

RESEARCH PROJECT TECHNICAL COMPLETION REPORT
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Application of Radioisotope Techniques to a Critical
Water Resources Problem Area - Namely
Nutritional Pollution

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by

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NOTE

A doctoral thesis entitled "The Criticality of the Nitrogen and Phosphorus Ratio to Aquatic Microorganisms, Namely Planktonic Algae" was developed by Robert A. Gearheart under this project. Though the essential elements of the research findings of Project A-011-OKLA are contained within this technical completion report, supplemental and related data are contained in the referenced document.

ABSTRACT

Eutrophication is characterized by excessive algal and bacterial growth. The extent of this growth is, within limits, dependent upon the concentrations of available nitrogen and phosphorus, their relative ratio to one another, and upon the rate at which these nutrients are assimilated by the algae-bacteria symbiosis.

This investigation defines optimum nitrogen to phosphorus ratios with respect to varying phosphorus concentrations, proposes a mathematical formulation which describes the relationship between the algae-bacteria symbiosis and the nutrient concentrations, and confirms the establishment of an equilibrated phosphorus cycle by the determination of exchange rates and turnover times of phosphorus compounds in the symbiotic cultures.

Algae-bacteria systems were cultured in 150 milliliter and 2 liter Erlenmeyer flasks with various concentrations of nitrogen and phosphorus. The ratio of nitrogen to phosphorus levels. The optimum ratio of nitrogen to phosphorus varied from 2:1 to 16:1 over a phosphorus level of 0.5 mg/l to 8.0 mg/l.

A multivariable regression analysis of the following linearized form gave a R^2 of 0.9216.

$$V(2)/\text{ALOG}(Z) = B_0 + B_1V(1) + B_2V(2)$$

Z = algae - bacteria biomass - mg/100 ml.
V(1) = nitrogen concentration - mg/ml.
V(2) = phosphorus concentration - mg/ml.
 B_0 , B_1 , and B_2 represent constants.

By using labeled phosphorus and appropriate radiochromatographic techniques it was found that six organic compounds, ATP, Glucose-1- PO_4 , Glucose-6- PO_4 , Fructose-6- PO_4 , Fructose-1, 6-Di- PO_4 and Phosphoglyceric Acid are released into the surrounding medium by the algae-bacteria symbiosis. In addition, by substituting experimentally determined data into the corresponding equilibrium and rate of change equations, exchange rates, (a, b_1 , b_2 , c), and turnover times, (the reciprocals of the exchange rates), were determined for cultures containing 0.2 mg/100ml phosphorus.

Average exchange rates were: .31 days⁻¹ for a, .26 days⁻¹ for b_1 , .04 days⁻¹ for b_2 , 1.7 days⁻¹ for c. The overall equilibrium equation was then written as follows:

Dissolved Inorganic Phosphorus (0.02 mg.)

b_1 $\left\| \begin{array}{l} \uparrow \\ \downarrow \end{array} \right.$ a .0062 mg P/Day in each direction

Particulate Phosphorus (0.156 mg.)

c $\left\| \begin{array}{l} \uparrow \\ \downarrow \end{array} \right.$ b_2 .0405 mg P/Day in each direction

Dissolved Organic Phosphorus (0.024 mg.)

Average turnover times were 3.2 days for DIP, 3.3 days for PP and 0.6 days for DOP.

APPLICATION OF RADIOISOTOPE TECHNIQUES TO A CRITICAL
WATER RESOURCES PROBLEM AREA - NAMELY
NUTRITIONAL POLLUTION

By

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Today a major water pollution problem is excessive enrichment or eutrophication of receiving waters by nutrient wastes. Several contemporary factors have combined to create this serious problem; the exponential growth of population, the trend toward urbanization, the increasing use of garbage grinders, the universal addition of synthetic detergents to domestic sewage, the increased use of agricultural inorganic fertilizers, and the conditioning of industrial cooling water. Thus, large quantities of mineral-enriched wastewater are discharged into relatively small geographic areas.

A paradox of modern wastewater treatment complicates the problem. As measured by the classical yardsticks of suspended solids and BOD removal, the degree of treatment has increased steadily. This has resulted in more efficient mineralization of the organic constituents of sewage. Sunlight readily penetrates the clear waters, and the minerals which have been released promote rapid and extensive growth of photosynthetic organisms,

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particularly algae. Thus, undesirable changes in natural bodies of water, which would have required thousands of years through normal eutrophication processes, have been brought about in five to ten years by wastewater discharge.

According to the work of numerous investigators over the past twenty years the nitrogen and phosphorus concentrations of the waste liquids have been found to be the most important factors causing this excessive enrichment -- this extraordinary growth of algae -- and the consequent problems arising from the discharge of sewage effluents into waterways. These problems, however are very complex and difficult to evaluate because of the lack of basic information concerning the role and interdependence of nitrogen and phosphorus in the growth of algae. Thus, a portion of the first phase of the study was concerned with the collection and evaluation of badly needed fundamental data.

The consequence of nutritional pollution is accelerated eutrophication by promotion of algal blooms. As an engineering problem, certain conditions must be defined and rational assumptions made. Also, numerical values must be associated with conditions, assumptions, and the ensuing relationship which develops. The purpose of this study was to evaluate those conditions shown by the literature to be most responsive to the promotion of algae growth.

When regulations are enacted concerning the effluent or stream standard of nitrogen and phosphorus, some reliability must be built into the standard to insure success in reduction of algae in the stream or reservoir. To develop reliability in this standard, the effect of various

concentrations of nitrogen and phosphorus must be known. This involves testing for minimum growth, maximum growth, and toxic values of the nutrients.

This project approached this particular problem from the standpoint of minimal concentrations of nitrogen and phosphorus necessary for the growth of algae. A radioactive phosphorus method was used as one of the parameters for biological mass produced and phosphorus uptake rate in an alga culturing unit. In this manner, some standard or standards, as the case may be, can be obtained to govern sewage treatment efficiency as far as nitrogen and phosphorus are concerned. This was achieved by holding one of the nutrients constant while varying the other and vice versa. All other necessary inorganic and organic nutrients, including vitamin B₁₂ and other growth metabolites, were supplied in excess to allow maximum algae reaction to the nitrogen and phosphorus.

Batch studies were carried out in 150 milliliter and 2 liter Erlenmeyer flasks. In the 150 ml. flask, 100 ml. of media were used, and 2 liter flasks contained 1 liter of media. The culture media was composed of well water from the 1500 foot level beneath Norman, Oklahoma. The well water was used because of its consistent water quality similar to that of surface water in the central Oklahoma area. The well water had the following significant water quality parameters: chlorides - 12.5 mg/l; sulfates - 33.0 mg/l; iron - 0.01 mg/l; Ca hardness - 19.0 mg/l as CaCO₃; carbonate alkalinity - 66 mg/l; bicarbonate alkalinity - 245 mg/l; orthophosphate - 0.04 mg/l as P; pH - 8.4; and nitrate - 0.00 mg/l. Various trace minerals and vitamins were supplied to the media by use of a commercial vitamin in dilute proportions.

Phosphorus was added in the form of orthophosphates associated with a sodium salt (Na_2HPO_4) which also served as the carrier for the radioactive phosphorus. The nitrogen was supplied to the algae in the form of sodium nitrate, (NaNO_3), as this represented the most highly oxidized form of nitrogen in natural waters. This would more closely parallel the recovery from organic pollution rather than a stage of active decomposition. Carbon was used as a parameter in several experiments and was added in the form of glucose ($\text{C}_6\text{H}_{12}\text{O}_6$).

The ^{32}P was counted, using a deep-well integral scintillation counter (photomultiplier tube, plastic phosphorus for Beta counting) with an efficiency of 37% and a background of 80 ± 4 cpm. Since geometry corrections present a problem in counting liquid samples, only 2 ml. of the batch culture was filtered and the radioactivity of the residue determined. However, after drying and counting the residue of many samples, it was found as accurate to determine the amount of radioactivity in the biological mass by taking the difference between the ^{32}P in the batch culture and the ^{32}P in the filtrate after membrane filtration (0.45u pore diameter Millipore filters were used). Therefore, radioactivity was determined on aliquots of 0.1 ml before and after filtration and the percent difference taken as the ^{32}P in the phytoplankton. Sampling and counting were done in duplicate, and all samples were counted to a reliable error.

The relationship between nitrogen, phosphorus and algal growth is expressed by a multiple regression analysis procedure, the Dolittle procedure. The Dolittle procedure determined the partial correlation coefficients and multiple correlation coefficients. The two independent variables

are nitrogen, X_1 , and phosphorus concentration, X_2 ; the dependent variable is algal mass, Y .

In general, the multiple regression analysis was of the following form:

$$\hat{Y} = B_0 + B_1X_1 + B_2X_2 \quad \text{or} \quad \hat{Y} = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3 X_1/X_2$$

Where \hat{Y} is the estimator of the true Y and B_0 represents the intercept, and B_1 and B_2 represent the partial coefficients of correlation, and X 's represent the independent variables. In this case, transformations were made to account for any interdependency between nitrogen and phosphorus.

To complete the first phase of the study, the effect of the nutrients nitrogen and phosphorus on algae was determined for the conditions selected in this study. Of importance is the symbiotic consideration with the heterotrophic bacteria in an ecosystem, such as a sewage treatment plant's effluent receiving stream. The carbon enriched nitrogen and phosphorus studies reveal the following:

1. A bacterial bloom occurs within the second or third day in contrast to the algae growth which usually requires six to eight days.
2. ³²P uptake studies show immediate uptake of the soluble phosphate, 80-90% in the first day.
3. The maximum yield of algae is the same for carbon enriched and non-enriched conditions.
4. Based on the above, phosphorus metabolic by-products are easily metabolized by the algae.

The effect of the nitrogen to phosphorus ratio, N/P , appears to manifest itself in different phosphorus levels. No straightforward relationship was developed by this study concerning the N/P ratio. Each series of N/P ratios tested produced an optimum concentration of nitrogen and phosphorus (Table 1).

Variation in the optimum ratio for an algae-bacteria symbiotic system based on this study could be due to the following:

1. The phosphorus is reused in the case of autotrophic organisms as the phosphorus is cycled through the system, promoting secondary populations of algae.
2. The physiological condition of the seed population of algae and bacteria, such as the age of the inoculate.
3. The luxuriant uptake of phosphorus by the algae-bacteria which would produce a lower optimum ratio.
4. Competition between the heterotrophic and autotrophic segments of the population.

The minimum concentration necessary for algae growth in this study was found to be above 0.01 mg/1 of $\text{PO}_4\text{-P}$ or lower. The 0.01 mg/1 level is the smallest concentration used in the experimental design. At a $\text{PO}_4\text{-P}$ concentration of 0.10 mg/1, the algal bloom condition threshold is reached. Regardless of the nitrogen concentration, this narrow range of sensitivity to the phosphorus concentration demonstrates the problem in attempting to control nutritional pollution. The ability of the phosphorus to control algae growth at this lower concentration could be due to several things:

1. Recycling of the nutrients as metabolic by-products sustaining growth.
2. N_2 fixation by bacteria in low nitrogen concentration conditions and high phosphorus conditions.
3. Variable efficiency by the algae in using phosphorus in the energy transformation reactions--in the restricted phosphorus in storage, more phosphorus in the high energy phosphate bonds.

A multivariable regression analysis, with the algae growth the independent variable and the nitrogen and phosphorus the dependent variable was attempted. Several linearized models based on possible functional relationships were fit with the experimental data. The correlation coefficient, R^2 ,

the number of interactions and power of polynomial were used to evaluate the models as to their applicability and to their goodness of fit.

The following linearized form gave a correlation coefficient, R^2 , of 0.9216 with no interaction inputs.

$$V(2) / \text{ALOG}(Z) = B_0 + B_1 V(1) + B_2 V(2)$$

Z = algae-bacteria biomass - mg/100 ml

V(1) = nitrogen concentration, mg/ml

V(2) = phosphorus concentration, mg/ml

B_0 = 0.0413

B_1 = 0.0060

B_2 = 0.2204

The F statistic for $F_{.01(2,44)} = 99.47$ while the computed value is 497.0; therefore the variance test is significant at this level. Since: $99.47 < 497.0$.

This equation demonstrates the sensitivity of algae to the phosphorus concentration in that low concentrations of nitrogen and low concentrations of phosphorus produce significant algae growth.

$$\text{ALOG}(Z) = V(2)/(0.0413 - .0060[V(1)] + .2204 [V(2)])$$

$$Z = 10^{V(2)/[0.0413 - .0060 V(1) + 0.2204 V(2)]}$$

This equation does not imply a functional relationship between nitrogen, phosphorus, and algae of this form. Quite possibly, many relationships could give a correlation coefficient of this magnitude. It does imply that the variation can be explained within the limits set by R^2 for the given variables.

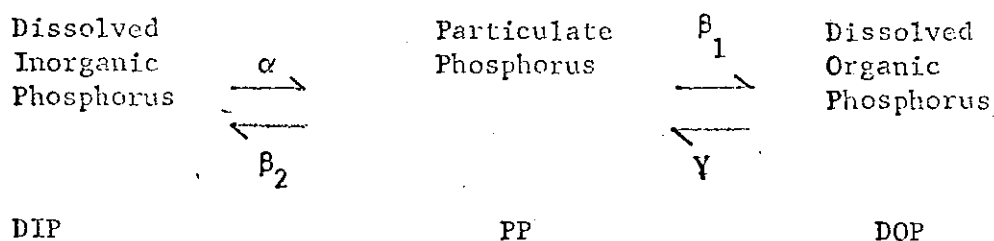
This research demonstrates the sensitivity of algae growth to the nutrient concentration and the interaction between an algae-bacteria symbiotic system in relation to the phosphorus concentration. Further studies combining other ecological conditions, such as turbidity, light, alkalinity, and harvesting herbivores in various combinations with the nutrient concentrations could be made; however, a correlation coefficient of .92 is very satisfactory. In addition, further studies aimed at establishing definite maximum and minimum N and P concentrations might prove useful. If information is to be obtained that can be incorporated into engineering planning studies of nutritional pollution, broad assumptions must be verified and qualified.

Phase two of the study was concerned with phosphorus exchange and turnover in algal cultures.

The extent of algal growth was shown in phase one to be directly related to the nutrient concentration. Likewise, algal growth is directly related to the availability of the nutrients.

If an essential nutrient, such as phosphorus, is present in limited quantities or if it is in some manner sequestered, algal growth is reduced, and is dependent on the concentration of the nutrient or on the rate at which the nutrient becomes available for algal uptake and assimilation.

A phosphorus system in water approach equilibrium when environmental conditions are constant and no net phosphorus exchanges can be measured. When a steady state is approximated the system can be treated as though it consisted of two simultaneously occurring reversible reactions:



where $DIP \rightarrow PP$ represents uptake of orthophosphate by living organisms.

α represents the rate of loss of material from the DIP phase; the reaction

$PP \rightarrow DOP$ represents the release of organic phosphorus from dead organisms

presumably as a result of bacterial attack and leaching out by water, β_1

represents the rate of loss from the PP phase; the return process $DOP \rightarrow PP$

represents the uptake of dissolved organic phosphorus compounds by bacteria

and possibly by the algae, γ being the rate of loss of material from the

DOP; finally $PP \rightarrow DIP$ is the result of several simultaneous processes:

1. The release of DIP from the organic compounds which have been broken down by bacteria.
2. The return of DIP to the water by algae and bacteria as a result of exchange in which inorganic phosphorus is constantly passing into and out of living cells.
3. Autodephosphorylation of labile organic phosphate contained within organisms which have died.

β_2 represents the rate of loss from PP to DIP.

The algae were cultured in 150 ml flasks under the following conditions:

- a. The media was 100 ml of water (well water) in which growth factors such as Vitamin B and trace elements were maintained at optimum concentrations. Phosphorus was added in the form of orthophosphate associated with a potassium salt (K_2HPO_4); nitrogen was in the form of sodium nitrate, $NaNO_3$. The N:P ratio was 4:1 with 8 mg/L N, and 2 mg/L P.
- b. An algal inoculum consisting principally of *Chlorella* was used. Two ml of inoculum (Percent transmittance = 31.5) were added to each flask.

- c. Bacteria: The algal inoculum was not bacteria free -- since there is a definite relationship between the algae and the bacteria in a natural environment, it was important in this study to consider them one phenomena.
- d. P-32 in the form of $K_2HP^{32}O_4$ was used.

Analytical procedures consisted of the following:

- a. PP-32 (Particulate Phosphorus) was taken as the difference between total P-32 in the culture and P-32 in the filtrate passed through a 0.45 pore diameter filter. Aliquots of 0.1 ml before and after filtration were counted in a deepwell integral scintillation counter with an efficiency of 37%. The percent difference was taken as the P-32 in the algae.
- b. The DIP-32 and DOP-32 in the filtrate were separated chromatographically and identified by their respective Rf's. The percent of each compound was determined by drawing the chromatogram strip in front of a Geiger-Muller gas flow counter and then determining the area under the curve with a planimeter. The chromatograms were prepared by placing 100 μ L samples of the filtrate on Whatman #1 paper, previously washed in 0.1N HCl and distilled water, and developed in methanol, formic acid, and water, (80:15:5), using unidimensional, descending chromatography. Of the many solvents tested this was selected as the best for a rapid separation of the phosphoric esters.
- c. The Phosphate compounds formed in the Algal Mass, (PP-32), were also analyzed chromatographically. For each run several samples were pooled, centrifuged, supernatant decanted; the phosphorus compounds were then extracted from the algal mass by the addition of boiling absolute ethanol, followed by 80% boiling ethanol. This mixture was taken down to dryness using a Flash Evaporator and then taken up in 2 ml of water. Samples were placed on chromatographic paper and developed in the same manner as the filtrate. The chromatograms were scanned and the percent of each compound determined.

By using P-32 and appropriate radiochromatographic techniques, it was determined that six organic phosphorus compounds, ATP, Glucose-1- PO_4 and Fructose-6- PO_4 , Fructose-1, 6Di PO_4 , Glucose-1 PO_4 and Phosphoglyceric Acid were released into the surrounding medium by the algae. In addition to qualitative data, experimentation has produced quantitative data which, when substituted into the corresponding equilibrium equation and differential

equation (App. 1) will result in an exchange rate from one phase to another. Turnover times then are the reciprocals of the exchange rates. Average exchange values found for our cultures were:

DIP	(0.02 mg)
⇕	0.0062 mg P/L/Day in each direction
PP	(0.156 mg)
⇕	0.0405 mg P/L/Day in each direction
DOP	(0.024 mg)

and average turnover times were:

DIP	= 3.2 days
PP	= 3.3 days
DOP	= .58 days

Figure 1 illustrates the average equilibration of dissolved inorganic phosphate and the simultaneous increase of particule phosphorus and dissolved organic phosphorus.

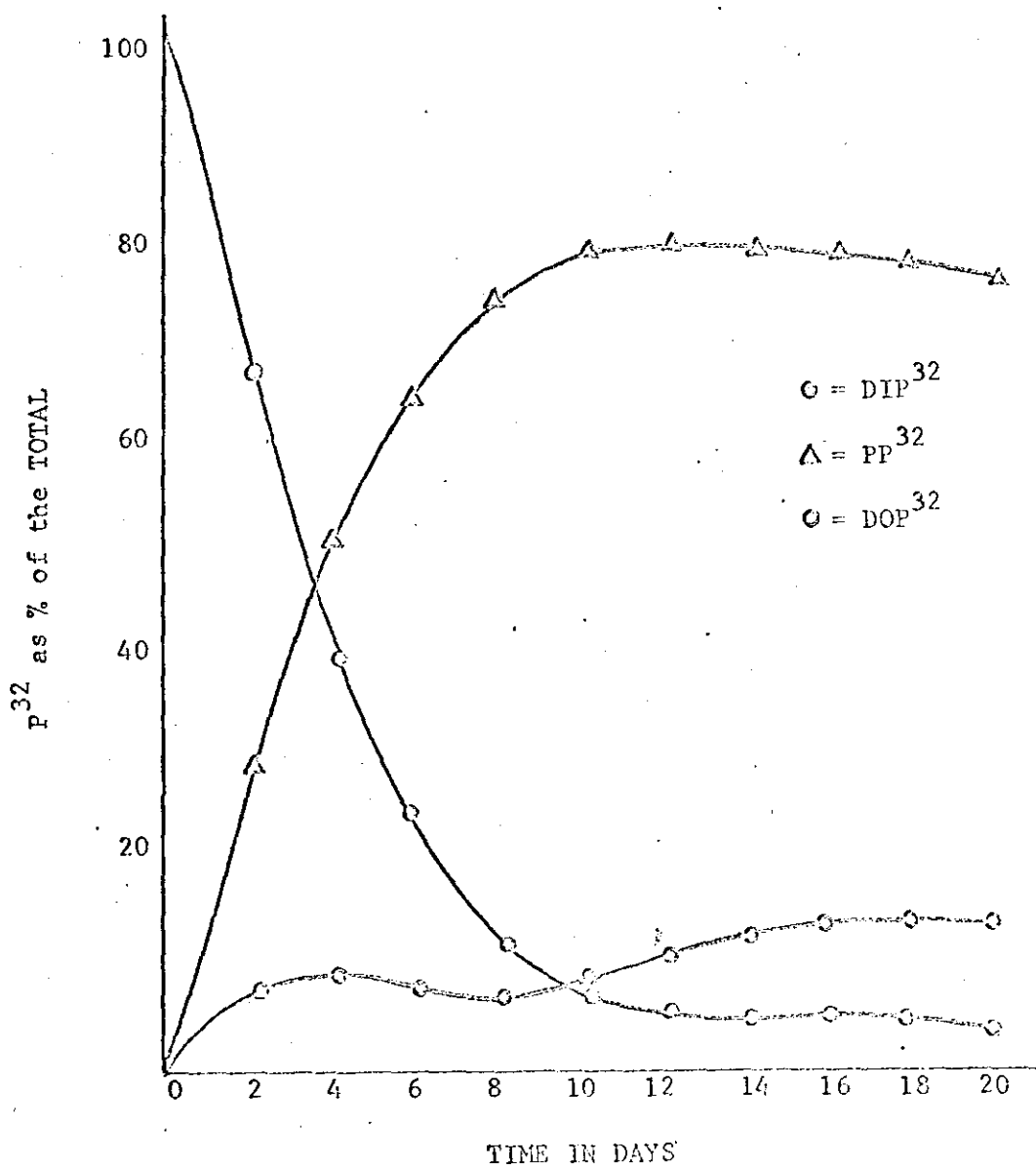
By establishing the exchange rate and turnover time of phosphorus at varying concentrations and coupling these data with existing data concerning limiting phosphorus concentrations, a mathematical model to predict exchange rates could be constructed. This knowledge would then be of value in predicting the algae growth potential of a particular body of fresh, surface water.

TABLE 1

Optimum Ratios of N/P at Various PO₄ - P Levels

<u>Phosphorus Level (mg/l)</u>	<u>Optimum Ratio</u>	<u>Yield (mg/100 ml)</u>
0.5	9/1	60
1.0	9/1	50
1.5	9/1	48
0.1	16/1	60
2.0	16/1	55
3.0	4/1	45
4.0	4/1	65
8.0	2/1	50

Figure 1



The Equilibration of DIP 32 in Batch Culture

Appendix 1

Equilibrium equation #1:

$$\alpha (\text{DIP}) = \beta_1 (\text{PP})$$

Equilibrium equation #2:

$$\beta_2 (\text{PP}) = \gamma (\text{DOP})$$

Rate of change equations:

$$d \frac{(\text{DIP-32})}{dt} = -\alpha (\text{DIP-32}) + \beta_1 (\text{PP-32})$$

for a finite time interval this equation is written:

$$\frac{(\text{DIP-32})_2 - (\text{DIP-32})_1}{\Delta t} = -\alpha \frac{(\text{DIP-32})_1 + (\text{DIP-32})_2}{2} + \beta_1 \frac{(\text{PP-32})_1 + (\text{PP-32})_2}{2}$$

$$\frac{d(\text{DOP-32})}{dt} = \beta_2 (\text{PP-32}) - \gamma (\text{DOP-32})$$