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.

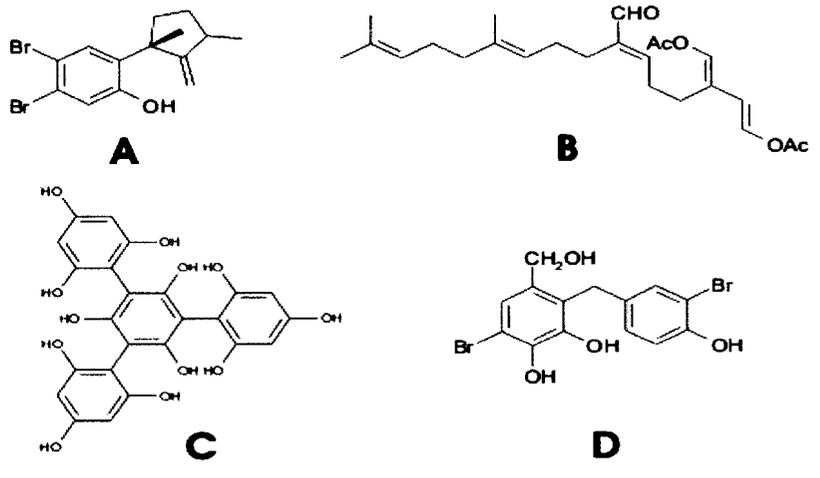

Figure 1.1 The structures of some compounds produced by macroalgae with growth inhibitory activities (Fenical, 1975; Hay, 1992; Paul, 1992b; Steinberg, 1992). **A** = isolaurinterol from *Laurencia intermedia* (Rhodophyta); **B** = Udoteal from *Udotea* spp. (Chlorophyta); **C** = tetrafucol B from *Fucus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

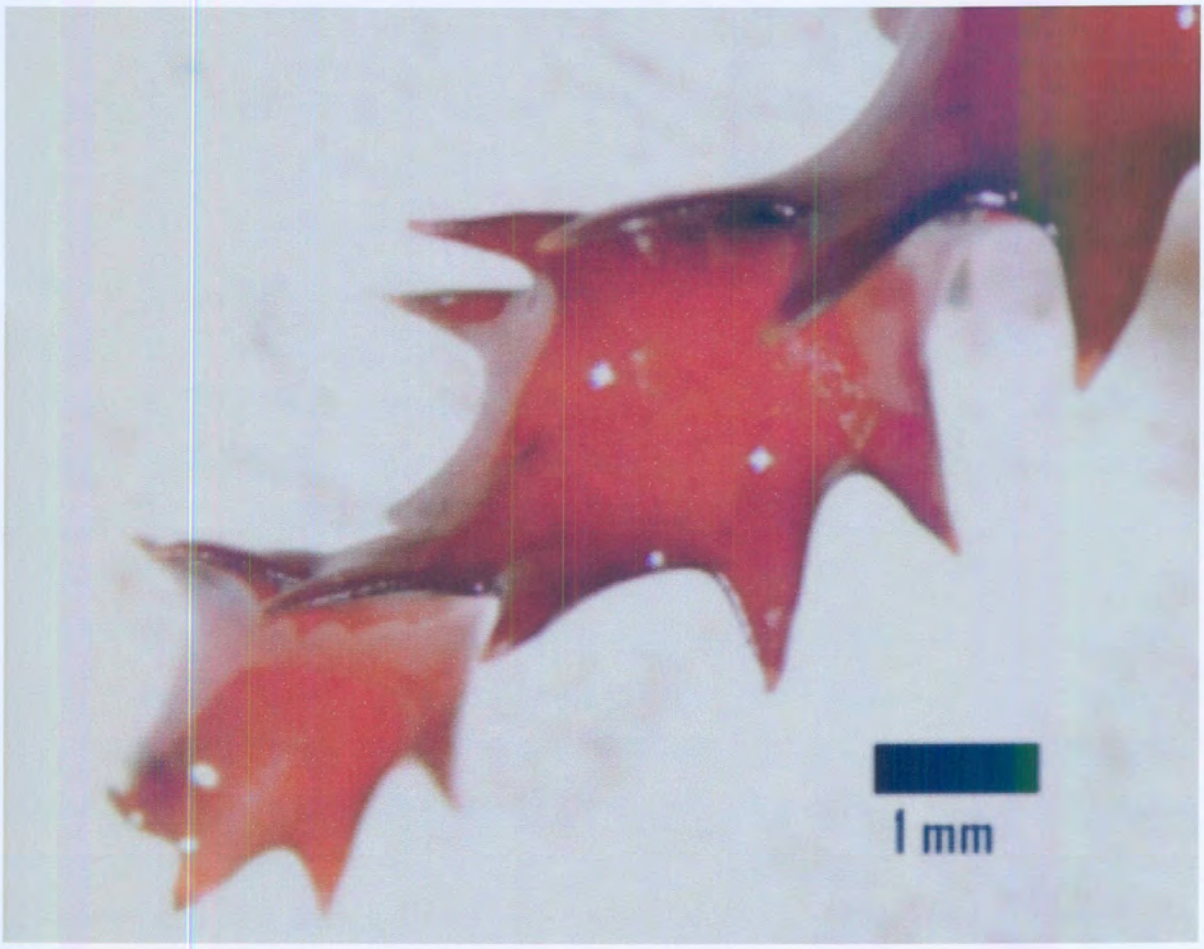

Figure 1.3 The spiralled and serrated blade of *Osmundaria serrata*. The lighter coloured tip is to the bottom left.

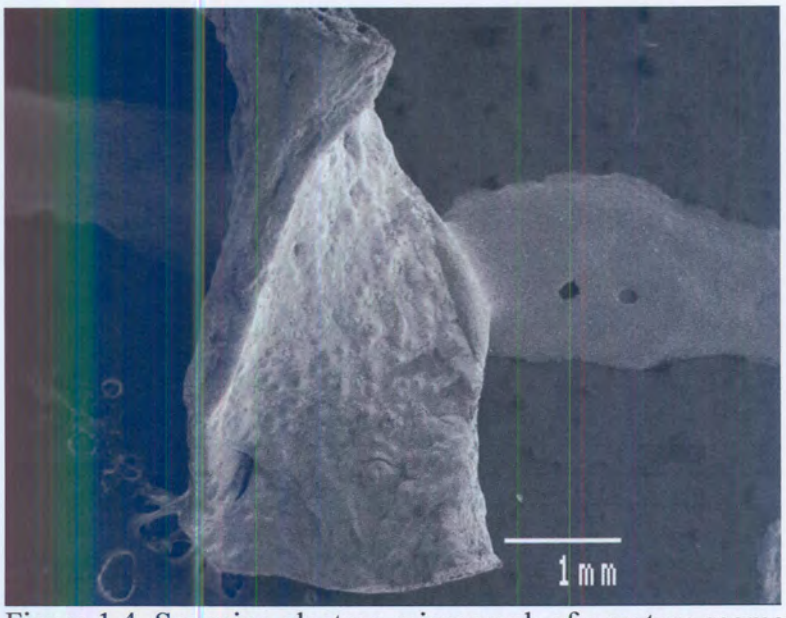

Figure 1.4 Scanning electron micrograph of a mature segment of *Osmundaria serrata* blade with no serrations and a thickened thallus.

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.

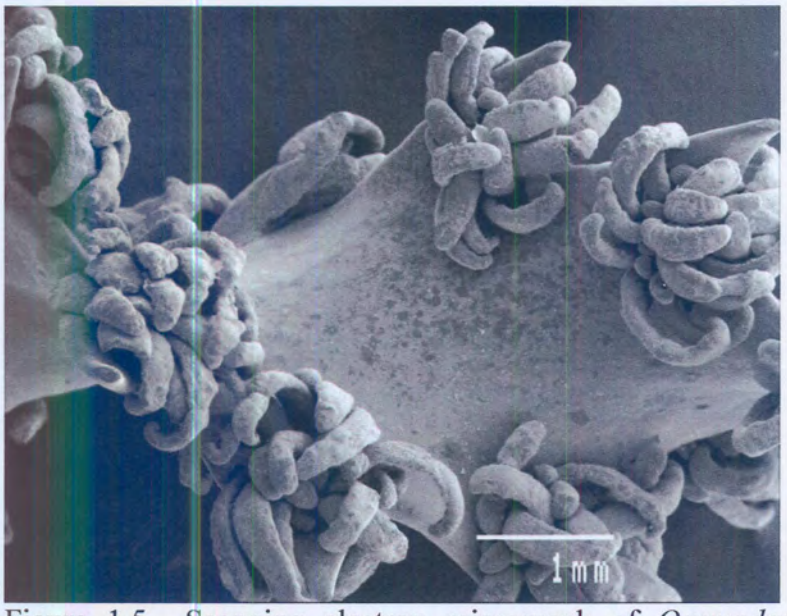

Figure 1.5 Scanning electron micrograph of *Osmundaria serrata* showing the tetraspores producing stichidia found along the edge of the blade.

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.

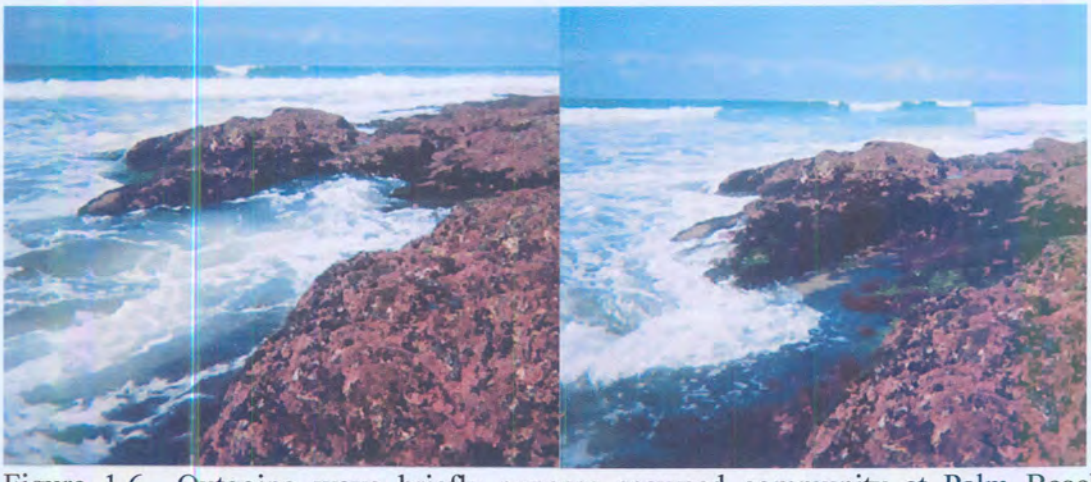

Figure 1.6 Outgoing wave briefly exposes seaweed community at Palm Beach, KwaZulu-Natal, South Africa during a Spring tide low.

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*	

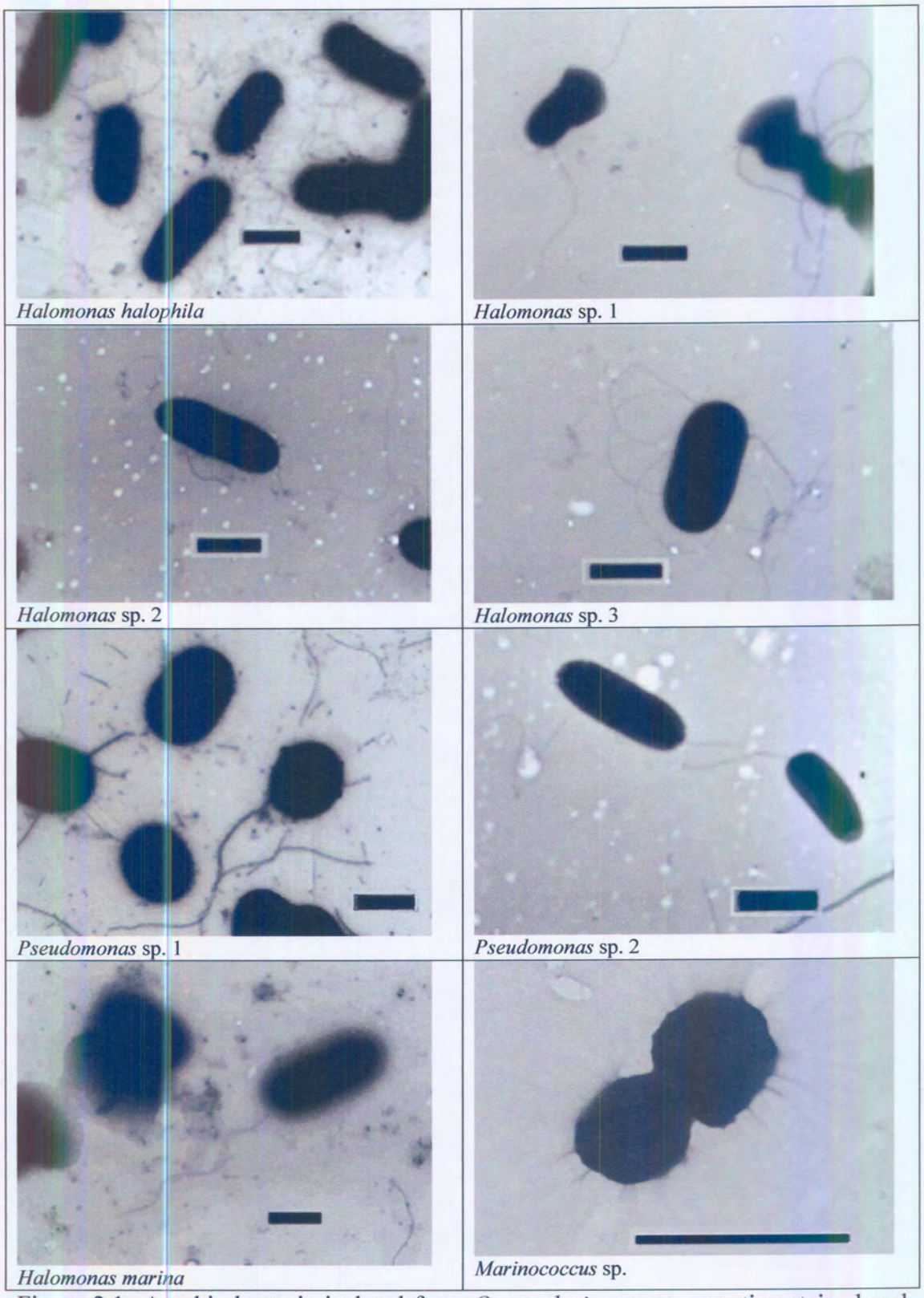
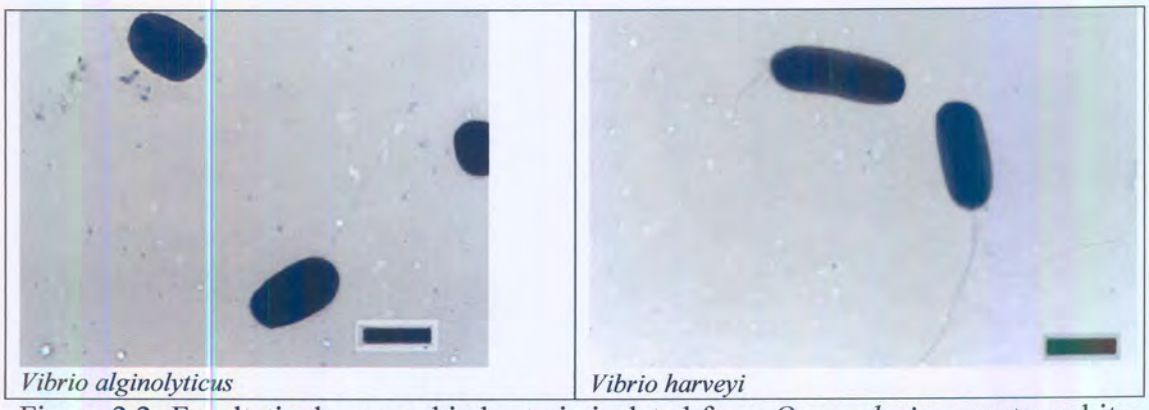


Figure 2.1 Aerobic bacteria isolated from *Osmundaria serrata* negative stained and viewed under a transmission electron microscope. Bars = 1 µm.

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).

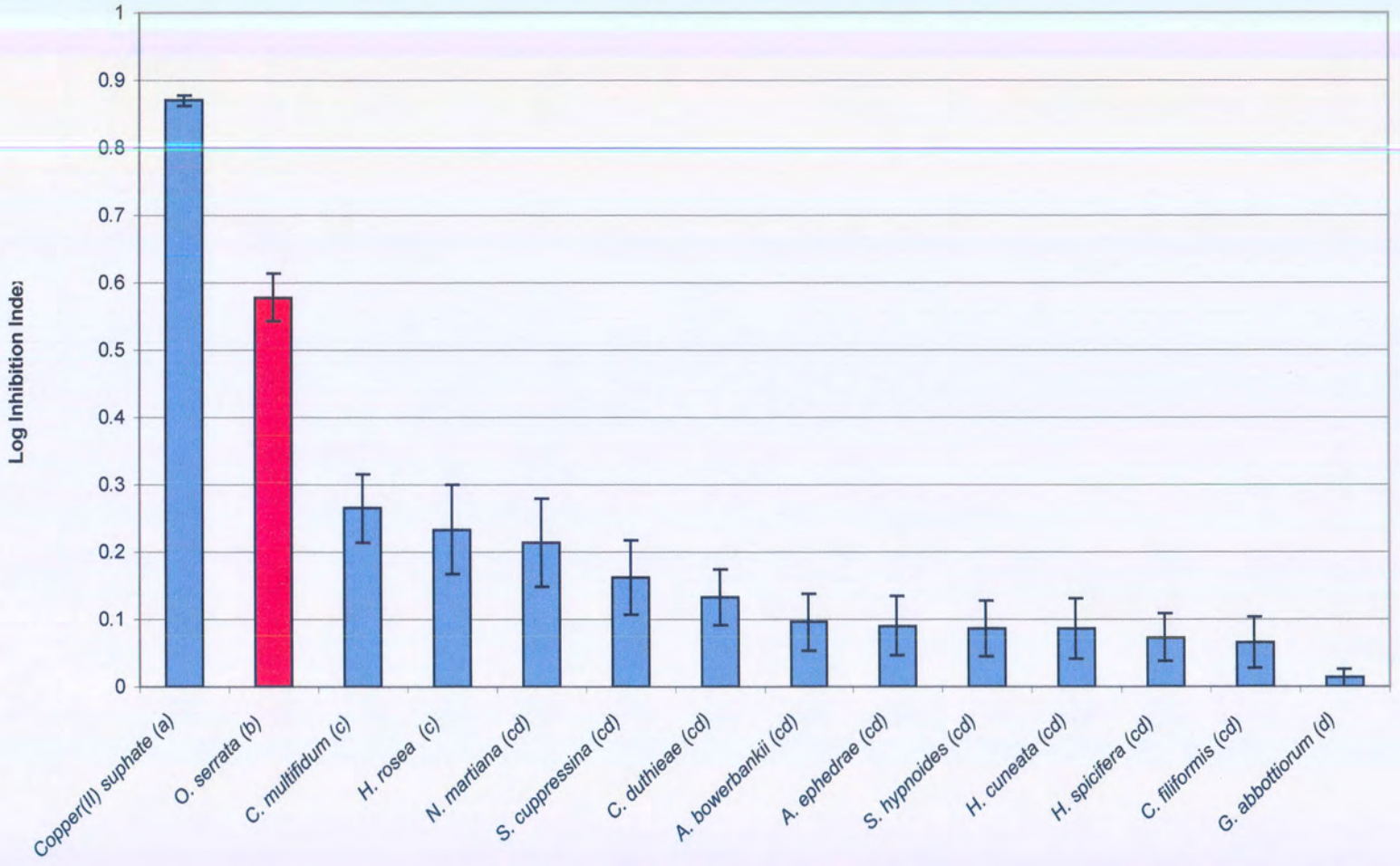

Figure 4.1 Biological activity of macroalgal extracts and $CuSO_4$ (positive control). High values indicate high inhibition of bacterial growth. Different letters indicate significant ($\alpha \leq 0.01$) differences between bacteria. Bars = SE, $n = 23$ different bacteria, see table 5.1 for full species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

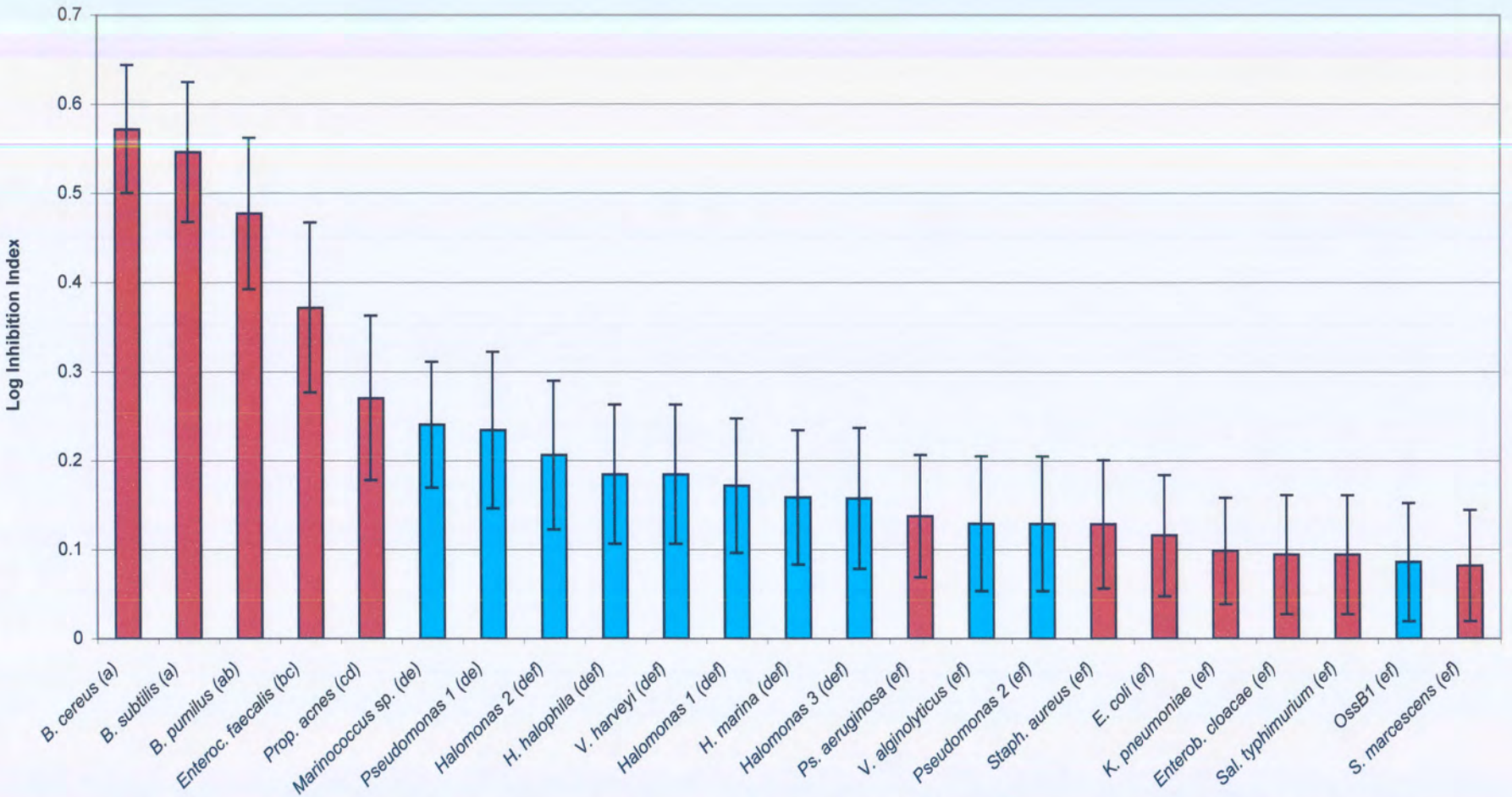

Figure 4.2 Average response of test bacteria to all macroalgal extracts and $CuSO_4$ (positive control). High values indicate high inhibition of bacterial growth. Different letters indicate significant ($\alpha \leq 0.01$) differences between bacteria. Bars = SE, n = 13 test substances, brown bars =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

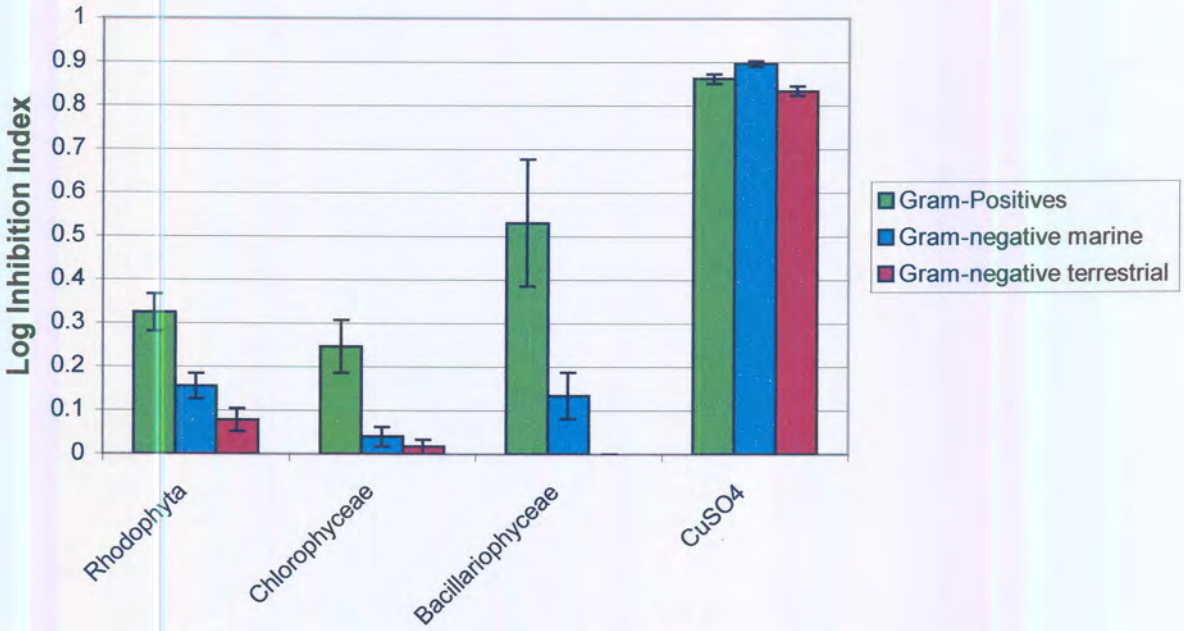

Figure 4.3 Biological activities of the groups of test substances used in the study against the groups of bacteria used in the bioassays. Bars = SE.

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.

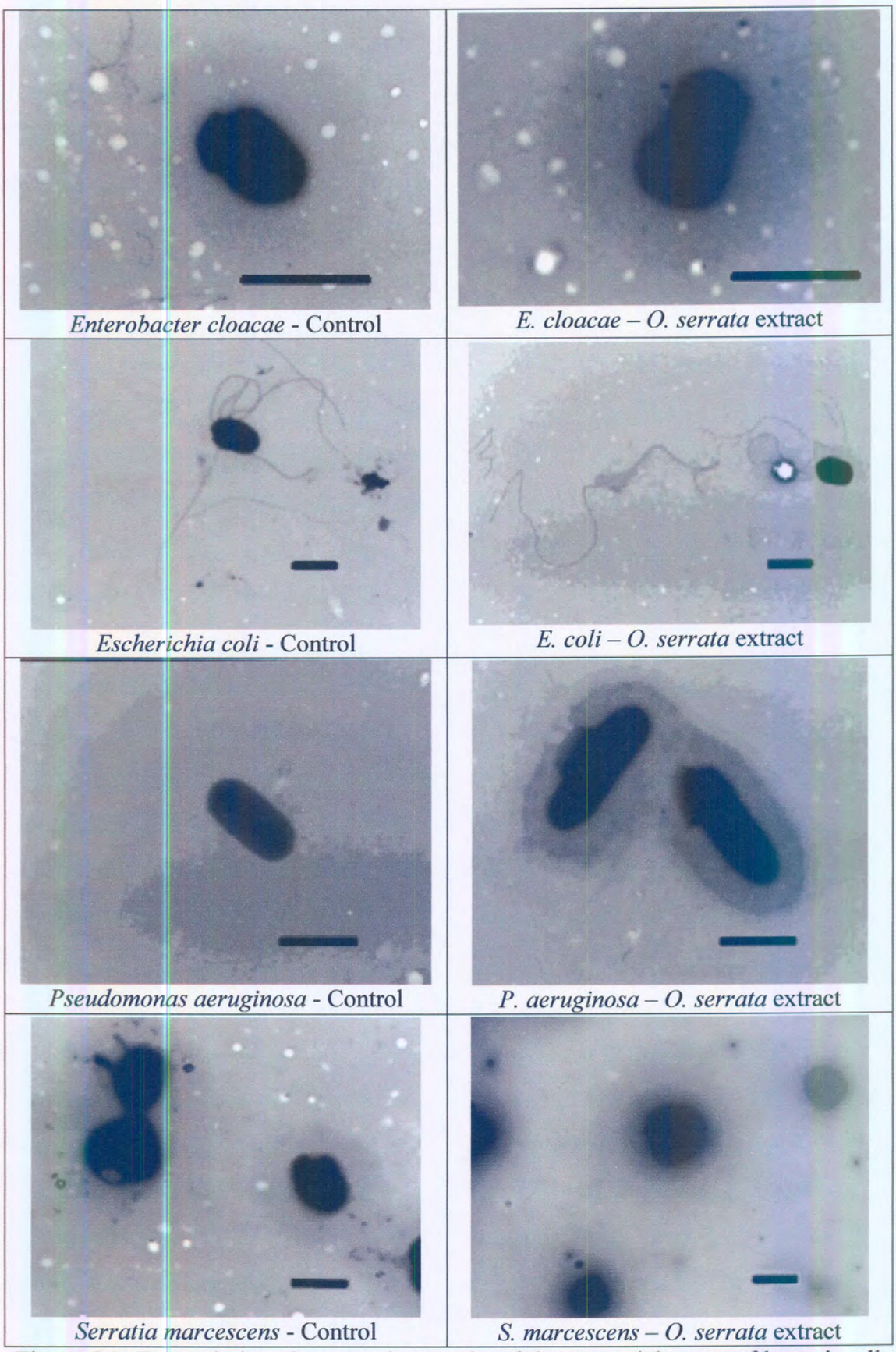

Figure 5.1 Transmission electron micrographs of the terrestrial group of bacteria, all potential human pathogens, in response to an extractive from *Osmundaria serrata*. Scale bars = 1 μm.

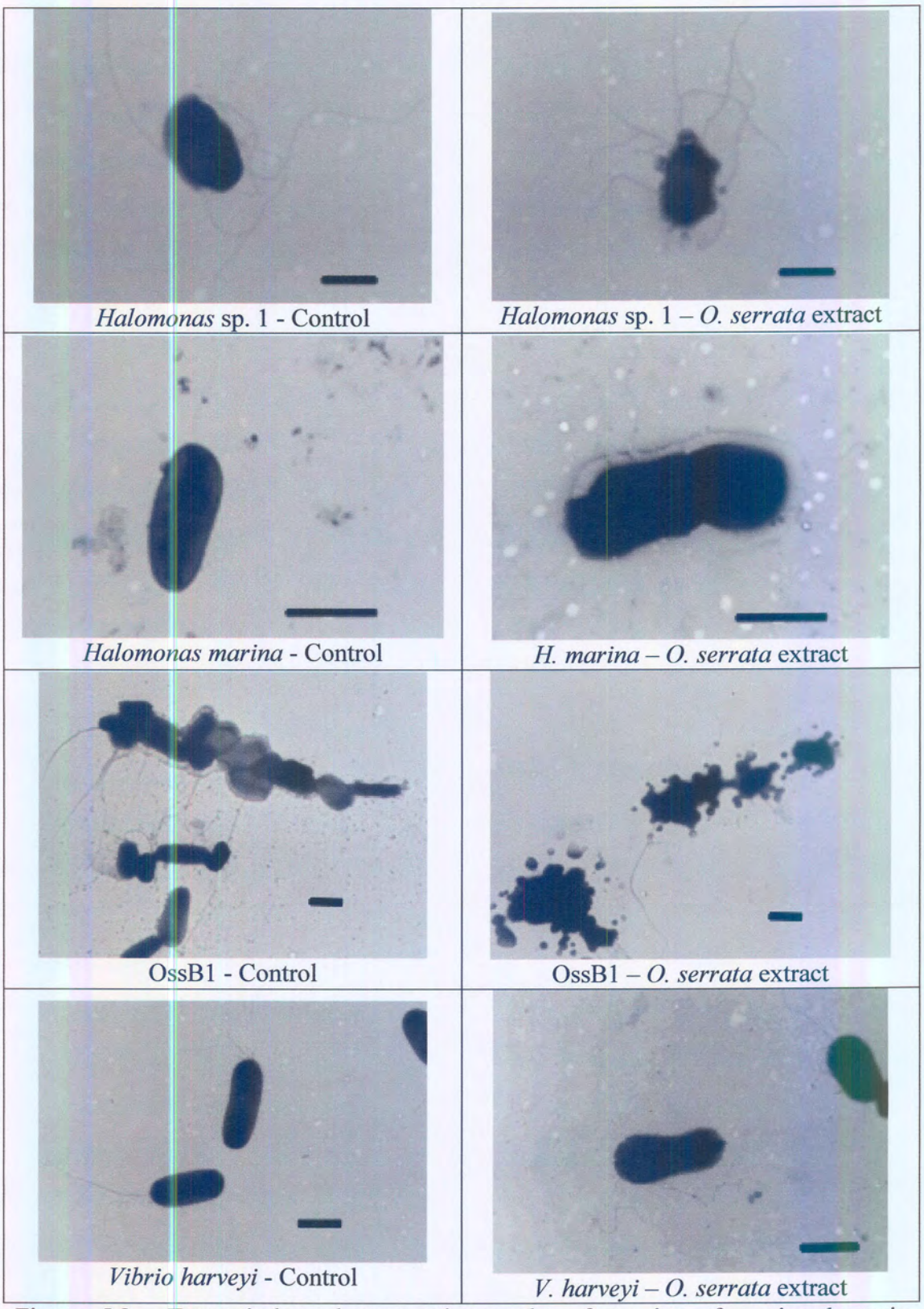

Figure 5.2 Transmission electron micrographs of species of marine bacteria *Halomonas* isolated from the surface of *Osmundaria serrata* in response to an extractive from the alga.

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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