Evaluation of phytoplankton pigments in a shallow coastal lake submitted to strong hydrodynamics: comparative analysis of spectrophotometric methods.

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ABSTRACT: Evaluation of phytoplankton pigments in a shallow coastal lake submitted to strong hydrodynamics: comparative analysis of spectrophotometric methods. Spectrophotometric methods were used to evaluate the variability on concentrations of phytoplankton pigments in Itapeva Lake, a shallow coastal lake on the northern coast of Rio Grande do Sul (Brazil). The comparison between methodologies of chlorophyll *a* showed that data from the trichromatic (OD 664 nm and 663 nm) and from the total pigment methods were significantly correlated to each other. Moreover, these methods achieved better results than the monochromatic methods, due to the small number of samples with a negative value. The results of both methods of pheopigments determination exhibited seasonally the same behavior. The relationships between optical densities, before and after acidification, were adequate to explain the physiological status of the samples, serving as a good indicator of the methods' adequacy for pigment determination. The phytoplankton pigments presented a spatial and temporal distribution according to wind action, in which fetch had a marked effect on hydrodynamics and on the phytoplankton community.
Key-words: pigment, shallow coastal lake, methodology, hydrodynamics

RESUMO: Avaliação de pigmentos fitoplanctônicos em lagoa costeira rasa: análise comparativa de métodos espectrofotométricos. Métodos espectrofotométricos foram usados na avaliação da concentração de pigmentos fitoplanctônicos na Lagoa Itapeva, uma lagoa costeira rasa no litoral norte do Rio Grande do Sul (Brasil). A comparação entre metodologias da análise de clorofila *a* mostrou que os dados obtidos pelos métodos tricromáticos (DO 664 nm e 663 nm) e pelo método do pigmento total estiveram significativamente correlacionados entre si. Além disso, estes métodos obtiveram melhores resultados em detrimento dos métodos monocromáticos, devido ao baixo número de amostras com valor negativo. Os resultados de ambos métodos na determinação de feopigmentos, geralmente, exibiram durante o ano o mesmo comportamento. As relações entre densidades ópticas, antes e após a acidificação, foram de extrema valia para a interpretação do estado fisiológico das amostras, servindo como um bom indicador da adequação dos métodos empregados na análise de pigmentos. Os pigmentos fitoplanctônicos exibiram uma distribuição espaço-temporal em função da ação dos ventos, onde o "fetch" teve um grande efeito na hidrodinâmica e sob a comunidade fitoplanctônica.
Palavras-chave: pigmento, lagoa costeira rasa, metodologia, hidrodinâmica.

Introduction

The extraction and measurement of chlorophyll are procedures with several steps, each of which can be performed in several different manners (Edler, 1979). The standardization of the chlorophyll analysis method has already been performed by APHA (1992) and CETESB (1990), and others. The difference in the methodologies include the type of solvent for extraction (acetone or methanol), porosity of the filtering membrane (cellulose acetate or fiberglass filters), the volume of the sample to be

filtered, the reading equipment (spectrophotometer, fluorometer or HPLC –"High Performance Liquid Chromatographic"), and the type of equations (mono or trichromatic). However, the method chosen will depend on the specific environmental conditions, the research goal and, especially, on existing laboratory conditions.

Different interpretations regarding the pigment analysis methodologies are discussed by several authors (Lorenzen, 1967; Edler, 1979; Rai, 1980; Barbosa, 1981; Schwarzbold et al., 1999). However these methods have been used without criticism. Relations in deep lakes are often valueless in shallow ones. Although the concentration of chlorophyll *a* in shallow lakes is broadly used as a phytoplankton biomass estimator, its general validity has not been submitted to detailed studies (Vörös & Padisák, 1991).

Pheophorbide *a* and pheophytin *a*, two common products of chlorophyll *a* degradation, interfere in the determination of chlorophyll *a* because they absorb light and fluoresce in the same region of the spectrum. If these pheopigments are present, they will produce significant errors in the chlorophyll *a* values (Moed & Hallegraeff, 1978; APHA, 1992). Often it is not possible to determine the main type of pheopigment measured, since chlorophyll *a* can be degraded along two pathways: chlorophyll *a* → pheophytin *a* → pheoforbide *a* or chlorophyll *a* → chlorophyllide *a* → pheophorbide *a* (Glooschenko et al., 1972). Fecal pellets produce pheoforbide before pheophytin (Lorenzen, 1967). Thus, the term pheopigment was used in place of pheophytin *a*.

Procedures for the trichromatic method are those most widely proposed (Vollenweider, 1974; Raí, 1980). The Strickland & Parsons equation (1968) for chlorophyll *a* is the one most commonly used. The differences between the trichromatic equations are negligible (<2.5%) to obtain chlorophyll *a*, but the differences between the equations are much greater, for the evaluation of chlorophyll *b* and *c* (Edler, 1979).

For both types of equation (monochromatic and trichromatic), the readings of the respective wavelengths should always be corrected for the reading made at 750 nm. Absorption at 750 nm is used to correct the absorption of other colored compounds and turbidity besides chlorophylls and should not exceed 0.005 (Holm-Hansen & Riemann, 1978; Golterman et al., 1978).

Regarding the trichromatic formula of chlorophyll *a*, a small change can occur as using wavelength 664nm, or also the wavelengths 663 and 665 nm (Jeffrey & Humphrey, 1975). So, the values of the optical density at 663nm were also be used to replace wavelength 664 nm in this trichromatic equation (Edler, 1979).

When chlorophyll *a* and pheopigment *a* are measured together by Lorenzen (1967) method, lower values of chlorophyll *a* are produced in comparison to the trichromatic method. However, in samples with high pheopigment content (sediments and bottom water), the trichromatic method overestimates the chlorophyll *a* content. Thus, the samples should also be measured according to the monochromatic equation, when this procedure involves little additional effort (Edler, 1979). Also chlorophyll *a* is overestimated by the trichromatic spectrophotometric equations when the proportion of pheophytin/chlorophyll increases (Rai, 1980). For future comparisons, it would be more useful to use monochromatic equations and to adjust the data of chlorophyll *a*.

In the method proposed by Lorenzen (1967), absorbance is measured only on two wavelengths (750 and 665 nm) before and after the acidification of the sample, and chlorophyll *a* and pheopigments can be calculated. This method can be applied to various types of aquatic environments (freshwater, estuarine and coastal waters), filtering a relatively small amount of water in a short time. Edler (1979) proposed the same range of wavelengths variations (from 663 to 665 nm) for the trichromatic and monochromatic methods.

Considering all the issues raised on the use of different methods to analyze pigments, it became necessary to learn to what extent this is also valid for Itapeva

Lake. This aquatic environment is a shallow coastal lake, with a rather high effective fetch (maximum value 19.8 km during autumn 1999 and winter 1999), due to morphometry and wind regime in the region (Cardoso, 2001).

The main goal of this study was to compare several pigment colorimetric analytical methods, at each sampling station in the lake. The other objectives were to determine the behavior of the trichromatic and monochromatic methods for chlorophyll *a* during each sampling; to present relations between trichromatic and monochromatic methods and the total pigment method; to find out which method maintains a more uniform and effective response during a long-term study; to compare the OD_{664} before/OD_{665} after and OD_{663}before /OD_{663}after acidification ratios; to compare the two methods pheopigment analysis and to present relations between the chlorophyll *a* and pheopigment methods.

Material and Methods

Three sampling stations were selected in Itapeva Lake (Fig.1). In North (0615690E-6747815N), Center (0603350E-6732254N) and South stations (0597474E-6725967N), water samples were taken at 3 depths (surface, middle and bottom) at 4 time intervals throughout the day (6 am, 10 am, 2pm and 6 pm).

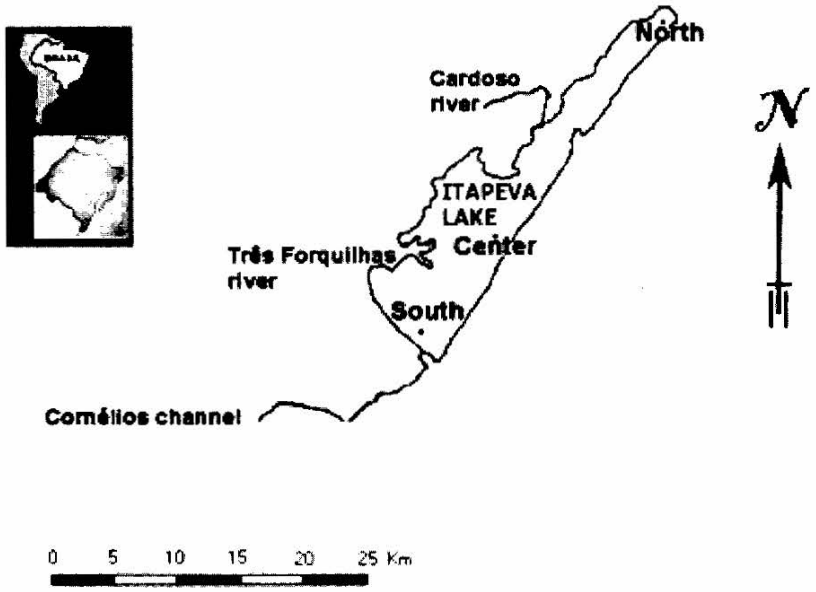

Figura 1: Location map of the study area, with the sampling stations (North, Center and South) in Itapeva Lake (northern coast of the state of Rio Grande do Sul – Brazil).

The samplings were performed during a long-term study: in spring (December 15-20, 1998), summer (March 2-7, 1999), autumn (May 21-26, 1999) and winter (August 14-19, 1999). However, water samples for pigment analysis were collected only three days, at 24 h intervals among them.

The methodology used for water sampling, filtration and extraction followed the recommendations of CETESB (1990) and APHA (1992). Water was collected at

each depth using a horizontal Van Dorn bottle. For each sample a volume of 250 mL of water was vacuum-filtered in a fiberglass filter (Ahlstrom nr. 151, 47 mm in diameter and 0.7 µm porosity). The fiberglass filter was immediately frozen and kept dry in the dark until the time of extractions for spectrophotometric determination (Bausch & Lomb Spectronic 1001 spectrophotometer). Acetone at 90% was used as a solvent for extraction. To obtain pheophytin *a*, 0.1 mL of HCl 0.1 N was added to the sample directly in the cuvette.

The following types of equations were used in order to compare the chlorophyll *a* concentrations: two trichromatic (APHA, 1992 and Edler, 1979) and three monochromatic equations (APHA, 1992; Golterman et al., 1978 and Edler, 1979 using a wavelength of 663nm). For pheopigments, two similar equations were used where only the wavelength read differed (APHA, 1992 and Golterman et al., 1978). Comparisons were also made between the ratios of optical densities involved (OD_{664}/OD_{665} and OD_{663}/OD_{663}), before and after acidification, for the indication of the physiological status of the sample. Finally, an equation to obtain the amount of total pigment in the sample (chlorophyll *a* + pheophytin) was also used to compare with the other methods (Golterman et al., 1978).

The concentrations of chlorophyll *a*, *b* and *c* can be obtained from the following trichromatic equations (CETESB, 1990 and APHA, 1992):

$$\text{Chlorophyll } a \text{ } (b, c) \text{ } (\mu g.L^{-1}) = \frac{C_{a\,b\,c} \times v}{V \times L} \quad (1)$$

V = volume (liters) of filtered water for extraction

v = volume (mL) of 90% acetone used

L = optical pathway (cm) of the cuvette used

$$C_a = 11.85 \times D_{664} - 1.54 \times D_{647} - 0.08 \times D_{630} \quad (2)$$

$$C_b = 21.03 \times D_{647} - 5.43 \times D_{664} - 2.66 \times D_{630} \quad (3)$$

$$C_c = 24.52 \times D_{630} - 7.60 \times D_{647} - 1.67 \times D_{664} \quad (4)$$

D_{664}, D_{647} and D_{630} = optical densities corrected by D_{750}

In order to obtain the chlorophyll *a* and pheophytin *a* concentrations in sample, the following monochromatic equations were used (CETESB, 1990):

$$\text{Chlorophyll } a \text{ } (\mu g.L^{-1}) = \frac{26.73 \, (D_{664} - D_{665}) \times (v)}{V \times L} \quad (5)$$

$$\text{Pheophytin } a \text{ } (\mu g.L^{-1}) = \frac{26.73 \, (1.7 \, D_{665} - D_{664}) \times (v)}{V \times L} \quad (6)$$

Another monochromatic equation was proposed by Golterman et al. (1978):

$$\text{Chlorophyll } (\mu g.L^{-1}) = \frac{10^3 \times 2.43 \, (U' - A') \times V_e}{l \times K_c \times V_s} \quad (7)$$

$$\text{Pheophytin } (\mu g.L^{-1}) = \frac{10^3 \, (2.43U' - 1.43A') \times V_e}{l \times K_p \times V_s} \quad (8)$$

$$\text{Total pigment } (\mu g.L^{-1}) = \frac{10^3 \, U' \times V_e}{l \times K_c \times V_s} \quad (9)$$

U' = optical density at 663, corrected ($OD_{663} - OD_{750}$), obtained before acidification; A' = optical density at 663, corrected ($OD_{663} - OD_{750}$) obtained after acidification; *l* = optical pathway (cm) of the cuvette used; V_s = volume of sample (L); V_e = volume of extract (mL); K_c = coefficient of chlorophyll absorption (K_c = 91) in 90% acetone; K_p = coefficient of absorption of pheophytin (K_p = 55) in 90% acetone.

The equations described by Golterman et al. (1978) provide a 10^3 error, besides the fact that the unit also presents an error of equal proportion. Using the *Golterman formulae* (1969), Aleixo (1981) used: a factor of 10^3 in the numerator (instead of 10^6); the filtered volume was expressed in L (instead of mL), and the result in $\mu g.L^{-1}$ or $mg.m^{-3}$ (instead of mg. L^{-1}).

Descriptive and regression analysis were performed using program STATISTICA ®. The simple linear regression analysis was made to show the relationships between the data obtained from the different methods. Abbreviations used in results were included in Tab. I.

Table I: Pigment values (%) obtained for each method tested in the seasonal cycle in Itapeva Lake.

Month		Tri664	Tri663	MonoA	MonoQ	Mono663	OD664/66	OD663/66	PheoA	PheoQ	TPig
Dec/1998	negative	0	0	24	24	24	13	14	52	53	0
	high	0	0	15	15	15	12	11	8	8	0
	No-sampling	6	6	6	6	6	6	6	6	6	6
	subtotal	6	6	44	44	44	31	31	66	67	6
	n	94	94	56	56	56	69	69	34	33	94
Mar/1999	negative	5	1	15	14	16	4	7	31	32	1
	high	1	0	1	1	0	3	0	3	2	0
	No-sampling	0	0	0	0	0	0	0	0	0	0
	subtotal	6	1	16	15	16	6	7	33	34	1
	n	94	99	84	85	84	94	93	67	66	99
May/1999	negative	1	4	18	15	15	1	4	13	19	4
	high	1	0	1	0	0	1	0	5	1	0
	No-sampling	1	1	1	1	1	1	1	1	1	1
	subtotal	3	5	19	16	16	3	5	19	20	5
	n	97	95	81	84	84	97	95	81	80	95
Aug/1999	negative	7	0	42	36	36	4	0	0	0	0
	high	0	0	0	0	0	0	0	0	0	0
	No-sampling	7	7	7	7	7	7	7	7	7	7
	subtotal	15	7	49	44	44	11	7	7	7	7
	n	85	93	51	56	56	89	93	93	93	93

(subtotal= negative values, extremely high values or without data; n= number of samples); Dec/1998= spring, Mar/1999= summer, May/1999= autumn, Aug/1999= winter
TRI664= 664 nm trichromatic method; TRI663= 663nm trichromatic method; MONOA= monochromatic method (APHA, 1992); MONOG= monochromatic method of Golterman et al. (1978); MONO663= 663nm monochromatic method; OD664/665 = ratio between optical densities at 664 and 665 nm; OD663/663= ratio between optical densities at 663 nm. PHEOA= pheopigments according to APHA (1992), PHEOG= pheopigment determination according to Golterman et al. (1978); TPIG= Total pigment (Golterman et al., 1978).

Results and Discussion

Analysis of chlorophyll *a*

Comparing the methods (Tab. I), the Tri664, Tri663 and TPig methods achieved the smallest source of error, due to the small number of samples with a negative value. On the other hand, in all monochromatic methods, negative values were always found in all seasons. The highest rate of negative values was recorded in winter, and this was linked to the senescence of the phytoplankton. Senescence may have occurred both due to intrinsic conditions in the community (life cycle, seasonal change) and as a function of adverse environmental conditions, such as high effective fetch, low temperature, increased turbidity and suspended solids, which reduced the euphotic layer (Cardoso, 2001). Negative values may be related to the 750 nm wavelength readings, because the interference caused by inorganic substances was no removed, since the turbidity values were rather high (mean of 55.4 to 276.6 NTU). Thus, Edler (1979) and Rai's (1980) suggestions to use monochromatic equations may not be applicable in an environment with the hydrodynamic characteristics of Itapeva lake. Extremely high values (much higher than 100 $\mu g.L^{-1}$) were recorded mainly for the spring, except using the Tri663 and TPig methods. The broadest range of data was recorded in spring and the smallest in summer (Tab. II and III). On the other hand, the behavior in autumn and winter was very similar (Tab. IV and V). The seasonal behavior was related to wind action on the lake hydrodynamics. During spring, the frequent

wind oscillations disturbed the system, reflected directly on changes in phytoplankton density. On the other hand, in summer the low velocity and the same wind direction (NE quadrant) promoted a hydrodynamically more stable environment. The greatest similarity of the phytoplankton pigments values between the cold seasons was related to the arrival of cold fronts in the region (Cardoso, 2001).

During the spring (Dec/1998) the results obtained using the Tri664, Tri663 and TPig methods were very similar between themselves; the same occurred between monochromatic methods (Fig. 2a). Higher values were obtained using the monochromatic methods, but lower data were found using Tri664, Tri663 and Tpig

Table II: Descriptive analysis of the pigment values (µg.L^{-1}) in relation to the tested methods, during the spring (Dec/1998), in Itapeva Lake (for the abbreviatures, see Table I).

Stations		Chlorophyll *a*					OD ratio		pheopigments		
		Tri664	Tri663	MonoA	MonoG	Mono663	664/665	663/663	PheoA	PheoG	TPig
NORTH	Mean	39.10	39.15	49.91	50.11	50.16	2.25	2.27	41.46	40.95	39.80
	Standard deviation	24.06	24.08	36.88	37.44	37.47	1.95	1.99	23.26	21.82	25.32
	Minimum	10.29	10.15	3.42	2.99	2.99	0.43	0.42	0.53	3.42	8.26
	Maximum	102.49	102.06	117.93	118.35	118.47	8.51	8.75	82.77	81.69	106.81
	N	**36**	**36**	**22**	**22**	**22**	**29**	**29**	**15**	**15**	**36**
CENTER	Mean	34.43	34.57	62.45	63.08	63.14	2.19	2.19	60.09	59.13	35.60
	Standard deviation	20.64	20.77	33.85	34.14	34.17	2.66	2.64	31.59	31.11	22.44
	Minimum	10.25	10.11	7.59	6.94	6.95	0.18	0.18	0.02	0.95	7.52
	Maximum	97.84	98.32	119.64	120.16	120.29	10.32	10.32	98.59	98.10	104.31
	N	**30**	**30**	**15**	**15**	**15**	**21**	**21**	**8**	**8**	**30**
SOUTH	Mean	30.38	30.47	47.49	47.98	48.03	2.21	2.25	34.97	36.68	31.45
	Standard deviation	14.72	14.89	30.42	30.54	30.57	1.92	1.98	37.60	36.78	15.97
	Minimum	5.15	4.81	5.77	6.52	6.52	0.09	0.09	0.63	1.72	5.23
	Maximum	58.11	58.44	107.56	108.31	108.42	6.97	7.49	98.38	96.77	60.92
	N	**36**	**36**	**23**	**23**	**23**	**25**	**25**	**14**	**13**	**36**

Table III: Descriptive analysis of the pigment values (µg.L^{-1}) as compared with the tested methods during the summer (Mar/1999) at Itapeva Lake (for the abbreviatures, see Table I).

Stations		Chlorophyll *a*					OD ratio		pheopigments		
		Tri664	Tri663	MonoA	MonoG	Mono663	664/665	663/663	PheoA	PheoG	TPig
NORTH	Mean	7.48	7.50	8.03	8.39	8.40	1.89	2.14	4.96	5.86	7.37
	Standard deviation	3.13	3.14	4.96	5.19	5.19	1.38	1.75	3.92	4.54	3.29
	Minimum	3.66	3.54	0.00	1.07	1.07	0.80	0.91	0.21	0.41	3.52
	Maximum	17.08	17.56	22.45	21.36	21.38	8.00	9.50	14.22	18.39	18.46
	N	**36**	**36**	**35**	**34**	**34**	**31**	**33**	**19**	**16**	**36**
CENTER	Mean	6.65	6.72	3.74	3.70	3.71	1.31	1.24	8.73	8.39	6.54
	Standard deviation	2.19	2.16	2.62	2.57	2.57	0.49	0.44	11.96	8.46	2.15
	Minimum	2.24	2.24	0.00	0.00	0.00	0.10	0.12	0.00	0.31	2.20
	Maximum	10.89	10.89	9.62	8.55	8.55	2.67	2.25	67.36	47.82	11.43
	N	**36**	**36**	**32**	**30**	**30**	**36**	**36**	**29**	**30**	**36**
SOUTH	Mean	9.05	10.03	6.55	11.97	10.55	1.35	1.32	12.09	8.40	10.33
	Standard deviation	5.05	6.05	4.39	16.67	17.40	0.71	0.59	8.35	8.50	6.31
	Minimum	1.94	1.77	0.00	1.07	1.07	0.08	0.07	1.39	0.09	1.76
	Maximum	29.32	35.48	18.18	85.45	85.54	3.50	2.67	34.86	25.25	34.73
	N	**30**	**35**	**23**	**24**	**22**	**29**	**29**	**20**	**21**	**30**

methods. Regression analysis showed a very strong linear relationship between Tri664 and Tri663 methods ($r=0.999$; $p<0.001$), Tri664 and TPig methods ($r=0.998$; $p<0.001$) and among the monochromatic methods ($r=0.999$ and 1; $p<0.001$) (Tab. VI). Significant relations were also found between method Tri664 and the monochromatic ones ($p<0.001$), but with a smaller correlation among these (Tab. VI).

Table IV: Descriptive analysis of the values of pigments ($\mu g.L^{-1}$) as to the tested methods, during the autumn (May/1999) at Itapeva Lake (for the abbreviatures, see Table I).

Stations		Chlorophyll a					OD ratio		pheopigments		
		Tri664	Tri663	MonoA	MonoQ	Mono663	664/665	663/663	PheoA	PheoQ	TPig
NORTH	Mean	15.44	14.82	8.93	8.69	8.70	1.27	1.29	12.75	16.04	14.19
	Standard deviation	5.75	5.88	5.58	5.81	5.82	0.23	0.24	7.45	16.99	5.70
	Minimum	3.04	3.52	0.00	0.00	0.00	0.86	0.67	1.71	1.85	3.08
	Maximum	32.45	33.40	21.38	22.43	22.45	1.88	1.87	28.55	73.19	32.09
	N	**35**	**31**	**31**	**29**	**29**	**35**	**31**	**34**	**33**	**31**
CENTER	Mean	6.54	8.47	5.54	6.52	6.53	1.32	1.49	7.59	5.12	7.06
	Standard deviation	3.61	7.15	4.81	4.48	4.49	0.47	0.46	9.49	5.26	3.85
	Minimum	0.95	0.95	0.00	0.00	0.00	0.31	0.67	0.21	0.20	0.88
	Maximum	15.26	43.13	22.45	17.09	17.11	2.40	2.50	43.09	22.55	16.70
	N	**34**	**36**	**27**	**29**	**29**	**34**	**34**	**29**	**25**	**34**
SOUTH	Mean	12.45	12.31	10.07	9.75	9.76	1.36	1.42	6.92	9.07	11.53
	Standard deviation	7.01	6.98	7.67	6.88	6.89	0.63	0.50	6.43	9.20	6.73
	Minimum	0.26	0.26	0.00	0.00	0.00	0.00	0.00	0.00	0.92	0.00
	Maximum	30.89	30.89	28.87	28.84	28.87	3.00	2.60	22.77	34.29	29.89
	N	**36**	**36**	**29**	**31**	**31**	**36**	**36**	**25**	**27**	**36**

Table V: Descriptive analysis of the pigment values ($\mu g.L^{-1}$) as to the tested methods, during the winter (Aug/1999) at Itapeva Lake (for the abbreviatures, see Table I).

Stations		Chlorophyll a					OD ratio		pheopigments		
		Tri664	Tri663	MonoA	MonoQ	Mono663	664/665	663/663	PheoA	PheoQ	TPig
NORTH	Mean	18.04	17.85	8.12	8.41	8.42	1.20	1.21	16.78	15.48	17.38
	Standard deviation	5.75	5.65	5.01	5.10	5.11	0.13	0.14	3.41	3.56	5.49
	Minimum	6.04	6.04	0.00	0.00	0.00	0.72	0.76	7.16	6.21	5.71
	Maximum	28.18	28.66	23.52	23.50	23.52	1.54	1.55	22.99	22.64	27.69
	N	**33**	**33**	**32**	**32**	**32**	**33**	**33**	**33**	**33**	**33**
CENTER	Mean	8.50	8.74	3.53	2.05	2.05	0.96	0.95	14.75	14.44	8.12
	Standard deviation	3.82	3.68	3.97	2.11	2.11	0.19	0.15	5.04	4.13	3.51
	Minimum	3.53	3.53	0.00	0.00	0.00	0.64	0.64	1.82	6.55	3.52
	Maximum	16.74	16.74	13.90	6.41	6.42	1.62	1.33	23.09	21.29	17.14
	N	**28**	**32**	**10**	**12**	**12**	**28**	**28**	**28**	**28**	**28**
SOUTH	Mean	7.53	7.41	1.23	0.99	0.99	0.94	0.90	14.52	15.29	7.35
	Standard deviation	2.57	2.80	1.22	0.81	0.81	0.14	0.16	5.07	5.18	2.82
	Minimum	3.60	3.21	0.00	0.00	0.00	0.71	0.40	3.96	7.27	3.52
	Maximum	12.38	13.33	4.28	2.14	2.14	1.36	1.10	31.86	32.01	14.07
	N	**35**	**35**	**13**	**13**	**13**	**35**	**35**	**35**	**35**	**35**

During the summer (Mar/1999), the values obtained among Tri664, Tri663 and TPig methods presented the same behavior of previous samplings (Fig. 2b). A large part of the values obtained by Tri663 method was closer to Tpig method. Higher correlations were found between Tri664 and Tri663 methods (r=0.984; p<0.001) and between MonoG and Mono663 methods (r=0.958; p<0.001), although significant relationships among the other methods were also recorded (Tab. VI).

In autumn (May/1999) (Fig. 2c, Tab. IV) and winter (Aug/1999) (Fig.5, Tab. V), very similar values among the methods were found. Linear behavior and similar correlations were detected. Using Tri664, Tri663 and TPig methods, values were higher in comparison to the others, both in autumn and in winter.

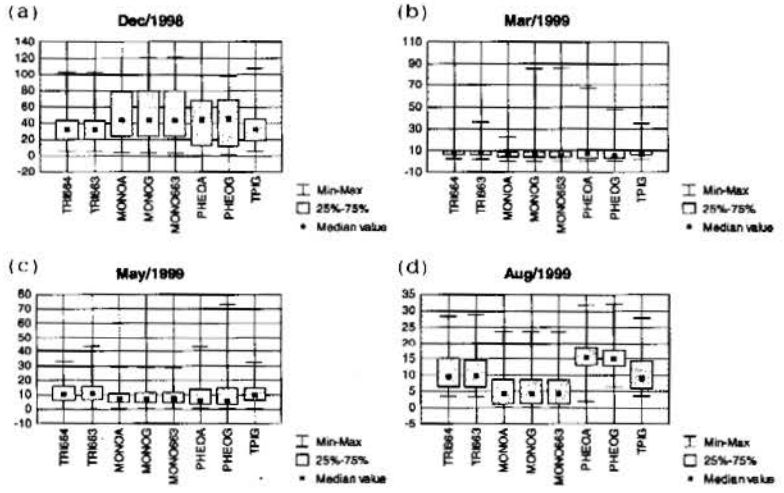

Figure 2: Distribution of the pigment values (µg.L^{-1}) for each method tested in Itapeva Lake: (a) spring (Dec/1998), (b) summer (Mar/1999), (c) autumn (May/1999), (d) winter (Aug/1999). (For the abbreviation, see the legend of Table I).

Regarding the spatial scale, some particularities were found. During the spring (Dec/1998), a decreasing gradient of values obtained by Tri664, Tri663 and Tpig methods was observed in the N→S axis. During the summer (Mar/1999), the gradient was inverted (Tab. II and III). The spring gradient was related to phytoplankton density. However, the summer conditions presented significant hydrodynamic influence (Cardoso, 2001). A higher concentration of nutrients (N and P) occurred in the South station and the phytoplankton community increased in terms of photosynthetic pigments, but not at density level. Concerning the monochromatic methods, the values were higher in the Center station during the spring, while in the summer a decrease was observed (Tab. II and III). High concentration of chlorophyll was related to the greater frequency of winds in the W direction. On the other hand, during the summer there was a marked displacement of the biomass from the Center to southwards, since the NE direction winds produced a fetch larger from C→S (15.6 km) than from the N→S (10.8 km). During the autumn (May/1999), lower concentrations were observed in the Center station, using all the methods (Tab. IV). Blooms of *Anabaena circinalis* were found in the autumn and the highest densities of phytoplankton occurred at the North and South sampling stations of the lake (Cardoso, 2001). During the winter (Aug/1999) similar data using all the methods were found, when the decreasing N→S gradient was also observed (Tab. IV). Thus, during the cold seasons of the year (May/1999 and Aug/1999), greater homogeneity of the values among the methods was observed.

while during the warm seasons (Dec/1998 and Mar/1999), there was a clear difference in the results between the trichromatic and monochromatic methods.

The close relationship between the Tri664 and Tri663 methods (Tab. VI) during all the samplings suggests that both trichromatic methods reproduced the same values. The trichromatic methods actually evaluated the amount of total pigment in the sample and not only chlorophyll *a* (CETESB, 1990; APHA, 1992). However, the TPig method was more advantageous in the case of Itapeva Lake due to: 1) the faster

Table VI: Correlation matrices (r – of Pearson $p<0.05$, $n=108$) between the pigment values of the tested methods during the seasonal cycle at Itapeva Lake (for the abbreviatures, see Table I).

Period		TRI664	TRI663	MONOA	MONOG	MONO663	OD664665	OD663663	PHEOA	PHEOG	TPIG
Spring (Dec/1998)	TRI664	1									
	TRI663	1.00	1								
	MONOA	0.530	0.534	1							
	MONOG	0.532	0.536	1.000	1						
	MONO663	0.532	0.536	1.000	1.000	1					
	OD664665	0.486	0.486	0.708	0.704	0.704	1				
	OD663663	0.483	0.484	0.709	0.706	0.706	0.998	1			
	PHEOA						-0.894	-0.892	1		
	PHEOG						-0.888	-0.887	0.999	1	
	TPIG	0.998	0.998	0.536	0.538	0.538	0.483	0.480			1
Summer (Mar/1999)	TRI664	1									
	TRI663	0.984	1								
	MONOA	0.281	0.254	1							
	MONOG	0.231	0.245	0.250	1						
	MONO663	0.685	0.753	0.481	0.958	1					
	OD664665			0.382			1				
	OD663663			0.569		0.519	0.858	1			
	PHEOA		0.261		0.287	0.564	-0.786	-0.628	1		
	PHEOG		0.253		-0.529	0.381	-0.427	-0.412	0.927	1	
	TPIG	0.502	0.454		0.752	0.243	-0.239				1
Autumn (May/1999)	TRI664	1									
	TRI663	0.958	1								
	MONOA	0.753	0.710	1							
	MONOG	0.780	0.792	0.900	1						
	MONO663	0.780	0.792	0.900	1.000	1					
	OD664665			0.488	0.332	0.332	1				
	OD663663			0.344	0.409	0.409	0.835	1			
	PHEOA	0.215	0.343				-0.635	-0.408	1		
	PHEOG	0.320	0.299				-0.319	-0.582	0.549	1	
	TPIG	0.954	0.995	0.723	0.805	0.805			0.345	0.250	1
Winter (Aug/1999)	TRI664	1									
	TRI663	0.990	1								
	MONOA	0.878	0.818	1							
	MONOG	0.901	0.900	0.973	1						
	MONO663	0.901	0.900	0.973	1.000	1					
	OD664665	0.780	0.724	0.888	0.909	0.909	1				
	OD663663	0.811	0.830	0.670	0.946	0.946	0.806	1			
	PHEOA	0.399	0.458						1		
	PHEOG	0.273	0.255					-0.225	0.734	1	
	TPIG	0.991	0.999	0.827	0.908	0.908	0.735	0.829	0.450	0.261	1

reading at, only two optical densities, as compared to four optical densities of the trichromatic method; and 2) the smaller error involved in the method. This was only true as compared to Tri664 method, since the Tri663 method reproduced the same percentages exhibited by data from Tpig method throughout the year. Thus, the 663nm wavelength was the determining factor to the improved performance of results.

Concerning the trichromatic method proposed by Jeffrey & Humphrey (1975), small change in the determination of chlorophyll *a* can occur when a measurement at the 664nm wavelength is made. They suggested that the reading can be made in the 663 and 665 nm range, using the peak value reading of one of these three lengths, depending on the sample. When the 665 nm wavelength reading was not available, the OD 663nm value was applied in the equation. Comparing the readings between OD664 and 663nm, the chlorophyll values obtained using a OD663 were higher than to those of OD664 (Fig. 3) in spring and summer, and the opposite occurring in autumn-winter. The close relations between the trichromatic methods are due to the high percentage of similar values of these optical densities, especially in summer and winter. Although in spring, the values obtained with OD663 were not significantly higher. In the autumn, the results between the trichromatic methods were further apart, and the same was observed for the ODs and the percentage of negative values recorded at OD663. It should be stationed out that in autumn cyanobacteria bloom occurred, which was a factor that influenced the peak reading of OD. Thus, no significant difference was found between the two trichromatic methods for Itapeva Lake.

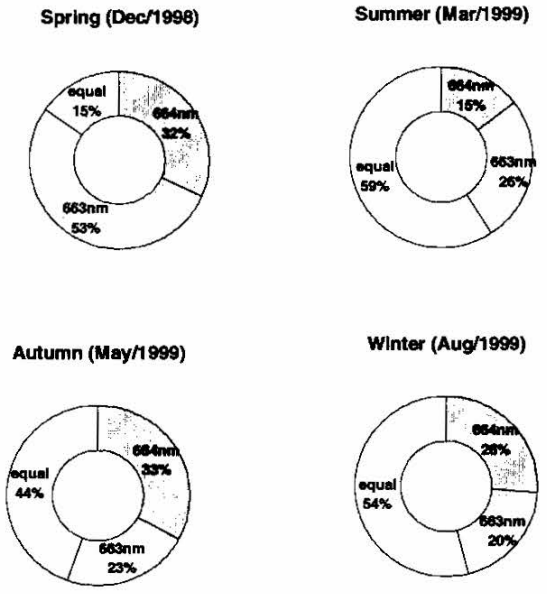

Figure 3: Frequency of maximum peak occurred between optical densities 663 and 664 nm, during each seasonal campaign at Itapeva Lake. (equal= values equal between OD663 and OD664).

The same interpretation for the optical densities of the trichromatic methods can be applied to the monochromatic methods. The high linear relationship between values from the MonoG and Mono663 methods was attributed because the same wavelength was used and the unsignificant difference in the equation (factor of 0.03). Due to the a high percentage of negative values in all the samplings, the monochromatic method is not recommended at least in the case of Itapeva Lake. It is a shallow, polymitic coastal lake, and also it is submitted to a strong fetch effect and thus, affecting the physiological state of the phytoplankton community (Cardoso, 2001). Therefore, the total pigment method(TPig) of Golterman et al. (1978) is considered the most effective spetrophotometric analysis.

Pheopigment Analysis

The methods used (Tab. I) presented the same error emphasized by Moed & Hallegraeff (1978). However, better results were found in winter. Negative and extremely high values occurred in spring due to the ratios concerning the optical densities readings. The errors were probably related to hydrodynamic aspects, which may have damaged the phytoplankton cells as a result of friction. As a result, the highest seasonal mean was produced in spring. The results of both methods tested usually presented the same behavior during the year (Figs. 2a to 2d). In winter (Fig. 2d), a large part of the values was located between the distribution limits considering the periods (Figs. 2a to 2c). In winter, the environmental conditions contributed to the great algal senescence. A highly significant linear relationship was observed between methods ($p<0.001$), and the highest correlations (Tab.VI) occurred in spring and in summer (Tab. II to V).

Both methods exhibited the same spatial behavior in the warm seasons. In spring, the highest mean values occurred in the Center station, the same behavior was observed for the monochromatic methods for chlorophyll *a* determination. Probably, the highly frequent W winds induced accumulation of chlorophyll at this site. There was also more disturbance because of the high effective fetch (14.7 km). In summer, both the values of the pheopigment methods exhibited the increasing gradient N→S, as a direct response to hydrodynamics, due to the more constant NE winds. For autumn and winter, the spatial distinction between the values was probably related to the presence of cyanobacteria blooms. Although it was more intense in autumn, in winter it was sporadically repeated in winter (Cardoso, 2001). This appears to indicate that algal growth and senescence are constantly fluctuating in the development of a bloom, and may also be concurrent. Thus, the pheopigment analysis methods during the blooms appear to be more sensitive than those of chlorophyll. Therefore, during a bloom it is essential not only to monitor chlorophyll *a* and the other pigments of the species involved, but also to determine pheopigments in order to verify the senescence of phytoplankton.

The results (Tab. I) confirmed the observations of Moed & Hallegraeff (1978) that it is difficult adopt acidification procedures in the routine analyses of pigments. Due to a number of uncertainties and complications, such as negative or very high pheopigment values and determining acidification rates over the value of 1.7 (upper limit proposed by Lorenzen, 1967), it appears to be a further indication that the reading at 750 nm did not exclude the high turbidity effect from the samples.

Relationships Between Pigments

The smallest mean values of Pheo/Chl *a* (APHA, 1992) were found in spring, increasing seasonally towards winter (Tab. VII). However, no spatial gradient was observed between the sampling stations as observed to chlorophyll *a* and pheopigments. The highest mean values occurred at the Center station in winter. The Pheo/Chl *a* was computed since chlorophyll *a* is overestimated by the spectrophotometric trichromatic equations, when the pheophytin/chlorophyll ratio increases (Rai, 1980). High increases were not observed in the North station because the data distribution was small during the year, and thus the chlorophyll *a* concentrations were not overestimated. On the other hand in the Center station, an overestimation occurred in summer and autumn, because the ranges and standard deviations were larger. In the South station, a larger range of the data occurred in spring and in autumn. However, the range during autumn, in the Center and South stations, can be linked to physiological aspects of cyanobacteria blooms and not to an overestimation of chlorophyll *a*.

Chlorophyll *a* concentrations are overestimated when the pheopigment ratios are high, as in the case of Saquarema Lagoon/RJ (Moreira, 1989). The overestimating of chlorophyll *a* must become critical when it is related to the relative densities of

Table VII: Pheopigments and chlorophyll *a* (Pheo/Chl *a*) and chlorophyll type ratios (*c*/*a* e *b*/*a*) in each station during the seasonal cycle at Itapeva Lake.

STATIONS/ LAKE		Spring Dec/1998			Summer Mar/1999			Autumn May/1999			Winter Aug/1999		
		Pheo/Chl *a*	Chl *c*/*a*	Chl *b*/*a*	Pheo/Chl *a*	Chl *c*/*a*	Chl *b*/*a*	Pheo/Chl *a*	Chl *c*/*a*	Chl *b*/*a*	Pheo/Chl *a*	Chl *c*/*a*	Chl *b*/*a*
Lake	Mean	0.81	0.03	0.68	0.97	0.09	0.30	1.07	0.02	0.36	1.67	0.01	0.25
	Sd	1.74	0.01	0.38	3.01	0.40	0.42	2.09	0.05	0.62	0.78	0.01	0.23
	Min	0	0	0.02	0	0	0.00	0	0	0.00	0.12	0	0
	Max	9.90	0.06	1.62	30.02	3.09	2.11	13.94	0.30	3.54	3.95	0.06	1.08
	N	102	90	92	102	72	68	105	36	36	96	81	86
NORTH	Mean	0.70	0.02	0.57	0.36	0.01	0.30	0.88	0.01	0.21	1.04	0.000	0.15
	Sd	1.20	0.01	0.31	0.56	0.01	0.32	0.60	0.000	0.11	0.47	0.01	0.12
	Min	0	0	0.13	0	0	0.04	0	0	0.02	0.26	0	0
	Max	3.94	0.04	1.09	2.63	0.04	1.09	2.24	0.01	0.33	3.12	0.03	0.48
	N	36	31	31	36	27	21	35	7	7	33	27	28
CENTER	Mean	0.88	0.03	0.77	1.60	0.01	0.19	1.52	0.02	0.58	1.97	0.01	0.30
	Sd	1.83	0.02	0.42	4.92	0.01	0.19	2.74	0.03	0.83	0.78	0.01	0.20
	Min	0	0	0	0	0	0.01	0	0	0.01	0.12	0	0
	Max	5.85	0.06	1.62	30.02	0.03	0.95	13.94	0.12	3.54	3.95	0.02	0.86
	N	30	26	27	36	23	26	34	16	18	28	21	24
SOUTH	Mean	0.85	0.03	0.74	0.95	0.26	0.45	0.82	0.03	0.10	2.02	0.01	0.29
	Sd	2.13	0.01	0.36	1.05	0.70	0.63	2.30	0.08	0.11	0.65	0.01	0.30
	Min	0	0	0.21	0	0	0	0	0	0	0.61	0	0.02
	Max	9.90	0.06	1.50	3.50	3.09	2.11	13.85	0.30	0.34	3.32	0.06	1.08
	N	36	33	33	30	22	21	36	13	11	35	33	34

phytoplankton. Another study in Saquarema Lagoon revealed that the high values of Chl *a*/Pheo were related to the time (days) after weather change, indicating a fast recovery of the community (Domingos, 1991).

However, an overestimation of the chlorophyll *a* concentration appear to have occurred only on two days during the summer in the South station: on March 2, at 2 pm on the surface (Chl *c*/*a*=1.3) and in the middle (Chl *c*/*a* =3.1) of the water column, when the ratio was greater than 1.0 as proposed by Loftus & Carpenter (1971). In fact the mean ratios were always low in all the samplings (Tab. VII), since the average value of chlorophyll *c* was also very low.

On the other hand, the overestimation of pheopigments was especially recorded in spring (Tab. VII), since the chlorophyll *b* to chlorophyll *a* (Chl *b*/*a*) ratio was higher than 0.4 (according to Holm-Hansen & Riemann, 1978). An overestimation was found at all sampling stations for over 50% of the values in spring. No overestimation of pheopigments occurred during the autumn at the North and South sampling stations. All the other Chl *b*/*a* ratios were higher than 0.4, especially at the Center station during the autumn, when a peak occurred. These data indicate that a large part of the pheopigments did not originate from the phytoplankton but from another plant source. In spring, the reproductive structures of the plants are formed and many of these are transported by the wind. The higher values at the Center station were related to the transport of the plant fragments through the drainage basin by Três Forquilhas river (Fig. 1). Other source is the small stand of aquatic macrophytes in the littoral zone.

OD664/665 versus OD663/663 Ratios

Throughout the year (Fig. 4), the $OD_{664/665}$ and $OD_{663/663}$ ratios were very similar and the minimum values were always recorded for the South station (Tab. II to V). The upper limit of the ratios was higher in spring and summer, and most of the data were closer to the lower limit. On the other hand, in autumn and winter the range of

the data was smaller and less asymmetrical, but the distribution differed greatly between the ratios.

Negative and extremely high values of the ratios were also found in spring and less frequently in autumn and winter (Tab. I). This ratio was adequate to speculate about the phytoplankton physiological status.

When a pure chlorophyll *a* solution is converted into pheophytin *a* by acidification, the proportion of the absorption peak ($OD_{664/665}$) of 1.70 is used to correct the apparent concentration of chlorophyll *a* to pheophytin *a*. Samples with a proportion of 1.70 for OD_{664}before/OD_{665}after are considered as not containing pheophytin *a* and as being in excellent physiological condition. Solutions of pure pheophytin do not show a reduction of OD_{665} on acidification, and have a 1.0 proportion of OD_{664}before/OD_{665}after. Thus, mixtures of chlorophyll *a* and pheophytin *a* have proportions of absorption peak between 1.0 and 1.7. These proportions are based on the use of 90% acetone as solvent (APHA, 1992).

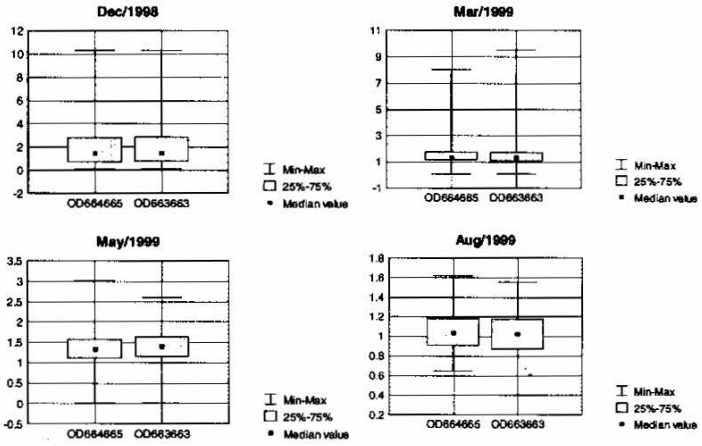

Figure 4: Distribution of the ratios between different optical densities (OD) in each seasonal campaign at Itapeva Lake. (OD664/665=ratio between optical densities at 664 and 665nm; OD663/663= ratio between optical densities at 663nm; Dec/1998=spring; Mar/1999=summer; May/1999=autumn; Aug/1999=winter).

The mean values and the frequency of the OD_{664}/OD_{665} ratio for spring and summer samples presented a better physiological condition (Fig. 5). This was directly related to a richness and diversity of phytoplankton community during this two seasons (Cardoso, 2001). However, during these two seasons, maximum values much higher than the ratio of 1.7 were also recorded (Fig. 4). On the other hand, autumn and winter samples were characterized by a greater mixture of these pigments, presenting a ratio similar to 1.7 and smallest deviations of the ratio were observed.

Smaller means occurred at the Center station during the hot seasons, indicating that physiological conditions of the phytoplankton at this site were disturbed by the lake hydrodynamics. On the other hand, N→S gradients occurred during autumn and winter, but in opposite directions. Since the cyanobacteria bloom was more intense in the South station during the autumn, higher ratios between optical densities would occur. The same is valid for winter, when the bloom occurred again in the North station (Cardoso, 2001).

Furthermore, the correlations between these pigments (r= 0.22 in autumn and r= 0.40 in winter) were significant ($p<0.05$) for these periods (Tab. VI). During autumn, the cyanobacteria bloom in the lake must have been the main factor of this mixture of pigments in the samples. In winter, the great predominance of pheopigments in the samples was produced by greater algal senescence.

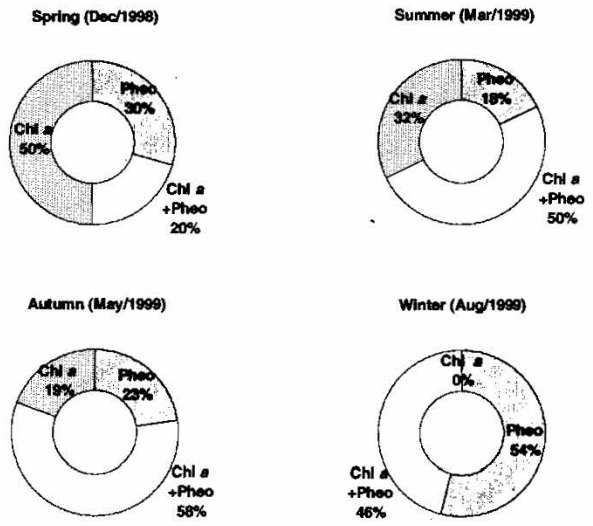

Figure 5: Relations between optical densities (OD $_{664/665}$) in the physiological interpretation of the samples, during the seasonal campaigns, in Itapeva Lake. (Pheo= predominance of pheopigments, Chl *a*= predominance of chlorophyll a, Chl *a*+Pheo= mixture of both pigments).

During spring the frequency of samples without pheophytin (according to ratio OD $_{664/665}$) was high and the same at all sampling stations. In fact, the proportion Chl *b*/ *a* showed an overestimation of pheopigments in spring at all stations (Tab. VII). Another overestimation of pheopigments occurred in autumn (only towards the Center station) and is also related to the very high maximum values of pheopigment (Tab. VII).

According to the values of Pheo/Chl *a*, an overestimation of the chlorophyll *a* concentration was recorded at the Center (summer and autumn) and South (spring and autumn) sampling stations. This was due to lower concentrations of chlorophyll *a* on the days of sampling and ratios OD $_{664/665}$<1.0. Therefore, a higher concentration of pheopigments over chlorophyll *a* occurred.

During the annual variation of chlorophyll *a*, there are significantly higher concentrations of pheophytin *a*, which was attributed to a deficiency in the nutritional conditions (Barbosa et al., 1988). Pheophytin *a* affects the physiological status of phytoplankton populations and causes a predominance of "old" cells, which are, therefore, less active. This was also observed for Itapeva Lake, except in autumn, when the increased concentration of chlorophyll *a* over pheopigments was directly related to cyanobacteria bloom.

The health of phytoplanktonic chlorophyll *a* is more closely correlated with cell size. Type of limitation, by light or nutrients, and trophic state may affect their relationship, but other factors such as taxonomic composition appear to be less important (Vörös & Padisak, 1991). However, George & Edwards (1976) found that when diatoms and chlorophytes were dominant, chlorophyll *a* was homogeneously distributed in the water column. During cyanobacteria bloom, dense accumulations of algae on the surface frequently appeared under calm conditions. At high wind speeds (>6 m.s^{-1}), cyanobacteria became more homogeneously distributed in the water column, because the turbulent mix overcame the tendency of highly floatable cells to remain on the surface. In general there is a close relationship between wind-induced turbulence and the development of agregates of cyanobacteria in the water profile. In Itapeva Lake, the dominance of diatoms and the higher concentration of

chlorophytes over spring occurred when the water column present a vertical heterogeneous distribution of chlorophyll *a*. During the cyanobacteria bloom in autumn, no significantly different distribution of chlorophyll *a* in the water column was found, probably due to the fact that the variance among the sampling stations and days was more representative (Cardoso, 2001). The highest concentration of chlorophyll *a* occurred in the middle of the water column and not on the surface. However, for pheopigments the system was vertically heterogeneous only in autumn, when the senescence of these algae along the profile was verified (Cardoso, 2001).

A highly significant linear relationship was observed between pheopigments and the optical densities ratios ($p<0.001$). The correlations (Tab. VI) present decreasing values from spring (Dec/1998) to winter (Aug/1999). The relationship was evident because the histograms of data distribution among the methods showed the same pattern during each sampling period (Cardoso, 2001), and the correlations between PheoA x OD_{664665} and PheoG x OD_{663663} were always negative, significant and decreasing from spring to winter (Tab. VI). The dependence of the phytoplankton physiological status with the ratios between the optical densities (Golterman et al., 1978; APHA, 1992) was confirmed. A reduction in these ratios implies a higher concentration of pheopigments than chlorophyll in the sample. In the same way, positive and significant correlations were found between the chlorophyll methods and the optical densities ratios (Tab. VI). These optical densities ratios become an excellent physiological indicator of algal senescence.

Thus both trichromatic (OD 664 nm and 663 nm) and the total pigment methods presented values significantly correlated to each other. In the case of Itapeva Lake, any one of these methods would be recommended to the monochromatic methods, due to the small number of samples with negative values. The data show that trichromatic methods do not account for only the chlorophyll *a*, but also the pheopigments.

Pheopigments analysis requires more attention for interpretation, due to many errors. Therefore, the interpretation must be made together with the values of the ratios between optical densities (before and after acidification). This ratio is valuable for the interpretation of the phytoplankton physiological status and serving as a good indicator of the methods suitability employed on the pigment analysis.

A more detailed investigation must be carried out in order to determine the wavelength appropriate to eliminate sample high inorganic turbidity. The 750nm OD showed that inorganic turbidity was the possible cause of the faulty data specially for coastal aquatic environments, under strong influence of the wind.

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