A COMPARISON OF CARBON-14 INCUBATION TECHNIQUES

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Abstract. A laboratory study with *Selenastrum capricornutum* has shown that the interpretation of C-14 uptake data can be influenced by the method of data presentation. The expression of C-14 data as rate of uptake rather than total uptake is more representative of the algal stimulation potential of the medium being investigated. Also, data expressed as a rate of uptake based on a four-hour incubation period are more sensitive to changes in algal activity than those based on rate of uptake for long-term incubation periods. Several examples are discussed where data were initially interpreted as showing stimulation, although conversion of the data to rate of uptake shows a lack of stimulation.

1. Introduction

The western arm of Lake Superior is presently receiving mining waste solids (taconite tailings) discharge of 67,000 t day^{-1} from a point source on the north shore. The algal stimulation potential of this discharge has been of concern. A number of investigators have used a C-14 (HCO_3^-) incorporation procedure to assess the response of algae to additions of the mining waste. Although variations of the same procedure were used in all cases, the stated conclusions were contradictory.

One technique consisted of comparing the net C-14 uptake during a constant exposure period. The method involved the addition of a known quantity of C-14 (HCO_3^-) to a constant volume of lake water or algal culture. Replicate samples were incubated for a 4 h period under constant light and duplicate samples were incubated for a similar period in the dark. Following incubation, samples were filtered and counted and net C fixation was calculated as average light bottle counts per minute minus average dark bottle counts per minute. Using this procedure, Plumb (1973) and Plumb and Lee (1973a,b,c) concluded that the mining waste discharge would not stimulate algal growth in Lake Superior.

The second technique was simpler and consisted of determining the total C-14 uptake by the test population as the test progressed. A known amount of C-14 (HCO_3^-) was added to a constant volume experimental culture at the beginning of the bioassay. On selected sample days, an aliquot of the culture was withdrawn and filtered. The activity of the sample was determined by counting C-14 incorporated into algal cells and reported as counts per minute. A plot of total C-14 uptake as a function of time after addition of C-14 to the original cultures yielded a positive sloped curve that was interpreted as

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stimulation by Shapiro (1970; 1973), Shapiro and Glass (1973), Goldman (1973) and McGee (1970).

In order to resolve the contradictory conclusions concerning the potential stimulatory effect of mining waste solids on algal growth in Lake Superior, between the two study approaches, a comparison was made using the same data presentation techniques. This paper presents the results of the studies on the potential stimulatory effects of mining waste solids on algae and a comparison of the contradictory results using the same experimental and data presentation techniques.

2. Procedures

The algal growth medium selected for use in this investigation was Provisional Algal Assay Procedure (PAAP) medium (U.S. Department of Interior, 1969). A series of stock solutions were prepared at 200 times the recommended concentration. The addition of 5 ml of each solution to 1 l of distilled water resulted in a medium containing all recommended concentrations of PAAP nutrients except phosphate. This phosphate deficient (PAAP-P) medium would not be expected to stimulate an algal population. This approach was used since phosphate has been identified as the element present in Lake Superior water which limits algal growth. A second aliquot of the phosphate-deficient medium received a separate addition from a stock phosphate solution to produce a final phosphate concentration of 80 μg PO_4-P l^{-1}. This complete medium would be expected to stimulate an algal population.

The test organism chosen for use in the investigation was *Selenastrum capricornutum.* The organism was cultured in the laboratory using PAAP medium discussed above. A phosphate-poor medium was used in culturing the stock organisms to insure that the algal cells would respond to available P during the testing phase of the study.

The purpose of the study was to compare observed C-14 (CHO_3^-) uptake in algal medium cultures of known stimulation potential using different incubation techniques. Therefore, one set of cultures was prepared in PAAP medium that would be expected to stimulate algal activity and a second set of cultures was prepared in PAAP-P medium that would not be expected to stimulate algal activity (Table I). In addition, all experimental cultures were prepared from the same stock culture and diluted to 1 l. Also, the same initial relative amount of C-14 was used in all cultures. This resulted in the addition of 80 μg $C^{14}C{-}CO_3^{-2}$ l^{-1} to the cultures monitored for total uptake as a function of incubation time (culture sets A, B, D, and E) and 2 μCi $^{14}CO_3^{2-}$ to 25 ml aliquots of the cultures monitored for C-14 uptake in a four-hour period (culture sets C and F).

Carbon-14 was purchased as bicarbonate in 1 or 5 mCi ampules. This material was diluted with a dilute sodium hydroxide solution to prepare a 2 μCi ml^{-1} working solution (pH 10.3). Carbon-14 was added to algal cultures at a rate of 2 μCi/25 ml (1 ml working C-14 solution/25 ml algal culture).

TABLE I

Selenastrum cultures prepared for comparison of incubation techniques

Culture	Additions	Replicates
A	PAAP medium + 80 μCi ^{14}C–CO_3^{-2} + 10 ml *Senastrum*	3
A[d]	PAAP medium + 80 μCi ^{14}C–CO_3^{-2} + 10 ml *Selenastrum*	1
B	PAAP media + 80 μCi ^{14}C–CO_3^{-2} + 10 ml *Selenastrum*	3
C	PAAP media + 10 ml *Selenastrum*	3
D	PAAP-P media + 80 μCi ^{14}C–CO_3^{-2} + 10 ml *Selenastrum*	3
D[d]	PAAP-P media + 80 μCi ^{14}C–CO_3^{-2} + 10 ml *Selenastrum*	1
E	PAAP-P media + 80 μCi ^{14}C–CO_3^{-2} + 10 ml *Selenastrum*	3
F	PAAP-P media + 10 ml *Selenastrum*	3

Samples designated [d] were prepared at the start of the experiment and used as dark bottle controls for the indicated samples.

All cultures were prepared from the same stock *Selenastrum* culture on the same day.

The amount of C-14 incorporation was determined by scintillation counting using a Packard 3330 Tri-carb Scintillation Spectrometer. A PPO-Dimethyl POPOP-Dioxane based flour was used to dissolve the sample filters which were counted for 20 min or 900,000 counts. Details of the procedure are given elsewhere (Plumb, 1973).

2. Results

One variation of the C-14 incorporation procedure is to add the radioactive C directly to the initial cultures at time zero (Shapiro, 1970, 1973; Shapiro and Glass, 1973; Goldman, 1973; McGee, 1970). Then, on selected sample days, an aliquot of the culture was removed and filtered. Samples were then counted to determine the extent of C-14 incorporation. All cultures processed using this technique (sets A and D) demonstrated an increased incorporation of C-14 into the *Selenastrum* cells with the passage of time (Figure 1). However, the test organisms were not able to distinguish between PAAP medium and PAAP-P medium used in the experiment. That is, the observed response was the same although one set of cultures (A) should have been stimulated and the other set (D) should not have been stimulated.

The range of replicate values on day 13 in the set D PAAP-P medium was 26,300 to 41,300 cpm. Set A PAAP medium values on the same day ranged from 24,700 to 30,000 cpm. It is interesting to note that the actual rate of C-14 fixation decreased in both sets. Set A PAAP cultures decreased from 3,245 cpm day^{-1} on day 3 to 2,160 cpm day^{-1} on day 13 and the set D PAAP-P cultures decreased from 2,930 cpm day^{-1} on day 3 to 2,610 cpm day^{-1} on day 13.

A second variation of the procedure also involved the addition of C-14 to the initial cultures. However, in this case, the culture aliquots were not filtered immediately on sampling days, but were placed in light bottled and dark bottles for an additional four-

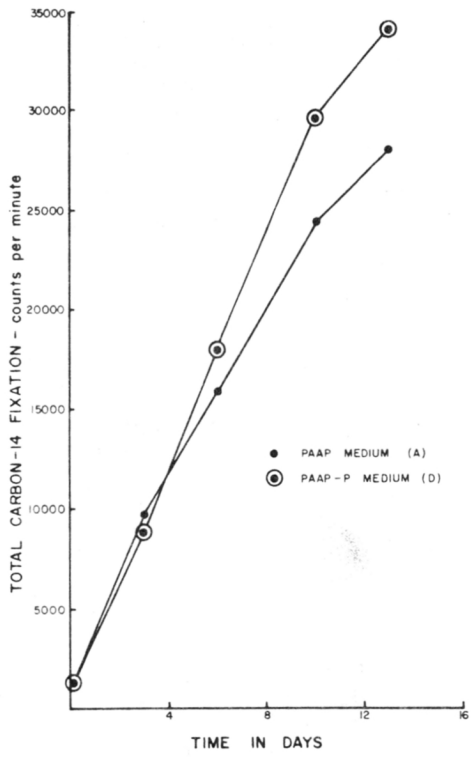

Fig. 1 Total C-14 uptake in *Selenastrum* cultures initially spiked with $^{14}C-CO_3^{-2}$. Aliquots filtered immediately.

hour incubation period (sets B and E). The purpose was to attempt to correct for previous incorporation and respiration. The aliquots were then filtered and counted. It appears that the *Selenastrum* population could distinguish between PAAP and PAAP-P medium, since the light bottle counts in set B were greater than the light bottle counts in set E, as would be expected (Figure 2). The B PAAP culture range was 30,400 to 38,200 cpm on day 12 compared to a range of 20,400 to 29,400 cpm in set E PAAP-P cultures on day 13.

When the light bottle results for culture sets B and E were corrected for dark bottle fixation, the relationship suggested in Figure 2 deteriorated. As shown in Figure 3, the light bottle minus dark bottle results suggest that when C-14 is added to the initial cultures, *Selenastrum* cannot distinguish between PAAP medium and PAAP-P medium.

The final method used in this study was that of not adding C-14 to the initial cultures. Aliquots of these cultures were withdrawn on sample days and placed in light bottles and dark bottles. At this point, C-14 of the same activity as that initially added to cultures A, B, D, and E, (2 μCi/25 ml) was added to the aliquots. The radioactive samples were incubated for an additional 4 h (sets C and F). The samples were filtered and the extent of C-14 incorporation determined by scintillation counting. The data

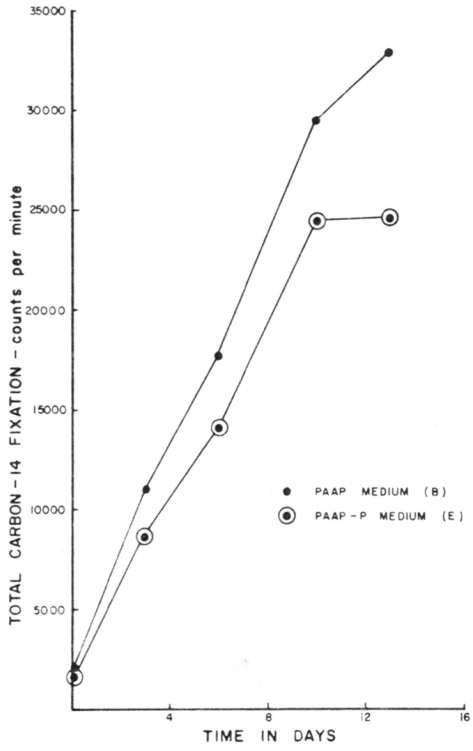

Fig. 2 Total C-14 uptake in *Selenastrum* cultures initially spiked with $^{14}C-CO_3^{-2}$ (gross light bottle fixation). Aliquots filtered after 4 h incubation period.

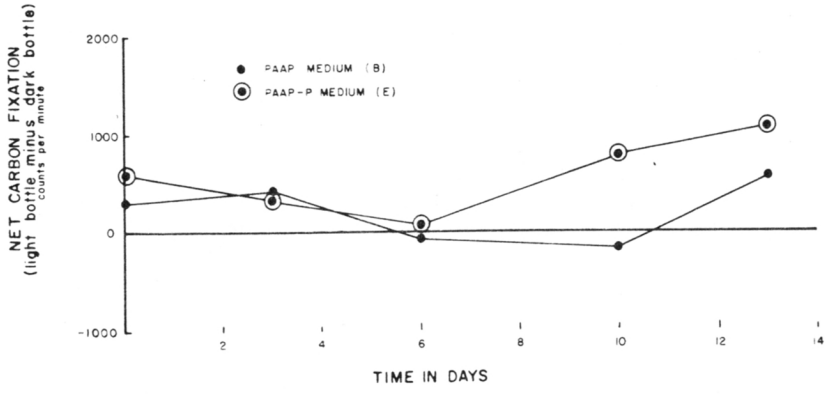

Fig. 3. Net C fixation for *Selenastrum* cultures initially spiked with $^{14}C-CO_3^{-2}$. Aliquots incubated an additional 4 h in light and dark bottles.

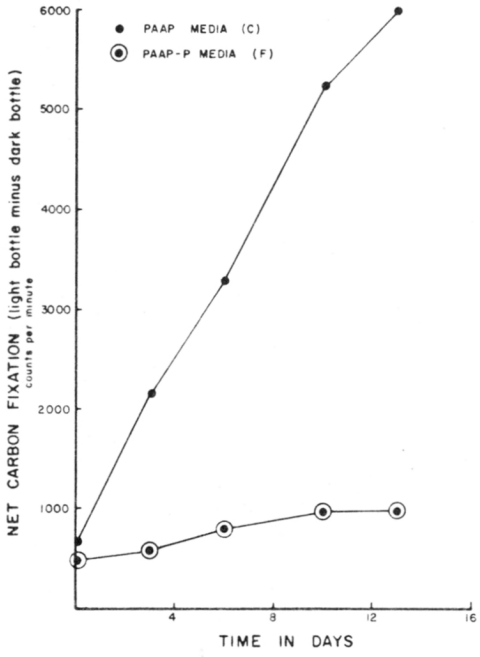

Fig. 4. Net C-14 fixation in *Selenastrum* cultures based on 4 h contact incubation time.

from this procedure are presented in Figure 4 and show a marked difference between PAAP and PAAP-P curves. The set C PAAP medium was clearly stimulating the *Selanastrum* population whereas the set F PAAP-P medium was not.

3. Discussion

The response of laboratory algal cultures to different growth medium was determined using several variations of the C-14 incorporation technique. One medium used was a complete PAAP supplement which would act as a stimulant and the other was PAAP-P medium which would be non-stimulatory. Results suggest that only the light bottle minus dark bottle method using a short incubation period could distinguish between the stimulation potential of the two mediums. This method can assure algal activity in a culture at any one time, and a plot of these values versus time is a measure of the change in culture activity. Such a plot would indicate whether an added substance was stimulating the test population.

Addition of C-14 to initial cultures, followed by an additional 4 h incubation period on sample days, was also attempted. This method could apparently distinguish between the phosphate levels based on gross C-14 fixation data (Figure 2). However, the net C-14 fixation data (light bottle minus dark bottle counts) could not distinguish between the

phosphate levels used. It is postulated that the long-term fixation of C-14 masks the amount of net C fixation that occurs in the 4 h period. Therefore, the addition of C-14 to the initial algal cultures has the effect of reducing the sensitivity of the radiocarbon fixation procedure since the counting error associated with the greater uptake of C-14 over the longer contact period limits the smallest change that can be measured. In addition, this approach cannot be used to calculate net C fixation since a culture kept in the dark since time zero is not a suitable control for a culture kept in the light for the same length of time.

Another aspect of the data should be stressed. Figure 4 shows there was no change in the 4 h C fixation value for algae grown in phosphate-deficient medium. However, the same medium produced a positive sloped curve when C-14 was added initially to the cultures (Figures 1 and 2). The plot of total uptake versus time, therefore, does not represent the stimulation potential of the experimental medium. The data, corrected for time of contact and expressed as rate of uptake, suggest that expected lack of stimulation. The rate of uptake in the PAAP-P medium using immediate filtration (culture D) decreased from 2,900 cpm day^{-1} on day 3 to 2,600 cpm day^{-1} on day 13. The rate of uptake in PAAP-P medium using an additional 4 h incubation period (culture E) decreased from 2,900 cpm day^{-1} on day 3 to 1,900 cpm day^{-1} on day 13 (Figure 5).

The correction of C-14 data for time becomes mandatory because the equation for calculating primary productivity from C uptake data is:

$$PP = \frac{1.06\,(\text{light bottle} - \text{dark bottle})(\text{C-12})}{(\text{C-14 added to sample})(\text{time of contact})}$$

where 1.06 is a factor to correct for selective isotopic uptake; light bottle is the average light bottle uptake in counts per minute; dark bottle is the average dark bottle uptake in

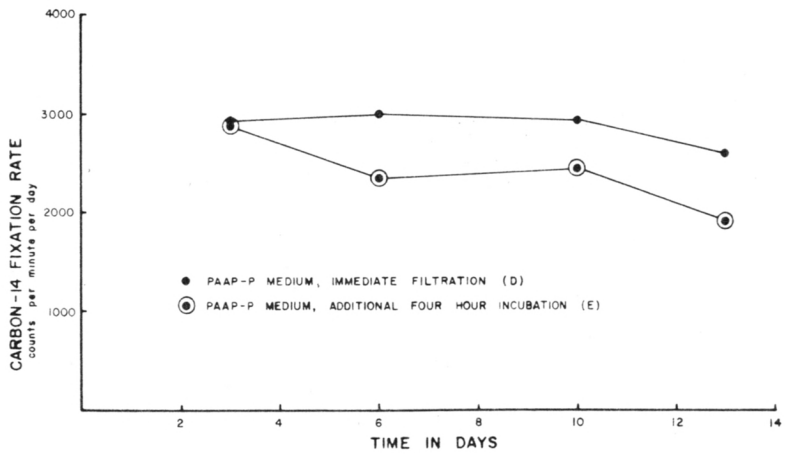

Fig. 5. Rate of C-14 fixation in *Selenastrum* cultures grown in PAAP-P medium and spiked initially with $^{14}C\text{-}CO_3^{-2}$.

counts per minute; C-12 is the amount of C-12 in the sample in mg l^{-1}; C-14 is the activity of the radioactive carbon added to the sample in decompositions per minute; time of contact is the length of time between addition of C-14 to the sample and filtration of the sample.

Thus, C-14 incorporation is proportional to primary productivity (and indirectly to the algal population) only when all other factors in the equation are held constant. When results are presented as total uptake versus time, C-14 uptake is not a measure of algal activity since the time factor in the equation also varies. It is only when the time of contact is held constant or the data are expressed as rate of uptake that C-14 uptake is proportional to algal activity.

Based on the results obtained in this investigation and the theoretical considerations presented above, it is determined that the data collected by Shapiro (1973) should be expressed as rate of C-14 uptake. In order to evaluate the reliability of the approach used by Shapiro (presenting results on the basis of total accumulated C-14 uptake), his reported values of total uptake were divided by the days of C-14 contact and expressed as counts per minute per day.

A comparison of the original Shapiro (1973) data with the time corrected Shapiro data (Table II) reveals that even though the total uptake increases with time, the actual rate of C-14 uptake decreases in cultures containing only mining wastes. For example,

TABLE II

Comparison of Shapiro (1973) C-14 data expressed as total uptake and rate of uptake

	Original Shapiro data (total uptake)* Days				
Culture additive	2	5	10	16	21
0% T.T.	2887	3771	5910	7968	8877
0.0001% T.T.	1852	3961	6431	8253	9247
0.001% T.T.	2065	4009	6871	9171	10373
0.01% T.T.	1826	4034	7081	10936	13834
10 $\mu g\ l^{-1}$ P	2388	6048	15510	29578	39358
20 $\mu g\ l^{-1}$ P	2192	5023	10431	22604	43355
8 $\mu g\ l^{-1}$ Mn	1938	3994	6191	8549	10457
Culture additive	Time corrected Shapiro Data (rate of uptake)** Days				
	2	5	10	16	21
0% T.T.	1443	754	591	498	422
0.0001% T.T.	926	792	643	515	440
0.001% T.T.	1032	801	687	573	493
0.01% T.T.	913	806	708	683	658
10 $\mu g\ l^{-1}$ P	1194	1209	1551	1848	1874
20 $\mu g\ l^{-1}$ P	1096	1004	1043	1412	2064
8 $\mu g\ l^{-1}$ Mn	969	798	619	534	497

*Total uptake expressed in counts per minute (cpm).

**Rate of uptake expressed in counts per minute per day (cpm day^{-1}).

Percentage refers to dilution of industrial discharge.

T.T. = taconite tailings.

total uptake in the 0.01% mining innoculated cultures increased from 1,836 to 13,834 cpm in 21 days, but the rate of uptake decreased from 913 to 658 cpm day^{-1}. Since a stimulatory effect should increase the population and increase the rate of uptake as shown in Figure 4, it must be concluded that the Shapiro (1973) data show no stimulation of algae by mining wastes. Cultures enriched with 10 and 20 $\mu g\ l^{-1}$ phosphate increased the total and rate of C-14 uptake which suggests that phosphate is limiting the algal population in Lake Superior. This agrees with results obtained by Plumb and Lee (1973a,b).

The data collected by Plumb (1973) and Plumb and Lee (1973a,b,c) were initially expressed as rate of uptake and suggested no stimulation. When data collected by Shapiro (1970, 1973), Shapiro and Glass (1973), Goldman (1973), and McGee (1970) were expressed as rate of uptake rather than total uptake, these data also suggest no stimulation. The opposite conclusion was reached by the original authors. Thus, the apparent contradiction in the conclusions of the taconite tailings–mining wastes bioassays is due to the different methods of data presentation and resultant interpretation by Shapiro (1970, 1973), Shapiro and Glass (1973), Goldman (1973), and McGee (1970).

Additional support for the conclusion that taconite tailings do not stimulate algal growth in Lake Superior is available from a Lake Superior algal survey (Meyer and Wheeler, 1973), and a Lake Superior primary productivity survey (Plumb and Lee, 1973c). These studies demonstrated that no increase in algal numbers or activity was occurring at or near where the industrial discharge was entering the lake. The only increase in the algal population in the Minnesota waters of Lake Superior occurs near Duluth, Minnesota (Meyer and Wheeler, 1973; Plumb and Lee, 1973c). This can be explained by the fact that an estimated 90% of the phosphate flux to the western arm of Lake Superior is from the Duluth, Minnesota–Superior, Wisconsin area (Plumb and Lee, 1973d, 1974).

4. Conclusions

The method of adding C-14 to algal samples and incubating for a short time interval of a few hours is more time-consuming than the method of adding the radioactive tracer at the beginning of an experiment and filtering sub-samples at desired times. However, this investigation has shown that the former method provides data which can reflect changes in the algal population during bioassays. In addition, this method is more sensitive and permits the detection of smaller changes. Finally, in order to calculate net fixation, the short-term incubation procedures can be corrected for changes in C-14 activity due to precipitation, sorption and algal respiration. The simplified method cannot be corrected since an algal culture kept in the dark for several days is not an adequate control for algae kept in the light for the same time period.

The apparent contradictions that led to the initiation of this study were due to different methods of data presentation and resultant interpretation. Presentation of total C-14 uptake versus time is not a direct measure of changes in algal populations. The present-

ation of data as rate of uptake, on the other hand, allows a rapid assessment of whether or not stimulation is occurring in the test population. The same information can be obtained from total uptake data by correcting for time of contact between C-14 and the test algae (calculating the slope of the total uptake curve). It should be pointed out that expressing total C-14 uptake in counts per minute is not rate of uptake. Counts per minute is the rate of C-14 decay (activity of the sample) and as such is only a measure of the amount of C-14 in the sample being counted. The rate of uptake is a function of C-14 in the sample and the time necessary for accumulation to occur. Rate of C-14 uptake should be expressed as counts per unit time.

It is suggested that investigators using the C-14 procedure in the future provide information on amount of C-14 added to samples, amount of C-14 eventually fixed in the samples, and time of contact between algal cells and radioactive carbon. Where possible, the results should be expressed as rate of uptake.

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