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***Sargassum* sp. act as a Large Regional Source of Marine Dissolved Organic Carbon and Polyphenols**

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Key Points:

- When exposed to natural sunlight, pelagic *Sargassum* species released large amounts of dissolved organic carbon (DOC).
- *Sargassum* DOC has a high phenolic content (~5-18% of solid phase extracted DOC).
- While phlorotannin stability should be evaluated, results highlight that *Sargassum* could be a substantive marine source of polyphenols.

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Abstract

Dissolved organic carbon (DOC) plays critical roles in marine carbon cycling, but its sources and sinks remain uncertain. In this study, we monitored DOC exudation rates of *Sargassum natans* under visible light ($\lambda > 390$ nm) and solar radiation. DOC release rates ranged from 7 to 10 $\mu\text{g C g}^{-1}_{\text{biomass}} \text{h}^{-1}$ (wet weight) under visible light, but increased to 23 to 41 $\mu\text{g C g}^{-1}_{\text{biomass}} \text{h}^{-1}$ when exposed to natural sunlight. Results indicate that DOC released by *Sargassum* could amount to 0.3 to 1.2 Tg C yr^{-1} , potentially contributing significantly to the marine DOC pool in the Gulf of Mexico and Western North Atlantic. We employed the Folin-Ciocalteu phenolic content method, nuclear magnetic resonance (NMR) spectroscopy, and ultrahigh resolution Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS) to characterize the diverse pool of organic compounds exuded from *Sargassum*. Results from these complementary methods showed that *Sargassum* release large quantities of phlorotannins, a class of polyphenols that have very similar properties to terrestrial DOC. These phlorotannins and their oxygenated phenolic derivatives exhibit a high hydrogen deficiency and functionalization (i.e. 4 to 6 oxygen atoms per aromatic ring), representing 5 to 18% of the released DOC isolated by solid phase extraction. Thus, *Sargassum* is the largest biological source of open ocean polyphenols recorded to date. The amount of polyphenolic DOC released by *Sargassum* challenges previous beliefs that all polyphenols found within the oceans are remnants of terrestrial organic matter, although the stability of phlorotannins and their derivatives needs to be further evaluated.

Plain Language Summary

Macro brown algae contribute dissolved organic carbon (DOC) to the coastal and open ocean, potentially influencing carbon cycles and redox chemistry. They also contain a very specific class of polyphenols known as phlorotannins; however, brown algae have not been considered a sizable source of marine polyphenols or DOC. Here we present evidence that pelagic *Sargassum* species release DOC with a high phenolic content (~5-18% of solid phase extracted DOC). Based on estimated literature values of annual *Sargassum* biomass and without accounting for possible sinks, *Sargassum* could potentially contribute approximately 0.3 to 1.2 Tg C yr^{-1} as DOC and 30 to 200 Gg C yr^{-1} as polyphenols to the Gulf of Mexico and western North Atlantic.

1 Introduction

Marine dissolved organic carbon (DOC) is one of Earth's major dynamic carbon reservoirs, yet its sources and reactivity remain poorly quantified. A recent synopsis evaluated the role of macro algae in global carbon sequestration, and estimated that 0.36 Pg C yr^{-1} is exported from macro algal beds as DOC, on the same scale as annual riverine DOC inputs into the ocean (Krause-Jensen & Duarte, 2016). While such estimates may have large error margins they nevertheless suggest a substantial contribution. Rivers constitute the largest allochthonous source of DOC to the ocean, delivering an estimated ~0.2 to 0.4 Pg C yr^{-1} (Raymond & Spencer, 2015). Terrestrial DOC exported by rivers contains a relatively large component that originates primarily from the degradation products of lignin

polyphenols (Boyle et al., 2009; Hernes, 2003; Hernes et al., 2008; Spencer et al., 2008, 2009; Stenson et al., 2003; Stubbins et al., 2010) and tannins (Maie et al., 2008; Sleighter & Hatcher, 2008). It has been suggested that terrestrial DOC is largely remineralized in coastal zones and this assumption is based on stable isotope compositions that suggest that marine DOC contains very little terrestrial character (Bauer, 2002; Beaupré, 2015) and because lignin biomarkers have very low abundance in the open ocean (Opsahl & Benner, 1997). However, the ultimate fate of terrestrially-derived DOC in the ocean is still under debate. Nevertheless, various fractions of terrigenous DOC are susceptible to rapid biodegradation, such as amino acids, and photodegradation, such as lignin phenols (Benner & Kaiser, 2011; Hernes, 2003; Spencer et al., 2008). Recent studies have challenged earlier carbon isotope work by analyzing fractions of marine DOC (Follett et al., 2014; Zigah et al., 2017), by characterizing molecular structures within DOC with NMR spectroscopy (Cao et al., 2018), or by detailed analysis of optical properties (Andrew et al., 2013; Cartisano et al., 2018), thereby painting a much more complex picture of sources that are likely constituents of the marine DOC pool.

Macro brown algae or Phaeophyceae, contain a class of marine polyphenols known as phlorotannins, which are exclusively formed by the polymerization of phloroglucinol (1,3,5-trihydroxybenzene) (Swanson & Druehl, 2002). Moreover, extracts of brown and green algae analyzed by Curie-point pyrolysis-gas chromatography contained alkylphenols, likely from alkylated phlorotannin-like compounds (Van Heemst et al., 1996). These alkylphenol pyrolysis products appear to result from refractory components of marine DOC, as the relative abundance of these compounds increased with depth in the water column (Van Heemst et al., 1996). As yet, the importance of macro brown algae to supply DOC and complex polyphenolic compounds to the oceans is unknown. Macro brown algae are distributed widely and are predominantly found in temperate and colder waters. An exception is the genus *Sargassum*, with approximately 250 species that are distributed throughout the temperate and tropical oceans. While benthic species can become pelagic after being removed from the seafloor, only two species, *Sargassum natans* and *Sargassum fluitans*, have been found to be truly holopelagic (referred to as *Sargassum* hereafter). These species live in the epipelagic zone of the Gulf of Mexico, Western Atlantic, and Sargasso Sea, where they can form extensive mats that are large enough to be observed by satellites and have a biomass that may exceed 2 Tg yr⁻¹ (Gower & King, 2011). While other biomass estimates vary between 4 and 11 Tg annually (Butler & Stoner, 1984; Parr, 1939), satellite data have

revealed an increasing trend in *Sargassum* abundance between 2000 and 2015 (Wang & Hu, 2016), and therefore annual *Sargassum* biomass may very well be even higher than previous estimates have indicated. A recent study estimated the biomass of *Sargassum* that has sedimented to the seafloor to be comparable to the biomass observed at the surface (Baker et al., 2017), which would require a quantitative annual removal of *Sargassum* and transport into the abyssal ocean, which is also supported by recent modeling work (Brooks et al., 2018).

Previous work has estimated that *Sargassum* fix 181 to 1,234 (328 on average) $\mu\text{g C g}^{-1}_{\text{biomass}} \text{h}^{-1}$ (dry weight), with 0 to 76% (25% on average) released as DOC (Hanson, 1977). DOC release is highly dependent on the brown alga species, with reported values ranging from under 1% to as much as 40% of net assimilation (Brylinsky, 1977; Hanson, 1977). Brylinsky (1977) measured exudation rates of DOC from holopelagic *Sargassum* ranging from 3 to 6 $\mu\text{g C g}^{-1}_{\text{biomass}} \text{h}^{-1}$ (dry weight), but these incubations were performed in glass containers and therefore may not reflect rates of DOC release under natural sunlight (i.e. including ultraviolet (UV) radiation). DOC exuded from two kelp species, *Macrocystis pyrifera* found in California coastal waters and *Laminaria hyperborean* found in Norwegian coastal waters, amounted to 14 to 26% of fixed carbon released as DOC, respectively, but was variable on monthly time scales (Abdullah & Fredriksen, 2004; Reed et al., 2015). Abdullah & Fredriksen (2004) also quantified carbohydrate and phenol concentrations in kelp exudates and while most of the DOC released was saccharides, the contribution of polyphenols varied between 6 and 19% of the DOC, determined using the Folin-Ciocalteu reagent and gallic acid standards. Although the proportion of phenolic compounds in DOC released by *Sargassum* is not currently known, these prior results suggest that polyphenolic compounds could be a major constituent (Abdullah & Fredriksen, 2004).

In this study, we monitored the increases in DOC concentration during controlled exudation experiments under natural sunlight (Bermuda, late September) and under artificial (visible light) irradiation in the laboratory. The Folin-Ciocalteu colorimetric test was employed to estimate phenolic content in the released DOC following isolation from seawater using solid phase extraction (SPE). Nuclear magnetic resonance (NMR) along with ultrahigh resolution Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS) were further employed to characterize the compounds exuded from *Sargassum*. These techniques

were used to evaluate the role of *Sargassum* as a marine source of polyphenols and other classes of organic compounds in the Gulf of Mexico and Northern Atlantic Ocean.

2 Materials and Methods

2.1 Sampling

Sargassum samples were collected along the ship side using a hand-held net aboard the R/V *Sharp* in the Sargasso Sea during July 2016 for controlled laboratory exudation experiments. *Sargassum* was housed onboard in a tank (<3 days) with continuously flowing seawater and transported back to the laboratory immediately upon return to another tank with filtered ambient bay water (adjusted to salinity = 35) that was circulated through a UV treatment system (Neotech Aqua Solutions, Inc.) to keep background DOC levels low. *Sargassum* samples were also collected aboard the R/V *Henry Stommel* 9 km off the coast of Bermuda in late September 2016 and were transferred to outdoor tanks housed at the Bermuda Institute of Ocean Sciences, with continuously flowing seawater within 2 h of collection to mimic “natural” conditions.

2.2 Exudation experiments

Three types of exudation experiments were conducted during this study. One set of experiments was conducted in the laboratory (listed as “indoors” in Table 1). For these experiments, *Sargassum* subsamples (approximately 100 g wet weight) were rinsed with 24 h UV-treated artificial seawater (Instant Ocean) and transferred to small tanks containing 7 L 24 h UV-treated artificial seawater. Each tank was equipped with a Radion LED lamp (Eco Tech Marine, spectral output in Supporting Information Figure S1). To mimic summertime conditions, lamps were set for a 14 h day/10 h night cycle and tanks were maintained at 29 °C with Eheim Jager TruTemp Quantum heaters. One tank containing no *Sargassum* served as a blank and was sampled for DOC concentrations as described in Section 2.3. To minimize any stress from transfer, the water was drained and replaced before monitoring dissolved organic carbon (DOC) exudation from the *Sargassum* samples.

Another set of exudation experiments was conducted in outdoor tanks at the Bermuda Institute of Ocean Sciences (listed as “outdoors” in Table 2). For outdoor incubations, *Sargassum* was placed in small tanks containing open ocean seawater within a large tank of continuously flowing seawater. Temperature (°C) and solar intensity (Lux) in the tanks were periodically monitored with HOBO® pendant temperature/light data loggers. For these experiments, temperatures ranged between 26 and 27.5 °C. To better understand the impacts of UV radiation on DOC exudation rates, tanks were either left uncovered and exposed to full

solar irradiation or tanks were covered with a Plexiglas cover that had irradiation cut off at 345 nm and UVA (320 to 400 nm) transmission reduced to 65%. Incubation volumes and *Sargassum* biomass for these experiments are listed in Table 1.

The third set of experiments was performed to investigate the impacts of stress and senescent conditions on the release of DOC by *Sargassum* (listed as “stress conditions” in Table 1). Although some of the incubation temperatures are unrealistic for the open ocean (e.g. temperatures reached up to 49 °C), the goal of these experiments was to understand the maximum potential for *Sargassum* DOC exudation, especially in light of the annual *Sargassum* inundation events that occur in coastal environments. It has been noted that 24 to 30 °C is the optimal temperature range for pelagic *Sargassum* sp. (Hanisak & Samuel, 1987), so one tank was kept indoors and left at 20 °C. Four experiments were conducted outdoors. One tank contained mid-senescent *Sargassum* (based on visual inspection) and was kept under low light (no direct sunlight) where the temperature did not change from 25 °C over the course of 12 h. The other tanks contained healthy *Sargassum* but were left outdoors for 12 h in direct sunlight with no temperature control. In these tanks the temperature ranged from 25 °C at the start of the experiment up to 49 °C after 12 h (Table 1).

2.3 Dissolved organic carbon and solid phase extraction

For all exudation experiments, subsamples of tank water at various time points were 0.2 µm filtered (Whatman 25 mm GD/X syringe filters) into clean combusted (500 °C) 40 mL amber glass vials. Samples were acidified to pH 2 using concentrated HCl (Sigma Aldrich 32 %, pura) and analyzed for DOC concentrations using a Shimadzu TOC-V. Samples were either analyzed immediately or stored at 4 °C until analysis (typically within 1 to 10 d of collection). DOC concentrations at these time points were used to determine DOC release rates over the day or entire exudation experiment (Table 1). At the end of all incubation experiments (Table 1), typically lasting 24 to 48 h, tank water housing *Sargassum* was filtered through pre-combusted Whatman 0.7 µm GF/F glass fiber filters, acidified to pH 2 using concentrated HCl and solid-phase extracted using Agilent Bond Elut PPL (5g) cartridges. This solid-phase extraction (SPE) technique was previously described in detail elsewhere (Dittmar et al., 2008). For our first extractions (i.e. indoor experiments), ~6 L of filtered tank water was passed through the cartridge ensuring that no more than 50 mg of DOC was loaded per 5 g cartridge. After extraction, cartridges were rinsed with 30 mL Milli-Q water followed by 30 mL 0.1% formic acid water (Optima™ LC/MS grade, Fisher) to remove salts. Cartridges were dried in a hood using a vacuum manifold and were eluted with 30 mL methanol (Optima™ LC/MS grade, Fisher). Extraction efficiencies were determined by

measuring DOC concentrations on SPE samples that were generated by completely drying methanolic extracts under a stream of N₂ and re-dissolving them in water. For these samples, extraction efficiencies were low (~30%), perhaps due to overloading the cartridge. For remaining experiments, 10 g cartridges were custom-packed and only 1 to 2 L of filtered tank water was extracted, improving extraction efficiency to ~40 – 60%, which is typical for marine dissolved organic matter (DOM) (Dittmar et al., 2008). However, extraction efficiency was always low for extractions of DOM from *Sargassum* incubated with no temperature control. The decreased extraction efficiency of the DOM could be due to the loss of highly polar monosaccharides and small organic acids, which are not efficiently retained by this solid-phase extraction method.

2.4 Phenolic content

Phenolic content in SPE-DOM was determined following minor modifications of the Folin-Ciocalteu method for the determination of phenolic content in solid-phase extracted marine DOM (Takeda et al., 2013). Briefly, SPE-DOM samples were created with 0.2 mL of each MeOH extract that dried completely under N₂ and re-dissolved in 20 mL Milli-Q water, sonicated for 5 min, and 0.2 µm filtered (Whatman 25 mm GD/X syringe filters). 3.6 mL of each sample was transferred to a combusted borosilicate vial containing 0.4 mL 1 M NaOH (J.T. Baker) and 0.2 mL Folin-Ciocalteu phenol reagent (Sigma-Aldrich), and reacted for 30 min at room temperature. Subsequently, 4 mL 2 M Na₂CO₃ (Fisher) and 1.8 mL Milli-Q water was added to the reaction mixture, the color was developed for 1 h in an oven at 40°C and the mixture's absorbance was monitored in a 1 cm quartz spectrophotometric cell at 720 nm using a Horiba Aqualog. Phenolic content was determined by a calibration curve of phloroglucinol (Aldrich) solutions ranging from 0 to 50 µM that were reacted in the same way as the samples. Thus, phenolic content reported here is reported as phloroglucinol equivalents. The unreacted SPE-DOM sample was analyzed for DOC concentration, as above. Phenolic content was simply determined by dividing the phenol content (as µM phloroglucinol equivalents) by the SPE-DOC concentration (µM) and reported as % phenolic content ($100 \times (\text{phenol content})_{\mu\text{M}} / (\text{DOC})_{\mu\text{M}}$) or as mol kg⁻¹ C ($(\text{phenol content})_{\mu\text{M}} / (\text{DOC})_{\text{mgC/L}}$). For select samples (SRNOM and *Sargassum* SPE-DOM), phenolic content was estimated by pH titration as in Ritchie & Perdue (2003), using an Orion 8220 BNWP microelectrode, µL additions of 0.1 N HCl and 0.1 N NaOH, and assuming that half the phenolic groups were titrated between a pH of 8 and 10.

2.5 FT-ICR MS analysis

Mass spectrometric analyses were undertaken at the Helmholtz Zentrum, Munich, Germany using a Bruker Solarix 12 Tesla Fourier transform ion cyclotron resonance mass spectrometer (FT-ICR MS) using both negative and positive electrospray ionization at 3600 V. The flow rate was 120 $\mu\text{L/h}$, 500 spectra were averaged and the resolution ($>500,000$ at m/z 400) and the mass error (<0.2 ppm) were sufficiently high to compute exact molecular formulae (Herzprung et al., 2014; Koch et al., 2007). Calibration was performed with arginine clusters and spectra were post-calibrated using known DOM m/z ions across the m/z range of 200-700 (Gonsior et al., 2009; Timko et al., 2015). The following chemical elements were used to compute exact molecular formulae: $^{12}\text{C}_{1-\infty}$, $^1\text{H}_{1-\infty}$, $^{16}\text{O}_{1-\infty}$, $^{14}\text{N}_{0-5}$ and $^{32}\text{S}_{0-2}$, and $^{79/81}\text{Br}_{0-2}$. Detailed descriptions how to accurately assign molecular formulae have been previously described (Herzprung et al., 2014; Koch et al., 2007) and the calibration of mass spectrometric data in this study was also previously explained in detail (Timko et al., 2015). Van Krevelen diagrams are effective in visualizing the chemical space of the assigned molecular formulae (van Krevelen, 1950) according to their hydrogen to carbon (H/C) and oxygen to carbon (O/C) ratios and were used throughout this study. Known phlorotannins (Isaza Martínez & Torres Castañeda, 2013) were plotted in Van Krevelen space to better visualize how these compounds compare to the entire SPE-DOM pool that can be characterized by FT-ICR MS. Likewise, nominal additions indicating oxygenation ($\pm\text{O}$), hydration ($\pm\text{H}_2\text{O}$) and carboxylations ($\pm\text{CO}_2$) to known phlorotannins were also included in Van Krevelen space to help visualize theoretical transformations of exuded phlorotannins.

The Kendrick mass defect (KMD) and the z^* values are independent parameters to unambiguously identify CH_2 -based homologues (Stenson et al., 2003).

$$\text{KM} = \text{Mass}_{\text{measured}} (14.0000/14.01565) \quad (1)$$

$$\text{KMD} = \text{nominal mass (NM)} - \text{KM} \quad (2)$$

$$z^* = \text{modulus}(\text{NM}/14) - 14 \quad (3)$$

In this study we used a modified Kendrick plot, where the ratio of KMD/z^* is plotted against the exact mass, which was previously introduced (Shakeri Yekta et al., 2012). This diagram can be used to visualize series of H_2 - ($\Delta m = 2.1057$ Da) and CH_2 -based ($\Delta m = 14.0156$ Da) homologous series spacing and the spacing created by the nominal exchange of 1 C and 4 H versus oxygen ($\Delta m = 36.39$ Da). Additionally, double bond equivalents (DBE) were computed as follows (Koch & Dittmar, 2006)

$$\text{DBE} = 1 + (2\text{C} - \text{H} + \text{N} - \text{Br})/2 \quad (4)$$

Two additional parameters, the aromaticity index (AI) (Koch & Dittmar, 2016; 2006) and the aromaticity equivalent (X_C) (Yassine et al., 2014), were also computed for all assigned molecular formulas containing carbon, hydrogen, and oxygen as follows

$$\text{AI} = (1 + \text{C} - m\text{O} - \frac{1}{2}\text{H})/(\text{C} - m\text{O}) \quad (5)$$

$$X_C = 1 + (2\text{C} - \text{H} - 2m\text{O})/\text{DBE} - m\text{O} \quad (6)$$

For equation 5 and 6, m is the number of oxygen atoms in double bonds and is therefore 1 for aldehydes and ketones and 0.5 for carboxylic acids and esters (Yassine et al., 2014). When the value of 0.5 is selected for AI this parameter is known as the modified aromaticity index (AI_{mod}) and assumes half of the oxygen atoms are located in double bonds (Koch & Dittmar, 2016; 2006).

2.6 Nuclear Magnetic Resonance Spectroscopy

Bruker Avance III spectrometers housed at the Helmholtz Center, Munich, Germany, and TopSpin 3.2/PL6 software were used to acquire ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra of re-dissolved *Sargassum* SPE-DOM (0.4-1.5 mg mg solid SPE-DOM in typically 50-75 μL CD_3OD (99.95 % ^2H), Aldrich, Steinheim, Germany; the consolidated SPE-DOM sample used for acquisition of ^{13}C and 2D NMR spectra was ~ 4 mg). Briefly, the re-dissolved sample was transferred to 2.0 mm Bruker Match tubes and sealed. A cryogenic inverse geometry 5 mm z-gradient $^1\text{H}/^{13}\text{C}/^{15}\text{N}/^{31}\text{P}$ QCI probe ($B_0 = 18.8$ T) was used for 1D ^1H NMR. 1D ^1H was run on individual samples, but pooling of the three samples (labeled 394-396, Table 1) was necessary to obtain high-quality 2D and ^{13}C NMR spectra (Supporting Information Figure S11). We computed the relative proportions of protonated carbon CH_{123} by comparison of ^{13}C DEPT-45 (show all CH_{123}), ^{13}C DEPT-135 (show CH_{13} with positive and CH_2 with negative amplitude) and ^{13}C DEPT-90 NMR (show CH only) spectra; a uniform coupling constant $^1\text{J}_{\text{CH}}$ of 150 Hz was used for magnetization transfer. We corrected the ^{13}C DEPT-90 NMR spectrum by subtracting a certain amount (commonly ~ 2 -3%) of the ^{13}C DEPT-45 NMR spectrum to attenuate leakage of CH_3 and CH_2 into the ^{13}C ^{13}C DEPT-90 NMR spectrum which arises from the unavoidable variance in $^1\text{J}_{\text{CH}}$ of SPE-DOM. We used (acquisition-time adjusted) linear combinations of the ^{13}C DEPT-45, -135 and -90 NMR spectra to compute the individual traces of CH (^{13}C DEPT NMR spectrum), CH_2 (^{13}C DEPT-45 minus ^{13}C DEPT-135) and CH_3 (^{13}C DEPT-45 plus ^{13}C DEPT-135] minus ^{13}C DEPT-90).

Then we determined the relative contributions of the individual spectra (CH_3 , CH_2 , CH_1) to the sum CH_{123} as observed in ^{13}C DEPT-45 NMR spectra with recognition of the individual transfer amplitudes which were as follows ($\text{CH}_3 = 1.06$; $\text{CH}_2 = 1.0$; $\text{CH} = 0.707$) (Sørensen and Jakobsen in Brey, 1988; Doddrell et al., 1982). We erroneously have not performed this correction in our previous publication Hertkorn et al. (2013); overall, computed fractions of methine carbon (CH) increased by $\sim 20\text{-}35\%$, those of methylene (CH_2) decreased by $\sim 5\text{-}12\%$ and methyl (CH_3) by $1\text{-}15\%$, depending on the fractions of the individual types of carbon (CH_{0123}) present in SPE-DOM. This implies that recognition of the individual transfer amplitudes in ^{13}C DEPT-45 NMR spectra computes higher proportions of aliphatic branching in SPE-DOM than the previous means of computation which had assumed uniform transfer amplitudes for CH_{123} . The increased spectral dispersion of ^1H , ^{13}C HSQC NMR spectra was used to constrain the relative proportions of phlorotannin-related methine carbon units in the “phlorotannin-section” of one-dimensional ^1H and ^{13}C NMR spectra. This approach could resolve the relative proportions of anomeric O_2CH groups and $=\text{C}_{\text{sp}^2}\text{H}$ groups from δ_{C} : $90\text{-}107$ ppm, and the relative proportions of conjugated and alkylated olefins and $=\text{C}_{\text{sp}^2}\text{H}$ units from δ_{H} : $5.8\text{-}6.5$ ppm and provided more accurate assessment of the phlorotannin contents in *Sargassum* extracts (Supporting Information Table S3, S4). See Supporting Information Table S7 for all acquisition parameters.

3 Results and Discussion

3.1 Dissolved organic carbon exudation rates

DOC release rates during controlled *Sargassum* incubation experiments were determined from a linear regression of all DOC measurements versus time for individual tanks (Table 1). DOC release monitored during controlled indoor incubation experiments (artificial LED light, no UV, Supporting Information Figure S1) ranged from 6.6 to $9.5 \mu\text{g C g}^{-1}_{\text{biomass}} \text{h}^{-1}$ over 28 to 36 h periods for individual experiments (r^2 values = 0.96 to 0.99 , Table 1). This range in DOC release rate may be due to the differences in incubation time, *Sargassum* biomass, tank volume or the health of the plant. However, with the exception of the DOC release rate of $9.5 \mu\text{g C g}^{-1}_{\text{biomass}} \text{h}^{-1}$, the majority of indoor DOC exudation rates were not significantly different from the rates of 5.0 to $6.6 \mu\text{g C g}^{-1}_{\text{biomass}} \text{h}^{-1}$ determined for outdoor tanks covered with Plexiglas (rates compared by analysis of covariance, ANCOVA, in Matlab®). Under these experimental conditions, DOC exudation rates likely underestimate those *in situ* because indoor tank incubations were carried out using artificial light with no UV exposure and lower measured irradiance than natural sunlight (Supporting Information

Figure S1) and Plexiglas tank incubations were not exposed to solar radiation <345 nm and were exposed to reduced UVA radiation. To better compare the potential release *in situ* we also monitored the release of DOC from *Sargassum* in uncovered tanks during outdoor exudation rate experiments in Bermuda between September 28 and October 3, 2016. Over 28 to 48 h, DOC exudation rates ranged between 10 to 14 $\mu\text{g C g}^{-1}_{\text{biomass}} \text{h}^{-1}$ for individual tanks and were more variable than to those measured indoors (r^2 values = 0.79 to 0.99, Table 1). This variability was likely due the large increases in DOC concentration observed during afternoon outdoors (Figure 1A). To assess these increases, DOC release rates were calculated for all experiments between 1100 and 1900 local time. For all but one uncovered tank, rates increased significantly over this time to 24 to 41 $\mu\text{g C g}^{-1}_{\text{biomass}} \text{h}^{-1}$ (p -value <0.001, rates compared by ANCOVA in Matlab®; Table 1). For comparison, DOC release was only 4.7 to 11 $\mu\text{g C g}^{-1}_{\text{biomass}} \text{h}^{-1}$ for indoor and Plexiglas covered tanks between 1100 and 1900. Increases in DOC for uncovered outdoor tanks tracked well with increases in solar irradiance (Figure 1A) and likely explain the larger range in biomass normalized DOC exudation rates as compared to the indoor incubation experiments and the Plexiglas covered experiments.

To better compare all experiments, a linear regression between DOC and time for all time points acquired in uncovered tanks outdoors provided a value of $12 \pm 1 \mu\text{g C g}^{-1}_{\text{biomass}} \text{h}^{-1}$ ($n = 4$, $r^2 = 0.85$), which was significantly higher than the value obtained from all indoor and Plexiglas incubations of $7.1 \pm 0.3 \mu\text{g C g}^{-1}_{\text{biomass}} \text{h}^{-1}$ ($n = 6$, $r^2 = 0.93$) (Figure 1B, p -value <0.001, ANCOVA). It should be noted that these more natural outdoor exudation rates were determined after the fall equinox, and may therefore be even higher during the summer months under maximum UV exposure. Reed et al. (2015) found that DOC release by giant kelp *Macrocystis pyrifera* increased significantly with irradiation over the year and was 80% higher during the day than at night. Furthermore, one study that monitored DOC release from *Sargassum* between the hours of 10 and 12 found that exudation rates increased from 46 $\mu\text{g C g}^{-1}_{\text{biomass}} \text{h}^{-1}$ (dry weight) in April to 287 $\mu\text{g C g}^{-1}_{\text{biomass}} \text{h}^{-1}$ (dry weight) in June (Hanson, 1977). During our experiments, even though water temperature increased by 1°C between daytime and nighttime hours (Figure 1B), the increase in temperature cannot explain the increased exudation rates in incubations exposed to full solar radiation because a Plexiglas covered tank experienced the same temperature increase, without comparable increases in DOC concentrations (Figure 1A). However UV exposure alone does not appear to completely explain the differences in DOC exudation, because rates were still variable even when normalized to integrated light dose, ranging from 0.8 to $1.5 \times 10^{-4} \mu\text{g C g}^{-1}_{\text{biomass}} \text{h}^{-1} \text{Lux}^{-1}$.

Hanson (1977) reported higher DOC release rates from 'unhealthy' plants (up to $2,550 \mu\text{g C g}^{-1}_{\text{biomass}} \text{h}^{-1}$ dry weight for plants lacking thalli and/or overgrown with algae). To evaluate the release of DOC from *Sargassum* under more stressful conditions (Table 1), plants were incubated for 12 h both indoors and outdoors at temperatures ranging from 20 to 49°C. At 20°C, the DOC release was $35 \pm 6 \mu\text{g C g}^{-1}_{\text{biomass}} \text{h}^{-1}$, 4.6 times higher than the non-stress temperature controlled incubations indoors, and not significantly different than non-stress outdoor incubations in full sunlight between 1100 and 1900 local time (p -value = 0.07, ANCOVA). On the other hand, incubations that reached 43°C and 49°C released DOC at significantly higher rates than all other experiments, with exudation rates of 900 ± 200 and $1700 \pm 300 \mu\text{g C g}^{-1}_{\text{biomass}} \text{h}^{-1}$ respectively (ANCOVA p -values = 0.01 and <0.01, respectively). It is possible that these high temperatures caused cell lysis and such a high increase in DOC concentrations. While pelagic *Sargassum* sp. live and grow optimally between 24 to 30°C (Hanisak & Samuel, 1987), these results suggest that DOC release by *Sargassum* may increase under heat stress especially when wracked in coastal areas during inundation events. Previous work indicated that *Sargassum* exudation rates are highest for mid-senescent *Sargassum* and lowest for severely senesced plants (Shank et al., 2010a), thus additional work is needed to better evaluate DOC release rates at all life stages.

3.2 Polyphenol content in *Sargassum* exudates

Sargassum DOM was isolated from 25 time points from our tank incubations using a SPE technique described above (Dittmar et al., 2008). DOC concentrations and phenolic content were then determined for SPE-DOM samples using the Folin-Ciocalteu (FC) reagent method (Takeda et al., 2013). Because concentrations cannot be determined for all phenolic compounds in a sample containing a mixture of compounds, the FC reagent was standardized with phloroglucinol, and results are reported in molar equivalents. Phenolic content correlated well with SPE-DOC concentrations determined from these same SPE-DOM (Supporting Information Figure S2), and average phenol concentration expressed as phloroglucinol equivalents was $5.3 \pm 0.4\%$ of the SPE-DOC concentration, as determined by a linear regression of phenolic content versus SPE-DOC concentration for indoor and outdoor controlled incubation experiments ($r^2 = 0.92$; Supporting Information Figure S2). This percentage corresponded to an average phenolic content per unit carbon of $4.9 \pm 1.5 \text{ mol kg}^{-1} \text{ C}$. For comparison, phenol content expressed as gallic acid equivalents in SPE-DOM riverine samples collected in the Hiroshima Prefecture, Japan ranged from 0.5 to $1.9 \text{ mol kg}^{-1} \text{ C}$ (Takeda et al., 2013). Although a different reversed-phase SPE cartridge as well as gallic acid standards were used in this study, this difference suggests that polyphenol content is much

higher for *Sargassum* DOM than that for riverine DOM. Albeit for only a few samples, the average proportion of phenolic content in *Sargassum* DOM when exposed to full sunlight (7%, n = 4, range = 5% to 10%) or high temperature (9%, n = 1) appears to increase, as average phenol concentrations were lower at 5% (n = 18, range = 2.6% to 6.5%) of the SPE-DOM for all indoor incubations (p -value < 0.01, one-way analysis of variance).

Although the FC reagent has been widely used, it has been criticized owing to a number of limitations and interferences. For instance, the FC reagent is more reactive towards polyphenols with higher reduction potentials (i.e. those with more free hydroxyl groups) (Parys et al., 2007), so the measured phenolic content can vary widely based on the dominant polyphenols in a sample. Although phloroglucinol standards are a logical choice for analysis of brown algae phlorotannins, the FC reagent also reacts with monophenols to different degrees so results will vary depending on the standard used (Singleton et al., 1998). The FC reagent can also react with non-phenolic reducing compounds (Singleton et al., 1998), thereby overestimating phenol concentrations. In more complex mixtures of polyphenols containing interfering substances such as saccharides, the Folin-Ciocalteu reagent can underestimate phenol concentrations by up to three-fold (Lester et al., 2012). Considering the oxygenated conditions of all *Sargassum* incubations, partial oxidation of the phlorotannins during release and extraction could also lower phenol yields, as measured by the FC reagent. Phenolic content estimated by potentiometric titrations of standard and reference materials from the International Humic Substances Society (IHSS) ranged from 1.8 to 4.2 milli-equivalents (meq) g⁻¹ C (Ritchie & Perdue, 2003). As an additional test, we titrated a 500 mg L⁻¹ solution of Suwannee River Natural Organic Matter (SRNOM, IHSS) from pH 3 to 10 and estimated a phenolic content of 4.0 meq g⁻¹ C, in agreement with the 3.9 meq g⁻¹ C determined in this previous study (Ritchie & Perdue, 2003). The same titration repeated for *Sargassum* SPE-DOM resulted in phenolic contents that ranged from 3.5 to 5.4 meq g⁻¹ C for indoor incubations but was 10.2 meq g⁻¹ C for *Sargassum* DOM collected from an outdoor incubation (Table 2), again suggesting a higher phenolic content in *Sargassum* DOM when compared to terrestrial DOM. Phenolic content may also be underestimated using this approach because it assumes that carboxyl groups and no phenolic groups are titrated to pH 8 and that half the phenolic groups are titrated between pH 8 and 10 (Ritchie & Perdue, 2003). The ratio of phenolic to carboxyl content was 0.4 for the SRNOM sample determined here and previously (Ritchie & Perdue, 2003). *Sargassum* DOM had a similar phenolic to carboxyl content ratio of 0.3 to 0.6, meaning that carboxyl groups are also

abundant in *Sargassum* DOM and phenolic content makes up 24 to 38% of titratable groups in *Sargassum* SPE-DOM.

3.3 Composition of *Sargassum* DOM analyzed by FT-ICR MS

We applied non-targeted ultrahigh resolution FT-ICR MS in both positive and negative electrospray ionization (ESI) modes and a total of 3,462 molecular formulas were assigned to m/z ions (Supporting Information Figure S3). The majority of assigned molecular formulas were found in all samples regardless of incubation conditions (Supporting Information Figure S3B) and only contained hydrogen, oxygen, and carbon (CHO, 54%), followed by CHO + nitrogen (CHNO, 39%), and CHO + sulfur (CHOS, 4%) (Supporting Information Table S1). The remaining assignments included brominated compounds (CHOBr, 2%) that were identified in both positive and negative modes and nitrogen and sulfur containing compounds (CHNOS, 1%) that were only found in positive ESI mode. CHO formulas were most abundant in negative ESI mode (83% of assignments), and exhibited a diverse composition of molecular formulas when plotted in van Krevelen space (Figure 2A). The intensity-weighted average O/C elemental ratio (O/C_{wt}) was 0.62 (Supporting Information Table S1), comparable to that determined for marine SPE-DOM collected in the open Atlantic Ocean ranging from 0.54 in surface waters to 0.67 at 5,500 m (Hertkorn et al., 2013). However, marine DOM is typically highly aliphatic with H/C ratios ranging from 1.2 to 1.7 (Hertkorn et al., 2013; Lechtenfeld et al., 2014) while *Sargassum* SPE-DOM was far more unsaturated, with an intensity-weighted H/C_{wt} and a double bond equivalent (DBE_{wt}) of ~0.83 and ~11, respectively (Supporting Information Table S1). *Sargassum* DOM analyzed in negative ESI mode also had a relatively high intensity-weighted average modified aromaticity index (AI_{mod_wt}) of 0.5 and aromaticity equivalent (X_{C_wt}) of 2.6 when using $m = 0.5$ in Equation 6 (Table S1). $AI/AI_{mod} \geq 0.5$ and $X_C \geq 2.5$ have been proposed as criteria for unequivocal identification of aromatic compounds in samples analyzed by FT-ICR MS (Koch & Dittmar, 2016; 2006; Yassine et al., 2014). While these parameters are useful in comparing formula assignments between samples within a study, the resulting % aromatic compounds vary dramatically depending on the parameter selected and how samples are analyzed. Using AI and $X_C(m = 1)$ results in 14% and 16% of negative ESI mode peak intensities identified as aromatic, respectively. However, using AI_{mod} and $X_C(m = 0.5)$ results in 44% (AI_{mod}) and 66% (X_C) of peak intensities being identified as aromatic compounds. *Sargassum* DOM analyzed in positive ESI mode was even more variable, with 3% of peak intensities having an AI value ≥ 0.5 , 16% having an AI_{mod} value ≥ 0.5 , 27% having an $X_C(m = 1)$ value ≥ 2.5 and 70% having an $X_C(m = 0.5)$ value ≥ 2.5 . Thus, use of

these parameters should be interpreted with caution. Nonetheless, the high O/C ratios and low H/C ratios in *Sargassum* DOM are indicative of a large contribution of hydrogen-deficient and highly oxygenated compounds typically associated with an undefined pool of polyphenols, similar to high DOM freshwater systems such as the Rio Negro in the Amazon Basin (Gonsior et al., 2016).

Because CHO formulas occupy the largest region of van Krevelen space (Figure 2A and Supporting Information Figure S4A-C), we grouped CHO formulas based on those common or unique between ESI negative and positive mode (Supporting Information Table S1 and Figure S4D). We found a high density of CHO compounds within the area of expected phlorotannins (Figure 2A) identified in both ESI positive and negative mode as well as those unique to ESI negative mode (Supporting Information Figure S4D). However, common formulas between both ESI modes had higher MW_{wt} (~ 420 m/z) than formulas unique to ESI negative mode (~ 350 m/z) (Supporting Information Table S1). Assigned formulas with identical molecular compositions in different mass spectra will probably not represent identical (single) compounds or specific ratios of more numerous compounds because very high numbers of isomers may be present for these sizable molecules (Hertkorn et al., 2007) that also will ionize differently in positive or negative ESI (Hertkorn et al., 2008). Phlorotannin molecular weights are expected to range between 126 (phloroglucinol monomer) and $>100,000$ Daltons (Da) (Van Altena & Steinberg, 1992); however, only phlorotannins with ≤ 18 rings and a molecular weight of 1,117 Da have been annotated using ESI-MS (Vissers et al., 2017). The observed mass range in our experiments covered m/z ions between 150-1,200 Da, but only m/z ions <700 m/z were observed within our FT-ICR MS data set. The average molecular characteristics were similar between common CHO formulas and those only found in negative ESI mode (Supporting Information Table S1), but in either case their high modified aromaticity index ($AI_{mod_wt} = 0.47$ for common CHO formulas) and high DBE_{wt} (11.4 for common CHO formulas) support the high abundance of phlorotannin-related polyphenolic compounds (Supporting Information Table S1). The modified Kendrick plot (Shakeri Yekta et al., 2012), or the ratio of Kendrick mass defect to z^* (KMD/z^*) versus exact mass (Figure 2B), further underlines the presence of multiple rather extended (≥ 6 members) homologous CH_2 -based series of CHO compounds, with Gaussian-like intensity profiles among homologous series (Figure 2B) (Gonsior et al., 2016). Phlorotannins are known strong antioxidants and hence can react rapidly with reactive oxygen species (Fujii et al., 2013; Kuda et al., 2006; Le Lann et al., 2016) to likely form new compounds containing additional oxygen (Schmitt-Kopplin et al., 1998; Zhang & Blough, 2016) which might in

particular reflect increased hydroxylation on the aromatic rings. To further investigate compositional relationships in *Sargassum* DOM, nominal transformations were tested originating from CHO formulas that matched known phlorotannins. Although structures are not known, additions of OH, H₂O and carboxylations are all conceivable (Figure 2C) given the oxygenated conditions and solar exposure during these experiments (Schmitt-Kopplin et al., 1998; Zhang & Blough, 2016). Thus, simple oxidative transformations of phlorotannins like oxygenation (nominal insertion of oxygen in -C-H bonds) and carboxylation (nominal insertion of CO₂ in -C-H bonds) will expand the chemical space of CHO compounds towards higher O/C ratios in van Krevelen diagrams, while leaving the H/C ratio unaffected (Figure 2A, C and Supporting Information Figure S4A, D). These reactions also support an increased propensity for ionization of transformed *Sargassum* polyphenolic compounds.

A number of aromatic brominated compounds was also confirmed in all *Sargassum* DOM samples (Figure 2A). CHOBr compounds (6% of ESI negative mode formula assignments) had a similarly high O/C_{wt} of 0.68, but lower H/C ratios (0.62) than CHO compounds given that Br often substitutes for H. Although the abundance of CHOBr compounds was low compared to CHO compounds (Figure 2), halogenated phlorotannins have been described (La Barre et al., 2010) and may well be involved in the synthesis pathway of phlorotannins which remains largely unknown (Berglin et al., 2004; Meslet-Cladiere et al., 2013; Salgado et al., 2009). Release of reactive volatile organic halogens from brown algae exposed to visible and ultraviolet radiation has also been documented (Laternus et al., 2010). Previous work has shown that the brown algae release bromophenolic compounds, but only halogenated phloroglucinol, monomeric bromophenols, and specific halogenated phlorotannins were targeted (Shibata et al., 2006). CHNO (3% of negative mode assignments) and CHOS (8% of ESI negative mode formula assignments) formulas both had (O/C)_{wt} ratios that centered around 0.4, but had higher (H/C)_{wt} for CHOS formulas (1.6) than for CHNO formulas (1.3). Their low abundance and presumably aliphatic nature indicate that these CHOS compounds could make up a pool of labile DOM that might be rapidly utilized (Hertkorn et al., 2013). It should be noted that these compounds may arise from *Sargassum* itself or from associated biomass (e.g., epiphytes). Therefore, our results indicate that SPE-*Sargassum* DOM has a high chemodiversity with a particularly complex composition of reduced and highly oxygenated compounds that may resemble a complex suite of phlorotannins. Although FT-ICR MS analysis is extremely information rich, it is at best a semi-quantitative approach and does not supply structural information beside that what can be inferred from assigned molecular formulas. Hence quantitative estimates of the polyphenol

content of *Sargassum* SPE-DOM were undertaken using ^1H and ^{13}C NMR spectra, as described below.

3.4 Structural characteristics of *Sargassum* SPE-DOM analyzed by ^1H and ^{13}C NMR spectroscopy

^1H and ^{13}C NMR spectroscopy were also used to estimate the phenolic component of *Sargassum* DOM, in order to independently and perhaps more accurately quantify the polyphenolic content in *Sargassum* SPE-DOM. As mentioned previously, phlorotannins carry an unique 1,3,5-trioxo-aromatic substitution, which is retained in virtually all of the ~150 known phlorotannins (Isaza Martínez & Torres Castañeda, 2013) of different size and oxygenation. Aromatic rings (C_{ar}) are either directly connected ($\text{C}_{\text{ar}}\text{-C}_{\text{ar}}$; biaryl) or form arylether bonds ($\text{C}_{\text{ar}}\text{-O-C}_{\text{ar}}$) (Isaza Martínez & Torres Castañeda, 2013). Key structural motifs comprise furans resulting from polymerization of C-C-bond hydroxylated aromatic compounds and 1,4-dioxines resulting from condensation of hydroxylated aryl ethers. Because at least four aromatic positions have to be occupied by non-hydrogen substituents (a minimum of three in terminal arylethers), all larger-size phlorotannins show considerable hydrogen deficiency (Figure 3B). NMR spectra show a notable distinction of non-exchangeable (e.g. -ZCH units; Z: any atom) and exchangeable (e.g. -XH units; X: O, S, N) hydrogen atoms in organic molecules which does not appear in common FT-ICR mass spectra. In methanolic solution of SPE-DOM, all exchangeable protons present combine with the residual water to produce a single rather broadened resonance at $\delta_{\text{H}} \sim 4.9 \pm 0.3$ ppm, which is rather well attenuated in ^1H NMR spectra acquired with solvent suppression as used here. All non-exchangeable protons except those which reside very close to the solvent NMR resonance will be observed in standard 1D and 2D NMR spectra. Hence, while common oxidative reactions (like oxygenation and carboxylation) will not change the H/C ratio of molecules in FT-ICR mass spectra (cf. above), the count of NMR-observable non-exchangeable protons decreases because -C-H units are being transformed into -C-O-H units. It is noteworthy that FT-ICR mass spectrometry-based methods exist to determine the extent and some structural attribution of exchangeable protons in organic matter (Zherebker et al., 2017); similarly, a wide range of NMR spectra readily detect abundance and chemical environments of exchangeable protons in organic molecules as well. Hence, polymerization and oxidation further decrease the abundance of non-exchangeable protons in molecules and make detection of evolved phlorotannins by ^1H NMR spectroscopy eventually impractical. The combined electron-donating resonance effects of two ortho- and one para-oxygen

substituents in 1,3,5-trioxo-phlorotannins produce NMR chemical shifts $\delta_{H/C}$ that are normally attributed to either olefinic protons ($=\underline{C}\underline{H}$; $\delta_H \sim 5.8\text{-}6.1$ ppm) or anomeric positions in carbohydrates ($O_2\underline{C}\underline{H}$; $\delta_C \sim 90\text{-}100$ ppm) but not to benzene derivatives.

Under realistic outdoor conditions comprising exposure to sunlight and oxygen, *Sargassum* exudates produced a highly diverse mixture of biomolecules produced by *Sargassum* itself, epiphytes and other microorganisms that have colonized the *Sargassum* plants. Hence, NMR spectra showed the expected fundamental primarily aliphatic metabolite classes of lipids (abundant), peptides (discernible but not prominent) and carbohydrates (abundant) (Supporting Information Figs. S7, S8). The presence of these labile compounds implies that *Sargassum* exudates support oceanic microbial foodwebs with a large diversity of readily bioavailable molecules (Hanson, 1977). In addition, *Sargassum* extracts showed fairly well developed signals that can be attributed to carboxyl-rich alicyclic compounds (CRAM) (Hertkorn et al., 2006), oxygenation products of carotenoids (Arakawa et al., 2017) and other oxyomethylene and oxomethine groups (Supporting Information Figure S8). General aromatic molecules, unsaturated natural products and specifically phlorotannins present in *Sargassum* extracts were altogether much more abundant than aromatic molecules in common aquatic and marine organic matter (Supporting Information Table S2 and Figs. S5, S6, S11) (Hertkorn et al., 2013).

The ^1H NMR-derived content of phlorotannins (for in-depth analysis of NMR spectra, see Supporting Information) ranged from 2.3%-6.1% for individual *Sargassum* extracts, while the consolidated extract showed 5.3% abundance (Table 2 and Supporting Information Table S3). Regarding the low H/C atomic ratios and paucity of aromatic C-H ($C_{ar}H$) groups in oxidized phlorotannins, these ^1H NMR-derived proportions are expected to underrepresent its actual abundance. Hence ^{13}C NMR spectra were needed and provided evidence of the consolidated *Sargassum* extract to have $\sim 18\%$ phlorotannin content, with a minimum of $>80\%$ quaternary carbon atoms (i.e. carbon atoms attached directly to 4 other carbon or oxygen atoms) which resonated from $\delta_C \sim 130\text{-}165$ ppm ($\underline{C}_{ar}\text{-O}$ units) and $\delta_C \sim 100\text{-}140$ ppm ($\underline{C}_{ar}\text{-C}$ units) (Supporting Information Table S4). In isolated 1,3,5-trioxo aromatic rings, these resonated at $\delta_C \sim 95\text{-}120$ ppm ($\underline{C}_{ar}\text{-C}$ units) while a high diversity of poly-oxygenated aromatic rings with ≥ 4 oxygen atoms produced $\delta_C \sim 140\text{-}165$ ppm ($\underline{C}_{ar}\text{-O}$ units) (Supporting Information). Notably, oxidized phlorotannins are more hydrogen-deficient than ordinary ones and therefore produce also smaller HSQC cross peaks at equal molar concentration. Terminal aromatic rings of phlorotannins often show local C2 symmetry which frequently causes two protons to resonate at identical frequencies (e.g. at 3,5- or 2,6-positions),

providing NMR resonances with double amplitude. The structural regularity of common phlorotannins with distinct trioxo aromatic rings separated by aryl-aryl or aryloether bonds allows for extrapolation of $\delta_{\text{H/C}}$ values. So far, HSQC cross peaks of any DOM investigated at our institute (> 300) with analogous $\delta_{\text{H/C}}$ have only been observed in Suwannee River water in which these probably represented catechins and other terrestrial derived tannins (Figure 7 in Perdue et al. (2007)).

Remarkably, at least 40% of exudated phlorotannins were oxidized moieties with ≥ 4 oxygen atoms per aromatic ring (section b₃, Figure 3 and Supporting Information Figure S12). This is in agreement with an efficient reaction with reactive oxygen species and the known very high antioxidant properties of phlorotannins. Hydroxylation and/or carboxylation of the aromatic rings would lead to the oxygenation of phlorotannins, in particular under irradiation in oxygenated conditions (Schmitt-Kopplin et al., 1998; Zhang & Blough, 2016). Individual *Sargassum* extracts showed considerable variance of composition for all functionalities, reflecting a complex temporal evolution of a multi-organism mini ecosystem under our experimental conditions (Supporting Information Table S2, S3 and Figs. S6-S12). Notably, a single sample showed near double the amount of phlorotannins compared with that found in the consolidated sample (Supporting Information Table S2, S3 and Figure S6), suggesting the capacity of *Sargassum* to produce even higher proportions of phlorotannins and DOM under certain conditions. Remarkably, high abundance of phlorotannins went along with low proportions of a group of aliphatic esters and vice versa (Supporting Information Table S2, S3 and Figure S6). In general, NMR spectra confirmed considerably higher proportions of oxygenated and unsaturated protons and a substantially lower extent of aliphatic branching in *Sargassum* extract when compared with DOM in Atlantic open ocean waters (Supporting Information). This conforms to a high share of metabolites present in *Sargassum* extract which could be eventually transformed into a more defunctionalized, evolved marine DOM.

3.5 *Sargassum* contribution to DOC and polyphenols in the Gulf of Mexico and North Atlantic

Previous work suggested that *Sargassum* colonies might be an important source of DOM to the blue waters of the Gulf of Mexico and Sargasso Sea (Shank et al., 2010a), so by simply assuming that annual biomass between 2 to 11 Tg (Butler & Stoner, 1984; Gower & King, 2011; Parr, 1939; Wang et al., 2018), and a DOC release rate of $12 \pm 1 \mu\text{g C g}^{-1}_{\text{biomass h}}^{-1}$ for one day (Figure 1B), *Sargassum* is capable of releasing 0.3 to 1.2 Tg C annually as

DOC, which amounts to approximately 10% of its annual biomass. The estimated production of 0.3 to 1.2 Tg C from *Sargassum* is modest when compared to terrestrial DOC inputs to the ocean via river systems of 200 to 400 Tg C yr⁻¹ (Raymond & Spencer, 2015). However, a recent study reported that *Sargassum* can account for ~18% of the particulate organic carbon in the Central Western Atlantic and the Caribbean Sea during bloom events, suggesting that *Sargassum* can contribute significant quantities of organic carbon that has not been previously considered in climate models (Wang et al., 2018). This work is an excellent example of utilizing field data with remote sensing data to improve estimates of *Sargassum* biomass and density (Wang et al., 2018), which will allow improved evaluation of *Sargassum*'s contribution to various carbon pools in the future.

Estimates of terrestrial DOC in the ocean based on lignin phenols ranged from ~0.7 to 2.4% in the Pacific and the Atlantic, respectively (Opsahl & Benner, 1997), but it is not likely that 1,3,5-trioxo aromatic rings of phlorotannins would be detected as lignin phenols. Recent work that has fractionated marine DOC by polarity found that low molecular weight hydrophilic DOC had depleted stable isotope values (-24 to -31‰) relative to hydrophobic and high molecular weight fractions (-23 to -21‰), especially below 1,000 m (Zigah et al., 2017) and evidence suggests that terrestrial DOM components persist in the deep Atlantic (Andrew et al., 2013) and Pacific (Cartisano et al., 2018). Zigah et al. (2017) attributed these depleted values in the hydrophilic fraction to allochthonous sources, such as terrestrial inputs, especially because lignin is typically 3 to 6‰ depleted relative to bulk DOC (Spencer et al., 2009). The stable carbon isotope values for phlorotannins did not differ from that of the plant body for three marine brown algae species, which ranged from -28.9 to -26.3‰ (Arnold & Targett, 1998). Therefore, polyphenols from marine sources could contribute autochthonous components of the large deep ocean refractory DOC pool.

Here we present evidence that polyphenols make up a significant portion of the DOM actively released by *Sargassum* (18% of the consolidated SPE-DOM sample, Supporting Information Table S4). Moreover, DOC release significantly increased in response to solar irradiation, likely serving a protective role against exposure to UV radiation. In all experiments performed, phenolic content appeared to increase in DOM released from *Sargassum* when exposed to solar radiation (Supporting Information Figure S2), and previous work has shown that the rates of DOM release from *Sargassum* increased under UV radiation when compared to dark controls (Shank et al., 2010a). Solid phase extraction is not 100% efficient, so the proportion of polyphenols in *Sargassum* DOC before extraction may be higher or lower. However, simply assuming 5 to 18% of *Sargassum* DOC is polyphenolic

would amount to a release 30 to 200 Gg C yr⁻¹ as polyphenols, suggesting that the exudation of DOC from *Sargassum* represents a significant source of polyphenolic material to the Gulf of Mexico and Western Atlantic that has not been previously quantified.

4 Conclusions

The fact remains that no one has observed local large sources of *Sargassum* DOM, implying that this DOM is either rapidly removed or diluted. Estimates on the lability of *Sargassum* DOC are uncertain, given that heterotrophic bacteria can utilize between 20 to 70% of the DOC released by brown algae (Brylinsky, 1977; Hanson, 1977). Previous work has also shown that DOM released by *Sargassum* is readily photodegraded when exposed to solar radiation (Shank et al., 2010b) and rapidly biodegraded (Carpenter & Cox, 1974). It has been demonstrated that aromatic compounds in riverine DOM (Stubbins et al., 2010) and deep ocean marine DOM (Medeiros et al., 2015; Stubbins & Dittmar, 2015) are readily photodegraded. Moreover, the abundance of phenolic macromolecules increased with depth in the ocean and are present in a suite of marine algae (Van Heemst et al., 1996), suggesting the production of recalcitrant compounds derived from phlorotannins (Van Heemst et al., 1996). We have demonstrated that *Sargassum* exudates evolve into DOM that is extremely rich in quaternary carbon (~47%, Supporting Information Figure S11). Because 44% of the quaternary carbon is associated with phlorotannins (Table 2) and with consideration of the high proportion of exchangeable protons, ¹H NMR spectra severely underestimate the content of phlorotannins. Autonomous underwater vehicle photography found that *Sargassum* biomass on the seafloor was much higher than that estimated from surface waters (Baker et al., 2017). Furthermore, Baker et al. (2017) estimated maximum sedimentation rates of 2 m min⁻¹ or only 2 days to reach 5,000 m depth; however, it is not yet known whether sinking *Sargassum* or that on the seafloor releases DOC. Although the fate of *Sargassum* exudates has yet to be defined, polyphenols have been implicated in the formation of colored dissolved organic matter (CDOM) (Sharpless & Blough, 2014; Del Vecchio & Blough, 2004) and the exudates are strongly colored (Shank et al., 2010a). Future work that investigates *Sargassum* DOM optical and chemical properties, bioavailability and photochemical reactivity will help constrain the nature and fate of this material in the marine environment and its contribution to marine CDOM.

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Table 1. Release rates of dissolved organic carbon (DOC) from *Sargassum*. Rates were determined by a linear regression of DOC concentration versus time during exudation experiments for individual tanks using various light conditions, wet weights, and tank volumes as described in Section 2.2. DOC concentrations (mg L^{-1}) were normalized to *Sargassum* biomass in wet weight ($\text{g}_{\text{biomass}}$) and tank volume (L). Rates ($\mu\text{g C g}^{-1}_{\text{biomass h}^{-1}} \pm 1$ SD) were either determined over the entire experiment (28 to 47 h) or between the hours of 1100 and 1900 local time corresponding r^2 values are listed in parentheses. For non-stress condition experiments, tanks were maintained at 26 to 29°C and for stress condition experiments, temperature ranged from 20 to 49°C. Non-stress experiments with full solar exposure ($n = 4$) and without full solar exposure ($n = 6$) were pooled and average rates were determined by linear regression of these pooled non-stress condition experiments (p -value < 0.001 , $F = 24.6$, determined by analysis of covariance in Matlab®). All indoor experiments were for *Sargassum* samples collected in July 2016 and all outdoor experiments were for *Sargassum* samples collected in September/October 2016, as described in the Methods section.

	Biomass / Volume (g L^{-1})	DOC ($\mu\text{g C g}^{-1}_{\text{biomass h}^{-1}}$) (entire experiment) ($r^2$)	DOC ($\mu\text{g C g}^{-1}_{\text{biomass h}^{-1}}$) 1100 to 1900 ($r^2$)
Non-stress conditions			
Indoor 28 h	16	9.5 ± 0.4 (0.98)	11 ± 2 (0.93)
Indoor 36 h	13	8.1 ± 0.6 (0.96)	9.6 ± 2.4 (0.88)
Indoor 36 h	15	6.6 ± 0.4 (0.97)	4.7 ± 2.2 (0.69)
Indoor 36 h	14	7.4 ± 0.4 (0.98)	5.8 ± 2.5 (0.73)
Outdoor Plexiglas 47 h	16	6.7 ± 0.5 (0.95)	11 ± 1 (0.98)
Outdoor Plexiglas 22 h	15	5.0 ± 1.0 (0.80)	10 ± 3 (0.81)
Pooled (without full solar exposure)		7 ± 0.3 (0.93)	7 ± 2 (0.35)
Outdoor no cover 47 h	20	11.8 ± 1.8 (0.79)	41 ± 12 (0.78)
Outdoor no cover 28 h*	16	11.0 ± 1.5 (0.88)	36 ± 3 (0.99)
Outdoor no cover 27 h*	43	9.9 ± 1.2 (0.93)	24 ± 2 (0.99)
Outdoor no cover 30 h*	32	14 ± 0.6 (0.99)	14 ± 0.4 (0.99)
Pooled (with full solar exposure)		12 ± 1 (0.85)	23 ± 8 (0.40)
Stress conditions			
Indoor 20°C	30	35 ± 5 (0.94)	52 ± 7 (0.98)
Outdoors 26 to 39°C	10	65 ± 17 (0.84)	50 ± 5 (0.99)
Outdoors (shade) 25°C	35	50 ± 21 (0.64)	9.3 ± 5 (0.75)
Outdoors 26 to 43°C	29	930 ± 160 (0.92)	1400 ± 310 (0.95)
Outdoors 26 to 49°C	6.2	1700 ± 350 (0.89)	2900 ± 530 (0.97)

*Solid phase extracts from these samples were analyzed by ^1H nuclear magnetic resonance spectroscopy (NMR) with the following sample identification noted in the Supporting Information: outdoor 28 h is sample 394, outdoor 27 h is sample 395, and outdoor 30 h is sample 396. These extracts were consolidated to provide sufficient material for both ^1H -based 2D and ^{13}C NMR spectroscopy.

Table 2. Estimated phenolic content (units listed in table) in *Sargassum* SPE-DOM from samples exposed to full sunlight using various methods. For ^1H NMR spectra 100% corresponds to total integral, with recognition of cross peak distribution in ^1H , ^{13}C HSQC NMR spectra. See Tables S2-S4 for additional details.

Techniques used to estimate relative phenol content in <i>Sargassum natans</i> SPE-DOM	Estimated relative phenolic content in <i>Sargassum natans</i> SPE-DOM (%)
Folin-Ciocalteu Colorimetric method	7.0% ($100 \times (\text{phenol content})_{\mu\text{M}} / (\text{DOC})_{\mu\text{M}}$)
^1H NMR $\delta(^1\text{H}) = 7.2 - 5.8$ ppm	5.3% total integral
^{13}C NMR $\delta(^{13}\text{C}) = 160 - 90$ ppm	18% total integral
^{13}CH DEPT NMR $\delta(^{13}\text{C}) = 160 - 90$ ppm	8.4% total integral
^{13}C NMR quat: quaternary carbon only; $\delta(^{13}\text{C}) = 160 - 90$ ppm	44% of the quaternary carbon is associated with phlorotannins. The average substitution within phlorotannins ranges near 5.5 per aromatic ring; i.e. only 0.5 protons are attached to phlorotannin aromatic rings on average, suggesting high oxygenation (hydroxylation and carboxylation)
pH titration experiments	10 milli-equivalents g^{-1} C or 38% of titratable groups ($100 \times (\text{phenol content}) / (\text{phenol content} + \text{carboxyl content})$)

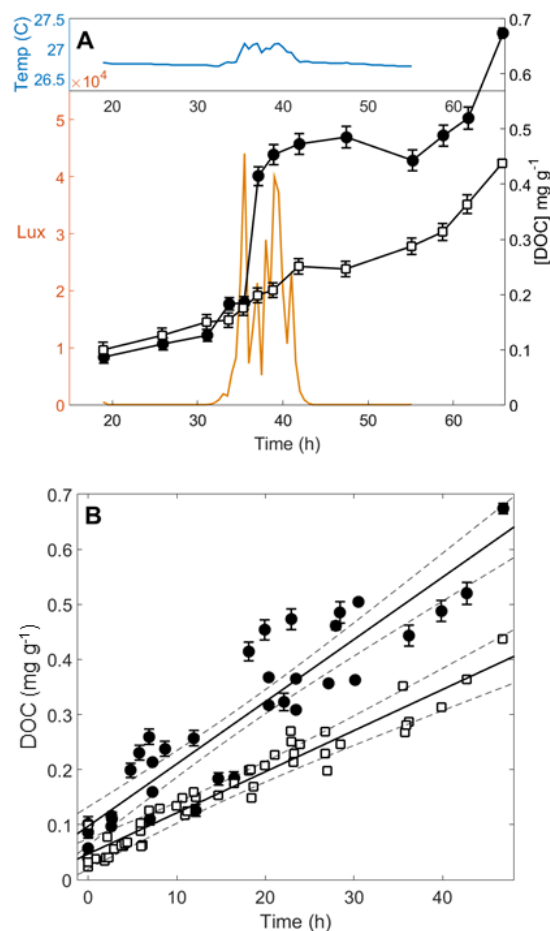


Figure 1. Dissolved organic carbon (DOC) concentrations during *Sargassum* exudation experiments. DOC ($\text{mg C g}_{\text{biomass}}^{-1}$, ± 1 SD, right axis) release over time during two outdoor incubation experiments (A). Black circles represent DOC from tanks exposed to full solar radiation and white squares represent DOC released in a tank with a Plexiglas cover (no UV exposure). The orange line is integrated visible solar irradiation (Lux, left axes) and the blue line is tank temperature ($^{\circ}\text{C}$, top left axes). DOC ($\text{mg g}_{\text{biomass}}^{-1}$) versus time (h) for four outdoor incubation experiments in full sunlight (black circles) and those for four indoor experiments with no UV exposure (white squares) (B). Linear regressions (solid lines), plotted with 95% confidence intervals (dashed lines), were used to determine DOC release rates regardless of start time or time of day (experiments ranging from 27 to 46 h).

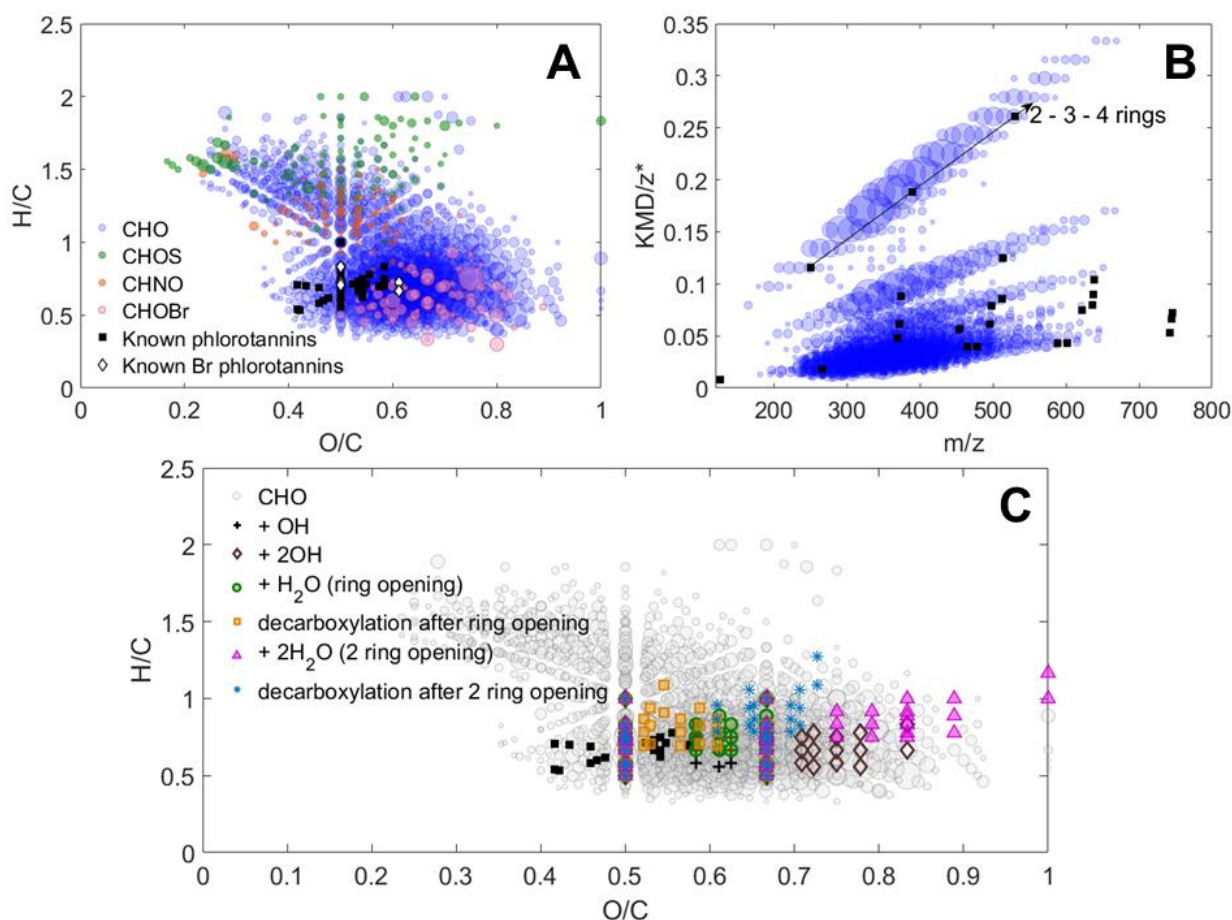


Figure 2. Formula assignments for an average (4 individual outdoor tanks) ultrahigh resolution mass spectrum of *Sargassum* DOM analyzed in negative ESI mode. **(A, C)** van Krevelen diagrams (hydrogen to carbon, H/C, versus oxygen to carbon, O/C, ratios) and **(B)** modified Kendrick plots, KMD/z* versus exact mass (m/z) found in negative mode FT ICR-MS data. In **(A)**, blue dots represent formulas that contain only carbon, hydrogen, and oxygen (CHO), orange dots represent CHO + nitrogen (CHNO) formulas, green dots represent CHO + sulfur (CHOS) formulas, pink dots represent CHNOS formulas, and purple dots represent CHO + bromine (CHOBr) and black squares and white diamonds represent known CHO and CHOBr phlorotannins, respectively. Bubble size corresponds to peak intensity. The arrow in **(B)** highlights known phlorotannins (black squares) plotted on top of CHO series that corresponds to increasing phloroglucinol rings in known phlorotannins. The van Krevelen diagram **(C)** illustrates hypothetical CHO compounds that can occur from nominal transformations of known phlorotannins plotted on top of CHO formulas (gray circles) and known phlorotannins (black squares). These transformations include additions of 1-2 OH, 1-2 H₂O, 1-2 decarboxylations after ring opening and suggest that phlorotannins and their transformation products can occupy a large area within van Krevelen space.

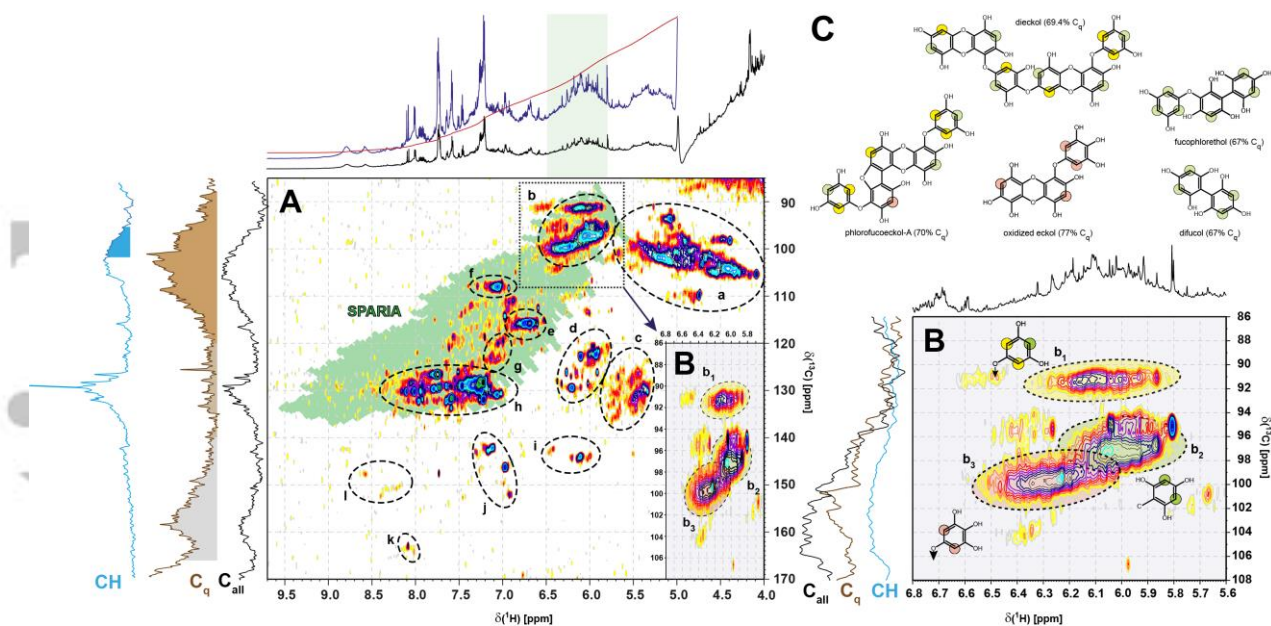


Figure 3. ^1H and ^{13}C nuclear magnetic resonance spectra of *Sargassum* DOM. **(A)** ^1H ^{13}C HSQC NMR spectrum (800 MHz, CD_3OD) of $\text{C}_{\text{sp}2}\text{H}$ units in consolidated *Sargassum* extract; with section of phlorotannins indicated; C_q denotes quaternary carbon atoms and shaded areas in DEPT (blue) and QUATD (brown) ^{13}C NMR spectra denote relative proportions of protonated and quaternary carbon, indicating less than 0.5 protons per aromatic ring in phlorotannins on average. The dotted box and inset shows three key substructures of phlorotannins also shown in **(B)** (b_1 - b_3 ; for full annotation cf. Supporting Information). Additional substructures highlighted with dashed circles are explained in Supporting Information Figure S9. Green shaded area indicates range of chemical shift that is accessible to single aromatic rings (SPARIA: substitution patterns in aromatic rings by increment analysis, Perdue et al., 2007). **(B)** highlights the section of 1,3,5-trioxoaromatic compounds (i.e. phlorotannins) with key substructures. **(C)** shows a few known phlorotannins, and while they may not be present in our *Sargassum* DOM samples, these model structures indicate ranges of $\delta_{\text{H/C}}$ according to three major classes of substitution indicated by color ($\delta_{\text{H/C}} \sim 5.9$ - 6.3 / 93 ± 1.5 ppm, $\delta_{\text{H/C}} \sim 5.8$ - 6.2 / 98 ± 2 ppm and $\delta_{\text{H/C}} \sim 6.0$ - 6.4 / 101 ± 2 ppm). These included an array of 3,5-dihydroxybenenes attached via oxygen at C1 and substituted 1,4-dioxins (section b_1 (also resonating in section b_2) 15%), carbon-bound 2,4,6-trihydroxybenzenes (section b_2 , 45%), and oxidized phlorotannins, with ≥ 4 oxygen atoms (section b_3 , 40%).

Acce