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# Potential effects of isotopic dilution on apparent respiration in $^{14}C$ heterotrophy experiments

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ABSTRACT: Patterns of [U-$^{14}C$]glucose uptake and $^{14}CO_2$ production by *Vibrio natriegens* in model experiments indicated that the percent respiration of a substrate can be underestimated when using radiotracers if effects of isotopic dilution are not considered. Percent respiration of radiolabelled glucose differed in broth cultures of *V. natriegens* that were similar except for the timing of addition to the cultures of tracer [U-$^{14}C$]glucose. Percent respiration was high (~ 45 to 50 %) in cultures to which tracer glucose was added in early log phase but was initially low and increased over time in cultures to which tracer glucose was added in mid-log or stationary growth phases. The observed patterns of [U-$^{14}C$]glucose respiration were probably due to changes in the specific activity of the immediate intracellular precursors of $^{14}CO_2$ in accordance with standard models of the phenomenon of isotopic dilution. One implication of these results is that percent respiration in field studies of bacterial heterotrophy may have been underestimated, since in many cases isotopic dilution has not been taken into consideration. Previously published studies of substrate uptake and transformation in the water column have been re-interpreted in view of these findings.

## INTRODUCTION

Recent discussions of carbon flow and trophic dynamics suggest that the role of bacteria in pelagic marine ecosystems is uncertain (Williams, 1983). It is well known that bacteria are active in the uptake and transformation of many organic solutes and algal exudates (Williams, 1970; Smith and Wiebe, 1976; Williams 1976; Williams and Yentsch, 1976; Billen et al., 1980; Iturriaga and Zolnay, 1981; Joint and Morris, 1982). However, it is not clear whether bacteria simply mineralize organic substrates or whether they represent a significant source of secondary production and therefore an important component of the marine food web (Pomeroy, 1974; Newell et al., 1982; Williams, 1983). Bacteria have been viewed traditionally as mineralizers (e.g. Rheinheimer, 1980) and there is certainly evidence to support this notion. Williams (1973, 1981) and Sorokin (1978) have reported relatively high rates of $CO_2$ production by bacterioplankton and Williams (1983), in a summary of the literature, indicates that bacteria are probably the major respiratory agents in marine systems. In addition, Lucas et al. (1981), Newell et al. (1981, 1982), and Stuart et al. (1981, 1982) suggest that bacterial biomass production is small relative to respiration in a kelp system. Robinson et al. (1982) have also shown relatively high percentages of bacterial respiration of kelp. In contrast, numerous other studies have indicated that in many environments diverse organic substrates are incorporated into biomass at relatively high efficiencies. Hobbie and Crawford (1969), Williams (1970), Gocke (1976), Smith and Wiebe (1976); Hanson and Wiebe (1977), Iturriaga and Hoppe (1977), Meyer-Reil et al. (1978), Keller et al. (1982) and others have reported that only about 30 % of added [$^{14}C$] labelled substrates are respired. Even lower levels of respiration (< 15 %) have been reported for marine and freshwater sediments (e.g. Meyer-Reil et al., 1978; Toerien and Cavari, 1982). These apparently high assimilation efficiencies suggest that bacteria could contribute significantly to secondary production. Clearly, the extent of biomass production *versus* respiration by bacteria must be established in order to understand mass flow in pelagic marine systems.

The application of radiotracer techniques has pro-

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vided a powerful tool for examining bacterial metabolism. The introduction of uptake studies by Parsons and Strickland (1962) and of kinetic analyses by Wright and Hobbie (1964) and Hobbie and Crawford (1969) have given marine ecologists important insights into the phenomena of uptake, incorporation and respiration. However, some questions have been raised about the use of radiotracers and about the interpretation of tracer data (Smith and Wiebe, 1976; Hanson and Wiebe, 1977; King and Klug, 1982).

In measurements of bacterial heterotrophy using $^{14}$C-organic substrates, the evolved $^{14}CO_2$ has generally been assumed to correspond directly with stable $CO_2$ formation, in spite of the fact that few studies have addressed the validity of this assumption. Though $^{14}CO_2$ undoubtedly arises from respiration, all sources of intracellular $^{14}CO_2$ must be in isotopic equilibrium with the added [$^{14}$C] labelled substrate before $^{14}CO_2$ production can be used to determine the mass flow of total substrate catabolism. Alternatively, $^{14}CO_2$ can be used to establish percent respiration if the specific activities of the immediate precursors of $^{14}CO_2$ are known. Without specifying precursor-product relations or otherwise assuring isotopic equilibrium, $^{14}CO_2$ production cannot be used to calculate a true percent respiration for the substrate of interest.

We report a study that illustrates the need to account for the extent of isotopic equilibrium in heterotrophy experiments. Time course assays of glucose uptake and respiration in cultures of a marine bacterium, *Vibrio natriegens,* indicate that respiration can be significantly underestimated even in rapidly growing cultures if the assay system does not attain or approach isotopic equilibrium. A comparison of the patterns observed in our model experiments with previously reported field data suggest that the respiration of marine and freshwater bacterioplankton may have been underestimated.

## METHODS

The effects of isotopic dilution on patterns of [U-$^{14}$C]glucose uptake and respiration were examined using cultures of *Vibrio natriegens* grown in a medium containing: glucose, 2.0 g; tryptone, 5.0 g; NaCl, 23.4 g; KCl, 0.76 g; $MgSO_4 \cdot 7H_2O$, 10.96 g; deionized water, 1000 ml; pH was adjusted to 7.0 with 0.1 M phosphate buffer. All chemicals were of reagent grade; tryptone was obtained from Difco Laboratories, Inc. (Detroit, MI). *V. natriegens* was grown in 500 ml Erlenmeyer flasks sealed with rubber stoppers. Each stopper was fitted with a port for sampling the flask contents, a gas inlet line to which an air stone was attached, and a gas outlet line. Replicate flasks containing 250 ml of the above medium were inoculated with 50 ml of a culture of logarithmically growing *V. natriegens.* After inoculation, $CO_2$-free air was bubbled through the sealed flasks to aerate and mix the cultures. Air flow was approximately 30 ml $min^{-1}$. The air stream from the gas outlet of each flask was channelled into 2 scintillation vials in series each of which contained 7 ml of 1 M KOH to trap $CO_2$ produced during growth. Samples of the flask contents were removed at intervals to assay for absorbance and cell number. Absorbance of the growth medium at 550 nm was measured using an Aminco spectrophotometer; cell numbers were determined by phase microscopy with a Leitz microscope and a Petroff-Hauser counting chamber. Changes in absorbance with time were used as an indication of the growth state of the cultures during the course of the experiments. Changes in cell number verified the patterns in absorbance.

Glucose uptake and respiration were measured by adding ~ 185 kBq [U-$^{14}$C]glucose (11.1 GBq/mmol-Amersham Searle) to each of the growth flasks. Growth within and incubation conditions of each of the flasks were identical but the [U-$^{14}$C]glucose was added at different times – at either the early log (EL), mid-log (ML) or early stationary (ST) phase of growth. Glucose concentrations were not significantly altered by the addition of [U-$^{14}$C]glucose at any time point because of the high initial glucose concentration in the medium (~ 10 mM). Subsequent to the addition of radiolabelled glucose, samples were removed to determine the activities of $^{14}$C particulate matter (cells), dissolved [U-$^{14}$C]glucose, dissolved $^{14}$C-glucose metabolites, and dissolved $^{14}$C-inorganic carbon ($H^{14}CO_3^-$). Of the flask contents, 2.0 ml portions were filtered through 0.22 µm Millipore filters. After washing with 2.0 ml of cold medium, the filters were dissolved in 10 ml of Scintiverse (Fisher Scientific, Inc.) for radioassay. The filtrate was collected and washed through a Dowex AG50x8 (100/200 mesh, $Cl^-$ form) anion exchange column as described by King and Klug (1982) to separate unreacted [U-$^{14}$C]glucose from $^{14}$C-glucose metabolites that may have been excreted during growth. Dissolved $H^{14}CO_3^-$ was determined by injecting 1.0 ml of an unfiltered sample of the flask contents in to a sealed 30 ml serum bottle containing 1 ml of 1 N $H_2SO_4$. Subsequently, 0.2 ml of phenethylamine was injected into a filter cup (Kontes Inc.) suspended within the serum bottle as described by Hobbie and Crawford (1969). After 48 h, the filter cup was placed in a scintillation vial containing Scintiverse for radioassay. $^{14}CO_2$ in the gas phase of the flasks was collected in the KOH traps previously described. Total respired $^{14}CO_2$ was calculated as the sum of $H^{14}CO_3^-$ and gas phase $^{14}CO_2$. Each of the above parameters (absorbance, cell numbers, $^{14}$C-particulate matter, etc.)

were determined in duplicate; good agreement was noted among replicates. Trapping efficiencies of both the serum bottles and KOH traps were > 90 %. Radioassays were performed with a Beckman LS7500 liquid scintillation counter; quench corrections were made using internal standards.

Glucose concentrations in the growth media were determined using triplicate samples for each interval assayed. Concentrations were estimated using an enzymatic technique resulting in the formation of $NADH+H^+$. Absorbance of $NADH+H^+$ at 340 nm was measured using a Spectronic 2000 (Bausch and Lomb). Reagents were obtained in kit form from Sigma Chemical Co.

## RESULTS

The potential effects of isotopic dilution on glucose respiration were examined by determining $^{14}C$ uptake and $^{14}CO_2$ production in broth cultures of *Vibrio natriegens* that were incubated with tracer [U-$^{14}$C]glucose for various periods of time. *V. natriegens* growth in each of 3 treatments was similar (Fig. 1), with generation times of approximately 20 min. Cell numbers reached a maximum of approximately $2 \times 10^{10}$ ml$^{-1}$ in all cultures, and total glucose concentrations decreased from 10 mM initially to < 8.5 mM after a 9 h incubation. The similarity of growth and cell number among treatments (Fig. 1) and the addition of [U-$^{14}$C]glucose at tracer levels (added glucose < 0.001% of the total glucose) allowed a comparison of [U-$^{14}$C]glucose respiration among cultures which differed only to the extent to which cells were in isotopic equilibrium.

Cultures to which [U-$^{14}$C]glucose was added in early log phase growth (EL) were used to establish baseline respiration values. $^{14}CO_2$ production and $^{14}C$ incorporation were measured about 2 h after addition of label to EL flasks. During this period cell numbers increased from approximately $1.4 \times 10^8$ ml$^{-1}$ to $1.0 \times 10^9$ ml$^{-1}$. This 7-fold increase in cell numbers insured sufficient glucose uptake and biomass production for the cells to approach or attain isotopic equilibrium; equilibrium was indicated by the linear uptake of [$^{14}$C]glucose and production of $^{14}CO_2$ during the assay period (Fig. 1 and 2) and by relatively constant apparent respiration values which varied between 45 to 53 % during the assay period (Fig. 2).

Total [U-$^{14}$C]glucose uptake exhibited patterns that were correlated with growth state (Fig. 1, insert). In EL cultures, total uptake per flask showed an initial lag followed by a linear increase during log phase (7226 dpm min$^{-1}$; $r^2 = 0.997$, n = 4) and a decrease during stationary phase. A similar pattern was observed in ML cultures with linear uptake during log phase growth

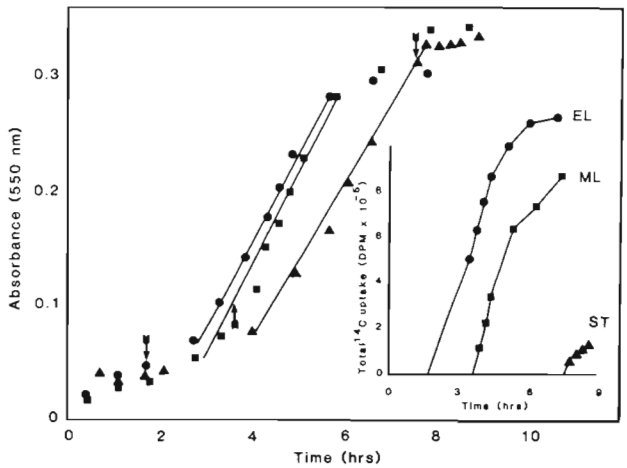


Fig. 1. *Vibrio natriegens*. Growth and glucose uptake in cultures. Growth as absorbance at 550 nm; glucose uptake as total [U-$^{14}$C]glucose dpm (uptake and respiration); ● early log $^{14}C$ additions (EL); ■ mid log $^{14}C$ additions (ML); ▲ stationary phase $^{14}C$ additions. Arrows: time of [U-$^{14}$C]glucose addition

(5942 dpm min$^{-1}$; $r^2 = 0.993$, n = 4) and a decrease during the stationary phase. ST cultures also exhibited linear uptake (1471 dpm min$^{-1}$; $r^2 = 0.991$, n = 4) but the rate of uptake was considerably less than for uptake during the log phase of either EL or ML cultures. Uptake rates were similar in the stationary phases of all 3 cultures (1443, 1929, 1471 dpm min$^{-1}$ in EL, ML, and ST treatments, respectively). Differences in the absolute amount of [U-$^{14}$C]glucose uptake in the EL, ML, and ST cultures at the termination of the incubations were simply due to the differences in the time at which label was added to the cultures.

Respiration of [U-$^{14}$C]glucose also differed among the treatments (Fig. 2). [U-$^{14}$C]glucose respiration was relatively low initially in the ML and ST cultures (32 and 36 %, respectively) and increased over time (58 and 64 %, respectively). During the period in which

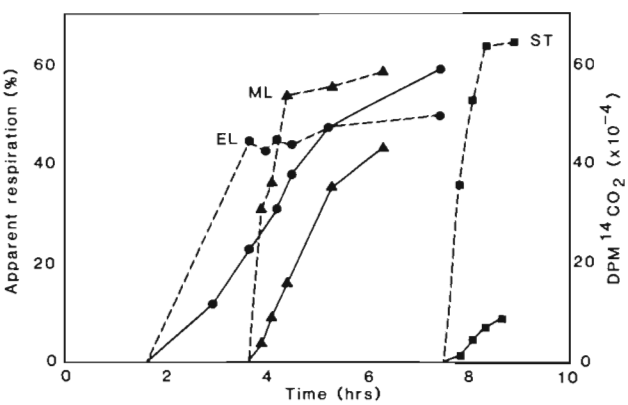


Fig. 2. *Vibrio natriegens*. Apparent respiration (% of total $^{14}C$ uptake) and total $^{14}CO_2$ production (dpm) in cultures. ● EL; ▲ ML; ■ ST. Dashed lines: apparent respiration; solid lines: total $^{14}CO_2$

respiration changed markedly in ML and ST cultures, respiration was relatively stable in the EL cultures (Fig. 2). These differences in [U-$^{14}$C]glucose respiration presumably resulted from greater isotopic dilution in the ML and ST treatments than in the EL treatment since other aspects of growth (such as cell density, as indicated by absorbance in Fig. 1) in the cultures were similar. Thus, we suggest that the [U-$^{14}$C]glucose taken up by cells in the ML and ST treatments was diluted by unlabelled intracellular pools of glucose and glucose metabolites to a greater extent than in the EL cultures. As the specific activity of the intracellular pools of glucose and glucose metabolites increased in ML and ST treatments, the percent respiration of tracer glucose also increased. Differences in tracer glucose respiration among treatments were not due to excretion or leakage of glucose metabolites since extracellular glucose metabolites were only a small percent of total uptake in all treatments (data not shown).

## DISCUSSION

The model experiments with *Vibrio natriegens* presented here demonstrate the potential for error in estimating the percent respiration of $^{14}$C substrates in heterotrophy experiments. The relative extent of respiration can be underestimated if all pools involved in respiratory pathways are not at isotopic equilibrium with added radiotracers. The well-described phenomenon of isotopic dilution, illustrated by the time course of respiration in the EL, ML, and ST cultures, results in distinctly different rates of mass flow [$^{12}$C] and radiotracer flow [$^{14}$C]. Radiotracer flow is of course necessarily less than mass flow until isotopic equilibrium is approached. This and other fundamental aspects of the use of radiotracers to determine the kinetics of mass flow in multi-compartment systems has been described in a number of basic treatises; the reader is referred to Bernhard et al. (1975) and Smith and Horner (1981) for especially lucid and useful versions of various simulation models which are beyond the scope of this paper.

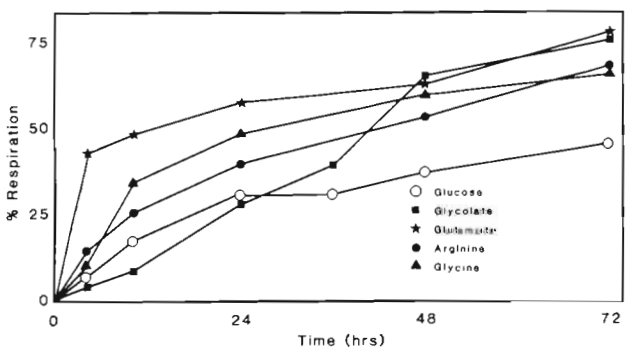


Fig. 3. Apparent respiration as percent of total $^{14}$C uptake from varied substrates added to water-column samples from Cape Lookout Bight, NC, USA. (Data from Iturriaga and Zolnay, 1981)

Although the principles of radiotracer studies have long been developed, relatively few studies of organic matter metabolism in natural systems have employed techniques suitable for a clear interpretation of $^{14}CO_2$ respiration data. Kinetic analyses of radiotracer data have been used to study other aspects of substrate uptake and transformation in a variety of systems (Smith and Wiebe, 1976; Wiebe and Smith, 1977; Lancelot, 1979; Larrson and Hagstrom, 1979; Smith and Horner, 1981; Cuhel et al., 1982; Dring and Jewson, 1982; Kirchman et al., 1982; Li and Harrison, 1982; Wolter, 1982). These studies all indicate the need for time course data or specific activities in order to interpret properly radiotracer data. As an example, Karl et al. (1981) have shown that incorporation of [$^{3}$H]adenine into RNA cannot be used to estimate bacterial biosynthesis unless the specific activity of [$^{3}$H]ATP is known. These authors have demonstrated that the specific activity of exogenous [$^{3}$H]adenine is diluted significantly by intracellular ATP, which is the immediate source of adenine in RNA, and that such dilution could lead to underestimation of biosynthesis.

Since most assays of microbial respiration in marine systems have not made use of time course analyses (typically only a single incubation period is used for determining uptake and respiration), it is difficult to assess the extent to which published values of % respiration may have been affected by isotopic dilution. Data of Iturriaga and Zolnay (1981) suggest that percent respiration may have been underestimated generally. Iturriaga and Zolnay (1981) have reported a time course analysis of the uptake and respiration of a number of substrates, including a variety of sugars and amino acids. In all cases, percent respiration increased from relatively low values initially to higher values after 12 to 24 h (Fig. 3). These increases in percent respiration are analogous to those we observed in cultures of *Vibrio natriegens* and may be explained as the result of isotopic dilution. It is important to note that the specific activity of extracellular substrates may have changed continually in the systems examined by Iturriaga and Zolnay (1981) since [$^{14}$C] substrates were added as a pulse to pools which probably turned over during the course of the experiments. As a result, the bacterioplankton may never have attained isotopic equilibrium though equilibrium may have been approached at the longer incubation points. King and Klug (1982) have shown in similar studies that mass flow of glucose and glucose respiration in lake sediments can be underestimated if isotopic dilution is not considered. Time course analyses of [U-$^{14}$C]glucose

uptake and [$^{14}C$]end product formation indicated that $^{14}CO_2$ production alone was not a reliable indicator of mineralization. King and Klug (1982) also noted that apparent respiration of [U-$^{14}C$]glucose increased from $< 10$ % to $> 60$ % during 6 h of incubation and to $> 80$ % after 24 h. These changes in apparent mineralization were attributed in part to isotopic dilution. Of course, the observed patterns could have arisen from the turnover of incorporated label or [$^{14}C$]macromolecules (Williams, 1983) and not entirely as a function of isotopic dilution. Neither alternative can be excluded at this time. It is clear though that the interpretation of percent respiration values may be somewhat arbitrary since no one point can be chosen as representative of true respiration without a resolution of the state of isotopic equilibrium and macromolecular turnover.

Parsons et al. (1980) have also reported data which suggest that the determination of percent respiration of substrates may be affected by isotopic dilution. Parsons et al. (1980) examined glucose uptake and respiration in pelagic mesocosms. Uptake and respiration were determined using both mass balance and radiotracer techniques. Estimates of percent respiration based on [U-$^{14}C$]glucose data (10 to 46 %) were about one-half of estimates from mass balance (55 to 66 %). The lower values from the $^{14}C$ additions were consistent with the effects of isotopic dilution and underscore the differences between mass flow and radioisotope flow.

Finally, we propose that isotopic dilution can account for the differences in percent respiration observed for planktonic versus sediment samples. Generally, percent respiration is lower in sediments than in the water column (e.g. Wood and Chua, 1973; Meyer-Reil et al., 1978; Toerien and Cavari, 1982). This systematic difference could arise if activity per cell is greater for planktonic than benthic bacteria and if planktonic bacteria approach isotopic equilibrium to a greater extent as a result of greater activity. Data from the only comprehensive comparison of bacterial populations and activities in the water column and sediment (Meyer-Reil et al., 1978) known to us support this hypothesis. Meyer-Reil et al. (1978) determined bacterial numbers and biomass and glucose pool sizes and uptake rates for sandy beach sediments and the associated water column. From these data (see their Table 1), one can calculate that gross, net, and actual glucose uptake rates per cell are considerably higher in planktonic *versus* benthic populations (mean of all actual uptake data = 2.09 fg cell$^{-1}$ h$^{-1}$ and 0.14 fg cell$^{-1}$ h$^{-1}$ for planktonic and benthic bacteria, respectively), in spite of the fact that turnover times were longer in the water column (mean of all samples reported = 46.4 h) than in the sediment (mean = 0.81 h). Although total cell numbers may be misleading since the numbers of cells active in uptake are unknown, the differences between sample types are striking. These differences are consistent with the notion that planktonic bacteria approach isotopic equilibrium more rapidly, thus accounting for at least part of the difference in percent respiration (mean of all samples = 34.4 and 6.7 % for planktonic and benthic samples, respectively; Meyer-Reil et al., 1978).

Clearly, the interpretation of radiotracer data is ambiguous if the state of isotopic equilibrium or precursor-product relationships are not specified. Therefore, we propose the term 'apparent respiration' as a more appropriate descriptor for $^{14}CO_2$ production. This term should be used whenever the state of isotopic equilibrium cannot be specified. Given the current debate over growth efficiencies of bacteria in natural systems (Williams, 1983), it is important to avoid equivocal interpretation of $^{14}CO_2$ data. The term 'apparent respiration' does not connote an absolute value for respiration or incorporation and therefore does not result in misleading conclusions about carbon flow. Even more important perhaps, the use of 'apparent respiration' emphasizes the need for more detailed studies of substrate metabolism by aquatic bacteria.

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