

A system to quantitatively recover bacterioplankton respiratory CO₂ for isotopic analysis to trace sources and ages of organic matter consumed in freshwaters

S. Leigh McCallister¹, François Guillemette, and Paul A. del Giorgio

Groupe Interuniversitaire en Limnologie (GRIL), Dépt. des sciences biologiques, Université du Québec à Montréal, CP 8888, Succ. Centre Ville, Montréal, Québec, Canada

Abstract

We present a new system and method to measure the natural abundance isotopic ($\delta^{13}\text{C}$, $\Delta^{14}\text{C}$) values of respiratory CO₂ produced by bacterioplankton, to directly assess the sources and ages of organic carbon (OC) respired by bacteria in aquatic ecosystems. The Respiratory Carbon Recovery System (ReCRoS) and operating procedure were designed to reduce background dissolved inorganic carbon values by > 98% and then quantitatively recover the CO₂ derived from bacterial respiration after incubation of freshwater samples. The 2-component ReCRoS consists of an airtight incubation system (20 L), for short-term regrowth incubations of filtered water samples inoculated with ambient bacteria, and a harvest system to recover the respiratory CO₂ produced during these incubations. The multistep operating procedure involves the following: (1) filling of incubation system with 0.2- μm filtered sample water; (2) addition of 1 N HCl (pH to ~2.8); (3) sparge with ultrahigh-purity (UHP) He to remove dissolved inorganic carbon; (4) sparge with UHP, volatile organic carbon-, CO₂-free air to replenish oxygen; (5) neutralization (1 N carbonate free NaOH), reinoculation with the ambient bacterial assemblage, and incubation (80–132 h); (6) acidification to pH 2.8; and (7) UHP He sparge for > 12 h. The evolved CO₂ is sent through 2 water traps (dry ice slurries) before cryogenic trapping in liquid N₂. Control incubations were processed concurrently to evaluate extraction efficiencies, potential methodological contamination, and fractionation artifacts. Collectively, our results suggest that respiratory CO₂ is quantitatively recovered and the isotopic fidelity ($\delta^{13}\text{C}$, $\Delta^{14}\text{C}$) between the OC respired and the CO₂ harvested is retained. Moreover, the system allows the recovery of sufficient C to measure both $\delta^{13}\text{C}$ and $\Delta^{14}\text{C}$ values, even in the most oligotrophic systems.

Introduction

Respiration is the major sink for organic carbon (OC) and the dominant biological source of CO₂ in the biosphere. Although there have been recent advances in our understanding of the magnitude and regulation of respiration in aquatic ecosystems

(summarized in del Giorgio and Williams 2005), there are still major gaps in our knowledge of this key aspect of ecosystem function. One of these gaps concerns the OC sources fueling aquatic respiration. Aquatic ecosystems have multiple internal sources of OC, including phytoplankton, periphyton, and macrophyte production. In addition, no aquatic ecosystem in the biosphere is completely closed, and most ecosystems receive and process nutrients and OC from surrounding systems. This is particularly true for lakes, rivers, estuaries, and coastal oceans, which often receive large amounts of terrigenous OC. Respiration in all these systems is thus based on a complex mixture of OC from various autochthonous and allochthonous sources. Determining the composition of the OC mixture respired, how it varies temporally within and spatially among ecosystems, and the factors that influence the supply of these various respiratory substrates are key to understanding the regulation of respiration and C cycling across multiple time and space scales in aquatic ecosystems.

Bacteria are responsible for processing much of this OC, and for a large fraction of total respiration in many aquatic ecosystems (Cotner and Biddanda 2002); thus, exploration of the OC

¹Corresponding author: Rutgers University, Institute of Marine and Coastal Sciences, 71 Dudley Road, New Brunswick, NJ 08901; Email: lmccalli@marine.rutgers.edu

Acknowledgments

We are grateful to Gilles St Jean and Paul Middlestead (Hatch Isotope Lab, University of Ottawa) for their invaluable advice, input, and expertise at all stages of method development. We thank Tom Guilderson and Paula Zermeno at the Lawrence Livermore National Lab for their help with radio-carbon analyses. We thank Jérôme Comte, Dominic Frechette, Marie Noelle Boivin, and Catherine Beauchemin for their assistance in the field and with sample analyses. Future modifications of the system for saline systems were conceived by Greg Rau at University of California, Santa Cruz. We thank Dr. Peter Raymond and three anonymous reviewers for constructive comments which greatly improved an earlier version of this manuscript.

base of aquatic respiration must begin by identifying the sources and ages of OC respired by aquatic heterotrophic bacteria. Researchers working in aquatic systems have, for the most part, employed indirect approaches to infer the sources of C fueling aquatic bacterial respiration. Simultaneous measurements of primary production and respiration (del Giorgio et al. 1999) and gas or organic mass balances (Dillon and Molot 1997; Prairie et al. 2002) have been used to infer the relative importance of autochthonous versus allochthonous sources to lakes and other ecosystems. Others have approached this question using carbon isotopes, through experiments employing isotopically enriched tracers (Pace et al. 2004), mass balance approaches (Hullar et al. 1996), and/or natural abundance isotope measurement of bacterial biomass (Coffin et al. 1990; Cherrier et al. 1999; McCallister et al. 2004).

Although each of these indirect approaches has yielded new insights, all have assumptions and limitations that weaken their capacity to quantitatively partition bacterial respiration into its various OC components. Metabolic measurements and OC or gas balances allow one to determine if the systems consume external organic matter but seldom allow total R to be quantitatively partitioned into its OC sources. Isotopic mass balance approaches are limited in their application by the additive errors associated with estimating multiple C pools which may significantly overwhelm the small differences in $\delta^{13}\text{C}$ (3‰ to 7‰) between OC sources in some systems (del Giorgio and France 1996). Bacterial biomass production, though one metabolic pathway, is frequently a secondary C sink relative to respiratory pathways (del Giorgio and Cole 1998). Further, our current level of knowledge does not justify the assumption that the OC sources supporting biomass production and respiration are the same (Kritzberg et al. 2005). Thus, in spite of recent advances in our understanding of carbon cycling in aquatic systems, the sources and ages of organic matter fueling aquatic bacterial respiration remain a major gap in our understanding of ecosystem function and global carbon biogeochemistry.

One way to avoid the above problems and effectively assess the nature of the organic matter respired by aquatic bacterial communities exposed to a complex pool of substrates with multiple sources and ages is to recover the resulting respiratory CO₂ for isotopic analysis. The ability to isolate and measure the natural abundance $\delta^{13}\text{C}$ and $\Delta^{14}\text{C}$ of respiratory CO₂ in terrestrial systems, to resolve the sources and ages of OC soil respiration (Trumbore 2000), has surpassed our progress in aquatic systems (del Giorgio and Williams 2005). The only published study to our knowledge to have attempted this approach in aquatic systems is that of Waichman (1996), who developed a technique to isolate bacterial respiratory CO₂; however, isotopic analyses were limited solely to $\delta^{13}\text{C}$. The absence of direct isotopic measurements of respiratory CO₂ in aquatic ecosystems probably reflects major technical difficulties that are intrinsic to these systems. Respiration rates in most aquatic systems are low compared with soil respiration,

and the bicarbonate buffering system provides the added analytical challenge of discerning small changes in CO₂ superimposed on a much greater background of dissolved inorganic carbon (dissolved inorganic carbon (DIC); CO₂(aq) + HCO₃⁻ + CO₃²⁻). Resolution of the isotopic signature of respiratory CO₂ in aquatic systems is further complicated by internal (CO₂(aq), HCO₃⁻, CO₃²⁻) and external (aquatic, atmospheric) exchanges and isotopic fractionations.

To fill this gap, and to directly assess the isotopic signature of CO₂ derived from aquatic bacterial respiration, we have designed the Respiratory C Recovery System (ReCRoS). The system and operating method described here allow quantitative recovery of bacterial respiratory CO₂ in aquatic systems for subsequent $\delta^{13}\text{C}$ and $\Delta^{14}\text{C}$ analysis to determine the sources and ages of the OC that was respired. The system was designed to overcome the problem of isolating small amounts of respiratory CO₂ in the context of the high natural background DIC pool, by completely stripping the sample water of DIC prior to incubation with the native bacterial assemblage. The bacterial respiratory CO₂ evolved during the incubation thus is not diluted by any ambient DIC and can be quantitatively recovered and purified for subsequent isotopic analysis. Further, because all of the inorganic C produced is recovered, we eliminate errors in $\delta^{13}\text{C}$ derived from fractionations between inorganic C species.

Materials and procedures

The ReCRoS consists of 2 distinct subsystems: The Incubation Module and the Gas Recovery Module (Figure 1). The Incubation Module allows (1) the preincubation manipulation of the sample, which involves lowering the pH of the sample to convert all DIC to CO₂, stripping of the CO₂ with ultrahigh-purity (UHP) He, adjustment of pH to initial values, reoxygenation with CO₂-free air, and reinoculation of the sample with the native bacterial community (Figure 2A); (2) the actual incubation of the sample to generate respiratory CO₂; and (3) the postincubation treatment of the sample to quantitatively strip the respiratory CO₂ from the water. The Gas Recovery Module, which is coupled to the Incubation Module, is then used to quantitatively capture the respiratory CO₂ evolved during the incubation (Figure 2B). Below, we describe in detail the hardware components as well as the operating procedure.

Incubation Module—ReCRoS was developed and engineered to quantitatively isolate respiratory C for isotopic analysis. The application of this system to resolve not only $\delta^{13}\text{C}$ but also the $\Delta^{14}\text{C}$ signature of respired C requires extreme precautions to ensure all seals and connections are airtight and to reduce areas of potential dead space within the ReCRoS. In addition, the system must generate sufficient CO₂ to allow $\Delta^{14}\text{C}$ analysis (> 200 $\mu\text{g C}$), so relatively large water samples must be incubated. The Incubation Module consists of round-bottom 20-L flasks (Chem Glass [CG]), which were custom modified (Verrierie de Precision Enr.) and fitted with (1) a bottom exit port (Figure 1, Port E), used to empty the flask and also to draw

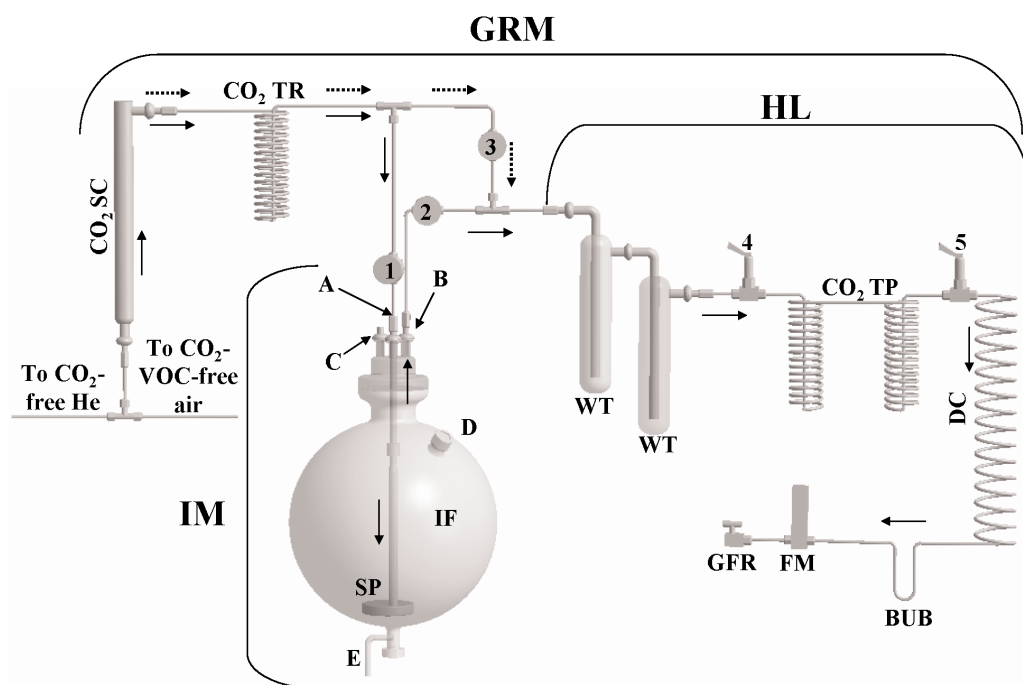


Fig. 1. Schematic of the ReCRoS and gas flow paths. Bracketed gas recovery module (GRM) and incubation module (IM) identify the 2 primary components of the ReCRoS system. The harvest line (HL) is a subcomponent of the GRM. Numbers designate system valves (1-5). Letters denote IM ports (A-E). The following components comprise the ReCRoS: (1) CO₂ scrubber (CO₂ SB), (2) gas impurity CO₂ trap (CO₂ IM), (3) incubation flask (IF), (4) sparge (SP), (5) water trap (WT), (6) CO₂ trap (CO₂ TP), (7) diffusion coil (DC), (8) bubbler check valve (BUB), (9) flow meter (FM), and (10) gas flow regulator (GFR). Solid arrows denote gas flow for sparge and recovery procedures. Dotted arrows denote alternate gas flow path for line preparation before gas recovery.

samples during processing and incubation (Teflon sealing stopcock, CG-560-03), (2) a side-mounted septum injection port (CG-194-03; Figure 1, Port D), used to add acid, base, and inoculum to the sample while maintaining a closed system, and (3) an airtight sealing lid with viton o-ring (Figure 1), which contains 3 gas ports (A-C) that connect the Incubation Module to the purge UHP He gas and the CO₂-free air, as well as to the Gas Recovery Module. To eliminate dead space inside the injection port (Figure 1, Port D), a Teflon insert with a 2-mm aperture to allow multiple punctures of a 22-gauge needle is fitted flush with the inside wall of the incubation flask (Figure 1, IF).

Three female glass rotulex (RF19, Pegasus Industrial) ports are mounted (Verrerie de Precision Enr.) to the ReCRoS lid (Figure 1, Ports A-C)). Two glass ports (Figure 1, A and B) are coupled to custom-manufactured stainless steel (SS) male rotulex adaptors (Ottawa) and then connected to Bellows valves (SS-4BK) via SS tubing (1/8-inch diameter) and Swagelock (SS-200-R4) adaptors. One SS male rotulex is modified with a SS extension (1/4-inch diameter, 15 cm length) fitted through the female port (Figure 1, Port A) and connected via a Cajon fitting (SS-6UT-64) to a glass sparge (7 cm diameter, coarse frit; Figure 1, SP) positioned at the center/bottom of the flask. The third glass port (Figure 1, Port C) is capped with a sealed-end male glass Rotulex (port available for future adaptations). An airtight seal between

the incubation flask and lid is maintained with an adjustable metal-reinforced wood (5 cm thick) pressure clamp (custom designed, UQÀM).

Gas Recovery Module—The Gas Recovery Module is designed to allow the incubation system to remain sealed while the harvest line is prepped and flushed, thus permitting multiple incubations to be simultaneously initiated, maintained, and harvested. A Bellows valve (SS-4BK; Figure 1, Valve 3) isolates the harvest line from the incubation system for independent operation. The harvest line is connected directly to the gas source

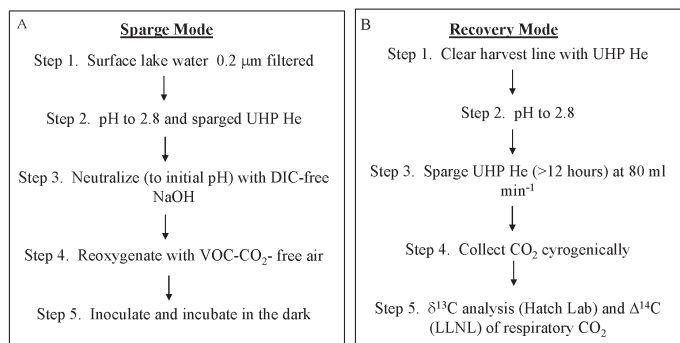


Fig. 2. Flow chart detailing the sequential steps of the ReCRoS operating procedure for the Sparge (A) and Recovery (B) modes, respectively.

UHP He and an additional CO₂ trap, comprised of a single 1/8-inch brass tubing coil, placed inline to remove trace impurities in the UHP carrier gas (Figure 1, CO₂ TR). The harvest line consists of dual inline water traps (400 mL; Figure 1, WT) connected via a glass to SS rotulex coupling to the respiratory CO₂ recovery traps. Respiratory CO₂ recovery traps (Figure 1, CO₂ TP) are composed of 3 m, SS 1/8-inch diameter gas chromatography grade tubing dual coiled (20 cm length) with toggle Bellows valves (SS-4BKT; Figure 1, Valves 4 and 5) sealing each end. The downstream valve (5) is attached to a 1/8-inch diameter 3 m length SS tubing to provide capillary flow resistance (Figure 1, DC) and prevent atmospheric diffusion into respiratory CO₂ recovery traps. Airflow through the line is monitored with a Labcor flowmeter (U-32457-40) attached inline to a bubbler flow check valve (Figure 1, BUB) to prevent atmospheric intrusion should airflow through the system be disrupted. As a final precaution, the line was connected to a terminal gas flow regulator (Figure 1, GFR) which can be used to seal the line as well as regulate gas flow when pressurized. All pneumatic valves (SS-4BK; Figure 1, valves 1-3) are operated at 60 psi and are electronically controlled from a central switchboard.

Sample Preparation—The ReCReS has been designed to operate with freshwater samples, although the operating procedure could be adapted to brackish and marine waters with little substantial change in the hardware. For the preliminary studies of ReCReS, 20-L lake water samples were taken at a depth of 0.5 to 1.0 m in acid-leached (10% HCl) Nanopure-rinsed polycarbonate bottles from several lakes in southeastern Québec and returned to the lab within 2 h of collection. The water sample was then filtered sequentially through a combusted (525°C for 4 h) Millipore AE glass fiber filter (1.0 µm nominal pore size) and an inline Gelman filter capsule (0.2 µm) to remove particulates and bacteria, respectively. The latter was used because previous work has shown no detectable DOC leaching from these capsules (S.L.M., unpublished data). Samples of the filtered water were stored at 4°C for DOC and fluorometric analysis of initial water conditions (see “Assessment”). A subsample (5 L) of AE (1.0 µm) filtered water was collected for subsequent preparation of inoculum (see below). Alternatively, deionized water (DI) for assessment experiments (see below) was dispensed directly from a Barnstead (model 1244) system through a 0.1 µm capsule filter.

All glass and Teflon components of the ReCReS incubation systems are cleaned overnight (minimum 12 h) with 10% HCl and rinsed with DI before use. The flasks are filled through the open lid with 19.0 to 19.5 L sample water, leaving a 1-L headspace. System lid, sparge, and all ports are connected and sealed. To ensure an airtight system, the Incubation and Gas Recovery modules are pressurized with 5 psi He and potential leaks located with a He leak detector (Alltech 60229) having a 0.01 mL min⁻¹ sensitivity.

ReCReS operating procedure—To overcome the handicap of resolving small respiratory changes in the δ¹³C and Δ¹⁴C signature of CO₂(aq) diluted by a significantly larger background

of DIC, we modified the approach of Waichman (1996), developed solely for δ¹³C, whereby the initial DIC pool is removed, oxygen replenished, and the system reinoculated before incubation (Figure 2).

Sparge Mode—The initial sparge sequence consists of the following steps (Figure 2A): (1) UHP He (< 0.1 ppm CO₂) is channeled through an in-line CO₂ scrubber (ascarite and drierite; Figure 1, CO₂ SB) to remove trace amounts of CO₂; (2) Valve 1 is opened to allow He flow to enter the ReCReS through Port A and initiate the sparge (Figure 1); (3) Valve 2 is opened to complete the circulation loop and allow the carrier gas to exit the system via the harvest line; and (4) to mobilize the majority of DIC (> 98%) into CO₂(aq) and thus strip it from the system, the pH is lowered to ~2.8 with the addition of 10% HCl (14 to 20 mL, depending on the alkalinity of the sample) through the septum Port D (Figure 1). Water samples are drawn from the bottom Port E to confirm the pH of the sample after acidification.

The system is allowed to sparge (He flow > 1000 mL min⁻¹) in a turbulent manner for a minimum of 3 h to ensure conversion of CO₂(aq) to CO₂(g) and the latter's complete removal from the headspace (see “Assessment”). The pH is next readjusted to initial values by the addition of carbonate-free, 1 N NaOH (stored in septum bottle under N₂ headspace) with an airtight syringe through septum Port D (Figure 1). Helium flow is closed at the tank and the volatile organic carbon (VOC)-free and CO₂-free air tank is concurrently opened. Oxygen levels are returned to starting values by bubbling with this VOC/CO₂-free air for approximately 45 min (Figure 2A). Water samples are drawn from Port E to confirm the final levels of oxygen (YSI O₂ probe).

Inoculum preparation and incubation initiation—The preliminary sample processing involves filtration through 0.2 µm to remove particles, planktonic organisms, and most bacteria, as well as the temporary elimination of O₂ and reduction of pH to < 3. These treatments most likely injure or kill bacteria that could have remained in the sample, so it is necessary to reinoculate the water with the native bacterial assemblage (Figure 2A). The inocula are prepared in the following way. A Pellicon Mini-ultrafiltration unit is fitted with a single precleaned (per manufacturer's specifications) regenerated cellulose (PLCMK) cartridge (300 kDa) and used to concentrate the AE filtrate (~ 5 L) retained from the above water filtration sequence. Particles (i.e., bacteria) are concentrated to ~100 mL final volume. Subsamples of the filtrate are taken for bacterial abundance and DOC concentration to ensure both the effectiveness of concentration (70%) and the absence of DOC contamination from ultrafiltration cartridge. Slight modifications were made for the control inoculum (see “Assessment”). To remove residual DIC in the ultrafiltration concentrate, the bacterial inoculum is further reduced in volume (25 mL) by vacuum filtration over an acid-cleaned (10% HCl) 0.2 µm polycarbonate filter. The concentrate volume is exchanged 3 times with DIC-free water removed from Port E of the ReCReS, and the inoculum is injected through

Port D with a gas tight syringe. Rinsing with this DIC-free sample water also ensures removal of any DOC that might have been concentrated in the ultrafiltration process.

To confirm minimal DIC contamination from the various injections (e.g., NaOH, bacterial inoculum), samples are removed for DIC analysis from Port E and stored in 40 mL septum-top bottles without a headspace until analysis (see below). Samples to evaluate potential changes in DOC as a consequence of pH adjustments are also removed from Port E and stored at 4°C until chemical and fluorometric analysis (see below). In preparation for incubation, Valve 2 is closed and the exit port sealed. The system is pressurized to 5 psi through Port A with VOC/CO₂-free air, and Valve 1 is closed. The incubation system is encased in foil to prevent any exposure to light, and the incubation is allowed to proceed for 80 to 132 h. Samples are periodically removed from Port E to monitor bacterial production throughout the incubation period (see "Assessment").

Recovery mode—Before harvesting the respiratory CO₂ generated during the incubation, Valve 3 (Figure 1) is opened and the recovery line is flushed with helium (minimum 40 min) to remove potential atmospheric contamination (Figure 2B). The harvesting procedure (Figure 2B) consists of the following steps: (1) The harvest line is closed at the outlet with the gas flow regulator (Figure 1 GFR); (2) coils of CO₂ TR trap (Figure 1) are immersed in liquid N₂ (−196°C) to prevent trace CO₂ impurities in He sparge gas from accumulating in sample CO₂ trap; (3) both water traps and twin coils of the sample CO₂ trap are immersed in dry ice/ethanol slurries (−78°C) and liquid N₂, respectively; (4) Valve 2 is opened and residual pressure from the incubation system is released via the terminal gas flow regulator (Figure 1 GFR) at a rate of 80 mL min^{−1} to quantitatively capture any CO₂(g) evolved in the headspace through exchange across the air/water boundary; and (5) sparge Valve 1 is opened and the pH adjusted to 2.8 through injection of 1 N HCl via septum Port D to mobilize all CO₂(aq) into the headspace [CO₂(g)] and eliminate fractionation artifacts between DIC constituents.

The purge gas containing the evolved CO₂ is sent through the 2 water traps (Figure 1, WT) before cryogenic trapping in liquid N₂ at a flow rate of 80 mL min^{−1}. The ReCREs sparges for a minimum of 12 h, throughout which time the temperature of both water and CO₂ traps is maintained. Upon harvest termination, toggle valves 4 and 5 on the ReCREs CO₂ trap are simultaneously closed and the trap removed (Figure 1). To verify complete conversion of CO₂(aq) to CO₂(g), samples for DIC are removed from Port E as previously described.

Analytical procedures—DIC and DOC were determined using an OI 2010 TIC/TOC Analyzer. Analytical precision was determined from multiple injections ($n = 3$) per sample and ranged from ± 0.003 to 0.08 ppm for the low and high range of concentrations, respectively. Stable carbon isotope ratios were performed at the G.G. Hatch Laboratory (University of Ottawa). Isotopic analysis ($\delta^{13}\text{C}$) of DIC and DOC was performed on a

modified OI Analytical model 1010 wet oxidation TOC analyzer interfaced with a Finnegan MAT Delta Plus IRMS with a CONFLO III continuous flow interface, as detailed by St-Jean (2003). Analytical precision was typically < 0.20‰. Results for stable isotope values are reported in standard δ notation as:

$$\delta^{13}\text{C} = [(R_{\text{sample}}/R_{\text{standard}}) - 1] \times 10^3 \quad (1)$$

where R is $^{13}\text{C}/^{12}\text{C}$.

Recovered CO₂ was extracted from ReCREs traps, split for $\delta^{13}\text{C}$ and $\Delta^{14}\text{C}$ isotopic analyses, and transferred to breakseals. ReCREs traps were connected via an o-ring connector to a vacuum line in the G.G. Hatch laboratories. The vacuum line was pressurized with 1 atm of UHP He, the second coil of the ReCREs was immersed in liquid N₂, and toggle Valve 5 on the ReCREs trap was opened. The recovered CO₂ was expanded, stripped of residual moisture, and quantified manometrically (Baratron, 0.5 μmol sensitivity) before transfer to breakseals. Breakseals for $\delta^{13}\text{C}$ analysis were then transferred into Exetainers and run in continuous flow via GasBench peripheral (Thermo Finnigan) interfaced to an Isotope Ratio Mass Spectrometer Delta XP (Thermo Finnigan) with an analytical precision of 0.10‰.

For natural abundance $\Delta^{14}\text{C}$ measurements, CO₂ from the breakseals was reduced to graphite in an atmosphere of H₂ over cobalt catalyst (Vogel et al. 1987). Graphite targets were analyzed at the Center for Accelerator Mass Spectrometry at Lawrence Livermore National Laboratory. $\Delta^{14}\text{C}$ is defined as the ‰ (per mil) deviation of a sample from the ^{14}C activity of 19th-century wood. All reported $\Delta^{14}\text{C}$ values were corrected for fractionation using the $\delta^{13}\text{C}$ values of the samples and the conventions of Stuiver and Polach (1977). Total measurement uncertainties for $\Delta^{14}\text{C}$ analyses of these samples were typically ± 5–8‰.

Spectrofluorometric characterization of initial and pH-adjusted (acidified/neutralized) DOC (e.g., humic constituents) was carried out according to McKnight et al. (2001). Fluorescence was measured on a RF-5301PC Spectrofluorometer (Shimadzu) with 150-W Xenon lamp at 5-nm excitation wavelength intervals between 220 and 450 nm and at emission ranging between 260 and 560 nm at 5-nm increments (Cammack et al. 2004) in a temperature-controlled (20°C) cuvette chamber.

Bacterial biomass and activity—Subsamples (2 mL) for bacterial abundance were preserved with filtered 25% glutaraldehyde diluted to a final concentration of 1% and enumerated using a Becton Dickinson FACScalibur flow cytometer and the nucleic acid stain Syto-13. Rates of bacterial production were determined by ³H-leucine incorporation as modified by Smith and Azam (1992).

Assessment

The two factors critical for an accurate measurement of the isotopic ($\delta^{13}\text{C}$, $\Delta^{14}\text{C}$) signature of bacterial respiratory CO₂ are (1) that all of the CO₂(g) recovered is derived primarily from bacterial respiration and (2) that all the DIC generated during the incubation is quantitatively recovered [as CO₂(g)] and the isotopic fidelity between the organic matter respired and the

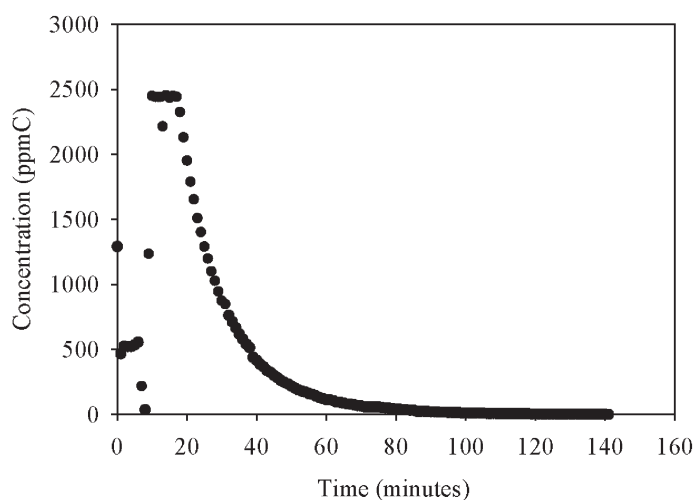


Fig. 3. Time course of CO₂ concentrations in the headspace of the ReCREs following Figure 2A, Step 2.

CO₂(g) collected is retained. In addition, because the main application of this work is to assess the sources of OC (from the C isotopic signatures) supporting in situ aquatic bacterial respiration, potential methodological artifacts affecting the lability or quality of the OC arising from sample manipulation (i.e., pH adjustments) must be evaluated.

Bacterial respiration as the primary source of recovered CO₂(g)—To evaluate the former criteria, one must consider first whether the majority of the initial DIC pool was removed and second whether the recovered CO₂ resulted predominantly from bacterial metabolism rather than methodological additions (e.g., atmospheric intrusion, NaOH, bacterial inoculum).

To verify quantitative removal of the initial DIC pool and the evolved CO₂(g), the terminal outlet of the harvest line was connected to a PP Systems (EGM 3) IR (sensitivity 0.5 ppm) to measure CO₂ concentrations in the headspace of the incubation system. An initial spike of CO₂ saturated the detector (> 2500 ppm) after acidification of sample to pH 2.8 (Figure 3). This peak of CO₂ quickly (< 20 min) dropped to a baseline value of ~25 ppm before gradually tailing to 0 ppm (140 min), indicating complete removal of CO₂(g) from the headspace (Figure 3). DIC concentrations measured from water collected initially and postsparge verified removal of > 98.7% of the initial DIC (Table 1). The same pattern was observed in 5 different trials performed.

Postinoculation samples were removed from Port E to monitor bacterial growth during the incubation. In all trials, there was a relatively long (> 48 h) lag phase in bacterial growth (Figure 4), after which the rates of bacterial production quickly increased. The lag phase that we observed in the ReCREs incubations was longer than that in parallel regrowth incubations conducted using the same water samples that had not undergone DIC stripping, suggesting the longer lag may be related to some aspect of the experimental manipulation. In particular, heterotrophic bacteria use external CO₂ for some meta-

Table 1. Removal of background DIC pool and respiratory CO₂ recovery.

Trial number	Initial DIC, ppm	Postsparge DIC, ppm	Recovered CO ₂ , μmol
1	8.3	0.08 ± 0.01	109
2	6.4	0.06 ± .01	93
3	5.6	0.07 ± .001	94
4	5.4	0.07 ± .001	65
5	4.3	0.06 ± .004	76

Data are means ± SD.

bolic pathways through the anaplerotic B-carboxylation reactions (Overbeck 1979), and the almost complete absence of DIC in the samples at the start of the incubations (< 0.1 ppm, Table 1) may temporarily limit bacterial growth. After this initial lag phase, the ReCREs incubations attained levels of leucine uptake similar to those of the parallel regrowth incubations. Thus, although incubations carried out to determine respiration rates in aquatic ecosystems are typically shorter than these ReCREs incubations (typically < 48 h; Pace and Prairie 2005), once the long initial lag phase (> 50 h) is accounted for, the rates of bacterial growth, as well as the dissolved organic matter (DOM) and inorganic nutrient consumption in the ReCREs incubations, are similar to those in unmanipulated samples.

Control experiments—The introduction of inorganic carbon [DIC, CO₂(g)] from sample processing was assessed quantitatively through control incubations processed concurrently with the sample. Initially, the ReCREs operating procedure detailed above was duplicated for a killed control with the addition of 50 mL of a saturated HgCl₂ solution through Port D following system inoculation. However, unaccountably high values of CO₂(g) (36 to 50 μmol) were recovered despite undetectable levels of bacterial production (data not shown). Further, no dis-

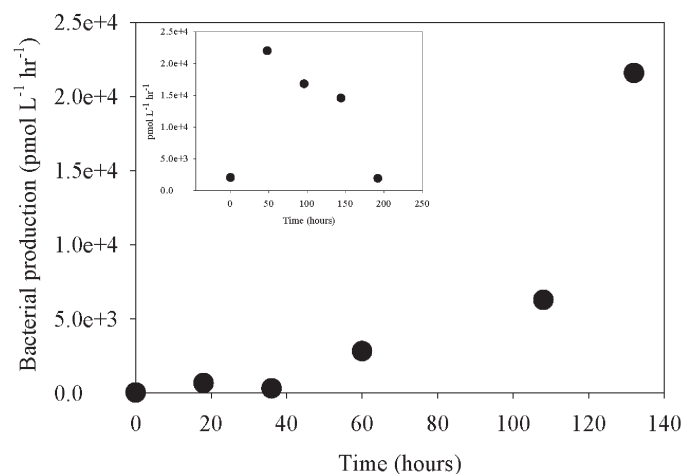


Fig. 4. Time course of bacterial production during regrowth incubation (Figure 2A, Step 5). Inset shows time course of bacterial production from parallel regrowth incubations.

Table 2. HgCl₂ killed and killed inoculum control incubation recoveries and δ¹³C values.

	Recovered CO ₂ , μmol	δ ¹³ C, ‰
HgCl ₂ control	36.0	-27.3
	39.0	-28.4
	39.5	-25.3
	49.5	-26.7
Killed inoculum	8.1	-29.0
	9.2	-22.8
	6.0	-28.6
	10.6	-30.2
	8.6	-27.2

cernible leaks in the incubation system or harvest line were identified with the He leak detector, nor did the isotopic signature of recovered CO₂(g) (-26.9‰ ± 1.3‰) reflect atmospheric values in the room (-13.3‰; Table 2). DIC concentrations of the HgCl₂ stock solution were minimal (0.04 ppm) and could not account for the amount of recovered CO₂(g).

Consequently, we carried out alternative controls, in which rather than poisoning the sample, we limited bacterial growth by inoculating the systems with a killed rather than a live inoculum. The latter consisted of the boiling and subsequent cooling of approximately 100 mL of the 300-kDa filtrate (i.e., bacteria-free) from the ultrafiltration unit. The sequence of DIC-free water exchange and injection was followed as above. Leucine incorporation in these “killed inoculum” controls was measurable but remained at < 10% of the sample. CO₂ recoveries for the killed inoculum control averaged 8.5 ± 1.7 μmol and were < 10% of the sample (Table 2). As the CO₂(g) recovered from the control may be attributed in part to some low level of bacterial respiration and also to methodological additions, we conducted additional experiments to better constrain the contributions from each. Control incubation systems were filled with filtered lake water, and each step of the inoculation operating procedure was performed additively (Step 1, Step 1 + Step 2, etc.; Figure 2). The

controls were harvested immediately (Figure 2B) so that the recovered CO₂ could be attributed solely to methodological manipulations. Methodological additions of DIC [as recovered CO₂(g)] were minimal (4.7 + 1.5 μmol), implying that up to 50% of the CO₂(g) recovered in the killed inoculum controls was derived from bacterial respiration. These results suggest that methodological additions of CO₂ are consistently < 5% of the total CO₂ recovered at the end of the incubations and thus have a small impact on the δ¹³C signature of this carbon.

Extraction efficiency and isotopic fidelity (δ¹³C, Δ¹⁴C) of source OC and recovered CO₂—Abiotic tests. Evaluating the recovery efficiency of the ReCREs is not straightforward. Bacterial consumption of a given amount of organic substrate will result in the production of bacterial biomass and CO₂, the relative proportion of each determined by bacterial growth efficiency (BGE) (del Giorgio and Cole 1998). Thus although we may manipulate the incubation conditions such that the total amount of OC added is metabolized, thereby alleviating isotopic fractionation effects, the proportion to be recovered as CO₂ remains uncertain. To circumvent the problem of inferring quantitative C recovery from estimates of BGE, we employed instead an abiotic assay to evaluate the CO₂ recovery efficiency of the ReCREs.

ReCREs flasks were filled with DI water and the system initialized according to Steps 1-5 of the sparge protocol (Figure 2A). A freshly prepared 200 ppm Na₂CO₃ (Sigma S-7795) solution (8 mL) was injected through septum Port D (Figure 1). The Na₂CO₃ was then recovered as CO₂(g) following the steps of the recovery protocol (Figure 2B), and the δ¹³C of the Na₂CO₃ stock solution and the recovered CO₂(g) was analyzed following the methods described above. Approximately 100% (134 ± 6 μmol; Table 3) of the initially added Na₂CO₃ was recovered as CO₂(g), the slight excess presumably attributed to methodological manipulations (see previous section). Further, the δ¹³C of harvested CO₂(g) (-5.3‰ ± 0.5‰) relative to the stock solution of sodium bicarbonate (-5.6‰) suggests that any fractionations resulting from incomplete recovery were well within the analytical error of the continuous flow measurement (Table 3).

Table 3. Quantitative recovery of CO₂ and isotopic fidelity.

Substrate	Added			Recovered		
	Amount, μmol	δ ¹³ C, ‰	Δ ¹⁴ C, ‰	CO ₂ , μmol	δ ¹³ C, ‰	Δ ¹⁴ C, ‰
Na ₂ CO ₃	133.3	-5.6	NA	134 ± 6	-5.3 ± 0.5	NA
Glucose	1979.2 ^a	-13.0 ± 0.6	74	119 ± 5	-14.6 ± 0.1	59 ^b
Lake 08/04/05	NA	NA	NA	78	-32.2	NA
Lake 09/01/05	NA	NA	NA	108	-32.5	NA

Data are means ± SD. NA, not applicable.

^aGlucose added at 1.25 ppm C concentration.

^bBlank corrected from the equation: $\Delta^{14}\text{C}_{(\text{sample} + \text{blank})} = \Delta^{14}\text{C}_{(\text{sample})} (x) + \Delta^{14}\text{C}_{(\text{blank})} (y)$, where $\Delta^{14}\text{C}_{(\text{sample} + \text{blank})}$ is the measured isotopic value for the recovered CO₂, $\Delta^{14}\text{C}_{(\text{blank})}$ is the isotopic signature from the control incubation, and x and y are the relative contributions of sample and blank CO₂, respectively, to the total CO₂ recovered. The equation was solved for the isotopic signature of the sample CO₂ ($\Delta^{14}\text{C}_{(\text{sample})}$).

To concurrently evaluate a potential source of sample CO₂ loss as well as the efficiency of the ReCREs trap, a second ReCREs trap was placed inline and downstream of the primary recovery trap. The incubation system was filled with lake water and the initial sparge and recovery procedure (Figure 2) was followed with both traps (4 coils) immersed in liquid N₂ to determine whether sample CO₂ was recovered in the secondary trap. Both traps were processed on the vacuum line as indicated above and 108 μmol C were measured in the initial trap, whereas no CO₂ was detected in the second trap (Baratron pressure gauge, ± 0.5 μmol), thus serving as an independent validation of harvest conditions (e.g., flow rate 80 mL min⁻¹, dual coil trap configuration).

Biotic tests. The final assessment of the ReCREs, and perhaps the most critical for applications of identifying the sources and ages of OC fueling bacterial respiration, is whether the natural abundance C isotopic (δ¹³C, Δ¹⁴C) signature of the organic substrates is preserved in the recovered CO₂(g). Our evaluation of the ReCREs in this aspect is potentially complicated by isotopic discrimination via bacterial enzymatic pathways. Previous research using a single OC source and one species of bacteria suggested that the respired C was generally more depleted (2-3‰) in δ¹³C than the substrate (Abelson and Hoering 1961; Blair et al. 1985). In contrast, Hullar et al. (1996) found similar values or slight enrichments in the δ¹³C of respired CO₂ relative to the initial δ¹³C of the DOC pool in a more complex system with a microbial consortium and mixed substrates. Consequently we anticipate potential slight positive or negative deviations in the δ¹³C, Δ¹⁴C of respired C relative to the isotopic value of the organic substrate as a result of bacterial fractionation (e.g., not fractionations from quantitative recovery).

The most direct evaluation of isotopic fidelity between OC substrate and respired C is through use of a sole C substrate. Artificial lake water was prepared according to Bastviken et al. (2004) with slight modifications to the concentration of N, P, and OC substrate to better duplicate previously measured rates of respiration and nutrient conditions in the lakes of interest (E.G., unpublished data). Glucose (Sigma G-7528) was added to a final concentration of 1.25 ppm (as C) and N and P as NH₄Cl and Na₂HPO₄ to 0.4 ppm and 0.08 ppm, respectively. Duplicate sample incubators were run in parallel using this artificial lake water with glucose added. The initialization and harvest procedures (Figure 2) were followed as described above and approximately 119 ± 5 μmol C were recovered in the ReCREs traps (Table 3). A slight 1-2‰ fractionation was calculated for the δ¹³C of respired C (14.6‰ ± 0.1‰) relative to the δ¹³C of the initial DOC in the incubation system (-13.0‰ ± 0.6‰; Table 3). The blank corrected (see Table 3) radiocarbon value of the respired C (+ 59‰) was equivalent to the starting substrate (glucose powder, + 74‰) considering the range of error (± 5-8‰) in radiocarbon measurements. Given the dynamic range of potential radiocarbon values in aquatic systems (~ -1000 to + 435‰), the ReCREs provides adequate accuracy to differentiate between OC sources fueling bacterial respiration.

Last, surface water from a moderately productive lake (chlorophyll *a* 5-7 μg L⁻¹) was collected (August and September 2004) and processed as detailed above, and the ReCREs's initialization and recovery procedures (Figure 2) were followed. Approximately 78 and 108 μmol of CO₂ were recovered for the August and September incubations, respectively (Table 3). The amounts are in agreement with the rates of respiration previously determined for this lake, once the initial lag phase is discounted (E.G., unpublished data). The δ¹³C of recovered CO₂, -32.2‰ and -32.5‰ for August and September, respectively, varied by only 0.3‰, demonstrating the consistency and reproducibility of the ReCREs for field samples containing a mixed OC substrate (Table 3).

Collectively, these results suggest that the respiratory DIC [as CO₂(g)] is quantitatively recovered and the isotopic fidelity (δ¹³C, Δ¹⁴C) between the OC respired and the CO₂(g) harvested is retained (Table 3). Furthermore, reproducibility of δ¹³C CO₂ measurement was 0.1‰, which is sufficient to discriminate between allochthonous and autochthonous pools of OC in these lakes (-27‰ to -35‰; McCallister and del Giorgio, in press).

Evaluation of potential artifacts from pH adjustments and methodological manipulations—Potential conformational changes to DOC arising from methodological manipulations were evaluated with 3 independent methods: (1) DOC lability was compared between manipulated and ambient incubations, (2) bacterial production was measured in manipulated and ambient incubations, and (3) DOC was assessed fluorometrically before and after methodological modifications. There were no significant differences resulting from pH changes in any of the parameters assessed. DOC lability determined using bacterial regrowth experiments over ~4 days of incubation was similar between acidified and non-acidified incubations (and other methodological manipulations), with approximately 0.20 ± 0.03 ppm and 0.21 ± 0.02 ppm used in the acidified vs. non-acidified incubations, respectively. Bacteria attained comparable peak levels of production irrespective of whether the sample was manipulated (e.g., harvest protocol; Figure 2), with 45.6 ± 0.6 nmol L⁻¹ h⁻¹ and 49.3 ± 1.3 nmol L⁻¹ h⁻¹ of ³H-Leucine incorporated for manipulated and nonmanipulated incubations, respectively. It is important to note that although comparable peak levels of bacterial production were attained in the manipulated and nonmanipulated incubations, the initial sparge removes volatile OC as potential bacterial substrates from the ReCREs incubations. Last, spectrofluorometric analysis (McKnight et al. 2001; Cammack et al. 2004) resulting in over 2500 individual scans per sample (see above for wavelength details) were used to assess differences between ambient DOC (0.2 μm filtered) samples and those that had undergone pH and gas manipulation (Figure 2A, Steps 1-5). Samples were taken from a humic stream draining a wetland to provide a robust evaluation of the acidification procedure. Potential variations were assessed by subtracting output values between samples and plotting these differences

in 3 dimensions against excitation and emission wavelengths. No significant peaks were observed, suggesting minimal alteration to the conformational structure of DOC as a result of methodological manipulations. Our findings are consistent with Moran and Hodson (1990), who concluded that alterations in pH did not affect the quality of OC and its bioavailability to bacteria.

Comments and recommendations

The protocol that we present involves the removal of all particulate OC (POC) before incubation. As a result, the method targets the consumption and respiration of dissolved rather than total OC. The samples are filtered to remove particulates, as the abrupt pH changes and vigorous bubbling necessary to strip the DIC could result in the rupture, lysis, and leaching of planktonic organisms and other particles, creating an artificial supply of substrates and potentially biasing the results. There is no question, however, that POC may play a significant role in aquatic metabolism and represent a source of substrates for microbial respiration, but it is important to note that our basic protocol could be easily modified to assess the consumption and respiration of different types of POC.

An additional reason that our standard protocol involves the removal of all particulates before incubation is so that we can recover the bacterial biomass that develops during the incubations, to address fundamental questions about the bioenergetics of bacterioplankton growth. A comparison of the isotopic composition of the recovered bacterial biomass and respiratory CO₂ can be used to evaluate the patterns in substrate allocation by heterotrophic bacteria. The initial removal of particulates ensures that recovered biomass at the end of the incubation is composed primarily of bacterial carbon. We deal in detail with the procedures involved in isolating and analyzing the bacterial biomass obtained during these incubations in a separate paper (McCallister et al., in press).

Our protocol involves substantial manipulation of both water samples and bacterial communities as well as rather long incubation times. In addition to the many technical challenges related to the recovery of small amounts of respiratory CO₂ in sufficient amounts and purity for isotopic analysis ($\delta^{13}\text{C}$, $\Delta^{14}\text{C}$), a key question is to what extent the results obtained are representative of in situ carbon consumption and metabolic processes in lakes and rivers. The ReCREs provides a snapshot of lake metabolism, as there is no replenishment of the organic matter consumed. In this regard, the ReCREs is not fundamentally different from traditional incubations used to measure the rates of respiration. Future modifications to the ReCREs might use Port C (free with current configuration; Figure 1) to develop an auxiliary component to replenish the system with fresh OC and better reproduce in situ conditions.

Last, slight procedural modifications in the removal of the initial background DIC pool, such as replacement of the initial

DIC by sparging with a DIC of a known isotopic signature (¹⁴C-dead-CO₂), would allow use of the ReCREs in brackish and saline waters. For example, bacterial respiration of terrigenous OC with its unique isotopic ($\delta^{13}\text{C}$, $\Delta^{14}\text{C}$) signature (Bauer et al. 2002) could be traced across the continental margin. The ReCREs traps were leak-tested after 6 weeks and the integrity of the seal confirmed; hence the system could be used for cruise deployment with the removable traps allowing multiple harvests per cruise. The ReCREs system opens the door to a new generation of studies that will allow researchers not only to quantify respiration rates in natural aquatic ecosystems, but more importantly to systematically assess the sources and ages of the OC respired, and thus study the links and lags between OC production, loading, and ecosystem metabolism.

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Submitted 6 October 2005

Revised 15 May 2006

Accepted 19 June 2006