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ADAPTA

Photo-oxidation processes, properties of DOC, reactive oxygen species (ROS), and their potential impacts on native biota and carbon cycling in the Rio Negro (Amazonia, Brazil)

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Abstract Given the reported degraded nature of DOC in the Rio Negro, and low oxygen, pH, and bacterial riverine levels, we hypothesized: (1) DOC would have strong humic and fulvic acid fluorescence signals with high aromaticity and large mean molecular weight; and (2) photo-oxidation rates would be slow, and reactive oxygen species (ROS) concentrations low, producing no oxidative stress in biota. We surveyed the environment and properties of DOC and explored DOC photo-oxidation and fish sensitivity to DOC products. DOC properties were investigated using absorption and fluorescence indices and parallel factor analysis (PARAFAC) of excitation–emission matrices. ROS concentrations were measured spectrophotometrically. A native fish, *Hemigrammus levis*, was exposed to photo-oxidizing DOC and its tissues (brain, gill, liver) assayed for changes in antioxidant and biotransformation enzymes. With respect to our hypotheses, (1) DOC was highly terrigenous, with high SAC_{340} values (aromaticity), high capacity to produce ROS, and high tryptophan-like fluorescence (bacterial, autochthonous signal); (2) photo-oxidation rates were appreciable, while products were related to mean UV-radiation levels (total radiation was constant). ROS levels were often higher than freshwater averages, yet fish experienced no oxidative stress. Results suggest photo-oxidation influences patterns in C-cycling, bacterial production and community dynamics between wet and dry seasons.

Keywords Photo-oxidation · EEMS · PARAFAC · Oxidative stress · Carbon cycling · Anavilhanas Archipelago

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Introduction

The degradation of dissolved organic carbon (DOC) through photolysis and microbial action drives heterotrophic food webs in aquatic ecosystems and is essential to carbon cycling in the ecosystem (Wetzel,

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2001; Cammack et al., 2004; Tucker & Williamson, 2011). The importance of DOC and DOC cycling cannot be overstated (e.g., Cory et al., 2014). DOC is composed of a complex mixture of organic compounds derived from the degradation of biological material (Thurman, 1985). Humic substances (i.e., humic and fulvic acids), which are the largest component of DOC (40–80%, Thurman, 1985; Wetzel, 2001), are responsible for many of its properties from fluorescence and color to its ability to regulate pH, complex metals, and nutrients, alter biological membranes, and cause oxidative stress in organisms (Thurman, 1985; Kullberg et al., 1993; Küchler et al., 1994; Vigneault et al., 2000; Steinberg et al., 2006; Winters et al., 2007: Wood et al., 2011). The net results of the ecological and physiological effects of DOC are alterations in species diversity, animal behavior, productivity, and food web structure.

Photolysis can either work directly on DOC, without the need for oxygen, and lead to the production of dissolved inorganic carbon (DIC, i.e., CO_2) (Patel-Sorrentino et al., 2004), or UV radiation (280–400 nm) can interact with the chromophores of DOC changing excitation states, producing reactive oxygen species (ROS) and degrading DOC, possibly also with conversion to CO_2 (Scully et al., 1996). This is known as photo-oxidation and requires the presence of oxygen. Hydrogen peroxide (H_2O_2), a strongly reactive ROS, is formed from the dismutation of the oxygen radical, superoxide, formed during photo-oxidation. H_2O_2 is a relatively stable molecule and proceeds to further alter DOC creating smaller organic molecules (Scully et al., 1996). H_2O_2 can also pass through cell membranes and cause oxidative stress in organisms (Abele et al., 1998; da Rosa et al., 2008).

The goal of studying DOC is to relate its structure and quality to its role in aquatic systems and carbon cycling. Two multidisciplinary, field expeditions under the ADAPTA Program (Adaptations of Aquatic Biota of the Amazon) provided an excellent opportunity to study the DOC of the Rio Negro, the largest tributary of the Amazon River. The expeditions were scheduled for December to accommodate the availability of the majority of participants, and corresponded with the end of the dry season/beginning of the wet season.

DOC in the Rio Negro is different from DOC in other Amazonian rivers: it is particularly altered in chemical structure due to aerobic degradation within the podzolic soils of its valley (Ertel et al., 1986). These soils have little adsorptive capacity and allow the colored, aromatic humic and fulvic acids produced from plant degradation (DOC) to leach into the river creating a blackwater river (Leenheer, 1980). The watershed of the Rio Negro is composed primarily of forests on weathered, white-sands which are nutrient poor and the forest is highly specialized in retaining nutrients (Jordan & Herrera, 1981). Thus, the river is also low in suspended solids and solutes (Forsberg et al., 1988; Konhauser et al., 1994; Aucour et al., 2002). Primary productivity is low and the system is net heterotrophic (Benner et al., 1995), and thus, the ecosystem is supported by DOC processing. Although DOC in the Rio Negro has been degraded more than that in other Amazonian rivers, it is still susceptible to photo-oxidation and microbial attack (Amon & Benner, 1995). Recent studies have shown that it has retained protective properties against osmoregulatory stress at low pH (Wood et al., 2003; Durate et al., 2016) and copper toxicity (Matsuo et al., 2005).

Our study examines the quantity and quality of the Rio Negro DOC at the end of the dry season using absorption and fluorescence indices and parallel factor analysis (PARAFAC) of excitation–emission matrices (c.f. Al Reasi et al., 2011). These data can provide information on the relative allochthonous (terrigenous) versus autochthonous (algal, microbial) character of DOC, its level of degradation as indicated by its aromaticity and mean molecular size, its protective potential compared with other known blackwaters, and its potential to produce ROS. At the same time, photo-oxidation experiments were performed to provide information on its lability through changes in composition with exposure to sunlight. Measurements of ROS (H_2O_2) concentrations were taken to determine the level of ROS exposure of organisms due to photo-oxidation of DOC. Water quality factors may also influence ROS production (pH, temperature, oxygen) (Scully et al., 1996; Bruskov et al., 2002a, b) and those potential relationships were assessed. Lastly, we asked whether exposure of a small, native, planktivorous fish, *Hemigrammus levis*, to the products of photo-oxidation and ROS production would affect its oxidative status, because ROS and some components of DOC can exert oxidative stress on organisms (Araujo-Lima et al., 1986; Abele et al., 1998; Steinberg et al., 2006).

Given the character of the river water (low pH and low oxygen levels), the highly degraded character of the DOC and the low bacterial biomass, we

hypothesized (1) that the DOC would show strong humic and fulvic acid fluorescence signals together with high levels of aromaticity and large mean molecular weights, and (2) the rates of photo-oxidation would be low due to the low oxygen content of the water, resulting in little change in DOC structure and low ROS water concentrations. We also hypothesized (3) that fish living in this environment would be adapted to high DOC concentrations, and the ROS concentrations and the DOC products produced by photo-oxidation.

Materials and methods

The Anavilhanas Archipelago is a region of long islands, lakes, and channels through which the Rio Negro flows about 110 km up river from Manaus, Brazil (Fig. 1). The research vessel, the Ana Clara, was docked at the floating ranger station (2°43′10.9″S, 60°45′18.8W). The study was carried out over two periods, Dec 5–14, 2013 and Dec 3–13, 2014, at the end of the dry season and beginning of the wet season.

Environmental assessments

DOC, ROS, and water quality variables were examined in the main channel (MC) of the archipelago, in a lake, and in the near-shore of the lake (LNS) where terrestrial vegetation extended into the lake (Fig. 1). Sampling always occurred in the mid-afternoon (3:00–4:45 pm). In the first year, each habitat was sampled at least twice, and in the second year, only once. Sites were generally located along a 5–6-point transect. Only on the first trip (Dec 8, 2013) was a single site sampled. It was in the MC at 100 m offshore, in addition to water quality measurements, three replicate surface samples (0.05 m depth) were taken for both ROS and DOC plus two samples for ROS from 15 m depth, one at the site and one further offshore. On Dec 14, 2013, an along-shore transect was set up in the MC approximately 100 m from shore. Only surface samples were collected due to the impending storm. The next year, on Dec 8, 2014, an across-channel transect was established in the MC, perpendicular to the along-shore transect, and sampled for DOC and water quality measures at the surface and at depths down to 10 m (DOC—at 0.05, 5.0, and 10.0 m; water quality—at 0.05, 0.50, 1.0, 2.0, 3.0, and 4.0 m). In the lake, a mid-lake transect was established which ran down the center of the lake from the source to the outflow. This transect was sampled on Dec 9—partial transect only due to weather, Dec 11 and 14, 2013 and Dec 3, 2014.

Single replicates for ROS and DOC were collected from 0.05 m depth at each site along the transects. ROS was examined only in 2013, all other variables

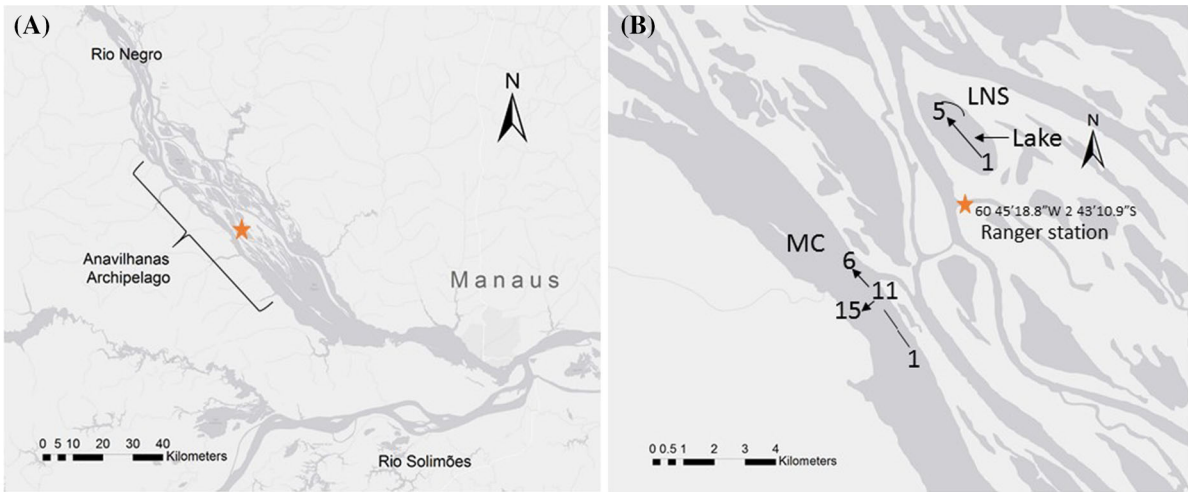

Fig. 1 **A** Map of the reach of the Rio Negro above Manaus showing the Anavilhanas Archipelago and the location of the ranger station where the research vessel was docked. **B** The research area indicating the locations of the along-shore (1–6) and across-channel (11–15) transects in the main channel (MC), and the mid-lake (1–5) transect and near-shore (LNS) sites within the lake. Maps were taken from Esri, HERE, DeLorme, MapmyIndia, © OpenStreetMap contributors, and the GIS user community

were measured in both years. For both ROS and DOC samples, water was filtered through a 0.45-μm syringe-tip filter (Acrodisc, Pall Corporation, Port Washington, USA) into a clean, sample-water rinsed, glass vial and held in the dark on ice until return to the ship. As noted above, DOC samples were also collected in the MC, at 5 and 10 m depths in 2014. Two liters of surface water were collected in dark, plastic bottles from the central site of each transect in 2014 for DOC photo-oxidation experiments.

At each site, oxygen concentration, pH, temperature, and conductivity were measured with a multimeter HI 9828 (Hanna Instruments, Woonsocket, USA; Coefficients of variation for each measure [readings every 20 min over 2 h under stable conditions] were 2.23, 0.02, 0.26, 0.0%). On four trips (MC: Dec 8, 2013 and Dec 8, 2014; lake: Dec 14, 2013 and Dec 3, 2014), vertical profiles were measured to a maximum depth of 4 m with the multimeter and transparency measured with a Secchi Disk. In the MC, a Van Dorn sampler was utilized to collect water from depths greater than 4 m. On the ship, DOC samples were transferred to black, light-proof bags and stored at 4°C for later analysis. Samples for ROS were analyzed immediately.

ROS concentrations were determined fluorometrically using black, 96-well plates and the Amplex UltraRed method (Molecular Probes, Eugene, USA), which measures H_2O_2. Excitation and emission wavelengths were set at 568 and 581 nm, respectively. The samples were held on ice until processed. Seven-point H_2O_2 (Life Brand, 3% H_2O_2 w/w, Shoppers Drug Mart, Vancouver, Canada) calibration curves were constructed from 0 to 10 μM using water from the same location which had been allowed to sit in the dark at air temperature for at least 12 h in order to eliminate any ROS in the water (e.g., Johannsson et al., 2014). The detection limit of this analysis is ≤80 nM (Molecular Probes, 2009).

DOC analyses

DOC concentrations of all environmental and experimental samples were measured on a high temperature Total Organic Carbon Analyzer (Shimadzu, Kyoto 604-8511, Japan). The TOC machine was calibrated using primary standard grade potassium hydrogen phthalate (KHP) as per manufacturer's instructions. In addition, periodic (i.e., every 10 samples) KHP tests were run during analysis to ensure consistent instrument response. KHP standards consistently agreed within 1% of expected values. Spectral profiles were determined at 10-nm intervals between 200 and 600 nm [2013: SpectraMax Plus 384 spectrophotometer (Molecular Devices, Sunnyvale, CA 94089, USA); Varian Cary 50 UV–Visible Spectrophotometer (Cary 50 Software-Scan) (Agilent Technologies Canada Inc., Mississauga, ON, Canada)]. A reference profile for MilliQ water was subtracted from the absorbance profiles. Fluorescence characteristics of DOC were investigated by exciting DOC at all wavelengths between 200 and 450 nm and recording the emission spectra between 250 and 600 nm. Fluorescence excitation versus emission fluorescence matrices (FEEM) were recorded using a Cary Eclipse fluorescence spectrophotometer (Agilent Technologies Canada Inc., Mississauga, ON, Canada). Fluorescence was measured on the samples at their ambient pH. A laboratory standard mixture of tyrosine and tryptophan and allochthonous carbon from Luther Marsh (at 5 mg C/L) was run as a quality control standard, to ensure instrument performance matched past measurements (Al Reasi et al., 2011, 2012). Fluorophore variation was less than 5% and no systematic variation was found in FEEM measurements over time. In addition, regular blank FEEM scans were performed (MilliQ water) to ensure the cuvette was not contaminated.

Properties of DOC were characterized in terms of both absorbance and fluorescence. With respect to absorbance, four indices were calculated:

(1) the specific absorbance at 340 nm (2.303 × absorbance at 340 nm DOC^{-1} × 1000) (SAC_{340}), which is a measure of aromaticity (cyclic rings in the humic substances), and of the protective potential of DOC against some metals and ionoregulatory disturbance (Al Reasi et al., 2011; Wood et al., 2011);
(2) the ratio of absorbance at 254 to 365 nm ($R_{254/365}$), which is a measure of the average molecular weight within DOC (Dahlén et al., 1996);
(3) the ABS DOC^{-1} (total absorbance of $DOC_{200–600}$ DOC^{-1}), which is a measure of the specific total chromophore absorbance; and
(4) absorption coefficient at 310 nm (2.303 × $absorption_{310}$/pathlength (cm)) (Ka_{310}), which has been related to the rate of ROS production during DOC photo-oxidation (Scully et al., 1996).

The fluorescence index, FI, a relative measure of the autochthonous versus allochthonous source material in the DOC, was calculated as the ratio of the emission at 450 to 500 nm upon excitation at 370 nm (McKnight et al., 2001). PARAFAC analysis was performed on the spectral fluorescence data using the PLS Toolbox (Eigenvector Research, Wenatchee, WA, USA) on a Matlab platform (The Mathworks, Inc., Natick, MA, USA) (DePalma et al., 2011; Al Reasi et al., 2012). The program was set to select the four dominant subgroups which typically correlate with the humic acids (HA), fulvic acids (FA), and tyrosine (Tyr)- and tryptophan (Tryp)-like moieties (Fig. 2). Information from PARAFAC analysis can shed light on the origin, composition, and degree of degradation of DOC (Hudson et al., 2007). Pure tyrosine and tryptophan FEEMs were included in the PARAFAC dataset as a means of "weighting" the data toward recovering fluorophores corresponding to these fluorescent amino acids. PARAFAC analysis explained 98.1% of the variability in the entire dataset using the four-component model.

Photo-oxidation experiments

Two light-exposure experiments were performed in 2014. The first experiment was run on Dec 4 and 5 using water collected from the lake on Dec 3. The second experiment took place on Dec 12 using water collected from the MC on Dec 8. In both cases, on the evening before the experiment, bulk water, collected at the mid-transect site, was filtered (0.45 μm) into eleven 130-ml rectangular, quartz bottles (no bubbles) and two 22-ml glass vials wrapped with aluminum foil. The latter vials constituted the cold, dark initial condition controls. The bottles were stored in the refrigerator overnight. In early morning, 5 of the 11 experimental bottles were wrapped in aluminum foil to serve as dark temperature controls and all bottles were laid horizontally in a white tray with full sun exposure, in a flowing water bath. Water covered the bottles by no more than 1 cm. The lake experiment ran from 5:40 a.m. Dec 4 to 1:45 p.m. Dec 5. It was extended to 2 days due to cloudy conditions and the fact that the experimental setup was shaded as of 1:30 p.m. Thus, the bottles were exposed to sunlight for 15.5 h. Temperature was maintained between 32 and 34.5°C, slightly above river temperature (31°C): river water flowed both around and through the tray to control temperature. The MC experiment was run on the upper deck with full sun exposure from 7:40 a.m. until 6:00 p.m., a total of 10.3 h of sunlight exposure: this location was not available earlier. Temperature ranged from 31.0 to 33.5°C. When the experiments were terminated, a 22-ml sample was saved from each bottle and stored in light-proof bags in the refrigerator

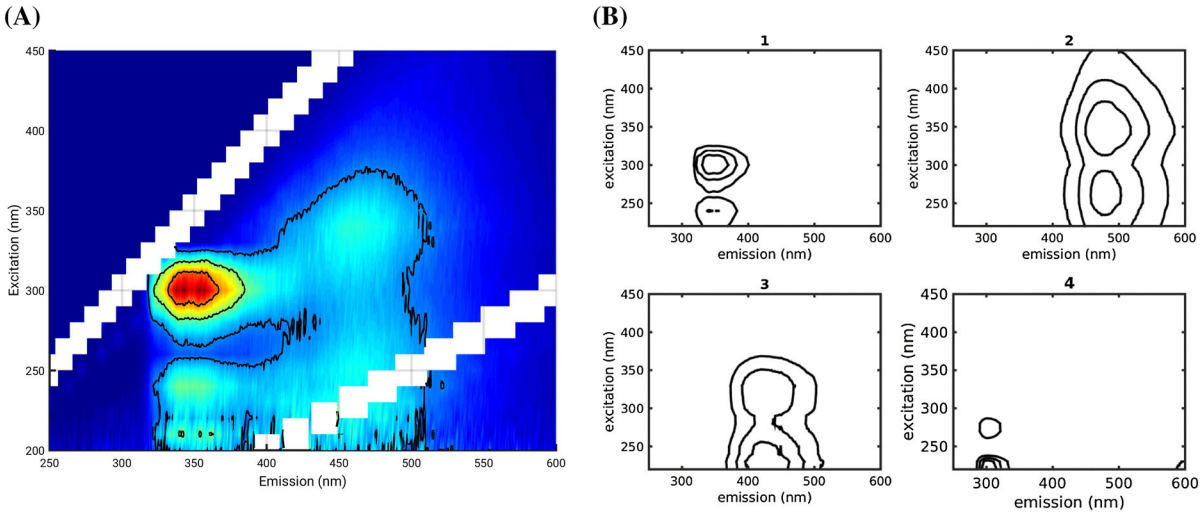

Fig. 2 **A** An example of an excitation–emission fluorescence matrix from the lake (site 2). The *white diamond lines* across the matrix are the limits imposed by Rayleigh–Tyndall effects. **B** Diagrams indicating the regions of emission fluorescence (*x*-axis) for four types of compounds upon excitation (*y*-axis). *1* Tryptophan (Tryp)-like compounds, *2* humic acids, *3* fulvic acids, and *4* tyrosine (Tyr)-like compounds

for later DOC analyses. In preliminary experiments in 2013, the loss of oxygen was minimal; therefore, oxygen was not measured during the 2014 experiments. ROS was measured at the end of the lake experiment, but was so low (<5 nM) that it was not measured at the end of the MC experiment.

Photo-oxidation and fish

Fish [*Hemigrammus levis*, mean wet weight 0.86 ± 0.07 g, total body length 3.35 ± 0.12 cm (20)] were collected by net from the local environment and held in a covered, aerated tank for 3 days prior to the experiment (water temperature ~30–35°C). The holding and experimental water was pumped directly from the river at the side of the boat (DOC 9.12 mg C l^{-1}; pH 5.25, and temperature ~30°C). Twenty fish were randomly distributed in individual aerated chambers of 300 ml. Ten chambers were covered (dark treatment), while the other ten were exposed to continuous sunlight (light treatment). The chamber lids allowed 63% of the UVA + B radiation to reach the internal water. The UVA + B measurements were taken with a UV513AB Digital Light Meter (General Tools and Instruments, New York, NY, USA: accuracy ±4%). The chambers were placed in a shallow water bath connected to a water recirculating system with a 150-l reservoir. A chiller maintained water temperature at 30°C. The fish were added to the chambers at 7:30 a.m. and euthanized at 7:30 p.m. Gills were taken immediately and placed on ice for processing in preparation for later measurement of oxidative stress indices.

The tissues were homogenized in a TRIS-HCl buffer (100 mM, pH 7.75) containing EDTA (2 mM) and $MgCl_2 \cdot 6H_2O$ (5 mM). After centrifugation for 20 min at 10,000×*g* at 4°C, the supernatant was frozen at −80°C for later analyses of total antioxidant competence against peroxyl radicals (ACAP), as well as antioxidant and biotransformation enzymes. The supernatants were analyzed for protein concentrations on the day of analysis using the Bradford method (Bradford, 1978) in order to standardize the assays to protein level. Catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione-s-transferase (GST) were measured spectrophotometrically using the methods of Beutler (1975, in Anderson et al. 2014), McCord & Fridovich (1969), Hopkins & Trudhope (1973), and Keen et al. (1976), respectively. ACAP was assessed fluorometrically using the method of Amado et al. (2009).

Statistical analysis

Statistical comparisons were performed using Systat 11.0 (Systat Software Inc., San Jose, USA). All distributions were checked for normalcy using the Shapiro–Wilcox test, and non-parametric or parametric procedures chosen accordingly. Statistical significance was designated as $P \leq 0.05$. Data are presented as means ± standard error of the mean (1SEM) sample size (*n*). If specific statistical results are not presented in the text, they are printed in the figures and figure legends.

Environmental parameters

Differences between years and among habitats, in surface water conditions (0.55 m depth), and DOC characteristics were investigated using the non-parametric Kruskal–Wallis one-way ANOVA as many of the distributions were not normally distributed. When habitats were significantly different, non-parametric Mann–Whitney *U* tests were used to explore the differences.

Patterns along transects and vertical profiles in DOC characteristics, ROS, and water conditions (oxygen, pH, temperature, and conductivity) were first explored graphically. Transects were then collapsed into a depth profile and differences associated with depth investigated with one-way ANOVA. Environmental and DOC data in the lake and MC (except pH, FI, DOC, and $R_{254/365}$) were not normally distributed and were assessed using non-parametric Kruskal–Wallis ANOVA followed by Mann–Whitney *U* tests. The remaining data were examined using parametric one-way ANOVA, followed by LSD post hoc tests. In the case of HA, the analysis was applied to the ΔHA values where HA at each depth was subtracted from its surface value. DOC profiles were only available for the MC. In the MC 2014 data, where several samples were collected at each site along the transect, across-river patterns could be evaluated statistically. Kruskal–Wallis non-parametric ANOVA was applied in all cases where any pattern was suspected (FI, FA, HA, and Ka_{310}). General linear modeling, a parametric analysis, was used to examine relationships between

ROS and environmental variables (temperature, pH, and oxygen) within habitats.

In a separate analysis, the fluorescence variables were divided by their associated DOC in order to determine if the characteristics of DOC, per se, differed among habitats. Differences were then compared using a non-parametric Kruskal–Wallis ANOVA.

Photo-oxidation experiment

In the photo-oxidation experiment, the source water was collected in two, 1-l bottles. One 22-ml cold, dark control was taken from each bottle. The data for each experimental bottle was referenced to its corresponding dark, cold control. In order to examine the response of treatments, the value at the end of the photo-oxidation period was subtracted from the value of its cold, dark control. The delta values were tested for difference from a mean of zero using a parametric one-sample *t* test in order to determine if daytime exposure to sun and warmth or just warmth (aluminum-wrapped bottles) had altered DOC. In order to determine if DOC response was the same in both habitats, a parametric two-tailed *t* test was applied to the delta values from the two experiments. The delta Ka_{310} and delta DOC values were standardized by their original DOC concentration for this comparison.

The qualities of DOC in cold, dark control samples from the MC and lake photo-oxidation experiments were compared using parametric two-tailed *t* tests. Measures, which were not ratios or already related to DOC concentration, were corrected for DOC concentration (e.g., Ka_{310} DOC^{-1} of the sample).

Fish exposure

Responses of ACAP, CAT, GPx, SOD, and GST were compared statistically between Dark and Light experimental treatments using a non-parametric Mann–Whitney rank sum test.

Results

Environmental conditions

Transparency in the Anavilhanas Archipelago was just less than 1 m. Average Secchi Disk depths were 91–97 cm in the lake with a slightly lower value of 76 cm at the site closest to the in flow, and 83–92 cm in the lake near-shore region (LNS) in 2013. In the main channel (MC), Secchi Disk depths average 96 ± 3 (*4*) cm across the channel with no pattern in 2014.

Surface water quality differed between the MC and lake systems (Fig. 3a–e): the statistical results are provided in the figure and figure legend. Temperature, oxygen, and pH were higher in the lake than in the MC, while DOC was lower, as was conductivity in 2013. The LNS was intermediate with respect to oxygen and temperature; otherwise, it resembled the lake. Conditions within the MC did not differ between years; however, in the lake, conductivity and pH were higher in 2014, while oxygen and DOC were lower in 2014 (Fig. 3a–c, e). Similar changes in DOC and conductivity were observed in the LNS (Fig. 3a, e). In the lake and LNS, DOC fell from 9.69 ± 0.07 *(12)* and 9.92 ± 0.48 (*6*), respectively, in 2013 to 8.35 ± 0.10 (*5*) and 8.65 ± 0.07 (*5*) in 2014. In the MC, DOC was 11.08 ± 0.07 (*10*) in 2013 and 10.65 ± 0.43 (*5*) in 2014.

Within habitat variability

In the lake, vertical mixing normally extended 1–2 m below the surface with oxygen, temperature, and pH decreasing below the mixed depth (Kruskal–Wallis ANOVA: $P = 0.003$, 0.004, and 0.038 ($n = 20$), respectively, in 2013, and not significant, 0.001 and 0.053 ($n = 11$) in 2014) (Supplemental Data Table 1A, F). The minimum values observed were 4.84 mg l^{-1}, 30.5°C, and 4.05 units for dissolved oxygen, temperature, and pH, respectively. Oxygen concentrations increased toward the outflow and were near 100% saturation (5.95–7.65 mg l^{-1}) (Supplemental Data Table 1A, B, F). pH decreased toward the outflow from 5.0 to 4.3 units in 2013, while no trend was observed in 2014 (Supplemental Data Table 1B, F).

In the MC, water was mixed to the bottom of the depths sampled as judged by temperature (Supplemental Data 1D, G): that is 15 m (2013) and 10 m (2014). In 2013, pH and oxygen also did not change with depth. Variables averaged 30.6 ± 0.07°C, 5.4 ± 0.07 mg l^{-1} oxygen, and 4.1 ± 0.07 units pH (10) (Supplemental Data 1D). In 2014, decreases in oxygen (0.3–0.4 mg l^{-1}, sites 12–14), and pH (0.6–0.9 units, sites 11–14) occurred over the top 4 m

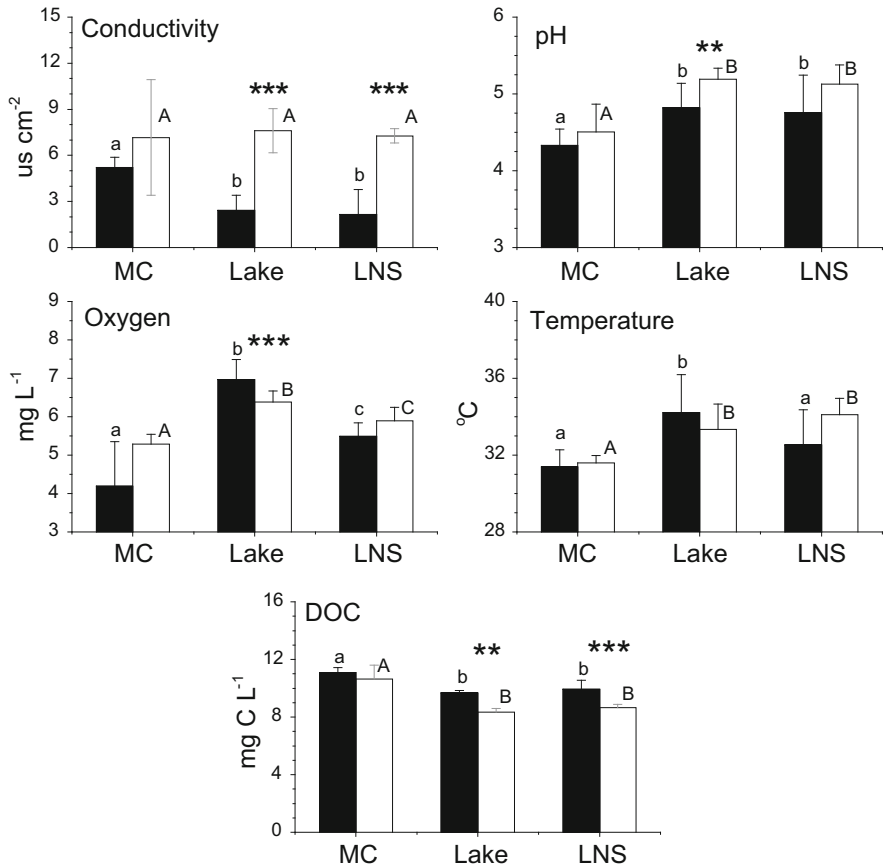

Fig. 3 A comparison among habitats of water quality measures in the surface waters (0.55 m depth) of the Rio Negro, Dec 2013 (*black bar*) and Dec 2014 (*white bar*). MC = main channel, LNS = lake near shore. Data expressed as mean ± 1SEM. $n = 5$–10 for 2014 data from each habitat, and for 2013, $n = 4$ for LNS and $n = 7$ for lake and MC data. Differences were assessed using the non-parametric Kruskal–Wallis one-way ANOVA followed by Mann–Whitney *U* tests when habitats were significantly different. *Letters* indicate significant differences between habitats: 2013 (*small letters*), 2014 (*capitals*). Significant differences between years are indicated as $^{*}P < 0.05$ and >0.01, $^{**}P < 0.01$ and >0.001, $^{***}P < 0.001$

(Supplemental Data 1G). Averaged across the transect, the decline in pH with depth was significant (ANOVA, $P = 0.005$ (*22*)), the change in oxygen was not. No absorbance measures of DOC changed with depth.

In the MC, conditions within the surface waters in the along-shore transect varied without pattern on Dec 13, 2013 (Supplemental Data 1E). Notably oxygen levels were significantly lower than on the day of the vertical profile: 2.88–4.16 versus 5.7 mg l^{-1} (two-tailed *t* test, $P = 0.008$ (*7*)). Along the across-channel transect on Dec. 8, 2014, pH declined from 5.1 units at site 11 to 4.2–4.5 units on the far side of the river: it also declined along the transect at all depths measured. No pattern was present in oxygen (5.1 ± 0.2 mg l^{-1} (*7*)) or temperature (31.6 ± 0.1°C (*7*)) (Supplemental Data 1G).

DOC measures of quality

Only $R_{254/365}$ and Ka_{310} differed significantly among habitats. In 2013, $R_{254/365}$ values were higher in the MC than in the lake and LNS—the lake and LNS were not significantly different: MC, 3.111 ± 0.009 (*9*); lake, 3.052 ± 0.007 (*12*); and LNS, 3.034 ± 0.018 (*6*) (Fig. 4a). $R_{254/365}$ values were similar across habitats in 2014. In 2013, Ka_{310} values differed among all habitats: MC, 0.716 ± 0.003 cm^{-1} (*8*); lake, 0.620 ± 0.003 cm^{-1} (*11*); and LNS, 0.639 ± 0.005 cm^{-1} (*6*). In 2014, Ka_{310} values in the MC were higher than those in the lake and LNS, which were not significantly different: MC, 0.723 ± 0.109 cm^{-1} (*5*); lake, 0.579 ± 0.005 cm^{-1} (*5*); and LNS, 0.583 ± 0.011 cm^{-1} (*5*)

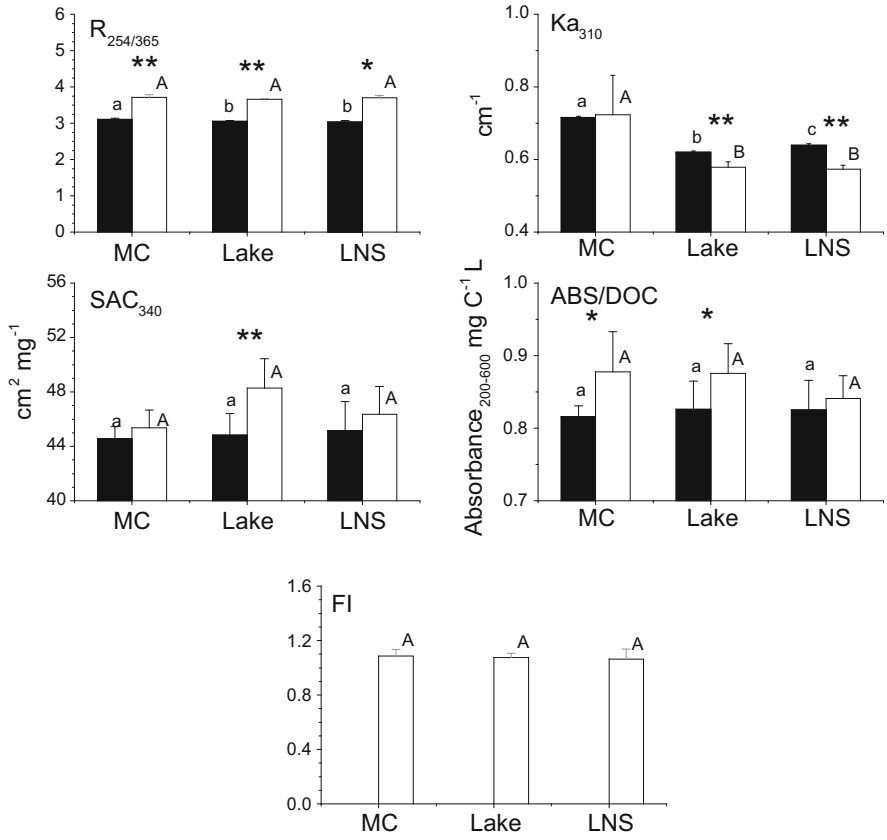

Fig. 4 A comparison among habitats of DOC absorbance indices in the surface waters (0.55 cm depth) of the Rio Negro, Dec 2013 (*black bar*) and Dec 2014 (*white bar*). MC = main channel, LNS = lake near shore. Data expressed as mean ± 1-SEM. $n = 5$ for 2014 data from each habitat, and for 2013, $n = 4$ for LNS data and $n = 7$ for the lake and MC data. Differences were assessed using the non-parametric Kruskal–Wallis one-way ANOVA followed by Mann–Whitney *U* tests when habitats were significantly different. *Letters* indicate significant differences between habitats: 2013 (*small letters*), 2014 (*capitals*). Significant differences between years are indicated as *asterisks*: $*P < 0.05$ and >0.01, $**P < 0.01$ and >0.001, $***P < 0.001$

(Fig. 4b). Ka_{310} is not standardized to DOC concentration. When it was, there were no differences among the three habitats.

Spatially, Ka_{310} was the only absorbance measure which changed along the across-channel transect in the main river (Kruskal–Wallis ANOVA, $P = 0.023$ (*14*)) (Fig. 5a). A step-drop occurred between sites 12 and 13 with Ka_{310} falling from 0.771 ± 0.030 (*6*) cm^{-1} (average of sites 11 and 12) to 0.663 ± 0.003 (*8*) cm^{-1} (average of sites 13–15). No spatial pattern was observed in the lake.

The quality of DOC also differed between years. Most absorbance measures appeared to be higher in 2014 than in 2013, although only some were significantly higher (Fig. 4a, c–e, Supplemental Data Table 5). $R_{254/365}$ increased in all habitats from an average of 3.068 ± 0.008 (*27*) in 2013 to 3.695 ± 0.015 (*15*) in 2014 (Fig. 4a). Ka_{310} decreased significantly in the lake and LNS (see values above) (Fig. 4b). In the lake, SAC_{340} rose from 44.83 ± 0.42 (*11*) to 48.28 ± 0.97 (*5*) $cm^2\ mg^{-1}$, and ABS DOC^{-1} from 0.826 ± 0.012 (*11*) to 0.875 ± 0.018 (*5*) $absorbance_{200–600}\ mg\ C^{-1}\ l$ (Fig. 4c–d). A similar increase in ABS DOC^{-1} occurred in the MC from 0.816 ± 0.006 (*7*) to 0.877 ± 0.025 (*5*) $absorbance_{200–600}\ mg\ C^{-1}\ l$ (Fig. 4d). SAC_{340} did not change between years in the MC and averaged 44.91 ± 0.32 $cm^2\ mg^{-1}$(*12*). FI was measured only in 2014 and averaged 1.076 ± 0.013 (*15*) (Fig. 4e).

The composition of the 2014 DOC, as described by fluorescence properties and quantified by PARAFAC

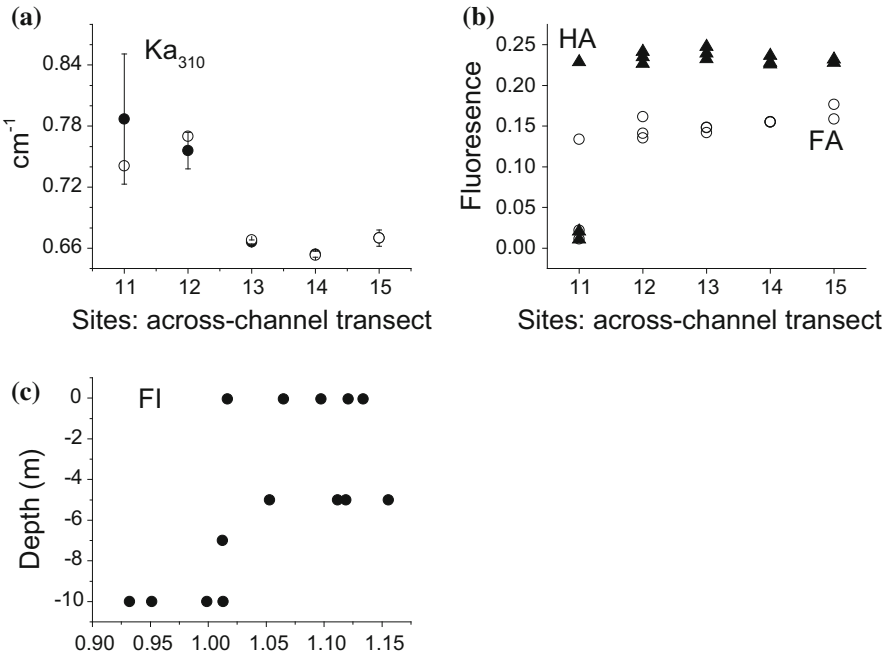

Fig. 5 Spatial patterns along the across-channel transect in the main river, Dec 8, 2014: **a** changes in the mean ± 1SEM (*black circle*) and median (*open circle*) of Ka_{310}, $P = 0.023$, non-parametric Kruskal–Wallis ANOVA, note that means and medians fall on top of eachother at sites 13 to 15; **b** patterns in humic acid fluorescence with depth (*black triangles*, $P = 0.018$) and fulvic acid fluorescence across the transect (*open circles*, $P = 0.044$), non-parametric Kruskal–Wallis ANOVA. Samples were collected at 0.05, 5.0, and 10.0 m. Humic and fulvic acids from 0.05 and 5.0 m depths are the two low fluorescent values at site 11, and **c** changes in the fluorescence index (FI) with depth, $P = 0.027$, non-parametric Kruskal–Wallis ANOVA. $n = 14$

Table 1 Mean fluorescence ± 1SEM (n) or median and percent composition of the fluorescent moieties (%) determined from PARAFAC analysis for each habitat: Main Channel (MC), Lake, Lake near shore (LNS)

Moiety (probability)	MC Median (n) (%)	Lake Mean ± 1SEM (n) (%)	LNS Mean ± 1SEM (n) (%)
TYR (0.100)	0.006 (14) (0.6)	0.009 ± 0.001 (5) (1.1)	0.009 ± 0.000 (5) (1.5)
TRYP (0.063)	0.546 (14) (58.7)	0.460 ± 0.047 (5) (54.8)**	0.226 ± 0.044 (5) (37.7)
FA (0.057)	0.148 (14) (15.9)	0.156 ± 0.001 (5) (18.7)	0.158 ± 0.002 (5) (26.2)
HA (0.010)	0.230 (14) (24.7)*	0.213 ± 0.002 (5) (25.4)*	0.208 ± 0.001 (5) (34.7)
Total fluorescence (fluorescence)	0.894 (14)	0.837 ± 0.049 (5)**	0.601 ± 0.043 (5)

The Lake and LNS values are based on means of the fluorescence values, and the MC data on medians because the latter data were not normally distributed. *Tyr* tyrosine-like, *Tryp* tryptophan-like, *FA* fulvic acids, *HA* humic acids. Kruskal–Wallis ANOVA was used to test for differences among all sites. Kruskal-Wallis probabilites are given in the first column. t tests were also used to examine the potential lake–LNS differences as they were normally distributed. An asterisk indicates that the Moiety is significantly different between the two adjacent locations, and the number of asterisks indicate the P value $P < 0.05 > 0.01$, $P < 0.01 > 0.001$

(for component spectra see Fig. 2), was similar in the lake and MC with respect to FA, Tyr-like, and Tryp-like moiety fluorescence (Table 1), while HA fluorescence was highest in the MC (Kruskal–Wallis ANOVA $P = 0.010$ (*15*); Mann–Whitney U $P = 0.021$ (*10*)) (Table 1). The lake had statistically higher fluorescence levels of HA and Tryp-like fluorescence than the LNS ($P = 0.048$ and 0.007, respectively, $n = 10$, two-sample t test). Tryp-like moieties contributed 54.8–58.7% of the total fluorescence in the lake and MC, and thus they were the dominant fluorescent group, while FA and HA contributed only 15.9–25.4%. HA fluorescence

averaged 0.202 ± 0.21, 0.213 ± 0.002, and 0.208 ± 0.001 fluorescence ($n = 5$) in the MC, lake, and LNS, respectively. In the LNS, the relative contribution to fluorescence of the Tryp-like moiety was lower and the HA and FA accordingly higher than in the lake and MC (Table 1). Total fluorescence also decreased from the lake to the LNS. When fluorescent components were examined relative to DOC content, specific HA and specific FA were highest in the lake (Kruskal–Wallis ANOVA $P = 0.008$ and 0.009, respectively, $n = 15$, 15). Specific Tryp-like and total florescence had borderline significance levels ($P = 0.067$ and 0.063, respectively, $n = 15$, 15) and were also highest in the lake.

In the MC, three spatial patterns were observed in fluorescence along the across-channel transect (Dec. 8th 2014): FA fluorescence increased from the north-east (site 11) to the south-west (site 15) side of the river (Kruskal–Wallis ANOVA $P = 0.044$ (*15*)) (Fig. 5b), while HA and FA fluorescence were very low in the top 5 m at site 11 on the north-east side of the transect producing a gradient across the transect (Kruskal–Wallis ANOVA $P = 0.018$ (*15*)) (Fig. 5b). The fluorescence index, FI, decreased between 5 and 10 m depth, from an average of 1.097 ± 0.015 (*9*) in the upper 5 m to 0.974 ± 0.019 (*4*) at 10 m (ANOVA $P = 0.027$ ($n = 14$)) (Fig. 5c).

One objective of the study was to determine if there were relationships between physical environmental conditions and quality of DOC. We did note that pH was lower in habitats and years with higher DOC, which accords with the regulation of pH by DOC at pH < 6.0. No other relationships between measures of DOC and environmental variables were noted within the Archipelago.

ROS concentrations

Observed concentrations of ROS were in the nM range in the lake and LNS. Distinct patterns were observed along the mid-lake transect with ROS concentration increasing from the inflow (site1) to the outflow (site 6) (Table 2). ROS concentrations in the lake differed between sampling dates, ranging from 0 to 98 nM on Dec 13 and 190–845 nM on Dec 11. Concentrations in the LNS were similar on the 2 days, averaging 323 ± 62 nM (*6*).

In the MC, ROS concentrations also differed between dates and were higher than those in the lake or LNS on Dec 8, but similar to those on Dec 14 (Table 2). Surface concentrations on Dec 8, at a single site, averaged 1,081 ± 203 nM (*3*) and did not decline with depth (15 m). Further offshore, the concentration dropped to 329 nM at 15 m. On Dec 13, ROS

Table 2 Concentrations of H_2O_2 (nM) in surface waters of the Rio Negro, Anavilhanas Archipelago

(A)

Habitat Site/date	Lake 11th	Lake 13th	LNS 11th	LNS 13th
1	301	4	273	271
2	190	0	224	154
3	330	68	511	510
4	373	30		
5	656	98		
6	845	92		

(B)

Depth (m)	Habitat Rep/Date	MC 8th	MC-2 8th	Site/date	MC 13th	Boat 7th
0.05	1	701		1	0	98
0.05	2	1,146		2	286	
0.05	3	1,395		3	240	
15.00	1	1,761	329	4	257	
				5	27	
				6	221	

Samples were collected between 3:00 and 4:45 p.m. with the exception of a sample collected beside the research boat at 7:00 a.m. (A) Lake and lake near-shore (LNS) surface water data (0.05 cm) by site and date, (B) surface and deep samples collected in the main channel (MC) on Dec 8 and on the along-shore transect Dec 13, as well as the 'boat' sample

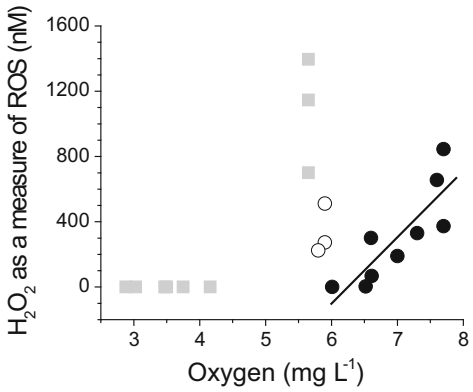

Fig. 6 Variation in the concentration of H_2O_2 with oxygen concentration across habitats in 2013. Samples collected in mid-afternoon (3:00 p.m.–4:45 p.m.). Main channel (MC) = *gray squares*, lake = *black circles*, and lake near shore (LNS) = *open circles*. The relationship in the lake was H_2O_2 (nM) = $-2501 + 401 \times$ oxygen (mg l^{-1}) $P = 0.005$, squared multiple $R = 0.706$ (9) (parametric regression analysis). Three values from the LNS and 2 from the lake were not collected as the oxygen probe failed on one cruise

concentrations in the along-shore transect varied from 0 to 286 nM, averaging 172 ± 51 nM (6).

General linear modeling found no relationship of ROS levels with pH or temperature. In the lake, a significant positive, linear regression relationship with oxygen was observed over the two sampling dates (parametric statistics): ROS (nM) = $-2501 + 401$ oxygen (mg l^{-1}), squared multiple $R = 0.706$, $P = 0.005$ (*9*) (Fig. 6). Similarly, oxygen levels were much lower in the MC when low levels of ROS were observed (Fig. 6), but the relationship was not linear.

Photo-oxidation experiments

DOC differed in both quantity and quality between the lake and MC. Therefore, the characteristics of the initial DOC in the two photo-oxidation experiments were compared using a two-sample *t* test (Table 3): Ka_{310}, HA, FA, Tryp-, and Tyr-like moieties were first standardized by DOC—other measures were ratios or already divided by DOC in their calculation. Lake DOC had a higher specific Tyr-like moiety than did MC DOC. In addition, the water collected for the lake experiment contained more oxygen (6.28 mg l^{-1}) at a higher pH (5.1 units) than the water collected for the MC experiment, oxygen (5.14 mg l^{-1}) and pH (4.6 units). As noted in the methods, the light regime also differed. The lake experiment was run for 15.5 h of daylight due to the misty/cloudy conditions and presence of shade after 1:30 p.m., while the MC experiment experienced 10.3 h of good sunshine. The lake experiment experienced a total of 336 W m^{-2} or 21.7 W m^{-2} h^{-1} of UVA + UVB exposure, while the MC experiment experienced at total of 340 W m^{-2} of UVA + UVB exposure or 33.0 W m^{-2} h^{-1} (UV data courtesy of Dr. Greg Goss, Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada).

One-sample *t* tests, comparing changes in DOC characteristics before and after exposure (delta values) to a mean of zero (expectation of no change in the DOC characteristic), detected significant declines in DOC after exposure of waters from the lake (6.4%) and MC (9.4%) to sunlight (Fig. 7a, Table 4). Ka_{310} declined by approximately 11% in both experiments,

Table 3 Initial quality of the DOC in the photo-oxidation experiments

Values of the DOC measures were standardized to the concentration of DOC in the cold, dark controls, where appropriate—initial experimental conditions. When the initial DOC quality differed between habitats, Mann–Whitney *U* probabilities are presented

Habitat	Lake		MC		Mann–Whitney
Variable/control vial	1	2	1	2	*P*
FI	0.116	0.125	0.103	0.113	
SAC_{340}	42.53	44.39	44.38	43.80	
$R_{254/365}$	3.70	3.65	3.71	3.71	
ABS DOC^{-1}	0.799	0.828	0.827	0.814	
Ka_{310}	0.061	0.063	0.064	0.063	
Tyr-like	0.0009	0.0010	0.0007	0.0006	*
Tryp-like	0.046	0.016	0.016	0.013	
FA	0.023	0.023	0.022	0.022	
HA	0.017	0.018	0.015	0.015	
Fluorescence	0.086	0.058	0.054	0.051	

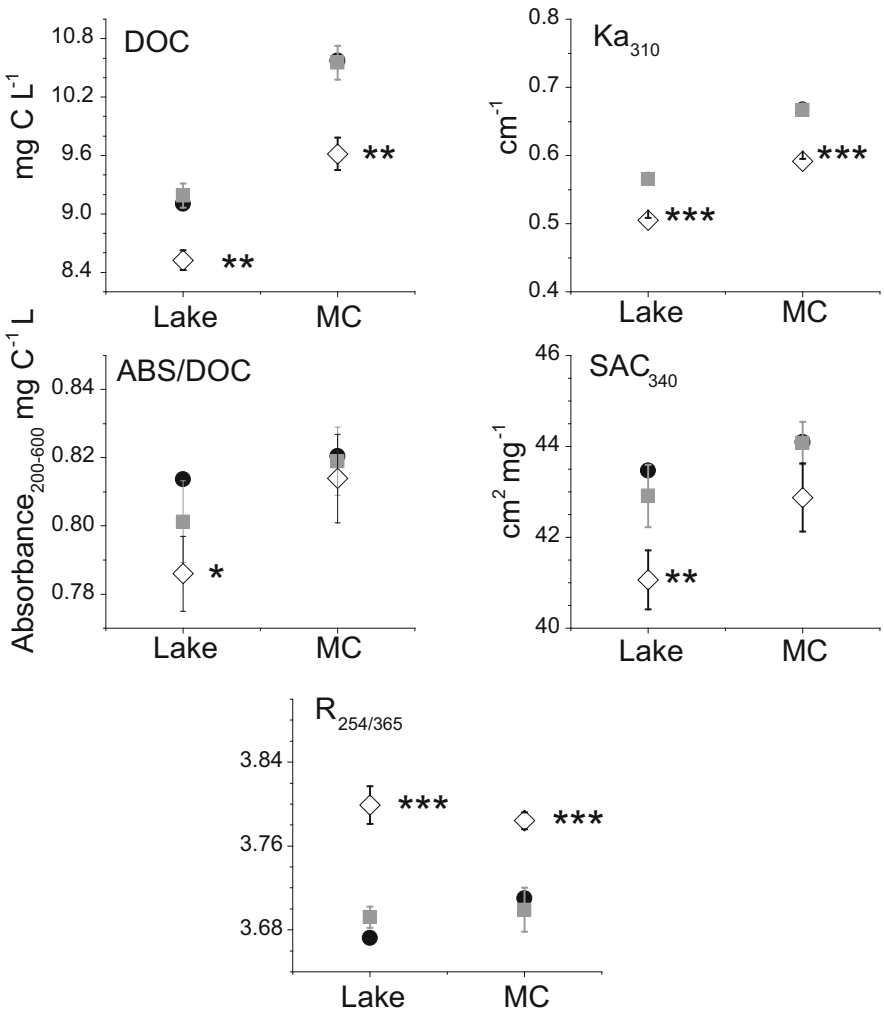

Fig. 7 Comparisons of measures of DOC quality and quantity before and after exposure to sunlight in experiments performed during Dec 2014. Lake and main channel waters were exposed to sunlight for 10.5 (MC) and 15.5 (lake) h (see text), using 5 dark temperature controls (*gray squares*) and 6 light-exposed (*open diamonds*) bottles. The responses were compared to initial conditions (*black circle*). Data expressed as means ± 1SEM Parametric one-tailed (Δ difference of light response from zero) and two-tailed (*dark* vs. *light* responses) tests were run. Significant change from control conditions is indicated by *asterisk* beside the changed bottle. $^{***}P < 0.001$, $^{**}P < 0.01$ and >0.001, $^{*}P < 0.05$ and >0.01

10.9% in the lake and 11.9% in the MC (Fig. 7b). ABS DOC^{-1} and SAC_{340} declined only in the lake (Fig. 7c, d); however, the percent decrease in absorbance at 340 nm (ABS_{340}) was not significantly different in the two experiments, averaging 11.7 ± 0.4% (12). The $R_{254/365}$ increased significantly in both experiments (Fig. 7e). PARAFAC analyses revealed significant declines in HA fluorescence and increases in FA and Tyr-like fluorescent moieties in both experiments (Fig. 8a–c). Notably, 18.6 and 13.2% of the HA fluorescence disappeared, while FA fluorescence increased by 17.2 and 16.5% and the Tyr-like moiety by 32.5 and 47.9%, respectively, in the lake and MC (Table 4). Tryp-like moiety showed a small but significant decrease in the MC (Fig. 8d). Total fluorescence declined significantly (12.5%) in the MC: no significant difference was observed in the lake (Fig. 8e). In the dark controls, HA varied slightly (∼2%) but significantly, increasing in one experiment (lake) and decreasing in the other (MC) (Fig. 7a). Only FI changed significantly and similarly in both the light and dark (Fig. 8f; Table 4) and the changes in the

Table 4 Percentage change in the fluorescence and absorbance variables, which altered significantly over the duration of the photo-oxidation experiments, calculated as (change/original × 100)

Habitat	Variable	Light (%)	1-Tailed *t* test *P*	Dark (%)	1-Tailed *t* test *P*	2-Tailed *t* test *P*
Lake	HA	−18.6 ± 0.7	≤0.001	2.6 ± 0.6	0.010	
	FA	17.2 ± 2.7	≤0.001			
	Tyr	32.5 ± 8.1	0.011			
	FI	−6.9 ± 0.9	0.001	−3.8 ± 1.2	0.036	
	SAC_{340}	−5.5 ± 1.2	0.006			
	$Ratio_{254/365}$	3.5 ± 0.3	≤0.001			0.002
	Ka_{310}	−10.9 ± 0.6	≤0.001			
	DOC	−6.4 ± 1.0	0.002			
	ABS DOC^{-1}	−3.4 ± 1.2	0.032			
	ABS340	−11.60 ± 0.57	≤0.001			
MC	Total fluorescence	−12.5 ± 1.8	≤0.001			
	HA	−13.2 ± 2.9	≤0.001	−2.3 ± 0.7	0.026	
	FA	16.5 ± 3.8	≤0.001			
	Tryp	−37.0 ± 9.4	≤0.001			
	Tyr	47.9 ± 21.6	0.026			
	FI	−9.0 ± 2.6	0.020	−4.9 ± 2.0	0.070	
	$Ratio_{254/365}$	2.0 ± 0.2	≤0.001			0.002
	Ka_{310}	−11.9 ± 0.6	≤0.001			
	DOC	−9.4 ± 1.8	0.003			
	ABS340	−11.84 ± 0.66	≤0.001			

One-tailed *t* tests report the probability of significant change in delta values during the experiment. Two-tailed *t* tests report the probability of a difference in response of delta values DOC^{-1} during the experiment between the two habitats, where a significant change was observed in delta values in both experiments. Mean ± 1SEM. $n = 6$ in the light treatment and $n = 5$ in the dark treatment. A negative value is a loss. No data are presented if the change was not significant. See Figs. 7 and 8 for details of values and significant differences. Variables which did not alter significantly were *Lake* total fluorescence, Tryp

MC SAC_{340}, ABS/DOC

light and dark bottles were not significantly different from one another (two-sample *t* test).

When a property of DOC changed significantly in both experiments, the degree of change was tested for different responses between the two experiments. The changes were standardized to their original DOC and assessed using a two-sample *t* test. Only $R_{254/365}$ responded differently in the two experiments, increasing relatively more in the lake than in the MC (two-sample *t* test of the delta values standardized to DOC $P = 0.002$, $n = 12$) (Table 4).

Photo-oxidation and fish

No significant differences were found in the levels of ACAP, GPx, CAT, SOD, or GST between the two groups of fish, one exposed to sunlit waters and the other held in the dark (Fig. 9).

Discussion

Through these studies, we have gained a better understanding of the water quality and thermal structure of the Rio Negro as it passes through the Anavilhanas Archipelago and of the structure, quality, and reactivity of its DOC. The composition of the DOC is predominantly terrigenous, as noted by earlier researchers (Thurman, 1985; Ertel et al., 1986), and confirmed by the low FI values. Given this composition and in agreement with hypothesis (1), Rio Negro DOC has high mean molecular weight (low $R_{254/365}$) and high SAC_{340} values, an index of the reactivity of

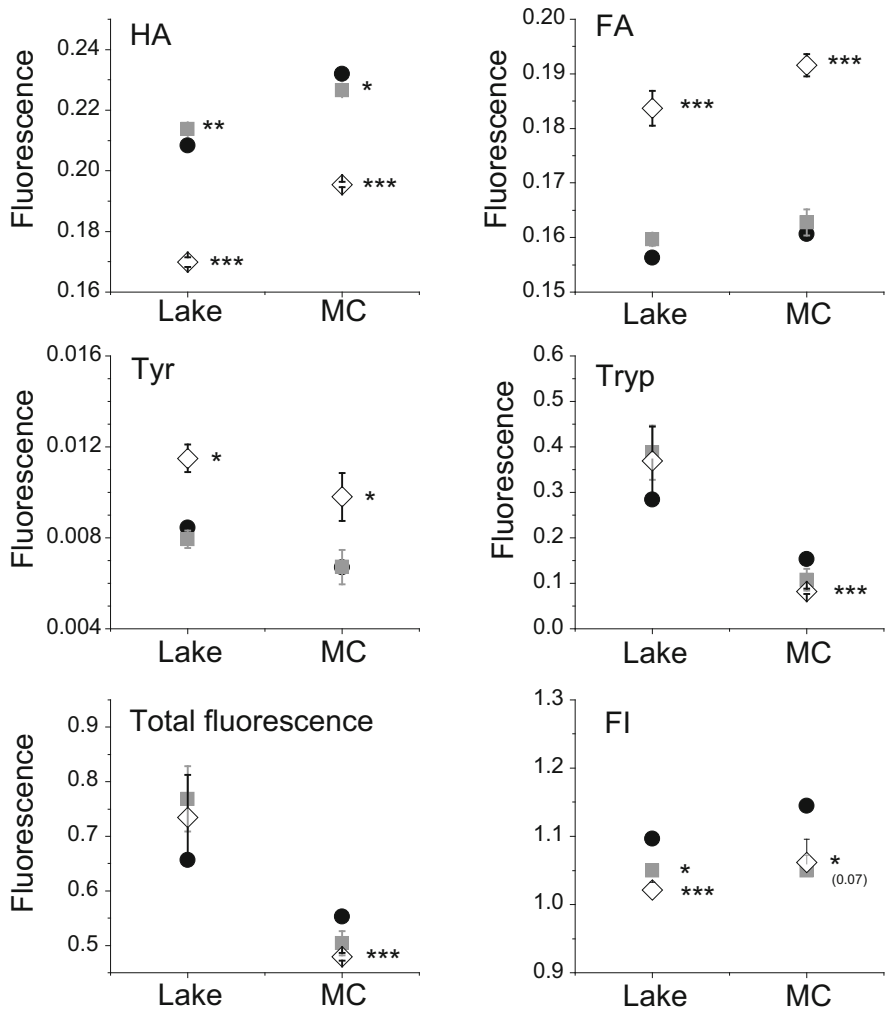

Fig. 8 Comparisons of measures of DOC fluorescence indices before and after exposure to sunlight in experiments performed during Dec 2014. Lake and Main Channel waters were exposed to sunlight for 10.5 (MC) and 15.5 (lake) h (see text) using 5 dark temperature controls (*gray squares*) and 6 light-exposed (*open diamonds*) bottles. The responses were compared to initial conditions (*black circle*). Data expressed as means ± 1SEM. Parametric one-tailed (Δ difference of light response from zero) and two-tailed (*dark* vs. *light* responses) tests were run. Significant change from control conditions is indicated by *asterisk* beside the changed bottle. $***P < 0.001$, $**P < 0.01$ and >0.001, $*P < 0.05$ and >0.01

the aromatic carbon structure. Interestingly, the microbial/algal (Tryp-like) fluorescence signal was stronger than the fluorescence signals of HA and FA. Unexpectedly (negating hypothesis (2)), the DOC index of the potential rate of ROS production, Ka_{310}, is high, and ROS concentrations accumulated to normal and above normal levels in the river by mid-afternoon. In agreement with the high levels of ROS attained, detectable changes in DOC were observed in the photo-oxidation experiments. These experiments also provided information on the paths of DOC degradation which differ in the quantity of small compounds produced. Exposure of the native fish, *Hemigrammus levis,* to the ROS and DOC components produced during photo-oxidation has revealed that the fish does not find these an oxidative challenge, in agreement with hypothesis (3).

Ros

Miller & Kester (1994) reported that the majority of ROS concentrations in aquatic systems were

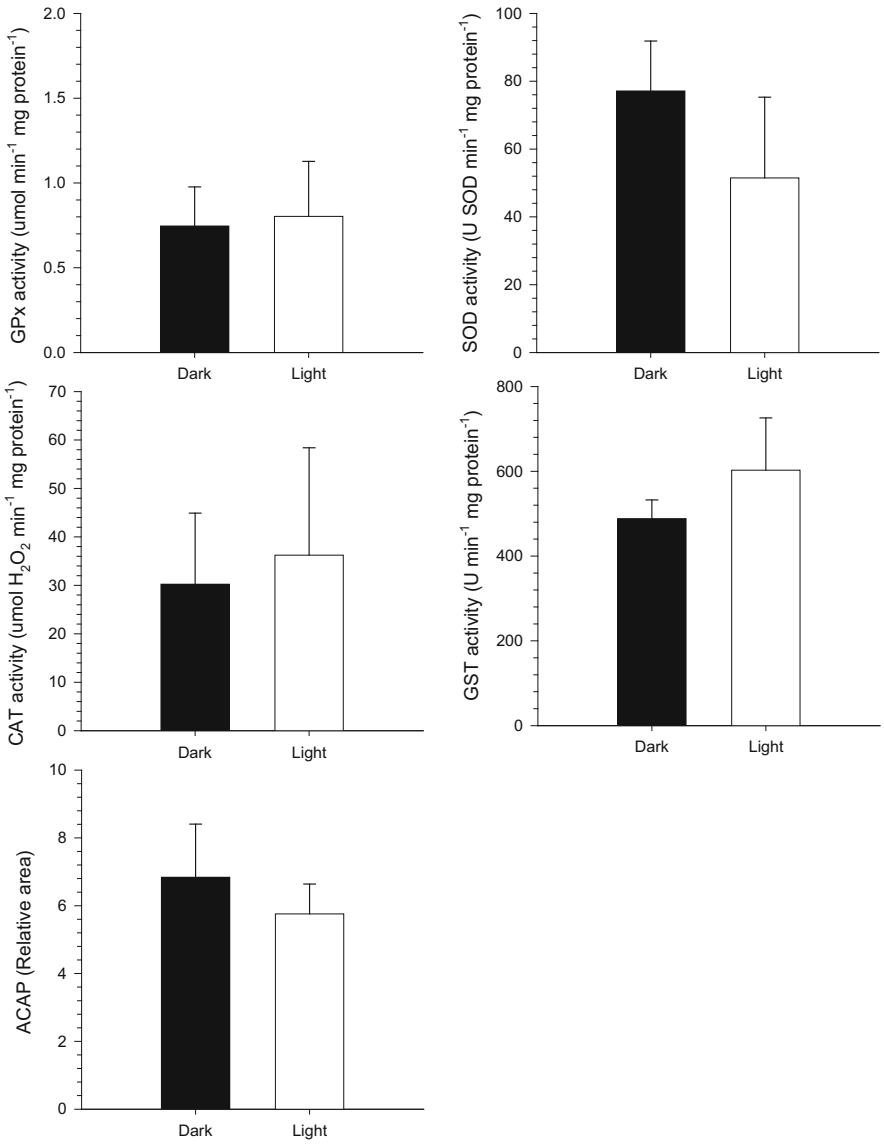

Fig. 9 Enzymatic activities of glutathione peroxidase (GPX), superoxide dismutase (SOD), catalase (CAT), and glutathione-s-transferase (GST) and antioxidant competence against peroxyl radicals (ACAP) in gill samples from *Hemigrammus levis* after 12-h exposure to Rio Negro water held either in the dark or in sunlight. The data are presented as means ± 1SEM. ($n = 10$ *Dark*, 8 *Light*)

<420 nM. At this time, much of the reported work was marine. The ROS concentrations observed in the Rio Negro ranged from 0 to 1.76 µM. The majority of them (70%) fell within this 'normal' observed range. Values >420 nM were observed 30% of the time, and occurred in both the lake and MC. More recently, ROS concentrations >1 µM have been reported from a range of more unusual, mainly freshwater habitats: arctic and boreal lakes with long summer photoperiods (Häkkinen et al., 2004; Febria et al., 2006); intertidal sandflats (Abele-Oeschger et al., 1997); small, highly alkaline Rift Valley lagoons (Johannsson et al., 2014); and an agricultural drainage ditch (Draper & Crosby, 1983).

The vast majority of ROS produced in aquatic ecosystems comes from the interaction of UV

radiation with DOC (Cooper et al., 1994). Scully et al. (1996) determined that the production rate of ROS was primarily dependent on the photon flux of UV radiation and the quality of DOC, best quantified by its fluorescence at 437 nm (excitation at 365 nm) or by its absorption coefficient at 310 nm (Ka_{310}). They compared 23 lakes from southern Canada to the high Arctic which varied in total DOC from 1.4 to 20 mg l^{-1} and included both clear and colored DOC. Their Ka_{310} values ranged from 0.54 to 89.63 m^{-1}: their ROS production relationship was developed only for Ka_{310} values $\leq$40 m^{-1}. They could not obtain good rates of H_2O_2 production from the high Ka_{310} bog samples due to the early start of H_2O_2 decay during the measurements; thus, these measures were excluded from development of the relationship. Rio Negro Ka_{310} values ranged from 56 to 77 m^{-1}, values which should indicate high ROS production but are beyond Scully et al. (1996) published relationship. The Ka_{310} values did differ among habitats in the Rio Negro; however, when they were standardized to DOC concentrations, the Ka_{310} values were no longer significantly different. Thus DOC, per unit weight, among habitats and likely throughout the Anavilhanas Archipelago has a similar capacity to interact with light and produce ROS.

ROS concentrations in the river are controlled by many factors, including the quality of the DOC. They are the net result of (1) rates of ROS production which are also pH, oxygen, temperature, and photo flux dependent (Scully et al., 1996; Bruskov et al., 2002a, b); (2) rates of ROS breakdown which can be dependent on temperature, bacterial activity, Fe^{++} concentration (Fenton reaction), and presence of reactive compounds; and (3) rate and depth of mixing of surface waters.

With respect to the Rio Negro, low pH and non-saturated oxygen levels, at least in the main channel, may lower potential ROS production, while high temperatures and DOC concentrations promote photo-oxidation and ROS production. ROS concentrations in the lake may be elevated by the shallow mixing depth (1–2 m) in that system and perhaps also by the higher oxygen concentrations that accumulate in the surface waters. ROS concentrations did show a positive relationship with oxygen levels in the lake (Fig. 6), which could indicate that the level of oxygen in the water may sometimes be limiting in the Rio Negro system. In the MC, surface waters were mixed to the bottom of sampling depths (10 and 15 m). Such deep mixing should lead to lower overall ROS concentrations as UV radiation does not penetrate very deeply into water. In humic waters in Finland, 99% of UVB radiation was removed in the top 10 cm and 99% of UVA radiation in the top 25 cm (Huovinen et al., 2003). In agreement with this prediction, low levels of ROS were observed on Dec 13, 2013. On the other hand, high levels of ROS were measured on Dec 8, 2013 both at the surface and at 15 m depth. We cannot explain these high values at both the surface and at depth.

The main channel of the river is not homogeneous across its width and the north-east, near-shore region which was the site of the unusual ROS values at depth, also exhibited unusual physical and DOC characteristics. The across-channel transect (Dec 8, 2014) showed distinct changes across the river in oxygen concentration, FA fluorescence, and Ka_{310} with the north-east side being the most different. HA and FA fluorescence was exceptionally low in the top 5 m on the north-east shore (Fig. 1B, site 11). These patterns suggest that the river is composed of a series of laminar flows across its breadth and that the north-east, near-shore region originates from a distinct and slightly different source. Perhaps the incoming waters ride near the surface when they join the main stream.

Doc

FI, an index of the relative contribution of autochthonous and allochthonous sources of DOC (McKnight et al., 2001), confirmed that DOC from the Rio Negro was highly allochthonous in nature. Thurman (1985) and later Ertel et al. (1986) reached similar conclusions based on the high acid to aldehyde ratio, low methoxyl content, and high vanillin degradation products of the lignin of Rio Negro DOC. Ertel et al. (1986) argued that the highly degraded nature was a result of aerobic process in the soil. Further degradation occurs in the river due to microbial and photo-oxidative processes. In the present study, FI did decrease in both light and dark bottles during photo-oxidation experiments. Yet FI values in surface waters of the MC were higher than those at depth. The higher FI values likely reflect autochthonous DOC produced in surface waters. Biotic activity is higher near the surface in aquatic systems as these organisms are dependent on light both to fuel primary production and break down DOC to provide energy/food.

The absorbance and fluorescence properties of the Rio Negro DOC compared well with other highly allochthonous sites, such as Luther Marsh (Ontario, Canada) and Suwannee River (Georgia, USA), which are used routinely to represent DOC of natural, highly allochthonous sources in North America (e.g., Cory & McKnight, 2005; Al Reasi et al., 2012). Luther Marsh FI, $R_{254/365}$, and SAC_{340} values were 1.19, 3.73, and 39.30 $cm^2\ mg^{-1}$, respectively (Al Reasi et al., 2012). Those in the Rio Negro were comparable at 1.08, 3.72, and 45.07 $cm^2\ mg^{-1}$. The FI of the Suwannee River was 1.3–1.4: no information on SAC_{340} or $R_{254/365}$ was given (McKnight et al., 2001). The high SAC_{340} values of Rio Negro DOC are indicative of the strong potential observed in its DOC to protect aquatic life in the river from metal toxicity (Cu, Ag, Pb) and from osmoregulatory problems associated with life at low pH (Galvez et al., 2008; Wood et al., 2011; Durate et al., 2016).

The most striking feature of Rio Negro DOC composition, as revealed from PARAFAC analysis of the fluorescence data, was the large and variable contribution of the Tryp-like moiety. The low pH conditions of the Rio Negro may have contributed to the depression of the HA and FA fluorescence signals (Hudson et al., 2007); however, even if they were corrected to a higher pH, the Tryp-like components would still be dominant. Duarte et al. (2016) recently reported on fluorescence signals from DOC concentrates from the upper and lower Rio Negro main channel. In these samples, HA and FA dominated, although the Tryp-like and Tyro-like signals were not insignificant. It should be noted that fluorescence is a very sensitive method: the high intensity of the Tryp-like moieties does not mean that they were a large portion of DOC by weight. Fluorescence reveals more about the activity and availability of DOC fluorophores than DOC composition by weight, and is influenced by the chemical conditions in the water (e.g., Patel-Sorrentino et al., 2002; Yan et al., 2014).

Tryp-like fluorescence is often associated with sewage effluent (Hudson et al., 2007); however, others have found high Tryp-like fluorescence signals which correlated with bacterial and algal populations and degradation products (Determann et al., 1998; Cammack et al., 2004; Huang et al., 2013) as well as extracts from floodplain top soils (Fasching & Battin, 2012) in natural environments. The river itself has little human development and is known as the 'hungry river' due to it generally low productivity. Therefore, it seems unlikely that the signal is produced or totally produced in the river. Structurally, the river runs through multiple archipelagos composed of wandering channels and lakes. Each year it floods much of that land and the adjacent forest (igapó) (Val & Almeida-Val, 1995). Microbial degradation of the flooded vegetation is sufficiently high that it can result in hypoxic conditions within the flooded lakes (Saint-Paul, 1996). Perhaps the bacterial activity during the flooded period provides part of the Tryp-like signal. Rai & Hill (1982) found that bacterial activity fluctuated from extreme oligotrophy to eutrophy within each of the three lakes they studied along the Rio Negro. Wissmar et al. (1981) noted the importance of bacterial carbon in all the riverine ecosystems of the Amazon River system. Thus, the annual flooding and draining of these lands and forests may provide the Tryp-like signal. Support for this idea comes from the work of Roelke et al. (2006) on the Cinaruco River, a tributary of the Orinoco River in Venezuela. This river has a similar annual cycle to the Rio Negro with seasonal flooding of an igapó forest and a river region of lagoons and channels. Fluorescence excitation–emission matrices showed Tryp-like structures when the water was declining, both in the main river and in a lagoon (Roelke et al., 2006, p. 191, Fig. 3d, f). They traced the source to near-shore and lagoon phytoplankton and bacterial production. In the Anavilhanas Archipelago, Tryp-like fluorescence was higher in the MC and lake than in the LNS. Assuming these values are representative of their habitats generally and the upstream signal is not different, this would suggest that some of the signal is produced in the river system itself. Duarte et al. (2016) suggest that the signal may originate from violacein, a purple pigment, produced by *Chromobacterium violaceum,* which is a common facultative, anaerobic bacterium in the water and soils of tropical regions (Davis, 1986 in Kumar, 2012).

Experimental DOC changes

HA, FA, and Tyr-like fluorescence all changed significantly in the photo-oxidation experiments and in the directions expected from degradation of complex organic molecules. However, the Tryp-like moiety in the Rio Negro did not change or decreased only slightly and paralleled changes in the dark bottles. This observation would suggest that the

majority of this material was not susceptible to photo-oxidation and not produced by photo-oxidation.

In the MC experiment which received higher rates of UV exposure, neither aromaticity (SAC_{340}) nor ABS DOC^{-1} decreased significantly, while DOC declined, suggesting that the structure of DOC had not changed greatly on exposure to light, although DOC was being broken down to CO_2 (Fig. 7a, c, d). Ka_{310}, an index of the capacity of the DOC to react with UV light and produce ROS and is not corrected to DOC concentration, declined at a similar rate to the loss of DOC (11.8 vs. 9.4%), further indicating that the structure of DOC had not changed substantially. The $R_{254/365}$ increased, indicating some decrease in mean molecular weight (Fig. 7e) which corresponded with the increase in fulvic acid molecules and decrease in humic acid molecules (Fig. 8a, b). Fulvic acid molecules are smaller than humic acid molecules (Thurman, 1985).

In the lake experiment, aromaticity (SAC_{340}), ABS DOC^{-1}, and DOC all decreased, while $R_{254/365}$ increased (Fig. 7a, c–e), indicating that the overall structure of DOC changed toward more smaller molecular weight compounds and that some DOC was lost to CO_2. Ka_{310} declined by 10.9%, compared with the 6.4% loss of DOC, also suggesting a structural change in DOC. The increase in the $R_{254/365}$ was greater in the lake experiment than in the MC experiment (Fig. 7e and results above). The percent loss of DOC was not significantly different in the two experiments (Table 4), although the degradation of DOC proceeded differently. Although both SAC_{340} and Ka_{310} increased in all habitats between 2013 and 2014, the photo-oxidation experiments show that these two measures do not necessarily move in tandem.

When DOC is photo-oxidized by UV radiation, ROS and small molecular weight compounds are produced (Cooper et al., 1994; Scully et al., 1996). ROS is then involved in the oxidation of the small molecular weight DOC, molecules which are otherwise utilized by bacteria (Lindell et al., 1995; Scully et al., 2003). If ROS are scavenged from the environment, DOC breakdown proceeds more slowly to produce DIC (CO_2) and more, low molecular weight compounds survive (Scully et al., 2003). DOC can also be broken down to DIC by UV radiation without the production of ROS as shown by Patel-Sorrentino et al. (2004) when they irradiated DOC in anoxic water. A comparison of the changes between the two experiments suggests that more ROS was produced in the MC experiment than in the lake experiment; that is, DOC was broken down more completely in the MC experiment, while more, smaller molecules accumulated in the lake experiment. The different patterns in the breakdown of DOC would explain the paradox that the SAC_{340} decreased in one experiment (lake) but not the other (MC) although the percent change in ABS_{340} was not different between the two experiments (Table 4).

ROS production is correlated with photon flux (Scully et al., 1996) and the net ROS concentrations are a balance between production and losses. Higher ROS concentrations were likely attained in the MC photo-oxidation experiment as the rate of photon flux and likely the proportion of UVB to UVA was higher during that experiment than during the lake experiment due to the lack of cloud cover. Total photon flux, which was essentially the same in the two experiments, may be more important for the rate of degradation of chromophores (e.g., at 340 nm, which was similar in the two experiments). ROS levels can be high in the river. Consequently, photo-oxidation of DOC in the Rio Negro under bright sunshine (high photon flux and ROS concentrations) should result in low production of smaller organic molecules and little or no increase in food resources for bacteria. Amon & Benner (1995) noted that bacterial growth did not increase in Rio Negro waters when exposed to sunlight; however, this work needs to be confirmed for, as they point out, 50% of UVB and 20% of UVA radiation was removed by the glass of their containers.

The rate of loss of *C* due to photo-oxidation reported by Amon & Benner (1995) was 0.049 mg l^{-1} h^{-1} (converted from μM), which was intermediate to the rates observed in the present experiments of 0.103 (MC) and 0.038 (lake) mg l^{-1} - h^{-1}. Given a correction for a 50% loss of UVA + UVB penetration, their predicted rate of *C* loss at the mouth of the Rio Negro would be very similar to that observed in the MC experiment.

The results of the present study emphasize the variability inherent in DOC degradation with its implications for the food web and changes in protective capacity. The rate of loss of DOC in the Rio Negro experiments was 3–10-fold greater than that observed in temperate studies [(Granéli et al., 1996, Sweden; Ma & Green, 2004, Lake Superior; and Winters et al., 2007, southern Ontario) (data calculated assuming logarithmic declines in DOC over Winters et al.'s 13-day exposure period)].

Blackwaters will limit the impact of UV radiation to the top half meter or less, depending on concentration, and dampen the loss rate of protective capacity within the water column.

Waters from the Anavilhanas Archipelago proved to be exceptionally useful for examining photo-oxidation. The bacterial numbers are reported to be low and no changes occurred in DOC concentration or measures of quality in the dark control bottles, with the exception of FI. This would indicate that any changes in DOC due to temperature or the few bacteria that might slip through the filter, were small, allowing for a cleaner assessment of changes in the DOC parameters due to photo-oxidation.

Photo-oxidation and fish

Dissolved organic carbon (DOC) molecules are known to help fish living in challenging environments, such as Rio Negro water (low pH and low ions concentrations), modifying the electrical properties of the gill membranes and helping in ion regulation (Wood et al., 2003; Galvez et al., 2008; Durate et al., 2016) and decreasing metal toxicity through decreasing its availability (De Schamphelaere et al., 2004; Ryan et al., 2004). On the other hand, DOC molecules were already reported to interact with biological membranes, leading to alterations of the antioxidant and biotransformation enzymes (Matsuo et al., 2005), lipid peroxidation (Timofeyev et al., 2004), and alterations on the Aryl hydrocarbon receptor. In the present work, no alterations in the activity of the antioxidants SOD, CAT, and GPx, biotransformation GST enzymes, and in the total antioxidant competence against peroxyl radicals (ACAP) were observed for the specimens of *H. levis* exposed, during 12 h, to Rio Negro MC water and natural sunlight.

UV radiation was reduced by 37% in the fish containers, a level similar to the decrease in photon flux rate between the two photo-oxidation experiments. The experimental day was sunny; therefore, the photo-oxidation of DOC within the chambers should have been similar to natural conditions in surface waters on a cloudy day. This lower photon flux rate favors production of smaller DOC molecules, such as fulvic acids and tyr-like compounds (Table 4, also see above). If *H. levis* were sensitive to increases in fulvic acids or other small components produced by photo-oxidation, the resultant oxidative stress should have been detectable. The gill antioxidant and biotransformation enzymes of specimens of *H. levis* were similar to background values found for two cichlid fish species (*Acarichthys heckelii* and *Satanoperca jurupari*) living in the Rio Negro (Sadauskas-Henrique, personal communication) which shows that *H. levis* were not oxidatively stressed by changes in DOC composition or production of ROS during the exposure of Rio Negro water to natural irradiance.

Concluding thoughts

This study contributes to our understanding of the role of DOC in the aquatic environment of the Rio Negro and similar tropical rivers, and the contribution of DOC to carbon cycling in the Rio Negro.

As we have discussed above, photo-oxidation of DOC is an iterative process influenced by photon flux and oxygen supply, as it relates to the formation of ROS. Our photo-oxidation results may provide insight into annual cycles in photo-oxidative processes in tropical regions typified by rainy and dry seasons. Rainy seasons experience more cloud cover for large periods of the day—whether high cloud cover, gathering storms, or storms, which reduce the photon flux of UV radiation to the earth's surface significantly. With reduced photon flux, degradation of DOC should result in more small compounds and less complete reduction to CO_2. Lower photo flux should also mean lower levels of ROS production and thus less direct breakdown of photo-oxidative products to CO_2. Bacteria should benefit under this regime from an increased food resource. This pattern of photo-oxidation results in lower SAC_{340} values. During the dry season, the cloud cover is much reduced and photon flux of UV radiation is high resulting in more complete DOC photo-oxidation and production of ROS (assuming adequate oxygen concentrations). This scenario of DOC degradation reduces the food for bacteria, but maintains the SAC_{340} levels due to the more complete oxidation of the breakdown products, and increases the production of CO_2 from the DOC. Therefore, we anticipate that the dry season is more important than the wet season for re-introducing CO_2 into the atmosphere from photo-oxidation. During the wet season, the carbon would take a more circuitous route through bacterial growth, consumption within the food

chain, and respiration before eventually entering the sediment or returning to the atmosphere as CO_2.

Could climate change affect this cycling of DOC photo-oxidative processes? In as much as warming temperatures may depress oxygen concentrations, some impact on ROS production might be expected. Our study would suggest that shallowly mixed regions like lakes and lagoons may be the most affected. Reductions in ROS might lengthen the degradation process which would be taken over more by bacteria. Climate change may have other larger impacts. Combined with deforestation, the weather patterns are expected to change to longer, hotter dry seasons in the majority of the central and eastern Amazon Basin (Duthie et al., 2015). If this occurs, then on an annual basis there may be more direct break down of DOC to CO_2 as the dry season would be longer. Climate change and deforestation may have other far reaching impacts on photo-oxidation; however, they are out of the scope of our work.

The question remains whether photo-oxidation, which is limited to surface water, is sufficient to have a significant effect on the system, per se, on bacterial production, carbon cycling, or the quality of DOC's protective properties? If it does, the impact is most likely to occur in the shallower reaches where a greater percentage of the water column DOC can be affected by photo-oxidation. If photo-oxidation extended to 5 cm with 5% loss of DOC C day^{-1} in the top 5 cm, 25 mg C m^{-2} would be lost per day as CO_2. That is one-sixth the rate of loss from temperate shallow wetlands (Bastviken et al., 2011), which are highly productive systems, suggesting that the potential importance is worthy of further investigation.

The Rio Negro supports a very diverse fish community and other studies have shown that its DOC not only sets the pH of the river but also protects the fish from the effects of low pH, osmoregulatory stress in the low ion environment, and metal toxicity (Galvez et al., 2008; Wood et al., 2011; Durate et al., 2016). We have shown that at least one fish, and likely others, do not suffer oxidative stress from the ROS, DOC, or DOC degradation products in the Rio Negro.

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