**CHESAPEAKE BAY PROGRAM DATA ANALYSIS ISSUES TRACKING SYSTEM**

**ISSUE TRACKING NUMBER:** 050

**CATEGORY CODE:** Analytical Methods (AM)

**ISSUE TITLE:** Comparison of field preservation methods for ammonium analyzed at the Department of Health and Mental Hygiene

**DATE OF INTRODUCTION OF THIS TO THE SYSTEM:** April 2011

**STATEMENT OF ISSUE:**
The procedures used by the Maryland Department of Natural Resources (DNR) for preserving non-tidal dissolved ammonium (NH4) samples do not conform to the EPA Region 3 policy for ambient surface water monitoring, which is to follow Clean Water Act requirements. According to Table 2 (Required Containers, Preservation Techniques, and Holding Times) in Title 40 Code of Federal Regulations Part 136, "Guidelines Establishing Test Procedures for the Analysis of Pollutants under the Clean Water Act", NH4 samples are required to be preserved by cooling to $\leq 6^{\circ}C$ and acidifying with sulfuric acid ($H_2SO_4$) to pH<2 (40 CFR, Part 136).

Rather than using acid preservation as specified by Title 40, samples are preserved either by icing them at $\leq 6^{\circ}C$ for short holding times or by freezing at $-20^{\circ}C$ for longer holding times. The regulation prohibits freezing of samples "unless data demonstrating that sample freezing does not adversely impact sample integrity is maintained on file and accepted by the regulatory authority", i.e., EPA Region 3. In 2010, DNR collected data to evaluate the impact of their preservation techniques on sample integrity.

DNR's Annapolis Field Office staff collects water samples at 55 non-tidal fresh water stations as part of the CORE/Trend monitoring program (Appendix A) and at 13 non-tidal fresh water stations as part of the non-tidal network (Appendix B) (Although there are 55 historical CORE/Trend, non-tidal stations one of these stations is no longer included in the analyses that are performed under the CORE/Trend program, so there were 54 candidate sites included in this study.) Funding for the CORE/Trend program and the non-tidal network is provided by the U.S. Environmental Protection Agency under Sections 106 and 117 of the Clean Water Act and the results and recommendations described herein only apply to samples collected under those two grants.

DNR has monitored non-tidal fresh water streams in Maryland since the early 1970s in response to the Clean Water Act. The original DNR lab that analyzed samples for NH4 and other nutrients implemented preservation methods based on refrigeration, since samples were analyzed within 24 hours. Subsequently, the Maryland Department of Health and Mental Hygiene (DHMH) laboratory, which assumed responsibility for laboratory work continued this practice as the monitoring program expanded. As a result, field office staff preserved samples by keeping them on ice as opposed to preserving them with acid. In addition, several parameters analyzed under the monitoring programs have a maximum holding time of 48 hours and are delivered to the lab

within 24 hours, so preservation by acidification did not seem warranted.

Field office staff adopted freezing as an option for preserving samples collected under the non-tidal network program following a study by the Chesapeake Biological Laboratory (CBL) in Solomons, MD that demonstrated there were no differences in the results between freezing samples and preserving them with acid (CBL used estuarine samples in their study, so their results may not be applicable to fresh water systems.) Freezing is occasionally used for the non-tidal network stations, because sample collection is not always pre-planned, which occasionally makes it impossible to deliver samples to the lab within 24 hours of collection (e.g., storm event samples may be collected during Fridays and weekends when it would not be possible to promptly deliver samples to the lab). Because they assumed that the refrigeration/freezing preservation methods also applied to fresh water samples, DNR field office staff never adopted the requirement described in 40 CFR Part 136 regarding acid preservation of NH4.

**PROPOSED SOLUTION:**
Three lines of evidence were pursued to test for differences in the concentration of NH4 based on the three preservation methods studied in this report: 1) ≤6°C for up to 24 hrs, 2) freezing, and 3) acidification. The first part of the study involved analyzing data collected from a subset of the 55 CORE/Trend stations that would provide a cross section of low to high NH4 concentrations. The second part of the study involved comparing the NH4 concentrations of de-ionized (DI) water that was used by field staff to clean sampling equipment. In the final part of the study, laboratory analytical results were compared to the concentrations of prepared known low and known high samples of NH4. All statistical analyses were performed and all graphs were prepared using SAS® software. All samples were analyzed by DHMH using the phenate method (EPA 350.1).

**Samples Analyzed**
*CORE/Trend Samples*
Ambient water quality samples were collected at five fresh water non-tidal stations in the CORE/Trend network per day on November 29th, November 30th and December 1st for a total of 15 stations (Data from a minimum of 15 stations were required to obtain sufficient power to statistically discern differences among the preservation methods.) The 15 stations were selected on the basis of providing a range of concentrations to ensure that differences among preservation methods were not a function of NH4 concentration. Using 15 stations also provided a range of environmental conditions. The range of NH4 in historical CORE/Trend data (1986-2009) was used as the basis for selecting the 15 stations used in this study.

To ensure that a suitable range of concentrations was obtained, the CORE/Trend data were first summarized across stations, by month and then across the months, by station to test for seasonal and spatial variability, respectively. The box plots of the natural log of NH4 plotted against month in Figure 1 and by station in Figure 2 show there is more spatial variability than seasonal variability. The comparison of seasonal and spatial variability indicated that there was sufficient variability among stations to obtain a wide range in concentrations regardless of the season.

The 15 stations used in this study were selected by first calculating the mean of the natural log of NH4 by station across the months and years of data. The station means were then ranked from 1 to 54 and blocked into 15 groups of three or four based on the similarity of the mean concentration. With the exception of block one, field office staff selected one station from each block to be included in the study. The three stations in block one were deemed unacceptable by the field office because they are located in western Maryland, which would have precluded getting the iced samples to DHMH in a timely manner. As a result, two stations were selected from block two.

One field crew consisting of two individuals collected and field-processed samples at the CORE/Trend stations over the three day sampling period. A three day time period was used to allow DHMH sufficient time to process the samples, while not introducing a "time effect" into the analysis. The field crew collected a carboy of water at each station, which was filtered and sub-sampled for each of the three preservation methods (iced, frozen, and acidified). The sub-samples for each preservation method and station (replicate 1 and replicate 2) allowed replicate variability (field plus laboratory) to be examined.

*De-ionized Water Samples*

De-ionized (DI) water is used by the field office to rinse filtration equipment before use, after use, and between samples. DI water is also used to rinse filter pads during filtration and as a final rinse after acid washing equipment. DI water is generated from tap water using a Thermo Scientific DIamond TII RO/DI system. Feed water is first run through a General Electric Smart Water external carbon pre-filter before entering the reverse osmosis system.

One iced, one frozen, and one acidified de-ionized water sample were generated each sampling day for a total of nine samples. Replicates of the DI treatments were not made. Analysis of DI water treatments provided a control for possible contamination of the field samples.

DHMH also analyzed blanks on each day samples were run in the laboratory. Iced samples, which had to be analyzed within 24 hours, were analyzed on 30 November, and on 1 and 2 December 2010. Frozen and acid-preserved samples were analyzed on 16 December 2010. The blank data for the field and DHMH are presented in Table 1 at the end of this report.

*Known Concentration Samples*

A comparison of field samples would have indicated whether or not there were differences among the methods and the magnitude of the differences, but would not have shown which method or methods produced the most accurate results. In order to test preservation methods for accuracy, CBL created two carboys with a known low and a known high concentration of NH4. The carboys were picked up at CBL by field office staff which created sub-samples that were preserved by each method with replication.

**Sample Treatment**

*Iced Samples*

Iced samples were put on ice immediately after they were filtered and sub-set. Depending on time-of-day considerations, the iced samples

were either delivered to DHMH by overnight courier, or delivered by field office staff early in the morning the day after the samples were collected. DHMH analyzed the iced samples within 24 hours of the collection time.

*Acid-preserved Samples*

A 95% concentrated 36 normal (gram equivalent weight of a solute per liter of solution) solution of sulfuric acid was added to the samples for acid preservation after they were sub-set. Of the 37 acid-preserved samples analyzed by the lab (30 field samples, 3 de-ionized water blanks, and 4 known concentration samples) 35 had three drops of acid added, one had four drops of acid added, and one had five drops of acid added. The amount of acid added was determined in the field by testing the pH of each 100 milliliter sample bottle with a strip of litmus paper to ensure that each sample pH was 2 or less. As a result the maximum amount of acid solution added to a sample was 0.25 milliliters, assuming one drop is approximately 0.05 milliliters. The acid-preserved samples were then placed in a cooler with ice and taken back to the field office.

After returning to the field office, the acid-preserved samples were stored in a refrigerator that is kept at approximately 2°C and then delivered to DHMH at the end of the week, which is the normal practice for the field office staff. The acidified samples were stored at DHMH at $\leq$6°C and analyzed 14 days after being received. Rather than neutralizing acidified samples prior to analysis, the standards, reagents, and carrier were acidified to match the pH of the samples i.e., DHMH matched the matrix of the samples.

*Frozen Samples*

The frozen samples were placed in a cooler along with the iced samples and brought back to the field office where they were stored in a freezer which is kept at approximately -20°C. The frozen samples were delivered to DHMH along with the acidified samples at the end of the week. Frozen samples were analyzed by DHMH 14 days after they were received, the typical holding time for frozen samples.

**DISCUSSION:**

*CORE/Trend Samples*

The results for the CORE/Trend stations are presented graphically in Figure 3. Twelve of the 15 stations sampled had concentrations of NH4 that were ranked in the same order. Acid-preserved NH4 samples were biased low in comparison to both frozen and iced samples and frozen samples exceeded iced. Acid-preserved samples appear to be an exact match with frozen and iced at two stations (DER0015 and GWN0115); however, the concentrations of NH4 at those stations are very low. Concentrations of acid-preserved samples were well below those of the frozen and iced samples at CAC0148 and MON0155. The larger bias associated with CAC0148 and MON0155 compared to the other stations and the lack of consistency in the bias associated with the acid-preserved samples appears to indicate that the problem may be associated with the acid-preserved samples and not frozen or iced.

The data were also analyzed statistically using SAS® PROC GLM, a General Linear Model. The model included two direct effects (StationName and PreservationMethod) and an interaction term (StationName*PreservationMethod). The inclusion of station in the

model controlled for the effect of station on differences in concentrations based on preservation method. The interaction term tested for consistency in the results for preservation methods across the stations.

P-values for all three terms were significant at $p<0.0001$. The significance of StationName was expected because the stations were selected to provide a cross-section of NH4 concentrations. The significance of PreservationMethod indicates that there are statistically discernible differences among the methods. The significance of the interaction term indicates that the differences among the methods are not consistent across the stations.

The cause of the significant interaction term was further explored by deleting the acid preservation method data from the analysis. With the acid-preserved samples deleted from the analysis, the p-value for the interaction term increased to 0.07, which is not considered significant. This indicates that the lack of consistency in the results can be attributed to the acid preservation method.

Differences among the preservation methods were also explored using Tukey's Studentized Range Test of Honest Significant Differences (HSD) (SAS, 1989). The Tukey HSD test compares the means of values after controlling for the Type 1 experimentwise error rate, which occurs when multiple comparisons are made (A type 1 error occurs when one incorrectly rejects the null hypothesis of no difference when it is true – a false positive. Performing multiple comparisons on the same data increases the likelihood that one of those comparisons will be significant when it is in fact not significant.) The Tukey HSD test grouped the mean of each preservation method separately with frozen having the highest mean (0.017 mg/L), iced next highest (0.015 mg/L), and acid having the lowest mean (0.012 mg/L).

To put these differences in context it is helpful to consider the method detection limit (MDL) for the laboratory method that was used to analyze the data (0.0051 mg NH4/L). The MDL is the concentration of a substance that can be measured and reported as greater than zero with 99% confidence. The MDL is typically calculated by multiplying the standard deviation of seven low level samples by 3.14, which is the value from the Student's t-table of critical points associated with six degrees of freedom and a 99% confidence level.

A direct measure of the MDL for this experiment can be calculated from the root mean square error found in the analysis of variance table for the comparison of the preservation methods. The root mean square error is an estimate of the standard deviation pooled across all of the stations and treatments in this study. The MDL calculated from the root mean square error of 0.001174 is 0.0037, which is less than the laboratory MDL of 0.0051. Thus, the level of precision associated with this experiment is greater than (a lower MDL) the level of precision calculated by the laboratory. As a result, this experiment is better able to detect small differences in the data, which contributes to detecting a small difference between frozen and iced samples.

It should also be pointed out that the difference between the means of frozen and iced samples (0.0017 mg/L) is less than the MDL for the laboratory (0.0051) and the experiment (0.0037). So, while

statistically significant, the difference between frozen and iced samples is unimportant in the context of management decisions which are based on estimating spatial and temporal trends that are subject to environmental variability as well as laboratory measurement error.

*Field and Laboratory Blank Samples*

The results for the field DI water blank samples are presented in Figure 4, which compares the concentrations of NH4 in the DI water, based on the preservation methods. Figure 4 shows that very low concentrations of NH4 were detected in two DI water samples that were prepared on 29 November. All other concentrations were in the negative range (Laboratory data are essentially random variables whose "true" concentrations are not known. The values of random variables occur within a probability distribution. When concentrations are very low, as they are with the DI water samples, the mean of the distribution may be zero, but the distribution of possible values can include negative, as well as positive numbers.)

There was some concern that adding acid to the field samples might introduce some contamination; however the data presented in Figure 4 clearly show that acid-preserved DI water samples have lower concentrations of NH4 than iced and frozen samples. The pattern in the results is somewhat different than the CORE/Trend data in that the concentrations of NH4 in the iced samples exceeded those of the frozen samples on two of the three days. Frozen samples exceeded iced samples on only one day. The figure also shows that NH4 concentrations for all three preservation methods are highest on 29 November.

DI water samples were also evaluated statistically using SAS® PROC GLM. The model included the main effects of PreservationMethod and SampleDate. The p-values for preservation method (0.0126) and sample date (0.0066) were significant. The significant results were somewhat surprising given the limited number of DI water samples that were collected. However, Figure 4 shows that acid-preserved samples are clearly lower than iced and frozen samples. The significant result for sample date was caused by the relatively higher concentrations of NH4 in the 29 November samples.

Tukey's HSD test ranks the mean concentration of iced samples highest (-0.001), frozen next (-0.002), and acid-preserved lowest (-0.007). The test assigned iced and frozen samples to the same group, which indicated that the mean concentrations of these preservation methods were not significantly different from each other. The mean of the acid-preserved samples is in a separate Tukey grouping, which indicates that concentrations of acid-preserved DI water samples were significantly different from iced and frozen samples. These results indicate that preserving samples on ice and by freezing produces similar results and that acid preservation produces significantly different (lower) results.

The results of the laboratory and field blanks are compared graphically in Figure 5. The purpose of the field blanks is to ensure that the various preservation treatments did not contaminate the sample. Results of the same-day lab blanks are presented to ensure that any elevated levels observed in the field are not due to laboratory contamination.

All field and laboratory blanks were below the MDL of 0.0051 mg NH4/L, indicating that contamination was not a problem in this set of samples. On two of the three analysis days, the iced field blanks were lower than most of the laboratory blanks and tied with the lowest laboratory blanks on days two and three. This may have been due to the different DI water sources used for the laboratory and field blanks, or different ambient NH4 levels in the field and laboratory environments.

All acidified field blanks showed a consistent negative bias, even greater than the negative bias that occurred in the acidified known concentration samples (Table 1). Therefore, none of the preservation treatments was seen to contaminate the samples, and in fact, the acid treatment appeared to bias the samples low.

*Comparison to Known Concentrations*

The results presented above demonstrated that acid-preserved samples are biased low compared to iced and frozen samples; however, they do not indicate which preservation method produces the most accurate results. To answer that question, known high and low concentrations of NH4 were prepared by CBL, preserved by DNR field office staff, and analyzed by DHMH. These data could not be evaluated statistically because of the small sample size, though the results are displayed graphically in Figure 6 and are presented in Table 2.

Figure 6 shows the analytical results for the mean of the replicate samples compared to reference lines for the known low and high concentrations. The mean of the replicates for the known low frozen (0.033 mg/L) and known low iced (0.031 mg/L) samples are very close to each other and they are almost the same for the known high concentrations (0.128 mg/L for frozen and 0.127 mg/L for iced). Both are very close to their respective known concentrations (0.0315 mg/L for low and 0.126 mg/L for high). The mean of the replicates for the acid-preserved samples (0.027 mg/L for low and 0.122 mg/L for high) are biased low relative to the known concentration samples and to the iced and frozen samples.

The magnitude of the differences between iced and acidified samples and between frozen and acidified samples is similar for the known and CORE/Trend water samples (Table 3). This implies that the acid effect is independent of the matrix and possibly affecting the chemistry or analytical system.

**SENSE OF THE RESOURCES NEEDED TO RESPOND:**

Members of the Analytical Methods and Quality Assurance Workgroup (AMQWA) and data analysts are requested to review this document and provide comments by 24 March 2011.

**PRIORITY RANKING:**

Three (medium). A decision regarding the need to change the historic methods for preserving samples (icing and delivering to the laboratory for analysis within 24 hours) or freezing is needed so that a waiver from the methods outlined in 40 CFR, Part 136, Table 2 can be requested from the U.S. Environmental Protection Agency, Region 3.

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**ACTIONS TO DATE:**

Presented results to the Analytical Methods and Quality Assurance Workgroup on 24 March 2011 (Appendix C). Prepared a final report on 6 April 2011. Analyses performed in PreservationStudy_NH4.SAS program.

**OVERALL RESOLUTIONS SUMMARY OF ACTIONS:**

**RECOMMENDED ACTIONS:**

Based on the analyses described in this report, it is the author's recommendation that field office staff continue to preserve filtered NH4 samples collected under Sections 106 and 117 of the Clean Water Act by keeping them on ice if they can be delivered to and analyzed by the laboratory within 24 hours or by freezing at -20°C if they cannot be delivered within 24 hours. This would provide consistency with the historical data and provides an accurate measure of ambient NH4. Changing preservation methods to acidification could result in a negative step trend in the data. In addition, this study shows that preserving NH4 with acid can result in samples that are biased low relative to the "true" concentration.

**ACTIONS NUMBER:**

1. Designated Respondent:
2. Action:
3. Resources Needed:
4. Due Date:
5. Action Item Resolution Summary:

Table 1. DHMH blanks and field blanks.

| Blank type | Date | NH4 (mg/L) | Preservation method |
|---|---|---|---|
| Field | 11/29/2010 | 0.001 | Iced |
| Field | 11/30/2010 | -0.002 | Iced |
| Field | 12/1/2010 | -0.003 | Iced |
| Lab | 11/30/2010 | -0.003 | Iced |
| Lab | 11/30/2010 | 0.001 | Iced |
| Lab | 11/30/2010 | 0 | Iced |
| Lab | 11/30/2010 | 0.002 | Iced |
| Lab | 12/1/2010 | 0.002 | Iced |
| Lab | 12/1/2010 | -0.002 | Iced |
| Lab | 12/1/2010 | 0.001 | Iced |
| Lab | 12/1/2010 | 0 | Iced |
| Lab | 12/1/2010 | 0 | Iced |
| Lab | 12/2/2010 | -0.003 | Iced |
| Lab | 12/2/2010 | 0.002 | Iced |
| Lab | 12/2/2010 | 0.002 | Iced |
| Field | 11/29/2010 | 0.002 | Frozen |
| Field | 11/30/2010 | -0.004 | Frozen |
| Field | 12/1/2010 | -0.004 | Frozen |
| Lab | 12/16/2010 | -0.002 | Frozen |
| Lab | 12/16/2010 | 0.002 | Frozen |
| Lab | 12/16/2010 | 0 | Frozen |
| Lab | 12/16/2010 | 0 | Frozen |
| Lab | 12/16/2010 | 0.001 | Frozen |
| Field | 11/29/2010 | -0.003 | Acid |
| Field | 11/30/2010 | -0.008 | Acid |
| Field | 12/1/2010 | -0.009 | Acid |
| Lab | 12/16/2010 | 0.001 | Acid |
| Lab | 12/16/2010 | 0.001 | Acid |
| Lab | 12/16/2010 | 0.002 | Acid |
| Lab | 12/16/2010 | 0 | Acid |
| Lab | 12/16/2010 | 0.003 | Acid |

Table 2. Analytical results for the individual known concentrations of NH4 compared to the reported concentrations.

| | Low level samples (mg/L) | High level samples (mg/L) |
|---|---|---|
| Known concentrations | 0.0315 | 0.126 |
| Iced (replicate 1) | 0.032 | 0.128 |
| Iced (replicate 2) | 0.030 | 0.126 |
| Frozen (replicate 1) | 0.033 | 0.129 |
| Frozen (replicate 2) | 0.032 | 0.126 |
| Acid (replicate 1) | 0.026 | 0.122 |
| Acid (replicate 2) | 0.027 | 0.121 |

Table 3. Comparison of mean differences between preservation methods.

| | Iced minus acid (mg/L) | Frozen minus acid (mg/L) |
|---|---|---|
| CORE/Trend | 0.003 | 0.005 |
| Known low | 0.005 | 0.006 |
| Known high | 0.006 | 0.006 |

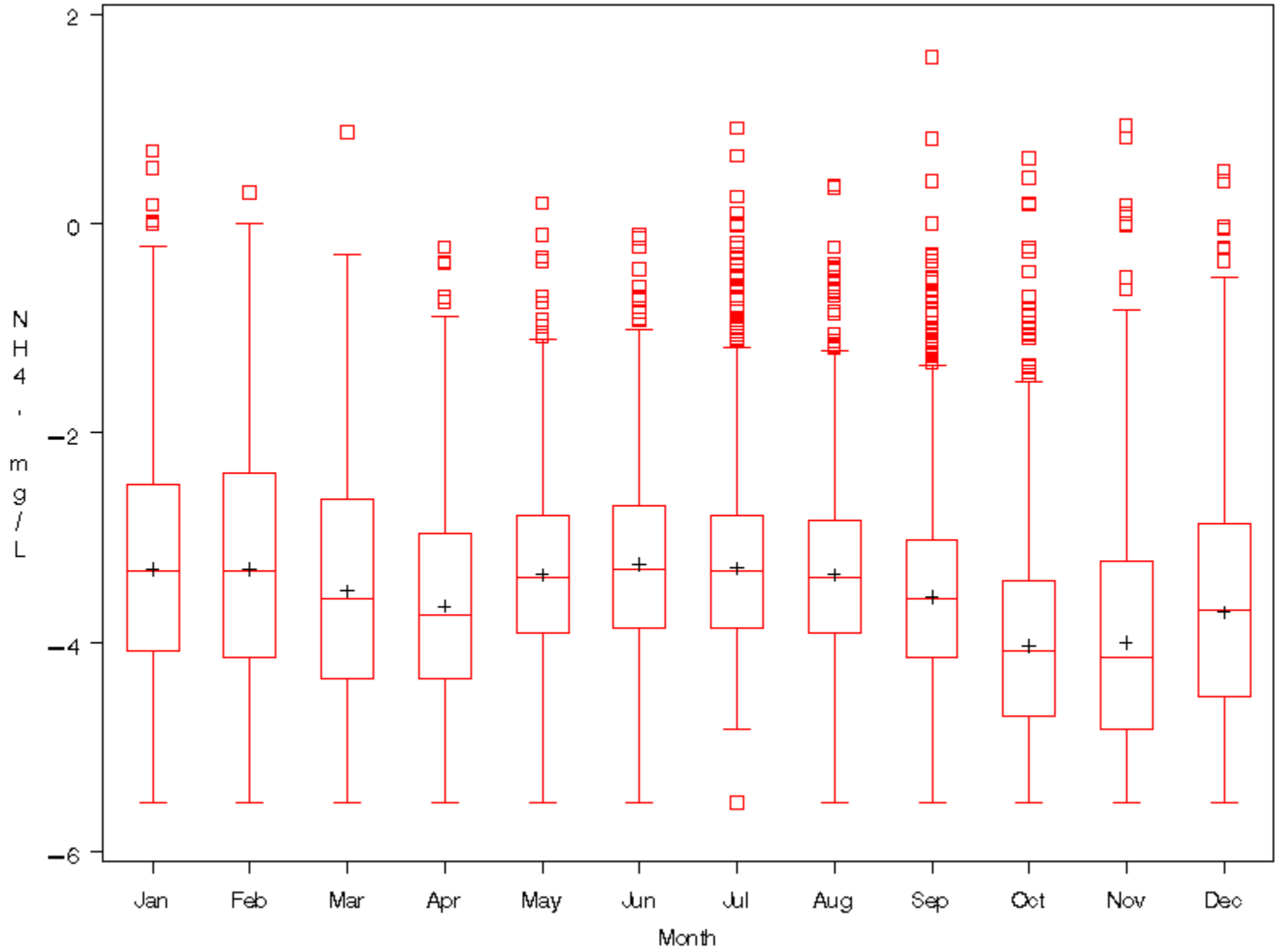


Figure 1. Natural log of ammonium concentrations averaged over the fifty-four CORE/Trend stations, by month.

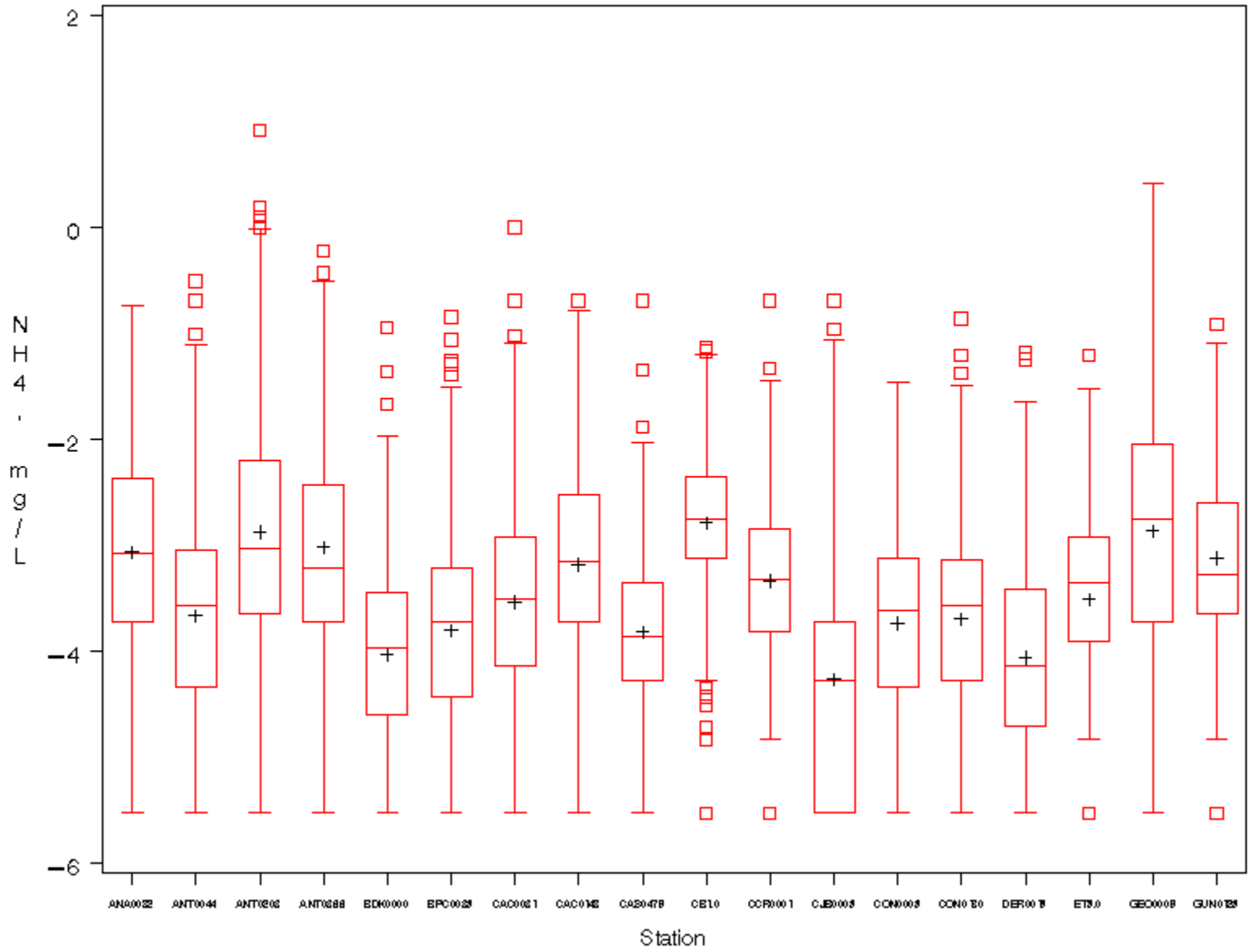


Figure 2. Natural log of ammonium concentrations averaged over the months, by station.

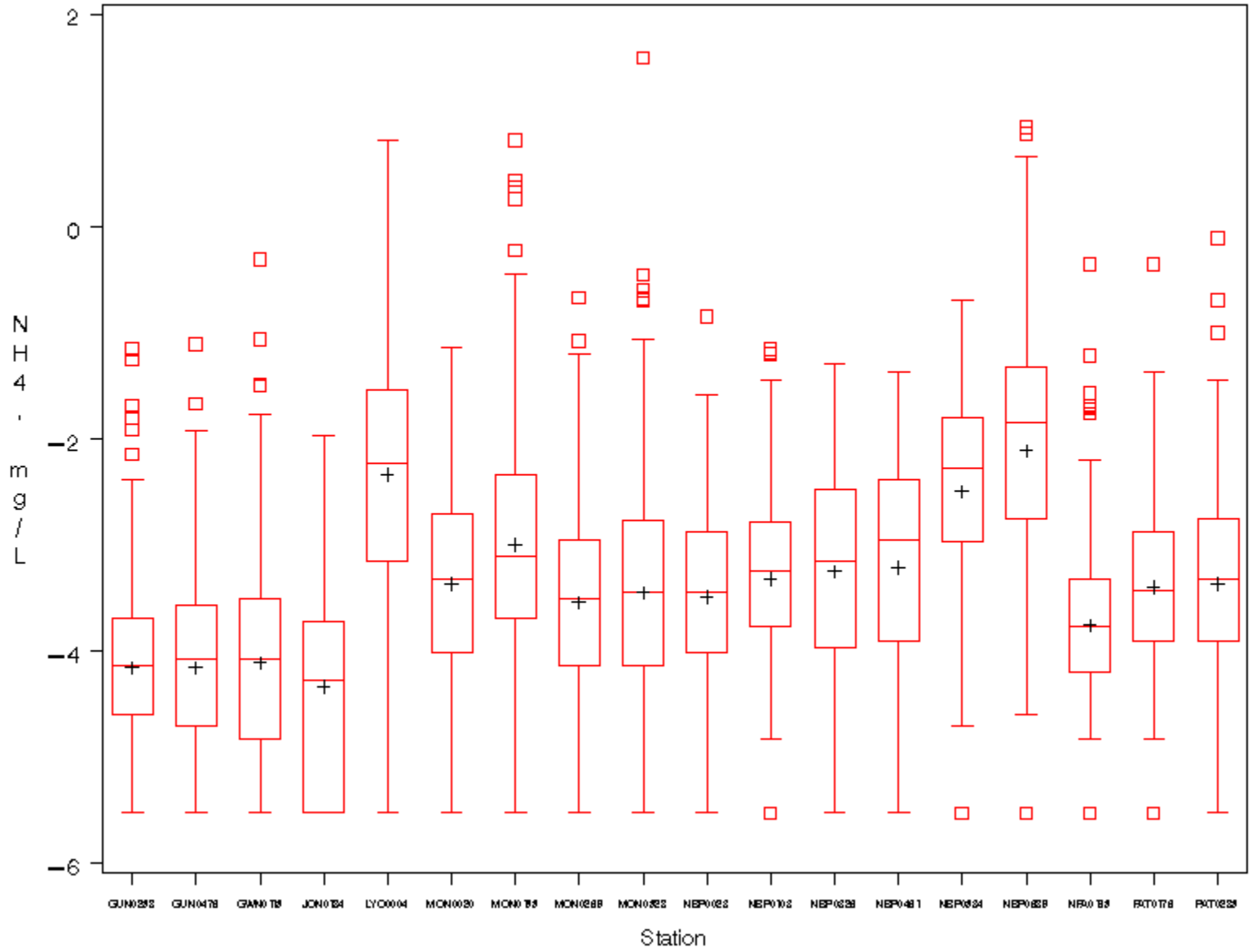


Figure 2. Natural log of ammonium concentrations averaged over the months, by station (continued).

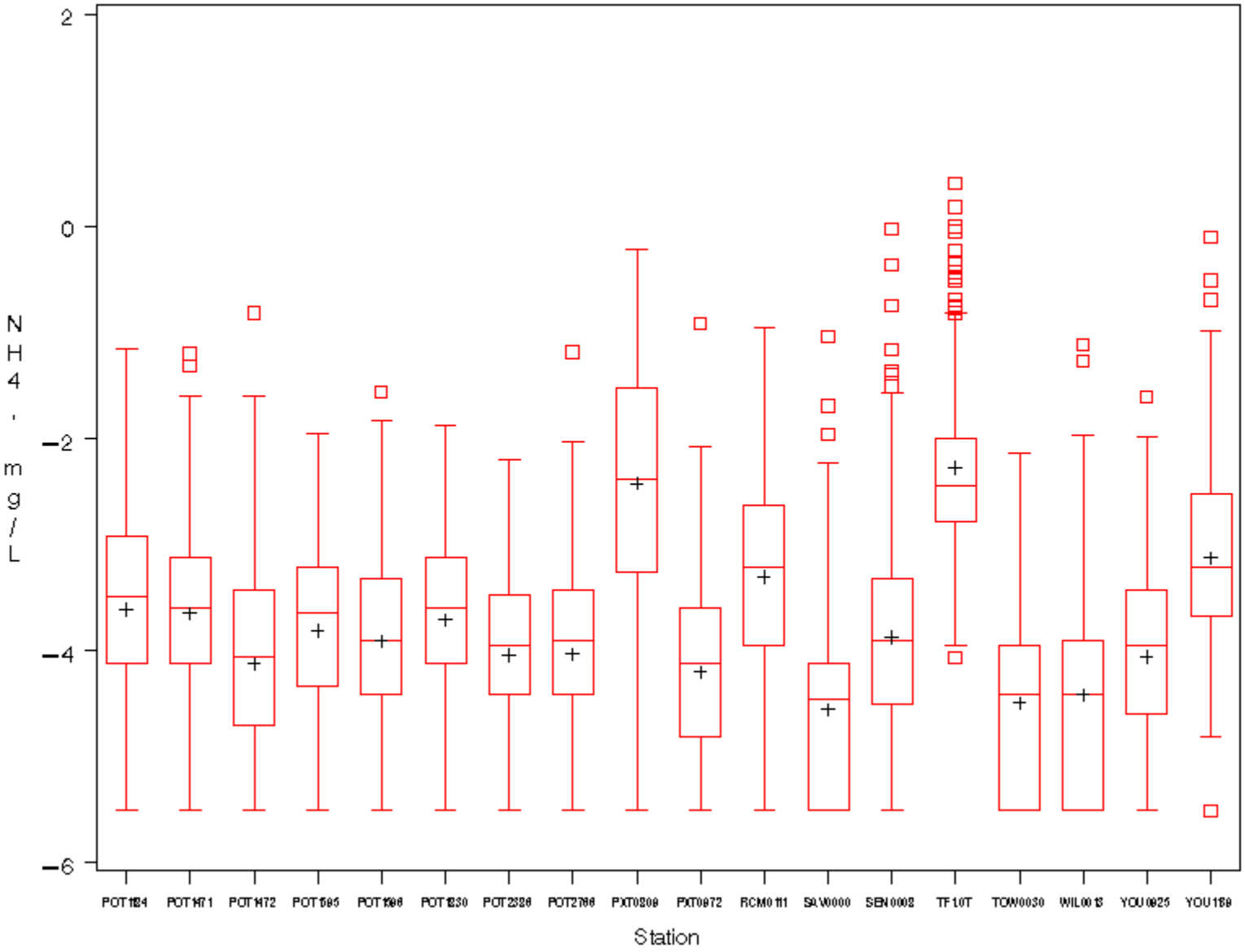


Figure 2. Natural log of ammonium concentrations average over the months, by station (continued).

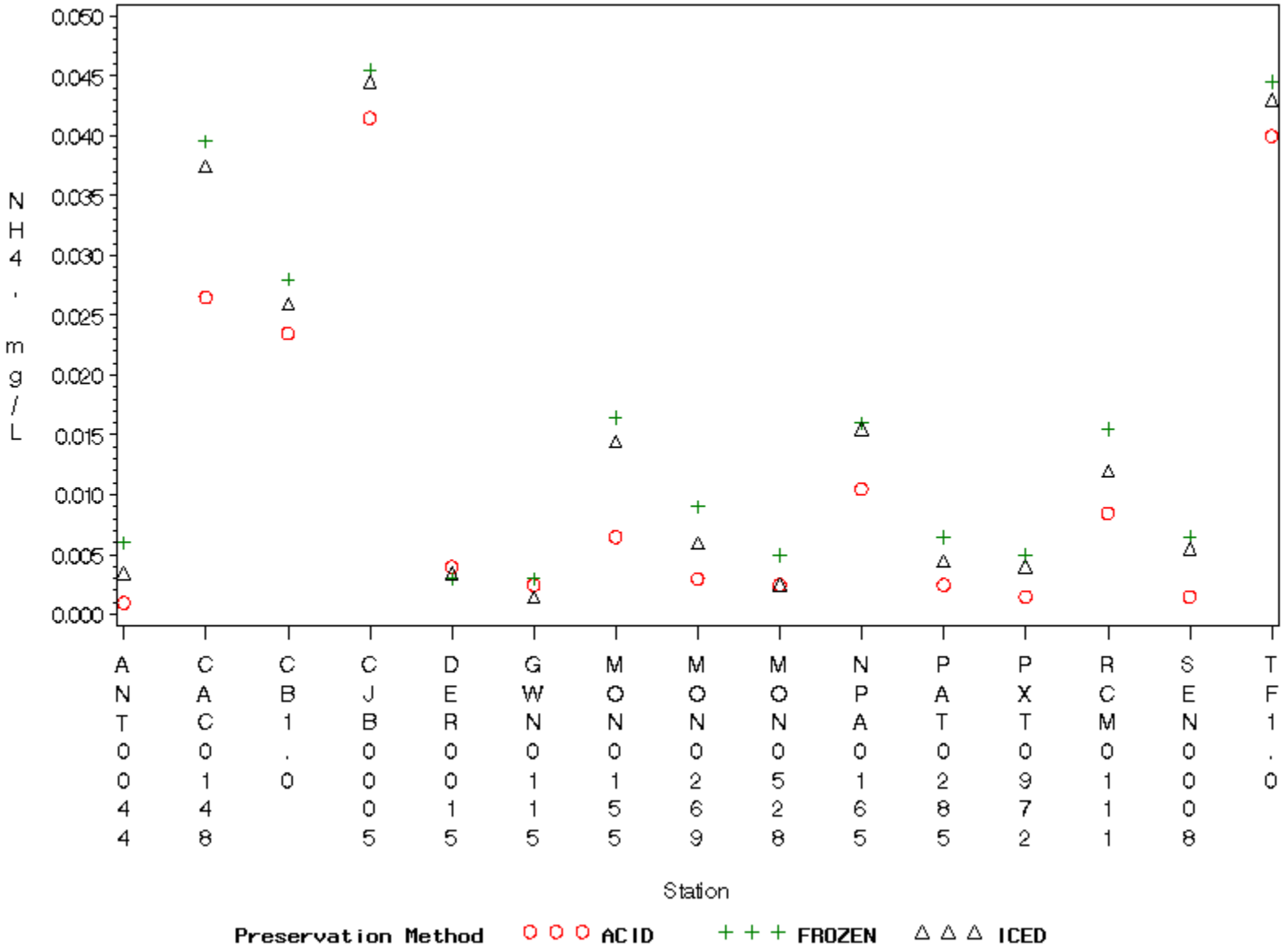


Figure 3. Comparison of preservation methods for the 15 CORE/Trend stations.

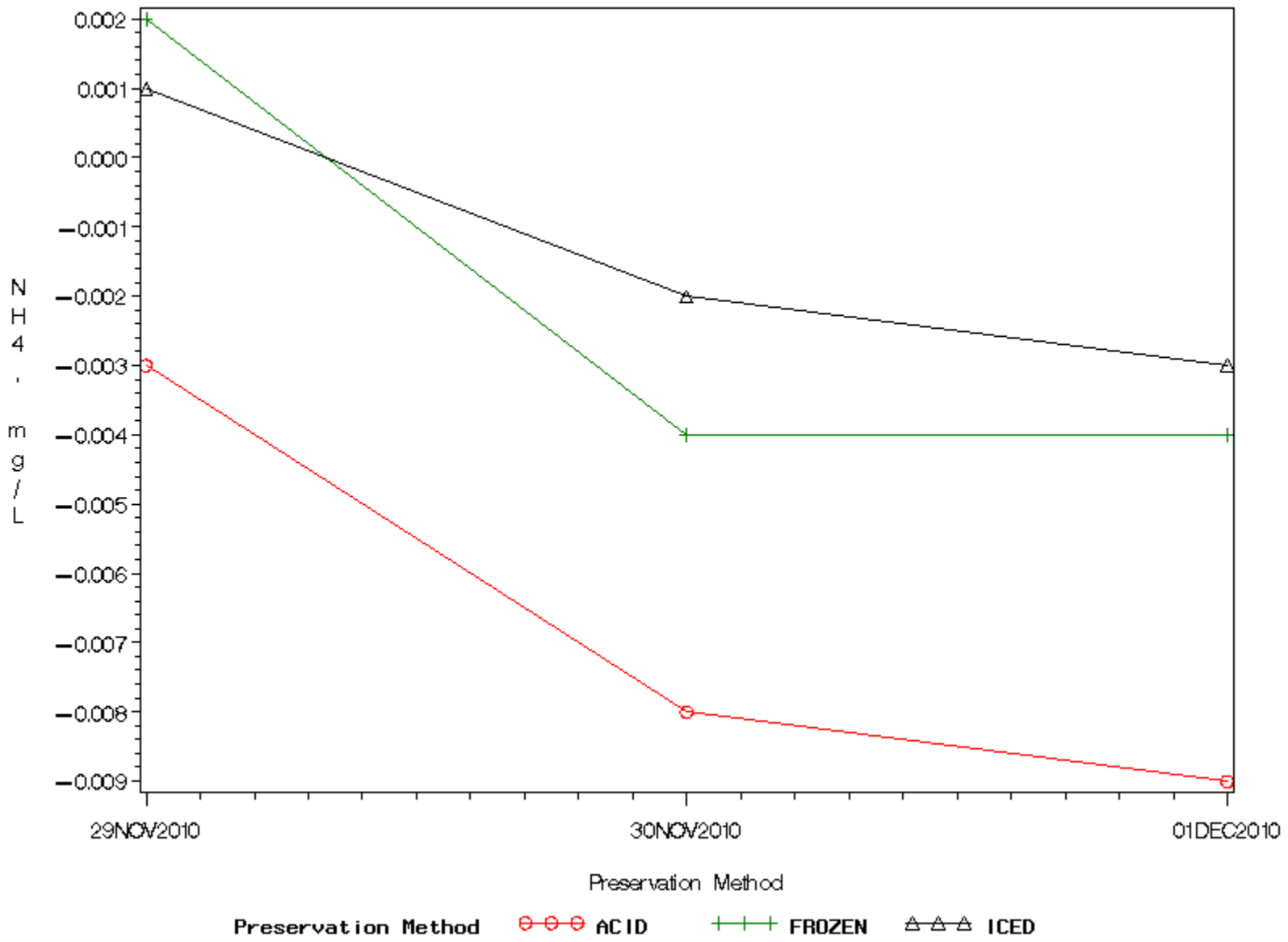


Figure 4. Comparison of sample preservation methods for field DI water blanks.

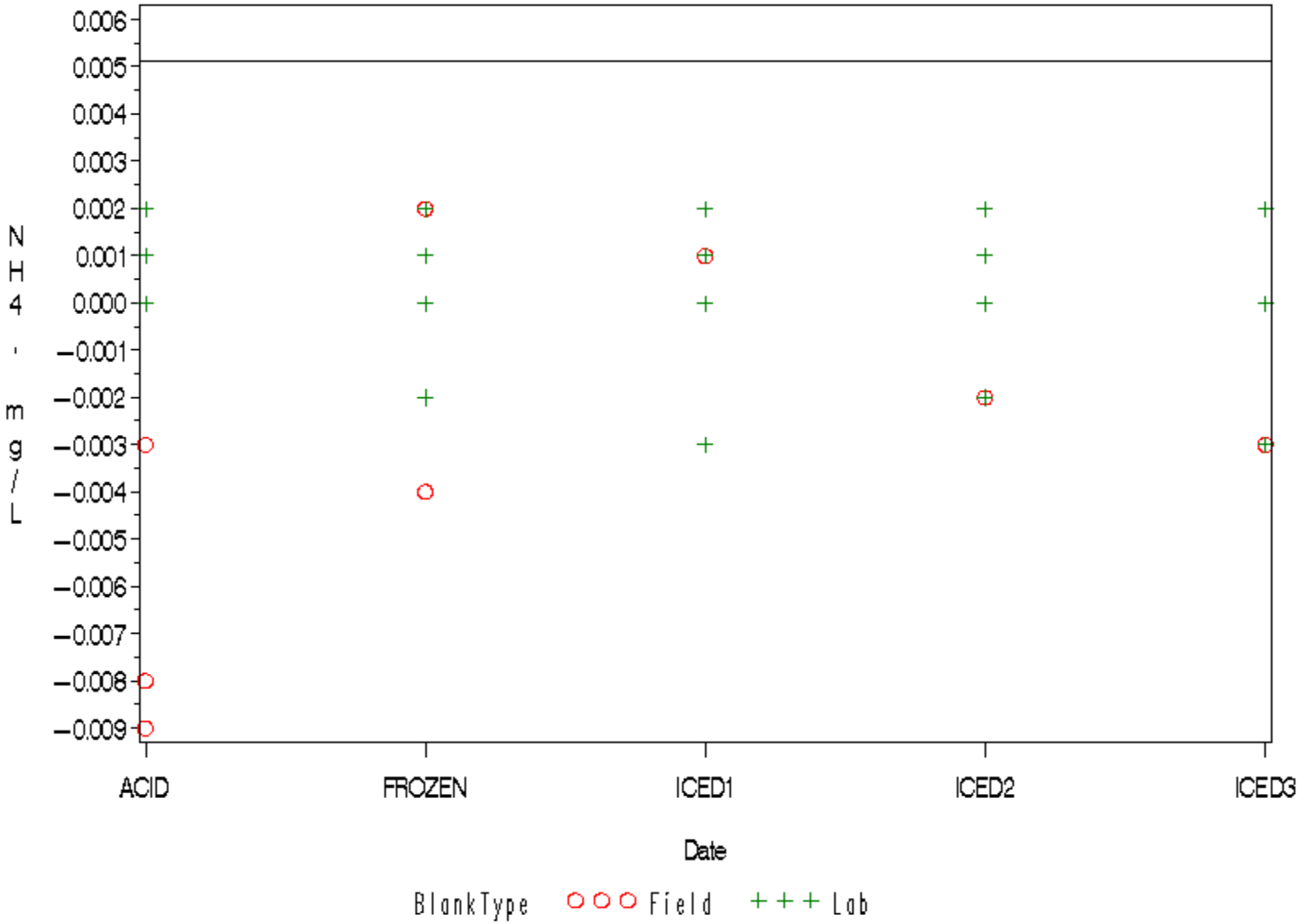


Figure 5. Comparison of DHMH and field blanks. Iced samples were analyzed within 24 hours of collection; acid-preserved and frozen were analyzed approximately two weeks after collection, all on the same day. Vertical axis reference line shows the method detection limit.


Figure 6. Mean of replicates for known concentrations - reference lines are true concentrations

**References**

Guidelines establishing test procedures for the analysis of pollutants, Title 40 Code of Federal Regulations, Part 136. July 1, 2003 edition.

SAS Institute, Inc., *SAS/Stat*® User's Guide, Version 6, Fourth Edition, Volume 2, Cary, NC: SAS Institute Inc., 1989, 846 pp.

**Appendix A**

1) ANA0082
2) ANT0044
3) ANT0203
4) ANT0366
5) BDK0000
6) BEL0053
7) BPC0035
8) CAC0031
9) CAC0148
10) CAS0479
11) CB1.0
12) CCR0001
13) CJB0005
14) CON0005
15) CON0180
16) DER0015
17) GEO0009
18) GUN0125
19) GUN0258
20) GUN0478
21) GWN0115
22) JON0184
23) LYO0004
24) MON0020
25) MON0155
26) MON0269
27) MON0528
28) NBP0023
29) NBP0103
30) NBP0326
31) NBP0461
32) NBP0534
33) NBP0689
34) NPA0165
35) PAT0176
36) PAT0285
37) PIS0033 (no longer analyzed as part of the CORE/Trend program)
38) POT1184
39) POT1471
40) POT1472
41) POT1595
42) POT1596
43) POT11830
44) POT2386
45) POT2766
46) PXT0809
47) PXT0972
48) RCM0111
49) SAV0000
50) SEN0008
51) TF1.0
52) TOW0030
53) WIL0013
54) YOU0925
55) YOU1139

**Appendix B**

1) ANT0047
2) BEL0053
3) CAC0148
4) DER0015
5) GEO0009
6) GUN0258
7) GWN0115
8) MON0546
9) NPA0165
10) PXT0972
11) TF1.2
12) TUK0181
13) WIL0013

**Appendix C**

Presentation to the Analytical Methods and Quality Assurance Workgroup