ANTIMICROBIAL ACTIVITY OF MACROALGAE FROM KWAZULU-NATAL, SOUTH AFRICA, AND THE ISOLATION OF A BIOACTIVE COMPOUND FROM *OSMUNDARIA SERRATA* (RHODOPHYTA)

by
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Abstract

The rhodophytes or red seaweeds are an ancient group of organisms that are related to plants. Like terrestrial plants, they use secondary compounds to protect themselves from microbial infection and grazing by herbivores. However, unlike terrestrial plants, they produce mostly halogenated secondary compounds and rarely alkaloids. *Osmundaria serrata* (Rhodophyta) is found along the eastern South African coast and the Maldive Islands. Its descriptive common name is "red spirals" and the species is adapted to live in habitats with high wave action. Extracts from this seaweed had previously shown to have antimicrobial activity, but ecologically irrelevant microbes were used to test the extracts. In this study, ten bacteria were isolated from the surface of *O. serrata* and its habitat, and identified. Mostly aerobic and Gram-negative bacteria were isolated (*Halomonas* and *Pseudomonas* species) along with facultatively anaerobic forms (*Vibrio* spp.) and a Gram-positive (*Marinococcus* sp.). These were used in bioassays to compare the activity of extracts made from *O. serrata* and other seaweeds that occur in the same habitat. Marine bacteria are the initial colonisers in biofilm formation and subsequent fouling of surfaces in marine environments. The study of these bacteria in relation to their macroalgal hosts may help to control biofouling of surfaces that cause economic losses worldwide.

A comparison was made between using agar dilution and microtitre methods for testing the antibacterial activity of an *O. serrata* extract. The microtitre method was found to be more sensitive than the agar dilution method. Possibly because some of the bacteria on the petri plates (in the agar dilution method) were not in direct contact with the toxicant in the growth medium, but were in direct contact in the liquid medium of the wells in the microtitre plates.

The extract from *O. serrata* was the most active of the thirteen species of macroalgae collected from the same habitat and tested for antibacterial activity. Deformities in bacteria were observed in response to the *O. serrata* extract. Increased capsule production and blebbing of the outer membranes were observed by transmission electron microscopy (negative staining).

Lanosol diethyl ether was isolated from *O. serrata* and tested for antibacterial activity. Lanosol is produced mainly by the rhodophytes, but it is also found in other macroalgae and fungi in lower concentrations. The compound inhibited the test bacteria with average MIC's of 0.27 ± 0.07 mg.ml^{-1} (bacteriostatic) and 0.69 ± 0.15 mg.ml^{-1} (bactericidal).

Different forms of biofilm were observed by scanning electron microscopy on the thirteen species of macroalgae. These ranged from a very little biofilm covering on the calcified reds to complex communities on the other macroalgae. The treatment with OsO_4 vapour before fixation in glutaraldehyde preserved the biofilm structure better than no treatment and indicated that lipids are important in maintaining biofilm structure.

Since a complex biofilm community was seen on the surface of *O. serrata*, it is unlikely that lanosol functions as an antifouling agent. This chemical seems to multifunctional with antimicrobial and feeding deterrent activities.

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Dedication

This thesis is dedicated to **Mrs. Swanepoel-Engelbrecht**,
my high school biology teacher, who instilled a love for the study of life in me.

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Publications

The following articles are in preparation for publication based on the research derived from this study:

1. Barreto, M. and J.J.M. Meyer, 2003. The antibacterial activity of extracts from selected macroalgae from KwaZulu-Natal, South Africa. [Incorporating Chapters 2, 4 and 5.]
2. Barreto, M. and J.J.M. Meyer, 2003. Isolation and antimicrobial activity of the ethyl ether derivative of lanosol, from Osmundaria serrata (Rhodophyta) [Chapter 6]
3. Barreto, M. and J.J.M. Meyer, 2003. A seaweed is more than the sum of its parts: SEM visualisation of biofilms on some seaweeds from KwaZulu-Natal, South Africa [Chapter 7]

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CHAPTER ONE

INTRODUCTION

The diversity of life along the South African coastline is remarkable. Along the western coast of the country the upwelling caused by the cold Benguela current supports an ecosystem that consists of large biomasses of seaweeds, seals, penguins and fish (Warne, 2002). The warm Agulhas current on the east coast supports a smaller biomass of organisms, but the species diversity is greater than that of the west coast. Algae are the primary producers in marine ecosystems and support communities of fish, turtles, dolphins and humans (Branch *et al.*, 1994).

There may not be as many species of macroalgae as there are land plants, but the long evolutionary history (1.3 to 2 billion years) of the seaweeds makes them fascinating (Woelkerling, 1990). The ancestors of the almost 4500 species of macroalgae living today survived four major calamities when mass extinctions occurred about 435, 370, 240 and 65 million years ago (Woelkerling, 1990; Miller, 1992). Up to 90% of marine life became extinct in some of these mass extinctions, but the present and ever-changing species composition is thought to have reached this point in the last 24 million years (Hommersand, 1986; Stanley, 1989).

The Rhodophyta, or red algae, are an unusual group of organisms because, besides being mostly red, the more than 2500 species do not produce flagella as all other eukaryotes do (Woelkerling, 1990). From mitochondria-encoded protein sequences it has relatively recently been found that they are related to land plants and are considered to be members of the plant kingdom, Plantae (Baldauf *et al.*, 2000).

However, unlike land plants, their secondary metabolites are often halogenated due to the abundance of halogens in seawater, but they rarely produce alkaloids because the amount of nitrogen in the oceans is usually limited (Hay and Fenical, 1992; Paul, 1992a). Nitrogen concentrations are higher near offshore sewage outlets.

Remnants of macroalgae, *Gracilaria* sp. (Rhodophyta) and *Durvillaea antarctica* (Heterokontophyta, Phaeophyceae or brown alga), have been discovered in a 12,500 year old archaeological site in Monte Verde, Chile, and were part of a chewing wad with land plants with analgesic and hallucinogenic activity (Gove, 1997). They were preserved by peat. What is most surprising is that *D. antarctica* is still used by Chileans, and *Gracilaria* species by Hawaiians, for food (Abbott, 1996). Other seaweeds are also eaten, especially in the East, and have important economic value.

1.1 SEAWEED NATURAL PRODUCTS

Seaweeds form a major part of the Japanese diet, but members of the Phaeophyceae are favoured above those of the Rhodophyta for food purposes. This may indicate traditional knowledge that many rhodophytes produce toxic secondary metabolites (Fenical, 1975). Other macroalgal compounds have growth promoting activity and are found in products that are used in the agricultural and horticultural industries. For example, Kelpak 66 (Kelp Products Ltd., Simon's Town, South Africa) is an extract from the kelp *Ecklonia maxima* (Phaeophyceae) and contains auxins, cytokinins and 1-aminocyclopropane-1-carboxylic acid as active constituents.

Compounds with growth inhibitory activities (figure 1.1) are probably used as defences against grazers, epiphytes and pathogens (Hay *et al.*, 1988; Jones, 1988;

Pesandro, 1989; Hay and Fenical, 1992; Lobban and Harrison, 1994). Many of these inhibitory compounds contain the halogens chlorine, bromine and iodine, which are toxic to many organisms (Fenical, 1975; Mehendale, 1992). Bromine is commonly found in members of the Rhodophyta as part of a wide variety of compounds ranging from relatively simple phenols and ketones, to more complex terpenes and fatty acids (Fenical, 1975; Dembitsky and Srebnik, 2002).

The brown seaweeds, or members of the Phaeophyceae, also produce halogenated compounds. However, this group of seaweeds most commonly produces polyphenolic compounds as defensive secondary compounds (Steinberg, 1992). The phenolic compounds released by red and brown seaweeds influence the life cycles of green seaweeds, Chlorophyta (Jones, 1988). This suggests complex chemical interactions between marine organisms.

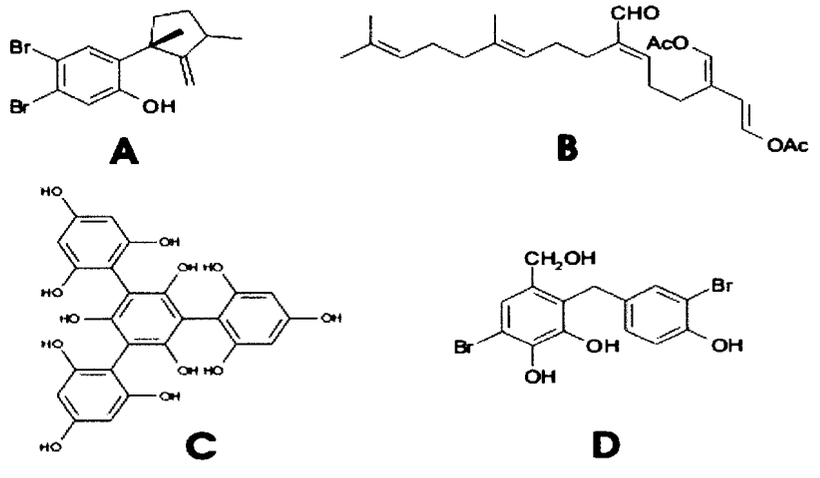

Figure 1.1 The structures of some compounds produced by macroalgae with growth inhibitory activities (Fenical, 1975; Hay, 1992; Paul, 1992b; Steinberg, 1992). **A** = isolaurinterol from *Laurencia intermedia* (Rhodophyta); **B** = Udoteal from *Udotea* spp. (Chlorophyta); **C** = tetrafucol B from *Fucus vesiculosus* (Pheophyceae); **D** = avrainvilleol from *Avrainvillea longicaulis* (Chlorophyta).

1.2 GENERAL DESCRIPTION OF *OSMUNDARIA SERRATA*

The macroalga, *O. serrata* (figure 1.2) is the largest species (up to 40 cm long) of the genus *Osmundaria* (≡ *Vidalia*) that occurs along the South African coast, the others being *O. oliveae* and *O. papenfussii* (Norris, 1991). The genus *Osmundaria* consists of eight species and are all distributed in the tropical, sub-tropical and warm temperate oceans of the World. *O. serrata* has been found along the eastern South African coast and also off the Maldive Islands (Norris, 1991). They have been collected from depths of 20 m and presumably occur at greater depths since other rhodophytes grow to depths of 200 m.

Figure 1.2 A complete specimen of *Osmundaria serrata*.

The common name of *O. serrata* is 'Red Spirals' (Branch *et al.*, 1994), named after the spiralled blades that form a right hand helix to the tips (figure 1.3). The blades are dark-red to brown with the tips becoming lighter. The edges of younger tissue are serrated with four spikes per turn of the helix (figure 1.3). Norris (1991) described

the blades as being dentate rather than serrate. The serrations are ground down by wave action where suspended sand particles act as sand paper. In older parts the serrations are completely missing and the edges are smooth (figure 1.4). These older parts are thickened due to secondary cortication, which forms a tough "woody" holdfast at the base. This cortication is thought to be seasonal and is up to three layers thick in mature branches, but many more in the holdfast (Norris, 1991). This implies that branches are regenerated after three years.

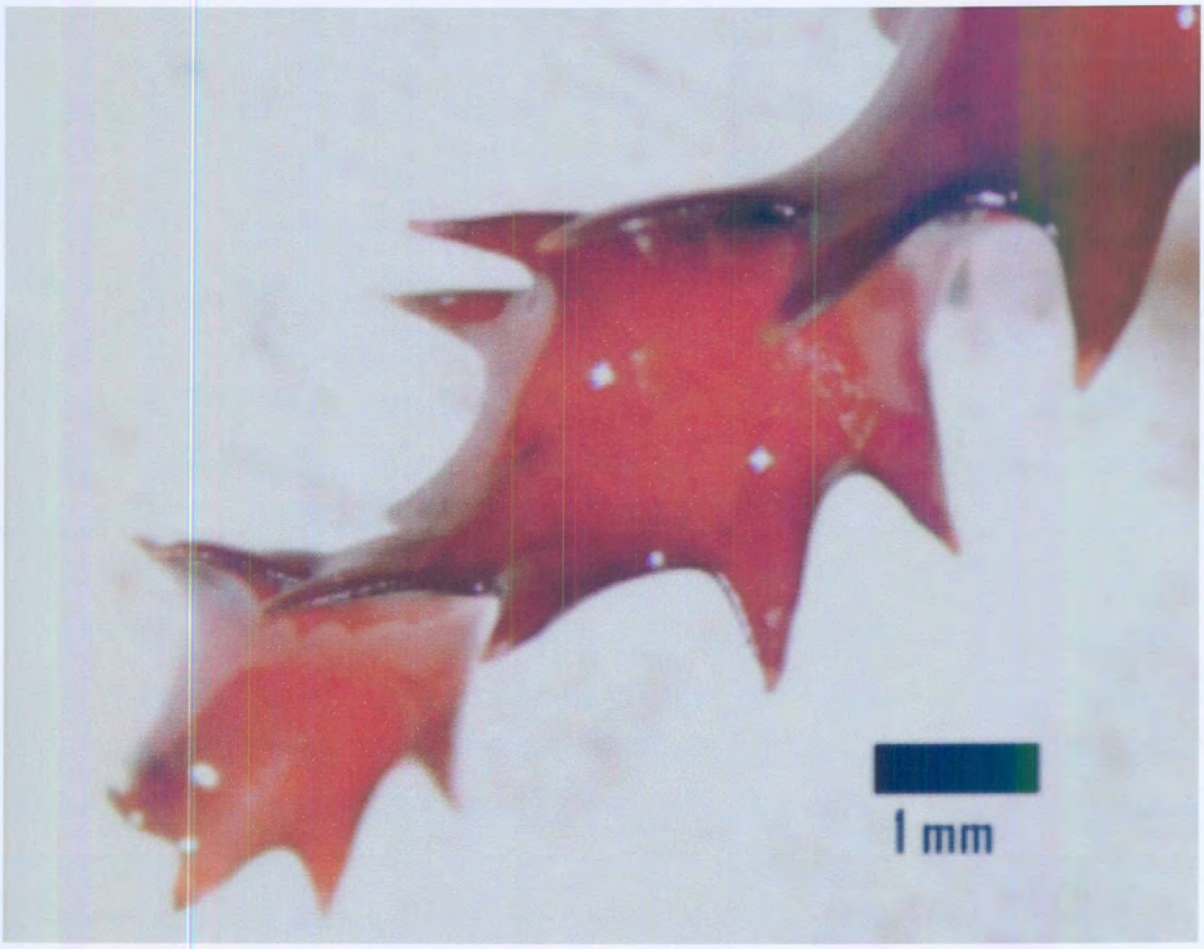

Figure 1.3 The spiralled and serrated blade of *Osmundaria serrata*. The lighter coloured tip is to the bottom left.

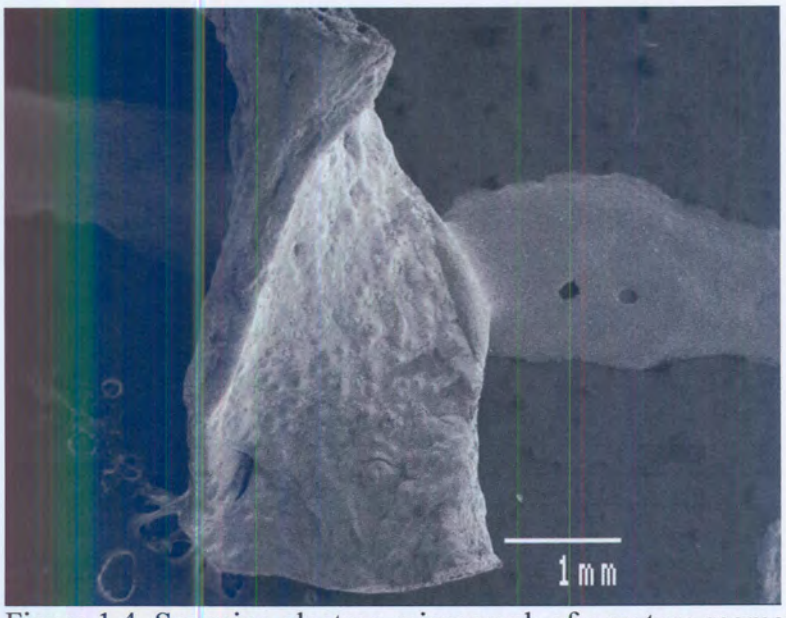

Figure 1.4 Scanning electron micrograph of a mature segment of *Osmundaria serrata* blade with no serrations and a thickened thallus.

The branches come off the edge of the blade and rarely off the blade's surface. Reproductive structures (stichidia which produce tetraspores - figure 1.5) were observed in winter and have a patchy distribution along the blade. These were not observed on older branches, but sometimes on younger ones.

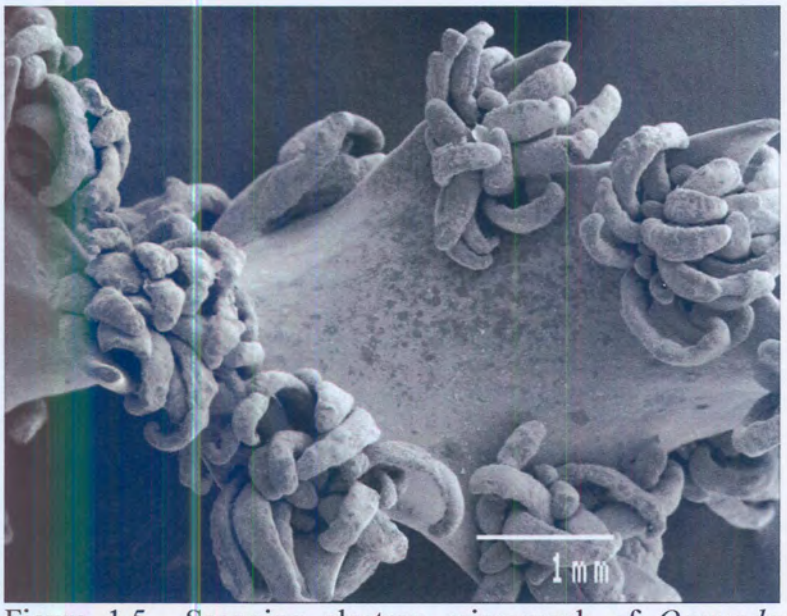

Figure 1.5 Scanning electron micrograph of *Osmundaria serrata* showing the tetraspores producing stichidia found along the edge of the blade.

1.3 ECOLOGY OF *O. SERRATA*

This macroalga occurs mostly in sub-tidal habitats (Norris, 1991). The edges of populations may be seen at low spring tides where outgoing waves briefly expose the plants (figure 1.6). Plants exposed for too long bleach to an orange colour (similar to material that has been extracted by ethanol and ethyl acetate). The population at Palm Beach, KwaZulu-Natal, South Africa, occurs in rocky gulleys in the very low inter-tidal region.

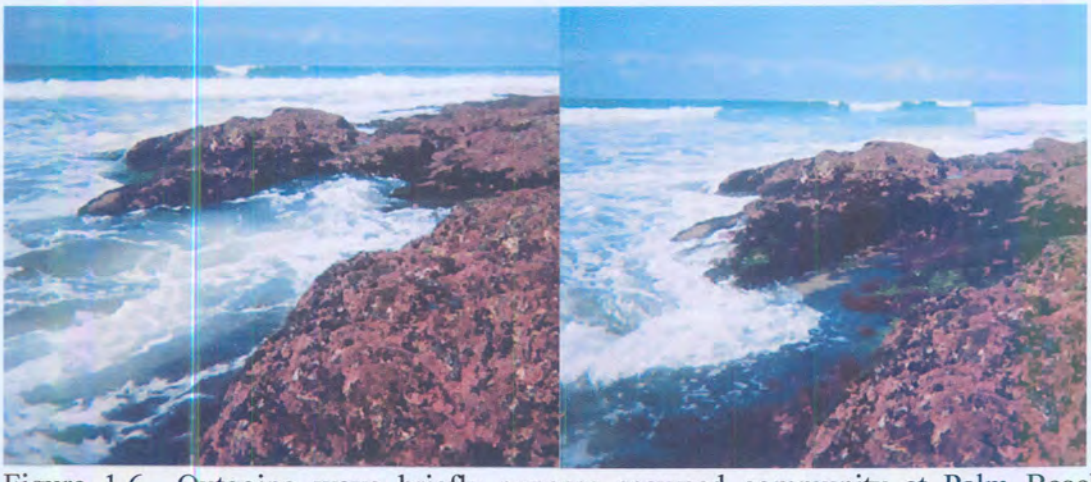

Figure 1.6 Outgoing wave briefly exposes seaweed community at Palm Beach, KwaZulu-Natal, South Africa during a Spring tide low.

The whole plant is tough, difficult to break, and well adapted to growing on rocky shores where strong wave action would destroy a more fragile thallus. Some of the other seaweeds that share its habitat flow with the waves as slimy and thin strap-like branches, e.g. *Caulerpa filiformis* of the Chlorophyta (figure 1.7). Others produce hard-calcified branches, e.g. the Corallines, *Amphiroa bowerbankii, A. ephedraea* and *Cheilosporum multifidum* (Rhodophyta). While others, e.g. *Hypnea spicifera* (Rhodophyta), have strong gelatinous thalli that are also adapted to being exposed to the waves.

Figure 1.7 Seaweed community growing on the rocky shores of Palm Beach, KwaZulu-Natal, South Africa. **A** = *Hypnea spicifera*, **B** = Corallines; *Amphiroa bowerbankii, A. ephedraea* and *Cheilosporum multifidum*, **C** = *Caulerpa filiformis*, **D** = *Osmundaria serrata*.

Some of the animals that occur along the eastern South African coast that may be important to the ecology of the macroalgae include various herbivorous fish, crabs, amphipods and isopods (Branch *et al*., 1994). Grazing pressure in marine habitats is intense and macroalgae have evolved various methods of defence. Some have calcified cell walls that make them unpalatable, while others produce toxic secondary metabolites (Hay, 1996; Steinberg *et al*., 1997).

1.4 OBJECTIVES

Some *Osmundaria* species are unpalatable to fish. *O. colensoi* from New Zealand is digested more slowly in the presence of fish digestive enzymes, and has been found to have a lower starch content than other palatable macroalgal species (Zemke-White and Clements, 1999). However, there may be other reasons that species of

Osmundaria are non-dietary to herbivores because many other plants produce anti-feeding chemicals for protection.

The main aim of this work was to investigate the ecological chemistry of *O. serrata* because its crude extracts had previously shown antibacterial (Vlachos *et al.*, 1997) and antifungal activity (Barreto *et al.*, 1997). However, these studies used ecologically irrelevant bacteria and fungi to test the extracts. Thus, bacteria were isolated from the surface of *O. serrata* and identified in this study (chapter 2). These bacteria (along with terrestrial forms for comparison) were then used in biological assays to determine whether using the agar dilution or microtitre method was the best to test extracts from *O. serrata* (chapter 3). Twelve other seaweeds growing in the same habitat as *O. serrata* were sampled and their extracts also tested for antibacterial activity (chapter 4). It had previously been noticed that extracts from this seaweed causes abnormalities in fungi (Barreto, 1995) and it was therefore decided to investigate if bacteria were similarly affected (chapter 5).

The extract from *O. serrata* had the highest antibacterial activity and an active compound was isolated from it. This compound was a lanosol derivative and the pure compound was tested against the growth of the same bacteria used in the other bioassays (chapter 6). In chapter 7 the surfaces of the thirteen seaweeds were examined under a scanning electron microscope to determine if there was a correlation between surface bacterial covering and biological activity of the seaweed. Although no visual correlation was found, a rich and diverse microbiological component to the ecosystem was observed. The thesis ends with a general discussion (chapter 8) and an appendix introducing the animations in the CD-ROM.

1.5 AIMS

The aims of this study were to:

1. Isolate and identify some epiphytic bacteria from *O. serrata*.
2. Compare the minimum inhibitory concentrations of the agar dilution and the microtitre methods of determining the antibacterial activity of an extract from *O. serrata*.
3. Investigate the biological activity of extracts made from macroalgae that grow in the same habitat as *O. serrata*.
4. Determine if the morphologies of bacteria are affected by an extract from *O. serrata*.
5. Isolate and identify the active compound(s) from *O. serrata*.
6. Determine the biological activity of the active compound(s) against the growth of ecologically relevant and other bacteria.
7. Explore the surfaces of the seaweed tested (aim 3) to determine whether there was a visual correlation between biofilm covering and biological activity.

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CHAPTER TWO

IDENTITIES OF SOME BACTERIA ISOLATED FROM THE SURFACE OF THE MACROALGA *OSMUNDARIA SERRATA* (RHODOPHYTA) AND ITS HABITAT

2.1 ABSTRACT

This chapter describes the isolation and identification of ten bacteria from the surface of *Osmundaria serrata* (Rhodophyta) and its habitat. Ninety percent of the bacteria isolated were Gram-negative, with most of these being non-fermentative. The representative genera were *Halomonas*, *Pseudomonas*, *Marinococcus* and *Vibrio*. Some species of these are biofilm formers and it was an aim of this study to isolate and identify bacteria to test for the biological activity of an extract and pure compound from *O. serrata*.

2.2 INTRODUCTION

Bacteria, the smallest cells in the oceans, are indirectly responsible for polluting harbours and shipping routes. Paints containing metallic compounds, mostly copper and tin, are painted onto the hulls of ships to prevent bacteria from colonising submerged surfaces to form biofilms. Mature biofilms cause significant economic losses due to corrosion and increasing fuel costs for shipping because of increased friction between the ship and water (Ponasik *et al.*, 1998; Armstrong *et al.*, 2000). Unfortunately, the metals biomagnify and bioaccumulate after they leach from the

paint into the marine environment causing severe ecological damage (Hellio *et al.*, 2001). Antifouling marine biochemicals are currently being investigated to replace these toxic paints.

There is some disagreement on whether there is a difference between bacterial species found in seawater and in biofilms on macroalgae. Kong and Chan (1979) did not detect bacteria from genera commonly found in seawater, e.g. *Aeromonas*, *Alcaligenes* and *Vibrio* [≡*Beneckea*], from the seaweeds that they studied in Hong Kong. Shiba and Taga (1980) found that members of the *Vibrionaceae* family, in general, were poorly represented in their study of bacteria on seaweeds from the Japanese coast. However, other workers (Jaffray *et al.*, 1997) have isolated *Aeromonas*, *Alcaligenes* and *Vibrio* species from the red alga *Gracilaria gracilus* from the West coast of southern Africa.

There is a current trend in using ecologically appropriate bacteria to test the biological activity of marine biochemicals (Hellio *et al.*, 2001). Bacteria occurring in biofilms are found on marine macroalgae and their use in bioassays of macroalgal products would be appropriate. In this chapter we describe the isolation and identification of some of the bacteria growing on *O. serrata* (Rhodophyta), from the East coast of South Africa.

2.3 MATERIALS AND METHODS

O. serrata samples were collected from Palm Beach (30° 59' 30" S, 30° 16' 30" E), KwaZulu-Natal, South Africa, during the full moon spring tide of September 2001 and rinsed with sterile seawater to remove unattached organisms. One centimetre segments of the seaweed and surface scrapings with a sterile needle were placed on growth medium consisting of 5 g peptone, 1 g yeast extract and 15 g agar per 1 litre seawater (pH adjusted to 7.6 before autoclaving).

Colonies were separated by repeated streaking and isolated on the basis of morphology and microscopic observation of Gram-stained samples. Cultures were stored in 50 % glycerol at - 20 °C and in agar slants under sterile mineral oil at 0 °C. All bacteria were grown at 23 ± 2 °C with light-dark cycle of 16:8 hours. Seawater used for culturing was obtained from the study area, aged for a week, filtered through Whatman's membrane filter (0.45µm pore size) and autoclaved. To test for sodium requirements the isolates were grown in medium made with distilled water instead of seawater.

Motility was determined by the using semi-solid agar growth medium (Tittsler and Sandholzer, 1936). The characteristics of the flagellum, cell morphology and size were determined by transmission electron microscope. Twenty four hour old colonies were suspended in a drop of seawater onto which copper grids were floated for 30 seconds. The grids were then blotted and floated on top of drops of uranyl acetate for 30 seconds. The grids were blotted again and dried for 10 min before being viewed under a Philips 301 TEM. Gram stains of the bacteria were made according to the method of Clark (1984).

Baumann and Baumann's (1981) methods were used to determine the ability of the isolates to ferment glucose, the presence of cytochrome oxidase, starch hydrolysis, catalase and lipase activity.

Further physiological tests were performed with the API 20 E and NE system of bioMérieux (Lyon, France) for fermentative and non-fermentative isolates respectively. Twenty four hour old cultures were suspended in sterile seawater and used in the test strips.

Bacterial strains were identified according to the ninth edition of *Bergey's Manual of Determinative Bacteriology* (Holt *et al.*, 1994). Nomenclature was confirmed according to the DSMZ-German Collection of Microorganisms and Cell Cultures website (*http://www.gbf.de/dsmz/bactnom/bactname.htm*).

2.4 RESULTS AND DISCUSSION

2.4.1 Generic Identification

Most of the ten strains chosen for identification were Gram-negative and strictly aerobic rod shaped cells (table 2.1). Four of these were identified as *Halomonas* species on the basis of their peritrichous flagellation and requirement of seawater for growth (Holt *et al.*, 1994). Some *Halomonas* species used to be known as *Deleya* and *Alcaligenes* until Dobson and Franzmann (1996) unified the genera into *Halomonas* on the basis of 16S rRNA data.

Three isolates were strictly aerobic with polar flagellation, required seawater for growth and were designated as a *Pseudomonas* species. They were able to utilise DL-malate and were thus differentiated from *Marinomonas* and *Pseudoalteromonas* [≡*Alteromonas*] (Holt *et al.*, 1994).

The two strains that were able to ferment glucose, required seawater for growth, and showed polar flagellation and were identified as members of the *Vibrionaceae*; either *Photobacterium* or *Vibrio* species. Even though one exhibited bioluminescence, they both differed from *Photobacterium* species because of their ability to utilise D-mannitol, and were therefore identified as *Vibrio* species (Holt *et al.*, 1994).

The remaining Gram-positive and non-motile isolate was identified as a *Marinococcus* species (table 2.2; figure 2.1) because it was aerobic, with a yellow-orange nondiffusing pigment, positive for catalase and negative for oxidase, non-motile and was isolated from the marine environment (Holt *et al.*, 1994). It was differentiated from *Planococcus* because of its lack of flagella.

Table 2.1 Characteristics of the strictly aerobic bacteria isolated from *Osmundaria serrata* (Rhodophyta).

Characteristic	*Halomonas halophila*	*Halomonas* sp. 1	*Halomonas* sp. 2	*Halomonas* sp. 3	*Halomonas marina*	*Pseudomonas* sp. 1	*Pseudomonas* sp. 2
Colonies	Light cream, iridescent, convex with lobate margins	Cream, iridescent, convex with entire margins	Clear, ingrowing into medium with entire margins	Beige, iridescent, raised with undulate margins	Cream, iridescent, convex with entire margins	Beige, iridescent, convex with entire margins	Cream, convex with entire margins
Gram reaction	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Cell shape	Rods	Rods	Rods	Rods	Rods	Cocci-bacillus	Rods
Cell size	0.8 x 1.3 – 2.0	0.6 x 0.6 - 1.3	0.7 x 1.7 - 2.8	1.0 x 2.0	0.9 x 1.7 - 2.2	01.2 x 1.2 - 1.8	0.5 x 1.0 - 1.6
Flaggelation	Peritrichous	Peritrichous	Peritrichous	Peritrichous	One polar	One polar	One polar
Capsule	Not seen	Yes	Not seen	Yes	Yes	Not seen	Yes
Cytochrome oxidase	+	+	+	-	-	+	+
Catalase	+	+	+	+	+	+	+
Amylase	+	-	-	+	-	-	-
Lipase	+	+	-	+	-	-	-
NO3 to NO2	+	+	-	-	-	+	-
NO3 to N2	-	-	-	-	-	-	-
Gelatinase	-	-	+	-	-	-	+
Acid from glucose	+	+	+	-	-	-	-
Indole production	-	-	-	-	-	-	-
Arginine dihydrolase	-	-	-	-	-	-	-
Urease	-	-	-	-	-	-	-
Beta-glucosidase	+	+	+	-	-	-	+
Beta-galactosidase	+	-	+	-	-	-	-
Glucose assimilation	+	+	+	+ Weak	+	+	-
Arabinose assimilation	+	+	-	-	Weak	Weak	+
Mannose assimilation	-	-	-	-	Weak	Weak	-
Mannitol assimilation	+	+	+	-	+	Weak	-
N-acetyl-glucosamine assimilation	+	+	+	-	Weak	Weak	+
Maltose assimilation	+	-	+	-	+	Weak	+
Gluconate assimilation	+	+	+	+	+	+	-
Caprate assimilation	-	-	-	-	+	-	-
Adipate assimilation	+	+	-	+	Weak	-	-
Malate assimilation	+	-	-	+	+	+	+
Citrate assimilation	+	-	-	+	+	Weak	-
Phenyl-acetate assimilation	+	-	-	-	-	Weak	-
Synonym(s)	*Deleya halophila*	*Deleya* sp.	*Deleya* sp.	*Deleya* sp.	*Pseudomonas marina; Deleya marina*		

2.4.2 Specific Identification

One of the *Halomonas* isolates was most similar to *H. halophila* (Vreeland *et al.*) Dobson and Fransmann (figure 2.1). *Halomonas* sp. 1 (figure 2.1) was very similar to this isolate, but differed from it in being negative for the assimilation of citrate, maltose, malate, phenyl-acetate and the presence of β–galactosidase (Holt *et al.*, 1994). *Halomonas* sp. 2 (figure 2.1) was similar to *H. pacifica*, but differed from it by being able to utilise maltose and mannitol. *Halomonas* sp. 3 (figure 2.1) was also similar to *H. pacifica*, but did not show cytochrome oxidase activity. However, these two isolates differed from each other in eleven phenotypic traits (table 2.1).

There were two strains similar to *Pseudomonas nautica* and this species has recently been transferred to *Marinobacter hydrocarbonaclasticus* (Spröer *et al.*, 1998). The isolates were designated *Pseudomonas* strains 1 and 2 (figure 2.1) and differed from each other in nine traits. The traits of the other *Pseudomonas* isolated were identical to those of *P. marina* (figure 2.1) (Krieg and Holt, 1984). This species was reclassified as a *Deleya* species (≡ *D. marina*) and most recently as a *Halomonas* species (≡ *H. marina* (Cobet *et al.*) Dobson and Fransman) (Holt *et al.*, 1994; Dobson and Franzmann, 1996). This bacterium is commonly found on seaweed surfaces and was isolated from other seaweeds by Kong and Chan (1979) in China. These seaweeds included: *Polysiphonia lanosa* (from which the secondary metabolite lanosol gets its name) is in the same family (Rhodomelaceae) as *O. serrata*; *Hypnea charoides*, which is a member of the Gigartinales of the Rhodophyta, while *O. serrata* is in a different order, the Ceramiales; and *Ulva lacuta*, which is a member of the *Chlorophyceae* and is more related to higher plants than to *O. serrata.*

Table 2.2 Characteristics of the facultatively anaerobic and Gram-positive bacteria isolated from *Osmundaria serrata* and its habitat.

Characteristic	*Vibrio harveyi*	*Vibrio alginolyticus*	*Marinococcus* sp.
Origin	Beach sand	Surface of *O. serrata*	Surface of *O. serrata*
Colonies	Beige, convex colonies with entire margins	Cream, iridescent, round to oval, raised with entire margins, swarming	Yellow-orange, convex with entire margins
Gram reaction	Negative	Negative	Positive
Cell shape	Rods	Rods	Cocci
Cell size	0.8 x 1.7 - 2.3	0.6 x 1.3 -2.3	0.5 – 0.8
Flaggelation	Two to several polar	One to few polar	None
Capsule			Yes
Cytochrome oxidase	+	+	-
Catalase	+	Weak	+
Amylase	+	+	-
Lipase	+	+	-
NO3 to NO2	+	+	+
NO3 to N2	-	-	-
Gelatinase	+	+	+
Acid from glucose	+	+	-
Indole production	+	+	-
Arginine dihydrolase	-	-	-
Urease	-	-	-
Beta-galactosidase	+	-	-
Glucose assimilation	+	+	-
Arabinose assimilation	-	-	-
Mannitol assimilation	+	+	-
Citrate assimilation	+	+	-
Lysine decarboxylase	+	+	-
Ornithine decarboxylase	-	+	-
H_2S production	-	-	-
Tryptophane deaminase	-	-	-
Acetoin production	-	-	-
Glucose fermentation	+	+	-
Mannitol fermentation	+	+	-
Inositol fermentation	-	-	-
Sorbitol fermentation	-	-	-
Rhamnose fermentation	-	-	-
Sucrose fermentation	-	-	-
Melibiose fermentation	-	-	-
Amyglalin fermentation	+	+	-
Arabinose fermentation	-	-	-
Synonym(s)	*Beneckea harveyi; Lucibacterium harveyi, Vibrio carchariae; V. trachuri*	*Beneckea alginolyticus*	

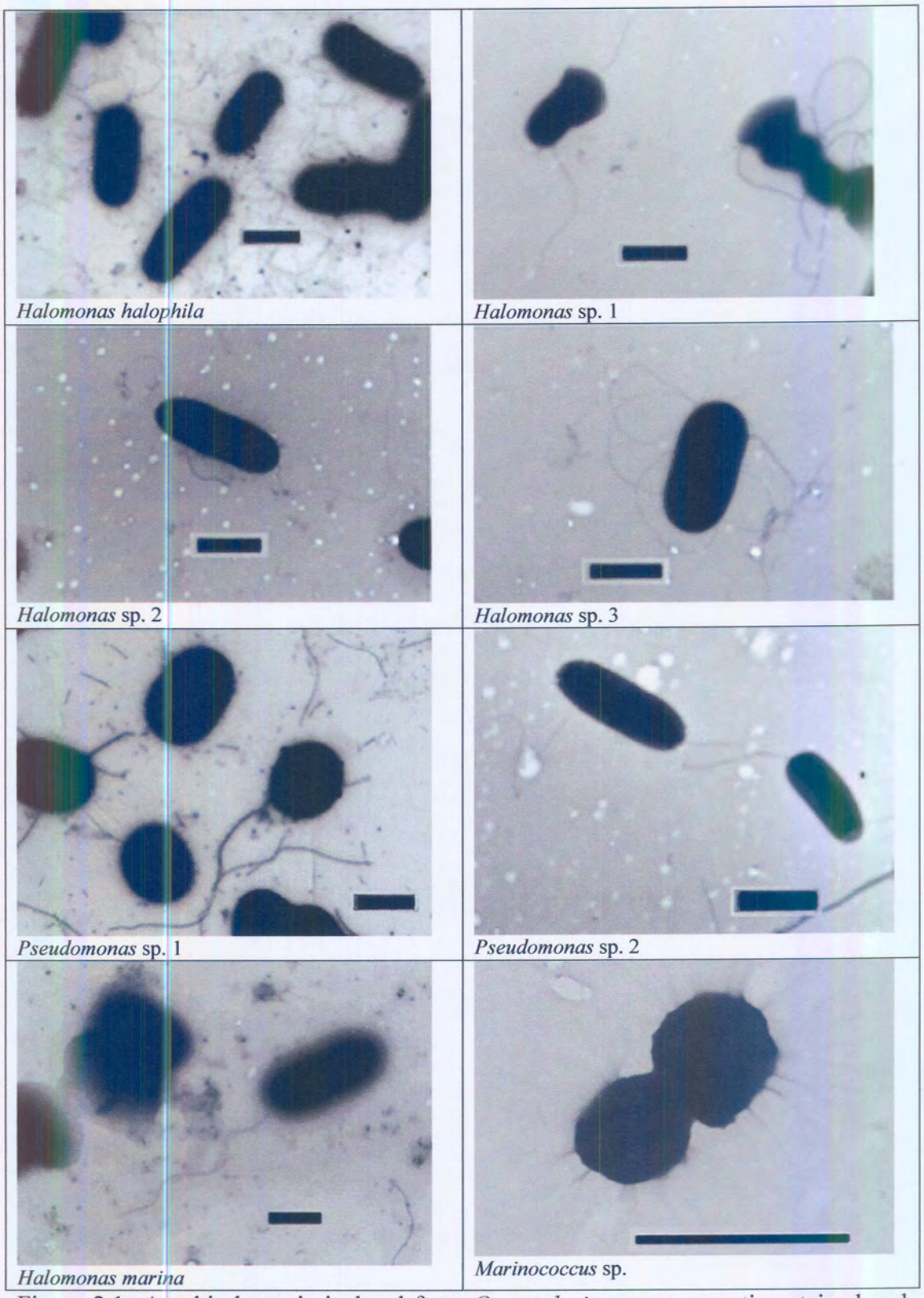
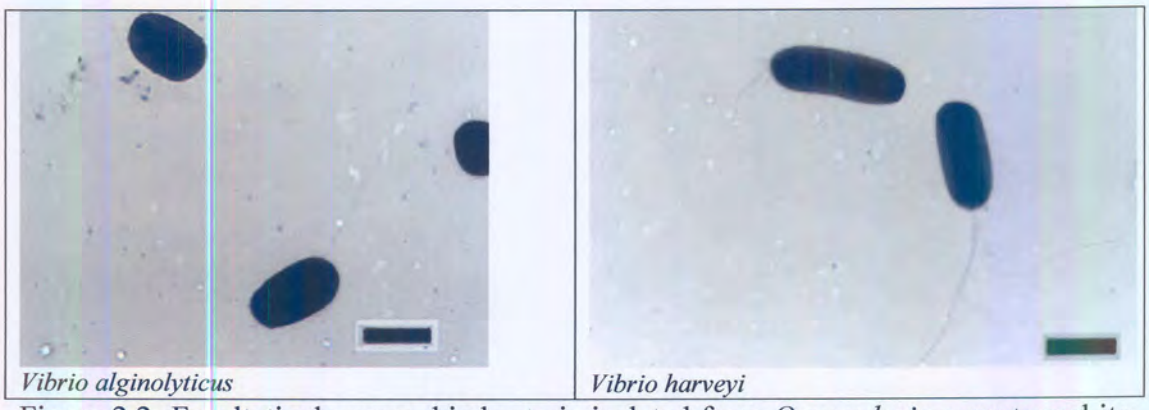


Figure 2.1 Aerobic bacteria isolated from *Osmundaria serrata* negative stained and viewed under a transmission electron microscope. Bars = 1 µm.

The *Vibrio* sp. that was isolated from beach sand was bioluminescent and was identified as *V. harveyi* (Johson and Shunk) Baumann *et al.* (figure 2.2). It has economic importance as a shrimp pathogen (Karunasagar *et al.*, 1996). The other *Vibrio* sp. was identified as *V. alginolyticus* (Miyamota) Sakazaki (figure 2.2) by the Biolog 20E system. However, this strain could not utilise caprate as most other *V. alginolyticus* strains do (Holt *et al.*, 1994). Because of a general lack of marine bacteria in the Biolog database, this was the only isolate to be identified using it.

The *Marinococcus* species did not match any of the three species in *Bergey's Manual* (Holt *et al.*, 1994) and was designated *Marinococcus* sp. (figure 2.1).



Vibrio alginolyticus *Vibrio harveyi*

Figure 2.2 Facultatively anaerobic bacteria isolated from *Osmundaria serrata* and its habitat. The cells were negative stained and viewed under a transmission electron microscope. Bars = 1 μm.

2.4.3 General Discussion

The high proportion of Gram-negative, non-fermenting rods isolated from the seaweed is in agreement with other studies (Kong and Chan, 1979; Jaffray *et al.*, 1997). Gram-negative bacteria are most common in marine environments (Baumann and Baumann, 1981). There is no shortage of oxygen on the surface of *O. serrata*

growing on the rocky shore because of wave action and oxygen released from the photosynthetic processes. It is therefore not surprising to find mostly aerobic bacteria associated with these environments.

Interestingly none of the bacteria isolated showed agarolytic activity, but *Halomonas* sp. 2 did grow into the agar medium. It was expected that some of the isolates would degrade agar and thus be potentially pathogenic to the macroalga (Jaffray *et al.*, 1997). Perhaps the method of homogenising seaweed material favours the isolation of agarolytic bacteria more than our methods. Although the name *Vibrio alginolyticus* implies that agar is degraded it is a misnomer because this species does not degrade agar (Holt *et al.*, 1994).

Other studies have shown that *Pseudoalteromonas* species are commonly found in marine habitat associated with eukaryotic hosts (Holmström and Kjelleberg, 1999). However, none were isolated from *O. serrata*. Laycock (1974) found that the numbers of *Vibrio* and *Pseudomonas* species associated with *Laminaria longicruris* (Phaeophyceae) were seasonal and this may explain the disagreement on the bacterial composition of seaweeds.

Species of *Halomonas*, *Pseudomonas*, and *Vibrio* are known to form biofilms (Laycock, 1974; O'Conner and Richardson, 1998; Davies, 2000). In fact, *H. marina* has been found to inhibit the settling of barnacle larvae *in vitro* (O'Conner and Richardson, 1998). Since this bacterium is found growing on *O. serrata* we may speculate that it, and other bacteria, protect the macroalga from epibiotic attachment (Egan *et al.*, 2001). It is likely that the species composition of the biofilm on *O.*

serrata is specifically cultivated by the alga for such protection. However, most of the bacteria in biofilms have never been cultured (Marshall, 2000).

Luna and co-workers (2002) found that most (*ca.* 70%) of the bacteria in marine sediments are either dead or dormant (in a non-culturable state) and only about five percent are actively growing. Those in a dormant state can be activated when supplied with nutrients. Large proportions of non-culturable bacteria exist in biofilms, but it is unknown what proportions of them occur in the biofilms on macroalgae (Marshall 2000).

We know from direct observations that biofilms are complex communities which respond to environmental cues (Costerton, 2000). An important cue for the biofilms on seaweeds is the state of the host. An actively growing macroalga is different to one in a reproductive state. It is likely that the biofilm community is able to sense the change and respond to it in appropriate ways. One of these may be by altering the composition of non-culturable cells in the community and this would affect the species of bacteria humans are able to isolate. Other phenotypic variations are thought to be due to differing ecological niches and may explain why two isolates can be so different phenotypically, but are identified as the same species (Ivanova *et al.*, 1998). Genetic exploration of the isolates would aid in our understanding of the species composition of the biofilm. However, genetic data gives only one facet of the picture and phenotypic characteristics are still required to give a more holistic view. This is especially important when physiological processes are be altered by genetic manipulation.

2.5 ACKNOWLEDGEMENTS

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CHAPTER THREE

COMPARISON BETWEEN AGAR DILUTION AND MICROTITRE METHODS OF TESTING FOR THE ANTIBACTERIAL ACTIVITY OF AN EXTRACT FROM *OSMUNDARIA SERRATA*

3.1 ABSTRACT

The minimum inhibitory concentration (MIC) of an extract from the red alga *Osmundaria serrata* was determined using two methods:

- The agar dilution technique with solid growth medium.
- The microtitre plate method in liquid medium.

The microtitre method gave lower MIC results (8.4 ± 1.7 mg.ml^{-1}) than the agar dilution method, where 77% of the results had values greater than 25 mg.ml^{-1}. The bacteria in the microtitre plates had more of their surface areas exposed to the toxicant in the growth medium than the bacteria growing in colonies on the agar medium. It is likely that some of the bacteria on the solid media were protected from the toxin because they were not in direct contact with the growth medium. It may be that in the wells of the microtitre plates a synergistic action between the solvent (acetone) and the seaweed toxin produced lower MIC results. With the agar dilution method the petri dishes were left to set and dry during which time most of the solvent had probably evaporated.

3.2 INTRODUCTION

Bioassays are used to determine the minimum inhibitory concentration (MIC) of substances and this information may be used to compare the biological activities of different compounds and their potential efficacy. Assays using petri dishes are familiar, but increasingly microtitre plates are being used. How do these methods compare? Eloff (1999) found the microtitre method to be eight times more sensitive than the agar diffusion method when using extracts from the leaves of *Combretum molle* against the growth of *Staphylococcus aureus*. While Afolayan and Meyer (1997) observed similar MIC values to Eloff's (1997) when testing galangin (an antimicrobial compound from *Helichrysum aureonitens*) against the growth of *S. aureus* even though the different workers used different methods.

In this chapter we compare the agar dilution method using petri dishes and the microtitre method by using an extract from *Osmundaria serrata* (Rhodophyta) that had previously been shown to have antimicrobial activity (Barreto *et al.*, 1997). The method that is most relevant considering the ecology of the test substance is also discussed.

3.3 MATERIALS AND METHODS

O. serrata was collected during a low spring tide in April 2002 from Palm Beach (30° 59' 30" S, 30° 16' 30" E), KwaZulu-Natal, South Africa. A voucher specimen of the macroalga (MB21) was deposited in the HGWJ Schweickerdt Herbarium (PRU) at the University of Pretoria. The fresh material was frozen within an hour of collection and stored in a frozen state before extraction. Eighty grams of frozen material was rinsed with sterile distilled water and homogenized in ethanol-ethyl acetate (1:1). The

material was left to extract on a shaker at 22° C for three days before being filtered through Whatman number 1 filter paper and then evaporated to dryness in a rotary evaporator at 40° C. The fresh extract was dissolved in 50% acetone to the desired concentration for use in the bioassays (Eloff, 1997).

The following potential human pathogenic bacteria were used in the bioassays: *Bacillus cereus* (Gram-positive (G+)), *B. pumilis* (G+), *B. subtilis* (G+), *Enterobacter cloacae* (G+), *Propionibacterium acnes* (G+), *Staphylococcus aureus* (G+), *Enterococcus faecalis* (Gram-negative (G-)), *Escherichia coli* (G-), *Klebsiella pneumoniae* (G-), *Pseudomonas aeruginosa* (G-), *Salmonella typhimurium* (G-), and *Serratia marcescens* (G-). These were grown in nutrient broth or nutrient agar (Biolab, Midrand, South Africa) made to the manufacturer's instructions and the bioassay method used. The following marine bacteria were also used: *Marinococcus* sp. (G+), *Halomonas* spp. 1, 2 and 3 (G-), *H. halophila* (G-), *H. marina* (G-), OssB1 (G-), *Pseudomonas* spp. 1 and 2 (G-), *Vibrio alginolyticus* (G-) and *Vibrio harveyi* (G-). These were grown in a marine growth medium that consisted of 5 g soy peptone, 1 g yeast extract, 15 g agar (for solid media) and 1 litre aged and filtered seawater (Schneider and Rheinheimer, 1988). The pH was adjusted to 7.6 before autoclaving.

For the agar dilution method 1 ml seaweed extract was added to 9 ml molten nutrient agar medium to final concentrations of 25, 12.5, 2.5 and 1.25 mg extract per ml medium. For the control petri dishes a 50% solution of acetone was used. The plates were left to set and dry for about an hour before being inoculated with one-day-old cultures of the bacteria. The plates were then incubated for a day at 23 ± 2 ° C for the

marine bacteria and 37 ± 2 ° C for the potential human pathogens. Three replicate plates were made of each treatment and control.

For the microtitre bioassay, the method of Eloff (1999) was used with modifications. Briefly, 50 µl of the appropriate liquid growth medium was added to the wells in the microtitre plates, after which 50 µl of the extract was added to the first well in the dilution series of 12. For the control 50 % acetone was used in the first well. Dilution proceeded by taking 50 µl from the first well and mixing it in the second well and so continuing until 50 µl was taken from the 12^{th} well and discarded. One day old bacteria in liquid cultures were diluted 1:100 with the appropriate growth medium, and 50 µl of this was added to each well. The concentration of extract in the first well was 25 $mg.ml^{-1}$, this was diluted serially to 0.01 $mg.ml^{-1}$ in the last well. Plates were incubated at appropriate temperatures for a day, as for the agar dilution method, after which 20 µl of tetrazolium salt (0.2 $mg.ml^{-1}$) was added to each well. The plates were then incubated again until a red colour developed in the wells where the bacteria were active (up to three hours in some cases). The colour and turbidity of each treatment well was compared visually to the controls to determine the MIC values.

3.4 RESULTS AND DISCUSSION

The MIC values from the two methods are given in tables 3.1 and 3.2. It is clear that the microtitre bioassay gave lower MIC values than the agar dilution method. In most cases the growth of the bacteria inoculated on the agar plates was not inhibited even at 25 mg/ml. From this we may deduce that the microtitre method is a more sensitive method than the agar plate method.

In the agar dilution method the extract was added to molten agar (temperature of approximately 40° C). The plates were then left to set and dry during which time most of the acetone evaporated off. In the microtitre plates, on the other hand, the acetone was neither added to heated medium, nor given a chance to evaporate before the bacteria were inoculated into the wells. The acetone still present in the microtitre wells may have influenced the results.

Staphylococcus aureus was the only bacterium that showed the same MIC values (table 3.1) with both methods and these results are in agreement with Afolayan and Meyer (1997). On the other hand, Eloff (1999) obtained contradictory results with this bacterium when testing the extracts from *C. molle*. It is likely that the specific toxicant being tested influences the results and indicates a need to use a variety of test bacteria to obtain a general view of a substance's toxicity profile.

Which method is more appropriate considering the ecology of the test organism? In human diseases the pathogen is usually surrounded by a liquid medium (blood, lymph and other body fluids) and it is only in some skin diseases that the pathogen may not be surrounded by fluid. In the case of infection in seaweeds this also occurs in liquid medium (seawater and cell sap) and some agarolytic bacteria may attach themselves to the cell wall and so live on a solid medium surrounded by fluid. For these reasons the microtitre method is generally more appropriate than the petri dish method.

Table 3.1 MIC ($mg.ml^{-1}$) of *Osmundaria serrata* extract on human pathogenic bacteria using the agar dilution and microtitre methods (- = no growth inhibition at 25 $mg.ml^{-1}$; ND = not determined).

Bacterial Species	**MIC values from agar dilution method**	**MIC values from microtitre method**
Bacillus cereus (G+)	12.5	0.4
B. pumilis (G+)	12.5	1.6
B. subtilis (G+)	12.5	1.6
Enterobacter cloacae (G+)	-	12.5
Propionibacterium acnes (G+)	ND	0.4
Staphylococcus aureus (G+)	12.5	12.5
Enterococcus faecalis (G-)	-	6.3
Escherichia coli (G-)	-	12.5
Klebsiella pneumoniae (G-)	-	25.0
Pseudomonas aeruginosa (G-)	-	12.5
Salmonella typhimurium (G-)	-	12.5
Serratia marcescens (G-)	-	25.0

Table 3.2 MIC ($mg.ml^{-1}$) of *Osmundaria serrata* extract on marine bacteria using the agar dilution and microtitre methods (- = no growth inhibition at 25 $mg.ml^{-1}$).

Bacterial Species	**MIC values from agar dilution method**	**MIC values from microtitre method**
Marinococcus sp. (G+)	-	0.16
Halomonas sp. 1 (G-)	-	0.6
Halomonas sp. 2 (G-)	-	2.5
Halomonas sp. 3 (G-)	12.5	3.1
H. halophila (G-)	-	6.3
H. marina (G-)	-	12.5
OssB1[1] (G-)	-	25.0
Pseudomonas sp. 1 (G-)	-	1.6
Pseudomonas sp. 2 (G-)	-	6.3
Vibrio alginolyticus (G-)	-	6.3
V. harveyi (G-)	-	6.3

1 - Isolated from *O. serrata* these bacteria resisted attempts to separate them and they were included as a simplified community from *O. serrata*'s biofilm.

A reason why the microtitre bioassay gave lower MIC values than the agar dilution method could be that in the wells of the microtitre plates the bacteria have more of their surface area in contact with the liquid medium and potential inhibitory substance than on the petri plates of the agar dilution method, where only part of the surface is in contact with the medium. This may increase the efficacy of the inhibitory substance in the liquid medium because there are more receptors available for it to interact with or the bacteria can absorb more of the substance. On solid medium only

the bacteria at the bottom of the colonies are in direct contact with the toxin and the medium. The bacteria near the top of these colonies are buffered from being directly influenced by the toxin. In liquid cultures they are all equally in contact with the medium and toxin, unless the bacteria form flocs or aggregations of bacterial cells. In the colony the bacteria "work together" and survive better as a whole, while in liquid cultures it is each to their own and survival drops. The saying "United we stand, divided we fall" comes to mind!

In addition, a synergism between the toxicant and acetone may be responsible for the sensitivity of the microtitre method and thus may not reflect the true MIC value of the test substance. However, because the microtitre method uses far less of the test substance than the petri dish method, it may be the best method to use when only very small quantities of an active natural product are available. In these cases a possible synergistic effect between the test product and the solvent must be considered.

Table 3.3 gives a comparison between the two methods. It is recommended that each method be considered in terms of the ecology of each test organism and tests be performed to evaluate possible synergism between the test substance and the solvent used.

Table 3.3 Summary of comparison between the microtitre and agar dilution bioassay methods.

Microtitre Method	**Agar Dilution Method**
Quick and relatively easy	Laborious
Very little test substance required (375 mg for 25 bacteria and three replicates was used)	Much more test substance is needed (619 mg for 25 bacteria and three replicates was used)
Cheaper than petri dish method?	More expensive than microtitre method?
More realistic model of reality	Less realistic model of reality
Expensive specialised equipment used (multichannel micropipette)	Standard equipment used
Can test more substances at many different dilutions against more bacteria in the same amount of time than the petri dish method	Method too laborious to test many test substances at many different dilutions as with the microtitre method
Results often not clear – green extracts may mask red of tetrazolium reaction	Results clear – bacterial growth easily observed

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CHAPTER FOUR

ANTIBACTERIAL ACTIVITY OF EXTRACTS FROM SELECTED MACROALGAE FROM KWAZULU-NATAL, SOUTH AFRICA

4.1 ABSTRACT

Macroalgae produce secondary compounds, some of which are thought to protect the seaweeds against microbial infection and grazing by herbivores. Crude extracts from *Osmundaria serrata* (Rhodophyta) had previously been found to be antimicrobial. This seaweed, and 12 other species that occur in the same intertidal zone in Palm Beach, KwaZulu-Natal, South Africa, where collected and extracted. The extracts were then tested against the growth of 12 terrestrial and 10 marine bacteria in pure culture, and a consortium of Gram-negative bacteria isolated from the biofilm growing on *O. serrata*. Copper(II) sulphate was used as a positive control and elicited an average minimum inhibitory concentration (MIC) for the test bacteria of 0.48 ± 0.05 $mg.ml^{-1}$. The average MIC value for the extract from *O. serrata* was 9.34 ± 1.54 $mg.ml^{-1}$ and was the most active seaweed tested. This is a first report on the antibacterial activities of extracts from *Caulerpa filiformis* (Chlorophyta), *Nitzchia martiana* (Bacillariophyceae), and the rhodophytes *Amphiroa bowerbankii*, *Cheilosporum cultratum* subsp. *multifidum*, *Hypnea rosea*, and *Spyridia hypnoides*.

4.2 INTRODUCTION

Seaweeds or macroalgae are an ancient group of organisms with a long evolutionary history. They have had plenty of time to evolve chemical defences against the

herbivores, pathogens and epiphytes that they share their habitats with. They are known to contain many unusual secondary compounds, many of which are halogenated (Jones, 1988). These compounds are thought to have chemical defensive roles against microbial infection and grazing by herbivores (Fenical, 1975).

The most common method of testing for biological activity *in vitro* is the effect on microbial growth with antibacterial activity predominating (Chesters and Stott, 1956; Caccamese and Azzolina, 1979; Rao, 1991), probably because of the ease and simplicity of antibacterial tests. However, other reported biological activity tests include antifungal, antiviral, antitumour, cytotoxic, and more specific assays like for antimalaria activity (Noda *et al.*, 1989; Pesando, 1990; Tariq, 1991; König *et al.*, 1994).

Osmundaria serrata (Rhodophyta) had previously been found to have antimicrobial activity and deserved further study since nothing was known about the chemistry of this species (Barreto *et al.*, 2001; Vlachos *et al.*, 2001). The aim of this chapter was to screen locally abundant seaweed that share the same local habitat with *O. serrata* (i.e. the area between the intertidal and subtidal zones) at Palm and Trafalgar Beaches within the Mpenjati marine reserve, KwaZulu-Natal, South Africa. These were tested against the growth of bacteria isolated from the habitat of the seaweed, and other bacterial species that are potential human pathogens.

4.3 MATERIALS AND METHODS

Samples of seaweed (table 4.1) were collected from Palm (30° 59' 30" S, 30° 16' 30" E) and Trafalgar Beaches (30° 57' 30" S, 30° 18' 00" E; both 30 30 CD), KwaZulu-

Natal, South Africa in June 2002. The macroalgae were identified using the methods of Simons (1976) and Branch *et* al. (1994). Voucher specimens were deposited in the HGWJ Schweickerdt Herbarium (PRU) at the University of Pretoria (table 4.1). The samples were cleaned of visible epiphytes, frozen and stored at about –5 °C until taken to the laboratory and extracted. From 10 to 100 g of macroalgal material was homogenized with ethanol and ethyl acetate (1:1) and left to extract for three days on a shaker. The extracts were then filtered and dried under reduced pressure at 40 °C. The ethyl acetate soluble fractions were used in the bioassay experiments dissolved in 50% acetone.

Table 4.1 Macroalgal species collected from the KwaZulu-Natal, South Africa coast. (Names and authorities after Silva *et al.*, 1986.)

Kingdom	**Division**	**Family**	**Genus and Species**	**Collection Site**	**Notes and Herbarium Specimen Numbers**
Protista	Heterokonta	Bacillariophyceae	*Nitzchia martiana* (Agardh) Van Heurk	Trafalgar	Colonial diatom; MB22
Plantae	Chlorophyta	Codiaceae	*Codium duthieae* Silva	Palm Beach	Siphonaceous; MB19
			Halimeda cuneata Hering	Palm Beach	Lightly calcified; used by local artists in collages; MB17
		Caulerpaceae	*Caulerpa filiformis* (Suhr) Hering	Palm Beach	Siphonaceous dominant in habitat; MB14
	Rhodophyta	Corallinaceae	*Amphiroa bowerbankii* Harvey	Palm Beach	Calcified; used by local artists in collages; MB12

			A. ephedraea (Lamarck) Decaisne	Palm Beach	Calcified; used by local artists in collages; MB13
			Cheilosporum cultratum (Harvey) Areschoug subsp. *multifidum* (Kützing) Johansen	Palm Beach	Calcified used by local artists in collages; MB14
		Gelidiaceae	*Gelidium abbottiorum* R.E. Norris	Palm Beach	Used in agar industry; MB20
		Hypneaceae	*Hypnea rosea* Papenfuss	Palm Beach	Epiphytic; MB15
			H. spicifera (Suhr) Harvey	Palm Beach	Forms dense mats, dominant in habitat; MB16
		Ceramiaceae	*Spyridia cuppressina* Kützing	Palm Beach	Small, growing on rocks; MB23
			S. hypnoides (Bory de Saint-Vincent) Papenfuss	Palm Beach	Small and soft, growing on rocks; MB24
		Rhodomelaceae	*Osmundaria serrata* (Suhr) R.E. Norris	Palm Beach	Dominant in habitat, leathery and tough; MB21

The bacteria in table 4.2 were used in the bioassays. They were grown for 24 hours at 21 ± 2 °C prior to being used in the experiments. The marine isolates (chapter 2) were grown in marine broth made with 5 g soy peptone (Sigma), 1 g yeast extract (Biolab, Midrand, South Africa) 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 prepared according to the manufacturer's instructions.

Table 4.2 Bacterial isolates used in the bioassays to determine the MIC's of the macroalgal extracts.

Bacterial Species	**Gram Reaction**
Isolated from marine sources	
Marinococcus sp.	Positive
Halomonas halophila	Negative
Halomonas marina	Negative
Halomonas sp. 1	Negative
Halomonas sp. 2	Negative
Halomonas sp. 3	Negative
OssB1	Natural consortium of gram negatives*
Pseudomonas sp. 1	Negative
Pseudomonas sp. 1	Negative
Vibrio alginolyticus	Negative
Vibrio harveyi	Negative
Obtained from Medical Microbiology Department of University of Pretoria	
Bacillus cereus	Positive
Bacillus pumilis	Positive
Bacillus subtilis	Positive
Enterococcus faecalis	Positive
Propionibacterium acnes	Positive
Staphylococcus aureus subsp. *aureus*	Positive
Enterobacter cloacae	Negative
Escherichia coli	Negative
Klebsiella pneumoniae	Negative
Pseudomonas aeruginosa	Negative
Salmonella typhimurium	Negative
Serratia marcescens	Negative

* - Isolated from *O. serrata* these bacteria resisted attempts to separate them and they were included as a representative community from *O. serrata*'s biofilm.

The method of Eloff (1999) was used to determine the minimum inhibitory concentration (MIC) of the extracts in a dilution series of 12 (from 25 to 0.01 mg.ml^{-1}). The negative control wells contained acetone (from 12.5 to 0.006 % dilution series), and Copper(II) sulphate ($CuSO_4 \cdot 5H_2O$) was used as the positive control (from 1.25 to 0.0006 mg.ml^{-1}). Overnight cultures of the bacteria were diluted 1:100 before being inoculated into the microtitre wells. The marine isolates were incubated at 23 ± 2 °C and the other bacteria at 37 ± 2 °C for 24 hours. MIC results were ranked from no activity at 25 mg.ml^{-1} (inhibition index = 1) to the highest activity at 0.4 mg.ml^{-1}

(inhibition index = 8). The data were then log-transformed and Student *t*, analysis of variance and Duncan's multiple range tests ($\alpha = 0.01$) were performed (French and Lindley, 2000; Gadsden and Sparks, 2000; Rothery, 2000).

4.4 RESULTS AND DISCUSSION

Data are presented as log inhibition index (figures 4.1, 4.2 and 4.3) because the mean ranked data could not be converted back to concentrations because there was no concentration value for no inhibition at 25 $mg.ml^{-1}$. High values of log inhibition index indicate a high inhibitory response while low values indicate the opposite.

Excluding Copper(II) sulphate, the extract made from *O. serrata* was the most active against the test bacteria (figure 4.1) and was chosen for further chemical investigation. Since only the Copper(II) sulphate and the extract from *O. serrata* inhibited all of the bacteria, it was possible to calculate their mean MIC concentrations: Copper(II) sulphate = 0.48 ± 0.05 $mg.ml^{-1}$ and *O. serrata* extract = 9.34 ± 1.54 $mg.ml^{-1}$. The other extracts showed at least 8 (*Cheilosporum cultratum* subsp. *multifidum*) to 22 (*Gelidium abbottiorum*) out of 23 determinations with no inhibitory response at 25 $mg.ml^{-1}$. The extracts from *C. cultratum* subsp. *multifidum* and *Hypnea rosea* (both Rhodophyta) elicited the next highest inhibitory reactions from the test bacteria.

Halimeda species have been found to convert halimedatetraacetate (a less toxic sequiterpenoid) into halimedatrial (more toxic compound) following cell damage (Proksch, 1999). This reaction probably did not take place while the sample of *Halimeda cuneata* was homogenised because the ethanol had deactivated the

enzyme(s) required for that reaction. Hence the low inhibitory action of the extract from this green seaweed.

The most sensitive bacteria toward the extracts were the Gram-positives (except *Staphylococcus aureus* – figure 4.2). The most resistant bacteria were Gram-negatives grown on the terrestrial medium. *Serratia marcescens* topped the list and has recently been found to cause a coral disease (Patterson, *et al.*, 2002). It is interesting that it is also the most resistant to the macroalgal products tested here. This enterobacterium is regularly dumped into the oceans in sewage, and it would probably not be strictly correct to call it a terrestrial bacterium. In this study it was called (and other bacteria) "terrestrial" because they were not grown on a seawater medium.

OssB1, the group of marine bacteria isolated from *O. serrata*, was more resistant to the extracts than any of the other marine isolates. The bacteria that made up the consortium (or floc) were more successful than the solitary isolates in tolerating the presence of the active compound. This is potentially a good example of a positive result from an association between members of a microbial community in a biofilm (Wimpenny, 2000).

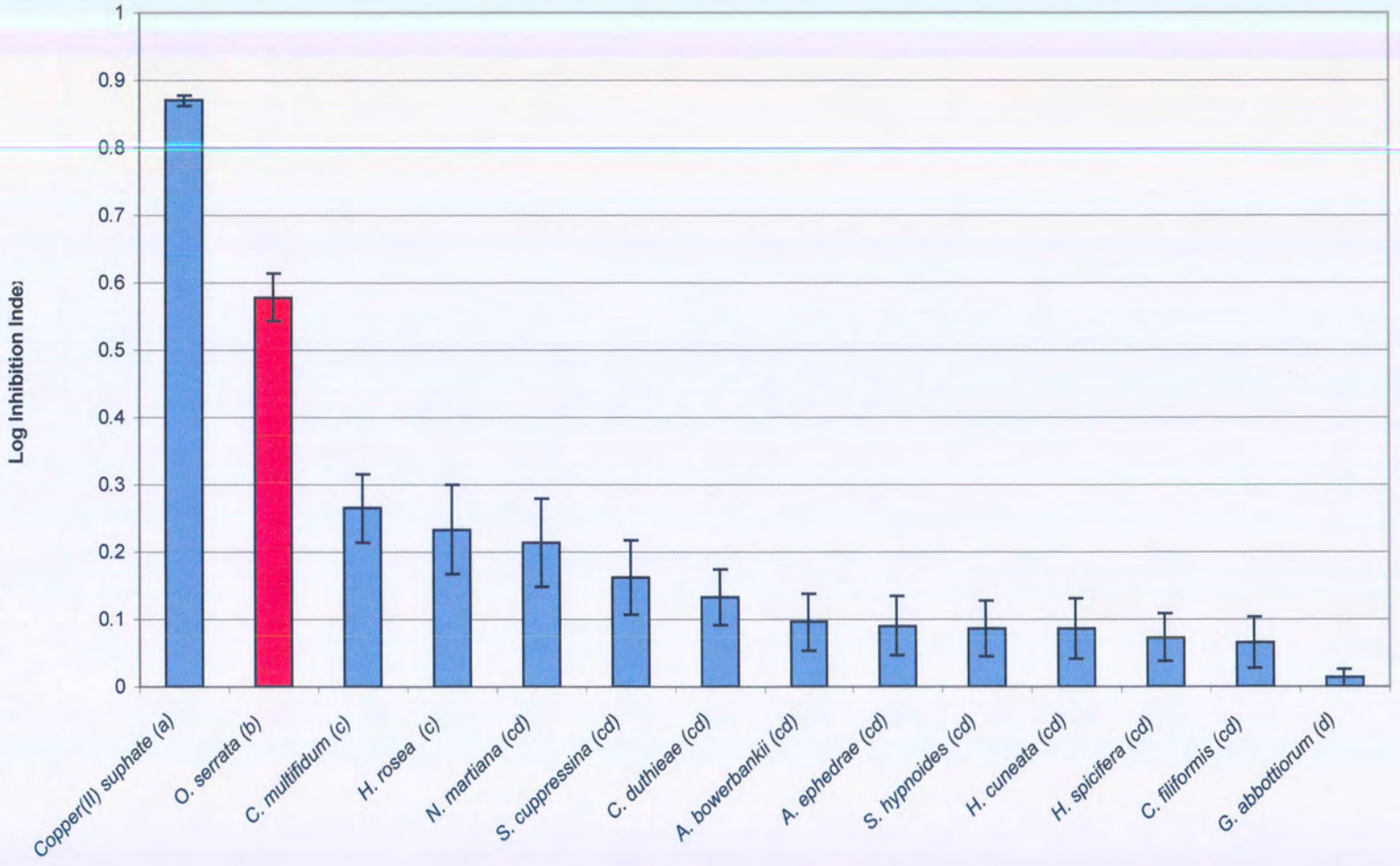

Figure 4.1 Biological activity of macroalgal extracts and $CuSO_4$ (positive control). High values indicate high inhibition of bacterial growth. Different letters indicate significant ($\alpha \leq 0.01$) differences between bacteria. Bars = SE, $n = 23$ different bacteria, see table 5.1 for full species names.

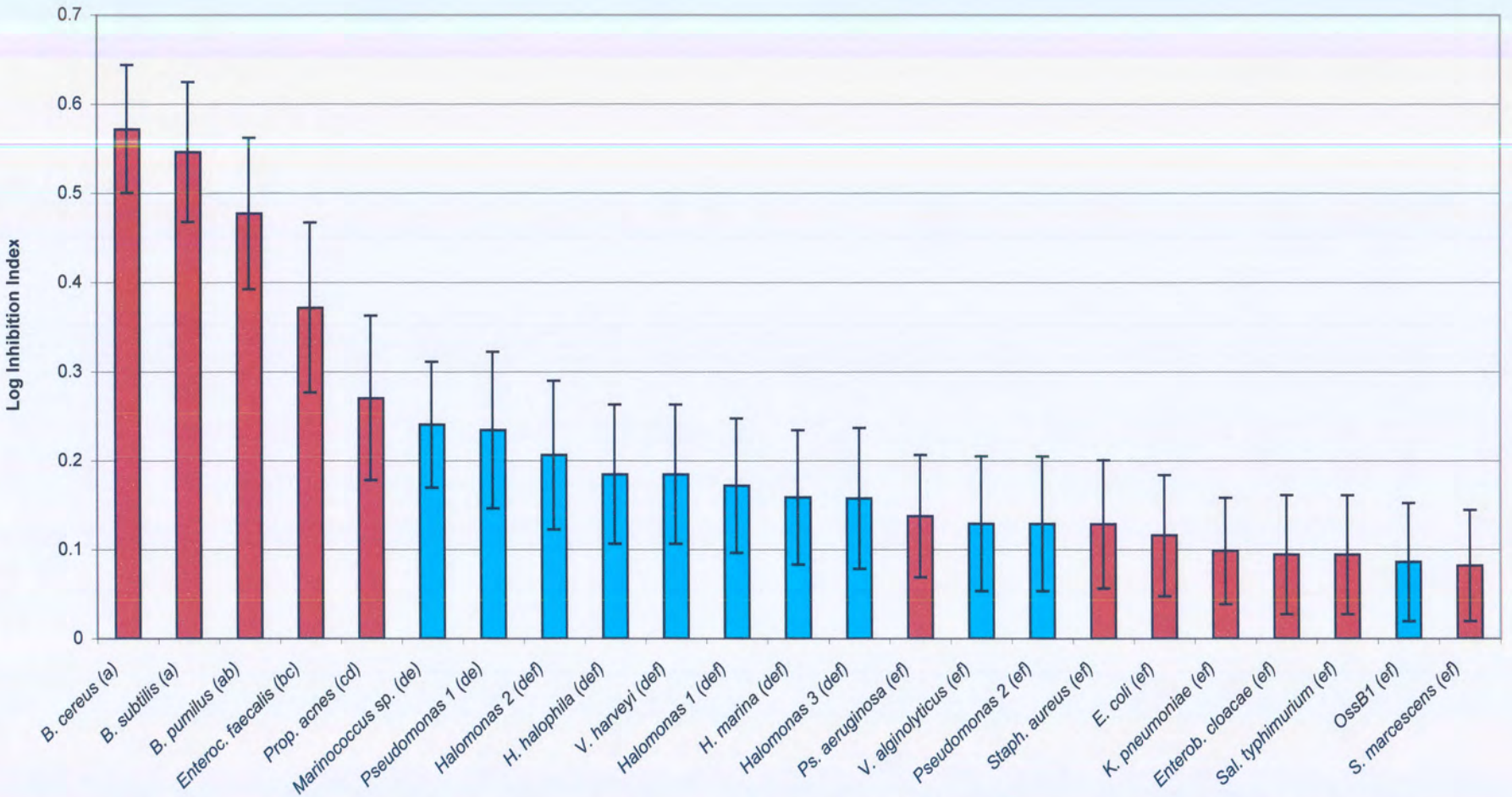

Figure 4.2 Average response of test bacteria to all macroalgal extracts and $CuSO_4$ (positive control). High values indicate high inhibition of bacterial growth. Different letters indicate significant ($\alpha \leq 0.01$) differences between bacteria. Bars = SE, n = 13 test substances, brown bars = terrestrial bacteria, blue bars = marine bacteria, see table 5.2 for full species names.

The Gram-positive bacteria tested were highly significantly ($p = 3.0 \times 10^{-7}$) more sensitive to the extracts than the Gram-negative bacteria (figure 4.2). This is due to differences in the cell walls of the two groups of bacteria. The Gram-negatives have a more complex multi-layered wall than the Gram-positives, which only have a thick single layer of peptidoglycan (Madigan *et al.*, 1997). In addition the multi-layered walls of the Gram-negatives have binding proteins in the periplasmic space that are involved in the transportation of substances into the cell. These proteins are lacking in the Gram-positives.

The terrestrial bacteria were more sensitive than the marine isolates to the extracts ($p = 0.0013$). This was as expected because the marine bacteria had been evolving with the macroalgae and would potentially have previously been exposed to the algal toxins. Thus, they would have had chance to evolve resistance to it.

On the basis of these differences in responses the bacteria were divided into three groups; the Gram-negative marine, the Gram-negative terrestrial, and the Gram-positives (because there was only one Gram-positive marine isolate and the difference in inhibition indices between it and the terrestrial Gram-positives was not statistically significant).

Analysing the data in these groups showed that there was no difference between the activities of the calcified and non-calcified red algae. However, using the total data set a slight difference ($p = 0.09$) between the calcified and non-calcified red algae was seen. By grouping the data we could clearly see that the difference was due to

differences between the bacteria within the algal groups and not the algal groups themselves.

In figure 4.3 the differences between the bacterial groups are shown in more detail. In contrast with the macroalgal extracts, copper sulphate inhibited the Gram-negative marine bacteria significantly (p = 0.009) more than the Gram-positives. The Gram-negative marine bacteria were generally also more sensitive to the macroalgal extracts than the terrestrial Gram-negatives, although this was not significant with the greens and this was only one diatom in the data set. It is unknown why the marine bacteria were more sensitive to copper sulphate than the terrestrial forms. This is, however, put to good use in copper containing paints that protect ships hulls and other surfaces from fouling in the marine environment (Hellio *et al.*, 2001). It is just unfortunate that these products also pollute marine ecosystems.

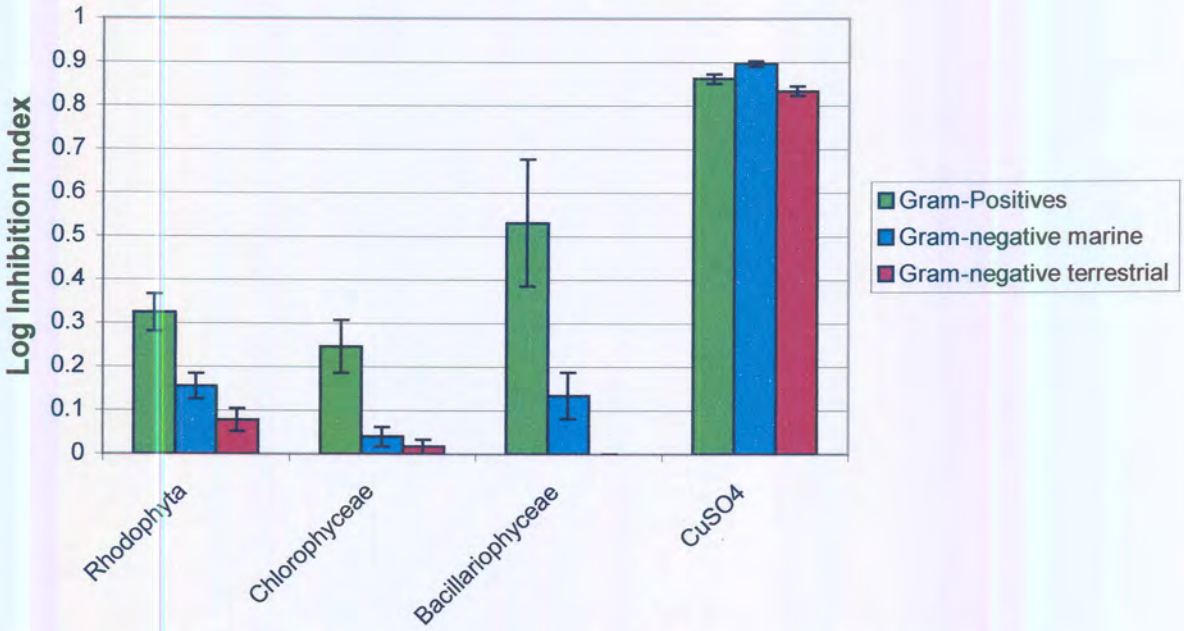

Figure 4.3 Biological activities of the groups of test substances used in the study against the groups of bacteria used in the bioassays. Bars = SE.

From figure 4.3 it may be seen that the Rhodophyta were generally more active against the bacteria than the Chlorophyta. In fact, the extracts from the Chlorophyta sampled were the least inhibitory toward the bacteria tested, with nearly 80% of the observations showing no activity at 25 $mg.ml^{-1}$. Other workers have found similar trends. Both Caccamese and Azzolina (1979) and Vlachos and co-workers (1997) found the reds more active than the greens. It is interesting to note that Caccamese and Azzolina (1979) found *O. volubilis* only slightly active against *Bacillus subtilis*, but not active against *Escherichia coli* and the fungi *Saccharomyces cerevisiae* and *Penicillium digitatum*, when *O. serrata* showed very good activity in this study. Further study is required to determine whether other species of *Osmundaria* also produce the same active compound.

The high inhibitory results obtained from the *O. serrata* extract contrasts with those obtained by Vlachos and co-workers (1997) who found that its activity was not exceptional. However, in a 1999 paper the same authors reported that "the antibacterial activity of *O. serrata* was relatively high compared to other southern African Rhodophyta. Nonetheless, the antibacterial activities of some of the other species of macroalgae of the Vlachos *et al.* paper (1997) overlapped with those tested in this study: *A. ephedraea*, *C. duthieae*, *G. abbottiorum*, *H. spicifera* and *S. cuppressina*. They were not considered to be highly active in either of the studies. This is a first report on the antibacterial activities of extracts from *A. bowerbankii*, *C. filiformis*, *C. cultratum* subsp. *multifidum*, *H. rosea*, *N. martiana*, and *S. hypnoides*.

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CHAPTER FIVE

DEFORMITIES INDUCED IN BACTERIA BY AN EXTRACT FROM *OSMUNDARIA SERRATA*

5.1 ABSTRACT

The crude extracts from the red alga *Osmundaria serrata* had previously been found to induce deformities in fungi. It was the aim of this study to determine whether bacteria are also affected by extracts from this macroalga. Negative staining and transmission electron microscopy (TEM) were used to view the bacteria; *Enterobacter cloacae*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Serratia marcescens*, *Halomonas* sp, *Halomonas marina*, *Vibrio harveyi* and a consortium of Gram-negative bacteria isolated from the biofilm that grows on *O. serrata*. The *E. cloacae* and *E. coli* showed little or no response to the seaweed extract, whereas *P. aeruginosa* and *S. marcenscens* showed morphological deformities. Increased glycocalyx production and cell deformities were observed with the *Halomonas* species. Blebbing of the outer membrane and cell leakage were with the consortium of bacteria and *H. marina*. The cells of *V. harveyi* were also deformed. The active compound in *O. serrata* is a brominated phenol, lanosol, and as phenolic compounds are known to interact with proteins, this could explain the cell deformations that were observed. The negative staining with the TEM technique gave good results of the morphological changes induced in bacteria by toxic chemicals. Scanning electron microscopy (SEM) is usually used to visualise the morphology of bacteria. However, structures such as the glycocalyx and flagella are mostly lost during sample preparation for SEM.

5.2 INTRODUCTION

Macroalgae, or seaweeds, attached to rocky shores live in a very dynamic environment. The crashing waves may carry sand that grazes the seaweed blades causing wounds where potential pathogens carried in the seawater may enter and cause infections. Seaweeds produce many different secondary compounds. Many of these are halogenated and protect the algae from microbial attack and grazers, and prevent the settlement of fouling organisms (Sieburth, 1968; Fenical, 1975; Hay and Fenical, 1992; Walters *et al.*, 1996).

The crude extracts from *O. serrata* have been found to induce deformities in fungi (Barreto, 1995). Culture characteristics were altered in *Verticillium* sp. and *Rhizoctonia solani* with prolific chlamydospore production observed in the latter.

This study is aimed to investigate whether the morphologies of bacteria are also affected by extracts of *O. serrata*. Negative staining and a transmission electron microscope was used to view the bacteria because this method is routinely employed to view the morphologies of bacterial cells for identification purposes (Baumann and Baumann, 1981).

5.3 MATERIAL AND METHODS

The methods were similar to those followed in chapter 3. Briefly, *O. serrata* extract in 50% acetone was incorporated into agar growth medium at 25 $mg.ml^{-1}$ and the bacteria listed below were inoculated onto the plates and incubated for 24 hours. For the controls only a 50% solution of acetone was added to the growth medium.

Colonies that grew were sampled and processed for viewing under a transmission electron microscope as described in chapter 2.

The following potential human pathogenic bacteria were used in the bioassays: *Enterobacter cloacae* (Gram-positive (G+)), *Escherichia coli* (Gram-negative (G-)), *Pseudomonas aeruginosa* (G-) and *Serratia marcescens* (G-). The following marine bacteria were also used: *Halomonas* sp. 1 (G-), *H. marina* (G-), OssB1[1] (G-), and *Vibrio harveyi* (G-).

5.4 RESULTS

A third of the bacteria tested showed no differences between the control and the treatment, while the rest showed varying degrees of glycocalyx production and cell deformities. The terrestrial bacteria, *E. cloacae* and *E. coli*, showed little or no cell deformations (figure 5.1). On the other hand, *P. aeruginosa* showed an increase in glycocalyx production and more elongated cells, while those of *S. marcescens* were more irregular in shape. The marine bacteria all showed morphological changes in response to the extract (figure 5.2). *Halomonas* sp. 1 and *H. marina* both showed increased glycocalyx production and cell deformities. However, in the case of *Halomonas* sp. 1, the differences between the control and treatment plates were dramatic, where the algal extract induced copious glycocalyx production and strange bubble-shaped objects. Similar structures were observed with the consortium of bacteria, OssB1, but not with the deformed cells of *V. harveyi* (figure 5.2). The typical polar flagellum of *H. marina* was absent in the presence of the seaweed toxin.

[1] Isolated from *O. serrata* these bacteria resisted attempts to separate them and they were included as a representative community from *O. serrata*'s biofilm.

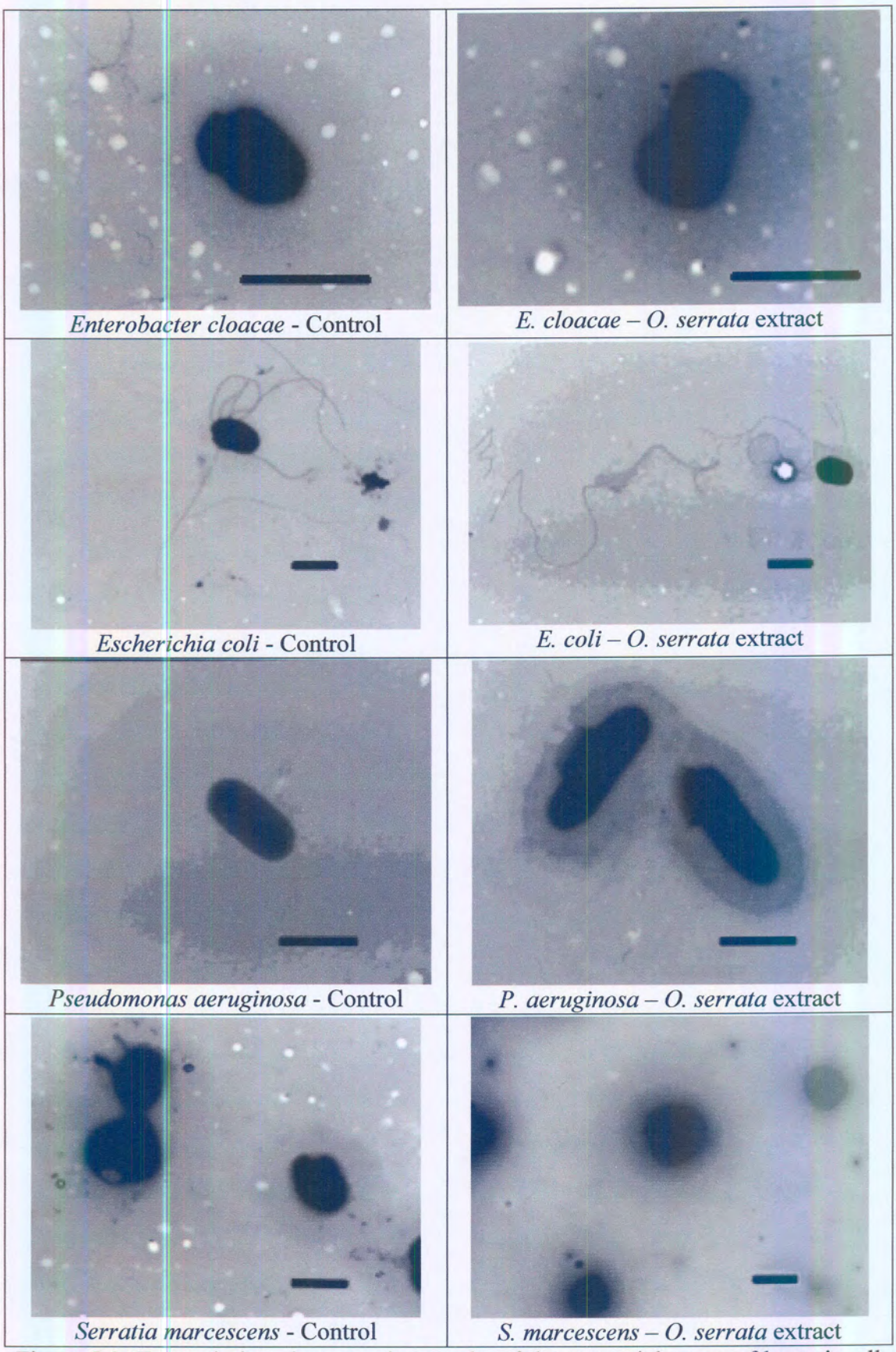

Figure 5.1 Transmission electron micrographs of the terrestrial group of bacteria, all potential human pathogens, in response to an extractive from *Osmundaria serrata*. Scale bars = 1 μm.

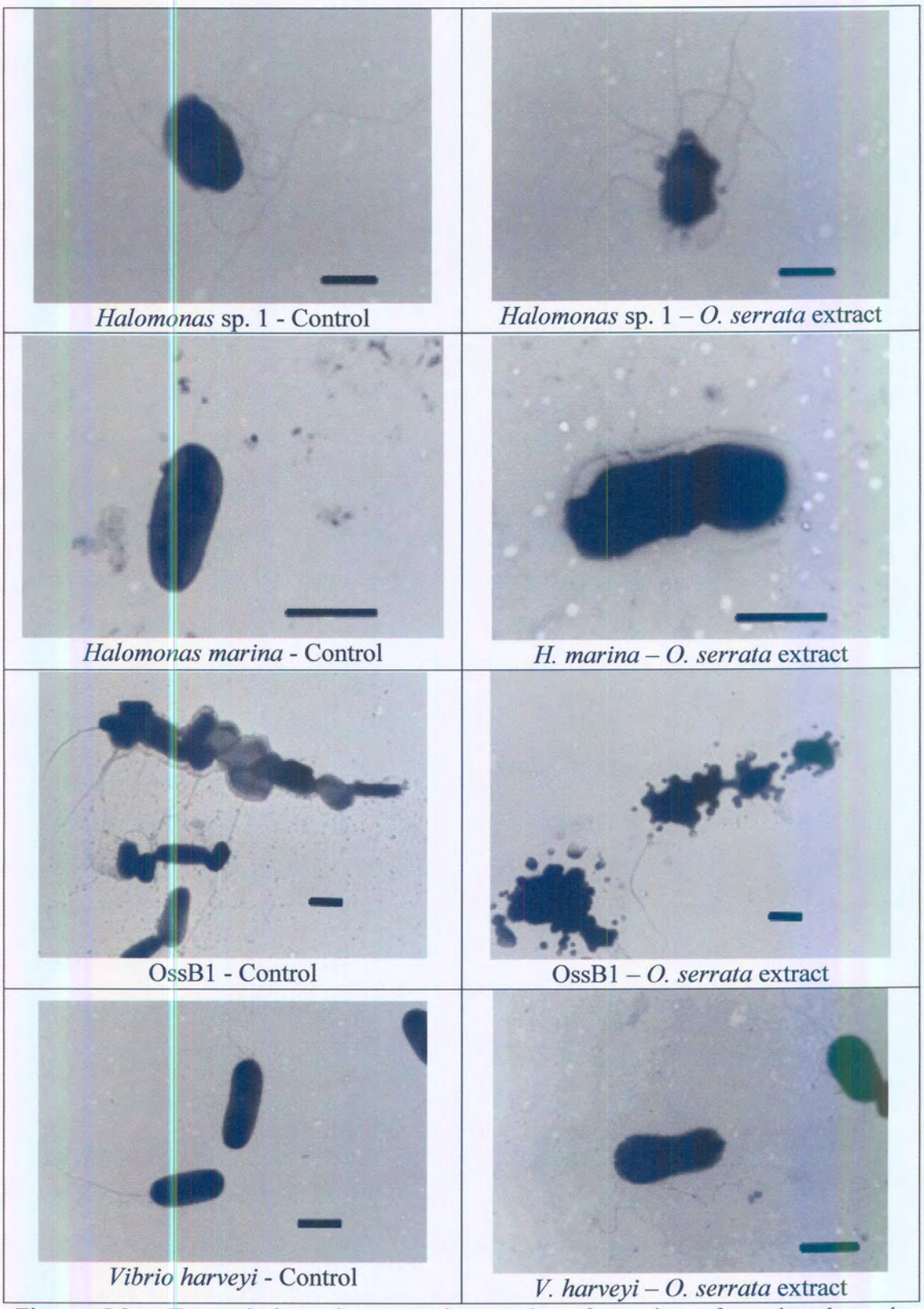

Figure 5.2 Transmission electron micrographs of species of marine bacteria *Halomonas* isolated from the surface of *Osmundaria serrata* in response to an extractive from the alga.

5.5 DISCUSSION

The strange bubble-shaped objects resemble severe blebbing of the outer membrane and leakage of cell contents (Beveridge *et al.*, 1991). This is probably linked to the precipitation effect that phenols have on proteins (Madigan *et al.*, 1997). Proteins (or enzymes) also control the formation of cell walls. Thus, the deformed cells are a reflection of the disruptive effect that the compounds in the extract had on the enzymes and structural proteins of the bacteria. It is unknown why some bacteria (e.g. *E. cloacae* and *E. coli*) should grow in the presence of the seaweed toxin showing little cell deformities, while others showed severe morphological changes because it is unlikely that these bacteria would have evolutionary contact with the seaweeds.

The production of glycocalyx (slime or capsule) is a common response of bacteria to toxicants (Gristina *et al.*, 1994). Glycocalyx consists of mostly polysaccharide and protein matrix, which is thought to prevent some toxicants from reaching the cells. It is also produced by bacteria in biofilms and is called extracellular polymeric substance (EPS). It is implicated in conferring the high resistance of biofilms to antimicrobials (Wimpenny, 2000). *Halomonas* sp. 1, *H. marina*, the consortium OssB1 and *P. aeruginosa* all showed increased glycocalyx production in response to the seaweed extract and they are all biofilm formers. However, *V. harveyi*, another biofilm former did not show glycocalyx production.

Negative staining is not normally used to investigate the effects that antimicrobial agents have on bacteria. Scanning electron microscopy (SEM) has also been used in this regard, but more detailed views are obtained with ultra-thin sectioned material

where membranes and wall structures are seen (Beveridge *et al.*, 1991; Li *et al.*, 2000). However, the negative staining technique is fast and simple. In addition, flagellae and glycocalyx are seen which are most often lost during the preparation of samples for SEM viewing, where these structures are easily lost during the dehydration steps. The negative staining technique gives a good view of the effects of the antimicrobial agent on the morphology of the bacteria.

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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.

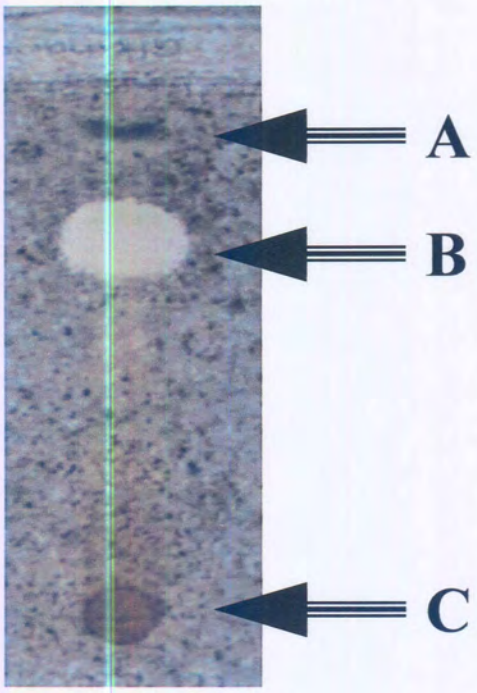

Figure 6.1 Sporulation of *Alternaria alternata* on thin layer silica gel chromatography plate to visualise the active compound from *Osmundaria serrata*. A = chlorophylls close to solvent front (hexane : ethyl acetate, 1:1); B = inhibition zone, where *A. alternata* did not sporulate; C = origin with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

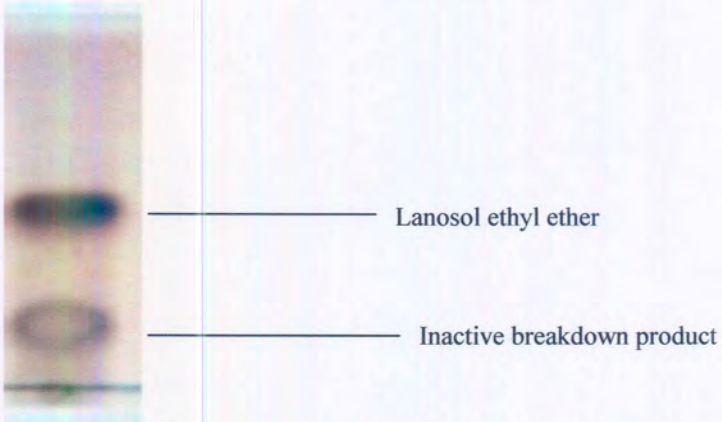

Figure 6.2 The active compound from *O. serrata*, lanosol ethyl ether, after five days storage at room temperature in the dark. Note the extra spot indicating the breakdown of the active compound. Hex and ethyl acetate (1:1) mixture was used to develop the TLC plate.

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.

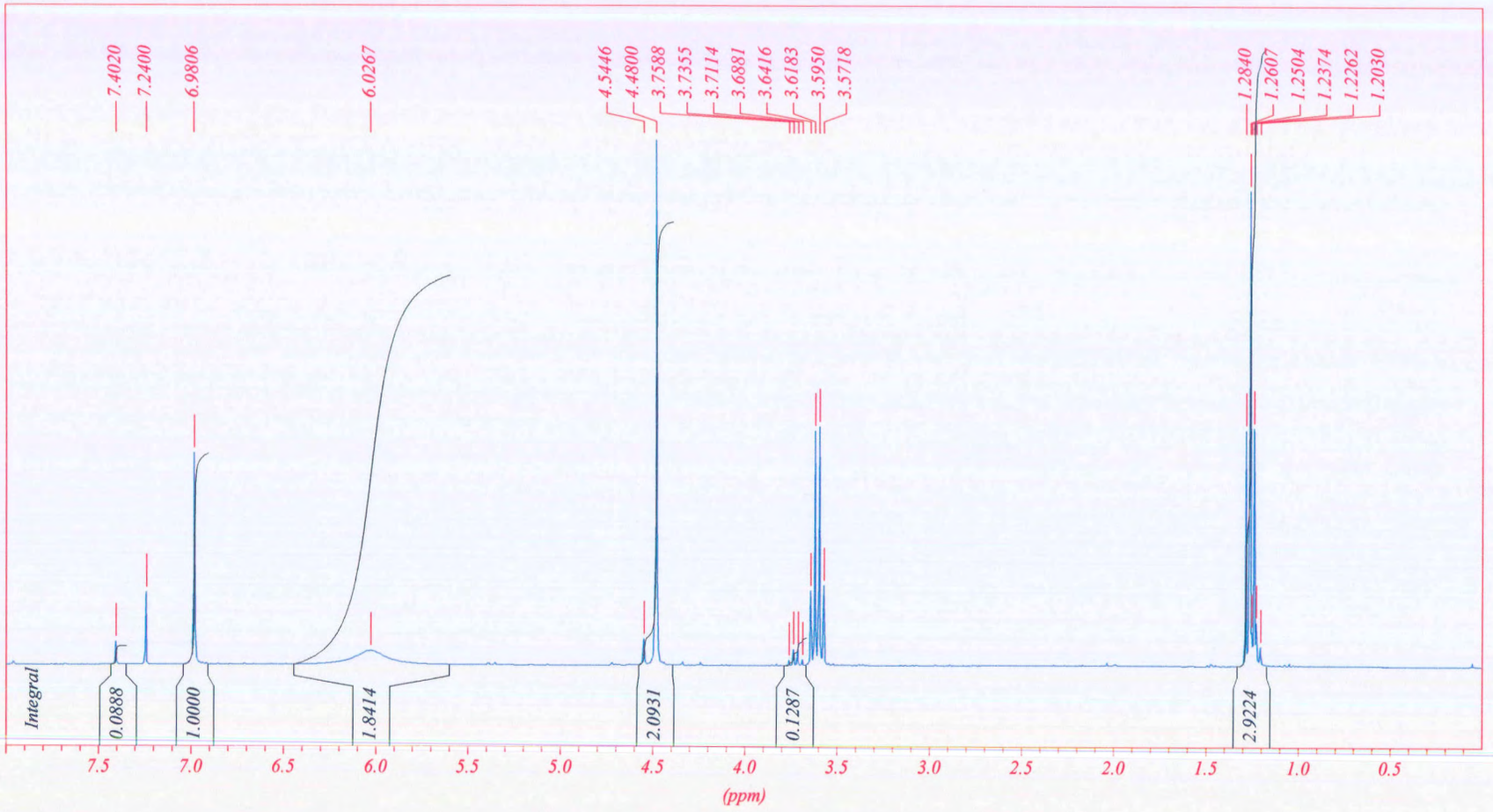

Figure 6.3 Proton NMR of the active compound, lanosol ethyl ether, isolated from *Osmundaria serrata.*

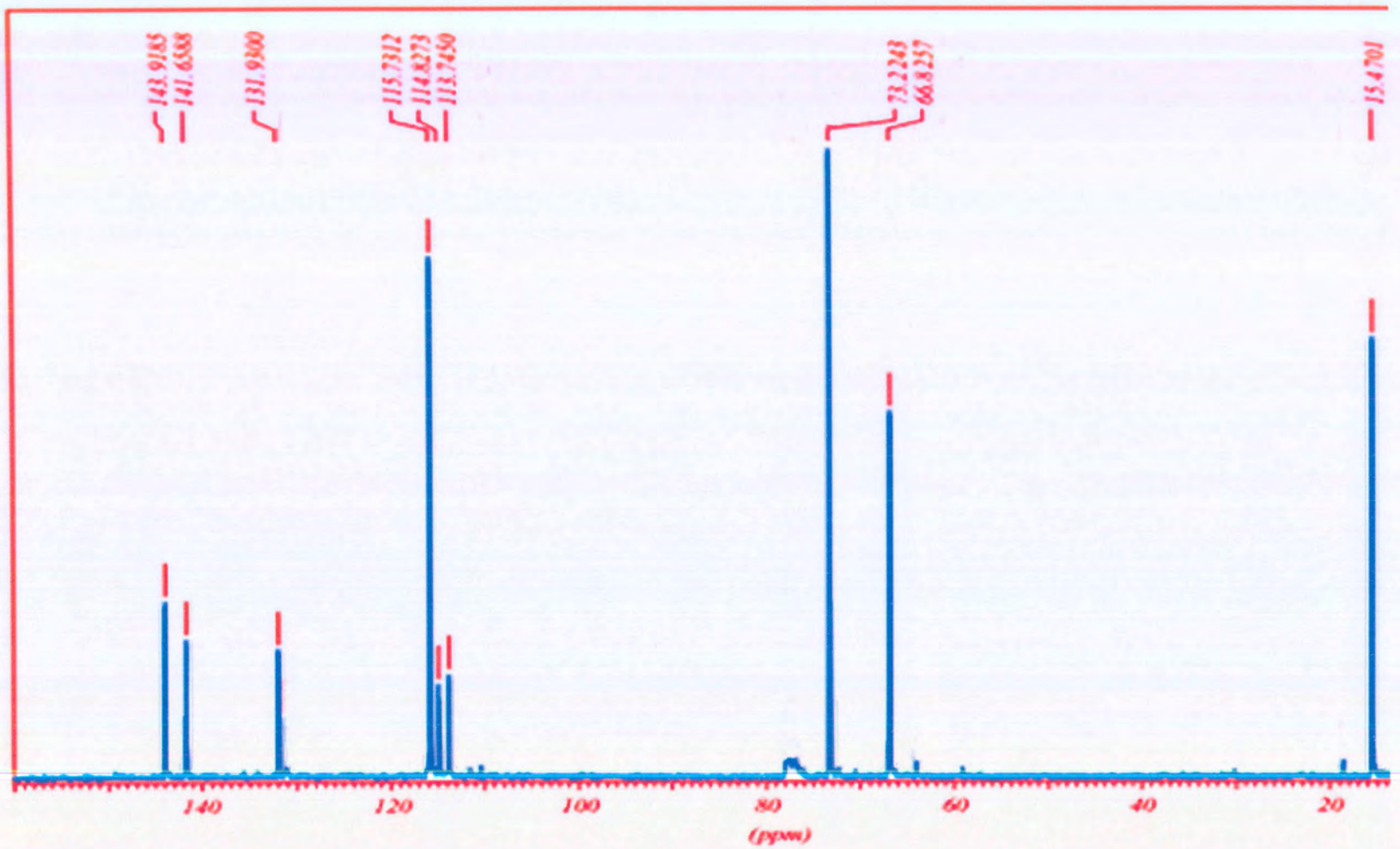

Figure 6.4 Carbon 13 NMR of the active compound, lanosol ethyl ether, isolated from *Osmundaria serrata.*

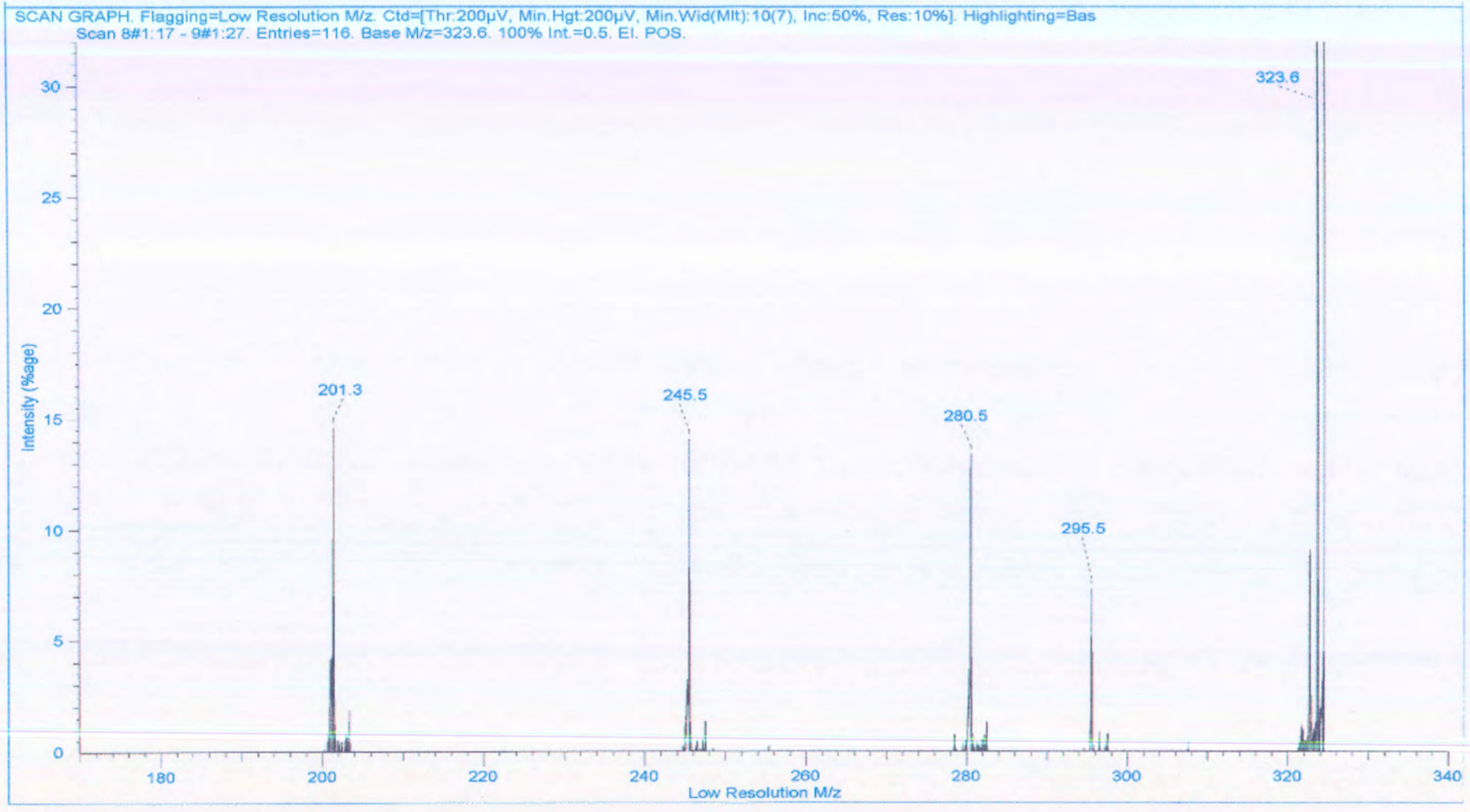

Figure 6.5 Mass spectrograph of lanosol diethyl ether isolated from *Osmundaria serrata*.

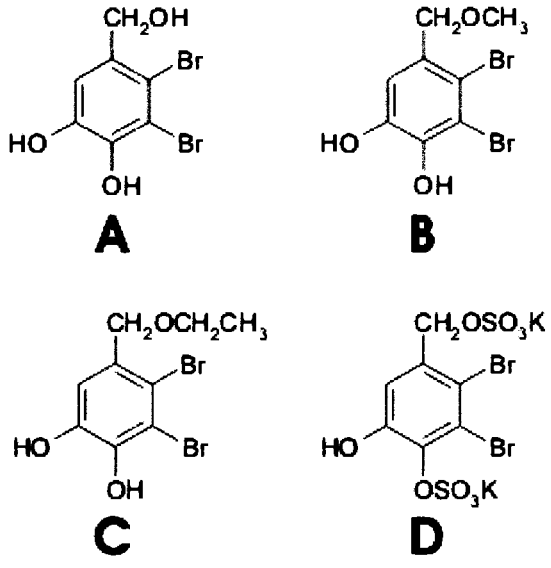

Figure 6.6 Chemical structures of lanosol and derivatives. **A** = Lanosol; **B** = lanosol-1'-methyl ether, **C** = lanosol-1'-ethyl ether; **D** = lanosol 1,4-disulfate ester.

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.

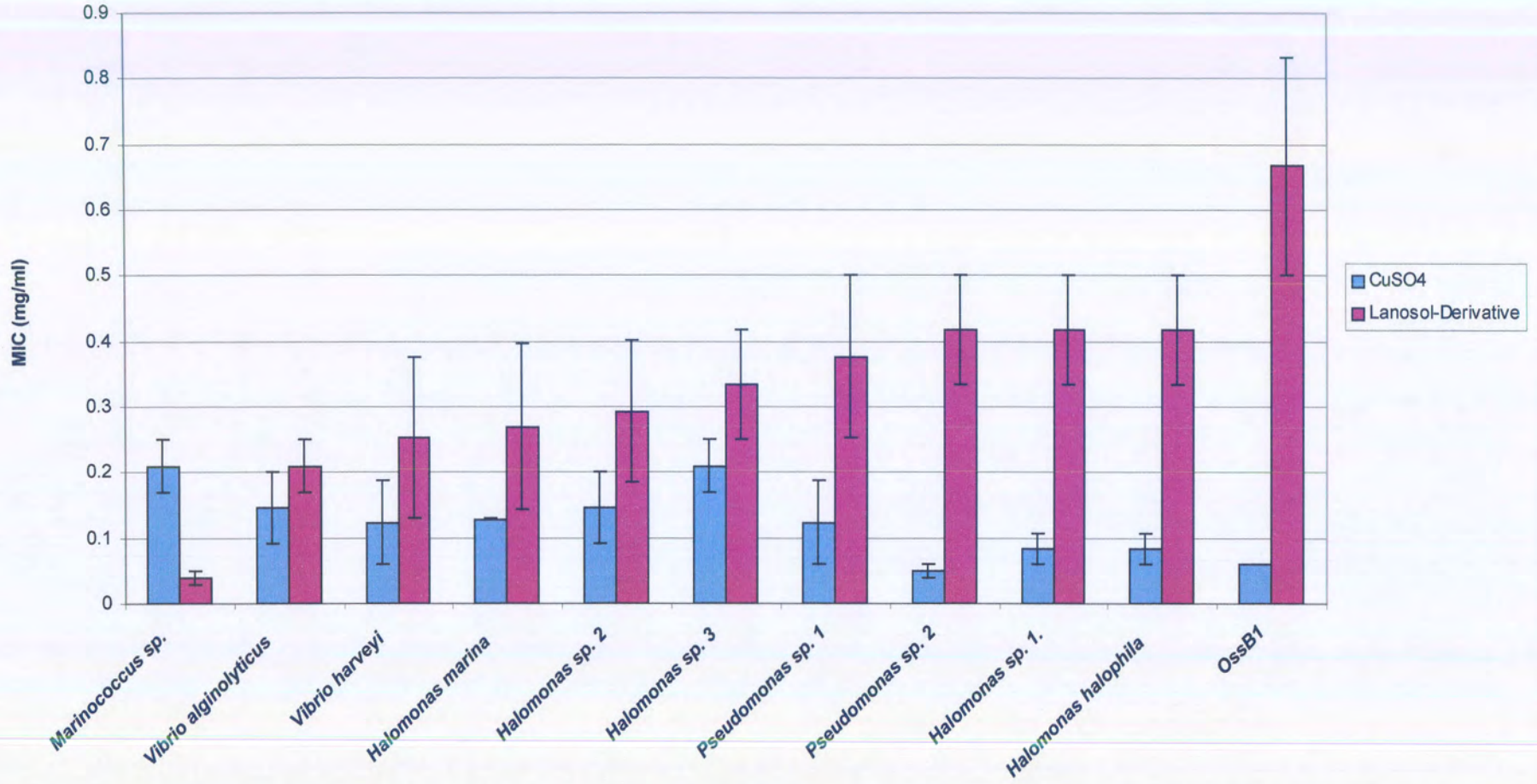

Figure 6.7 Minimum inhibitory concentrations (bacteriostatic) of lanosol ethyl ether against the growth of marine bacteria isolated from the habitat of *Osmundaria serrata*. Bars = SE, $n = 3$.

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).

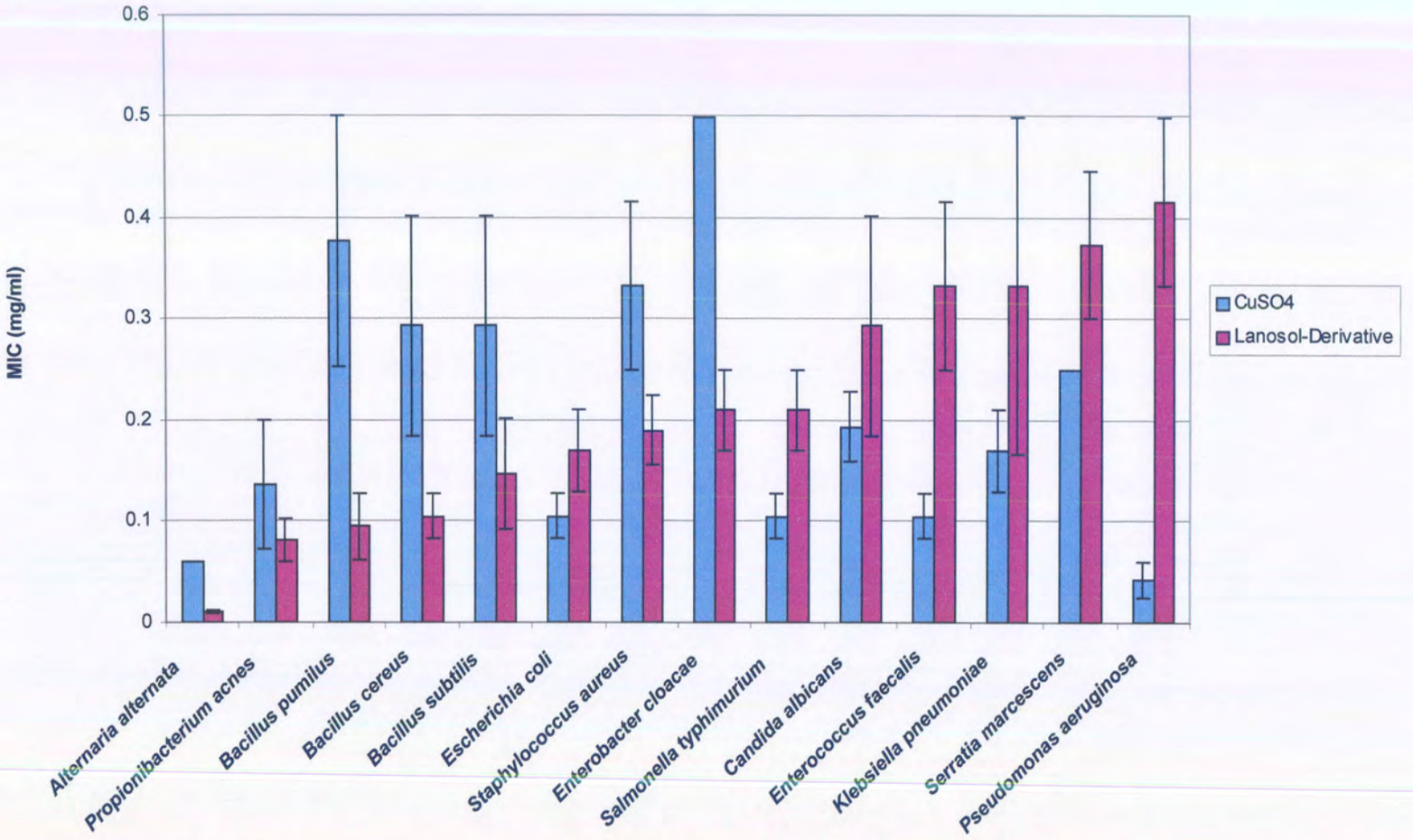

Figure 6.8 Minimum inhibitory concentration (bacteriostatic) of lanosol ethyl ether against the growth of terrestrial bacteria and fungi. Bars = SE, $n = 3$.

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.

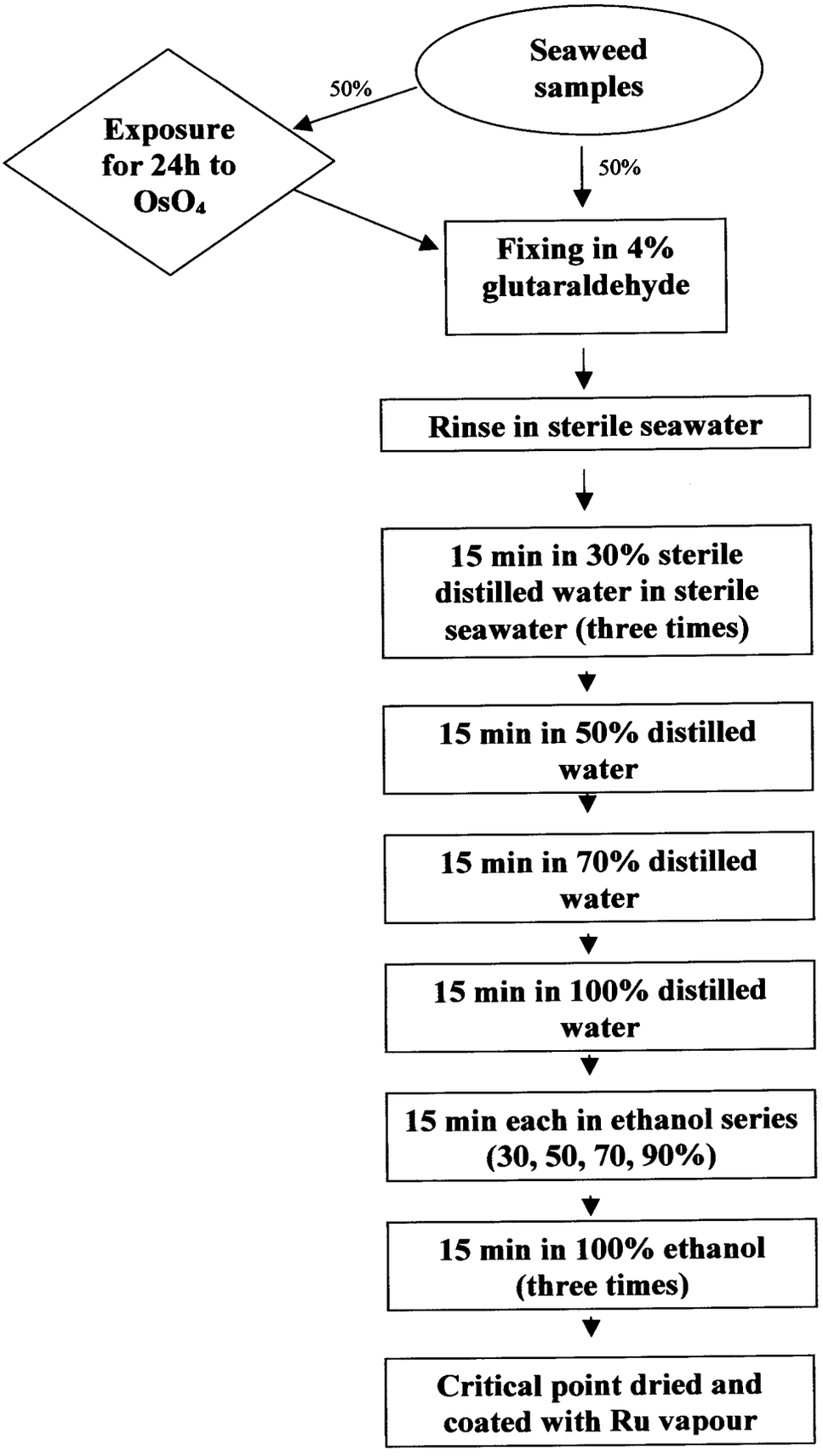
7.3 MATERIALS AND METHODS

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).

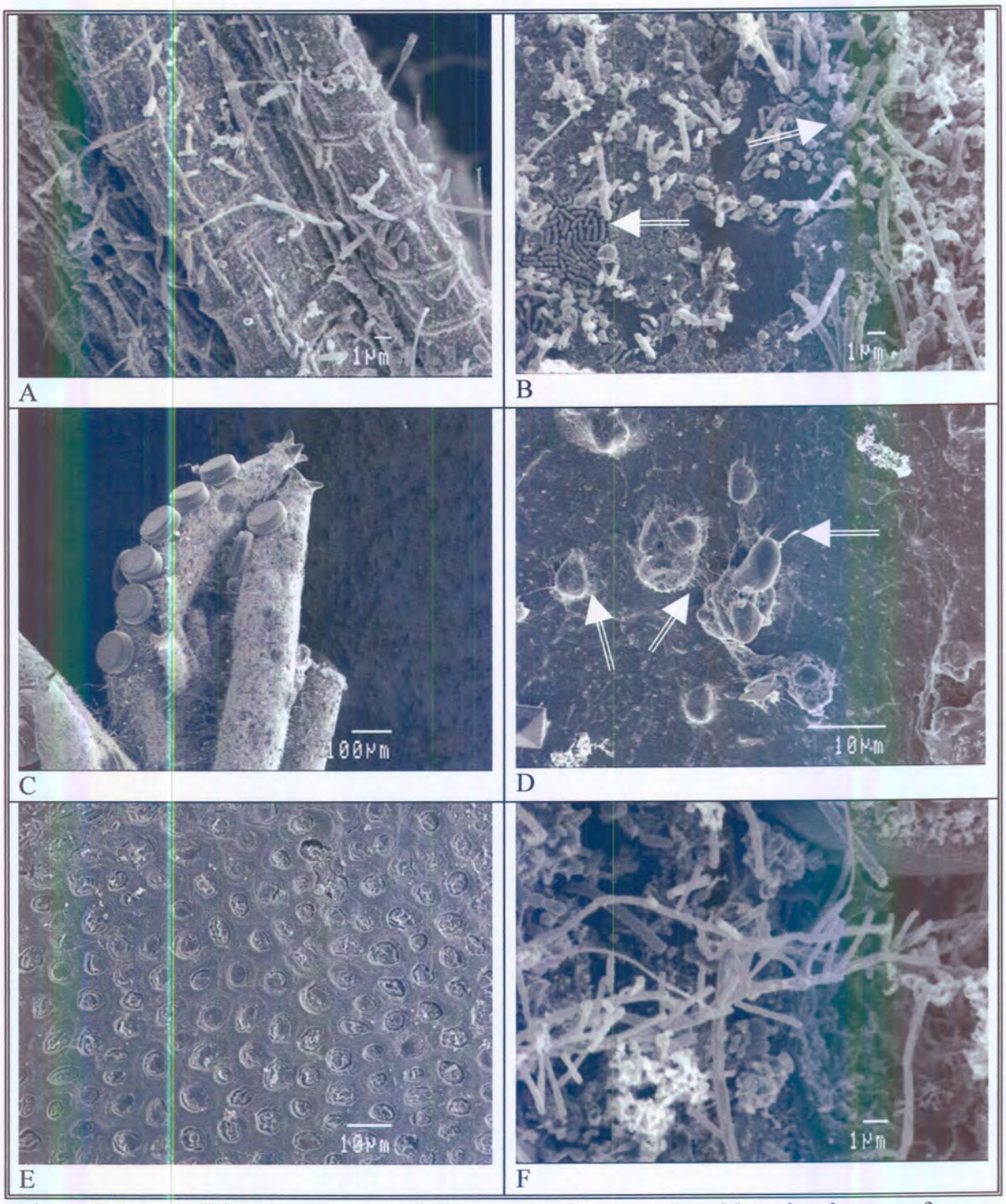


Figure 7.2.A. Epibiota on the colonial diatom *Nitzchia martiana*. Note the absence of cocci.

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*.

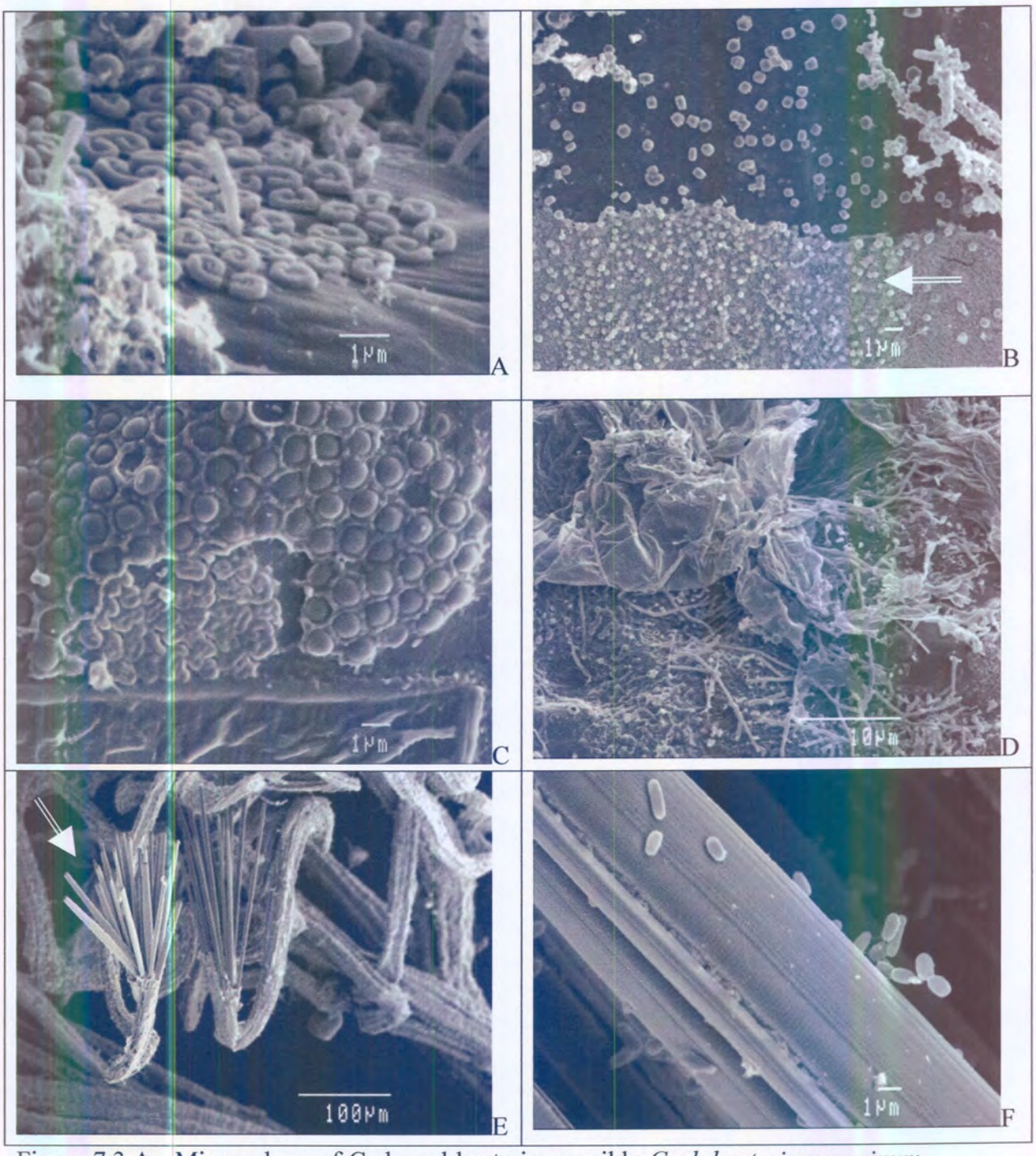


Figure 7.3.A. Microcolony of C-shaped bacteria, possibly *Cyclobacterium marinum*, on *Gelidium abbottiorum*.
B. Very small cocci (arrow) growing on *Codium duthieae*.
C. Encapsulated cocci on *Osmundaria serrata*. Note the thickened glycocalyx surrounding the cells and holding them in place.
D. Remnants 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).

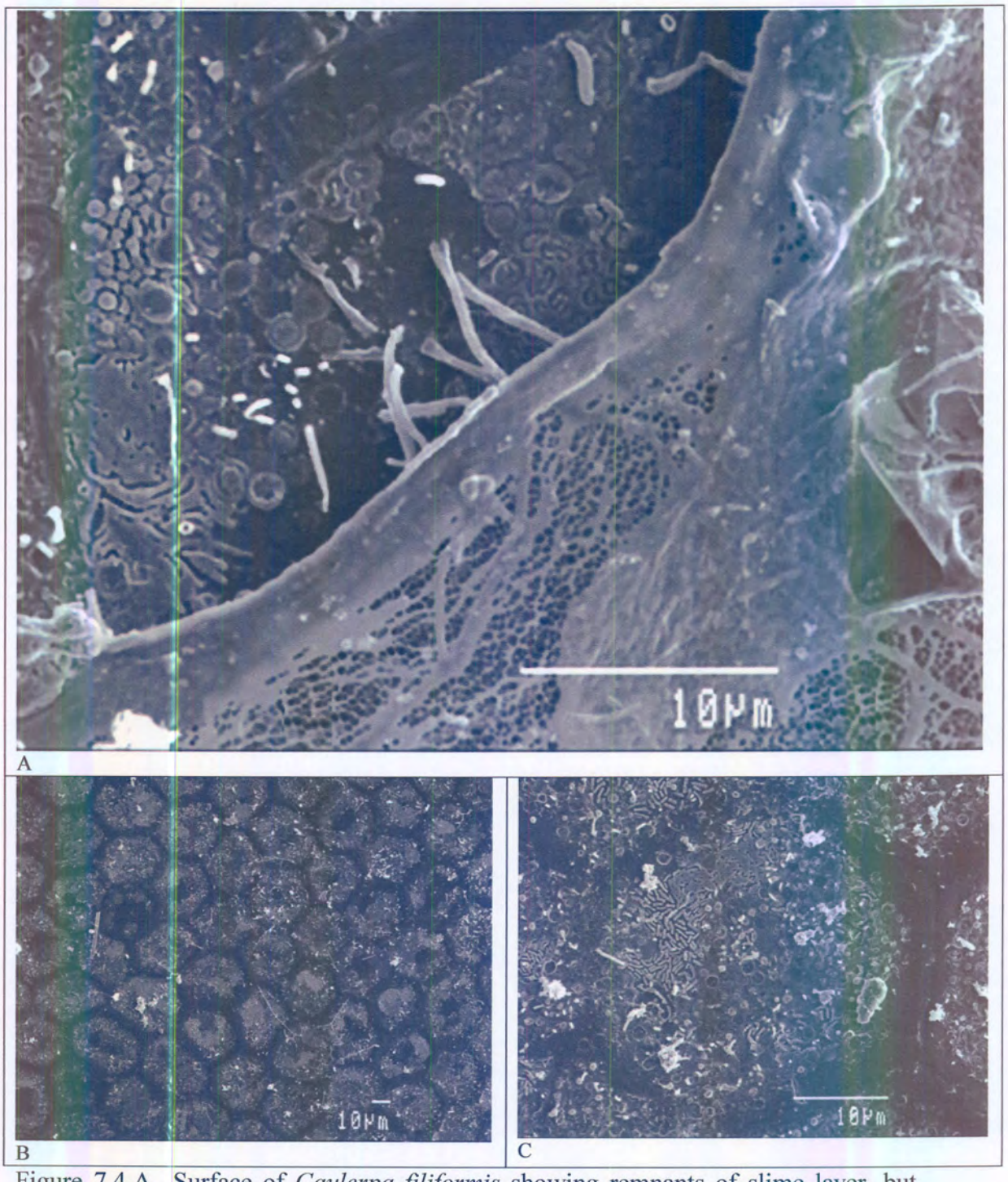


Figure 7.4.A Surface of *Caulerpa filiformis* showing remnants of slime layer, but removal of cocoid cells during processing of the sample. The tissue was treated with OsO_4 vapour.

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

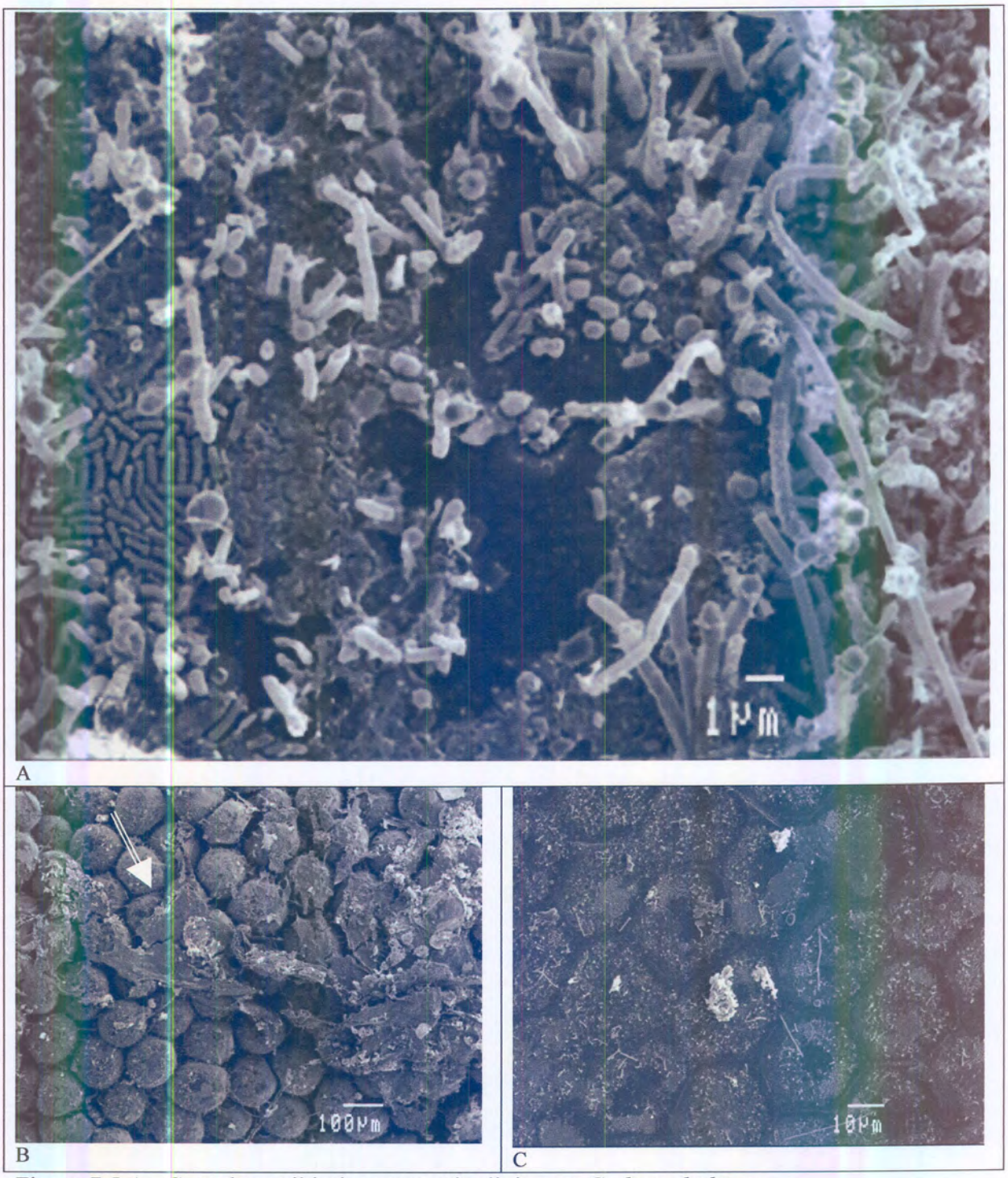


Figure 7.5.A. Complex epibiotic community living on *Codium duthieae*.
B. Remnants of slime layer over utricles (arrow) of *C. duthieae* treated with OsO_4 before glutaraldehyde fixation.
C. Outline of *Halimeda cuneata* epithelial cell. No bacteria were living in the margins of the algal cells.

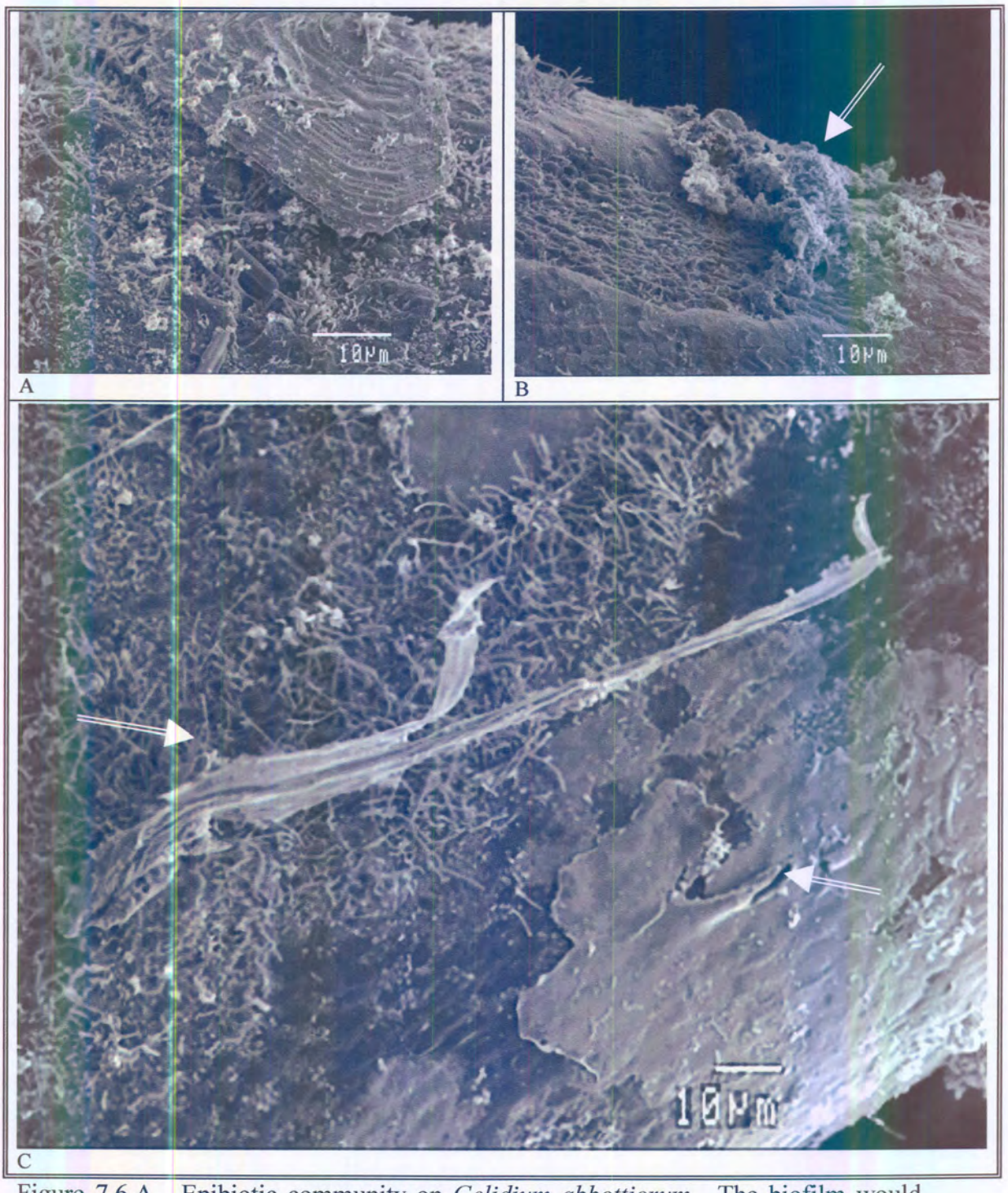


Figure 7.6.A. Epibiotic community on *Gelidium abbottiorum*. The biofilm would have been covered by the EPS, the remnants of which are visible near the top of the micrograph.
B. Micro-community living in a depression on the surface of the red alga *G. abbottiorum*. Note the remnants of the EPS (arrow).
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).

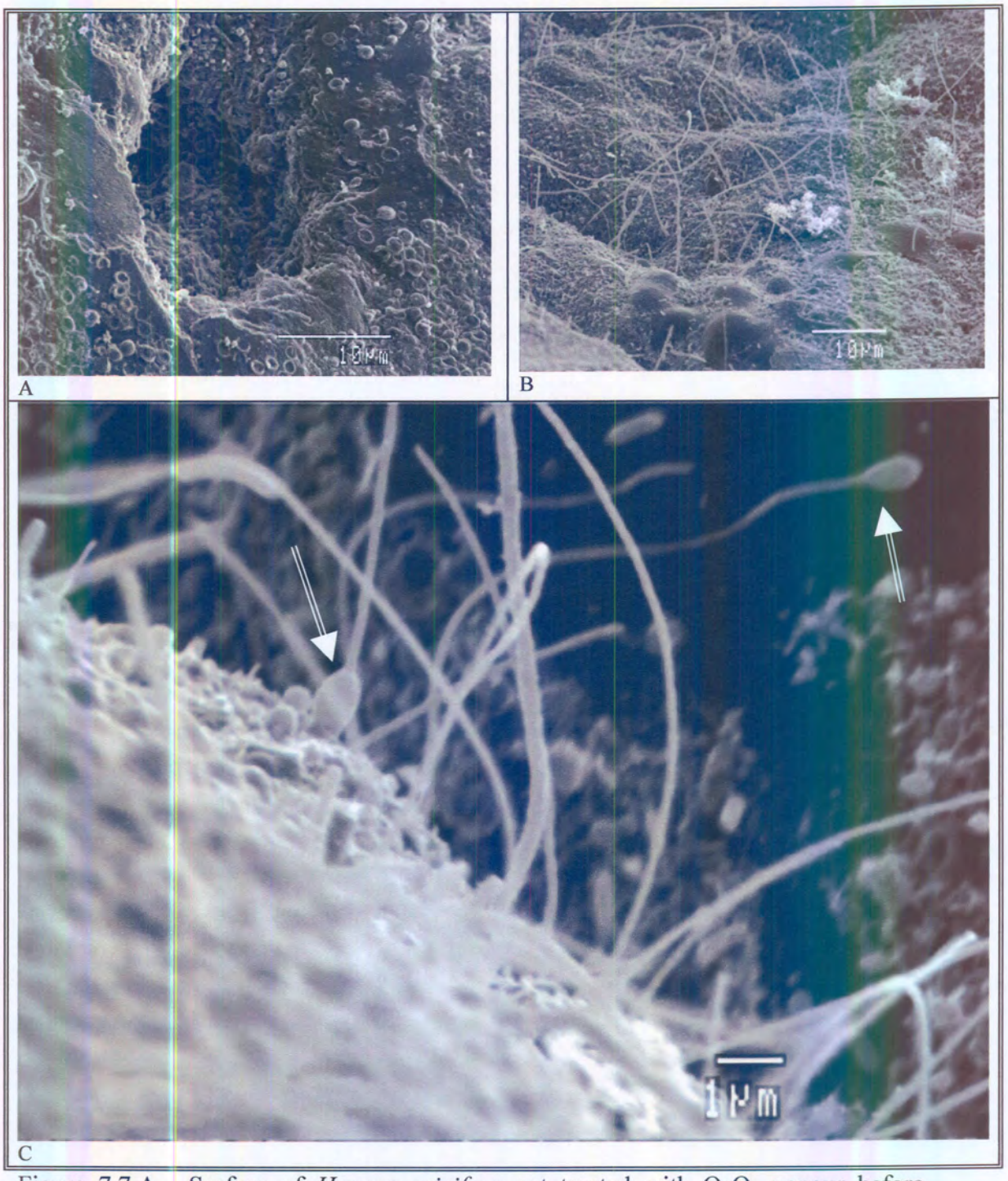


Figure 7.7.A. Surface of *Hypnea spicifera* not treated with OsO_4 vapour before glutaraldehyde fixation.
B. Surface of *H. spicifera* treated with OsO_4 vapour before glutaraldehyde fixation.
C. Sperm cells (arrows) found on the surface of the red alga *H. spicifera*. This may represents an important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

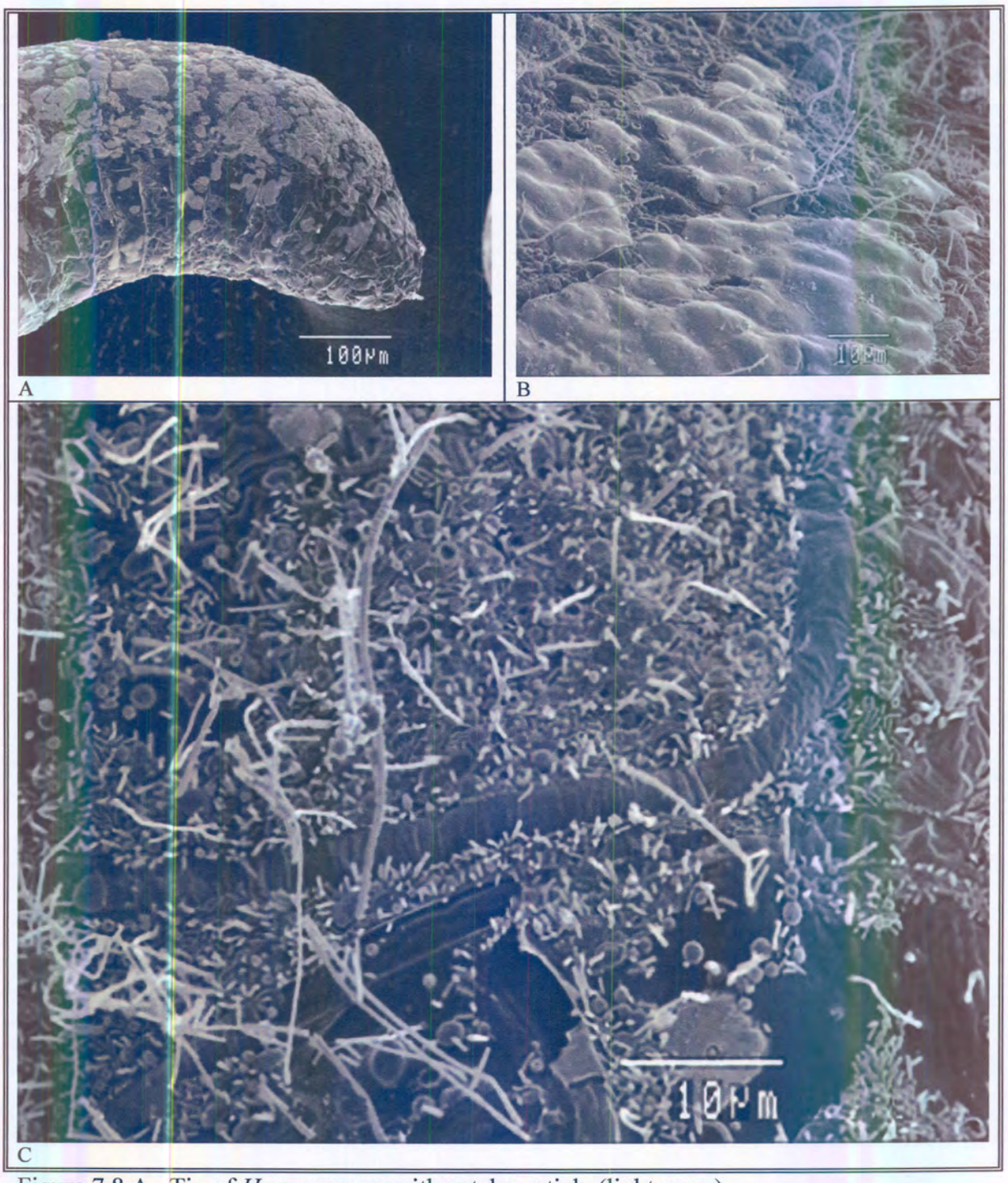


Figure 7.8.A. Tip of *Hypnea rosea* with patchy cuticle (light areas).
B. Close-up of cuticle patches on *H. rosea*. No bacteria were growing on the cuticles, whereas next to them there is a biofilm.
C. Surface of *H. rosea* with track made through biofilm. This grazing of the biofilm indicates a complex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

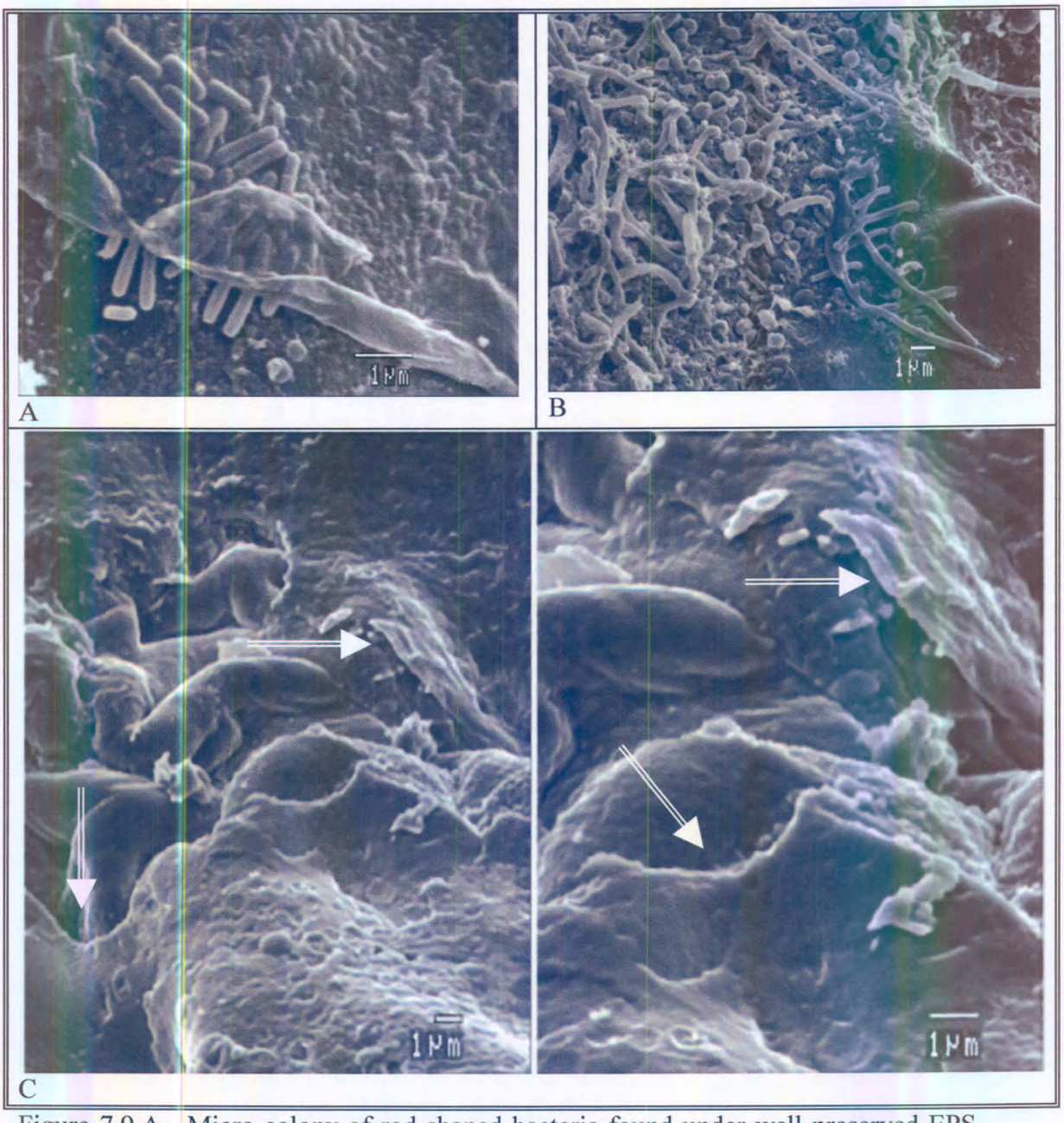


Figure 7.9.A. Micro-colony of rod-shaped bacteria found under well preserved EPS layer on the surface of *Osmundaria serrata*. The layer had folded back to expose the bacteria. This tissue was treated with OsO_4 before glutaraldehyde fixation.
B. Community of bacteria found on *O. serrata*. The slime 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).

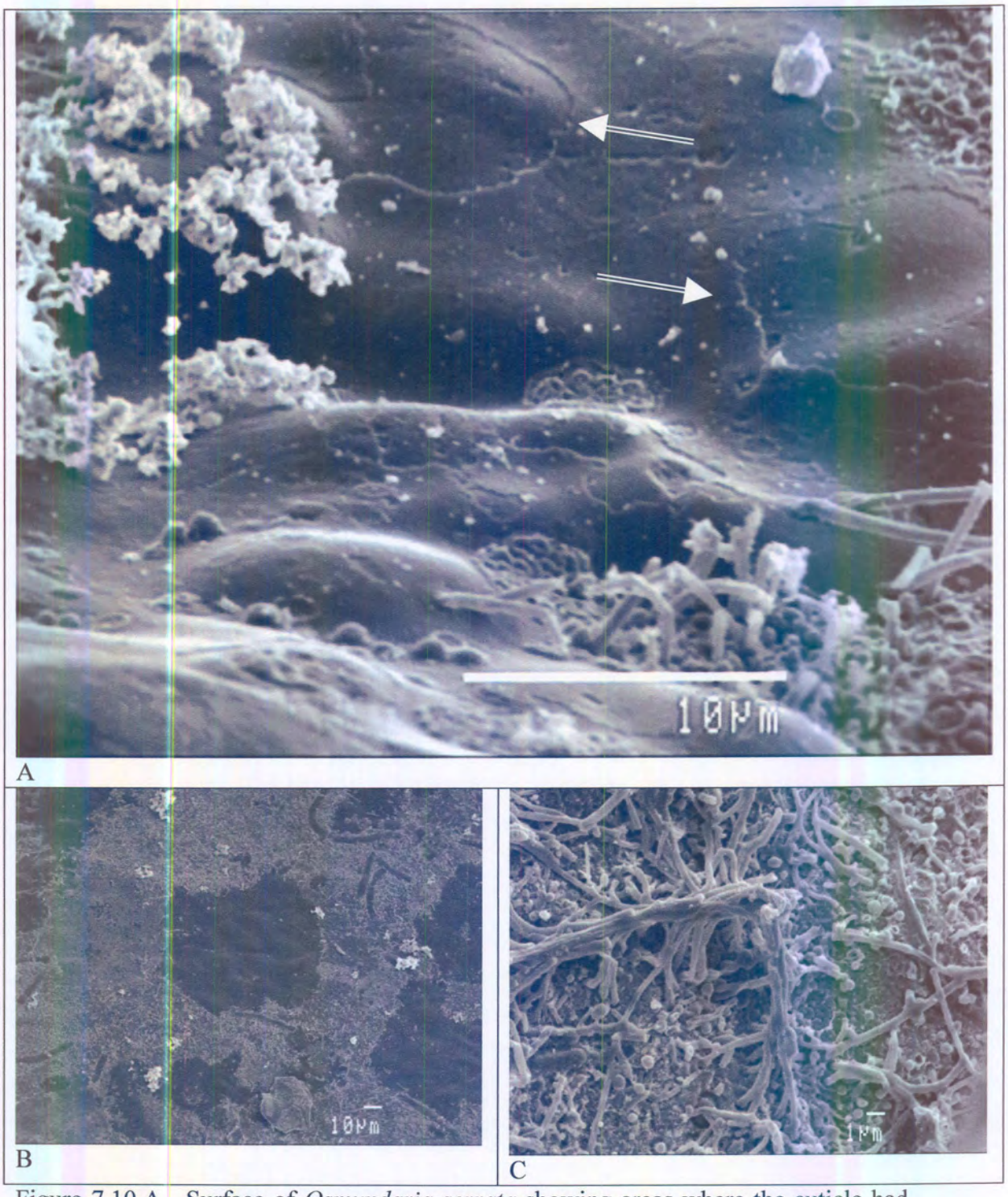


Figure 7.10.A. Surface of *Osmundaria serrata* showing areas where the cuticle had peeled off (arrows).
B. Surface of *O. serrata* showing relatively large patches (dark areas) free of epibiota. The lighter areas are part of a (possibly a snail; ZoBell and Feltmam, 1938).
C. Complex epibiotic community 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.

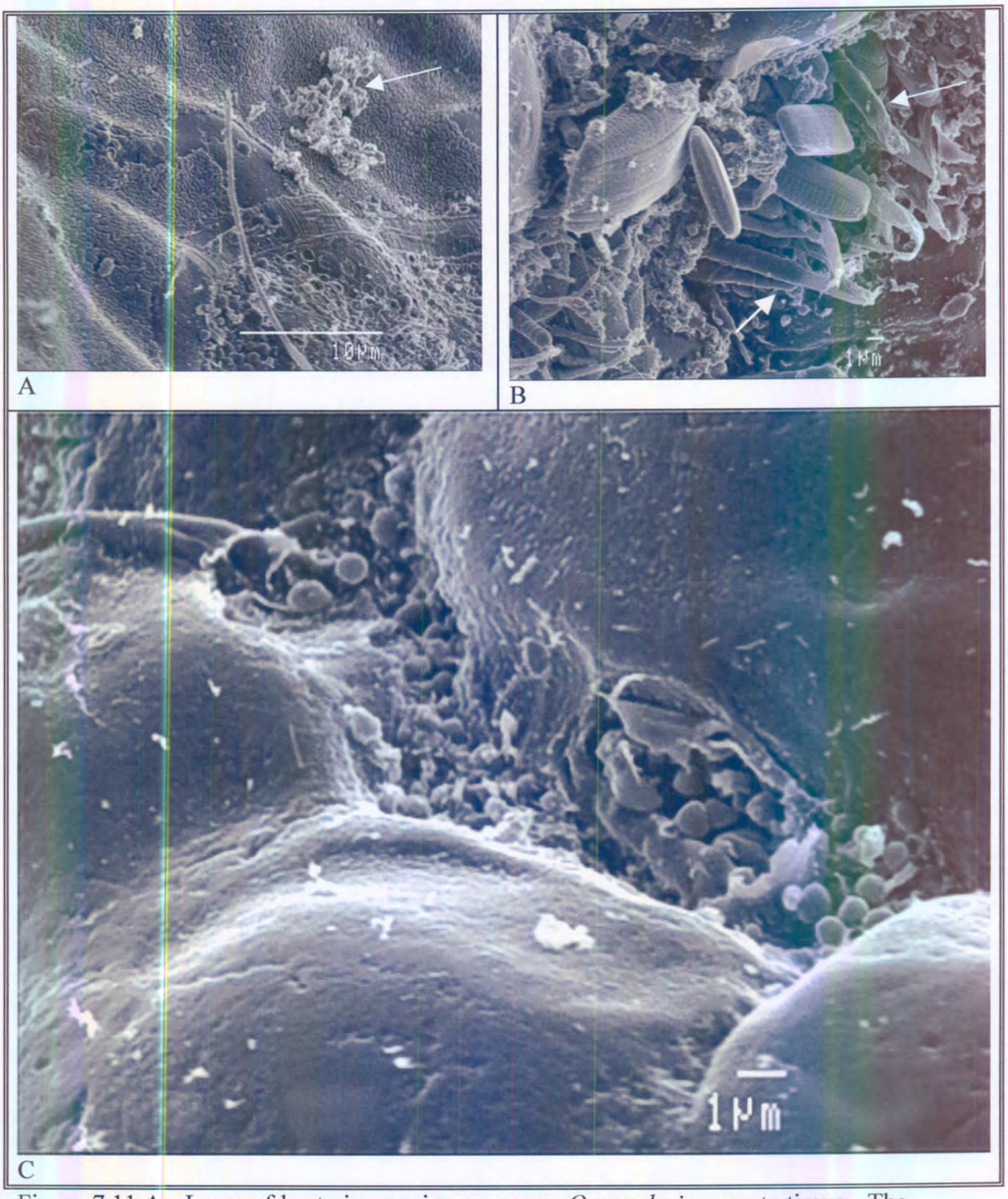


Figure 7.11.A. Lawn of bacteria growing on young *Osmundaria serrata* tissue. The slime layer was removed (remnant – arrow) and exposed the bacteria. The C-shaped bacteria in the upper portion of the micrograph resemble *Cyclobacterium marinum*.
B. Part of the complex biofilm on *O. serrata*. Micro-colonies 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.

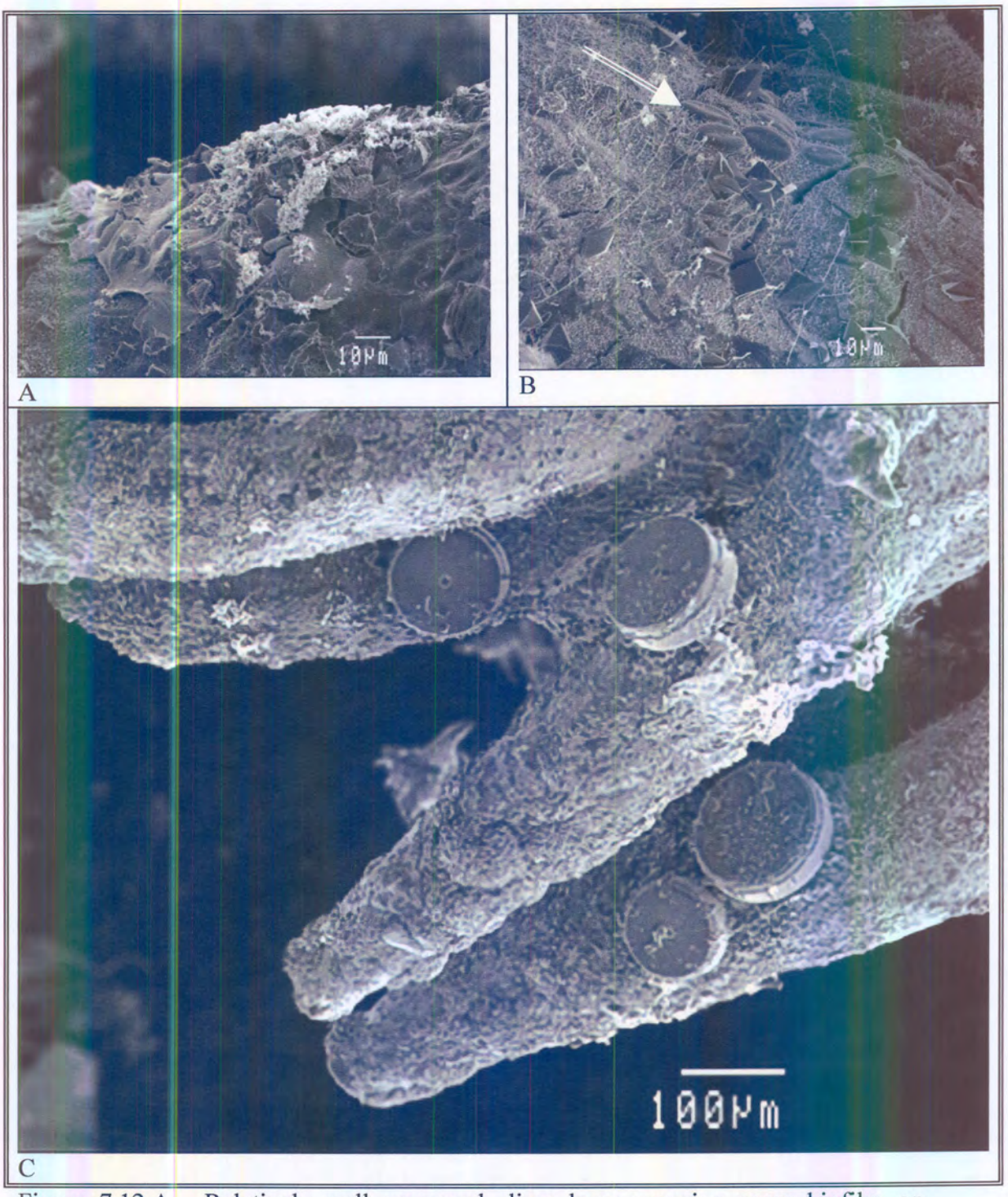


Figure 7.12.A. Relatively well preserved slime layer covering some biofilm on *Spyridia cuppressina* tissue treated with OsO_4.
B. Micro-colony of the diatom *Campyloneis* sp. (arrow) embedded in the biofilm on *S. cuppressina*.
C. Round diatoms of the genus *Thalassiosira* found on near the tips of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

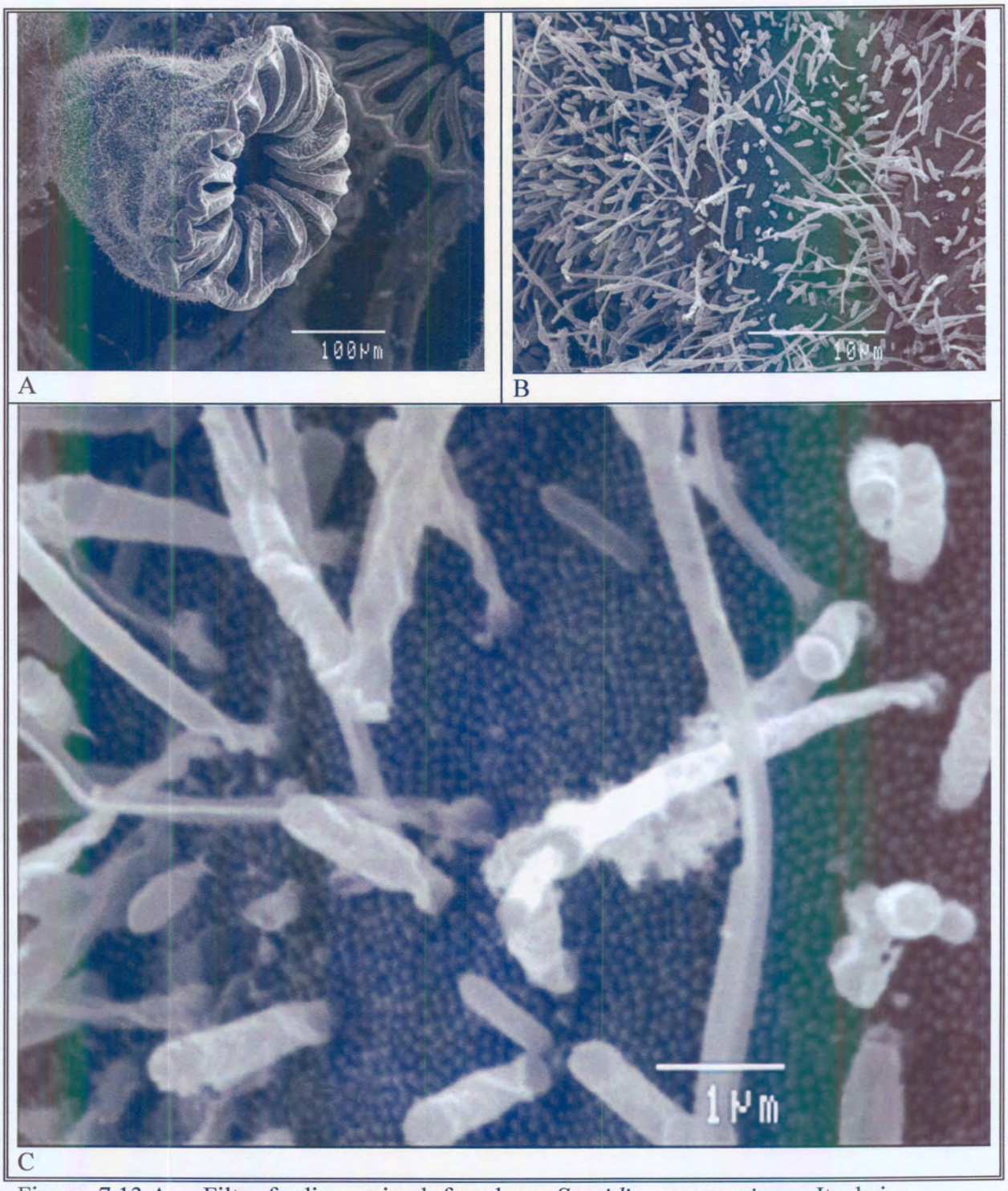


Figure 7.13.A. Filter-feeding animal found on *Spyridia cuppressina*. Its hairy appearance is due to filamentous bacteria growing on its surface.
B. Filamentous bacteria growing on the surface of a filter-feeding animal that was found on *S. cuppressina*.
C. Close-up view of the surface of the filter-feeding 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.

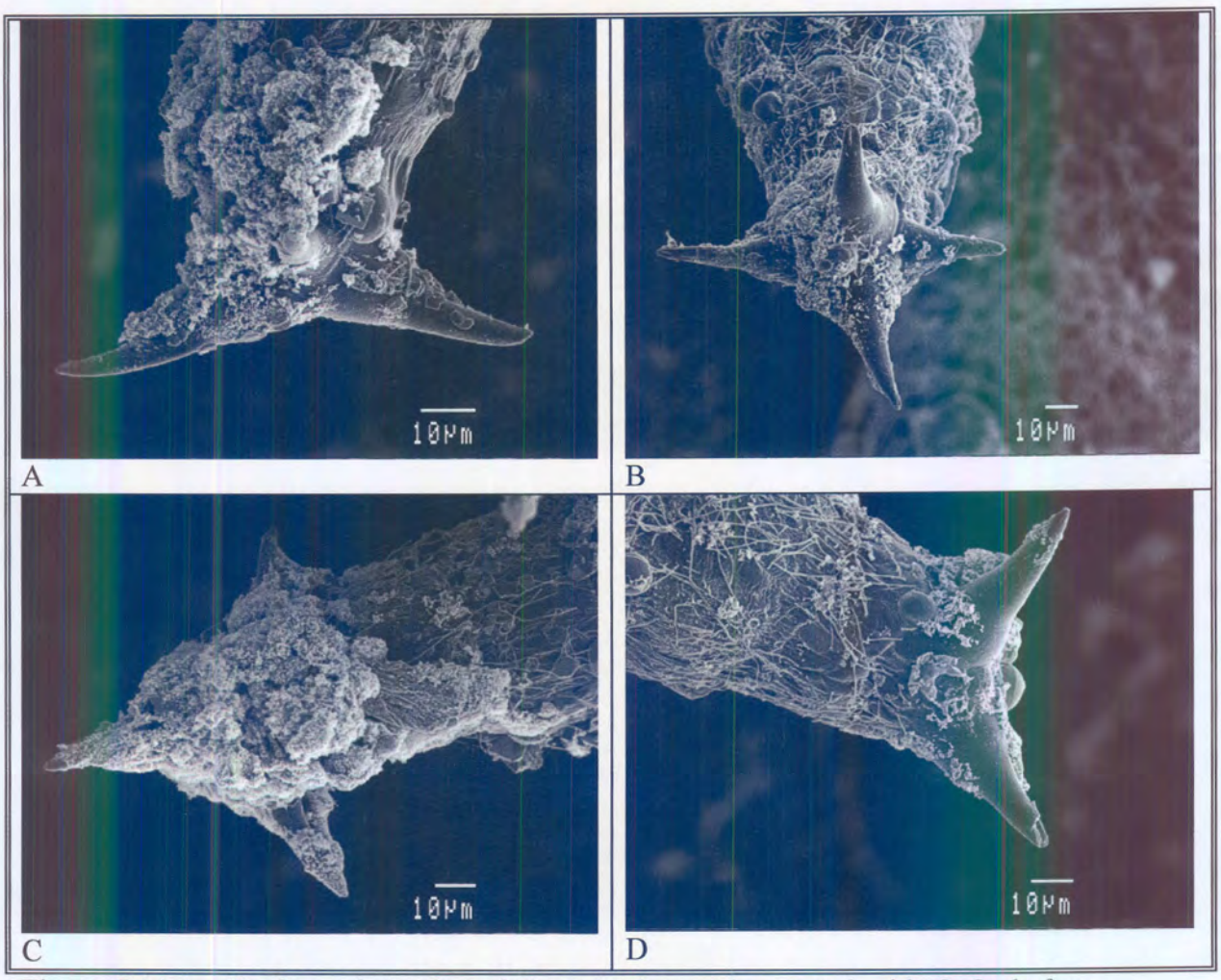


Figure 7.14.A. Surface of the tips of *Spyridia hypnoides* treated with OsO_4 before fixation in glutaraldehyde. A very thick biofilm nearly covering the structure. The slime layer had dried in places to form "fluffy" objects.

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

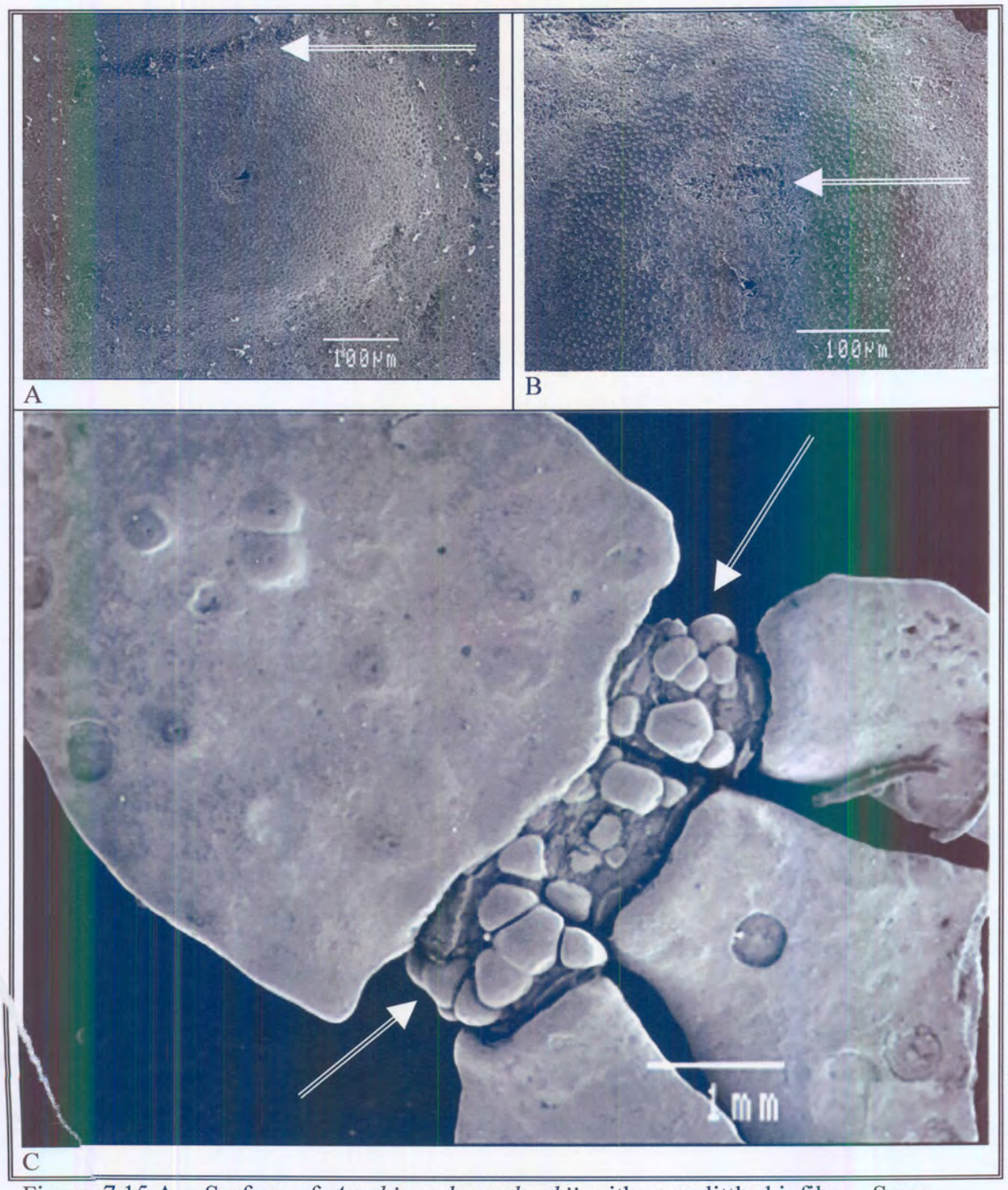


Figure 7.15.A. Surface of *Amphiroa bowerbankii* with very little biofilm. Some bacterial cells were growing in a protected pocket at the edge of the conceptacle (arrow).
B. Surface of *A. ephedraea* with almost no biofilm. Some bacterial cells are seen near the pore of the conceptacle (arrow).
C. Macro-view 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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APPENDIX

PRESENTATIONS ON CD-ROM

The CD-ROM in the pocket attached to the back of the thesis contains three animations created using Macromedia Flash MX. The seaweed material was prepared in the same way as in chapter 7.

In the first animation the viewer zooms into the surface of a segment of *Osmundaria serrata*. The diversity of a small portion of the biofilm on this seaweed is shown. The second animation zooms into the tip of a serration of *O. serrata* to show damage by waves and sand. In the third animation the viewer zooms into the surface of *O. serrata* where sand particles embedded in the biofilm is seen. The image then pans across the surface of the seaweed showing more of the biofilm bacteria.

A Microsoft PowerPoint presentation is also included and is a synthesis of three conference presentations of the work: the South African Association of Botanists meeting in Johannesburg, South Africa (SA), 2001, the Phycological Society of Southern Africa meeting in Cape Town, SA, 2002 (where the SASCA award was won) and the Indigenous Plant Use Forum meeting in George, SA, 2002.

To view the animations Flash software has to be installed on your computer. If it is not then you should be directed by your browser to the site where you may download it. If your browser does not direct to the Flash download site then please go to the following site:

http://download.macromedia.com/pub/shockwave/cabs/flash/swflash.cab#version=6,0,29,0

The animations may run slowly if you have less than 128 MB of RAM in your computer. If this is the case please try to increase the size of the virtual memory (the amount of memory that Windows can use on your hard drive as RAM) your computer uses. To do this go to Control Panel > System Properties > Performance Options > Advanced (this may be slightly different for different versions of Microsoft Windows.) This should speed up memory hungry applications (like those that use graphics), but makes the hard drive work harder. It is not recommended for long-term use because of the wear on the parts of the hard drive.

The animations and PowerPoint presentation supplement the work in the thesis and is intended to enrich the viewer's experience of the thesis. With the animation an extra dimension is added to the still micrographs; the illusion of movement over the seaweed surface. In this way the viewer may easily compare different areas and become aware of the size of the biofilm bacteria compared to the seaweed. The latter was the initial motivation for making the animations.