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Measuring the *in situ* carbon isotopic composition of distinct marine plankton populations sorted by flow cytometry

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Abstract

The carbon isotope ratio (δ^{13}C value) of marine particulates is a potentially useful tracer for elucidating pathways of carbon flow in the marine environment. Different species of phytoplankton vary in fractionation vs. CO_2 by up to 24‰ in laboratory cultures under varying nutrient and growth conditions, a signal that should propagate through the microbial food web. However, such contrasts have been difficult to confirm in field measurements due to analytical limitations. Here, we combine fluorescence-activated cell sorting (FACS) with a specialized micro-combustion interface and isotope-ratio mass spectrometry (SWiM-IRMS) to provide some of the first direct measurements of whole-cell δ^{13}C values for specific phytoplankton populations in the wild. For three samples collected off Scripps Pier in 2010–2011, *Synechococcus* averages δ^{13}C values of -25.7 ± 2.0‰, *Prochlorococcus* averages -23.0 ± 1.3, and diatoms average -20.8 ± 1.7‰. Diatoms were ~3‰ enriched in ^{13}C when measured during a bloom (March 2011) as compared with mid-summer (July 2010). Sorted particles thought to represent living heterotrophic bacteria averaged -25.4 ± 2.5‰, whereas total filterable particles averaged -19.6 ± 1.0‰, indicating a strong similarity to diatom biomass. These variations demonstrate that in situ differences in δ^{13}C among different populations of particles can be exploited to follow carbon flow through successive trophic levels, and throughout organic matter remineralization, sinking, and preservation.

The stable carbon isotopic composition (expressed as δ^{13}C) of organic material in the marine environment is a potentially useful tracer of the origins and fate of fixed carbon within the ecosystem. When measured in aggregate, marine particulate organic carbon (POC) typically yields δ^{13}C values ranging from −20‰ to −22‰ (Druffel et al. 1992; Bauer 2002), reflecting the net fractionations of ribulose 1,5-bis-phosphate carboxylase/oxygenase (RubisCO) and β-carboxylases during photosynthetic carbon fixation by phytoplankton (Goericke et al. 1994) together with heterotrophic recycling (Azam et al. 1983; Blair et al. 1985). However, there is considerable evidence from culture-based studies that individual populations of phytoplankton might vary in isotopic fractionation—and thus δ^{13}C in the marine environment—over a larger range. Factors contributing to such variable fractionation include cell size and geometry (Popp et al. 1998), growth rate (Laws et al. 1995), and growth conditions, as well as differing forms of RubisCO (Roeske and O'Leary 1985; Guy et al. 1993; Robinson et al. 2003; Scott et al. 2004, 2007; Boller et al. 2011, 2015). Most notably, the concentration of CO_2(aq) and the transport of CO_2 into the cell have a significant effect on ^{13}C fractionation (Rau et al. 1989, 2001; Laws et al. 1995, 1997). In laboratory cultures, carbon isotopic fractionation has been shown to differ among algal species by up to 24‰ (Popp et al. 1998; Burkhardt et al. 1999a), and is affected by light levels, day length, growth phase, and nutrient availability (Burkhardt et al. 1999a,1999b; Rost et al. 2002; Brutemark et al. 2009). To the extent that heterotrophs feed on specific phytoplankton sources, such C-isotopic signals should also propagate into their biomass, providing an opportunity to more directly assess this important but microscopic trophic level.

Bulk carbon isotopic analysis of POC is typically accomplished via a combustion elemental analyzer (EA) coupled to an isotope-ratio mass spectrometer (IRMS). Typical sample-size requirements are in the range of 10–100 μg C (Werner et al. 1999; Polissar et al. 2009), which is too large to allow for analysis of hand-picked or sorted phytoplankton. Analyses

Additional Supporting Information may be found in the online version of this article.

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have thus been largely confined to size-fractionated POC (e.g., Rau et al. 1990; Kukert and Riebesell 1998), which necessarily confound both multiple types of organisms, as well as detrital particles. Nevertheless, POC samples taken from areas or times where specific phytoplankton populations are dominant (Fry and Wainwright 1991; Pancost et al. 1997) do provide insight into $\delta^{13}C$ variations among plankton groups in situ. For instance, POC from diatom-dominated plankton blooms can be enriched in ^{13}C (Fry and Wainwright 1991; Kukert and Riebesell 1998) and various size fractions can differ by up to 5‰ (Rau et al. 1990), thus knowledge of the composition of POC samples can be important for evaluating $\delta^{13}C$ values in a biogeochemical context.

Compound-specific isotopic analyses of characteristic (biomarker) lipids can potentially provide more targeted insight into variations in $\delta^{13}C$ (Evershed et al. 2007). For example, some phospholipid fatty acids (PLFAs) can be diagnostic of certain types of bacteria and eukaryotes such as methanotrophs (16:1ω8c, 18:1ω8c), sulfate reducers (i17:1, 10Me18:0), and algae (20:5ω3, 18:3ω3) (Boschker and Middelburg 2002). Ether-linked lipids are unique to archaea (De Rosa et al. 1986; De Rosa and Gambacorta 1988), long-chain alkenones to certain haptophytes (Volkman et al. 1980; Marlowe et al. 1984), and chlorophyll and other pigments to specific groups of phytoplankton (Jeffrey et al. 2003). However, the significant drawbacks of biomarker analysis are that (1) many important species do not have characteristic biomarkers, (2) they cannot reliably be followed through trophic interactions, and (3) they are not necessarily representative of the flows of total fixed carbon. In practice, the first of these limitations is often the most severe.

It would clearly be beneficial to have the ability to measure $\delta^{13}C$ of total organic carbon in discrete populations of plankton. Toward this goal, we take advantage of a microcombustion device based around a continuously spooling nickel wire (SWiM; Brand and Dobberstein 1996; Sessions et al. 2005) that is interfaced with an IRMS. The primary advantage of this system for our present purpose is that it is capable of measuring as little as 10–100 ng C, a range that is realistically achievable by fluorescence-activated cell sorting (FACS). The utility of FACS for detecting and isolating specific groups of planktonic cells has been demonstrated previously for primary production (Li, 1994; Jardillier et al. 2010; Grob et al. 2011), phosphorus (Zubkov et al. 2007; Larsen et al. 2008; Casey et al. 2009; Michelou et al. 2011; Duhamel et al. 2012), and nitrogen (Zubkov et al. 2003; Casey et al. 2007; Fawcett et al. 2011), and is reviewed extensively in Lomas et al. (2011). The ability of FACS to target specific populations is essentially limited only by the ability to mark that population with a suitable fluorescent stain.

The first proof-of-principle demonstration of coupled FACS and SWiM-IRMS in the laboratory was reported by Eek et al. (2007), who separated two strains of laboratory-grown yeast, one of which expressed green fluorescent protein. They reported $\delta^{13}C$ precision better than 0.2‰ for samples containing only 50 ng C, which corresponds to $\sim 10^4$ yeast cells. Here, we take the next step of applying FACS and SWiM-IRMS to populations of natural phytoplankton sorted from seawater. We are able to resolve *Prochlorococcus* and *Synechococcus* cells based on their size and autofluorescence, and diatoms by use of a silica-targeted stain. We show that their $\delta^{13}C$ values can differ by up to 7‰ at a given time.

Materials and procedures

Sample collection and processing

Particulate material was collected off the Scripps Institution of Oceanography (SIO; La Jolla, California) pier in June 2010, July 2010, and March 2011. Samples were concentrated up to 125× by filtration through 5-μm (polyvinylidene fluoride; Millipore Durapore) and/or 0.1-μm (polyethersulfone; Pall Supor) pore size 47-mm diameter disc filters, then resuspended in filtered seawater for sorting. All samples were pipetted through 35-μm cell strainers prior to cell sorting. To identify non-autofluorescing heterotrophic cells, the nucleic acid stain 4′,6-diamidino-2-phenylindole (DAPI) was added 1 : 100 to concentrated seawater samples before sorting. The silica frustules of diatom cells were stained by adding fluorescein-5-isothiocyanate(FITC)-silane at 1 : 100 (Desclés et al. 2008) to previously FACS-sorted chlorophyll-containing phytoplankton. The sample was then centrifuged for 5 min at 5000 rpm (Eppendorf Centrifuge 5415C) to pellet cells and remove excess stain before resuspending in filtered seawater and re-sorting based on FITC fluorescence. Care was taken to minimize light exposure to the fluorescent stains and stained samples. The potential effects of these stains on sample $\delta^{13}C$ values are addressed below.

Fluorescence-activated cell sorting

Cells were sorted using an Influx cell sorter (BD Biosciences) located at SIO equipped with five lasers [488-nm (200 mW), 457-nm (300 mW), 532-nm (150 mW), 355-nm (100 mW), and 640-nm (50 mW)] using a 70-μm nozzle and a sheath pressure of 227.5 kPa. Sheath fluid was made up with pre-combusted NaCl (3.4% by weight) and purified (Milli-Q) water, then filtered through 0.2-μm Sterivex filters (Millipore). Laser alignment, optimization, and quality control was performed daily using Ultra Rainbow calibration beads (Spherotech). Events were triggered off of forward light scatter at rates ranging from 2000 to 3000 events/s with a differential pressure of about 6.9 kPa, and sort gates were established based on detected chlorophyll (692 ± 40 nm), phycoerythrin (580 ± 30 nm), FITC (530 ± 40 nm), and DAPI (460 ± 50 nm) fluorescence, depending on the population of interest (see Figs. 1 and 3 for examples). The instrument was run in purity-yield mode, and sorts were checked qualitatively on the Influx as well as by epifluorescence microscopy. Populations of interest were sorted as either single sorts or two at a time, and sorting times are listed in Table 1. The drop delay for all sorts was 33.2 ± 2.6 (average ± one standard deviation),

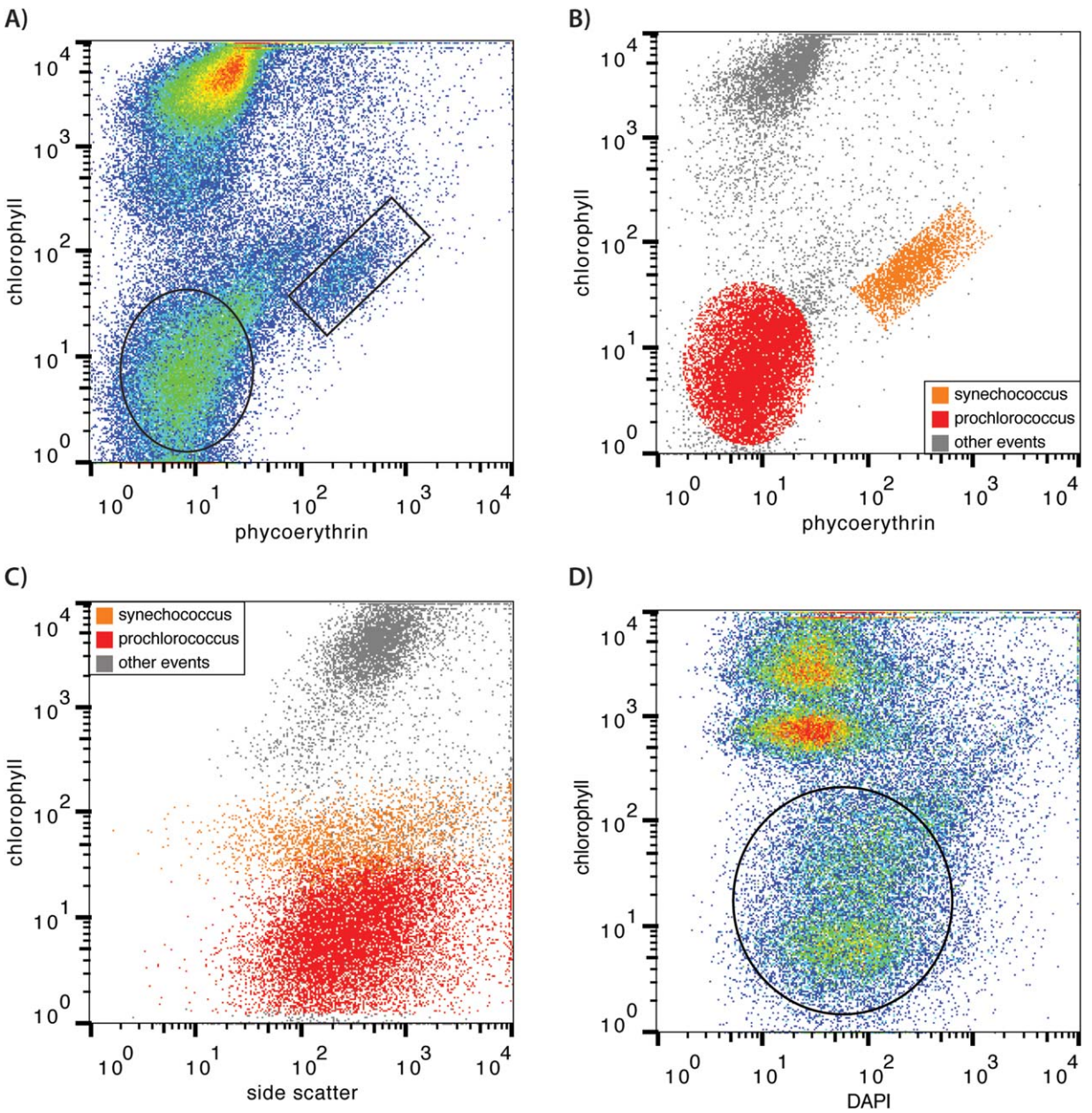

Fig. 1. Sample cytograms of seawater and specific plankton populations gated for sorting. Gated *Prochlorococcus* and *Synechococcus* populations in seawater (**A**) and color-coded by gate in (**B**) are identified based on their natural autofluorescence from chlorophyll and phycoerythrin pigments. Without phycoerythrin, it would be difficult to distinguish between these two populations based on chlorophyll and light scatter alone (**C**). This particular sample had a small *Synechococcus* population relative to *Prochlorococcus*, and the majority of high chlorophyll events were sorted as "other chlorophyll-containing plankton" cells. In a separate sample, presumed heterotrophs (**D**) are stained by adding DAPI to seawater prior to sorting.

determined as "virtual drops" either manually or using Accu-Drop beads (BD Biosciences). Drop frequency varied slightly between sorts but averaged 64.3 ± 3.9 kHz.

Cell concentration and recovery

Sorted cells were collected in suspensions of approximately 10^5 cells mL^{-1} sheath fluid, roughly two orders of magnitude too dilute for carbon isotope analysis by SWiM-IRMS. Cell suspensions were filtered using a pre-combusted glass filtration tower onto 25-mm diameter 0.2-μm pore size polycarbonate disc filters (Millipore Isopore) that were pre-cleaned with ethanol and rinsed with purified water. Cells were then resuspended off the filter in microcentrifuge tubes with 1 mL purified Milli-Q water by vortexing for 15 min (VWR Vortex Genie 2, speed = 3) and sonication in an ultrasonic bath (VWR Model 50T) for 30 min at room temperature. At this stage, cells

Table 1. Summary of sorting times for sample populations analyzed for $\delta^{13}C$.

Sample	Sampling date	# Cells sorted ($\times 10^6$)	Sort time (hh:mm:ss)
Synechococcus	June 2010	14.6	12:20:18
Other chl-containing plankton	June 2010	6.2	03:17:25
Heterotrophs	June 2010	14.5	04:50:00
Synechococcus	July 2010	2.6	03:56:01
Prochlorococcus	July 2010	2.6	01:29:35
Diatoms	July 2010	8.4	07:22:51
Other chl-containing plankton	July 2010	n.a.	n.a.
Heterotrophs	July 2010	6.4	00:25:38
Synechococcus	March 2011	11.8	18:56:02
Diatoms	March 2011	2.4	05:39:12
Other chl-containing plankton	March 2011	11.0	16:37:23
Heterotrophs	March 2011	10.0	02:20:21

n.a., not available.

were potentially disrupted by these steps and no longer intact. However, no further filtration steps were employed, and isotopic measurements were made on the total nonvolatile carbon present in solution, including both intact cells and disrupted cellular components Thus disruption of cells during resuspension should not affect the recovery of cellular carbon. As necessary, samples were further concentrated by drying with a CentriVap centrifugal concentrator (Labconco).

SWiM and carbon isotope analysis

The carbon isotopic composition of sorted cells was measured using the SWiM device with a DeltaS IRMS (Finnigan MAT). Details of this system have been previously reported by Eek et al. (2007). Briefly, suspensions of sorted cells are applied to and dried on a spooling nickel wire, and then the deposited sample residue is combusted to CO_2 for isotopic analysis. Four to ten aliquots of cell suspensions, each 1–2 μL and spaced ~60 s apart, were analyzed for each sample and were bracketed by peaks of reference CO_2 gas. Sample carbon content was calculated using a peak area calibration curve for *m/z* 44 based on a dilution series of sodium acetate. Reported $\delta^{13}C$ values are the mean and standard deviation of the aliquots (analytical replicates), reported as permil (‰) deviations from the V-PDB reference standard. Student's *t*-test, analysis of variance (ANOVA), and Tukey's Honestly Significant Difference (HSD) tests were performed in *R* (R Core Team 2014).

Assessment and discussion

Identifying and sorting specific plankton populations

Specific plankton populations sorted from coastal seawater included cyanobacteria *Prochlorococcus* and *Synechococcus*, which were identified based on their natural autofluorescence from chlorophyll and phycoerythrin pigments (Fig. 1). Other chlorophyll-containing plankton were also sorted as a single, heterogeneous group. Putative heterotrophic cells were sorted on the basis of DAPI fluorescence and a lack of chlorophyll fluorescence.

Sorting diatoms from other chlorophyll-containing phytoplankton was at first challenging, but was ultimately achieved by staining the silica frustules with FITC-silane as detailed in Desclés et al. (2008). The staining method was first tested on cultures of *Thalassiosira oceanica* and *Thalassiosira weissflogii* (Fig. 2), and then adapted to seawater samples. Due to fluorescence overlap between phycoerythrin and FITC compounded by the presence of excess dye, FITC-stained diatoms could not be positively sorted directly from seawater. Instead, cells containing chlorophyll but not phycoerythrin were first sorted from concentrated bulk seawater samples (Fig. 3), then FITC-silane was added to these sorted cells. Samples were centrifuged to pellet cells and remove excess dye before being resuspended for sorting. Stained and sorted diatoms from July 2010 seawater samples were confirmed by epifluorescence microscopy and are shown in Fig. 3. The significant size difference between phycoerythrin-containing *Synechococcus* cells and the FITC-stained diatoms should allow for both populations to be sorted simultaneously without the initial sorting step; however, in our experience it was necessary to remove excess dye first to clearly distinguish the diatom population.

Several methods for fixing cells were tested, including the use of formaldehyde, ethanol, and DMSO. However, all fixatives lead to significant contamination of the cells, causing both dramatic increases in the amount of cellular carbon present and altered $\delta^{13}C$ values (Supporting Information Table S1). Therefore, only freshly collected samples were used for FACS in this study. Future investigations of samples collected further from the laboratory will need to grapple with this issue of preservation, and develop optimized fixation protocols that do not add significant amounts of carbon.

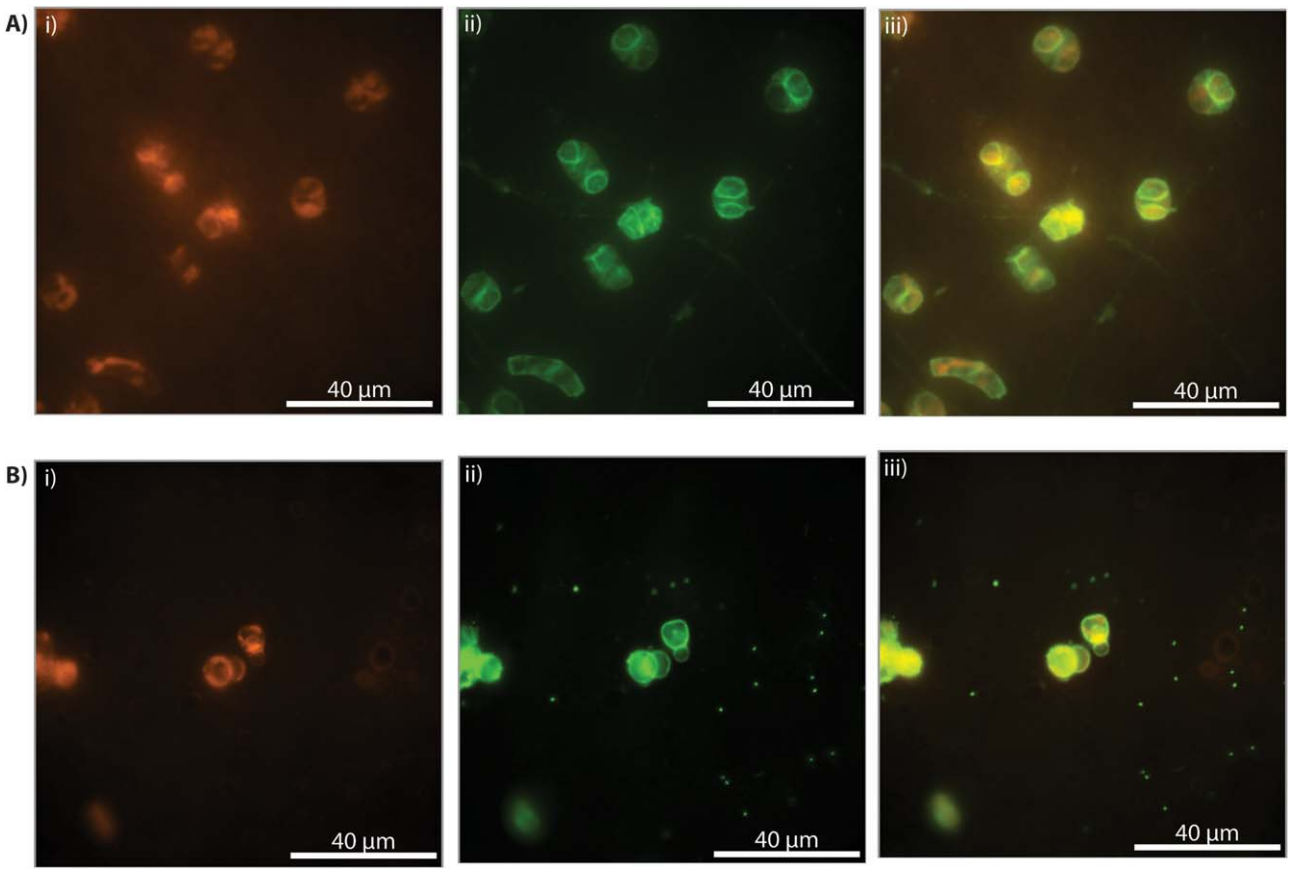

Fig. 2. Diatom cultures of *T. weissflogii* (**A**) and *T. oceanica* (**B**) stained with FITC-silane (Desclés et al. 2008) under an epifluorescence microscope (i, chlorophyll filter; ii, FITC filter; iii, combined chlorophyll and FITC filters).

Cell concentration and recovery

Because cells were sorted in sheath fluid at concentrations too dilute for $\delta^{13}C$ analysis by SWiM-IRMS (generally 10^5 cells mL^{-1}), we developed a method to concentrate cells in suspension. Although pelleting cells by centrifugation may be a good option for larger phytoplankton cells or microbial cultures as in Eek et al. (2007), many marine bacteria are too small to recover easily and efficiently by this method. We opted to filter cells onto 25-mm diameter, 0.2-μm nominal pore size polycarbonate filters and then recover these cells from the filter by vortexing and sonication. Carbon recovery was greater from polycarbonate filters (82.1%) as compared with those made from Teflon (56.4%). Inspection of DAPI-stained filters by epifluorescence microscopy after resuspension revealed that the more fibrous Teflon filters appeared to trap more cells than the more uniform surface of the polycarbonate filters.

To minimize the contribution of blank carbon from the polycarbonate filters, a variety of cleaning methods were tested including soaking in ethanol, 1% HCl, and 0.1N NaOH. The smallest carbon blank was achieved by first soaking filters in ethanol and then rinsing with purified water. The blank from this cleaning method was determined to be 1.04 ± 0.56 ng C/μL, as compared with nearly 5 ng C/μL for unwashed filters (Supporting Information Table S2). Our blank was further reduced to 0.4 ± 0.2 ng C/μL by flushing with sheath fluid from the sorter, and its $\delta^{13}C$ value varied by up to 1‰. This blank is about 1–10% of typical sample sizes.

To test our cell concentration and recovery method, natural seawater communities of heterotrophic microbes were grown on fructose ($\delta^{13}C = -11.0$‰) or acetate (-25.4‰). $\delta^{13}C$ values of whole cells recovered by pelleting with centrifugation and by filtration onto ethanol-cleaned 0.2-μm polycarbonate filters are shown compared with substrate $\delta^{13}C$ in Table 2. Discrepancies between substrate and cellular $\delta^{13}C$ values may be due to metabolic ^{13}C fractionation, particularly for those grown on acetate.

Process blanks

Procedural blanks were evaluated to test the effects of sample processing and cell sorting, concentration, and recovery methods on $\delta^{13}C$ analyses. These blanks included cultures of a heterotrophic bacterium, *Escherichia coli*, and a diatom, *T. weissflogii*, as well as 1-μm fluorescent polystyrene latex beads (yellow-green; Polysciences Fluoresbrite). As shown in Fig. 4, the blank-corrected $\delta^{13}C$ values of unsorted

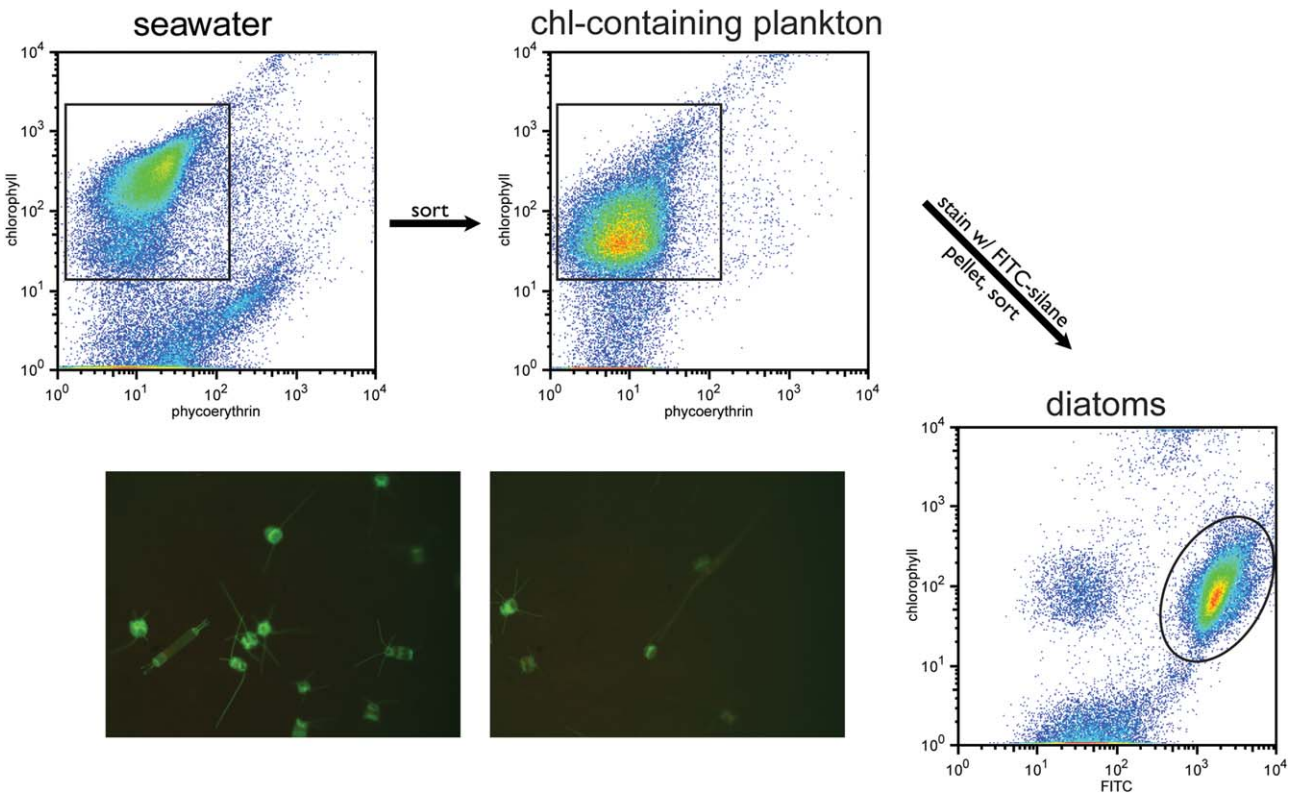

Fig. 3. Diatoms in seawater are identified by adding FITC-silane to sorted chlorophyll-containing plankton, which binds to silica frustules (Desclés et al. 2008) giving them a green fluorescence. Sorted groups are confirmed using epifluorescence microscopy (FITC-silane stained and sorted diatoms isolated from bulk seawater shown).

Table 2. $\delta^{13}C$ values of carbon sources and concentrated microbial cells, as determined by SWiM-IRMS.

Sample	Carbon source	$\delta^{13}C$ (‰)
Fructose		-11.0 ± 0.2
Pelleted cells	Fructose	-12.5 ± 0.8
Filtered/recovered cells	Fructose	-13.0 ± 1.1
Acetate		-25.4 ± 0.6
Pelleted cells	Acetate	-20.2 ± 0.7
Filtered/recovered cells	Acetate	-23.0 ± 1.2

and sorted samples of both unstained and stained *E. coli* and *T. weissflogii* cultures were in good agreement. Additionally, the fluorescent beads showed similar results. Thus, our two staining methods appear to add negligible amounts of carbon that affect C-isotopic measurements. $\delta^{13}C$ values of sorted samples measured by SWiM-IRMS were corrected for blanks associated with sorting, concentration, and recovery determined by collecting sheath fluid from the sorter and then proceeding with the established concentration and preparation method before SWiM-IRMS analysis. These tests with the cultures and fluorescent beads indicate our blank corrections suitably account for any carbon added during sample processing.

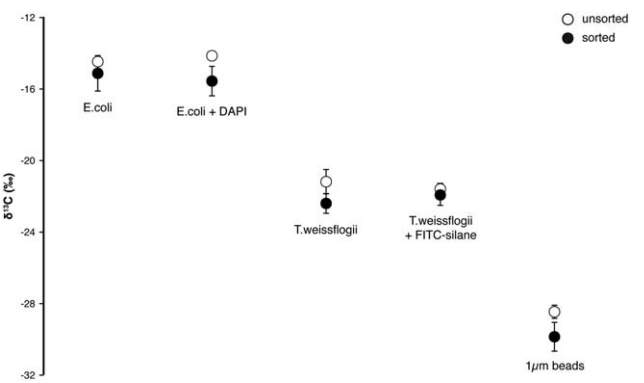

Fig. 4. $\delta^{13}C$ values of procedural blanks measured using SWiM-IRMS before (open circles) and after (closed circles) sorting by FACS: cultured *E. coli* cells (heterotrophic bacterium), *E. coli* cells stained with DAPI, cultured *T. weissflogii* cells (diatom species), *T. weissflogii* cells stained with FITC-silane, and fluorescent 1-μm beads. Error bars are $\pm$ one standard deviation of the mean, and if not visible are smaller than the marker.

Carbon isotopic signatures of sorted populations

Results for natural populations of cells as measured by SWiM-IRMS are shown in Fig. 5. The precision of $\delta^{13}C$ measurements by SWiM-IRMS improved as a function of sample size (Fig. 6; Eek et al. 2007). Generally, obtaining sufficient heterotrophic biomass for precise measurements was difficult

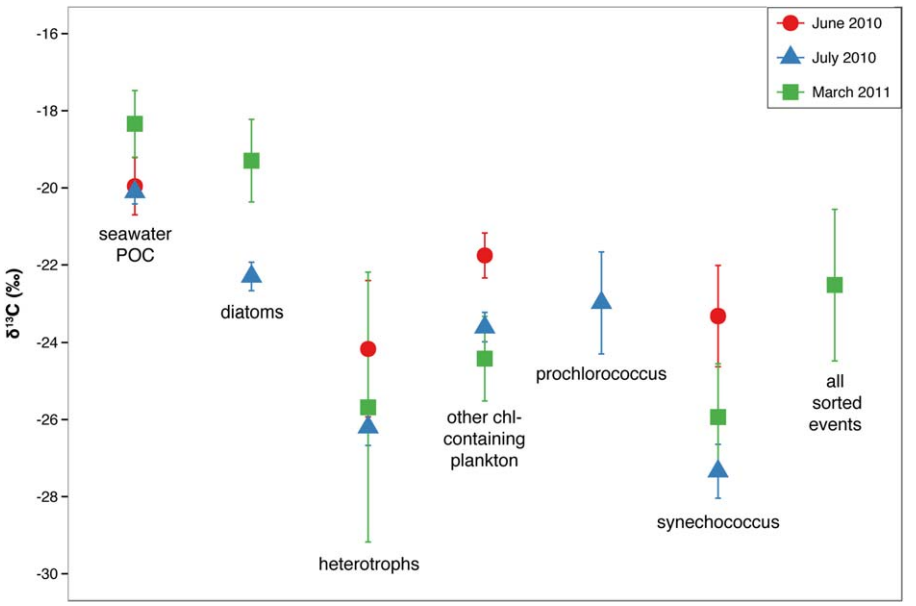

Fig. 5. $\delta^{13}C$ values of groups of cells sorted by FACS and measured using SWiM-IRMS. Cells were sorted from seawater collected off the SIO pier at three different time points: June 2010 (circles), July 2011 (triangles), and March 2011 (squares). Error bars are ± one standard deviation of 5–10 measurements, propagated through blank corrections.

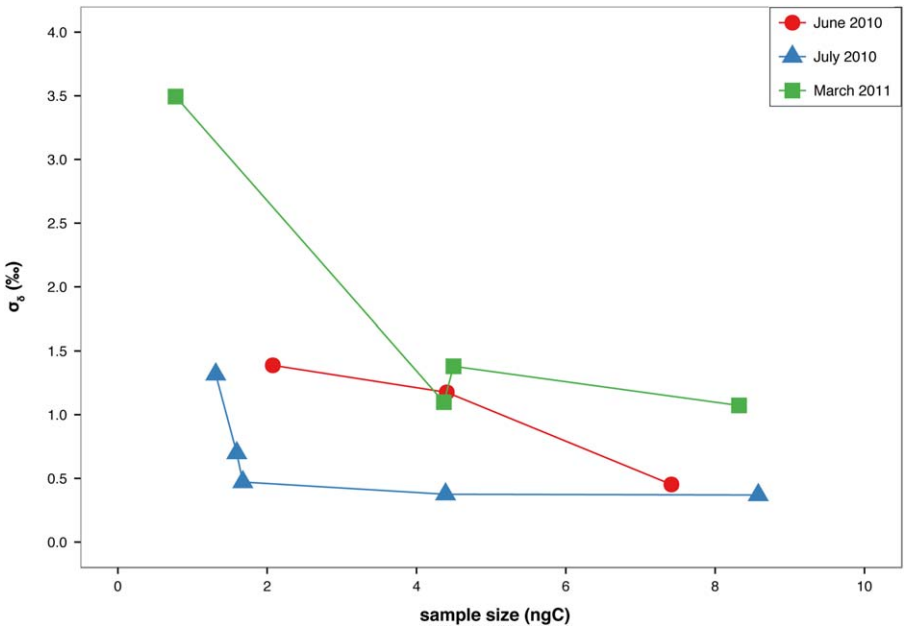

Fig. 6. Precision of blank-corrected $\delta^{13}C$ measurements (σ_δ) by SWiM-IRMS as a function of sample size for groups of cells sorted in June 2010 (circles), July 2010 (triangles), and March 2011 (squares).

so our $\delta^{13}C$ values for this group have significant error bars (>1.5‰) at all time points and cannot be confidently distinguished from other sampled populations. In particular, our smallest sample size (1.1 ng C/μL) for heterotrophs in March led to a large (>3‰) uncertainty in $\delta^{13}C$. Overall larger uncertainties for March samples are driven in part by a larger variability in the $\delta^{13}C$ of the sorter blank at that time point (1‰; Fig. 7).

Although we have few time points, and in some cases relatively large uncertainties in measured $\delta^{13}C$ values, several patterns are apparent. First, diatoms have higher $\delta^{13}C$ values than either *Synechococcus* or other chlorophyll-containing

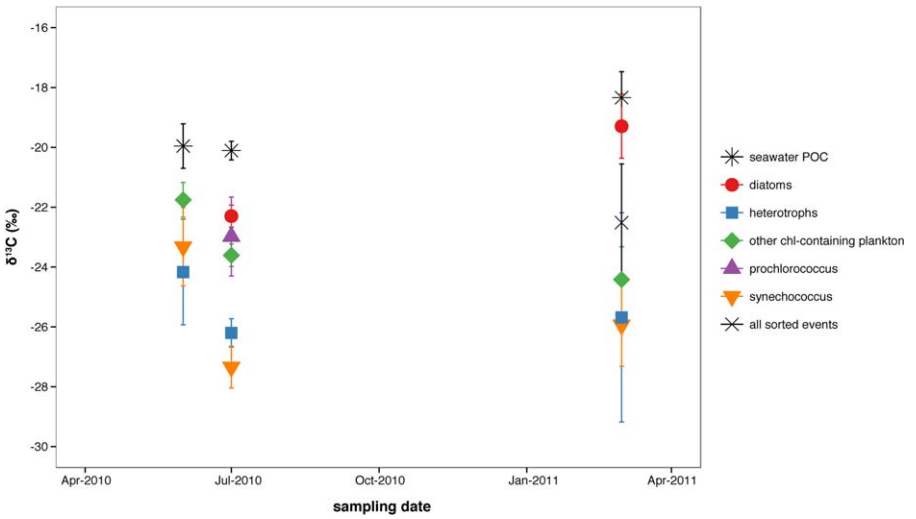

Fig. 7. $\delta^{13}C$ values of groups of cells sorted by FACS and measured using SWiM-IRMS as shown in Fig. 5, plotted against sampling date. Error bars are ± one standard deviation of 5–10 measurements, propagated through blank corrections.

plankton at the same time (Tukey's HSD, $p < 0.01$), and have higher $\delta^{13}C$ during the March 2011 bloom period than during July 2010. In two of the three time points, *Synechococcus* is remarkable for being quite ^{13}C-depleted ($-27.3‰$ and $-25.9‰$ in July 2010 and March 2011). Second, *Prochlorococcus* has a $\delta^{13}C$ value—during the single time period it was isolated—that is significantly ^{13}C-enriched relative to *Synechococcus*, but indistinguishable from other phytoplankton. Third, total POC is significantly ^{13}C-enriched relative to all other particles, and is slightly enriched relative to diatoms. Its isotopic composition cannot be explained solely by the populations of primary producers sampled here. Fourth, putative heterotrophic bacteria are ^{13}C-depleted, having $\delta^{13}C$ values similar to *Synechococcus*; however, given the large error bars for this group, they cannot be confidently distinguished from other plankton populations. Fifth, the $\delta^{13}C$ values of specific groups can vary temporally, as all populations that were measured more than once, aside from the heterotrophs, had significant differences in $\delta^{13}C$ between sampling time points (ANOVA, $p < 0.001$). In sum, application of this method demonstrates the ability to measure significant $\delta^{13}C$ variations between sorted populations of phytoplankton. While our preliminary dataset is not sufficiently large to reach robust conclusions about inter-species patterns of isotopic fractionation, it does hint at some interesting patterns. Next we explore some possible explanations for these observed $\delta^{13}C$ values primarily to highlight potential applications of the method in the marine environment, and the ecological or biogeochemical implications that could be addressed through a more comprehensive study.

Although all cells in a small volume of seawater are generally subject to the same physical and chemical conditions (e.g., temperature, salinity, nutrient and $CO_{2(aq)}$ concentrations), specific groups may be at different phases of their growth cycle, growing at different rates, or comprised of different species with varying cell morphology, which could lead to differences in isotope fractionation and thus measured $\delta^{13}C$ values (Laws et al. 1995; Bidigare et al. 1997; Laws et al. 1997; Burkhardt et al. 1999a,b; Brutemark et al. 2009). Popp et al. (1998) suggested that cell geometry, specifically surface area-to-volume ratios, can explain observed ^{13}C fractionation variations with growth rate and $[CO_{2(aq)}]$ for some eukaryotic phytoplankton species, although these differences were not observed for cultured *Synechococcus*. These differences in fractionation among species may be partially due to different forms of the enzymes involved in inorganic carbon fixation, specifically RubisCO. For example, marine cyanobacteria *Prochlorococcus* and *Synechococcus* possess form IA of the enzyme, whereas form ID is prevalent in many marine eukaryotes such as diatoms and coccolithophores. Although very limited in scope, high-precision measurements of species-specific RubisCO ^{13}C fractionation factors indicate that form ID fractionates less than form IA—Scott et al. (2007) determined a ^{13}C fractionation factor of $24‰$ for *Prochlorococcus marinus MIT9313* compared with $18.6‰$ for the diatom *Skeletonema costatum* (Boller et al. 2015) and $11.1‰$ for the coccolithophore *Emiliania huxleyi* (Boller et al. 2011). These RubisCO fractionation factors combined with growth condition effects can potentially explain our ^{13}C-enriched diatoms, as well as high $\delta^{13}C$-POC values from blooms dominated by diatoms previously observed (Kukert and Riebesell 1998; Fry and Wainwright 1991).

The more ^{13}C-depleted values, relative to the other groups measured and total POC, for *Synechococcus* are consistent with previously observed size-fractionated $\delta^{13}C$-POC results, where the smallest size fraction (<3 μm) had the lowest $\delta^{13}C$

value (−25.3‰; Rau et al. 1990). In this context, we would expect the single sample of *Prochlorococcus* from July 2010 to also be depleted in ^{13}C, rather than the −23.0‰ value we measured, which was more similar to diatoms and other chl-containing plankton than *Synechococcus*. *Prochlorococcus* is not always found in the waters off the SIO Pier (Worden et al. 2004) and was absent during our other sampling time points, so we do not know if this more ^{13}C-enriched value is typical for this species group in situ. Differences have been identified in carboxysome-associated components of the carbon dioxide-concentrating mechanisms between *Prochlorococcus* and *Synechococcus* (Ting et al. 2015), which could be responsible for the different $\delta^{13}C$ values for cyanobacterial groups sorted from the same seawater sample. Alternatively, the *Prochlorococcus* population sampled could have been at a more advanced growth stage (i.e., stationary; Brutemark et al. 2009), or assimilating carbon from organic substrates such as amino acids (Zubkov et al. 2003; Michelou et al. 2007).

Although we would expect the $\delta^{13}C$ values of heterotrophic bacteria to reflect their carbon source (Blair et al. 1985), presumably dissolved organic material released from the particulate pool, this group was much more ^{13}C-depleted than total POC and more similar to measured *Synechococcus* $\delta^{13}C$ values. Heterotrophs cannot yet be distinguished isotopically from the other groups due to large measurement errors, but their lighter $\delta^{13}C$ values could be explained by a tight coupling between *Synechococcus*-produced carbon and heterotrophic bacteria. Physical *Synechococcus*-bacteria associations have been observed off SIO pier using atomic force microscopy (Malfatti and Azam 2009; Malfatti et al. 2010).

Temporal variation in $\delta^{13}C$ was observed for plankton groups and POC (Fig. 7). The total seawater POC $\delta^{13}C$ value shifted from -20.0 ± 0.7‰ and -20.1 ± 0.3‰ in June and July 2010 to -18.3 ± 0.9‰ in March 2011. Average water temperature (± one standard deviation) measured from the SIO pier was identical over the days sampled in June and July 2010 (19.1 ± 0.5°C and 19.1 ± 0.7°C, respectively), but colder at 14.6 ± 0.7°C in March 2011. Goericke and Fry (1994) found that the $\delta^{13}C$ of marine POC is depleted at lower temperatures, but this relationship is weak over the temperature range indicated here. Nevertheless, temperature differences could contribute to variation in phytoplankton growth rates (Boyd et al. 2013), and thus affect ^{13}C fractionation within specific populations (Burkhardt et al. 1999a,b). Although total cell counts as determined by the flow cytometer were similar for all three sample periods, the in situ community composition clearly varied. Chlorophyll concentration averaged 1.68 ± 0.57 μg/L and 1.63 ± 0.49 μg/L over the days sampled in June and July 2010, respectively, but was 1.47 ± 0.24 μg/L in March 2011. Additionally, the proportion of *Synechococcus* cells was approximately three times higher in June 2010 compared with March 2011, in agreement with seasonal variation previously observed at this location (Tai and Palenik 2009). Changes in the $\delta^{13}C$ signatures of specific groups and their relative contributions to the total POC pool could be expected to drive the values of seawater POC $\delta^{13}C$, notably the diatoms which measured -22.3 ± 0.4‰ in July 2010 and -19.3 ± 1.1‰ in March 2011.

A curious result is that the $\delta^{13}C$ of seawater POC has a higher $\delta^{13}C$ value than all sorted groups at each sampling point. Seawater POC $\delta^{13}C$ was measured by concentrating and recovering particulate material as all other samples before analysis by SWiM-IRMS, but without first being sorted on the Influx. Therefore, it is possible that a component of blank carbon added to sorted samples exists that we have not properly accounted for; however, the results from our procedural blanks indicate that this is not the case. Additionally, the inclusion of carbonate particles in the total POC samples could result in higher $\delta^{13}C$ values; however, separate POC samples collected on pre-combusted glass fiber filters in March 2011 that were acidified to remove carbonates and then analyzed more traditionally by EA-IRMS had $\delta^{13}C$ values within the range reported for the SWiM-IRMS samples. An alternative explanation could be that larger-sized living and detrital particles included in the seawater sample are more enriched in ^{13}C and are responsible for the more positive $\delta^{13}C$ value. All samples run on the cell sorter are first put through a cell strainer, so we are only sorting particulate material < 35 μm. When all sorter events from March 2011 seawater were collected, concentrated, and measured by SWiM-IRMS, the $\delta^{13}C$ value was -22.5 ± 2.0‰ (Fig. 5), more ^{13}C-depleted than the -18.3 ± 0.9‰ measured from total seawater that month (Student's *t*-test, $p < 0.001$), lending some credence to this possible discrepancy between $\delta^{13}C$ of seawater POC and sorted groups. This is further supported when we calculate the mass-weighted $\delta^{13}C$ value of the four populations sorted from March 2011 (diatoms, other chlorophyll-containing plankton, *Synechococcus*, and heterotrophs) as -22.5 ± 0.2‰, equal to that of all sorted events.

Identifying $\delta^{13}C$ signatures for specific plankton groups or populations could potentially enable the tracing of these organisms' carbon as it sinks through the water column, becomes remineralized, or is incorporated into higher trophic levels. These results demonstrate measurable differences in the $\delta^{13}C$ values of sorted plankton populations and indicate the possibility of using differences in $\delta^{13}C$ to trace the flow of carbon from specific groups in the marine environment.

Comments and recommendations

The current method was developed for freshly collected samples, as common cell fixatives introduced significant amounts of additional carbon and altered $\delta^{13}C$ values; however, to apply this method more broadly in the field, some form of fixation may be required. Other cell fixation

methods not tested here may introduce less carbon, or further analyses of commonly used fixation chemicals might allow for systematic correction of $\delta^{13}C$ values. The use of sea-going cell sorters for immediate sorting of freshly collected samples would not require cells to be fixed.

Additional staining or labeling techniques used to identify plankton populations not analyzed here will require evaluation to determine the presence and isotopic effects of added blank carbon. Some potentially useful staining methods for marine plankton can be those that label a cellular component specific to a certain type of population, such as the silica frustules of diatoms we used in this study. These could include staining the acidic organelles of heterotrophic protists (LysoTracker Green; Rose et al. 2004) or the calcium carbonate structures of foraminfera or coccolithophores (calcein; Bernhard et al. 2004), although this cellular carbonate would then need to be removed by acidification prior to organic carbon analysis. Additionally, species or cell types within a specific group with distinct sizes or morphologies, such as the several different diatom species we sorted (Fig. 3), could be further separated based on their light scatter properties.

Fluorescent in situ hybridization (FISH) can be used to identify and fluorescently label marine planktonic cells down to the species level based on specific oligonucleotide probes, but the modified technique typically required for adequately labeling marine prokaryotes (catalyzed reporter deposition, or CARD-FISH; Pernthaler et al. 2002; Sekar et al. 2004) introduces carbon-containing horseradish peroxidase and tyramide molecules into the cells that would have to be corrected for following $\delta^{13}C$ analysis. Additionally, current CARD-FISH methods require cell fixation. Standard FISH protocols may ultimately be easier to adapt for FACS-SWiM analysis (Simon et al. 1995; Rice et al. 1997).

Sorting time will always be dependent on in situ abundances of populations of interest. Bulk seawater samples can be pre-concentrated by filtration to maximize the sorting capabilities of the instrument used, but the relative proportion of cells to be sorted will ultimately determine how much time is required to acquire enough material for isotopic analysis. Additional targeted pre-concentration steps such as size fractionation for populations within a specific size-range could help reduce sorting time. Table 1 shows some of the variability in sorting times for sample collection, which ranged from 25 min to nearly 19 h and was dependent on several factors including the degree of pre-concentration, relative abundance of each population of interest, and the number of cells sorted. Sorting larger cells with higher per cell carbon content will certainly require fewer cells for $\delta^{13}C$ measurements, and increasing measured sample sizes increases $\delta^{13}C$ precision (Fig. 6).

The SWiM device employed here is not currently available as a commercial instrument, potentially limiting the broad applicability of this method. The nano-EA/IRMS system described by Polissar et al. (2009) has a reported sensitivity down to a few μg, roughly 10–50 times larger than for the SWiM system, and could serve as a partial replacement. Although sorting bacteria and very small eukaryotes will be impractical for this system, larger phytoplankton and protists should be accessible with just a few hours of sorting if these large particles are preconcentrated. Alternatively, secondary ionization mass spectrometers (SIMS) would be able to analyze smaller samples even than those reported here, albeit with lower precision and accuracy (see Orphan 2011). Primary drawbacks of this approach include the expense and difficulty of operating the instrumentation, and the need to develop further protocols for preparing FACS-sorted cells for SIMS analysis. Finally, building copies of the SWiM interface should be well within the capabilities of any lab with the expertise to run an IRMS and access to a machine shop. Interested parties are encouraged to contact the authors for further details.

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