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Methodological Comparisons for Nitrogen and Chlorophyll Determinations in Estuarine Water Samples

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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 (± 1.04) + TKN * 0.153 (± 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 ± 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.

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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

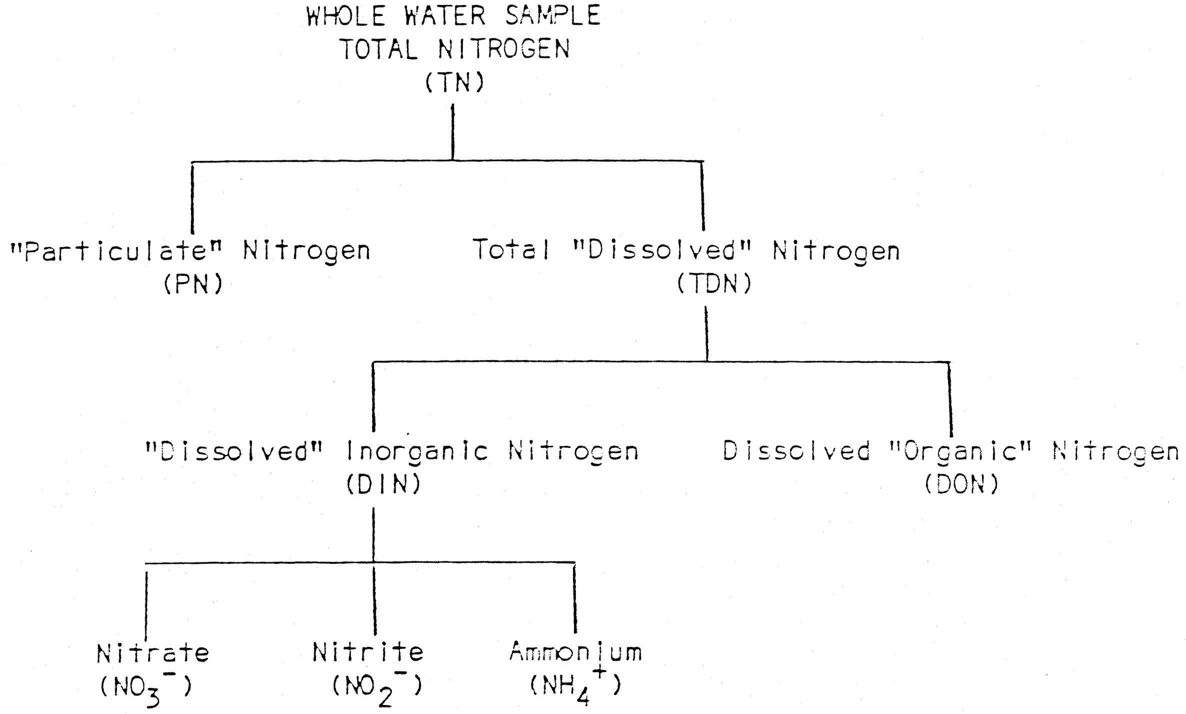
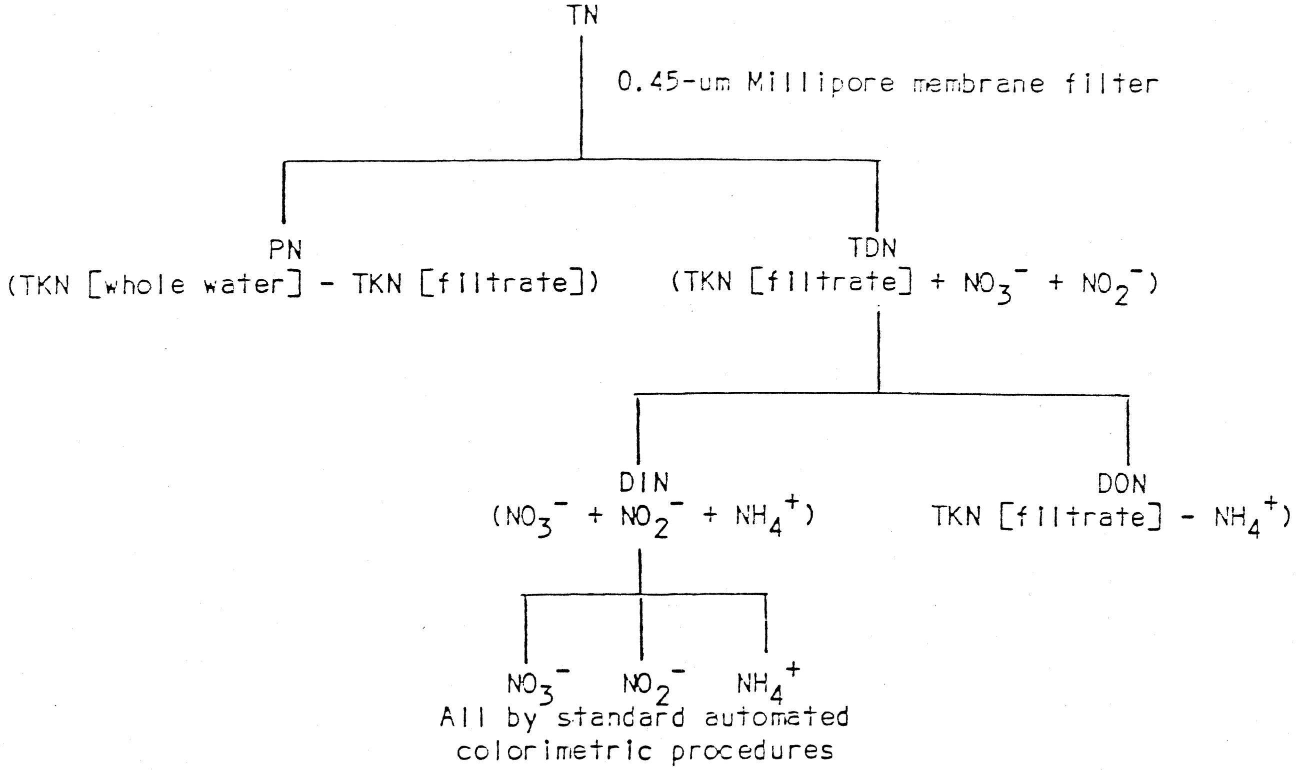


Figure 1-1. N fractions determined in typical water quality studies.

A. Standard EPA



B. Typical Oceanographic (CBL)

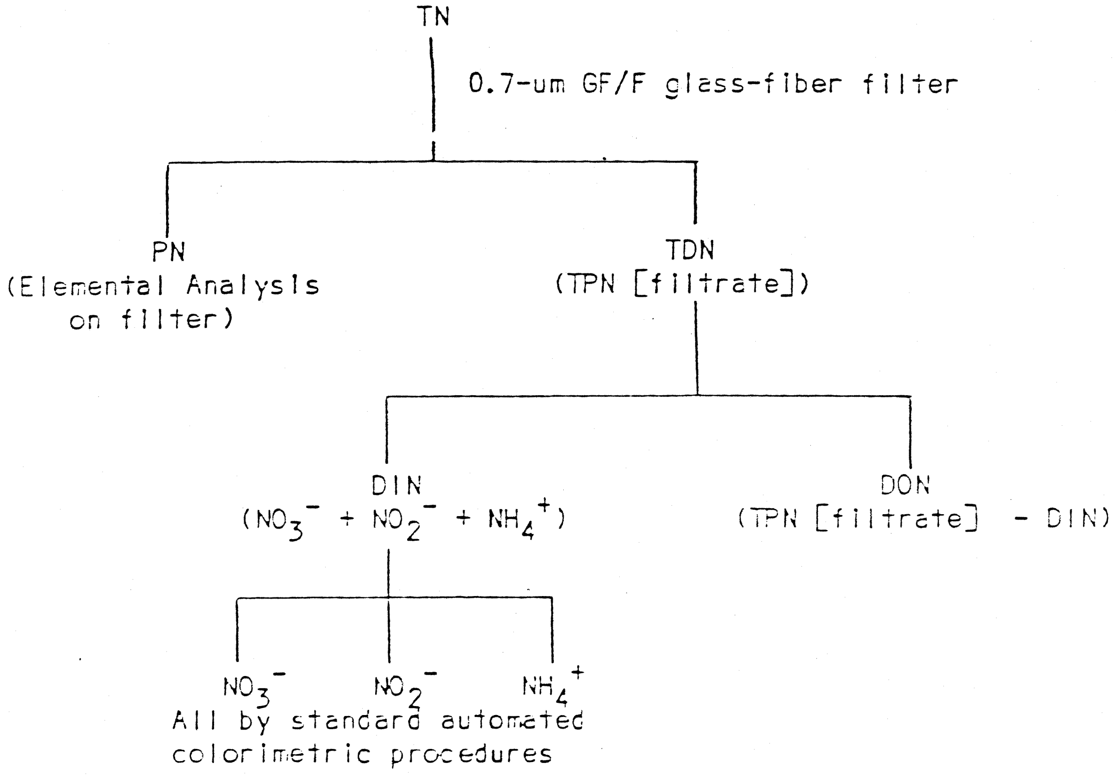


Figure 1-2. Comparison of standard EPA and typical oceanographic (CBL) nitrogen determinations.

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 $100^{o}C$ 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 $680^{o}C$ 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

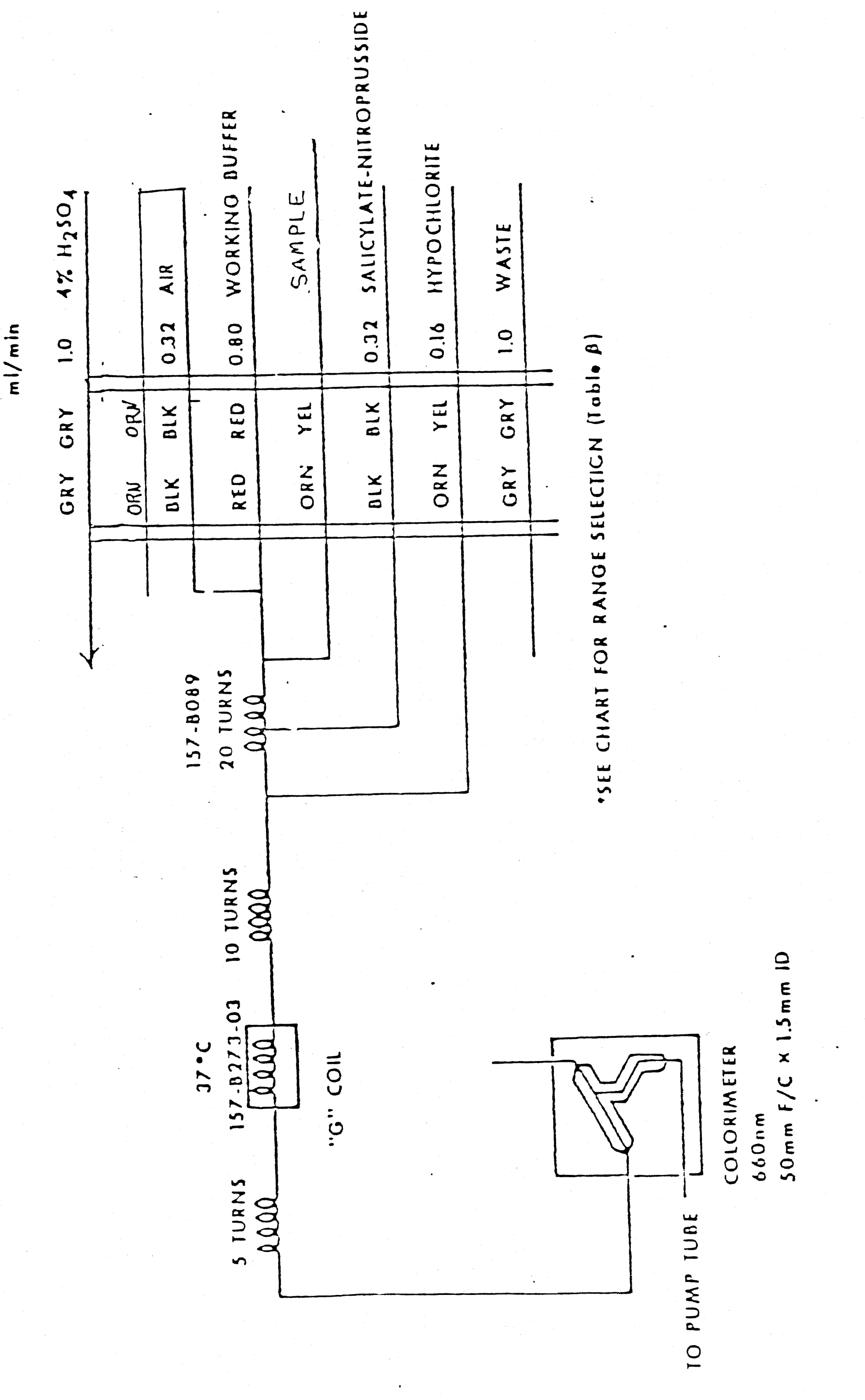


Figure 1-3. Ammonium manifold used in conjunction with block digestor for the semiautomated TKN procedure at EPA's Central Regional Laboratory in Annapolis.

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.

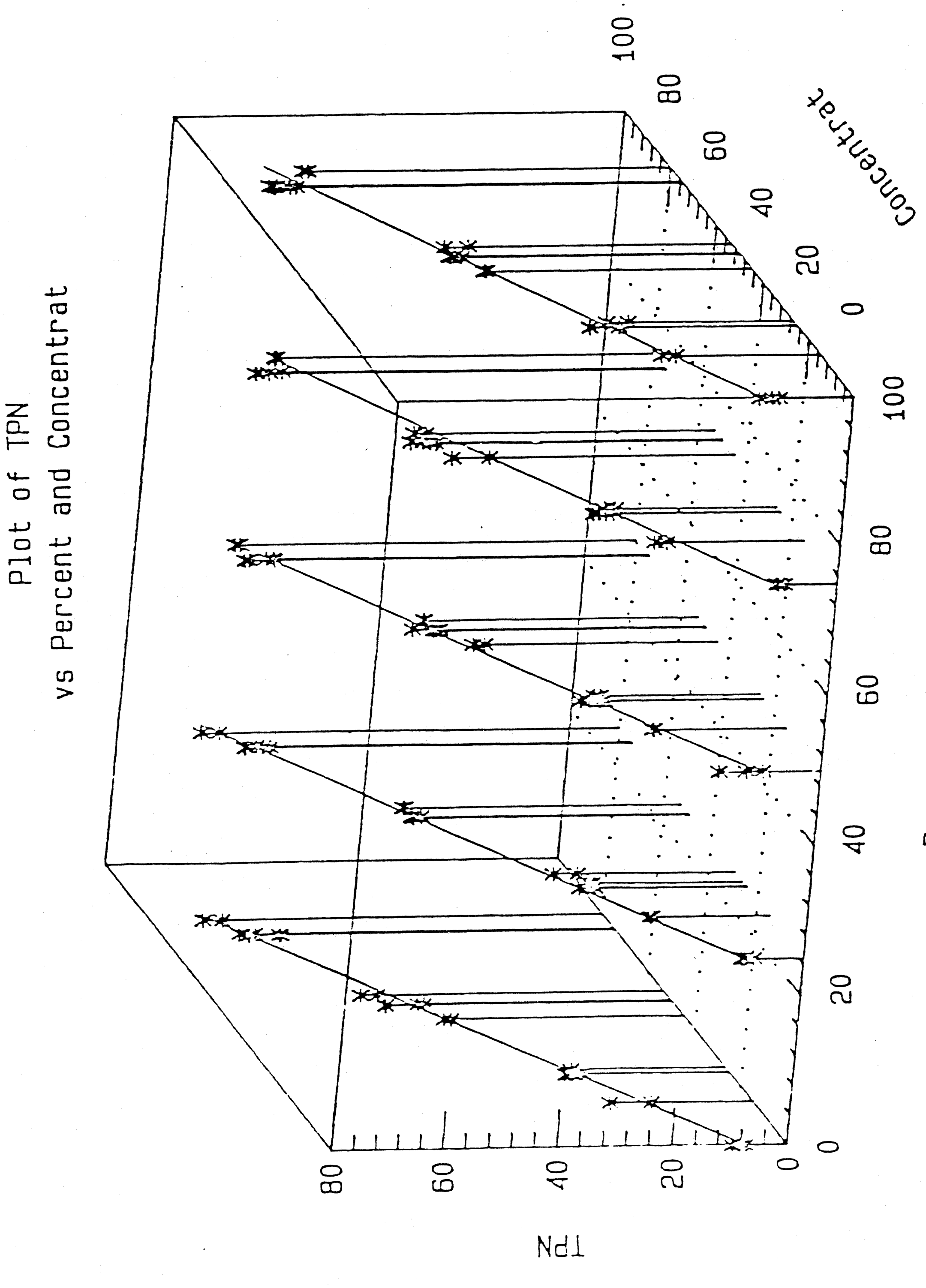


Figure 1-4. Three dimensional plot of TPN-determined concentration of standards (umol N L^{-1}) vs. percent seawater vs. expected concentration of spiked reference standards (umol N L^{-1}).

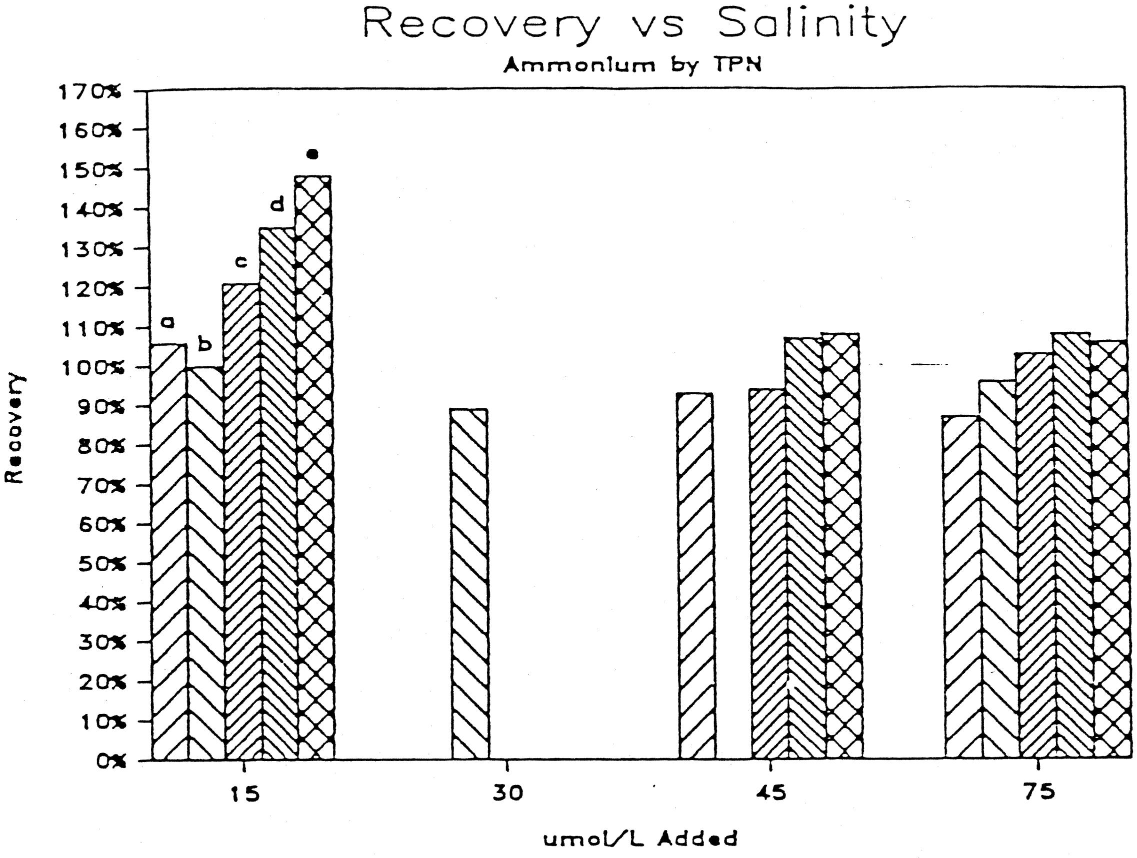


Figure 1-5a. Percent recovery by TPN method vs. concentration of ammonium (umol N L^{-1}) in different salinity water (a = 0% seawater, b = 25 % seawater, c = 50 % seawater, d = 75 % seawater, e = 100% seawater).

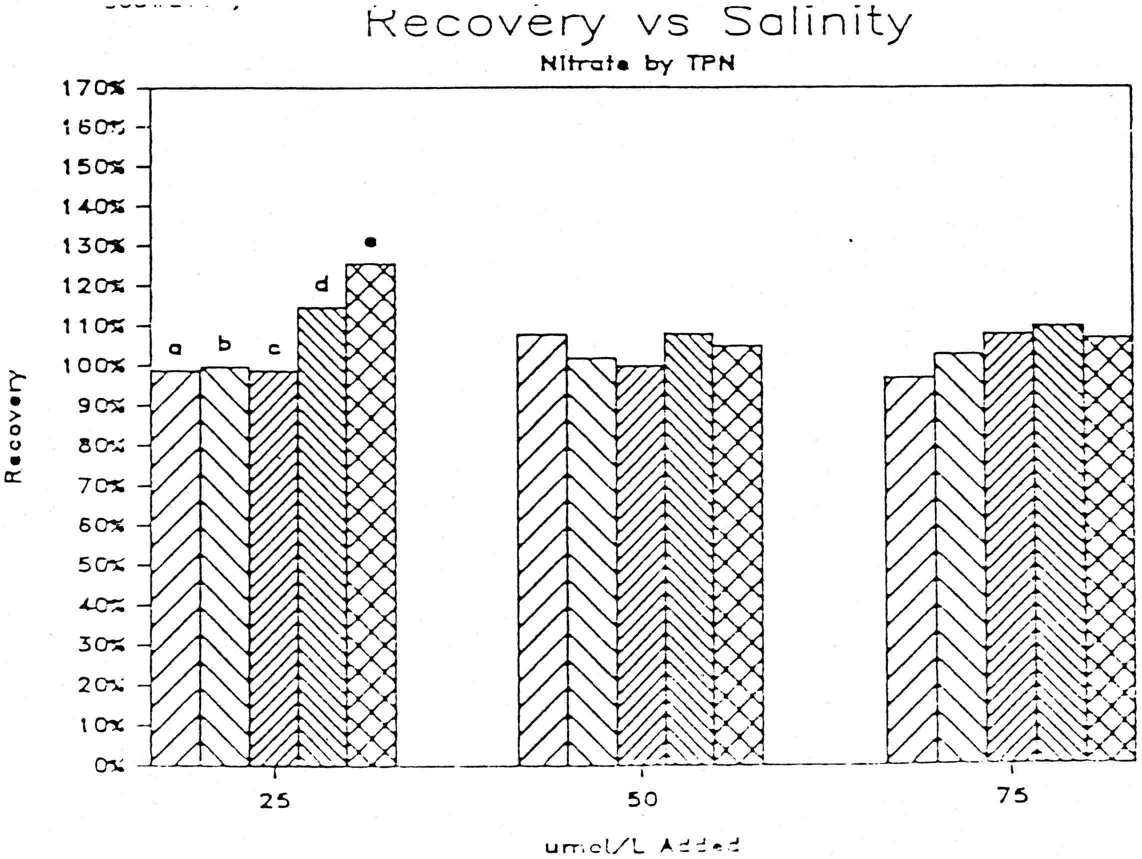


Figure 1-5b. Percent recovery by TPN method vs. concentration of nitrate (umol N L^{-1}) in different salinity water (a = 0% seawater, b = 25% seawater, c = 50 % seawater, d = 75 % seawater, e = 100 % seawater).

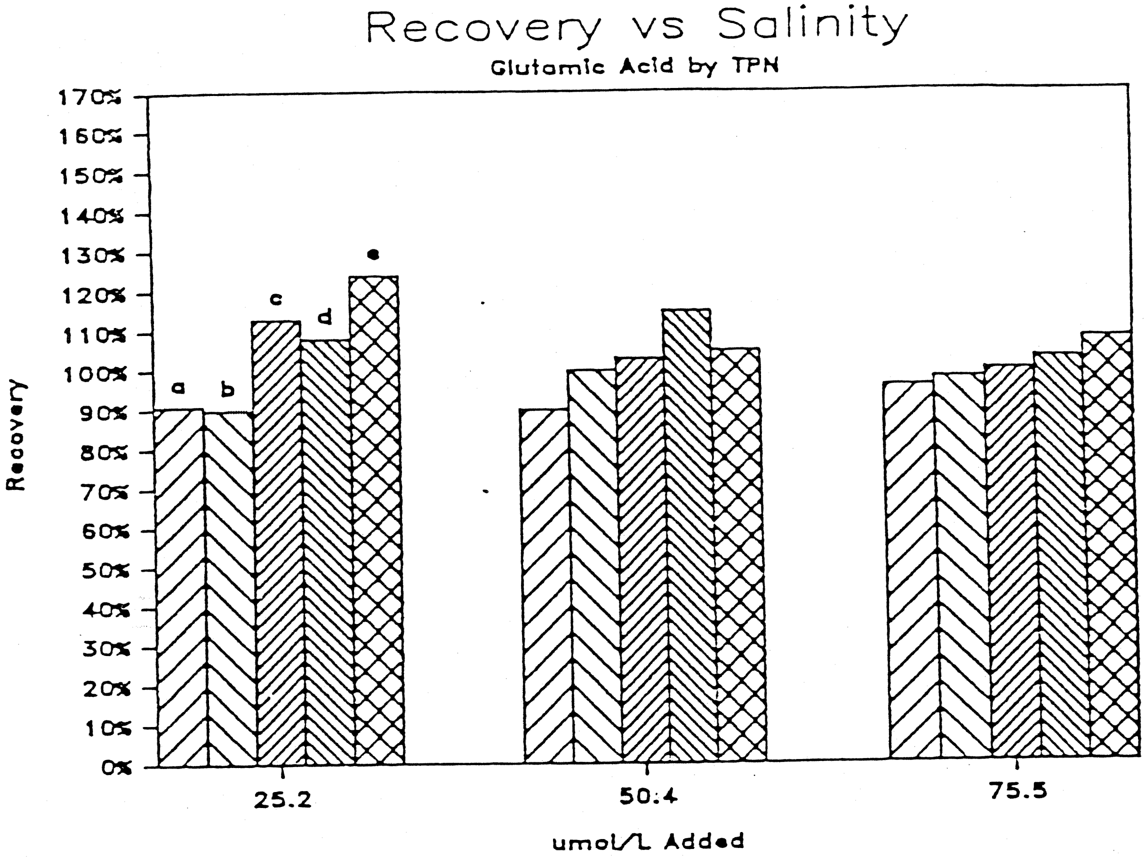


Figure 1-5c. Percent recovery by TPN method vs. concentration of glutamic acid (umol N L^{-1}) in different salinity water (a = 0% seawater, b = 25% seawater, c = 50 % seawater, d = 75% seawater, e = 100% seawater).

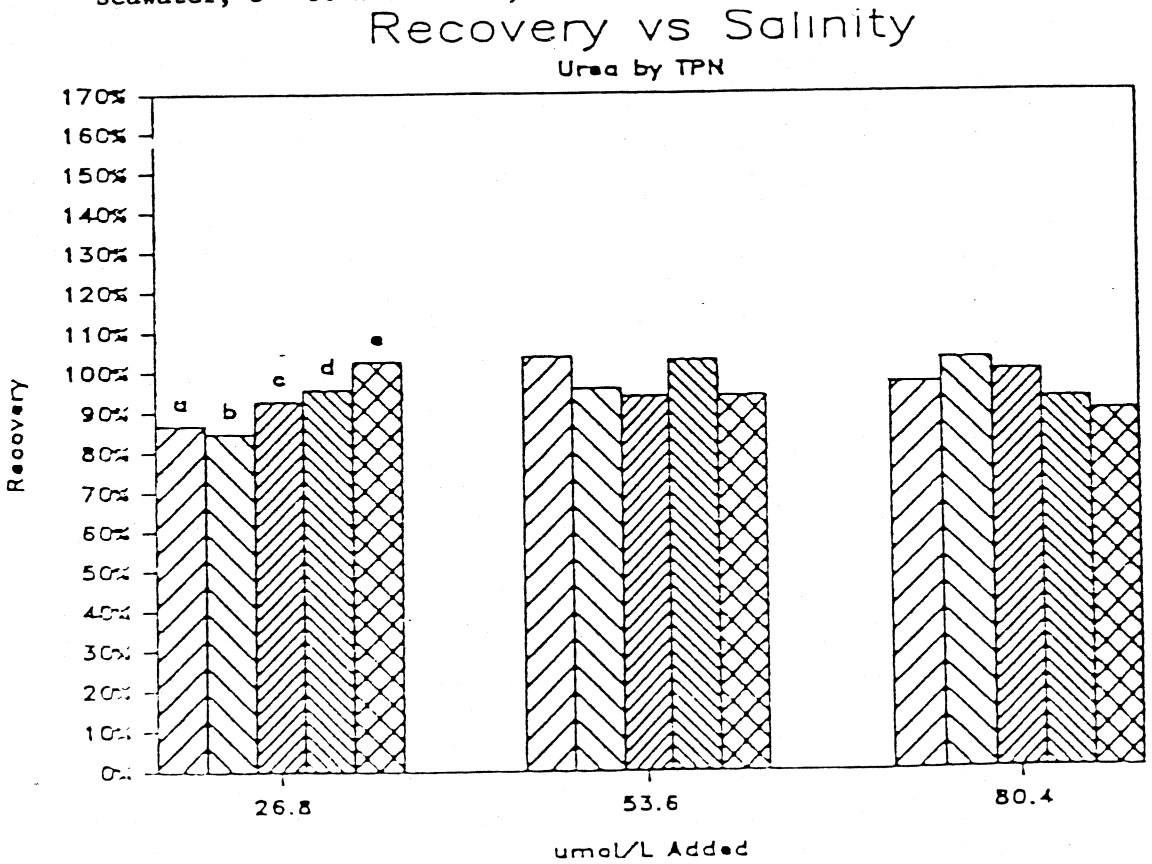


Figure 1-5d. Percent recovery by TPN method vs. concentration of urea (umol N L^{-1}) in different salinity water (a = 0% seawater, b = 25% seawater, c = 50% seawater, d = 75% seawater, e = 100% seawater).

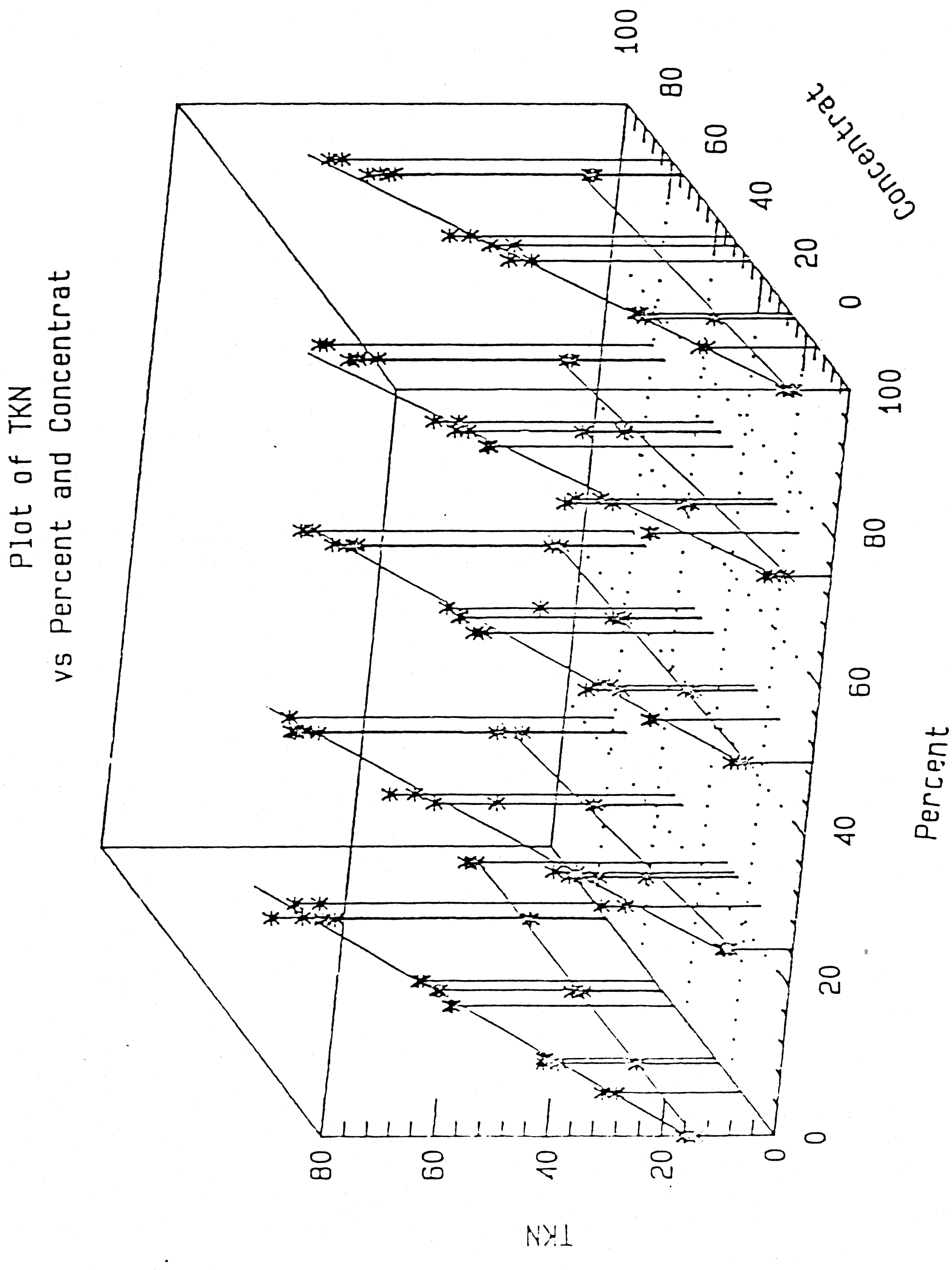


Figure 1-6. Three dimensional plot of TKN-determined concentration of standards (umol N L^{-1}) vs. percent seawater vs. expected concentration of spiked reference standards (umol N L^{-1}).

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 (Reay, 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: $TPN - NO23 = 21.79(\pm 1.04) + TKN*0.153$ (± 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

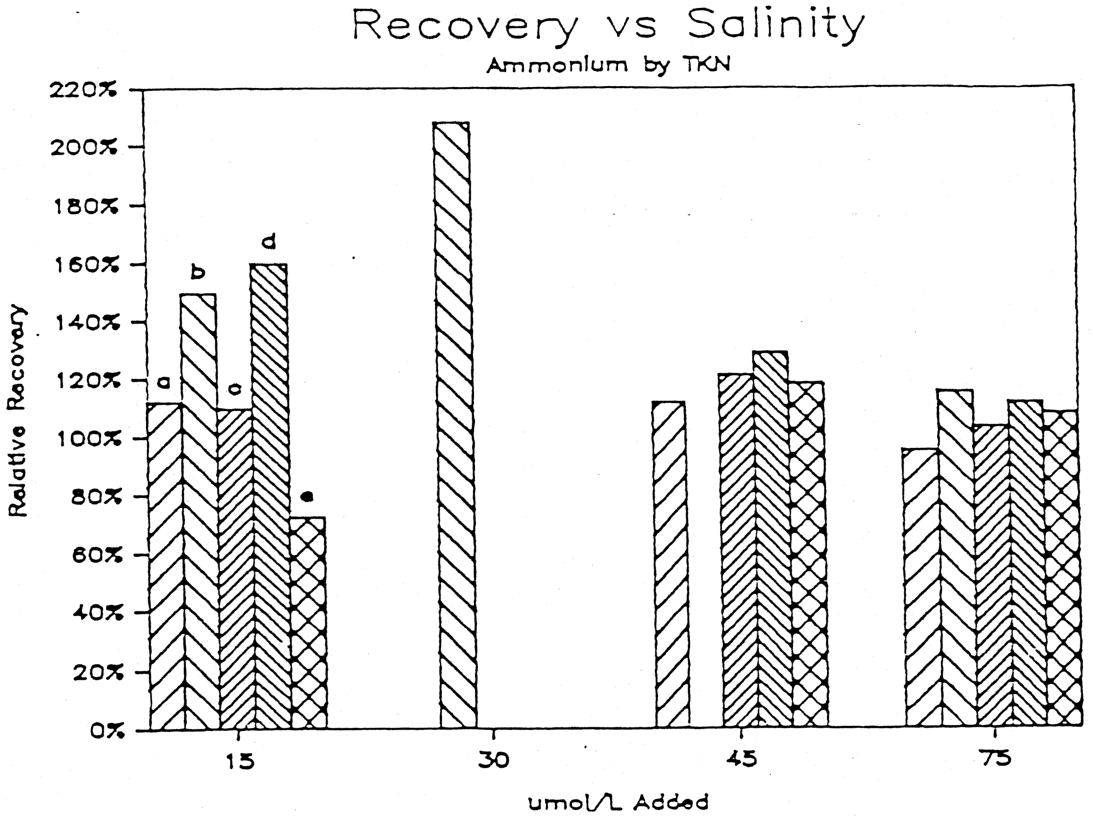


Figure 1-7a. Percent recovery by TKN method vs. concentration of ammonium (umol N L^{-1}) in different salinity water (a = 0% seawater, b = 25% seawater, c = 50% seawater, d = 75% seawater, e = 100% seawater).

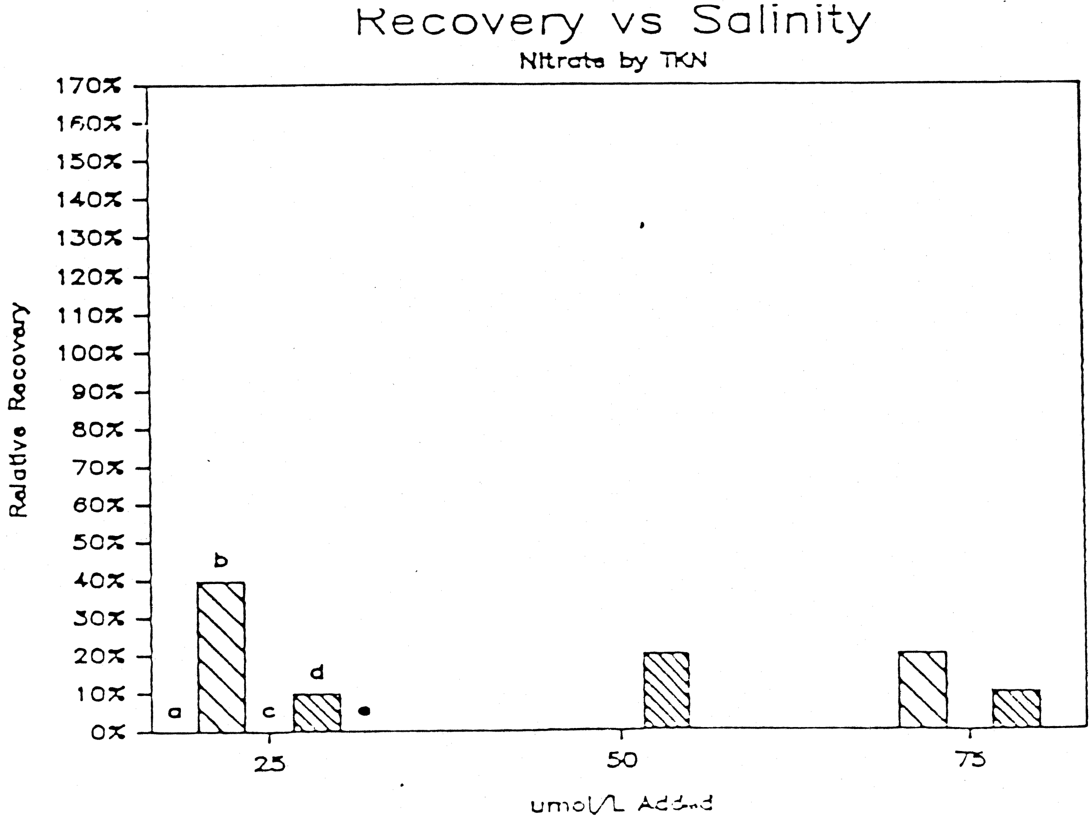


Figure 1-7b. Percent recovery by TKN method vs. concentration of nitrate (umol N L^{-1} in different salinity water (a = 0% seawater, b = 25% seawater, c = 50% seawater, d = 75% seawater, e = 100% seawater).

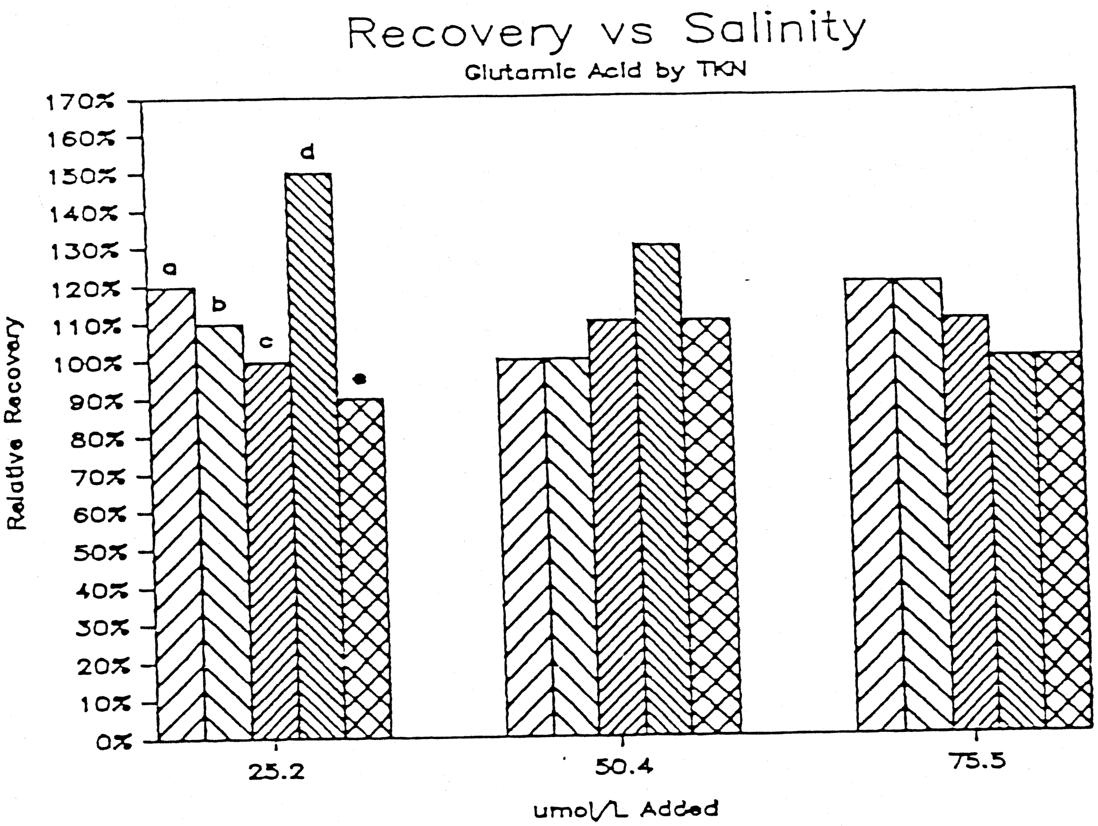


Figure 1-7c. Percent recovery by TKN method vs. concentration of glutamic acid (umol N L^{-1}) in different salinity water (a = 0% seawater, b = 25% seawater, c = 50% seawater, d = 75% seawater, e = 100% seawater).

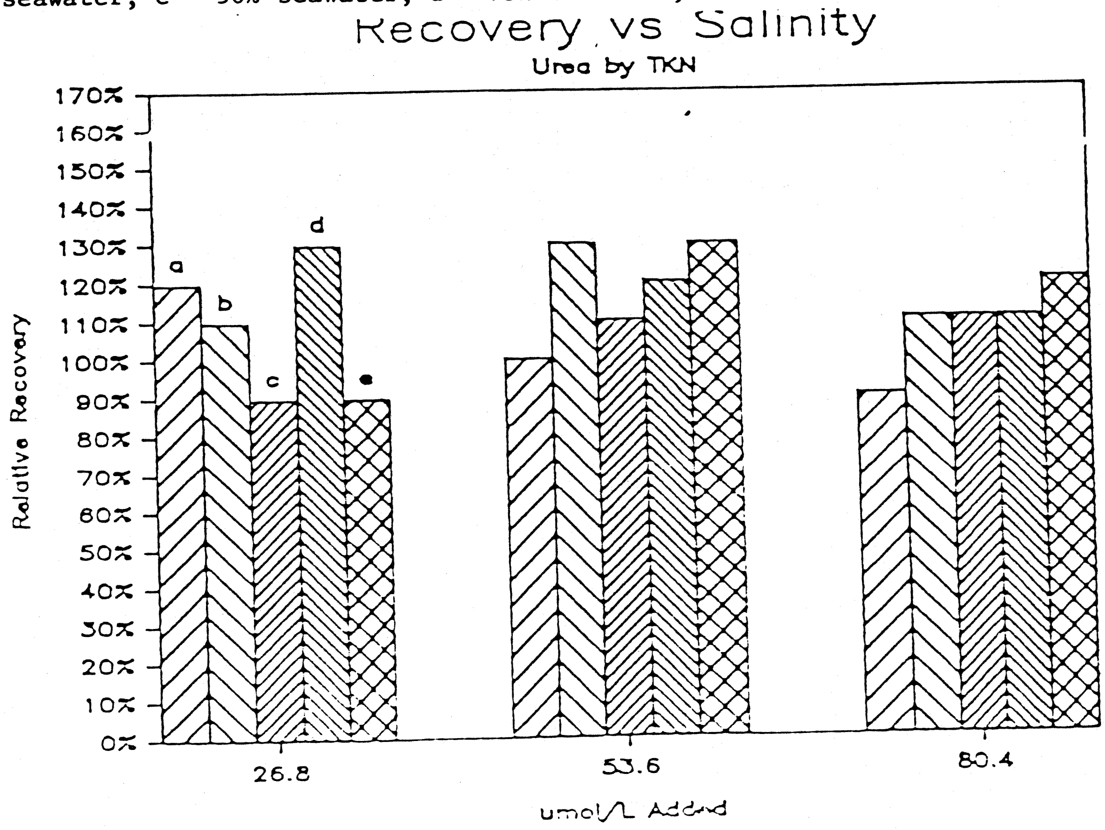


Figure 1-7d. Percent recovery by TKN method vs. concentration of urea (umol N L^{-1}) in different salinity water (a = 0% seawater, b = 25% seawater, c = 50% seawater, d = 75% seawater, e = 100% seawater).

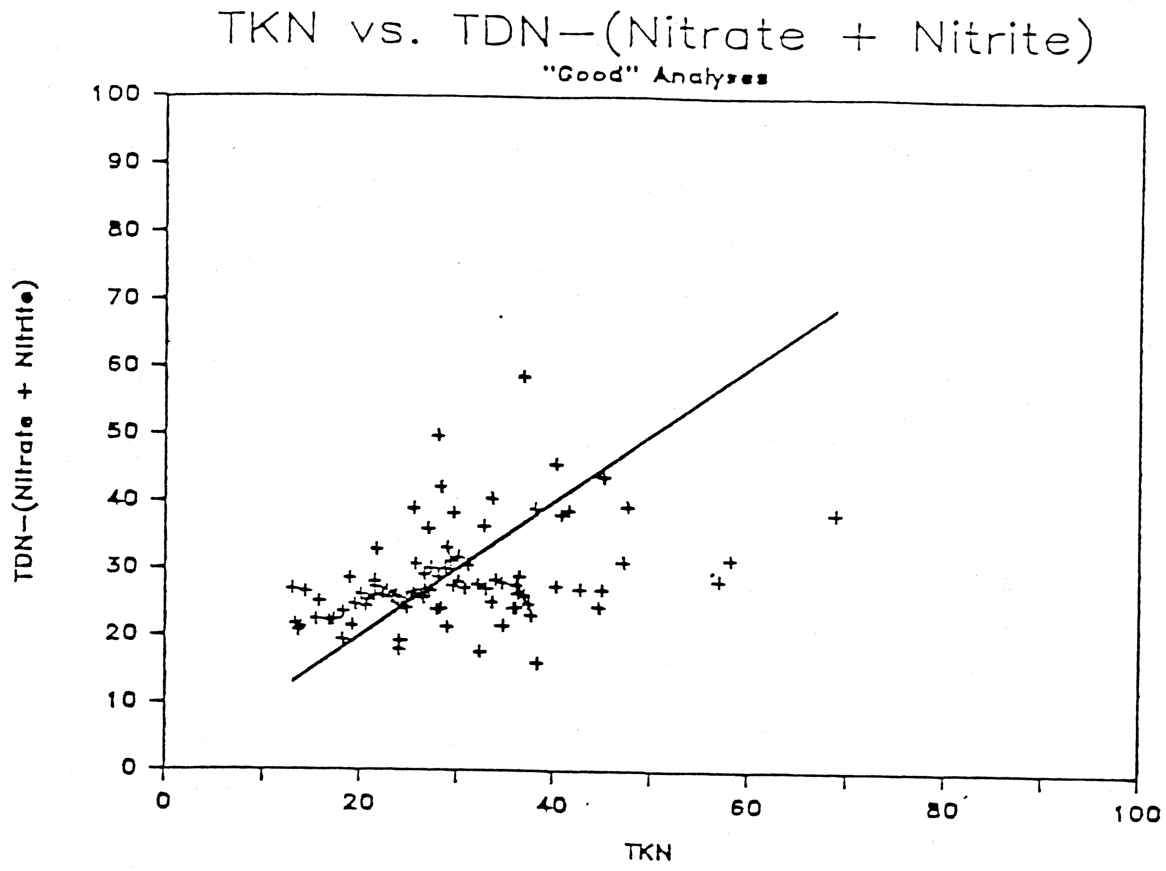


Figure 1-8a. TDN - (nitrate + nitrite) vs. TKN determinations of estuarine samples for analyses without bumping and splattering ("good" data).

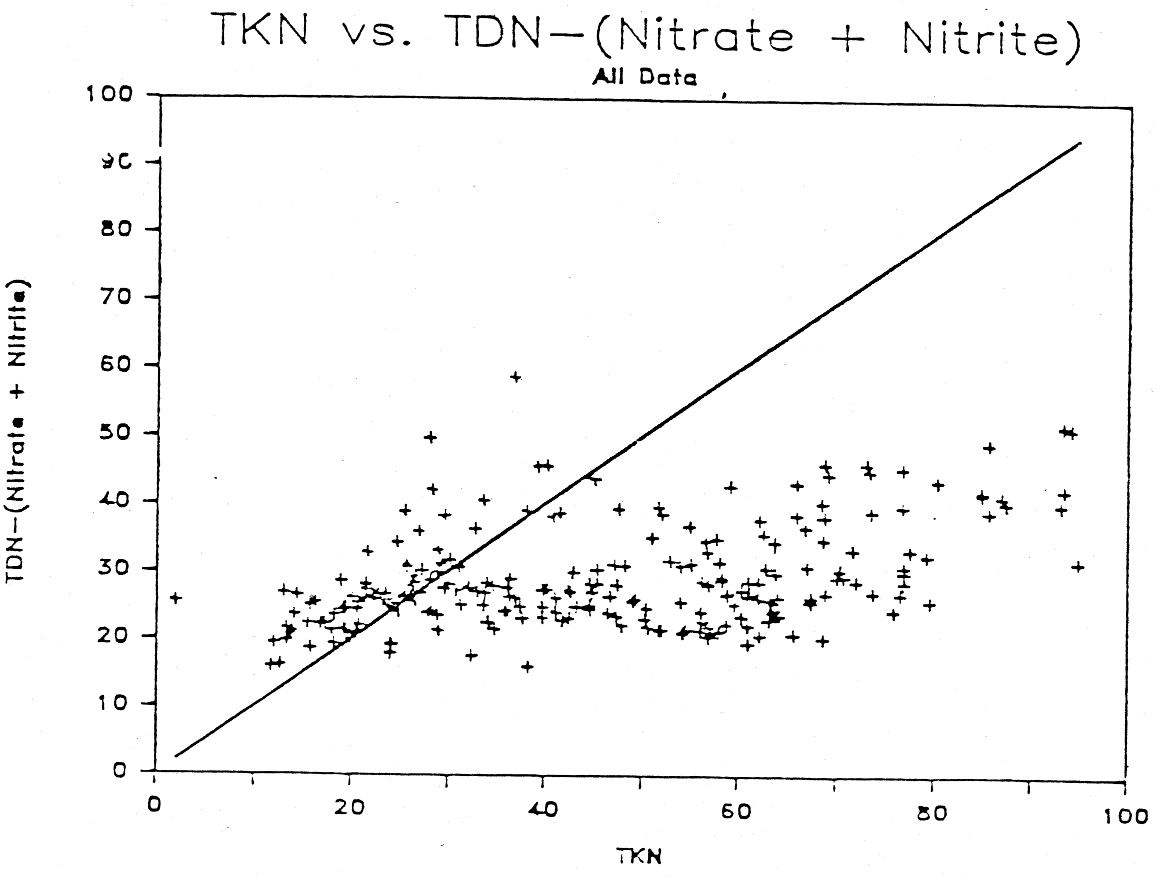


Figure 1-8b. TDN - (nitrate + nitrite) vs. TKN determinations of estuarine samples for all analyses preformed.

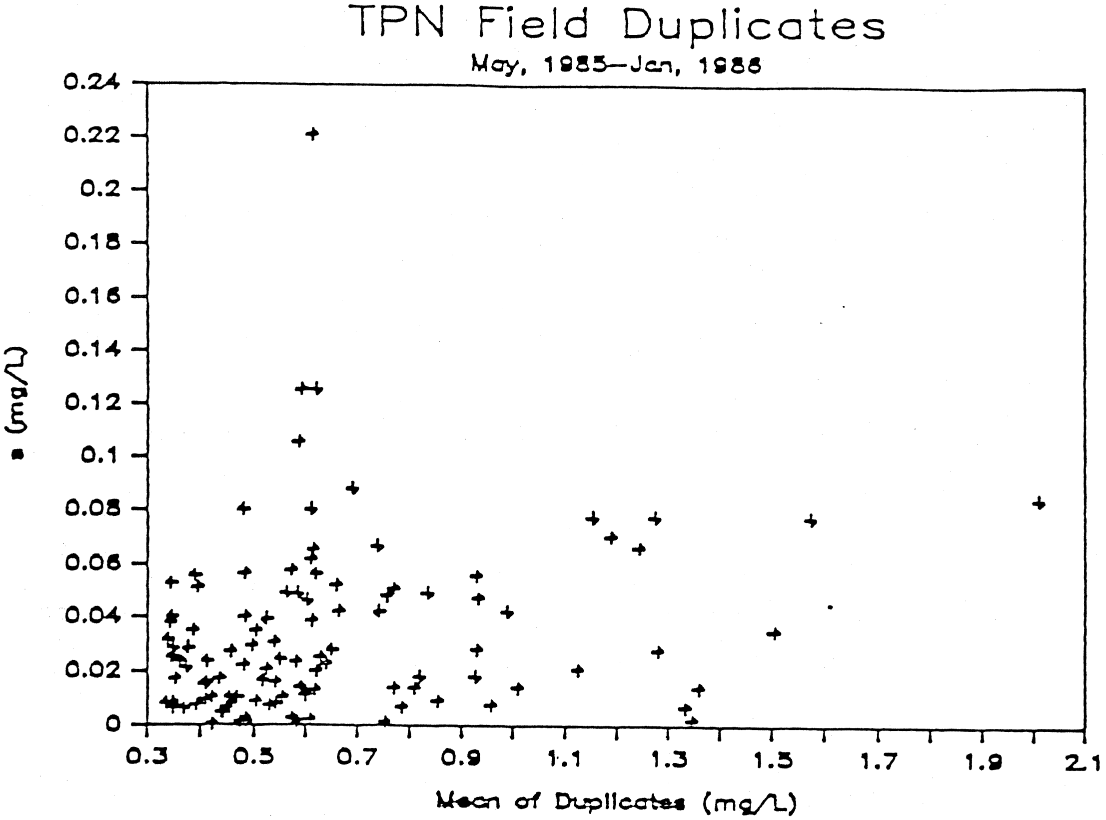


Figure 1-9a. Standard deviation of duplicates vs. mean concentration of field duplicates for bay-wide EPA-sponsored monitoring program.

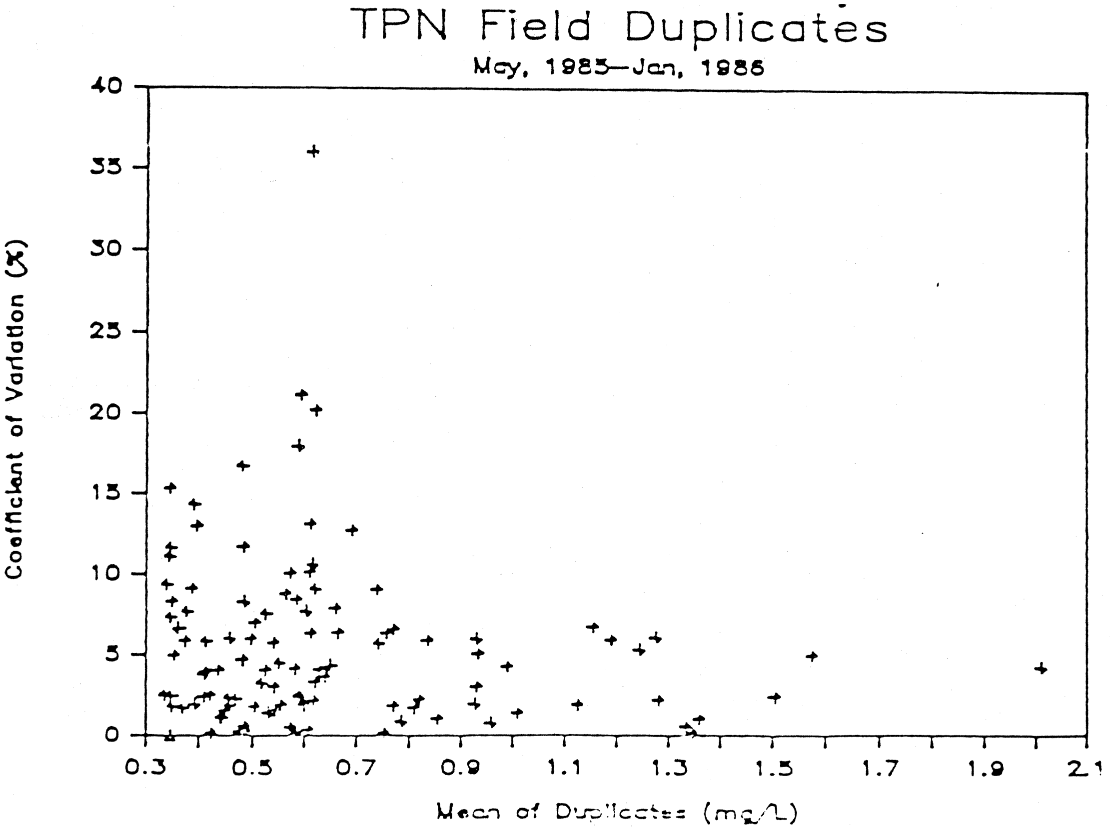


Figure 1-9b. Coefficient of variation vs. mean concentration of field duplicates for bay-wide EPA-sponsored monitoring program.

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, 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.

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

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			Holm-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

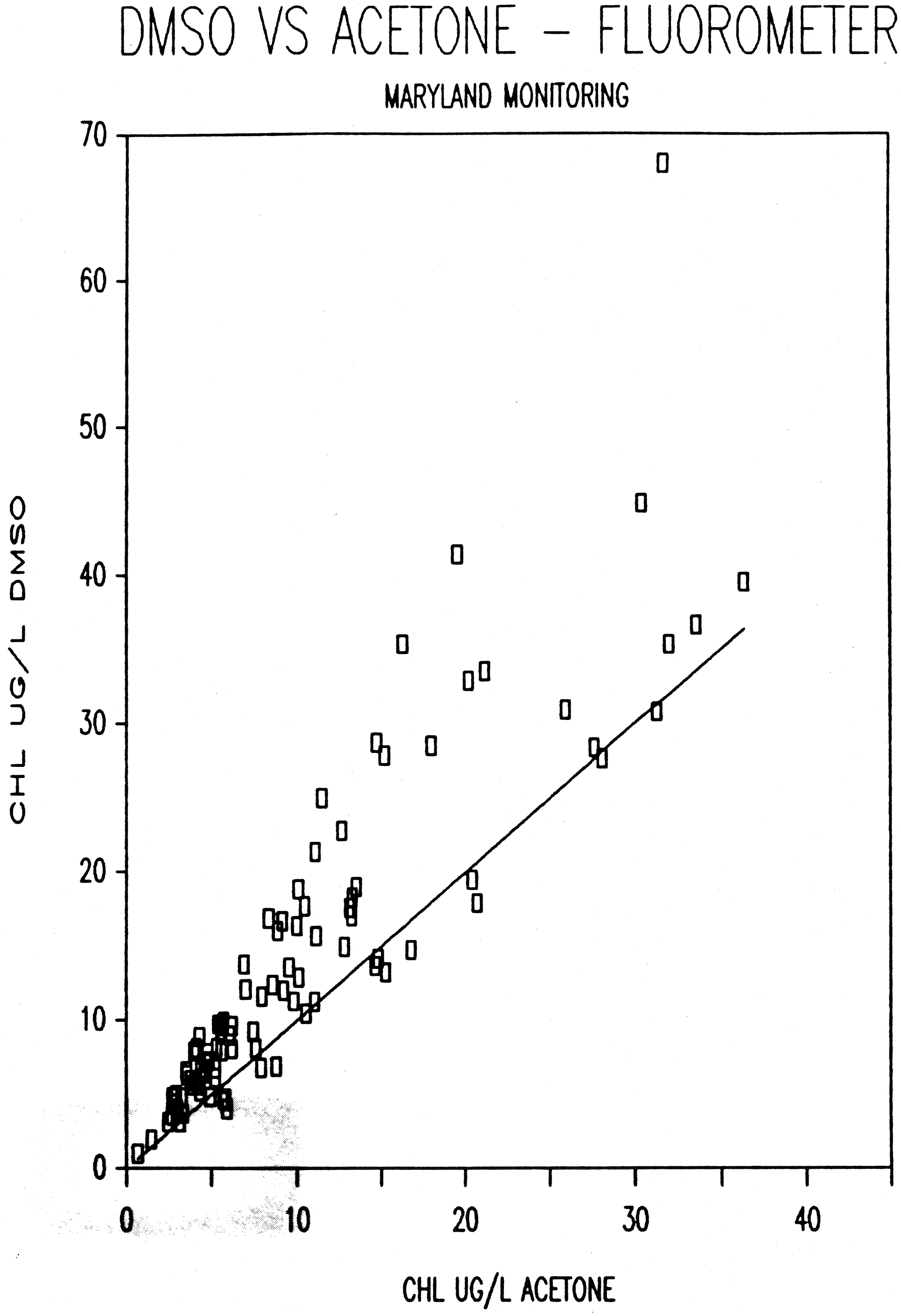


Figure II-1. Maryland EPA Monitoring Program Samples: CBL-DMSO extract measured by fluorometer compared to samples frozen and analyzed later at VIMS by grinding in acetone for extraction and fluorometer determination. Both data sets are calculated without phaeo-pigment corrections.

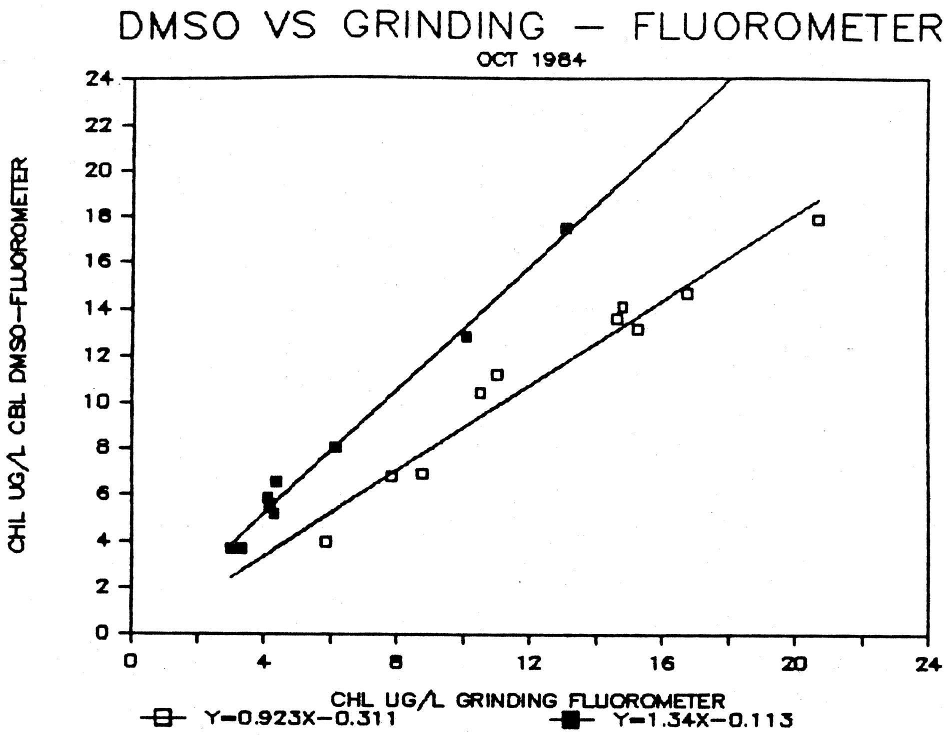


Figure II-2. October, 1984, Maryland samples frozen for two different times. Grinding fluorometric analysis using Turner Model 111 (□) frozen 5 months, (■) using Turner Designs, frozen 11.5 months.

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 $\mu g\ l^{-1}$.

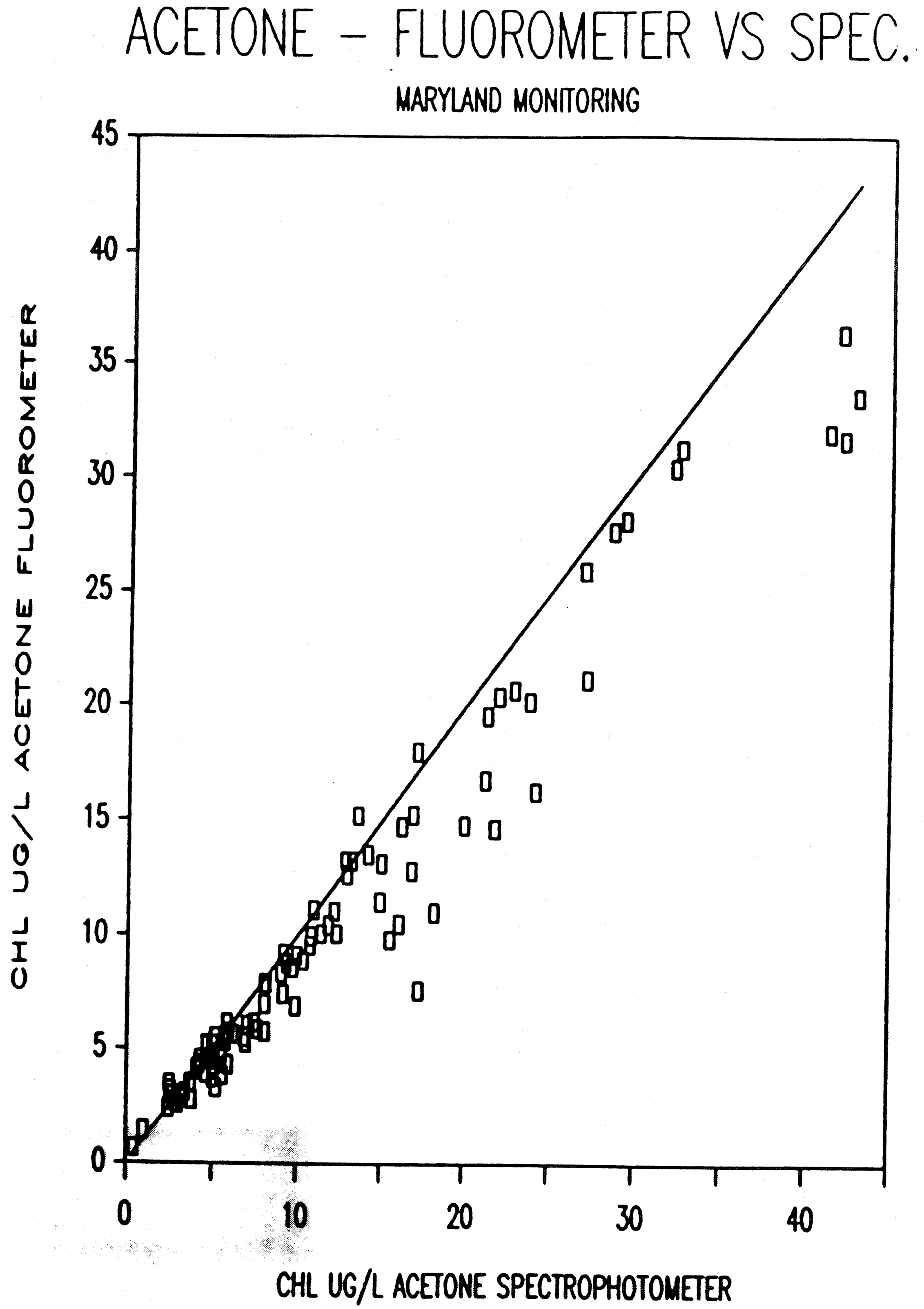


Figure II-3. Maryland EPA Monitoring Program Samples: samples frozen and analyzed later at VIMS by grinding in acetone for extraction and analyzed by fluorometer and spectrophotometer determination. Both data sets are calculated without phaeopigment corrections. The spectrophotometric data are calculated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

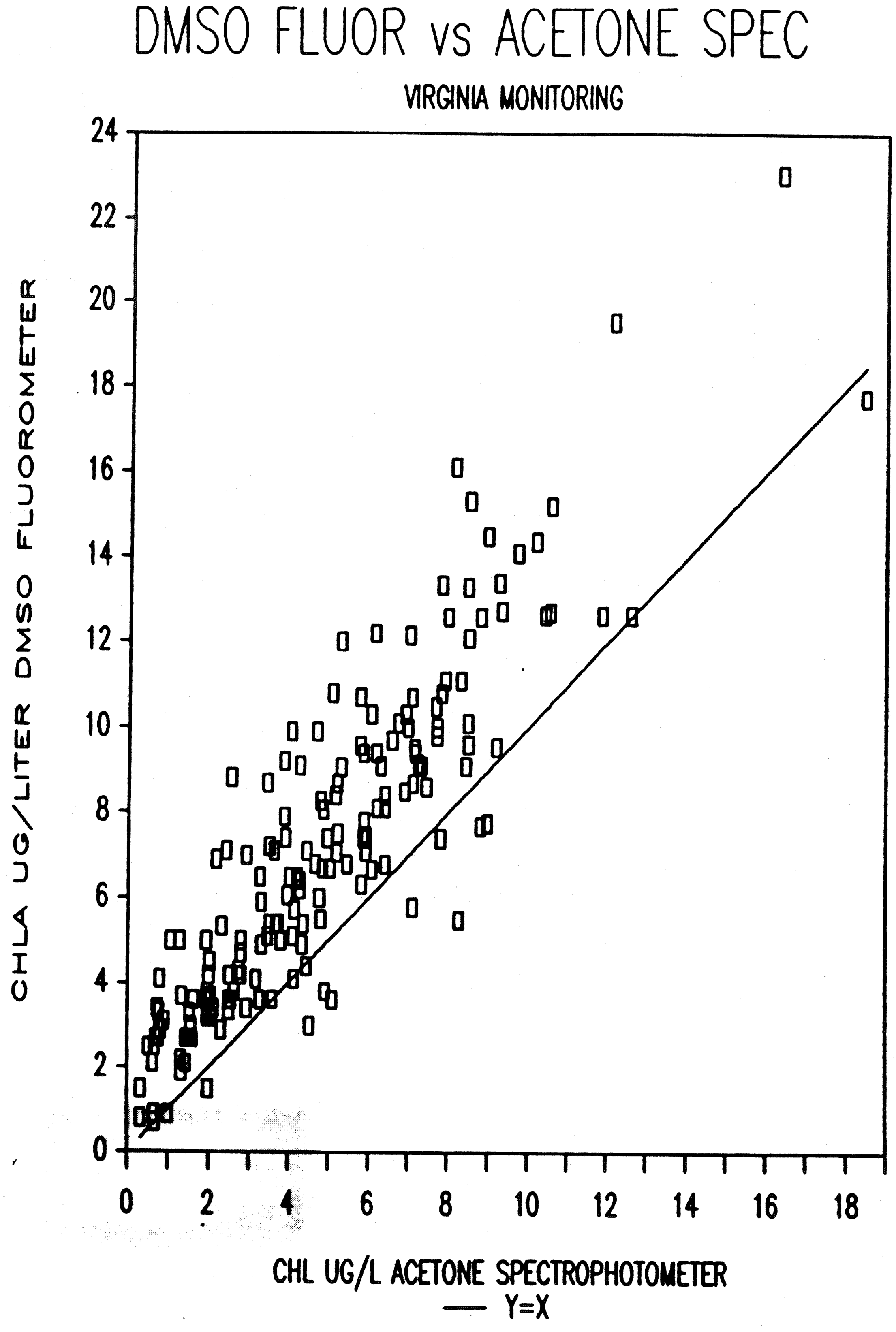


Figure II-4). Virginia EPA Chesapeake Bay Monitoring samples comparing freshly extracted by DMSO fluorometric determinations (means of pairs), with single 90% acteone extracts with grinding after freezing. The 90% acetone extracts were read on the spectrophotometer and calculated by the Jeffrey and Humphrey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

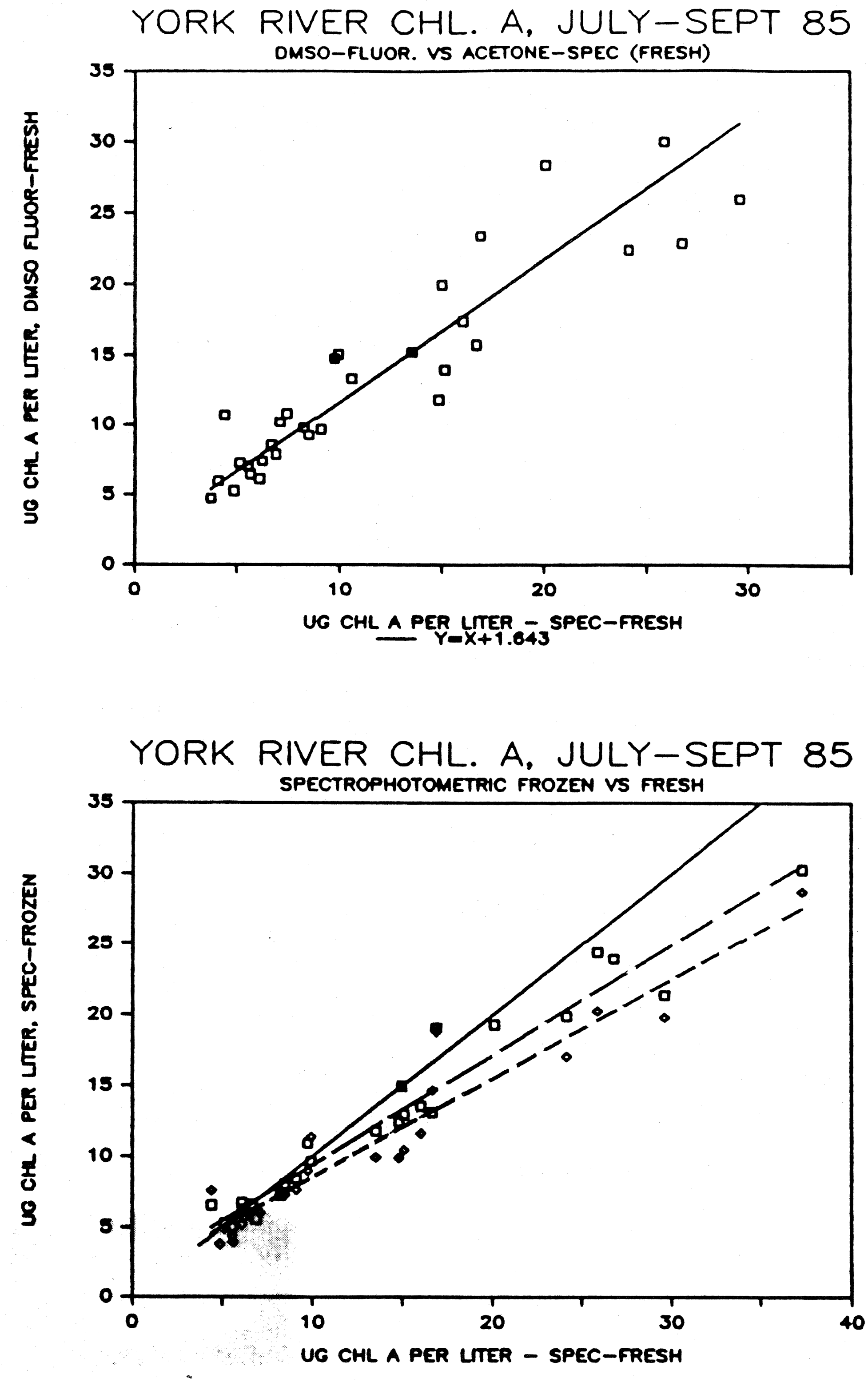


Figure II-5. The spectrophotometric data are calculated with the trichromatic equations of Jeffrey and Humphrey (1975) for chl a, b, and c. VIMS Coast Guard Pier samples, July-Sept 1985.

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

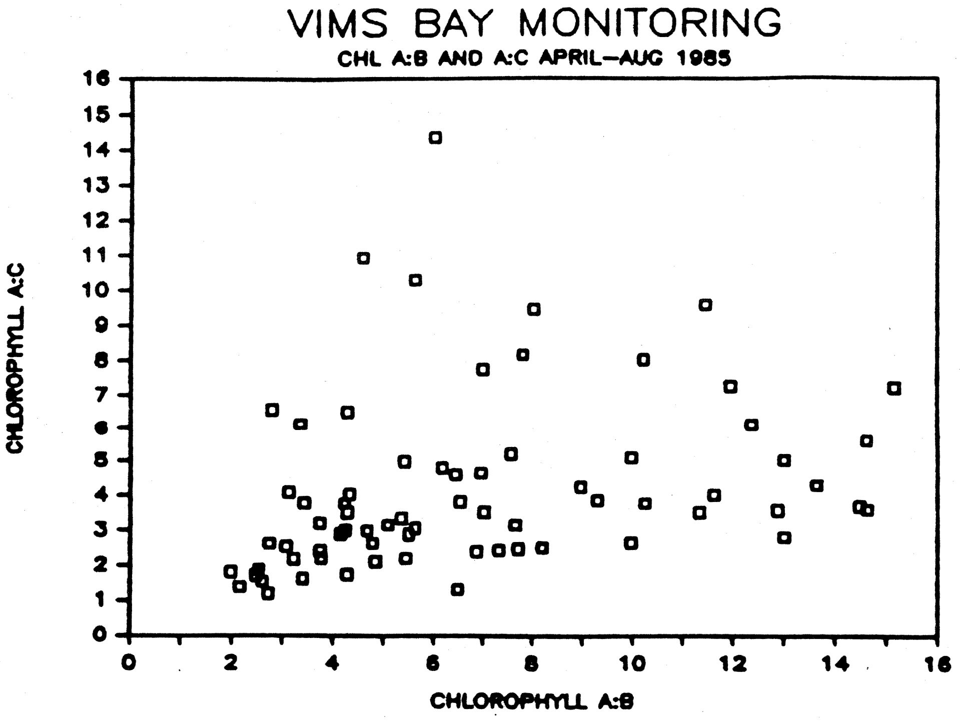


Figure II-6. The spectrophotometric data were calculated with the trichromatic equations of Jeffrey and Humphrey (1975) for chl a, b, and c and the values below 0.2ug/ml extract were deleted. The remaining values are plotted as a:b and a:c ratios.

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

Light Harvesting Pigments	Chlorophyll a	Chl b	Chl c1	Chl c2	Fucoxanthin	Peridinin	Phycobiliproteins	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.

URI
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 $^{o}/oo$) and filtered (precompusted Glass fiber-filters). The samples were then analysed using both Kjeldahl and Persulfate techniques.

		Total dissolved nitrogen (µg-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	Conc., µM	Recovery %	TPN PK ht	Conc., µM	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-L^{-1})	CV%	Concentration (μM)	Mean (mg-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 ($mg-L^{-1}$)	CV%	Mean ($mg-L^{-1}$)	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