CHAPTER SIX

ISOLATION AND ANTIMICROBIAL ACTIVITY OF THE ETHYL ETHER DERIVATIVE OF LANOSOL, FROM *OSMUNDARIA SERRATA* (RHODOPHYTA)

6.1 ABSTRACT

This is the first report on the isolation of a compound from a South African seaweed with antimicrobial activity. The active compound from *Osmundaria serrata* (Rhodophyta) was isolated by column chromatography and analysed by nuclear magnetic resonance spectroscopy and mass spectroscopy, and shown to be lanosol ethyl ether (lanosol^{ee}). Lanosol is commonly found in seaweeds of the family Rhodomelaceae, Rhodophyta. Another aim of this study was to determine the biological activity of the purified compound against the growth of ecologically relevant marine bacteria, potential human patterns (terrestrial bacteria) and the fungi *Alternaria alternata* and *Candida albicans*. Copper(II) sulphate was the positive control and was most active against the test bacteria and fungi with an average MIC value of 0.17 ± 0.016 mg.ml^{-1}, while the average MIC value of lanosol^{ee} was 0.27 ± 0.023 mg.ml^{-1}. The Gram-negative marine bacteria were significantly more sensitive to the copper sulphate than the lanosol^{ee}. In contrast, the Gram-negative terrestrial bacteria showed little difference in response to the toxicants. There is significantly less copper in seawater than in rivers and it is likely that the terrestrial bacteria had built up tolerance to copper because they had been exposed to higher levels of the metal in their environment than the marine bacteria. The ecological significance of

lanosol is discussed and it is concluded that lanosol is a multifunctional secondary metabolite.

6.2 INTRODUCTION

In 1975 Weinstein and co-workers reported that the chemical study of the seaweeds of British Columbia and Washington had been lacking. The same can be said of the seaweeds of South Africa up until the early 2000's. After an extensive literature search only one paper was found where the diterpenes and sterols of *Bifurcaria brassicaeformis* and *Bifurcariopsis capensis* (both Phaeophyceae) collected near Cape Town, South Africa, were isolated for taxonomical purposes (Daoudi *et al.* 2001). Papers on the antimicrobial activity of crude extracts made from southern African seaweeds have been published, but no active compounds were isolated and tested (Barreto *et al.*, 1997, 2001; Vlachos *et al.*, 1996, 1997, 1999, 2001).

An extract from *O. serrata* had in previous sections been shown to potently inhibit the growth of bacteria isolated from the seaweed and its habitat (chapter 3), and bacteria that are potentially pathogenic to humans (chapter 4). Therefore, the active compound from this alga was isolated and tested against marine and terrestrial microbes, some of which are biofilm formers (table 6.1). Since this compound had not previously been tested against the growth of marine bacteria this may indicate the possible function of the related compound, lanosol, as a chemical defence against microbes by *O. serrata* and the other macroalgae and fungi that produces it (Katsui *et al.*, 1967; Stoffelen *et al.*, 1972; Pedersén *et al.*, 1974; Weinstein *et al.*, 1975; Saenger *et al.*, 1976; Pedersén *et al.*, 1979; Demoulin, 1985; Kurata *et al.*, 1997).

Bacteria involved in the formation of biofilms in the marine environment have been used in bioassays of macroalgal extracts (Hellio *et al.*, 2001). This is because of the need to find substitutes to using highly toxic antifouling paints that are presently used on ships hulls and other surfaces. The use of these paints is problematic because the toxic active components (e.g. tributyltin and copper) are accumulating along shipping routes and in harbours. Here they cause detrimental ecological effects (for example toxicity to mammals), and pose a direct risk to humans through the marine food chain (Ponasik *et* al., 1998; Hellio *et al.*, 2001; Horiguchi *et al.*, 2002). Active research into discovering alternatives to these toxic compounds will contribute to more environmentally responsible maritime industries.

Table 6.1 Bacterial isolates used in the bioassays to determine the MIC values of $CuSO_4$ and lanosol ethyl ether isolated from *Osmundaria serrata* (Krieg and Holt, 1984; Holt *et al.*, 1994).

Bacterial Species	**Notes**
Isolated from biofilm on *O. serrata*[1]	
Marinococcus sp.	Gram-positive
Halomonas halophila	Gram-negative
Halomonas marina	Gram-negative
Halomonas sp. 1	Gram-negative
Halomonas sp. 2	Gram-negative
Halomonas sp. 3	Gram-negative
OSSB1	Natural consortium of Gram-negatives[2]
Pseudomonas sp. 1	Gram-negative
Pseudomonas sp. 2	Gram-negative
Vibrio alginolyticus	Gram-negative
Vibrio harveyi	Gram-negative, bioluminescent, shrimp pathogen
Terrestrial bacteria	
Bacillus cereus	Gram-positive, forms endospores, widespread, found in foods and may cause food poisoning
Bacillus pumilis	Gram-positive, ubiquitous endospores
Bacillus subtilis	Gram-positive, forms endospores, widespread, causes ropey (slimy) bread
Enterococcus faecalis	Gram-positive, inhabits intestinal tracts of most animals (including humans), pathogen in urinary tract infections, known

	biofilm former
Propionibacterium acnes	Gram-positive, inhabits human skin and is considered a pathogen
Staphylococcus aureus subsp. *aureus*	Gram-positive, found on warm-blooded animals, potential human pathogen, known biofilm former
Enterobacter cloacae	Gram-negative, wide distribution in environment, opportunistic pathogen in infections of urinary tract, wounds, burns and meningitis
Escherichia coli	Gram-negative, normal inhabitants of warm-blooded animals' colons, pathogenic: diarrhoea, urinary tract infections and meningitis, known biofilm former
Klebsiella pneumoniae	Gram-negative, opportunistic pathogen: pneumonia, meningitis, urinary tract and other infections, known biofilm former
Pseudomonas aeruginosa	Gram-negative, inhabits soil and water, opportunistic pathogenic: wound, urinary tract and burn infections, known biofilm former
Salmonella typhimurium	Gram-negative, pathogenic to humans and other animals, agent of gastroenteritis in humans
Serratia marcescens	Gram-negative, important opportunistic pathogen
Fungi	
Alternaria alternata	Common saprotroph. Degrader of fruit and vegetables.
Candida albicans	Human pathogen. Prevalent in HIV-AIDS patients, known biofilm former

1 – *Vibrio harveyi* was isolated from sea-sand from the habitat of *O. serrata.*
2 - Isolated from *O. serrata*, these unidentified bacteria resisted attempts to separate them and were included here because they had previously shown relatively high resistance to the crude extract from *O. serrata* and were representative of the biofilm on *O. serrata* (chapter 5).

6.3 MATERIALS AND METHODS

6.3.1 Isolation of active compound from *Osmundaria serrata*

To isolate the active compound from *O. serrata*, 1 kg of air-dried material was homogenized with ethanol and extracted for one week on a shaker at 21 ± 2 °C. The extract was filtered, dried under reduced pressure at 40 °C and stored under nitrogen at 0 °C. The seaweed material was re-extracted with ethanol-ethyl acetate (1:1) for

another week, and again with ethyl acetate for another week. The extracts were combined, dried and stored as before. The ethyl acetate soluble fraction was separated on a silica gel 60 F_{254} (Merck) column eluted with gradient steps of hexane and ethyl acetate. The activity of the fractions was determined by bio-autography on silica gel thin layer chromatography (TLC) plates, developed with hexane-ethyl acetate (1:1). Once dried, the TLC plates were sprayed with a spore suspension of *Alternaria alternata* in malt extract broth. The plates were then incubated at 25 °C for three days. The active fraction was separated further in another silica gel column eluted with hexane-ethyl acetate (9:1). The active fractions were combined and repeatedly separated in a Sephadex LH-20 column eluted with ethanol to give pure compound.

The proton, carbon and two dimensional NMR data for the purified compound in $CDCl_3$ was obtained at 300 MHz for the proton and 75 MHz for the carbon spectra.

6.3.2 Bioassay of active compound from *O. serrata*

Table 6.1 shows the species of bacteria and fungi that were used in the bioassays. They were grown for 24 hours at 21 ± 2 °C. The marine isolates were grown in marine broth made with 5 g soy peptone (Sigma), 1 g yeast extract (Sigma) and 1 litre filtered (0.45 µm pore size) seawater. The pH of the medium was adjusted to 7.6 before autoclaving. The other bacteria were grown in nutrient broth supplied by Biolab, Midrand, South Africa and made according to the manufacturer's instructions. The *A. alternata* spores were suspended in malt extract broth and their absorbance's read at 660 nm to ensure consistent spore concentrations.

The method of Eloff (1999) was used to determine the bacteriostatic minimum inhibitory concentration (MIC) of the extracts in a dilution series of 12 (from 25 to 0.01 $mg.ml^{-1}$). Copper(II) sulphate ($CuSO_4 \cdot 5H_2O$) was used as a positive control (dilution series from 1.25 to 0.0006 $mg.ml^{-1}$), while the negative control wells contained acetone (from 12.5 to 0.006 %). Overnight cultures of the bacteria were diluted 1:100 before being used to inoculate the multiwells. The experiment was repeated three times on separate days with three replicates in each experiment. The marine isolates were incubated at 23 ± 2 °C, the other bacteria, and *C. albicans*, at 37 ± 2 °C, all for 24 hours. *A. alternata* spores were incubated at 25 ± 2 °C for 72 hours. Student *t*-tests were used to analyse the data (Rothery, 2000).

The same plates were incubated for another week during which the lanosol^{ee} had oxidised to an inactive compound and allowed viable bacteria and fungi to grow. The average bactericidal MIC value was then calculated from these plates.

6.4 RESULTS AND DISCUSSION

6.4.1 Isolation of lanosol ethyl ether from *O. serrata*

The active compound showed up as a light area against a dark background of *A. alternata* spores on the TLC plates (figure 6.1). Using the *A. alternata* spores to visualise the active compound worked well for this general antimicrobial compound and was a safe and cheap alternative to spraying with potential human pathogenic bacteria and then with tetrazolium salts. However, it would be inadequate for a study on a compound with more specific activity without testing it against other organisms first. The compound is unstable (figure 6.2) and was stored under a nitrogen atmosphere at –20 °C.

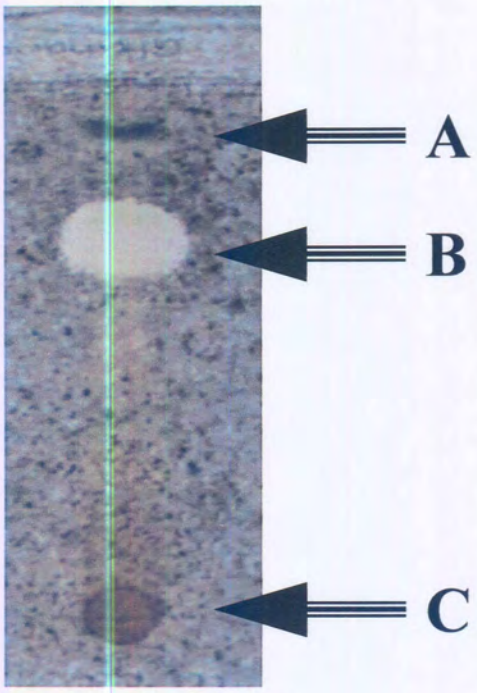

Figure 6.1 Sporulation of *Alternaria alternata* on thin layer silica gel chromatography plate to visualise the active compound from *Osmundaria serrata*. A = chlorophylls close to solvent front (hexane : ethyl acetate, 1:1); B = inhibition zone, where *A. alternata* did not sporulate; C = origin with spot of polar compounds.

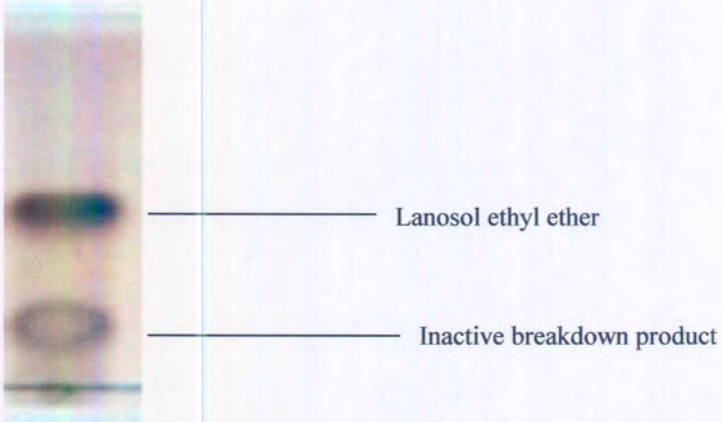

Figure 6.2 The active compound from *O. serrata*, lanosol ethyl ether, after five days storage at room temperature in the dark. Note the extra spot indicating the breakdown of the active compound. Hex and ethyl acetate (1:1) mixture was used to develop the TLC plate.

The proton NMR spectrum (figure 6.3) of the purified active compound (yield ≈ 0.1%) from *O. serrata* showed peaks at δ 1.22 (3H, t, J = 6 Hz), 3.57 (2H, dd, J = 7 Hz), 4.44 (2H, s), 5.99 (2H, br) and 6.94 (1H, s). The nine ^{13}C NMR peaks (figure 6.4) were as follows: δ 143.9, 141.7, 131.9, 115.7, 114.8, 113.7, 73.2, 66.8 and 15.5.

The proton NMR spectrum indicated that the compound contained a substituted aromatic ring (δ 6.94). Two hydroxyl groups were present on this aromatic ring (δ 5.99). It was suspected that the compound was halogenated because such compounds are commonly isolated from red algae (Fenical, 1975). Bromine was suspected of being covalently bonded to the aromatic ring. The other proton peaks indicated an ethyl ether group (δ 1.22 and 3.57) (Macomber, 1998). The peaks in the ^{13}C spectrum also pointed to a substituted aromatic ring and an ethyl ether group. The structure was confirmed by GC-MS analysis (figure 6.5) as being lanosol ethyl ether (lanosol^{ee}) with a chemical formula of $C_9H_{10}0_3Br_2$ (MW = 323.9). The structure is shown in figure 6.6.

This is the first report of lanosol and lanosol^{ee} in *O. serrata*. Simple brominated compounds such as these have been isolated from other taxonomically diverse seaweed such as the brown *Fucus vesiculosis*, and the reds *Lenormandia prolifera*, *Odonthalia corymbifera*, *Polisiphonia lanosa*, and *Rhodomela larix* (Katsui *et al.*, 1967; Stoffelen *et al.*, 1972; Weinstein *et al.*, 1975; Saenger *et al.*, 1976; Pedersén *et al.*, 1979; Kurata *et al.*, 1997). It is also found in low levels in fungi, but its occurrence is concentrated in the Rhodomelaceae, Rhodophyta (Pedersén *et al.*, 1974; Demoulin, 1985). Weinstein and co-workers (1975) suggested that lanosol and its derivatives are artefacts of the extraction procedure with the solvents water, methanol

and ethanol, which give lanosol, the methyl and ethyl ether forms respectively. The potassium sulphate salt of the compound is thought to be constitutive in the seaweed (Weinstein *et al.*, 1975).

Lanosol and its derivatives potently inhibited the feeding of sea urchin and abalone, while the salt showed no activity (Kurata *et al.*, 1997). The salt is probably the inactive form stored in the seaweed and is converted to lanosol upon injury. This reaction occurs too slowly at ambient temperatures to be considered an effective defence mechanism and an enzyme probably catalyses the reaction (unpublished data). In addition, lanosol was isolated from seawater taken from the habitat of *Polysiphonia brodiei*, a seaweed that also produces lanosol (Pedersén *et al.*, 1974). Therefore we may assume that lanosol occurs naturally, but that its methyl and ethyl derivatives might be artefacts of the extraction procedure.

It was decided to use the lanosol[ee] that had been isolated because it had similar activity to lanosol in the antifeeding activity study of Kurata *et al.* (1997). In addition, lanosol and related derivatives had similar antibacterial activities (Glombitza *et al.*, 1974). The ethyl ether derivative of lanosol, however, had not previously been tested for antimicrobial activity.

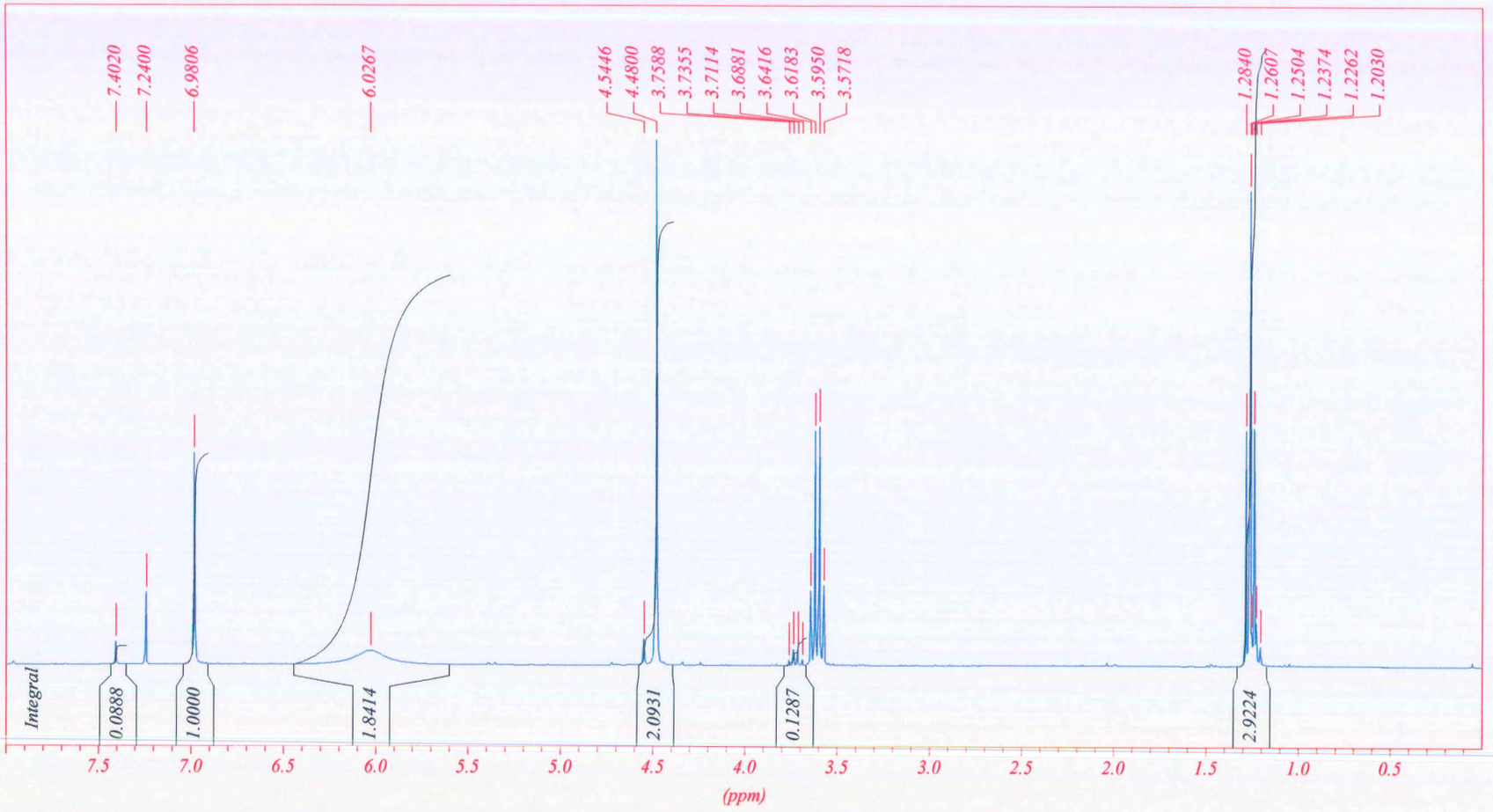

Figure 6.3 Proton NMR of the active compound, lanosol ethyl ether, isolated from *Osmundaria serrata.*

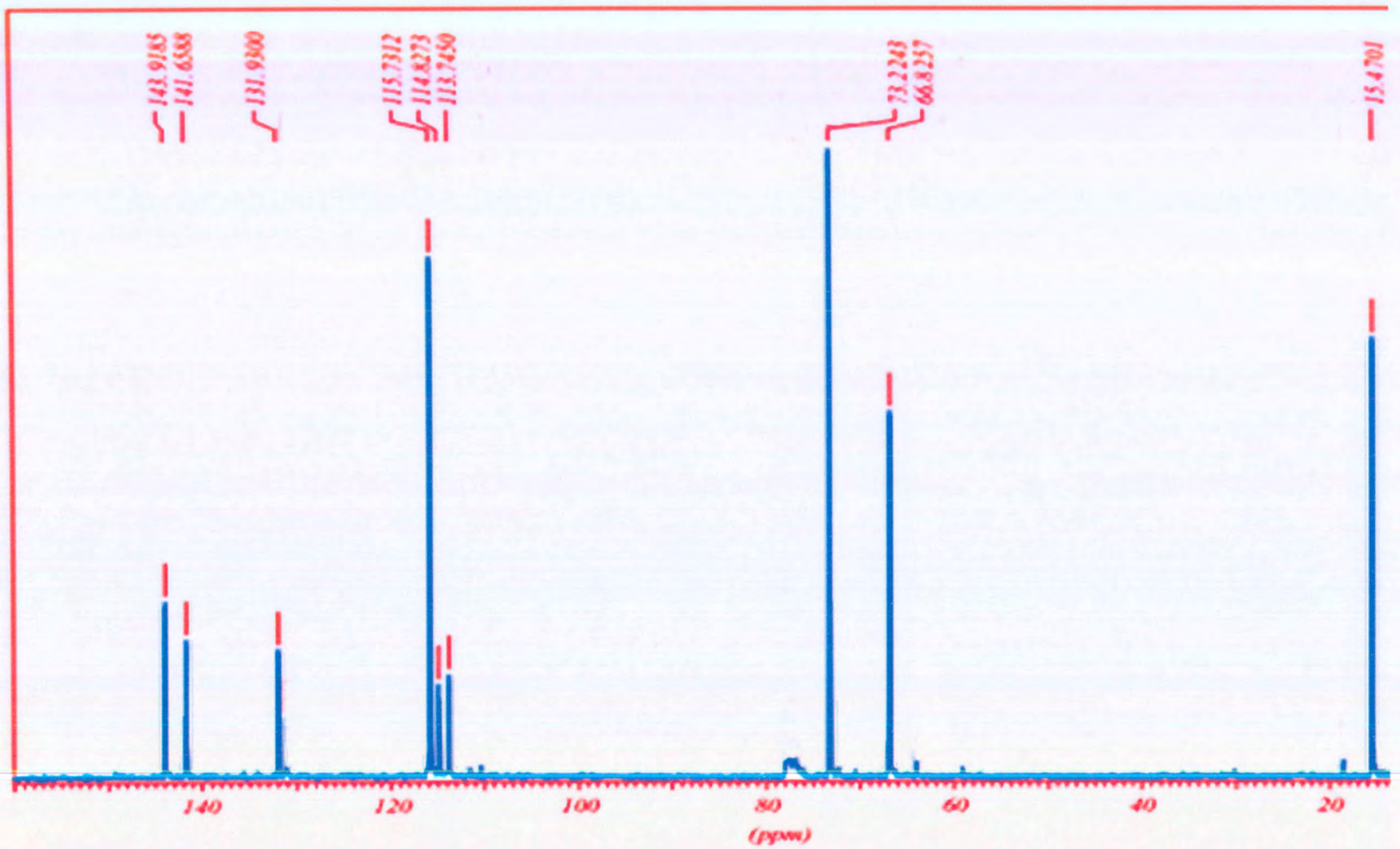

Figure 6.4 Carbon 13 NMR of the active compound, lanosol ethyl ether, isolated from *Osmundaria serrata.*

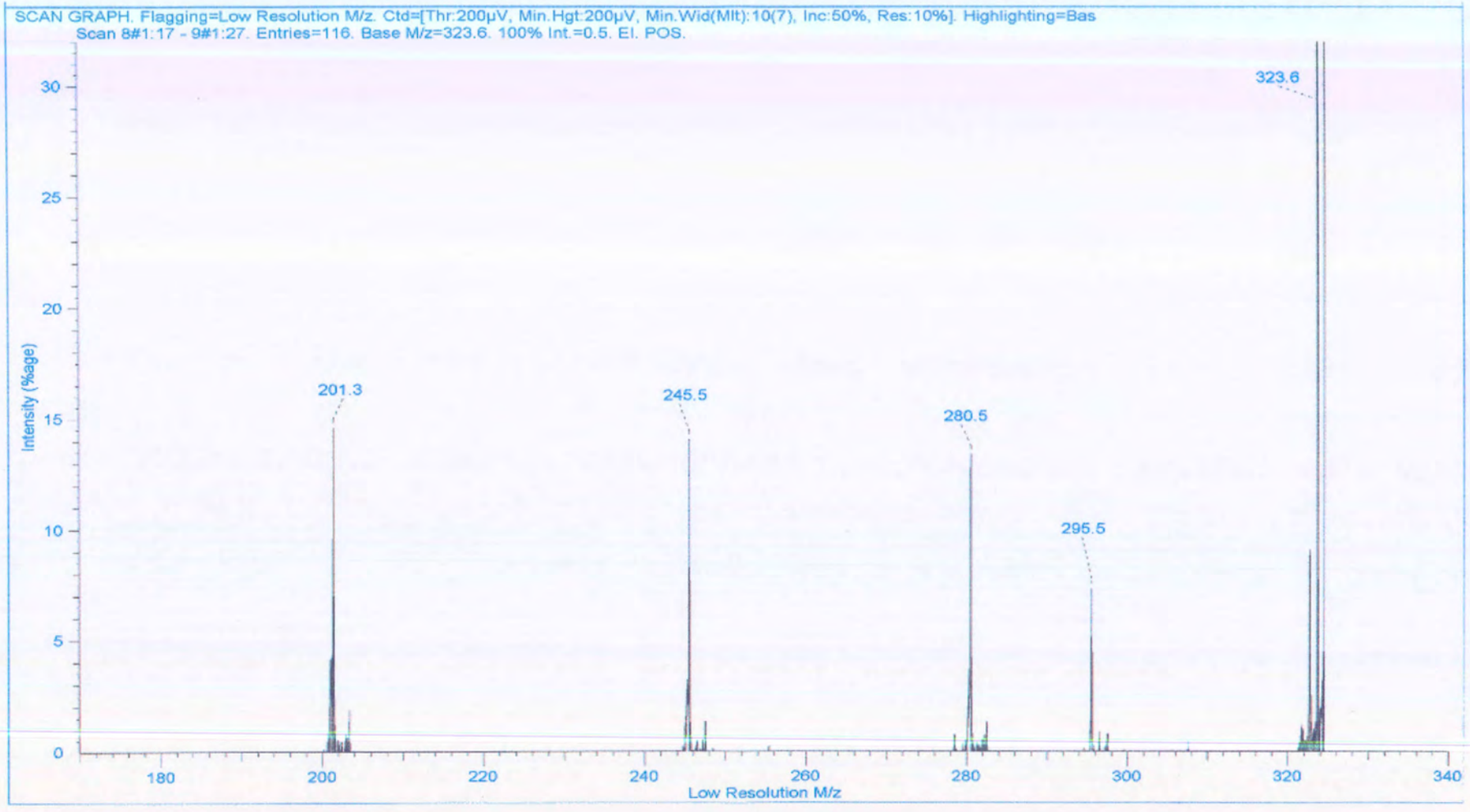

Figure 6.5 Mass spectrograph of lanosol diethyl ether isolated from *Osmundaria serrata*.

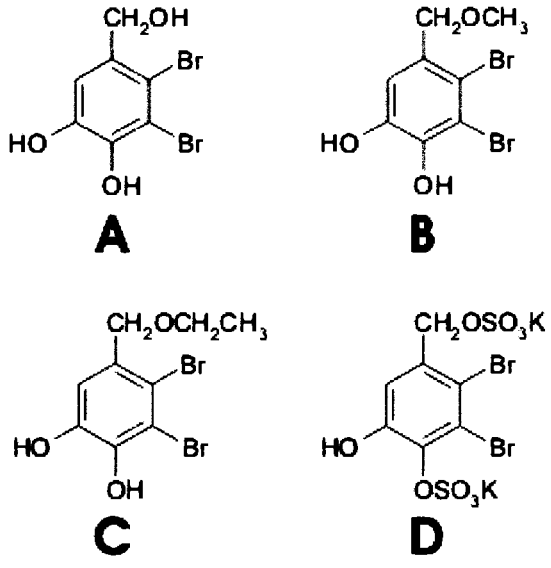

Figure 6.6 Chemical structures of lanosol and derivatives. **A** = Lanosol; **B** = lanosol-1'-methyl ether, **C** = lanosol-1'-ethyl ether; **D** = lanosol 1,4-disulfate ester.

6.4.2 Bioassays of lanosol ethyl ether

Copper(II) sulphate inhibited the test organisms more than lanosol^{ee} (table 6.2). Looking at the bacteriostatic data in more detail it is evident that the Gram-positive and negative bacteria responded differently to treatments. The Gram-negatives were far more sensitive to copper(II) sulphate than lanosol^{ee}. In contrast, the Gram-positives were more sensitive to lanosol^{ee}, and macroalgal extracts in general (chapter 4), than copper(II) sulphate. Differences in the cell walls of these groups of bacteria might explain these results (Madigan *et al.*, 1997).

There was no difference in response to lanosol^{ee} between the terrestrial and marine bacteria. This had also been found with the crude extract (chapter 4). However, the marine bacteria as a group responded differently to the different toxicants. Copper(II) sulphate inhibited their growth significantly more than lanosol^{ee} (table 6.2). OssB1,

from the marine group, was most resistant to lanosol^{ee} (0.67 ± 0.17 mg.ml^{-1}) and very sensitive to copper(II) sulphate (0.06 ± 0.00 mg.ml^{-1}). This trend was also observed with *Halomonas* sp. 1, *H. halophila*, *Pseudomonas* sp. 1 and 2 (figure 6.7). However, the terrestrial bacteria responded similarly to the treatments. The sensitivity of marine bacteria toward copper is well known and it has been found that tolerance builds up after exposure (Hellio *et al.*, 2001). The levels of copper in coastal seawater range from 0.069 to 20.0 µg.l^{-1}, and in rivers 0.11 – 200 µg.l^{-1} (Crompton, 1997). Terrestrial bacteria are thus more likely to have built up a tolerance to copper than marine bacteria because they had previously been exposed to higher levels of the metal than the marine bacteria.

Table 6.2 Student *t*-test results of comparing $CuSO_4$ and lanosol^{ee} within the different groups of bacteriostatic data.

Groups	Variables	Ave. MIC ± SE (mg.ml^{-1})	*p*-Values[1]
All	$CuSO_4$	0.17 ± 0.016	0.0005
	Lanosol^{ee}	0.27 ± 0.023	
Gram-positive bacteria	$CuSO_4$	0.25 ± 0.034	0.007
	Lanosol^{ee}	0.14 ± 0.024	
Gram-negative bacteria	$CuSO_4$	0.15 ± 0.017	3.5×10^{-8}
	Lanosol^{ee}	0.34 ± 0.027	
Marine bacteria	$CuSO_4$	0.12 ± 0.014	1.6×10^{-6}
	Lanosol^{ee}	0.34 ± 0.037	
Terrestrial bacteria	$CuSO_4$	0.23 ± 0.027	0.46
	Lanosol^{ee}	0.22 ± 0.025	
All bacteria	$CuSO_4$	0.18 ± 0.017	0.0003
	Lanosol^{ee}	0.28 ± 0.023	
All fungi	$CuSO_4$	0.13 ± 0.034	0.39
	Lanosol^{ee}	0.15 ± 0.080	
Gram-positive marine bacteria	$CuSO_4$	0.21 ± 0.040	0.03
	Lanosol^{ee}	0.04 ± 0.010	
Gram-positive terrestrial bacteria	$CuSO_4$	0.26 ± 0.040	0.02
	Lanosol^{ee}	0.16 ± 0.026	
Gram-negative marine bacteria	$CuSO_4$	0.12 ± 0.014	8.0×10^{-8}
	Lanosol^{ee}	0.37 ± 0.036	
Gram-negative terrestrial bacteria	$CuSO_4$	0.20 ± 0.037	0.05
	Lanosol^{ee}	0.29 ± 0.037	

1 - $p > 0.10$ = no difference between variables (i.e. accept null hypothesis H_0); $0.10 > p > 0.05$ = slight evidence for a difference between variables (rejecting H_0); $p < 0.05$ = moderate evidence for rejecting H_0; $p < 0.01$ = strong evidence for rejecting H_0 (Rothery, 2000).

With OSSB1 we see a group of bacteria that were isolated from *O. serrata* being very tolerant (relative to bacteria in pure culture) to a toxin that their macroalgal-habitat produces and probably releases. It is known that bacteria in a biofilm are more resistant to toxicants and this characteristic makes their control so difficult (Allison *et al.*, 2000). The extracellular polymeric substances (EPS) surrounding the cells in the biofilm protect them from antibiotics. However, chlorine degrades EPS effectively and controls biofilms in drinking water (Characklis, 1990). Bromine, another halogen, is covalently bonded to the phenolic ring in lanosol and its derivatives. McLachlan and Craigie (1966) demonstrated the anti-algal activity of lanosol and stated that the addition of bromine onto a phenol did not increase its toxicity, but it did increase when chlorine was added. Bromine is less reactive than chlorine, but it seems unlikely that it would have no affect on the toxicity of the compound since the carbon-bromine bond is more potent in initiating free radical reactions and producing lipid peroxidation than the chlorine-carbon bond (Mehendale, 1992).

Interestingly the fermentative isolates (*V. alginolyticus* and *V. harveyi*) and the Gram-positive *Marinococcus* sp. were the most sensitive to lanosol[ee] of the marine isolates (figure 6.7). It is unknown why, however, they would benefit the most from being in a biofilm in an environment exposed to lanosol.

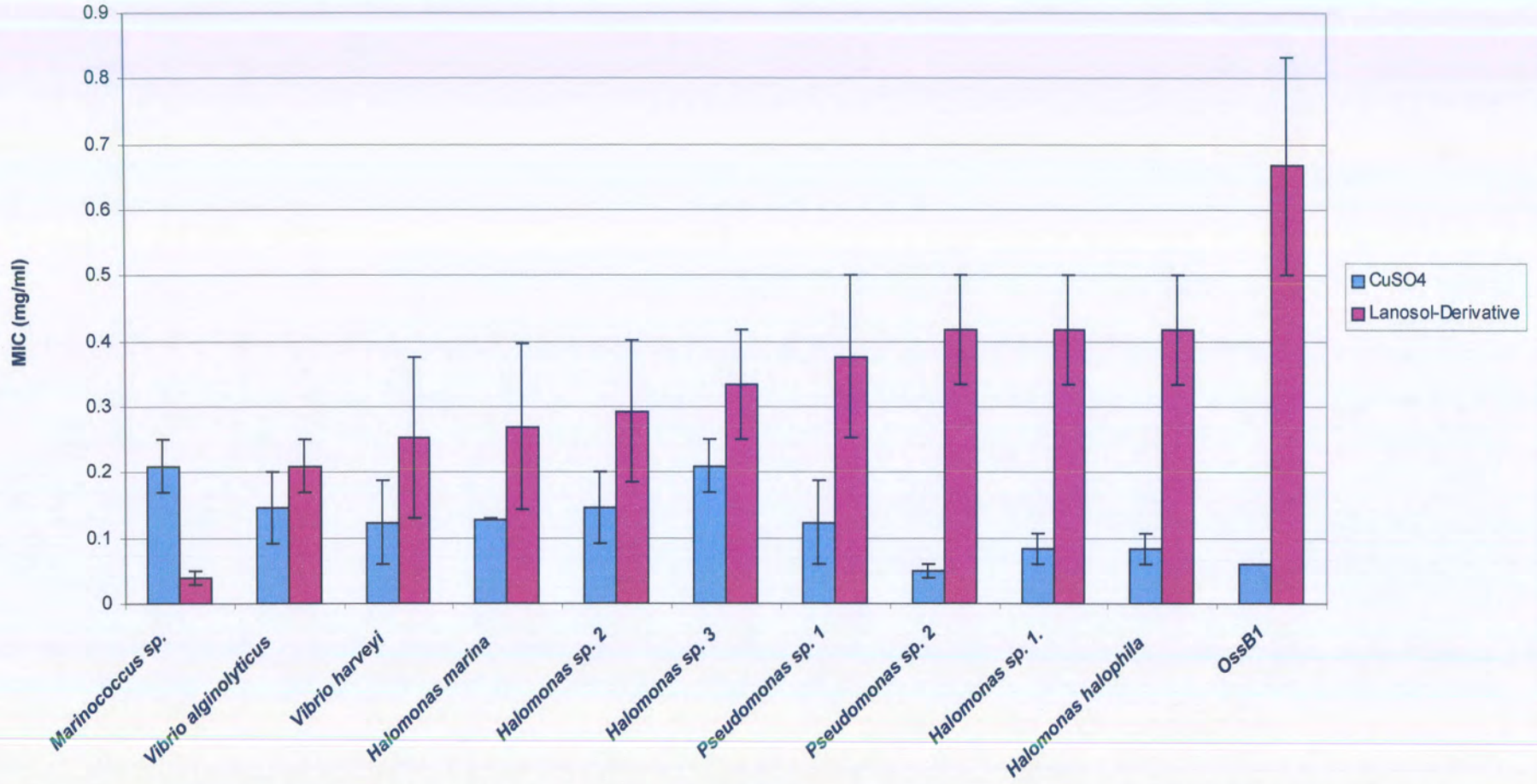

Figure 6.7 Minimum inhibitory concentrations (bacteriostatic) of lanosol ethyl ether against the growth of marine bacteria isolated from the habitat of *Osmundaria serrata*. Bars = SE, $n = 3$.

Within the terrestrial group, *P. aeruginosa* was most resistant to lanosol^{ee}, but very sensitive to copper(II) sulphate (figure 6.8). The Gram-positive *S. aureus* was not as sensitive as the other Gram-positive species. These results are similar to those from the extract of *O. serrata* (chapter 5). In addition, Weinstein and co-workers (1975) found that the salt of lanosol (figure 6.6 - **D**) was ineffective against *Staphylococcus* sp., but showed activity against other bacteria. Resistance to phenols and halogens are a feature of the genus *Staphylococcus* (Krieg and Holt, 1984).

The inhibition of the growth of OssB1 by lanosol^{ee} indicates that the biofilm bacteria would be more resistant to the chemical than planktonic forms as predicted (Marshall, 2000). It was beyond the scope of this study, but future work could test the effect lanosol has on the initial stages of biofilm formation. Other seaweed products, e.g. halogenated furanones from *Delisea pulchra* (Rhodophyta), are known for their antifouling activities and it may be that lanosol^{ee} has a biofilm regulatory function (McLachlan and Craigie, 1966; De Nys and Steinberg, 2002).

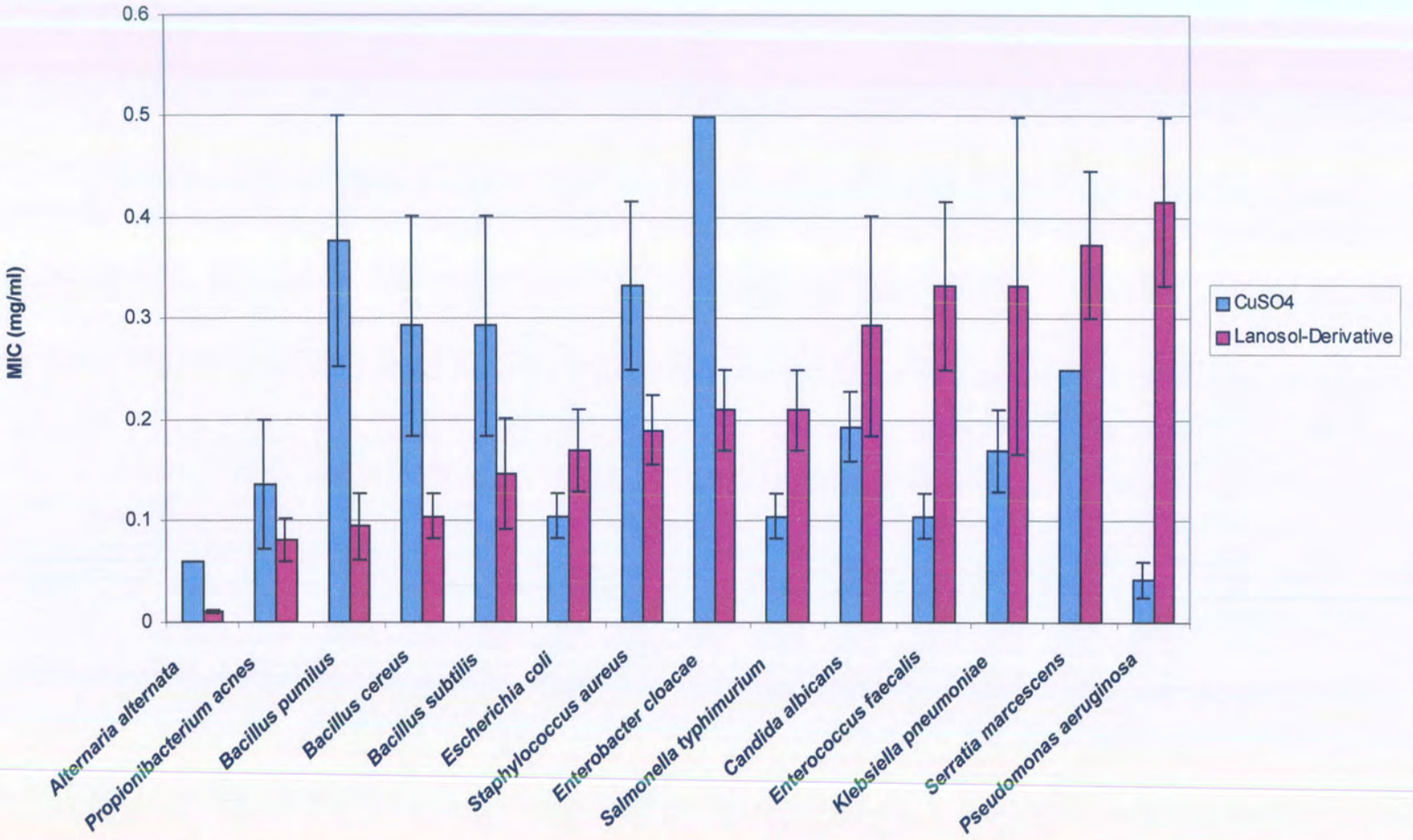

Figure 6.8 Minimum inhibitory concentration (bacteriostatic) of lanosol ethyl ether against the growth of terrestrial bacteria and fungi. Bars = SE, $n = 3$.

What other role may lanosol play in the ecology of *O. serrata*? About 0.1% (1 $mg.g^{-1}$) of the seaweed consisted of lanosol. We may speculate that a specimen *O. serrata* that is wounded by sand and waves (see chapter 1, section 1.2) would release lanosol into the wounded area. If seawater typically has a density of 1026 kg/m^3 then the concentration of lanosol in the direct vicinity of the wound would be about 0.97 $mg.ml^{-1}$. As already mentioned the highest MIC value for lanosol[ee] was 0.67 $mg.ml^{-1}$ against OssB1. Thus if these bacteria were in the vicinity of a wound in *O. serrata*, they would be challenged with about 30 % more than the lowest concentration that inhibits them! Localised concentrations of lanosol in the alga would thus be effective in preventing bacterial infection in wounded tissue. In addition, the mean bactericidal results (0.69 ± 0.042 $mg.ml^{-1}$) were higher than the bacteriostatic results (0.27 ± 0.030 $mg.ml^{-1}$, table 6.3). This indicates that although lanosol[ee] is effective at inhibiting the growth of bacteria and fungi at low concentrations, it kills them only at relatively high concentrations (e.g. those found inside the seaweed tissue).

The determination of the location of bromine (related to lanosol) in the thallus of *O. serrata* was attempted using X-ray microanalysis, but none was found in the cuticle, epithelial and cortical cells of the samples that were looked at. Thin sections (100 nm) were looked at and vesicles containing the bromine rich lanosol may have been easily missed. Pedersén *et al.* (1979) used the same technique and found bromine in all parts of the red alga *Lenormandia prolifera* and concluded that brominated phenols in macroalgae are involved in the sloughing off of the outer cell walls of macroalgae because they build it up by polymerising in those parts. McLachlan and Craigie (1966) on the other hand suggested that the brominated penolic compounds "may regulate the occurrence and abundance of endo- and epiphytes". Provasoli (1965 –

quoted in Mclachlan and Craigie, 1966) found that phenols (presumably halogenated because they were produced by red algae) were necessary for the normal growth and development of the green algae *Ulva* and *Monostroma*. As already mentioned, lanosol and its derivatives also display antifeeding activity in sea urchins, but have no effect on chitons (Kurata *et al*., 1997; DeBusk *et al*., 2000).

Table 6.3 Comparing the bacteriostatic and bactericidal results of lanosol ethyl ether against the growth of the test marine and terrestrial bacteria (± SE). The fungistatic and fungicidal results for *Alternaria alternata* and *Candida albicans* are also shown.

Bacteria	**Bacteriostatic** ($mg.ml^{-1}$)	**Bactericidal** ($mg.ml^{-1}$)
Alternaria alternata	0.01 ± 0.00	0.41 ± 0.10
Marinococcus sp.	0.04 ± 0.01	1.00 ± 0.00
Propionibacterium acnes	0.08 ± 0.02	0.50 ± 0.29
Bacillus pumilus	0.10 ± 0.03	0.67 ± 0.17
Bacillus cereus	0.10 ± 0.02	0.67 ± 0.17
Bacillus subtilis	0.15 ± 0.06	1.00 ± 0.00
Escherichia coli	0.17 ± 0.04	1.00 ± 0.00
Staphylococcus aureus	0.19 ± 0.03	0.67 ± 0.33
Salmonella typhimurium	0.21 ± 0.04	0.67 ± 0.33
Vibrio alginolyticus	0.21 ± 0.04	0.67 ± 0.33
Enterobacter cloacae	0.21 ± 0.04	1.00 ± 0.00
Vibrio harveyi	0.25 ± 0.12	0.83 ± 0.17
Halomonas marina	0.27 ± 0.13	0.83 ± 0.17
Candida albicans	0.29 ± 0.11	0.67 ± 0.17
Halomonas sp. 2	0.29 ± 0.11	0.67 ± 0.17
Enterococcus faecalis	0.33 ± 0.08	0.67 ± 0.17
Halomonas sp. 3	0.33 ± 0.08	0.67 ± 0.33
Klebsiella pneumoniae	0.33 ± 0.17	0.33 ± 0.17
Serratia marcescens	0.37 ± 0.07	0.37 ± 0.07
Pseudomonas sp. 1	0.38 ± 0.12	0.38 ± 0.12
Pseudomonas aeruginosa	0.42 ± 0.08	0.42 ± 0.08
Halomonas sp. 1.	0.42 ± 0.08	0.67 ± 0.17
Halomonas halophila	0.42 ± 0.08	0.83 ± 0.17
Pseudomonas sp. 2	0.42 ± 0.08	1.00 ± 0.00
OssB1	0.67 ± 0.17	0.67 ± 0.17
Mean	**0.27 ± 0.07**	**0.69 ± 0.15**

It is possible that lanosol may have more than one function. Hay (1996) mentions that secondary compounds with "broad bioactive effects" may also defend the producing macroalgae from microbial pathogens. In the same paper he states that phenols have multiple effects when released into seawater; from affecting water colour (and presumably the amount of light reaching primary producers) to chelating ions. Chemicals with more than one function are indeed remarkable products of millions of years of evolution and indicate a highly complex ecological chemistry.

6.5 ACKNOWLEDGEMENTS

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CHAPTER SEVEN

A SEAWEED IS MORE THAN THE SUM OF ITS PARTS: SEM VISUALIZATION OF BIOFILMS ON SOME SEAWEEDS FROM KWAZULU-NATAL, SOUTH AFRICA

7.1 ABSTRACT

Biofilms are found wherever bacteria grow. They may be beneficial, e.g. in vinegar manufacture, or they may have detrimental effects, such as the fouling of surfaces in the marine environment. Antifouling paints containing toxic metallic compounds protect surfaces in seawater from fouling. Unfortunately these compounds accumulate and cause severe ecological problems. Alternatives are being researched. One of these is by studying marine organisms that keep themselves mostly free from fouling. Twelve macroalgae were collected from Palm Beach, KwaZulu-Natal, South Africa and viewed under a scanning electron microscope (SEM). The colonial diatom, *Nitzchia martiana*, had a biofilm that consisted mostly of rods and filamentous bacteria with no cocci forms. The calcified reds (Corallines) had poorly developed biofilms except where the segments join. Here the biofilm was relatively well developed presumably because the calcification of the algal tissue is thinnest here and the seaweeds excrete the nutrients that the biofilm bacteria live on. In comparison to the calcified reds, the non-calcified reds and green seaweed had very well developed biofilms growing on them that consisted of a diversity of mostly bacterial cells.

Treating the seaweed tissue with osmium tetroxide (OsO_4) prior to fixation with glutaraldehyde preserved the structure of the biofilm. In the untreated samples most of the extracellular polymeric substance (EPS) was removed by the processing of the sample for SEM viewing. Since OsO_4 interacts with lipids it is implied that the lipid component of the EPS of biofilms is important in maintaining cohesiveness of the biofilm.

7.2 INTRODUCTION

Biofilms are ubiquitous. They are found in the oceans and on our teeth. They consist mostly of various types of bacteria, but at maturity may also contain algae, fungi and invertebrates depending on where the biofilm is growing. Biofilms form complex communities where consortia of bacteria work together to utilize resources and even protect each other from antimicrobial agents (Marsh and Bowden, 2000). This increased resistance to antimicrobials causes problems when trying to control the unwanted biofilms that foul surfaces in water (e.g. boats, ships and industrial cooling systems).

Biofilms on implanted medical devices are also problematic because they act as sources for recurring infections in the body (Stewart and Costerton, 2001). Antibiotics kill only the free-floating bacteria, while the EPS or slime that surrounds the cells in the biofilm, also protects them from toxins and desiccation (Madigan *et al.*, 1997). However, biofilms also have beneficial uses, such as in filtration systems and vinegar production (Wimpenny, 2000).

Biofilms on the hulls of ships and boats mature into thick communities, which may include macroalgae (e.g. *Enteromorpha* spp.) and animals (e.g. invertebrates such as barnacles, e.g. *Balanus amphitrite*) (Zachary *et al.*, 1980; Lüning, 1990). These cause increased friction and result in increased fuel consumption by the vessel (Evans *et al.*, 2000). Paints containing copper and tributyltin compounds are presently used to control these biofilms. Unfortunately, these chemicals are accumulating along shipping routes and in harbours. They are toxic and have been linked to the deaths of cetaceans in these areas and humans are also at risk via the food chain (Ponasik *et al.*, 1998). Tributyltin has been banned for use on small boats because of its toxic effects on ecosystems.

Alternatives to using these toxic chemicals are being actively researched (De Nys and Steinberg, 2002). One way is by looking at how marine life defends itself against fouling. Some macroalgae, and other organisms like sponges and tunicates, are mostly free from fouling organisms. Understanding how they do this may help us to deliver sustainable and ecologically responsible alternatives to using tributyltin and other toxic compounds.

The adherence of biofilms on chicken skin have been shown to be preserved by a pre-treatment of osmium tetroxide (OsO_4) vapour before the specimens were fixed and dehydrated for scanning electron microscopic (SEM) viewing (McKeekin *et al.*, 1979). It was an aim of this study to determine if this was also the case with biofilms on macroalgae. McKeekin and co-workers (1979) suggested that the OsO_4 stabilised the lipids in the EPS of the biofilm. Fixation in glutaraldehyde was thought to wash the untreated biofilm off the chicken skin. The biofilms on seaweeds are in an

aqueous environment and have to tolerate high shear forces from wave action. This might make a pre-treatment with OsO_4 vapours unnecessary. However, the interactions between the carbohydrates, proteins and DNA in the EPS is thought to maintain the cohesiveness of the EPS (Mayer *et al.*, 1999; Flemming *et al.*, 2000). The role of lipids in the EPS is not generally known, nor considered important in maintaining the cohesiveness of the EPS.

The seaweeds collected for this study had also been extracted and tested for antibacterial activity (chapter 4). Another aim was to determine whether there was a general visual correlation between biological activity and biofilm cover of seaweeds. Some seaweeds produce potent antimicrobial agents, e.g. *Osmundaria serrata* (Rhodophyta) produces a simple brominated phenol that shows activity against fungi and bacteria isolated from the habitat of the seaweed (Chapter 6). Other researchers have suggested that sessile macroalgae that are free from fouling organisms have antifouling mechanisms (Hellio *et al.*, 2001). Thus, if *O. serrata* is relatively free from epibiota, then we may assume that its active product, lanosol, is involved in antifouling.

In this study, a general exploration of the surfaces of some seaweeds growing along the KwaZulu-Natal coast, South Africa was undertaken. This had never been done before. Scanning electron microscopy was used to visualize the biofilms. Unfortunately drying the specimen before viewing creates artefacts due to shrinkage of tissue. While this may not be ideal, it might still give us a good, if shrunken, picture of the epibiota on the seaweeds.

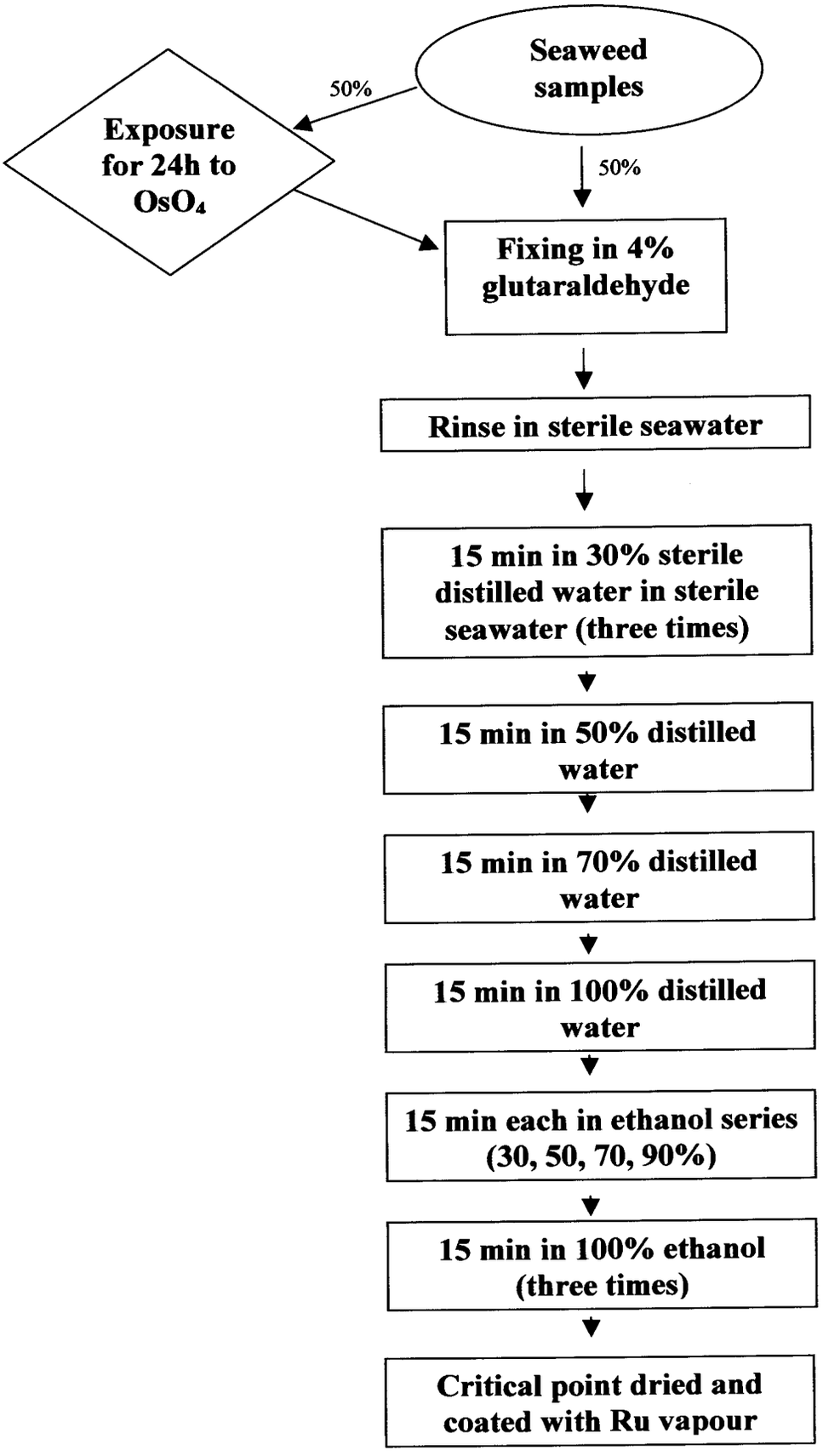
7.3 MATERIALS AND METHODS

The seaweeds (table 7.1) were collected from Palm Beach, KwaZulu-Natal, South Africa. The samples were divided and half were fixed in glutaraldehyde (4 % in sterile seawater) for three hours. The other half were exposed to OsO_4 vapours for 24 hours before fixation in glutaraldehyde. The processing procedures are shown in figure 7.1 and were performed at about 4° C. The samples were stored in 100% ethanol at about 4° C before being critical point dried and exposed to ruthenium vapour for three hours (van der Merwe and Peacock, 1999). They were then mounted onto aluminium stubs for viewing under a Jeol JSM-840 SEM with an accelerating voltage of 5 kV.

Table 7.1 Species of colonial diatom and macroalgae collected and their surfaces viewed using a scanning electron microscope.

Kingdom	**Division/Class**	**Family**	**Genus and Species**
Protista	Heterokonta	Bacillariophyceae	*Nitzchia martiana*
Plantae	Chlorophyceae	Codiaceae	*Codium duthieae*
			Halimeda cuneata
		Caulerpaceae	*Caulerpa filiformis*
	Rhodophyta	Corallinaceae	*Amphiroa bowerbankii*
			A. ephedraea
			Cheilosporum multifidum
		Gelidiaceae	*Gelidium abbottiorum*
		Hypneaceae	*Hypnea rosea*
			H. spicifera
		Ceramiaceae	*Spyridia hypnoides*
		Rhodomelaceae	*Osmundaria serrata*

Figure 7.1 Processing procedure of seaweed samples for scanning electron microscope viewing.



7.4 RESULTS AND DISCUSSION

7.4.1 General Observations

The seaweeds were divided into three groups on the basis of the overall features of the biofilms found on them. The colonial diatom (*N. martiana*) had mostly rods and filaments growing on it with no coccoid cells (figure 7.2.A). The biofilms on the second group were well developed and contained a large diversity of mostly bacterial cells (figure 7.2.B). Diatoms were also seen here (figure 7.2.C), and more rarely what looked like yeast cells (figure 7.2.D). There were no obvious differences between the biofilms on the reds and the greens in this group. Relatively poorly developed biofilms were found on the third group, the calcified reds (figure 7.2.E). However, a diverse community of bacterial cells was found where the segments of the thalli joined (figure 7.2.F).

Several distinctive bacteria were seen on the macroalgae. Cocci, rod-shaped and filamentous cells dominated, but C-shaped bacteria were also seen on some seaweeds (figure 7.3.A). These latter were most likely *Cyclobacterium marinum*, because they grow on macroalgae, form coils and have rounded ends (Holt *et al.*, 1994). The filamentous cells were initially thought to be *Leucothrix mucor*, which is commonly found on seaweeds (Harold and Stanier, 1955; Holt *et al.*, 1994). However, *Erythrobacter longus*, another filamentous bacterium, is also commonly found on seaweeds (Holt *et al.*, 1994). To complicate things further, some bacteria, e.g. *Escherichia coli*, are rod shaped in normal cultural conditions, but form filaments up to 1 mm long in response to sub-MIC levels of toxicants (Beveridge *et al.*, 1991). Since many algae release toxic chemicals (Hellebust, 1974), some of the filamentous bacteria seen on macroalgae may grow as different shaped cells in culture (assuming

that they could be isolated at all). Some of the filaments may also have been fungal hyphae.

Very small cocci (<0.3 μm) were seen (figure 7.3.B) and these are common in the general environment including marine habitats (Kieft, 2000). Larger coccoid-shaped cells were also observed and these were heavily encapsulated (figure 7.3.C). It was almost impossible to tell which type of bacteria these were because some bacteria can change cell shape depending on environmental conditions (Byrd, 2000).

In some cases remnants of the EPS in which the members of the biofilm are embedded was found (figure 7.3.D). In most cases this was better preserved by the OsO_4 treatment and is consistent with the results of McKeekin and co-workers (1979). In other cases no differences were seen between the treatments, but in no cases were the untreated tissues better preserved than the treated ones.

7.4.2 Biofilm on *Nitzchia martiana* (Bacillariophyceae)

The older parts of the filaments of this colonial diatom were covered by a biofilm (figure 7.3.E). Rod shaped cells were even present on younger silica frustules that had not yet gained an EPS covering (figure 7.3.F).

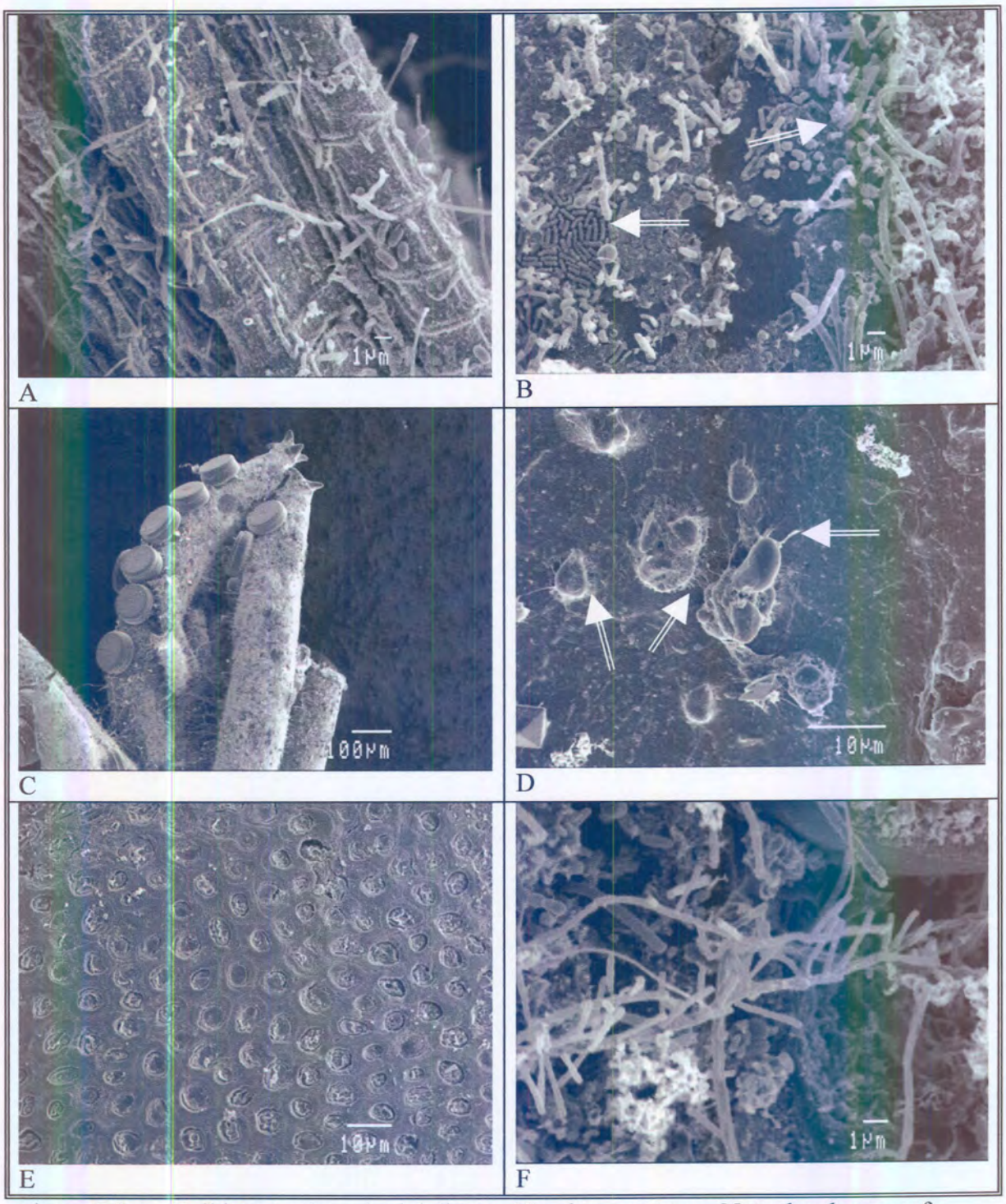


Figure 7.2.A. Epibiota on the colonial diatom *Nitzchia martiana*. Note the absence of cocci.

B. Diversity of bacterial cells growing on *Codium duthieae*. Microcolony of rod-shaped cells (left arrow) with mostly filamentous cells (right arrow).

C. Diatom cells growing on *Spyridia cuppressina*. The frustules of these *Thalassiosira* sp. cells are remarkable clear of epibiota compared with the macroalga.

D. Yeast-like cells growing on *Caulerpa filiformis*. Extracellular polymeric substance found around the cells that anchored them to the seaweed (arrows).

E. Surface of *Amphiroa ephedraea* with almost no epibiota.

F. Epibiota living in the joints between the segments of *A. bowerbankii*.

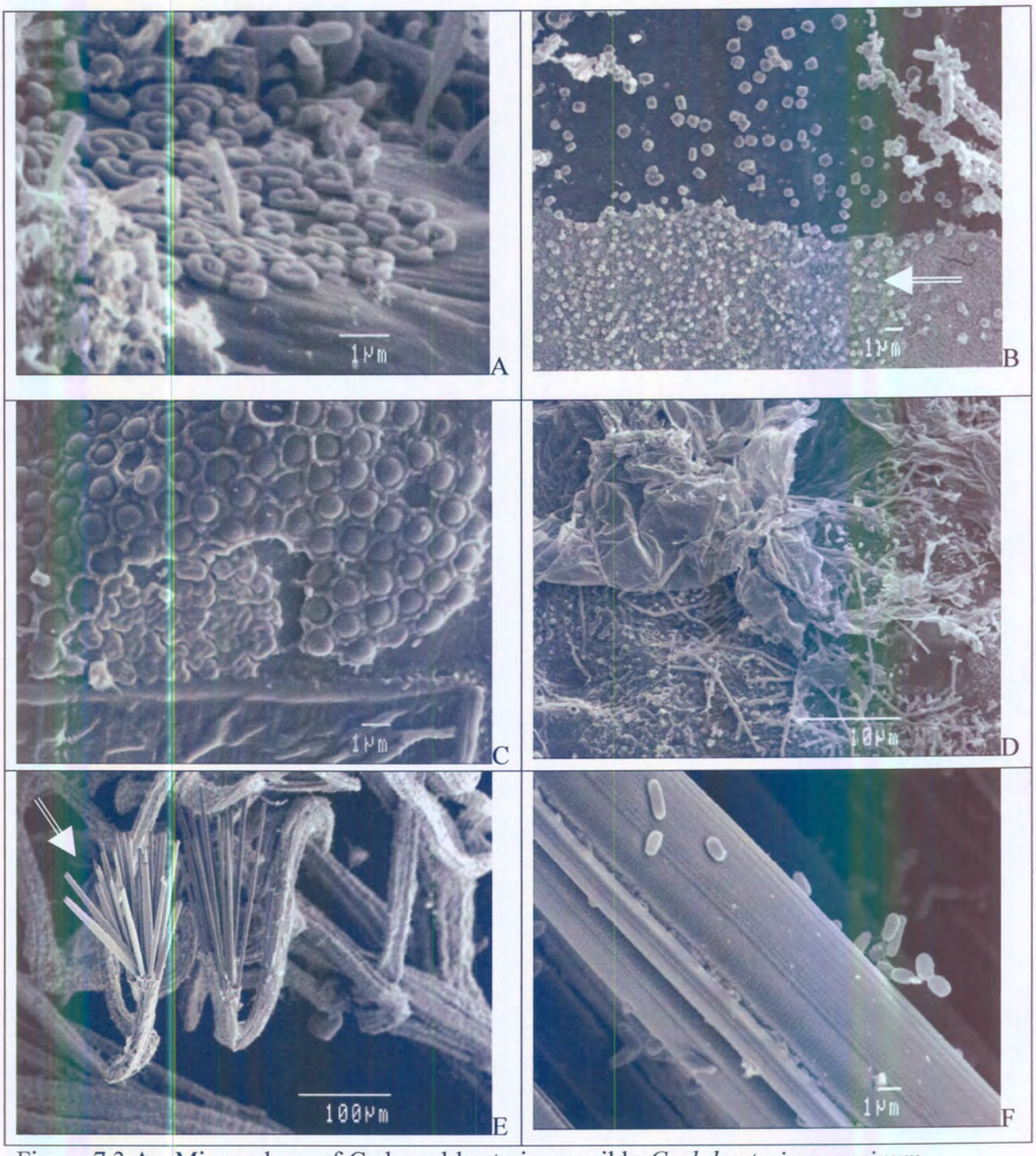


Figure 7.3.A. Microcolony of C-shaped bacteria, possibly *Cyclobacterium marinum*, on *Gelidium abbottiorum*.
B. Very small cocci (arrow) growing on *Codium duthieae*.
C. Encapsulated cocci on *Osmundaria serrata*. Note the thickened glycocalyx surrounding the cells and holding them in place.
D. Remnants of slime layer on *O. serrata*. Bacteria are visible where the slime had peeled away during processing of the sample for viewing by the electron microscope.
E. The ends of *Nitzchia martiana* cells (arrow) protruding from the sheaths that form the colonies of this unusual diatom.
F. Rod-shaped bacteria on naked frustules of the colonial diatom *N. martiana*.

7.4.3 Well Developed Biofilms on the Chlorophyceae and the Remaining Rhodophyta

No obvious differences in the species composition of the biofilms were observed between these species. However, the same bacterial species may have different morphologies depending on environmental conditions, and different bacterial species may have very similar morphologies. It is difficult to make direct comparisons and this discussion is limited to being somewhat descriptive. Nonetheless, the epibiota on this group of seaweeds resembled what was growing on the corallines (calcified reds), except that here the overall cell densities appeared much greater.

Caulerpa filiformis

Remnants of the slime that covered this macroalga were clearly seen on the treated sample (figure 7.4.A), but were completely removed from the untreated one (figure 7.4.B) where hexagonal shaped epithelial cells were seen. Epibiota were found in the lighter areas of the hexagons, while the darker outlines appear clean (figure 7.4.C). This suggests that the slime was extruded from in between the cells. Hence no epibiota were found here because the stream of slime would constantly remove them. This strategy is also used by other macroalgae to keep them free from epibiota (Steinberg *et al.*, 1997). If we assume that the bacteria also contribute to the slime layer, as is the case in all biofilms, then we have here a slime layer made by both the epibiota and the host alga. One wonders whether the slime from the alga is as important to the epibiota as saliva is to the bacteria that form biofilms on teeth, where bacteria are more prone to colonise enamel surfaces that have been conditioned with saliva, than unconditioned surfaces (Kolenbrander *et al.*, 2000).

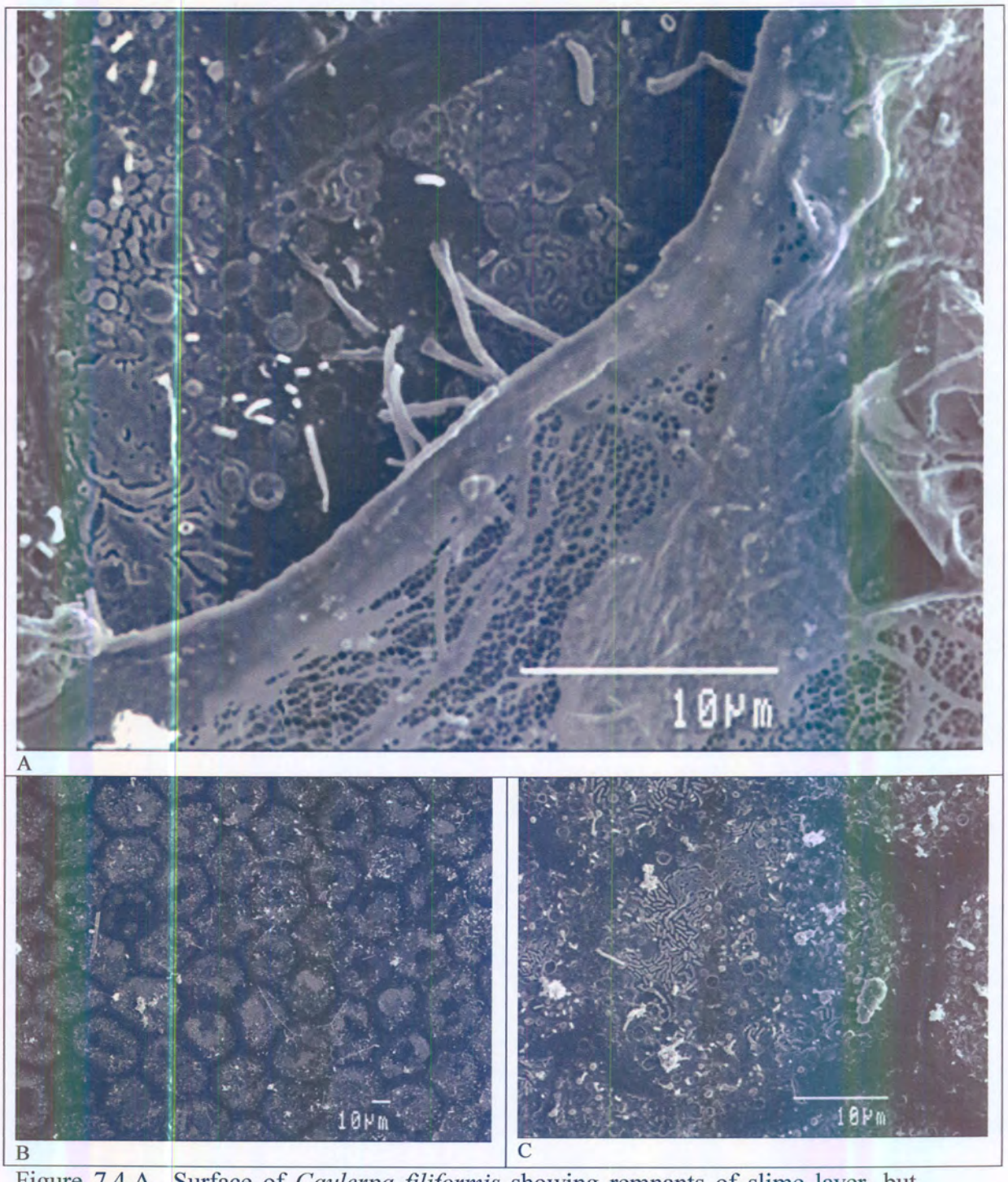


Figure 7.4.A Surface of *Caulerpa filiformis* showing remnants of slime layer, but removal of cocoid cells during processing of the sample. The tissue was treated with OsO_4 vapour.

B. Surface of *C. filiformis* not treated before fixing in glutaraldehyde. No slime layer, but hexagonal epithelial cell outlines visible.

C. Close-up of epithelial cell outline of *C. filiformis*. Note the absence of epibiota around the algal cells, but their presence within the outline. Some cocci were removed during processing of the sample.

Codium duthieae

A complex epiphytic community was observed on this macroalga with filamentous bacteria found next to microcolonies of smaller rod-shaped cells and a great diversity of other cells (figure 7.5.A). Remnants of a slime layer were visible on the utricles of the treated sample (figure 7.5.B), but not on the untreated tissue (not shown).

Halimeda cuneata

Unlike the other green algae, not much difference in biofilm cover was observed between the treated and untreated surfaces of this lightly calcified green. However, hexagonal cell outlines (similar to those on *C. filiformis* – figure 7.4.B) were observed with the epibiota occurring only toward the centre and not near the edges (figure 7.5.C). This may indicate that the *C. cuneata* also secretes a substance that inhibits the settling and growth of epibiotic organisms like *C. filiformis*. However, unlike *C. filiformis*, *C. duthieae* is not slimy and some other inhibitory substance is implied. Since the extract from this seaweed was not remarkably bioactive (see chapter 5) further investigation is suggested.

Gelidium abbottiorum

A complex micro-community was found on this seaweed (figure 7.6.A) with many different bacterial forms. In some areas, bacteria were seen living in depressions on the algal surface (Figure 7.6.B). In figure 7.6.C there is evidence for two types of coverings on the seaweed. The thinner one is associated with a biofilm, but the surface is clean under the thicker, carpet-like layer. This was probably the cuticle of the seaweed. It was found to have a patchy distribution. No bacterial cells were found on the cuticle and may indicate localised antibiotic action. Chemicals with

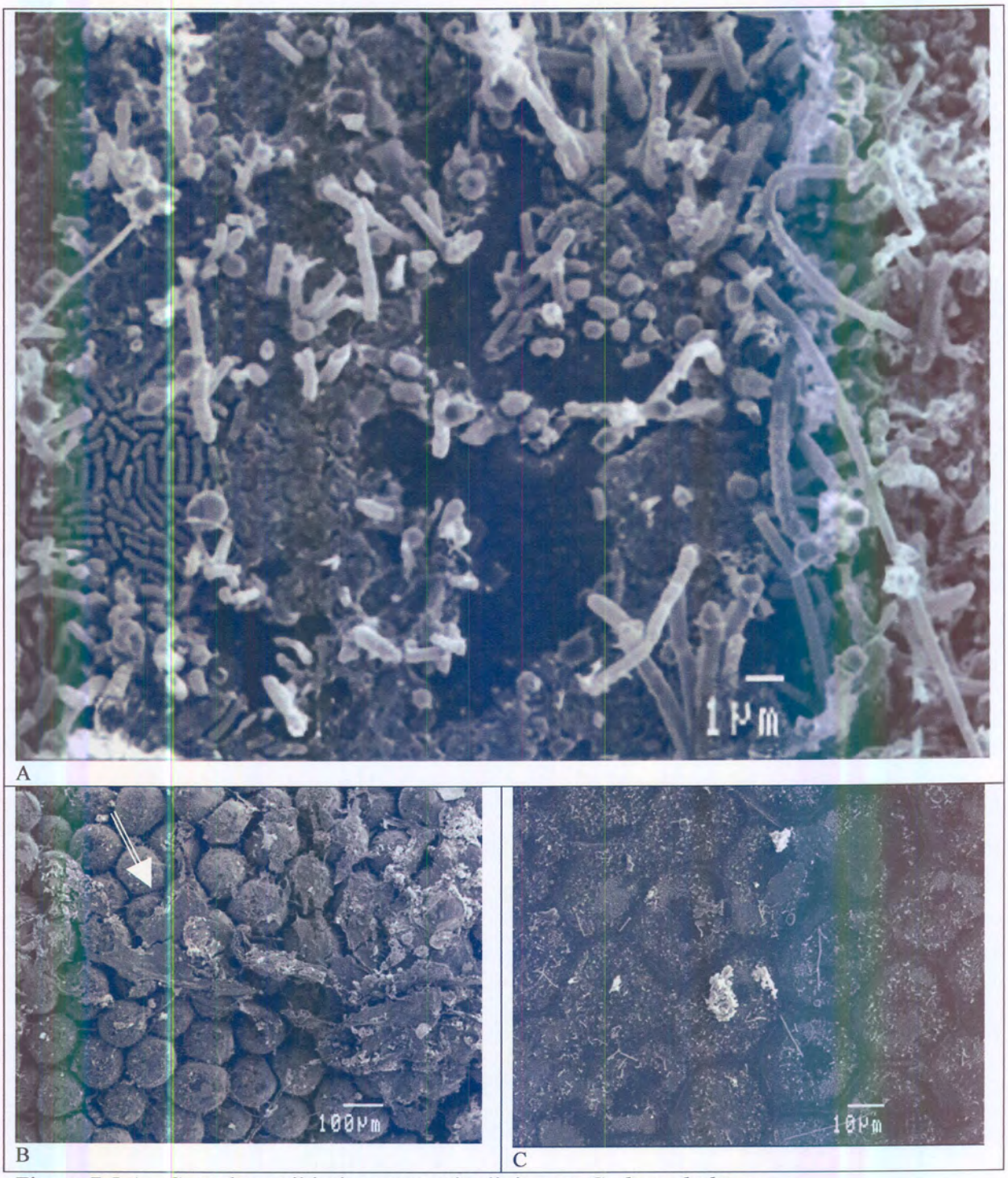


Figure 7.5.A. Complex epibiotic community living on *Codium duthieae*.
B. Remnants of slime layer over utricles (arrow) of *C. duthieae* treated with OsO_4 before glutaraldehyde fixation.
C. Outline of *Halimeda cuneata* epithelial cell. No bacteria were living in the margins of the algal cells.

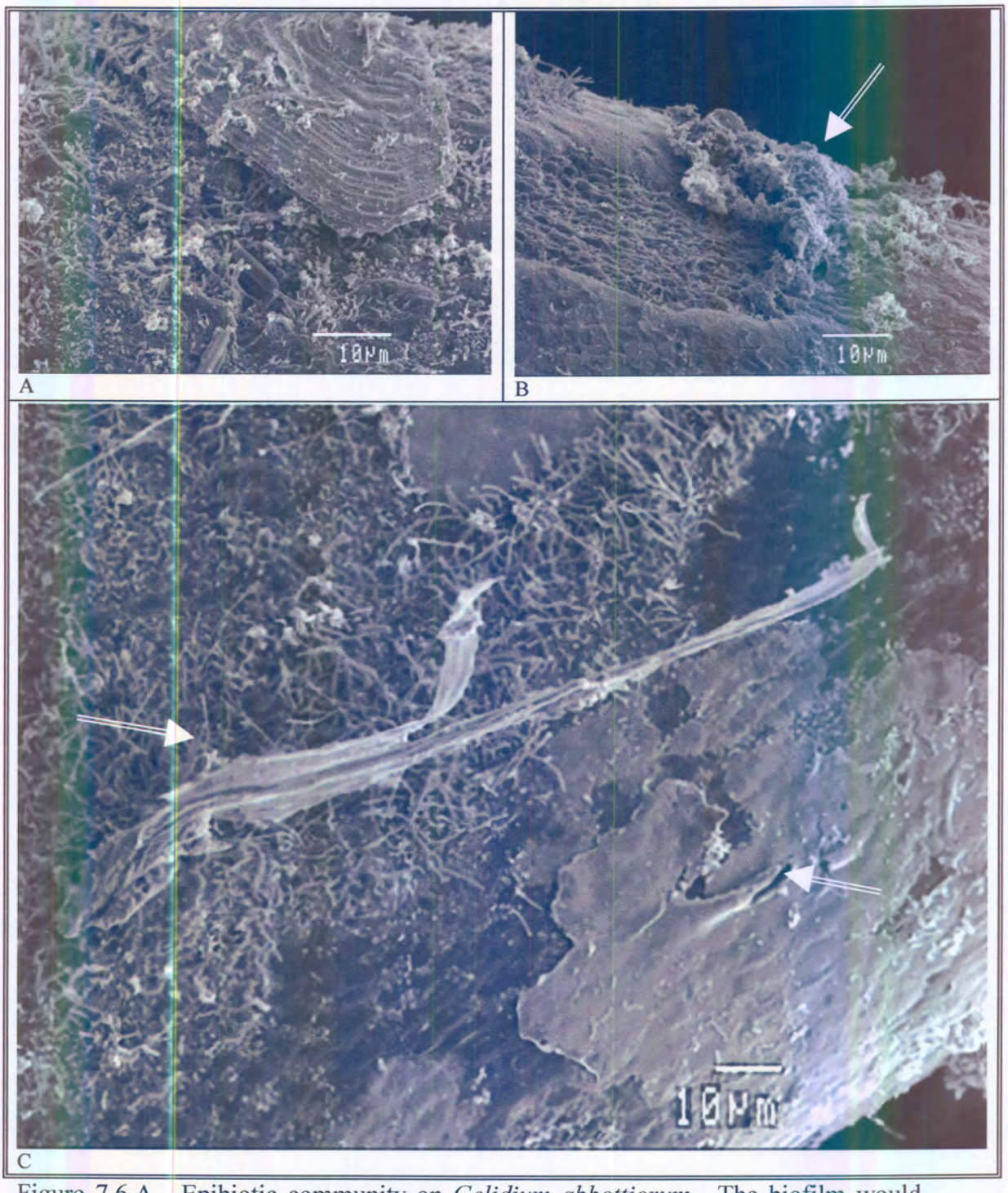


Figure 7.6.A. Epibiotic community on *Gelidium abbottiorum*. The biofilm would have been covered by the EPS, the remnants of which are visible near the top of the micrograph.
B. Micro-community living in a depression on the surface of the red alga *G. abbottiorum*. Note the remnants of the EPS (arrow).
C. Surface of *G. abbottiorum*. The thick, carpet-like layer is the cuticle (right arrow), while the thinner layer is the remnants of the EPS (left arrow) that covered the biofilm. Note no bacterial cells on the cuticle.

antibiotic and antifouling effects have been found on the surfaces of other red seaweeds and may also be present in *G. abbottiorum* (Pedersén, 1979; Steinberg *et al.*, 1997). The extracts made from this alga had very low biological activity (chapter 5). However, the active agent(s) may not have been antibiotic, but antifouling instead (Steinberg *et al.*, 1997). The EPS was better preserved on the treated tissue than on the untreated one (not shown).

Hypnea spicifera

There were far less epibiota on the untreated surface (figure 7.7.A) than on the treated one (figure 7.7.B). The OsO_4 vapour had preserved the biofilm by preventing much of the cells from being removed while the tissue was being processed for SEM viewing. Sperm cells were also found on this seaweed and perhaps represents an input of nutrients for the biofilm system (figure 7.7.C). This type of nutrient input is important to the dynamic biofilms that are constantly importing and exporting cells, organic and inert materials (Wimpenny, 2000).

Hypnea rosea

A patchy cuticle was observed on this red alga (figure 7.8.A and B). Again, no bacteria were found on the patches indicating the possibility of antifouling compounds in the cuticle. The extract from this epiphytic seaweed was unremarkably antibiotic (chapter 5). A clear track in figure 7.8.C indicated grazing and a complex micro-ecosystem associated with the biofilm where some bacteria are preyed on most likely by snails (ZoBell and Feltham, 1938).

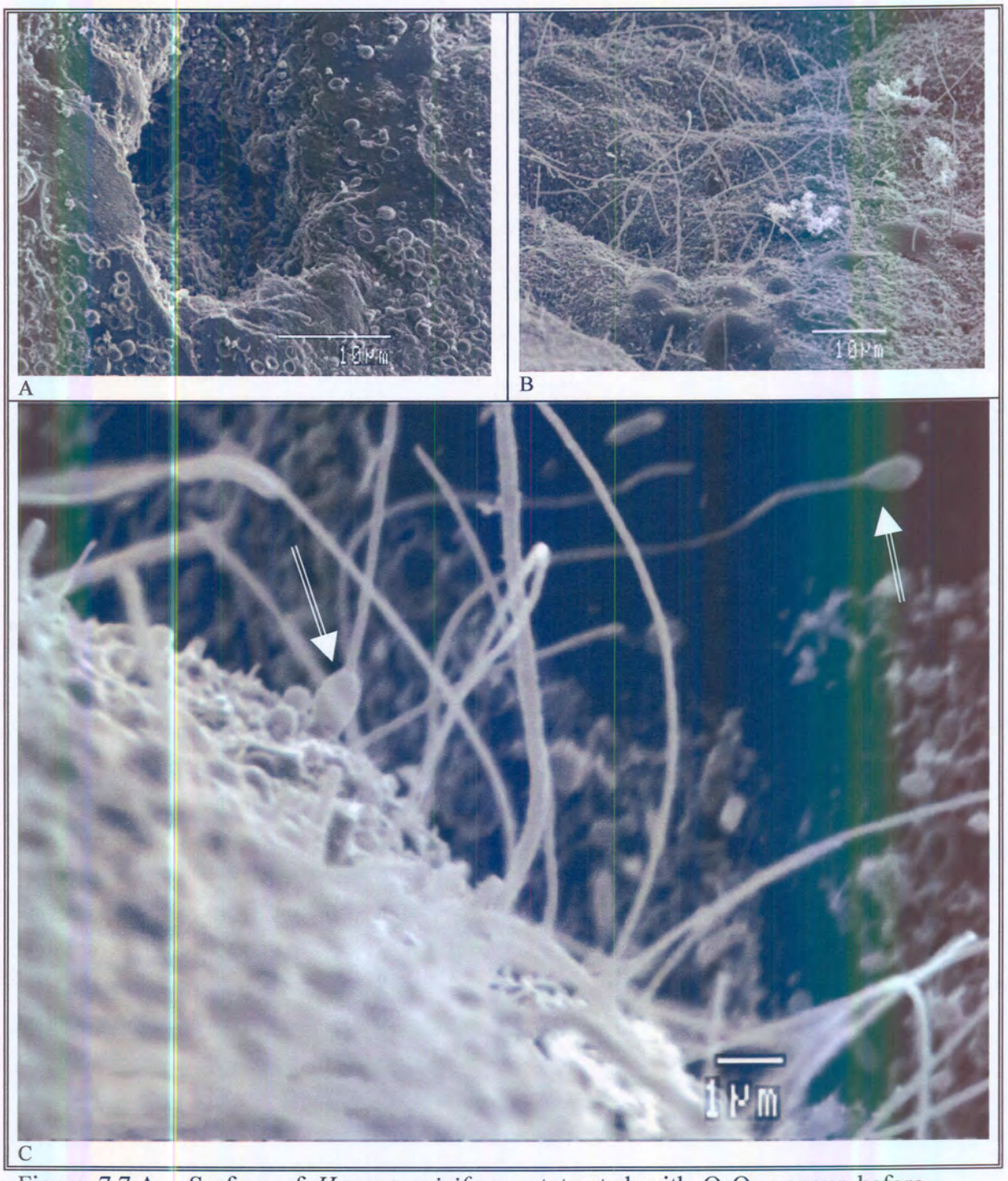


Figure 7.7.A. Surface of *Hypnea spicifera* not treated with OsO_4 vapour before glutaraldehyde fixation.
B. Surface of *H. spicifera* treated with OsO_4 vapour before glutaraldehyde fixation.
C. Sperm cells (arrows) found on the surface of the red alga *H. spicifera*. This may represents an important nutrient import into the biofilm system on the seaweed.

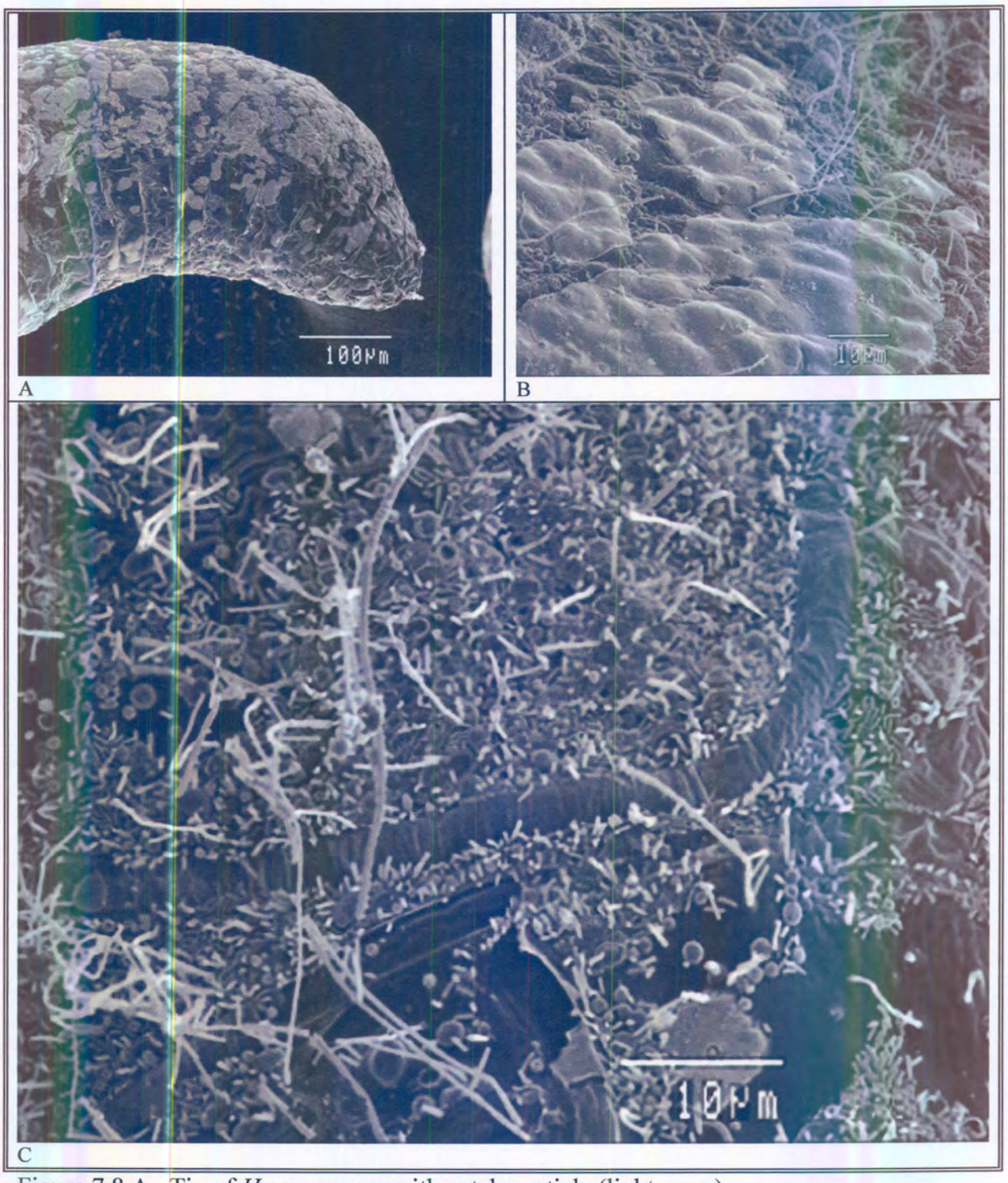


Figure 7.8.A. Tip of *Hypnea rosea* with patchy cuticle (light areas).
B. Close-up of cuticle patches on *H. rosea*. No bacteria were growing on the cuticles, whereas next to them there is a biofilm.
C. Surface of *H. rosea* with track made through biofilm. This grazing of the biofilm indicates a complex community.

Osmundaria serrata[1]

On the samples treated with OsO_4 the EPS was preserved to some extent (figure 7.9.A). On the other hand, no EPS was found on the untreated samples of *O. serrata* (figure 7.9.B). A relatively thick, almost blanket like, cuticle was evident that consisted of several layers (figure 7.9.C). Up to 17 layers have been found in other red algae (Craigi, 1990). In other macroalgae the cuticle is continuously sloughed off, and helps to keep their surfaces clear of epibiota (Pedersén *et al.*, 1979; Steinberg *et al.*, 1997). Evidence for this process occurring on *O. serrata* is presented in figures 7.10.A and B.

The epiphytic community on *O. serrata* was complex (figure 7.10.C). This was unexpected from a macroalga that produces a potent antimicrobial agent (chapters 5 and 6). Near complete lawns of cells were found on tissue taken from near a growing tip (figure 7.11.A). Even diatoms were seen on young tissue (figure 7.11.B). Figure 7.11.C shows what appears to be an infection of the young tissue. It is interesting that such a diversity of epibiota was seen on such young tissue when other workers have found the growing tips of another red alga, *Delisea pulchra*, clean of epibiota (Maximilien, 1995 – quoted in Steinberg *et al.*, 1997). It is suggested that algae (and other eukaryotes) control the biofilms that grow on them by chemical means. Lanosol may be one of these chemicals because although it is produced by *O. serrata* and it is released into seawater, this macroalga has a biofilm covering. However, in the case of *O. serrata* at least, the control of its biofilm is not due only to chemicals, as the sloughing off of outer cell wall layers is probably also important.

[1] See appendix and CD-ROM for animated views of the surface of this macroalga.

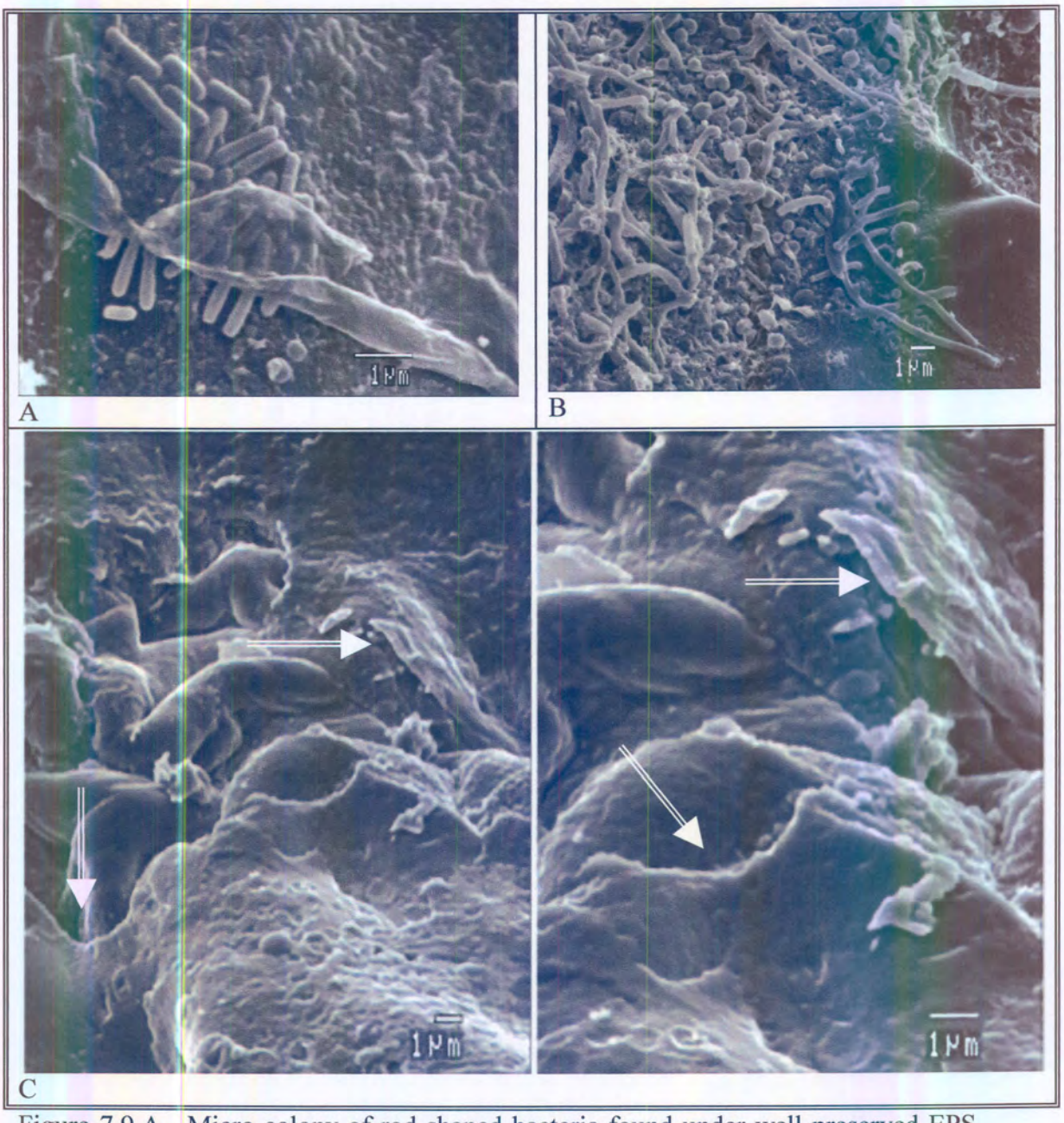


Figure 7.9.A. Micro-colony of rod-shaped bacteria found under well preserved EPS layer on the surface of *Osmundaria serrata*. The layer had folded back to expose the bacteria. This tissue was treated with OsO_4 before glutaraldehyde fixation.
B. Community of bacteria found on *O. serrata*. The slime layer was not preserved in this tissue because it was not treated with OsO_4 vapour before to glutaraldehyde fixation.
C. Damaged surface of *O. serrata* showing the layers of the outer cell wall or cuticle (arrows).

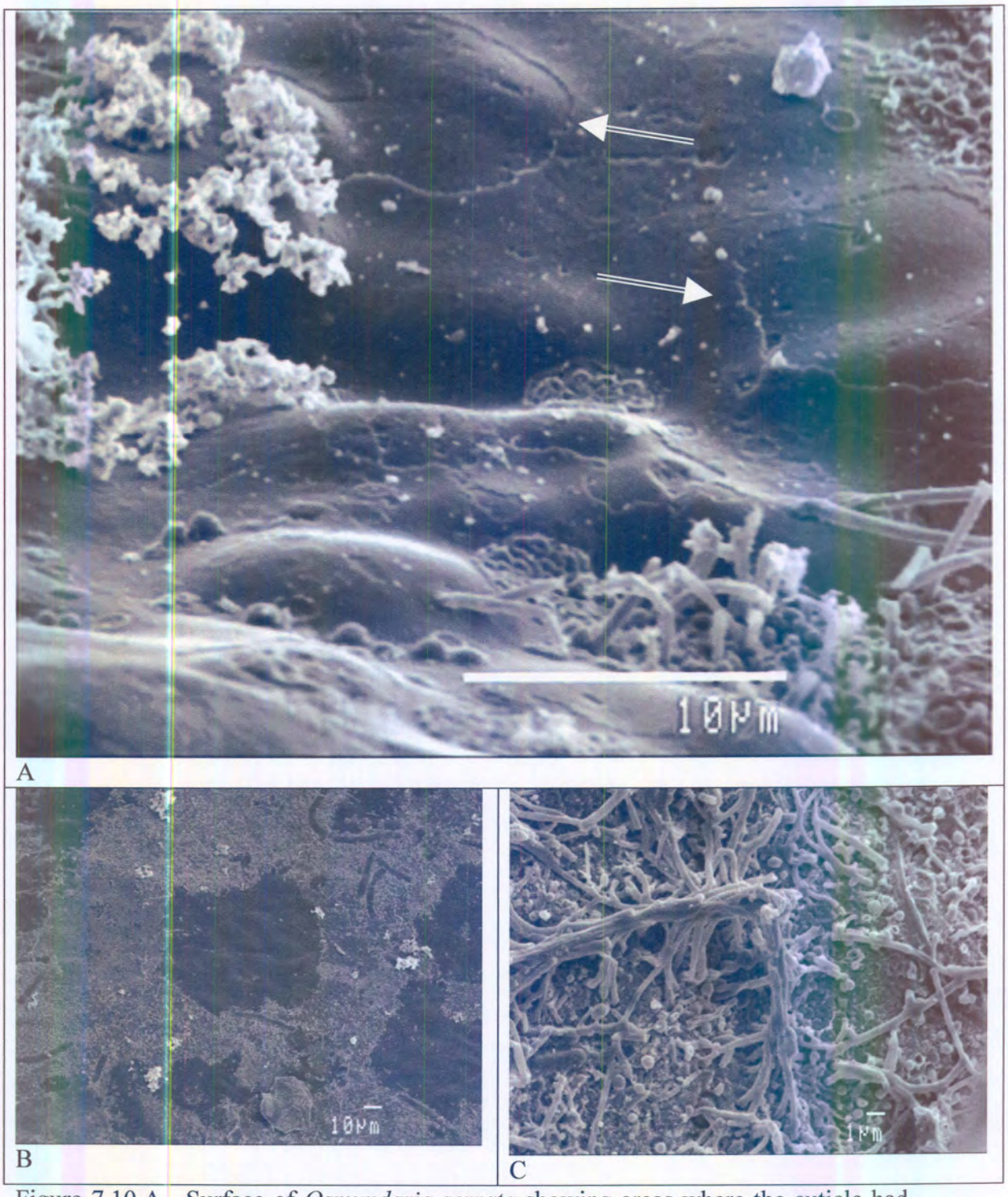


Figure 7.10.A. Surface of *Osmundaria serrata* showing areas where the cuticle had peeled off (arrows).
B. Surface of *O. serrata* showing relatively large patches (dark areas) free of epibiota. The lighter areas are part of a (possibly a snail; ZoBell and Feltmam, 1938).
C. Complex epibiotic community on *O. serrata*. Note the aggregations of filamentous cells that growing with bacteria of various forms. These cells were exposed because the slime layer that usually covers them was lost during processing of the tissue for SEM viewing.

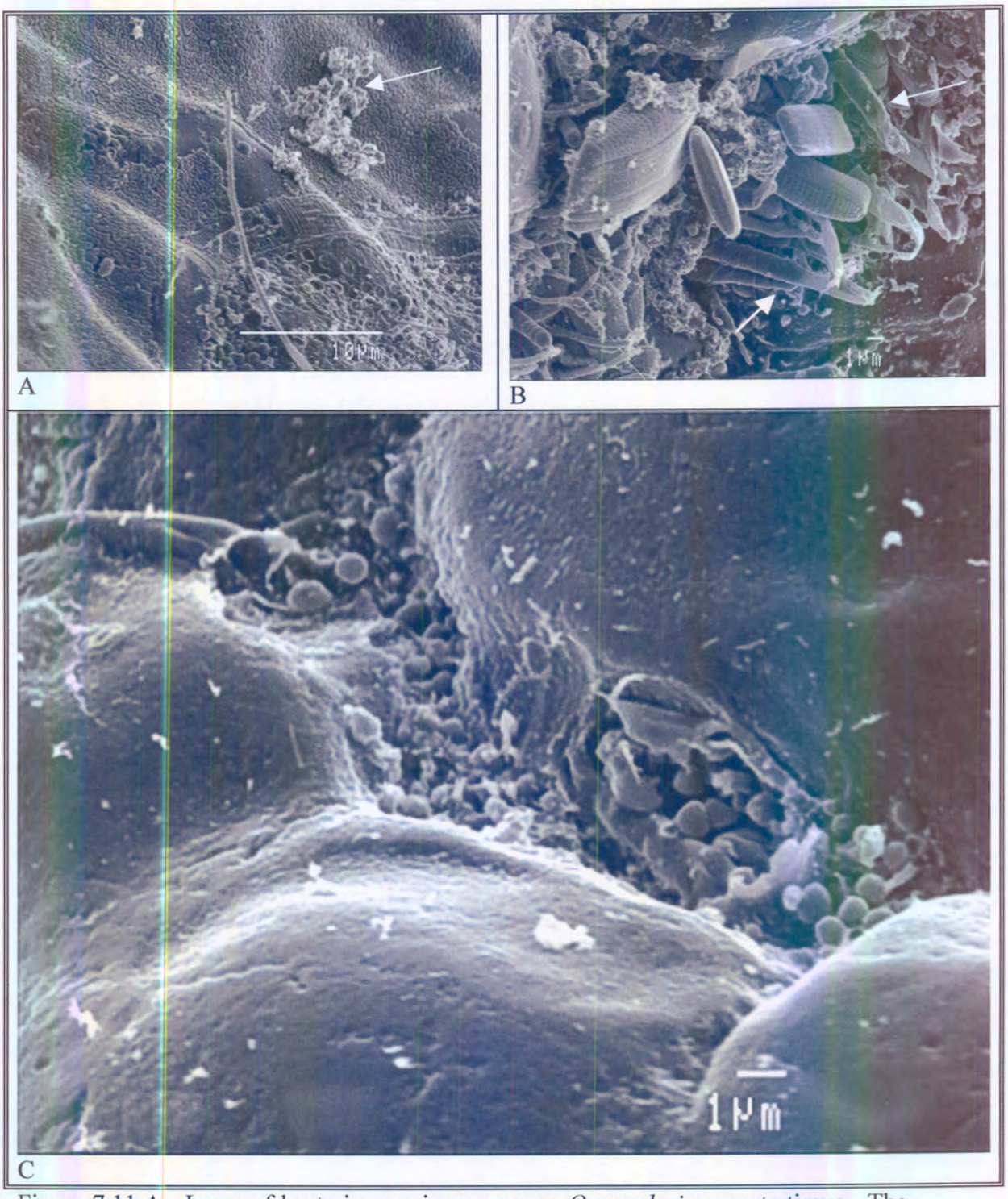


Figure 7.11.A. Lawn of bacteria growing on young *Osmundaria serrata* tissue. The slime layer was removed (remnant – arrow) and exposed the bacteria. The C-shaped bacteria in the upper portion of the micrograph resemble *Cyclobacterium marinum*.
B. Part of the complex biofilm on *O. serrata*. Micro-colonies of the diatom *Plagiogramma* sp. growing with the remains of what resembles fungal hyphae (arrows).
C. Cocci bacteria living in a wound on *O. serrata*. This may be an early infection where only the outer cell walls are affected. The cells occur in a depression, which may have been a weak area on the alga.

Spyridia cuppressina

The slime layer was again preserved by the treatment with OsO_4 (figure 7.12.A). The biofilm on this alga was the most complex and well developed of the alga sampled in this study. A diversity of epibiota, including diatoms (figure 7.12B and C) and a filter-feeding animal (figure 7.13.A) were found. The animal had a thin biofilm composed mostly of filamentous cells (figure 7.13.B) and a close up (figure 7.13.C) revealed nano-structures on its surface. Nanostructures found on marine mammals are thought to help to keep their surfaces relatively clean by preventing attachment of bacteria (Baum *et al.*, 2002). The surface of the animal in figure 7.13.A was much cleaner in comparison to the alga, with only the relatively small ends of the filamentous bacteria able to attach onto the nano-rough surface. No nanostructures were found on any of the algae used in this study, but it is interesting that both filter feeders and cetaceans have nanostructures for controlling biofilm formation.

Spyridia hypnoides

The sample treated with OsO_4 had a fair amount of biofilm covering (figure 7.14.A). However, when compared with a tip from an untreated sample (figure 7.14.B) the osmium treatment clearly preserved more of the biofilm. More evidence of this is presented in figures 7.14.C and D.

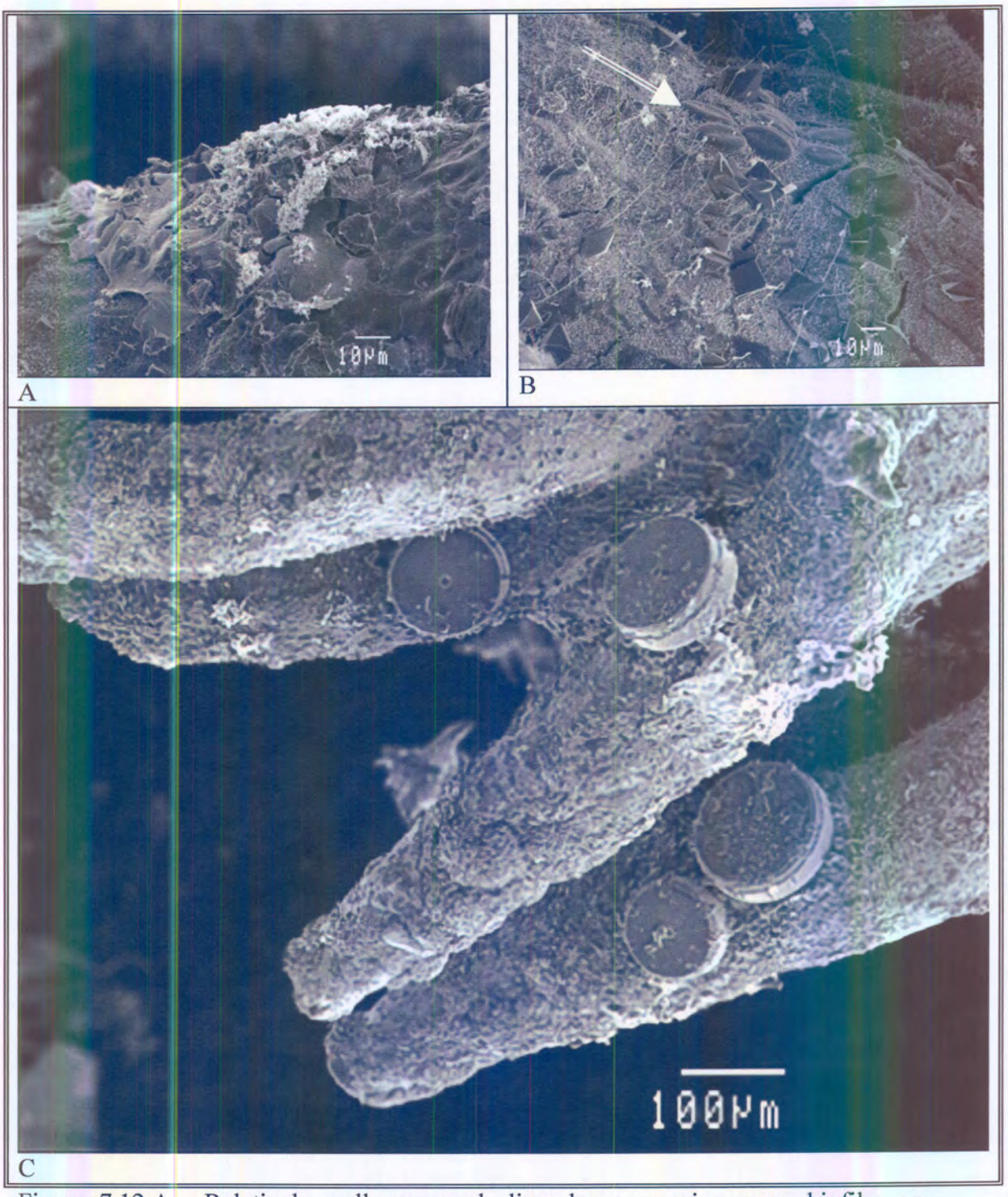


Figure 7.12.A. Relatively well preserved slime layer covering some biofilm on *Spyridia cuppressina* tissue treated with OsO_4.
B. Micro-colony of the diatom *Campyloneis* sp. (arrow) embedded in the biofilm on *S. cuppressina*.
C. Round diatoms of the genus *Thalassiosira* found on near the tips of *S. cuppressina*. Note that the surface of the diatom appear rough, indicating a biofilm covering of their own.

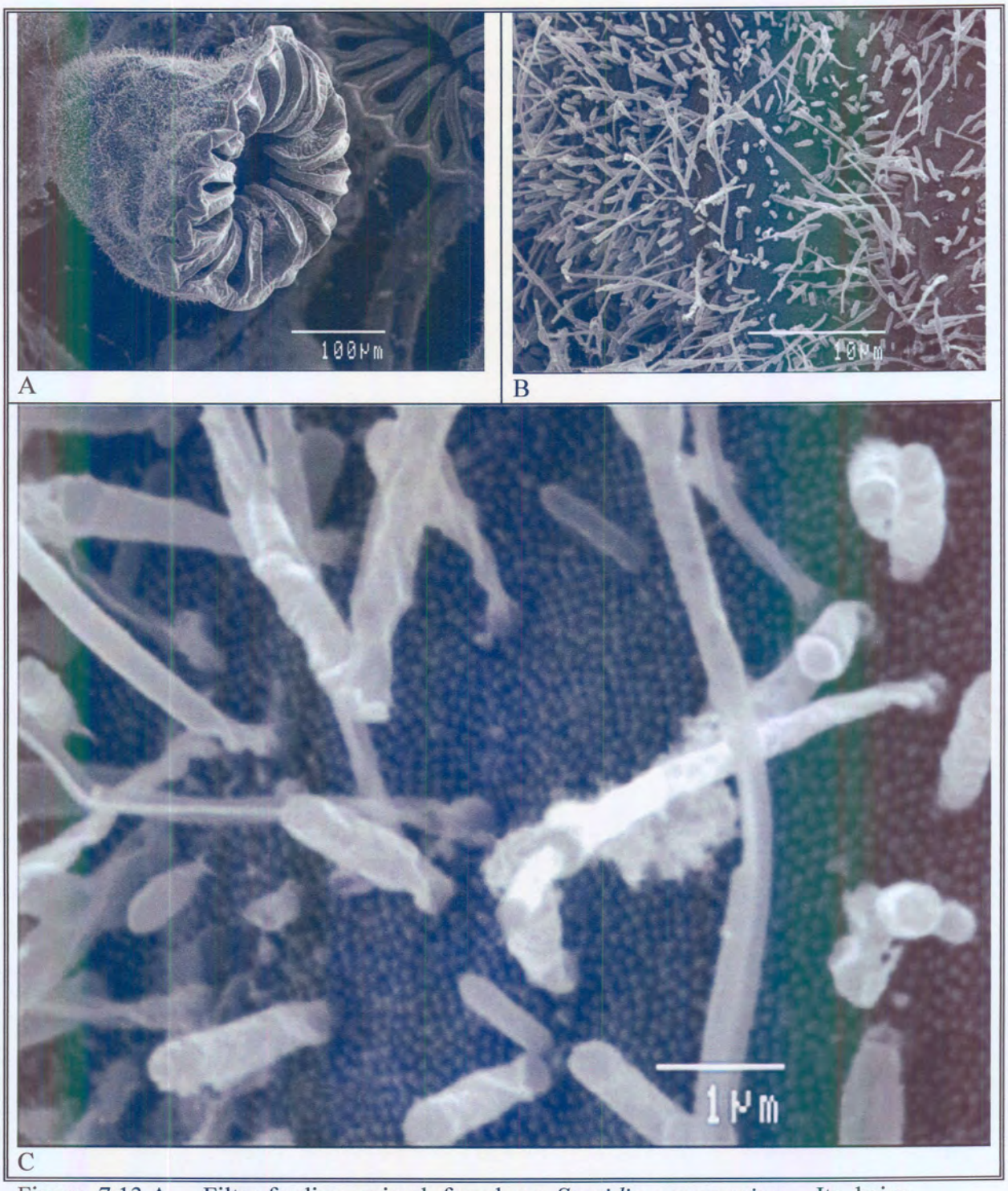


Figure 7.13.A. Filter-feeding animal found on *Spyridia cuppressina*. Its hairy appearance is due to filamentous bacteria growing on its surface.
B. Filamentous bacteria growing on the surface of a filter-feeding animal that was found on *S. cuppressina*.
C. Close-up view of the surface of the filter-feeding animal found on *S. cuppressina*. The nano-rough surface is evident and prevents most bacteria from attaching themselves. Only the ends of filamentous bacteria were able to attach to the nano-rough surface.

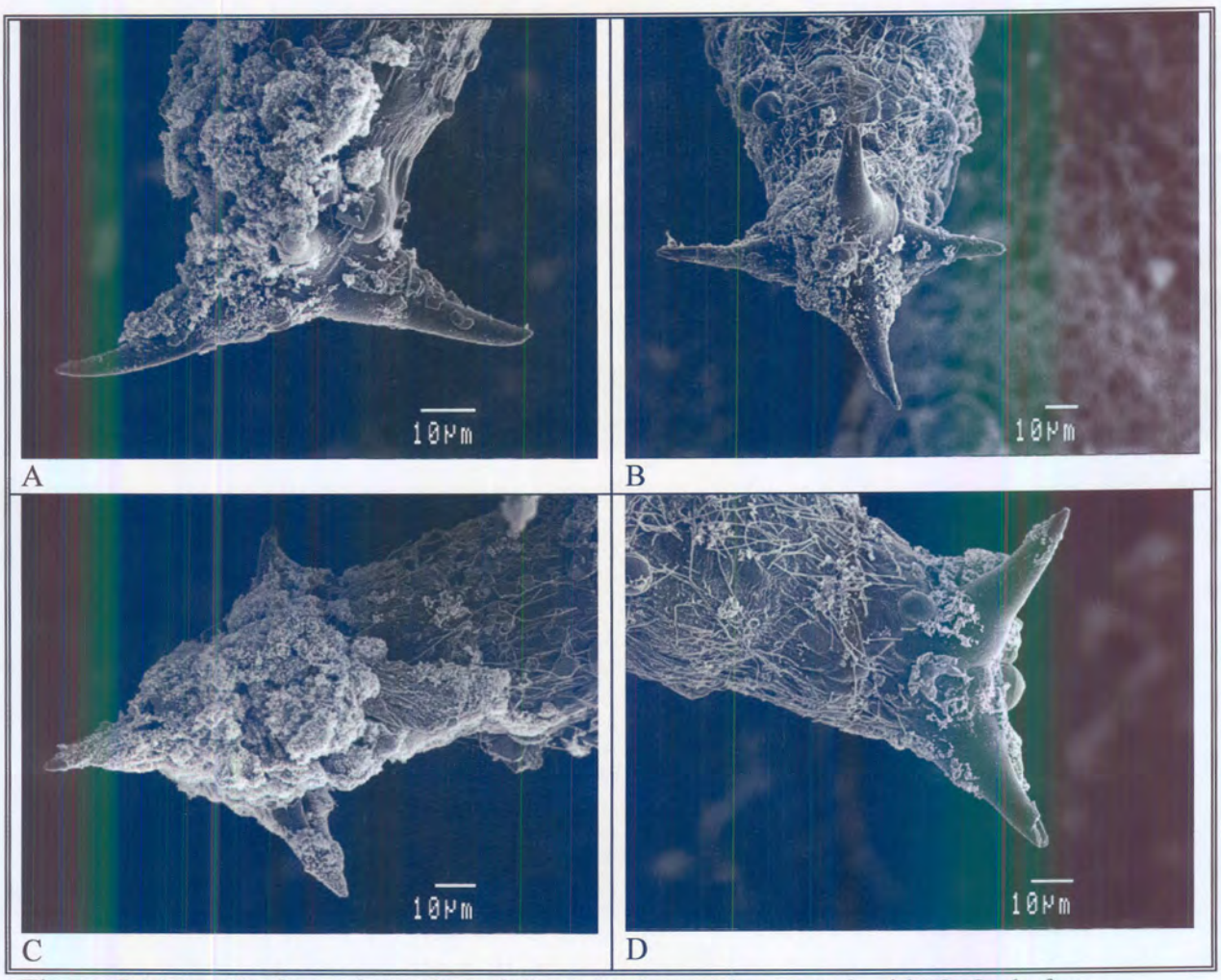


Figure 7.14.A. Surface of the tips of *Spyridia hypnoides* treated with OsO_4 before fixation in glutaraldehyde. A very thick biofilm nearly covering the structure. The slime layer had dried in places to form "fluffy" objects.

B. Surface of *S. hypnoides* not treated with OsO_4 before glutaraldehyde fixation. Much of the biofilm had been lost during processing of the sample and no remnants of the slime layer remained.

C. Surface of the tips of *Spyridia hypnoides* treated with OsO_4 before fixation in glutaraldehyde. A very thick biofilm nearly covering the structure. The slime layer had dried in places to form "fluffy" objects.

D. Surface of *S. hypnoides* not treated with OsO_4 before glutaraldehyde fixation. Much of the biofilm had been lost during processing of the sample and no remnants of the slime layer remained.

7.4.4 Poorly Developed Biofilms on the Corallinaceae (Calcified Rhodophyta)

The surfaces of these macroalgae all appeared very similar; for example, compare figures 7.15.A and B. Well-developed biofilms that were observed where the segments of the thalli joined (see figure 7.15.C) may indicate that extracellular products are released from the macroalgae in these areas. The calcification of the cells is thinnest at these junctions to allow for movement of the thallus and to prevent breakage due to the strong wave action that characterises the habitats of these macroalgae. It is likely that the seaweed releases various products at these points, and these are then utilised by the bacteria that live there. There were no obvious differences between the material treated and untreated with OsO_4.

7.4.5 General Discussion

Since the extract of *O. serrata* was most inhibitory towards bacterial growth it was expected to see much less bacteria on it. However, this was not the case, and bacteria covered even young tissue! Therefore lanosol does not seem to prevent the formation of biofilms. It may, however, function to control the biofilm and protect damaged tissue from infection. Epiphytic bacteria have been discovered that prevent the settlement of invertebrate larvae and spores from marine algae (Egan *et al.*, 2001). It would thus seem that the biofilms on macroalgae are beneficial, by preventing the fouling of the macroalgae. Other benefits may include the bacteria detoxifying inhibitory substances and nutritional advantages for example nitrogen fixation (Sieburth, 1968). It is thought that some of the secondary metabolites that macroalgae produce and release may function to control their epiphytic bacteria (McLachlan and Craigie, 1966). Further study with lanosol is required and may tie in with the use of

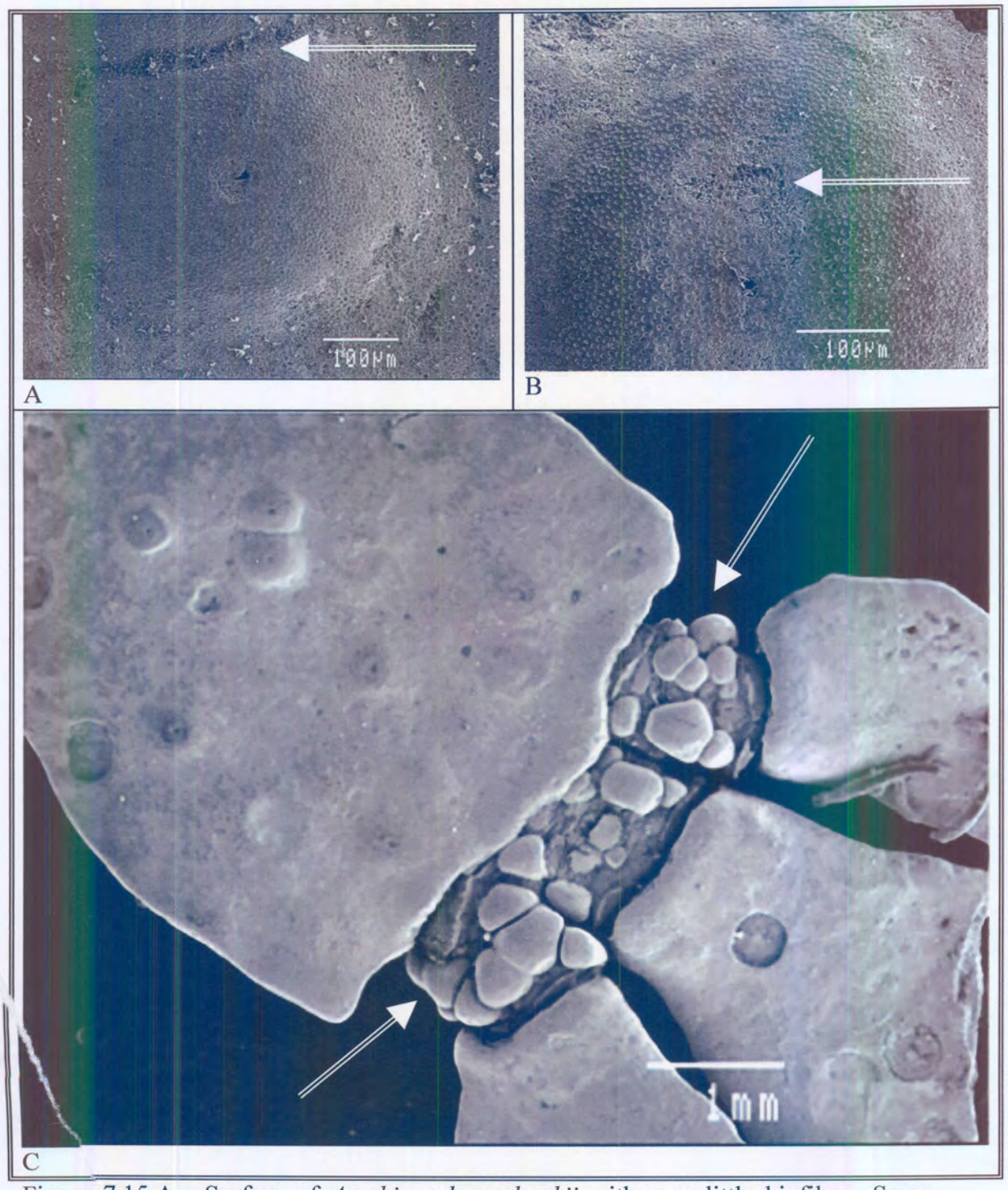


Figure 7.15.A. Surface of *Amphiroa bowerbankii* with very little biofilm. Some bacterial cells were growing in a protected pocket at the edge of the conceptacle (arrow).
B. Surface of *A. ephedraea* with almost no biofilm. Some bacterial cells are seen near the pore of the conceptacle (arrow).
C. Macro-view of *A. bowerbankii* showing the relatively poorly calcified joints (arrows) of the segmented thallus. Most of the biofilm on the calcified red algae were found on these joints.

artificial biofilms that are currently being developed to keep surfaces in marine environments free from fouling organisms (De Nys and Steinberg, 2002).

It must be noted that *L. mucor* was found to grow significantly faster on the red alga *Antithamnion sarniense* in pure culture than alone (Brock, 1967). This suggests that a ship's hull in the future could be made of a composite material consisting of an engineered symbiosis between algal and bacterial cells (reminiscent of a lichen with its fungal and algal symbionts). The algal cells photosynthesising and providing nutrients to the bacteria, while the bacteria fix nitrogen for the algae and liberate antifouling compounds as long as they are fed, and so keeping the surface clean. Such an antifouling system would be environmentally friendly and, hopefully, not too tasty for grazers.

While this study gave a good general picture of the biofilm covering on some of the macroalgae from KwaZulu-Natal, several limitations in using SEM were encountered. The most important being the inability to identify the bacteria that were seen. Other methods such as confocal scanning laser microscopy (CSLM) used in combination with fluorescent probes could be used to identify individual species in a biofilm (Wimpenny, 2000). This would deliver very detailed results. It is even possible to use fluorescent probes that give information on pH and the distribution of polysaccharides. The three-dimensional images that result would greatly improve our understanding of the processes in living biofilms on macroalgae. The analysis of rRNA may also be used to identify the bacteria in the biofilm without the need for cultivation (Amann *et al.*, 1995).

Biofilms on seaweeds have been evolving with the macroalgae that they live on for millions of years. In terms of functionality, the biofilm may be perceived as part of the cuticle of the seaweed just like the biofilm on terrestrial plants being part of its cuticle (C. van der Merwe pers. comm.) This makes the seaweed more than a collection of cells containing seaweed DNA. We have known about biofilms since the early twentieth century, and we must consider that the line between what we perceive as a macroalga and its epibiotic bacteria has to be changed because it is based only on structural details. Functionality is just as important and also needs to be considered. Just as human bodies are made of more than just cells containing human DNA, seaweeds also have microscopic symbionts.

An altered definition of "seaweed" is required: A seaweed (or macroalga), in the holistic sense, is made up of both eukaryotic and prokaryotic cells. The latter usually forms a patchy layer around the former, but endophytes are known and the bacteria sometimes invade the algal cells and utilize them as food. The algal cells export biochemicals that are used by the bacteria as nutrients. Antimicrobial substances are released by some of the bacteria, and prevent the settlement and growth of algae, invertebrates, and other fouling organisms that could prevent light from reaching the photosynthetic cells of the macroalga. The seaweed may also release antimicrobial substances that control the biofilm. A similar broadening of the definition of other organisms is also required, for example it is well known that a human body consist of much more than just eukaryotic cells containing *Homo sapiens* DNA. This fact cannot be ignored and a shift in the perception of what constitutes an organism is thus implied.

The morphologically distinct remnants of the EPS indicate differences in their nature (Fleming et al., 2000). At least four different types of EPS on the surfaces of macroalgae was observed in this study:

1. Sponge or fluffy type (figure 7.14.C)
2. Thin membranous (figure 7.9.A)
3. Thicker membranous with web-like structure (figure 7.4.A)
4. Thick slime matrix (figure 7.3.C).

One of the main functions of EPS is to hold bacteria next to each other forming microbial consortia (Flemming *et al.*, 2000). These are able to live in environments that would be toxic to the individual members in isolation. It was previously seen that the consortium of bacteria, OssB1, was more resistant to the seaweed extracts (chapter 5) and seaweed product (chapter 6) than individual bacteria in pure culture. The creation of similar flocs may be a good way to study the physiology of biofilms. Pure cultures of biofilm forming bacteria give limited information, whereas a stable culture of a consortia of bacteria isolated from a biofilm would behave more like the real thing. The individuals of such a consortium may be identified by 16S rRNA analysis. OssB1 was characterised on the basis of 27 physiological traits. This may be a convenient method of distinguishing between different consortia. A future experiment may include other consortia from different biofilms (and characterise them differently). These would be very useful to test the activity of antifouling agents because we would gain a better idea of their activities in the environment than with individual bacteria in pure cultures.

The fact that the EPS is preserved with a prefixation treatment of exposure to osmium vapour strongly suggests that lipids are important structural components of the various types of EPS because osmium stabilises lipids (McKeekin *et al.*, 1979). Studies on the cohesiveness of EPS have focused on weak binding forces associated with the major components (carbohydrates and proteins) of the EPS. In fact, a recent review (Flemming *et al.*, 2000) on the cohesiveness of the biofilm matrix EPS did not even mention the possible function of lipids in the EPS.

In addition, the fact that proteins are stabilised by glutaraldehyde and fixation in glutaraldehyde does not effectively preserve the EPS structure (Richards and Turner, 1984), strongly suggests that the protein component of EPS has little to do with the its structural integrity.

The biofilms on the macroalgae used in this study had one thing in common; they were all exposed to strong sheer forces due to wave action. Perhaps the EPS that they produce has a different lipid composition that is critical in maintaining its cohesiveness. Perhaps the removal, or even the control of, marine biofilms that lead to biofouling can benefit from this information. One certainty is that the high sheer forces shape the morphology of biofilms, which tend to consist of closely packed cells (Sutherland, 2001). These were seen on the macroalgae exposed to strong wave action (figure 7.3.C). In addition, bacteria tend to move downstream in response to turbulent flow and in the case of *O. serrata*, the young tissue at the tips are downstream. This is where we were surprised to find nearly complete lawns of bacteria growing. Bacterial adhesion has also been found to be enhanced by shear forces through the protein *FimH* (Thomas *et al.*, 2002). The biofilms on seaweeds are

a complex and dynamic system of interactions between the epibiota in the biofilm, the environment and the seaweed substrate. The seaweeds and epibiota are interdependent and although communism may not have worked in the 20th century for humans, it works in the oceans on the macroalgae.

7.5 ACKNOWLEDGMENTS

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CHAPTER EIGHT

GENERAL DISCUSSION

8.1 DEFENCE OF *OSMUNDARIA SERRATA*

Osmundaria serrata needs defence from the herbivorous animals that it shares its habitat with. These include the decorator crab (*Dehaanius dentatus*), green turtle (*Chelonia mydas*), Natal rock crab (*Grapsus grapsus tenuicrustatus*), bronze-bream (*Pachymetopon grande*), and stone-bream (*Neoscorpis lithophilus*) (Branch *et al.*, 1994). This macroalga has well developed mechanical defences, to not only larger predators, but also to smaller potentially fouling organisms. If these epiphytes were permitted to settle and grow, they would block all-important sunlight from reaching the photosynthetic epithelial cells. The thallus is leathery, tough and difficult to break. This toughness, combined with the spikes along the margins, probably act as feeding deterrence much like thorns do in land plants.

The spiralled form of the blades may protect the seaweed from being settled on by epiphytes. Caro *et al.* (2002) found that the spiralled structure of vessels conduct liquid in such a way as to keep the vessels washed clean (quoted by Watts, 2002). The water washing over *O. serrata* blades by wave action is swirled and probably prevents the settlement of organisms not adapted to conditions of such high shear forces. In addition to these mechanical defences, *O. serrata* produces lanosol in high enough concentrations to act as a antimicrobial agent and feeding deterrent (Kurata *et al.*, 1997). It is likely that other species of *Osmundaria* also produce this compound because it is found in such a wide variety of seaweeds. It would be interesting to determine the range of macroalgae that produce it. Seaweeds have had a much longer

natural history in[1] this planet than land plants. And although land plants produce a great diversity of secondary compounds, seaweeds have been producing their own unique compounds for millions of years longer than their distant relatives on land.

Since lanosol has been found in seawater it is probably released by seaweed and a concentration gradient exists. The concentration of lanosol in the seaweed tissue is bactericidal (0.97 $mg.ml^{-1}$ – chapter 6, section 6.4.2), while at lower concentrations it is bacteriostatic for some bacteria, but other bacteria are able to tolerate these levels and grow (figure 8.1). Further from the seaweed is the concentration of lanosol is too low to inhibit bacterial growth, but the chemical may serve as an attractant to "desirable" species of bacteria. The sizes of these zones would be dependent on local currents and rate of lanosol release by the seaweed.

Figure 8.1 Hypothetical lanosol concentration zones of *Osmundaria serrata*. **a** = bactericidal concentrations of lanosol in seaweed cells; **b** = bacteriostatic or tolerant concentrations of lanosol in 'phycosphere' surrounding seaweed; **c** = very low concentrations of lanosol further away from seaweed thallus (see text for details).

[1] The planet Earth consists of more than just a lithosphere, but also has a hydrosphere, an atmosphere and a magnetosphere extending beyond the solid rocky mass. It is thus more correct to say that we live ***in*** the planet (with the seaweeds) rather than on top of it. Technically there is no 'on' any planets with magnetospheres because they taper off gradually, as all magnetic fields do.

8.2 IMPORTANT RESULTS

All the aims of this thesis were met and the main findings were as follows:

8.2.1 Identities of some bacteria isolated from the surface of the macroalga *Osmundaria serrata* (Rhodophyta) and its habitat

A high proportion of aerobic Gram-negative bacteria were isolated from *O. serrata*. This is in agreement with other studies. However, this is no reflection of the actual species composition of the biofilm living on the seaweed because only a small fraction of these will grow in laboratory media. Other techniques, for example rRNA probes, would give a better picture of species composition. The isolation method was appropriate in this case because it delivered ecologically relevant bacteria that could be used in bioassays of seaweed products. However, we are left not knowing how the other, non-culturable bacteria would respond to the compounds. This is significant because these other bacteria represent the majority of the population in the biofilm. This aim was successfully fulfilled.

8.2.2 Comparison between agar dilution and microtitre methods of testing for the antibacterial activity of an extract from *O. serrata*

The microtitre method was found to be more sensitive than the agar dilution method in petri dishes. This finding was similar to those of Eloff's (1999). The microtitre method was more appropriate considering the liquid environment that the test bacteria usually live in (the watery marine environment as opposed to the artificial solid-air environment of the petri dishes). Another possible reason for the increased sensitivity of the microtitre method is that in a liquid medium more surface area of bacteria is exposed to the medium and toxicant than on the solid medium. On solid medium

some members of the colony are not in direct contact with the toxicant and may thus grow while the bacteria at the bottom of the colony are killed. In liquid media, unless the bacteria form flocks, all the cells are equally in contact with the medium and toxicant. We, therefore, see a greater sensitivity. There was success in meeting this aim, but questions arose which need answering regarding the different environments of the solid and liquid media.

8.2.3 Antibacterial activity of extracts from selected macroalgae from KwaZulu-Natal, South Africa

The extract made from *O. serrata* was the most active of the macroalgae tested. However, the others did show some activity, i.e. there were no cases of absolutely no activity. Therefore, all seaweeds seem to produce some antibiotic substances, and it would be interesting to isolate and identify the other antibacterial products. There is much work to be done in this regard as there are over four hundred species of seaweeds on the KwaZulu-Natal coast alone. This aim was successfully met, but further investigation is required

8.2.4 Deformities induced in bacteria by macroalgal extracts

There were morphological deformities in the bacteria in response to the extract from *O. serrata*. This confirms a previous study with fungi. Increased capsule production and blebbing of the outer membranes were observed. However, some bacteria (i.e. *Enterobacter cloacae* and *Escherichia coli*) showed no morphological distortions even though their growth rates were negatively affected by the extract. It is unknown why this should have been and further investigation is required. There was again success in this aim and the negative staining method was considered good to use for

viewing general morphological changes in bacteria in response to toxicants. The method was considered superior to the SEM method because, during sample preparation for the latter, flagella and capsules are most often lost during the dehydration steps.

8.2.5 Isolation and antimicrobial activity of the ethyl ether derivative of lanosol, from *Osmundaria serrata* (Rhodophyta)

The active compound was successfully isolated and identified. It was the ethyl ether derivative of lanosol. Lanosol is found naturally in the seaweed, but lanosol ethyl ether (lanosol^{ee}) may be an artefact from using ethanol in the extraction (Weinstein *et al.*, 1975). However, other workers have found lanosol and its derivatives to have similar biological activities. Lanosol is found in a diversity of macroalgae, but its production is concentrated mostly in the Rhodophyta. It is not known whether other members of the genus *Osmundaria* also produce it. The biological activities of lanosol and lanosol^{ee} are similar (Kurata *et al.*, 1997; Glombitza *et al.*, 1974).

Lanosol^{ee} inhibited the test bacteria with an average MIC of 0.27 mg.ml^{-1}. This was significantly higher than that of copper sulphate with 0.17 mg.ml^{-1}. Interestingly the 'consortium' of marine bacteria that was included in the bioassays was consistently more resistant to lanosol^{ee} and the seaweed extracts than the individual bacteria. These results were consistent with the finds that the bacteria in biofilms are more resistant than their planktonic forms. It is thought that the extracellular polymeric substance produced by the biofilm protects them.

8.2.6 A seaweed is more than the sum of its parts: SEM visualisation of biofilms on some seaweeds from KwaZulu-Natal, South Africa

Three different general groups of biofilms were observed.

1. The diatom: No cocci were seen here, only rod and filamentous bacteria.
2. The calcified reds: Little biofilm covering except where the segments of the thalli joined. It is suspected that the macroalgae release nutrients at these points because calcification is thinnest here to allow for movement of the macroalgae. Rigid structures would break due to wave action
3. The non-calcified reds and green algae showed a complex biofilm community on their surfaces. There was even evidence of grazing of the bacteria. There were also differences between the seaweeds and it would be interesting to determine whether different seaweeds have a unique biofilm with respect to species composition.

The treatment with OsO_4 vapour before fixation in glutaraldehyde preserved the biofilm structure well. Therefore it is recommended that OsO4 vapour be used in processing samples for SEM viewing and confirms the findings of other workers. These results suggest that lipids are important structural components of extracellular polymeric substance that surrounds biofilms.

The SEM technique had limits. One of these was the inability to identify any of the bacteria because of inadequate information. Confocal scanning laser microscope used in combination with various probes would give a better understanding of the biofilm in general.

A complex biofilm community was seen on the surface of *O. serrata*. This indicated that lanosol was unlikely to have functioned as an anti-fouling agent as other chemicals, e.g. lactones, do in other seaweeds. It is more likely that lanosol functions as an antimicrobial agent that protects the alga against infection. Since other workers have found it to deter the feeding of herbivores it has more than one function in seaweed.

This thesis is a drop in the ocean of scientific knowledge. Much is not known of ecosystems, especially those in the marine environment. However, our awareness and understanding of the universe is broadened by all work done with organisms that have never been investigated before.

8.3 REFERENCES

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