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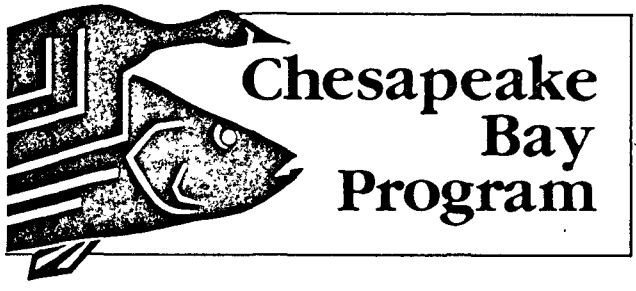
Nitrogen and Phosphorus Determinations in Estuarine Waters: A Comparison of Methods Used in Chesapeake Bay Monitoring



NITROGEN AND PHOSPHORUS DETERMINATIONS IN ESTUARINE WATERS: A COMPARISON OF METHODS USED IN CHESAPEAKE BAY MONITORING[1,2]

Christopher F. D'Elia[3], Robert E. Magnien[4], Carl F. Zimmermann[3], Pauline A. Vaas[4], Nancy L. Kaumeyer[3], Carolyn W. Keefe[3], Diane V. Shaw[3], and Kathryn V. Wood[3]

TABLE OF CONTENTS

[1]A report prepared for the Chesapeake Bay Program Liaison Office, Region III, U.S. Environmental Protection Agency, 410 Severn Ave., Annapolis, MD 21403.

[2]Reference No. UMCEES 87-19 CBL.

[3]Nutrient Analytical Services Laboratory, Chesapeake Biological Laboratory, Center for Environmental and Estuarine Studies, University of Maryland, Solomons, MD 20688-0038.

[4]Water Management Administration, Office of Environmental Programs, Department of Health and Mental Hygiene, 201 W. Preston St., Baltimore, MD 21201.

ABSTRACT

This study was performed to compare standard EPA techniques for determining nitrogen and phosphorus concentrations in natural waters with oceanographic techniques typically employed by estuarine and marine scientists. The following comparisons were made using samples collected over a four-month period and a wide range of salinities from Chesapeake Bay: (1) particulate nitrogen derived from direct determination (oceanographic) on particulate matter collected on filters *vs* particulate concentrations derived "by difference" (EPA), i.e. by determining total Kjeldahl nitrogen on whole and filtered water samples and subtracting the two to obtain particulate nitrogen; (2) an analogous direct determination (oceanographic) for particulate phosphorus *vs* a "by difference" determination (EPA); (3) dissolved nitrogen and phosphorus determination using alkaline persulfate digestion (oceanographic) *vs* Kjeldahl and acid persulfate digestion (EPA).

Direct determinations of particulate N and P were more precise (sensitive) than "by difference" techniques. For example, field duplicates for particulate nitrogen by the direct approach gave a mean concentration of 0.184±0.011 mg N/L *vs* 0.172±0.125 mg N/L using the "by difference" technique. This represents a coefficient of variation (CV%) of 5.8% *vs* 72.7%. Alkaline and acid persulfate digestions yielded comparable precision for dissolved P, but comparisons of two dissolved N techniques were inconclusive owing to the high variance of each.

Field duplicates from all sampling periods showed that the Kjeldahl determination gave a mean concentration of 0.434±0.068 mg N/L and alkaline persulfate determination (less nitrate) a mean concentration of 0.433±0.062 mg N/L. However, for a given sampling period the two techniques gave comparable accuracy, at other times not. The alkaline persulfate technique provided precision (CV% = 9.71%) superior to that for the Kjeldahl technique (CV% = 15.81%).

A cost comparison showed that the more precise oceanographic protocols provide a better than 30% savings over EPA-required techniques.

The above technical findings and cost comparisons suggest that in estuarine waters the oceanographic procedures provide equivalent or superior results to those obtained by EPA procedures at a considerable cost savings.

INTRODUCTION

The Chesapeake Bay Program (CBP) was established in 1976 by the Environmental Protection Agency (EPA) as a comprehensive estuarine research and management project. One of the first major goals of CBP was to determine historical changes in water quality parameters such as nitrogen and phosphorus (Heinle et al. 1980).

In 1984, the scope of effort was increased to include comprehensive monitoring of bay-wide water quality parameters of which the various forms of nitrogen and phosphorus are of prime importance. This comprehensive program involves not only EPA, but the U.S. Geological Service and the state agencies of Maryland (Office of Environmental Programs, OEP), Virginia (Water Control Board), and Pennsylvania (Department of Environmental Resources) as well as local agencies including the District of Columbia and the Metropolitan Washington Council of Governments. The program includes coordinated monitoring of both the mainstem of the Bay and major tributaries. Data collected from the monitoring program will provide useful information for making historical comparisons, characterizing baseline conditions and projecting future trends with respect to the "health" of the bay. The data should also provide information for developing mathematical models and nutrient budgets and identify the important processes which affect water quality. Such scientific information will aid managers in decisionmaking.

In establishing the monitoring program, much attention was paid to selecting sampling times and stations, but the selection of analytical methods received less scrutiny, because it was assumed that standard EPA procedures are satisfactory. However standard EPA methods are oriented to legal requirements. Accordingly, these standard methods are often not appropriate for the first choice in research studies, particularly when low environmental concentrations or unusual sample matrices are expected. Thus, the quandry is that most EPA methods are designed to meet legal constraints for effluent discharges with high nutrient concentrations. EPA methods have not been evaluated in the context of precision, accuracy, cost or suitability to estuarine samples. [Precision is defined as the repeatability of a given measurement (e.g. the standard deviation of a series of replicate analyses), and accuracy refers to the correctness of the data values (Fig. 1)].

Such methodological distinctions are clearly made in Sections 106 and 308 of the Clean Water Act. Methods used for Section 308 requirements pertain to legally sensitive aspects of the National Pollutant Discharge Elimination System (NPDES) effluent monitoring, while Section 106 requirements, which are applicable to pollution research, are more flexible.

The Chesapeake Bay Monitoring Program falls under the latter category, but state and Federal labo-

ratories involved in analyzing both Section 308 and Section 106 samples prefer to analyze both types of samples using the Section 308 mandated methods. The rationale is that it is more convenient and less expensive for them to analyze all samples, regardless of source, using one method than to utilize a variety of different methods. Accordingly, these laboratories are reluctant to vary from standard Section 308 methods.

EPA-CBP currently requires all contractors in the monitoring program to use only EPA-approved, Section 308 methods to ensure that comparability is maintained both with historical data and among present programs. Although comparability is clearly a valid concern, it can be argued that if historical methods are inadequate, then comparability is a moot point. In fact, many of the historical studies in Chesapeake Bay, funded in part by EPA, have used the oceanographic techniques. Furthermore, use of seemingly comparable methods by different laboratories or by the same laboratory at different times may also present intercalibration-related problems, although the oceanographic literature, at least, shows surprising consistency for observations made by different groups (Kamykowski and Zentara 1985, 1986).

Because the goals of maintaining historical continuity and obtaining precise and accurate data are not necessarily compatible, the adequacy and appropriateness of using EPA-mandated techniques alone has been questioned. Clearly, the most precise and accurate data practically obtainable are required to obtain adequate scientific information to make sound management decisions and thereby reach program objectives.

Four important concerns that relate to the bay monitoring program are as follows:

1. Standard EPA limits of detection (i.e. precision) should be improved because they may be inadaquate for many parameters, over much of the Bay, during much of the year. Differences between EPA protocols and more precise ones, in most cases, involve only trivial changes (such as increasing the path length of colorimeters in autoanalyzers). In other cases, alternate protocols should be used.

2. Standard EPA approaches to the determination of particulate nitrogen (PN) and phosphorus (PP) may not be satisfactory. Significant improvements may be realized if these constituents are determined directly by a single analysis rather than "by difference" of a pair of analyses, as required by EPA.

3. Kjeldahl nitrogen determination is not well established as precise or accurate enough to provide reliable data for estuarine and marine samples. Alternative techniques, which have seen favor in oceanography, such as the total alkaline persulfate technique, although more precise, are not well estab-

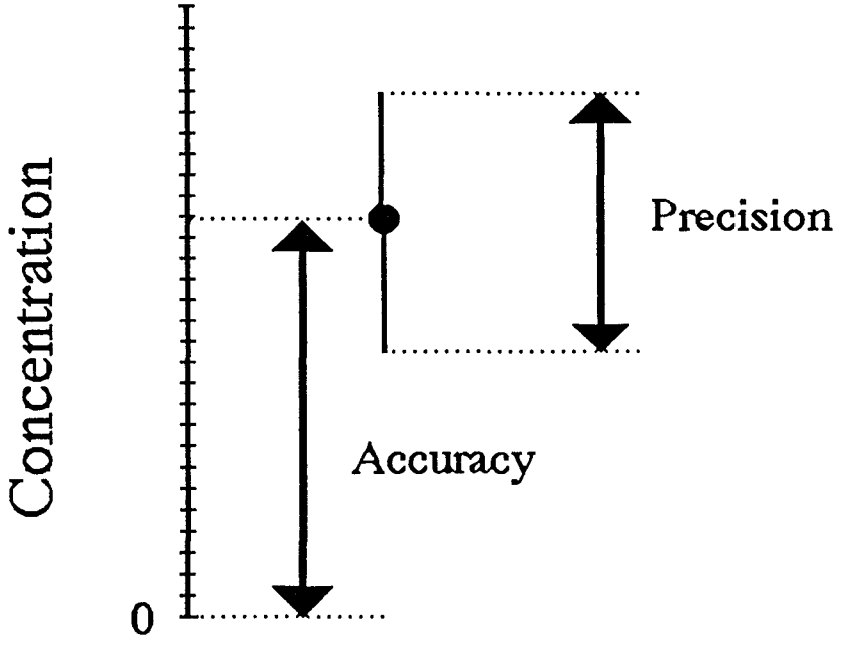


Figure 1. Diagram to show the difference between precision and accuracy of an analytical determination. Precision is the repeatability of a given measurement–since there is statistical error associated with any analytical procedure, repeated determinations on subsamples of water will not be exactly the same, but will instead fall in a given range. Accuracy, on the other hand relates to the degree of conformity to a standard, in essence, the correctness of the determination.

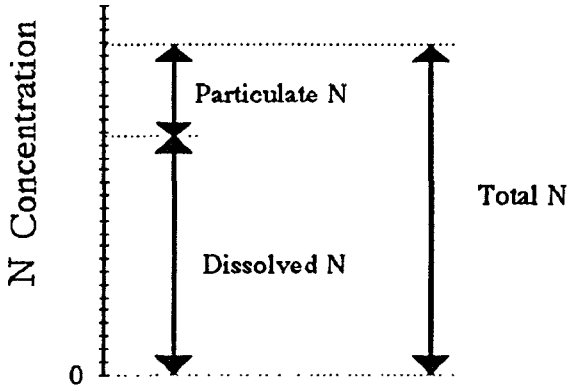


Figure 2. Partitioning of a water sample into particulate and dissolved fractions: particulate N can be determined directly or by determining total N and dissolved N and subtracting the latter from the former.

lished as accurate EPA standard methods. In the words of Head (1985), "[the Kjeldahl] method is not without problems when applied to saline samples and the alkaline digestion procedure developed by Koroleff (see Grasshoff et al. 1983) seems to offer considerable advantages." Such alternative techniques should be considered for inclusion in the bay monitoring program.

4. The analytical costs associated with standard EPA-approved protocols may substantially exceed acceptable alternatives (D'Elia et al. 1986). The use of acceptable, more cost-effective techniques should be considered by EPA.

EPA/CBP provided funds for analytical work so that CBL and OEP could compare oceanographic analytical techniques with EPA techniques for Chesapeake Bay samples from June through September, 1986 to evaluate the above concerns. In addition, monitoring data collected between July, 1984 and May, 1986 was also used in the analysis. This report was assembled at CBL's initiative to summarize the results of the methodological comparisons.

Nutrient Fractions in Estuarine Waters

To understand the analytical questions at hand, one must understand clearly how to determine nutrient concentrations in the particulate and dissolved phases. For a given water sample, the particulate phase is operationally defined as that part which is retained on a filter pad with a nominal pore size in the range of 0.45-1.2 um (Strickland and Parsons 1972). The water which passes through the filter, the filtrate, is defined as the dissolved phase of a given water sample. Figure 2 demonstrates the partitioning of a water sample into different nitrogen fractions. The same rationale is applied for particulate and dissolved carbon and phosphorus.

The oceanographic, coastal and estuarine scientific communities, have largely chosen direct measurement of particulate fractions collected on inert filters using elemental analysis (Sharp 1974; Williams 1985; Gardner et al. 1984) or other combustion methods (e.g. Flemer and Biggs 1971) for N and C, and by combustion (Solorzano and Sharp 1980) or wet oxidation (Grasshoff et al. 1983; Williams 1985) for P. Elemental analysis is extremely precise and offers the advantage of being a direct measurement of the particulate fraction collected on an inert filter. Total nutrient concentrations are then obtained by summing the concentrations found in the particulate and dissolved fractions. But probably the most important reason for collecting and measuring the particulate fraction directly is the information that would be lost if only a whole-water sample were analyzed. The particulate fraction includes the biological part of the ecosystem and the temporal and spatial variations associated with this fraction could be overlooked were the analysis not made (Head 1985). Moreover, unlike dissolved solute phases which mix conservatively between different water masses, particulate phases may, through sedimentation, repartition nutrients nonconservatively between one water mass and another.

Another way to determine the amount of a nutrient present in the particulate fraction is "by difference." This is calculated by subtracting the results obtained from a filtered sample from the results obtained from the original unfiltered sample. This is the standard EPA protocol. Figure 3 illustrates how analytical error may result in negative values for

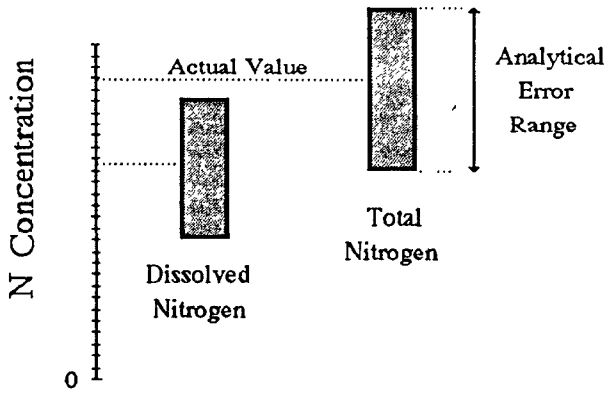


Figure 3. Analytical error can cause negative values for particulate N when the "by difference" approach is used. Negative particulate N values occur when normal analytical error results in higher dissolve than total N values.

particulate N when these are determined "by difference". When high dissolved nitrogen concentrations are present, there is a greater chance of obtaining negative values for PN concentrations. Analytical errors in analysis of carbon and phosphorus fractions may also yield negative particulate values. Figure 4 shows nitrogen and phosphorus fractions typically required in studies of nutrient-related processes and Figure 5 compares N and P fractions determined using standard EPA protocols (Fig. 5A) and using the typical oceanographic protocols employed at and advocated by CBL (Fig. 5B).

Purpose of Project

The major purpose of this project was to use field data to compare "direct" and "by difference" procedures for determination of PN and PP. We compared results obtained by direct analysis of PN and PP with those obtained by subtraction (EPA methods) on water samples collected from eight cruises from June-September, 1986. We also compared the dissolved Kjeldahl nitrogen with an alkaline persulfate nitrogen technique and the acid persulfate technique with the alkaline persulfate technique for dissolved phosphorus. By conducting all analyses on the same water samples, we were better able to compare the various methods.

Data from EPA's Central Regional Laboratory (CRL) in Annapolis, which analyzed Maryland mainstem samples from June, 1984 to May, 1985, are also presented to provide additional comparisons.

Specific questions addressed in this report are:

1. Is the value derived from subtracting dissolved from unfiltered Kjeldahl analyses comparable to that obtained by the direct measurement of PN with an elemental analyzer?
2. Is the precision obtained using the dissolved Kjeldahl technique comparable to that obtained using the alkaline persulfate dissolved N technique?
3. Are whole water nitrogen concentrations obtained by these alternative techniques comparable?
4. Is the value derived from subtracting dissolved acid persulfate P values from the same whole-water sample comparable to the direct measurement of PP using a combustion technique?
5. Are the results obtained using the dissolved acid persulfate technique for dissolved P comparable to those obtained using the alkaline persulfate technique?
6. Are whole-water phosphorus concentrations obtained by these alternative techniques comparable?

MATERIALS AND METHODS

Study Site and Duration

A total of 22 stations located in the Maryland mainstem portion of the Chesapeake Bay were sampled on eight occasions from June to September, 1986 at approximate two-week intervals. This portion of the Bay spans the range of conditions normally found in Chesapeake Bay from tidal freshwater to salinities exceeding 20 ppt. Samples were collected at surface, bottom, and above and below the pycnocline. Field duplicates (subsamples from one water sample) were also taken, yielding a total of 92 samples for each of the analyses for each of the eight cruises.

Sample Collection and Analysis

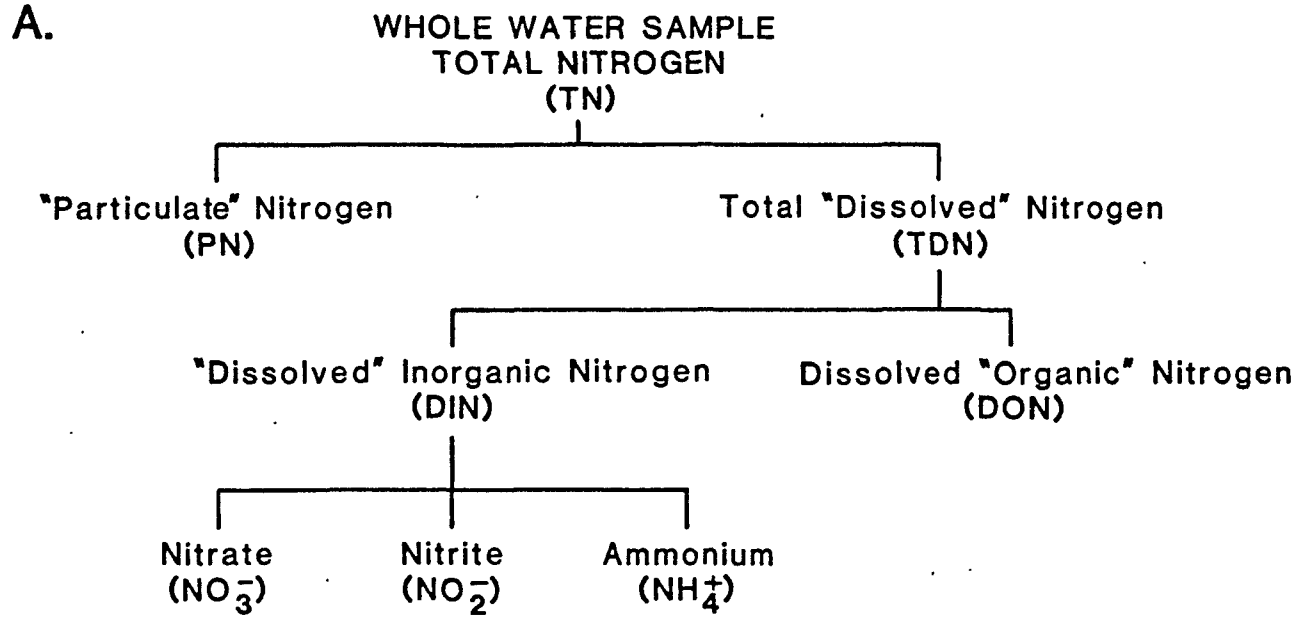
All water samples were collected using a submersible pump system. Where applicable, samples were filtered through GF/F glass fiber filters (nominal pore size, 0.7-um). Particulate samples on filters were kept frozen until analysis (usually less than 20 days).

Kjeldahl Nitrogen. Filtered and unfiltered samples were placed in acid washed 50-ml plastic, screw-cap, centrifuge tubes and two drops of concentrated sulfuric acid were added as a preservative. The samples were then refrigerated at 4 degrees C until digestion. Twenty-five ml samples were digested using a 40-tube block digestor and analyzed for nitrogen according to EPA method 351.2 (U.S.E.P.A. 1979) for the June samples. The July-September samples were analyzed using a slight modification employed by Old Dominion University personnel of EPA method 351.2, in which Teflon boiling balls were substituted for boiling chips in the digestion.

Alkaline Persulfate Dissolved Nitrogen and Phosphorus. Filtered, ten-ml samples were placed in 30-ml glass test tubes and frozen until analysis. The method used is based on that of D'Elia et al. (1977), Glibert et al. (1976), and Ebina et al. (1983) where nitrate and phosphate are hydrolyzed from organic N and P compounds by oxidation with potassium persulfate.

Acid Persulfate Phosphorus. Twenty ml of filtered or unfiltered water were placed in 30-ml screw-cap test tubes and frozen until analysis. The procedure used was that of Menzel and Corwin (1965) and EPA method 365.2, where phosphate is hydrolyzed from organic P compounds by persulfate oxidation.

Particulate Nitrogen. A known volume of sample (usually >200 ml) was filtered through a precombusted 25-mm GF/F filter. Particulate analyses



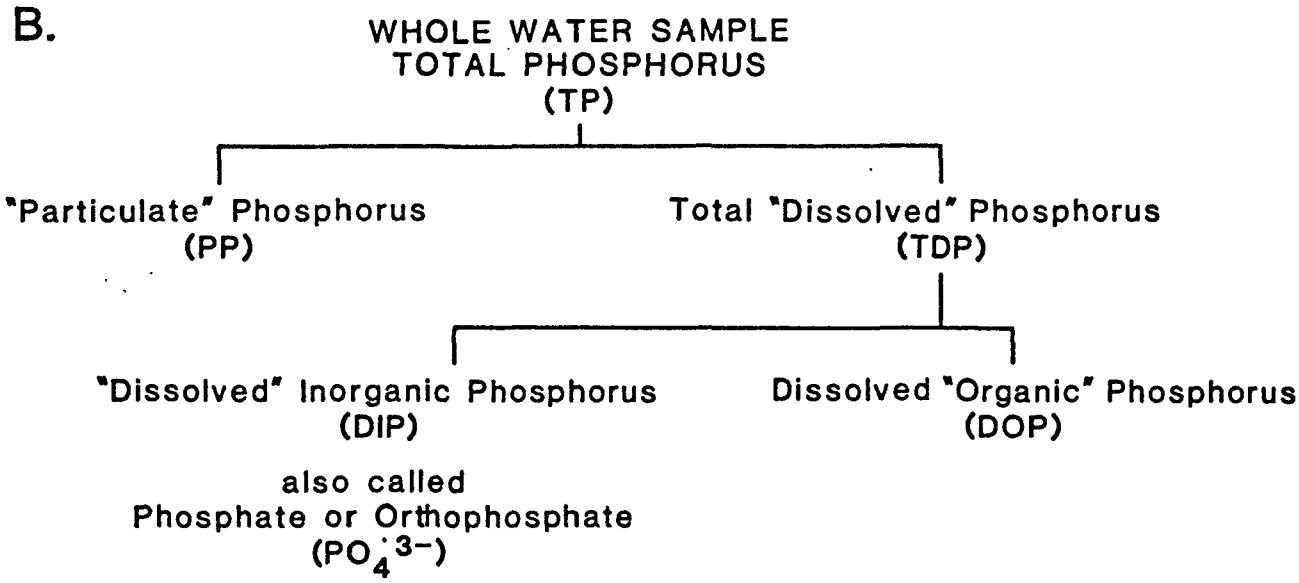


Figure 4. Nitrogen (A) and phosphorus (B) fractions typically determined in water quality studies.

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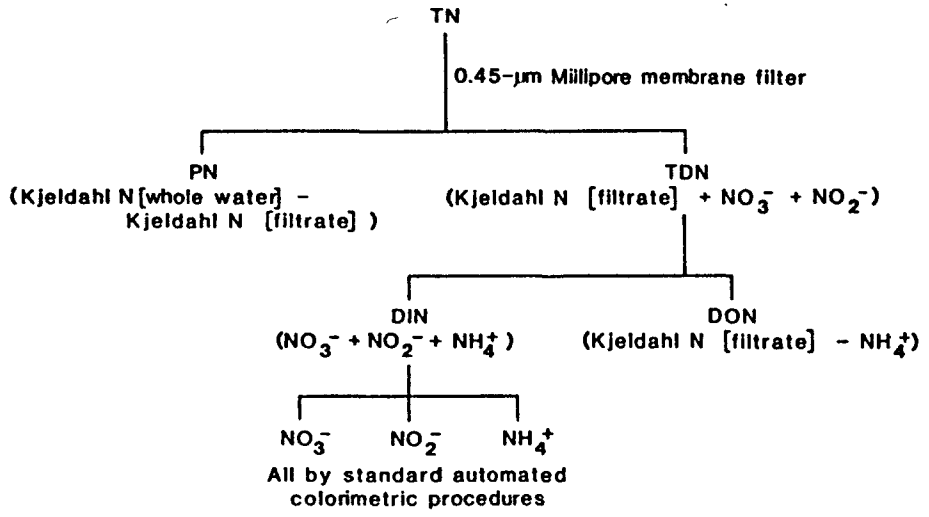
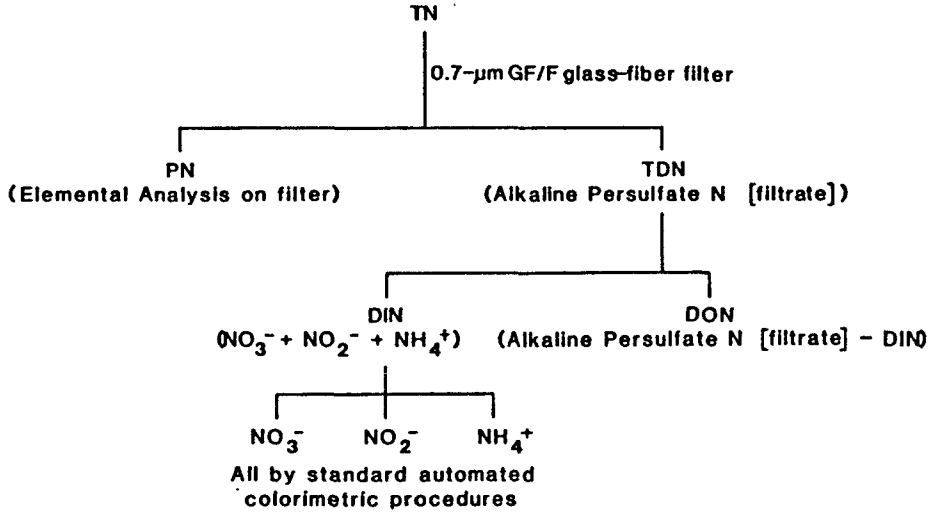
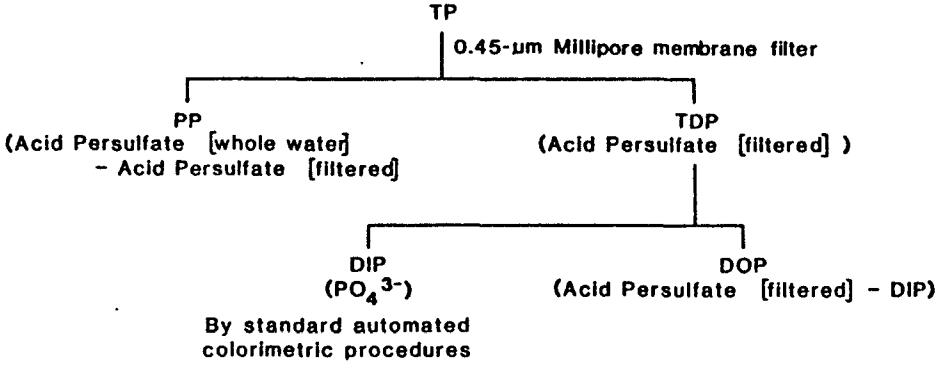


B. TYPICAL OCEANOGRAPHIC (CBL)



C. STANDARD EPA



D. TYPICAL OCEANOGRAPHIC (CBL)

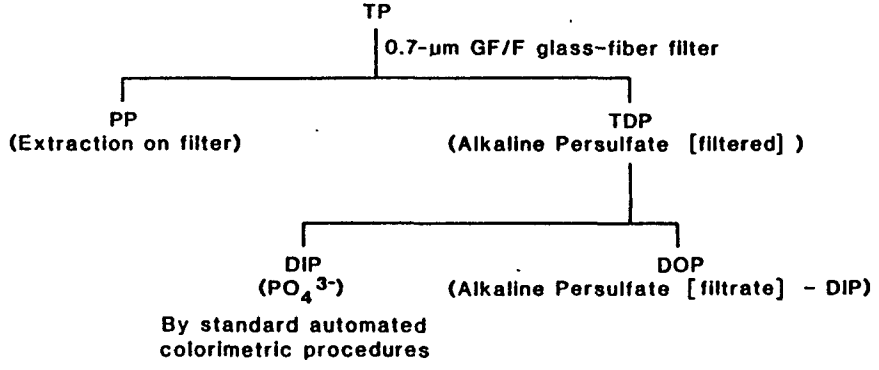


Figure 5. Comparison of analytical procedures used to obtain nutrient fractions shown in Fig. 4. (A) Standard EPA nitrogen determinations. (B) Typical oceanographic nitrogen determinations (as practiced at CBL). (C) Standard EPA phosphorus determinations. (D) Typical oceanographic phosphorus determinations (as practiced at CBL).

were performed using a Control Equipment Inc. Model 240-XA Elemental Analyzer. Combustion of the sample occurs in pure oxygen at high temperature. The combustion products (N_2, CO_2, and H_2O) are then analyzed automatically in a self-integrating, steady-state, thermal conductivity analyzer. All results and calculations are processed by a Hewlett-Packard model 150 computer.

Particulate Phosphorus. A known volume of water (>250 ml) was filtered through a 47-mm GF/F filter. Prior to extraction, the filter pad was combusted at 550 degrees C for 1.5 h, cooled overnight and then extracted in 10 ml of 1.0 N HCl for at least 24 hours (Aspila et al. 1976). The supernatant was then analyzed for phosphate.

A complete description of the methods utilized in this study can be found in the CBL Nutrient Analytical Services Laboratory methods book (Appendix C) and D'Elia et al. (1986--Appendix D).

RESULTS AND DISCUSSION

Nitrogen

Direct vs By Difference" Particulate Nitrogen Determination. The direct measurement of particulate nitrogen (PN) gave better precision and consistently more plausible values than did the "by difference" technique. This result is to be expected on the basis of statistical considerations alone. When dissolved inorganic nitrogen values are high, more negative particulate values are likely to occur with the "by difference" technique because taking the difference between two large values with relatively great percentage error often yields negative values (Fig. 3).

Table 1 is based on CBL/OEP Quality Assurance/ Quality Control (QA/QC) data from duplicate field samples collected during June-Sept. 1986; it presents comparative values obtained from the direct measurement of particulate N and from the "by difference" technique (see also Appendix A). The use of field blanks in determining detection limits has been recommended by experts in QA/QC (Analytical Methods Committee 1987). The overall mean particulate N concentrations as determined by both methods were close, although the direct measurement of PN yielded more than an order-of-magnitude increase in precision. Increased precision may be very desirable if one wants to characterize the particulate material in bay waters (see below).

The comparison of PN concentrations determined by the two techniques on the same water samples between June and September, 1986 is presented in Figure 6. Negative particulate values occurred when they were calculated "by difference" from the Kjeldahl measurements but only positive values were obtained from the direct measurements. The slope of this line is 0.610 (July - Sept. 1986; Appendix B).

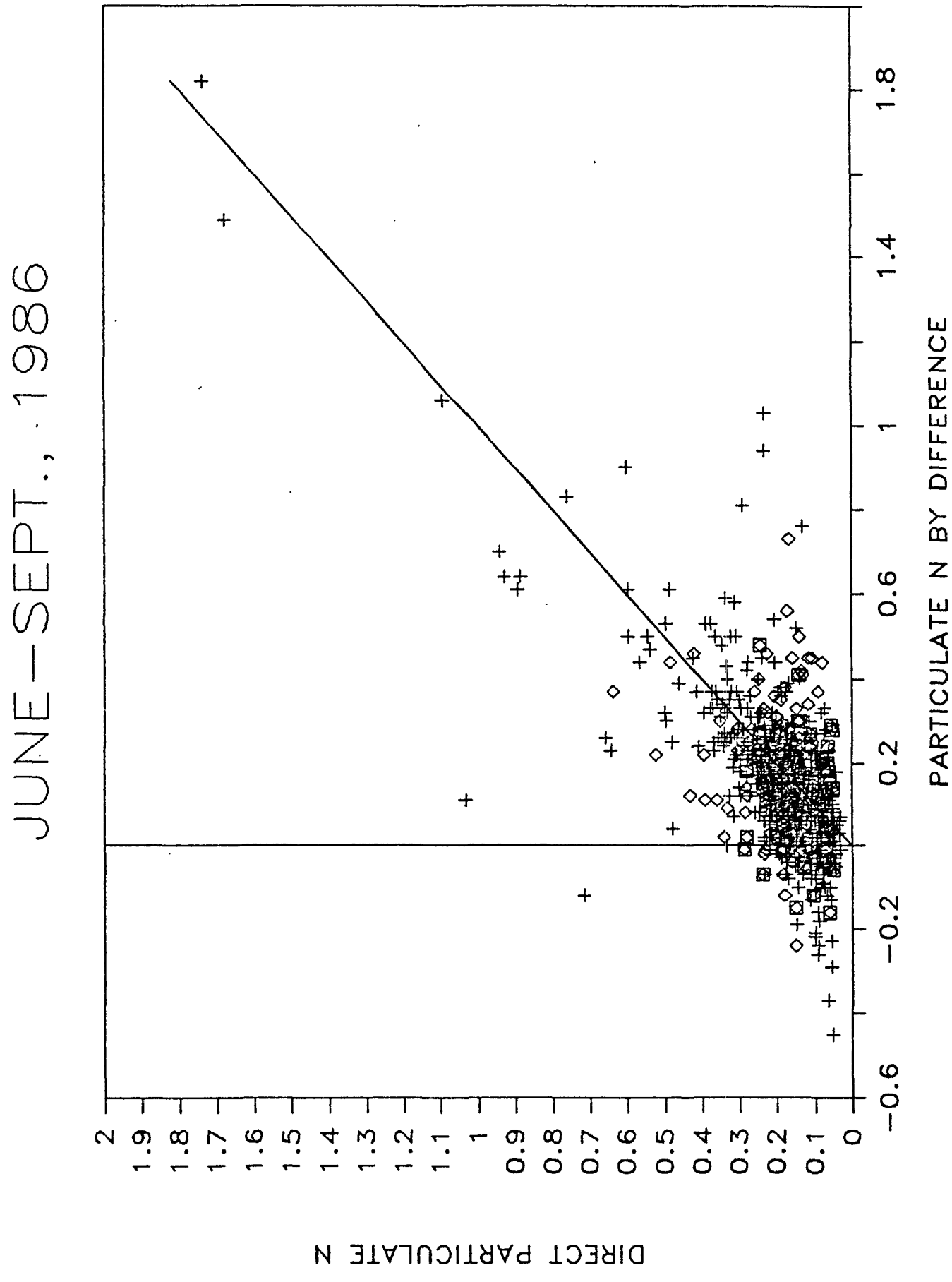
Comparability varied with sampling period. The July data, for which an improved Kjeldahl technique had been implemented, show fewer data points in the negative range and the data clearly follow the line of 1:1 comparability (Fig. 7A). Note, however, that July is the period of highest particulate and lowest dissolved nitrogen levels (i.e. the highest "signal-to-noise" relationship), when the "by difference" technique would be expected to produce the best data. Data for other months have lower PN concentrations relative to total N, and show more negative "by difference" values and poorer correlations with PN determined directly (Fig. 7B). Clearly then, the range of concentrations found affects the results. When concentrations are higher, such as for the July data, the relationship between the two methods is good. However, the relative variation in the EPA method is much more evident in the lower concentration ranges because the Kjeldahl method is imprecise and "by difference" errors propagate additively (Table 1).

Figure 8A presents data analyzed at EPA/CRL from June, 1984 to May, 1985 in which PN concentrations are calculated "by difference" between whole-water and dissolved Kjeldahl nitrogen values on duplicate subsamples of single, field samples. This is part of the CBL/OEP-QA/QC program and ideally should result in 1:1 correspondence, high corelation (r=1.0), and no negative values. However, the correlation obtained by regression of PN duplicates by difference is obviously low (r^2 = 0.03) and many negative values occur.

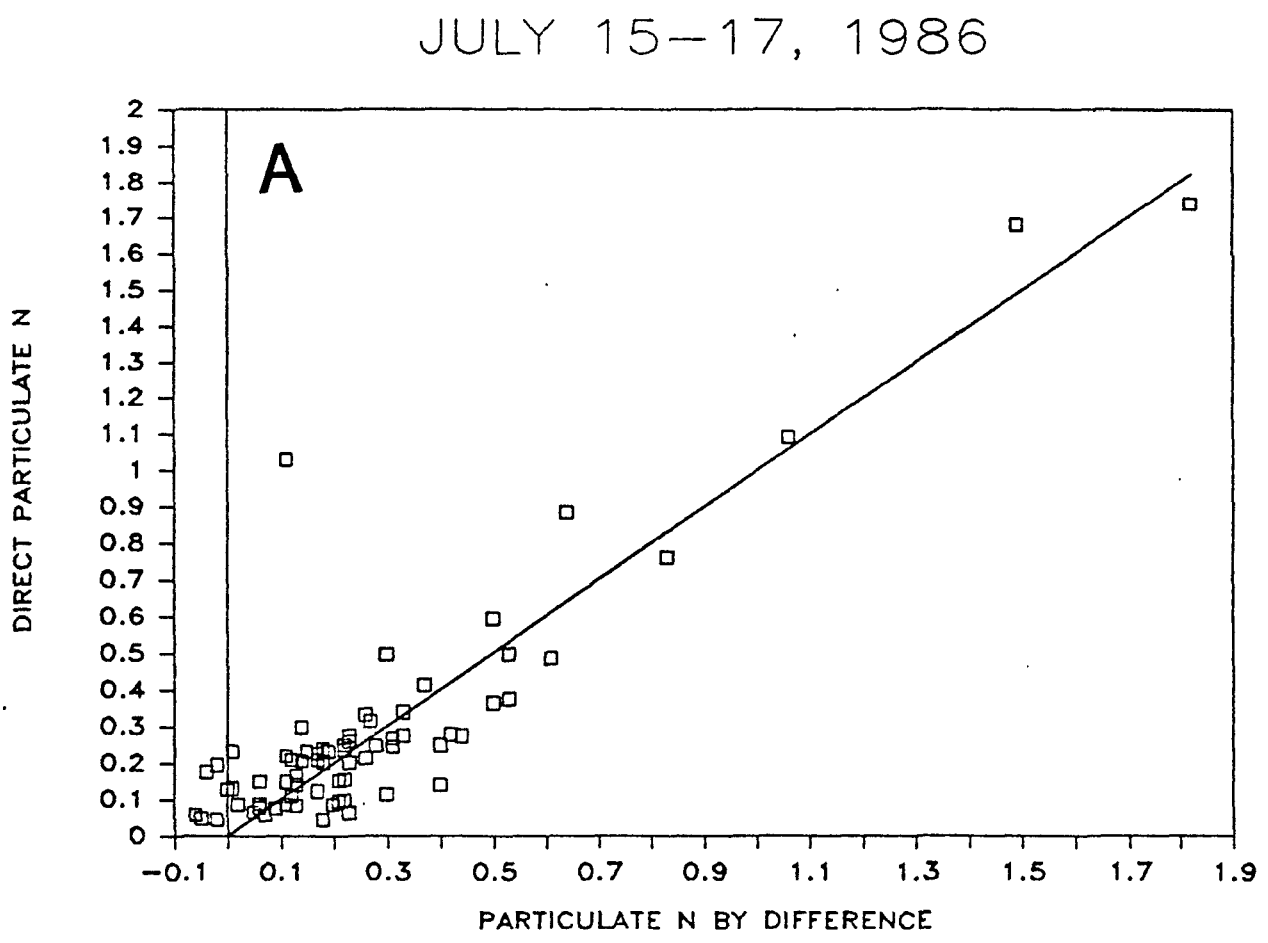
Figure 8B presents PN data analyzed at CBL from May, 1985 to June, 1986 on duplicate samples using the direct measurement technique. The most important differences in protocol between the study periods represented in Fig. 8A and 8B are the analytical techniques used and the laboratory performing the analyses–all field sampling activities remained identical. As it should in theory do, the direct determination resulted in a high correlation coefficient (r^2 = 0.91) and no negative values. Thus, precision in the determination of particulate fractions is vastly improved by direct determination.

A way to test the accuracy of analytical results is to compare the data obtained with an independent variable. Fig. 9 shows the results of scatterplots of the above PN data against corresponding PC data. Fig. 9A gives the June, 1984 to May, 1985 data when PC was also obtained using a by-difference technique. Fig. 9B gives comparable data analyzed by elemental analysis at CBL for the study period May, 1985 to June, 1986. Clearly, the data in Fig. 9A demonstrate

Figure 6. Scatterplot of particulate nitrogen determined by the "by difference" (EPA) vs "direct" (CBL) procedures.



cedures: (A) During July, 1986, and (B) August, 1986.



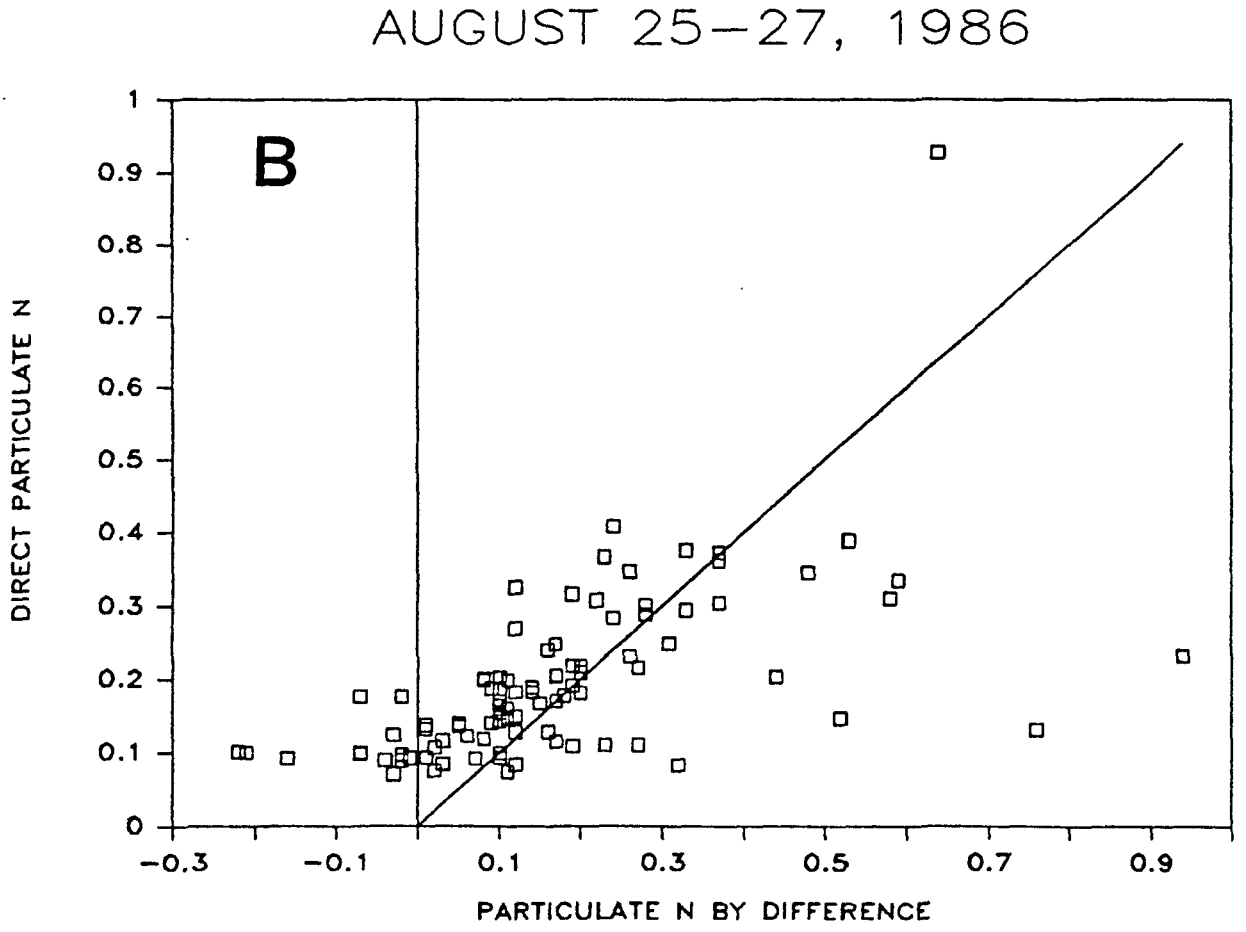
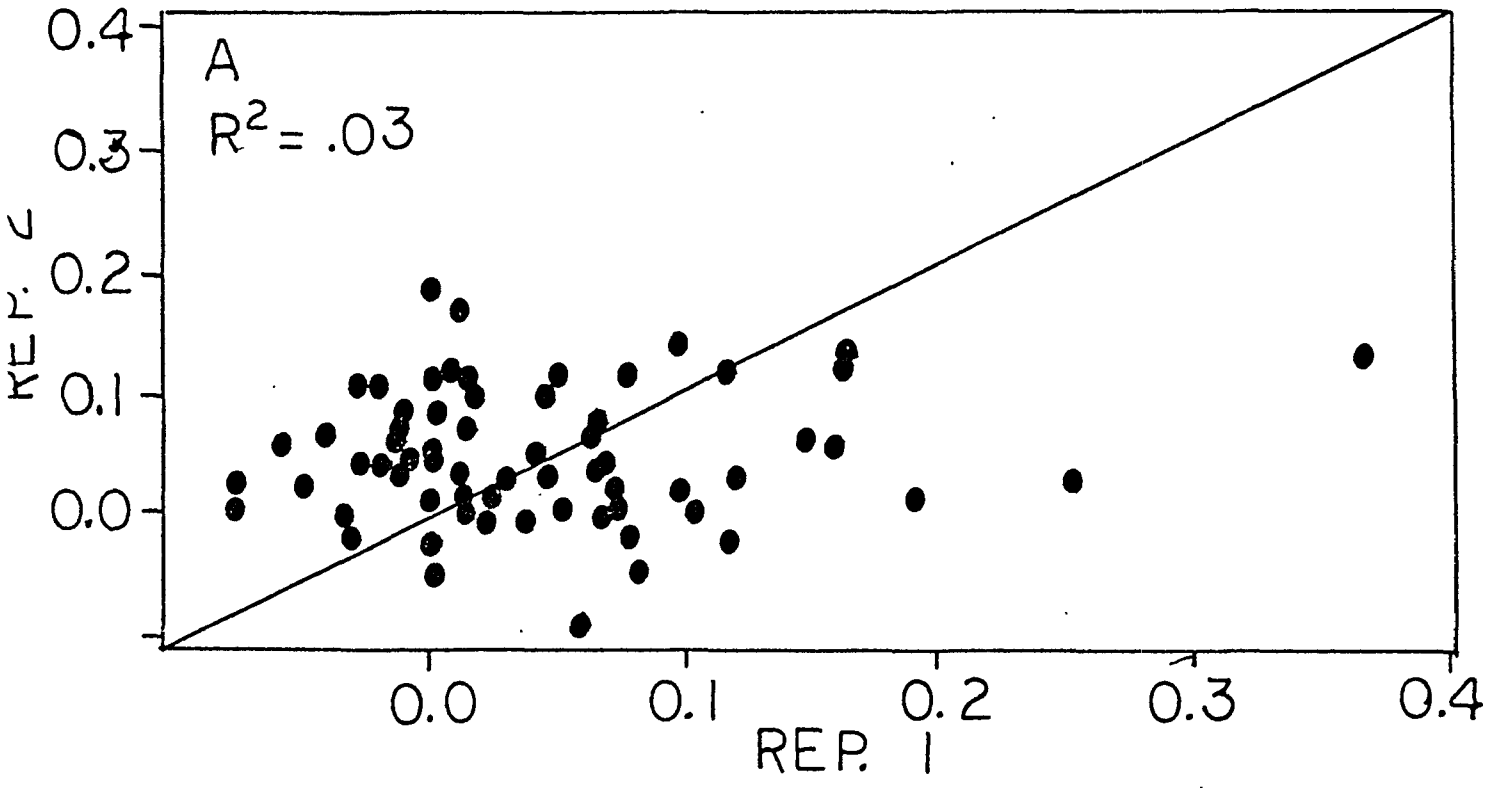
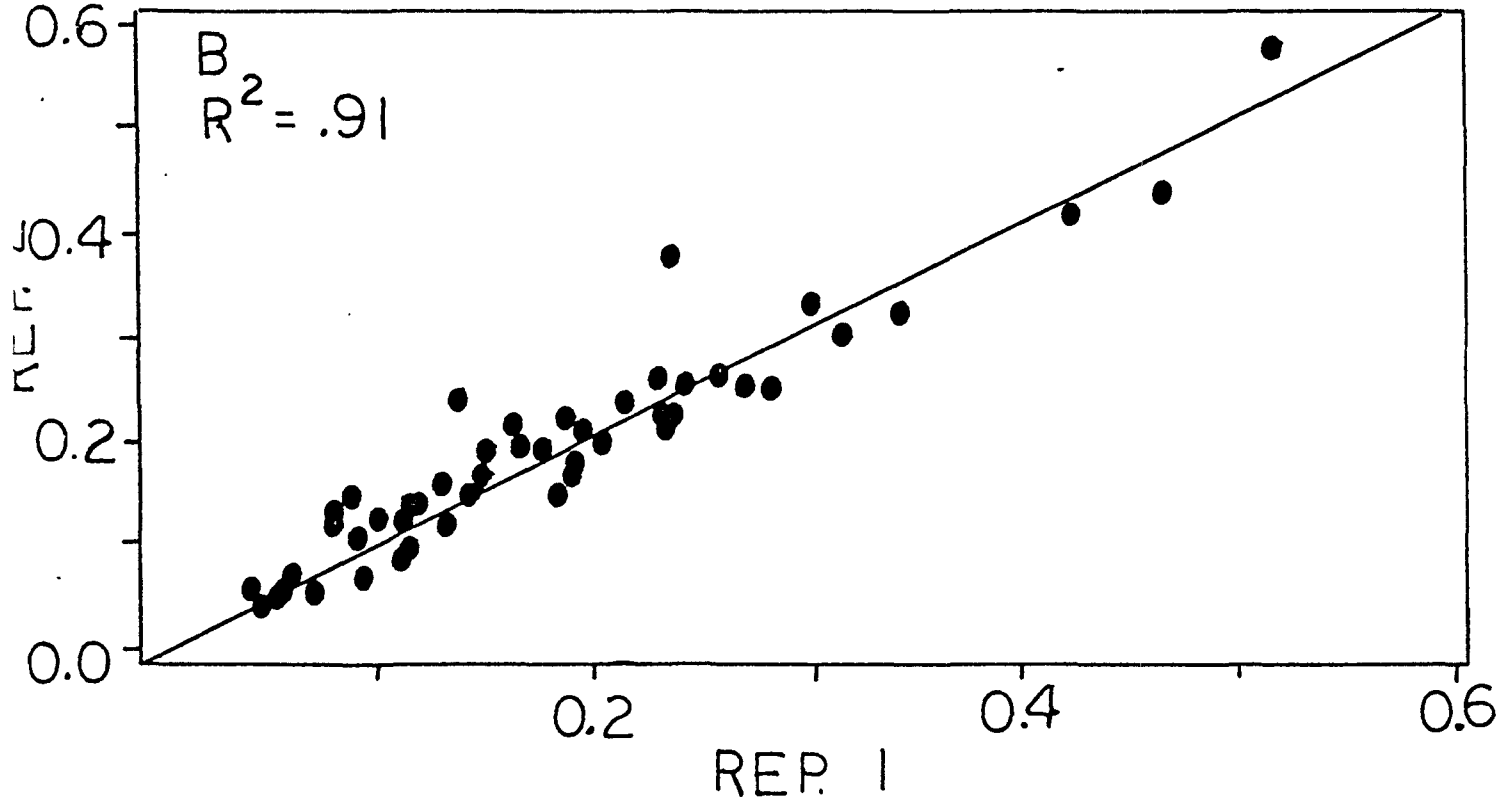


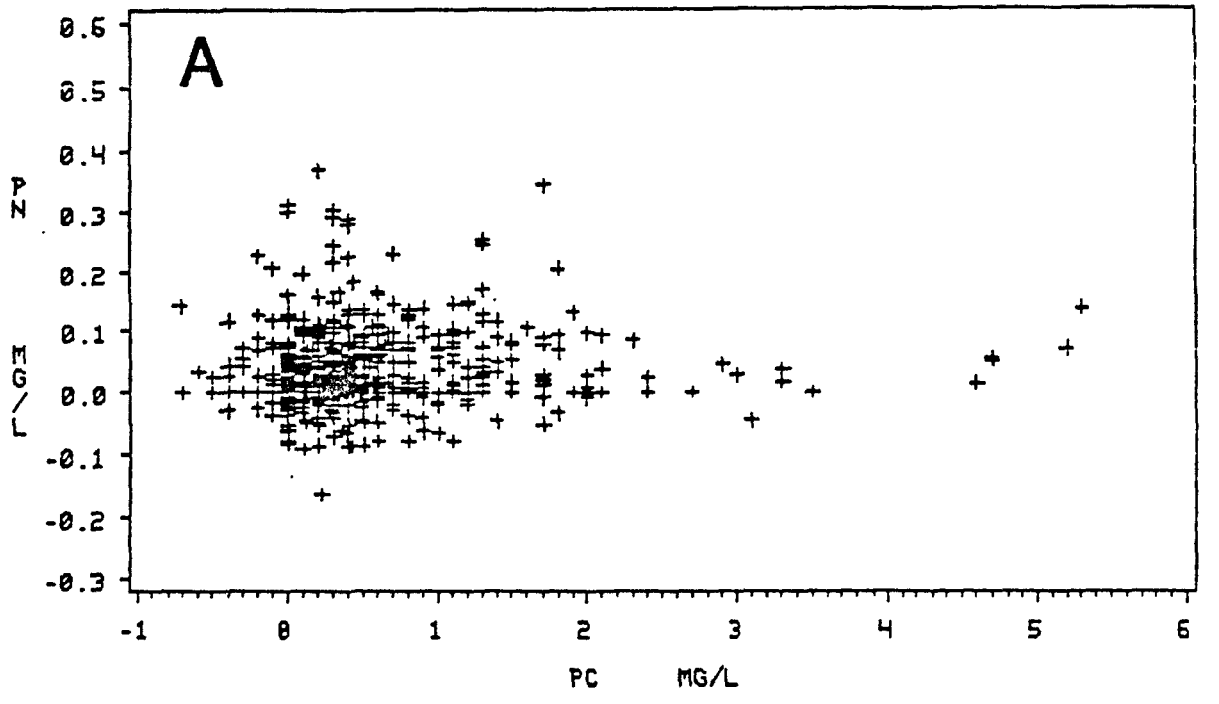
Figure 8. Scatterplots of field duplicates (i.e. two independent determinations on duplicate samples) for particulate nitrogen determined by (A) the "by difference" (EPA) and (B) "direct" (CBL) procedures. Note that the ideal relationship would be 1:1 with an r^2 of 1.0.



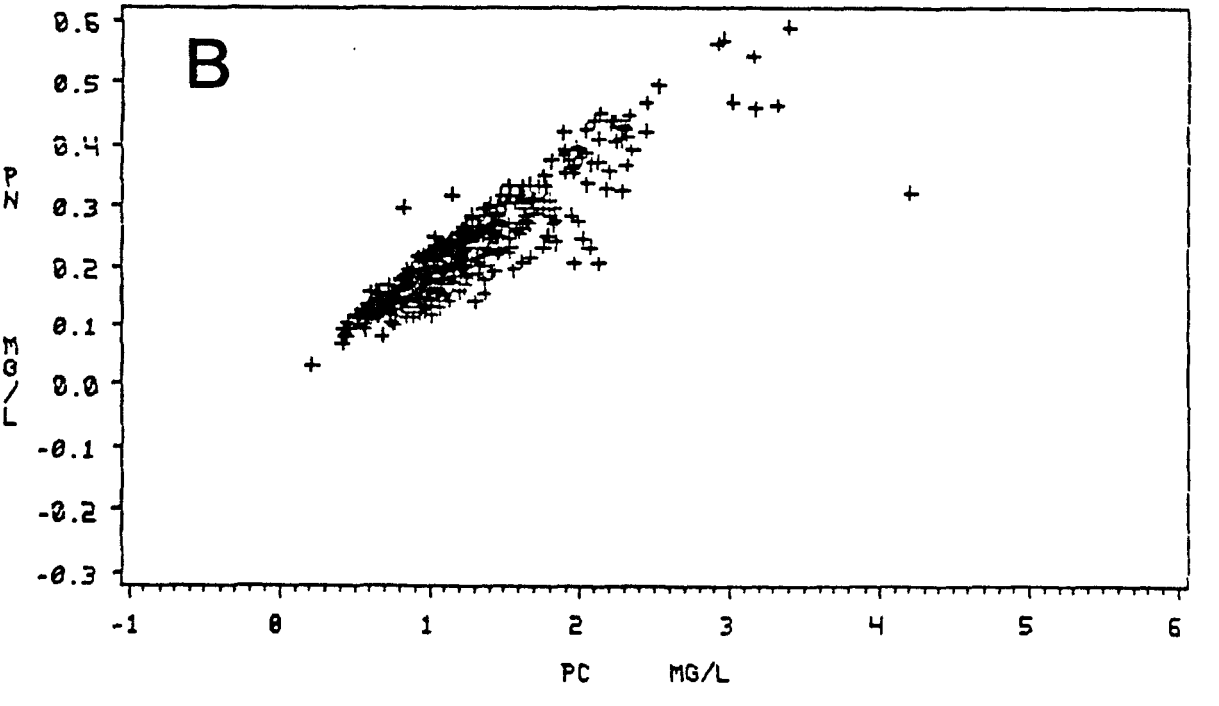


ship between PC and PN for a given sample should normally be approximately 6:1 or higher. S = Surface and AP = Above Pyconocline.

PN VS PC (S + AP VALUES)-- 01JUL84-15MAY85



PN VS PC (S + AP VALUES)-- 16MAY85-30SEP85



the high variability of the two "by difference" techniques and show no obvious correlation. On the other hand, the data in Fig. 9B show a strong correlation between PC and PN, as expected from previous studies (Flemer and Biggs 1971). Correlations between PC and PN obtained by difference seem implausible, whereas those obtained directly provide interpretable results.

Dissolved Kjeldahl Nitrogen vs Dissolved Alkaline Persulfate Nitrogen. Monthly comparisons between the dissolved Kjeldahl methodology and alkaline persulfate total dissolved methodology (minus nitrate and nitrite, for comparability, since the Kjeldahl digestion cannot convert these oxidized forms to ammonium) are presented in Figure 10.

We felt unsatisfied with our Kjeldahl methodology through June, which was the same as practiced at EPA Central Regional Laboratory and as discussed by D'Elia et al. (1986). Colleagues at Old Dominion University recommended that we use their slight modifications to the Kjeldahl method for the July-September samples; these modifications improved the analyses immensely. The July-September data set demonstrates closer comparability of these two methods. When alkaline persulfate dissolved N was compared to dissolved Kjeldahl N plus nitrite and nitrate, a slope of 0.849 was determined (Appendix B, Fig. B.3). The range of dissolved N measured by the two methods was similar, from approximately 0.2 to 0.8 mg N/L, with the majority of values between 0.3 to 0.6 mg N/L (Fig. 10A); corresponding total dissolved N values ranged from ca. 0.2 to 1.5 mg N/L (Fig. 10B). Accordingly, analytical variance (Table 1) is high relative to the range of values encountered. This variance greatly complicates the comparison.

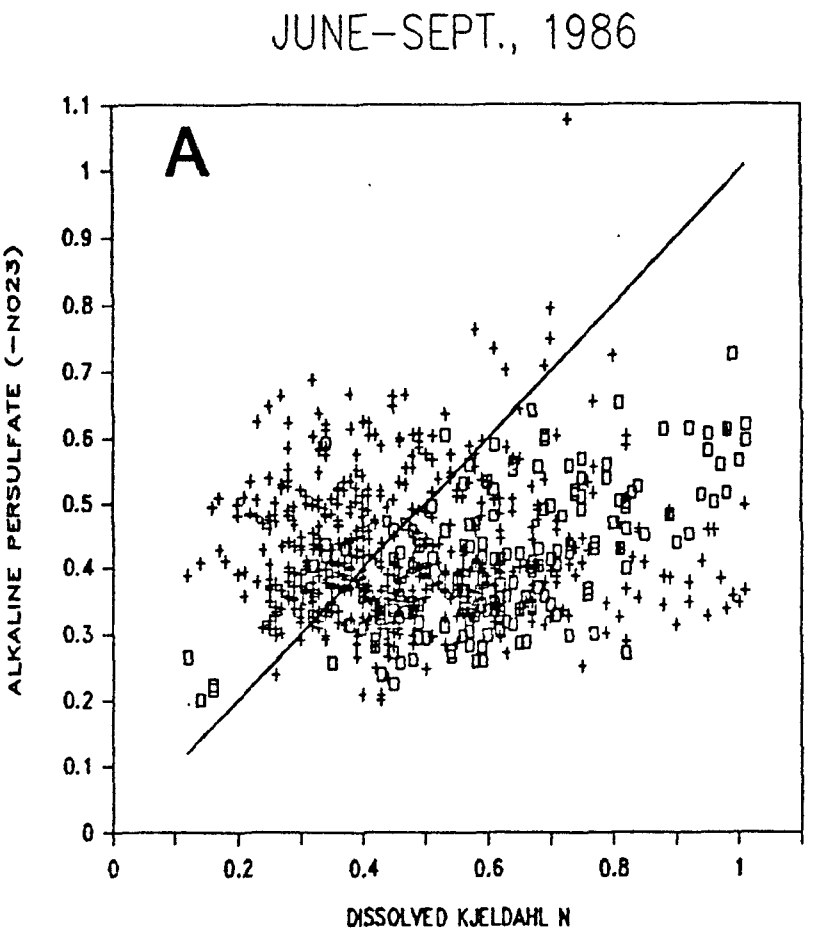
A convenient way to examine analytical error as a function of analyte concentration is to plot the percent coefficient of variation (CV%) of replicates *vs* mean replicate concentration. Fig. 11 shows that for field duplicates, the CV% decreases from approximately 15% at 0.3 mg N/L to less than ca. 10% at 1.0 mg N/L. In contrast, for Kjeldahl dissolved N, the CV% appears much higher at all concentrations.

Total Nitrogen Comparisons. Total nitrogen comparisons were made from CBL data collected between July and Sept., 1986 after implementation of improved Kjeldahl techniques. Total nitrogen was calculated first by summing the alkaline persulfate total dissolved nitrogen and PN, and then by summing dissolved Kjeldahl N, nitrate plus nitrite and PN. Each of these two calculated values were compared with the total nitrogen calculated from the sum of whole water Kjeldahl analysis plus nitrate and nitrite. These comparisons are shown in Fig. 12 for log-transformed data. This transformation

Table 1. Comparison of field duplicate samples for particulate nitrogen and phosphorus determined by the direct and "by difference" techniques, June - Sept., 1986. Also compared are dissolved phosphorus samples using the alkaline and acid persulfate techniques, and dissolved nitrogen using the Kjeldahl and alkaline persulfate techniques for the same time period. The paired comparisons reflect equivalent determinations. "Kjeldahl" [in quotes] signifies dissolved organic nitrogen plus ammonia, while Kjeldahl [no quotes] signifies the Kjeldahl procedure specifically.

	Number of Paired Samples	Mean (mg/L)	Standard Deviation	Coefficient of Variation	Coefficient of Determination
Particulate N					
Direct	63	0.180	0.011	5.80	0.995
"By Difference"	60	0.172	0.125	72.7	0.659
Particulate P					
Direct	64	0.021	0.002	10.6	0.990
"By Difference"	61	0.025	0.006	23.9	0.835
Dissolved N					
Alkaline Persulfate	63	0.615	0.060	9.71	0.889
Kjeldahl + Nitrate	61	0.633	0.070	11.0	----
"Kjeldahl" Dissolved N					
Alk. Pers. - Nitrate	63	0.433	0.062	14.4	----
Kjeldahl	61	0.434	0.068	15.8	0.905
Dissolved P					
Alkaline Persulfate	63	0.0238	0.0049	20.7	0.929
Acid Persulfate	62	0.0276	0.0037	13.3	0.958

Figure 10. Scatterplots of (A) dissolved Kjeldahl nitrogen (i.e. dissolved organic nitrogen plus ammonium) determined by Kjeldahl digestion (x-axis) or by alkaline persulfate digestion minus nitrate+nitrite (y-axis), and (B) dissolved nitrogen (i.e. dissolved inorganic plus organic nitrogen) determined by Kjeldahl digestion plus nitrate+nitrite (x-axis) and alkaline persulfate digestion.



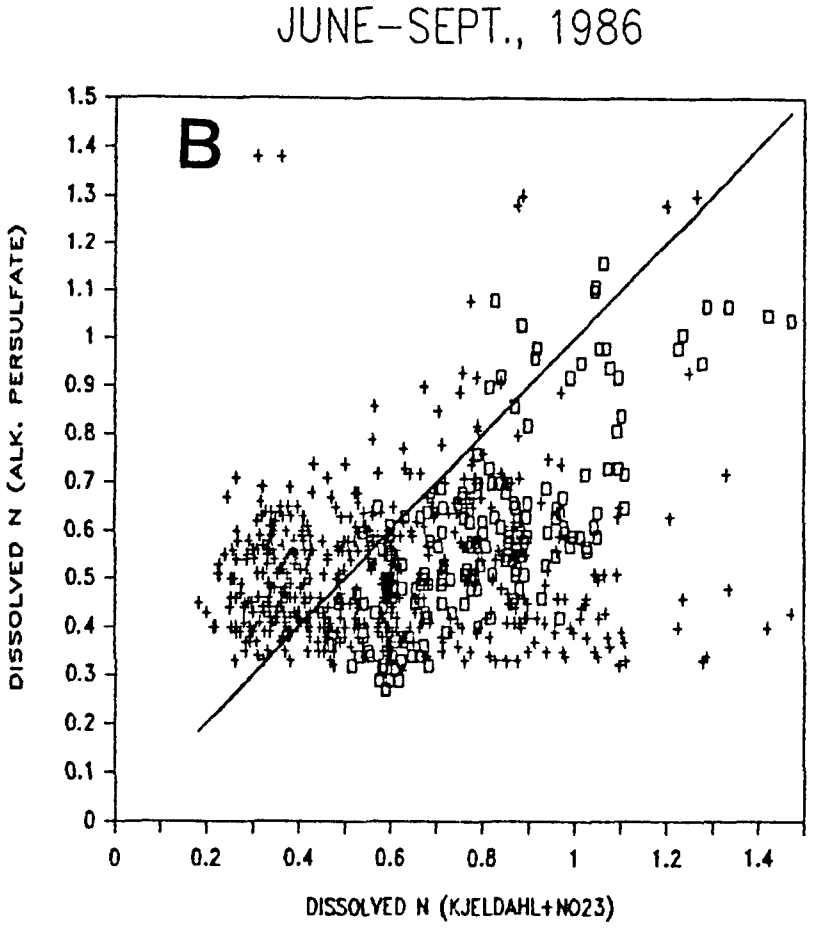
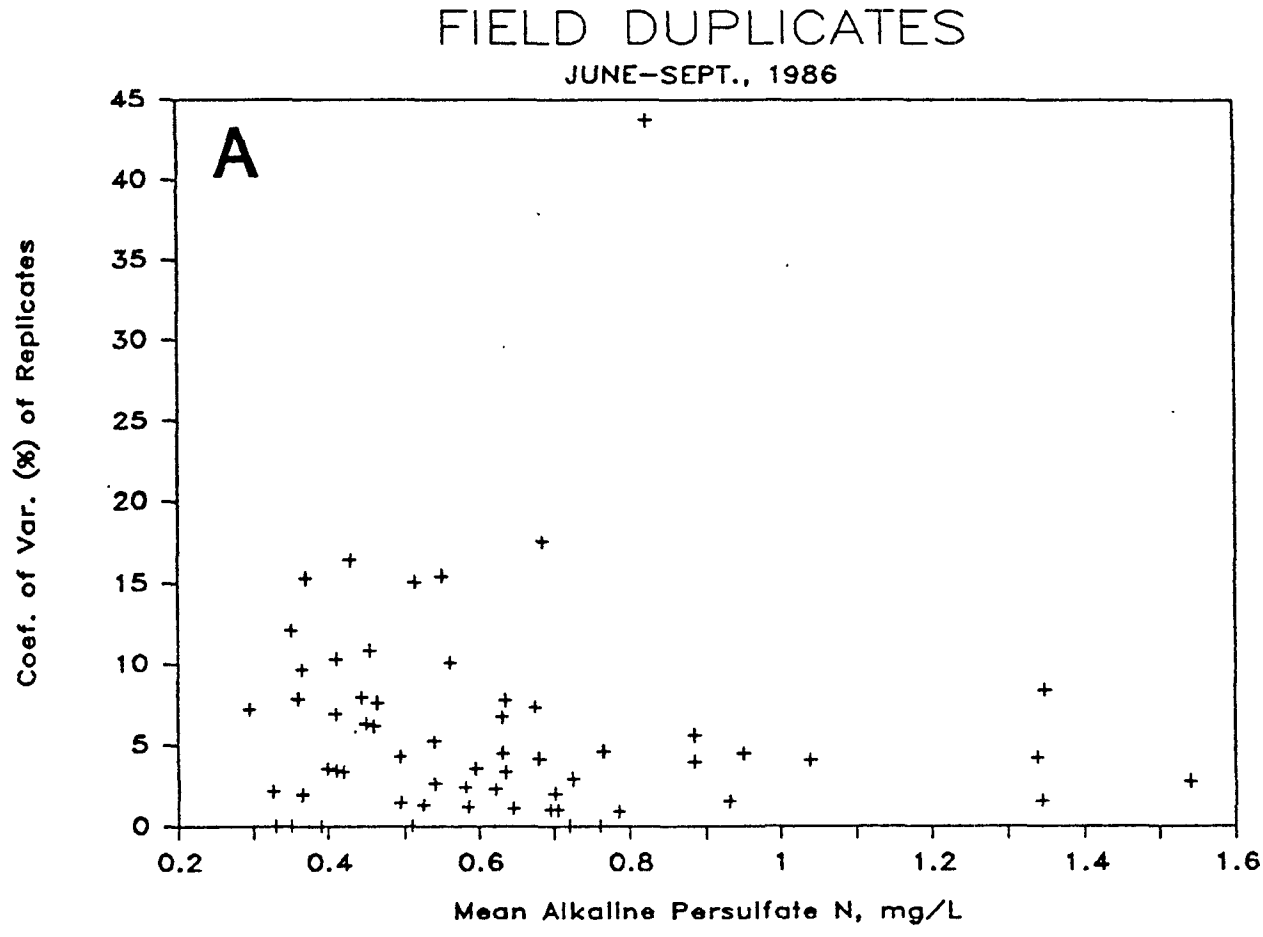
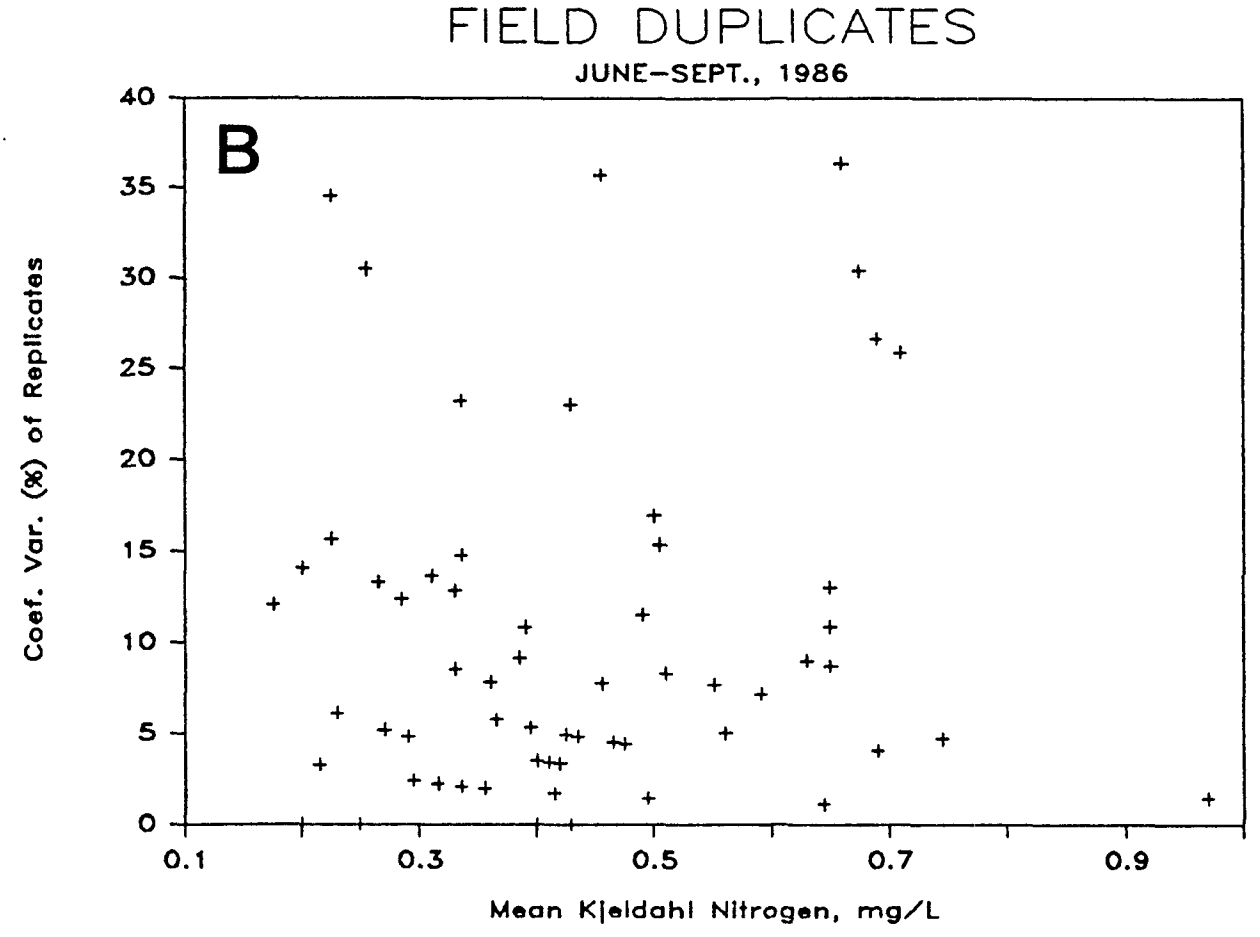


Figure 11. Coefficient of variation *vs* concentration for field duplicates using (A) alkaline persulfate nitrogen determination, and (B) Kjeldahl nitrogen determination.





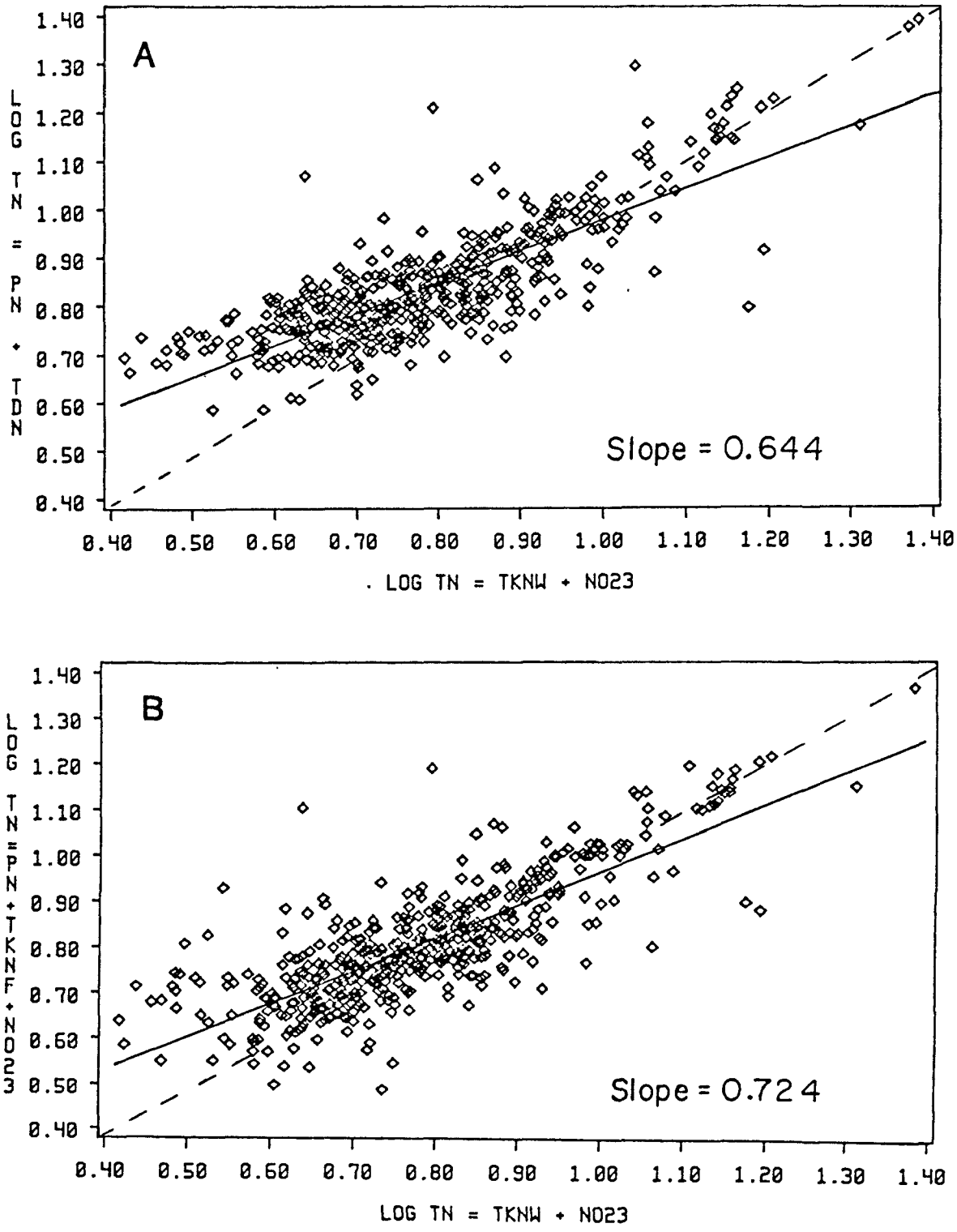


Figure 12. Comparison between (A) total nitrogen determined by total Kjeldahl plus nitrate+nitrite determ nation (x-axis) and bydirect particulate determination plus dissolved nitrogen by alkaline persulfate d termination, and (B) direct particulate N determination plus dissolved nitrogen by Kjeldahl and n trate+nitrite determinations. Values have been $\log_{10}$ transformed. Solid line is least-squares best fi Dashed line denotes equivalence.

Table 2. Slope Coefficients for Log/Log Models with unrestricted intercept terms. DF= degrees of freedom; r^2 = coefficient of determination; LB = Lower Bound of 95% Confidence Interval for the Slope parameter; UB = Upper Bound of 95% C.I. for Slope. TN = Total Nitrogen, PN = Particulate Nitrogen (Direct), TDN = Alkaline Persulfate Dissolved Nitrogen, TKNT = Kjeldahl Nitrogen unfiltered, NO23 = Nitrate+Nitrite, TKND = Kjeldahl Nitrogen Dissolved, TP = Total Phosphorus, PP = Particulate Phosphorus (Direct), TDP = Alkaline Persulfate Dissolved Phosphorus, APUP = Acid Persulfate Phosphorus unfiltered, APDP = Acid Persulfate Phosphorus Dissolved.

Comparison	D.F.	r^2	Slope	LB	UB
(TN = PN + TDN) vs. (TN = TKNT + NO23)	526	0.66	0.644	0.604	0.684
(TN = TKND + NO23 + PN) vs. (TKNT + NO23)	527	0.66	0.724	0.678	0.769
(TP = PP + TDP) vs. APUP	731	0.85	1.150	1.108	1.192
(TP = PP + APDP) vs. APUP	730	0.88	0.988	0.957	1.019

stabilized variance as well as provided a more even distribution of data along the tested range of values. Coefficients of determination, slopes and 95% confidence intervals of the slope for linear regressions of this comparison are presented in Table 2. The appropriate model for comparing these values is a bivariate normal correlation model which does not assume dependent or independent variables. However, the equivalence of this model with the normal error regression model permits conditional inferences to be made using standard regression techniques (Neter and Wasserman 1974, p. 402-403).

The slopes for both comparisons are significantly different from 1 (Table 2) and the least-squares regression line intersects the equivalence line somewhere near the median values. The total nitrogen calculation using dissolved Kjeldahl nitrogen (Fig. 12B) appears to provide somewhat better correspondence, although spurious correlation problems may be responsible for this since both X and Y values may include nitrate as a major component. For both comparisons, the differences between techniques are greatest for either low or high values, suggesting a concentration-related effect. The causes for this divergence have not yet been examined. The Kjeldahl analysis would more likely be affected by changes in concentration than would direct particulate analysis. This occurs because the quantity of particulate material retained on filters is determined by the quantity of particulates in the water at the time the sample is collected. More water is passed through the filters when particulate samples are low than when concentrations are high. Thus, the range in amount of material actually retained on the filter pad is much lower than the range of concentrations present in the sample.

Phosphorus

Direct vs "By Difference" Particulate Phosphorus Determination. Comparison of these two methods showed the direct measurement (r^2 = 0.990) to be more precise than "by difference" (r^2 = 0.835, Table 1). Concentrations determined by the direct measurement of particulate P were generally slightly lower than those values obtained "by difference," but followed the line of 1:1 comparability quite well (Fig. 13) and a slope of 0.702 was obtained for this comparison (Appendix B). Several negative values occurred for PP determined "by difference," but no negative values resulted from direct PP determination.

National Bureau of Standards (NBS) reference material 1646 (estuarine sediment) was analyzed using the direct measurement protocol. The certified concentration of phosphorus (weight %) was reported by NBS to be 0.054±0.005. CBL obtained a value of 0.049, which is within the standard deviation of the analysis.

A graphical and statistical comparison of duplicate analyses of water samples for particulate P is presented in Figure 14. Figure 14A presents PP data determined "by difference" at EPA/CRL for the period June, 1984 - May, 1985. Fig. 14B presents data

Figure 13. Scatterplots of particulate phosphorus determination by "by difference" determination (x-axis) vs by "direct" determination (y-axis).

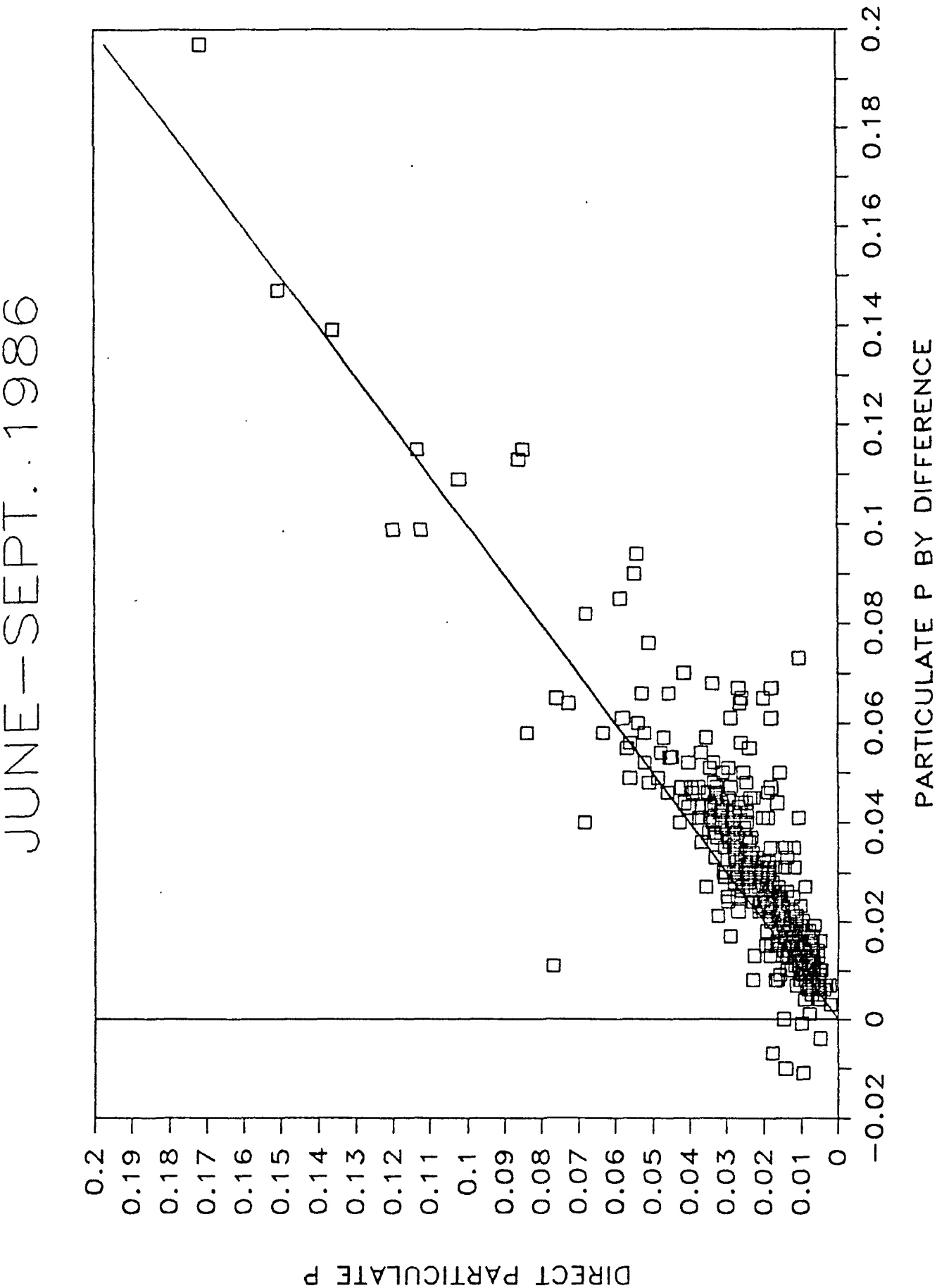
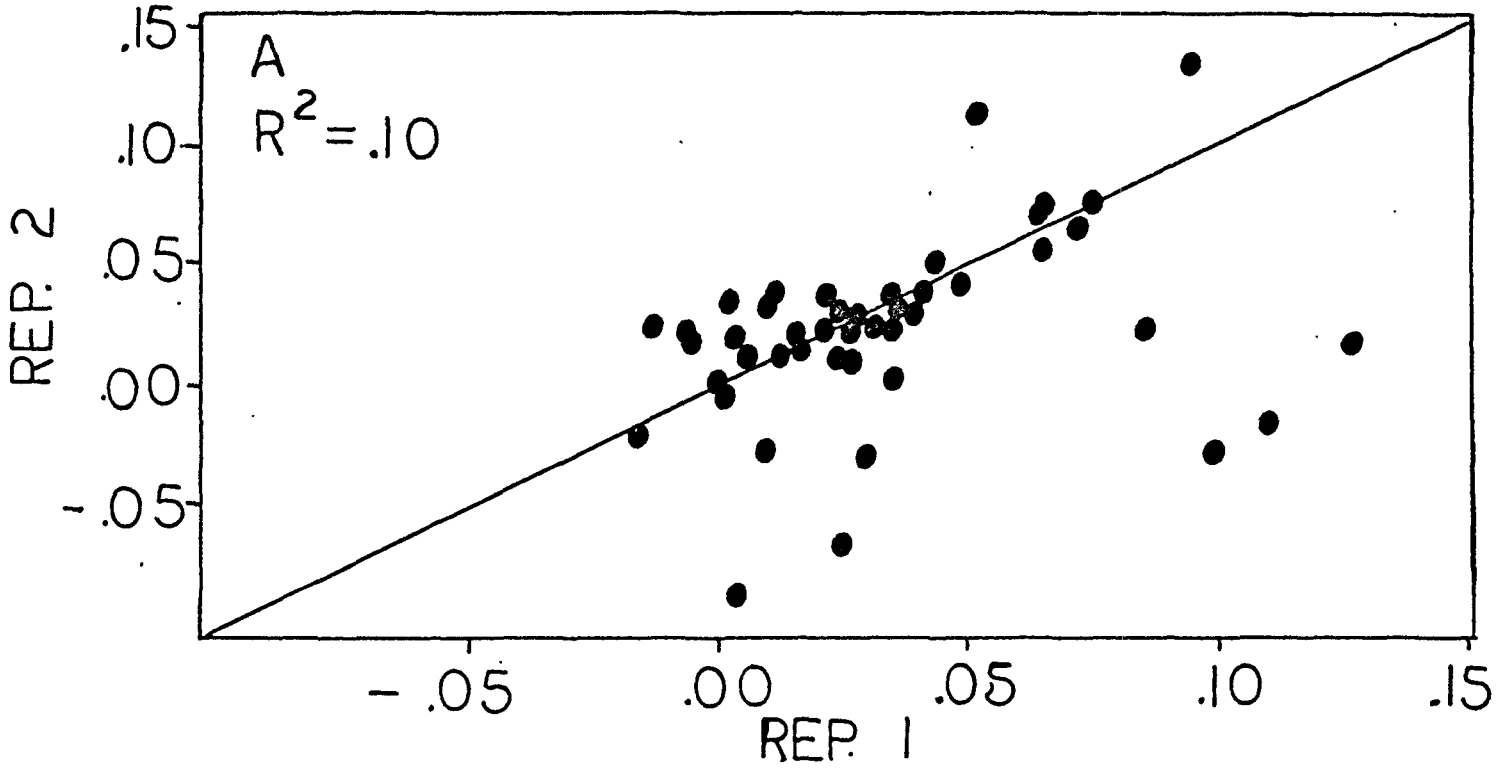
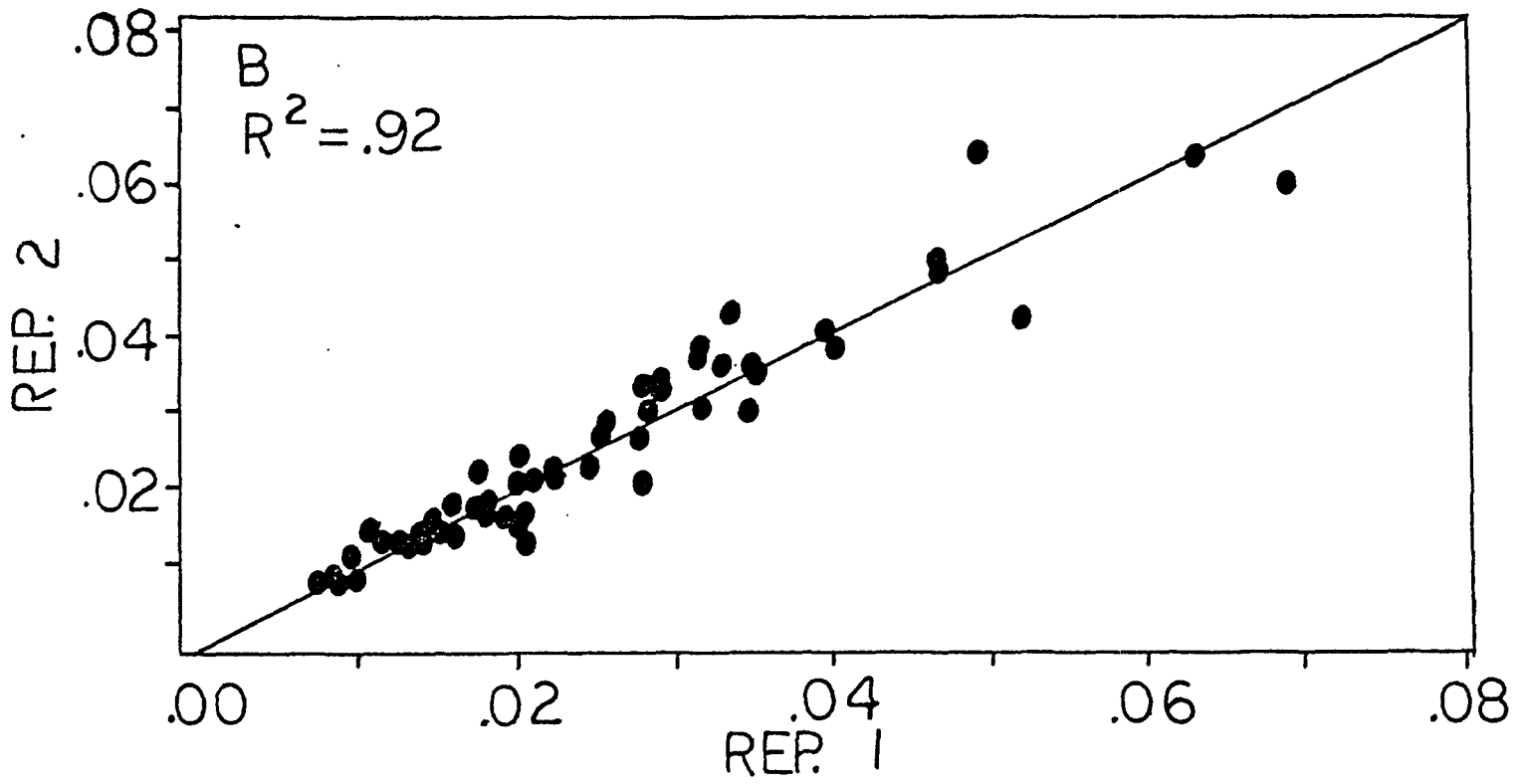
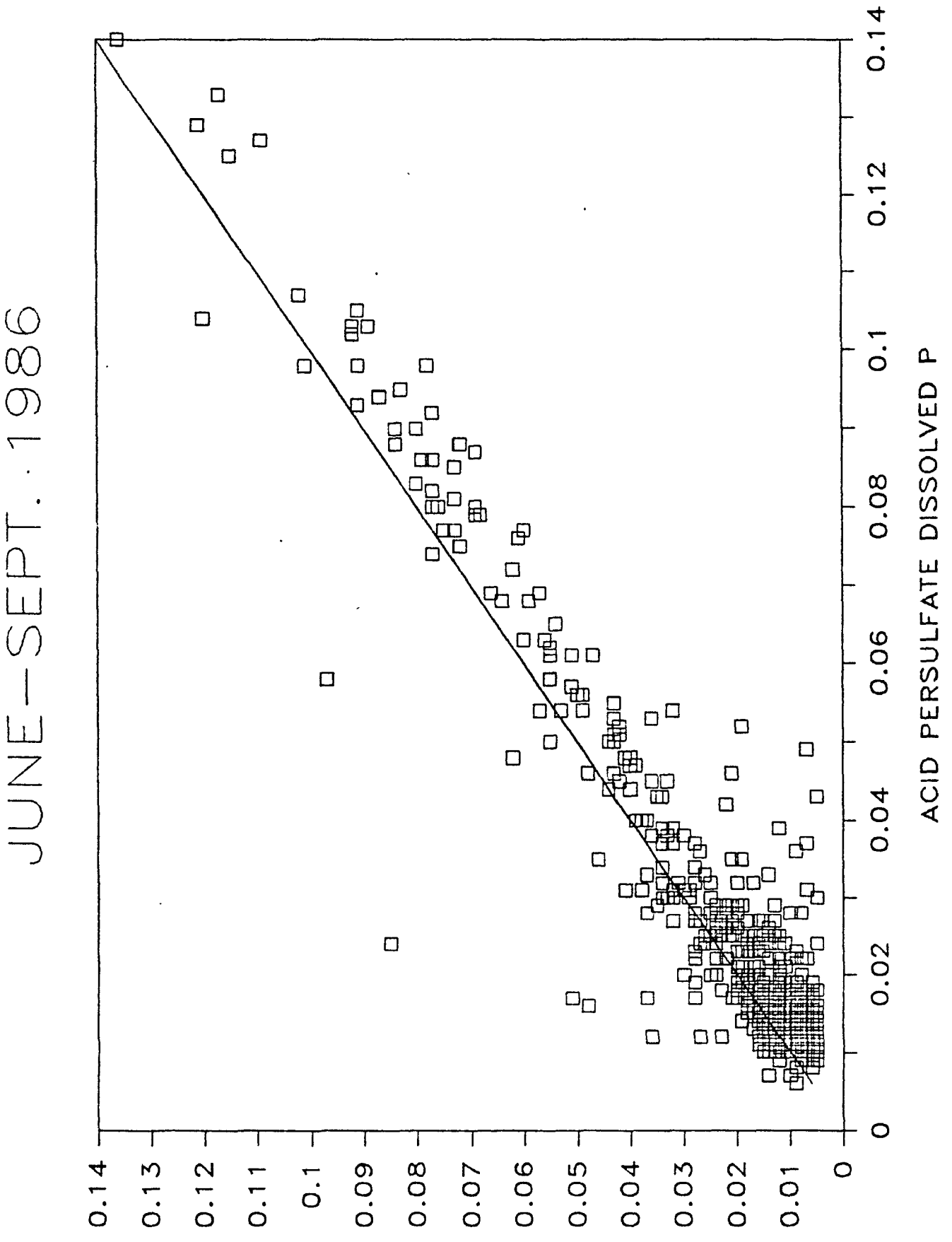


Figure 14. Scatterplots of field duplicates (i.e. two independent determinations on duplicate samples) for particulate phosphorus determined by (A) the "by difference" (EPA) and (B) "direct" (CBL) procedures. Note that the ideal relationship would be 1:1 with an r^2 of 1.0.





and alkaline persulfate dissolved P determination (y-axis) on duplicate samples. Solid line denot
equivalence.



analyzed at CBL using direct PP determination for the period May, 1985 - June, 1986. The data for PP duplicates "by difference" produces many negative values and is obviously more variable (r^2 = 0.10) than PP determined directly (r^2 = 0.92). Thus precision is vastly improved by PP direct determination. Appendix A presents r^2 values of field duplicates for the present study.

Acid Persulfate Dissolved Phosphorus vs. Alkaline Persulfate Dissolved Phosphorus. Data from the two alternative methods for determining total dissolved phosphorus are presented in Table 1 and Figure 15. A slope of 0.954 indicates a strong equivalence between the two methods. The acid persulfate dissolved phosphorus technique produced slightly higher values than the alkaline persulfate dissolved P method. Although both methods give excellent percent recoveries of organic phosphorus compounds, the difference may relate to the internal dilution of the alkaline persulfate method. An internal dilution factor of 2.85 is used to extend the analytical range in this method as it is routinely performed at CBL. For low concentration periods of the year, the precision is less than that obtained using acid persulfate digestion. However, in practice, the differences encountered are slight and the internal dilution factor can be adjusted as needed for future work.

Total Phosphorus Comparisons. Comparisons between total phosphorus determined directly by the acid persulfate digestion on a whole water sample and total phosphorus obtained by summing the direct determination of the particulate fraction with dissolved phosphorus determined with either the alkaline persulfate or acid persulfate methods were very comparable. Comparisons of the two methods with total acid persulfate P are shown in Fig. 16 for log-transformed data collected on cruises between June and Sept., 1986. This transformation stabilized variance as well as provided a more even distribution of data along the tested range of values. Coefficients of determination, slopes and 95% confidence intervals of the slope for linear regressions of this comparison, are presented in Table 2. The slopes of both regression lines are very close to 1 and, in the case of the acid persulfate dissolved plus particulate P comparison, a slope of 1 is included in the 95% confidence interval. The very high number of degrees of freedom produce very tight confidence intervals, and these intervals should be judged in light of other sources of variation inherent in the nutrient determinations. The regression lines are also very close to the equivalence lines indicating very close correspondence between these alternative techniques.

Comparison of PP derived "by difference" (Fig. 17A) and directly (Fig. 17B) with an independent variable, PN, clearly showed the superiority of direct determination for reasons discussed above.

Cost Comparisons

In comparison with EPA-mandated methods, those recommended by CBL and routinely performed in our laboratory (direct measurement of PC, PN, and PP; alkaline persulfate dissolved N and P) can result in a substantial savings of analytical costs while improving sensitivity and turnaround time.

The reasons are twofold. First, unlike the alkaline persulfate method, the analysis of Kjeldahl nitrogen is a very time-consuming, tedious and hazardous process: the cost per sample reflects this (D'Elia et al. 1986). An additional carbon analysis is also required for the whole-water C fraction. Secondly, particulate C and N concentrations are determined simultaneously, thus eliminating the need for two separate analyses. The same is true for the alkaline persulfate technique that is used to digest dissolved N and P together.

A cost breakdown is presented in Table 3. During a one year (20-cruise period), a savings of $76,000 (including 20% overhead) could be realized in CBL's contract alone. Any additional start-up costs

Table 3. Analytical costs associated with CBL-recommended methods and EPA-required methods based on present per-sample charges and requirements of Chesapeake Monitoring Program.

	CBL	EPA
Inorganic Nutrients[a,b]	$17.50	$17.50
Suspended Solids[a,b]	3.75	3.75
Dissolved Organic C[a,b]	15.00	15.00
Whole-water Organic C[a]	---	15.00
Dissolved Kjeldahl N[a]	---	18.50
Whole-water Kjeldahl N[a]	---	18.50
Acid Persulfate Dissolved P[a]	---	8.00
Acid Persulfate Whole-water P[a]	---	8.00
Particulate C and N[b]	10.00	---
Alkaline Persulfate N and P[b]	11.50	---
Particulate P[b]	11.75	---
Total:	$69.50	$104.25

Percent Savings: 33.3%

[a]Required by EPA.

[b]Recommended by CBL and OEP.

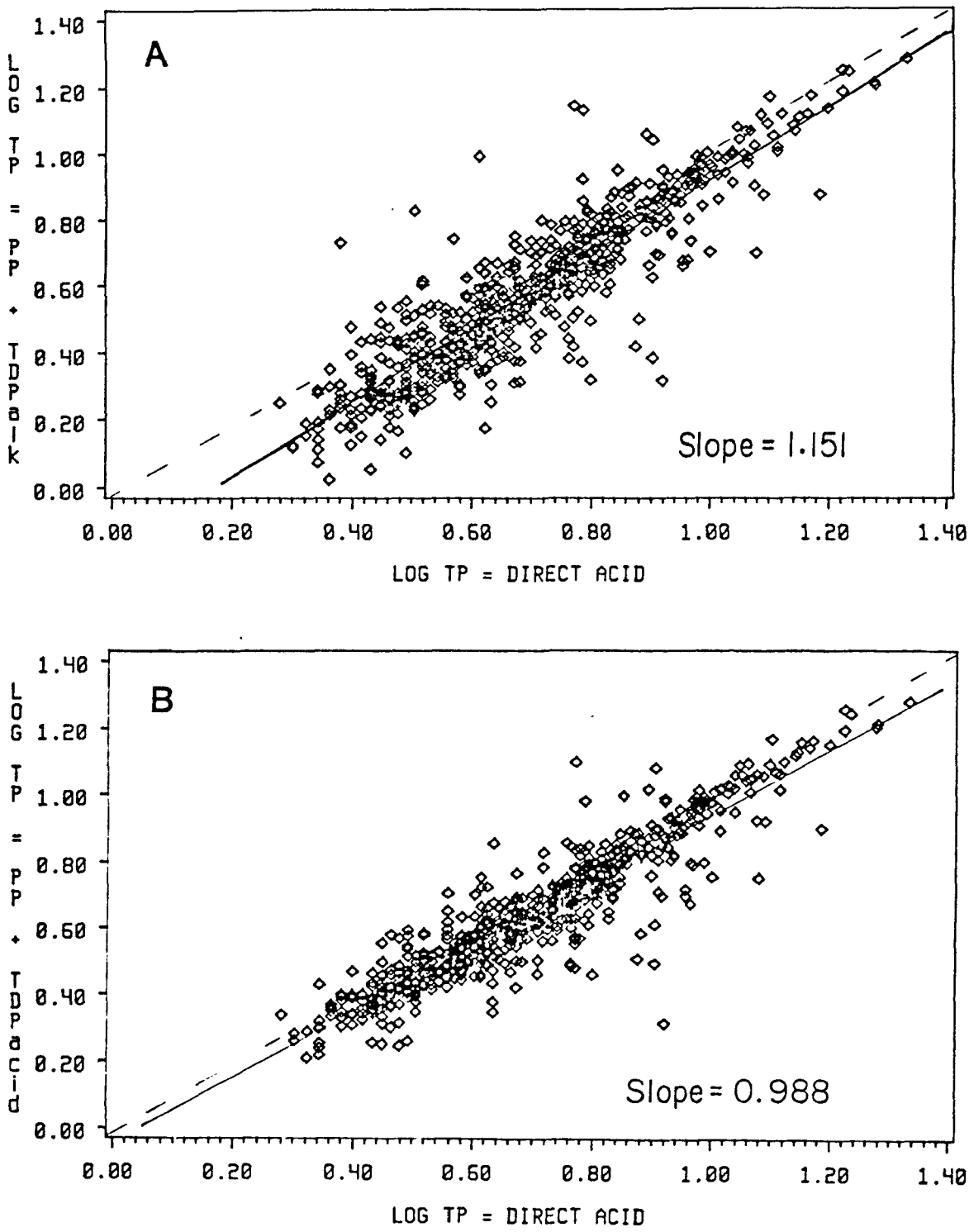
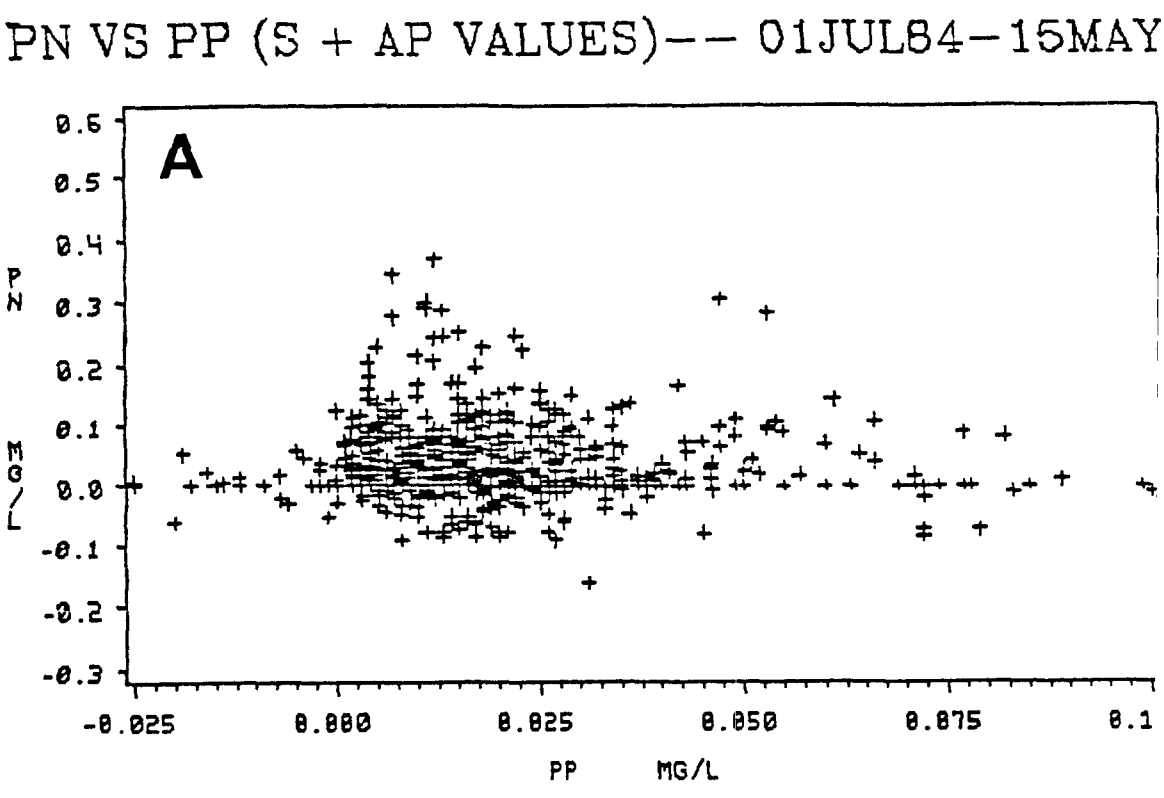


Figure 16. Comparison between (A) total phosphorus determined by the whole-water acid persulfate phosphorus procedure (x-axis) and by direct particulate phosphorus determination plus dissolved phosphorus by alkaline persulfate determination, and (B) total phosphorus determined by the whole-water acid persulfate phosphorus procedure (x-axis) and by direct particulate phosphorus determination plus dissolved phosphorus by acid persulfate determination. Values have been $\log_{10}$ transformed. Solid line is least-squares best fit. Dashed line denotes equivalence.



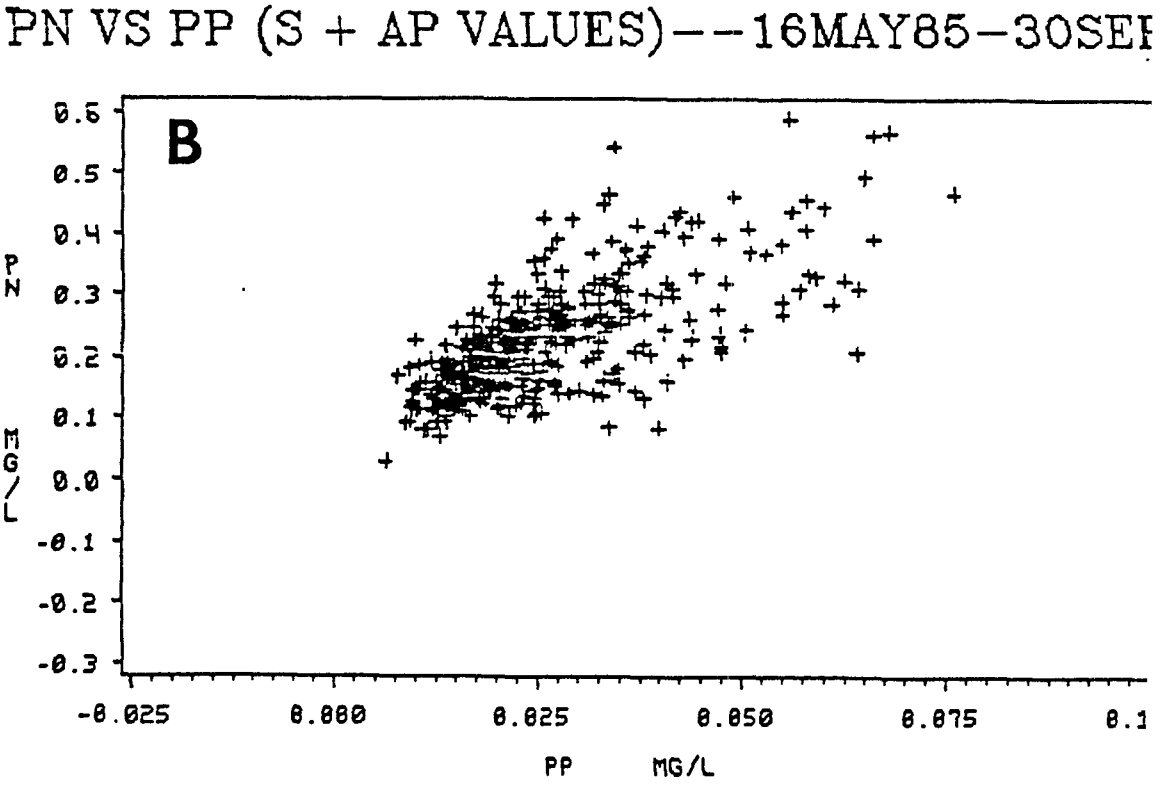


Figure 17. Scatterplots of particulate nitrogen (PN) *vs* particulate phosphorus (PP) on duplicate samples using (A) "by difference" and (B) "direct" determinations. There should be no negative values, and the relationship between PN and PP for a given sample should normally be approximately 7:1 or higher. S = Surface and AP = Above Pynocline

required for initiating elemental analysis are quickly offset through more efficient personnel use and higher sample throughput and convenience. Furthermore, modern instruments for elemental analysis are more reliable and easy to operate than those introduced two decades ago.

Detection Limits

We emphasize that although using the alternative techniques recommended by CBL reduces costs, it does not reduce analytical sensitivity: in fact, it generally increases precision (Table 4), and accuracy is not affected (Appendix E).

SUMMARY

1. Field replicate data indicates that the direct measurement of particulate N is more precise (for replicates, r^2=0.995) than the "by difference" technique (for replicates, r^2=0.621). When the two methods were compared to each other, a slope of 0.610 was obtained, an artifact of the unequal variances of the two methods.

2. Kjeldahl and persulfate N techniques determine different things, and therefore are not directly comparable without correction. A slope of 0.724 was obtained when total nitrogen (direct particulate N, dissolved Kjeldahl N and nitrite plus nitrate) was compared to Total Kjeldahl N and nitrite plus nitrate while total nitrogen (direct particulate N and alkaline persulfate dissolved N) compared to total kjeldahl N and nitrite plus nitrate yielded a slope of 0.644. The persulfate technique is more precise than the Kjeldahl technique, however, when derivative total N or dissolved organic N values are obtained, the precision of the two is nearly equal. The persulfate technique is much easier to perform and costs less. Based on the present study, neither can be relied upon to yield quantitative recovery of dissolved organic nitrogen, and it is erroneous to assume that the Kjeldahl technique is a true standard for comparison with other techniques.

3. Comparison of whole water total nitrogen methods indicated that the precision of field replicates analyzed by the direct measurement of particulate N combined with dissolved kjeldahl N and nitrite plus nitrate were exactly the same as total N calculated with particulate N (direct) plus alkaline persulfate dissolved N (r^2=0.954).

4. Field replicate data indicate that the direct measurement of particulate P is more precise (r^2=0.990) than the "by difference" technique (r^2=0.835). When the data for both methods were plotted against each other; a slope of 0.702 was obtained. On the average, the acid persulfate method

Table 4. Comparison of CBL/OEP field detection limits, based on actual field duplicates (see Table 1). Field detection limits are based on 3 standard deviations of field duplicates, accordingly, actual *analytical* detection limits at CBL are lower for all analytes. Note that the Chesapeake Bay Monitoring Program analytical detection limits, which are shown for reference, are more stringent than those required by EPA.

Parameter	CBL/OEP Field Duplicate (mg/L)	(uM)	Monitoring Program (mg/L)	(uM)
Dissolved Nitrogen				
Kjeldahl[a]	0.204	14.6	0.20	14.2
Alkaline Persulfate[b]	0.18	12.9		
Dissolved Phosphorus				
Acid Persulfate[a]	0.011	0.355	0.01	0.32
Alkaline Persulfate[b]	0.015	0.484		
Particulate Nitrogen				
By Difference[b]	0.375	26.8	0.40	28.4
Direct[b]	0.033	2.36		
Particulate Phosphorus				
By Difference[a]	0.018	0.581	0.02	0.64
Direct[b]	0.006	0.194		

[a]Required by EPA.
[b]Recommended by CBL and OEP.

of determining particulate P "by difference" yielded marginally higher results than those values obtained by the direct analysis. However, with direct measurement, the greater volume filtered yields a more representative sample and negative values cannot occur as they do in the "by difference" technique. As would be predicted from *a priori* statistical considerations, negative particulate values were apparent in virtually every data set where determined "by difference." Most importantly, direct measurement results in at least an order-of-magnitude improvement in limits of detection for N and more than twofold improvement for P.

5. Field replicate data indicate that the precision of the alkaline persulfate dissolved P method (r^2=0.929) is virtually identical to that of the acid persulfate dissolved P method (r^2=0.958) and that when data for both methods were compared with each other, a slope of 0.954 was obtained; indicating that these methods are of comparable accuracy. The acid persulfate dissolved P technique produced slightly higher values than the dissolved alkaline persulfate dissolved P method. In practice, the differences probably relate to a dilution factor used to increase the range of determination. The internal dilution factor of the alkaline persulfate procedure can easily be adjusted for future work. If the alkaline persulfate procedure for N is adopted, a concomitant alkaline persulfate method for P should prove satisfactory.

6. Comparison of whole water total phosphorus methods indicated that the precision of field replicates of acid persulfate unfiltered (r^2=0.964) was almost identical to whole water P determined by direct particulate P plus acid persulfate dissolved P (r^2=.972) and particulate P plus alkaline persulfate dissolved P (r^2=0.949). Slopes of 0.988 and 1.15 were determined when direct particulate plus alkaline persulfate dissolved P were compared to total acid persulfate phosphorus, respectively.

RECOMMENDATIONS

1. Better precision methods should be adopted as soon as possible.

2. The direct measurement of particulate N and P is more precise than determination "by difference." We strongly recommend that measurement of particulate N and P be performed by direct measurement. This also applies to particulate C, which we did not address in this study; improvements in precision should approach those obtained for direct analysis of particulate N. A proof of this obvious conclusion should not require additional study.

3. Dissolved Kjeldahl N and alkaline persulfate dissolved N values correlated better than in a previous study (D'Elia et al. 1986), giving mean concentrations of 0.433 and 0.434 mg N/L on over 60 field duplicates. However, scattergrams of the two parmeters plotted against each other show the high variance of dissolved N, and for given paired comparisons, one cannot expect close correspondence from these high-variance procedures. Accordingly, we recommend that the accuracy of the Kjeldahl determination on salt-matrix samples receive further scrutiny. In any case, despite our use of certain improvements in technique, we do not feel satisfied with the presently used Kjeldahl technique and recommend that more suitable modifications be sought for samples with a saline matrix. It cannot be relied on as an adequate standard with which to compare other methods.

4. The persulfate N technique should receive further scrutiny against an independent (non-Kjeldahl) dissolved nitrogen determination in order to provide an adequate test of its accuracy. It may prove to be more accurate than the Kjedahl N technique.

5. Despite the uncertainties involved, we recommend adoption of the alkaline persulfate digestion and simultaneous determination of dissolved N and P on the digest, which will result in substantial cost savings for the monitoring program without compromising data quality.

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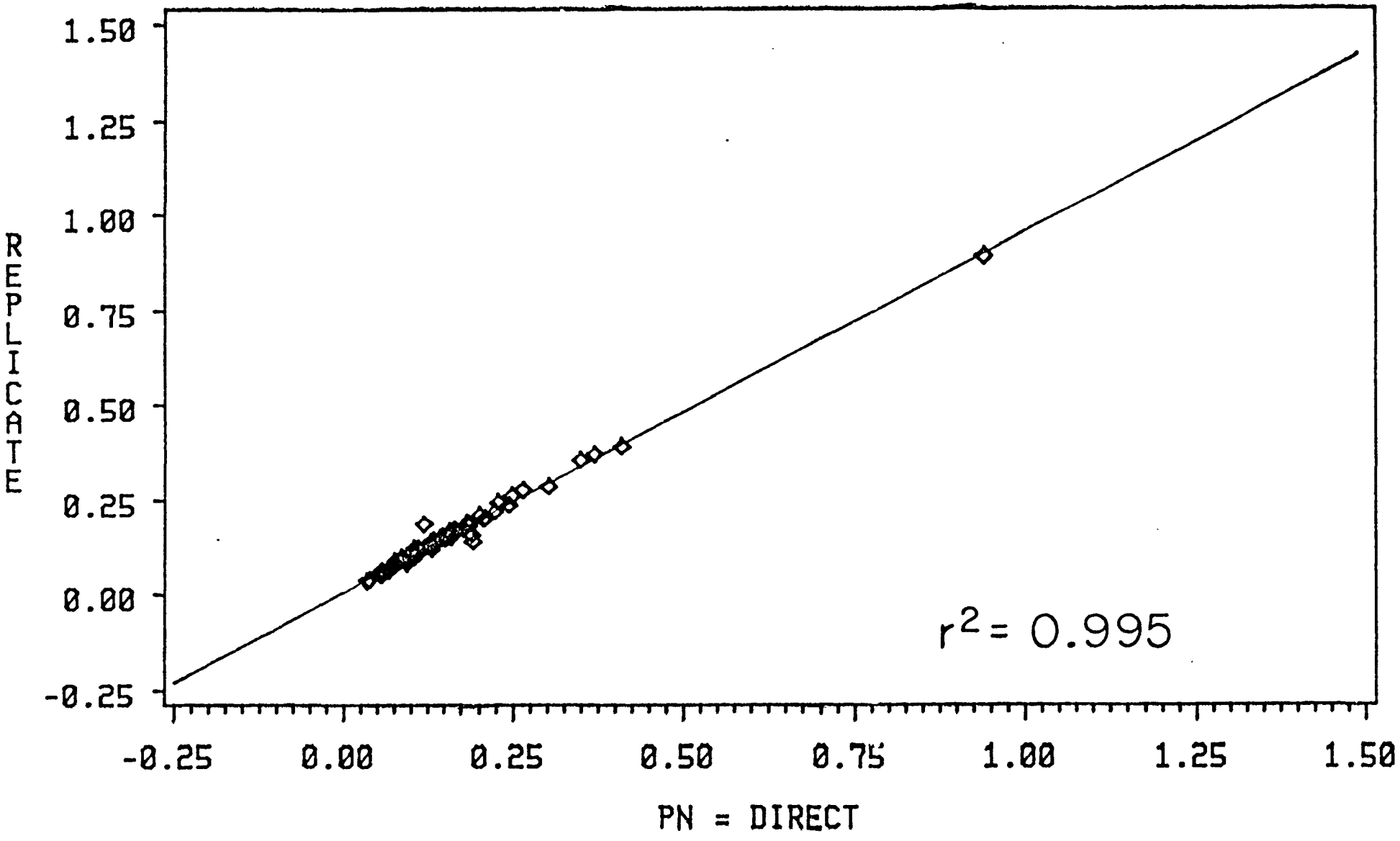
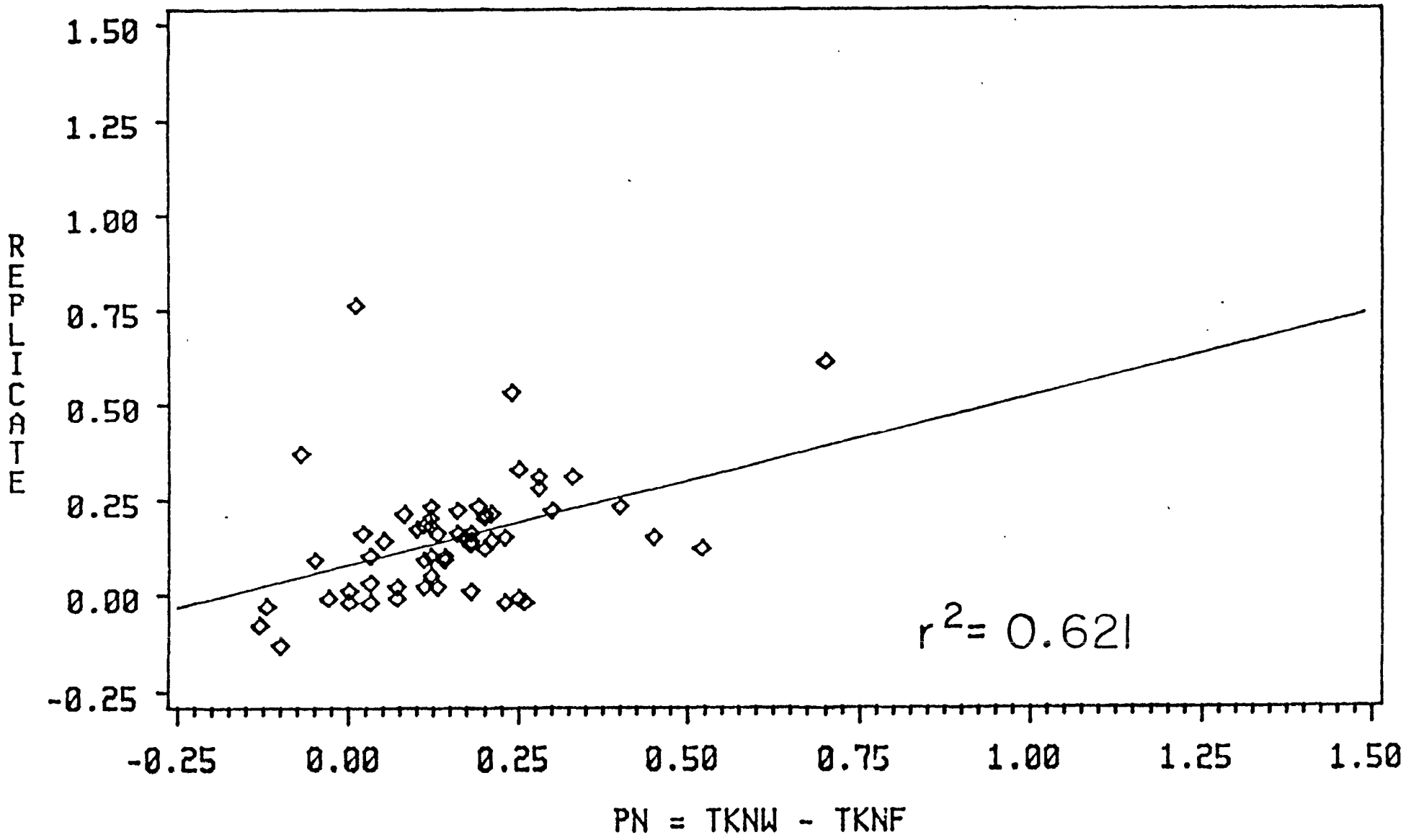
APPENDIX A

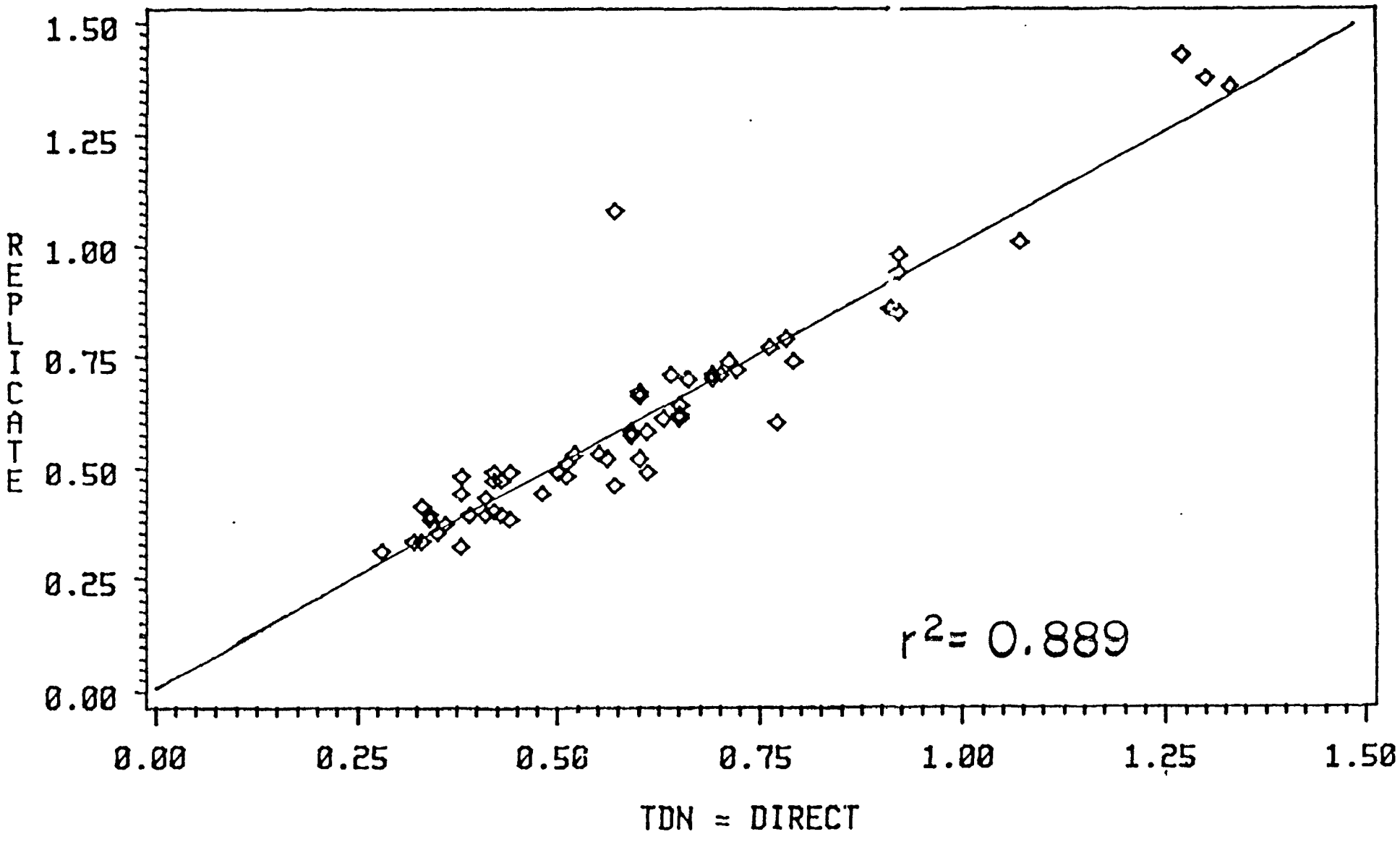
FIELD REPLICATE GRAPHS WITH COEFFICIENTS OF DETERMINATION (r^2) FOR DISSOLVED AND PARTICULATE N AND P



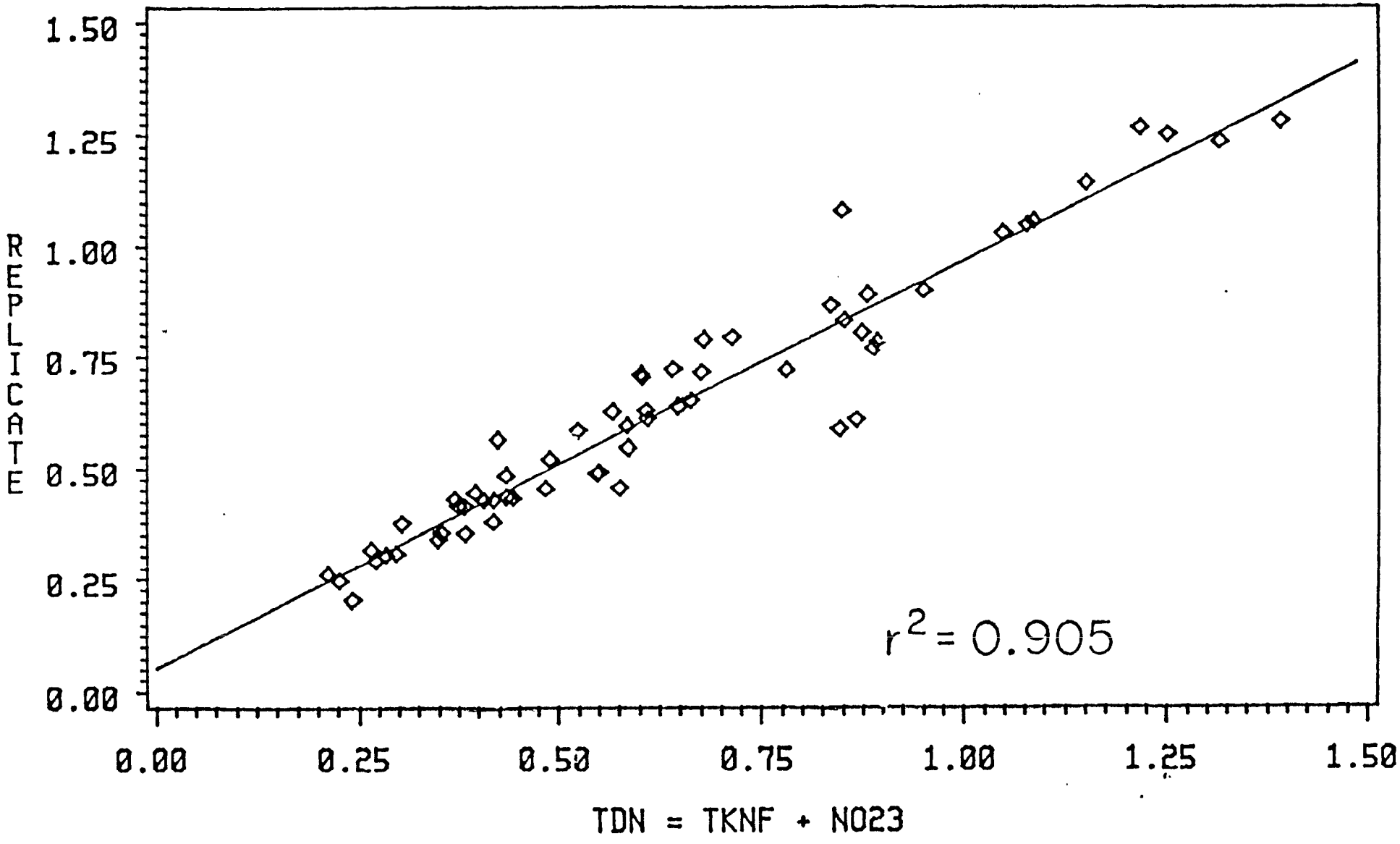
A.1. Field replicate data for the direct determination of particulate nitrogen (June-September, 1986). Concentration in mg N/l.



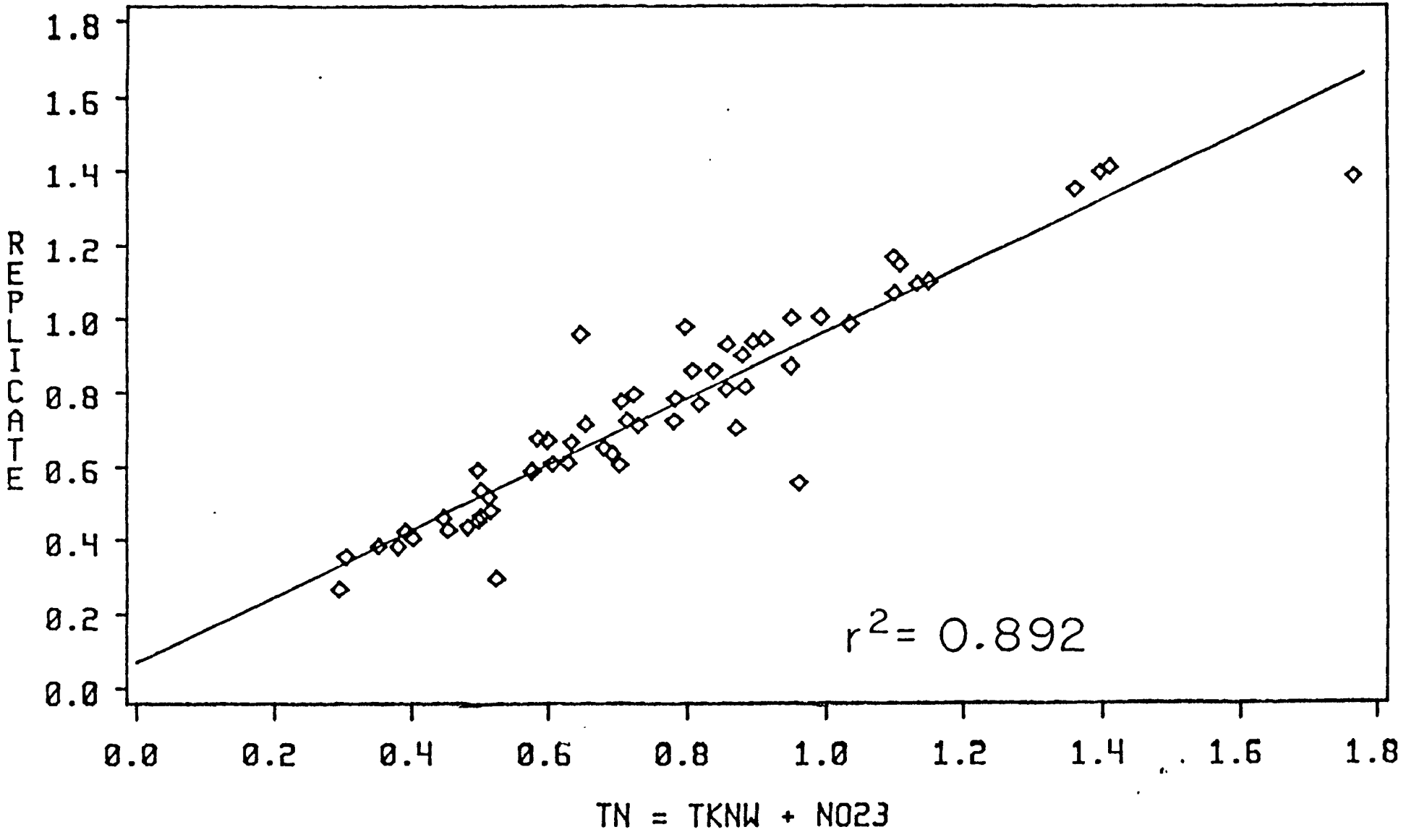
A.2. Field replicate data for the determination of particulate Nitrogen by difference (July-September, 1986). Particulate nitrogen = Total Kjeldahl N - unfiltered Kjeldahl N. Concentration in mg N/l.



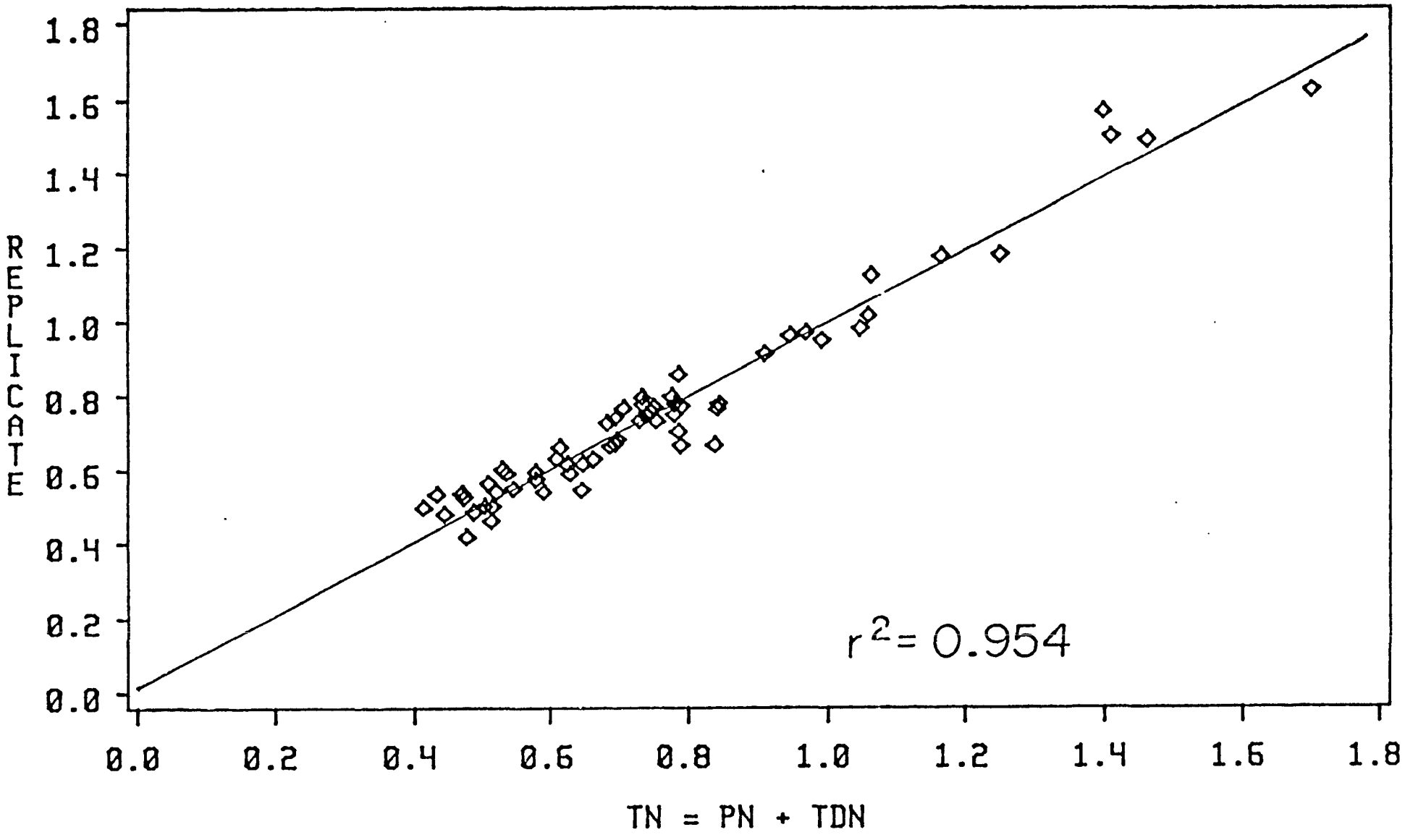
A.3. Field replicate data for the determination of total alkaline persulfate dissolved N (June-September, 1986). Concentration in mg N/l.



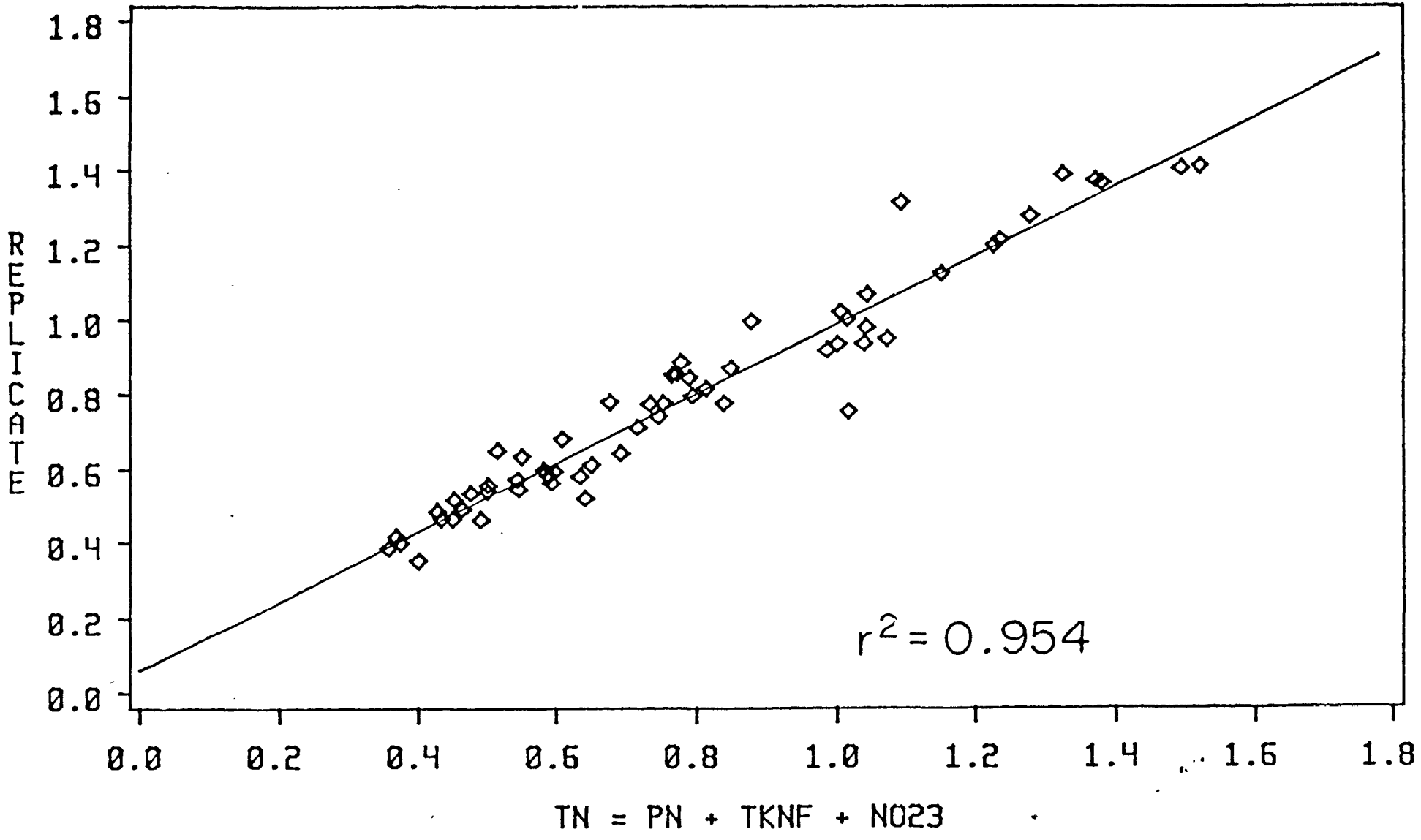
A.4. Field replicate data for the determination of total dissolved N (July-September, 1986). Total dissolved N = dissolved Kjeldahl N + nitrite plus nitrate-N. Concentration in mg N/l.



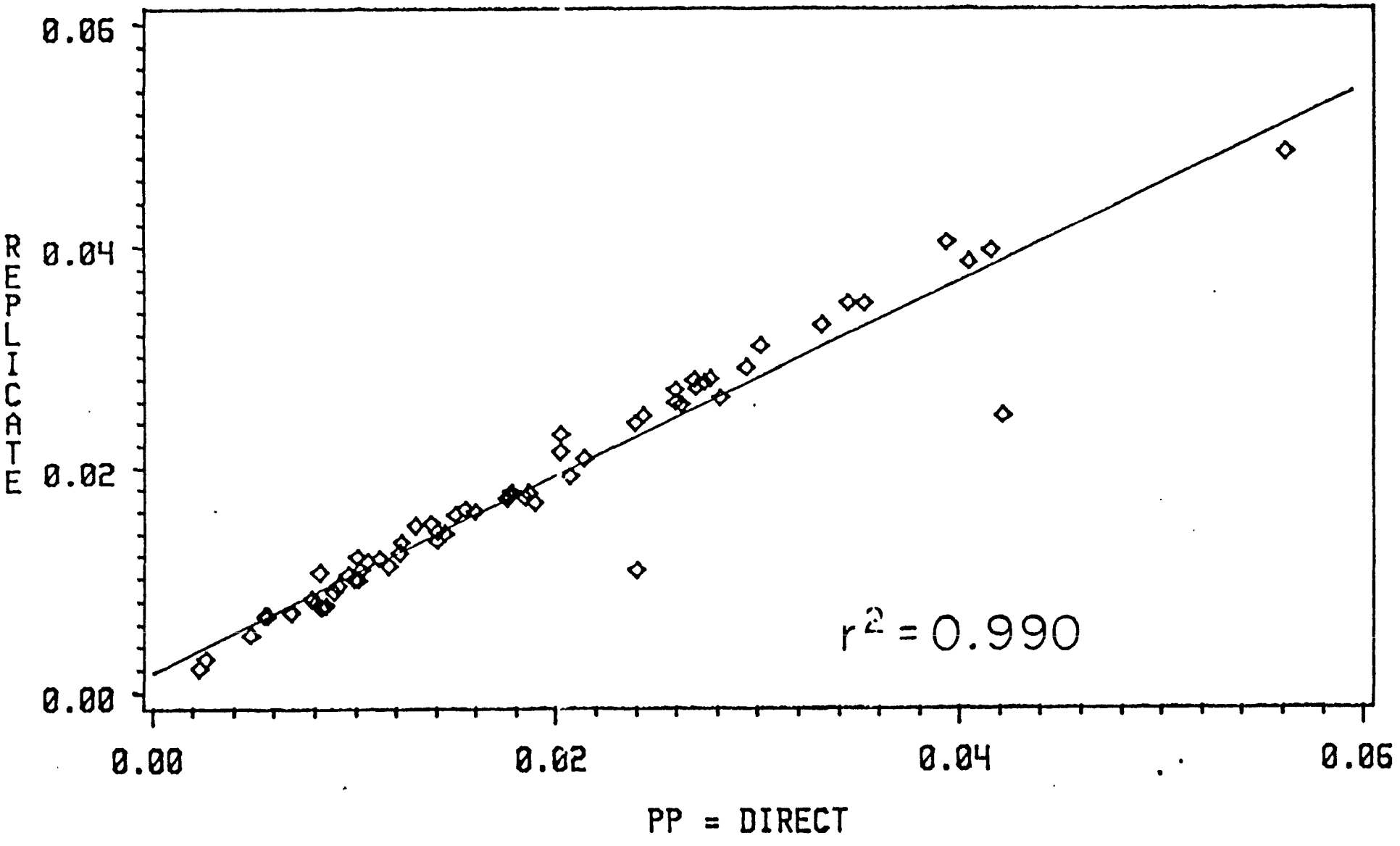
A.5. Field replicate data for the determination of total N (July-September, 1986). Total N = total Kjeldahl N + nitrite plus nitrate-N. Concentration in mg N/l



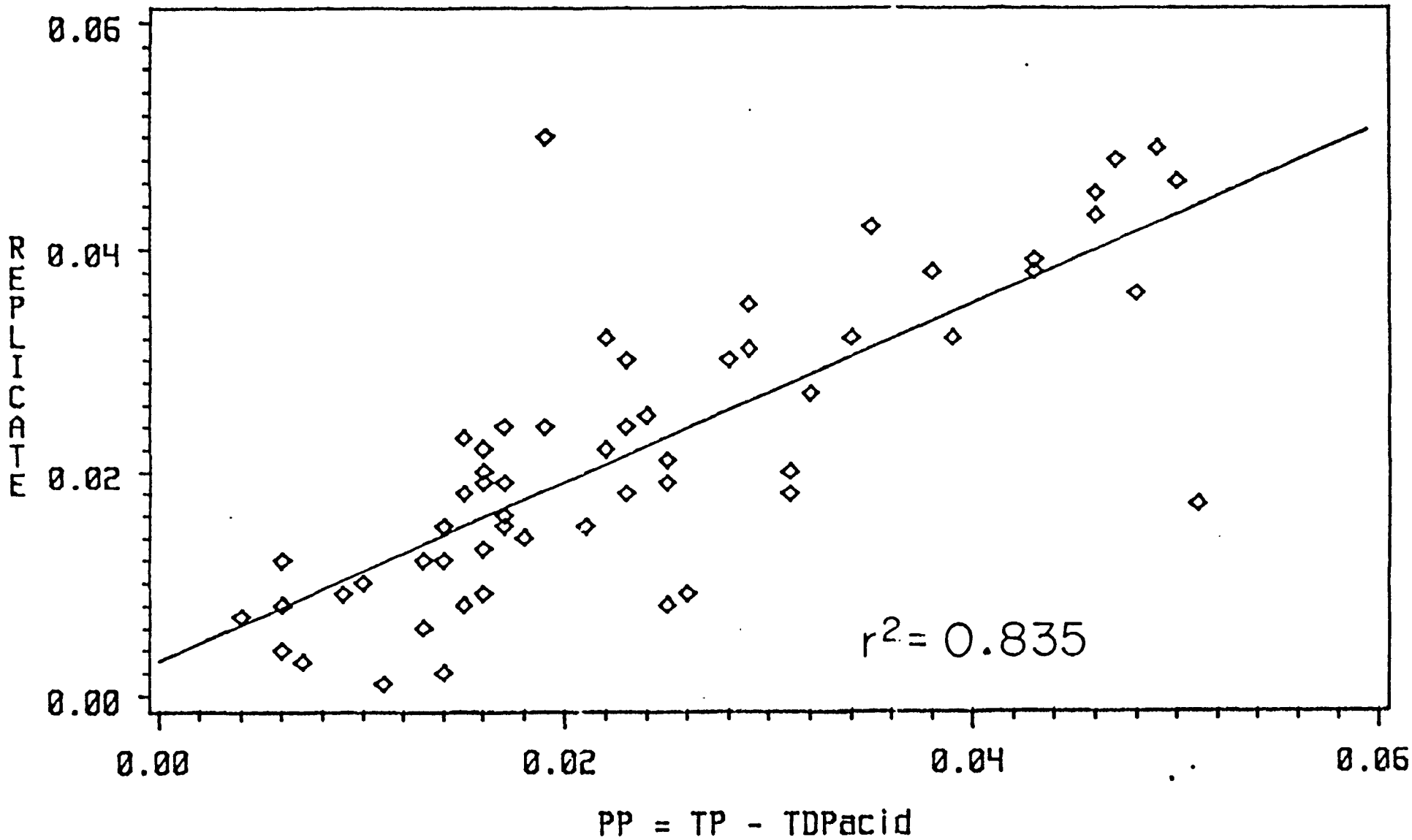
A.6. Field replicate data for the determination of total N (June-September, 1986). Total N = particulate N (direct method) + alkaline



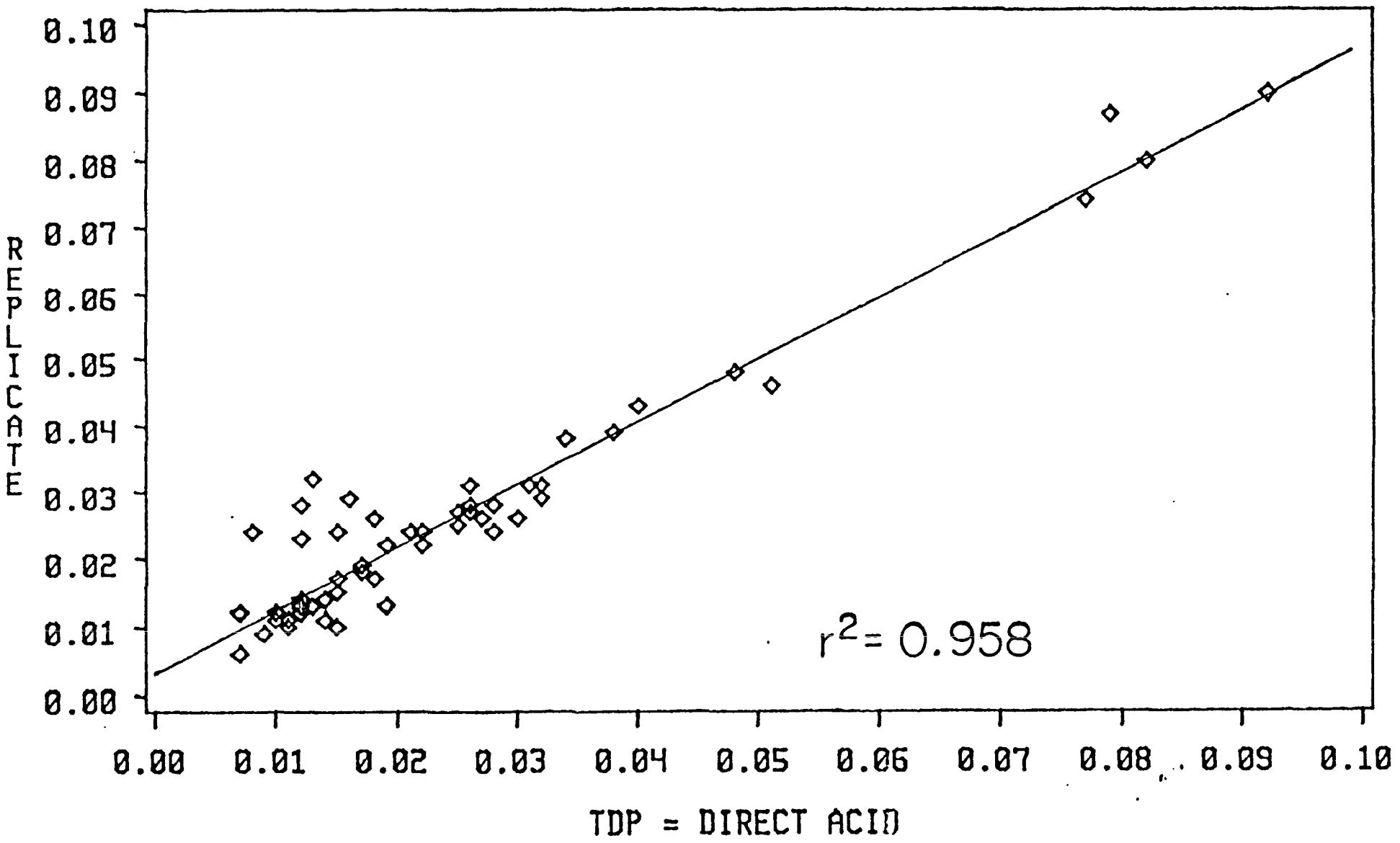
A.7. Field replicate data for the determination of total N (July-September, 1986). Total N = dissolved Kjeldahl N + particulate N (direct method) + nitrite plus nitrate-N. Concentration in mg N/l.



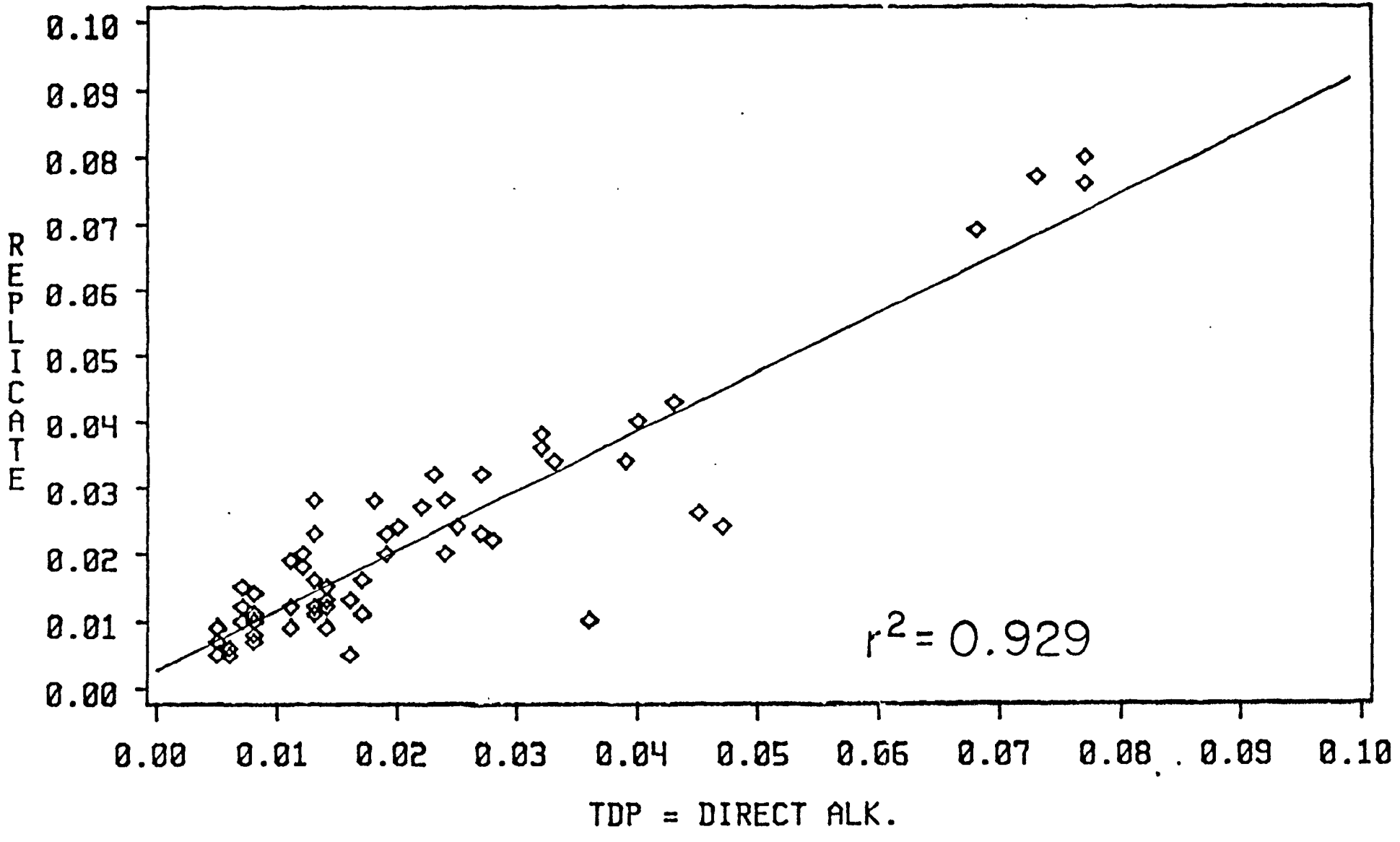
A.8. Field replicate data for the direct determination of particulate phosphorus (June-September, 1986). Concentration in mg P/l.



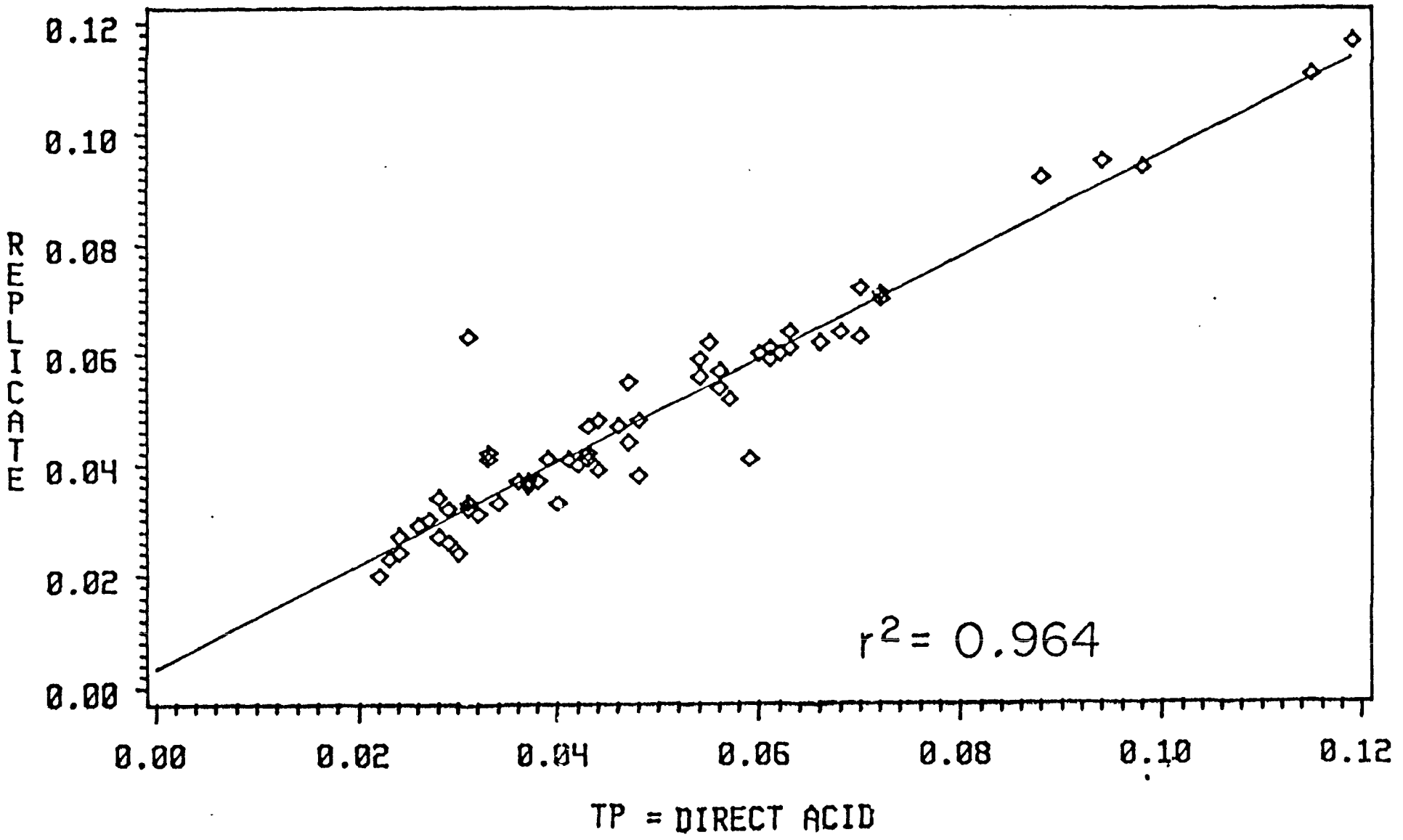
A.9. Field replicate data for the determination of particulate P by difference (June-September, 1986). Particulate phosphorus = total acid



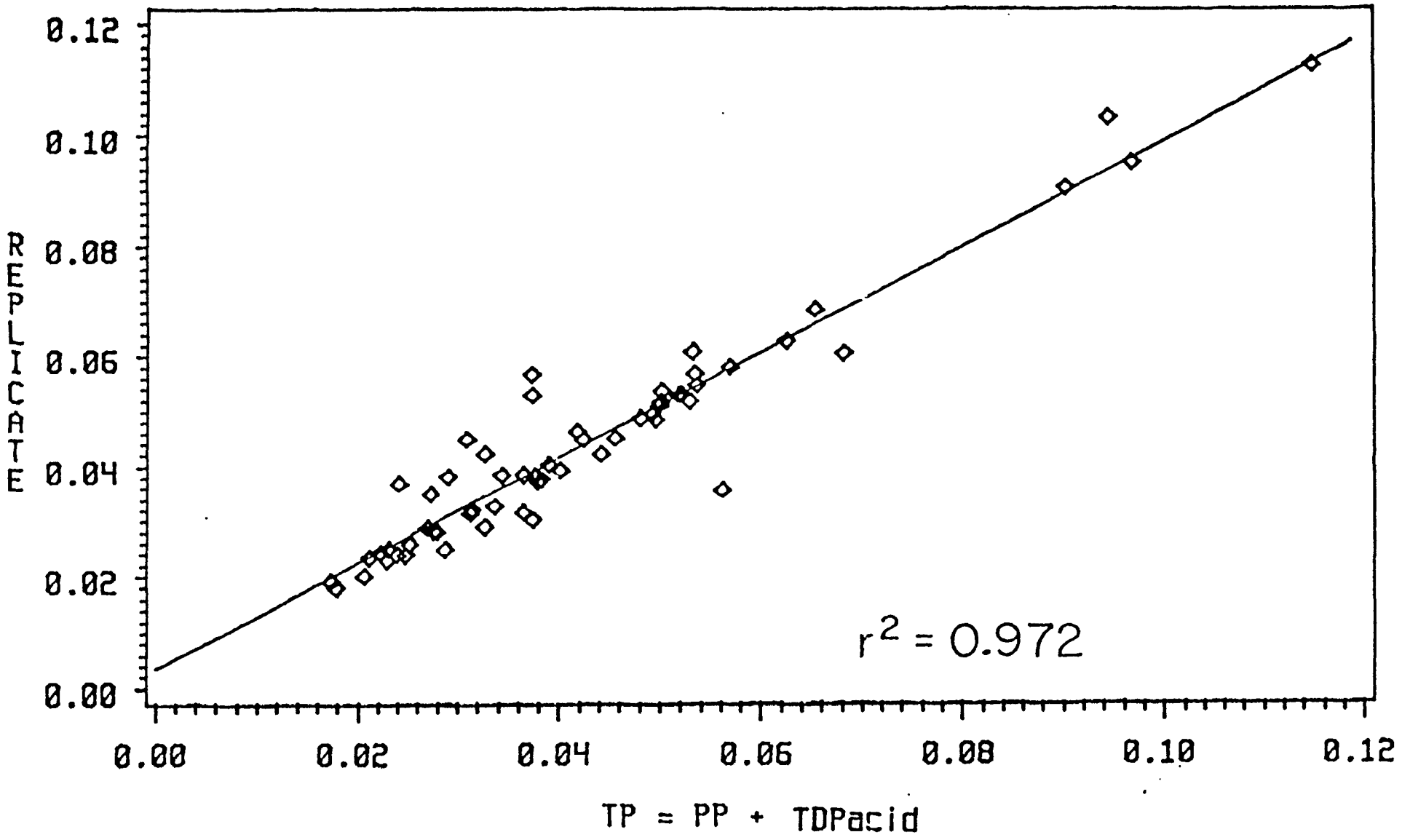
A.10. Field replicate data for the determination of dissolved P by the acid persulfate method (June-September, 1986). Concentration in mg P/l.



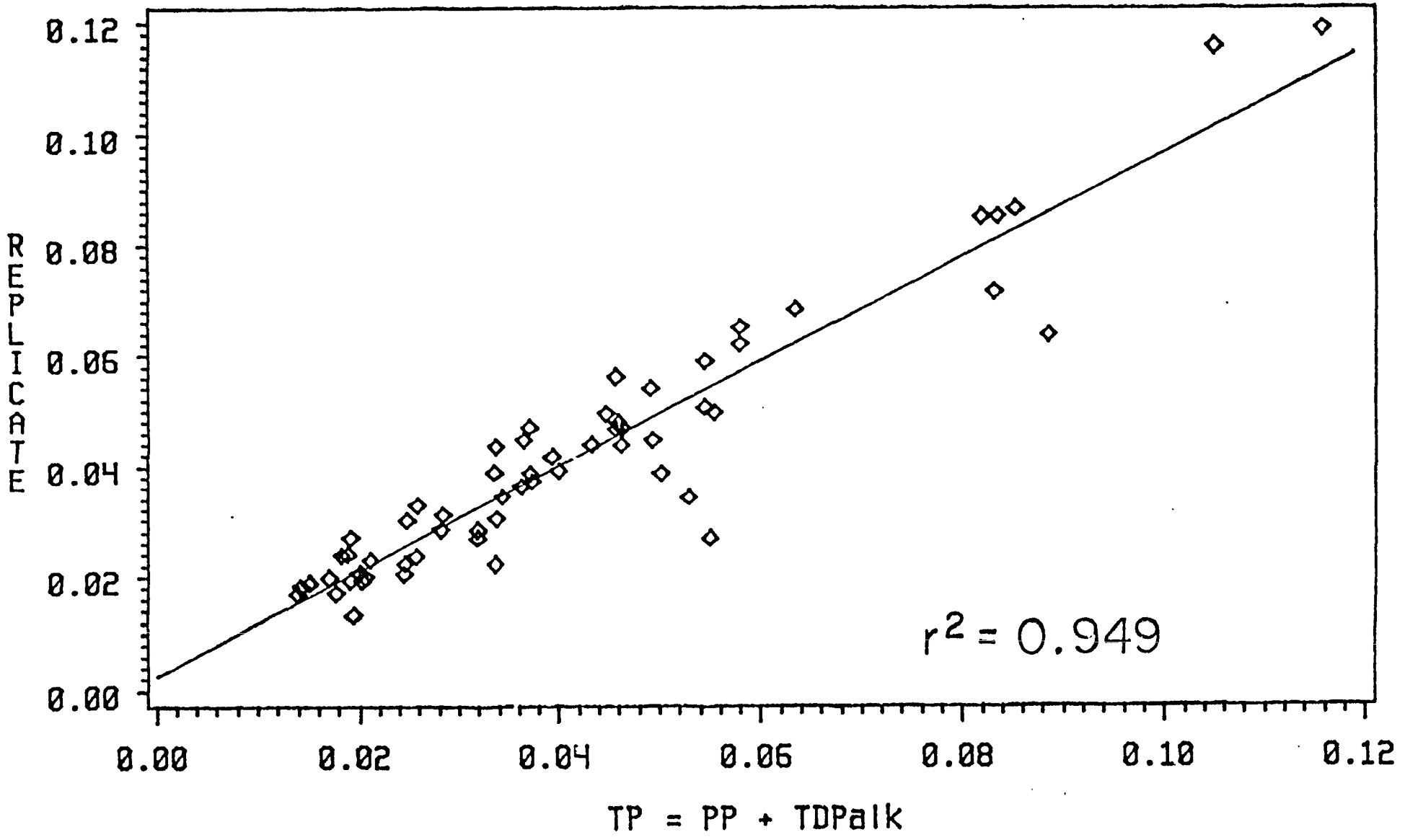
A.11. Field replicate data for the determination of dissolved P by the alkaline persulfate method (June-September, 1986). Concentration in mg P/l



A.12. Field replicate data for the determination of total P by the acid



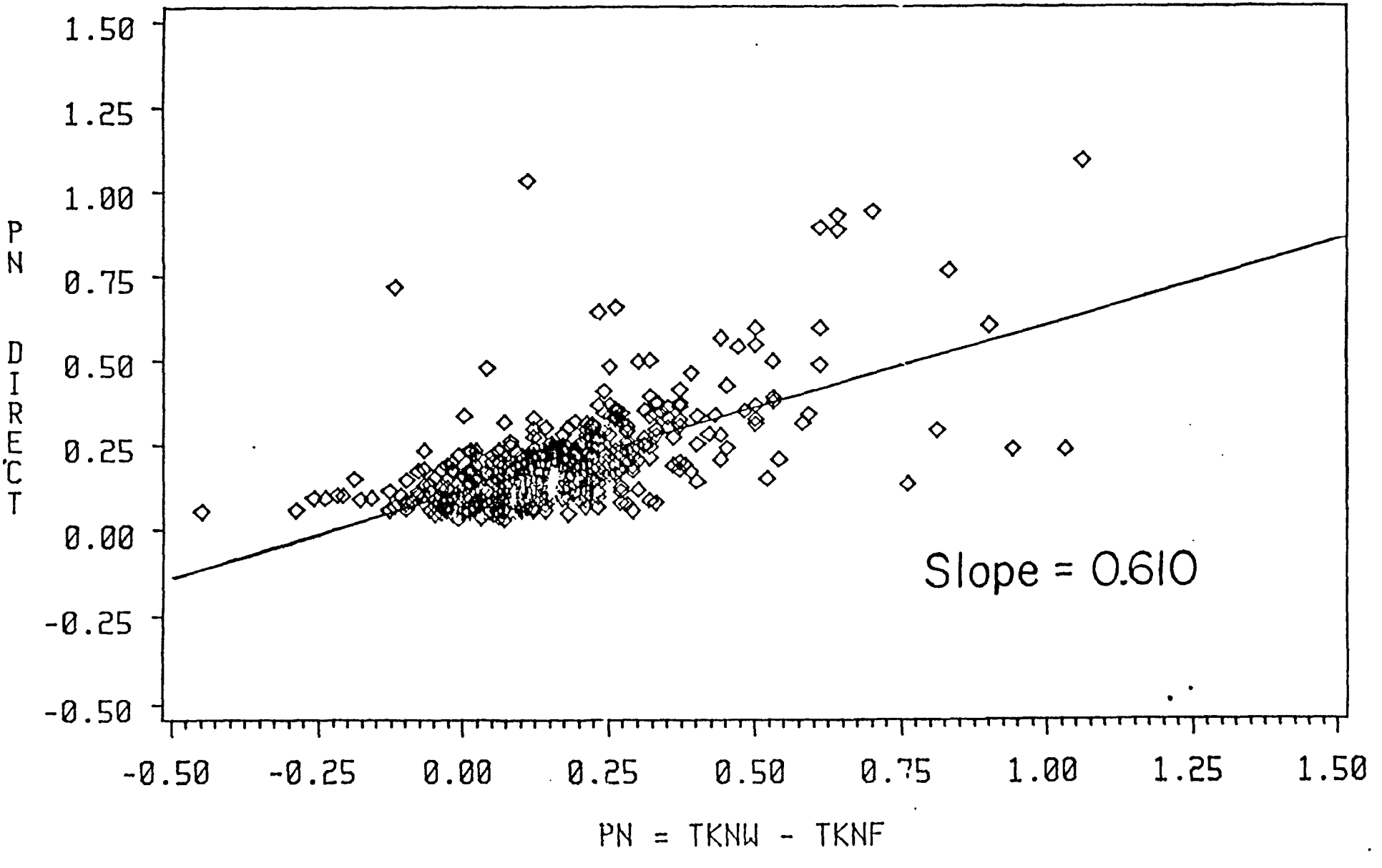
A.13. Field replicate data for the determination of total P (Juen-September, 1986). Total P = particulate P (direct) + acid persulfate

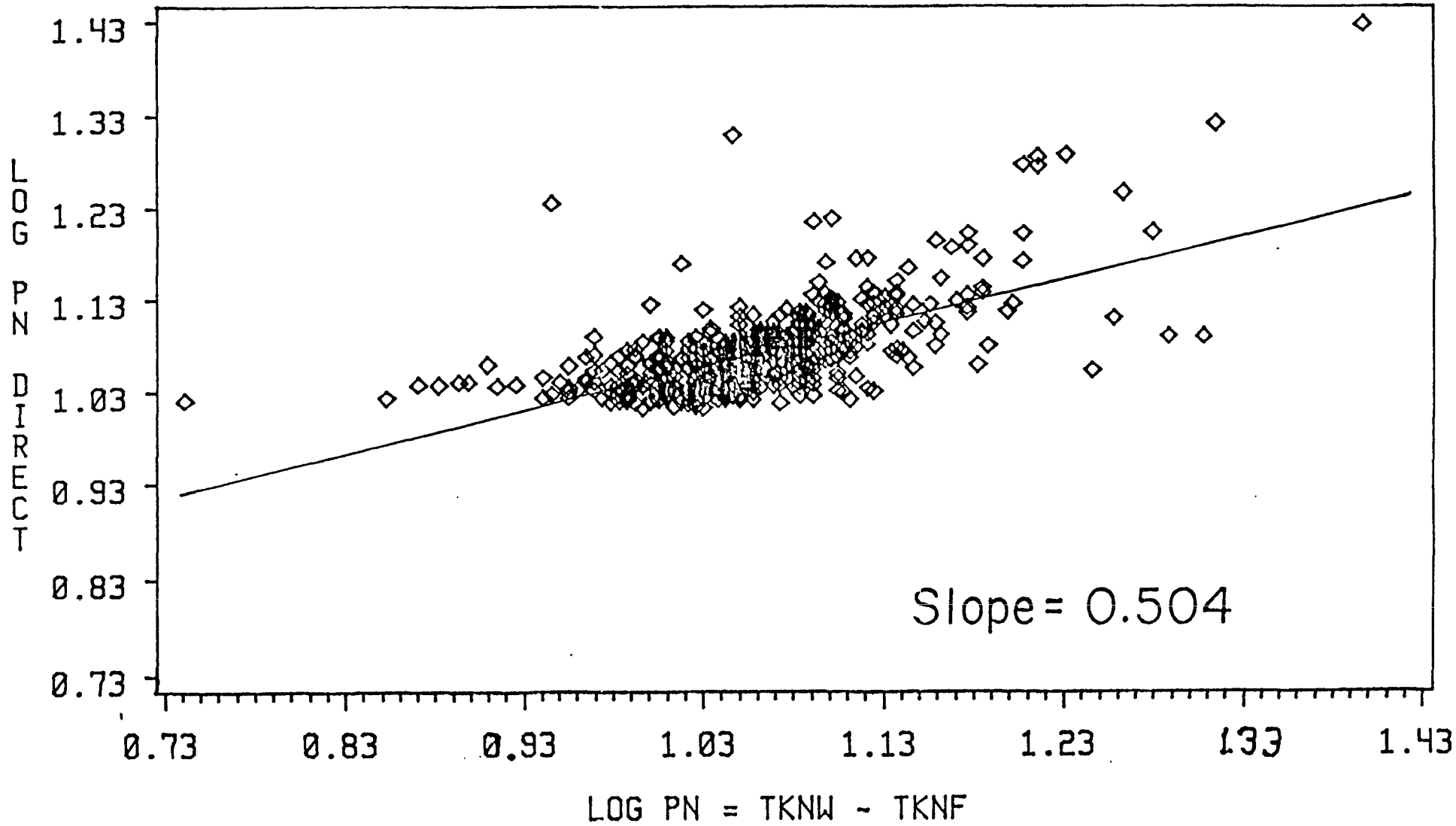


A.14. Field replicate data for the determination of total P (June-September, 1986). Total P = particulate P (direct) + alkaline persulfate

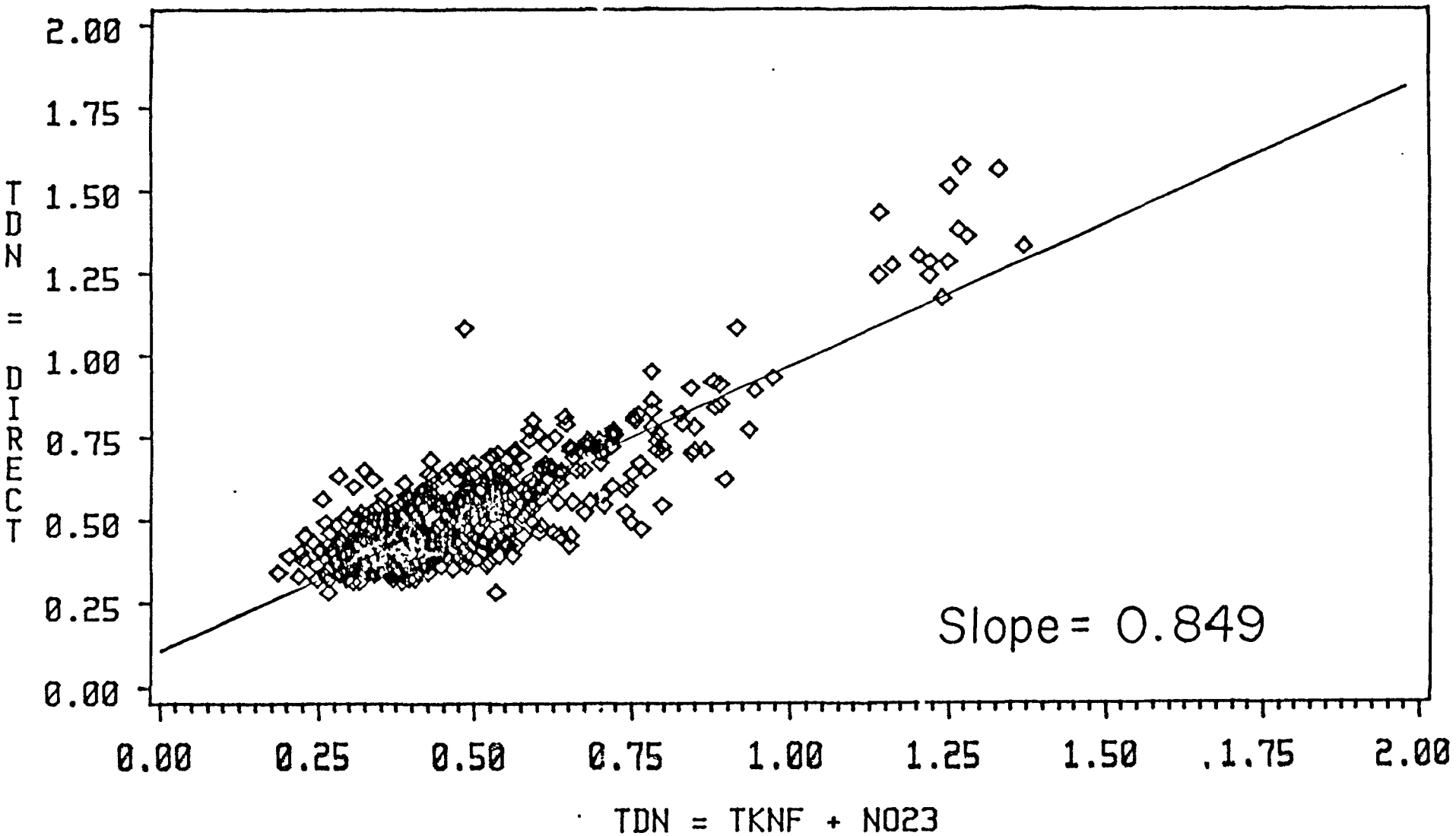
APPENDIX B

NITROGEN AND PHOSPHORUS COMPARISONS WITH REGRESSION ANALYSES

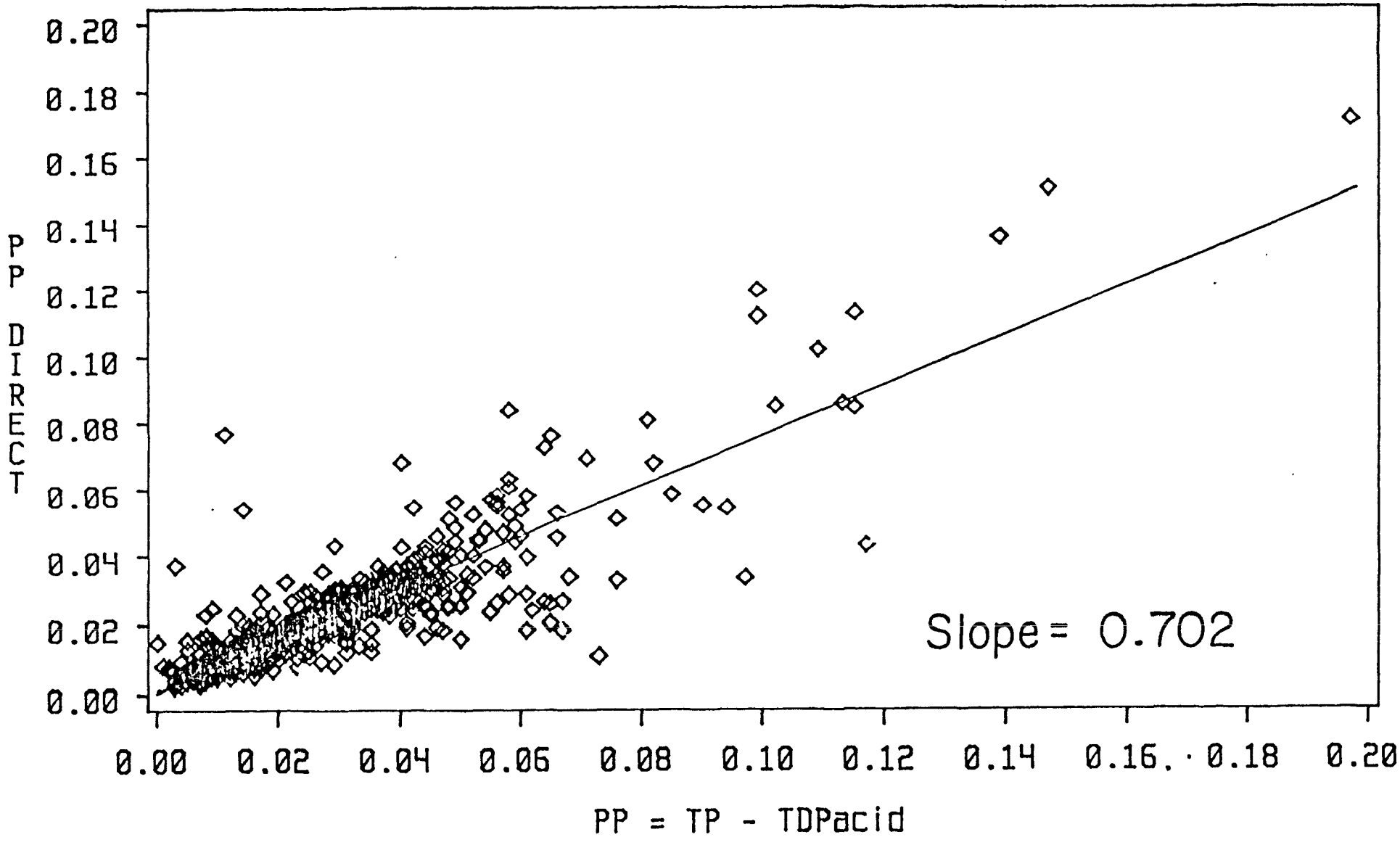

1.50
1.25
1.00
0.75
0.50
0.25
0.00
-0.25
-0.50
PN DIRECT
-0.50
-0.25
0.00
0.25
0.50
0.75
1.00
1.25
1.50
PN = TKNW - TKNF
Slope = 0.610



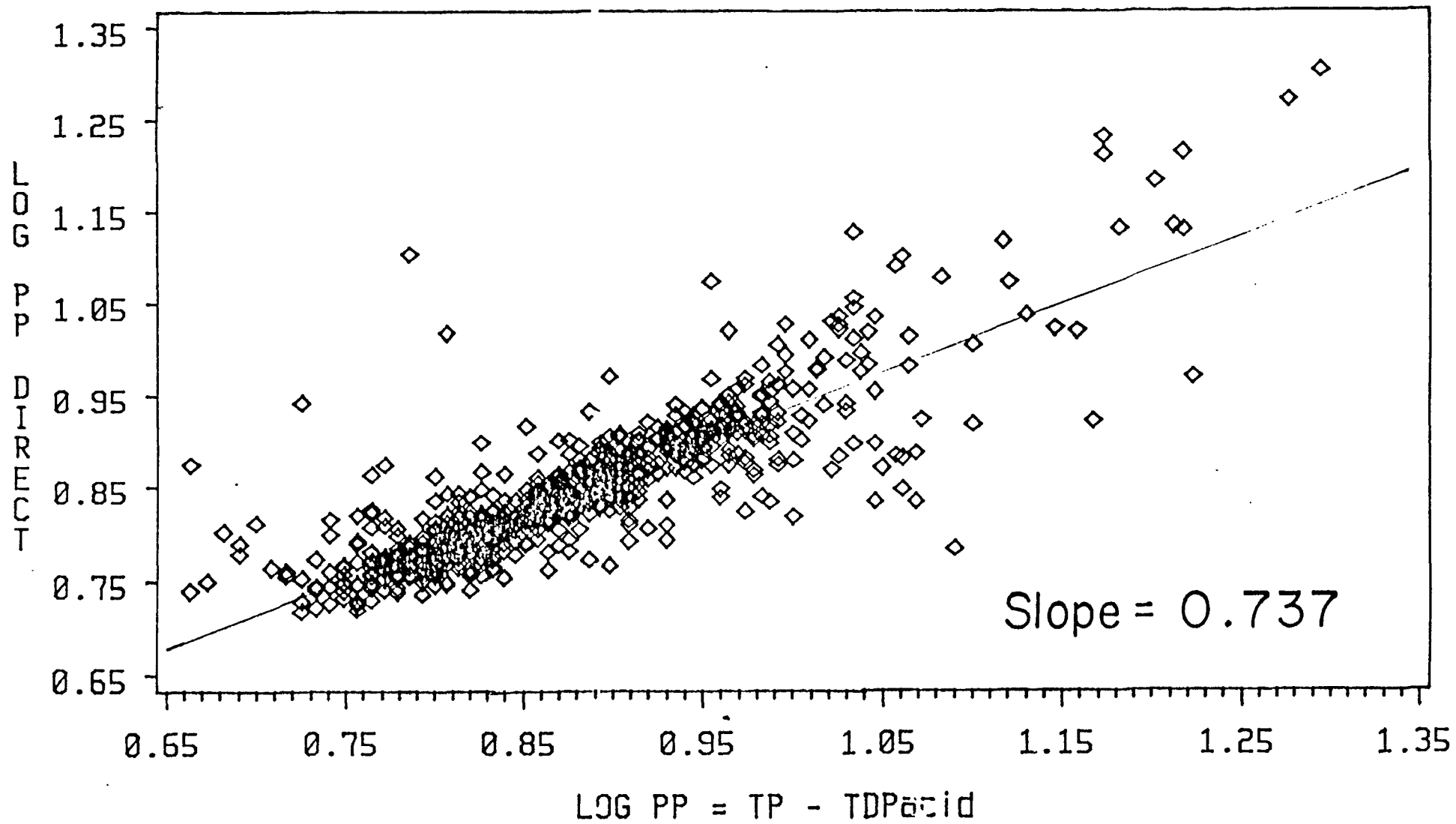
B.2. Comparison of Log direct particulate N (Y axis) vs Log particulate N by difference (Total minus dissolved Kjeldahl N) for the period July-



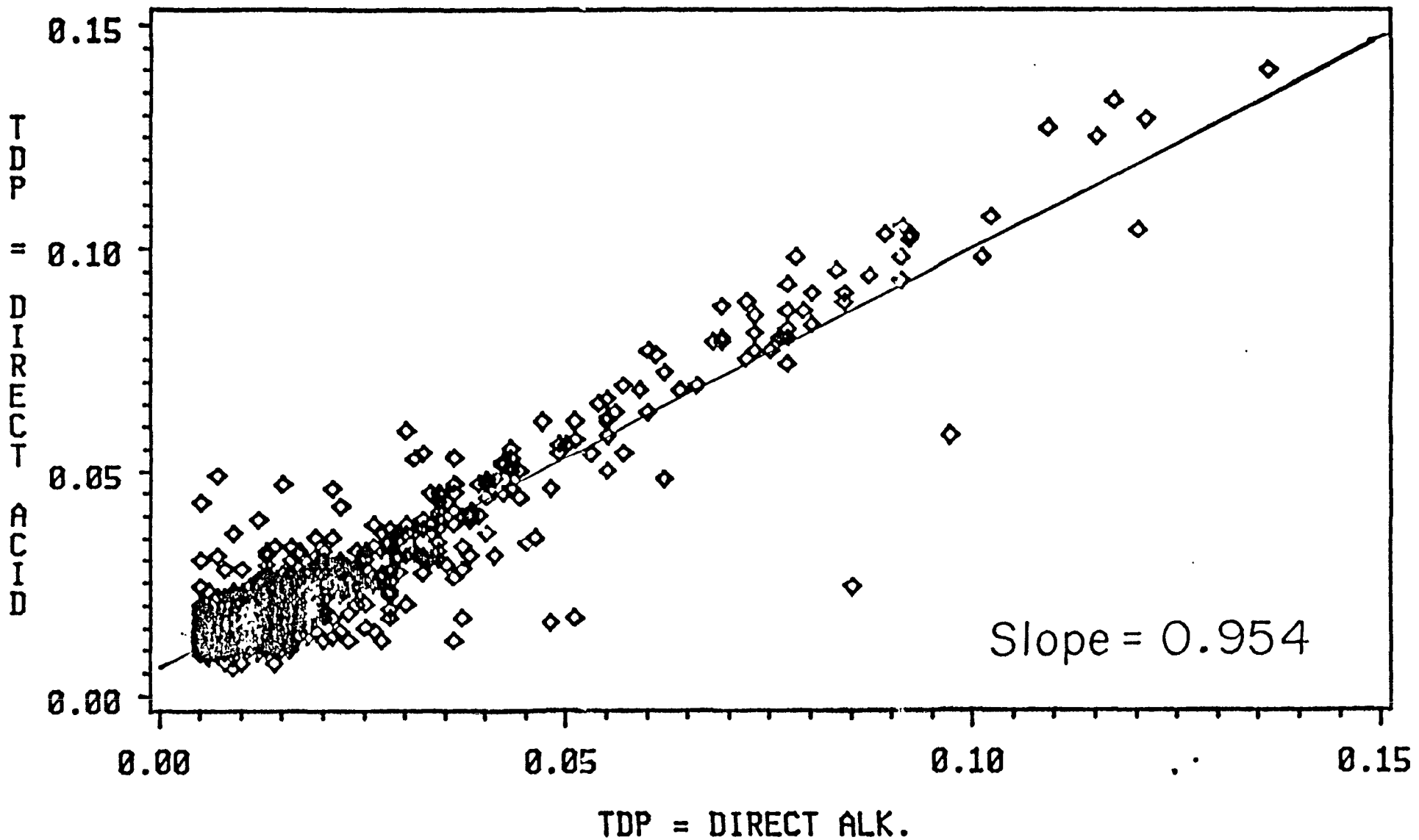
B.3. Comparison of alkaline persulfate dissolved N (Y axis) vs dissolved Kjeldahl N + nitrite plus nitrate-N for the period July-September, 1986.



B.4. Comparison of direct particulate P (Y axis) vs particulate P by difference (Total minus dissolved acid persulfate P) for the period June-



B.5. Comparison of Log direct particulate P (Y axis) vs log particulate P by difference (Total minus dissolved acid persulfate P) for the period June-September, 1986. Concentration in mg P/l



B.6. Comparison of acid persulfate dissolved P (Y axis) vs alkaline persulfate dissolved P for the period June-September, 1986. Concentration in mg P/l.

APPENDIX C

ANALYTICAL PROCEDURES USED IN THIS STUDY

Taken from CBL's Nutrient Analytical Services Laboratory Methods Book

1. ORTHOPHOSPHATE
2. NITRITE+NITRATE
3. TOTAL DISSOLVED N AND P
4. TOTAL P (ACID PERSULFATE)
5. KJELDAHL N
6. PARTICULATE N (DIRECT)
7. PARTICULATE P (DIRECT)

Nutrient Analytical Services Laboratory

STANDARD OPERATING PROCEDURES

Christopher F. D'Elia
Nancy L. Kaumeyer
Carolyn L. Keefe
Diane L. Shaw
Kathryn V. Wood
Carl F. Zimmermann

Chesapeake Biological Laboratory (CBL)
University of Maryland
Box 38
Solomons, Maryland 20688

April 1987

Nitrate ± Nitrite:

Filtered samples are passed through a granulated copper cadmium column to reduce nitrate to nitrite. The nitrite (originally present plus reduced nitrate) is then determined by diazotizing sulfanilamide and coupling with N-1-napthylethylenediamine dihydrochloride to form a colored azo dye. Nitrate is obtained subtracting NO_2+ NO_3 from NO_2 values.

Methodology: Technicon Industrial Method: 158-71 W/A
EPA. 1979. Chemical Analysis of Water and Waste
USEPA-600/4-79-020. Method #353.2.

Manifold Assembly: See figure ?.

Standard Calibration Settings:

Yellow/Orange Sample Tubes: 2.0, 1.0, 0
Black/Black Sample Tubes: 9.0, 6.0, 2.0

Damp: Normal

Sampling Rate: 40 hours 9:1 sample/wash ratio

Filter: 550 nm

Phototube: 199-B021-01 Flowcell: 50 mm

Interferences: Metal ions may produce a positive error if pres in sufficient concentrations. The presence of large concentrations of sulfide and/or sulfate will cause a large loss of sensitivity to the copper-cadmium column.

Reagents:

1. Ammonium Chloride Reagent:

Ammonium Chloride (NH_4Cl)	10.0 g
Alkaline Water	1000 ml

Dissolve 10.0 g (NH_4Cl) in alkaline water and dilute one liter. Alkaline water is prepared by adding ~ 2 concentrated Ammonium hydroxide to one liter of deion water. Should attain a pH balance of 8.5.

2. Color Reagent:

Sulfanilamide ($C_6H_8N_2O_2S$)	20.0 g
Concentrated Phosphoric Acid (H_3PO_4)	200.0 ml
N-1-naphthylethylenediamine dihydrochloride ($C_{12}H_{14}N_2$ * 2HCl)	1.0 g
Deionized Water	2000 ml
Brij-35	1.0 ml

To approximately 1500 ml of deionized water, carefull
200 ml of concentration H_3PO_4 and 20 g of sulfanilami
Dissolve completely (heat necessary). Add 1.0 g of N
naphthylethylenediamine dihydrochloride and dissolve.
Dilute to 2 liters with deionized water and add 1.0 m
Brij-35. Store in a cold, dark place.

Preparation of copper-cadmium column:

1. Use good quality cadmium filings (25-60 mesh size).

2. Ten grams of cadmium are cleaned with 50 ml of 6 N HCl
one minute. Decant the HCl and wash the cadmium with
another 50 ml portion of 6 N HCl for one minute.

3. Decant the HCl and wash the cadmium several times with
distilled water.

4. Decant the distilled water and add 50 ml of 2% (W/V) C
* $5H_2O$. Wash the cadmium until no blue color remains
solution.

5. Add another 50 ml of 2 $CuSO_4$ * $5H_2O$ and wash the cadmi
until no blue color remains in solution.

6. Decant and wash throughly (approximately 10 times) wit
deionized water.

7. Fill the reductor column with ammonium chloride reagen
transfer the prepared cadmium particles to the column
a Pasteur pipette. Be careful not to allow any air
bubbles to be trapped in the column. The column is a
cm length of 0.110" ID tubing.

8. When the entire column is fairly well packed with gran
insert glass wool plugs at both ends of the column, wi
reagents running through the system attach the column.
Remember to have no air bubbles in the valve and to at
the column to the intake side of the valve first.

9. Check for good flow characteristics (good bubble patte
If the column is packed too tightly, you will get an
inconsistent flow pattern will result.

Prior to sample analysis, condition the column with approximate
100 mg N (nitrate)/l for 5 minutes followed by 100 mg N (nitrite)/l
10 minutes.

Standards

A. Stock Standard: Dissolve 0.5055 g KNO_3 into one liter deionized water (1 ml = 5 ug-at N).

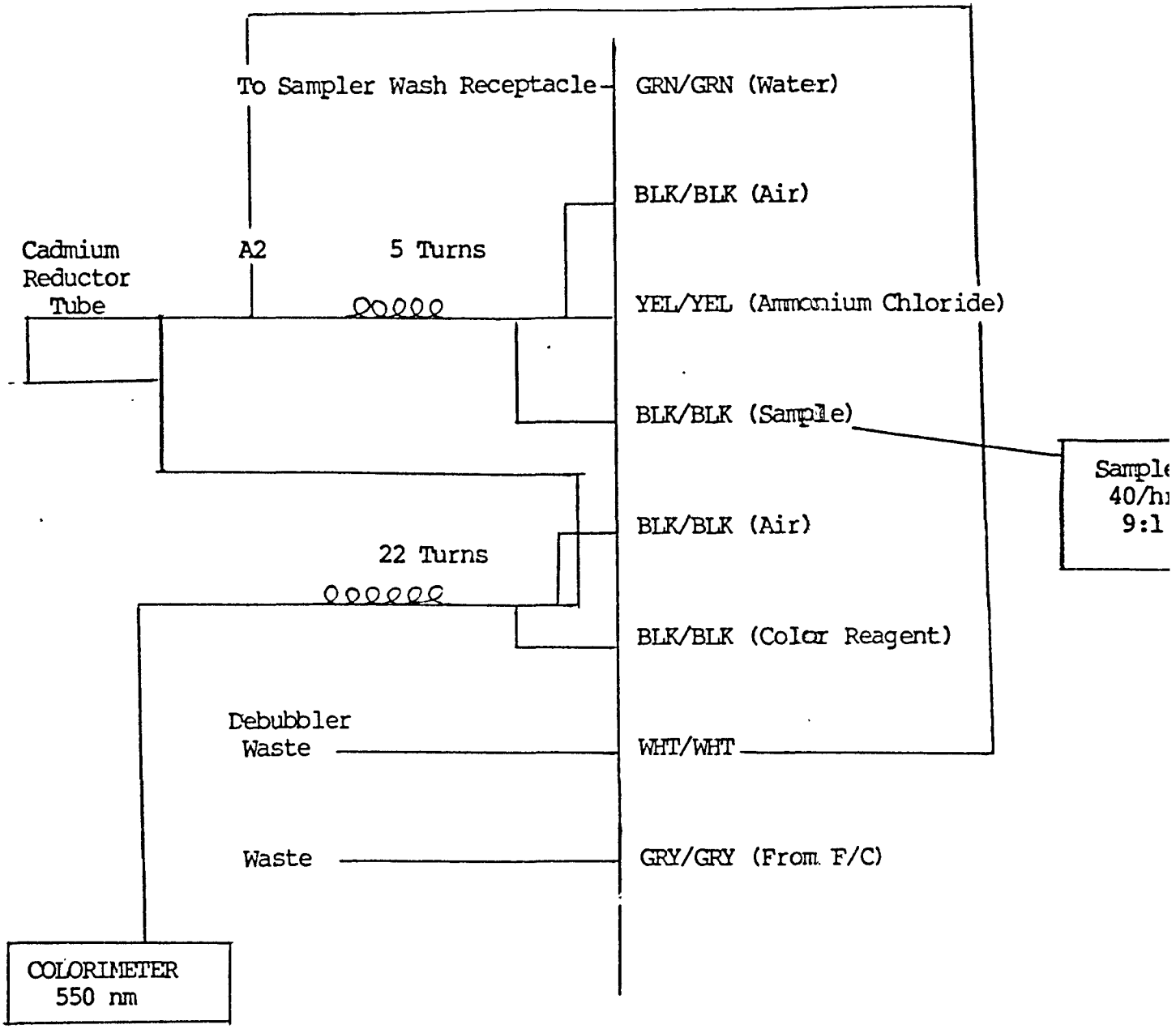
B. Working Standard A: 0.8 mls of stock standard up to 1(with deionized water yields 40 ug at N/l (0.56 mgN/l).

C. Working Standard: 0.8 mls of stock standard up to 200 with deionized water yields 20 ug at N/l (0.28 mgN/l).

1.0 mls and 1.5 mls of stock standard up to 100 ml wit deionized water yields 50 and 75 ug at N/l, respective (.70 and 1.05 mg N/l) for use with the orange-yellow s tube and yellow-blue NH_4Cl tube employed with sample concentrations < 0.56 mg N/l (NO_3^- + NO_2^-).

2.5, 5.0, 10.0, 15.0, 25.0 mls of working standard A u 100 ml with deionized water yields 1.0, 2.0, 4.0, 6.0 10.0 ug at N/l or .014, .028, .056, .084, and .14 mg N respectively.

MANIFOLD CONFIGURATION FOR NITRATE



50 mm F/C x 1.5 mm ID

199-B021-01 Phototube

Note: If sample concentration >.56 mgN/l
substitute:YEL/BLU for Ammonium Chloride
ORN/YEL for Sample

Orthophosphate:

Ammonium molybdate and antimony potassium tartrate react in an acid medium to form an antimony — phosphomolybdate complex which is reduced to an intensely blue colored complex by ascorbic acid.

Methodology: Technicon Industrial Method No. 155-71W
EPA. 1979. USEPA-600/4-79-020. Method #365.1

Manifold Assembly: See figure ?.

Standard Calibration Settings: 9.0, 6.0, 3.0

Damp: Normal

Sampling Rate: 40/hr. 9:1 sample/wash ratio

Filter: 880 nm

Phototube: 199-B021-04 Flowcell: 50 mm

Interferences: Silicon at a level of 100 ug at Si/l causes an interference equivalent to approximately 0.04 ug at P/l.

Reagents:

1. Sulfuric Acid (4.9N):

Sulfuric Acid (H_2SO_4), concentrated (sp. gr. 1.84)	136 ml
Deionized Water (QS to)	1000 ml

Add 136 ml conc. H_2SO_4 to approximately 800 ml good quality deionized water while cooling (cold water bath). After the solution is cooled, dilute to one liter with deionized water.

2. Ammonium Molybdate:

Ammonium Molybdate [$(NH_4)_6\ Mo_7O_{24} * 4\ H_2$]	40 g
Deionized Water	1000 ml

Dissolve 40 g of ammonium molybdate in 800 ml of deionized water. Dilute to one liter with deionized water. Store in plastic bottle away from direct sunlight.

3. Ascorbic Acid:

Ascorbic Acid ($C_6H_8O_6$)	18.0 g
Deionized Water	1000 ml

Dissolve 18 g. of ascorbic acid in 800 ml. deionized water. Dilute to one liter with deionized water and dispense (approx.) 40 ml. into clean polybottles and freeze.

4. Antimony Potassium Tartrate:

Antimony Potassium Tartrate [$(K(SbO)C_4H_4O_6 * 1/2\ H_2]$	3.0 g
Deionized Water	1000 ml

Dissolve 3.0 g antimony potassium tartrate in 800 ml deionized water. Dilute to one liter with deionized water.

5. Sodium Lauryl Sulfate (SLS):

Sodium Lauryl Sulfate (Sodium Dodecyl Sulfate M.W. = 288.38; Phosphate $\leq$ 0.0001%)	3.0 g
Deionized water	100 ml

Dissolve 3.0 g SLS in 80 ml deionized water. Dilute to 100 ml with deionized water.

6. Working Reagents:

a. Reagent A:

Sulfuric Acid (4.9N)	50 ml
Ammonium Molybdate	15 ml
Antimony Potassium Tartrate	5 ml + 1 ml SLS

b. Reagent B: Ascorbic Acid 30 ml + 0.3 ml SLS

Standards

A. Stock Standard: Dissolve 1.632 g KH_2PO_4 into one liter deionized water and add 1.0 ml chloroform as a preservative (1 ml = 12 ug at P).

B. Secondary standard: Take 1.0 ml of stock standard and dilute to 100 ml with deionized water (0.12) ug at P/ml).

C. Working Standards: 0.1, 0.25, 0.5, 2.5 and 5 mls of B up to 100 ml with deionized water yield concentrations of 0.12 ug at/l (0.00372 mg/l), 0.3 ug at/l (0.0093 mg/l), 0.6 ug at/l (0.0186 mg/l), 1.2 ug at/l (0.0372 mg/l), 3.0 ug at/l (0.093 mg/l) and 6.0 ug at/l (0.186 mg/l).

Organic Analytes

Rationale:

Dissolved organic Carbon, Nitrogen and Phosphorus are described below. All procedures except Kjeldahl require the addition of potassium persulfate to a sample, which when under heat and pressure break down the organic constituents to inorganic forms. Inorganic fractions are then subtracted from the total dissolved sample to yield the dissolved organic concentration. (Figure ___ and ___).

Sampling and Storage:

Surface, bottom, above and below pycnocline water samples are collected via a submersible pump system. Collected water samples are filtered through GF/F filters (nominal pore size 0.7 um) and placed in appropriate containers and preserved (Table ____).

Analyte	Volume	Storage
Dissolved Organic Carbon	~20	Freeze
Dissolved Nitrogen/Phosphorus	10	Freeze
Dissolved Phosphorus (Acid Persulfate)	20	Freeze
Dissolved Kjeldahl	~50	H_2SO_4

Total Dissolved Nitrogen and Phosphorus:

The method utilized is that of D'Elia, et al. 1977. This method is a persulfate oxidation technique for nitrogen and phosphorus where, under alkaline conditions, nitrate is the sole N product and phosphate is the sole P product.

Manifold Assembly: Same as nitrate and phosphate.

Damp: Normal

Sampling Rate: 40/hr 9:1 sample/wash ratio

Filters: 550 nm for nitrate; 880 nm for orthophosphate

Phototubes: 199-B021-01 for nitrate; 199-B021-04 for orthophosphate

Flowcells: 50 mm

Interferences: Metal ions may produce a positive nitrate error if present in sufficient concentrations. The presence of large concentrations of sulfate will cause a large loss of sensitivity to the copper-cadmium column. Silicon at a level of 100 ug at Si/l causes an interference equivalent to approximately 0.04 ug al P/l.

Outline

1. Ten mls of filtered water (GF/F, 0.7 um) is placed in a 30 ml screw cap test tube and frozen.

2. When ready to analyze, thaw samples and bring to room temperature.

3. Add 15.0 ml oxiding reagent ($Mg(OH)_2$). A precipitate will form with seawater samples. Test tubes are capped fairly tightly.

4. Samples are then autoclaved at 100-110$_0$C (between 3-4 psi) for 30 minutes and slowly brought back to atmospheric pressure.

5. Tubes are removed and cooled to room temperature (samples can be stored at this point).

6. Add 1.5 ml 0.3N HCl to each sample.

7. Mix with Vortex mixer until precipitate dissolves.

8. Add 2.0 ml buffer solution to each tube. The pH of the sample should be 7-8 after the addition of the buffer solution.

9. Analyze for NO_2^- + NO_3^- and PO_4^- (see dissolved inorganic section).

Reagents

1. Buffer solution:

   30.9 g H_3BO_3 (Boric Acid) dissolved in approximately 800 ml deionized water. Add 101 ml of a 1M NaOH solution (40 g NaOH/1) to the H_3BO_3 solution and bring up to one liter with deionized water. The solution is stable for many weeks.

2. 0.3N HCl:

   2.5 ml concentrated HCl brought up to 100 ml with deionized water.

3. Oxidizing Reagent:

   3.0 g NaOH and 6.7 g of low N (<0.001%) potassium persulfate ($K_2S_2O_8$) are dissolved in one liter of deionized water just before use.

Notes:

1. The use of internal organic standards (glutamic acid and glycerophosphate) allows to check for percent recovery and is routinely used at CBL.

2. The procedure includes an internal dilution factor of samples and standards due to addition of reagents of 2.85.

3. Reagent Blanks: Reagents only are digested in 30 ml test tubes, neutralized and buffered. The analyzed peak heights of NO_3 and PO_4^{-3} are normalized to the sample + reagent volume by multiplying by 18.5/28.5. The resultant normalized reagent blank peak height is then subtracted from the sample peak heights before calculating the concentrations based on the peak heights of the standards.

Preparation of Internal Standards:

A. Stock Glutamic Acid Standard: Dissolve .3705 g glutamic acid in approximately 400 ml deionized water and then bring up to 500 ml with deionized water. Add 0.5 ml chloroform to act as a preservative.

B. Working Glutamic Acid Standard: 1 ml of A up to 100 mls with deionized water will yield 50.4 ug at N/l (0.7056 mg N/l).

C. Stock Glycerophosphate Standard: Dissolve 0.0473 g B-Glycerophosphoric Acid, Disodium Salt, 5-Hydrate in approximately 400 ml deionized waterand then bring up to 500 ml with deionized water. Add 0.5 ml chloroform to act as a preservative.

D. Working Glycerophosphate Standard: 1 ml of C up to 100 mls with deionized water will yield 3.09 ug at P/l (0.096 mg P/l).

Preparation of Working Inorganic Standards:

A. Stock Nitrate Standard: From nitrate method.

B. Working Nitrate Standards: 0.5, 1.0 and 1.5 ml of Nitrate Stock Standard A up to 100 ml with deionized water will yield 25 ug at N/l (.35 mg N/l), 50 ug at N/l (.70 mg N/l) and 75 ug at N/l (1.05 mg N/l), respectively.

C. Stock Orthophosphate Standard: From othophosphate method.

D. Secondary Orthophosphate Standard: From othophosphate method.

E. Working Orthophosphate Standards: 0.5, 1.0 and 2.5 mls of Secondary Orthophosphate Standard D up to 100 ml with deionized water will yield 0.6 ug at P/l (.0186 mg P/l), 1.2 ug at P/l (.0372 mg P/l) and 3.0 ug at P/l (.093 mg P/l), respectively.

Total Phosphorus (Acid Persulfate):

The method used by CBL personnel is that of Menzel, D.W. and N. Corwin (1965).

Outline

1. Prepare 0 - 5% solution of $K_2S_2O_8$.

   a. 25g $K_2S_2O_8$ up to 500 mls with deionized water.
   b. 12.5 g $K_2S_2O_8$ up to 250 mls with deionized water.

2. To each 20 ml of sample (in 30 ml screw cap test-tube) add 3.2 ml of the 5% $K_2S_2O_8$ solution and shake.

3. Place tubes in pressure cooker at 3-4 psi for one hour.

4. 20 mls of standards (3 replicates) are placed in 30 ml test-tube and treated in exactly the same manner as the samples.

5. Blanks (3 replicates) consist of 20 ml deionized water and then treated in exactly the same manner as the samples.

6. Aliquot of cooled, shaken sample transferred to AutoAnalyzer cup with Pasteur pipette.

7. Phosphate analyzed.

Methodology: Menzel, D.W. and N. Corwin. 1965. The measurement of total phosphorus in seawater based on the liberation of organically bound fractions by persulfate oxidation. Limnol. Oceanogr. 10:280-282.

Manifold Assembly: See figure ?

Damp: Normal

Sampling Rate: 40/hr 9:1 Sample/Wash Ratio

Filter: 880 nm

Phototube: 199-B021-04

Reagents:

1. Deionized Water Diluent:

   Add .5 g sodium lauryl sufate (SLS) to 500 ml good quality deionized water. Mix well!

2. Sulfuric Acid:

   From orthophosphate method.

3. Ammonium Molybdate:

   From orthophosphate method.

4. Ascorbic Acid:

   From orthophosphate method.

5. Antimony Potassium Tartrate:

   From orthophosphate method.

6. Sodium Lauryl Sulfate (SLS):

   From orthophosphate method.

7. Working Reagents:

   From orthophosphate method.

Standards:

A. Stock Standard: KH_2PO_4; from Orthophosphate method.

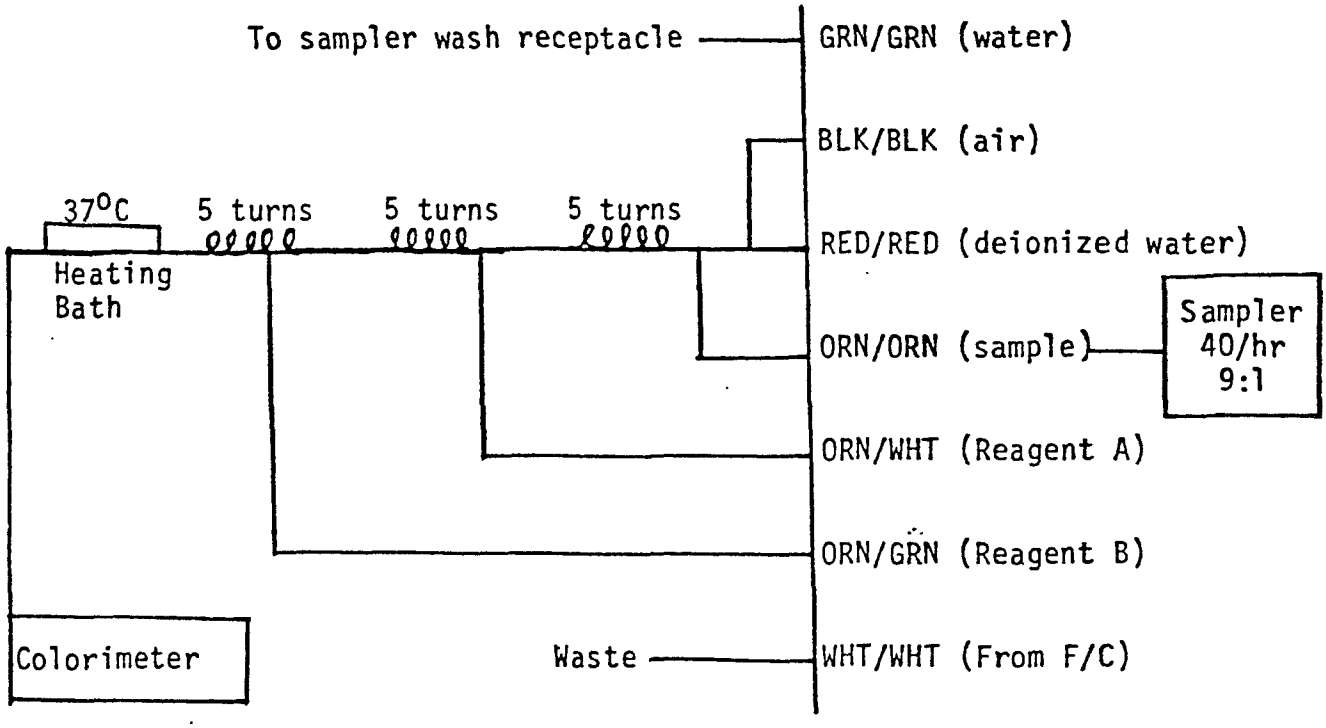
B. Secondary Standard: from Orthophosphate method.

C. Working Standards: Take 0.5, 1.0, 2.0, 2.5 ml of Secondary Standard B and dilute each to 100 ml with deionized water which will yield 0.6 ug at P/l (.0186 mg P/l); 1.2 ug at P/l (.0372 mg P/l); 2.4 ug at P.l (.0744 mg P/l) and 3.0 ug at P/l (.093 mg P/l).

D. Stock Glycerophosphate Standard: From alkaline persulfate method.

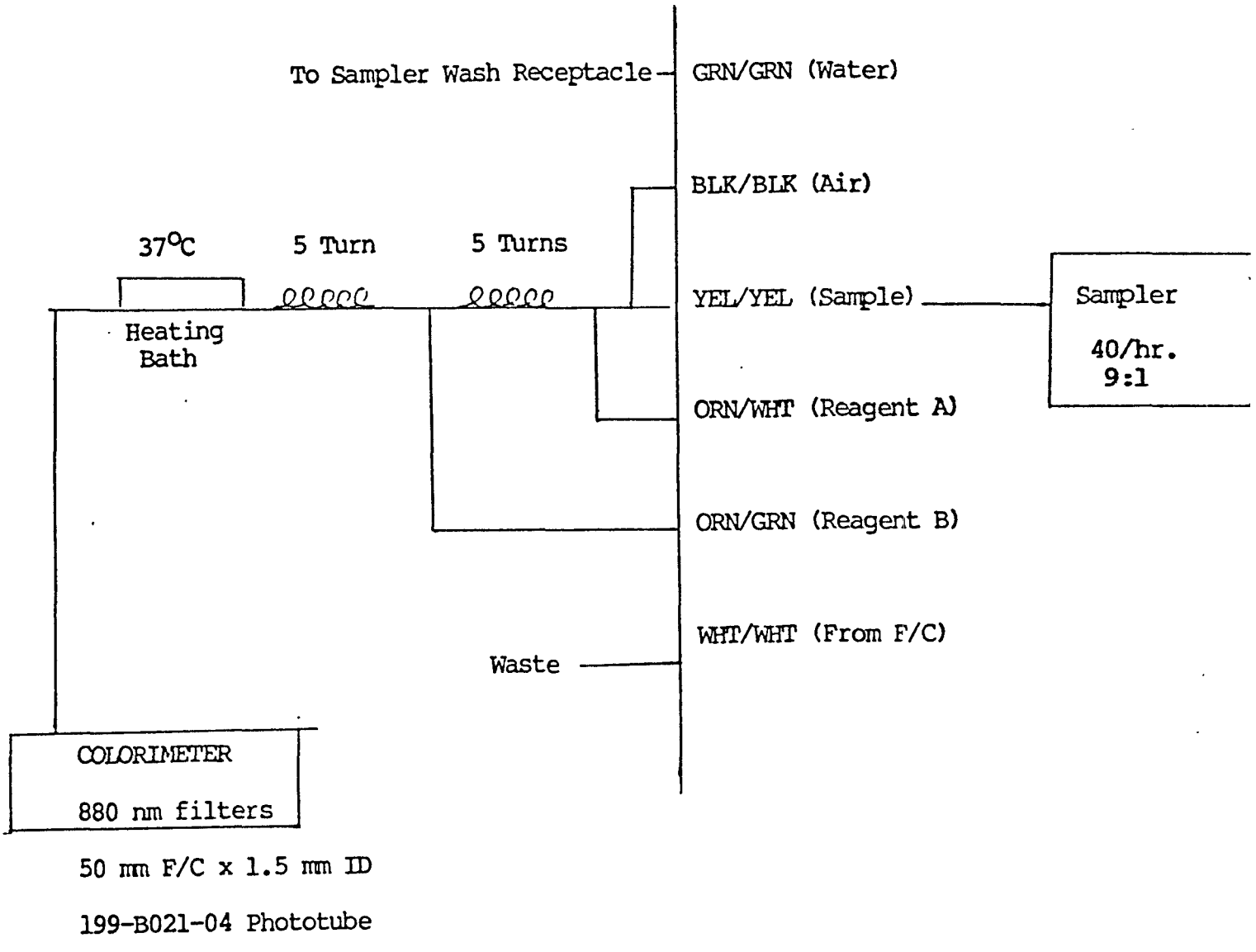
E. Working Glycerophosphate Standard: Take 1.0 ml of Stock Glycerophosphate Standard B and dilute to 100 ml with deionized water which will yield 3.09 ug at P/l (.096 mg P/l).

Manifold Configuration for Total Phosphorus
(Acid Persulfate)



880 nm filters
50 x 1.5 flow cell
199-B021-04 Phototubes

MANIFOLD CONFIGURATION FOR PHOSPHATE



Kjeldahl Nitrogen:

The sample is heated with a teflon boiling ball in the presence of sulfuric acid, potassium sulfate and mercuric sulfate for 3.5 hours. The residue is cooled, diluted to the original volume and is then analyzed for ammonium. The ammonium determination is based on a colormetric method in which an emerald-greem color is formed by the reaction of ammonia with sodium salicylate, sodium nitroprusside and sodium hypochlorite in a buffered alkaline medium at a pH of 12.8-13.0. The ammonia salicylate complex is read at 660 nm using an automated analyzer.

Digestion

Reagents:

1. Stock Mercuric Sulfate:

Mercuric Oxide, Red (HgOO	8 g
Sulfuric Acid, (H_2SO_4); concentrated	10 ml

Diluted to 100 ml with ammonia free deionized water.

2. Digestion Solution:

Potassium Sulfate (K_2SO_4)	135 g
Sulfuric Acid (concentrated)	200 ml
Stock Mercuric Sulfate	25 ml
Distilled Water	qs 1000 ml

Dissolve 135 g of K_2SO_4 in approximately 500 ml deionized water and slowly add 200 ml concentrated H_2SO_4. Add 25 ml mercuric sulfate solution, let cool and dilute to 1000 ml with deionized water.

Digestion Procedure

1. A 25 ml sample is added to each digestion tube.

2. Five (5 ml) of digestion solution and two teflon boiling balls (Fisher Scientific) are then addded to each tube and mixed with a vortex mixer.

3. SILICONE AIRTIGHT PLUGS ARE INSERTED IN THE DIGESTION TUBE WHENEVER THEY ARE NOT BEING HEATED.

4. The digestion tubes are then heated in a block digestion at 200°C for 1 hour and then at 360°C for 2.5 hours.

5. The tubes are then taken off the digestion and allowed to cool for 15 minutes. Approximately 15 mls of deionized water are then added to each tube (to dissolve any precipitate) and capped. Allow to stand overnight.

6. The following day, bring up to 25 ml volume with deionized water (digestion tubes have been pre-marked).

Cleaning Digestion Tubes: 25 mls of deionized water are added to each tube and boiled at 200°C until dry. You may need to rinse the tubes with 20% NaOH followed by numerous deionized water rinses.

Analysis

Reagents:

A. Sulfuric Acid Sampler Wash Solution:

Potassium Sulfate (K_2SO_4)	34 g
Sulfuric Acid	50 ml
Deionized water up to	1 ml

To approxmiately 800 ml deionized water add 34 g K_2SO_4 and dissolve. Slowly add 50 ml concentrated H_2SO_4 an dilute to 1 liter with deionized water.

B. Sodium Chloride Diluent Solution:

Sodium Chloride	10 g
Deionized water	qs 1000 ml

C. Sodium Hyudroxide Solution:

Sodium Hydroxide	200 g
Deionized water	qs 1000 ml

To approximately 600 ml deionized water **CAREFULLY** and **SLOWLY** add 200 g NaOH. Please wear goggles! A great deal of heat will be liberated. After the solution has cooled, dilute to 1 liter with deionized water.

D. Sodium Salicylate/Sodium Nitroprusside Solution:

Sodium Salicylate	70.0 g
Sodium Nitroprusside	0.3 g
Deionized water	qs 1000 ml
BRIJ - 35	1 ml

E. Sodium Hypochloride Solution:

Sodium Hypochlorite (Clorox)	12 ml
Deionized waer	qs 200 ml

F. Stock Buffer Solution:

Sodium Phosphate, dibasic ($Na_2 HPO_4 7H_2O$)	134 g
Sodium Hydroxide	20 g
Deionized water	qs 1000 ml

Heat to dissolve 134.0 g of sodium phosphate, dibasic ($Na_2 H PO_4$) in approximately 800 ml deionized water. Add 20.0 g of sodium hydroxide and dilute to 1 liter.

G. Working Buffer:

* Sodium Potassium Tartrate	50 g
Stock Buffer solution	200 ml
NaOH solution (20% w/v)	100 ml
BRIJ	0.3 ml
Deionized water	qs 1000 ml

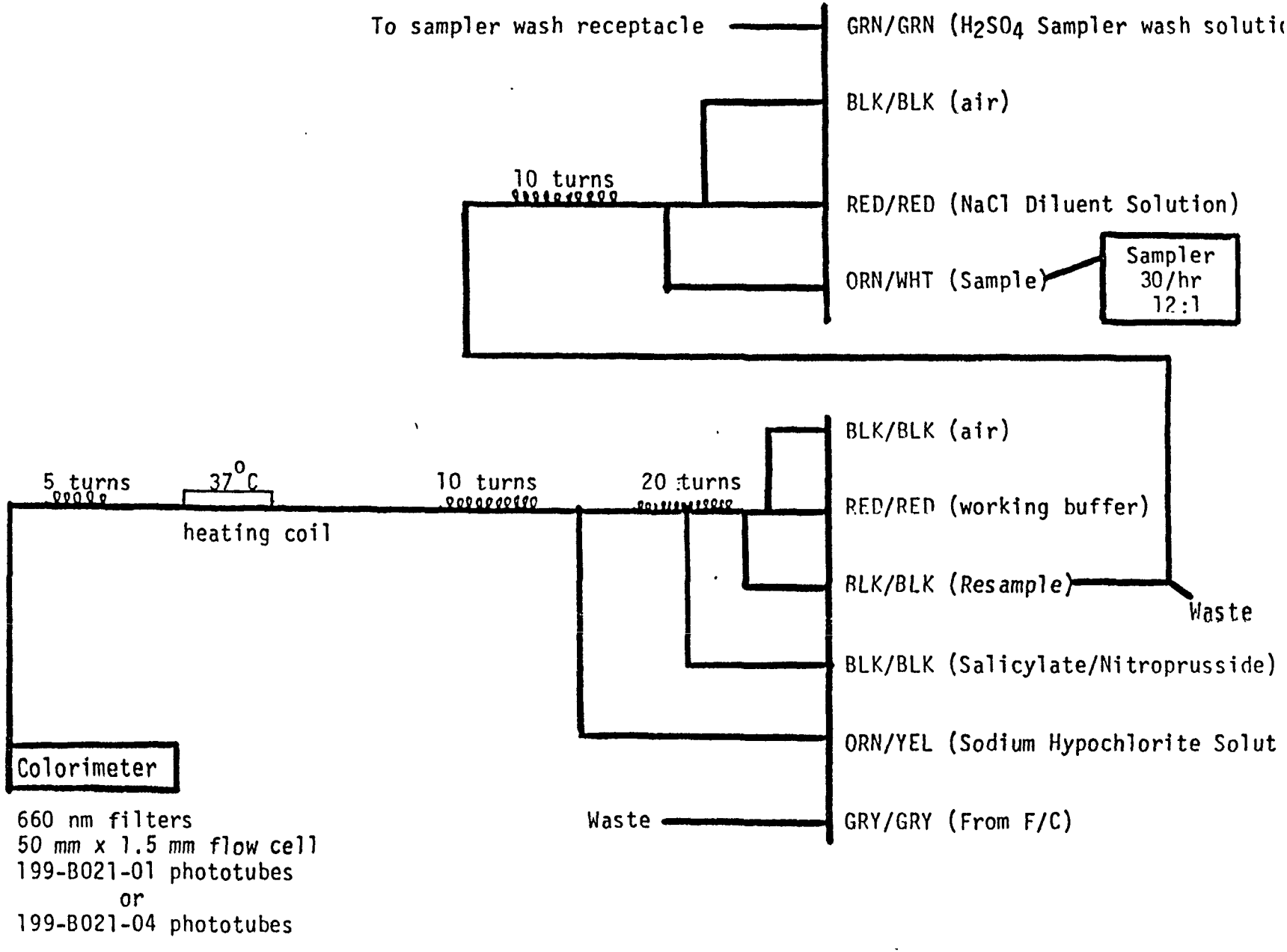
* Fifty (50) grams of Sodium Potassium tartrate is added to approximately 600 ml deionized water. (This is added as a solid to avoid the rapid formation of mold during storage of a 20% w/v Sodium Potassium Tartrate Stock Solution.) 200 ml of Stock buffer, 100 ml of sodium hydroxide solution are then added. Deionized water is used to dilute to 1 liter and 0.3 ml BRIJ is added as the wetting agent.

Analysis Procedure:

1) With the system pumping and deionized water flowing through the system, add all the reagent lines **EXCEPT** the Salicylate/ Nitroprusside Line. After approximately ten minutes, add the Salicylate/Nitroprusside line. If the pH of the flow stream is low, the sodium salicylate reagent will precipitate.

2) Prepare standards and blanks in exactly the same manner as samples — taking them all through the digestion procedure.

Manifold Configuration for Kjeldahl Nitrogen



660 nm filters
50 mm x 1.5 mm flow cell
199-B021-01 phototubes
or
199-B021-04 phototubes

Particulate Analytes

Rationale:

The direct measurement of particulate C, N & P is the preferred method used in this laboratory. It is felt that the greater volume filtered onto the pad yields a more representative sample. The alternative, subtraction of the dissolved from the total sample to determine the particulate concentration often yields negative values is totally unacceptable. Direct mesurement is rapid, more sensitive more precise.

Sampler and Storage:

Surface, bottom, above and below pycnocline water samples are collected via a submersible pump system. A known volume of the coll water is filtered through GF/F filters (nominal pore size 0.7 um), t filter folded, placed in aluminum foil and frozen until analysis.

Particulate Carbon and Particulate Nitrogen Analysis:

Outline

1. A known volume of water is filtered onto a 25 mm precombust GF/F (nominal pore size 0.7 um) filter pad.
2. Duplicate sample taken
3. Samples are folded in half, wrapped in aluminium foil, labelled and frozen for later analysis.
4. Before actual analysis the pads in aluminium foil are place in a drying oven overnight at 45°C.
5. Samples, standards and blanks are then loaded into sample wheel and analysis begins.

Instrument: Control Equipment Corp. Model 240-XA Elemental Anlyzer

1. CHN Analysis - Carbon (CO_2), hydrogen (H_2O) and nitrog (N_2) content in organic and inorganic compounds can be determined
   a. Combustion of the weighed or filtered sample occur pure oxygen under static conditions (see figure ?)
2. Helium is used to carry the combustion products throug analytical system to the atmosphere. Helium is also u for purging the instrument. It is a chemically inert relative to tube packing chemicals and has a high coefficient of thermal conductivity.

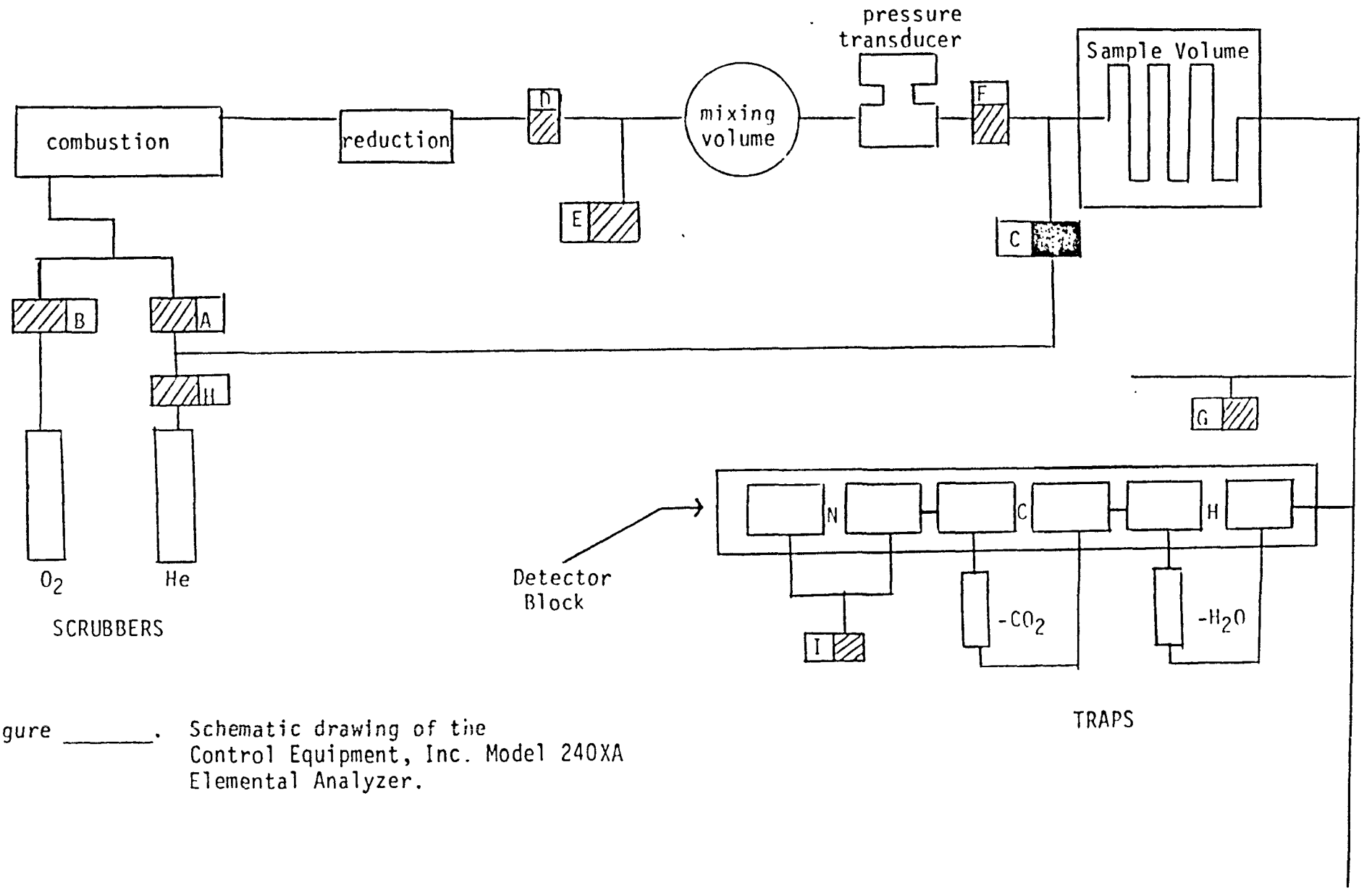


Figure _____. Schematic drawing of the Control Equipment, Inc. Model 240XA Elemental Analyzer.

a. Solenoids A-G control the gas flow through the sys
valves H and I - are used for automatic leak test

3. The products of combustion are passed over suitable r
in the combustion tube to assure complete oxidation.
reduction tube, oxides of nitrogen are converted to
molecular N and residual N_2 is removed. The CO_2, wat
vapor and nitrogen are then flushed into a mixing volu
where they are thoroughly homogenized at a precise vo
temperature and pressure. This mixture is then releas
the sample volume into the thermal conductivity detec

4. Between the first of three pairs of thermal conductiv
cells an absorption trap removes water from the sampl
The differential signal read before and after the tra
reflects the amount of water (hydrogen) in the origin
sample. A similar measure is made of the signal outp
second pair of thermal conductivity cells between whi
trap removes CO_2. The remaining gas only consists of
nitrogen and helium. This gas passes through a therm
conductvity cell and the output signal is compared to
reference cell through which pure helium flows. This
the nitrogen concentration.

Standard Run Cycle:

1. At the start of each run, the entire system is flushe
helium at a high flow rate while the sample is in the
zone.

2. The injection box is automatically purged using the P
valve.

3. The combustion train is then filled with oxygen and t
sample is injected.

4. Shortly after sample injection, D valve closes to sea
the combustion train from the rest of the analytical
system, which is still being flushed with helium.

5. Combustion occcurs under static conditions in an exce
oxygen at about 950°C.

6. During this time the mixing volume is being purged wi
and F valves open.

7. Then F closes to allow the pressure in the mixing vol
reach atmospheric pressure.

8. Close to the end of the combustion period, a high
temperature heat coil around the combustion tube vapo
any condensates at the entrance of the combustion tub
which may have been produced by diffusion of the samp
during initial stages of combustion.

9. To assure complete combustion, the ladle is retracte a small amount of O_2 is added and the ladle is fully injected.

10. During high heat, valve E closes, A and D reopen, an combustion products are completely flushed from the combustion train into the mixing volume.

11. When a pressure of 1500 mm Hg is reached, valve D cl trapping the sample gas in the mixing volume.

12. The time required to reach this pressure is called t fill time (usually 60-100 seconds).

13. The combustion train remains under positive pressure the end of the complete cycle.

14. While the sample gases are mixing, pure helium flows valve C through the sample volume and through the detectors.

15. The signal from each detector bridge is read and sto memory to provide a baseline reading with no sample the detector.

16. After mixing is complete and baseline reading has be F and G open which allows the sample gas captured in mixing volume to expand through the sample volume to atomsophere. During this time valve C is closed and is low flow through the detector.

How C, H, N is measured

17. When sample gases are near atomospheric pressure, va and G close and C opens. The water, carbon dioxide a nitrogen concentrations of the sample are measured b displacing the sample gas through the detectors to t atomosphere.

18. The volume of sample gas in the system is large enou that the helium flow allows measurement of the conte each detector in sequence, under steady state condit for at least 30 seconds.

19. The sample gas passses through the detectors at a co flow, pressure and temperature. This eliminates any variation in water vapor pressure or water vapor concentration due to changes in water adsorption of t walls of the pneumatic system.

20. While the sample gas is displaced through the detect the output signals are recorded.

21. The difference in microvolts between each "read" sign the "baseline" level for the same detector is in dire proportion to the concentration of the sample gas mea

22. At the end of a cycle, the exhaust valves are opened allow the sample gases to escape to the atmosphere.

23. The HP-159 DATA HANDLER then prints out the calculate results, places the instrument in STANDBY with C valv open, and waits for the next command.

24. With the HA automatic injector the results are printe after each run, but the run cycle continues until the selected number of runs have been completed.

Definition of Terms

BLANKS	Blank value = blank read minus blank zero. An indicator of the stability of the system.
BOAT	Platinum container used to inject sample into combu furnace.
CAPSULE	Aluminum, tin, or silver container. Used for seali samples with an accurate weight and maintains integ prior to combustion.
COMBUSTION TIME	Time for sample to fully combust in oxygen environm
COMBUSTION TUBE	Quartz tube used for packing reagents and for sampl combustion.
DETECTOR	The heart of the analyzer consisting of three brid Determines the percentages of carbon, hydrogen, and nitrogen in the sample via thermal conductivity.
DETECTOR OVEN	Keeps the temperature of the detector, pressure transducer, mixing volume, and sample volume consta
DOUBLE DROP	On HA automation, two samples are dropped for one r used for filter and inorganic applications. Sample requires a + prefix.
FILL TIME	Time required to build up the pressure in the mixi volume to 1500 mmHg.
FURNACE	Heats the reduction and combustion tubes to operat temperature.
INJECTION	Moving the ladle, containing a boat or capsule wit sample into the combustion furnace.

INJECTION BOX	For the HA automation, the box assembly that houses sample wheel.
K-FACTOR	Instrument sensitivity factor in microvolts per microgram, calibrated using a chemical standard.
LADLE	Transports the boat or capsule with the sample into combustion furnace.
MIXING VOLUME	Spherical bottle in which sample gases become homog
MOTHER BOARD	The main printed circuit board. All 240-XA power supplies are located here.
READ SIGNAL	Steady state signal produced by detector when sampl gases are present in stable concentration.
REDUCTION TUBE	Quartz tube with reduced copper that removes excess from the sample gas and reduces oxides of nitrogen free nitrogen.
RUN	One sample analysis from start to finish, including printout.
RUN CYCLE	Typically a day of operation - the entire analytica sequence of runs from the first run to the last run including the transfer of the run cycle data to the
SAMPLE VOLUME	Tube where sample gas is echausted from the mixing pricr to entering the detector.
SCRUBBERS	Removes water and CO_2 from the gas supplies.
TRAPS	Used for removing water and CO_2 from the sample gas
ZERO VALUE	Bridge signal with only pure helium flowing through detector.

Calibration:

The following formula is used to calculate K factors, as well and H concentrations in unknown samples.

$$\% = \frac{1}{K} * \frac{1}{W} * (R-Z-B) * 100$$

where: K = Calibration factor for the instrument
W = Sample weight
R = Read signal of sample gas
Z = Zero reading or instrument baseline
B = Blank signal (instrument, ladle and capsules)

Standard Used: Acetanilide

Composition: C = 71.09%
H = 6.71%
N = 10.36%

Conditioner: The conditioner coats the walls of the system surfaces (especially the mixing and sample volume) with water vapor, carbon dioxide and nitrogen which simulate actu sample running conditions.

Blanks: Should be run immediately after a conditioner.

K-factors: Always run a conditioner before a standard and before a after a blank.

K factors vary greatly from instrument to instrument, b should be within the following microvolt/microgram rang

KC = 15 to 25
KH = 44 to 76
KN = 6 to 10

Particulate Phosphorus (PP):

The method used by CBL personnel is that of Aspila, et al. (19

OUTLINE

1. Known volume of water passed through Whatman prcombus mm GF/F filter (0.7 um pore size).
2. Frozen
3. Dried at 50°C overnight
4. Muffled at 550°C for 1.5 hours.
5. Cooled overnight
6. Combusted filter placed in a labelled 50 ml plastic s cap centrifuge tube and 10 ml 1N H Cl added.
7. Capped and shaken several times during a 24 hour peri
8. Supernatant extract transferred to AutoAnalyzer cup w Pasteur pipette.
9. Phosphate (that was extracted into the 1N H Cl) analy
10. Blank filter pads are carried through the procedure a

Methodology: Aspila, I., H Agemian, and A. S. Y. Chau. 1976. semi-automated method for the determination of inorganic, organic and total phosphate in sedime Analyst. 101:187-197.

Manifold Assembly: See figure ?.

Damp: Normal

Sampling Rate: 40/hour 9:1 Sample/Wash ratio

Filter: 880 nm

Phototube: 199-B021-04

Interferences: Silicon at analysis temperature > 40°C and or N H_2SO_4 in the mixed reagent solution causes interference in the concentration range of > .05 mg/ml silicon in the extract. These conditions are avoided by maintaining an acid concentration of 2.45 N H_2SO_4 in the reagents analysis at 37°C.

Reagents:

1. 1N Hydrochloric Acid:

Hydrochloric Acid (HCl), concentrated (sp. gr. 1.19)	86 ml
Deionized water (QS to :)	1000 ml

Add 86 ml conc. HCl to approximately 800 ml good qua deionized water while cooling (cold water bath). Af the solution is cooled, dilute to one liter with dei water.

2. Deionized Water Diluent:

Add .5 g sodium lauryl sulfate (SLS) to 500 ml good quality deionized water. Mix well!

3. Sulfuric Acid (4.9 N):

From orthophosphate method

4. Ammonium Molybdate:

From orthophosphate method

5. Ascorbic Acid:

From orthophosphate method

6. Antimony Potassium Tartrate:

From orthophosphate method

7. Sodium Lauryl Sulfate (SLS):

From orthophosphate method

8. Working Reagents:

From orthophosphate method

Standards

A. Stock Standard: From orthophosphate method

B. Secondary Standard: Take 0.1, 0.25, 0.5, 1.0 ml of s standard A and dilute each to 100 ml with 1N HCl whic yield 12 ug at P/l (.372 mg P/l); 30 ug at P/l (.93 P/l); 60 ug at P/l (1.86 mg P/l) and 120 ug at P/l (P/l).

Calculation of Concentration:

$$\text{mg P/l} = \frac{[\%\text{ on AA Chart of Blank}] * F * \text{HCl extraction volume (ie.,}}{\text{Volume filtered (l) onto the filter pad}}$$

(F is the mean of $\frac{\text{standard concentration}}{\%\text{ on AA Chart of standard}}$)

Total Suspended Solids (TSS):

The method used by CBL personnel is basically that of APHA met 208D (Total Nonfiltrable Residue) dried at 103-105°C and EPA method Residue, Total-Non-Filterable with some modification. Washing of f pads with aliquots of deionized water has not been included. TSS i retained material on a standard glass fiber filter disk after filtr of a well mixed sample of water. Results are expressed in mg/l.

Methodology:

1. Whatman 47 mm GF/F filter pads (0.7 um pore size) are numbered and then weighed to 4 decimal places.

2. The pads are then placed in an oven at 103°C for one

3. Pads are then weighed.

4. In the field, a known volume of water is filtered thr the pad.

5. Upon returning to the laboratory, these pads are froz

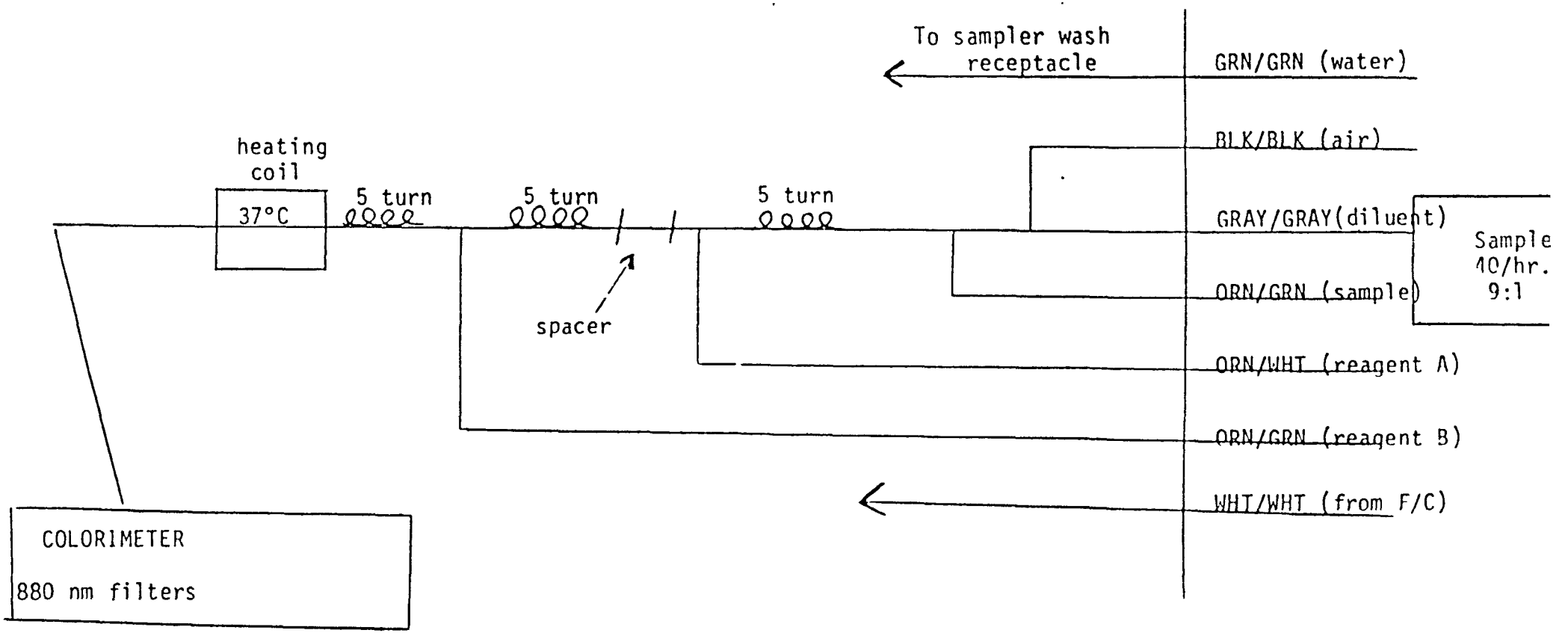
Day of Analysis

6. Filters are dried for one hour at 103-105°C and then weighed and the weights recorded. A few pads in that are weighed again one hour later to check for any additional weight loss. If there is more than a 0.5 r weight loss between the same filter all pads are then dried and re-weighed.

7. Calculation

$$\text{mg TSS/l} = \frac{(\text{weight of filter + residue}) - (\text{weight of filter}) * }{\text{mls of sample filtered}}$$

MANIFOLD CONFIGURATION FOR PARTICULATE P



50 mm x 1.5 mm I.D. Flowcell
199-B021-04 Phototube

Note: A teflon sample probe with glass shield

APPENDIX D

METHODOLOGICAL COMPARISONS FOR NITROGEN DETERMINATION IN ESTUARINE WATER SAMPLES

Methodological Comparisons for Nitrogen and Chlorophyll Determinations in Estuarine Water Samples

by

Christopher F. D'Elia[1]

Kenneth L. Webb[2]

Diane V. Shaw[1]

Carolyn W. Keefe[1]

[1]Nutrient Analytical Services Laboratory, Chesapeake Biological Laboratory. Center for Environmental and Estuarine Studies, University of Maryland, Solomons, MD 20688-0038.

[2]Virginia Institute of Marine Science, Gloucester Point, VA 23062

Submitted to:

Power Plant Siting Program
Department of Natural Resources
State of Maryland
Annapolis, Maryland

and

Chesapeake Bay Liaison Office
U.S. Environmental Protection Agency
Annapolis, Maryland

ABSTRACT

This study was undertaken to compare results obtained with "standard" and "alternative, new" techiques for total nitrogen and chlorophyll determination in estuarine water samples.

The standard technique for total nitrogen (TN) determination recommended by the U.S.E.P.A. involves the total Kjeldahl nitrogen (TKN) procedure in which TKN + nitrate + nitrite gives TN. The EPA TKN procedure using the Technicon Block Digestor proved difficult to implement with estuarine water samples: the block digestor heated samples unevenly and continous flow analyzer baselines were unstable. However, standard "spikes" with a variety of analytes yielded quantitative recovery and exhibited no salinity effect. The alternative, the total persulfate nitrogen (TPN) technique, gives TN directly and is easier to perform. More samples can be run per day using the TPN procedure. TPN determination on standard spikes, like TKN, yielded quantitative recovery and no salinity effect. A comparison of values obtained using both techniques on natural, estuarine water samples collected from a variety of locations in the Chesapeake Bay over an annual cycle yielded equivocal results. The regression equation TPN (less nitrate & nitrite) = 21.79 ($\pm$ 1.04) + TKN * 0.153 ($\pm$ 0.021), best fitted the data. At low TKN and TPN values the two techniques gave comparable results, but as TKN values increased, TKN gave consistently higher values. Whether this discrepancy results from an over-recovery by TKN or under-recovery by TPN cannot be determined at present. Additional comparative work is continuing using a modified TKN procedure to improve continous flow analyzer baseline stability.

The standard technique for chlorophyll a determination recommended by the U.S.E.P.A. involves grinding a glass-fiber filter, extraction with 90% acetone and spectrophotometric determination of pigment concentration. The alternative technique we tested involved extracting the filter with dimethylsulfoxide(DMSO):acetone:water (9:9:2) and reading pigment concentrations using a fluorometer calibrated with chlorophyll a from a commerical supplier. The results indicated that the fluorometric and spectrophotometric methods for chlorophyll a estimations in general use have a low accuracy (approximately $\pm$ 30%) due to storage and interference problems. The DMSO-based technique allows for the immediate extraction of pigments from plankton samples and prevents the loss of chlorophyll a due to storage and subsequent grinding and extraction with 90% acetone. In one comparison, reduction in recovery after storage was nearly one-third. Chlorophyll b, which has been shown in the literature to interfere with the determination of chlorophyll a, was shown to occur in Chesapeake Bay phytoplankton. For convenience, cost, rapid extraction, and prevention of storage loss of pigments, we recommend the DMSO-extraction technique followed by fluorometric determination within several days. An acceptable alternative is to extract and read the samples spectrophotometrically, within a few days of sampling in cuvettes of appropriate path length (1-10cm), with and without acidification for phaeophytin correction. If truly high accuracy, high precision results are required, an HPLC method is desirable.

Table of Contents

OVERVIEW

The following report is submitted jointly to the Maryland Department of Natural Resources' Power Plant Siting Program (PPSP) and the Environmental Protection Agency's Chesapeake Bay Liaison Office. The work reported on was performed at the request of these agencies to compare (1) total Kjeldahl nitrogen (TKN) determination using a semi-automated block digestor procedure with a semi-automated alkaline persulfate nitrogen (TPN) digestion determination and (2) several alternative methods of chlorophyll a determination. These determinations are of considerable interest with regard to water quality monitoring programs on the Chesapeake Bay. The TKN vs. TPN comparisons were done in the Analytical Services laboratory of Chesapeake Biological Laboratory (CBL) which typically uses the TPN procedure, and the chlorophyll a determinations were performed primarily by the Virginia Institute of Marine Science (VIMS) with assistance by CBL.

The funding agencies solicited this work to ensure that the adoption of alternative, non-standard methods would provide data comparable to those obtained using standard, EPA-approved methods.

SECTION 1

COMPARISON OF TPN AND TKN METHODS

General Description of N Fractions in Natural Waters

Figure 1-1 shows the nitrogenous fractions typically determined in water quality studies. Also shown are the abbreviations typically used for these fractions.

The distinction between "particulate" and "dissolved" nitrogen is necessarily arbitrary. Particulate N (PN) is assumed to be that retained on a filter having a nominal pore size between 0.45 and 1.2 um. Total dissolved N (TDN) is that passing through such filters, and undoubtedly contains some small particulates and colloidal compounds, regardless of the filter used. In most cases, the difference between that retained on different filters in that range of nominal pore sizes is negligible, although the filter matrix used may have an effect--organic "membrane" filters are more prone to contamination than glass fiber filters.

Figure 1-2 and Table 1-I present all abbreviations used in this report and give a comparison of how the different N fractions are determined using standard EPA methods and the commonly used oceanographic measurements employed by CBL. In Table 1-I all determinations of a given fraction done directly, i.e. not by difference or sum of other fractions, is indicated in **boldface**.

The major differences between the standard EPA and commonly used oceanographic procedures are that the latter (1) measure PN directly by elemental (CHN) analysis of particulate material filtered onto glass fiber filters, and (2) determine TDN using alkaline persulfate oxidation (TPN analysis). Oceanographers have adopted the alternate procedures for the following reasons. Elemental analysis is extremely precise and offers the

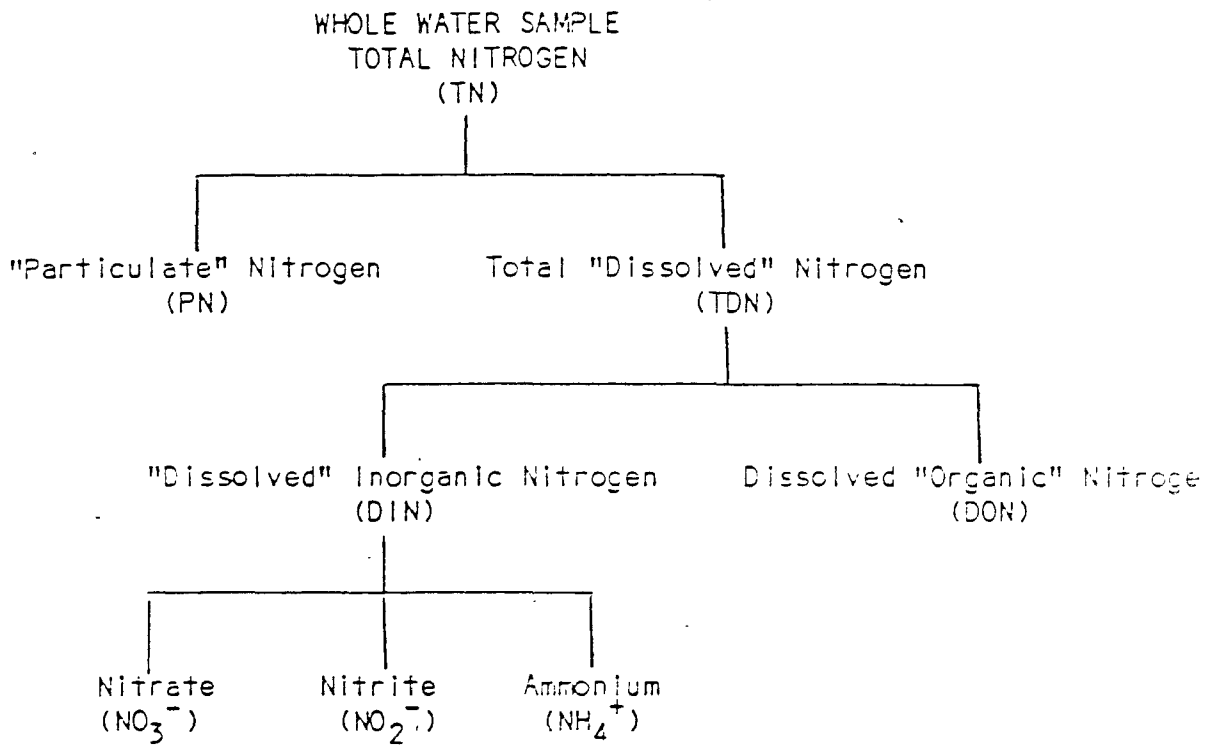
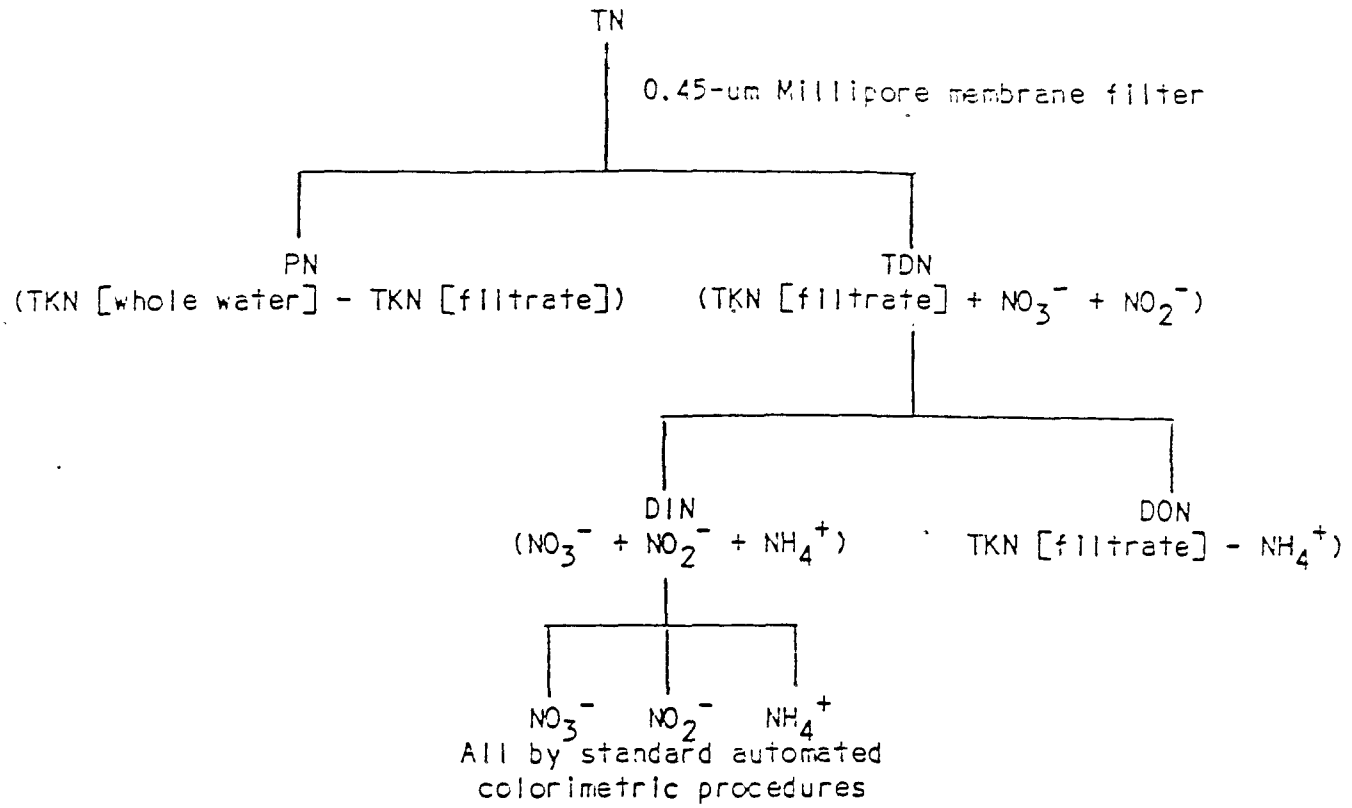


Figure 1-1. N fractions determined in typical water quality studies.

A. Standard EPA



B. Typical Oceanographic (CBL)

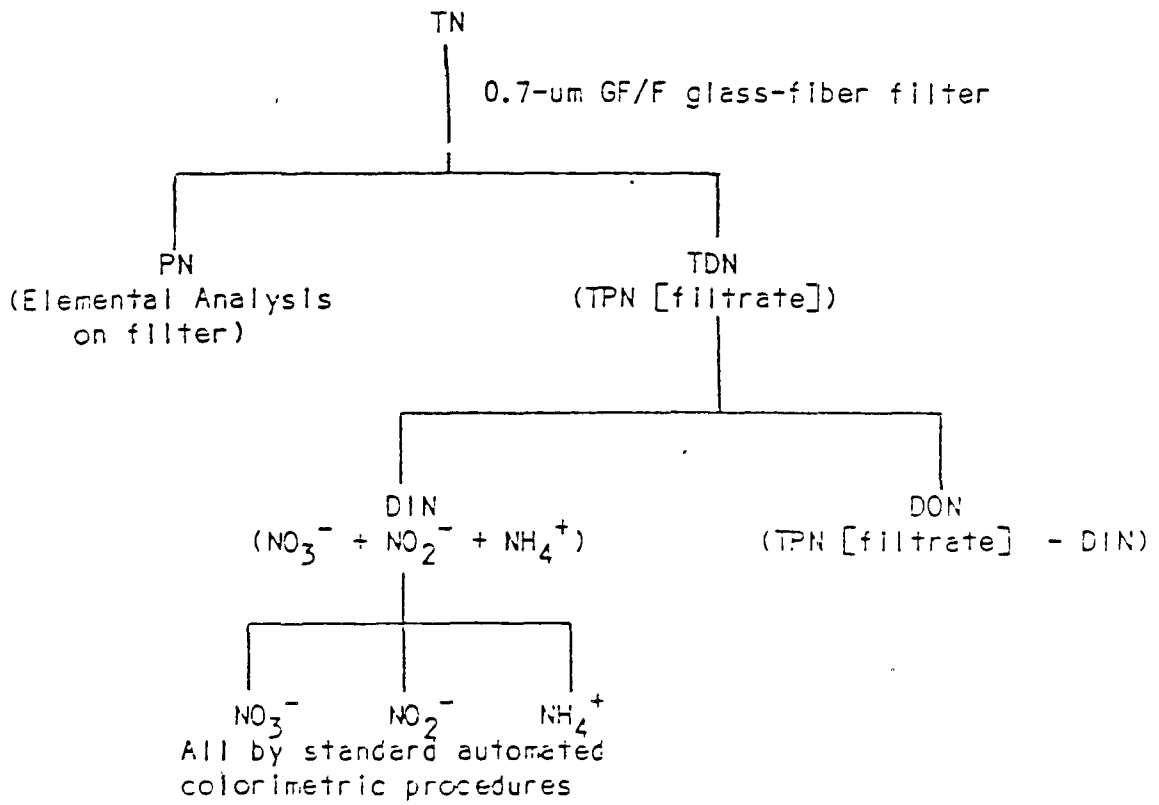


Figure 1-2. Comparison of standard EPA and typical oceanographic (CBL) nitrogen determinations.

Table 1-1. Comparison of standard EPA and typical oceanographic (CBL) procedures. Fractions measured directly are boldfaced.

Fraction	EPA	Typical Oceanographic (CBL)
TN	TKN (whole water) + NO_3^- + NO_2^-	PN + TDN
PN	TKN (whole water) minus TKN (filtrate)	PN
TDN	TKN (filtrate) + NO_3^- + NO_2^-	TPN (filtrate)
DIN	NO_3^- + NO_2^- + NH_4^+	Same as EPA
NO_3^-	**NO_3^- (Colorimetric)**	Same as EPA
NO_2^-	**NO_2^- (Colorimetric)**	Same as EPA
NH_4^+	**NH_4^+ (Colorimetric)**	Same as EPA
DON	TKN (filtrate) minus (NO_3^- + NO_2^-)	TDN minus DIN

advantage of being a direct, rather than indirect determination of that fraction. TPN digestion is much simpler and easier to perform than TKN analysis, costs less to analyze per sample, and provides a direct measurement of total dissolved nitrogen (TDN).

Background and Literature Review

Oxidation procedures utilized in TKN and TPN methods are used primarily to oxidize N-containing organic compounds, i.e. dissolved organic nitrogen (DON). The following discussion pertains to these and similar oxidation procedures for DON, and is provided here for general background information. Much of this was exerpted from D'Elia (1983).

As was shown in Figure 1-2, DON is determined by difference between total dissolved nitrogen (i.e. nitrate + nitrite + ammonia + organic nitrogen) and dissolved inorganic nitrogen (i.e. nitrate + nitrite + ammonia) or by difference between Kjeldahl nitrogen (ammonia + dissolved organic nitrogen) and ammonia. A variety of oxidation procedures have been used to oxidize and quantify DON.

1. Wet Oxidation Procedures

a. Kjeldahl Oxidation. Most of the earlier procedures for DON determination lacked adequate sensitivity, and involved the traditional but tedious Kjeldahl wet oxidation procedure (Kjeldahl, 1883). This approach consists of an initial evaporation step followed by an oxidation with concentrated sulphuric acid. It is generally regarded as difficult to perform, and lends itself neither to shipboard use or to automation. In early work, ammonium produced by the digestion process was determined by titration (Barnes, 1959), while more recently colorimetric procedures have been used (Strickland and Parsons, 1972; Webb et al. 1975; Webb, 1978). A number of semiautomated procedures are in use in which samples are oxidized by a manual Kjeldahl procedure with subsequent ammonia determination on the digests being performed by autoanalysis using photometric (Faithfull, 1971; Scheiner, 1976; Jirka et al., 1976; Conetta et al., 1976; Adamski, 1976) or electrometric procedures (Stevens, 1976).

b. Photo-oxidation. The photochemical oxidation procedure first developed by Armstrong et al. (1966) has generally superceded the Kjeldahl oxidation procedure in most marine applications. A small quantity of hydrogen peroxide is added to a sample contained in a quartz reaction vessel, and high wattage mercury lamps are used to produce ultraviolet light to photo-oxidize organic nitrogen, nitrite and ammonia to nitrate; nitrate is then determined as described previously. The procedure is considerably less tedious than the Kjeldahl procedure, can be performed at sea, and unlike other procedures for DON oxidation, is relatively easy to automate (Afghan et al., 1971; Lowry and Mancy, 1978). However, it does have some shortcomings. Workers testing this method in freshwaters have found that the photochemical reaction is very pH-sensitive and may not completely oxidize compounds such as ammonia and urea (Afghan et al., 1971; Henriksen, 1970; Lowry and Mancy, 1978). Lowry and Mancy (1978) found that ultraviolet digestion gave good results decomposing C-N but not N-N bonds, yet felt that most compounds implicated in biological processes would be recovered satisfactorily. Obviously, for samples containing a large amount of nitrate plus nitrite, such as those from the deep ocean, the precision of DON determination by use of photo-oxidation will be less than that of a

modern Kjeldahl procedure.

c. Persulfate Oxidation. Koroleff (1970; 1976) developed an alternative wet oxidation procedure for total nitrogen determinations that is becoming more widely used. He found that under alkaline conditions at 100°C and in the presence of excess potassium persulfate, organic nitrogen in a seawater sample is oxidized to nitrate. Nitrate is then determined by the standard photometric procedures used for nitrate determination. D'Elia et al. (1977) and Smart et al. (1981) have shown that organic nitrogen determinations by the persulfate and Kjeldahl techniques yield comparable results and precision for both sea and freshwater samples; they also discussed the advantages and disadvantages of persulfate oxidation relative to Kjeldahl oxidation and photo-oxidation. Nydahl (1976) and Solorzano and Sharp (1980) have suggested some improvements to Koroleff's original procedure that alter reaction pH, lower blanks, and provide for the requisite excess of peroxydisulfate. Nydahl (1976) noted that errors may result when using persulfate oxidation on turbid samples; he also provided an in-depth study of reaction kinetics and percentage recovery at varying oxidation temperatures. Valderrama (1981) reported the simultaneous determination of total N and total P using alkaline persulfate oxidation. Goulden and Anthony (1978) have studied kinetics of the oxidation of organic material using persulfate and have thus provided a basis for still further refinement of the procedure such that simultaneous determination of C, N and P may ultimately be possible on the same sample. As in the case of photochemical oxidation, determination of DON by the persulfate technique will have poor precision in the presence of large quantities of nitrate or nitrite.

The original Koroleff procedure has been improved by Koroleff (see Grasshoff et al., 1973) and modified recently to provide for increased precision (Kalff and Bentzen, 1984) and for semiautomation and simultaneous determination of both N and P (Glibert et al., 1977; Ebina et al., 1983), and for determining N and P in particulate matter (Lagner and Hendrix, 1982). Both reports indicated that satisfactory recoveries were obtained with most organic nitrogen compounds.

2. Dry Combustion Procedures

Dry combustion procedures have been generally disappointing or impractical for determining DON, although a recent report (Suzuki et al., 1985) suggests that a practical alternative may be at hand. Gordon and Sutcliffe (1974) reported a dry combustion procedure in which a seawater sample is freeze dried and the salt residues subsequently ignited in a CHN analyzer. The obvious disadvantage of this is the need for a freeze drier and the time involved in sample preparation. Other procedures have been developed in which small volumes of sample are injected directly into a combustion tube for evaporation and combustion (Van Hall et al., 1963; Fabbro, et al., 1971; Hernandez, 1981), but these have not found wide use by oceanographers because expensive and specialized equipment is required and sea salt accumulation in the combustion chamber may reduce oxidation efficiencies.

Recently, Suzuki et al. (1983) reported on a high-temperature catalytic oxidation method in which nitrogenous compounds in liquid samples are oxidized on a platinum catalyzer at 680°C under oxygen atmosphere and the generated nitrogen dioxide (NO_2) is absorbed into a chromogenic reagent,

followed by a spectrophotometric determination. These authors report that the TPN procedure yielded from 30-90% of the recovery afforded by their pyrolysis technique. Unfortunately, the required instrumentation for this procedure, the Sumitomo TN-200 total nitrogen analyzer is not available in the U.S., and there have been no other published comparisons between results of this dry combustion technique and wet oxidation procedures. However, given the results of the Suzuki, et al. (1985) study, more comparisons should be made between their dry combustion and other oxidation procedures.

Methods

1. Sampling and experiments. Samples for comparing TKN and TPN determinations derived from three sources: (1) samples collected by the "SONE" program of W.R. Boynton, et al.; (2) samples collected from the large scale outdoor continuous culture system operated by the Academy of Natural Sciences at Benedict, MD; (3) samples prepared in an experiment to compare recovery of spikes of standard compounds in water of different salinity.

All samples were frozen as soon as possible after collection and were thawed immediately before analysis.

2. TPN procedure. TPN determination was basically that of D'Elia et al. (1977), with the following exceptions: (a) the oxidation was done on 10 ml samples in 30-ml glass screw-cap test tubes, and (b) the method used to determine the nitrate concentration in the digest was the EPA-approved AutoAnalyzer method (353.2)(USEPA, 1979).

This method with the above modification has been in use at CBL for the past five years, although some improvements in the methodology have been proposed by others (e.g. Valderrama, 1981; Solórzano and Sharp, 1980) that may help further improve the method.

a. General Description. 15 ml of alkaline persulfate reagent is added to the 10 ml sample in the 30-ml screw-cap test tube. Samples are autoclaved at 100-110°C for one half hour and slowly brought back to room temperature. Each digested sample is neutralized by the addition of 1.5 ml of 0.3 N HCl and mixed with a vortex mixer. Two ml of borate buffer is then added to the sample and vortexed. The nitrate concentration of the buffered samples is then determined.

b. Reagents. Reagents were prepared as follows:

o Oxidizing reagent: 3.0 of NaOH and 6.7 g of low N (<0.0003%) potassium persulfate, $K_2S_2O_8$, are dissolved in 1 liter with nitrogen-free distilled water just before use.

o 0.3 N HCl

o Borate buffer solution: 30.9 g of H_3BO_3 are dissolved in distilled water, 101 ml of 1 N NaOH are added, and the solution brought to 1 liter with distilled water.

3. TKN procedure. We used a semiautomated total Kjeldahl nitrogen (TKN) procedure--EPA method 351.2 (colorimetric, semi-automated block digestor, AutoAnalyzer II). The TKN procedure we employed was as close to that used by the EPA's Central Regional Laboratory in Annapolis (U.S.E.P.A., 1979) as possible. On several occasions, we used the identical equipment used by EPA for analyses. This was done to obtain the most comparable TKN data.

a. General Description. The sample is heated with a boiling chip in the presence of sulfuric acid, potassium sulfate, and mercuric sulfate for four and one-half hours. The residue is cooled, diluted to the original volume and placed on the continuous flow analyzer for ammonia determination. The determination of ammonia-N is based on a colorimetric method in which an emerald-green color is formed by the reaction of ammonia with sodium salicylate, sodium nitroprusside, and sodium hypochlorite in a buffered alkaline medium at a pH of 12.8-13.0. The ammonia salicylate complex is read at 660 nm using a continuous-flow analyzer photometer.

b. Reagents. Reagents were as follows:

o Digestion mixture: 25 ml Hg_2SO_4 + 200 ml conc. sulfuric acid + 133 g K_2SO_4 are diluted to 1 liter with ammonia-free distilled water. H_2SO_4 solution: 8 g HgO + 10 ml conc. H_2SO_4 diluted to 100 ml with ammonia-free DW.

o Sulfuric acid solution (4%): add 40 ml of conc. sulfuric acid to 800 ml of ammonia-free distilled water, cool and dilute to 1 liter.

o Stock Sodium Hydroxide (20%): Dissolve 200 g of sodium hydroxide in 900 ml of ammonia-free distilled water and dilute to 1 liter.

o Stock sodium potassium tartrate solution (20%): Dissolve 200 g potassium tartrate in about 800 ml of ammonia-free distilled water and dilute to 1 liter.

o Stock buffer solution: Dissolve 134.0 g of dibasic sodium phosphate (Na_2HPO_4) in about 800 ml of ammonia free water. Add 20 g of sodium hydroxide and dilute to 1 liter.

o Working buffer solution: Combine the reagents in the stated order; add 200 ml of stock buffer solution to 250 ml of stock sodium potassium tartrate solution and mix. Add 120 ml sodium hydroxide solution and dilute to 1 liter.

o Sodium salicylate/sodium nitroprusside solution: Dissolve 150 g of sodium salicylate and 0.3 of sodium nitroprusside in about 600 ml of ammonia free water and dilute to 1 liter.

o Sodium hypochlorite solution: Dilute 6.0 ml sodium hypochlorite solution to 100 ml with ammonia-free distilled water (reagent is made daily).

c. Digestion procedure. 20- or 25-ml samples are mixed well, rinsed 3x with ammonia-free DW and the sample plus rinse water are added to the digestion tube for each sample. 5 ml of digestion solution and 4-8 Teflon boiling stones are added to each tube, which is then mixed on a tube

vortex mixer. With the block digestor in the "manual" mode, the low and high temperatures are set at 160°C and preheated until temperature is reached (verified with a thermometer in sample of digestion solution alone). Tubes are placed in digestor and heated at 160°C for 1 hour. After 1 hour the "manual" mode is reset to 380°C and samples are heated for 2.5 hours longer. At the end of 2.5 hours the block digestor is shut off manually.

Samples are cooled to room temperature at which time approximately 20 ml of ammonia-free distilled water is added. Samples are then placed in a sonicator (Astrason, Ultrasonic Cleaner, Model 13-H) for one-half hour to break up precipitate. Each sample is mixed with a tube vortex mixer until complete dissolution of all digestion residue and complete absence of layers of solutions in the tubes. Ammonia-free distilled water is then used to dilute samples back to the 25 ml initial sample volume.

During measurement of ammonia-N on the continuous-flow analyzer (Scientific Instruments Corporation CFA 200) one set of reagents is used during each sampling series. The continuous-flow analyzer is fitted with a Kjeldahl manifold (Scientific Instruments Corporation TKN Cartridge No. 116-540-0), which is used without the dilution loop (Figure 1-3). Reagent lines are added to the manifold in the order: Working buffer, 4% sulfuric acid, hypochlorite solution, and nitroprusside. The system is allowed to equilibrate after the addition of each reagent and prior to running samples.

d. Standards and Blanks. TKN determinations included the following standards and blanks:

- Ammonium sulfate standards: 0.0, 15.0, 45.0, 75.0 umol N L^{-1}.
- Urea standards: 0.0, 10.7, 32.1, 42.8 umol N L^{-1}.

4. Experimental Comparisons. We analyzed samples collected in the field and samples prepared in the laboratory to compare TPN and TKN recovery efficiencies. Since TKN analysis yields organic nitrogen and ammonium nitrogen and TPN analysis also determines nitrate, nitrite and ammonium, direct comparisons cannot be made. Accordingly, we also performed nitrate and nitrite determinations on all samples. The value obtained by subtracting nitrate and nitrite from TPN is then comparable with TKN. Our comparative studies included samples from: (1) The SONE program (August and October, 1984; May, June, August, October, 1985); (2) An experiment in which standards were added to samples of seawater diluted with distilled water to different salinities; and (3) A wide range of N concentrations in the outdoor large-scale continuous cultures at the Academy of Natural Science's Benedict Estuarine Research Laboratory.

Results and Discussion

1. General Observations

TKN determination with the EPA-approved block digestor method proved to be tedious and difficult. We chose to use this block digestion method because it is often used when large numbers of samples must be processed and because this is the method used by EPA in the monitoring program. We do not use this procedure routinely in our laboratory, so much of our work was done at the Central Regional EPA Laboratory in Annapolis, particularly until we were able

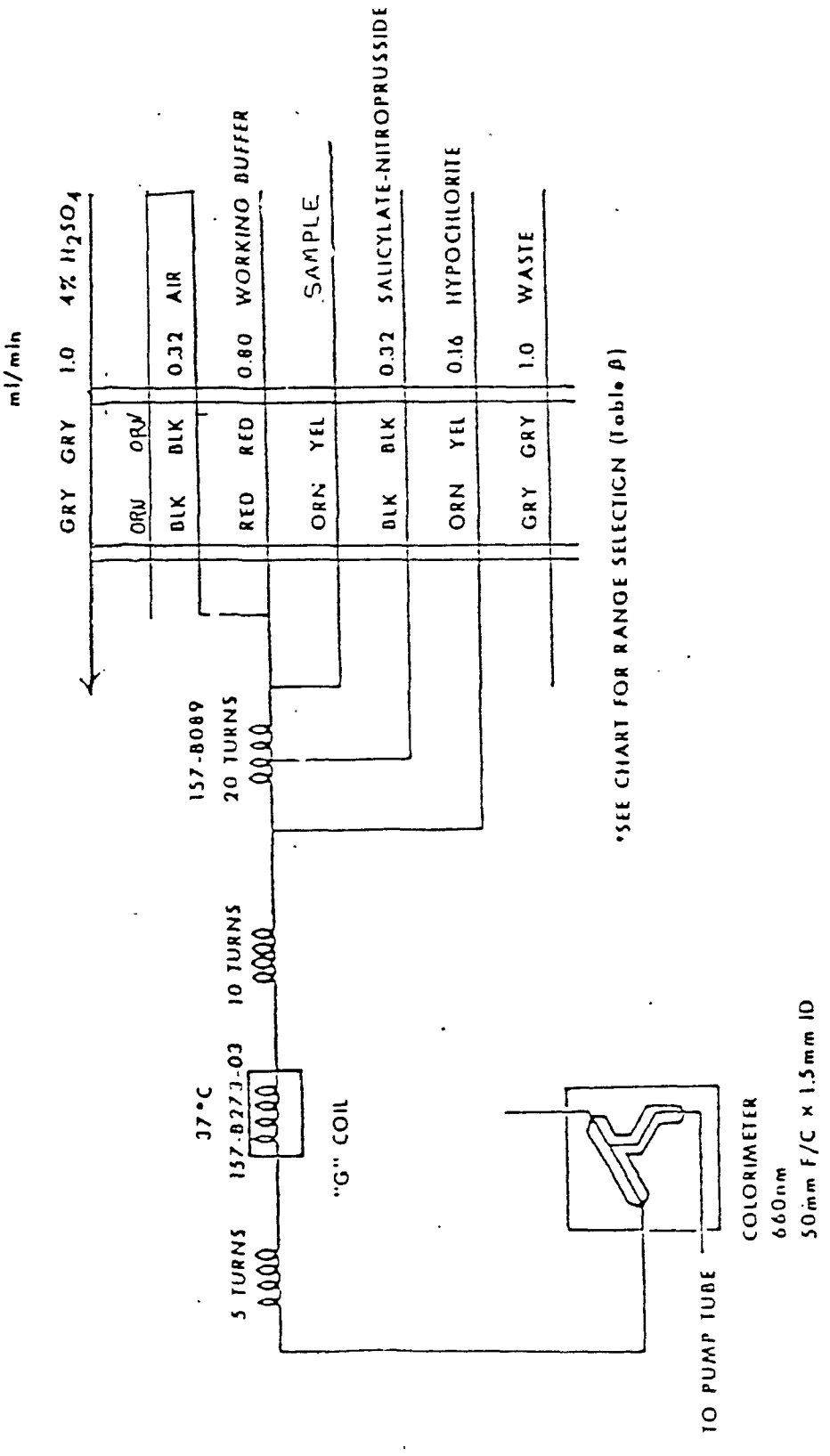


Figure 1-3. Ammonium manifold used in conjunction with block digestor for the semiautomated TKN procedure at EPA's Central Regional Laboratory in Annapolis.

to gear up fully at CBL. We encountered a great number of problems particularly with the digestion phase. The brand-new Technicon Block Digestor we used failed to heat samples evenly and took a long time to reach temperature. Analysts at EPA have also reported similar difficulties with their block digestor. Once we had successfully determined block digestor preheating times and had calibrated the temperature regime achieved in each individual position in the digestor, we encountered further problems. The principal problem was with the use of the Teflon boiling chips recommended in the EPA procedure. On samples containing appreciable salinity, at the latter phases of the digestion procedure after most water had boiled off, the chips floated and failed to prevent bumping and splattering. Such problems are discussed in greater detail below.

a. Block digestor temperature control. Verification of exact temperature settings and timing for the block digestor were made by filling each heating cell with sand and measuring the temperature of the cells during heating. The temperatures of selected cells were further verified by measuring the temperature of a sample of digestion solution during heating.

Initially, the proper temperatures were attained and maintained by the digestor according to the proper temperature schedule. However, when the control was set on "automatic" the control box sporadically turned the block heater off during heating, as well as boiled some samples dry (loss of boiling chips and sample, which we termed "melt down"). Melt downs did not appear predictable, i.e. they did not occur in the same block hole nor did they occur during every digestion run. Samples were run on "manual" to avoid the problems with the "automatic" setting. The occasional sample loss due to melt downs could not be prevented. Due to these inconsistent differences in temperature and melt downs between successive digestion runs, standard curves based on ammonium sulfate and urea were constructed for each set of samples digested.

b. Standards. The EPA Standard Operating Procedure for TKN Determination recommends the following working standards of ammonium sulfate: 0.2, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0 mg N L^{-1}. A standard curve of these concentrations is non-linear at the higher concentrations and requires a dilution loop. However, the concentration of total Kjeldahl nitrogen in field samples is typically much lower than the lowest EPA standard (20 - 70 umol N/L) and the dilution loop, if used considerably reduces the analytical precision of the TKN method. Due to the previous problems the following standard curve was used: 0.0, 15.0, 45.0, 75.0 umol N L^{-1} (0.0, 0.21, 0.63, and 1.05 mg N L^{-1}) based on an ammonium sulfate primary standard. Standard curves were linear and field sample concentrations consistently fell within this standard range.

The EPA procedure presents the data of one accuracy test which showed 100% recovery of organic-N from ammonium standards spiked with N-nicotinic acid. Recovery of organic nitrogen depends upon the digestion history of the sample, therefore each digestion run should include an accuracy test for organic nitrogen recovery. For this reason each TKN run contained a urea standard curve of 0.0, 10.7, 31.2, 42.8 umol N L^{-1} (0.0, 0.15, 0.45, 0.6 mg N L^{-1}).

c. Teflon boiling chips. The EPA method recommends cooling samples 15 minutes, then adding water to the digestion tube up to the initial volume before digestion (25 ml). The precision of estimation of

ammonia-N is unavoidably affected because the boiling chips cannot be removed from the samples before diluting to 25 ml.

d. Dilution loops. The standard Kjeldahl digestion manifold (Scientific Instruments, TKN Cart. 116-540-01) for ammonia-N determinations dilutes each sample with distilled water in a dilution loop prior to the introduction of reagents. Output curves recovered from the manifold with the digestion loop appeared noisy with standards and samples almost indistinguishable from background noise. Exclusion of the dilution loop from the rest of the Kjeldahl manifold produced very distinct peaks for both samples and standards (0.0 - 75.0 umol N L^{-1}; 0.0 - 1.05 mg N L^{-1}) which were clearly above background noise. See Figure 1-3 for a diagram of revised Kjeldahl manifold.

2. TPN and TKN Recovery Efficiencies vs Salinity

Once we had obtained satisfactory performance with our Kjeldahl procedure, we performed the following experiment to compare TPN and TKN recoveries at different salinities and concentrations. Low-nutrient, continental shelf seawater and various dilutions thereof were spiked with reference compounds (ammonium, urea, glutamic acid, and nitrate) at concentrations ranging from 0 to 75 uM. The original data are presented in Appendix II, with correlation coefficients for the standard curves in Appendix III. Precision of the total N determination by TKN and TPN taken from the literature are compared by coefficients of variation in Appendix IV. For future work with reference compounds, more-difficult-to-oxidize compounds such as caffeine should also be tested (Suzuki et al., 1985).

a. TPN. Figure 1-4 shows peak heights obtained by the TPN (x-axis) procedure plotted against seawater dilution (y-axis) and spike concentration (z-axis). All peak height data are included for a given percent seawater dilution and spike concentration, regardless of the nitrogen compound used in the spike. Curves are fitted by eye to the concentration data for a given seawater dilution--in effect, representing a standard curve for each dilution. Precision is obviously good at all seawater dilutions, and the "standard curves" appear linear.

Figure 1-5A through 1-5D present the percentage recoveries of spiked compounds relative to nitrate standard curves in distilled water for the same data lumped together in the previous figure. With the exception of recoveries at the lowest spike concentrations which exceeded 100% (function of ammonium contamination of the seawater used for the experiment that can be corrected by subtracting a blank value determined for each salinity), essentially 100% recovery occurred at all concentrations and dilutions.

To determine the upper range of the persulfate method, recoveries of glutamic acid and urea were also determined on 150-750 umole spikes in the given seawater dilutions. Essentially 100% recovery occurred at all concentrations and dilutions.

b. TKN. Figure 1-6 shows peak heights obtained for TKN plotted as a function of seawater dilution and spike concentration. As with the TPN determination, there was no obvious salinity effect for the TKN procedure--all standard curves clearly had similar slopes and intercepts on the y axis.

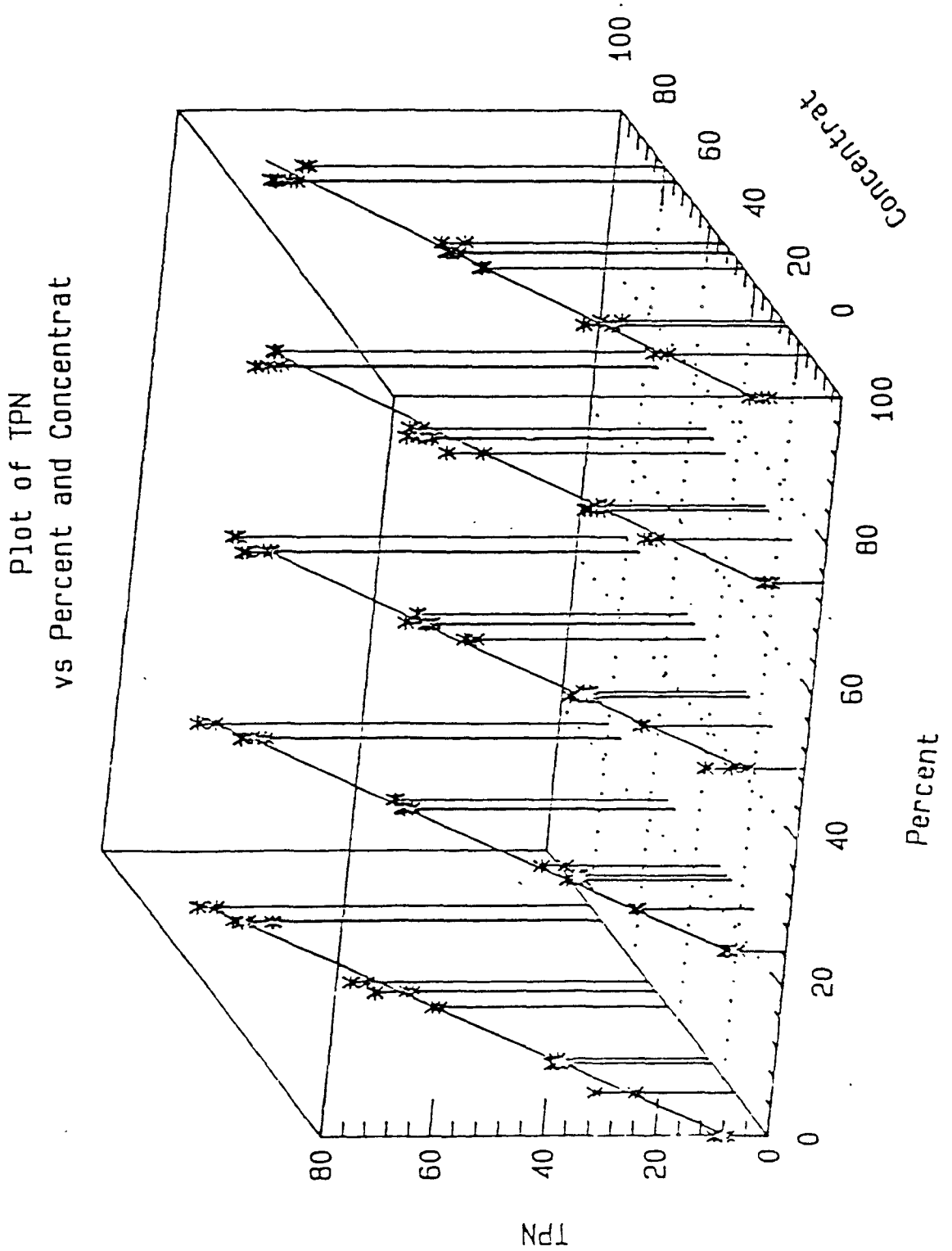


Figure 1-4. Three dimensional plot of TPN-determined concentration of standards (umol N L^{-1}) vs. percent seawater vs. expected concentration of spiked reference standards (umol N L^{-1}).

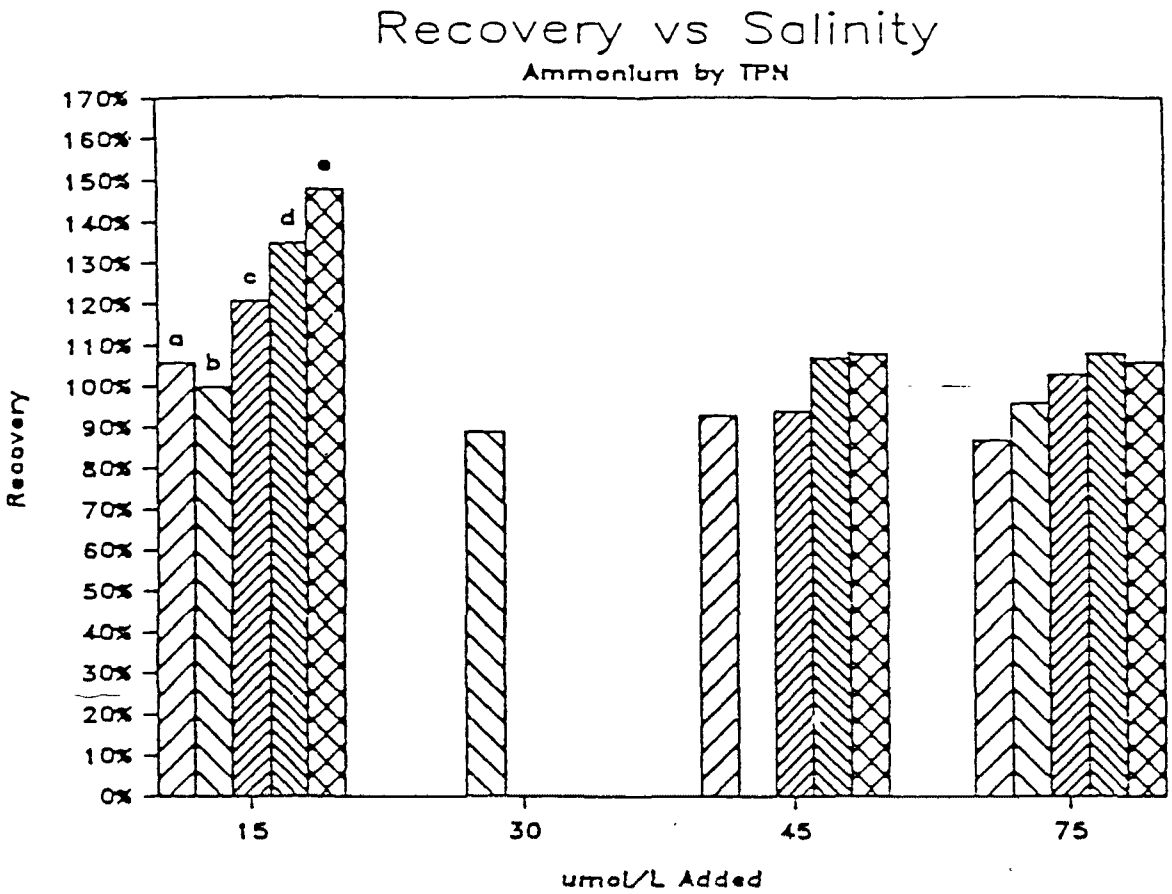


Figure 1-5a. Percent recovery by TPN method vs. concentration of ammonium (umol N L^{-1}) in different salinity water (a = 0% seawater, b = 25 % seawater, c = 50 % seawater, d = 75 % seawater, e = 100% seawater).

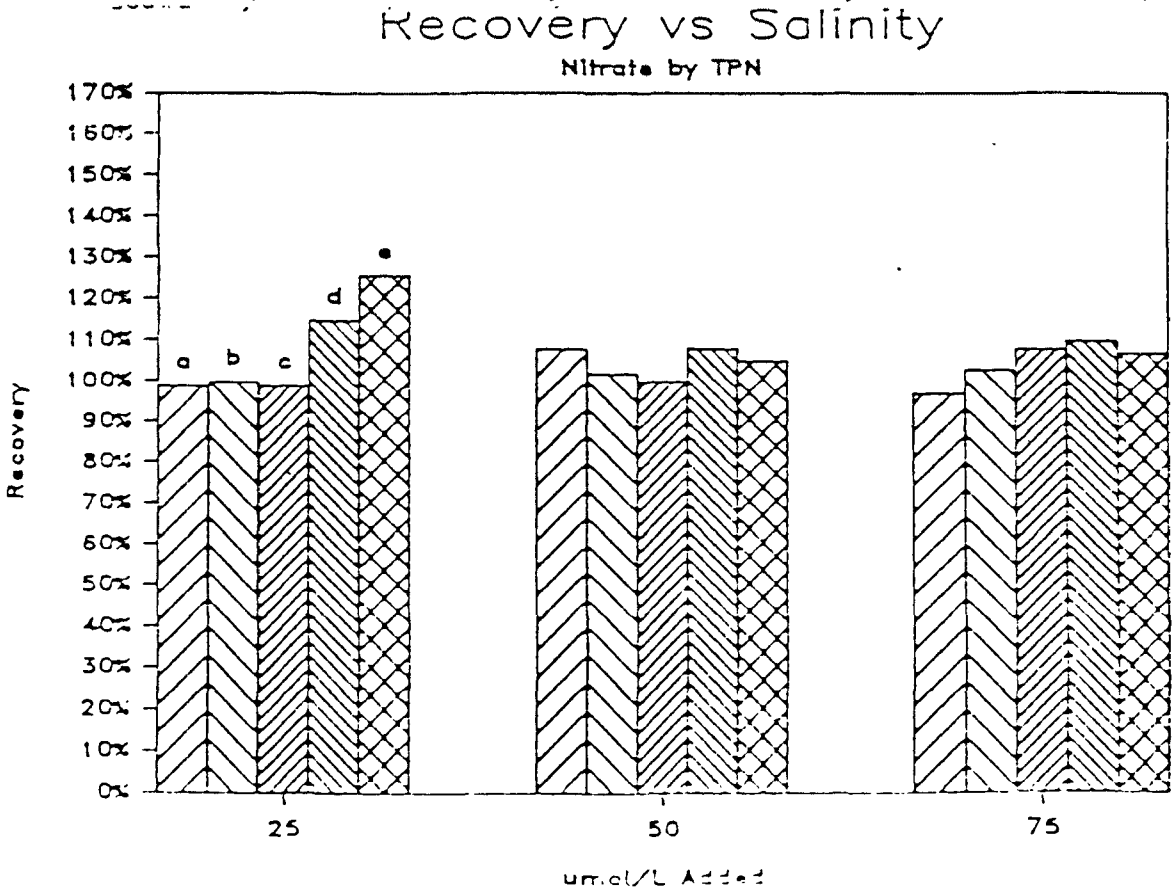


Figure 1-5b. Percent recovery by TPN method vs. concentration of nitrate (umol N L^{-1}) in different salinity water (a = 0% seawater, b = 25% seawater, c = 50 % seawater, d = 75 % seawater, e = 100 % seawater).

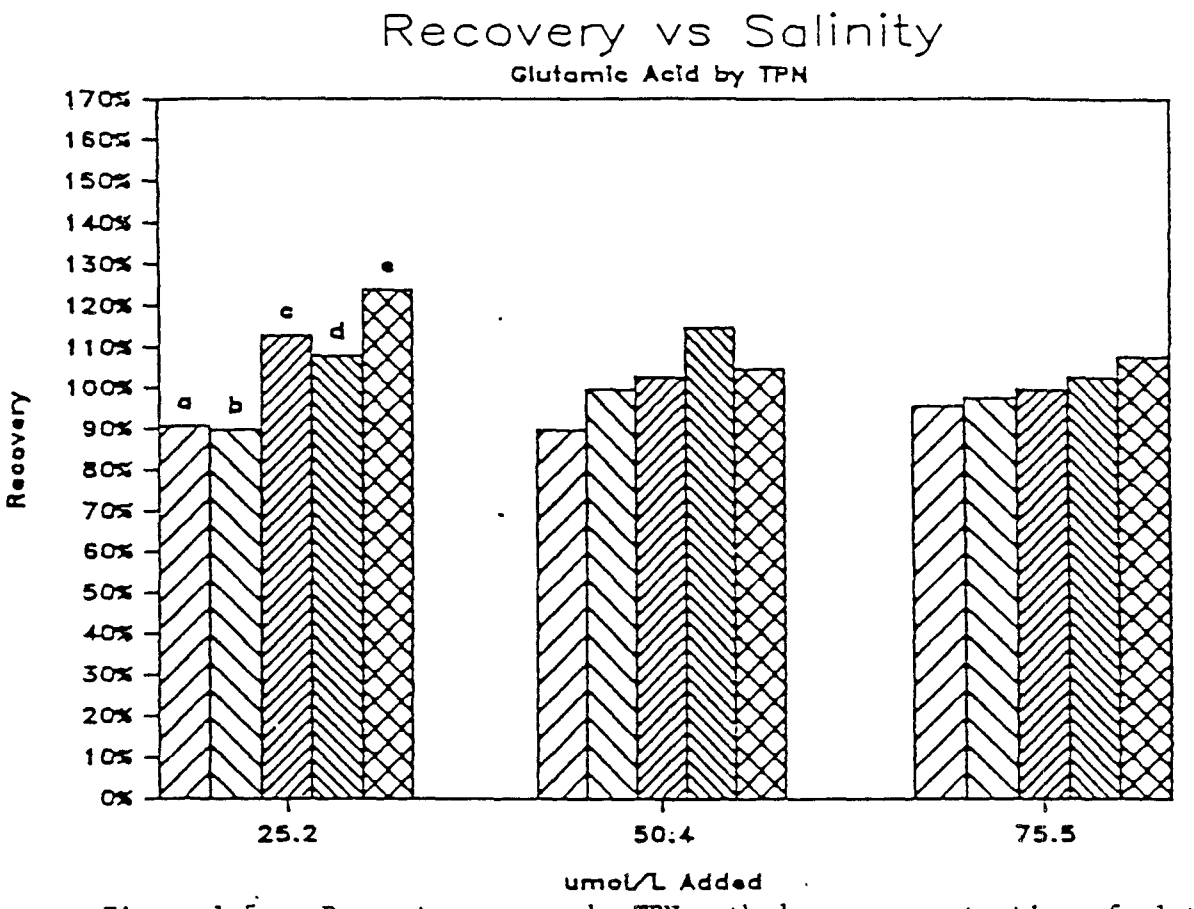


Figure 1-5c. Percent recovery by TPN method vs. concentration of glutamic acid (umol N L^{-1}) in different salinity water (a = 0% seawater, b = 25% seawater, c = 50 % seawater, d = 75% seawater, e = 100% seawater).

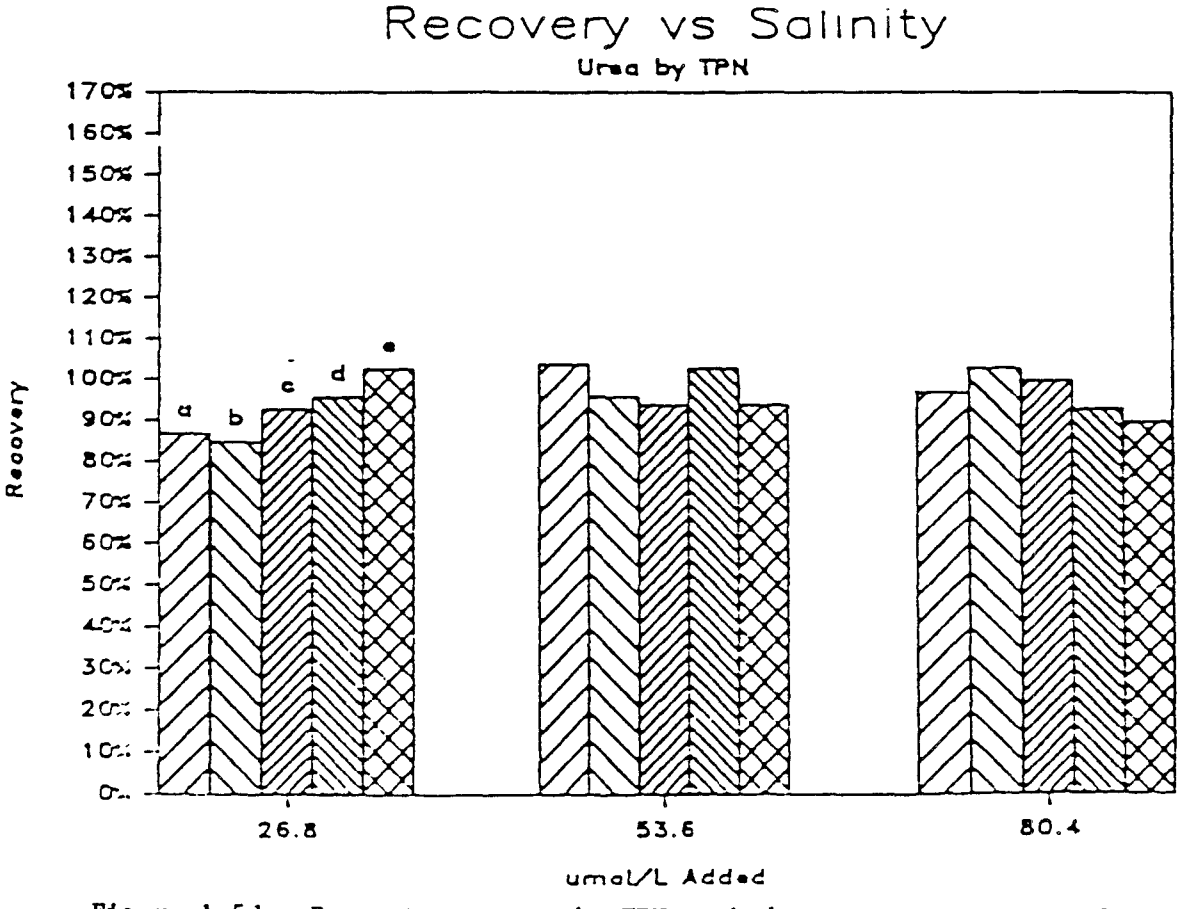


Figure 1-5d. Percent recovery by TPN method vs. concentration of urea (umol N L^{-1}) in different salinity water (a = 0% seawater, b = 25% seawater, c = 50% seawater, d = 75% seawater, e = 100% seawater).

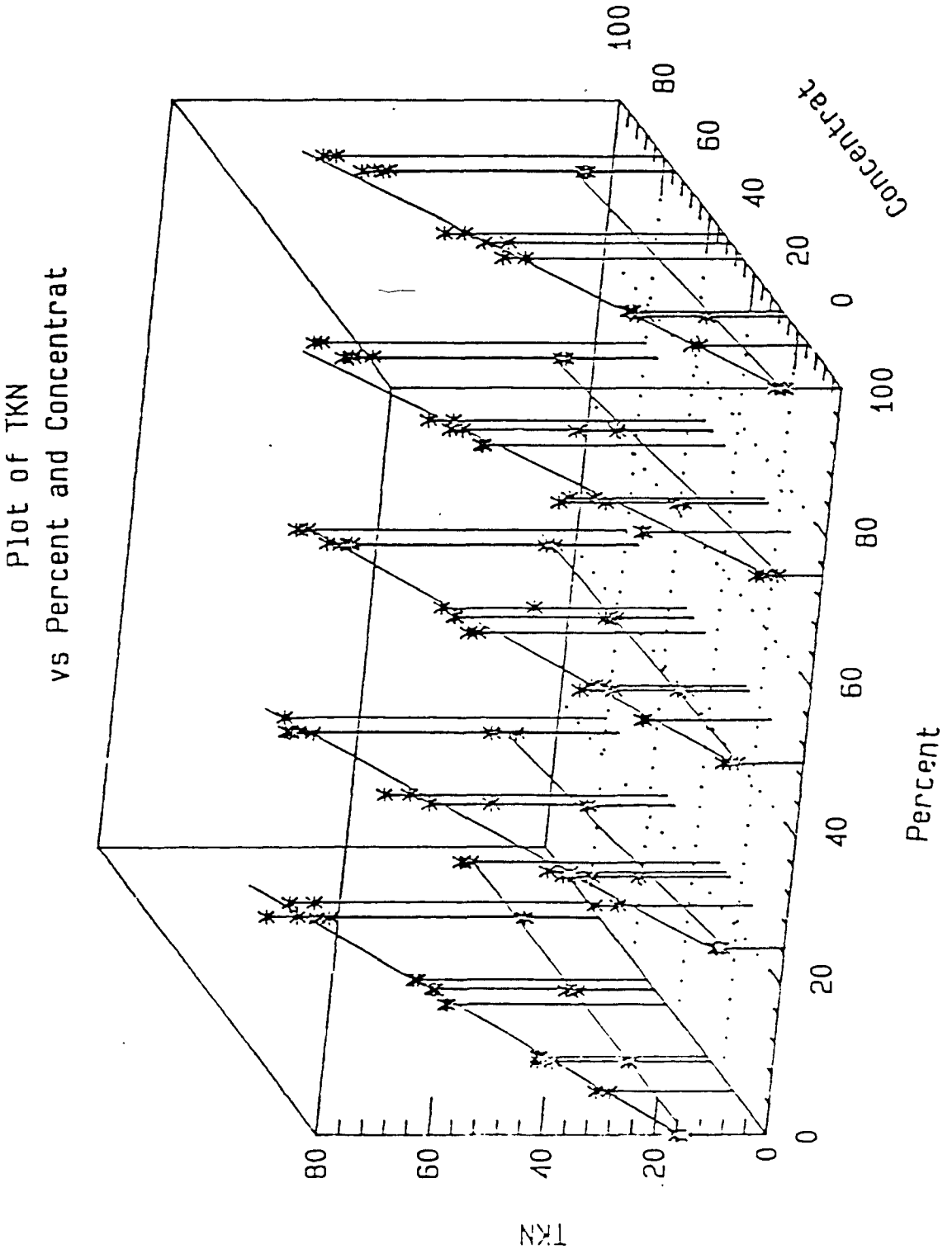


Figure 1-6. Three dimensional plot of TKN-determined concentration of standards (umol N L^{-1}) vs. percent seawater vs. expected concentration of spiked reference standards (umol N L^{-1}).

However, precision clearly was not as good by TKN as it was for TPN, and as expected for the procedure, nitrate was not recovered. The nitrate points are connected by additional lines fitted to the data.

Figures 1-7A through 1-7D presents the percentage recoveries of the individual spiked compounds relative to ammonium standard curves in distilled water analyzed by the TKN method. Clearly the precision was less than for the TPN analysis, but recoveries appeared complete at all salinities and spike concentrations. However, a small amount of nitrate appeared to have been recovered in some samples--this is anomalous because TKN should not reduce nitrate to ammonium, and is probably explained by contamination. Nonetheless, there is the interesting prospect of some unexplained nitrate reduction occurring, which would be difficult to explain chemically.

3. Comparison of TPN and TKN Determinations on Estuarine Water Samples

Samples over a range of salinities were collected from August, 1984 through December, 1985 for comparison of results obtained using TPN and TKN determinations. These data were obtained from the "SONE" monitoring program conducted for the State of Maryland and in large-scale continuous cultures drawing water from the mesohaline region of the Patuxent River.

The results of these comparisons were poor and the explanations for the lack of comparability between TKN and TPN - nitrate + nitrite (comparable values) is as yet unresolved, despite exhaustive checking and rechecking of all procedures and calculations. We wish it were as simple as having ignored that ammonium sulfate standard has two moles of N per formula weight, but we did not make that error . We also are aware that refractive index problems can affect results (Froelich and Pilson, 1978) and that pH adjustment of the acid digest is critical for proper color development (Keay, 1985). Figure 1-8a shows the comparison of data from digestions we deemed "good" according to the criterion of low rates of bumping and splattering. Figure 1-8b shows the comparison of data from all digestions and determinations we performed. While comparisons of samples containing less than 30 uM Kjeldahl nitrogen seem close, there appears to be a systematic difference between the two procedures. The regression equation best fitting this relationship is: TPN - NO23 = 21.79($\pm$1.04) + TKN*0.153 ($\pm$0.021). It is not clear from this study whether the discrepancy between the TPN and TKN data in Figs. 1-8 and 1-8b is "real" or due to a contamination problem.

4. Precision of TPN Determinations on Replicate Samples

The CBL nutrient analytical services laboratory has been conducting TPN analyses for the bay-wide EPA-sponsored monitoring program since May, 1985. These analyses are conducted over a wide range of salinities and total dissolved nitrogen concentrations and are subjected to a rigorous QA/QC protocol, as dictated by EPA. To illustrate the achievable precision of the TPN determination on duplicate samples (each involving separate filtration, aliquoting and storage), it seemed appropriate to present here the results from the QA/QC program. Figures 1-9A and 1-9B show the EPA QA/QC plots for standard deviation of duplicates vs. mean concentration and for coefficient of variation vs. mean concentration. The mean coefficient of variation for all samples is approximately 8%, an excellent value considered that it represents more than analytical error alone. Typical coefficients of

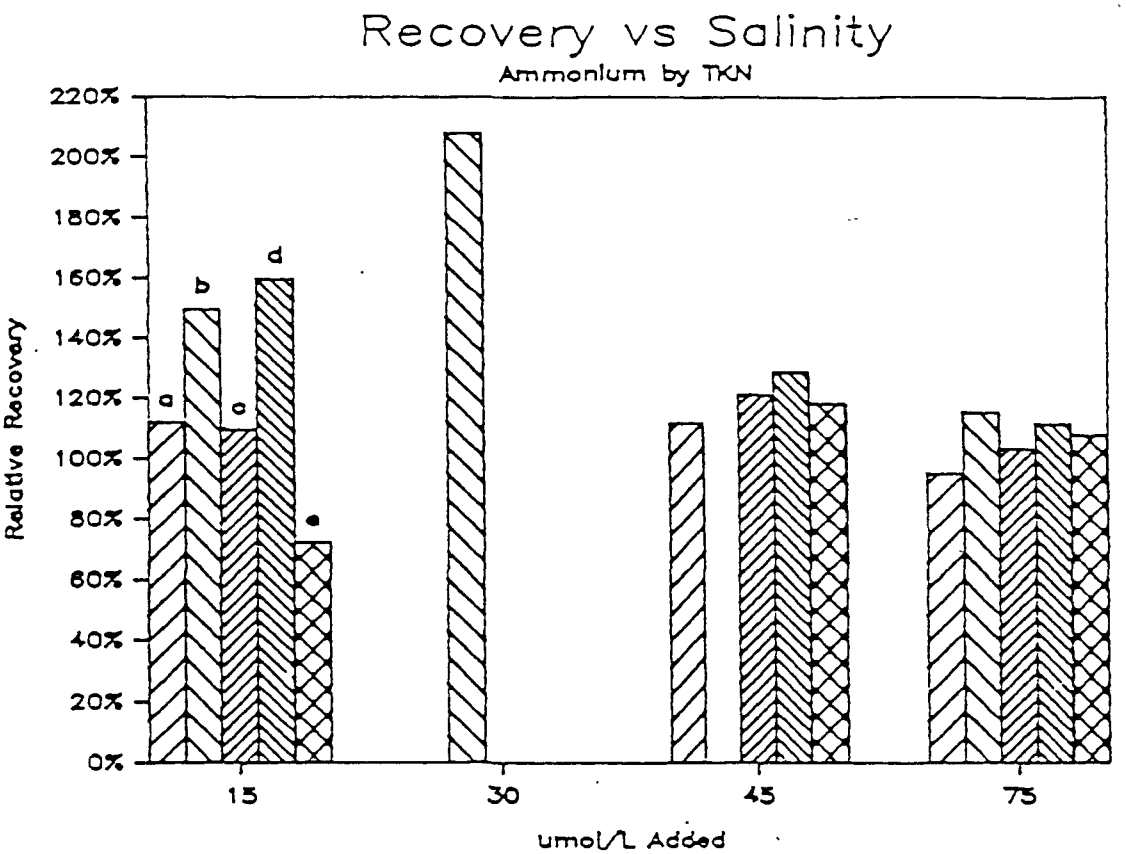


Figure 1-7a. Percent recovery by TKN method vs. concentration of ammonium (umol N L^{-1}) in different salinity water (a = 0% seawater, b = 25% seawater, c = 50% seawater, d = 75% seawater, e = 100% seawater).

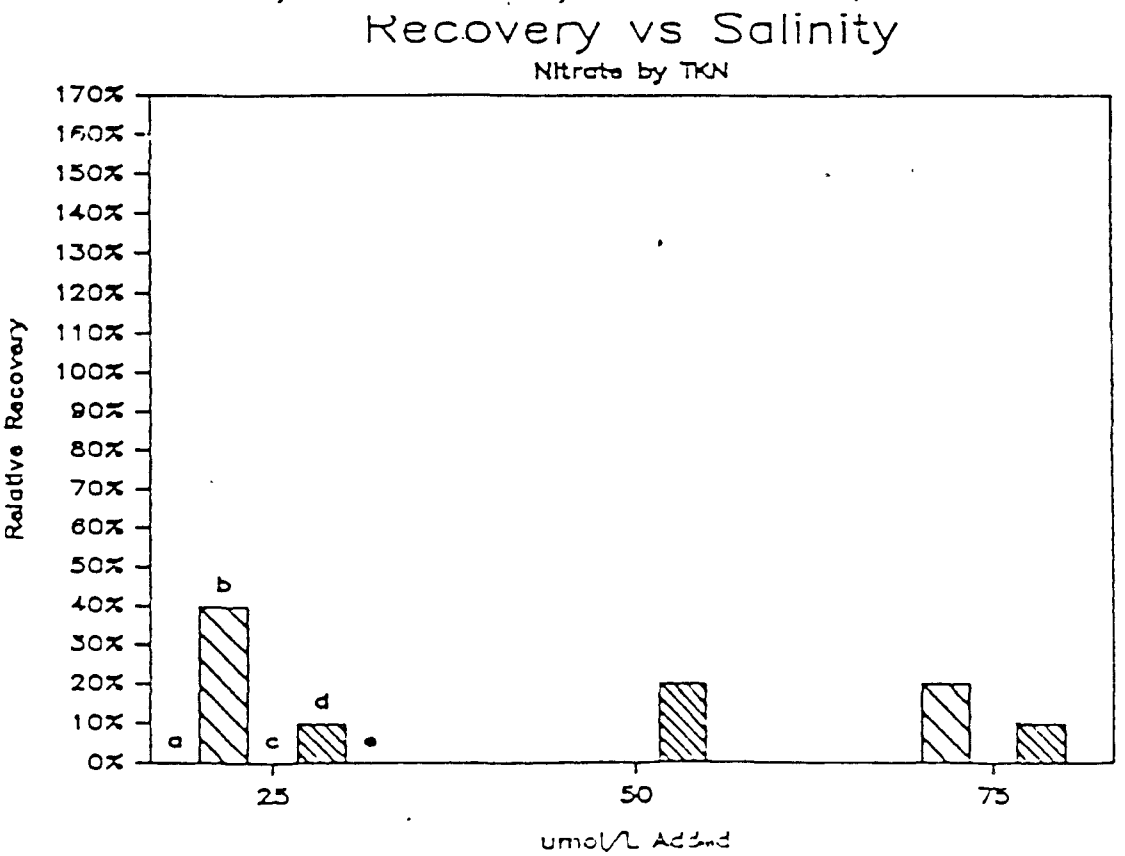


Figure 1-7b. Percent recovery by TKN method vs. concentration of nitrate (umol N L^{-1} in different salinity water (a = 0% seawater, b = 25% seawater, c = 50% seawater, d = 75% seawater, e = 100% seawater).

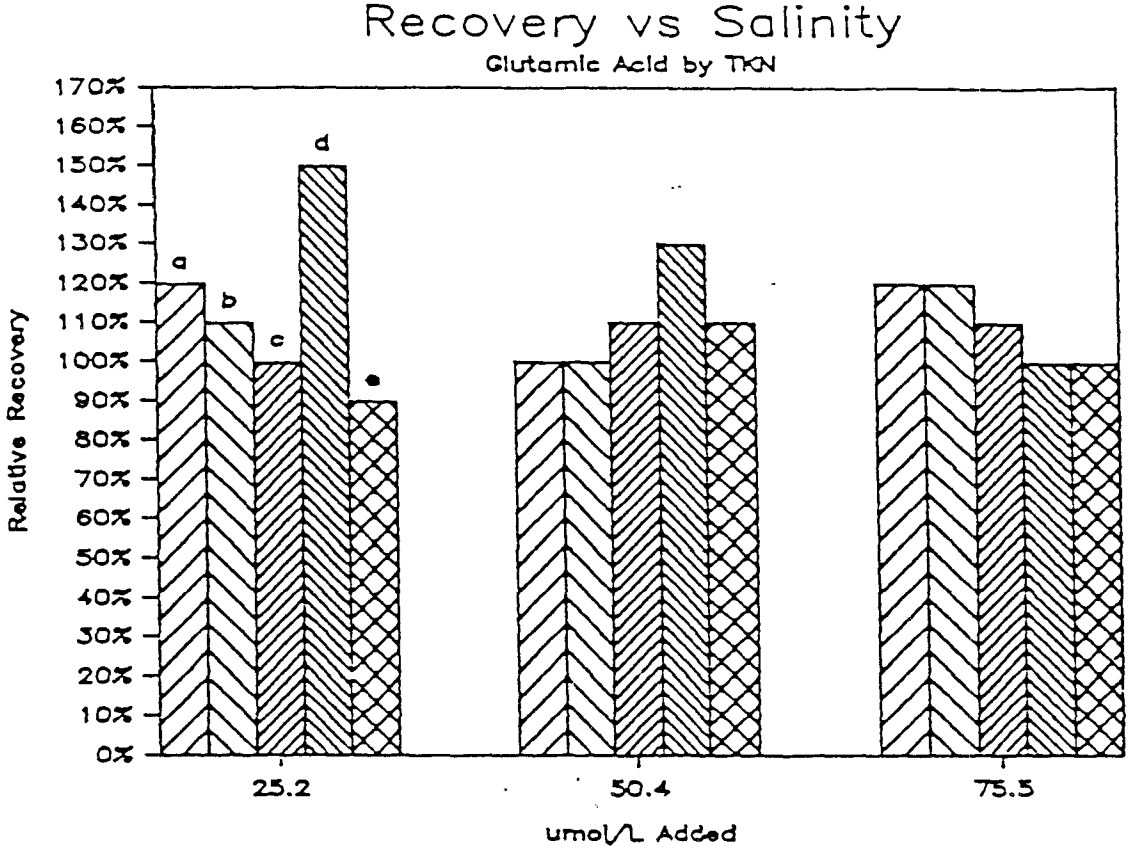


Figure 1-7c. Percent recovery by TKN method vs. concentration of glutamic acid (umol N L^{-1}) in different salinity water (a = 0% seawater, b = 25% seawater, c = 50% seawater, d = 75% seawater, e = 100% seawater).

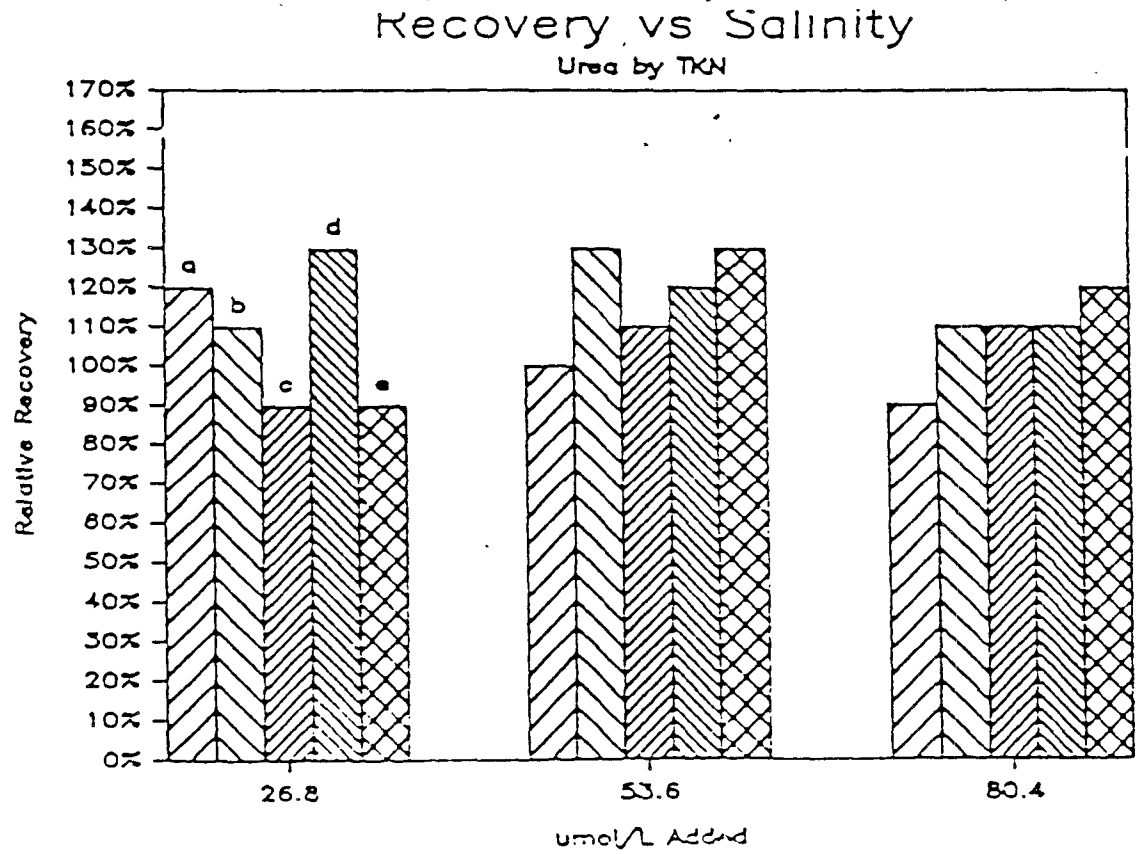


Figure 1-7d. Percent recovery by TKN method vs. concentration of urea (umol N L^{-1}) in different salinity water (a = 0% seawater, b = 25% seawater, c = 50% seawater, d = 75% seawater, e = 100% seawater).

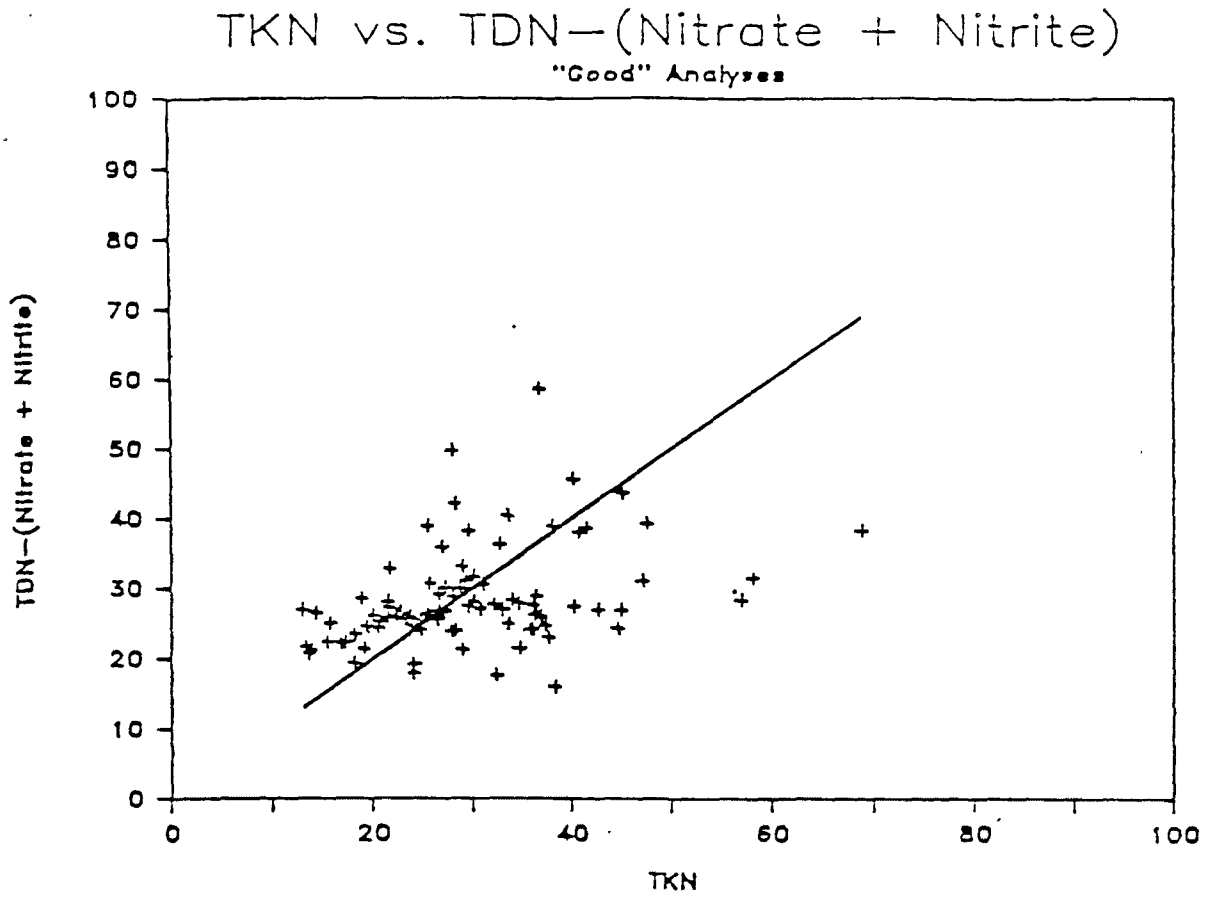


Figure 1-8a. TDN - (nitrate + nitrite) vs. TKN determinations of estuarine samples for analyses without bumping and splattering ("good" data).

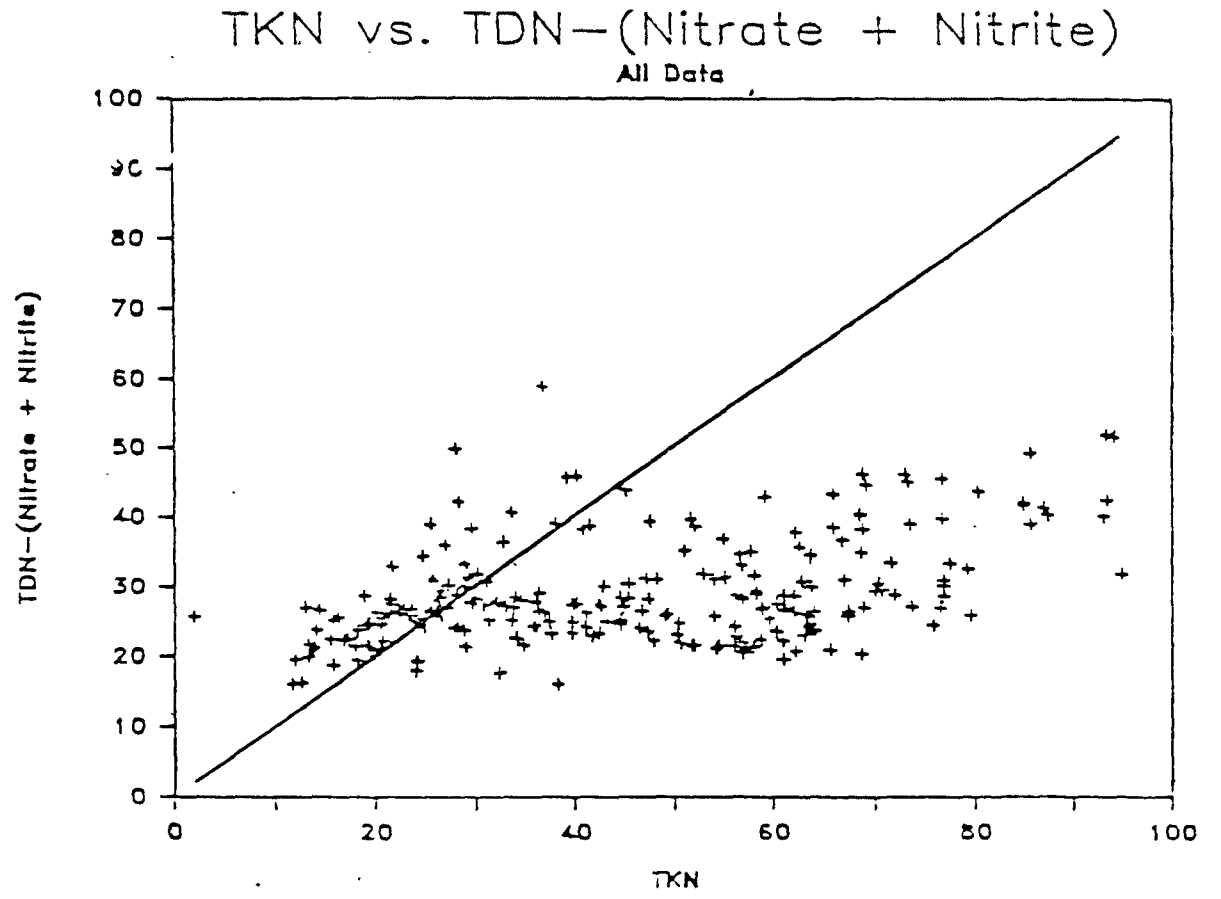


Figure 1-8b. TDN - (nitrate + nitrite) vs. TKN determinations of estuarine samples for all analyses preformed.

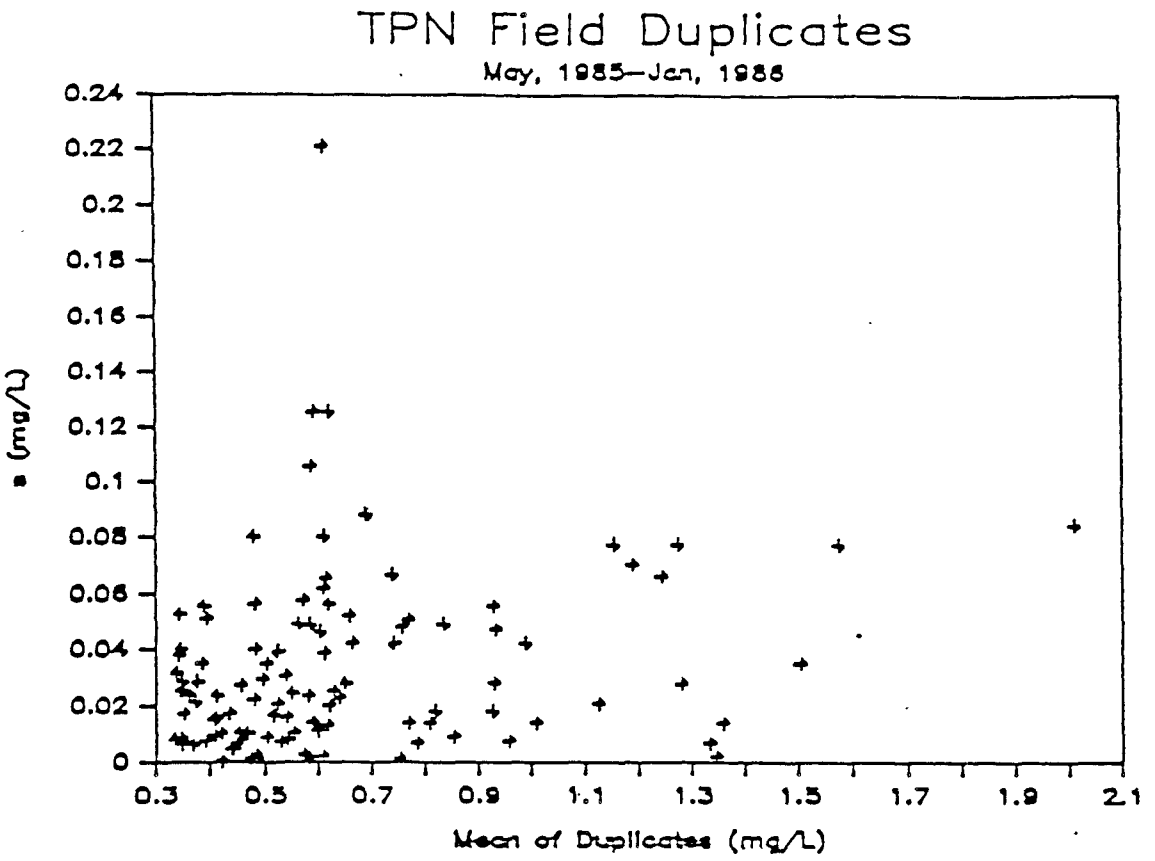


Figure 1-9a. Standard deviation of duplicates vs. mean concentration of field duplicates for bay-wide EPA-sponsored monitoring program.

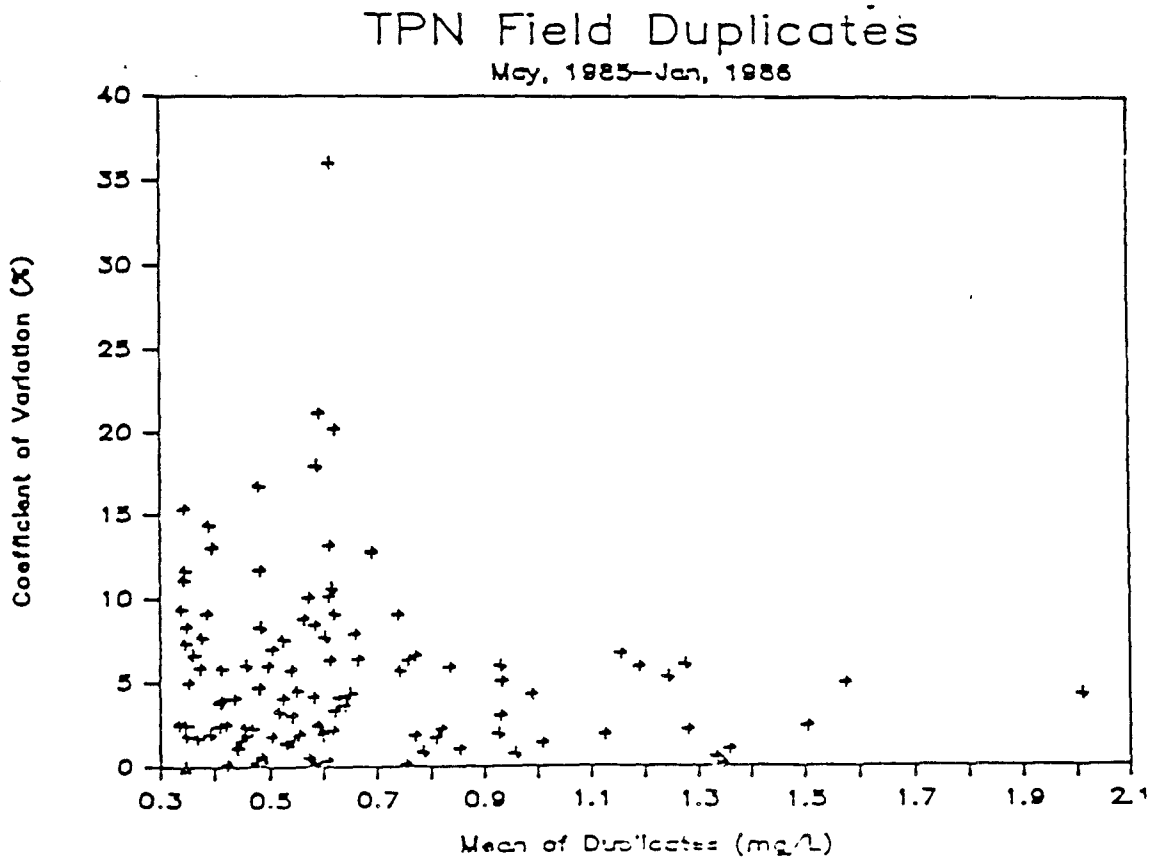


Figure 1-9b. Coefficient of variation vs. mean concentration of field duplicates for bay-wide EPA-sponsored monitoring program.

variation for Kjeldahl analyses are given in Appendix IV.

5. Advantages and disadvantages of the two methods.

While this work has clearly not shown the equivalence of the two analytical determinations, we believe that our analytical inexperience with the TKN procedure and the poor semiautomated TKN protocol are responsible for the lack of comparability. We recommend that further comparisons be made between TKN and TPN determinations. In addition, we also recommend that a laboratory that routinely runs TKN analysis, not with the block digestor, split samples with us, so that we can do TPN determinations for comparison.

It is important to emphasize why it is worthwhile to pursue the comparative work further. TPN analysis offers a number of advantages over Kjeldahl analysis that make it a highly desirable alternative to TKN. Such advantages in cost, ease of use, and excellent precision (cf. Fig 1-9A and 1-9B) means that TPN determination deserves further comparison.

Table 1-II shows the analyst's time and steps involved in processing a series of TKN samples. Table 1-III shows a comparison of the analyst's time and steps involved in processing a series of TPN and TKN samples.

Table 1-III summarizes the advantages and disadvantages of the two procedures.

6. Further Considerations

Although there have been reports by Japanese workers that the alkaline persulfate digestion technique substantially underestimates total nitrogen in seawater compared to the oxidative pyrolysis technique, several points should be made regarding comparability between the two methods. First, results have not been reproduced by others, probably due to the unavailability of the Japanese instrument in other countries. Secondly, while the Japanese workers did not state the temperatures at which their oxidation was carried out, the temperature used may have exceeded that recommended for optimum digestion. Goulden and Anthony (1978) and others have cautioned that high temperatures will cause too rapid a breakdown in the persulfate and poor oxidations.

One criterion that Suzuki et al. (1985) used in criticism of the persulfate technique was that it yielded poor recoveries of caffeine. However, B. Nowicky and M. Pilson (pers. comm.--cf. Appendix I) have obtained complete recovery of nitrogen in caffeine.

The persulfate oxidation procedure could be optimized still further--especially worth checking are (1) the heat of combustion and speed with which the samples are brought up to temperature, and (2) the ability of the procedure to oxidize complex rings.

Table 1-II. Comparison of analyst's time and steps required for the TPN and TKN methods.

Method	Day	Step and Activity	Time Involved (hours)
TPN	1	1. Thaw 100 samples (10 ml in 30-ml tubes)	
		2. Make up standards and put in 30-ml tubes.	0.4
		3. Make up 2 L oxidizing reagents.	0.1
		4. Add 15 ml oxidizing reagents to all standards and samples.	1.0
		5. Autoclave at 100 - 110 degrees C.	0.5
		6. Cool in autoclave.	1.0
		7. Remove from autoclave and cool to room temperature.	1.0
		8. Make up 0.3 N HCl and borate buffer.	0.1
		9. Add 1.5 ml 0.3 N HCl and vortex mix.	1.0
		10. Add 2.0 ml borate buffer and vortex mix.	1.0
	2	1. Set up continuous flow analyzer.	1.0
		2. Prepare and run nitrate standard curves.	0.5
		3. Run samples and standards.	3.0
		4. Shut down auto analyzer.	0.5
		5. Read charts and calculate concentrations.	2.0
		6. Wash tubes and caps.	1.5
		Total	14.6
		Time/Sample	9 min

Table 1-II, cont'd.

Method	Day	Step and Activity	Time Involved (hours)
TKN	1	1. Thaw 45 samples (20-25 ml in 30-ml tubes) and put in Kjeldahl digestion tubes.	0.4
		2. Prepare ammonium standards.	0.1
		3. Put 25 ml samples and standards in Kjeldahl digestion tubes.	1.0
		4. Add 5 ml digestion solution to all standards and samples.	0.25
		5. Add 2 boiling chips to each sample and vortex mix.	0.25
		6. Digest standards and samples in block digestor at the following temperatures and times:	
		Temperature (degrees C)	
		90	0.25
		120	0.5
		150	0.5
		180	0.5
		200	0.5
		230	0.5
		360	2.5
		7. Let cool in digestor.	1.0
		8. Remove from digestor and cool to room temperature.	2.0
		9. Dilute cooled samples and standards to 25 ml with distilled water and vortex mix.	1.0
	1 or 2	10. If solid develops and persists after dilution to volume, sonicate covered samples to break up solid, then allow samples to settle.	2.0 to 3.0
	2	1. Set up continuous flow analyzer.	1.0
		2. Run digested ammonium standard curve.	0.5
		3. Run digested samples in duplicate.	2.0
		4. Shut down continuous flow analyzer	0.5
		5. Read charts and calculate concentrations.	2.0
		6. Wash tubes and caps.	1.5
		Total	20.75
		Time/Sample	28 min

Table 1-III. Comparison of the TKN and TPN methods for the procedures we used and assuming the availability of an autoanalyzer colorimeter, sampler, pump and chart recorder.

Characteristic or Feature	TKN	TPN
Estimated Cost		
Startup	$504	$250
Block Digestor	$3395	-
Pressure Cooker	-	$ 80
Autoanalyzer manifold	$1000	$430
Total		
Per Sample Charge in our Laboratory	$18.00	$5.75
Special Equipment	Fume Hood Block Digestor AutoAnalyzer Kjeldahl Tubes	Pressure Cooker AutoAnalyzer Test tubes
Ease of Use	Not easy	Very Easy
Samples per Day	20	50
Precision (CV%)	>10%	~ 3%
Comments	Seawater samples are more difficult --proper boiling chips must be used	DON not precisely determined in the presence of high nitrate concentrations

Summary and Recommendations:

1. The persulfate total nitrogen procedure is easier to perform, yields better routine precision, requires less expensive and sophisticated digestion apparatus, and requires less analyst time per sample. This procedure deserves further evaluation as a potential standard digestion procedure for total dissolved nitrogen by EPA.

2. Both methods yielded expected and complete recoveries of laboratory-spiked samples over a wide salinity range. However, results obtained comparing natural estuarine samples appeared to yield a systematic difference between the two procedures that is as yet unresolved.

3. The block digestor for the TKN procedure does not perform well and proved difficult to use, particularly in the hands of technicians inexperienced in its use. Differential heating of different locations on the digestor must be accounted for. The heating characteristics of the digestor seem to depend on external factors such as location in the hood, laboratory temperature and warm-up time. Such factors need to be accounted for if the block digestor is to be used.

4. The residue remaining in the digestion tubes after block digestion of TKN samples is very difficult to redissolve in high salinity samples. Sonication may be required as well as long sitting times. Contamination may occur during such sitting times. A better re-dissolution procedure should be developed for high salinity samples.

5. Additional comparisons should be made between the two procedures using split samples from the natural environment. We recommend that a laboratory not using the block digestor and achieving TKN results satisfactory to EPA share samples with us so that we can perform additional TPN analyses.

6. Organic N standards in seawater should be used for standard curves. Such standards should include difficult-to-oxidize nitrogen-containing reference materials, e.g. nicotinic acid, caffeine.

SECTION II

COMPARISON OF CHLOROPHYLL METHODS

General Description of Chlorophyll Rationale

Many aquatic investigations utilize one or more estimates of photoautotrophic plankton biomass, e.g. cell counts, total cell volume estimates, protein determinations, dry weight, cell carbon, nitrogen, phosphorus or silica and pigment analyses including chlorophyll a determinations. The use of chlorophyll a, especially fluorometric determinations, has become widespread, possibly to the point of indiscriminate use, because the method is relatively fast, simple and reproducible. The use of this biomass measure has been questioned because it may vary by an order of magnitude relative to other biomass measures, e.g. dry weight, cell volume or cell protein. Eppley (1977) reported 10-fold variation in cell carbon:chlorophyll a ratio of phytoplankton. The failure of the fluorometric method to provide any information about population structure as well as the observed interference problems from accessory pigments and phaeo-pigments are largely overlooked.

Any monitoring or other routine sampling program for chlorophyll pigment must address certain criteria such as: (1) design of sampling scheme, e.g. frequency, depths, replicates, etc., (2) technique of sampling, e.g. by pump, bottle, rossette sampler, etc., (3) sample treatment, e.g. filtration, including types of filters and filter holders or the use of whole unfiltered water samples, (4) possible storage of samples either before and/or after filtration or extraction, (5) extraction techniques including solvent composition, temperature and or physical treatment (sonication or grinding) and duration of extraction, (6) quantification method such as spectrophotometric, fluorometric or spectrofluorometric determinations on the gross extract, and (7) how the calculations are made after the raw data are gathered.

Recently a variety of solvent systems containing dimethyl sulfoxide (DMSO) has been suggested for the extraction of chlorophyll type pigments from freshwater phytoplankton (Shoaf and Lium, 1976; Stauffer et al., 1979). Burnison (1980) has described a method using pure DMSO at 65 C followed by dilution with 90% acetone; Speziale et al. (1984) subsequently compared this method to N,N-Dimethylformamide (DMF) and 90% acetone extractions on natural samples and cultured freshwater phytoplankton. Both DMF and DMSO were better extractants than 90% acetone, with DMF being very slightly better with chlorococcalean species. No work has been published concerning the use of DMSO:acetone solvent systems with marine plankton species, although Seely et al. (1972) reported using DMSO as part of a serial extraction method for brown algae and a modified method is suggested for marine macrophytes generally (Duncan and Harrison, 1982). Although there is reason to predict that DMSO:acetone solvents are more effective in extracting marine samples than present acetone methods, the method should be evaluated before it is utilized extensively. We have recommended a DMSO technique as the procedure of choice for the EPA-Chesapeake Bay Monitoring program because it is easy, requires a minimum of handling,

storage as a separate step isn't required, and it gives results identical to the 90% acetone extraction with grinding for an uncorrected (for phaeo-pigments) chlorophyll a value by fluorometry.

The original scope of this work was to further investigate extraction techniques for chlorophyll a; it was expanded to include some aspects of sample storage (freezing) and a comparison of spectrophotometric and fluorometric determinations in order to assist the interpretation of the data.

Background and Literature Review

1. Calculations:

Methods manuals (e.g. APHA, 1985; ASTM, 1979; Parsons et al., 1984) appear to be in consensus that the accepted methods for spectrophotometric determination of chlorophylls involves the use of the trichromatic equations of Jeffrey and Humphrey (1975). The spectrophotometric determination of phaeo-pigments utilizes readings taken at 665 or 664 nm before and after acidification and the formulae of Lorenzen (1972) for the calculations. The formulae for a 1 cm cell are as follows:

Jeffrey and Humphrey (µg chl/ml extract for 1 cm cell)

Chlorophyll a = 11.85 E(at 664nm)-1.54E(at647nm)-0.08E(at 630nm)
Chlorophyll b = 21.03 E(at 647nm)-5.43E(at664nm)-2.66E(at 630nm)
Chlorophyll c = 24.52 E(at 630nm)-1.67E(at664nm)-0.08E(at 760nm)

where E is the absorbance at different wavelengths corrected by a blank reading at 750 nm. Chl per unit seawater is then calculated by:

Chlorophyll µg/l = (Chl x v)/V

where v is the extract volume in ml and V is the sample volume in liters

Lorenzen (for 1 cm cell)

Chlorophyll a (µg/l) = [26.7(665b-665a)v]/V
Phaeo-pigments (µg/l) = [26.7(1.7(665a)-665b)v]V

where 665a and b are after and before acidification respectively and V and v are as above. The b reading is listed at 664 in APHA (1985) and ASTM (1979), while the original articles (Lorenzen, 1967) and Parsons et al., 1984) cite 665nm for both the b and a readings. In this presentation we use the above equations although Speziale et al. (1984) indicates that the Lorenzen equations cause underestimations by about 6%, i.e. the 26.7 of the above equations should be replaced by 28.4.

The above equations are often utilized directly from manuals without consulting the original volumes. Thus, one may not realize that Jeffrey and Humphrey published four sets of equations, for differing kinds of populations: 1) Chl a and b for higher plants and chlorophyta, 2) Chl a and c1, c2 for diatoms, chrysomonads and brown algae, 3) Chl a and c2 for dinoflagellates and cryptomonads, and 4) the above equations for mixed populations of phytoplankton. Chl a was well recovered by all equations (98-102%). The specific equations for a + b and a + c gave similarly good values for all the pigments, however the mixed plankton equation gave good results for b and c only when these pigments were abundant relative to chl a, i.e., a:b or a:c ratios of less than 4:1.

2. Interference by phaeo-pigments and accessory chlorophylls:

The use of all of these equations assumes that the solution analyzed is a mixture of pure pigments and contains no decomposition products. The colored phaeo-pigments, Table II-1, in contrast to the colorless ones, show up in these data as chlorophyll a. Prior to 1978,

Table II-1. Chlorophyll breakdown products (phaeo-pigments)

	Absorption peak	Absorption coefficient	Reference
Phaeophytin a	667nm	51.2	Score
Chlorophyllide a	664nm	127	Score
Phaeophorbide a	667nm	74.2	Score

phaeophytin a was thought to be found only in traces in natural marine samples; this was subsequently found not to be true. Pheaophytin a is formed by removal of magnesium from the chlorophyll a molecule, chlorophyllide, by removal of the phytol chain, and phaeophorbide by removal of both Mg and phytol. Opening of the porphyrin ring of any of these molecules will result in a colorless product. Light, enzymes, acid, oxygen and high temperatures are known to produce degradation.

Some relevant data from the literature are presented in Table II-2. These data seem to support the assumption that phaeo-pigments (chlorophyllide and phaeophorbide) don't interfere significantly with the trichromatic determination of chlorophylls b and c, only with chlorophyll a. It is unfortunate that Lorenzen and Jeffrey (1980) did not recognize the importance of phaeophytin and include it in their determinations because Moss (1967) indicates that its presence should make the trichromatic calculation of chlorophylls b and c particularly unreliable. Phaeo-pigments are often calculated by using before and after acidification values, either from fluorometry or spectrophotometry. Chlorophyll b and to a lesser extent, chlorophyll c show up in these calculations as phaeo-pigments. The data of Lorenzen and Jeffrey (1980) and those of Gibbs (1979) do not agree on the extent of the chl b interference with the fluorometric determination. Gibbs suggests that an artifact of 2.5 times the real chl b shows up as phaeo-pigment in the Holm-Hansen et al. (1965) calculation, compared to a range from 0.89 to 2.05 for Lorenzen and Jeffrey (1980). In sparse data for the Lorenzen (1967) spectrophotometric calculation of phaeo-pigments, the Lorenzen and Jeffrey (1980) data indicate an interference of 0 to 0.26, i.e. chl b is read as phaeo-pigments. It thus appears that the accessory chlorophylls interfere with the spectrophotometric or fluorometric determination of phaeo-pigments and likewise, the presence of phaeo-pigments interferes with the determination the chlorophylls, especially chlorophyll a.

Table II-2. Comparison of fluorometric and spectrophotometric methods of pigment mixtures of known composition. Data are reproduced from the literature, lines 1-10 are from Lorenzen and Jeffrey (1980) Table 3, lines 11-16 are from Gibbs (1979) Table 2. This table shows interference by Chl b on determination of phaeopigments and interference of spectrophotometric determination of Chl a by phaeopigments.

	Known concentrations ug/ml						Jeffrey & Humphrey (1975) calculation			Hola-Hansen et al. (1965)			Lorenzen (1967)	
No.	chl a	chl b	chl c	Ph 1	Ph 2	a:b	Chl a	Chl b	Chl c	UChl a	CChl a	Phaeo-	Chl a	Phaeo-
1	1.836	-	-	-	-	-	1.84	-0.034	-0.007	1.76	1.72	0.07	1.84	-0.25
2	-	1.947	-	-	-	-	0.024	2.014	-0.711	0.36	-0.55	1.74	-0.13	0.5
3	-	-	1.972	-	-	-	0.002	-0.024	1.98	0.59	0.75	0.31	0.0	0.35
5	1.836	0.389	0.296	-	-	4.72	1.85	0.392	0.227	1.87	1.63	0.45	1.74	-0.06
6	-	-	-	1.765	-	-	1.485	-0.01	-0.115	1.2	0.14	2.03	0.16	2.05
7	-	-	-	-	1.264	-	1.84	-0.199	0.088	1.69	1.76	-0.11	1.84	-0.25
8	1.836	0.779	-	-	-	2.36	1.869	0.771	-0.21	1.87	1.45	0.47	1.79	-0.03
9	1.836	0.389	-	0.833	-	4.72	2.583	0.241	-0.044	2.34	1.63	1.35	1.79	0.89
10	1.947	-	-	-	0.632	-	2.703	1.94	-0.078	2.56	1.79	1.46	2.47	0.27
11	1.0	0.00	-	-	-	-	-	-	-	1.0	1.0	0.00	-	-
12	0.95	0.05	-	-	-	19.0	-	-	-	0.96	0.9	0.12	-	-
13	0.7	0.03	-	-	-	23.3	-	-	-	0.79	0.4	0.77	-	-
14	0.6	0.4	-	-	-	1.5	-	-	-	0.72	0.22	1.01	-	-
15	0.5	0.5	-	-	-	1.0	-	-	-	0.65	0.02	1.26	-	-
16	0.0	1.0	-	-	-	-	-	-	-	0.3	-0.97	2.53	-	-

Pigment identifications:
Ph 1 = pheophorbide a
Ph 2 = chlorophyllide a
UChl a = Chl a uncorrected for phaeopigments
CChl a = Chl a corrected for phaeopigments
Phaeo- = phaeopigments

3. Storage, Freezing:

The effect of storage conditions on chlorophyll determinations are not well documented in the literature. Most methods use magnesium carbonate on the filters to prevent acid conditions from causing chlorophyll degradation. The recommended DMSO method uses 0.1% by volume of diethylamine to maintain alkaline conditions. Jeffrey and Hallegraeff (1980) froze filters in liquid nitrogen and then held them at -20C until extraction. This method resulted in a 5-10% loss of chlorophyll a in 6 weeks of storage with a gain of 2-3% phaeophytin, presumably the major breakdown product was colorless.

Some publications suggest that stored extracts or extracting tissue show less degradation of chlorophyll than do plankton samples stored on frozen filters. For example, Wood (1985) reported 11-21% loss of chlorophyll from samples stored dry when compared to those stored in extracting solvent for 9 days. Similarly Moran and Porath (1980), reported no loss of chlorophyll in N,N-Dimethylformamide with dark storage at 4C. Inskeep and Bloom (1985), however, reported no difference between stored soybean leaf disks with and without solvent. Logic suggests that extracting solvents such as DMSO may denature enzymes which denature chlorophyll and that, consequently, combinations of tissue and extracting solvent may remain stable for chlorophyll concentration even at room temperature.

Methods:

1. EPA Chesapeake Bay Study, July 1980

a.) Sampling. Samples for the extraction method comparison, between DMSO and 90% acetone with grinding, were taken from a field study in the York River (USA) (37'15'40" N. Lat, 76'23'28" W. Long) and from 4 stations on a transect across Chesapeake Bay along Long 37' 20', July 8-16, 1980. These field samples consisted of the surface samples (1 m depth) processed by standard fluorescence methods (Yentsch and Menzel, 1963) with freezing for less than a week, in triplicate (and were a subset of a larger sample set) and additional samples in duplicate from the 1 m water samples for extraction with dimethylsulfoxide (DMSO): acetone:water (9:9:2) with 0.1% by volume of diethylamine (DEA); insofar as possible the samples were taken twice a day at the five stations for 9 consecutive days. Whatman GF/F filters were used because they retain more chlorophyll than a number of other filters tested.

b.) DMSO extraction technique. A measured volume of sample sufficient to produce visible color on the filter disc was filtered through a Whatman GF/F 2.5 cm filter. For estuarine water 5-10 ml is usually sufficient. The filter was folded with the sample side inward and placed in a 16x100 mm glass culture tube which had been coated (see below) to exclude as much light as possible. The tube contained a 10 ml aliquot of DMSO and a minimum of air space. The tube was closed with a teflon lined screw cap and the filter was extracted for at least 2 hours at ambient temperature. Filters were always manipulated with forceps. It was not necessary to filter or centrifuge the sample before measuring fluorescence.

c.) Tube coating technique. To exclude light from the culture tubes during extraction, the tubes were dipped twice in a mixture of lampblack and plastic "tool grip compound" obtained from Brookstone Company, Peterborough, NH. About 70 cc of lampblack was added to each 16 oz. can of red compound and mixed thoroughly. Approximately three dozen tubes were coated from each can.

d.) Fluorometry. Fluorescence measurements were taken with G.K. Turner Associates Model 111. Purified chlorophyll a, (Sigma Chemical Company, product no. C-5753, lot number 39C-9690) was used for calibration. Concentrations were verified spectrophotometrically using the equations of Jeffrey and Humphrey (1975). Spectrophotometric measurements were taken with a Bausch and Lomb Spectronic 710. The Sigma standard was dissolved in 100% acetone and then diluted so that final concentrations of solvents matched those of the extraction systems.

e.) Storage. To test the effect of storage on extracted material, a second repetition of some of the DMSO samples were extracted in the original sample tubes at room temperature for varying periods up to 32 days after the first repetition was read.

f.) Calculations. The pigment concentration (µg l-1) values were calculated as follows: (1) uncorrected (for phaeophytin a) chl a equivalents directly from before acidification fluorescence values (Strickland and Parsons, 1972, page 201) and (2) corrected chl a and phaeophytin from the before and after acidification values (Yentsch and Menzel, 1963). Because sample variance was significantly correlated with sample mean, a log transform was performed before analysis (Snedecor and Cochran, 1967, page 329). All statistical analyses were performed using Statistical Analysis System GLM, CORR, SUMMARY,and MEANS procedures (SAS, 1979).

The comparisons were made on paired sets (i.e. data from two methods on the same water sample) in duplicate, the duplicate values for the standard method were produced arbitrarily by choosing the first two values in the data set from the existing triplicate values. The second of the DMSO duplicates was analyzed in a time series fashion, i. e. 0, 1, 2, 10, 16 or 32 days after its pair, in order to allow testing for extraction time/storage time effects.

2. State of Maryland Chesapeake Bay Monitoring

Approximately 80 samples were collected for chlorophyll analysis on each of five cruises (August and October 1984 and May, June and August 1985) for a total 388 individual samples. At each station samples were taken from two depths, surface and bottom, in quadruplicate. Sample volume varied from 50 to 1000 ml depending upon the apparent chlorophyll in the sample. Samples were filtered onto 47 mm Whatman GF/F filters and frozen for the duration of each cruise, 1-5 days. Two of each set of replicates were analyzed by the CBL laboratory following the DMSO extraction technique described above but starting with frozen samples.

The two remaining replicates from each station were kept frozen and transported to the Virginia Institute of Marine Science (VIMS) for analysis by the method (Strickland and Parsons, 1968) of grinding in 90%

acetone, allowing to stand overnight in the refrigerator, centrifuging and reading on either a Turner Model 111 or Turner Designs fluorometer. Most extracts were sufficiently concentrated to be analyzed by spectrophotometry; such was done using a 1-cm cell in a Cary Model 15 spectrophotometer. Spectrophotometric readings were taken at 750, 665, 664, 647, 630 nm and at 665 nm after acidification. The trichromatic equations of Jeffrey and Humphrey (1975) were used to calculate chlorophylls a, b, and c. The assumption is made that no phaeo-pigments are present when these equations are used. Chlorophyll a and phaeo-pigments were also calculated with the 750 nm and the 665 nm before and after acidification readings by the equations of Lorenzen (1967). Chlorophyll b interferes with this evaluation.

3. Virginia EPA Chesapeake Bay Monitoring

We accompanied the VIMS Bay monitoring cruises on 8 consecutive cruises from mid-April through mid-August 1985. Sampling procedure in this Virginia counterpart to the Maryland monitoring program was as follows. A large volume sample (200 to 800 ml) was collected, filtered onto a GF/F 2.5 cm filter on board the vessel with the addition of a few drops of a magnesium carbonate suspension. The filter was held on water ice until returning to the lab when it was frozen. In one case (May 6, 1985), ice was not available and the samples were held in a dark insulated box until returning to the lab. At a later date the samples were processed and data calculated as described above (Methods Heading 2) for spectrophotometric samples (i.e. by the method (Strickland and Parsons, 1968) of grinding in 90% acetone, allowing to stand overnight in the refrigerator, centrifuging and reading), with the exception that the Lorenzen equation used a 664nm before acidification reading rather than the 665.

For fluorometric readings, samples of either 5 or 10 ml were taken in duplicate and processed as described above (Methods Heading 1) with 8 ml of the DMSO solvent on the vessel and read 3-7 days after the cruise. Calculations were made without a correction for phaeo-pigments although after acidification readings were taken for possible future use.

4. VIMS York River Plankton Monitoring

This monitoring program followed plankton-related parameters from the Coast Guard Pier near the mouth of the York River for the winter/spring bloom period and during the summer. Samples were collected three times a week at high slack water. A surface sample was constructed from equal parts of water from 1, 3, and 5 meters collected by bottle and a bottom sample was collected by means of a pump. Water samples from this study were placed in a cooler and returned to the laboratory within 30 minutes for processing. Chlorophyll samples were taken for this study from the surface sample, July through September, 1985. Fluorometric samples were taken in 5 ml duplicate samples on 25 mm GF/F filters, extracted with DMSO and read 5 - 7 days later. Samples for spectrophotometric readings were in duplicate, 800 ml or less in volume, filtered onto 47 mm GF/F filters with several drops of a saturated magnesium carbonate suspension, and immediately ground with 90% acetone, held until the next day in refrigeration, centrifuged and read. One or two additional duplicate sets of samples were taken for spectrophotometric

analysis. One set was frozen for two weeks and one remained frozen for 4 to 8 weeks before analysis; the freezer temperature was -12 C.

Results

1. Comparison of solvents (DMSO and 90% acetone) for extraction by fluorometry.

In the 1980 Chesapeake Bay data set, the DMSO extraction method produced chl *a* values under those test conditions which were equally as good as those from the 90% acetone extraction with grinding. Using a total of 136 pairs of observations, the two extraction methods produced values which were statistically indistinguishable (Table II-3, lines 1 and 3), although there is less variation in the values uncorrected for phaeophytin.

Table II-3. Comparison of two methods of extracting and calculating chl *a* values. Values are (ln DMSO - ln 90% acetone).

	Samples	Mean Difference Between Extractions	t	PROB>|t|	N
1980 Chesapeake Bay Study					
1.	Corrected chl *a*	-0.05096	-1.05	0.2985	68
2.	Phaeophytin	0.32321	4.88	0.0001	68
3.	Uncorrected chl *a*	-0.002579	-0.07	0.9450	68
4.	Uncorrected vs corrected chl *a*	0.0853	2.09	0.041	68
1984-85 Maryland Chesapeake Bay Monitoring.					
5.	Uncorrected chl *a*	0.3208	11.4	0.0001	95

Calculated phaeophytin values from the two solvents are highly significantly different with the DMSO method producing higher values (Table II-3, line 2). Uncorrected DMSO chl *a* values are significantly higher than the corrected 90% acetone values (line 4). Thus DMSO seems to extract chlorophyll *b* (chl *b*) more completely from these samples, i.e. an increase in the chl *b* interference would reduce the corrected chl *a* values and increase the calculated phaeophytin.

The comparison of the DMSO with the 90% acetone extraction methods during the 1984-85 Maryland Chesapeake Bay Monitoring (Table II-3, line 5 and Figure II-1) proved to be highly significantly different with the DMSO values being approximately 145% of the 90% acetone values. The reason for this significant difference proved to be related to storage conditions rather than analytical techniques. This can be best illustrated by October 1984 samples where approximately half the samples

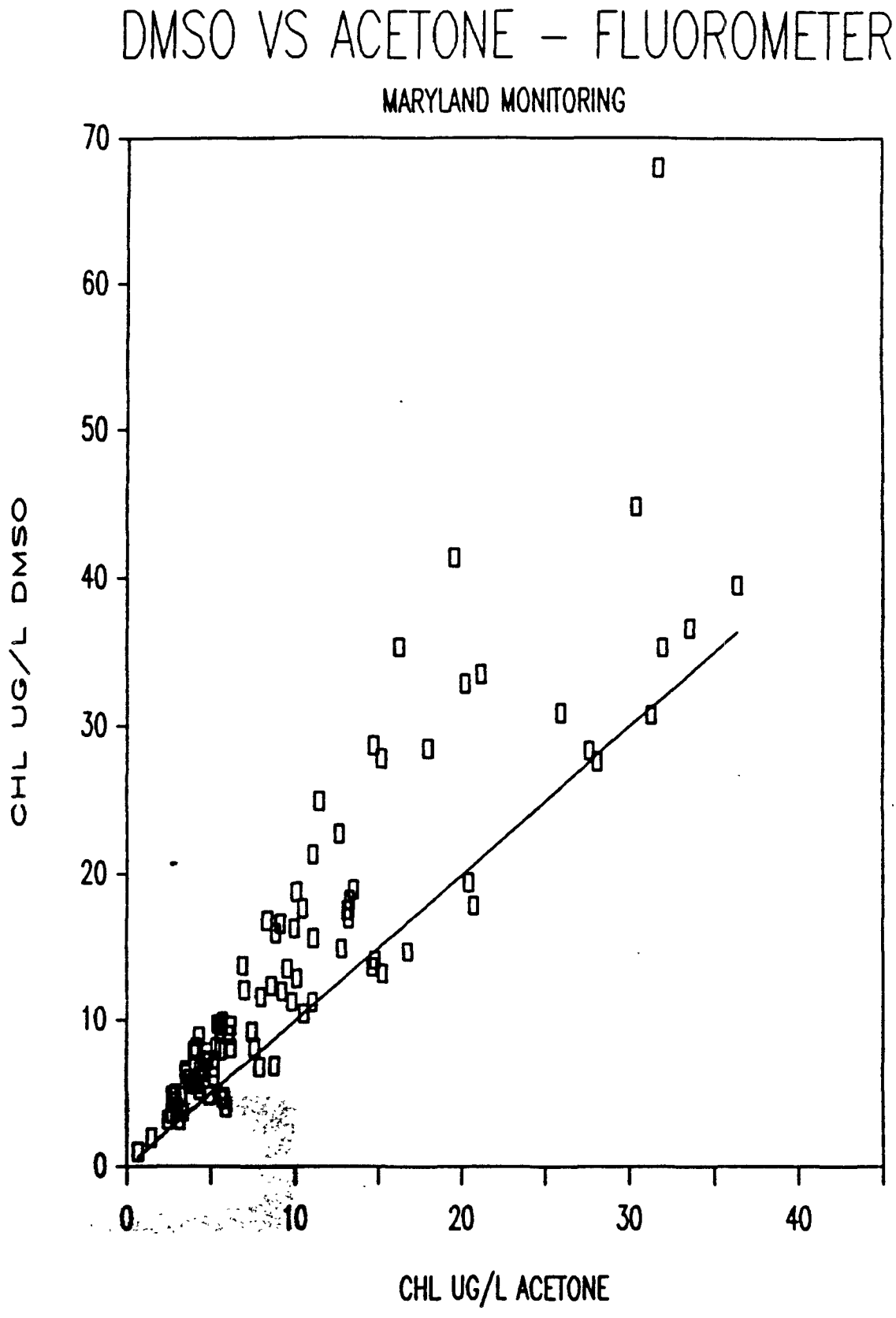


Figure II-1. Maryland EPA Monitoring Program Samples: CBL-DMSO extract measured by fluorometer compared to samples frozen and analyzed later at VIMS by grinding in acetone for extraction and fluorometer determination. Both data sets are calculated without phaeo-pigment corrections.

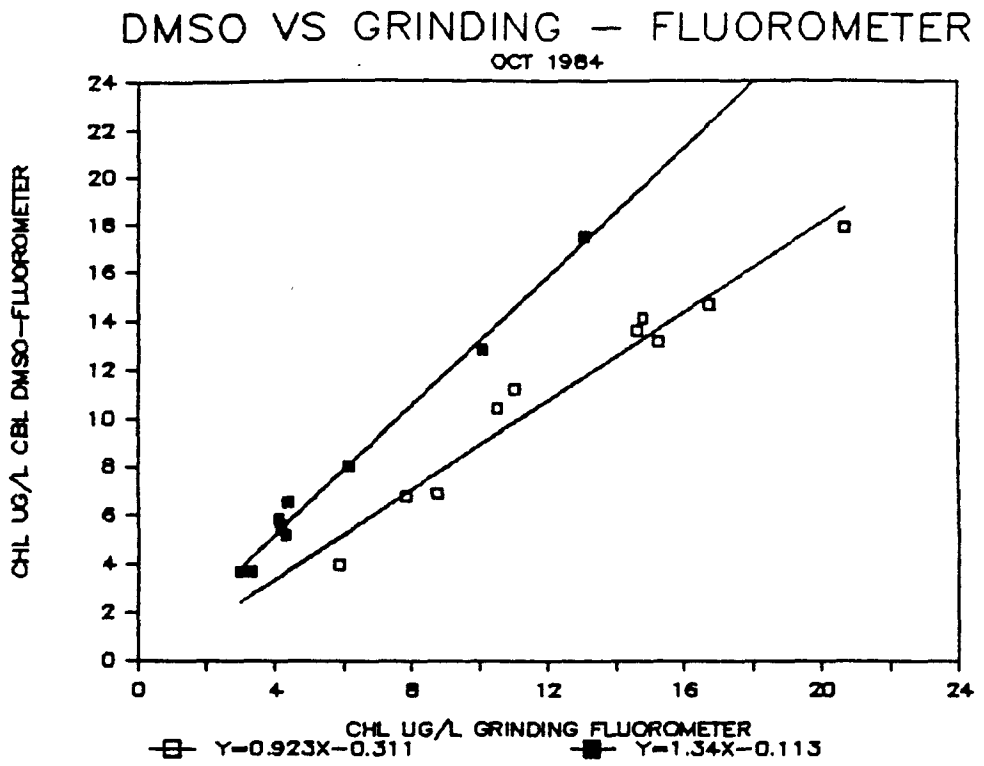


Figure II-2. October, 1984, Maryland samples frozen for two different times. Grinding fluorometric analysis using Turner Model 111 (□) frozen 5 months, (■) using Turner Designs, frozen 11.5 months.

were stored for 5 months whereas the other half were stored for 11.5 months (Figure II-2). The amount of measured chlorophyll clearly declined with time.

2. Comparison of fluorometry with spectrophotometry.

2a. 90% Acetone with grinding.

Many of the Maryland Chesapeake Bay Monitoring samples were large enough to produce 90% acetone extracts which could be read on the spectrophotometer. Figure II-3 shows the relationship between the fluorometric and spectrophotometric determinations on the same extracts (90% acetone with grinding). Since the fluorometer was calibrated with known chl a measured on the same spectrophotometer, one would expect to see data like that of a calibration curve where the two values are essentially identical. For these samples, which were stored for several months and undoubtedly contained chlorophyll breakdown products, the fluorometric values averaged about 85% of the spectrophotometric value. The two determinations are significantly different (Table II-4, line 1). The fluorometric samples which are above about 15 $\mu g\ l^{-1}$ chl a on the spectrophotometer seem to deviate more than those with < 15 μg. These results may be dependent upon the breakdown products resulting from storage but are unexplained at the time of this writing.

2b. DMSO/fluorometry compared to acetone/spectrophotometry

Data from the Virginia EPA Chesapeake Bay Monitoring are shown in Fig II-4. The majority of these data show DMSO fluorometer values about 10% greater than those for the 90% acetone/spectrophotometric values and are significantly different (Table II-4, line 2). The acetone/spectrophotometer samples were stored frozen for one to 3.5 weeks before analysis whereas the DMSO/fluorometer samples were extracted on board the research vessel and analyzed a few days later. Loss during storage to a colorless breakdown product or a colored product with a lower absorbance could produce the greater fluorometer values.

The VIMS York River Plankton Monitoring provided the opportunity to carry out a similar comparison with all processing carried out by the same laboratory personnel. Figure II-5a compares these data from the DMSO fluorometer procedure with that of the 90% acetone grinding spectrophotometer, all analyses carried out on fresh samples without a storage period. The fluorometer values were significantly higher (Table II-4, line 3) and appeared to be offset by a constant value rather than a percentage of the spectrophotometric value. Subtracting a value of 1.643 from the fluorometric values (line in Fig. II-5a) produced data which were not significantly different (Table II-4, line 4). Without data between 0 and 5 $\mu g\ l^{-1}$ it is impossible to tell if in fact a zero spectrophotometer reading could give a fluorometer reading of 1.6 $\mu g\ l^{-1}$.

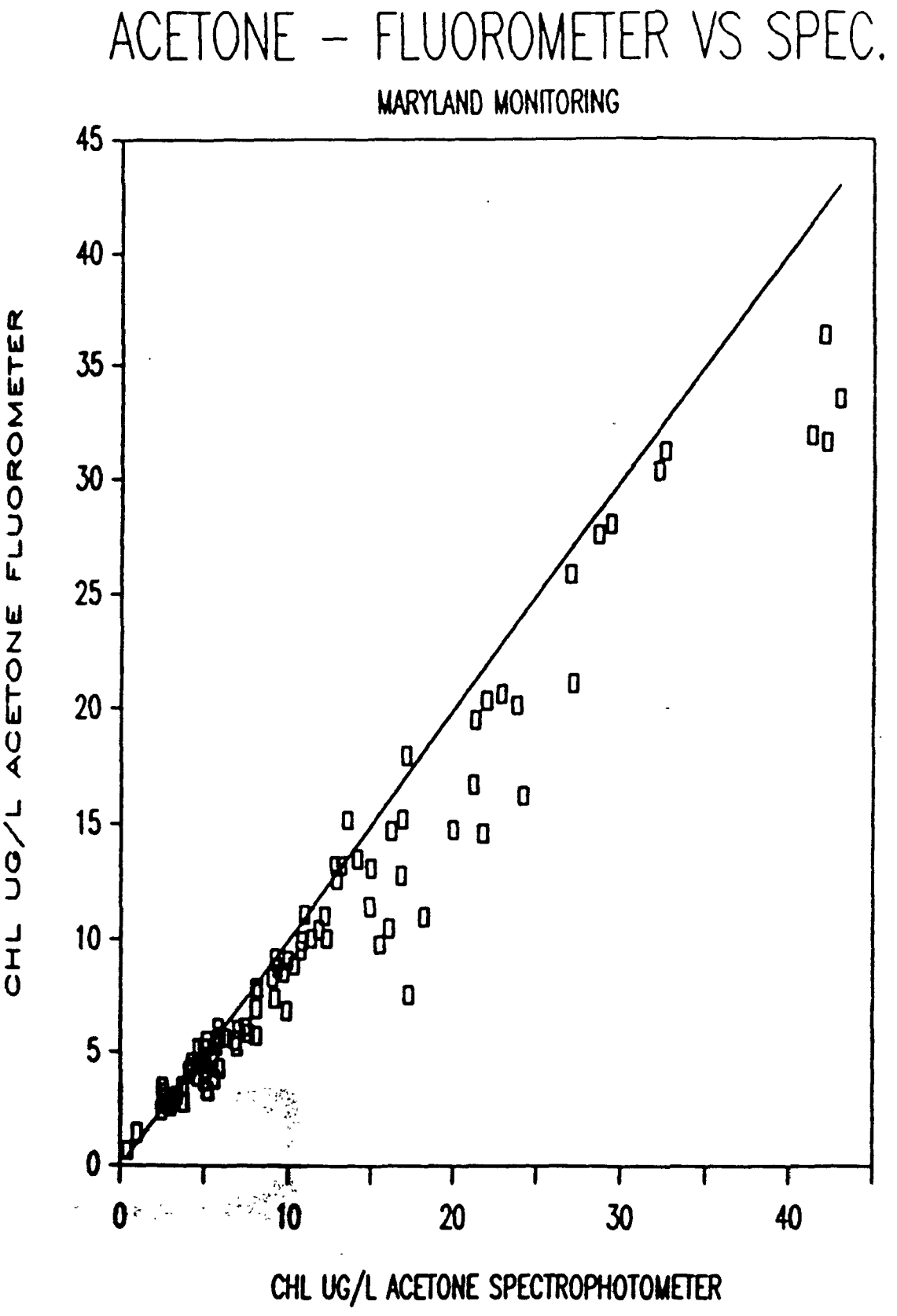


Figure II-3. Maryland EPA Monitoring Program Samples: samples frozen and analyzed later at VIMS by grinding in acetone for extraction and analyzed by fluorometer and spectrophotometer determination. Both data sets are calculated without phaeopigment corrections. The spectrophotometric data are calculated with the trichromatic equations of Jeffrey and Humphrey (1975) for chl *a*, *b*, and *c*.

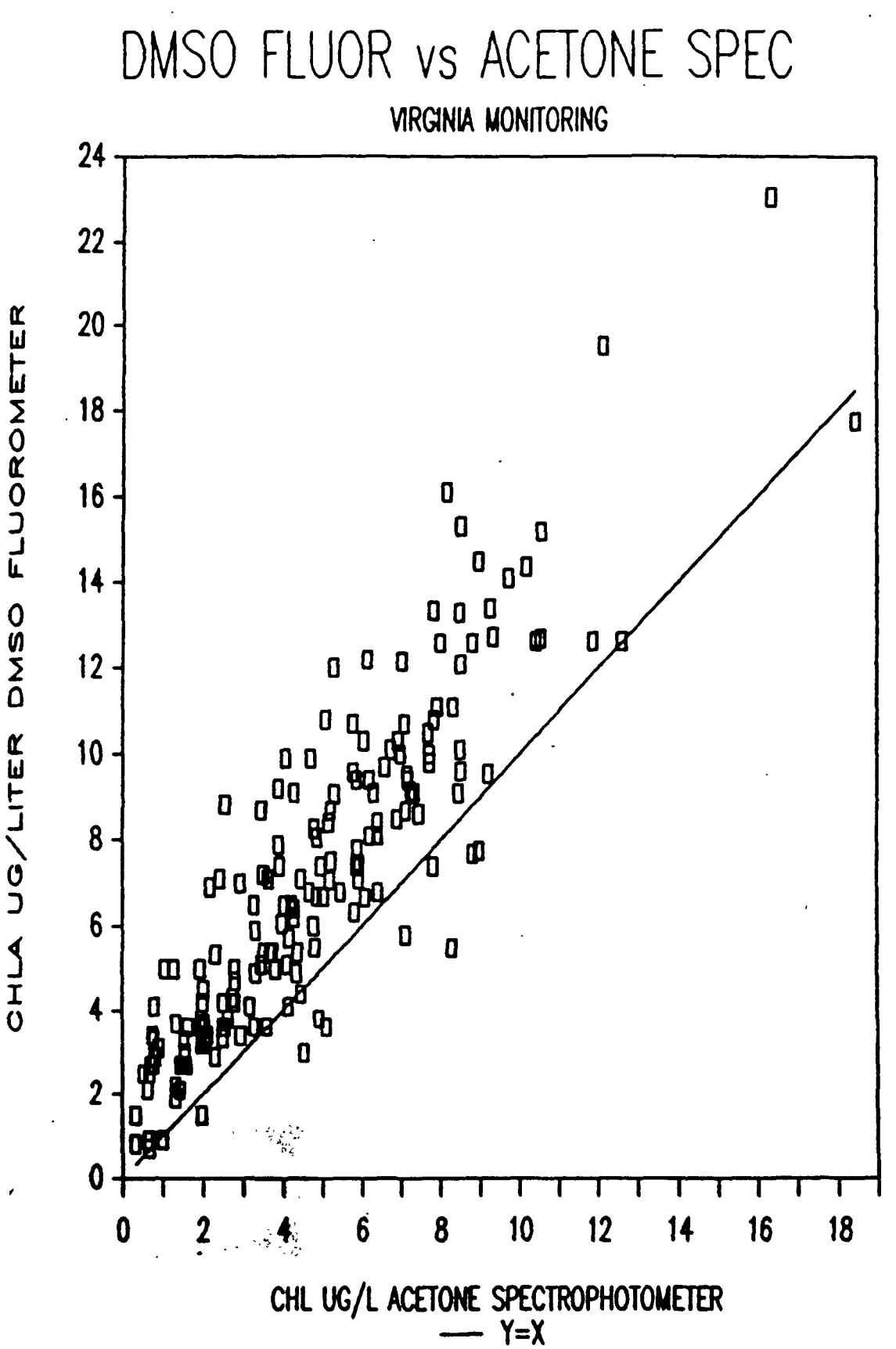
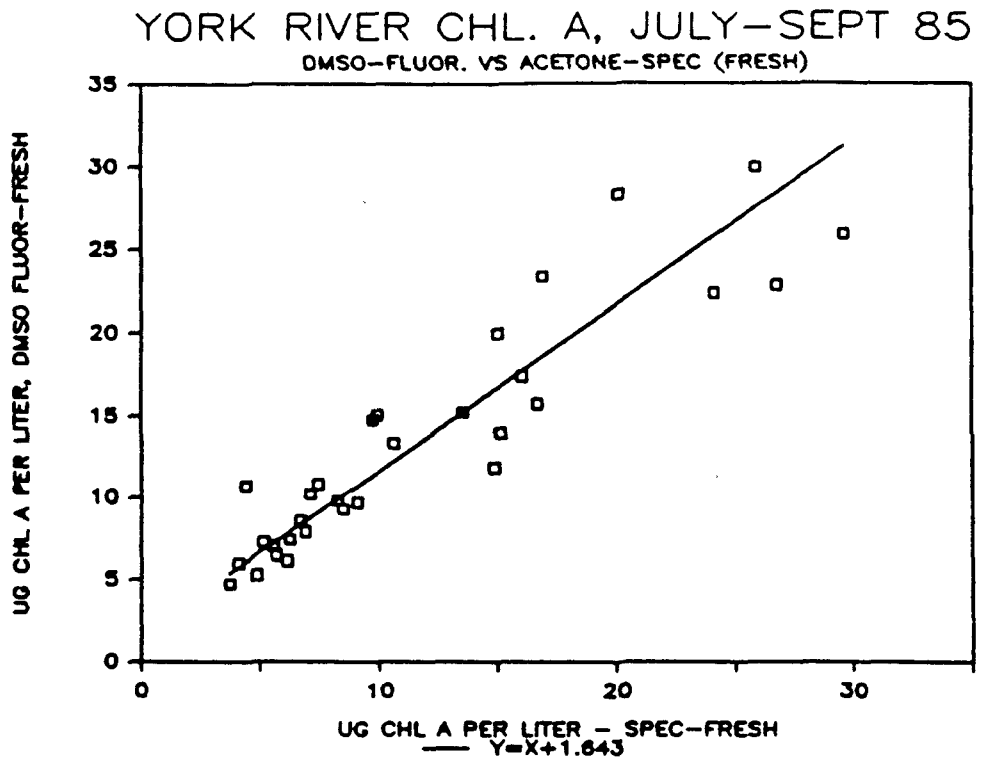


Figure II-4). Virginia EPA Chesapeake Bay Monitoring samples comparing freshly extracted by DMSO fluorometric determinations (means of pairs), with single 90% acteone extracts with grinding after freezing. The 90% acetone extracts were read on the spectrophotometer and calculated by the Jeffrey and Humphrey (1975) equations for chl a, b, and c.



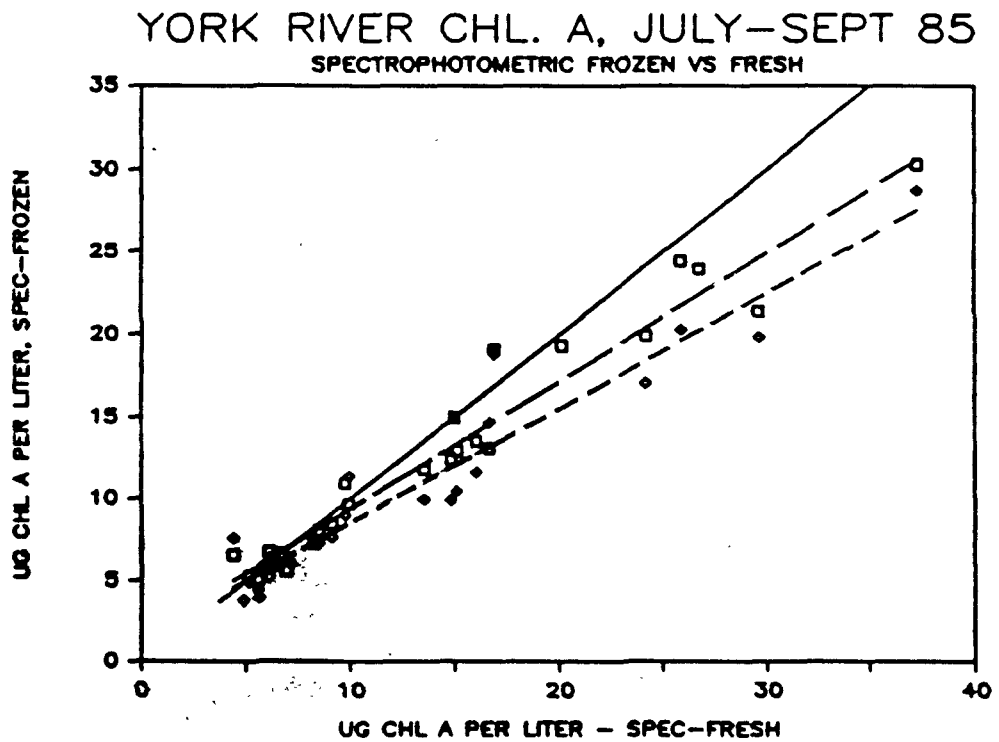


Figure II-5. The spectrophotometric data are calculated with the trichromatic equations of Jeffrey and Humphrey (1975) for chl a, b, and c. VIMS Coast Guard Pier samples, July-Sept 1985.

A) Comparison of DMSO fluorometer, with 90% acetone with grinding spectrophotometric data on fresh samples.

B) Effect of freezing; (———) fresh samples, (□ — — —) frozen 2 weeks Y=0.789X+1.59, (◇ — — —) frozen 4-6 weeks Y=0.699X+1.54

Table II-4. Comparison of fluorometry with spectrophotometry for determining chl *a* values. Values are (ln Fluorometer - ln spectrophotometer).

Samples	Mean Difference Between Methods	t	PROB>|t|	N
1984-85 Maryland Chesapeake Bay Monitoring.				
1. Uncorrected chl *a*	-1.116	-5.11	0.0001	95
1985 Virginia Chesapeake Bay Monitoring.				
2. Uncorrected chl *a*	0.4734	15.8	0.0001	177
1985 Virginia York River Plankton Monitoring.				
3. Uncorrected chl *a*	0.177	4.42	0.0001	31
4. (Fluorometer -1.643)	0.000017	0.0004	0.99	31

3. Storage effects.

Early in the study we observed a difference between values determined at CBL and those at VIMS. This persisted after complete renovation and recalibration of equipment. During one trip between the laboratories we made 12 replicates of DMSO plankton sample extracts, i.e. the same water sample was divided and filtered onto 12 filters which were placed in the DMSO tubes for extraction. Six of the tubes were transferred to CBL and, the samples at VIMS and CBL were read the same afternoon. The VIMS results were 3% higher numerically but not significantly different from the CBL values (VIMS = 7.53, S.D. 0.52; CBL = 7.30, S.D. 0.36; d.f. 10, t 0.819). As a result of this experience we designed a simple frozen storage experiment (see methods). Results are presented in Fig. II-5b. These data indicate a loss of chlorophyll of about 20% during the first 2 weeks and an additional 10% loss in the next 2-4 weeks. This loss could indicate either a partial conversion to a colorless breakdown product or a combination with almost a complete conversion to a colored form which should have an absorption coefficient about 85% of that of chl *a*.

4. Presence of chlorophyll *b* and *c*

The spectrophotometric data allow chlorophylls *b* and *c* to be calculated as well as *a* using the Jeffrey and Humphrey (1975) equations. This was done for all the extracts with a chlorophyll concentration 0.2 µg/ml or above for the Virginia Chesapeake Bay monitoring program. Below the concentration of 0.2 µg/ml extract values are unreliable (Lorenzen &

Jeffrey, 1980). These values are plotted as a:b and a:c ratios (Figure II-6). Samples with low a:b ratios should have populations dominated by Chlorophyceae (green algae), and samples with low a:c ratios should have populations dominated by diatoms or dinoflagellates (see Table II-5). There are no cell counts for these samples to verify these observations; however, such analyses were attempted with the VIMS Coast Guard samples. This attempt proved unsuccessful, presumably because the taxonomic divisions of the counts were not detailed enough, i.e. categories were too inclusive.

5. Precision of DMSO method.

The results from the 1980 Chesapeake Bay study indicate no significant change in the determined values (P=0.99), nor in coefficient of variation associated with the interval of storage (P=0.55). Presumably if either additional materials were extracted with time or the extracted pigment decomposed to colorless products during the storage period the data would be more variable with longer storage/extraction time. Thus if chl a is breaking down to phaeophytin a or to other colored decomposition products, this method registers the product as chl a. It is therefore practical to place the filters in the extraction tubes in the field and read them in the lab at a later date.

Discussion

The July 1980 EPA Chesapeake Bay study showed to our satisfaction that DMSO:acetone:water (9:9:2) was a satisfactory solvent when compared to 90% acetone with grinding. The comparison was made with fluorometric determinations uncorrected for phaeo-pigments. The main advantages of this method were ease of sampling handling and storage (no grinding, refrigeration, dilution). The samples are filtered, the filter placed in solvent to extract, and the extract is decanted into the fluorometer tube for the reading. The extracting sample can be stored at room temperature for several weeks without affecting the results. This approach gives one a value which amounts to chl a plus phaeo-pigments (including any which were produced during storage), and may not be appropriate if phaeo-pigment values are desired, however, it may be a perfectly adequate index of phytoplankton biomass, i.e. living plus recently dead (or eaten) phytoplankton.

It is apparent from a literature review that accessory pigments, especially chlorophyll b, interfere with both the fluorometric and the spectrophotometric determination of phaeo-pigments and, conversely, the presence of phaeo-pigments may interfere with the determinations of the chlorophylls, especially chl a. Chlorophyll b has been shown to occur in Virginia Bay Monitoring samples. Thus if either of these techniques is used to measure pigments, compromises will have to be made. It is thus apparent that if one really needs to know the amount of chlorophyll a or other pigments present, it (they) will have to be separated from interfering substances prior to their determination. It is feasible to do this with chromatographic procedures. Several investigators have reported using thin layer chromatography (e.g. Garside and Riley, 1969; Jeffrey, 1975). High Performance Liquid Chromatography (HPLC) is a

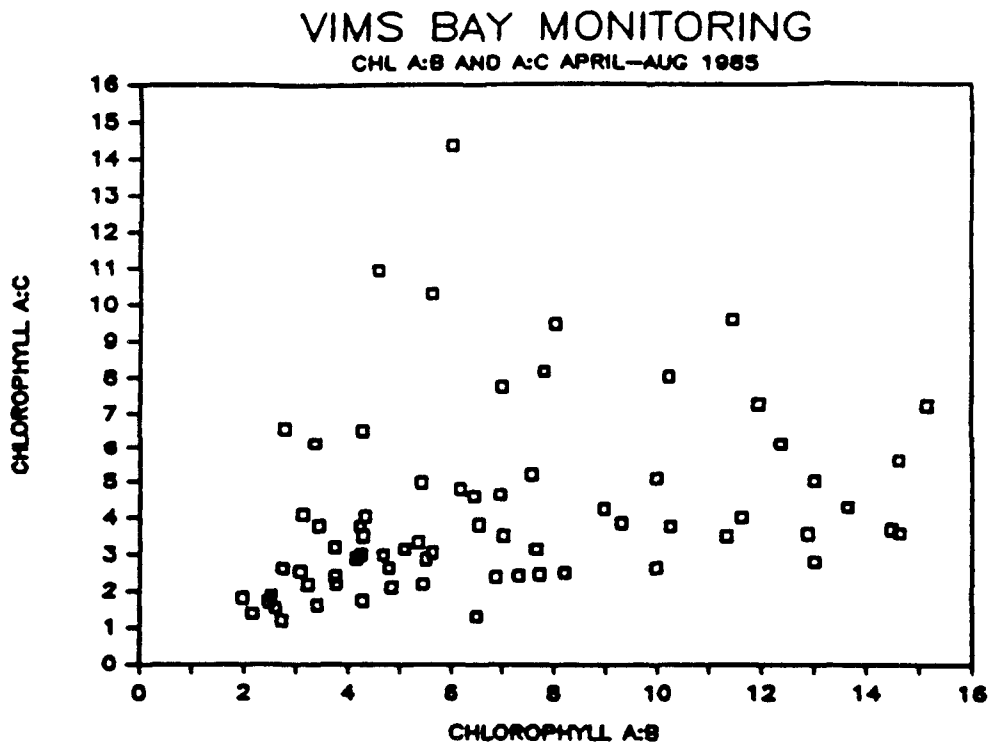


Figure II-6. The spectrophotometric data were calculated with the trichromatic equations of Jeffrey and Humphrey (1975) for chl a, b, and c and the values below 0.2ug/ml extract were deleted. The remaining values are plotted as a:b and a:c ratios.

Table II-5 Major light-harvesting pigments of the algae.

Light Harvesting Pigments	Chlorophyll a	Chl b	Chl c1	Chl c2	Fucoxanthin	Peridinin	Phycobili-proteins	Siphonaxanthin
Algal Class								
Bacillariophyceae (Diatoms)	+		+	+	+			
Chlorophyceae (Green algae)	+	+						+ (not planktonic forms)
Chrysophyceae (Golden algae)	+		+	+	+			
Cryptophyceae (Cryptomonads)	+			+			+	
Cyanobacteria (Blue-green algae)	+						+	
Dinophyceae (Dinoflagellates)	+			+				
	+		+	+	+	+		
Phaeophyceae (Brown Algae)	+		+	+	+			
Prochlorophyta	+	+						
Rhodophyceae (Red Algae)	+						+	

better choice in that it can be automated to a large degree. Numerous investigators have published using HPLC for chlorophyll determinations (e.g. Abaychi and Riley, 1979; Brown, et al., 1981; Gieskes and Kraay, 1983; Goeyens, L. et al., 1982; Knight and Mantoura, 1985; Mantoura and Llewellyn, 1983; Pearl et al., 1983; Shioi et al., 1983).

In summary, it appears that the fluorometric and spectrophotometric methods for chlorophyll a estimations in general use have a fairly low accuracy (optimistically perhaps within 30%) due to interference and storage problems. A logical approach to chlorophyll a estimation is to use a fast simple extraction, such as the proposed DMSO approach which involves a minimum of handling, possible storage at room temperature and, thus, should improve precision no matter how the extract is analyzed. The method of choice for extract analysis clearly is the use of a chromatographic method to separate the pigments so that they can be measured with less interference and greater accuracy. If this technique isn't available, the individual investigator can use any or all of several fluorometric and spectrophotometric methods to estimate the chlorophyll pigments, including bulk breakdown products, at a sacrifice in accuracy.

Comments on Interim Guidance on Quality Assurance/Quality Control (QA/QC) for The Estuarine Field and Laboratory Methods.

The "Interim Guidance on Quality Assurance/Quality Control (QA/QC) for The Estuarine Field and Laboratory Methods" (USEPA, 1985) provides a standard operating procedure (SOP) for chlorophyll which essentially paraphrases Strickland and Parsons (1972) for sample collection, and processing and storage; it further recommends the fluorometric method detailed in Strickland and Parsons (1972, Section IV.3.IV) based on 90% acetone extractions, the implied use of the Turner Model 111 fluorometer and calibration by pigment extracts from a combination of algal cultures.

Storage time: Strickland and Parsons (1972) suggest that filters with chlorophyll samples may be stored "in the dark in a desiccator frozen to -20 C but only for a few weeks. This procedure almost always leads to low results and makes the extraction of chlorophyll more difficult; filters should be extracted without delay if at all possible." Our results agree with the loss of chlorophyll with weeks, e.g. 20% within 2 weeks. Our proposed solvent extraction technique using DMSO is easily started immediately after filtering the sample in the field; we recommend it over the acetone extraction because it eliminates the problems of sample storage, grinding etc., while performing equally well.

Calibration: The Interim Guidance (USEPA, 1985) follows Strickland and Parsons' (1972) recommendation that healthy cultures and a "mixture of about equal amounts (by pigment) of Skeletonema costatum, Coccolithus huxleyii, and Peridinium trochoidium be used as a source of spectrophotometrically determined chlorophyll for calibration of the fluorometer. It is our recommendation that commercially available chlorophyll, not generally available in 1972, be used in the calibration. Strickland and Parsons (1972) in fact state that calibration "must be done on extracts from marine phytoplankton as pure chlorophyll a is

difficult to obtain." Using pure chlorophyll should reduce interlaboratory calibration differences and be an easily reproducible frame of reference within a laboratory. Any potential advantage of calibrating with a pigment mixture very similar to that of the sample population quickly disappears in an estuarine environment having rapidly changing pigment complements throughout the year. The use of chlorophyll quality control (QC) samples available from the Environmental Monitoring and Support Laboratory - Cincinnati (EMSL-Cincinnati) should be incorporated into routine analyses programs.

The above comments generally apply also to the APHA (1985) Method 1001G2 which is essentially the same as Strickland and Parsons (1972). The Interim Guidance should be more inclusive, or general, to include other fluorometers such as the Turner Designs which is coming into widespread use. For estuarine work, units of µg per liter are more appropriate than mg per cubic meter. The possibility of using HPLC to separate the pigments before analysis should be both allowed and encouraged. An evaluation of the costs of obtaining accurate and informative data through automated HPLC techniques should be carried out.

Recommendations for the Chesapeake Bay Program

1. Take small samples 5-15 ml depending on chlorophyll concentration and place them in the DMSO solvent on board the ship.

2. After 24 hours or upon return to port several days later, the samples are read on the fluorometer and calculated without a phaeo-pigment correction.

It should be recognized that this method although fast and easy, will give the best data on euphotic zone samples which have few chlorophyll decomposition products. Samples from near the bottom or which contain sediments, fecal pellets, etc., will give values which are inflated by the decomposition products.

Alternative Recommendation.

1. Take samples of 200-1000 ml and extract as in the above recommendation.

2. Read the sample before and after acidification in a spectrophotometer using a 1 cm cell only if the concentrations are above a fixed threshold such as 0.25 µg/ml. For lower concentrations, small volume longer light path (5 or 10 cm) cuvettes should be required.

3. An option to step 2 is to read the extract at multiple wavelengths as well as before and after acidification and report all the pertinent data so that users can make whatever calculations they wish, i.e. station data, sample and extract volumes, and spectrophotometric readings and length of light path.

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Appendix I. Letter from B. Nowicky at the University of Rhode Island summarizing her comparisons of the TKN and TPN techniques as well as the recovery of caffeine-N using the TPN technique.

University of Rhode Island, Narragansett, Rhode Island 02882
Graduate School of Oceanography, Narragansett Bay Campus

February 6, 1986

Dr. Christopher D'Elia
Chesapeake Biological Laboratory
P. O. Box 38
Solomons, Maryland 20688

Dear Dr. D'Elia:

I haven't forgotten your request for data comparing the Kjeldahl technique with the Persulfate digestion for total nitrogen, I'm afraid that locating that work (done some eight or nine years ago) is proving more difficult than I expected. I've enclosed a brief table which may be of some help. As the table shows, I first noticed that I got consistently higher values for the Persulfate digestion than with the Kjeldahl technique. When I checked my percent recovery of standard additions of various organic compounds (urea, glycine, EDTA) to seawater, I found I got better recovery with the Persulfate Technique. In addition I found that my precision was much better using a persulfate digestion. The "caffeine recovery experiment" was done after Suzuki et al. (Mar. Chem. 16, (1985) 83-97) published an article questioning the ability of the persulfate digestion to deal with ring nitrogen compounds. My decision to switch to persulfate digestions was made after quite a lot of "playing around" with the various techniques. Unfortunately, I never published the data (or intended to) and it sits in my lab notebooks in disarray. The tables I'm sending are some bits and pieces. I hope they're of use.

Sincerely,

Barbara Nowicki

Barbara Nowicki

BN/d
Enc.

Six different samples were taken from the MERL experimental mesocosms (salinity = 30 °/oo) and filtered (precompusted Glass fiber-filters). The samples were then analysed using both Kjeldahl and Persulfate techniques.

		Total dissolved nitrogen ($\mu g\text{-at } L^{-1}$)	
Tank #	Time	Kjeldahl technique	Persulfate digestion
5	9 a.m.	10.9	15.3
5	noon	10.8	14.7
5	3 p.m.	11.7	13.7
7	9 a.m.	12.0	15.0
7	noon	14.4	18.3
7	3 p.m.	11.3	15.3

Kjeldahl technique - precision of duplicate estuarine samples.

Sample	Total dissolved Nitrogen (μg at L^{-1})		$\bar{x}$	± 1 s.d.
Brushneck Cove mouth	#1	31.91	31.2	1.05
	#2	30.42		
Brushneck Cove head	#1	46.51	47.1	0.8
	#2	47.60		

Persulfate digestion - precision of six replicate estuarine samples from the MERL mesocosms.

	Total N	Total P
	$\bar{x}$ ± s.d.	$\bar{x}$ ± s.d.
Unfiltered samples	60.3 ± 0.3	2.0 ± 0.08
Filtered samples	31.7 ± 0.3	1.16 ± 0.04

A check on percent recovery of various organic N compounds added to artificial seawater using the persulfate digestion technique.

Compound	AA chart units (mean of 4 replicates)	% recovery relative to NO_3^-
10 μM NO_3^-	11.63	--
10 μM Glycine	11.54	99%
10 μM Urea	11.55	99%
10 μM Caffine	11.34	99%

Appendix II. Raw data for TKN and TPN analysis performed on continental shelf seawater spiked with standard.

Salinity %		Standard conc., µM	TKN Pk ht	TKN Conc., µM	TKN Recovery %	TPN PK ht	TPN Conc., µM	TPN Recovery %
0	BLANK	0.0	16.0	2.22		7.3	0.00	
0	BLANK	0.0	15.8	1.81		8.2	0.00	
0	BLANK	0.0	14.1	-1.74		9.4	0.60	
0	GLU	25.2	28.0	27.24	1.08	25.4	21.50	0.85
0	GLU	25.2	30.6	32.66	1.30	27.8	24.60	0.98
0	GLU	50.4	39.6	51.43	1.02	44.4	46.20	0.92
0	GLU	50.4	38.9	49.97	0.99	43.2	44.70	0.89
0	GLU	75.5	59.3	92.51	1.23	63.4	71.00	0.94
0	GLU	75.5	53.7	80.83	1.07	66.2	74.60	0.99
0	NH4	15.0	24.3	19.53	1.30	17.4	20.60	1.37
0	NH4	15.0	21.8	14.32	0.95	24.7	11.10	0.74
0	NH4	45.0	39.3	50.81	1.13	40.5	41.20	0.92
0	NH4	45.0	38.8	49.76	1.11	41.8	42.80	0.95
0	NH4	75.0	48.1	69.15	0.92	59.0	65.20	0.87
0	NH4	75.0	50.3	73.74	0.98	59.4	65.80	0.88
0	NO3-	25.0	14.4	-1.11	-0.04	27.3	24.00	0.96
0	NO3-	25.0	14.5	-0.90	-0.04	28.5	25.50	1.02
0	NO3-	50.0	13.9	-2.16	-0.04	50.3	53.90	1.08
0	NO3-	50.0	15.9	2.01	0.04	50.2	53.80	1.08
0	NO3-	75.0	13.6	-2.78	-0.04	64.5	72.40	0.97
0	NO3-	75.0	13.3	-3.41	-0.05	64.7	72.60	0.97
0	UREA	26.8	30.0	31.41	1.17	26.6	23.10	0.86
0	UREA	26.8	29.6	30.58	1.14	26.8	23.30	0.87
0	UREA	53.6	41.5	55.39	1.03	53.4	57.90	1.08
0	UREA	53.6	40.8	53.93	1.01	50.4	54.00	1.01
0	UREA	80.4	48.7	70.40	0.88	70.7	80.50	1.00
0	UREA	80.4	53.1	79.58	0.99	67.3	76.00	0.95
25	BLANK	0.0	11.3	-7.58		10.1	1.50	
25	BLANK	0.0	12.1	-5.91		10.8	2.40	
25	BLANK	0.0	12.5	-5.07		8.2	0.00	
25	GLU	25.2	29.5	30.37	1.21	26.6	23.00	0.91
25	GLU	25.2	27.3	25.78	1.02	26.2	22.40	0.89
25	GLU	50.4	32.5	36.63	0.73	46.7	49.10	0.97
25	GLU	50.4	43.5	59.56	1.18	48.6	51.60	1.02
25	GLU	75.5	59.1	92.09	1.22	66.2	74.50	0.99
25	GLU	75.5	56.9	87.50	1.16	64.9	72.80	0.96
25	NH4	15.0	23.6	18.07	1.20	20.7	15.30	1.02
25	NH4	15.0	27.8	26.83	1.79	20.1	14.50	0.97
25	NH4	30.0	44.0	60.60	2.02	31.6	29.50	0.98
25	NH4	30.0	45.8	64.36	2.15	27.3	23.90	0.80
25	NH4	75.0	54.5	82.50	1.10	64.1	71.80	0.96
25	NH4	75.0	58.6	91.05	1.21	64.1	71.80	0.96
25	NO3-	25.0	16.1	2.43	0.10	27.6	24.30	0.97
25	NO3-	25.0	24.4	19.74	0.79	20.9	26.10	1.04
25	NO3-	50.0	15.5	1.18	0.02	48.3	51.20	1.02
25	NO3-	50.0	15.3	0.76	0.02	48.3	51.20	1.02
25	NO3-	75.0	22.7	16.19	0.22	68.3	77.20	1.03
25	NO3-	75.0	18.3	7.02	0.09	68.5	77.50	1.03
25	UREA	26.8	31.6	34.75	1.30	26.8	23.20	0.87
25	UREA	26.8	27.6	26.41	0.99	25.9	22.10	0.82
25	UREA	53.6	45.6	63.94	1.19	48.4	51.30	0.96
25	UREA	53.6	50.1	73.32	1.37	48.8	51.90	0.97

25	UREA	80.4	NA	NA	NA	70.9	80.60	1.00
25	UREA	80.4	57.5	88.75	1.10	74.1	84.80	1.05
50	BLANK	0.0	14.4	-1.11		10.1	1.40	
50	BLANK	0.0	13.1	-3.82		17.6	11.10	
50	BLANK	0.0	11.9	-6.33		13.0	5.20	
50	GLU	25.2	29.9	31.21	1.24	30.0	27.30	1.08
50	GLU	25.2	23.8	18.49	0.73	31.7	29.50	1.17
50	GLU	50.4	42.4	57.27	1.14	51.4	55.10	1.09
50	GLU	50.4	42.0	56.43	1.12	46.3	48.50	0.96
50	GLU	75.5	55.1	83.75	1.11	66.2	74.40	0.99
50	GLU	75.5	50.8	74.78	0.99	67.5	76.10	1.01
50	NH4	15.0	22.5	15.78	1.05	23.1	18.30	1.22
50	NH4	15.0	22.8	16.40	1.09	22.9	18.10	1.21
50	NH4	45.0	41.9	56.23	1.25	40.7	41.20	0.92
50	NH4	45.0	40.3	52.89	1.18	42.7	43.80	0.97
50	NH4	75.0	51.3	75.83	1.01	70.7	80.50	1.07
50	NH4	75.0	53.0	79.37	1.06	66.2	74.60	0.99
50	NO3-	25.0	11.3	-7.68	-0.31	28.3	25.10	1.00
50	NO3-	25.0	12.6	-4.87	-0.19	27.6	24.20	0.97
50	NO3-	50.0	15.5	1.18	0.02	47.8	50.50	1.01
50	NO3-	50.0	13.6	-2.78	-0.06	47.5	50.10	1.00
50	NO3-	75.0	14.9	-0.07	.00	71.5	81.30	1.08
50	NO3-	75.0	16.3	2.85	0.04	71.5	81.30	1.08
50	UREA	26.8	25.1	21.20	0.79	28.6	25.50	0.95
50	UREA	26.8	27.1	25.37	0.95	27.6	24.20	0.90
50	UREA	53.6	43.3	59.15	1.10	48.1	50.80	0.95
50	UREA	53.6	26.8	24.74	0.46	47.8	50.50	0.94
50	UREA	80.4	58.8	91.46	1.14	71.1	80.80	1.00
50	UREA	80.4	56.8	87.29	1.09	70.2	79.60	0.99
75	BLANK	0.0	10.5	-9.24		1.0	0.20	
75	BLANK	0.0	8.0	-14.46		1.9	1.30	
75	BLANK	0.0	11.9	-6.33		2.3	1.80	
75	GLU	25.2	37.0	46.01	1.83	31.4	28.90	1.15
75	GLU	25.2	28.6	28.49	1.13	28.9	25.70	1.02
75	GLU	50.4	44.1	60.81	1.21	54.7	59.20	1.17
75	GLU	50.4	46.5	65.82	1.31	52.5	56.40	1.12
75	GLU	75.5	50.4	73.95	0.98	67.7	76.20	1.01
75	GLU	75.5	54.0	81.46	1.08	70.0	79.10	1.05
75	NH4	15.0	26.3	23.70	1.58	23.7	18.90	1.26
75	NH4	15.0	25.9	22.87	1.52	25.8	21.60	1.44
75	NH4	45.0	43.0	58.52	1.30	49.3	52.20	1.16
75	NH4	45.0	42.6	57.69	1.28	42.8	43.70	0.97
75	NH4	75.0	55.8	85.21	1.14	72.4	82.30	1.10
75	NH4	75.0	54.1	81.66	1.09	70.2	79.40	1.06
75	NO3-	25.0	15.1	0.35	0.01	30.3	27.50	1.10
75	NO3-	25.0	16.0	2.22	0.09	32.4	30.20	1.21
75	NO3-	50.0	16.8	3.89	0.08	50.2	53.40	1.07
75	NO3-	50.0	24.1	19.11	0.38	51.2	54.70	1.09
75	NO3-	75.0	17.0	4.31	0.06	72.6	82.50	1.10
75	NO3-	75.0	16.2	2.64	0.04	72.6	82.50	1.10
75	UREA	26.8	30.0	31.41	1.17	28.0	24.90	0.93
75	UREA	26.8	34.5	40.80	1.52	29.8	26.80	1.00
75	UREA	53.6	44.5	61.65	1.15	50.5	53.80	1.00
75	UREA	53.6	49.0	71.03	1.33	52.8	56.80	1.06
75	UREA	80.4	57.5	88.75	1.10	67.1	75.40	0.94
75	UREA	80.4	58.9	91.67	1.14	66.6	74.70	0.93

100	BLANK	0.0	10.5	-9.24		13.1	5.00	
100	BLANK	0.0	10.0	-10.29		16.6	9.60	
100	BLANK	0.0	11.8	-6.53		14.2	6.40	
100	GLU	25.2	24.8	20.57	0.82	35.9	34.70	1.38
100	GLU	25.2	26.3	23.70	0.94	30.8	28.00	1.11
100	GLU	50.4	43.6	59.77	1.19	49.6	52.50	1.04
100	GLU	50.4	39.4	51.01	1.01	50.6	53.80	1.07
100	GLU	75.5	50.6	74.37	0.98	72.9	82.80	1.10
100	GLU	75.5	53.3	80.00	1.06	71.5	81.00	1.07
100	NH4	15.0	19.5	9.52	0.63	25.1	20.60	1.37
100	NH4	15.0	20.8	12.23	0.82	27.5	23.70	1.58
100	NH4	45.0	42.5	57.48	1.28	46.2	48.10	1.07
100	NH4	45.0	38.4	48.93	1.09	46.7	48.70	1.08
100	NH4	75.0	55.8	85.21	1.14	68.3	76.80	1.02
100	NH4	75.0	51.8	76.87	1.02	72.1	81.80	1.09
100	NO3-	25.0	14.3	-1.32	-0.05	31.0	28.30	1.13
100	NO3-	25.0	14.3	-1.32	-0.05	36.0	34.80	1.39
100	NO3-	50.0	NA	NA	NA	48.6	51.20	1.02
100	NO3-	50.0	NA	NA	NA	50.5	53.70	1.07
100	NO3-	75.0	15.5	1.18	0.02	69.4	78.30	1.04
100	NO3-	75.0	16.2	2.64	0.04	72.4	82.20	1.10
100	UREA	26.8	27.3	25.78	0.96	31.8	30.00	1.12
100	UREA	26.8	26.5	24.12	0.90	28.6	25.20	0.94
100	UREA	53.6	49.5	72.07	1.34	46.0	47.80	0.89
100	UREA	53.6	45.8	64.36	1.20	50.1	53.10	0.99
100	UREA	80.4	58.2	90.21	1.12	65.1	72.70	0.90
100	UREA	80.4	60.4	94.80	1.18	64.1	71.40	0.89

Appendix III. Regression curves for TKN and TPN analyses performed on continental shelf seawater spiked with standard.

Salinity %	Standard	Method	Intercept	SEM	Slope	SEM	r
0	glutamic acid	TKN	15.11	1.217	0.529	0.027	0.991
		TPN	7.97	0.775	0.740	0.017	0.998
0	ammonia	TKN	15.99	0.881	0.462	0.021	0.993
		TPN	9.42	1.187	0.678	0.028	0.994
0	nitrate	TKN	15.29	0.447	-0.020	0.010	-0.605
		TPN	8.87	1.032	0.768	0.123	0.997
0	urea	TKN	16.24	0.960	0.446	0.020	0.993
		TPN	7.99	1.150	0.772	0.024	0.997
25	glutamic acid	TKN	11.94	1.949	0.587	0.044	0.981
		TPN	9.17	0.711	0.747	0.016	0.998
25	ammonia	TKN	16.44	3.230	0.593	0.083	0.937
		TPN	9.25	0.758	0.724	0.020	0.998
25	nitrate	TKN	13.41	1.982	0.092	0.045	0.611
		TPN	8.17	1.524	0.791	0.035	0.993
25	urea	TKN	12.81	1.507	0.604	0.037	0.989
		TPN	-0.71	1.593	1.008	0.034	0.996
50	glutamic acid	TKN	13.44	1.206	0.537	0.027	0.991
		TPN	13.40	1.281	0.706	0.029	0.994
50	ammonia	TKN	14.19	1.068	0.531	0.026	0.992
		TPN	12.54	1.538	0.719	0.037	0.991
50	nitrate	TKN	12.52	0.689	0.036	0.016	0.661
		TPN	11.77	1.702	0.763	0.039	0.991
50	urea	TKN	11.98	2.884	0.527	0.061	0.956
		TPN	11.96	1.575	0.704	0.033	0.992
75	glutamic acid	TKN	13.21	2.659	0.570	0.060	0.963
		TPN	4.04	1.951	0.907	0.044	0.992
75	ammonia	TKN	13.12	1.821	0.593	0.044	0.982
		TPN	5.10	2.139	0.902	0.051	0.989
75	nitrate	TKN	11.54	1.801	0.106	0.041	0.699
		TPN	3.64	1.423	0.939	0.032	0.996
75	urea	TKN	12.28	1.868	0.604	0.040	0.985
		TPN	3.83	1.762	0.826	0.037	0.993
100	glutamic acid	TKN	11.31	1.041	0.558	0.023	0.994
		TPN	14.27	1.098	0.750	0.025	0.996
100	ammonia	TKN	11.44	1.134	0.586	0.027	0.993
		TPN	14.66	0.828	0.733	0.020	0.998
100	nitrate	TKN	11.35	0.597	0.066	0.014	0.902
		TPN	14.47	1.137	0.738	0.026	0.996
100	urea	TKN	11.09	1.222	0.623	0.026	0.994
		TPN	14.32	0.909	0.624	0.019	0.997

Appendix IV. Tables from literature comparing precision of the total N determinations by TKN and TPN.

(A) Seawater field samples. (D'Elia et al., 1977)

	TPN			TKN + NO_3^- and NO_2^--N		
Concentration (μM)	Mean (μM)	N*	CV%	Mean (μM)	N (pairs)	CV%
20	14.2	23	8.7	14.3	12	5.3
20-40	26.9	14	5.9	27.1	12	6.9
40-60	50.7	11	8.6	47.3	3	7.3
60-80	70.9	20	5.2	70.1	3	2.2
80-100	88.2	12	3.2	----	---	---
100-120	110.9	16	3.7	----	---	---

*n = # pairs of samples analyzed

(B) Standard samples (NH_4^+-N) (Smart et al., 1981)
(3 samples analyzed for each measurement)

	TPN			TKN	
Concentration (μM)	Mean ($mg\text{-}L^{-1}$)	CV%	Concentration (μM)	Mean ($mg\text{-}L^{-1}$)	CV%
0.16	0.17	20.05	0.10	0.11	25.52
0.36	0.39	4.07	0.20	0.57	10.84
0.51	0.49	2.22	0.30	0.36	6.16
0.81	0.83	7.85	0.60	0.53	4.66
1.12	1.08	4.24	0.80	0.66	16.91
1.22	1.21	3.33	1.20	1.28	1.10
1.42	1.51	3.04	1.40	1.30	3.81
1.76	1.84	4.69	1.60	1.72	14.36
2.20	2.17	2.02	2.00	1.88	2.55
2.42	2.48	4.85	2.40	2.83	5.35

(C) Freshwater field samples. (Smart et al., 1981)
(3 samples analyzed for each measurement)

	TPN		TKN	
Sample Sites	Mean ($mg\text{-}L^{-1}$)	CV%	Mean ($mg\text{-}L^{-1}$)	CV%
Bear Creek above site	0.22	5.72	0.18	10.65
Silver Fork Creek	0.41	6.49	0.36	19.29
Mississippi River	0.80	6.22	0.55	5.79
Salt River	0.76	3.23	0.59	25.31
Hinkson Creek	0.69	4.46	0.61	9.90
Ted Shanks Marsh No. 8	1.05	2.28	0.61	25.25
Bear Creek Below Site	0.82	9.44	0.72	7.37
Ted Shanks Marsh No. 2	1.20	5.11	0.75	11.24
Cedar Lake	1.10	6.04	0.87	2.89
LeFevre Pond	4.83	6.88	4.39	9.49

APPENDIX E

RESULTS OF EPA AUDIT #WP481 PERFORMED BY CBL

A CHECK OF ACCURACY FOR DISSOLVED NITROGEN AND PHOSPHORUS

25. March 1987

Dr. Robert Magnien
Office of Environmental Programs
Water Management Administration
Dept. of Health and Mental Hygiene
201 W. Preston St.
Baltimore, Md. 21201

Dear Rob:

I am enclosing the results of quality control samples from EPA unknowns WP481 performed by CBL in conjunction with the February 1987 mainstem samples. The actual concentrations of these unknowns were kn only to myself and I had no part in the analyses.

Nutrient	CBL	EPA	95% C.I. report by EPA
Ammonia-N	0.281	0.28	0.23-0.3
Nitrate-N	0.142	0.14	0.11-0.17
Orthophosphate-P	0.045	0.05	0.04-0.06
Total Kjeldahl-N	0.34	0.32	0.18-0.48
*Alkaline Persulfate-N	0.311	--	---
Total-P	0.107	0.10	0.07-0.13
*Alkaline Persulfate-P	0.104	--	---

All concentrations are reported in mg/l

Alkaline persulfate N and P were also performed on these unknowns and the results are reported above. Again, in each case, the values obtained by the different methods are nearly identical.

These results will become part of our continuing QA/QC program for 1987. We are all very pleased with the results and should you have any questions, please call us at your convenience.

Sincerely yours,

Carl F. Zimmermann

cc: Dr. C.F. D'Elia
Mr. R. Batiuk
Ms. B. Fletcher
Nutrient Analytical Services file