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Biogeosciences Discussions

Interactive comment on "Technical Note: Comparison between a direct and the standard, indirect method for dissolved organic nitrogen determination in freshwater environments with high dissolved inorganic nitrogen concentrations" *by* D. Graeber et al.

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Proof of minor effects of different numbers of measurement replicates in the study \\ \\ Daniel Graeber and Bjorn Gücker on behalf of all authors of the manuscript, August 8, 2012 \\ \\

1 Background

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In our study, we used different numbers of measurement replicates for the indirect determination of DON concentrations. This was criticized by anonymous referee # 2. We have stated in our reply to anonymous referee 2 that: "In the HTCO measurements, additional measurement replicates were performed by the equipment, when the standard deviation was above a threshold value. We could simply not report these values, but we decided to include all available replicates in order to improve data quality." In this additional comment, we show that different numbers of measurement replicates had only minor effects on the indirect DON concentration determinations and did not affect the conclusions of our study.

In the manuscript, DON concentration for the indirect standard method were calculated as either DON = total dissolved nitrogen (TDN) - (NO3- + NO2-) - NH4+ or as DON = TDN - (NO3- + NO2-), depending on the experiment performed. To calculate the uncertainty of these DON determinations, means and standard deviations were calculated by Monte Carlo (MC) simulations. These MC simulations are based on the mean and standard deviation of separate measurements of TDN, NO3- + NO2- and NH4+. In this additional comment to reviewer # 2, we calculated the accuracy of the means and standard deviations for different numbers of measurement replicates in order to test if different numbers of measurement replicates had an effect on the MC simulations as hypothesized by the reviewer.

2 Methodology used to test the effect of different numbers of measurement replicates

We used a bootstrap procedure based on 10000 iterations to calculate the mean, standard deviation, as well as the accuracy of both for different replicate numbers, ranging between the extreme case of 2 measurement replicates and the real number of measurement replicates of each sample reported in the original manuscript. Such bootstrap procedures to investigate the accuracy of statistical estimates (such as for example mean, standard deviation or standard error) are decribed in detail in Efron and Tibshirani' s book on boostrap techniques (Efron and Tibshirani: An Introduction to the Bootstrap, Chapman & Hall, 1994). The bootstrap was performed for TDN, NO3-

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+ NO_2^- and NH_4^+. The samples used for the boostrap were the ones which were used in the manuscript to compare the indirect determination of DON to the direct determination of DON by size-exclusion chromatography (NOM, Agricultural ditch and Agricultural stream). These samples were chosen because they exhibited the largest number of measurement replicates (5 - 11) which makes it possible to analyze a large gradient of error estimates (n = 2 - 11).

The bootstrap procedure works as following: For each number of measurement replicates n (n = 2, 3, ..., real number of measurement replicates), the mean and standard deviation is calculated 10000 times subsequently, randomly selecting n measurement replicates with replacement. From these 10000 bootstrap samples of mean and standard deviation, the mean is calculated Moreover, the accuracy of each mean and standard deviation is calculated as boostrap estimate of standard error (see page 13 in Efron and Tibshirani: An Introduction to the Bootstrap, Chapman & Hall, 1994 for details on the calculation of this estimate).

3 Results and discussion

3.1 Nitrate + nitrite

For different replicate numbers in NO_3^- + NO_2^- only very small and unsystematic changes in the mean occurred (Table 1). For standard deviation, as slight increase was found with increasing numbers of measurement replicates (Table 1). The bootstrap estimate of standard error (SE) of the mean and the standard deviation decreased with an increasing number of measurement replicates (Table 1).

The factor of increase of accuracy for the mean from 2 to 6 measurement replicates is roughly 1.7 and for the standard deviation it is roughly 2.2. In the manuscript, we used 2 measurement replicates for the MC simulation of the screening samples. Thus, for these we have to assume a higher uncertainty in the comparison to the other samples measured with more measurement replicates. However, since we used 99 independent replicates for the screening of ambient samples, we believe that our conclusions on the

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relationship between DON determination accuracy and DIN:TDN ratio are justified. For all other samples, we used either 5 or 6 measurement replicates for NO3- + NO2-. For these samples differences in accuracy of the mean and standard deviation was very low when comparing 5 and 6 measurement replicates (Table 1).

In two samples, NO3- + NO2- was very low or below detection limit, respectively. For these, the boostrap SEs are very low (Table 1)

3.2 Ammonium

For different sample sizes of NH4+ measurements, changes in SDs and SEs only occurred for 4 samples, since the NH4+ concentrations of 2 samples were below detection limit (Table 2, ditch and NOM + NO3-). Since all of these samples had the same value (0.015 mg N / L) which is half of the detection limit, bootstrap SEs and standard deviations were zero (Table 2).

For the samples, in which NH4+ occurred in measurable concentrations (Table 2), similar patterns as for NO3- were observed (see section 3.1). Thus, effects of different sample sizes in NH4+ measurements were also low and did not affect the conclusions of our study.

3.3 Total dissolved nitrogen

For different replicate numbers of TDN measurements, similar differences as for nitrate and ammonium were observed (sections 3.1, 3.2). The mean was stable, independent of the number of measurement replicates and the standard deviation was slightly lower at lower numbers of measurement replicates (Table 3). Moreover, changes of the bootstrap standard errors (SE) were similar to that of NO3- and NH4+ measurements for the comparison of 2, 5 and 6 measurement replicates (see section 3.1 for details).

One sample was measured accidently twice for TDN (NOM). Thus we measured 11 replicates. From this sample, between 2 and 11 measurement replicates, the bootstrap SE changed by a factor of 2.4 for the mean and by a factor of 2.7 for the standard

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deviation. Between 6 and 11 measurement replicates differences in bootstrap SE were lower: the bootstrap SE of the mean and standard deviation changed by a factor of 1.4 only. For this NOM sample, the increase in the calculated standard deviation was very also low when comparing 6 and 11 measurement replicates. It was 0.070 for 6 measurement replicates and 0.072 for 11 measurement replicates.

4 Conclusions

We found undirected, very small effects of different numbers of measurement replicates in our study on mean values, but a slight increase in standard deviation with increasing numbers of measurement replicates.

The bootstrap SEs of both means and standards deviation revealed an increase in accuracy with increasing numbers of measurement replicates. This increase was high when comparing 2 and 6 measurement replicates and very low, when comparing 5 and 6 or 6 and 11 measurement replicates.

From this analysis, only for the screening of 99 ambient samples in our manuscript – for which both NO_3^- + NO_2^- and NH_4^+ were measured with 2 measurement replicates – a potential effect of low replicate numbers on our conclusions seems possible. However, the relationship between DIN:TDN ratio and the uncertainty of the indirect DON determination by the standard method is still well represented due to a high number of ambient samples (n=99) that compensates for the low analytical replication (n=2). Since we found the lowest standard deviations in our comparison analysis (tables 1 to 3) with 2 measurement replicates, we probably still underestimated the uncertainty in the DON determinations by the standard method in the screening. Thus, the main conclusions in our are valid, irrespective of the number of measurement replicates used.

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Table 1: Mean, standard deviation and bootstrap standard error (SE) of the accuracy of the two statistical estimates for NO3- measurements in dependence on the number of measurement replicates. Based on a bootstrap procedure with 10000 iterations for each sample and estimator (mean, standard deviation). The minimum number of measurement replicates (n) is 2 and the maximum is the original number of measurement replicates for each of the samples.

Sample	n	Mean conc.	Boostrap SE of mean conc.	Standard deviation	Boostrap SE of standard deviation
Agricultural ditch	2	18.362	0.108	0.121	0.092
Agricultural ditch	3	18.362	0.088	0.141	0.063
Agricultural ditch	4	18.363	0.076	0.145	0.048
Agricultural ditch	5	18.364	0.069	0.149	0.039
Agricultural ditch	6	18.363	0.063	0.149	0.034
Agricultural stream	2	7.079	0.131	0.141	0.121
Agricultural stream	3	7.080	0.108	0.165	0.086
Agricultural stream	4	7.078	0.093	0.174	0.066
Agricultural stream	5	7.082	0.084	0.177	0.054
NOM	2	0.011	0.001	0.001	0.001
NOM	3	0.011	0.001	0.001	0.000
NOM	4	0.011	0.000	0.001	0.000
NOM	5	0.011	0.000	0.001	0.000
NOM	6	0.011	0.000	0.001	0.000
NOM + NH4+	2	0.005	0.000	0.000	0.000
NOM + NH4+	3	0.005	0.000	0.000	0.000
NOM + NH4+	4	0.005	0.000	0.000	0.000
NOM + NH4+	5	0.005	0.000	0.000	0.000
NOM + NH4+ + NO3-	2	2.411	0.034	0.036	0.031
NOM + NH4+ + NO3-	3	2.412	0.027	0.042	0.023
NOM + NH4+ + NO3-	4	2.412	0.024	0.044	0.018
NOM + NH4+ + NO3-	5	2.412	0.022	0.046	0.016
NOM + NH4+ + NO3-	6	2.412	0.020	0.046	0.014
NOM + NO3-	2	4.703	0.058	0.064	0.053
NOM + NO3-	3	4.704	0.048	0.074	0.037
NOM + NO3-	4	4.703	0.041	0.077	0.029
NOM + NO3-	5	4.703	0.037	0.079	0.023
NOM + NO3-	6	4.704	0.034	0.080	0.020

Fig. 1.

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Table 2: Mean, standard deviation and bootstrap standard error (SE) of the accuracy of the two statistical estimates for NH4+ measurements in dependence on the number of measurement replicates. Based on a bootstrap procedure with 10000 iterations for each sample and estimator (mean, standard deviation). The minimum number of measurement replicates (n) is 2 and the maximum is the original number of measurement replicates for each of the samples.

Sample	n	Mean conc.	Boostrap SE of mean conc.	Standard deviation	Boostrap SE of standard deviation
Agricultural ditch	2	0.0150	0.0000	0.0000	0.0000
Agricultural ditch	3	0.0150	0.0000	0.0000	0.0000
Agricultural ditch	4	0.0150	0.0000	0.0000	0.0000
Agricultural ditch	5	0.0150	0.0000	0.0000	0.0000
Agricultural ditch	6	0.0150	0.0000	0.0000	0.0000
Agricultural stream	2	0.0445	0.0011	0.0012	0.0011
Agricultural stream	3	0.0445	0.0009	0.0014	0.0007
Agricultural stream	4	0.0445	0.0008	0.0015	0.0006
Agricultural stream	5	0.0445	0.0007	0.0015	0.0004
Agricultural stream	6	0.0445	0.0007	0.0016	0.0004
NOM	2	0.0480	0.0099	0.0108	0.0089
NOM	3	0.0479	0.0080	0.0122	0.0068
NOM	4	0.0480	0.0069	0.0126	0.0057
NOM	5	0.0480	0.0062	0.0129	0.0049
NOM	6	0.0481	0.0057	0.0131	0.0045
NOM + NH4+	2	4.5662	0.0328	0.0351	0.0302
NOM + NH4+	3	4.5666	0.0270	0.0403	0.0220
NOM + NH4+	4	4.5660	0.0230	0.0430	0.0171
NOM + NH4+	5	4.5661	0.0206	0.0441	0.0141
NOM + NH4+ + NO3-	2	2.4122	0.0251	0.0274	0.0227
NOM + NH4+ + NO3-	3	2.4130	0.0203	0.0319	0.0159
NOM + NH4+ + NO3-	4	2.4126	0.0178	0.0337	0.0119
NOM + NO3-	2	0.0150	0.0000	0.0000	0.0000
NOM + NO3-	3	0.0150	0.0000	0.0000	0.0000
NOM + NO3-	4	0.0150	0.0000	0.0000	0.0000
NOM + NO3-	5	0.0150	0.0000	0.0000	0.0000

Fig. 2.

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Table 3: Mean, standard deviation and bootstrap standard error (SE) of the accuracy of the two statistical estimates for total dissolved nitrogen (TDN) measurements in dependence on the number of measurement replicates. Based on a bootstrap procedure with 10000 iterations for each sample and estimator (mean, standard deviation). The minimum number of measurement replicates (n) is 2 and the maximum is the original number of measurement replicates for each of the samples.

Sample	n	Mean conc.	Boostrap SE of mean conc.	Standard deviation	Boostrap SE of standard deviation
Agricultural ditch	2	18.476	0.051	0.057	0.045
Agricultural ditch	3	18.477	0.042	0.065	0.032
Agricultural ditch	4	18.476	0.037	0.068	0.025
Agricultural ditch	5	18.477	0.032	0.069	0.021
Agricultural ditch	6	18.477	0.030	0.070	0.018
Agricultural stream	2	6.187	0.177	0.191	0.167
Agricultural stream	3	6.189	0.146	0.223	0.120
Agricultural stream	4	6.188	0.126	0.234	0.094
Agricultural stream	5	6.190	0.114	0.240	0.077
Agricultural stream	6	6.190	0.103	0.244	0.065
NOM	2	1.261	0.053	0.057	0.047
NOM	3	1.260	0.042	0.064	0.036
NOM	4	1.260	0.037	0.068	0.030
NOM	5	1.260	0.033	0.069	0.026
NOM	6	1.260	0.030	0.070	0.024
NOM	7	1.260	0.028	0.071	0.022
NOM	8	1.260	0.026	0.071	0.020
NOM	9	1.260	0.025	0.071	0.019
NOM	10	1.260	0.023	0.072	0.018
NOM	11	1.260	0.022	0.072	0.017
NOM + NH4+	2	6.971	0.463	0.503	0.427
NOM + NH4+	3	6.958	0.374	0.578	0.308
NOM + NH4+	4	6.959	0.325	0.610	0.241
NOM + NH4+	5	6.967	0.293	0.621	0.200
NOM + NH4+	6	6.966	0.269	0.629	0.173
NOM + NH4+ + NO3-	2	7.331	0.244	0.263	0.222
NOM + NH4+ + NO3-	3	7.328	0.198	0.312	0.152
NOM + NH4+ + NO3-	4	7.331	0.173	0.327	0.111
NOM + NH4+ + NO3-	5	7.330	0.155	0.334	0.086
NOM + NO3-	2	6.964	0.251	0.270	0.220
NOM + NO3-	3	6.960	0.203	0.309	0.162
NOM + NO3-	4	6.962	0.176	0.323	0.133
NOM + NO3-	5	6.965	0.155	0.332	0.112
NOM + NO3-	6	6.961	0.142	0.337	0.103

Fig. 3.

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