

**Direct dissolved
organic nitrogen
measurement**

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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

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Abstract

Research on dissolved organic nitrogen (DON) in aquatic systems with high dissolved inorganic nitrogen (DIN, the sum of NO_3^- , NO_2^- and NH_4^+) concentrations is often hampered by high uncertainties of the determined DON concentration. The reason is that DON is determined indirectly as the difference between total dissolved nitrogen (TDN) and DIN. In this standard approach to determine DON concentrations, even small relative measurement errors of the DIN and TDN concentrations propagate into high absolute errors of DON concentrations at high DIN:TDN ratios. To improve the DON measurement accuracy at high DIN:TDN ratios, we investigated the DON measurement accuracy of this standard approach in dependence of DIN:TDN ratio and compared it to the direct measurement of DON by size-exclusion chromatography (SEC). For this, we used standard compounds and natural samples with and without DIN enrichment. We show that for the standard approach, large errors of the determined DON concentrations at DIN:TDN ratios >0.6 occur for both standard compounds and natural samples. In contrast, measurements of DON by SEC always resulted in low errors at high DIN:TDN ratios due to the successful separation of DON from DIN. For SEC, DON recovery rates were 90.7–107.9 % for five pure standard compounds and 89–103 % for two standard compounds, enriched with DIN. Moreover, SEC resulted in 93–101 % recovery rates for DON concentrations of natural samples at a DIN:TDN ratio of 0.8. With 2.5 h of measurement time per sample, SEC is a moderately fast and accurate alternative to the standard approach for the determination of DON concentrations in freshwaters with DIN:TDN ratios >0.6 . The direct DON measurement by SEC will enable the scientific community to gather accurate information on DON concentrations, especially in anthropogenically disturbed systems with high DIN concentrations.

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1 Introduction

Dissolved organic nitrogen (DON) constitutes an important pool of nitrogen in freshwater (Jørgensen, 2009) and marine ecosystems (Berman and Bronk, 2003). DON may also play an important role in agricultural catchment nitrogen fluxes. For example, the mean agricultural soil leachate DON export reported in 16 studies was $12.7 \text{ kg ha}^{-1} \text{ yr}^{-1}$ and made up 26 % of the total dissolved nitrogen (TDN) export (van Kessel et al., 2009). However, research on DON in agricultural and otherwise anthropogenically-influenced systems is often hampered by high concentrations of dissolved inorganic nitrogen (DIN, the sum of NO_3^- , NO_2^- and NH_4^+) which can strongly reduce the measurement accuracy of DON at high DIN:TDN ratios (e.g., Lee and Westerhoff, 2005).

The reason for this reduced measurement accuracy is that TDN, NO_3^- , NO_2^- , and NH_4^+ need to be measured to calculate DON as $\text{DON} = \text{TDN} - (\text{NO}_3^- + \text{NO}_2^-) - \text{NH}_4^+$. In this standard approach, the subtraction of multiple independent measurements propagates the analytical variance of the single measurements into the finally determined DON concentration and at a high DIN:TDN ratio, small errors of TDN or DIN measurements can result in a high error of the calculated DON concentration (Lee and Westerhoff, 2005). Such high errors at high DIN:TDN ratios were found for DON concentrations in forest soils (Vandenbruwane et al., 2007) and canal water (Lee and Westerhoff, 2005). Further errors may be a result of systematic under- or overestimation of the single measurements needed to determine the DON concentration. For example, a systematic underestimation of TDN concentrations may result from incomplete conversion of the different nitrogen forms into the measured form (e.g. NO_x gas in high-temperature catalytic oxidation, Bronk et al., 2000). Also, systematic errors of up to 2 and 4 % were reported for standard spectrophotometric measurements of $\text{NO}_3^- + \text{NO}_2^-$ (Rutkoviene et al., 2005) and NH_4^+ (Verdouw et al., 1978), respectively. Under- or overestimations of these single measurements can even lead to negative determined DON concentrations as was shown in two studies of agricultural and forest soils, in which 15% and 13% of the determined DON concentrations were negative, respectively

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(Siemens and Kaupenjohann, 2002; Solinger et al., 2001). But even if determined DON concentrations are positive, they still might be prone to under- or overestimations which are only detectable with high effort due to the different potential measurement errors of the independent measurements.

5 A possibility to reduce DON determination errors would be the removal of DIN prior to analysis or the direct measurement of DON concentrations. Removal of DIN reduces the errors propagated into DON concentrations because the error of the DON concentration should only or, if some DIN is left, nearly only depend on the error of the TDN measurement (Crumpton et al., 1992; Lee and Westerhoff, 2005). Moreover, the direct
10 measurement of DON would also result in reduced errors, because propagation of errors associated with TDN and DIN measurements would not apply. However, until now, there is no direct method for DON quantification (Worsfold et al., 2008).

Two approaches to remove DIN prior to indirect DON measurements have been proposed in the literature: anion-exchange and dialysis pretreatment. Anion-exchange pretreatments were used to remove NO_3^- and NO_2^- from standard compounds in order to increase DON measurement accuracy (Crumpton et al., 1992; Schlueter, 1977).
15 However, this method cannot be applied for removal of NH_4^+ (Crumpton et al., 1992; Schlueter, 1977). Dialysis pretreatment was shown to be a simple and cheap method to reduce DIN concentrations in order to reliably increase the measurement accuracy
20 of DON (Lee and Westerhoff, 2005). Different variants of this method have been shown to work for forest sites (Vandenbruwane et al., 2007) and several surface water sites (Lee and Westerhoff, 2005). However, the dialysis pretreatment is time consuming, because the removal of DIN takes minimum 24–48 h (Lee and Westerhoff, 2005; Vandenbruwane et al., 2007). Another disadvantage of the dialysis pretreatment is the
25 potential loss of a part of the DON during dialysis (Lee and Westerhoff, 2005). Potential loss mechanisms are adsorption to the dialysis membrane, loss of small molecules that can permeate through the pores of the dialysis membrane (Lee and Westerhoff, 2005) and uptake of bioavailable molecules by bacteria (Vandenbruwane et al., 2007). Moreover, both anion-exchange and dialysis pretreatment still rely on the subsequent

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indirect determination of DON by the standard approach, as often DIN is not completely removed from the sample and NH_4^+ is principally not removed by anion-exchange pretreatment.

A potential direct method to measure DON could be size-exclusion chromatography (SEC). SEC separates molecules by molecular size and polarity (Huber et al., 2011b). This separation should allow for the direct measurement of DON at high NO_3^- , NO_2^- and NH_4^+ concentrations, as NO_3^- , NO_2^- and NH_4^+ are separated from DON by their smaller molecular size (Huber et al., 2011b). Hence, this method would overcome the aforementioned problems of indirect DON determination at high DIN:TDN ratios. Moreover, SEC could be superior to the anion-exchange pretreatment, which cannot remove NH_4^+ from a sample and should be faster than the dialysis pretreatment, as one sample can be measured within 2.5 h (Huber et al., 2011b). However, it has not yet been tested whether SEC efficiently separates DON and DIN by molecular size and thus can be used to directly measure DON. Moreover, the SEC utilizes a UV reactor to oxidize all nitrogen to NO_3^- , which is then measured by an UV detector (Huber et al., 2011b). The oxidation efficiency of the UV reactor and accuracy of the UV detector used in SEC systems have to be tested thoroughly for various DON standard compounds and natural substances, because UV oxidation methods may result in low DON recovery rates (Bronk et al., 2000) and the accuracy of the UV detector has previously only been tested for pure standard compounds (Huber et al., 2011b).

The aim of this study is to assess errors of DON measurements using the standard approach and to compare these to the novel, direct DON measurement by SEC. We used both model DON substances and natural samples from surface waters for the comparison.

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2 Materials and methods

2.1 Measurements of total dissolved nitrogen, $\text{NO}_3^- + \text{NO}_2^-$ and NH_4^+ for the standard approach

We measured the concentrations of TDN (determination limit = 0.1 mg NI^{-1}) using High Temperature Catalytic Oxidation (HTCO, multi N/C 3100, Jena Analytik), after acidifying the sample to pH 2–3 with HCl and sparging for 5 min with synthetic air. Samples were oxidized with a platinum catalyst at 700°C in a synthetic air stream, and TDN was measured as NO_x gas with a chemiluminescence detector. These measurements are hereafter referred to as HTCO-TDN measurements. $\text{NO}_3^- + \text{NO}_2^-$ (determination limit = 0.01 mg NI^{-1}) and NH_4^+ (determination limit = 0.03 mg NI^{-1}) were measured using standard spectrophotometric methods (ISO 13395 for $\text{NO}_3^- + \text{NO}_2^-$ and ISO 11732 for NH_4^+) with a SAN++ continuous flow analyzer (Skalar Analytical B.V., Breda, Netherlands).

2.2 Size-exclusion chromatography

For size-exclusion chromatography (SEC), we used the Liquid Chromatography–Organic Carbon–Organic Nitrogen Detection system (determination limit = 0.05 mg NI^{-1}), manufactured by DOC-Labor Dr. Huber (Karlsruhe, Germany, www.doc-labor.de). The system is driven by an HPLC pump (S-100, Knauer, Berlin) and consists of an auto-sampler (MLE, Dresden, Germany) and a chromatographic column ($250 \text{ mm} \times 20 \text{ mm}$, TSK HW 50S, Toso, Japan).

Within the SEC, a part of each sample is measured after bypassing the chromatographic column and a second part is passing the chromatographic column. Both parts are measured at first by an UV detector (254 nm , S-200, Knauer, Berlin, Germany) and then the flow is further divided into two streams. One stream goes to a UV reactor to measure nitrogen. The UV reactor is a helical silica capillary of 4 m length and 1 mm inner diameter, fused into the electric discharge arc of a low-pressure mercury lamp

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(emitting at 185 and 254 nm, DOC-Labor, Karlsruhe). In this reactor, nitrogen is oxidized to NO_3^- , which is then measured with an in-line UV-detector at 220 nm (K-2001, Knauer, Berlin, Germany). The second stream goes to a thin-film reactor where DOC is oxidized to CO_2 and then goes to an infrared CO_2 detector (for details on this part of the SEC see Huber and Frimmel, 1991). The used mobile phase was a phosphate buffer of pH of 6.85 ($2.5 \text{ g KH}_2\text{PO}_4 + 1.5 \text{ g Na}_2\text{HPO}_4 \times 2\text{H}_2\text{O}$ to 1 l, Fluka, #30407 and #30412). See Huber et al. (2011b) for further details of the system.

In the the weak cation-exchange chromatographic column of the SEC, molecules are separated by molecular size and polarity (Huber et al., 2011b). The column has a separation range of 0.1 to 10 kDa, resulting in the fast elution of hydrophilic high-molecular weight substances of 10 kDa or higher. This fraction is followed by a fraction similar to extracted humic and fulvic acids. Subsequently low-molecular weight acids are eluted as a compressed peak ahead of NO_3^- and NH_4^+ . NO_3^- itself is separated from NH_4^+ by its polarity. The part of the nitrogen which elutes before NO_3^- or NH_4^+ will hereafter be referred to as SEC-DON.

For natural water samples, the nitrogen value measured in the chromatographic column bypass can exceed the value obtained for the hull curve of the chromatogram. The difference is organic matter that remains on the column (Huber et al., 2011b). For the samples of our study, we will either report this difference or, for standard compounds, the difference between known concentrations and concentrations measured as SEC-DON.

2.3 Calculation of errors and statistics

In order to calculate the random error of the determined DON concentration, we conducted Monte-Carlo (MC) simulations with 100 000 realizations with the mc2d package (version 0.1-9, Pouillot and Delignette-Muller, 2010) in R (version 2.14.2, R Development Core Team, 2012). For the MC simulations, normal distribution of the input variables was assumed. Depending on the experiment, the input variables were HTCO-TDN, NO_3^- and/or NH_4^+ and hence in the MC simulations, DON was calculated

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as $\text{DON} = \text{HTCO-TDN} - (\text{NO}_3^- + \text{NO}_2^-) - \text{NH}_4^+$, as $\text{DON} = \text{HTCO-TDN} - (\text{NO}_3^- + \text{NO}_2^-)$ or as $\text{DON} = \text{HTCO-TDN} - \text{NH}_4^+$. To investigate the source of the uncertainty of DON measurements, we used a sensitivity analysis (tornado function, mc2d package in R), which is a Pearson correlation between the variability of each of the input variables and the variability of the calculated DON concentration, whereby the variability of all other input variables is kept stable (Pouillot and Delignette-Muller, 2010).

Recovery rates were calculated as recovery rate (%) = $100 \times \text{measured concentration} / \text{true concentration}$. This calculation was done for various standard compounds with known DOC and DON concentrations and also for the natural samples for which the concentration could be measured with high accuracy (see Samples and treatments for details).

In order to test for significant differences between MC simulation results or to compare MC simulation results to directly measured SEC-DON concentrations, a pairwise, two-sided, exact bootstrap test with 9999 bootstrap resamples (Efron and Tibshirani, 1993) was conducted in R ($\alpha = 0.05$, see a detailed description in Appendix A). The reason for choosing this test was that MC simulations with 100 000 iterations deliver 100 000 simulated cases to test. However, the number of real measurement replicates for the input variables of the MC simulation (HTCO-TDN, DIN) was much smaller, depending on the experiment (5–11 replicates in our case). Hence, the bootstrap test was needed to test for significant differences between different treatments for a realistic number of measurement replicates (we used $n = 5$ or 6). To control for the α error in multiple pairwise comparisons, we used a Sequential Bonferroni correction (Quinn and Keough, 2002).

2.4 Samples and treatments

All natural samples used in the assessment were filtered by a $0.45 \mu\text{m}$ filter with $8 \mu\text{m}$ pre-filter prior to the measurements (cellulose-actetate membrane filters, Sartorius,

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Göttingen). Filters were rinsed with 1 l of DI water and 300 ml of sample water before filtration of the samples.

2.4.1 Errors of the standard approach

To assess the reliability of the standard approach in dependence on the DIN:TDN ratio of natural samples, we conducted a screening of 99 streams and rivers draining agricultural, forested and wetland catchments in Northeast Germany. For each of the screening samples, HTCO-TDN was measured with three measurement replicates, and $\text{NO}_3^- + \text{NO}_2^-$ as well as NH_4^+ were measured with two measurement replicates.

To test the recovery rates of the DON concentrations determined by the standard approach in dependence of DIN:TDN ratio, we used two standard compounds and one natural sample from a wetland outflow: L-tyrosine (100 % purity, Sigma Chemical CO, St. Louis, USA) and imidazole (>99 % purity, Merck, Darmstadt, Germany). Both standard compounds were enriched with NO_3^- (CertiPUR, Merck, Darmstadt, Germany) to reach DIN:TDN ratio levels of 0.2, 0.4, 0.5, 0.6, 0.7 and 0.8. In contrast, the natural sample was characterized by a high NH_4^+ concentration (0.6 mgNI^{-1}) compared to the TDN concentration (1.5 mgNI^{-1}); therefore, the NO_3^- enrichment started with a DIN:TDN ratio of 0.4. Samples were enriched to DIN:TDN ratios of 0.5, 0.6, 0.7, 0.8 and 0.9. At all levels of enrichment, the TDN concentration was 2 mgNI^{-1} for the standard compounds and 1.5 mgNI^{-1} for the natural sample. At each level of enrichment, $\text{NO}_3^- + \text{NO}_2^-$ and HTCO-TDN were measured for the standard compounds, and $\text{NO}_3^- + \text{NO}_2^-$, NH_4^+ and HTCO-TDN were measured for the natural sample. Each variable was measured with six measurement replicates at each level of enrichment.

Recovery rates for the standard compounds were calculated based on the known, true concentration and the recovery rate for the sample from the wetland outflow was calculated as the percent deviation from the concentration measured at a DIN:TDN ratio of 0.4. In these calculations, dilution of the sample by the added NO_3^- was included.

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No recovery rates were calculated for the samples of the screening due to unknown DON concentrations.

We used MC simulations to calculate the random error of the determined DON concentrations, as described previously. For the samples of the screening, the input variables for the MC simulation were H₂CO₃-TDN, NH₄⁺ and NO₃⁻ + NO₂⁻. For the standard compounds, the input variables were H₂CO₃-TDN and NO₃⁻ + NO₂⁻ and for the sample from the wetland outflow, the input variables were H₂CO₃-TDN, NH₄⁺ and NO₃⁻ + NO₂⁻. For all samples, we used the sensitivity analysis described previously to investigate the source of the random error for the determined DON concentrations.

2.4.2 Comparison of standard approach and size-exclusion chromatography

To compare the reliability of size-exclusion chromatography (SEC) to that of the standard approach across a range of concentrations (0.5, 4 and 8 mgNI⁻¹), we used four standard compounds: L-tyrosine, imidazole, nicotinic acid (>99% Merck, Darmstadt, Germany) and glycine (>99.7%, Merck). For these, SEC-DON as well as H₂CO₃-TDN were measured to compare both methods. Because we used pure standard compounds, H₂CO₃-TDN equals DON.

In a second step, we enriched L-tyrosine and imidazole with NO₃⁻ to reach DIN:TDN ratios of 0.2, 0.4, 0.5, 0.6, 0.7 or 0.8. TDN was kept stable at 2 mgNI⁻¹ at all levels of enrichment. SEC-DON, H₂CO₃-TDN and NO₃⁻ + NO₂⁻ were measured and DON was calculated by the standard approach from H₂CO₃-TDN and NO₃⁻ + NO₂⁻ measurements. For each of the enrichment levels, recovery rates were calculated as the difference between true concentrations of the standard compounds and the DON determinations by SEC-DON or the standard approach, respectively.

To compare the accuracy of the results from the standard approach and SEC-DON measurements for natural samples, we used natural organic matter (NOM) extracted by reverse osmosis from a pond with a high percentage of organic soils in the catchment. We measured pure NOM as SEC-DON and H₂CO₃-TDN and then added DIN to adjust

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the NOM sample to a DIN:TDN ratio of 0.8. For this either nitrate (NOM + NO_3^-) or ammonium (NOM + NH_4^+), or both (NOM + NH_4^+ + NO_3^-) were added, whereby the DON concentration was kept at the same level for all treatments. The treatments with added DIN were measured by SEC-DON and by the standard approach. Moreover, we measured a water sample from an agricultural stream and a water sample from an agricultural tile drain by SEC-DON and the standard approach to compare both. For the standard approach, H₄CO₃-TDN, NO_3^- + NO_2^- and NH_4^+ were measured with 6–11 measurement replicates for each of the 4 treatments (NOM, NOM + NH_4^+ , NOM + NH_4^+ + NO_3^- , NOM + NO_3^-). For the agricultural stream and tile drain H₄CO₃-TDN, NO_3^- + NO_2^- and NH_4^+ were measured with 5–8 measurement replicates. SEC-DON was measured with 5 measurement replicates for each of the NOM treatments, as well as the agricultural stream and tile drain.

An MC simulation, using the respective input variables (H₄CO₃-TDN, NO_3^- + NO_2^- and/or NH_4^+) was conducted for the NOM treatments with added NO_3^- and/or NH_4^+ as well as the agricultural tile drain and stream to determine the uncertainty of the DON concentration calculated by the standard approach. The pairwise bootstrap test described above was used to test for significant differences in the DON concentration between the NOM treatments for each the standard approach and SEC-DON. This test was also used to check for significant differences between the standard approach and SEC-DON measurement within each NOM treatment. For the agricultural stream and tile drain, the same test was used to test for differences in the DON concentration determined by the standard approach or SEC-DON within the sites.

3 Results

3.1 Errors of the standard approach

In the screening of 99 sites, the mean DIN:TDN ratio of all samples was 0.75, and above a DIN:TDN ratio of 0.8 the random error of the calculated DON concentration

was often high (Fig. 1). Moreover, some samples exhibited negative DON concentrations with DIN:TDN ratios >1 (Fig. 1). The sensitivity analysis of the MC simulation revealed a high correlation between the variability of DON and HTCO-TDN (mean $r = 0.94 \pm 0.18$ 1 SD, $n = 99$). In contrast, variability of DON correlated much less with that of $\text{NO}_3^- + \text{NO}_2^-$ (mean $r = -0.22 \pm 0.27$ 1 SD, $n = 99$) or NH_4^+ (mean $r = -0.08 \pm 0.1$ 1 SD, $n = 99$).

To investigate the recovery rate of the standard approach in dependence on DIN:TDN ratio, we used two standard compounds and one natural sample from a wetland outflow. The systematic error increased with the DIN:TDN ratio, and recovery rates deviated increasingly from 100 % with larger deviations when the DIN:TDN ratio was >0.6 – 0.8 (Fig. 2a). For the standard compounds (L-tyrosine, imidazole) DON was underestimated, and for the natural sample DON was overestimated.

As for the screening, a sensitivity analysis of the MC simulation was used to determine the source of this random error. It revealed a high correlation between the variability of the DON and the HTCO-TDN concentrations, with a minimum Pearson r of 0.88 for all samples and enrichment levels (Fig. 2b). In contrast, the correlation between DON and $\text{NO}_3^- + \text{NO}_2^-$ concentrations (L-tyrosine, imidazole, wetland outflow) or DON and NH_4^+ concentrations (wetland outflow) was weak (maximum of Pearson $r = -0.48$, Fig. 2b).

3.2 Comparison of standard approach and size-exclusion chromatography

For pure standard compounds, the range of recovery rates was 90.7–107.9 % (Table 1) for SEC-DON. In contrast, the range of recovery rates was 96.4–123.5 % for HTCO-TDN. The HTCO-TDN measurements especially overestimated concentrations at 0.5 mg NI^{-1} and the recovery rate was not improved by using calibration curves for low concentrations only (0.1 – 1 mg NI^{-1}). When excluding the measurements at 0.5 mg NI^{-1} , we found recovery rates between 96.4 and 104.8 % for HTCO-TDN.

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For the DIN-enriched standard compounds, the mean recovery rate of SEC-DON was often closer to 100 % than for the standard approach (Table 2). This was always the case at DIN:TDN ratios > 0.4.

In the tests of DIN-enriched natural organic matter (NOM), SEC clearly separated the peaks of DON from the ones of NO_3^- and NH_4^+ (Fig. 3). Moreover, we found low measurement errors (a low standard deviation of the error propagation) for the NOM samples with and without added DIN, as well as low measurement errors for the agricultural stream and tile drain (Fig. 4). In contrast, the standard approach resulted in high measurement errors for the NOM treatments with added DIN, as well as high measurement errors for the agricultural stream and tile drain (Fig. 4). Moreover for the agricultural stream, the SEC detected a DON concentration of 0.84 mg NI^{-1} , whereas with -0.94 mg NI^{-1} the DON concentration was wrongly determined by the standard approach (Fig. 4).

The recovery rates for the HTOCO-TDN measurements were calculated using the pure NOM treatment as reference. For the SEC-DON measurements, the recovery rates amounted to 93–101 % (Fig. 4a) for NOM treatments with added NO_3^- and/or NH_4^+ . In contrast, with recovery rates of 187–209 %, the standard approach substantially overestimated the DON concentration for the NOM treatments with added NO_3^- and/or NH_4^+ . These overestimations were significantly different from pure NOM for the NOM + NH_4^+ + NO_3^- and the NOM + NO_3^- treatment (Fig. 4a).

The main reason for the overestimation of DON concentrations for the NOM treatments was the overestimation of HTOCO-TDN. The recovery rates of the HTOCO-TDN concentrations were 123.1 % for both the NOM + NH_4^+ and the NOM + NO_3^- treatment and 135.2 % for the NOM + NH_4^+ + NO_3^- treatment when comparing them to the pure NOM treatment (data not shown). In contrast to the pure NOM treatment the treatments with added NO_3^- and NH_4^+ were measured diluted, with HTOCO-TDN concentrations of $0.70\text{--}0.76 \text{ mg NI}^{-1}$ for the diluted sample. At similarly low concentrations (0.5 mg NI^{-1}), the HTOCO-TDN measurement also resulted in overestimations of the TDN concentrations for the standard compounds (Table 1).

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The amount of nitrogen remaining on the SEC column was low for the standard compounds, as can be seen from their high recovery rates (Tables 1, 2). For the NOM treatments, the total nitrogen measured after SEC column passage was compared to the total nitrogen measured after bypassing the SEC column. Recovery rates of 93–104 % were found for this comparison (data not shown).

4 Discussion

4.1 Errors of the standard approach

The measurement error of DON is strongly influenced by the DIN:TDN ratio if DON is calculated as $\text{DON} = \text{TDN} - (\text{NO}_3^- + \text{NO}_2^-) - \text{NH}_4^+$ in the standard approach. Frequently this high error even lead to negative determined DON concentrations, yielding DIN:TDN ratios above 1. The error of the DON determination was increasing with DIN:TDN ratio due to the fact that similar errors of $\text{NO}_3^- + \text{NO}_2^-$, NH_4^+ and TDN measurements are larger relative to the DON concentration at higher DIN:TDN ratios. We showed that high errors of the determined DON concentrations are a common problem in the measurement of stream water samples at high DIN:TDN ratios >0.6 – 0.8 .

According to our data, the HTCO-TDN measurement is the main source error of DON concentrations determined by the standard approach. Similar relationships between the DIN:TDN ratio and the DON measurement error were found in another study using HTCO (Lee and Westerhoff, 2005), and also for persulfate digestion (Vandenberg et al., 2007). Hence, the problem of high DON random measurement errors seems to be a common problem for many types of TDN measurements, since even small random errors of TDN measurements propagate into high DON random errors at high DIN:TDN ratios. At low concentrations ($\approx 0.5 \text{ mg N l}^{-1}$), we consistently found an overestimation of HTCO-TDN measurements for standard compounds and natural samples. We could not determine the reason for this and even a calibration curve for low concentrations did not improve the recovery rates for the standard compounds.

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No such problems were found in previous publications on HTCO-TDN measurements (Bronk et al., 2000) which makes an unknown systematic measurement error of the HTCO measurements at low TDN concentrations likely for our study. However, the increasing error of the DON determination with increasing DIN:TDN ratio remains also without this error. According to our data, an accurate TDN measurement would strongly improve the accuracy of the DON determination by the standard approach. However, different TDN measurement techniques (Thermo Flash, HTCO, persulfate digestion) have not yet been systematically compared as to their ability to accurately determine DON concentrations in relation to varying DIN:TDN ratios for model and natural substances.

Already at a DIN:TDN ratio >0.6 , larger errors occurred for the determined DON concentration, a finding which is in accordance with another study (Lee and Westerhoff, 2005). The large errors of the DON concentration at high DIN:TDN ratios found in our study are a common problem that exists in several freshwater systems (Lee and Westerhoff, 2005; Siemens and Kaupenjohann, 2002; Solinger et al., 2001; Vandenbruwane et al., 2007). This suggests that future studies in freshwaters should apply measures to increase DON measurement accuracy at high DIN:TDN ratios.

4.2 Comparison of standard approach and size-exclusion chromatography

We could show that for pure standard compounds, size-exclusion chromatography (SEC) results in high recovery rates, similar to the measurements of TDN by HTCO. For standard compounds, high recovery rates of SEC were also shown by Huber et al. (2011b), even for EDTA which is considered to be difficult to be oxidized and hence to be measured (Bronk et al., 2000). Moreover, SEC resulted in high recovery rates of DON, irrespective of the DIN:TDN ratio for both standard compounds and NOM. These high recovery rates are a result of the clear separation of the DON peaks from the DIN peaks by the chromatographic column of the SEC. In addition, we could show that even in cases where no DON seems to exist in the sample according to the indirect determination by the standard approach, we found a well detectable DON concentration with the

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SEC. Overall, from these results, we clearly recommend SEC for DON measurement for samples with high DIN:TDN ratios (>0.6). In addition, the SEC not only separates DON from DIN but also allows to separate low- and high-molecular weight fractions within DON (Huber et al., 2011b). This can be used to assess DON composition for example in studies on sources and processing of DON in freshwaters.

With the mobile phase of the SEC used in our study, urea elutes together with NO_3^- and NH_4^+ and thus may falsely be attributed to DIN. However, for the analysis of urea, the same system setting can be used except for the mobile phase which is five times weaker in ionic strength. This results in a clearly resolved urea peak and allows a quantitative detection of urea concentrations (Huber et al., 2011a). Thus in freshwaters where urea is to be expected, this alternative setting should be used.

Within the SEC-DON measurement, a part of the DON can remain on the SEC column and is therefore not measured by the nitrogen detector (Huber et al., 2011b). In our study, we could not find a detectable influence of this potential measurement error since we have found no consistent underestimations of the SEC-DON concentrations for the standard compounds or the NOM treatments. Moreover, for the standard compounds, recovery rates of SEC-DON were similar or even closer to 100% than those of the HTCO-TDN measurements, implying that the error of the SEC-DON measurement was not increased by DON remaining on the SEC column. From these results, the amount of DON remaining on the column is small and does not affect the accuracy of the SEC-DON measurement. Moreover, in order to evaluate the quantity of DON remaining on the SEC column for future samples, the SEC system used in our study allows to measure the nitrogen in a sample after passing the column as well as bypassing the column.

The time of measurement for SEC-DON is higher (2.5 h per sample) than for the single measurements of the standard approach. However, for the standard approach three measurements (TDN , $\text{NO}_3^- + \text{NO}_2^-$, NH_4^+) need to be conducted to determine DON concentration for one sample. This, in connection with the preparation of the samples, as

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well as maintenance and calibration of the instruments, sums up to a amount of time which is roughly comparable to that of one SEC-DON measurement.

The price of the SEC system used in our study is higher than for standard TDN and DIN measurement equipment. This high price results mainly from the simultaneous oxidation of carbon and nitrogen, as well as the simultaneous detection of carbon, nitrogen and UV₂₅₄ with on-line detectors (Huber et al., 2011b). To reduce the costs of such a system for the purpose of direct DON measurement, a more simple system could be set up that only contains an auto-sampler, an auto-collector, a HPLC pump and a column. After column passage, water containing only DON could be collected and measured with a TDN analyzer, such as HTCO. However, such a system would have to be developed and tested, and especially the full separation of DON and DIN has to be assured. An alternative to SEC could be the application of the dialysis pretreatment which is cheaper and was also thoroughly tested but is more time consuming and can result in a loss of DON from the sample (Lee and Westerhoff, 2005; Vandenbruwane et al., 2007).

5 Conclusions

For high DIN:TDN ratios (>0.6), the standard approach results in high errors. Therefore, we strongly suggest that the standard approach is not used when high DIN:TDN ratios are to be expected and that future studies in freshwaters with high DIN:TDN ratios should apply measures to increase DON measurement accuracy. Moreover, we suggest that literature values of DON concentrations determined by the standard approach at high DIN:TDN ratios are regarded with caution.

We recommend SEC for DON measurement in samples with high DIN:TDN ratios (>0.6) because this technique is able to directly measure the DON concentration with high accuracy, which makes the DON determination independent from the concentration of DIN in the sample. With this novel technique, the scientific community will be

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able to gather more information on DON concentrations especially for anthropogenically disturbed systems such as freshwaters in agricultural and urban areas.

Appendix A

Description of two-sided, exact bootstrap pairwise test

5 The null hypothesis of the bootstrap pairwise test is that, with the probability β , the difference between the means of two samples is lower than the difference of two means randomly generated from the combination of the two distributions. In order to test this null hypothesis for a pair of samples with n and m replicates, the following steps are conducted to generate 9999 bootstrap resamples: n values are randomly sampled with
10 replacement from the first sample of the tested pair and m values are randomly sampled with replacement from the second sample of the tested pair. These are then merged to a list of $n + m$ values. From this list, two samples of the sizes n and m are sampled with replacement and the difference between their means is calculated. After generating the 9999 bootstrap resamples, the test statistic is calculated as follows
15 (Efron and Tibshirani, 1993):

$$\rho(\hat{\tau}) = \frac{1}{B} \cdot \sum_{j=1}^B I(|\tau_j| > |\hat{\tau}|)$$

$\rho(\hat{\tau})$ is the significance level, B is the number of bootstrap samples, τ_j is the difference of the random means calculated for each of the bootstrap resamples and $\hat{\tau}$ is the difference between the mean of the two samples. I is an indicator function, which is
20 1 if $\tau_j > \hat{\tau}$ or 0, if this is not the case. See “bootstrap_test.zip” in the Supplement for the R script.

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Supplementary material related to this article is available online at:
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Table 1. Measured concentrations and recovery rates of HTCO-TDN (6–15 measurement replicates) and SEC-DON (2–7 measurement replicates) for four standard compounds at three standard concentration levels (Std. conc.).

Std. compound	Std. conc. mgNI ⁻¹	HTCO-TDN		SEC-DON	
		mgNI ⁻¹	% Recovery	mgNI ⁻¹	% Recovery
Glycine	0.5	0.58	116.1	0.48	95.6
Glycine	4.0	3.87	96.7	3.75	93.8
Glycine	8.0	7.94	99.3	8.00	99.9
Imidazole	0.5	0.53	105.9	0.45	90.7
Imidazole	4.0	3.90	97.6	4.13	103.3
Imidazole	8.0	7.7	96.4	7.40	92.4
Nicotinic acid	0.5	0.58	117.0	0.53	105.2
Nicotinic acid	4.0	4.19	104.8	4.31	107.9
Nicotinic acid	8.0	8.29	103.6	8.18	102.3
L-tyrosine	0.5	0.62	123.5	0.50	100.4
L-tyrosine	4.0	3.97	99.1	4.04	101.0
L-tyrosine	8.0	8.24	103.0	8.36	104.5

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Table 2. Recovery rates (%) of DON standard compounds determined by the standard approach (Std. approach: H₂CO₃-TDN minus NO₃⁻, 6 measurement replicates) and SEC-DON measurements (2 measurement replicates) for L-tyrosine and imidazole at different DIN:TDN ratios, adjusted by the addition of NO₃⁻.

DIN:TDN ratio	Imidazole		L-tyrosine	
	Std. approach	SEC-DON	Std. approach	SEC-DON
0.2	102.2	94.1	102.0	92.9
0.4	92.1	99.2	96.1	96.9
0.5	98.8	100.4	86.6	90.1
0.6	93.4	99.6	86.5	89.3
0.7	71.5	102.1	85.7	97.5
0.8	90.6	102.9	90.6	96.9
Mean	91.4	99.7	91.2	93.9

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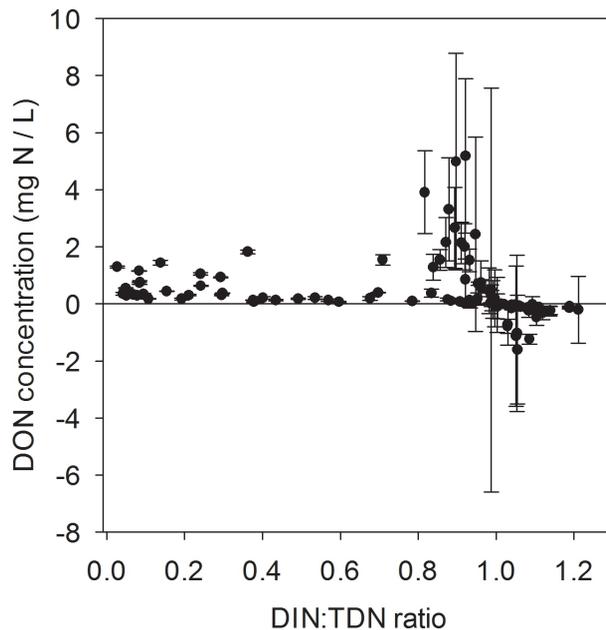


Fig. 1. Mean and 1SD of DON concentrations versus the DIN:TDN ratio measured by the standard approach (TDN – DIN) for the screening of 99 streams and rivers draining Northeastern German catchments. Means and uncertainties were determined by using MC simulations for the standard approach with 3 measurement replicates for HCO₃⁻-TDN and 2 measurement replicates for NO₃⁻ + NO₂⁻ and NH₄⁺.

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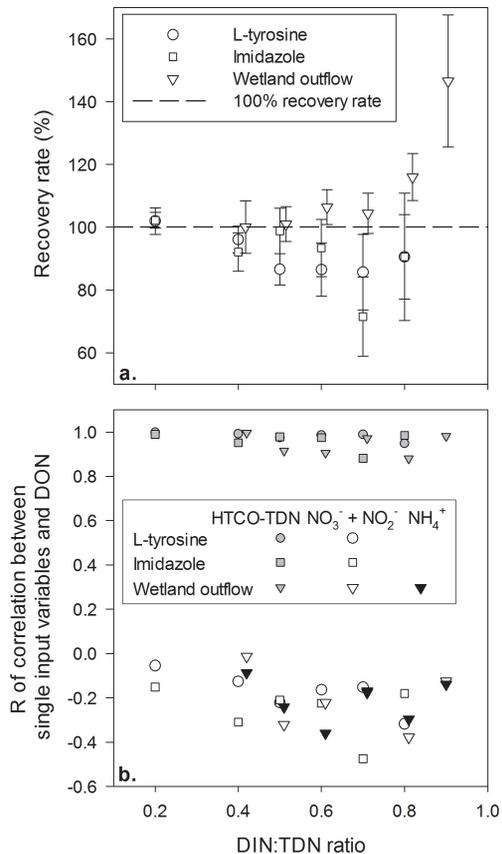


Fig. 2. Effect of NO_3^- enrichment on the recovery rate of DON (mean \pm 1 SD of 6 measurement replicates) determined by MC simulations for the standard approach (**a**) and results from the sensitivity analysis of the MC simulations (**b**). Correlation coefficients (R = Pearson r) are given for correlations between the variability of the input variables and the variability of the calculated DON concentration.

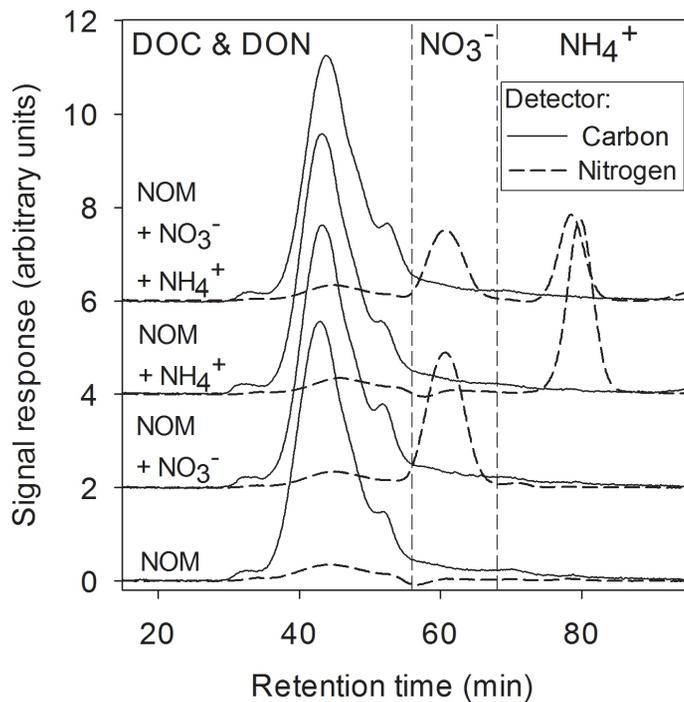


Fig. 3. Size-exclusion chromatograms of natural organic matter (NOM) extracted from a pond with added NO_3^- and/or NH_4^+ .

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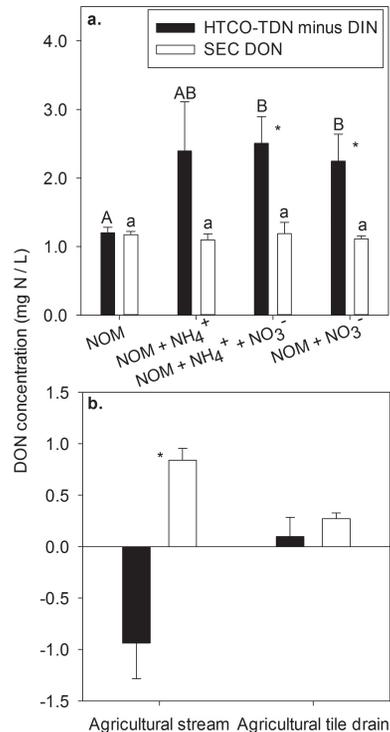


Fig. 4. Mean (\pm 1SD) of DON calculated by the standard approach (HTCO-TDN minus DIN, 6–11 measurement replicates) or measured by size-exclusion chromatography (SEC-DON, 5 measurement replicates). **(a)** Concentrations for the different natural organic matter (NOM) treatments are displayed, **(b)** shows concentrations for the agricultural stream and tile drain. Means and uncertainties of the standard approach were calculated by MC simulations. Letters indicate significant differences between the treatments: capital letters for the standard-approach determinations and small letters for the SEC-DON measurements. Asterisks indicate significant differences between the results from the standard-approach and SEC-DON determinations (pairwise bootstrap test).

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