Supplement to "Technical Note: Comparison between a direct and the standard, indirect method for dissolved organic nitrogen determination in freshwater environmenmts with high dissolved inorganic nitrogen concentrations"

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1 Size-exclusion chromatograms

Five chromatograms of the blank measurements (MilliQ water) show the low background noise occurring during the size-exclusion chromatography (SEC) measurements (Fig. 1).

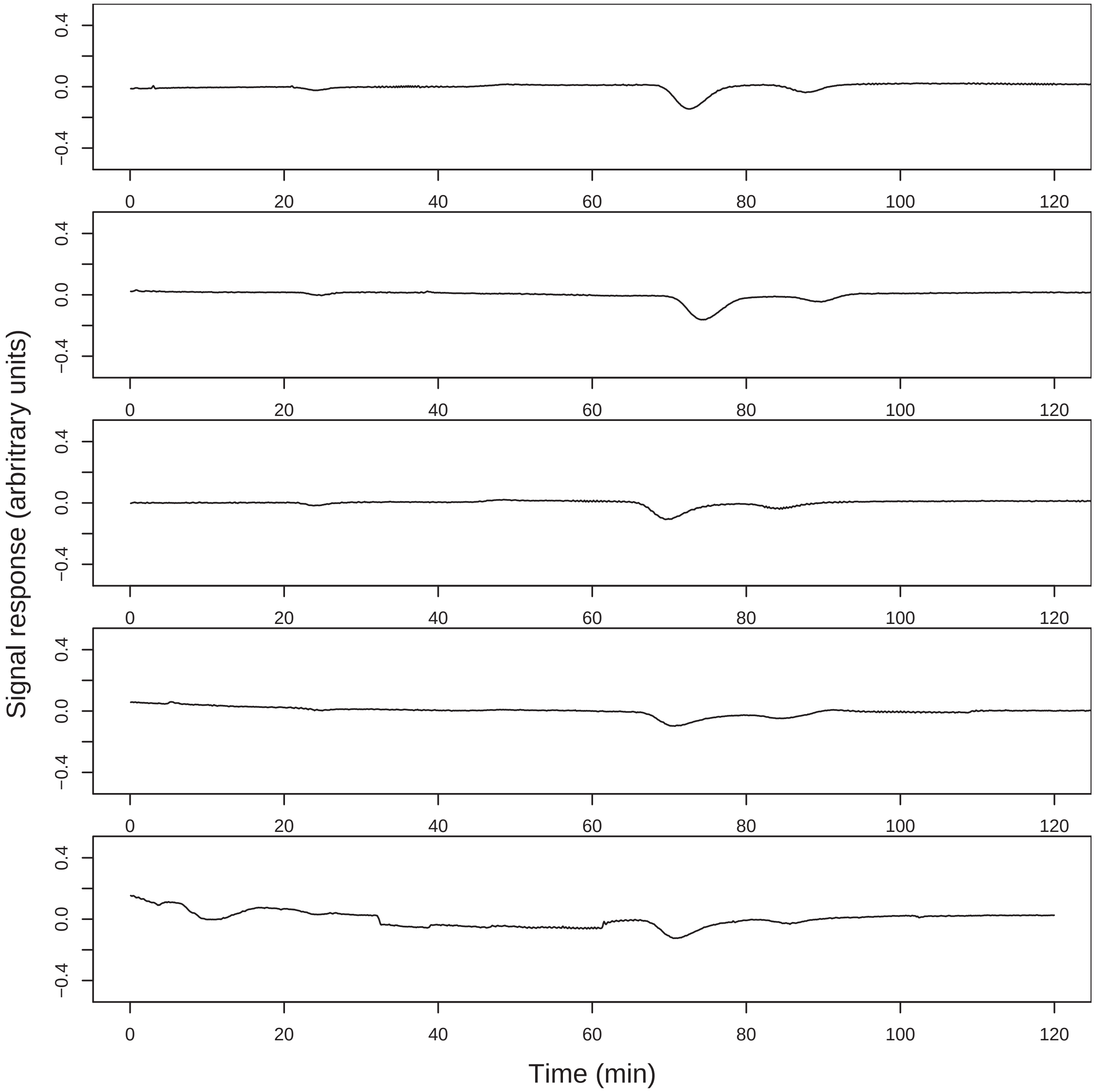

Figure 1: Chromatograms of five blank measurements from size-exclusion chromatography.

The chromatograms for the agricultural streams and tile drain show the working principle of size-exclusion chromatography (Fig. 2). Dissolved organic nitrogen (DON) is separated by molecular size from dissolved inorganic nitrogen (DIN) and is then directly measured in an UV-detector after oxidation to NO_3^- This also works for the tested forest streams (Fig. 3a, b), waste water samples (Fig. 3c, d) and the NOM samples (Fig. 4, 5).

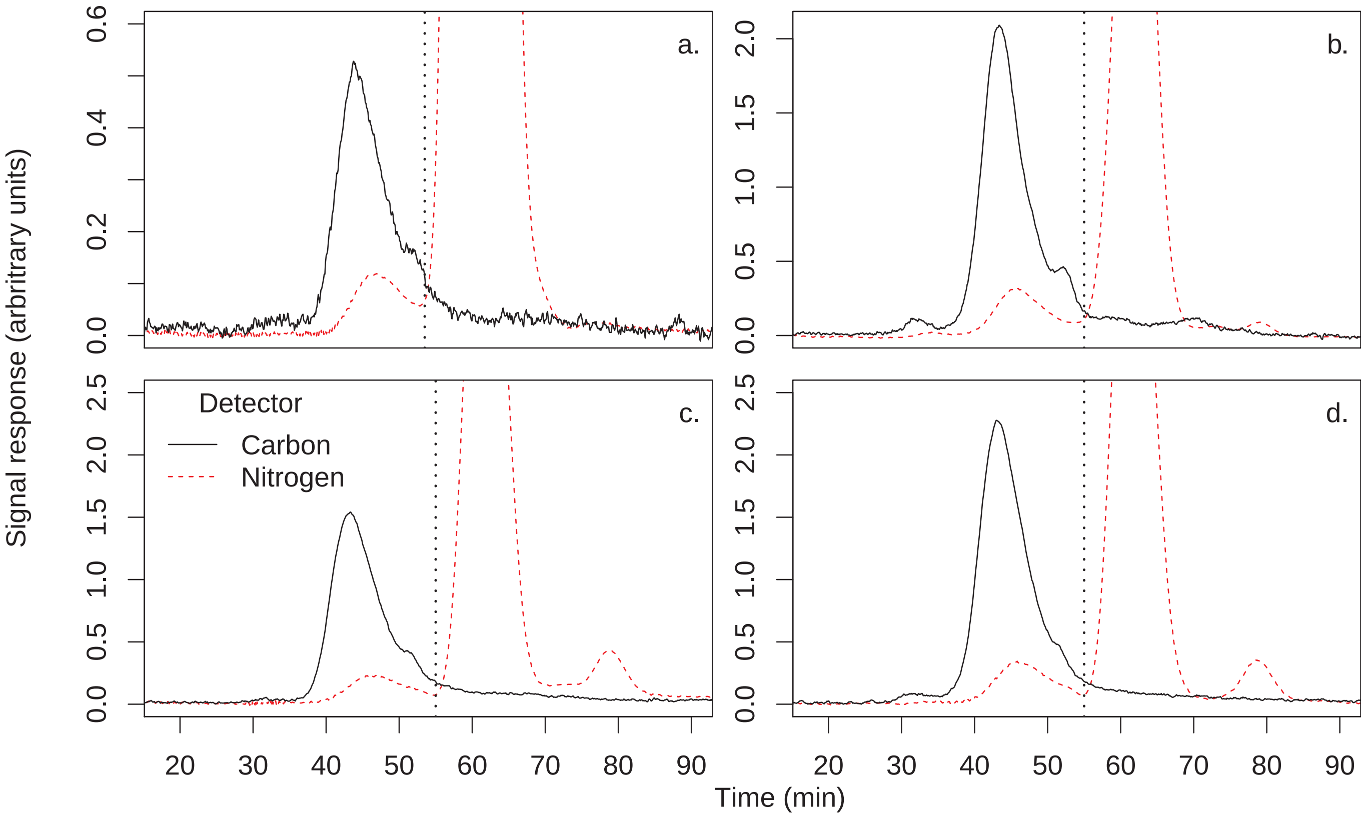

Figure 2: Chromatograms of four samples from size-exclusion chromatography. **(a)** GER agricultural tile drain, **(b)** GER agricultural stream, **(c)** DK agricultural stream 1, **(d)** DK agricultural stream 2. The dotted vertical line indicates the separation between DON (left) and DIN (right).

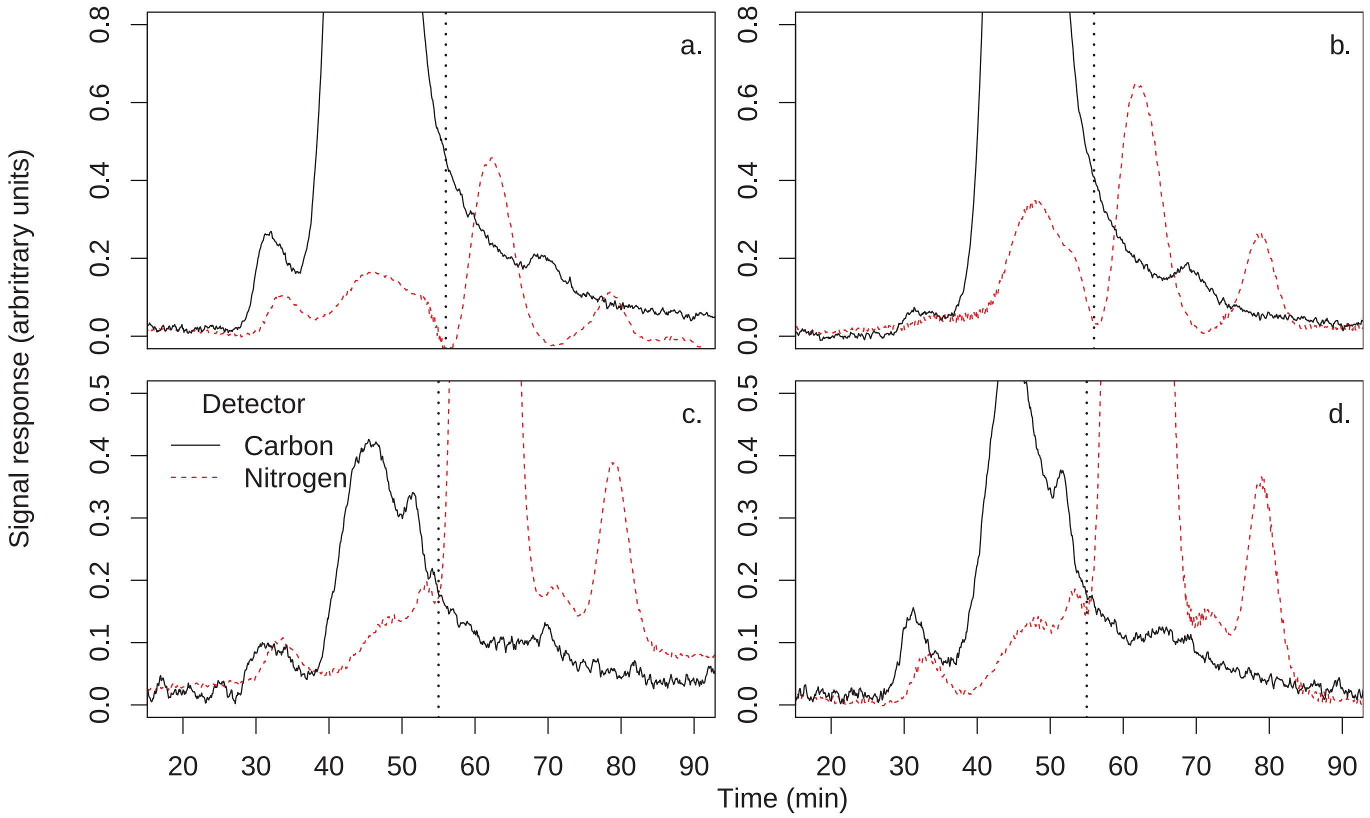

Figure 3: Chromatograms of four samples from size-exclusion chromatography. **(a)** Forest stream 1, **(b)** Forest stream 2, **(c)** Waste water 1, **(d)** Waste water 2. The dotted vertical line indicates the separation between DON (left) and DIN (right).

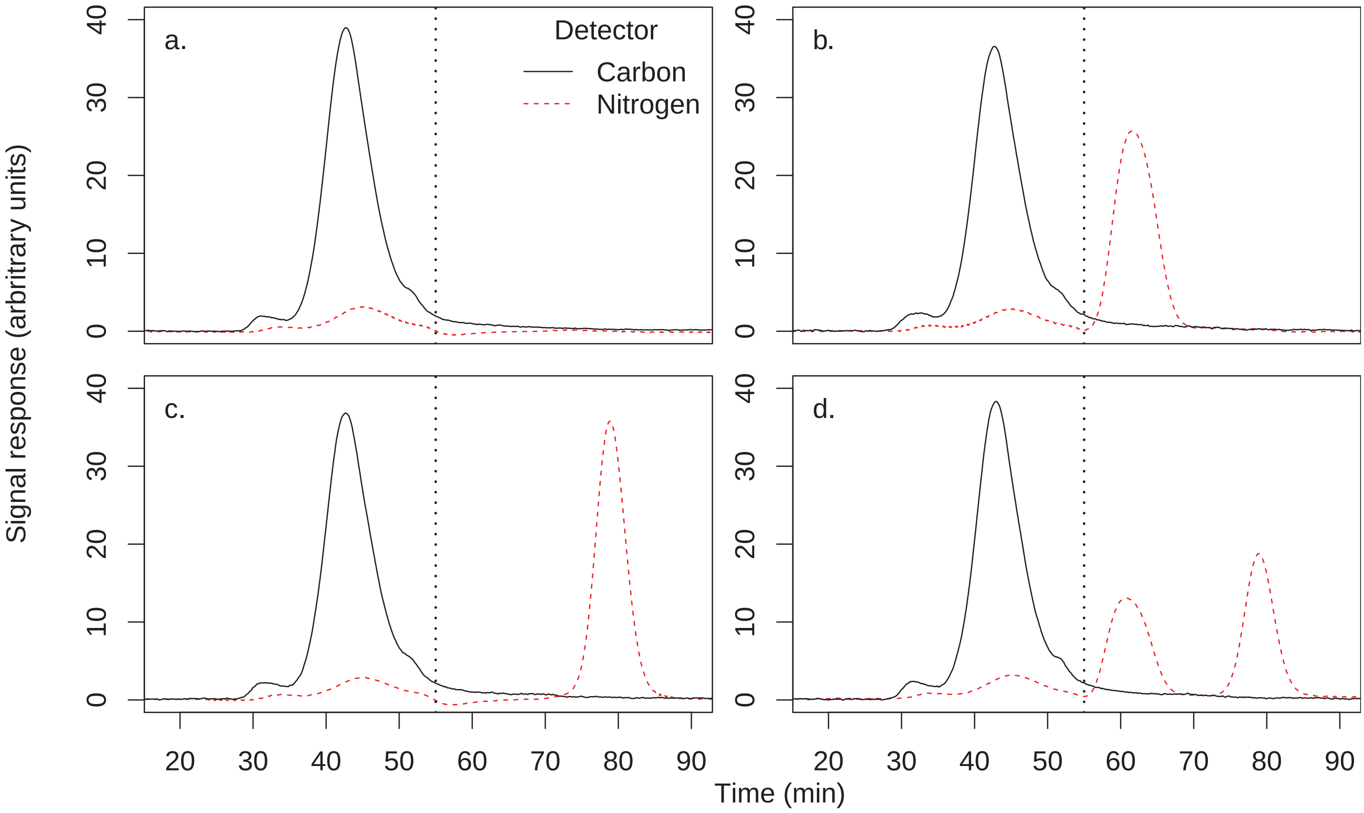

Figure 4: Chromatograms of four samples from size-exclusion chromatography. **(a)** pure Schwarzer See NOM, **(b)** Schwarzer See NOM + NO_3^- , **(c)** Schwarzer See NOM + NH_4^+ , **(d)** Schwarzer See NOM + NO_3^- + NH_4^+ The dotted vertical line indicates the separation between DON (left) and DIN (right).

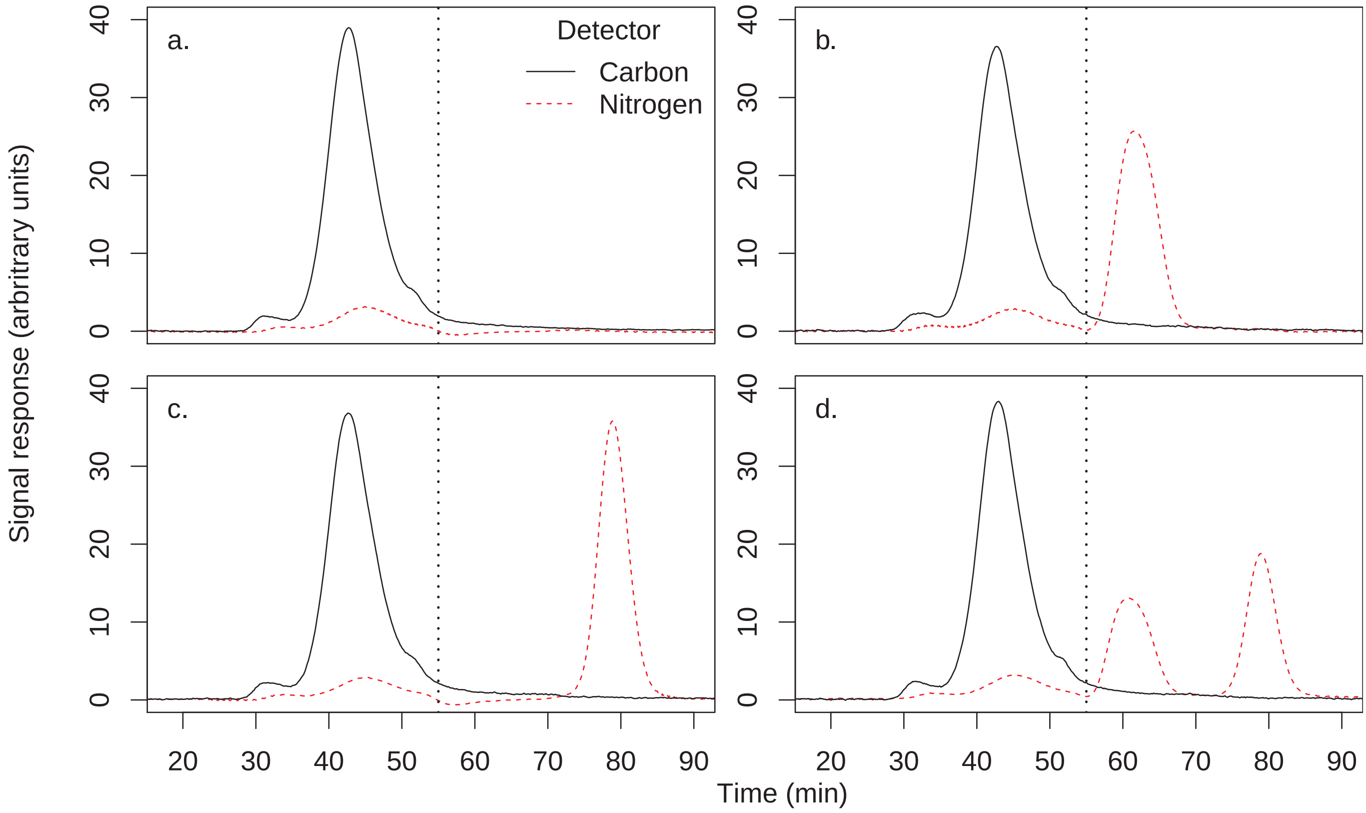

Figure 5: Chromatograms of four samples from size-exclusion chromatography. **(a)** pure wetland outflow sample, **(b)** Wetland outflow + NO_3^- , **(c)** Wetland outflow + NH_4^+ , **(d)** Wetland outflow + NO_3^- + NH_4^+ The dotted vertical line indicates the separation between DON (left) and DIN (right).

2 Map, geographical coordinates and concentrations of the screening samples

Table 1: Geographical coordinates and concentrations (mean and standard deviation (SD)) of the screening samples used as input for the Monte Carlo simulation

Site	Latitude	Longitude	TDN mean	TDN SD	NO_3^- mean	NO_3^- SD	NH_4^+ mean	NH_4^+ SD	DIN:TDN
			$mg\,N\,L^{-1}$						
1	N 50°44.376’	E 013°43.477’	0.90	0.02	0.90	0.00	0.01	0.00	1.02
2	N 50°50.041’	E 013°35.503’	2.30	0.06	2.47	0.02	0.03	0.00	1.09
3	N 52°13.124’	E 014°03.133’	2.25	0.14	1.81	0.00	0.06	0.00	0.83
4	N 52°34.935’	E 014°6.839’	1.95	0.01	1.77	0.00	0.04	0.00	0.93
5	N 52°34.734’	E 014°7.224’	0.16	0.03	0.05	0.00	0.05	0.00	0.60
6	N 52°34.898’	E 014°6.691’	0.43	0.05	0.22	0.00	0.01	0.00	0.53
7	N 52°27.639’	E 014°13.126’	37.17	7.09	36.14	0.03	0.56	0.01	0.99
8	N 52°27.813’	E 014°13.645’	46.08	3.39	43.63	0.10	0.01	0.00	0.95
9	N 52°24.366’	E 014°12.299’	25.17	1.42	22.48	0.00	0.01	0.00	0.89
10	N 52°22.126’	E 014°11.673’	24.33	0.63	25.04	0.36	0.01	0.00	1.03
11	N 52°21.953’	E 014°13.381’	22.33	0.38	20.79	0.10	0.01	0.00	0.93
12	N 52°13.590’	E 014°03.993’	25.17	0.80	24.70	0.06	0.01	0.00	0.98
13	N 52°13.643’	E 014°03.391’	27.25	1.80	23.93	0.06	0.01	0.00	0.88
14	N 52°12.750’	E 014°03.026’	66.00	2.70	60.72	0.00	0.07	0.01	0.92
15	N 52°10.621’	E 014°10.801’	15.23	0.06	14.51	0.00	0.01	0.00	0.95
16	N 52°26.861’	E 014°14.943’	29.25	1.89	30.82	0.25	0.03	0.00	1.05
17	N 52°25.888’	E 014°8.305’	22.08	2.45	23.21	0.06	0.01	0.00	1.05
18	N 52°25.888’	E 014°8.305’	54.50	1.00	54.29	0.00	0.01	0.00	1.00
19	N 52°25.057’	E 014°8.342’	23.92	0.38	23.61	0.06	0.01	0.00	0.99
20	N 52°27.854’	E 014°13.558’	48.25	3.78	43.25	0.19	0.01	0.00	0.90
21	N 52°25.408’	E 014°14.110’	10.70	0.36	9.14	0.03	0.01	0.00	0.86
22	N 52°24.324’	E 014°12.300’	14.47	0.12	15.58	0.13	0.12	0.01	1.09
23	N 52°22.453’	E 014°13.211’	12.33	0.96	12.35	0.10	0.01	0.00	1.00
24	N 52°21.950’	E 014°13.373’	18.17	0.67	17.97	0.06	0.04	0.01	0.99
25	N 52°13.588’	E 014°03.011’	21.33	1.44	17.39	0.05	0.03	0.00	0.82
26	N 52°13.335’	E 014°03.313’	23.92	0.72	21.76	0.06	0.01	0.00	0.91
27	N 52°10.621’	E 014°10.801’	16.67	0.87	14.50	0.02	0.01	0.00	0.87
28	N 52°26.317’	E 014°15.670’	24.83	0.80	22.80	0.00	0.03	0.00	0.92
29	N 52°34.898’	E 013°44.837’	19.13	0.76	18.37	0.03	0.01	0.00	0.96
30	N 52°38.779’	E 013°42.647’	19.53	2.73	20.52	0.03	0.06	0.00	1.05
31	N 52°25.879’	E 014°8.340’	27.25	0.25	27.99	0.00	0.05	0.00	1.03
32	N 52°25.057’	E 014°8.341’	23.75	0.43	23.75	0.06	0.09	0.00	1.00
33	N 52°23.467’	E 014°12.694’	5.29	0.18	3.69	0.00	0.06	0.01	0.71
34	N 52°20.726’	E 014°11.814’	1.67	0.09	0.09	0.00	0.14	0.00	0.14
35	N 52°17.664’	E 014°02.824’	1.39	0.04	0.17	0.00	0.16	0.01	0.24
36	N 52°17.679’	E 014°02.996’	0.35	0.02	0.01	0.00	0.01	0.00	0.07
37	N 52°17.689’	E 014°03.006’	0.36	0.03	0.01	0.00	0.01	0.00	0.06
38	N 52°17.697’	E 014°03.018’	0.58	0.01	0.01	0.00	0.01	0.00	0.05
39	N 52°17.715’	E 014°03.056’	0.52	0.01	0.06	0.00	0.01	0.00	0.15
40	N 52°35.251’	E 013°44.075’	1.31	0.01	0.90	0.00	0.01	0.00	0.70
41	N 52°30.626’	E 014°4.956’	1.33	0.03	0.00	0.00	0.04	0.01	0.03
42	N 52°30.649’	E 014°5.000’	2.85	0.07	0.98	0.00	0.05	0.00	0.36
43	N 52° 28.803’	E 13° 57.841’	1.19	0.05	0.92	0.00	0.12	0.00	0.88
44	N 52°20.659’	E 013°47.825’	1.26	0.00	0.04	0.00	0.06	0.00	0.08
45	N 52°20.549’	E 013°48.063’	0.36	0.05	0.00	0.00	0.01	0.00	0.04
46	N 52°27.127’	E 013°56.855’	1.49	0.09	1.46	0.00	0.01	0.00	0.99
47	N 52°27.111’	E 013°56.824’	1.01	0.08	1.05	0.00	0.01	0.00	1.05
48	N 52°26.899’	E 013°56.445’	0.64	0.03	0.58	0.00	0.01	0.00	0.93

Site	Latitude	Longitude	TDN mean	TDN SD	NO_3^- mean	NO_3^- SD	NH_4^+ mean	NH_4^+ SD	DIN:TDN
			$mg\,N\,L^{-1}$						
49	N 52°30.971'	E 013°50.677'	0.57	0.07	0.27	0.00	0.12	0.00	0.68
50	N 52°31.448'	E 013°51.030'	0.35	0.04	0.11	0.00	0.03	0.00	0.40
51	N 52°31.520'	E 013°51.299'	0.32	0.04	0.01	0.00	0.01	0.00	0.08
52	N 52°19.858'	E 013°43.892'	0.44	0.03	0.05	0.00	0.08	0.00	0.29
53	N 52°20.156'	E 013°43.906'	0.30	0.02	0.00	0.00	0.01	0.00	0.05
54	N 52°18.575'	E 013°45.497'	0.54	0.01	0.10	0.00	0.06	0.00	0.30
55	N 52°18.702'	E 013°49.164'	0.82	0.06	0.00	0.00	0.07	0.00	0.09
56	N 52°19.280'	E 013°54.124'	1.32	0.02	0.29	0.00	0.10	0.00	0.29
57	N 52°9.722'	E 013°34.849'	0.38	0.02	0.01	0.00	0.03	0.00	0.09
58	N 52°21.318'	E 013°48.408'	0.83	0.02	0.03	0.00	0.17	0.00	0.24
59	N 52°23.972'	E 013°43.740'	0.80	0.03	0.03	0.00	0.04	0.00	0.08
60	N 52°21.815'	E 013°47.768'	0.20	0.01	0.01	0.00	0.01	0.00	0.11
61	N 52°22.154'	E 013°47.465'	0.39	0.02	0.05	0.00	0.03	0.00	0.21
62	N 52°18.913'	E 014°06.878'	0.86	0.05	0.74	0.00	0.01	0.00	0.88
63	N 52°6.564'	E 013°36.716'	0.21	0.03	0.03	0.00	0.01	0.00	0.19
64	N 52°10.452'	E 013°59.759'	0.20	0.02	0.03	0.00	0.05	0.00	0.38
65	N 52°10.452'	E 013°59.759'	1.09	0.02	1.06	0.00	0.01	0.00	0.99
66	N 52°6.432'	E 014°28.787'	3.57	0.19	3.39	0.09	0.01	0.00	0.95
67	N 52°6.432'	E 014°28.787'	0.88	0.07	0.86	0.02	0.06	0.00	1.04
68	N 52°6.432'	E 014°28.787'	4.01	0.02	4.15	0.01	0.01	0.00	1.04
69	N 52°6.103'	E 014°28.949'	2.80	0.23	2.94	0.05	0.09	0.00	1.08
70	N 52°6.103'	E 014°28.949'	4.64	0.27	5.10	0.07	0.01	0.00	1.10
71	N 52°6.103'	E 014°28.949'	0.29	0.04	0.00	0.00	0.16	0.00	0.57
72	N 52°6.187'	E 014°28.819'	3.76	0.02	3.89	0.06	0.01	0.00	1.04
73	N 52°12.433'	E 012°19.979'	0.17	0.02	0.15	0.16	0.01	0.00	0.93
74	N 52°12.414'	E 012°19.949'	0.85	0.02	0.84	0.01	0.01	0.00	1.01
75	N 52°12.380'	E 012°27.289'	0.93	0.02	1.00	0.03	0.01	0.00	1.09
76	N 52°14.118'	E 012°32.802'	0.10	0.01	0.02	0.00	0.01	0.00	0.38
77	N 52°14.118'	E 012°32.802'	0.08	0.01	0.00	0.00	0.07	0.00	0.94
78	N 50°44.557	E 013°43.421	1.07	0.02	1.17	0.01	0.01	0.00	1.11
79	N 50°43.254	E 013°41.710	0.43	0.01	0.50	0.00	0.01	0.00	1.19
80	N 50°44.449	E 013°40.978	0.79	0.03	0.91	0.01	0.03	0.00	1.19
81	N 52°25.208'	E 014°13.678'	14.40	0.26	13.81	0.13	0.04	0.01	0.96
82	N 52°24.312'	E 014°12.773'	7.95	0.44	6.61	0.10	0.05	0.00	0.84
83	N 52°22.208'	E 014°11.689'	10.60	0.36	10.41	0.03	0.08	0.00	0.99
84	N 52°20.642'	E 014°11.330'	10.87	0.71	9.89	0.06	0.12	0.01	0.92
85	N 52°15.508'	E 014°04.504'	0.34	0.02	0.14	0.00	0.03	0.00	0.49
86	N 52°34.755'	E 014°5.994'	0.66	0.03	0.58	0.00	0.04	0.01	0.94
87	N 52°30.588'	E 013°49.492'	0.43	0.01	0.33	0.00	0.01	0.00	0.78
88	N 52°30.556'	E 013°49.458'	0.80	0.02	0.71	0.00	0.01	0.00	0.91
89	N 52°7.574'	E 014°28.161'	1.53	0.06	1.61	0.02	0.01	0.00	1.06
90	N 52°7.001'	E 014°28.721'	0.29	0.02	0.25	0.00	0.01	0.00	0.92
91	N 52°6.441'	E 014°28.703'	1.73	0.20	1.87	0.01	0.01	0.00	1.09
92	N 52°6.741'	E 014°28.926'	1.83	0.17	2.06	0.01	0.01	0.00	1.14
93	N 52°6.672'	E 014°28.928'	2.70	0.19	3.00	0.13	0.01	0.00	1.12
94	N 52°6.074'	E 014°28.922'	1.70	0.13	1.92	0.04	0.01	0.00	1.14
95	N 52°5.834'	E 014°29.113'	0.22	0.02	0.08	0.00	0.01	0.00	0.43
96	N 52°11.907'	E 012°30.384'	0.29	0.02	0.28	0.28	0.04	0.00	1.09
97	N 52°14.127'	E 012°32.856'	0.98	0.01	1.17	1.17	0.01	0.00	1.21
98	N 50°44.391	E 013°43.474	1.13	0.03	1.18	0.00	0.01	0.00	1.06
99	N 50°48.321	E 013°36.410	1.67	0.03	1.76	0.01	0.01	0.00	1.06

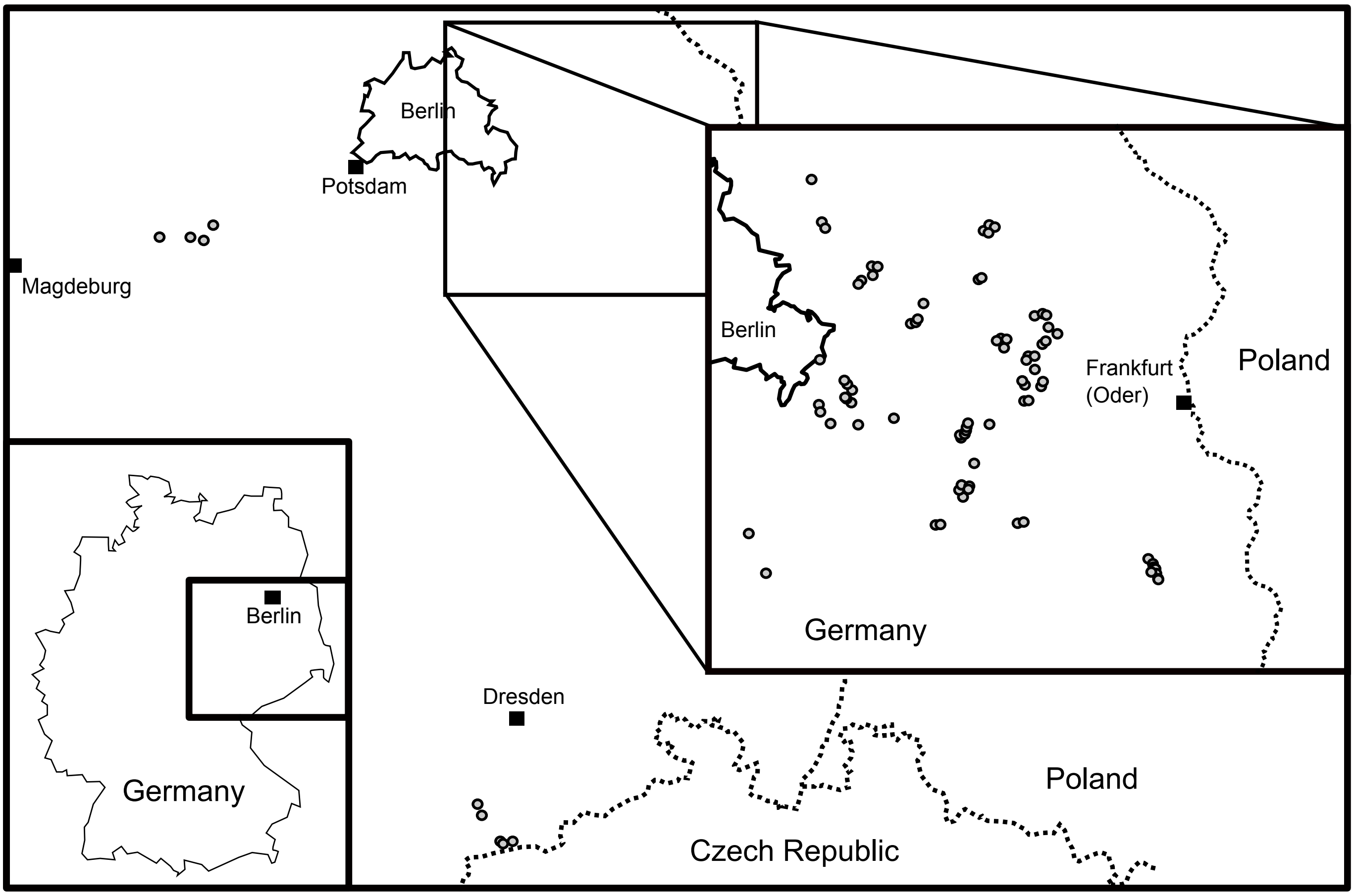

Figure 6: Map of sampling sites of the screening. The sampling sites are indicated by small circles. Some sites were close to each other and hence are only visible as one circle. Please see Table 1 for the exact geographical coordinates.

3 Effects of different numbers of measurement replicates

3.1 Background

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 have reported these values, but we decided to include all available replicates in order to improve data quality." In this addition to the supplement, 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 this study, DON concentration for the indirect standard method were calculated as either DON = total dissolved nitrogen (TDN) - (NO_3^- + NO_2^-) - NH_4^+ or as DON = TDN - (NO_3^- + NO_2^-), 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, NO_3^- + NO_2^- and NH_4^+ . In this addition to the supplement and to comment on 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.

3.2 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 described 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, NO_3^- + 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 follows: 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 the boostrap estimate of the 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).

3.3 Results and discussion

3.3.1 Nitrate + nitrite

For different replicate numbers in (NO_3^- + NO_2^-) only very small and unsystematic changes in the mean occurred (Table 2). For the standard deviation, as slight increase was found with increasing numbers of measurement replicates (Table 2).

The bootstrap 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, we have to assume a slightly higher uncertainty in the comparison to the other samples measured with more measurement replicates for these measurement. However, since we used 99 independent replicates for the screening of ambient samples, we believe that our conclusions on the relationship between DON determination accuracy and DIN:TDN ratio are justified. For all other samples, we used either 5 or 6 measurement replicates for (NO_3^- + NO_2^-). As can be seen from the samples with 6 measurement replicates, the difference in accuracy of the mean and standard deviation is very low for these samples. Both change by a factor of roughly 1.1 due to different replicate numbers (Table 2).

In two samples, NO_3^- was very low or below detection limit, respectively. For these, the boostrap SEs are very low (Table 2).

Table 2: Mean, standard deviation and bootstrap standard error (SE) of the accuracy of the two statistical estimates for NO_3^- 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 + NH_4^+	2	0.005	0.000	0.000	0.000
NOM + NH_4^+	3	0.005	0.000	0.000	0.000
NOM + NH_4^+	4	0.005	0.000	0.000	0.000
NOM + NH_4^+	5	0.005	0.000	0.000	0.000
NOM + NH_4^+ + NO_3^-	2	2.411	0.034	0.036	0.031

Sample	n	Mean conc.	Boostrap SE of mean conc.	Standard deviation	Boostrap SE of standard deviation
NOM + NH_4^+ + NO_3^-	3	2.412	0.027	0.042	0.023
NOM + NH_4^+ + NO_3^-	4	2.412	0.024	0.044	0.018
NOM + NH_4^+ + NO_3^-	5	2.412	0.022	0.046	0.016
NOM + NH_4^+ + NO_3^-	6	2.412	0.020	0.046	0.014
NOM + NO_3^-	2	4.703	0.058	0.064	0.053
NOM + NO_3^-	3	4.704	0.048	0.074	0.037
NOM + NO_3^-	4	4.703	0.041	0.077	0.029
NOM + NO_3^-	5	4.703	0.037	0.079	0.023
NOM + NO_3^-	6	4.704	0.034	0.080	0.020

3.3.2 Ammonium

For different sample sizes of NH_4^+ measurements, changes in SDs and SEs only occurred for 4 samples, since the NH_4^+ concentrations of 2 samples were below detection limit (Table 3, ditch and NOM+NO_3^-). For the samples, in which NH_4^+ occurred in measurable concentrations (Table 3), similar patterns as for NO_3^- were observed (see section 3.3.1). Thus, effects of different sample sizes on NH_4^+ measurements were also negligible and did not affect the conclusions of our study.

Table 3: Mean, standard deviation and bootstrap standard error (SE) of the accuracy of the two statistical estimates for NH_4^+ 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 + NH_4^+	2	4.5662	0.0328	0.0351	0.0302
NOM + NH_4^+	3	4.5666	0.0270	0.0403	0.0220
NOM + NH_4^+	4	4.5660	0.0230	0.0430	0.0171
NOM + NH_4^+	5	4.5661	0.0206	0.0441	0.0141
NOM + NH_4^+ + NO_3^-	2	2.4122	0.0251	0.0274	0.0227
NOM + NH_4^+ + NO_3^-	3	2.4130	0.0203	0.0319	0.0159
NOM + NH_4^+ + NO_3^-	4	2.4126	0.0178	0.0337	0.0119
NOM + NO_3^-	2	0.0150	0.0000	0.0000	0.0000
NOM + NO_3^-	3	0.0150	0.0000	0.0000	0.0000
NOM + NO_3^-	4	0.0150	0.0000	0.0000	0.0000
NOM + NO_3^-	5	0.0150	0.0000	0.0000	0.0000

3.3.3 Total dissolved nitrogen

For different replicate numbers of TDN measurements, similar differences as for nitrate and ammonium were observed (sections 3.3.1 and 3.3.2). 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 4).

Also, changes of the bootstrap standard errors (SE) were similar to that of NO_3^- and NH_4^+ measurements for the comparison of 2, 5 and 6 measurement replicates (see section 3.3.1 for details). One sample was accidently measured twice for TDN (NOM). Thus , we measured 11 replicates for this sample (Table 4). For this sample, the bootstrap SE changed by a factor of 2.4 for the mean and by a factor of 2.7 for the standard deviation. Between 6 and 11 measurement replicates differences in bootstrap SE were lower and the bootstrap SE of the mean and standard deviation changed by a factor of 1.4 only.

For the NOM sample, the increase in the calculated standard deviation was very low when comparing 6 and 11 measurement replicates. It was 0.070 for 6 measurement replicates and 0.072 for 11 measurement replicates.

Table 4: 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 + NH_4^+	2	6.971	0.463	0.503	0.427
NOM + NH_4^+	3	6.958	0.374	0.578	0.308
NOM + NH_4^+	4	6.959	0.325	0.610	0.241
NOM + NH_4^+	5	6.967	0.293	0.621	0.200
NOM + NH_4^+	6	6.966	0.269	0.629	0.173
NOM + NH_4^+ + NO_3^-	2	7.331	0.244	0.263	0.222
NOM + NH_4^+ + NO_3^-	3	7.328	0.198	0.312	0.152
NOM + NH_4^+ + NO_3^-	4	7.331	0.173	0.327	0.111
NOM + NH_4^+ + NO_3^-	5	7.330	0.155	0.334	0.086
NOM + NO_3^-	2	6.964	0.251	0.270	0.220
NOM + NO_3^-	3	6.960	0.203	0.309	0.162
NOM + NO_3^-	4	6.962	0.176	0.323	0.133
NOM + NO_3^-	5	6.965	0.155	0.332	0.112
NOM + NO_3^-	6	6.961	0.142	0.337	0.103

3.4 Conclusions

This analysis shows that different numbers of measurement replicates did not affect the conclusions of our study. 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 low when comparing 2 and 6 measurement replicates and very low, when comparing 5 and 6 or 6 and 11 measurement replicates.

Thus, for the samples, which were measured with 5 or more measurement replicates we do not expect effects on the results of the Monte-Carlo simulations (which are based on mean and SD) according to the above shown analysis.

This analysis indicates that 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 only – 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=2–3). Since we found the lowest standard deviations in our comparison analysis (Tables 2, 3.3.2, 4) with 2 measurement replicates, we probably still underestimated the uncertainty in the DON determinations by the standard method in the screening. Thus, our main conclusions are valid.