Heterotrophic utilisation of a labelled algal extract in water samples from a subtidal reef

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ABSTRACT: The major primary food source found on a typical Natal reef in South Africa is macrophytic detritus, largely of marine origin. Rapid release of soluble contents of the detritus is caused by vigorous surf action on the reefs; this was demonstrated experimentally. Free-living bacteria are the most common microorganism found in the water column and they appear to be heterotrophically the most active. It was thus postulated that they must use this dissolved substrate; if uniformly labelled, it should provide a suitable tracer for studying their heterotrophic activity. A salt-tolerant Chlorella sp. was cultured with NaH¹⁴CO₃ for sufficient duration to permit uniform labelling and its soluble cell contents and excretory products were extracted for this purpose. Extracts of specific activity 0.67 pCi mg⁻¹ organic carbon and 58.07 yCi mg⁻¹ organic carbon were prepared and used in heterotrophic potential experiments. The results are evaluated and compared with results of glucose uptake measured in parallel experiments and during previous seasonal sampling. The results indicate that microbial heterotrophic activity exceeded primary production by phytoplankton in the study area.

INTRODUCTION

A study on a nearshore subtidal reef in Natal, South Africa (Schleyer, 1980a, 1981), established that the major primary food source is seaweed, most of which enters the detritus food chain. Terrestrial macrophytic material is also introduced to the marine environment by rivers swollen by summer rains, making a further contribution to the detrital pool. The combined material accumulates in eddies adjacent to reefs such as the one studied and disintegrates rapidly in the rough surf. Soluble contents leach rapidly from it, a fact demonstrated in an experiment reported here in which the isotope released from sand-churned, labelled seaweed was measured. Free-living bacteria are the most common microorganism found in the water column; heterotrophically they appear to be the most active. It was thus postulated that they must use this dissolved substrate and, if uniformly labelled, the substrate should provide a suitable tracer to study their activity in heterotrophic potential experiments. Preliminary work using a labelled algal extract (LAE) for this purpose showed promise (Schleyer, 1980a) and the results of further experiments are presented in this paper.

MATERIALS AND METHODS

Study area

The reef studied is situated in front of the Oceanographic Research Institute (ORI), Durban, and is known as ORI Reef. It consists of sandstone, measures approximately 80 × 20 m and runs straight out to sea. It is subjected to heavy and continuous surf action and only at some spring low tides is a portion of the reef exposed. At high tide the reef is covered by 1 to 1.8 m of water and its top is usually about 1 m above the surrounding sand. Water conditions in the area are subtropical with an annual mean temperature of 21.8 °C (1957–1974); mean salinity is 35.2 ‰. ORI Reef is densely populated by the mussel Perna perna and is also inhabited by other suspension feeders, mainly the oyster Saccostrea margaritacea and the tunicate Pyura stolonifera, all of which are typical of this habitat in Natal. P. perna is capable of filtering particles as small as the free-living bacteria mentioned above (Schleyer, 1980b; Berry and Schleyer, 1983). Thus these bacteria probably constitute an important link in the food chain.
Demonstration of the release of dissolved organic carbon (DOC) from sand-churned seaweed

Freshly collected tufts of *Halimeda cuneata* and *Jania rubens* were incubated for one 24 h cycle (6L:12D:6L) at 22 °C in sterile-filtered seawater containing 1 μCi ml⁻¹ NaH¹⁴CO₃. Natural daylight from an east-facing window was used for incubation, after which the seaweeds were rinsed twice for 15 min in sterile-filtered seawater. They were divided in half and each half was separately placed in a 1 L Erlenmeyer flask containing 75 ml of washed, boiled beach sand and 200 ml sterile-filtered seawater. One in each pair of flasks was agitated for 2 s in every 10 s with a good shake and vigorous swirl to simulate churning surf. The other was retained as a control and was only gently agitated to ensure mixing before the removal of 1 ml subsamples of seawater. These were withdrawn periodically to monitor the release of label as a measure of DOC release from the seaweed by radio-active counting in 10 ml Insta-gel. Radio-active counts for early work described in this paper were performed using a Packard Model 3380 liquid scintillation counter fitted with automatic external standardisation and an Absolute Analyser Model 544 for conversion of cpm to dpm. This was later replaced with a similarly equipped LKB Model 1211 Minibeta liquid scintillation counter.

These 2 seaweeds were chosen for the study since their thalli are tough and they are persistent in debris adjacent to the OR1 Reef. If DOC release is demonstrable in these forms, it follows that it would be more evident in softer, more delicate species.

Production of labelled algal extract (LAE)

Production and use of LAE was similar to the methods described by Schleyer (1980a). A salt-tolerant *Chlorella* sp., obtained from an outdoor saltwater pond and adapted to continuous light irradiation of 2000 lux, was incubated at ambient temperature in a boro-silicate bottle at a dilution of 1:2 with 2% Alga-gro seawater medium (Carolina Biological Supply Company, Burlington, North Carolina, USA). Alga-gro is a nitrate and phosphate enriched algal growth medium free of carbonates and bicarbonates. At this, the normal application of Alga-gro (James, 1974), the high lipid levels stored by *Chlorella* in nitrogen-deficient media (Spoehr and Milner, 1949; Strickland, 1960; Round, 1965) were not expected. NaH¹⁴CO₃ of high specific activity (680 μCi mg⁻¹) was added to the culture at a final concentration of 10 μCi ml⁻¹ at the start of incubation. The labelled bicarbonate is diluted by carbonates and bicarbonates in seawater so the absence of these ions in the Alga-gro is considered an advantage. After incubation for 3 d to permit uniform labelling of the *Chlorella* cells, the culture was centrifuged and the pellet was reconstituted in a small quantity of sterile-filtered seawater. To it was added the residual particulate fraction from the incubation medium. This was collected on a 0.2 μm pore-size polycarbonate membrane filter and carefully removed with a clean razor blade by gently scraping it off after placing the filter on a flat sheet of clean glass. Algae excrete a wide range of organic substances (Heylebusch, 1974); excreted DO¹⁴C left in the medium was extracted by successively passing it through a SEP-PAK C₁₈ chromatographic cartridge (Waters Associates Inc., Milford, Massachusetts 01757, USA). The organic material was eluted with 2 ml of methanol and the process was repeated 5 times to ensure maximum extraction of the DO¹⁴C. The methanol was evaporated in a hot water bath at 45 °C and the dissolved organic material was also added to the reconstituted pellet. This was sonicated in an ice bath at 100 to 150 W for 20 min using an Ultrasonic SP914 probe sonicator and then repeatedly frozen and thawed to achieve maximum disruption of cellular components. Finally it was centrifuged at 1800 G for 30 min and the remaining ¹⁴CO₂ was flushed out by bubbling ¹²CO₂ through the supernatant which was then membrane-filtered (0.2 μm pore size) and frozen in a sterile container until use. DOC content of the resultant LAE was analysed using a Beckman Model 915A Carbon Analyser. A specific activity for the LAE of 58.07 μCi mg⁻¹ organic carbon was established by liquid scintillation counting, making it satisfactory as a heavily-labelled tracer according to the criteria of Wiebe and Smith (1977).

Measurement of heterotrophic potential

The method of Hobbie and Crawford (1969), adapted from the original method of Parsons and Strickland (1962), was employed with a few modifications (Schleyer, 1980a) to measure heterotrophic potential using tracer quantities of D-[U-¹⁴C]glucose and LAE in parallel experiments. Twenty-five ml seawater samples from OR1 Reef were inoculated to 5 final concentrations of between 19.3 and 96.6 μg C l⁻¹ LAE or 0.645 and 3.226 μg l⁻¹ glucose in sealed, heat-sterilized 125 ml Erlenmeyer flasks. These were incubated for 1 h in the dark at sea temperature; the assimilated particulate and respired fractions of ¹⁴C were then separately collected for counting. Sterile-filtered seawater samples with added labelled substrate were used as controls for background counts and for monitoring sterility of the substrates. At the end of incubation, addition of 1 ml of 2N H₃PO₄ to stop biological activity and release
RESULTS

DO\(^{14}\)C release from labelled seaweeds which were artificially sand-churned was 2.5 to 3 times faster than from controls which were gently agitated (Fig. 1). The release from *Jania rubens* was greater and by the end of the experiment the sand-churned fronds were breaking into fragments. The sand-churned lobes of *Halimeda cuneata* were not as severely damaged; they did not disintegrate but were well polished and were becoming pitted.

CAUSE precipitation of a component of the LAE; maximum filtrate counts were found in the controls in which no LAE was assimilated by bacteria. Therefore, filtration was performed immediately after incubation without fixation or, when large numbers of samples were handled, biological activity was effectively reduced by sample dilution with sterile-filtered seawater. Particulate material in each sample was filtered onto a 0.2 \(\mu\)m pore-size polycarbonate filter which was then digested by 100 \(\mu\)l phenylethylamine introduced into a capless vial suspended in each Erlenmeyer flask. After 1 h the contents of the vials were separately flushed into scintillation vials with three 3.3 ml aliquots of Instagel for counting. The efficiency of this system was calibrated for correction of the results by collection of the labelled gas released by acid from small amounts of NaH\(^{14}\)CO\(_3\). Linear regressions of Hanes-Woolf transformations (Segel, 1975) of the data for total uptake according to Michaelis-Menten enzyme kinetics (Wright and Hobbie, 1965, 1966) provided values for: \(V_{\text{max}}\), the theoretical maximum rate of uptake of the substrate; \(K+S\), the Michaelis-Menten transport constant and natural substrate concentration; and \(T\), the turnover time of the substrate. These experiments were performed at all seasons.

The uptake of labelled glucose and LAE measured in parallel heterotrophic potential experiments provided the mean seasonal results presented in Table 1. Previously published results (Schleyer, 1980a) on preliminary uptake work using labelled glucose and a similarly prepared LAE of lower specific activity are included for comparison. Data only of significance at

<table>
<thead>
<tr>
<th>Season</th>
<th>Temp. (°C)</th>
<th>(V_{\text{max}}) ((\mu)g C l(^{-1}) h(^{-1}))</th>
<th>(K+S) ((\mu)g C l(^{-1}))</th>
<th>(T) (h)</th>
<th>(V_{\text{max}}) ((\mu)g C l(^{-1}) h(^{-1}))</th>
<th>(K+S) ((\mu)g C l(^{-1}))</th>
<th>(T) (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spring 1979</td>
<td>22.0</td>
<td>47.6</td>
<td>1698.5</td>
<td>41.2</td>
<td>1.24 (1.3)</td>
<td>4.7 (7.7)</td>
<td>5.6 (5.4)</td>
</tr>
<tr>
<td>Winter 1981</td>
<td>20.9</td>
<td>2.5</td>
<td>94.9</td>
<td>37.8</td>
<td>0.12 (0.1)</td>
<td>4.8 (3.9)</td>
<td>44.5 (59.1)</td>
</tr>
<tr>
<td>Summer 1982</td>
<td>24.6</td>
<td>2.3</td>
<td>40.3</td>
<td>16.9</td>
<td>0.19 (0.5)</td>
<td>1.4 (5.0)</td>
<td>7.8 (13.7)</td>
</tr>
<tr>
<td>Autumn 1982</td>
<td>20.4</td>
<td>1.2</td>
<td>16.8</td>
<td>24.1</td>
<td>0.17 (0.5)</td>
<td>0.7 (2.7)</td>
<td>4.6 (7.6)</td>
</tr>
<tr>
<td>Winter 1982</td>
<td>21.1</td>
<td>4.5</td>
<td>100.1</td>
<td>22.5</td>
<td>0.18 (0.1)</td>
<td>2.0 (3.9)</td>
<td>17.4 (59.1)</td>
</tr>
<tr>
<td>Spring 1982</td>
<td>21.5</td>
<td>2.9</td>
<td>19.7</td>
<td>8.0</td>
<td>0.27 (1.3)</td>
<td>1.7 (7.7)</td>
<td>6.2 (5.4)</td>
</tr>
<tr>
<td>Total</td>
<td>21.7</td>
<td>12.2</td>
<td>401.8</td>
<td>26.4</td>
<td>0.38 (0.6)</td>
<td>2.8 (5.2)</td>
<td>16.9 (21.4)</td>
</tr>
</tbody>
</table>

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Table 1. Mean seasonal results of the uptake of labelled glucose and LAE measured in parallel heterotrophic potential experiments. Values are presented for \(V_{\text{max}}\), the theoretical maximum attainable rate of uptake, \(K+S\), the Michaelis-Menten transport constant and natural substrate concentration and \(T\), the turnover time of the substrate. Values for glucose in parentheses are seasonal results from a previous year's fortnightly results (Summer 1978-Autumn 1979).
the 95% level or better when tested with the F ratio test (Fisher and Yates, 1963) were used in the compilation of the table. Nine percent of the results for LAE and 22% for glucose were rejected as being not significant. Regressions from 55% of the LAE results had F ratios greater than 4 times the critical F value at the 95% level of confidence, indicating that they were predictive as well as significant (Draper and Smith, 1966). Values for uptake of LAE were consistently higher than those for glucose.

The preliminary work performed in spring 1979 yielded higher values for both substrates, and values for glucose uptake measured fortnightly in the previous year (Schleyer, 1981) were also elevated (figures in parentheses in Table 1). The data for $V_{\text{max}}$ from which Table 1 is derived are presented in Fig. 2 where the difference between the results obtained before and after 1980 is clearly portrayed. During 1980 extensive and prolonged dredging of a local river, the Mgeni, was initiated (indicated in Fig. 2 by an arrow), adversely increasing turbidity in the sea by a considerable amount and probably influencing the results.

Aspects of the work with results similar to those fully reported and discussed by Schleyer (1980) will not be repeated here, e.g. the amount of substrate respired, being ~ 22% of the glucose and ~ 1.5% of the LAE taken up.

![Fig. 2. $V_{\text{max}}$ values of labelled glucose and LAE from which Table 1 is derived. Prior to 1980 high values were obtained for uptake of both substrates which were not found in later work. During 1980 extensive and prolonged dredging of a local river, the Mgeni, was initiated (indicated by arrow). This increased the turbidity in the sea considerably and probably influencing the results.](image)

**DISCUSSION**

Use of LAE to trace microbial heterotrophy on the ORI Reef is only valid if seaweed-derived DOC is in fact released as a natural food resource. Although such release is considered an obvious consequence of damage to seaweed (Wangersky, 1978), it was nevertheless demonstrated in the experiment in which robust seaweeds from the reef were artificially sand-churned (Fig. 1). The release would undoubtedly have been greater with softer algal species.

Since seaweed appears to be the main source of the natural dissolved substrate, LAE derived from this source would be most suitable for uptake studies. Careful selection of the alga for this purpose would be important as the biochemistry of the chosen species should be typical of seaweeds which contribute to debris on Natal reefs. Green and red algae are the most common seaweeds on the Natal coast (Jackson, 1976) and besides the composition of their pigments, which comprise only a small proportion of their mass (Strickland, 1965), the major difference between the 2 groups is in their storage products – starch and floridean starch, respectively (Boney, 1966; Lewin, 1979). Both of these are α-(1,4)-linked glucans and are readily hydrolysed by amylases (Craigie, 1974). They consist largely of amylopectin, but floridean starch also contains a small number of α-(1,3)-linkages and chlorophyte starch a small amount of amylose (Boney, 1966; Craigie, 1974). The 2 algal groups are biochemically similar in other respects except in their composition of structural polysaccharides which are of no significance in the production of a soluble LAE. Thus a suitable representative from either should satisfy the biochemical criteria required for LAE production. The only exception to the above is the family Ulvaceae, members of which occur on the reef but would be unsuitable as they atypically produce sucrose as a storage product (Boney, 1966; Percival and Smestad, 1972).

Uniformity in labelling is another criterion essential for successful use of LAE in the present work. Since seaweeds are differentiated with meristematic regions this would be difficult to achieve, except by incubating for a long period to permit considerable growth. Attempts to do this with suitable seaweeds proved unsuccessful (Schleyer, 1980b) and it was decided that the problem of uniformity in labelling could largely be surmounted by using a healthy unicellular phytoplankton species such as the chlorophytes, prymnesiophytes and diatoms. However, the storage product of the latter 2 is the β-(1,3)-glucan, chrysosaminarin (Lewin, 1974; Sieburth, 1979), and never starch (Boney, 1966). Thus their biochemistry...
cannot be considered representative of that of Natal reef seaweeds and they too were rejected.

Finally the well-known phytoplankter, *Chlorella*, was chosen and labelled with considerable success. High lipid levels are stored by many algae when grown in nitrogen-deficient media (Round, 1965) and *Chlorella* is no exception (Spoehr and Milner, 1949; Strickland, 1960; Round, 1965). However, it appears to be biochemically typical of the chlorophytes when cultured in non-deficient media (Milner, 1961) and care was taken to provide it with optimum growth conditions. LAE considered to be analogous to seaweed-derived DOC was thus produced.

Schleyer (1980a) presented evidence that the $V_{\text{max}}$ values for LAE uptake provide an approximate measure of the bulk of microbial heterotrophic production in OR1 Reef water samples. In this evaluation of the preliminary work, uptake of the *Chlorella* LAE was linear, even though the LAE was a complex mixed substrate taken up by morphologically different bacteria which possibly had different uptake rates (Schleyer, 1980a). A similar finding in the present work is indicated by the high statistical validity of the LAE results. Using the same technique, Bell (1980) found the uptake of labelled phytoplanktonic extracellular products was also linear according to enzyme kinetics. Such extracellular products generally consist predominantly of a few compounds of low molecular weight (Hellebust, 1965, 1974), and Bell suggested that linearity of uptake of the tracer indicated that an identical substrate was supporting natural heterotrophic activity *in situ*. His argument was that a bacterial interaction with the substrate had developed by adaptation and enzyme induction in the natural environment and natural heterotrophic activity was measured using the labelled extracellular products. Bell’s interpretation of non-linear uptake or of regressions with zero slope would be that the tracer was of minimal importance to the bacteria relative to their utilization of other nutrient sources. This he demonstrated by adapting the natural bacteria to a foreign substrate before incubation with the algal extracellular products or by incubation with extracellular products from a foreign source. In the present and previous work, uptake of the LAE commenced and continued without fluctuation from the start of the short incubation period which was of insufficient duration to permit adaptation to the substrate. Thus the linearity of LAE uptake suggests that it was representative of uptake of the complex natural substrate to which the bacterial population on OR1 Reef had already adapted, and the measured rate of uptake was an average for different rates which may have occurred within the population.

Uptake of LAE was considerably greater than that of $^{14}$C glucose (Table 1) which merely provides an index of heterotrophic activity. Although increase or decrease in the uptake of one of these was generally accompanied by a similar change in the uptake of the other, there was no significant mathematical relationship between the rates of uptake of the 2 labelled substrates. This was true of both *Chlorella* extracts despite the fact that they were separately produced and used at entirely different concentrations.

Environmental disturbance seems to have influenced the results collected after 1980 (Fig. 2) but it is unlikely that increased turbidity would have affected the activity of microbial heterotrophs to the extent to which it appears depressed. A more likely suggestion is that the turbidity impaired primary production by seaweeds, thus reducing the major source of autochthonous detritus on reefs in the study area.

Even though the measurements taken after 1980 were probably depressed (Fig. 2), a mean daily value of 293 $\mu$g C l$^{-1}$ d$^{-1}$ calculated from the $V_{\text{max}}$ of LAE exceeds that of phytoplanktonic primary production measured in the study area in previous years ($\approx$154 $\mu$g C l$^{-1}$ d$^{-1}$; Schleyer, 1981). The results for LAE also show that assimilable DOM is turning over in a relatively short time ($\approx$T = 26.4 h). This supports earlier findings that microbial heterotrophs are more important than phytoplankton in energy transfer to suspension-feeders on Natal reefs. Much of the detritus on which the microheterotrophs depend becomes available to them as DOM in accordance with a model, based largely on work on coastal ecosystems, in which half or more of the primary production is channelled in this manner (Williams, 1981). In the present case the situation is produced, or at least encouraged, by the turbulent surf conditions in the study area, hence the greater abundance and activity of free-living bacteria than their counterpart attached to detritus particles in the water column (Schleyer, 1981).

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**LITERATURE CITED**


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