COMMENT

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Does adenine incorporation into nucleic acids measure total microbial production?: A response to comments by Fuhrman et al.[1]

The uptake and incorporation of [^{3}H]adenine as a measure of total microbial (bacteria and unicellular algae) nucleic acid synthesis was first used in oceanographic field experiments by Karl (1979). Since that time, the method has been substantially improved (Karl 1981, 1982; Karl et al. 1981*a*,*b*) and its accuracy evaluated with cultures of bacteria and unicellular algae (Winn and Karl 1984) and under field conditions (Laws et al. 1984; Karl and Bossard 1985*b*). The accuracy of the method depends on the validity of several assumptions that have been presented and discussed elsewhere (Karl 1979; Karl et al. 1981*a*; Karl and Winn 1984).

A unique characteristic of the [^{3}H]adenine method is the ability to measure the specific radioactivity (nCi pmol^{-1}) of the immediate precursor pool of the adenine incorporated into nucleic acids. This measurement permits correction for isotope dilution when the added radiotracer mixes with exogenous pools of structurally related compounds before uptake and with endogenous pools after transport into the cells. The immediate precursors to [^{3}H]adenine incorporation into cellular RNA and DNA are ATP and dATP. Since the intracellular dATP pool is in isotopic equilibrium with the ATP pool (Karl 1981; Winn and Karl 1984), one can determine the specific radioactivity of both precursor pools by measuring that of the intracellular ATP pool (Karl 1981). If the specific activity of the precursor pool is not measured, then it is impossible to extrapolate incorporation measurements to estimates of nucleic acid synthesis with any degree of accuracy. Even comparisons of "relative" rates are probably not justified since differences in the measured rates of RNA and DNA labeling may result either from variations in the specific radioactivity of the precursor pool or from actual differences in the rates of nucleic acid synthesis.

Fuhrman et al. (1986) conclude that [^{3}H]adenine cannot be used to measure total microbial production either in the epipelagic or mesopelagic zone of the ocean. They reach this conclusion on the basis of two experiments performed during spring bloom conditions in the Choptank River and Long Island Sound. In addition to questions concerning the validity of using [^{3}H]adenine to measure microbial DNA synthesis, they also conclude that our extrapolations to carbon production, based on a C:DNA ratio of 50, overestimate actual rates of carbon production by an order of magnitude. We cannot accept their conclusions regarding the ability of [^{3}H]adenine to measure total microbial production in the oligotrophic oceans for three reasons: their experiments did not follow the protocol which we have carefully developed over the past several years; their data were not derived from oceanic environments; and our many experiments in both the epipelagic and the mesopelagic zones of the ocean indicate that the adenine method does measure total microbial production. We also do not agree with their conclusion that growing marine bacteria have C:DNA ratios approximately equal to 5.

We will respond to the paper of Fuhrman et al. (1986) with a review of our data which, we believe, justifies the use of the [^{3}H]adenine protocol in marine environments. In developing our case, we will refer to previously published results, as well as extensive unpublished data collected over the past 5 years during the VERTEX research program in experiments with samples collected at depths ranging from 0 to 2,000 m in coastal upwelling and oligotro-

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phic oceanic ecosystems. We will also present the results of two experiments at Palmer Station, Antarctica, during the annual spring bloom.

Fuhrman et al. (1986) question the validity of the [^{3}H]adenine protocol because they believe that algae cannot compete successfully with bacteria for nanomolar concentrations of exogenous adenine. They suggest that our procedure for measuring the specific radioactivity (nCi pmol^{-1}) of the nucleic acid precursor pool is inappropriate since the numerator of the equation is determined only by the "[^{3}H]adenine responsive" microorganisms while the denominator includes all viable microbial cells. As evidence to support their view they present quantitative data from two size-fractionation experiments of [^{3}H]adenine incorporation and discuss qualitative results from [^{3}H]adenine microautoradiography on these samples. However, autoradiography is based on a threshold activity principle, so that the results of these latter experiments can only be used to evaluate the relative radioactivity of individual groups of microorganisms (i.e. labeled vs. unlabeled) and cannot be used as rigorous proof that a particular subpopulation is nonresponsive to a specific substrate. In fact, similar microautoradiographic studies (Moriarty and Pollard 1982) showed that [^{3}H]adenine is assimilated by diatoms and cyanobacteria even when added at nanomolar concentrations.

To begin with, the [^{3}H]adenine uptake protocol used by Fuhrman et al. bears little, if any, resemblance to the procedure that we routinely follow. The first major difference is that we do time-course experiments whenever possible (as was possible for the surface water samples analyzed by Fuhrman et al.) and then calculate nucleic acid synthesis rates by dividing the label incorporated into nucleic acid by the integral of the precursor-specific radioactivity (Karl and Winn 1984; Winn and Karl 1984). We also recommend incubation periods of 6–8 h in order to eliminate potential problems caused by isotopic disequilibrium and kinetic compartmentalization (Karl et al. 1981*a,b*; Karl and Winn 1984; Winn and Karl 1984). Unfortunately, no ATP-pool-specific radioactivities were reported, and apparently none measured, by Fuhrman et al. Measurement of the specific radioactivity of the ATP pool is central both to our protocol and to the criticisms that they raise. Without this information, it is impossible to compare the data they present with any of our previous laboratory or field measurements. We find it hard to understand how such sweeping conclusions can be made about the [^{3}H]adenine method without measuring its most important parameter—the specific radioactivity of the ATP pool.

At least two interpretations of the data presented by Fuhrman et al. (1986) are possible. First, it is possible to interpret these data, as they do, as evidence that algae in the Choptank River and Long Island Sound do not assimilate adenine, at least not under spring bloom conditions. In our view, however, it is at least as likely that both algae and bacteria assimilate adenine, but that in these environments algae simply synthesize nucleic acids more slowly than bacteria per unit biomass. Time-course uptake experiments and specific radioactivity measurements would have distinguished between these two alternate hypotheses.

Our experiments in various aquatic environments over the past several years have led us to conclude that adenine is assimilated by both algae and bacteria. Perhaps the simplest method of evaluating whether bacteria and algae assimilate [^{3}H]adenine is to compare the magnitude of ATP-pool-specific radioactivities measured in the laboratory and in the field. Since we routinely use the same initial concentration of adenine (0.1 μCi ml^{-1} seawater) this comparison should provide at least a qualitative evaluation of the assimilative capacity of different microbial assemblages. Our previously published data show ATP-pool-specific activities from 0.25 to 2.5 nCi pmol^{-1} in cultures of bacteria and algae, with specific activities of similar magnitude in field-collected samples. Obviously, if a large proportion of the microbial population in nature (i.e. phytoplankton) does not assimilate [^{3}H]adenine, then the specific radioactivities of the total microbial ATP pool measured in nature should be consistently lower than those measured in laboratory experiments. Furthermore, our work with

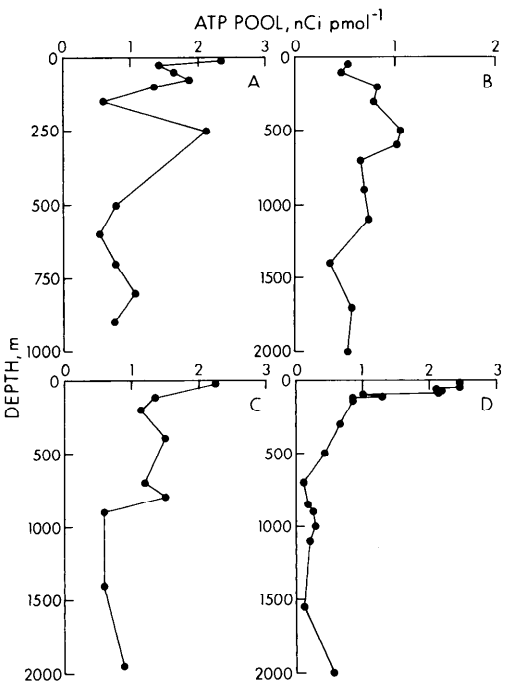


Fig. 1. ATP-pool-specific radioactivity vs. water depth for samples collected at four geographically distinct hydrostations in clean Niskin bottles and incubated in situ in clean 2.5-liter polycarbonate bottles for 6–8 h following the addition of 100 μCi [2,^{3}H]adenine liter^{-1} (15.5 Ci mmol^{-1}; New England Nuclear Corp.). Station locations: A—18°44′N, 156°50′W; B—35°45′N, 123°45′W; C—15°41′N, 107°29′W; D—28°N, 155°W.

phytoplankton cultures also supports our conclusion that both algae and bacteria can assimilate adenine, even at nanomolar concentrations: all 15 strains of phytoplankton that we investigated could assimilate adenine (Karl and Winn 1984), and accurate rates of nucleic acid synthesis can be derived from experiments with cultures (Winn and Karl 1984).

Fuhrman et al. correctly point out that the "important question" is not whether algae can assimilate [^{3}H]adenine, but whether they can compete effectively with heterotrophic bacteria under field conditions. If algae cannot compete with bacteria for the uptake of adenine in field experiments, then one might predict that algae would assimilate [^{3}H]adenine only at relatively high concentrations, as has been shown for the uptake of some radiolabeled organic compounds (e.g. glucose: Wright and Hobbie 1966). We have measured the ATP-pool-specific radioactivities and calculated rates of RNA and DNA synthesis for natural samples in the presence of [^{3}H]adenine additions ranging over two orders of magnitude (Karl 1982; Craven and Karl 1984). If algae did not compete successfully with heterotrophic bacteria at the low concentrations (≤6.67 nM) but did at higher concentrations (>300 nM) then we would have seen a systematic decrease in the calculated rates of total microbial RNA and DNA synthesis. Instead we found rates that were statistically indistinguishable, irrespective of the concentration of [^{3}H]adenine added (Karl 1982; Craven and Karl 1984). Our data support the assumption that algae compete successfully with heterotrophic bacteria for nanomolar concentrations of adenine.

The uptake of [^{3}H]adenine can also be evaluated indirectly by examining the magnitude of the mean ATP-pool-specific radioactivity in oceanic depth profiles. The maximum specific radioactivity of the total microbial community ATP pool measured in our field experiments is determined by the amount of isotope added relative to the ambient concentration of structurally related compounds (i.e. the external specific radioactivity of the introduced tracer), the relative proportion of salvage to de novo synthesis of adenine, and the percentage of the total microbial population that is [^{3}H]adenine responsive. If the amount of [^{3}H]adenine is kept constant (as it is in most of our field experiments), then the measured specific radioactivity will be a function of the percentage of [^{3}H]adenine-responsive cells and of the ambient pool of adeninelike compounds, most likely in that order of importance. Although we do not know of any attempts to measure the total concentration of adenine-containing compounds in seawater, we do know that the concentration of dissolved ATP (which is likely to represent a large proportion of the "total adenine" pool) is greater in surface waters than in the mesopelagic zone, by a factor of about two (Azam and Hodson 1977).

If algae cannot compete with heterotrophic bacteria for the uptake of [^{3}H]adenine, then one might expect a systematic increase in the specific radioactivity of the total mi-

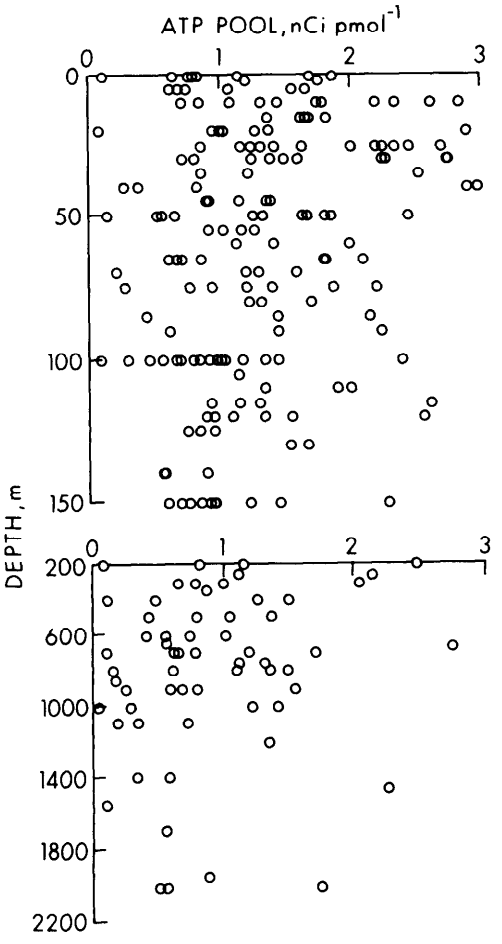


Fig. 2. Plot of ATP-pool-specific radioactivity vs. water depth for 234 individual water samples collected during the VERTEX research program. Not shown are data for 14 samples where the measured ATP pool was >3 nCi pmol^{-1}. Of these 14 samples, 7 were from surface waters (<150 m) and 7 were from depths >200 m. The correlation coefficient, $r^2 = 0.037$ ($n = 248$), indicates that there is no statistical relationship between ATP-pool-specific radioactivity and water depth.

crobial community ATP pool with increasing water depth. Figure 1 shows four individual oceanic profiles of the ATP pool (nCi pmol^{-1}). It is evident from these data that no such trend exists; if anything, the specific radioactivity of the total microbial ATP pool decreases with depth. A comprehensive summary of all VERTEX data (Fig. 2) shows no correlation at all between ATP-pool-specific radioactivity and water depth. Indeed, the variability observed in the measured value of the equilibrated ATP pools is precisely the reason why one must measure the precursor pool labeling in order to calculate accurate rates of nucleic acid synthesis.

Table 1. The relationship between cell size and the assimilation of [^{3}H]adenine for samples collected in the North Pacific Ocean.

Sample depth (m)	Size fraction*	ATP (ng liter^{-1})	ATP pool (nCi pmol^{-1})	[^{3}H]adenine incorporation (nCi liter^{-1})	
				RNA	DNA
20	nano	18.42	1.47	1,249	130
	pico	25.52	1.58	724	48
125	nano	7.66	0.70	680	47
	pico	13.63	0.76	382	29

* Nano is operationally defined as the 2–20-μm size fraction and pico as the fraction <2 μm. Samples were collected at 0500 on 30 June 1984 during VERTEX 5 (33°N, 140°W), prescreened through 20-μm Nitex mesh before size separation by reverse flow filtration (Pomeroy and Johannes 1968) with a 2-μm Nuclepore membrane. The samples were incubated in situ for about 9 h after addition of 100 μCi [^{3}H]adenine liter^{-1}. All samples were processed with GF/F filters (Karl et al. 1981*a*).

Experiments designed to evaluate the effects of inorganic nutrient addition on the ATP-pool-specific radioactivity of natural microbial assemblages (Karl et al. 1981*b*) also support the conclusion that both bacteria and unicellular algae assimilate [^{3}H]adenine in surface seawaters. In these selective enrichment experiments, the addition of inorganic nutrients resulted in a biomass increase of 52% (presumably algae) over a control, but the autotrophic biomass increase had no measurable effect on the specific radioactivity of the ATP pool. If algae did not assimilate [^{3}H]adenine, the specific radioactivity of the algal-enriched sample should have been lower than that of the control sample.

Some of the evidence presented above is indirect, since one cannot hope to assess the magnitude of phytoplankton assimilation of [^{3}H]adenine by simply examining data on total microbial ATP-pool-specific radioactivity with depth or determining the magnitude of changes in specific activity following selected nutrient enrichments. As an independent evaluation of the assumption that the [^{3}H]adenine protocol measures total microbial production in seawater, Karl and Bossard (1985*b*) compared rates of nucleic acid synthesis with $^{32}PO_4^{3-}$ and [^{3}H]adenine. Since PO_4^{3-} is unquestionably assimilated by both bacteria and algae, a comparison of the results can be used to test the hypothesis that [^{3}H]adenine measures

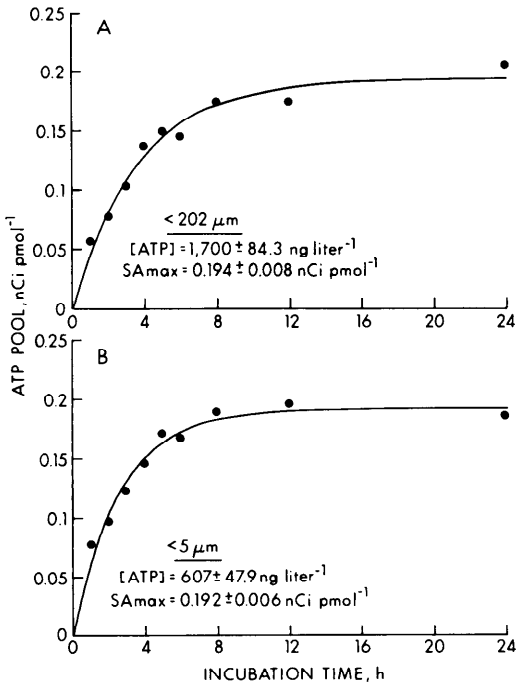


Fig. 3. Time-course of labeling for the ATP pools of microorganisms in two different size fractions following the addition of 100 μCi [^{3}H]adenine liter^{-1}. The samples were collected from a station near the Joubin Islands, Antarctica (64°46'S, 64°22'W), and were transported to Palmer Station where they were incubated under in situ light and temperature conditions. SA_{max} represents the specific radioactivity of the ATP pool at isotopic equilibrium as determined by a nonlinear least-squares analysis of the experimental data fit to an exponential equation (Karl and Bossard 1985*a*,*b*).

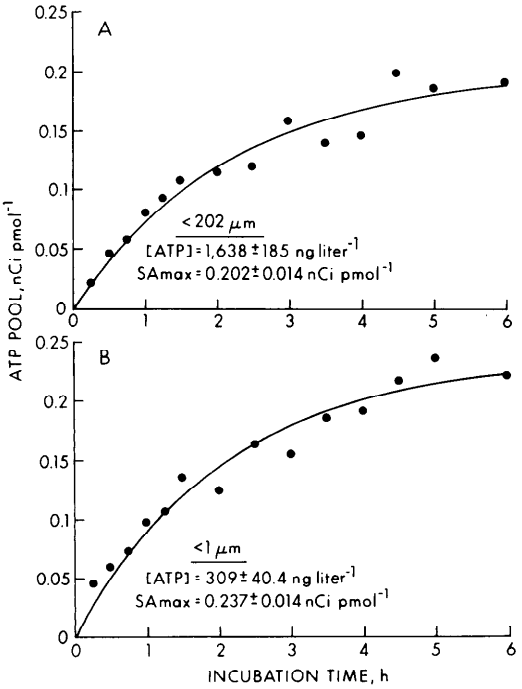


Fig. 4. As Fig. 3.

total microbial production. The rates of nucleic acid synthesis calculated from these two independent precursors of nucleic acid were statistically indistinguishable, again supporting the conclusion that adenine is assimilated by both algae and bacteria and that our adenine protocol measures total microbial production.

We have also done size-fractionation experiments to evaluate bacterial and algal assimilation of [^{3}H]adenine under field conditions (Table 1). There is no evidence that the ATP pool of the nanoplankton fraction (which contains larger phytoplankton and most protozoans) had a substantially lower specific radioactivity than the bacteria-enriched picoplankton fraction at either depth shown. The average ratio of nanoplankton: picoplankton ATP-pool-specific radioactivity measured for the entire cruise was 0.96 ± 0.39 ($n = 14$ experiments), again indicating that the assimilation of [^{3}H]adenine by all microorganisms <20 μm is about in proportion to their biomass (i.e. ATP) contribution. These experiments were identical to those reported by Fuhrman et al. (1986) except for two important points: we measured precursor-specific activities in addition to incorporation, and our incubations were for 9 h.

We have also measured the ATP-pool-labeling characteristics of size-fractionated microbial communities sampled from the Joubin Islands, Antarctica, during an intensive spring bloom (Figs. 3–4). Our first experiment was a 24-h time-course of ATP pool labeling comparing the kinetics of the <202- and the <5-μm size fractions. The <5-μm fraction contained 36% of the total ATP and presumably all of the bacteria, with some phytoplankton. The <202-μm fraction was dominated by large phytoplankton cells, characteristic of the spring bloom in this region.

Two features were evident that bear di-

rectly on the conclusions of Fuhrman et al. First, the mean ATP pool does not achieve isotopic equilibrium until about 8–10 h into the incubation period. Clearly, attempts to compare size-fractionated incorporation of [^{3}H]adenine into nucleic acids without concomitant measurements of the ATP-pool-specific radioactivity are difficult, if not impossible, to interpret. The size-fractionated uptake results of Fuhrman et al. could easily be explained by a transient isotopic disequilibrium between the two subpopulations studied. This potential source of error is especially relevant to their experiments because their incubations were relatively short (i.e. 0.5–1.5 h). The second relevant aspect of our ATP-pool-labeling experiment is the observation that the maximum specific radioactivity (SA_{max}, i.e. the value achieved at isotopic equilibrium) is indistinguishable between the two different size classes of microorganisms (Figs. 3 and 4). Once again these data support our assumption of an equivalent per capita uptake of [^{3}H]adenine by all microorganisms, irrespective of size.

In a second size-fractionation experiment during bloom conditions in Antarctic waters we assessed the kinetics of short term labeling in more detail and compared a <1-μm size fraction with the total (<202 μm) microbial population (Fig. 4). The picoplankton populations had labeling kinetics similar to those for the total microbial community. This implies that they also had similar specific growth rates (Karl and Bossard 1985*a*,*b*). Although the <1-μm fraction contained $<20\%$ of the total ATP, the magnitude of ATP pool labeling (as determined by SA_{max}) was not substantially different from that of the total microbial community. If the phytoplankton in Antarctic waters could not assimilate appreciable amounts of [^{3}H]adenine, then the measured SA_{max} of the <1-μm fraction would have exceeded that of the <202-μm fraction by an order of magnitude since the algal biomass of the <1-μm fraction was $<10\%$ of that in the <202-μm fraction (O. Holm-Hansen pers. comm.).

From the considerable evidence presented above we conclude that adenine is assimilated by both algae and bacteria and that our adenine protocol measures total microbial production. It is conceivable that, for some as yet unknown reason, the dominant algae in the samples analyzed by Fuhrman et al. did not assimilate [^{3}H]adenine. However, we once again stress that without direct measurements of ATP-pool-specific radioactivity in the various size fractions, one cannot comment on the capacity for [^{3}H]adenine assimilation.

In addition to suggesting that our adenine protocol cannot measure total microbial production in surface waters, Fuhrman et al. also conclude that our estimates of mesopelagic zone production are probably in error because our measured data on ATP-pool-specific radioactivity are underestimates of the subpopulation that is responsive to [^{3}H]adenine, due to the presence of sinking algae and resident protozoan populations. Frankly, we are puzzled about how these conclusions can be derived from experiments done in the Choptank River and the surface waters of Long Island Sound. Nevertheless, Fuhrman et al. conclude, without supporting data, that anomalous production estimates in the mesopelagic are due, in part, to sinking algae. We consider the potential contribution of viable algae to the total microbial biomass in the mesopelagic to be negligible (*see below*). Although the presence of protozoan populations in the mesopelagic zone is more difficult to evaluate quantitatively, we know of no data that would suggest that protozoa introduce significant error in our measurements of DNA synthesis. There are relatively few data available to estimate the average ratio of protozoan : bacterial biomass beneath the euphotic zone. Mel'nikov (1977) examined phytoplankton, microzooplankton, and bacteria by direct microscopy at several stations in the eastern Pacific Ocean and found no phyto- and microzooplankton deeper than 200 m. Watson and Hobbie (1979) compiled a summary of estimates of bacterial biomass obtained by two independent methods along with total microbial biomass (bacteria, protozoa, sinking algae, etc.) measured by ATP concentrations. For deep water samples (200–2,000 m) from a station in the South Atlantic Ocean, bacterial biomass was estimated to be $119 \pm 38\%$

Table 2. Measurements of particulate ATP, pigments, and microbial cell numbers for two water samples collected at the Natural Energy Laboratory, Keahole Point, Hawaii. Water was collected at the Hawaii OTEC facility through 0.29-m-diam PVC pipes extending from the shore to water depths of 30 and 600 m off the point. The transit time of water in the warm (26°C) and cold (8°C) water pipes was 14 and 45 min. The increase in deep water temperature was <1°C during transit.

Parameter measured	Water depth (m)	
	30	600
ATP concn (ng liter^{-1})	16.5±3.7	1.28±0.32
ATP-pool-specific radioactivity (nCi pmol^{-1})	1.70*	0.55*
Bacteria × 10^8 (cells liter^{-1})	3.40±0.81	0.64±0.22
Autofluorescence × 10^6 (cells liter^{-1})	3.30±3.90	0.011±0.012
Chl *a* (ng liter^{-1})	38.0±1.8	0.60±0.02
Pheopigments (ng liter^{-1})	30.0±2.2	4.80±0.31
Flagellates × 10^5 (cells liter^{-1})	2.97±1.17	0.20±0.49

* Measured after 6 h of incubation at in situ temperature following addition of [^{3}H]adenine (100 μCi liter^{-1}).

of the total microbial biomass by the lipopolysaccharide method and 85±39% by direct microscopy.

Our own comparative data from an oligotrophic ocean site off Keahole Point, Hawaii, clearly indicate that the mesopelagic zone sample (600 m) is enriched in bacterial cells relative to either Chl *a* or flagellate numbers (Table 2). The percentages of total microbial biomass, bacterial cells, autofluorescent cells, Chl *a*, and flagellate cells at 600 m of those at 30 m were 7.8, 18.8, 0.3, 1.6, and 6.7%. There was no evidence that the bacteria collected at 600 m were any smaller than those from 30 m. Therefore, we find, on the basis of data currently available, no compelling evidence to support the criticisms presented by Fuhrman et al. regarding either the importance of sinking phytoplankton or the presence of substantial populations of microzooplankton in the mesopelagic zone of the ocean.

Table 3. Incorporation of [^{3}H]adenine into microbial communities collected from the North Pacific Ocean. A comparison of Whatman GF/F and Millipore GS filters.

Sample depth (m)	ATP (ng liter^{-1})	[^{3}H]adenine incorporation* (nCi liter^{-1})		
		GF/F	GS	GF/F:GS
10	52.1†	6,600	6,570	
		5,667	6,941	
		$\bar{x}$ = 6,134	6,756	0.91
50	72.1	7,155	6,843	
		7,021	6,740	
		$\bar{x}$ = 7,088	6,792	1.04
75	45.5	2,190	2,380	
		2,331	2,406	
		$\bar{x}$ = 2,261	2,393	0.94
100	38.6	1,149	1,185	
		1,328	1,238	
		$\bar{x}$ = 1,239	1,212	1.02

* Samples were collected at 0600 in August 1984 during VERTEX 4 (28°N, 155°W) and incubated in situ for about 10 h. The data reported are values for the incorporation of [^{3}H]adenine into the [RNA + DNA] fraction by the methods of Karl (1981).
† ATP concentrations were measured with GF/F filters.

In discussing our experimental methods and results, Fuhrman et al. criticize our choice of filters (Reeve Angel 984-H and Whatman GF/F) which, in their opinion, allow most bacteria to pass through. If significant, this would lead to an underestimation of total microbial DNA synthesis and not a "large overestimate of production" as they claim. For our samples from Keahole Point, the percentage of total bacterial cells that passed a Whatman GF/F filter but was captured on a 0.2-μm Nuclepore membrane was 7% for the 30-m and 6.9% for the 600-m sample. Since these cells were, by definition, smaller than most bacteria in the samples, their collective contribution to total microbial biomass was probably negligible (≤5%). During the VERTEX 4 cruise in the oligotrophic North Pacific Ocean, we also compared the retention of Whatman GF/F filters with 0.22-μm porosity Millipore GS membrane filters and found no evidence that we were missing any microorganisms responsive to [^{3}H]adenine (Table 3). These results are consistent with data of Cuhel et al. (1983) for radiolabeled organic compounds and Orrett and Karl (1987) for the uptake of $^{32}PO_4^{3-}$.

Fuhrman et al. (1986) also question the use of [^{3}H]adenine for experiments with in situ sediment traps. They base their concern on data of Silver et al. (1984) which suggest that ciliates are numerically abundant on sinking detritus. However, recent quantitative analyses of the population structure of particle-associated microorganisms indicate that bacteria comprise $\geq 75\%$ of the total microbial biomass, ciliates $<1\%$, and flagellates $<25\%$ (Taylor et al. 1986).

Another major conclusion of Fuhrman et al. relates not to the measurement of DNA synthesis but to the extrapolation of this value to an estimate of carbon production. Because most microbial ecologists use carbon as the basic unit of living biomass, production, and flux among trophic levels, it is desirable to use laboratory or field-derived conversion factors to translate the in situ measurement of DNA synthesis into a more universal ecological metric. As methods evolve and become more refined, the extrapolation factors also change. At present we use a C:DNA ratio of 50 and a DNA:ATP ratio of 5 to extrapolate our DNA synthesis rates (expressed as pg DNA liter^{-1} h^{-1}) to estimates of carbon production and specific growth rate (Winn and Karl 1983; Karl and Winn 1984; Laws et al. 1984); these factors are based on laboratory data for unicellular algae (Holm-Hansen 1969) and bacteria (Mandelstam and McQuillen 1968; Jeffrey and Paul 1986).

Fuhrman et al. argue that our C:DNA factor is too high for natural populations of marine bacteria and suggest that the ratio is probably closer to 5: implicit is the suggestion that our DNA:ATP ratio is also in error by an order of magnitude. They base their conclusion on two lines of indirect evidence. First, they cite the argument of Fuhrman and Azam (1982) that very small marine bacteria should have a DNA content high relative to carbon, since there is a theoretical minimum genome size for viability. While this is likely to be true, there is little evidence to indicate whether these very small marine bacteria are actually viable. Moreover, Fuhrman et al. believe that we are probably excluding the small bacteria from our analyses due to the use of Whatman GF/F filters rather than a membrane with a 0.2-μm porosity (such as Millipore GS or Nuclepore). Consequently, it is not clear to us why we should adopt a C:DNA factor biased toward the very small bacteria of uncertain viability which are not even sampled by our current procedures.

The second indirect line of evidence cited by Fuhrman et al. to support their suggested C:DNA ratio of 5 is the data of Fuhrman and Azam (1982) and Paul and Carlson (1984) for the DNA content of submicron particulate matter in seawater. To estimate the DNA content of marine bacteria, Fuhrman and Azam (1982) filtered seawater through 0.6-μm Nuclepore filters (which they acknowledged selected against certain bacteria) and then concentrated a portion of the filtrate onto 0.2-μm filters for direct counts of bacteria. A second portion of the 0.6-μm filtrate was concentrated onto a 0.45-μm Gelman filter for fluorometric determination of DNA. These data (their table 4, p. 117) are expressed as DNA/cell, not DNA/cell carbon. Therefore, it seems odd that this paper is cited as evidence for a C:DNA ratio of 5 when, in fact, bacterial carbon was never even measured. Furthermore, they acknowledge the potential presence of detrital DNA which would result in an overestimate of "bacterial DNA." This may be a major uncertainty in the interpretation of data like those of Fuhrman and Azam (1982) since much of the particulate DNA in seawater may be nonreplicating (Winn and Karl 1986).

Paul and Carlson (1984) measured DNA and counted bacteria in the 0.2–1-μm fraction of various seawater samples. The ratio of total (living plus nonliving) particulate organic carbon to total (living plus nonliving) particulate DNA (i.e. the C:DNA ratio) ranged from 2.3 to 17, with a mean value of 3.8. These results are difficult to reconcile with previously published data for growing bacteria. Paul and Carlson (1984) suggest that the relatively high DNA content of bacteria-sized particles is consistent with the hypothesis that many marine bacteria exist under starvation conditions. However, starved, debilitated, or otherwise inactive cells probably do not assimilate [^{3}H]adenine (Karl 1979). Again we must question the validity of selecting a C:DNA ratio biased

toward the subpopulation that is nonresponsive.

One unique advantage of the [^{3}H]adenine method is its ability to measure rates of RNA and DNA synthesis simultaneously (Karl 1981). Under conditions of balanced growth, the ratios of synthetic rate are equivalent to those of concentration of RNA and DNA in the microorganisms that are [^{3}H]adenine responsive. Perhaps the most compelling direct evidence that the C:DNA ratio of marine microorganisms that are [^{3}H]adenine responsive must be substantially >5 is a summary of the RNA:DNA data collected over the past 5 years from various oceanic habitats. In surface waters (<150 m) the mean microbial RNA:DNA ratio was 11.0 (median = 8.6, n = 157) and for samples collected in the mesopelagic zone (150–2,000 m) was 9.3 (median = 7.9, n = 39): thus the microorganisms we typically measure in our field experiments contain about 10 times as much RNA as DNA. These results are consistent with the expected distributions of nucleic acid in growing microorganisms (Rosset et al. 1966; Dennis and Bremer 1974; Brunschede et al. 1977; Dortch et al. 1983). If the mean C:DNA ratio was actually as low as 5, as suggested by Fuhrman et al., and if the RNA:DNA ratio is 10, as indicated by our extensive data summary, then the implied C:RNA ratio would be 0.5. Clearly cells cannot contain twice as much RNA as total carbon, considering that RNA itself is about 35% carbon (by wt) and that other constituents like protein, lipids, and polysaccharides exceed the mass of nucleic acids by a factor of 3–5 (Lehninger 1971; Stouthamer 1973). We therefore must conclude that the C:DNA ratio of 5 cannot be correct when applied to the populations that are [^{3}H]adenine responsive as measured in our field experiments. When direct and comprehensive data are available to indicate that the average C:DNA ratio of microorganisms that are [^{3}H]adenine responsive is significantly different from the currently used factor of 50, we will certainly adopt the new factor.

Finally, we feel that a logical way to evaluate the accuracy of ecological data is to compare results obtained by several independent methods. Whereas quantitative agreement between different techniques does not guarantee the accuracy of the derived estimates, the approach is, nevertheless, useful. During the PRPOOS I experiment, the [^{3}H]adenine method gave results similar to other "standard" methods for estimating plankton production and growth rate (Laws et al. 1984). In two experiments off the leeward coast of Oahu, Hawaii, the rate of total microbial carbon production derived from measurements of DNA synthesis (and an assumed C:DNA ratio of 50) were 129 and 97% (stations C and E) of the ^{14}C estimates of primary production. Clearly, our method of extrapolation does not appear to be overestimating microbial production in these surface seawaters by an order of magnitude. The total microbial carbon production measured at a station in Mamala Bay (station B: Laws et al. 1984) exceeded the rate of photosynthesis by a factor of 4.3, which we interpret to represent a heterotrophic-bacteria-dominated ecosystem. The specific growth rate of the total microbial community extrapolated from measurements of DNA synthesis (with a DNA:ATP ratio of 5) was indistinguishable from those calculated from ^{14}C assimilation into Chl *a*, ^{14}C assimilation/biomass C estimates, ^{14}C assimilation into protein or $^{15}NH_4^+$ incorporation/biomass N (Laws et al. 1984). Furthermore, our data for depth-integrated production of microbial carbon in the euphotic zone of coastal upwelling and oligotrophic ocean habitats, 1.85 and 400 mg C m^{-2} d^{-1} (Karl and Knauer 1984; Winn and Karl 1983), do not differ greatly from other field data (Knauer et al. 1984).

Specific growth rates of the total microbial community estimated by DNA synthesis (with a DNA:ATP ratio of 5) and by turnover rates of the adenine nucleotide pool yield comparable results (Novitsky and Karl 1985; Karl et al. in prep.). A unique aspect of this comparison is the fact that the adenine nucleotide pool turnover method measures only those microorganisms responsive to [^{3}H]adenine and is totally unaffected by the presence of subpopulations that are nonresponsive (Karl and Bossard 1985*a*,*b*). That the two methods yield equivalent estimates of specific growth rate indicates that the majority of viable microorganisms assimilate

[^{3}H]adenine under in situ conditions and that the DNA:ATP factor of 5 must represent the microorganisms present in these environmental samples.

Accurate measurements of microbial biomass, metabolic activity, productivity, and specific growth rate are essential for testing hypotheses in microbiological oceanography. Progress in our discipline is limited by methods. Ideally, each new method should be evaluated and thoroughly calibrated before it is used routinely in field studies, but in practice most methods are introduced prematurely and then evolve with extensive field testing and thorough scientific review. This evolution and refinement is clearly documented for many of our "standard" methods (e.g. the ^{14}C method for measuring primary productivity, the use of radiolabeled organic compounds to measure heterotrophic activity, the use of direct microscopic counts, and measurements of bacteria).

It is much easier to criticize an existing method than to conceive, develop, and evaluate a novel experimental approach. The use of [^{3}H]adenine to estimate rates of microbial nucleic acid synthesis in aquatic ecosystems is a relatively new and potentially useful approach for the study of marine microbial ecology. The controversy regarding the proper interpretation of the derived field data serves to focus attention on both the method and the ecological hypotheses under investigation. To a certain point this is healthy and exerts a positive influence, but beyond that point further criticism in the absence of comprehensive data from which to argue is counterproductive and, in our opinion, tends to stifle advances in our understanding of microbial processes in nature.

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