Molecular changes of aquatic humic substances formed from four aquatic macrophytes decomposed under different oxygen conditions

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Molecular changes of aquatic humic substances formed from four aquatic macrophytes decomposed under different oxygen conditions

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ABSTRACT
Aquatic macrophytes’ decomposition is a source of recalcitrant carbon in the long term contributing to humic substances (HS) formation. Understanding the influence of plant detritus quality and oxygen availability over molecular changes of these compounds provides ecological information related to their cycling. This study described the molecular variation of dissolved HS from Eichhornia azurea, Egeria najas, Oxycaryum cubense and Salvinia molesta decomposition under aerobic and anaerobic conditions. The aquatic HS formed from the four aquatic macrophytes showed similar features (e.g. molecular weight and aromaticity). This fact indicates little influence of the detritus quality or availability of oxygen on the fulvic acids (FA) and humic acid characteristics. Under aerobic condition a decrease in the polysaccharides content in FA occurred. HS from E. najas were related to less-recalcitrant features, while HS from S. molesta were related to recalcitrant.

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KEYWORDS
E4/E6; dissolved organic carbon; dissolved oxygen; fulvic acid; FTIR; humic acid

1. Introduction
Dissolved organic carbon (DOC) consists of heterogeneous mixtures of carbon compounds from allochthonous and autochthonous sources constituting the major organic carbon pool in aquatic ecosystems [1]. Aquatic macrophytes can be responsible for up to 50% of the total input of carbon and nutrients in these environments, contributing to the maintenance of biogeochemical cycling of elements, especially in coastal regions [2,3].

In the early stages of aquatic macrophyte decomposition, the oxidation and dissolution of protoplasmic fractions and soluble compounds are the main factors responsible for mass loss [4]. The lignin and other components (i.e. structural compounds) are the major constituents of plant cellular walls [5] and are precursors of humic substances (HS) [6–8].
HS are natural non-living organic substances that are ubiquitous in the environment formed by the humification process. Humification can be metabolically expressed as a prolonged stabilisation of organic substances with slow biodegradation [9]. One theory points out that humification occurs by supramolecular associations of heterogenic molecules, linked by weak bonds of big molecules [10].

Operationally, aquatic HS can be divided into two fractions: fulvic acids (FA) and humic acids (HA). The HA are characterised mainly by compounds from lignin degradation added to their structures during humification [11] and usually have an aromatic content and higher molecular weight compared to FA [11,12].

HS have important roles in aquatic environments. They affect the water transparency, they are assimilated by microbial metabolisms and are used as electron acceptors or donors during biogeochemical cycles [13–17]. Furthermore, HS can mobilise heavy metals by complexation and insecticides by absorption, they can be adsorbed on surfaces of many particles influencing dissolution and have acid–base properties with a wide range of pK values [18–20].

Concerning HS characteristics, we hypothesised that the source of organic matter (aquatic macrophyte species) and the environment condition (e.g. oxygen availability) act as determinant factors to configure the chemical properties of the dissolved HS. Identifying the molecular modification in HS is important information concerning their development and rearrangement over time during decomposition providing ecological information about the cycling of these compounds, which are usually refractory in aquatic environments. Therefore, this study aimed to describe the molecular variation of dissolved HS from Eichhornia azurea (Sw.) Kunth., Egeria najas Planch, Oxycaryum cubense (Poepp. and Kunth) and Salvinia molesta (Mitchell) decomposition under aerobic and anaerobic conditions.

2. Methods

2.1 Sampling and handling

Plants and water were collected from the Óleo Lagoon (21°36′S and 47°49′N), an oxbow lake that belongs to the Mogi-Guaçu River floodplain (400 m from the river) and is classified as oligotrophic and has little anthropogenic interference [21,22]. The weather in the region is classified as AW (Köppen) with rainy and dry seasons. The maximum depth of the Óleo Lagoon is 5.1 m and it has an area of 19,470 m². The mean water temperature is 22.1°C ± 1°C and it has low concentrations of dissolved oxygen (3.57 ± 2.18 mg L⁻¹) and DOC (3.05 ± 0.98 mg L⁻¹) [23].

Water samples were collected from the surface and bottom of the lagoon using Van Dorn samplers. The sampled water was filtered using cellulose acetate membrane (0.45 µm pore). An aliquot of the water sample was reserved in natura to be used as an inoculum. This inoculum was mixed with the filtered water immediately before preparing the incubations (200 mL of inoculum per 20 L of filtered water). The adult specimens of E. azurea (rooted floating), E. najas (submersed), O. cubense (emergent) and S. molesta (floating) were chosen due to their abundance and dominance in Óleo Lagoon and their different ecological types that influences their tissue composition (i.e. content of recalcitrant compounds such as lignin). Collected plants were washed on site and in the laboratory. Subsequently, they were dried under 45°C until reaching a constant weight. The plants were cut into pieces of about 5 cm.
2.2 Aquatic plants’ incubations

The incubations were performed in 1 L glass flasks containing 9 g of dried plant (detritus) for 900 mL of water (filtered + inoculum). The flasks were maintained in the dark at 22°C. The anaerobic incubations were kept sealed and opened only on the respective sampling day, while the aerobic incubations were continuously aerated. On the sampling days (days 1, 3, 6, 10, 15, 20, 30, 45, 60, 90, 120 after the incubation preparation), the bottles were opened in triplicate. The particulate and dissolved fractions were separated by filtrations using cellulose acetate membrane (0.45 µm pore). From the dissolved fraction, the DOC and total dissolved nitrogen (TDN) (total organic carbon analyser – SHIMADZU TOC-LCSH/CPH) were determined and a visible spectroscopy analysis was carried out to determine the E4/E6 ratios (465/665 nm). The carbon/nitrogen ratio (C/N) was calculated by dividing the DOC/12 per the TDN/14.

2.3 HS acquisition and spectroscopy analysis

Moreover, on sampling days 15, 30, 65, 90 and 120, HS were extracted using a column of DAX-8 resin [24]. After extraction, samples were acidified until pH 1, and the supernatant (FA) was centrifuged and separated from the precipitate (HA) [25]. Consecutively, samples were dialysed (3500 Daltons – FISHER) and lyophilised. From the lyophilised samples, Fourier-transform infrared spectroscopy analysis from 400 to 4000 cm\(^{-1}\) was carried out (Perkin-Elmer/Spectrum 1000). Each spectrum obtained was the average of 32 readings (transmittance) for each sample, with a resolution of 4 cm\(^{-1}\). After freeze-drying, the samples were dissolved in 0.05 N NaHCO\(_3\) solution [26] at concentrations of 1 mg mL\(^{-1}\), and then the wave scan of UV–visible spectrum (UV–VIS; from 200 to 800 nm) analysis with a 1.0 cm quartz cuvette was performed. Deionised water was used as a blank and measurements were taken for every eight samples.

From the UV–VIS results, ratios of particular wavelengths were calculated in order to verify the DOC and HS characteristics:

- E4/E6 ratio (465/665 nm) – higher values of this ratio indicate lower molecular weight molecules, less aromaticity and less humification [26,27];
- E2/E4 ratio (270/407 nm) – lower values indicate higher participation of porphyrin structures (407 nm = Soret band), while higher values indicate participation of lignin-like structures [28].

The treatments were compared using analysis of variance repeated measures with 5% of significance.

3. Results

Higher C/N values occurred in *E. azurea* aerobic incubations (*p* < .01) and lower values in *S. molesta* anaerobic incubations (*p* = .01). *E. najas* and *O. cubense* incubations showed similar C/N values under both incubations (*p* = .12 and *p* = .76, respectively). The dissolved fraction of *E. azurea* presented the highest C/N values due to high DOC formation and low N concentrations. In the *E. najas* incubations, C/N higher values occurred in the first 20–30
days with a decreasing trend until the end of the incubation. Both *O. cubense* and *S. molesta* incubations showed low C/N values with an increasing trend over time. Incubations with *S. molesta* presented the lowest dissolved carbon concentrations (Figure 1).

The dissolved fraction of all incubations showed an increase in molecular weight during the initial days of decomposition of plants detritus. *E. azurea* and *O. cubense* samples showed the highest molecular weight under aerobic conditions during the whole incubation period (*p* < .01 and *p* = .02, respectively), while for *E. najas* and *S. molesta* samples, both conditions presented similar values (*p* = .61 and *p* = .83, respectively). However, anaerobic incubations had higher E4/E6 ratio values in early incubation and lower values in the final days (Figure 2).

E4/E6 ratio of dissolved HS samples were higher than E4/E6 ratios of DOC. Little variation occurred during incubations and generally oxygen availability did not interfere

![Figure 1](https://example.com/figure1.png)

**Figure 1.** The dissolved organic carbon/total dissolved nitrogen (C/N) ratios of the leachate formed during aquatic macrophytes decomposition under aerobic (white circle) and anaerobic (black triangle) conditions (*A* = *Eichornia azurea*; *B* = *Egeria najas*; *C* = *Oxycaryum cubense*; *D* = *Salvinia molesta*).
with molecular weight, except for FA formed from *E. azurea* (*p* = .03) and FA and HA formed from *Oxycaryum cubense* (*p* = .01 and *p* < .01, respectively).

During the incubation period, all samples showed few variations of E2/E4 ratios (Table 1). There were no differences among treatments for all species, except for HA formed during *E. najas* and *O. cubense* incubations (*p* = .04 and *p* = .03, respectively). This indicates that the content of lignin-like products in humic compounds was not affected by decomposition under aerobic or anaerobic conditions.

Generally, the intensity of bands occurred in similar regions for all the samples: ~3300 cm$^{-1}$ – related to N–H or O–H stretching of phenols, alcohols, carboxylic acids

**Table 1.** Averages and standard deviations (SD) of E2/E4 ratios (207/407 nm) of aquatic humic compounds formed from aquatic macrophyte decomposition under aerobic and anaerobic conditions.

<table>
<thead>
<tr>
<th></th>
<th>Fulvic</th>
<th>Humic</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Aerobic</td>
<td>Anaerobic</td>
</tr>
<tr>
<td>Average</td>
<td>SD</td>
<td>Average</td>
</tr>
<tr>
<td><em>E. azurea</em></td>
<td>4.86</td>
<td>0.14</td>
</tr>
<tr>
<td><em>E. najas</em></td>
<td>5.06</td>
<td>0.39</td>
</tr>
<tr>
<td><em>O. cubense</em></td>
<td>5.20</td>
<td>0.34</td>
</tr>
<tr>
<td><em>S. molesta</em></td>
<td>5.19</td>
<td>0.14</td>
</tr>
</tbody>
</table>

**Figure 2.** The E4/E6 (465/665 nm) ratios of the dissolved fraction formed during aquatic macrophytes decomposition under aerobic (white circle) and anaerobic (black triangle) conditions (A = *Eichhornia azurea*; B = *Egeria najas*; C = *Oxycaryum cubense*; D = *Salvinia molesta*).
and/or other substances which contain hydroxyl groups; ∼2900 cm⁻¹ – related to aliphatic (C–H)n stretches, such as fatty acids and waxes; ∼1720 cm⁻¹ – related to C = O stretches of – COOH, aldehydes and ketones; ∼1620 cm⁻¹ aromatic C = C vibration, carbon–carbon bonds, symmetric stretching of COO⁻ groups, and H-bonded C = O of conjugated ketones; ∼1400 cm⁻¹ – related to O–H deformation and C–O stretching of phenolic OH; and ∼1050 cm⁻¹ – related to C–O stretching of polysaccharides or derivatives [29–33] (Figures 4 and 5).

Dissolved FA formed during E. azurea degradation had a similar infrared response under aerobic and anaerobic conditions. However, under aerobic conditions, the band related to polysaccharides (∼1050 cm⁻¹) showed lower intensity over time, indicating

![E4/E6 ratios (465/665 nm) of dissolved humic substances formed from aquatic macrophyte decomposition under aerobic (white circle) and anaerobic (black triangle) conditions.](image)

Figure 3. E4/E6 ratios (465/665 nm) of dissolved humic substances formed from aquatic macrophyte decomposition under aerobic (white circle) and anaerobic (black triangle) conditions.
processing of this molecular group. In addition, under anaerobic conditions, the band related to N–H (∼1400 cm⁻¹) became more intense over time and similar to aerobic FA in the last days of incubation (Figure 4).

The dissolved FA formed during *E. najas* degradation had similar characteristics to *E. azurea*. The ∼1050 cm⁻¹ region also became less intense over time under aerobic conditions.
conditions. The main differences are a more intense band at $\sim 2900 \text{ cm}^{-1}$, indicating more aliphatic content and less recalcitrance, and a lower intensity at $\sim 1720 \text{ cm}^{-1}$, indicating less formation of ketones and aldehydes due to polysaccharides degradation (Figure 4).

**Figure 5.** The infrared spectroscopy (FTIR) of aquatic humic acid formed during aquatic macrophyte decomposition under aerobic and anaerobic conditions (A = *Eichhornia azurea*; B = *Egeria najas*; C = *Oxycaryum cubense*; D = *Salvinia molesta*).
The FA formed during *O. cubense* degradation showed low intensity in the ∼2900 cm\(^{-1}\) region showing a low presence of aliphatic structures. The *E. najas* samples showed less intensity for the ∼1720 cm\(^{-1}\) band. However, the main difference was observed in the ∼1050 cm\(^{-1}\) region, where an increase in intensity over time occurred indicating an increase in the polysaccharide content and humification process. In this region, the aerobic conditions favoured a spread peak even when the intensity increased. The dissolved FA formed during *S. molesta* decomposition showed the lowest intensity in all infrared regions. The low intensity in ∼3300 and ∼2900 cm\(^{-1}\) regions may indicate that the FA from *S. molesta* has more aromatic features than other plants. In addition, the low intensity of the ∼1050 cm\(^{-1}\) region indicates less polysaccharide content (Figure 4).

The dissolved HA formed from macrophyte decomposition showed similar peak regions to dissolved FA samples. The clearest difference was a decrease in the intensity of the ∼1050 cm\(^{-1}\) peak in HA, indicating a lower presence of C–O stretches of polysaccharides or derivatives and an advanced stage in the humification process (Figure 5).

Dissolved HA formed from aerobic *E. azurea* degradation did not change over time. The only evidence of intensity modification is an increase around the ∼1400 cm\(^{-1}\) region, showing an increase in carboxylate. However, the HA formed under anaerobic conditions showed an increase in intensity around the ∼2900, ∼1400 and ∼1050 cm\(^{-1}\) regions. This fact shows evidence of a decrease in humification and an increase in polysaccharide content. These bands had higher intensities under anaerobic treatments compared to aerobic treatments (Figure 5).

The dissolved HA formed from *E. najas* decomposition had the highest intensity in the ∼2900 cm\(^{-1}\) region compared to the others species, showing that this compound has high aliphatic content and is less recalcitrant. Under aerobic conditions, it can be observed that there was an increase in intensity around the ∼1400 cm\(^{-1}\) region, while under anaerobic conditions an increase around the ∼1525 cm\(^{-1}\) region showed the presence of aromatic C = C and/or amides C = N (Figure 5).

Dissolved HA formed from *O. cubense* decomposition presented bands in similar regions to *E. azurea* and *E. najas*. However, the region from around ∼1525 to ∼800 cm\(^{-1}\) showed undefined peaks with low intensity, indicating high humification and low proportions of polysaccharides and derivatives. HA formed under aerobic and anaerobic conditions had similar band regions. For dissolved HA formed in the *S. molesta* decomposition, the lowest intensity occurred around the ∼3000 cm\(^{-1}\) region compared with other species. This showed high aromaticity of HA compounds. It can also be observed that there was a peak around the ∼1400 cm\(^{-1}\) region and a low-intensity band around the ∼1050 cm\(^{-1}\) region, indicating a high carboxylate formation and a low content of polysaccharides and derivatives (Figure 5).

**4. Discussion**

In general, dissolved HS formed from leachates of four aquatic macrophyte species showed similar features of each other (e.g. high molecular weight, same functional groups) [34]. Different methods to characterise molecular features of HS were important to evaluate qualitative changes resulting due to macrophyte species or oxygen availability [35].
In a previous study [36], it became clear that O. cubense and S. molesta detritus are more recalcitrant than E. azurea and E. najas detritus. These recalcitrant detritus generated less DOC and promoted higher contents of dissolved HS, mainly under aerobic conditions. Besides, dissolved humic compounds represented a small fraction of the total detritus of each plant. However, due to their recalcitrance, the dissolved HS can accumulate in aquatic environments [36].

Although availability of oxygen increases detritus mineralisation and generates higher amounts of HA, the presence or absence of oxygen did not seem to be a determinant factor for the features of the dissolved organic carbon, including the HS yields. C/N ratio did not show a pattern according to oxygen availability, but this variable is an indicator of the detritus quality. E. azurea and E. najas dissolved fraction showed higher values of C/N compared to O. cubense and S. molesta (Figure 1). This was an expected result since E. azurea and E. najas have higher contents of the labile/soluble fraction [36]. Besides that emergent macrophytes (e.g. O. cubense) usually present a lower C/N content than floating and submerged macrophytes (e.g. E. najas) (submerged = 21.2–30.5; emergent = 13.0–22.4; [31]).

Under anaerobic conditions, mineralisation promotes an accumulation of intermediate compounds, since fermentative processes partially degrade organic molecules [37]. Furthermore, anaerobic bacteria has limitations to hydrolyse complex molecules and aromatic compounds [38]. These facts agree with E4/E6 ratios of DOC, where low values under anaerobic conditions occurred indicating higher molecular weight in this condition (Figure 2).

Although, generally E4/E6 and E2/E4 ratios of FA and HA showed similar patterns under both conditions (Figure 3 and Table 1), FA from E. najas and FA and HA from O. cubense incubated in anaerobic conditions favoured higher molecular weight. HS can present a decrease in E4/E6 and E2/E4 ratios indicating changes in composition and/or content of high weight and lignin-like molecules [28,39]. However, our results indicate that the molecular weight and lignin-like content in dissolved HA and FA compositions experience a low influence of aquatic macrophyte species (i.e. detritus composition). In addition, these HS presented few changes in these characteristics during aerobic and anaerobic degradation.

The main molecular groups presented in FA and HA were similar regardless of the detritus source and oxygen availability (Figures 4 and 5) [34]. The main variation observed in the FA samples was the decrease in polysaccharides molecules, mainly under aerobic conditions [33]. The decrease in intensity of the bands related to polysaccharide content indicates that FA were being processed. As polysaccharides are compounds with the relevant energetic content, the metabolisation of these compounds is expected [27,40].

Dissolved FA derived from E. najas showed closer relation to higher aliphatic content while the opposite was observed for S. molesta (Figure 4). Moreover, FA derived from E. najas was the only FA sample where an increase in the phenolic O–H content was not observed (~1400 cm⁻¹). This agrees with the fact that E. najas has more labile content and presents higher mass decay than the other studied species [36]. However, this fact seems to not influence FA mass loss [36].

In general, HA samples from different species detritus had similar features (Figure 5). An increase in the polysaccharide content occurred in HA from E. azurea. This could be related to cellulose degradation [40]. Moreover, HA from E. azurea presented higher mass decay
than other plants [36]. The HA from E. najas were also related to more aliphatic content, but under aerobic conditions a decrease in the aliphatic content was observed (Figure 5). This might be related to lower cell wall fraction and lignin content than the other plants [41–45].

Generally, aromaticity in the macrophyte-derived DOM is lower than that in DOM from the allochthonous sources, sediment or the HS, but similar to or higher than that in the phytoplankton derived from DOM [31]. Although some studies show higher decays of HA than FA [36,39,46], the spectroscopy analysis demonstrated that HA are more resistant to molecular changes than FA, once less qualitative variations were noticed in the HA samples (Figures 4 and 5).

5. Conclusions

Based on adopted experiments, it can be concluded that aquatic HS formed from four aquatic macrophytes leachates presented similar features. This shows little influence of the detritus quality or availability of oxygen on the FA and HA characteristics. Anaerobic condition resulted mainly in a DOC with a higher molecular weight. Aerobic condition favoured a decrease in polysaccharides content of FA from all plants. FA and HA derived from E. najas were related to less-recalcitrant features and higher aliphatic molecule content. FA and HA from S. molesta were related to recalcitrant structures and presented low chemical change than HS from other plants.

Disclosure statement

No potential conflict of interest was reported by the authors.

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