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Ultrastructural observations on the marine fouling diatom *Amphora*

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ABSTRACT: Ecological and Scanning electron microscope (S. E. M.) studies indicated that the diatom *Amphora* was an important constituent in the initial colonization of test panels coated with a copper antifouling composition. *Amphora* was also found as the dominant fouling diatom species on paint samples from "in-service" supertankers and yachts. Associated with the diatom was copious amounts of mucilaginous material, which often encapsulated the cells. Histochemical analysis of the mucilage indicates that it is predominantly polysaccharide in nature. Using the Transmission electron microscope (T. E. M.) and electron microscope cytochemistry the intracellular origin of the adhesive was investigated. T. E. M. and S. E. M. observations of acid-cleaned-cells indicate that the mucilage may be secreted through specialized regions of the frustule. Material isolated from antifouling panels was compared with laboratory cultured *Amphora* spp. for copper resistance and internal accumulation using TEMSCAN – X ray analytical equipment.

INTRODUCTION

The development of a marine fouling community is a sequential process with the formation of a "primary" or "slime" film constituting a very early phase. This primary film is composed of an intricate association of micro-organisms, their secretion products and other organic and inorganic particulate matter. Predominant amongst the micro-organisms are the bacteria (Zobell, 1946; Zobell & Allen, 1935; Waksman et al., 1943; Corpe, 1977; Gerchakov et al., 1978) and diatoms (Coe & Allen, 1937; Harris, 1946; Hendey, 1951; Wood, 1950, 1967) although protozoa, dinoflagellates, fungi and algal spores may also be present (Sechler & Gunderson, 1973; Gerchakov et al., 1977; Marszalek et al., 1979).

The majority of earlier authors considered that the primary film was inevitable and of little consequence to the overall frictional resistance of ships. Emphasis was given to eradication of the plant (algae) and animal macrofouling. In latter years, with the considerable expansion of shipping and the necessity for better antifouling compositions, more attention has been directed towards film development, its chemical and physical effects on paint performance and whether it is a necessary pre-requisite for macro-foulers (Wood, 1953; Crisp & Ryland, 1960; O'Neill & Wilcox, 1971; Corpe, 1973; Milanovich et al., 1975).

Although large numbers of diatom species have been recorded colonizing test

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surfaces throughout the world (Coe & Allen, 1937; Pyefinch, 1951; Hendey, 1951; Anon, 1952; Crosby and Wood, 1959; Skerman, 1956, 1958, 1959; Karajeva, 1964/65; Lebedev et al., 1964/65) comparatively few genera can be regarded as major foulers, i. e. ones which occur regularly on the hulls of in-service shipping as well as on inshore toxic test surfaces.

Pre-eminent amongst the fouling diatoms is the genus *Amphora*, which like the fouling macro-algae *Ectocarpus* and *Enteromorpha*, has a cosmopolitan distribution and an ability to grow in a wide range of conditions. Bishop et al. (1974) recorded *Amphora* spp. on Australian naval shipping while Hendey (1951) and Crosby & Wood (1959), found it colonising test panels coated with cuprous and mercuric oxides. Indeed Hendey regarded *Amphora* as the most resistant and troublesome diatom on cuprous oxide test surfaces. Further species have been observed by Phillip (1973) (illustration Figure 1, page 17 – identified by Phillip as *Nitzschia* sp.) and Callow et al. (1978) recorded members on organotin coatings.

The attachment of several species of marine fouling macro-algae, is achieved through spores becoming secured by the secretion of extracellular adhesives (Evans & Christie, 1970; Baker & Evans, 1973a, b; Chamberlain & Evans, 1973). Diatoms are known to produce mucilaginous materials which may afford attachment (Hendey, 1964; Chamberlain, 1976), although there is little information on their intracellular origin or secretion, especially in *Amphora*.

The ability to settle and proliferate on antifouling surfaces necessitates not only rapid adhesion but also some form of "defence" mechanism or protection against toxins. Hendey (1951) considered that protection in *Amphora* may be afforded by the production of copious mucilage although the possible interaction of metallic ions and porphyrin-based pigments as postulated by Bishop et al. (1972) would infer some form of intracellular immobilization.

The present investigation involved the study of *Amphora* films developed on ships' hulls and toxic surfaces. This is correlated with *in vitro* observations on cultured material with reference to extrafrustular secretion and intracellular origin. Histochemical techniques were applied to secreted mucilage in an attempt to identify the components involved with reference to heavy metal binding. Finally use of a transmission electron microscope X-ray analytical system allowed investigation into possible intracellular immobilization as a means of copper resistance.

MATERIAL AND METHODS

Scanning electron microscopy (S. E. M.)

Antifouling paint surfaces

Paint fragments recovered from supertankers (courtesy of Hempel Marine Paints), yachts and test panels were fixed for 2–3 hours in 4 % v/v glutaraldehyde in 0.1 M sodium cacodylate buffer containing 0.25 M sucrose or 3 % sodium chloride at pH 7.2. After rinsing in the buffer and sucrose solution post-fixation was carried out in 2 % w/v osmium tetroxide in 0.1 M sodium cacodylate (pH 7.2). Following thorough rinsing in deionized water, the material was plunged into liquid nitrogen and freeze-dried using an Edwards E. F. 6 drier. The freeze-drying technique was developed to prevent speci-

men shrinkage and cracking normally incurred in air-drying and also to avoid the use of acetone normally used in the critical point drying procedure which affects paint films.

Laboratory cultures

Amphora cells isolated from test panels were settled on 'Thermanox' coverslips (Lux Scientific Corporation Inc.) and were maintained in F/4 enriched seawater (Guillard & Ryther, 1962) at 15 °C with a 14–10 hr photoperiod and a light intensity of 2 K lux. Specimen material was fixed as above and dehydrated using an ethanol series and transferred via 2 : 1, 1 : 1, 1 : 2 ethanol-acetone mixtures to absolute acetone. After a period of one hour the material was critical point dried using liquid CO_2 and a Polaron E 3000 drier.

Cleaned frustules

Specimens scraped from antifouling coatings were acid-cleaned according to the technique of Hasle & Fryxell (1970) and pipetted onto either polycarbonate membranes (Bio-Rad Laboratories, U.S.A.) or copper grids bearing a carbon coated 'parlodion' film for subsequent S. E. M. or T. E. M. examination respectively.

In all cases material was mounted on stubs and coated with gold using a Polaron sputter coater. Final examination was in a Jeol T 20 scanning electron microscope.

Table 1. Histochemical techniques utilized

Component	Stain/test	Use	Reference
Proteins	Ninhydrin/Schiff reagent	L. M.	Yasuma & Ichikawa (1953)
	Bromophenol blue	L.M.	Pearse (1968)
	Dinitro-fluoro-benzene (D.N.F.B.)	L.M.	Danielli (1953)
	Acrolein/Schiff reagent	L.M.	Pearse (1960)
	Fast green F.C.F.	L.M.	Ruthmann (1970)
Carbohydrates	Periodic acid-Schiff reagent (P.A.S.)	L.M.	McCully (1966)
	Periodic acid-thiosemicarbazide silver proteinate (P. a-tsc-S.p)	E.M.	Thièry (1967)
Acidic groups	Ruthenium red	E.M.	Blanquet (1976)
Anionic macro-moelcules	Toluidine blue 0 at pH 5.6; 4.2 and 1.0	L.M.	Chayen et al. (1973)
Sulphate and carboxyl groups	Alcian blue/alcian Yellow	L.M.	Parker & Diboll (1966)
	Critical electrolyte concentration (C.E.C.) method	L.M.	Scott & Dorling (1965)
Sulphate groups	Tetrazonium-sulphonate method	L.M.	Pearse (1968)
	Heath's sulphate method	L.M.	Heath (1961)
	High and low iron diamine methods	L.M.	Spicer (1965)
Sulphate esters	Acriflavine-dimethyl amino-benzaldehyde	L.M.	Hollander (1964)
Lipids	Sudan black B	L.M.	Chayen et al. (1973)
	Mild methylation	L.M.	Spicer (1960)

Light microscope histochemistry

For histochemical observations laboratory cultures of *Amphora* spp. were fixed at pH 7.2 in either 10 % aqueous acrolein or 4 % formaldehyde in seawater. The different procedures utilized are outlined in Table 1.

Transmission electron microscopy (T. E. M.)

Material stripped from antifouling coatings or cultured on cover slips was fixed according to the S. E. M. procedure. After thorough washing in buffer the cells were dehydrated in an ethanol series followed by propylene oxide and flat embedded in Spurr's resin (1969). Ultrathin sections were cut using a Huxley ultramicrotome fitted with a diamond knife. Sections were collected on grids bearing a carbon coated 'parlodion' film and stained according to Reynolds (1963). Final examination took place in a Jeol 100 B electron microscope. For electron cytochemistry the technique of Thièry (1967) was employed on unstained sections mounted on 'parlodion' coated gold grids.

T.E.M.-Scan X-ray analysis

Primary films stripped from antifouling coatings and *Amphora* films cultured in solutions of copper chloride were processed as for normal T.E.M. but omitting post osmication. The *Amphora* isolates were derived from CuO antifouling coatings, and maintained in the laboratory for at least a month prior to testing. The culture medium was again based on Guillard & Ryther (1962) but with omission of EDTA and the addition of 0, 0.75 or 1.5 ppm $CuCl_2$. For analysis gold sections were collected on aluminium grids bearing a 'parlodion' film which was carbon coated before and after picking up sections. Unstained sections were examined using Jeol T.E.M.-scan 200 CX (accelerating voltage 100 Kv, beam current 5 μ amps, spot diameter approximately 10 nm). For correlation sections were also stained and observed as described above.

Test panels

Unplasticized PVC sheet was cut to give 20 × 10 cm test panels. These were primed and coated with a range of antifouling (A/F) compositions but at different particle sizes to yield paints of low-high copper leaching rates. Additional panels were coated with an A/F composition containing CuO and triorganotin compound(s) as a booster. The panels were exposed on rafts in Langstone Harbour, Hampshire, U.K. by the kind courtesy of the Admiralty Exposure Trials station.

RESULTS

Observations on the colonisation of A/F paint surfaces

Test panels coated with slow-leaching cuprous oxide anti-fouling paint and immersed in Langstone Harbour, U.K. were soon colonised by a range of diatoms including several species of *Amphora* (Table 2). After one month, a visible film could be

detected on the slow-leaching panels but on rapid-leaching panels, 2–3 months were required before discrete 1–2 cm diameter *Amphora* colonies could be detected. Initially, colonies were golden brown in colour, but later turned grey-green. The films varied from 20–400 μm in thickness (Fig. 1: *1–4*) and originated from distinct foci of colonisation.

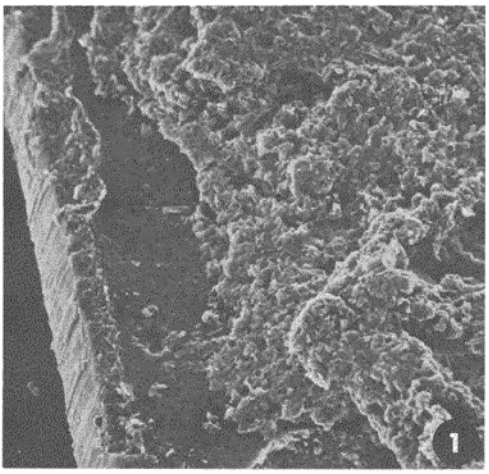
The apparent importance of *Amphora* spp., especially *A. veneta* (Fig. 1: *5, 6*), was reinforced when samples from "in service" supertankers and yachts were also examined (Table 2, Fig. 2: *7–12*). However, the tankers were mainly colonised by *Amphora* species not present on test-panels, namely *A. bigibba* (Fig. 2: *11*) and *A. (delicatissima?)* (Fig. 2: *9*). These films achieved 200–250 μm in thickness (Fig. 2: *7, 8*). In some samples, the films were disrupted, presumably by exfoliation, but normally in such cases, a residual, or perhaps recolonising layer was present (Fig. 2: *8*). The settlement and colonisation by *Amphora* spp. was quite rapid and they may be dominant on test-panels after 4 weeks, some ship samples (Table 2) show that they can remain the most important micro-fouling organisms even after 18 months.

The *Amphora* films were always associated with copious mucilage (Fig. 2: *3, 5*; Fig. 3: *9, 10*) and particulate matter. This was expecially so on panels coated with cuprous oxide paints containing organotin boosters. These panels appeared to encourage bacterial colonisation and *Amphora* settlement occurred on a bacterial (pre-conditioning?) film. However, further observations showed that *Amphora* settlement can occur, on copper-containing paints at least, without the presence of such a layer. The presence of copious mucilage unites the micro-organisms into a cohesive mass.

Figures 1 and 2 not only demonstrate the gross changes in surface topography, but also indicate how the "slime" film may affect the performance of both paint and craft. Although many other diatoms, particularly members of the genera *Navicula* and *Nitzschia* (Fig. 2: *11, 12*) were observed in fouling films almost unialgal populations of *Amphora* were frequently encountered. In view of this, a more detailed examination of *Amphora* species both *in situ* and isolated from fouling samples was carried out.

Table 2. The occurrence of *Amphora* spp. on toxic A/F coated test surfaces (T/S) and 'in-service' ships

Date	Location	Toxin in use	Species	Period of immersion (months)
1978	Langstone Harbour U.K. T/S	Cuprous oxide (rapid leaching and slow leaching)	*A. exigua* *A. veneta* *A. coffeaeformis*	1–6
1978	Supertanker (Rotterdam-Persian Gulf)	Cuprous oxide	*A. bigibba* *A. coffeaeformis*	1–18
1979	Supertanker (Australia-Persian Gulf)	Cuprous oxide/ tri-organotin	*A. sp (delicatissima?)*	1–3
1979	Yachts (Gosport harbour, U.K.)	unknown	*A. veneta* *A. exigua* *A. coffeaeformis*	probably one fouling season only
1980	Langstone Harbour U.K.T./S	Curprous oxide/ tri-organotin	*A. coffeaeformis*	1–3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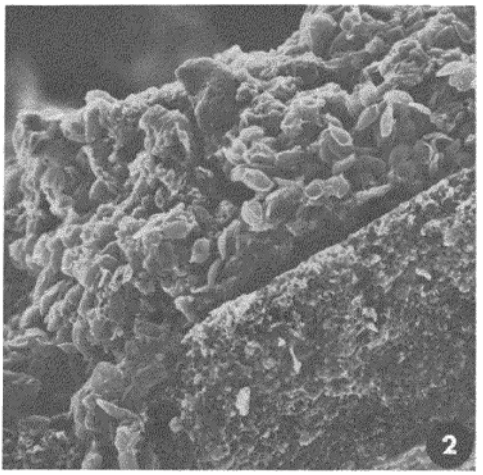
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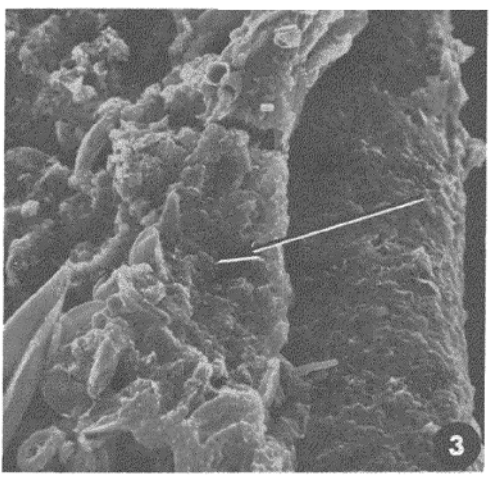
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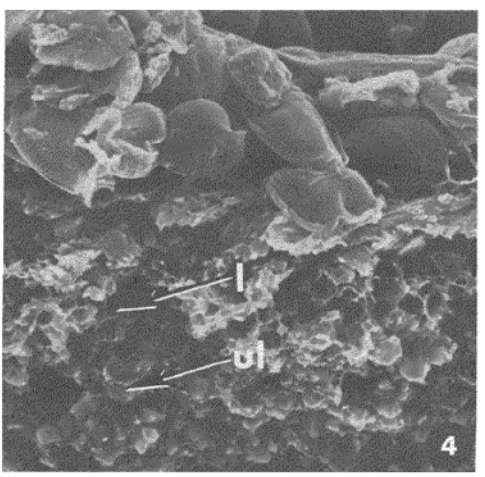
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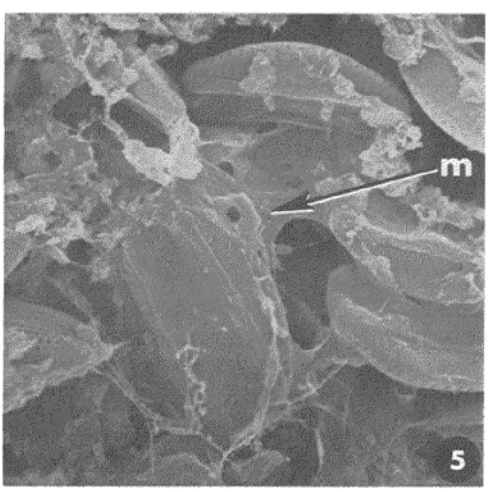
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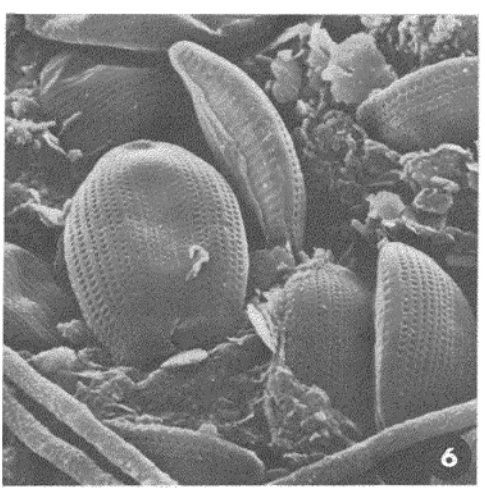
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Gross morphology and mucilage secretion

Amphora is a member of the Biraphidineae and hence possesses two raphes. However, the cells, although symmetrical on their transapical axis, are asymmetrical on the apical axis. The valves are sub-lunate (Fig. 3: *13, 14*) and are united into a complete frustule with a greatly enlarged dorsal and reduced ventral girdle (Fig. 2: *12*; Fig. 3: *15*; Fig. 4: *21*).

The girdle is composed of numerous connecting bands (Fig. 2: *12*; Fig. 4: *21*) which are much wider on the dorsal side, a feature responsible for individuals being usually observed in this view (Fig. 4: *21*). As a result, the ventral margins of the valves and thus the two raphes lie, almost parallel on the ventral surface (Fig. 3: *15*; Fig. 4: *22*). This is believed to have an important effect on attachment since a proportion of mucilage appears to be secreted through them (Fig. 4: *19, 22*).

In the species investigated, the raphes were straight rather than biarcuate, possessing a prominent central nodule and two polar nodules (Fig. 3: *15, 16*). Under the S.E.M. the valve surface appears striate with numerous simple pores constituting each of the striae. With the increased resolution of the T.E.M. these pores are seen to be closed by a finely perforated velum (Fig. 3: *17*). The pores of the connecting bands are similarly constructed.

In comparison with other fouling diatoms, *Amphora* spp. are comparatively small. Thus, examination of mucilage extrusion was most easily examined by scanning electron microscopy. Cells initially isolated from A/F test-panels were cultured and the development of a "slime" film followed. Recently settled cells are highly motile and lay-down mucilaginous trails. However, once attachment has occurred, quantities of adhesive mucilage are found located around the lower margins, particularly the polar region (Fig. 4: *18, 19*).

If adhering cells are detached from the substrate, they leave a characteristic elevated pattern of mucilage complementing the underside of the cell. This demonstrates that the secretion is associated with polar, raphe and valve margin areas (Fig. 4: *18–23*). Often distinct lines of adhesive, indicative of the position of the raphes, are located along the longitudinal axis of the attachment 'mould' (Fig. 4: *19, 20*). The adhesive at the centre of this mould is scanty; further evidence for the secretion's origin being associated with the raphes and valve margin areas. Some cells, however, have been observed with large quantities of mucilage secreted directly from the margins,

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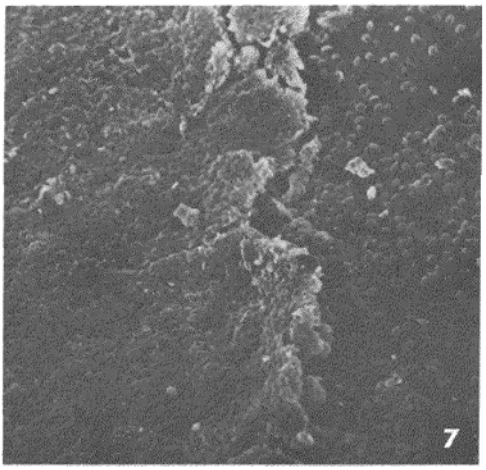
Fig. 1. *1–6:* S. E. M. of paint fragments from test panels immersed in Langstone Harbour, U. K. *1:* Low power micrograph showing heavy settlement of *Amphora* sp. on a slow-leaching cuprous oxide paint (× 280). *2:* Cross-section of cuprous oxide paint showing thickness of *Amphora* film in comparison with paint (× 800). *3:* Shows peeling from the surface of a primary film. Notice the underside of the film (arrowed) which is amorphous and probably bacterial in origin (× 1,520). *4:* Cross-section of a cuprous oxide paint with *Amphora* cells adpressed to the surface. The leached (l) and unleached (ul) layers of the paint are apparent (× 1,760). *5:* Showing the underside of an *Amphora* film. Note the intricate meshwork and copious mucilage (m) involved in holding the film together (× 2,600). *6:* High power micrograph showing *A. veneta* one of the most prominent diatom foulers found in this study (× 3,000)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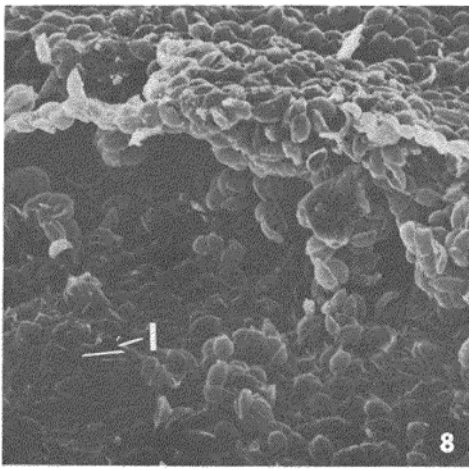
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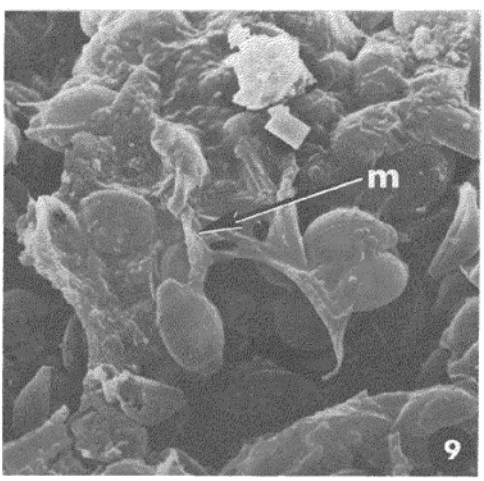
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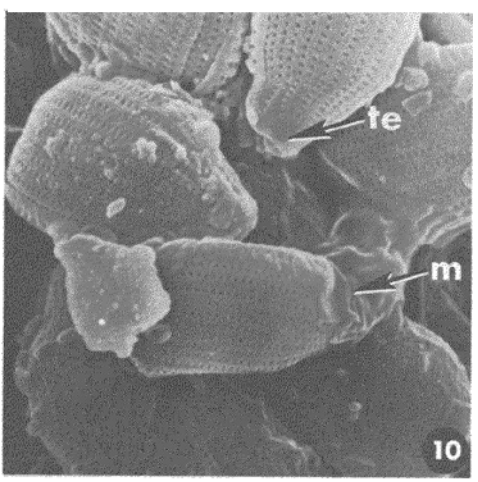
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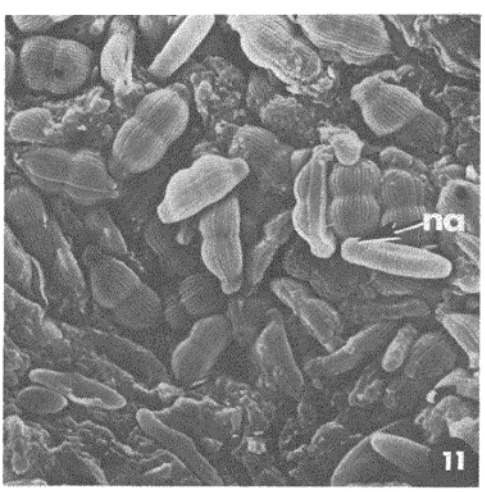
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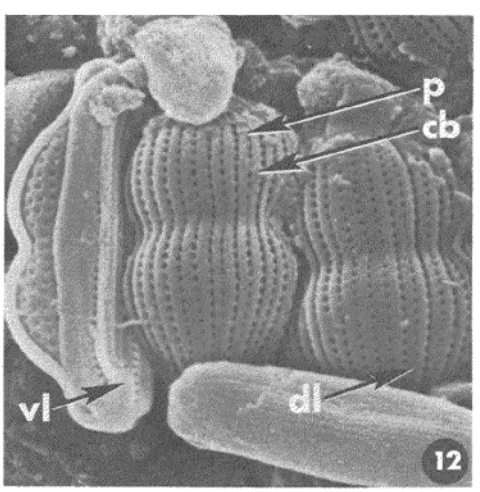
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either outer (Fig. 4: *21*) or inner (Fig. 4: *22*) whilst others have small quantities over the entire frustule, as seen in some films from A/F paint surfaces.

The copious mucilage secreted, the shape and size of the cells, the position of the raphes and the large surface contact area no doubt all contribute to its success in remaining attached.

Light microscope histochemistry

The results are summarised in Table 3. Histochemical investigations were carried out on cultures of *A. veneta* which were established on glass or "Thermanox" coverslips and fixed *in situ*.

The basic thiazine dye toluidine blue 0 was used to detect anionic macromolecules as specified by Chayen et al. (1973). This dye has had widespread use (McCully, 1966; Feder & O'Brien, 1968; Evans & Holligan, 1972) and is known to react metachromatically with free carboxyl, sulphate and phosphate groups. By changing the pH of the dye solution the acidic nature of the adhesive polymer was determined. The moderate reaction at pH 1.0 indicates that sulphate and phosphate groups are present. The very strong metachromatic reaction at pH 4.2 indicates that carboxyl groups may also exist. Further evidence for the presence of acidic groups (Blanquet, 1976) was obtained from the intense staining with ruthenium red at both light and electron microscope levels.

Evidence for the presence of carboxylic and sulphated polysaccharides was obtained using the alcian dyes technique of Parker & Diboll (1966). The results suggest that sulphated groups predominate although carboxyl groups may exist due to the weak reactions at pH 2.5 and the blue/green colour resulting from the alcian sequence.

The C.E.C. series is a more stringent method for the differentiation of acidic substances. Essentially, in conjunction with alcian blue, both sulphated mucins and glycosaminoglucuromoglycans containing carboxyl groups stain in the presence of low concentrations (below 0.3 M) of magnesium chloride whereas only sulphated types do so at higher concentrations (above 0.8 M). Once again the results indicate that both groups are present, with the most intense reactions occurring between 0.10 and 0.5 M magnesium chloride, the molarity at which sulphated and carboxyl groups are likely to add to the staining.

Further more specific methods including the Tetrazonium-sulphonate, Heath's stain

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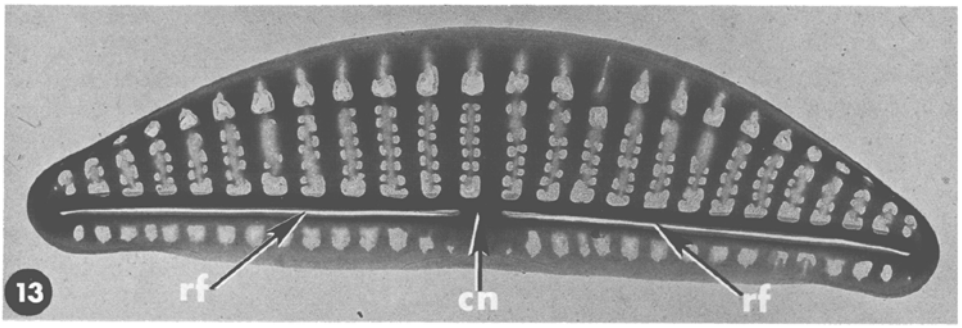
Fig. 2. *7–12:* S. E. M. of paint fragments from 'in-service shipping'. *7:* Low power micrograph showing an *Amphora* film. (Yacht, Gosport harbour, U. K.) (× 160). *8:* Unialgal *Amphora* film from a Supertanker on route from the Persian Gulf to Australia. Although the major part of the film has been sloughed-off a residual or recolonising layer (1) appears adpressed to the paint surface (× 480). *9:* High power of 8 showing the copious mucilage (m) surrounding and often encapsulating the cells (× 1,200). *10:* Detailed S. E. M. of 9 showing mucilaginous material (m) associated with the truncated ends (te) of the cells (× 4,160). *11: Amphora bigibba* film on a cuprous oxide paint surface. Note the shape of the cells and their investment in amorphous material within a surface cranny. Members of the Naviculaceae are also present (na) (Supertanker on route from Rotterdam to the Persian Gulf) (× 1,200). *12:* Detailed S. E. M. of *11*. The ventral (vl) and dorsal (dl) structure of the cells is evident. Note the complexed dorsal girdle composed of punctae (p) bearing connecting bands (cb). (× 4,400)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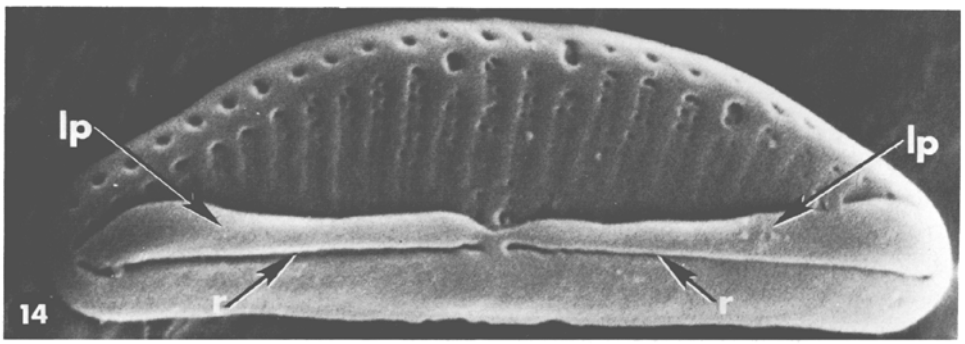
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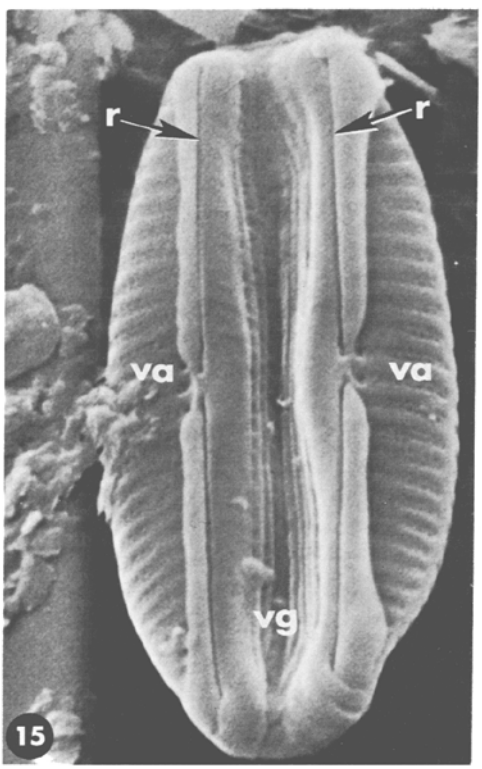
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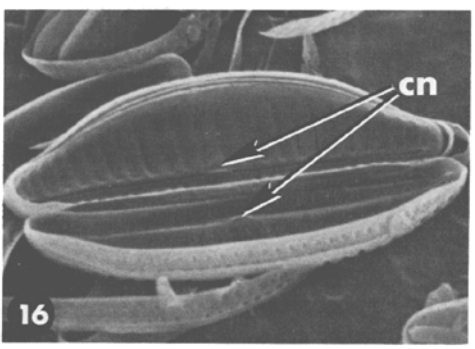
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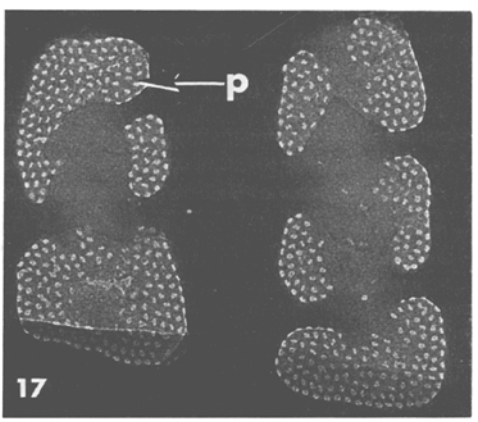
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and the Diamine techniques illustrated the presence of sulphate groups. Finally, the definitive acriflavine DMAB test for sulphated esters resulted in moderate staining proving beyond doubt the presence of sulphate.

The P.A.S. reaction is a diagnostic histochemical test for the presence of polysaccharides with free vicinal hydroxyl groups (Hotchkiss, 1948). Staining of *A. veneta* gave weak to negative results indicating that these groups were not present, or that they were masked in some way. At the E.M. level the P.A.S. equivalent reaction, P.a-tsc.-S.p also proved negative, giving similar indications. However, after methylation a slightly

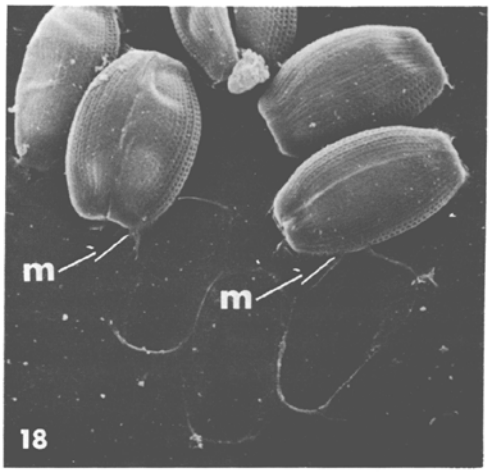
Table 3. Results of histochemical staining of adhesive material secreted by *Amphora veneta*

Stain/test	Reaction	Colour
P.A.S.	−	−
P.A.S. after methylation	+/−	Pinkish
P.a-tsc-S.p	−	−
Ruthenium red	+++	Red
Toluidine pH 5.6	+++	γ Red/pink
blue 0 pH 4.2	+++	γ Red/pink
pH 1.0	++	β Purple/pink
Alcian blue pH 1.0	+++	−
Alcian yellow pH 2.5	+	−
Alcian blue/yellow sequence	++/+++	Blue/green
C.E.C. Series 0.025 M	++	Blue
0.05 M	++	Blue
0.10 M	++	Blue
0.45 M	+++	Deep blue
0.80 M	+	Blue
1.00 M	+/−	Light blue
Tetrazonium method	+/++	Reddish/violet
Heath's method	++/+++	Red/purple
Hi Diamine method (HID)	++	Purple/black
Low Diamine method (LID)	++	Purple
Acriflavine DMAB method	+/++	Orange

Reactions are graded: +++ very strong
++ moderate
+ weak
− negative

All protein and lipid tests proved negative

Fig. 3. *13–17:* Electron micrographs of acid cleaned frustules of *Amphora* sp. *13:* T. E. M. of the valvar face of *A. veneta*. Note its sub-lunate shape and the presence of two raphe fissures (rf) separated by a central nodule (cn) (× 8,560). *14:* Using the S. E. M. the valvar face is seen to bear a distinct lip-like (lp) process continuous with the raphe (r) (× 9,600). *15:* S. E. M. showing the underside of an entire *Amphora* frustule. The two valves (va) bearing 4 raphes (r) are united by the central girdle (vg) (× 7,200). *16:* S. E. M. showing an *Amphora* cell split open along its dorsal face. The central nodules (cn) are observed as distinct humps projecting into the complete cell (× 4,480). *17:* T. E. M. of the valvar marginal area. The pores are not homogenous but contain distinct punctae (p) (× 75,850)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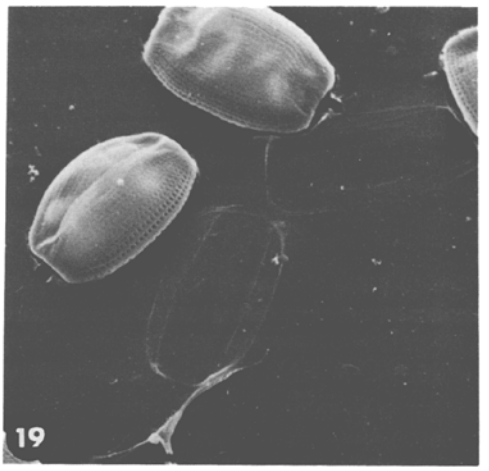
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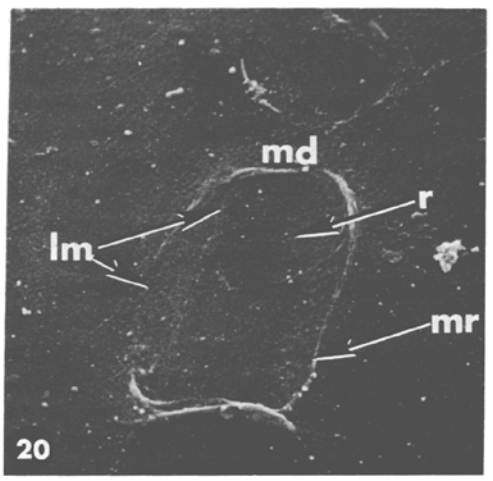
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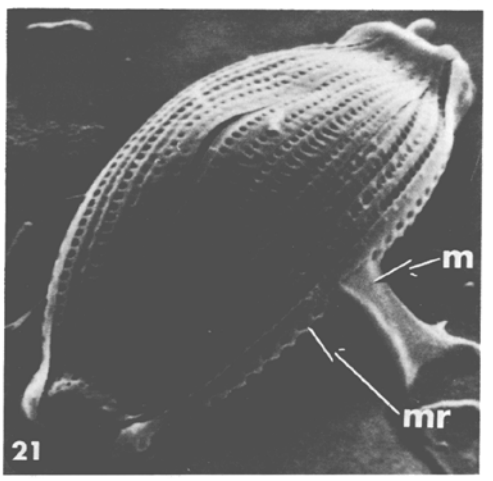
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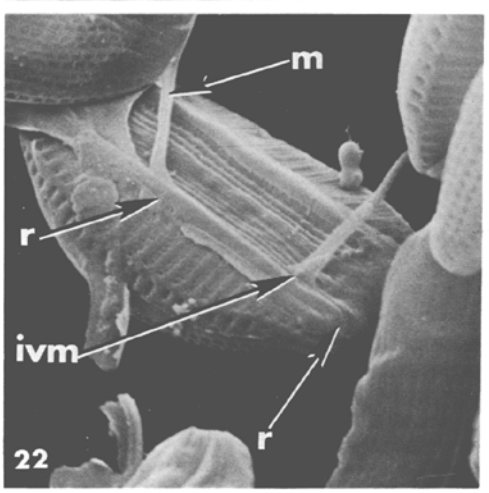
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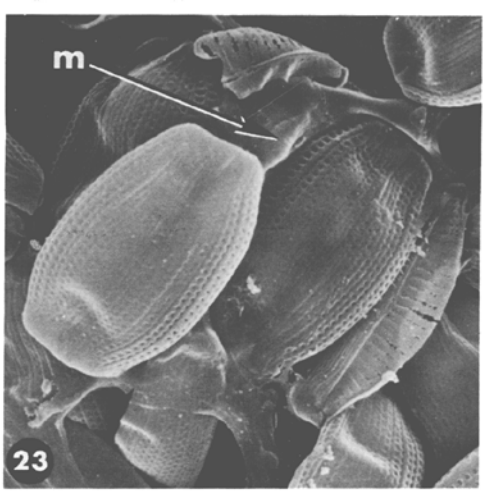
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positive result was achieved. As methylation is known to remove sulphate groups it is tentatively proposed that these may be substituting on the vicinal hydroxyl groups and masking them.

All protein tests proved negative, as did the Sudan Black B test for lipids. Very few reports of proteins in diatom adhesives exist although the present author (unpublished results), Lewin (1958) and Paulsen et al. (1978) have noted its presence in *Berkeleya rutilans*, a tube-forming species.

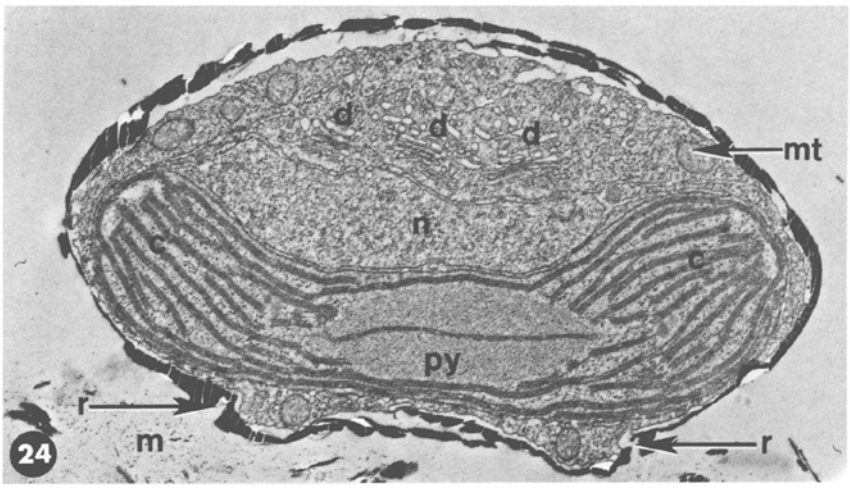
Staining highlighted the mucilaginous trails laid down during movement. However, the most intense reactions were associated with the cells, particularly the regions beneath the raphes (especially the sites of the central and polar nodules) and along the ventral margins. This was correlated by similar staining intracellularly suggesting secretion from these regions.

Transmission electron microscopy of cultured *Amphora veneta*

Transverse median sections are shown in Figure 5: *24, 25*. The centrally placed nucleus is bounded by the chloroplast and Golgi apparatus. The several mitochondria are arranged peripherally mainly beneath the plasmalemma. The nucleus contains a prominent nucleolus and is surrounded by a double membrane the outer of which is continuous with outermost membrane surrounding the chloroplast. Large vacuoles normally observed towards the ends of the cells often contain electron-dense storage bodies, depending on the age and activity of the cell. The chloroplasts are characterized by lamellae (Fig. 5: *24, 25*) running the length of the cell each consisting of three thylakoids. Within the chloroplast is a pyrenoid composed of dense granular material surrounded by a thin membranous line. Traversing the pyrenoid is a single lamella, an extension from one of the plastid lamellae.

On its dorsal side the nucleus is bounded by an endomembranous system consisting of dictyosomes, endoplasmic reticulum and numerous perinuclear vesicles. The dictyosomes, usually 4–6 per cell, consist of 5–8 cisternae and are always located in a perinuclear position. The cisternae adjacent to the nucleus constitute the forming face of the dictyosome and are thought to form from the fusion of vesicles produced by the outer nuclear membrane (Fig. 5: *24*; Fig. 6: *26*). In accordance, the outer cisternae represent the mature face of the dictyosome.

Fig. 4. *18–23:* S. E. M. of cultured *Amphora* cells on "Thermanox" coverslips. *18:* Low power showing recently attached cells. Mucilage (m) is located around the peripheral margins of the cells (× 2,000). *19:* Showing cells and respective mucilaginous moulds. Note the characteristic pattern of mucilage which complements the underside of the cell (× 2,000). *20:* An adhesive 'mould' (md) remaining after removal of the cell. The mould, with scanty secretion in the centre, is characterised by distinct lines of mucilage (lm) in the region of the raphes (r) and margin (mr) (× 3,360). *21:* High power showing the sub-lunate shape of the cell and adhesive material (m) extruding from the margin (mr) (× 6,000). *22:* The underside of an *Amphora* cell. Note the copious secretion (m) associated with the raphes (r) and the inner valve margin (ivm) (× 5,360). *23:* Low power of a group of *Amphora*. The cells are held together in a colony by mucilaginous material (m) secreted through the ventral regions of the cells (× 3,000)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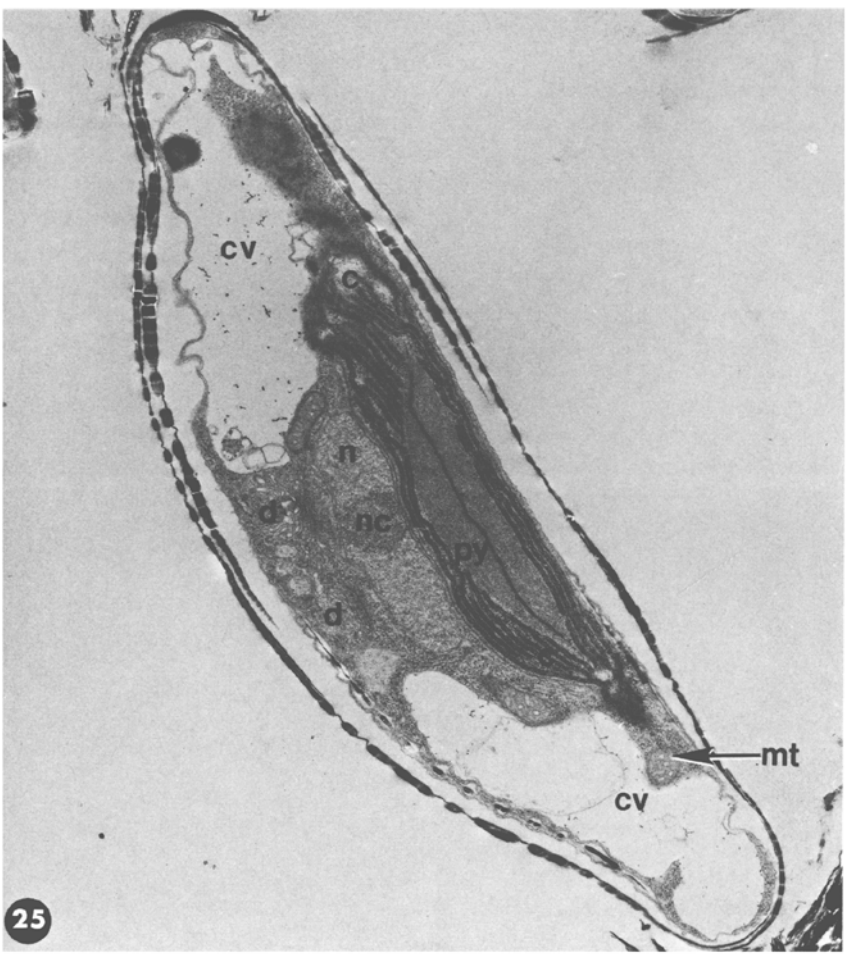
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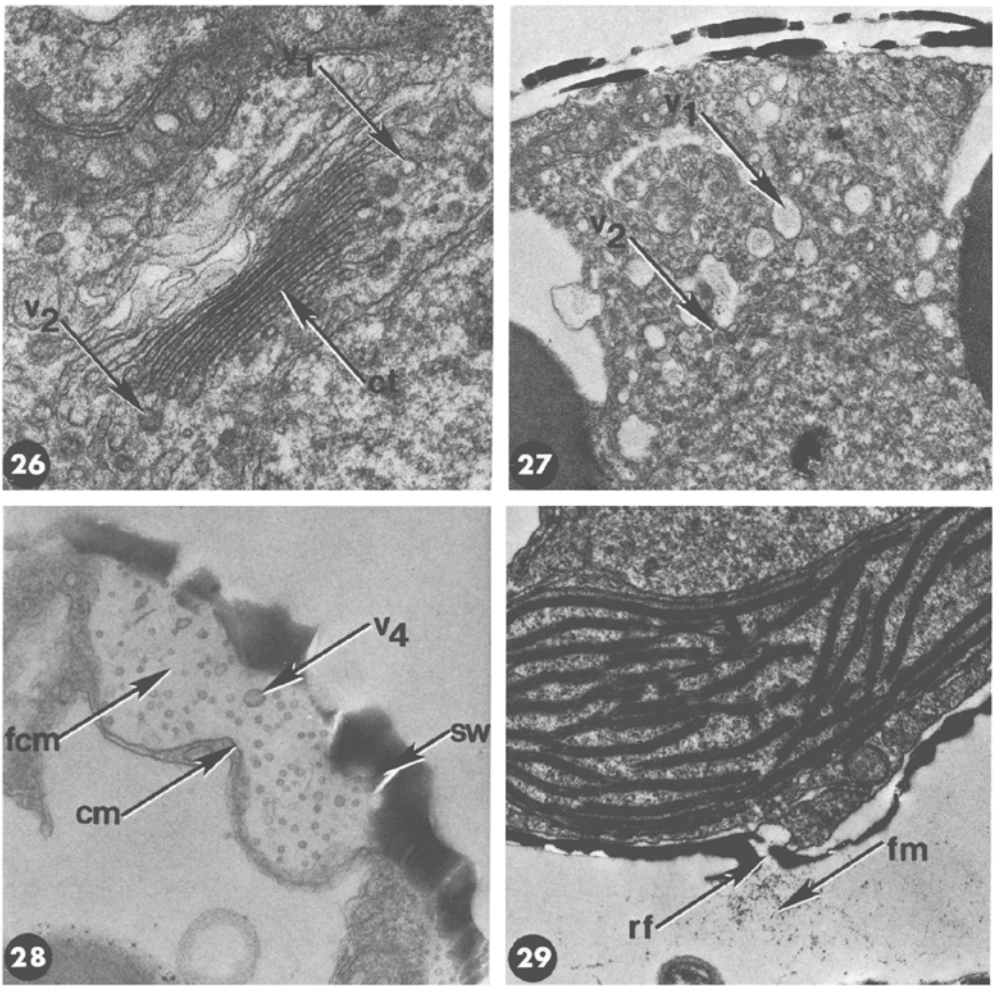


Fig. 6. *26–29:* T. E. M. of thin sections of *Amphora veneta*. *26:* T. S. through a perinuclear dictyosome. The dictyosome is composed of 8 cisternae (ct) which are blebbing fibrous (v_1) and smaller granular (v_2) vesicles (× 44,000). *27:* Oblique L. S. through the central region. Irregularly shaped fibrous (v_1) and smaller granular vesicles (v_2) are apparent (× 32,400). *28:* T. S. part of cell to show frustule-cell-membrane interface (fcm). Large numbers of vesicles (v_4) are present outside the cell membrane (cm) but within the silica wall (sw) (× 33,000). *29:* T. S. showing fibrous material (fm) extruding from a raphe fissure (rf) (× 22,400)

Fig. 5. *24:* T. S. (T. E. M.) through the central region of a typical *Amphora veneta* cell showing position of nucleus (n), chloroplast (c), pyrenoid (py), dictyosomes (d) and peripheral mitochondria (mt). Note the position of the raphes (r) and the mucilage (m) which is seen in close proximity (× 14,650). *25:* Median longitudinal section (T. E. M.) through an *Amphora veneta* cell showing nucleus (n), nucleolus (nc), chloroplast (c), pyrenoid (py), dictyosomes (d) and the large cell vacuoles (cv) (× 11,000)

The cisternae are seen to bud vesicles which are liberated into the surrounding cytoplasm (Fig. 6: *26*). Two main types of intracellular vesicles were observed:

(a) Large, smooth vesicles (v_1) produced by the more mature cisternae. These vesicles were irregular in shape and contained dispersed fibrous material (Fig. 6: *26, 27*).

(b) Smaller electron-dense vesicles (v_2) containing granular material. These are usually in the range of 0.05 μm in diameter and were produced by the forming face of the cisternae (Fig. 6: *26*).

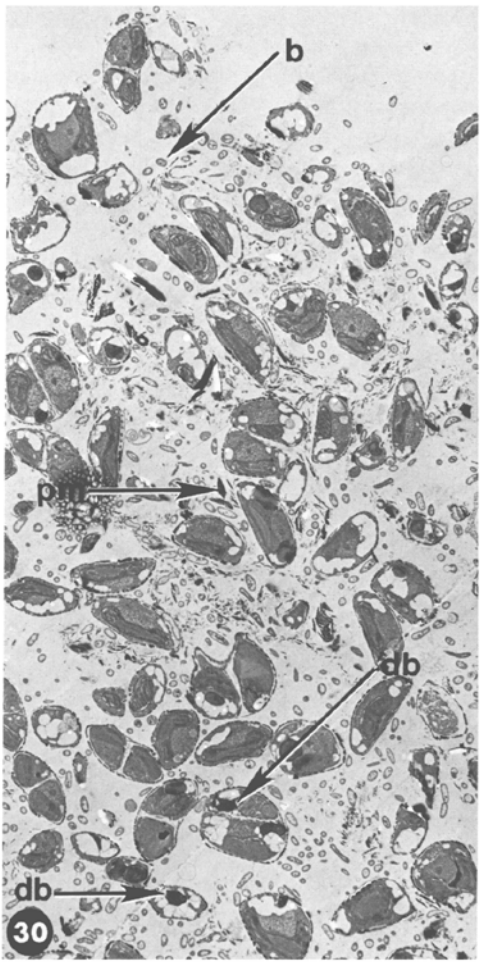
Tentatively a third vesicle type (v_3) with granular contents but with a densely staining membrane was occasionally observed. However these were very similar to (v_2) apart from size. Other smaller vesicles and multivesicular bodies are often found associated with the cisternal ends, however it was not possible to determine whether they were liberated by them.

Vesicle types v_1 and v_2 are observed throughout the peripheral and central cytoplasm. The irregular vesicles (v_1) are often within and protruding into the large cell vacuoles (Fig. 6: *27*).

Smaller vesicles (v_4) are often seen outside the cell membrane at the frustule-membrane interface (Fig. 6: *28*). Aggregation of vesicles in this position is a common feature and may occur around the entire periphery of the cell. However, larger numbers are usually found in the ventral regions of the cell, often in close proximity to the raphe fissures. Above the raphe the cell membrane is usually contracted away from the frustule edge leaving a region continuous with the exterior. Often fibrous material is observed extruding from these fissures and is believed to be the mucilaginous material recognised with the S.E.M. (Fig. 6: *29*). Since vesicles can be found around the entire frustule-cell membrane interface one cannot preclude mucilage secretion from any part of the cell, but the raphe fissures do offer a direct route to the outside.

An attempt was made to determine the chemical composition of the Golgi vesicles contents using the E.M. technique of Thièry (1967). However, the test, like its L.M. counterpart, proved negative, indicating that none of the vesicles contained material with free vicinal OH groups. Further tests are in progress to determine the composition of these vesicles.

Fig. 7. *30–33: Amphora* cells stripped from cuprous oxide panels. *30:* T. S. of an *Amphora* film stripped from a cuprous oxide panel. The cells in L. S. and T. S. are in association with bacteria (b) and particulate matter (pm) all held together by extracellular mucilage. Characteristically vacuolated, the cells also contain electron dense bodies (db) (× 1,440). *31:* Oblique section through a dead *Amphora* cell, a probable result of copper poisoning. The chloroplast lamellae (cl) are typically distended and dense inclusions are observed within it. Dense irregular bodies (db) are also observed within the disrupted cytoplasm (× 4,560). *32:* High power T. E. M. showing a dense body from the vacuolar region. The body appears membrane bound and contains numerous electron dense inclusions (di) (× 27,760). *33:* X-ray spectrum (0–10 KeV) of the dense granular inclusions. Note the copper Kα, Kβ and Lα peaks at 8.04 eV, 8.90 eV and 0.928 eV. Peaks for Kα phosphorus (2.01 eV), Kα sulphur (2.30 eV), Kα calcium (3.68 eV), Kα silicon (1.74 eV), Kα chlorine (2.62 eV) and Kα aluminium (1.49 eV) are also evident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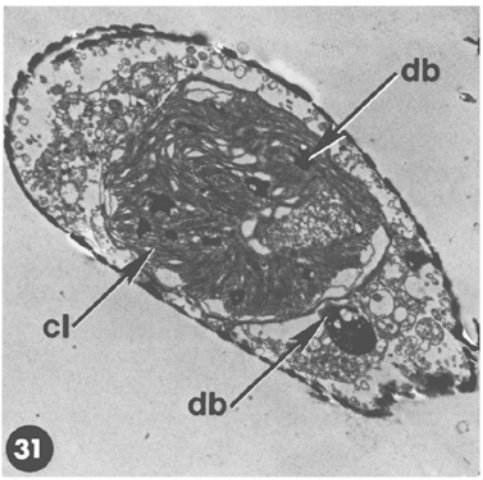
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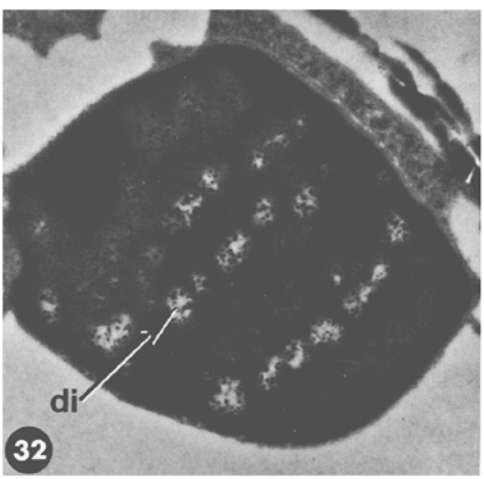
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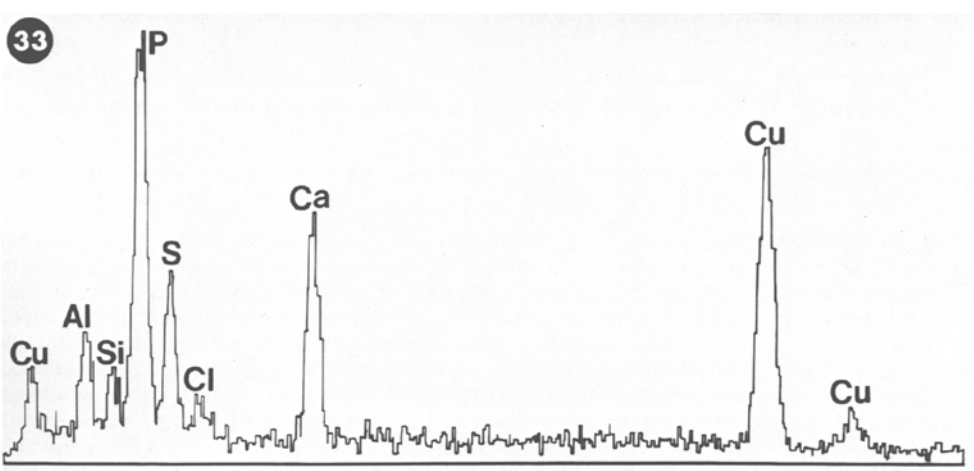
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T.E.M.-scan X-ray microanalysis of intracellular inclusion bodies

When *Amphora* slime-films are processed for T.E.M. the intricate relationship between the cells and bacteria becomes apparent (Fig. 7: *30*). This film was approximately 100 μm thick and removed from a slow leaching cuprous oxide panel two months after immersion in Langstone Harbour, U.K. It was laden with *Amphora* cells, bacteria, mucilage and associated particulate matter. In surface profile the films are usually corrugated with greatest thickness occurring at the centres of colonization.

The cells seen in L.S. and T.S. are vacuolated with a single chloroplast, nucleus and pyrenoid. Some of the cells are actively dividing whereas other appear dead with loss of internal structures. Particularly conspicuous within the cells are dense spherical bodies (0.4–2.0 μm in diameter) usually within or protruding into the large cell vacuoles although smaller irregular bodies also occur (Fig. 7: *30, 31*). Up to five such bodies may be observed in any one cell although two or three are the norm. Similarly dense bodies have been observed within many of the film bacteria.

Dense bodies often occur when osmium tetroxide is used as a fixative and is normally taken as an indication of lipid material. If the bodies are observed in detail smaller dense inclusions are seen (Fig. 7: *32*). To determine the elemental composition of the inclusions thin sections of unosmicated material were analysed using an energy dispersive spectrometer capable of analyzing X-ray energies up to 10 KeV. The presence of copper in the inclusions was indicated by the characteristic peaks at 8.04 KeV (Kα-line), 8.90 KeV (Kβ-line) and 0.928 KeV (Lα-line) respectively (Fig. 7: *33*). Other peaks observed were silicon (Kα 1.74 KeV), phosphorus (Kα 2.01 KeV), sulphur (Kα 2.30 KeV) and calcium (Kα 3.68 KeV). The peaks for chlorine (Kα 2.62 KeV) and aluminium (Kα 1.49 KeV) result from the embedding resin and the support grid respectively.

To investigate further the presence of these inclusions *Amphora* cells isolated from toxic panels were cultured in the laboratory and subsequently treated with 0, 0.75 and 1.5 ppm copper chloride ($CuCl_2$). Cells were treated for one week and subsequently fixed in 4 % glutaraldehyde in sea-water and processed as for normal T.E.M.

Amphora films treated with 1.5 ppm $CuCl_2$ became grey-green and the cells died within three days. Films treated with 0.75 ppm $CuCl_2$ were greeny-brown but the cells retained motility albeit sluggish. The control cells retained motility and films remained golden-brown.

Thin sections of cells treated with 0.75 ppm $CuCl_2$ were characterised by large granules (Fig. 8: *34, 35*), as many as five per cell. They were normally located within or

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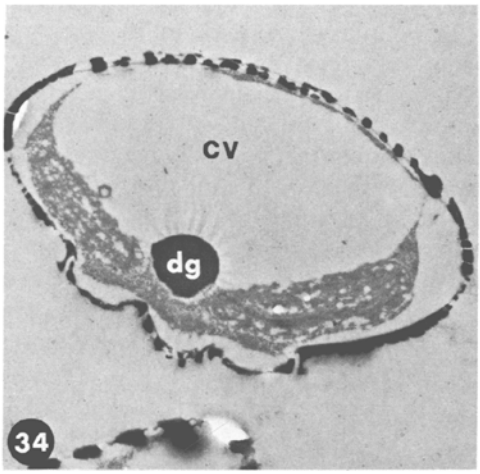
Fig. 8. *34–38: Amphora* cells treated with varying concentrations of $CuCl_2$. *34:* T. S. of an *Amphora* cell treated with 0.75 ppm $CuCl_2$ for one week. A dense granule (dg) is observed protruding into the cell vacuole (cv) (× 11,200). *35: Amphora* cell (oblique T. S.) treated as above. Two large granules are visible within the cell vacuoles (cv) and appear attached to peripheral cytoplasm (pc) (× 8,160). *36:* High power of an intracellular granule. Lighter regions (arrows) may be observed within the structure (× 37,850). *37: Amphora* cell treated with 1.5 ppm $CuCl_2$. Note the large amounts of mucilage (m) in close proximity to the cell, the contraction from the cell wall of the cytoplasm and cell membrane and the disorganised chloroplast (dc) (× 38,640). *38:* X-ray energy spectrum (0-10 KeV) from an intracellular granule. Note the characteristic copper, sulphur, silicon, calcium, aluminium and chlorine peaks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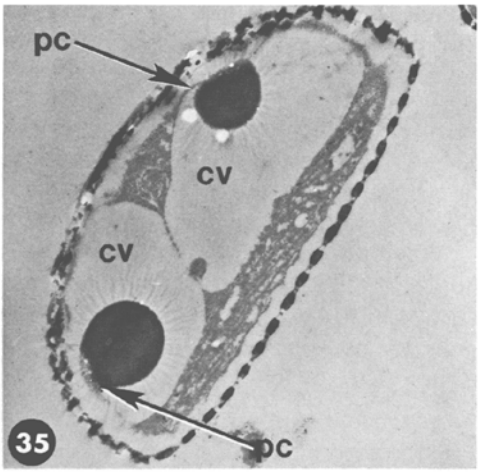
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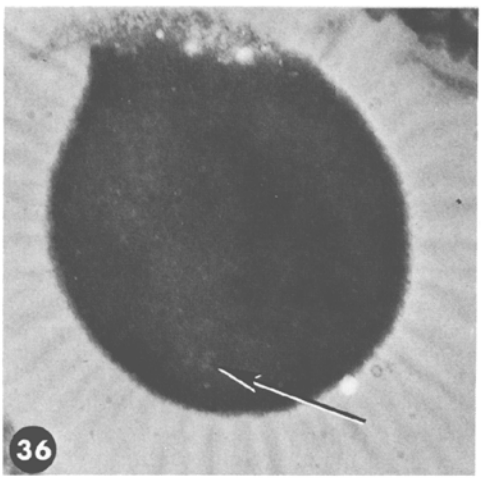
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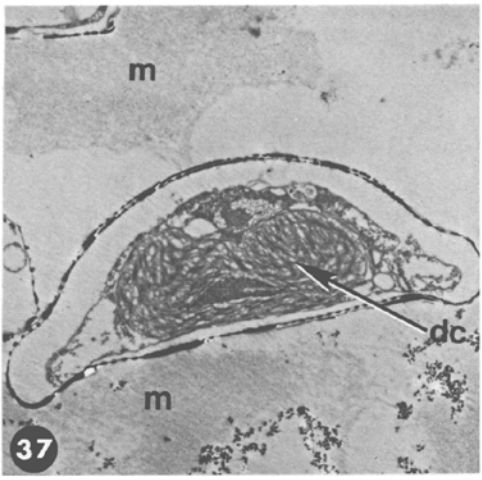
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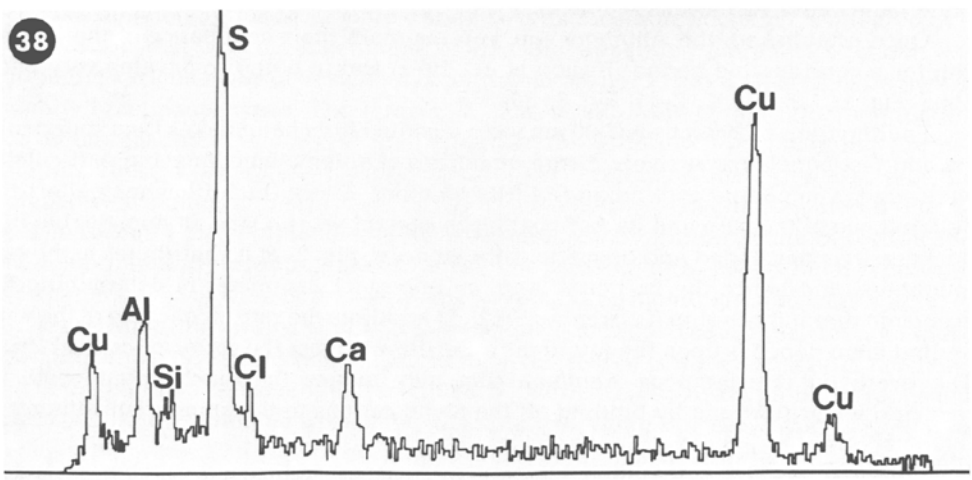
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protruding into the large cell vacuoles as in the 'wild' type cells. Granules as large as 1.2 μm were seen, usually bound to the peripheral cytoplasm (Fig. 8: *35, 36*). However, it was not possible to determine whether the granules were membrane bound. Other subcellular structures were similar to the control. Cells treated with 1.5 ppm $CuCl_2$ showed disorganised chloroplasts, characteristically abnormal nuclei and contraction of the cytoplasm and cell membrane from the edge of the silica frustule (Fig. 8: *37*). Also copious amounts of mucilage were observed in close proximity to all cells.

When analysed these granules gave copper peaks at Kα (8.04 KeV), Kβ (8.90 KeV) and Lα (0.928 KeV) (Fig. 8: *38*). Characteristic peaks for sulphur (Kα 2.30 KeV), silicon (Kα 1.74 KeV) and aluminium (Kα 1.49 KeV) were recorded. Unlike the 'wild' situation peaks for phosphorus (Kα 2.01 KeV) and chlorine (Kα 2.62 KeV) were low although calcium (Kα 3.68 KeV) was evident.

DISCUSSION

The preponderance of *Amphora* spp. in fouling samples from widely separated geographical locations examined in this study indicate their cosmopolitan nature. The occurrence and frequent dominance of *Amphora* on anti-fouling coatings agrees with the results of Hendey (1951), Crosby & Wood (1959) and Bishop et al. (1972) (see Table 4). Predictably, the major species of *Amphora* encountered depends upon the location and previous history of the sample, thus *A. bigibba, A. (delicatissima?)* and *A. coffeaeformis* were recorded from shipping while *A. exigua, A. veneta* and *A. coffeaeformis* were dominant on cuprous oxide bearing test panels. Hendey (1951) recorded similar observations for cuprous oxide paints exposed in southern U.K. waters. The ability of *Amphora* to grow on biocides other than copper is shown by their occurrence on A/F paints containing tri-organotin compounds (Table 2 and Callow et al., 1978), and mercuric oxide (Table 4, Crosby & Wood, 1959). Indeed, Stanbury (1944) in laboratory experiments found that the levels of biocide required to prevent growth of *A. exigua* were 1.0 ppm for copper and 1.85 ppm for mercury. Crosby & Wood (1959) working in Australia found *A. arcta* the only diatom species growing on both mercuric and cuprous oxide paints after two weeks (Table 4).

Once established, the *Amphora* spp. can maintain their dominance of the fouling film for a considerable period (Bishop et al., 1972) and in doing so develop very thick films (Fig. 1: *1–6*; Fig. 2: *7–11*; Fig. 7: *30*).

Fouling films of 200 μm and 300 μm were recorded from paint flakes from supertankers and test panels respectively. Large quantities of anionic mucilage and particulates were always important in binding the film together. These thick films may affect the performance of the ship and its A/F coating in several ways. Once an appreciable film thickness has developed and begun to exfoliate (e. g. Fig. 2: *8*) it contributes to the hull roughness, and hence the frictional drag, an important parameter in determining the economic running of a ship (Lackenby, 1962). In addition, the rate of leaching of the anti-fouling toxin depends upon the turbulent water-flow accross the surface (Marson, 1969). The overlying mucilaginous *Amphora* film may reduce the leaching rate both by reducing water-flow and by binding off the metal cations to the anionic substituents of the mucilage.

Observations using scanning electron microscopy indicate a number of factors

which contribute to the tenacity of attachment of *Amphora* cells. These include the position of the four raphes, the small size of the cells and the turtle-like hydrodynamic shape. Harper & Harper (1967) measured both adhesive and tractive forces for a number of epipsammic diatoms including *Amphora ovalis* and obtained values of 400 millidynes and 48 millidynes respectively. *A. ovalis* could also withstand 100 dynes cm^{-2} over a protracted period in a laminar flow apparatus. As *A. ovalis* is very similar in attributes to the fouling *Amphora* spp., these results indicate the very large forces with which such cells may adhere to surfaces.

The histochemical results indicate that the mucilaginous material responsible for adhesion of *Amphora* cells comprises a complex polysaccharide with uronic acid and sulphate substituents; no protein or lipid was detected. This correlates with the histochemical results of Chamberlain (1976) and the gross chemical analysis of Allen et al. (1974) which demonstrated xylose, galactose and glucose in mucilage from an unspecified *Amphora*. In lacking a proteinaceous component, the *Amphora* adhesive differs from those of many macro-fouling algal spores; *Enteromorpha* (Christie et al., 1970), *Ectocarpus* (Baker & Evans, 1973a), *Ceramium* (Chamberlain & Evans, 1973).

Some indications of the route of secretion of the adhesive mucilage have been obtained from scanning and transmission electron microscopy. Figure 4: *18–20* show the imprint of adhesive left after cell removal. The lack of material between the raphes is due to the concavity of the ventral girdle region (Fig. 3: *15*; Fig. 4: *22*). The main points of secretion appear to be the raphe fissures (Fig. 4: *18–20*), the large punctae of the dorsal valve mantle (Fig. 4: *21*) and the large punctae of the ventral valve mantle (Fig. 4: *22*). What effect the finely perforated velum closing at least some of these punctae (Fig. 3: *17*) may have, is unknown, but if the adhesive was released into the region between plasmalemma and silica frustule, the individual polysaccharide molecules could easily exude to the outside. Although a thin layer of organic material coats the whole frustule, there is no evidence of massive mucilage secretion through the punctae of the dorsal connecting bands, the bulk appears to be ventrally discharged.

Despite the ease with which adhesive mucilage secretion can be detected by scanning electron microscopy, localisation and identification of the material in thin sections examined by T.E.M. is more difficult. As extracellular secretion in eukaryotes is usually associated with the functioning of the Golgi apparatus, particular attention was paid to the various types of vesicle of dictyosomal origin. There appeared to be two, or possibly three, major types. The largest, V, (Fig. 6: *27*), were derived from the mature dictyosome cisternae, and were often irregular in outline whilst containing fibrillar material. These vesicles were widely distributed throughout the cytoplasm and the contents were similar in morphology to the extracellular mucilaginous material (Fig. 6: *29*). The second type, V_2 (Fig. 6: *26*), were smaller, more regularly round in section and contained granular electron-dense material. The possible third intracellular type were very similar to V_2 but were bounded by a densely staining membrane and were similar to vesicles in *Melosira varians* which Crawford (1973) termed "coated vesicles". Of these types, the largest $V_1$1 vesicles are thought to be the source of the extracellular mucilage (Fig. 6: *27*) and that the small, extracellular, V_4, vesicles (Fig. 6: *28*) probably represent extraneous lipid membrane associated with the exocytotic process. Vesicles similar to the V_1 type were also noted by Dawson (1973) in the freshwater, mucilage-stalk producing *Gomphonema parvulum*. Crawford (1973) suggested that in *Melosira* muci-

lage secretion occurred via pyriform vesicles derived from nuclear blebbing. No such vesicles were recorded in *A. veneta.*

Settlement and subsequent development on A/F surfaces necessitates some form of resistance to or protection from the incorporated biocides. The minimum leaching rate necessary to prevent animal and plant fouling on cuprous and mercuric oxide paints was calculated as 10 μg cm^{-2} day^{-1} and 2 μg cm^{-2} day^{-1} respectively (Harris, 1946; Anon, 1952). Since *Amphora* cells colonise A/F paints soon after immersion, they must possess a means of adaptation or resistance to the high levels of toxins within the surface boundary layer. Previous work on a variety of micro-organisms and algae has implicated three main mechanisms in overcoming toxic conditions:

(1) Extracellular interaction and binding by mucilaginous material and cell walls (Fogg & Westlake, 1955; Ashida, 1965; Jones, 1967; Fitzgerald, 1967; Button, 1968; Nielsen & Kamp-Nielsen, 1970; Corpe, 1972, 1974; Silverberg, 1975).

(2) Active exclusion (Foster, 1977).

(3) Intracellular immobilization with the development of subcellular granules or complexes (Silverberg et al., 1976; Walker, 1977).

The present investigation gives evidence for the latter, with the development of intracellular copper-containing granules. Both laboratory cultures grown in copper solutions (0.75 ppm $CuCl_2$ in seawater) and *Amphora* cells stripped from cuprous paints contained obvious granular inclusions usually within the two polar cell vacuoles. Up to five such granules per cell have been recorded and were similar in structure and morphology to those recorded by Walker (1977) in the prosomal region of *Balanus balanoides.* Energy dispersive x-ray analysis of these structures demonstrated the presence of copper in association with sulphur, phosphorus and calcium.

Walker (1977) records high concentrations of sulphur within the copper granules of *Balanus balanoides* and further indicated from histochemical and solubility tests that they were organically complexed with proteins, i. e. metallothioneins rich in sulphur-containing residues. A similar situation may exist within the *Amphora* cells studied since recent work by Cloutier-Mantha & Brown (1980) indicated that a metallothionein as well as a low molecular weight substance may play a role in the storage and detoxification of heavy metals in the marine diatom *Skeletonema costatum.*

Some of the granules, particularly in "wild" *Amphora* cells, had high concentrations of phosphates. Polyphosphates are often found as storage bodies within diatom cells (Crawford, 1973; Dawson, 1973) and may offer a convenient site for detoxification since they are usually located in the vacuolar regions. Ashida (1965) also suspected polyphosphates as a possible site for sequestration of copper in copper-tolerant yeasts.

In addition to this internal sequestration and hence protection, the extracellular mucilage with its strongly anionic character probably also binds the metal cations. However, x-ray analysis of the mucilage failed to show appreciable amounts of copper, possibly a result of removal during processing for the E. M. Nevertheless, this mechanism of metal binding, albeit reversible, is likely to function in *Amphora* and afford a primary line of "defence". Other micro-organisms with mucilages of similar chemical composition do bind heavy metals (Fogg & Westlake, 1955; Corpe, 1972, 1974), as do the highly sulphated algal polyaccharides, fucoidan and carragheenan (Paskins-Hurburt et al., 1978; Veroy et al., 1980). Veroy *et al.* (1980) were able to correlate metal binding capacity with degree of sulphation. Such a binding of the heavy metal toxins by

Table 4. Previous reports on the occurrence of *Amphora* on toxic A/F coated test-surfaces (T/S) and 'in-service' ships

Location	Toxin in use	Species	Period of immersion	Author
Chichester Harbour, U.K. T/S	Cuprous oxide	*A. turgida* *A. veneta* *A. exigua* *A. coffeaeformis*	1–4 weeks	Hendey (1951)
Australia T/S	Cuprous oxide/ mercuric oxide	*A. arcta*	1–2 weeks	Crosby & Wood (1959)
Australian naval ships	Cuprous oxide	*A.? bigibba* *A.? coffeaeformis* *A.? delicatissima*	6–18 months	Bishop et al. (1972)
Britain T/S Newton Ferrers, U.K.	Organotin	*A. proteus*		Callow et al. (1978)

Amphora mucilage will also have the effect of reducing the leaching rate by affecting the diffusion boundary layer.

It has been shown that *Amphora* species are extremely successful as fouling micro-organisms (see Table 4) and have achieved this position as a result of several factors. A number of these including shape, size, wide tolerances of many environmental factors, position of raphes and strength of adhesion, the production of anionic mucilage and the capability for internal metal sequestration have been discussed already. Additional factors may also be important. Thus, Lewin (1963) found eight strains of *A. coffeaeformis* capable of heterotrophic growth and Anderson (1975) showed resistance to dessication by the same species due to the formation of resting cells when unfavourable conditions prevailed. In conclusion, it appears that *Amphora* is admirably equipped to tolerate and grow on anti-fouling compositions and no doubt, benefits from exploiting an environment which is relatively free of competing diatoms.

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LITERATURE CITED

Allan, G. G., Medcalf, D. G., Lewin, J., Brannon, J. H. & Scott, J. R., 1974. Polysaccharides from unicellular algae and diatoms. – Int. Seaweed Symp. *8*, abstr. B 8.

Anderson, R. O., 1975. The ultrastructure and cytochemistry of resting cell formation in *Amphora coffeaeformis*. – J. Phycol. *9*, 50–61.

Anon, 1952. Species recorded from fouling. In: Marine fouling and its prevention. United States Naval Institute, Annapolis, 165–207.

Ashida, J., 1965. Adaptation of fungi to metal toxicants. – A. Rev. Phytopath. *3*, 153–174.

Baker, J. R. J. & Evans, L. V.,1973a. The ship-fouling alga *Ectocarpus*. I. The ultrastructure and cytochemistry of plurilocular reproductive stages. – Protoplasma *77*, 1–13.

Baker, J. R. J. & Evans, L. V., 1973b. The ship-fouling alga *Ectocarpus*. II. Ultrastructure of the unilocular reproductive stages. – Protoplasma *77*, 181–189.

Bishop, J. H., Marson, F. & Silva, S. R., 1972. Microfouling on antifouling coatings. – Aust. OCCA Proc. News *9*, 4–6.

Bishop, J. H., Silva, S. R. & Silva, V. M., 1974. A study of micro-fouling on antifouling coatings using electron microscopy. – J. Oil Colour Chem. Ass. *57*, 30–35.

Blanquet, P. R., 1976. Ultrahistochemical study on the ruthenium red surface staining. Part 1. Processes which give rise to an electron dense marker. – Histochemistry *47*, 63–78.

Button, D. K., 1968. Some factors influencing kinetic constants for microbial growth in dilute solutions. In: Symposium on organic matter in natural waters. Ed. by D. W. Hood. Univ. of Alaska, College, Alaska. 537–547.

Callow, M. E., Wood, K. R. & Evans, L. V., 1978. The biology of slime films. Part 3. – Shipp. Wld *171*, 133, *5*, 9.

Chamberlain, A. H. L., 1976. Algal settlement and secretion of adhesive materials. In: Proceedings of the 3rd international biodegradation symposium. Ed. by J. M. Sharpley & A. M. Kaplan. Applied Science Publ., London, 417–432.

Chamberlain, A. H. L. & Evans, L. V., 1973. Aspects of spore production in the red alga *Ceramium*. – Protoplasma *76*, 139–159.

Chayen, J., Bitensky, L., Butcher, R. G. & Poulter, L. W., 1973. A guide to practical histochemistry. Lippincott, Philadelphia, 271 pp.

Christie, A. O., Evans, L. V. & Shaw, M., 1970. Studies on the ship-fouling alga *Enteromorpha* II. The effect of certain enzymes on the adhesion of zoospores. – Ann. Bot. *34*, 467–482.

Cloutier-Mantha, L. & Brown, D. A., 1980. The effects of mercury exposure on intracellular distribution of mercury, copper and zinc in *Skeletonema costatum* (Grev.) Cleve. – Botanica. mar. *23*, 53–58.

Coe, W. R. & Allen, W. E., 1937. Growth of sedentary marine organisms on experimental blocks and plates for nine successive years. – Bull. Scripps Instn. Oceanogr. tech. Ser. *4*, 101–136.

Corpe, W. A., 1972. Periphytic marine bacteria and the formation of microbial films on solid surfaces. In: Effect of the ocean environment on microbial activities. Ed. by R. R. Colwell & R. Y. Morita. Univ. Park Press, Baltimore, 397–417.

Corpe, W. A., 1973. The role of primary film forming marine bacteria. In: Proceedings of the 3rd international congress on marine corrosion and fouling. Ed. by R. F. Acker, B. F. Brown, J. R. Depalma & W. P. Iverson. Northwestern Univ. Press, Evanston, Ill., 598–609.

Corpe, W. A., 1974. Detachment of marine periphytic bacteria from surfaces of glass slides. – Devs. ind. Microbiol. *15*, 281–287.

Corpe, W. A., 1977. Primary bacterial films and marine microfouling. In: Proceedings of the 4th international congress on marine corrosion and fouling. Ed. by V. Romanovsky. Centre de Recherches et d'Etudes Océanographiques, Boulogne, 97–100.

Crawford, R. M., 1973. The protoplasmic ultrastructure of the vegetative cell of *Melosira varians* C. Agardh. – J. Phycol. *9*, 50–61.

Crisp, D. J. & Ryland, J. S., 1960. Influence of filming and of surface texture on the settlement of marine organisms. – Nature, Lond. *185*, 119.

Crosby, L. H. & Wood, E. J. F., 1959. Studies on Australian and New Zealand diatoms. II. Normally epontic and benthic genera. – Trans. R. Soc. N. Z., *86*, 1–58.

Danielli, R., 1953. Cytochemistry: A critical approach. Wiley, New York, 139 pp.

Dawson, P. A., 1973. Observations on the structure of some forms of *Gomphonema parvulum* Kutz. II. The internal organization. – J. Phycol. *9*, 165–175.

Evans, L. V. & Christie, A. O., 1970. Studies on the ship-fouling alga *Enteromorpha*. I. Aspects of the fine structure and biochemistry of swimming and newly settled zoospores. – Ann. Bot. *34*, 451–466.

Evans, L. V. & Holligan, S. M., 1972. Correlated light and electron microscope studies on brown

algae. I. Localization of alginic and sulphated polysaccharides in *Dictyota*. – New Phytol. *71*, 1161–1172.
Feder, N. & O'Brien, T. P., 1968. Plant microtechnique: Some principles and new methods. – Am. J. Bot. *55*, 123–142.
Fitzgerald, G. P., 1967. The algistatic properties of silver. – Wat. Sewage Wks *114*, 185–189.
Fogg, G. E. & Westlake, D. F., 1955. The importance of extracellular products of algae in fresh water. – Verh. int. Verein. theor. angew. Limnol. *12*, 219–232.
Foster, P. L., 1977. Copper exclusion as a mechanism of heavy metal tolerance in a green alga. – Nature, Lond. *269*, 322–323.
Gerchakov, M. S., Marszalek, D. S., Roth, F. J. & Udey, L. R., 1977. Succession of periphytic microorganism on metal and glass surfaces in natural seawater. In: Proceedings of the 4th international congress on marine corrosion and fouling. Ed. by V. Romanovsky, Centre de Recherches et d'Etudes Océanographiques, Boulogne, 203–211.
Gerchakov, M. S., Marszalek, D. S., Roth, F. J., Sallmann, B. & Udey, L. R., 1978. Observation on microfouling applicable to OTEC systems, In: Proceedings of the OTEC biofouling and corrosion symposium. Ed. by R. H. Gray. Seattle, Washington Pacific Northwest Laboratory, Richland, Washington, 63–75.
Guillard, R. R. L. & Ryther, J. H., 1962. Studies on marine planktonic diatoms. I. *Cyclotella nana* Hustedt and *Detonula confervaceae* (Cleve.) Gran. – Can. J. Microbiol. *8*, 229–239.
Harper, M. A. & Harper, J. F., 1967. Measurements of diatom adhesion and their relationship with movement. – Br. phycol. Bull. *3*, 195–207.
Harris, J. E., 1946. Report on anti-fouling research, 1942–44. – J. Iron Steel Inst. *154*, 296–333.
Hasle, G. R. & Fryxell, G. A., 1970. Diatoms: Cleaning and mounting for light and electron microscopy. – Trans. Am. microsc. Soc. *89*, 469–474.
Heath, I. D., 1961. Staining of sulphated mucopolysaccharides. – Nature, Lond. *191*, 1370–1371.
Hendey, N. I., 1951. Littoral diatoms of Chichester Harbour with special reference to fouling. – Jl. R. microsc. Soc. *71*, 1–86.
Hendey, N. I., 1964. An introductory account of the smaller algae of British coastal waters. – Fishery Invest., Lond. (Ser. 4) 5,1–317.
Hollander, H., 1964. The histochemistry of sulphated esters with Trypaflavin. – Histochemie *3*, 387–395.
Hotchkiss, R. D., 1948. A microchemical reaction resulting in the staining of polysaccharide structures in fixed tissue preparations. – Arch. Biochem. *16*, 131–141.
Jones, G. E., 1967. Growth of *Escherichia coli* in heat and copper-treated synthetic sea water. – Limnol. Oceanogr. *12*, 167–172.
Karajeva, N. I., 1964–1965. Data on the flora of diatom algae in marine fouling along the east coast of the Caspian sea. In: Marine fouling and borers. Ed. by I. V. Stavostin, U. S. Naval Oceanographic Office, Washington, D. C., 41–53. (Translation. 221.)
Lackenby, H., 1962. Resistance of ships, with special reference to skin friction and hull condition. – Proc. Instn mech. Engrs *176*, 981–1014.
Lebedev, Y. M., Permitin, Y. Y. & Karajeva, N. I., 1964–1965. The problem of fouling on panels in the Black Sea. In: Marine fouling and borers. Ed. by I. V. Stavostin, U. S. Naval Oceanographic Office, Washington, D. C., 390–398. (Translation. 221.)
Lewin, J. C., 1963. Heterotrophy in marine diatoms. In: Marine micro-biology, Ed. by C. H. Oppenheimer. Thomas, Springfield Ill., 229–235.
Lewin, R. A., 1958. The mucilage tubes of *Amphipleura rutilans*. – Limnol. Oceanogr. *3*, 111–113.
Marson, F., 1969. Antifouling paints. I. Theoretical approach to leaching of soluble pigments from insoluble paint vehicles. – J. appl. Chem. *19*, 93–99.
Marszalek, D. S., Gerchakov, S. M. & Udey, L. R., 1979. Influence of substrate composition on Marine microfouling. – Appl. environ. Microbiol. *38*, 987–995.
McCully, M. E., 1966. Histological studies on the genus *Fucus*. I. Light microscopy of the mature vegetative plant. – Protoplasma *62*, 287–305.
Milanovich, F. P., Wilson, D. W. & Yeh, Y., 1975. Detoxifying effect of yellow substance on *E. coli* in media containing copper. – Nature, Lond. *253*, 460–461.
Neilsen, E. S. & Kamp-Nielsen, L. K., 1970. Influence of deleterious concentrations of copper on the growth of *Chlorella pyrenoidosa*. – Physiol. Pl. *23*, 828–840.

O'Neill, T. B. & Wilcox, G. I., 1971. The formation of a "primary film" on materials submerged in the sea of Port Hueneme, California. – Pacif. Sci. *25*, 1–12.

Parker, B. C. & Diboll, A. G., 1966. Alcian stains for histochemical localization of acid and sulphated polysaccharides in algae. – Phycologia *6*, 37–46.

Paskins-Hurburt, A. J., Skoryna, S. C., Tanaka, Y., Moore, W. & Stara, J. F., 1978. Fucoidan: its binding of lead and other metals. – Botanica mar. *21*, 13–22.

Paulsen, B. S., Haug, A. & Larsen, B., 1978. Structural studies of a carbohydrate containing polymer present in the mucilage tubes of the diatom *Berkeleya rutilans* (Trent). Grün. – Carbohydrate Res. *66*, 103–111.

Pearse, A. G. E., 1968. Histochemistry: theoretical and applied I. Churchill, London, 759 pp.

Phillip, A. T., 1973. Marine science aids the development of antifouling coatings. – Aust. OCCA Proc. News 17–22.

Pyefinch, K. A., 1951. Seaweeds as fouling organisms. In: Seaweed utilization. Ed. by L. Newton. Sampson-Low, London, 146–156.

Reynolds, E. S., 1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. – J. Cell Biol. *17*, 208–213.

Ruthmann, A., 1970. Methods in cell research. Bell, London, 368 pp.

Scott, J. E., Dorling, J., 1965. Differential staining of acid glycosaminoglycans (Mucopolysaccharides) by alcian blue in salt solutions. – Histochemie *5*, 221–233.

Sechler, G. E. & Gundersen, K., 1973. Role of surface chemical composition on the microbial contribution to primary films. In: Proceedings of the 3rd international congress on marine corrosion and fouling. Ed. by R. F. Acker, B. F. Brown, J. R. De Palma & W. P. Inverson. Northwestern Univ. Press, Evanston, Ill., 610–616.

Silverberg, B. A., 1975. Ultrastructural localization of lead in *Stigeoclonium tenue* (Chlorophyceae, Ulotrichales) as demonstrated by cytochemical and x-ray microanalysis. – Phycologia *14*, 265–274.

Silverberg, B. A., Stokes, P. M. & Ferstenberg, L. B., 1976. Intranuclear complexes in a copper-tolerant green alga. – J. Cell Biol. *69*, 210–214.

Skerman, T. M., 1956. The nature and development of primary films on surfaces submerged in the sea. – N. Z. Jl. Sci. Technol. *38*, 44–57.

Skerman, T. M., 1958. Marine fouling at the port of Lyttelton. – N. Z. Jl. Sci. *1*, 224–257.

Skerman, T. M., 1959. Marine fouling at the port of Auckland. – N. Z. Jl. Sci. *2*, 57–95.

Spicer, S. S., 1960. A correlative study of the histochemical properties of rodent acid mucopolysaccharides. – J. Histochem. Cytochem. *8*, 18–35.

Spicer, S. S., 1965. Diamine methods for differentiating mucosubstances histochemically. – J. Histochem. Cytochem. *13*, 211–235.

Spurr, A. R., 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. – J. Ultrastruct. Res. *26*, 31–43.

Stanbury, F. H., 1944. Experiments on the growth of marine plants with special reference to the effects of copper and mercury salts. – Marine corrosion Sub. Com. Rept. Iron and Steel Corr. Comm. (M. S.) 10 pp.

Thièry, J. P., 1967. Mise en evidence des polysaccharides sur coupes fines en microscopie electronique. – J. Microsc. *6*, 987–1018.

Veroy, R. L., Mantano, N., Cuzman, M. L. B., Laserna, E. C. & Cajipe, J. B. G., 1980. Studies on the binding of heavy metal to algal polysaccharides from Philippine seaweeds. I. Carrageenan and the binding of lead an cadmium. – Botanica mar. *23*, 59–62.

Waksman, S. A., Johnstone, D. D., Carey, C. L., 1943. The effect of copper upon the development of bacteria in seawater and the isolation of specific bacteria. – J. mar. Res. *5*, 136–152.

Walker, G., 1977. "Copper" granules in the barnacle *Balanus balanoides*. – Mar. Biol. *39*, 343–349.

Wood, E. J. F., 1950. Investigations in under water fouling. I. The role of bacteria in the early stages of fouling. – Aust. J. mar. Freshwat. Res. *1*, 85–91.

Wood, E. J. F., 1953. Heterotrophic bacteria in marine environments of eastern Australia. – Aust. J. mar. Freshwat. Res. *4*, 160–200.

Wood, E. J. F., 1967. Some economic aspects of water microbiology. In: Microbiology of oceans and estuaries. Ed. by E. J. F. Wood, Elsevier, Amsterdam, 206–225.

Yasuma, A. & Ichikawa, T., 1953. Ninhydrin-Schiff and alloxan-Schiff staining. A new histochemical staining method for proteins. – J. Lab. clin. Med. *41*, 296–299.
Zobell, C. E., 1946. Marine microbiology. Chronica Botanica Co., Waltham, Mass., 230 pp.
Zobell, C. E. & Allen, E. C., 1935. The significance of marine bacteria in the fouling of submerged surfaces. – J. Bacteriol. *29*, 239–251.