exposure to benzene, which can be inhaled by cleanup workers. Inhalation of benzene can induce hematotoxicity, reproductive and developmental abnormalities, and leukemia.<sup>56</sup> Volatile aromatic hydrocarbons, including benzene and alkylated monoaromatic compounds, were detected at 68  $\mu\text{g/L}$  in subsurface water samples following the Gulf oil spill.<sup>19</sup> A retrospective study of exposed and unexposed Gulf residents detected elevated levels of phenol, a biomarker for benzene exposure, in the urine of exposed workers. Clinical laboratory findings included decreased platelet counts, increased serum enzyme markers of liver injury, and reduced kidney function in the exposed workers consistent with benzene-induced toxicity.<sup>57</sup>

Benzene and particle coexposure experiments show that the CB cannot mitigate benzene toxicity, which is consistent with the small amounts adsorbed at the particle concentrations used in the biological experiments ( $< 1000$  mg/L). PAC, in contrast, can mitigate free benzene toxicity when present at  $\sim 250$  mg/L, due to its much higher engineered surface area. Activated carbon, however, is not known to be active as a Pickering dispersant and would likely function primarily as a sorbent in this system. The secondary role of CB-based Pickering dispersants as sorbents was not realized here, and further work is needed to see if the adsorptive effects may be more pronounced for higher-MW polycyclic aromatics.

These preliminary studies suggest that functionalized hydrophilic CB particles under development as petroleum dispersants exhibit low toxicity to the marine model organism, *Artemia franciscana*. Future studies are needed to evaluate a wider range of particulate and polymeric dispersants under development as alternatives to existing chemical dispersants. The low toxicity of these functionalized nanoparticles demonstrates that co-optimization for dispersant performance and low adverse environmental impacts can be integrated together for the surface engineering of future dispersants. The brine shrimp model presented here is suitable for evaluation of both lethal and sublethal acute toxicity end points of novel, particle-based dispersants for marine oil spills, individually or as coexposures with aromatic hydrocarbons. This model system can be used to assess potential inhibitory, additive, or synergistic effects of combined exposure to physical stressors (particle-based dispersants) plus chemical stressors (petrogenic aromatic hydrocarbons), which is a challenging problem in human risk assessment.<sup>58</sup> Continued work is required to identify and validate more sensitive molecular biomarkers to assess chronic toxicity and multigenerational impacts of particle-based dispersants with and without adsorbed hydrocarbons at lower concentrations that are more relevant for environmental exposures following marine oil spills.<sup>47,59,60</sup>

## ■ ASSOCIATED CONTENT

### 📄 Supporting Information

Additional characterization data of the CB particles, adsorption kinetics, protocol details for brine shrimp protein isolation and analysis, and images of brine shrimp coexposed to PAC and as-produced CB particles. This material is available free of charge



via the Internet at <http://pubs.acs.org>. Additional data are available through the Gulf of Mexico Research Initiative Information and Data Cooperative (GRIIDC) data repository.

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### Notes

The authors declare no competing financial interest.

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## ABBREVIATIONS

CB	carbon black
hsp70	heat shock protein 70
MSB	menadione sodium bisulfite
MW	molecular weight
OMA	oil-mineral aggregates
PABA	para-amino benzoic acid
PAC	powdered activated carbon
PAH	polycyclic aromatic hydrocarbons
PTFE	poly(tetrafluoroethylene)

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