

Comparison of three methods for Chlorophyll determination: Spectrophotometry and Fluorimetry in samples containing pigment mixtures and spectrophotometry in samples with separate pigments through High Performance Liquid Chromatography.

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ABSTRACT: Comparison of three methods for Chlorophyll determination: spectrophotometry and fluorimetry in samples containing pigment mixtures and spectrophotometry in samples with separate pigments through high performance liquid chromatography. **Chlorophyll concentration is one of the most used forms for the determination of phytoplanktonic organisms biomass. Despite the fact that this substance concentration varies due to both, the cells physiological state and species composition, it permits a faster evaluation of the natural phytoplanktonic organisms biomass, than microscopic methods. Therefore it is a useful tool in scientific studies and in hydric resources monitoring. Because of the wide utilization, some methods have been considered to determine the chlorophyll concentration, with different extraction and quantification forms. This research compares different forms of chlorophyll a concentration quantification: spectrophotometry, fluorimetry and spectrophotometry after high performance liquid chromatography separation (HPLC). From two environments (Lobo and Salto Grande Reservoir), 120 samples had been used to compare these methods. Although they have a very great correlation, two of them, spectrophotometric and fluorimetric, had overestimated chlorophyll concentration. This overestimate was more significant in the Lobo reservoir.**

Key-words: chlorophyll, phytoplankton, spectrophotometry, fluorimetry, HPLC.

RESUMO: Comparação de três métodos para determinação de clorofila: espectrofotometria e fluorimetria em amostras contendo mistura de pigmentos e espectrofotometria em amostras com pigmentos separados através de cromatografia líquida de alta precisão. **A concentração da clorofila a é uma das formas mais utilizadas para determinar-se a biomassa de organismos fitoplanctônicos. Apesar da concentração dessa substância variar em função do estado fisiológico das células e da composição de espécies, ela permite avaliar mais rapidamente a biomassa de organismos fitoplanctônicos no ambiente, que métodos mais diretos de microscopia, tornando-se uma ferramenta útil em estudos científicos e de monitoramento de recursos hídricos. Em função da ampla utilização, vários métodos foram propostos para determinação da concentração de clorofila-a, com diferentes formas de extração e de quantificação. O presente trabalho compara as diferentes formas de quantificação da concentração de clorofila a: espectrofotometria, fluorimetria e espectrofotometria após separação por cromatografia líquida de alta precisão (HPLC). Para comparar os três métodos foram utilizadas 120 amostras, de dois ambientes: Reservatório do Lobo e de Salto Grande. Apesar dos métodos terem uma correlação muito grande, tanto a espectrofotometria quanto a fluorimetria superestimaram a concentração de clorofila a. Esta superestimação é mais significativa no reservatório do Lobo.**

Palavras-chave: clorofila, fitoplâncton, espectrofotometria, fluorimetria, HPLC

Introduction

Chlorophyll-a concentration is one of the most used variables in limnology to determine the phytoplanktonic community biomass, to characterize environments, in experimental works and even in monitoring programs with the purpose of aquatic ecosystem management.

The methodologies of determination of the chlorophyll-a concentration have always been submitted to some criticism. These methodological discussions include both the filter type, used in samples concentration, and the real meaning of the pheophytin presence in aquatic environments (Rai, 1980).

However, reliable data are often difficult to be obtained, because of the great amount of interferers (Rai, 1980). The use of the chlorophyll quantification as the biomass estimate is very criticized, because chlorophyll content can vary according to the species and the cell physiological state (Sakshaug, 1981).

The first accepted spectrophotometric methods for chlorophyll-a determination were established about 40 years ago, such as the ones of Strickland & Parsons (1968) and Lorenzen (1967), and are used until today. At this same time, the fluorimetry also started to be used for the determination of chlorophyll concentration (Yentsch & Menzel, 1963). Despite the great sensitivity of the fluorimetric method, the biggest difficulty is the pigment's complexity of natural communities.

The liquid chromatography is the most recently developed method for chlorophyll-a determination and other vegetal pigments, whereas the first research is about 20 years old (Arar, 1997b). However the high cost of the equipment and time still be a disadvantage for the everyday use of this method.

The first attempt of standardization of chlorophyll determination was in 1966, with the "Determination of photosynthetic pigments in seawater" UNESCO publication. After, many researchers, have tried to improve the determination methodology of this important substance in the aquatic ecology research (Nush, 1980).

In 1980, Rai in a survey of problems on chlorophyll analysis suggested a new approach for the standardization of the methods of photosynthetic pigment determination in continental aquatic environments. However, despite the valuable suggestions, (Marker et al., 1980), many doubts still persist on which is the best methodology for specific conditions.

The present work aims to evaluate the most appropriate methodology (spectrophotometry, fluorimetry or high performance liquid chromatography - HPLC) for chlorophyll determination in two environments, with different trofic levels and, consequently, with different phytoplanktonic organisms predominance. This work also intends to find the protocol standardization for the BIOTACE (Laboratory of Biototoxicology in Continental Waters and Sewage in Department of Hydraulic and Sanitation for School of Engineering at São Carlos, University of São Paulo) chlorophyll analyses.

Material and methods

To compare the methods, 120 samples proceeding from two reservoirs: Lobo (an oligo-mesotrophic reservoir) and Salto Grande (a hipereutrophic reservoir) were used. Sixty (60) samples from subsurface of 15 different stations of each reservoir were used. The samples concentrations smaller than 3 $\mu\text{g. L}^{-1}$ (the detection limit of spectrophotometric method, according to Arar, 1997a) were disregarded for statistics analysis.

The samples were filtered in Whatmann GF/C filters and the filtered volumes varied between 0.25 L and 0.50 L. The filters were stored at -20°C and in the dark until extraction.

Some researchers use acetate filters, for chlorophyll extraction, because the filters are dissolved in organic solvents for the extraction. Lenz & Fritsche (1980) concluded that

there is no quantitative difference between the cells number retained in the acetate filter as well as in the fiberglass one. The fiberglass filter seems to be less harmful to the sample turbidity.

Schwartzbold et al (1999) had found a significant difference between samples filtered in filters GF/C and GF/F, being that filters GF/F had been efficient in the retention of the algal cells. The present work did not intend to compare the similarity of the samples and yes the differences between the determination methods. As the same sample was used for the three methods, the use of filters GF/C does not have significant influence in the final result.

Pigment extraction was carried out through the methodology described by Arar (1997a), extracted by 90% acetone, in cold. The filter was removed from the freezer and kept in the dark. After 12 hours, the filter was cut in small pieces, macerated in porcelain recipient, and transferred to centrifuge tubes. The final volume of the extract was standardized in 12.0 mL. After the filter had been converted to a slurry, it was kept at 4°C and in the dark during 12 hours, in the minimum, or 16 hours, in the maximum. The slurry than was centrifuged at 1,000 rpm during 15 min at 4°C. The supernatant was transferred an inert bottle. To reduce the turbidity interference from filter particles, the extract was filtered, with glass syringe through Milipore membrane (HV-Durapore of PVDF) of 0.45 µm porosity and 13 mm diameter. The filtered extract was separated in two portions: 1.5 ml for the chlorophyll determination by liquid chromatography and the remaining portion for determination by the fluorimetry and spectrophotometry analysis.

Although some authors (Bowles et al., 1985; Shoaf & Lium, 1976; Webb et al., 1992) reported that some types of chlorophyll and carotenoid are extracted completely with the use of methanol or dimetil-sulphate, the acetone is the best choice when there is no information on the specific composition of the community in the sample, besides to prevent the increase the chlorophyll degradation products (Mantoura & Llewellyn, 1983; Prezz & Bates, 1991). Due to the samples source (two environments, with different phytoplanktonic compositions), and the toxicity of the other chemical products (like methanol and dimethyl-sulphate) we chose acetona solvent.

For spectrophotometric analysis the HATCH spectrophotometer, model DR 4,000, and cuvette of 1 cm diameter was used, that were tested with 90% acetone solution to identify possible differences.

Values of the sample absorbancy were measured at four wavelengths (630, 647, 664 and 750 nm) were used in tricromatic equations. For the pheopigments concentration, the absorbancy values were measured at 750 and 665 nm with and without acidification samples (0.1 N HCl). According to Arar (1997a), this procedure does not require the calibration with pure chlorophyll-a solution.

For the determination of chlorophyll and pheopigments concentrations, the equations presented in Arar (1997a) were used, with some modifications in the specific chlorophyll absorption coefficient, according to Lorenzen (1967). Tricromatic equations of Jeffery & Humphrey (1975) were also used.

Chlorophyll-a determination by fluorescence followed the methodology proposed by Arar & Collins (1997). Fluorimeter Turner Designs U-10 model was used, with light bulb "day light", excitation filter of 350 nm to 500 nm, emission filter above of 665 nm, filter of neutral density reference, attenuating and 10.0 mm cuvettes. The detection limits of this method is 0.082 mg.L⁻¹, in the sample extract.

The fluorimeter was calibrated with Sigma.Co (CASRN 479-61-8) pure chlorophyll solution. The calibration curve was achieved with the following concentrations: 2; 5; 10; 20; 50, and 100mg.L⁻¹. The chlorophyll-a concentration was computed according to Arar & Collins, (1997).

Chlorophyll-a determination with high performance liquid chromatography (Shimadzu) was made according to the methodology described by Arar (1997b) with some modifications. This method uses reverse phase column (C18) with guard column, detector in the visible band, flow in 1.0 mL.min⁻¹, and 200µL sample.

The mobile phase was a gradient mixture of three eluents. They are (a) methanol: 0.5 M ammonium acetate/80:20 (v:v, pH 7.2), (b) acetonitrile: water/ 90:10 (v:v), and (c) 100% ethyl acetate. The eluent gradient program is listed in Arar (1997b).

Although the method recommends the absorbancy value at 440 nm, for determination of chlorophylls a (chl a) and b (chl b) and separation the pigments from a complex pigment mixture, we used 429 nm, a wavelength better absorbed by chlorophyll-a, to avoid interference of the others pigments (Li et al, 2002).

According Arar (1997a) the detection limits in the sample extract in HPLC is 0.080 mg.L⁻¹.

The standards for the calibration curve were the same used in the fluorimetric method. The standards concentrations were determined by tricromatic equation (Jeffery & Humphrey, 1975). The calibration curve was achieved with the following concentrations: 2; 5; 15; 25; 50, and 100 mg.L⁻¹.

Results and discussion

In the optical methods, spectrophotometry and fluorimetry, the presence of other pigments (as chlorophyll b, c, and the respective degradation products) are the main interferers in chlorophyll-a determination. Some authors (Brunt et al., 1992; Sartory, 1985; Trees et al., 1985) assert that the spectrophotometry and fluorimetry can underestimate or overestimate, significantly, chlorophyll concentration. This situation can happen, partly, because the bands absorbancy overlapping and chlorophyll fluorescence with accessory pigments and degradation products can occur. The two main chlorophyll degradation products are pheophytin and chlorophyllide, that can be degraded in a third substance type, the pheophorbide. This substance can affect the quantification of chlorophyll pigments because they fluoresce and absorb light in the same wavelengths (Carson & Simpson, 1996).

The phytoplanktonic community of the Lobo reservoir (oligo-mesotrophic and very mixed) is composed predominantly by Chlorophyceae and Bacillariophyceae. In the Salto Grande reservoir (hipereutrofic), the phytoplanktonic community is dominated by Cyanobacteria, being the Chlorophyceae the second most abundant group (Fig. 1).

The phytoplankton community composition in both ecosystems is related to pigment concentration determined by the tricromatic equations (Fig. 2).

Pigment composition differs in the two environments, because these pigments are not uniformly found in all the phytoplankton groups. In Cyanobacteria only chlorophyll a is present, whereas in Chlorophyceae, is found chlorophyll a as well as chlorophyll b and in Bacillariophyceae is present too chlorophyll c, e c₂, but not chlorophyll b.

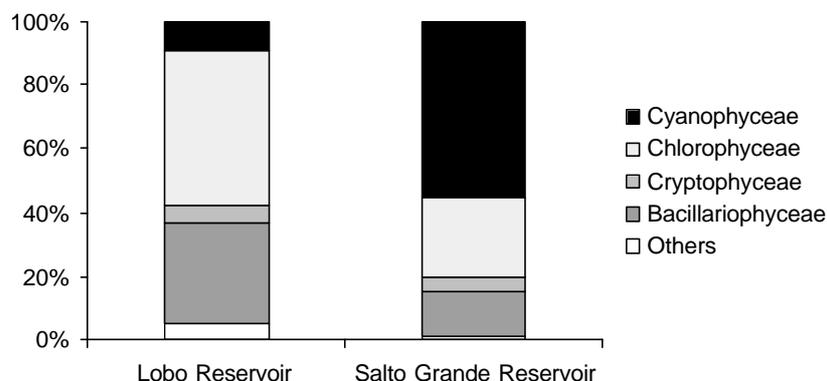


Figure 1: Average phytoplanktonic distribution in the Lobo and the Salto Grande reservoirs between October 1999 and July 2000.

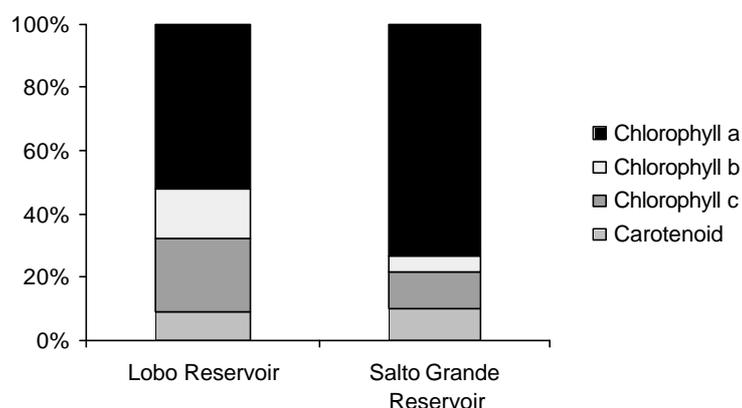


Figure 2: Relation between the average concentrations of chlorophyll a, b, c, and carotenoid, in the Lobo and Salto Grande reservoir between October 1999 and July 2000.

Chlorophyll a predominance in the Salto Grande reservoir is due to the higher Cyanobacteria and Chlorophyceae concentration. In the Lobo reservoir, the relative increase of chlorophyll c concentration occurs because of the higher Bacillariophyceae abundance.

The amount of chlorophyllide-a is associated with the cells physiological state (Sigleo et al., 2000). Mature cells can have from 40% to 50% of their pigments in this form (Hallengraf, 1981; Klein & Sournia, 1987). The phytoplanktonic community biomass, right after bloom occurrence, can contain from 40% to 60% of pheophorbide-a (Bidigare et al., 1996). The chlorophyll a and the other accessory pigments ratio apparently was constant (at approximately 1:1) in whatever environmental conditions (Trees et al., 2000).

Chlorophyll a is overestimated by the tricromatic equations of Jeffrey & Humphrey (1975), when the pheophytin is present. The modified equation of Lorenzen (1967) can underestimate chlorophyll a concentration when chlorophyll b is present. According to Arar (1997a), chlorophyll b concentration depends on chlorophyll-a and pheophytin concentrations. With the increment of chlorophyll a concentration, an increasing chlorophyll b underestimate can be observed. If the concentration of chlorophyll a is from 4 to 10 times higher than chlorophyll b, the underestimation will fluctuate between 13% to 38%.

Chlorophyll a concentrations determined by the tricromatic method in comparison with the ones by fluorimetry and after chromatographic separation presented values about 33% higher than the concentrations (Tab. I). The difference between the concentrations computed with tricromatic and Lorenzen equations are correlated positively with the pheophytin concentration (Fig. 3).

The fluorimetric and spectrophotometric calibrations after chromatographic separation depend on the concentrations determined by Jeffrey & Humphrey's equation (1975), after spectrophotometry. The calibration curves obtained by fluorimetric and spectrophotometric methods, in the visible band, are shown in the Fig 4.

Table I: Chlorophyll concentrations (mg.L^{-1}) determined by the tricromatic and the Lorenzen (1967) equations.

Concentrations	Tricromatic	Lorenzen
Average	22.31	13.43
Minimum	4.97	3.21
Maximum	73.29	63.51
Standard deviation	17.22	13.80
Variation coefficient (%)	77	103

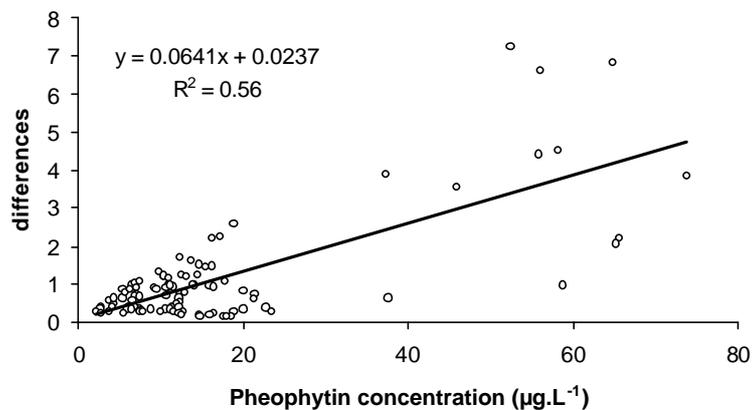


Figure 3: Linear correlation between the differences between chlorophyll a concentrations determined by the tricromatic and Lorenzen (1967) equations, and pheophytin concentrations.

The Salto Grande reservoir samples presented higher variation coefficients than the Lobo reservoir samples (Tab II), however they are very close in the three studied methods. The biggest average concentrations were the ones determined by the fluorimetric method. Through the spectrophotometric method at 429 nm, and after chromatographic separation, the least average concentrations in the samples of the two environments were obtained.

In the Lobo and Salto Grande reservoirs, the three methodologies were positively correlated (Tab. II). However, the correlations between the spectrophotometry in visible, after the chromatographic separation (HPLC), the spectrophotometry - UV and the fluorimetry were bigger in the reservoir of Salto Grande samples (Tab. I).

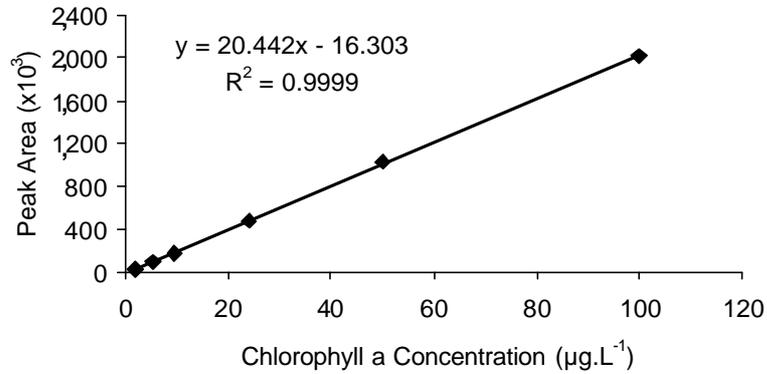
The use of the high performance liquid chromatography has increased in phytoplanktonic community studies (Brunet et al., 1992). Although expensive, slow and demanding specialized technicians, the information obtained through this method can be important when pigment separations in the sample are necessary. This separation allows to suppose the taxonomic composition of phytoplankton (Proença, 1997), to quantify the diverse groups through the key-pigment analysis (Breton et al., 2000; Flander et al., 2000; Barlow et al., 1998; Andersen et al., 1996; Preez & Beats, 1991), and to determine the relative concentration of degradation products in communities submitted to nutritional stress. In these communities, the cells

Table II: Descriptive statistics and Pearson correlations for chlorophyll concentrations (mg. L^{-1}) obtained through the three tested methods: spectrophotometry-UV (SPECT), fluorimetry (FLUOR), and the spectrophotometry at 429nm after separation for high performance liquid chromatography (HPLC) of the two environments samples (Lobo and Salto Grande reservoirs)

	Lobo Reservoir			Salto Grande reservoir		
	SPECT	FLUOR	HPLC	SPECT	FLUOR	HPLC
Average	11.26	20.04	7.24	28.70	44.89	22.77
Maximum	23.87	40.80	15.05	73.29	97.65	66.42
Minimum	6.69	11.51	3.38	1.68	2.71	2.84
Standard Deviation	3.33	5.68	2.10	19.64	27.44	16.45
Variation coefficient (%)	30	28	29	68	61	72

	Pearson Correlations					
	SPECT	FLUOR	HPLC	SPECT	FLUOR	HPLC
Spectrophotometry	1.00			1.00		
Fluorimetry	0.98	1.00		0.97	1.00	
Chromatography	0.87	0.93	1.00	0.98	0.95	1.00

(1)



(2)

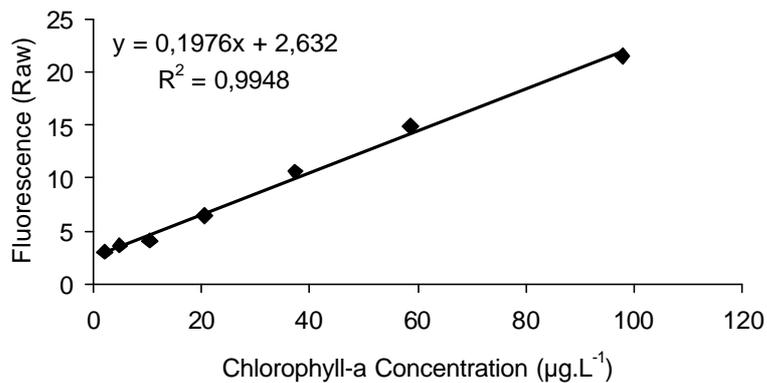


Figure 4: Relation between absorbancy (= 429nm) (1) and fluorescence (2) values, and different chlorophyll a concentrations.

senescence carries to a change in the pigment content (Yoshihito & Yasuhiro, 2000; La-Giraudiere, 1987).

Since the chlorophyll determination through liquid chromatography, is subjected to low interference, the method is considered the most precise and sensible because it makes the separation diverse chlorophyll types and degradation products. However, it is expensive and slow (Brown et al., 1981; Murray et al., 1986).

The correlations between the data gotten in the spectrophotometric-UV and fluorimetric methods with the HPLC ones, in the Lobo reservoir (Fig. 5), were smaller of that in the Salto Grande reservoir (Fig. 6)

In spite of the high correlation linear and Pearson coefficients obtained in the Lobo reservoir, the overestimate occurred in all the samples. Larger average value for the fluorimetric than for the spectrophotometric method in the Salto Grande reservoir, was recorded without chromatographic separation, overestimated and underestimated chlorophyll concentrations. However, the average value indicated overestimate for the two methods, being higher in the fluorimetric. When we compared the two environments, the overestimate was smaller in the Salto Grande reservoir (Tab. III).

The comparison between the three methods confirms previous data (Sigleo et al., 2000; Brunet et al., 1992; La-Giraudiere, 1987; Trees et al.,1985; Gowen et al., 1983). Among the three used methodologies, the fluorimetry, despite being quick and requiring

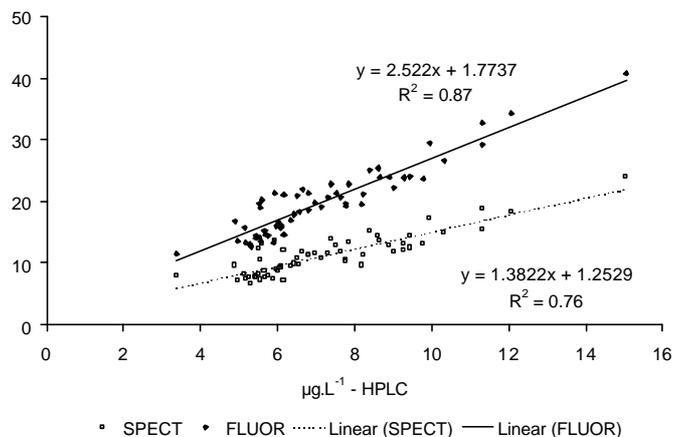


Figure 5: Relationship between chlorophyll concentrations ($\mu\text{g.L}^{-1}$) obtained by the spectrophotometric-UV fluorimetric and HPLC methods in the samples from Lobo reservoir

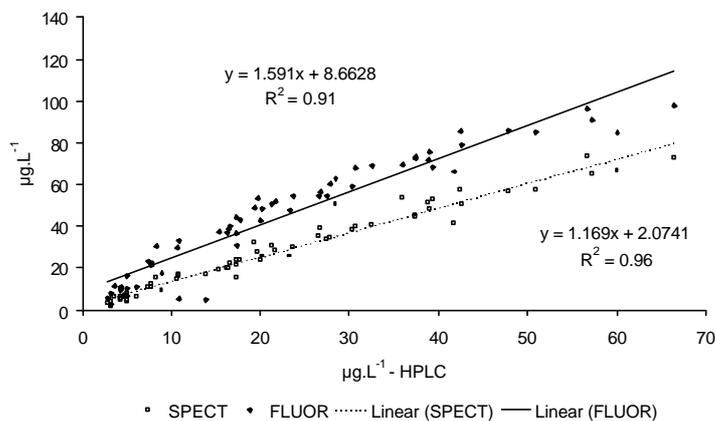


Figure 6: Relationship between chlorophyll concentrations ($\mu\text{g.L}^{-1}$) obtained by the spectrophotometric-UV fluorimetric and HPLC methods, in the samples from Salto Grande reservoir.

smaller sample volume, was the one that most overestimated chlorophyll concentrations. In the presence of chlorophyll b, other accessory pigments, and degradation products, chlorophyll a is underestimated (Brown et al., 1981).

The differences between the concentrations obtained by spectrophotometry-UV, fluorimetry, and spectrophotometry in visible, after the chromatography, can be significant if degradation products are present. The pheophytin determination using Lorenzen's equation 2 (Lorenzen, 1967) should be studied, mainly to reduce the effect of the turbidity after the sample acidification. Trees et al (2000), studying the relation between accessory

Table III: Percentage (%) of over (+) or underestimate (-) of chlorophyll a concentration by the spectrophotometric-UV (SPECT) and fluorimetric (FLUOR) methods in relation to the spectrophotometric with chromatographic separation (HPLC), in the two reservoir samples.

	Lobo Reservoir		Salto Grande Reservoir	
	SPECT	FLUOR	SPECT	FLUOR
Maximum	+133	+261	+87	+270
Minimum	+17	+125	-48	-63
Average	+57	+179	+28	+113

pigments and chlorophyll a in 7,000 samples of oceanic and coastal regions, for HPLC, found a small relation (0.035) between pheophytin and chlorophyll-a and recommended that the determination by traditional methods, spectrophotometry-UV and fluorescence, should be better studied. According to Barlow (1990), reliable data of the pheopigments concentrations can only be obtained through chromatography (HPLC).

Myens et al. (1994), comparing the spectrophotometric and chromatographic (HPLC) methods, in samples from a temperate lake, concluded that the difference among chlorophyll concentrations determined by the two methods vary during the year and is related to the production of other forms of chlorophyll and degradation products during the phytoplanktonic succession. So, no conversion factor between these methods was developed.

The optical methods without pigment separation (spectrophotometric-UV and fluorimetric) are inefficient for the determination of the diverse types of chlorophyll (a, b, c₁, and c₂) and its degradation products (Pheophytin, Chlorophyllide and Pheophorbide), as with the chromatographic method. Some authors suggest that the term chlorophyll a must be abandoned when the determination is not made through chromatographic methods. So, it is advisable to use only chlorophyll or still total pigments, even if the tricromatic equations (Carlson & Simpson, 1996) were used. In environments dominated by cyanobacteria (as the Salto Grande reservoir), most of the present pigments is in the form of chlorophyll a, and the spectrophotometric method can be used, considering that it is less subjected to interferences. In environments with a more diversified composition of species and Chlorophyceae and Bacillariophyceae dominance (as the Lobo reservoir) the presence of chlorophyll b and c can overestimate significantly the data obtained by UV spectrophotometry.

In spite of the high correlation among chlorophyll concentrations obtained by the different methods, the overestimate percentages request for a better care in the optical methods utilization (spectrophotometry and fluorimetry). As the chromatographic method separates the sample components, it should be considered the most reliable method. Only through this method it is possible to obtain more real values for each sample pigment type.

The use of the UV spectrophotometry can be recommended for environments whose composition is predominantly of Cyanobacteria, as the Salto Grande reservoir. In environments with great species diversity, and with Bacillariophyceae and Chlorophyceae presence in large amount, data obtained by UV spectrophotometry should be considered like a total chlorophyll estimation. In these environments, the use of chromatography (HPLC) is recommended.

In all the analyzed aspects, the fluorimetric method was the least efficient. The greatest advantage of this method is its quickness and sensibility. However, due to the possible interferers, it is more appropriate for the total pigments estimation.

Selection of the most appropriated method takes into account diverse factors: a) analysis accuracy; b) application facility (infrastructure and financial aspects), c) comparison with other works and d) adequacy to the research objectives.

The best method for chlorophyll-a determination is the chromatography. However the analysis cost and the time make the adoption of this methodology difficult in routine analysis. When chlorophyll is the subject of the investigation, as in phytoplanktonic community and physioecologic studies, this method must be used.

In monitoring programs, and for comparison between environments, chlorophyll a can be look at as total chlorophyll or total pigments; spectrophotometry can be used, mainly in environments dominated for cyanobacteria. Despite the inherent errors of the method, it still is the most used worldwide, providing legitimate comparisons.

The fluorimetry is only indicated in two situations: when the time analysis is a limitation for the work objectives, as in the effluent monitoring or simultaneous studies in wide scale, hydrodynamic studies, for example; or when the chlorophyll measure is a comparative data in unialgae samples, as algal growth measurement in ecotoxicological tests.

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