THE EXPERIMENTAL CULTIVATION AND ALGINATE PROPERTIES OF SOUTH AFRICAN KELPS.

by

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DECLARATION

The work described in this thesis was carried out under the supervision of Prof. D.W. Keats of the Botany Department, University of the Western Cape, and Dr. R.J. Anderson, of Marine and Coastal Management.

I declare that "Experimental cultivation and alginate properties of South African kelps" is my own work and has not been submitted in this or any other form to another university. Where use has been made of the work of others, it has been acknowledged by means of complete references.

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Signature: M. N. Harper

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To my family, the guiding influence in my life and to Shawn.

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CHAPTER 1:

1.1. LITERATURE REVIEW

1.1.1. INTRODUCTION

Seaweeds belong to the three classes, Chlorophyceae, Rhodophyceae and Phaeophyceae with the last two classes being the most important commercially. Seaweeds form a raw material that is easily available has been used for a multitude of purposes either in food or industrial applications. Asian countries, especially Japan and China, are the largest consumers of seaweed products; more than 400 species are commercially used (Critchley *et al*, 1998). More and more research is being done on seaweeds and their extracts and advances in this research will promote the sustainable use of coastal waters.

There are three types of phycocolloids namely agar, carrageenan and alginate; these are used as thickeners, emulsifyers and gelling agents in the different industries (Anderson *et al*, 1989). Alginate is the main compound produced from brown algae on a commercial basis (Heydraud *et al*, 1990). Alginates are anionic polysaccharides composed of homopolymeric regions of β-D-mannuronic (M) and α-L-guluronic (G) acids (called "M-blocks" and "G-blocks") interspaced with regions of mixed sequence ("MG-blocks"). Alginate is an important product in the hydrocolloid as well as in the biomedical industry. This is to ensure that products obtained will be reproducible. Therefore it may be useful to develop a method to grow kelp in mariculture to improve the quality and quantity of alginate produced.

1.1.2. USES OF ALGINATES

Alginates are used in large quantities by various industries. They are widely used as additives in various pharmaceutical products. In the medical field, alginates provide a protective covering to capsules and tablets and are used in the making of casts and bandages (Kasloff, 1990). In the dental profession alginate impression material is used

to make dental casts for diagnostic purposes. When mixed with water sodium alginate forms a sol (water-like solution), then mixed with a reactor like Calcium it produces an insoluble calcium – alginate in the form of a gel. This gel is then used to make intraoral impressions from which gypsum casts can then be made. The dentist then used the casts to plan dental procedures and design prosthetic devices (Phillips, 1982).

Alginate-based wound dressings, haemostatic agents or swabs have been used on burn victims for many years. Calcium alginate, which is insoluble in water, has been used in the manufacture of a medical dressing very suitable for burns and extensive wounds where a normal dressing would be extremely difficult to remove (Kasloff, 1990). The calcium alginate is extruded to make a fibre, which is then woven into a gauze-like product; alginates with a high proportion of guluronic acid blocks are most suitable for this purpose (Kasloff, 1990). When applied to either a wound or burn, a network is formed around which a healthy scab may form; the bandage may be removed with a sodium chloride solution, which renders the alginate soluble in water. Before this development the physical removal of dressings caused considerably pain and disruption to wounds (Kasloff, 1990; Reynolds and Prasad, 1982). Many medical advances using alginates have taken place during the last few years including bioimplantation and tissue engineering.

It has been shown that membranes made from pure alginate hydrogels may protect encapsulated cells or tissues from a foreign immune system (Colton, 1995). This immuno-isolation by semi-permeable alginate membranes shows a potential strategy to overcome the host immune response that is observed when transplanting allogenic or xenogenic graphs (Pfefferman *et al*, 1996; Colton, 1995). However, in the past few years *in vivo* and *in vitro* studies of biocompatibility of alginates have been performed, and mitogenic stimulations of lymphocytes, induction of pro-inflammatory cytokines, foreign body reaction and fibrosis have been reported (Pfefferman *et al*, 1996; Klöck *et al*, 1994). Microencapsulation of hormone-producing cells in Ca2+ - and Ba2+ - cross-linked alginates has also been used in the treatment of Diabetes mellitus, liver and parathyroid diseases. However, experiments with animals have shown that foreign-body reactions

occur after implantation of alginate-based capsules. These may result in failure of cellular functions of the transplants some time after in-vivo application (Klöck *et al*, 1994; Pfefferman *et al*, 1996; Hasse *et al*, 1997). Hasse *et al* (1998) has reported on the first clinical application of microencapsulation technology. Microencapsulated parathyroid tissue was transplanted into rats with parathyroid disease affecting calcium metabolism. A third of the rats received parathyroid tissue within mitogenic alginate, a third coated with amitogenic alginate and a third uncoated. The amitogenic alginate was obtained using patented electrophoretic methods (Zimmerman *et al*, 1992; Klöck *et al*, 1994). After 6 months the transplants were excised and animals that received nonmicroencapsulated parathyroid glands showed a transient increase of serum calcium, those with microencapsulated parathyroid tissue showed calcium levels within the normal range. Animals in the amitogenic group showed calcium levels slightly lower than those in the mitogenic group until the 11th week after transplantation. After this the median concentrations of calcium in those animals with parathyroid tissue encapsulated in amitogenic alginate were consistently higher than those of the mitogenic group. Six months after the transplant all animals in the amitogenic group showed normal levels of calcium compared to 7 animals in the mitogenic group (Hasse *et al*, 1997). The pure or amitogenic alginate showed better results.

For transplantation the alginate gels must show mechanical and chemical stability, defined (and narrow) pore size distribution, and a absence of immunogenic compounds. The first two requirements are fulfilled when alginates are cross-linked with Ba2+ ions. Alginates cross-linked with Ba2+ ions lead to gel matrices, which are chemically stable under both *in vivo* and *in vitro* conditions (Klöck *et al*, 1994). However commercial alginate, containing phenolic and other mitogenic compounds were used for immobilization of Islets of Langerhans in Ca2+ or Ba2+ cross-linked alginates. The presence of these contaminants was presumably the primary reason for certain processes occurring, which induced fibrotic overgrowth of the beads. Fibrotic overgrowth is cause by the alginate itself, which stimulates the liberation of tumor necrosis factor alpha and interleukins thereby stimulating fibroblast activation (Klöck *et al*, 1994). Several laboratories have examined alginate gel beads for the transplantation of islets of

Langerhans. Such insulin-producing tissue is required to treat diabetes mellitus. Klöck *et al* (1994) reported that microencapsulated Islets of Langerhans implanted beneath the kidneys of rats became unstable due to fibrotic overgrowth and mitogenic activity. This resulted in the random failure of islets some time after implantation. Zimmerman *et al*, (1992) demonstrated that commercial alginate contain impurities that exhibit mitogenic activity in an *in-vitro* test by encapsulating islets in raw alginate hydrogel beads. Random failure of the islets occurred some time after transplantation. Using free flow electrophoresis it was found that commercial alginates contained 10 – 20 fractions that showed mitogenic activity. Removal of these contaminants resulted in alginate preparations that did not provoke foreign-body reactions after cross-linkage with Ba2+ ions, at least 2 weeks after implantation into the peritoneal cavity of rodents (Zimmermann *et al*, 1992).

1.1.3. ECONOMICS OF KELP

In 1993 about 6.36 million tonnes (t) of seaweeds (wet weight) were produced worldwide of which about 65% was used as food. Phaeophyceae accounted for 4 million t of which Chinese production of the food grade kelp *Laminaria japonica* was 2.7 million t and has since climbed to over 3.5 million t (Guiry, 1997). Japan also produces *Laminaria japonica* and in 1995 together with China the total production of this kelp was about 4 million t making *L. japonica* the largest single-species crop produced by aquaculture in the world. Norway processes about 15 000 dry weight t of *Laminaria hyperbororea*, manufacturing about 10 000 t of alginates. Japan produces about 1 400 t alginates from 33 000 t of weed, mainly Laminariales. China, a relatively recent addition to the alginate manufacturers of the world, produced about 10 000 t of alginates in 1994 from cultivated *Laminaria japonica*. The total worth of the international seaweed business exceeds US$ 4.5 billion (Guiry, 1997). Therefore, with an increasing demand for kelp and kelp products South Africa may be able to expand its seaweed industry.

South African seaweeds have been commercially exploited for about 45 years. The industry developed during World War II (Critchley *et al*, 1998), because of a need

for colloids. The kelps *Ecklonia, Laminaria*, and the agarophytes *Gracilaria* and *Gelidium* are the major economically important seaweed resources in South Africa (Share *et al*, 1996). Over the last 3 years South Africa have been importing agar, carrageenan and alginic acids from China, Japan and Norway since South Africa does not produce any alginate locally. South Africa has then been re-exporting them to other African countries (*SARS, 2001).

Kelp beds in South Africa occur from Cape Agulhas along the entire west coast (Critchley *et al*, 1998). In 1994, 456.5 dry tonnes of kelp were collected in South Africa, representing a market value of US $ 2.8 million (Share *et al*, 1996). S. Africa exports dried kelp to China, Japan, the United States of America and Europe for alginate production. In 1999, South Africa exported 58 842 kg of alginic acid to various African countries, earning South Africa R1 067 380,00 in revenue (SARS, 2001) (Fig1.1). The industry is not always so profitable; on average South Africa only exported about 15 000 kg of alginic acid annually over the last 8 years (SARS, 2001) (Fig1.1). In 2000, South Africa imported 107 030 kg of alginic acid for the manufacturing industry (Fig. 1.2), this cost South Africa R3 915 598,00 (SARS, 2001). The cost of importing alginic acid is high (Anderson *et al*, 1989) yet there is presently no local alginate production industry. This is probably due to a lack of investment finance and infrastructure.

Some kelp products are produced locally in Cape Town (Simons Town), by a company called Kelp Products Ltd. They produce fertilizers, a biostimulant for improved plant growth and various other seaweed products. The biostimulant is called Kelpak and is made by a internationally patented Cold Cellular Burst process developed by Kelp Products Ltd., Kelpak is a non-toxic natural product manufactured from *Ecklonia maxima*. It is also biodegradable, beneficial to soils and efficient in stimulating root development and increasing yields of plants (Internet 1, 2003).

* SARS- South African Revenue Service

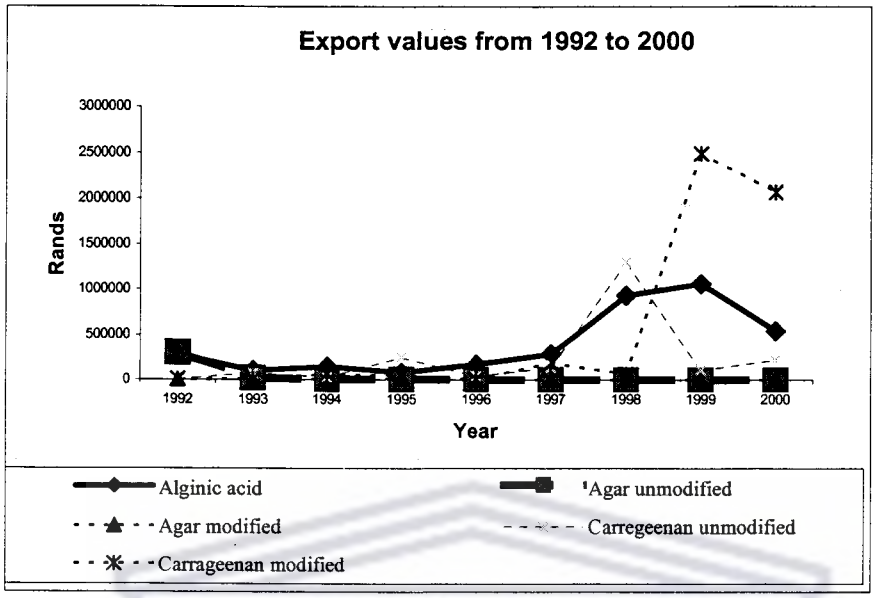

Figure 1.1: Graph of export values for phycocolloids from 1992-2000.

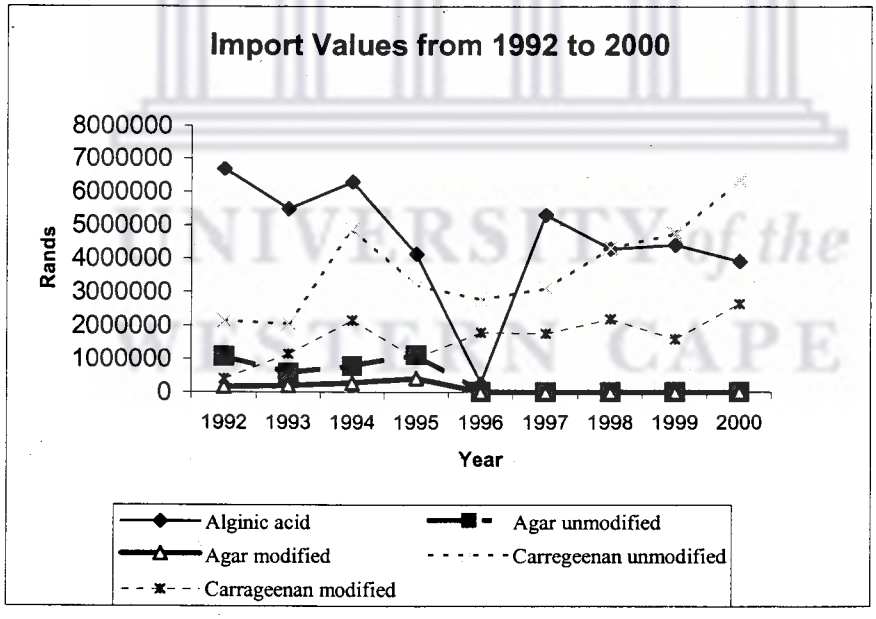

Figure 1.2: Graph of Import values for phycocolloids from 1992-2000.

1.1.4. PHENOLICS

Seaweeds contain phenolic substances which affect their taste, examples of such substances are the phenol-like substances in brown algae and halogenated substances like the brominated phenols in red algae (Lüning, 1990). Within the Phaeophyceae, the plant reduces digestibility by the deployment of polymers of phloroglucinol (Steinberg, 1985). These polymers are present in all orders of brown algae. The main contribution of polyphenols to brown algal fitness is through the deterrence of herbivores. In the kelp *Alaria marginata* from the North Pacific the sporophylls have the highest concentrations of phenolic compounds and are thus better protected from grazers than the vegetative parts (Steinberg, 1984). In *Laminaria pallida,* from South Africa polyphenol levels are high in the meristoderm tissue of the holdfast, stipe and meristem (Steinberg, 1984), which helps deter grazers from feeding on these parts of the plant. Polyphenol levels were significantly lower in internal tissues, sporogenous and vegetative tissue. Polyphenolic levels in phaeophyta have been reported as typically ranging from 0 to 5% dry weight. These polyphenols are present in the alginate extract and contaminate the alginate, causing undesirable effects in medical procedures (Klöck *et al*, 1994; Pfefferman *et al*, 1996; Hasse *et al*, 1997). Therefore the polyphenols have to be removed from alginate for it to be used in the medical field.

1.1.5. TAXONOMY

This study uses the kelp *Macrocystis angustifolia* for a cultivation experiment and the kelp *Laminaria pallida* for a study on alginate properties. *Macrocystis angustifolia* and *Laminaria pallida* belong to the Division Phaeophyta, Class Phaeophyceae, Order Laminariales and Family Lessoniaceae (North, 1971)

1.1.6. *MACROCYSTIS* BIOLOGY

The *Macrocystis* life cycle is typical of the Laminariales, it involves an alteration between a haploid microscopic gametophyte generation and a macroscopic diploid

sporophyte generation (Fig. 1.3), (North, 1971; North, 1994; Anderson *et al*, 1989). The sporophyte is the large plant growing in the field while the gametophyte phase is a minute, filamentous body that can only be seen with the aid of a microscope (Papenfuss, 1942).

In *Macrocystis pyrifera* sporophylls are the site of meiosis, producing haploid zoospores and initiating the reproductive cycle. *Macrcrocystis angustifolia* has a similar reproductive cycle as *Macrocystis pyrifera*. Sporophylls are specialized blades located at the base of the plant, branching off of the stipes just above the holdfast. Biflagellated spores are liberated from the fruiting blades (sporophylls) of the adult sporophyte. The spores are single cells that are the units of dispersal into the environment (North, 1994, Papenfuss, 1942). The spores travel by their own swimming activity or water movement and attach to a suitable substratum. After settling and attachment the spore produces a germination tube within a few hours and the cell contents pass through this to form a pigmented mass at the distal end (Fig. 1.3). Gametophytes develop in a few days (North, 1994; Gherardini and North, 1972). Maturation and gametogenesis occur in both males and females and one ovum is produced per cell of the female. The male gametophytes develop outgrowths (antheridia) that liberate motile biflagellate sperm (antherozooids). The females exude a large egg cell that develops into an embryo after fertilization. A zygote is produced which undergoes rapid cell division to produce an embryonic sporophyte (Cole, 1968). Small sporophytes of 1-2mm long may develop in a few weeks as tiny bladelets (North, 1994). The time required from spore release to the appearance of juvenile *Macrocystis pyrifera* sporophytes in nature was estimated to vary from about 80 days in summer to 120 days in winter (North, 1971).

In *Macrocystis pyrifera* small sporophytes 1 or 2 mm long develop after a few weeks as a single lanceolate blade. After a few weeks, development of an additional blade, which displays slight transverse corrugations on it, occurs. Small depressions develop on either side of this blade just distal to the junction with the stipe. The depressions deepen producing a hole that constituent the basal cleft. The blade is divided into two equal halves as the cleft elongates towards the tip (North, 1994). The two blades

continue growing producing additional basal clefts that yield four, then eight blades and so on (Fig. 1.3). The two outermost blades become frond initials while the inner constitute the first basal meristems. The basal portion of the apical meristem thickens and becomes stipe tissue. The stipe tissue elongates rapidly along the longitudinal axis of the frond and thus separates the small bladelets from each other. Sporophytes become reproductively mature in 6-12 months (North, 1994; North, 1971).

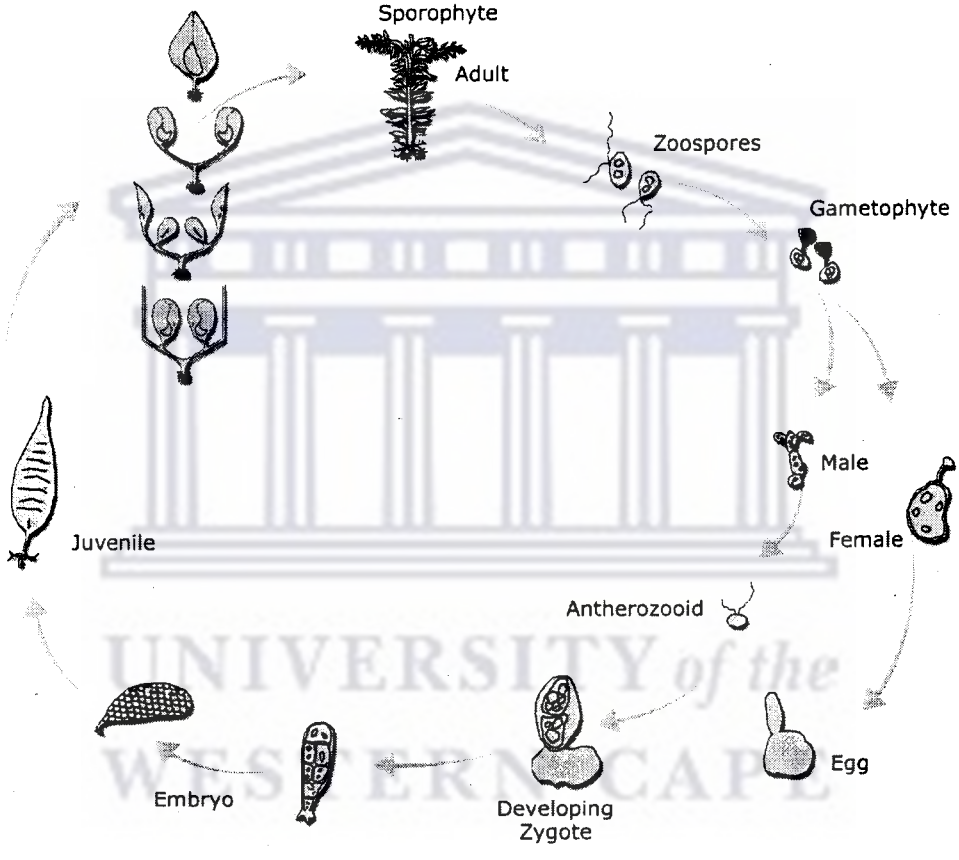

Figure 1.3: Life cycle of *Macrocystis angustifolia*, showing stages of development and growth. (Source: R. Frans, Graphic artist Interntional Ocean Institute)

1.1.7. DISTRIBUTION

Generally *Macrocystis* can be described as a temperate genus, inhabiting regions where the summer monthly mean temperature are commonly 12 - 16 °C, (as found on the

west coast of South Africa) (Fig. 1.4) with a mean in the warmest months in the range 13 - 19 °C and in the coldest months to be 11 - 14 °C (Bolton, 1986; Bolton and Anderson, 1987). *Macrocystis* has a disjunct geographic distribution; it has a circumpolar cold-temperate distribution in the Southern Hemisphere and is limited to the North Pacific Ocean in the Northern hemisphere. *Macrocystis pyrifera, M. angustifolia* and *M. integrifolia* occur in the Southern Hemisphere, while in the Northern Hemisphere only *Macrocystis pyrifera* and *M. integrifolia* occur (Lewis and Neushul, 1994).

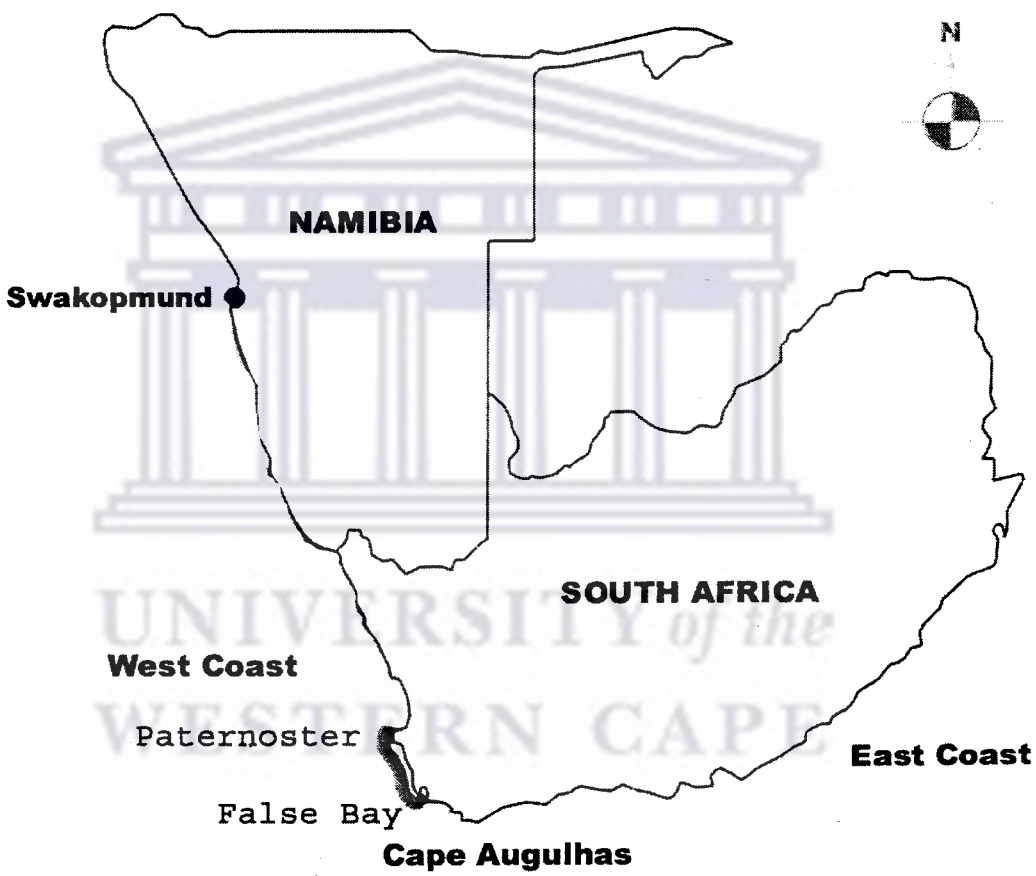

Figure 1.4: Distribution of *Macocystis angustifolia* beds along the South African coast.

The general distribution of *Macrocystis* along the coast of British Columbia was shown to be related to seasonal seawater temperatures and salinity regimes (Druehl, 1978) *Macrocystis* is also found in southern Australia and Tasmaniais (Stegenga *et al*,

1997). *Macrocystis* usually located in areas where there is little seasonal variations of temperature or salinity or in areas where the lower salinities are encountered during the cooler winter months. To determine the effects of surface temperature – salinity regimes, plants were transplanted from Bamfield to Vancouver Harbour in November and growth was monitored. Reciprocal transplants between various stations and Scotts Bay was also done. The plants that were transplanted to Vancouver grew until April the next year, but by June only discoloured basal portions remained and from data it could be seen that plants died during a period of decreased salinity and increased temperature. Stations situated near to *Macrocystis* beds had lower annual temperatures and higher annual salinities than stations further away from *Macrocystis* beds (Druehl, 1978). The single transplant of *Macrocystis integrifolia* from Bamfield to Vancouver, an area where the lower salinity occurs during summer, supported the supposition that the absence of *M. integrifolia* reflected seasonal changes in seawater temperature-salinity and not the ability of this species to immigrate (Druehl, 1978).

Macrocystis angustifolia is found on the coast of Southern Africa, from Cape Point (False Bay) to Paternoster (along the west coast) (Fig.:1.4) (Stegenga *et al*, 1997), where it occurs only in moderately wave-exposed areas and nowhere is it extensive. The west coast of South Africa is influenced by upwelling, which brings nutrients from the deep water to the surface where it fertilizers the kelp beds and enhances their growth (Anderson *et al*, 1989).

The gametophytes of *Macrocystis angustifolia* grow better at temperatures ranging from 13 -17 °C, in an experiment conducted by Deysher and Dean (1986) gametophytes grew well at all temperatures above 11°C except 19 °C and 20 °C. Fain and Murray (1982) as well as Tianjing *et al* (1984) found temperatures in excess of 20 °C were destructive to gametophyte stages. Gametophytes were cultured at temperatures ranging from 10 to 25 °C in a PES medium and showed decreased photosynthetic activity at temperatures of 10 °C and exceeding 20 °C (Fain and Murray, 1982; Tianjing *et al*, 1984). In the culture experiment conducted by Tianjing *et al* (1984) *Macrocystis pyrifera*

gametophytes and sporophytes show optimal growth at temperatures ranging between 13 -17 °C.

1.1.8. NUTRIENT AVAILABILITY AND EFFECT ON GROWTH

Water motion influences growth and production of marine macrophytes, especially when nutrient availability is low. The uptake rates of nitrogen, other macronutrients, various micronutrients and CO_2 are potentially limiting to growth and production of *Macrocystis* species (Gerard, 1982a). Gerard (1982b) conducted an experiment where *Macrocystis* tissue attached to a whole plant was place in a bag with ambient seawater *in situ*; the bag was not filled which allowed flexibility and water motion within the bag. The bag was then incubated and at the end of the period the experimental tissue was removed, the volume of water measured and a water sample taken. The total nitrogen of the experimental tissue was determined. Those tissues that were close to the surface water took up more nitrogen more rapidly than frond tissues located deeper in the water column (Gerard, 1982b; Wheeler and North, 1981).

Internal N reserves are accumulated in kelp frond tissues when ambient NO^-_3 concentrations are high and are used to support kelp growth when external N availability is low (Gerard, 1982c). Dissolved NO^-_3 is a major nitrogen source for *Macrocystis pyrifera*. Nitrate concentrations fluctuate and are high during periods of upwelling or terrestrial runoff. In an experiment performed by Gerard (1982c) an adult *Macrocystis pyrifera* sporophyte was moved from an inshore forest to an offshore, low nitrogen environment, it maintained high growth rates for 2 weeks in the absence of a significant external nitrogen supply. Although frond elongation rates decreased and plant growth rates slowed (Gerard, 1982c) rapid reduction in tissue nitrogen contents indicated that internal N-reserves were being utilized during this period (Gerard, 1982c; Wheeler and North, 1980; Gerard and North, 1981). After the depletion of the nitrogen reserves, the nitrogen content of the lamina and stipe tissues of *M. pyrifera* averaged 1.1 and 0.7% dry weight, respectively. The plant was then exposed to higher ambient nitrogen concentrations for 2 weeks. The rates of frond elongation increased, but nitrogen content

in frond tissues remained low. Of the total nitrogen contained in the frond tissue 58% was used to support growth in the absence of significant external nitrogen supply (Gerard, 1982c; Wheeler and North, 1980; Jackson, 1977). The accumulation and utilization of internal reserves depend on rates of N uptake and assimilation. However NO^-_3 is not the only source of Nitrogen available to the kelp, other sources of N are NH^+_4, which contribute significantly to kelp nutrition even at low ambient N concentrations.

1.1.9 TANK AND SPRAY CULTIVATION

Growing seaweeds in tanks has several advantages over pond or open-water farming because there is a greater control over the conditions to which the seaweeds are exposed. Productivity per unit area is higher than in other types of farming and the production process can be effectively controlled (Ugarte and Santelices, 1992; Friedlander and Levy, 1995). This method however requires a higher capital input than some of the other methods and can only be feasible if high quality kelps or alginate products can be produced. Until now, no one has produced kelp by this method for alginate collection or any other purposes, probably because the kelps grow too large for the making of tanks the required size to be profitable.

Experimental tank cultivation of *Gracilaria conferta* has been developed in Israel (Friedlander and Levy, 1995). The size and shape of the tanks are important for the plants light and nutrient requirements and will determine what quantity of seaweeds will be produced (McLachlan, 1991). The designs of the tanks vary from small, round PVC tanks of 40 liters to rectangular tanks with straight walls of PVC or concrete with aeration pipes at the base (Friedlander and Levy, 1995; Hanisak and Ryther, 1984). When growing *Gracilaria tikvahiae* production rates of 34.8 g dry weight were achieved (Hanisak and Ryther, 1986). The design of the tanks has to take into account the specific requirements of the species being cultivated. Growth of seaweeds in tanks are often light limited due to self-shading and absorption of light by the water. Tank cultures of *Ulva lactuca* became light limited at biomasses higher than 0.8kg m^{-2} (Lignell *et al*, 1987). The

decrease in growth rates when the density of seaweeds increased in both tank and spray cultures illustrates the influence of self-shading on the light conditions for growth (Lignell *et al*, 1987).

In Sweden the cultivation of seaweeds using a spray or mist has been a new culture technique that has been the subject of various experimental trials (Lignell and Pedersen, 1986; Pickering *et al*, 1995; Indergraad *et al*, 1986). Moeller *et al* (1984) attempted such a technique with *Ascophyllum nodosum* and Lignell and Pedersen (1986) showed that this method was suited to seaweeds with rigid thalli. *Ascophyllum nodosum* plants were collected and placed on nets in a tiered fashion in a box-like structure. Sprayed water applications are used on the multiple layered tiers in order to maintain a film of seawater on the seaweed surface (Lignell and Pedersen, 1986). Using this technique Lignell and Pedersen (1986) showed that cultivation of *Ascophyllum nodosum* under green house conditions resulted in an average growth rate of 2.8% weight per day over nine months. Preliminary trials for *Gracilaria* and *Ascophyllum* have proved successful (Lignell and Pedersen, 1986; Pickering *et al*, 1995

1.1.10. RAFT CULTIVATION

There are many different types of raft culture and techniques for the construction of artificial seaweed beds; the different raft techniques used are dependent on what type of seaweed you want to culture. The different methods range from low fixed raft culture to deep water, long tethered floating rafts (Santelices & Doty, 1989). A floating frame is designed to which ropes containing seeded material is attached; seeded material are ropes or nets that have been impregnated with spores or juvenile plants. These ropes may be in the form of nets or simple monofilaments. The ropes are hung vertically downwards from a main line at 2m intervals or the ropes can be stretched across the frame. These are called the vertical hanging method and the "longline" method respectively (Kawashima, 1993; Dawes, 1995). The seaweeds used in this method are always submerged. *Laminaria* sp. (Kombu) is cultivated using this method and blade lengths increase rapidly until the plant attains a length of about 5m and maximum growth rates of 3.6 cm per day

(Kawashima, 1993). There are several advantages of rope farming which include the positioning of algae in optimal environments, crop predictability and possible epiphytic and grazing control (Santelices and Doty, 1989; Dawes, 1995). This method is widely used in countries such as China, Namibia and Venezuela (Critchley and Ohno, 1998).

There are several advantages to using these floating methods of cultivation. Grazing by herbivores is minimized and plants near the surface are exposed to more light. Also the seaweeds are exposed to moderate water movement that is needed for growth; however, this method is not recommended for areas with strong wave action.

1.1.11. DISEASES AND EPIPHYTISM

Diseases and pests are major problems in the seaweed mariculture industry and they must be controlled so as to reduce damage to the overall production in the industry. Growth under artificial conditions will usually render the host plant susceptible to attack and large monocultures provide ideal conditions for the spread of diseases and contaminants. High temperatures and low seaweed densities increase the development of epiphytes. Epiphytes proliferate on tank walls and in the water medium of cultures (Friedlander and Levy, 1995). Epiphytes have a negative effect on the growth of seaweeds. The main problems of epiphytes are: competition for space in the habitat, attack by pathogens such as bacteria and viruses; growth of epiphytes and endophytes and the biggest problem is the contamination of the plant. Epiphytes also add weight to the host algae causing the detachment of the entire host plant, especially in systems that where there is strong wave action or currents (Buschmann and Gomez, 1993; Fletcher, 1995). Epiphytic growth gives the most cause for concern at present (Fletcher, 1995) and there have been reports that epiphytes promote sedimentation (Rebello *et al*, 1996). The epiphytes most people are concerned about belong to the genera *Ceramium* and *Ulva* (Fletcher, 1995).

In tank and pond experiments on *Gracilaria* sp. (Friedlander and Levy, 1995), the appearance on epiphytes on the seaweed and on tank walls was a major problem. High

temperatures and low seaweed density increased the development of epiphytes. Epiphyte biomass was only inhibited in some of the tank treatments. The experiments where low irradiance and pulse fed nitrogen was given the epiphyte biomass did not increase, while in treatments with high irradiance and continuous feeding, epiphyte biomass increased significantly. Several practical means for limitation of epiphyte development are: limited irradiance by increasing the seaweed density, high pulse feed N concentration, night fertilization, pH control, water pretreatment with chlorine (Ugarte and Santelices, 1992; Fletcher, 1995), vigorous aeration and filtered circulation, temporary salinity decrease, decrease in seawater exchange, supply of low nutrient seawater, and mechanical cleaning of the pond (Friedlander and Levy, 1995).

1.2. PROJECT OVERVIEW

In South Africa the seaweed industry is small compared to the worldwide industry, few commercial species are collected from the shore or harvested for export. Currently there is no commercial seaweed farm in South Africa, although there is a small one in Namibia and there are a number of commercial abalone farms in South Africa that grow seaweeds for abalone feed. There is a market available for alginate and other seaweed products and South Africa may be able to take advantage of this natural resource.

This study is an attempt to develop a method suitable for cultivating *Macrocystis angustifolia* from spores through to harvestable sporophytes *in situ*. This is to ascertain whether the method could be used to populate certain areas of the coast with kelps and so to start an industry that could be sustained. Also part of the study is to determine if alginate quality of *Laminaria pallida* can be improved by a controlled extraction method.

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CHAPTER 2

JOURNAL:

Journal of Phycology

Experimental Cultivation of *Macrocystis angustifolia.*

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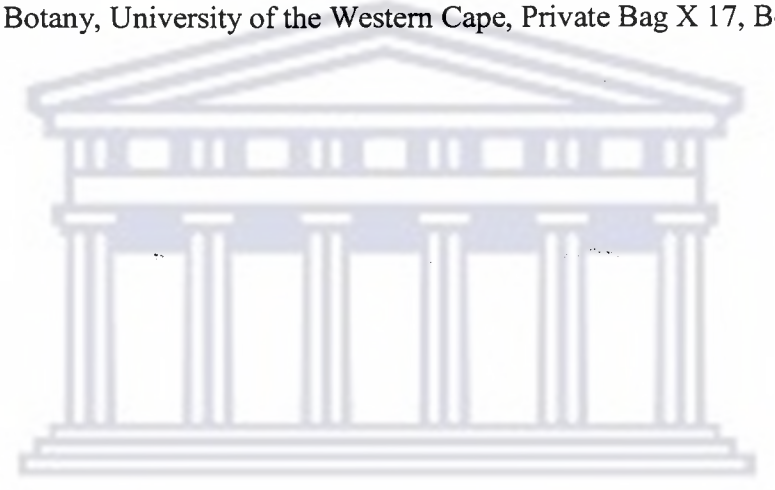

2.1. ABSTRACT

Kelps are the basis for a number of commercial products, including alginate, plant growth substances, cosmetics, food additives and abalone feed. The potential of low - volume, high - value products such as medical alginate, as well as the available local market for abalone feed suggests that the commercial cultivation of kelps may be economically viable in some areas. *Macrocystis angustifolia* was grown using various techniques such as laboratory, tank, mist, spray and raft cultivation. Laboratory cultivation of the gametophyte stage to the sporophyte stage was done using nutrient enriched seawater. A mean growth rate of 0.037% day^{-1} for gametophytes was experienced over 4 weeks. A mean growth rate of 0.021% day^{-1} for sporophytes was experienced over 20 weeks. Sporophyte growth rate fluctuated due to crowding in culture dishes. Problems were experienced with tank and mist cultivation, mainly due to the location at which the experiment was conducted. The specimens had developed weak holdfast during the laboratory cultivation and thus did not attach successfully to the ropes in the tank and mist experiments. Also the seaweed received an insufficient water supply due to problems with the spray nozzles in the cabinets. Cultured and field collected juvenile kelp was transplanted to a raft in St Helena Bay, but after 4 weeks all specimens had disappeared. A new set of specimens was set up and growth was measured after 3 weeks. It was found that although some growth of specimens had taken place, many of the specimens were diseased or had died. Adjacent specimens growing on the dock also appeared necrotic and covered in silt. The difficulties of growing kelp at this site underscores the importance of species – specific site selection.

Keywords: *Macrocystis angustifolia*, cultivation, spray culture, raft culture, St. Helena Bay.

2.2. INTRODUCTION:

Kelps such as *Macrocystis, Ecklonia* and *Laminaria* are the basis of various commercial products including plant growth substances, alginate, beauty products, food additives and animal feed. For the mariculture of kelp to succeed in South Africa, it is essential to develop high- value, low- volume kelp products, such as medical alginate. Kelps have recently become sought after as feed for the abalone mariculture industry in South Africa. This local market, as well as the possible high- value alginate production, suggests that the commercial cultivation of kelps may be economically viable in some areas. Currently the demand for seaweed is being met by producers of seaweed over the world but should demand increase or some natural disasters occur natural stocks could be damaged or wiped out (McHugh, 1991; Critchley, 1993). Therefore it would be advantagous to develop a cultivation method that would produce good quality alginate and high volumes of kelp. The purpose of this study is to develop such a method of cultivating the kelp *Macrocystis angustifolia.*

Various methods of cultivation of different species have been developed (Friedlander and Levy, 1995). Different designs of tank cultivation have been developed, such as small round PVC tanks or longer concrete tanks supplied with aeration (Friedlander and Levy, 1995). Other designs use a spray or mist technique that allows sprayed water applications over tiers in order to maintain a film of seawater on the seaweed surface (Lignell and Pedersen, 1986; Pickering *et al*, 1995). The use of spray cultivations has proved successful for *Gracilaria* and *Ascophyllum* and the seaweeds are exposed to optimum levels of light and nutrients for growth. The advantages of tank cultivations are numerous because of the degree of control over the process and also mechanization (Friedlander and Levy, 1995). However, there are economic limitations as there are high costs in terms of seawater pumping and water movement.

Another method of cultivation is raft culture and techniques for this type of culture varies from low fixed raft culture to deep water long tethered floating rafts (Santelices and Doty, 1989). The floating rafts are designed with ropes; these ropes

contain seeded material and can be hung vertically from the raft or straight across the raft (Kawashima, 1993; Dawes, 1995). Advantages to rope farming include positioning of algae in optimal environments, crop predictability and epiphytic control (Dawes, 1995).

The objectives of this study is firstly to develop a method of cultivating *Macrocystis angustifolia* in a laboratory successfully, then to establish if artificial spray or raft cultivation will work using sporophytes grown in the laboratory; and secondly,to establish if juvenile *Macocystis angustifolia* collected *in situ* will grow on a raft.

2.3. MATERIALS AND METHODS:

Seawater was collected and filtered twice to remove fine particles by using a Büchner funnel with Scheicher and Schuell filter paper no. 0860, 100 over a gentle vacuum. Filter paper was changed regularly. Seawater was sterilized in a water bath at 90°C for 1 hour, cooled down afterwards for 1 hour and then heated again for 1 hour. Filtered seawater was stored in covered glass jars at 4°C.

Fertile sporophytes of *Macrocystis angustifolia* were collected at Kommetjie. Zoospore release was stimulated by first wiping the fertile area on the blade with a paper towel. The blades were then placed in between the paper towel (moistened with sterilized sea water) in a growth chamber at 10°C overnight. The following morning fertile pieces of the blades, approximately 8 by 4 cm, were placed in a beaker with sterilized seawater for 1-3 hours to release zoospores into a spore suspension medium.

Cotton string for spore attachment was wound around one of two microscope slides in each of 6 Petri dishes (Fig 2.1) for spore attachment. An enriched nutrient medium was prepared by using the Provasoli (1968) method. The nutrient medium was autoclaved to sterilize it completely and 20 ml of nutrient medium was added to 1 lt. of sterilized seawater. Then 50 ml of the nutrient medium was added to the Petri dishes, and 2 ml of spore suspension was added to each of the Petri dishes. The Petri dishes were

then placed into growth cabinets at 15 ° C (Fig. 2.2). The nutrient medium was renewed every 7 days thereafter.

The length and width of male (Fig. 2.3) and female gametophytes (Fig. 2.4) were measured. When the sporophyte stage (Fig. 2.5) occurred, the sporophytes were measured as well. An eyepiece micrometer and a stage micrometer were used to calibrate the compound and dissecting microscopes to measure the spore lengths. Measurements took place weekly.

The specimens were then transported to Marine and Coastal Management research aquarium in Sea Point. Here the total length of sporophytes were measured and seeded onto rope, by twisting the nylon rope open and then inserting the holdfasts of the *M. angustifolia* in between the nylon (Fig. 2.6). The specimens were then placed in two different spray cultivations: fine mist, coarse spray. The spray systems consisted of two rectangular, clear perspex cabinets fitted with a series of pipes, which supplied water (Fig. 2.7). The pipes had spray nozzles fitted approximately 10 cm apart, which faced directly down and sideways into the cabinets. Normal, filtered seawater was supplied 24 hours a day.

Juvenile *Macrocystis angustifolia* were collected at Kommetjie and transplanted to a suspended raft in St. Helena Bay on the west coast of South Africa (Fig. 2.8). Specimens were transported using cooled iceboxes and ice packs to keep the temperature of the water between 15-20 °C. Specimens were tied to the ropes using numbered cable ties; they were tied in a criss-cross pattern around the holdfast of the seaweed. Specimens were placed approximately 20 cm apart and the ropes were placed horizontally across the raft and vertically down from the raft to a depth of about 5 m.

2.4. RESULTS

Both gametophyte and sporophyte stages of *M. angustifolia* grew well at 15 °C. During the development of the gametophytes the percentage growth rate increased steadily over 4 weeks. The development of male and female gametophytes took place

during the first two weeks. The highest growth rate of 0.05% day^{-1} took place during weeks 3 and 4 and some gametophytes were fertilized during this period (Fig. 2.9). A steady increase in length of the gametophytes occurred over the 4 week period (Fig. 2.9). Sporophyte growth rates fluctuated during the experimental period. The percentage growth rate of sporophytes experienced 3 major peaks during development (Fig. 2.10). Sporophyte lengths grew consistently until the last 3 weeks when a dramatic increase in length was experienced (Fig. 2.10).

The laboratory cultivation of sporophytes was successful. The specimens were transplanted to Marine and Coastal Management Research Aquarium for the different cultivation methods. Some difficulties with the mist and coarse sprays were experienced and specimens died. Although new experiments were set up, the specimens after two weeks appeared necrotic and died. Several attempts were made to get viable results from the spray cultures but the specimens never lasted more than 2-3 weeks. The failure of the sprays was probably due to the lack of sufficient water supply and build-up of sediment. Various design flaws in the spray systems contributed to the experiments failure. Five different spray and mist nozzles were tried and all eventually failed due to silt build up within the nozzle. The spray and mist cultivations could have succeeded if the cabinets and nozzles were cleaned every day. However, that was not possible considering the location of the spray cultures and my regular place of work. No data for both the mist and coarse sprays are available as each time the material died.

Raft cultivation of juvenile *Macrocystis angustifolia* was unsuccessful. The relative growth rates of juveniles on the horizontal ropes (Fig. 2.11) were slightly higher than those on the vertical ropes (Fig. 2.12). For the horizontal ropes growth of specimens increased for the first 4 weeks but the specimens were not healthy and after week 4 almost all specimens died or were washed away. Although growth rates on vertical ropes increased rapidly for the first 2 weeks, the average length of the specimens increased slowly. After week 4 however most specimens were washed away or appeared necrotic.

2.5. DISCUSSION:

The results obtained in this paper are comparable to those obtained by Tianjing *et al* (1984). These authors found that the optimal temperature ranges for gametophyte and sporophyte production was between 13 and 17 °C and that after 4-5 weeks sexual reproduction occurred and viable sporophytes were produced. This study showed that after a further 1 or 2 weeks sporophytes grew to about 0.5-1 cm and this can also be compared with a 1cm growth of *Macrocystis pyrifera* found by Tianjing *et al* (1984) after the same time period. Stekoll and Else (1990) also found that after fertlization sporophytes took another 2 weeks of growth to reach a length of 0.5cm. The cultures can be sustained for a long period with weekly water changes and nutrient medium. Growth rates of specimens showed a positive production at all times yet growth rates for both gametophytes and sporophytes were low. This could be attributed to specimens becoming stressed over nutrients and space. The improvement in growth rates and lengths is attributed to the separation of specimens into separate beakers. The growth rate was thought to decrease again after the peaks due to competition for space and nutrients in the beakers. Specimens last for a few weeks in the beakers once they reached about 5cm in length, after this they become stressed and appear necrotic. If not transplanted to a larger container they start eroding and died.

In the raft cultivation some growth took place on both vertical and horizontal ropes, after 4 weeks 50% of the specimens had washed away and the other 50% appeared necrotic and were covered in silt. This can be due to epiphytism (Rebello *et al*, 1996) and insufficient water movement at this site. The site selection of the raft is suitable for *Gracilaria gracilis* cultivation, but not for *Macrocystis angustifolia*. The time of year in which the experiment took place could also be a contributing factor to the raft failure. The experiment took place in summer when temperatures range between 19 and 25° C (Brown, 1999) and are not suitable for *M. angustifolia* (Bolton, 1986; Bolton and Anderson, 1987), also the water movement in the bay is low during summer. Due to time constraints the experiment was stopped; however, a few samples were placed on the raft during winter to test the theory of water movement. After 4 weeks the ropes were taken

out and most specimens had grown and appeared healthy with little sedimentation. If water movement in winter is greater then seasonal farming of kelp may be possible, but this requires further investigation.

The spray cultivations clearly indicated that proper equipment and maintenance of the experiment is essential and that this method may work on a larger scale. It was also clear that raft cultivation would work if proper site selection is done and that water movement is very important to kelps.

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2.7. FIGURE CAPTIONS:

Figure 2.1: Petri dish containing two slides, one with cotton string around and the other not. This was done so that the slide with no string could be used to take weekly measurements with. (s=slide with string, n=Slide without string)

Figure 2.2: Slides were kept in a growth cabinet at 15 ° C. (p=petri dish with slides inside)

Figure 2.3: A male gametophyte of *Macrocystis angustifolia*. (s=antherozooids inside cell) Mag.: 1x40

Figure 2.4: A female gametophyte of *Macrocystis angustifolia* showing extruding eggs. (o=oogonium, e=egg) Mag.:1x40

Figure 2.5: A sporophyte of *Macrocystis angustifolia*.(s=sporophyte)Mag.:1x10

Figure 2.6: *Macrocystis angustifolia* sporophytes were seeded onto nylon ropes by twisting the rope open and inserting the holdfast into the rope.

Figure 2.7: The spray cabinets at Sea Point Research Aquarium showing how the ropes with sporophytes were attached. (r=ropes with sporophytes attached)

Figure 2.8: The floating raft at St. Helena Bay on the west coast of South Africa. (r = horizontal ropes attached to raft)

Figure2.9: Graph of relative growth rate and lengths of male and female gametophytes over 4 weeks at 15 °C

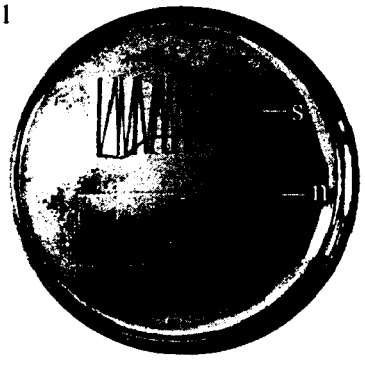
Figure 2.10: Graph of relative growth rate and lengths of sporophytes over 16 weeks at 15 °C

Figure 2.11: Graph of relative growth rate and length of juvenile *Macrocystis angustifolia* on horizontal ropes on the raft in St. Helena Bay.

Figure 2.12: Graph of relative growth rate and length of juvenile *Macrocystis angustifolia* on vertical ropes on the raft in St. Helena Bay.

2.8. FIGURES:

2.1



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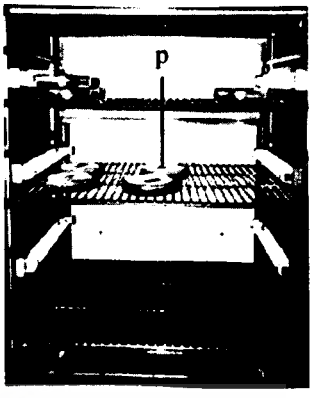

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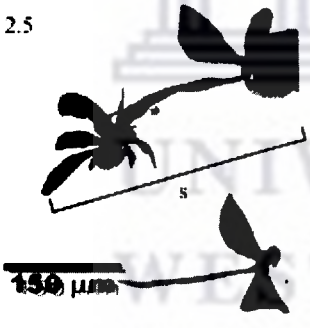

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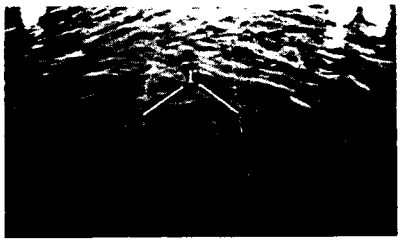

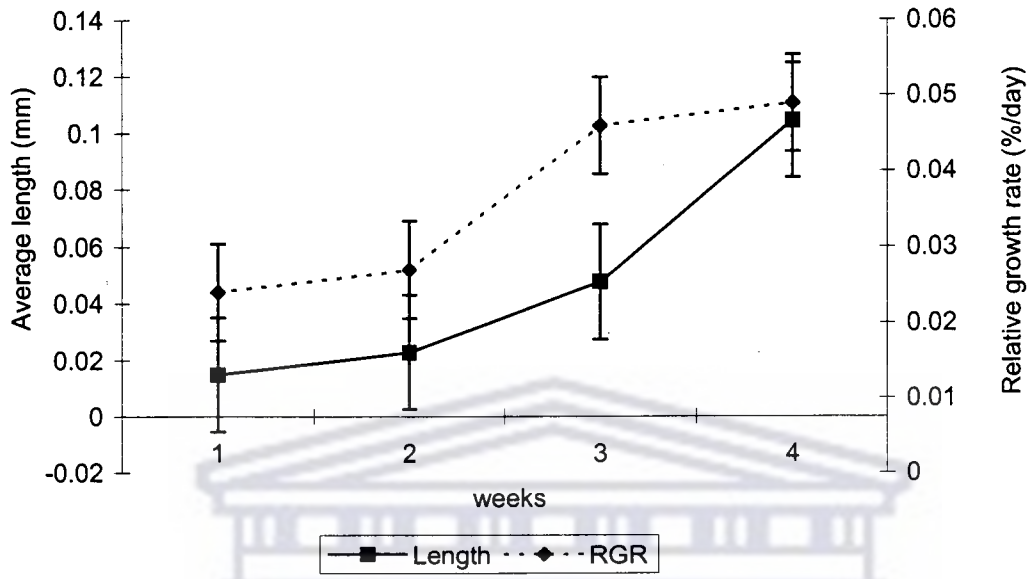

Figure 2.9

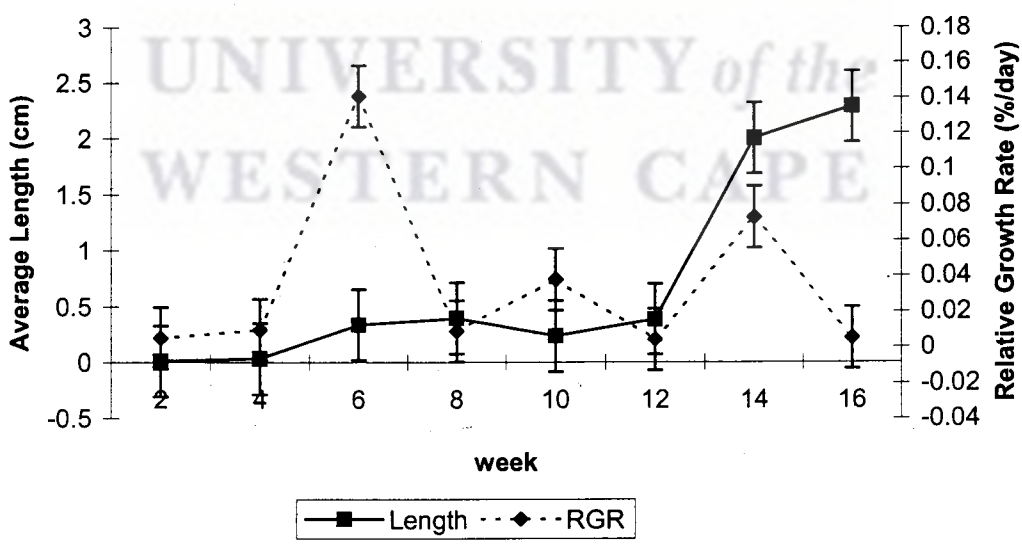

Figure 2.10

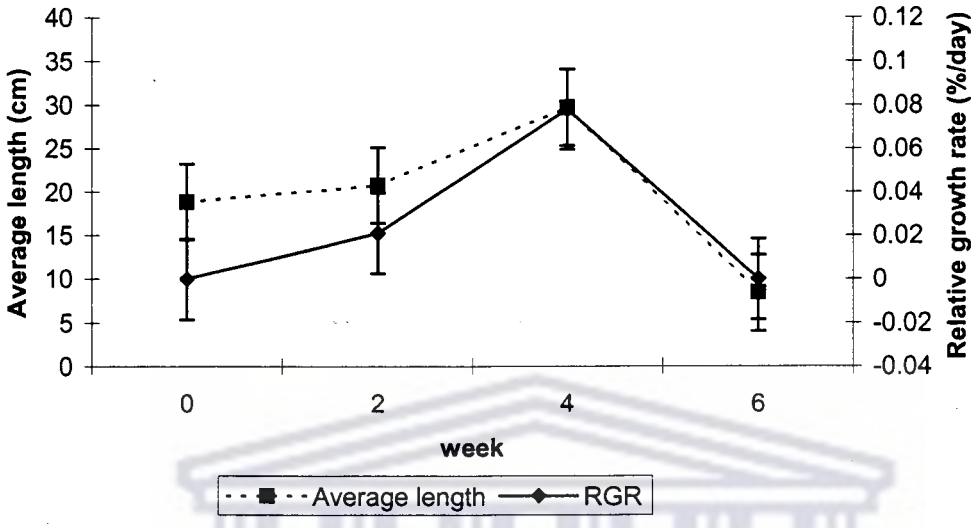

Figure 2.11

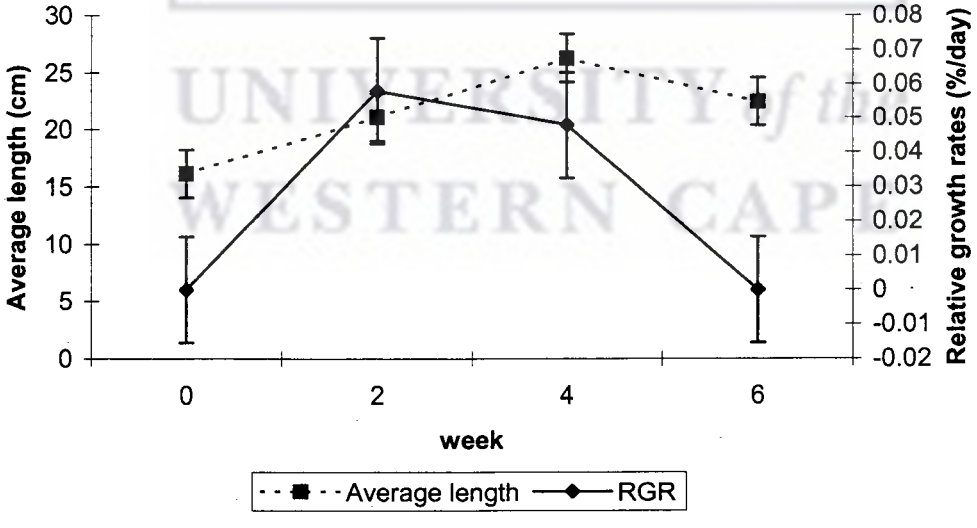

Figure 2.12

CHAPTER 3

JOURNAL:

Journal of Phycology

Alginate Properties of *Laminaria pallida.*

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3.1. ABSTRACT

Alginates are used in various industries and the quality of alginates used must be controlled. Alginates used for medical purposes must be of a very high quality. The cost of obtaining high-quality alginate is high and very time-consuming. A simple procedure is described for the purification and extraction of alginate from the kelp *Laminaria pallida*. The kelp was divided into different sections, including the blades. The stipes were cleaned and some were peeled and extractions were made of all the different sections including the peels. Analysis of the purified alginate showed a protein content below detectable levels and a very low polyphenol content. The purified alginate was highly viscous, with 24 - 29 centipoise for a 0.1 % solution. Using this method a higher quality alginate can be obtained from any section of the kelp and this will help in more medical advances.

Keywords: Alginate, extraction, *Laminaria pallida*, phenols, viscosity.

3.2. INTRODUCTION

Alginates are used in large quantities by the textile, food and pharmaceutical industries. The quality alginates used in these different industries have to be controlled. The alginate used in the food industry has to be of a higher quality that that used in the textile industry and the alginate used in the pharmaceutical and medical fields must be of the highest quality/grade. Alginate of such high quality is a very expensive product and difficult to produce; it's used in the manufacture of medical dressings to treat burns and wounds and in the making of tablets and capsules (Kasloff, 1990; Reynolds and Prasad, 1982). Another use for highly purified alginate is in implantation and tissue engineering (Pfefferman *et al*, 1996; Klöck *et al*, 1997). It has been shown that membranes from alginate hydrogels can protect encapsulated cells or tissues from a foreign host's immune system (Colton, 1995; Pfefferman *et al*, 1996). There have been many advances in the medical field where alginates can be used and it is important that methods to extract quality alginate from kelps must be perfected. The question then is: can a simple purification and extraction method yield high quality alginate products and can any section of the kelp be used? Jork *et al* (2000) developed the extraction and purification method and conducted their study on the inner stipes of *Laminaria pallida*. They also tested protein content, polyphenol content and viscosity of the inner stipes. The objective of this study is to show how high quality alginate can be obtained and that any section of the kelp can be used.

3.3. MATERIALS AND METHODS

RAW MATERIAL

Alginate was extracted from *Laminaria pallida* plants collected from Kommetjie on the west coast of Southern Africa in July 2000. SCUBA divers cut mature kelp plants just above the holdfast at a depth of between 5-10m. The specimens were cleaned of epiphytes and cut or the outer layer of stipes were peeled as required (Fig. 3.1; 3.2; 3.3

and 3.4). The material was then dried in an oven at 50°C. Extraction was done on nine different sections of the plant:

1. Top section of blade
2. Middle section of blade
3. Bottom section of blade

The stipe was divided into an upper and lower section

4. Inner stipe (upper section)
5. Stipe (upper section)
6. Inner stipe (lower section)
7. Stipe (lower section)
8. Peels of stipe (upper section)
9. Peels of stipe (lower section)

EXTRACTION AND PURIFICATION

The extraction and purification experiments were conducted at room temperature. All specimens were dried in an oven at 50°C. For each extraction, 7 g of powdered material were stirred overnight in 500 mL of 5% Na_2CO_3 / 50mM EDTA at pH 8.5 to extract the alginate (Jork *et al*, 2000). The alginate solution was filtered through 0.45 μm strainers to remove all solid particles (phase 1, crude extract). KCl was used to convert the solution to 0.13 M KCl (Jork *et al*, 2000). Then 350 mL of 90% ethanol (99% dried with acetone) was added and the solution was vigorously stirred to precipitate the alginate. The alginate was then dissolved in 250 mL of 0.5 M KCl / 10 mM EDTA solution (Jork *et al*, 2000) (phase 2). The above precipitation was repeated with 200 mL ethanol (Jork *et al*, 2000). The extracted alginate was dissolved in 250 mL of 0.5 M KCl / 10 mM EDTA solution and the resulting solution were subjected to dialysis for removing ionic components. The dialysis tubing was boiled three times in distilled water to remove sulfur and other potential contaminants. Dialysis was conducted over three days including six changes of water. Dialysis tubes were suspended in an excess of distilled water (phase 3). Following dialysis, 0.13 M KCl was added to facilitate the

precipitation of the alginate. The alginate solution was added to 250 mL of ethanol, and the entire solution (consisting of precipitated alginate, KCl and ethanol) was poured through a sterile strainer to separate the precipitated alginate from the remaining solution. The resulting alginate sample was then dried under sterile conditions in a laminar flow cabinet. Once the alginate was dry it was weighed. The dried alginate was then dissolved in 250 mL of 0.5 M KCl / 10 mM EDTA (phase 4). After each phase (purification step) the presence of proteins and phenolics was determined and measured. Two plants were used for the extractions and the mean value was then calculated (n = 2). There was no significant difference between the results of the two plants.

PROTEIN DETERMINATION

Bradford (1976) has developed a fairly quick and sensitive method for the determination of small protein quantities in solutions. This method is based on the fact that the maximum light absorption of Coomassie Brilliant Blue dye at various wavelength experiences a shift from 465 to 595 nm when allowed to bind to protein. An increase in protein concentration results in a corresponding increase in optical density or light absorbency. According to Bradford the relationship is fairly linear with only a slight curvature. Using the Bradford Protein Assay (1976) method the total protein concentration of alginate samples was determined. The Bradford Protein Assays (as used in Jork *et al*, 2000) uses a standard protein curve to which all subsequent measurements are based. The procedure involves the reaction of a color reagent with the specific amino acids of the protein. Bovine Serum Albumin (BSA) was used in the preparation of a standard curve. Approximately 2ml of the Bradford-alginate sample solution was transferred to separate cuvettes. Readings were taken at 595nm by using a UV-160A-visible Recording Spectrophotometer (Shimadzu make).

VISCOSITY

The term viscosity is used to describe thickening or resistance to flow (Guist, 1990). Viscosity can be used to predict the biocompatibility and stability of alginate

beads, and it enables an estimation of the molecular weight of the alginate (Matinsen *et al*, 1991). The viscosity of the alginate was measured by using a Hasse Viscometer, model, VT6/7, serial number L: 3-2000000 mPa. Spindle number 1 was used on all 27 samples. Each sample was measured three times.

POLYPHENOLIC DETERMINATION

To determine the presence of polyphenols we used the Ferric–to–Ferrous Reduction method (VanMiddlesworth and Cannell, 1998). Polyphenols are able to reduce Fe^{3+} to Fe^{2+} with different efficiencies and the production of these Fe^{2+} ions can be monitored by the formation of Prussian blue. The assay sample solution was transferred to separate cuvettes. Readings were taken at 700nm by using a UV-160A-visible Recording Spectrophotometer (Shimadzu make).

3.4. RESULTS

During the first phase of testing most of the sections showed levels of protein between 2 –10 ug ml^{-1} (Fig.3.7). After the second phase of the extraction and purification procedure the protein content of all the sections tested were according to the Bradford test below detectable levels (Fig.3.7). Using the Ferric to Ferrous Reduction procedure the phenolic content of all sections were tested. In the first phase of testing the phenolic content in all sections were high (Fig 3.8). After each subsequent purification the phenolic levels dropped notably and after the 4^{th} phase in the procedure (Fig 3.8) the phenolic content of all sections was below detectable levels. Phenolic content of alginate after each purification is shown visually in figures 3.9 - 3.14.

The viscosity (Fig. 3.) of a 0.1 % solution of alginate extracted from all section ranged from 24 - 29 centipoise, with only the bottom peels of a stipe at 17 centipoise. All measurements were taken with the same Hasse viscosity meter at a temperature of 19 °C.

3.5. DISCUSSION

The results presented here indicate that it is possible to produce biocompatible alginate by using stipes and blades of *Laminaria pallida*, and that this can be done reproducibly using a simple extraction and purification procedure. Jork *et al* (2000) used only peeled stipes, whereas peeled and unpeeled stipes as well as the blades of the *Laminaria pallida* were used in this study. I also performed the tests on the peels collected from the plant. According to Tugwell and Branch (1989) polyphenol levels are highest in meristoderm tissue of holdfast, stipe and meristem and are lower in infertile vegetative tissue. The purified alginate in all sections showed no detectable levels of proteins or polyphenolic compounds. This is in agreement with results obtained by Jork *et al* (2000). These authors also found that after the first precipitation step most of the polyphenolic compounds were removed and that after the third precipitation most of the phenolic compounds were below detectable levels. Similarly the Bradford test in the Jork *et al* (2000) study was also below detectable levels. Using peeled or unpeeled stipes has no effect on the purity of the alginate obtained and any section of the plant can be used to extract alginate from plants using this method.

The purified alginate in all sections showed a high viscosity (Table I) and this is by far better than commercial alginate solutions of the same concentration. Commercial alginate solutions of 0.1% show a viscosity of 6 centipoise for solutions of low and medium viscosity and 8 centipoise for a commercial alginate solution of high viscosity. All viscosity measurements were taken on the same day with the room temperature of 19 °C. Jork *et al* (2000) reported a viscosity reading of 12mPa on a 0.1% solution; and their findings cannot properly be compared with those reported here as the temperature at which they took their readings is not stated.

This purified alginate should be tested in various experiments, for example: to determine whether this purified alginate with high viscosity will be suitable for bead formation and whether the beads will be stable enough for use in implantation experiments. It is known that alginates with a high viscosity have higher molecular

weights and alginates with high molecular weights are more stable and this is important in creating alginate beads that encapsulate tissues or cells for long-term implantation in humans, thereby overcoming the rejection of foreign tissue by the host immune system (Hasse *et al*, 1997). The alginate beads should be further tested by implantation of Islets of Langerhans or parathyroid tissue in special rats. Using a purified alginate in mitogenic and immunological tests has shown a reduction in fibrotic reactions and that the purified alginate can be used in long term immunoisolation of allogenic and xenogenic tissue implanted in human patients (Jork *et al*, 2000).

3.6. REFERENCES:

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3.7. FIGURE CAPTIONS:

Figure 3.1: A blade of *Laminaria pallida* cut into a t= top, m= middle and b= bottom section.

Figure 3.2: A stipe being peeled from its outer layer. (s=stipe, p=peels)

Figure 3.3: A cleaned stipe. (s=stipe)

Figure 3.4: A stipe with its outer layer intact and one without an outer layer. (un=not peeled stipe, p=peeled stipe)

Figure 3.5: Filtration of ethanol and alginate solution to obtain clean alginate.

Figure 3.6: An uncontaminated piece of alginate. (A=cleaned alginate)

Figure 3.7: Graph of protein content in various sections of *Laminaria pallida.*

Figure 3.8: Graph of phenolic content in various sections of *Laminaria pallida.*

Figure 3.9: Bottom blade of Laminaria pallida after first purification treatment. The Prussian blue is clearly visible as an indicator of phenols. (A=alginate with phenols present)

Figure 3.10: Bottom blade of Laminaria pallida after second purification treatment. (A=alginate with slight phenolic presence)

Figure 3.11: Bottom blade of Laminaria pallida after final purification treatment. As you can see no blue is visible. (A= alginate with no detectable phenols present)

Figure 3.12: Bottom stipe without peels after first purification treatment. The Prussian blue is clearly visible as an indicator of phenols. . (A=alginate with phenols present)

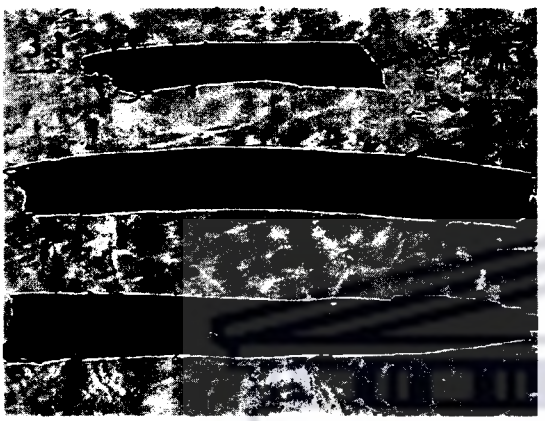
Figure 3.13: Bottom stipe without peels after second purification treatment. (A=alginate with slight phenolic presence)

Figure 3.14: Bottom stipe without peels after final purification treatment. There is no blue visible. (A= alginate with no detectable phenols present)

3.8. TABLE CAPTION:

Table I: Table of viscosity content of 0.1% solution of alginate in various sections of *Laminaria pallida* at temperature of 19 ^{O}C.

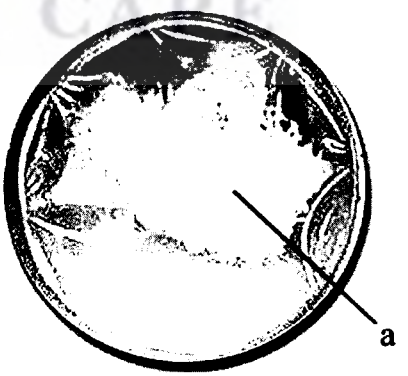
3.9. FIGURES:



3.2

3.5

3.6



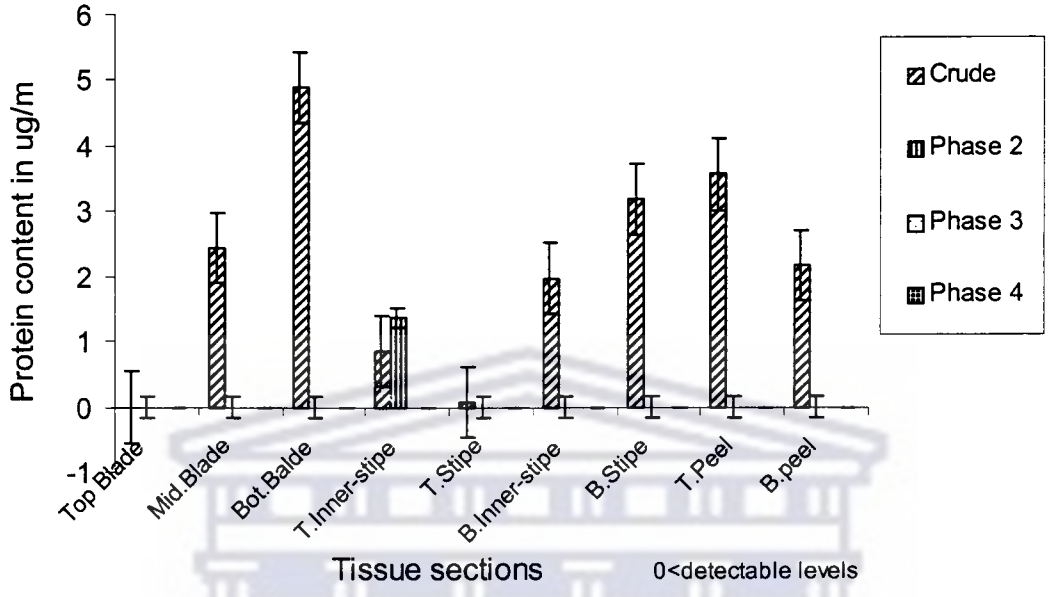


Figure 3.7

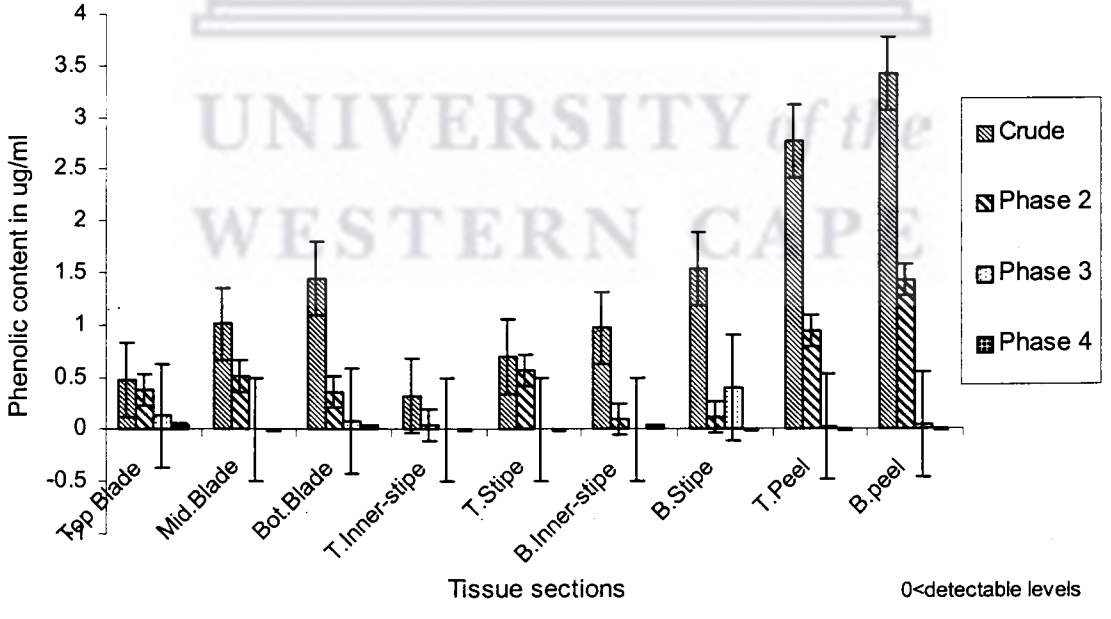
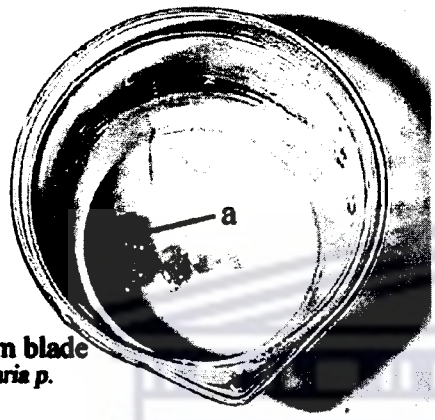


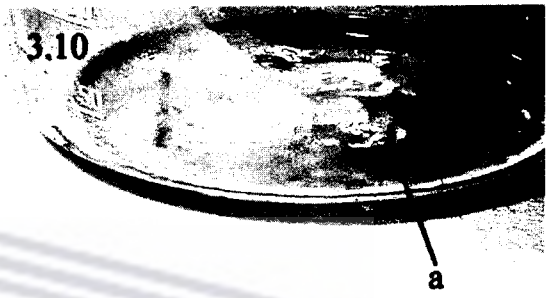
Figure 3.8

3.9



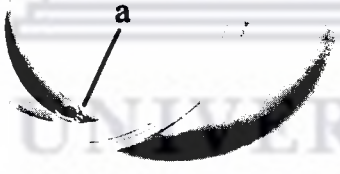
Bottom blade
Laminaria p.

3.10



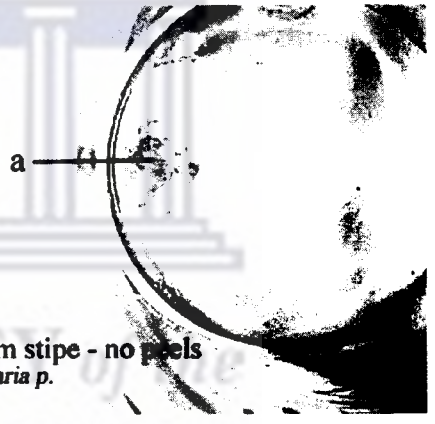
Bottom blade
Laminaria p.

3.11



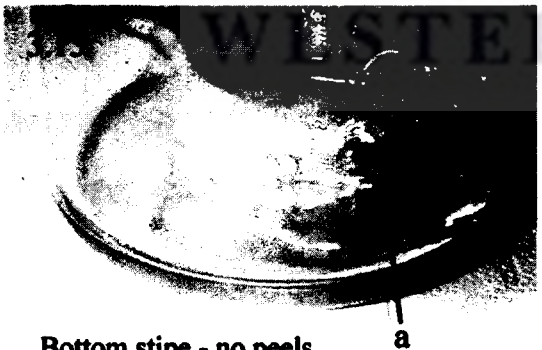
Bottom blade
Laminaria p.

3.12



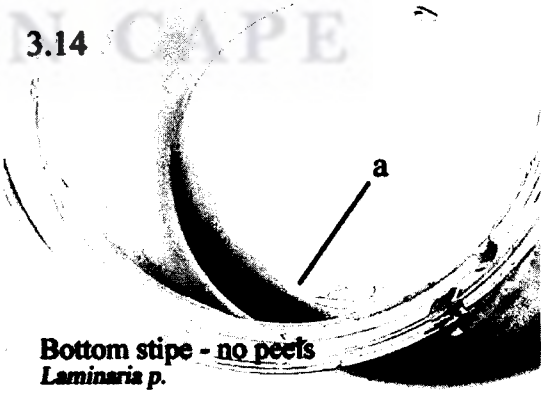
Bottom stipe - no peels
Laminaria p.

3.13



Bottom stipe - no peels
Laminaria p.

3.14



Bottom stipe - no peels
Laminaria p.

3.10. Table I.

Tissue Sections	**Viscosity in centipoise (mPa)**
Top Blade	29
Middle Blade	27
Bottom Blade	27
Top Inner- stipe	27
Top stipe	26
Bottom Inner-stipe	25
Bottom stipe	24
Top peels	25
Bottom Peels	17

JOURNAL OF APPLIED PHYCOLOGY

Instructions for authors

General

Journal of Applied Phycology publishes original articles relevant to practical uses of algae, including fundamental research, development of techniques and commercial applications. The scope includes algal biotechnology and genetic engineering, tissue culture, culture collections, commercially useful micro-algae and their products, marine culture, algal inoculation and soil fertility, pollution and fouling, monitoring, toxicity tests, toxic compounds and antibiotics. Each part of the journal also includes a section of camera-ready copy for brief notes and information on new products, patents and company news.

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4. Gene Bulletin: 1–(usually)2 pages. Data on sequencing insufficient for full-length paper. (For examples, see 8: 563–564 and 8: 565–567.)
5. Brief notes for the camera-ready appendix to each number of the journal on topics of general interest such as new products, patents and company news.

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Manuscripts will first be checked for language, presentation and style. Substandard manuscripts will be returned to authors without review. Such manuscripts may be resubmitted after all necessary adjustments have been made.

Papers which conform to journal scope and style are sent to outside referees.

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Manuscripts should conform to standard rules of English grammar and style. British or American spelling may be used, but must be consistent throughout the article.

Conciseness improves greatly the readability of a paper. Ambiguous statements, vague expressions and long and pointless series of adjectives should be avoided. Do not include long lists of references to make a single scientific point. Authors are also warned against a sloppy use of scientific expressions. Examples are such terms as physicochemistry where physical and chemical properties (of water) are meant, the non-interchangeable use of variables (or variates, or environmental factors) and parameters etc. Authors are warned against the erroneous use of period and comma in numerical values. Remember that in English ten thousand is written 10,000 while ten, exact to three decimals, is written 10.000. Do not include a comma in four-digit numbers.

THREE COPIES of the manuscript should be submitted. They should be free of handwritten corrections. They should be double spaced throughout, typed on only one side of the paper, with 3 cm left-hand margin. Do NOT adjust vertically the right-hand margin. Tables and illustrations should also be submitted in triplicate, but there is a need for only one high-quality original.

White paper of good quality and standard size (21 × 29 cm) should be used. Word-processed manuscripts should be printed in letter quality or near-letter quality mode.

The contents of the manuscript should be well organized. Page one should show the title of the contribution, name(s) of the author(s), address(es) or affiliation(s) and up to ten key words. An appropriate phone, fax and email number (in parentheses) may follow the corresponding author's address. The abstract should appear on page two. The body of the text should begin on page three. It should be free of footnotes and divided into sections and subsections.

A typical organization might be:

Introduction
Materials and methods
Results
Discussion
Acknowledgements
References
Tables
Figure captions.

The Introduction of a research paper should usually end with a clear statement of aims (and not a repeat abstract). Results and Discussion must be separated, unless there is a scientific reason to amalgamate them. Approximate locations for tables and figures should be indicated in the left margin of the text.

Words to be published in italics should be presented in italics.

Tables

Tables should not duplicate figures or *vice versa*. They should be numbered consecutively in Arabic numerals, and bear a descriptive legend on top. Tables should b presented on separate sheets of white paper. Author should plan tables to fit either one or two column widths of the journal.

Vertical lines are not to be used and horizontal one should be kept to a minimum.

Figures

All figures should be numbered in Arabic numerals, either on top or on the back and identified by the author's name. The top of the figure should also be indicated. Figure captions should be grouped on a separate sheet(s) of paper, after the tables. Do not type captions on the figures themselves.

Photographs should be original, glossy prints, and not cuttings from books or papers.

Colour photographs will only be accepted if the author agrees to pay for the extra cost (about US$ 700.00).

Figures will mostly be printed to fit one column width of the journal, so it is important to consider how well the figure will reduce. Particular features to consider are to use thick lines, large symbols and text and sufficient space between text and lines to permit satisfactory reduction. (90% of authors fail to follow these simple guidelines and the figures have to be returned for re-drawing.)

As far as possible, avoid the use of shading. If it is essential for clarity, use types of shading that will reduce satisfactorily.

Do not surround figures with unnecessary boxes or add upper and right-hand lines to graphs, unless these lines are needed to provide additional scales.

While some computer graphics packages are excellent, several widely used ones are inadequate to produce journal-quality figures. If you do not have access to a good software package, you will need to add additional lettering and delete nonsense such as 0.00 for 0 on the axes.

Quantities, units, symbols and their abbreviations

Standard international units (S.I. system) are in general the only ones acceptable, but L is used for liter and other units are used for time besides 's'. These are common examples:

1. Basic quantities, units and their symbols

Quantity	Unit	Symbol	Wrong (derived) symbols or wrong abbreviations
length	meter	m	mikron, Å, mile, foot, in.
mass	kilogramme	kg[1]	kgr, ounce, . . .
time	second	s[2]	sec
electrical current	ampere	A	
amount of matter	mole	mol[3]	

(1) The gramme, g, equals 10^{-3} kg and is a widely used unit of mass, although only the kg has a definition as a *basic* unit in the S.I. system.

(2) In wide use, and bearing separate names, are the following multiples of the second: minute (min); hour (h); day (d); year (annum): (yr, a).

(3) The dimension of the mole is that of a *number*, of a given entity, which is to be specified (molecules, atoms, ions, . . .). In practical water chemistry, the concentration of matter (mol m^{-3}) is used, and often not distinguished from the mole itself.

2. Some decimal prefixes for S.I.-units

giga	G	10^{9}
mega	M	10^{6}
kilo	k	10^{3}
deci	d	10^{-1}
centi	c	10^{-2}
milli	m	10^{-3}
micro	μ	10^{-6}
nano	n	10^{-9}
pico	p	10^{-12}
femto	f	10^{-15}

3. Some derived SI-units with and without a name and their symbols

	Unit	Symbol	Wrong unit or symbol	Comments
surface	square metre	m^2	acre	hectare (ha) is tolerated
volume	cubic metre	m^3	gallon	the volume taken by 1 kg of water is 1 dm^3 but litre (L) is tolerated, as well as mL (cm^3), but not cc
speed	metre per second	$m\ s^{-1}$	knots, miles per hour	
concentration	mole per cubic metre	$mol\ m^{-3}$	in ionic balances, $meq\ L^{-1}$	
B(C_B)force	newton	$N = kg\ m\ s^{-2}$	dyne	
pressure	pascal	$Pa = N\ m^{-2}$	bar, atm, torr, mm Hg, mm H_2O ...	
energy, work, amount of heat	joule	$j = Nm$	cal, kWh, erg HP, CV ...	
power	watt	$W = J\ s^{-1}$		
electrical tension	volt	$V = W\ A^{-1}$		
electrical resistance	ohm	$\Omega = V\ A^{-1}$		
(electrical) conductance (conductivity)	Siemen	$S\ cm^{-1}$	mho, mho cm^{-1}	in freshwaters, the range is mostly $\mu S\ cm^{-1}$ (at a specified temperature)
light intensity	photon flux density (or irradiance)	$\mu mol\ photon\ m^{-2}\ s^{-1}$		usually given for PAR (Photosynthetically Active Radiation)
radioactivity (1)	bequerel	$Bq = s^{-1}$	1 curie (Cu) = 37.10^9 Bq	
equivalent absorbed dose (1)	Sievert	$Sv = J\ kg^{-1}$	rem = 10^{-2} Sv	

4. Combined expressions in text, tables and figures should be presented using negative exponents. Examples are given in the table below. (It is recommended that L be used for litre.)

Preferred	Rejected
$g\ C\ m^{-2}\ h^{-1}$	$g\ Cm^2/h$
$kg\ m^{-2}\ s^{-1}$	$kg{\cdot}m^{-2}{\cdot}s^{-1}$
	$kg \times m^{-2} \times s^{-1}$
	$kg/m^2/s$
$meq\ L^{-1}$	meq/L^{-1}
‰, $g\ L^{-1}$, $g\ kg^{-1}$	ppt
$\mu g\ L^{-1}$, $\mu g\ kg^{-1}$	ppb
$mg\ L^{-1}$	ppm

5. Chemical symbols

Ions: PO_4^{3-} is preferred over PO_4^{---}

Fe^{2+} is preferred over Fe^{++}

Equivalents (or milliequivalents) and moles (or millimoles) are both acceptable, if properly defined.

Compounds: more and more abbreviations are being introduced for chemical compounds. Some, like DO, EDTA, HEPES are widely known, but it is advisable to give a full statement of the meaning when first used in the text. Such usage is compulsory for less familiar acronyms. Excessive use of acronyms and other abbreviations is strongly discouraged.

6. Biological nomenclature

Authors are urged to comply with the rules governing biological nomenclature, as expressed in the

International Code of Botanical Nomenclature, the International Code of Nomenclature of Bacteria and the International Code of Zoological Nomenclature.

Authors are urged to check the correct spelling of all scientific names appearing in their texts.

When a species name is used for the first time in an article it should be stated in full. The authority for a species used for research purposes should be given in Materials and methods.

7. Chemical nomenclature

The conventions of the International Union of Pure and Applied Chemistry, and the recommendations of the IUPAC-IUB Combined Commission on Biochemical Nomenclature should be applied.

References to the literature

1. Citation in the text:

Use the name and year system: Adam (1983) or (Adam, 1983). For two authors, use: Adam and Eve (1982), not Adam & Eve or Adam et Eve. For more than two authors, use et al.: Adam et al. (1982). Initials should be used in the case of personal communications (pers. comm.), which need not be repeated in the reference list. Reference can also be made to a particular page, table or figure in published work, as follows: Brown (1966: p. 182) or Brown (1966: p. 182, Figure 2).

2. Citation in the list of references:

All publications cited in the text, and only these, should be listed alphabetically after first authors. If an author published several papers in the same year, they should appear as Adam, 1980a, 1980b, ... This also applies to citations in the text. If an author has published both alone and with (a) co-author(s), the papers which he authored alone should be ranked first, followed by the ones with one co-author alphabetically after the name of the co-authors (not chronologically), followed by the ones with two-co-authors, etc.

Initials of authors should always follow family names:

Casey RP, Lubitz JA, Benoit RJ, Weissman BJ, Chau H (1963) Mass culture of *Chlorella*. Food Technology 17: 85–89.

Prospective authors are urged to give attention to details of punctuation in this example.

Compound names: alphabetization by first word of the family name is preferred: Von Stroheim, Van Straelen should appear under V, De Ridder and Du Plessis under D. Authors should carefully check and conform to capitalization and spacing in such names. For non-European names, where the use of a family name is substituted by other systems (as in Arabic and several Asiatic cultures), authors are requested to indicate clearly on their manuscript which name they wish to use as the homologue to a family name. However, Chinese names should usually be written in full, starting with the family name (1-syllable name), followed by the given name (2-syllable name) written without hyphen and no capital for second syllable, e.g. Liu Chungchu.

Publications should always be cited in their original language, except if in a non-Latin alphabet. For the latter a Latin letter-by-letter transliteration is preferred, but an English translation of the title may be added with the original language indicated between square brackets at the end of the reference.

Papers which are unpublished or in press should be cited only if formally accepted for publication. Unpublished, internal reports are not acceptable in reference lists, unless they are available for general distribution.

Avoid the use of 'Anonymous'. If no author is ascertainable, list reference by name of sponsoring body, or name of editor.

In a continuous series of article citations from a single journal, do not use *ibid.* instead of the journal abbreviation.

3. Journal citations and abbreviations

3.1 If the title of a journal is a single word, *do not* abbreviate. Examples: Behaviour, BioScience, Biotechnology, Experienta, Growth, Hydrobiologia, Photosynthetica. Do not insert a comma between the name of the journal and the volume number.

3.2 Journals and book series that appear on a regular basis should be abbreviated (example 1). Several systems are in use but *Journal of Applied Phycology* uses a standard which is based on the 'World List of Scientific Periodicals', published by Butterworths, London, with certain simplifications.

Note that adjectives are only capitalized if they are the first word of a journal's title. Abbreviated words are followed by a period (Journal = J.), contracted words are not (Board = Bd. not Bd.; other examples of contractions are: Doctor = Dr, circa = ca.

Issue numbers should be added only (between brackets) if every issue starts with page one. Volume numbers should be expressed by Arabic numbers in all cases.

3.3 Edited symposia, special volumes or issues, etc., published in a periodical.

Author(s), year of publication. Title of paper. In editor(s), title of special volume, periodical (abbreviated, cf. supra), vol: pp. (example two).

3.4 Books

Author(s), year. Title. Publisher, city: pp. (example three).

3.5 Multi-author books

Author(s) of chapter, year, title of chapter. In editor(s), title of book. Publishers, city: pp.

Examples

Edwards P (1980) The production of micro-algae on human wastes and their harvest by herbivorous fish. In Shelef G, Soeder CJ (eds), Algae Biomass. Production and Use. Elsevier/North Holland Biomedical Press, Amsterdam: 191–203.

Harvey W (1988) Cracking open marine algae's biological treasure chest. Biotechnology 6: 487–495.

Hutchinson GE (1975) A Treatise on Limnology, 3. J. Wiley & Sons, New York, 660 pp.

Metting B (1988) Micro-algae in agriculture. In Borowitzka MA, Borowitzka LJ (eds), Micro-algal Biotechnology. Cambridge U.P., Cambridge: 288–304.

Final recommendations

Before mailing a manuscript to *Journal of Applied Phycology*, proofread the final version thoroughly and correct any left-over errors. In particular, check the spelling of all scientific terms, Latin names of animals and plants, figure captions and tables. Are all units S.I.? Is all lettering properly composed and will it be readable after reduction? Are all numerical values and mathematical symbols exact? Are locations of figures and tables indicated in the margin? Make certain that every reference is abbreviated correctly, and appears both in the text and reference list.

To those scientists who use English as a foreign language, we strongly recommend that their manuscript be read by a native English-speaking colleague.

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