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# 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 and Hongve 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; Amarowicz 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 (•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 $^1O_2$ 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 $^3$DOM 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 $^1O_2$ by means of photosensitizers follows an energy transfer type II reaction while the formation of $H_2O_2$ or $OH^•$ follows an electron transfer

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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-picryhydrazyl and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid, DDPH and ABTS assays, respectively)) 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 neccesary 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 redisolution 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 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 *Conocarpus 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* and *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 (TB) through Harney River (H) and followed upstream from Ponce de León Bay (PLB) through Shark River (S).

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 $N_2$ gas and the retained DOM eluted with 40 mL 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 ($ABTS^{\bullet+}$). The DPPH assay was applied as Brand-Williams et al. (1995) reported and modified for DOM as follows. A volume of 0.8 mL of a methanol solution DPPH• ($7.09 \times 10^{-5}$ M), with an absorbance of 0.8 ± 0.03 AU measured at 515 nm, 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