Kinetic aspects of humic substances bleaching during biological mineralization

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Abstract: Kinetic aspects of humic substances bleaching during biological mineralization. Assays were carried out to describe the kinetic aspects of biological bleaching of humic substances during its mineralization. Samples of aquatic plant and water (*Oxycaryum cubense*) were collected in the Infernão lagoon (21º 35' S and 47º 05' W; São Paulo State, Brazil). Experiments were carried out using fulvic acid (FA) and humic acid (HA) derived from leachates of *O. cubense* decay (120 days). FA and HA were added to 450 mL of lagoon water and incubated under high and low dissolved oxygen concentrations and at three temperatures. The optical density was monitored (95 days) and the data were fitted to first-order kinetic model. The processes involved with bleaching were favored with increasing temperature only during HA mineralization at low dissolved oxygen availability, indicating that in the Infernão lagoon, the biological bleaching of HA is more effective between November and April. Overall, FA presented higher bleaching coefficients than HA. Low HA bleaching coefficients are probably due to the complexity of the chemical structures and its transformation in FA.

Key words: *Oxycaryum cubense*, humic acid, fulvic acid, bleaching coefficients, kinetic aspects.

Resumo: Aspectos cinéticos da descoloração de substâncias húmicas durante a mineralização biológica. Ensaios foram realizados para descrever aspectos cinéticos da descoloração de substâncias húmicas (SH) durante sua mineralização biológica. Amostras de água e de planta aquática (*Oxycaryum cubense*) foram coletadas na lagoa do Infernão (21º 35'S e 47º 05'W; estado de São Paulo, Brasil). Os experimentos foram realizados usando ácidos fúlvico (AF) e húmico (AH) obtidos da degradação (120 dias) dos lixiviados de *O. cubense*. Os AF e AH foram adicionados a 450 mL de água da lagoa e incubados sob condições de altas e baixas concentrações de oxigênio dissolvido e em três temperaturas. A densidade óptica foi monitorada (95 dias) e os dados foram ajustados a um modelo cinético de primeira ordem. Os processos envolvidos com a descoloração das SH foram favorecidos com o aumento da temperatura, somente na mineralização dos AH sob condições de baixa disponibilidade de oxigênio dissolvido; indicando que na lagoa do Infernão, a descoloração biológica dos AH seja favorecida de novembro a abril. De maneira geral, os AF apresentaram coeficientes de descoloração mais altos que os HA. Os coeficientes baixos de descoloração dos AH provavelmente relacionaram-se com a complexidade das estruturas químicas e com sua transformação em AF.

Palavras-chave: *Oxycaryum cubense*, ácido húmico, ácido fúlvico, coeficientes de descoloração, aspectos cinéticos.

Introduction

Humic substances (HS) are ubiquitous in the environment and can be readily isolated from soils, waters and sediments. HS are formed from microbiological or chemical (abiotic) transformation of dead tissues of plants or animals. In most natural waters, the major portion of dissolved organic carbon (DOC) is dominated by dissolved HS, which corresponds to up to 60% of DOC (Thurman, 1985). Even though HS is normally assumed to be inert to bacterial degradation, a fraction of aquatic humic substances may be available as a primary bacterial substrate (Carlsson *et al.*, 1998; Coates *et al.*, 2002). The *Geobacteraceae* species might be

important in humic-reducing organisms especially in sediments (Coates *et al.*, 1998). It is clear that any process influencing the fate and chemistry of DOC will provide a feedback on secondary production through microbial loop (Miller, 2000). The rates that HS are cycled and accumulated depend mainly on the balance between the immobilization and mineralization processes. Immobilization of a given element or carbon is the incorporation or maintenance in the organic form. In aquatic systems this process generally occurs due to the action of phytoplankton and microorganisms, by absorption into the roots of aquatic plants and by chemical interactions involving humic substances formation and adsorption processes of organic compounds. Mineralization occurs when inorganic forms of a given element are released by chemical or photochemical reactions or during the catabolism of an organic resource (Mackensen and Bauhus, 1999). In general, the aerobic decomposition is faster than under anaerobic conditions, acting on a wider spectrum of substrata, generating stable compounds and supporting a large number of microbial cells (Davis and Cornwell, 1991).

Aquatic humic substances are polar, amorphous, polymeric and straw-colored organic acids (Thurman and Malcolm, 1981). The HS is considered as polymeric products from carbohydrate degradation, lignin, proteins and fats in different age of decomposition. There is a consensus that HS are produced either by biochemical degradation of plant (Cunha and Bianchini Jr., 2001) or animal residues or by polycondensation of relatively small organic molecules released during decay, and that microorganisms are related to both processes (Lu *et al.*, 2001). It can also be formed by photodegradation process (Bertilsson and Tranvik, 2000). Novel theory about nature of HS suggests that rather than macromolecular polymers, they are supramolecular associations of heterogeneous molecules bounded by weak forces in contiguous hydrophobic and hydrophilic domains of large molecular sizes (Picollo *et al.*, 2000). HS may be separated into three fractions: humic, fulvic acid (FA) and humic acid (HA); these compounds are categorized on the basis of its solubility on alkali and acid media.

In natural waters, most organic molecules lack optical activity in the visible range; a prominent exception is HA and FA, which absorb in the blue range of the spectrum, and provides hue to water (Yacobi *et al.*, 2003); consequently HS reduces light transmission through water, affecting plankton metabolism within aquatic systems (Håkanson, 2002). HS are often considered chemically stable and biologically refractory; however, there is evidence that they are chemically reactive in aquatic environments and that a large fraction of HS degrades on scales of weeks to months (Brezonik, 1994). The bleaching effect of sunlight on dissolved organic color has been known for many decades; Hutchinson (1957) reported studies dating back to the late 19th century that demonstrated substantial loss of color in lake water incubated in bottles near the lake surface. The presence of dissolved oxygen has been considered essential for the bleaching reaction and that the loss of color and DOC from lake water incubated in the light follows first order kinetics; half-time of color and DOC decay were c.a. 35 to 45 days (Zepp *et al.*, 1977; Brezonik, 1994). The availability of oxygen also affects the microbial community and indirectly the metabolic routes adopted for mineralization (Cunha-Santino and Bianchini Jr., 2002a). In this context, this study aimed at evaluating the bleaching of humic substances (HA and FA) during its biological mineralization.

Material and methods

Formation and separation of humic substances

HS were extracted from 120 days leachate from decomposing aquatic macrophyte, the *Oxycaryum cubense* (Poepp e Kunth) Lye (Cyperaceae). For HS generation, *O. cubense* and water samples were collected from Infernão lagoon (21º 35' S and 47º 05' W; Ecological Reserve of Jataí; municipality of Luiz Antônio, São Paulo State, Brazil). The plant material was washed under tap water to remove attached matter, oven-dried (c.a. 45ºC) to constant weigh and grounded. For humic compounds acquisition, the plant material was placed in acid-washed 5-liter flasks with water lagoon, producing dry weight concentration of 10 g L^{-1}. The flasks were incubated in the dark, under aerobic conditions (by constant clear air flux) and at room temperature. After 120 days, the chambers contents were fractionated into dissolved (DOM = whole leachate) and particulate organic matter (POM) according to the procedures described by Wetzel and Likens (1991).

Isolation of humic substances

The HS were isolated and fractionated into FA (fulvic acid) and HA (humic acid) from DOM using analytical procedures described by Cunha-Santino and Bianchini Jr. (2002b), based on solubility differences in acid and alkaline media.

Mineralization assays of humic substances

Samples of FA and HA from O. cubense leachate were incubated in the dark, in flasks containing filtered (fiber glass; 1.2 μm Millipore) lagoon water (concentration of FA and HA in carbon basis are presented in Table 1) and under different redox conditions: high and low dissolved oxygen concentrations (DO). High (HDO) were maintained by constant bubbling air flux. Low (LDO) were obtained by periodically bubbling N2 on FA and HA incubation flasks (without headspace). The DO of each flask were monitored using an oxygen-meter (Metrohm Herisau AGCH-9100/E-637); for HDO condition, when the dissolved oxygen concentrations were above 2.0 mg L-1, the flasks were aerated again, until the DO reached the saturation value according to temperature incubation. The mean DO for FA flasks for LDO conditions were 1.47 mg L-1 (SD = 0.32, n = 15) and for HA was 1.73 mg L-1 (SD = 0.24, n = 15). For HDO conditions this value was 8.10 mg L-1 (SD = 0.77; n = 15) for FA and 7.61 mg L-1 (SD = 0.81; n = 15) for HA.

The incubation temperatures simulated those within the Infernão oxbow lake (Antonio and Bianchini Jr., 2000). Two flasks maintained in HDO and two in LDO conditions (using FA and HA as substrate) were incubated at 16.0 ± 1.7°C; 22.4 ± 1.2°C and 26.5 ± 2.1°C. Initial and final concentrations of HA and FA (on carbon basis) were determined by high temperature combustion with a Shimadzu TOC-5000A analyzer. The kinetic of HS blanching were measured as optical density (OD) after incubation periods of 0, 2, 4, 6, 8, 10, 15, 20, 25, 30, 40, 56, 65, 80 and 95 days; the sampling days totalizing 15 measurements for each redox condition. Considering the HS bleaching mechanisms, related to the decomposition of the organic resources, follow a first order kinetics (Brezonik, 1994) the color decreasing can be described in agreement to the following equation:

$$\frac{dOD}{dt} = -k_B OD \qquad (1)$$

Where:

OD = optical density of a given wavelength (absorbance);

k_B = bleaching constant rate (day^{-1}).

The optical density was measured as absorbance (optical path length = 1 cm, wavelength = 450 nm; Toledo, 1973; Stevenson, 1982) using spectrophotometer (Pharmacia LKB - Novaspec II). The blanching coefficient (k_B) was calculated by fitting the values of OD decreasing fitted to first-order kinetic model (Equation 1) using a non-linear method (Levenberg-Marquardt iterative algorithm) according to Press *et al.* (1993). According to USEPA (1985), the k_B was utilized to estimate the effect of temperature on the bleaching reactions. To test differences among treatments (dissolved oxygen availability and temperature), the Kruskal-Wallis test was used ($\alpha > 0.05$).

Results and discussion

Although the sunlight has been often considered the main factor related with the DOC bleaching, the dynamics of organic carbon cycling in aquatic ecosystems is affected by bacterioplankton (Cotner, 2000). Dissolved organic matter contributes to detrital pool for microbial heterotrophy, and such decomposition is very important to higher trophic levels in feedback processes, both positively as in nutrient recycling and utilization by primary producers, and negatively as in oxygen consumption and production of fermentative metabolic end products (Wetzel, 1995). Microbial degradation of humic substances is an important part of humus turnover and therefore essential for maintaining the global carbon cycle; implicit in this process, the bleaching of these substances is often registered (Blondeau, 1989; Cunha-Santino and Bianchini Jr., 2004a). The OD values decreased (*i.e.* the samples lost color) during FA and HA mineralization (Figures 1 and 2). In relation to temperature, differences between treatments were only observed in HA mineralization at 16.0°C and 26.5°C (LDO) (Kruskal-Wallis test; $p < 0.05$); the other treatments showed no significant differences ($p > 0.05$).

In HA mineralization just one peak in the initial phase of the experiment was observed. After this a continuous decrease in the OD values was observed.

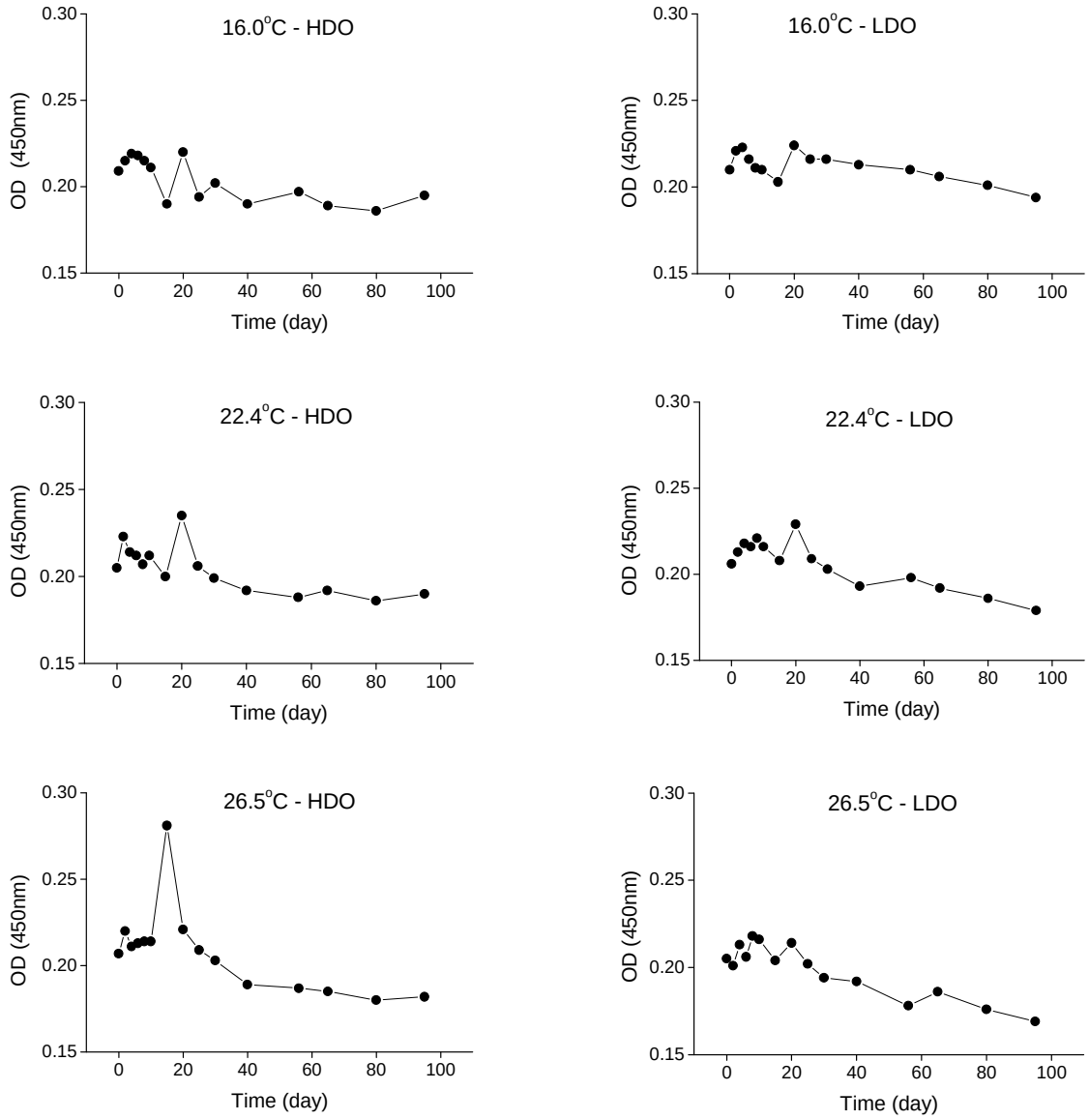

Figure 1. Optical density variation during the HDO and LDO mineralization from HA at different temperatures.

Until the 20th day, under HDO conditions an increase in the OD values was observed with increasing temperatures. A quite different behavior was observed for the FA mineralization, in which three peaks of increasing color were observed until approximately the 20th day. The peak intensity increased with increasing temperatures, except under LDO conditions at 26.5°C where the peaks are not clear. The OD values registered for the HA flasks were higher (from 0.15 to 0.25) in comparison with AF flasks that ranged from 0.014 to 0.08. The changes in the OD values, verified in the first 3 weeks, probably were associated with the reactions of consumption and formation of chromophores groups (re-synthesis) evolved in mineralization of the labile fraction of FA and HA molecules; which accounts for approximately 25% of these substances (Cunha-Santino and Bianchini Jr., 2002b). During the mineralization of HS, the bleaching process could be associated with specific attack to the chromophores groups of the HS molecules; these chromophores are color centers with both phenolic quinones and conjugated double bounds (Thurman, 1985).

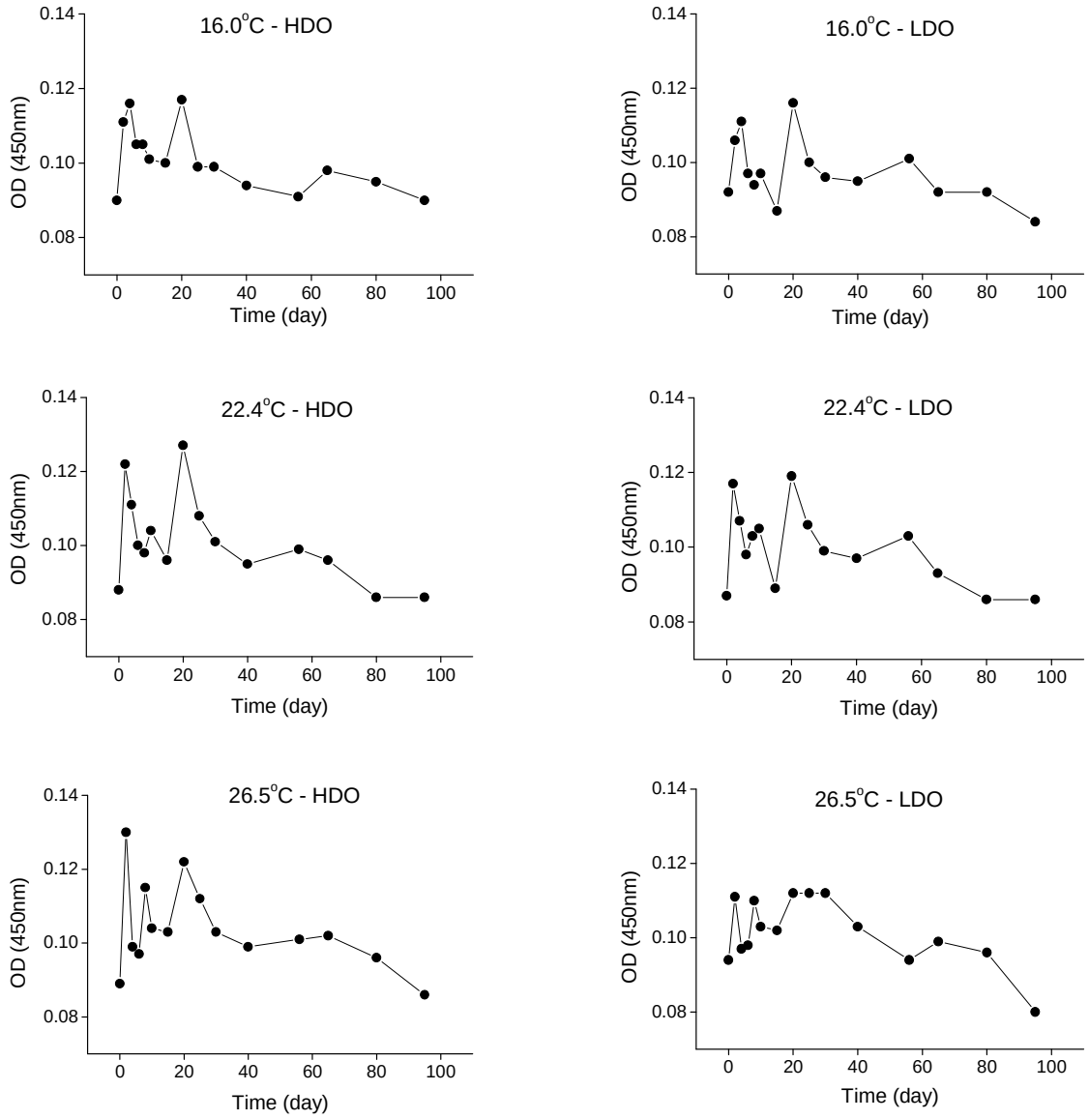

Figure 2. Optical density variation during the HDO and LDO mineralization from FA at different temperatures.

The bleaching of HA and FA can be compared by the k_B (Table 1). For HA, k_B was calculated from the beginning of the experiment until the 95th day. For FA, these coefficients were estimated since the 20th day. Such coefficients were also used to evaluate Q_{10} and the half-time ($t_{1/2}$) of the bleaching process. These rates ranged from 0.00091 to 0.00255 day^{-1} for HA and 0.00217 to 0.00424 day^{-1} for FA. The higher bleaching coefficients were observed for FA. The half-time of the bleaching process ($t_{1/2}$) ranged from 163 to 760 d (HA: 271 to 760 days and FA: 163 to 319 days). The kinetic fittings from the experimental results presented determination coefficients (r^2) that varied from 0.39 to 0.87 (Table 1). Comparing k_B values and the amounts of consumed organic carbon ($[COD]_i$-$[COD]_f$) it was possible to verify that the rates of bleaching processes are proportional to carbon loss derived from mineralization reactions; this relationship tended to be linear for HA and exponential for FA acid. This biological bleaching half-time was greater (at least 3.6 times) than that reported by Brezonik (1994); who attributed to the effect of solar radiation in the loss of water color (photochemical bleaching).

Table 1. Bleaching coefficients (k_B), bleaching half-time ($t_{1/2}$) at different temperatures and redox conditions (HDO and LDO). Error from the kinetic fitting and r^2 = determination coefficient. [COD]i = initial concentrations of HA and FA (on carbon basis); [COD]f = final concentrations of HA and FA (on carbon basis).

Substrata	Condition	Temp. (°C)	k_B (day^{-1})	Error	$t_{D1/2}$ (day)	r^2	[COD]i ($mg\ L^{-1}$)	[COD]f ($mg\ L^{-1}$)
HA	HDO	16.0	0.00150	0.00040	462	0.52	53.85	37.68
FA	HDO	16.0	0.00217	0.00099	319	0.45	44.70	38.12
HA	LDO	16.0	0.00091	0.00026	760	0.49	53.82	35.35
FA	LDO	16.0	0.00282	0.00092	245	0.61	43.32	33.21
HA	HDO	22.4	0.00167	0.00044	414	0.53	53.52	33.29
FA	HDO	22.4	0.00424	0.00114	163	0.71	42.96	31.68
HA	LDO	22.4	0.00198	0.00033	349	0.75	53.69	32.24
FA	LDO	22.4	0.00360	0.00088	192	0.74	43.71	30.65
HA	HDO	26.5	0.00255	0.00091	271	0.39	53.99	31.05
FA	HDO	26.5	0.00337	0.00082	205	0.75	44.13	34.71
HA	LDO	26.5	0.00239	0.00032	290	0.83	54.00	30.72
FA	LDO	26.5	0.00389	0.00062	178	0.87	44.07	22.78

The Q_{10} values (ratio of reaction rates at 10°C temperature increments) showed greater influence of temperature on the HA bleaching under LDO condition (Q_{10} = 2.57) while for HA at HDO and FA (LDO and HDO) the Q_{10} values varied from 1.24 to 1.61; these coefficients corroborated with the statistical results, showing similarity among treatments (Table 2).

Table 2. Coefficients Q_{10} and θ from bleaching process (HDO e LDO) from fulvic and humic acids.

	HDO condition		LDO condition	
Bleaching	Q_{10}	θ	Q_{10}	θ
HA	1.24	1.022	2.57	1.098
FA	1.61	1.049	1.36	1.032

The values of θ (= $Q_{10}^{0.1}$), an empirical coefficient often used in mathematical models for description of the temperature effects on biochemical reactions (USEPA, 1985), are also presented in Table 2. According to the incubations made with samples of water of the Infernão lagoon (Antonio and Bianchini Jr., 2002), the Q_{10} values for bleaching under HDO were close to that obtained for aerobic glucose decay (1.12) and those related with LDO were smaller than reported to the anaerobic glucose decay (3.3). With the progressive increasing of temperature, a proportional increase in bleaching of HA at LDO occurred, as denoted by bleaching coefficients (k_B). The increase in bleaching is probably due to an increase of metabolic activity of the heterotrophic community acting on humic substances molecules. Experiments with *P. chrysosporium* demonstrated that grown in low ionic strength also decolorized the humic acids, but this bleaching was a result of the binding of humic acid on fungus mycelia caused by the reduction of charge on these molecules induced by acidification of the media (Blondeau, 1989). FA bleaching coefficient was 1.83 higher than HA. Thus, the HA chemical properties contributed to the inhibition of the bleaching process due to the aromaticity and less reactivity of this molecule. In relation to HA the re-synthesis reactions lead to a new colored compound. The conversion process of HA resulted in the formation of lower-molecular-mass FA and carbon dioxide; the bleaching of different high molecular mass HA has been reported to be a result of white-rot fungi (Steffen *et al.*, 2002). The fungi not only bleached HA but also changed their physicochemical properties towards higher polarity.

It should be stressed that during the k_B kinetics fittings the gap used for FA and HA just refereed to bleaching process. The color increment was not considered. The average values obtained during the bleaching process suggested a great influence on metabolic activities of the heterotrophic community during the LDO process. In spite of the aerobic process energetic yield is more efficient than anaerobic process, the microbiota of Infernão lagoon are well adapted to anaerobic conditions (Antonio and Bianchini Jr., 2002) becoming the anaerobic process more effective in the removal of HS molecules. For the FA flasks, oscillations in the optical density were observed in the initial phase of the experiment. These peaks probably indicate that FA molecule re-synthesis by the action of microorganisms in the beginning of experiment, resulting in an increase in color intensity (Stevenson, 1982).

In summary, considering the statistical analyses and the Q_{10} results we may conclude that the temperature affected the velocity of bleaching of humic acids at LDO conditions; meaning that the bleaching process linked with the biological reactions was more sensitive to higher temperature. In the Infernão lagoon, the higher temperatures were observed in the rainy season (Freitas-Lima and Godinho, 2000; Suzuki and Esteves, 2000), indicating that the biological bleaching of HA is more effective from November to April. In this period, it must also be considered the input of allochtonous recalcitrant material from the catchments areas. Overall, FA molecules presented higher bleaching coefficients than HA molecules. Low HA bleaching coefficients are probably due to the complexity of the chemical structures and its transformation in FA. For the Infernão lagoon, the temporal variation of HS mineralization suggests that the HS decay is a slow process. Therefore, these compounds are expected to be extensively incorporated in the sediments (Cunha-Santino and Bianchini Jr., 2004b), suggesting that the bleaching process will occur within the sediment of this system. Infernão lagoon is located at c.a. 250 m from

principal channel of the Mogi-Guaçu river, and joined to the river only in the rainy season (Feresin and Santos, 2000); the Mogi-Guaçu river presented unimodal inundation regime, *i.e.,* potential conditions for inundations once a year, this event usually occurs from december to february (Ballester and Santos, 2001). During this period, exportation of HS due to flood and bleaching process due to higher temperatures are the events that probably dominate.

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