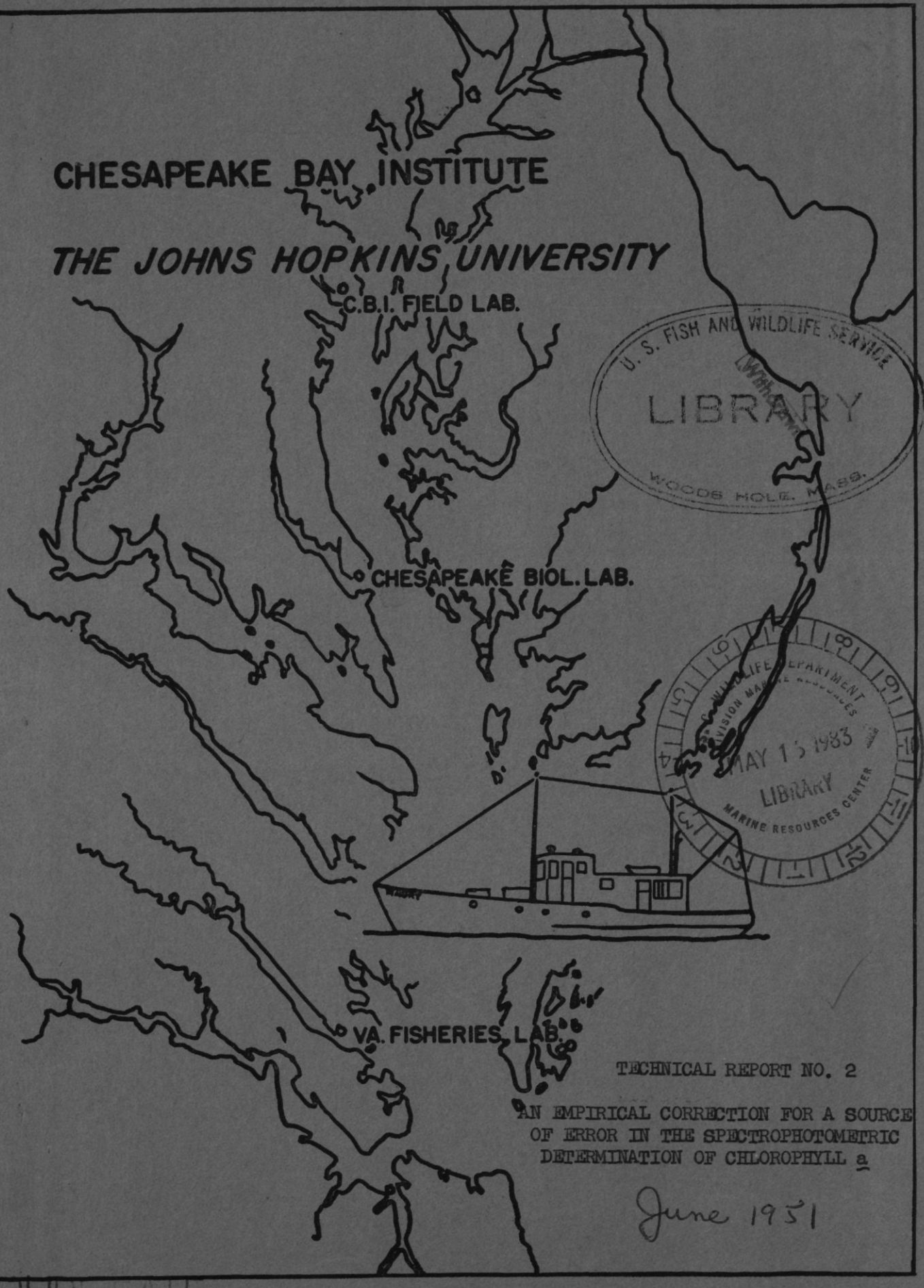


TECHNICAL REPORT NO. 2

AN EMPIRICAL CORRECTION FOR A SOURCE OF ERROR IN THE SPECTROPHOTOMETRIC DETERMINATION OF CHLOROPHYLL a

June 1951

CHESAPEAKE BAY INSTITUTE

THE JOHNS HOPKINS UNIVERSITY

TECHNICAL REPORT

AN EMPIRICAL CORRECTION FOR A SOURCE OF ERROR IN THE SPECTROPHOTOMETRIC DETERMINATION OF CHLOROPHYLL *a*

This report is the second of a series of technical reports prepared by the Chesapeake Bay Institute containing results of work carried out for the Office of Naval Research of the Navy Department under research project NR-083-016, Contract N6onr-243, Task Order 10; the State of Maryland (Department of Research and Education); and the Commonwealth of Virginia (Virginia Fisheries Laboratory); under contract with The Johns Hopkins University.

Technical Report No. 2
Dayton E. Carritt and
James H. Carpenter

June, 1951

ABSTRACT

An 80% aqueous acetone extract of the suspended solids from Chesapeake Bay waters yields a solution which contains finely divided solids in addition to the extracted plant pigments. The suspended solids appear to be formed from organic constituents during extraction. There is no correlation between the natural turbidity of the untreated raw water sample and the turbidity in the extract.

Spectrophotometric analysis of the extracts for chlorophylls gives results which are high by an amount proportional to the amount of suspended materials. A method is described which provides a measure of chlorophyll a alone.

Details of the concentration and extraction procedure are given.

Chlorophyll analyses have been used by many investigators as measures of phytoplankton population, which in turn are used as indexes for the amount of organic carbon that may be fixed during photosyntheses.

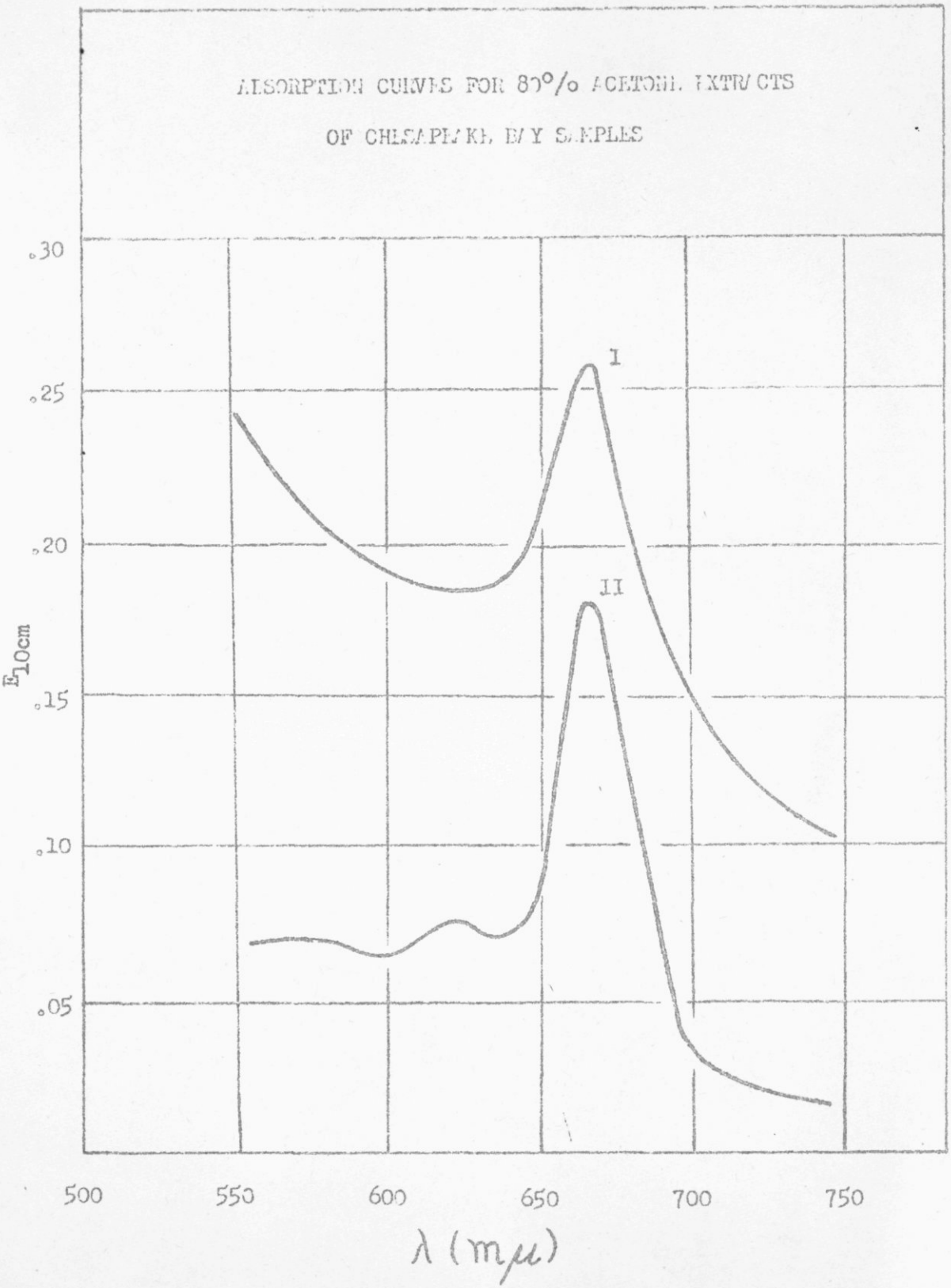
The visual comparison of acetone, methyl alcohol or diethyl ether extracts of collected suspended material agninst permanent inorganic colorimetric standards (1, 2, 3) can give only an integrated value for all of the pigments present. When the ratios of the pigments vary, comparison is difficult because of ''off-color'' in the extracts.

In principle, spectrophotometric methods provide a means of analysis for each component of a multicomponent system, provided, of course, that each component has a characteristic absorption curve. We had hoped that application of these methods would provide a better measure of chlorophyll without interference by other pigments.

Existing absorption curves for pigments likely to be found in acetone extracts of marine plant materials indicate that the 667 $m\mu$ absorption peak of chlorophyll a is nearly free of interference from other pigments. MacKinney (4) indicates that the light absorption by crude leaf extracts above 530 $m\mu$ can be accounted for by chlorophylls alone. Strain and Manning (5) show that brown algae and diatoms contain chlorfucine (chlorophyll c), but that it has little absorption at 667 $m\mu$. They also found little evidence of the presence of chlorophyll b in these algae. Thus, it would appear that spectrophotometric analysis, at 667 $m\mu$, should provide a method for quantitative analysis for chlorophyll a.

Absorption curves for acetone extracts of the solids filtered from Chesapeake Bay samples are shown in Figure 1. They show the characteristic chlorophyll a absorption peak at 667 $m\mu$. However, comparison with a curve for pure chlorophyll a (Figure 2, Curve I) shows that at other

Figure 1


ABSORPTION CURVES FOR 80% ACETONE EXTRACTS
OF CHESAPEAKE BAY SAMPLES
.30
.25
.20
.15
.10
.05
E_{10cm}
I
II
500
550
600
650
700
750
λ (mμ)

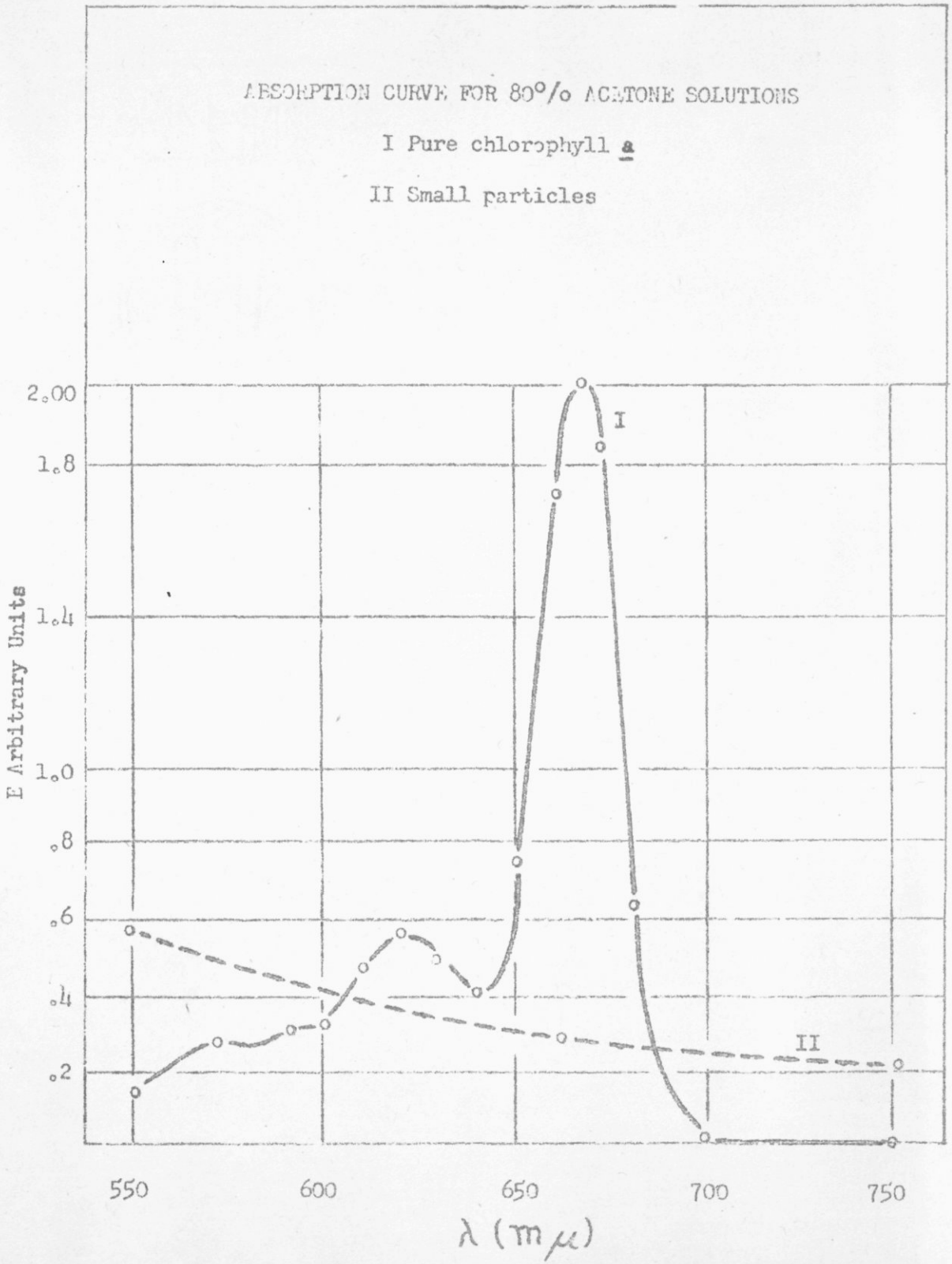
wave lengths, and especially at shorter wave lengths, the extracts of Bay samples have an abnormally high absorption which cannot be accounted for adequately by assuming other pigments to be present.

The curves in Figure 1 illustrate the source of error. Considering just the extinction measurements at 667 $m\mu$, the concentration of chlorophyll a from curve I would appear greater than that from curve II. However, when compared with the curve for pure chlorophyll a, it is apparent that both I and II have been displaced (and distorted) to higher extinctions. A qualitative estimate of the displacement is given by dropping each curve so that the extinction at 750 $m\mu$ is very nearly zero, a condition that would exist if only chlorophyll a were present. When treated quantitatively, by the procedure described below, the concentration of chlorophyll a in solution II is shown to be greater than that in solution I.

It was noticed that many of the samples contained a slight turbidity. At first it was thought to be caused by some of the particulate materials of the water passing the filter during filtration of the extract. However, no correlation between the abnormally high absorption at 750 $m\mu$ and normal turbidity of raw unfiltered samples could be found.

A comparison of the extract curve with a chlorophyll a curve, (Figure 2, curve I) and a curve for a suspension of small particles (Figure 2, curve II) suggests that the extract curve results from measuring chlorophyll a in the presence of a suspension of finely divided solids. Thus, absorption measurements at 667 $m\mu$ include the effects of both chlorophyll and suspended particles. The problem then is to obtain enough additional data to make it possible to make a correction for the effects of suspended materials on the 667 $m\mu$ measurement which would then give a measure of the chlorophyll alone.

Figure 2


ABSORPTION CURVE FOR 80°/o ACETONE SOLUTIONS
I Pure chlorophyll a
II Small particles
E Arbitrary Units
2.00
1.8
1.4
1.0
.8
.6
.4
.2
I
II
550
600
650
700
750
λ (mμ)

The correction system, to be described below, requires extinction (optical density) measurements at 550, 667, and 750 $m\mu$. At 550 and 750 $m\mu$, the extinction for pure chlorophyll a is only 6% and 2% respectively of the 667 $m\mu$ value. An increase in the 550 to 667 or 750 to 667 ratios is assumed to be caused by the presence of the suspended material only.

The elements of the correction system are shown schematically in Figure 3. Routinely, points ABC are measured with the spectrophotometer. If no turbidity were present, the distance FB would be a measure of the chlorophyll a. The curve AJC is a generalized turbidity curve. The extinction corresponding to chlorophyll a, in the presence of turbidity, is equal to the distance FB minus the distance FJ. However, no direct measure of point J is obtainable.

As a first approximation, the line ADC can be constructed, locating a point D, and the distance FB minus FD may be used as the corrected extinction for chlorophyll a at 667 $m\mu$. However, when this is done for samples having low chlorophyll concentrations, negative values for chlorophyll are obtained, i.e., FD>FB.

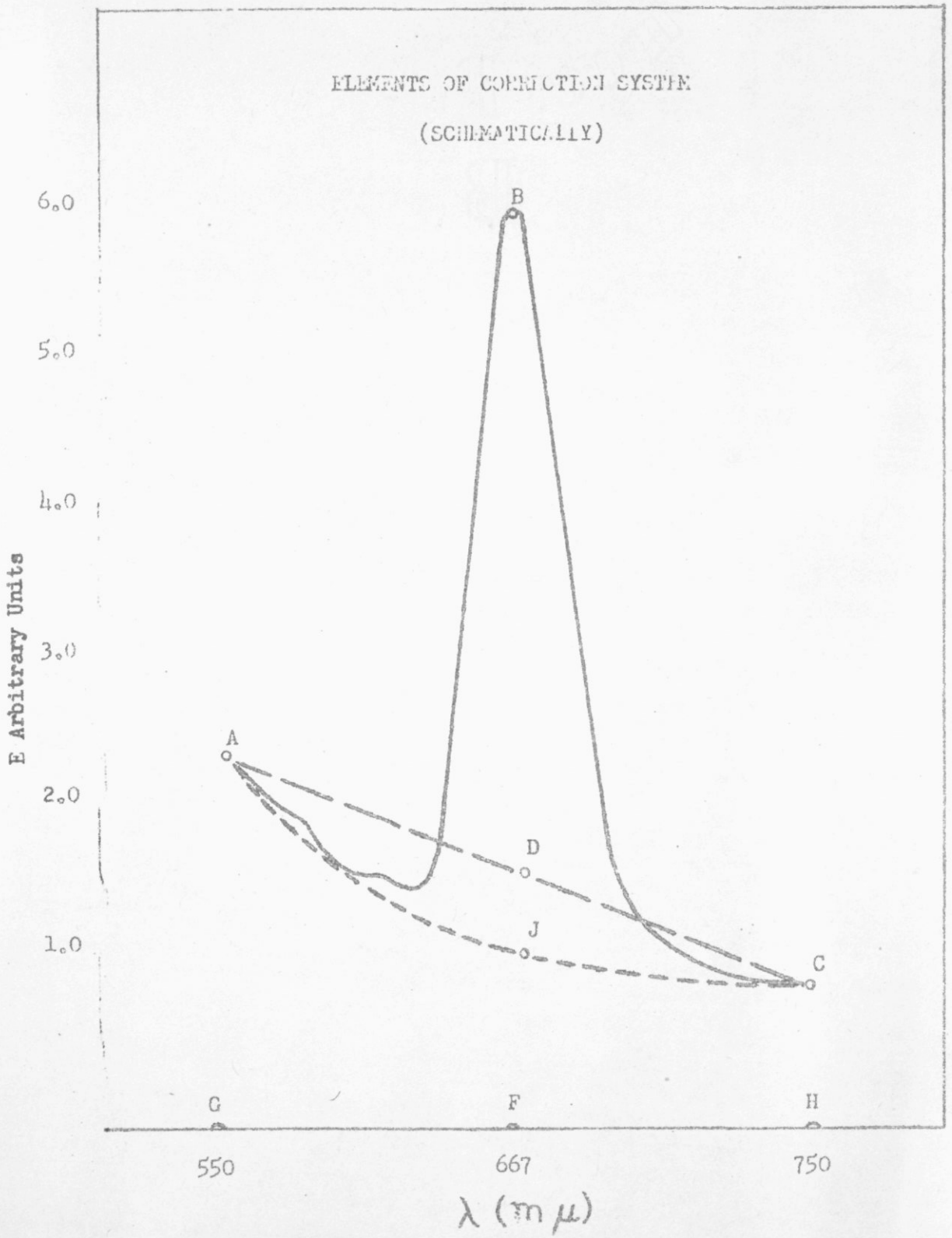
Our correction has an empirical nature which consists of a method used to locate point J. Absorption curves for extracts which showed no peak at 667 $m\mu$ and so presumably contained negligible amounts of chlorophyll, were examined to determine the ratio FJ : FD. From 25 samples so examined it was found that:

$$FJ = 0.90\ FD \qquad (I)$$

Graphically FD can be obtained as:

$$FD = HC + 83/200\ (GA - HC)$$

Figure 3


ELEMENTS OF CORRECTION SYSTEM
(SCHEMATICALLY)
6.0
5.0
4.0
3.0
2.0
1.0
E Arbitrary Units
A
B
C
D
J
G
F
H
550
667
750
λ (mμ)

In terms of extinction measurements at 550 and 750 $m\mu$:

$$FD = E_{750} + 0.415\,(E_{550} - E_{750})$$

An approximation of the extinction of 667 $m\mu$, due to chlorophyll a alone is

$$JB = FB - FJ \qquad \text{(II)}$$

or

$$JB = E_{667} - 0.90\,\left[\,E_{750} + 0.415\,(E_{550} - E_{750})\,\right]$$

The extinction at 667 $m\mu$ for chlorophyll a alone is given by (III), after correcting (II) for the absorption at 550 $m\mu$ by chlorophyll a. This correction amounts to 6% of the term JB. Thus, the final equation is in the form of a series of approximations. Retaining only the first two terms, since they are the only ones of numerical significance, the extinction at 667 $m\mu$ for chlorophyll a alone becomes:

$$E_{667\ chl.} = E_{667} - 0.9\Big(E_{750} + 0.415\Big[E_{550} - 0.06\Big\{E_{667} - 0.9\Big[E_{750} + 0.415\,(E_{550} - E_{750})\Big]\Big\} - E_{750}\Big]\Big) \qquad \text{(III)}$$

Collecting terms and simplifying gives:

$$E_{667\ chl.} = (1.002)\,E_{667} - (0.486)\,E_{750} - (0.382)\,E_{550} \qquad \text{(IV)}$$

Applying the result of (IV) to the Beer-Lambert Law, using MacKinney's (4) value for the specific extinction of chlorophyll a in 80% acetone at 677 mμ of 80.5 liter $gm^{-1}cm^{-1}$, and making the extinction measurements in a 10 cm cell on the material from a one-liter sample, the concentration of chlorophyll a in the sample is given by:

$$\text{Concentration} = \frac{\text{Extinction}}{\text{Specific extinction} \times \text{Light path}}$$

$$C = \frac{(1.002)\, E_{667} - (0.486)\, E_{750} - (0.382)\, E_{550} \times 10^6}{(80.5)\,(10)\,(20)} \; \mu g/\text{liter}$$

The 20 in the denominator results from concentrating the chlorophyll from a liter raw sample to a fifty ml sample.

$$C = (62.6)\, E_{667} - (30.4)\, E_{750} - (23.9)\, E_{550}. \; \mu g/\text{liter} \qquad (V)$$

Equation (V) can be used to calculate the concentration of chlorophyll a from optical density measurements at 550, 667, and 750 mμ .

DISCUSSION

The advantages and limitations of spectrophotometric methods as compared to visual colorimetric procedures are well known (7). The high sensitivity of modern spectrophotometers should provide the basis for an analysis scheme which is rapid and accurate and permits the use of relatively small samples. All of these features are desirable if large numbers of samples are to be analyzed routinely. It is undoubtedly possible to obtain a pigment extract free from suspended solids by repartition of the extract into other solvents. However, such a procedure results in the loss of pigment and makes the procedure more cumbersome. Richards' (8) procedure for pigment analysis requires that the collected solids be dried in a vacuum desiccator prior to extraction. His absorption curves appear to be free from the interference of suspended materials.

Two assumptions are implied in the proposed correction scheme. The validity of equation I depends upon the particle size distribution of the suspended material being constant from sample to sample. Of the 25 samples examined to obtain the constant, which we report as 0.9, the values ranged from 0.84 to 0.95 with a standard deviation of .20. This amounts to an error of about 0.1 micrograms chlorophyll per liter in the correction term which, on the average, is about 2.0 micrograms per liter. The concentration of chlorophyll in our samples has been as high as 40 micrograms per liter but usually is less than 10.

We have assumed that chlorophyll c is either absent or in low concentration in our samples. Chlorophyll c absorbs little if any at 667 and 750 $m\mu$ but considerable at 550 $m\mu$. Thus, if appreciable chlorophyll c is present, the proposed correction will be in error. The extent of this small negative error is impossible to establish at present as the specific extinction coefficient for this compound has not been reported.

EXPERIMENTAL METHODS

Method of Concentrating Phytoplankton and Extracting Chlorophyll*

A. Vacuum filter a one liter sample of water in a modified Seitz filter through Whatman #5 paper.

B. Immerse the filter paper and collected solids in a vial containing approximately 15 ml of neutral 80% aqueous acetone. Stopper vial with cork which has been soaked in at least two changes of 80% acetone. (If the samples are to be held for more than a half day before further processing, store under refrigeration and in the dark.)

C. Filter contents of the vials through a 1.5 cm circle of Whatman #5 paper. Collect filtrate in a 50 ml volumetric flask and dilute the 50 ml mark with 80% acetone. Mix the solution thoroughly.

D. Read percent transmission (or extinction) of the solution from (C) in a 10 cm cell, at 550, 667 and 750 mμ .

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* A modification of the method used by Dr. M. Sargent, Scripps Institution of Oceanography, La Jolla, California.

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