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# Utilisation of seaweed carbon by three surface-associated heterotrophic protists, *Stereomyxa ramosa*, *Nitzschia alba* and *Labyrinthula* sp.

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ABSTRACT: In view of the abundance of protists associated with seaweeds and the diversity of nutritional strategies displayed by protists in general, the ability of 3 closely associated protists to utilise seaweed carbon was investigated. *Stereomyxa ramosa*, *Nitzschia alba* and *Labyrinthula* sp. were cultured with seaweed polysaccharides as well as seaweed itself. *N. alba* and *Labyrinthula* sp. were found to utilise seaweed polysaccharides in axenic culture. All 3 protists were capable of penetrating intact but 'damaged' (autoclaved) seaweed particularly when bacteria were present. The possibility that these and other heterotrophic protists are directly removing macroalgal carbon in the field is discussed.

KEY WORDS: Protist · Heterotroph · Seaweed · Carbon · Utilisation

## INTRODUCTION

Heterotrophic protists are abundant on seaweed surfaces (Rogerson 1991, Armstrong et al. 2000) because seaweeds are rich in bacterial prey (Laycock 1974, Shiba & Taga 1980). But given the abundance of protists and the diversity of nutritional strategies displayed by these organisms, it is possible that some heterotrophic protists are capable of utilising dissolved or particulate seaweed carbon directly. Carbon fixed by seaweed during photosynthesis is used to produce structural and storage products; however, excess is released to the surrounding waters as dissolved carbon or sloughed off as particulate carbon. Sieburth (1969) indicated that this loss of photosynthetic product may be considerable, perhaps as high as 40% of daily photosynthate production. It is generally accepted that bacteria are the main scavengers of dissolved carbon and that they can rapidly utilise any soluble products (Lucas et al. 1981, Rieper-Kirchner 1989). This conversion of dissolved carbon into particulate forms (as bacterial biomass) is thought to be a major link to higher trophic levels (Newell et al. 1980). To compete effectively with bacteria, heterotrophic protists would need to occupy a more favourable location. Spatial partitioning on seaweeds has been noted before. For example, *Navicula endophytica* can be isolated from seaweeds by squeezing the tips of several undamaged species of brown macroalgae (Wardlaw & Boney 1984), and flagellates and ciliates have been found within *Codium bursa* (Vaqué et al. 1994). This seaweed consists of hollow, water-filled spheres that provide a refuge for these protists.

Seaweed cell walls are composed of a complex array of carbohydrates that form the crystalline phase (the skeleton) and the amorphous phase (the matrix). The skeletal component is embedded in the matrix polysaccharides which are also called phycocolloids. The phycocolloids that make up the wall vary considerably among species (Kloareg & Quatrano 1988) although most are based on glucose and galactose. Amounts of phycocolloid also vary as a function of age, life history

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stage, physiological status, habitat and season. The storage products of seaweeds can be monomers or polymers, frequently mannitol and glucans.

Despite the fact that bacteria are efficient scavengers of dissolved carbon and that particulate algal carbon is composed of complex polysaccharides, making it difficult to digest, a few studies have suggested that some protists may be capable of utilising macroalgal carbon directly in the field. For example, Sherr (1988) demonstrated that some estuarine flagellates can use high molecular weight polysaccharides found in surface waters, and one amoeba, *Trichosphaerium sieboldi*, has been shown to be capable of digesting intact seaweeds (Polne-Fuller et al. 1990, Rogerson et al. 1998).

In light of the above, it follows that any protists capable of competing with bacteria for algal carbon will be closely associated with the seaweed surface. Over the course of a 1 yr study investigating protists inhabiting seaweeds (Armstrong et al. 2000) 3 isolates were identified as 'intimately associated' protists that may be capable of utilising seaweed product. The first protist chosen was a colourless, heterotrophic diatom, *Nitzschia alba*. Heterotrophic diatoms have been noted previously on seaweeds (e.g. Li & Volcani 1987), but only 7 species have been described. Compared to photosynthetic diatoms, little is known about their biology. The second protist was *Labyrinthula* sp. which occurred throughout the year on different seaweeds. *Labyrinthula* species have a structure that consists of a network of tubes through which the spindle-shaped cells glide. The extensive nature of this surface-attached slime network suggested a direct association with the seaweed surface. Moreover, the parasitic properties of this organism are of interest since it is the causative agent of eelgrass wasting disease (Short et al. 1987). The third protist was a branched naked amoeba, *Stereomyxa ramosa*. This flattened amoeba has a large surface area and the potential to secure nourishment over a relatively large area. Although most amoebae are bacterivores, this does not exclude the possibility that some amoebae obtain additional nutrients from seaweed exudates or particulate seaweed carbon.

## MATERIALS AND METHODS

**Cultures.** The 3 heterotrophic protists *Nitzschia alba*, *Labyrinthula* sp. and *Stereomyxa ramosa* were isolated from seaweeds collected from the Clyde Sea area, Scotland. All isolates were maintained routinely on MY75S medium (0.1 g malt extract, 0.1 g yeast extract in 1 l 75% seawater, Page 1983) with attendant mixed bacteria.

Axenic cultures of *Labyrinthula* sp. were prepared by growing cells on serum seawater agar. This was prepared by dissolving 12 g technical agar in 940 ml seawater. The mixture was left to cool before adding 50 ml filter-sterilised foetal bovine serum and 10 ml of antibiotic/antimycotic mix (Sigma). Plates were inoculated with *Labyrinthula* sp. that migrated over the agar surface. Cells from the growing front were transferred to new agar plates. After several such transfers, the cultures were free of bacteria and were maintained on serum seawater agar without antibiotics.

Axenic cultures of *Nitzschia alba* were prepared in a similar manner except MY90 agar (0.1 g malt extract, 0.1 g yeast extract in 90% filtered seawater, 12 g technical agar, and 10 ml antibiotic/antimycotic mix added after autoclaving) was used instead of serum seawater agar. All attempts to get *Stereomyxa ramosa* into axenic culture using antibiotics were unsuccessful. Therefore, the only experiment carried out with this amoeba was one to assess its ability to invade intact algal tissue.

**Polysaccharides.** A range of soluble and gel-forming polysaccharides was used to mimic some of the carbohydrates found in seaweeds (Oxoid and Sigma). Carboxymethyl cellulose was used to represent the skeletal structure. This is similar to the cellulose in seaweeds but has additional methyl groups at positions 2,3 and 6, or 2,3,4, and 6 of each carbon residue. Technical agar, purified agar, agarose (the non-sulphated constituent of agar), carrageenan type I, which contains predominantly kappa and lesser amounts of lambda carrageenans, and carrageenan type II, which consists of mostly iota carrageenans, were used to represent the matrix phase phycocolloids. Other matrix components used were fucoidan and ascophyllan. Laminarin was used as an example of a storage product. Dextran sulphate and D-glucose were used to simulate the dissolved organic carbon released by seaweeds.

**Culture on 'solid' polysaccharides.** To look for evidence of direct utilisation of seaweed polysaccharides by *Nitzschia alba* and *Labyrinthula* sp., and to investigate the effect of competing bacteria, 3 different experiments were conducted. The first examined the migration rate of cells on different 'solid' polysaccharides. Migration was considered as the combined motion resulting from both the migration rate and the division rate of cells. Secondly, light microscopy and scanning electron microscopy (SEM) were used to look for disturbance of the agar surface. Thirdly, staining methods were used to detect the removal of polysaccharide substrates. All these experiments were conducted in triplicate using axenic cells and under monoxenic conditions in the presence of the bacterium *Planococcus citreus*.

To determine the migration rates of cells on different gels, agar blocks (0.5 $cm^2$) containing cells of *Nitzschia alba* or *Labyrinthula* sp. were cut from exponentially growing stock cultures and used to inoculate the cen-

tre of a range of polysaccharide plates. These plates contained MY90S medium (malt and yeast extract in 90% seawater) in the case of diatoms, or serum seawater in the case of *Labyrinthula* sp., with one of the following solidifying polysaccharides; technical agar, agarose, carrageenan type I, carrageenan type II or purified agar. Cultures were monitored regularly by microscopy over a 2 wk experimental period to assess the rate of migration of cells over the surface of the plates.

During the growth and migration of cells, the gel was examined by light microscopy for the presence of burrows extending down through the gel. Parallel trials were set up to enable the gel surface to be observed by SEM. After 2 wk incubation, blocks of gel were fixed for 2 h in 4% glutaraldehyde made up in 0.1 M cacodylate buffer. Blocks were dehydrated through an acetone series and the solvent was removed by transferring through 2 changes of hexamethlydisilizane (HMDS; Sigma Chemical Co.). Prepared material was air-dried, coated with gold palladium and examined in a JEOL JSM-5200 SEM. Gel blocks were compared adjacent to the inoculation site and at the growing edge.

To assess whether *Nitzschia alba* and *Labyrinthula* sp. were removing the polysaccharides, gels were made without the malt/yeast enrichment. These contained polysaccharide (1.2%) made up in artificial seawater medium (ASW; Provasoli 1964). Preliminary trials showed that survival of *Labyrinthula* sp. required serum, hence foetal bovine serum was added to these cultures (50 ml $l^{-1}$). After 2 wk, the plates were flooded with toluidine blue (50 mg $l^{-1}$) for 30 min to stain the polysaccharide. Significant utilisation of substrate was indicated by the presence of stain-free zones on the gel surface (Fig. 1c).

**Growth of *Nitzschia alba* on liquid polysaccharides.** The growth rates of *N. alba* were determined using a method similar to that used by Uchida & Kawamura (1995). Rate determinations were not made with *Labyrinthula* sp. since survival (and growth) of this organism required the presence of serum. Purified carbohydrates (0.1%) were dissolved in 90% artificial seawater and filter sterilised (0.22 µm pore size, Millipore™). The carbon sources used were: laminarin, fucoidan, ascophyllan, carrageenan type I, carrageenan type II, dextran sulphate, methyl cellulose and glucose. Seaweed extracts were also prepared by blending 5 g aliquots of *Fucus serratus* in 100 ml of 90% artificial seawater before autoclaving. To mimic exudates from damaged tissue, some of these extracts were partially degraded by exposing them to mixtures of bacteria isolated from seaweed surfaces (incubation at 18°C for 3 d in the dark). Larger particles were removed by centrifugation and the supernatants (with the soluble seaweed extracts) were filter sterilised.

Diatoms were harvested from liquid axenic cultures (MY90S) and washed twice with artificial seawater to remove traces of the malt and yeast extracts. Growth experiments were carried out in 96-well microtitre plates containing 10 µl of inocula (ca 20 cells), 100 µl of 90% artificial seawater and 100 µl of polysaccharide solution (0.1% in 90% artificial seawater). Five replicates were set up for each substrate tested. Additional treatments included diatoms in artificial seawater, diatoms in natural seawater and diatoms in MY90S medium. Cultures were incubated at 18°C in the dark and counts were made over the exponential phase of growth to enable 5 replicate maximum growth rates to be calculated for each substrate.

**Direct utilisation of seaweed.** Pieces of the seaweeds *Fucus serratus*, *F. spiralis*, *Laminaria digitata*, and *Palmaria palmata* were incubated with each of the protists in liquid culture (18°C, in the dark). After 1 and 2 wk of incubation the seaweed was thick-sectioned to look for evidence of penetration by the protists into the body of the tissue. Blocks for sectioning were prepared by simultaneously fixing the seaweed in 5% glutaraldehyde and 0.5% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.2). Tissue was dehydrated through an alcohol series, embedded in Spurr resin and thick-sectioned (ca 1 µm thick). Sections were heat-fixed onto glass slides and examined at ×1000 using phase contrast optics. Autoclaved tissue was used in the above experiments. Autoclaving (121°C, 15 min) produced some intracellular damage; however, the structural integrity of the thickened seaweed walls was retained.

*Stereomyxa ramosa* and *Labyrinthula* sp. that had grown in culture with these seaweed pieces were harvested and the contents of the protists' food vacuoles examined by transmission electron microscopy (TEM). In this case, fixation was in glutaraldehyde (2.5%) made up in a 50:50 mix of 0.1 M cacodylate buffer and 3.5% saline for 30 min. Post-fixation was in 2% osmium tetroxide in 0.1 M buffer for 2 h. Dehydration and embedding was with alcohol and Spurr resin, respectively.

Invasion into living tissue was investigated in the case of *Fucus serratus*. Rocks with attached, healthy *F. serratus* were placed in tanks with constant running seawater and 12:12 h illumination with green light, which approximated light levels in the sea. Pieces of 'infected' *F. serratus* that had been cultured with *Nitzschia alba*, *Labyrinthula* sp. or *Stereomyxa ramosa* were attached to the surface of the seaweed using split tubing 'clamps' (method of Muehlstein et al. 1988). After 2 wk incubation, tissue blocks from the attachment site were fixed, thick-sectioned and examined by light microscopy.

## RESULTS

*Nitzschia alba* and *Labyrinthula* sp. grew on all of the polysaccharide gels in both axenic culture and monoxenic culture with *Planococcus citreus*. However, the migration characteristics on the different gels varied (Table 1). In the case of the diatoms, migrations ranged from 12 to 45 mm from the inoculation site (over 2 wk). The presence of bacteria increased the migration rate in 2 cases (i.e. with carrageenan type I and agarose) and tended to reduce the final density of cells. The presence of bacteria also promoted burrow formation through the technical agar and the carrageenan type II. Examination of these burrows by epifluorescence microscopy, using the DNA-specific fluorochrome DAPI to stain cells, showed that diatoms were at the front of the burrows ahead of any bacteria. This clearly suggests that diatoms were primarily responsible for the formation of the burrows. The presence of bacteria was detrimental to the diatoms and the more rapid migration rates, lower final cell densities and burrow formations all suggest that diatoms were attempting to spatially avoid bacteria and reduce competition for food or to escape any toxic exudates from the bacteria.

In almost all cases, *Labyrinthula* sp. cells penetrated the gel surfaces, forming extensive networks, regardless of whether bacteria were present or not (Fig. 1a). With the exception of agarose, which did not promote the migration of cells, the presence of bacteria markedly increased the migration rate. However, in this case the increased migration rates were due to increased growth of cells. Although *Labyrinthula* sp. cannot phagocytose bacterial-sized particles, they were benefiting from the presence of bacteria and cleared them from the gel surface (Fig. 1b).

Staining of gels with toluidine blue showed that there was a clear zone beneath the axenic diatoms (Table 1, Fig. 1c), except in the case of agarose. This shows that diatoms were removing substantial amounts of polysaccharide from the gel matrix. There was no such evidence that *Labyrinthula* sp. utilised polysaccharides by this method. On the other hand, examination of the gel surface by SEM showed some evidence of surface penetration in both organisms, regardless of whether the cultures were axenic or monoxenic (Table 1, Fig. 1a,d).

*Nitzschia alba* grew rapidly in control trials with MY90S liquid medium (Fig. 2). Equivalent growth rates were achieved in ASM with added fucoidan, methyl cellulose and seaweed extract previously exposed to bacteria. Moderate growth was found in the case of the polysaccharides laminarin, ascophyllan, and carrageenan type II and seaweed extract. Glucose and natural seawater also permitted moderate growth. No growth was found with artificial seawater, carrageenan type I or dextran sulphate.

Thick-sectioning was used to look for evidence of penetration into seaweed tissue. It is important to note that the tissue used in the experiments had been damaged by autoclaving, although the cell walls were intact. Moreover, since the seaweed pieces had been dissected from fronds they had 4 cut (i.e. damaged) edges, allowing organisms to invade seaweed without penetrating the outer protective cuticle. Even so, further penetration into the intact tissue required the microbes to penetrate the thickened cell walls of the algae.

The results of the thick-sectioning experiments are shown in Table 2. Invasion into 4 seaweed species was investigated after 2 and 4 wk of exposure to a mixture of bacteria alone, as well as exposure to axenic *Labyrinthula* sp. and *Nitzschia alba* cultures. Since *Stere-*

Table 1. Migration distance of *Nitzschia alba* and *Labyrinthula* sp. (with and without the bacterium *Planococcus citreus*) after incubation for 2 wk on different polysaccharide gels. Information is given regarding the ability of protists to penetrate the agar surface (as judged by SEM) and form burrows down into the gel. Gel utilisation was assessed on the basis of polysaccharide staining by toluidine blue (axenic cultures only). +: an effect; –: no effect; nd: no data

| Organism | Gel type | Migration (mm) | Surface penetration | Burrow formation | Gel utilisation |
|---|---|---|---|---|---|
| *N. alba* (axenic) | Technical agar | 45 | + | – | + |
| | Carrageenan I | 12 | nd[a] | – | + |
| | Carrageenan II | 43 | nd | – | + |
| | Purified agar | 37 | + | – | + |
| | Agarose | 14 | + | – | nd |
| *N. alba* (bacteria) | Technical agar | 41 | + | + | |
| | Carrageenan I | 37 | nd | – | |
| | Carrageenan II | 43 | nd | + | |
| | Purified agar | 43 | + | – | |
| | Agarose | 21 | + | – | |
| *Labyrinthula* sp. (axenic) | Technical agar | 8 | + | + | – |
| | Carrageenan I | 9 | nd | + | – |
| | Carrageenan II | 10 | nd | + | – |
| | Purified agar | 4 | + | + | – |
| | Agarose | 7 | + | + | nd |
| *Labyrinthula* sp. (bacteria) | Technical agar | 60 | + | + | |
| | Carrageenan I | 20 | nd | + | |
| | Carrageenan II | 14 | nd | + | |
| | Purified agar | 32 | + | + | |
| | Agarose | 1 | + | – | |

[a]Carrageenan did not survive the preparation processes for SEM

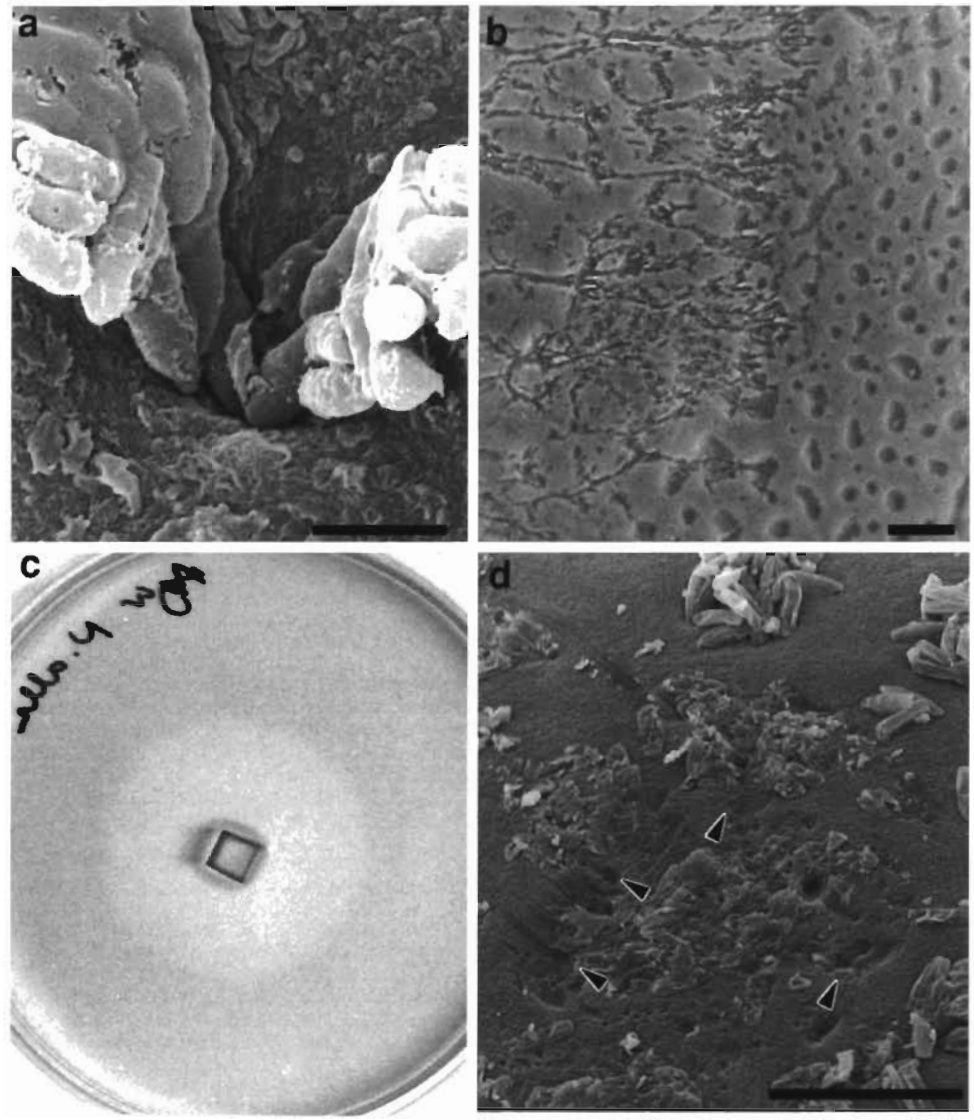


Fig. 1. (a) Scanning electron micrograph of axenic *Labyrinthula* sp. grown on technical agar. Ropes of cells could clearly be seen disappearing into burrows created in the agar surface. Scale bar = 5 µm. (b) Light micrograph of *Labyrinthula* sp. grown in monoxenic culture with *Planococcus citreus* on carrageenan type II. Bacterial clearance from areas where *Labyrinthula* sp. had colonised had clearly taken place. Scale bar = 35 µm. (c) Light micrograph of a petri dish containing axenic *Nitzschia alba* growing on technical agar after staining with toluidine blue. The white area in the centre was clear of stain while the greyish area was pink. The diatoms had therefore utilised the agar. (d) Scanning electron micrograph of purified agar after axenically grown *N. alba* were washed off the surface. Pitting in the agar was evident where the diatoms had been situated (arrowheads). Scale bar = 50 µm

*omyxa ramosa* utilises bacteria in its diet and the migration of *Labyrinthula* sp. is enhanced by the presence of bacteria, treatments included *Labyrinthula* sp. with bacteria and *S. ramosa* with bacteria.

When incubated with bacteria alone, all seaweeds showed evidence of invasion after 4 wk. This bacterial penetration was through the cut edges, the outer cuticle remained intact in all seaweeds (e.g. Fig. 3a). Axenic *Labyrinthula* sp. were unable to penetrate *Fucus serratus* tissue (the only axenic treatment undertaken) even after 4 wk incubation. However, when incubated with bacteria, *Labyrinthula* sp. penetrated all 4 seaweeds within just 2 wk (Fig. 3b). Moreover, there was evidence of synergistic invasion through the outer cuticle (Fig. 3c). When examined by TEM, *Labyrinthula* sp. contained no food vacuoles, supporting the view that this protist is osmotrophic (Young 1943). Presumably it is capable of releasing extracellular enzymes to digest particulate material, in this case bacteria and algal material (Watson 1957).

The amoeba *Stereomyxa ramosa*, in conjunction with bacteria, invaded tissue only via the cut edges.

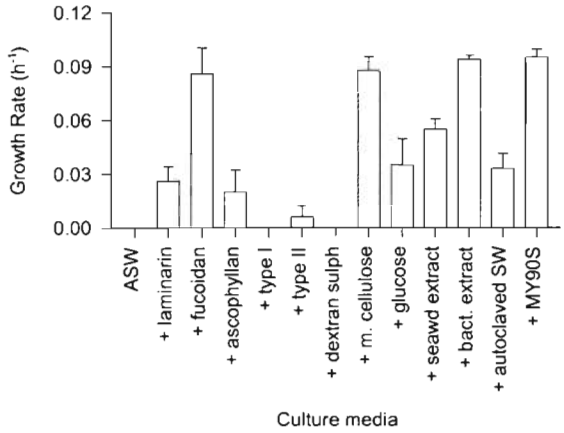


Fig. 2. Growth rates of *Nitzschia alba* with artificial seawater (ASW) and artificial seawater and various polysaccharides, glucose and seaweed extracts (e.g. + laminarin). Error bars = standard error of the mean. (m. cellulose = methyl cellulose, bact. extract = seaweed extract previously exposed to bacteria)

Once inside the tissue, *S. ramosa* was capable of moving both between and through cell walls. Fig. 3d shows several amoebae cells moving through the tissue and digesting intracellular contents. When the food vacuoles of these cells were examined by TEM, some contained bacteria whereas others had unidentifiable material, thought to be remnant macroalgal tissue. It is interesting to note that both *Labyrinthula* sp. and *S. ramosa*, when accompanied by bacteria, were capable of penetrating the thickened walls of *Laminaria digitata* after only 2 wk incubation. Bacteria alone required 4 wk to penetrate this seaweed.

Axenic diatoms penetrated both *Fucus* species as well as *Laminaria digitata* after 4 wk; however, they failed to invade *Palmaria palmata*. No evidence of invasion through the cuticle of these species was found and it is likely that initial entry was via the cut edges. Inside the tissue, these axenic diatoms were capable of migrating several cells' depth into the seaweeds.

Additional experiments conducted with the red alga *Porphyra umbilicalis* incubated with diatoms and bacteria showed that some diatoms migrated under the outer bacterial layer (Fig. 3e) and even penetrated the thin cuticular layer of *P. umbilicalis* (Fig. 3f). These invading diatoms appeared to lose their siliceous walls after penetrating the tissue; an interesting observation that needs to be verified by further experimentation. Such a phenomenon is not without precedent since endosymbiotic diatoms in foraminiferans are reportedly sequestered without shells (Lee 1983). Moreover, when the surface of *Fucus spiralis* was observed by SEM, after incubating with bacteria and diatoms, several indentations of the cuticular surface were observed. This suggested that diatoms were migrating beneath the bacterial layer and partially digesting the algal surface.

There was no evidence of invasion into living *Fucus serratus* with infected seaweed pieces clamped to the fronds. However, experimental incubations had to be short (2 wk) to maintain the health of the *F. serratus* in the tanks with flowing seawater.

## DISCUSSION

Axenic *Nitzschia alba* migrated to different extents on the various polysaccharide gels, perhaps reflecting varying abilities to utilise different phycocolloids. Alternatively, since additional nutrients were present in these MY90S or serum seawater gels, the physical

Table 2. Penetration of protists and bacteria into seaweed tissue after 2 and 4 wk incubation. Thick sections were scanned by light microscopy for the presence of invading microbes. +: invasion; –: no invasion; nd: no data available

| Seaweed | Organism | Invasion 2 wk | Invasion 4 wk |
|---|---|---|---|
| *Fucus serratus*[a] | Bacterial mixture | + | + |
| | *Labyrinthula* sp. (axenic) | –[b] | – |
| | *Nitzschia alba* (axenic) | – | + |
| | *Labyrinthula* sp. plus bacteria | + | + |
| | *Stereomyxa ramosa* plus bacteria | + | + |
| *Fucus spiralis* | Bacterial mixture | + | + |
| | *Labyrinthula* sp. (axenic) | nd | nd |
| | *Nitzschia alba* (axenic) | – | + |
| | *Labyrinthula* sp. plus bacteria | + | + |
| | *Stereomyxa ramosa* plus bacteria | + | + |
| *Laminaria digitata* | Bacterial mixture | – | + |
| | *Labyrinthula* sp. (axenic) | nd | nd |
| | *Nitzschia alba* (axenic) | – | + |
| | *Labyrinthula* sp. plus bacteria | + | + |
| | *Stereomyxa ramosa* plus bacteria | + | + |
| *Palmaria palmata* | Bacterial mixture | + | + |
| | *Labyrinthula* sp. (axenic) | nd | nd |
| | *Nitzschia alba* (axenic) | – | – |
| | *Labyrinthula* sp. plus bacteria | + | + |
| | *Stereomyxa ramosa* plus bacteria | + | + |

[a]When *F. serratus* was sterilised by immersion in ethanol rather than autoclaving, the results were similar

[b]Although there was no invasion of seaweed tissue the *Labyrinthula* sp. cells were found thickly covering the seaweed while not very abundant on the culture vessel

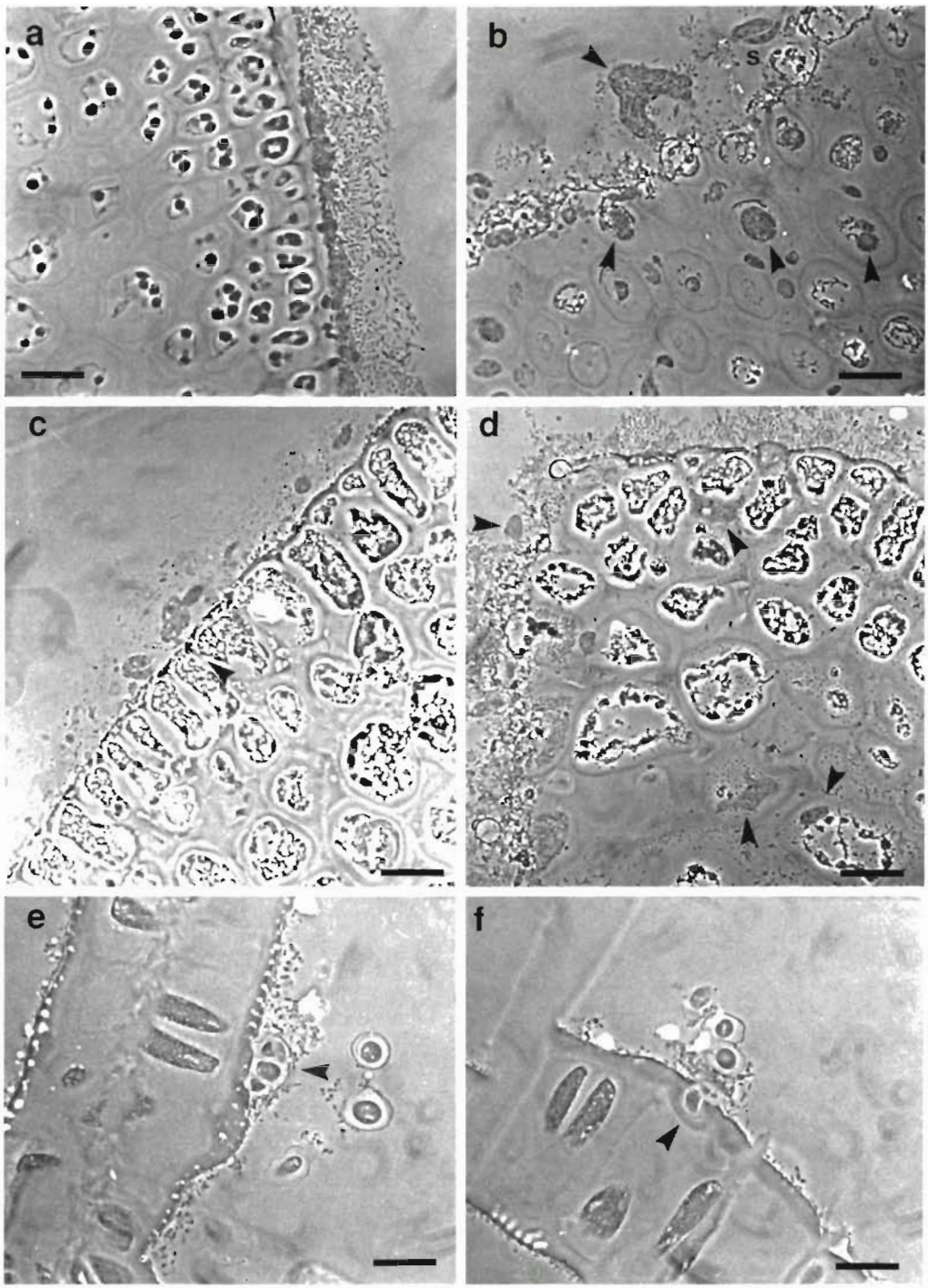


Fig. 3. Light micrographs of: (a) a section through *Laminaria digitata* after incubation with bacteria for 2 wk. A thick layer of bacterial growth covered the tissue surface. Scale bar = 10 µm; (b) a section through *Fucus spiralis* after incubation with *Labyrinthula* sp. for 2 wk. Protistan cells (arrowheads) were abundant within the cells of the seaweed tissue and at the cut seaweed edge. Spindles (s) were easily visible. Scale bar = 10 µm; (c) a section through *F. spiralis* after incubation with *Labyrinthula* sp. for 2 wk. There is some indication that the *Labyrinthula* sp. cells may be penetrating the seaweed cuticle (arrowhead). Scale bar = 10 µm; (d) a section through *F. spiralis* after incubation with *Stereomyxa ramosa* for 4 wk. This area was at the cut edge of the seaweed. Amoebae (arrowheads) were seen within seaweed cells. Scale bar = 10 µm; (e) a section through *Porphyra umbilicalis* incubated with *Nitzschia alba* for 4 wk. Diatoms were attached to the seaweed surface under the bacterial layer (arrowhead). Scale bar = 10 µm; (f) a section through *P. umbilicalis* incubated with *N. alba* for 4 wk. A diatom (arrowhead) was penetrating the seaweed cell wall. Scale bar = 10 µm

properties of the gels may have accounted for the different mobilities. Migration was least in the agarose and carrageenan type I gels, which appeared more rigid than the other gels. These 2 gel types contained less sulphate groups than the other gels, although it is not known whether levels of sulphate influence motility in diatoms. Sulphate can form up to 20% of the agaropectin found in agars, and carrageenan type II contains twice as much sulphate as carrageenan type I. When grown with bacteria, *N. alba* migrated further on agarose and carrageenan type I, suggesting that sulphate, which is needed for sulphur amino acid synthesis, may have been more available as a result of bacterial action. However, the most interesting result with this experiment was the burrowing activity displayed by *N. alba* in the presence of bacteria. It is likely that this was a mechanism adopted by diatoms to reduce competition with bacteria, resulting in spatial separation of the 2 populations. Rogerson et al. (1993) observed similar results with a different heterotrophic diatom, *N. albicostalis*. Similar strategies may be occurring in field populations since some of the sections showed that *N. alba* burrowed down beneath the bacterial layer on the surface of seaweeds. It is interesting to note that burrows were not found in the case of agarose and carrageenan type I, supporting the notion that interaction with bacteria, rather than avoidance, may have nutritional benefits in these low sulphate gels. Observations by SEM of algal surfaces (Armstrong 1998) with all 3 protists showed evidence of migration below the bacterial layer and onto the seaweed surface. Since bacteria are likely to utilise seaweed DOC as soon as it is released (Linkins 1973), the ability to reside under the bacterial film would impart a clear competitive advantage.

Burrows were not detected in the axenic gels where spatial separation of the microbial populations is not required. Even though burrows were not formed, the toluidine staining showed evidence of polysaccharide utilisation, as did the surface penetration observed by SEM. The secretion of enzymes by *Nitzschia alba* to digest macromolecules was suggested by Linkins (1973), who grew this diatom on microcrystalline cellulose, agar and chitin. Moreover, 2 phototrophic diatoms, *N. frustulum* and *N. filiformis*, were shown to form pits on agar surfaces (Lewin & Lewin 1960), an observation attributed to extracellular enzyme production.

*Nitzschia alba* failed to grow in artificial seawater alone but grew when seawater was supplemented with various polysaccharides, particularly fucoidan and methyl cellulose. It is possible that some of the less highly purified polysaccharides also contained small amounts of other compounds such as proteins that promoted the diatom growth. Growth was also possible with glucose as the substrate, which was previously reported by Linkins (1973) for the same diatom. He concluded that glucose, galactose and fucose share a common uptake system. This accounts for the uptake of seaweed carbohydrates which are rich in these sugars. *N. alba* also grew readily on seaweed extract previously exposed to bacteria. Uchida & Kawamura (1995) found similar results with phototrophic diatoms. Rapid growth was presumably due to the fact that these exudates contained simpler, partially digested saccharides. However, the more rapid growth may also be due to bacteria eliminating an anti-algal factor in the extract (Uchida & Kawamura 1995).

*Labyrinthula* sp. migrated and grew better in the presence of bacteria, suggesting that these protists used bacteria in their diet. The clearing of bacteria from around cells in monoxenic culture supports this view. In all cases, regardless of gel type and bacterial status, *Labyrinthula* sp. formed extensive networks throughout the gels. It is likely that this was a consequence of extracellular enzyme action, since the burrows were considerably wider than the width of the labyrinthulid network. Burrowing by physically pushing the gel matrix aside is therefore highly unlikely. Although the toluidine staining failed to show any significant clearing of the gels with *Labyrinthula* sp., it is likely that they were utilising polysaccharides in their diet and were burrowing to exploit larger areas of substrate. The lack of staining in the axenic cultures was probably due to the low migration and hence low growth rates in these cultures which required bacteria for vigorous growth.

Evidence of direct invasion into seaweed tissue was provided by the thick-sectioning experiments. Unfortunately, to distinguish between bacterial and protistan action it was necessary to autoclave the tissue before experimentation. Autoclaving cells did disrupt cellular membranes but left the cell walls intact, suggesting that little damage occurred in those areas where the phycocolloids were located. Given the correct circumstances, all protists were found to be able to invade seaweed tissue. The only organism to achieve invasion under axenic conditions was *Nitzschia alba*, and this was only possible through the cut (i.e. damaged) edge of the tissue. Generally, the presence of bacteria facilitated the invasion process. In the presence of bacteria, *N. alba* was even capable of penetrating the outer cuticle, at least in the case of *Porphyra umbilicalis*. This required a degree of synergistic action since bacteria alone were unable to penetrate the outer surface. Likewise, *Labyrinthula* sp. penetrated seaweed cells only when bacteria were present, suggesting that bacterial enzymes were required to initiate invasion of the tissue, even at damaged edges. It also suggests that there was a nutritional advantage to *Labyrinthula* sp. invading tissue, otherwise it would

prey on the far more abundant bacteria on the outer surface.

*Stereomyxa ramosa* could only be tested in the presence of accompanying bacteria since it proved impossible to develop conditions for the axenic cultivation of this amoeba. However, the results were similar in that amoebae with bacteria could penetrate and digest macroalgal cells, at least when offered a damaged edge. Examination of the digestive vacuole contents by TEM showed what appeared to be algal material.

Another amoeba, *Trichosphaerium sieboldi*, has been shown to be capable of digesting a large range of seaweed species, even under axenic conditions (Polne-Fuller 1987, Polne-Fuller et al. 1990, Rogerson et al. 1998). Moreover, unidentified amoebae-like cells penetrated both cortical and medullary cells of *Gracilaria chilensis* and digested the protoplasm, causing the seaweed tissue to soften and fragment (Correa & Flores 1995).

There was no evidence in the present study of protists invading healthy growing seaweed. However, there was evidence that they can utilise phycocolloids or phycocolloid-like polysaccharides and that they can invade damaged tissue, particularly when bacteria are present. Given the degree of damage to seaweed fronds as a result of herbivore grazing, wave damage and erosion, it is likely that direct removal of macroalgal carbon is occurring in the field. What remains to be determined is the importance of this heterotrophy within the carbon budget of coastal ecosystems.

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