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Laboratory Analytical Procedure (LAP)

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1 Introduction

- 1.1 Total carbon content can be divided into two major categories: inorganic carbon and organic carbon. Inorganic carbon is primarily composed of carbonate, bicarbonate, and dissolved CO₂. Organic carbon includes all carbon atoms covalently bound in organic molecules and can further be divided into the following: dissolved organic carbon (DOC), the fraction of organic carbon that passes through a 0.45- μ m filter; purgeable organic carbon (POC) or volatile organic carbon (VOC), the fraction of total organic carbon (TOC) removed from an aqueous solution by purging with VOC free gas under specified conditions; and non-purgeable (NPOC) or non-volatile organic carbon (NVOC), the fraction of TOC not removed by purging with VOC free gas [1].
- 1.2 TOC can be measured by analyzing a sample for total carbon (TC) and inorganic carbon (IC) and then subtracting the IC to obtain a calculated value for TOC. This method is applicable when TOC concentrations are a significant fraction of the TC concentration, otherwise calculated TOC results may be negative or inaccurate due to error propagation [1, 2]. Alternatively, TOC can be measured as NPOC by acidifying the sample and purging with VOC free gas to remove any volatile carbon before analyzing. NPOC results are often equivalent to TOC due to relatively low amounts of POC. The method described in this laboratory analytical procedure focuses on the calculated value of TOC using the subtraction of IC from TC.
- 1.3 Biological cultures (e.g., algae cultures) provide a unique challenge for measuring carbon because samples may remain biologically active after sampling, potentially affecting the carbon results. Preliminary data suggest that heat-treating samples immediately following sampling halts biological activity and stabilizes carbon results.
- 1.4 The measurement of organic carbon in biological cultures is complex and requires the measurement of TOC in the whole sample (cells and media) as well as the supernatant (media without cells). To calculate TOC for the cells alone, the TOC result from the supernatant is subtracted from the TOC result of the whole sample.

2 Scope

- 2.1 This procedure is used to quantify the fraction of total carbon, organic carbon, and inorganic carbon present in biological culture samples (e.g., algae cultures), fermentation samples, and chemically treated samples such as hydrolysate after acid treatment.

- 2.2 Portions of this procedure are specific to a Shimadzu TOC-LCPH analyzer with corresponding software (TOC-Control L) using a combustion method and suspended solids kit and may vary depending on laboratory-specific setup.
- 2.3 Samples with high alkalinity (high inorganic carbon) will damage the combustion tube over time due to the accumulation of salts. Care should be taken to minimize the amount of inorganic carbon added to the combustion tube. A rigorous preventative maintenance schedule will help preserve the instrument and component lifetime [1].
- 2.4 This procedure is **not** intended for the analysis of solid or sludge samples, only liquid samples with suspended solid particles less than 0.8 mm in diameter.

3 Terminology

- 3.1 *Total Carbon (TC)*: The sum of organic and inorganic carbon, including elemental carbon.
- 3.2 *Total Inorganic Carbon (TIC or IC)*: All carbon not covalently bound in organic molecules. Predominately composed of carbonate, bicarbonate, and dissolved carbon dioxide [1].
- 3.3 *Total Organic Carbon (TOC)*: The sum of carbon atoms covalently bonded in organic molecules [1].
- 3.4 *Dissolved Organic Carbon (DOC)*: Organic carbon remaining in the sample after it has been filtered through a 0.45- μm filter [1, 3].
- 3.5 *Volatile Organic Carbon (VOC) or Purgeable Organic Carbon (POC)*: The sum of carbon that is converted to CO_2 by purging with VOC free gas under specified conditions [1].
- 3.6 *Non-Volatile Organic Carbon (NVOC) or Non-Purgeable Organic Carbon (NPOC)*: The sum of carbon that is not converted to CO_2 when purged with VOC free gas [1, 4].
- 3.7 *Calibration Standard*: A set of standards, each at a known concentration, used to determine a detector response. The calibration standards must include the analytes of interest and be run in series with a sample set. The detector response can then be used to predict the concentration of an analyte in a sample.
- 3.8 *Calibration Verification Standard (CVS)*: A standard made from the same parent standard as the calibration for each analyte (IC and TOC) and analyzed every 10 samples to monitor instrument functionality during the run.

- 3.9 *Low-Level Calibration Verification Standard (LLCVS)*: A standard made from the same parent standard as the calibration for each analyte (IC and TOC) at the lowest calibration point to measure long-term variability at the low end of the curve.
- 3.10 *Second Source Standard*: An independent standard analyzed after a calibration to verify calibration standards.

4 Significance and Use

- 4.1 This procedure is used to quantify the fraction of total carbon, organic carbon, and inorganic carbon present in biological culture samples (e.g., algae cultures) and chemically treated samples (e.g., hydrolysate after acid treatment).
- 4.2 This procedure may be used in conjunction with other methods to characterize and track carbon content and other metabolites such as the National Renewable Energy Laboratory (NREL) laboratory analytical procedures “Determination of Total Solids and Ash in Algal Biomass” [5], “Determination of Total Carbohydrates in Algal Biomass” [6], and “Determination of Total Lipids as Fatty Acid Methyl Esters (FAME) by *in situ* Transesterification” [7].

5 Interferences

- 5.1 High amounts of inorganic carbon relative to low amounts of organic carbon may result in increased error in the calculated TOC measurement if the TOC result is a calculated value determined by subtracting IC from TC. Reporting results with a calculated error may be useful if this analysis method is used. Refer to Section 11.6 for the related equation [2].
- 5.2 High concentrations of inorganic carbon may result in incomplete purging due to the buffering capacity of bicarbonate, possibly leading to erroneously high TOC results. High concentrations of salts associated with high alkalinity may also damage the combustion tube after prolonged use [3].
- 5.3 Samples are heat-treated in sealed vials with unpunctured septa to minimize the loss of volatile carbon.
- 5.4 Certain instruments are equipped with suspended solids kits and an autosampler equipped with a stir plate to homogenize the sample during analysis. Suspended solids kits keep particles suspended and therefore increase the accuracy of the measurement. They are also equipped with larger-diameter tubing, which allow larger particle sizes to be analyzed. This procedure is applicable only to homogeneous samples with suspended solid particle sizes less than instrument specifications. Particle sizes larger than the tubing will be excluded and will not be counted as part of the total carbon content. They may also block sample lines and damage the instrument.

- 5.5 Elemental carbon may not be oxidized at lower combustion temperatures (<680°C); however, this is generally not present in biological samples [1].

6 Apparatus

- 6.1 Analytical balance, accurate to 0.1 mg
- 6.2 Water bath, set to 75°C–80°C
- 6.3 Centrifuge (Thermo Scientific Sorvall ST 16R or equivalent) capable of reaching 4,100 relative centrifugal force (RCF)
- 6.4 Magnetic stir plate
- 6.5 TOC analysis instrument (Shimadzu TOC-LCPH or equivalent) and all associated parts, including required chemical reagents and VOC free gas. If biological cultures are to be analyzed, the system must be equipped with a suspended solids kit.

7 Reagents and Materials

7.1 Reagents

- 7.1.1 Hydrochloric acid (HCl), concentrated ACS reagent grade (36.5%–38%) (CAS # 7647-01-0)
- 7.1.2 Phosphoric acid (H₃PO₄), ACS reagent grade (≥85 wt % in H₂O) (CAS # 7664-38-2) *This reagent is specific to the Shimadzu TOC-LCPH system and may not be required depending on laboratory-specific setup.*
- 7.1.3 Water, 18.2 megaohm (MΩ)
- 7.1.4 Potassium hydrogen phthalate (KHP), ≥99.95% (CAS # 877-24-7)
- 7.1.5 Sodium bicarbonate (NaHCO₃), 99.5%–100.5% (CAS # 144-55-8)
- 7.1.6 Sodium carbonate (Na₂CO₃), anhydrous, ACS reagent (≥99.5%) (CAS # 497-19-8)
- 7.1.7 Second source organic carbon standard (recommended 20 ppm or mid-range standard) (KHP or equivalent).

7.2 Materials

- 7.2.1 TOC vials, clear, 40 mL (ESS # TOC040-0300 or equivalent, certified to <10 ppb TOC)

- 7.2.2 pH paper, range 0–13
- 7.2.3 Pasteur pipettes
- 7.2.4 Adjustable pipette, covering ranges from 0.5–40 mL
- 7.2.5 Stir bars, size 4.5 × 12 mm (for TOC vials) (Fisherbrand Octagon Spinbar #14-513-57 or equivalent)
- 7.2.6 100-mL volumetric flasks, glass (class A), cleaned with hot water and rinsed with 18.2-MΩ water. It is best to have dedicated glassware for TOC analysis, free of contaminants.
- 7.2.7 Stir bars (for standards preparation).

8 Environmental Safety and Health Considerations and Hazards

- 8.1 Hydrochloric acid is toxic and corrosive and should be handled with care.
- 8.2 Phosphoric acid is corrosive and should be handled with care.
- 8.3 Potassium hydrogen phthalate is an irritant and may be harmful if absorbed through the skin; wear appropriate personal protective equipment (PPE).
- 8.4 Follow all applicable chemical and biohazard handling procedures.

9 Sampling, Test Specimens, and Test Units

- 9.1 Care must be taken to ensure that a representative sample is taken for dilution and analysis.
- 9.2 Samples and standards must be prepared in unused, 40-mL glass TOC vials with unpunctured septa and clean volumetric glassware.
- 9.3 A stir bar (4.5 × 12 mm) must be added to TOC sample vials that contain any suspended particles to ensure a representative aliquot is injected into the analyzer for analysis. *This applies to a Shimadzu TOC-LCPH system equipped with a suspended solids kit and may not be applicable in certain laboratory-specific setups.*
- 9.4 Time should be limited between sampling of live biological samples and either analysis or heat treatment. These samples are biologically active and TOC/TIC values may change over time. A procedure to halt biological activity is included in Section 10.3 as an example.

- 9.5 If IC analysis is NOT of interest, samples should be preserved with hydrochloric acid to a pH < 2 at time of sampling and stored between 0°C and 4°C. Freezing is not recommended. Samples preserved with hydrochloric acid must be analyzed within 28 days [3].

10 Procedure

- 10.1 Prepare reagents and standards as follows:

- 10.1.1 Prepare a 25% (w/w) phosphoric acid solution as an IC reagent. Dilute 50 mL of 85% phosphoric acid to a total volume of 250 mL with 18.2-MΩ water.
- 10.1.2 Prepare a 0.05-M HCl solution for use in the B-type halogen scrubber. Add 10 mL of 1-M HCl to 190 mL of 18.2-MΩ water. *The B-type halogen scrubber is specific to the Shimadzu TOC-LCPH system and may not be required depending on laboratory-specific setup.*
- 10.1.3 Prepare a 1,000-ppm potassium hydrogen phthalate stock standard for TOC analysis (store at 4°C, stable for 1 month). Using an analytical balance, weigh *exactly* 2,125.0 mg of potassium hydrogen phthalate into a 1-L, class A, glass volumetric flask. Dilute to volume with 18.2-MΩ water.
- 10.1.4 Prepare a 1,000-ppm inorganic carbon standard for IC analysis (this solution must be prepared fresh daily). Using an analytical balance, weigh *exactly* 1,748.5 mg of sodium bicarbonate and *exactly* 2,206.0 mg of sodium carbonate into a 500-mL, class A, glass volumetric flask. Dilute to volume with 18.2-MΩ water.

- 10.2 Prepare a series of calibration and CVS for organic and inorganic carbon analysis:

- 10.2.1 Prepare 100-ppm intermediate calibration standards, one each for TC and IC:
- Add 10 mL of the 1,000-ppm stock TC standard to a 100-mL, class A, glass volumetric flask. Dilute to volume using 18.2-MΩ water.
 - Add 10 mL of the 1,000-ppm stock IC standard to a 100-mL, class A, glass volumetric flask. Dilute to volume using 18.2-MΩ water.

NOTE: TC and IC calibration standards are auto-diluted by the Shimadzu TOC-LCPH analyzer using the manually prepared 100-ppm intermediate standards. Refer to Table 1 for suggested calibration levels.

Table 1. Calibration Standard Concentrations and Dilution Factors from Intermediate Stock Standard Solutions

Calibration Standard Concentration (ppm)	Dilution Factor (performed by instrument)	Diluted from IC/TC Standard (ppm)
0	1	18.2-M Ω Water
2	50	100
5	20	100
10	10	100
25	4	100
50	2	100
100	1	100

10.2.2 Prepare CVS and LLCVS for organic and inorganic carbon analysis. Standards should be prepared in 100-mL, class A, glass volumetric glassware. Dilute standards to volume using 18.2-M Ω water. Refer to Table 2 for CVS/LLCVS preparation and suggested concentrations.

Table 2. CVS and LLCVS Preparation Diluted to Volume with 18.2-M Ω Water

CVS/LLCVS Concentration (ppm)	TOC/IC 1,000-ppm Stock Solution (mL)	Final Volume (mL)
2	0.2	100
40	4	100

Sample Preparation

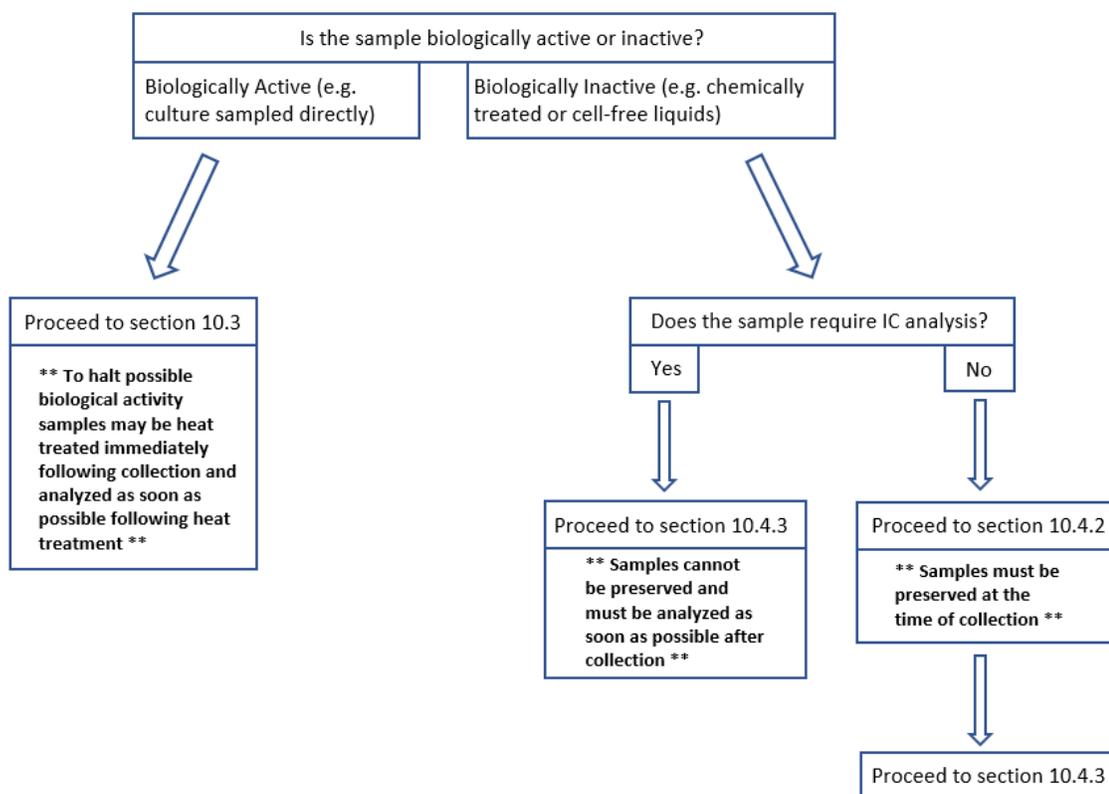


Figure 1. Flow chart for determining sample treatment based on sample type

10.3 Analysis of biologically active samples (e.g., live algal cultures):

10.3.1 Based on preliminary data, biologically active samples can be heat-treated immediately after sample collection in order to minimize potential changes in TOC/TIC concentrations following sampling. After heat-treating, samples should be stored in a refrigerator until analysis and ideally analyzed as soon as possible. A stability study is ongoing to determine the length of time samples can be stored prior to analysis. Perform heat treatment of samples using the following steps:

NOTE: Live cultures may be analyzed in two parts: (1) whole sample (sample containing cells and culture medium) and (2) supernatant (cell-free liquid), collected after centrifugation of the whole sample. If only the whole sample is analyzed, do not follow instructions for centrifuging and diluting the supernatant.

- Fill a water bath with enough water to cover 40-mL TOC vials to the shoulder and heat water bath to 75°C–80°C.

- Ensure that enough undiluted whole sample is available to analyze the supernatant, and then prepare whole sample dilutions directly into 40-mL TOC analysis vials using a calibrated adjustable pipette and 18.2-M Ω water as a diluent.
- Add stir bar (4.5 \times 12 mm) to sample vial prior to capping and heat-treating.
- Place capped 40-mL sample vial containing the diluted whole sample with stir bar into a rack and into the preheated 75°C water bath for 15 minutes.
- After 15 minutes, remove vial from water bath and immediately place in 4°C refrigerator until analysis. **Do not** open TOC sample vial after heat-treating and prior to analysis.
- Centrifuge the remaining whole sample that was not heat treated at up to 4,100 RCF until a pellet forms and the supernatant is clear and free of cells.
- Dilute and heat-treat the supernatant as previously described for the whole sample. For the supernatant, a stir bar is not needed in the analysis vial.

10.4 Keep the following in mind for analysis of samples that are *not* biologically active (e.g., hydrolysates, sterile filtered fermentation samples):

- Per standard method guidelines, unpreserved samples must be analyzed as soon as possible after sample collection [1].
- If samples have particulates, add stir bars.
- Once diluted, place TOC vials into a 4°C refrigerator until analysis.
- If diluting a preserved sample, check the pH of the dilution to ensure a pH < 2.

10.4.1 Upon sample receipt, determine if samples can be preserved with hydrochloric acid prior to analysis:

- If IC analysis is required, samples *cannot* be preserved (proceed to Section 10.4.3).
- If IC analysis is *not* required, samples *can* be preserved (complete Section 10.4.2 before proceeding to Section 10.4.3).

10.4.2 If samples can be preserved, proceed through the following steps before proceeding to Section 10.4.3:

- Preserve samples with concentrated hydrochloric acid to a pH < 2 by adding acid one drop at a time. Mix the sample thoroughly after adding each drop and check pH using a suitable pH strip until the desired pH is reached. Most samples require very little acid for preservation, and therefore the volume added is considered negligible.
- If sample analysis will not be performed the same day, store samples in a refrigerator at 4°C until analysis.

10.4.3 If samples cannot be preserved or have already been preserved, perform necessary sample dilution directly into a 40-mL TOC vial using 18.2-M Ω water as diluent.

10.5 TOC Instrument Setup

NOTE: This section is specific to the Shimadzu TOC-LCPH system and software. Instrument and quality control specifications will vary depending on the laboratory-specific setup.

10.5.1 Create calibration curves in the instrument software for IC and TC.

- Injection volume: 150 μL
- Number of injections: 3 out of 4
- Calibration: linear regression.

10.5.2 Create sample sequence according to laboratory-specific Quality Assurance Plan (QAP) [8]. Analyze CVS at determined frequency (recommended every 10 samples). Multiple wash blanks are recommended prior to calibration.

10.5.3 Perform all instrument maintenance and daily checks in accordance with manufacturer recommendations.

11 Calculations

11.1 Create a calibration curve for TC using linear regression. From this curve, determine the concentration in mg/L of the total carbon present in the samples.

11.2 Create a calibration curve for IC using linear regression. From this curve, determine the concentration in mg/L of the inorganic carbon present in the samples.

11.3 Calculate the amount of each TC/IC CVS or LLCVS recovery using the following calculation:

$$\%CVS\ recovery = \frac{\text{conc. detected by TOC (mg/L)}}{\text{known conc. of standard (mg/L)}} \times 100$$

11.4 Export the results from the TOC software and calculate TOC/TC/IC results as mg/L for each sample:

$$\text{Total Organic Carbon} = \text{Total Carbon} - \text{Total Inorganic Carbon}$$

11.5 Calculate TOC of the biomass in live biological culture samples:

$$\begin{aligned} \text{TOC in biomass (mg/L)} \\ = \text{TOC in whole (mg/L)} - \text{TOC in supernatant (mg/L)} \end{aligned}$$

- 11.6 Calculate the uncertainty in the TOC measurement using the following calculation, where % RSD (relative standard deviation) is calculated based on data from the three reported replicates for each sample injection [2]:

$$\begin{aligned} & \textit{Uncertainty in TOC measurement} \\ & = (TC \text{ (mg/L)} * \%RSD) + (IC \text{ (mg/L)} * \%RSD) \end{aligned}$$

12 Report Format

- 12.1 Report data for all samples for TC, IC, and the calculated values on a mg/L (ppm) basis. Avoid reporting values below the calculated minimum detection limit (MDL). Results between the limit of quantitation (LOQ) and MDL should be flagged as estimated values.

13 Precision and Bias

- 13.1 Precision and bias need to be determined by data quality objectives and a laboratory-specific QAP.

14 Quality Control

- 14.1 *Reported Significant Figures:* Figures need to be determined by data quality objectives and laboratory-specific QAP.
- 14.2 *Replicates:* The Shimadzu TOC-LCPH analyzer software reports TC and IC values for each sample as an average of the most closely aligned three of four replicate injections.
- 14.3 *Blank:* 18.2-M Ω water—the same water used for standard and sample preparation. This water source must contain less reported TC and IC than the lowest-level calibration point.
- 14.4 *Calibration Verification Standard:* CVS and LLCVS are prepared from the same stock standard as the calibration standards
- 14.5 *Second Source Standard:* A source independent of the calibration source that should be analyzed with every calibration.
- 14.6 *Sample Size:* Dependent on the expected concentration of carbon in each sample; required sample volumes will vary. Samples low in carbon that do not require a dilution will need at least 40 mL for analysis.

- 14.7 *Sample Storage:* The U.S. Environmental Protection Agency (EPA) recommendation for TOC analysis of drinking water is to preserve samples to a pH < 2 and store between 0°C and 4°C for no more than 28 days before analysis. Freezing samples is not recommended [3].
- 14.8 *Standard Preparation:* All calibration and check standards should be prepared using 18.2-MΩ water as described in the procedure.
- 14.9 *Standard Storage:* Total carbon standards should be stored in a 4°C refrigerator and are stable for one month. Inorganic carbon standards must be prepared fresh daily [9].
- 14.10 *Definition of a Batch:* A batch is any number of samples that are analyzed together. The maximum size of a batch will be limited by the equipment constraints or laboratory-specific QAP.
- 14.11 *Method Detection Limit (MDL):* The MDL is the concentration at which the analyst has >99% confidence that the analyte can be detected but the concentration is too low to quantify the analyte within a specified acceptable recovery. This concentration is determined using the EPA guidelines for MDL calculations and will vary depending on the instrument and laboratory [10].

15 Appendix

Table 3. Quality Control Specifications (May Vary Depending on Laboratory-Specific QAP)

Quality Control	Frequency	Acceptance Criteria
Total Carbon Calibration	Once per sequence	$r^2 \geq 0.999$
Inorganic Carbon Calibration	Once per sequence	$r^2 \geq 0.999$
Blank	Every 10 samples	<2 ppm
LLCVS (TOC/IC)	Once, immediately after calibration	±50%
CVS (TOC/IC)	Every 10 samples	±10%
Second Source CVS (TOC)	Once, immediately after calibration	±10%

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