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Potential Impact of Biochar Water-Extractable Substances on Environmental Sustainability

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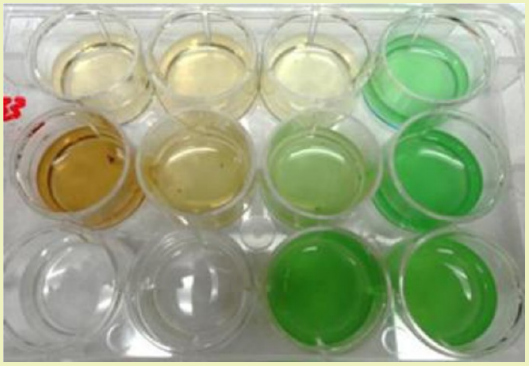
Supporting Information

ABSTRACT: Application of biochar as a soil amendment could be a significant approach for carbon sequestration to possibly control climate change for energy and environmental sustainability. However, more studies are needed in a number of research areas, including the development of clean biochar materials free of any harmful substances, before this approach could be implemented at a global scale. In this study, biochar water-extractable substances were tested for their potential harmful effects on the growth of aquatic photosynthetic microorganisms including both blue−green alga (cyanobacteria *Synechococcus*) and eukaryotic green alga (*Desmodesmus*) that represent the primary photosynthetic producers of the aquatic environment. The water extracts from three different biomass-derived biochar materials varied widely in their dissolved organic and inorganic contents, as well as in their characteristics including their pH values. Bioassays with pinewood-derived biochar water extract showed a significant inhibitory effect on aquatic photosynthetic microorganism growth in a dose-dependent manner, while chicken litter and peanut shell-derived biochar water extracts showed no signs of growth inhibition. The pinewood-derived biochar water-extracted substances were further separated into three fractions based on their molecular sizes and electric charges through an electrodialysis separation process using a cellulose−acetate membrane with a 500-delta cutoff pore size. Our analysis showed that the active component of pinewood-derived biochar water-extracted substances that are toxic to both blue−green alga (cyanobacteria *Synechococcus*) and eukaryotic green alga (*Desmodesmus*) is likely a 500-delta (or smaller) organic chemical species that carries at least one carboxyl group. This finding is important to engineering a high-tech biochar that can be free of any undesirable substances for its soil applications toward agricultural and environmental sustainability.



KEYWORDS: *Water-extractable biochar toxin, Biochar inhibitory factor, Biochar harmful substance, Biochar side effects on algae, Biochar environmental sustainability, Biochar and aquatic photosynthetic microorganisms, High-tech biochar, Biochar soil amendment and carbon sequestration*

INTRODUCTION

The world currently faces a systemic energy and environmental problem of increasing CO_2 emissions and global climate change. Solving this problem requires a comprehensive portfolio of research and development efforts with multiple strategies. Among these, the approach of using advanced biomass pyrolysis with reduced emissions to produce biochar soil amendments, in addition to biosyngas and biofuels, appears to be a particularly promising pathway to sustainability. The central idea is that biochar, produced cleanly and sustainably by pyrolysis of biomass wastes and used as a soil amendment, would "lock up" biomass carbon in a form that can persist in soils for hundreds to thousands of years, while at the same time helping to retain nutrients in soils and reduce the runoff of agricultural chemicals.[1] This approach is receiving increased worldwide attention.[2−4] Globally, each year about 6.6 gigatons (Gt) of dry matter biomass (e.g., crop stovers, dead leaves, waste woods, and rice straws) are produced but not effectively utilized.[5] Application of this approach could turn this type of waste into valuable biochar, biosyngas, and biofuel products at gigaton scales in a distributed manner.[6] Worldwide, this approach could result in a net reduction of greenhouse-gas emissions by about 1.8 Gt of CO_2−C equivalent emissions per year, which is about 12% of the current global anthropogenic emissions.[7] This is a unique "carbon-negative" bioenergy system approach, which on a life-cycle basis could not only reduce but also reverse human effects on climate change.[8] However, much more studies are needed before this approach can be considered for widespread implementation. First of all, biochar occasionally shows inhibitory effects on plant growth.[9−11] Organic species including possibly inhibitory and benign (or stimulatory) chemicals are produced as part of the biomass pyrolysis process.[12−15] Thus far, very little is known about the mechanisms of these factors. If biochar were to be globally used as a soil amendment and carbon sequestration agent at gigatons of carbon (GtC) scales, the release of

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potentially toxic compounds into soil and associated hydrological systems (rivers, lakes, and oceans) might have negative consequences in both agro-ecosystems and aquatic environmental systems. It is essential to produce this knowledge and mitigate against any undesirable effects in order for biochar to be used as a soil amendment and carbon-sequestration agent at gigaton scales.

In the past, researchers have studied the effects of biochar on soil biota in both harmful and beneficial aspects,[16] but to our knowledge, little attention has been paid to the possible effects of biochar water-extractable substances on aquatic microorganisms. Water-extractable organic carbon and inorganic nutrient species have been reported in a number of biochar materials.[17−19] Through soil rainwater runoff and leaching, significant amounts of biochar water-soluble chemical species could enter the hydrologic system, possibly affecting the aquatic microorganisms in nearby rivers, lakes, etc., especially if biochar materials were to be used as a soil amendment and carbon-sequestration agent at gigaton scales. Here, we report our recent study on extraction and examination of biochar water-extractable substances (BWES) and their effect on the growth of aquatic photosynthetic microorganisms including both blue−green alga (cyanobacteria *Synechococcus*) and eukaryotic green alga (*Desmodesmus*), which represent the primary photosynthetic producers in the aquatic environment, relevant to energy and environmental sustainability. The main objective of this study was to assess the possible effect of BWES on aquatic microorganisms including both prokaryotic and eukaryotic algae by monitoring their growth via absorbance measurements at the algal chlorophyll peak wavelength. The findings reported here may have practical implications in developing better biochar materials with higher cation change capacity and free of any undesirable substances for soil applications toward energy, agricultural, and environmental sustainability.[20]

MATERIALS AND METHODS

Biochar Materials. Three different biochar samples were used in this study: (i) peanut shell-derived biochar (PSB), (ii) chicken litter-derived biochar (CLB), and (iii) pinewood-derived biochar (PWB). The peanut shell- and chicken litter-derived biochar materials were produced from pelletized peanut shells and chicken litter through pyrolysis at 411 and 424 °C, respectively, using a superheated steam pyrolysis unit at Eprida Inc. (Athens, GA). The pinewood-derived biochar was produced at Auburn University. Pinewood chips were obtained from a local wood chipping plant in Opelika, AL. Pinewood chips obtained from the plant were clean (no bark and leaves). Wood chips were dried in a conventional oven for 24 h at 75 °C and ground using a hammer mill (New Holland Grinder model 358) fitted with 3.175 mm (1/8 in.) screen size. After drying and size reduction of wood chips, the wood chips that were fractionated using a sieve analysis and the particles in the range of 0.841−1.41 mm (U.S. Sieve no. 14−20) were used in this study. The pinewood biomass was converted into bio-oil and biochar through the fast pyrolysis process using an auger reactor, designed and fabricated at Auburn University.[21] Average yield of biochar from the pinewood was about 33 wt % at the pyrolysis temperature of 450 °C measured on the outside of the reactor. Figure S1 (in the Supporting Information) shows the side-by-side comparison of the three different biochar samples prior to extraction of water-extractable substances.

Extraction of Biochar Water-Extractable Substances. Extraction of water-soluble substances from biochar was performed by soaking 50 g of biochar sample material (mentioned above) in 200 mL of ultraquality Milli-Q water (Millipore). The biochar−water mixture was placed on a shaker platform operating at 100 rpm for at least 24 h. The biochar−water mixture was transferred to 500-mL centrifuge tubes and centrifuged at 5000 rpm (×4682*g*) with JLA-10.5 rotor at 4 °C for 15 min using a Beckman Coulter Avanti high-speed centrifuge (model no. J-26 XP). The supernatants and pellets were collected separately. Then, the biochar−water mixture was vacuum-filtered through a Whatman grade-1 qualitative filter paper (11 μm pore size) via a 12-cm diameter Buchner funnel. The collected supernatants were then frozen and freeze-dried (170 Torr, −75 °C) to obtain the biochar water-extractable substances in dryness as shown in Figure S2 in the Supporting Information. The dry mass of the BWES that are shown in Figure S2 (Supporting Information) was 1.693 g of BWES/50 g of pinewood-derived biochar, 5.334 g of BWES/50 g of chicken litter, and 0.247 g of BWES/50 g of peanut shell-derived biochar. These BWES were redissolved in a smaller amount of Milli-Q-deionized water to make their stock solutions at the following concentrations: 28.2 g of pinewood BWES/L, 28.2 g of chicken-litter BWES/L, and 14.1 g of peanut-shell BWES/L (this lower concentration was made owing to its limited dry mass of 0.247 g). The pH values of the pinewood BWES, chicken-litter BWES, and peanut-shell BWES solutions were determined to be 3.94, 8.87, and 9.09, respectively. These three stock solutions were used to conduct biochar assays in duplicates for 3 experiments (total six replications). To determine whether the pinewood BWES solution's acidity (pH = 3.94) could be an influencing factor in the bioassay, a portion of the pinewood BWES solution was neutralized with sodium hydroxide to pH 7.83. The neutralized pinewood BWES solution was used in a separate bioassay in duplicate as well.

Bioassay of Biochar Water-Extractable Substances. Bioassays of biochar water-extractable substances with both blue−green (prokaryotic) and green (eukaryotic) alga cells were conducted using Corning Costar 12-well culture plates with 5-mL capacity. The blue−green alga *Synechococcus elongatus* (ATCC-33912) used in the assay was obtained from the American Type Culture Collection (ATCC). BG-11 medium (ATCC, 616 medium) was prepared and used as the *Synechococcus* culture medium for the bioassay experiments. A small amount (ranging from 0 to 100 μL) of BWES solution was then added into the 5-mL bioassay well containing 2250 μL of liquid algal culture. Any liquid volume difference due to the addition of stock solution was properly balanced with small amounts of deionized water (ranging from 150 to 250 μL) to give a total volume of 2.5 mL for each assay well. The final BWES concentrations (doses) in the bioassay liquid medium were 1.13, 0.564, 0.282, 0.0564, and 0 g/L (control). More specifically, the total liquid volume of each well was limited to 2.5 mL (half volume of a 5-mL well) to minimize any possible spillover (cross-contamination) between wells. On each plate, 2 wells were designated as blanks and filled with 2.25 mL of uncultured BG-11 medium and 0.25 mL of Milli-Q water. All 12-well culture plates were placed on a shaker platform operating continuously at 100 rpm. The algal culture bioassay plates were illuminated at an actinic intensity of about 25 $\mu E/m^2 \cdot s$ provided by use of daylight fluorescent lamps above the shaker platform. The actinic intensity was measured at the position of the algal bioassay plates using a Li-Cor quantum photometer (LI-250A). Similar bioassay of the biochar water-extracted substances were conducted with eukaryotic alga, *Desmodesmus* strain 4N2, isolated locally in Virginia by Drs. Harold G. Marshall and Andrew Gordon of ODU's Biology Department. The *Desmodesmus* bioassay conditions were essentially the same as those of *Synechococcus* except that a *Desmodesmus*-specific culture medium (RLH-2) was used instead of the BG-11 medium.

Furthermore, bioassays with equivalent dissolved organic carbon (DOC) dose (0.158 g of DOC/L) of the pinewood BWES and peanut-shell BWES were subsequently conducted on *Synechococcus*. More details of the experimental procedures are reported in section 1.2 and Figure S10 in Supporting Information.

Algal Growth Monitoring. The algal growth in every well of the multiwall plates was monitored by measuring the change in absorbance at 680 nm (algal chlorophyll absorbance peak) and by recording other observable changes (color and transparency) via photographs. At the beginning (day 0 or 1), middle (day 7 or 8), and end (day 14, 15, or 16) of the experiments, absorbance spectra in a wavelength range from 300 to 750 nm were measured using BioTek Synergy HT multimode microplate reader. Absorbance measurement readings at 680 nm were

taken daily for all bioassay wells for each plate using the same BioTek Synergy HT multimode microplate reader as well. The absorbance reading for a 12-well plate was programed to take about 1−3 min at temperatures ranging from 25 to 28 °C inside the microplate reader instrument. For more detailed absorbance measurement information, refer to the Supporting Information.

Electrodialysis Separation of Biochar Water-Extracted Substances. The procedures for electrodialysis separation of biochar water-extracted substances were followed directly from the methods for a mini-electrodialysis system for desalting small-volume saline samples for Fourier transform ion cyclotron resonance mass spectrometry.[22] Figure S4a and S4b (Supporting Information) shows the mini-electrodialysis (ED) system (Harvard Apparatus) that was used to desalt/separate the biochar water-extracted substances to isolate the possible inhibitor substance. For each mini-ED system desalting run, 1.5 mL of BWES stock solution was placed into a 1.5-mL Teflon sample chamber (Supporting Information, Figure S4d) in which two cellulose acetate membranes (Supporting Information, Figure S4c) with a 500-Da molecular weight cutoff (MWCO) pore size were placed at both ends. Prior to their use, cellulose−acetate membranes were stored in 0.05% sodium acetate solution and were rinsed and soaked in Milli-Q water. After the sample chamber was placed into the mini-ED system, 600 mL of Milli-Q water was filled in the larger chamber where the anode electrode resides, which will be referred to as the anode chamber. The other smaller chamber with the cathode will be referred to as the cathode chamber and is roughly equivalent to half of the anode chamber size (Figure S5, Supporting Information, shows the schematic diagram of the mini-ED system and a detailed description of the electrodialysis process). The cathode chamber was filled with 300 mL of Milli-Q water, rendering an equal water level in both chambers. A small power supply (maximum 200Vdc, 100 mA) was attached to two electrodes in which 200 V was applied as shown in Figure S4a, Supporting Information. The salinity in each of the two chambers was monitored (Hanna Primo conductivity meter) daily until their salinity became constant near the end of the electrodialysis (separation) experiment. Unlike that of Chen et al.,[22] no additional water was added to compensate for the water loss due to evaporation and water electrolysis (O_2 and H_2 gas production) at the anode and cathode. The anode and cathode chamber water (containing electrodialysis-separated biochar water-extracted substances) were collected and stored separately in the freezer, while the resulting BWES stock solution inside the 1.5-mL Teflon sample chamber (now referred to as the center chamber retained substances) was stored in the refrigerator (6 °C). The frozen anode and cathode chamber water was freeze-dried (170 Torr, −75 °C). The dry masses of each chamber, which were then referred to as anode-isolated and cathode-isolated substances, were redissolved in 10 mL of Milli-Q water to be used as stock solutions for the dissolved organic carbon (DOC) analysis and additional bioassays.

Dissolved Organic Carbon Analysis. Samples of biochar water-extracted substance stock solutions were analyzed for their dissolved organic carbon (DOC) concentrations by high-temperature catalytic combustion to CO_2 via Shimadzu TOC-VCPH total organic carbon analyzer, calibrated with potassium hydrogen phthalate (KHP). Dissolved inorganic carbon (DIC) was removed prior to combustion by acidifying samples to pH 2 and sparging. Measurements were taken in triplicate, and the average and standard deviation of the three measurements are shown in Tables 1 and 2.

Bioassay of Electrodialysis-Separated Biochar Substances. Bioassays were conducted using electrodialysis-separated biochar substances with a similar procedure as mentioned in the section Bioassay of Biochar Water-Extractable Substances. The separated pinewood BWES fractions resulting from the electrodialysis-separation process include the center chamber retained substances, anode-isolated substances, and cathode-isolated substances. The center chamber retained (pinewood BWES) substances were assayed in the same way as that of the pinewood BWES described in the section Bioassay of Biochar Water-Extractable Substances in which the same volumes of center chamber retained substances (100, 50, 25, 5, 0 μL) as those of the pinewood BWES were used. Unlike in the section Bioassay of Biochar Water-Extractable Substances, the total solute content (including both organic matter and salts) of the center chamber retained pinewood BWES solution was likely reduced because of the electrodialysis-separation process. Meanwhile, organic matters can be better represented by dissolved organic carbon (DOC) measurement. Therefore, DOC measurements were used to represent the sample concentrations shown in Figure 3 and Figures S3 and S11, Supporting Information. Following the center chamber retained substances assay, the bioassays of the anode-isolated substances and cathode-isolated substances were conducted in a similar manner. The stock solutions of anode-isolated and cathode-isolated substances (mentioned above) were considerably more dilute than the original pinewood BWES solution, and therefore the volumes of tested substances solutions and cultured medium were adjusted. Adjusted aliquots (volumes ranging 0−666 μL) of anode-isolated and cathode-isolated substances solutions and the corresponding Milli-Q water (ranging from 666 to 0 μL to balance the total volume) were added to 1.834 mL of culture medium, resulting in a total volume of 2.5 mL. As for the center chamber retained substances, DOC measurements were used to represent sample concentrations in Figure 3 and Figures S3 and S11, Supporting Information. Figure S3e and S3f, Supporting Information, shows the diagrams of the algal culture plates used in the bioassays in more details.

Table 1. Dissolved Organic Carbon (DOC) in Biochar Water Extracts

biochar water extract	total solute (g/50 g of biochar)	dissolved organic carbon (g/50 g of biochar)	% DOC in total solute	% non-DOC in total solute
pinewood derived (pH 3.94)	1.693 ± 0.001	0.953 ± 0.003	56.2%	43.8%
peanut shell derived (pH 8.87)	0.247 ± 0.001	0.0264 ± 0.0001	10.7%	89.3%
chicken litter derived (pH 9.09)	5.334 ± 0.001	0.0539 ± 0.0001	1.01%	98.99%

Table 2. Electrodialysis Separation of Pinewood Biochar Water-Extracted Substances (BWES) Containing about 23.8 mg of Dissolved Organic Carbon (DOC)

fractions of pinewood-derived BWES	dissolved organic carbon (mg of carbon)	percentile distribution of DOC after separation
center ch. retained substances	7.61 ± 0.09	31.4%
anode-isolated substances	15.4 ± 0.1	63.4%
cathode-isolated substances	1.25 ± 0.01	5.2%

RESULTS AND DISCUSSION

Biochar Water-Extracted Substance Bioassay. The bioassay results showed that the water-soluble substances extracted from pinewood-derived biochar can inhibit both cyanobacteria (*Synechococcus elongatus*) and eukaryotic alga (*Desmodesmus*) culture growth, while those extracted from peanut shell-derived biochar and chicken litter-derived biochar appear to contain no inhibitory factor. Figure 1a presents the observed growth curve of the *Synechococcus elongates* culture in BG-11 liquid medium with addition (1.13 g/L) of water-extractable substances from biochars derived from three different biomasses: pinewood, peanut shell, and chicken litter. As shown in Figure 1a, the cyanobacterial culture density for the treatment with pinewood BWES (1.13 g/L) as measured by absorbance at 680 nm (chlorophyll absorbance peak) remained

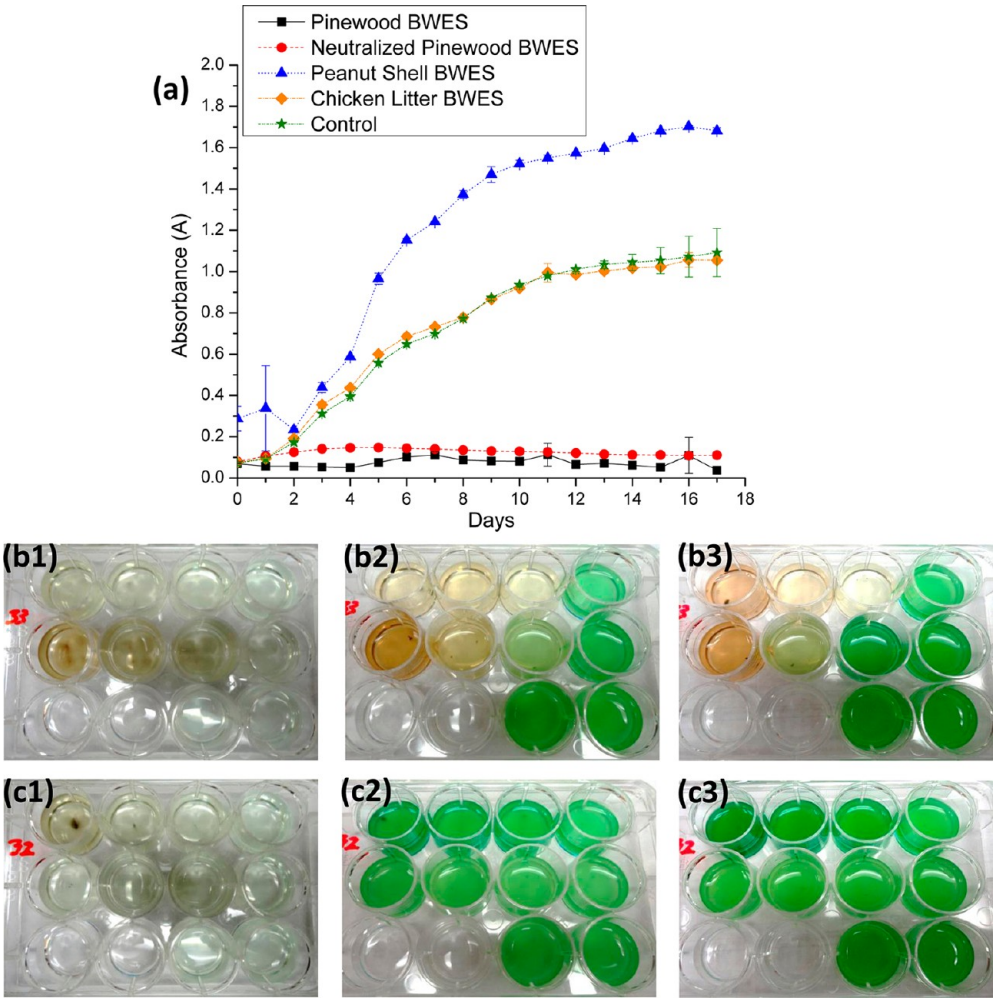

Figure 1. Bioassays of biochar water-extracted substances (BWES) with blue−green alga, *Synechococcus*, in BG-11 culture medium. Graph (a) plots the algal growth (absorbance measurements at 680 nm) curves with the presence of 1.13 g of BWES/L from pinewood (black, square), neutralized pinewood (red, dot), peanut shell- (blue, triangle) and chicken litter- (orange, diamond) derived biochar, in comparison with the 0.0 g of BWES/L control (green, star); photographs on the left (b1 and c1) show the *Synechococcus* assays at day 0 (beginning of assay) whereas the photographs in the middle (b2 and c2) and the right (b3 and c3) show the assay plates at days 5 and 9, respectively. Plate b: from column 1 to 4 row (i) pinewood-derived BWES 1.13, 0.564, 0.282, and 0.0564 g/L; row (ii) neutralized pinewood BWES 1.13, 0.564, 0.282, and 0.0564 g/L; row (iii) 2 blanks (BG-11 only) and 2 controls (alga in BG-11 without BWES). Plate c: from column 1 to 4 row (i) peanut shell-derived BWES 1.13, 0.564, 0.282, and 0.0564 g/L; row (ii) chicken litter-derived BWES 1.13, 0.564, 0.282, and 0.0564 g/L; row (iii) 2 blanks (BG-11 only) and 2 controls (alga in BG-11 without BWES).

unchanged (flat) during the entire assay period (17 days), which indicated no growth (completely inhibited), whereas the treatment with peanut-shell BWES (1.13 g/L) showed sigmoidal growth better than the control cyanobacterial culture (with no biochar substance). The treatment with chicken-litter BWES (1.13 g/L) displayed a sigmoidal growth curve almost identical to the control cyanobacterial culture. These experimental results indicated that the water-extracted substances from the pinewood-derived biochar may possess certain chemical species toxic, or inhibitory, to cyanobacterial cells, whereas both peanut-shell BWES and chicken-litter BWES are essentially benign. In fact, the addition of peanut-shell BWES (1.13 g/L) resulted in a somewhat stimulated cyanobacterial growth curve (better than the control) and could possibly be attributed to an increase in nutrient content from the peanut-shell BWES for cyanobacterial cells. These observations were further documented through the photographs of the bioassay plates in Figure 1b and 1c as well.

The bioassay of the biochar water-extractable substances was completed also with eukaryotic alga, *Desmodesmus* strain 4N2 isolated locally in Virginia, which demonstrated similar results (Figure S6, Supporting Information) consistent with those observed in cyanobacteria *Synechococcus* culture growth (Figure 1a). Figure S6 shows that, upon addition of pinewood BWES, complete inhibition of *Desmodesmus* culture growth was observed, while peanut-shell and chicken-litter BWES showed no observable inhibitory effects. The observation was documented through photographs of the bioassay culture plates in Figures S6b as well. This is a significant result because it indicated that not only prokaryotes (cyanobacteria *Synechococcus*) but also eukaryotic algae (*Desmodesmus*) seem to be sensitive to certain biochar substances such as the pinewood-derived biochar water-extracted substance. Both cyanobacteria and eukaryotic algae are significant members of the aquatic environment. If biochar material is to be used globally as a soil amendment and carbon-sequestration agent at GtC scales, significant amounts of biochar water-extractable

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substances may leach, or run off, into the surrounding aquatic ecosystems. Therefore, although we believe application of biochar material as a soil amendment and carbon sequestration agent is likely to be the most effective strategy in helping control global climate change, it is also important to consider addressing the possible side-effects including certain undesirable component(s) associated with some of the biochar materials, such as the inhibitory factor to aquatic photosynthetic microorganisms shown in this study.

Biochar Water-Extracted Substance pH and Dose Effects. This work also demonstrated that different biochar materials may contain quite different amounts of water-extractable substances. For example, we noticed that chicken litter-derived biochar contains much more water-extractable substance (5.334 g/50 g of biochar) than the pinewood-derived biochar (1.693 g of WES/50 g of biochar) and peanut shell-derived biochar (0.247 g of WES/50 g of biochar). Furthermore, the characteristics of the water-extractable substances such as pH value can also be quite different. After the BWES were extracted, freeze-dried, and redissolved, it was learned (Table 1) that pinewood-derived BWES stock solution had an acidic pH of 3.94, which was much lower than both the chicken litter-derived biochar water extract (pH 9.09) and peanut shell-derived biochar water extract (pH 8.87). Because it is known that cyanobacteria do not grow at a pH lower than 4 or 5,[23] an immediate question was naturally raised: was the observed inhibitory effect on algal growth (Figures 1 and S6 (Supporting Information)) due to the acidic pH (3.94) of the pinewood-derived biochar water extract? To answer this question, the pH value of the pinewood-derived biochar water extract was neutralized to 7.83. The pH-neutralized pinewood-derived biochar water extract was then used in repeating the bioassay with cyanobacteria *Synechococcus* and eukaryotic alga *Desmodesmus*. The subsequent assay results (Figure S9a) demonstrated that pH-neutralized pinewood-derived biochar water extract displayed inhibitory effect on algal growth similar to the original pinewood-derived biochar water extract (Figures 1 and S6). Therefore, the pH value of the pinewood-derived biochar water extract was not the contributing factor to the observed algal growth inhibition. Not only did we consider the water-extract pH but we also checked that the pH of the BG-11 culture media (2.5 mL) after adding the small aliquot (100 μL) of the original pinewood-derived biochar water extract (pH 3.94) was still about 7.5 because of the culture medium's buffering capacity. The liquid culture medium pH (7.5) is well above the critical pH of 4 or 5 for algal growth.

Figure 2 shows the pinewood BWES' inhibition on cyanobacteria *Synechococcus* growth as a function of the BWES concentration. Data shows that, within the first 3–4 days after introducing pinewood-derived BWES into the cyanobacteria culture medium, its inhibition on growth can be observed at a dose as low as 0.0564 g of pinewood BWES/L. After 4 days, the cyanobacteria growth at the lowest dose (0.0564 g of pinewood BWES/L) could fully recover and, in most cases, grew slightly better than the control. With the dose of 0.282 g of pinewood BWES/L, the cyanobacteria culture initially showed inhibition but started to grow after about 10 days. Only at doses 0.564 and 1.13 g of pinewood BWES/L did cyanobacteria culture growth exhibit complete inhibition during the entire assay period (17 days). The pinewood BWES dose-dependent inhibition on cyanobacteria growth can also be seen in Figure S7, Supporting Information, which shows the absorbance spectra (from 530 to 750 nm) of the *Synechococcus*

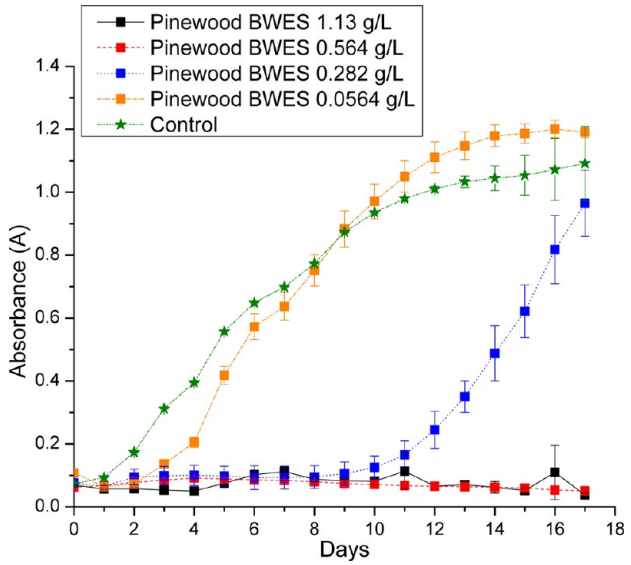

Figure 2. Blue–green alga *Synechococcus* growth (measured as the absorbance at 680 nm) with pinewood-derived biochar water-extracted substances (BWES) with the following dose: 1.13g/L (black), 0.564 g/L (red), 0.282 g/L (blue), and 0.0564 g/L (orange), in comparison with 0.0 g/L control (green, star).

culture at days 1, 8, and 14. The pinewood BWES dose-dependent response was observed also in the assay with eukaryotic alga, *Desmodesmus*, shown in Figure S8 (Supporting Information). Furthermore, the bioassay with the neutralized pinewood BWES samples (Figure S9a, Supporting Information) also showed similar dose-dependent inhibition effect on cyanobacteria *Synechococcus* growth in which full inhibition of algal cell growth was observed at the dose of 1.13 g of neutralized pinewood BWES/L. These results indicated that the pinewood BWES toxin(s) may have a limited active lifetime or the cyanobacteria could somehow slowly overcome its toxic effect when its dose is relatively low.

As shown in Figure S9b and S9c (Supporting Information), various doses in a range from 0.0564 to 1.13 g BWES of peanut shell-derived and chicken litter-derived biochars were tested on cyanobacteria *Synechococcus* growth. The data showed that neither peanut shell-derived BWES nor chicken litter-derived BWES had any inhibitory effect on algal culture growth at any of the doses tested.

Note that the concentration of DOC (0.635 g of DOC/L) in the treatment with the 1.13 g/L of pinewood BWES was significantly higher than that (0.121 g of DOC/L) of the 1.13 g/L peanut shell BWES treatment. To answer the question of whether or not the concentration of DOC in the treatment is the factor that inhibits algal growth, we performed an additional bioassay using the same amounts of DOC from the pinewood-derived biochar as that from the peanut shell-derived biochar. Figure S10a (Supporting Information) shows the effects of pinewood BWES and peanut-shell BWES at equivalent DOC concentrations (0.158 g of DOC/L) on the growth of cyanobacteria *Synechococcus* over a 10-day growth period. Pinewood BWES (at a concentration with 0.158 g of DOC/L) showed inhibition through day 4, similar to the results observed in both Figure 2 and Figure S8 (Supporting Information), whereas peanut-shell BWES (0.158 of g of DOC/L) did not show any sign of growth inhibition when compared to the control (0.0 g of DOC/L). These experimental results

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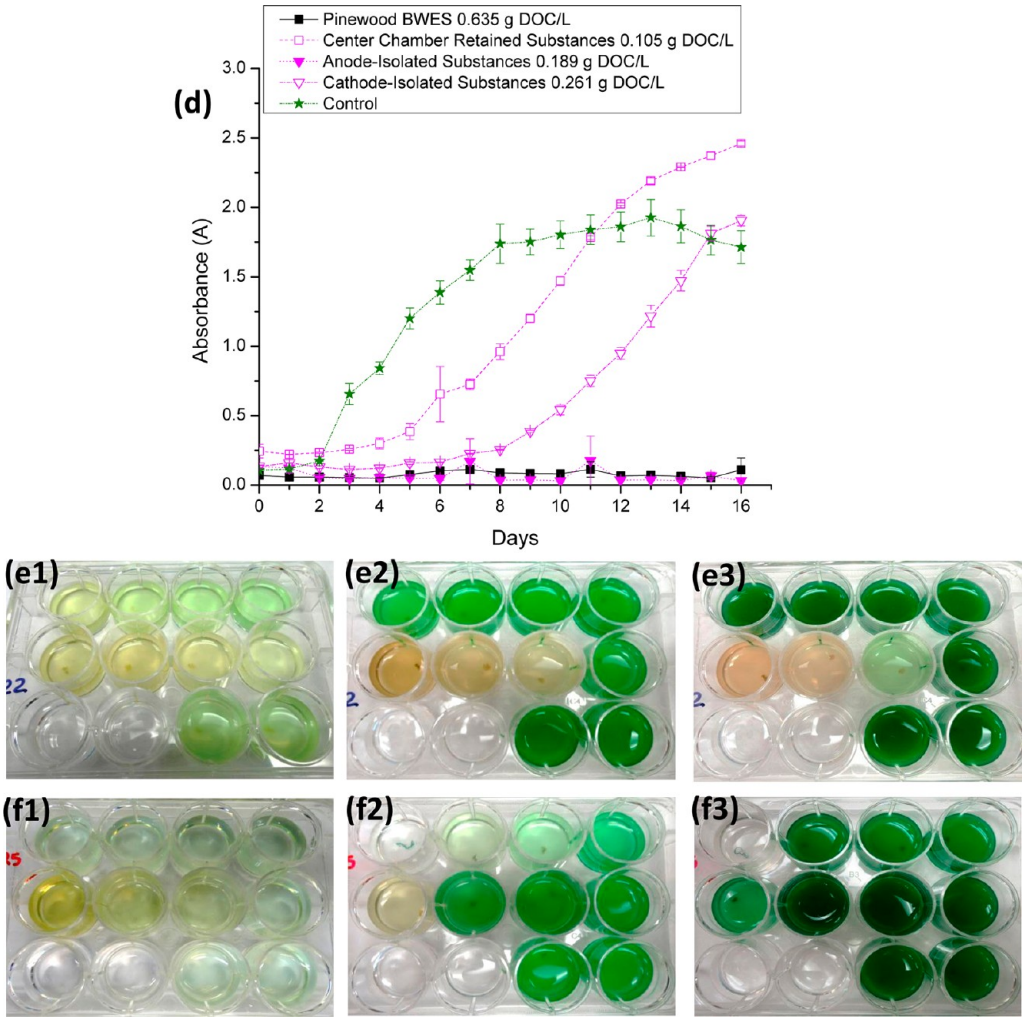

Figure 3. Assays of electrodialysis-separated biochar substances with *Synechococcus* in BG-11 culture medium. Graph (d) plots the algal growth (measured as absorbance at 680 nm) curves with pinewood BWES (635 mg of DOC/L, black square), center chamber retained substances (105 mg of DOC/L, pink hollow square), anode-isolated substances (189 mg of DOC/L, pink inverted triangle), cathode-isolated substances (261 mg of DOC/L, pink inverted hollow triangle), and control (0.0 g of DOC/L, green star). Plate photographs on the left (e1 and f1) show the *Synechococcus* assays at day 2 (e1) and day 0 (f1) (beginning of assay) whereas the photographs in the middle (e2, f2) and the right (e3, f3) show the assay plates at day 7 (e2), day 4 (f2), day 12 (e3), and day 11 (f3), respectively. Plate e: from column 1 to 4 in row (i) center chamber retained pinewood BWES substances with dose DOC mg/L: 105, 52.3, 26.2, 5.23; row (ii) pinewood BWES with dose DOC mg/L 635, 317, 159, 31.7; row (iii) 2 blanks (BG-11 only) and 2 controls (alga in BG-11 without BWES). Plate f: columns 1−4 in row (i) anode-isolated substances with DOC mg/L:189, 94.8, 47.4, 9.48; row (ii) cathode-isolated substances with DOC mg/L: 261, 131, 65.3, 13.1; row (iii) 2 blanks (BG-11 only) and 2 controls (alga in BG-11 without BWES).

indicated that the amount of DOC per se does not necessarily correlate with the observed inhibitory effect; it is the specific chemical species within the type of DOC from the pinewood-derived biochar that apparently causes the inhibition effect.

In the bioassay (shown in Figure S10a), we also noticed that the cyanobacteria *Synechococcus* culture seem to overcome the inhibitory effect by the treatment with pinewood BWES at the concentration of 0.158 g of DOC/L (equivalent to about 0.282 g of BWES/L) in a few (3−5) days in a manner somewhat similar to that observed in the experiment of Figure 2. To answer the question of whether this phenomenon is because the cyanobacteria *Synechococcus* culture somehow developed tolerance to the pinewood BWES or because the culture could somehow detoxify the biochar toxin, we took a portion of the cyanobacteria *Synechococcus* culture that had been exposed to the pinewood BWES treatment at the concentration of 0.158 g of DOC/L (equivalent to about 0.282 g of BWES/L) of Figure S10a at day 10 and used it as an inoculating culture for another subsequent bioassay (Figure S10b). If the growth of the pinewood BWES-treated *Synechococcus* culture after day 4 (in Figure S10a) was somehow due to development of tolerance to the pinewood BWES, reintroduction of the same pinewood BWES treatment at the same dose (0.158 g of DOC/L) would show no inhibition. The assay result (Figure S10b) demonstrated that the reintroduction of the pinewood BWES treatment resulted in nearly identical inhibition effect on the growth of *Synechococcus* that had been pre-exposed to the pinewood BWES treatment at the concentration of 0.158 g of DOC/L as observed in Figure S10a. This is also a significant experimental observation, because it indicated that the algal cells could somehow overcome the pinewood BWES inhibition effect, probably through some type of detoxification process when its dose is relatively low (0.158 g of DOC/L).

Electrodialysis-Separated Biochar Substances and Their Bioassay. A portion (e.g., 1.5 mL) of the original BWES solutions such as the pinewood-derived BWES stock solution (28.2 g/L) was treated with the electrodialysis process, resulting in three separate fractions: (i) anode-isolated substances, (ii) cathode-isolated substances, and (iii) center chamber retained substances. After the anode-isolated substances and the cathode-isolated substances were collected and freeze-dried separately, their dissolved organic carbon (DOC) contents were determined along with those for the original BWES stock solutions and the center chamber retained BWES substances. The results of the DOC analysis are listed in Tables 1 and 2.

As shown in Table 1, the DOC contents in different biochar water extracts are very different. About 56.2% of the pinewood-derived BWES mass (1.693 g/50 g of biochar) is its DOC content (0.953 g/50 g of biochar), whereas the contents of chicken litter-derived BWES DOC (0.0539 g/50 g of biochar) and peanut shell-derived BWES DOC (0.0264 g/50 g of biochar) represent only 1.01% and 10.1% of their BWES masses (5.334 g/50 g of biochar and 0.247 g/50 g of biochar), respectively. In other words, the pinewood BWES is highly rich in organic compounds content, whereas chicken litter-derived BWES and peanut shell-derived BWES contain very little organic compounds. The majority of the contents in chicken litter-derived BWES and peanut shell-derived BWES are non-DOC materials, such as inorganic salts.

Table 2 presents a typical distribution of the DOC content when 1.5 mL of pinewood-derived BWES stock solution (1.5 mL × 15.869 mg of DOC/mL) was separated with the mini-ED system resulting in the three fractions: (i) anode-isolated substances (63.4%: 15.4 mg of DOC), (ii) cathode-isolated substances (5.2%: 1.25 mg of DOC), and (iii) center chamber retained substances (31.4%: 7.61 mg of DOC).

Note that, during the electrodialysis separation process, the cations (of a size less than 500 Da) of the BWES in the charge-neutral 1.5 mL sample chamber would pass through the cellulose−acetate membrane into the cathode chamber under the influence of the electrophoretic field created by the cathode (refer to Figure S5, Supporting Information). Meanwhile, the anions (of a size less than 500 Da) of the BWES in the charge-neutral 1.5 mL sample chamber would pass through the cellulose−acetate membrane into the anode chamber under the influence of a positive electric field exerted by the anode (refer to Figure S5). When the electrodialysis-separation process is completed, what remains in the charge-neutral 1.5 mL sample chamber should mostly be charge-neutral chemical species and/or compounds greater than 500 Da. Therefore, the data presented in Table 2 indicate that about 63.4% DOC of the pinewood-derived BWES separated into the anode chamber and are most likely from organic compounds that possess at least one negatively charged functional group, such as a deprotonated carboxyl group (a deprotonated carboxyl group is the most likely negatively charged organic functional group when water is used as the solvent). About 5.2% DOC of the pinewood-derived BWES separated into the cathode chamber and can most likely be attributed to chelated organic−metal cation complexes that carry net positive charge. The remaining 31.4% DOC of the pinewood-derived BWES represents organic chemical species with neutral charge and/or molecular weight of greater than 500 Da.

Figure 3 presents the bioassay results of the electrodialysis-separated fractions in comparison with those of the original pinewood-derived BWES. As shown in Figure 3, both the center chamber retained and cathode-isolated pinewood-derived BWES displayed only relatively little inhibition on cyanobacteria growth whereas the anode-isolated substances showed complete inhibition on cyanobacteria growth during the entire assay period (18 days). Figure S11a (Supporting Information) shows that the anode-isolated substances not only displayed complete algal growth inhibition at the concentration of 189.7 mg of DOC/L but also manifested a similar dose-dependent inhibition at DOC of 94.8, 47.4, and 9.48 mg/L, which was comparable to the observation in the bioassay with the original pinewood-derived BWES. Meanwhile, the cathode-isolated substances (at DOC of 261, 131, 65.3, and 13.1 mg/L) and center chamber retained substances (at DOC of 105, 52.3, 26.2, and 5.23 mg/L) showed relatively little or no inhibition (Figure S11b and S11c, Supporting Information) and, in most cases, displayed slight stimulation of growth when compared to the control. These bioassay results indicated that the majority of the pinewood-derived BWES inhibitory chemical species was in the anode-isolated substances, while relatively small amounts of them are in the cathode-isolated substances and center chamber retained substances. These observations were documented through photographs of the bioassay plates shown in Figure 3 as well.

Furthermore, the bioassay results also indicated that the inhibitory effect is unlikely due to the total amount of DOC per se, but likely owing to certain specific chemical species within the DOC content. For example, the bioassay treatment of cathode-isolated substances with larger amounts of DOC (261 mg/L) displayed a relatively small inhibition effect whereas the bioassay of anode-isolated substances with smaller amounts (roughly 30% less) of DOC (189.7 mg/L) severely inhibited algal growth.

These experimental observations support our hypothesis that the inhibitory component of the pinewood-derived BWES is likely to be some type of 500-dalton (or smaller) phenolic organic chemical species that contains at least one carboxyl group. Its carboxyl group may make the inhibitory organic species negatively charged in aqueous environment. This predicted feature with a carboxyl group could explain a number of the observations: (i) why the original pinewood-derived BWES was acidic (pH 3.94): carboxyl group represents an organic acid; (ii) how the majority of the pinewood-derived BWES inhibitory chemical species can pass through the 500-dalton-cutoff cellulose−acetate membrane of the charge-neutral 1.5 mL sample chamber and predominantly enter the anode chamber under the influence of the positive electrophoretic field produced by the anode: because carboxyl groups can predominantly become negatively charged by deprotonating in water; (iii) why some of the pinewood-derived BWES inhibitory chemical species could also enter the cathode chamber under the influence of the negative electric field from the cathode: negatively charged carboxyl group can chelate with certain higher valent cations, such as Fe^{2+} or Fe^{3+}, forming a chelated organic acid−metal cation complex such as $[R{-}COO\ Fe]^{+}$ that carries a net positive charge enabling it to transport through the cellulose−acetate membrane into the cathode chamber; and (iv) small amount of the pinewood-derived BWES inhibitory chemical species may still remain in the charge-neutral center sample chamber: because a small fraction of the carboxyl groups may remain charge-neutral (protonated) or the electrodialysis separation was not fully complete.

Overall, the biochar assays showed that some of the biochar substances, such as the pinewood-derived BWES, exhibited inhibitory effects on both prokaryotic (*Synechococcus*) and eukaryotic (*Desmodesmus*) photosynthetic microorganisms that are significant members of the aquatic ecological community, whereas other biochar materials, such as chicken litter-derived and peanut shell-derived BWES, seem benign to these aquatic organisms. The pinewood-derived BWES inhibitory factor that was demonstrated in this work is likely due to some type of 500-dalton (or smaller) organic chemical species that contains at least one carboxyl group. The biochar materials used in this study were made from three different types of biomass materials with somewhat different pyrolysis conditions at different locations in the United States. Therefore, the origin or formation of the inhibitory organic chemical species could be related to both the sources of the different biomass materials and/or their processing (including pyrolysis) conditions. Although the precise chemistry of the inhibiting compound(s) extracted from pinewood-derived BWES has not yet been fully determined, it is apparent from the research presented in this paper that biochar materials must be closely examined before implementation for large-scale environmental applications. Further work in this line of research will be directed to both isolate and investigate the source of the inhibiting compound(s), hopefully leading to a new generation of biochar materials completely free of any undesirable chemical species to accomplish the envisioned mission of biochar soil amendment and carbon sequestration at GtC scale to mitigate global climate change.

■ ASSOCIATED CONTENT

Supporting Information

Supporting Information includes the expanded materials and methods section, as well as the additional figures and experimental data referenced in this report. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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