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Seasonality of abundance, reproduction and epiphytism in *Gracilaria cliftonii* Withell, Millar & Kraft, 1994 from Western Australia

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ABSTRACT

The Government of Western Australia (RIDC) has identified seaweed cultivation as an alternative activity to utilise inland saline water bodies in rural areas. *Gracilaria cliftonii* has been identified as a potential species for inland saline water aquaculture and is high agar yield (62%). However, information related to its seasonal variations from natural populations has not yet been reported. The aim of the present research was to determine the seasonality in biomass, abundance, epiphytism and chemical properties of *Gracilaria cliftonii* collected from the wild. *G. cliftonii* samples were collected during 6 seasons from Autumn 2008 to Winter 2009 from Point Peron, Western Australia. Maximum biomass of *G. cliftonii* was observed in Winter (42.2 g m^2) while maximum total abundance occurred in Summer (3.4 nh/m^2). Three reproductive stages viz. tetrasporophyte, carposporophyte and male gametophyte were observed all year round.

The biomass and abundance of tetrasporophyte and carposporophyte stages from *G. cliftonii* in Autumn 2008 were significantly higher than other seasons while the biomass of the vegetative stage was significantly higher in Spring and Summer. Abundance showed a strong correlation (R^2=0.96; $p<0.05$) with day length. Over the study period, tetrasporophyte and carposporophyte were significantly longer ($p<0.05$) than male gametophyte and vegetative stages.

Epiphytism was a common phenomena observed in *G. cliftonii*. Epiphytic biomass was significantly higher in Autumn and Winter 2008, while, epiphytic load was significantly higher in Spring 2008. Over entire study period twenty four macroalgae epiphytes were recorded, with the *Ceramiales* as the most dominant group and *Hypnea episcopalis* and *Polysiphonia forfex* the most dominant epiphytes. The epiphytes of *G. cliftonii* are attached in different ways to its thallus. Although, most of the epiphytes were attached superficially to the surface, *Polysiphonia* sp., and *Ceramium* sp. were penetrated into the host tissue.

The chemical composition of *G. cliftonii* varied among life stages and seasons and consisted mainly of carbohydrates followed by ash and protein. The physicochemical properties of *G. cliftonii* were mainly influenced by seasons and all the values obtained were higher than previous recorded for other macroalgae. Finally, yield and properties of the agar of *G. cliftonii* were also demonstrated to be influenced by seasons and life stages.

ABSTRACT

The present study shows that biomass of *G. cliftonii,* epiphytism, and chemical compositions are highly variable and are result of the effect of seasons and life stages. Therefore, the effects of seasons and life stages have to be considered for culture and exploitation of the species.

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SEM	Scanning electron microscope
nh	Number of holdfasts
%db	Percentage dry basis
ETOH	Ethanol
HMDS	Hexamethyldisilizane
GMA	Glycol methacrylate
WRC	Water retention capacity
ORC	Oil retention capacity
S.E.	Standard error
N/A	Not applicable
PES	Provasoli Enriched Seawater
ppt	Parts per thousand

1. LITERATURE REVIEW

1.1. Macroalgae

Macroalgae are a heterogeneous group of photosynthetic, nonvascular organisms that contain chlorophyll a and have simple reproductive structures (Vadas, 1979). They include macroscopic multicellular marine red, brown and green algae (Lobban and Harrison, 1994). Macroalgae are benthonic organisms with a cosmopolitan distribution within the photic zone of the oceans (Lüning, 1990) and play an important role in marine communities as primary producers and in the transference of energy to higher trophic levels of aquatic ecosystems (Lobban and Harrison, 1994). As components of aquatic ecosystems, macroalgae reflect the health of the environment through their population parameters such as density and abundance (Stevenson *et al.*, 1996). In addition, macroalgae also provide shelter and substratum for other organisms creating microhabitats and nursery areas (Chapman *et al.*, 1987).

From the commercial point of view, macroalgae are important source of food, fertilisers, hydrocolloids, pigments, fodder and secondary metabolites (Gellenbeck and Chapman, 1983). These applications have created a seaweed industry which provides a wide variety of products and is estimated to provide total annual value of US\$ 5.5-6 billion (Critchley *et al.*, 2006, McHugh, 2001).

1.2. Gracilaria

Gracilaria is the third largest genus in the Rhodophyta, with over 150 described species (Armisen, 1995). It is cosmopolitan, with the greatest standing stocks and numbers of species occurring in temperate seas (Abbott, 1999). *Gracilaria* species are commercially important in the production of the phycocolloid agar (Tseng and Xia, 1999). According to Bixler and Porse (2010) worldwide agar production in 2009 was estimated to be 9600 tones with a value of US \$ 173 million. Main producers of agar are China, Chile and Indonesia with an average price of US \$18 k^{-1} of agar.

Many studies have been undertaken on their taxonomy and suitability for commercial exploitation (Bird, 1995). Over the past 45 years most of the studies have focused on comparative morphological descriptions (Chang and Xia, 1963, Edelstein *et al.,* 1978, Bird and McLachlan, 1982, Bird and McLachlan, 1984,

Fredericq and Hommersand, 1989, Withell *et al.,* 1994, Byrne *et al.,* 2002). At the same time, surveys of the *Gracilaria* floras in different locations were conducted, which have helped to clarify species concepts and biogeographic distribution (Yamamoto, 1978, Dinh, 1992, Zhang and Xia, 1992, Womersley, 1956).

In Australia, the first extensive anatomical and taxonomic study of the genus *Gracilaria* was made by May (1948), who dealt mostly with terete species and described nine species of *Gracilaria* (Withell *et al.,* 1994). It was until 1994 when studies on *Gracilaria* were attempted again (Withell *et al.,* 1994, Byrne *et al.,* 2002, Womersley, 1996). Latest report on *Gracilaria* sp. reports 30 species distributed in Australia (Millar, 1997) with few only few studies on the genus *Gracilaria* conducted for the last 10 years.

1.3. Gracilaria cliftonii

1.3.1. Taxonomy

As in rest of the Gracilariales, the taxonomy of *Gracilaria cliftonii* is not clear and has been reviewed several times (Withell *et al.,* 1994, Agardh, 1876, May, 1948). Before Withell *et al.,* (1994) revision, *G. cliftonii* was known as *G. furcellata* Harvey based on the morphological descriptions made by Harvey (1863) and Agardh (1876). In 1863, Harvey (1863) described *G. cliftonii* as *Gracilaria furcellata* based on descriptions provided by Montagne (1850) for *Plocaria furcellata*. In 1876, Agardh (1876) found the specimens from Harvey to be typical of *Gracilaria* but different from *P. furcellata.* Therefore, Agardh (1876) excluded *P. furcellata* as the basionym of Harvey's species (*G. furcellata*) but continued using the name of *G. furcellata* with Harvey as the only authority for the specie. Later, Papenfuss and Edelstein (1974) considered *Plocaria furcellata* and *G. furcellata* (Montagne) Zanardini as synonyms of *Sarconema filiformis* (Sonder) Kylin. Finally, Withell *et al.,* (1994) revised the previous names and proposed the new name of *Gracilaria cliftonii* which is the current accepted name for the species.

The current classification for *G. cliftonii* is as following (FloraBase, 2009):

Phylum: Rhodophyta

Class: Florideophyceae

Order: Gracilariales

Family: Gracilariaceae

Genus: *Gracilaria*

Species: *Gracilaria cliftonii*

1.3.2. Distribution

Ten *Gracilaria* spp. are found in Western Australia; *Gracilaria cliftonii, G. blodgettii, G. verrucosa, G. perissana, G. urvillei, G. comosa, G. salicornia, G. eucheumatoides, G. canaliculata* and *G. flagelliformis* (Womersley, 1996, FloraBase, 2009). *G. cliftonii* is distributed from Geraldton (28°46′44″S 114°36′25″E) to Esperance (43° 19' 60″ S 147° 4' 0″ E) and is high in agar content (52 % db) (Byrne *et al.*, 2002)(Plate 1.1).
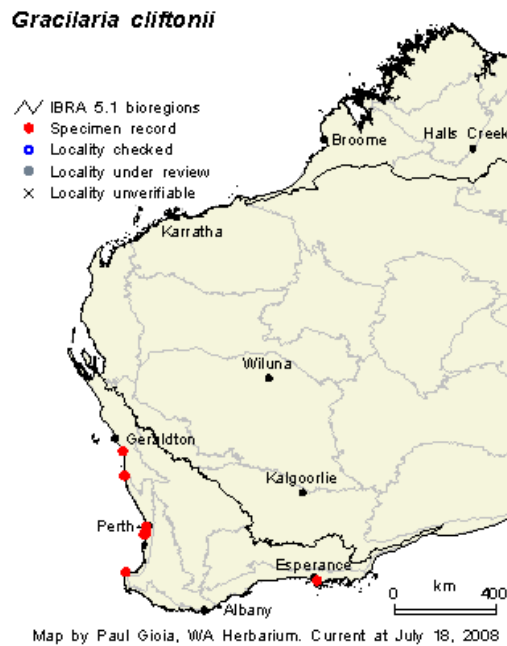
Plate 1.1 Distribution of *Gracilaria cliftonii* in Western Australia (FloraBase, 2009).

1.3.3. External morphology

The morphology of *G. cliftonii* has been described by Withell *et al.,* (1994). Thalli are terete up to 18 cm in length arising either singly or in dense clusters from an encrusting holdfast. The base of the main axis has a diameter of 0.6-1.5 mm which decreases gradually towards the apices. The thalli when arising from individual holdfasts are usually robust but once they are consolidated they decrease

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in height and diameter. Main axes and branches of the thalli usually are dichotomously divided. Bases of branches are usually unconstricted. Liquid-preserved specimens are firm and pliable in texture, while dried specimens are firm but brittle. Specimens usually remain terete although sometimes can become compressed. The colour of *G. cliftonii* thalli varies from light brown to dark brown-red.

1.3.4. Vegetative anatomy

The vegetative thalli of *Gracilaria* spp. consist of cortex and medulla (Withell *et al.*, 1994). The cortical cells are pigmented cells and are smaller than the cortical cells. The medulla comprises of large parenchymatous cells. The layers of cortex, the size and number of medullary cells and the change of cells from cortex to medulla are used for identification of this species (UNDP/FAO, 1990). The transition of cell size from cortex to medulla is gradual throughout the thallus. Medullary cell walls are 8-15 μm thick and filled with starch grains. Secondary pit-connections are conspicuous in all sub-surface layers and are rare in outermost cells. The cortex of larger branches is 2-4 cells thick and the cells stain densely. The outermost cells are 9-13 μm long by 6-8 μm in diameter while the medulla is 9-14 cells wide. The medullary cells are polygonal to spherical in shape increasing gradually in size towards the centre (160-280 μm in diameter).

1.4. Reproduction and life cycle

Gracilaria species reproduce asexually, through vegetative growth and sexually including meiosporogenesis as well as gametogenesis and mating. Asexual reproduction allows population increase, but allows no variation, whereas sexual reproduction allows variation but can be expensive under cultivation because of the wasted gametes that fail to meet and thus fertilise (Cole and Sheath, 1990). The switch from vegetative growth to reproduction often depends on environmental factors and is characterised by a reduction of the thallus growth (Lobban and Harrison, 1994).

Sexual reproduction is oogamus in *Gracilaria* spp., and involves the union of a non-flagellate male gamete, the spermatium, with the trychogyne of the carpogonia of the female. The sexual system is linked to the life cycle through different stages.

Populations of *Gracilaria* species have complex haploid-diploid life cycles that are referred as triphasic or "*Polysiphonia*" type and consist of three observable stages (Plate 1.2). The three phases are a diploid phase (tetrasporophyte), a haploid (gametophyte), usually dioecious phase and an additional diploid, zygote-derived sporangium (carposporophyte) phase (Kain and Destombe, 1995). While the gametophyte and tetrasporophyte develop as independent thalli, the carposporophyte stage develops on the gametophyte thallus.

As in all sexual life cycles, the three phases are interconnected through meiosis and syngamy. Meiosis takes place on the tetrasporophyte thallus, giving rise to haploid tetraspores. Tetraspores develop into gametophytes, which produce gametes by mitosis. Fertilisation occurs on the female gametophyte and the fertilised female gamete develops into a carposporophyte thalli. Once fertilised, the cystocarps develop as macroscopic hemispherical swellings on the surface of female branches within which the carposporophyte produces thousands of diploid carpospores. Finally, completing the cycle, each carpospore develop into a new tetrasporophyte organism (Engel *et al.*, 2001).

Although *Gracilaria* is characterised by a *Polysiphonia*-type life cycle with an alternation of generations, deviations from this typical life cycle have been described for many species of *Gracilaria* (Kain and Destombe, 1995, Plastino and Oliveira, 1996, Engel *et al.*, 2001). Mixed reproductive phases have been reported, such as the presence of male and/or female reproductive structures on tetrasporophyte thalli or as spermatangia conceptacles and cystocarps developing in the same thallus (Destombe *et al.*, 1989, Guimarães *et al.*, 1999, Polifrone *et al.*, 2006, Kim, 1970). However, no information is available on the ecophysiological advantage of these deviations on any natural populations.
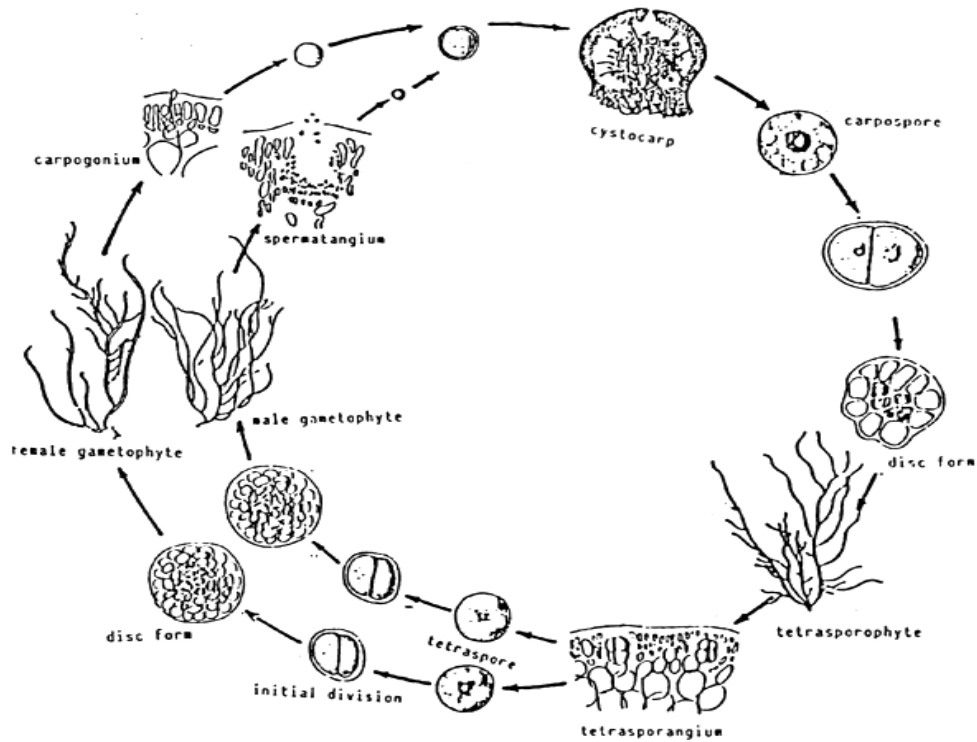
Plate 1.2 *Gracilaria* spp. triphasic life cycle (UNDP/FAO, 1990)

1.4.1. Tetrasporophyte

Tetrasporangia are structures which produce tetraspores. They are usually found on free range (unattached) tetrasporophyte thalli typically giving rise to four uninucleate equal -sized spores (Cole and Sheath, 1990). Tetrasporangia in *Gracilaria* are decussate, cruciate and occasionally have been reported as tetrahedral (Edelstein *et al.*, 1978) or regularly divided (Yamamoto, 1978). Withell *et al.,* (1994) described tetrasporangia of *G. cliftonii* as cruciately divided (Plate 1.3), 29-48 µm long and 20-27 µm wide which are borne in branches on the upper half of the thallus. The cortex surrounding mature tetrasporangia consists of 3-6 cell layers. The cortex cells are frequently larger and more elongate than cells in vegetative portions of the thallus.

1.4.2. Male gametophyte

Spermatogenesis in male gametophytes takes place within specialised structures known as spermatangia (Cole and Sheath, 1990). Spermatangia are obovoid to globose, 3-5 µm long by 3-4 µm in diameter. Spermatangia are borne in

ovoid verrucosa-type conceptacles or very rarely deep, cavern-like polycavernosa-type conceptacles. The surrounding cortical cells are frequently elongated compared to the cortical cells in vegetative portions of the thallus (Bird, 1995). Three basic types of spermatangia are recognized: 1) verrucosa-type, 2) textorii-type and 3) chorda-type (Bird and McLachlan, 1984). Additionally, different variations from the three main types, including symmetrica-type and henriquesiana-type can be observed for *Gracilaria* spp. (Bird, 1995) (Plate 1.3). As described by Withell *et al.,* (1994), *G. cliftonii* conceptacles are 55-80 μm deep by 40-50 μm across. They are continuous when mature and borne on the entire thallus except basal and extreme apical portions. Thalli are dioecious and can bear few cystocarps on predominantly male thalli.
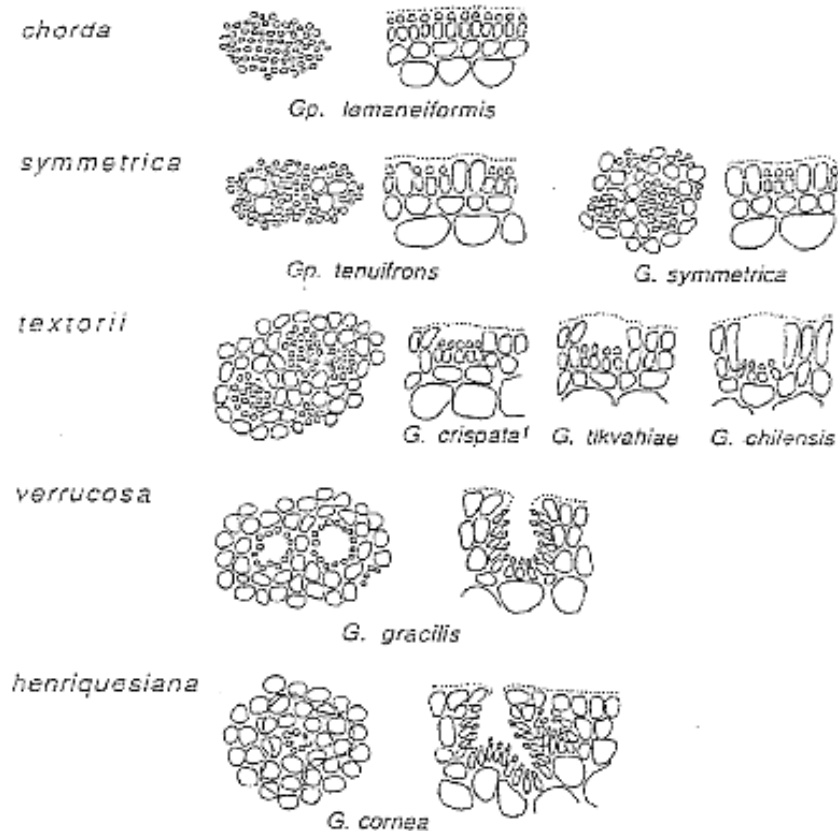
Plate 1.3 Spermatangia types found in Gracilariaceae
G = *Gracilaria,* Gp= *Gracilariopsis* (Bird, 1995)

1.4.3. Female gametophyte

For *G. cliftonii*, carpogonial branches are present in the cortex of younger branches and borne on sub cortical cells that also bear cortical filaments. The mature carpogonial branch is two-celled thick, the hypogynous cell transversely ovoid to sub spherical and the carpogonium conical to ovoid bearing distally a short, lightly staining trichogyne that penetrates to the cuticle (Withell *et al.*, 1994).

1.4.4. Carposporophyte

After fertilization, a large lobed fusion cell forms which remains conspicuous at the base of the carposporophyte throughout subsequent development. Mature cystocarps ostiolate are 0.7-1.2 mm high by 1.0-2.0 mm wide, occasionally basally constricted and/or beaked. Pericarp is 200-420 μm thick, consisting of 15-18 cell layers. Gonimoblast parenchyma is moderately abundant with basal cells 75-90 μm long by 43-55 μm wide.

Carposporophyte thalli produce haploid spores known as carpospores. Carpospores are ovoid to obovoid, 32-46 μm long by 16-32 μm diameter, containing a central chloroplast and borne terminally on the gonimoblast filaments. The carposporophyte sometimes fails to develop beyond a fusion-cell stage; nevertheless, the pericarp enlarges to the size found in mature cystocarps. Thalli with aborted carposporophytes also bear cystocarps of normal size and form (Withell *et al.,* 1994).

1.5. Spore release

The mechanism of spore release has already been described for some Rhodophytes (Pacheco-Ruíz *et al.*, 1989, González and Meneses, 1996, Santos and Duarte, 1996, West and McBride, 1999) and some *Gracilaria* spp. (Rao, 1976, Rama Rao and Thomas, 1974, Orduña-Rojas and Robledo, 1999) (Table 1.1). Spore release, settlement and germination of *Gracilaria* spp. have been studied to determine dynamics of natural populations, mainly recruitment. The number of spores released represents a measure of the reproductive potential of the species and indicates the time when the environmental factors are more suitable for the species reproduction (Pacheco-Ruíz *et al.*, 1989). However, most of the work has been focused on the carpospore release due to the rapid development of the carposporophyte under laboratory conditions making it ideal for experimentation on

patterns of spore development and release (West and McBride, 1999).

Table 1.1 Tetraspore and carpospore release of *Gracilaria* spp.

Species	Tetraspores	Carpospores	References
Gracilaria chilensis	N/A	405-554 /cystocarpic thallus /cm^2	Alveal *et al.* (1997)
G. cornea	171,691 g /day	9000/cystocarp/day	Guzmán-Urióstegui and Robledo (1999)
G. cornea	N/A	2465/cystocarp/day	Orduña-Rojas and Robledo (1999)
G. corticata	N/A	4911/cystocarp/day	Rao (1976)
G. foliifera	3,050 cystocarpic thallus/cm^2	200/0.1 ml	Friedlander and Dawes (1984)
G. pacifica	30-50,000 g /day	150, 00 g /day	Garza-Sánchez *et al.* (2000)
G. parvispora	N/A	18,600/cystocarpic thallus/cm^2	Glenn *et al.* (1996)
G. verrucosa	N/A	19,700 / cystocarpic thallus	Oza and Krishnamurthy (1968)

Different environmental factors have been cited as critical in the development of carpospores (Guzmán-Urióstegui and Robledo, 1999) and tetraspores (Ye *et al.*, 2005) and different studies on *Gracilaria* spp. have been carried out with the aim of establishing different propagation methods. The effect of some environmental factors on *Gracilaria* spp. spore release and development is summarised in Table 1.2, however, it is not fully understood how these factors influence spore release in *Gracilaria* spp.

Table 1.2 Studies on the effect of environmental factors on the spore development *Gracilaria spp.*

Species	Reproductive structure	Factor	Development stage	References
Gracilaria asiatica	Carpospores	Temperature	Germination	(Chen and Ren, 1987) (Fengjuan *et al.*, 2006)
G. chilensis	Carpospores		Release Attachment Germination	(Alveal *et al.*, 1997)
G. cornea	Carpospores	Temperature Day length Photon irradiance	Release	(Guzmán-Urióstegui and Robledo, 1999)
G. cornea	Carpospores	Irradiance Temperature	Release Growth	(Orduña-Rojas and Robledo, 1999)
G. corticata	Tetraspores	Air exposure	Release	(Rao, 1976)
	Carpospores	Photoperiod		
G. foliifera	Carpospore	Light intensity Temperature Salinity	Release Growth Photosynthesis	(Friedlander and Dawes, 1984)
G. lemaneiformis	Tetraspores	Temperature Irradiance	Germination	(Ye *et al.*, 2005)
G. pacifica	Carpospores Tetraspores	Irradiance Temperature	Release Attachment Survival	(Garza-Sánchez *et al.*, 2000)
G. parvispora	Carpospores	Substratum	Attachment	(Glenn *et al.*, 1996)
	Carpospores	Water motion	Release Attachment Development	(Ryder *et al.*, 2004)

1.6. Spore germination

The development of sporelings in *Gracilaria* is composed of four stages: primary division stage, hemisphere body stage, basal disc stage, and young sporeling stage (Fengjuan *et al.*, 2006, Orduña-Rojas and Robledo, 1999). Once the reproductive cells (carpospores/tetraspores) have been released from the cystocarps

and tetrasporangia, three events are distinguished in the development of the cells into adults: settlement, attachment and germination. After the spores have contacted and adhered to the surface, they begin to improve their adhesion to the substratum and developing rhizoids. Once the cell is attached to the substratum the cell starts a process of cell division forming a basal disc which develops into a holdfast (Lobban and Harrison, 1994, Cole and Sheath, 1990).

Germination is an oriented process as algae cells have polarities, especially apico-basal polarities that distinguish holdfast from the thallus. Normally, the cell undergo further divisions and the basal cells form the rhizoid while the apical cells form the thallus and develops into a new differentiated thallus (Lobban and Harrison, 1994).

1.7.   Reproductive phenology

Studies on seasonal variation of reproduction in tropical (Rao, 1973, Hoyle, 1978c, Orduña-Rojas and Robledo, 2002) and temperate (Jones, 1959, Pickering *et al.,* 1990, Penniman *et al.,* 1986) of *Gracilaria* species in different part of the world have been previously reported (Table 1.3). The occurrence of gametophytic and tetrasporophytic thalli from *Gracilaria* spp. populations have been reported in various geographical locations. A maximum peak of biomass and abundance of reproductive stages has been observed in Summer and has been reported to be dependent on water temperature (Boraso de Zaixso, 1987). In the contrary, some *Gracilaria* populations have reproductive thallus throughout the year (Hay and Norris, 1984, Hoyle, 1978c) suggesting that reproductive development is species specific. Since the occurrence of reproductive stages from *Gracilaria* spp. is highly variable in time and space, further investigation is required to understand the life cycle of each species.

Table 1.3 Monthly variability of the occurrence of *Gracilaria* spp. from natural populations

Species	Locations	Main stage	Reproductive peak	Reference
Gracilaria spp.	Caribbean	C	November	(Hay and Norris, 1984)
G. bursapastoris	Hawaii	T	July	(Hoyle, 1978c)
G. cornea	Mexico	T	May-August	(Orduña-Rojas and Robledo, 2002)
G. coronipofolia	Hawaii	T	January-February July-August	(Hoyle, 1978c)
G. domingensis	Caribbean	T	November August	(Hay and Norris, 1984)
G. heteroclada	Philippines	T C	April-May January	(Luhan, 1996)
G. sordida	New Zealand	T C	December	(Pickering *et al.*, 1990)
G. sordida	New Zealand	T	Summer	(Pickering *et al.*, 1990)
G. tikvahiae	New Hampshire	V	June-August	(Penniman *et al.*, 1986)
G. verrucosa	British Columbia	T	July	

Abbreviations: T: tetrasporophyte, C: Carposporophyte, V: Vegetative

1.8. Gracilaria productivity

1.8.1. Photosynthesis and respiration

With the aim of increasing the productivity of *Gracilaria*, several studies have been undertaken to determine the effect of different environmental factors on the photosynthetic and respiration rates of *Gracilaria* spp. (Table 1.4).

Table 1.4 Studies of environmental factors on the photosynthesis of *Gracilaria* spp. under different cultivation conditions

Species	Variable tested	Conditions	References
Gracilaria spp.	Photon fluence rate Light spectrum	L	(Beer and Levy, 1983)
Gracilaria spp.	CO_2 addition	L	(Gao *et al.*, 1993)
G. chilensis	CO_2 addition	L	(Gao *et al.*, 1993)
G. chilensis	Natural solar radiation	F	(Gomez *et al.*, 2005)
G. conferta	Carbon acquisition	L	(Andría *et al.*, 1999)
G. cornea	Seasonality	F/L	(Israel and Beer, 1992)
G. cornea	Temperature, salinity, irradiance	L	(Orduña-Rojas and Robledo, 2002, Orduña-Rojas *et al.*, 2002)
G. gaditana	Carbon acquisition	L	(Dawes *et al.*, 1999)
G. lemaneiformis	Use as biofilter	F	(Yang *et al.*, 2005)
G. lemaneiformis	Inorganic carbon	L	(Zou *et al.*, 2004)
G. tenuistipitata	Carbon supply	L	(García-Sánchez *et al.*, 1994)
G. tenuistipitata	Carbonic anhydrase	L	(Haglund *et al.*, 1992)
G. tenuistipitata	pH	L	(Haglund and Pedersen, 1992)
G. tikvahiae	Light / temperature	L	(Lapointe *et al.*, 1984b)
G. tikvahiae	[P] / [N]	F	(Lapointe, 1987)
G. tikvahiae	Hypo-osmotic shock	O	(Lapointe *et al.*, 1984a)
G. vermiculophylla	Photosynthetic responses	L	(Phooprong *et al.*, 2008)
G. verrucosa	PO_4^- / NH_4^+	L	(Dawes *et al.*, 1984)
G. verrucosa	Salinity	L	(Koch and Lawrence, 1987)

Abbreviations: L: Laboratory, O: outdoor, F: Field

1.8.2. Biomass

Productivity of *Gracilaria* spp. has been reported in numerous experimental systems and from different natural populations (Table 1.5). Most of the studies have been determined over relatively short periods of time since it has been difficult to

sustain production for prolonged periods. Natural populations have shown productivity values of 1-2 g db m^{-2} day^{-1} with yields not exceeding 5 t ha^{-1} year^{-1}. During Summer, productivity may be relatively high but is generally regulated by seasonal conditions which declines during Winter (McLachlan and Bird, 1986).

Table 1.5 Productivity of *Gracilaria* species under field cultivation conditions

Species	Productivity g m^{-2} day^{-1} (db)	Cultivation method	Location	Reference
G. asiatica	29 max. 49 (fw)	Raft cultivation	China	(Li *et al.*, 1984)
G. lemaneiformis	3.8 -1.06	Subtidal, free-living population	Chile	(Santelices *et al.*, 1984)
G. secundata	0.8-2.7	Free living/ subtidal	New Zealand	(Luxton, 1981)
G. sjostedtii	10-19 max. 27 (fw)	Raft cultivation	China	(Li *et al.*, 1984)
G. verrucosa	4.7	Intertidal free living	British Columbia	(Saunders and Lindsay, 1979)
	2	Subtidal attached		
G. verrucosa	40 t year^{-1}	N/A	N/A	(Chiang, 1981)
G. verrucosa	6.6	Field	Israel	(Friedlander and Lipkin, 1982)
G. terete	5 t year^{-1}	Cultivation	West Indies	(Smith *et al.*, 1984)

Biomass of *Gracilaria* spp. is extremely variable (Table 1.6). In free-range (unattached) populations of *Gracilaria*, up to several kilograms of wet weight in each square meter are commonly reported. In these situations, *Gracilaria* is usually the most abundant species establishing monocrops; however, much taxonomic identifications are questionable. Attached populations, range from widely scattered to

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a few thalli in each square meter or several hundred grams fresh-weight, and *Gracilaria* is the most common species in the habitat. Because of low densities, hence low production, there have been relatively few attempts to exploit populations of attached thallus (McLachlan and Bird, 1986).

Table 1.6 Biomass of *Gracilaria* spp. from natural populations

Species	Location	Biomass (kg/m^2)	Habitat	Reference
Several, intermixed	Belize		Tropical reef; attached, seasonal variation	(Hay and Norris, 1984)
Gracilaria spp.	Central Chile	0.12	Attached, seasonal variation maximum Winter	(Pizarro and Barrales, 1986)
Gracilaria spp.	British Columbia	4.8	Coastal, attached, subtidal	(Saunders and Lindsay, 1979)
Gracilaria spp.	British Columbia	0.1-1.0	Bay, free-living, intertidal	(Saunders and Lindsay, 1979)
Gracilaria spp.	Central Chile	0.7-5.1	Bay, free-living, subtidal, density related to storms	(Black and Fonck, 1981)
Gracilaria spp.	Central Argentina	2.0	Mixed population, free-living and attached	(Mayer, 1981)
G. lemaneiformis	Central Chile	0.019-0.192	Bay, free-living, subtidal, seasonal variation	(Santelices *et al.*, 1984)
G. verrucosa	Central California	0.7-2.9	Attached, seasonal variation	(Abbott, 1980)
G. verrucosa	Massachusetts	0.02-4.63	Shallow embayment, free-living, seasonal variation	(Conover, 1958)
G. tikvahiae	Nova Scotia		Embayment, free-living, monthly and site variation	(Bird *et al.*, 1977a)
G. tikvahiae	Nova Scotia		Rapidly flowing current, attached, Summer	(Hay and Norris, 1984)

1.8.3. Seasonality

Several studies have been directed towards examining the community structure from macroalgae populations and understanding the main factors contributing to this structure. The significance of factors such as light, energy and interspecific competition has been demonstrated for macroalgae (McQuaid, 1985) but there has been debate over the relative importance of environmental and biological factors (Connel, 1972). This interaction between environmental and biological factors has created temporal and spatial individual patterns of growth, morphology and reproduction in macroalgae (Norall *et al.*, 1981).

There are studies showing the effect of environmental factors on the growth and biomass of *Gracilaria* spp., but a few studies (Table 1.7) have reported the seasonality in biomass and productivity from natural populations (Santelices *et al.*, 1984) and reproductive phenology (Hoyle, 1978c). Previous research on *Gracilaria* spp. productivity and reproductive phenology has produced different results among species around the world (Luhan, 1996, Nelson, 1989, Orduña-Rojas and Robledo, 2002, Penniman *et al.,* 1986, Pickering *et al.,* 1990, Pondevida and Hurtado-Ponce, 1996a, Whyte *et al.,* 1981, McLachlan and Bird, 1986).

In addition, *Gracilaria* spp. growth and reproduction has been reported to be dependent on water temperature (Boraso de Zaixso, 1987, Luhan, 1996), light intensity (Orduña-Rojas and Robledo, 1999), day length (Pickering *et al.*, 1990), water movement (Ryder *et al.*, 2004), nutrient concentration (Pondevida and Hurtado-Ponce, 1996a), salinity and rainfall (De Castro *et al.*, 1991).

Table 1.7 Seasonality of *Gracilaria* biomass

Species	Country	Study Time Month	Sampling method	Population Parameters	Peaks	Environmental parameter
Gracilaria spp.[1]	Japan	13	2500 m^2 (n=3)	Biomass	Summer Spring	N/A
Gracilaria spp.[2]	Brazil	12	0.25 m^2 quadrats Transect	Biomass	January	Temperature Rainfall
Gracilaria spp.[3]	Philippines	11	45 cm Ø iron ring	Biomass	Feb-Sep	Rainfall
Gracilaria spp.[4]	Kenya			Biomass	Sep-Dec	
Gracilaria spp.[5]	Chile	12	Transect 1 m^2 quadrats	Biomass	January	N/A
G. asiatica[5]	China	8	Random samples	Biomass Thallus Length	April	N/A
G. changii[7]	Philippines	12	45 cm Ø iron ring	Biomass	March	Rainfall
G. cornea[8]	Mexico	15	50 m transects 1 m^2 quadrats	Biomass Abundance Thallus length	Jun-Sep	Irradiance
G. heteroclada[8]	Philippines	12	0.25 m^2 quadrats	Biomass	February	Temperature
G. manilaensis[6]	Philippines	12	45 cm Ø iron ring	Biomass	May	N/A
G. sordida[10]	New Zealand	12	0.5 m^2 quadrats	Growth Density	Summer	Temperature Sunshine hr
G. sordida[11]	New Zealand	20	0.25 m^2 quadrats	Biomass Abundance	Summer	N/A
G. tikvahiae[12]	USA	18		Growth	June-August	Temperature
G. verrucosa[13]	Argentina	10	0.25 m^2 quadrats	Weight Thallus Length	Spring Winter	Temperature
G. verrucosa[14]	Canada	5	0.25 m^2 quadrats Transect	Biomass	July	Solar radiation

1 (Chirapart and Lewmanomont, 2004); 2 (Silva *et al.*, 1987); 3 (De Castro *et al.*, 1991); 4 (Oyieke and Kokwaro, 1993); 5 (Wang *et al.*, 1984); 6 (Santelices *et al.*, 1984); 7 (Pondevida and Hurtado-Ponce, 1996a); 8 (Orduña-Rojas and Robledo, 2002), 9 (Luhan, 1996); 10 (Pickering *et al.*, 1990); 11 (Nelson, 1989); 12 (Penniman *et al.*, 1986); 13 (Boraso de Zaixso, 1987), 14 (Whyte *et al.*, 1981)

1.9. Environmental factors

The following environmental variables are commonly regarded as the most important factors affecting distribution and ecology of *Gracilaria* spp. (Kim, 1970).

1.9.1. Sediments

Sediments include a wide range of materials like silt, sand and gravel from different origins that is, terrigenous and marine. Sediments can affect macroalgae assemblages by scouring the surface or abrading the tissues and removing them from the benthos (Cole and Sheath, 1990). Suspended particles and the deposition of fine sediments can interfere with settlement, growth and photosynthetic activity. Sediments can also interfere with settlement and other processes in early life histories of many algae (Schiel *et al.*, 2006).

Attached populations of *Gracilaria* spp. grow on solid substratum (i.e. rocks, coral) which are usually accompanied by sand or other poorly consolidated sediments. More commonly, *Gracilaria* thalli are attached to shells, small stones, pebbles or small objects, which in turn are stabilized among loose sediments. *Gracilaria* may grow with the basal portion of the thalli buried in the sediment (Bird *et al.,* 1977a, Mayer, 1981, Doty *et al.,* 1983). The large commercial populations of *Gracilaria* are found on intertidal or shallow subtidal, wave-sheltered, horizontal or slightly inclined plane surfaces where there is unconsolidated, sandy to muddy sediment. *Gracilaria* spp. often forms monogeneric populations in these habitats, where competition is reduced due to reduction of survival of other macroalgae (Kim, 1970).

The most productive populations of *Gracilaria* are found in Chile, New Zealand, Malaysia, Thailand, The Philippines, Indonesia and China (Santelices and Doty, 1989). *Gracilaria* productivity has been reported to be decreased when there is sand displacement due to water turbulence (Pizarro and Barrales, 1986) and has also been reported to affect spore adhesion (Glenn *et al.*, 1996). However, sediment effects on *Gracilaria* are one of the most neglected due to the difficulty of measuring this parameter in the field.

1.9.2. Water movement

Water movement is an important parameter which affects the growth, distribution and composition of algae populations (Cole and Sheath, 1990). Water movement can affect directly species distribution through spore dispersion, and indirectly affecting the quality and quantity of light into the water column, sedimentation processes of aquatic systems and availability of substratum due to the mobility of lose stones (Lobban and Harrison, 1994).

In addition, water movement supplies nutrients and gases, removes waste products and prevents the settling of silt on *Gracilaria* thalli. However, water motion can negatively affect by imposing mechanical stress (i.e. tension, shear, removal) to *Gracilaria* thalli (Lüning, 1990). Furthermore, water motion affects the morphology of macroalgae thalli. In *G. verrucosa*, the downstream deflection of the thallus caused by the flowing water increases the thallus growth rate by presenting the thallus perpendicular to the incident light (Jones 1959). As a result of these phenomena many thalli achieve increased lengths when growing under steady tidal conditions.

Studies on the effect of water movement on *Gracilaria* populations are reduced comparing to the effect of other environmental factors (i.e. light, temperature) and has mainly determined under laboratory conditions (Gonen *et al.*, 1993) and in few cases under cultivation conditions (Ryder *et al.*, 2004). The effect of water motion on different parameters from red algae is summarized in Table 1.8.

Table 1.8 Effect of water motion on *Gracilaria* spp.

Species	Conditions	Effect/Relation	Reference
Gracilaria cornea	Tanks	Water velocity and incident light are function of thallus shape	(Gonen *et al.*, 1996)
G. conferta	Laboratory	Increased by 30 %-50% on photosynthetic rates	(Gonen *et al.*, 1993)
G. conferta	Tanks	Water velocity and incident light are function of thallus shape	(Gonen *et al.*, 1994a)
G. foliifera	Outdoor	Growth dependant on flow rate	(Lapointe and Ryther, 1979)
G. parvispora	Ocean	Thallus growth rate and spore development positively correlated to water motion	(Ryder *et al.*, 2004)
G. parvispora	Ocean	Growth not significantly affected by water motion	(Glenn *et al.*, 1999)

1.9.3. Temperature

Temperature is one of the most important environmental factors which determines the distribution of marine macroalgae (van den Hoek, 1982, Druehl, 1981). It has been reported that diversity of *Gracilaria* spp.*,* decreases with increasing latitude (Oliveira, 1984, McLachlan and Bird, 1984), thus, it is generally accepted that *Gracilaria* is primarily a warm-water genus (Dawson, 1961, Lawson and John, 1982).

McLachlan and Bird (1986) observed that most *Gracilaria* spp. were limited to regions where water temperatures were 20°C or higher for at least three months of the year.

Furthermore, under laboratory conditions the majority of *Gracilaria* spp. tested have shown a maximum growth within the range 20-28 °C (Kim, 1970).

Some examples of responses to temperature are given in Table 1.9, where it is indicated that most species grow well when at temperatures around 20 °C or higher. This includes species from both tropical or warm-water regions and some species from temperate areas. Species from warm-water areas show maximum growth, and presumably production, between 25 and 30 °C. There are no reports of *Gracilaria* spp. showing maximum growth rates at temperatures exceeding 30 °C and temperatures higher than 30 °C can be lethal to macroalgae (Wang *et al.,* 1984). However, in tropical areas, where there is little fluctuation in temperatures, some species possibly exist at or near supra-optimal conditions throughout the year.

Many habitats in subtropical and temperate areas experience marked seasonal variations in temperature, which during Summer can exceed 30 °C, especially in shallow waters (Wang *et al.*, 1984). Increasing temperatures reduce growth and biomass (Wang *et al.,* 1984, Li *et al.,* 1984) and for many *Gracilaria* spp. productivity is limited to a single period during Spring-early Summer. During Winter, low temperatures limit *Gracilaria* productivity which occurs only in a short period of the year (Bird *et al.*, 1977a). Many of these species show little growth or production when the temperature is less than 15 °C (Simonetti *et al.,* 1970, McLachlan and Bird, 1984, McLachlan and Bird, 1986).

There are temperate areas where there is a variation of less than 10 ^{o}C in the water temperature (Westermeier *et al.*, 1991) or no seasonal fluctuation in temperature

(Lindsay and Saunders, 1980). In these temperate regions, *Gracilaria* can reach biomass of 6.4 kg/m^2 (Hansen, 1984), with relatively high levels of production.

1.9.4. Light

The importance of light related to *Gracilaria* productivity is a difficult parameter to assess. *Gracilaria* spp. occurs throughout a wide variety of habitats, from clear tropical oceanic waters to extremely turbid estuaries and bays. Generally, best growth occurs at or near the surface of the water at irradiances of nearly full sunlight (Kim, 1970), and production usually declines with increasing depth. Laboratory studies show that *Gracilaria spp* are able to maintain net productivities under low levels of light and the compensation point as determined experimentally for *G. tikvahiae* (Rosenberg and Ramus, 1982, Lapointe and Duke, 1984). This may be one of the reasons that species of *Gracilaria* spp. are able to thrive in turbid habitats with low levels of illumination and where these species form essentially monospecific communities (McLachlan and Bird, 1986).

Field studies have shown that maximum biomass and abundance occurs when day length is longer and increase in irradiance (Conover, 1958, Orduña-Rojas and Robledo, 2002). Comparison between various species as documented by previous studies is difficult as optimum light ranges have been reported for *Gracilaria* spp. without specifying the individual species (Table 1.9) and also the light units and methods used to measure the intensity of light differ (Lapointe and Duke, 1984, Gonen *et al.,* 1994b, Beer and Levy, 1983). Light can be expressed with different variables that are related somehow to the amount and quality of light that the thallus is receiving (i.e. irradiance, intensity, day length, photon fluence rate, light spectrum)(Lüning, 1990). Rarely the amount of light absorbed by the thalli has been measured (Lüning, 1990).

1.9.5. Salinity

Gracilaria spp. show a broad tolerance for salinity, growing within ranges of 4-47 ppt (Haglund and Pedersen, 1992, Bird and McLachlan, 1986). The effect of salinity on growth, photosynthesis and agar yield has been previously reported for *Gracilaria* spp. (Israel *et al.,* 1999, Choi *et al.,* 2006, Daugherty and Bird, 1988)

(Table 1.9). The effect of salinity on *Gracilaria* parameters is not fully understood and evidence suggests that it is species specific (Israel *et al.,* 1999, Daugherty and Bird, 1988). However, contradictory effects can be observed for the same species too (Bird, 1988). For *G. verrucosa*, productivity and agar yield decreased with increase of salinity and temperature (Daugherty and Bird, 1988), while, for same species higher agar yields were observed with decreasing salinities (Bird, 1988).

In some cases, under laboratory conditions, a strong correlation can generally be found between *Gracilaria* growth with varying salinity ranges similar to their natural habitats (Friedlander *et al.,* 1993, Choi *et al.,* 2006). However, other *Gracilaria* spp. growth and biomass production do not vary with salinity changes supporting that *Gracilaria* spp. is a euryhaline genus (Pondevida and Hurtado-Ponce, 1996a, Chirapart and Ohno, 1993, Orduña-Rojas and Robledo, 2002).

Under laboratory conditions, Kumar (2008) determined the effect of two salinities (25 and 33 ppt) on *G. cliftonii* growth and agar yield cultivated in ocean and inland saline water. Growth and agar from *G. cliftonii* did not vary with salinity (Kumar, 2008). However, no information is available on the effect of salinity on *G. cliftonii* life stages from natural populations.

As temperature, light and salinity are interdependent variables and are some of the most important productivity factors for *Gracilaria* spp. the effect of these parameters is often reported as a multivariate approach rather than isolated (Table 1.9). In areas where salinity is stable (25 ppt), studies suggest that reduced growth of *Gracilaria* spp. below the infra and supra optimal concentrations is only indirectly affected by salt concentration, with CO_2 and O_2 being limiting (Bird *et al.*, 1979).

Table 1.9 Effect of temperature, light and salinity on different variables from *Gracilaria* spp.

Species	Environmental parameter	Response parameter	Optimum range
Gracilaria sp.[1]	Salinity	Distribution	15-38 ppt
Gracilaria sp.[2]	Photon fluence rate Light spectrum	Growth Photosynthesis Pigment content	100 µmol m^{-2} s^{-1}
G. chilensis[3]	Natural radiation	Photosynthesis	N/A
G. chorda[4]	Temperature Irradiance	Growth	18–24 °C 60–120 µmol m^{-2} s^{-1}
G. chorda[5]	Temperature Salinity	Growth	17–30 °C 15–30 ppt
G. cornea[6]	Temperature Irradiance	Carpospore release Carpospore growth	25- 28 °C 10 -50 µmol m^{-2} s^{-1}
G. cornea[7]	Temperature Salinity Irradiance	Photosynthetic and respiratory responses	N/A
G. gracilis[8]	Temperature Salinity	Growth	18 °C, 30 ppt
G. pacifica[9]	Temperature Irradiance	Spore release Attachment Survival	21 – 24 °C
G. parvispora[10]	Salinity Temperature	Growth	N/A
G. tenuistipitata[11]	Salinity Temperature Light intensity	Growth Agar yield	N/A
G. tenuistipitata[12]	Irradiance	Pigment composition	N/A
G. tikvahiae[13]	Irradiance Temperature	Growth	25-30 °C
G. tikvahiae[14]	Temperature Light Salinity	Growth	10-40 ppt 20-25 °C
G. verrucosa[15]	Temperature Salinity	Growth	17–30 °C 15-30 ppt
G. verrucosa[16]	Temperature Light	Growth Photosynthesis Agar	N/A
G.verrucosa[17]	Temperature Salinity	Productivity Salinity	25-33 ppt
G. cliftonii[18]	Salinity/ionic profiles	Growth Agar	N/A

1 (Bird and McLachlan, 1986); 2 (Beer and Levy, 1983); 3 (Gomez *et al.*, 2005); 4 (Kakita and Kamishima, 2007); 5 (Choi *et al.*, 2006); 6 (Orduña-Rojas and Robledo, 1999); 7 (Dawes, 1998); 8 (Rebello *et al.*, 1996); 9 (Garza-Sánchez *et al.*, 2000); 10 (Glenn *et al.*, 1999), 11 (Israel *et al.*, 1999); 12 (Carnicas *et al.*, 1999); 13 (Lapointe *et al.*, 1984b); 14 (Bird *et al.*, 1979);15 (Choi *et al.*, 2006); 16 (Levy *et al.*, 1990); 17(Daugherty and Bird, 1988); 18 (Kumar, 2008)

1.9.6. Nutrients

The nutrient requirements of macroalgae are similar but the variability within macroalgae species makes it difficult to specify their nutritional requirements (Cole and Sheath, 1990). Previous studies on *G. cliftonii* suggested that different nutrient media and supplementations have a significant effect on the growth, physicochemical and agar properties of *G. cliftonii* culture under laboratory conditions (Kumar, 2008). The effect of nitrogen on *G. cliftonii* growth rate depended on the nitrogen source (NH_4^+ or NO_3^-) in the culture media, while phosphorus supplementation increased *G. cliftonii* growth and net yield (Kumar, 2008). However, there is no information available on the effect of nutrient concentration on *G. cliftonii* growth, biomass and chemical properties on natural populations.

Nutrient supply has been investigated under laboratory and outdoor cultivation conditions for *Gracilaria* spp. (Lignell and Pedersén, 1987, Hanisak and Ryther, 1984, Friedlander, 2001, Ryther *et al.,* 1981). Nutrition of algae usually includes nitrogen and phosphate fertilisers (Lobban and Harrison, 1994) however; different investigations have provided contrasting results for both nutrients (Table 1.10). The influence of nitrogen, mainly as ammonium, into the cultivation media is associated with an increase on growth rates (apical and specific) and high yields (Lapointe, 1985, Smit *et al.,* 1996, Navarro-Angulo and Robledo, 1999, Capo *et al.,* 1999). In addition, it is reported that *Gracilaria* spp. have higher absorption rate for NH_4^+ than for NO_3^- or NO_2^-. Since NH_4^+ without undergoing further reductions is directly incorporated into the amino acid pool, it is expected to show higher growth rates comparing to the other nitrogen reduced forms (Haglund and Pedersen, 1993).

The effect of phosphorus on *Gracilaria* spp. has been less investigated comparing to nitrogen (Lapointe, 1987, Friedlander, 2001, Lewis and Hanisak, 1996) as its status as limiting factor is not clear (Smith, 1984). However, productivity can decrease without phosphorus enrichment in some *Gracilaria* spp. (Lewis and Hanisak, 1996).

Table 1.10 Effect of nitrogen and phosphorus on *Gracilaria* growth and productivity

Species	Nutrient	Effect
Gracilaria spp.[1]	N, P	Productivity reduced without P supply
G. conferta[2]	NH_4^+, PO_4^-, SO_4^-	Starvation affect growth and agar properties
G. conferta[3]	$NH_4^+ + NO_3^-$	Inverse relationship of yield with [N] > 20 μm
G. conferta[4]	NH_4^+, NO_3^-, PO_4^-	High phosphate levels inhibited growth
G. foliifera[5]	$NH_4^+ + NO_3^-$	NH_4^+ supports exponential growth
G. sordida[6]	NH_4^+	Growth affected by [NH_4^+] at 25 °C
G. tenuistipitata[7]	$NH_4^+ + NO_3^-$	Maximum growth at 4 μm
G. tikvahiae[8]	$NH_4^+ + NO_3^-$	NH_4^+ uptake faster than NO_3^-
G. tikvahiae[9]	$NH_4^+ + PO_4^-$	Positive correlation between uptake and concentration
G. tikvahiae[10]	Nitrogen Phosphorus	Maximal yields occurred at low nutrient enrichments (10-100 μm N and 1-10 μm P)
G. tikvahiae[11]	NO_3^-	NO_3^- /light limitation, growth was related to C:N
G. tikvahiae[12]	$NH_4^+ + NO_3^-$	Above minimal N load yield independent to N source
G .verrucosa[13]	NH_4^+	0.5 mg [N] adequate for growth
G. cliftonii[14]	Nitrogen Phosphorus	Its effect on growth depend on the concentration ratio

1 (Lewis and Hanisak, 1996); 2 (Friedlander, 2001); 3 (Friedlander, 1991); 4 (Friedlander and Ben-Amotz, 1991); 5 (Lapointe and Ryther, 1979); 6 (Liang *et al.*, 1989); 7 (Chaoyuan *et al.*, 1984); 8 (Ryther *et al.*, 1981); 9 (Friedlander and Dawes, 1985); 10 (Hanisak and Ryther, 1984); 11 (Lapointe and Duke, 1984); 12 (Lapointe and Ryther, 1978); 13 (Chaoyuan *et al.*, 1984); 14 (Kumar, 2008)

1.9.7. pH

The effect of seawater pH on *Gracilaria* growth is poorly understood (De Busk and Ryther, 1984, Lignell and Pedersen, 1989) (Table 1.11). However, it has been demonstrated that net photosynthesis from *Gracilaria* decreases when the seawater pH is increased above 8.0 (Lignell and Pedersen, 1989). These results have suggested that some species like *G. secundata* are unable to utilize the bicarbonate ion (HCO_3^-) as the carbon source (Lignell and Pedersen, 1989).

Table 1.11 Effect of pH on *Gracilaria* spp.

Species	Conditions	Effect	Reference
Gracilaria corticata	Laboratory	Growth occurs only between 7-8 pH	(Singh *et al.*, 1980)
G. secundata	Laboratory	Maximum growth occurred at pH 8.0	(Lignell and Pedersen, 1989)
G. tenuistipitata var. *liui*	Outdoor Laboratory	pH above 9.0 decrease growth rates by 50% High growth rates at pH above 9.0	(DeBusk and Ryther, 1984, Haglund and Pedersen, 1992)
G. tikvahiae	Outdoor	Growth rates were reduced at pH 8.0 without carbon supplementation	(Israel *et al.*, 1999)
G. verrucosa	Laboratory	Photosynthetic rate declined rapidly at pH above 8.5 and below 6.5	(Menendez *et al.*, 2001)

1.10.   Epiphytism

The occurrence of macroalgal species growing on other macroalgae (epiphytes) has been widely reported (Goff and Hommersand, 1982, Fletcher, 1995, Correa, 1996); however, the nature of the relationship (that is, competition, parasitism, and symbiosis) is not well understood for most of the cases. Epiphytism in *Gracilaria* spp. has been observed from natural populations (Kim, 1970, Santelices and Doty, 1989, Leonardi *et al.,* 2006, Kuschel and Buschmann, 1991) and under cultivation conditions (Shacklock and Doyle, 1983, Friedlander, 1991, Alveal *et al.,* 1997). Under cultivation conditions, epiphytes affect *Gracilaria* productivity by decreasing growth rates, increasing loss of biomass and decreasing the value of *Gracilaria* harvest (Buschmann *et al.,* 1994, Kuschel and Buschmann, 1991, Buschmann and Gómez, 1993).

Studies of epiphytism on *Gracilaria* spp. have reported the seasonality of epiphytic biomass and abundance from natural populations (Buschmann *et al.,* 1997, Kuschel and Buschmann, 1991, González *et al.,* 1993) and under laboratory conditions (Friedlander, 1991). Maximum epiphytic abundance occurs in Summer (Buschmann *et al.,* 1997, Westermeier *et al.,* 1991, Westermeier *et al.,* 1993). Morphological and anatomical studies have been carried out to understand the mechanisms of interaction between epiphyte-*Gracilaria* (Buschmann and Gómez,

1993, Leonardi *et al.,* 2006, Dawes *et al.,* 2000). However, few studies provide physiological evidence for designating such relationship (Evans *et al.*, 1973) and are focused on the interaction with Chlorophyta epiphytes (i.e. *Ulva, Enteromorpha, Cladophora*) (Friedlander, 1992, Svirski *et al.,* 1993, Santelices and Varela, 1993a, Peckol and Rivers, 1995).

The macroalgal epiphytes found on *Gracilaria* include members of the Rhodophyta, Phaeophyceae, and Chlorophyta (Table 1.12). Although, many epiphytes have been described for *Gracilaria* species eight macroalgal genera have been identified as the most common epiphytes (Leonardi *et al.,* 2006, Buschmann *et al.,* 1997, Friedlander, 1991). These genera include *Polysiphonia, Antithamnionella, Chondria, Ceramium* (Rhodophyta); *Ulva, Enteromorpha* (Chlorophyta); *Giffordia and Ectocarpus* (Phaeophyta). These epiphytes attach in different ways to *Gracilaria* host. Whereas, epiphytes such as *Hincksia, Ectocarpus, Acrochaetium, Antithamnionella, Colpomenia* attach superficially to the host; *Polysiphonia* and *Ceramium* species can penetrate into the host via rhizoids (Leonardi *et al.,* 2006, Hurtado *et al.,* 2006). Leonardi *et al.,* (2006) has classified *Gracilaria* epiphytes into five categories depending on the level of penetration by the epiphyte into its host. The only information available on *G. cliftonii* epiphytism (described as *G. furcellata*) reports one parasite: *Holmsella australis* (Wetherbee and Quirk, 1982).

Table 1.12 Macroalgae epiphytes recorded for *Gracilaria* spp. after Fletcher (1995)

Species	Epiphytes	Reference
Gracilaria chilensis	*Giffordia* sp. *Ulva* sp. *Enteromorpha* sp. *Rhizoclonium sp* *Polysiphonia* sp. *Ceramium rubrum* *Callithamnion* sp.	(Buschmann *et al.*, 1997)
G. chilensis	*Sahlingia subintegra* *Acrochaetium* sp. *Antithamnionella* sp. *Chondria californica* *Acrosorium corallinarum* *Ceramium rubrum* *Ceramium secundatum* *Polysiphonia harveyi* *P. flaccidissima* *Fosliella* sp. *Ulva lactuca* *Ulothrix flacca* *Ectocarpus acutus* *Hincksia mitchelliae* *H. granulose* *Colpomenia sinuosa*	(Leonardi *et al.*, 2006)
G. conferta	*U. lactuca*	(Friedlander *et al.*, 1996)
G. cornea	*U. lactuca*	(Dawes *et al.*, 2000) (Friedlander *et al.*, 2001)
G. gracilis	*Ceramium diaphanum* *Ulva* sp. *Polysiphonia* sp.	(Anderson *et al.*, 1998) (Dural *et al.*, 2006)
G. lemaneiformis	*U. lactuca*	(Friedlander *et al.*, 2001)
G. parvispora	*Acanthophora spicifera* *Enteromorpha intestinalis*	(Glenn *et al.*, 1996)
G. tikvahiae	*Ulva lactuca*	(Dawes *et al.*, 2000)
G. verrucosa	*Ulva lactuca*	(Anderson *et al.*, 1998, Anderson *et al.*, 1996)
	Enteromorpha compressa *Polysiphonia sp* *Ceramium* sp.	(Choi *et al.*, 2006)

1.11. Proximate composition

The nutritional properties of all species of seaweeds are not completely known yet and they are usually estimated from their chemical composition (Darcy-Vrillon, 1993, Mabeau and Fleurence, 1993). Compared to land plants, the chemical composition of seaweeds has been poorly investigated and most of the available information only deals with few species of seaweeds (Burtin, 2003). The chemical composition of macroalgae varies with species, habitat, maturity and environmental conditions (Wong and Cheung, 2000). In general, macroalgae are rich in non-starch polysaccharides, minerals and vitamins (Darcy-Vrillon, 1993, Mabeau and Fleurence, 1993). As seaweed polysaccharides cannot be entirely digested by human intestinal enzymes, they are regarded as a new source of dietary fibre and food ingredients (Lahaye, 1991, Mabeau and Fleurence, 1993).

Macroalgae are a valuable food source as they contain protein, fat, vitamins and minerals (Burtin, 2003) (Table 1.13). Seaweeds are not only a useful food source to humans, whole plants and seaweed mixes have been used in animal nutrition (Burtin, 2003) and fish feed (McHugh, 2003). The high vitamin and mineral contents of edible seaweeds make them nutritionally valuable. In addition to vitamins and mineral nutrients, macroalgae are also potentially good sources of proteins, polysaccharides and fibre (Lahaye, 1991, Darcy-Vrillon, 1993). However, few of the world's available macroalgae species are used commercially for specific nutritional purposes. In Asia , people have a long tradition of consuming macroalgae as part of their diet while, in the Western countries, the principal uses of macroalgae are as sources of phycocolloids, thickening and gelling agents for various industrial applications including uses in foods (Abbott, 1996, Darcy-Vrillon, 1993, Mabeau and Fleurence, 1993).

Table 1.13 Proximate composition of *Gracilaria* spp. (% dry basis)

Species	Ash	Protein	Fat	Carbohydrate	References
G. cliftonii	31.2	11.1	<0.05	57.3	(Kumar, 2008)
G. coronipofolia	53.4	10.5	2.1	15.2	(McDermid and Stuercke, 2003)
G. parvispora	48.1	7.6	2.8	22.9	
G. salicornia	52.9	5.6	2.4	20	
G. cervicorni[2]	10.5	19.7	0.4	631 (g kg^{-1})	(Marinho-Soriano and Bourret, 2005)
G. changii	22.7	6.9	3.3	N/A	(Norziah and Ching, 2000)
G. lemaneiformis	16.66	20.87	0.87	61.6 (µg 100gdb)	(Wen *et al.*, 2006)
G. tikvahiae	50.3	8.3	1.6	17.1	(McDermid and Stuercke, 2004)

Proximate composition of *G. cliftonii* has been reported for the tetrasporophyte, carposporophyte and vegetative stages from natural populations. However, proximate composition of the male and female gametophyte from *G. cliftonii* and its seasonality was not determined (Kumar, 2008). Table 1.14 summarizes the proximate composition for *G. cliftonii* life stages.

Table 1.14 Mean proximate composition (%db) from life stages of *G. cliftonii* from natural populations. Source: (Kumar, 2008)

Life stage	Ash	Protein	DF	SDF	TDF
Tetrasporophyte	36.2	13.1	5.1	44.3	49.5
Carposporophyte	33.3	14.5	5.2	46.8	52.0
Vegetative	31.2	11.1	4.9	51.5	56.4

1.12. Physicochemical properties

The physiological effects of dietary fibre and its applications are related to their physicochemical properties which depend on the chemical structure of the constituent polysaccharides. Fleury and Lahaye (1991) reported that the physicochemical properties of macroalgae powder reflect those of the fibre present. In addition, since macroalgae proteins are closely related to the cell wall polysaccharides (Fleurence, 1999, Jordan and Vilter, 1991), proteins may also play a role in the physicochemical properties such as water-holding (Schneeman, 1998).

The physicochemical properties of *G. cliftonii* were determined by Kumar (2008) for the tetrasporophyte, carposporophyte and vegetative stages. The water retention capacity (WRC) from vegetative stages (10 g/g) was observed to be higher than tetrasporophyte and carposporophyte stages (4-5 g/g). For oil retention capacity (ORC), vegetative stage was higher (4 g/g) than carposporophyte stage (2.5 g/g). However, physicochemical properties from gametophyte stage were not determined and seasonality was not considered.

In addition, Kumar (2008) determined that WRC of *G. cliftonii* was higher when cultured with nutrient supplemented ocean water and that *G. cliftonii* physicochemical properties vary as a function of nutrient source (i.e. f2, PES). However, *G. cliftonii* physicochemical properties were determined without considering life stage.

1.13. Agar

Different agar extraction techniques from *Gracilaria* species have been described in literature. The general extraction process is shown in Plate 1.4. In general, agar extraction is done in a water bath or autoclave and there is no account mentioned of any preference of one over another except Buriyo and Kivaisi (2003) who reported that agar yield is higher using an autoclave than water bath. Extraction parameters like soaking time, seaweed to water ratio, extraction temperature and time have been considered to affect agar extraction process, but no information is available on the effect of these variables on the extraction of agar from *Gracilaria* spp. There are lot of discrepancies in the agar extraction process even for the same species. Extraction variables in the process for different *Gracilaria* species are summarized in Table 1.15.

```
┌─────────────────────────────────────────┐
│         Gracilaria cliftonii sample      │
└─────────────────────────────────────────┘
                     │
                     ▼
┌─────────────────────────────────────────┐
│          Soaking time = 1 hour           │
│       Soaking temperature = 30 °C        │
└─────────────────────────────────────────┘
                     │
                     ▼
┌─────────────────────────────────────────┐
│       Seaweed to water ratio = 1:150     │
└─────────────────────────────────────────┘
                     │
                     ▼
┌─────────────────────────────────────────┐
│      Extraction temperature = 100 °C     │
│         Extraction time = 3 hours        │
└─────────────────────────────────────────┘
                     │
                     ▼
┌─────────────────────────────────────────┐
│   Freezing overnight, thawing, oven drying │
└─────────────────────────────────────────┘
                     │
                     ▼
┌─────────────────────────────────────────┐
│    Reconstitution by boiling in excess water │
└─────────────────────────────────────────┘
```

Agar yield   Gel strength   Gelling temperature   Melting point

Sulphate
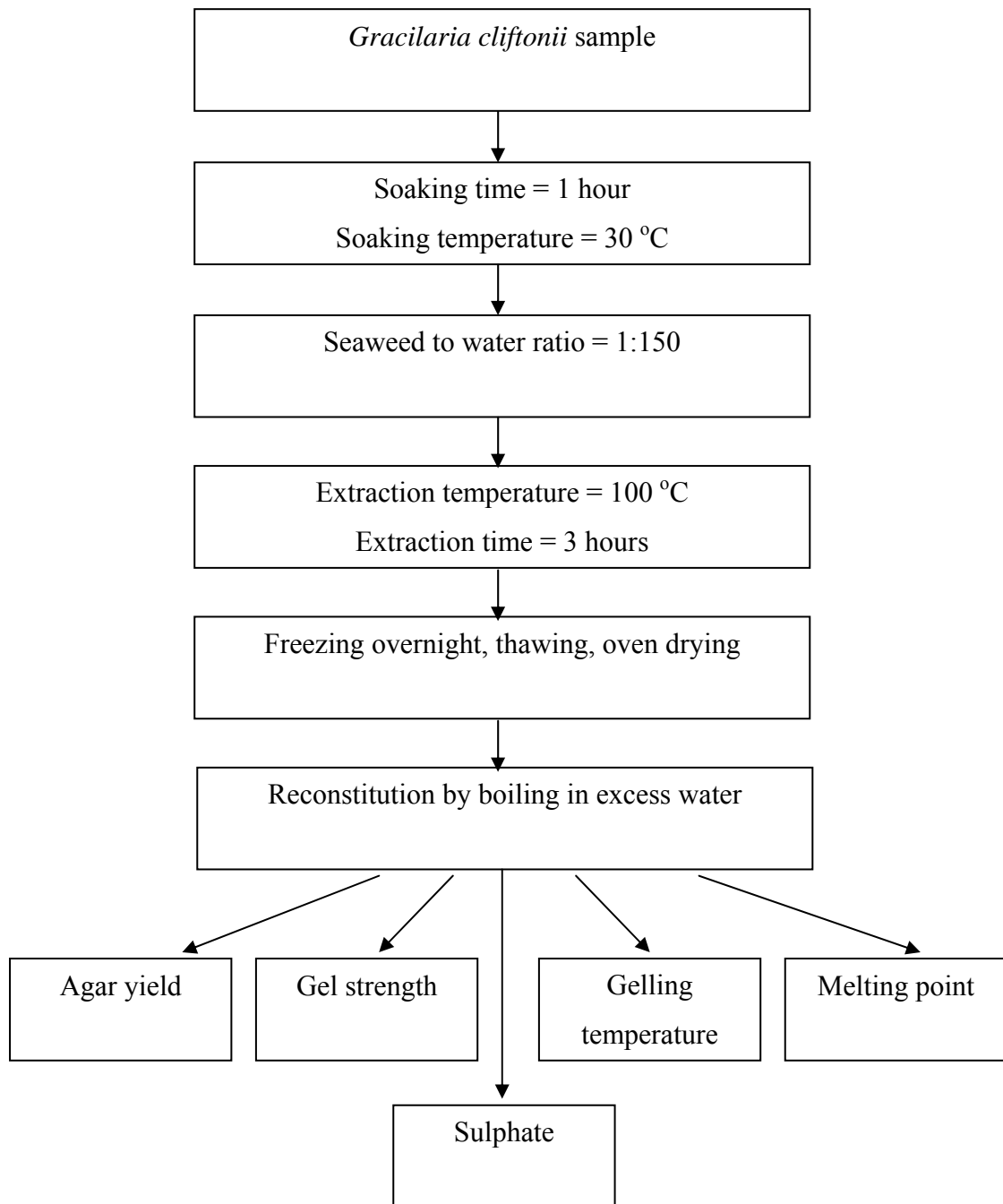
Plate 1.4 Agar extraction process for agarophytes
Source: (Kumar, 2008)

Table 1.15 Extraction variables for extraction of native agar from *Gracilaria* spp.

Species	Equipment	Extraction		Reference
		Temp. (°C)	Time (h)	
G. arcuata	Autoclave	120 /100	1 / 3	(Tako *et al.*, 1999)
G. bursapastoris	Autoclave	110	1	(Marinho-Soriano *et al.*, 1999)
G. eucheumoides	Autoclave	100	1	(Takano *et al.*, 1995)
		121	1	
G. vermiculophylla *G. gracilis*	Autoclave	121	1	(Mollet *et al.*, 1998)
G. gracilis *G. edulis* *G. chilensis* *G. tenuistipitata*	Waterbath	Boiling	2	(Rebello *et al.*, 1997)
G. gracilis *G. dura* *G. bursapastoris*	Autoclave	110	1	(Marinho-Soriano, 2001)
G. maramae *G. edulis* *G. arcuata*	Pressure cooker	120	0.5	(Falshaw *et al.*, 1999)
G. cervicornis *G. blodgettii* *G. crassissima*	Waterbath	Boiling	1.5	(Freile-Pelegrin and Murano, 2005)
G. tenuistipitata *G. arcuata*	N/A	Boiling	1	(Montaño *et al.*, 1999)
G. lemaneiformis	Autoclave	115	2	(Li *et al.*, 2009)
G. dura	Autoclave	110	1	(Marinho-Soriano and Bourret, 2005)
G. damaecornis *G. ferox* *G. domingensis* *G. compressa* *G. debilis* *G. foliifera*	Waterbath	100	3	(Duckworth *et al.*, 1971)
G. corticata *G. crassa* *G. millardetii* *G. salicornia* *G. verrucosa* *Gracilaria* sp.	Waterbath	95	3	(Oyieke, 1993)
G. cliftonii	Waterbath	95-100	1	(Byrne *et al.*, 2002)
G. cliftonii	Waterbath	60, 70, 80	1-3	(Kumar and Fotedar, 2009)

Most of the literature available for *Gracilaria* is related to agar yield and its properties (Table 1.16). Agar yields are highly variable between species and in many cases the physicochemical properties have not been determined. It is difficult to compare previous results due to differences on the parameters of agar extraction (i.e. soaking time, temperature) and places of collection.

Table 1.16 Agar yield and its physicochemical properties for different *Gracilaria* spp.

Species	Agar yield	Gel strength	Melting point	Gelling temp.	Sulphate content
	% db	g/cm^2	^{o}C	^{o}C	% db
G. arcuata[1]	33.2	N/A	N/A	Room temp.	N/A
G. bursapastoris[2]	36-39	22-42 g	N/A	30-36	N/A
G. eucheumoides[3]	N/A	N/A	121	N/A	N/A
G. gracilis[4]	11-19	120-240	N/A	N/A	4.4-6.6
G. gracilis[5]	N/A	N/A	N/A	N/A	2.9-3.4
G. edulis[5]	N/A	N/A	N/A	N/A	2.3
G. chilensis[5]	N/A	N/A	N/A	N/A	2.2
G. tenuistipitata[5]	N/A	N/A	N/A	N/A	2.3
G. gracilis[6]	30.0	630	N/A	N/A	1.13
G. dura[6]	33.5	318	N/A	N/A	1.05
G. bursapastoris[6]	34.8	22.2	N/A	N/A	1.7
G. maramae[7]	23	N/A	N/A	N/A	N/A
G. edulis[7]	37	N/A	N/A	N/A	N/A
G. arcuata[7]	21	N/A	N/A	N/A	N/A
G. cervicornis[8]	39.3	<50	54-67	36-37	5.3
G. blodgettii[8]	~35	<800	86-88	42-45	~3
G. crassissima[8]	~30	180	~82.5	~40	~4
G. tenuistipitata[9]	32.9	304	86.5	42.3	1.18-3.9
G. arcuata[9]	17.2	161	96.2	63.5	4.17 1.17
G. dura[11]	32-35	263-600	N/A	38-43.25	0.97-1.1

G. damaecornis[12]	N/A	10 g	N/A	N/A	6.0
G. ferox[12]	N/A	30 g	N/A	N/A	7.0
G. domingensis[12]	N/A	14 g	N/A	N/A	6.0
G. compressa[12]	N/A	41 g	N/A	N/A	4.1
G. debilis[12]	N/A	140 g	N/A	N/A	3.4
G. folifera[12]	N/A	20 g	N/A	N/A	2.5
G. corticata[13]	8.8-20.5	<60	82-87.3	29.3-34.9	N/A
G. crassa[13]	12.7-25.3	125-205	91-94.8	36.2-37.2	N/A
G. millardetii[13]	8.7-17.2	60-99	86.3-92.5	30.7-34.5	N/A
G. salicornia[13]	9.2-15.1	60-198.3	85.3-94.5	33.6-38.8	N/A
G. verrucosa[13]	29.1-30.3	205-220	90-91.3	28.9-29.5	N/A
Gracilaria sp.[13]	14.2-16.8	199.4-203.3	90.3-91.5	39.9-40.4	N/A
G. cliftonii[14]	52	N/A	N/A	N/A	N/A
G. cliftonii[15]	62	180	87.9	33.3	5.5

1 (Tako *et al.*, 1999), 2. (Marinho-Soriano *et al.*, 1999), 3. (Takano *et al.*, 1995), 4. (Mollet *et al.*, 1998), 5. (Rebello *et al.*, 1997), 6. (Marinho-Soriano, 2001), 7. (Falshaw *et al.*, 1999), 8. (Freile-Pelegrin and Murano, 2005), 9. (Montaño *et al.*, 1999), 10. (Li *et al.*, 2009), 11. (Marinho-Soriano and Bourret, 2005), 12. (Duckworth and Yaphe, 1971b, Duckworth *et al.,* 1971), 13. (Oyieke and Kokwaro, 1993), 14. (Byrne *et al.*, 2002); 15 (Kumar, 2008)

Previous studies have reported high agar yields from *G. cliftonii* collected from natural populations (Byrne *et al.,* 2002, Kumar and Fotedar, 2009) (Table 1.17). However, both studies did not consider agar yield fluctuations due to seasonality and site of collection. Although, Kumar and Fotedar (2009) determined agar yield for different life stages, agar yield from gametophyte stage are yet to be determined.

Table 1.17 Agar yield and properties from different life stages from *G. cliftonii.*
Source: (Kumar, 2008)

Life Stage	Agar yield (%db)	Gel strength (g/cm^2)	Gelling temperature (^{o}C)	Melting point (^{o}C)	Sulphate content (%db)
Carposporophyte	56.7	164	37	89	6.7
Tetrasporophyte	59.9	159	35	87	5.7
Vegetative	49.5	181	38	86	7.9

1.14. Seasonality

The biochemistry of agar is known to change in response to several factors (Marinho-Soriano, 2002). The agar yield and quality not only depend on its specific characteristics, but are closely related to season (Chirapart and Lewmanomont, 2004, Price and Bielig, 1992), environmental parameters (Bird, 1988, Daugherty and Bird, 1988) and growth (Christiaen *et al.*, 1987) and reproductive cycle of *Gracilaria* (Whyte *et al.,* 1981, Marinho-Soriano *et al.,* 1998).

Besides the seasonality observed in growth and biomass of *Gracilaria* spp., seasonal changes on agar yield and its properties have been reported (Table 1.18). For *Gracilaria* spp., the seasonality in the chemical and physical properties of agar are a combination of species specific characteristics (Patwary and Van Der Meer, 1983) and environmental factors (Orduña-Rojas and Robledo, 2002, Marinho-Soriano and Bourret, 2005).

Table 1.18 Seasonality of agar from *Gracilaria* spp.

Species	Agar yield (% db)	Peak	Country	Reference
Gracilaria sp.	26	June	Israel	(Friedlander *et al.*, 1987)
Gracilaria sp. (*chorda* type)	33	August	Japan	(Chirapart and Ohno, 1993)
Gracilaria sp. (*verrucosa* type)	30	August (tetrasporic/cystocarpic)	Canada	(Whyte *et al.*, 1981)
	25	May-September (vegetative)		
	27	July (male)		
G. arcuata	20	September December	Philippines	(Calumpong *et al.*, 1999)
G. bursapastoris	36	Summer	France	(Marinho-Soriano and Bourret, 2003)
	19.6	Minimum in Winter	Hawaii	(Hoyle, 1978a)
G. blodgetti	20	October	Philippines	(Calumpong *et al.*, 1999)
	25.7	November	USA	(Bird and Hinson, 1992)
G. cervicornis	20	February	Brazil	(Marinho-Soriano *et al.*, 2001)
G. conferta	2 g/m^2/week	May	Israel	(Friedlander, 1991)
G. cornea	31.6	July-October	Mexico	(Freile-Pelegrin and Robledo, 1997)
			Mexico	(Orduña-Rojas and Robledo, 2002)
G. coronipofolia	26.2	March	Hawaii	(Hoyle, 1978a)
G. corticata	25	November	India	(Oza, 1978)
G. domingensis	36	February, April, September	Brazil	(Durairatnam *et al.*, 1990)
G. edulis	26	Summer	Australia	(Price and Bielig, 1992)

G. eucheumoides	29	May	Philippines	(Villanueva *et al.*, 1999)
G. gracilis	45	Winter	South Africa	(Wakibia *et al.*, 2001)
	30	Spring	France	(Marinho-Soriano and Bourret, 2003)
G. multipartita	30	August	Morocco	(Givernaud *et al.*, 1999)
G. salicornia	~15	November March	Philippines	(Calumpong *et al.*, 1999)
G. sordida	16-23	N/A	New Zealand	(Pickering *et al.*, 1990)
G. verrucosa	24-43	Summer	Turkey	(Yenigül, 1993)
G. tikvahiae	23	October	USA	(Penniman and Mathieson, 1987)

2. INTRODUCTION

Macroalgae are among the marine resources that are commercially exploited with 221 species utilised for different purposes, including human consumption (145 spp.) and as raw material for the extraction of colloids (101 spp.). In addition, algae provide ecological services as primary producers (Chand *et al.*, 2000) and some species can reach biomass of 1-7 kg/m^2 (Simonetti *et al.*, 1970) making them important marine resources in many locations.

Traditionally, the exploitation from marine resources has been without proper management strategies based on the scarce knowledge on the availability and abundance of the resource. In many instances management practices did not take account of the temporal variability of the resource thus resulting in the overexploitation of the resource (Orduña-Rojas, 2000).

Macroalgae like many other aquatic organisms respond to changes in environmental parameters. This response can be at metabolic levels influencing growth, photosynthesis, proximate composition and properties of extracted products (agar), and in turn can have consequences at population levels in terms of biomass and reproductive characteristics (Lobban and Harrison, 1994, Cole and Sheath, 1990).

Gracilaria spp. are of commercial importance for the phycocolloid industry and a major source of agar (Santelices and Doty, 1989). *Gracilaria* is one of the most studied genera for aquaculture and agar production (Friedlander and Levy, 1995). *Gracilaria* spp. show many positive attributes which make them important for commercial cultivation. Some of these attributes are rapid growth rates, broad tolerances to environmental parameters and species diversity present in tropical and temperate waters of the world (Abbott, 1995, Bird, 1995).

In Western Australia, ten *Gracilaria* spp. are found. These are including *Gracilaria cliftonii, G. blodgettii, G. verrucosa, G. perissana, G. urvillei, G. comosa, G. salicornia, G. eucheumatoides, G. canaliculata and G. flagelliformis* (FloraBase, 2009). However, most of the existing literature deals with anatomical and taxonomic aspects of *Gracilaria* spp. (Byrne *et al.*, 2002, May, 1948, Withell *et al.*, 1994, Womersley, 1996, Huisman, 2000, FloraBase, 2009) and only two reports by Byrne *et al.* (2002) and Price and Bielig (1992) determined the agar content in *Gracilaria*. *G. cliftonii* was reported to contain 52 % agar, (Byrne *et al.*, 2002) however, recent

studies reported its agar content to be as 62 % (Kumar and Fotedar, 2009).

The growth of *G. cliftonii* has been determined under indoor laboratory conditions. The effect of different ionic profiles of inland saline and ocean water has been investigated by Kumar (2008). Although, he reported the agar content, proximate composition and physicochemical properties of *G. cliftonii*, no information is available on the growth and chemical properties of male and female gametophyte of natural populations of *G. cliftonii* from Western Australia. Furthermore, the effects of seasonality on the biomass, abundance, reproduction and competition (epiphytism) of *G. cliftonii* remain unknown. Except for morphological and taxonomical descriptions provided by Withell *et al.,* (1994) and Byrne *et al.* (2002), the basic biology of *G. cliftonii* which is distributed in Australia from Geraldton to Esperance (FloraBase, 2009, Huisman, 2000) has not been investigated yet.

Research on the biomass and growth rates of other *Gracilaria* spp. has produced varying results (Luhan, 1996, Nelson, 1989, Orduña-Rojas and Robledo, 2002, Penniman *et al.,* 1986, Pickering *et al.,* 1990, Pondevida and Hurtado-Ponce, 1996a, Whyte *et al.,* 1981, McLachlan and Bird, 1986). *Gracilaria* spp. productivity is dependent on water temperature (Boraso de Zaixso, 1987, Luhan, 1996), irradiance (Orduña-Rojas and Robledo, 2002), day length (Pickering *et al.*, 1990), water movement (Ryder *et al.*, 2004), phosphate concentration in seawater (Pondevida and Hurtado-Ponce, 1996a), salinity and rainfall (De Castro *et al.*, 1991). Similarly, the seasonality in abundance and thallus length of *Gracilaria* spp. from natural populations is reported to be highly variable. The abundance can range from widely scattered thalli to a few thalli per square meter (Santelices and Fonck, 1979) while thallus length of reproductive stages can be maximum in Spring and/or Summer (Pickering *et al.,* 1990, Orduña-Rojas and Robledo, 2002).

In natural populations of *Gracilaria* spp., tetrasporophyte thallus has been reported as the major reproductive fraction while male gametophytes are rarely observed (Hoyle, 1978c, Jones, 1959, Rao, 1973). Although, a peak for reproductive thallus has been observed in Summer (Penniman *et al.*, 1986, Boraso de Zaixso, 1987), it can occur all year round. Hoyle (1978c) suggested that the seasonality and development of reproductive stages is species dependant rather than environment dependant.

In macroalgae community analysis, little attention has been paid to population dynamics as distinct from biomass or productivity studies (Lobban and Harrison, 1994). Descriptions of demographic analysis are based on the measurement of parameters such as germling and adult mortality, age or time of beginning of reproduction, reproductive life span, proportion between reproductive and vegetative stages and fecundity of the species (Engel *et al.*, 2001).

Previous studies have determined that *Gracilaria* spp. exhibit a *"Polysiphonia"* type of life history with an alternation of isomorphic generations (Ogata *et al.*, 1972, Bird *et al.*, 1977a, Oliveira, 1984, Kain and Destombe, 1995, Engel *et al.*, 2001). Populations of *Gracilaria* consist of four stages: tetrasporophytes, female gametophytes and male gametophytes and carposporophyte (Engel *et al.*, 2001). Under culture environments the life history has been demonstrated in several species of *Gracilaria* (Bird *et al.*, 1977a, Oliveira, 1984, Kain and Destombe, 1995, Guimarães *et al.*, 1999). However, under outdoor field conditions the populations of *Gracilaria* have shown different patterns of life history. In natural populations, there are fewer males than female gametophytes or different proportions of gametophytes and tetrasporophytes (Kain and Destombe, 1995, Engel *et al.*, 2001). In other cases, the ratio between tetrasporophyte and gametophyte stages can be equal (Destombe *et al.*, 1989) or tetrasporophyte stage can dominated over the other stages (Engel *et al.*, 2001).

Moreover, some *Gracilaria* spp. show small gametophytic thalli growing as epiphytes on parental tetrasporophytes thalli and male and/or female structures occur on the same thallus (Svirski *et al.*, 1993, Buschmann and Gómez, 1993, Kuschel and Buschmann, 1991). In some cases spermatangial conceptacles are found close to carpogonia and cystocarps on the same thallus (van der Meer and Todd, 1977, Guimarães *et al.*, 1999). Another important aspect of the biology of *Gracilaria* is the fecundity of the species (Santelices, 1990, Diaz-Pulido and McCook, 2005, Santos and Duarte, 1996). The quantity of spores released represents a measure of the reproductive potential of *Gracilaria* and indicates the environmental conditions which favour reproduction activity (Pacheco-Ruíz *et al.*, 1989).

The population structure of macroalgae is not only affected by intraspecific parameters like reproduction and fecundity but also depends on biotic interactions like competition, epiphytism, parasitism and grazing (Lobban and Harrison, 1994,

Lüning, 1990).

Epiphytism is as common phenomena observed for *Gracilaria* spp. under natural and cultivation conditions (Fletcher, 1995) which adversely affects the productivity and quality of *Gracilaria*. Epiphytes decrease the host growth rates and results in loss of biomass through direct competition with the host algae for space, nutrients and inorganic carbon from the water column (Kuschel and Buschmann, 1991, Buschmann and Gómez, 1993, Svirski *et al.*, 1993).

Identification and temporal abundance of epiphytes from *Gracilaria* spp. have been poorly reported and majority of the research is focused on *G. chilensis* (Kuschel and Buschmann, 1991, Fletcher, 1995, González *et al.*, 1993, Buschmann *et al.*, 1997). Several epiphytes have been described for *Gracilaria* spp. but eight macroalgae genera such as *Polysiphonia, Antithamnionella, Chondria, Ceramium, Ulva, Enteromorpha, Giffordia* and *Ectocarpus* have been identified as the most common epiphytes. In addition, maximum peaks of epiphytic abundance are reported in Summer (Fletcher, 1995).

Previous studies have characterised different types of interactions between macroalgae and its epiphytes (Goff and Cole, 1973, Linskens, 1976) which can occur at biochemical, cellular and physiological levels. These interactions can be influenced by shape, texture and thickness of the cortex and/or chemical composition of the host cells, and production of metabolites and enzymes (Dawes *et al.*, 2000). Despite the commercial interest of *Gracilaria* spp.*,* the interactions with their epiphytes are poorly defined. The most complete characterisation of *Gracilaria*-epiphyte interaction was prepared by Leonardi *et al.* (2006) based on the degree of infection and penetration by epiphytes into *Gracilaria* thallus.

Analysis of macroalgae populations can provide the basis for management and conservation of marine resources (Poblete and Inostroza, 1987). However, studies on macroalgae physiological and biochemical responses under laboratory conditions are required to complement and understand observations from the field. These responses include the analysis of the chemical composition of macroalgae which can determine the potential application of a species in the food industry (Burtin, 2003). Macroalgae are rich in polysaccharides, minerals, and vitamins (Mabeau and Fleurence, 1993) and constitute potential sources of dietary fibre that differ chemically and physicochemically from terrestrial plants (Ruperez and Saura-

Calixto, 2001). It is important to determine the proximate and physicochemical properties of macroalgae as they can influence their nutritional status for human consumption.

The proximate composition of wild populations of *G. cliftonii* is mainly nitrogen free extract (NFE) which is mainly carbohydrates followed by ash and protein (Kumar, 2008). K^+ is the main mineral component (7.8- 9.9 %db) of *G. cliftonii*, while, water retention capacity and oil retention capacity range is 4-10 (g/g) for WRC and 2-4 (g/g) for ORC (Kumar, 2008).

The main application of *Gracilaria* spp. is as a source of agar for the food and pharmaceutical industry (Armisen and Galatas, 1987). Numerous studies have shown that agar production and quality are influenced by extrinsic factors like seasons (Hoyle, 1978a) and intrinsic factors such as life stage (Penniman and Mathieson, 1987, Marinho-Soriano *et al.,* 1999).

The agar properties depend on its structure, particularly the number and location of the sulphate groups in the polysaccharide chain (Duckworth *et al.,* 1971, Lahaye, 1991, Craigie and Jurgens, 1989, Andriamanantoanina *et al.,* 2007). Melting temperature is affected by the pyruvic acid content (Young *et al.,* 1971) while, gelling temperature is related to the methoxyl content and its location in the agar structure (Rebello *et al.,* 1997, Guiseley, 1970). Therefore, agar properties may be characterised by its sulphate content and other constituents (Asare, 1980, Zanlungo, 1980). *G. cliftonii* agar yield and properties have been reported (Kumar and Fotedar, 2009) but its seasonality of agar and physicochemical properties are not known.

The Shoalwater Islands Marine Park (32° 19' 21.08" S 115° 41' 52.22" E) is located 50 kilometres south of Perth, adjacent to the City of Rockingham and covers an area of approximately 6658 hectares (DEC, 2007). The park starts in the south at Point Becher, it extends approximately 3 kilometres offshore and encompasses the chain of limestone rocks and islands (including Penguin Island) that runs parallel with the coastline. It contains the waters of Shoalwater Bay, Warnbro Sound and a small southern section of Cockburn Sound.

The northern boundary of the park is located just north of Point Peron. The marine flora and fauna of the marine park is a mixture of tropical and temperate species, the former carried south by the Leeuwin Current from tropical northern waters and the latter carried north by the Capes Current from the cool temperate waters of the south

(Koslow *et al.*, 2008, Waite *et al.*, 2007). There is a diverse range of habitats within the marine park, including seagrass meadows, subtidal and intertidal macroalgal limestone reefs and the silty basin of Warnbro Sound (Wilson *et al.*, 1978). In the north of the marine park, a broken chain of islands and reefs protects the coast from south-westerly swell and waves, making this area accessible most of the year. In addition, its wide diversity of habitats and close proximity to Perth makes the area a valuable and important ecological and social resource (DEC, 2007).

The outcomes of this research will provide baseline information on the biology and ecology of *G. cliftonii*. It will provide a better understanding of the effect of environmental parameters on the life cycle of locally available *Gracilaria* spp. It will also provide information on the population structure and its interactions of *Gracilaria* spp. from another region of the world. Finally, this research will assist in the management and conservation of *G. cliftonii* as a marine resource.

2.1. Aim

The aim of this research project was to investigate the seasonal variability of the abundance, epiphytism and chemical properties of *G. cliftonii* at Point Peron, Shoalwater Marine Park, Western Australia.

2.2. Objectives

➤ Identify the sampling site for *Gracilaria cliftonii* around Perth based on the pre determine selection criteria.

➤ Determine biomass and abundance of various life stages of *G. cliftonii* from the selected site.

➤ To investigate the fecundity and spore development of *G. cliftonii* under laboratory conditions.

➤ To identify and characterise the mechanisms of attachment of the epiphytes from *G. cliftonii*.

➤ To determined the seasonality of epiphytic load and biomass of *G. cliftonii*.

➤ To determine the seasonal variations in the physicochemical properties and proximate composition of various life stages of *G. cliftonii*.

➤ To determine the seasonal variations in the agar yield and its physiochemical properties of various life stages of *G. cliftonii*.

➤ To investigate the effect of environmental variables on the abundance, epiphytism and chemical composition of *G. cliftonii*.

3. METHODOLOGY

3.1. Sampling site

3.1.1. Site Selection

To determine the natural distribution of *Gracilaria cliftonii* populations along Perth coast, preliminary surveys were undertaken at 9 locations from November 2008 to January 2008. The locations surveyed were Point Peron (32°16'S, 115°41'E), Cottesloe (31°59'S, 115°45'E); Triggs (31°52′S, 115°45′E), North-beach (31°51′40″S 115°45′29″E), Watermans (31°51′S 115°45′E), Marmion (31°50′S 115°45′E), Hillary's (31°48′S 115°44′E), Mullaloo (31°46′S 115°44′E) and Burns–beach (31°43′S 115°43′E). The surveys were performed by swimming along a 50 m perpendicular transect to the shore. The substrate characteristics in the vicinity of *G. cliftonii* populations were recorded and triplicate seawater samples were randomly taken for further nutrient analysis.

G. cliftonii biomass within 1 m^2 quadrat in triplicate was harvested randomly and weighed (fresh weight ± 0.01 g) to determine the biomass at each site. Point Peron at Shoalwater Marine Park was selected as the sampling site for this study. The criteria for selection of site were based on the adequate availability of *G. cliftonii* biomass, exposure of site to winds and currents and accessibility to the area. A license for collection of *G. cliftonii* was obtained from the Department of Environment and Conservation, Western Australia (Ref: SW012725) to collect the *G. cliftonii*. A permanent 30 m x 10 m plot was established situated 5 m from the shore over a subtidal population of *G. cliftonii* at Point Peron; Shoalwater Marine Park, Western Australia was earmarked for future regular samplings.

3.2. Environmental parameters

3.2.1. Sediment size composition analysis

Sediment samples (0.5 kg cores) were collected randomly in triplicate within the sampling site (Section 3.1.1). Particle size distribution analysis was performed as an indirect measure of water movement of the study site following the methodology proposed by Keulen and Borowitzka (2003) with minor modifications. The samples were rinsed with tap water and oven dried at 60 ˚C till the sample was completely dried. Samples of 100 g were sieved through seven different mesh sizes: ≥1 mm, 0.7

mm, 0.6 mm, 0.5 mm, 0.4 mm, 0.3 mm and <0.3 mm. The sieves were shaken for 10 minutes and samples trapped in each sieve were collected and weighed (±0.01 g). The weight of sediments from each sieve size was expressed as percentage of total weight of the sediments. Histograms percentage/sieve sizes were created in order to determine the major particle size contributor to the sample. The sediments were classified accordingly to Pettijohn *et al.,* (1987).

3.2.2. Water quality

Each month seawater samples (n=3) were taken randomly at sampling site and transported to the Curtin Aquatic Research Laboratories (CARL). Seawater samples were stored in refrigerator at 5ºC and then analysed for NO_2^-, NO_3^-, NH_4^+ and PO_4^- contents by colorimetric methods (AOAC, 1995) using low range nutrient kits for seawater analysis (HACH®, Perth Scientific). Temperature, osmolality, pH and rainfall were recorded monthly from March 2008 to August 2009.

Seawater temperature was recorded by placing temperature data loggers (HOBO® Onset, USA) *in situ* and replaced every month. Salinity expressed as osmolality (osmol/kg) was measured with an osmometer (Osmomat 030D®, Perth Scientific). pH was determined with a digital pH meter (YSI®, Perth Scientific). Monthly rainfall and day length data were obtained from Department of Environment and Conservation, Western Australia (Address: 17 Dick Perry Avenue, Technology Park, Kensington, Western Australia. Postal address: Locked Bag 104 Bentley Delivery Centre 6983).

3.3. Sampling

G. cliftonii was collected over a period of six seasons from Autumn 2008 to Winter 2009, each season consisting of three samplings (one each month). Monthly samplings per performed at intervals of 25 days ± 2 days at the beginning of the month to avoid sampling in the same month. The samplings were undertaken as following seasons:

Autumn 2008 – March 2008 to May 2008

Winter 2008 – June 2008 to August 2008

Spring 2008 – September 2008 to November 2008

Summer 2009 – December 2008 to February 2009

Autumn 2009 – March 2009 to May 2009

Winter 2009 – June 2009 to August 2009

Collections were made at 1–2 m depth by free diving from within the preselected plot (Section 3.1.1). Randomly, thirty quadrats (1 m^2) were placed within the plot ensuring quadrats were not replaced at the same place. All *G. cliftonii* thalli from each quadrat was harvested and placed in a labelled plastic bag and transported to Curtin Aquatic Research Laboratories, Curtin University of Technology, Western Australia for further analysis.

3.3.1. Preparation of samples

In the laboratory, thalli were rinsed with seawater to remove any sand, blotted dry and weighed (fresh weight $\pm$ 0.1 g) to determine the total fresh weight per sample (*Gracilaria* + epiphytes). Visible epiphytes were removed manually and with forceps under the binocular microscope (Olympus SZH[®]). *Gracilaria* samples (n=30) free of epiphytes were then weighed again to determine the fresh weight (fresh weight $\pm$ 0.1 g) of *Gracilaria* and total number of thalli for each sample (N). Each thallus was analysed under a binocular microscope (Olympus SZH[®]) for visible reproductive structures. Hand cross sections from *G. cliftonii* thalli bearing reproductive structures were observed under a light microscope (Olympus BH2[®]) to confirm observations from the binocular microscope.

G. cliftonii thalli were separated into carposporophyte, tetrasporophyte, male gametophyte and vegetative stages. Carposporophyte thalli were identified by the presence of protruding cystocarps. Tetrasporophyte thalli were identified by the presence of tetrasporangia and male gametophyte thallus by spermatangial conceptacles. Thalli without reproductive structures were considered at vegetative stage.

Fresh weight samples of *G. cliftonii* were rinsed with tap water, dried for 8 h at 60 ^{o}C in oven (Contherm[®], Perth Scientific) and then stored in sealed plastic bags (Ziplock [®]) for further chemical analysis.

3.3.2. Total biomass and abundance

Total *G. cliftonii* biomass and abundance were calculated using equation 1 and 2 respectively.

Biomass (g/m^2) = *Gracilaria* fresh weight (g) / total sampled area (1)

Abundance (nh/m^2) = Number of holdfasts (nh) / total sampled area (2)

3.3.3. Photosynthesis and respiration

Photosynthetic and respiration rates of *G. cliftonii* were determined following day of sampling using oxygen evolution method (BOD) described by Dawes (1998) with minor modifications. Clean and free of epiphytes (1 g fresh weight) of *G. cliftonii* was placed in 300 mL transparent glass bottles and dark bottles with filtered seawater (1 μm filter 35 ppt) from the collection site to determine photosynthesis and respiration rates respectively. Bottles containing only seawater were used as control samples. The dissolved oxygen (DO) of the water was measured (YSI®, Perth Scientific) in all the bottles before placing samples inside the bottles. All the bottles were placed in a waterbath at 21 ± 0.5 ^{o}C at 1500 lux. Light was provided using halogen tubes (40 W). After 5 h, the DO in the bottles was measured while stirring the water and samples oven dried at 60 ^{o}C. Experiments were performed with four replicates and readings corrected for possible fluctuations using 3 blank flasks.

The photosynthetic and respiration rates, gross and net photosynthesis were calculated considering initial and final oxygen levels for each bottle as following equations:

$$\text{Photosynthesis (mg O}_2 \text{ g db h}^{-1}) = \frac{(DO_{LF} - DO_{L0})}{\text{Sample dry weight (mg) x Time (h)}} \qquad (3)$$

$$\text{Respiration (mg O}_2 \text{ g db h}^{-1}) = \frac{(DO_{DF} - DO_{D0})}{\text{Sample dry weight (mg) x Time (h)}} \qquad (4)$$

$$\text{Gross photosynthesis (mg O}_2 \text{ g db h}^{-1}) = \text{Photosynthesis} + \text{Respiration} \qquad (5)$$

$$\text{Net photosynthesis (mg O}_2 \text{ g db h}^{-1}) = \text{Photosynthesis} - \text{Respiration} \qquad (6)$$

Where: DO_{LF} is the dissolved oxygen (mg/L) in light bottle at the end of experiment; DO_{L0} is the dissolved oxygen (mg/L) in light bottle at the beginning of experiment.

3.3.4. Biomass, abundance and thallus length per life stage

The fresh weight and number of thalli for each life stage viz. tetrasporophyte, carposporophyte, male gametophyte and vegetative were recorded monthly to determine biomass and abundance for each life stage (equation 1 and 2).

Thallus length was determined every month by measuring the total mean length ($\pm$ 0.1 cm) of 10 thallus in each life stage.

3.3.5. Occurrence

The occurrence defined as the frequency of each life stages was calculated using equation 7.

$$\text{Occurrence (\%)} = [N_{stage} / N_{total}] \times 100 \qquad (7)$$

Where, N_{stage} is total number of individuals per stage, and N_{total} is total number of individuals.

3.4. Spore release

3.4.1. Cystocarp size

Two cystocarp sizes were compared by selecting cystocarps of 1.5 mm and 1 mm Φ from different apical branches from mature *G. cliftonii* carposporophyte thalli under the binocular microscope. Selected cystocarps were rinsed for 10 sec each with 0.5 % sodium hypochlorite solution and 0.5 % iodine solution and finally rinsed with filtered sterile seawater (1 μm filter, 35 ppt) as described by Guzmán-Urióstegui and Robledo (1999).

One cystocarp of each size was placed in one sterile plastic Petri dish containing 20 mL of filtered sterile seawater (1 μm filter, 35 ppt) (n=6). The dishes were left under natural conditions without direct sunlight at room temperature (25 $\pm$ 1 ^{o}C). After 24 h dishes were agitated to break up carpospore clumps and spores were allowed to settle.

After 1 h, the number of carpospores released was quantified following the methodology described by Pacheco-Ruiz *et al.,* (1989) with minor modifications. Spores were counted under a stereomicroscope (Olympus$^{®}$) by placing 0.25 mm^2 lined graph paper under the Petri dish. The area of Petri dish (56 mm^2) was divided into square blocks. Randomly 10 of these blocks were selected and all spores inside these blocks were counted. The total number of spores released by cystocarp was

calculated by multiplying total area by mean number of spores per mm^2. To avoid counting spores from same release, cystocarps were transferred every 24 hr to a new Petri dish and kept under same conditions described above. Carpospore release was quantified until the cystocarps started disintegrating or no more spores were observed.

3.4.2. Nutrient media

Mature branches of *G. cliftonii* bearing cystocarps and tetrasporangia respectively, were selected and cleaned following procedure described by Guzmán-Urióstegui and Robledo (1999). One gram of apical branches (n=5) were placed in 250 mL beakers containing 100 mL of PES and kept under natural laboratory conditions (500 lux, 25 ± 1 Cº). After 24 h, the branches were transferred to new beakers as previously described. The number of spores released was counted as described for carpospore release. The branches were transferred daily to new beakers and tetraspores counted until no spores were released as described earlier.

3.5. Spore survival

Spore survival was calculated according to methodology described by Pacheco *et al.* (1989):

$$\text{Spore survival (\%)} = N_{Tf} * 100 / N_{T0} \tag{8}$$

Where N_{T0} is the number of spores released after 24 h and N_{Tf} is the number of spores which survived after 30 days from the first release (N_{T0}).

3.6. Spore size

To determine the carpospore and tetraspore diameter at the time of release, a drop of seawater containing released carpospores was observed under the microscope. Diameter of ten spores was measured and mean calculated.

3.7. Spore germination

To determine spore development of *G. cliftonii,* the first lot of released spores (Day 1) was utilised. A sample (1 mL) of water containing spores was taken every 24 hr, placed on a slide and observed under the light microscope (Olympus®, USA). Spore diameter, height and number of cell divisions were recorded and photographed with a Leica® digital camera attached to the microscope. The mean spores released

and the size was plotted against time.

3.8.   Reproductive anatomy

Sections from *G. cliftonii* thalli bearing reproductive structures were selected to determine the anatomy of reproductive *G. cliftonii*. Sections were fixed in glutaraldehyde-seawater 2.5 % solution and processed for light microscopy studies according to Fedder and O'Brien (1968). After fixation, the sections were rinsed 3 times for 30 min each with 0.5 M phosphate buffer followed by alcohol dehydration series of methoxypropanol at 100 % for 24 h, ethanol (ETOH) at 100 % for 24 h, n-butanol at 100 % for 24 h and propanol 100 % for 24 h.

After dehydration, the samples were immersed in 50 % glycol methacrylate (GMA) solution for 24 h. Finally, the samples were changed 3 times with 100 % GMA after 24, 48 and 72 h respectively. The samples were then embedded in a solution of 100% GMA-polyethylene glycol-benzoil peroxide and polymerized overnight at room temperature under nitrogen atmosphere and UV light conditions.

Sectioning and preparation of slides was performed at the Centre for Microscopy, Characterisation and Analysis (CMCA), University of Western Australia, Perth, Western Australia. The embedded samples were sectioned with dry glass knife at 2.5 µ using a Sorvall® microtome. The sections were mounted on glass slides and then stained with 0.5% toluidine blue (pH 4). The slides were observed under a light microscope (Olympus BX51) and sections of reproductive structure photographed with a digital camera attached to the ocular of the microscope.

3.9.   Epiphytism

G. cliftonii thalli bearing epiphytes were collected randomly from within the preselected plot as described in section 3.1.1. Epiphytes growing on *G. cliftonii* were removed physically under the binocular microscope and epiphyte biomass and epiphytic loads were calculated as described by Buschmann and Gómez (1993):

Epiphytic biomass (g/m^2) = E / total sampled area   (9)

Epiphytic load (%) = (E / G) x 100   (10)

Where, E represents fresh weight (g) of epiphyte and G represents fresh weight (g) of *G. cliftonii*.

3.9.1. Characterization of attachment *Gracilaria*-epiphyte

Thalli of *G. cliftonii* with attached epiphytes were observed under the binocular microscope. Then sections of 2 mm thickness where the epiphytes were entangled or attached to *G. cliftonii* cortex were selected and processed for histological studies. The epiphytes were removed, fixed in 5% formalin-seawater solution and taxonomically identified. Based on the strength required to remove the epiphyte from host, the strength of attachment was categorised as easy epiphyte and hard to be removed. The distribution of the epiphytes, based on the location of the epiphyte on *G. cliftonii* thalli defined as the different zones where epiphytes were attached to *G. cliftonii* thallus was recorded as basal, intermediate and apical according to Arrontes (1990).

Sections of 0.5 mm length from *G. cliftonii* thallus where epiphytes were attached were selected for histological studies. Thallus sections were fixed in 2.5% glutaraldehyde-phosphate buffer 0.5 M solution under vacuum conditions of 1.7-2 bar and then processed for light microscopy as described in section 3.9. The slides were observed under a light microscope (Olympus SZH [®]) and areas where the epiphyte was attached or penetrated into *G. cliftonii* thallus were photographed with a digital camera (Olympus BX51[®]).

G. cliftonii sections were processed for scanning electron microscopy (SEM) according to methodology described by Dunlap and Adaskaveg (1997) with minor modifications. The fixed material was rinsed 3 times for 5 min each with 0.5 M phosphate buffer, rinsed with distilled water for 5 min and immersed in 2% OsO_4 for 2 h. The samples were rinsed with distilled water and dehydrated using series of ethanol series: 50% ETOH - 50% H20 (5 min); 75% ETOH - 25% H20 (5 min); 95% ETOH - 5% H20 (5 min) and 100% ETOH, 3 times for 5 min respectively.

Finally, the samples were dried using series of hexamethyldisilizane (HMDS) washes: 50% HMDS-50% ETOH for 5 min; 75% HMDS-25% ETOH for 5 min and 2 times 100% HMDS for 5 min each, and dried at room temperature overnight. After drying the sections were mounted on double sided carbon adhesive tapes, fixed to aluminium stubs and then gold coated. Micrographs from the samples were obtained with Scanning Electron Microscope Phillips X30® at 15 kV.

Characterisation of the attachment between *G. cliftonii* and its epiphytes was established following criteria such as type of attachment of epiphyte to host,

thickness of *G. cliftonii* cortical layer, depth of penetration by the epiphyte into *G. cliftonii* tissue and morphological changes at the site of attachment.

3.10. Proximate composition

Proximate composition analysis of *G. cliftonii* was performed in terms of temperature and photoperiod seasons in triplicate for each life stage.

Moisture, ash and protein content were determined according to AOAC Standard Methods (1995).

For protein content analysis a sample of *G. cliftonii* (1 g db) was weighed and placed into a Kjeldahl tube followed by the addition of 10 mL of a digestion acid (orthophosphoric acid/sulphuric acid in ratio 5:95 and the addition of 1 Kjeldahl tablet (BDH®). One tube containing 10 mL of digestion acid solution and 1 Kjeldahl tablet without *G. cliftonii* sample was used as a blank. The tube placed into a heating digester (DK20®, Rowe Scientific) at 420 ^{0}C until a clear colourless solution was obtained. After the digestion of the samples, the tube was removed and cooled for 30 min inside the fume hood. Approximately, 75 mL of distilled water were added to the tube and placed into a Kjeltec 1030® auto analyser for nitrogen content of the sample. Finally, the volume of sample titre was recorded and the protein content determined a correction factor of 6.5.

Carbohydrate content was determined as the weight difference using protein, lipid, and ash content as described by Marinho-Soriano and Bourret (2005).

3.11. Physicochemical properties

Clean and dried *G. cliftonii* samples from different life stages were used to determine their physicochemical properties. Swelling capacity (SC), water retention capacity (WRC) and oil retention capacity (ORC) were determined monthly in triplicate according to procedure described by Rupérez and Saura-Calixto (2001) with minor modifications.

To determine swelling capacity (SC), 500 mg of *G. cliftonii* dried sample was weighed in a 10 mL measuring cylinder (0.1 mL graduations) and 10 mL distilled were added. The mixture was stirred gently to eliminate any trapped air bubbles and left overnight at room temperature to allow the sample to settle. Next day, the volume (mL) occupied by the sample was measured and SC was expressed as mL

per g of dry sample.

To determine water retention capacity (WRC), 10 mL of distilled water were added to 150 mg of dry macroalgae in a 10 mL centrifuge tube. The sample was stirred and left at room temperature (22 ± 1 ^{o}C) for 1 h. After centrifugation at 3000 x g for 20 min, the supernatant was discarded, the residue was weighed and WRC calculated as equation:

$$WRC \ (g/g) = Ww \ (g)/ \ Ws \ (g) \tag{11}$$

Where, Ww is weight of wet sample in g and Ws is weight of dry sample in g.

To determine oil retention capacity (ORC), the protocol described for WRC was followed with minor modifications. To determine ORC commercial olive oil (Coles$^{®}$) was used instead of water, and then *G. cliftonii* sample was processed as described earlier for WRC. ORC was calculated using equation:

$$WRC \ (g/g) = Wo \ (g)/ \ Ws \ (g) \tag{12}$$

Where, Wo is weight of wet sample in g and Ws is weight of dry sample in g.

3.11.1. Agar

Agar extractions in triplicate were performed from tetrasporophyte, carposporophyte, male gametophyte and vegetative life stages of *G. cliftonii* from every sample collected monthly. Agar yield and agar properties *viz.* melting point, gelling point and sulphate content were determined using the methodology described by Kumar (2008) .

3.12.   Statistical analysis

Data were analysed in terms of seasons based on temperature and photoperiod changes. Temperature seasons were defined grouping 18 months (March 2008 to August 2009) into three temperature categories: Group 1 included all the months recorded seawater temperatures below 18 ^{o}C; Group 2 included those months with seawater temperatures between 18 to 21 ^{o}C and Group 3 included those months with seawater temperatures above 21 ^{o}C. Since, months were grouped in same way as Australian seasons, data were analysed in the same way: Autumn (March to May), Winter (June to August), Spring (September to November) and Summer (December to February). To identify differences in temperature between same seasons (i.e. Autumn 2008 and Autumn 2009), independent samples T-test analysis was

performed at 95 and 99 % confidence interval. Data was analysed in terms of temperature seasons over one year period (Autumn 2008 to Summer 2009).

Photoperiod seasons were defined by groupings of 18 months (Autumn 2008 to Summer 2009) in two groups. Group 1 included all months which had photoperiod ≤ 12 hours light and Group 2 included those months with photoperiod above 12 hours light. In this basis, three photoperiod seasons were defined: PS1 (March 2008 to September 2008); PS2 (October 2008 to March 2009) and PS3 (April 2009 to August 2009).

To identify differences among temperature and photoperiod seasons on biomass, epiphytism and chemical composition of *G. cliftonii* life stages; data was tested for homogeneity of variances (Levene's). Data were subjected to one way ANOVA with least significant difference (LSD) post-hoc test at significance level of $p < 0.05$ using SPSS V. 16 for Windows.

Linear relationship between seasonal biomass, epiphytism and chemical composition and with environmental factors was performed calculating correlation coefficients (R^2) using Microsoft® Excel V. 2003.

To determine significant differences on epiphyte occurrence among temperature seasons, non parametric Chi-square test was performed ($p < 0.05$).

4. RESULTS

4.1. Description of the study site

According to Shoalwater Islands Marine Park management plan, the Department of Environment and Conservation (DEC) (2007), the geomorphology of the area is characterised by limestone ridges and reef platforms which protect the coast from south-westerly swell and waves. The study area has a Mediterranean climate with hot, dry Summers and mild, wet Winters. Mean minimum and maximum air temperatures vary between 18 to 30 °C in Summer and 7 to 16 °C in Winter season. Annual rainfall is approximately 750 mm, with most falling from May through to October.

Local oceanographic studies, summarised as part of the Southern Metropolitan Coastal Waters Study, the Department of Environmental Protection (1996) have identified that the parameters such as wind, stratification, sea and swell, tide, coastal evaporation and atmospheric heating and cooling of the water are key environmental factors influencing the hydrodynamics of the area.

The chain of islands and reefs protects the marine park from wave action, which minimizes transport of sediments through to the coast except where breaks occur in this limestone barrier. Regional coastal currents are predominantly northwards in Summer and are more variable but generally southwards in Winter.

The marine flora and fauna in the marine park are generally representative for those described for sandy bays and reefs along the metropolitan coast. The park is dominated by beach and rocky shoreline habitats and includes six major marine benthic habitat types; seagrass, subtidal mobile sand, bare reef macroalgae and silt (Bancroft, 2002). Intertidal reef platforms are characterized by diverse algal communities that support large populations of invertebrates such as *Haliotis roe*, *Thais orbita*, *Acanthopleura hirtosa* and *Turbo torquatus*. Subtidal reefs are dominated by large macrophytes, such as kelp "*Ecklonia radiata"* recognised as one of the most important contributors to primary production of the marine communities in the area (DEC, 2007). Point Peron is located adjacent to the Perth metropolitan area (Figure 4.1).
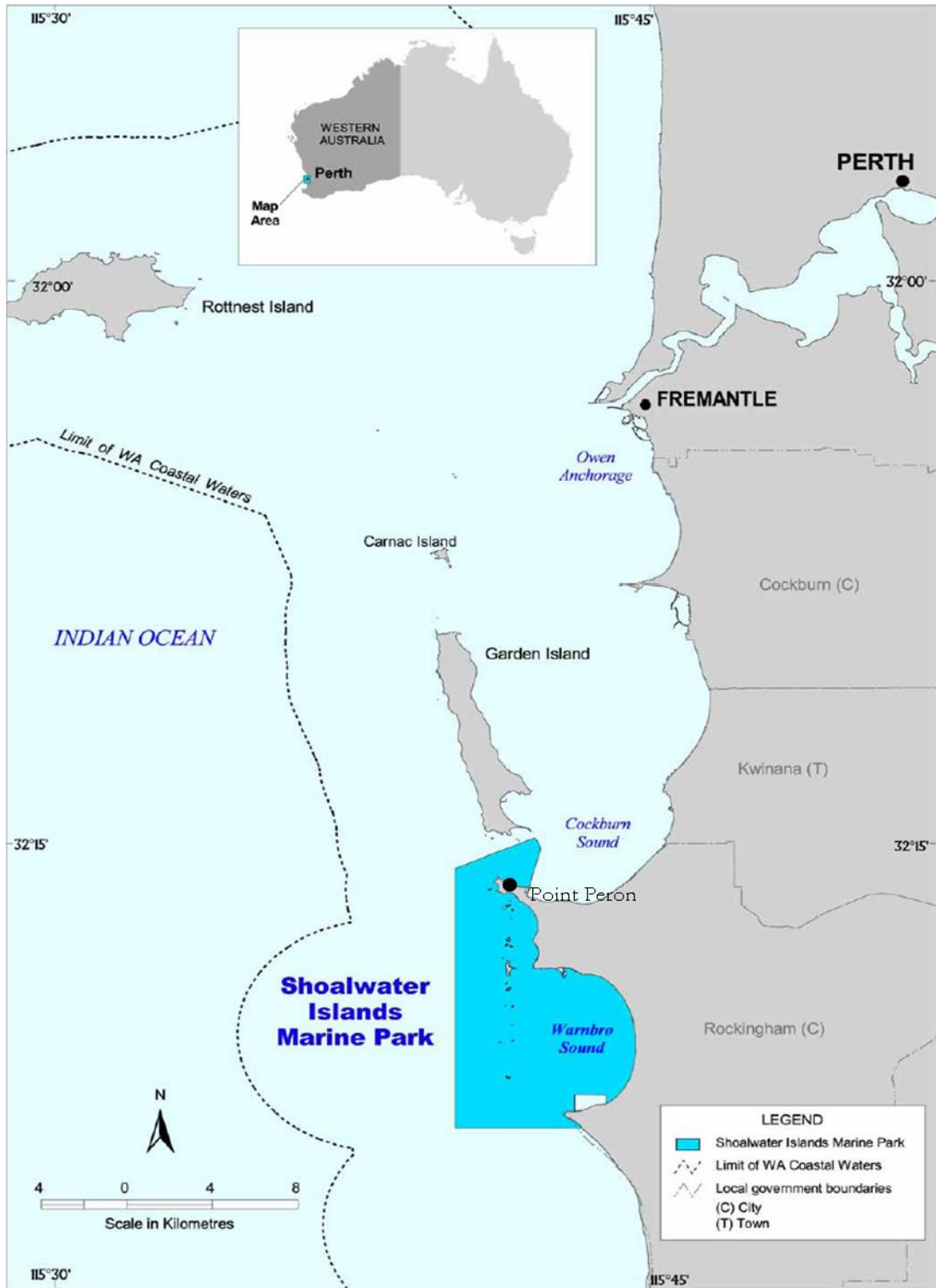
Figure 4.1 Locality of Point Peron, Shoalwater Islands Marine Park, Western Australia.
Source: Department of Environment and Conservation (DEC) (2007)

4.2.   Life cycle

4.2.1. Reproductive anatomy

In the present study, vegetative (infertile) and three reproductive stages *viz.* tetrasporophyte (Plate 4.1A), carposporophyte (Plate 4.2A) and male gametophyte (Plate 4.3A) were observed.

Tetrasporangia from tetrasporophyte thalli consisted of cruciate tetrasporangia which were located in the outer cortical cell layer (Plate 4.1B). Furthermore, tetrasporangia were born on the upper half of the thallus and were 30-50 μm long by 27-30 μm and wide.
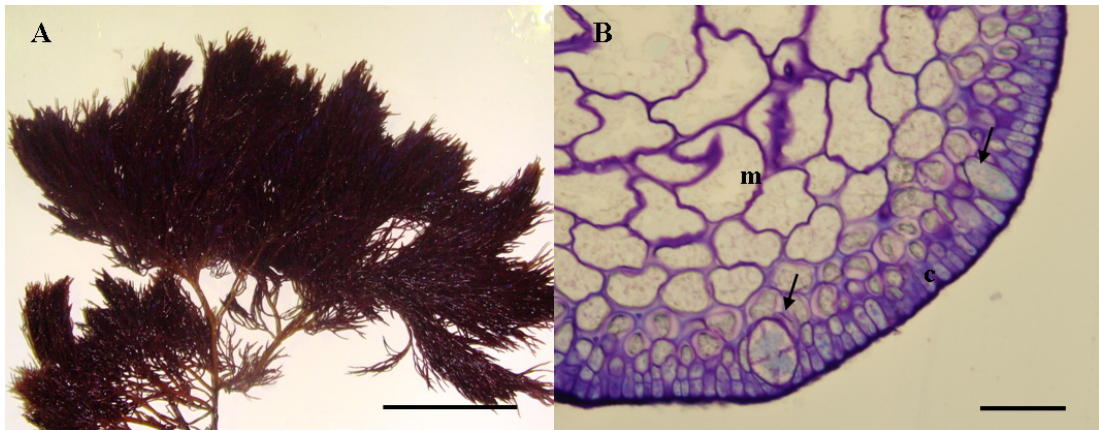
Plate 4.1 Morphology of tetrasporophyte thallus of *G. cliftonii*
A. *G. cliftonii* tetrasporophyte thallus. Bar = 5 cm. B. Cross section of *G. cliftonii* tetrasporophyte thallus indicating the presence of cruciate tetrasporangia (arrows) growing on the outer cortical cell layer. Bar=50 μm. Abbreviations: m=medulla, c = cortex.

The cystocarps of carposporophyte thalli were distributed on upper half of the thallus. In addition, mature cystocarps were 0.5-1.5 mm high (Plate 4.2A) and consisted of a well developed gonimoblast, pericarp and ostiole (Plate 4.2B).
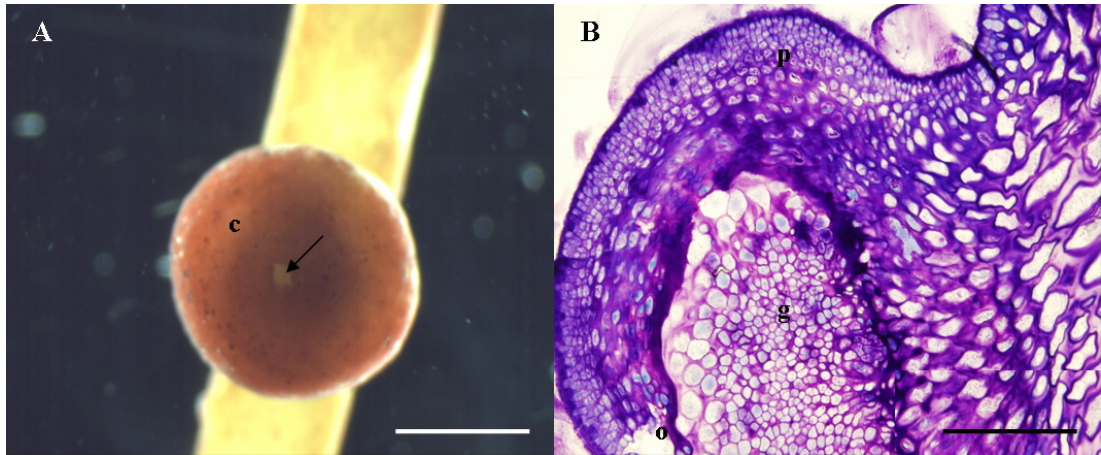
Plate 4.2 Carposporophyte of *G. cliftonii*
A. Mature cystocarp (c) bearing a defined ostiole (arrow). B. Cross section of a
mature cystocarp presenting a well developed gonimoblast (g), pericarp (p) and
ostiole (o). Bar=200 µm.

The male gametophyte thalli were characterised by the presence of
"*verrucosa type*" spermatangia which were born in few branches of the thallus.
Mature spermatangia were 55-80 µm deep and 40-50 µm across (Plate 4.3B).
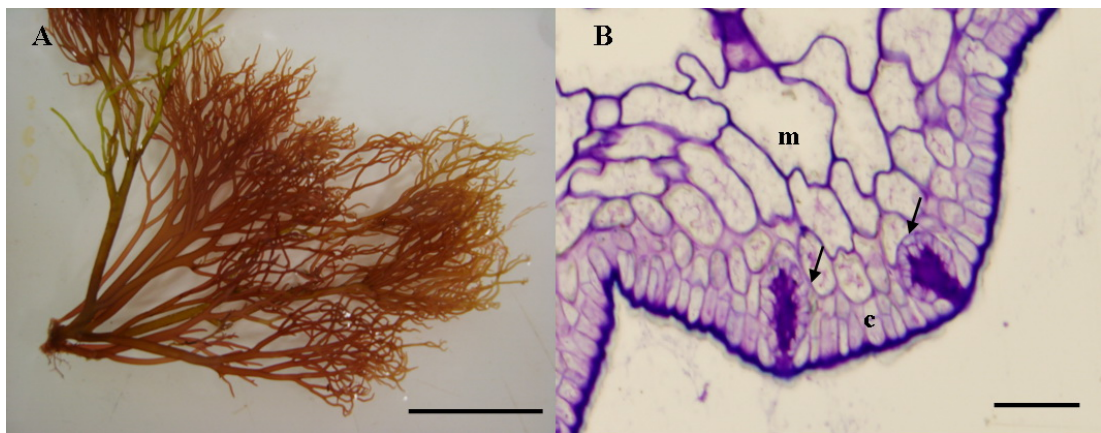
Plate 4.3 Male gametophyte of *G. cliftonii*
A. Fresh thallus of a male gametophyte. Bar = 3 cm. B. Cross section bearing mature
spermatangia "*verrucosa type*" (arrows) growing on the cortex. Bar=50 µm.
Abbreviations: m=medulla, c = cortex.

Mixed reproductive stages were also observed as tetrasporangia and
cystocarps growing on same thallus (Plate 4.4).
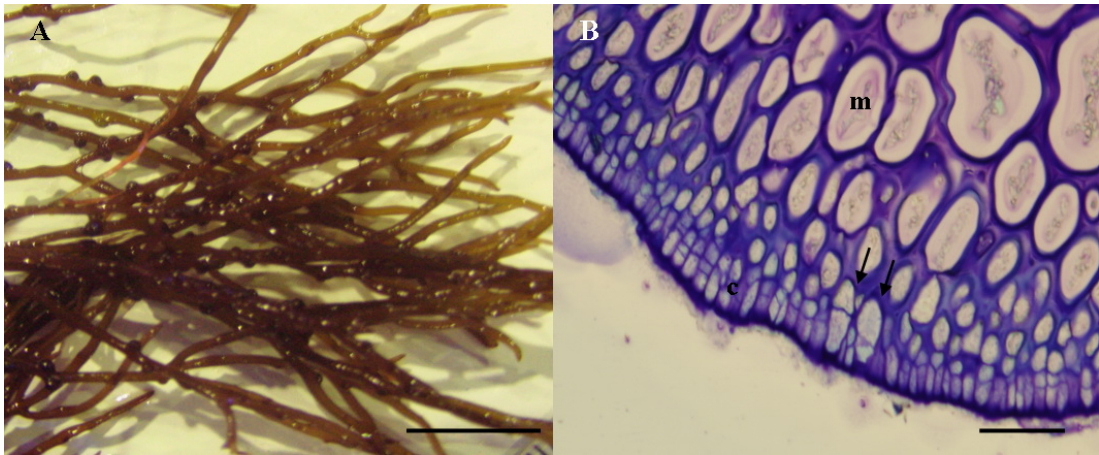
Plate 4.4 Deviations from typical life cycle of *G. cliftonii*
A. Thallus identified as carposporophytic due to the presence of cystocarps. Bar = 2 cm. B. Cross section of *G. cliftonii* carposporophyte thallus bearing tetrasporangia structures. Bar=50 μm. Abbreviations: m=medulla, c = cortex.

4.2.2. Spore release

4.2.2.1.   Cystocarp size

Number of spores released per cystocarp was independent ($p>0.05$) of time (Figure 4.2). However, on the fourth day the number of carpospores released from 1.5 mm cystocarps were significantly higher ($p<0.05$) than from cystocarps of 1 mm size. There was no influence ($p>0.05$) of cystocarp size on the number of spores released on the same day.
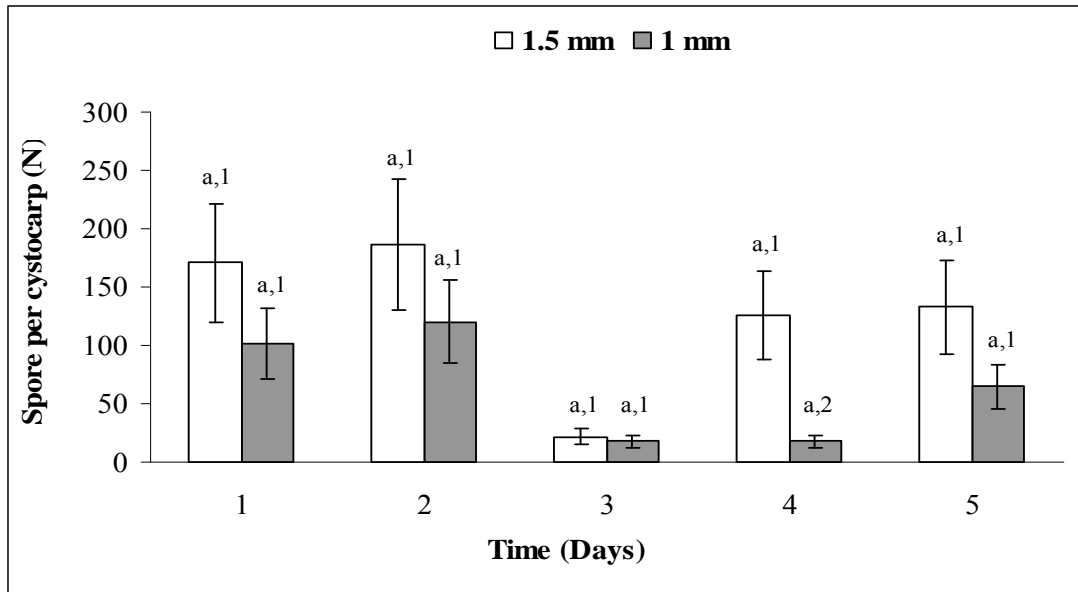
Figure 4.2 Mean daily carpospore release of *G. cliftonii* per cystocarp size over a period of 5 days

Error bars represent standard error of the mean

Different letters (a,b) indicate significant differences between days, while different numbers (1,2) indicate significant differences between cystocarp size at the level of $p<0.05$

4.2.2.2.   Nutrient media

Cystocarps released carpospores in both OW and PES up to a period of 5 weeks independently of cystocarp size. After this time, the cystocarp started disintegrating in both OW and PES. Number of carpospores released per cystocarp in PES were significantly higher ($p<0.05$) than in OW for third and fourth week of experiment (Figure 4.3).

The number of carpospores released in PES during third week were significantly higher ($p<0.05$) than other weeks. Number of spores released in OW in the first and second weeks were significantly higher ($p<0.05$) than the other weeks (Figure 4.3).
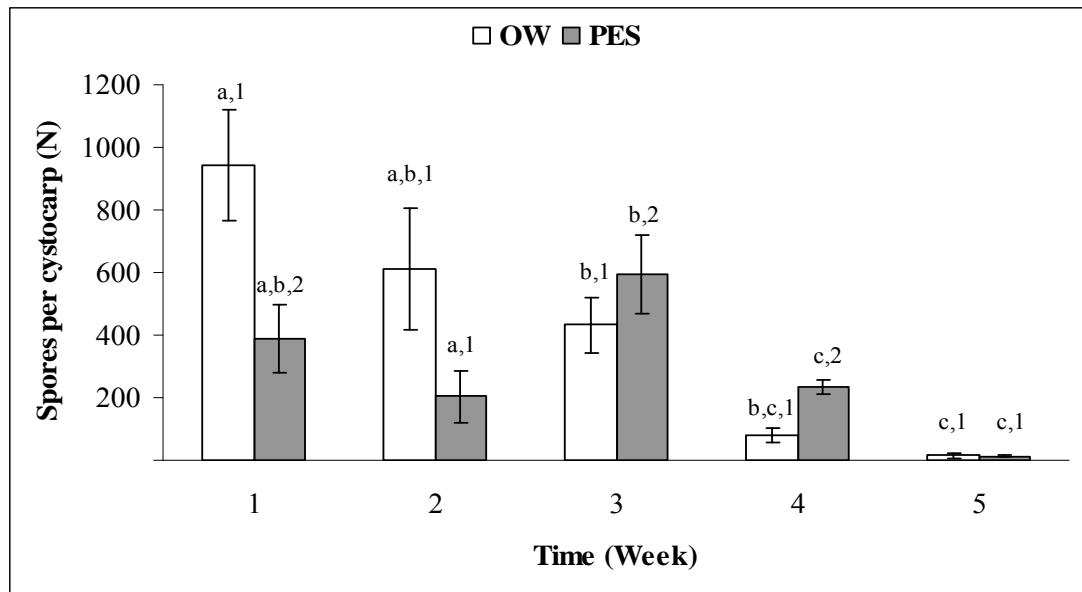
Figure 4.3 Mean weekly carpospore release by cystocarp of *G. cliftonii* with and without nutrient addition

Error bars indicate standard error of the mean

Different letters (a,b,c) indicate significant differences between days, while different numbers (1,2) indicate significant differences between treatments at the level of $p < 0.05$

The number of carpospores released per gram of cystocarpic material in OW were significantly higher ($p < 0.05$) than in PES during the entire experimental period. The number of carpospores released on third day in PES and OW were significantly higher ($p < 0.05$) than the rest of the days (Figure 4.4).
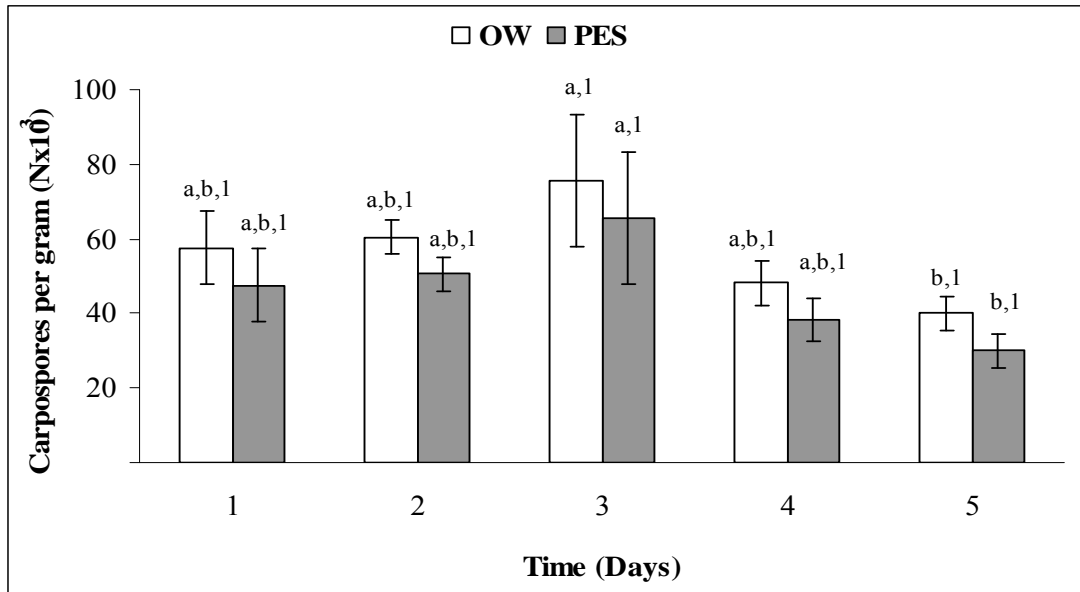
Figure 4.4 Mean daily carpospore release of *G. cliftonii* with and without nutrient addition

Error bars indicate standard error of the mean

Different letters (a,b) indicate significant differences between days, while different numbers (1,2)

indicate significant differences between treatments at the level of $p < 0.05$

The number of tetraspores released in OW and PES decreased gradually every day with significantly higher ($p < 0.05$) numbers released on first day. On first, second and third day, number of tetraspores released in OW were significantly higher ($p < 0.05$) than in PES (Figure 4.5).
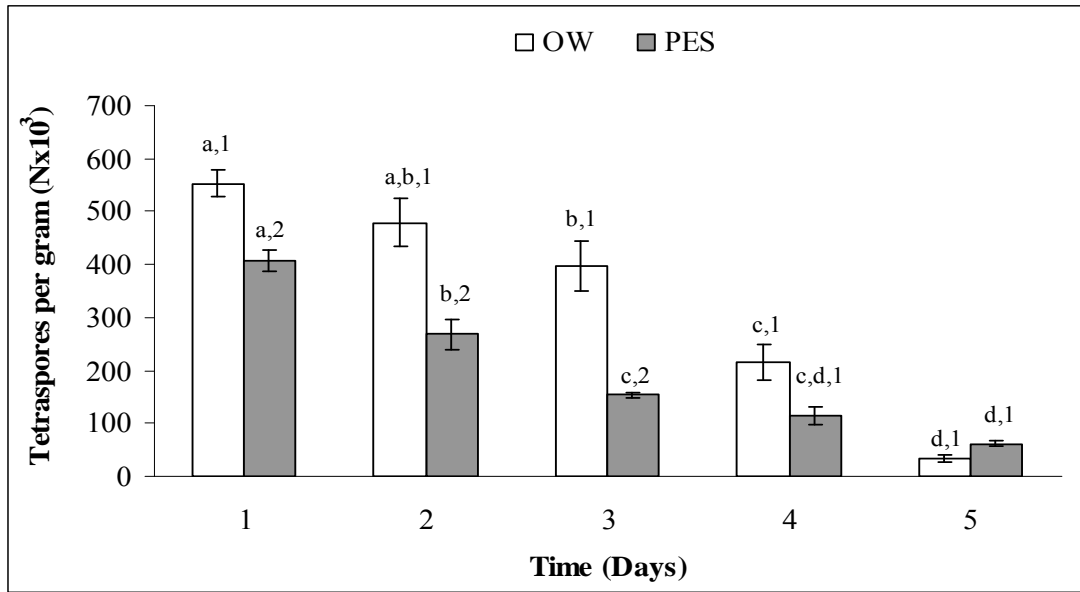
Figure 4.5 Mean daily tetraspore release of *G. cliftonii* with and without nutrient addition

Error bars indicate standard error of the mean Different letters (a,b) indicate significant differences between days, while different numbers indicate significant differences between treatments at the level of $p < 0.05$

In addition, over a period of 5 days, number of tetraspores released in ocean water were significantly higher ($p < 0.05$) than the number of carpospores released (Figure 4.6).
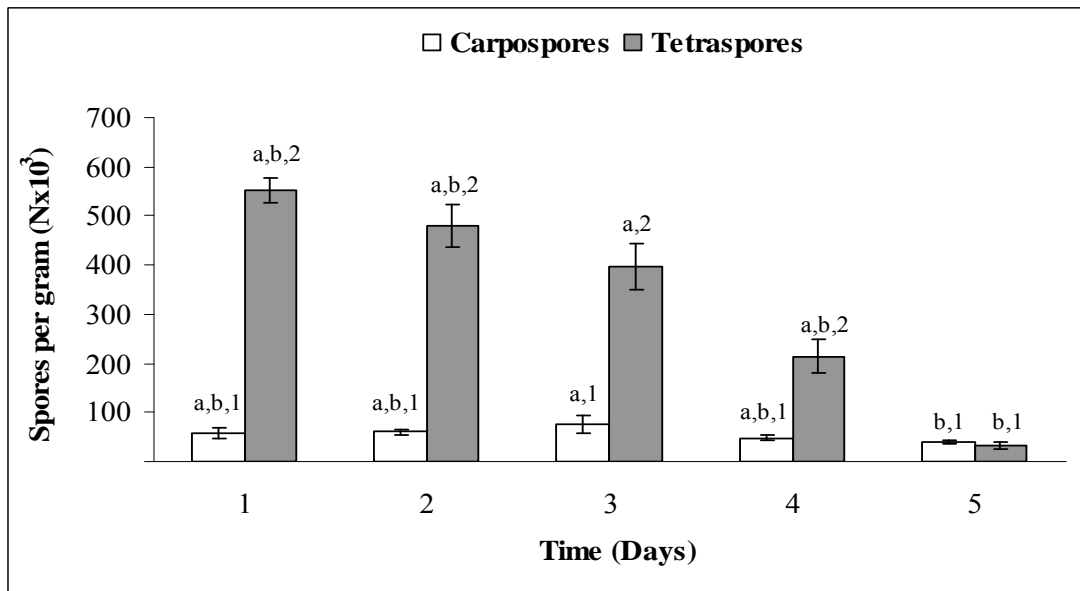
Figure 4.6 Tetraspore and carpospore of *G. cliftonii* released in ocean water over a five days experiment

Error bars indicate standard error of the mean

Different letters (a,b) indicate significant differences between days, while different numbers (1,2) indicate significant differences between type of spore at the level of $p < 0.05$

4.2.3. Survival rate

Survival rate of tetraspores and carpospores after one month of culture was significantly higher (p<0.05) in PES than in OW. However, survival rates of tetraspores and carpospores were similar (p>0.05) in the same media (Table 4.1).

Table 4.1 Percentage survival rate of carpospores and tetraspores of *G. cliftonii* 30 days post release

Treatment	Carpospores	Tetraspores
OW	$_1 27.0 \pm 3.4^a$	$_1 31.9 \pm 3.8^a$
PES	$_2 63.6 \pm 8.1^a$	$_2 69.8 \pm 5.9^a$

Different letters (a) indicate significant differences between type of spore, while different numbers (1,2) indicate significant differences between treatment at the level of p<0.05

4.2.4. Spore germination

In the present study, *G. cliftonii* carpospore development consisted of four stages: primary division stage, hemisphere body stage, basal disc stage and young sporeling stage. Carpospores were released as clumps surrounded by a mucilaginous sheath (Plate 4.5A). Mean cell diameter was 20 μm and had round to ovoid shape (Plate 4.5B). During the first 5 days post release, the carpospores began a process of germination which initiated with a transversal cell division (Plate 4.5C).
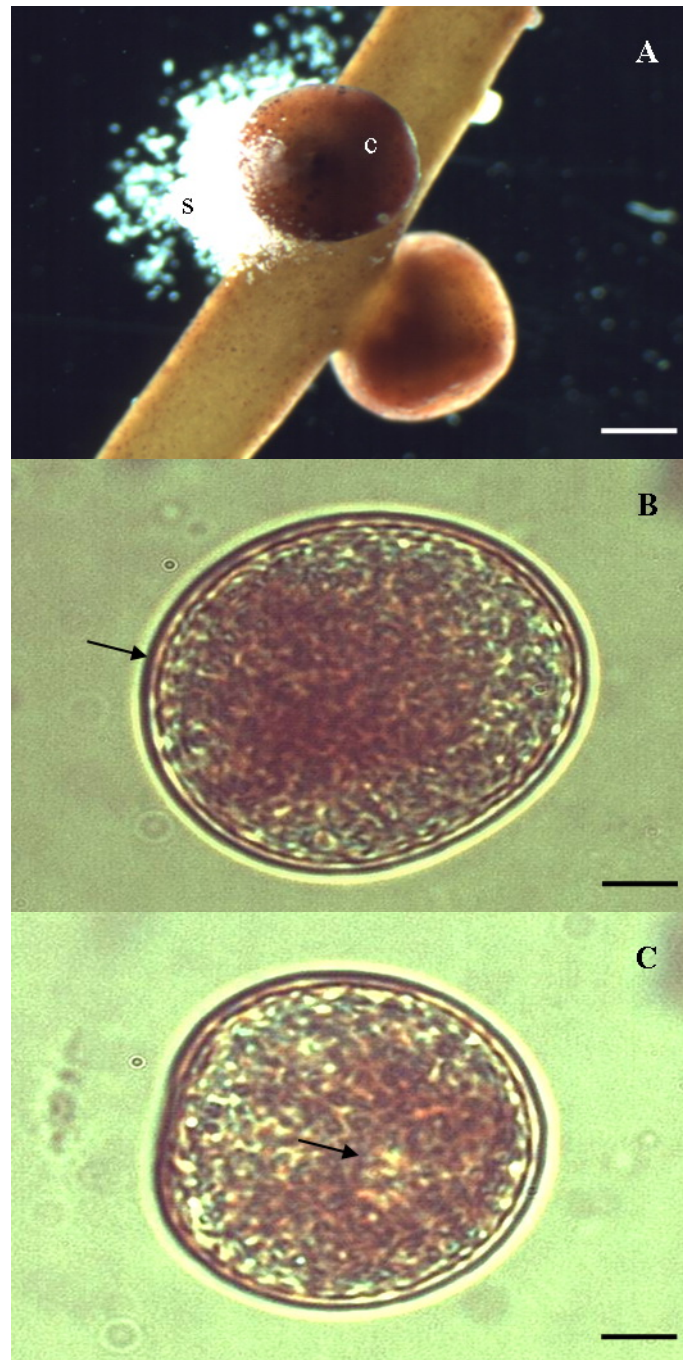
Plate 4.5 *G. cliftonii* carpospore development I

A. *G. cliftonii* mature cystocarp releasing carpospore. Bar= 1 mm. B. Carpospore released after 24 h. Arrow indicates the presence of a cell wall. Bar = 5 μm. C. Carpospore beginning germination. Arrow indicates the migration of the cellular contents. Bar = 5 μm.

After 24 h, 50 % of the carpospores released initiated the first transverse cell division (Plate 4.6A) followed by second (Plate 4.6B) and third cell divisions (Plate 4.6C).
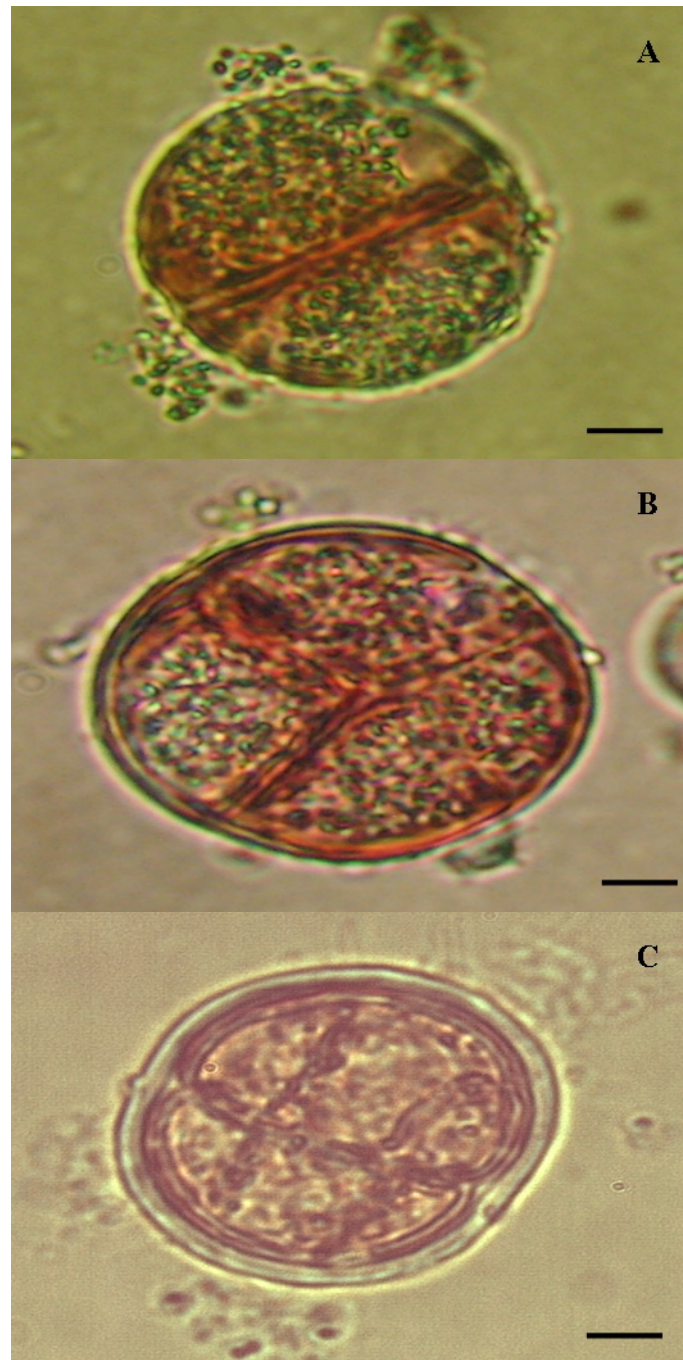
Plate 4.6 *G. cliftonii* carpospore development II

A. Carpospore initial cell transverse division (indicated by arrow). B. Carpospore second division (4 cells). C. Carpospore third division (6 cells). Bar = 10 μm.

The germinative carpospore then developed into a multicellular young sporeling (Plate 4.5A). This multicellular sporeling became oval shaped and commenced the development of a basal disc (Plate 4.5B-C).
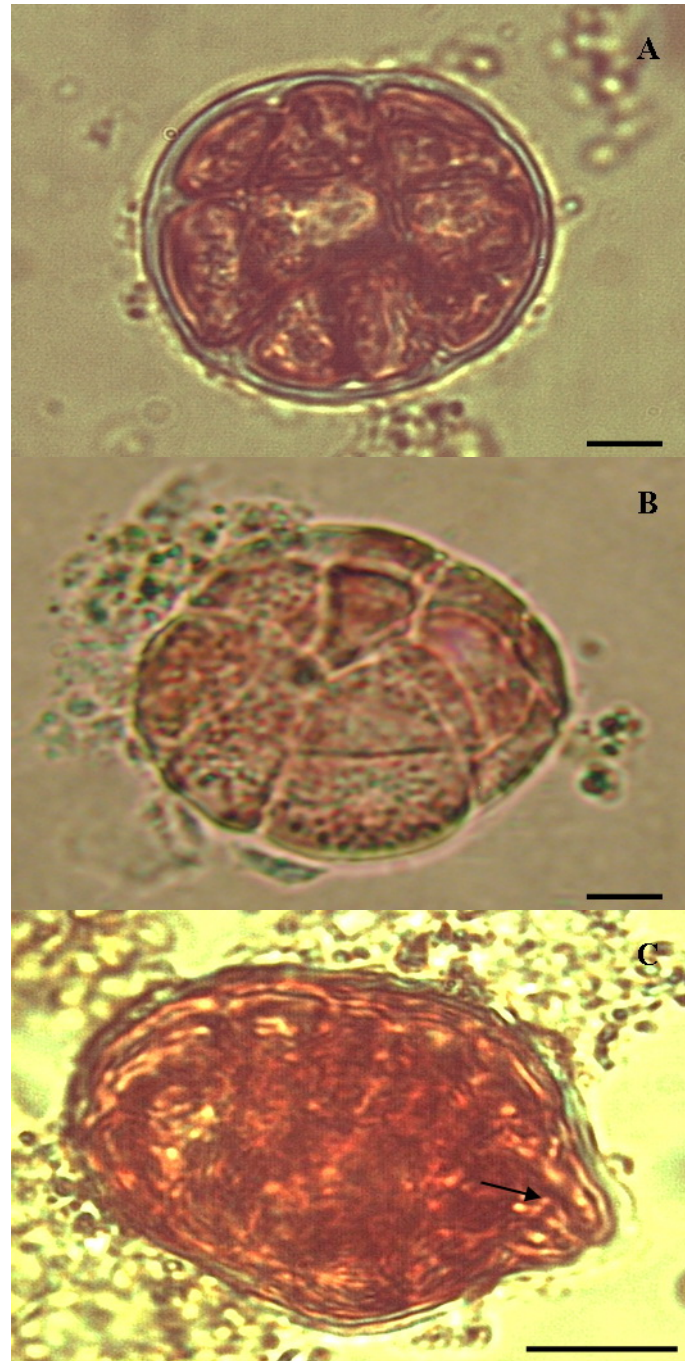
Plate 4.7 *G. cliftonii* carpospore development III
A. Carpospore germination developed into a young multicellular thallus. B. Carpospore seventh cell division. Bar = 10 μm. C. Formation of basal disc (arrow). Bar = 10 μm. Bar = 20μm.

The basal disc stage was observed 10 ± 2 days after the settlement and attachment of spore to the substratum (Petri dish). The cells at the top which were in the centre of the disc began to arch and formed the first branch (Plate 4.8A) and grew up to size of 100 μm (Plate 4.8B). The fusion or coalescence of 2 to 4 basal discs was

also observed in the present study (Plate 4.8C). In addition, it was observed that the plantlets which originated from coalescent spores develop faster into young thallus.
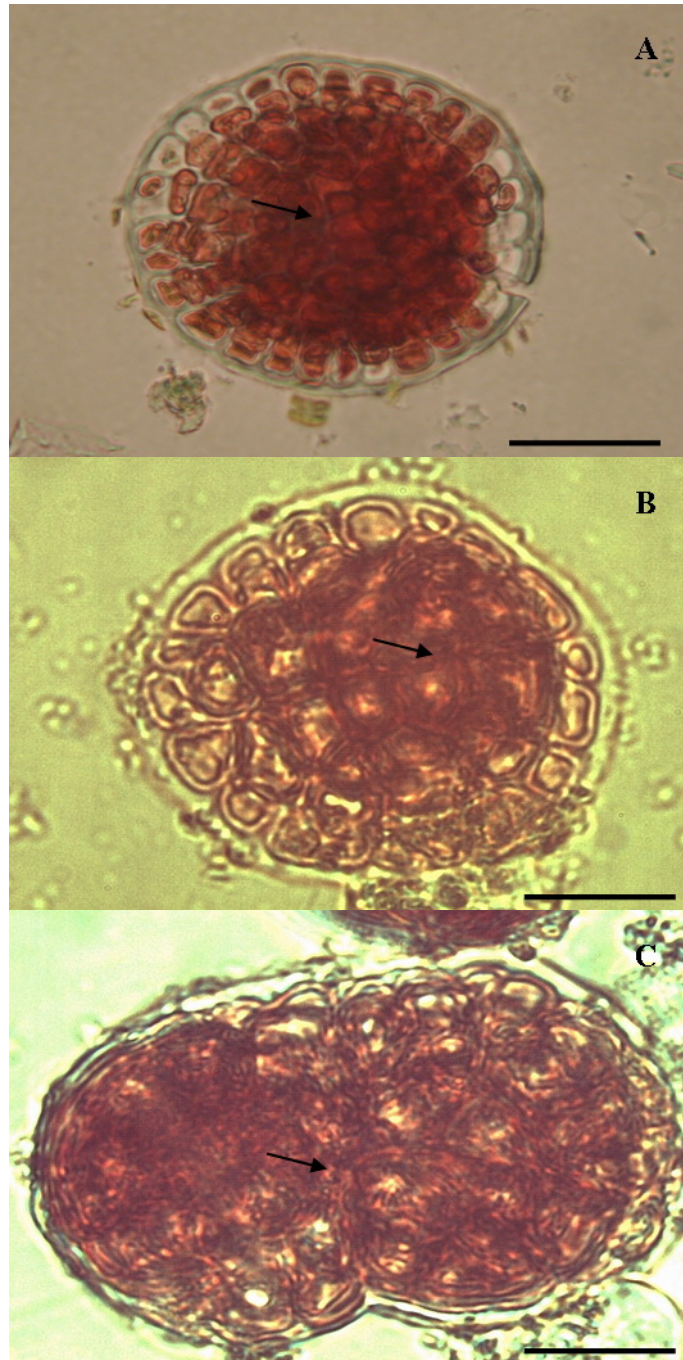
Plate 4.8 *G. cliftonii* carpospore development IV

A. Top view of a germinated carpospore initiating the formation of basal disc. Bar = 30 μm.

B. Basal disc presenting the development of the first branch. Bar = 30 μm. C Coalescence or fusion of two basal discs. The arrow indicates the area of the fusion of two discs. Bar = 30 μm.

The carpospores developed into the young sporeling stage with a well defined branch (Plate 4.9A). After 15 days the spore developed into a thick multicellular,

cone shaped structure (Plate 4.9B) and reached up to 0.5 mm height in 30 days (Plate 4.9C).
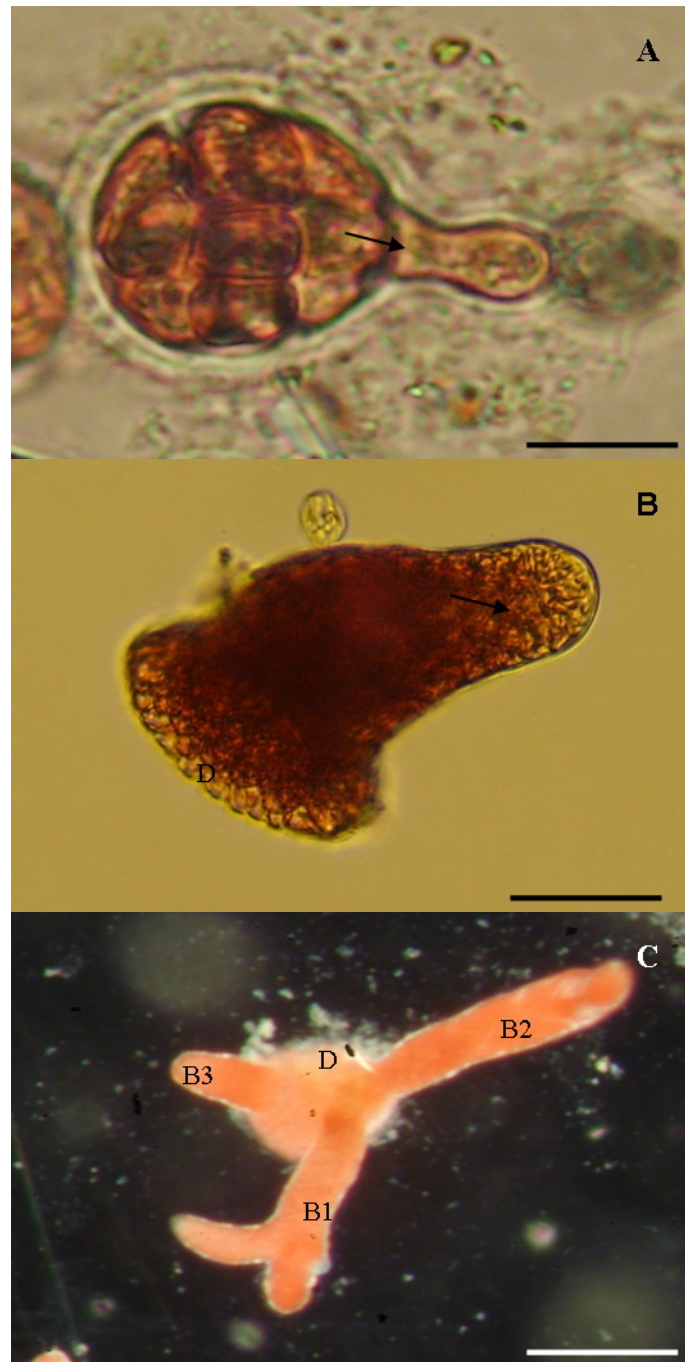
Plate 4.9 *G. cliftonii* carpospore development IV

A. Lateral view of a germinated carpospore initiating the first branch development. Bar = 40 µm. B. Young thallus with apical branch. Bar = 100 µm. C. Young thallus one month after release bearing 3 branches arising from a holdfast. Bar = 500 µm.

The tetraspores released were spherical in shape with mean diameter of 29-33 µm and were surrounded by a thin mucilaginous sheath which dissolved after liberation of spores (Plate 4.10). Germination of tetraspores began 24 hr after release without a resting period and followed similar pattern like carpospores and the fusion of two or three discs was observed.

Tetraspore size (Plate 4.10A) was significantly higher (p<0.05) than the carpospore size and tetraspores grew faster than carpospores. After 3 months of culture, germinated tetraspores were 2 mm long while carpospores mean length was 1.2 mm. In addition, germinated tetraspores showed more branches as compared to young carposporophytes (Plate 4.10B).
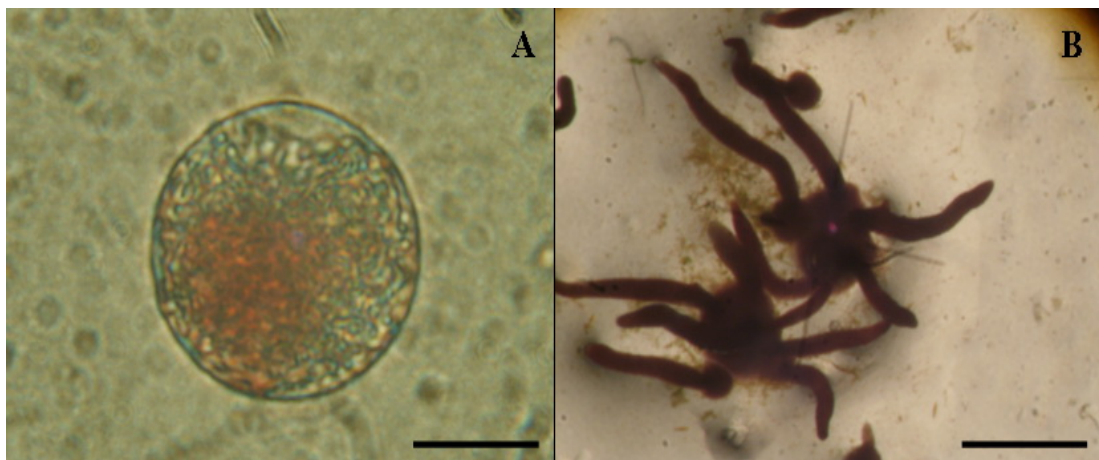
Plate 4.10 Germinated tetraspores from *Gracilaria cliftonii*
A. Tetraspores released after 24 h. Scale bar: 15 µm B. Young tetrasporophyte thallus after 3 months cultured in full PES. Scale bar: 2 mm

4.3. BIOMASS

4.3.1. Sediment composition

4.3.1.1. Seasons based on temperature

The overall composition of sediment size from the sampling site revealed higher proportion of coarse to very coarse sediments (particle size 1 and 0.7 mm) (Figure 4.7). An examination of the size fraction distribution data showed two patterns. First pattern was observed in Autumn with a higher proportion of fine fraction sediments (0.3 and <0.3 mm) (Figure 4.7A). While in Winter, Spring and Summer seasons higher proportion of 1 and 0.7 mm sediments, with very coarse particles (1 mm) as the most dominant size (Figure 4.7B).
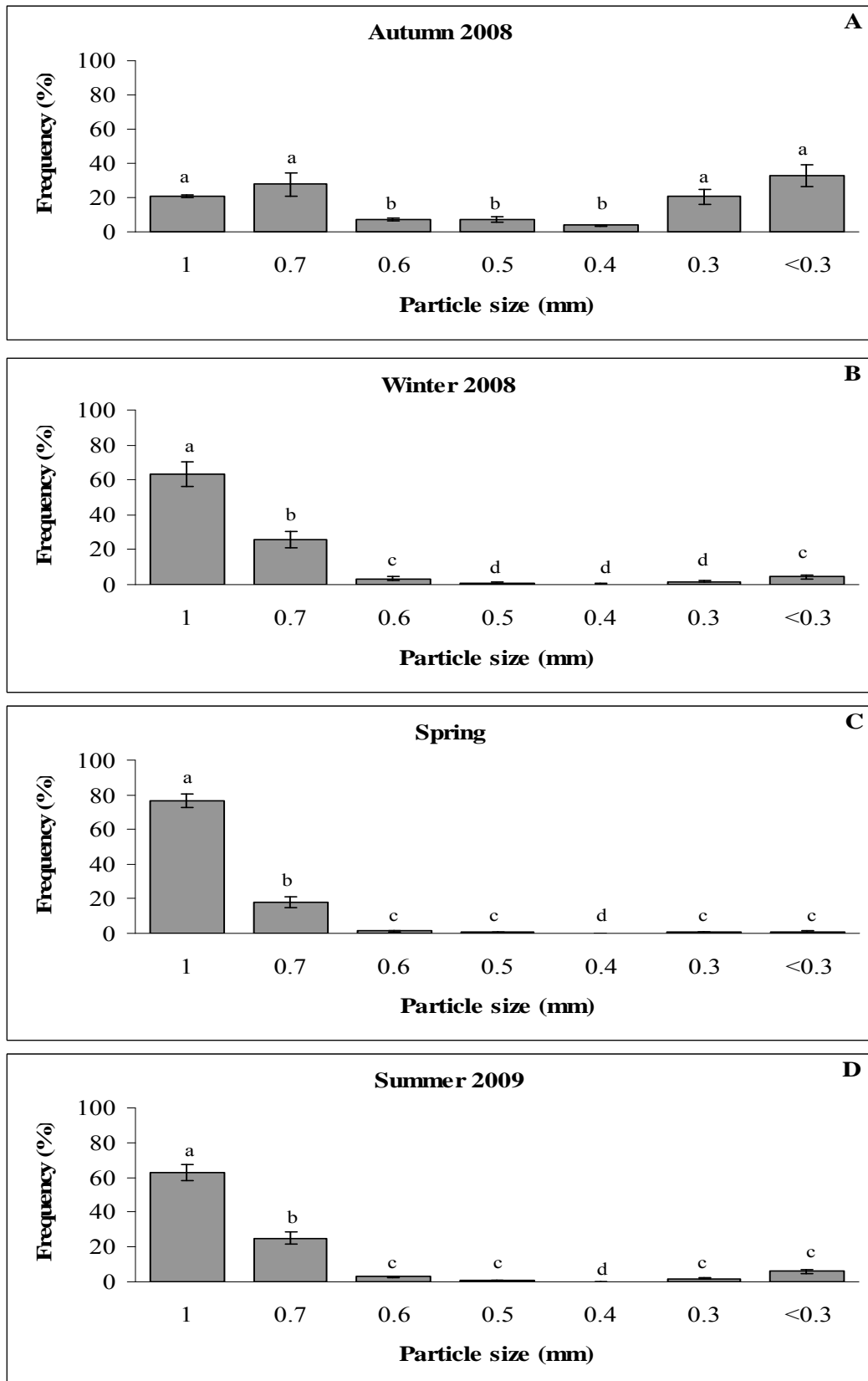
Figure 4.7 Composition of sediment (%) by particle size over one year study period from Point Peron, Western Australia

Error bars indicate standard error of the mean. Different letters indicate significant differences between sediment sizes (p<0.05)

Particle size of the sediment from the study site was influenced by the year (Table 4.2). In Autumn and Winter of 2009, grain sizes of 1mm were more frequent than in Autumn and Winter of 2008.

Table 4.2 Frequency (%) (Mean ±SE) of particle size composition of sediment in Autumn and Winter from 2008 and 2009

Sediment size	Autumn		Winter	
	2008	**2009**	**2008**	**2009**
1 mm	$20.8 \pm 0.8_a$	$64.3 \pm 4.7_b$	$63.1 \pm 6.9_a$	$78.1 \pm 7.4_b$
0.7 mm	$27.8 \pm 6.9_a$	$28.6 \pm 4.0_a$	$25.5 \pm 4.6_a$	$11.4 \pm 3.9_b$
0.6 mm	$7.2 \pm 0.5_a$	$2.4 \pm 0.4_b$	$3.4 \pm 0.9_a$	$3.4 \pm 1.5_a$
0.5 mm	$7.1 \pm 1.6_a$	$0.9 \pm 0.2_b$	$1.1 \pm 0.3_a$	$1.9 \pm 0.9_a$
0.4mm	$4.0 \pm 0.4_a$	$0.1 \pm 0.0_b$	$0.3 \pm 0.1_a$	$0.1 \pm 0.0_a$
0.3 mm	$20.5 \pm 4.3_a$	$1.1 \pm 0.2_b$	$1.7 \pm 0.5_a$	$2.3 \pm 1.3_a$
<0.3 mm	$33.0 \pm 6.3_a$	$3.6 \pm 1.2_b$	$4.5 \pm 1.3_a$	$1.8 \pm 1.0_b$

Subscript letters (a) represent significant differences between same seasons at a level of $p<0.05$, t-test

4.3.1.2. Seasons based on photoperiod

The sediment composition analysis from the study area showed that sediments were influenced by seasons based on photoperiod. For all the seasons, sediments with particle size of 1mm and 0.7 mm were significantly more frequent than rest of the sediments sizes. In PS3 sediments sizes of 1mm were more frequent than in PS1 ($p<0.05$) while in PS1 sediments sizes of 0.6 to <0.03 mm were significantly more frequent than PS2 (Table 4.3).

Table 4.3 Frequency (%) of sediment composition (Mean ± SE) for three seasons based on photoperiod

Sediment size	PS1	PS2	PS3
1 mm	$_1$52.0 ± 6.8[a]	$_{1,2}$66.0 ± 3.0[a]	$_2$72.7 ± 5.3[a]
0.7 mm	$_1$24.9 ± 4.2[b]	$_1$24.7 ± 2.2[b]	$_1$18.8 ± 4.0[b]
0.6 mm	$_1$4.5 ± 0.8[c]	$_2$2.4 ± 0.4[c]	$_{1,2}$2.9 ± 0.9[c]
0.5 mm	$_1$2.1 ± 0.6[c]	$_2$0.8 ± 0.1[c]	$_{1,2}$1.5 ± 0.5[c]
0.4 mm	$_1$1.4 ± 0.4[c]	$_2$0.1 ± 0.0[c]	$_2$20.1 ± 0.0[c]
0.3 mm	$_1$5.5 ± 1.8[c]	$_2$1.2 ± 0.2[c]	$_2$1.8 ± 0.7[c]
<0.3 mm	$_1$10.1 ± 3.1[c]	$_2$4.0 ± 0.9[c]	$_2$2.6 ± 0.9[c]

Superscript letters (a,b,c) indicate significant differences among sediment sizes while, subscript numbers indicate significant differences between seasons ($p < 0.05$)

4.3.2. Physical parameters

4.3.2.1.   Seasons based on temperature

Seawater temperature and photoperiod varied ($p < 0.05$) as a function of season with maximum temperature and photoperiod observed in Summer. Rainfall in Winter season was significantly higher ($p < 0.05$) than in Summer (Table 4.4).

Table 4.4 Seawater physical parameters (Mean ± SE) from Autumn 2008 to Summer 2009 at Point Peron, Western Australia

Season	Temperature °C	Photoperiod* hours	Rainfall* mm
Autumn	21.5 ± 0.1[a]	11.2 ± 0.1[a]	75.8 ± 41.0[a,b]
Winter	17.4 ± 0.1[b]	10.3 ± 0.0[b]	116.4 ± 45.6[a]
Spring	19.0 ± 0.1[c]	12.6 ± 0.1[c]	57.3 ± 10.8[a,b]
Summer	22.2 ± 0.1[d]	13.7 ± 0.1[d]	6.7 ± 3.4[b]

Superscript letters (a,b,c) represent significant differences between seasons at a level of $p < 0.05$.

* Source: Bureau of Meteorology (Goldberg and Kendrick, 2004)

Years did not influence seawater temperature, photoperiod and rainfall at the study site (Table 4.5).

Table 4.5 Seawater physical parameters (Mean ± SE) of Autumn and Winter for 2008 and 2009

Parameter	Autumn 2008	Autumn 2009	Winter 2008	Winter 2009
Temperature (^{o}C)	$21.5 \pm 0.10_a$	$20.7 \pm 0.10_a$	$17.4 \pm 0.1_a$	$17.0 \pm 0.1_a$
Photoperiod (h)	$11.2 \pm 0.10_a$	$11.2 \pm 0.10_a$	$10.3 \pm 0.0_a$	$10.3 \pm 0.0_a$
Rainfall (mm)	$75.8 \pm 41.0_a$	$19.3 \pm 13.4_a$	$116.4 \pm 45.6_a$	$129.3 \pm 23.7_a$

Subscript letters (a) represent significant differences between same seasons at a level of $p<0.05$, t-test

4.3.2.2.   Seasons based on photoperiod

Table 4.6 shows the mean temperature, photoperiod and rainfall grouped on season based on photoperiod. For temperature and photoperiod all the seasons were significantly different from each other. In PS1 rainfall was significantly higher ($p<0.05$) than PS2.

Table 4.6 Seawater physical parameters (Mean ± SE) over three seasons based on photoperiod

Season	Temperature ^{o}C	Photoperiod hours	Rainfall mm
PS1	19.0 ± 0.2^a	10.7 ± 0.0^a	106.4 ± 24.6^a
PS2	21.1 ± 0.1^b	13.2 ± 0.1^b	20.5 ± 9.2^b
PS3	18.3 ± 0.2^c	10.5 ± 0.0^c	$87.8 \pm 29.3^{a,b}$

Different letters (a) represent significant differences between seasons at a level of $p<0.05$

PS1: March to September 2008, PS2: October 2008 to March 2009, PS3: April to August 2009

4.3.3. Chemical parameters

4.3.3.1.   Seasons based on temperature

Ammonium concentration [NH_4^+] in ocean water was significantly higher ($p<0.05$) in Winter and Spring season than in Autumn and Summer. Nitrites concentration [NO_2^-] in Winter was significantly lower ($p<0.05$) than the rest of the seasons. Orthophosphate concentration [PO_4^-] in Spring season was significantly higher ($p<0.05$) than rest of the seasons (Table 4.7).

Table 4.7 Seawater nutrient concentration (mg/L) (Mean ± SE) from Autumn 2008 to Summer 2009 at Point Peron, Western Australia

Season	NH_4	NO_3	NO_2	PO_4
Autumn	1.14 ± 0.03^a	0.013 ± 0.001^a	0.002 ± 0.000^a	0.097 ± 0.005^a
Winter	1.34 ± 0.05^b	0.013 ± 0.001^a	0.004 ± 0.000^b	0.110 ± 0.005^a
Spring	1.40 ± 0.07^c	0.014 ± 0.001^a	0.004 ± 0.000^b	0.296 ± 0.032^b
Summer	1.25 ± 0.05^d	0.014 ± 0.001^a	0.005 ± 0.000^b	0.021 ± 0.002^c

Superscript letters (a,b,c) represent significant differences between seasons at a level of p<0.05.

Nutrient concentration was dependant on year. $[NO_3^-]$ and $[NO_2^-]$ in Autumn of 2009 were significantly higher than Autumn of 2008. While $[PO_4^-]$ was significantly higher (p<0.05) in Autumn of 2008 than Autumn of 2009 but significantly lower in Winter of 2008 than Winter of 2009 (Table 4.8).

Table 4.8 Seawater nutrient concentration (mg/L)(Mean ± SE) from Autumn 2008 and Winter 2009 at Point Peron, Western Australia

Nutrient	Autumn 2008	Autumn 2009	Winter 2008	Winter 2009
NH_4^+	$1.140 \pm 0.030_a$	$1.250 \pm 0.050_a$	$1.340 \pm 0.050_a$	$1.590 \pm 0.050_a$
NO_3^-	$0.013 \pm 0.001_a$	$0.020 \pm 0.002_b$	$0.013 \pm 0.001_a$	$0.022 \pm 0.001_a$
NO_2^-	$0.002 \pm 0.000_a$	$0.004 \pm 0.000_b$	$0.004 \pm 0.000_a$	$0.003 \pm 0.000_a$
PO_4^-	$0.097 \pm 0.005_a$	$0.182 \pm 0.013_b$	$0.110 \pm 0.005_a$	$0.275 \pm 0.012_b$

Subscript letters (a) represent significant differences between same seasons at a level of p<0.05, t-test

Osmolality and pH of seawater was influenced by seasons based on temperature. Osmolality of seawater was significantly different (p<0.05) among all seasons while, in Summer pH of seawater was significantly higher (p<0.05) than Winter and Spring (Table 4.9).

Table 4.9 Seawater osmolality and pH (Mean ± SE) from Autumn 2008 to Summer 2009 at Point Peron, Western Australia

Season	Osmolality (Osmol/kg)	pH
Autumn	1.24 ± 0.01^a	$7.94 \pm 0.01^{a,b}$
Winter	0.99 ± 0.02^b	7.94 ± 0.01^a
Spring	1.06 ± 0.00^c	7.89 ± 0.02^b
Summer	1.12 ± 0.01^d	8.07 ± 0.03^c

Superscript letters (a,b,c) represent significant differences between seasons at a level of $p<0.05$

Osmolality and pH of seawater was dependant on year (Table 4.10). Osmolality was significantly higher ($p<0.05$) in Autumn 2008 than Autumn 2009. In Autumn and Winter 2009, pH was significantly higher ($p<0.05$) than Autumn and Winter 2008.

Table 4.10 Seawater osmolality (osmol/kg) and pH (Mean ± SE) in Autumn and Winter for 2008 and 2009

Parameter	Autumn 2008	Autumn 2009	Winter 2008	Winter 2009
Osmolality	$1.24 \pm 0.01_a$	$1.17 \pm 0.02_b$	$1.24 \pm 0.01_a$	$1.15 \pm 0.01_a$
pH	$7.94 \pm 0.01_a$	$8.04 \pm 0.04_b$	$7.94 \pm 0.01_a$	$8.02 \pm 0.02_b$

Subscript letters (a,b) represent significant differences between same seasons at a level of $p<0.05$, t-test

4.3.3.2. Seasons based on photoperiod

Nutrient concentration in seawater was influenced by seasons based on photoperiod (Table 4.11). $[NH_4^+]$ and $[NO_3^-]$ was significantly higher ($p<0.05$) in PS3 than PS1 and PS2. $[NO_2^-]$ was significantly higher ($p<0.05$) in PS3 than PS2 while $[PO_4^-]$ was significantly higher ($p<0.05$) in PS3 than PS2.

Table 4.11 Nutrient concentration (Mean ± SE) over three seasons based on photoperiod

Season	NH$_4$ mg/L	NO$_3$ mg/L	NO$_2$ mg/L	PO$_4$ mg/L
PS1	1.28 ± 0.03^a	0.013 ± 0.001^a	$0.004 \pm 0.000^{a,b}$	0.179 ± 0.020^a
PS2	1.29 ± 0.04^a	0.013 ± 0.001^a	0.005 ± 0.000^a	0.116 ± 0.012^b
PS3	1.50 ± 0.04^b	0.023 ± 0.001^b	0.004 ± 0.000^b	0.219 ± 0.011^a

Superscript letters (a) represent significant differences between seasons at a level of $p<0.05$

PS1: March to September 2008, PS2: October 2008 to March 2009, PS3: April to August 2009

Osmolality in PS3 was significantly higher ($p<0.05$) than PS1 and PS2 while in PS2 pH was significantly higher ($p<0.05$) than other seasons (Table 4.12).

Table 4.12 Osmolality and pH of seawater (Mean ± SE) over three seasons based on photoperiod

Season	Osmolality Osmol/kg	pH
PS1	1.09 ± 0.02^a	7.93 ± 0.01^a
PS2	1.10 ± 0.01^a	8.06 ± 0.02^b
PS3	1.17 ± 0.01^b	7.97 ± 0.02^a

Superscript letters (a,b) represent significant differences between seasons at a level of $p<0.05$

PS1: March to September 2008, PS2: October 2008 to March 2009, PS3: April to August 2009

4.3.4. Total biomass and abundance

4.3.4.1. Seasons based on temperature

Total biomass of *G. cliftonii* varied from 10.9 to 42.2 g/m^2, while, total abundance varied from 1.7 to 3.4 nh/m^2. Significantly lower ($p<0.05$) biomass was observed in Spring then the rest of the year (Figure 4.8). Total abundance was not influenced by any season ($p>0.05$) (Figure 4.9).
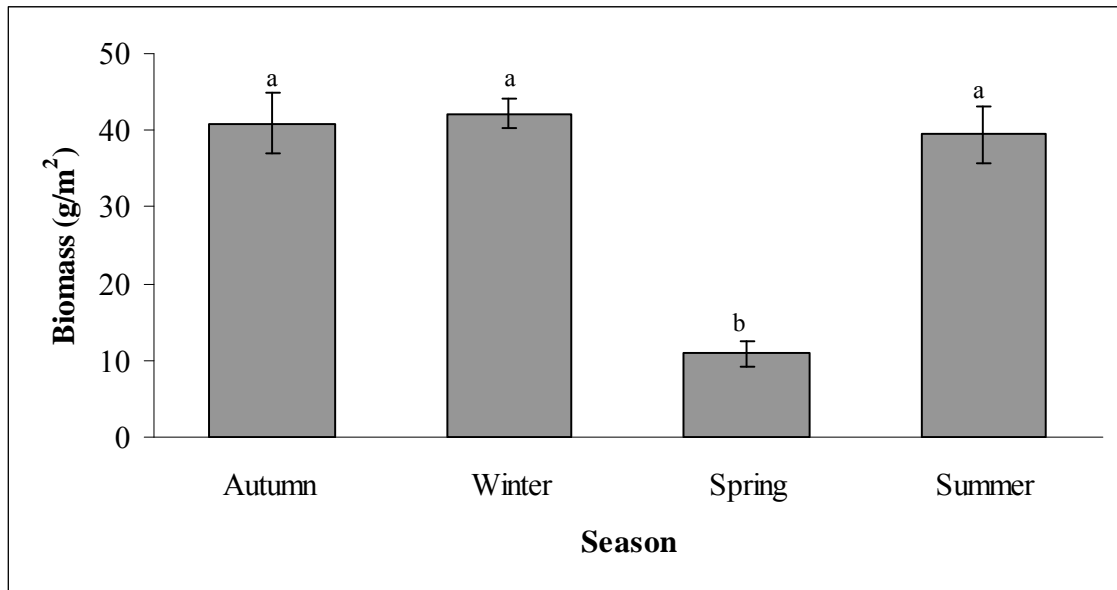
Figure 4.8 Mean total biomass of *Gracilaria cliftonii* based on temperature seasons at Point Peron, Western Australia

Error bars represent standard error of the mean.

Bars with different letters (a,b) indicate significant differences between seasons from Autumn 08 to Summer.
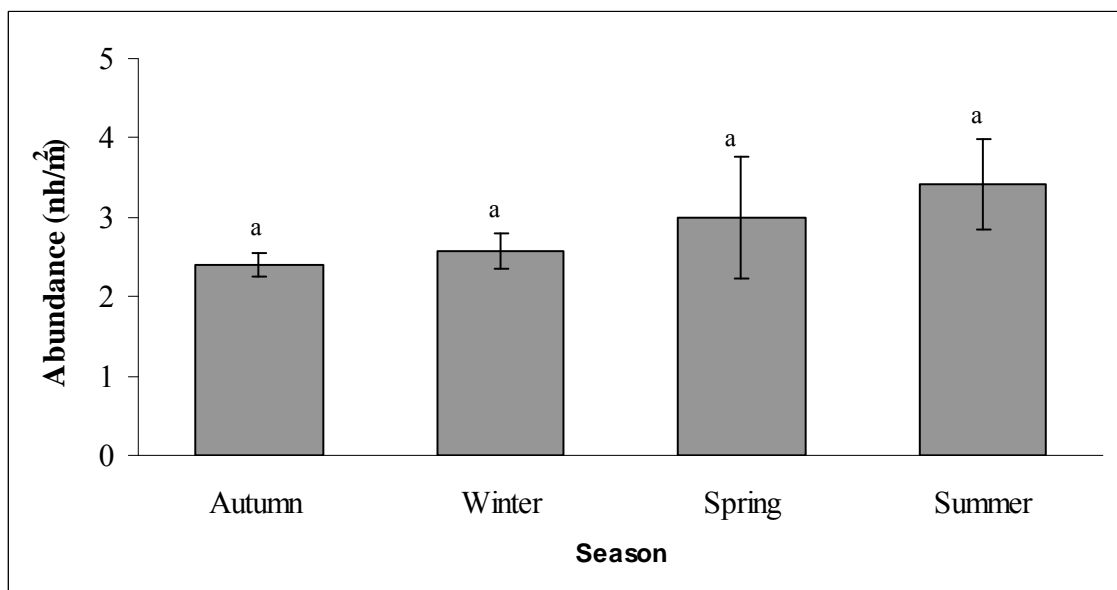
Figure 4.9 Mean total abundance of *Gracilaria cliftonii* based on temperature seasons at Point Peron, Western Australia

Error bars represent standard error of the mean.

Bars with different letters (a,b) indicate significant differences from Autumn 08 to Summer.

Total biomass and abundance in Autumn and Winter of 2008 were similar (p>0.05, t-test) to Autumn and Winter of 2009 respectively (Table 4.13)

Table 4.13 Table Total biomass and abundance (mean ± S.E) in Autumn and Winter from 2008 and 2009.

Parameter	Autumn 2008	Autumn 2009	Winter 2008	Winter 2009
Biomass (g/m^2)	$40.8 \pm 4.0_a$	$40.7 \pm 4.2_a$	$42.2 \pm 1.9_a$	$44.2 \pm 2.0_a$
Abundance (nh/m^2)	$2.4 \pm 0.2_a$	$1.8 \pm 0.1_a$	$2.6 \pm 0.2_a$	$1.7 \pm 0.1_a$

Subscript letters (a,b) represent significant differences between same seasons at a level of $p<0.05$, t-test

4.3.4.2.   Seasons based on photoperiod

Total biomass of *G. cliftonii* varied from 32.2 to 40.6 g/m^2, while total abundance varied from 1.7 to 3.2 nh/m^2 over three photoperiod seasons.
Total biomass (Figure 4.10) and abundance (Figure 4.11) of *G. cliftonii* were not influenced (p>0.05) by photoperiod seasons.
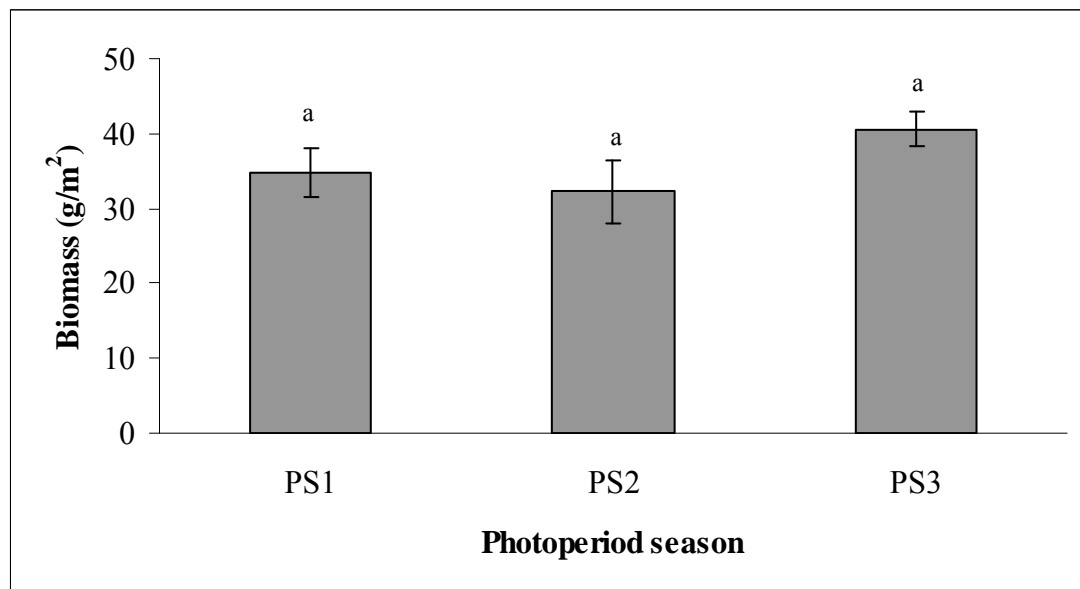
Figure 4.10 Mean total biomass of *Gracilaria cliftonii* based on photoperiod seasons at Point Peron, Western Australia
Error bars represent standard error of the mean

Bars with different letters (a) are significantly different (p<0.05)

PS1: March to September 2008, PS2: October 2008 to March 2009, PS3: April to August 2009
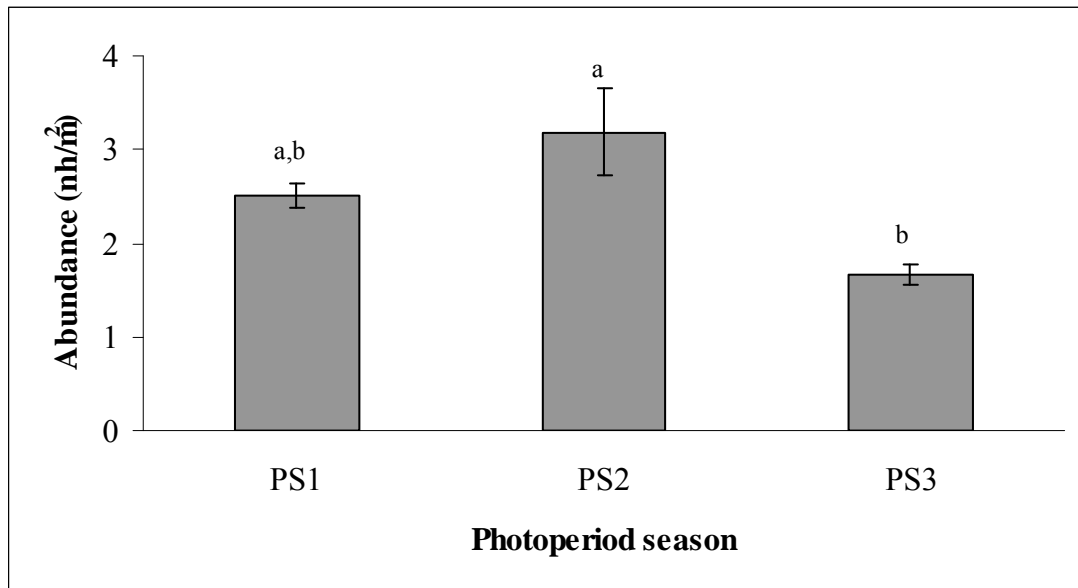
Figure 4.11 Mean total abundance of *Gracilaria cliftonii* based on photoperiod seasons at Point Peron, Western Australia

Error bars represent standard error of the mean.

Bars with different letters (a) are significantly different (p<0.05).

PS1: March to September 2008, PS2: October 2008 to March 2009, PS3: April to August 2009.

Total abundance of *G. cliftonii* showed a strong negative correlation (R^2=0.88; p<0.05) with [NO_3^-] and positive correlation (R^2=0.90; p<0.05) with [NO_2^-] in seawater. Total biomass was also found to be strongly positively correlated to day length (R^2=0.96; p<0.05).

4.3.5. Photosynthesis and Respiration

4.3.5.1.   Seasons based on temperature

Photosynthetic rates of *G. cliftonii* were in range 1.1 to 1.9 mg O_2/ g db/h while, respiration rates varied from -0.2 to -1.0 mg O_2/ g db/h. Photosynthetic rates in Summer were significantly higher (p<0.05), while, respiration rates were significantly lower (p<0.05) than Winter (Figure 4.12).
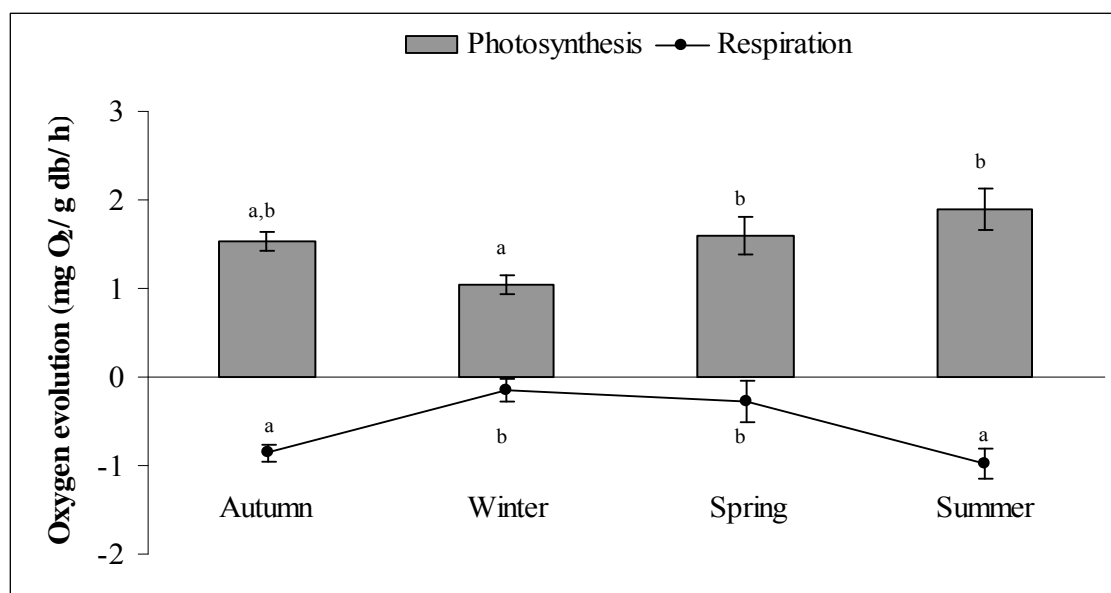
Figure 4.12 Mean photosynthetic and respiration rates of *Gracilaria cliftonii* based temperature seasons.

Error bars represent standard error of the mean.

Different letters (a,b) indicate significant differences among seasons from Autumn to Summer 2008 at p<0.05. Different numbers (1,2) indicate differences among same seasons (t-test; p<0.05).

Photosynthetic rates were not influenced by year while, respiration rates in Winter of 2008 were higher than in Winter of 2008 (Table 4.14).

Table 4.14 Mean photosynthetic and respiration rates (mg O_2/ g db/h) of *Gracilaria cliftonii* in Autumn and Winter for 2008 and 2009

Parameter	Autumn 2008	Autumn 2009	Winter 2008	Winter 2009
Photosynthesis	$1.5 \pm 0.1_a$	$1.5 \pm 0.2_a$	$1.1 \pm 0.1_a$	$1.5 \pm 0.1_a$
Respiration	$-0.9 \pm 0.1_a$	$-0.8 \pm 0.1_a$	$-0.2 \pm 0.1_a$	$-0.4 \pm 0.3_b$

Subscript letters (a,b) represent significant differences between same seasons at a level of p<0.05, t-test

4.3.5.2. Seasons based on photoperiod

Photosynthetic and respiration rates of *G. cliftonii* did not vary as a function of seasons (p>0.05) based on photoperiod (Figure 4.13).
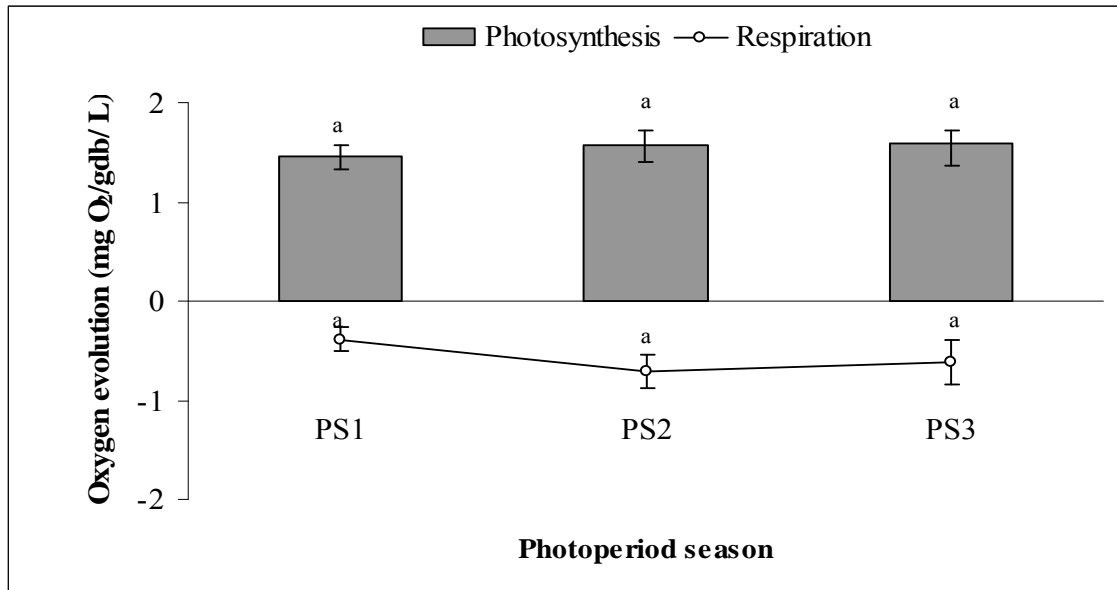
Figure 4.13 Mean photosynthetic and respiration rates of *Gracilaria cliftonii* based on photoperiod seasons

Error bars represent standard error of the mean

Different letters indicate significant differences among seasons at the level of $p<0.05$

PS1: March to September 2008, PS2: October 2008 to March 2009, PS3: April to August 2009

Photosynthesis rate of *G. cliftonii* showed a negative linear correlation ($R^2=0.83$; $p<0.05$), whereas, respiration showed a positive linear correlation ($R^2=0.70$; $p<0.05$) with rainfall.

4.3.6. Gross and net photosynthesis

4.3.6.1. Seasons based on temperature

Gross photosynthesis in Summer and Autumn was significantly higher ($p<0.05$) than Winter and Spring (Figure 4.14). However, net photosynthesis was not influenced ($p>0.05$) by seasons. Net photosynthetic and gross photosynthetic rates were not influenced by year (Table 4.15).
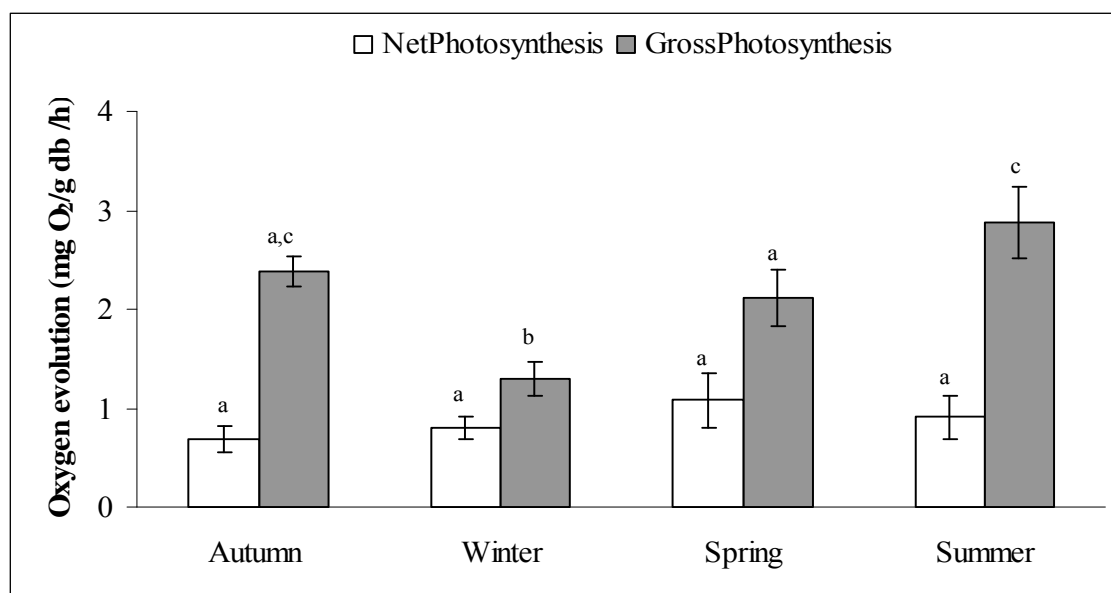
Figure 4.14 Mean net photosynthesis and gross photosynthesis of *Gracilaria cliftonii* based temperature seasons

Error bars represent standard error of the mean

Different letters (a,b,c) indicate significant differences among seasons from Autumn 2008 to Summer at the level of p<0.05.

Table 4.15 Mean photosynthetic and respiration rates (mg O_2/ g db/h) of *Gracilaria cliftonii* in Autumn Winter for 2008 and 2009

Parameter	Autumn 2008	Autumn 2009	Winter 2008	Winter 2009
Gross photosynthesis	$2.4 \pm 0.1_a$	$2.3 \pm 0.3_a$	$1.3 \pm 0.1_a$	$2.5 \pm 0.2_a$
Net photosynthesis	$0.7 \pm 0.2_a$	$0.8 \pm 0.2_a$	$0.8 \pm 0.2_a$	$0.5 \pm 0.1_a$

Subscript letters (a,b) represent significant differences between same seasons at a level of p<0.05, t-test

4.3.6.2.   Seasons based on photoperiod

Gross and net photosynthesis of *G. cliftonii* varied from 1.9 to 2.6 and from 0.6 to 1 mg O_2/ g db/ h respectively. Gross photosynthesis was not influenced (p>0.05) by photoperiod seasons, while net photosynthesis was significantly higher (p<0.05) in PS1 season than other photoperiod seasons (Figure 4.15).
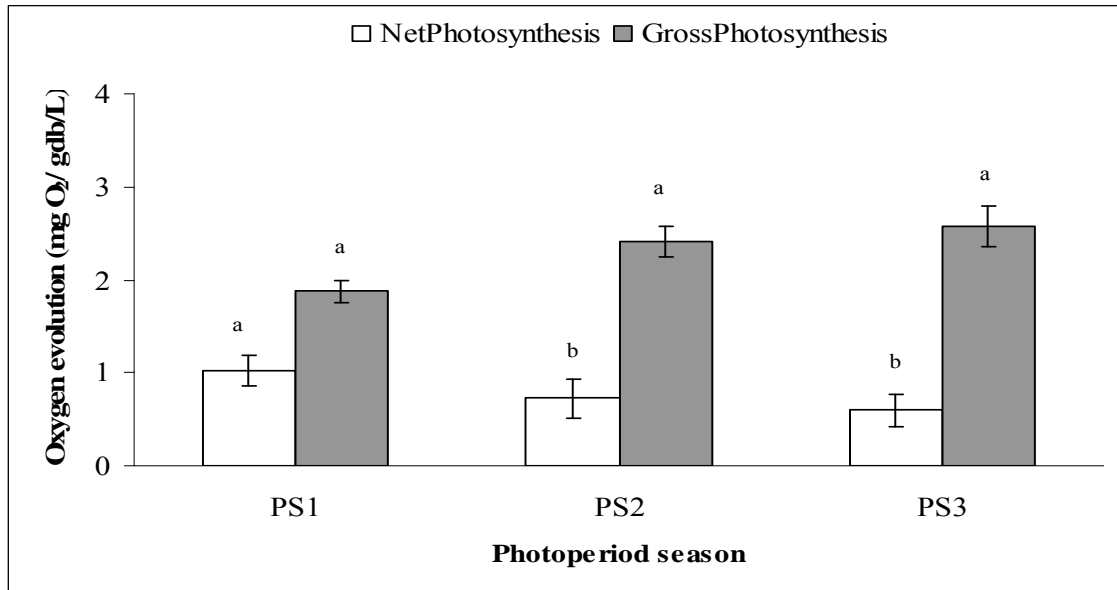
Figure 4.15 Mean net photosynthesis and gross photosynthesis of *Gracilaria cliftonii* based on photoperiod seasons

Error bars represent standard error of the mean

Different letters indicate significant differences among seasons at the level of $p<0.05$

PS1: March to September 2008, PS2: October 2008 to March 2009, PS3: April to August 2009

4.3.7. Life stages

4.3.7.1. Occurrence

4.3.7.1.1. Seasons based on temperature

Over the study period, four life stages were observed; tetrasporophyte, carposporophyte, male gametophyte and vegetative stage.

Occurrence of tetrasporophyte and carposporophyte stages in Autumn and Winter was significantly higher ($p<0.05$) than Spring (Table 4.16). Occurrence of male gametophyte stage was not influenced ($p<0.05$) by any season. Occurrence of vegetative stage in Spring and Summer was significantly higher ($p<0.05$) than Autumn and Winter.

Biomass of tetrasporophyte and carposporophyte stage showed a strong linear correlation ($R^2= 0.99$ and $R^2= 0.94$; $p>0.05$) to the total biomass of *G. cliftonii*.

Table 4.16 Occurrence (%) (Mean ± SE) of the life stages from *Gracilaria cliftonii* from Autumn 2008 to Summer 2009

Season	Tetrasporophyte	Carposporophyte	Male	Vegetative
Autumn	$_1 35.5 \pm 5.2^a$	$_{1,2} 25.6 \pm 3.4^a$	$_2 16.7 \pm 3.8^a$	$_{1,2} 22.3 \pm 4.0^a$
Winter	$_1 29.5 \pm 2.6^a$	$_{1,2} 22.6 \pm 2.1^a$	$_2 21.3 \pm 3.3^a$	$_{1,2} 26.7 \pm 2.4^a$
Spring	$_1 8.1 \pm 2.3^b$	$_1 6.3 \pm 1.7^b$	$_1 16.6 \pm 4.8^a$	$_2 69.0 \pm 7.1^b$
Summer	$_1 25.9 \pm 5.0^a$	$_1 17.7 \pm 4.1^a$	$_1 12.4 \pm 4.6^a$	$_2 44.0 \pm 8.3^c$

Different letters (a,b) indicate significant differences from Autumn 2008 to Summer, while different numbers indicate significant differences between life stages at the level of p<0.05

Occurrence of tetrasporophyte and vegetative stages were dependant on year (Table 4.17. In Autumn of 2008, occurrence of tetrasporophyte was significantly higher (p<0.05, t-test) than Autumn of 2009. In Winter 2009, tetrasporophyte occurrence was significantly higher than Winter 2008 (p<0.05).

Table 4.17 Occurrence of the life stages of *G. cliftonii* in Autumn and Winter 2008 and 2009

Life stage	Autumn 2008	Autumn 2009	Winter 2008	Winter 2009
Tetrasporophyte	$35.5 \pm 5.2_a$	$53.7 \pm 4.7_a$	$29.5 \pm 2.6_a$	$47.6 \pm 6.8_b$
Carposporophyte	$25.6 \pm 3.4_a$	$25.1 \pm 2.2_a$	$22.6 \pm 2.1_a$	$32.5 \pm 3.8_a$
Male gametophyte	$16.7 \pm 3.8_a$	$8.2 \pm 1.3_a$	$21.3 \pm 3.3_a$	$12.3 \pm 5.7_a$
Vegetative	$22.3 \pm 4.0_a$	$13.1 \pm 2.4_b$	$26.7 \pm 2.4_a$	$47.6 \pm 2.2_a$

Different letters (a,b) represent significant differences between same seasons at a level of p<0.05,t-test

4.3.7.1.2. Seasons based on photoperiod

Occurrence from the life stages of *G. cliftonii* also varied among photoperiod seasons (Table 4.18). In photoperiod season PS3, occurrence of tetrasporophyte and carposporophyte stage was significantly higher (p<0.05) than other seasons. In photoperiod season PS1 occurrence of male gametophyte stage was significantly higher (p<0.05) than other season. In photoperiod season PS2 occurrence of vegetative stage was significantly higher (p<0.05) than other seasons.

Table 4.18 Occurrence (%) (Mean ± S.E.) of different life stages from *Gracilaria cliftonii* over three photoperiod seasons

Season	Tetrasporophyte	Carposporophyte	Male	Vegetative
PS1	$_1 29.5 \pm 3.1^a$	$_2 21.0 \pm 1.6^a$	$_2 20.4 \pm 2.5^a$	$_1 29.1 \pm 2.9^a$
PS2	$_1 23.4 \pm 4.4^a$	$_1 14.6 \pm 2.9^a$	$_1 11.1 \pm 0.5^b$	$_2 51.0 \pm 7.2^b$
PS3	$_1 51.0 \pm 4.8^b$	$_2 29.0 \pm 2.7^b$	$_3 10.3 \pm 3.4^b$	$_3 9.8 \pm 2.0^c$

Different letters (a,b) indicate significant differences, while different numbers indicate significant differences between life stages from Autumn to Summer 2008 ($p < 0.05$)

PS1: March to September 2008, PS2: October 2008 to March 2009, PS3: April to August 2009

4.3.7.2. Biomass

4.3.7.2.1. Seasons based on temperature

Biomass of tetrasporophyte stage (1.8 to 23.2 g/m^2), carposporophyte stage (1.8 to 9.3 g/m^2), male gametophyte (1.9 to 8.8 g/m^2) and vegetative stage (4 to 11.1 g/m^2) are shown in Figure 4.16. Biomass of tetrasporophyte and carposporophyte stage in Spring was significantly lower ($p < 0.05$) than rest of the seasons. In addition, tetrasporophyte stage biomass in Autumn and Winter was significantly higher ($p < 0.05$) than Spring and Summer. Biomass of male gametophyte stage in Winter and vegetative stage in Summer was significantly higher ($p < 0.05$) as compared to rest of the seasons.

In Autumn, Winter and Summer, biomass of the tetrasporophyte stage was significantly higher ($p < 0.05$) than other stages. In Spring, biomass of vegetative stage was significantly higher ($p < 0.05$) as compared to rest of the stages.
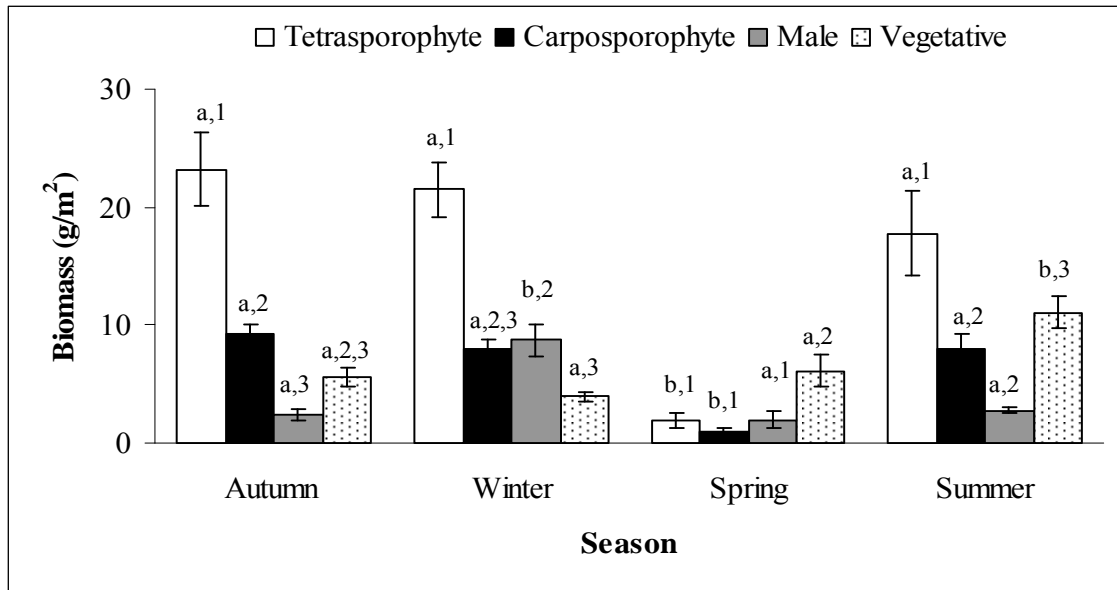
Figure 4.16 Biomass (g/m^2) of life stages from *Gracilaria cliftonii* based on temperature seasons at Point Peron, Western Australia

Error bars represent standard error of the mean

Different letters (a,b) indicate significant differences among seasons, while different numbers indicate significant differences between life stages from Autumn 2008 to Summer at the level of p<0.05

Biomass of the life stages of *G. cliftonii* was influenced by year (Table 4.19). In Winter of 2009, biomass of tetrasporophyte was significantly higher (p<0.05); while biomass of male gametophyte and vegetative stages were significantly lower (p<0.05) than in Winter of 2008.

Table 4.19 Biomass (g/m^2) of life stages from *Gracilaria cliftonii* during Autumn 2009 and Winter 2009

Life stage	Autumn 2008	Autumn 2009	Winter 2008	Winter 2009
Tetrasporophyte	$23.2 \pm 3.2_a$	$25.6 \pm 2.6_a$	$21.5 \pm 2.3_a$	$28.9 \pm 0.8_b$
Carposporophyte	$9.3 \pm 0.8_a$	$8.1 \pm 1.3_a$	$8.0 \pm 0.8_a$	$11.5 \pm 0.6_a$
Male gametophyte	$2.4 \pm 0.5_a$	$1.9 \pm 0.4_a$	$8.8 \pm 1.4_a$	$1.6 \pm 0.4_b$
Vegetative	$5.6 \pm 0.8_a$	$3.4 \pm 0.8_a$	$4.0 \pm 0.4_a$	$2.6 \pm 1.0_b$

Subscript letters (a,b) represent significant differences between same seasons at a level of p<0.05, t-test

4.3.7.2.2.   Seasons based on photoperiod

Biomass of life stages from *G. cliftonii* also varied as a function of photoperiod seasons. Biomass from tetrasporophyte and carposporophyte stages in PS3 season was significantly higher ($p < 0.05$) than other seasons. Biomass from male gametophyte stage in PS1 season was significantly higher ($p < 0.05$) than other seasons. Biomass from vegetative stage in PS2 season was significantly higher ($p < 0.05$) than other seasons (Figure 4.17).

During entire study period biomass from tetrasporophyte stage was significantly higher ($p < 0.05$) than other stages.
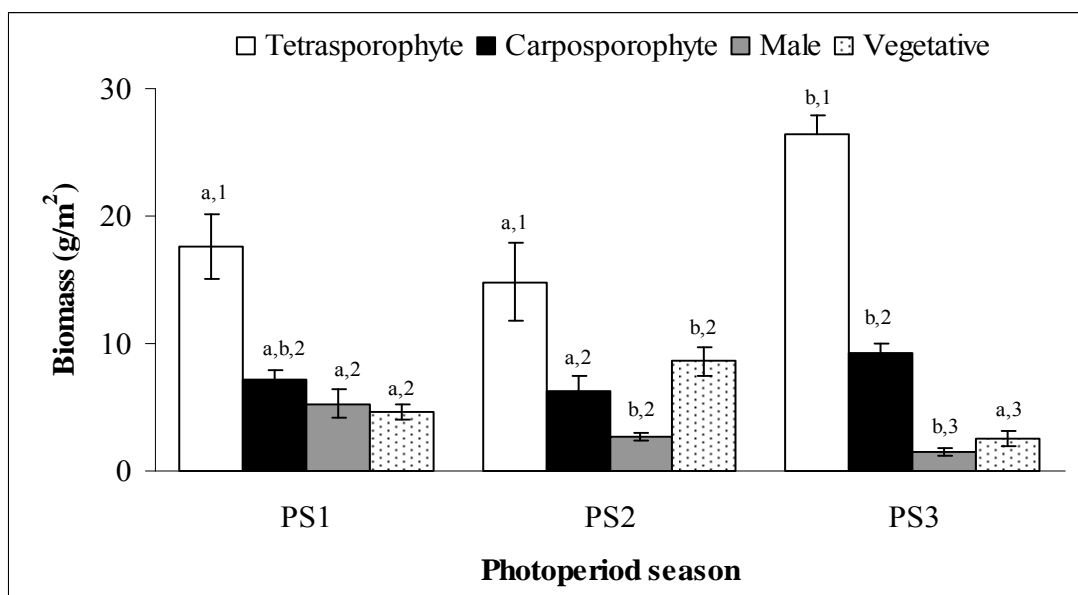
Figure 4.17 Mean biomass from life stages of *Gracilaria cliftonii* during three photoperiod seasons at Point Peron, Western Australia

Error bars represent standard error of the mean

Bars with different letters (a,b) represent significant differences among seasons, while different numbers (1,2,3) represent significant differences among life stages at a level of $p < 0.05$.

PS1: March to September 2008, PS2: October 2008 to March 2009, PS3: April to August 2009

4.3.7.3.   Abundance

4.3.7.3.1.   Seasons based on temperature

Abundance of *G. cliftonii* of tetrasporophyte stage (0.22 to 1.01 nh/m^2), carposporophyte stage (0.15 to 0.63 nh/m^2), male gametophyte (0.25 to 0.56 nh/m^2) and vegetative stage (0.51 to 2.28 nh/m^2) are shown in Table 4.20. Abundance of

tetrasporophyte stage in Autumn and Summer season was significantly higher (p<0.05) than Winter and Spring. Abundance of carposporophyte stage in Spring was significantly lower (p<0.05) than rest of the seasons. Abundance of male gametophyte in Winter was significantly higher (p<0.05) than Summer. Abundance of vegetative stage in Spring and Summer was significantly higher (p<0.05) than Autumn and Winter seasons.

In Autumn and Winter, abundance of tetrasporophyte stage was significantly higher (p<0.05) than carposporophyte and male gametophyte. In Spring and Summer, abundance of vegetative stage was significantly higher (p<0.05) as compared to other stages (Table 4.20).

Table 4.20 Abundance (Mean ± S.E.) (nh/m^2) of life stages from *Gracilaria cliftonii* from Point Peron, Western Australia

Season	Tetrasporophyte	Carposporophyte	Male	Vegetative
Autumn	$_1$1.01 ± 0.09^a	$_2$0.63 ± 0.05^a	$_2$0.32 ± 0.06^{a,b}	$_{1,2}$0.51 ± 0.12^a
Winter	$_1$0.76 ± 0.09^b	$_1$0.57 ± 0.06^a	$_2$0.56 ± 0.12^a	$_{1,2}$0.68 ± 0.08^a
Spring	$_1$0.22 ± 0.06^c	$_1$0.15 ± 0.04^b	$_1$0.43 ± 0.11^{a,b}	$_2$2.23 ± 0.79^b
Summer	$_1$0.93 ± 0.10^{a,c}	$_1$0.55 ± 0.06^a	$_1$0.25 ± 0.04^b	$_2$1.78 ± 0.55^{a,b}

Different letters (a,b,c) represent differences among seasons, while numbers (1,2) represent significant differences between life at a level of p<0.05

In Autumn of 2008, abundance of vegetative was significantly higher (p<0.05, t-test) than Autumn of 2009, while, in Winter of 2008 male gametophyte abundance was significantly higher than Winter of 2009 (Table 4.21).

Table 4.21 Abundance (Mean ± S.E.) (nh/m^2) of different life stages from *Gracilaria cliftonii* during Autumn and Winter of 2008 and 2009

Life stage	Autumn 2008	Autumn 2009	Winter 2008	Winter 2009
Tetrasporophyte	1.01 ± 0.09$_a$	0.93 ± 0.08$_a$	0.76 ± 0.09$_a$	0.89 ± 0.05$_a$
Carposporophyte	0.63 ± 0.05$_a$	0.46 ± 0.07$_a$	0.57 ± 0.06$_a$	0.55 ± 0.05$_a$
Male gametophyte	0.32 ± 0.06$_a$	0.16 ± 0.03$_a$	0.56 ± 0.12$_a$	0.13 ± 0.03$_b$
Vegetative	0.51 ± 0.12$_a$	0.24 ± 0.05$_b$	0.68 ± 0.08$_a$	0.14 ± 0.04$_a$

Different letters (a,b) represent significant differences between same seasons at a level of p<0.05,t-test

4.3.7.3.2. Seasons based on photoperiod

Abundance of tetrasporophyte and carposporophyte stages were not influenced ($p > 0.05$) by the seasons based on photoperiod, whereas, abundance of male gametophyte in PS1 season was significantly higher ($p < 0.05$) than other photoperiod seasons. In photoperiod season PS2 abundance of vegetative stage was significantly higher ($p < 0.05$) than other seasons (Table 4.22).

Biomass and abundance were found to be dependant on $[NO_3^-]$ and $[NO_2^-]$, while male biomass was dependant ($p < 0.05$) on rainfall and osmolality.

Table 4.22 Abundance (Mean ± SE) (nh/m^2) of life stages from *Gracilaria cliftonii* based of Seasons based on photoperiod at Point Peron, Western Australia

Season	Tetrasporophyte	Carposporophyte	Male	Vegetative
PS1	$_117.6 \pm 2.5^a$	$_27.2 \pm 0.8^{a,b}$	$_25.3 \pm 1.1^a$	$_24.6 \pm 0.5^a$
PS2	$_114.8 \pm 3.1^a$	$_26.2 \pm 1.3^a$	$_22.7 \pm 0.4^b$	$_28.6 \pm 1.1^b$
PS3	$_126.5 \pm 1.4^b$	$_29.2 \pm 0.8^b$	$_31.5 \pm 0.3^b$	$_32.5 \pm 0.7^a$

Different letters (a,b) represent differences among seasons, while numbers (1,2,3) represent differences between life stages at $p < 0.05$

PS1: March to September 2008, PS2: October 2008 to March 2009, PS3: April to August 2009

4.3.7.4. Thallus length

4.3.7.4.1. Seasons based on temperature

Thallus length of tetrasporophyte and carposporophyte stage in Spring was significantly shorter ($p < 0.05$) while, thallus length of male gametophyte in Winter was significantly longer ($p < 0.05$) than other seasons (Table 4.23). Thallus length of vegetative stage in Summer was significantly longer ($p < 0.05$) as compared to rest of the seasons.

In Autumn, Spring and Summer thallus length of tetrasporophyte and carposporophyte was significantly longer ($p < 0.05$) than male gametophyte and vegetative stages (Table 4.23).

Table 4.23 Thallus length (Mean ± SE) (cm) of life stages from *Gracilaria cliftonii* based on seasons based on temperature at Point Peron, Western Australia

Season	Tetrasporophyte	Carposporophyte	Male	Vegetative
Autumn	$_1 20.0 \pm 1.1^a$	$_1 19.5 \pm 0.9^a$	$_2 14.1 \pm 1.1^a$	$_2 13.5 \pm 1.0^a$
Winter	$_1 21.0 \pm 0.9^a$	$_2 18.1 \pm 0.8^a$	$_2 18.0 \pm 0.6^b$	$_3 12.7 \pm 0.6^{a,b}$
Spring	$_1 16.4 \pm 0.6^b$	$_{1,2} 14.0 \pm 0.8^b$	$_2 13.4 \pm 1.5^a$	$_3 10.3 \pm 1.0^b$
Summer	$_1 20.9 \pm 2.0^a$	$_1 19.4 \pm 1.6^a$	$_2 12.9 \pm 1.6^a$	$_2 14.6 \pm 1.0^a$

Different letters (a,b) represent differences among seasons, while numbers (1,2,3) represent differences between life stages at the level of $p < 0.05$

Thallus length of the carposporophyte stage was influenced by year, in Autumn of 2009 thallus length was significantly longer ($p < 0.05$) than Autumn of 2008 (Table 4.24).

Table 4.24 Thallus length (Mean ± SE) (cm) of life stages from *Gracilaria cliftonii* during Autumn of 2009 and Winter of 2009

Life stage	Autumn 2008	Autumn 2009	Winter 2008	Winter 2009
Tetrasporophyte	$20.0 \pm 1.1_a$	$23.3 \pm 2.1_a$	$21.0 \pm 0.9_a$	$21.0 \pm 0.8_a$
Carposporophyte	$19.5 \pm 0.9_a$	$21.9 \pm 2.0_b$	$18.1 \pm 0.8_a$	$20.0 \pm 0.8_a$
Male	$14.1 \pm 1.1_a$	$14.1 \pm 1.7_a$	$18.0 \pm 0.6_a$	$13.2 \pm 0.8_a$
Vegetative	$13.5 \pm 1.0_a$	$13.6 \pm 0.9_a$	$12.7 \pm 0.6_a$	$12.6 \pm 0.6_a$

Different letters (a,b) represent significant differences between same seasons at a level of $p < 0.05$, t-test

4.3.7.4.2.   Seasons based on photoperiod

Thallus length of tetrasporophyte and male gametophyte was not influenced by the photoperiod seasons ($p > 0.05$). Thallus length of carposporophyte in PS3 season was significantly longer ($p < 0.05$) than other seasons. Thallus length of vegetative stage in PS2 was significantly longer ($p < 0.05$) than PS1 season.

During all photoperiod seasons plant length of tetrasporophyte was significantly longer ($p < 0.05$) than other stages (Figure 4.18).
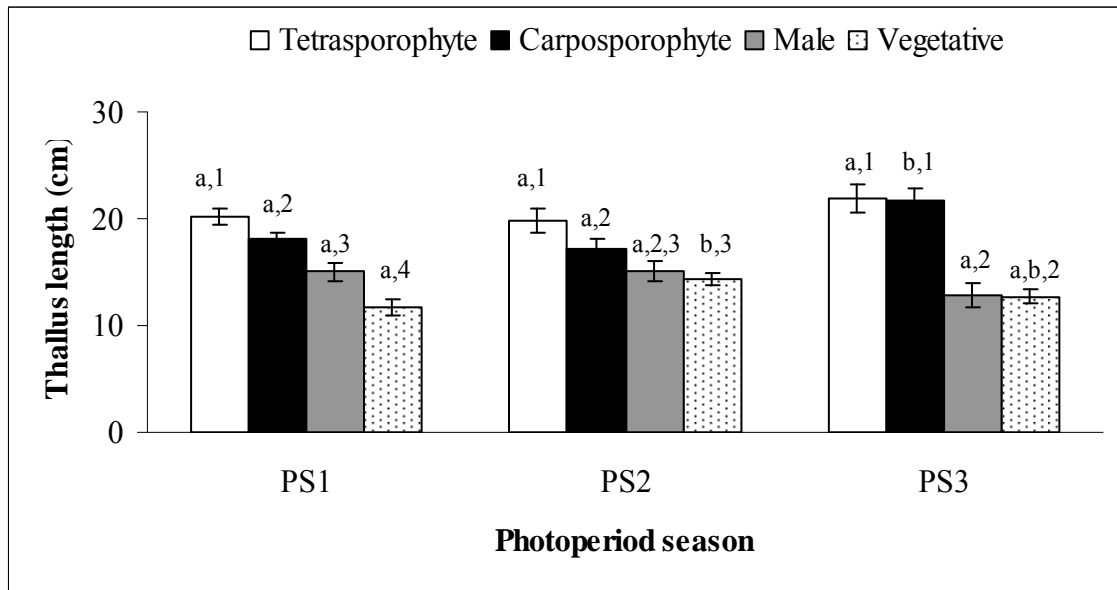
Figure 4.18 Mean thallus length (cm) of life stages from *Gracilaria cliftonii* over three photoperiod seasons at Point Peron, Western Australia

Error bars represent the standard error of the mean

Different letters (a,b) represent differences among seasons, while numbers (1,2,3) represent differences between life stages at p<0.05

PS1: March to September 2008, PS2: October 2008 to March 2009, PS3: April to August 2009

4.4. EPIPHYTISM

4.4.1. Epiphytic biomass

Epiphytic biomass of *G. cliftonii* varied from 3.1 to 11.2 g/m^2 as shown in Figure 4.19. Epiphytic biomass in Winter was significantly higher (p<0.05) than in Autumn and Spring season. Epiphytic biomass showed strong correlation (R^2= 0.89 and R^2= 0.97; p<0.05) with biomass of tetrasporophyte and carposporophyte stages.
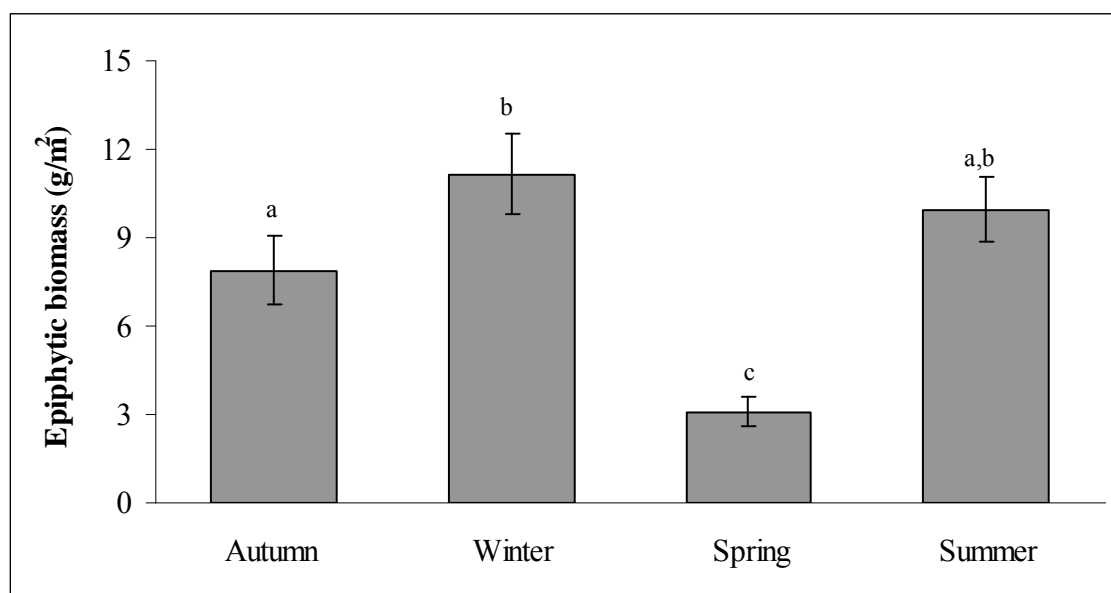
Figure 4.19 Epiphytic biomass (Mean ± SE) of *Gracilaria cliftonii* based on temperature seasons at Point Peron, Western Australia

Error bars represent standard error of the mean

Bars with different letters (a,b) represent significant differences among seasons from Autumn 2008 to Summer; different number indicate significant differences between same season (p<0.05)

Epiphytic biomass and loads of *G. cliftonii* were not influenced by year (Table 4.25).

Table 4.25 Epiphytic biomass and loads (Mean ± SE) of *G. cliftonii* during Autumn of 2009 and Winter of 2009

Parameter	Autumn 2008	Autumn 2009	Winter 2008	Winter 2009
Epiphytic biomass (g/m²)	$7.9 \pm 1.2_a$	$10.7 \pm 0.8_a$	$11.2 \pm 1.4_a$	$11.2 \pm 0.8_a$
Epiphytic load (%)	$20.5 \pm 3.0_a$	$28.0 \pm 2.4_a$	$26.4 \pm 2.7_a$	$25.4 \pm 1.7_a$

Different letters (a,b) represent significant differences between same seasons at a level of p<0.05,t-test

4.4.2. Epiphytic load

Epiphytic load of *G. cliftonii* varied from 20.5 to 39.8 % (Figure 4.20). Epiphytic load in Spring was significantly higher (p<0.05) than in Autumn. Epiphytic load showed a strong negative correlation (R^2= 0.96; p<0.05) with total biomass of *G. cliftonii*.
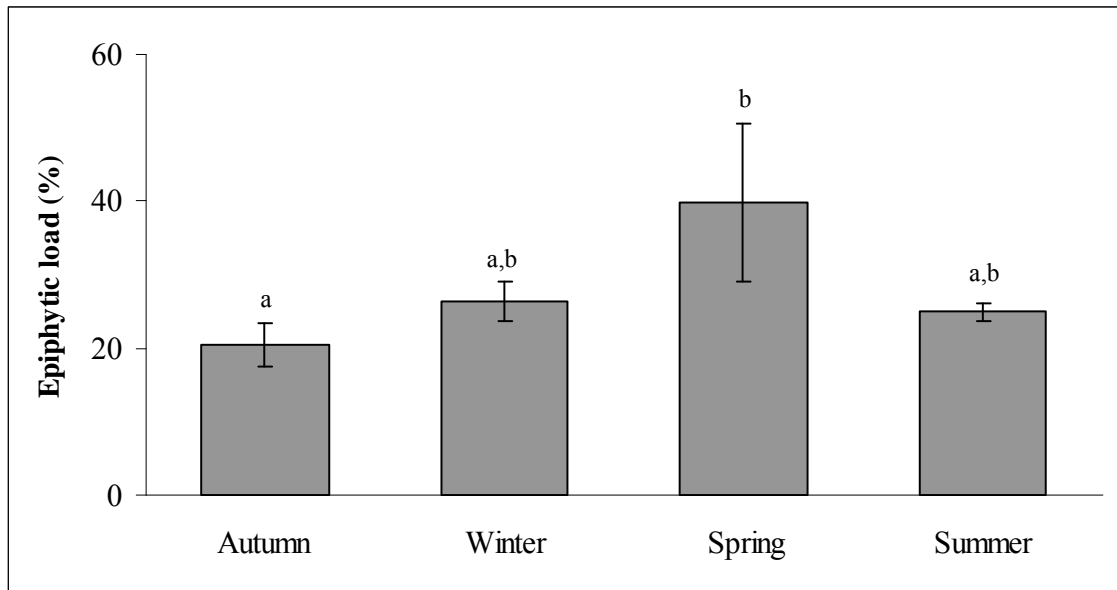
Figure 4.20 Epiphytic load (Mean ± SE) of *Gracilaria cliftonii* based on temperature seasons at Point Peron, Western Australia

Error bars indicate standard error of the mean

Bars with different letters (a,b) represent significant differences among seasons (p<0.05)

4.4.3. Epiphytic composition

Twenty four macroalgae species were recorded as *G. cliftonii* epiphytes during the study period (Table 4.10). Twenty one species belonged to the Rhodophyta and 3 to the Chlorophyta divisions. The Ceramiales was the most dominant group with four *Ceramium* species (*C. puberulum, C. pusillum, C. isogonum and C. minuta*) and two *Polysiphonia* species (*P. forfex* and *P. spinosissima*). However, most common species observed were *Hypnea episcopalis* and *Polysiphonia forfex* which were present most of the seasons except in Autumn 2009 and Winter 2009 respectively. *H. episcopalis* and *P. forfex* occurred more frequently (p = 0.004) than rest of the epiphyte species (Table 4.10). Winter and Summer were the seasons when higher number of epiphytes was recorded, while Spring 2008 was the season with less number of epiphytes observed (Table 4.26).

Table 4.26 Seasonal epiphytic incidence of *G. cliftonii* epiphytes at Point Peron, Western Australia indicating presence (1) and absence (0) of epiphytes from Autumn 2008 to Winter 2009.

Epiphyte	2008				2009	
	AUT	WIN	SPR	SUM	AUT	WIN
Rhodophyta						
Hypnea episcopalis	1	1	1	1	0	1
Ceramium puberelum	1	1	1	0	0	1
Ceramium pusillum	1	1	1	1	0	0
Ceramium isogonum	1	0	0	1	0	0
Ceramium minuta	1	1	1	0	0	1
Polysiphonia forfex	1	1	1	1	0	1
Polysiphonia spinosissima	0	1	1	0	1	0
Laurencia clavata	1	1	0	1	0	0
Leveillea jungermannioides	1	1	0	1	0	0
Dasyclonium incisum	0	0	1	1	1	0
Echinotamnion hystrix	0	0	0	0	1	0
Ptilota hannafordi	0	0	0	0	1	0
Champia parvula	0	0	0	1	0	1
Wrangellia spp.	0	0	0	0	1	0
Dasya haffiae	0	0	0	0	1	0
Mazoyerella australis	0	0	0	0	0	1
Callithamnion spp.	0	0	0	0	1	0
Griffithsia sp	0	0	0	0	0	0
Griffithsia ovalis	0	0	0	1	0	1
Helminthocladia australis	0	0	0	0	0	0
Plocamium mertensii	0	0	0	0	1	0
Chlorophyta						
Ulva lactuca	1	1	0	0	0	1
Bryopsis plumosa	0	1	0	1	0	0
Cladophora	0	1	0	1	0	0

4.4.4. Characterization of attachment *Gracilaria*-epiphyte

The epiphytes such as *Polysiphonia* spp. and *Hypnea episcopalis* were growing on *G. cliftonii* covering more than 50% of the thallus (Plate 4.11).
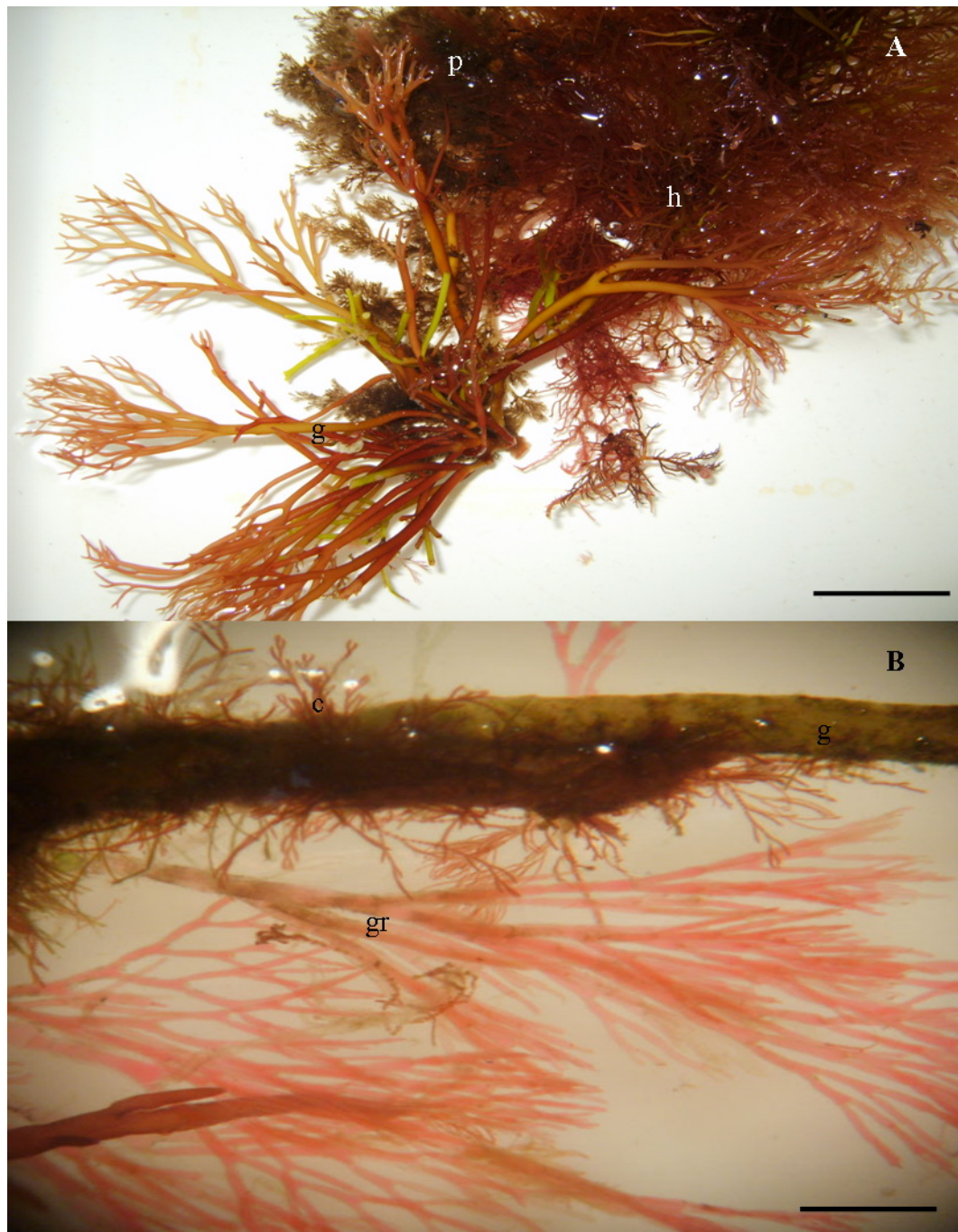
Plate 4.11 Epiphytism of *G. cliftonii*

A. *Polysiphonia* spp. (p) and *Hypnea episcopalis* (h) entangled with *G. cliftonii* thallus (g). Scale bar: 4 cm. B. *Ceramium* spp. (c) and *Griffithsia* spp. (gr) growing on the surface of *G. cliftonii* (g). Scale bar: 0.5 cm.

The epiphytes like *Ulva lactuca* (Plate 4.12A) grew individually on basal portions of *G. cliftonii*, while epiphytes like *Polysiphonia* spp. and *M. australis* (Plate 4.12B) where observed growing together. *Helminthocladia australis, Polysiphonia* spp., *Ulva lactuca* were observed arising from wounded areas of *G. cliftonii* cortex (Plate 4.12C), while *H. episcopalis* and *B. plumosa* where entangled to *G. cliftonii* thallus (Plate 4.12D).
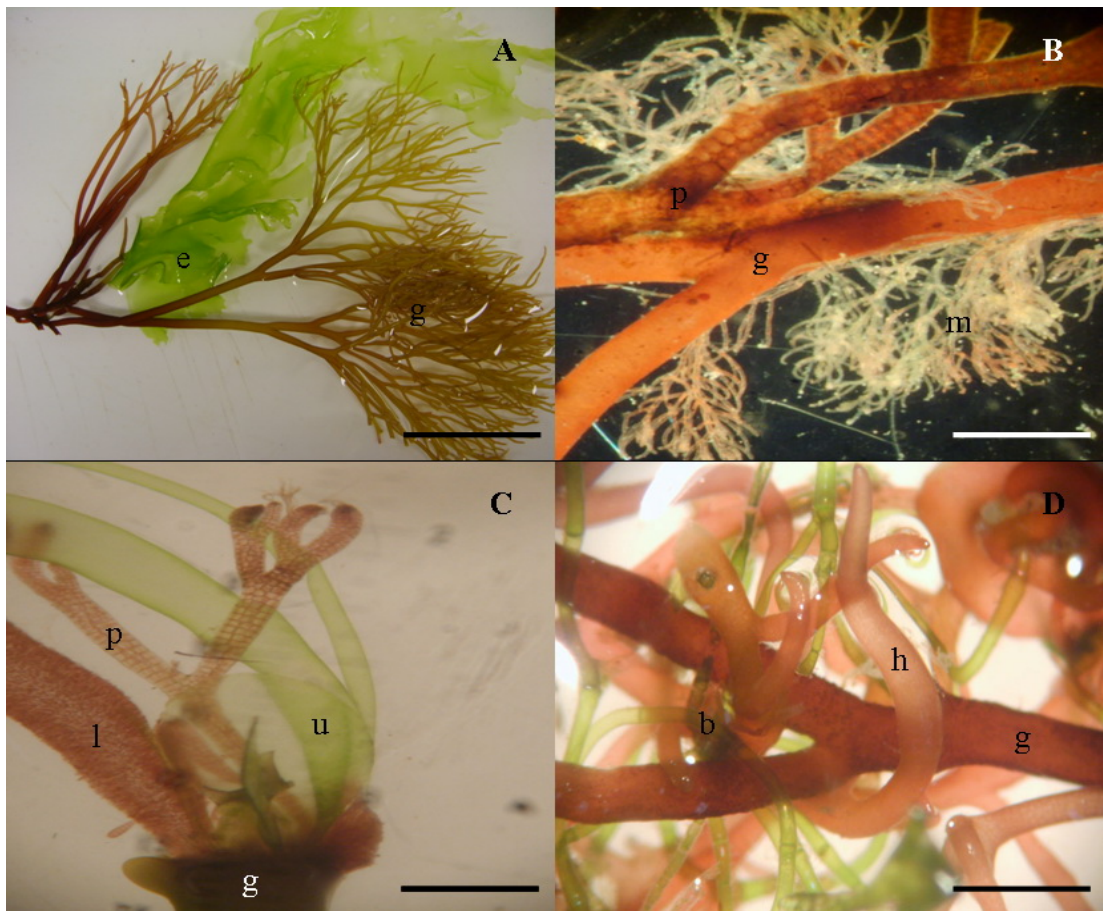
Plate 4.12 Epiphytes of *G. cliftonii*

A. *Ulva lactuca* attached to the basal portion of *G. cliftonii* thallus. Scale bar: 3 cm. B. *Mazoyerella australis* (m) and *Polysiphonia* spp. (p) growing on *G. cliftonii* thallus (g). Scale bar: 1 cm. C. *Helminthocladia australis* (l), *Polysiphonia* spp. (p) and *Ulva* spp. (u) growing on the wounded portion of an apical branch (g). Scale bar: 0.5 cm. D. *Hypnea episcopalis* (h) and *Bryopsis plumosa* (b) entangled with *G. cliftonii* thallus (g). Scale bar: 1 cm.

Different types of attachment were observed between epiphytes and *G. cliftonii*. *H. episcopalis* was anchored to *G. cliftonii* through the apical hooks (Plate 4.13A), however, it was easily removed by hand without any superficial damage. *Leveillea jungermannioides* (Plate 4.13B), *Champia parvula* (Plate 4.13C), *Dasya haffiae* (4.13D) were observed as an erect thallus arising from the surface of *G. cliftonii* and were harder to remove from the host.
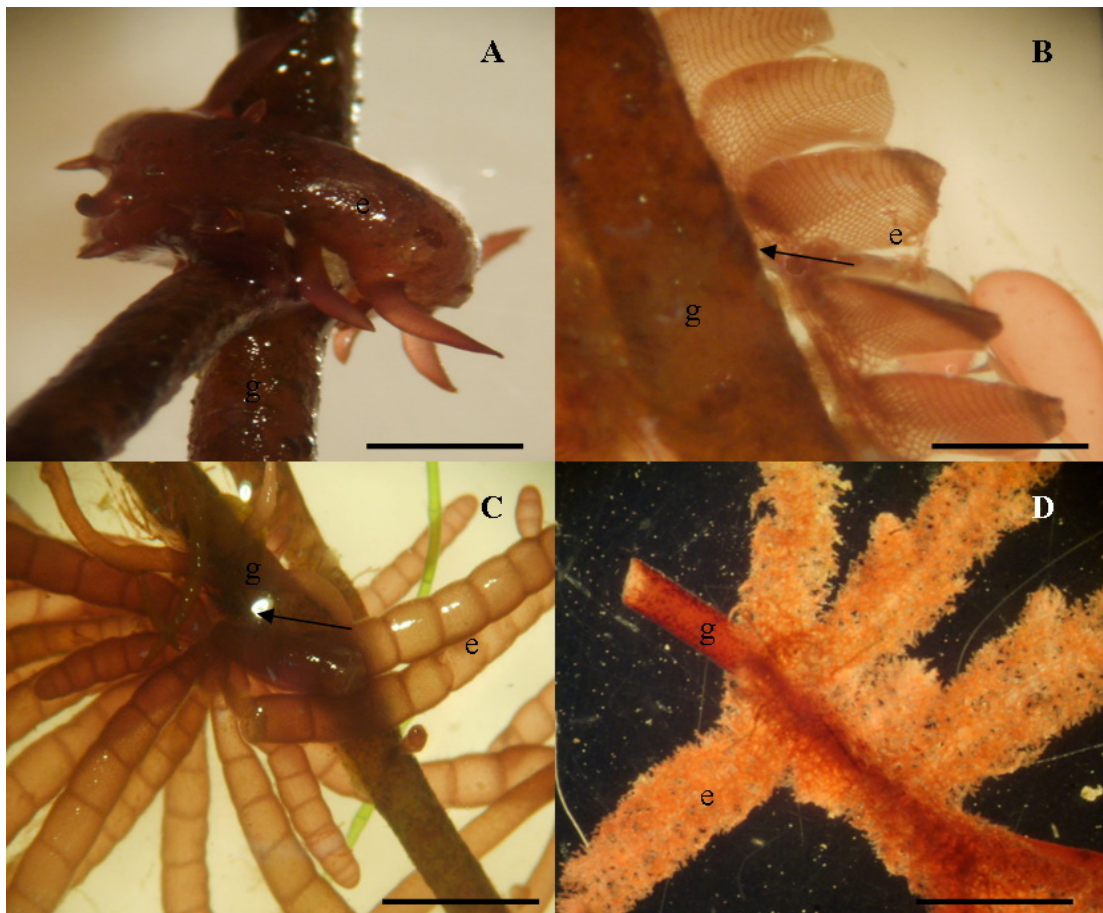
Plate 4.13 Sites of epiphyte attachment to *G. cliftonii* I
A. *Hypnea episcopalis* hooks anchored to *G. cliftonii*. Scale bar: 5 mm. B. *Leveillea jungermannioides*. Scale bar: 0.5 cm. C. *Champia parvula*. Scale bar: 1 cm. D. *Dasya haffiae*. Scale bar: 1 cm. The arrow indicates the site of attachment. Abbreviations: e-epiphyte, g-*Gracilaria.*

The areas of attachment of *Dasyclonium incisum* (Plate 4.14A), *Laurencia clavata* (Plate 4.14B), *Ceramium minuta* (Plate 4.14C) and *Griffithsia ovalis* (Plate 4.14D) were easily distinguished under the binocular microscope.
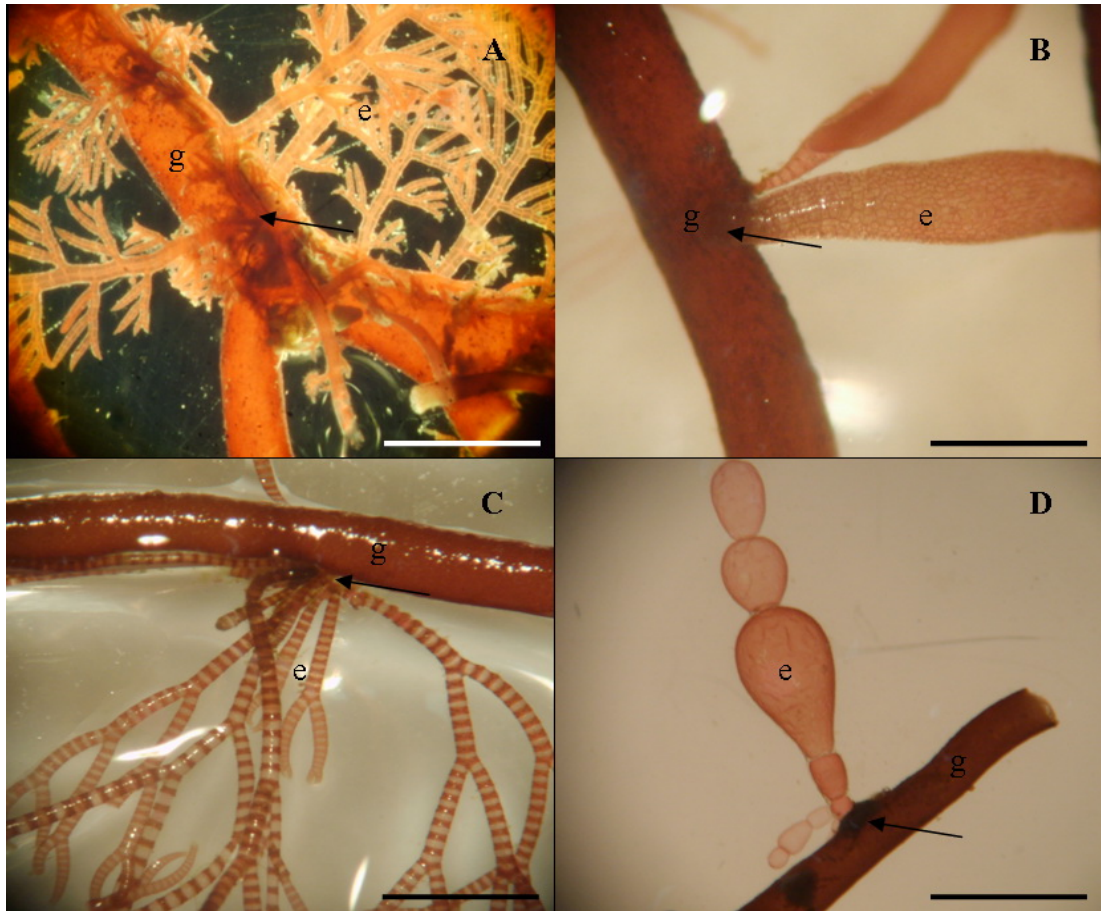
Plate 4.14 Sites of epiphyte attachment to *G. cliftonii* II
A. *Dasyclonium incisum.* Scale bar: 1 cm. B. *Laurencia clavata.* Scale bar: 2 mm. C. *Ceramium minuta.* Scale bar: 1 cm. D. *Griffithsia ovalis.* Scale bar 2 mm. The arrow indicates the site of attachment. Abbreviations: e-epiphyte, g-*Gracilaria*

Most of the cases *P. forfex* was covering more than 50 % of *G. cliftonii* thallus (Plate 4.15A) and was arising as an erect thallus (Plate 4.15B) with branches hooked around the branches of the host (Plate 4.15C). The epiphytes of *G. cliftonii* had an even distribution over its thallus but five species deviated from this pattern *P. forfex, C. parvula, L. clavata, M. australis* (Plate 4.15D) and *L. jungermannioides. P. forfex* and *M. australis* were found growing mainly on the apical tips of *G. cliftonii*, while *C. parvula, L. clavata, Ulva lactuca* and *L. jungermannioides* were observed mostly in the basal portions of the thallus.
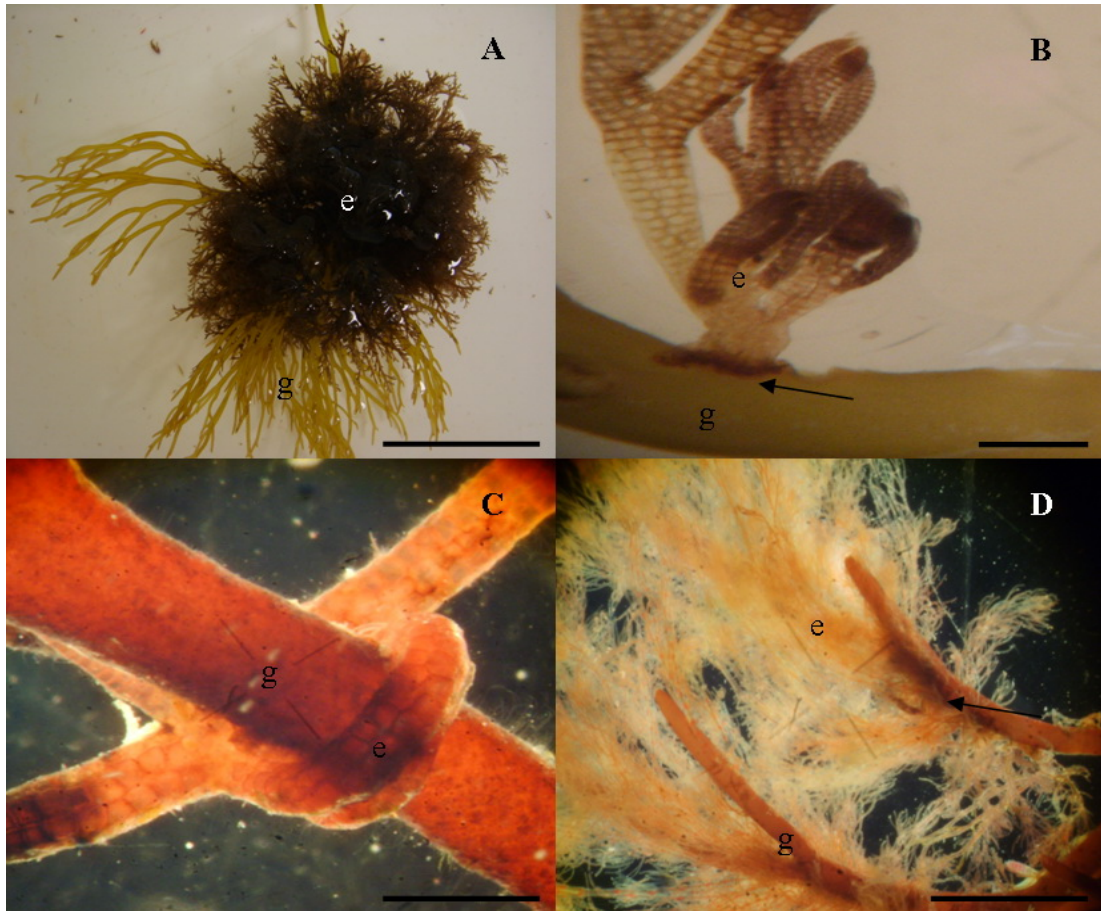
Plate 4.15 Sites of epiphyte attachment to *G. cliftonii* III

A. *Polysiphonia forfex* covering *G. cliftonii* thallus (g). Scale bar: 2 cm. B. *P. forfex* attached to *G. cliftonii* thallus. Scale bar 1 mm. C. *P. forfex* branch hooked around *G. cliftonii* thallus. Scale bar: 0.5 cm. D. *Mazoyerella australis* growing on the apical branches of *G. cliftonii*. The arrow indicates the site of attachment. Abbreviations: e-epiphyte, g-*Gracilaria*.

4.4.5. Light microscopy

Light microscopy revealed three types of attachment for *Gracilaria* to its epiphytes (Plate 4.16). First, epiphytes such as *M. australis* (Plate 4.16A) and *L. jungermannioides* (Plate 4.16B) were growing only on the surface without penetration into the host's cortex. No alteration of the host cortical cells was observed (2-4 cells in non infected thalli). Second type of attachment was characterised by alteration of the cellular structure at the attaching site. Cellular disorganisation of the cortical cell layer at the area of attachment was observed with *B. plumosa* (Plate 4.15C). The host cortical cells were elongated and were characterised with a high content of polysaccharides around the area. Similarly, the

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site of attachment with *L. clavata* (Plate 4.15D) presented 6-8 cortical cells layers comparing with 2-4 cortical cells in the non infected areas.

The third type was penetration of epiphyte into the host tissue and was observed with *C. isogonum*. *C. isogonum* was found growing along *G. cliftonii* surface anchoring in those areas with presence of cleavages (Plate 4.15E) and attaching to the host through rhizoids. The rhizoids penetrated the cortex up to the outer medullary cells. The epiphyte rhizoid wall was clearly defined and the penetration area was characterised with a high content of polysaccharides (Plate 4.15F).

4.4.6. Scanning electron microscopy

Scanning electron microscopy revealed penetration of *P. forfex* (Plate 4.16A) and *C. isogonum* into *G. cliftonii* tissue (Plate 4.16B) thus confirming the histological results. *C. isogonum* was attached to *G. cliftonii* through *adhesive pad* like structures (Plate 4.16C). The sites of attachment were also characterised by the presence of diatoms and/or lacunae on *G. cliftonii* surface (Plate 4.16D-E). Epiphytes were also observed arising from damaged areas from the surface of *G. cliftonii* (Plate 4.16F)
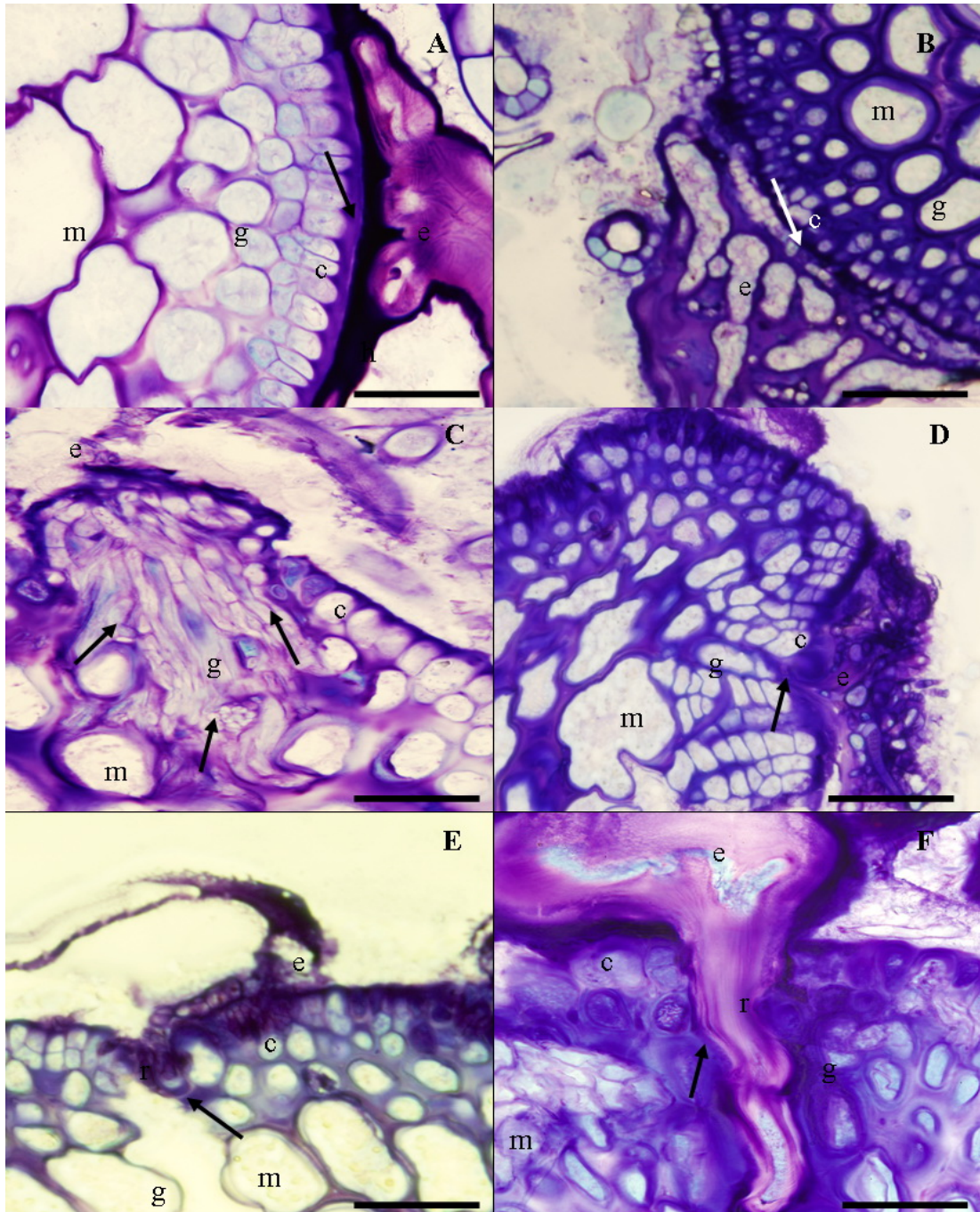
Plate 4.16 Light microscopy studies of the sites of attachment between *G. cliftonii* and different epiphytes

A. *Mazoyerella australis* growing on *G. cliftonii* surface. Scale bar 50 μ. B. *Leveillea jungermannioides* growing on *G. cliftonii* surface without penetration showing 3-4 cells cortical layer. Scale bar 100 μ. C. Elongated cortical cells (arrows) at the site of attachment with *Bryopsis plumosa*. Scale bar 50 μ. D. *G. cliftonii* cortex consisting of 6-7 cells (arrow) at the site of attachment with epiphyte *Laurencia clavata*. Scale bar 50 μ. E. *Ceramium isogonum* growing into *G. cliftonii* surface cleavages. Scale bar 200 μ. F. *C. isogonum* rhizoid penetrating into the host cortex and outer medulla. Scale bar 50 μ.

Plate 4.17 Scanning electron micrographs showing damage and penetration by epiphytes to *G. cliftonii*

A. Penetration by *Polysiphonia forfex* with presence of diatoms at the site of penetration. Scale bar 10 μ. B. Penetration by *Ceramium isogonum*. Scale bar 10 μ. C. *C. isogonum* attached to *G. cliftonii* through rhizoids. Scale bar: 50 μ. D-E Lacunae on *G. cliftonii* surface observed under the presence of *C. isogonum.* Scale bar 10 μ (D) and 50 μ (E). F. Damage of surface of *G. cliftonii* by its epiphytes. Scale bar: 50 μ. Arrow indicates the site of penetration or damage to the surface of *G. cliftonii.*

4.5. CHEMICAL PROPERTIES

4.5.1. Moisture

4.5.1.1. Seasons based on temperature

Moisture content of tetrasporophyte and carposporophyte stage in Autumn was significantly higher ($p<0.05$) than Spring and Summer. Moisture content of vegetative stage in Summer was significantly higher ($p<0.05$) than Winter and Spring.

In Autumn and Winter, moisture content of tetrasporophyte and carposporophyte was significantly higher ($p<0.05$) than male and vegetative stage (Table 4.27)

Table 4.27 Moisture (% db) (Mean ± S.E.) life stages of *Gracilaria cliftonii* for temperature seasons

Season	Tetrasporophyte	Carposporophyte	Male	Vegetative
Autumn	$_186.7 \pm 0.5^a$	$_186.1 \pm 0.7^a$	$_284.0 \pm 0.7^a$	$_{1,2}84.9 \pm 0.6^{a,b}$
Winter	$_186.3 \pm 0.7^a$	$_{1,2}84.6 \pm 0.3^{a,b}$	$_284.1 \pm 0.9^a$	$_284.1 \pm 0.8^a$
Spring	$_183.9 \pm 0.2^b$	$_182.7 \pm 0.9^b$	$_183.3 \pm 0.7^a$	$_182.2 \pm 0.7^c$
Summer	$_{1,2}84.1 \pm 1.0^b$	$_183.6 \pm 1.0^b$	$_{1,2}84.9 \pm 0.7^a$	$_286.1 \pm 0.5^b$

*Different letters (a,b) represent significant differences between seasons, while, different numbers (1,2) represent significant differences between life stages ($p<0.05$)

Moisture content of different life stages of *G. cliftonii* was not influenced by year (Table 4.28).

Table 4.28 Moisture (% db) (Mean ± S.E.) life stages of *Gracilaria cliftonii* in Autumn and Winter 2009

Life stage	Autumn 2009	Autumn 2009	Winter 2009	Winter 2009
Tetrasporophyte	$86.7 \pm 0.5_a$	$82.2 \pm 0.9_a$	$86.3 \pm 0.7_a$	$83.2 \pm 0.7_a$
Carposporophyte	$86.1 \pm 0.7_a$	$82.2 \pm 0.9_a$	$84.6 \pm 0.3_a$	$83.2 \pm 0.7_a$
Male gametophyte	$84.0 \pm 0.7_a$	$80.6 \pm 1.6_a$	$84.1 \pm 0.9_a$	$84.7 \pm 0.9_a$
Vegetative	$84.9 \pm 0.6_a$	$81.3 \pm 0.3_a$	$84.1 \pm 0.8_a$	$82.1 \pm 0.9_a$

Different letters (a,b) represent significant differences between same seasons at a level of $p<0.05$, t-test

4.5.1.2.   Seasons based on photoperiod

Moisture of life stages from *G. cliftonii* was also affected by seasons based on photoperiod. Moisture content of tetrasporophyte, carposporophyte and vegetative stages in PS1 were significantly higher ($p < 0.05$) than PS3 season.

In PS1 season, moisture content of tetrasporophyte was significantly higher ($p < 0.05$) than rest of the stages (Table 4.29).

Table 4.29 Moisture (% db) (Mean ± S.E.) life stages of *Gracilaria cliftonii* for photoperiod seasons

Season	Tetrasporophyte	Carposporophyte	Male	Vegetative
PS1	$_186.0 \pm 0.4^a$	$_284.3 \pm 0.5^a$	$_283.9 \pm 0.5^a$	$_283.7 \pm 0.5^a$
PS2	$_184.1 \pm 0.5^b$	$_183.5 \pm 0.7^a$	$_182.8 \pm 1.0^a$	$_183.9 \pm 0.7^a$
PS3	$_{1,2}82.3 \pm 0.6^c$	$_181.8 \pm 0.4^b$	$_283.8 \pm 0.7^a$	$_181.9 \pm 0.5^b$

Different letters (a,b) represent differences among seasons, while numbers (1,2) represent differences between life stages at $p < 0.05$

PS1: March to September 2008, PS2: October 2008 to March 2009, PS3: April to August 2009

4.5.2. Ash

4.5.2.1.   Seasons based on temperature

Ash content of tetrasporophyte stage in Summer was significantly lower ($p < 0.05$) than other seasons while ash content of carposporophyte and male gametophyte stages was not influenced ($p > 0.05$) by any season.

In Spring, ash content of vegetative stage was significantly higher ($p < 0.05$) than Summer and Winter.

Ash content of tetrasporophyte in Autumn and Winter was significantly higher ($p < 0.05$) than male gametophyte and vegetative stages (Table 4.30).

Table 4.30 Ash (% db) (Mean ± S.E.) life stages of *Gracilaria cliftonii* for temperature seasons

Season	Tetrasporophyte	Carposporophyte	Male	Vegetative
Autumn	$_126.0 \pm 0.8^a$	$_{1,2}23.7 \pm 1.0^a$	$_222.2 \pm 1.2^a$	$_222.7 \pm 1.1^{a,b}$
Winter	$_126.6 \pm 1.2^a$	$_222.7 \pm 1.0^a$	$_222.3 \pm 0.4^a$	$_221.8 \pm 0.5^a$
Spring	$_125.0 \pm 1.2^a$	$_122.7 \pm 0.9^a$	$_124.5 \pm 1.2^a$	$_125.0 \pm 0.9^b$
Summer	$_121.3 \pm 0.8^b$	$_121.2 \pm 0.8^a$	$_122.0 \pm 1.3^a$	$_121.1 \pm 0.8^a$

Different letters (a,b) represent significant differences between seasons, while, different numbers (1,2) represent significant differences between life stages (p<0.05)

Ash content of carposporophyte was influenced by year (Table 4.31). In Autumn of 2008, ash content of carposporophyte was significantly higher (p<0.05) than in of Autumn 2009.

Table 4.31 Ash (% db) (Mean ± S.E.) life stages of *Gracilaria cliftonii* during Autumn 2009 and Winter 2009

Life stage	Autumn 2008	Autumn 2009	Winter 2008	Winter 2009
Tetrasporophyte	$26.0 \pm 0.8_a$	$21.7 \pm 0.6_a$	$26.6 \pm 1.2_a$	$22.6 \pm 1.2_a$
Carposporophyte	$23.7 \pm 1.0_a$	$21.7 \pm 0.6_b$	$22.7 \pm 1.0_a$	$22.6 \pm 1.2_a$
Male gametophyte	$22.2 \pm 1.2_a$	$23.8 \pm 1.0_a$	$22.3 \pm 0.4_a$	$22.2 \pm 1.3_a$
Vegetative	$22.7 \pm 1.1_a$	$22.9 \pm 1.1_a$	$21.8 \pm 0.5_a$	$25.5 \pm 0.9_a$

Different letters (a,b) represent significant differences between same seasons at a level of p<0.05,t-test

4.5.2.2.   Seasons based on photoperiod

In addition, ash content of different life stages from *G. cliftonii* were influenced by seasons based on photoperiod (Table 4.32). Ash content of tetrasporophyte stage in PS1 was significantly higher (p<0.05) than PS3 season. Ash of vegetative stage in PS3 season was significantly higher (p<0.05) than other seasons.

In PS1 and PS3 seasons, ash of tetrasporophyte stage was significantly higher (p<0.05) than vegetative stage (Table 4.32).

Table 4.32 Ash content (% db) (Mean ± SE) among photoperiod seasons and life stages of *Gracilaria cliftonii* from Point Peron, Western Australia

Season	Tetrasporophyte	Carposporophyte	Male	Vegetative
PS1	$_1 25.3 \pm 0.8^a$	$_2 22.4 \pm 0.6^a$	$_2 21.8 \pm 0.3^a$	$_2 21.9 \pm 0.4^a$
PS2	$_1 23.6 \pm 0.8^{a,b}$	$_1 23.5 \pm 0.9^a$	$_1 23.6 \pm 1.0^a$	$_1 22.6 \pm 0.8^a$
PS3	$_1 21.8 \pm 0.8^b$	$_{1,2} 23.4 \pm 1.0^a$	$_{1,2} 22.6 \pm 0.9^a$	$_2 24.7 \pm 0.8^b$

Different letters (a,b) represent differences among seasons, while numbers (1,2) represent differences between life stages at p<0.05

PS1: March to September 2008, PS2: October 2008 to March 2009, PS3: April to August 2009

4.5.3. Protein

4.5.3.1.   Seasons based on temperature

Protein content of tetrasporophyte, male gametophyte and vegetative stages were significantly higher (p<0.05) in Winter than Summer.

In Winter, protein content of male gametophyte was significantly higher (p<0.05) than carposporophyte stage. In Spring, protein content of tetrasporophyte and carposporophyte stages was significantly higher (p<0.05) than male and vegetative stages (Table 4. 33).

Table 4.33 Protein (% db) (Mean ± S.E.) life stages of *Gracilaria cliftonii* for temperature seasons

Season	Tetrasporophyte	Carposporophyte	Male	Vegetative
Autumn	$_1 10.8 \pm 0.2^a$	$_1 11.1 \pm 0.5^a$	$_1 11.6 \pm 0.4^{a,b}$	$_1 11.7 \pm 0.4^{a,b}$
Winter	$_{1,2} 12.1 \pm 0.4^{a,b}$	$_1 11.7 \pm 0.3^{a,b}$	$_2 12.9 \pm 0.6^a$	$_{1,2} 11.9 \pm 0.4^a$
Spring	$_1 13.3 \pm 0.8^b$	$_1 12.6 \pm 0.6^b$	$_2 10.7 \pm 0.3^b$	$_2 10.7 \pm 0.3^b$
Summer	$_1 10.7 \pm 0.3^c$	$_1 10.8 \pm 0.6^a$	$_1 11.3 \pm 0.5^b$	$_1 10.8 \pm 0.4^b$

Different letters (a,b) represent significant differences between seasons, while, different numbers (1,2) represent significant differences between life stages (p<0.05)

Protein content of vegetative stages was influenced by year being significantly higher (p<0.05) in Winter 2009 than Winter 2008 (Table 4.34).

Table 4.34 Protein content (% db) (Mean ± S.E.) life stages of *Gracilaria cliftonii* during Autumn 2009 and Winter 2009

Life stage	Autumn 2009	Autumn 2009	Winter 2009	Winter 2009
Tetrasporophyte	$10.8 \pm 0.2_a$	$11.3 \pm 0.4_a$	$12.1 \pm 0.4_a$	$12.9 \pm 0.6_a$
Carposporophyte	$11.1 \pm 0.5_a$	$11.3 \pm 0.4_a$	$11.7 \pm 0.3_a$	$12.9 \pm 0.6_b$
Male gametophyte	$11.6 \pm 0.4_a$	$11.0 \pm 0.2_a$	$12.9 \pm 0.6_a$	$13.2 \pm 0.7_a$
Vegetative	$11.7 \pm 0.4_a$	$12.2 \pm 0.5_a$	$11.9 \pm 0.4_a$	$13.9 \pm 0.8_b$

Different letters (a,b) represent significant differences between same seasons at a level of $p<0.05$,t-test

4.5.3.2. Seasons based on photoperiod

Protein content was also influenced by photoperiod seasons. In season PS2 protein of male gametophyte was significantly lower ($p<0.05$) than other seasons (Table 4.35), while in PS3, vegetative protein content was significantly higher ($p<0.05$) than other seasons.

Table 4.35 Protein content (% db) (Mean ± SE) among photoperiod seasons and life stages of *Gracilaria cliftonii* from Point Peron, Western Australia

Season	Tetrasporophyte	Carposporophyte	Male	Vegetative
PS1	$_1 11.9 \pm 0.4^a$	$_1 11.4 \pm 0.3^a$	$_1 12.2 \pm 0.4^a$	$_1 11.6 \pm 0.3^a$
PS2	$_1 11.7 \pm 0.5^a$	$_1 11.7 \pm 0.5^a$	$_1 10.9 \pm 0.3^b$	$_1 11.0 \pm 0.3^a$
PS3	$_1 12.1 \pm 0.4^a$	$_1 12.4 \pm 0.4^a$	$_1 12.3 \pm 0.5^a$	$_1 13.2 \pm 0.6^b$

Different letters (a,b) represent differences among seasons, while numbers (1,2) represent differences between life stages at $p<0.05$

PS1: March to September 2008, PS2: October 2008 to March 2009, PS3: April to August 2009

4.5.4. Carbohydrate

4.5.4.1. Seasons based on temperature

In Summer, carbohydrate content of tetrasporophyte, carposporophyte and vegetative stages was significantly higher ($p<0.05$) than Spring. Carbohydrate content of tetrasporophyte in Winter was significantly lower ($p<0.06$) than rest of the life stages (Table 4.36).

Table 4.36 Carbohydrate (% db) (Mean ± S.E.) life stages of *Gracilaria cliftonii* for temperature seasons

Season	Tetrasporophyte	Carposporophyte	Male	Vegetative
Autumn	$_1 63.3 \pm 0.9^a$	$_1 65.2 \pm 0.9^{a,b}$	$_1 66.2 \pm 1.5^a$	$_1 65.6 \pm 1.1^{a,b}$
Winter	$_1 61.3 \pm 1.4^a$	$_2 65.6 \pm 1.0^{a,b}$	$_2 64.9 \pm 0.9^a$	$_2 66.4 \pm 0.7^{a,b}$
Spring	$_1 61.7 \pm 1.2^a$	$_1 64.7 \pm 1.3^a$	$_1 64.7 \pm 1.1^a$	$_1 64.4 \pm 1.0^a$
Summer	$_1 68.0 \pm 0.9^b$	$_1 68.0 \pm 1.0^b$	$_1 66.6 \pm 1.7^a$	$_1 68.1 \pm 1.1^b$

Different letters (a,b) represent significant differences between seasons, while, different numbers (1,2) represent significant differences between life stages ($p < 0.05$)

Carbohydrate content from all life stages was not influenced by year ($p < 0.05$, t-test) (Table 4.37).

Table 4.37 Carbohydrate content (% db) (Mean ± S.E.) from the life stages of *Gracilaria cliftonii* during Autumn 2009 and Winter 2009

Life stage	Autumn 2008	Autumn 2009	Winter 2008	Winter 2009
Tetrasporophyte	$63.3 \pm 0.9_a$	$67.0 \pm 0.8_a$	$61.3 \pm 1.4_a$	$64.5 \pm 0.9_a$
Carposporophyte	$65.2 \pm 0.9_a$	$67.0 \pm 0.8_a$	$65.6 \pm 1.0_a$	$64.5 \pm 0.9_a$
Male gametophyte	$66.2 \pm 1.5_a$	$65.2 \pm 1.0_a$	$64.9 \pm 0.9_a$	$64.6 \pm 1.5_a$
Vegetative	$65.6 \pm 1.1_a$	$64.9 \pm 1.3_a$	$66.4 \pm 0.7_a$	$60.6 \pm 1.2_a$

Different letters (a,b) represent significant differences between same seasons at a level of $p < 0.05$, t-test

4.5.4.2. Seasons based on photoperiod

Carbohydrate content was dependant on seasons based in photoperiod. Carbohydrate content of tetrasporophyte stage in PS3 was significantly higher ($p < 0.05$) than PS1 season. Carbohydrate of vegetative stage in PS3 season was significantly lower ($p < 0.05$) than PS2 season.

In season PS1, carbohydrate of tetrasporophyte stage was significantly lower ($p < 0.05$) than other stages, while in season PS3 was significantly higher ($p < 0.05$) than vegetative stage (Table 4.38).

Table 4.38 Carbohydrate content (% db) (Mean ± SE) among photoperiod seasons and life stages of *Gracilaria cliftonii* from Point Peron, Western Australia

Season	Tetrasporophyte	Carposporophyte	Male	Vegetative
PS1	$_162.8 \pm 0.9^a$	$_266.2 \pm 0.6^a$	$_266.0 \pm 0.6^a$	$_266.5 \pm 0.4^a$
PS2	$_164.8 \pm 1.1^{a,b}$	$_164.8 \pm 1.0^a$	$_165.4 \pm 1.1^a$	$_166.4 \pm 0.8^a$
PS3	$_166.0 \pm 0.7^b$	$_{1,2}64.2 \pm 0.8^a$	$_165.2 \pm 1.0^a$	$_262.1 \pm 1.1^b$

Different letters (a,b) represent differences among seasons, while numbers (1,2) represent differences between life stages at p<0.05

PS1: March to September 2008, PS2: October 2008 to March 2009, PS3: April to August 2009

Carbohydrate content showed a strong inverse relationship with ash content (R^2=0.92; p<0.05) and with protein content (R^2=0.89; p<0.05).

4.5.5. Swelling capacity

4.5.5.1.　　　Seasons based on temperature

Swelling capacity of tetrasporophyte, carposporophyte and male gametophyte stages in Winter were significantly higher (p<0.05) than Summer. Swelling capacity of vegetative stage was not influenced (p>0.05) by seasons.

In Spring, swelling capacity of male gametophyte stage was significantly higher (p<0.05) than carposporophyte stage. However, swelling capacity was not influenced (p>0.05) by any other life stage (Table 4.39).

Table 4.39 Swelling capacity (mL/g db) (Mean ± S.E.) life stages of *Gracilaria cliftonii* for temperature seasons

Season	Tetrasporophyte	Carposporophyte	Male	Vegetative
Autumn	$_18.9 \pm 0.6^a$	$_19.9 \pm 1.0^{a,b}$	$_110.3 \pm 0.8^{a,b}$	$_110.9 \pm 0.8^a$
Winter	$_111.9 \pm 0.5^b$	$_111.1 \pm 0.3^a$	$_112.2 \pm 0.3^b$	$_111.3 \pm 0.5^a$
Spring	$_{1,2}10.7 \pm 0.4^{b,c}$	$_19.8 \pm 0.3^{a,b}$	$_212.3 \pm 1.1^b$	$_{1,2}10.6 \pm 0.8^a$
Summer	$_110.2 \pm 0.6^{a,c}$	$_19.3 \pm 0.6^b$	$_19.6 \pm 0.5^a$	$_19.9 \pm 0.7^a$

Different letters (a,b,c) represent significant differences between seasons, while, different numbers (1,2) represent significant differences between life stages (p<0.05)

Swelling capacity of different life stages were not influenced by year (Table 4.40).

Table 4.40 Swelling capacity (mL/g db) (Mean ± S.E.) life stages of *Gracilaria cliftonii* in Autumn 2009 and Winter 2009

Life stage	Autumn 2008	Autumn 2009	Winter 2008	Winter 2009
Tetrasporophyte	$8.9 \pm 0.6_a$	$9.9 \pm 0.7_a$	$11.9 \pm 0.5_a$	$9.5 \pm 0.5_a$
Carposporophyte	$9.9 \pm 1.0_a$	$9.9 \pm 0.5_a$	$11.1 \pm 0.3_a$	$9.4 \pm 0.4_a$
Male	$10.3 \pm 0.8_a$	$10.0 \pm 0.4_a$	$12.2 \pm 0.3_a$	$10.2 \pm 0.5_a$
Vegetative	$10.9 \pm 0.8_a$	$9.8 \pm 0.4_a$	$11.3 \pm 0.5_a$	$9.3 \pm 0.5_a$

Different letters (a,b) represent significant differences between same seasons at a level of $p<0.05$, t-test

4.5.5.2. Seasons based on photoperiod

Swelling capacity of life stages from *G. cliftonii* was also influenced by seasons based on photoperiod. In season PS1, swelling capacity of carposporophyte stage was significantly higher ($p<0.05$) than PS2 season. Swelling capacity of vegetative stage in PS3 season was significantly lower ($p<0.05$) than rest of the seasons. Swelling capacity of tetrasporophyte and male gametophyte stages was not influenced by seasons based on photoperiod ($p>0.05$).

In season PS2, swelling capacity of male gametophyte stage was significantly higher ($p<0.05$) than carposporophyte stage (Table 4.41).

Table 4.41 Swelling capacity (g/mL) (Mean ± SE) among photoperiod seasons and life stages of *Gracilaria cliftonii* from Point Peron, Western Australia

Season	Tetrasporophyte	Carposporophyte	Male	Vegetative
PS1	$_1 10.9 \pm 0.4^a$	$_1 10.6 \pm 0.5^a$	$_1 11.2 \pm 0.5^a$	$_1 11.1 \pm 0.5^a$
PS2	$_{1,2} 10.1 \pm 0.5^a$	$_1 9.5 \pm 0.3^b$	$_2 11.4 \pm 0.7^a$	$_{1,2} 10.7 \pm 0.5^a$
PS3	$_1 10.1 \pm 0.4^a$	$_1 9.8 \pm 0.3^{a,b}$	$_1 9.9 \pm 0.3^a$	$_1 9.3 \pm 0.4^b$

Different letters (a,b) represent differences among seasons, while numbers (1,2) represent differences between life stages at $p<0.05$

PS1: March to September 2008, PS2: October 2008 to March 2009, PS3: April to August 2009

4.5.6. Water retention capacity

4.5.6.1. Seasons based on temperature

Water retention capacity (WRC) of tetrasporophyte stage in Winter was significantly higher ($p<0.05$) than Autumn. WRC of vegetative stage in Autumn was

significantly higher (p<0.05) than Spring and Summer seasons.

In Autumn, WRC of male gametophyte and vegetative stage was significantly higher (p<0.05) than tetrasporophyte stage. In Summer, WRC of tetrasporophyte and male gametophyte stage was significantly higher (p<0.05) than vegetative stage (Table 4.42).

Table 4.42 Water retention capacity (g/g) (Mean ± S.E.) life stages of *Gracilaria cliftonii* for temperature seasons

Season	Tetrasporophyte	Carposporophyte	Male	Vegetative
Autumn	$_1 5.0 \pm 0.2^a$	$_{1,2} 5.9 \pm 0.2^a$	$_2 6.1 \pm 0.4^a$	$_2 6.8 \pm 0.5^a$
Winter	$_1 6.4 \pm 0.6^b$	$_1 6.1 \pm 0.6^a$	$_1 6.7 \pm 0.5^a$	$_1 6.2 \pm 0.3^{a,b}$
Spring	$_1 6.0 \pm 0.4^{a,b}$	$_1 5.4 \pm 0.3^a$	$_1 5.8 \pm 0.4^a$	$_1 5.5 \pm 0.2^b$
Summer	$_1 5.6 \pm 0.2^{a,b}$	$_{1,2} 5.1 \pm 0.3^a$	$_1 5.7 \pm 0.2^a$	$_2 4.8 \pm 0.2^c$

Different letters (a,b,c) represent significant differences between seasons, while, different numbers (1,2) represent significant differences between life stages (p<0.05)

WRC of different life stages were influenced by year (Table 4.43). In Autumn of 2008, WRC of vegetative stages was significantly higher (p<0.05) than Autumn of 2009 while, in Winter 2008, WRC of male gametophyte was significantly lower (p<0.05) than Winter 2009.

Table 4.43 Water retention capacity (g/g) (Mean ± S.) life stages of *Gracilaria cliftonii* in Autumn 2009 and Winter 2009

Life stage	Autumn 2008	Autumn 2009	Winter 2008	Winter 2009
Tetrasporophyte	$5.0 \pm 0.2_a$	$6.4 \pm 0.2_a$	$6.4 \pm 0.6_a$	$6.1 \pm 0.3_a$
Carposporophyte	$5.9 \pm 0.2_a$	$5.5 \pm 0.4_a$	$6.1 \pm 0.6_a$	$5.3 \pm 0.3_a$
Male	$6.1 \pm 0.4_a$	$5.3 \pm 0.2_a$	$6.7 \pm 0.5_a$	$5.3 \pm 0.2_b$
Vegetative	$6.8 \pm 0.5_a$	$5.3 \pm 0.3_b$	$6.2 \pm 0.3_a$	$5.3 \pm 0.4_a$

Different letters (a,b) represent significant differences between same seasons at a level of p<0.05,t-test

4.5.6.2. Seasons based on photoperiod

WRC of *G. cliftonii* varied as a function of seasons based on photoperiod.

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WRC of carposporophyte, male gametophyte and vegetative stages in PS1 season was significantly higher ($p<0.05$) than other seasons. In PS2 and PS3 season, WRC of tetrasporophyte stage was significantly higher ($p<0.05$) than other stages.

WRC showed a strong inverse relationship with carbohydrate content ($R_2=0.92$; $p<0.05$) and positive relationship with ash content ($R_2=0.74$; $p<0.05$) (Table 4.44).

Table 4.44 Water retention capacity (g/g) (Mean ± SE) among photoperiod seasons and life stages of *Gracilaria cliftonii* from Point Peron, Western Australia

Season	Tetrasporophyte	Carposporophyte	Male	Vegetative
PS1	$_16.0 \pm 0.4^a$	$_15.9 \pm 0.3^a$	$_16.6 \pm 0.3^a$	$_16.6 \pm 0.3^a$
PS2	$_15.7 \pm 0.1^a$	$_25.1 \pm 0.2^b$	$_15.4 \pm 0.2^b$	$_25.0 \pm 0.2^b$
PS3	$_16.2 \pm 0.2^a$	$_25.5 \pm 0.3^{a,b}$	$_25.3 \pm 0.2^b$	$_25.5 \pm 0.3^b$

Different letters (a,b) represent differences among seasons, while numbers (1,2) represent differences between life stages at $p<0.05$

PS1: March to September 2008, PS2: October 2008 to March 2009, PS3: April to August 2009

4.5.7. Oil retention capacity

4.5.7.1. Seasons based on temperature

Oil retention capacity (ORC) of tetrasporophyte, male gametophyte and vegetative stages in Autumn was significantly higher ($p<0.05$) than Spring.

In Autumn , ORC of male gametophyte stage was significantly higher ($p<0.05$) than other stages, however, ORC was not influenced ($p>0.05$) by other life stages (Table 4.45).

Table 4.45 Oil retention capacity (g/g) (Mean ± SE) life stages of *Gracilaria cliftonii* for temperature seasons

Season	Tetrasporophyte	Carposporophyte	Male	Vegetative
Autumn	$_13.2 \pm 0.2^a$	$_12.6 \pm 0.2^a$	$_24.1 \pm 0.3^a$	$_13.1 \pm 0.3^a$
Winter	$_12.7 \pm 0.3^{a,b}$	$_12.9 \pm 0.5^a$	$_13.3 \pm 0.6^a$	$_13.0 \pm 0.5^a$
Spring	$_12.4 \pm 0.2^b$	$_12.1 \pm 0.3^a$	$_12.2 \pm 0.2^b$	$_11.8 \pm 0.2^b$
Summer	$_12.5 \pm 0.3^{a,b}$	$_12.5 \pm 0.3^a$	$_12.6 \pm 0.3^c$	$_12.7 \pm 0.2^a$

Different letters (a,b) represent significant differences between seasons, while, different numbers (1,2) represent significant differences between life stages ($p<0.05$)

In Winter of 2008, WRC of vegetative stage was significantly higher (p<0.05) than in Winter of 2009 (Table 4.46).

Table 4.46 Oil retention capacity (g/g) (Mean ± S.E.) life stages of *Gracilaria cliftonii* in Autumn 2009 and Winter 2009

Life stage	Autumn 2008	Autumn 2009	Winter 2008	Winter 2009
Tetrasporophyte	$3.2 \pm 0.2_a$	$3.2 \pm 0.3_a$	$2.7 \pm 0.3_a$	$2.5 \pm 0.2_a$
Carposporophyte	$2.6 \pm 0.2_a$	$3.0 \pm 0.2_a$	$2.9 \pm 0.5_a$	$2.9 \pm 0.2_a$
Male gametophyte	$4.1 \pm 0.3_a$	$3.0 \pm 0.3_a$	$3.3 \pm 0.6_a$	$2.6 \pm 0.4_a$
Vegetative	$3.1 \pm 0.3_a$	$3.7 \pm 0.6_a$	$3.0 \pm 0.5_a$	$2.6 \pm 0.2_b$

Different letters (a,b) represent significant differences between same seasons at a level of p<0.05,t-test

4.5.7.2.   Seasons based on photoperiod

No significant differences (p<0.05) on ORC were observed among seasons based on photoperiod and different life stages, only in PS1, ORC of male gametophyte was significantly higher (p<0.05) than PS3 (Table 4.47).

A strong relationship was observed between ORC and agar content (R^2=0.95; p<0.05).

Table 4.47 Oil retention capacity (g/g) (Mean ± SE) among photoperiod seasons and life stages of *Gracilaria cliftonii* from Point Peron, Western Australia.

Season	Tetrasporophyte	Carposporophyte	Male	Vegetative
PS1	$_1 2.6 \pm 0.2^a$	$_1 2.5 \pm 0.3^a$	$_1 3.4 \pm 0.4^a$	$_1 3.0 \pm 0.3^a$
PS2	$_1 2.8 \pm 0.2^a$	$_1 2.6 \pm 0.2^a$	$_1 2.8 \pm 0.2^{a,b}$	$_1 2.9 \pm 0.4^a$
PS3	$_1 2.6 \pm 0.2^a$	$_1 2.9 \pm 0.2^a$	$_1 2.6 \pm 0.2^b$	$_1 2.7 \pm 0.2^a$

Different letters (a,b) represent differences among seasons, while numbers (1,2) represent differences between life stages at p<0.05

PS1: March to September 2008, PS2: October 2008 to March 2009, PS3: April to August 2009

4.5.8. Agar yield

4.5.8.1.   Seasons based on temperature

Tetrasporophyte agar yield in Autumn was significantly higher (p<0.05) than in Spring while, agar yield of carposporophyte and vegetative stages was

significantly higher (p<0.05) than Winter and Spring season. Agar yield of male gametophyte stage in Summer was significantly higher (p<0.05) than other seasons. In Summer, agar yield of tetrasporophyte stage was significantly lower (p<0.05) than male gametophyte stage. In Autumn, agar yield of male gametophyte was significantly higher (p<0.05) than vegetative stage (Table 4.48).

Table 4.48 Agar yield (% db) (Mean ± S.E.) life stages of *Gracilaria cliftonii* for temperature seasons

Season	Tetrasporophyte	Carposporophyte	Male	Vegetative
Autumn	$_{1,2}57.5 \pm 1.3^a$	$_{1,2}57.8 \pm 1.2^a$	$_157.5 \pm 1.5^a$	$_259.6 \pm 1.1^a$
Winter	$_155.1 \pm 0.7^{a,b}$	$_154.5 \pm 1.0^b$	$_153.6 \pm 0.8^a$	$_153.3 \pm 0.7^b$
Spring	$_154.1 \pm 0.6^b$	$_153.6 \pm 0.5^{b,c}$	$_154.0 \pm 0.8^a$	$_155.0 \pm 0.6^b$
Summer	$_155.2 \pm 1.2^{a,b}$	$_{1,2}56.6 \pm 1.1^c$	$_258.6 \pm 0.7^b$	$_{1,2}57.7 \pm 0.9^a$

Different letters (a,b) represent significant differences between seasons, while, different numbers (1,2) represent significant differences between life stages (p<0.05)

Agar yield of the carposporophyte stage was influenced by year (Table 4.49). In Winter of 2009, agar yield was significantly higher (p<0.05) than in Winter of 2008. Yield and sulphate content of agar from *G. cliftonii* showed inverse correlation with epiphytic load (R^2=0.95, 0.79; p<0.05).

Table 4.49 Agar yield (% db) (Mean ± S.E.) life stages of *Gracilaria cliftonii* in Autumn 2009 and Winter 2009

Life stage	Autumn 2008	Autumn 2009	Winter 2008	Winter 2009
Tetrasporophyte	$57.5 \pm 1.3_a$	$55.1 \pm 1.2_a$	$55.1 \pm 0.7_a$	$56.6 \pm 1.0_a$
Carposporophyte	$57.8 \pm 1.2_a$	$56.1 \pm 1.3_a$	$54.5 \pm 1.0_a$	$57.3 \pm 0.8_b$
Male gametophyte	$55.5 \pm 1.5_a$	$55.5 \pm 1.1_a$	$53.6 \pm 0.8_a$	$58.2 \pm 0.7_a$
Vegetative	$59.6 \pm 1.1_a$	$56.4 \pm 0.9_a$	$53.3 \pm 0.7_a$	$57.9 \pm 0.8_a$

Different letters (a,b) represent significant differences between same seasons at a level of p<0.05,t-test

4.5.8.2. Seasons based on photoperiod

Agar yield of male gametophyte stage in PS3 season was significantly higher (p<0.05) than in PS1 season. However, agar yield was not influenced by other seasons or life stages (p>0.05) (Table 4.50).

Table 4.50 Agar yield (% db) (Mean ± SE) among seasons based on photoperiod for different life stages of *Gracilaria cliftonii*

Season	Tetrasporophyte	Carposporophyte	Male	Vegetative
PS1	$_1 55.4 \pm 0.6^a$	$_1 55.7 \pm 0.9^a$	$_1 54.1 \pm 0.9^b$	$_1 56.3 \pm 0.9^a$
PS2	$_1 55.0 \pm 0.8^a$	$_1 55.6 \pm 0.8^a$	$_1 56.3 \pm 0.7^{a,b}$	$_1 56.4 \pm 0.6^a$
PS3	$_1 55.5 \pm 0.8^a$	$_1 56.5 \pm 0.9^a$	$_1 57.3 \pm 0.8^a$	$_1 57.1 \pm 0.7^a$

Different letters (a,b) represent differences among seasons, while numbers (1,2) represent differences between life stages at p<0.05

PS1: March to September 2008, PS2: October 2008 to March 2009, PS3: April to August 2009

4.5.9. Gelling temperature

4.5.9.1. Seasons based on temperature

Gelling temperature of agar from tetrasporophyte stage in Spring was significantly higher (p<0.05) than Summer.

In Winter, gelling temperature of agar from male gametophyte stage was higher (p<0.05) than carposporophyte stages. In Spring, gelling temperature of agar from tetrasporophyte stage was higher (p<0.05) than carposporophyte and male gametophyte stages (Table 4.51).

Table 4.51 Gelling temperature (^{o}C) (Mean ± S.E.) life stages of *Gracilaria cliftonii* for temperature seasons

Season	Tetrasporophyte	Carposporophyte	Male	Vegetative
Autumn	$_1 34.6 \pm 0.9^{a,b}$	$_1 34.1 \pm 0.8^a$	$_1 35.1 \pm 0.6^a$	$_1 34.8 \pm 0.5^a$
Winter	$_{1,2} 35.2 \pm 0.4^{a,b}$	$_1 34.2 \pm 0.3^a$	$_2 35.7 \pm 0.6^a$	$_{1,2} 34.4 \pm 0.5^a$
Spring	$_1 36.0 \pm 0.6^a$	$_2 34.6 \pm 0.3^a$	$_2 34.3 \pm 0.6^a$	$_{1,2} 35.2 \pm 0.4^a$
Summer	$_1 34.0 \pm 0.4^b$	$_1 34.5 \pm 0.6^a$	$_1 34.2 \pm 0.5^a$	$_1 35.0 \pm 0.4^a$

Different letters (a,b) represent significant differences between seasons, while, different numbers (1,2) represent significant differences between life stages (p<0.05)

Gelling temperature of the agar from different life stages were not influenced by year (Table 4.52).

Table 4.52 Gelling temperature (^{o}C) (Mean ± S.E.) of agar from different life stages of *Gracilaria cliftonii* in Autumn and Winter from 2008 and 2009

Life stage	Autumn 2008	Autumn 2009	Winter 2008	Winter 2009
Tetrasporophyte	$34.6 \pm 0.9_a$	$35.5 \pm 0.7_a$	$35.2 \pm 0.4_a$	$34.7 \pm 0.9_a$
Carposporophyte	$34.1 \pm 0.8_a$	$35.2 \pm 0.5_a$	$34.2 \pm 0.3_a$	$36.2 \pm 0.6_a$
Male	$35.1 \pm 0.6_a$	$35.0 \pm 0.7_a$	$35.7 \pm 0.6_a$	$35.4 \pm 0.4_a$
Vegetative	$34.8 \pm 0.5_a$	$35.5 \pm 0.4_a$	$34.4 \pm 0.5_a$	$34.3 \pm 0.8_a$

Different letters (a,b) represent significant differences between same seasons at a level of p<0.05,t-test

4.5.9.2.   Seasons based on photoperiod

Gelling temperature of the agar from *G. cliftonii* was not influenced by seasons based in photoperiod (Table 4.53). In PS1, gelling temperature of the agar from male gametophyte stage was significantly higher (p<0.05) than carposporophyte stage. Gelling temperature showed a strong relationship with ash content (R^2=0.92; p<0.05).

Table 4.53 Gelling temperature of the agar (^{o}C) (Mean ± SE) from different life stages of *Gracilaria cliftonii* over three seasons based on photoperiod

Season	Tetrasporophyte	Carposporophyte	Male	Vegetative
PS1	$_{1,2}34.9 \pm 0.5^a$	$_234.0 \pm 0.3^a$	$_135.5 \pm 0.3^a$	$_{1,2}34.7 \pm 0.4^a$
PS2	$_134.6 \pm 0.5^a$	$_134.6 \pm 0.3^a$	$_134.6 \pm 0.5^a$	$_135.2 \pm 0.3^a$
PS3	$_135.5 \pm 0.6^a$	$_135.8 \pm 0.4^a$	$_134.7 \pm 0.4^a$	$_134.6 \pm 0.5^a$

Different letters (a,b) represent differences among seasons, while numbers (1,2) represent differences between life stages at p<0.05

PS1: March to September 2008, PS2: October 2008 to March 2009, PS3: April to August 2009

4.5.10. Melting point

4.5.10.1.   Seasons based on temperature

Melting point of agar from tetrasporophyte in Spring and Summer was significantly higher (p<0.05) than Winter. Melting point of agar from male gametophyte in Winter was significantly higher (p<0.05) than in Autumn.

Only in Winter, melting point of agar from male gametophyte was significantly higher ($p<0.05$) than carposporophyte stage (Table 4.54).

Table 4.54 Melting point of the agar ($^{\circ}$C) (Mean $\pm$ S.E.) from different life stages of *Gracilaria cliftonii* for temperature seasons

Season	Tetrasporophyte	Carposporophyte	Male	Vegetative
Autumn	$_1 80.7 \pm 2.0^{a,b}$	$_1 80.3 \pm 1.7^{a}$	$_1 80.2 \pm 1.2^{a}$	$_1 81.0 \pm 1.5^{a}$
Winter	$_1 77.0 \pm 2.6^{a}$	$_{1,2} 78.0 \pm 3.8^{a}$	$_2 85.1 \pm 1.1^{b}$	$_{1,2} 79.4 \pm 2.3^{a}$
Spring	$_1 84.3 \pm 0.5^{b}$	$_1 80.3 \pm 1.5^{a}$	$_1 81.4 \pm 1.7^{a,b}$	$_1 83.4 \pm 2.2^{a}$
Summer	$_1 82.9 \pm 1.8^{b}$	$_1 82.0 \pm 0.9^{a}$	$_1 81.6 \pm 1.5^{a,b}$	$_1 83.1 \pm 1.7^{a}$

Different letters (a,b) represent significant differences between seasons, while, different numbers (1,2) represent significant differences between life stages ($p<0.05$)

Melting point of the agar from different life stages were not influenced by year (Table 4.55).

Table 4.55 Melting point ($^{\circ}$C) (Mean $\pm$ S.E.) life stages of *Gracilaria cliftonii* in Autumn and Winter 2009

Life stage	Autumn 2008	Autumn 2009	Winter 2008	Winter 2009
Tetrasporophyte	$80.7 \pm 2.0_a$	$83.1 \pm 1.8_a$	$77.0 \pm 2.6_a$	$81.0 \pm 1.5_a$
Carposporophyte	$80.3 \pm 1.7_a$	$79.9 \pm 2.4_a$	$78.0 \pm 3.8_a$	$82.2 \pm 1.3_a$
Male	$80.2 \pm 1.2_a$	$80.0 \pm 2.0_a$	$85.1 \pm 1.1_a$	$82.5 \pm 1.0_a$
Vegetative	$81.0 \pm 1.5_a$	$77.0 \pm 2.5_a$	$79.4 \pm 2.3_a$	$79.4 \pm 1.1_a$

Different letters (a,b) represent significant differences between same seasons at a level of $p<0.05$, t-test

4.5.10.2.   Seasons based on photoperiod

Melting point of the agar from *G. cliftonii* was not influenced by photoperiod seasons ($p>0.05$) (Table 4.56).

Table 4.56 Melting point (^{o}C) (Mean ± SE) of the agar from different life stages of *Gracilaria cliftonii*

Season	Tetrasporophyte	Carposporophyte	Male	Vegetative
PS1	$_{1}80.5 \pm 1.6^{a}$	$_{1}79.7 \pm 2.0^{a}$	$_{1}83.1 \pm 1.1^{a}$	$_{1}80.5 \pm 1.5^{a}$
PS2	$_{1}83.2 \pm 0.9^{a}$	$_{1}81.4 \pm 0.9^{a}$	$_{1}81.4 \pm 1.0^{a}$	$_{1}82.4 \pm 1.4^{a}$
PS3	$_{1}81.9 \pm 1.4^{a}$	$_{1}80.6 \pm 1.6^{b}$	$_{1}81.7 \pm 1.1^{a}$	$_{1}79.0 \pm 1.5^{a}$

Different letters (a,b) represent differences among seasons, while numbers (1,2) represent differences between life stages at p<0.05

PS1: March to September 2008, PS2: October 2008 to March 2009, PS3: April to August 2009

4.5.11. Sulphate

4.5.11.1. Seasons based on temperature

Sulphate of the agar from different life stages of *G. cliftonii* varied from 5.9 to 6.6 % db but was not influenced by seasons (p>0.05). Except in Winter of 2008, sulphate content of agar from carposporophyte and male gametophyte stages were significantly higher (p<0.05) than tetrasporophyte stage (Table 4.57).

Table 4.57 Sulphate content (% db) (Mean ± S.E.) life stages of *Gracilaria cliftonii* for seasons based on temperature

Season	Tetrasporophyte	Carposporophyte	Male	Vegetative
Autumn	$_{1}5.9 \pm 0.3^{a}$	$_{1}6.5 \pm 0.3^{a}$	$_{1}6.3 \pm 0.4^{a}$	$_{1}6.4 \pm 0.4^{a}$
Winter	$_{1}6.3 \pm 0.2^{a}$	$_{2}6.2 \pm 0.3^{a}$	$_{2}6.5 \pm 0.4^{a}$	$_{1,2}6.6 \pm 0.2^{a}$
Spring	$_{1}5.9 \pm 0.3^{a}$	$_{1}6.2 \pm 0.3^{a}$	$_{1}6.5 \pm 0.4^{a}$	$_{1}6.5 \pm 0.3^{a}$
Summer	$_{1}6.4 \pm 0.3^{a}$	$_{1}6.2 \pm 0.3^{a}$	$_{1}6.3 \pm 0.3^{a}$	$_{1}6.4 \pm 0.3^{a}$

Different letters (a,b,c) represent significant differences between seasons, while, different numbers (1,2) represent significant differences between life stages (p<0.05).

Sulphate of agar from different life stages of *G. cliftonii* was not influenced by year (Table 4.58).

Table 4.58 Sulphate content of agar (% db) (Mean ± S.E.) from different life stages of *Gracilaria cliftonii* in Autumn and Winter of 2008 and 2009

Life stage	Autumn 2008	Autumn 2009	Winter 2008	Winter 2009
Tetrasporophyte	$5.9 \pm 0.3_a$	$6.7 \pm 0.3_a$	$6.3 \pm 0.2_a$	$5.9 \pm 0.3_a$
Carposporophyte	$6.5 \pm 0.3_a$	$6.7 \pm 0.4_a$	$6.2 \pm 0.3_a$	$6.3 \pm 0.2_a$
Male	$6.3 \pm 0.4_a$	$6.6 \pm 0.2_a$	$6.5 \pm 0.4_a$	$5.9 \pm 0.3_a$
Vegetative	$6.4 \pm 0.4_a$	$6.2 \pm 0.2_a$	$6.6 \pm 0.2_a$	$6.1 \pm 0.3_a$

Different letters (a,b) represent significant differences between same seasons at a level of p<0.05,t-test

4.5.11.2.   Seasons based on photoperiod

In season PS1, sulphate content of the agar from male gametophyte stage was significantly higher (p<0.05) than tetrasporophyte stage (Table 4.59). However, sulphate content of agar was not influenced by other photoperiod seasons (p>0.05).

Table 4.59 Sulphate content of the agar (% db) (Mean ± SE) among photoperiod seasons and life stages of *Gracilaria cliftonii* from Point Peron, Western Australia

Season	Tetrasporophyte	Carposporophyte	Male	Vegetative
PS1	$_16.1 \pm 0.2^a$	$_{1,2}6.3 \pm 0.2^a$	$_26.7 \pm 0.3^a$	$_{1,2}6.3 \pm 0.2^a$
PS2	$_16.3 \pm 0.2^a$	$_16.4 \pm 0.2^a$	$_16.4 \pm 0.2^a$	$_16.3 \pm 0.2^a$
PS3	$_16.3 \pm 0.2^a$	$_16.4 \pm 0.2^a$	$_16.1 \pm 0.2^a$	$_16.2 \pm 0.2^a$

Different letters (a,b) represent differences among seasons, while numbers (1,2) represent differences between life stages at p<0.05

PS1: March to September 2008, PS2: October 2008 to March 2009, PS3: April to August 2009

5. DISCUSSION

Marine red algae, or Rhodophyta, comprise the largest and most diverse assemblage of the marine plants. They are an important component of aquatic ecosystems as primary producers and provide shelter and substratum to other organisms. They play an important role in the marine environment and are used as raw materials for the extraction of valuable products. Furthermore, they are important source of food, phycocolloids and secondary metabolites. *Gracilaria* genus is distributed along the shores of Australia (Womersley, 1996), but the biology and environmental requirements for the culture of the various species are not known. However, the seasonality of the growth, biomass, proximate composition and agar has been previously reported for other *Gracilaria* spp. around the world (see Literature review). However, most of the studies have been performed under laboratory, outdoor tank or field cultivation conditions. The *Gracilaria* genus in Australia is poorly understood especially *Gracilaria cliftonii,* which is an exceptional high agar yielding macroalgae (Armisen, 1995). There is no information available regarding to its basic biology.

The taxonomic knowledge of *Gracilaria* is based primarily on comparative morphological studies. One driving force to understand the species of *Gracilaria* is the commercial uses and demands for the colloid agars produced within its cell walls. Despite the progress made, many questions still abound related to taxonomy of the Gracilariaceae, specially concerning the inter and intra generic relationships with regard to *Gracilaria sensu lato*. Taylor (1960) suggested that the identification of *Gracilaria* based on morphology alone should not be attempted. The ambiguities found on *Gracilaria* taxonomy are the result of morphological plasticity, which generates high level of homoplasies among many species. While clarification and understanding for many *Gracilaria* species has been clarified, others continue to be elusive (Bird and McLachlan, 1984, Bird and McLachlan, 1982, Fredericq and Hommersand, 1989).

This is the case of *G. cliftonii* which taxonomy has been mainly examined through anatomy and morphological criteria (May, 1948, Agardh, 1876, Withell *et al.,* 1994) and in one case on molecular basis (Byrne *et al.*, 2002). Withell *et al.,* (1994) have emphasised the necessity of utilising reproductive criteria on *G. cliftonii*

taxonomy. These characteristics include size and type of tetrasporangia for the tetrasporophyte stage, verrucosa type spermatangia for male gametophyte stage and size/shape of cystocarp for carposporophyte stage. Present study confirms the previous descriptions of reproductive structures of *G. cliftonii* (Byrne *et al.*, 2002, Withell *et al.*, 1994) and confirms the importance of utilising criteria based on the reproductive morphology for taxonomic classification of *G. cliftonii.*

From the literature available few studies deal with the reproductive phenology of *Gracilaria* spp. (Penniman *et al.*, 1986, Whyte *et al.*, 1981, Orduña-Rojas and Robledo, 2002, Luhan, 1996, Hay and Norris, 1984, Hoyle, 1978c, Pickering *et al.*, 1990). Only two articles are available from a similar geographic region as *G. cliftonii*. Both studies are on *G. sordida* from New Zealand (Pickering *et al.*, 1990, Nelson, 1989) where different environmental parameters are operating. In addition, the information available on the seasonality of chemical properties provides contradictory and variable patterns for *Gracilaria* spp. making it difficult to compare the available results. Compared to ecological studies, more concentrated efforts have been put to identify the chemical properties of *Gracilaria* spp. under natural and cultivation conditions. Particular attention has been paid to polysaccharide yield and its properties due to the importance of *Gracilaria* spp. as a source of agar.

Several studies deal with the seasonality of the agar of *Gracilaria* spp. under natural and laboratory conditions (see Literature review). Out of 130 articles revised, only 9 have considered the effect of life stages on agar variability (Pickering *et al.*, 1990, Whyte *et al.*, 1981, Marinho-Soriano *et al.*, 1999, Shi Sheng *et al.*, 1984, Penniman and Mathieson, 1987, Penniman, 1977, Hoyle, 1978b, Kim and Henriquez, 1979, Durairatnam and Nascimento, 1985). Moreover, only Whyte *et al.*, (1981) showed agar variability among all life stages of *Gracilaria* (*viz.* tetrasporophyte, carposporophyte, male gametophyte, female gametophyte and vegetative stages). The chemical composition and agar characteristics of *G. cliftonii in* wild populations have been reported only for the tetrasporophyte and carposporophyte stages (Kumar, 2008) but the effect of seasonality on them has not been studied.

The lack of information on the reproductive phenology of *Gracilaria* spp. in many cases is related to the absence of reproductive thalli in the field (Byrne *et al.*, 2002), the difficulty to identify male and female reproductive structures (Withell *et*

al., 1994) and the reduced availability of biomass which from these stages to extract agar (Shi Sheng *et al.*, 1984).

The results of this study have contributed to the existing knowledge of the biomass and reproduction of *Gracilaria* spp. A point that must be reiterated is the lack of information from different areas of distribution of *G. cliftonii* and enough replication within the study area of this research. Therefore, care must be taken comparing these results to other populations of *G. cliftonii*.

5.1.   Life cycle

The results have demonstrated that *G. cliftonii* deviates from the typical *"Polysiphonia"* life cycle as mature cystocarps were observed developing on mature tetrasporophyte thalli unlike previous descriptions (Withell *et al.,* 1994, Byrne *et al.,* 2002). Although, *Polysiphonia*-type life cycle has been described for many *Gracilaria* spp. deviations from this type of life cycle is not uncommon (Guimarães *et al.,* 1999, Polifrone *et al.,* 2006, Plastino and Oliveira, 1988). Gametophytes developed *in situ* on tetrasporophytes have been previously reported for other *Gracilaria* spp. (Polifrone *et al.,* 2006). Different explanations are provided for the occurrence of mixed life cycle in *Gracilaria* including mechanical and genetical explanations.

One explanation is that basal discs arising from spores which germinate close to each other can coalesce and become one plant (*a chimaera*). This has been recorded in *G. gracilis* (as *G. verrucosa*) (Jones, 1956), *G. tikvahiae* and *G. chilensis* (Maggs and Cheney, 1990, Muñoz and Santelices, 1994). Sporelings of the last two species even coalesced with each other (Maggs and Cheney, 1990). Second explanation is that during their development, cystocarps close to each other fuse resulting in a single cystocarp which contain carpospores that are not genetically identical. However, this event, shown in *G. chilensis*, is rare (3%) (Santelices and Varela, 1993b).

The deviations observed for *G. tikvahiae* (as *Gracilaria* sp.) have been explain in terms of genetical origin (Kain and Destombe, 1995). The tetrasporophytes sometimes produce functional diploid male and female gametes which, when appropriately mated, can produce polyploids. The mechanism for this

was shown (van der Meer and Todd, 1977) to be mitotic recombination, a switching of parts of chromosomes during normal nuclear division, allowing a heterozygous condition to become homozygous for mating type in resulting cells and their progeny. Another genetical explanation suggests that mixed reproductive phases are as a consequence of a naturally occurring mutation at a locus where female expression is repressed in males. This allows carpogonia to be formed on gametophyte thalli which are therefore bisexual (van der Meer, 1986). Self-fertilization in such plants produces carpospores developing into diploid bisexual individuals. This life history anomaly again allows the production of polyploids. However, these explanations have originated only from laboratory experiments and may not apply for naturally occurring populations.

Environmental factors can also affect the deviations of *G. cliftonii's* life cycle. Under culture conditions it has been observed that aeration induce the direct development of the sporangial contents into spermatangia, or stimulate mitotic recombination (Plastino and Oliveira, 1988) and that water movement affects the distribution of spores into the water column (Ryder *et al.*, 2004). In this study, the deviation observed could be due to an *in situ* germination of tetraspores on the tetrasporophyte thallus and the faster growth rate exhibited by these tetraspores under laboratory conditions as compared to the tetraspores released into the water column under outdoor natural conditions (Guimarães *et al.*, 1999, Kain and Destombe, 1995).

The significance of this mechanism needs to be further investigated in nature, however, a possible advantage for *Gracilaria* spp. is the reduction of gametophytes that would increase the chances of fertilization and higher adaptability of tetrasporophytic as compared to gametophytic thalli (Oliveira and Plastino, 1994). Studies under laboratory conditions are required to understand the deviation on the life cycle of *G. cliftonii*. The life history of *G. gracilis* (Ogata *et al.*, 1972, Rueness *et al.*, 1987)*, G. tikvahiae* (Bird *et al.,* 1977b, Bird *et al.,* 1977a), *G. debilis* (Oliveira, 1984) and *G. chilensis* (Plastino and Oliveira, 1988) has been completed in culture conditions taking 5-12 months to complete. Although, information on spore release was obtained in this study, it was not possible to complete the life cycle of *G. cliftonii* under laboratory conditions. The lack of growth and further development of

sporelings into adult thalli was not possibly due to the environmental conditions maintained in the laboratory which were not the optimal for the development of *G. cliftonii.*

It has been observed that morphology, growth and physiology of *Gracilaria* spp. (Guimarães *et al.*, 1999) vary when cultured under laboratory conditions. For many *Gracilaria* spp., the interaction between temperature and irradiance is one of the most important factors affecting sporelings growth (Orduña-Rojas and Robledo, 1999, Friedlander and Dawes, 1984, Guzmán-Urióstegui and Robledo, 1999). Optimal growth for some tropical *Gracilaria* spp. occurs under a narrow range (25-28 °C) and transition between growth and heat damage is within 1 °C. However, it is also known that warm-water species of *Gracilaria* have narrow limits of thermal tolerance when compared to cold water species. Optimal conditions for *G. foliifera* (Friedlander and Dawes, 1984) carpospores growth were found at light intensities of 9 to 17 µmol photon m^{-2} s^{-2}, the ability to growth under low light conditions represents an ecological adaptation to survive at the base of the marine vegetation. *G. cliftonii* is usually found in caves or shaded areas and similar mechanism must be acting on the spore development. Since the carpospores released of *G. cliftonii* survived but did not grow at the set conditions, it is possible that carpospores might growth faster under lower temperatures and different light conditions than those one maintained under laboratory conditions. However, further laboratory trials under different conditions are required to achieve further spore growth and development and completion of its life cycle.

Although, the life cycle of *G. cliftonii* was not completed, spore release can provide important knowledge on its recruitment processes and provide the basis for spore micropropagation. The release of carpospore and tetraspores from *G. cliftonii* occurred during first days of experiment similar to previous observations on carpospore release of *G. cornea* (Guzmán-Urióstegui and Robledo, 1999, Orduña-Rojas and Robledo, 1999), *G. edulis* (Rama Rao and Thomas, 1974), *G. corticata* (Rao, 1976) and *G. verrucosa* (Oza and Krishnamurthy, 1968) where spores were released during the first 5 days. Furthermore, it was also observed that one cystocarp can release spores continuously for a period of 5 weeks before disintegration. In red algae, carpospores originate from the gonimoblast of the cystocarp (Cole and Sheath, 1990) and many cases the gonimoblast shows successional development (West and

McBride, 1999) suggesting that spore release occurs repeatedly as sporangia mature.

In *G. cliftonii* a similar mechanism might be operating and is strongly dependant on environmental conditions especially nutrient availability. This provides evidence to the long held assumption that the carposporophytes are nutritionally dependent throughout their development on the female gametophyte (Hommersand and Fredericq, 1990). The reduced availability of nutrients after 5 weeks of culture might reduce the total reproductive potential of the cystocarps from *G. cliftonii* including: the number of spores released overall by each cystocarp, the number of spore discharge events and the spore size. In addition, the differences in the maturity of the cystocarp thus number of spores, can also explain the large standard error observed in all the experiments of *G. cliftonii* carpospore release.

A variety of environmental factors have been cited as critical in the release and growth of spores from macroalgae (Burns and Mathieson, 1972, McLachlan, 1977, Charnofsky *et al.,* 1982, Lefebvre *et al.,* 1987). Three abiotic factors, light, temperature and nutrient concentration have been found to stimulate spore production of macroalgae under laboratory conditions (Santelices and Ugarte, 1987, Oza and Sreenivasa Rao, 1977). The most commonly demonstrated factor is photoperiodism as a component of light. Photoperiodic regulation of gamete or spore production is by far best represented among the red algae, but Dring (1984) has suggested that this is merely the reflection the intense interest in recent years in life history studies on red algae, rather than any real difference between the red algae and other groups of seaweeds. Species which show photoperiodic response have heteromorphic life cycles (i.e *Gracilaria*). An explanation for this response suggests that one of the two phases can only survive part of year, so that the initiation of this phase, including spore formation and release, has to be timely accurately (Lüning, 1981).

Temperature is consider one of the most important factors affecting formation, release and germination of spores on *Gracilaria* spp. (Guzmán-Urióstegui and Robledo, 1999, Rama Rao and Thomas, 1974). Optimal conditions for carpospore release in *G. foliifera* were found to be 20 ^{o}C in the dark or low light conditions (Friedlander and Dawes, 1984). This suggested that exposure of spores to higher light intensities and temperature can be lethal and that spore release might

occur more frequently at night and during the cooler periods of the year. The maximum seawater temperature in the study site was 22 °C, temperature lower than the optimal range reported for spore release of other *Gracilaria* spp. (Rao and Rangaiah, 1991, Guzmán-Urióstegui and Robledo, 1999). Rao and Rangaiah (1991) reported a decrease in spore release at temperature below 26 °C. However, according to Guzmán-Urióstegui and Robledo (1999), temperature and photon irradiance together can be lethal for *Gracilaria* carpospores and affects carpospore release when irradiance is above 50 μmol/m^2/s^2 and temperature above 31°C (Santelices, 1990) but contradicts findings by Garza-Sanchez (2000) who observed higher spore release at light intensities (140 μmol/m^2/s^2). The variability observed when comparing to previous results of *Gracilaria spp.* and other red algae spore release indicates that the effects and tolerance limits of environmental factors depend on geographical distribution of each species. The proposed optimal range on low light intensity provided by Santelices (1990) for spore release has considered mainly tropical algae which live where the variations of the seasonal light and temperature in the field are smaller than in temperate zones and other factors like abrupt changes in salinity or light conditions could be more important for signalling the timing for the release of spores (Hoffmann, 1987).

The onset of sexual and asexual reproduction has been frequently attributed to nutrient availability (DeBoer, 1981), although, it is not well documented, some species have particular nutrient requirement to reproduce. This is the case of *Petalonia fascia* (Hsiao, 1969) which requires iodine in order to reproduce. The other relates specifically to nitrogen perhaps because is one of the most studied nutrient. The evidence suggest that both nitrogen enrichment and nitrogen depletion can induce the production of different types of reproductive cells (Rao and Rangaiah, 1991, Oza and Sreenivasa Rao, 1977). Low concentrations of N and P in seawater are not only essential for tetrasporangia production (Guiry and Dawes, 1992, Oza and Sreenivasa Rao, 1977) but different nutrients in cultural environment could influence the release of tetraspores and carpospores differently (Jones, 1957), this is confirmed by the increment observed in the number of tetraspores released in PES as compared to the number of carpospores released of *G. cliftonii*.

The number of spores produces and the precise timing of spores discharge depend on at least three variables: 1) the physiological state of the parent plant, 2) the degree of maturation of the developing spores and 3) the modifying effect of the environmental factors triggering the process. The number of carpospores released per cystocarp on daily and weekly basis were lower than the number of carpospores released by *G. cornea* (2475) (Orduña-Rojas and Robledo, 1999) and *G. corticata* (4911) (Rao, 1976). However, the number of carpospores released per gram of cystocarpic material was higher than that reported for *G. verrucosa* (19700 carpospores/cystocarpic plant) (Oza and Krishnamurthy, 1968). The difference in number of carpospore released by cystocarp and weight of cystocarpic material could be due to self shedding of the cystocarpic material as compared to the exposed cystocarp, to time of collection of the cystocarpic *G. cliftonii* thalli and to the maturation stage of thalli. The number of carpospores released decrease with a reduction in cystocarp thickness in *G. verrucosa* (Oza and Krishnamurthy, 1968) and *Gelidium sesquipedale* (Santos and Duarte, 1996). This supports observations made for *G. cliftonii* where higher number of carpospores was released by cystocarps of 1.5 mm as compared to 1 mm size. The higher number of spores produced represent a higher fecundity for *G. cliftonii* and higher recruitment of new organism to the population (Guzman-Uriostegui, 2004).

The maximum rate of carpospore viability in *G. cliftonii* recorded in the present study was similar to *G. dura* (36%) (Mantri *et al.*, 2009) and *G. pacifica* (29% carpospore and 34% tetraspore (Garza-Sánchez *et al.*, 2000). The higher survival rate, number of spores and growth rates for tetraspores than carpospores observed could be due to a higher settlement and germination percentage of tetraspores than carpospores. Oza (1976) reported similar differences for *G. corticata* and correlated it to environmental factors and natural distribution of species, while Jones (1959) observed higher growth rate for tetraspores than carpospores for *G. verrucosa*.

Carpospore and tetraspore germination of *G. cliftonii* was similar to the pattern described as *Dumontia*-type germination described by Guiry (1990). In addition, the spore germination process in the present study is also in agreement with previous studies for other *Gracilaria* spp. (Ogata *et al.,* 1972, Oza, 1975, Oza and Krishnamurthy, 1968, Bird *et al.,* 1977a, Rabanal *et al.,* 1997, Yokoya and Oliveira,

1993). The unipolar germination observed in *G. cliftonii* secures the attachment of the spore prior to development of an erect thallus. Considering the distribution of *G. cliftonii* in area with high exposure to water movement, a strong attachment to the substratum is of vital importance to avoid drifting of the thalli (Mantri *et al.*, 2009).

However, few studies have been undertaken to determine the factors involved in the germination process in *Gracilaria* spp. (Waaland, 1990) and no literature is available on *G. cliftonii*. The expulsion of carpospores and tetraspores within a mucilage layer is similar to the process described by Jones (1957) and Boney (1978) for *G. verrucosa* and *Rhodymenia pertusa*, respectively. This is advantageous from the ecological point of view as cohesion via mucilage would increase the sedimentation rate (Hoffmann and Camus, 1989) and reduce drift from the parental habitat. It would also permit a greater residence time on the settling substratum for fixation (Pacheco-Ruíz *et al.*, 1989).

In present study, the coalescence of more than two spore discs was commonly observed and thalli which originated from coalescent spores showed higher thallus length as compared to non-coalescent spores. Coalescence of germinated spores is a common phenomena observed in other red algae (Maggs and Cheney, 1990, Tveter and Mathieson, 1976) and *Gracilaria* spp. (Muñoz and Santelices, 1994, Jones, 1959). This could represent a mechanism to enhance growth of *G. cliftonii* as larger basal area represents larger attachment surface for the sporeling which further results in growth. Sporeling coalescence has been considered as a mechanism to enhance sporeling growth as these spores initiate faster development of erect branches (Santelices *et al.*, 1996). Faster growth rates provide competitive advantage over other growing sporelings in a limited resource environment i.e. low in nutrients and substrate. Early development of sporeling would also reduce the time required to grow beyond the boundary layer or to emerge from the sand (Muñoz and Santelices, 1994).

In field studies, each clump has traditionally been assumed to be unisporic or to represent only one genet independent of its ontogenic origin (Scrosati and De Wreede, 1999). Therefore, at present we lack quantitative information on the demographic consequences of coalescence, either among recruiting sporelings or among field established populations (Santelices and Alvarado, 2008).

Several factors indicate that coalescence should be considered in population studies of macroalgae.

Inter-individual fusions simultaneously reduce the numbers and increase the sizes of individuals in a population. If the individuals cannot be properly defined, then individual-based demographic models cannot be used (Raymundo and Maype, 2004). Similarly, since coalescence also results in modifications of size, size-based demographic models are also of little use. Furthermore, since each sporelings of a coalescing species can either live, die, or coalesce, their survivorship curves would be expected to be more complex than those traditionally described for unitary macroalgae. Both the environment and the number of spores coalescing to form the sporelings seem to influence growth rates in these individuals. Higher survival rate, number of spores and growth rates for tetraspores can provide advantages over carpospores resulting in a dominant tetrasporophyte stage. The characteristics of tetraspores might play a major role in *G. cliftonii* population dynamics, however, more studies are required to confirm and compare observations under natural and laboratory conditions.

5.2.   Biomass

The selection of *Gracilaria* strains should be based in certain traits like adaptability to wide environmental variations, high biomass production and better quality and quantity of agar. In order to select those strains basic knowledge on the growth rates and biomass production under natural conditions under varied environmental conditions is a requisite. The biomass and abundance of the life stages from *G. cliftonii* showed a strong seasonality. Populations of *G. cliftonii* occur in tidal shallow creeks. In this fluctuating environment, macroalgae are subjected daily to emersion cycles and continuous and abrupt changes in temperature, pH, alkalinity, irradiance and nutrient levels, thus it is important to analyse the combined effects of C and N levels in this species (Andria *et al.,* 1999).

The vegetative biomass peak of *G. cliftonii* observed in Spring and Summer is likely to be associated with the high production and liberation of spores from reproductive stages in Autumn and Winter. According to Pickering *et al.,* (1990) a peak in vegetative thalli abundance is expected after the peaks in carposporophyte

and tetrasporophyte abundance due to an increase in spore release from these stages. These spores when settled germinate and undergo a period of active growth which is induced by the rise in seawater temperature and longer photoperiods (Penniman *et al.,* 1986). The favourable conditions of photoperiod and temperature experienced in Spring and Summer could contribute to the growth of *Gracilaria* juveniles followed by their development into reproductive thalli in Autumn. Similar seasonal variability in biomass and abundance of the life stages from *Gracilaria* spp. has were observed in temperate populations (Santelices and Fonck, 1979, Boraso de Zaixso, 1987), where vegetative thalli decline with an increase in the number of reproductive thalli (Whyte *et al.,* 1981, Boraso de Zaixso, 1987).

Tetrasporophyte stage was the most dominant reproductive stage in this study coinciding with the period of maximum total biomass of *G. cliftonii.* Similar observations have been reported (Nelson, 1989, Penniman *et al.,* 1986, Jones, 1959) for other *Gracilaria* spp. The dominance of the tetrasporophyte stage observed in present study could be due to differences in fertility and survivorship of tetraspores and carpospores as reported by Hoyle (1978c) and Destombe *et al.,* (1992, 1993) in *Gracilaria* spp. of temperate and tropical environments. Similar observations on growth and survival between tetraspores and carpospores were observed in *G. cliftonii* spore culture under laboratory conditions (Section 5.1). The differences in occurrence between tetrasporophyte and carposporophyte have been explained in terms of a decay phase (Dixon, 1965), ecological distribution limits (Hoyle, 1978c), thermal tolerance (Santelices, 1990, Destombe *et al.,* 1992, Destombe *et al.,* 1993) and differences in fertility and survivorship of carpospores and tetraspores (Kain and Destombe, 1995). According to Kain and Destombe (1995) the predominance of tetrasporophytes in *Gracilaria* spp. is due to higher survival capacity and growth rates of this stage. This capacity has been demonstrated in *G. verrucosa* where tetraspores had twice the survival rate than the carpospores (Boraso de Zaixso, 1987) and supports the higher growth and survival observed in present study for tetraspores from *G. cliftonii.* Tetrasporophytic (diploid) thalli have a large advantage in enriched culture medium as well as under toxic and mutagen conditions; tetrasporophytic juveniles have a higher growth and sometimes a greater viability (Destombe *et al.,* 1993).

This supports the genetic hypothesis in favour of diploidy advantage as dominance of this phase can protect against the deleterious effects of recessive mutations (Crow and Kimura, 1965). However, the advantage of diploidy (tetrasporophytic) dominance for *G. cliftonii* and the environmental factors which can favour this stage still has to be determined.

In addition, both the tetrasporophyte and carposporophyte occurrences at the same time supports that fertility of *G. cliftonii* is a seasonal event. This suggests that a fertilization period might occur at the same time when the tetrasporophyte is in its most active growth period reaching maximum abundances in Autumn and Winter. However, this result contradicts the observation made by Borazo de Zaixso (1987), who observed a negative correlation between *G. verrucosa* tetrasporophyte and carposporophyte biomass.

Unlike previous studies on *Gracilaria* spp. (Penniman *et al.,* 1986, Pickering *et al.,* 1990, Hoyle, 1978c, Nelson, 1989) highest biomass of the male gametophyte from *G. cliftonii* occurred after the peak of biomass of the carposporophyte stage. In this study, the highest biomass and abundance of the male gametophyte stage observed in Winter was similar to *G. sordida* from New Zealand (Pickering *et al.*, 1990). According to Pickering *et al.,* (1990) and Whyte *et al.,* (1981) a gap between the biomass peak of male gametophyte stage and the peak of the carposporophyte stage is observed. Same authors described the highest peak of the biomass from carposporophyte stage in Spring after the peak of the male gametophyte stage assuming Spring is a fertilisation period for *Gracilaria* spp. Following the same logic fertilisation could have occurred in previous seasons (Summer) as highest carposporophyte biomass was observed in Autumn. The gap between carposporophyte and male gametophyte peaks can be due to differences in the fertility of the male gametophyte of *G. cliftonii.* The low biomass of the male gametophyte stage observed in Autumn and Summer seasons can be the result of the increased difficulty in recognising males during the early developmental stages (Hay and Norris, 1984). In addition, the lowest biomass exhibited by *G. cliftonii* can be explained by the shading offered by other longer stages, like tetrasporophyte thalli, which have higher surface are for light harvesting (Araño *et al.*, 2000)

In present study, total biomass of *G. cliftonii* was significantly lower in Spring when total abundance was higher. The higher abundance of short thalli

confirms that Spring is a period of active growth for *G. cliftonii* sporelings due to an increase in day length and temperature (Littler *et al.*, 1979). The seasonal variations on the photosynthetic and respiratory rates of *G. cliftonii* provides additional evidence that Spring is a period of active growth and explains the higher abundance of vegetative thalli in Spring.

Photosynthetic and respiration rates from *G. cliftonii* were different than previously reported for *G. cornea* (Orduña-Rojas *et al.*, 2002) and *G. tikvahiae* (Penniman and Mathieson, 1985). These differences might be attributed to the natural distribution of these species as compared to the temperate distribution of *G. cliftonii* where environmental conditions (i.e. light intensity, temperature and irradiance) vary from one site to another. Furthermore, intrinsic factors such as thallus morphology (Penniman *et al.,* 1986, Nelson, 1989) and circadian rhythms of macroalgae (Jones, 1959) might also affect photosynthetic and respiration rate in *G. cliftonii.*

In previous studies, the seasonality of the biomass and reproductive phenology of *Gracilaria* spp. has been mainly attributed to changes in light by Penniman (1977) and seawater temperature by Boraso de Zaixso (1987), however, the influence of these parameters on *Gracilaria* spp. is not clear. In this study, vegetative biomass of *G. cliftonii* was related to day length. It is known that *Gracilaria* spp. show higher photosynthetic efficiency which allows them a rapid growth under high light intensities (Lapointe, 1981). This efficiency on light utilisation could have produced higher growth and photosynthetic rates of the juvenile *G. cliftonii* thalli in Summer and utilise most of the energy for growth. Similar results were observed by Whyte *et al.,* (1981) where optimum growth of *G. verrucosa* was positively correlated to light and by Liang *et al.,* (1989) for *G. sordida* grown under laboratory conditions which shown higher growth rates at higher light intensities. Similarly, *G. lemaneiformis* thalli grown at low irradiances compared to the higher irradiances resulted in a weakened transport of Ci towards Rubisco within the cell during photosynthesis (Zou and Gao, 2009).

The importance of light for *Gracilaria* spp. is related to the natural distribution of this group. *Gracilaria* sp. which grows on the subtidal level are subjected to great variations in light intensities, sometimes due to the turbidity of

water, cloudy sky, or overcrowding by other algae and distribution on the substratum. Exposure to full light in tidal pool has deleterious effects on *Gracilaria*, especially young plants. Mature thalli develop well in the infra-littoral zone and have been reported to grow luxuriantly in the intertidal zone (Boraso de Zaixso, 1987).

The negative correlation between photosynthesis and rainfall and positive between respiration and rainfall provides additional evidence of the importance of light for vegetative biomass of *G. cliftonii*. The conditions of cloudiness observed due to an increase in rainfall in Winter could reduce the availability of light for *G. cliftonii* during these seasons.

The effect of nutrients on *Gracilaria* growth is known to be dependant on other environmental factors like light, salinity and temperature (Buschmann *et al.*, 1996). The uptake rate of nitrogen *G. tenuistipitata* var. liui reached as high as 0.32-0.36 $\mu mol.g^{-1} h^{-1}$ at favourable light intensity of 800-2400 μmol photon $m^{-2} s^{-1}$, while that of phosphorus reached 0.017-0.018 $\mu mol\ g^{-1} h^{-1}$ at 320-1600 μmol photon m-2 s^{-1}. The nutrient uptake rate reached the maximum at pH 8.0. In addition, significant difference of N uptake rate at different temperatures or salinities and that of P uptake rate at different temperatures was observed (Xu *et al.*, 2001).

The importance of environmental factors on *G. cliftonii* can be explained by the negative correlation observed between photosynthesis and $[NO_3^-]$ that can be due to a preference of *G. cliftonii* of ammonium as nitrogen source over nitrates/nitrites as former was the inorganic nitrogen form most available. Limitation of algal growth by N availability results in a decrease in N:C ratios, photosynthetic pigments and protein content (Lapointe and Duke, 1984). Previous studies on *Gracilaria* spp. (Dixon, 1965, Smit, 2002) have reported a higher affinity of $[NH_4^+]$ over $[NO_3^-]$ and a reduction of NO_3^- uptake rate by up to 38% in the presence of NH_4^+. Nutrient uptake is reported to be dependant of the concentration of other nutrient being taken up and ionic status, the molecular form of the element and on environmental factors like temperature (Lobban and Harrison, 1994). In addition, a marked decreased in nitrate uptake and affinity was observed by *Laminaria longicruris* (Harlin and Craigie, 1978) and for *G. gracilis* (Smit, 2002) with a decreased in seawater temperature.

NH_4^+ was the inorganic nitrogen source with higher concentration in seawater and might be the main nitrogen source involved in the growth of *G. cliftonii*. Higher affinity of NH_4^+ by macroalgae is a common phenomena observed in nitrogen limited environments (Fujita, 1985). Furthermore, the mechanisms of nitrogen uptake and affinity to different nitrogen sources by *G. cliftonii* can represent an important ecological adaptation to its environment. The enhancement of nutrient uptake rate following stress in the form of N limitation has often been proposed as a mechanism that enables algae to maintain growth in an environment with fluctuating nutrient concentrations (Fujita, 1985). Similarly, the affinity of *Gracilaria* for NH_4^+ as nitrogen source can explain the negative correlation observed between tetrasporophyte and carposporophyte biomass with $[NO_3^-]$ respectively. Furthermore, in Winter, highest biomass of tetrasporophyte and carposporophyte coincided with highest concentration of NH_4^+ in seawater. The availability of NH_4^+-nitrogen in Winter during the period of reproduction of *G. cliftonii* might be promoting the uptake of NH_4^+ over NO_3^- and incorporating it into the amino acids and protein pool (Lignell and Pedersén, 1987, Bird *et al.,* 1982).

Besides the availability of nitrogen ions in seawater, distinct differences in nutrient uptake also occur between early life stages and maturity of the thalli of the same species. Ammonium and nitrate uptake rates for *Fucus distichus* germlings were 8 to 30 times higher than those for the mature thalli (Thomas *et al.*, 1985). The presence of NH_4^+ inhibited NO_3^- uptake in mature plant but not in germlings. These characteristics of nutrient uptake indicate that germlings are better adapted for procurement of nitrogen than are mature thalli. This difference in uptake ability is due to the large proportion of storage and support tissues in mature thalli, tissue that do not actively require nitrogen (Thomas *et al.*, 1985). It was also observed that adult thalli can loose of the ability to take up NO_3^- as compared to younger thalli, which is metabolically active and require both forms of nitrogen. For *G. cliftonii*, similar mechanism might be operating as mature reproductive thalli might be selective for NH_4^+ in Winter, while young plants in Summer might have a higher requirement for NO_3^- nitrogen forms.

The effect of $[PO_4^-]$ on *Gracilaria* spp. is limited but indicates that phosphorus (P) limitation occurs only when the internal concentration of the plant decreases to critical levels (Friedlander, 2001, Friedlander and Dawes, 1985, Lewis

and Hanisak, 1996, Dawes *et al.,* 1984, Navarro-Angulo and Robledo, 1999). Productivity of *Gracilaria* sp. was reduced after 4 weeks of culture under zero P supply and productivity was P-limited only after internal P reserves were depleted below a critical level of 0.07% dry weight. (Lewis and Hanisak, 1996). Furthermore, *G. tikvahiae's* phosphate uptake showed three uptake phases: two saturation phases at 0 -0.2 and 0 -2 µM and a linear phase at 0-11 µM which were related to the seasonality on [PO_4^-] in seawater. *G. tikvahiae* was phosphorus limited in Summer and nitrogen and phosphorus limited in Winter (Friedlander and Dawes, 1985). The positive correlation between [PO_4^-] in seawater and thallus length of carposporophyte and tetrasporophyte indicates that *G. cliftonii* productivity might be also limited by phosphorus.

In addition, some studies indicate that phosphorus enrichment affect the productivity of *Sargassum* by increasing its growth and photosynthetic rates (Lapointe, 1986) providing evidence that P is also a limiting nutrient in marine environments. In present study, highest plant length did not coincide with highest [PO_4^-] in seawater indicating that other parameters like temperature might be involved in *G. cliftonii* development. More than [PO_4^-] itself, the relation of this element with nitrogen and N:P ratio might be regulating *G. cliftonii* growth as observed in previous studies (Kumar, 2008, Lapointe, 1987, Navarro-Angulo and Robledo, 1999). Furthermore, previous studies indicate that higher growth and agar characteristics are obtained when *G. cliftonii* is cultured with supplemented N-P-K in ratio of 1:2:1 (Kumar, 2008). Similar study has shown that *G. cornea* growth rate is affected by the N:P at 10:1 ratio (Navarro-Angulo and Robledo, 1999) where lower ratios reduced the growth due to a reduction on the photosynthetic capacity of the thalli.

The interaction with nutrients like K^+ also affect *G. cliftonii* growth and chemical properties probably influencing nitrogen absorption and storage enhancing nutrient absorption (Kumar, 2008). Although, ions like K^+, Na^{2+} and Cl^- are not limiting nutrients for macroalgae, they affect uptake rates of other nutrients (N and P) and are interfere in osmoregulatory processes (Lobban and Harrison, 1994). The role of K^+ in ionic relations in seaweeds is non-specific as it is only one of the several monovalent cations involved in osmoregulation. K^+ has a more specific role

as an enzyme activator and in many protein syntheses (O'Kelley, 1974). Gutknecht (1965) reported active efflux of Na^+ and active influx of K^+ due to ionic regulation to maintain equilibrium between thalli and water in *G. foliifera.* Similar results were observed by Kumar (2008) with a similar $[K^+]$ and $[Na^+]$ of thalli and water under different ionic profiles suggesting *G. cliftonii* also maintains the equilibrium of K^+ and Na^+ by active influx of K^+ and Na^+ from water to thalli.

Changes in ion concentration and osmotic pressure in macroalgae is mainly due to changes in salinity of seawater (Lobban and Harrison, 1994). The relation observed between male gametophyte biomass with rainfall and osmolality respectively, might be due to the optimum salinity range for the male gametophyte. The reduction in growth, thus biomass, of the male gametophyte when salinity is altered could be a result of osmoregulation activity of the thallus. As the thallus has to maintain ionic equilibrium most of the energy could be utilised for this purpose rather than growth. However, because of the difficulty of unravelling the roles of environmental factors which control *G. cliftonii* productivity from field data alone, further studies under laboratory conditions are required to determine the effect of these parameters on *G. cliftonii* growth and reproduction.

The productivity of *Gracilaria* spp. has been reported to be affected by the sediments characteristics but information is limited (Pizarro and Barrales, 1986, Glenn *et al.,* 1996) mainly due to the difficulty of determining this parameters in the field (Littler and Littler, 1985). However, information on the sediment characteristics can provide indirect information of the water movement in areas where *Gracilaria* populations grow.

The sediments at the study site were mostly characterised as coarse sediments and it was also evident that a significant seasonal effect existed. The Autumn samples, with higher components of fine sediments, suggest a decrease level of mixing over the rest of the seasons. This suggests that water movement was reduced during Autumn, allowing finer sediments to settle out of the water column; Winter storms resuspended and exported finer sediments, resulting in a higher proportion of coarse sediments in the following seasons. Similar patterns on sediment distribution in Western Australia have been previously reported by Keulen and Borowitzka (2003) and by Kendrick *et al.,* (1998) and is mainly associated to local wind waves

and ocean swells which increase during Winter storms.

Water movement has long been recognised as a prime factor regulating the growth and distribution of submerged aquatic macrophytes (Madsen *et al.*, 2001). Pizarro and Barrales (1986) observed a decline of *Gracilaria* sp. biomass due to sand displacement and surfacing of the sandstone substratum caused by water turbulence. Parameters of *G. cliftonii* like distribution, biomass and abundance might be related to other characteristics of the sediments as observed for *Gracilaria* spp. from Namibia (Molloy and Bolton, 1995). One of the disadvantages of the sediment analysis gives particle sizes for each sample but this does not relate directly to the texture of the sediment because organic matter and detritus is excluded from the analysis (Anderson, 2007). Wanless (1981) noted a correlation between increased productivity in seagrasses and an increase in the contribution of fines to sediment grain size distribution. He proposed that this correlation might be the result of seagrass detritus breaking down and *fining-up* of the sediments within meadows. This is unlikely to be the case of this study because of overall higher wave energies than those of studied by Wanless (1981). In similar studies, Keulen and Borowitzka (2003) indicated a very low retention of detrital material within *Posidonia sinuosa* meadows distributed within the Shoalwater Marine Park (study site in this research).

The high levels of wave energy experienced by seagrass meadows prevent the accumulation of large amounts of such detritus, with much material being washed up on adjacent shorelines where it decomposes. This might explain why *G. cliftonii* biomass and abundance were not correlated to particle size of the sediment of the study site. Further studies are required to understand the effect sediment on *G. cliftonii* populations. However, sediments might be affecting in two ways to *G. cliftonii*: 1) distribution due to spore attachment and 2) productivity due to interaction with nutrient availability (Kelderman, 1984).

The concentration of nutrients in seawater from the study area was similar to previous records and reflects the conditions of oligotrophic waters in Western Australia (Pattiaratchi and Imberger, 1991). The higher concentration of NH_4^+ and PO_4^- in Winter and Spring can be due to the higher mixing of the water column, and reflects an increase of water movement due to storms and stronger winds (Goldberg and Kendrick, 2004). Similarly, temperature, rainfall and photoperiod recorded at the

study site are similar to previous reports and are within the range expected for the four seasons defined in Western Australia (Pearce *et al.,* 1999, Pearce and Pattiaratchi, 1999).

5.3.   Epiphytism

The effect of environmental factors on *Gracilaria* spp. has been widely reported in the literature. Despite the importance of biological interactions (i.e. competition, epiphytism) on the population structure of macroalgae, little is known for *Gracilaria* spp. Furthermore, out of fifty references reviewed (Fletcher, 1995) which described and identified epiphytes of *Gracilaria* spp. only a few have considered the seasonality of epiphytic loads (Buschmann *et al.,* 1997, Leonardi *et al.,* 2006, Kuschel and Buschmann, 1991, Friedlander, 1991, González *et al.,* 1993) and interaction mechanisms between *Gracilaria* and its epiphytes (Quirk and Whetherbee, 1980, Dawes *et al.,* 2000, Leonardi *et al.,* 2006).

Most of the epiphytes belong to the Rhodophyta according to Whittick (1983) as observed in *G. cliftonii*. These epiphytes have been previously reported as *Gracilaria* epiphytes (Ugarte and Santelices, 1992, Fletcher, 1995, Westermeier *et al.,* 1993, Westermeier *et al.,* 1991). Furthermore, the great diversity of red macroalgae at the study site (Roberts *et al.*, 2002) can explain the dominance of red algae epiphytes for *G. cliftonii*. In addition, the epiphytes observed in present study are reported for seagrass *Amphibolis* sp. distributed around Western Australia coastline (Ducker *et al.*, 1977) suggesting that the epiphytes observed are not specific to *G. cliftonii* and reflects the algal vegetation of the local environment.

Higher epiphytic loads observed in Spring can be due to the environmental factors and epiphyte specific characteristics. Water temperature, light intensity and water movement during Spring and Summer enhance epiphyte growth while it gets decreased towards Winter. In addition to increased water movement conditions developed due to wind and waves during Winter months might also drift the epiphytes away from *G. cliftonii* (Norderhaug *et al.*, 2002).

Epiphytes can reduce the amount of light reaching the host algae, compete for nutrients and dissolved gas in the seawater, add weight to the host alga thereby promoting the detachment of the entire, or part of the host alga (Kuschel and Buschmann, 1991) and release exudates that can promote or be detrimental to the host alga. The resistance to epiphyte growth reflected by lowest epiphytic load in Winter can be attributed to the higher growth rates of *G. cliftonii* similar to observed by *Gracilaria firma* and *Gracilaria* sp. or due to a change in the pH at the thallus surface caused by a rapid metabolic rate. It was also observed that when the growth of *Gracilariopsis bailinae* and *Gracilaria* sp. decreased epiphytes presented inverse pattern increasing their growth (Araño *et al.*, 2000), thus it was suggested that fast growing species are less prone to epiphyte contamination.

Epiphytism of *Gracilaria* has been observed, mainly peaking in Spring and Summer and are associated to increase water temperature and irradiance (Kuschel and Buschmann, 1991). In contrast, Pizarro and Santelices (1993) reported that Winter favoured epiphytes decreasing with the approach of Spring and renewed growth of *Gracilaria*. Similarly, in Western Australia, two peaks of diversity and abundance of epiphytes have been observed one in Summer and second maximum peak in late Winter (Womersley, 1978).

However, seasonal abundance is highly variable and varies with the epiphyte species. Seasonal differences have also been reported on *Gracilaria* natural populations (Westermeier *et al.,* 1991, Westermeier *et al.,* 1993) for example *Giffordia* spp. is very abundant in Spring, while *Ceramium* spp. is more abundant in the Summer. Pizarro and Santelices (1993) associated this variability to changes in environmental conditions such as surface water temperature and light intensity.

The *Ceramium* species observed have been previously reported as common epiphytes on macroalgae from the study site (Womersley, 1978). The dominance of the Ceramialean epiphytes has been reported for *G. chilensis* (Buschmann *et al.*, 1997). This group is known for their invasion success as their life cycle is dominated by an asexual stage. This clonal morphology provides them with a method of vegetative propagation that has important ecological consequences, thus resulting in a rapid occupation of a large amount of space (Santelices *et al.*, 1995).

Similarly, the presence of *P. forfex* and *H. episcopalis* during most of the year can be related to the absence of a seasonal cycle of growth and reproduction observed in *Polysiphonia* species for the first case. The occurrence of *H. episcopalis* might be due to two important characteristics observed in this group. First, the presence of multiple meristems and abundant production of spores provides them a rapid vegetative growth. Second, the presence of hook like tips allows this species from being drifted away from the attaching to host during Winter months (Cecere *et al.*, 2004).

G. *cliftonii* epiphytes presented a differential distribution on its thallus attaching in three different ways, epiphytes growing on the surface without penetration, alteration of the cellular structure at the attaching site and penetration into the host tissue. Two explanations for these differences in distribution and penetration by epiphytes are provided. The first one is related to the ability of the epiphyte to digest the host wall and medullary cells. The interruption of the epiphyte wall and no apparent tearing of the host wall indicate that penetrative rhizoidal cells from *P. forfex* and *C. isogonum* can penetrate *G. cliftonii* degrading its wall enzimatically as observed for *Harveyella mirabilis* (Goff and Cole, 1973). Secondly, the differences in host wall construction affect the type of epiphyte attaching and penetration. Species with thicker outer cortical wall and more structurally diffused deck lamellae are more resistant to epiphytism as reported for *G. tikvahiae* and *G. cornea* and its epiphyte *Ulva lactuca* (Dawes *et al.*, 2000). Besides host intraindividual characteristics might be operating and can explain the two types of attachment observed for *B. plumosa* (Prasad, 1986).

5.3.1. Proximate composition

The present study shows that the chemical composition of *G. cliftonii* is similar to previous studies (Wen *et al.,* 2006, McDermid and Stuercke, 2003, Norziah and Ching, 2000, Friedlander *et al.,* 1987, Penniman and Mathieson, 1987) i.e. rich in ash, protein and carbohydrates, which depend on seasons (temperature and photoperiod) and life stages.

Present study is the first report for moisture content of *G. cliftonii* but there are few reports on moisture content of *Gracilaria* spp. (Wen *et al.,* 2006, McDermid and Stuercke, 2003, McDermid and Stuercke, 2004). Moisture content is the most important factor affecting the quality of food products when seaweeds are exploited for phycocolloid extraction as it is required to contain minimum of 35 % of moisture content to assure good quality and price of the harvested product (Foscarini and Prakash, 1990). However, the variations observed in present study are due to various factors like season and stages. The difference in methodology adopted that is, time of cleaning the thalli and sample size utilised (Kumar 2008, pers. comm) could be the major factor influencing the surface area of exposure in the oven and thus drying time and moisture content of the thalli. A variation in proximate composition of thalli is observed when maintained under culture conditions or modified environmental parameters, thus seaweed collected from field should be analysed immediately in the laboratory (Dawes, 1998). Other differences in moisture content could be due to method of drying like oven versus freeze drying as former method is more efficient than latter (Stegen *et al.*, 1998). Other variations in moisture content analysis are due to sampling location, time of collection and biological factors (health, age and reproductive status) which could influence allocation of resources within the thalli thus resulting in variation of seaweeds nutritional composition (McDermid and Stuercke, 2004).

High moisture content observed for tetrasporophyte and carposporophyte in Autumn and Winter and for vegetative stage in Summer could be related to the morphology and reproductive status of the thalli. During these seasons, carposporophytic and tetrasporophytic thalli attain the maximum size and biomass resulting in an increase of tissue available for water storage. An increase in tissue affects the uptake rate and concentration of ions which form compound bonds with water molecules which also explains higher moisture content observed in PS1 compared to PS3.

Variation in ash content from different life stages of *G. cliftonii* between 2008 and 2009 cannot be concluded as it was only observed for carposporophyte stage in Autumn of each year. However, it might be due to the fact that carposporophyte stage has different physiological responses to environmental changes as compared to

other life stages and that factor such as temperature and day length were optimal for carposporophyte.

The lower ash content of the tetrasporophyte stage of *G. cliftonii* in Summer provides evidence to support that ash content of *Gracilaria* spp. is dependent on the reproductive state of thalli and each stage might respond distinctly to changes in environmental factors (Richmond, 1986). The lower ash content observed can be further explained through lower nutrient uptake rate and growth of *G. cliftonii* in Summer. Higher biomass of reproductive tetrasporophytic thalli was observed in Autumn and Winter indicating period of highest activity and thus growth. In addition, higher concentration of ammonium, nitrites and phosphates in Winter result in an increase in nutrient uptake rates of tetrasporophytic thalli of *G. cliftonii*.

The seasonality in ash content might also be due to cellular adjustments to osmotic stress (De Castro Araújo and Tavano Garcia, 2005) and/or osmoregulation. Kumar *et al.,* (2009) observed that *G. cliftonii* ash content and mineral composition is due to ionic regulation between internal thalli and external medium of ions like K^+, Ca^{2+}, Mg^{2+} and Fe^{2+}. Table 5.1 shows the variation in mineral composition of cultured *G. cliftonii* with change in ionic concentrations and salinity. Thus, osmoregulation and nutrient uptake along with growth could be an important adaptative mechanism for *G. cliftonii* in response to changes in salinity and ionic concentrations in the environment.

Table 5.1 Mineral composition (%db) (Mean ± S.E.) of *G. cliftonii* from natural populations and culture under different ionic and salinity profiles

Ionic profiles	Na	K	Ca	Mg	Fe	S
Natural population	$5.0 \pm 0.2^{a,b}$	9.5 ± 0.4^{a}	0.6 ± 0.0^{a}	$1.1 \pm 0.1^{a,b}$	0.015^{a}	2.0 ± 0.2^{a}
$^{35}ISW_0$	5.5 ± 1.5^{a}	6.6 ± 1.2^{b}	0.5 ± 0.1^{a}	$1.3 \pm 0.2^{a,b}$	0.019^{a}	2.3 ± 0.0^{a}
$^{35}ISW_{33}$	$4.4 \pm 0.9^{a,b}$	6.9 ± 0.8^{b}	0.6 ± 0.1^{a}	$1.3 \pm 0.2^{a,b}$	0.021^{a}	2.2 ± 0.1^{a}
$^{35}ISW_{66}$	6.4 ± 0.4^{a}	$7.1 \pm 0.9^{a,b}$	0.6 ± 0.1^{a}	1.5 ± 0.1^{a}	0.045^{b}	2.1 ± 0.1^{a}
$^{35}ISW_{100}$	$4.8 \pm 0.7^{a,b}$	6.8 ± 0.9^{b}	0.6 ± 0.1^{a}	$1.3 \pm 0.2^{a,b}$	$0.024^{a,b}$	2.0 ± 0.2^{a}
$^{25}ISW_0$	3.9 ± 1.1^{b}	6.0 ± 0.5^{b}	1.1 ± 0.2^{b}	1.0 ± 0.1^{b}	$0.026^{a,b}$	2.2 ± 0.1^{a}
$^{25}ISW_{33}$	2.6 ± 0.6^{b}	$7.1 \pm 0.8^{a,b}$	2.1 ± 0.8^{b}	1.1 ± 0.1^{b}	$0.034^{a,b}$	2.3 ± 0.2^{a}
$^{25}ISW_{66}$	3.6 ± 0.5^{b}	$7.6 \pm 0.5^{a,b}$	1.4 ± 0.9^{b}	1.2 ± 0.1^{b}	$0.028^{a,b}$	2.2 ± 0.2^{a}
$^{25}ISW_{100}$	3.1 ± 0.3^{b}	6.5 ± 1.0^{b}	2.7 ± 0.8^{b}	1.0 ± 0.0^{b}	$0.031^{a,b}$	1.9 ± 0.1^{a}

Modified Source: (Kumar and Fotedar, 2009)

Other parameters like water and nutrient content of sample can affect ash content in *G. cliftonii* as suggested by Ekman *et al,.* (1991). They observed that thalli contained less water when subjected to elevated salinity, while, raised algal nitrogen content, influenced the osmoregulation pattern of *G. sordida*. The changes in pools of low molecular weight metabolites, such as free amino acids or floridoside, are quantitatively small in the tissue, but their contribution to cytoplasmic osmotic acclimation can still be significant (Kirst, 1990). Prasad (1986) also observed that ash content of *Gracilaria* spp. was related to changes in salinity. In addition, a reduction of salinity has been observed to result in a decrease in ash content of macroalgae (Zarodnik, 1973, Munda and Kremer, 1977, Durako and Dawes, 1980)

The differences observed in ash content with previous studies could be due to difference in drying time and method that is, oven drying process for 8 hours utilised in present study as compared to freeze drying with unknown time used by Kumar (2008).

In addition, the cleaning methodology and presence of seawater on *G. cliftonii* samples can be a source of variation as presence of seawater and calcareous organisms can contribute increasing the salt content of the sample (Özer *et al.*, 2004). Higher protein content observed for tetrasporophyte, carposporophyte and vegetative stages of *G. cliftonii* in Winter than Summer can be due to breakdown of protein structure and interference with enzyme regulators at higher temperatures (Renaud *et al.*, 2002). These metabolic changes have been explained to be related to protein-carbohydrate ratios of *Gracilaria verrucosa*. Bird (1984) explained that there is shift between carbohydrates syntheses during Summer to protein synthesis during Winter which is associated to changes in water temperature. In addition, they found a zone of transition or critical protein-carbohydrate ratio at 0.38 when growth rates were highest. They observed that the highest protein-carbohydrate ratios were found in Winter coinciding with the highest biomass of *G. verrucosa*, minimum water turbidity, lower temperature and higher total inorganic concentrations. Similar seasonality of protein content has been observed for *G. cornea* (Orduña-Rojas *et al.*, 2002). Higher protein values in Winter can also be associated to decreasing salinities and increased rainfall (Banerjee *et al.*, 2009).

Biomass of tetrasporophyte and carposporophyte stages coincided with the highest protein values in Winter. The relation between biomass and growth with protein synthesis explain the variation on reproductive stages of *G. cliftonii*. The higher availability of NH_4^+ in Winter have promoted its uptake by *G. cliftonii* incorporating it into the amino acids, triggering the synthesis of proteins over carbohydrates (Lignell and Pedersén, 1987) thus resulting differences among life stages. Kumar (2008) cultured *G. cliftonii* under different ionic profiles, and explained that protein content under culture conditions was higher than natural conditions due to changes in ion concentration, ionic stress and availability of nitrogen ions (Aderhold *et al.*, 1996, Caliceti *et al.*, 2002).

Overall, protein content of reproductive stages of *G. cliftonii* was significantly higher when water temperatures were lower. However, the lag observed in the protein peaks among reproductive stages with higher values for the male gametophyte in Winter and tetrasporophyte and carposporophyte stages in Spring can be the result of differences in responses to light and temperature among stages.

In *G. sesquipedale* the male gametophyte thalli showed the highest content of phycoerythrin and total soluble in response to a reduction in light (Carmona and Santos, 2006) and had faster growth rates as compare to the carposporophyte stage. The higher growth rates observed in *G. verrucosa* by Destombe *et al.,* (1993) under higher UV radiation support the higher protein contents of tetrasporophyte in Spring. The low protein content of male gametophyte in PS2, coinciding with longer daylength, provides additional evidence that protein variation in *G. cliftonii* is the results of the interaction of life stages and light.

Protein content of *G. cliftonii* was similar to previous reports (Kumar, 2008) but higher than reported for *G. cornea* (Orduña-Rojas *et al.*, 2002), *G. parvispora, G. salicornia* and *G. tikvahiae* (McDermid and Stuercke, 2003) and lower than *G. cervicornis* (Marinho-Soriano *et al.*, 2006). Variations can be due to differences between species and geographical distribution as previously reported (Robledo and Freile-Pelegrin, 1997, Wong and Cheung, 2000).

Carbohydrate content was not influenced by life stages but varied in both temperature and photoperiod seasons. Carbohydrate content showed negative trend to protein content as higher carbohydrate values were observed in Summer and lower in Winter. Increasing seawater temperature as well as day length in Summer might be triggering carbohydrate biosynthesis over protein for *G. cliftonii* which is in confirmation with previous studies on *G. cornea* (Orduña-Rojas *et al.*, 2002) and *G. cervicornis* (Marinho-Soriano *et al.,* 2006). Carbohydrate synthesis is known to be favoured by light intensity, temperature and decrease of nitrogen (as in Summer) whereas, protein synthesis occur when light intensity and temperature decreases (as in Winter) (Rosenberg and Ramus, 1982). In addition, carbohydrate synthesis is also related to periods of active growth and increased photosynthetic activity (Rosenberg and Ramus, 1982). The higher photosynthetic rates observed in Summer due to increase temperatures an photoperiod and observations in the field suggest that Summer is a period of active growth for *G. cliftonii*.

The inverse relationship observed between carbohydrates and protein content of *G. cliftonii* is similar to previous studies for other macroalgae (Banerjee *et al.,* 2009, Kaehler and Kennish, 1996, Rosenberg and Ramus, 1982, Hernández-Carmona *et al.,* 2009), however, this pattern is not clearly defined for many other

species (Renaud and Luong-Van, 2006). The metabolic pathways of C and N are highly coordinated and coupled, since CO_2 fixation and N assimilation compete for assimilatory power and C skeletons.

The inverse correlation observed between carbohydrate and ash content indicates that nutrients, salinity and pH might be contributing to the carbohydrate seasonality. Several authors (Marinho-Soriano *et al.,* 2006, Munda and Kremer, 1977, Perfeto, 1998, Banerjee *et al.,* 2009, Perfeto *et al.,* 2005) have found that salinity, phosphate and pH affect carbohydrate synthesis. Kumar (2008) also observed this relationship for *G. cliftonii* and attributed it to changes in ionic concentrations. The increment in carbohydrate content with increase salinity has been observed as a response to maintain osmoregulation. This osmotic balance can be achieved through the synthesis of organic solutes or synthesising polysaccharide (Perfeto *et al.*, 2005).

Another important factor affecting growth of seaweed cultures is the high seawater pH resulting from photosynthetic uptake of inorganic carbon (Maberly, 1990). Lower concentrations of HCO^{-3} and CO_2 at high pH and low diffusion rates of CO_2 in seawater can in several occasions reduce rates of CO_2 uptake, photosynthesis and algal growth (Beer, 1994). The effect of pH on algal metabolism (i.e. carbohydrate biosynthesis) suggests that parameters such as temperature and salinity are also involved. Growth experiments on *G. tenuistipitata* recorded a broad tolerance range of salinities with higher growth rate with increasing salinity (Israel *et al.*, 1999). However, decreasing temperatures inhibited the growth of plants in 20 ppt and 30 ppt salinities, whereas growth rates were unaffected in regular seawater. In addition, Israel *et al.,* (1999) growth was strongly stimulated at 39 ppt and at a pH of 6.5 and 7.0 possibly due to relatively high CO_2 concentrations at such pH values. While algal growth can be made optimal through control of salinity or pH in the laboratory, a cost-effective outdoor cultivation system at higher salinities (i.e. inland saline water) could also support high growth in combination with abundant light and constant mixing through aeration and seawater flow outdoors.

Low water exchange rates conditions similar to those found in Summer seasons can lead to a rapid rise in ambient pH, followed by inorganic carbon (Ci) limitation, particularly at high densities (Friedlander and Levy, 1995).

Growth can be inhibited when pH values are 9.0, or above, which commonly occur in culture systems. However, high growth rates have been measured in non-aerated pond cultures of *G. tenuistipitata* in which the pH quickly increased to values around 10 (Haglund and Pedersen, 1993). In this study, highest biomass of *G. cliftonii* was maximal at a pH below 8.07, similar to observed by Israel and Friedlander (1998) for *Gelidiopsis* sp. grown under high seawater pH. They observed four times higher growth of *G. tenuistipitata* in outdoor tanks due to nutrient and Ci limitations, or water motion in laboratory setups. In this study, higher carbohydrate content in Summer coincided with higher pH and salinity which could affect the photosynthetic rate and growth of *G. cliftonii* regulating its carbohydrate synthesis.

In addition, the inverse relation between carbohydrate and ash content could be due to differences in the caloric content of these components. Thalli with faster growing rates have higher energy demands have a preference for carbohydrate synthesis over ash which has low caloric values (Shivji, 1985) Use of ash content and caloric values then can provide useful indices for estimating growth rates of seaweeds both in culture and in the field. Lapointe (1981) observed that ash content was negatively correlated to growth and positively correlated to the carbon-phycoerythrin ratio of *Gracilaria* sp. A 5% change in ash content reflected a twofold change in the growth rates.

Higher carbohydrate content of *G. cliftonii* in PS3 coincided with higher values of osmolality and phosphate concentration of the seawater. Enrichment of the culture media with inorganic phosphorus results in an increase in carbohydrate content (Chopin and Wagey, 1999). Chopin *et al.,* (1991) suggested that his element can play a role in carbon flux regulation affecting the synthesis of glucose, galactose and carrageen. This positive relation between phosphorus and carbohydrate content of the thalli can be an ecological strategy of energy storage to be utilised when this nutrient is limited.

5.3.2. Physicochemical properties

The present study demonstrates that the variations on the physicochemical properties *viz.* swelling capacity, water and oil retention capacity of *G. cliftonii* are dependent on interaction of year, seasons and life stages. Several authors suggest that the physicochemical properties are influenced by polysaccharide biosynthesis and its chemical structure, however, no explanation is provided to support the statement (Ruperez and Saura-Calixto, 2001, Fleury and Lahaye, 1991, Jimenez-Escrig and Sanchez-Muniz, 2000, Lahaye, 1991). Furthermore, the physicochemical properties of macroalgae have been determined in fewer occasions to understand the nature of their variations (Jimenez-Escrig and Sanchez-Muniz, 2000).

Swelling capacity in Winter of reproductive stages (tetrasporophyte, carposporophyte and male gametophyte) was higher than in Summer. The higher swelling capacity in Winter coincided with a decrease in the seawater temperature and photoperiod which in turn affect carbohydrate biosynthesis. This can also be supported by the analysis made in terms of photoperiod seasons. In addition, the inverse correlation was observed between swelling capacity with osmolality of seawater (R^2=0.99; p<0.05). Kumar (2008) reported that physicochemical properties are dependent on the culture media salinity and nutrient concentration. Therefore, swelling capacity of *G. cliftonii* can be dependent on osmoregulation and ion content within the thallus rather than carbohydrate content.

Difference in the swelling capacity between male and carposporophyte stages can be due to tissue rupture of the carposporophyte (i.e. cystocarps) during spore liberation which affects water absorption. The male gametophyte has reproductive structures which are internal and smaller than the cystocarps and could contribute in less degree to tissue damage (Hommersand and Fredericq, 1990). In addition, differences in herbivory between male and carposporophyte could also affect the strength and toughness of the tissue which influence the capacity of tissue breakage. A response of algae to herbivory results on a change of the mechanical properties of the thallus by changing the thickness of the cell wall or cell layers, the structure of the polysaccharide blocks and the packing density of load bearing cells as compared to the amount of intercellular matrix (Lowell *et al.*, 1991).

In addition, other factors like the presence of water soluble materials and sample handling could be affecting this property like protein of the cell wall and free ion of the cellular wall can also make bonds with water molecules. Specially, matrix structure breakdown in experimental condition which is known to affect swelling capacity (Robertson *et al.,* 2000). The swelling capacity of *G. cliftonii* was higher than previously recorded for *Fucus* spp.*, Chondrus* spp. and *Porphyra* spp. (Ruperez and Saura-Calixto, 2001) which could be attributed to difference in chemical composition i.e. alginates (*Fucus*) and carrageen (*Chondrus, Porphyra*) and/or species physiological characteristics.

Water retention capacity (WRC) and oil retention capacity (ORC) was significantly in Autumn and PS1 seasons which could be due to reduction of osmolality in Winter and PS1 seasons. In addition, the strong positive correlation observed between WRC and ash content and the negative correlation with carbohydrate content of *G. cliftonii* explains that osmoregulation plays a major role on WRC and ORC due to changes in ion concentrations within the thallus (Kumar, 2008) and could undermine the effect of charged polysaccharides on the physicochemical properties.

The differences in WRC and ORC among life stages could be related to differences in energy distribution and utilisation by each life stage, polysaccharide biosynthesis and growth. The higher WRC values for male gametophyte and vegetative stage in Autumn coincide with the period of lower growth and biomass production for these stages. During this time male gametophyte and vegetative stage do not synthetise polysaccharides for tissue building, thus can utilise more energy for osmoregulation rather than growth, whereas, tetrasporophyte stage has to utilize most of the energy for growth or reproduction.

Although, the methodology used to determine WRC and ORC in present study has been widely utilised due to the consistency of results (Robertson *et al.*, 2000), however, differences can occur due to losses of sample due to pellet loss from the centrifuge tube during draining procedures. In addition, differences between present study and previous observations of *G. cliftonii* (Kumar, 2008) might be related to the smaller sample size used in present study (see Methodology). WRC measurement is based on the sample weight recovered and assumes to be an

insoluble matrix. However, contribution from soluble material retained by the pellet can occur at increasing sample size affecting the final weight of the sample (Robertson and Eastwood, 1981).

Overall, WRC and ORC of *G. cliftoni*i observed in present study were higher than reported for other macroalgae (Ruperez and Saura-Calixto, 2001, Fleury and Lahaye, 1991, Lahaye *et al.,* 1993), but were lower than *Laminaria* spp., *Undaria* spp., and *Chondrus* spp. (Ruperez and Saura-Calixto, 2001). Comparisons with previous studies are inconclusive due to different variables which influence the physicochemical properties. First, the effect of environmental conditions such as pH, ionic strength and nature of the ions which influence the hydration values of fibres containing polyelectrolytes (charged groups such as carboxyl in fibres rich in pectins, carboxyl and sulphate groups in fibres from algae) (Guillon and Champ, 2000) are not reported for earlier studies. Secondly, previous reports does not explain experimental conditions such as temperature, time, centrifugation, and sample preparation which are reported to influence WRC and ORC of dietary fibres (McConnell *et al.,* 1974). Finally, specific characteristics such as age and morphology of the thallus (Eastwood, 1973) can also influence these properties are not reported in previous studies.

Fibres are used as texturing and bulking agents, particularly in the making of low calorie foods. These properties are essentially dependent on the ability of fibres to absorb and hold water (Jimenez-Escrig and Sanchez-Muniz, 2000). The higher values observed on swelling capacity and ORC of *G. cliftonii* provides a technological interest for this species.

5.3.3. Agar

In red algae the major sinks of photosynthetically fixed carbon are floridean starch, floridoside and cell wall polysaccharides (e.g. agar and carrageen). Starch and floridoside are both transient products, which are continuously accumulated and degraded, whereas the cell wall polysaccharides are believed to be end products with no or little possibility for degradation and recovery of the carbon (Macler, 1986). Because of the economic importance of red algal cell wall polysaccharides and the negative effect of starch on agar extraction, properties and the effects of

environmental factors such as light, nutrient supply, salinity, and temperature on the content of starch and agar have been investigated extensively (Ekman, 1990). For *G. cliftonii*, agar yield, gelling point, melting point and sulphate content of agar was observed to be influenced by year, life stages and seasons.

Present study showed that agar yield of tetrasporophyte was higher in Autumn while carposporophyte and vegetative stages were higher in Winter as compared to Spring season. In Winter, the conditions of low photoperiod, low salinity and nutrient concentration could have promoted agar synthesis over other polysaccharide forms. Similar results were observed by Rotem *et al.,* (1986) who obtained higher agar yields under reduced light conditions, whereas starch content increased with increased light intensity. Furthermore, direct exposure to sunlight and high tropical temperatures can lead to suppression of agar formation (Bird, 1988). Similarly, Araño (2000) observed that agar yields of *Gracilaria* spp. grown under high light level were lower than those obtained from plants grown under lower light levels. He suggested that higher light intensities promote production of protoplast (affecting rapid growth) over wall material production (Devlin and Witham, 1983), resulting in lower agar deposition. On the other hand, lower light intensities favour cell wall formation rather than protoplast synthesis.

In contrast, Sousa Pinto *et al.*, (1999) showed that the yield of soluble agar molecules increased with increasing light intensity, while, the molecular weight and the degree of methylation of agar molecules decreased with increasing light intensity. They observed that, when growth is limited by light, starch accumulation was directly proportional to light in the range 10–168 μmol photons m^{-2} s^{-1}. It is known that floridean starch, is the major carbon storage polymer in red algae, and floridoside, the principal low molecular weight photoassimilate, are accumulated during the light phase of photosynthesis and utilised during the dark phase, for the synthesis of cell wall polysaccharides. The effect of photoperiod on agar yield was not clear in this study, as it differences were only observed for the male gametophyte in PS3. It is more likely, that agar synthesis is affected by other parameters such as temperature, salinity and nutrients. The effect of temperature is mainly related to the optimal temperature for photosynthesis and productivity (Freile-Pelegrin *et al.,* 1995).

Although, both salinity and temperature interact affecting the overall agar content, the way they affect is not clear (Daugherty and Bird, 1988) and studies have provided variable results. In outdoor cultivation trials, agar yields decreased in a period from early May to mid-July (Guerin and Bird, 1987). During this period, average water temperature increased from 26 to 34 °C. In contrast, maximum agar yields have been correlated to increasing water temperature during the Summer months (Freile-Pelegrin *et al.,* 1995, Freile-Pelegrin and Robledo, 1997, Marinho-Soriano *et al.,* 2001).

The location of polysaccharides like starch granules and floridoside in the cytosol of red algae (Nyvall Collen *et al.,* 2004) suggest that this components are also implicated in osmoregulation processes (Reed *et al.,* 1981). Yields of agar from *G. cliftonii* were higher during the rainy season (Winter), which is attributed to conditions of hyposalinity (Luhan, 1996). Bird (1988) demonstrated that higher agar is produced at low salinity, whereas floridoside content increases at high salinity (Reed *et al.,* 1981). However, Daugherty and Bird (1988) showed that *G. verrucosa* cultured under controlled environmental conditions (where light intensities were 20 % of full sunlight) showed salinity effects of 17 vs. 33 ppt only when salinity interacted with extreme temperatures and nutrient limitation.

Both starch and floridoside are accumulated during nutrient deficiency with a decrease after fertilisation events indicating that both compounds may function as carbon storage (Nyvall Collen *et al.,* 2004). The higher agar content in *G. tenuistipitata* during nutrient limitation was twice that observed in *G. chilensis* exposed to seawater with a salinity of 70 ppt. These concentrations are likely to have an osmotic effect that the algae somehow have to counteract, because the salinity of the growth medium was kept constant. In addition, the enzymes involved in floridoside synthesis and breakdown, FPS and a-galactosidase, respectively, show a similar pattern of activity as fertilization (Ekman *et al.,* 1991). In Autumn and Winter, ammonium was higher but lower concentration of nitrates and phosphates were observed.

Agar yield seasonality showed a similar trend with biomass of *G. cliftonii*. Previously, Friedlander (1991) observed a correlation between agar production and maximum growth rates for *G. tenuistipitata* and for *G. conferta*. However, they demonstrated that growth and chemical constituents were affected by concentration

of nutrients rather than by light levels. This could result from low light saturation point for growth of *G. conferta*, which seems to occur at about 330 μmol photon m^{-2} s^{-1} (corresponding to 20% of 1,650 μmol photon m^{-2} s^{-1}, the average sunlight during the experimental period). However, Bird *et al.* (1981) demonstrated that an increase of nitrogen fertilizer led to decreased agar yield in *G. tikvahiae*. Similarly, in Autumn, the low concentration of NO$_3^-$, NO$_2^-$ and PO$_4^-$ could have promoted higher agar yields of *G. cliftonii* as a response to nutrient storage under low nutrient conditions.

Similarly results were observed by Lewis and Hanisak (1996) where limiting concentrations of phosphate under high nitrogen feeding of *Gracilaria* sp. showed higher native agar concentrations than under high nitrogen and high phosphate concentrations. In addition, agar isolated from *G. verrucosa* cultured under nitrogen limited conditions showed large non-polar components (ethanol soluble), minor amounts of methylation and presence of Floridian starch, whereas nitrogen supplemented cultures produced more polar agar (hot water soluble) with little starch (Chiles *et al.*, 1989).

One of the most important parameters in polysaccharide synthesis are the C:N ratios which are affected by the overall metabolism of the thalli. The high C:N ratios found in *G. tenuistipitata* at low levels of N and P can provide information of the nutritional status of the thallus since low nitrogen levels would limit uptake, intracellular levels and algal growth (Friedlander and Ben-Amotz, 1991). Also, high C:N ratios in both micro and macroalgae are correlated with high CO$_2$ concentrations in the seawater medium (Burkhardt and Riebesell, 1997). However, the magnitude of C:N change caused by CO$_2$ availability is moderate compared to effects generally observed under severe N or P limitation. Red algae inhabiting nutrient-rich seawater in an upwelling system showed low C:N values of 7–12, similar to *G. tenuistipitata* grown in 40 m^3 tanks with N-rich brackish water (Haglund and Pedersen, 1993).

The higher agar yields of *G. cliftonii* observed in Winter can be also the result of a lag effect of the higher salinity observed in Autumn seasons. Changes in agar yield are found after post-treatment in altered salinity, being caused by changed agar solubility and/or changed agar biosynthesis. Ekman *et al.,* (1991) demonstrated in a nutrient enriched culture of *G. sordida* accumulation of floridoside upon transfer to hypersaline conditions in darkness. The α-galactosidase activity is increased when

thalli was subsequently transferred to hyposaline conditions, while starch degradation was enhanced under the same conditions (Israel *et al.*, 1999). It is suggested that a flow of carbon derived from starch degradation may be diverted through floridoside and recycled within the thallus for use in biosynthesis, since total thallus carbon content is almost unchanged.

Furthermore, higher agar yields in Winter could also be a physiological adaptation of *G. cliftonii* as thalli have to withstand physiological and physical stress due to increase water movement, thus developing tougher cell walls. Besides the multiple environmental parameters that affect agar yield, inherent characteristics of *G. cliftonii* such as age, reproduction, morphology and biological interactions (i.e. epiphytism and herbivory) can cause variations in metabolic responses. Agar yields also vary with the age status of the thalli of *Gracilaria* spp., with mature parts of the thallus showing higher yields of native agar than young parts as observed by Craigie and Wen (1984). It has been demonstrated that young tissues (tips and short lateral branches) are characterised by higher growth activity containing less substituted agar. The agar from young parts of the thallus also contained a higher proportion of 3,6 anhydrogalactose than did agar from mature parts of the thallus. This suggests a gradual biosynthetic pathway which ends only in the mature plants of *G. tikvahiae*. The higher agar yield of *G. conferta* appeared in Summer, when the proportion of young tissue was higher, suggesting that the biosynthetic pathway in this species terminates earlier (Araño *et al.*, 2000). This observation explains the higher agar yield for the male gametophyte stage in Summer and vegetative in Winter but not for the rest of the stages from *G. cliftonii*.

Yield and sulphate content of agar from *G. cliftonii* showed inverse correlation with epiphytic load. The effect of epiphytism is poorly known specially related to its effect on agar yields. Dawes *et al.* (2000) showed *G. cornea* responds against the epiphytism of *Ulva lactuca* by increasing the thickness of its cortical cells. Other defence mechanisms are the physiological changes of *G. conferta* against oligoagars from cell degrading bacteria (Weinberger and Friedlander, 2000). These physiological changes include higher oxygen consumption and bleaching of the tips. These mechanisms involve re-allocation of resources within the thallus including higher production of matrix polysaccharides and increasing metabolic rates (Lobban and Harrison, 1994). This explains the lower agar yield in Summer when epiphytic

load was highest. In addition, Friedlander (1991) observed an inverse correlation between agar yield and epiphyte load which was the results of the interaction with temperature, pH and nitrogen supply. Higher epiphytic biomass was observed in Summer, with pH above 8.8, increasing light intensities and ammonium supply which resulted in a decrease in agar yield from *G. conferta*.

The low agar yields observed in Spring can be due to the habitat and morphology of juveniles of *G. cliftonii* which grow in high densities close to the substrate. Previous studies demonstrated that thalli that grow as tufts or cushions on rocky surfaces generally have low agar yields (Oyieke, 1994). The low yield is due to the effects of water motion which is decreased when thalli grow in patches (juveniles) as compared to the solitary forms (i.e. adult *G. cliftonii* thalli), since more energy is expended by the former to survive the harsher physiological conditions (Taylor and Hay, 1984)

Agar yield from *G. cliftonii* varied as a function of life stages similar to previous reports (Marinho-Soriano *et al.*, 1999, Penniman, 1977, Penniman and Mathieson, 1987, Pickering *et al.*, 1990, Durairatnam and Nascimento, 1985). However, present results contradict observations made for *G. cliftonii* (Kumar, 2008) and *G. verrucosa* (Kim and Henriquez, 1979) which showed higher agar yields for the tetrasporophyte and carposporophyte stages. Differences on the agar yields can be due to variation in site of collection and extraction technique (Villanueva and Montano, 1999, Villanueva *et al.*, 1999), while, the variations observed between agar yield of *G. cliftonii* tetrasporophyte and vegetative stages could be due to the structure of agar (Andriamanantoanina *et al.*, 2007, Craigie and Jurgens, 1989, Duckworth and Yaphe, 1971a, Lahaye and Rochas, 1991, Murano, 1995).

Quantity and quality of agar are difficult parameters to assess (McLachlan and Bird, 1986). Both are modified by environmental conditions and problems in identifying the source-species correctly have already been mentioned. Different procedures of extraction and expression of results are additional source of variation.

The yield of agar observed was within the range reported by Kumar (2008) for *G. cliftonii* collected from wild populations but was higher than previously reported by Byrne *et al.,* (2002) for the same species. Details of the extraction method for the agar of *G. cliftonii* were not fully provided in previous studies (Byrne *et al.*, 2002), however, variables like soaking time and soaking temperature might

have influenced the agar yield. The higher agar yields observed in present study could be due to shorted periods of soaking time used in present study (see Methodology) as compared to overnight soaking time used by Byrne *et al.,* (2002). Longer soaking times can result in diffusion of some agar into water resulting in lower yields as observed by Kumar and Fotedar (2009). In addition, differences on the methods of filtration and drying of the agar could have the quantity of agar recovered (Cote and Hanisak, 1986, Durairatnam, 1987), thus affecting the final yield of *G. cliftonii*.

In addition, agar yield is usually expressed as per cent algal dry weight, assuming dry weight to be more or less constant. However, Ekman *et al.,* (1991) demonstrated that conditions of altered salinity and changed nutrient availability can affect dry weight composition of *G. sordida*. Agar yield of *G. cliftonii* may thus be influenced by the same conditions, if ash and/or starch is a quantitatively significant component of dry weight. Changes in the amounts of other quantitatively important components of algal dry weight can additionally contribute to the observed changes in agar yield of *G. cliftonii*. This was also suggested as a possible explanation for increased agar yields in *Gelidium coulteri* (Macler, 1988).

Despite the importance of gelling temperature and melting point of agar, most of the literature available focus only on the seasonality of agar yield, gel strength, anhydrogalactose or sulphate content (Whyte *et al.,* 1981, Pickering *et al.,* 1990, Hoyle, 1978a, Rebello *et al.,* 1996, Luhan, 1992, Durairatnam, 1987, Lahaye and Yaphe, 1988, Asare, 1980, Yenigül, 1993, Friedlander *et al.,* 1987, Price and Bielig, 1992, Romero *et al.,* 2007). Reports on seasonal variation of gelling and melting temperatures of agar show either clear seasonal variation (Marinho-Soriano and Bourret, 2003)(Villanueva *et al.,* 1999, Freile-Pelegrin and Robledo, 1997, Romero *et al.,* 2007) or very little or no seasonality at all (Marinho-Soriano *et al.,* 2001, Bird and Hinson, 1992, Shi Sheng *et al.,* 1984, Hoyle, 1978b, Chirapart and Ohno, 1993).

The seasonality of the gelling point of agar from *G. cliftonii* observed in present study also did not showed a clear pattern. However, higher gelling temperatures of agar were observed in Spring and PS1 seasons. Differences could be due to the effect of light and nitrogen concentration which has been observed to affect gelling temperatures of *G. sordida* (Christeller and Laing, 1989, Bird, 1988). However, it has been suggested that the seasonality of the gelling temperature of agar

of *Gracilaria* spp. is mostly due to species specific characteristics rather than environmental factors (Marinho-Soriano and Bourret, 2003, Oyieke, 1993, Marinho-Soriano *et al.,* 2001, Villanueva *et al.,* 1999).

In present study, gelling temperature of agar varied as a function of life stages and were in similar range reported for *G. cliftonii* (Kumar, 2008) and other *Gracilaria* spp. (Oyieke, 1993). However, higher gelling temperature of agar were observed for tetrasporophyte stage of *G. cliftonii,* which is in contradiction to previous observations (Kumar, 2008). This difference in gelling temperature of agar from tetrasporophyte stage could be due to seasonality of *G. cliftonii* which was not considered in previous study. In addition, differences could be due to changes in chemical structure of agar which includes location of methoxyl group along with its molecular weight and distribution (Andriamanantoanina *et al.,* 2007, Rebello *et al.,* 1997, Guiseley, 1970).

The seasonality of melting point of agar from *G. cliftonii* was clearly defined for the tetrasporophyte and male gametophyte stages. Former showed higher melting point of agar in Spring and Summer while latter was higher in Winter. The seasonality of melting point observed can be due to reproduction processes as reported by Givernaud *et al.* (1999). In addition, melting point of agar from male gametophyte was significantly higher than carposporophyte stage, however, present results cannot be compared with previous studies on *G. cliftonii* and/or other *Gracilaria* spp. as no information was available for the male gametophyte stage (Kumar, 2008, Shi Sheng *et al.,* 1984, Pickering *et al.,* 1990, Whyte *et al.,* 1981, Hoyle, 1978b, Kim and Henriquez, 1979, Penniman and Mathieson, 1987).

Differences observed between different life stages of *G. cliftonii* could be due to intrinsic life stages characteristics and differences in the pyruvic acid content of agar which is known to influence melting point of agar of *Gracilaria* spp. (Young *et al.,* 1971).

Melting point of agar from *G. cliftonii* was in similar range reported by Kumar (2008) and showed small variation due to seasons. These observations are in agreement with previous reports on the seasonality of melting point of agar (Marinho-Soriano *et al.,* 2001, Bird and Hinson, 1992, Shi Sheng *et al.,* 1984, Hoyle, 1978b, Chirapart and Ohno, 1993).

The agars from *G. cliftonii* was considered as a bacto-agar, which is

characterised by a gelling temperature of 32-36 ^{o}C and a melting temperature of 85-86 ^{o}C (Armisen and Galatas, 2000). Despite the production of industrial-grade agar from this alga, it remains untapped due to the lack of a protocol for its rational exploitation.

Considerable more attention has been paid to the sulphate content of agar from *Gracilaria* spp. as the amount and location of sulphate esters substituents in the agar chain strongly affect the physical properties of the gel (Freile-Pelegrin and Murano, 2005, Asare, 1980). No seasonality was observed in the sulphate content of agar from *G. cliftonii* and contradicts the strong seasonality observed by other authors. Asare (1980) and Romero *et al.,* (2007) observed higher sulphate content of agar in Summer and Winter seasons respectively. However, the former only observed this seasonality for infertile vegetative thalli from *G. tikvahiae* while in the latter reproductive stage of the sample was not mentioned. The constant sulphate content of *G. cliftonii* could be due to the occurrence of an invariable level of alkali-stable sulphates in the agar polymer as observed for *G. eucheumoides* (Villanueva *et al.,* 1999).

Alkali treatment of native agar increases the anhydro-sugar content and decreases the sulphate content (Lahaye and Yaphe, 1988). These changes in the present study were not significant and this could be attributed to the fact that the samples were hot-water extracts and it is most likely that the alkali-labile sulphate compounds had been lost during the freeze-thawing process as they are known to be soluble in cold water (Yaphe, 1984). The inverse relationship between sulphate content and agar yield exhibited by *G. eucheumoides* suggests the compensation of quality with quantity and *vice versa*, similar to other *Gracilaria* spp. which also behaved in such manner (Luhan, 1992, Chirapart *et al.,* 1995).

Although, seasonality of sulphate content was not observed in this study, sulphate content of agar from carposporophyte and male gametophyte stages were higher than tetrasporophyte stage of *G. cliftonii*, similar to reported by Kumar (2008). On the contrary, Craigie and Wen (1984) reported that the agar yield (less sulphate) from mature parts of a thallus was double that from apical parts or the young lateral branches.

In addition, high sulphate content was observed one *Gracilariopsis bailinae* during periods of slow growth. Pondevida and Hurtado-Ponce (1996b) also observed

that low sulphate content coincided with maximum biomass (with more younger thalli). Cote and Hanisak (1986) on the other hand, speculated that the agar polysaccharide precursors, present in younger thalli, were highly substituted with sulphate, pyruvate and methoxyl groups, which are later enzymatically modified to give a less substituted and less charged polymer in the mature thalli.

The lack of seasonality of sulphate content of agar can be related to nutrient concentration, particularly ammonium. Ammonium is a cation which forms bonds with sulphate maintaining a constant equilibrium with the external medium. Sulphate is not considered a limiting factor for *Gracilaria* spp. (Lobban and Harrison, 1994). It is present in proteins, polysaccharides, sulfolipids and low molecular sulphated compounds (DeBoer, 1981). Cell division has a special requirement for sulphate in the growth medium (O'Kelley, 1974) however, the effect of sulphate starvation on agar biosynthesis has not been studied. Craigie and Wen (1984) showed that during nitrogen and phosphate starvation the synthesised agar accumulated more the sulphated agarose form, rather than its neutral agarose one. The higher sulphate content and lower molar ratio in Summer reflects the high biosynthetic activity of non-mature highly sulphated agar, although Summer conditions affected this seaweed experiment only in the pre-experiment outdoor culture.

The sulphate content of agar from *G. cliftonii* observed in present study was within the range reported for *G. cliftonii* (Kumar, 2008) but higher than other *Gracilaria* spp. (Oyieke, 1993, Shi Sheng *et al.,* 1984, Bird and Hinson, 1992, Romero *et al.,* 2007, Rebello *et al.,* 1997). Differences on sulphate content of agar observed might be due to differences in the methodology used to determine this property and seasonality which previous researchers did not document.

6. CONCLUSIONS

➢ The natural population of *G. cliftonii* from Point Peron, Western Australia has a triphasic life cycle with reproductive and vegetative stages present all year round. However, deviation in the life cycle of *G. cliftonii* is observed as cystocarps and tetrasporangia develop in the same thallus.

➢ Tetrasporophyte is the most dominant reproductive stage of the population of *G. cliftonii* from Point Peron, Western Australia. Under laboratory conditions, the number of tetraspores released and growth is higher than carpospores supporting observations from the naturally occurring populations.

➢ Biomass of *G. cliftonii* varies with the alternation of life stages within the population. Vegetative stage dominates in Spring and Summer while, reproductive stages dominate in Autumn and Winter. This shift is triggered by the changes in temperature and photoperiod.

➢ Epiphytic loads are inversely correlated to the biomass of *G. cliftonii*. Maximum epiphytic loads are shown in Summer, while maximum biomass is attained in Autumn and Winter.

➢ There is more than one way in which epiphytes attach to *G. cliftonii* thallus.

➢ The biomass and abundance of *G. cliftonii* from Point Peron is too low to support any commercial exploitation from natural populations. Aquaculture of this species can be considered as an alternative to harvest from the wild.

➢ The physicochemical properties of *G. cliftonii* indicated that this species has a potential for health food development, particularly as a source of dietary fibre.

➢ *G. cliftonii* is high in agar yield, ash and protein content; however, the time of harvest and harvested life stages have to be considered to assure maximum yield and quality.

7. RECOMMENDATIONS FOR FURTHER RESEARCH

➢ Biomass and abundance assessment of *G. cliftonii* populations from different locations need to be researched in order to have a comparison with the population of Point Peron.

➢ Research on the reproductive phenology and effect of environmental factors under laboratory conditions and under out-door conditions need to be investigated. Particularly, deviations from the typical "*Polysiphonia*" life cycle needs further investigations.

➢ Vegetative and spore propagation of *G. cliftonii* under laboratory and outdoor conditions need to be studied.

➢ Physiological studies need to be conducted in order to determine the environmental parameters affecting the metabolism of *G. cliftonii,* particularly, productivity and agar production.

➢ Studies on the effect of epiphytism and the interaction with environmental factors on the productivity of *G. cliftonii* need to be researched.

➢ Further studies on the characterisation of attachment between epiphyte–host are required to understand the interactions between *G. cliftonii* and its epiphytes and its managements under cultivation conditions.

➢ The mechanisms of epiphytic attachment to *G. cliftonii* thallus have to be considered for future studies, particularly while considering the effect on the agar yield.

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APPENDICES

Appendix I Provasoli Enriched Seawater

Appendix II Publications originated from this research

APPENDIX I

PROVASOLI ENRICHED SEAWATER (PES)

Enrichment Stock Solution

To prepare the enrichment stock solution, begin with 900 mL of dH_2O, add the following components (vitamins should be added last, after mixing other ingredients), bring the final volume to 1 L with dH_2O and pasteurize (do not autoclave). To prepare PES medium add 20 mL of the enrichment stock solution to 980 mL of filtered natural seawater. Pasteurize.

Enrichment Stock Solution

Component	Stock solution g/L dH_2O	Quantity used	Concentration in Final Medium (M)
Tris base	-	5.0 g	8.26×10^{-4}
$NaNO_3$	-	3.5 g	8.24×10^{-4}
Na_2 β-glycerophosphate H_2O	-	0.5 g	4.63×10^{-5}
Iron-EDTA solution	(See following recipe)	250 ml	-
Trace metal solution	(See following recipe)	25 ml	-
Thiamine HCl (Vitamin B_1)	-	0.500 mg	2.96×10^{-8}
Biotin (Vitamin H)	0.005	1 ml	4.09×10^{-10}
Cyanocobalamin (Vitamin B_{12})	0.010	1 ml	1.48×10^{-10}

Iron EDTA solution

Into 900 mL of dH$_2$O, dissolve EDTA and then the iron sulphate. Bring the final volume to 1 L. Pasteurize and store refrigerated.

Iron EDTA solution

Component	Stock solution g/L dH$_2$O	Quantity used	Concentration in Final Medium (M)
Na$_2$EDTA 2H$_2$O	-	0.841 g	1.13 x 10^{-5}
Fe(NH$_4$)$_2$(SO$_4$)$_2$ 6H$_2$O	-	0.702 g	8.95 x 10^{-6}

Trace metal solution

Into 900 mL of dH$_2$O, dissolve EDTA and the individually dissolve the following components. Bring the final volume to 1 L and store refrigerated.

Trace metal solution

Component	Stock solution g/L dH$_2$O	Quantity used	Concentration in Final Medium (M)
Na$_2$EDTA · 2H$_2$O	-	12.74 g	1.17 x 10^{-4}
FeCl$_3$ · 6H$_2$O	-	0.484 g	8.95 x 10^{-6}
H$_3$BO$_3$	-	11.439 g	9.25 x 10^{-5}
MnSO$_4$ · 4H$_2$O	-	1.624 g	3.64 x 10^{-5}
ZnSO$_4$ · 7H$_2$O	-	0.220 g	3.82 x 10^{-6}
CoSO$_4$ · 7H2O	-	0.048 g	8.48 x 10^{-7}

Source:(Andersen, 2005)

APPENDIX II

Publication generated from this research

1 **PUBLICATION FINAL DRAFT**

2

3 **Epiphytism of *Gracilaria cliftonii* (Withell, Millar & Kraft) from Western Australia**

4

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Abstract

Epiphytism in *Gracilaria* is a common phenomena observed in natural populations and under culture conditions. Generally, epiphytes are attached superficially to the surface of the host however, genera such as *Polysiphonia spp.* and *Ceramium spp.* can penetrate into the host tissue affecting its growth and hence productivity. The present paper aims to identify and quantify epiphyte abundance and characterize their attachment and penetration on the natural populations of *Gracilaria cliftonii* collected from Shoalwater Marine Park, Perth, Western Australia. *G. cliftonii* samples were collected monthly for 1 year and their epiphytes were identified. Histological studies of *G. cliftonii* were also performed on epiphyte attachment sites. Twenty-four species of macroalgae epiphytes were recorded, 21 belonging to Rhodophyta and 3 to Chlorophyta. *Hypnea episcopalis* and *Polysiphonia forfex* were the dominant (p<0.05) species during the sampling period. The maximum epiphytic load was observed in October (40%), however, the maximum diversity of epiphytes was recorded in September and February (n=14). Light and scanning electron microscopy studies of the epiphyte attachment sites revealed that *Polysiphonia forfex* and *Ceramium isogonum* penetrated into the cortex and outer medulla of *G. cliftonii*, while *Bryopsis plumosa* and *Laurencia clavata* altered the cellular structure of the cell wall of *G. cliftonii*.

Key words

Epiphytes, penetration, *Gracilaria*, epiphytic load

Introduction

Epiphytism is as common phenomena for *Gracilaria spp.* in the natural environment and under cultivation conditions (reviewed by Fletcher, 1995). It is a major problem affecting the productivity and quality of *Gracilaria spp.* Epiphytes decrease the host growth rates and result in loss of biomass through direct competition with the host algae for space, nutrients and inorganic carbon from the water column (Buschmann and Gómez 1993; Kuschel and Buschmann 1991; Svirski et al. 1993).

Identification and reports of temporal abundance of epiphytes have been poorly reported for natural populations of *Gracilaria spp.* with most of the research being focused on *Gracilaria chilensis* (Buschmann et al. 1997; Fletcher 1995; Gonzalez et al. 1993; Kuschel and Buschmann 1991). Several epiphytes have been described for *Gracilaria* species but eight macroalgae genera such as *Polysiphonia, Antithamnionella, Chondria, Ceramium, Ulva, Enteromorpha, Giffordia* and *Ectocarpus* have been identified as the most common epiphytes for *Gracilaria* species (Fletcher 1995; Kuschel and Buschmann 1991; Leonardi et al. 2006) with maximum abundance observed in summer (Buschmann et al. 1997; Westermeier et al. 1993; Westermeier et al. 1991).

Several types of interactions that occur at different levels are possible between host and epiphytes. These interactions can be influenced by shape, texture and thickness of the cortex and/or chemical composition of host cells and production of metabolites and enzymes (Dawes et al. 2000; Leonardi et al. 2006). However, the interactions of *Gracilaria spp.* with their epiphytes are poorly defined with the only comprehensive study provided by Leonardi et al. (2006) who characterized epiphyte-*Gracilaria* interactions depending on the degree of infection by epiphytes.

In Australia, seven *Gracilaria* species have been identified, five of these are found in Western Australia (Huisman 2000). However, most of the literature available for Australian *Gracilaria spp.* (Byrne et al. 2002; May 1948; Withell et al. 1994; Womersley 1996) is related to taxonomic aspects. *Gracilaria cliftonii,* is distributed around the Perth coastline and is reported to have the highest agar content (52 % db) (Byrne et al. 2002). Recently, *G. cliftonii* has been cultivated experimentally under indoor conditions (Kumar 2008) as a source for agar extraction (Kumar and Fotedar 2009) however, no information is available for *G. cliftonii* epiphytism. The present paper aims to identify and determine the temporal abundance and characterize the attachment of epiphytes on a natural population of *G. cliftonii* found at Point Peron, Shoalwater Marine Park, Western Australia.

Material and Methods

103   *G. cliftonii* samples were collected from a subtidal population located at Point Peron,

104   Shoalwater Marine Park, Western Australia (32° 15' S, 115° 41' E). The substrate in this area

105   consist of limestone ridges and reef platforms (DEC 2007). These ridges and platforms

106   protect the coast from south-westerly swell and waves creating a semi-enclosed near shore

107   area with a tidal monthly mean variation from 0.5 to 1 m (DT 2008)

108   *G. cliftonii* samples were collected for 12 months from April 2008 to March 2009.

109   Preliminary site trials established the size of the permanent 30 x 3 plot which was located 5 m

110   from the shore at 1-2 m depth. Ten quadrats (1 m^2) were randomly sampled within the pre-

111   selected permanent plot by free diving. *G. cliftonii* thalli within each quadrat were removed

112   by hand from the substrate, placed in labelled plastic bags and transported to Curtin Aquatic

113   Research Laboratories, Curtin University of Technology, Western Australia for further

114   analysis.

115   In the laboratory *G. cliftonii* samples were rinsed in seawater, blotted dry and weighed

116   (wet weight = ±0.1 g). Epiphytes observed growing on *G. cliftonii* were then removed

117   physically and the total weight of epiphytes recorded (wet weight = ±0.1 g). The epiphytic

118   load was calculated according to Buschmann and Gómez (1993) (Equation 1).

119   Equation 1   Epiphytic load (Epiphyte/*Gracilaria*) = epiphyte fresh weight (g)   x 100

120   *Gracilaria* fresh weight (g)

121

122   The epiphyte occurrence was defined as the frequency of observation of each species per

123   month. Epiphyte diversity was defined as the total number of epiphytes recorded for each

124   month.

125   **Characterization of attachment**

126   Thalli of *G. cliftonii* were observed under the binocular microscope and sections (2

127   mm) where the epiphytes were entangled or attached to its cortex were selected and processed

128   for histological studies. The epiphytes were removed, fixed in 5% formalin-seawater solution

129   for taxonomic identification. Based on the level of force required to remove the epiphyte

130   when the epiphyte removed from *G. cliftonii* surface, the strength of attachment was

131   categorised as epiphyte easy and/or hard to be removed. Epiphyte distribution was determined

132   recording the epiphytes distribution on *G. cliftonii* thallus (ie basal, intermediate and apical)

133   (Arrontes 1990).

134   **Histological studies**

135   **Light microscopy studies**

136   *G. cliftonii* sections were processed for light microscopy according to Fedder and

137 O'Brien (1968). Selected sections of *G. cliftonii* thallus (0.5 mm) where epiphytes were

138 attached were fixed in 2.5% glutaraldehyde-phosphate buffer (0.5 M) solution under vacuum

139 conditions (1.7-2 Bar). After fixation, the sections were rinsed 3 times for 30 min each with

140 0.5 M phosphate buffer followed by an alcohol dehydration series (methoxypropanol 100%

141 for 24 h, ethanol (ETOH) 100% for 24 h, n-butanol 100% for 24 h and propanol 100% for 24

142 h).

143   After dehydration, the samples were immersed in 50% glycol methacrylate (GMA)

144 solution for 24 h. Finally, samples were placed in 100% GMA and refreshed after 24, 48 and

145 72 h. The samples were then embedded in a solution of 100% GMA-polyethylene glycol-

146 benzoil peroxide and polymerized overnight at room temperature under a nitrogen atmosphere

147 and UV light conditions.

148   Sectioning and preparation of slides was performed at the Centre for Microscopy,

149 Characterisation and Analysis, University of Western Australia, Perth, Western Australia. The

150 embedded samples were sectioned with a dry glass knife at 2.5 µm using a Sorvall[®]

151 microtome. The sections were mounted on glass slides and stained with 0.5% toluidine blue

152 (pH 4). The slides were observed under a light microscope (Olympus SZH[®]) and areas where

153 the epiphytes were attached to *G. cliftonii* were photographed with a digital camera (Olympus

154 BX51[®]).

Scanning electron microscope

156   *G. cliftonii* sections were processed for scanning electron microscopy (SEM)

157 according to Dunlap and Adaskaveg (1997) with minor modifications. The fixed material was

158 rinsed 3 times for 5 min each with 0.5 M phosphate buffer, rinsed with distilled water for 5

159 min and immersed in 2% OsO_4 for 2 h. The samples were rinsed with distilled water and

160 dehydrated using a series of ethanol washes: 50% ETOH - 50% H_2O (5 min); 75% ETOH -

161 25% H_2O (5 min); 95% ETOH - 5% H_2O (5 min) and 100% ETOH, 3 times for 5 min each.

162   Finally, the samples were dried using series of hexamethyldisilizane (HMDS) washes:

163 50% HMDS-50% ETOH for 5 min; 75% HMDS-25% ETOH for 5 min and 2 times 100%

164 HMDS for 5 min each, and dried at room temperature overnight. Then the sections were

165 mounted on double sided carbon adhesive tape, fixed to aluminium stubs and gold coated.

166 Micrographs of the samples were obtained with a Phillips X30 Scanning Electron Microscope

167 at 15 kV.

Statistical analysis

169   All data was analysed using SPSS V. 16 for Windows. Epiphytic load data was tested

170   for homogeneity of variance using Levene's test. The data was then subjected to a one way

171   ANOVA with least significant difference (LSD) post-hoc tests at a significance level of

172     $p < 0.05$. To determine significant differences on epiphyte occurrence, a Chi-square test was

173   performed ($p < 0.05$) on the data.

174   **Results**

175   **Epiphyte composition and abundance**

176   Twenty four macroalgae species were recorded as *G. cliftonii* epiphytes during the

177   study period (Table 1). Twenty one species belonged to the division Rhodophyta and 3 to

178   Chlorophyta. The Ceramiales was the most dominant group with four *Ceramium* species (*C.*

179   *puberulum, C. pusillum, C. isogonum and C. minuta*) and two *Polysiphonia* species (*P. forfex*

180   and *P. spinossissima*) identified. However, the most common species observed were *Hypnea*

181   *episcopalis*, *Polysiphonia forfex* and *Cladophora spp.*, which were present during most of the

182   months except for March 2009. *H. episcopalis* and *P. forfex* occurred more frequently

183   ($p < 0.05$) as compared to other epiphytes (Table 1). The maximum number of epiphytes was

184   observed in October 2008 and February 2009 with 14 species recorded for both months while

185   the minimum was observed in July 2008 and January and March 2009 (n=6).

186   The epiphytic load (Fig. 1) was significantly higher ($p < 0.05$) in October 2008 (40%)

187   while May and November 2008 were significantly lower ($p < 0.05$) as compared to all other

188   months.

189   **Characterization of attachment**

190   Different types of attachment between epiphyte and *G. cliftonii* were observed.

191   *Hypnea episcopalis* was found anchored to *G. cliftonii* through the apical hooks that

192   characterize this genus (Fig. 2A), however, it was easily removed by hand and no superficial

193   damage was observed to the host. *Ceramium pusillum* (Fig. 2B)*, Polysiphonia forfex* (Fig.

194   2C)*, Leveillea jungermannioides* (Fig. 2D), *Champia parvula* (Fig. 2E) and *Griffithsia spp.*

195   (Fig. 2F) were observed growing as an erect thallus arising from the surface of *G. cliftonii* and

196   were harder to remove from the host. The majority of *G. cliftonii* epiphytes were distributed

197   evenly over the thallus. Four species deviated from this distribution that is, *P. forfex, C.*

198   *parvula, Laurencia clavata* and *L. jungermannioides. P. forfex* was found growing mainly in

199   the apical tips of *G. cliftonii*, while *C. parvula, L. clavata, Ulva lactuca* and *L.*

200   *jungermannioides* were observed mostly in the basal portions of the fronds.

201   **Histological studies**

202   **Light microscopy studies**

203   Light microscopy revealed three types of epiphytic attachment to *G. cliftonii*. The
204 epiphytes such as *Bryopsis plumosa*, *Mazoyerella australis* (Fig. 3A) and *L. jungermannioides*
205 (Fig 3B) were growing on the host surface without penetration. No alteration of the host
206 cortical thickness was observed as compared to 2-4 cells thickness found in non infected
207 thalli. The second type of attachment was characterized by alteration of the cellular structure at
208 the site of attachment. Cellular disorganization of the cortical cell layer, observed mainly as
209 elongation of the cortical cells at the area of attachment, was observed with *Bryopsis plumosa*
210 (Fig. 3C). Similarly, the site of attachment with *L. clavata* (Fig 3D) presented 6-8 cortical cell
211 layers compared with 2-4 cortical cells in the non infected areas.

212   The third type of attachment was penetration of the epiphyte into the host tissue and
213 was observed with *C. isogonum* (Fig. 3E-F). *C. isogonum* was found growing along the
214 surface of *G. cliftonii*, anchoring in those areas with the presence of cleavages (Fig. 3E) and
215 attaching to the host through rhizoids. The rhizoids penetrated the cortex to the depth of the
216 outer medullary cells and its wall was clearly defined (Fig. 3F). Scanning electron microscopy
217 revealed the penetration of *P. forfex* (Fig. 4A) and *C. isogonum* (Fig. 4B) into *G. cliftonii*
218 tissue thus confirming the histological results. The sites of penetration by *C. isogonum* were
219 also characterized by the presence of diatoms and/or lacunae on *G. cliftonii* surface (Fig.4C).

220 **Discussion**

221 **Epiphyte composition and abundance**

222   This study demonstrates that *G. cliftonii* is host to a wide range of epiphytes with
223 Rhodophyta the most dominant group. The species of epiphytes observed during the study
224 period (Table 1) have been previously reported as *Gracilaria* epiphytes (Fletcher 1995)
225 (Table 2). The dominance by red algae epiphytes reflects the great diversity and abundance of
226 the Rhodophyta at the study site (Roberts et al. 2002). In addition, the epiphytes observed in
227 this study (*Hypnea episcopalis, Plocamium mertensii, Ulva lactuca, Enteromorpha*
228 *intestinalis, Champia parvula, Ceramium spp., Dasya spp., Chondria spp.*) have been
229 reported for the seagrass *Amphibolis spp.* which is distributed around the Western Australian
230 coastline (Ducker et al. 1977). This suggests that *G. cliftonii* epiphytic composition might be a
231 result of the natural distribution and occurrence of these epiphytes in the study site.

232   The maximum diversity of epiphytes was observed in October (austral spring) which
233 could be due to the effect of environmental factors and epiphyte specific characteristics.
234 Furthermore, epiphytism of *Gracilaria spp.* has been observed, mainly peaking in spring and
235 summer and is associated with an increase in water temperature and irradiance (Fletcher 1995;
236 Ugarte and Santelices 1992; Westermeier et al. 1993; Westermeier et al. 1991). In contrast,

237 Buschmann et al. (1994) reported an increase in epiphyte abudance during winter and suggested

238 this could be induced by the approach of spring and renewed growth of *Gracilaria*. In

239 Western Australia, results supporting both studies were observed by Ducker (1977) who

240 reported two peaks of diversity and abundance of epiphytes, one in summer and one in late

241 winter. In this study, epiphyte occurrence was highly variable with no significant pattern in

242 seasonality being detected for any epiphyte species. Such temporal variations have been

243 previously reported for *Gracilaria spp.* due to changes in environmental conditions such as

244 surface water temperature and light intensity (Kuschel and Buschmann 1991).

245 The *Ceramium* species observed have been previously reported as common epiphytes

246 on macroalgae distributed in Australia (Womersley 1978) and the dominance of the

247 Ceramialean epiphytes has been reported for *G. chilensis* (Westermeier et al. 1993;

248 Westermeier et al. 1991). Species belonging to the Ceramiales are known for their invasion

249 success and life cycle dominated by an asexual stage. This clonal morphology provides them

250 with a method of vegetative propagation that has important ecological consequences such as

251 rapid occupation over a large area (Collado-Vides 2001).

252 The occurrence of *P. forfex* during most of the year in this study can be related to the

253 absence of a seasonal cycle of growth and reproduction observed in *Polysiphonia spp.*

254 Similarly, the occurrence of *H. episcopalis* during most of the year might be due to two

255 important characteristics observed in this species that is, the presence of multiple meristems

256 and abundant production of spores, enabling rapid vegetative growth all year round. Also, it is

257 likely that the presence of hook like tips found in this species strengthen the attachment

258 between host and epiphyte, resulting in a decrease in the likelihood of separation during the

259 generally rougher conditions of winter (Kong and Ang 2004; Russell 1992).

260 **Histological studies**

261 In this study the epiphytes of *G. cliftonii* were observed growing on the thallus surface

262 in three ways that is, i) without penetration, ii) alteration of the cellular structure at the

263 attachment site and iii) with penetration into the host tissue. These differences could be due to

264 the ability of different epiphytes to digest the cortex and medullary cells of the host

265 (Leonardi et al. 2006). The interruption of the epiphyte wall and no apparent tearing of the

266 host wall indicates that penetrative rhizoidal cells from *P. forfex* and *C. isogonum* may

267 penetrate *G. cliftonii* degrading its wall enzymatically as observed with *Harveyella mirabilis*

268 (Goff and Cole 1975). This might also explain why epiphytes such *C. parvula, L. clavata,*

269 *Ulva lactuca* and *L. jungermannioides* were growing on the basal portion *G. cliftonii* thallus

270 while rest of the epiphytes species were growing on the apical tips of the host. Secondly, the

271 differences in host wall construction may affect the type of epiphyte attaching and
272 penetration. Species with a thicker outer cortical wall and more structurally diffused deck
273 lamellae are more resistant to epiphytism as reported for *G. tikvahiae* and *G. cornea* and its
274 epiphyte *Ulva lactuca* (Dawes et al. 2000). The two types of attachment observed with *B.*
275 *plumosa* to *G. cliftonii* might be due to differences on individual thalli characteristics as
276 observed for *G. chilensis.* Parameters such as thallus age, tissue susceptibility or physical
277 break-up of the thallus might determine a localized infection in some of *G. chilensis* thalli
278 causing intra-clonal or intra individual phenotypic variations (Santelices et al. 1995).

279   This is the first study which reports epiphytism on *G. cliftonii* and provides new
280 information about *Gracilaria* epiphytism in a different region of the world. Our study
281 suggests that the epiphyte composition observed is not specific for *G. cliftonii* and depends on
282 the distribution of epiphytes in the study area. Also, our study shows that more than one type
283 of attachment between epiphyte-*G. cliftonii* may operate at the same time and that the way
284 epiphytes attach to their host depends on epiphyte and host characteristics. However, further
285 studies on characterization of attachment between epiphyte-host are required to understand
286 these interactions between *G. cliftonii* and its epiphytes.

287 **Acknowledgments**

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441 Table 1 Epiphytic occurrence of *G. cliftonii* epiphytes at Shoalwater Marine Park, Western Australia from April 2008 to March 2009 indicating
442 presence (+) and absence (-). *Significant differences p<0.05.

| | 2008 | | | | | | | | | 2009 | | | |
	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Chi-square
Rhodophyta													
Hypnea episcopalis	+	+	+	+	+	+	+	+	+	+	+	-	0.004*
Ceramium puperelum	+	-	+	+	-	-	+	+	-	-	+	-	1.000
C. pusillum	+	-	-	-	-	-	-	+	+	+	+	-	0.564
C. isogonum	+	+	+	-	-	-	-	-	+	-	+	-	0.564
C. minuta	+	-	-	-	+	-	-	+	-	-	+	-	0.248
Polyshiphonia forfex	+	+	+	+	+	+	+	+	+	+	+	-	0.004*
P. spinossisima	-	+	+	+	+	+	+	-	-	+	-	+	0.248
Laurencia clavata	+	+	+	+	+	-	+	-	+	-	+	-	0.248
Leveillea jungermannoindes	+	-	+	-	+	-	-	+	+	+	+	-	0.564
Dasyclonium incisum	-	+	+	+	-	+	+	-	+	-	+	-	0.564
Echinotamnion hystrix	-	+	+	-	-	-	+	-	+	-	-	+	0.564
Ptilota hannafordi	-	+	-	-	+	-	+	-	-	-	+	-	0.248
Champia parvula	-	+	-	-	+	+	-	-	+	+	+	-	1.000
Wrangellia spp.	-	+	-	-	-	-	+	-	-	-	-	+	0.083
Dasya haffiae	-	-	-	-	-	-	+	-	-	-	-	+	0.021*
Mazoyerella australis	-	+	-	-	+	+	+	-	-	-	+	-	0.564
Callithamnion spp.	-	+	-	-	-	-	+	-	-	-	-	+	0.083
Griffithsia spp.	-	-	-	-	-	+	+	-	-	-	+	-	0.083
Griffithsia ovalis	-	-	-	-	+	+	-	-	+	-	+	-	0.248
Helminthocladia australis	-	-	-	-	+	-	+	-	-	-	-	-	0.021*
Plocamium mertensii	-	-	-	-	-	-	-	+	-	-	-	+	0.021*
Chlorophyta													
Ulva lactuca	+	-	+	+	+	+	+	+	-	-	+	-	0.248
Bryopsis plumosa	-	+	+	+	+	-	+	+	+	+	-	-	0.248
Cladophora spp.	-	+	+	+	+	-	+	-	-	+	+	-	0.564

Table 2. A list of macroalgae epiphytes reported on *Gracilaria spp.* (*post 1995*).

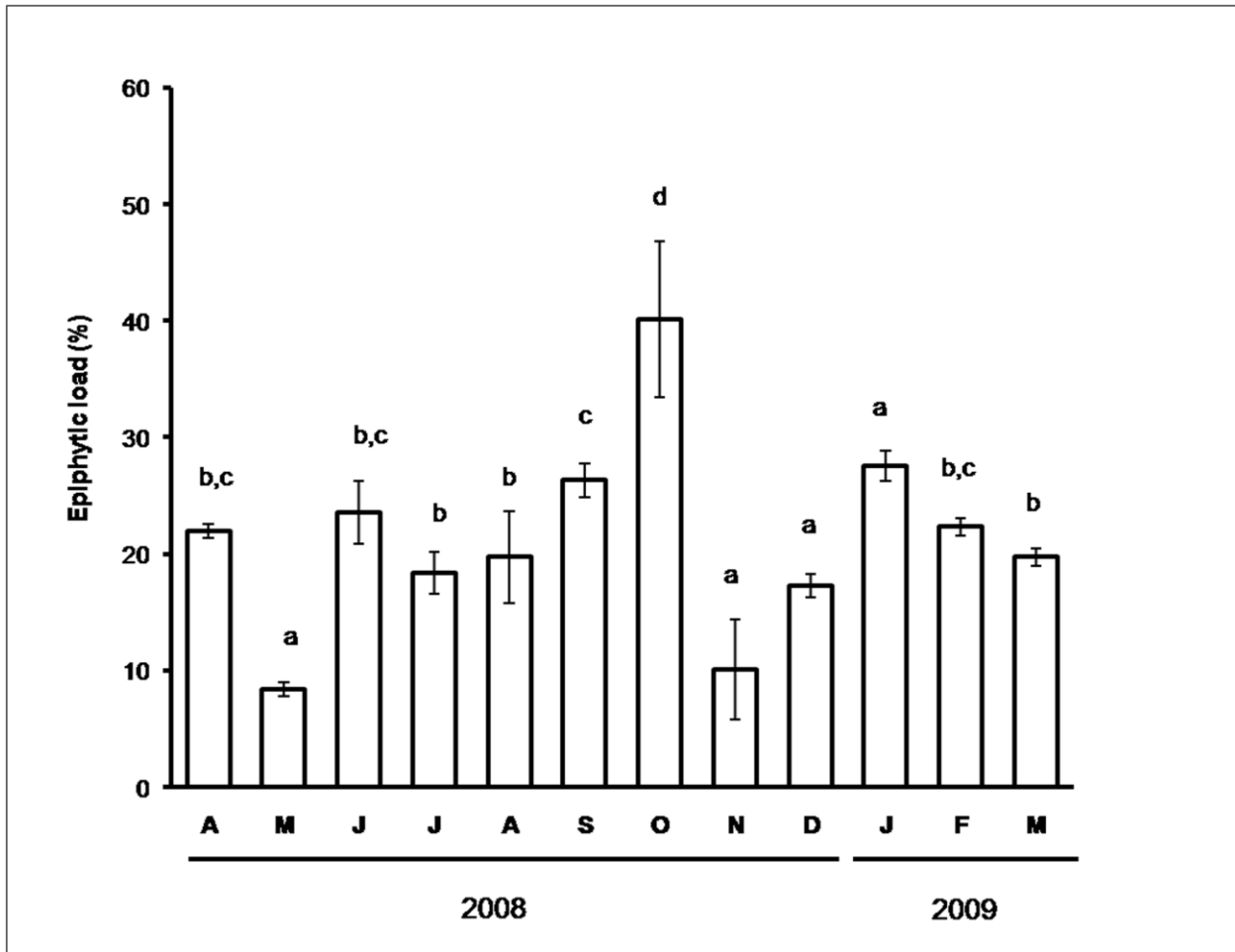| Species	Epiphytes	Reference
G. chilensis	*Giffordia spp.*	Buschmann et al. (1997)
	Ulva spp.	
	Enteromorpha spp.	
	Rhizoclonium sp	
	Polysiphonia spp.	
	Ceramium rubrum	
	Callithamnion spp.	
	Sahlingia subintegra	Leonardi et al. (2006)
	Acrochaetium spp.	
	Antithamnionella spp.	
	Chondria californica	
	Acrosorium corallinarum	
	Ceramium rubrum	
	Ceramium secundatum	
	Polysiphonia harveyi	
	P. flaccidissima	
	Fosliella spp.	
	Ulva lactuca	
	Ulothrix flacca	
	Ectocarpus acutus	
	Hincksia mitchelliae	
	H. granulose	
	Colpomenia sinuosa	
G. conferta	*Ulva lactuca*	Friedlander et al. (1996)
G. cornea	*Ulva lactuca*	Dawes et al.(2000)
		Friedlander et al. (2001)
G. gracilis	*Ceramium diaphanum*	Anderson et al. (1998)
	Ulva spp.	Dural et al.(2006)
	Polysiphonia spp.	
G. lemaneiformis	*Ulva lactuca*	Friedlander et al. (2001)
G. parvispora	*Acanthophora spinifera*	Glenn et al.(1996)
	Enteromorpha intestinalis	
G. tikvahiae	*Ulva lactuca*	Dawes et al. (2000)
G. verrucosa	*Ulva lactuca*	Anderson et al. (1996)
	Enteromorpha compressa	Choi et al. (2006)
	Polysiphonia sp	
	Ceramium spp.	

Fig. 1. Mean epiphytic load of *Gracilaria cliftonii* during April 2008 to March 2009 (weight epiphytes / weight *G. cliftonii*) expressed in percentage. Bars indicate standard error. Different letters indicate epiphytic load significant differences (p<0.05).
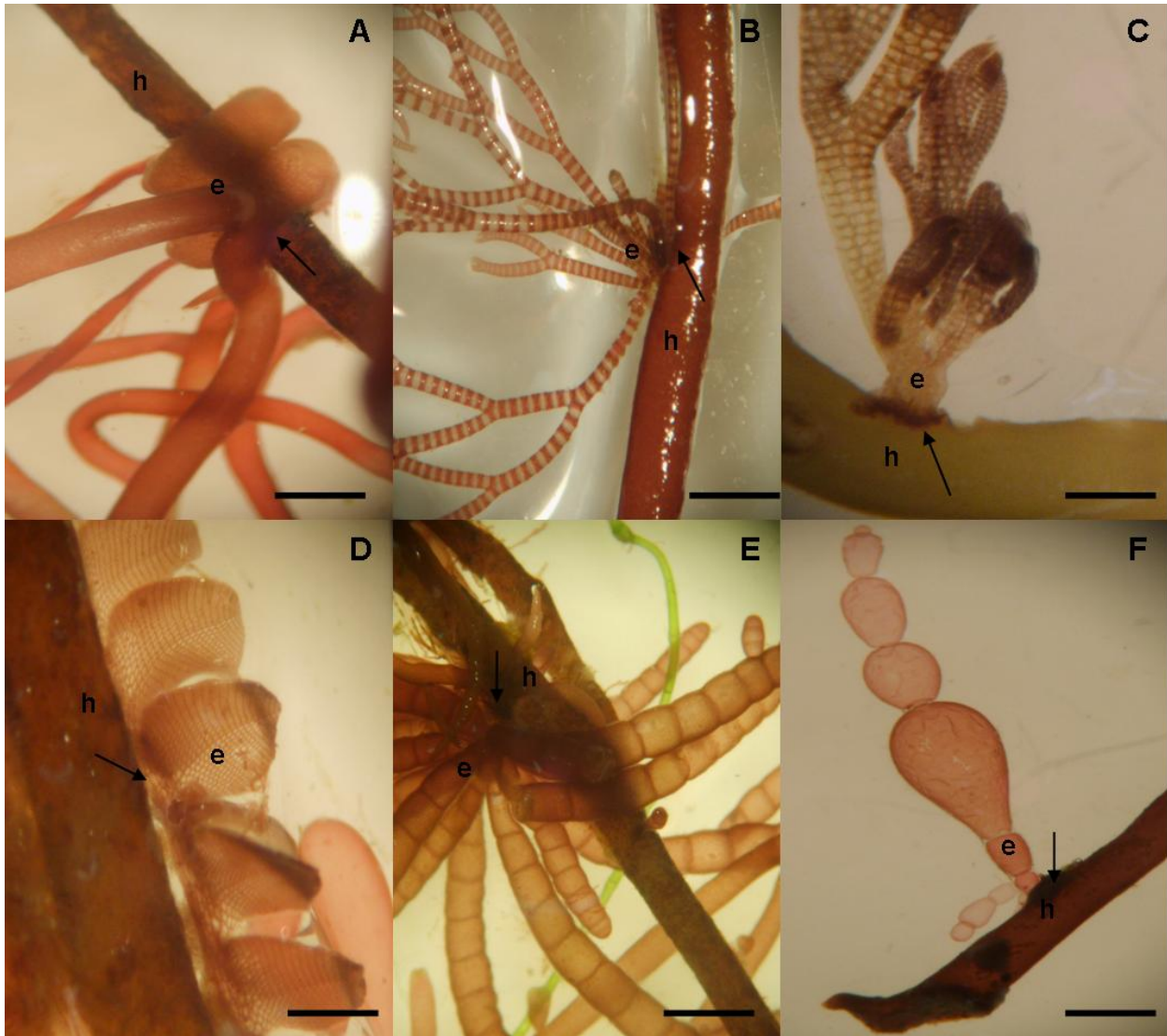
Fig. 2. Sites of attachment epiphytes to *Gracilaria cliftonii*. A. *Hypnea episcopalis* hooks anchored to *G. cliftonii*. B. *Ceramium minuta*. C. *Polysiphonia forfex*. D. *Leveillea jungermannioides*. E. *Champia parvula*. F. *Griffithsia ovalis*. Scale bar 1 mm.

Abbreviations: h: host, e: epiphyte. The arrow indicates the site of attachment.
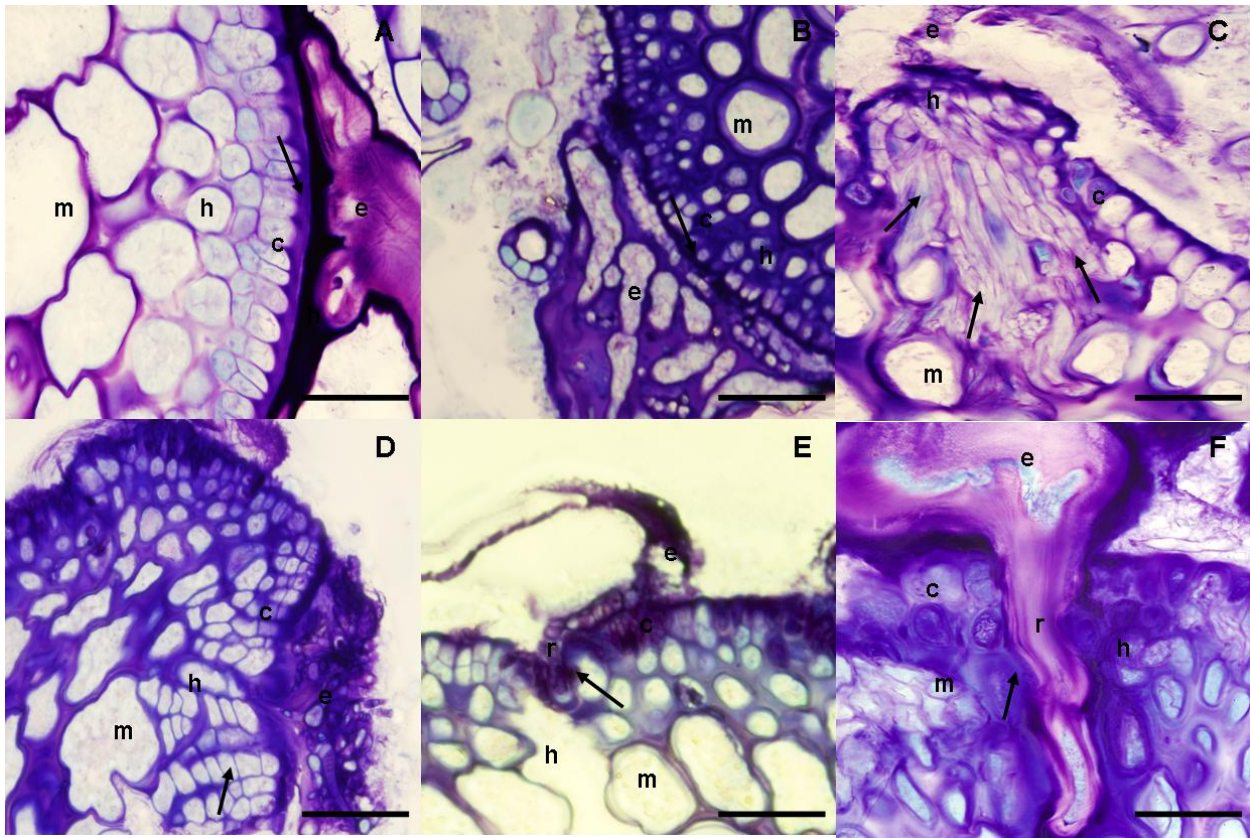
Fig. 3. Light microscopy studies of the sites of attachment between *G. cliftonii* and different epiphytes. A. *Mazoyerella australis* growing on *G. cliftonii* surface. Scale bar 50 μ. B. *Leveillea jungermannioides* growing on *G. cliftonii* surface without penetration showing 3-4 cells cortical layer. Scale bar 100 μ. C. Elongated cortical cells (arrows) at the site of attachment with *Bryopsis plumosa*. Scale bar 50 μ. D. *G. cliftonii* cortex consisting of 6-7 cells (arrow) at the site of attachment with epiphyte *Laurencia clavata*. Scale bar 50 μ. E. *Ceramium isogonum* growing into *G. cliftonii* surface cleavages. Scale bar 200 μ. F. *C. isogonum* rhizoid penetrating into the host cortex and outer medulla. Scale bar 50 μ. Abbreviations: e: epiphyte, h: host, r: rhizoid, c: cortex, m: medulla
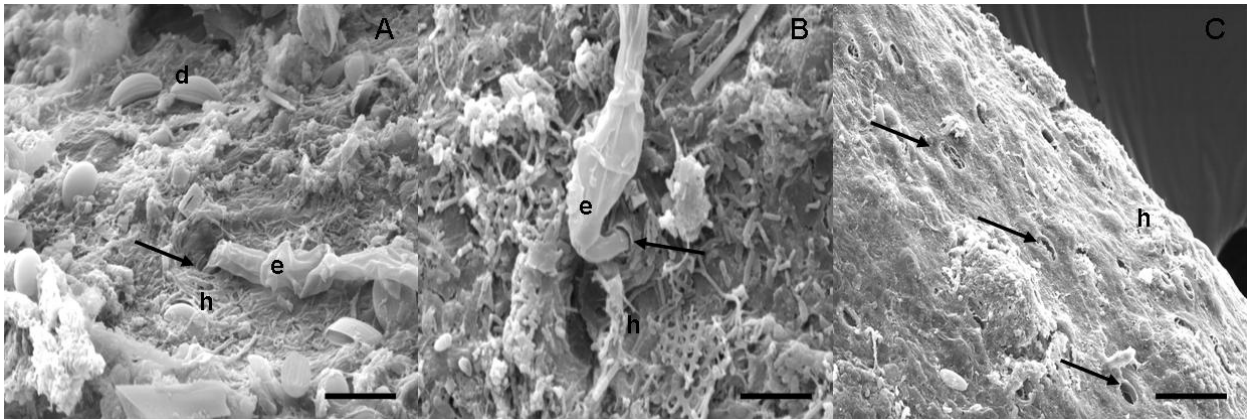
Fig. 4. Scanning electron micrographs showing the penetration of epiphytes into the *G. cliftonii*. A. Penetration by *Polysiphonia forfex*. Scale bar 10 μ. B. Penetration by *Ceramium isogonum* with presence of diatoms at the site of penetration. Scale bar 10 μ. Arrow indicates the site of penetration. C. Lacunae on *G. cliftonii* surface observed under the presence of *Ceramium isogonum.* Scale bar 50 μ.

Abbreviations: h: host, d: diatoms, e: epiphyte. Arrow indicates lacunae on host surface.