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Estimates of bacterial production using the leucine incorporation method are influenced by differences in protein retention of microcentrifuge tubes

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Abstract

The most widely used methods to determine bacterial production involve measuring the incorporation of radioactive precursors, such as leucine, into macromolecular pools. The leucine method that involves incubation and extraction within a single microcentrifuge tube has become a widely used technique because of its relative convenience, precision, and low cost. We observed a discrepancy in parallel determinations of leucine incorporation for the same water samples that lead us to explore aspects of the method including tube-washing methods, operator differences, and differences among tube brands. Operators and washing methods had minimal effects on rate measurements, but results were strongly dependent on tube brands. Differences in tube performance were observed consistently in comparisons from a variety of freshwater and marine environments. Microcentrifuge tubes differed in protein retention with the consequence that estimates of leucine incorporation in a given sample could vary by as much as 60% depending on the tube used. There was no simple relationship between tube plastics or manufacturer and tube performance. We advise researchers to check the protein retention of tubes and to use the same brand of tube during field studies to minimize this potential source of variation.

Smith and Azam (1992) introduced a version of the leucine incorporation method (Kirchman et al. 1985) for measuring bacterial production where the entire procedure is conducted in microcentrifuge tubes. In this assay an environmental sample is mixed with radiolabeled leucine in a tube and incubated. Incorporation of the label into protein is measured by counting the retention of radioactivity on the walls of the microcentrifuge tube after extraction and washing of the sample with trichloroacetic acid. This method allows rapid bacterial production estimates at reduced cost and decreases the quantity of radioactive waste generated. In addition, the variance of replicate determinations is lower with the microcentrifuge method than with the previous method, which required concentrating samples on filters (Kirchman 2001). Because of these advantages, the microcentrifuge method of measuring leucine incorporation and estimating bacterial production has been widely adopted among aquatic microbial ecologists.

The ease and speed of the microcentrifuge method offers the possibility of measuring rates of bacterial production (BP) at greater spatial and temporal resolution. We assessed variation in BP spatially in a large aquatic ecosystem by conducting a series of transects of the Hudson River (New York, USA). We were surprised in these surveys to find large differences between a duplicate series of replicate measurements on samples from the same set of stations done by two of the authors. Leucine incorporation rates measured on the same water samples by analyst 1 were consistently higher than those done by analyst 2 (Fig. 1).

These results motivated us to examine several aspects of the microcentrifuge leucine incorporation method. In this article we explore possible differences in techniques and supplies that might affect measurements made with the method. We assessed tube-washing and preparation methods, differences among operators, and results obtained with different brands of microcentrifuge tubes. We found consistent differences in measurements of leucine incorporation made with different brands of tubes in a variety of freshwater and marine environments. We also report experimental measurements of protein retention in tubes and evaluate the

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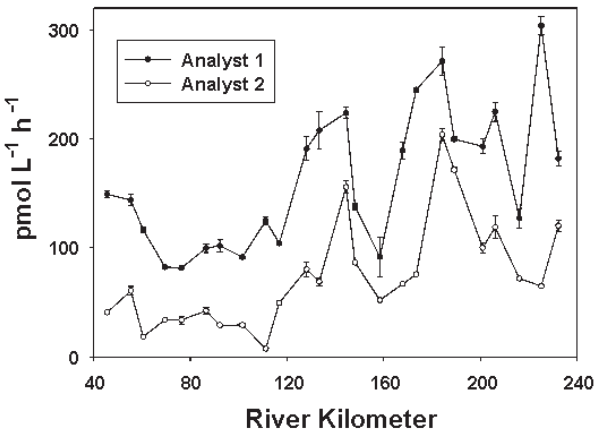

Fig. 1. Results of leucine incorporation measurements made by two analysts on samples from a Hudson River transect. *River kilometer* is distance from the mouth of the river at Battery Park, Manhattan, NY, USA. Values are means ± 1 standard deviation. The two series were correlated ($r = 0.67$, $P < 0.0003$), whereas the average difference between analysts was 87 pmol L^{-1} h^{-1}.

impact of differential retention on estimates of leucine incorporation.

Materials and procedures

Microcentrifuge method—Throughout this study, we employed the original leucine incorporation method of Kirchman et al. (1985) as modified by Smith and Azam (1992). Briefly, 1.5 mL water samples were dispensed in microcentrifuge tubes containing ^{3}H-leucine (~40 Ci $mmol^{-1}$; Perkin-Elmer NET 135H). Final leucine concentration varied from 40 nM in open ocean samples to as high as 60 nM in estuarine samples. Samples were incubated 60 min in the dark, and then uptake was terminated by adding 0.3 mL of cold 50% trichloroacetic acid (TCA). Samples were mixed and immediately centrifuged at 14,000 rpm (relative centrifugal force 17,000*g*) in a microcentrifuge for 10 min. The supernatant was removed by running a Pasteur pipette attached to an aspirator down the center of the tube. After discarding the supernatant, 1.5 mL of 5% cold TCA was added, and the sample was mixed and immediately centrifuged again. The supernatant was again discarded and 1.5 mL of scintillation cocktail was added (Scintiverse BD). The samples were mixed thoroughly and the microcentrifuge tubes placed in glass 20-mL scintillation vials. Samples were counted, usually within 24 h, on a liquid scintillation counter (Beckman LS6500). We found no differences in one batch of samples that we recounted after 96 h, to examine the possibility that longer holding times might lead to higher counts because of greater protein dissolution from tube walls (data not shown). Counts per minute were converted to disintegration per minute using a relationship between the H number (Horrocks 1977) and the counting efficiency determined for the specific reagents, scintillation cocktail, and sample containers used. We periodically measured uptake of radiolabel in control samples that were killed at the beginning of incubations with 0.3 mL of 50% TCA. Uptake in killed controls was always low (typically <5%, often ~1% of "live samples") and unrelated to the treatments. Reported values are not corrected.

Table 1. Label, supplier, plastic type, and manufacturer of the microcentrifuge tubes used in this study

Tube label	Supplier	Catalogue number	Plastic type	Manufacturer
A	Phenix	max-820	Homopolymer	Axygen
B	VWR	20902-540	Copolymer	Eppendorf
C	Fisher	02-681-344	Homopolymer	Porex
D	Phenix	scs-020f	Copolymer	Axygen
E	Phenix	sax-020	Homopolymer	Axygen
F	Fisher	05-664-35	Copolymer	Bioplas
G	VWR	20170-140	Copolymer	Labcon
H	VWR	20170-271	Copolymer	Labcon

Microcentrifuge tubes—Our initial comparisons in the Hudson River were done with 2.0-mL screw cap microcentrifuge tubes supplied by Fisher Scientific and VWR. We subsequently explored possible differences among microcentrifuge tubes by purchasing a variety of tubes. Microcentrifuge tubes are made from polypropylene polymers, either with a mixture of plastics referred to as copolymers or from a single plastic referred to as homopolymer (based on discussions with technical representatives of suppliers and manufacturers). We compared both types of plastic, as well as tubes from a variety of manufacturers and suppliers (Table 1). Tube brands are referred to throughout this article by letter (A to H) as in Table 1. In some cases, tubes were from the same supplier and manufacturer, although they differed in catalogue number (e.g., Tubes G and H, Table 1).

Tube preparation—We initially hypothesized that differences between measurements made on the Hudson River transect (Fig. 1) were related to tube preparation. One set of tubes had been acid-washed; another had not. In addition, we noted that the two operators were using different brands of tubes, C and G (Table 1). We compared brands C and G with three types of preparation. Tubes were acid-washed in 10% 0.1 N HCl and air-dried; acid-washed and oven-dried; or used directly without preparation. We measured leucine incorporation for the three treatments with two tube types (C and G) using surface water from the Hudson River. Three replicates were run for each preparation treatment and tube brand combination. Data were analyzed using two-way analysis of variance (ANOVA) ($P < 0.05$) with treatments—tube preparation and brand. A single analyst carried out the tube preparation experiment using samples from the Hudson River.

Field comparison of tubes in different environments—To test if the differences observed in the Hudson River (Fig. 1) were independent of the Hudson environment, we measured leucine

incorporation rates in tubes C and G in another estuarine system (Monie Bay, MD, USA). Monie Bay is a sub-estuary located on the eastern shore of Chesapeake Bay that consists of an open bay and three tidal creeks. Samples were taken at three stations along a salinity gradient (1 to 12 ppt) of one of the creeks. Five replicates were performed for each tube type at each station.

We compared leucine incorporation rates for eight different tube brands (Table 1) in various freshwater and marine environments. For the freshwater experiments we used tubes A through G. Experiments were done using surface water from Minniwaska, Sylvan, Mohonk, Tyrell, Upton, and Chodokee lakes as well as the Hudson River. Four replicates were run for each tube type in each lake. The lakes are located in the Hudson River Valley of New York, USA, and range from oligotrophic (Minniwaska) to eutrophic (Chodikee) as previously described (Cole et al. 1993; Baines and Pace 1994; Cole and Pace 1995). For the marine experiments, we used tubes A through H (excluding tube E). Tubes were compared for two sites and two depths on a cruise in the northeastern Pacific Ocean with six or seven replicates run for each tube type at each site-depth combination. Samples were taken at 10 m and in the pycnocline of a station on the outer shelf of the Oregon coast (44°39′N, 124°38′W) and in the northeast Pacific gyre (44°39′N, 125°22′W). Tube brands were compared within each environment using one-way ANOVA.

Protein retention experiment—To test the protein retention properties among tube brands, we diluted 2.5 µCi (82.5 kBq) of ^{14}C-labeled bovine serum albumin (specific activity = 14 µCi mg^{-1} [518 kBq mg^{-1} NEC 719]) into 73.5 mL of Nanopure® water and dispensed 1.5 mL of this mixture to 6 replicates of tubes A through G. After 1 h, the labeled albumin solution was extracted following the standard procedure for the leucine incorporation method. We measured radioactivity in the initial volume, the supernatant from the first extraction, the supernatant from the second extraction, and the remaining activity absorbed in the microcentrifuge tube after the second extraction.

Washing and extraction procedures—Two aspects of the leucine microcentrifuge procedure were briefly evaluated primarily to test if procedures affected differences between tube brands. In one experiment, leucine incorporation was measured for a sample from the Hudson River in tubes C and G for six replicates of each. In this experiment we added an ethanol (80%) wash. Ethanol helps remove non-protein molecules such as unincorporated 3H-luecine (Wicks and Robarts 1988). In a second experiment, we added NaCl (final concentration 3.5% as recommended by Kirschner and Velimirov 1999) to C and G tubes (six replicates each, Hudson River sample). Kirschner and Velimirov (1999) provide evidence that NaCl promotes precipitation of labeled macromolecules.

Assessment

Acid-washing and drying methods did not affect rates of leucine incorporation (ANOVA: tube preparation $P = 0.15$), but there was a strong difference between tube brands C and G (Fig. 2; ANOVA: brands $P < 0.0001$). There was no interaction between tube brand and tube preparation (ANOVA: interaction $P = 0.36$) indicating tube preparation did not differentially affect tubes C and G. The magnitude of the difference between brands was consistent with the Hudson River transects and suggested that the initial differences in results (Fig. 1) were related to tube brands, not to differences in tube preparation.

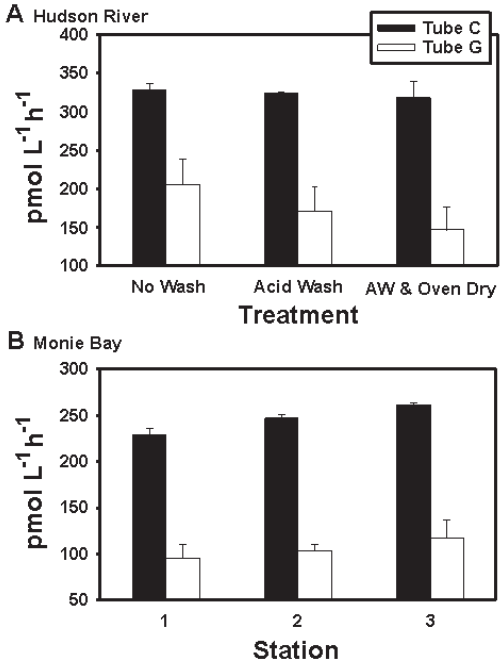

Fig. 2. (A) Comparisons of tubes C and G for samples from the Hudson River incubated with three tube preparation treatments. (B) Comparisons of tubes C and G for three stations along a salinity gradient in Monie Bay, MD, USA. Values are means ± 1 standard deviation.

We examined whether a different environment would result in the same differences observed between C and G tubes in the Hudson River by comparing the tubes at three stations in Monie Bay. A single analyst carried out the Monie Bay experiment. Consistent differences between tube brands C and G were observed at the three stations (Fig. 2) along the salinity gradient (ANOVA: brands $P < 0.0001$). These results suggest that the differences between C and G tube brands were not specific to conditions in the Hudson River.

The results of these two experiments also indicate indirectly that the analysts were not responsible for differences observed in leucine incorporation in the Hudson River (Fig. 1). Different analysts conducted the Monie Bay and tube preparation experiments and independently measured strong differences of a similar magnitude related to tube brands. We further confirmed that analysts had insignificant effects by running parallel experiments after having analyst exchange equipment (aspirators, centrifuge) and finding no differences (data not shown) other than those related to microcentrifuge tubes.

Table 2. Means and standard errors (SE) in pmol L^{-1} h^{-1} for leucine incorporation rates measured using different tube types

	Tube A		Tube B		Tube C		Tube D		Tube E		Tube F		Tube G		ANOVA*
Freshwater	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	*P*
Upton Lake	1046	21.4	950	22.2	936	13.1	944	8.7	884	13.7	875	15.5	540	49.2	0.0102
Tyrell Lake	803	22.3	741	38.0	749	14.8	769	13.8	733	20.3	718	16.7	601	29.7	<0.0001
Sylvan Lake	468	10.6	466	15.9	461	8.5	455	6.5	436	29.2	440	12.3	305	12.7	<0.0001
Mohonk Lake	501	16.2	478	6.8	464	14.9	507	2.6	522	20.6	535	24.1	132	17.7	<0.0001
Lake Minnewaska	346	15.2	316	7.8	323	2.9	335	9.3	178	10.7	321	13.2	83	11.3	<0.0001
Chodikee Lake	1074	84.1	1061	45.8	1188	8.9	1192	23.2	1023	76.1	1016	32.5	417	34.7	<0.0001
Hudson River	312	0.3	282	1.8	256	27.2	305	5.0	280	23.9	289	10.5	94	7.4	<0.0001
Average Rank	2.1		3.4		3.4		2.9		4.1		5.0		7.0		

	Tube A		Tube B		Tube C		Tube D		Tube F		Tube G		Tube H		ANOVA*
Marine	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	*P*
NE Pacific Gyre, 10 m	166	5.4	132	3.4	153	16.5	192	1.9	170	4.4	130	4.9	198	13.0	<0.0001
NE Pacific Gyre, Pycno	117	3.9	83	2.9	113	4.4	136	4.8	110	2.1	97	3.4	145	4.4	<0.0001
Oregon Shelf, 10 m	150	3.5	139	6.2	142	9.5	159	2.2	158	5.4	117	9.4	183	4.8	<0.0001
Oregon Shelf, Pycno	66	2.4	60	2.2	59	2.9	74	2.3	72	3.1	69	2.6	79	5.6	0.0005
Average Rank	4.0		6.3		5.3		2.0		3.5		6.0		1.0		

*ANOVAs are the results for one-way analyses to test among tube differences at each site.

When tube brands were compared among a series of freshwater and marine environments, highly significant differences were observed in all cases (Table 2). For the freshwater comparisons, lowest rates were always observed for tube G, where, in some cases, rates were 30% or less of the tubes with highest rates. If tube G is removed from the data set, there are still significant differences ($P < 0.05$) among rates in all comparisons. Thus, the differences among brands are not just the result of low rates measured for brand G. The rank of tubes from highest (1) to lowest (7) was fairly similar among the freshwater experiments. For the freshwater sites, tube A had the highest rates most often and lowest average rank across experiments (Table 2). Tubes B, C, and D were fairly similar in average rank whereas tubes E, F, and G typically had lower rates as reflected in their respective average ranks (Table 2). The magnitude of the differences among tubes was often large. For example, in the Upton Lake experiment, rates were highest in tube A whereas tubes E and F had rates that were 85% and 84%, respectively, of A (Table 2), reflecting an absolute difference of >150 pmol L^{-1} h^{-1}. In the marine comparisons, tube H (not used in the freshwater experiments) had the highest rates, and the ordering of tubes from highest to lowest rates was somewhat different than what was observed in the freshwater comparisons (Table 2). However, the lowest rates in the marine comparisons were measured for tube G in three of four cases (Table 2). Tube D performed well in both marine and freshwater.

We checked for possible differences in quenching among tubes brands by adding known concentrations of ^{3}H-leucine to each. We observed no differences in counts among tubes, implying that counting efficiency was the same irrespective of tube brand (data not shown).

The leucine incorporation method measures the incorporation of all the radioactively labeled protein that is retained on the walls of the centrifuge tubes, while soluble, low molecular weight compounds are extracted during the TCA washes. In the protein retention experiment, we observed differential retention among brands (Fig. 3). For all tubes, some radioactivity was lost during the first washing and a minor loss also occurred in the second washing (Fig. 3b). Differences among tubes were primarily related to the amount of material lost during the first washing. Overall, brands A, B, C, and D retained 80% or more of the added protein (ratio of initial radioactivity to final radioactivity). Brands E and F retained >70% whereas brand G only retained 55% (Fig. 3). The pattern of differential retention of protein is similar to the differences observed in BP among brands (Table 2).

We checked for recovery of the added radioactivity by summing counts of the two extractions with the counts remaining in the tube (protein retention) and compared this value to the measured value at time zero. For all brands of tubes except G, recovery of radioactivity was close to 100% (range 94% to 99%). For brand G, only 74% of the added radioactivity was measured in the sum of the extracts plus final value. One possibility for the loss of radioactivity in tube G is that some protein strongly adsorbed to the tube walls, although not all the protein was strongly adsorbed, as indicated by the greater loss of counts during the first TCA wash for tube G (Fig. 3). When the scintillation fluid was added to the tubes and mixed, some protein in the G tubes might have remained firmly attached

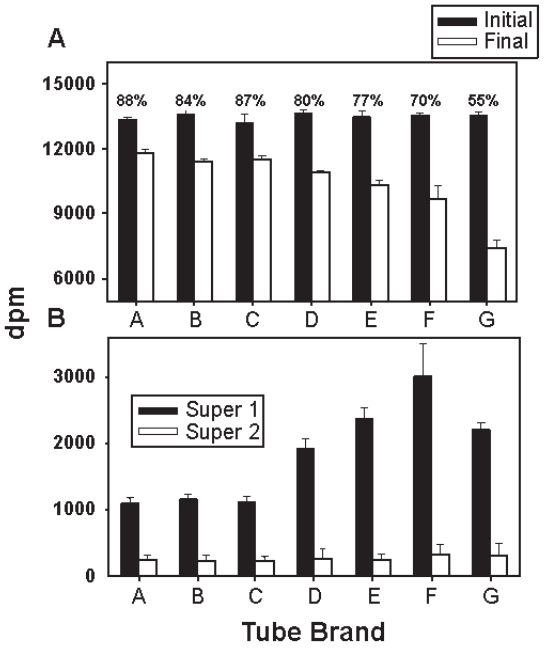

Fig. 3. Protein retention in different tube brands. (A) Comparison of initial and final disintegrations per min (dpm) and (B) dpm lost in the supernatant of the first and second trichloroacetic acid washes. Values are means ± 1 standard deviation.

such that the radioactive protein and scintillation fluid did not interact. This mechanism could explain the missing radioactivity and account for the low protein "retention" measured for tube G.

Measured leucine incorporation was about 10% lower (Table 3) when an ethanol rinse was added to the microcentrifuge method (ANOVA rinse: $P < 0.0001$) and strong differences remained in both treatments between the tube brands (ANOVA brand: $P < 0.0001$). The ethanol rinsing did not affect the differences between tubes (ANOVA brand × rinse interaction $P = 0.79$). Addition of NaCl to the extraction procedure did not affect measured leucine incorporation rates (ANOVA NaCl: $P = 0.42$), whereas differences between brands were similar to previous experiments (Table 3; ANOVA brand: $P < 0.0001$).

Correction of BP estimates for differential protein retention—We divided estimates of BP by the measured protein retention efficiencies to assess whether correction for differences among tubes would produce similar rate estimates. This calculation generally produced rates that were similar although, in some cases, differences remained. For example, the corrected BP estimates for Sylvan Lake were in the range of 528 to 567 pmol L^{-1} h^{-1} for all tubes except F where the corrected mean was 615 pmol L^{-1} h^{-1} (Fig. 4). One-way ANOVA using the Sylvan Lake corrected values was significant when tube F was included ($P = 0.007$), but not significant when tube F was deleted ($P > 0.05$).

Table 3. Comparison of tube brands C and G with different washing and extraction procedures

Tubes*	C	G
Without rinse	962 ± 17	671 ± 20
With ethanol rinse	877 ± 5	595 ± 14
Without NaCl extraction	755 ± 13	452 ± 8
With NaCl extraction	729 ± 12	499 ± 15

*Tubes were compared with and without an 80% ethanol rinse and with and without extraction with an NaCl solution. Values are means ± 1 SE in pmol L^{-1} h^{-1}.

Discussion

Our tests revealed surprising differences among tube brands in rates of leucine incorporation and protein retention. One tube brand (G) had particularly low retention and also yielded low rate estimates. Differences among tube brands were not related to plastic type or manufacturer. The reason for differential protein retention is not known but is a consistent feature. Kirschner and Velimirov (1999) found that protein may not precipitate efficiently with the standard microcentrifuge method of Smith and Azam (1992), which we used in this study. They recommended that NaCl or a humic extract be used to promote precipitation. The differences among microcentrifuge tubes we observed, however, were not simply the result of inefficient protein precipitation. First, we observed no effects on rates in freshwater Hudson River samples incubated in tubes C and G when we followed Kirschner and Velimirov's protocol for adding NaCl to promote precipitation. Second, we observed strong tube brand effects in freshwater, estuarine, and oceanic samples, whereas Kirschner and Velimirov (1999) found protein precipitation was primarily a problem in freshwater systems. In addition, we observed differential protein retention among tubes when a labeled protein was added and where protein precipitation in TCA should have been uniform among tubes. The differences among

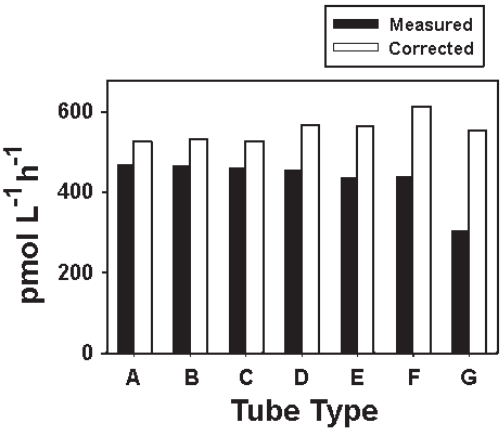

Fig. 4. Comparison of leucine incorporation rates for Sylvan Lake before and after correction for differential protein retention efficiency. Values are means of 4 replicates.

counts in the first TCA extraction (Fig. 3) suggest that differential adsorption of protein among tube brands is significant. Finally, the loss of radioactivity observed for tube G possibly indicates stronger adsorption of some of the protein during centrifugation that could not be recovered with standard mixing after addition of the scintillation fluid. This suggested reason for the loss of radioactivity, however, is speculative and requires further analysis to confirm.

Hientanen et al. (2001) also measured recovery of radioactivity for the leucine centrifugation method and found that they could not account for "around 20%" of the added label but offered no explanation. The quantity of missing activity in their experiments was essentially the same as we observed for tube G. Some of the differences they report between leucine incorporation measured with filtration and centrifugation techniques might have been related to the performance of the microcentrifuge tubes used in their study.

Comment and recommendations

Differences among tube brands can be easily checked using protein retention experiments. These comparisons at least provide qualitative assessments of tube performance that are consistent with field observations. It is uncertain if the retention of protein we measured by adding radiolabeled albumin is closely comparable to the retention of bacterial proteins that occurs during a leucine assay of an environmental sample. Bacterial proteins include a broad spectrum of sizes and shapes. This spectrum of properties probably influences protein adsorption and is likely more complex and variable than the adsorption and retention of a single protein such as albumin. Future experiments should consider measuring total protein in cultured bacterial cells and determining how much protein is lost versus retained in microcentrifuge tubes during the steps of the leucine assay.

In theory, all of the radiolabeled albumin should have been adsorbed in the retention experiments. In practice, a substantial amount was lost during the first extraction (Fig. 3). One possibility for this loss is that the radioactivity in albumin undergoes some exchange and/or degradation during the experiments and so is not present in protein. We cannot exclude this possibility, but we suspect the primary loss was related to inefficient adsorption of the label to tube walls. In any case, the question of whether or how to correct bacterial production estimates for possible losses of protein during extraction requires further attention.

Like all other techniques, the leucine incorporation method for measuring BP has some limitations and concerns (Kirchman 1993). Foremost among these is the problem of converting measured rates of radioactive leucine incorporation into estimates of bacterial carbon production (Kirchman and Ducklow 1993; Ducklow et al. 2000). Other concerns include saturating bacterial uptake and measuring the contribution of unlabeled leucine that dilutes the isotope added by the investigator (Simon and Azam 1989; Cole and Pace 1995), uptake of leucine by non-target organisms (Kamjunke and Jahnicen 2000), differences in rates associated with light and dark incubation (Moran et al. 2001), and efficient precipitation of proteins (Kirschner and Velimirov 1999). Applications of the method, heretofore, have not, to our knowledge, evaluated protein retention and the possibility of differential retention by microcentrifuge tubes.

In general, differences in rates among tube brands were <30% when tube G was excluded, but these differences are still quite significant because, frequently, studies attribute differences of this magnitude to ecological and environmental conditions. We could not identify a characteristic of the plastic, supplier, or manufacturer that predicted tube performance. For example, tubes from the same supplier and manufacturer (i.e., tubes G and H) and made from the same type of plastic performed very differently. Researchers commonly change tube types according to availability and price, so it is possible that some of the variability in published rates of bacterial production reflect differences in the microcentrifuge tubes used within and among studies. We advise researchers to be consistent with the type of tube used during experiments and surveys, because switching tubes may cause large differences that are unrelated to real changes in bacterial production. To minimize these problems, we suggest that researchers test protein retention of tubes and consider the need to correct bacterial production estimates for losses of labeled protein during extraction.

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