Carbon-isotopic analysis of microbial cells sorted by flow cytometry

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ABSTRACT

One of the outstanding current problems in both geobiology and environmental microbiology is the quantitative analysis of *in situ* microbial metabolic activities. Techniques capable of such analysis would have wide application, from quantifying natural rates of biogeochemical cycling to identifying the metabolic activity of uncultured organisms. We describe here a method that represents one step towards that goal, namely the high-precision measurement of ^{13}C in specific populations of microbial cells that are purified by fluorescence-activated cell sorting (FACS). Sorted cells are concentrated on a Teflon membrane filter, and their ^{13}C content is measured by coupling an isotope ratio mass spectrometer (IRMS) with a home-built spooling wire microcombustion (SWiM) apparatus. The combined instrumentation provides measurements of $\delta^{13}C$ in whole cells with precision better than 0.2‰ for samples containing as little as 25 ng of carbon. When losses associated with sample handling are taken into account, isotopic analyses require sorting roughly 10^4 eukaryotic or 10^7 bacterial cells per sample. Coupled with ^{13}C-labelled substrate additions, this approach has the potential to directly quantify uptake of metabolites in specific populations of sorted cells. The high precision afforded by SWiM-IRMS also permits useful studies of natural abundance variations in ^{13}C. The approach is equally applicable to specific populations of cells sorted from multicellular organisms.

INTRODUCTION

The realization that less than 1% of known microorganisms have been isolated in laboratory culture (e.g. Staley & Konopka, 1985; Keller & Zengler, 2004) provides a strong motivation for the development of new methods to study *in situ* metabolic activities. For some organisms, the consumption or production of specific substrates or metabolites provides a window into their activity that can be correlated with cell numbers. Methanotrophy, sulfate reduction, and nitrogen fixation are prominent examples of such activities (Barnes & Goldberg, 1976; Hoehler *et al.*, 1994; Karl *et al.*, 1997). Yet for most of this uncultured majority, their basic metabolic processes – and thus influence on ecology and biogeochemical cycling – remain largely unknown. Connecting phylogenetic identity with metabolic function in environmental samples therefore remains a top priority for geobiologists, microbial ecologists, and biogeochemists alike. Even for those microbes amenable to current culture techniques, biochemical processes and rates studied in cultures cannot easily be extrapolated to natural, more complex ecosystems. Studies of these organisms also stand to benefit from methods for assessing *in situ* metabolism. We describe here a method for analysing the carbon-isotopic composition of whole microbial cells sorted from environmental samples by flow cytometry, one that should provide a valuable new tool in these pursuits.

A variety of techniques for assaying *in situ* metabolic activity are currently under development. Within the discipline of molecular biology, approaches such as mRNA analysis (Pernthaler & Pernthaler, 2005) and antibody-based enzyme assays (Krieger *et al.*, 2000) hold promise, but are generally qualitative rather than quantitative indicators. DNA stable isotope probing (DNA-SIP) can provide a definitive link between *in situ* metabolic activity and taxonomy, but requires high levels of ^{13}C incorporation into DNA and is thus rather insensitive to slowly growing organisms (Friedrich, 2006). The combination of radioactive substrates, microautoradiography, and fluorescent *in situ* hybridization (MAR-FISH; Lee *et al.*, 1999; Ouverney & Fuhrman, 1999) allows for both detection of a specific metabolic activity and its phylogenetic origin (Nielsen

et al., 1999), as well as enumeration of the population carrying out the activity (Ito *et al.*, 2002; Nielsen *et al.*, 2003). 'Selective polymerase-biotinylation and capture' (SNAP-BAC) provides quantitation of bacterial cell production in a selected phylum by measuring [^{3}H-methyl] thymidine incorporation (Van Mooy *et al.*, 2004), but does not allow introduction of varied substrates.

Analyses of the stable-isotopic composition (including ^{2}H, ^{13}C, ^{34}S, ^{15}N, and ^{18}O) of biological constituents have been used with considerable success to study *in situ* microbial processes (Conway *et al.*, 1994; Zyakun, 1996; Boschker & Middelburg, 2002; Chidthaisong *et al.*, 2002; Johnston *et al.*, 2005). Methods for analysis of ^{13}C are the most fully developed, and so have received the heaviest use in studies of microbial metabolism. This report focuses exclusively on ^{13}C, while the potential for its extension to other isotopes (particularly ^{15}N) is obvious. Environmental studies utilizing ^{13}C offer two potential benefits. First, ^{13}C-enriched tracers can be added to incubated samples without the safety concerns associated with radioactive substrates. Aerosols generated by fluorescence-activated cell sorting (FACS) instruments preclude the use of radioactive isotopes without elaborate safety precautions. Second, variations in the natural abundance of ^{13}C can be used to infer the presence of specific biochemical reactions or intermediaries, such as carbon fixation or methanotrophy (Whiticar, 1999; Hinrichs *et al.*, 2000; Hayes, 2001).

Analytical methods that facilitate such C-isotopic experiments share the following common properties. First, they must isolate and measure chemical fractions that can be attributed to specific organisms or groups of organisms. Second, they must be sensitive enough to work with typically small samples, while (third) be sufficiently accurate to distinguish natural variations in ^{13}C content. The first of these requirements has, thus far, proven to be the most stringent. Indeed, isolation of specific sample fractions remains the single greatest challenge in applying most isotopic methods to complex environmental samples.

Some organisms produce distinctive membrane lipids or pigments (Volkman *et al.*, 1998; Green & Scow, 2000), and in these cases analysis of individual compounds by coupled gas chromatography-isotope ratio mass spectrometry (GC-IRMS) is the most direct approach. For example, Hinrichs *et al.* (2000) were able to document methanotrophy by a group of novel marine archaea by measuring δ^{13}C values of the lipid archaeol. Zhang *et al.* (2005) demonstrated, based on δ^{13}C measurements of phospholipid fatty acids, heterotrophic growth on non-methane hydrocarbons by *Beggiatoa* mats in the Gulf of Mexico. Unfortunately, such biomarkers are rarely specific at the genus level, nor can they be definitively attributed to organisms that have never been cultured. Moreover, the majority of bacteria do not produce any diagnostic lipids. Pel *et al.* (2003, 2004) introduced a hybrid approach in which they measured δ^{13}C of fatty acids extracted from whole microbial cells that had been isolated by FACS. The biomass requirements for

such techniques are relatively high (~2 μg) because fatty acids are only a minor component of cell biomass. A similar method developed by Minor *et al.* (1998, 1999) also utilizes FACS to separate cellular material from detritus in natural waters, but uses direct temperature-resolved mass spectrometry (DT-MS) and so does not provide isotopic compositions.

A method for analysis of ^{13}C in microbial nucleic acids was developed by Coffin *et al.* (1990), and has been used to assess sources of carbon for estuarine bacteria (Coffin *et al.*, 1990; Coffin & Cifuentes, 1999; Grey *et al.*, 2001; Colaco *et al.*, 2002). However, the nucleic acids collected in these studies are derived from all bacteria rather than from specific groups. A much higher level of specificity can potentially be obtained through the separation of ribosomal RNA by probe-capture techniques (MacGregor *et al.*, 2002; Pearson *et al.*, 2004), an approach that appears promising. Because nucleic acids are non-volatile, the sample fractions isolated by these procedures cannot – regardless of how they are purified – be analysed by GC-IRMS and hence have high sample requirements. Improved methods for isotopic analysis of non-volatile carbon, such as the one described here, will make all such methods much more practical.

Orphan *et al.* (2001, 2002) measured the carbon-isotopic composition of whole cells by secondary ion mass spectrometry (SIMS). Cells were identified by FISH and epifluorescence microscopy prior to isotopic analysis, thus enabling the direct connection of phylogeny (ANME-1 and 2) with metabolic function (methanotrophy). The use of SIMS provides exquisite sensitivity, with sample requirements as low as a single cell, and is extremely promising. There are several drawbacks, however. First, FISH-SIMS requires expensive instrumentation operated by trained technicians, and access to such instrumentation is a significant issue for many researchers. Second, the accuracy of SIMS analyses, particularly for very small samples, is relatively poor and difficult to quantify. Although reported precision for replicate δ^{13}C measurements of homogeneous standards is often as low as 1‰ (Orphan *et al.*, 2001), issues of standardization lead to larger uncertainties in the absolute accuracy of such measurements.

As a complement to such approaches, we have developed a new method in which whole cells are isolated by FACS, and their ^{13}C content is measured with high precision using a custom-built, spooling wire microcombustion (SWiM) device coupled to a conventional IRMS. This method offers several advantages, including (i) utilization of existing, widespread technology for cell sorting, (ii) maximizing the use of limited biomass by avoiding isolation of minor components, and (iii) the ability to measure very small samples, containing <1 μg biomass, with very high isotopic precision. The approach is amenable to either enriched-tracer or natural abundance types of experiments. While SWiM devices are not yet commercially available, they are simple enough to allow fabrication in any laboratory with access to an IRMS.

One current limitation of our approach is that flow cytometry is only able to positively sort cells that exhibit a unique

fluorescence. Isolating a particular species or group of organisms from environmental samples thus requires selective staining. While this is possible for some organisms using FISH probes (Kalyuzhnaya *et al.*, 2006), it is generally difficult to achieve adequate fluorescence intensity (Sekar *et al.*, 2004). One promising development is the amplification of fluorescence using the CARD-FISH protocol (Ishii *et al.*, 2004; Sekar *et al.*, 2004). For specific targets that have been cultured, immunofluorescent probes offer an alternative (Toledo & Palenik, 2003; West *et al.*, 2006).

MATERIAL AND PROCEDURES

Spooling wire microcombustion device

Whole cells are combusted to CO_2 for isotopic analysis using a home-built microcombustion device in which samples are loaded and combusted on a continuously spooling wire. A previous version of this interface was described by Sessions *et al.* (2005), where it was referred to simply as a 'moving-wire device'. The instrumentation discussed here was built at Caltech and is optimized for the analysis of discrete samples, rather than as an interface for liquid chromatography (Brand & Dobberstein, 1996). We have adopted the new acronym SWiM to emphasize this distinction. While the two devices are principally the same, a few improvements are described in detail below.

Operation of spooling-wire-type devices for analysis of discrete samples is described in detail by Sessions *et al.* (2005). In brief, a single droplet of liquid sample is placed onto a continuously, slowly spooling wire. The sample is carried by the wire through a heated tube where the solvent evaporates, and then into a combustion reactor where remaining non-volatile organic matter is quantitatively oxidized to CO_2, H_2O and $N_2 + NO_x$. A fraction of the product gases is carried to an IRMS for continuous measurement of the $^{13}C/^{12}C$ ratio of the CO_2 produced.

The system employed by the present study is shown in Figure 1. Nickel wire is guided through the system by ceramic pullies (Cosmos North America, Carmel, IN, USA) mounted on an optical breadboard, which serves as a base for the interface. Careful alignment of components reduces drag and thus tension on the heated wire, eliminating most wire breakage. Wire is cleaned of organic residue by spooling through a 60-cm-long oven (2×30 cm ceramic fibre tube heaters, WATLOW, Columbia, MO, USA) open to the air at both ends and heated to 900 °C. We use a 0.25-mm-diameter nickel wire with carbon content less than 20 p.p.m. (*N201*, AMETEK, Specialty Metal Products Division, Wallingford, CT, USA). Wire speed is typically 0.7 cm s^{-1}, a compromise between longer cleaning and drying time and slower analysis. Because the wire maintains a significant C content even after cleaning, the stability of the wire speed also impacts noise on the CO_2 background. To maintain precise control of wire speed, a
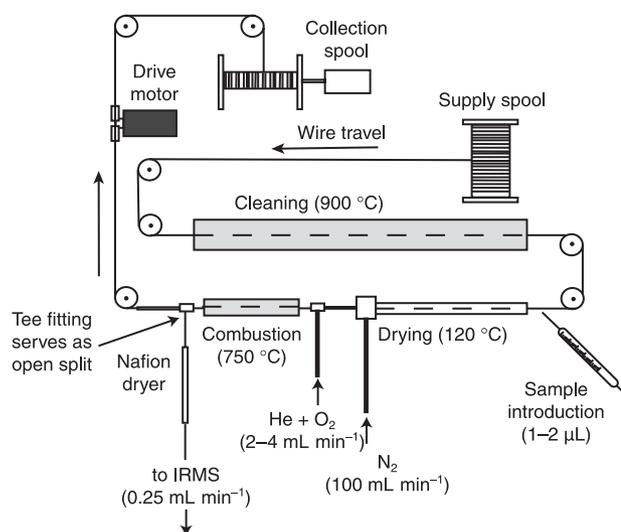

Fig. 1 Schematic drawing of the SWiM device. The system is mounted horizontally on a 60 by 90 cm optical breadboard. Operation of the components is described in the text.

highly regulated brushless DC servomotor (model #3056, Minimotor SA/Faulhaber, Croglio, Switzerland) with a planetary gearhead (model 30/1, Faulhaber) is employed. The overall effect of these improvements is to reduce background CO_2 signals in our device to approximately 40% of those reported by Sessions *et al.* (2005).

As the wire exits the cleaning oven, 1 μL of sample suspension is transferred onto the wire from a syringe. The sample droplet then traverses a 35-cm-long glass tube (1.25 cm inner diameter) heated to 120 °C (Fig. 1) where all solvent is evaporated. The tube is flushed with 100 mL min^{-1} dry N_2 counter-current to wire travel to aid evaporation and to limit contamination by airborne dust. Next, the non-volatile sample residue is carried into a combustion reactor consisting of a 20-cm-long quartz glass tube, 3.2 mm OD and 0.8 mm ID, heated to 750 °C by a ceramic-fibre tube heater (WATLOW). One 10-cm-long Pt wire and four Cu wires (all 0.25 mm diameter) are twisted together and inserted into the reactor to serve as catalytic surface and oxidant, respectively, for sample combustion. Cu wires are oxidized in place using 100% O_2 at 500 °C for 24 h prior to use of the analyser. After its initial oxidation, the reactor can be used for about a month with occasional overnight re-oxidation. A standard – in this case lyophilized *Escherichia coli* cells – is used to regularly test for reactor oxidation capacity.

Helium, supplemented with a small flow of O_2, is used as the carrier gas. O_2 is added to ensure excess oxidizing capacity during sample combustion (Merritt *et al.*, 1995). The flow rate of O_2 is set to produce a signal at the IRMS for *m/z* 32 between 3.3 and 6.7 nA (~1 V in the mass 44 detector). Since both ends of the reactor must be open to allow passage of the wire, the flow rate of He is adjusted to minimize air entering
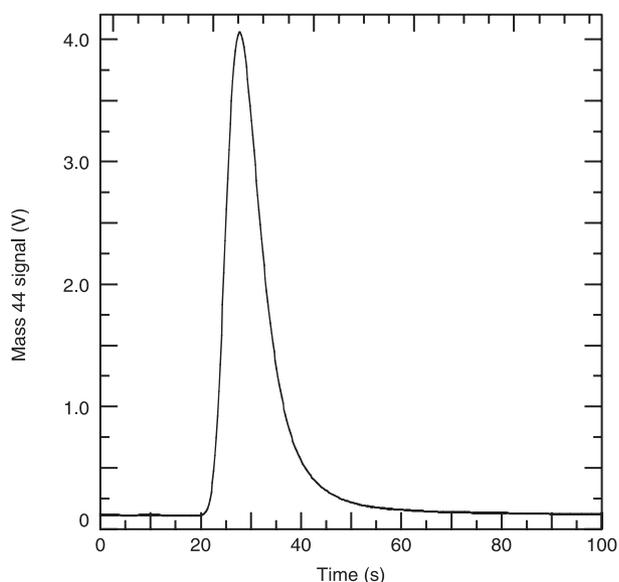
Fig. 2 Typical peak recorded by the SWiM-IRMS. This CO_2 peak represents 60 ng carbon from an *Eescherichia coli* whole-cell suspension. Peak width at 50% height is 8 s, the signal returns to baseline in ~60 s.

the IRMS. This arrangement also serves to buffer pressure fluctuations caused by the combustion of analytes, thus the tee fitting at the exit end of the combustion reactor (see Fig. 1) is effectively an open split. The earlier moving-wire device was equipped with a separate open split, resulting in roughly two-to fourfold lower transfer efficiency of combustion gases to the IRMS (Brand & Dobberstein, 1996; Sessions *et al.*, 2005). We have not observed any problems using the combustion reactor itself as the open split.

Peaks of CO_2 produced by the SWiM device have full widths at half maximum from 5 to 10 s depending on sample size (Fig. 2). Moderate tailing of the peaks is present, likely due to unswept volume in the exit union. Both peak width and tailing decrease with increasing carrier gas flow rate, but this also lowers sensitivity by decreasing the fraction of CO_2 transmitted to the mass spectrometer. At optimal sensitivity some tailing of peaks is thus unavoidable.

Carbon isotope analysis

The carbon-isotopic composition of whole cells was measured by coupling the SWiM to a Finnigan MAT DeltaS IRMS via a fused silica capillary. Isotopic analyses were then performed in an analogous fashion to a conventional elemental analyser (EA)-IRMS system. Pulses of CO_2 reference gas, used to standardize all analyses, were produced using a Finnigan Conflo interface. Values of $\delta^{13}C$ for samples were calculated by ISODAT 2.0 software (Thermo-Fisher Scientific, Bremen, Germany) and are reported in permil (‰) units relative to the VPDB standard. To further reduce analytical uncertainties, four to six aliquots of each sample are typically analysed in rapid succession, with

each aliquot separated by 90–120 s. This sequence of peaks is bracketed fore-and-aft by reference gas pulses. The isotopic composition and analytical uncertainty of the sample are then reported as the arithmetic mean and standard error ($\sigma/\sqrt{n}$), respectively, of the replicate aliquots.

Cell cultures and standards

Escherichia coli MG1655 was cultured at 37 °C in 3 mL LB broth or in 10 mL of M9 minimal media (Miller, 1972) with glucose as sole carbon source. The non-fermenting bacterium *Shewanella oneidensis* MR-1 was cultured in 3 mL LB broth at 30 °C. To produce bacterial biomass with varying $\delta^{13}C$ values, a small amount of ^{13}C-labelled pyruvate (Cambridge Isotope Laboratories Inc, MA, USA) was added along with glucose to a minimal media culture. Overnight cultures in the LB broth typically reached cell density of 10^9 cells mL^{-1} at the time of harvest, while cultures in minimal media reached 10^7 cells mL^{-1}. Cell densities were calculated from optical density at 600 nm measured with a DU 7400 spectrophotometer (Beckman Coulter, Fullerton, CA, USA) and calibrated with *E. coli* and CFU counts. At harvest, cells were washed three times with a saline solution (0.85% NaCl prepared from deionized water and precombusted NaCl) by pelleting at 12 000 *g* for 2 min in Eppendorf tubes, removing the liquid and resuspending in fresh saline solution.

The eukaryotic yeast *Saccharomyces cerevisiae* RDB505/ RJD863 was grown in SD media, in some cases with the addition of ^{13}C-labelled glucose. For cell-sorting purposes two different strains were used, one of which produced green fluorescent protein (GFP). Cultures of cyanobacteria were grown in BG11 media bubbled either with CO_2 from a tank (*Synechococcus* sp. PCC 6301) or room air (*Synechococcus* sp. PCC 7942).

Several cultures were grown in larger volumes (250 mL to 1 L) for use as analytical standards. They were harvested and washed as above, rinsed once with deionized water and then lyophilized. The resulting dry cell cakes were gently crushed with a solvent-cleaned pestle and mortar, and stored in a dessicator. The $\delta^{13}C$ values of these standards were measured in triplicate using an ECS 4010 Elemental Analyser (Costech, Valencia, CA, USA) connected to the same IRMS and using the same CO_2 reference gas as for the SWiM-IRMS system.

Fluorescence-activated cell sorting

Cell sorting was performed at the Beckman Institute Flow Cytometry Facility (Caltech) with a FACSAria flow cytometer (Becton-Dickinson, San José, CA, USA) using a 488-nm (13 mW) excitation laser and detection at 530 ± 30 nm by a photomultiplier. Sorting rates varied from 1000 to 5000 events per second, with events triggered on the forward scatter signal. Sort criteria included the intensity of forward- vs. side-scattered light for particle size and green fluorescence

intensity. The instrument was run in 'high purity' mode wherein any droplet suspected of containing more than one particle was discarded. Timing was calibrated with 3 μm Accudrop Beads and the emission optics were calibrated with Rainbow Fluorescent Particles (Becton Dickinson Biosciences, Franklin Lakes, New Jersey, USA). Prepackaged sheath fluid was found to contain a high concentration of non-volatile carbon, which constitutes a blank for subsequent SWiM analyses. Therefore a 0.85% NaCl solution, prepared with deionized water and precombusted NaCl, was filtered through a 0.1-μm polycarbonate membrane filter and used as sheath fluid. Sorted cells were collected in 15 mL polypropylene Falcon tubes. Depending on cell size, $5 \cdot 10^6$ (*S. cerevisiae*) to $1 \cdot 10^7$ (*E. coli*) cells were accumulated in each sample destined for isotopic analysis. Volumes of the final isolates were 15–30 mL, with cell concentrations equivalent to ~0.05 ng C μL^{-1}. This concentration is far below the detection limit of the SWiM, so a cell concentration step was required prior to analysis.

Concentration and recovery of cell suspensions

Suspensions of 10^5 cells mL^{-1} were used to mimic FACS isolates for the purpose of testing cell concentration methods. The suspensions were prepared fresh for each experiment from an overnight culture, with cell concentration estimated from optical density. Each experiment consisted of three replicate suspensions of cells in 30 mL of 0.85% NaCl solution, plus a blank (30 mL saline solution), all subjected to identical treatment. Cell recovery was estimated by comparing the C content of both the original cell suspension and the recovered samples after blank subtraction.

Two different methods for concentrating cell suspensions were tested (see Results and Discussion). In the first, samples were transferred into 50 mL polypropylene Falcon tubes and centrifuged for 60 min at 1000 *g* (Beckman Coulter Allegra X-15R centrifuge equipped with swinging bucket rotor SX4750). The supernatant was carefully removed by suction through a glass pipette, leaving 1.5 mL liquid. Cells were resuspended and the suspension was transferred into a 1.5-mL Eppendorf tube and centrifuged at 12 000 *g* for 4 min (Eppendorf centrifuge 5415D, fixed rotor). A syringe was used to remove most of the supernatant, leaving 10–30 μL in which the cells were resuspended. The volume of the remaining suspension was estimated by weight after transfer to a preweighed 200 μL glass vial, and its C content was measured by SWiM-IRMS.

In the second method, samples were filtered onto a 0.2-μm nominal pore size, 5-mm-diameter Teflon membrane disk (Omnipore™ Membrane Filters, Millipore, Billerica, MA, USA). The funnel of a Millipore glass microanalysis filter holder, 25 mm in diameter, was adapted to hold the membrane by fusing a 3-mm ID glass tube to the inside of the filter funnel. During filtration a precleaned 25-mm-diameter GF/F filter was used as support for the Teflon membrane. The suspension was filtered under slight vacuum (0.2 psi). The Teflon mem-

brane was then transferred with forceps into a 200-μL conical glass vial and 10 μL of clean water was added. This water was used to wash cells off the membrane by repeatedly squirting the membrane with a syringe, and the rinsed membrane was then removed from the vial. Sample volume and C content were then measured as above.

Blanks and cleaning

The most important sources of C contamination during SWiM analysis are the glass vessels and syringes used to hold and transfer samples. For large sample sizes, e.g. cultured cells where the volume of the suspension to be analysed could be as large as 1 mL, simply baking glassware at 450 °C for 6 h produces a blank contribution that is typically 0.3–0.5 ng μL^{-1}, roughly 1% of sample C. However, for samples where final volumes are only 10–20 μL, i.e. sorted cells following concentration, the blank contribution from a baked 200 μL conical glass vial can be up to 1.1 ng C μL^{-1}. To remedy this, vials were individually heated in the flame of a Bunsen burner immediately prior to use, bringing the blank contribution down to ~0.1 ng C μL^{-1}. To minimize contamination arising from syringes, they were cleaned before each use by removing the plunger and rinsing the syringe barrel and needle with water, methanol and dichloromethane.

The $\delta^{13}C$ values of glassware and syringe blanks typically varied between −21 and −25‰. A Barnstead Nanopure Infinity system (APS Water Services Co., Van Nuys, CA, USA) provided the water used for cleaning and sample dilution. The measured C content of this water was less than 0.3 ng C μL^{-1}. All data reported below are corrected for blank contributions based on the procedural blanks processed for each experiment.

RESULTS AND DISCUSSION

Measurement precision and accuracy

The DeltaS IRMS used in this study is nearly two decades old. To determine whether its performance might be limiting the attainable isotopic precision of our SWiM-IRMS method, reference gas peaks of constant height, superimposed on a steady He background, were measured repeatedly. For peak areas larger than 10 Vs – roughly the same signal produced by a 25 ng sample – the precision of $\delta^{13}C$ values was <0.1‰. Peak areas smaller than 10 Vs resulted in a precision for $\delta^{13}C$ of 0.15–0.35‰. These values are comparable to those obtained for analyses of whole cells, suggesting that the IRMS, rather than the SWiM device, remains the fundamental limitation on isotopic precision.

The precision of $\delta^{13}C$ measurements for whole cells was estimated by measuring cell suspensions of three different cultured organisms, as well as dissolved sodium acetate (Fig. 3A). Stock cultures were diluted to yield a range of approximately 10–100 ng C μL^{-1}. For whole-cell samples, precision ranged
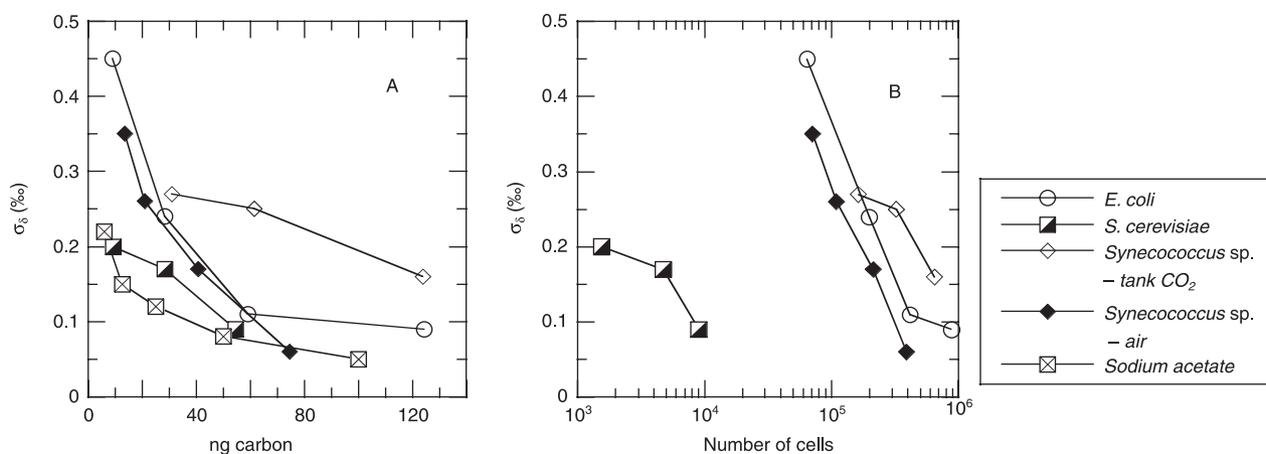
Fig. 3 (A) Precision of $\delta^{13}C$ measurements (σ_δ) by SWIM-IRMS as a function of sample size. *x*-axis refers to the mass of C deposited on the wire in each 1 μL aliquot. Precision is calculated as the standard deviation of six replicate aliquots of each sample. Measurements of microbial strains represent analyses of whole-cell suspensions. (B) Precision of $\delta^{13}C$ measurements as a function of cell numbers. This is the same data as plotted in panel A, with the *x*-axis transformed using typical carbon contents for the different species: *Escherichia coli:* 140 fg carbon/cell (Neidhardt *et al.*, 1990), *Synecococcus:* 200 fg carbon/cell (Bertilsson *et al.*, 2003), *Saccharomyces cerevisiae* 7200 fg carbon/cell (Sherman, 2002)

Table 1 Comparison of carbon-isotopic compositions of whole cells measured by SWiM-IRMS and by EA-IRMS

	Elemental Analyser		SWiM	
	$\delta^{13}C$ (‰)	Standard error (‰)	$\delta^{13}C$ (‰)	Standard error (‰)
Escherichia coli (glucose +^{13}C enriched pyruvate)*	+ 9.1	0.1	+ 8.9	0.1
Saccharomyces cerevisiae (glucose)	− 9.2	0.2	− 9.5	0.2
E. coli (glucose)	− 11.5	0.1	− 11.6	0.1
Shewanella oneidensis (LB medium)	− 23.2	0.1	− 23.2	0.1

*Growth substrate is given in parentheses.

from <0.1‰ at the highest concentrations up to 0.5‰ for more dilute suspensions of 10 ng C μL^{-1}. Sodium acetate yielded the best precision of 0.2‰ at a concentration of 10 ng C μL^{-1}, within a factor of 4 of the theoretical shot noise limit of 0.05‰ (calculated as in Sessions *et al.*, 2005). Measurements of smaller bacterial cells (*E. coli* and *Synecococcus*) are systematically less precise than those of the larger yeast cells even when carbon contents are identical. The reasons for this discrepancy are still unknown.

Isotopic analyses of *Synecococcus* grown on tank CO_2 were systematically less precise than for those grown in air. Values of $\delta^{13}C$ for the former cells were −50‰, while those for the latter were −24‰. Procedural blanks, including contributions from the water used to wash and suspend cells as well as from the syringe, have typical $\delta^{13}C$ values varying from −21‰ to −25‰. The decrease in precision for tank-grown *Synecococcus* is thus likely attributable to the larger effect of blank carbon on its isotopic composition (Sessions *et al.*, 2005).

We relate our estimates of isotopic precision to absolute cell numbers, a unit more relevant for cell sorting, using literature values for the carbon content of the different organisms (Fig. 3B). Based on these estimates, a final sample concentra-

tion of $0.5-1.0 \times 10^6$ bacterial cells μL^{-1} is needed to attain isotopic precision of 0.1‰, while only 10^5 cells μL^{-1} is required for precision of 0.5‰. For larger cells, such as yeast, a mere 1000 cells μL^{-1} is sufficient to obtain precision <0.2‰. In practical terms, samples must have a volume of at least 10–20 μL for efficient handling, and so the corresponding total requirements are thus ~10^7 bacterial and ~10^4 yeast cells.

The accuracy of whole-cell analyses was evaluated by measuring four cultures with differing isotopic compositions. Dried biomass from each culture was measured both by EA and by SWiM. Biomass was resuspended in deionized water for SWiM analyses. Within the range tested, the measured values of $\delta^{13}C$ from the SWiM system are virtually identical to those obtained using the conventional EA (Table 1). Therefore, as for dissolved organic analytes (Sessions *et al.*, 2005), the SWiM device is capable of quantitatively converting whole cells to CO_2 without isotopic fractionation or contamination.

Sessions *et al.* (2005) showed that the presence of inorganic salts can adversely affect wire-based isotopic measurements, decreasing both precision and accuracy. Sodium chloride is the most abundant salt in both culture media and most environmental samples, so our tests of microbial cells focused
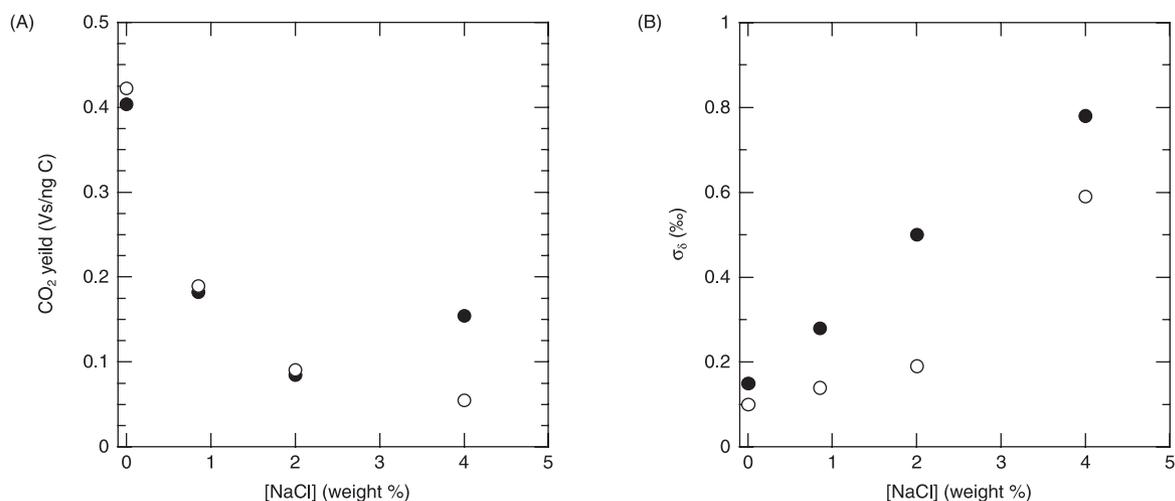
Fig. 4 CO_2 yield (A) and precision of $\delta^{13}C$ values (B) for SWiM-IRMS measurements as a function of NaCl concentration. A suspension of *Escherichia coli* standard culture ($\delta^{13}C = -10‰$) containing 50 ng C μL^{-1} (filled circles) or 100 ng C μL^{-1} (open circles) was used for the measurements.

specifically on NaCl. A single stock culture of *E. coli* cells was diluted into waters of increasing NaCl concentration analysed by SWiM-IRMS (Fig. 4). Saline samples create two problems. First, the yield of CO_2 (moles CO_2 produced per mole of cellular C introduced) drops dramatically as the concentration of NaCl increases (Fig. 4A). This is presumably due to the formation of macroscopic salt crystals, which may both inhibit combustion by occluding organic matter and lead to losses if salt crystals fall off the wire. Second, the precision of isotopic analyses decreases with increasing concentration of NaCl. This effect can partly, though not entirely, be explained by sample loss and thus lower signal intensity. Regardless of the causes, the degradation of analytical performance places important constraints on methods for concentrating cells. Lyophilization, which would concentrate any dissolved salts, is not a viable option.

Methods for concentrating cells

While the absolute mass requirements for SWiM-IRMS are quite low, samples must also be concentrated in very small volumes. The absolute cell concentration needed for acceptable precision is thus moderately high, corresponding to 1–10 mM C. Cell suspensions produced by the cell sorter are much more dilute, 1–10 μM C, and must be concentrated 1000-fold prior to analysis. Although concentrating cells to a small volume by centrifugation is relatively straightforward, it is difficult to do so with high recovery for a small number of cells.

The detection limits reported above for bacteria (~10^7 cells) would require, for example, roughly 3 h of cell sorting at 1000 cells s^{-1} assuming 50% abundance of the target cells. If, 80% of those cells were lost during concentration, then the sorting requirement would jump to 15 h. A lower abundance of target cells in the sample would further increase sorting time. Given the expense of operating cell sorters, and the likelihood that

collecting 1000 cells s^{-1} from most environmental samples is optimistic, the speed of cell sorting remains a significant limitation of our method for analyses of bacterial-sized cells. We thus focused considerable effort on optimizing the efficient recovery of cells from dilute solutions into small volumes. Two methods were tested: filtration and centrifugation.

The highest cell recoveries in a final volume of 10–30 μL were obtained by capturing cells on a 0.2-μm Teflon membrane filter, averaging $49 \pm 24\%$ for three replicate samples (Table 2). After rinsing, each of the filter membranes were stained with DAPI and examined by epifluorescence microscopy. This examination revealed numerous cells remaining embedded in the membranes, which presumably represents the largest loss of cells. Attempts to further remove cells using organic solvents (methanol, dichloromethane), concentrated acid (1 M HCl), and ultrasonication served only to increase the procedural blank, not the cell recovery. Experiments with polycarbonate filter materials produced similar results.

Concentration of cells by centrifugation must involve several steps to achieve the required ~1000-fold volume reduction. As a preliminary measure, we tested single-step concentration of larger samples to a final volume of 1 mL in order to examine the influence of centrifuge design and speed. First we compared centrifugation using swinging-bucket vs. fixed angle rotors. Cell suspensions containing 10^8 *E. coli* cells were spun down in a single step to a final volume of 1 mL, which was analysed directly on the SWiM. Specific experimental conditions are summarized in Table 2. These tests demonstrated slightly better recovery of cells using swinging-bucket ($66 \pm 21\%$) as opposed to fixed-angle ($51 \pm 10\%$) rotors. There was no significant improvement in recovery at speeds above 930 *g* or for spinning times longer than 1 h.

The number of cells being concentrated by these methods is too low to form a visible pellet. This likely contributes to

Table 2 Summary of results of trial cell concentration protocols

Method	No. of cells	n*	Centrifuge speed (10^3 g)	Time (min)	Final volume	% Recovery[†]
Single-step centrifugation fixed angle rotor[‡]	10^8	6	48	60	1 mL	51 ± 10
Single-step centrifugation fixed angle rotor[‡] + clay	10^8	3	48	60	1 mL	68 ± 9
Single-step centrifugation swinging buckets[§]	10^8	6	0.93	60	1 mL	66 ± 21
Two-step centrifugation[¶]	2.5×10^7	3	48/12	60/5	10–30 μL	18 ± 3
Five-step centrifugation**	2.5×10^7	3	48/12/4/4/4	60/30/30/15/5	10–30 μL	12 ± 9
Teflon membrane filtration	10^7	3	–	–	10 μL	49 ± 24

*Number of replicate trials.
[†]Uncertainties are indicated as one standard deviation of replicate experiments.
[‡]Beckman Coulter Avanti J-E centrifuge with fixed angle rotor (JA-25.50).
[§]Beckman Coulter Allegra X-15R centrifuge with swinging bucket rotor (SX4750).
[¶]Step 1, Allegra X-15R; step 2, Eppendorf 5415D.
**Step 1–4, Avanti J-E; step 5, Eppendorf 5415D.

resuspension of cells following centrifugation, and makes it impossible to visually locate a cell pellet within the tube when removing the supernatant. To increase the volume of the resulting cell pellet, we added 10 mg of montmorillonite clay (SAz-1 clay reference material, University of Missouri-Columbia Source Clay Mineral Repository, Columbia, MO, USA) as an organic-free carrier to each suspension prior to centrifugation. Although this did produce a visible pellet, the increase in cell recovery was negligible (Table 2) and produced additional difficulties in transferring the sample via syringe.

Next we tested the efficiency of two multistep centrifugation protocols designed to achieve the required 1000-fold volume reduction. In the first, a dilute cell suspension (2.5×10^7 cells in 50 mL) was concentrated in two sequential steps, first from 50 mL to 1 mL and then from 1 mL to 10–30 μL. Experimental conditions are summarized in Table 2. Finally, the cells were resuspended and transferred into a preweighed 200 μL conical glass vial for analysis. This protocol produced a net recovery (all steps) of 18 ± 3%.

To examine whether leaving a larger supernatant volume would minimize cell losses, the second tested protocol used five successive centrifugation steps. At each step, supernatant was removed, cells were re-suspended and transferred into a smaller centrifuge tube by pipette. The supernatant volume remaining after each successive step was: 10, 5, 3, 1.5, and finally 0.5 mL. After the final step, cells were re-suspended in 10-μL water and transferred into a preweighed 200 μL glass vial for isotopic analysis. This protocol yielded a poorer recovery (12 ± 9%) than the much simpler two-step protocol (Table 2).
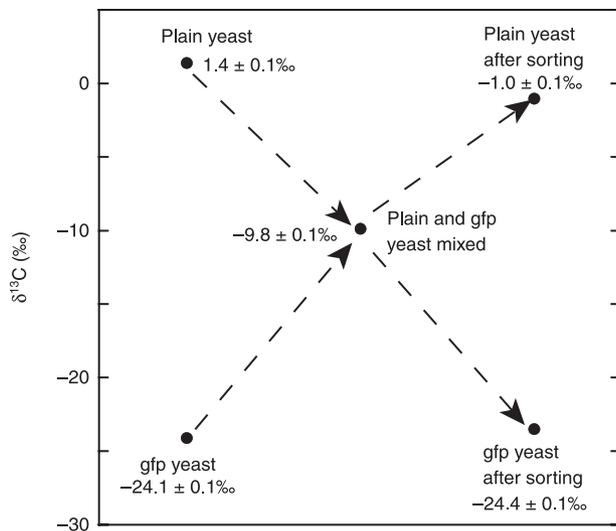
In summary, concentration of cells by multistep centrifugation yielded cell recoveries from a dilute suspension, such as that produced by FACS cell sorting, of no greater than 18%. Concentration by filtration produced higher, though more variable, recovery of 49%. Given the typical FACS sorting rates of ~1000 cells s^{-1}, recovery of sufficient bacterial biomass for

analysis with the highest possible precision currently requires 10 h or more of continuous, high-speed cell sorting. Improving the efficiency of cell recovery could lower this to a few hours, and this remains an important target for future work. Smaller samples of bacteria-sized cells can also be analysed in cases where poorer isotopic accuracy is acceptable, such as when ^{13}C-enriched tracers are employed. Because eukaryotic cells contain roughly 10^3 more carbon per cell, sufficient sample for high analytical accuracy can be recovered with only ~1 h of cell sorting, even with relatively low recovery efficiency.

Isotopic analyses of sorted cells

To demonstrate the accuracy and feasibility of cell sorting coupled to SWiM-IRMS analysis, we mixed two cultures of laboratory-grown *S. cervesiae* cells with differing δ^{13}C values. One culture expressed GFP, whose fluorescence served as the basis for cell sorting. Two cell populations were sorted and collected using the FACS-Aria, one yielding a green fluorescence signal of >10^3 and another with green fluorescence of <10^2. Isolates from the cell sorter were concentrated by filtration on Teflon membranes. To determine the blank contribution, 30 mL sheath fluid was collected from the cell sorter and processed identically to the cell suspensions. Aliquots of the original cultures and the cell mixture were diluted in deionized water to a suitable concentration (~100 ng C μL^{-1}), and the isotopic compositions of all fractions were measured at the same time. Results are shown in Figure 5.

The δ^{13}C value of yeast cells sorted by positive gating (i.e. those expressing green fluorescence) was within 0.3‰ of that for the pure culture. This experiment demonstrates that sorted cells can be analysed by SWiM-IRMS with sufficient accuracy for natural-abundance ^{13}C studies of microbes, as well as for those consuming ^{13}C-enriched substrates. For yeast cells collected by negative gating (i.e. those expressing no green

with appropriate fluorescent stains and ^{13}C-labelled substrates, this combined method has the potential to directly quantify consumption of specific substrates by microbes in environmental samples. The approach is equally applicable to populations of cells sorted from multicellular organisms.

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Fig. 5. Isotopic composition measured by SWiM-IRMS of yeast cells prior to, during and after FACS cell sorting. Experimental details are described in the text. The 'gfp yeast' fraction was collected with positive gating on green fluorescence, the 'non-gfp yeast' fraction was collected as all nonfluorescing cells.

fluorescence), the discrepancy was slightly larger (2.4%) with the sorted cells depleted in ^{13}C relative to the pure culture. The non-fluorescent cell fraction must also include any cells from the GFP culture that produced weak or no fluorescence, but which would nevertheless have an isotopic composition ($\delta^{13}C = -24.1‰$) much different from the original non-GFP yeast. The offset between sorted, non-fluorescent cells and the non-GFP culture can thus be readily attributed to mixing of the two populations. Indeed, isotopic mass balance suggests that the non-fluorescent cell fraction contained ~10% cells derived from the GFP yeast culture, and is consistent with the proportion of non-fluorescing cells (~17%) observed by FACS for a sample of the pure GFP yeast culture.

CONCLUSIONS

We have developed a new method for determining the carbon-isotopic composition of specific populations of whole microbial cells. The target cells are isolated using conventional FACS sorting based on appropriate fluorescence signals. Approximately 10^7 bacterial cells or 10^4 eukaryotic cells must be collected. The sorted cells are then concentrated to a volume of 10–30 µL by filtration onto a 0.2-µm Teflon membrane, with an average cell recovery of 49%. The $\delta^{13}C$ value of collected cells is then measured using a custom-built spooling wire microcombustion (SWiM) device coupled to a conventional IRMS. Precision for these analyses is roughly 0.5‰ for samples containing 10 ng C µL^{-1}, corresponding to approximately 10^5 bacterial cells µL^{-1}. For larger samples containing 50–100 ng C µL^{-1} (0.5–1·10^6 bacterial cells µL^{-1}, or 3500–7000 eukaryotic cells µL^{-1}), precision is <0.1‰. When combined

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