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Title: Production of Biologically-Refractory Dissolved Organic Carbon by Natural Seawater Microbial Populations

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David J. Carlson

Small amounts of carbon from glucose and leucine added at natural concentrations to seawater were biologically transformed to higher molecular weight (MW) dissolved materials which persisted through six months of incubation. These materials were resistant to biological utilization: only 1 to 17% of the higher MW carbon was respired when re-incubated with seawater microbial populations. Over the same time span, 40 to 75% of the monomers were respired. *In situ* transformations of biologically-available carbon may be important mechanisms for the production of refractory dissolved organic carbon in the oceans.

# PRODUCTION OF BIOLOGICALLY-REFRACTORY DISSOLVED ORGANIC CARBON BY NATURAL SEAWATER MICROBIAL POPULATIONS

by

Jennifer Elaine Brophy

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Dean of Oceanography

Redacted for privacy

Dean of Graduate School

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To my parents

Jere and Elaine Brophy

who have inspired in me

an appreciation of the natural world

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conventional methods because of an an apparent heteropolycondensate nature (Degens, 1970; Gagosian and Lee, 1981).

Thus, the marine DOC pool can be described as being composed of two major fractions, each having a very different reactivity with respect to biological and chemical degradation: a smaller portion which is biologically-labile and a larger portion which is refractory in the sense that it is only slowly utilized or removed by biological and chemical processes (Bada and Lee, 1977). Menzel and Ryther (1970) hypothesized that the depth profile of oceanic DOC concentration reflects rapid cycling of newly produced DOC in the euphotic zone leaving small fractions of refractory material as contributions to the overall DOC pool.

Constituents of the labile DOC pool include small compounds leaked, secreted, or excreted by live oceanic organisms or released during feeding processes or senescence. The relatively low concentrations of identified biochemicals in seawater indicate that most biogenic organic carbon is transformed or removed at rates comparable to production (Gagosian and Lee, 1981).

Sources and composition of refractory DOC are essentially unknown. The wide disparity between the large amount of DOC in the oceans and the small amount characterizable in biochemical terms can be reconciled by postulating _in situ_ transformations of recognizable to unrecognizable DOC (Degens, 1970; Gagosian and Lee, 1981) or by emphasizing riverine inputs of complex soil-derived DOC (Mantoura and Woodward, 1983; Hedges et al., 1986). In either process the end products, unrecognizable or complex, are assumed to be biologically-refractory

(Barber, 1968; Menzel, 1970) and thus stable, at least on the thousand-year scales of oceanic circulation.

*In situ* processes forming refractory DOC from labile compounds may include condensation-type reactions with polyphenolic compounds exuded by macroalgae (Sieburth and Jensen, 1969; Carlson et al., 1985a) or with hydrophobic DOC (Carlson et al., 1985a), or biologically-mediated alterations (Iturriaga and Zsolnay, 1983). Only recently have transformation processes been examined at realistic concentrations (Carlson et al., 1985a); it has yet to be shown that products of transformation are more resistant to biological utilization than are the reactants. This investigation is the first to show that a portion of DOC produced during normal oceanic processes is resistant to heterotrophic utilization, and that its production rate is consistent with the apparent age of oceanic DOC.

# MATERIALS AND METHODS

## Experimental design

The procedures followed in this investigation were adapted and expanded from Carlson et al. (1985a) and are outlined in Figure 1. In overview, seawater samples to which small amounts of uniformly- (U-) $^{14}C$-labelled monomeric substrates had been added were incubated for several months in the dark. Transformations of labelled carbon to larger molecular weight (MW) compounds were detected by chromatographic separations using size exclusion gels. Heterotrophic utilization of the transformation products was then determined by measuring their respiration and incorporation by freshly-collected natural microbial populations.

## Collection and preparation of samples and controls

Five samples were collected off the Oregon and Washington coasts in the Pacific Ocean and another was collected from Yaquina Bay, Oregon. Sampling information is summarized in Table 1. Surface (15 m) and deep (2000 m) waters were collected with Niskin bottles in July 1985 (46.83°N; 124.45° and 125.37°W), and Teflon-lined Go-Flo bottles in October 1985 (43.67°N; 125.38°W), on cruises aboard R/V *Wecoma*, and immediately filtered through 62 μm Nitex mesh. At the first station (Sample 01), a portion of the sample was filtered through 1.0 μm Nuclepore polycarbonate filters. In March 1986 surface (0.3 m) water was collected in Nalgene flasks at high tide from Yaquina Bay, OR, and was not filtered.

Buffer and seawater controls were prepared at each station.

Figure 1. Outline of procedures used in these investigations, after Carlson et al. (1985a). Procedures specific to samples or seawater and buffer controls are outlined separately at the top. Once $^{14}C$-labelled monomers had been added, sample and control vials were treated identically. After size-exclusion chromatography, fractions were either counted by liquid scintillation spectrophotometry (LSC) to provide a MW profile or were pooled for incubation with natural microbial populations. All materials which were counted by liquid scintillation are indicated by the notation "--> LSC", identified at the right margin, and described in Appendix 2a.

**PREPARATION**

**SAMPLES** **CONTROLS**

SEAWATER BORATE BUFFER

**FILTER** (0.2 Nuclepore)

<-- ADD: $NaN_3$

ADD: U-$^{14}$C MONOMER --> 

----> LSC .....**initial total $^{14}C$**

**TRANSFORMATION**

------------> **INCUBATE**

(*in situ* temperature, dark, 1-163 days)

REPEAT

**SUBSAMPLE**

----> LSC ... **total recovered $^{14}C$**

**FILTER** --> LSC ............ **$PO^{14}C_{(1)}$**

----> LSC ................ **$DO^{14}C$**

----------------->

**CHROMATOGRAPH**

REPEAT

----> LSC ........... **MW profile**

**UTILIZATION**

**POOL**

----> LSC .... **pooled initial $^{14}C$**

**RE-INCUBATE** with seawater microbes

(some supplemented, one week or three months)

**TRAP $CO_2$** --> LSC ................ **$^{14}CO_2$**

----> LSC ......... **residual $^{14}C$**

**FILTER** --> LSC .............**$PO^{14}C_{(2)}$**

**DISPOSE**

Figure 1.

**Table 1: SAMPLING DATA**

| Sample Number[a] | Date | Location | Depth (m) | Temperature ($^oC$) | Chl-a[b] (ug/l) |
|---|---|---|---|---|---|
| 01 | 28 July 1985 | $46.83^oN$, $124.45^oW$ | 15 | 11.0 | 5.31 |
| 02 | 29 July 1985 | $46.83^oN$, $125.37^oW$ | 15 | 14.8 | 1.86 |
| 03 | 29 July 1985 | $46.83^oN$, $125.37^oW$ | 1925 | 3.0 | ---- |
| 04 | 26 October 1985 | $43.67^oN$, $125.38^oW$ | 15 | 13.0 | 0.94 |
| 05 | 26 October 1985 | $43.67^oN$, $125.38^oW$ | 2000 | 3.8 | ---- |
| 06 | 26 March 1986 | $44.63^oN$, $123.05^oW$ | 0.3 | 12.5 | ---- |

[a] Individual vials were identified by sample number, followed by "G" or "L" for U-$^{14}$C-glucose or U-$^{14}$C-leucine, respectively. If replicate vials were monitored, vial identification numbers included "(a)" or "(b)".

[b] Chlorophyll-a measured at 15 m depth; data from Kokkinakis (1987) for July stations and from Wheeler (unpublished data) for the October station.

Borate buffer (Appendix 1) and freshly-collected seawater were filtered through 0.2 µm Nuclepore filters and poisoned with $NaN_3$ (0.02% final concentration). Controls were then treated identically to the samples.

Samples and controls were transferred to glass containers: scintillation vials for July and October samples, and 250 ml bottles for March samples. Sample and control volumes were 10 ml in July, 15 ml in October, and 50 ml in March. Within an hour of collection, U-$^{14}C$-labelled glucose or leucine (New England Nuclear) which had specific activities of 257.7 and 344 mCi/mmole, respectively, were added to samples and controls. The monomers were added at concentrations representative of seawater glucose and leucine concentrations: approximately 100 nM U-$^{14}C$-glucose and 50 nM U-$^{14}C$-leucine (Keller et al., 1982; Mopper et al., 1980). Actual initial activity ("initial total $^{14}C$") for each vial was determined by triplicate liquid scintillation (LSC) counts of 0.200 ml subsamples. The samples to which $^{14}C$-glucose was added are designated "SW+Glc samples"; those to which $^{14}C$-leucine was added are "SW+Leu samples." Azide-poisoned, 0.2 µm filtered seawater controls are designated "SW+Glc controls" or "SW+Leu controls," and poisoned, filtered borate buffer controls are "Buffer+Glc controls" or "Buffer+Leu controls."

Transformation

Once label was added, vials were incubated in the dark near *in situ* temperatures: in cold rooms at 5°C (deep) and 15°C (surface) for July and October samples, and in the laboratory at 19°C for the March sample. Incubations lasted 70 to 163 days.

During incubations, containers were removed briefly from incuba-

tion conditions, opened, and subsamples removed to determine utilization and transformation of radiolabelled carbon (Figure 1; Appendix 2). Triplicate 0.200 ml subsamples were taken to determine "total recovered $^{14}C$." Next, 1.5 ml subsamples were withdrawn, using glass syringes fitted with silicone tubing, and filtered through 0.2 μm Nuclepore filters. The filters were counted for radiolabelled particulate organic carbon ("$PO^{14}C_{(1)}$"), and triplicate 0.200 ml subsamples were taken of the filtrates to determine radiolabel in dissolved fractions ("$DO^{14}C$"). Additional 0.200 ml portions of the filtrates were chromatographed to determine the MW spectrum of the dissolved radiolabelled components ("MW profile").

MW profiles were generated by size exclusion chromatography on gels with a MW fractionation range of approximately 100-1800. Typical MW profiles of dissolved radiolabelled materials from buffer controls and seawater samples are superimposed in Figure 2 to show chromatographic resolution and relative amounts of higher and lower MW materials. Monomeric materials eluted consistently in peaks centered at retention ratios ($V_e/V_o$) of approximately 2.75. Transformations of monomeric carbon (MW = 131 for leucine and 180 for glucose) into higher MW compounds were evident when radiolabelled materials eluted in smaller peaks at ratios of approximately 1.5. Details of chromatographic procedures are given below.

Utilization

$^{14}C$-labelled materials from samples or controls were separated for incubation with natural microbial populations; these incubations

Figure 2. Elution profiles of a SW+Glc sample (solid line) and a Buffer+Glc control (dashed line) chromatographed on size exclusion columns. Ratios of elution volume to exclusion volume ($V_e/V_o$) indicate retention of materials relative to 2.0 ml exclusion volume ($V_o$). Peaks centered at retention ratios of 2.75 represent radiolabelled monomer in controls and primarily $^{14}CO_2$ in samples. The peak centered at a retention ratio of 1.5 represents higher MW $DO^{14}C$.

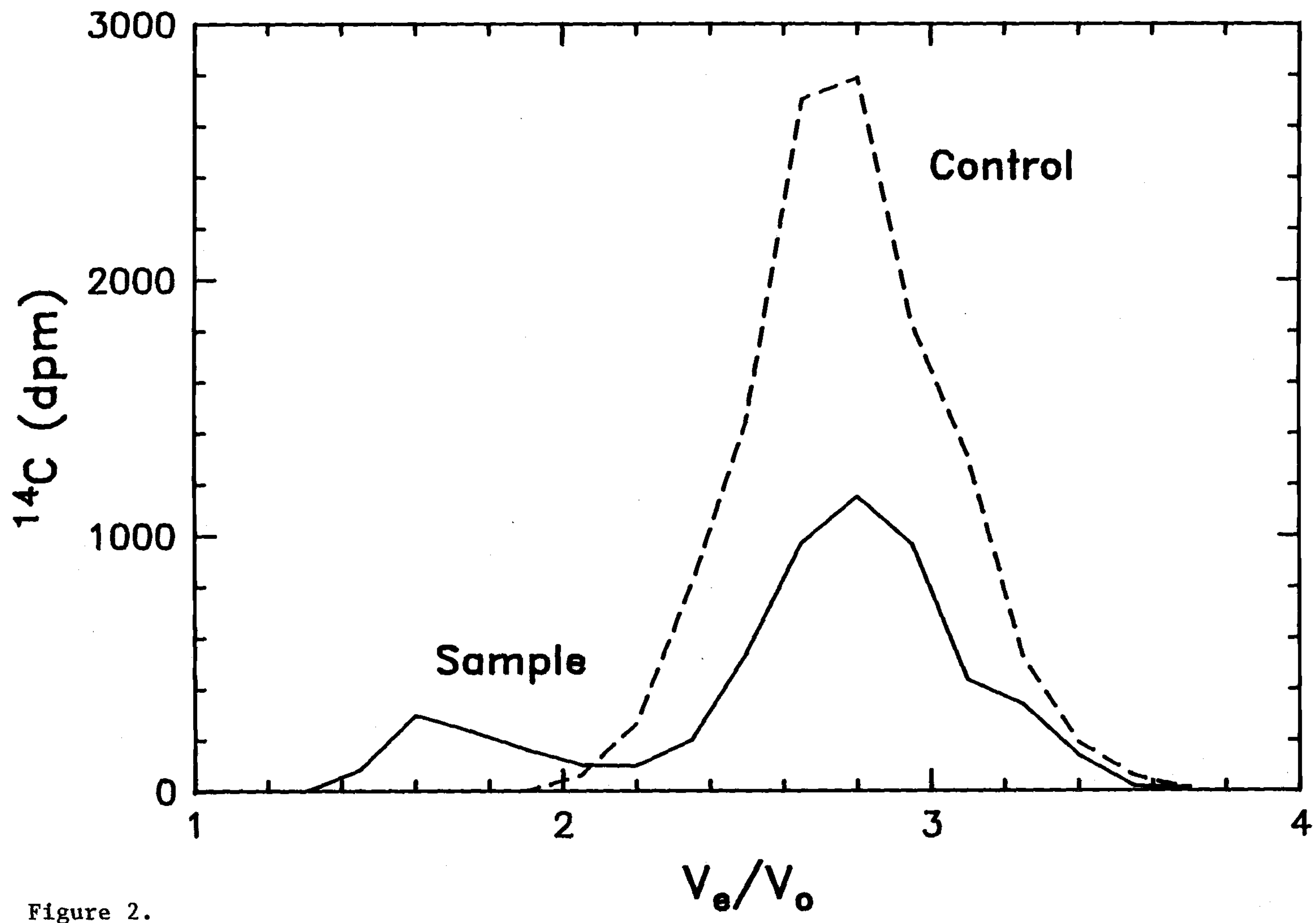


Figure 2.

will be referred to as "secondary incubations" to avoid confusion with the original incubations in which transformation products were formed. Dissolved higher MW materials were separated from several sequential sample elutions and then pooled for incubation. $PO^{14}C$ was collected from Sample vial 04G(b) by filtration onto a 0.2 μm Nuclepore membrane and used as substrate in one secondary incubation. Controls were stock radiolabelled glucose or leucine solutions or low MW fractions separated chromatographically from buffer controls (monomers from samples were mostly respired).

The separated labelled materials were added to aged deep-seawater in glass vials. The aged water, collected in July from 2000 m and aged in a darkened bottle at 5°C, was used to provide a consistent and representative matrix for secondary incubation. Parallel secondary incubations were supplemented with 2.5% (final concentration) Lib-X bacterial enrichment media (Baross et al., 1972; Appendix 1). Total volume of labelled materials plus deep-seawater, plus enrichment media, if present, was 20 ml. Triplicate 0.500 ml subsamples were counted to determine initial concentrations of higher or lower MW $DO^{14}C$ ("pooled initial $^{14}C$"). The initial amount of $PO^{14}C$ added was estimated by counting filtered material from a replicate volume of vial 04G(b).

Natural microbial populations were used to assess the metabolic availability of radiolabelled materials. Populations were filtered onto Whatman GF/F filters within 24 hours of collection at high tide from Yaquina Bay. The filters were then immediately immersed in secondary incubation vials. The vials containing aged deep-seawater,

microbial populations (on the GF/F filters), and monomeric or high MW materials were sealed with rubber septa fitted with Kontes center wells holding dry, fluted chromatography papers (Harrison et al., 1971). The vials were then incubated in the dark at room temperature on a shaker table for one week or three months.

$^{14}CO_2$ production was measured following procedures given by Hobbie and Crawford (1969) and Griffiths et al., (1982). Secondary incubations were terminated with addition of 1 ml 10N NaOH and shaken for one hour. Next, the chromatography papers were soaked with 0.2 ml $\beta$-phenethylamine, the $HCO_3^-$ was driven out of solution by acidification with 2 ml 10N $H_2SO_4$, and the $^{14}CO_2$ resulting from respiration of radiolabelled substrate was trapped on the chromatography paper while vials were shaken for at least another hour. Chromatography papers and 0.500 ml triplicate subsamples were counted for "$^{14}CO_2$" and radiolabelled organic materials ("residual $^{14}C$"), respectively. After $^{14}CO_2$ was removed, some samples were filtered through Whatman GF/F filters and those filters counted to determine $PO^{14}C$ resulting from incorporation during incubation or condensation upon acidification ("$PO^{14}C_{(2)}$").

**Additional details of procedures and calculations**

Prior to use, all GF/F filters, incubation containers, and other glassware were detergent-washed, rinsed with MilliQ water, and then combusted at 500°C. Pipette tips were washed with detergent and rinsed with methanol, and syringes were detergent-washed and rinsed with MilliQ water.

Radioactive material was assayed by liquid scintillation spec-

trophotometry on a Beckman LS-1800. Data were automatically corrected for counting efficiency calculated from quench curves. Materials were counted until two standard deviations equalled ± 2.0% of a count, but counting was terminated after 10 or 30 minutes for materials with counts near background radiation levels if such precision could not be reached. Transformed materials were counted for a maximum of 10 minutes, and in that time counting errors of ± 100% were reached at roughly 10 dpm above background; secondary incubation materials, which contained much less radiolabel, were counted for 30 minutes and reached counting errors of ± 100% at about 4 dpm above background. Best counting precision and reproducibility were obtained using Ecolume Scintillation Cocktail (ICN Radiochemicals). Care was taken to minimize dust, fingerprints and static; vials with anomalous counts were recounted.

Dissolved radiolabelled materials were chromatographed on 0.7 cm (ID) by 20 cm columns of Bio-Gel P2 (Bio-Rad) gel, a hydrophilic polyacrylamide gel with fractionation range of approximately 100 to 1800. They were eluted with borate buffer at rates of 0.20 ml/min into eighteen 0.30 ml fractions. Molecules eluted in order of decreasing size, or, if the shape of the molecules was relatively constant, roughly by decreasing MW (Freifelder, 1982). Ratios of elutant volume to exclusion volume ($V_e/V_o$) indicated retention of materials relative to 2.0 ml exclusion volumes. Monomeric glucose and leucine had retention ratios of approximately 2.75, which corresponded to observed monomer peaks such as in Figure 2. Higher MW materials were not retarded by gels as much as monomers and eluted at $V_e/V_o$ of

approximately 1.5. MW distributions of radiolabelled materials in seawater samples were calculated relative to retention of monomers in chromatographs of buffer control solutions (Appendix 2a, 2b). Column recoveries were calculated (Appendix 2b) for every chromatography run and averaged $97.0 \pm 15.08\%$ ($\bar{x} \pm$ s.d., n = 63) for samples and $97.0 \pm 7.1\%$ (n = 44) for buffer controls.

$^{14}C$ adsorbed to container walls was measured in the only vial (Sample vial 01G(a)) emptied over the course of this investigation. After that vial was drained, decontamination detergent was swirled in it, scintillation cocktail was added, and the vial was sealed and counted.

Filterable materials ("$PO^{14}C$"), which may have included particles larger than filter pore sizes but also smaller surface-active substances, were measured by counting $^{14}C$ retained by 0.2 µm Nuclepore membranes ("$PO^{14}C_{(1)}$") or Whatman GF/F filters ("$PO^{14}C_{(2)}$"; see Figure 1 and Appendix 2). The appearance of label in $PO^{14}C$ is referred to in this thesis as "incorporation" but does not necessarily reflect only biological activity.

Loss of $^{14}C$ during transformation incubations was calculated (see Appendix 2b) for all samples and controls. Respiration of monomers during transformation incubations was measured in several vials: $^{14}CO_2$ was trapped from low MW fractions separated chromatographically from sample vials 04G(a) and 04L, seawater control vials 02L and 04L, and buffer control vials 01G, 06G and 02L. $^{14}CO_2$ was not directly measured in other vials. $^{14}CO_2$ could not be contained during these incubations because vials were closed with teflon screw-caps, not

sealed with rubber septa, to avoid contamination with carbon from degraded rubber.

Because $H^{14}CO_3^-$ was found to elute in low MW fractions, respiration during secondary incubations had to be distinguished from $^{14}CO_2$ inadvertently added with monomers: amounts of $^{14}CO_2$ trapped after the secondary incubations ("$^{14}CO_2$") were corrected for "initial $^{14}CO_2$" trapped from replicate vials (never greater than 0.7% of "pooled initial $^{14}C$") to calculate respiration (Appendix 2b). Respiration was then reported as percentages of "pooled initial $^{14}C$". Efficiency of the $CO_2$ trapping procedure is reported to be 93-95% (Griffiths et al., 1982).

Precision of calculated values (Appendices 2b and 2c) was affected by natural counting variability (described above, and see Appendix 2a), and also by counting reproducibility and pipetting errors. Each standard deviation reported in Tables 2 through 8 represents the worst case of those individual errors within a measurement. Precisions were poorer for materials with lower radioactivities. Hence, the least precise values calculated in this investigation were the results of secondary incubations of higher MW materials; nevertheless, they were precise to within a factor of two.

# RESULTS

The results of these experiments can be summarized as follows. First, carbon from glucose and leucine was rapidly incorporated into particulate material or oxidized to $CO_2$ in seawater samples but not in buffer controls. During those processes, some of the carbon was transformed into higher MW material. When transformation products were separated and re-incubated with natural microbial populations, they were not respired to $CO_2$ to the same extents as monomeric carbon in controls.

## Heterotrophic uptake and respiration

Carbon from leucine and glucose was rapidly incorporated into $PO^{14}C$ in seawater samples: over 40% of initially dissolved glucose and leucine $^{14}C$ was filterable within one day. Amounts of $PO^{14}C$ decreased quickly thereafter (Figure 3). At the end of incubations, $PO^{14}C$ averaged 15.0 nmoles $^{14}C$/liter, representing $2.4 \pm 2.1\%$ ($\bar{x} \pm$ s.d., n = 5) of initial glucose-carbon and 18.6 nmoles $^{14}C$/liter, representing $6.4 \pm 5.6\%$ (n = 5) of initial leucine-carbon (Table 2 and 3); highest values were from deep water samples. In the less than 1.0 µm fractions of Sample 01 incorporation of leucine-carbon into $PO^{14}C$ was only slightly increased over incorporation in the corresponding unfiltered sample, but incorporation of glucose-carbon was enhanced by a factor of 10 over average incorporation in corresponding unfiltered vials (Tables 2 and 3; Figure 4).

SW+Glc controls and Buffer+Glc and Buffer+Leu controls rarely contained $PO^{14}C$ totalling more than 1% of initial labelled carbon, but

Figure 3. Incorporation of $^{14}C$ from monomers into particulate materials (closed symbols) and subsequent transformation of monomeric to higher MW dissolved materials (open symbols) in March samples to which glucose (squares) and leucine (triangles) had been added.

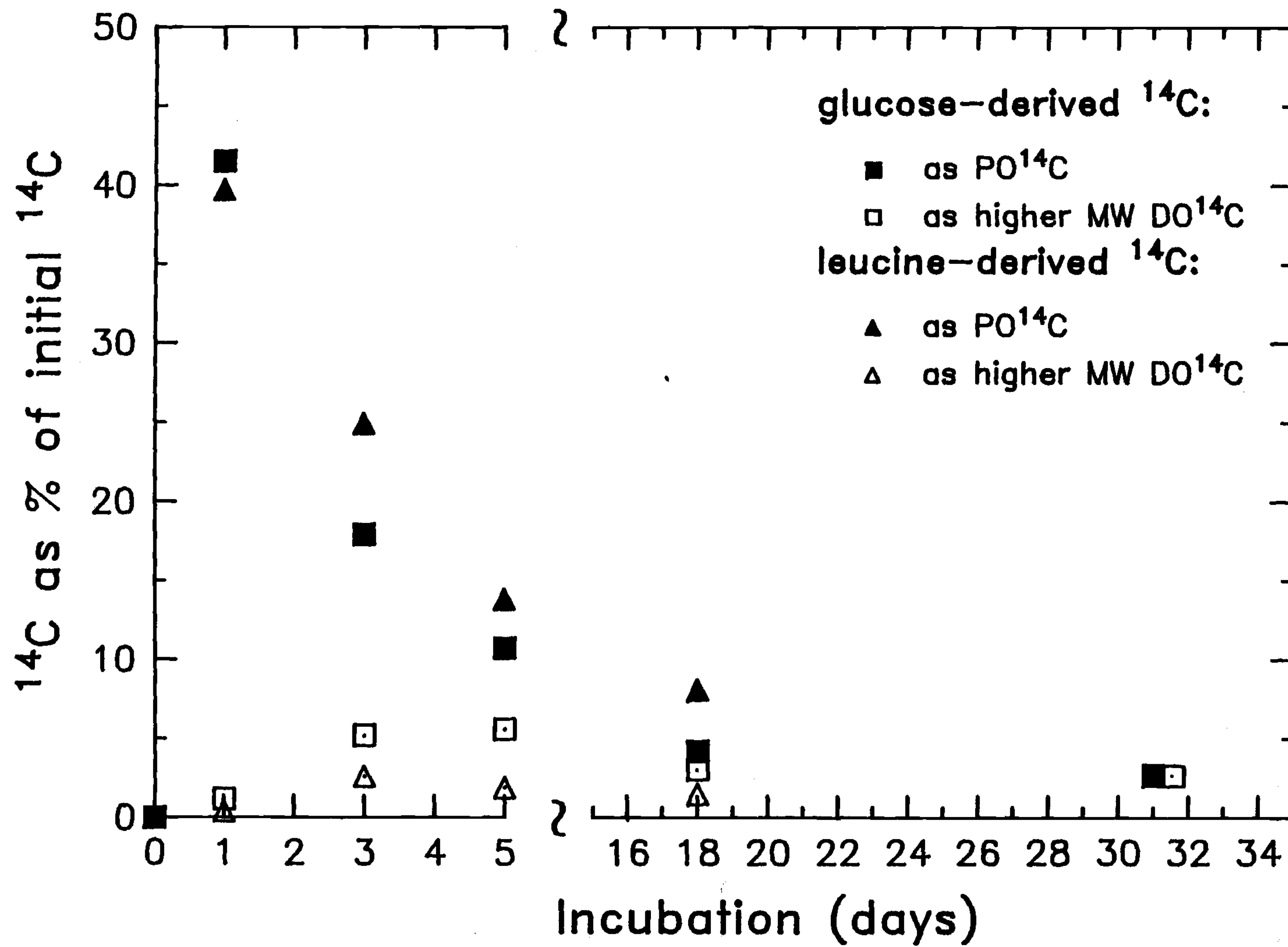


Figure 3.

**Table 2: END-POINT SUMMARY: SEAWATER SAMPLES + GLUCOSE**

| Sample Number | Initial Total $^{14}C$ (nmol $^{14}C/l^{a}$) | Incubation (days) | Times Opened | Total recovered $^{14}C$ (nmol $^{14}C/l^{b}$; | %) | Higher MW $DO^{14}C$ (nmol $^{14}C/l^{c}$; | %) | $PO^{14}C$ (nmol $^{14}C/l^{d}$; | %) |
|---|---|---|---|---|---|---|---|---|---|
| July surface | | | | | | | | | |
| 01G(a) | 626.4 | 130 | 4 | 140.4 | 22.4 | 22.0 | 3.5 | 12.3 | 2.0 |
| 01G(b) | 788.2 | 94 | 2 | 348.3 | 44.2 | 18.3 | 2.3 | 6.3 | 0.8 |
| 01G,<1µm[e] | 643.0 | 111 | 3 | 227.9 | 35.4 | 7.9 | 1.2 | 91.3 | 14.2 |
| 02G | 643.8 | 116 | 2 | 327.9 | 50.9 | 15.0 | 2.3 | 11.6 | 1.8 |
| July deep | | | | | | | | | |
| 03G | 633.8 | 129 | 1 | 364.7 | 57.5 | 32.4 | 5.1 | 39.3 | 6.2 |
| October surface | | | | | | | | | |
| 04G(a) | 597.8 | 70 | 3 | 342.2 | 57.2 | 41.3 | 6.9 | 7.2 | 1.2 |
| 04G(b) | 574.9 | 163 | 2 | 388.0 | 67.5 | 41.8 | 7.2 | 10.3 | 1.8 |
| October deep | | | | | | | | | |
| 05G | 579.1 | 163 | 2 | 358.2 | 61.9 | 48.2 | 8.3 | 6.9 | 1.2 |
| March surface | | | | | | | | | |
| 06G | 619.3 | 108 | 7 | 88.0 | 14.2 | 11.0 | 1.7 | 9.3 | 1.5 |
| | | | | **MEAN**[f] | 47.6 | 30.2 | 5.0 | 15.0 | 2.4 |

[a] Standard deviation range: 6.3 to 17.3 nmoles $^{14}C$/liter
[b] Standard deviation range: 1.6 to 7.3 nmoles $^{14}C$/liter
[c] Standard deviation range: 0.3 to 0.4 nmoles $^{14}C$/liter
[d] Standard deviation range: 0.1 to 0.4 nmoles $^{14}C$/liter
[e] < 1 µm fraction of Sample 01. Values not included in calculation of means.
[f] See Appendix 2c.

**Table 3: END-POINT SUMMARY: SEAWATER SAMPLES + LEUCINE**

| Sample Number | Initial Total $^{14}C$ (nmol $^{14}C/l$[a]) | Incubation (days) | Times Opened | Total recovered $^{14}C$ (nmol $^{14}C/l$[b]; | %) | Higher MW $DO^{14}C$ (nmol $^{14}C/l$[c]; | %) | $PO^{14}C$ (nmol $^{14}C/l$[d]; | %) |
|---|---|---|---|---|---|---|---|---|---|
| July surface | | | | | | | | | |
| 01L | 288.1 | 130 | 4 | 140.2 | 48.7 | 4.1 | 1.4 | 12.7 | 4.4 |
| 01L,<1μm[e] | 296.5 | 111 | 3 | 134.0 | 45.2 | 3.6 | 1.2 | 13.6 | 4.6 |
| 02L | 299.4 | 116 | 2 | 151.9 | 50.7 | 3.3 | 1.0 | 9.3 | 3.1 |
| July deep | | | | | | | | | |
| 03L | 304.5 | 129 | 2 | 169.7 | 55.7 | 7.1 | 2.3 | 26.8 | 8.8 |
| October surface | | | | | | | | | |
| 04L | 287.2 | 163 | 4 | 159.4 | 55.5 | 3.3 | 1.1 | 4.9 | 1.7 |
| October deep | | | | | | | | | |
| 05L | 283.6 | 163 | 2 | 169.3 | 59.7 | 4.6 | 1.6 | 42.5 | 15.0 |
| March surface | | | | | | | | | |
| 06L | 291.2 | 108 | 6 | 44.9 | 15.4 | 2.9 | 1.0 | 7.6 | 2.6 |
| | | | | **MEAN**[f] | 47.2 | 4.3 | 1.4 | 18.6 | 6.4 |

[a] Standard deviation range: 3.0 to 7.7 nmoles $^{14}C$/liter
[b] Standard deviation range: 1.4 to 3.5 nmoles $^{14}C$/liter
[c] Standard deviation range: 0.2 to 0.3 nmoles $^{14}C$/liter
[d] Standard deviation range: 0.1 to 0.4 nmoles $^{14}C$/liter
[e] < 1 μm fraction of Sample 01. Values not included in calculation of means.
[f] See Appendix 2c.

Figure 4. Amounts of $PO^{14}C$ (closed symbols) and higher MW $DO^{14}C$ (open symbols) derived from glucose (a) and leucine (b) in July Sample 01 whole seawater samples (squares) and $< 1$ μm size-fractionated samples (triangles).

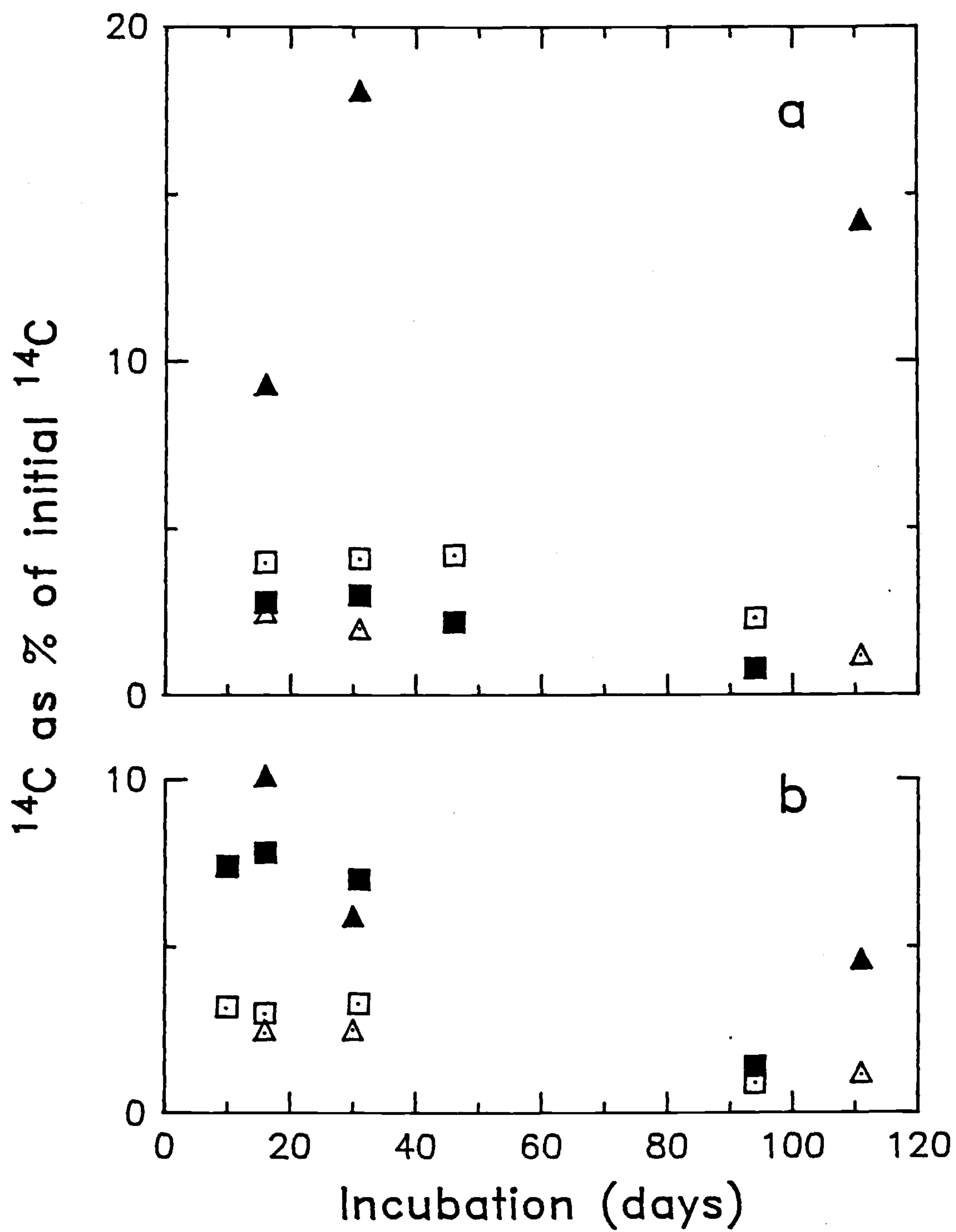


Figure 4.

SW+Leu controls frequently contained greater amounts, averaging 5.2 ± 4.3% (n = 7 vials) of initial leucine carbon remaining as $PO^{14}C$ (Tables 4 and 5).

After incorporation, most of the labelled carbon was oxidized to $^{14}CO_2$. Low MW peak fractions of seawater samples were found to be nearly 100% $^{14}CO_2$ (n = 2) whereas buffer control low MW peaks contained less than 0.2% $^{14}CO_2$ (n = 3) after several months of incubation. Though $^{14}CO_2$ was not specifically measured in other seawater samples, its escape from solution probably caused progressive loss of label from samples, which averaged 59 ± 4.7% (n = 17) in all samples and replicates (see Tables 2 and 3) by the end of incubations. Loss from buffer controls averaged only 4.5 ± 1.1% (n = 15, glucose and leucine not significantly different). Loss of label from seawater controls was greater than average losses from buffer controls only in conjunction with significant $PO^{14}C$ formation (see Table 4).

Loss of label from seawater samples was strongly influenced by how many times vials were opened (Figure 5), which supports the hypothesis that escape of $^{14}CO_2$ had occurred. Adsorption is not believed to have been a major sink for the missing label. For example, only 3% of the initial total $^{14}C$ was adsorbed to container walls after Sample vial 01G(a) was emptied; adsorption can not account for the 78% loss of label from the solution in that vial over the 130 days of incubation (Table 2).

**Transformation**

In addition to the $PO^{14}C$ and $^{14}CO_2$ formed during incubation, small

portions of $^{14}C$ were transformed to higher MW dissolved materials. These materials eluted at approximately $V_e/V_o$ = 1.5 (Figure 2), indicating the presence of extracellular materials with relatively large molecular size. The MW of these materials is estimated to be in the range of 700 - 1400 daltons from reported retention characteristics of the size exclusion gels (Carlson et al., 1985a).

Transformation products were formed in all natural seawater samples (but not in 0.2 μm filtered, azide-poisoned buffer controls). The higher MW $DO^{14}C$ was formed in as little as five days (Figures 3 and 6) and persisted for the duration of the 70-163 day incubations. An average of 30.2 nmoles $^{14}C$/liter, representing 5.0 $\pm$ 2.8% (n = 5) of carbon originally derived from labelled glucose, and 4.3 nmoles $^{14}C$/liter, representing 1.4 $\pm$ 0.5% (n = 5) of carbon derived from labelled leucine persisted as higher MW $DO^{14}C$ (Figure 6, Tables 2 and 3). Filtration of seawater from July Sample 01 through 1.0 μm Nuclepore membranes prior to addition of radiolabel reduced carbon transformation to 41 and 86% of the transformations in corresponding unfiltered seawater to which glucose and leucine, respectively, had been added (Tables 2 and 3; Figure 4).

More carbon was transformed to persistent higher MW materials from glucose in October samples than in July or March samples. October surface samples averaged 41.6 nmoles $^{14}C$/liter, while July and March surface samples averaged 17.6 and 11.0 nmoles $^{14}C$/liter, respectively. The October deep sample also showed greater transformation than the July deep sample by a factor of 1.6. These differences in amounts of higher MW carbon remaining between the three different

**Table 4a: END-POINT SUMMARY: FILTERED SEAWATER + AZIDE + GLUCOSE**

| Sample Number | Initial Total $^{14}C$ | Incubation | Total recovered $^{14}C$ | | Higher MW $DO^{14}C$ | | $PO^{14}C$ | |
|---|---|---|---|---|---|---|---|---|
| | (nmol $^{14}C$/l) | (days) | (nmol $^{14}C$/l; | %) | (nmol $^{14}C$/l; | %) | (nmol $^{14}C$/l; | %) |
| July surface | | | | | | | | |
| 01G | 637.1[a] | 111 | 605.6(10.7)[b] | 95.1 | 0.8(.2) | 0.1 | 0.7(.01) | 0.1 |
| 02G | 642.2 | 116 | 591.2 (5.9) | 92.1 | 1.0(.3) | 0.1 | 1.9(.02) | 0.3 |
| July deep | | | | | | | | |
| 03G | 631.1 | 115 | 608.2 (7.8) | 96.4 | 1.5(.4) | 0.2 | 3.8(.04) | 0.6 |
| October surface | | | | | | | | |
| 04G | 576.2 | 92 | 582.3 (8.4) | 101.0 | 0.6(.2) | 0.1 | 0.8(.01) | 0.1 |
| October deep | | | | | | | | |
| 05G | 576.6 | 94 | 578.8 (5.8) | 100.4 | 0.4(.2) | 0.0 | 0.5(.07) | 0.0 |
| March surface | | | | | | | | |
| 06G | 578.4 | 108 | 580.6 (5.8) | 100.4 | 2.4(.3) | 0.4 | 10.4(.10) | 1.8 |
| | | | **MEAN**[c] | 98.4 | 1.2 | 0.2 | 3.4 | 0.5 |

[a] Standard deviation range: 5.8 to 12.0 nmoles $^{14}C$/liter
[b] Standard deviation in parentheses.
[c] See Appendix 2c.

**TABLE 4b: END-POINT SUMMARY: FILTERED SEAWATER + AZIDE + LEUCINE**

| Sample Number | Initial Total $^{14}C$ | Incubation | Total recovered $^{14}C$ | | Higher MW $DO^{14}C$ | | $PO^{14}C$ | |
|---|---|---|---|---|---|---|---|---|
| | (nmol $^{14}C$/l) | (days) | (nmol $^{14}C$/l; | %) | (nmol $^{14}C$/l; | %) | (nmol $^{14}C$/l; | %) |
| July surface | | | | | | | | |
| 01L | 291.9[a] | 111 | 281.3(6.4)[b] | 96.4 | 0.3(.2) | 0.1 | 2.6(.04) | 0.9 |
| 02L | 304.3 | 116 | 108.9(11.3) | 35.8 | 3.7(.2) | 1.2 | 22.7(.3) | 7.3 |
| July deep | | | | | | | | |
| 03L | 295.3 | 115 | 293.2 (5.9) | 99.0 | 0.4(.1) | 0.1 | 3.0(.05) | 1.0 |
| October surface | | | | | | | | |
| 04L(a) | 280.9 | 94 | 155.9(22.1) | 55.5 | 6.8(.2) | 2.4 | 27.5(.3) | 9.8 |
| 04L(b) | 280.7 | 98 | 196.6 (3.8) | 70.0 | 3.1(.2) | 1.0 | 21.1(.2) | 7.5 |
| October deep | | | | | | | | |
| 05L | 264.8 | 94 | 281.8 (2.8) | 106.4 | 0.3(.2) | 0.1 | 0.5(.06) | 0.2 |
| March surface | | | | | | | | |
| 06L | 231.8 | 108 | 197.4 (4.5) | 85.2 | 3.5(.2) | 1.4 | 22.7(.2) | 9.8 |
| | | | **MEAN**[c] | 83.9 | 2.2 | 0.8 | 12.6 | 4.8 |

[a] Standard deviation range: 2.3 to 6.1 nmoles/liter

[b] Standard deviation in parentheses.

[c] See Appendix 2c.

**Table 5a: END-POINT SUMMARY: FILTERED BUFFER + AZIDE + GLUCOSE**

| Sample Number | Initial Total $^{14}C$ (nmol $^{14}C/l^{a}$) | Incubation (days) | Total recovered $^{14}C$ (nmol $^{14}C/l^{b}$; | %) | Higher MW $DO^{14}C$ (nmol $^{14}C/l^{c}$; | %) | $PO^{14}C$ (nmol $^{14}C/l^{d}$; | %) |
|---|---|---|---|---|---|---|---|---|
| July surface | | | | | | | | |
| 01G(a) | 632.3 | 130 | 589.9 | 93.3 | 2.0 | 0.3 | 0.6 | 0.1 |
| 01G(b) | 640.5 | 31 | 642.2 | 100.3 | 3.5 | 0.6 | 2.6 | 0.4 |
| 02G | 633.0 | 116 | 581.0 | 91.8 | 3.4 | 0.5 | 5.1 | 0.8 |
| July deep | | | | | | | | |
| 03G | 657.6 | 115 | 578.8 | 88.0 | 5.1 | 0.7 | 1.3 | 0.2 |
| October surface | | | | | | | | |
| 04G | 581.6 | 94 | 553.6 | 95.2 | 4.9 | 0.8 | 0.6 | 0.1 |
| October deep | | | | | | | | |
| 05G | 593.9 | 163 | 584.8 | 98.5 | 1.0 | 0.1 | 1.2 | 0.2 |
| March surface | | | | | | | | |
| 06G | 590.1 | 108 | 566.7 | 96.0 | 3.5 | 0.6 | 17.7 | 3.0 |
| | | | MEAN[e] | 94.4 | 3.5 | 0.5 | 4.8 | 0.8 |

[a] Standard deviation range: 5.8 to 10.7 nmoles $^{14}C$/liter
[b] Standard deviation range: 5.5 to 10.9 nmoles $^{14}C$/liter
[c] Standard deviation range: 0.3 to 0.5 nmoles $^{14}C$/liter
[d] Standard deviation range: 0.01 to 0.2 nmoles $^{14}C$/liter
[e] See Appendix 2c.

**Table 5b: END-POINT SUMMARY: FILTERED BUFFER + AZIDE + LEUCINE**

| Sample Number | Initial Total $^{14}C$ (nmol $^{14}C/l^{a}$) | Incubation (days) | Total recovered $^{14}C$ (nmol $^{14}C/l^{b}$; | %) | Higher MW $DO^{14}C$ (nmol $^{14}C/l^{c}$; | %) | $PO^{14}C$ (nmol $^{14}C/l^{d}$; | %) |
|---|---|---|---|---|---|---|---|---|
| July surface | | | | | | | | |
| 01(a) | 294.4 | 130 | 274.4 | 93.3 | 0.7 | 0.2 | 0.9 | 0.3 |
| 01(b) | 297.5 | 16 | 314.7 | 105.8 | 4.2 | 1.4 | 4.8 | 1.6 |
| 02 | 298.1 | 116 | 266.1 | 89.3 | 0.2 | 0.1 | 0.6 | 0.2 |
| July deep | | | | | | | | |
| 03 | 299.0 | 92 | 276.4 | 92.4 | 1.5 | 0.5 | 0.9 | 0.3 |
| October surface | | | | | | | | |
| 04 | 286.2 | 94 | 285.2 | 99.7 | 0.6 | 0.2 | 0.6 | 0.2 |
| October deep | | | | | | | | |
| 05 | 285.8 | 163 | 271.6 | 95.0 | 0.4 | 0.1 | 0.6 | 0.2 |
| March surface | | | | | | | | |
| 06 | 224.3 | 108 | 236.3 | 105.3 | 2.0 | 0.9 | 2.5 | 1.1 |
| | | | **MEAN**[e] | 97.4 | 1.2 | 0.4 | 1.3 | 0.5 |

[a] Standard deviation range: 2.2 to 4.5 nmoles $^{14}C$/liter
[b] Standard deviation range: 2.3 to 9.5 nmoles $^{14}C$/liter
[c] Standard deviation range: 0.2 to 0.5 nmoles $^{14}C$/liter
[d] Standard deviation range: 0.01 to 0.05 nmoles $^{14}C$/liter
[e] See Appendix 2c.

Figure 5. Amount of radiolabel lost during incubations (expressed as percentages of initial $^{14}C$-label) as a function of how many times incubation vials had been opened. Squares - seawater samples to which glucose had been added; triangles - seawater samples to which leucine had been added.

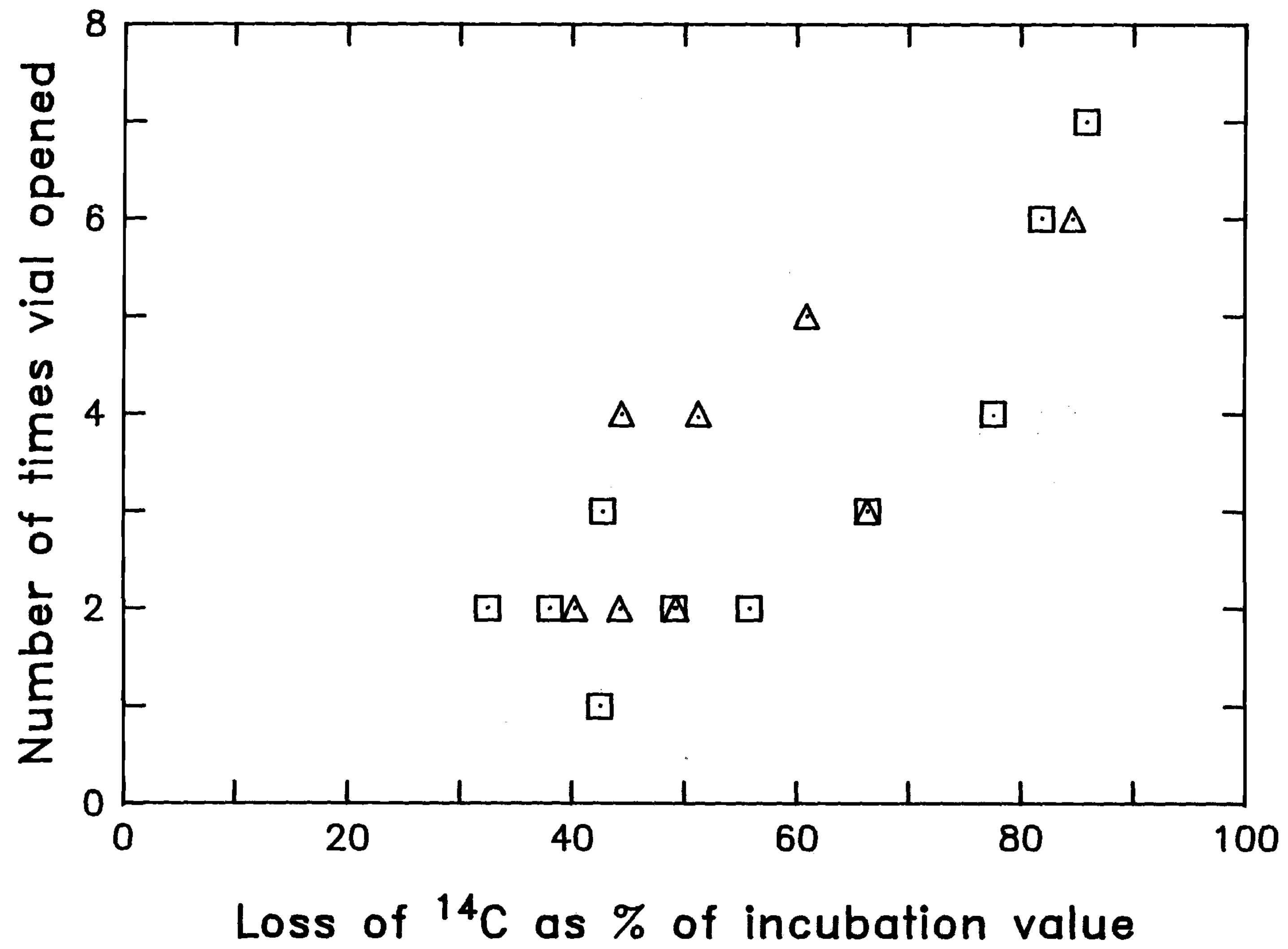


Figure 5.

sampling periods were greater than the 34% difference between the two July surface samples (01 and 02), the 20% difference between replicates of July Sample 01, or the 1% difference between replicates of October Sample 04 (Table 2). In contrast, there were no detectable differences among amounts of transformed carbon remaining in samples to which leucine had been added.

Transformed glucose-carbon in July and October samples persisted through the incubations at roughly the same amounts present early in incubations, whereas transformed glucose-carbon in March (vial 06G) exhibited a value equivalent to October values after five days of incubation but then decreased rapidly to a lower stable value that was only 30% of the maximum value (Figure 6). Transformed leucine-carbon in all surface samples peaked early in incubations and then exhibited decreases similar to the March SW+Glc sample. Deep water samples, incubated at lower temperatures than surface samples, showed less transformation to high MW carbon than corresponding surface samples after 5 days but equivalent transformations after longer incubations (Tables 2 and 3, Figure 6).

Filtered, azide-poisoned seawater controls exhibited sporadic transformation activity (Table 4). SW+Leu controls averaged 2.2 nmoles $^{14}C$/liter representing $0.8 \pm 0.7\%$ ($n = 5$) of initial carbon transformed to higher MW $DO^{14}C$. The seven vials in this series of controls (listed individually in Table 4b) exhibited two distinctly different patterns: three had only 0.1% of initial $^{14}C$ transformed while the other four had considerably more, averaging $1.5 \pm 0.6\%$, identical to the average amount of higher MW $DO^{14}C$ present in SW+Leu

Figure 6. Amounts of higher MW $DO^{14}C$ derived from glucose (a) and leucine (b) present in each sample as a function of incubation duration, expressed as nmoles carbon per liter. Open circles - July, surface (n = 2); closed circles - July, deep; open squares - October, surface; closed squares - October, deep; open triangles - March, surface.

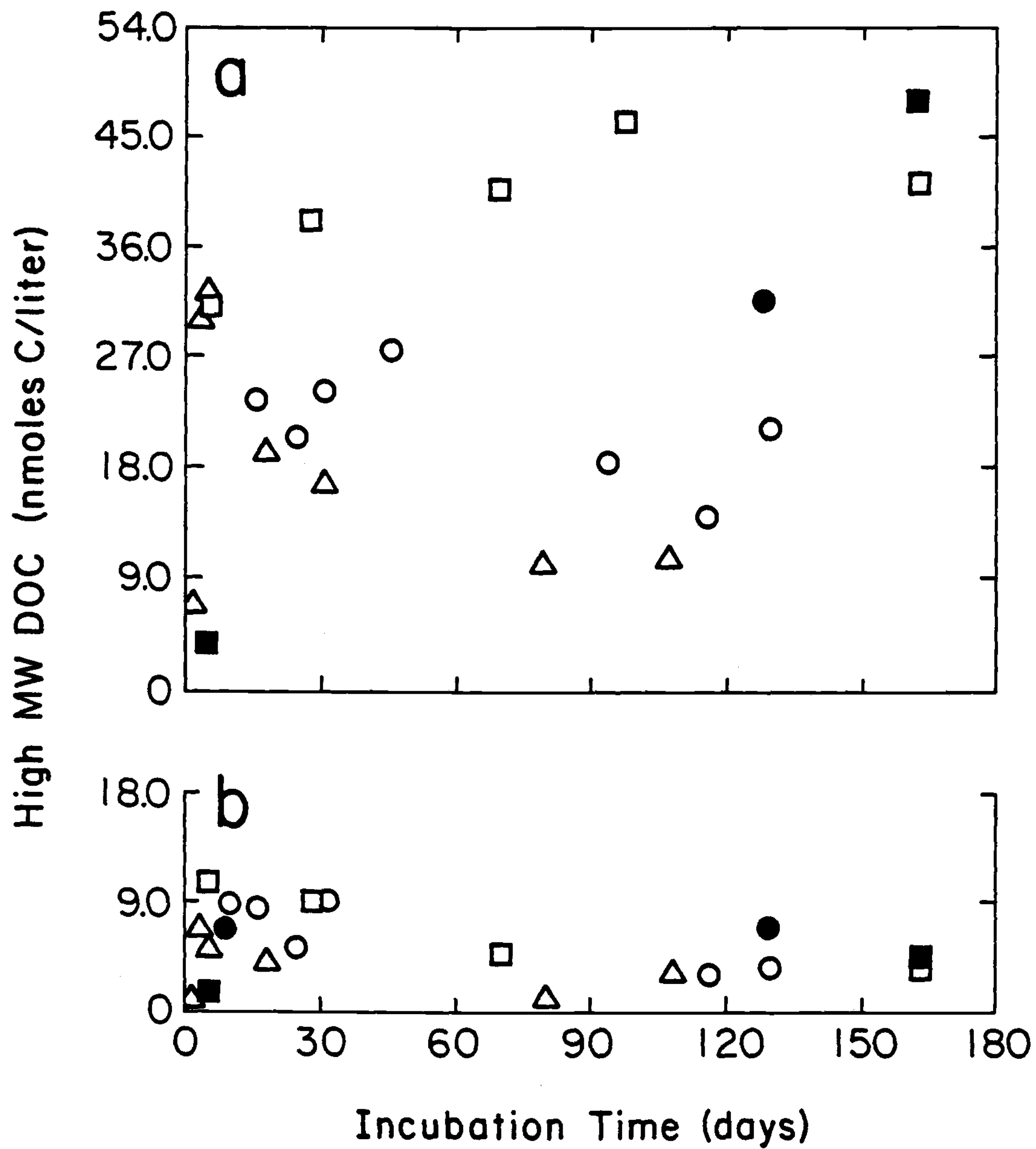


Figure 6.

samples. These four vials also averaged 8.6% of initial $^{14}C$ as $PO^{14}C$ and 38.4% of initial $^{14}C$ lost from solution, and are suspected to have contained microorganisms not excluded by 0.2 μm filtration or inhibited by sodium azide (see Discussion). The three vials with low transformation averaged only 0.7% $PO^{14}C$ and had little losses of $^{14}C$ label. Little activity was found in SW+Glc controls, which averaged only 0.2 ± 0.2% (n = 5) transformed, or in buffer controls, which altogether averaged 0.6 ± 0.3% (n = 10) of initial carbon transformed. Only one buffer control vial, O1L(b), appeared to have been biologically-contaminated, with 1.4% of initial $^{14}C$ transformed.

**Heterotrophic utilization of transformation products**

Higher MW materials formed in these experiments were found to be dramatically less utilizable than monomers in secondary incubations (Figure 7). Only 1.0 ± 0.8% (n = 5) of glucose-derived, chromatographically-separated higher MW $DO^{14}C$ and 3.0 ± 1.1% (n = 2) of leucine-derived higher MW $DO^{14}C$ were respired in one week (Tables 6 and 7). Over the same amount of time, 41.2 ± 7.4% (n = 4) of the similarly-separated monomers, 40.3% (n = 1) of fresh glucose (Table 8), and 51.5% (n = 1) of labelled particulate material (Table 6) were respired. Supplementation with bacterial enrichment media (Appendix 1) enhanced respiration of monomeric carbon but had little effect on respiration of higher MW fractions: in one week, 61.7% of monomeric carbon was respired while 2.1 ± 1.1% (n = 5) of glucose-derived and 4.1 ± 2.4% (n = 2) of leucine-derived higher MW carbon was respired.

Little of the dissolved carbon, monomeric or higher MW, appeared in particulate fractions during the secondary incubations: only 10.4 ±

Figure 7. Percentage of unrespired $^{14}C$ after secondary incubation of monomeric or high MW materials with natural microbial populations. Unsupplemented vials contained monomer or high MW materials and microbial populations in aged deep seawater; supplemented vials included 2.5% yeast extract enrichment media. Duration of incubations was one week.

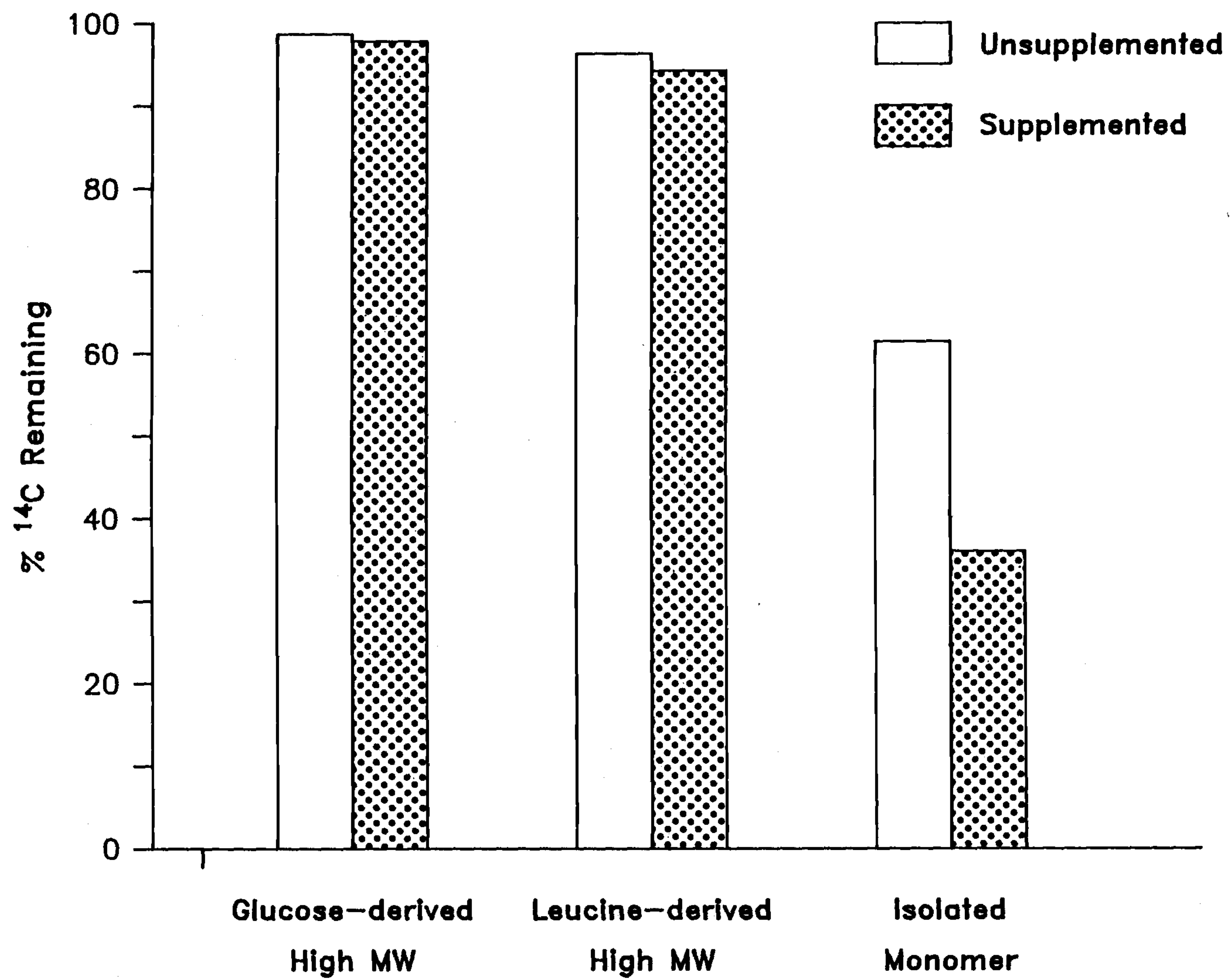


Figure 7.

**Table 6: HETEROTROPHIC UTILIZATION OF GLUCOSE-DERIVED HIGHER MW $DO^{14}C$ AND $PO^{14}C$**

| Sample Number | Sample age (days) | Incubation (days) | Unsupplemented: Pooled initial total (nmol $^{14}C$/l) | Unsupplemented: Resp. (%) | Unsupplemented: $PO^{14}C$ (%) | Supplemented: Pooled initial total (nmol $^{14}C$/l) | Supplemented: Resp. (%) | Supplemented: $PO^{14}C$ (%) |
|---|---|---|---|---|---|---|---|---|
| **Higher MW $DO^{14}C$** | | | | | | | | |
| | One week | | | | | | | |
| July | | | | | | | | |
| 01G(b)[a] | 46 | 11 | 0.8 (.3)[b] | 3.8(.2) | --- | --- | --- | --- |
| 01G(b) | 72 | 7 | 0.3 (.6) | 2.2(.4) | 18.4(.7) | 0.45(.07) | 1.9(.4) | 14.3(.6) |
| October | | | | | | | | |
| 04G(a)[a] | 10 | 7 | 1.0 (.2) | 0.8(.1) | 13.5(.3) | 1.00(.17) | 2.6(.2) | 7.6(.3) |
| 04G(a) | 75 | 7 | 0.69(.03) | 0.9(.2) | 5.1(.2) | 0.70(.04) | 2.9(.2) | 6.6(.2) |
| 04G(b) | 100 | 7 | 0.72(.05) | 1.1(.1) | 3.4(.2) | 0.61(.04) | 1.3(.2) | 3.9(.7) |
| 05G | 176 | 7 | 0.08(.05) | 1.0(1.5) | --- | 0.14(.11) | 3.6(.9) | --- |
| March | | | | | | | | |
| 06G[a] | 5 | 7 | 0.25[c] | 4.2(.5) | --- | 0.28[c] | 5.8(.8) | --- |
| 06G | 31 | 7 | 0.21(.04) | 0.0 | --- | 0.21(.04) | 0.8(.3) | --- |
| | | | **MEAN**[d] | 1.0 | | | 2.1 | |
| | Three months | | | | | | | |
| March | | | | | | | | |
| 06G | 31 | 85 | 0.24(.07) | 17.3(.6) | --- | --- | --- | --- |
| **$PO^{14}C$** | | | | | | | | |
| October | | | | | | | | |
| 04G(b) | 100 | 7 | --- | --- | --- | 1.06(.01) | 51.5(.6) | 6.9(.2) |

[a] Not included in calculation of mean value; see Appendix 2c.
[b] Standard deviation in parentheses.
[c] Calculated from final concentrations assuming 80% (min) recoveries.
[d] See Appendix 2c.

**Table 7: HETEROTROPHIC UTILIZATION OF LEUCINE-DERIVED HIGHER MW $DO^{14}C$**

| Sample Number | Sample age (days) | Incubation (days) | Unsupplemented: Pooled initial total (nmol $^{14}C$/l) | Unsupplemented: Resp. (%) | Unsupplemented: $PO^{14}C$ (%) | Supplemented: Pooled initial total (nmol $^{14}C$/l) | Supplemented: Resp. (%) | Supplemented: $PO^{14}C$ (%) |
|---|---|---|---|---|---|---|---|---|
| One week | | | | | | | | |
| October | | | | | | | | |
| 04L | 75 | 7 | 0.09(.03)[a] | 3.7(1.4) | 12.9(4.8) | 0.07(.02) | 5.8(1.9) | 16.3(5.3) |
| March | | | | | | | | |
| 06L | 5 | 7 | 0.21(.03) | 2.2 (.4) | 5.7 (.9) | 0.25(.06) | 2.4 (.6) | 2.8 (.7) |
| | | | **MEAN**[b] | 3.0 | | | 4.1 | |
| Three months | | | | | | | | |
| 06L | 5 | 109 | 0.13(.03) | 12.1(2.9) | --- | --- | --- | --- |

[a] Standard deviation in parentheses.
[b] See Appendix 2c.

**Table 8: HETEROTROPHIC UTILIZATION OF LOWER MW $^{14}$C-CARBON FROM BUFFER CONTROLS AND STOCK SOLUTIONS**

| Sample Number | Sample age (days) | Incubation (days) | Unsupplemented: Pooled initial total (nmol $^{14}$C/l) | Unsupplemented: Resp. (%) | Unsupplemented: PO$^{14}$C (%) | Supplemented: Pooled initial total (nmol $^{14}$C/l) | Supplemented: Resp. (%) | Supplemented: PO$^{14}$C (%) |
|---|---|---|---|---|---|---|---|---|
| *One week* | | | | | | | | |
| **Glucose** | | | | | | | | |
| July 01G | 177 | 7 | 2.18(.06)[a] | 34.8(1.0) | 18.0(.5) | 2.31(.11) | 69.8(3.3) | 7.9 (.4) |
| Mar 06G[b] | 5 | 7 | 1.07(.06) | 64.3(3.6) | 5.5(.3) | 0.75(.05) | 55.4(3.8) | 5.4(1.3) |
| Mar 06G | 31 | 7 | 0.60(.04) | 46.1(3.1) | --- | 0.77(.10) | 49.8(6.2) | --- |
| 2° stock | -- | 7 | 25.0 (.3) | 40.3 (.4) | 9.6(.1) | 15.3 (1.1) | 64.3(5.5) | 6.6(2.8) |
| **Leucine** | | | | | | | | |
| July 02L | 178 | 7 | 1.07(.04) | 34.8(1.4) | 16.1(.6) | 1.13(.04) | 72.5(5.3) | 9.7(.4) |
| Mar 06L | 5 | 7 | 1.6 (.3) | 48.9(8.9) | 4.8(.9) | 1.09(.13) | 54.7(6.7) | 7.7(.9) |
| 2° stock[b] | -- | 7 | --- | --- | --- | 58.3 (.6) | 82.8 (.8) | 6.8(.07) |
| | | | **MEAN**[c] | 41.2 | | | 61.7 | |
| *Three months* | | | | | | | | |
| **Glucose** | | | | | | | | |
| Mar 06G | 31 | 109 | 0.75(.07) | 73.7(6.6) | --- | --- | --- | --- |
| **Leucine** | | | | | | | | |
| Mar 06L | 5 | 109 | 1.56(.06) | 67.8(4.2) | --- | --- | --- | --- |

[a] Standard deviation in parentheses.
[b] Not included in calculations of overall mean values.
[c] See Appendix 2c.

6.0% (n = 9) was filterable after one week of secondary incubations. This filterable material may have been formed by biological incorporation or by condensation of DOC induced by acidification during $CO_2$ trapping procedures.

After three months, 68 to 74% of the monomers (n = 2) but only 12 to 17% of higher MW materials derived from glucose and leucine in March (06) samples (n = 2) had been respired. Despite significantly greater utilization after three months than after one week, higher MW materials still were not utilized to the same extents as monomeric or particulate materials.

# DISCUSSION

Radiolabelled carbon from glucose and leucine was utilized heterotrophically to form $PO^{14}C$ and $^{14}CO_2$ in seawater samples and in some controls. In addition, small amounts of monomeric radiolabelled carbon were transformed to higher MW $DO^{14}C$. Because higher MW $DO^{14}C$ production occurred only in conjunction with biological $PO^{14}C$ and $^{14}CO_2$ formation, transformations were probably biologically-mediated and not procedural artifacts or abiotic processes.

The higher MW $DO^{14}C$ formed in these experiments was apparently refractory to heterotrophic utilization under natural seawater concentrations of organics and microorganisms. Higher MW $DO^{14}C$ persisted in incubations for nearly six months and, when re-incubated with natural seawater microbial populations, was not incorporated into $PO^{14}C$ or respired to $^{14}CO_2$ to the same extent as monomeric forms of carbon. Supplementation with bacterial enrichment media only slightly increased respiration of transformation products, suggesting that biological utilization of transformation products was not restricted by nutrient or energy limitation.

## Monomer utilization

Oxidation of $^{14}C$-labelled glucose and leucine to $^{14}CO_2$ following incorporation was consistent with normal heterotrophic activity (Hobbie and Crawford, 1969). Indeed, under the conditions of these experiments, heterotrophic metabolism was likely the only mechanism by which labelled $CO_2$ could have been formed. Oxidation of compounds such as glucose, though thermodynamically favored, has such high

activation energy that environmentally unrealistic temperatures or oxidizing conditions are required for chemical combustion to occur. Under natural seawater conditions in the absence of light, oxidation of glucose can only proceed at detectable rates with the aid of enzymes (Goodwin and Mercer, 1983); production of $^{14}CO_2$ in any samples or controls must have resulted from biological utilization and respiration of the radiolabelled monomers.

In contrast, formation of $PO^{14}C$ may not have been exclusively a biological process: adsorption of monomers or transformed materials to particles or formation of "sticky" complexes (Carlson et al., 1985a) may have caused $^{14}C$ label to have become filterable on 0.2 µm Nuclepore membranes. However, biological activity seemed the primary mechanism responsible for $PO^{14}C$ formation because the label was, for the most part, only temporarily in filterable form (Figure 3). This pattern was consistent with results of other, albeit shorter time-course, investigations of heterotrophic uptake and metabolism of simple substrates (Wright and Hobbie, 1966; Williams and Yentsch, 1976; Billen et al., 1980; Iturriaga and Zsolnay, 1981; Wolter, 1982). The small fractions of initial $^{14}C$ that remained as $PO^{14}C$ after several months may have resulted from actual cellular assimilation or from abiotic adsorption. Whatever the processes, that residual particulate $^{14}C$ was still available to heterotrophic utilization when reincubated with natural microbial populations.

**Biological transformation**

Transformations of monomeric to higher MW $DO^{14}C$ were evidently

consequences of biological activity: they occurred subsequent to $PO^{14}C$ formation and in conjunction with respiration. Loss of label, presumably as $^{14}CO_2$ (inferred from two direct measurements), and formation of $PO^{14}C$ always accompanied transformation of monomer-derived $^{14}C$ to higher MW materials in samples and biologically-contaminated seawater controls, whereas buffer controls never had appreciable formation of labelled higher MW material or of $PO^{14}C$ or $^{14}CO_2$. The correlation of higher MW $DO^{14}C$ production with biological $^{14}CO_2$ production and $PO^{14}C$ formation implied that transformation, even in filtered, poisoned controls, also resulted from biological activity.

Iturriaga and Zsolnay (1981, 1983) investigated transformations of dissolved substrates by microbial utilization and also observed considerable respiration of monomeric substrates before appearance of label in macromolecular materials. However, their procedures may have induced condensations of low MW substrates because initial monomer concentrations were higher than natural substrate levels and because filtrates were concentrated by evaporation or ultrafiltration before chromatographic analysis (see Carlson et al., 1985b). Their results therefore do not definitively establish that transformation of monomeric carbon to higher MW $DO^{14}C$ results from natural seawater heterotrophic activity. In contrast, my investigations were conducted at near-ambient concentrations of monomers and microbiota and involved no concentration steps. Other than containment, a condition necessary in studies using radiolabel tracers, my experiments were representative of natural conditions. Therefore, transformation of monomeric to higher MW carbon may be concluded to be a result of normal microbial

heterotrophic activity.

The general absence of transformations in buffer and seawater controls, and the loss of $^{14}C$ label from all samples and formation of $^{14}CO_2$ in at least two of the seawater controls in which transformation occurred, led me to reassess earlier conclusions (Carlson et al., 1985a) that such transformations were abiotic. Carbon transformation in seawater controls presumably was due to activity of cells which passed through 0.2 $\mu m$ filters or were introduced after filtration, and which were not inhibited by $NaN_3$. The controls that exhibited transformation activity were primarily seawater controls to which leucine had been added (Table 4b). Because biological contamination should have occurred with similar frequency in all control treatments, abiotic processes, such as hydrophobic interactions of $^{14}C$-leucine with seawater DOC (Carlson et al., 1985a), which might preferentially affect leucine in seawater, may have caused development of higher MW $DO^{14}C$ in those seawater controls. Transformations should not have resulted from handling artifacts because glassware was combusted prior to incubations to remove other carbon sources and samples were never shaken to avoid physically inducing condensation. However, abiotic transformation processes, if they occurred, were sporadic in seawater controls and can not be concluded to be consistently effective or important in seawater. Moreover, biological mediation of transformation of leucine to higher MW $DO^{14}C$ is strongly implied by the presence of $^{14}CO_2$ in those four seawater control vials.

The biological activities responsible for transformations were microbial and heterotrophic processes. Removal of large ($> 1$ $\mu m$)

cells by filtration only partly reduced the extent of transformation. All vials were kept in the dark so heterotrophic rather than phototrophic processes were implicated in carbon transformation. Moreover, because vials were kept in the dark, higher MW materials could not have been produced by photosynthetic organisms utilizing $^{14}CO_2$ respired by heterotrophs. Transformation of monomers to higher MW materials occurred at slower rates than incorporation of monomers to $PO^{14}C$ (Figure 3) or reported microbial assimilation rates of glucose or leucine (Keller et al., 1982), suggesting that monomers were taken up by heterotrophs and altered during incorporation or metabolism before the altered forms appeared in solution as higher MW materials.

Appearance of higher MW $DO^{14}C$ seemed to be facilitated by activities of organisms larger than 1 µm. Size-fractionation of Sample 01 resulted in less higher MW $DO^{14}C$ produced in $< 1$ µm fractions than in corresponding unfiltered seawater, which suggests that some of the heterotrophs responsible for higher MW $DO^{14}C$ production were larger than 1 µm. Despite removal of large cells, $PO^{14}C$ formation increased in the 1.0 µm filtered SW+Glc sample to 10 times the corresponding unfiltered seawater average value. Microflagellate bacteriovores (which range in size from roughly 3 to 10 µm, Fenchel, 1982) would have been excluded by 1.0 µm filtration, so increased $PO^{14}C$ in bacteria-size biomass may reflect lack of turnover of bacterial standing stock or reflect bacterial growth unrestricted by predation. $DO^{14}C$ production was not equally stimulated by this incorporation of carbon to biomass: it actually decreased relative to unfiltered seawater. This suggests that transformation products were not simply by-products

of assimilation of labelled carbon to biomass but resulted from the cycling of biomass carbon through one or more trophic levels.

Transformed products were probably not artifacts of cell breakage during filtration (Goldman and Dennett, 1985) because $DO^{14}C$ was not always found when $PO^{14}C$ was found (Figure 3). The definition of "dissolved material" is operational and in this thesis refers to all substances that pass an 0.2 μm membrane and thus includes colloids, pieces of cells and small bacteria that are not truly dissolved (Sharp, 1973). Chromatographically-separated "dissolved" materials, however, must have been smaller than the approximate 1800 MW exclusion limit, certainly smaller than polymers (such as DNA) or than cell fragments. In addition, they were less utilizable than filterable $PO^{14}C$.

**Variations in transformation activity**

Variations in amounts of carbon transformed between replicate vials of samples (relative differences of 35% in Sample 01 and 1% in Sample 04, Table 2) may have been caused by several factors, including pipetting errors (although triplicate measurements had coefficients of variation that rarely exceeded 2.0%) or the presence or absence of small microflagellate bacteriovores in each vial.

Overall, less $DO^{14}C$ and more $PO^{14}C$ were formed from leucine-carbon than from glucose-carbon, a pattern that may reflect preferential conservation of leucine or, more generally, of compounds containing nitrogen. A notable exception to this pattern was in $< 1$ μm size-fractioned seawater, in which $PO^{14}C$ derived from glucose-carbon was very high (Table 2, Figure 6).

Seasonal variations in transformation activity were evident: more transformation products derived from glucose persisted in October samples than in July or March samples by factors of two and four, respectively. In addition, higher MW $DO^{14}C$ formed early in March samples was apparently partially utilized during incubations, whereas that formed in July and October samples persisted through the incubations. Leucine-derived higher MW $DO^{14}C$ seemed less persistent than glucose-derived higher MW $DO^{14}C$, especially in March. There was apparently little seasonal difference among amounts of leucine-derived transformed carbon that persisted throughout incubations but slight differences may have been obscured by variability at low radioactivity levels.

In coastal waters, bacterial activity has seasonality (Bolter, 1981; Larsson and Hagstrom, 1979; Wolter, 1982), probably in response to dissolved substrate abundances which might to be related to phytoplankton seasonality (Azam et al., 1983; Larsson and Hagstrom, 1979). Chlorophyll-a content (Kokkinakis, 1987; Wheeler, unpublished data; Table 1) was higher in surface waters in July than in October due to dense phytoplankton blooms in response to transient upwelling in July. If there had been a direct relationship between phytoplankton abundance and bacterial transformation activity, July's samples should have had the greater transformation activity, with July Sample 01 much greater than July Sample 02. Alternatively, sampling location or incubation temperature may have caused differences among samples: bay water might contain organisms with different metabolic capabilities than open ocean organisms, and higher temperatures might inhibit or

enhance particular enzyme systems. The causes of variations between sampling periods can not be explained from these limited numbers of samples and treatments.

Higher MW $DO^{14}C$ formation varied with depth only in that rates were slower in deep water samples. After 5 days, transformation products had reached nearly maximal quantities in surface samples but were still minimal in deep water samples. Over the long-term, however, surface and deep waters had similar potential for DOC transformation. The lag may reflect influences of incubation temperature on enzymatic activities, or recovery from dormancy (Wright, 1984) or from shock of sudden pressure change. Less respiration (implied from lower loss of label) and generally more incorporation of label into $PO^{14}C$ occurred in deep waters than in corresponding surface waters, which may reflect greater efficiency or temperature suppression of metabolic activities of microbial populations.

**Refractory nature of transformation products**

Transformation products persisted throughout incubation periods of nearly 6 months, indicating a degree of biological unavailability or refractivity. Results of the secondary incubations confirmed this refractivity. Very little transformed carbon was incorporated or respired when presented to natural microbial populations, populations which respired fresh monomeric carbon as well as monomeric and particulate carbon remaining from initial incubations. Further alteration of higher MW $DO^{14}C$ could not be determined because concentrations of dissolved radiolabel remaining after secondary incubations were too

low to permit chromatographic analysis.

Persistence of transformed materials is consistent with evidence that marine DOC resists further microbial oxidation (Barber, 1968). However, formation and chemical nature of transformation products in these experiments were not influenced by ultrafiltration artifacts which might have been present in Barber's investigation (see Carlson et al., 1985b). The persistence of transformed materials and their rates of respiration (1 - 2% in one week and 12 - 17% in three months) are comparable to persistence and degradation of other naturally-occurring materials such as soil humic acids or polyaromatic compounds generally considered refractory (Alexander, 1973; Martin et al., 1972) and of dissolved organic macromolecules from lake water (Geller, 1986).

Persistence of the higher MW $DO^{14}C$ formed in these experiments was probably due to its chemical composition (Alexander, 1973) because other constraints on biological utilization were addressed under these experimental conditions. Nutrient and energy limitation have been suggested to restrict microbial growth and utilization of organic substrates (Jannasch, 1967). However, populations supplemented with media containing trace elements and energy-rich compounds did not greatly increase utilization of higher MW $DO^{14}C$. An alternate hypothesis is that other more easily or preferentially utilized carbon sources may have been available. This was probably not the case because the bulk solution in secondary incubations was deep seawater which generally has low DOC concentration (Williams, 1975), and because utilization of higher MW $DO^{14}C$ was slightly increased rather than decreased by addition of supplemental carbon substrate. It is

also possible that higher MW $DO^{14}C$ may have been too dilute for microbial utilization, yet microbial uptake of very dilute substrates has been demonstrated (Morita, 1984), and monomers added at carbon concentrations similar to higher MW $DO^{14}C$ (within an order of magnitude) were utilized to much greater extents. Finally, although degradation of complex materials often requires induction of appropriate enzymes, longer incubations only slightly increased utilization of higher MW $DO^{14}C$.

Little is known about the chemical nature of the higher MW $DO^{14}C$ formed in these incubations other than chemical origin, approximate MW, and persistence. Further chemical characterization of similarly formed higher MW $DO^{14}C$ would require large amounts of transformation products, unrealistically high monomer concentrations, or concentration of radioactive materials after incubation. Geller (1986) cultured freshwater bacteria in mineral salt solutions supplemented with high concentrations of glutamic acid, and reported the formation of dissolved UV absorbing materials (e.g., phenolic compounds or proteins with aromatic amino acid residues). It is not known, however, whether the formation of UV-absorbant materials that occurred in Geller's experiments represented transformation of carbon from glutamic acid to refractory DOC, or whether it would have occurred under natural seawater conditions.

The properties of transformation processes and products were consistent with corresponding properties of marine DOC and marine conditions. Transformations were relatively rapid and the higher MW products persistent, consistent with the hypothesis that labile carbon

rapidly cycles near sites of production leaving small residual amounts that degrade very slowly (Menzel and Ryther, 1970). The molecular weight of transformation products (700 - 1400 MW) was in the range suggested to include a majority of marine DOC (Carlson et al., 1985b). Transformations of labile carbon to refractory carbon occurred with natural concentrations of microbes and organic materials, and have now been demonstrated in Atlantic (Carlson et al., 1985a) and Pacific waters. The inventory of monomeric carbon compounds transformed to higher MW materials includes not only glucose and leucine but also palmitic acid, alanine, valine (Carlson et al., 1985a), glycine (Carlson et al., 1985a; Iturriaga and Zsolnay, 1981), arginine (Iturriaga and Zsolnay, 1981) and glutamic acid (Iturriaga and Zsolnay, 1981; possibly Geller, 1986). Persistence of complex materials formed from monomers other than glucose and leucine has not been tested.

The higher MW materials were probably not simple polymers of glucose or leucine or associations of intact monomers with pre-existing oceanic DOC because monomers were likely to have been biologically utilized, suggested by the high incorporation and oxidation rates of the monomers prior to appearance of transformation products. Extracellular associations of biologically-altered materials with oceanic DOC may have occurred. Alternatively, higher MW $DO^{14}C$ may have been microbially produced and exuded during metabolism or leached after death or during predation.

Little is known of the fate of higher MW $DO^{14}C$. Most was not utilized in three months, but adsorption of some $^{14}C$ to container walls after six months may indicate surface reactivity of transformed

materials (or the assimilation of radiolabel by attached bacteria). Eventual heterotrophic degradation, abiotic condensation, flocculation or adsorption to particles that would sink out of the water column may remove some of these materials from solution.

**Ecological significance**

Microbial mediation of DOC transformation has important implications for marine microbial ecology. Marine microbiota have classically been regarded as remineralizers, responsible for oxidizing organic matter and recycling nutrients to primary producers, but their role as efficient secondary producers is now considered important in food-chain dynamics. Bacterial utilization of organic matter is now thought to be a major pathway for material and energy flow in pelagic food webs (Azam et al., 1983; Fenchel, 1982; P.J.LeB. Williams, 1971). Marine bacteria are apparently more efficient with their carbon resources than originally thought, converting up to 80% of ingested carbon to biomass that can be passed up the food chain; the remainder of absorbed carbon is mostly respired and only a negligible amount assumed to be excreted (Azam et al., 1983). Despite the efficiency of microbial metabolisms, the results of this study suggest that microbes produce refractory materials.

Biological production of refractory carbon had been postulated though direct evidence was lacking. Then, Iturriaga and Zsolnay (1983) reported microbial alteration of phytoplankton exudates to higher MW dissolved compounds that had turnover times longer than 70 hours. The experiments reported here confirm that biological transformations of labile DOC occur under seawater conditions and

demonstrate that the higher MW dissolved transformation products are resistant to heterotrophic utilization for periods of up to six months.

This production of refractory materials from labile substrates by marine microbial populations apparently conflicts with the current understanding of ecological roles of marine microbes. Why should marine bacteria, whose growth may often be limited by supply of carbon and nutrients, produce resistant compounds from substrates they might use for growth or maintenance? Why have they not evolved mechanisms to utilize these products or, for that matter, mechanisms to utilize more of oceanic DOC? Resolution of these questions requires better understanding of conditions under which molecules are not available to microbial utilization and of chemical characteristics that render materials resistant to microbial attack (Alexander, 1973).

**Geochemical significance**

This demonstration of biological transformation of DOC from utilizable to refractory forms at natural seawater concentrations of reactants and biota verifies a suspected mechanism for producing refractory DOC. The extent to which global carbon cycles are affected by these transformation processes depends on the *in situ* rates of transformation relative to the amounts and composition of reactants. The time and volume scales of these experiments were necessarily shorter and smaller than those of *in situ* processes. From the results of these experiments, however, it is possible to estimate *in situ* production of refractory DOC. The average transformation of 5.0% of

glucose carbon and 1.4% of leucine carbon from single inputs of tracers constituted a total transformation of 415.2 ng carbon to higher MW material per liter of seawater. Of that amount, some 83 to 99% was refractory. These values extrapolate to 4.7 - 5.6 x $10^{14}$ g refractory carbon produced per year over the world ocean volume (1370 x $10^6$ $km^3$), an amount equivalent to the entire oceanic DOC pool (7 x $10^{17}$ g, P.M. Williams, 1971) in approximately 1250 to 1500 years. The similarity between this estimate and the estimated 3400 year average age of oceanic DOC (Williams et al., 1969) emphasizes the potential importance of these transformations as mechanisms for the production of refractory DOC. To determine their actual importance, however, it will be necessary to quantify the production rates from repetitive inputs of transformation precursors and the rates of processes by which transformation products are removed from solution (e.g., particle adsorption, Carlson et al., 1985a).

## CONCLUSIONS AND RECOMMENDATIONS

Rapid biological transformations of small amounts of labile carbon substrates to materials resistant to heterotrophic oxidation has been demonstrated to occur under natural seawater concentrations of organics and biota. Such transformations may be important mechanisms for *in situ* production of refractory marine DOC. The conditions which regulate rates of production and accumulation of transformed carbon, the mechanisms of transformation, and the chemical composition of the transformed carbon compounds are not yet known.

For future studies, I recommend that samples be injected with several pulses of radiolabelled substrates to evaluate accumulation of transformation products. I suggest that other organic substrates, such as additional amino acids and sugars, lipids, and nucleic acids, be added to samples to better estimate total carbon transformation. Temperature effects on microbial mediation of transformation and utilization of transformation products should be investigated. Enzymatic degradation of transformation products using enzymes produced by marine organisms and specific to polymeric forms of the monomers could be used to elucidate microbial responses to incubation conditions and yield structural information about transformation products. I also recommend that larger volumes and more replicates be monitored.

There is obviously much to learn. However, from results of experiments presented here, it is clear that transformation of labile to bio-refractory carbon may constitute a quantitatively and qualitatively important autochthonous input to the marine DOC pool.

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# APPENDICES

## Appendix 1: COMPOSITION OF REAGENTS.

A. Borate buffer (Perrin and Dempsey, 1974, p.147), pH = 8.2.

| | |
|---|---|
| Boric acid | 3.092 g |
| Potassium chloride | 3.727 g |
| Sodium hydroxide | 0.24 g |
| Sodium azide (as required) | 0.2 g |
| Filtered MilliQ water | to 1.0 liter |

Filter through pre-combusted Whatman GF/F immediately and again before use.

B. Lib-X broth (Baross et al., 1972):

| | |
|---|---|
| Bacto-yeast extract | 1.2 g |
| Sodium citrate | 0.3 g |
| L-glutamic acid | 0.3 g |
| Sodium nitrate | 0.05 g |
| Ferrous sulfate | 0.005 g |
| Rila marine salts | 33.0 g |
| Distilled water | to 1.0 liter |

Adjust pH to 8.2. Autoclave.
Filter through pre-combusted Whatman GF/F before use.

**Appendix 2a: CALCULATION OF PRIMARY DATA**

Conversion of LSC data (dpm) to $^{14}C$ concentration (nmol $^{14}C/l$) using specific activity (sp. act.):

$$\text{nmol } ^{14}C/\text{liter} = \frac{(\text{dpm/subsample volume}) \, / \, \text{sp. act.}}{(2.2 \times 10^6 \text{ dpm/uCi})}$$

**Transformation**

counting precision: ± 2% of count, or vial counted for 10 minutes.

**initial total $^{14}C$**: mean of three 0.200 ml subsamples prior to incubation

**total recovered $^{14}C$**: mean of three 0.200 ml subsamples after incubation

**$PO^{14}C_{(1)}$**: $^{14}C$ retained by 0.2 µm Nuclepore membrane

**$DO^{14}C$** (amount $DO^{14}C$ applied to column): mean of three 0.200 ml subsamples after filtration

**MW profile**: $DO^{14}C$ chromatographed into eighteen 0.3 ml fractions

**Utilization**

counting precision: ± 2% of count, or vial counted for 30 minutes.

**pooled fractions**: from three to five sequential chromatographs, relative to monomer peak centered at $V_e/V_o$ = 2.75 (i.e., fractions 11 and 12)

higher MW $DO^{14}C$: fractions 1 through 5
lower MW $DO^{14}$: fractions 9 through 14

**pooled initial $^{14}C$**: mean of three 0.500 ml subsamples prior to re-incubation

**initial $^{14}CO_2$**: $CO_2$ trapped with β-phenethylamine from replicate incubation vial prior to incubation

**$^{14}CO_2$**: $CO_2$ trapped with β-phenethylamine after secondary incubation (efficiency of method: greater than 93%)

**residual $^{14}C$**: mean of three 0.500 ml subsamples after $CO_2$ removal

**$PO^{14}C_{(2)}$**: $^{14}C$ retained by Whatman GF/F filter after $CO_2$ removal

## Appendix 2b: CALCULATIONS FROM PRIMARY DATA

**column recovery** (as % of $DO^{14}C$ applied to column):

$$(\sum \text{MW profile} / DO^{14}C) \times 100$$

**$^{14}C$ lost during incubation** (as % of initial total $^{14}C$):

$$\frac{(\text{initial total } ^{14}C - \text{total recovered } ^{14}C)}{\text{initial total } ^{14}C} \times 100$$

**higher MW $DO^{14}C$** (as % of initial total $^{14}C$):

$$\frac{(\sum \text{fractions 1 through 5 of MW profile})}{\text{initial total } ^{14}C} \times 100$$

**$PO^{14}C$ formed** (as % initial total $^{14}C$, after primary incubation):

$$\frac{PO^{14}C_{(1)} / \text{volume filtered}}{\text{initial total } ^{14}C} \times 100$$

(as % of pooled initial $^{14}C$, after secondary incubation):

$$\frac{PO^{14}C_{(2)} / \text{volume filtered}}{\text{pooled initial } ^{14}C} \times 100$$

**respiration** (as % of pooled initial $^{14}C$):

$$\left[\frac{(^{14}CO_2 / \text{volume incubated})}{\text{pooled initial } ^{14}C} - \frac{(\text{initial } ^{14}CO_2 / \text{volume of replicate})}{\text{replicate pooled initial } ^{14}C}\right] \times 100$$

**Appendix 2c: CALCULATION OF SUMMARY DATA**

**mean $^{14}C$ transformation** in the five sample groups: July surface*, July deep, October surface, October deep and March surface.

higher MW $DO^{14}C$ / 5

* to calculate mean value for the July surface sample group, Sample 01G replicates "a" and "b" were first averaged, and the result was averaged with Sample 02G

**mean $PO^{14}C$ formation** in the five sample groups:

$PO^{14}C_{(1)}$ / 5

**mean respiration** for one-week incubations:

respiration / # vials assayed**

** if several trials were performed from the same sample vial, only the result of the final trial was used in calculations of means

Standard deviations given in Tables 2 through 8 are worst cases estimated from standard deviations of counting data, replicate data and triplicate subsamples.