A numerical simulation of trichromatic equations in chlorophyll estimation using the spectrophotometric technique[1]

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Abstract

A numerical simulation of trichromatic pigment equations is made with the aid of a computer utility program. Significant quantitative differences in the estimates of pigment concentration result from using different sets of trichromatic equations. Estimates of chlorophylls *a, b,* and *c* were found highly correlated with the application of the equations, even though the absorbance values used as input for the stimulation are not correlated.

Introduction

There has been a marked increase in the use of pigment estimations, especially in the use of chlorophylls *a, b* and *c*, for assessing the abundance of planktonic algae and in studies on primary productivity. Large variances have been observed with respect to estimates of the chlorophylls *a, b,* and *c,* based on differences in the use of analytical methodologies, extraction solvents, and extraction procedures in the spectrophotometric technique. A significant portion of the variance in the spectrophotometric technique for chlorophyll analysis can be also accounted for by the trichromatic equations used in the computation of chlorophyll concentration. Significant quantitative differences in the estimates of pigment concentration result from using different sets of trichromatic equations, and significant correlations are found between estimates of chlorophylls a, b, and c by the application of the equations, even though the absorbance values used as input for the simulation are completely uncorrelated. The numerical simulation of these equations is made with the aid of a computer utility program. The trichromatic equations of Strickland & Parsons (1965), UNESCO (1966), and Richards & Thompson (1952) are under consideration in this study.

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Procedure

Three groups of random values are selected from a random values generating table (Gibra 1973). Each group contains 20 sets of chlorophyll absorbances at wavelengths of 665 mu, 645 mu, and 630 mu. The first two groups correspond to the sets 0.001–0.009 and 0.01–0.09, respectively. The values for the third group are mostly in the range of 0.10–0.99; the fact that a few values fall below 0.10 is attributed to the use of the random digit generating technique. The values 0.001–0.009, 0.01–0.09, and 0.10–0.99 are used to simulate the low, moderate, and high absorbances, respectively, of pigment concentration occurring in nature. The three groups

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Table 1. The trichromatic equations of Strickland & Parsons, UNESCO, and Richards & Thompson. E665, E645, and E630 stand for the absorbance at wavelengths of 665 mu, 645 mu and 630 mu. A, B, and C refer to chlorophylls *a, b,* and *c* respectively; the *2nd* letter denotes the equation used (R = Richards & Thompson, S = Strickland & Parsons, U = UNESCO).

Strickland & Parsons

Chl. *a* (AS) = 11.6[E665] - 1.31[E645] - 0.14[E630]
Chl. *b* (BS) = 20.7[E645] - 4.34[E665] - 4.42[E630]
Chl. *c* (CS) = 55[E630] - 4.64[E665] - 16.3[E645]

UNESCO

Chl. *a* (AU) = 11.64[E663] - 2.16[E645] + 0.1[E630]
Chl. *b* (BU) = 20.97[E645] - 3. 94[E663] - 3.66[E630]
Chl. *c* (CU) = 54.22[E630] - 14.81[E645] - 5.53[E663]

Richards & Thompson

Chl. *a* (AR) = 15.6[E665] - 2.0[E645] - 0.8[E630]
Chl. *b* (BR) = 25.4[E645] - 4.4[E665] - 10.3[E630]
Chl. *c* (CR) = 109[E630] - 12.5[E665] - 28.7[E645]

of values are then entered independently into an SPSS simulator computer program written specifically for this research. The equations of Strickland & Parsons, UNESCO, and Richards with Thompson are used and are shown in Table 1. Since the optical difference resulting from an estimate at frequencies of 663 mu and 665 mu is small and insignificant (Banse & Anderson 1967), the same optical absorbances are assumed for these two frequencies.

The results of this simulation are presented in Tables 2a, 2b, and 2c. Multiple t-comparisons between the results of Strickland & Parsons, UNESCO, and Richards & Thompson for chlorophylls *a, b,* and *c* are made as shown in Table 3. Correlations matrixes between chlorophylls *a, b,* and *c* for each method are presented in Table 4.

Results

Significant differences are shown between the estimates made using the Strickland & Parsons

Table 2A. The simulated absorbance values of low pigment concentration and the results of using the equations of Strickland & Parsons (S), UNESCO (U) and Richards & Thompson (R). E665, E645, and E630 stand for the absorbance at the wavelengths of 665 mu, 645 mu and 630 mu. A, B and C refer to chlorophylls *a, b,* and *c* respectively; the *2nd* letter denotes the equation used (R = Richards & Thompson, S = Strickland & Parsons, U = UNESCO).

Cases	E665	E645	E630	AS	BS	CS	AU	BU	CU	AR	BR	CR
1	0.007	0.001	0.007	.08	-.04	.34	.08	-.03	.33	.10	-.08	.65
2	0.004	0.006	0.009	.04	-.07	.38	.03	.08	.38	.04	.04	.76
3	0.009	0.007	0.009	.09	.07	.34	.09	.08	.33	.12	.05	.67
4	0.000	0.002	0.008	.00	.01	.41	.00	.01	.40	-.01	-.03	.81
5	0.008	0.005	0.005	.09	.05	.16	.08	.06	.15	.11	.04	.30
6	0.006	0.006	0.004	.06	.08	.09	.06	.09	.09	.08	.08	.19
7	0.007	0.002	0.001	.08	.01	-.01	.08	.01	-.01	.10	.01	-.04
8	0.005	0.008	0.003	.05	.13	.01	.04	.14	.02	.06	.15	.03
9	0.003	0.002	0.009	.03	-.01	.45	.03	.00	.44	.04	-.06	.89
10	0.008	0.002	0.002	.09	.00	.04	.09	.00	.03	.12	.00	.06
11	0.008	0.003	0.001	.09	.02	-.03	.09	.03	-.03	.12	.03	-.08
12	0.002	0.008	0.008	.01	.12	.30	.01	.13	.30	.01	.11	.62
13	0.003	0.009	0.007	.02	.14	.22	.02	.15	.23	.02	.14	.47
14	0.003	0.004	0.003	.03	.06	.09	.03	.06	.09	.04	.06	.17
15	0.006	0.005	0.000	.06	.08	-.11	.06	.08	-.11	.08	.10	-.22
16	0.008	0.003	0.000	.09	.03	-.09	.09	.03	-.09	.12	.04	-.19
17	0.009	0.007	0.006	.09	.08	.17	.09	.09	.17	.12	.08	.34
18	0.003	0.008	0.008	.02	.12	.30	.02	.13	.30	.02	.11	.60
19	0.00	0.007	0.003	-.01	.13	.05	-.01	.14	.06	-.02	.15	.13
20	0.006	0.009	0.006	.06	.13	.16	.05	.14	.16	.07	.14	.32

Table 2B. The simulated absorbance values of moderate pigment concentration and the results of using the equations of Strickland & Parsons (S), UNESCO (U), and Richards Thompson (R). E665, E645, and E630 stand for the absorbance at the wavelengths of 665 mu, 645 mu, and 630 mu. (Refer to Table 2A for notations used.)

Cases	E665	E645	E630	AS	BS	CS	AU	BU	CU	AR	BR	CR
1	0.09	0.03	0.08	.99	-.12	3.49	.99	.02	3.40	1.28	-.46	6.73
2	0.02	0.01	0.04	.21	-.06	1.94	.22	-.02	1.91	0.26	-.25	3.82
3	0.04	0.01	0.04	.39	0.68	1.20	.36	.74	1.21	0.49	0.68	2.43
4	0.04	0.05	0.00	.40	0.86	-1.00	.36	.89	-.96	0.52	1.09	-1.94
5	0.04	0.02	0.00	.44	0.24	-.51	.42	.26	-.52	0.58	0.33	-1.07
6	0.05	0.09	0.07	.45	1.34	2.15	.39	1.43	2.19	0.54	1.34	4.42
7	0.04	0.07	0.06	.36	1.01	1.97	.32	1.09	2.00	0.44	0.98	4.03
8	0.07	0.02	0.07	.78	-.20	3.20	.78	-.11	3.11	1.00	-.52	6.18
9	0.07	0.07	0.06	.71	0.88	1.83	.67	.97	1.83	0.90	0.85	3.66
10	0.05	0.00	0.03	.58	-.35	1.42	.59	-.31	1.35	0.76	-.53	2.65
11	0.09	0.07	0.03	.95	0.93	0.09	.90	1.00	0.09	1.24	1.07	0.14
12	0.04	0.04	0.01	.41	0.61	-.29	.38	.64	-.27	0.54	0.74	-.56
13	0.04	0.08	0.02	.36	1.39	-.39	:29	1.45	-.32	0.45	1.65	-.62
14	0.01	0.05	0.07	.04	0.68	2.99	.02	.75	3.00	0.00	0.51	6.07
15	0.08	0.06	0.08	.84	0.54	3.05	.81	.65	3.01	1.06	0.35	6.00
16	0.09	0.07	0.05	.95	0.84	1.19	.90	.93	1.18	1.22	0.87	2.32
17	0.03	0.06	0.03	.27	0.98	0.53	.22	1.03	.57	0.32	1.08	1.17
18	0.04	0.04	0.09	.40	0.26	4.11	.39	.35	4.07	0.47	-.09	8.16
19	0.08	0.09	0.01	.81	1.47	-1.29	.74	1.54	-1.23	1.06	1.83	-2.49
20	0.06	0.08	0.02	.59	1.31	-.48	.53	1.37	0.43	0.76	1.56	-.87

Table 2C. The simulated absorbance values of high pigment concentration and the result of using the equations of Strickland & Parsons (S), UNESCO (U), and Richards & Thompson (R). E665, E645, and E630 stand for the absorbance at the wavelengths of 665 mu, 645 mu, and 630 mu. (Refer to Table 2A for notations used).

Cases	E665	E645	E630	AS	BS	CS	AU	BU	CU	AR	BR	CR
1	0.19	0.29	0.54	1.75	2.79	24.09	1.64	3.36	23.93	1.95	.97	48.16
2	0.20	0.59	0.43	1.49	9.44	13.11	1.10	10.01	13.47	1.60	9.68	27.44
3	0.21	0.54	0.86	1.61	6.47	3.75	21.36	7.35	37.47	1.51	3.92	75.62
4	0.25	0.96	0.93	1.51	14.68	34.34	0.93	15.74	34.82	1.24	13.70	70.69
5	0.26	0.65	0.91	2.04	8.30	38.25	1.71	9.28	38.28	2.03	5.99	77.29
6	0.29	0.54	0.96	2.52.	5.68	42.65	2.31	6.67	42.45	2.68	2.55	85.52
7	0.06	0.04	0.06	0.64	0.30	2.37	0.62	0.38	2.33	0.81	0.13	4.64
8	0.39	0.51	0.03	3.85	8.73	-8.47	3.44	9.05	-8.08	5.04	10.93	-16.24
9	0.51	0.26	0.87	5.45.	-.68	41.25	5.46	0.26	40.50	6.74	-4.60	80.99
10	0.56	0.49	0.07	5.84	7.40	-6.74	5.47	7.81	-6.56	7.70	9.26	-13.43
11	0.59	0.05	0.14	6.76	-2.14	4.15	6.77	-1.79	3.59	8.99	-2.77	6.45
12	0.59	0.40	0.47	6.25	3.64	16.59	6.05	4.34	16.30	8.03	2.72	32.37
13	0.69	0.93	0.35	6.74	14.71	.89	6.06	15.50	1.39	8.62	16.98	2.83
14	0.78	0.21	0.21	8.74	0.03	4.51	8.65	0.56	3.96	11.58	-.26	7.11
15	0.80	0.24	0.12	8.95	.97	-1.02	8.81	1.44	-1.47	11.90	1.34	-3.81
16	0.80	0.43	0.85	8.60	1.67	36.03	8.47	2.75	35.29	10.94	-1.35	70.31
17	0.86	0.44	0.37	9.35	3.74	9.19	9.10	4.48	8.79	12.24	3.58	16.95
18	0.90	0.29	0.13	10.04	1.52	-1.75	9.86	2.06	-2.22	13.36	2.07	-5.40
19	0.94	0.75	0.16	9.90	10.74	-7.79	9.34	11.44	-7.63	13.04	13.27	-15.84
20	0.96	0.16	0.33	10.88	-2.31	11.09	10.86	-1.63	10.21	14.39	-3.56	19.38

Table 3. Multiple t-comparisons between the results of Strickland & Parsons (S), UNESCO (U), and Richards & Thompson (R) for chlorophylls *a*, *b* and *c* at low, moderate and high absorbance levels (* – significant at 0.05, ** – significant at 0.01).

	Low				Moderate				High		
	AS	AU	AR		AS	AU	AR		AS	AU	AR
AS				AS				AS			
AU	**			AU	**			AU	**		
AR	**	**		AR	**	**		AR	**	**	
	BS	BU	BR		BS	BU	BR		BS	BU	BR
BS				BS				BS			
BU	**			BU	**			BU	**		
BR		*		BR		*		BR			
	CS	CU	CR		CS	CU	CR		CS	CU	CR
CS				CS				CS			
CU				CU				CU			
CR	**	**		CR	**	**		CR	**	**	

equation, the UNESCO equation, and the Richards & Thompson equation for chlorophyll *a* under simulated conditions of low, moderate, and high absorbances. Of these differences, the ones between chlorophyll *a* estimates by using the UNESCO equation and the Strickland & Parsons equation are relatively small. Nevertheless, they are statistically significant ($P < 0.01$, Table 3). The differences in chlorophyll *a* between estimates derived from the *Unesco* equation and the Richards & Thompson equation, and between those derived from the Strickland & Parsons equation and the Richards & Thompson equation, not only are significant ($P < 0.01$), but are about six times greater in magnitude than the differences between Strickland & Parsons and UNESCO estimates. The differences in percentage remain constant throughout the simulation. Significant differences in chlorophyll *b* estimates are also found betweenthe results of the equations of Strickland & Parsons and those of UNESCO at low, moderate, and high levels of absorbance ($P < 0.01$) and between the results of the equations of UNESCO and those of Richards & Thompson at low and high levels of absorbance ($P < 0.05$, Table 3). Significant chlorophyll *b* estimates are also found between the comparison between the estimates derived from the Strickland & Parsons equations and the Richards & Thompson equations, and between those from the UNESCO equation and the Richards & Thompson equation at all levels of absorbance, but the differences in percentage remain constant throughout the simulations.

Correlation coefficients between chlorophylls *a*, *b*, and *c* are examined using each set of equations. Significant negative coefficients are found between chlorophylls *a* and *b* at the low level of absorbance, chlorophylls *a* and *c* at the moderate level of absorbance, and chlorophylls *b* and *c* at the high level of absorbance when applying the Strickland & Parsons equations. These significant negative correlation coefficients are also noticed between chlorophylls *a* and *b* at the low level of absorbance, between chlorophylls *a* and *b*, and *a* and *c* at the moderate level of absorbance, and *b* and *c* at the high level of absorbance using the UNESCO equations. They are also found between chlorophylls *a* and *c* at the low and moderate levels of absorbance, and between chlorophylls *b* and *c* at the high level of absorbance using the equations of Richards Thompson.

Discussion

Since pigment concentration has been widely used to assess changes in water quality and photosynthetic potential of planktonic populations, it is important to know whether the extent of this difference is determined by the organisms or by the

Table 4. Multiple correlations between chlorophylls *a*, *b*, and *c* for each method at the simulated absorbances of low, moderate or high concentrations. The top value indicates the correlation coefficient, the bottom value in parentheses indicates the probability of this occurrence.

	Low				*Moderate*				*High*		
	AS	BS	CS		AS	BS	CS		AS	BS	CS
AS				AS				AS			
BS	-0.41			BS	-0.36			BS	0.07		
	(0.036)				(0.059)				(0.378)		
CS	-0.38	-0.11		CS	-0.45	0.00		CS	0.04	-0.53	
	(0.051)	(0.319)			(0.022)	(0.496)			(0.42)	(0.008)	
	AU	BU	CU		AU	BU	CU		AU	BU	CU
AU				AU				AU			
BU	-0.46			BU	-0.41			BU	-0.13		
	(0.021)				(0.037)				(0.285)		
CU	-0.37	-0.05		CU	-0.46	0.06		CU	0.08	-0.48	
	(0.052)	(0.415)			(0.022)	(0.394)			(0.367)	(0.016)	
	AR	BR	CR		AR	BR	CR		AR	BR	CR
AR				AR				AR			
AR	-0.29			BR	-0.22			BR	-0.06		
	(0.106)				(0.179)				(0.139)		
CR	-0.44	-0.31		CR	-0.51	-0.25		CR	-0.01	-0.65	
	(0.026)	(0.089)			(0.01)	(0.148)			(0.47)	(0.001)	

application of the equations. Chlorophyll estimates using different sets of equations yield results that are of significant statistical difference, particularly with the application of the equations of Richards & Thompson; in such cases, estimates are up to 35 per cent higher than those based on other equations (Wartenberg 1978). Therefore, if these concentration estimates are used with other variables in a statistical analysis, conclusions derived from such analysis can vary markedly among themselves.

Strong negative correlations are shown between the estimates of chlorophylls *a*, *b*, and *c* for each method when the absorbances are a set of random values. Since there is no evidence in the literature to show that increasing the concentration of chlorophyll *a* would inhibit the production of chlorophylls *b* and *c* or vice versa, or that decreasing the concentration of chlorophyll *a* would stimulate the production of chlorophylls *b* and *c*, it is believed that this discrepancy is a result of the equations used. This discrepancy may well explain the fact that when trichromatic equations are applied, the results can show that a certain pigment is always present in good quantity irrespective of whether or not the pigment is contributed by the organisms (Rai 1973). For example, indications of the presence of chlorophyll *c* in *Scenedesmus quadricauda* based on these equations are contrary to the findings in the literature and chromatographic studies indicating that this species does not contain this pigment (Rai 1973). Extensive impacts can result from these significant negative correlations, in particular when chlorophylls *a*, *b*, and *c* are used as the major indicators of aquatic systems. Since significant correlations imply a strong dependence between the estimates of chlorophyll *a*, *b*, and *c*, and since the fundamental basis for the statistical analysis is an independence between the estimates, the strong dependent relationship can cause significant biases when comparisons are made between chlorophyll concentrations computed from tichromatic equations. Furthermore, any attempt to correlate the chlorophyll concentrations with environmental variables as in the study by Schwartzkopf & Hergenrader (1978) may include the artificial correlations, which primarily result form the equations used. However, if it is necessary to use statistical comparisons and correlations analysis, an increase in sample size and reduction of sampling error I to $P \leq 0.01$ must be seriously considered before a conclusion is derived from such analysis.

Conclusions

(1) Chlorophyll estimates using different sets of equations yield results that are of significant statistical difference. If these results are used with other variables in a statistical analysis, conclusions derived from the analysis can vary markedly among themselves. (2) Strong negative correlations are found between the estimates of chlorophylls *a, b,* and *c* for each method. These correlations indicate a discrepancy resulting from the application of the trichromatic equations.

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