

## Characterization of humic substances and their distribution of XAD fractions by absorption spectroscopy in the Godavari estuary, India

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**Humic substances (HS) are formed in estuarine regions by biogeochemical processes of terrestrial and *in situ* produced organic matters. Their structure and distribution may vary seasonally and spatially. To examine this, HS were isolated from Godavari estuarine waters using ion-exchange resins XAD-8 followed by XAD-4 during 2014–2015. The structural differences between the two fractions were characterized by  $E_2/E_3$  ratio ( $a_{250}/a_{365}$ ), spectral slope ( $S_{275-295}$ ), and spectral slope ratio ( $S_R$ ,  $S_{275-295}/S_{350-400}$ ), which were derived from UV-visible absorbance spectra. Lower values of  $E_2/E_3$  ratio,  $S_{275-295}$  and  $S_R$  for XAD-8 fractions than XAD-4, indicate higher aromaticity and higher molecular weight of dissolved organic compounds retained on the former resin. The  $E_2/E_3$  ratio for XAD-8 and  $S_R$  for XAD-4 fractions were found to decrease gradually from post-monsoon to monsoon, indicating that the biological process controls the production of organic matter in upstream waters. Lower molecular weight organic compounds formed by bacterial decay and photodegradation during pre-monsoon and higher molecular weight organic compounds formed during the post-monsoon season was attributed to the freshly exudated organic matter from phytoplanktons dominated at the mouth of the estuary.**

**Keywords:** Absorbance spectra,  $E_2/E_3$  ratio, Godavari estuary, humic substances, spectral slope ratio.

DISSOLVED organic matter (DOM) of hydrosphere is one of the largest pools of Earth's carbon reservoirs<sup>1,2</sup>. Only about 30% of this DOM is structurally characterized as compounds belonging to carbohydrate, amino acid, hydrocarbon, phenol and other structural classes<sup>3</sup>. The remaining 70%, uncharacterized at the molecular level<sup>4</sup>, is collectively termed as gelbstoff (humic material) or yellow substance<sup>5</sup>. HS are complex polymers with molecular weights ranging from 500 to >10,000 amu (atomic mass units) formed from decomposition products of lignin or from breakdown products of plant and animal tissues by chemical and biological processes<sup>6</sup> and by the formation of melanoidins by condensation reactions between sugars and aminoacids<sup>7</sup>. HS constitute a major part of DOM in aquatic environments and play an important role in biogeochemical processes<sup>8,9</sup>. The isolation method

of HS from freshwater was established by the International Humic Substances Society (IHSS), which involves its adsorption on XAD-8 resin column at pH 2 followed by elution with NaOH. The HS are later separated into humic and fulvic acids (HA and FA), desalted and freeze-dried<sup>10-12</sup>. Since XAD-8 resin adsorbed only hydrophobic organic compounds (HPO) and not hydrophilic organic compounds, this method was modified using XAD-4 resin in tandem with XAD-8 for the recovery of hydrophobic as well as hydrophilic organic compounds in aquatic environments<sup>13</sup>. The XAD-4 fraction can recover hydrophilic acids (HPhA), which are more aliphatic in nature and have higher O/C, H/C, N/C ratios<sup>12-15</sup>.

Chromophoric dissolved organic matter (CDOM) is a fraction of DOM<sup>16</sup>, which includes HS characterized by UV-visible absorption spectra.  $E_2/E_3$  ratio, spectral slope [ $S(\text{nm}^{-1})$ ] and spectral slope ratio ( $S_R$ ), derived from the absorbance spectra, have been widely used to characterize DOM in terms of aromaticity, molecular size, composition and molecular weight in aquatic environments<sup>17-19</sup>. Similar characterization is also done for isolated fractions of XAD-8 (HA, FA and HPO) and XAD-4 (HPhA) in natural, lake, river, estuary, coastal and marine waters<sup>14,20,21</sup>. The composition of HS in estuary is predominated by terrestrially derived high molecular weight and *in situ* produced lower molecular weight organic compounds during wet and dry seasons respectively.

Very few studies related to HS are available on Indian estuaries. For example, metal-HS interaction studies were conducted in Cochin backwater<sup>22-24</sup> and the Godavari estuarine<sup>25-28</sup> sediments. However, there are no studies related to aquatic HS and their structural characterization in the Godavari estuary. This estuary experiences terrestrially derived DOM during peak discharge and the *in situ* produced DOM during lean/nil discharge times<sup>29</sup>. Since HS contribute a major part of DOM in estuarine environment, their structural characterization and distribution are necessary to define the status of the ecosystem. Towards this objective, an initial study was done for characterization and distribution of HS in estuarine environment by UV-visible absorbance spectroscopy.

Godavari, the largest peninsular river in India, originates near Trayambak in Maharashtra and runs about 1480 km and flows eastwards and merges with Bay of Bengal. This river flows through six states (Maharashtra, Andhra Pradesh, Madhya Pradesh, Chhattisgarh, Orissa and Karnataka) and lies between 73°24' to 83°07'E long. and 16°16' to 22°36'N lat. Its catchment area is about  $3.1 \times 10^5$  sq. km, occupying ~9.5% of total geographical area of India<sup>30</sup>. Godavari basin receives more rainfall during south-west monsoon (~84%) than north-east monsoon<sup>31</sup>. Before the river merges with Bay of Bengal, a century old low dam at Dowlaiswaram, intercepts the flow into two main tributaries namely Vasista and Gauthami (Figure 1). The latter, being a major tributary, receives >70% of terrestrial freshwater in flux during peak

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monsoon season. This estuary acts as a source for carbon dioxide emission<sup>32</sup> during peak discharge period while carbon dioxide uptake happens during moderate to nil discharge periods due to phytoplankton blooms<sup>33</sup>. Recent observations on flushing times and salinity showed an intrusion of saline water upstream, beyond 36 km from the mouth of the estuary<sup>34</sup>. In order to study HS in the entire estuarine region, we examined four sampling stations from the mouth of the estuary up to a point where tidal influence took place in upstream (Figure 1).

Sampling was carried out during three different seasons, viz. post-monsoon (December 2014), pre-monsoon (April 2015) and monsoon (October 2015) at four stations from the estuarine mouth to head, namely Kapleswarapuram (station 1), Kotipalli (station 2), Yanam (station 3) and Bhairavapalem (station 4), using small mechanized boats. Bulk quantity (~10 l) of water samples was collected from the surface (~1 m) using a 5 l external spring Niskin sampler and transferred to pre-washed 20 l high density polyethylene (HDPE) Nalgene carboy (treated with 2 M HCl and thoroughly washed with Milli-Q water) for the isolation of HS.

Water samples were filtered through pre-combusted (~450°C) glass fibre filters (0.7 µm GF/F, Whatman) using glass filtration unit (millipore) and the filtrate obtained was acidified to pH 2 with HCl. Glass fibre filters were preferred instead of membrane filters as they are binder-free and avoid contamination of the samples<sup>13,35,36</sup>. The

resins (75 g each of XAD-8 and 4) were purified<sup>13,15,21</sup> and filled in 1 litre glass columns separately, supported by pre-washed glass wool. Before isolating the samples, the resin columns were washed thoroughly with 0.1 M NaOH followed by 0.1 M HCl and 0.01 M HCl. Also, flow rates of the two columns were adjusted with stop cocks by passing the Milli-Q water and care was taken to avoid the effect of air on flow rate by maintaining the same flow rate for input and output of samples through the columns. About 10 l of acidified water samples were passed through XAD-8 resin with a flow rate of ~8 ml/min and the effluent was collected and passed through XAD-4 resin with the same flow rate. Then the two columns were washed with Milli-Q water until all chlorides were removed. After that, desorption of XAD-8 and XAD-4 fractions was collected (in 125 ml pre-combusted glass bottles) by performing back elution with 120 ml of 0.1 M NaOH with an elution rate of ~2.5 ml/min.

The eluent fractions obtained from XAD-8 and XAD-4 resin columns were scanned between 200 and 800 nm with an interval of 1 nm to obtain an absorption spectra using Shimadzu UV-visible 1800 double-beam spectrophotometer with 1 cm path length quartz cuvettes and Milli-Q water as reference. Since XAD fractions have significant absorbance above 700 nm, to maintain the uniformity, a null point correction was applied using absorbance at this wavelength<sup>37,38</sup>. Absorption coefficients were calculated using the equation

$$a_{\lambda} = 2.303A/l, \quad (1)$$

where  $a_{\lambda}$  is the absorption coefficient ( $\text{m}^{-1}$ ),  $A$  the absorbance and  $l$  is the path length (m).

$E_2/E_3$  ratio was calculated as the ratio of absorption coefficients at 250–365 nm (refs 17, 39).

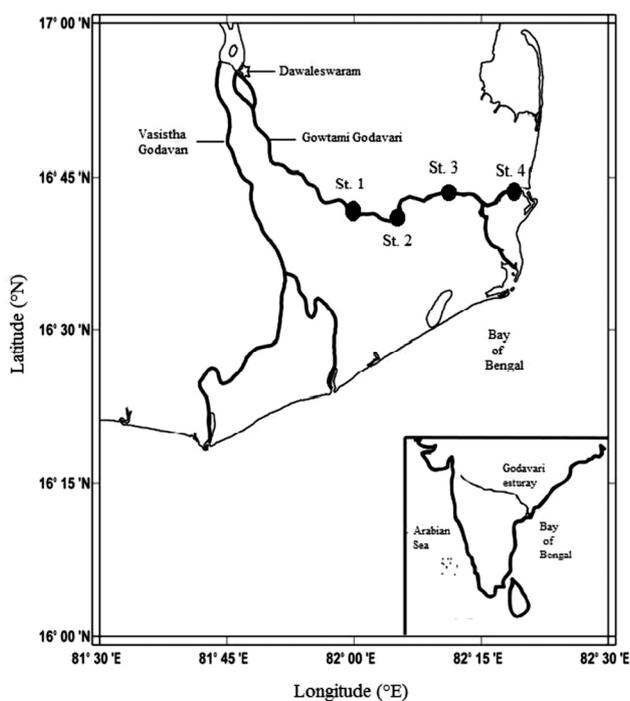
Spectral slope ( $S$ ) was calculated for the regions 275–295 and 350–400 nm using an exponential regression model between the absorption coefficient and wavelength

$$a(\lambda) = a(\lambda_0)e^{-S(\lambda-\lambda_0)}, \quad (2)$$

where  $a(\lambda)$  is the absorption coefficient at wavelength  $\lambda$ ,  $\lambda_0$  the reference wavelength and  $S$  is the spectral slope. The spectral slope ratio ( $S_R$ ) was then calculated as the ratio of  $S_{275-295}$  to  $S_{350-400}$  (ref. 19).

Absorption coefficient of XAD-8 and XAD-4 fractions decreased as the wavelength increased from 200 to 800 nm. However, XAD-4 fraction showed significant lower absorbance when compared with XAD-8 (Figure 2). This is attributed to adsorptive removal of HS by an earlier column (XAD-8) in the series.  $E_2/E_3$  ratio,  $S_{275-295}$ ,  $S_{350-400}$  and  $S_R$  were derived from these spectra.

$E_2/E_3$  ratio of XAD-8 was significantly lower than XAD-4 fraction (Figure 3) irrespective of the season.



**Figure 1.** Study area of sampling stations. Station 1, Kaplileswarapuram; Station 2, Kotipalli; Station 3, Yanam; Station 4, Bhairavapalem.

This indicated that the moderately high molecular weight and aromatic-nature compounds<sup>17</sup> retained on XAD-8 resin might be due to its structure containing polymeric structure of acrylic esters with intermediate polarity nature. Previous studies based on gas chromatography technique<sup>40</sup> and <sup>13</sup>C NMR analysis proved that the organic acids contained five carbons with a carboxyl functional group and were hydrophobic enough to be retained by XAD-8 resins. It was also concluded that the major composition of the retained fraction was constituted with high concentration of aromatic, greater heteroaliphatic ketones and carboxylic compounds<sup>13</sup>. No significant spatial variability was observed in the distribution of  $E_2/E_3$  ratio. However, a significant decrease in  $E_2/E_3$  (5.67–3.91) was found from post-monsoon to monsoon season (Figure 3a) from stations 1 to 3. This may be due to the formation of non-degradable low molecular weight organic compounds during post-monsoon as a consequence of bacterial metabolic activity<sup>41</sup>. Whereas, during monsoon the estuary received enormous loads of terrestrially derived organic matter (mostly from the plant origin) from upstream<sup>30,42</sup> and thus formed non-degradable high molecular weight organic matter. However, higher values of  $E_2/E_3$  ratio for XAD-4 fraction inferred that the lower

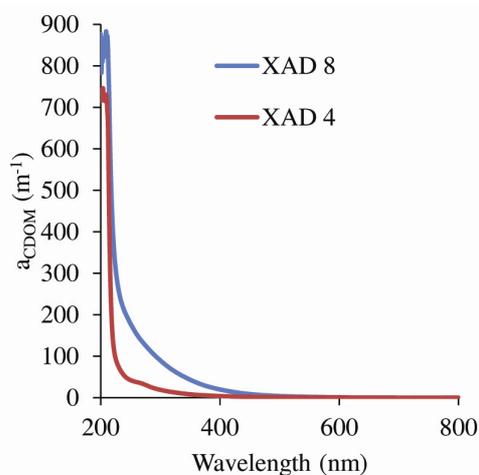


Figure 2. Absorbance spectra of XAD-8 and 4 fractions.

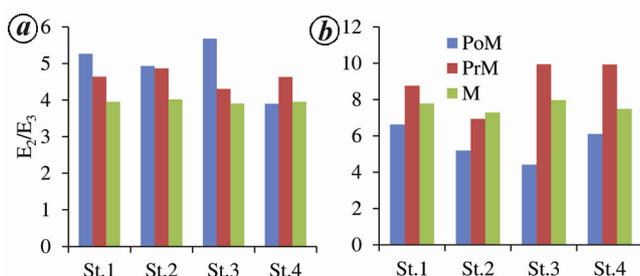


Figure 3. Variation of  $E_2/E_3$  of humic substances fractions: a, XAD-8; b, XAD-4 (PoM, Post-monsoon; PrM, Pre-monsoon; M, Monsoon).

molecular weight organic compounds retained on this resin might be due to its structure made of styrenedivinyl benzene, which is more hydrophilic in nature. This ratio was found to be low during post-monsoon than pre-monsoon for all the stations and decreased from stations 1 to 3. This may be due to exudation by phytoplankton<sup>43</sup> or formation of phytoplankton blooms<sup>29</sup> during post-monsoon and their extensive release of organic matter (as a function of extracellular metabolic activity) into the estuarine environment.

$S_{275-295}$  was lower than  $S_{350-400}$  for XAD-8 fraction in all the seasons (Figure 4) due to the adsorption of high molecular weight organic matter derived from terrestrial sources. However, exactly opposite trend ( $S_{350-400} < S_{275-295}$ ) was observed for XAD-4 fractions (Figure 5) during post- and pre-monsoon seasons due to lower molecular weight organic matter produced from *in situ*

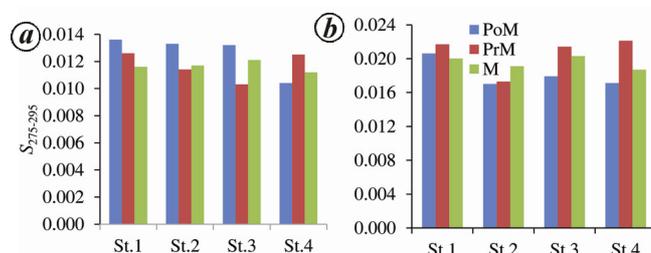


Figure 4. Variation of  $S_{275-295}$  ( $\text{nm}^{-1}$ ) of humic substances fractions: a, XAD-8; b, XAD-4.

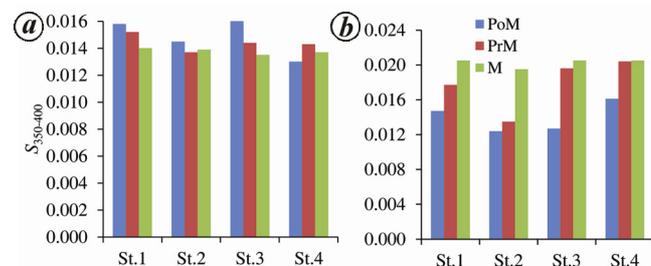


Figure 5. Variation of  $S_{350-400}$  ( $\text{nm}^{-1}$ ) of humic substances fractions: a, XAD-8; b, XAD-4.

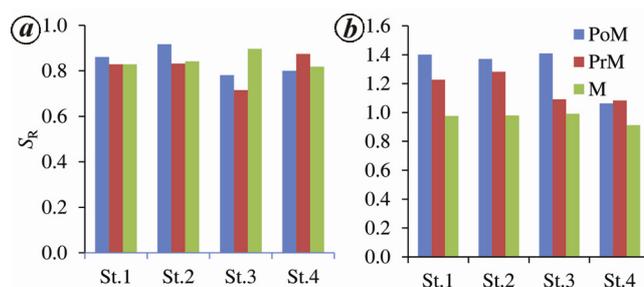


Figure 6. Variation of  $S_R$  of humic substances fractions: a, XAD-8; b, XAD-4.

process. Similar trend was not found during monsoon which could be due to the fact that the entire estuary was saturated with terrestrially derived organic matter during high riverine runoff from upstream<sup>19</sup>.  $S_{350-400}$  was significantly increasing trend from post-monsoon to monsoon season (Figure 5 b). This may be due to the *in situ* produced organic matter released from the extracellular products of phytoplankton decreases from post-monsoon to monsoon season. The results were similar to the spectral slope ratio ( $S_R$ ), which was consistently lower for XAD-8 fractions (Figure 6 a). For XAD-4 fractions, it showed a significant decreasing pattern from post-monsoon to monsoon for all the stations except station 4 (Figure 6 b). The results also agreed with studies conducted at open ocean, proved that the higher values of  $S_R$  indicated the presence of newly produced CDOM, formed during microbial degradation process<sup>44</sup>. This was also evident from the reported values of chlorophyll *a* concentrations (moderate discharge > no discharge > peak discharge) at the study area<sup>29</sup>.

HS isolated from the mouth (i.e. station 4) of Godavari estuary showed contrasting spectral characteristics ( $E_2/E_3$  and  $S_R$ ) for XAD-8 fractions compared to upstream stations (1–3) during post- and pre-monsoon seasons (Figures 3 a and 6 a). Here the retention of higher molecular weight aromatic organic compounds on XAD-8 resin during post-monsoon might be due to phytoplankton blooms with a surplus of nutrients involved during photosynthesis. Consequently, extracellular release of organic compounds took place from phytoplanktons, viz. combined amino acids, protein-like (tyrosine and tryptophan) compounds<sup>45</sup> and humification of organic matter<sup>46</sup>. However, during pre-monsoon high molecular weight humic like compounds was involved in photo-degradation and formed lower molecular weight organic compounds due to the presence of intense solar radiations<sup>47,48</sup>. Experimental studies based on photochemical degradation of pre-concentrated dissolved humic substances also resulted in the formation of lower molecular weight compounds<sup>19</sup>.  $S_R$  was lower during post-monsoon for XAD-4 fraction due to the increase of dissolved organic matter produced from phytoplanktons. This is attributed to the decrease in microbial activity or abundance of phytoplanktons at the mouth of the estuary. Similar results with high density of autotrophic pico planktons were observed at the mouth of tropical<sup>49,50</sup>, subtropical<sup>51</sup> estuaries and coastal environments.

The UV-visible absorbance spectra and their derived parameters, e.g.  $E_2/E_3$  ratio, spectral slopes ( $S_{275-295}$  and  $S_{350-400}$ ) and  $S_R$  of the two (XAD-8 and XAD-4) fractions of dissolved HS are useful to identify spatiotemporal variations in the Godavari estuary. DOM molecular weight, highest during monsoon in the entire estuary, is attributed to river flux and the accompanying terrestrially derived organic matter. While at the mouth of the estuary, DOM molecular weight decreased during pre-monsoon

than post-monsoon, the reverse happened in upstream water. These are attributed to photo (and bacterial) degradation and phytoplankton production respectively.

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