

Utilisation of bacteria as nitrogen resource by kelp-bed mussel *Choromytilus meridionalis*

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ABSTRACT: The crystalline style of *Choromytilus meridionalis* Krauss contains a bacteriolytic enzyme capable of lysing the majority of free-living bacteria in the adjacent water column. Estimates of the carbon-to-nitrogen ratio (3.7:1) of free-living bacteria, and of the filtration capabilities of the mussels, indicate that bacteria could meet the nitrogen requirements of the mussels. The bacteriolytic agent in the style is subject to considerable adaptive changes in activity, correlated with water temperature. Water temperatures < 10 °C are associated with induction of the bacteriolytic agent. During upwelling, cold water depleted in particulate matter but containing significant numbers of bacteria occurs commonly amongst the kelp beds. It is suggested that low water temperature (or an associated environmental parameter) results in stimulation of bacteriolysis. This in turn permits efficient utilisation by the mussel of free-living bacteria which compensate for the depletion of phytoplankton available in the water column.

INTRODUCTION

There have been many studies of the potential significance of bacteria in the nutrition of marine animals (recent reviews: Watson, 1978; Mann, 1982). Although some filter-feeding bivalves such as the bay scallop *Argopecten irradians* utilise phytoplankton rather than the microbial component of plant detritus (Kirby-Smith, 1976), many other filter-feeders including both bivalves (Sorokin, 1973) and sponges (Reiswig, 1971) may derive a major proportion of their diet from bacteria and associated sub-particulate material. A wide range of deposit-feeding organisms, especially those exploiting detrital food resources with high C:N ratios such as occur near to saltmarshes and macroalgae, have also been shown to derive a significant proportion of their diet from the ingestion of the bacterial component of decomposing plant detritus. Such organisms include polychaetes (Tenore, 1977a, b), gastropods (Newell, 1965; Wetzel, 1976), amphipods (Fenchel, 1970), mysids (Foulds and Mann, 1978), prawns (Moriarty, 1976, 1977), holothurians (Yingst, 1976), and fish (Moriarty, 1976).

Some of these deposit-feeders may actively select smaller-sized particles from the deposits and thus enrich the bacterial component of their diet compared with non-selective deposit-feeders (review: Newell,

1979). Prieur (1981) showed, in addition, that the gut microflora may divide several times during passage through the gut of the mussel *Mytilus edulis*, so that the yield from bacteria could be considerably in excess of the standing stock or biomass of bacteria ingested with the diet. Complex interactions between gut microflora and detritus utilisation by the host have also been implicated in studies on the nutrition of *Mysis sterolepis* (Foulds and Mann, 1978) and *Strongylocentrotus* (Fong and Mann, 1980). More recently, Newell and Field (1983), in a study of carbon and nitrogen flux through kelp beds, estimated that utilisation of bacteria associated with detritus could contribute as much as 69 % of the nitrogen requirements of the consumer community as a whole. Both the interactions of the gut microflora with the ingested detrital diet and the utilisation of free-living bacteria ingested with particulate debris are thus potentially implicated in the nutrition of detritivores.

There have, however, been very few investigations of the digestive enzymes actually involved in the utilisation of detritus and its associated microflora by invertebrates. Kristensen (1972) and Yingst (1976) have reviewed the evidence that comparatively few deposit-feeding invertebrates possess the necessary enzymes to digest the structural carbohydrates which make up the bulk of aged plant detrital material.

However, Stuart (1982) and Stuart et al. (1982) have recently shown that the kelp-bed mussel *Aulacomya ater* is capable of absorption of sterilised kelp detritus with an efficiency of approximately 50%. Again, Seiderer et al. (1982) have shown that 2 mussels, *Choromytilus meridionalis* and *Perna perna*, both possess the necessary style carbohydrases to digest plant detrital material. Furthermore, the rate of digestion by the style enzymes was linked to the gut passage time in such a way that the carbon requirements of the mussels could be met from a detrital diet alone.

Apart from the work of McHenery et al. (1979), and McHenery and Birkbeck (1979, 1982) there have been very few studies of the digestive enzymes which are required for the utilisation of the bacterial component of such detrital diets. McHenery et al. (1979) described the occurrence of a lysozyme-like enzyme in the bivalves *Mytilus edulis*, *Modiolus modiolus*, *Chlamys opercularis* and *Mya arenaria* and suggested that its primary role is in the utilisation of bacteria. McHenery and Birkbeck (1982) subsequently showed that the enzyme from *M. edulis* is a true N-acetylmuramyl-hydrolase capable of degrading the cell walls of a variety of bacteria including *Micrococcus luteus*, *Escherichia coli* and *Bacillus subtilis*.

Clearly, the occurrence and activity of such lysozyme-like enzymes is of importance in estimating the significance of bacteria as a carbon, and above all, a nitrogen resource for consumer organisms which are exploiting detrital diets with a high C:N ratio. The relative resistance of the resident gut microflora to such lysozyme-like enzymes compared with free-living bacteria ingested with the food may yield some insight into the role of free-living as opposed to resident gut microflora in the nutrition of the consumer. The following work was therefore undertaken to examine the occurrence and role of lysozyme-like enzymes in the utilisation of bacteria as a protein resource by the kelp-bed mussel *Choromytilus meridionalis* whose carbon balance on a detrital diet has already been established in previous publications (Seiderer and Newell, 1979; Seiderer et al., 1982).

MATERIALS AND METHODS

Sampling and preparation of style extract

Specimens of the black mussel *Choromytilus meridionalis* Krauss were collected from a rocky intertidal reef at Bloubergstrand, West Coast of South Africa, between Nov 1982 and Feb 1983 and transferred to the laboratory for immediate extraction of the crystalline style. The styles of 20 specimens were removed, rinsed and homogenised over ice with a

glass tissue grinder in 12 ml 20 mM phosphate buffer, pH 7.0, containing 150 mM NaCl (see also Langton, 1977; Seiderer and Newell, 1979; Seiderer et al., 1982). All glassware had been sterilised immediately prior to use. The homogenate was centrifuged at $15,000 \times g$ for 5 min and the supernatant diluted to 1.00 mg protein ml^{-1} (Groves et al., 1968) with phosphate buffer before using in lysozyme and protease assays.

Isolation and culture of bacterial strains

In order to isolate water-column bacteria, samples of seawater were taken from Oudekraal, also on the West Coast of South Africa, and incubated in the laboratory at 10 °C with 0.5 g l^{-1} sterilised powdered kelp detritus. This had been prepared by grinding freeze-dried tips of the fronds of *Laminaria pallida*, sieving to obtain particles from 43 to 63 μm diameter, followed by sterilisation for 24 h under a UV light (see also Stuart et al., 1981). Subsamples of 1 ml were taken after 3 d of incubation and pipetted onto agar plates made of 1.5% agar in seawater growth medium. The liquid growth medium comprised three parts 0.45 μm filtered seawater, one part glass-distilled water and 0.5% w/v peptone (Oxoid), 0.1% w/v yeast extract (Difco) and 0.01% w/v ferric phosphate (Mazure, 1977). Plates were then incubated at 25 to 30 °C for 24 to 48 h. Twenty five strains were isolated and restreaked at least 5 times before being assigned to collections and stored on slants of seawater agar at 4 °C.

Bacterial isolates were also prepared from the gut contents of *Choromytilus meridionalis*. The guts of 4 mussels were removed and the contents cultured on seawater-agar plates at 20 °C after treatment with 1% trypsin for 30 min at 30 °C. Cultures were also made from homogenised gut tissues and a total of 20 strains were isolated and stored on seawater agar at 4 °C.

Each of the isolates in the collections was then incubated in an orbital shaker at 30 °C for 10 h in 2 ml seawater liquid growth medium. These were then reinoculated into 50 ml growth medium and grown at 30 °C for a further 12 h before being used for preparation of experimental agarose plates.

Preparation and incubation of plates

The activity of lysozyme-like enzymes on free-living bacteria and on bacteria isolated from the gut of *Choromytilus meridionalis* was assayed on 0.8% agarose plates containing heat-killed target bacteria (McHenery et al., 1979). The bacterial suspensions were centrifuged at $8,000 \times g$ for 15 min and the

pellets resuspended in 2 ml sterile seawater. The suspensions were then adjusted with sterile seawater so that the final optical density in the agarose medium would be 0.122 at 600 nm, and added to 20 ml agarose medium. The media were then heat-shocked at 65 °C for 10 to 15 min to kill the bacteria, and poured into 8.4 cm diameter petri dishes. Wells of equal diameter were cut into each agarose plate to receive the enzyme extract.

Eight serial 10-fold dilutions of enzyme extract (p. 110) were prepared and aliquots of 10 µl of each of the dilutions were placed individually into the wells and incubated at 20 °C for 45 h. Intact bacteria were stained by addition of 5 ml of 0.12 % crystal violet to each plate. After 2 h, the plates were rinsed with distilled water and the diameter of the clear zones of lysis was measured. From the number of bacteria per unit surface area of plates, the area of the clear zone could be expressed both as number of bacteria lysed per 45 h of incubation at 20 °C, and its equivalent in bacterial protein. The equations for the regressions were: bacterial number lysed ($\times 10^5$) $\hat{y} = -0.92 + 0.11 X$; bacterial protein (µg) $\hat{y} = -0.36 + 0.05 X$ (where X = zone diameter in mm).

Interference contrast micrographs

An isolated water column pseudomonad was cultured in seawater growth medium (p. 110) and rinsed in 5 ml 10 mM ammonium acetate buffer pH 7.0 to remove salts. To the experimental bacteria, a 1-in-5 volume of style extract which was known to have bacteriolytic activity was added. In the control tube the style extract was replaced with phosphate buffer, pH 7.0. Micrographs were taken after 30 min using a Zeiss microscope equipped with interference contrast optics.

CHN-analysis

Particulate organic carbon and nitrogen measurements were made on seawater which had been initially filtered through a 200 µm mesh net to remove larger particulate material. A measured volume of 100 to 1,000 ml, depending on the particulate load, was filtered under a vacuum of < 12 cm Hg through 25 mm Whatman GF/C glass fibre filters which had been preashed at 400 °C for 6 h. The filters were stored at -20 °C and then oven-dried at 55 °C prior to analysis with a Heraeus model CHN-Mikro Universal combustion analyser calibrated with cyclohexanone (Monar, 1972).

Bacterial carbon and nitrogen measurements were made on five isolates of bacteria which had been

cultured in both nutrient-rich (18 h incubation at 30 °C) and nutrient-poor (6 d incubation at 15 °C) media. The nutrient-rich medium consisted of liquid seawater broth, and the nutrient-poor medium of artificial seawater (Sieburth, 1979) to which 25 µg atoms Nitrate N ml⁻¹ and 6.25 mg Mannitol l⁻¹ had been added. This nutrient level and incubation temperature is thought to approximate that found under upwelling conditions in the kelp bed. The cultured bacteria were harvested by centrifugation at 7,000 × g for 10 min, resuspended in ammonium acetate buffer pH 7.0, and lyophilised to constant weight. These weighed samples were combusted in the Heraeus combustion analyser to obtain C:N ratios for locally isolated bacteria.

RESULTS

Lysis of target bacteria

The zones of lysis which formed the basic assay for lysozyme activity in extracts of the crystalline style from *Choromytilus meridionalis* are shown in Fig. 1. The assay for the activity of this factor is extremely well reproducible and sensitive, due to the use of crystal violet to enhance contrast of low levels of target bacteria. The bacterium used as the target organism throughout this study was isolated from the water column near the sampling site at Oudekraal and iden-

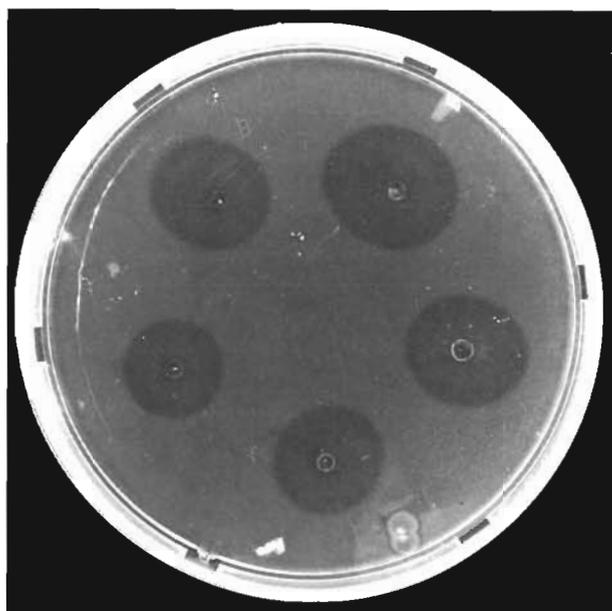


Fig. 1. *Choromytilus meridionalis*. Plate assay for lysozyme-like activity in crystalline style extracts in which heat-killed target bacteria suspended in sea-water agarose are lysed by serial 1:10 dilutions of style extract

tified as a pseudomonad. Several bacterial isolates from the water column displayed similar sensitivities, independent of bacterial genus; on average 57 % of the water column bacteria were susceptible to lysis. Bacterial isolates from onshore sampling sites, in particular from the gut of *C. meridionalis*, displayed varying, and in some cases complete, resistance to the lytic activity in active style extracts (Muir et al., in prep.).

The lysozyme appears to induce breakdown of the outer membrane and cell walls, as shown in Fig. 2, and results in the liberation of the cell contents of the water column bacteria which are thus available for absorption by the mussel. The diameter of the zone of lysis, and hence the number of target bacteria lysed, can be calculated from the regression: bacterial number lysed ($\times 10^5$) = $-0.92 + 0.11 X$. The equivalent protein liberated from bacterial breakdown is related to the diameter of the zone of lysis by the regression: bacterial protein (μg) = $-0.36 + 0.05 X$ (where X = zone diameter in mm; see also p. 111).

Variations in bacteriolytic activity

One of the striking features of bacteriolytic activity from the crystalline style of *Choromytilus meridionalis* is that it is very variable, reaching a minimum during periods of onshore wind with associated downwelling of phytoplankton-rich water which is commonly from 14 to 15 °C. Conversely, during offshore wind when cold upwelled water of approximately 9 °C impinges on the kelp bed, there is a considerable reduction in phytoplankton available for consumption by the filter-feeders (see Field et al., 1977, 1980, 1981). Turnover of style enzymes can take place within 18 h (Seiderer et al., 1982) and bursts of lytic activity were observed within 24 h of the onset of upwelling. These temporal variations in the activity of lysozyme-like enzymes in the style therefore appear to be actively induced by changes in environmental conditions during the upwelling-downwelling cycle.

The relation between the bacteriolytic activity of crystalline style extracts and the temperature of the water during a complete upwelling-downwelling cycle in March, 1983, is shown in Fig. 3. There is evidently an inverse correlation between seawater temperature and the activity of lysozyme-like enzymes in the style, periods of upwelling and relatively low phytoplankton abundance being associated with high bacteriolytic activity by the style. The relation between bacteriolysis and water temperature is described by the following linear regression: cells lysed by 1 μl style

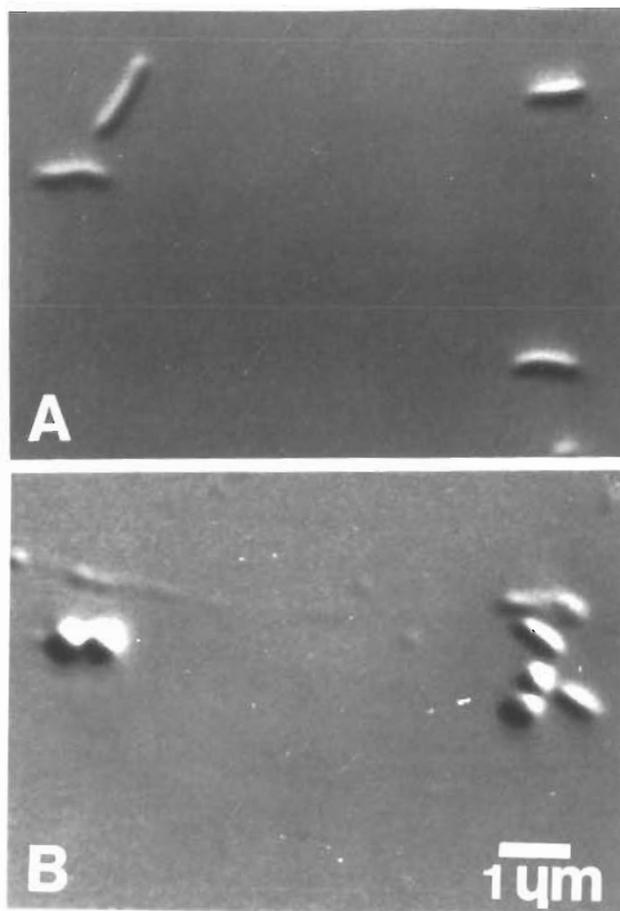


Fig. 2. Interference contrast micrographs showing an intact *Pseudomonad* (A) and the same *Pseudomonad* after 30 min exposure to a 1-in-5 volume of style extract of *Choromytilus meridionalis* (B)

extract ($\times 10^5$) = $ke^{-1.24T}$, where T = seawater temperature °C; $k = e^{14.88}$ cells.

Using direct microscopy, mean bacterial numbers were 4×10^5 cells ml^{-1} during upwelling and 2 to 3×10^6 cells ml^{-1} during downwelling conditions (Linley*, pers. comm.). Table 1 shows C:N ratios of particulate material from macrophytes, phytoplankton and bacteria. From this it is clear that the bacteria which are likely to be lysed primarily during periods of upwelling of cold phytoplankton-poor water represent a relatively nitrogen-rich nutritional resource. Periods of downwelling of phytoplankton-rich water (Hutchings, 1981; Mann, 1982) are likely to be associated with a higher C:N ratio in the potential food supply in the water column.

The extent to which these trophic resources could meet the carbon and nitrogen requirements of the consumer mussels can be calculated from the concentration of materials in the water column, coupled with some estimates of the consumer demands based on the carbon and nitrogen budgets for individual mussels.

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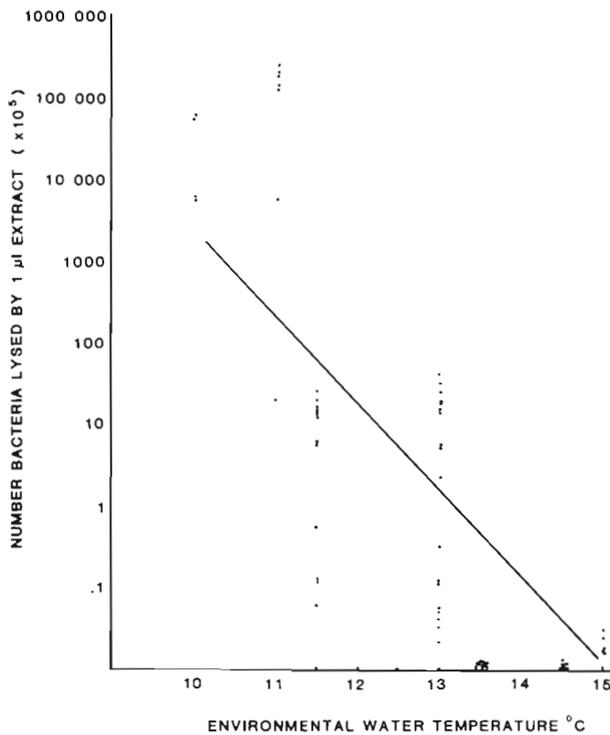


Fig. 3. *Choromytilus meridionalis*. Numbers of bacterial cells lysed by 1 ml of crystalline style extract, plotted as a function of environmental sea-water temperature. Regression equation: cells lysed = $ke^{-1.124T}$, where $T = ^\circ\text{C}$; cells lysed = the number of cells lysed by 1 μl style extract ($\times 10^5$) and $k = e^{14.88}$ cells

Quantitative significance of style bacteriolytic activity in relation to nitrogen budget

The data presented above allow some estimates to be made of the total bacteriolytic activity of the style, and of its potential ability to meet the nitrogen requirements of mussels of different sizes, provided that the total style protein in relation to body size is known.

The following regression (Seiderer et al., 1982) relates total style protein (mg) to *Choromytilus*

Table 1. Comparative data on the C:N ratios of particulate or sub-particulate organic matter associated with kelp beds

Particulate organic matter	C:N	Source
Macrophytes	15.6	Dieckmann (1978), Koop et al. (1982)
Phytoplankton	6.6 7.3	Bailey (pers. comm.) Bishop et al. (1978)
Bacteria: Nutrient rich	3.5	Present study
Nutrient poor	3.9	Present study

meridionalis shell length (mm); $\hat{Y} = -3.47 + 0.16 X$ ($r = 0.89$, $n = 20$). This can be used to relate the bacteriolytic activity of the proteins to the nitrogen requirements of the mussels in the following way. The protein concentrations of the style extracts were standardised to $1 \mu\text{g} \mu\text{l}^{-1}$, and the cell lysis shown in the agarose plates (Fig. 1) was expressed in terms of the number of bacteria lysed per μl of extract in 45 h. This can be converted to the amount of bacterial nitrogen made available ($N = \text{protein}/6.5$) (Newell and Field, 1983) by a single crystalline style and related to the size of the mussel. The style turnover time for *C. meridionalis* is approximately 18 h (Seiderer et al., 1982); hence the final figure of maximum lytic capacity of the crystalline style has been expressed as $\mu\text{g N}$ made available in 1 h. An estimate of the nitrogen requirements of different-sized mussels, using oxygen consumption data of Griffiths (1980), gave a mean value for respiration (R) of 74% of the absorbed ration (A).

Values for the oxygen consumption and its nitrogen equivalent, calculated using C:N ratio of 4.74 (taken from Hawkins, 1983, for *Mytilus edulis*) and the absorbed ration at $1.35 \times$ the respiration are summarised in Table 2.

Table 2. *Choromytilus meridionalis*. Nitrogen requirements at different sizes (shell length, mm). Data for nitrogen requirements recalculated as $\mu\text{g h}^{-1}$ from oxygen consumption ($\mu\text{l h}^{-1}$) and absorbed ration (A) in Griffiths (1980) using a C:N ratio of 4.74 (taken from Hawkins, 1983, for *Mytilus edulis*)

Shell length (mm)	Respiration (R)		Absorbed ration (A)
	($\mu\text{lO}_2 \text{ h}^{-1}$)	($\mu\text{gN h}^{-1}$)	($\mu\text{gN h}^{-1}$)
20	65	7.342	9.9
40	220	24.873	33.6
60	390	44.072	59.5
80	670	75.717	102.2
100	1000	113.228	152.8

Data for filtration rate and bacterial yield from the water column for *Choromytilus meridionalis* of different sizes are summarised in Table 3. The filtration rates, recalculated from Griffiths (1980), are related to body size by the equation: Filtration rate (l h^{-1}) = $6.44 \times 10^3 \cdot (\text{shell length, mm})^{1.5764}$. Since the bacterial numbers in the water column were 4×10^5 cells ml^{-1} and 2 to 3×10^6 cells ml^{-1} during upwelling and downwelling respectively (Linley, pers. comm.), the protein equivalents were 1.876 and $13.70 \mu\text{g}$ protein ml^{-1} or 288.6 and $2,106.9 \mu\text{g N l}^{-1}$, respectively, using a protein:nitrogen ratio of 6.5:1. This value has been used to estimate the gross nitrogen yield available by

Table 3. Bacteria as a potential nitrogen resource for *Choromytilus meridionalis*. Filtration rates ($l\ h^{-1}$) of mussels of different sizes (mm shell length) are from Griffiths (1980). Direct counts of bacterial numbers in the water column (bacteria ml^{-1}) (Linley, pers. comm.) were converted to bacterial protein ($\mu g\ protein\ ml^{-1}$) and thence to bacterial nitrogen ($\mu g\ N\ l^{-1}$). Filtration rates were used to convert the amount of bacterial nitrogen in the water column to the amount available to the mussel, assuming that only 57% of the bacteria are susceptible to lysis (Muir et al., in prep.) and that the retention efficiency of 0.5 μm bacteria by *C. meridionalis* is 10% (Stuart, 1983). Maximum lytic activity ($\mu g\ N$ made available h^{-1}) was calculated from Fig. 3

Shell length (mm)	Filtration rate ($l\ h^{-1}$)	Bacterial N from water		N required by mussel ($\mu g\ N\ h^{-1}$)	Maximum lysis ($\mu g\ N\ h^{-1}$)
		Minimum ($\mu g\ N\ h^{-1}$)	Maximum ($\mu g\ N\ h^{-1}$)		
20	0.724	12	87	10	—
40	2.160	36	259	34	125
60	4.092	67	491	60	261
80	6.441	106	774	102	339
100	9.156	151	1099	153	535

filtration by each size class of mussel. However, as pointed out on p. 111, only 57% of the water column bacteria were found to be susceptible to lysis. This and a retention efficiency of only 10% of 0.5 μm bacteria by *C. meridionalis* (Stuart and Klumpp, 1984) were corrected for in the final calculation (Table 3).

From this it can be seen that the nitrogen yield potentially available from lysis of the susceptible component of bacteria in the water column shows a general correspondence with the estimated nitrogen requirements of each size class of mussel. In addition, the maximal lytic activity which could be achieved at high bacterial concentrations, and which is shown in the final column of Table 3, is almost 4 times the nitrogen required by the mussels. It seems likely, therefore, that under upwelling conditions when the phytoplankton and macrophyte detritus loads are low, the nitrogen requirements of *C. meridionalis* can be met by the activity of the bacteriolytic enzymes of the style.

CONCLUSIONS

Lysozyme-like enzymes present in the style of the mussel *Choromytilus meridionalis* are capable of lysing approximately 57% of free-living bacteria in the water column adjacent to kelp beds. Estimates of the biomass of such bacteria through an upwelling-downwelling cycle and of the filtration rate of different-sized mussels suggest that free-living bacteria could meet the estimated nitrogen requirements of the mussels.

* Dr. A. J. Hawkins (Inst. for Marine Environmental Research, Plymouth) pointed out that a more direct method would be to use the O:N ratio. He has recently shown that in *Mytilus edulis* this ratio varies seasonally from 21 to 107 in well-fed mussels but is generally low in starved individuals. Use of the O:N ratio for *M. edulis* yields values similar to those which were calculated in Table 2 from the C:N ratio

The bacteriolytic activity of the crystalline style is, however, in a remarkable and apparently adaptive equilibrium with the food resources available for exploitation in the water column during upwelling and downwelling conditions. When upwelling occurs, the water temperature is approximately 9 °C and is poor in phytoplankton. Under these conditions, lysozyme-like activity is at its maximum and bacteria predominate as a nitrogen-rich exploitable food resource with a C:N ratio of approximately 3.7:1. These phases of active upwelling are interspersed with an opposite flow of phytoplankton-rich warm surface water which occurs often within 24 h following an upwelling phase (Field et al., 1977). Under these conditions lysozyme-like activity of the crystalline style is at a minimum. Nutritional requirements are then met by the activity of the carbohydrases in the crystalline style which are capable of digesting the cell walls of phytoplankton and thus making protein available from the cell contents, as well as meeting the carbon requirements of the mussels from a detrital diet alone (Seiderer et al., 1982).

Whether these adaptive changes in the enzyme activity of the style are induced by the qualitative differences in particulate matter available in the water column, or by abrupt temperature changes associated with the upwelling-downwelling cycle is at present unknown. The rate of turnover of the style compared with the response time for the appearance of lysozyme-like activity suggests, however, that specific enzymes may be incorporated into the style to meet relatively short-term changes in nutritional conditions in the water column near to kelp beds.

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