Use of high temperature catalytic oxidation (HTCO) to measure carbon content of microorganisms

Silvia P. Pelegri*, John Dolan, Fereidoun Rassoulzadegan

ABSTRACT: High temperature catalytic oxidation (HTCO) was used for the first time to determine carbon content of heterotrophic protists (the helioflagellate *Pteridomonas danica*, the dinoflagellate *Oxyrrhis marina*, and the scuticociliate *Uronema* sp.) and bacteria (*Escherichia coli*). This technique has the advantage, over the conventional CHN analysis of glass-fiber filters, of measuring total organic carbon in the liquid phase. Failing to retain small organisms on filters and cell rupture during filtration can thus be avoided. Cell volumes were measured on live (Coulter Counter) and Lugol's iodine- or formaldehyde-preserved cells and used to obtain a carbon:biovolume conversion factor for each species. Carbon:biovolume conversion factors ranged from 123 to 175 and from 121 to 864 fg C pm⁻³ for live and preserved cells, respectively. A linear regression of cell carbon content versus live biovolume gave a mean carbon biovolume conversion factor of 125.3 ± 7.6 (SE) fg C pm⁻³ for the 4 species studied with biovolumes ranging from 0.69 to 1590 pm³. The data obtained in this study is compared to live and preserved cells data obtained from the literature and the possibility of using a single carbon:biovolume conversion factor for bacteria and protozoa is discussed.

KEY WORDS: Carbon content, Bacteria, Protozoa, Biovolume, Conversion factor

INTRODUCTION

Estimating the biomass of microorganisms is essential to evaluating their role in food webs and material cycling. Carbon biomass is usually estimated from population biovolumes (cell count multiplied by average cell biovolume) by using carbon:biovolume conversion factors. A wide range of carbon:biovolume conversion factors have been published for each microbial assemblage, ranging over 3 orders of magnitude (see ‘Discussion’). These differences could be attributed to species-specific carbon:biovolume ratios, nutritional state of the organisms and the methodology employed. A number of studies have reported that preserved cell volumes are significantly different from live cell volumes (Bersheim & Bratbak 1987, Choi & Stoecker 1989, Verity et al. 1992). Most of the data on carbon content of microorganisms reported in the literature has been obtained by concentrating the organisms on pre-combusted Whatman GF/F filters which are subsequently burned on a CHN analyser (e.g. Bratbak 1985, Lee & Fuhrman 1987). Few studies have used X-ray analysis of single cells in the scanning electron microscope (Norland et al. 1987), HPLC of hydrolysed amino acids (Simon & Azam 1989) or radiolabelling techniques (Pett & Stoecker 1989).

We present here a new method of measuring carbon content of microorganisms by high temperature catalytic oxidation (HTCO). This method has the advantage of measuring carbon content directly on liquid samples. Collecting the microorganisms on filters is not required, thus the risks involved in this manipulation are reduced (e.g. filter blank subtraction, cell destruction and loss). Cell rupture may result in underestimation of the microbial biomass because cell residues and soluble pools can pass through the filters (Nagata & Kirchman 1990). In addition, it is very difficult to retain efficiently the smallest organisms on filters. For example, Whatman GF/F filters have a nominal retention
size of 0.7 μm. Consequently, they cannot collect a significant fraction of the bacterial cells (Lee & Fuhrman 1987, Lee 1993).

We used the HTCO technique to measure the carbon content of the helioflagellate Pteridomonas danica Patterson & Fenchel (1985), the dinoflagellate Oxyrrhis marina Dujardin, the scutococciolate Uronema sp., and the enterobacteria Escherichia coli HB10B. Although we found in the literature a number of references on carbon:biovolume conversion factors for bacteria (e.g. Norland et al. 1995, Fagerbakke et al. 1996, Troussellier et al. 1997), only few studies have been conducted on protozoa (Börshem & Bratbak 1987, Putt & Stoecker 1989, Ohman & Snyder 1991). Carbon:biovolume conversion factors for protozoa have often been based on empirical equations developed for similarly sized microalgae or on theoretical assumptions about their specific gravity, dry weight content, and carbon content in dry weight (Beers & Stewart 1970, Smetacek 1981, Fenchel & Finlay 1983).

**MATERIAL AND METHODS**

We used for our experiments monospecific laboratory cultures of protozoa and bacteria.

**Establishment of cultures.** (1) Escherichia coli HB10B was grown on Luria-Bertani medium at 37°C and with vigorous shaking. The culture, which had reached ~1 unit optical density at 600 nm, was centrifuged (Centrikon T-124) at 9000 rpm during 10 min. The supernatant was substituted by filtered (consistently Whatman GF/F, 0.8 μm and 0.22 μm Millipore filter units) and autoclaved seawater. Each centrifuge tube was sonicated for 1 min and centrifuged again. This procedure was repeated 5 times. This bacterial suspension, containing 2 to 3 × 10^9 cells ml^-1, was then diluted and used in our experiments.

Escherichia coli was added to filtered and autoclaved seawater to a final concentration of 17.4 ± 1.1 × 10^9 cells ml^-1. This culture was then diluted with filtered and autoclaved seawater from 0 to 10 times in duplicate (n = 22). Ten ml of each sample was collected in 20 ml ignited Pyrex glass tubes and treated as described for Escherichia coli. The exponential growth phase culture contained 3.1 ± 0.1 × 10^9 O. marina and 668 I. galbana ml^-1. Cells of O. marina were not detected in the filtrate. The number of I. galbana in the original culture did not differ significantly (p > 0.5) from that in the filtrate.

(3) Uronema sp. was fed Escherichia coli. When the culture had reached the exponential growth phase, we enumerated the E. coli left. The culture was then diluted from 0 to 10 times in duplicate (n = 22) with filtered and autoclaved seawater containing an equal number of E. coli. Ten ml from each of these samples was collected in 20 ml ignited Pyrex glass tubes and treated as described for E. coli. The exponential growth phase culture contained 1.7 ± 0.0 × 10^5 Uronema sp. and 1.9 × 10^6 E. coli ml^-1.

(4) Pteridomonas danica was fed natural bacteria isolated from Villefranche Bay. When the culture had reached the exponential growth phase, we enumerated the bacteria left. The culture was then diluted from 0 to 10 times in duplicate (n = 22) with filtered and autoclaved seawater containing an equal number of bacteria. Ten ml from each of these samples was collected in 20 ml ignited Pyrex glass tubes and treated as for Escherichia coli. The original culture contained 1.0 ± 0.1 × 10^5 P. danica and 1.1 × 10^6 bacteria ml^-1.

**Cell number determination.** Cell number was determined with an electronic particle sizer on live samples (see below), and microscopically on formaldehyde- or Lugol's iodine-preserved samples.

Buffered formaldehyde-preserved samples (2% final concentration vol./vol.) were stained with 4,6-diamidino-2-phenylindole (DAPI, Sigma Chemical Co., 5 mg l^-1 final concentration), filtered onto black 0.2 μm pore-size Nuclepore polycarbonate filters (Porter & Feig 1980), and examined under an epifluorescence Zeiss microscope. Escherichia coli was enumerated in quintuplicate at a magnification of ×2500 and Pteridomonas danica in triplicate at a magnification of ×1250. A total of 700 and 1500 cells were counted, respectively.

Lugol's iodine-fixed samples (2% final concentration vol./vol.) were counted on a Zeiss inverted microscope at a magnification of ×400. A total of 2000 Oxyrrhis marina and Uronema sp. cells were counted in triplicate.

**Biovolume determination.** Biovolumes were measured with an electronic particle sizer on live samples, and microscopically on a minimum of 60 formaldehyde- or Lugol's iodine-preserved cells. In addition, unfixed DAPI-stained Escherichia coli were measured microscopically on a minimum of 60 cells.
Live cell volumes were measured with the Coulter Counter for all species. The Coulter Counter may give imperfect measures of live cell volume when cells are nonspherical (Harbison & McAlister 1980, Nilsson 1990, Montagnes et al. 1994). However, if a sufficiently high number of particles is counted to ensure an averaging of orientations of particles traversing the aperture, an accurate estimate of cell volume can be obtained. For this study, we measured a minimum of 16,000 cells (Table 1) with a Coulter Counter (Multisizer) analysing system equipped with either a 20 µm (for Escherichia coli and Pteridomonas danica) or 100 µm (for Oxyrrhis marina and Uronema sp.) aperture tube. The Coulter Counter was calibrated with 2.09 and 19.32 µm latex microspheres, respectively.

The biovolume of Escherichia coli was determined microscopically (x2500) on DAPI-stained samples (as described in the preceding subsection). This determination was conducted on both live and formaldehyde-preserved samples. Width (W) and length (L) were measured with a calibrated ocular micrometer. The shape of the cells was approximated to a cylinder with a hemisphere at each end (Bratbak 1985) and biovolumes were calculated as $\frac{1}{4} \pi (L - W/3) \times W^2$.

The biovolume of Pteridomonas danica was determined on formaldehyde-preserved samples. Immediately and 1 and 48 h after formaldehyde addition, P. danica's diameter was determined microscopically (x1250) on DAPI-stained samples (as described in the preceding subsection). Cell biovolume was calculated as for a sphere, corresponding to the geometry of P. danica.

Biovolumes of Oxyrrhis marina and Uronema sp. were determined on Lugol's iodine-preserved samples. One hour after Lugol's iodine addition, cell width (W) and length (L) were measured on a Zeiss inverted microscope (x400). Biovolume was determined as for Escherichia coli.

**Cell carbon determination.** Total organic carbon was determined on 10 ml samples by high temperature catalytic oxidation (HTCO) (e.g. Sharp et al. 1995). Samples were sparged with an artificial air mixture (AGA, France) (containing ≤0.1 ppm CO, CO$_2$ or hydrocarbons) in order to eliminate inorganic carbon, and measured on a Shimadzu TOC-5000 instrument equipped with a high sensitivity catalyst. Total organic carbon concentrations were calculated with the instrument software and a 4-point standard calibration curve made with potassium biphthalate. The coefficient of variation of triplicate injections was always <2%.

A linear regression analysis ($y = ax + b$) was used to describe the relationship between the number of organisms ($X$) and the corresponding concentration of carbon ($y$) for each species. The slope of these linear regressions ($a$) is the carbon content per cell, and the intercept ($b$) is the background carbon corresponding to seawater and prey items. A carbon:biovolume conversion factor was calculated for each species by dividing carbon content per cell by mean cell biovolume.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Method</th>
<th>Size [µm]</th>
<th>Volume [µm$^3$]</th>
<th>N</th>
<th>Carbon [g C org.$^{-1}$]</th>
<th>N</th>
<th>Carbon:biovolume [fg C pm.$^{-3}$]</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Oxyrrhis marina</em></td>
<td>Coulter</td>
<td>L 18.8 ± 4.6 W 12.4 ± 1.9</td>
<td>1590 ± 860</td>
<td>25018</td>
<td>196 × 10$^{-12}$</td>
<td>22</td>
<td>123</td>
</tr>
<tr>
<td></td>
<td>Lugol</td>
<td>L 18.5 ± 2.3 W 8.6 ± 1.2</td>
<td>846 ± 157</td>
<td>22850</td>
<td>121 × 10$^{-12}$</td>
<td>22</td>
<td>143</td>
</tr>
<tr>
<td><em>Uronema sp.</em></td>
<td>Coulter</td>
<td>L 18.8 ± 4.6 W 12.4 ± 1.9</td>
<td>1619 ± 780</td>
<td>64</td>
<td></td>
<td></td>
<td>121</td>
</tr>
<tr>
<td></td>
<td>Lugol</td>
<td>L 18.5 ± 2.3 W 8.6 ± 1.2</td>
<td>727 ± 221</td>
<td>64</td>
<td></td>
<td></td>
<td>166</td>
</tr>
<tr>
<td><em>Pteridomonas danica</em></td>
<td>Coulter</td>
<td>4.2 ± 0.8</td>
<td>39 ± 30</td>
<td>16439</td>
<td>52 × 10$^{-13}$</td>
<td>22</td>
<td>124</td>
</tr>
<tr>
<td></td>
<td>Form. 0 h$^a$</td>
<td>3.9 ± 0.4</td>
<td>32 ± 9</td>
<td>64</td>
<td></td>
<td></td>
<td>163</td>
</tr>
<tr>
<td></td>
<td>48 h</td>
<td>3.4 ± 0.3</td>
<td>21 ± 5</td>
<td>64</td>
<td></td>
<td></td>
<td>248</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>Coulter</td>
<td>L 2.1 ± 0.6 W 0.6 ± 0.04</td>
<td>0.69 ± 0.3</td>
<td>584554</td>
<td>121 × 10$^{-15}$</td>
<td>22</td>
<td>175</td>
</tr>
<tr>
<td></td>
<td>DAPI$^b$</td>
<td>L 2.1 ± 0.6 W 0.6 ± 0.04</td>
<td>0.53 ± 0.2</td>
<td>70</td>
<td></td>
<td></td>
<td>228</td>
</tr>
<tr>
<td></td>
<td>Formaldehyde</td>
<td>L 0.9 ± 0.3 W 0.5 ± 0.05</td>
<td>0.14 ± 0.1</td>
<td>64</td>
<td></td>
<td></td>
<td>864</td>
</tr>
</tbody>
</table>

$^a$Size was measured just after formaldehyde addition. $^b$Non-fixed samples, just stained with DAPI
RESULTS

Figs. 1 to 4 show the relationship between cell number and carbon concentration for the 4 species studied. A significant linear relationship (p < 0.0001) was found between both parameters in all of them. The slope of the regression line represents the carbon content per cell and the intercept represents the background level, corresponding to prey items and seawater.

Table 1 shows the parameters involved in the calculation of the carbon:biovolume conversion factors for the 4 species. Carbon:biovolume conversion factors are calculated for live and preserved cells. The large standard deviations of biovolumes of all 4 species were attributed to growth-related size variations within the population.

We found no significant (p > 0.3) differences between live (Coulter Counter) and preserved *Oxyrrhis marina* cell volume. However, that was not the case for *Uronema* sp., *Escherichia coli* and *Pteridomonas danica*.

*Uronema* sp. volume decreased by fixation from 846 ± 157 to 727 ± 221 μm³ (p < 0.001), increasing carbon:biovolume conversion factors from 143 to 166 fg C μm⁻³.

*Escherichia coli* decreased in volume from 0.69 ± 0.3 (live, Coulter Counter) to 0.53 ± 0.2 μm³ (23%) after DAPI staining (p < 0.001). Biovolume decreases were even more important when formaldehyde-preserved...
cells were DAPI stained, from 0.69 ± 0.3 to 0.14 ± 0.1 μm³ (80%). As a result of these changes in cell volume, the carbon:biovolume conversion factor increased from 175 fg C μm⁻³ for live E. coli cells to 228 and 864 fg C μm⁻³ for DAPI-live and DAPI-preserved cells, respectively.

*Pteridomonas danica* volume decreased by fixation with formaldehyde. The initial volume of 39 ± 30 μm³ (live, Coulter Counter) was reduced to 32 ± 9 μm³ (17%) just after fixation (p < 0.001), to 28 ± 9 μm³ (n = 64) (28%) 1 h after fixation and to 21 ± 5 μm³ (n = 64) (46%) 48 h later (data not shown). Consequently, carbon:biovolume conversion factors increased from 124 to 248 fg C μm⁻³.

Taking into account only live biovolumes, we found a good correlation between biovolume (x, x10² μm³) and carbon content (y, pg C cell⁻¹) of all 4 species (y = 3.0 + 125.3x, r² = 0.994, p < 0.004), giving a mean conversion factor for bacteria and protozoa of 125.3 ± 7.6 (mean ± SE) fg C μm⁻³.

**DISCUSSION**

In this study, the HTCO technique was applied for the first time to the measurement of carbon content of microorganisms. By using this technique, we obtained a carbon:biovolume conversion factor of 125 fg C μm⁻³ for organisms ranging in volume from 0.69 to 1590 μm³: bacteria, nanoflagellates, dinoflagellates and ciliates. Below, we compare this carbon:biovolume conversion factor and each species-specific conversion factor to the values reported in the literature. In addition, we discuss the effect of cell preservation on cell volume and consequently on carbon:biovolume conversion factors.

Live *Escherichia coli* carbon:biovolume conversion factor (175 fg C μm⁻³) did not greatly differ from others reported previously in the literature (90 to 170 fg C μm⁻³, Norland et al. 1995; 92 to 160 fg C μm⁻³, Fagerbakke et al. 1996). Live *Uronema* sp. carbon:biovolume conversion factor (124 fg C μm⁻³) was half of Ohman & Snyder’s (1991) conversion factor (323 fg C μm⁻³, 710 μm³), while our carbon:biovolume conversion factor for *Pteridomonas danica* (124 fg C μm⁻³) was close to the only other reference found for live nanoflagellates (96.1 fg C μm⁻³ for Monas sp., Børshem & Bratbak 1987).

This study provides the first carbon:biovolume conversion factor for non-preserved heterotrophic dinoflagellates (123 fg C μm⁻³, *Oxyrrhis marina*). Mullin et al. (1966) had measured a carbon:biovolume conversion factors of 154 fg C μm⁻³ (calculated from Mullin et al. data) for the autotrophic dinoflagellate *Peridinium trochoideum*. In their study, cell biovolume was determined on live cells (Coulter Counter) and cell carbon content was measured by wet oxidation.

Lugol’s iodine preservation (2% final concentration vol./vol.) did not affect significantly live (Coulter Counter) cell volumes of *Oxyrrhis marina*. If there was any difference in volume between live and preserved cells, it was masked by the wide range of cell volumes of the population. However, a slight decrease in volume was observed in *Uronema* sp. Lugol’s iodine-preserved cells. Various authors have reported Lugol’s iodine (1 to 10% vol./vol.) underestimation of live protozoa and algae cell volumes (Choi & Stoecker 1989, Puitt & Stoecker 1989, Montagnes et al. 1994).

Cell preservation with formaldehyde (2% final concentration vol./vol.) did decrease *Pteridomonas danica* live cell volume by 17, 28 and 46% immediately, 1 h and 48 h after fixation, respectively. Formaldehyde has been reported to underestimate (Børshem & Bratbak 1987) but also to overestimate (Putt & Stoecker 1989) live cell volumes of protozoa. Børshem & Bratbak (1987) reported the nanoflagellate *Monas* sp. cell volume to decrease by 27 and 53% 2 h and 2 wk after formaldehyde (5% final concentration) addition, respectively.

DAPI staining live *Escherichia coli* resulted in lower cell volumes by 23%, while DAPI staining of formaldehyde-preserved bacteria resulted in lower cell volumes by 80%. DAPI is not entirely specific for DNA (Mosta-Rentsch 1998). Fixatives have been reported to cause a significant cell volume reduction in bacteria (Trueba & Woldringh 1980, Fuhrman 1981, Kogure & Koike 1987, Fagerbakke et al. 1996), although no significant effect was observed of formaldehyde addition (0.2%) by Fry & Davies (1985).

Our data indicates that preservation with Lugol’s iodine or formaldehyde leads to cell shrinkage. Cell volume reduction was more important for smaller compared to larger organisms. For the former organisms, cell preservation led to overestimated carbon:biovolume conversion factors. We compared previously reported preserved and live cell means of carbon:biovolume conversion factors calculated for bacteria, 517 ± 423 versus 90 ± 40 fg C μm⁻³, and concluded that preservation leads to higher carbon:biovolume conversion factors (p < 0.001) (Table 2). In these studies, preservation was conducted with glutaraldehyde (0.5–25%), formaldehyde (2–4%) and Lugol’s iodine (2%). Not enough data was available for testing the effect of preservation on carbon:biovolume conversion factors for small flagellates and ciliates. However, both mean literature conversion factors and changes in factors due to preservation-related shifts in cell volumes are in agreement with our results.
A log-log representation was used to allow 8 orders of magnitude in cell carbon and volume to be plotted on a single graph. We analysed the data with linear regression techniques.

A good correlation was obtained for live cells: bacteria volume versus carbon content ($x = \log(\mu m^3 cell^{-1})$, $y = \log(pg C cell^{-1})$, $y = -1.036 + 1.153x$, $r^2 = 0.905$, $n = 13$, $p < 0.0001$), bacteria and small flagellate volume versus carbon content ($y = -1.051 + 1.114x$, $r^2 = 0.953$, $n = 15$, $p < 0.0001$), and volume of all organisms versus their carbon content ($y = -1.046 + 1.058x$, $r^2 = 0.991$, $n = 21$, $p < 0.0001$). The slopes of the regression lines were not significantly different from unity ($p > 0.3$) indicating that there was a similar carbon content of the organisms as a function of volume. Carbon:biovolume conversion factors ranged from 106 to 115 fg C \(\mu m^{-3}\).

When plotting our live-cell experimental data on a log-log representation we obtained a carbon:biovolume conversion factor of 96.4 ± 1.6 (mean ± SE) fg C \(\mu m^{-3}\) ($x = \log(\mu m^3 cell^{-1})$, $y = \log(pg C cell^{-1})$, $y = -0.778 + 0.964x$, $r^2 = 0.999$, $n = 4$, $p < 0.0004$), which is close to the above calculated carbon:biovolume conversion factors.

A weaker correlation was observed for preserved organisms: bacteria volume versus carbon content ($x = \log(\mu m^3 cell^{-1})$, $y = \log(pg C cell^{-1})$, $y = -0.912 + 0.274x$, $r^2 = 0.04$, $n = 38$, $p > 0.2$), bacteria and small flagellate volume versus carbon content ($y = -0.485 + 0.874x$, $r^2 = 0.718$, $n = 47$, $p < 0.0001$), and volume of all organisms versus their carbon content ($y = -0.488 + 0.869x$, $r^2 = 0.968$, $n = 63$, $p < 0.0001$). The slope of the regression line was significantly lower than unity ($p < 0.002$) for bacteria.

Thus, according to these data smaller bacteria would have a higher carbon content than larger bacteria. Some authors reported that carbon:biovolume for bacteria changed from small to big bacteria, the former containing more carbon per unit of volume (Norland et al. 1987, Lee & Fuhrman 1987, Simon & Azam 1989), whereas such a trend was not found by others (Fagerbakke et al. 1996). The former studies were conducted on preserved cells but the latter on
live cells. Thus, cell preservation may be the explanation for these observed differences and for the higher carbon:biovolume conversion factors reported by Bratbak (1985).

We conclude that high temperature catalytic oxidation (HTCO) can provide accurate carbon contents of microorganisms. HTCO presents the advantage over the conventional CHN analysis of directly measuring liquid samples; filtration can thus be avoided. Our study indicates that a single carbon:biovolume conversion factor (125 fg C μm⁻²) could be applicable to bacteria and protozoa. The high variability in carbon:biovolume conversion factors reported in the literature would result from different degrees of cell shrinkage by preservation rather than from species-specific physiological or taxonomic differences. Shrinkage of cells after fixation, which may be variable depending on the species, should be taken into account when calculating microbial biomasses.

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