

## Low volume quantification of dissolved organic carbon and dissolved nitrogen

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### Abstract

A method for the quantification of dissolved organic carbon (DOC) and total dissolved nitrogen (TDN) in aqueous samples of less than 600  $\mu\text{L}$  is described. Analysis of low volumes is achieved through modification of a Shimadzu HTC TOC analyzer, which in its conventional configuration requires at least 7 mL sample. The modification allows manual injection of sample directly into the high temperature combustion column, bypassing much of the instrument's dead volume. Instrument performance (blank, precision, and response factor) in the low volume configuration was equal to that of the instrument in its native larger volume, auto-analyzer configuration. The low volume modification is simple, low cost (less than €25) and reversible, taking a matter of minutes to install or remove. It allows routine high precision and accuracy measurement of DOC and TDN in cases where sample volume is limiting. When sample volumes are not limiting, the standard configuration of the Shimadzu is recommended due to the ability to use the auto-analyzer and reduce the labor required per sample run.

Dissolved organic matter (DOM) plays a major role in key biogeochemical processes, providing sustenance at the base of aquatic food webs and transporting carbon (C), nutrients, and trace elements between the land and sea. The pool of DOM in the oceans (~700 Pg C; Hedges 1992) holds approximately the same amount of C as is present in the atmospheric  $\text{CO}_2$  pool. Thus, minor changes in the dynamics of oceanic DOM can impact the global ecosystem, particularly the balance between ocean C-storage and atmospheric  $\text{CO}_2$  concentrations.

Due to the significance of DOM in the global C-cycle, a great deal of effort has been put into developing accurate, precise, and reproducible methods for the quantification of both dissolved organic carbon (DOC) and nitrogen (e.g., Menzel and Vaccaro 1964; Sharp 1973; Benner and Hedges 1993; Benner and Strom 1993; Hedges et al. 1993; Wangersky 1993; Sharp et al. 1995; Qian and Mopper 1996; Sharp et al. 2002a, 2002b). The labors of the past provide current researchers with instruments that are reliable and easy to use, facilitating the

wealth of studies that continue to improve our comprehension of the role of DOM and particulate organic matter (POM) in aquatic systems.

Today, the instruments most widely used for the measurement of DOC and total (i.e., dissolved and suspended) organic carbon (TOC) in marine and other saline waters are the Shimadzu series of high temperature catalytic (HTC) oxidation Total Organic Carbon (TOC) analyzers ([www.ssi.shimadzu.com](http://www.ssi.shimadzu.com)). These instruments use HTC oxidation to convert TOC to  $\text{CO}_2$ . The concentration of  $\text{CO}_2$  is then determined by infrared absorbance detection. The Shimadzu instruments are relatively simple to use and provide a level of oxidation efficiency, accuracy, and precision that is sufficient for the study of TOC dynamics in many environments (Wangersky 1993; Sharp et al. 1993; Hansell and Carlson 2002). With the addition of a Total Nitrogen Measuring unit (TNM-1; Shimadzu), these instruments can simultaneously determine TOC and total dissolved nitrogen (TDN).

The high sensitivity configurations of Shimadzu's TOC/TDN analyzers have quoted detection limits of 0.33  $\mu\text{M}$  C for TOC and 4.2  $\mu\text{M}$ -N for TDN in a sample volume as small as 100  $\mu\text{L}$ , where the sample volume is defined as the volume reaching the HTC oxidation column (TOC-V<sub>C<sub>PH</sub>/C<sub>PN</sub></sub> user's manual for TOC-Control V ver.2, Shimadzu). Despite this potential for small sample volume injections, in practice the plumbing of the instrument increases the required sample volume to at least 7 mL. The extra sample is required to rinse the portions

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### Acknowledgments

This work was supported by a Fellowship from the Hanse Institute for Advanced Studies (HWK, Delmenhorst, Germany) granted to Stubbins. Matthias Friebe provided dissolved organic carbon and total dissolved nitrogen standard curves acquired in the conventional, auto-analyzer configuration of the Shimadzu TOC analyzer.

DOI 10.4319/lom.2012.10.347

of the instrument, which routinely come into contact with the sample. The main components requiring rinsing are the instrument tubing and the “multi-function sample pretreatment/injection system” (2.1.7 Flow Diagram, TOC-V<sub>CPH/CPN</sub> user’s manual for TOC-Control V ver.2, Shimadzu).

Shimadzu offers both automatic and manual injection options. However, the instrument’s internal machinations are rinsed in both modes, such that the sample volume requirement remains at approximately 7 mL. It is possible to purchase a manual injection kit directly from Shimadzu that reduces sample volume requirements to 100  $\mu$ L. This unit costs approximately €360, and to the authors’ knowledge, no protocol or appraisal for the analysis of marine samples with this setup exists in the peer reviewed literature. Here we present a low cost (< €25) adaptation to a Shimadzu TOC analyzer (TOC-V<sub>CPH</sub>). The adaptation allows for the routine analysis of small sample volumes (tens of microliters depending upon sample OM concentrations). Analytical performance in this modified setup is equivalent to that acquired by using the Shimadzu TOC-V<sub>CPH</sub> in its native, automated, larger volume configuration.

## Materials

### Standards and deep seawater reference material

Standards for both DOC and TDN were prepared by the volumetric dilution of a stock solution containing 500  $\mu$ M-DOC (potassium hydrogen phthalate, Carl Roth Germany, p.a. quality) and 500  $\mu$ M-TDN (potassium nitrate, Carl Roth Germany, p.a. quality) to produce the following series of standards: 0, 2, 5, 8, 10, 25, 50, 75, 100  $\mu$ M-DOC and TDN. In addition to standards, deep seawater reference material from the Consensus Reference Material Project (CRM; <http://yyy.rsmas.miami.edu/groups/biogeochem/CRM.html>) was used to determine the precision and accuracy of the low volume DOC/TDN method for a well-studied environmental sample. The CRM sample analyzed came from Batch 10, Lot# 05-10. The consensus DOC and TDN concentrations for this sample were 41-44  $\mu$ M DOC and 31-33  $\mu$ M TDN, respectively.

### Instrumentation

A Shimadzu TOC-V<sub>CPH</sub> analyzer with a TNM-1 add-on at the Max Planck Research Group for Marine Geochemistry (Oldenburg, Germany) was used throughout. The TNM-1 unit is installed downstream of the TOC-V<sub>CPH</sub> and quantifies TDN through the chemiluminescent detection of nitrogen monoxide (NO) produced during the HTC of DOM. The TOC-V<sub>CPH</sub> analyzer with a TNM-1 add-on is routinely used in conjunction with a Shimadzu ASI-V autosampler in nonpurgable organic carbon (NPOC) mode, following the methodology recommended by Shimadzu. In routine running of the instrument, up to five replicate injections are made from each sample. Injections proceed until the coefficient of variation for three replicate injections is  $\leq 2\%$ . If this is not achieved, then the average for the three closest values from five replicate injections is reported. In this method, approximately 7 mL

sample is consumed, the bulk of which is used to rinse the tubing, pumping, and degassing apparatus of the instrument.

## Procedures

### Sample degassing

As the Shimadzu TOC-V<sub>CPH</sub> oxidizes TOC to CO<sub>2</sub> and then quantifies the latter, inorganic carbon must be removed from the sample before HTC oxidation. This is achieved by acidifying the sample to pH 2 with concentrated hydrochloric acid (Carl Roth Germany, p.a. quality) as recommended by Shimadzu (TOC-V<sub>CPH/CPN</sub> user’s manual for TOC-Control V ver.2, Shimadzu). Hydrochloric acid can be added upon sample collection, as is often the case for marine samples, or the TOC-V<sub>CPH</sub> can make the addition as part of the automatic sample analysis. Acidification causes carbonic acid in the sample to deprotonate to dissolved CO<sub>2</sub>, which is then purged from the sample with a carbon clean stripping gas. In the routine setup of the TOC-V<sub>CPH</sub>, CO<sub>2</sub> is purged from the sample by the instrument in its injection syringe (~5 mL). The flushing and filling of this syringe is one reason the instrument consumes sample volumes greater than those injected into the HTC reactor. For the small volume adaptation, acidification and degassing of approximately 600  $\mu$ L standards and CRM seawater were conducted offline in precombusted (6 h at 450°C) 2 mL Wheaton V glass vials (Fig. 1). To acidify samples concentrated hydrochloric acid was added to the 600  $\mu$ L sample using a 0.5  $\mu$ L syringe with a 0  $\mu$ L dead volume (7000 series, Hamilton) until sample pH dropped to approximately pH 2 (determined



**Fig. 1.** Purging of CO<sub>2</sub> from a 600  $\mu$ L sample.

by using the 0.5  $\mu\text{L}$  syringe and pH paper). For open ocean samples, this requires addition of approximately 0.6  $\mu\text{L}$  concentrated hydrochloric acid (i.e., 1 part acid to 1000 parts seawater). Volumes of less than 0.05  $\mu\text{L}$  are readily dispensed with sufficient accuracy to allow adjustment of pH in less buffered waters. Carbon-free Argon gas (Air Liquide Germany, 5.0 quality) was then bubbled from the base of the sample through a 1/16-inch PEEK tube. The flow rate was set to provide the maximum flow of bubbles achievable without causing the sample to overflow (Fig. 1). A test was conducted to determine the time required to strip  $\text{CO}_2$  from a sample of the deep sea reference material before immediate injection into the HTC reactor.

#### Small volume adapter assembly

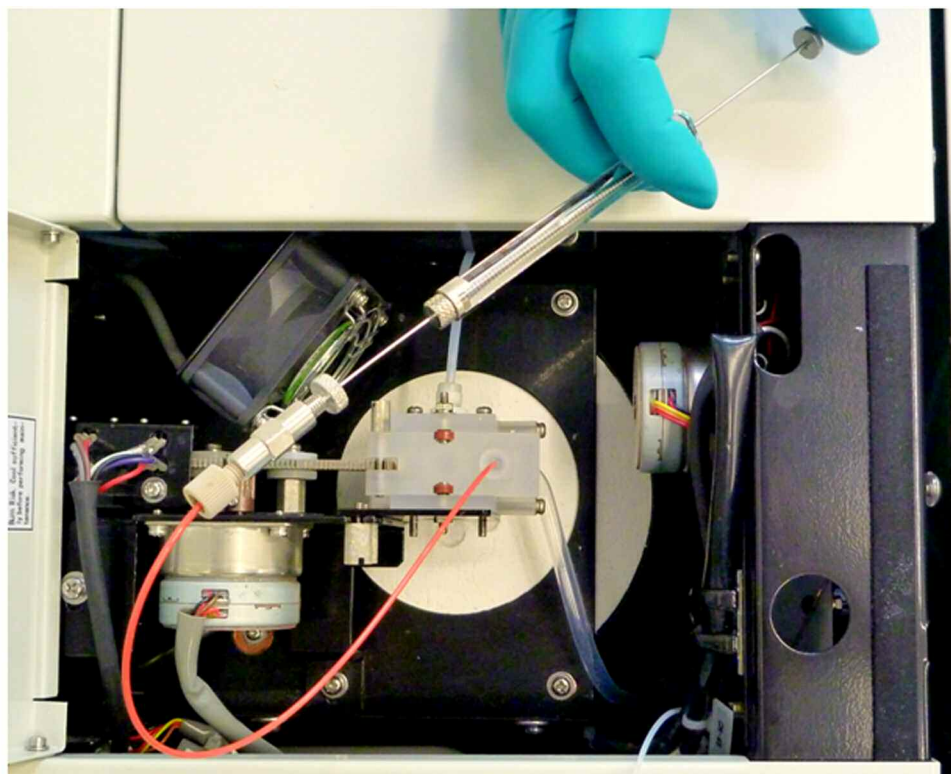
To allow multiple injections to be made from a small volume (< 600  $\mu\text{L}$ ), all components before HTC reactor of the TOC- $V_{\text{CPH}}$  were bypassed. This was achieved by coupling a piece of PEEK tubing (length 10 cm, outer diameter 1/16-inch, inner diameter 0.005-inch; €1.50) directly to the inlet of the HTC reactor (Fig. 2). The PEEK tubing was ~10 cm in length, resulting in a dead volume of 1.3  $\mu\text{L}$ . The PEEK tubing was coupled to the inlet of the HTC reactor using a stainless steel union (0.010-inch outer diameter; Bruker Daltonics part no. 21151; €3.10). An injection port assembly (Bruker Daltonics part no. 18218; €19.00) was connected to the other end of the PEEK tubing to allow injection through a 100  $\mu\text{L}$  standard glass micro-syringe (Hamilton part no. 80600; dead volume of

stainless steel needle 0.90  $\mu\text{L}$ ). Following use, the syringe was rinsed with acidified ultrapure water, then with ultrapure water alone to clean and reduce corrosion of the needle. The TOC- $V_{\text{CPH}}$  was run in *Manual Injection* mode to immobilize the sliding cover plate above the HTC reactor and to enable manual actuation of the run start. Before analyses and between samples, the syringe was rinsed three times with 2% hydrochloric acid in ultrapure water (Millipore, Milli-Q) and three times with approximately 10  $\mu\text{L}$  sample. The syringe was then overfilled with sample drawn from the base of the vials while Argon continued to bubble through the sample. Care was taken to avoid drawing bubbles into the syringe and any small bubbles were expunged from the syringe before a volume of exactly 100  $\mu\text{L}$  sample was injected into the HTC reactor. The TOC- $V_{\text{CPH}}$  software was used to trigger the instrument run immediately before injection. As in the automatic mode, five injections of each sample were made.

#### Assessment

##### Manual sample degassing

Without degassing, analysis of the deep sea Consensus Reference Material (CRM) yielded an apparent DOC concentration of approximately 1700  $\mu\text{M}$  C due to the presence of dissolved inorganic carbon in the sample. This is despite the reference material being supplied pre-acidified. After 5 min of degassing, the measured value for CRM DOC was 42  $\mu\text{M}$  (i.e., within the 41-44  $\mu\text{M}$  range expected for CRM). Purging for up



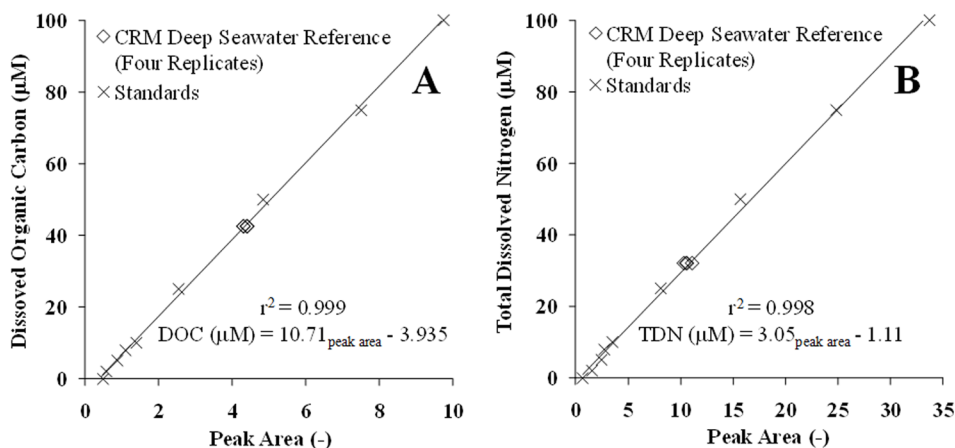
**Fig. 2.** The small volume manual injection modification to the Shimadzu TOC analyzer.

to 30 min longer resulted in no further decrease in recorded DOC concentration.

**Small volume DOC and TDN precision and accuracy**

The responses of both the TOC-V<sub>CPH</sub> and TNM-1 were linear over the investigated range of DOC<sub>CPH</sub> and TDN concentrations, with small offsets from zero on the x-axis (peak area) due to an omnipresent analytical blank (Fig. 3). The resultant standard curves were consistent with those attained using automatic injections with the same instrument. The blank offset for the DOC standard curve was 3.9 μM C (Fig. 3A; Table 1). For comparison, offsets ranged from 0.2 to 6.4 μM C (mean = 2.1 ± 1.1 μM C) for 10 standard curves acquired using the auto-injection setup in the 4 weeks before and after the small volume injection tests were conducted (Table 1; Note: No outliers were removed from the auto-injection dataset). Using the small volume method, the slope of the standard curve was 10.7 (Fig. 3A; Table 1). The slope for 10 auto-injection standard curves ranged from 10.7-11.5 (Table 1; mean = 11.0 ± 0.1), indicating the instrument response factor is near-identical using the manual (small volume) injection method

or the automated method, both of which ultimately inject a volume of 100 μL on to the HTC column. The standard error associated with the slope of the standard curve was 1.32% of the DOC concentration (SigmaPlot 10.0, Systat Software Inc.) when using the small volume method and ranged from 0.2-5.4% for the 10 auto-injection runs (Table 1; mean = 1.7 ± 0.5%). Minimum detection limits (MDL) were calculated following the root mean square error (RMSE) method described by Corley (2003), which is recommended for determinations of MDLs from linear calibration curves. The MDL was 3.6 μM C for the small volume method and ranged from 0.4-9.2 μM C (mean = 2.8 ± 0.3 μM C) for the ten auto-injection runs (Table 1). This compares to a typical detection limit for the Shimadzu system of 8 μM C (Wurl and Min Sin 2009). At the measured mean concentration of the CRM (42.7 μM C), the 1.3% error associated with the slope of the low volume standard curve would result in analytical precision of ± 0.6 μM C. The precision calculated as the standard error from four repeat analyses of CRM was actually slightly lower (± 0.3 μM C; Table 1). The four analyses of CRM ranged from 42.1-43.3 μM C



**Fig. 3.** Standard curves and values for quadruplicate analysis of the Deep Seawater Consensus Reference Material (CRM) using the low volume manual injection method. Panel A: Results for dissolved organic carbon (DOC). Panel B: Results for total dissolved nitrogen (TDN).

**Table 1.** Summary of analytical performance of the Shimadzu TOC/TDN analyzer in conventional, auto-analyzer configuration and in small volume manual injection mode.

Method	Standard Curve Slope (μM/Peak Area)	Blank (μM)	Detection limit* (μM)	Precision† (%)	Deep seawater reference‡ (μM)
Conventional DOC§	10.6–11.0	0.2–6.4	0.6–14.8	0.2–5.4	Consensus value: 41–44
Small volume DOC	10.7	3.9	3.4	1.3	42.7 ± 0.3
Conventional TDN§	3.00–3.34	0.1–2.7	0.6–11.9	0.2–4.4	Consensus value: 31–33
Small volume TDN	3.05	1.1	4.5	1.8	31.4 ± 0.3

\*Detection limit was calculated from standard error of the slope of the standard curve according to DIN 38402-1:2011-09 and using SigmaPlot 10.0 (Systat Software Inc.)

†Precision reported is the standard error of the slope of the standard curve (SigmaPlot 10.0, Systat Software Inc.)

‡Deep seawater reference material analyzed was from Batch 10, Lot# 05-10 of the Consensus Reference Material Project. Consensus concentrations reported for these references are taken from <http://yyy.rsmas.miami.edu/groups/biogeochem/CRM.html>.

§Data from ten standard curves acquired using the conventional instrument setup in the 4 weeks before and after the small volume manual injection tests were conducted. No outliers were removed.

with a mean of 42.7  $\mu\text{M C}$  (Table 1; Fig. 3A). These values fall within the range of consensus concentrations for this batch of CRM (41-44  $\mu\text{M C}$ ; <http://yyy.rsmas.miami.edu/groups/bio-geochem/CRM.html>).

The standard curve for TDN had a blank offset of 1.1  $\mu\text{M N}$  and slope of the 3.05 (Fig. 3B). The slope error was 1.8% of the measured concentration and the detection limit of 4.5  $\mu\text{M N}$  (Table 1). Offsets for 10 auto-injection standard curves ranged from 0.1-2.7  $\mu\text{M N}$  (mean =  $0.9 \pm 1.1 \mu\text{M-N}$ ), their slopes from 3.00-3.34 (mean =  $3.23 \pm 0.06$ ), their slope errors ranged from 0.2-4.4% (mean =  $0.8 \pm 0.4\%$ ), and detection limits ranged from 0.6-11.9  $\mu\text{M N}$  (Table 1; mean =  $2.9 \pm 0.2 \mu\text{M N}$ ). The TDN concentrations for four separate analyses of CRM ranged from 30.4-32.8  $\mu\text{M N}$  with a mean of 31.4  $\mu\text{M N}$  and a standard error of  $\pm 0.26 \mu\text{M N}$  (Table 1; Fig. 3B). The consensus concentration range for this batch of CRM is 31-33  $\mu\text{M N}$ .

These results indicate that the presented small volume technique provides a precision and accuracy of measurement indistinguishable from the automated assessments of DOC and TDN concentrations employed by the laboratories in the CRM Project and the marine organic matter community generally.

### Comments and recommendations

#### Purging carbon dioxide

Sample purging takes less than 5 min, and a sample injection run takes approximately 5 min. When the first injection is made, the sample should be continuously purged until the last of the five injections per sample is made. After this the tubing carrying the purge gas can be rinsed with ultrapure water and placed into the next sample. By the time the previous sample run is complete, the next sample will be purged and ready for injection. Based upon our experience switching between higher standards (100  $\mu\text{M C}$ ), ultrapure water, and the CRM, rinsing the purge gas line with ultrapure water between runs is sufficient to prevent sample carryover. When not in use, the end of the gas line that goes into the sample should be kept in a 2% aqueous solution of hydrochloric acid. If contamination is observed, the reader may try rinsing the tubing with an aqueous solution of hydrogen peroxide (up to 30%) or sodium hydroxide (up to 1 M).

Any organic and inorganic carbon-free gas can be used for purging. The type of gas used, as well as the sample temperature, sample pH, and gas flow rate will likely impact the purge time required. Therefore, each investigator should perform a simple purge time test to ensure that they employ appropriate purge times for their samples and configuration. In addition, the vial holding the sample during purging should be suitable for aggressive cleaning (ideally glass combustible at 450°C) and shaped so that sample does not overflow during bubbling, but can readily be reached with the Hamilton needle once purged. In the current study, 2 mL Wheaton V glass vials (Fig. 1) were used.

#### Consistency of user and technique

As with any manual injection technique, consistency is required of the operator. Users should develop a consistent technique and ensure that they are getting good quality data for standard curves and CRM (or an expendable and relevant environmental sample) before proceeding to analyze valuable samples. Multiple operators using a single instrument are recommended to prepare individual standard curves. The same syringe, needle, and injector port assembly should be used for both standards and samples. Any bubbles in the syringe must be expunged before determining the syringe volume. In the current study, we used a syringe volume of 100  $\mu\text{L}$  as this provided sufficient signal when injecting the CRM. However, the instrument can handle both smaller and larger volume injections and a lower volume could certainly be injected for samples with higher carbon or nitrogen concentrations. The lower limit in volume size would be determined by the user's ability to degas the samples and quantitatively transfer it to the HTC column. For volumes smaller than 100  $\mu\text{L}$ , we would recommend further reductions in the dead volumes of the tubing and syringes to below 5% of the volume to be analyzed. For lower concentrations, caution should be applied as injection of too much water can lead to cooling of the HTC reactor, reducing the efficiency of organic matter oxidation and degrading data quality. Therefore, users should consult the instrument manual to check the maximum injection volume recommended for their specific system. In the case of the TOC- $V_{\text{CPH}}$ , the software limits the automatic injection volume to 150  $\mu\text{L}$ , and we do not recommend exceeding this limit without consulting with Shimadzu and empirically determining whether HTC efficiency declines above this injection volume. Finally, for repeatable results, it is imperative that the rate of sample injection into the HTC reactor be constant and that a smooth syringe action is used as variations in the rate of sample injection may generate variations in peak shape and integration.

#### Applications

The proposed method delivers the same performance as the standard volume automatic and manual configurations of the Shimadzu TOC- $V_{\text{CPH}}$  and TNM-1. Therefore, we recommend the use of the presented method for applications classically catered to by the Shimadzu TOC/TDN instruments but where sample volume is limiting. In our laboratories, we have found cause to use the low volume technique to determine DOC and TDN concentrations in valuable aquatic samples from deep sea vents, porewater, rainwater, dew, frost, and individual hailstones. We expect the method presented here to find application wherever the high quality data of the Shimadzu system is required, but sample volume is limiting. However, when sufficient sample volume is available to run DOC and TDN analyses using the larger volume, auto-sampler configuration of the Shimadzu, we recommend this standard configuration due to savings in user time.



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Submitted 15 September 2011

Revised 11 January 2012

Accepted 6 April 2012