Free radical scavenging (antioxidant activity) of natural dissolved organic matter

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Abstract

Free radicals are produced in aquatic environments through photochemical reactions. They can affect the concentration and composition of organic matter and have negative effects on aquatic organisms. Free radical scavengers (antioxidants) can remove these highly reactive species from the media. Some dissolved organic matter (DOM) constituents are widely known to present antioxidant properties (e.g. phenols and hydroquinones). However, little is known about the free radical scavenger capacity of DOM. Here we applied two simple, analytical assays (ABST and DPPH) to assess the antioxidant capacity of aquatic DOM, after their validation against a more complex electrochemical technique. These assays were applied to DOM from various environmental settings, including freshwater marshes, fringe mangrove estuaries and a coastal bay in Everglades National Park, Florida. All the samples presented different degrees of antioxidant activity depending on their origin and thus DOM quality. Samples associated with mangrove areas presented the highest antioxidant activity, possibly due to the presence of tannins, which are known to be powerful antioxidants. The free radical scavenging capacity or antioxidant properties of DOM may have important implications in aquatic photochemistry as well as in microbial processes.

1. Introduction

Antioxidants are compounds that can scavenge free radicals by interrupting radical chain reactions, or even prevent the reactive oxidants from being formed in the first place (Huang et al., 2005). Adverse effects of free radicals on aquatic organisms have been reported elsewhere. For example, the presence of the free radical precursor H_2O_2 reduced bacterial activity in short

term experiments (Anesio et al., 2005) and negatively affected cyanobacteria physiology (Leunert et al., 2013). It was suggested that bacterial metabolism was also inhibited by oxidizing reagents (e.g. hydroxyl radicals) produced after UV irradiation of humic substances (Lund et al., 1994). Therefore, the presence of antioxidants in the aquatic environment could have implications on photochemical reactions as well as be beneficial for aquatic organism health, since they can remove the harmful free radicals.

Polyphenols, such as flavonoids, tannins and lignins are known for their antioxidant capacity (Rice-Evans et al. 1996; Dizhbite et al., 2004; Amarovicz, 2007). All these compounds are commonly found in the DOM pool. In general, the antioxidant properties of these compounds are related with the presence of hydroxyl groups in their aromatic rings. Commonly, the larger their number the higher the antioxidant activity of the compound. However, the position of these OH moieties in the structure of the molecule is also determinant of their antioxidant activity (Symonowicz and Kolanek, 2012). On the other hand, the antioxidant activity of a compound in natural water could also depend on environmental conditions (e.g., ionic strength) and conformation size in the same way that these variables affect other characteristics of the DOM (i.e., optical properties, Pace et al., 2012).

Considering the known presence of polyphenols such as tannins and lignin in DOM, studies about the antioxidant activity of DOM are surprisingly scarce. The role of DOM as a sink of hydroxyl radical ($\cdot\text{OH}$) in freshwaters has been reported (Vione et al., 2006; Page et al. 2014). However, free radicals are not the only species to be quenched by DOM. It has been shown that some DOM constituents can also quench the harmful singlet oxygen (Cory et al., 2009). Cory and coworkers showed that both terrestrially and microbially derived fulvic acids react with $^1\text{O}_2$ through a mechanism involving the incorporation of oxygen into DOM (Cory et al., 2009). The

quenching capacity of DOM is linked to its redox properties, which are mainly attributed to hydroquinone and phenol moieties (Aeschbacher et al., 2012), and therefore can be driven by source and composition. However, DOM can also act as a photosensitizer producing ^3DOM and reactive oxygen species (Page et al. 2014, Janssen et al. 2014). Triplet DOM reactivity is mainly attributed to excitation of aromatic ketones, aldehydes or quinone moieties (Golanoski et al., 2012). On the other hand, the production of $^1\text{O}_2$ by means of photosensitizers follows an energy transfer type II reaction while the formation of H_2O_2 or $\text{OH}^\bullet$ follows an electron transfer type I mechanism (Glaeser et al., 2010a). Therefore, DOM can exert a dual role as both sensitizer and quencher (antioxidant), the strengths of which can be controlled by specific photooxidation conditions and driven by type (source/composition) of DOM (Janssen et al. 2014). While environmental relevance of the quenching process is clearly established, the quantitative assessment in the antioxidant capacity of DOM needs further development.

Two recent studies have reported a method to determine the electron donating (i.e., antioxidant) capacity of the humic substances (Aeschbacher et al., 2010, 2012). Aeschbacher et al. (2012) applied electrochemical techniques using 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid, ABTS), as an electron transfer mediator, to characterize several humic and fulvic acids and two natural organic matter standard solutions from the International Humic Substances Society (IHSS). They reported that the electron donating capacity was in general higher for aquatic humic substances compared to their soil counterparts. Similarly, the antioxidant activity of organic matter from soils has been determined using the ABTS free radical assay (Rimmer, 2006; Rimmer and Smith, 2009; Cardelli et al., 2012). Rimmer and Smith (2009) observed a general decrease of antioxidant activity with depth in soil profiles, seemingly related to an

increase in the degree of humification of the soil OM with depth. Since some common constituents of the aquatic DOM pool are known antioxidants, e.g., lignins, tannins and humic acids (Dizhbite et al., 2004; Aeschbacher et al., 2012), it would be expected that aquatic DOM presented a varying degree of free radical scavenging (antioxidant activity) depending on source and degree of diagenetic degradation. However, to our best knowledge, information about this general subject remains limited, likely in part due to the lack of simple, easily accessible methodologies for their determination.

Two of the most popular methods used to measure the antioxidant activity of plant extracts and isolated compounds are based on the reaction of a colored solution of a free radical, 2,2-diphenyl-1-picrylhydrazyl (DPPH assay) and 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS assay), with the antioxidant in methanol solution (Brand-Williams et al., 1995; Re et al., 1999). The disappearance of color as the free radical is reacting with the antioxidant is measured spectrophotometrically. Both are simple, easy and fast methods but, to our knowledge, they have never been applied to natural DOM. To study the chemical characteristics of aquatic DOM, it is usually necessary to concentrate the sample. Aquatic DOM is composed of very different and, mostly, uncharacterized compounds and their low concentration make its chemical characterization a challenge (Benner, 2002). Solid phase extraction (SPE) with PPL cartridges, a modified styrene divinyl benzene polymer type sorbent, has been recently proposed as a good concentration method of DOM, with a recovery of ~60% (Dittmar et al., 2008; Green et al., 2014). It retains polar to nonpolar substances from large volumes of water and the extract is believed to be well representative of the overall DOM pool. The DOM retained in the PPL cartridge is usually recovered by elution with methanol (Dittmar et al., 2008).

Since both free radical assays, DPPH and ABTS, can be directly applied to alcoholic solutions (DPPH[•] is not soluble in water), they could be used on PPL extracts without previous steps of drying and redissolution of the sample, rendering a fairly simple, high sample throughput approach. Thus, the aim of this study was: 1) to assess the effectiveness of the free radical DPPH and ABTS assays as techniques to measure the antioxidant activity of DOM; 2) to test if aquatic DOM extracted by SPE present antioxidant properties; 3) to assess the differences in the free radical scavenging capacity (antioxidant activity) of DOM from different environmental settings and characteristics.

2. Materials and methods

2.1. Sampling

Individual bulk surface water samples were collected in Everglades National Park (ENP) as part of the Florida Coastal Everglades Long Term Ecological Research (FCE-LTER) in June 2013 (Fig. 1a). Eight samples were collected along the established Taylor Slough (TS/Ph) transect and six along the Shark River Slough (SRS) transect. Sites TS/Ph1-3 and SRS1-3 are characterized as freshwater marsh environments with the sawgrass *Cladium jamaicense* and abundant periphyton as dominant vegetation. The tidally-influenced estuarine sites, SRS4-6, are dominated by mangroves. At SRS4 site, *Rhizophora mangle*, *Laguncularia racemosa* and *C. erectus* are the prevalent mangrove species while at downstream sites, SRS5-6 *R. mangle*, *Avicennia germinans* and *L. racemosa* dominate. Sites TS/Ph6-7 are also located in the fringe mangrove zone, seasonally freshwater-dominated during the wet season and receiving wind driven estuarine inputs during the dry season. Local vegetation is dominated by *R. mangle*, and

C. erectus with some strands of *C. jamaicense*, *Eleocharis* sp. in the northern sections of TS/Ph6 and seasonal growth of *Chara* sp. Finally, three sites are located at shallow (< 3 m) Florida Bay waters (TS/Ph9-11) where the seagrass *Thalassia testudinum* dominates (Ewe et al., 2006). In addition, the distribution of free radical scavenging activity based on gradual DOM source changes was determined along a salinity gradient in two rivers (Shark River and Harney River) inside the ENP. They were sampled in March 2013 (Fig. 1b) and represent an additional 13 samples ranging from the estuarine freshwater to the marine end-member. Salinity was collected with an YSI meter just below the water surface (approximately 0.2 m depth). The nomenclature of the samples here follows the sampling order. It started in Tarpon Bay through Harney River and followed upstream from Ponce de León Bay through Shark River.

All surface water samples were collected in 2 L acid pre-rinsed brown high density polyethylene (Nalgene) bottles and kept on ice until their return to the laboratory. Once there, the samples were immediately filtered through glass microfiber filter (GF/F). One fraction of the filtered sample was extracted by solid phase extraction (SPE) while the remainder was used for dissolved organic carbon (DOC), absorbance and fluorescence measurements.

2.2. Solid phase extraction

The extraction of the DOM was achieved using PPL cartridges according to Dittmar et al. (2008). One liter of 0.2 µm filtered sample (Durapore, Millipore) was acidified to pH 2 with concentrated HCl. PPL cartridges (1 gr size) were conditioned with methanol and acidified MilliQ water (concentrated HCl until pH 2) before extracting the sample. The samples were loaded onto the PPL cartridges by gravity. The cartridges were rinsed with MQ water (pH 2) after the extraction, to remove salt. After that, they were dried with N₂ gas and the retained DOM eluted with 40mL of methanol. The extracts were stored in amber glass vials at -20°C until

analysis within 2 weeks. All the extracts presented pH=7 after methanol evaporation and re-dissolution in MQ water (see section 2.4.).

2.3 Antioxidant measurements

The antioxidant activity of the PPL extracts was determined using two spectrophotometric assays: the free radical DPPH (DPPH[•]) and the free radical ABTS (ABTS^{•+}). The DPPH assay was applied as Brand and Williams et al. (1995) reported and modified for DOM as follows. A volume of 0.8 mL of a methanol solution DPPH[•] (7.09×10^{-5} M), with an absorbance of 0.8 ± 0.03 AU measured at 515nm, was mixed with 0.4 mL of each methanol PPL extract (eluate collected from the cartridge). After the addition of the DPPH[•] reagent, the samples were shaken and left reacting in the dark and at room temperature (21 °C) during 15 min (t=15).

The second free radical assay used here, ABTS, was also applied to the PPL extracts based on Re et al. (1999) and modified for DOM as follows. A solution of 7 mM of ABTS was prepared in MQ water. A volume of 9 mL of that solution was mixed with 1 mL of 24.5 mM potassium persulphate. The mix was left to stand in the dark for 12-16 hours at room temperature to permit the formation of the free radical cation (ABTS^{•+}). The mix was then diluted 50 times until reaching an absorbance of ~0.7 at 734 nm (~0.126 mM). A volume of 0.4 mL of each methanol PPL extract (eluate collected from the cartridge) was added to 0.8 mL of the ABTS^{•+} solution and left reacting in the dark at room temperature (21 °C) during 6 min (t=6).

For both methods, the absorbance decrease, after the reaction time, was measured in a Varian Cary 50 bio spectrophotometer. Each solution was measured by triplicate and the results reported as an average of the 3 measurements. A blank of 0.4 mL of methanol mixed with 0.8 mL of the corresponding free radical solution, was also measured at time 0 (t=0). Since the methanol

extracts present some color, a blank of each one (0.4 mL of each solution mixed with 0.8 mL of methanol) was also measured at t=0. The antioxidant activity or quenching percentage is expressed as:

$$AxA = [1 - (A_A - A_C) / A_B] \times 100 \quad (1)$$

where A_A is the absorbance of the antioxidant sample at the end of the reaction time; A_C is the absorbance of the sample blank at t=0; and A_B is the absorbance of the methanol blank (0.4 mL MeOH + 0.8 mL DPPH[•] or ABTS^{•+} solution) at t=0. The absorbance of the samples measured with ABTS assay at 734 nm was not significantly different from the methanol blank and did not interfere with the measurements, $A_C=0$ for the ABTS assay.

The antioxidant activity results for the DPPH assay were expressed in two ways: 1) as AxA_{total} , the total antioxidant activity of the methanol extract; 2) AxA_{100} and AxA_{50} , the antioxidant activity for 100 mg L⁻¹ and 50 mg L⁻¹ of DOC of the methanol extract, respectively, determined using a calibration curve. In the same way, the antioxidant activity results from ABTS assay were expressed as AxA_{100} and AxA_{50} . This allows comparison of the antioxidant capacity between samples for the same DOC concentration. See Appendix for more details.

We contrasted both colorimetric methods with the electron donating capacity (EDC) method reported by Aeschbacher et al. (2012). The EDC measured by these authors was quantified with an electrochemical oxidation over a wide range of pH values and applied redox potentials (E_h) using ABTS as an electron transfer mediator. We used the same five standards acquired from the International Humic Substances Society (IHSS) used by them (Fig. 2). The standards were Suwannee River HA Standard II (SRHA), Pony Lake FA Reference (PLFA), Suwannee River NOM (SRNOM), Pahokee Peat Fulvic Acid Standard II (PPFA) and Nordic

Aquatic Fulvic Acid (NLFA). The antioxidant butylated hydroxytoluene (BHT) was used as reference.

2.4. Dissolved organic carbon (DOC) and optical measurements

To analyze the DOC of the extracts, an aliquot (100-200 μ L) of each one was dried with N_2 gas. To ensure the complete dryness of the samples, they were also subject to vacuum during 2 h. Once dry, the samples were re-dissolved with a weighted volume of MQ water (10 mL) and their DOC concentrations determined. The pH of the extracts re-dissolved in MQ water was checked rendering pH=7 for all of them. The DOC concentration was measured by high temperature combustion with a Shimadzu TOC-5000 analyzer. Each sample was acidified with 10 μ L of concentrated HCl and sparged for 5 min with nitrogen to remove inorganic carbon. The mean of 3–6 injections (coefficient of variation < 2%) was reported for each sample. The system was standardized daily with a potassium hydrogen phthalate standard.

DOC, absorbance and fluorescence of the GF/F filtered samples, before the SPE extraction procedure, were also measured. EEMs were measured using a Horiba Jovin Yvon SPEX Fluoromax-3 spectrofluorometer equipped with a 150W continuous output Xe arc lamp. Slits were set at 5.7 nm for excitation and 2 nm for emission. Forty-four emissions scans were acquired in a 1 cm quartz cell at excitation wavelength (λ_{ex}) between 240 and 455 nm every 5 nm. The emission wavelengths were scanned from $\lambda_{ex} + 10$ nm to $\lambda_{ex} + 250$ nm (i.e., between 250 and 705 nm) in 2 nm steps. Fluorescence signals were acquired in signal over reference ratio mode (S/R) to eliminate potential fluctuation of the Xe lamp. More detailed information of post-acquisition steps for correction (inner filter effects, instrumental bias) and unit conversion to

221 quinine sulfate units can be found elsewhere (Chen et al., 2010). Fluorescence scans were
222 collected in the signal/reference ratio mode with an integration time of 0.25 s and reported in
223 quinine sulfate units (QSU). EEMs were corrected for instruments optics and inner-filter effects
224 according to Ohno (2002) and Raman normalized and blank subtracted using Matlab 7.0.4.
225 software. UV-Vis analysis was performed using a Varian Cary 50 bio spectrophotometer with a
226 1 cm quartz cuvette, scanning from 250 nm to 800 nm. Measurements were performed at
227 constant room temperature (21 °C). Milli-Q water was used as a blank. The absorption
228 coefficient at several wavelengths, $a_{\text{CDOM}}(\lambda)$ (in m^{-1}), was calculated as $2.303 \times [\text{Abs}(\lambda) - \text{Abs}$
229 $(600-700)]/l$, where $\text{Abs}(\lambda)$ is the absorbance at wavelength λ , l is the path length (m) and
230 $\text{Abs}(600-700)$ the average absorbance between 600 and 700 nm, which corrects for the
231 background signal.

232 PARAFAC modeling statistically decomposes the complex fluorescent matrix into
233 individual, quantifiable components without any assumptions regarding their spectral shape or
234 their number (Stedmon et al. 2003). PARAFAC modeling applied here was achieved by fitting
235 the EEMs of all the samples to an already established PARAFAC model for surface water from
236 the Everglades (Yamashita et al. 2010; Chen et al., 2010). The characteristics of each component
237 have been reported by Yamashita et al. (2010) as C1 (<260(345)/462) ubiquitous humic-like; C2
238 (<260/454) terrestrial humic-like possibly photorefractory; C3 (<260(305)/416) terrestrial
239 humic-like, fulvic acid-type; C4 (<260(305)/376) microbial humic-like; C5 (275(405)/>500)
240 terrestrial humic-like, humic acid-type; C6 (325/406) ubiquitous humic-like, possibly photo-
241 labile, a product of microbial activity, and agricultural land use derived; C7 (275/326) and C8
242 (300/342) protein-like. PARAFAC component spectral characteristics and split-half validation
243 can be found elsewhere (Chen et al., 2010). The analysis was carried out in MATLAB 7.0.4.

(Mathworks, Natick, MA) with the DOMFluor toolbox (Stedmon and Bro 2008). No residual peaks were observed after fitting EEMs to established PARAFAC model.

Principal component analysis (PCA), using SPSS software, was applied to the results from the PARAFAC modeling and to the antioxidant activity for 100 mg L⁻¹ of DOC (for both methods separately). The values obtained after the PCA analysis were identical no matter which of these two methods was used. Therefore, for simplicity, we only show here those calculated with ABTS assay.

3. Results

The antioxidant activity or quenching percentage for 50 mg L⁻¹ of DOC (AxA₅₀) measured using both free radical assays, DPPH and ABTS, significantly correlated ($R^2 = 0.91$; $p < 0.05$ and $R^2 = 0.95$; $p < 0.005$) with the electron donating capacity (EDC) reported by Aeschbacher et al. (2012) for the same humic and fulvic standard substances (Fig. 2a and b). This supports the validity of the free radical scavenger methodology used here, suggesting that both approaches could be used as proxies of DOM antioxidant activity. Among the humic, fulvic and natural organic matter (NOM) standards used here, Suwannee River HA Standard (SRHA) showed the highest AxA₅₀ at 32.1% (DPPH assay) and 40% (ABTS assay), while Pony Lake Fulvic Acid (PLFA) showed the lowest one, 4.8% and 8.9%, for DPPH and ABTS assays, respectively. Suwannee River NOM showed an intermediate value of 16.8% and 18%, for DPPH and ABTS assays, respectively. We also calculated the AxA₅₀ for the reference antioxidant butylated hydroxytoluene (BHT) with the DPPH assay and it presented the highest value with

49.1% of A_{x50} . The results from both assays presented a high and significant correlation between them, $R^2 = 0.70$; $p < 0.01$ (Fig. 2c).

The recovery of the PPL extractions (calculated as the fraction of DOC eluted from the whole DOC of the sample) was on average $68 \pm 3\%$, a value close to the average of 62% of efficiency of PPL extraction reported by Dittmar et al. (2008). The DOC concentrations of the LTER whole samples oscillated between 4.46 mg L^{-1} for marine samples (Florida Bay) and 16.18 mg L^{-1} for samples from mangrove zones (Table 1). These values are within the DOC concentration range measured in the Everglades National Park (ENP) LTER program during the wet season (Chen et al., 2013). The $a_{\text{CDOM}}(340)$ in the LTER sampling were lowest in the Florida Bay samples ($2.04\text{-}5.34 \text{ m}^{-1}$) and highest in the mangrove areas ($29.11\text{-}35.13 \text{ m}^{-1}$, Table 1). Fluorescent PARAFAC components of these sites have already been reported (Chen et al., 2013). In general, PARAFAC component fluorescence decreased from fresh to marine waters similarly to DOC and CDOM with some high values in the fringe mangrove zone (see also Chen et al., 2013; Cawley et al., 2013; Timko et al., 2014). PARAFAC humic-like components in the LTER sampling showed the highest relative abundance in the Shark River Slough samples and in TS/Ph-7. The lowest values were found in the marine Florida Bay samples. The protein-like components presented the highest relative abundance for the marine areas.

DOC, CDOM and FDOM behavior along the salinity transects (Harney and Shark Rivers) were similar to those previously published in the same rivers (Cawley et al., 2013; Timko et al., 2014). Briefly, DOC presented a general inverse relationship with salinity, decreasing with the increase of salinity (Fig. 3a). It behaved non-conservatively in both rivers, specially in the Harney River (Fig. 3a). Values of $a_{\text{CDOM}}(340)$ in samples from the Shark River were higher than those from Taylor River. In both rivers, $a_{\text{CDOM}}(340)$ presented a similar trend

than DOC as well as a non-conservative behaviour (Fig. 3b). The influence of the mangroves was observable in the high values of $a_{\text{CDOM}}(340)$ of samples collected in those areas. Also most PARAFAC fluorescent components showed a decrease of fluorescence intensity with the increasing of salinity and a non-conservative mixing behaviour. Our results of the PARAFAC fluorescent components agree with those published for the same rivers, Shark River and Harney River, during March 2010 and April 2013 (Cawley et al., 2013; Timko et al., 2014).

LTER samples showed a wide range of $\text{AxA}_{\text{total}}$ (DPPH assay) from 11.0% for seagrass-dominated Florida Bay (TS/Ph-9) to 74.4% for TS/Ph-7, at the Taylor River mouth where mangroves are the dominant vegetation (Table 2). The $\text{AxA}_{\text{total}}$ (DPPH assay) was dependent on DOC concentration since both variables correlated significantly ($R^2 = 0.75$; $p < 0.005$; $N = 14$). When the antioxidant activity was calculated for 100 mg L^{-1} of DOC, LTER samples could be clasified in three areas attending to their origin: mangrove, marine and freshwater marsh zones. Samples from mangrove zones presented the highest AxA_{100} , with a range of 23.7- 29.6% (DPPH assay) and 32.5%-44.9% (ABTS assay). Samples from freshwater and marine zones presented similar values (14.8-19.3% with DPPH assay and 10.03-22.12 with ABTS assay) with the lowest value for the Florida Bay TS/Ph-9 sample (11.3% and 7.5%, with DPPH and ABTS assays, respectively). The three Florida Bay samples presented an increase of the antioxidant activity with increasing salinity (Table 2).

For samples from Harney and Shark River transects, $\text{AxA}_{\text{total}}$ showed a similar pattern than for DOC, decreasing with salinity (Table 2, Fig. 3c). Harney River values presented a more pronounced non-conservative behaviour than in Shark River. In general, samples from Harney River presented a higher $\text{AxA}_{\text{total}}$ than those from Shark River. However, values of antioxidant activity calculated for 100 mg L^{-1} of DOC, AxA_{100} , with both assays showed a different

distribution with a trend to increase with salinity (Table 2, Figs. 4d-e). Samples from mangrove areas showed the highest values of $Ax A_{100}$. Two samples from the transect in Shark River (March 2013), S2 and S3, were geographically close to LTER sites SRS6 and SRS5, respectively, and their $Ax A_{100}$ were similar. Samples from mangrove zone collected along the transects presented values of $Ax A_{100}$ within the range of those found for mangrove areas collected during LTER sampling in June.

Principal component analysis (PCA) including PARAFAC components and $Ax A_{100}$ of the LTER data showed that PC1 accounted for 62% and PC2 accounted for another 18% of the variance (Fig. 4). Components C1, C3, C5 and $Ax A_{100}$ presented negative values of PC1 while the rest were positive. The $Ax A_{100}$ loading was very close to those of the ubiquitous humic-like components C1 and C5. Samples from mangroves zones were placed in the more negative side of PC1, those from freshwater marsh areas showed values of PC1 close to zero, and marine samples were on the positive side of PC1.

4. Discussion

The free radical assays used here can be applied in methanol or ethanol solutions. Since DOM extracts using PPL cartridge are commonly eluted with methanol, these assays are very appropriate for analyzing the antioxidant properties of those DOM extracts. The common use of PPLs in DOM research (Dittmar et al., 2008), the general availability of UV-Vis spectrophotometers, and the general technical simplicity of the proposed assays, makes them ideal for studies of antioxidant activity of marine and freshwater DOM.

It has been reported that the reactivity of $DPPH^{\bullet}$ free radical is more strongly influenced by steric effects than reactivity of $ABTS^{\bullet+}$, so it has to be carefully used when the studied

samples present antioxidants of different structural classes (Apak et al., 2013). However, the two free radical assays used here appropriately worked for the measurement of antioxidant activity of the DOM. Both methods presented significant and high correlation ($R^2 > 0.9$) with the electron donor capacity analyzed by Aeschbacher et al. (2012, Fig. 2). The results from both assays, DPPH and ABTS, showed the same trend between samples and a significant correlation ($R^2 = 0.70$; $p < 0.01$) between them. An advantage of the ABTS assay is that, unlike DPPH assay that can be only applied to alcoholic solutions due to its insolubility in water, the ABTS can also be applied to aquatic solutions. In both cases, it is necessary to measure the sample at different concentrations and build a calibration curve as it was done here (see Appendix).

4.1. Distribution of the antioxidant activity

Since the AA_{total} is dependent on DOC concentration ($R^2 = 0.43$; $p < 0.001$; $N = 27$), the calculation of the antioxidant activity for 100 mg L^{-1} of DOC (or 50 mg L^{-1} of DOC as appropriate) was necessary to compare the quality of the DOM from different sites. DOM from different areas of the Everglades presented a different antioxidant activity for the same concentration of DOC, indicating a different quality and composition of the DOM. The differences in the quality of DOM from different sites of the Everglades had been shown in previous works using optical techniques (e.g., Yamashita et al., 2010; Chen et al., 2013). The range of values of AxA_{100} for all our samples was within the range of the measured humic and fulvic acid standards (Fig. 2), between Suwanee River Humic Acid (SRHA) and Pony Lake Fulvic Acid (PLFA) values. In the LTER study, the highest antioxidant activity corresponded to those samples from mangrove areas. This is also observable in both salinity transects along

Harney and Shark Rivers. Mangroves are known to produce and release a significant amount of polyphenols, e.g., tannins (Maie et al. 2006). Polyphenols, and tannins in particular, are known antioxidants (Amarovicz, 2007). Therefore, the high free radical scavenging activity found in mangrove area samples is probably due to a high presence of such compounds in the DOM.

PCA of the EEM-PARAFAC and AxA₁₀₀ (ABTS assay) data shows that PC1 is controlled by the source of the samples. The same results were observed using DPPH assay instead (data not shown). Marine samples (TS/Ph9-11), whose predominant species is the seagrass *T. testudinum*, presented positive values of PC1, indicating that they are enriched in microbial and protein-like components (C4, C7, C8) compared to the rest of the samples (Chen et al., 2013; Maie et al., 2012). Samples from the freshwater marsh and mangrove estuary, with sawgrass and mangroves as predominant vegetation, respectively, are more enriched in humic-like compounds (C1, C3 and C5), especially the latter. This agrees with the affirmation that terrestrial humic-like materials are the main components of the mangrove CDOM contributing to Shark and Harney River (Cawley et al., 2013). Plotting PC1 values versus AxA₁₀₀ (Fig. 4c) we observed a significant negative correlation ($AxA_{100}=0.24-0.08\cdot PC1$; $R^2=0.50$; $p < 0.005$; $N=14$) between both variables, with samples grouped as: 1) marine; 2) freshwater marsh; 3) mangrove. From that graph, we concluded that the antioxidant activity of the DOC increased, according to the source of the sample, in the following order: marine $\leq$ marsh < mangrove. For the marine samples collected in Florida Bay (TS/Ph9-11) an increase in antioxidant activity was observed with salinity, increasing off-shore. The relatively high antioxidant activity at these sites is interesting, and probably due to the presence of polyphenols released by the seagrass (Maie et al., 2006). The higher antioxidant activity of DOM at sites TS/Ph-10 and, particularly, TS/Ph-11 may be related to higher cover of healthy seagrass beds at these sites compared to TS/Ph-9

(Fourqurean and Robblee, 1999). An increment in the signal of seagrass-derived OM in sediments along a similar transect in Florida Bay has also been reported (Xu et al., 2006).

It is known that the solvent variables, like pH or ionic strength, affect the chemical properties (i.e., optical, conformation size) of DOM and that those properties also condition key physico-chemical reactions in aquatic systems (Pace et al., 2012; Baalousha et al., 2006). Actually, humic substances and FDOM are thought to be supramolecular associations of relatively small heterogeneous molecules held together by weak dispersive forces that can be reversibly affected by a pH change (Piccolo et al., 2001; Romera-Castillo et al., 2014). For that reason, the antioxidant behavior of the DOM in natural water could also be affected by such environmental variables. All the methanol extracts in this study presented the same pH value (ca. 7) and ionic strength, so the antioxidant activity measurements were not affected by them. In this study, the pH values in the sampling sites (7.2-8.4), are thought to not affect the supramolecular structure of the humic acids which is more susceptible to change at lower pH (Piccolo et al., 2001; Romera-Castillo et al., 2014). However, further investigations are needed to better understand to which extent the antioxidant behavior of aquatic DOM is affected by variables like pH or ionic strength in nature.

Photoproducts from irradiated DOM have been suggested to inhibit bacterial growth (Reche et al., 1998; Ortega-Retuerta et al., 2007). These include the generation of inhibitory compounds such as reactive oxygen species (Zepp et al., 1977; Cooper et al., 1989). Antioxidants or free radical quenchers associated with DOM could have beneficial effects on microorganisms, preventing cell damage caused by free radicals and affecting microbial loop dynamics. Some examples of the reported beneficial effects of the antioxidants on the microbial activity can be found in the literature. Scully et al. (2003) showed that bacterial growth increased in the presence

of a ROS scavenger in a water lake sample. Kaiser and Sulzberger (2004) reported a decrease of 80% of the DOC bacterial utilization after sunlight exposure of several DOM fractions that was significantly reduced with the addition of free radical scavengers. However, the presence of DOM photolysis products, such as $^1\text{O}_2$, has been also shown to favor some bacterioplankton phylotypes. These are believed to adapt to the $^1\text{O}_2$ exposure and take advantage of the simultaneously generated low molecular weight substances (Glaeser et al., 2010b). Therefore, DOM as photosensitizer could also have a positive role on microorganism health that will depend on the species and, likely, the type of photolysis product that it generates. The study of the dual role of DOM as photosensitizer and antioxidant and how it affects the microbial population is an interesting and important research subject, but beyond the scope of this study.

This work contributes to the understanding of the composition of DOM and the role of its constituents in the biogeochemical cycle. It is a first step in the knowledge of the distribution of the antioxidant activity of the DOM and its causes. The methodology presented herein may be ideal to move this field forward. We have shown evidence that natural aquatic DOM presents free radical scavenging capacity in different degree depending on its origin. However, the role of the antioxidants in the biogeochemical processes is an open field that need to be explored in future studies.

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Table 1. Values of salinity, DOC (mg L⁻¹), $a_{\text{CDOM}}(340)$ (m⁻¹) of samples collected during LTER program in June 2013.

Sample	Salinity	DOC whole sample (mg L ⁻¹)	$a_{\text{CDOM}}(340)$ (m ⁻¹)
TS/Ph-1	0	8.4	15.06
TS/Ph-2	0	6.23	9.98
TS/Ph-3	0	8.0	13.74
TS/Ph-6	0.8	13.05	19.65
TS/Ph-7	5	16.18	35.13
TS/Ph-9	33.36	5.21	2.22
TS/Ph-10	35.16	4.46	2.04
TS/Ph-11	37.79	6.63	5.34
SRS-1	0	13.32	25.54
SRS-2	0	13.11	27.66
SRS-3	0	12.7	29.11
SRS-4	0.9	12.97	29.63
SRS-5	2	14.14	33.13
SRS-6	14.2	11.33	26.59

Table 2. Values of total antioxidant activity and antioxidant activity calculated for 100 and 50 mg L⁻¹ of DOC of extract using DPPH and ABTS assays. AxA is expressed as a percentage, so it is unitless.

Sample	AxA _{total} DPPH	AxA ₁₀₀ DPPH	AxA ₅₀ DPPH	AxA ₁₀₀ ABTS	AxA ₅₀ ABTS
TS/Ph-1	37.1 ± 0.2	17.3 ± 0.8	12.0 ± 1.0	22.12 ± 0.5	15.53 ± 0.6
TS/Ph-2	30.3 ± 1.1	16.9 ± 0.9	10.6 ± 1.0	21.66 ± 0.4	12.35 ± 0.4
TS/Ph-3	31.8 ± 0.5	19.2 ± 0.3	14.23 ± 0.5	18.61 ± 0.7	11.71 ± 0.3
TS/Ph-6	52.0 ± 0.4	24.9 ± 0.4	14.36 ± 0.2	39.67 ± 1.3	23.99 ± 0.8
TS/Ph-7	74.4 ± 0.5	29.2 ± 0.3	22.3 ± 0.3	44.49 ± 0.5	31.42 ± 0.8
TS/Ph-9	11.0 ± 0.3	11.3 ± 0.2	6.0 ± 0.2	7.48 ± 0.4	5.28 ± 0.4
TS/Ph-10	14.0 ± 0.2	15.4 ± 0.5	9.3 ± 1.0	11.85 ± 0.4	8.55 ± 0.1
TS/Ph-11	16.9 ± 0.2	18.7 ± 0.1	13.5 ± 0.3	18.78 ± 0.1	13.34 ± 0.1
SRS-1	36.4 ± 0.4	14.8 ± 0.5	10.3 ± 0.4	10.03 ± 0.0	5.21 ± 0.3
SRS-2	42.2 ± 0.5	16.2 ± 0.2	11.2 ± 0.4	13.06 ± 0.3	8.85 ± 0.4
SRS-3	44.2 ± 0.4	19.3 ± 0.1	15.2 ± 0.6	17.98 ± 0.5	11.71 ± 0.1
SRS-4	63.1 ± 0.4	23.7 ± 0.1	15.2 ± 0.1	33.52 ± 1.0	23.07 ± 0.6
SRS-5	70.3 ± 0.3	28.3 ± 0.0	16.6 ± 0.5	36.34 ± 0.1	25.09 ± 0.3
SRS-6	67.3 ± 0.4	29.6 ± 0.4	17.7 ± 0.3	32.56 ± 0.2	19.48 ± 0.4
TB	58.7 ± 0.6	14.0 ± 0.6	6.0 ± 0.0	19.64 ± 0.5	10.64 ± 0.9
H1	69.5 ± 0.1	18.7 ± 0.4	11.1 ± 0.5	32.19 ± 0.3	21.46 ± 1.0
H2	71.2 ± 0.1	20.1 ± 0.8	13.0 ± 0.2	34.39 ± 0.9	22.86 ± 1.0
H3	72.5 ± 0.2	23.0 ± 0.9	15.0 ± 0.7	37.69 ± 0.1	24.40 ± 0.7
H4	69.8 ± 0.9	25.7 ± 0.4	16.5 ± 0.8	38.49 ± 0.4	21.67 ± 0.4
H5	64.7 ± 0.7	28.9 ± 0.2	19.7 ± 0.4	38.88 ± 0.1	22.25 ± 0.6
PLB	24.0 ± 0.5	18.3 ± 0.4	12.3 ± 0.5	15.85 ± 0.6	7.80 ± 0.6
S1	28.2 ± 0.4	21.2 ± 0.2	11.9 ± 1.5	19.37 ± 0.3	8.83 ± 0.7
S2	50.9 ± 0.2	28.0 ± 0.3	17.1 ± 0.1	33.16 ± 1.1	18.73 ± 0.6
S3	51.9 ± 0.1	25.9 ± 0.6	16.3 ± 0.7	26.12 ± 0.9	15.82 ± 0.4
S4	63.7 ± 0.7	25.8 ± 0.8	15.7 ± 0.1	32.63 ± 0.5	22.86 ± 0.8
S5	64.7 ± 0.2	24.3 ± 0.4	15.5 ± 0.4	28.42 ± 0.2	19.50 ± 0.7
S6	67.2 ± 0.5	18.5 ± 0.8	13.3 ± 0.1	27.58 ± 0.2	17.44 ± 0.3

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571 **Figure legends**

572 **Fig. 1** Map of the sampling sites. Everglades LTER program sites (a) and Salinity transects along
573 the Harney River and Shark River (b). Harney River transect (TB: Tarpon Bay; H: Harney River;
574 PLB: Poncel de León Bay); Shark River Transect: S. Panel b) is a subset of panel a).

575 **Fig. 2.** Relationships between the antioxidant activity measured by mediated electrochemical
576 oxidation method (data from Aeschacher et al. 2012) and that measured by the free radical: a)
577 DPPH assay; and b) ABTS assay, for five humic and fulvic standards from IHSS. c) Relationship
578 between DPPH assay versus ABTS assay for all the samples used in this study.

579 **Fig. 3** Distribution along the salinity gradient of: (a) DOC concentration, (b) $a_{CDOM}(340)$, (c)
580 total % antioxidant activity of each extracted sample normalized for 1000 Kg of extracted water;
581 (d) proportion of antioxidant activity for 100 mg L⁻¹ of DOC extract (AxA₁₀₀) measured with
582 DPPH assay; e) proportion of antioxidant activity for 100 mg L⁻¹ of DOC extract (AxA₁₀₀)
583 measured with ABTS assay. Antioxidant activity is expressed as percentage.

584 **Fig. 4** Principal component analysis (PCA): a) loadings plot; b) Score plots, for PC1 and PC2
585 which represent 62.3 and 16.5% of the variability, respectively. c) PCA score plots for PC1 as a
586 function of AxA₁₀₀ (ABTS assay) for the LTER samples.

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5 **Fig. 1**

Fig. 2.

Fig. 3

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5 **Fig. 4**

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