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Prokaryotic cells separated from sediments are suitable for elemental composition analysis

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Abstract

Cell-sediment separation methods can potentially enable determination of the elemental composition of microbial communities by removing the sediment elemental contribution from bulk samples. We demonstrate that a separation method can be applied to determine the composition of prokaryotic cells. The method uses chemical and physical means to extract cells from benthic sediments and mats. Recovery yields were between 5% and 40%, as determined from cell counts. The method conserves cellular element contents to within 30% or better, as assessed by comparing C, N, P, Mg, Al, Ca, Ti, Mn, Fe, Ni, Cu, Zn, and Mo contents in *Escherichia coli*. Contamination by C, N, and P from chemicals used during the procedure was negligible. Na and K were not conserved, being likely exchanged through the cell membrane as cations during separation. V, Cr, and Co abundances could not be determined due to large (>100%) measurement uncertainties. We applied this method to measure elemental contents in extremophilic communities of Yellowstone National Park hot springs. The method was generally successful at separating cells from sediment, but does not discriminate between cells and detrital biological or noncellular material of similar density. This resulted in Al, Ti, Mn, and Fe contamination, which can be tracked using proxies such as metal:Al ratios. With these caveats, we present the first measurements, to our knowledge, of the elemental abundances of a chemosynthetic community. The communities have C:N ratios typical of aquatic microorganisms, are low in P, and their metal abundances vary between hot springs by orders of magnitude.

Better understanding of the coupling of major biogeochemical cycles requires knowledge of the cellular elemental composition of microbes (e.g., Williams and Fraústo da Silva 2000; Haraguchi 2004; Morel 2008). Examples of connections between trace metals and macronutrient cycling by microbes abound. Nitrogen is cycled using enzymes that contain Fe, Cu, and Mo (Morel and Price 2003). Inorganic carbon fixation by photosynthesis requires electron transfer intermediates containing Fe (Morel and Price 2003), and the pigment chlorophyll contains Mg. Reactive radicals resulting from photosynthesis are managed by superoxide dismutase enzymes with Mn, Fe, Ni, Cu, or Zn cofactors (Bowler et al. 1992). Metalloenzymes with a Zn, Co, or Cd metal center assist microbes with inorganic carbon assimilation (Morel and Price 2003). Organically bound phosphorus can be made bioavailable by phosphatase enzymes containing Zn, Mg, or Fe (e.g., Stec et al. 2000).

Because of the importance of these microbial processes, we are motivated to study major element cycling in situ by measuring the elemental composition of microbial cells extracted directly from their environment. Carbon, macronutrients (e.g., nitrogen and phosphorus), and micronutrients (e.g., trace metals) need to be taken up from the environment by microbes. In Earth's oceans, the availability of metals, P, and N varies with space and time (Anbar 2008; Falkowski 1997; Papineau et al. 2009) and is known to influence element use by cells (Morel 2008; Dupont et al. 2010). However, in extreme environments such as hot springs, the distribution of elements, their use by extremophilic microorganisms, and the

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influence of extremophiles on element cycling remain largely unexplored. Hot springs at temperatures above 72°C are also environments where primary production is supported by chemosynthetic processes rather than photosynthesis. Chemosynthesis also involves metals as enzyme cofactors, such as Fe and Ni in hydrogen oxidation. Determining the elemental contents of microbes in such settings will likely advance our understanding of modern biogeochemical processes and potentially those in the past as well.

However, in benthic sediments and microbial mats such as those found in hot springs, it is difficult to determine the composition of microbes because of the contributions of mineral grains and particles. It is especially difficult to determine the microbial content of metals such as Fe, Ni, Cu, Zn, and Mo, which are also found in sediment. However, these elements need to be quantified to extend the stoichiometric analysis beyond the standard "Redfield C:N:P ratio" to a broader suite of elements of biological importance (Twining et al. 2011; Nuester et al. 2012). Removing the sediment contribution from elemental analyses of microbial communities, extremophilic or otherwise, requires either effective isolation of cells from complex mineral matrices (Havig et al. 2011; Steenbergh et al. 2013) or in situ microscopic analyses (Heldal et al. 2003, Steenbergh et al. 2013).

There is a need to verify that such cell separation procedures do not alter cellular elemental composition before analysis. Cell separation methods usually involve chemical (use of detergents and chelating agents) and physical means (stirring or blending and/or sonication) of disrupting microbe-mineral bonds, followed by density gradient centrifugation (Lindahl and Bakken 1995; Amalfitano and Fazi 2008; Kallmeyer et al. 2008; Amalfitano et al. 2009; Poté et al. 2010, Morono et al. 2013). Amalfitano and Fazi (2008) compared combinations of chemical and physical separations. They found that the most efficient method of detaching cells from sediment comprised three steps: (1) stirring of the sample slurry in an extraction mixture containing a detergent and a chelating agent, (2) gentle sonication of the resulting suspension, and (3) centrifugation through a cushion of the density gradient medium Nycodenz (Rickwood et al. 1982) to isolate the cells, which have lower density than sediment. Alternative methods to that of Amalfitano and Fazi (2008) involve blending, dispersion by rotary pestle, or shaking with glass beads (e.g., Lindahl and Bakken 1995); all of which could contaminate the separated cells with trace metals. This trace metal contamination risk from hardware is minimized in the procedure of Amalfitano and Fazi (2008), which can be carried out entirely in acid-washed plasticware.

For all cell separation methods, there is a trade-off between efficiently breaking the strong cell-sediment bonds and preserving cell integrity (Lindahl and Bakken 1995). Preserving cell integrity is essential to conserve cellular elemental contents. The method of Amalfitano and Fazi (2008) can affect cellular integrity (Amalfitano et al. 2009), which could lead to problems with measurements of cellular elemental composition. We tested the ability of the method of Amalfitano and Fazi (2008) to produce accurate information about the elemental contents of *Escherichia coli* cells by comparing elemental abundances in cells that had undergone separation to those in cells that had not. We then separated cells from sediments and mats collected in hot springs of Yellowstone National Park (USA) and measured their elemental makeup using Isotope Ratio Mass Spectrometry (IRMS) for C and N, and Inductively-Coupled Plasma Mass Spectrometry (ICP-MS) for the other elements.

Materials and procedures

To evaluate the ability of the cell separation method to preserve the elemental content of cells, wild-type *Escherichia coli* in LB broth was cultured to late log or early stationary phase, then divided into 1-mL aliquots and kept frozen at –80°C without addition of any cryoprotectant. Freezing and thawing could result in cell damage. However, when observing thawed *E. coli* cells using epifluorescence microscopy for cell counting, we noticed no physical damage (at least at this limited resolution). Furthermore, cells underwent only one freeze-thaw cycle, and care was taken to thaw cells slowly (10°C, 20-40 min). Freezing is necessary for field samples because cell separations cannot be performed in situ, and in some cases, the distance of field sites from laboratory facilities necessitates sample transport; we therefore applied the same procedure to *E. coli*. We measured the abundances of C, N, P, Na, Mg, Al, K, Ca, V, Cr, Fe, Co, Ni, Cu, Zn, and Mo in *E. coli* before and after separation. We did not mix *E. coli* with sterile sediment before separation because no significant cell-sediment bonding would likely occur (Lindahl and Bakken 1995). Cell separations, cell counts, and elemental analyses of *E. coli* were performed on a minimum of three but typically four replicates.

Cell-sediment separation procedure

The separation procedure is the combined $C_{1+2} + P_{1+2}(N)$ procedure of Amalfitano and Fazi (2008), followed by washing and drying of separated cells before elemental analysis. Amalfitano and Fazi's $C_{1+2} + P_{1+2}(N)$ separation procedure consists of two steps: (a) physical and chemical detachment of cells from sediment by vortexing and sonication of the samples in a buffer containing a detergent and a chelating agent, and (b) isolation of the cells by centrifugation of the suspension through a gradient of Nycodenz (Rickwood et al. 1982). Throughout the procedure, polypropylene containers and spatulas were used to prevent sample contamination by trace metals from glass or metallic items. These items were acid-washed and autoclaved before use; care was taken to avoid any physical contact between the plasticware and metals from the autoclave. All solutions were sterilized by autoclaving except sodium pyrophosphate, which was filter-sterilized. Although autoclaving is not considered best practice for trace metal work (Price et al. 1989), the trace metal content of all solutions was negligible compared with abundances measured in *E. coli* (Web Appendix A).

Cell extraction buffer was prepared as follows. To prepare 50 mL buffer, we used 0.05 g of the chelating agent sodium pyrophosphate (0.1% wt./vol. final concentration), 0.25 mL Tween 20 detergent (0.5% vol./vol. final concentration), and 5 mL phosphate-buffered saline (PBS), brought up to 50 mL with 18 MOhm-cm MilliQ water. The 5 mL PBS contained 1.3 M sodium chloride (0.13 M final concentration in the extraction buffer), 70 mM sodium phosphate dibasic (7 mM final concentration), and 30 mM sodium phosphate monobasic (3 mM final concentration); the pH of PBS was adjusted to 7.4 with a few drops of concentrated hydrochloric acid or sodium hydroxide. Sodium pyrophosphate was purchased from Sigma-Aldrich (Cat. No. 221368), Tween 20 from Bio-Rad (Cat. No. 170-6531), sodium chloride from Fisher Scientific (Cat. No. S271-500), sodium phosphate dibasic from Mallinckrodt (Cat. No. 7914), sodium phosphate monobasic from EMD Millipore (Cat. No. SX0710-1), and Nycodenz from Axis-Shield (Cat. No. 1002424).

The procedure is depicted in Fig. 1A. Aliquots of *E. coli* were gently thawed in a centrifuge (6000 g, 10°C, 20-40 min). The supernatant was removed. Samples were weighed and transferred into 20-mL beakers. A different beaker was used for approximately every 2 g sediment or microbial mat to avoid overflow during vortexing. In each beaker, extraction buffer (5 mL) was added. The resulting suspensions were then stirred on a large capacity stirrer for 30 min at 720 rpm using acid-washed, autoclaved stir rods. Each sample subsequently underwent sonication in an ice-cold bath (Branson Sonifier 450) with the following parameters: 40% output, 35% duty cycle, 2 min, to not lyse the cells. Amalfitano and Fazi (2008) sonicated cell suspensions for only 1 min; however, initial observations using optical and epifluorescence microscopy showed that more cells were detached from sediment particles in suspensions sonicated for 2 min than in those sonicated for 1 min.

Each suspension was gently homogenized and carefully layered on top of 5 mL of Nycodenz in 15-mL centrifuge tubes. The Nycodenz solution was prepared by dissolving 8 g Nycodenz in 10 mL MilliQ water, which yields a density of 1.3 g/mL (Poté et al. 2010). The density of the Nycodenz solution, which is intermediate between cells and sediment, allows sediment to migrate through Nycodenz while cells remain on top. Each sample was centrifuged at 5000*g* and 4°C for 30 min using a swing-out rotor. Centrifugation was repeated for an additional 30 min to 1 h until the Nycodenz layer was clear, and distinct phases could be seen. The phases of a separated field sediment sample are shown in Fig. 1B. From bottom to top, these phases are sediment pellet (absent when the procedure is applied to a sediment-free *E. coli* culture); Nycodenz; a thin layer of separated cells (with possible residual detritus of similar density); and the supernatant. The cell layer was pipetted out, transferred into a clean 2-mL tube, and washed three times to remove any Nycodenz or cell extraction buffer, using 0.85% sodium chloride (NaCl) to limit osmotic stress. For each wash, cells were resuspended in 1 mL NaCl solution, then centrifuged (10,000*g*, 5 min, 4°C), and the supernatant was discarded.

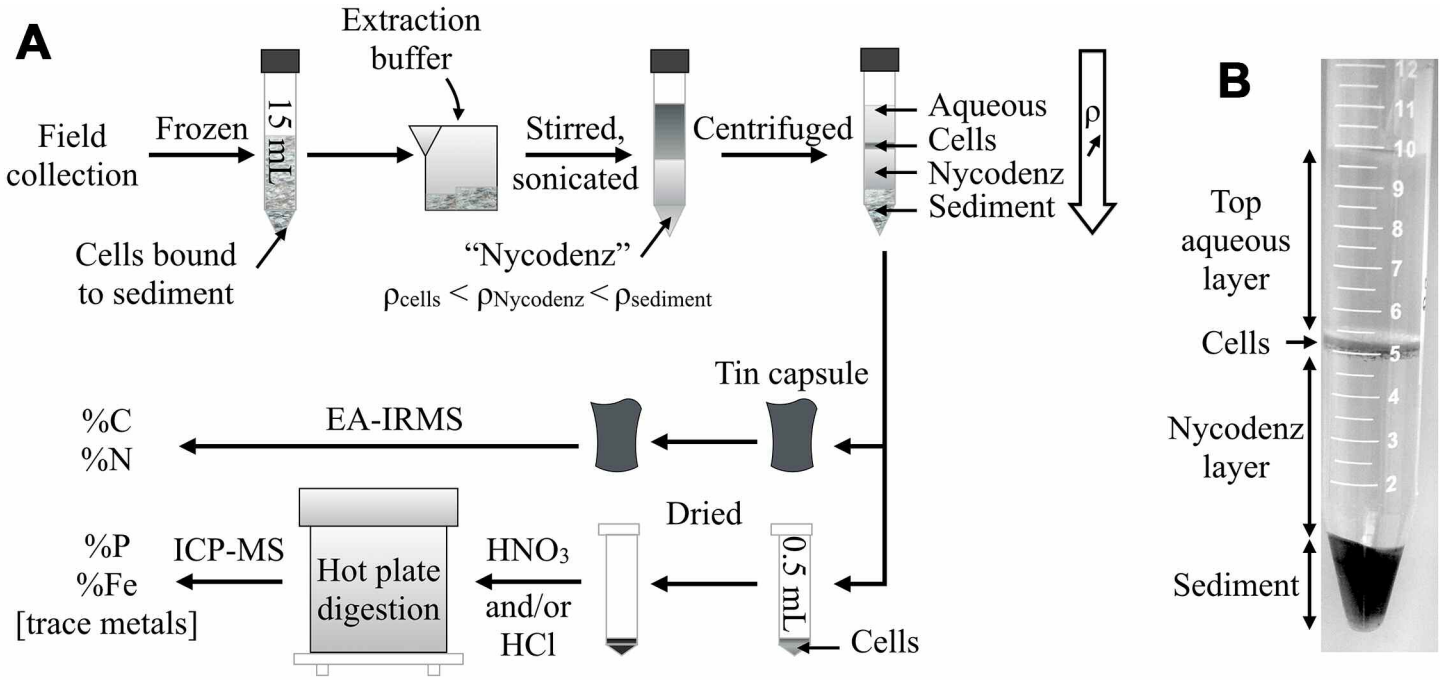

Fig. 1. A. Schematic of the cell-sediment separation and preparation for elemental analysis (see text for details). B. Four phases of a separated sediment sample: top aqueous layer, interface containing cells, Nycodenz layer, and bottom sediment pellet, ideally no longer containing cells. This sample was collected at an outflow of the hot spring in Yellowstone we unofficially named 'Bison Pool'.

Separated cells, resuspended in a minimal amount (200 to 1000 μL) of 18 MOhm-cm MilliQ water to allow pipetting, were divided into aliquots of 50 μL (IRMS replicates), and 300 μL (ICP-MS replicates) and dried (60°C, overnight) before elemental analysis. Obtaining dry masses of cell fractions was essential to quantify elemental contents in terms of dry cellular mass. *E. coli* cells that did not undergo separation were thawed, washed three times with either 0.85% NaCl or cell extraction buffer (to assess the capacity of these solutions to preserve the cellular makeup), resuspended in a minimal volume (200 to 1000 μL) of 18 MOhm-cm MilliQ water, and dried.

Cell counting

Cell counting using epifluorescence microscopy helped determine a cell recovery yield. Yields were determined by comparing the amount of cells present before and after centrifugation through Nycodenz. 45-μL aliquots of cell suspensions were removed immediately after the sonication step and again at the end of each NaCl wash. Cells were fixed using 5 μL formaldehyde (37%, final concentration 3.7%) and stored at 4°C until counting. They were stained using 10 μg/mL 4′,6-diamidino-2-phenylindole (DAPI), resuspended in 10 mL sterile 18 MOhm-cm MilliQ water, and filtered on a 0.22-μm polycarbonate membrane for enumeration via epifluorescence microscopy at 1000-fold magnification.

Elemental analysis

Carbon and nitrogen

We used elemental analysis–isotope ratio mass spectrometry (EA-IRMS) to determine the elemental and isotopic abundances of C and N. A Costech EA-IRMS (Costech International S.p.a.) was used to analyze all samples, calibration standards, and blanks. Before combustion, samples were loaded into 5 × 9 mm tin capsules (Costech). Freshly separated cells were resuspended in as little 18 MOhm-cm MilliQ water as possible (200 to 1000 μL) and pipetted into tin capsules in 50 μL aliquots, then dried overnight at 60°C. Serial pipetting-drying cycles were required for dilute suspensions; cells awaiting drying were stored at 4°C.

A calibration curve for C and N contents was built using a tomato leaf standard (0.2, 0.5, 1.0, 2.0, 5.0, and 7.0 mg). Glycine standards (0.7 mg) of known light, medium, and heavy C and N isotopic composition were analyzed between samples to determine the accuracy of isotopic determinations. These in-house standards were characterized using the following standards: USGS 40, USGS 41, IAEA NBS 18, and IAEA NBS 19.

Phosphorus and trace elements

P, Na, Mg, Al, K, Ca, V, Cr, Fe, Co, Ni, Cu, Zn, and Mo abundances were determined by Inductively-Coupled Plasma Mass Spectrometry (ICP-MS). All samples, appropriate multi-element calibration standards, and blanks were analyzed on an X Series quadrupole ICP-MS (Thermo Scientific). Sample preparation was carried out in a Class-10 clean laboratory, using 1 × distilled nitric and hydrochloric acids. Dilutions were made with 18 MOhm-cm water.

Cells dried in microcentrifuge tubes were transferred to acid-cleaned (using concentrated nitric acid followed by concentrated hydrochloric acid) Teflon vessels and digested overnight in 3 mL concentrated nitric acid on a hot plate at 100 to 150°C. The vessels were then uncapped and heated until sample volume decreased to approximately 0.5 mL. The samples were finally diluted to 3.5 mL (the minimum volume required by the ICP-MS to measure the 14 elements specified) with 0.32 M nitric acid in 15 mL acid-cleaned centrifuge tubes. The final dilution volume was weighed to obtain a dilution factor between initial dry mass and mass of solution analyzed.

A calibration curve was built using a phosphorus-enriched black shale standard solution of known elemental composition (characterized using the certified reference material USGS Devonian black shale SDO-1), serially diluted (1:15, 1:35, 1:150, 1:1000, 1:7500, 1:25000) with nitric acid (0.32 M). This standard was chosen because its elemental composition reasonably matched that of (sometimes widely varying) Yellowstone sediment samples and *E. coli*. The standard was not digested along with the samples to test for element recoveries; however, it is routinely run in parallel with samples, with generally excellent recovery, accuracy, and reproducibility. We performed sample-free digestions to check for contamination during digestion, and subtracted the average of the measured values on these digestions from the measured value of each *E. coli* replicate.

Assessment

Cell recovery yields

The yield for *E. coli* aliquots was consistently around 95%. Therefore, only a small portion of cells was either lysed during separation or lost to the aqueous or Nycodenz phases. Amounts of cells washed away in the three washing steps were about 1%, 0.1%, and 0.01%, respectively.

Preservation of elemental content

The elemental abundances of unseparated and separated *E. coli* cells are shown in Web Appendix A, and the relative element enrichments or depletions (i.e., [abundance in separated cells/abundance in non-separated cells] – 1) in *E. coli* due to cell separation are shown in Fig. 2. The measured elemental content of unseparated *E. coli* cells did not depend on whether these cells were washed with 0.85% NaCl or with cell extraction buffer (Web Appendix A).

C and N contents were systematically depleted by about 10% in separated cells, whereas two elements present as cations in the cells (Na and K, not plotted) were severely depleted in some separated replicates but highly enriched in others. P and trace metal (except V, Cr, and Co) contents were unchanged within 30% or less. V, Cr, and Co contents appeared to be conserved through the separation just as well as Ni, Cu, Zn, or Mo but the precision of our ICP-MS measurements did not allow reliable determination of their abundance (Web Appendix A). While V, Cr, and Co were present at quantifiable levels (>10 × blank level), the spread between replicates was high.

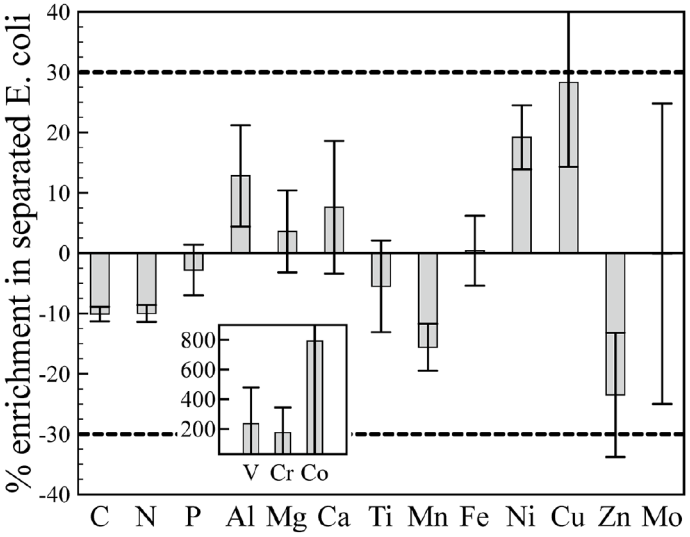

Fig. 2. Relative enrichment or depletion of elements in cultured wild-type *E. coli* that underwent separation, compared with the same culture without separation ([abundance after separation in μg/(g dry mass)/abundance before separation in μg/(g dry mass)] × 100 – 100%). Error bars show one standard deviation over four replicates (i.e., 2 × the standard error on the mean over four replicates, approximately a 95% confidence limit); standard deviations were propagated through the calculation. The dashed lines at +30% and –30% bracket the range of relative enrichments or depletions.

We note that *E. coli* grown in LB has a high metal content, up to an order of magnitude more than *E. coli* grown in minimal medium (Outten and O'Halloran 2001). This could mask small variations in metal content due to potential contamination, which may thus have been underestimated. Further investigations may wish to repeat this experiment with *E. coli* grown in minimal medium to assess potential contamination on a lower scale.

Gain of elements (contamination)

Artificially high elemental abundances could have resulted if the cells were contaminated by autoclaved solutions used during separation (Steenbergh et al. 2013), or during acid digestion. P- and Na-rich PBS and sodium pyrophosphate, C- rich Tween 20, and C- and N- rich Nycodenz are used in the separation procedure, raising the possibility of contamination by these elements. However, we observed a modest depletion in C and N after separation (Fig. 2), whereas P abundance remained unchanged within the limits of analytical uncertainties. In addition, the C and N isotopic compositions of *E. coli* did not change during separation ($\delta^{13}C_{VPDB}$ = –21.8 ± 0.5‰, $\delta^{15}N_{AIR}$ = 4.0 ± 0.4‰), and were very different from those of Nycodenz ($\delta^{13}C_{VPDB}$ = –30.3 ± 0.7‰, $\delta^{15}N_{AIR}$ = 0.0 ± 0.4‰), further supporting the inference that cell contamination by Nycodenz or Tween 20 was negligible. The isotope measurement errors were determined by measuring the isotopic content of glycine standards of known isotopic composition. Constant P abundances also suggest no contamination from the cell extraction buffer.

Furthermore, the Nycodenz molecule contains 3 atoms of iodine (I), which makes up 46% of its mass. Steenbergh et al. (2013) noted that measuring I in separated cells can help estimate Nycodenz contamination, and thus its contribution to the measured C and N. Microorganisms contain 1 to 100 ppm I (Bowen 1979). Up to 1.4% I by mass (roughly 3% Nycodenz) was measured in the field samples, equivalent to 0.83% C and 0.15% N enrichments from Nycodenz. This falls well within measurement uncertainties (1.6% for C and 0.6% for N). Since Nycodenz contributes the most C and N of the solutions used in the procedure, we infer that contributions of other chemicals were negligible as well.

The trace element contents of the solutions used in the procedure are shown in Web Appendix A. Most elemental signals were too noisy (below ten times the instrumental uncertainty on the analytical blank value) to be accurately quantified. The only signals clearly above the noise level were for one isotope of Cu, ^{63}Cu (not confirmed in noisy ^{65}Cu measurements below or around quantification limit) and for K, which was not conserved in *E. coli*. We therefore infer that trace metal contamination due to autoclaving of the solutions used in the cell separation procedure is negligible.

Metal contamination could also occur during sample digestion. We carried out sample-free digestions for each ICP-MS analysis and measured the trace element signal of these procedural blanks. This signal is compared with that of *E. coli* digestions in Table 1, where it can be seen that contamination at this step was negligible.

Loss of elements

Loss of elements from the cells can occur either if cell integrity is compromised by freeze-thaw, osmotic, or mechanical shocks, or if elements can leave through the cell membrane. Indeed, the cell separation procedure of Amalfitano and Fazi (2008) used in this study was found to result in membrane damage for 7 to 30% of cells separated from sediment (Amalfitano et al. 2009).

Separated *E. coli* had C and N abundances that were systematically lowered by about 10%. This depletion might reflect loss of some molecular pools in the cells, where compounds such as sugars, organic acids, nitrates, or amino acids are stored for future use after synthesis or uptake from the environment. Britten and McClure (1962) showed that *E. coli* amino acid pools can be emptied following freeze-thaw or osmotic shocks, and that the C and N contents of these pools can account for 10% of organismal abundances. Regardless of the source of the depletion, the consistent loss of C and N suggests that a correction factor of ~10% should be used to adjust C and N contents of samples analyzed with this procedure.

Epstein and Schultz (1965) and Rhoads and Epstein (1978) showed that osmotic shock during cold cell washing steps deplete the K content of *E. coli*, because intracellular K concentrations depend on osmolarity, and to a lesser extent, temperature of the external medium. This can explain the depletion in *E. coli* cellular K that we measured after some

Table 1. Comparison between the trace element signal from sample-free digestions and from separated *E. coli*, in part per billion per mass of solution analyzed (i.e., digestion diluted to 3.5 mL with 2% HNO_3). These values were automatically produced by the mass spectrometer software, which first subtracted the signal from an analytical blank (nondigested, trace metal-free 2% HNO_3 solution) from the physical mass spectrometer measurement, then averaged over three measurements of the same sample. The uncertainty represents the standard deviation between these three measurements of a given sample.

Element	Sample-free digestion				Example-separated
(ppb)	16 December 2011	21 April 2012	4 June 2012	20 November 2012	*E. coli*
^{23}Na	95 ± 25	–0.3 ± 1.1	–20.3 ± 9.5	14.9 ± 0.9	(2.6 ± 0.009) × 10^7
^{24}Mg	0.4 ± 0.2	0.5 ± 0.1	0.6 ± 0.2	1.31 ± 0.04	(9.4 ± 0.04) × 10^5
^{27}Al	0.3 ± 1.3	1.9 ± 0.2	7.6 ± 5.1	2.23 ± 0.03	(6.4 ± 0.3) × 10^3
^{31}P	68 ± 14	41.6 ± 0.9	118 ± 4	42.8 ± 3.6	(1.9 ± 0.02) × 10^7
^{39}K	5.3 ± 5.8	–7.7 ± 11.2	–38 ± 22	–7.8 ± 1.0	(2.5 ± 0.02) × 10^6
^{44}Ca	119 ± 58	–6.2 ± 5.8	–63 ± 10	21 ± 11	(2.0 ± 0.2) × 10^5
^{48}Ti	N/A	0.40 ± 0.06	3.5 ± 0.4	0.05 ± 0.04	(1.4 ± 0.2) × 10^3
^{51}V	0.004 ± 0.012	0.006 ± 0.024	0.076 ± 0.024	–0.098 ± 0.014	43.3 ± 8.3
^{52}Cr	0.042 ± 0.038	0.075 ± 0.034	0.42 ± 0.03	0.22 ± 0.06	8.3 ± 9.1
^{55}Mn-H_2	N/A	N/A	N/A	0.032 ± 0.008	(5.1 ± 0.2) × 10^3
^{55}Mn	N/A	N/A	N/A	0.024 ± 0.034	(4.5 ± 0.02) × 10^3
^{54}Fe	1.1 ± 0.7	4.7 ± 1.6	137 ± 4	2.1 ± 0.6	(2.1 ± 0.2) × 10^5
^{56}Fe	0.25 ± 0.14	2.0 ± 0.2	0.25 ± 0.79	1.4 ± 0.2	(2.1 ± 0.04) × 10^5
^{57}Fe	0.32 ± 0.24	1.9 ± 1.1	–26 ± 4	1.6 ± 1.3	(2.1 ± 0.2) × 10^5
^{59}Co	0.10 ± 0.02	–0.01 ± 0.02	–0.03 ± 0.01	0.000 ± 0.002	(2.6 ± 0.04) × 10^3
^{58}Ni	0.27 ± 0.08	0.03 ± 0.19	–0.12 ± 0.01	0.007 ± 0.018	(2.7 ± 0.07) × 10^3
^{60}Ni	0.7 ± 0.2	–0.8 ± 0.2	–0.85 ± 0.05	0.007 ± 0.016	(3.2 ± 0.04) × 10^3
^{63}Cu	–0.08 ± 0.02	0.63 ± 0.03	2.2 ± 0.7	–0.98 ± 0.04	(5.3 ± 0.09) × 10^3
^{65}Cu	0.04 ± 0.02	0.37 ± 0.05	0.9 ± 0.7	–0.59 ± 0.04	(5.0 ± 0.04) × 10^3
^{66}Zn	–0.18 ± 0.12	0.38 ± 0.04	0.9 ± 0.3	0.4 ± 0.1	(4.6 ± 0.05) × 10^4
^{68}Zn	–0.003 ± 0.044	N/A	0.79 ± 0.39	0.35 ± 0.06	(4.2 ± 0.07) × 10^4
^{95}Mo	0.11 ± 0.01	–0.12 ± 0.02	0.07 ± 0.01	0.37 ± 0.01	(4.8 ± 0.09) × 10^3

separations. When cell separation caused a K enrichment in *E. coli* cells, we suspect a contamination by trace K^+ from the extraction buffer: we measured 10-fold differences in K abundances in solutions between the buffer and supernatant phases, where most buffer should end up. Finally, the mismatch in *E. coli* Na is not surprising, given its ubiquity in the buffer and wash solution and the fact that Na^+ can pass through the cell membrane.

Application to field samples

Cell-sediment separation

Due to ongoing research projects at hot springs within Yellowstone National Park (Cox et al. 2011; Havig et al. 2011), we sought to determine cellular elemental ratios in samples from the area's diverse hot spring ecosystems. Field sediment samples were collected in July and August 2009 at hot springs with temperatures ranging from 20°C to boiling (~93°C). The pH of the springs ranged from 1 to 9. Four replicate 15-mL tubes were filled with sediment to 5 mL. Sediment samples without a cryoprotectant added were kept on ice in the field and then frozen at –20°C until transport back to Arizona State University, where samples were transferred to a –80°C freezer.

Cells were separated using the same method as for the *E. coli* samples. Visual examination of the freshly separated samples confirmed the applicability of the cell-sediment separation method for hot spring sediment samples (Fig. 1B). Four phases—aqueous, cell, Nycodenz, and sediment—could be seen after centrifugation of all samples, although the cell phase was at times difficult to distinguish in samples with low bulk biomass content.

For each cell separation of field-collected samples, we also separated a 1-mL *E. coli* aliquot, not mixed with any sediment, from the same culture as that used in the above element conservation studies. This extra *E. coli* separation was an independent check on the integrity of the method, allowing us to track anomalous element variations or recovery yields.

Cell recovery yields

Cell recovery yields were estimated for a few samples using the same method as for *E. coli* cells. Cell counting also provided an opportunity to examine the isolated and resuspended cell fraction to check for the presence of detrital and sedimentary particles. Cell counts for the field-collected samples indicated recovery yields between 5 and 40% (Table 2). These rates, lower than the 95% *E. coli* recovery rate, likely indicate that a significant fraction of the cells was still bound to sediment. Different sediment characteristics, such as humic content and grain size, may affect the cell separation efficiency. Moreover, the 0.85%

Table 2. Cell recovery yields for several separated samples collected at hot springs of Yellowstone National Park, as well as cultured *E. coli*. Uncertainties are one standard deviation over three replicates and were propagated for the recovery yields. Note that the names given to sampling sites are unofficial and correspond only to our own sampling program. All samples were frozen following sampling and thawed before separation.

Sample	T_{spring} (°C)	pH_{spring}	Site description	Cell concentration in unseparated sample (per g sediment)	Cell concentration in separated sample (per g sediment separated)	Cell recovery yield (±SD)
'Bison Pool'	66.2	8.1	Outflow of siliceous sinter spring, phototrophic site	$(3.6 \pm 2.8)\ 10^8$	$(9.8 \pm 6.0)\ 10^7$	28 ± 27%
'Green Cheese'	57.4	6.1	Phototrophic site	$(2.9 \pm 1.2)\ 10^8$	$(1.5 \pm 0.1)\ 10^7$	5 ± 2%
'Tinkerbell'	61.4	4.7	Red, turbid, Al-rich spring, chemotrophic site	$(7.5 \pm 2.4)\ 10^8$	$(2.8 \pm 0.9)\ 10^7$	37 ± 16%
'Mr. Clean'	76.4	6.1	Black, turbid spring, chemotrophic site	$(3.7 \pm 1.3)\ 10^8$	$(2.8 \pm 1.2)\ 10^7$	8 ± 4%
E. coli	N/A	N/A	Cell culture	$(2.4 \pm 0.03)\ 10^{10*}$	$(2.2 \pm 0.03)\ 10^{10\dagger}$	95 ± 2%

*Sum of the numbers of *E. coli* cells in all four separation phases and all three washes for 1 g *E. coli* culture separated.
†Number of *E. coli* cells in cell layer only (Fig. 1B) for 1 g *E. coli* culture separated.

NaCl wash solution may not always closely match the osmolarity of field samples given their considerable compositional diversity; the resulting osmotic stress may have lysed some cells. However, the reported yield percentages are in line with literature data (Lindahl and Bakken 1995; Kallmeyer et al. 2008; Morono et al. 2013).

Elemental composition of cells separated from sediment

When the separated cell phase was too small for quantitative analysis by both IRMS and ICP-MS, determination of C and N content by IRMS of three to four replicates was given priority, but we still kept one replicate for ICP-MS analysis. This was the case for seven Yellowstone samples out of the eleven separated.

Sample preparation for elemental analysis was carried out as described previously for *E. coli*. Samples for ICP-MS analysis were considered digested if the solution was clear. Turbid solutions were digested again by repeating the addition of 3 mL nitric acid and heating overnight (usually 2 repeats). If turbidity was still observed, aqua regia (1 mL of concentrated nitric acid and 3 mL of concentrated hydrochloric acid) was added and samples heated overnight for up to 10 repeats, until the turbidity disappeared. Rarely, samples contained particles that resisted digestion. These particles, considered residual sediment, were weighed and discarded; their mass was subtracted from those of the samples to calculate final element concentrations but was usually negligible.

Exemplary results are presented in Figs. 3 and 4 and Web Appendix A, in which all elemental abundances are reported as a percentage or part-per-million (ppm) of dry mass. The C:N:P relationships for four communities are shown in Fig. 3. C and N abundances were corrected to account for the 10% loss measured in *E. coli*. The hot spring photosynthetic communities sampled had C:N:P ratios in the range normally reported for microorganisms (*see* Sterner and Elser [2002] for a review). The chemosynthetic community sampled had C:N:P molar ratios of 850:109:1. Thus, its C:N ratio of 7.8 is similar to that measured by Redfield (1958) for marine phytoplankton (C:N = 106:16 = 6.6). To our knowledge, these data constitute the first measurement of the C:N:P ratios of a hot spring chemosynthetic community.

The abundances of metals in Yellowstone cells collected at a dozen sites are shown in Fig. 4. Most metal abundances (Mg, Al, Ca, Ti, Mn, Fe, Cu, and Mo) varied from site to site by orders of magnitude whereas others (Ni and Zn) were similar across all the sites. Al, Ca, and Fe tended to be the most abundant metals. The Al content of cells should be on the order of 1 ppm (Bowen 1979; Bruland et al. 1991) but was measured at 0.1 to 1% in the samples. Such artificially high abundances can result from non-biomass sediment matter (e.g., particles with similar densities to cells) remaining in the cell fraction. In the bulk sediment, the amount of Al was 1 to 5% of the dry mass, ten times higher than in the separated cell fraction. If 100% of the Al measured in the cell fraction was sediment, then sediment comprised 10% of the dry mass of separated sample analyzed, the rest being cells. This 10% mineral contribution is roughly represented by the segmented line in Fig. 4, calculated as the geometric average over all field samples of the sediment contribution, assuming sediment makes up 10% of the dry mass of separated sample analyzed. Because sediment abundances vary significantly between field samples, this line should only be taken as a guide to the eye. As shown on Fig. 4, sediment accounts for most of the measured Ti and Mn in the separated samples, and part of the measured Mg, Ca, and Fe. However, sediment contribution is negligible for C, N, P, Ni, Cu, Zn, and Mo. For all bioessential metals (Mg, Ca, Mn, Fe, Ni, Cu, Zn, and Mo), the metal:Al ratio is several-

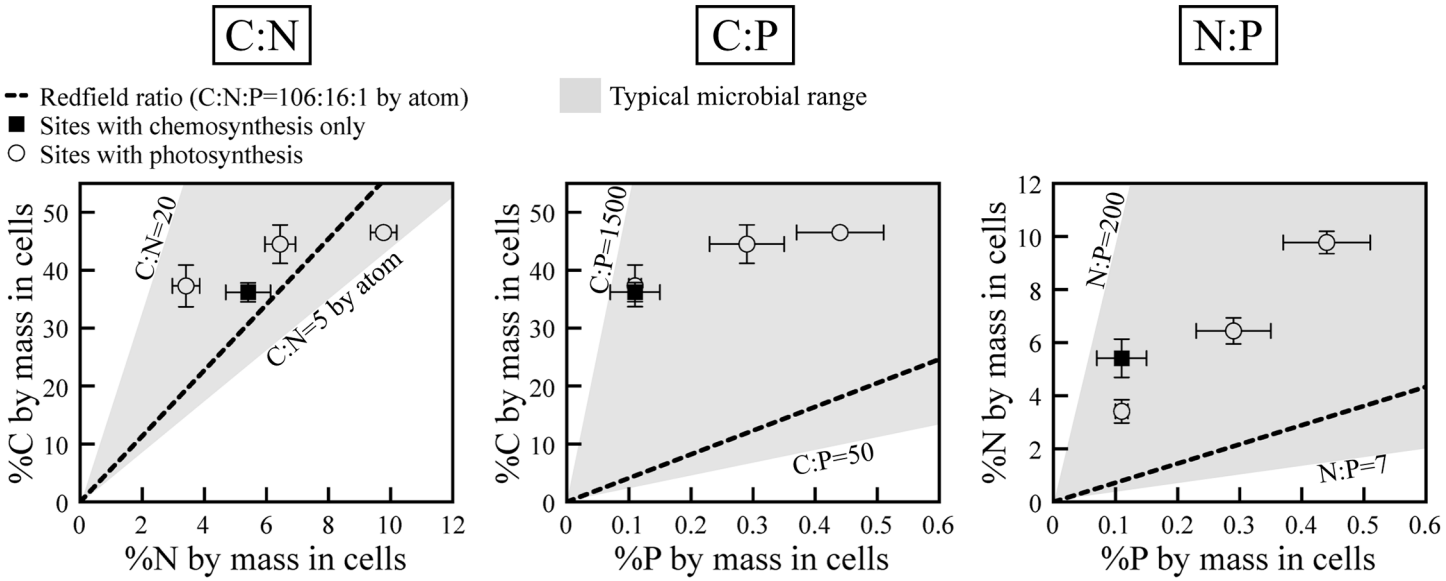

Fig. 3. C:N, C:P, and N:P ratios of cells separated from sediment collected at photosynthetic- and chemosynthetic-dominated Yellowstone hot springs. Error bars show one standard deviation uncertainties determined from both instrumental uncertainties by comeasurement of standards with the samples, and from one standard deviation over a minimum of three replicates. C and N abundances were corrected for a 10% loss during cell-sediment separation. Elemental abundances are reported as percentages of dry mass.

fold higher in separated samples compared with bulk sediment; whereas the Ti:Al is several-fold lower. We take this to indicate removal of at least part of the sediment contribution to measured elemental abundances in separated samples.

Discussion

These data show that the cell separation method we applied is successful at separating microbial cells from hot spring sediments, and based on the *E. coli* assessments, promising for retaining composition across a broad suite of biologically important elements.

The cell recovery yield was low for some field sites. Because our original samples were relatively large, these recovery yields were satisfactory to obtain sufficient material to determine an elemental composition profile of the sediment community. However, some microorganisms in a given community might be more easily separated than others from the sediment matrix; therefore there is likely to be further heterogeneity in elemental composition that this method cannot resolve. Such data can only be obtained by methods such as single-cell analyses via X-ray fluorescence (Heldal et al. 2003; Steenbergh et al. 2013).

This technique cannot separate detrital material (biogenic or otherwise) with a density similar to cell material, but a different element stoichiometry, from intact cells. In some samples, detrital and sedimentary particles were observed along with cells even after separation; however, the amount of detritus was much smaller compared with unseparated samples.

Another point of uncertainty arises from possible cell damage due to freezing and thawing of the samples before separation. We minimized this factor by freezing the samples only once, in the field, since separations cannot be performed there; we also thawed the samples gently.

Cellular C and N contents can be determined but appear to require correction of measured abundances for a 10% depletion during separation. Abundances of P, Mg, Al, Ca, Ti, Mn, Fe, Ni, Cu, Zn, and Mo were conserved during separation and could be determined by ICP-MS with an accuracy of 30% (1 SD) or better. Whereas V, Cr, and Co contents are likely conserved as well during separation, their cellular abundances were too low in our samples to obtain an accurate determination by ICP-MS.

Trace element abundances in field-collected samples show that there remains significant sediment contamination for elements enriched in sediments and depleted in cells, such as Al, Ti, Mn, and Fe, after the separation procedure (Fig. 4). Provided that the mineralogy of the sediment samples is accurately known, it may be possible to apply an assumed correction factor for sedimentary contribution, for example by using Al or Ti as a lithogenic tracer (Frew et al. 2006). We attempted to use Al, assumed nonbiological in origin, to estimate the extent of mineral contamination of purified cells. Aluminum abundances by mass range between 1 ppm and 0.1% in microorganisms, but between 0.9% and 30% in rocks, soil, and sediment (Bowen 1979; Bruland et al. 1991). *E. coli*, not mixed with sediment, had an average Al content of 5 ± 2.5 ppm, both with and without separation. (Uncertainties for both *E. coli* and field samples are 1 SD). These amounts are consistent with Al trapped in the cell membrane of *E. coli*, as measured by Coughlin et al. (1983). Al could also have been

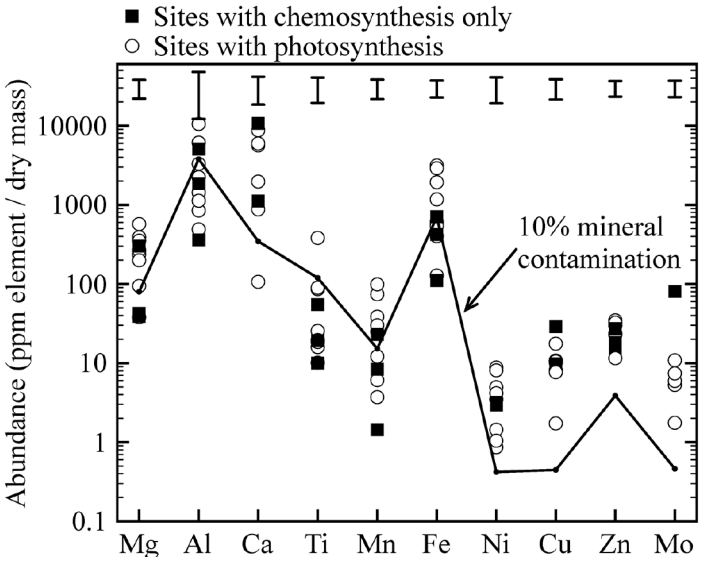

Fig. 4. Metal abundances in cells extracted from sediment samples collected at a dozen photosynthesis- and chemosynthesis-dominated Yellowstone hot springs. Elemental abundances are reported as ppm of dry mass. To avoid clutter, indicative error bars show typical one standard deviation uncertainties for this sample set, determined from both instrumental uncertainty by co-measurement of a standard with the samples, and from one standard deviation over at least three replicates. If not enough replicates were available, uncertainties were determined from one standard deviation over at least four replicates of *E. coli* separated and prepared along with the samples. As can be seen in Web Appendix A, the variability between replicates for *E. coli* and field samples is comparable and tends to scale with abundance. Therefore, the error bars provide an indicative estimate of the variability between replicates anywhere on the y-axis. The segmented line represents the mineral contribution to elemental abundances if the samples analyzed consist of 10% sediment and 90% cells, as estimated from the Al content measured in separated samples compared to that measured in bulk sediment (see text for details).

picked up in minute amounts during autoclaving of the LB solution, although Al is not part of LB medium itself. Al was present in sample-free digestions at levels of about 0.1% of that measured in *E. coli*, which makes the possibility of contamination during digestion unlikely. In half of the field samples, Al abundances were above 0.1%, reaching up to 1 ± 0.5%. The bulk field sediment samples contained 1 to 5% Al. Therefore, assuming 100% of the Al in separated cell fractions is from sediment, the separated cell fractions contained about 10% (up to 20%) sediment. However, the widely varying mineralogy of Yellowstone National Park hot springs complicates the determination of corrections based on metal:Al ratios in sediment and cells. We consider Al a worst-case contamination proxy, because Al^{3+} ions are more easily mobilized than other metal ions by ligands, such as the pyrophosphate used in the procedure (Williams 1996).

Another useful criterion is to reject data for samples or elements for which the metal:Al ratios closely mimic sedimentary ratios. Cu, Zn, Mo, and (to a lesser extent) Ni are systematically much higher than the sediment level (even if sediment constituted up to 50% of the separated fraction). For these metals, analyses can be interpreted relatively unambiguously in terms of cellular content, provided that supporting data (such as high Cu:Al, Zn:Al, and Mo:Al compared with sediment) are reported.

For the purposes of determining the C:N:P stoichiometry of samples, rejection of separated samples with a C content less than typical for microbes (35% to 50%, Sterner and Elser 2002) should suffice. This assumes that carbonates are not a major component of the sediment sample; if they are, a carbonate removal step before separation can be added (Kallmeyer et al. 2008). In practice, detrital biological material with a C:N:P stoichiometry different from that of the cells but with a similar density could also bias the measured C:N:P ratios of cells; this is a fundamental limitation of this method.

Thus, with further refinement, this cell separation method enables the determination of elemental contents, in mass per dry cell mass, of C, N, P, and key trace biological elements in sediment communities. This procedure is complementary to laborious electron microscopy methods that investigate the elemental composition of single cells (Heldal et al. 2003; Steenbergh et al. 2013). However, using both approaches would allow comparison of element distributions across broad components of a microbial community with those of the individual cells comprising the biomass.

This method will help quantify the multi-element stoichiometric envelope within which microbes operate (Sterner and Elser 2002; Ho et al. 2003; Twining et al. 2011; Cameron et al. 2012; Nuester et al. 2012). It will also allow comparisons of microbial elemental compositions with those of their environment to determine their mutual influence (Havig et al. 2011).

Comments and recommendations

Overall, we suggest that this method for separating microbial cells from sediment and analyzing their elemental contents at the community level should be applicable for samples from a wide variety of environments, such as soils, soil crusts, or lacustrine or riverbed sediments. Because high temperatures and pH extremes of hot springs result in low biomass of microbial cells, the abiotic contents of key elements can be unusually high, increasing the risk of elemental contamination of cells during separation. The possibility of contamination is likely lower for samples collected in milder conditions. This method should also be applicable for water samples containing high concentrations of suspended sediments, provided that enough sample is collected and filtered to obtain sufficient biomass for elemental analyses.

Based on our studies, here is a set of recommendations for future applications:

(1) Energy parameters for the sonication step should be considered as approximate values. As Lindahl and Bakken (1995) pointed out, the sonicator output depends on the age of the electromechanical converter, due to changes in its crystal structure.

(2) Precise measurement of cellular dry masses is key to the normalization of elemental abundances to mass and eventual determination of elemental ratios. Mass spectrometers measure the elemental masses in a sample more precisely than most balances measure dry masses of a few milligrams. Thus, we recommend to use a balance of microgram precision to measure the dry mass of recovered cell phases.

(3) For future analyses of samples from a specific location, standards used to determine the ICP-MS calibration curve and digestion recovery should be chosen as close to sample composition as possible to yield measurements as accurate as possible.

(4) As a coarse means of screening out contamination, we suggest discarding samples of C content too low to be biological (lower limit of about 35 to 40%, Sterner and Elser 2002). However, even minute amounts of sediment can significantly alter the metal content of the recovered cell fraction. Therefore, the use of complementary, more precise contamination indicators is advised. We recommend developing a correction factor for metal abundances based on lithogenic tracers such as Al or Ti (Frew et al. 2006) if the mineralogy of the samples is relatively uniform. Reporting of metal:Al or metal:Ti ratios is also advised to assess the reliability of a measurement.

(5) Finally, we recommend that follow-up work focuses on determining the extent of the selective biases due to overrepresentation of microorganisms that are more easily separated from sediment grains relative to those that contribute the most to the measured community composition. Energy-dispersive X-ray spectroscopy of single cells that remain in the sediment fraction may help (Heldal et al. 2003; Steenbergh et al. 2013).

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