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Nitzschia albicostalis : An Apochloritic Diatom Worthy of Ecological Consideration

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Abstract : The obligate heterotrophic diatom, *Nitzschia albicostalis,* was isolated from the surfaces of *Fucus serratus* between October 1991 and April 1992 but was not detected in samples taken between April and August (1992). This suggests that diatoms were only numerically significant over the winter months when they averaged 10.8 cells cm^{-2} of algal surface. Peak abundances, up to 35.4 cells cm^{-2} were noted in March. These apochloritic diatoms compete with bacteria for carbon and nutrients and consequently showed reduced growth rates and yields when cultured with bacteria. In the laboratory, the presence of bacteria stimulated diatoms to burrow down into agar and carrageenan, presumably through the localised release of diatom ectoenzymes. This tunnelling behaviour may be an adaptation to spatially separate competing microbial populations. Moreover, in some monoxenic cultures, the release by *Nitzschia* of a probable antimicrobial agent was noted. The ecological significance, and value of these laboratory observations is discussed.

Résumé: La diatomée hétérotrophique *Nitzschia albicostalis*, a été isolée de la surface de *Fucus serratus* entre octobre 1991 et avril 1992 mais n'a pas été décelée dans les échantillons récoltés entre avril et août 1992. Ceci laisse supposer que les diatomées étaient numériquement significatives seulement pendant les mois d'hiver quand leur moyenne était de l'ordre de 10.8 cellules par cm^2 de surface algale. Des pics d'abondance jusqu'à 35.4 cellules par cm^2 ont été décelés en mars. Ces diatomées apochloritiques, en compétition avec les bactéries pour le carbone et les éléments nutritifs, montrent donc des taux d'accroissement et de production réduits quand elles sont cultivées avec des bactéries. Au laboratoire, la présence de bactéries incite les diatomées à creuser des galeries dans l'agar et les carragènes, probablement grâce à la décharge localisée d'ectoenzymes des diatomées. Ce comportement de fouisseur doit être une adaptation aux populations microbiennes concurrentes séparées dans l'espace. Cependant, dans les cultures monoxéniques on a noté la décharge par *Nitzschia* d'un probable agent anti microbien. La signification écologique et l'évaluation de ces observations de laboratoire sont discutées ici.

INTRODUCTION

Research effort into the biology of colourless diatoms, those lacking photosynthetic pigments, peaked in the late sixties and early seventies with a wealth of studies detailing their nutritional requirements (e.g. Lewin & Lewin, 1967 ; Lewin & Hellebust, 1970 ; Linkins, 1973). These apochloritic diatoms are obligate heterotrophs capable of utilizing a wide range of organic carbon substrates ranging from simple monomers, such as glucose through to complex polysaccharides like chitin and cellulose. In some cases the valve morphology is similar to described photosynthetic species (Lewin & Lewin 1967), however, their inability to manufacture chlorophylls and their mode of nutrition suggests major modifications to their genome (Li & Volcani, 1987) and has justified the naming of seven apochlorotic species, all within the genera *Nitzschia* and *Hantzchia.*

Despite an early report suggesting that these colourless diatoms are common on seaweed surfaces (Pringsheim, 1951) virtually nothing is known about their ecological significance

on such epiphytic habitats. Indeed, while there is much information on bacterial populations adhering to seaweeds (e.g. Chan & McManus 1967, 1969 ; Linley *et al.*, 1981) few workers have considered any of the epiphytic protistan community. Linley *et al.* (1981) looked at the protistan types that developed in laboratory seawater cultures supplemented with kelp mucilage and found a succession of microbial populations culminating in flagellates and ciliates whose combined biomass reached some 6 to 10 % of that of bacteria. More recently, Rogerson (1991) investigated the abundance of naked amoebae on seaweeds and found up to 23 cells cm^{-2} of algal surface implying that intertidal algal stands can support up to 3.3×10^6 amoebae per m^2. Many of these protists graze the abundant bacterial flora of these surfaces and thus indirectly benefit from the release of extracellular algal carbon, which in the case of *Fucus vesiculosus* amounts to 30 % of total C fixed in a day (Sieburth, 1969). The ability of some protists to also utilize dissolved organic carbon directly is being realized. Sherr (1988) has demonstrated that some colourless estuarine nanoflagellates are osmotrophic and can take up high molecular weight compounds and some amoebae, such as *Trichosphaerium* sp., have enzymes to digest seaweeds cell walls (Polne Fuller *et al.*, 1990 ; Rogerson, 1991). The nutritional role of heterotrophic diatoms within the microbial assemblage may be similar given the facts that they are common, particularly on decaying seaweeds (Lewin & Hellebust, 1970), and that they can utilise a wide range of carbohydrate molecules (Linkins, 1973).

Sieburth (1969) commented that because the amount of extracellular carbon exported from macroalgal stands is considerable, this must be taken into account when studying carbon flux in in-shore regions. This, together with the current interest in heterotrophic protists in the context of the microbial loop (Azam *et al.*, 1983) justifies studies on seaweed associated protists. The present study investigates some of the laboratory growth characteristics of a common apochloritic diatom and uses these results to draw attention to these neglected protists and to explain how these diatoms compete within the seaweed microbial consortium.

METHODS

Cultivation

Cells of the diatom *Nitzschia albicostalis* were isolated from *Fucus serratus* fronds collected from the shoreline adjacent to the Marine Station, Millport, Scotland. Diatoms were cultured from washings of the algal tissue after inoculation onto agar plates (MYSWA) made with 1.5 % non-nutrient agar and 90 % natural seawater (diluted with distilled water) supplemented with 0.01 % malt and yeast extract. These cultures were incubated at 18 C in the dark for 3 weeks. Heterotrophic diatoms reproduced and migrated over the agar surface and were washed off the agar and rinsed with sterile seawater. Cells were plated onto agar plates (MYSWA) containing a solution (10 ml l^{-1}) of antibiotic/antimycotic mix (Sigma Chemical Co., England, U.K.) and allowed to migrate over the surface. Individual cells

were picked up on the blade of a sterile scalpel from the periphery of the migrating zone. These were inoculated onto fresh agar plates to initiate axenic, clonal cultures.

Subsequent axenic and monoxenic clonal diatom cultures, containing an unidentified bacterium (isolated with the original diatoms) were routinely maintained on all four of the media used in the experimental treatments. These were malt/yeast 90 %-seawater agar (MYSWA), natural 90 %-seawater agar (NSWA), artificial 90 %-seawater agar (ASWA), made with a commercially purchased salt mixture (Sigma Chemical Co., England, U.K.), and natural 90 %-seawater with carrageenan (NSCAR). In all cases the concentration of the phycocolloid base was 1.5 %.

Enumeration of diatoms

The number of *N. albicostalis* on the surface of *Fucus serratus* was determined on 20 occasions between October 1991 and August 1992. An enrichment cultivation method was used, similar to the method described by Rogerson (1991) for the enumeration of seaweed-associated amoebae. At least 24 squares (1 mm^2) of algal tissue were dissected from freshly collected *Fucus* and added, singly, to 2 ml of seawater containing 5 drops of soil extract (Page, 1983). Cultures were incubated in the dark at 18 °C for three weeks and then scored for the presence or absence of *Nitzschia*. From the frequency of positive results and the surface area of alga dissected it was possible to estimate the number of diatoms per unit area of algal surface. It should be noted that this method underestimates diatom abundance since it assumes that a positive culture developed from a single diatom on the dissected algal block.

Microscopy

Diatoms were identified by SEM and TEM. In both cases, frustules were acid cleaned in equal volumes of HCl (10N) and HNO_3 (10N) for 30 min at 100 °C and washed several times in distilled water. For TEM examination, acid-washed material was air-dried onto formvarcoated grids and examined in a Zeiss 902 TEM. Material for SEM was gold/palladium-coated and examined in a JEOL JSM-5200 SEM. Diatom cells examined intact by SEM were fixed in 2 % glutaraldehyde, washed in distilled water, dehydrated through an acetone series and critical-point dried.

Some samples were examined by fluorescence microscopy. Here, diatoms and bacteria were stained with the DNA-specific fluorochrome DAPI (4',6-diamidino-2-phenylindole, Sigma Chemical Co., England). The surface of agar cultures was examined at 1000x magnification using incident light epifluorescence ; UV excitation.

Migration rates

The spread of diatoms from the inoculation point on an agar or carrageenan surface provided an index of migration/growth rate. An agar block with diatoms from an exponentially growing culture was placed in the centre of an agar (or carrageenan) plate. The distance migrated by the reproducing diatom front was measured every 12 h. Diatoms, axenic and monoxenic, were inoculated onto artificial seawater agar, natural seawater agar, natural sea-

water agar supplemented with malt and yeast and natural seawater based carrageenan. Any disruption of the agar or carrageenan surface was noted. The effect of different species of bacteria in monoxenic diatom cultures was examined using *Micrococcus* NCIMB 365, *Moraxella* NCIMB 213, *Flavobacterium* NCIMB 411, *Coryneform* NCIMB 8 and *Planococcus citreus* NCIMB 1493.

Growth rates

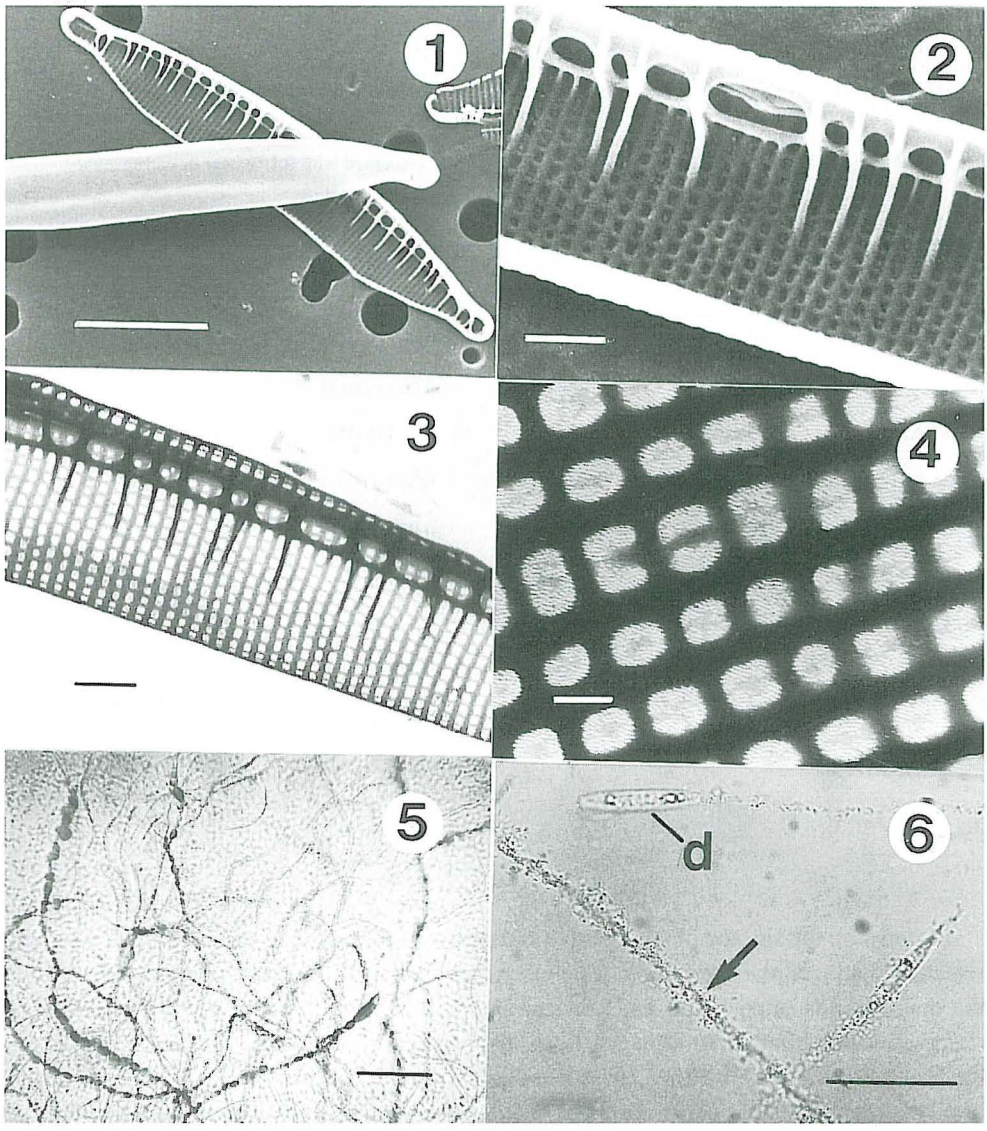
Diatoms from exponentially growing cultures were innoculated into the following liquid medium : artificial seawater (ASW), natural seawater (NSW) and natural seawater supplemented with malt and yeast (MYSW) all containing 0.1 % dissolved agar. A fourth medium contained natural seawater plus 0.1 % carrageenan (NSCAR). Cultures (n = 5) were incubated in the dark at 18 °C and diatoms in 10 randomly selected fields of view counted every 12 h using an inverted microscope. Specific growth rates (h^{-1}) were calculated from semi-logarithmic regressions (ln) of cell count against time.

RESULTS

The isolate of colourless diatom isolated from *Fucus* was morphologically identical to the obligate heterotroph, *Nitzschia albicostalis* as described by Li and Volcani (1987). Features of diagnostic note include the lanceolate shape of the valves (Fig. 1), the subcapitate apices, the prominent fibulae extending into the valve (Figs 2 and 3) with the median fibulae further apart than others (Fig. 2) and the velum perforated with concentrically arranged pores (Fig. 4). Diatoms ranged in length from 20.5 to 31.2 µm (which is close to the range reported by Li and Volani, 23 to 32 µm). Features which did not agree with the original diagnosis were the ability of our isolate to replicate faster and for them to grow without the vitamins thiamine and cobalamin. Despite these differences we consider this apochloritic diatom to be a strain of *N. albicostalis*.

On 9 occasions (out of 20) *N. albicostalis* was successfully isolated quantitatively from the surface of *Fucus serratus*. Positive results were only recorded between October and April suggesting temporal variation in abundance with highest numbers occuring over the winter months. The average number of *Nitzschia* on the surface of *Fucus* over these months was 10.8 diatoms cm^{-2} of tissue surface with peak abundances in March of 35.4 cells cm^{-2}. Assuming 1 g of *Fucus* has a surface area of 24.6 cm^2 (Rogerson, 1991) and the standing crop of *Fucus* per m^2 is 2130 g (Blinks, 1955) these diatom abundances imply that a 1 m^2 stand of *Fucus* averages 0.6 x 10^6 *Nitzschia* and on occasion can harbour up to 1.8 x 10^6 of this heterotrophic diatom.

When cultured on agar, these diatoms displayed characteristics which may help to explain their success in the field. The most obvious feature was their ability to form burrows (or tunnels) throughout the agar or carrageenan (Fig. 5) through which diatoms migrated (Fig. 6). Regardless of media, burrows were abundant in monoxenic cultures, radiating



Figs. 1 - 6 : Micrographs of *N. albicostalis*. Figs. 1 and 2. Scanning electron micrographs showing valve morphology. Scale bars = 5 µm and 1 µm, respectively. Figs 3 and 4. Transmission electron micrographs of diatom valves. Scale bars = 1 µm and 0.2 µm, respectively. Fig. 5. Burrows extending throughout agar plate (Bar = 100 µm). Fig. 6. Diatoms (d) submerged in agar forming burrows (arrowed). (Bar = 20 µm).

out from the site of inoculation, but far less so in axenic cultures (Table I). Frequently in these bacterized cultures, older diatom burrows became lined with bacteria.

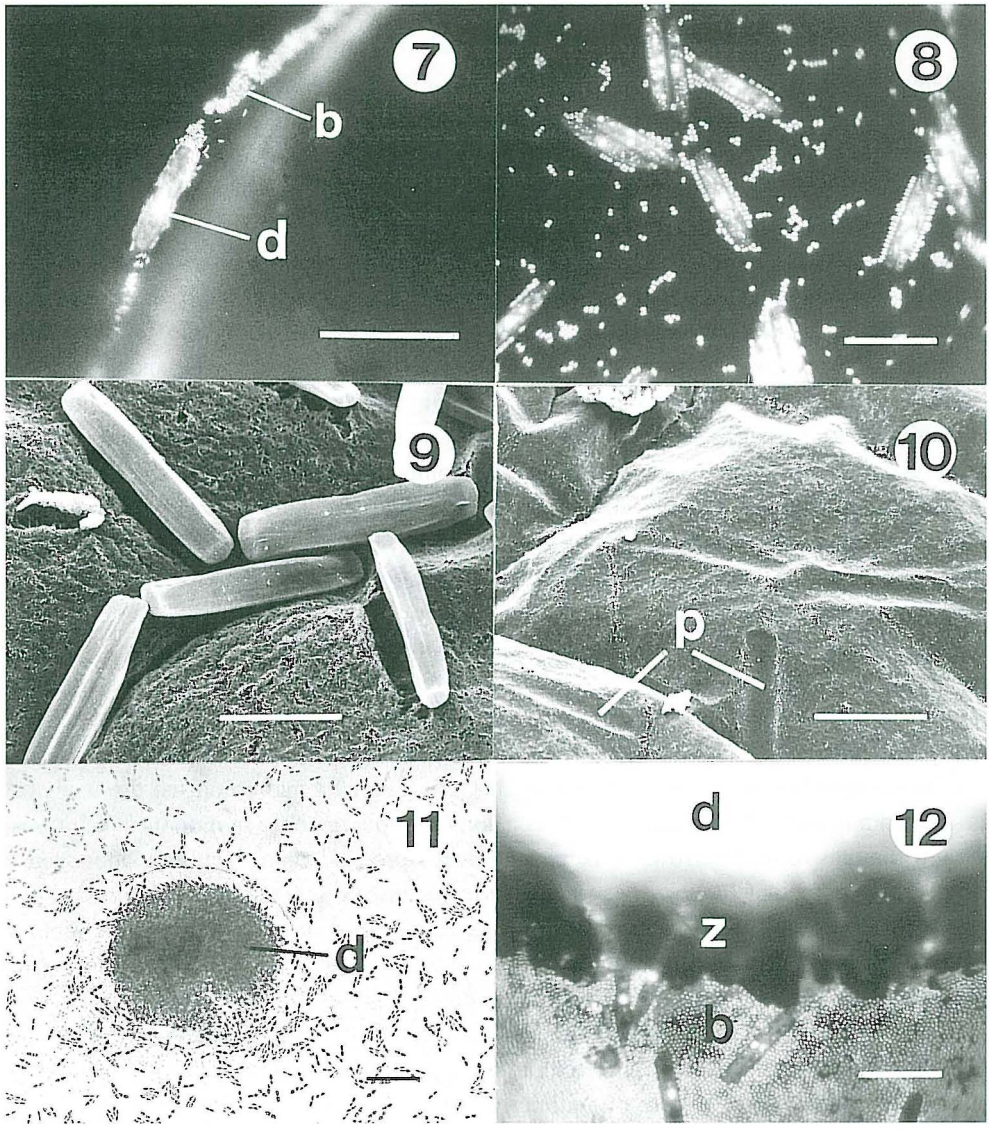
TABLE I

Comparison of *N. albicostalis* migration rate (n = 6) and growth (n = 5) on different media under axenic (A) and monoxenic (M) conditions. The media used was either artificial seawater (ASW), natural seawater (NSW), malt/yeast supplemented seawater (MYSW), or natural seawater with carrageenan (NSCAR). Migration rate and burrowing frequency was measured on agar or carrageenan plates, and specific growth rate, generation time and culture yield was in liquid media supplemented with 0.1 % agar or carrageenan.

Culture Condition	Migration Rate (mm h^{-1})	Burrowing Frequency	Specific Growth Rate (h^{-1})	Generation Time (h)	Culture Yield ($x10^6$)
ASW (A)	n.d.[1]	few	0.069 (0.018)[2]	10.04	0.73
ASW (M)	n.d.	many	0.061 (0.013)	11.36	0.42
NSW (A)	0.38 (0.05)[2]	few	0.058 (0.022)	11.95	0.71
NSW (M)	0.49 (0.05)	many	0.040 (0.005)	17.33	0.38
MYSW (A)	0.52 (0.13)	few	0.074 (0.011)	9.33	3.40
MYSW (M)	0.77 (0.22)	many	0.063 (0.009)	11.00	0.80
NSCAR (A)	0.34 (0.13)	few plus	0.056 (0.023)	12.37	0.48
NSCAR (M)	0.41 (0.18)	many plus	0.026 (0.013)	26.65	0.15

[1] n.d. = no data
[2] standard deviation

Diatoms on the surface of the agar (or carrageenan) formed pits, presumably through the release of extracellular enzyme. In the monoxenic cultures, the area of phycocolloid around the diatoms was often liquid and concentrations of moving bacteria could frequently be observed surrounding diatoms in this liquid depression (Fig. 8). These pits on the surface of the agar were also seen by SEM (Figs 9 and 10). The specific growth rate of diatoms in culture was rapid (Table I) particularly for axenic cultures where the mean rate, regardless of medium, was 0.064 h^{-1} corresponding to a generation time of only 10.8 h at 18 °C. In all cases, the presence of bacteria reduced the growth rate, for instance the overall mean was 0.047 h^{-1} (generation time, 14.7 h) for the monoxenic cultures. The presence of bacteria reduced the culture yield, by 68 % when the data are pooled regardless of culture conditions, and increased the migration rate of the diatom front by 34 % on the agar surface (Table I). The species of bacteria in the monoxenic cultures had no bearing on the rate of diatom migration or growth, however, in cultures containing *Planococcus citreus* dense clumps of diatoms on the agar surface were apparent in some relicates (Fig. 11). Clear zones devoid of any bacterial cells surrounded these diatom accumulations (Fig. 12).



Figs. 7 - 12 : Micrographs of *N. albicostalis*. Fig 7. DAPI-stained burrow in monoxenic culture showing submerged diatom (d) and bacteria (b) lining the burrow. Scale bar = 20 µm. Fig. 8. DAPI-stained preparation of diatoms on surface of agar in a monoxenic culture. Note bacteria surrounding individual diatom cells. Scale bar = 20 µm. Figs 9 and 10. SEM of agar surface showing diatoms (Fig 9) and pits (p) in the agar (Fig. 10, arrowed) with the same dimensions as the cell. Scale bars = 10 µm. Fig. 11. Diatoms on agar surface (with the bacterium *Planococcus citreus*). Dense accumulations of diatom cells (d). Scale bar = 100 µm. Fig. 12. DAPI-stained preparation showing edge of brightly fluorescing diatom accumulation (d). Note the clear zone (z) devoid of bacteria (b). Scale bar = 20 µm.

DISCUSSION

Heterotrophy, in one form or another, is common in pennate diatoms (Sieburth, 1979). Obligate heterotrophy, however, has only been found in seven species, most of which were isolated from organically-rich habitats such as the surface of macroalgae. In this rich epibiotic habitat bacteria thrive, typically reaching levels of 47 x 10^6 per cm^2 on *Fucus* (Rogerson, 1991), and presumably compete for available resources. The present laboratory studies showed that diatoms in the presence of bacteria grew slower than their axenic counterparts, migrated at a faster rate and attained lower culture yields. Clearly, in monoxenic culture, diatoms were not benefitting from bacterial exudates, as is true for *Navicula muralis* in the presence of some *Flavobacterium* (Jolley & Jones, 1977), and these rate reductions were a consequence of diatoms competing with bacteria for the same nutrient and carbon sources. Nutritional studies on the obligate heterotroph, *N. alba* (Linkins, 1973) have suggested that diatoms cannot effectively compete with bacteria for free dissolved glucose and acetate and that on seaweed surfaces *N. alba* probably utilizes proximal non-dissolved polysaccharides as a primary carbon source. Clearly, any mechanism or behaviour to minimise competition with the competing heterotrophic bacteria would be ecologically advantageous to the diatoms.

The increase in migration rate is, in itself, an adaptation to reduce competition, however, the laboratory studies on the growth of *Nitzschia* have suggested additional strategies to limit competition. When in the presence of bacteria, a significant proportion of the diatom population formed tunnels in the agar or carrageenan which spatially separated bacterial and diatom populations although eventually bacteria migrated and colonised the walls of these diatom tunnels. As expected, this was a common strategy only in monoxenic cultures and few diatoms formed burrows in axenic cultures. Moreover, diatoms cultured monoxenically in the presence of *Planococcus citreus*, formed dense accumulations of diatoms on the agar surface surrounded by a zone devoid of bacteria. This zone of bacterial inhibition, or perhaps cell lysis, was presumably due to the release of a growth inhibiting substance by the diatoms similar to the bacteriocidal agents found in the extracellular products of some other algae (Chrost, 1975 ; Linkins, 1973) and for at least one other diatom (Gauther, 1969) where the excretion of a specific antibacterial fungal agent was suspected. The environmental triggers promoting the release of anitmicrobial agents in *Nitzschia* cultures is unknown. However, since inhibition was only evident around dense accumulations of diatoms, surface crowding may be an important factor. Growth rates of *Nitzschia* were high regardless of culture conditions with cells replicating in as little as 9.3 h. This is consistent with the reported rates for other obligate heterotrophs (Li & Volcani, 1987) and with the notion that to effectively complete with other heterotrophs (predominantly bacteria) in organically-rich habitats, heterotrophic diatoms would have to reproduce rapidly (Lewin & Lewin 1967). The temporal variation in *Nitzschia* abundance is interesting, but unexplained, and could be related to reduced competion with autotrophic protists for essential nutrients, to temperature or to availability of macroalgal breakdown products.

In all the culture experiments, diatoms were presumably utilizing agar or carrageenan as a carbon source ; Linkins (1973) showed that *N. alba* could utilize the polysaccharides cellulose, agar and chitin for growth. Presumably, heterotrophic diatoms secrete extracellular enzymes to hydrolyse macromolecules prior to uptake of their constituents. Studies with other eukaryotic algae have indicated that ectoenzymes are secreted through the plasma membrane where they bind to the outer surface of the membrane or cell wall surface (Chrost, 1990). The ability to form burrows in the phycocolloids is a striking example of this controlled release of extracellular enzymes, as are the pits around diatoms seen in the SEM and in the LM as areas of low viscosity in the agar/carrageenan surface. Two faculative diatoms, *N. frustulum* and *N. filiformis* were presumed to secrete enzymes since they also formed pits on agar (Lewin & Lewin, 1960), however, agar (or carrageenan) disruption has never been reported for any obligate diatom species. Indeed, the degree of enzymatic-disruption found in this study and the ability to form burrows has never been demonstrated previously for any diatom species.

The increased frequency of burrows in monoxenic relative to axenic cultures (Table I) suggests that the presence of bacteria stimulates the production, and release of, ectoenzymes by *Nitzschia*. Since pure cultures of bacteria failed to show any obvious depressions or disruptive effects on the agar (or carrageenan) it is unlikely that these enzymes were of bacterial origin. In support of this, when the washings of monoxenic and axenic diatom cultures as well as bacterial cultures were incubated with a 1 % carrageenan solution, reducing sugars from the cleavage of polysaccharides were only detected in the case of monoxenic diatoms (spectrophotometric method of Kidby and Davidson, 1973).

Despite the fact that these are all laboratory-based observations, it is possible that pennate diatoms may also secrete enzymes in the field. This notion is supported by reports on the spatial localisation of some seaweed-associated facultative diatom species. For instance, diatoms have been found as endophytes in the intracellular substance of the receptacles of *Fucus* and *Ascophylum* and from the interior mucilage of the red alga *Dumontia incrassata* (Tassen, 1972 ; Baarsdeth & Tassen 1973).

One advantage of being able to migrate below the surface bacterial assemblage is that the diatoms could gain early access to the abundant dissolved organic compounds released by macroalgae (Sieburth, 1969) enabling heterotrophic diatoms to outcompete attendant bacteria. Secondly, the ability to burrow into the polysaccharide matrix of the outer wall may be an adaptation to help diatoms cope with the effects of desiccation or excessive salinity fluctations experienced in the intertidal zone.

Heterotrophy is widespread in pennate diatoms with, for example, one study showing that 28 of 44 diatom clones possessed some capacity for heterotrophic growth (Lewin & Lewin 1960, 1967). By investigating novel culture characteristics of just one diatom species, we have gained an insight into how heterotrophic diatoms may compete within the crowded epibiotic community and have highlighted a need for future studies to elucidate their full ecological role. Moreover, the production of ectoenzymes by heterotrophic diatoms with an affinity for phycocolloids has biotechnological significance since there is a

need for novel enzymes for the mass production of macroalgal protoplasts. Without these, the improvement of commercially important seaweeds is being hindered.

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