ECOPHYSIOLOGICAL STUDIES OF THREE SOUTH AFRICAN *ULVA* SPECIES FROM INTEGRATED SEAWEED/ABALONE AQUACULTURE AND NATURAL POPULATIONS

By

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DECLARATION

I declare that this thesis is my own, unaided work except for amino acid analysis that was carried out by the University of Cape Town, Cell and Molecular Department, Carbon and Nitrogen analysis that was carried out by the University of Cape Town, Chemistry Department and elemental and heavy metals that was carried out by the commercial feed laboratory (BEMLAB, analysis laboratory, Stellenbosch).

This thesis has not been submitted in this or any form to another university. Where use has been made of the research of others, it has been duly acknowledged in the text. Experimental work discussed in this thesis was carried out under the supervision of Prof. J. J. Bolton of the Department of Botany, University of Cape Town and Assoc. Prof R. J. Anderson of UCT/Department of Agriculture, Fishereies and Foresty.

Signed by candidate

Signature removed

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February 2011

DEDICATION

This thesis is dedicated to my parents and grandmother

For their love and support

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Diina Shuuluka, John J. Bolton, Robert J. Anderson and Michael S. Stekoll. Ecophysiological studies of a new aquaculture crop, *Ulva capensis* (Chlorophyta). Presentation at South African Marine Science Symposium, Cape Town, South Africa, 2008.

Diina Shuuluka, John J. Bolton and Robert J. Anderson. Influence of Irradiance, Temperature and Nutrients on Growth and Photosynthesis of intertidal *Ulva capensis* (Chlorophyta) cultivated in the Laboratory**.** Presentation at Phycological Society of Southern Africa, Durban, South Africa, 2008.

PUBLICATIONS:

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ABSTRACT

In South Africa, *Ulva* cultivation is of paramount importance to the marine aquaculture industry. Three local *Ulva* species (*Ulva lactuca* Linnaeus, *Ulva rigida* C. Agardh and *Ulva capensis* Areschoug) were selected for this research. The first two are currently cultivated on abalone farms for abalone feed and for use as bio-filters, and *Ulva capensis* was included because it is morphologically and biogeographically distinct from *Ulva rigida* in nature, despite the inability of molecular methods to separate them. *Ulva rigida was* collected at I & J farm and from nature at Kommetjie on the southwest of the Cape Peninsula, and *U. lactuca* was exclusively collected from I & J farm because it could not be found at sites where it had previously been recorded. *Ulva capensis* was exclusively collected from Kommetjie as this morphological species has not been recorded on abalone farms. The research also aimed to compare *U. capensis* with *U. rigida* on a variety of different measures, as molecular studies have suggested that they may represent a single polymorphic species.

In the laboratory, the growth rate of *Ulva* species under a wide range of environmental conditions was investigated to determine if these species have different responses given that they are collected from different environments (west coast shore and aquaculture farm). *Ulva. rigida* and *Ulva capensis* grew fastest at the irradiance of 80 µmol photons m^{-2} s^{-1}, whilst *U. lactuca* grew fastest at a higher irradiance (160 µmol photons m^{-2} s^{-1}). All species had the same growth pattern with differing light quality, growing best in white, about half as fast in blue, and poorly in red and green light. All species grew fastest at 15^{o} C. *Ulva rigida* and *Ulva lactuca* survived at 0 PSS salinity + Provasoli nutrients for 15 days while *U. capensis* plants died at salinities below 5 PSS after 6 days. Salinities of 25 – 30 PSS yielded the best growth rate for *U. rigida* whilst *U. capensis* and *U. lactuca* demonstrated highest growth in natural sea-water salinities of 35 PSS. For all species maximal growth rates were recorded at 21 µM NH_4^+. *Ulva capensis* had maximal growth at 66 µM NO_3^- whereas *U. lactuca* whilst *U. rigida* had maximal growth at 32.7 µM NO_3^- . *Ulva capensis* and *U. rigida* had the maximal growth at 6.7 µM PO_4^{3-} and *U. lactuca* at 12.9 µM PO_4^{3-}.

This study also focused on comparing the photosynthesis of two closely related taxa which grow close together but in different habitats, and studying their temporal patterns of production. The photosynthetic characteristics of *U. rigida* and *U. capensis* varied seasonally, with higher photosynthetic productivity in spring and summer and lower in winter. Overall,

Ulva rigida had higher photosynthesis parameters (maximal photosynthetic rate, irradiance saturation photosynthetic efficiency and dark respiration rate) than *U. capensis*.

In a semi-closed re-circulating integrated abalone/seaweed farm an *Ulva* production target had been initially proposed of 2.5 t per raceway pond per month in summer and 1.5 t per raceway pond per month in winter. *Ulva* production for 2008 was well below summer and winter targets while production for 2009 achieved the winter target but not the summer target. The set targets were not changed as they can be reachable with further modifications of light and/or nutrients in the system. Prior to this study raceway ponds were covered with 80% shade cloth during spring/summer to reduce epiphyte infection but such heavy shading during summer potentially reduces total production by reducing light. Our recommendation is 30 - 50% shade cloth in both summer and winter, allowing *U. lactuca* to dominate in summer during high irradiances, without growing too fast for the available nutrients, and also minimizing *Myrionema* infections and allowing *U. rigida* to dominate during low light conditions in winter. Laboratory experiments showed that infection of *Ulva* by *Myrionema strangulans* can be prevented even during high irradiances by providing sufficient nutrients. These results have been adopted at I & J farm and have become a valuable tool to help avoid infection and improve productivity of *Ulva*.

This study examined the heavy metals Pb, Cd, Hg and As in all three *Ulva* species, as they are important potential pollutants in seaweeds for human nutrition and aquafeed. The levels of heavy metals (Pb, Cd, Hg and As) in *Ulva* species collected at Kommetjie were higher than in cultivated *Ulva*. All values measured fell below the limits set for heavy metals in France except the average values of cadmium in wild *U. rigida* and *U. capensis*. Although these *Ulva* species are generally safe for animal and human consumption, use of seaweed as abalone feed suggests that South Africa implement a systematic program of seaweed safety monitoring, with regulations on the maximum levels of pollutants in seaweeds commercialised for human and animal consumption.

Temporal variation in nutritional quality, especially biochemical and mineral content of wild *U. capensis* and *U. rigida* and cultivated *U. lactuca* and *U. rigida* used as abalone feed was investigated. In the wild material, temporal changes in the contents of the various mineral elements were more gradual than in cultivated material, where changes were rapid and often haphazard. The ash (mineral) content of wild *Ulva* species showed a seasonal trend with an

increase during the late-winter to spring months. The farmed material generally had less ash (mineral) content than wild material. The crude fibre content of wild and farmed *Ulva* species ranged from 3.11 to 5.71 % dry weight and was similar to that of soy meal. Farmed *Ulva* species had higher carotenoids contents than wild species (inversely related to nitrogen level). The C:N ratio for both wild and farmed *Ulva* species ranged from 7.9 to 15.7, indicating that these species contain sufficient N for an animal diet. *U. rigida* and *U. capensis* had slight but significant differences in their chemical composition and mineral content.

Temporal variation in amino acid composition of wild and farmed *Ulva* was studied, and nitrogen to protein conversion factors for wild *U. capensis* (5.58) and *U. rigida* (5.12) as well as farmed *U. lactuca* (5.65) was established. An average N-Prot factor of 5.45 provides a more accurate estimate of the protein content of the *Ulva* species studied. There were no temporal variations in the amino acid content for all species, although the amino acid profile of *U. capensis* showed no cysteine, whereas *U. rigida* and *U. lactuca* had cysteine during all the analysed months. In all species the values of crude proteins were always significantly higher (by 58.9 -77.1%) than the protein contents directly determined by the Bradford method.

The findings of this thesis will help *Ulva* farmers (*e.g.* at I&J farm) to improve their cultivation methods in terms of productivity, nutritional quality, seasonal species preference and the control of epiphytes. The generally similar seasonal nutritional profiles of *Ulva* species in the present study are of great benefit for using *Ulva* as feed as its nutritional quality is consistent. In addition, the findings on the ecophysiology of *U. capensis* and *U. rigida* will complement the molecular studies of these two species which have different habitats and morphology but are very closely related, if not conspecific.

Chapter 1

General Introduction

1.1 The genus *Ulva*

The genus *Ulva* was described by Linnaeus in 1753 together with three other genera *Fucus*, *Conferva* and *Chara*. *Ulva* was later confined to consist of green seaweeds with distromatic blades, and *Enteromorpha* Link was recognized for tubular forms (Hayden *et al*., 2003). Hayden *et al.* (2003) determined the phylogenetic relationships among taxa currently recognized as *Ulva*, *Enteromorpha*, *Umbraulva* Bae *et* I.K. Lee and the monotypic genus *Chloropelta* C.E. Tanner. Findings of these authors, by DNA analysis combined with earlier molecular and culture data, gave strong evidence that *Ulva*, *Enteromorpha* and *Chloropelta* are not distinct evolutionary entities and should not be recognized as separate genera. Furthermore, since *Ulva* is the oldest name, *Enteromorpha* and *Chloropelta* were reduced to synonymy with *Ulva* (Hayden *et al*., 2003). Although *Ulva* now includes distromatic and monostromatic tubular forms, all the species used in the present study are distromatic forms (two cells thick).

From www.algaebase.org, *Ulva* belongs to the order Ulvales and family Ulvaceae; there are 557 species names in the database including synonyms, of which 97 are currently accepted taxonomically. There are 8 families in the order Ulvales including Ulvaceae and 11 genera in the family Ulvaceae including *Ulva*. The macroalgae in the family Ulvaceae are well known for their simple morphology, which partially contributed to the difficulties encountered in their identification (Silva *et al.*, 1996). In addition, species of *Ulva* are known to display a high degree of physiological and morphological plasticity in a wide variety of environments (Lobban and Harrison, 1997). Morphological and cytological characteristics that are used to identify species are not always consistent since they vary with season, wave energy, latitude and geographical location, even within a single population at a given time (Steffensen, 1976;

Tanner, 1986; Woolcott and King, 1999). For instance, wave energy was found to cause stunted growth in *U. fasciata* (Mshigeni and Kajumulo, 1979) and *U. fenestrata* (Titlyanov *et al.*, 1975) as well as affecting the size and morphology of *U. californica* (Tanner, 1986). Provasoli and Pintner (1980) showed that *Ulva lactuca* plants had an atypical "pincushion" morphology when grown axenically. When these unusual forms were exposed to bacteria, the plants reverted to a normal "foliaceous" morphology. Similar effects on morphology have also been observed when bacteria are excluded from cultures of U. *linza* and *U. compressa* (Fries, 1975) and *Ulva pertusa* (Nakanishi *et al*., 1996). Therefore, due to all these shortcomings with identification based on morphological characteristics only, the application of molecular techniques in addition to detailed morphological characteristics has been found to provide more reliable results. The use of molecular data is more feasible because it represents the genotype rather than the phenotype (John and Maggs, 1997). Recently, nuclear ribosomal DNA internal transcribed spacer (ITS) sequences have been used for the identification of *Ulva* species (Coat *et al*., 1998; Malta *et al*., 1999) and these rDNA data can be used for comparison among *Ulva* species (Blomster, 1998).

The taxonomy of *Ulva* species from the west coast of South Africa has been studied by Joska (1992) and documented by Stegenga *et al.* (1997). The latter authors provide descriptions of five species for the region: *U. capensis, U. fasciata, U. lactuca, U. rigida, U. unicialis* and *U. rhacodes*. The species of *Ulva* are recognised based on morphological and anatomical features. A study by Kandjengo (2003) was the first to investigate the taxonomy of local *Ulva* species at a molecular level, and the results from his study have provided more insights on the taxonomic status of the local *Ulva* species. It emerged that there are more *Ulva* species than previously documented and suggested that some of these species may have been introduced into South Africa waters in recent times (Kandjengo, 2003). This author also

reported that there is no difference between *U. capensis* and *U. rigida* according to ITS analysis and it was clearly shown by TCS analysis that these species could be haplotypes of a single genetically diverse species. TCS is a computer program designed by Templeton *et al.* (1992) to estimate gene genealogies for closely related species, those that produced polytomies under maximum parsimony. The TCS software program is more suitable for population level analyses (Clement *et al.,* 2000). The program treats taxa as haplotypes and a network of clusters is produced or else if distantly related taxa the "haplotypes" are not joined. The resulting graph also clearly indicates the number of steps connecting two haplotypes (Clement *et al.*, 2000).

Ulva is an opportunistic genus that is typically found in intertidal and estuarine habitats (Adams, 1994; Blomster *et al.*, 1998) and it has a wide variety of growth strategies (Vermaat and Sand-Jensen, 1987). Several characteristics enable species of *Ulva* to dominate a variable environment: they are both euryhaline and eurythermal, and desiccation and temperature-tolerant (Vermaat and Sand-Jensen, 1987; Fong and Zedler, 1993; Poole and Raven, 1997; Fong *et al.*, 1998). A new species of freshwater *Ulva, Ulva limnetica* has been recorded from the Ryukyu Islands, Japan (Ichihara *et al*., 2009), the first record of freshwater distromatic *Ulva*. Furthermore, in comparison with most other macroalgae, *Ulva* is fast growing (Björnsäter and Wheeler, 1990), has a high SA: V ratio (Rosenberg and Ramus, 1984; Taylor *et al.,* 1998; Taylor *et al.,* 1999), has high rates of ammonium uptake (Fujita, 1985; Thomas and Harrison, 1985; Taylor *et al.*, 1998; Taylor *et al.*, 1999) as well as high photosynthetic performance (Litter, 1980).

1.2. The biogeographical distribution of *Ulva*

Factors such as temperature regime of habitat and temperature requirements for growth, reproduction and survival are known to determine the biogeographical distribution of marine

benthic algae (van den Hoek 1982a, b; Lüning 1984; Bolton 1986; Breeman 1988). The genus *Ulva* is well known for its wide distributions from marine to fresh water all over the world (van den Hoek *et al*., 1995). Most of the species in the genus are found in near-shore marine and estuarine waters, upper to mid- intertidal (eulittoral and supralittoral zones), and in some locations may be found in the subtidal zones. They are normally epilithic but some species, such as *Ulva rhacodes*, may be epiphytic (Stegenga *et al.*, 1997). *Ulva* species often form extensive mats in highly dynamic environments, for instance where the rocks or boulders are frequently covered and then uncovered by shifting sands in the intertidal zone (Dickinson, 1963).

Due to the difficulties in the identification of members of this genus, many species names have been misapplied (Silva *et al.*, 1996) and this has resulted in artificially inflated ranges for many of the species. According to Stephenson (1948), Silva *et al*. (1996) and Stegenga *et al.* (1997), *U. lactuca* Linnaeus is a good example of a species name that has often been misapplied. The records of *U. lactuca* Linnaeus, shows this species to have a wide distribution from Arctic, Ireland, Europe, Atlantic Islands, North America, Central America, South America, Africa, Indian Ocean Islands, Asia, South-east Asia, Australia and New Zealand, Pacific Islands, Antarctic and subantarctic (www.algaebase.org). Thus, Stegenga *et al.* (1997) suggested that most records are probably not correct and there is molecular evidence that South African *U. lactuca* is not genetically identical to the original European *U. lactuca* (Kandjengo, PhD unpublished data). In addition, currently three species of *Ulva* are being grown commercially in South Africa. At Irvin & Johnson (I & J) farm, one of the species is called *U. lactuca* but isn't, one is called *U. rigida* but cannot be separated from *U. capensis* by ITS, and the other species grown at Wild Coast abalone currently has no name. Furthermore, only the *U. rigida* morphology has ever been observed in the farm cultures,

never the *U. capensis* morphology (*sensu* Stegenga *et al*., 1997) and these two species grow together on the west coast, and their morphologies appear distinct in nature.

1.3 Life history of *Ulva*

Species of the genus *Ulva* generally colonise open substrates and are often considered fouling organisms. This ecological role is a characteristic of their simple adult morphology and their fecundity (Beach *et al*., 1995). Carter and Anderson (1991) showed that in the lower eulittoral zone at Port Alfred (South African south coast), *Ulva rigida* was always the first macroalga to colonise cleared rock from which limpets had been excluded. Furthermore, Dye (1993) obtained similar results on the South African southeast coast (Transkei): cleared areas were rapidly colonized by species of the genera *Ulva, Ralfsia* and *Iyengaria*. Spores and gametes of *Ulva* species are known to have a net carbon balance which provides for the energy needs of unicells for motility (Beach *et al*., 1995). Moreover, during warmer months, a significant portion of *U. lactuca*'s biomass is allocated to the formation of zoospores and gametes (Niesenbaum, 1988). In nature *Ulva* can take 3–4 months to attain maximum size (Oza and Rao, 1977). However, this time may be shorter in upwelling areas, since *Ulva* has higher nitrogen uptake rates (Duke *et al*., 1989; Pedersen and Borum, 1997) and higher growth rates when compared to perennial species. The typical life history of *Ulva* is composed of two isomorphic generations: a haploid gametophyte and a diploid sporophyte (Raven *et al.*, 1992; Lobban and Harrison, 1997). Both haploid and diploid phases are multicellular and vegetative (Fig.1.1) (van den Hoek *et al.*, 1995).

Another form of reproduction that is common in the Ulvaceae is parthenogenesis whereby gametes develop into parthenosporophytes (Tanner, 1981; van de Hoek *et al.*, 1995). Multiplication of the plants by vegetative fragmentation is also very common in this group (Tanner, 1981; van de Hoek *et al*., 1995). Reproductive structure formation is influenced by

environmental factors, such as nutrients and tidal regime. Under rich supplies of nitrate *Ulva fasciata* shows fast sporogenesis and sporulation (Mohsen *et al*., 1974). In addition, temperatures below 20°C favor the production of gametophytes (Mohsen *et al*., 1974). Algal reproduction is also affected by light as has been shown in a variety of algal species (Lüning, 1981; 1990). Light of different wavelengths has been reported to influence macroalgal reproduction (Dring, 1988). In *Ulva lactuca*, the degree of sporulation was almost twice as much in blue or red as in green light (Lüning and Dring, 1985). In addition, spore release in intertidal *Ulva* species is driven by tidal/ lunar rhythms (Smith 1947, Christie and Evans 1962, Lüning, 1990). For instance, Lüning *et al.* (2008) showed that under constant conditions in the laboratory a free running rhythm was observed, with reproductive peaks of *U. pseudocurvata* appearing approximately every 7 days. However, when artificial moonlight was provided every 4 weeks, fewer reproductive events occurred and the reproductive rhythm becomes synchronized to artificial moonlight.

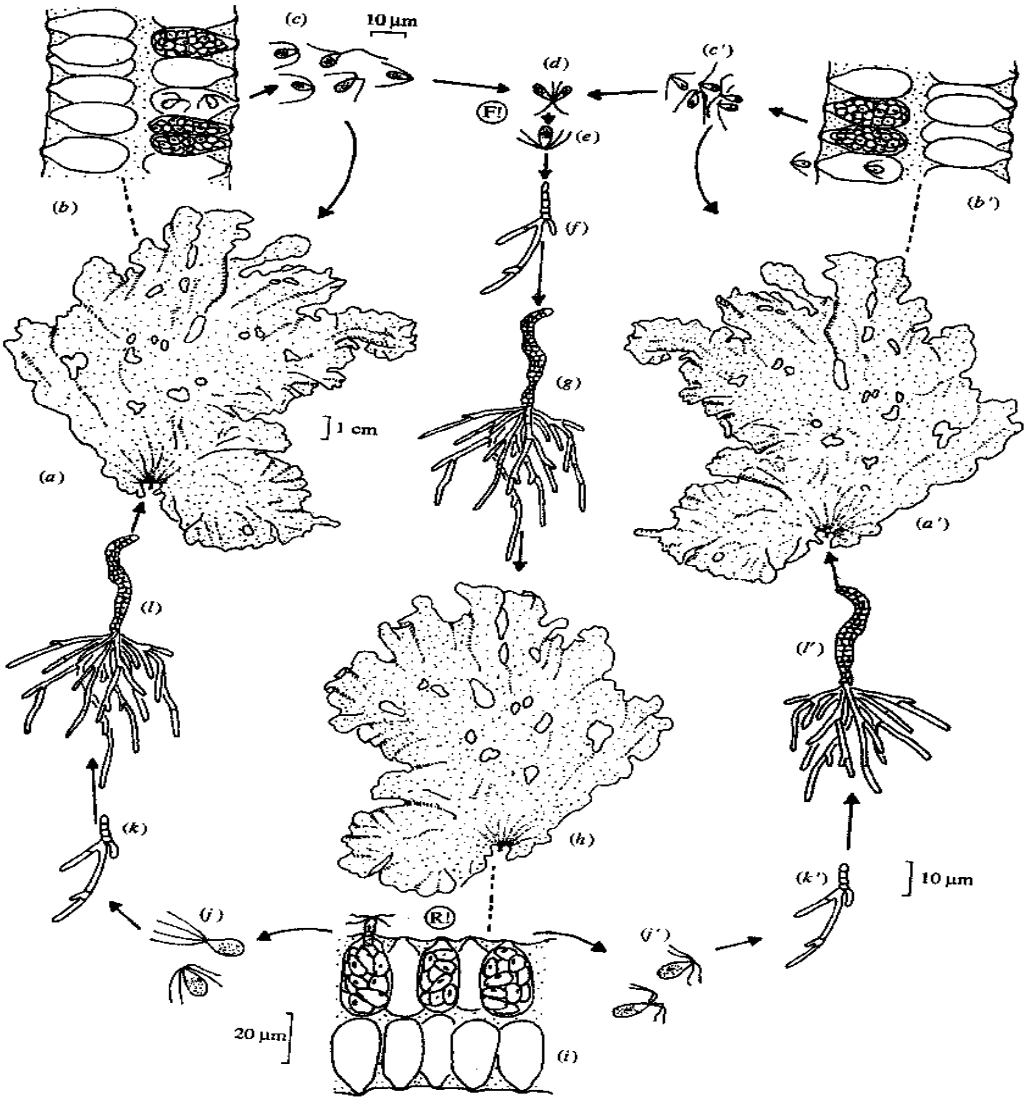


Figure 1.1: The life history of *Ulva*. (*a*, *a'*) Flat blade-like gametophytes. (*b*, *b'*) Division of the cell contents into biflagellate gametes; these are unequal, copulation being anisogamous. (*c*) Female gametes. (*c'*) Male gametes. (*d*) Anisogamous copulation. (*e*) Quadriflagellate planozygote. (*f*) Uniseriate filamentous germling of sporophyte generation attached via branched rhizoids. (*g*) Tubular germling of sporophyte generation. (*h*) Fully developed blade-like sporophyte (diploid). (*i*) Meiotic division of sporophyte cells to form haploid quadriflagellate zoids (meiospores). (*j*, j') Quadriflagellate meiospores. (*k*, k') Uniseriate filamentous germlings of the female and male gametophytes. (*l*, *l'*) Tubular germlings of the female and male gametophytes. F! = fertilization; R! = reduction division (meiosis). Source: van de Hoek *et al.*(1995). Pp 404 -405.

1.4 *Ulva* mariculture in South Africa

Interest in seaweeds as novel foods with potential nutritional benefits (Darcy-Vrillon, 1993), and animal fodder (Davies *et al*., 1997) is expanding. This interest has led to the development of extensive mariculture systems for seaweeds in the ocean, ponds and also intensive land based systems using tanks (Lignell *et al*., 1987). Cultivation techniques are being improved with the main objective of obtaining higher algal biomass that exhibits specific qualities (Lobban and Harrison, 1994). In South Africa, shortages of algal derived colloids during World War II sparked interest into commercialization of seaweeds (Isaac, 1942; 1953; Isaac and Molteno, 1953; Anderson *et al*. 1989; Critchley *et al.* 1998, Anderson *et al.*, 2003a) and most of the seaweed resource used is based on material from the wild (Critchley and Rotmann, 1991). Unfortunately, the exposed nature of the South African coastline makes it difficult to cultivate seaweeds in open-water systems, except in a few protected bays (Aken *et al*., 1992; Wakibia *et al*., 2001). However, since the late 1990's seaweed mariculture experiments were carried out using *Gracilaria* on an experimental sub-surface raft at Saldanha Bay (Anderson *et al*., 1996) based on the methods developed at Luderitz Bay, Namibia (Rotmann, 1987; Dawes, 1995).

Recently, seaweed demand has increased with the rapid development of the South Africa abalone industry, since seaweed (harvested wild kelp) initially formed the primary diet of the farmed abalone and is still the main diet on many farms. Unfortunately, harvest of wild kelp (*Ecklonia maxima* and *Laminaria pallida*) for abalone feed is now approaching limits of sustainable harvesting in kelp concession areas where abalone farms are concentrated (Robertson-Andersson, 2007). Thus, *in situ* cultivation of seaweed on the farms is desirable in order to deal with growing requirements of feed, and this has led to the initiation of collaborative research projects on the mariculture of various seaweeds in South Africa (Troell *et al*., 2006).

More recently, according to Bolton *et al.* (2008), Wild Coast Abalone farm (at Haga Haga near East London on the southeast coast) has become a world leader in *Ulva* cultivation, and the current estimated figure of *Ulva* cultivation in South Africa is about 1,100 tonnes wet weight per annum. This farm grows most of the South Africa's *Ulva* and a main impetus for this culture was the lack of available kelp in the East London region. In early 2006, an integrated abalone/*Ulva* system with partial re-circulation was set up at a farm on the southwest coast (Irvin & Johnson West Coast Abalone in Gansbaai). This system has been running commercially since January 2006 (Robertson-Andersson *et al*., 2008). Currently, the seaweeds cultivated on local abalone-seaweed farms are mainly *Ulva* species and at least four of different species are being cultivated (Bolton *et al*., 2008). A study by Bolton *et al.* (2008) discussed in details the benefits and drawbacks of *Ulva* cultivation in South Africa. The local cultivation of *Ulva* has proven successful so far, and the flow-through systems at Wild Coast Abalone (Haga Haga) farm produce about 2 t of *Ulva* per working day throughout the year, in a series of 32 large-scale ponds growing seaweed in effluent from the flow-through abalone tanks (Bolton *et al*., 2008). At I & J farm, the semi-closed seaweed integrated seaweed/abalone system comprises a platform with tanks of abalone connected to four seaweed raceways. The nutrient-rich effluent water from the abalone tanks passes through the seaweed raceways where most of the nutrients are absorbed by the seaweed *Ulva* spp. (Bolton *et al*., 2008). The recirculation rate in the system at the time of the present study was about 67%, with about 360 m^3 of fresh seawater and 720 m^3 of re-circulated water being pumped into the header tank each hour (Eric Dahl, pers. comm.).

1.5 *Ulva* as feed for aquatic animals

The long-term sustainability of aquaculture may be threatened by its present over-dependence on fish meal and fish oil (FAO, 2002). Therefore during these last decades efforts have been made to evaluate the potential of alternative protein sources in aquafeeds (Alexis, 1997). Studies on the diet performance on snakehead fish (*Channa striatus*) showed that the incorporation of 5% *Ulva spp*. into the formulated diet resulted in increased growth rate, feed efficiency and feed consumption, but the same level of *Gracilaria spp.* had no significant effect on those parameters (Hashim and Mat Saat, 1992). A study by Valente *et al.* (2006) suggested that the inclusion of up to 10% of *G. bursa-pastoris* and *U. rigida* in a fish-formulated diet can be considered as very useful for sea bass juveniles, as no negative consequences on growth performance, nutrient utilization or body composition were observed. Moreover, Cruz-Suarez *et al.* (2009) showed that the *Ulva* intake by shrimp improved the artificial feed conversion ratio and the growth rate.

Abalones (*Haliotis* spp) are herbivorous invertebrates that naturally feed on macroalgae (Sales and Britz, 2001) and are sometimes referred to as algivorous animals (Shpigel *et al*., 1999). A variety of seaweeds are naturally consumed by abalone (Britz, 1995) and kelp forms the primary food source for these animals on the west coast of South Africa (Rosen *et al*., 2000). The growing abalone industry depends on a steady supply of feed resources. In South Africa fresh seaweeds are being used as abalone feed in conjuction with the formulated feeds: these seaweeds include *Ecklonia* and *Laminaria*, *Ulva* and *Gracilaria.* Abfeed® (Marifeed Pty Ltd, South Africa) is a formulated feed containing fishmeal (55 %), starch, *Spirulina* spp. (10 %), vitamins and minerals (Fleming *et al*. 1996). The approximate analysis of Abfeed® is 34.6 % protein, 43.3 % carbohydrates, 5.3 % fat, 1.2 % Crude fibre, 5.7 % ash and ± 10 % moisture (Deborah Robertson-Andersson, pers. comm.) The formulated feeds are aimed to

fulfil the nutritional requirements of the abalone and such requirements include carbohydrates (Nelson *et al*. 2002); lipids (Durazo-Beltran *et al*., 2003) and protein (Fleming *et al*., 1996). Kelp is also used as a primary feed for cultured abalone because of its abundance in the south west and west coast of South Africa (Bolton *et al.*, 2009). A study by Naidoo *et al*. (2006) showed that South African *H. midae* grew best on a mixed diet of kelp plus other seaweeds compared to those fed with artificial feed exclusively. A diet of several seaweeds is more beneficial in aquaculture feed. For instance, Wahbeh (1997) analysed the nutritional quality of several algae and detected some amino acid deficiencies (for instance, *Ulva lactuca* lacked cysteine) and high ratios of n-6 to n-3 PUFAs. The author suggested that a mixture of several algae species could provide fish with an adequate supply of all essential amino acids and a fatty acids composition that would result in increased growth. Furthermore, it has been shown that abalone fed on seaweeds have high Food Conversion Ratio (FCR) values due to the high water content and relatively low protein content of macroalgae (Hahn, 1989). There is ongoing work in our laboratory on the inclusion of *Ulva* in the formulated diet of sea urchins and preliminary results are positive.

1.6 Factors affecting *Ulva* growth

In recent decades, research on the physiology and ecology of marine macroalgae has increased due to potential commercial usage (Bird and Benson, 1987). In the wild, the growth of seaweeds is affected by various chemical, physical and biological factors (Pedersen *et al*., 2004). The growth rate of cultured seaweeds has also been shown to be regulated by environmental parameters such as irradiance, salinity, temperature and nutrient supply (Lüning, 1990; Lobban and Harrison, 1994). To date, however, very little is known about the ecophysiology of South Africa's *Ulva* species. Such information would help us to understand

optimal growth conditions of these species in order to reduce the duration of cultivation and to ensure the greatest yield.

1.6.1 Salinity

Salinity fluctuation has been considered to be the primary factor limiting the growth of macroalgae from rocky intertidal regions and estuaries (Lobban and Harrison, 1997). The tidal cycle is the main factor in controlling variation in salinity in estuarine environments. Changes in freshwater runoff, fluctuations in the currents, storms, winds and the solar cycle, also affect salinity (Yarish and Edwards, 1982). It is becoming increasingly important to understand how algae respond and adapt to salinity stress (Fan-Lu *et al*., 2006). The physiological and biochemical responses of algae to salinity stress, including osmotic adjustment, ion homeostasis, metabolite accumulation, photosynthesis, respiration, growth and development have been extensively studied (Kirst, 1990; Parida and Das, 2005). Species in estuarine environments must withstand relatively high changes in salinity over a short time period. These rapid changes can have marked effects on macroalgal growth. This is a harsh environment in which only a few well adapted species can flourish (Josselyn and West, 1985). *Ulva* which has adapted to this environment has a salinity tolerance ranging from 3 PSS to 115 PSS (Lobban and Harrison, 1997). However, some former *Enteromorpha* species, which are now included in *Ulva*, grow in freshwater (<1 PSS). Moreover, *Ulva* plants are able to regulate the amounts of dissolved internal salts, keeping their internal osmotic pressures somewhat higher than the surrounding medium, allowing them to maintain a constant turgor (Lobban and Harrison, 1997). For instance, Blunden and Gordon (1986) found that the tertiary sulfonium β-dimethylsulfoniopropionate (DMSP) is involved in the salinity responses of *U. lactuca*; Karsten *et al* (1991) reported that the major osmolytes in *U. rigida* from Antarctica and South Chile were K^+ and DMSP at increasing salinitites; *U.*

fasciata accumulates free proline in response to hypersalinity (Liu *et al*., 2000), and similar findings on *U. pertusa* were reported by Kakinuma *et al.* (2001a). Moreover, *Ulva* can be negatively affected by low salinity, for instance, a study by Murthy *et al* (1988) found decreased growth of *U. lactuca* at salinities below 30 PSS and similar findings were reported by Friedlander (1992) who also found reduction in growth of *U. lactuca* at salinities below 20 PSS. In addition, *U. fasciata* growing in intertidal areas and experiencing salinity fluctuations has developed a defence system by increasing the availability of antioxidants and the activities of antioxidant enzymes to cope with the oxidative stress induced by salinity changes (Fan-Lu *et al*., 2006).

1.6.2 Light

Light is another major factor limiting the growth of macroalgae in the marine environment (Hanisak, 1983). It is also known to affect algal development in various ways such as photosynthetic production of biomass, photo-stimulation of growth direction, and photoinduction of the different stages of development in an algal life history (Dring, 1988; Lüning, 1990). Commonly, the light requirements of seaweed species tend to correspond to their geographical position, with lower requirements in Polar Regions and higher requirements in warmer regions (Roleda *et al*., 2006). Species growing in Polar Regions tend to be adapted to shade conditions (Wiencke *et al*., 2006). It a well known phenomenon that species that are subjected to low light have developed compensatory mechanisms to survive successfully in these conditions. Such mechanisms include high pigment content, high accessory pigment: chlorophyll *a* ratios (Reiskind *et al.*, 1989), mechanisms or morphological differences to cope with the varying wavelengths that are received in deeper waters and low photosynthetic saturation levels (Hanisak, 1979). Beer *et al.* (2000), found that most of the light absorbed by *Ulva* thalli is due to photosynthetic pigments, and that reflection from the

thallus surfaces is very low, particularly if the light is provided perpendicular to the thallus surface. Moreover, *Ulva lactuca* adapts efficiently to low light by increasing chlorophyll concentration (and therefore light absorption) and continuing to grow at a very low irradiance of 0.6 µE m^{-2} s^{-1}. This irradiance corresponds to minimum light requirements of deep-living marine macroalgae and phytoplankton growing under ice (Vermaat and Sand-Jensen, 1987). The light compensation point for growth of *U. pertusa* was found to be between 1 and 5 µmol m^{-2} s^{-1}, which is similar to the minimum light required by several understorey algae for growth (Leukart and Lüning, 1994; Han and Kain, 1996). *Ulva* is able to photoacclimatise within days to lower light levels and can maintain growth rates even if total irradiance is reduced slightly e.g by self shading (Vandermeulen and Gordin, 1990; Altamirano *et al*., 2000b). The duration of light availability has also a marked effect on seaweed growth rates, and the growth is generally better during summer months with the increased day length, and growth often decreases in winter (Rosenberg and Ramus, 1984a; Israel *et al*., 1993; Fillit, 1995).

1.6.3 Light quality

Light is subject to momentary, diurnal, seasonal and global changes both in irradiance and in spectral distribution (Talarico and Maranzana, 2000). Light reaching the water shows great variations as a result of scattering and absorption by the atmosphere. The total irradiance is also affected by the sun angle, decreasing as the sun moves towards the horizon (Lobban and Harrison, 1994). Four factors have been described that relate to the interaction of light with the ocean (Larkum and Barrett, 1983, Drew 1983):

1) heterogeneity of light and water over time
2) the effect of the water surface on light penetration
3) spectral changes of light with depth

4) attenuation of light with depth and depths to which seaweeds can grow.

Variable spectral proportions (i.e., red:far-red; blue:red; green:red; blue:green) are known to affect the relative pigment composition of seaweed, possibly acting as photomorphogenic 'signals' (Lopez-Figueroa, 1991). In seaweeds, light quality has been known to affect growth, photosynthesis and morphogenesis (McLachlan and Bidwell, 1983; Lüning and Dring, 1985). The spectral quantum yield in *U. lactuca* was found to be higher in red than in blue light (Lüning and Dring, 1985). It has been shown that green algae tend to form not only more chlorophyll but selectively more chlorophyll b with increasing depth and shade in the sea (Yokohoma and Misonou, 1980). In *U. pseudocurvata* Koeman et Hoek, blue light caused an immediate reduction of thallus area and growth rate whereas green light and red light resulted in an initial peak in growth rate followed by inhibition 60 minutes after the onset of light (Lüning, 1992). However, there are species of *Ulva* known to have pigments that absorb light in the green region of the visible spectrum. A study by Yokohama and Kageyama (1977) discovered an absorption peak at 540 nm in *U. japonica*. The pigment absorbing light in that region was identifed chromatographically as siphonaxanthin, and this pigment is also characteristic of many siphonous greens (Kleinig, 1969).

1.6.4 Temperature

It has been shown that individual seaweed species' biogeographic distributions are often limited by seawater temperature regimes (Breeman, 1988). Temperature tolerances of different species of seaweeds are at least partly responsible for patterns in their geographical distributions (Lüning, 1990) and seasonal blooms (Lobban and Harrison, 1994). van den Hoek (1975) used a system to describe the phytogeographic provinces in the North Atlantic and used the following definition: 'cold temperate' regions have water temperatures of > 10 ^{o}C in the summer but < 10 ^{o}C in the winter; 'warm temperate' is defined as regions with

temperatures > 15 ^{o}C in summer but < 20 ^{o}C in the winter. However, the temperature regimes in the Benguela marine province are strongly affected by cool water upwelling from the Benguela current system (Andrews and Hutching, 1980; Chapman and Shannon, 1985, Stegenga *et al*., 1997). Although the temperature of this upwelled water can be below 10 ^{o}C, the phenomenon of upwelling is variable and usually of short period, and has never been found to cause inshore monthly means to fall below 10 ^{o}C, which is the critical temperature for cold temperate biota (Bolton, 1986; Anderson and Bolton , 1985, 1989). In addition, at upwelling centres, monthly mean temperatures have never been recorded to rise above 15 ^{o}C, which is a criterion for warm temperate biota (Bolton, 1986). The South African west coast biota is thus generally described as 'cool temperate', whereas that of the south coast is 'warm temperate' (Bolton and Anderson, 1997).

Studies on the patterns of Southern Africa's seaweed communities on a biogeographic scale have been shown to correlate with temperature (Bolton and Anderson, 1990). Moreover, Bolton and Anderson (1990) found seaweed samples from the warmest region of False Bay to be similar to those from geographically distant sites with comparable temperature regimes. Temperature requirement for growth is known to vary with the life stages, for example, a study by Anderson and Bolton (1989) found young annual sporophytes of *Desmarestia firma* to grow best at 12 ^{o}C in laboratory culture, a temperature typical of summer upwelling, while the gametophytes grew best at 15 ^{o}C, which is consistent with winter sea temperatures. Other studies on temperature had been carried out on South African seaweeds: *e.g.* Engledow and Bolton (1992) studied *Gracilaria verrucosa* in culture and found its optimum growth temperature to be between 15 – 25 ^{o}C. In addition, Anderson and Bolton (1985) found the sporelings of both gametophyte and sporophyte phases of the red seaweed *Suhria vittata*

grew best at 15 and 20 ^{o}C. However, no experimental work on temperature has been done on South African *Ulva*, especially on the relationship between temperature and growth rate.

According to Shelford's "zone of tolerance" concept, growth rates also have a species-dependent optimal temperature (Ricklefs and Miller, 2000). The temperature tolerance curve usually exhibits a quick decline near the species-specific upper lethal limit (Lüning, 1990). The possible causes of algal death at high temperatures include processes such as denaturation of proteins, and damage to heat-labile enzymes or membranes (Lüning, 1990). Temperatures of 33-35 °C represent the long-term upper survival-limit for tropical benthic algae, with a low temperature tolerances ranging from 5-14 °C (Lüning, 1990). Bolton and Lüning (1982) observed an upper survival temperature of 23 ^{o}C for *Laminaria saccharina* gametophytes from the Canadian Arctic, which gave evidence for the temperate origins of the taxon. Furthermore, Lüning (1984) detected an upper survival limit of 28 ^{o}C for *Ulva spp* from Helgoland.

A study by de Casabianca *et al* (2002) showed that *U. rigida* from four different Mediterranean sites (Thau lagoon - France, Lido, Sacca and Fusina stations - Venice lagoon, Italy) had a maximum growth at 17 ^{o}C with limitation in growth below 7 ^{o}C and above about 25 ^{o}C. Hirata and Kohirata (1993) showed that *Ulva* sp. cultivated in a polyculture system (seaweed and fish) in Japan had growth that was closely related to the water temperature and the optimum value was between 20 and 28 ^{o}C. In addition, temperature has also an important influence on photosynthesis; for instance, Henley and Ramus (1989) found that *Ulva rotundata* growth was photoinhibited at irradiances above 40% sunlight at temperatures below 15 ^{o}C but not above 20 ^{o}C.

1.6.5 Nitrogen

With the exception of kelps, the nutrient physiology of west coast seaweeds has not been studied (Stegenga *et al*., 1997). Probyn and McQuaid (1985) showed that *Ecklonia maxima* takes up NO_3 nitrogen in direct proportion to ambient concentrations up to 20 g at N^{-1}, indicating that this kelp has higher tissue nitrogen content during upwelling events. Studies of nitrogen uptake are important since nitrogen (N) is the primary limiting nutrient for macroalgal growth in temperate coastal marine waters (Ryther and Dunstan, 1971; Smith, 1984) and many studies have focused on the uptake and metabolism of N and its effects on growth rates (Topinka and Robbins, 1976; DeBoer *et al*., 1978, Hanisak 1979a; Morgan and Simpson 1981; Wheeler and Weidner, 1983). Ammonium (NH_4^+) and nitrate (NO_3^-) are considered to be the most important sources of N for macroalgae as they are the generally available forms, and both are easily assimilated (Hanisak, 1983). The acquisition rate of the different N forms is affected by environmental conditions including light and temperature (Lapointe and Ryther, 1978; Valiela, 1984). Ammonium is often taken up by seaweeds at higher rates than nitrate (D'Elia and DeBoer, 1978; Haines and Wheeler, 1978; Hanisak and Harlin, 1978; Topinka, 1978; Morgan and Simpson, 1981; Wallentinus, 1984; Thomas and Harrison, 1985; Rees, 2003), despite the fact that nitrate is almost always the more abundant source of inorganic nitrogen in coastal waters (DeBoer, 1981). Thus, ammonium (NH_4^+), which can be toxic or inhibitory for some seaweeds at concentrations higher than 30–50 μM, is the preferred nitrogen form for *Ulva* and other species of macroalgae (Lobban and Harrison, 1994).

In general, nutrient-poor regions are characterised by slow-growing species of macroalgae while fast-growing species are dominant in nutrient-rich waters (Pedersen and Borum, 1997) The relationship between growth and nutrient uptake is complicated by changes in nutrient supply that may vary on time-scales ranging from hours (*e.g.* tidal) to months (*e.g.* seasonal)

(McGlathery *et al.*, 1996). In winter, internal nitrogen reserves in slow-growing, perennial seaweeds can be two or three times higher than in summer (Chapman and Craigie, 1977; Hanisak, 1983; Hanisak and Samuel, 1983). One example is the relationship between the uncoupling of uptake of nitrogen and growth is the kelp, *Laminaria*. In the summer when seawater nitrate levels are low and photosynthesis is most active, carbon is stored, which can then be used for growth in the autumn when nitrate levels increase (Mann, 1972). However, this isn't always the case in all species of *Laminaria*; for instance, in South Africa's *Laminaria pallida* there are almost no seasonal variations in carbon content and tissue nitrogen varies only slightly, indicating that this kelp does not accumulate carbon or nitrogen in order to survive periods of low nutrients (Dieckmann, 1978). Moreover, Wheeler and North (1980) found that nitrate and ammonium are not accumulated in the tissue of *Macrocystis pyrifera* from Southern California and free amino acids account for a major portion of soluble nitrogen, and juvenile *M. pyrifera* sporophytes does not store nitrogen (Wheeler and North, 1980).

In a study of the relationship between N-supply and growth, Pedersen and Borum (1997) concluded that fast-growing seaweeds, including *Ulva lactuca*, which are characterised by high SA: V (surfacea area: volume) ratios and high rates of uptake and growth have a high nitrogen requirement. Most of this requirement is due to a high demand for nitrogen associated with their high growth rate and, to a lesser extent, because of their high tissue-N content. Growth of *Ulva* and other Chlorophycean species can be sustained by N accumulated over such periods of excess availability (Hanisak, 1979; Rosenberg and Ramus, 1982; Fujita, 1985). Efficient nitrogen uptake even at low substrate concentrations has been reported for *Ulva* species (Hein *et al*., 1995; Pedersen and Borum, 1997). Moreover, nitrogen depletion

enhanced gamete formation in *Ulva fasciata* whereas vegetative growth and asexual reproduction was favoured by higher nitrogen concentrations (Mohsen *et al*., 1974).

1.6.6 Phosphorus

Phosphorus (in the form of orthophosphate) is an important component of sugar phosphates, nucleic acids, phospholipids, and ATP-related reactions, as well as Calvin cycle enzymes and carbon metabolism intermediates (Bieleski, 1973, Müller *et al*., 2005, Ghannoum and Conroy, 2007; Sanchez, 2007). The nitrogen-to-phosphorus (N:P) ratio is an important nutritional component for algal growth and the optimum ratio varies from species to species and may provide a basis for competitive elimination and co-existence of algal species in water (Wu and Suen, 1985). According to Björnsäter and Wheeler (1990) the growth rates of *U. fenestrata* decrease faster under phosphorus-limitation than during nitrogen-limitation. Generally, the growth of seaweeds in natural populations is often limited by phosphorus in spring (Howarth, 1988; Flores-Moya *et al*., 1997) when there is low P-concentration in the seawater (Chopin *et al*., 1990a; Lobban and Harrison, 1997). In addition, Chopin *et al*. (1990b) and Lapointe *et al.* (1992) found that macroalgae from carbonate-rich tropical waters were significantly depleted in phosphorus compared with macroalgae from temperate waters.

1.7 Pigments

Accessory pigments differ in the green (Chlorophyta), brown (Ochrophyta, Phaeophyceae), and red (Rhodophyta) algae (Raven and Richardson, 1986). The accessory pigments, in conjunction with chlorophyll a, are collectively termed the light-harvesting complex (Dring, 1988; Lobban and Harrison, 1994). Both genotypic and phenotypic variation in pigment composition occurs among individuals and species (Dring, 1988). Algae vary in their pigment

content in relation to the light environment, and shift in photosynthetic efficiency with relation to light and pigment content. This variation is known to occur in seaweeds in all three major groups, such as *Ulva rotundata* Bliding (Henley and Ramus, 1989), *Laminaria saccharina* Lamour (Gerard, 1988) and *Gracilaria tikvahiae* (Lapointe, 1982). Light is the factor that is directly controlling pigment content, in that the higher the light intensity, the lower the pigment and when the light intensity decreases, the pigment content of individual thalli starts increasing (Fillit, 1995). For instance, *Ulva fasciata* blades grown under low light conditions have twice the Chl-a content of blades grown under high light conditions (Lapointe and Tenore, 1981); *Ulva lactuca* growing under lower illumination than in natural conditions also contained high chlorophyll a levels (Israel *et al*., 1995). Furthermore, pigment concentrations in algal tissue provide a strong indication of the concentration of N available from the water column. Vergara and Niell (1993) demonstrated that in *Corallina elongata* the proportion of pigmented and non-pigmented proteins varies with N concentrations and light availability. This appears to function as a mechanism for storage of N for subsequent periods, when nutrients are limiting. While plant pigments are primarily light-absorbing substances, during times of nutrient limitation they are often used as a source of protein (Lapointe, 1982; Bird *et al*., 1982).

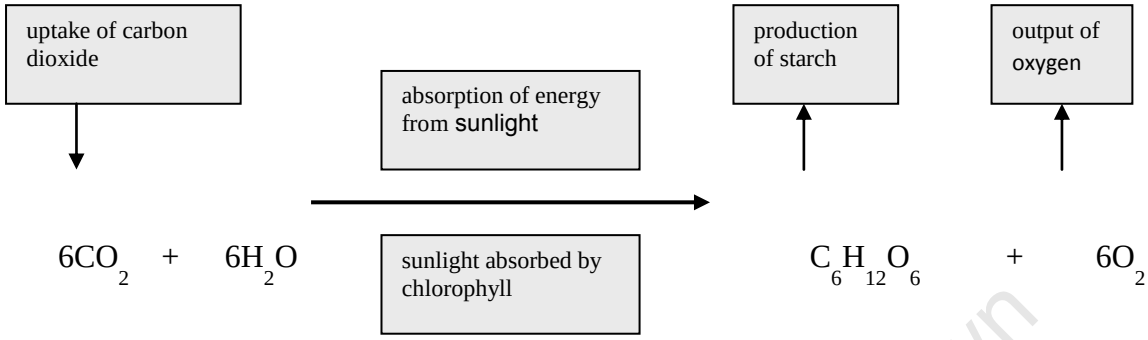
Yokohama (1981) studied pigment content of 47 species of green algae and found siphonaxanthin and siphonein in two deep-water species of *Ulva* (*U. japonica* and *U. olivascens*): this is an order where this pigment and its ester had not previously been detected. However, Yokohama and kageyama (1977) considered siphonaxanthin to be important as a light-harvesting pigment in green algae growing in shade or deep water, although it also occurs in *Codium* sp. growing in high light.

Apart from their potential nutritional value as protein substitutes, seaweeds can also be utilized as colouring agents (Sommer *et al*., 1992; Gouveia *et al*., 2002). One of the pigment classes that is very important in aquaculture is the carotenoids and they have been extensively studied due to their important biological functions in animals as well as their function in plants (Pinheiro-Santana *et al*., 2007) In plants, carotenoids serve as antenna pigments (Siefermann-Harms, 1987), stabilize chlorophyll (Chl) - protein complexes (Humbeck *et al*., 1989; Marquardt, 1998), and protect the organism against excessive light (Demmig-Adams, 1990). The antioxidant activity of carotenoids is mainly attributed to scavenging activity against superoxide and hydroxyl radicals, chelating ability, quenching singlet and triplet oxygen, and reducing power (Ruberto *et al*., 2001; Athukorala *et al*., 2006). The relationship between carotenoids and Vitamin A has been found as far back as 1919, and during 1930 it was established that some carotenoids have proVitamin A activity which could be transformed to Vitamin A inside the animal body (Della-Monica and McDowell, 1965; Carvalho, 1993). *Ulva* is known to accumulate high contents of carotenoids, and El-Baky *et al.* (2008) found *U. lactuca* to be a rich source of carotenoids which could be used as a natural colorant as well as a natural preservative in food.

1.8 Photosynthesis

In both terrestrial and marine plants, photosynthesis is a major, easily measured metabolic process, and is routinely used to gauge the effects of environmental conditions on algae (Lobban and Harrison, 1994). The process produces energy using certain wavelengths of light, involving two photosystems, PSI and PSII which are mostly active at 680 and 700nm wavelengths. Other wavelengths are also peaks in the action spectrum for photosynthesis. Autotrophs use CO_2 and energy from the sunlight to synthesize organic molecules (such as glucose). Plants are autotrophs, which mean they are able to synthesize food directly using

carbon dioxide gas, water and light to produce sugars and oxygen gas. For instance, the production of glucose can be simply represented in an overall chemical equation;



Even though this equation may appear simple, it is actually a summary of very complex processes (Falkowski and Raven, 1997). Studies on photosynthetic light-response curves are widely reported for seaweeds (Ramus, 1978; Platt *et al*., 1980; Jimenez *et al*., 1998, Henley, 1993; Perez-Llorens *et al*., 1996; Rodrigues *et al*., 2000). These studies developed the measurement techniques, under controlled conditions, of the photosynthesis and dark respiration parameters, which then can be compared among species or varying environmental conditions (temperature, light, salinity, nutrients, inorganic carbon, etc.). According to Platt *et al.* (1977), most of the variance in primary production is accounted for by fluctuations in light and seaweeds have different light requirements depending on the species and the environment where they grow. For instance, Rosenberg and Ramus (1982) found *Ulva curvata* to have an I_k of 465 μmol $m^{-2}s^{-1}$; Beach *et al.* (1995) found values of 130-160 μmol $m^{-2}s^{-1}$ for *Ulva fasciata* and Han *et al.* (2003) found I_k to occur at about 131–165 μmol $m^{-2}s^{-1}$ for *U. pertusa*. A study by Vergara *et al.* (1997) showed that the light-saturated photosynthetic rates in *Ulva rotundata* and *Ulva curvata* were positively correlated with periods with high concentrations of chlorophyll and tissue nitrogen. *Ulva lactuca* growing under lower illumination than in natural conditions contained high chlorophyll a levels and showed higher photosynthesis

rates at a lower rate of photon flux density, but had a lower maximal rate of photosynthesis (Israel *et al*., 1995).

It is also known that tissue differentiation in the seaweeds results in different photosynthetic capacities (Gao and Umezaki, 1988; Gao, 1991). The thallus of *Ulva* is also reported to contain a low percentage of non-pigmented components and all the cells are photosynthetic (Littler and Littler, 1980). Physiological studies on the effect of light on *Ulva* have been done by various researchers (*e.g.* Haxo and Clendenning, 1953, Ramus, 1978; Littler and Littler, 1980; Henley and Ramus, 1989a, b; Henley *et al.*, 1991a, b; Perez-Llorens *et al*., 1996, Altamirano *et al*., 2000). *Ulva* and other membranous forms comprise a group of macroalgae with considerably higher photosynthetic performances than other functional form groups (Littler and Littler, 1980). Similar findings were obtained by Levitt and Bolton (1991) on five intertidal seaweed species from False Bay (South Africa), who demonstrated that thin-sheet like forms were the most photosynthetically productive compared to thick-branched forms.

On the coasts of South Africa, *Ulva* species form dense growth from the intertidal to upper subtidal zones, and these plants experience contrasting light environments. Levitt and Bolton (1990) studied the photosynthesis of three subtidal red algae at Oudekraal and five intertidal algae from False Bay. These authors found that photosynthesis in these seaweeds reaches a maximum in spring, when ambient irradiance and photoperiod increases and it slightly decreases in summer when ambient irradiance and photoperiod are at their highest levels. This is the first study in South Africa to examine the photosynthetic physiology of *Ulva* species, which are common and abundant along the South African coastline and are an important product of marine aquaculture.

1.9 Seasonal variations in chemical composition

Several studies have shown seasonal changes in chemical composition of various South African west coast seaweeds (Stegenga *et al*., 1997). The effects of upwelling of cold water from the Benguela current are well documented for the South African west coast inshore ecosystems (Bolton and Levitt, 1987; Stegenga *et al*., 1997) and these upwelling events have an influence on the chemical composition of South Africa's seaweed flora. For instance, in *Ecklonia maxima* seasonal patterns have been shown in the nitrogenous uptake rates in which tissue nitrogen increased during upwelling events (Probyn and McQuaid, 1985). This is agreement with Dawes (1998) who showed that seaweeds show great variation in nutrient content, which are related to several environmental factors such as water temperature, salinity, light and nutrients. Dhargalkar (1986) reported seasonal changes in biochemical composition of *U. reticulata* from Goa coast in India which could be related to the chemical and morphological changes associated with some metabolic processes in the alga. Similar findings on seasonal biochemistry have also been reported for *Ulva sp.* from Kwazulu Natal, South Africa, which had a pattern of increasing elemental concentrations in winter and a decrease from winter to spring and summer (Misheer *et al*., 2006).

1.10 Accumulation of metals

Monitoring changes in the levels of metal contaminants in coastal environments has historically been done by direct measurement of water column metals. However, instantaneous seawater samples, even when taken regularly, may not give good information on changes in biologically significant metal concentrations in environments that are prone to tidal and other variations (Björnsäter and Wheeler, 1990; Jones *et al.*, 1996; Valiela *et al.*, 1997; Fong *et al.*, 1998). As a result of the shortcomings of conventional seawater monitoring techniques, the use of seaweeds as biological indicators has been examined using

Chlorophyta (Ho, 1990; Brown *et al*., 1999 and Villares *et al*., 2002), Rhodophyta (Muse *et al*., 1995) and Phaeophyta (Muse *et al*., 1995). Many studies use *Ulva* species as a biological indicator of metal contamination due to their simple morphology and high capacity for metal uptake (Ho, 1990; Villares *et al*., 2001; Besada *et al*., 2009). Seasonal variations can influence the range of metal uptake of marine organisms. If the temperature decreases, the metabolic activity of the organism also diminishes, and the active uptake may be less (Lozano *et al*., 2003). Thus, concentrations are generally low in spring/summer when growth rates are high and the accumulated metals are diluted and high in winter when the metabolic processes have slowed down (Villares *et al*., 2001). Seasonal fluctuations in metal concentrations in *Ulva rigida* have been attributed to growth, age of tissue and abiotic factors (such as salinity and temperature), as well as to variations in metal concentrations in the environment (Villares *et al*., 2002). Also, *Ulva lactuca* mainly takes up solubilised metals because of its laminar structure with a relatively high surface area (Villares *et al*., 2001). However, metal concentrations in algae are strongly dependent on the environmental parameters of the sampling sites (salinity, temperature, pH, light, nutrient concentrations, oxygen, etc) (Zbikowski *et al*., 2006). The capacity of algae to accumulate metals depends on a variety of factors such as bioavailability of metals in the surrounding water (Sanchez-Rodriguez *et al*., 2001). Malea and Haritonidis (2000) found significant positive correlations between lead (Pb) concentrations in *U. rigida* and in seawater. Additionally, as *Ulva* species are locally used as abalone feed, the monitoring of metal contents in their tissues would provide an early warning system of pollution that could not be achieved solely by the analysis of water samples. Permitted elemental levels ($\mu g.g^{-1}$ dw) in ingestible seaweeds in France are <5.0 for Pb, As, <0.5 for Cd and <0.1 for Hg whereas in Australia and New Zealand the maximum level for Cd is 0.2 $\mu g.g^{-1}$ dw and 1.0 $\mu g.g^{-1}$ dw for inorganic arsenic (Almela *et al*., 2002; 2006; Besada *et al*., 2009). These regulations in addition to the potential nutritional

properties of seaweeds allow the food industry to include seaweeds as raw or semi-processed materials in the formulation of seafood products and aquafeed. In South Africa, even though *Ulva* has been successfully utilized as abalone feed, little information is available on the metal concentrations in *Ulva* species grown in aquaculture effluent as well as from nature.

1.11 Amino acid composition

The amino acid composition of seaweeds has been frequently studied and compared to that of other proteinaceous foods such as eggs or soybeans (Fleurence, 1999). Amino acids are divided into essential and non essential amino acids. Essential amino acids cannot be synthesized by the organism and therefore must be supplied in the diet, whereas non-essential amino acids can be synthesized in the body and do not have to be obtained from the diet. The essential amino acid composition of soybean protein generally comprises 36.0% of total amino acids (Galland- Irmouli *et al*., 1999) and this concentration of essential amino acids is lower than that reported for *U. fasciata*, which has been reported as 41.4% of total amino acids (Lourenço *et al*., 2002). For most seaweed, aspartic and glutamic acids constitute a large part of the amino acid fraction and they are non-essential amino acids. In *Ulva rigida* and *Ulva rotundata*, the levels of these two amino acids can represent up to 26 and 32% of the total amino acids, respectively (Fleurence *et al*., 1995).

In aquaculture, the ratio of essential and non essential amino acids in the feed is as important as the protein content itself (Sales and Britz, 2001) and is often the first consideration when new processed feeds are formulated and the insufficiency of some important amino acids in seaweed could make it less suitable for abalone feed than the processed alternative (Mai *et al.*, 1995). When discussing the ideal protein concept, each of the amino acids in the feed is related to the requirement for lysine. There are several reasons for selecting lysine as the reference amino acid. Firstly, lysine has only one major function in the animal body and that

is for protein tissue deposition, so its requirement is not influenced by other metabolic roles (Miles and Chapman, 2007). Excess proteins are sometimes put into feeds because the protein is not very digestible (so that more has to be added to meet the amino acid requirements) or because specific essential amino acid requirements are not known (Miles and Chapman, 2007).

1.12 Protein content in *Ulva* and nitrogen to protein conversion factor

Proteins are a nutritional component of particular importance to animal aquaculture, and protein is typically the most costly component of formulated feed (Miles and Chapman, 2007). The protein content in abalone feed has been investigated in several studies because of its importance for growth and high cost for the farmer (Sales *et al.,* 2003). For many years quantification of crude protein content for most feed materials has been determined by a conversion factor of 6.25 multiplied by nitrogen content (Jones, 1931; Dintzis *et al*., 1988). This factor is based on the assumption that the samples contain protein with 16% nitrogen and an insignificant amount of non-protein nitrogen. However, plant materials normally show higher amounts of non-protein nitrogen (Conklin-Brittain *et al*., 1999). Conversely, it is common to find plant materials showing total protein with less than 16% nitrogen in the amino acid components (Yeoh and Wee, 1994). Thus, the calculation of protein content by N × 6.25 may tend to over or under estimate the actual protein content in the sample (Ezeagu *et al.*, 2002).

The seaweed generally consumed by abalone contains less than 20% crude protein (Nisizawa *et al*., 1987). The protein content of seaweeds differs from one species to another and it also varies seasonally (Fleurence, 1999). *Ulva* species have a crude protein content ranging from 10 to 26% (dry weight) of the plant (Fleurence, 1999). Several studies have looked at the protein content of *Ulva* species. For instance *Ulva pertusa*, which is frequently consumed

under the name of ``ao-nori'' by Japanese people, has a crude protein level between 20 and 26% DW (Arasaki *et al*., 1984), *Ulva lactuca,* 27.2 % DW (Oritz *et al*., 2006); *U. oxysperma;* 6 to 10% DW whilst *Ulva lactuca* and *Ulva fasciata* have levels of 13 and 18% DW respectively (de Padua *et al*., 2004). However a study by Wong and Cheung (2000) found lower crude protein content in *U. lactuca*, (7.06% DW) obtained from Tung Ping Chau, in the northeast of Hong Kong and the lowest was obtained from *U. lactuca* found in the Phillipines which had crude protein content of 4.20% DW (Medina and Matibag, 1983). Furthermore, crude protein content in *Ulva* can increase to over 32% when cultivated in nutrient-rich water (e.g Msuya and Neori, 2002; Robertson-Andersson, 2003; Viera *et al*., 2005). All of these authors have used a traditional factor of 6.25 (Jones, 1931) to determine the crude protein of *Ulva*.

Nevertheless, several researchers have also quantified protein contents in algae using the conventional extraction methods of Lowry *et al* (1951) and Bradford (1976). However, protein contents obtained from these extractions are very difficult to compare among algae because of methodological differences (Berges *et al*., 1993) and may be compromised by a large amount of interference (Stoscheck, 1990). Numerous substances such as phenol and phenolases (Mattoo *et al.*, 1987) and flavonoids (Compton and Jones, 1985) interfere with both the Lowry and Bradford methods. Differences in cell wall composition of algae also affect proper quantification of protein (Fleurence, 1999).

Another method of calculating protein content is the establishment of specific nitrogen-to-protein conversion factors (N-Prot factors) and this is a major subject in nutritional science today. The use of N-Prot factors provides an accurate end result and allows better comparisons of protein results among researchers, since protein is estimated without

conventional protein extraction (Lourenco *et al*., 2002b). A standard way of determining N-Prot conversion factors is based on the quantification of the nitrogen obtained from amino acids after hydrolysis (Huet *et al*., 1988; Salo-Väänänen and Koivistoinen, 1996; Lourenço *et al*., 2002). The total amount of amino acid residues permits the quantification of total proteins in a sample, and knowing the percentage nitrogen of each amino acid, the amount of nitrogen in the protein fraction can be calculated (Sosulski and Imafidon, 1990). Based on this approach, the determination of the N-Prot conversion factor relies on quantification of the protein content by the sum of amino acid residues and determination of the amount of nitrogen in the total protein, considering the individual contribution of each amino acid (Lourenco *et al*., 2002b).

1.13 Effect of epiphytes and diseases on *Ulva* cultivation

As long ago as 1977, Ryther stated that epiphytic growth is probably the single greatest problem and constraint on commercial seaweed culture, while Wheeler *et al.* (1981) stated that the control of epiphytes and provision of nutrients are two major problems for would-be macroalgal farmers. It is difficult to control epiphytes when seaweeds are cultured under non-unialgal conditions. Epiphytes are seen as opportunistic because they tolerate a wide range of environmental conditions such as temperature and light levels (Fletcher, 1995). When epiphytes remove nutrients and inorganic carbon from the water column they can significantly reduce production of the cultivated seaweed (Fletcher, 1995).

Although epiphytes can be very important in seaweed aquaculture, there is very little literature on epiphytes in *Ulva* aquaculture. In South Africa, the first record of the epiphyte species *Myrionema strangulans* (Phaeophyceae) was made in October 2001 when the small brown crusts of the epiphyte were observed growing on experimental tank cultures of *Ulva* at Irvine & Johnson Cape Abalone farm and four stages of infection were described (Robertson-

Anderson, 2003). **Stage 1** shows a healthy *Ulva* thallus, usually dark green in colour, with 5-10 brown spots. In, **Stage 2,** the spots increase in number and cover the entire thallus surface. The *Ulva* thallus becomes yellowish-green in colour and thinner. In **Stage 3,** the **s**pots continues to increase in number, consequently the thallus becomes very light in colour and very fragile. Finally, in **Stage 4,** the thallus breaks up into small pieces and once this happens, the culture populations never recover.

Also, Colorni (1989) observed a disease affecting a local *Ulva spp*. in culture at Eilat (Israel, Red Sea) which resulted in green spots dotting the algal thallus and gradually enlarged into perforations. Involvement of epibiontic microorganisms in the disease outbreak was ruled out and it was suggested that the onset of the lesions was possibly triggered by distressing events to which the alga was subjected in the culture conditions.

1.14 Background of the study

The high value of abalone has stimulated considerable effort into the development and optimisation of intensive abalone culture (Shpigel *et al*., 1999). Cultivation of *H. midae* is still a growing industry in South Africa because this species is highly desirable and fetches comparatively high prices on Asian markets (Oakes and Ponte, 1996), and South Africa has been described as the largest producer of abalone outside Asia (Troell *et al*., 2006). According to Oakes and Ponte (1996) South Africa produced 600 tons of *H. midae* in 1993 and subsequent demand for *H. midae* has increased and the current estimated annual production is 1100 tons (Bolton *et al*., 2009). At present there are five local mariculture farms that concurrently culture abalone and *Ulva.* Three out of those five farms produce *Ulva* in small quantities (Bolton *et al*., 2009). Wild Coast Abalone farm near East London grows by far the most, followed by I & J Abalone farm at Gansbaai and then the rest (including Abagold in Hermanus and Marine Growers in Port Elizabeth). Wild Coast and I&J Abalone

farms cultivate *Ulva* in effluent whereas the other farms grow the seaweed in fertilized seawater. This study also investigates *Ulva* material from Irvin & Johnson (I & J) farm at different levels which includes productivity, heavy metal accumulation, biochemical and mineral content as well as amino acid profile. Currently the main abalone feed used at I & J farm is the kelp, *Ecklonia maxima* collected from the wild complemented with fishmeal-based Abfeed® and cultivated *Ulva spp*. It has been reported that various natural and formulated feeds have varying results, with some tending to affect lipid composition, and consequently taste and texture of abalone meat negatively, hence reducing the value of the product (Dunstan *et al*., 1996; Smit *et al*., 2010). Also, the incorporation of fishmeal protein in these formulated feeds increases nutrient waste and reduces the water quality in abalone tanks (Troell *et al*., 2006). Furthermore, local farmers have observed higher infestation rates of sabellid polychaete parasites in abalone fed on Abfeed® and suggested that the diet could be responsible for these high infestation levels (Chalmers, 2002) The only commercially available all-seaweed based feed in South Africa is a formulated dried feed called "Midae Meal MM-1c" (Eric-Piet (Pty) Ltd, Luderitz Bay, Namibia). This formulated feed is manufactured for Taurus Products (Pty) Ltd and the ingredients are mainly *L. pallida* and *E. maxima* (stipes and fronds) and it also contains *Gracilaria* spp., *Gelidium* spp. (including the kelp epiphyte *G. vittata*), *Porphyra capensis* and "agar agar" (Robertson-Andersson, 2007). The wet seaweed: dry pellets ratio is 6 - 7:1 and protein content is ca. 18 % which gives the abalone meat a natural taste, colour and smell (Robertson-Andersson, 2007). In addition, sabellid infections have not been observed with this all-seaweed based feed and it has improved the abalone shell color (Robertson-Andersson, 2007). Unfortunately, this feed had limited success in a series of growth trials producing poor growth rates in abalone when compared against other formulated feeds (Dlaza, 2006; Robertson-Andersson, 2007).

Until the development of abalone farming, the only kelp directly harvested from beds in South Africa was used as a liquid growth stimulant for agricultural crops (Anderson *et al.* 2003). Currently, harvest of wild kelp (*Ecklonia maxima* and *Laminaria pallida*) for abalone feed is now approaching limits of sustainable harvesting in kelp concession areas where abalone farms are concentrated (Sales and Britz, 2001, Troell *et al.*, 2006; Anderson *et al.*, 2006). In 2003, coastal management authorities (Marine and Coastal Management, Department of Environmental Affairs and Tourism, Cape Town) estimated that a maximum sustainable yield of kelp was approached in parts of the two main areas of abalone farming; from Quoin Point to Cape Hangklip and in the Cape Columbine area (Anderson *et al.*, 2003) Under the Marine Living Resource Act (1998) seaweed resources are managed on an area basis, with 23 monitored concession areas between Namibian border and the southern border of Kwazulu - Natal (Fig 1.2).

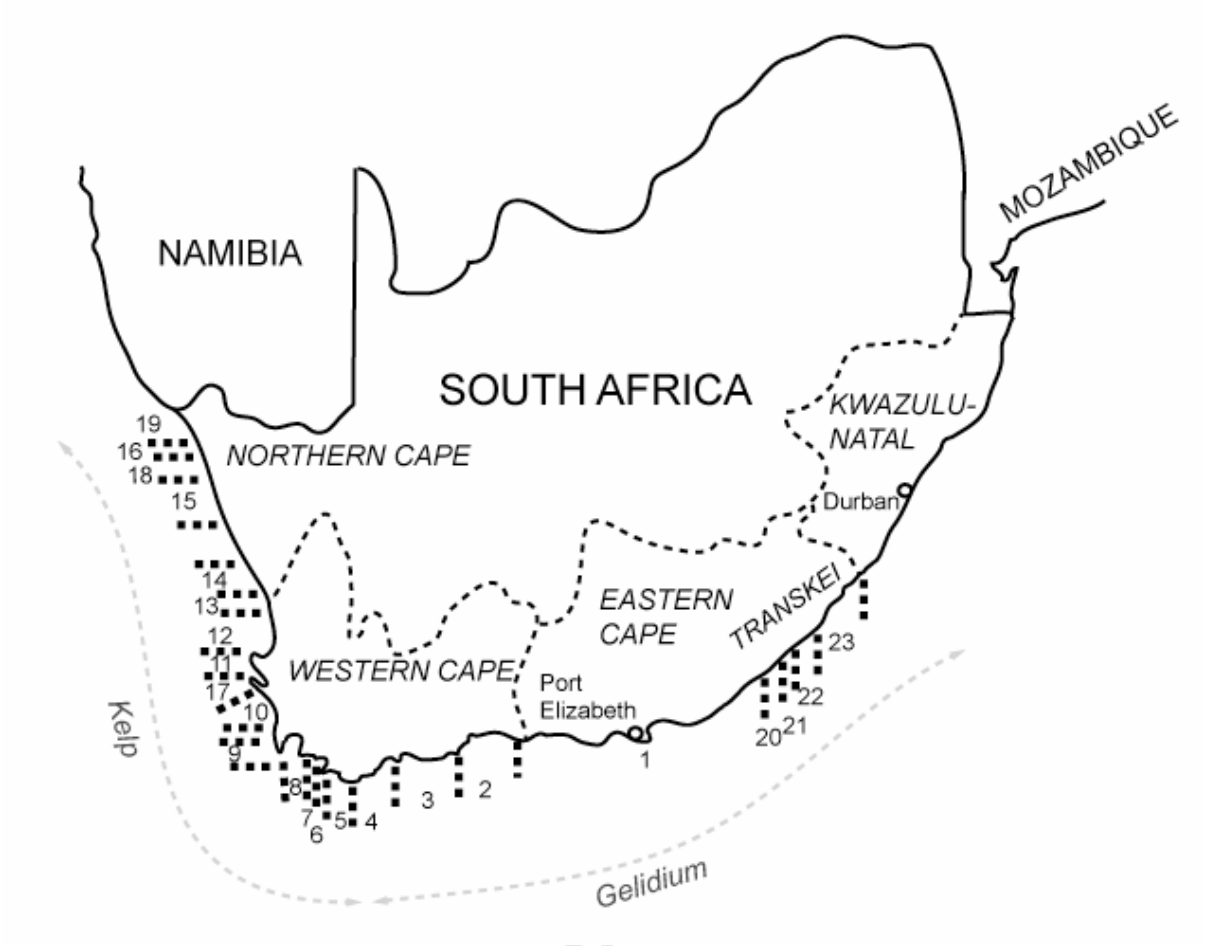


Figure 1.2: Map of South Africa showing the Seaweed Concession areas (Anderson *et al*. 2003). Areas 1, and 20-23 are where *Gelidium* is currently collected. Kelp is collected in areas 5-9, 11-16 and 18-19. No seaweed is currently collected from areas 2-4, 10, 20, 22, and 23. Lines separate concession areas.

As a result of the above shortcomings, *in situ* cultivation of seaweed on the farms has been developed as a cost effective and environmentally friendly alternative in global aquaculture (Neori *et al*., 2004; Troell *et al*., 2006; Naidoo *et al*., 2006). On South African abalone farms the main cultivated seaweed is *Ulva*, and to a smaller extent *Gracilaria* which is predominantly grown as a specific feed for juvenile abalone.

Although *Ulva* is a well studied genus worldwide, there is a need to study the local species. The are many gaps in ecophysiological information and this study is designed to provide farmers with data that will improve their cultivation methods in terms of productivity, nutritional quality, seasonal species preference and the control of epiphytes. It is also possible that the *Ulva* market in South Africa may develop beyond abalone feed to other applications such as ingredients in sea urchins and fish diets, cosmetics and health supplements. Furthermore, the study was also designed:

1. To investigate the success of cultivation in improving seaweed quality through aquaculture
2. To investigate whether there is really any difference between *U. capensis* and *U. rigida* (one of which is the species mostly grown at I&J), by comparing natural populations of *U. rigida* and *U. capensis* with cultivated *U. rigida*. .

1.15 Collection sites and *Ulva* species used in the research project

Three South African *Ulva* species (*Ulva lactuca* Linnaeus, *Ulva rigida* C. Agardh and *Ulva capensis* Areschoug) were selected for the experiments conducted for this research project. The first two were chosen as they are currently cultivated on abalone farms and they are utilized as abalone feed and used as biofilters, and *U. capensis* was included because it is morphologically and biogeographically distinct from *U. rigida* in nature, despite the inability of molecular methods to separate them.

There are number of factors that make *Ulva* an ideal candidate for cultivation:

(i) it is one of the simplest seaweeds to cultivate as it can grow from vegetatively propagated thalli (Tanner, 1981, van de Hoek *et al*., 1995)

(ii) a number of studies have been carried out on its biofiltration capacity for removal of dissolved nitrogen compounds in an integrated abalone aquaculture system (Cohen and Neori, 1991; Neori *et al.*, 1991; Jimenez Del Rio *et al.*, 1996; Neori *et al.*, 1996; Goldberg *et al*., 1998, Msuya and Neori, 2002; Robertson-Andersson, 2003; 2007) and all these authors have concluded that *Ulva* is a good biofilter.

(iii) *Ulva* species are commonly used in aquaculture as feed since they contain protein, pigments, vitamins and minerals (Nakagawa *et al*., 1987; Hashim and Maat-Saat, 1992; Wassef, 2001; Valenta *et al*., 2006, Cruz-Suarez *et al*., 2009).

Ulva capensis and *Ulva rigida* have been suggested to represent a single polymorphic species. Therefore ecophysiological studies are necessary to investigate if these species have similar or different ecological growth requirements.. *U. capensis* and *U. rigida* were collected from Kommetjie (34º09′06″S, 18º19′22″E) along the southwest coast of the Cape Peninsula, Cape Town and *U. lactuca* was exclusively collected from I & J farm because it was not possible to find free-living populations of *U. lactuca,* despite frequent searches at sites were the species had previously been recorded (*e.g.* Simon's Town, Saldanha Bay; Stegenga *et al*., 1997). In addition, I & J farm also cultivates *U. rigida*, therefore material of *U. rigida* was also collected from the farm and was used for analysis, to compare its metal contents and biochemical constituents to those of wild *U. rigida*.

1.16 Aims of this study

Both the seaweed and abalone industries bring important economic benefits to South Africa. They generate export earnings, boost local and regional economies and provide employment among poor coastal communities (Troell *et al*., 2006). If the South African abalone industry continues to grow at the current rate, there is a need to improve the cultivation of *Ulva* to increase productivity in order to meet the feed demand.

The goal of the first part of the study was to investigate the effect of environmental parameters (irradiance, salinity, temperature, nutrients, and light quality) on the growth rate of two of South Africa's *Ulva* species *U. rigida* and *U. lactuca* that are currently used in local farms as both bio-filters and feed for abalone. This information will aid in the cultivation of *Ulva* to produce a crop that is more reliable in terms of volume and quality.

The study also focused on the photosynthetic responses of *U. rigida* and *U. capensis* from natural populations at Kommetjie on the West Coast of South Africa to determine the extent of physiological adaptation to various light levels. The ability to withstand reduced or higher light levels should reflect the extent to which populations of *U. rigida* and *U. capensis* can prosper in fluctuating light levels in the intertidal zone. These physiological studies are of great interest since thalli in the intertidal conditions are exposed to low or high light levels for shorter or longer periods. Therefore a broader light tolerance will be a significant adaptive trait.

Furthermore, the study also focused on the temporal nutritional and chemical composition of the locally abundant *U. capensis and U. rigida* from the wild, to investigate how fluctuations in seawater conditions cause variation in the nutritional composition of these local seaweeds. In addition, material of *U. rigida and U. lactuca* cultivated in a large scale re-circulated abalone-seaweed system was also biochemically analysed to determine how fertilization and

abalone effluents affect their nutritional composition, and to compare to wild material. An estimate of chemical composition of seaweed is essential to determine its nutritional value, especially as it relates to abalone culture.

The second part of the study was designed to measure the temporal variation in metals in both wild *U. capensis* and *U. rigida* as well as farmed *U. rigida* and *U. lactuca* and to ensure that the metal contents of *Ulva* did not exceed safe limits for human and or/abalone feed. This is important because maximum metal contents in edible seaweed are limited in certain countries, which could have a negative effect on their value. Only a very small number of papers analysing heavy metals in South Africa seaweeds have been published. This study focussed on the seasonal variation in metals (Cd, Pb, Hg, As) in *Ulva* from the wild (at Kommetjie on the Cape Peninsula) and from an abalone farm (I&J, Danger Point) where the *Ulva* is grown in re-circulated abalone water. The food safety of these *Ulva* species in terms of metal contents will also be assessed.

The third part of the study focussed on *Ulva* species cultivated in a re-circulated abalone-seaweed system at I & J farm and the effect of epiphytes on its cultivation. An epiphytic outbreak in farmed *Ulva* at Irvine & Johnson farm was first reported in 2001 by Robertson-Andersson (2003). However, this problem received little attention until 2007 when epiphyte outbreaks increased and impacted production.

Furthermore, according to Bolton *et al.* (2009), there is a seasonal change in species dominance between two *Ulva* species growing in recirculation tanks at I &J farm. *U. rigida* (reported as *U. capensis* due to confusion over possible co-specificity of the two taxa) grew well during winter and its proportion in the ponds reduced during summer. Conversely, *U.*

lactuca proportions decreased during winter and increased during summer. However, to understand the underlying environmental factors required for the growth of these two species, ecophysiology data on both are required.

Therefore the major objectives and hypotheses of this study were:

1. To identify the culture conditions that promotes the best growth of these species. In this study we asked whether there are distinct differences in nutrient use and preference; light levels, salinity and temperature between the three *Ulva* species, *U. rigida, U. capensis* and *U. lactuca* and can this explain the dominance of each species at different localities.
2. To examine the temporal photosynthetic characteristics of *U. rigida* and *U. capensis* from wild populations with respect to light. Does their position in the intertidal zone cause them to have different physiological responses to varying light intensity or will these two species have similar responses as they are suggested to be morphological forms of a variable species?
3. To evaluate the temporal chemical composition and mineral content of wild and farmed *Ulva* species to assess the nutritional quality of each species. Does the nutritional content of *Ulva* species vary temporally in both nature and on farms, and do differing conditions provide different patterns in the two systems (wild and farm)?
4. To measure temporal variations in metal concentration in order to provide information on the concentrations of several harmful metals if present. Do wild *U. capensis*, wild *U. rigida*, farmed *U. rigida* and wild *U. lactuca* have different metal accumulation patterns? To propose conversion factors from nitrogen to total protein concentrations for each species based on amino acid composition and nitrogen content.

5. To evaluate the seasonal productivity of *Ulva* cultivated in a re-circulated abalone-seaweed system in order to investigate the relationships between environmental factors and growth, and aid in the prediction of seasonal growth.
6. To assess the occurrence of the epiphyte, *Myrionema strangulans* and to determine environmental conditions that may trigger its occurrence. We hypothesize that during summer, when the outbreak is severe, *Ulva* is growing fast and thus using its tissue nutrients, and where there is no sufficient external supply of nutrients the *Ulva* is unhealthy and becomes susceptible to this epiphyte.

Chapter 2

Effects of irradiance, light quality, temperature, salinity and nutrients on the growth rate of *U. rigida, U. capensis* and *U. lactuca* in culture

2.1 Introduction

The green algal genus *Ulva*, including species previously placed in the genus *Enteromorpha* (Hayden *et al*. 2003) is well known for its wide distribution in marine, freshwater and brackish environments throughout the world (Reed and Russell, 1979; van den Hoek *et al*., 1995; Tanner and Wilkes, 2005). The marine environment of the west coast of South Africa is dominated by upwelling which strongly influences the growth of seaweeds by its effect on temperature, light and nutrients (Bolton and Levitt, 1987). The growth of a number of South Africa's seaweed species has been studied in culture, although many studies have focused on juvenile plants (Stegenga *et al*., 1997). In the last few years there has been a growing interest in cultivation of *Ulva* as a feed supplement for the local abalone farming industry and as a biofilter for abalone effluent. In 2007 South African abalone farms cultivated over 1000 t fresh weight of *Ulva* for feed (Bolton *et al.*, 2008). Currently, at least three *Ulva* species are cultivated on commercial local abalone-seaweed farms (Bolton *et al*., 2008; L. Kandjengo pers. comm.). However, in spite of the rapid development of the *Ulva* industry in South Africa, basic data are not available on the growth of local *Ulva* species in response to environmental variables.

Ulva species are ecologically and physiologically opportunistic: they are usually the first colonizers on open substrata, and their cosmopolitan presence is attributed to their tolerance of a wide range of environments and opportunistic life strategy (Hernández *et al*., 1997; Ménesguen and Cugier, 2006). Several authors have studied how changing salinity affects the

growth and photosynthetic capacity of *Ulva* species (*e.g.* Steffensen, 1976; Reed, 1983; Einav *et al*., 1995). Blomster *et al*. (1998) clearly showed, using molecular phylogenetic analyses that *U. intestinalis* samples grow close to freshwater outlets. Pringle (1986) collected *U. intestinalis* from brackish water and was able to culture it in less than salinity of 2 PSS. Kamer and Fong (2000) suggested that *U. intestinalis* can tolerate short-term fluctuations in salinity but can be negatively impacted by persistent salinity reductions. However, some former *Enteromorpha* species, which are now included in *Ulva*, grow in salinity (<1 PSS) (Lobban and Harrison, 1997). Salinity change significantly affects the growth and photosynthesis of *Ulva* (Lartigue *et al*., 2003; Taylor *et al*., 2001; Fong *et al*., 1996; Floreto *et al*., 1994). Einav *et al.* (1995) confirmed that *Ulva lactuca* from Israel exhibited a decline in net oxygen production following a change in salinity, and similar results were observed by Lartigue *et al*. (2003). Moreover, populations of *Ulva* can also persist through fragmentation alone (Blomster *et al*., 2002) and lack of reliance on spores translates to broader salinity tolerance (Burrows, 1959). A new species of freshwater *Ulva, Ulva limnetica* has been recorded from the Ryukyu Islands, Japan (Ichihara *et al*., 2009), and is the first record of freshwater distromatic *Ulva*.

Algae are known to exhibit seasonal changes in their requirements for, and tolerance of temperature, as is known for temperature resistance in higher plants (Larcher, 1975; Bannister, 1976). In addition, Snoeijs (1992) found *Ulva* species (former *Enteromorpha*) to be tolerant of very cold temperatures in Finland, but to also thrive in a warm-water discharge area near a nuclear power plant on the Bothnian Sea. The Japanese species *Ulva pertusa* has its temperature optimum for growth at 20-25 °C (Ohno, 1977). Sfriso (1995) found the optimum temperature for growth of *Ulva* rigida C. Ag. from the Venice lagoon in Italy to be between 23 -24 ^{o}C. Enright (1979) found the temperature optimum for growth of Canadian *Ulva lactuca* at 20 °C. Temperature can also have an effect on growth form of *Ulva*. Tanner

(1986) showed that *U. californica* from south Point Conception in California forms densely tufted turfs when the culture temperature is above 15 ^{o}C.

Nitrogen and phosphorus are the two most common nutrients limiting macroalgal growth (Hanisak, 1979). Nitrogen has been described as the principal limiting nutrient (Wheeler and Björnsäter, 1992; Larned, 1998), but in some places phosphorus supply may limit macroalgal production (Chopin *et al*., 1990a, b; Lapointe, 1997; Villares and Carballeira, 2004). Many species of *Ulva* grow best in eutrophic habitats (Sousa *et al*., 2007) and nutrient-enriched water (Fong *et al*., 2004). Nutrients present in the water column can be found in a wide variety of forms, and not all are biologically available. Although nitrate, nitrite, ammonia and urea are all potential sources of nitrogen in the water column, nitrate (NO_3^-) and ammonia (*i.e.* NH_3 plus NH_4^+) provide the two forms of inorganic nitrogen most important to algae (Parsons and Harrison, 1983). Phosphorus exists in seawater as inorganic orthophosphate, metallophosphate complexes, polyphosphates and organic phosphorus compounds (Cembella *et al*., 1983), but is principally acquired by marine algae as free orthophosphate (Raven, 1980). In South Africa, levels of these nutrients are generally higher on the west coast than on the south coast due to upwelling events (Stegenga *et al*., 1997). *U. capensis* and *U. rigida* used in this study were collected from Kommetjie on the West Coast where upwelled water commonly contains about 6-8 µM phosphate and 10-20 µM nitrate as well as low levels of ammonia, nitrite and trace elements (Stegenga *et al*., 1997). In addition, nutrient levels are very variable on the aquaculture farm where *U. lactuca* was collected and during the course of this chapter, there were signs of nutrient starvation (loss of pigments and low nitrogen content) of *Ulva* in the farm system. In addition, *Ulva* species from aquaculture farms is free-floating and such species growing in a tumble culture could have different cellular characteristics. Therefore it was important that these species were routinely analyzed for

anatomical characteristics. Furthermore, while *Ulva* exhibits relatively rapid nutrient uptake (Rosenberg and Ramus, 1984; Rivers and Peckol, 1995; Neori *et al*., 2003; Robertson-Andersson, 2003.) and subsequent growth in relation to other macroalgal species, it may have a limited capacity to store inorganic nutrients (Fujita, 1985). Despite this limitation, the genus *Ulva* comprises highly productive species that are often responsible for a large percentage of total intertidal and estuarine primary production (Sfriso *et al*., 1987; Sfriso and Marcomini, 1996).

In seaweeds, the effect of light quality (wavelength) has been shown to affect growth, photosynthesis and morphogenesis (McLachlan and Bidwell, 1983; Lüning and Dring, 1985). The green alga, *U. pseudocurvata* Koeman et Hoek grew slowly in blue light whereas green light and red light resulted in an initial peak in growth rate followed by inhibition of growth (Lüning, 1992). However, some *Ulva* species such as *U. japonica* and *U. olivescens* have the pigment siphonaxanthin which absorbs light in the visible green region of the spectrum (Yokohama and Kageyama, 1977).

Interesting findings were shown by Bolton *et al.* (2008) of a change in *Ulva* species dominance during different seasons grown in a commercial farm in abalone effluent. A higher proportion of *U. rigida* (misnamed as *U. capensis*) was found during winter whereas a higher proportion of *U. lactuca* appeared during summer, in the I & J integrated seaweed/abalone system. This clearly shows that these two species have different environmental responses. It has been suggested by Robertson-Andersson (2003) that the dominance changes exhibited by *U. rigida* and *U. lactuca* might be an ecological niche strategy to avoid competition.

Ulva is one of the most studied seaweed genera worldwide. However it is important to study local species, as the taxonomy of *Ulva* is very complex, and it is likely that names which are used around the world may be used for different taxonomic entities in different biogeographical regions (Coat *et al*., 1998; Blomster *et al*., 1998; Malta *et al*., 1999). Until now, studies on local *Ulva* species have centred on taxonomy (*e.g.* Stegenga *et al*., 1997); reproduction (of *U. fasciata* and *U. rigida*, see Steyn, 2000); bioremediation (using *U. lactuca*, see Robertson-Andersson, 2003) or molecular taxonomy (Kandjengo, 2003). According to Kandjengo (2003), molecular systematic analysis on the relationship between *U. rigida* and *U. capensis* suggests that the taxa could not be separated using the ITS gene region. However, according to TCS analysis, these species could be haplotypes of a diverse species. These taxa differ both ecologically, morphologically and biogeographically (Stegenga *et al*., 1997). Therefore due the complexity of *Ulva* taxonomy, ecophysiological studies of local *Ulva* species will provide much needed information on whether they have different ecological requirements, and whether they conform to the ecophysiological data for *Ulva* species collected elsewhere in the world.

In the present study, we investigated growth rates of *U. rigida* and *U. capensis* collected from Kommetjie as well as *U. lactuca* from an integrated seaweed/abalone system at I & J farm. The three *Ulva* species in the current study have different morphological and anatomical characteristics as described in Stegenga *et al*. (1997) in Table 2.1 below.

Table 2.1 Descriptions of the investigated specimens based on morphological, anatomical and cytological characters (T/S = Transverse Section and S/V = Surface View).

Species name	Thallus morphology	Cell shape (T/S)	Cell shape (S/V)	Cell size (T/S) µm	Cell size (S/V) µm	Thallus thickness (µm)	Cell arrangement	No. of Pyrenoids
U. capensis	Dentate (double), porous, wrinkled around perforations, undulate, thick lamina, branched, tough basally, ovate, dull and rough, lanceolate, dark patches	Rectangular (rounded angular), slender – bullet, spindle shaped, squarish-roundish,	Rectangular-rounded, polygonal or irregular, bean shaped and well paired	(8-17) X (27-67)	(4-17) X (8-23)	72-209	Curved rows in part	1-4 (-5)
U. lactuca	Porous, thin, undulate, (un)branched, floating unattached thalli form flat sheets, rough, tougher basally	Rounded to rectangular	Rectangular, rounded-polygonal	(8-15) X (15-25)	(6-15) X (10-21)	38-86	short rows in part	1 -2 (-3)
U. rigida	Thick, consistency firm, tufty at the base, incised, entire margins, smooth and shiny, flat sheet, perforated,	Rectangular (rounded angular) – slender	Rectangular-rounded, polygonal, irregular, bean shaped well paired	(6-27) X (21-53)	(6-21) X (10-29)	57-247	Short rows to none	1-3 (-4)

In addition, *U. rigida* is found growing from the upper intertidal to shallow subtidal, from the Cape peninsula eastward into tropical East Africa whereas *U. capensis* grows from the mid-littoral to the shallow subtidal from Namibia to Cape Agulhas (Stegenga *et al*., 1997). Furthermore, only a few recent collections of South African *Ulva* have been identified as *U. lactuca*, and, Stegenga *et al.* (1997) suggested that most records of this species are probably incorrectly identified. There is now conclusive evidence that South African *U. lactuca* is not genetically identical to the original European *U. lactuca* (L. Kandjengo, unpublished data).

At present, there are no published studies on South Africa's *Ulva* species with respect to cultivation conditions that are relevant for aquaculture. Local seaweed farmers have raised concerns whether *Ulva* species grown on their farms have different ecological growth requirements. In the current study I measured the responses of three *Ulva* species to varying temperatures and irradiances as well as to known amounts of added nutrients to determine which nutrients may limit growth. Such information is required to increase our understanding of the growth requirements of this commercial crop and to develop management methods for successful mass cultivation.

This study aimed to investigate:

1. The growth rate of three local *Ulva* species under a wide range of environmental conditions in laboratory culture. Do these species have different responses to varying temperature, salinity, light and light quality given that they are collected from different environments (west coast shore and aquaculture farm)? Do they prefer the same or different sources of nitrogen (nitrate or ammonium)?

2. Whether the growth of *U. capensis* and *U. rigida* in response to varying environmental factors can give an insight into whether these two represent a single polymorphic species, or have some ecophysiological phenotypic differentiation?
3. Whether temperature tolerances for growth of local *Ulva* relates to their biogeographical distributions in nature.

2.2 Materials and Methods

2.2.1 Plant collection

Fresh mature thalli of *U. capensis* and *U. rigida* were collected from Kommetjie (34º09′06″S, 18º19′22″E) along the West coast of the Cape Peninsula during low tide, whilst *U. lactuca* was collected from I & J farm (34º37′38″S, 19º17′44″E) from a land-based re-circulating *Ulva*/abalone cultivation system. *U. lactuca* was collected from the farm because free-living populations could not be found despite numerous searches at sites were the species had previously been recorded (*e.g.* Simon's Town, Saldanha Bay; Stegenga *et al*., 1997). At Kommetjie, *U. capensis* and *U. rigida* occupy the same intertidal zone with similar physical and chemical hydrological conditions. However, *U. capensis* is mostly found growing in tidal pools and *U. rigida* on exposed rocks. These species were visually identified based on morphological characters used by Stegenga *et al.* (1997). The algae were returned to the laboratory, washed and cleaned to remove any surface contaminants and epiphytes. The species were identified according to Stegenga *et al.* (1997) using anatomical characteristics. The plant materials were examined in section and surface view under the light microscope with magnification up to 400 X. Sections for examination were cut from the holdfast, mid thallus and apical regions of the specimens. Cross sections of *U. lactuca* holdfast regions could not be made because *U. lactuca* from raceway culture is free-floating and had no

holdfast region. For acclimation, the plants were kept in the aquarium with circulating seawater at 14 °C, with continuous dim light. This was done to keep the plants in the same condition (an equilibration period) prior to the experiment.

2.2.2 Culture methods

2.2.2.1 Irradiance, salinity, temperature and light quality experiments

Algae were cultured in one-third Provasoli medium (Provasoli, 1968) under a wide range of environmental conditions. Irradiances of 20, 40, 80, 120 and 160 µmol photons m^{-2} s^{-1} (all ± 0.5 µmol photons m^{-2} s^{-1}) were provided by cool-white fluorescent tubes with a 16:8, Light: Dark photoperiod. Various irradiance levels were created by adjusting the number of fluorescent tubes and irradiance was measured using a Skye Quantum Sensor. Dark treatments were done by keeping the Petri dishes with *Ulva* discs in a box wrapped with black plastic bags.

A total of nine salinities were used: 0, 5, 10, 15, 20, 25, 30, 35 and 40 PSS (all ± 0.1 PSS) Treatments with salinities less than 30 PSS were prepared by mixing natural seawater with deionised freshwater, while salinities greater than 30 PSS were achieved by evaporating seawater. Mean dissolved inorganic N (NH_4 + NO_3) in the initial seawater was 15.31 µM. Sodium nitrate was added to the deionised freshwater to make the mean inorganic N in the reduced salinity treatments the same as in the seawater treatments. One-sixth Provasoli ES nutrients were added to each salinity treatment. This strength of Provasoli ES was chosen to ensure that the salinity is altered minimally, and growth medium salinity was checked regularly using an optical refractometer.

Culture rooms and a growth chamber were set up to provide constant temperatures of 5, 10, 15, 20, 25 and 30 ^{o}C (all ± 0.1 ^{o}C).

The individual light colours: red (600-700 nm), green (500-570) and blue (400-500 nm) were created by coloured glass attached on top of the growth box. The irradiance for all light colours (red, green, and blue) and white light (as reference) was 20 µmol photons m^{-2} s^{-1} and temperature was 15 (± 0.1) ^{o}C.

2.2.2.2 Nitrogen and phosphorus experiments

The effects of nutrients on algal growth were investigated using artificial seawater made from commercial sea salt. This sea salt contains all essential major and trace elements in optimal proportions and it does not contain phosphate and nitrate. This water was used to produce a basic culture medium that consisted of Provasoli nutrients with no added Nitrogen (nitrate and ammonium) or phosphorus. Enrichments of PO_4^{3-} -P (0, 2.1, 6.7, 12.9, 25.9 and 56.8 µM); NO_3^--N (0, 8.3, 32.7, 66, 132 and165 µM) and NH_4^+-N (0, 5.24, 10.5, 21, 41.9 and 83.8 µM) were then added (as NaH_2PO_4 .$2H_2O$, $NaNO_3$ and NH_4Cl, respectively), to the basic culture medium in various concentrations and all nutrient concentrations were ± 0.5 µM. The salinity level for all treatments was 35 PSS and all plants were grown at 15 ^{o}C and 80 µmol m^{-2} s^{-1}.

2.2.2.3 Growth measurements

Before each experiment, thalli were rinsed with sterile seawater, and cleaned with low-lint absorbent papers (Kimwipes, Kimberly-Clark) to remove any epiphytes. Discs of 10 mm diameter were cut from thalli using a stainless steel cork borer. Vegetative material of each species was cultured in glass Petri dishes containing 150 mL of the appropriate medium, in culture rooms or growth chambers, under the appropriate environmental conditions, and there were 6 *Ulva* discs in each Petri-dish. Triplicate Petri-dish were set up for each treatment.

Petri dishes were regularly repositioned to ensure uniform irradiance was received by all culture dishes. The potential presence of contaminants in the medium was checked by viewing the culture medium using a light microscope.

Growth measurements were taken and culture medium replaced in all cultures every 3 days for a total experimental period of 15 days. Growth was measured as the increase in disc area with time using a measuring ruler, across two perpendicular diameters (including the longest diameter) and averaged. Growth rates were determined as Specific Growth Rate using the equation: $SGR = 100 (\ln (A_0/A_n)/t)$

where A_i is the initial area of the disc and A_n is the area of the disc on the day of observation and t is time (De Boer *et al*., 1978).

2.2.3 Statistical analysis

Data from all experiments were tested for normality and homogeneity prior to statistical treatment using analysis of variance (ANOVA). Comparisons after ANOVA were made using the post hoc Tukey test at 95% of significance to individualise specific differences (Zar 1999).

2.3 Results

2.3.1 Effect of salinity on growth

Growth of all algae was significantly affected by salinity ($p < 0.05$), and growth increased with increasing salinity up to 35 PSS. According to ANOVA ($p < 0.05$), there were significant differences in growth rates between *U. rigida*, *U. capensis* and *U. lactuca* at different salinities tested (Fig. 2.1). *U. rigida* and *U. lactuca* survived for 15 days in fresh water (0 PSS) with Provasoli nutrients, with *U. rigida* showing active growth of 0.7% d^{-1}.

However at 0 and 5 PSS, *U. capensis* discs started to bleach within 2 to 3 days and the plants died after 5 to 6 days. Moreover, *U. capensis* growth was also reduced at other low salinities; attaining SGR of 0.3% d^{-1} at 10 PSS and discs from low salinity were thinner than those from high salinity when wet weights were compared. The growth rates of *U. capensis* and *U. lactuca* were lower at salinities below 25 PSS. Salinities of 25 – 30 PSS yielded the best growth rate of 3.7- 4.0% d^{-1} for *U. rigida* whilst *U. capensis* and *U. lactuca* demonstrated highest growth in their natural sea-water salinities of 35 PSS, reaching SGR's of 2.8 and 3.6% d^{-1}, respectively.

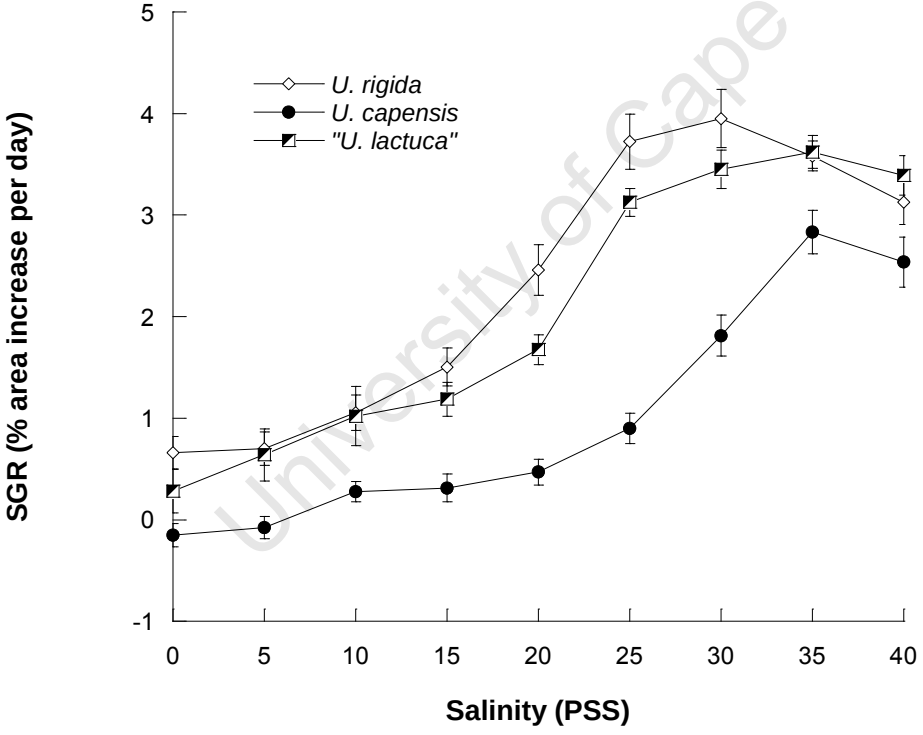


Figure 2.1: Effect of salinity levels on the specific growth rate of *U. rigida*, *U. capensis* and *U. lactuca* grown at 15 ^{o}C and 80 µmol m^{-2} s^{-1}. Growth measurements were taken after 15 days of experimental treatment. Each point represents the mean value of 18 plants. Vertical bars indicate SE.

2.3.2 Effect of irradiance on growth

Although all species grew at all irradiances tested, there were significant differences in growth at different irradiance levels (Fig. 2.2). For *U. rigida* and *U. capensis*, the relationship between growth rate and irradiance was best described by a polynomial function (Fig. 2.3) with the highest growth found at 80 µmol photons m^{-2} s^{-1} reaching SGR of 4.6 and 3.8% d^{-1}, respectively. However, when the irradiance was further increased to 120 µmol photons m^{-2} s^{-1}, less growth was observed for these two species whilst *U. lactuca* growth increased with increasing irradiance tested attaining SGR of 5.9% d^{-1} at 160 µmol photons m^{-2} s^{-1}. This suggests that *U. lactuca* may be tolerant of even higher light levels. All species grew at the lower irradiances (40 µmol photons m^{-2} s^{-1}) but there was no growth in the dark. All species survived in darkness for 15 days.

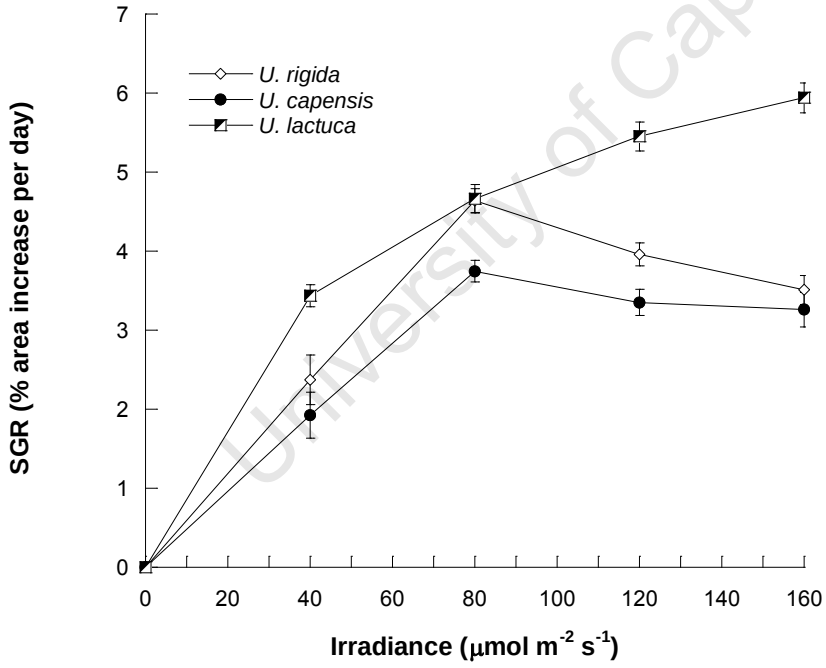


Figure 2.2: Effect of irradiance on the specific growth rate of *U. rigida*, *U. capensis* and *U. lactuca* grown at 15 ^{o}C. Growth measurements were taken after 15 days of experimental treatment. Each point represents the mean value of 18 discs. Vertical bars indicate SE.

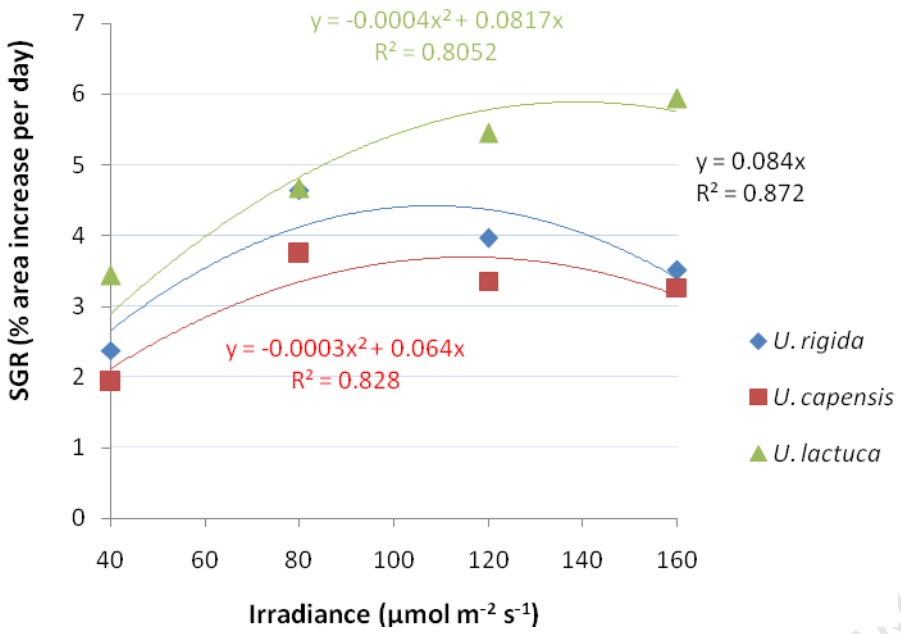


Figure 2.3: Polynomial function of effects of irradiance on the specific growth rate of *U. rigida*, *U. capensis* and *U. lactuca* grown at 15 ^{o}C. Growth measurements were taken after 15 days of experimental treatment. Each point represents the mean value of 18 discs. Vertical bars indicate SE.

2.3.3 Effect of light quality on growth

In comparison to white light (Fig. 2.4) other light spectra tested resulted in lower growth rates (p<0.05). The growth was minimal under red, green and blue: after 15 days, the growth rates of *U. rigida*, *U. capensis* and *U. lactuca* were around 0.4, 0.4 and 0.8 % d^{-1} for red light, 0.1, 0.4 and 0.7 % d^{-1} for green light, 0.9, 0.6 and 1.0 % d^{-1} for blue light, respectively. Blue light yielded significantly higher growth as compared to other spectra (red and green) (p<0.05) whereas green light and red light resulted in an initial peak in growth rate in the first week followed by no growth in the second week. All species had significantly higher growth under white light (p<0.05). Overall, all species had the same growth pattern , growing best in white, about half that in blue, worse in red and green.

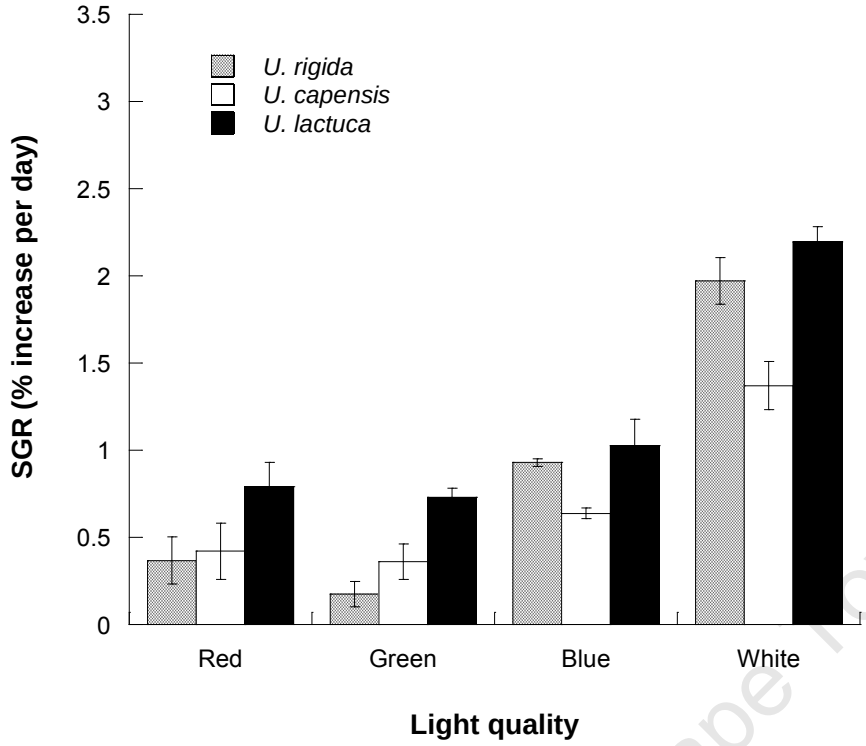


Figure 2.4: Effect of light quality on the specific growth rate of *U. rigida*, *U. capensis* and *U. lactuca* grown at 15 ^{o}C and 20 µmol photons m^{-2} s^{-1}. Growth measurements were taken after 15 days of experimental treatment. Each point represents the mean value of 18 plants. Vertical bars indicate SE.

2.3.4 Effect of temperature on growth

As shown in figure 2.5, all species survived and grew over the tested temperature range of 5 - 25 ^{o}C but all died at 30^{o}C, before the end of the experimentation period. At low temperatures the specific growth rate of all species was low and it increased with temperature, reaching its maximum at 15 ^{o}C (Fig. 2.5). The SGR of *U. rigida*, *U. capensis* and *U. lactuca* was 5.7, 4.1 and 5.2% d^{-1}, respectively at 15 ^{o}C. A temperature of 25 ^{o}C promoted rapid growth in the first week but growth rate declined towards the end of the experimental period. In addition at the end of the experiments *U. rigida* and *U. capensis* grown at 25^{o}C were generally coarse and

crinkled compared with thalli from lower temperatures. Overall, all species had the same growth pattern with *U. rigida* and *U. lactuca* growing faster than *U. capensis*.

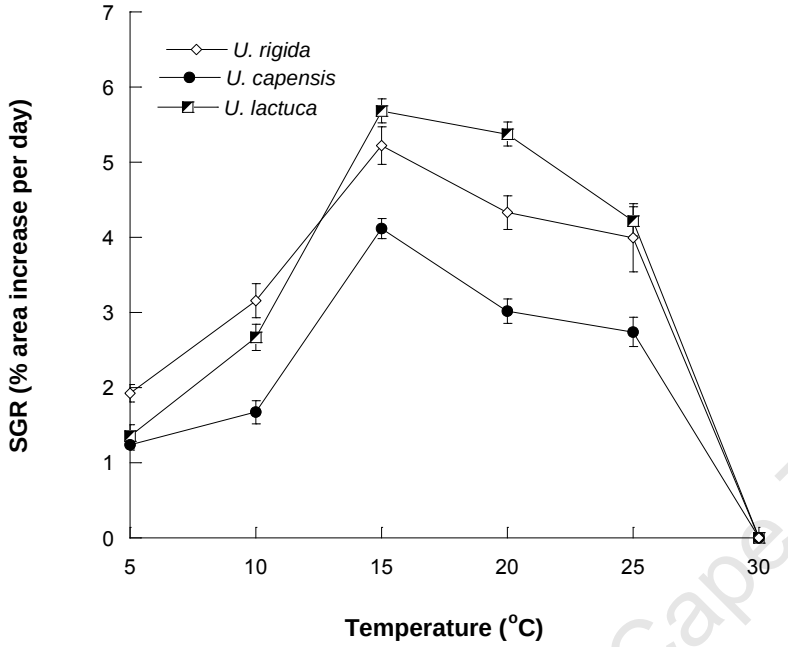


Figure 2.5: Effect of temperature on the specific growth rate of *U. rigida*, *U. capensis* and *U. lactuca* grown at 80 µmol m^{-2} s^{-1}. Growth measurements were taken after 15 days of experimental treatment. Each point represents the mean value of 18 plants. Vertical bars indicate SE.

2.3.5 Effect of nutrient enrichment on growth

All treatments with nitrogen enrichment significantly increased the growth rate of all species compared with the control treatments (p<0.05) (Fig. 2.6), where all species showed almost zero growth in seawater medium with no N-enrichment. A regression analysis over different nitrogen sources indicated a significant increase in specific growth rate (SGR) with increasing molar N concentration until an optimum concentration.

The highest growth rate was recorded at 21 µM NH_4^+-N for *U. rigida* (5.8% d^{-1}), *U. capensis* (4.1% d^{-1}) and *U. lactuca* (5.1% d^{-1}), while 40µM resulted in declining growth, and tissue

loss. . When nitrate was used as N-source, the highest growth rate for *U. rigida* (5.4% d^{-1}) was obtained at 32.7 µM NO_3^--N whilst for *U. capensis* (4.1% d^{-1}) and *U. lactuca* (7.7% d^{-1}) it was attained at 66 µM NO_3^--N. Most rapid growth of these three species occurred in the lower to mid-range (32.7 -132 µM) of NO_3^- concentrations although all algae showed a broad tolerance to higher concentrations. For *U. rigida* and *U. capensis*, analysis showed that differences in growth due to the two N-sources were not significant (p>0.05) and for *U. lactuca* the highest growth was recorded when NO_3^- was used as N-source. There was no evidence of spore production in any cultures and no spores were found in the culture medium.

As shown in figure 2.7, all algae showed a broad tolerance to phosphate with growth occurring at concentrations up to 56.8 µM (PO_4^{3-}-P). In *U. lactuca*, growth rates were significantly affected by PO_4^{3-}-P concentration ($p < 0.05$), with growth increasing with increasing PO_4^{3-}-P concentration up to an optimum level of 12.9 µM. The highest growth rate for *U. lactuca* (7.7% d^{-1}) was recorded at 12.9 µM PO_4^{3-}-P whilst *U. rigida* (5.1% d^{-1}) *and U. capensis* (4.1% d^{-1}) grew most rapidly at 6.7 µM PO_4^{3-}-P. All species grew at low rates in seawater unenriched with PO_4^{3-}-P.

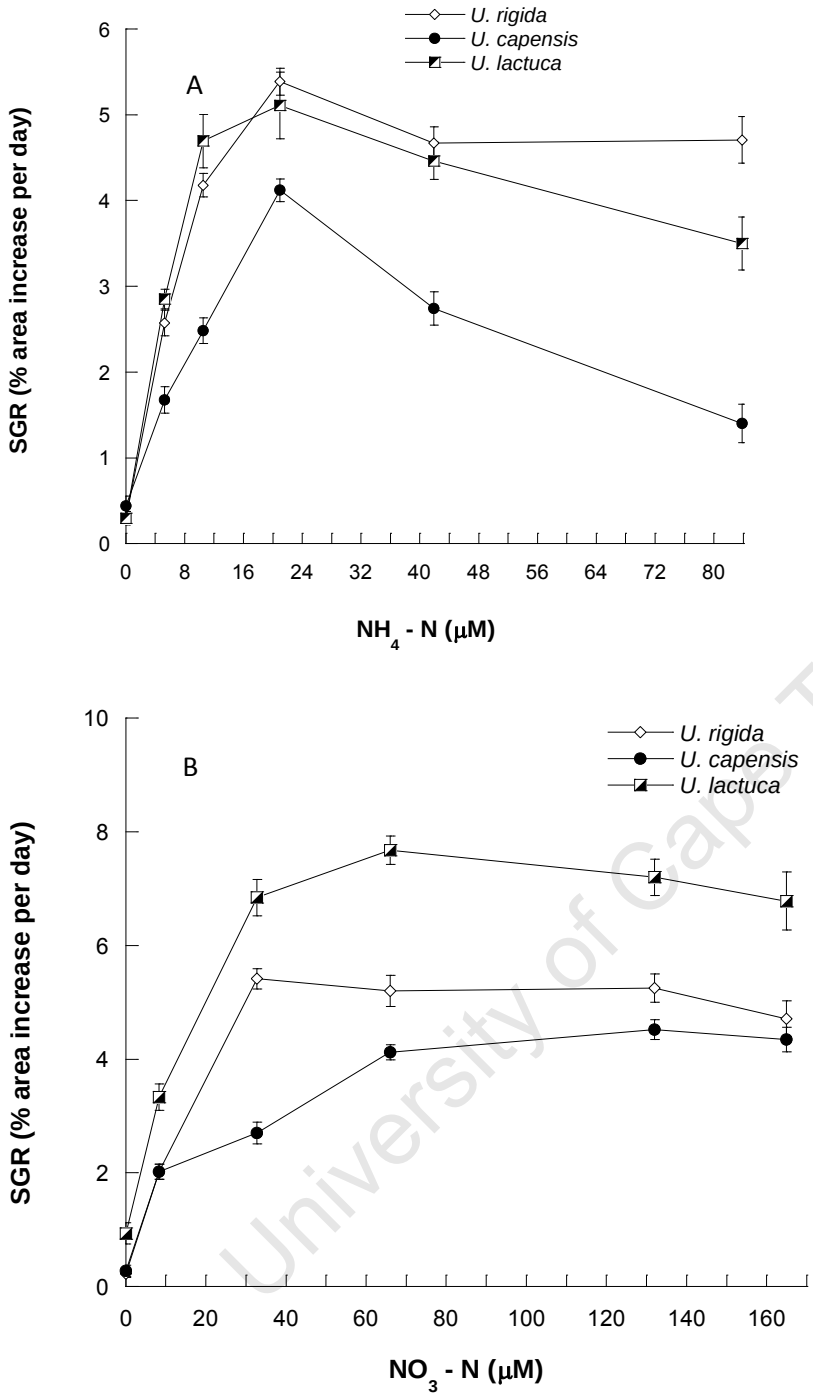


Figure 2.6: Effect of nitrogen (A. ammonium and B. nitrate concentrations) on the specific growth rate of *U. rigida*, *U. capensis* and *U. lactuca* grown at 15 ^{o}C and 80 µmol m^{-2} s^{-1}. Growth measurements were taken after 15 days of experimental treatment. Each point represents the mean value of 18 plants. Vertical bars indicate SE.

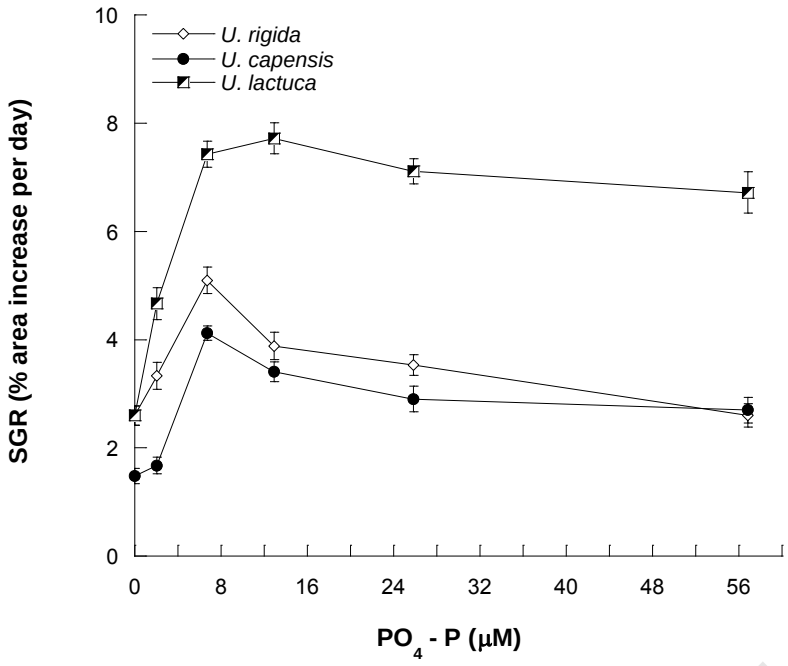


Figure 2.7: Effect of phosphate concentrations on the specific growth rate of *U. rigida*, *U. capensis* and *U. lactuca* grown at 15 ^{o}C and 80 µmol m^{-2} s^{-1}. Growth measurements were taken after 15 days of experimental treatment. Each point represents the mean value of 18 plants. Vertical bars indicate SE.

2.3.6 Optimum growth parameters

Figures 2.1 to 2.5 show the effect of environmental parameters on algal growth. Figures 2.6 to 2.7 show the effect of the different nutrients and these data are summarised in Table 2.2, which lists the conditions promoting the highest growth rates.

Table 2.2 Environmental parameters found to promote the highest growth rates of three *Ulva* species in laboratory culture

	Salinity (PSS)	Irradiance ($\mu molm^{-2}s^{-1}$)	Temperature (^oC)	PO_4^{3-}-P (μM)	NO_3^--N (μM)	NH_4^+-N (μM)
U. capensis	35	80	15	6.7	66	21
U. rigida	25-30	80	15	6.7	32.7	21
U. lactuca	30-35	160	15	12.9	66	21

2.4 Discussion

In South Africa, there has been little or no research on growth in laboratory culture of *Ulva* under controlled conditions. The aim of this chapter was to determine the optimal growth factors and to gain an understanding of these factors in the culture environment. The *in situ* cultivation of seaweeds for abalone feed is an increasing practice in South Africa; therefore in order to maintain a constant supply of feed it is important to attain good yields. Ecophysiology studies are essential, with the main objective of providing information which could lead to the production of algal biomass which exhibits specific (*e.g.* nutritional) qualities. Laboratory-based growth experiments were conducted to determine these growth conditions, and to further our understanding of fundamental physiological processes.

Under exposure to hypotonic or hypertonic conditions, algal cells may alter their internal osmotic pressure by pumping ions across cell membranes or by interconverting monomeric and polymeric metabolites (Hellebust, 1976; Dickson *et al*., 1982; Lobban and Harrison, 1994). In the current study, all species exhibited broad salinity tolerances with respect to

growth, and grew between 5 PSS and 40 PSS. The relationship between specific growth rates and salinity followed an optimum curve with a maximum growth observed at 20 - 30 PSS for *U. rigida* and 35 PSS for *U. capensis* and *U. lactuca*. Growth rates at these salinity ranges are common for most *Ulva* spp. (Bliding, 1968; Koeman and van den Hoek, 1981, Fong *et al*., 1996).

For *U. capensis*, 10 PSS seems to be the minimum tolerance limit; salinity lower than 10 PSS resulted in death after 5 to 6 days. Although *Ulva* species have demonstrated an ability to regulate turgor pressure, Martins *et al*. (1999) also found that *U. intestinalis* died after exposure to 0 PSS for as little as 6 days. In addition, *Ulva intestinalis* inhabiting isolated rock pools along the Swedish Atlantic coast died at salinity less than 1 PSS (Bjork *et al*., 2004). However, *U. rigida* demonstrated tolerance of low salinities by actively growing at 0 PSS plus Provasoli ES nutrients, and Ohno *et al*. (1999) reported that *U. prolifera* in the Shimanto River, Kochi Prefecture, can survive in freshwater to 18 PSS conditions. Moreover, *U. lactuca* showed almost no growth but was able to tolerate 15 days in the non-saline medium and resumed normal growth when transferred to full seawater. Murthy *et al*. (1988) found decreased growth of *U. lactuca* below 30 PSS as did Friedlander (1992) who also found that *U. lactuca* lost biomass at salinity below 20 PSS. Higher and lower optimal salinities have been reported for various *Ulva* species *e.g.* 35±40 PSS for *U. lactuca* (Friedlander, 1992); 25 PSS for *U. pertusa* (Floreto *et al*., 1994) and *U. fasciata* (Morand and Briand, 1996). Furthermore, species-specific differences and ecotypic variation in salinity tolerance can occur in *Ulva* (Friedlander, 1992; Floreto *et al*., 1994). Therefore, we conclude that *U. capensis* and *U. lactuca* are probably capable of surviving in brackish waters, but not in freshwater environments compared to *U. rigida*. In addition, our results suggest that *U. rigida* can tolerate lower salinity than *U. capensis* and this physiological response could be

attributed to their position in the intertidal zone. *U. capensis* grows in tidal pools and *U. rigida* on the open rock nearby. Evaporation will not cause a rapid salinity changes in tidal pools but will increase the salinity of water in the surface films on *U. rigida* on open rock surfaces. Similarly, during rainy seasons the salinity of water in the surface films on *U. rigida* will be significantly reduced, therefore this species has adapted to a broader salinity tolerance than *U. capensis*. Furthermore, the ability of these *Ulva* species to withstand salinity of 0 – 10 PSS for more than a day has proven useful for the control of epiphytes and grazers. Washing *Gracilaria* with fresh water has been found to control the growth of amphipods in South African *Gracilaria* aquaculture (Smit *et al*., 2003). Similarly, a study by Hansen *et al.* (2006) used the same practice to control the growth of the keyhole limpet *Fissurella mutabilis* in *Ulva* aquaculture systems.

Light represents one of the most important governing factors for land-based seaweed cultivation (Bidwell and McLachlan, 1985) and light directly controls growth rates in nature (Lapointe and Tenore, 1981). The effect of light with growth of these three *Ulva* species was determined in order to develop a predictive equation on production. Understanding species-specific light requirements under culture conditions is an important practice in managing land-based seaweed culture systems. *Ulva* is known to cope with pulses of high light as natural light levels are widely fluctuating (Sand-Jensen, 1988).

Both *U. capensis* and *U. rigida* are found in the intertidal zone at Kommetjie and that could explain their similar growth responses to various irradiance levels. In the present study, the growth of *U. lactuca* was not saturated even at the highest light intensity used in this experiment (160 µmol photons $m^{-2}s^{-1}$). Lapointe and Tenore (1981) obtained similar results, with a growth of *U. lactuca* in tank cultivation showing a linear response without any signs of

light saturation up to a light intensity of 160 µmol photons $m^{-2}s^{-1}$. This shows that *U. capensis* and *U. rigida* have adapted to low light requirements whereas *U. lactuca* is adapted to relatively high light. Our result agrees with those of Ramus (1978) who described the anatomy of *Ulva lactuca* as being adapted to high light environments. Furthermore, Fortes and Lüning (1980) found that the growth of *Ulva lactuca* is not inhibited at irradiances up to 225 µmol photons $m^{-2}s^{-1}$. Due to confusion in the taxonomy of *Ulva* species it is very unlikely that the above three studies are dealing with the same species (Malta *et al*., 1999; Blomster, 2000; Hayden *et al*., 2003; Kandjengo, 2003). The results showed that *U. capensis*, *U. rigida* and *U. lactuca* were able to maintain growth under low irradiances (20 and 40 µmol photons $m^{-2}s^{-1}$). Rosenberg and Ramus (1982) and Duke *et al*. (1989) found that irradiances between 35 and 80 µmol photons $m^{-2}s^{-1}$ are not limiting to *Ulva* growth. *Ulva* is capable of maintaining growth rates at reduced irradiances induced by factors like self shading at high densities (Altamirano *et al.,* 2000) and this is attributed to their thin thallus which enables them to reduce self-shading both within and among the photosynthetic tissue (Sand-Jensen, 1988). In *Ulva* spp., positive values for growth were found at irradiances as low as 0.6–2.5 µmol photons $m^{-2}s^{-1}$ (Vermaat and Sand-Jensen, 1987).

Studies by Vergara *et al. (*1997) found that as *Ulva* biomass increases, light is attenuated by the thalli so that lower layers receive less light than those in close proximity to the surface of the mat. Browne (unpublished data) obtained similar findings for *Ulva rigida* from I & J farm. In the raceways at I & J farm, *Ulva* is constantly aerated and mixed using a paddle wheel, and thus each plant is exposed to pulses of light from time to time. However, this will depend on the stocking density and flow rate. In addition, Browne (unpublished data) showed that irradiance penetration in the raceway system decreases with increasing *Ulva* density and the bottom of the raceways with *Ulva* biomass of approximately 2000 kg (immediately pre-

harvest) receives an average irradiance at midday of 54.9 µmol photons $m^{-2}s^{-1}$. Our results showed that *U. rigida, U. capensis* and *U. lactuca* were able to maintain growth even at 20 and 40 µmol photons $m^{-2}s^{-1}$, although their optimum levels for growth were respectively at 80, 80 and more than 160 (the latter was not saturated) µmol photons $m^{-2}s^{-1}$. Therefore, *Ulva* biomass of 2000 kg in the raceway is not light-limited for growth at midday. It is also important to note that with water movement in the raceways plants do not reside on the bottom of the raceways continuously.

Photoadaptation occurs both in response to changes in photon flux density and spectral quality or light source. However, much more is known about responses to changes in light intensity than light quality. The growth of *U. rigida, U. capensis* and *U. lactuca* in different wavebands of low irradiance (20 µmol photons $m^{-2}s^{-1}$) seems to agree well with the absorption spectrum of the thallus and the action spectrum for photosynthetic activity of *Ulva* species that decline markedly in green as compared with blue or red wavelengths (Lüning and Dring, 1985). Blue light promoted better growth of all three *Ulva* species when compared to red and green light, a result comparable to those from studies of other algae (Dring and Lüning, 1985). However, contrasting findings were obtained in *U. pseudocurvata* Koeman et Hoek by Lüning (1992) who found blue light to cause an immediate reduction in growth rate. Nevertheless, the same species grown in green and red light showed an initial peak in growth followed by growth inhibition.

When irradiance penetrates a canopy of *U. rigida, U. capensis* or *U. lactuca* it will be reduced, and the wavelength altered. According to Beach *et al.* (1995) this is the result of selective attenuation by chlorophylls and accessory pigments within the thallus that absorb most of radiation of 400–500 or 600–700 nm. The growth response of all three species could

possibly be related to differential photosynthetic activities under different light qualities. Although all species had the same pattern, growing best in white, about half that in blue, worse in red and worst in green**,** *U. lactuca* grew better than the other species in red and green light. Moreover, a study by Yokohama and Kageyama (1977) on deep water green seaweed and shallow water species discovered an absorption peak at 540 nm in deep-water species which was not present in shallow water species. The pigment absorbing light in that region was identified chromatographically as siphonaxanthin and this pigment is a characteristic of many siphonous greens (Kleining, 1969). *U. japonica* carries the pigment siphonaxanthin and Yokohama *et al.* (1977) found that the photosynthetic efficiency for *U. japonica* from a depth of 20 m was equal in green light to that in white light, and the efficiency was 50% greater than *U. pertusa* from shallow water which contains no siphonaxanthin. Moreover, these authors found that *U. pertusa* was 30% less efficient in green light than in white light. A spectrophotometric analysis from 480 nm to 750 nm was carried out, and none of the *Ulva* species in the present study had an absorption peak in the green portion of the visible spectrum. Therefore *Ulva* species in our study had no siphonaxanthin and possibly this is possibly why they all had poor growth in wavelengths other than white light.

Temperature is an important part of the ecological niche, a concept that is often used to describe the range of tolerance (Lampert and Sommer, 1997). The water-temperature tolerances of different species of seaweeds are partly responsible for the patterns of geographic distribution of adult plants (Lüning, 1990). In the present study, *U. capensis, U. rigida* and *U. lactuca* grew well at temperatures similar to the water temperatures in their natural habitat but they also grew better at higher temperatures than occur in their habitat and this is a common pattern with most seaweeds. Moreover, geographic distribution is often

limited by temperature range for reproduction rather than growth (Lüning, 1984). The west coast has a lower annual mean sea water temperatures (12 - 16ºC) than the south west coast (17 – 19ºC), a result of strong south easterly winds generating a semi-permanent upwelling system in which cold nutrient-rich water is brought to the surface in summer (Dieckmann, 1980; Bolton, 1986). Although growth was highest at 15 °C, the results obtained at other temperatures should also be noted. Plants died at 30 °C and the low temperature of 5 °C limited growth. It appears therefore that the three *Ulva* species have an upper survival limit somewhere just above 25, but below 30 °C. Furthermore, these species grew well at 25 °C, which is quite high for an algal species from the cold to warm temperate region (Fortes and Lüning, 1980; Lüning, 1990). Fries (1966) found the optimum temperature for growth of three red algae in axenic culture to be around 20-25 °C whereas the water temperature in their natural habitat rarely rose above 15 °C. This author hypothesized that this may have been due to the absence of bacteria from the culture since there is a physiological optimum temperature for the alga alone and there is an ecological optimum temperature in nature where it is interacting with bacteria and fungi. However, cultures in the present study were not axenic, so lack of bacteria cannot explain this phenomenon here.

Moreover, Biebl (1958) in Fortes and Lüning (1980) tested short term (12 hours) survival of different seaweed species under varying temperatures and found that *Agarum cribrosum* and *Laminaria saccharina* from West Greenland had upper survival temperature of 24 °C, well above natural water temperatures. An upper survival temperature of 23 °C (also well above ambient water temperatures) was observed in gametophytes of *Laminaria saccharina* from the Canadian Arctic, exposed for weeks to varying temperatures by Bolton and Lüning (1982). Biebl (1962)) in Fortes and Lüning (1980) found that tropical sublittoral algae have a lower lethal temperature as high as 14 °C and suggested that seaweeds change their

temperature characteristics radically given a lengthy geological time period. From our current study, despite *U. rigida* appearing to have a much wider geographical distribution into warmer waters (Stegenga *et al*., 1997), material from Kommetjie had similar temperature tolerances to material of *U capensis* from the same site. Alternative hypotheses are that these are the same species, differing in morphological form in different temperature regions, or that they are different species, with the distribution of *U. capensis* being limited by a factor other than the temperature tolerance of the adults.

From an ecological point of view, this study found *U capensis* and *U rigida* to have similar temperature tolerances but they are reported to have different biogeographical distributions. On this basis it seems *Ulva* species on the south coast are not *U. rigida* or *U. capensis* because the growth rates of these two species declined towards the end of the experimental period when grown at 25 ^{o}C and plants started to have a different morphological form which is crinkled. This may be true since according to Kandjengo (pers. comm.) all the species growing on the south coast are not *U. rigida* or *U. capensis* and probably will be called *U. uncialis*.

The positive correlation between nutrient availability in seaweed culture media and the specific growth of *Ulva* has been well documented (Duke *et al.,* 1989). Both P and N enrichment resulted in an increase of biomass after 3 days of incubation and the biomass continued increasing over the 15 days of the experiments. Nitrogen supply in *Ulva* is one of the most important factors regulating growth and photosynthesis (Pedersen, 1994).

All species had almost zero growth in seawater medium unenriched with N but were able to survive in this medium; however bleaching occurred at the end of the experimental period. Similar findings were reported by Robertson-Andersson *et al.* (2009), who found that

cultivated *U. lactuca* changed colour from green to green-yellow at a tissue nitrogen content of between 1.5 and 1.7% DW. Green indicated nitrogen-replete plants, and green-yellow indicated nitrogen starvation. This suggests that, for a limited time period at least, the algae used in the present study were able to rely on internal nutrient reserves for survival. The utilisation of accumulated internal nutrient reserves has been shown in *Ulva* by Fujita (1985); Rosenberg and Ramus (1982) and Duke *et al*. (1986). In *Gracilaria,* bleaching is a result of pigments being metabolized as a source of protein (Smit *et al*., 1997) and it is possible that similar metabolism can occur in these *Ulva* species during nitrogen starvation.

Koutropoulos *et al.* (1991) found *Ulva* spp. to grow better when NH_4^+ was used as the N-source rather than NO_3^-, but in the current study there was no significant difference in the growth of *U. capensis* and *U. rigida* when grown in low levels (> 66 or 21 µM.) of NO_3^- or NH_4^+, respectively. However, *U. lactuca* achieved highest growth rates when NO_3^- was used. From the current results it is clear that NH_4^+ - N concentrations somewhat higher than 21 µM can produce adverse effects on the physiological processes of growth in *U. rigida, U. capensis* and *U. lactuca*. In addition, *U. capensis* and *U. rigida* were very sensitive to increased NH_4^+ and started losing tissue at a concentration of 84.5 µM NH_4^+ - N. Waite and Mitchell (1972) found that *U. lactuca* photosynthesis was inhibited at ammonium concentrations over 60 µM. Depression or inhibition of algal performance under high-nutrient conditions is known to depend on nutrient type, nutrient concentration and the degree of tolerance or adaptation to high nutrient levels (Fong *et al*., 1996). A better understanding of the relationship between growth rate and nitrogen concentration would improve fertilizer application in the cultivation of commercial seaweeds. Furthermore, abalones excrete ammonium, so this is very relevant to the use of *Ulva* as a biofilter on abalone farms.

Even though nitrogen is believed to be the primary limiting nutrient for many seaweeds, evidence suggests that phosphorus may be as, or more important in certain waters. The results of in situ macro-algal bioassays in Florida reveal that inorganic phosphorus was quantitatively more important than nitrogen in limiting both photosynthetic capacity and growth of *Gracilaria tikvahiae* MacLachlan (Lapointe, 1986). Based on our results from this study, *U. lactuca* showed different responses to increasing concentration of PO_4^{3-} -P as compared to *U. capensis* and *U. rigida*, suggesting that the first species is adapted to more eutrophic waters while the latter two species are adapted to lower phosphate concentrations.

Ecophysiological results of this study found *U. capensis* and *U. rigida* to respond similarly to variations in irradiance, light quality, N (NH_4^+) and P enrichments. However, they showed different responses to salinity and N (NO_3^-), where *U. rigida* was very tolerant to low salinity and maintained highest growth at lower N (NO_3^-) concentrations than *U. capensis.* Another important difference was that *U rigida* grew faster than *U capensis* in all experiments. If the findings by Kandjengo (unpublished data) conclude that these two species represent one polymorphic species, the data from this study shows that perhaps these species have evolved two forms suited to different habitats. *U. rigida* generally grows on open rock whereas *U. capensis* is commonly found growing in nearby pools. Therefore, these habitats could match with their salinity tolerances and NO_3^- concentrations.

From the three *Ulva* species investigated in the current study, the best choice of *Ulva* species for cultivation would be *U. lactuca* as:

- It has a high growth rate
- Takes up more N and P
- Tolerates high light intensity

U. rigida would be the best second choice and *U. capensis* would be last as it has a slower growth rate.

Chapter 3

Seasonal variation in photosynthetic production in intertidal *Ulva capensis* and *Ulva rigida* from the west coast of South Africa

3.1 Introduction

Most of the research on South Africa's *Ulva* species has been on taxonomy (Stegenga *et al*., 1997) and aquaculture (Robert-Andersson *et al*., 2007, 2008; Bolton *et al*., 2009; Smit *et al*., 2010), and there are no studies that directly measured these species' primary production. This chapter is focused on comparing the photosynthesis of two closely related taxa which grow close together but in different habitats, and studying their temporal patterns of production. Light is one of the main abiotic factors that regulate benthic seaweed abundance and distribution, depth limits and seasonal growth patterns (Gerard, 1988; Hanelt *et al.*, 1997). *Ulva* are highly adaptable plants and are able to readily adjust to changes in light availability. The plants are capable of maintaining growth rates at reduced irradiances that can be caused by factors like self-shading at high densities (Altamirano *et al.*, 2000). Algae living at any location along the shore must be better adapted to live, grow, and reproduce in that environment, and therefore physiological differences between species of intertidal macroalgae exist (*e.g*; Littler, 1980; Brown, 1987). High light levels can reduce photosynthetic activity (cause photo-inhibition) in seaweeds (Ramus and Rosenberg, 1980; Coutinho and Zingmark, 1987; Henley *et al*., 1991a, 1992).

Seasonal submersed P-I curves of five species of intertidal seaweeds of differing morphologies from different shore zones on a South African rocky shore have been measured by Levitt and Bolton (1991). These authors reported that the thin, sheet-like *Porphyra capensis* which occurs high on the shore was the most photosynthetically productive species

either when submersed or when emersed. Among these species, fleshy forms with flattened thalli were also highly photosynthetically productive, whilst the terete branched forms were the least photosynthetically productive. They also found that these species have highest productivity in spring and is lowest in winter. In another local study by Levitt (1993) on the annual primary productivity of three littoral and three understorey sublittoral seaweed species between Cape Point and Yzerfontein, South Africa, the author found that the understorey species fix 68 t $C.yr^{-1}$ and the intertidal species 541 t $C.yr^{-1}$ along this portion of coast compared to 36 220 t $C.yr^{-1}$ fixed by kelps.

Primary production on an area basis, using the hole-punching method of Parke (1948), was measured in the kelps *Ecklonia maxima* (Mann *et al*., 1979) and *Laminaria pallida* (Dieckmann, 1980). These authors found that *E. maxima* in a west coast kelp bed produces 4.1 and 7.8 kg dry mass m^{-2} y^{-1} in seral and climax stands respectively, whereas total biomass of *L. pallida* turns over twice per year and produces ca. 27.9 kg dry mass m^{-2} y^{-1} in the kelp beds that were studied. Anderson and Hay (1986) used a biomass-based method to show that *Desmarestia firma* understorey populations at Oudekraal on the Cape Peninsula produce about 23 g dry mass m^{-2} y^{-1}.

Specimens of *U. capensis* and *U. rigida* used in this study were collected from Kommetjie on the West Coast of South Africa, where they grow on the intertidal zone and are subject to fluctuations in light level. The former grows in intertidal pools whereas the latter is found growing on the open rock, exposed to the air at low tides. Besides growing in different habitats, these two species have different morphology but are extremely closely related according to ITS analysis and it was clearly shown by TCS analysis that these species could be haplotypes of a single genetically diverse species. (L. Kandjengo, pers. comm.),

Furthermore, *U. rigida* grows from the upper intertidal to shallow subtidal, from the Cape Peninsula eastward into tropical East Africa, whereas *U. capensis* grows from the mid-littoral to the shallow subtidal from Namibia only as far east as Cape Agulhas, the southernmost point of Africa (Stegenga *et al.*, 1997). The different habitats and geographical distributions of these two entities suggested that they may be expected to differ with respect to their photosynthetic response to light.

This study aimed to:

(i) examine seasonal variations in photosynthesis and respiration in *U. capensis* and *U. rigida.*

(ii) to test whether these species have different photosynthetic performances.

3.2 Materials and Methods

3.2.1 Collecting site

U. capensis and *U. rigida* plants were collected every month from January 2008 to May 2009 from Kommetjie on the Cape Peninsula, South Africa (34º09′06″S, 18º19′22″E). These two *Ulva* species are found on the same intertidal zone with *U. capensis* growing in tidal pools and *U. rigida* growing on the open rock. *U. rigida* has irregular incised or lobed thalli and is often found as small, firm rosettes a few centimetres in diameter; *U. capensis* has ovate to broadly lanceolate, often irregularly laciniate thalli, sometimes with numerous holes and dentate edges (Stegenga *et al.*, 1997). Three replicates were collected each time and photosynthesis measurements were carried out on three plants. Three replicates were averaged on each sampling occasion.

3.2.2 Tissue preparation and photosynthesis measurements

Ulva samples were placed in plastic bags filled with seawater and carried to the laboratory in a cooler box. By hand section and microscopic observation, anatomical characteristics were determined using characters in Stegenga *et al.* (1997). Experiments were carried out immediately after the algal samples were returned to the laboratory. Photosynthetic production of *Ulva* species was measured using the gas exchange method which has been used by several authors (*e.g.* Littler and Littler, 1980; Henley and Ramus, 1989a, b; Altamirano *et al*., 2000). Measurements of photosynthetic O_2 evolution were performed using a Clark-type oxygen electrode (YSI Model), with a water jacket connected to a cooling circulator for temperature control (Fig. 3.1). Filtered seawater (pH 8.2, total dissolved inorganic carbon concentration 5 mM) was used as the medium for photosynthetic measurements. To avoid an eventual disruption of the oxygen probe due to super saturation, at the beginning of each experiment, the seawater inside the chamber was bubbled with nitrogen in order to lower the oxygen concentration to about 50% of saturation.

Before each experiment, thalli were rinsed with sterile seawater and cleaned with low-lint absorbent papers (Kimwipes, Kimberly-Clark) to remove any epiphytes. Discs of 10 mm diameter were cut from thalli using a stainless steel cork borer and a total of 6 thallus discs were introduced into the chamber containing 150 mL of filtered seawater, which was magnetically stirred to ensure sufficient mixing inside the chamber. The temperature within the chamber was maintained at 15 ^{o}C by a water cooling jacket, illumination was provided by cool white fluorescent lamps, and photon irradiance was measured with a Skye Quantum light meter at the surface of the chamber. The plant discs were subjected to increasing photon flux densities (PFDs), ranging from 0 for dark respiration measurement to 400 µmol photons m^{-2} s^{-1}. Irradiance was varied by altering the distance between the chamber and the light

source. At each PFD, oxygen concentration measurements were taken over a 10-min period, which was long enough to observe a constant rate of change of O_2 concentration and the rate calculations were made after a few minutes, when the slope stabilized. Dark respiration (R_d) was measured by completely blacking out the chamber. At the end of the experiment the discs were rinsed and dried for 24 h at 60 ^oC for dry weight (DW) determination. This procedure was repeated for each of three replicates.

The photosynthesis parameters derived from the photosynthesis versus irradiance (P-I) curves were measured as follows: irradiance-saturated maximum net photosynthetic rate (P_{max}) was calculated as the mean of values in the asymptote region of the P-I curve. Apparent photosynthetic efficiency (α) was estimated as the irradiance-limited slope of the P-I curve, and the intersection of the slope and P_{max} is defined as the saturation irradiance (I_k), according to Henley (1993).

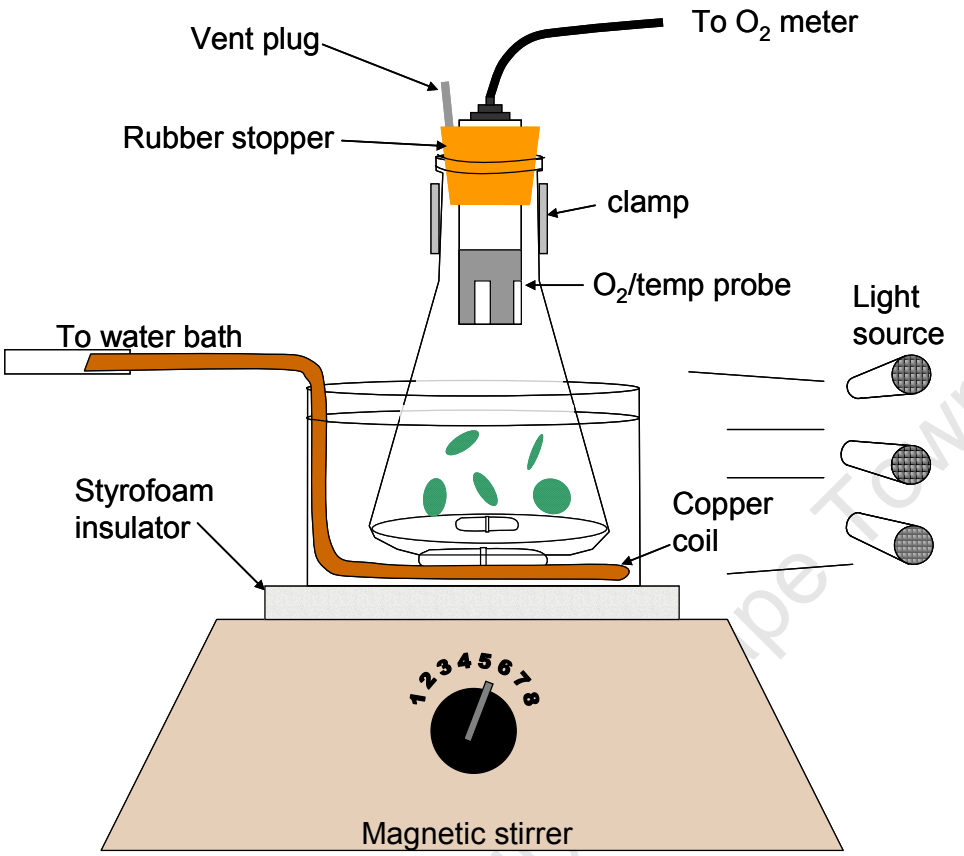


Figure 3.1: Diagram illustrating equipment used for photosynthesis measurements. The copper coil contained cooled water pumped from a controlled temperature bath.

3.2.3 Statistical analysis

All data are expressed as means ± Standard Errors. All data were regarded significant at $p < 0.05$. Photosynthetic rates between the species of each light treatment were compared using one-way ANOVA. The maximum photosynthetic rate, dark respiration, photosynthetic efficiency and saturation irradiance were compared between *U. rigida* and *U. capensis* in each season and among seasons in each species by ANOVA using STATISTICA 8.0.

3.3 Results

The seasonal P-I curves for *U. rigida* and *U. capensis* are shown in figure 3.2 and the derived photosynthesis parameters are shown in figure 3.3. Light saturation of P-I curves occurred at 240 - 400 µmol photons $m^{-2}s^{-1}$ in both species in each season. Furthermore, the photosynthetic rates at 100 – 400 µmol photons $m^{-2}s^{-1}$ was mostly higher in *U. rigida* than those of *U. capensis* in winter, spring and summer, while in autumn at 170 -400 µmol photons $m^{-2}s^{-1}$ the rates were about the same between the 2 species. Moreover, both species had almost similar photosynthetic rates at 9 – 90 µmol photons $m^{-2}s^{-1}$ in winter, spring and summer, while in autumn *U. capensis* had higher rates (Fig. 3.2). As shown in figure 3.3A, the maximum photosynthesis rate (P_{max}) in *U. rigida* was significantly greater than in *U. capensis* (2-way ANOVA, $p < 0.05$) in each season except in autumn when *U. capensis* had higher P_{max}. Moreover, *U. rigida* had higher P_{max} in spring and summer than in autumn and winter, whereas *U. capensis* had higher P_{max} in autumn which decreased in winter and increased slightly in spring and summer (Fig. 3.3A). Light saturation intensities (I_k) differed more within seasons and between species. In both species, saturating irradiance was at its lowest during winter ($p<0.05$) (Fig. 3.3B). A pronounced seasonal difference in photosynthetic efficiency (α) was observed in both species ($p < 0.01$; Fig. 3.3C). Photosynthetic efficiency was greater in winter than in other seasons. Photoinhibition of photosynthesis was not observed in either *U. capensis* or *U. rigida* over the range of irradiances tested (0 to 400 µmol photons m^{-2} s^{-1}) (Fig. 3.2).

As shown in figure 3.3D, there was no seasonal variation in the dark respiration of *U. capensis*, but *U. rigida* showed variations, with the lowest values during spring and high values during autumn. There were significant differences in the dark respiration of these species ($p<0.05$) with higher values in *U. capensis*.

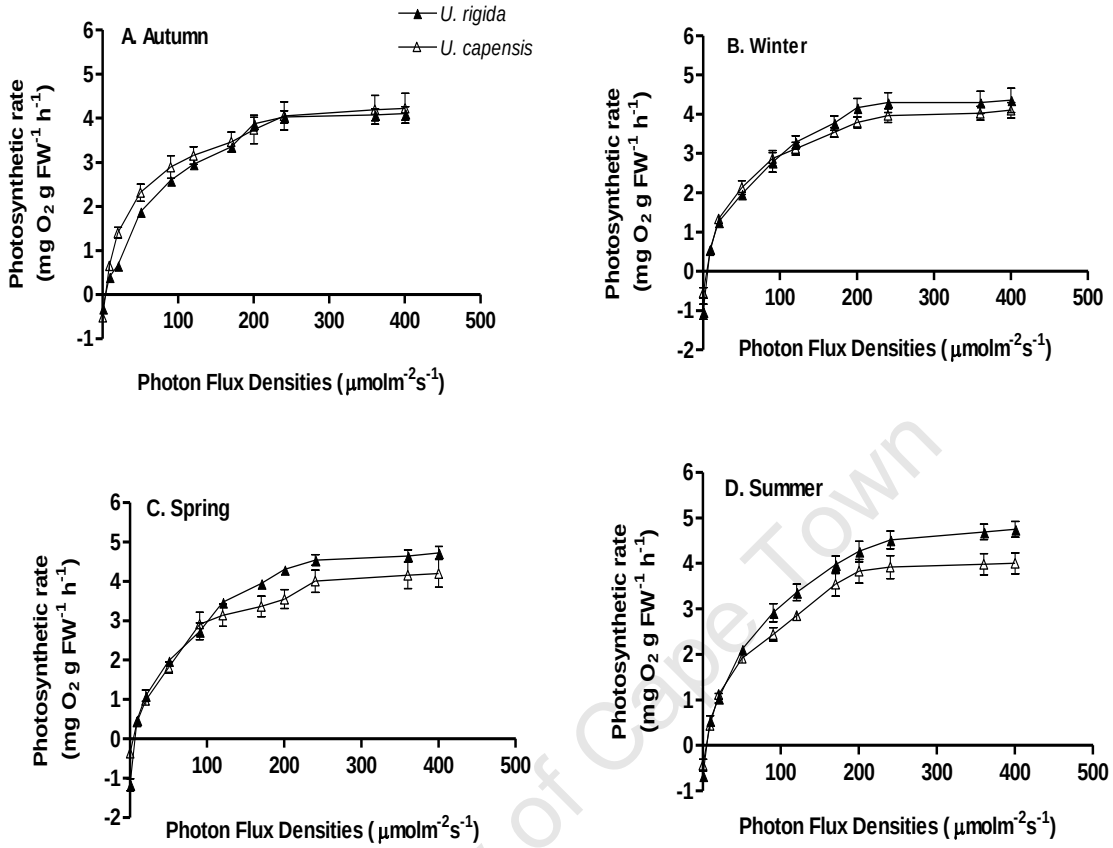


Figure 3.2: Photosynthesis-Irradiance curve for *U. rigida* and *U. capensis* during A: autumn; B: winter; C: spring and D: summer. *U. rigida* (solid triangles) and *U. capensis* (open triangles). For each season, data are expressed as the mean ± SE (n=9).

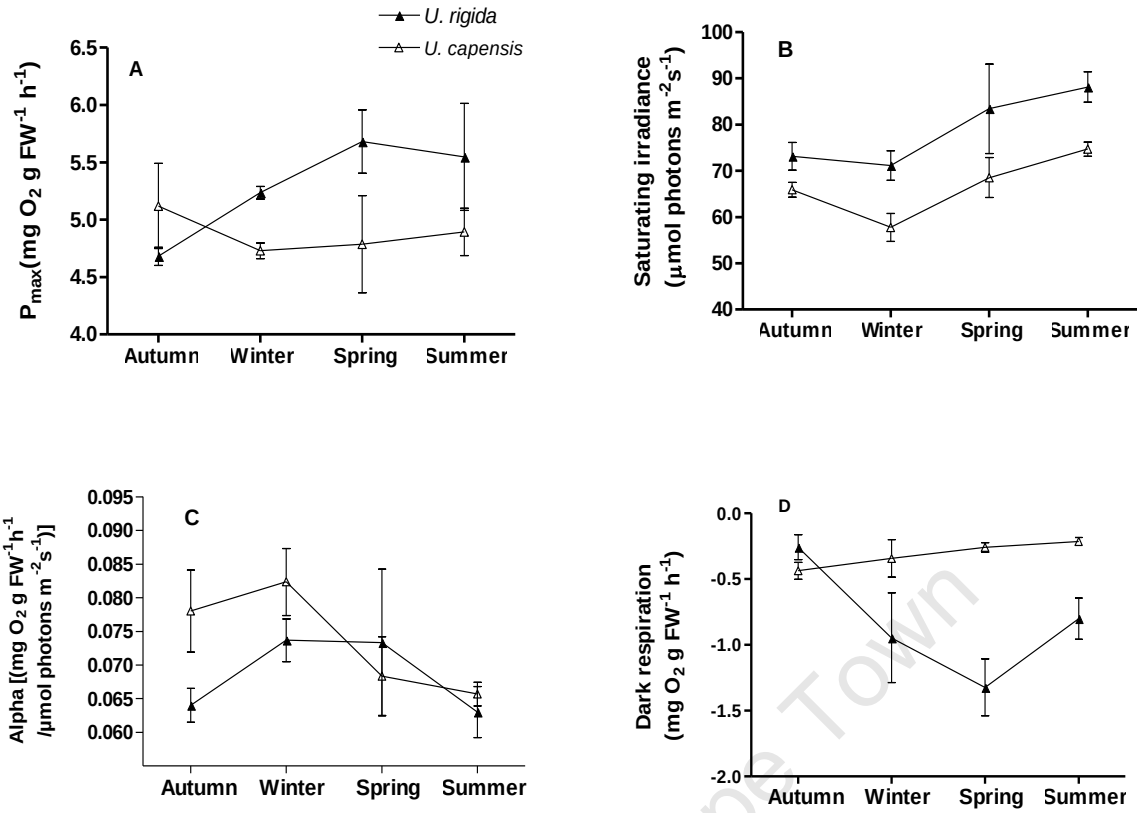


Figure 3.3: Seasonal variations of the parameters of photosynthesis-irradiance (P-I) curves; **A**: P_{max} (maximum photosynthetic rate); **B**: I_k (irradiance saturation); **C**: α (photosynthetic efficiency) and **D**: R_d (dark respiration rate). *U. rigida* (solid triangles) and *U. capensis* (open triangles). For each season, data are expressed as the mean ± SE (n=9).

3.4 Discussion

The two *Ulva* species (*U. rigida* and *U. capensis*) showed distinct seasonal variations in P-I curve with differences in P_{max}, I_k, α and R_d. As can be seen in Table 2.2, in both species light saturation of growth occurred at considerably lower irradiances (see Chapter 2) than light-saturation for photosynthesis and a similar pattern has been reported in other seaweed species.

Table 3.1 Light saturation (µmol photons $m^{-2}s^{-1}$) for photosynthesis and growth.

Species	Photosynthesis	Author	Growth	Author
U. rigida	240 - 400	This study	80	This study
U. capensis	240 - 400	This study	80	This study
U. lactuca	200	Yokohama, 1973	70	Fortes and Lüning, 1980
Laminaria saccharina	150	Lüning, 1979	70	Fortes and Lüning, 1980

Lüning (1980) concluded that the difference in saturating photon flux densities for photosynthesis and growth indicates that processes besides photosynthesis may limit growth and in this context it can be misleading to extrapolate optimum irradiances for growth from short-term measurements of photosynthesis. Furthermore, higher photosynthesis productivity in spring or summer and lower in winter has been reported in many seaweed species by several authors (Littler 1980; Levitt and Bolton, 1991; Henley, 1993; Vergara *et al*., 1997). The same trend was observed in the present study in both species. There were differences in the maximum photosynthetic rates between the species, with *U. rigida* having the highest values. This is possibly because *U. rigida* was found higher on the shore and on open rock, and showed greater net photosynthetic rates than *U. capensis* which is found in tidal pools. This is not surprising as a study by Zaneveld (1969) showed that upper shore algae, especially during spring tides, may lose sufficient water so as to arrest productivity, and so

they tend to have high rates of photosynthesis during periods when they are immersed as an adaption to their shorter daily opportunity for photosynthesis. *U. capensis* photosynthesis is saturated at a relatively low irradiance, probably because the plants occur in tidal pools. No photoinhibition was detected in either, probably due to the short incubation times and relatively low irradiances tested. Low nitrogen levels have been found to intensify photoinhibition in *U. rotundata* (Henley and Ramus, 1989), but the short duration of the experiments would reduce such likelihood here.

Saturating irradiances (I_k) showed a seasonal pattern in both species and I_k was reduced during winter and increased in spring and summer. The average I_k values from this study was 78.9 µmol photons $m^{-2}s^{-1}$ for *U. rigida* and 68.0 µmol photons $m^{-2}s^{-}1$ for *U. capensis* which is somewhat lower than the range of 150-250 µmol photons $m^{-2}s^{-1}$ previously reported for upper- and mid-sublittoral species (Lobban and Harrison, 1994). For instance, Rosenberg and Ramus (1982) found *Ulva curvata* to have an I_k of 465 μmol $m^{-2}s^{-1}$; Beach *et al.* (1995) found values of 130-160 μmol $m^{-2}s^{-1}$ for *Ulva fasciata* and Han *et al.* (2003) found I_k to occur at about 131–165 μmol $m^{-2}s^{-1}$ for *U. pertusa*; Figueroa *et al*. (2003) found *U. olivascens* to have I_k values of 116.35 μmol $m^{-2}s^{-1}$ and the same author found subtidal *U. rotundata* I_k to occur at 23.72 μmol $m^{-2}s^{-1}$, which is much lower than values obtained from our study. It has been suggested that a low value of I_k usually indicates the inefficient use of high irradiance rather efficient use of low irradiance (Henley, 1993).

In both species, values of α varied with seasons and α was reduced during spring and summer suggesting a reduction in the efficiency of light harvesting and energy conversion. Therefore the patterns of α and I_k suggest that *U. rigida* and *U. capensis* maintained a relative constant

efficiency at low irradiances, but an increasingly efficient use of high irradiances during spring and summer.

Although the growth phases of these two species at Kommetjie have not yet been studied, from the photosynthesis experiments, a relationship between the growth phases and photosynthesis can be extrapolated from figure 3.3A. The P_{max} for *U. rigida* was lowest in autumn whereas for *U. capensis* it was lowest during winter and this could be their static growth phase. In addition P_{max} was low in *U. rigida* during winter, but higher than in autumn, indicating active growth. Furthermore, P_{max} was higher in spring and summer for *U. rigida* and higher in *U. capensis* during autumn possibly indicating their reproductive or thallus producing phases.

Assessment of the overall photosynthetic performance showed that these two species appear to differ significantly with respect to photosynthetic response to light.

Chapter 4

Productivity and species dominance of *Ulva* in outdoor culture raceways at Irvin & Johnson abalone farm

4.1 Introduction

In South Africa the first abalone farms were built in the early 1990s and since then the demand for fresh kelp fronds for feed has increased. South Africa is currently the largest producer of cultured abalone outside Asia (Troell *et al*., 2006), producing ca. 1,100 t of abalone in 2007 (Robertson-Andersson, 2007). Marine Growers, near Port Elizabeth, were the first to cultivate seaweed in tanks as feed for abalone, partly using the expertise of students who carried out research into land-based cultivation techniques. Later, Wild Coast Abalone set up a large scale seaweed system (in raceway ponds) using a system based on research conducted in Israel by Friedlander and Levy (1995). Subsequently, Irvin and Johnson (I&J) farm built a re-circulating system with seaweed raceway ponds in 2005 (Robertson-Andersson, 2003; Bolton *et al*., 2008). In recent years, many countries have embarked on seaweed cultivation due to the challenge of obtaining fresh feed for abalone. For instance; in Chile, there has been a strong demand for fresh seaweed, mainly *Macrocystis*, in order to feed the abalone and currently both the red abalone (*Haliotis rufescens*) and the Japanese abalone (*Haliotis discus hannai*) are being cultured (Machiavello *et al*., 2010). Vásquez *et al.* (2006) estimated that these farms require an average of 500 t fresh algae per month and this amount is continuously increasing. It has been roughly calculated that close to 1,000 t of abalone will be produced in Chile by 2010.

Currently, cultured seaweeds represent most of the world's seaweed production, which is about 10 million tons fresh weight (FW) worldwide (Lüning and Pang, 2003; Chopin and

Sawhney, 2009). Wild Coast Abalone at Haga Haga near East London has become world leaders in commercial land-based *Ulva* cultivation (Bolton *et al*., 2008). Over 1,000t fresh weight of *Ulva* was cultivated on South African abalone farms in 2007, primarily for feed, but in one case to allow partial re-circulation by nutrient removal (Bolton *et al*., 2008). Furthermore, in the Pacific, the red seaweed *Palmaria mollis* or Pacific dulse is used as both a bio-filter and feed in temperate, land-based marine aquaculture of abalone and finfish (Demetropoulos and Langdon, 2003).

In South Africa, a green macroalga, *Ulva* is successful utilized as both feed for abalone and bio-filter in land-based aquaculture operations. Research has shown that abalone aquaculture has a relatively low environmental impact compared to other forms of aquaculture because abalone are mainly fed on kelp or feeds with relatively low fishmeal contents (Troell *et al*., 2006). However, although abalone farm effluent generally has a relatively low dissolved nutrient concentration; studies have shown that release of nutrient particulate loading is significant and could have negative effects on the local marine environment (Brandt, 2006). In Israel, macroalgae are used for nutrient removal: waste water from sea bream ponds is treated with *Ulva* (Cohen and Neori, 1991), demonstrating that it is biologically, technically and economically feasible (Chopin *et al.* 2001, Troell *et al.,* 2003, Neori *et al.,* 2004). *Ulva spp*. are efficient bio-filters and are able to remove up to 90% of dissolved nitrogen from aquaculture effluent (Neori *et al*., 1998). A local study by Robertson-Andersson (2003) showed that *Ulva* has high nutrient uptake rates, removing 90 % of ammonium during the day and 80 % of the ammonium during the night from abalone water. These results were similar to findings by Goldberg *et al.* (1998), who found *Ulva* to remove about 90 % of ammonium from fish effluent in Israel.

As a feed, farm-grown *Ulva* has been found to be rich in protein and to provide for good growth of *Haliotis tuberculata* (Neori *et al*., 1998; Shpigel *et al*., 1999), *Haliotis discus hannai* (Shpigel *et al*., 1999) and *Haliotis roei* (Boarder and Shpigel, 2001) and to improve the growth of *Haliotis midae* in conjuction with other seaweed and artificial feed (Dlaza, 2006; Naidoo *et al*., 2006; Robertson-Anderson, 2007).

The current study was conducted at Irvin & Johnson (I & J) Abalone Farm which is a land-based intensive mariculture operation that cultivates primarily abalone (*Haliotis midae*). The farm also grows seaweed (mainly *Ulva* and some *Gracilaria*); and has a fish hatchery, with pilot commercial culture of Kob (*Argyrosomus japonicus*). Previous research on *Ulva* cultivation at I & J farm showed promising results for commercial venture (Robertson-Andersson, 2003; 2007, Bolton *et al*., 2008) and *in situ* cultivation of seaweeds for abalone feed is carried out on a small number of abalone farms in South Africa. This chapter focuses on *Ulva* productivity at I & J Farm, where the investigated system is a semi-closed seaweed integrated abalone farm comprising a platform with tanks of abalone, connected to four seaweed raceway ponds. At present at least three different species of *Ulva* have been grown commercially on South African abalone farms (Lineekela Kandjengo, pers. comm.) but only two (*U. rigida* and *U. lactuca*) have been recorded at I & J farm. Previous records of *U. capensis* (Bolton *et al*., 2008) were a misidentification of *U. rigida*. Extrapolating from experiments conducted at I & J farm (Robertson-Andersson, 2007), an *Ulva* production target of 2.5 t per raceway pond per month in summer and 1.5 t per raceway pond per month in winter was proposed (Bolton *et al*., 2008). This system has been running since January 2006, and production data per raceway per month were recorded up to date. Bolton *et al.* (2008) included production data for 2007 and the production has been increasing, and is approaching proposed target production. The average monthly yield per raceway throughout 2007 was 1,419 kg, although yields fell below target particularly in the early months of the year

(January–March) and this problem is associated with an epiphyte growth on the *Ulva*. This chapter will focus on investigating the actual *Ulva* production in the raceways, relative to production targets from 2008 -2009, to update data of Bolton *et al.* (2008).

It had been noted that the mixture of *Ulva* species used in the raceways changes its relative proportions seasonally: data for 2007 showed that *U. lactuca* was more abundant from July - late November, and *U. rigida* from December – April (Bolton *et al*., 2008). Due to this change in species dominance, ecophysiological properties of these species were investigated in Chapter 2.

A SWOT analysis by Bolton *et al.* (2008) showed that commercial cultivation of *Ulva* for abalone feed has been productively carried out in South Africa. However, the activity is relatively new, and further research on the taxonomy and ecology of *Ulva* is required to improve understanding of the biology and cultivation ecology of this lucrative crop. Although much research has been done on growth rates of seaweeds in integrated aquaculture systems, (see: Neori *et al*., 1996; 1998, Msuya and Neori, 2002; Schuenhoff *et al*., 2002; Robertson-Andersson, 2003; 2007), most of these studies are based on experimental small and pilot-scale systems and the management constraints experienced on a commercial farming scale are invariably different. Therefore this study was designed to:

(i) investigate actual *Ulva* production in the raceways, relative to production targets, over 2 years.

(ii) determine if species dominance changes in the raceway ponds persist.

(iii) investigate seasonal differences in physico-chemical variables in the raceways (light intensity, temperature, dissolved oxygen and pH) and their influence on the SGR of the *Ulva* species, and try to explain any changes in species dominance.

4.2 Materials and Methods

4.2.1 System description

The system is a semi-closed seaweed integrated abalone farm where a platform with tanks of abalone is connected to four seaweed raceway ponds. The nutrient-rich effluent water from the abalone tanks passes through the seaweed raceway ponds where part of the nutrients are absorbed by the *Ulva*. The cultivation system consists of a pump house, a header tank where pumped seawater is mixed with the re-circulated water from the recirculation dam, a platform with 128 tanks of abalone, a drum filter, five seaweed raceway ponds (four in which *Ulva* was cultivated and one that was out of production), and a recirculation dam (Fig 1). The abalone platform is connected to the seaweed raceway ponds through a channel, and the four *Ulva* raceway ponds receive abalone waste water from the 120 tonnes abalone platform through a drum filter where 50% of water from the *Ulva* raceway ponds is re-circulated back to the abalone farm unit (Nick Loubser, pers. comm.). The water exchange rate is about 13.3 volume exchanges per day (Deborah Robertson-Andersson, pers. comm.).

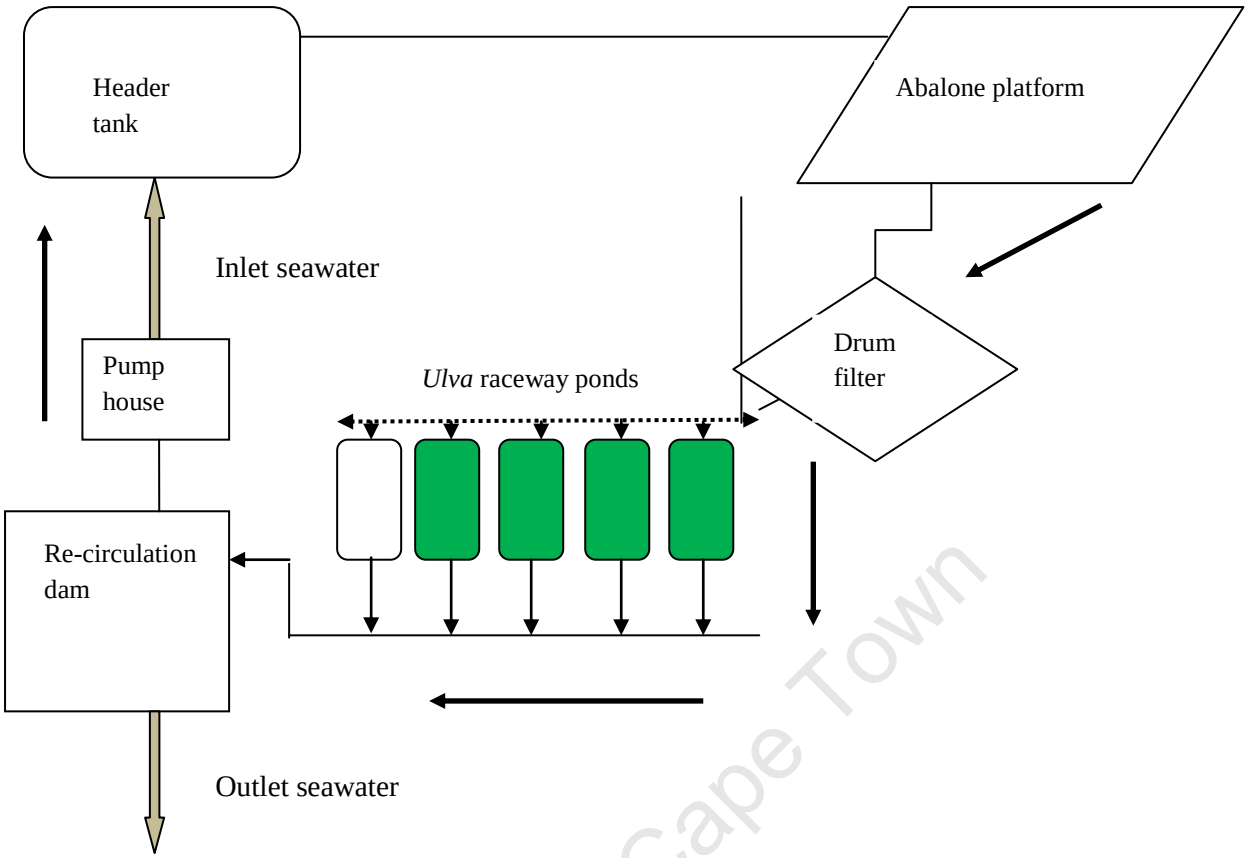


Figure 4.1: The integrated aquaculture system at I & J farm.

4.2.2 *Ulva* raceway ponds

Table 4.1 shows the dimension of raceway ponds at I & J farm, and each raceway pond shares a paddle wheel with the raceway pond next to it. The rotating speed of the paddle wheels is nine turns per minute (Lize Schoonbee, pers. comm.). The wheels keep the water in motion and break the *Ulva* into pieces that can move around and absorb light efficiently. The raceway ponds are lined with a high density (750 micron) polyethylene liner, to facilitate easy cleaning and maximize light reflection (Lize Schoonbee, pers. comm.). Once a week, each raceway pond is fertilized with 1.5 kg of ammonium sulphate and"Supergrow"® (P: 203g/kg, S: 23,6g/kg, Ca: 171, 4g/kg) in a ratio of 6:1 (Luvuyo September, pers. comm.). The

fertilizer is added in the late afternoon after disconnecting the raceway pond from the rest of the system by closing the inlet- and outlet sluice gates. The fertilization of all raceway ponds took place every Tuesday and the amount of nutrients added was the same for all raceway ponds, and thus independent of seaweed density in the raceway ponds. Each month, harvesting took place on a Monday, the day before fertilization. The harvested seaweed was fed to young abalone (12mm- 40mm; 1.5g – 30g) at the farm (Lize Schoonbee, pers. comm.).

As a common practice at the farm, all the raceway ponds were covered with an 80 % shade cloth from September to March to prevent high light intensity as well as *Myrionema* epiphyte disease outbreaks (Lawrence Ansara, pers. comm.).

Area (m^2)	282.62
Volume (m^3)	110.22
Stocking weight (kg)	500.00
Stocking density (kg/m^2)	1.8
Stock/volume (kg/m^3)	4.54
Average depth of the raceway pond (m)	0.39

Table 4.1: I & J raceway pond dimensions and stocking values.

4.2.3 *Ulva p*roduction

Production of *Ulva* was examined from January 2008 to December 2009 and *Ulva* material was harvested by farm employees from the raceway ponds at the end of every month; each raceway has an underground drain pipe which surfaces from the raceways on the sea side. During harvest, the pipe gate is opened to drain out the *Ulva* from the raceway ponds and

Ulva is harvested in perforated baskets to allow the water to drain through. The *Ulva* harvested from each raceway pond was weighed and the weight was estimated as the number of baskets of an assumed standard weight. Following weighing, the raceway ponds were immediately reseeded with 500 kg of the *Ulva.*

Specific growth rates were calculated from the monthly wet weight values using the following formula: $SGR = 100[\ln(W_t) - \ln(W_i)]/t$

where W_t=final weight (kg wet weight); W_i= initial weight (kg wet weight) and t= time (days)

4.2.4 Proportion of *U. lactuca* and *U. rigida* in each raceway pond

Proportions of each species from four raceway ponds were investigated during 2008 (from January 2008 to November 2008) and 2009 (from February 2009 to August 2009). Random *Ulva* samples (approximately 200 g each) were sampled from each raceway pond every two weeks and a total of three samples were taken from each raceway. The data for each species are presented on a monthly basis (n= 4 raceway ponds x 3 samples x every 2 weeks x 1 month). The samples were delivered to the laboratory, cleaned of epiphytes and sorted out according to species. By hand section and microscopic observation, anatomical characteristics were determined using diagnostic characters in Stegenga *et al.* (1997), and thallus colour was also noted using the Methuen Handbook of Colour (Konerup and Wanscher, revised by Pavey, 1978)). Throughout September to March, the raceway ponds were covered with 80% shade cloth to reduce the light intensity to approximately 207.9 ± 70.6 µmol $m^{-2}s^{-1}$and possible *Myrionema* infection (see Chapter 5).

4.2.5 Physico-chemical measurements

In situ measurements of physico-chemical variables in *Ulva* raceways were carried out from January 2008 – December 2009. Luminous intensity, temperature, dissolved oxygen and pH were recorded ever day for long term monitoring in the seaweed raceway ponds. Luminous intensity was measured using a HOBOware light meter which ran throughout the day and night and information was downloaded every week. During September to March, the raceway ponds were covered with a shade cloth and light measurements were taken under the shade cloth.

Dissolved oxygen and pH was measured twice daily, before 10:00 am and after 15:00 pm, using an Oxyguard dissolved oxygen and pH meter, and for each month there are 60 measurements averaged. Luminous intensity which is measured in lux does not measure photosynthetically available light, and so values were converted to light intensity and the conversion between lux and µmol photons m^{-2} s^{-1} is a wavelength dependent conversion and should be approximated only: e.g X µmol photons m^{-2} s^{-1} = lux x ~0.0165 or 1000 lux =16-20 µmol photons m^{-2} s^{-1} (Hershey, 1991).

4.2.6 Statistics

The analysis for this study was done using STATISTICA 8.0. One way ANOVA's were used to determine significant differences in SGR and physico-chemical parameters between the different seasons, and 2- way ANOVA's were used to determine significant differences in species dominance at different seasons. Correlation between species dominance and light intensity was determined using Pearson correlation.

4.3 Results

4.3.1 Annual physico-chemical parameters

As shown in figure 4.2, the light intensity did not show a clear trend. During January to March 2008, the raceway ponds were still covered (and had been since September 2007) and the light intensity ranged from 133.9 -348.9 µmol $m^{-2}s^{-1}$ with mean values of 207.9 ± 70.6 µmol $m^{-2}s^{-1}$. Interestingly, during September to December 2008, the light intensity was higher, ranging from 338.9 -505.3 µmol $m^{-2}s^{-1}$ with a mean value of 384.1± 81.0 µmol $m^{-2}s^{-1}$ despite covering of raceway ponds with a shade cloth. Furthermore, in January to March 2009, (raceway ponds still covered since September 2008) the light intensity was twice as high as in the same period during 2008, with values ranging from 227.9 – 536.0 µmol $m^{-2}s^{-1}$ with a mean value of 419.8 ± 96.6 µmol $m^{-2}s^{-1}$. In addition, during September to December 2009, the light levels were significantly lower with values ranging from 21.6 – 187.3 µmol $m^{-2}s^{-1}$ with a mean value of 78.8 ± 38.1 µmol $m^{-2}s^{-1}$. The light was measured under the shade cloth and the light was higher in the summer than winter, even though they were shaded in the summer.

Water temperatures (Fig. 4.3) showed a seasonal trend during 2008 and 2009 with temperature increasing from spring to summer and the overall range of temperatures was 10.6 – 23.8 ºC. There was no statistical difference between the average winter temperatures recorded in 2008 (14.3 ± 0.5 ºC) and 2009 (14.4 ± 0.3 ºC); between summer temperatures recorded in 2008 (17.3 ± 0.4 ºC) and 2009 (17.9 ± 0.7 ºC); between spring temperatures recorded in 2008 (14.4 ± 0.9 ºC) and 2009 (15.3 ± 0.5 ºC) and between autumn temperature recorded in 2008 (16.3 ± 0.1 ºC) and 2009 (16.4 ± 0.1 ºC). There were significant difference between winter, spring temperatures and summer, autumn temperatures. However, there were no significant differences between winter and spring temperatures.

Mean dissolved oxygen of water in *Ulva* raceways showed no significant seasonal variation (9.4 – 10.6 mg/l) with mean values of 9.9 ± 0.3 mg/l in 2008 and 8.8 ± 0.2 mg/l in 2009. The range was slightly higher in summer and autumn; however there was no clear seasonal pattern (Fig. 4.4).

Figure 4.5 shows the monthly pH values in *Ulva* raceway ponds during 2008 and 2009. There was no seasonal trend in the pH values and there was no statistical difference between pH values of 2008 and 2009 ($p>0.05$) and the pH mean values were 7.8 ±0.1 in 2008 and 7.5 ± 0.1 in 2009.

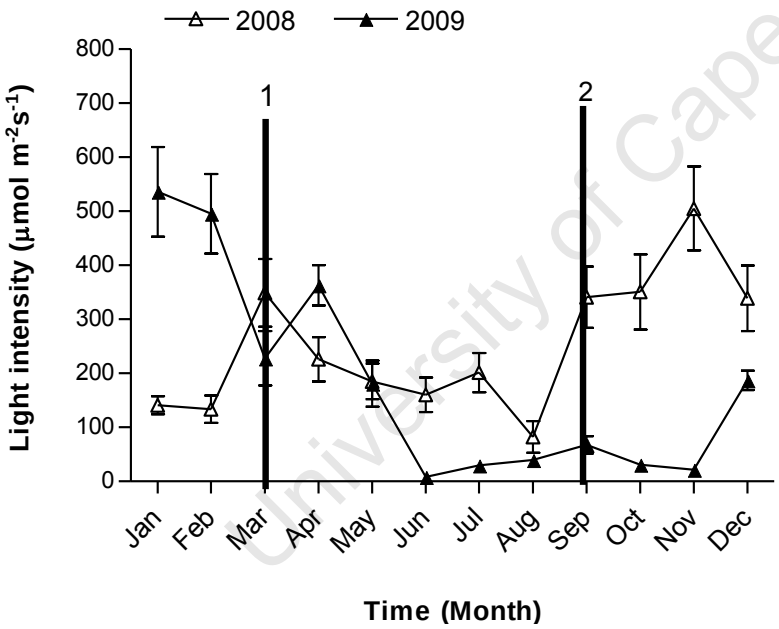


Figure 4.2: Monthly average light intensity of the raceway ponds for 2008 and 2009 at I & J farm. From the left, Bar 1 shows where shading ended and Bar 2 shows where shading started.

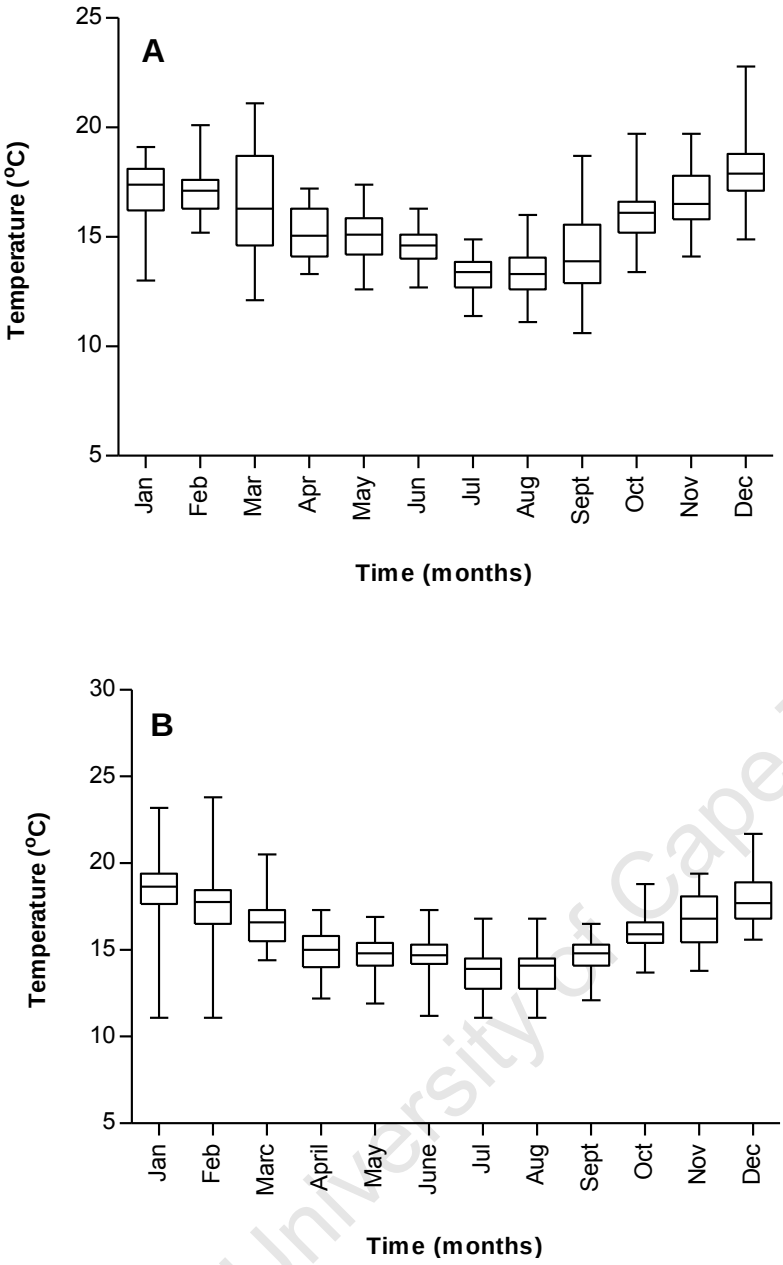


Figure 4.3: Monthly average water temperature of the raceway ponds for A. 2008 and B. 2009 at I & J farm. The vertical bar show maximum and minimum values, while the box shows the standard deviation from the mean (n = 60).

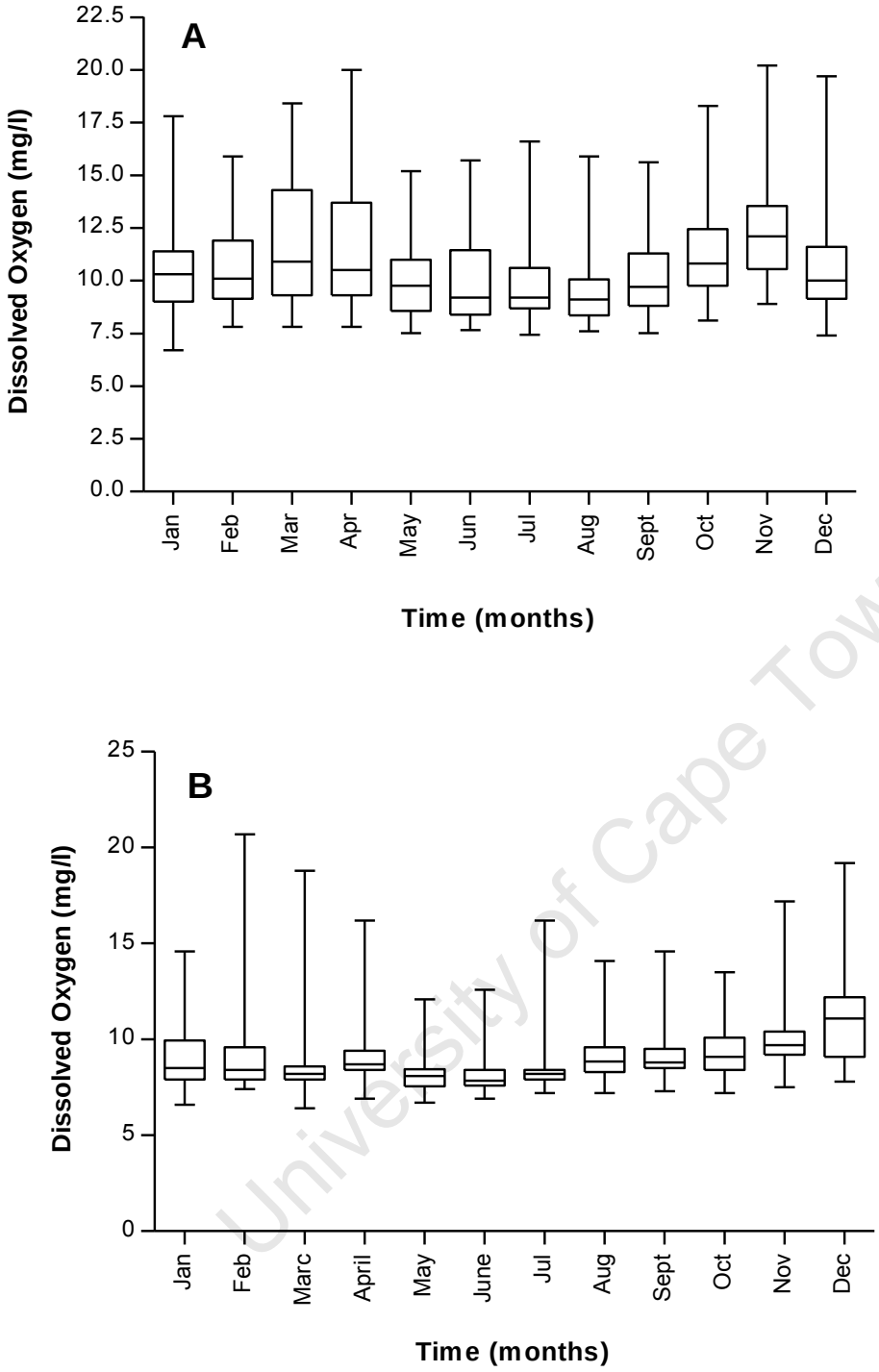


Figure 4.4: Monthly average dissolved oxygen in the raceway ponds water for A. 2008 and B. 2009 at I & J farm. The vertical bars show give maximum and minimum values, while the box shows the standard deviation from the mean (n = 60).

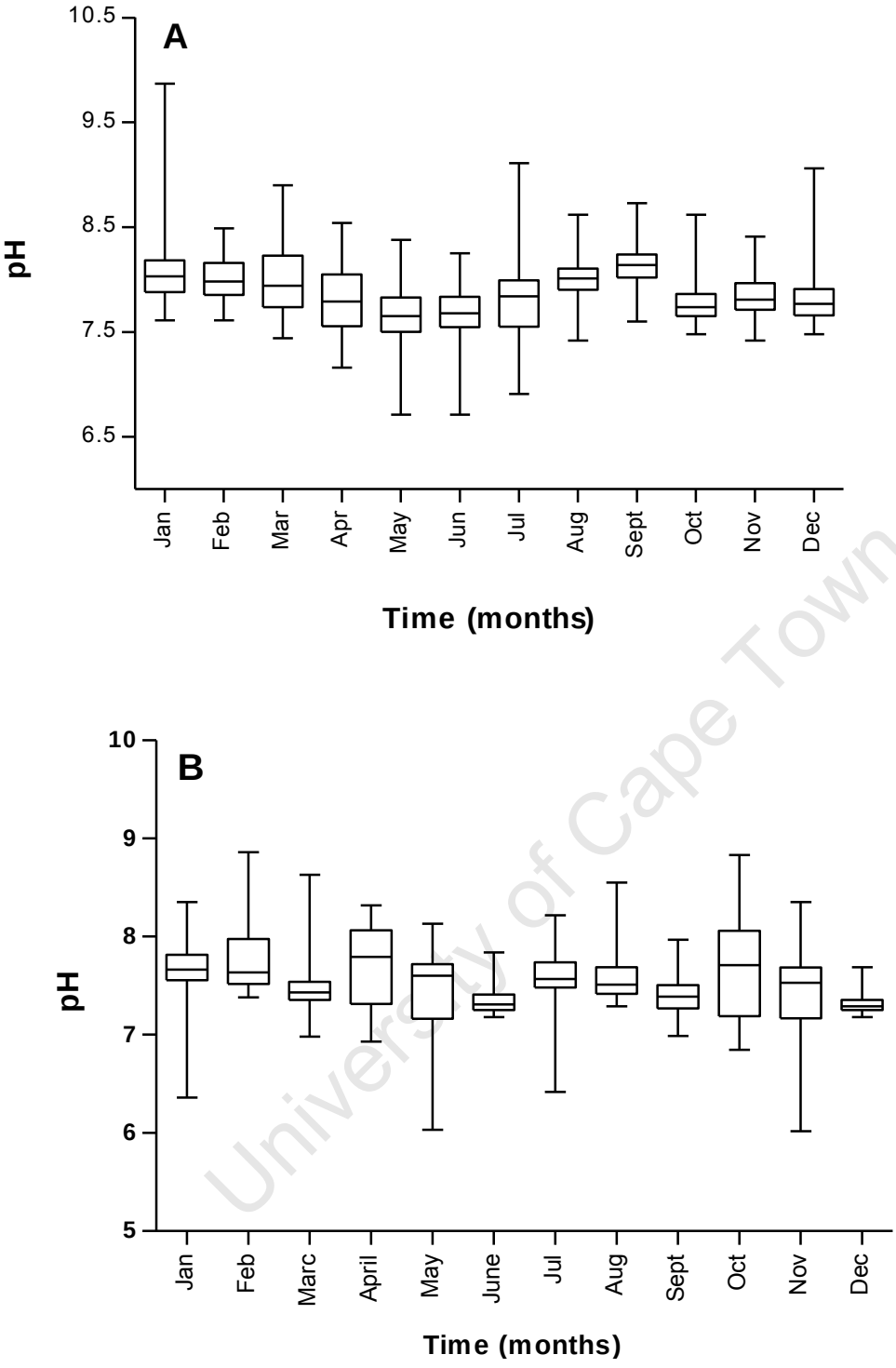


Figure 4.5: Monthly average water pH in the raceway ponds for A. 2008 and B. 2009 at I & J farm. The vertical bars show maximum and minimum values, while the box shows the standard deviation from the mean (n = 60).

4.3.2 *Ulva* production

In January 2008, raceway 1 and 2 were undergoing re-construction therefore harvest in January was missed (Fig 4.6A). In July 2008, raceways 1 and 4 overflowed resulting in the loss of some *Ulva* and as a consequence harvest was missed from raceway 4 and raceway 1 had a low production. Therefore, it was decided that these two raceway ponds should be restocked with 250 kg which is 50% of their usual stocking density and this led to a low harvest for August 2008 compared to other raceways. During September 2008, the raceways were covered with a shade cloth to lower the light intensity and *Myrionema* on *Ulva*. There was no harvest from raceway 2 during October 2008 as the raceway was destroyed by a storm.

Furthermore, as shown in figure 4.6B, during June 2009 there was no harvest from raceway ponds 1 and 3 and again no harvest from raceway 3 during July 2009, since the farm was conducting maintenance work. In August 2009 *Ulva* from raceway pond 1 was harvested earlier than the normal harvesting time and this led to low production. In addition, as shown in Fig 4.6 A and B, *Ulva* production fell below target particularly in the early months of the year (January–March). In the past this problem has been associated with an epiphyte, *Myrionema* on the *Ulva*, however this problem has been effectively managed since 2008. Moreover, during 2008 *Ulva* production was still below the winter and summer proposed target, but during 2009 the proposed target for winter was approached but yield was still below the target proposed for summer.

The average production values per 4 working raceway per month from January 2008 until December 2009 are shown in Figure 4.7. The production has been increasing and the average

monthly production per 4 working raceway throughout 2008 was 1567 kg and for 2009 was 1728 kg (Fig. 4.7).

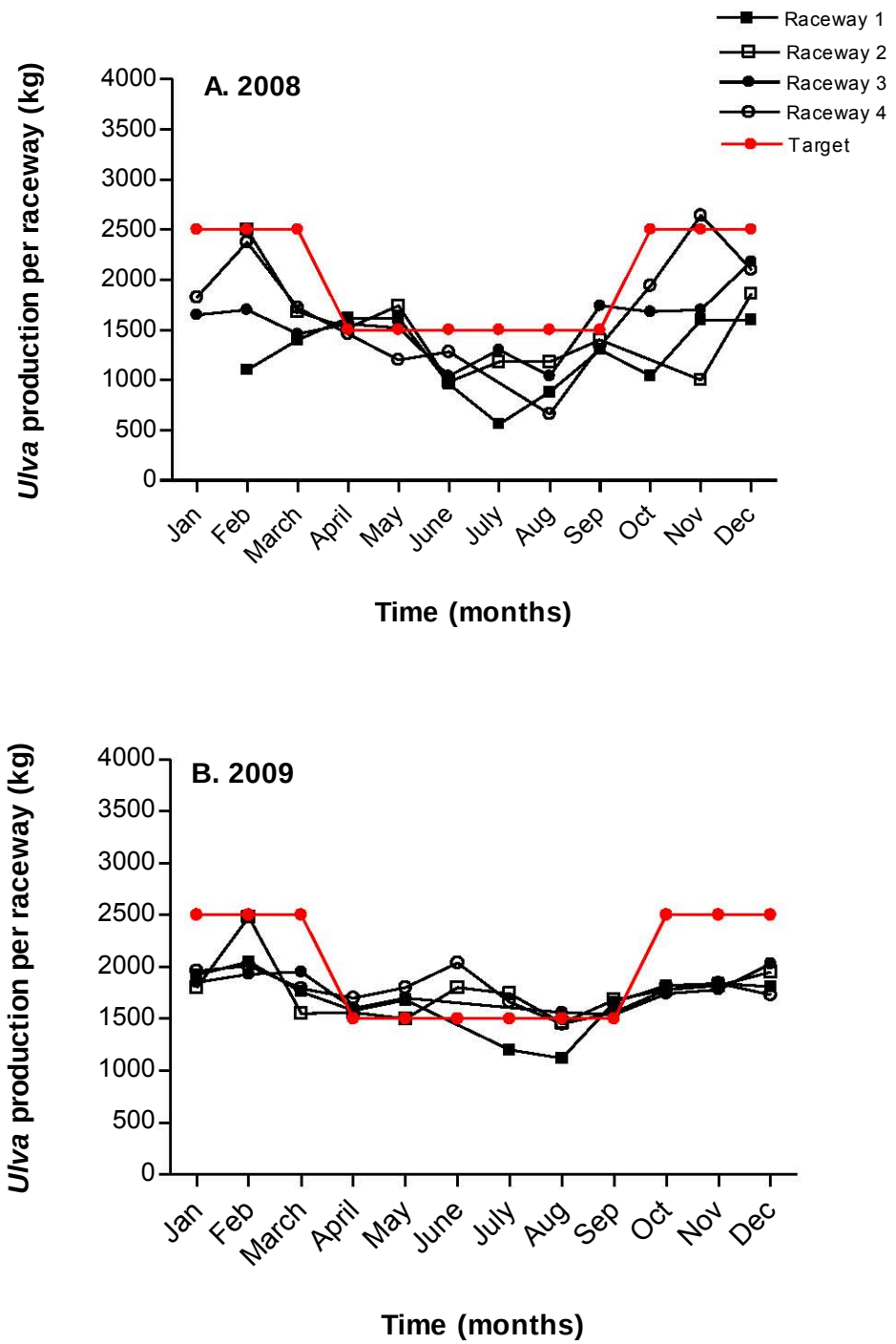


Figure 4.6: Monthly production of *Ulva* per working raceway pond at I & J Abalone Farm during A. 2008 and B. 2009. The graph includes the initial proposed target production by Robertson-Andersson (2007).

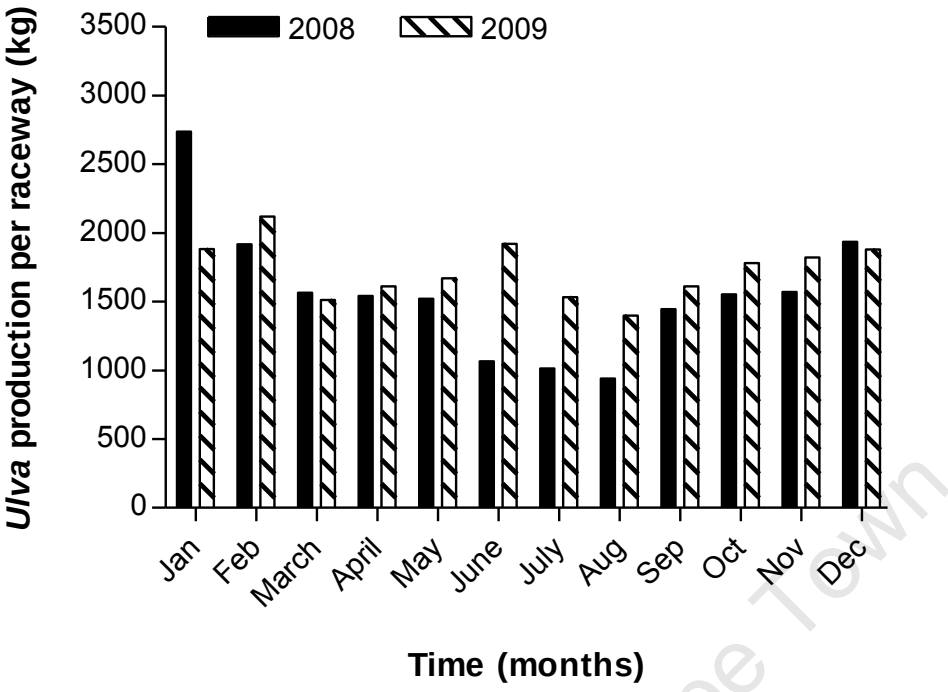


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Figure 4.7: Monthly average production of *Ulva* per working raceway ponds at I & J Abalone Farm from January 2008 to December 2009.

Seasonal growth data for *Ulva* per working raceway pond were expressed as percentage growth per day calculated on a wet weight basis (Table 4.2). *Ulva* growth rates were different in each raceway due to different culture conditions and the maintenance status of the raceway. In 2008, there were problems with re-construction and water overflow in some raceways and this affected the growth patterns at different seasons. A more consistent pattern of growth was observed in 2009.

As shown in Table 4.3, there was a significant statistical difference in the overall growth rate of *Ulva* at different seasons during 2008 and 2009 ($p<0.05$). In 2008, the best growth was obtained during summer and autumn with low growth during winter. Conversely, during 2009, the best growth was recorded during spring and summer, and there were no difference

in growth during autumn and winter. Furthermore, winter of 2009 had similar growth rates to those of spring of 2008.

Table 4.2. Average SGR (% wwt.day^{-1}) of *Ulva* production per working raceway pond at I & J farm over four seasons with Standard Errors in brackets.

Year	Spring	Summer	Autumn	Winter
2008				
Raceway 1	2.50(±0.38)	3.90 (±0.00)	3.40(±1.50)	1.40(±1.27)
Raceway 2	3.15(±0.28)	2.23 (±1.26)	4.50(±0.63)	3.10 (±0.56)
Raceway 3	3.55(±0.55)	4.32 (±0.29)	3.91 (±0.24)	3.11 (±0.37)
Raceway 4	2.91(±1.05)	4.88 (±0.36)	4.42 (±0.59)	3.03 (±0.11)
2009				
Raceway 1	3.66(±0.50)	3.47 (±0.51)	3.00(±1.50)	3.48(±0.56)
Raceway 2	3.94(±0.20)	3.47 (±0.41)	3.04(±0.61)	3.26 (±0.67)
Raceway 3	3.90(±0.13)	3.52 (±0.44)	3.51 (±0.68)	2.34 (±1.74)
Raceway 4	3.86(±0.20)	3.44 (±0.56)	3.53 (±0.72)	3.55 (±0.59)

Table 4.3. Average SGR (% wwt.day^{-1}) of *Ulva* at I & J farm with Standard Errors in brackets, obtained in each year for each season.

Year	Spring	Summer	Autumn	Winter
2008	3.02 (±0.57)	3.83 (±0.93)	4.05 (±0.45)	2.65 (±0.58)
2009	3.84 (±0.26)	3.48 (±0.48)	3.27 (±0.88)	3.16 (±0.88)

4.3.3 Species proportions in the raceways

As shown in Fig. 4.8, there was always more *U. rigida* in the culture raceways, except in September 2008 when proportions of the two species were equal, and in October and November 2008 when there was more *U. lactuca*. The two years have different patterns, and the only overall pattern is that the culture system is dominated by *U. rigida* almost throughout. Analysis showed (Fig. 4.9) that there was a strong correlation between the light intensity and proportion of *U. lactuca* in the raceways during the entire period of 2008 and 2009 (r^2=0.73). *U. rigida* predominated throughout the year and it is likely that if the raceway ponds were not shaded during summer, *U. lactuca* could also predominate during that season.

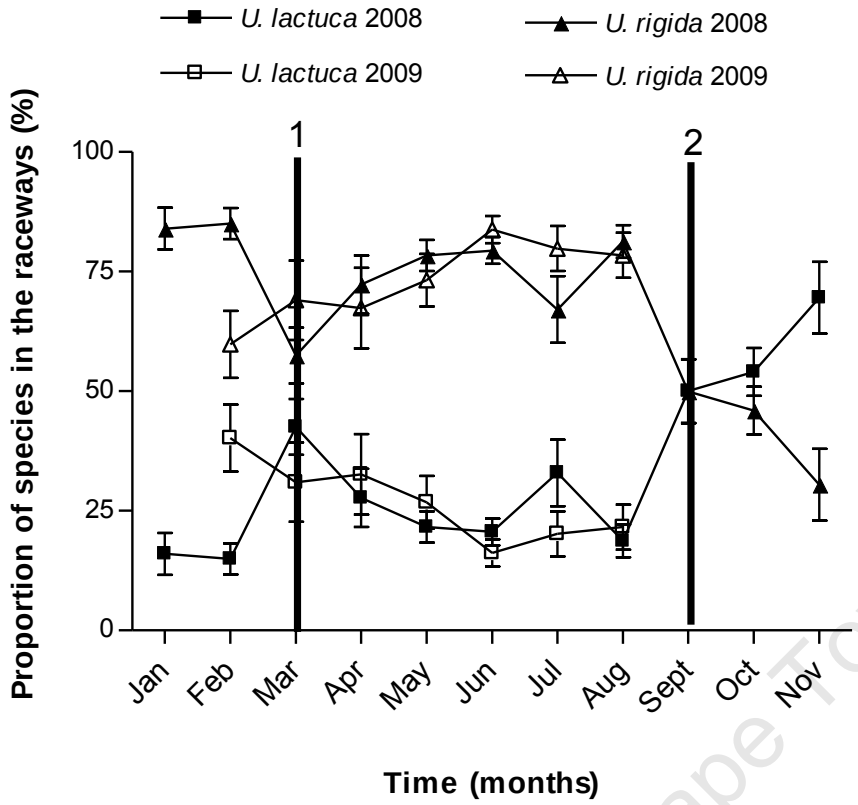

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Figure 4.8: Proportion of *U. lactuca* and *U. rigida* in the culture raceways during Jan 2008 to November 2008 and February 2009 to August 2009. From the left Bar 1 shows where shading ended and Bar 2 shows where shading started. Bars denote Standard Error, (n=48).

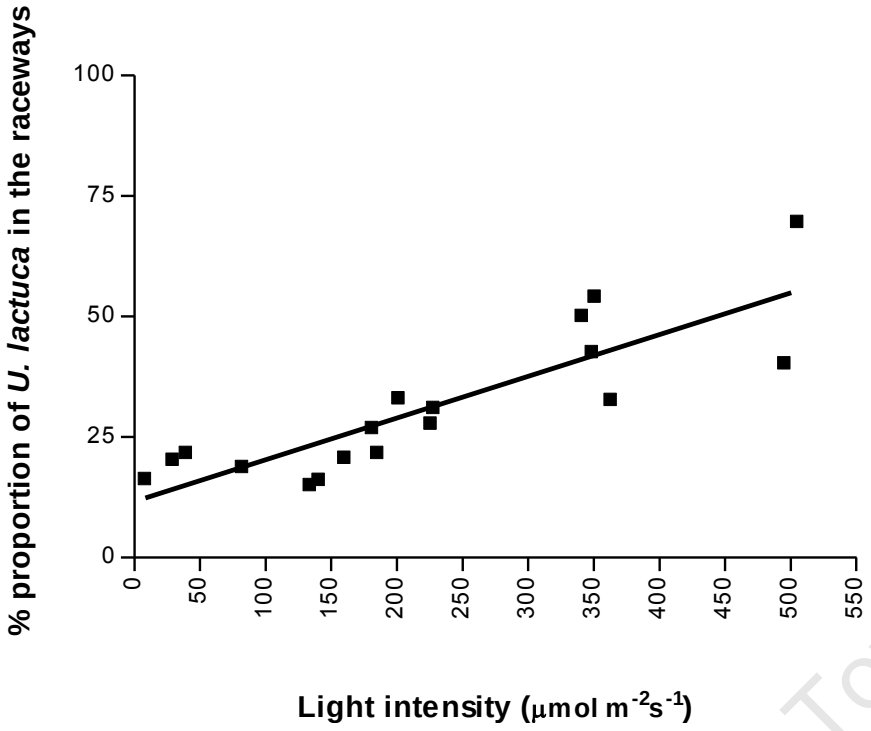

Figure 4.9: Correlation between percentage by weight of *U. lactuca* in the raceways and average monthly light intensity during January 2008 to November 2008 and February 2009 to August 2009 (n=11 for 2008 and n=7 for 2009).

4.4 Discussion

Measurements of *Ulva* yield each season are very important for farm management. The current study has contributed information on *Ulva* production throughout the year and conducting this study over 2 years was necessary to observe seasonal changes in the system performance in terms of *Ulva* production. The average monthly yield per raceway throughout 2006 was 1,877 kg and for 2007 was 1,419 kg and *Ulva* production was below the proposed target production for winter and summer (Bolton *et al*., 2008). Comparing our data to those obtained by Bolton *et al.* (2008), it's clear that the average monthly yield per raceway throughout 2006 – 2009 didn't increase significantly. However, from the present results it is evident that *Ulva* production has approached targets proposed for winter but still

is below the proposed target production for summer and this raised a question whether the target for summer was realistic. A more realistic target should be based on real production per raceway. From the data in this chapter, a realistic target for summer for 2008 would have been as follows:

- Stocking density = 500 kg raceway $^{-1}$
- Summer SGR =3.83 % wwt.day^{-1} of *Ulva* raceway $^{-1}$
- Summer target = 1.9 t per month

Similarly, summer target for 2009 would have been 1.7 t per month. The set targets will remain the same as they can be reachable with further modifications of light and/or nutrients in the system. .

As can be seen in Bolton *et al.* (2008), in years prior to this study it was noted that *Ulva* health can vary considerably throughout the year and during spring/summer there were outbreaks of an epiphyte infection which can cause severe damage to *Ulva* and reduce the production significantly. Covering of raceway ponds with shade cloth during spring/summer has been a farm management tool to prevent or reduce epiphyte infection. In 2009, the proposed target productions for winter were achieved possibly because they were realistic or there were more favourable growth conditions in the raceway system during the winter period.

Monitoring for pH, temperature and dissolved oxygen in seaweed raceways is an important part of farm management. This is because pH is an indicator of CO_2 levels in the water and pH greater than 9 is known to be unfavourable for seaweed growth (DeBusk *et al.,* 1986). A study by Lapointe (1981) and DeBusk *et al.* (1986) found prolonged periods of high pH to decrease growth rates and pigment content of seaweed. In a recirculation system, pH and

temperature are two of the most important variables. Experiments by Boyd (1998) showed that pH and temperature determine the amount of ammonia that is in the non-ionic (toxic) form. The ratio between ionised ammonium (non-toxic) and non-ionized (toxic) ammonia can change rapidly depending on the pH. During this study period pH remained well below 9 which indicated that *Ulva* was growing under favourable pH conditions.

It is also important to note that the range of mean dissolved oxygen obtained from this study is above critical levels for abalone respiration at night (6mg/l) (Lyon, 1995), indicating that a key requirement of the integrated abalone/seaweed system is met.

The mean temperatures recorded in the raceways during the study period (14.3 ^{o}C to 17.9 ^{o}C) are within the ranges that promote the best growth for *U. lactuca* and *U. rigida* (see Chapter 2). Temperatures of 5 ^{o}C prevent growth in these species and when temperature was increased to 30 ^{o}C, death occurs. In addition, the mean abalone effluent water temperatures in which *Ulva* is grown fell within the optimum temperature ranges for maximal growth rates of these species and other temperate *Ulva* species.

In order to achieve the proposed target production, water flow rates should be kept high to increase the amounts of available nutrients. DeBusk *et al.* (1986) conducted experiments on the effect of seawater exchange rates on the SGR of several species of *Ulva* and the results indicated that higher production rates were obtained in systems that have high water exchange rates (20 volumes d^{-1}) compared to systems that had low water exchange rates (4 volumes d^{-1}). They concluded that reduced carbon availability was the limiting factor in *U. lactuca* growth at low water exchanges. Similarly, Parker (1981) found that ammonium uptake and growth rates increased with increasing water velocity, due to the rapid

replacement of nutrients in the culture system, as nutrient-depleted seawater is constantly renewed. In the current system, low productions of *Ulva* were recorded in raceways that had low water flow which was later resolved by making necessary repairs to the affected raceways. The physico-chemical parameters were in the optimal range for seaweed cultivation; however, adequate water velocity and turnover should be maintained at all times because it can negatively affect those parameters, and hence the growth rates of *Ulva.* Overall, the fine balance has to be found between water exchange rates, productivity and biomass.

The increased proportion of *U. lactuca* during months of high light intensities suggests that *U. lactuca* is better adapted to high light. This hypothesis is further supported by the findings in Chapter 2 that compared the SGR of *U. lactuca, U. rigida and U. capensis* grown under varying irradiances and showed that *U. lactuca* grew well at the highest irradiance tested. The present study shows that light intensity is one of the important environmental factors determining the proportion of *U. lactuca* in the raceways. Consequently, it can be thought that *U. lactuca* increases during periods of high light intensity irrespective of the season whilst *U. rigida* is predominant all year round. Then it can be said that *U. lactuca* dominated in summer unless the raceway pond was shaded. Similar findings were obtained by Bolton *et al.* (2008) from pilot commercial tank cultures on a west coast farm (Jacobsbaai Sea Products). The tanks were subjected to 80% shade cloth in the summer (December) and the *Ulva* composition changed rapidly from a dominance of *U. lactuca* to *U. capensis* (which was misidentified as *U. rigida*). On removal of the shade cloth, this dominance reversed. Thus in our present study it is likely that, if the raceways were not shaded, *U. lactuca* would have dominated.

In addition, according to Bolton *et al.* (2008) the raceway ponds at I & J farm were also shaded with 20% shade cloth which resulted in *U. lactuca* becoming infected with an epiphyte because the shading was too little and when 80% shade cloth was applied it resulted in *U. rigida* dominating in summer. During the present study 80% shade cloth was applied during summer which resulted in the dominance of *U. rigida* and it is likely that such heavy shading during summer potentially reduces total production because the plants are receiving insufficient light. Perhaps in the future less dense shade cloth may be used to ensure that *Ulva* is receiving sufficient light intensity.

It is noteworthy that the integrated *Ulva*/Abalone system at I & J farm produces healthy *Ulv*a year-round in substantial amounts. Therefore it can be concluded that *Ulva* production at I & J farm is successful and with the completion of this whole research project, data on the biology of *Ulva* species cultivated in integrated re-circulating systems on commercial abalone farms is available. This will allow better management of this lucrative feed crop in terms of yield and disease control. However it must be clear that there is a danger in extrapolating the findings on *Ulva* performance in the studied system at I & J farm to other different systems. Some integrated systems use bottom-aerated seaweed tanks or ponds to oxygenate and mix the seaweed and this causes vertical movement of the seaweed fronds (Neori *et al*., 2004), which may alter some of the conditions under which this study was done.

Chapter 5

Infection of farmed *Ulva* with the epiphyte *Myrionema strangulans*: dependence of recovery on dissolved nutrients in the culture medium and on irradiance

5.1 Introduction

The green algal genus *Ulva* is locally cultivated as a feed supplement and a bio-filter on some abalone farms in South Africa. However, despite this seaweed being a major source of abalone feed that is successfully used in integrated mariculture, there is still a problem that affects its productivity: infestation by an epiphyte, *Myrionema strangulans* (Phaeophyta) on one of South Africa's *Ulva* farms (Bolton *et al*., 2008). Competition between hosts and their seaweed epiphytes has been demonstrated under natural and artificial conditions of growth (Arrontes, 1990; Friedlander and Ben-Amotz, 1991; Svirski *et al*., 1993), and the extent of the damage is determined by the intensity of the infections (Cancino *et al*., 1987; Buschmann and Gomez, 1993). Epiphytes are usually defined as organisms that grow on plants, but do not derive nutrients from their hosts (Linskens, 1976). According to Linskens (1976), holo-epiphytes are those attached to the outer layers of the host, whereas amphiepiphytes (sometimes called endophytes) are deeply anchored in the tissues of their hosts. The damage caused by an epiphyte to its basiphyte can be highly variable, and is mainly influenced by the type of anatomical association and the extent of the epiphyte (Fletcher, 1995).

As long ago as 1977, Ryther stated that epiphytic growth is probably the single greatest problem and constraint on commercial seaweed culture. Wheeler *et al.* (1981) considered that the control of epiphytes and provision of nutrients are two major problems for the macroalgal farmer. Epiphytism is a problem since, if excessive, it will deprive the seaweed of light and

will also decrease the amount of available nutrients. Epiphytes can lower growth rates and the quality of the product (Collen *et al*., 1995).

In South Africa, the first record of the epiphyte species *Myrionema strangulans* was made when the epiphyte was observed growing on experimental cultures of *Ulva* at I & J farm in October 2001 by Robertson-Anderson (2003), who described four stages of infection. Infection of *M. strangulans* observed since 2001 has resulted in tremendously reduced biomass production of *Ulva* during spring. In South Africa, other than the report of Robertson-Andersson (2003) no other documentation of *M. strangulans* epiphytism is known in other abalone farms or in nature. This species grows widely in temperate seas, and can produce profuse growth on *Ulva* elsewhere (Kornmann and Sahling, 1983). According to Kornmann and Sahling (1983), *M. strangulans* can grow very quickly, producing fertile plants from spores in 11 days in the laboratory. The present study results from a call for assistance from a local farm to assess the occurrence of this epiphyte further and to determine environmental conditions which could prevent its occurrence. This information is important for farming operations to reduce the level of infection and to diminish the direct negative effects of the epiphytes on the host.

The current study was designed:

(i) To monitor *Myrionema strangulans* relative abundance and variability during the seasons when infection is higher.

(ii) To investigate the response of the epiphyte to changes in light and nitrogen concentrations. It was hypothesized that by late spring and summer *Ulva* is growing rapidly, thereby utilizing its internal nitrogen and becoming subject to sustained epiphyte attack.

(iii)To investigate the tissue nitrogen and carbon content of *Ulva* infected with the epiphyte.

5.2 Materials and Methods

5.2.1 Collecting site

Ulva material was collected from I & J Abalone farm every two weeks from 2nd October to 13th November 2007, but after this the cultures collapsed and the raceways were restocked with new material from the tank where 'parent' stock is kept. In order to determine the prevalence and severity of infection by *Myrionema strangulans*, ten *Ulva* thalli were collected from each raceway (there are 4 raceways). The thalli were collected from different parts of the raceway in order to avoid sampling at the same place. Prevalence (i.e. percentage of thalli in the farm that were infected) and severity of infection (i.e. mean abundance of epiphytes on each *Ulva* thallus) were then estimated. A Braun-Blanquet (B-B) percentage cover-abundance scale was used to estimate percentage cover of *M. strangulans* on individual thalli (Robertson-Andersson, 2003). The range of the B-B values given as percentage cover of epiphyte contamination on the thallus is as follows: 1 = no coverage; 2 =1 - 10 % coverage; 3 =11 - 25 % coverage; 4 =26 - 50 % coverage; and 5= > 50 % coverage

5.2.2 *Ulva* tissue nitrogen, carbon and crude protein content

Fresh *Ulva* material from the farm was dried in the oven at 60 ^{o}C for 72 h and dry material was ground using a mechanical grinder with a maximum mesh size of 1 mm. The milled dry *Ulva* samples were sent to the Chemistry Department of the University of Cape Town where they were analysed for carbon and nitrogen using a CHNS elemental analyser (Thermo 1112 CHNS).

5.2.3 Experimental design

Thallus discs (diameter 10 mm) of *Ulva* were punched from thalli infected with *M. strangulans*. The brown spots on each disc were counted prior to the experiment and 6 discs were placed in each crystallizing dish containing 150 ml of either seawater or one-fifteenth Provasoli medium or one-third Provasoli medium under varying conditions of irradiance (40, 80 and 160 μmol photons m^{-2} s^{-1}). Three replicate crystallizing dishes were used for each treatment, with six-thalli discs sharing the same medium in each therefore n=3 for each treatment. The change in number of spots was recorded, under long days (16 h light) and the culture medium was replaced every 3 days in all treatments and the number of spots per disc was counted after 7 and 14 days.

Table 5.1: Dissolved Nitrogen concentration of culture media used in the experiment:

	NO_3^- (µM)	**NH_4^+ (µM)**	**Total N (µM)**
Seawater	15.02	0.29	15.31
$\frac{1}{15}$ PES + Seawater	54.42	7.29	61.71
$\frac{1}{3}$ PES + Seawater	212.02	35.29	247.31

*PES – Provasoli enriched seawater

The percentage change in number of spots per unit time (P_C) was calculated as:

$P_C = \frac{1}{t} \times \frac{(S_o - S_t)}{S_o} \times 100$, where S_o = initial number of spots per disc, S_t = final number of spots per disc, and t = time in days.

5.2.4 Estimation of nitrogen concentration in farm raceways

The surface area of each *Ulva* raceway is 282.62 m^2 (Browne, Honours project 2009) and the raceways are normally filled up to half a meter deep with water, thus the volume of seawater in the raceways when filled is about 141 m^3 (Sakkie Otto, pers. comm.). During the study period October to November 2007, each raceway was equally fertilized with 2 kg of N and P once a week and the ratio of N and P was 1:10. Ammonium sulphate and "Supergrow" (P: 203g/kg, S: 23,6g/kg, Ca: 171, 4g/kg) were used as fertilizer and all the raceways were fertilized every Tuesday afternoon and the amount of nutrients added to the raceways was independent of *Ulva* biomass (Lawrence Ansara, pers. comm.). The theoretical concentration of NH_4^+ in each raceway after fertilization was calculated as follows:

Ratio of N: $\frac{1}{11}$ x 2 kg =0.182kg or 182 g

Molar mass of NH_4^+ in $(NH_4)_2SO_4$: 36g/mol

Moles of NH_4^+ in 2 kg of fertilizer: $\frac{36g}{mol}$ x $\frac{1}{182g}$ = 0.198 moles

Concentrations of NH_4^+ in each raceways: 141.31 m^3 = 141310 Litres

$\frac{0.198 moles}{141310 Litres}$ = 1.401x10^{-6} moles/ L (M) = 1.401 µM NH_4^+

During the study period, the average N concentration recorded from *Ulva* raceways (Jonell, 2008) was as follows:

1. The average concentration of NH_4^+ in each raceways was approximately 0.89 ± 0.68 µM
2. The average NO_3^- concentration in each raceway was 0.38 ± 0.28 µM

5.2.5 Data treatment and statistical analysis

Data were analyzed by one- way analysis of v**a**riance (ANOVA). When significance was found, the Tukey test was applied to test for significant differences between individual treatments. Two-way ANOVA was used to compare treatments under different irradiances and dissolved N concentration in the culture medium.

5.3 Results

5.3.1 Prevalence and severity of infection

During October to November 2007, high prevalence of *Myrionema strangulans* was observed on sampled thalli, indicating a serious degree of infection. The epiphyte was homogeneously distributed within four *Ulva* raceways at the farm. The severity of epiphyte infection had a similar pattern to infection prevalence (Fig. 5.1). Therefore after the 13 November 2007 it was decided to replace the *Ulva* with clean stock from the "parent" stock.

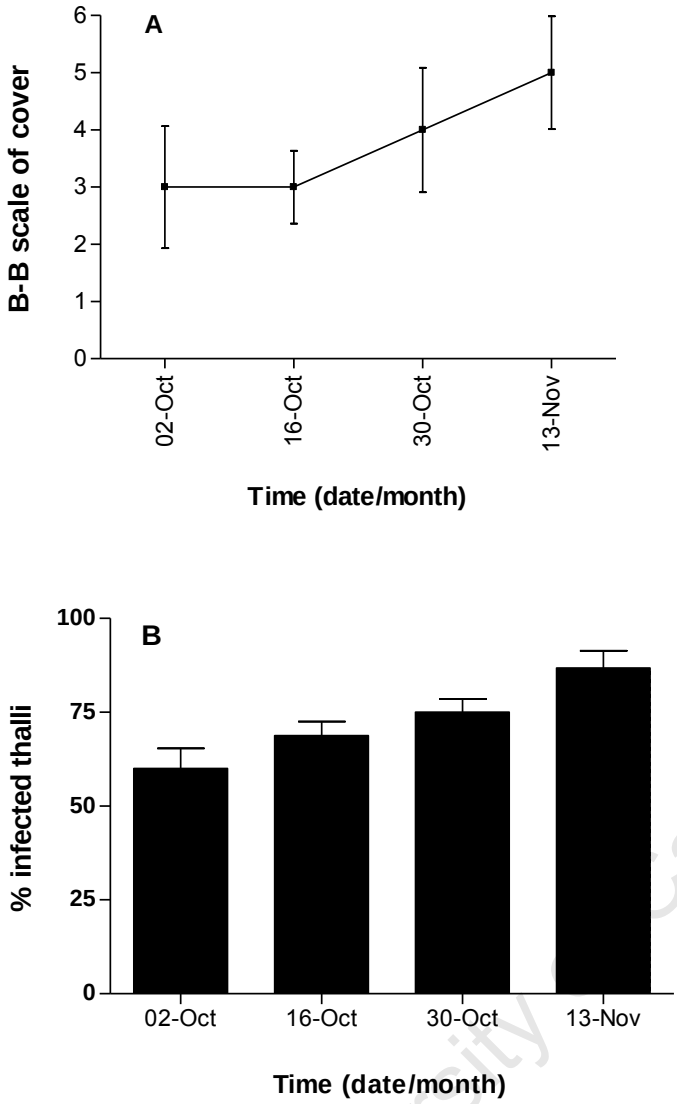

Figure 5.1: **A.** Infection severity (cover scale) and **B.** prevalence (% infected thalli) of the epiphyte, *M. strangulans* on *Ulva* grown in raceways receving abalone effluent at I & J farm from 02 October to 13 November 2007 (n=4 replicate raceways).

Based on the extent of *Ulva* damage by *M. strangulans*, the infections were subjectively classified as follows:

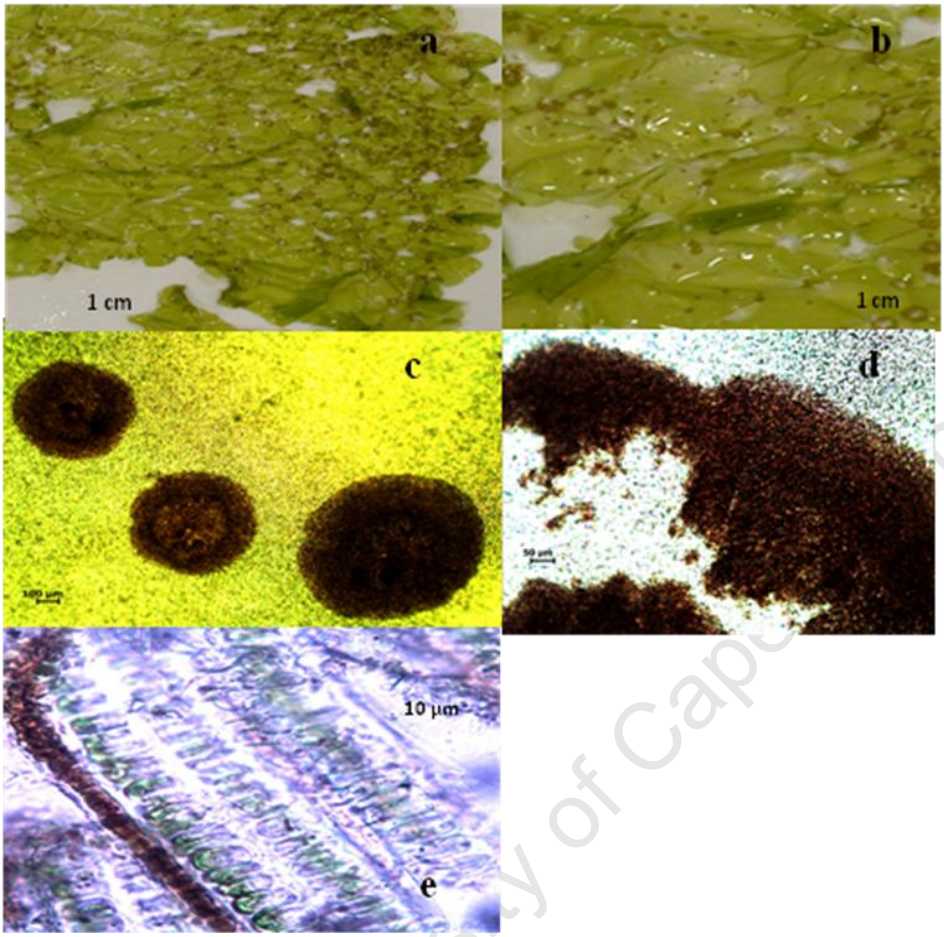

Fig 5.2a-e: a) *Myrionema strangulans* covers the *Ulva* thallus in the area where it grows, b) This leads to a weakening of the *Ulva* thallus and the thallus, which becomes light in colour and thinner, c) Circular spots increase in size and number and early tissue degradation could be noticed with the appearance of tiny perforations around the centre of the circular spots, d) Further progression leads to the joining of such perforations and eventually to disintegration of the thallus. e) Although the filamentous thalli of *M. strangulans* appeared to be clearly in contact with the cell wall of *Ulva*, they were never observed penetrating *Ulva* cells. However, due to some unknown processes, *M. strangulans* infection seems to be associated with destruction of *Ulva* cells in the area directly affected.

5.3.2 Tissue carbon, nitrogen and C:N ratio

High C:N values were observed in algae collected on the 30 October and the ratio increased until 13 November when values approached 52, indicating nitrogen limitation (Fig. 5.3). As shown in Figure 5.4, tissue content of carbon ranged from 22 to 23.8% and nitrogen tissue values decreased from 1.27% on 02 October to 0.45% on 13 November. All samples measured had low tissue nitrogen content. There appeared to be a relationship between thallus colour and C:N ratio. Material obtained on 02 October was green, with a lower C:N ratio, whereas material obtained on 30 October to 13 November was yellowish-green and had a higher C:N ratio.

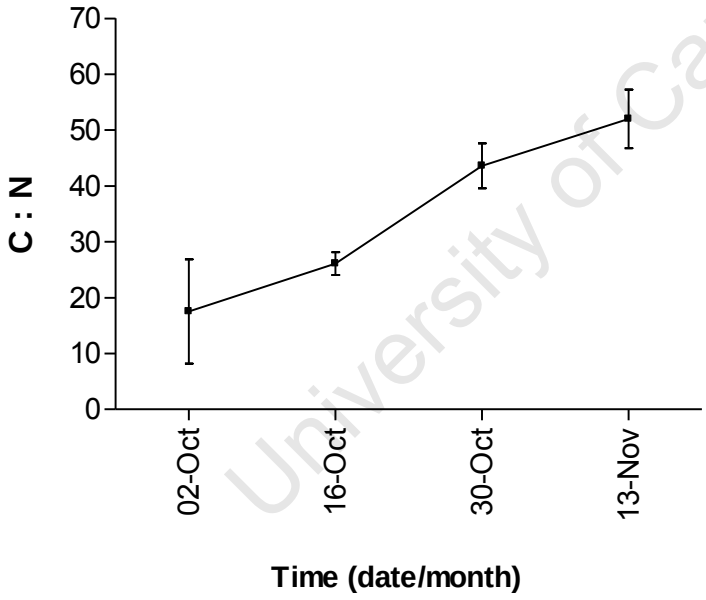

Fig 5.3: Tissue C:N ratio of *Ulva* infected with *Myrionema strangulans* (brown spots) during October – November 2007. Bars represent standard errors (n=4).

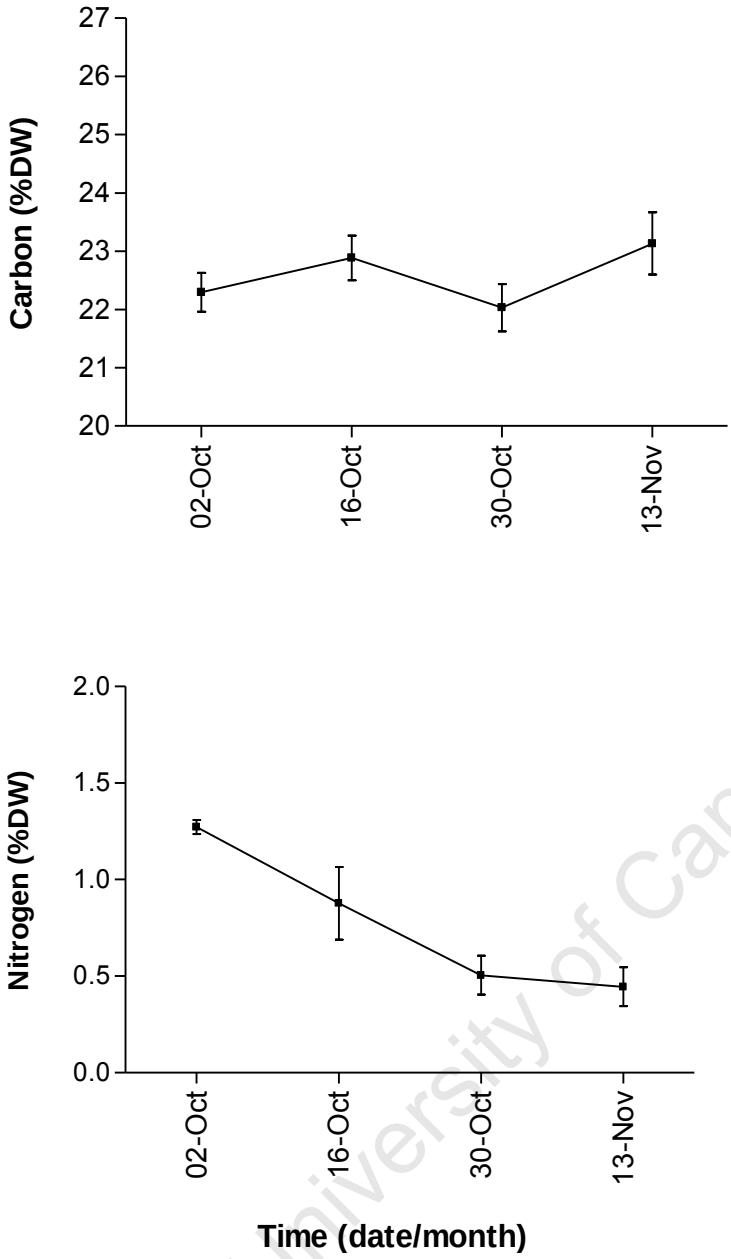

Figure 5.4: Tissue carbon and nitrogen content of *Ulva* infected with *Myrionema strangulans* (brown spots) during October – November 2007 Bars represent standard errors (n=4).

5.3.3 Change in number of epiphyte spots on *Ulva* thallus discs

In all treatments the mean number of epiphyte spots decreased (Fig. 5.5) after 7 d and 14 d in laboratory culture. However, in all cases except 7 d at 40 µmol m^{-2} s^{-1}, the reduction was significantly greater in PES than in seawater, and there was no significant difference between treatments with one-third and one-fifteenth PES. In one-third Provasoli and one-fifteenth Provasoli, after 14 d, *Ulva* discs had few to no spots. In seawater, after 14 days, the greatest reduction in *Myrionema* spots occurred at the lowest light level of 40 µmol m^{-2} s^{-1}.

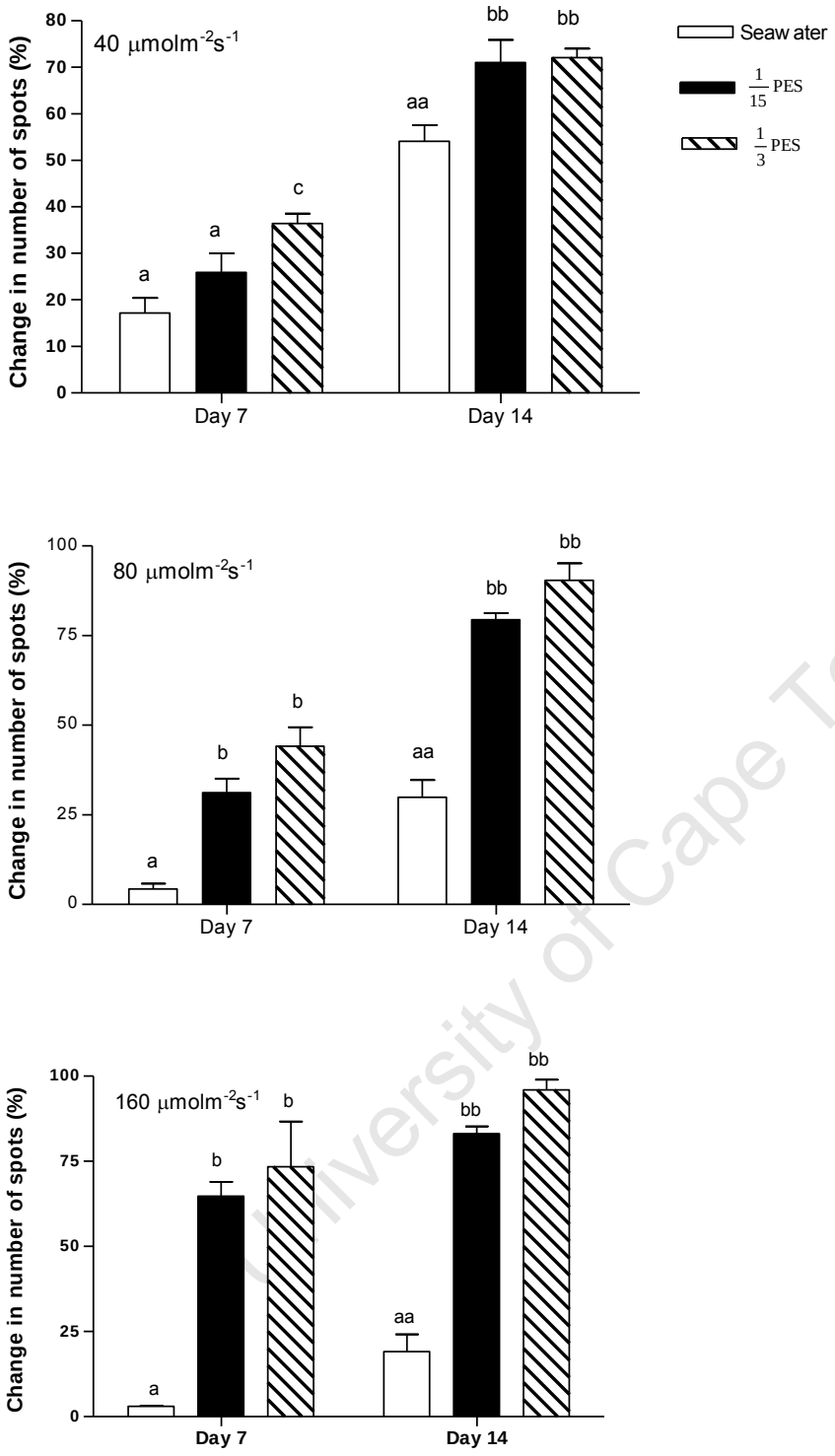

Figure 5.5: Change in number of *Myrionema strangulans* (brown spots) on *Ulva* discs cultured in seawater, 1/15 Provasoli medium and 1/3 Provasoli medium under varying irradiances.

5.4 Discussion

The spread of the epiphyte *Myrionema strangulans* is rapid and has posed a major threat to the success of *Ulva* cultivation in South Africa and it is therefore important to devise means of controlling its infections. *Myrionema strangulans* occurs on *Ulva* thalli in the United Kingdom (Fletcher, 1987) and in wild populations of *U. lactuca* in False Bay (Robertson-Andersson, 2003). Parallel to this study, *Ulva* was collected from natural populations at Kommetjie (West Coast) for two years but no *Myrionema* was found growing on it.

Myrionema outbreaks have been reported during spring and summer at I & J farm. The epiphyte species causes severe infection on *Ulva* and eventually the *Ulva* thalli disintegrate (Bolton *et al*., 2008). Interestingly, outbreaks of the epiphyte have not been observed during winter, possibly because *Ulva* is growing slower and there is luxury storage of nitrogen. Boyd *et al* (1985) showed that there is an increase in nitrogen in the seawater in the Walker Bay area in autumn and winter and this area is close to I & J farm. This increase is possibly related to processes occurring in the early winter e.g die-offs of plankton or stirring of sediments by early winter storms (Chapman and Shannon, 1985; Mitchell-Innes and Walker, 1991). The occurrence of the epiphyte during spring and summer coincided with low tissue nitrogen in *Ulva* growing in the raceways. This supports our hypothesis that during periods of fast growth (spring and early summer), nitrogen in the raceways is used rapidly and when it becomes limiting *Ulva* loses its ablity to withstand *Myrionema* infections. Observations in the present study showed that *M. strangulan*s grows very quickly: results accord with a study by Kornmann and Sahling (1983), who showed that *M. strangulans* produces fertile plants from spores in 11 days in the laboratory.

From the theoretical calculation of nitrogen in the farm raceways as well as the nitrogen levels measured in the raceways during the present study, it is clear that the nitrogen level was critically low when compared to the optimal nitrogen for growth discussed in Chapter 2. Nitrogen control is critical for the intensive cultivation of algae due to its role in growth, and regulation of metabolism (Smit *et al*., 1997). Depletion of nitrogen in the culture medium causes important cellular responses in algae. Excess nitrogen, as well as other nutrients, can be stored and used for growth during nutrient limited periods by a number of macroalgae including *Ulva fenestrata* and *Enteromorpha intestinalis* (Björnsäter and Wheeler, 1990). The laboratory results show that under sufficient N concentration (one-third and one-fifteenth Provasoli media), *Ulva* can defend itself against the epiphyte under varying light conditions. However, it must be borne in mind that other macro-and micro-nutrients in the Provasoli medium may have played a role in reducing the infection. Nevertheless, it is clear that some form of nutrient stress is implicated in *Ulva*'s susceptibility to infection by *Myrionema*. However, we do not have sufficient evidence to say that the levels of N nutrition are directly linked to the control of the infestation by *M. strangulans*. Much more work would be needed.

Infected *Ulva* thalli growing in natural seawater lost more than 50% of *Myrionema* when grown under low light, however, when light was increased to 80 and 160 µmol m^{-2} s^{-1}, there was no significant reduction in the epiphyte infection. This result is in agreement with a study by Robertson-Andersson (2003) on the same system at I & J farm which showed that shading during the period of spring and summer significantly reduces the infection of *Ulva* by *Myrionema*. From our results it is evident that at low or high light *Ulva* is resistant to *Myrionema* infection under sufficient N concentration. It is, however, possible that high N concentration could be toxic to *Myrionema*. It is known that ammonium (NH_4^+), can be toxic or inhibitory for some seaweeds at higher concentrations whilst it is the preferred nitrogen

form for *Ulva* (Lobban and Harrison, 1994). In addition, Gschwend *et al.* (1985) suggested that under certain conditions production of volatile halogenated compounds is part of some seaweed defence system and these compounds have allelopathic effect, reducing the amount of epiphytes. For instance, *Eucheuma denticulatum* produces other volatile halogenated compounds (VHCs), which might protect against unfavorable interaction with other organisms (Mtolera *et al*., 1995). It is possible that under the laboratory treatments (natural seawater at low light; all light levels and Provasoli media), *Ulva* produced compounds that reduced the amount of *Myrionema* growing on it. *Ulva* cultivated at I & J farm can resist *Myrionema* infection provided it is grown under adequate nitrogen, irrespective of light conditions.

Diseases and epiphytes on seaweeds cause physiological changes in the host. For example, infected *Ulva* plants appeared yellow in colour, indicating loss of pigments, and this has also been observed in diseased *Kappaphycus alvarezii* which exhibited low photosynthesis rates and decreased pigment concentration (Ganzon-Fortes *et al*., 1993). The infection of *Porphyra tenera* by a white wasting disease leads to a remarkable change in phosphorus metabolism in the tissue: *Porphyra* cells growing in seawater containing high phosphorus had a high tissue P-content and as the concentration of phosphorus increases in the cells, the oxidation-reduction potential becomes reductive and the white wasting disease does not develop on the frond (Kato and Watanabe, 1971).

One of the main objectives of this project was to assess the suitability of *Ulva* as a food source for the abalone (discussed in details in Chapter 7 and 8); therefore it is important to determine the nutritional content of the cultivated *Ulva*. Nitrogen starved seaweeds do not provide a good quality feed (Neori *et al*., 1998, 2004; Troell *et al*., 2003) and *Ulva* material

collected at I & J farm in this chapter had low tissue nitrogen content. This means that if severe infections were to lower the tissue nitrogen of *Ulva* and this would reduce the quality of *Ulva* as a feed source. During the period of October to November 2007, the crude protein content estimated using N x 6.25 was significantly lower than the recommended dietary protein for abalone. In addition, the C:N ratio in the present study was significantly higher than the ratio of 17 or less recommended by Russell-Hunter (1970) as necessary for proper nutrition of animals.

The C:N ratio is the most commonly used indicator of nitrogen limitation in marine algae, with low values indicative of nitrogen replete growth conditions (Wiencke *et al*., 2006). C:N values close to 10 have been described as optimal or normal for the nitrogen status of algae and a ratio greater than 10 indicates N-limitation in *Gracilaria* (Lapointe and Ryther, 1979). Mean C:N ratio for temperate and tropical marine plants is approximately 19 (Atkinson and Smith, 1983). In the present study, *Ulva* exhibited much higher C: N values, ranging from 18 to 52. Generally, C: N ratios are regarded as depending on nutrient availability (Atkinson and Smith, 1983; Levitt and Bolton, 1990). Red algae from the South African West coast, a nutrient-rich upwelling system, showed low C:N ratios of between 7: l and 12: l (Levitt and Bolton, 1990). Therefore, the low N contents and high C:N ratios determined from this study may be interpreted as the result of the low ambient N availability in the raceway water, during the study period.

Chapter 6

Bioaccumulation of heavy metals by *Ulva* from natural populations and from an integrated aquaculture system

6.1 Introduction

Many studies of contaminants and their effects on marine macroalgae have been published since the beginning of the 1960's (see Lobban and Harrison, 1994). Determination of heavy metal concentration in marine algae samples is usually preferred to the use of seawater and sediment samples (Forstner, 1985) as a method of measuring environmental contamination. Heavy metal concentrations in seawater are very low and show wide fluctuations, and heavy metal concentrations in sediment samples can be affected by organic matter content, grain size composition and pH (Forstner, 1985). Seaweeds have been known to be good indicators of heavy metal contamination in marine ecosystems since the first studies of Black and Mitchell (1952) on brown algae. The use of seaweeds as biological indicators of marine pollution in coastal areas has also been documented and reviewed by several researchers using Chlorophyta (Ho, 1990; Constantini *et al*., 1991; Kucuksezgin and Balci, 1994; Gnassia- Barelli *et al*., 1995; Haritonidis and Malea, 1995; Fityanos *et al*., 1999; Villares *et al*., 2002; Storelli *et al*., 2001; Misheer *et al*., 2006; Kamala-Kannan *et al*., 2008; Pérez *et al*., 2007; Besada *et al*., 2009), Phaeophyta **(**Preston *et al*., 1972; Stenner and Nickless, 1975; Muse *et al*., 1995) and Rhodophyta (Malea *et al*., 1994; Muse *et al*., 1995).

Heavy metal refers to any metallic chemical element that has a relatively high density and is toxic or poisonous at certain concentrations. Examples of heavy metals include mercury (Hg), cadmium (Cd), arsenic (As), chromium (Cr), thallium (T) and lead (Pb). Heavy metals in surface water systems can be from natural or anthropogenic sources and high levels of these

metals can act as ecological toxins in aquatic and terrestrial ecosystems (Guilizzoni, 1991). Sewage effluents and industrial wastewater discharges are obvious sources of a variety of pollutants including excessive concentrations of nutrients such as nitrogen and phosphorus (Valiela *et al.*, 1997). According to Rai *et al.* (1981), the order of metal toxicity to algae varies with algal species and the experimental conditions, however the general order is Hg > Cd > As > Pb.

Mercury is the most toxic metal, as it interacts with enzyme systems and inhibits their functions, particularly enzymes with reactive sulfhydryl groups (Van Assche and Clijsters, 1990), thereby causing cessation of growth, inhibition of photosynthesis, reduction in chlorophyll content and increased cell permeability (Rai *et al.*, 1981). In addition, cadmium is a pollutant particularly in coastal waters near industrial areas where concentrations may increase from normal levels of 0.1 µg/L to several micrograms per liter (Lobban and Harrison, 1994). Markham *et al.* (1980) studied Cd uptake and its effect on the growth, pigment content and carbon assimilation of *Ulva lactuca* and *Laminaria saccharina*. These authors reported that the growth rate for sporophytes of both species diminished to 50% of the control value when grown in a Cd concentration of 2000 µg/L, which is however higher than concentrations in most polluted areas. Furthermore, Markham *et al.* (1980) reported that at Cd concentrations greater than 2300 µg/L, the blades showed a sharp loss of pigment in their distal regions and at a sublethal concentrations >2000 µg/L, sharp reductions in photosynthesis and growth rates were observed.

Arsenic is an environmental contaminant and can arise from natural sources such as rocks and sediment as well as from anthropogenic sources. Furthermore, levels of arsenic are higher in aquatic environments as it is fairly water-soluble and can be washed out of arsenic-bearing rocks. Seaweeds are known to contain relatively high concentrations of arsenic as they can accumulate the arsenic they derive from seawater (Norman *et al.*, 1987) and hence

play an important role in its cycle. Accumulation of total arsenic concentrations (including organic and inorganic forms) in macroalgae is higher in brown algae than in green and red algae (Phillips, 1990). It is well-known that organic and inorganic forms of As differ widely in their toxicity (Oygard *et al.,* 1999), inorganic forms being in general more toxic than organic ones (López *et al.,* 1994) On the other hand, lead (Pb) is a less toxic heavy metal, and there has been little research on its effect on algal metabolism (Lobban and Harrison, 1994). Stewart (1977) reported the effect of lead on four small, finely branched red algae: *Platythamnion pectinatum*, *Platysiphonia decumbens*, *Pleonosporium squarrulosum* and *Tiffaniella snyderae*. He found significant reductions in growth to occur only at unrealisticly high lead concentration of 10 mg/L as lead chloride ($PbCl_2$).

Bioavailability and bioaccumulation of heavy metals in aquatic ecosystems is gaining tremendous significance worldwide, and there are number of toxic heavy metals whose increasing levels in the environment are of serious concern today. *Ulva* has been used as a bio-monitor of coastal contamination because its metal concentrations reflect the bio-available levels of metals in the water, and it is distributed worldwide, has a simple morphology and high capacity to accumulate metals (Ho, 1990; Haritonidis and Malea, 1995, 1999; Fityanos *et al*., 1999). Table 6.1 shows the heavy metal concentrations of *Ulva* recorded by different authors at different locations. Physiological changes and growth can affect concentrations of metals in the seaweed tissue (Huerta-Diaz *et al.,* 2007). The concentrations are usually low in summer when growth rates are high and the accumulated metals are diluted and high in winter when the metabolic processes slow down (Villares *et al.,* 2002). Heavy metals are potentially toxic to organisms when natural concentrations are exceeded (Almela *et al*., 2002; 2006).

In South Africa, *Ulva* species have been successfully produced in aquaculture and utilized as feed for abalone (Steyn, 2000; Robertson-Andersson, 2003, 2007; Bolton *et al*., 2008). In previous years *Ulva* was collected from natural populations on the South African west coast to add to dietary salt. This is currently not being carried out, as permits have not been issued for *Ulva* collection by the Government authorities (RJ Anderson, pers. comm.). Despite the nutritional value of *Ulva*, little information is available on the metal concentrations in the *Ulva* species grown in abalone farms or from the natural habitat. Metal contamination is an aspect that could affect the safety of *Ulva* as a feed. In addition, there have been very few studies on heavy metal accumulation of South African *Ulva* species. The only data from South Africa are an analysis of a species identified as *U. lactuca* (but probably referable to either *U. rigida* or *U. uncialis* (Lineekela Kandjengo, pers. comm.) from four sites on the South African east coast with different degrees of exposure to urban and industrial pollution (Misheer *et al*., 2006: see table 6.1).

In most countries except for France, Australia, New Zealand and Japan there is currently no legislation specific relevant to the heavy metal content of edible seaweeds. France was the first European country to set up regulations on the use of seaweeds for human consumption as non-traditional foods, and French limits for heavy metals in edible seaweeds are: Pb <5 $\mu g.g^{-1}$ dw; Cd <0.5 $\mu g.g^{-1}$ dw; Hg <0.1 $\mu g.g^{-1}$ dw; and inorganic As <3 $\mu g.g^{-1}$ dw (Besada *et al.*, 2009) whereas in Australia and New Zealand the maximum level for Cd is 0.2 $\mu g.g^{-1}$ dw and 1.0 $\mu g.g^{-1}$ dw for inorganic Arsenic (Almela *et al*., 2002; 2006; Besada *et al*., 2009). South Africa has no specific laws for the control of pollutants in edible seaweed, and therefore in order to evaluate the food safety of *Ulva* samples in the present study, it is necessary to refer to regulations of countries such as France where maximum limits for the content of the Pb, Cd, Hg and As have been established.

Table 6.1 Comparison of heavy metal contents in *Ulva* species reported by different authors

	Location	Pb ($\mu g\ g^{-1}$ dw)	Cd ($\mu g\ g^{-1}$ dw)	Hg ($\mu g\ g^{-1}$ dw)	As ($\mu g\ g^{-1}$ dw)	Author
U. lactuca	Beirut (Lebanon)	<7.5 – 37.5	<0.8 – 2.3			Shiber (1980)
U. lactuca	Hong Kong (China)	0.59 – 0.75	9 – 41			Ho (1990)
U. rigida	Thermakois Gulf (Greece)	6.3 – 29.8	0.1 – 2.5			Haritonidis and Malea (1999)
U. lactuca	South Adriatic Sea (Italy)	0.84	0.20			Storelli *et al* (2001)
U. lactuca	Kwazulu Natal (South Africa)			0.06 - 0.4	6.2	Misheer *et al* (2006)
U. lactuca	Pulicat Lake (India)	11.56	38.07			Kamala-Kannan *et al* (2007)
Ulva sp.	Gulf of San Jorge (Argentina)	0.82 - 1.72	0.17 - 1.03		2.98 - 5.61	Pérez *et al* (2007)
U. rigida	Spain	1.00 - 1.05	0.031 - 0.033	0.018 - 0.019	6.41 - 7.06	Besada *et al* (2009)

This study examined Pb, Cd, Hg and As levels because they are important potential pollutants in seaweeds for human nutrition and aquafeed. Therefore this study aims to:

(i) provide information on the concentrations of these potentially harmful heavy metals present in farmed *Ulva* species as well as *Ulva* from natural populations.

(ii) report on the degree of heavy metal contamination in the selected sites in order to contribute to quality controls on seaweed within South Africa.

6.2 Materials and Methods

6.2.1. Sampling sites

Ulva was collected from two sites:

(i) I & J Abalone farm, where *Ulva* is grown in abalone effluent in four raceways within an integrated *Ulva*/abalone aquaculture system, and is utilized as feed for abalone. Once a week, each raceway is fertilized with 1.5 kg of ammonium sulphate and "Supergrow" (P: 203g/kg, S: 23,6g/kg, Ca: 171, 4g/kg) in a ratio of 6:1 (Luvuyo September, pers. comm.).. (ii) Kommetjie on the Cape Peninsula, near Cape Town (34º09′06″S, 18º19′22″E), which is in a Marine Protected Area. The small bay where *Ulva* was collected gets some road runoff during rain, washing down approximately less than 1 km^2 of suburb. Furthermore, during several months of the year there is a lot of launching of small motor boats in a neighbouring bay about 300 m away (RJ Anderson, pers. comm.).

6.2.2 Sampling methods

U. rigida and *U. lactuca* were collected every three months for this Chapter (see below) from four raceways during January 2008 to May 2009. *Ulva* material was transported in water in a

cooler box from the farm to the laboratory. *U. rigida* and *U. capensis* were collected from Kommetjie every three month from January 2008 to May 2009 for this Chapter, and the same transportation of materials was used. All *Ulva* samples were briefly washed with freshwater to remove any epiphytes and weighed to determine the wet weight. The samples were then dried in an oven for 48 hours at 60°C, after which they were weighed for dry weight determination and milled to a fine homogenous powder through a 0.5 mm sieve. For the present study analyses were done every three months (Jan 08, Apr 08, Jul 08, Oct 08, Feb 09 and May 09) using four replicates for the farm materials and three replicates of wild material.

6.2.3 Analytical measurements

Milled, dry *Ulva* samples were sent to an element analyzing laboratory (BemLab (Pty) LTD, Stellenbosch, South Africa) for Pb, Cd, As and Hg analysis. Four heavy metals: arsenic (As), cadmium (Cd), lead (Pb) and mercury (Hg), were analyzed in wild *U. rigida* (WUR), wild *U. capensis* (WUC), farmed *U. rigida* (FUR) and farmed *U. lactuca* (FUL).

BemLab used the following method for analysis: Pb, As, Hg and Cd were determined by electro thermal atomic absorption spectrometry (Varian spectra ICP-OES) with a radial torch. Calibration curves were obtained with multi-elemental standards prepared by appropriate serial dilution of commercially available Pb, As, Hg and Cd stock standard solutions in 1.5 mol L^{-1} nitric acid (HNO_3). It must be noted that the arsenic analysed in this study was total As, which includes inorganic and organic arsenic.

6.2.4 Statistical analysis

The results were presented as micrograms per gram dry weight (μg g^{-1}d wt). Data were analysed by a 2-way analysis of variance to determine the effects of two variables (species and location) using STATISTICA 8.0. Furthermore, XLSTAT Version 2010.3.02,

Addinsoft™, USA was used for Principal Component Analysis and cluster analysis to see if there was any observable difference between species and environment in overall heavy metal accumulation. Pearson correlation was used to determine correlations between different metal contents in *Ulva* species using XLSTAT.

6.3 Results

6.3.1 Heavy metal content

Figures 6.1 to 6.4 show the average values of heavy metals in *Ulva* species every three months (Jan 08 to May 09) at the two locations (Kommetjie and I & J Abalone farm). There was no significant difference in the average values of Pb accumulated by *Ulva* species from the two locations ($p>0.05$). Wild *U. capensis* did not accumulate Hg at all or the content of Hg was below the detection limit (Fig. 6.2). A 2-way ANOVA showed no significant difference in the average values of Hg for the remaining sites and species. Wild populations tended to have more cadmium than cultured plants and the differences between wild and farmed species were statistically significant (Fig. 6.3). Both farmed *Ulva* species and wild *U. capensis* did not accumulate arsenic. However, wild *U. rigida* accumulated arsenic with values ranging from 0.13 – 0.38 $\mu g.g^{-1}$ dw (Fig. 6.4).

In all species, the order of element concentration was As>Cd>Pb>Hg (table 6.2). The Kruskal-Wallis ANOVA detected significant differences ($p<0.05$) between the various heavy metals during the different months for all species and thus supported the observed order of element content.

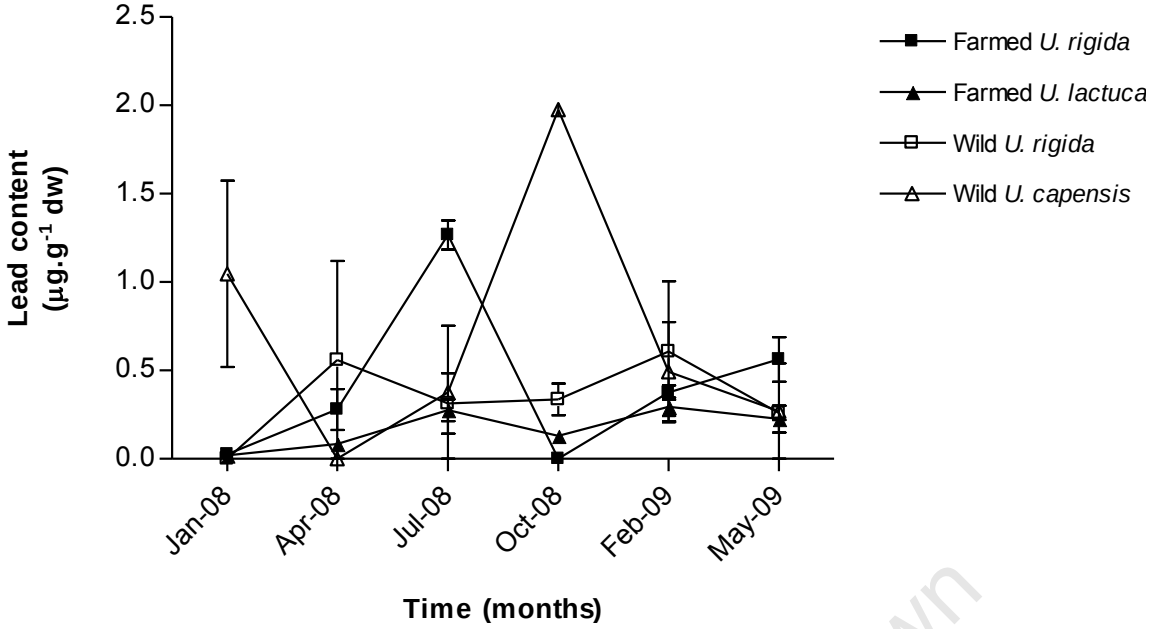

Figure 6.1: Mean (± SE) lead content in *Ulva* species from the natural population at Kommetjie and *Ulva* species from culture raceways at I & J farm.

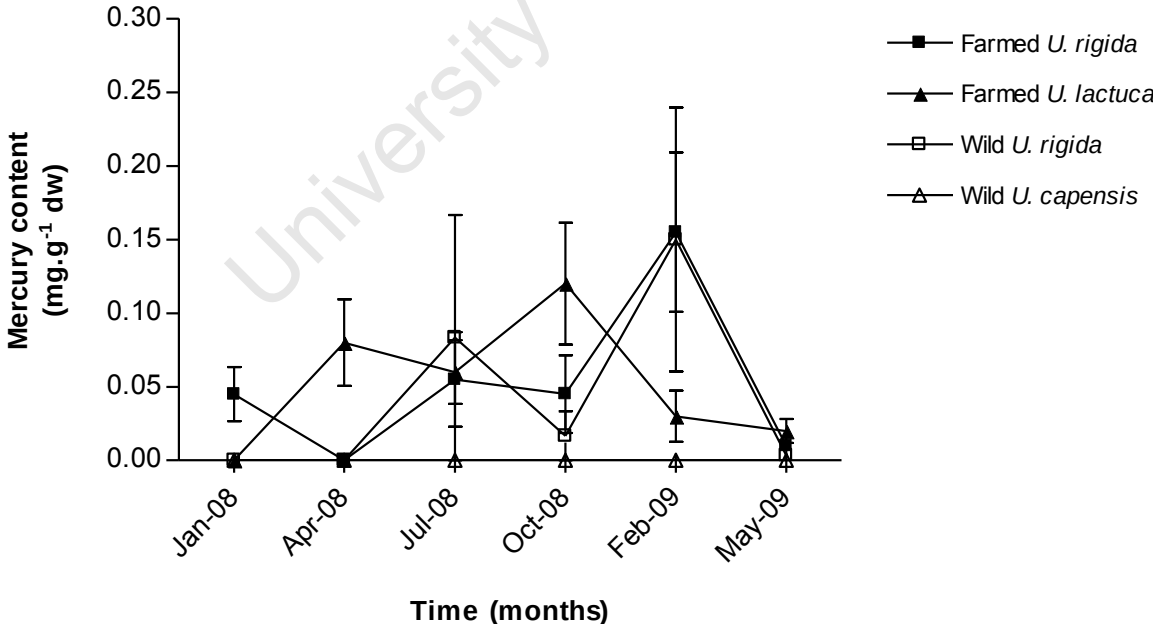

Figure 6.2: Mean (± SE) mercury content in *Ulva* species from the natural population at Kommetjie and *Ulva* species from culture raceways at I & J farm.

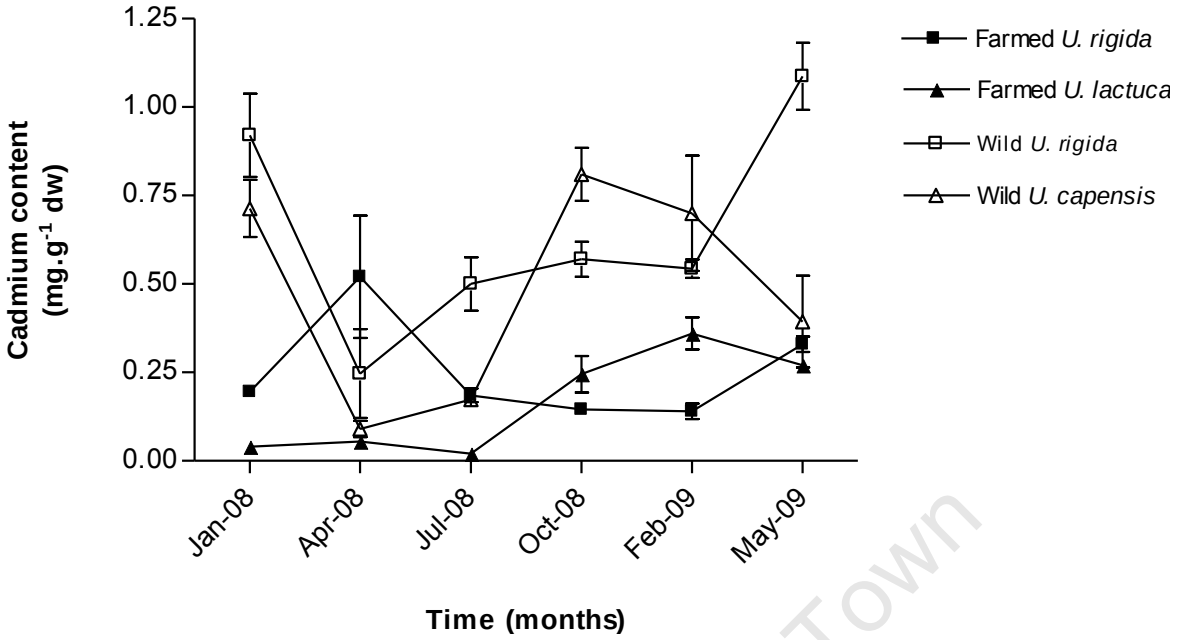

Figure 6.3: Mean (± SE) cadmium content of *Ulva* species from natural populations at Kommetjie and from culture raceways at I & J farm.

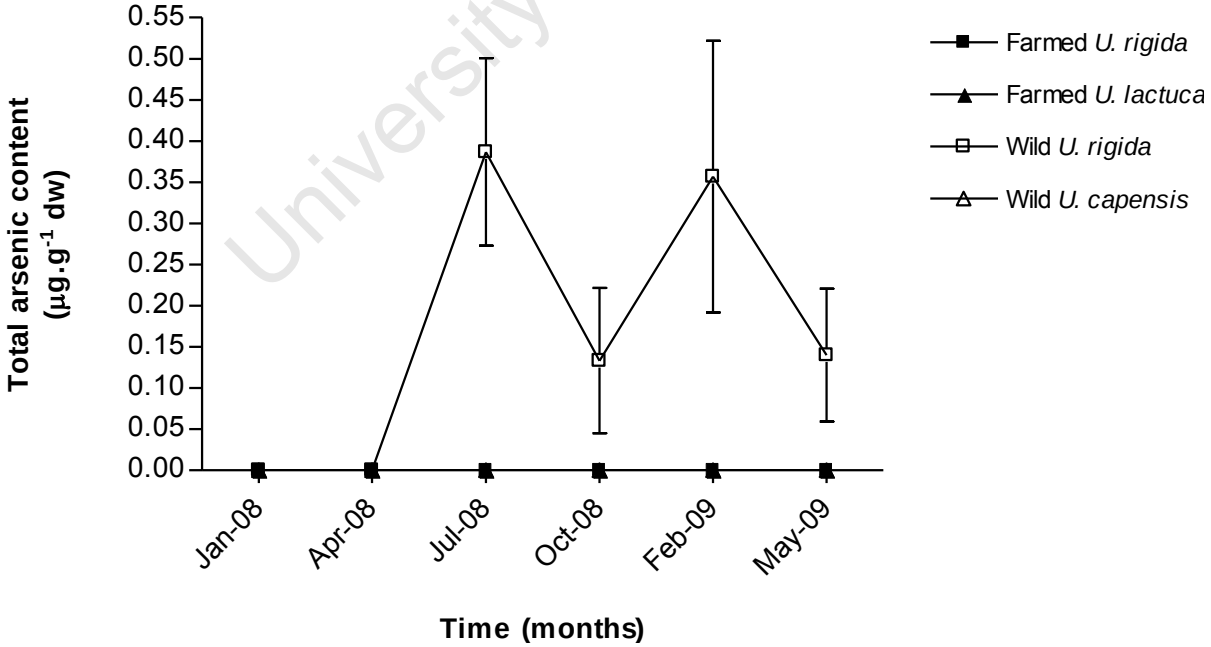

Figure 6.4: Mean (± SE) arsenic content in *Ulva* species from the natural population at Kommetjie and *Ulva* species from culture raceways at I & J farm.

Table 6.2. The average heavy metal content of three *Ulva* species for the analysed months. Values are expressed as means ± SE of dry weight (n=24 for farmed *U. rigida* and *U. lactuca*, n=18 for wild *U. rigida* and *U. capensis*).

	Heavy Metals (μg g^{-1} dw)			
	Hg	**Cd**	**Total As**	**Pb**
Farmed *U. rigida*	0.05±0.02	0.25±0.06	0.00±0.00	0.42±0.03
Farmed *U. lactuca*	0.06±0.02	0.17±0.01	0.00±0.00	0.17±0.02
Wild *U. rigida*	0.04±0.02	0.64±0.03	0.17±0.03	0.35±0.12
Wild *U. capensis*	0.00±0.00	0.48±0.03	0.00±0.00	0.69±0.07

6.3.2 Multivariate analyses

6.3.2.1 Principal component analysis (PCA)

PCA (Fig. 6.5) revealed that the wild *U. rigida* had high As and/or Cd, wild *U. capensis* had a range of Cd and Pb values (and low As) and the farmed *U. rigida* (and farmed *U. lactuca*) had generally low levels of metals, with one or two points with higher Hg. The main observation here seems that cultured plants at I & J farm tend to have lower levels of heavy metals than plants from the wild population.

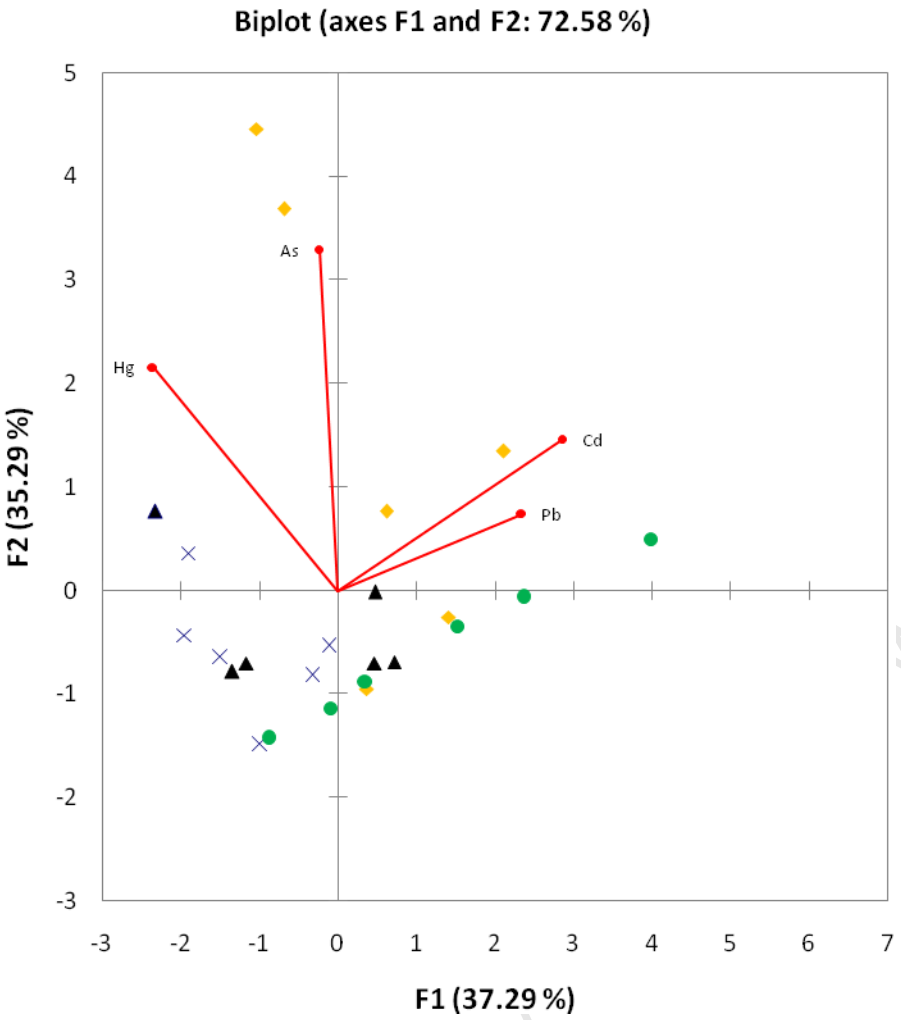

Figure 6.5: Principal Component Analysis for the monthly variation in heavy metal content (Hg, Pb, As and Cd) in *Ulva* species from the natural population of Kommetjie and *Ulva* species from culture raceways of I & J farm. Yellow data points represent wild *U. rigida*, green for wild *U. capensis;* black for farmed *U. rigida* and blue for farmed *U. lactuca*.

6.3.2.2 Cluster analysis

The dendrogram validated the main results obtained from both the PCA and the ANOVAs (Fig. 6.6). Three sample groups were observed: Group 1, associated only with *Ulva* species samples with high Pb concentrations (Wild *U. capensis* during January and October 2008; Farmed *U. rigida* during July 2008); Group 2 clustered all *Ulva* species samples with the lowest values in most metals and Group 3 is associated with samples that accumulated high Hg (Wild *U. rigida* in May 2009) and As (Wild *U. rigida* in July 2008 and February 2009).

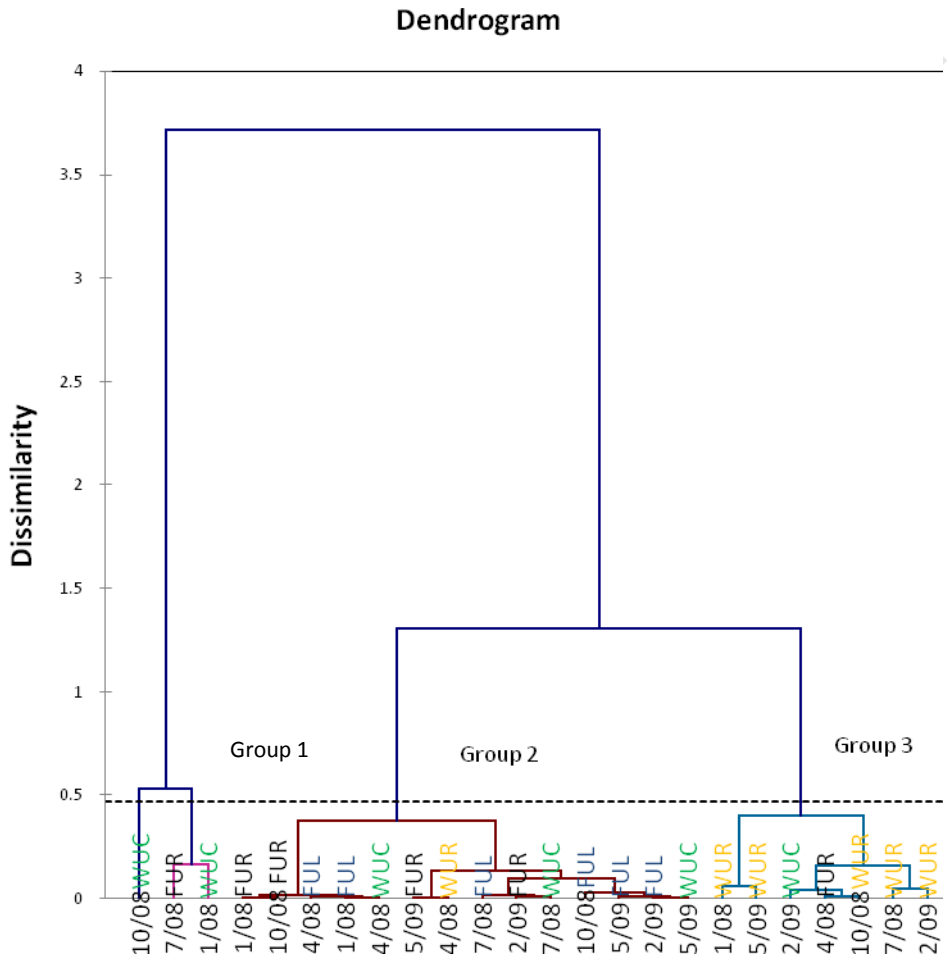

Figure 6.6: Dendrogram obtained using auto-scaled data and the Ward grouping method (WUR-wild *U. rigida*; WUC-wild *U. capensis*; FUR-farmed *U. rigida* and FUL-Farmed *U. lactuca*).

6.3.2.3 Correlation test

As shown in Figure 6.7, there was a significant positive correlation between the concentrations of Cd and Pb (r^2=0.5, Pearson correlation matrix) in farmed *U. lactuca*. There were no significant correlations found in metals accumulated by farmed *U. rigida* (Fig. 6.8). Similarly, wild *U. rigida* showed no significant correlations in accumulated metals (Fig. 6.9). Moreover, wild *U. capensis* showed positive correlations between contents of Cd and Pb (Fig. 6.10, r^2=0.792).

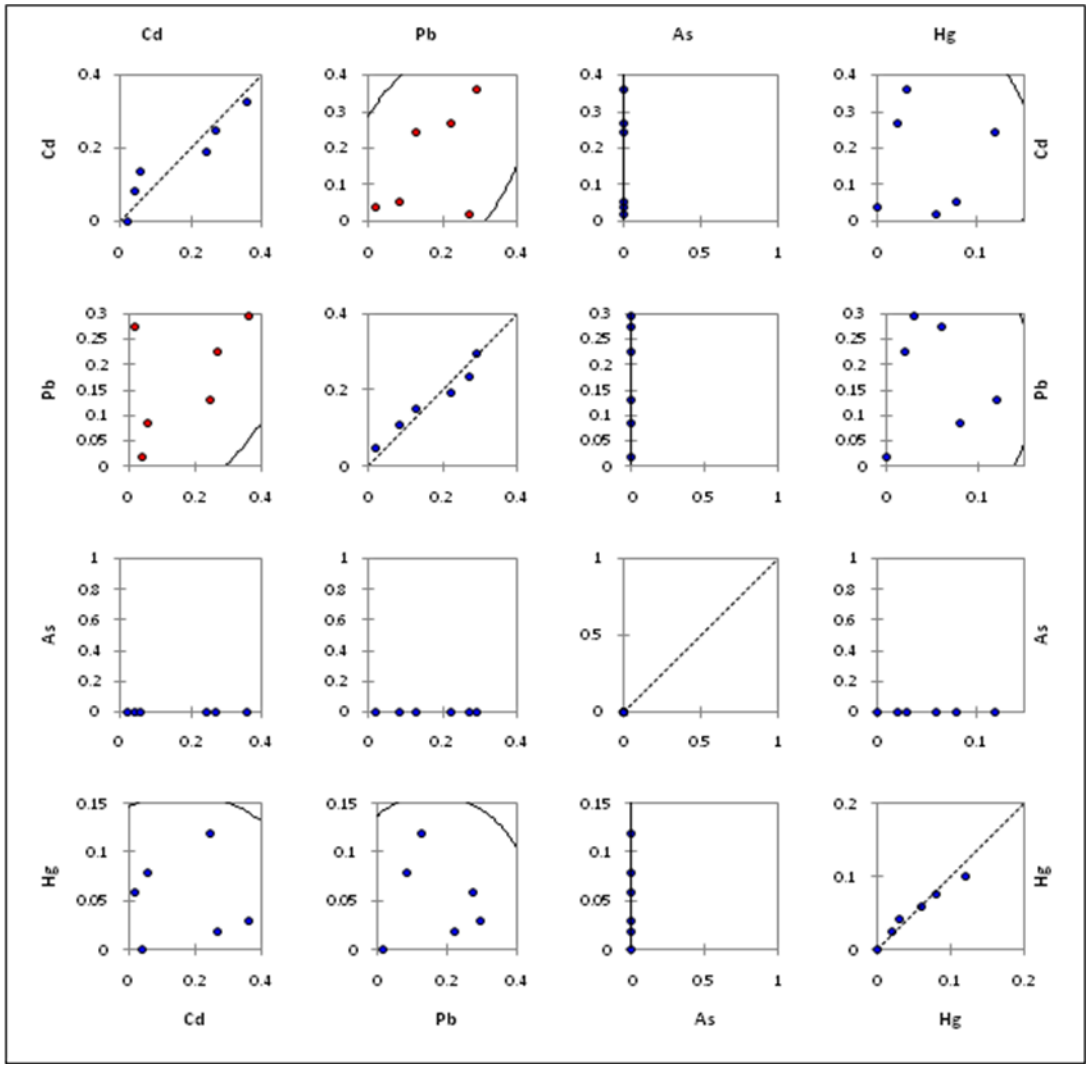

Figure 6.7: Scatter plots of farmed *U. lactuca* showing correlation of each metal against other metals. Red shows significant correlations.

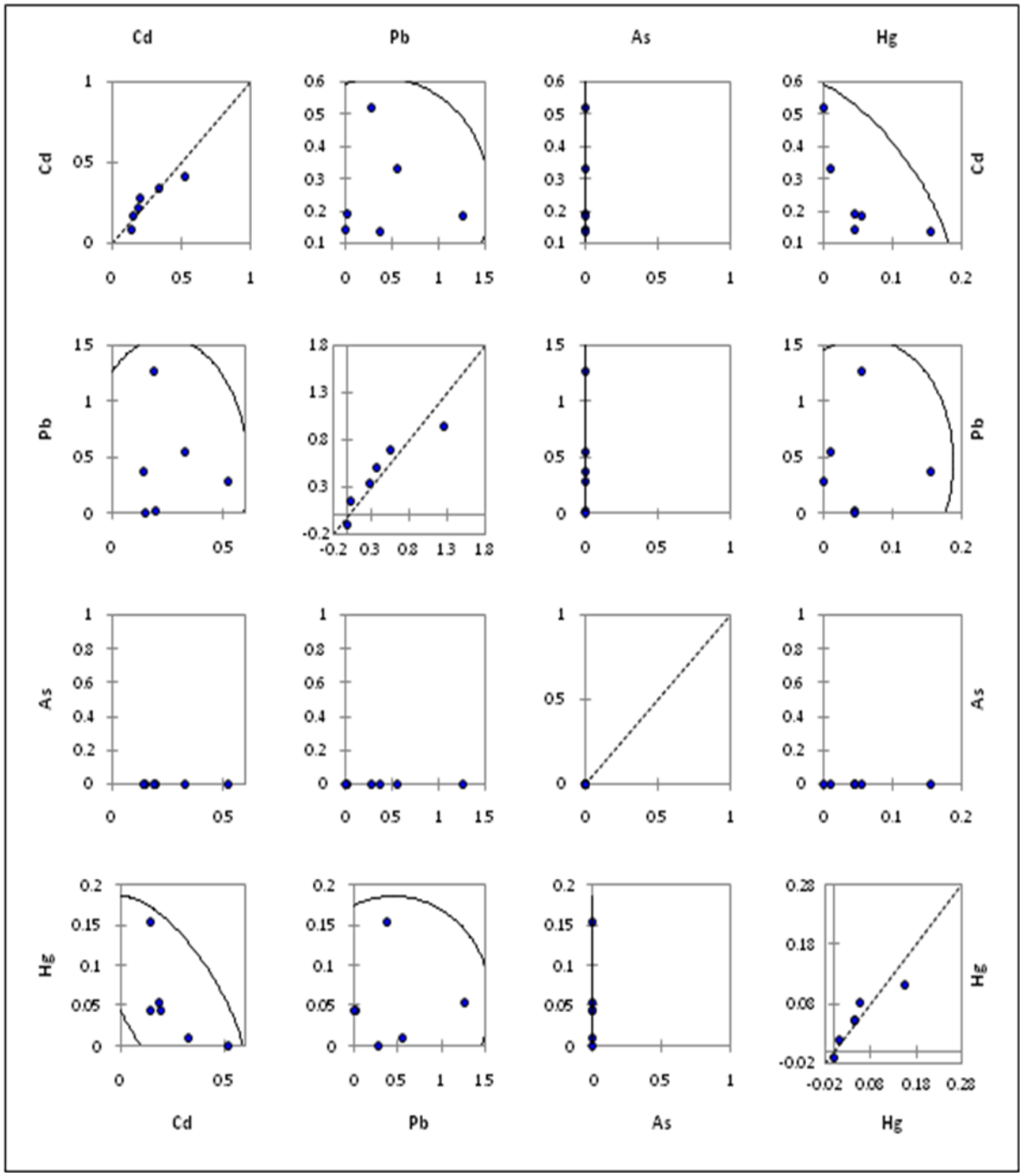

Figure 6.8: Scatter plots of farmed *U. rigida* showing correlation of each metal against other metals. No correlations were significant.

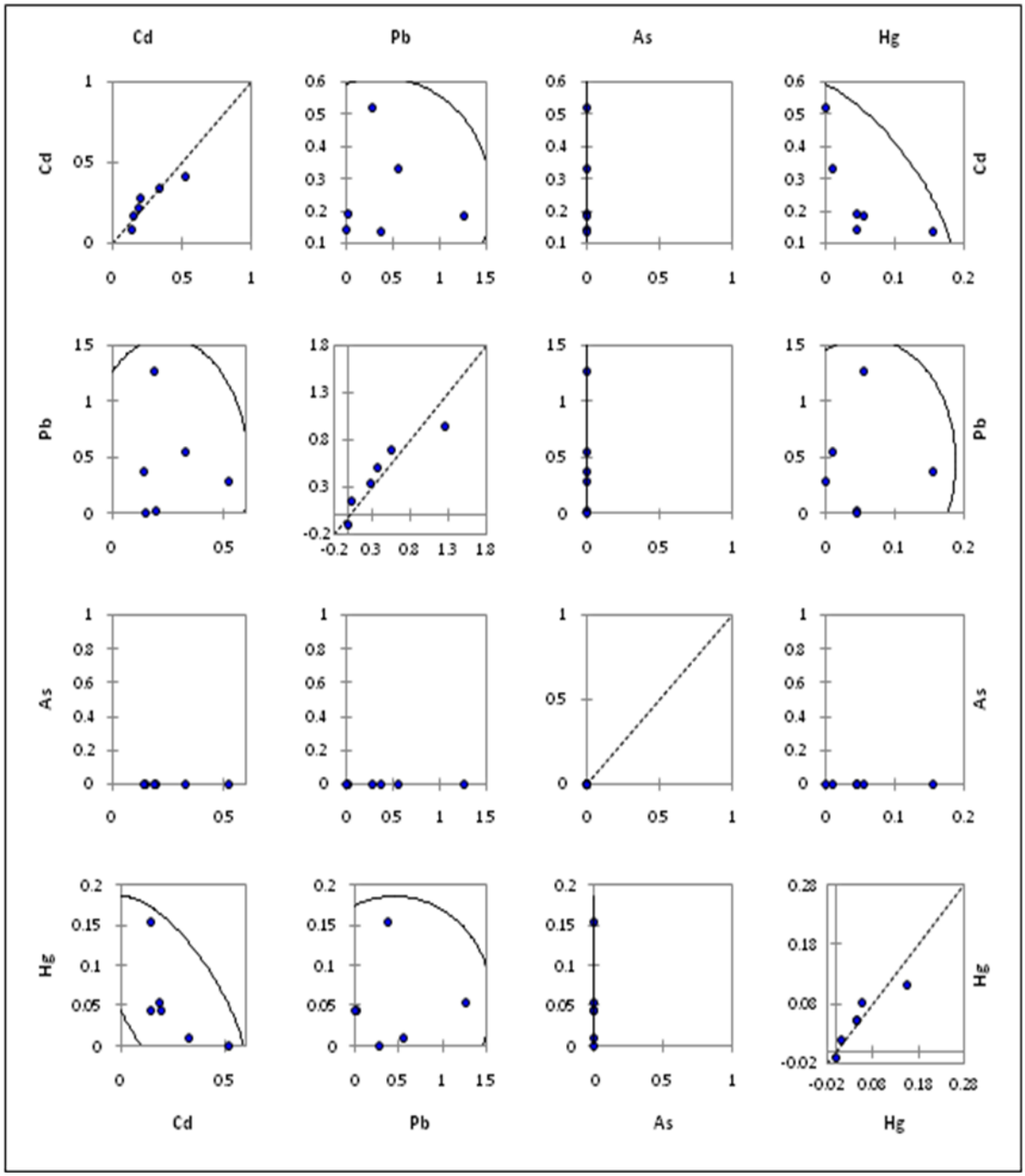

Figure 6.9: Scatter plots of wild *U. rigida* showing correlation of each metal against other metals. No correlations were significant.

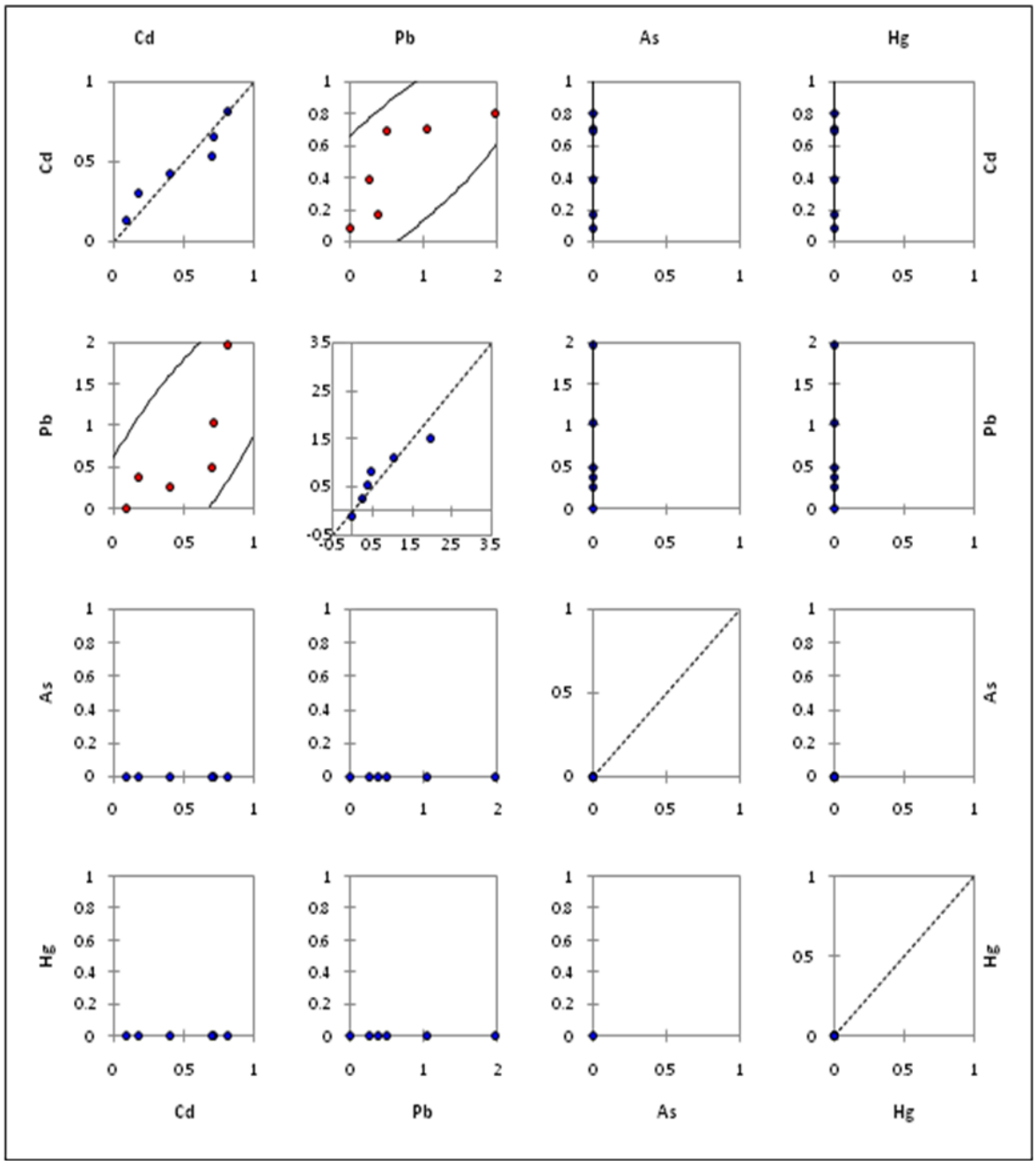

Figure 6.10: Scatter plots of wild *U. capensis* showing correlation of each metal against other metals. Red shows significant correlations.

6.4 Discussion

Although nutritionally valuable, seaweeds may accumulate high levels of heavy metals which may be toxic to the consumer (Almela *et al*. 2002; 2006). In addition, macroalgae that are widely used as abalone forage, can significantly accumulate toxic metals, thus there is an increasing concern for metal trophic transfer in abalones (Huang *et al*., 2008). The fact that samples collected from Kommetjie had the highest cadmium, lead and arsenic contents suggests that there might be an urban water outlet nearby with an outflow to the sea or some other anthropogenic source, which is releasing heavy metals. Because there is no industry in the immediate area where samples were collected (RJ Anderson, pers. comm.), this probably comes from road runoff from the large stormwater pipe draining directly into the bottom of the nearby small embayment, known locally as "The Kom".

According to Prosi (1983), the usual content for lead in algae ranges from 2 to 3 $\mu g.g^{-1}$ dw, with a minimum of 0.05 $\mu g.g^{-1}$ dw. In the current study, Pb ranges between 0.00 – 1.27 $\mu g.g^{-1}$ dw for farmed *Ulva* species with average values of 0.17 – 0.42 $\mu g.g^{-1}$ dw and 0.26 – 1.98 $\mu g.g^{-1}$ dw for *Ulva* species from the natural population of Kommetjie with average values of 0.35 – 0.69 $\mu g.g^{-1}$ dw. This is in a similar range to samples from *Codium tomentosum* collected from the Mediterranean which had Pb contents ranging from 0.32–0.92 $\mu g.g^{-1}$ (Constantini *et al*., 1991). In addition, Pb contents obtained in this study for wild *Ulva* species fall in the same range as those reported by Stenner and Nickless (1975) in species of *Fucus* from the Bay of Cadiz and *Enteromorpha* (now *Ulva*) from Atlantic Spain which had average contents of 1.7 and 0.8 $\mu g.g^{-1}$ dw, respectively. In industrialized and polluted areas of Venice Lagoon in Italy values of Pb reached levels of 17.6 $\mu g.g^{-1}$ dw in *Ulva sp.* (Caliceti *et al*., 2002) which is significantly higher than levels of Pb obtained in this study for all *Ulva* species.

Low levels of Cd were detected in *Ulva* species from I & J Abalone farms and these were in a similar range to Cd values (0.02 $\mu g.g^{-1}$ dw) of *Ulva sp* from the Black Sea, Turkey (Topcuoglu *et al*., 2003). The Cd contents obtained in this study for *Ulva* species from the natural populations are in the same ranges as those found by Paez-Osuna *et al.* (2000) in the gulf of California (Sonora) where content of Cd was 0.9 $\mu g.g^{-1}$ dw in *U. lactuca* populations. In addition, Cd content from our study fell in the same range as those for *U. lactuca* from Hong Kong (0.49-1.3 $\mu g.g^{-1}$ dw), and *U. rigida* from Greece (1.0 $\mu g.g^{-1}$ dw) (Haritonidis and Malea, 1999). However, the average Cd values in *U. rigida* from Kommetjie exceeded the French limit for human nutrition purposes (0.5 $\mu g.g^{-1}$ dw) by 14%.

Macroalgae accumulate moderate amounts of total As, contents are higher in brown algae than in green and red algae (Phillips, 1990) and kelps are very important in the arsenic cycle in coastal waters. In the current study, *Ulva* species collected from I & J farm and from Kommetjie had no detectable As at all, except for *U. rigida* from Kommetjie. This is surprising, since *U. rigida* and *U. capensis* were collected from the same locality at Kommetjie. In addition, wild *U. rigida* accumulated low As content compared to values obtained by Misheer *et al.* (2006) for *´U. lactuca'* sampled at Zinkwasi, Kwazulu-Natal on the east coast of South Africa (in winter) which recorded a content of As of 6.2 $\mu g.g^{-1}$dw. Caliceti *et al.* (2002) reported As contents in *Ulva sp.* in Italy to range from 4 to 65 $\mu g.g^{-1}$ dw, with a mean of 30 $\mu g.g^{-1}$ dw. This content was much higher than contents obtained in the current study for wild *U. rigida* or those reported for *U. lactuca* from Zinkwasi by Misheer *et al*. (2006).

The total values for Hg obtained in this study were very similar to those reported by Netten *et al.* (2000) and Almela *et al.* (2002) and clearly within the French limit of 0.1 $\mu g.g^{-1}$ dw (Almela *et al.*, 2002). *Ulva lactuca* from Treasure Beach near Durban, South Africa

accumulated mercury levels > 400 ppb (0.4 µg.g^{-1} dw) during the spring season (Misheer *et al.,* 2006) which was much higher than Hg levels obtained from all *Ulva* species used in this study. However, Misheer *et al.* (2006) recorded Hg content of 61.3 ppb (0.0613 µg.g^{-1} dw) in *U. lactuca* sample at Zinkwasi (in winter) which is in a similar range to the current study.

Overall, comparisons of data on heavy metals accumulated by seaweeds from different localities should be interpreted with care. Haritonidis and Malea (1995) reported that differences in the physiochemical factors (such as temperature, pH, salinity, wave exposure) and in the metallic contaminants between different localities and differences in the seasons of collection can influence the accumulation capacity of heavy metals by different seaweed species. Positive correlations between Hg and Pb; Hg and As and Pb and As in wild *U. rigida* were obtained, perhaps indicating that Hg, As and Pb enter the Kommetjie area from the same source or sources following a similar distribution pattern, Haritonidis and Malea (1999) have, however, suggested that such positive correlations between metal concentrations might also be a result of synergistic interaction for the binding sites in the plant.

Average levels of all heavy metals in *Ulva* species from Kommetjie were generally higher than in *Ulva* species from I & J Abalone farm, despite the fact that the cultured species are grown in abalone effluent, to which fertiliser is sometimes added. However, from both locations, the heavy metals were below the recommended levels for edible seaweed, except Cd levels in wild *U. rigida* and *U. capensis* which show mean values higher than the French limit.

The use of bio-indicators turned out to be very valuable for I & J Abalone farm where *Ulva* is cultivated as feed addition for abalone. The results indicated that the consumption of *Ulva* species cultivated at I & J Abalone farm does not seem to pose a risk for the abalone's health

at least with respect to their content of Hg, As, Cd and Pb, which were well within the allowable value for humans in France. I & J Abalone farm might be considered as a clean site with respect to heavy metals, and safe for the cultivation of *Ulva*. However, a study by Huang *et al*. (2008) quantified the route and rate of Ag, Cd and Hg uptake in the abalone *Haliotis diversicolor* fed on different macroalgae and showed that under typical conditions, dietary uptake is the dominant route for Ag, Cd and Hg accumulation in abalones. Their results suggest that metals in abalones can be a food safety issue due to the potential for trophic transfer from macroalgae. Therefore, it is critical that South Africa implement a continuous and systematic program of seaweed safety monitoring regarding inorganic pollutants.

Although the ecology and physiology of the different species of *Ulva* studied in Chapter 2 are very similar, there are differences with respect to the accumulation of metals. This shows that environment (including culture conditions) and species are important in looking at heavy metal accumulations. Thus the paper of Misheer *et al.* (2006), which analyzed *U. lactuca* growing along the KwaZulu-Natal coastline, suffers from the problem that the *Ulva* species was not properly identified and the worldwide problem of applying the name *U. lactuca* indiscriminately makes the interpreatation of such results difficult.

According to the limits laid down by the French legislation, the *Ulva* species in the present study attained the following:

(a) All samples fell below the limits set for heavy metals except for wild *U. rigida* and *U. capensis* which exceeded the limit set for cadmium

(b) Wild *U. rigida* is the only species in the present study that accumulated arsenic; however it did not exceed the arsenic set limit.

Chapter 7

Seasonal variation in chemical composition and mineral content of wild and cultivated *Ulva*

7.1 Introduction

The local cultivation of *Ulva* has proven successful so far and more recently Wild Coast Abalone farm has become a world leader in *Ulva* cultivation: the current estimated figure of *Ulva* cultivation in South Africa is about 1,100 tonnes wet weight (Bolton *et al*., 2008). Currently, at least three *Ulva* species (*U. lactuca, U. rigida* and *U. linza*) are being cultivated on local farms (Bolton *et al*., 2008). Although *Ulva* has been successfully utilized as abalone feed and as an aquaculture biofilter (Robertson-Andersson *et al*., 2007; Bolton *et al*., 2008), there is a lack of information on the nutritive value of these seaweeds. In China, Japan, USA, France, and Chile, seaweeds are eaten by humans, for example, *Ulva* is harvested to prepare ‘‘aonori’’ (Nisizawa *et al*., 1987), which is included in a great variety of dishes, including raw salads, soups, cookies, meals, and condiments (Ohno and Critchley, 1993). During the last few decades, efforts have been made to evaluate the potential of seaweed protein in aquafeeds (Alexis, 1997) due to the present over-dependence on fishmeal and fish oil which may threaten the long-term sustainability of aquaculture (FAO, 2002). Studies have shown that the inclusion of seaweeds in aquafeed has resulted in increased growth rate, feed efficiency and feed consumption in several species of fish (*e.g.* Hashim and Mat Saat, 1992; Valente *et al*., 2006). In addition, a study by Naidoo *et al.* (2006) showed that the abalone *Haliotis midae* grew best on a mixed diet of kelp plus other seaweed compared to those fed with artificial feed exclusively.

Chlorophycean algae generally contain the same set of major carotenoids as higher plants, namely β-carotene, α-carotene, lutein and violaxanthin (Goodwin and Britton, 1988). The addition of pigmenting carotenoids is an essential process in the aquafeed industry in relation to salmonid fish culture to ensure that the characteristic pink flesh colouration is maintained during the production of farm-raised trout and salmon in many countries (Foss *et al*., 1984; Bjerkeng, 1992; Choubert and Heinrich, 1993; Buttle *et al*., 2001). Pigmentation is one of the important qualities of the fish for consumer acceptability and marketing as they fetch a higher price in the commercial market (Skrede and Storebakken, 1986; Gupta *et al*., 2006). This is normally achieved by supplementing fish feeds with carotenoids (Nickell, 1998) as fishes cannot synthesize their own colouring pigments. These are synthesized by some plants, algae and microorganisms, and therefore they need to be incorporated in their diet (Johnson and An, 1991; Davies, 1985). The same applies to the culturing of sea urchins. According to Robinson *et al.* (2002) feeding cultured sea urchins an artificial diet with no pigments will result in large but pale coloured gonads that are commercially unacceptable. These authors found that natural β-carotene derived from algae produced significantly better gonad colour than that derived from synthetic β-carotene. Similarly, Shpigel *et al.* (2005) showed that seaweed carotenoids were more effective at producing good gonad colour than comparable concentrations of synthetic carotenoids. Carotenoids serve important functions as pro-vitamin A, antioxidants and immunoregulators and they are mobilized from muscles to ovaries in salmonids (Nakano *et al*., 1999b; Bell *et al*., 2000). It has also been observed that fishes with high level of carotenoids are more resistant to bacterial and fungal diseases (Shahidi *et al*., 1998). Fish feeds are commonly supplemented with synthetic carotenoids (Bjerkeng, 1992) which are known to have deleterious effects on the environment (Gupta *et al*., 2006). Synthetic carotenoids are considered to be very expensive and have contributed significantly to the production costs of salmonid aquaculture (Torrissen *et al*., 1990), fueling

a growing demand for the inclusion of pigmenting agents based on natural carotenoid sources. Consequently, there is much interest in developing new pigment-rich natural sources such as those obtained from algae and yeast products for potential application in aquafeed (Davies, 2004). Interestingly, *Ulva* is known to contain significant amounts of carotenoids (Abd El-Baky *et al*., 2008) and a study by Cruz-Suarez *et al.* (2009) showed that shrimp body carotenoid was significantly higher when the animals were fed on an *Ulva* diet, suggesting that *Ulva* carotenoids were efficiently assimilated and metabolized and may also be involved in growth enhancement. Due to all the benefits of carotenoids in the diet, it is essential to determine the carotenoid contents of local *Ulva* species as they are used as feed supplements. Although no information is available on the role of carotenoids in abalone culture, one would expect that carotenoids might affect the colour of the flesh. Although there are different types of carotenoids, in this study we did attempt to characterize them, and instead measured "total carotenoids".

The nutritional properties of seaweeds and their seasonal variation are not well understood and normally are evaluated from the chemical composition (Mabeau and Fleurence, 1993). The chemical composition of seaweeds includes the macroelements and microelements (Chan *et al*., 1997). Macroelements are described as being "required" or "essential" and are those elements upon which organisms depend on for normal physiological functions such as growth, reproductive success and survival and freedom from certain clinical/metabolic disorders (Combs, 1996). Microelements are sometimes referred to as beneficial elements (Combs, 1996) or trace elements (Goldhaber 2003) and are essential for seaweed metabolism since they may limit algal growth at low external concentrations (Reed and Gadd, 1990, Lobban and Harrison, 1994).

Studies have shown *Ulva* species to contain variable amounts of a number of elements. Brown *et al.* (1999) studied copper and zinc concentrations in *U. intestinalis* and *U. lactu*ca from various sites within Otago Harbour, southern New Zealand, and found spatial variation in the concentrations of both elements, with increasing levels of zinc in winter and lower levels in summer. In addition, Flodin *et al.* (1999) showed that both bromophenol content and bromoperoxidase activity exhibit seasonal variation in *U. lactuca*, with high values in summer which decrease in winter. Moreover, Villares *et al.* (2001) studied seasonal variation in the contents of different elements (Cu, Fe, Mn, Ni and Zn) in *Ulva* from 22 sites on the northwest coast of Spain. The seasonal variation in the different elements appeared to be caused by dilution during the period of maximum growth and concentration during periods of slow growth. Temporal differences, in the chemical composition of local *Ulva* species, whatever their causes, might be expected to affect their quality as abalone feed.

Studies on local *Ulva* species have been on taxonomy (Stegenga *et al*., 1997); reproduction of *U. fasciata* and *U. rigida* (Steyn, 2000); bioremediation using *U. lactuca* (Robertson-Andersson, 2003) and molecular taxonomic studies (Kandjengo, 2003). However there has been no research on the seasonal variations in chemical composition. Cultivated material may have a different mineral content (and hence different nutritive value) than wild material, and this may be important in nutritive value as feed.

Therefore this study aims:

(i) To investigate whether wild *Ulva* has seasonal variations in chemical constituents, and whether such patterns differ from those in cultivated *Ulva*.

(ii) To investigate the chemical composition of two apparent species in the wild (*U. rigida* and *U. capensis*), which have different habitats and morphology but are thought to be very closely related, if not conspecific.

(iii)To compare the chemical constituents of *U. rigida* in the wild with the same species growing in aquaculture effluent at I & J farm.

(iv)To determine if cultivated *Ulva* is a potentially better feed than wild *Ulva*, and whether cultivated *Ulva* varies less (seasonally) than wild *Ulva*.

(v) To compare the nutritional content of these with other *Ulva* species and with the nutritional requirements for *Haliotis midae*.

7.2 Materials and Methods

7.2.1 Preparation of *Ulva* samples for chemical analysis

U. rigida and *U. lactuca* were collected every two weeks from four raceways within an integrated *Ulva*/abalone aquaculture system at I & J Abalone farm. Once a week, each raceway was fertilized with 1.5 kg of ammonium sulphate and "Supergrow" ® (P: 203g/kg, S: 23,6g/kg, Ca: 171, 4g/kg) in a ratio of 6:1 (Luvuyo September, pers. comm.). *Ulva* material was transported in seawater in a cooler box from the farm to the laboratory. In addition, *U. rigida* and *U. capensis* were collected from Kommetjie every month from January 2008 to May 2009 and transported to the laboratory in seawater in a cooler box. All *Ulva* samples were washed briefly with freshwater to remove epiphytes and weighed to determine the wet weight. The samples were then dried in an oven for 72 hours at 60°C, after which they were weighed to get the dry weight and milled to a fine homogenous powder through a 0.5mm sieve. Milled dry *Ulva* samples were then analysed for elemental

composition, crude fibre and ash content, whereas total carotenoids was determined using fresh *Ulva* material.

7.2.2 Macro- and microelement analysis

Milled dry samples of *U. rigida* and *U. capensis* collected from the wild and *U. lactuca* and *U. rigida* from I & J farm were sent to a commercial feed laboratory (BEMLAB, analysis laboratory, Stellenbosch) for elemental analysis. Analysis were done every 3 month (Jan, Apr, Jul, Oct 2008 and Feb, May 2009) for each *Ulva* species. Elemental analysis on *Ulva* samples for the trace elements and macro elements involves a process whereby samples are digested, diluted and then analysed on an Atomic Absorption Spectrophotometer and UV-VIS spectrophotometer. For the determination of Ca, Mg and K it involves a process whereby lanthanum or strontium is added to an air - acetylene flame to suppress interference from PO_4. A strontium chloride - perchloric acid diluent removes interference due to protein acetate and PO_4. For the determination of Cu, Zn, and Fe, trichloroacetic acid is used to remove interference caused by proteins in the serum samples. Samples are analysed for specific trace elements on the AA spectrophotometer (Cu, Zn, Ca, Mg). In the determination of PO_4 the specimen is de-proteinised by iron tri chloro acetic acid reagent. The supernatant is mixed with molybdic acid to form molybdiphosphate which is reduced by Fe^{2-} to produce molybdenum blue. Absorbance is read on the UV-VIS spectrophotometer.

Calculations of concentrations of the element used the following formula.

$$\text{Macro analyte concentrations} = \frac{a * 50}{10000 * m} \%$$

$$\text{Micro / trace analyte concentration} = \frac{a * 50}{m} \text{ mg/kg}$$

Where a = the measured analyte concentration (mg/l) in the extract.

m = sample mass

7.2.3 Crude fibre analysis (Lignocellulose complex)

Crude fiber is the organic residue remaining after digesting with 0.255N H2SO4 and 0.313N NaOH. Crude fibre is generally regarded as the non-digestible carbohydrate component of the feed ingredient; within plant materials it is usually composed of a mixture of cellulose, hemicelluloses and lignin (the latter not being a carbohydrate but rather a complex aromatic compound) (FAO, 2009). In the cell wall of *Ulva* spp., ulvan is present with cellulose (Lahaye *et al*., 1995). Crude fibre in milled dry *Ulva* samples was analysed using an Ankom220 Fibre Analyzer (ANKOM Technology, New York, USA). For these analyses, Ankom220 Fibre Analyzer manufacturer methodology was used (ANKOM Technology Method 2008). The samples were hydrolysed in filter bags (F 57, pore internal dimension 50 µm, ANKOM Technology, New York, USA) by using 0.255N H_2SO_4 and 0.313N NaOH separately for 45 minutes. Then, the filter bags containing the samples were washed in water (three times) and in acetone (once, 3 min). After acetone evaporation, the bags were dried at 105°C (4 h) and then incinerated in a muffle furnace at 550°C (5 h). All samples were analysed in triplicate. The crude fibre content was calculated according to the equation:

$$\% \text{ Crude Fiber} = 100 \times \frac{(W_3 - (W_1 \times C_1))}{W_2}$$

Where: W_1 = Bag tare weight

W_2 = Sample weight

W_3 = Weight of Organic Matter (Loss of

weight on ignition of bag and fiber)

C_1 = Ash corrected blank bag factor

(Loss of weight on ignition of blank bag/original blank bag)

7.2.4 Total carotenoid content

For pigment analysis, 5mL of dimethyl sulfoxide (DMF) was added to 30 mg fresh weight of *Ulva* samples. The extraction was left overnight at 4 ^{o}C. A glass extraction plate was used and absorbance was read at 480, 646.4, 663.8 and 750 nm using a Multiskan Spectrum spectrophotometer, Version 1.2. Chl a, b and carotenoids were calculated using the equation of Wellburn (1994) below. This method calculates values for chl a and b and these values are required in the calculation of total carotenoids, however, for this study only carotenoid data were reported.

Chl a = 12(A663.8-A750)-3.11(A646.8-A750)

Chl b = 20.78(A646.8-A750)-4.88(A663.8-A750)

Carotenoids = (1000*A480-1.12Chla-34.07Chlb)/245

All samples were analysed in triplicate and values averaged. Data were expressed on a dry weight basis using 80% water content.

7.2.5 Moisture content

After washing the *Ulva*, the samples were spun in a salad spinner for 1 minute and weighed on an OHAUS electronic balance, oven dried (60 °C, 72 hours) and then re-weighed. Moisture content was determined by calculating the difference between wet and dry weights (A.O.A.C., 1970): All samples were analysed in triplicate.

% Moisture content = [(ww – dw) / ww] * 100

where ww and dw are wet and dry weights respectively.

7.2.6 Ash content

The ash contents of milled dry *Ulva* samples were determined by burning 1g of powdered algae at 600 ^{o}C, for 5h (A.O.A.C., 1970). All samples were analysed in triplicate and values averaged. The ash content was determined as follows:

% Ash = weight of ash/weight of sample x 100.

7.2.7 Statistical analysis

Data were analysed by a 2-way analysis of variance to determine the effects of two variables (species and temporal variation). Comparisons after ANOVA were made using the post hoc Tukey test to individualize specific differences (Zar, 1999) using STASTICA 8.0.

7.3 Results

Table 7.1 below shows average values for all parameters measured throughout the Chapter. A 2-way ANOVA was applied to test for significant differences (*) in location (wild or farmed), species and months. Water content is expressed as percentage wet weight whereas chemical constituents, crude fibres and total carotenoids are expressed on a dry weight basis.

Table 7.1: Averages and standard errors of chemical constituents, crude fibre, water and total carotenoids content for *Ulva* samples from the farm and natural populations.

	Wild *U. rigida*	Wild *U. capensis*	Farmed *U. rigida*	Farmed *U. lactuca*	Sig. in location	Sig. in species	Sig. in month
Moisture content	79.0± 0.7	85.2 ± 1.0	80.6 ± 0.7	80.8 ± 0.7		*	
Ash	20.1 ± 0.3	22.7 ± 0.3	19.3 ± 0.6	17.8 ± 0.7	*	*	
Crude fibre	3.7 ± 0.1	5.3 ± 0.1	3.5 ± 0.1	3.5 ± 0.0		*	
Total carotenoids	4.8 ± 0.1	4.9 ± 0.2	6.7 ± 0.2	7.3 ± 0.2	*	*	
Carbon	31.5 ± 0.2	31.3 ± 0.2	31.3 ± 0.2	30.6 ± 0.1			
Nitrogen	3.3± 0.1	3.4 ± 0.	2.70 ± 0.2	2.1 ± 0.1			
Phosphorous	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1± 0.00			*
Potassium	2.3±0.07	2.3±0.51	2.5± 0.25	2.7 ± 0.3		*	*
Calcium	0.1± 0.1	0.7 ± 0.1	0.8 ± 0.4	0.5 ± 0.1			*
Magnesium	2.2 ± 0.2	2.6± 0.1	2.5 ± 0.1	2.8 ± 0.1		*	*
Manganese	0.00068 ± 0.5	0.00099± 0.4	0.00071 ± 0.7	0.00066 ± 0.5			*
Iron	0.01068 ± 8.3	0.00938± 6.6	0.00758±12.6	0.00623 ± 5.9		*	*
Copper	0.00017 ± 0.2	0.00012 ± 0.1	0.00013 ± 0.3	0.0001 ± 0.1		*	*
Zinc	0.00088 ± 0.7	0.00142 ± 0.8	0.00073 ± 0.8	0.00053 ± 0.4			
Boron	0.00501 ± 4.1	0.00462 ± 5.9	0.00425 ± 2.2	0.00423 ± 1.6		*	*

7.3.1 Moisture content

Wild *U. capensis* had significantly higher moisture content than wild *U. rigida* (Fig. 7.1; $p<0.001$). However, the water contents of farmed *U. lactuca* and *U. rigida* were not statistically different from each other (2-way ANOVA, $p<0.05$). Moreover, there was no significant difference in the moisture content of *U. rigida* from the farm and wild. There were no clear seasonal patterns in the moisture content. However, wild *U. rigida* has water contents that were always lower than wild *U. capensis*. *U. rigida* was the only species that was collected out of the water, and its water content was not different from that of the farmed material.

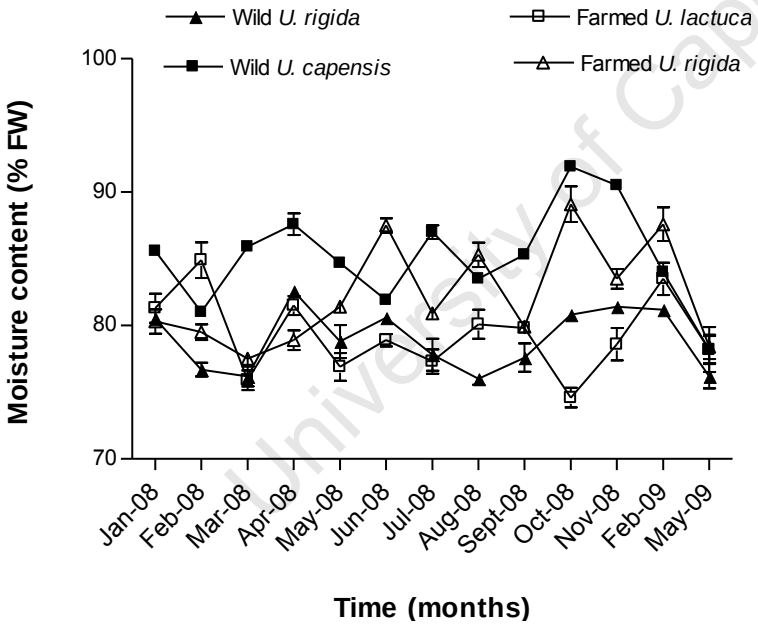

Figure 7.1: Monthly water content of *U. capensis* and *U. rigida* collected from Kommetjie and *U. lactuca* and *U. rigida* collected from I & J farm (growing in aquaculture effluent).

7.3.2 Ash content

The ash content (Fig. 7.2) of *U. capensis* from Kommetjie ranged from 21.5% (January, 2008) to 22.9% (May, 2009), significantly higher than *U. rigida* from the same locality which ranged from 19.3% (January, 2008) to 19.9% (May, 2009) (2-way ANOVA, $p<0.01$). The ash contents of wild and farmed *U. rigida* were not significantly different from each other ($p>0.05$). Ash levels of *U. lactuca* and *U. rigida* from I & J farm ranged from 19.5% and 19.3% (January, 2008) to 20.7% and 19.9% (May, 2009), respectively and were not statistically different from each other ($p>0.05$). The average for wild *U. capensis* was higher than other species and the average for all the samples was higher in the wild compared to the farm (Table 7.1). There were no clear seasonal patterns except perhaps a tendency for an increase in concentration during the late-winter spring months, reaching a maximum around September and October 2008 (Fig 7.2). However, farmed *U. lactuca* showed a pattern that was not clear due to rapid fluctuations (similar to its moisture content).

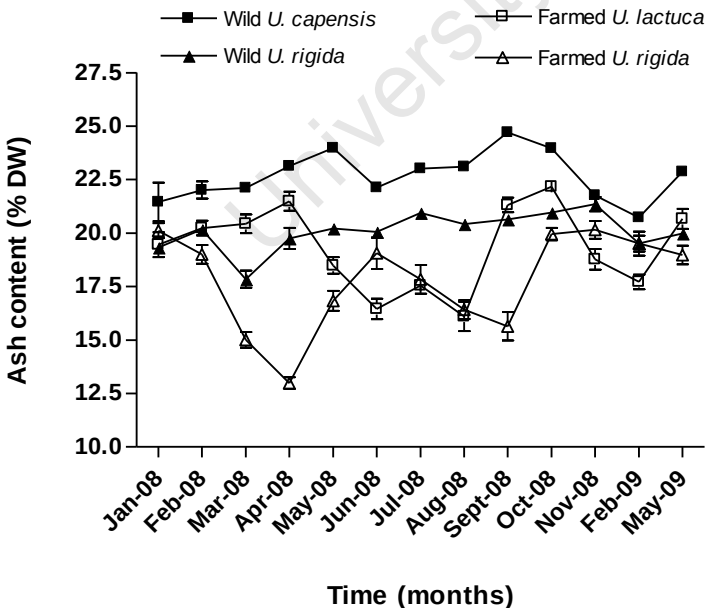

Figure 7.2: Monthly ash content of *U. capensis* and *U. rigida* collected from Kommetjie and *U. lactuca* and *U. rigida* from I & J farm (grown in aquaculture effluent).

7.3.3 Crude fibre content

As shown in figure 7.3, the crude fibre content of wild *Ulva* species ranged from 3.11 to 5.71 % dw, and *U. capensis* had significantly higher crude fibre than *U. rigida* (ANOVA, $p<0.0001$). The crude fibre did not differ significantly between farmed *Ulva* species, and there was no significant difference among months ($p>0.05$) (Fig. 3). Fibre differed significantly (ANOVA, $p< 0.05$) between locations with much higher average values for the wild compared to the farm (Table 7.1). However, when comparing *U. rigida* from the wild and farm, there was no significant difference in their crude fibre content. Overall, wild *U. capensis* had significantly higher crude fibre content than farmed *Ulva* and wild *U. rigida* ($p<0.05$). As can be seen in Table 7.2, the mean crude fibre obtained from wild and farmed *Ulva* species was compared with other crops.

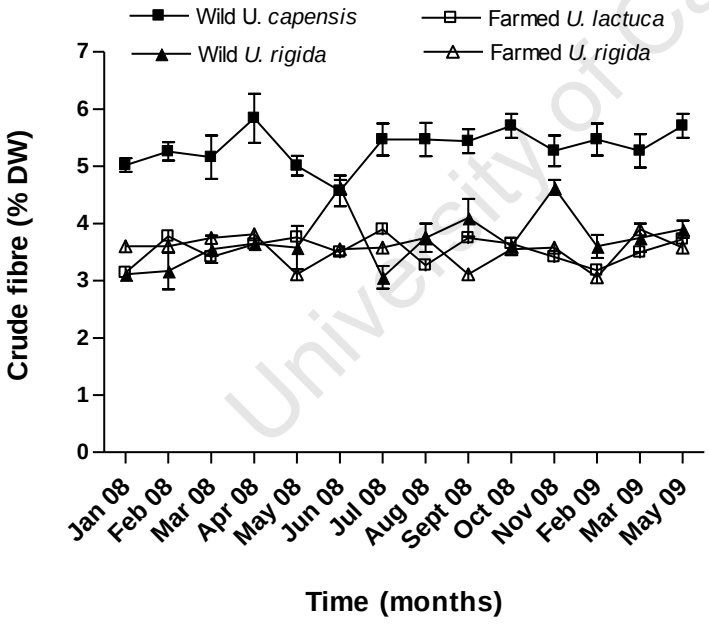

Figure 7.3: Monthly crude fibre content of *U. capensis* and *U. rigida* collected from Kommetjie and *U. lactuca* and *U. rigida* collected from I & J farm (growing in aquaculture effluent).

Table 7.2: Comparisons of mean crude fibre obtained from wild and farmed *Ulva* species, with other crops.

Sample type	Whole corn*	Soy meal*	Wild *U. rigida*	Wild *U. capensis*	Farmed *U. rigida*	Farmed *U. lactuca*
Crude fibre (% DW)	1.69 ± 0.16	3.70 ± 0.20	3.73 ± 0.12	5.34 ± 0.08	3.49 ± 0.05	3.51 ± 0.04

*** Results of the international collaborative study of the Filter Bag Technique for crude fibre (AOCS Ba 6a-05, 2005).**

7.3.4 Total carotenoid concentration

Total carotenoid concentrations in *U. rigida* and *U. capensis* from natural populations were not significantly different from each other and there was no significant difference among months (Fig. 7.4)**.** There was no significant difference between the total carotenoid concentrations in farmed *U. rigida* and *U. lactuca* during the study period. Farmed *U. rigida* had higher total carotenoids contents compared to wild *U. rigida*. Total carotenoids differed significantly (ANOVA, $p < 0.05$) between locations with much higher average values for the farm compared to the wild (Table 7.1).

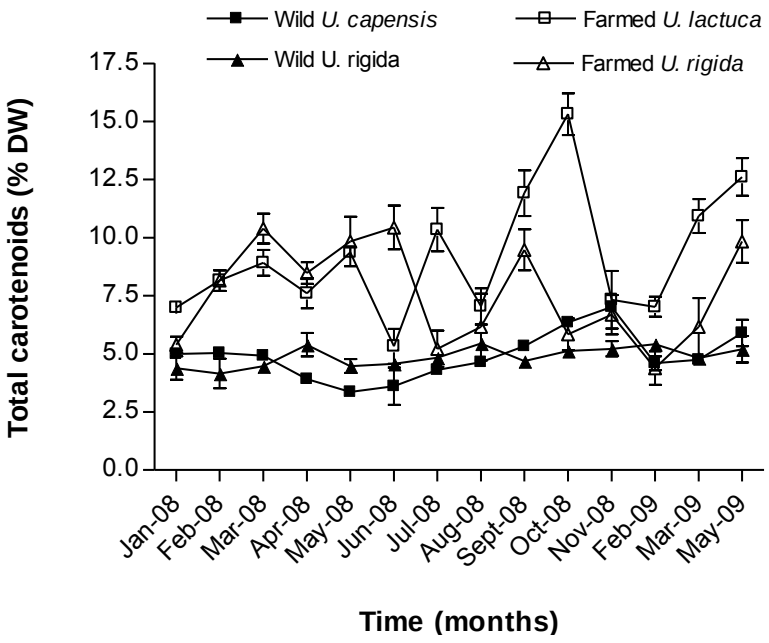

Figure 7.4: Monthly total carotenoids content of *U. capensis* and *U. rigida* collected from Kommetjie and *U. lactuca* and *U. rigida* collected from I & J farm (growing in aquaculture effluent).

7.3.5 Carbon content

The carbon contents were not significantly different ($p < 0.05$) between locations and species (Table 7.1). Carbon contents differed significantly among months (Fig 7.5) implying temporal variation in concentration.

7.3.6 Nitrogen content

Nitrogen contents differed significantly between locations ($p < 0.05$) (Table 7.1). The average nitrogen content of wild *Ulva* was higher than farmed *Ulva*. Moreover, wild *Ulva* did not show any seasonal pattern, and contents remained relatively constant throughout the year. Farmed *U. lactuca* tended to show a seasonal pattern with higher nitrogen during winter and

there were variations in the nitrogen content of farmed *U. rigida*, but the pattern was not clear due to rapid and random fluctuations (Fig. 7.5).

7.3.7 C:N ratios

As shown in figure 7.5, C:N values of both wild and farmed *Ulva* species ranged from 8 to 15.7. There was a slight temporal pattern in the C:N ratio of the wild species which was caused by the seasonal pattern of carbon. Contents tended to increase during May and October. However, for the rest of the year the ratio was fairly constant as a result of the lack of temporal variation in nitrogen content (Fig 7.5). Farmed *Ulva* species had rapid variations on the C:N ratio due to variations in their carbon and nitrogen contents. Nevertheless, C:N ratio did not differ significantly (ANOVA, $p>0.05$) between locations.

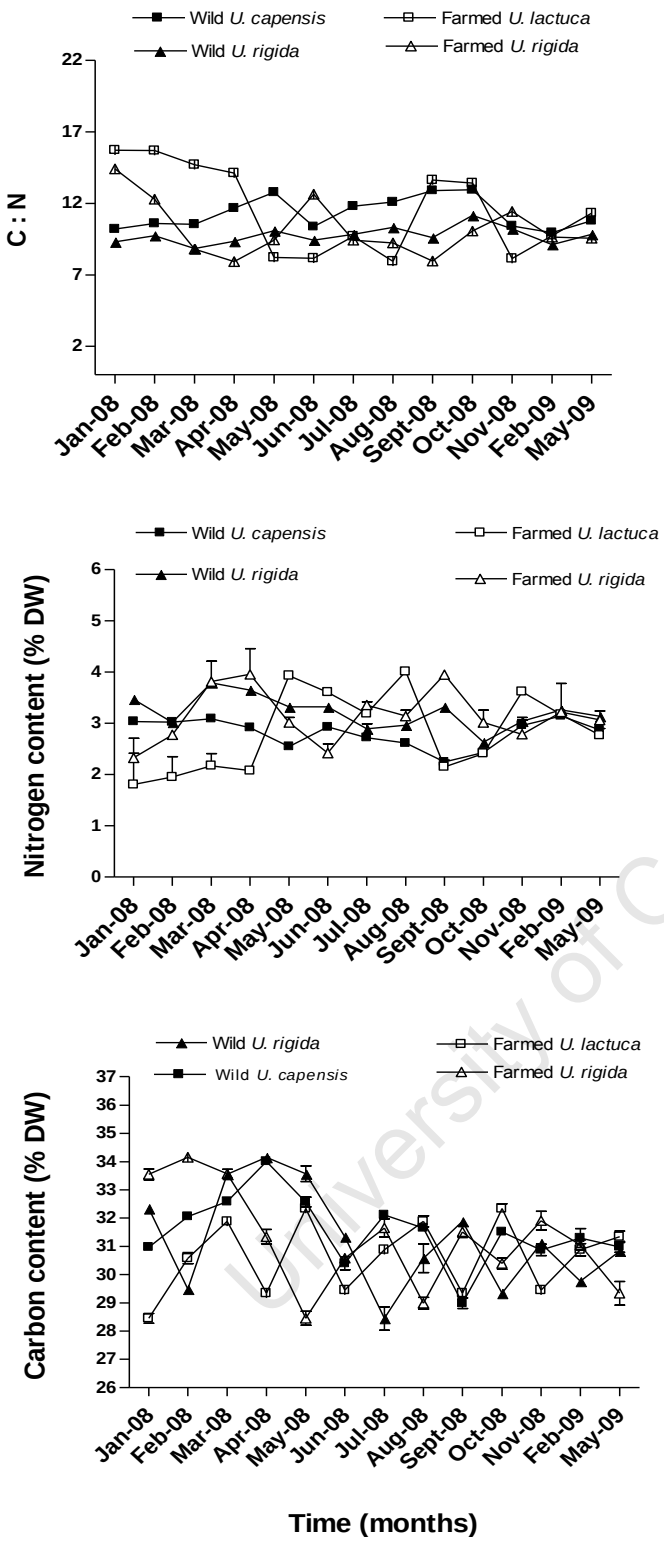

Figure 7.5: Monthly C:N ratios, nitrogen and carbon content of *U. capensis* and *U. rigida* collected from Kommetjie and *U. lactuca* and *U. rigida* collected from I & J farm (growing in aquaculture effluent).

7.3.8 Phosphorus content

Average contents of phosphorus were not significantly different between locations (ANOVA, $p>0.05$) (Table 7.1). The concentrations remained relatively constant for farmed *U. lactuca* and the other species showed some variations (Fig 7.6).

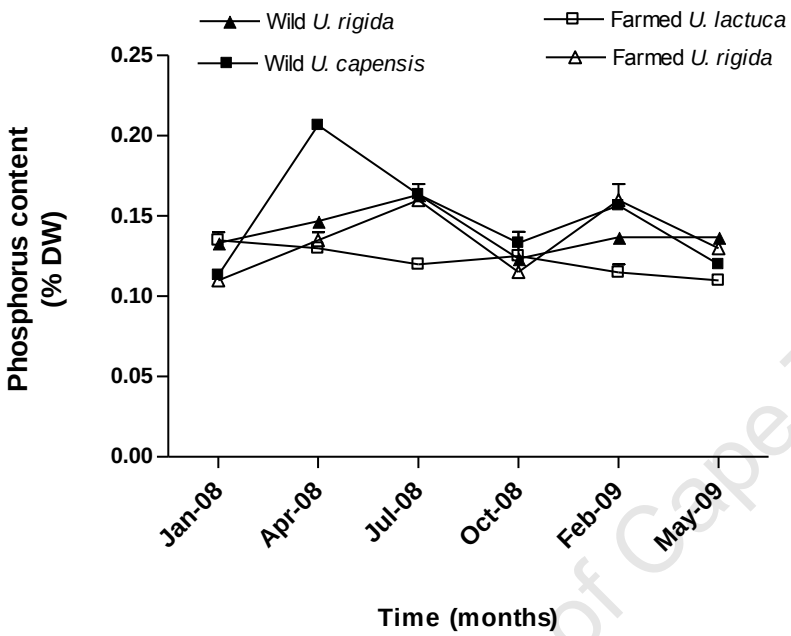

Figure 7.6: Phosphorus content of *U. capensis* and *U. rigida* collected from Kommetjie and *U. lactuca* and *U. rigida* collected from I & J farm (growing in aquaculture effluent).

7.3.9 Potassium content

The potassium content of both wild *Ulva* species ranged from 1.3 to 3.8% DW. The potassium contents were significantly different for both month and location ($p < 0.05$) with higher average values for the farm (Table 7.1). The contents tended to be quite variable throughout the year (Fig. 7.7).

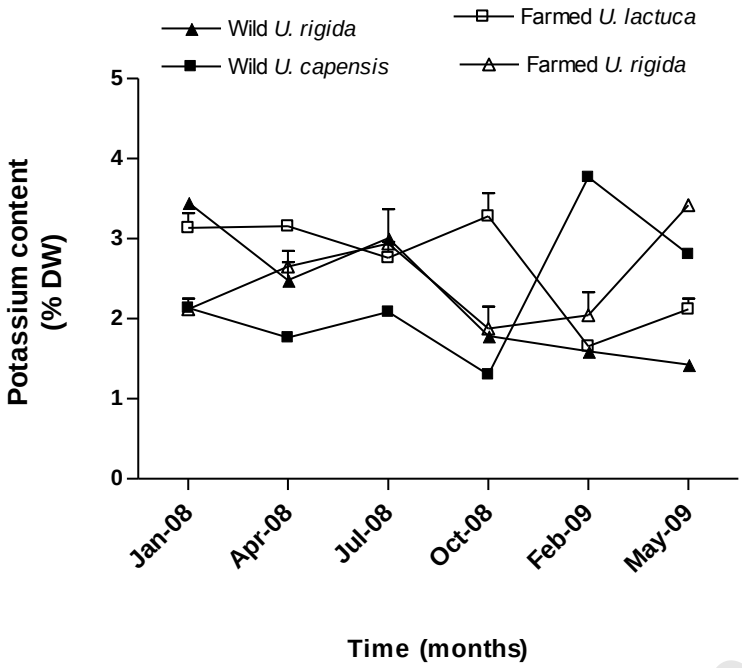

Figure 7.7: Potassium content of *U. capensis* and *U. rigida* collected from Kommetjie and *U. lactuca* and *U. rigida* collected from I & J farm (growing in aquaculture effluent).

7.3.10 Calcium content

The calcium content of wild and farmed *Ulva* species was not significantly different from each other (Fig. 7.8) ($p > 0.05$) and the contents did not appear to show any clear seasonal pattern.

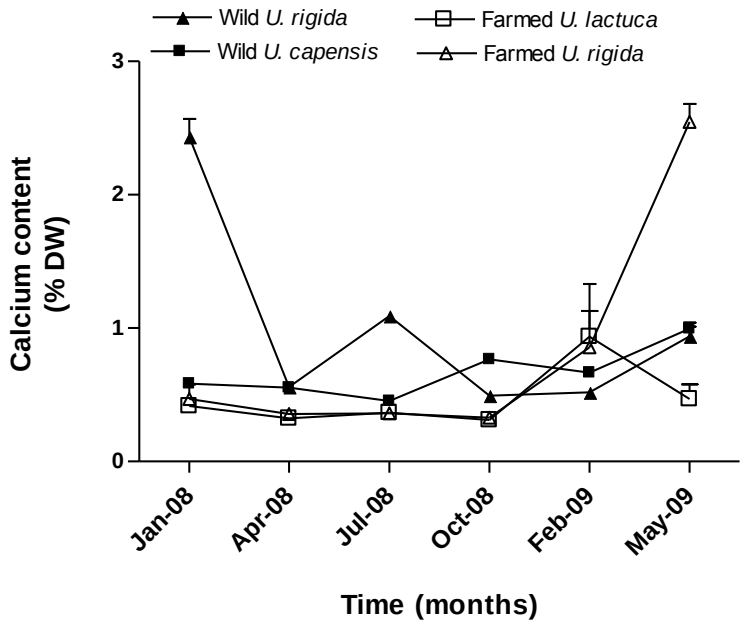

Figure 7.8: Calcium content of *U. capensis* and *U. rigida* collected from Kommetjie and *U.lactuca* and *U. rigida* collected from I & J farm (growing in aquaculture effluent).

7.3.11 Magnesium content

The average magnesium content was not significantly different for location ($p > 0.05$) (Table 7.1), however, there were variations among the months and species ($p < 0.05$) (Fig. 7.9). Wild *U. capensis* had higher average magnesium content of 2.6 ± 0.2 compared to wild *U. rigida* which had an average of 2.2 ± 0.1. Moreover, farmed *U. rigida* accumulated higher contents than wild *U. rigida* with an average of 2.5 ± 0.1.

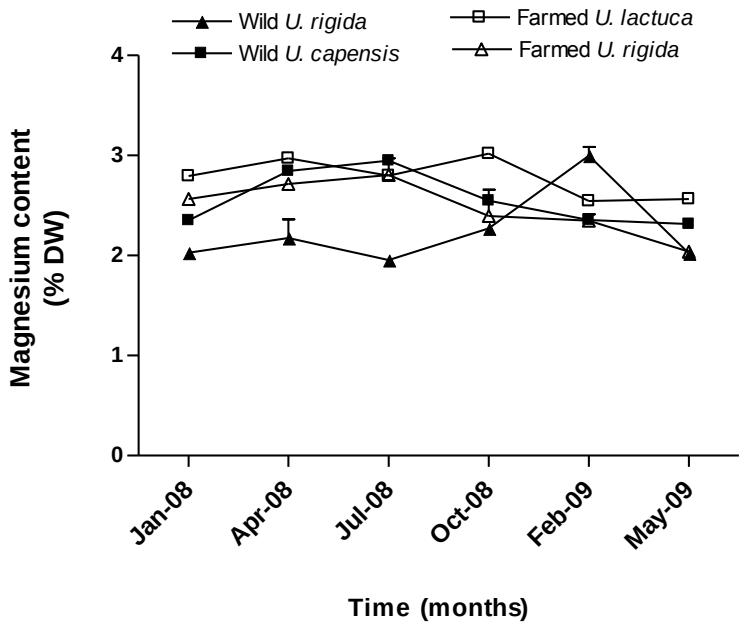

Figure 7.9: Magnesium content of *U. capensis* and *U. rigida* collected from Kommetjie and *U. lactuca* and *U. rigida* collected from I & J farm (growing in aquaculture effluent).

7.3.12 Manganese content

The averaged manganese content was not significantly different for location ($p > 0.05$) (Table 7.1), however, there were variations among the months and species ($p < 0.05$) (Fig. 7.9). For instance, wild *U. rigida* had higher manganese content in February 2009 and there was no significant difference in the manganese content of wild *Ulva* species during other months. Farmed *U. lactuca* had a higher manganese content during January 2008 whilst farmed *U. rigida* had higher contents during February 2009, however there was no significant difference in the manganese content of both farmed *Ulva* species during other months ($p > 0.05$).

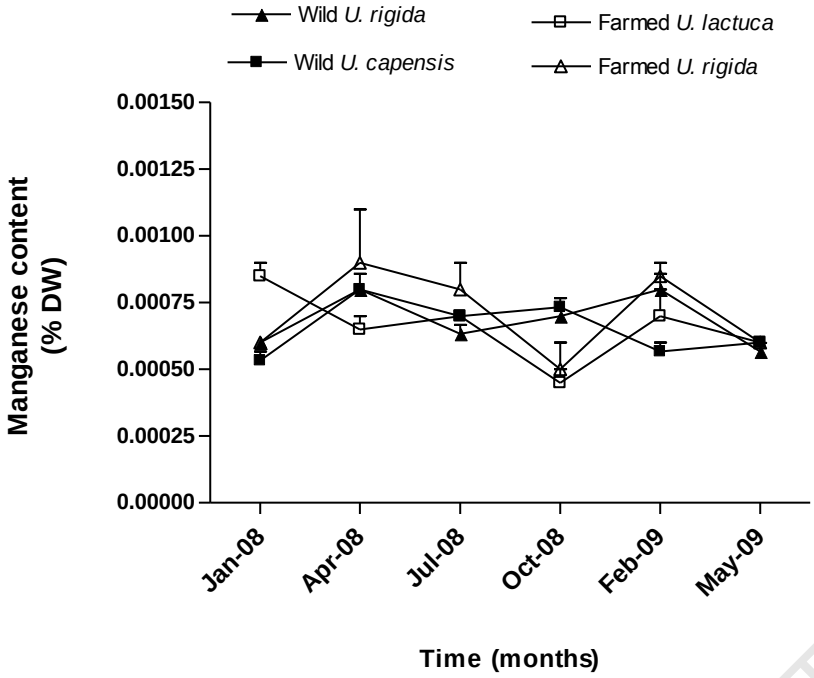

Figure 7.10: Manganese content of *U. capensis* and *U. rigida* collected at Kommetjie and *U. lactuca* and *U. rigida* collected from I & J farm (growing in aquaculture effluent).

7.3.13 Iron content

The iron content of wild *Ulva* ranged from 0.007 to 0.013 % DW and there was no statistical difference in the iron content of wild *Ulva* species ($p>0.05$) (Fig. 7.11) except in January 2008 when wild *U. rigida* had significantly higher iron content. Furthermore, farmed *U. rigida* had significantly higher iron content during October 2008 and May 2009 as compared to farmed *U. lactuca* (2-way ANOVA, $p<0.05$). When comparing the average iron content of wild and farmed *Ulva* species there were no significant differences (Table 7.1).

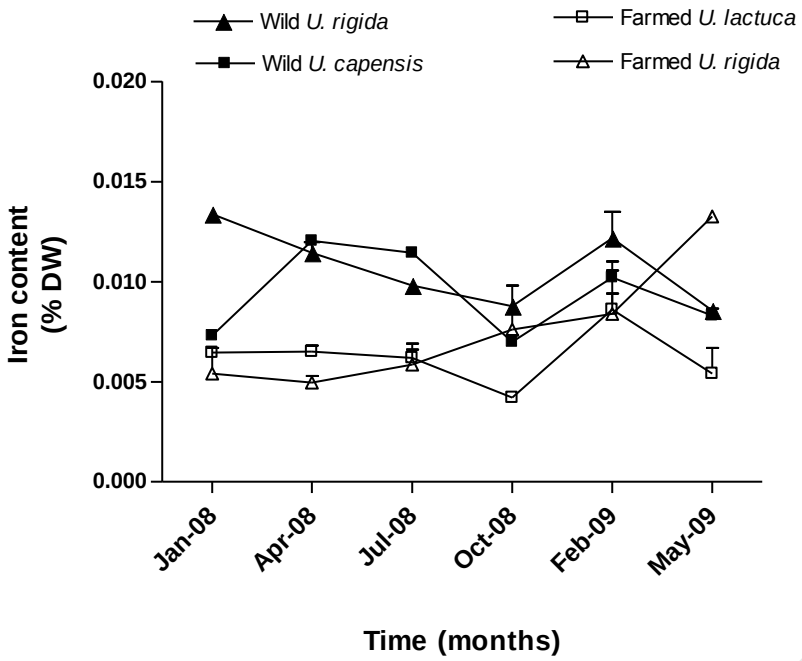

Figure 7.11: Iron content of *U. capensis* and *U. rigida* collected from Kommetjie and *U. lactuca* and *U. rigida* collected from I & J farm (growing in aquaculture effluent).

7.3.14 Copper content

The copper content of wild *U. rigida* was significantly higher during January 2008 compared to wild *U. capensis* and farmed *Ulva* species (2-way ANOVA, $p<0.05$), however, there was no significant difference during other months ($p>0.05$) (Fig. 7.12). There was no significant difference in the copper content of farmed *Ulva* species except during May 2009 when *U. rigida* had significantly higher copper content than *U. lactuca*. When comparing *U. rigida* from the wild and farm, during January 2008, wild *U. rigida* had higher copper content than farmed *U. rigida*. However, farmed *U. rigida* had higher copper content during May 2009.

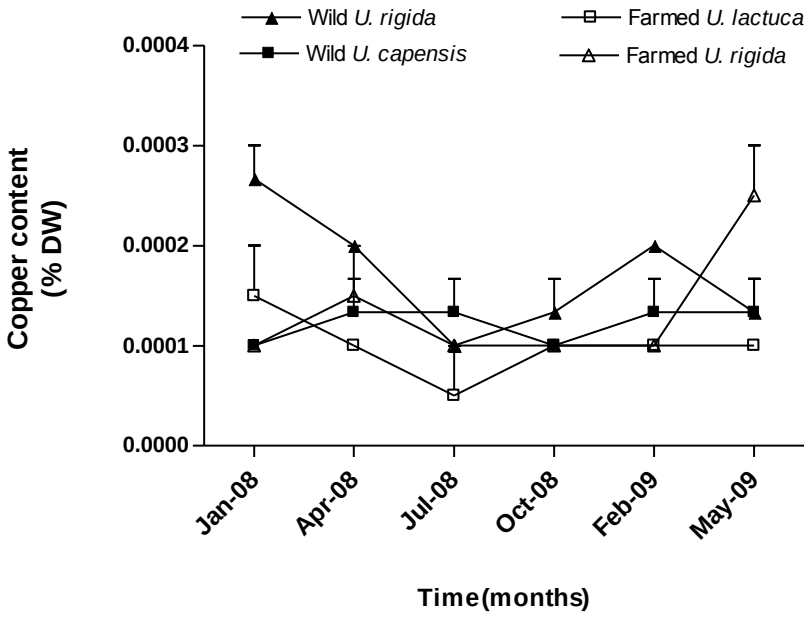

Figure 7.12: Copper content of *U. capensis* and *U. rigida* collected from Kommetjie and *U. lactuca* and *U. rigida* collected from I & J farm (growing in aquaculture effluent).

7.3.15 Zinc content

As shown in figure 7.13, the zinc content of both wild and farmed *Ulva* species ranged from 0.00047 to 0.01% DW and 0.0004 to 0.00105% DW respectively. During February 2009, farmed *U. rigida* had significantly higher zinc content (2-way ANOVA, $p < 0.05$). However, there was no significant difference in the zinc content of both wild and farmed *Ulva* species during other months (2-way ANOVA, $p > 0.05$).

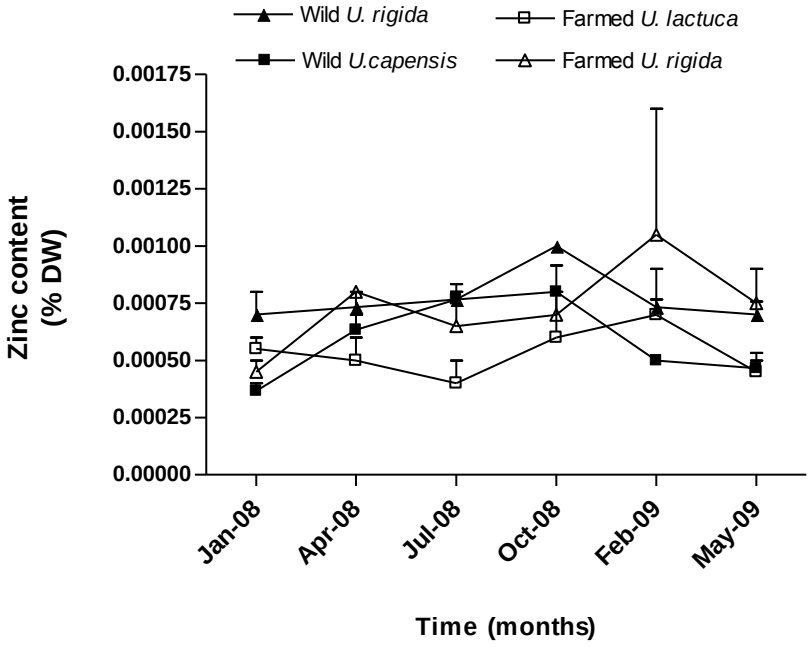

Figure 7.13: Zinc content of *U. capensis* and *U. rigida* collected from Kommetjie and *U. lactuca* and *U. rigida* collected from I & J farm (growing in aquaculture effluent).

7.3.16 Boron content

When comparing *U. rigida* from the two locations, wild *U. rigida* had significantly higher boron content than farmed *U. rigida* (Fig. 7.14) with average contents of 0.00501 ± 0.00034 and 0.00425 ± 0.00019, respectively. . There was a significant difference in the boron content of wild *Ulva* species ($p<0.05$) during April, October 2008 and February, May 2009. There was no significant difference in the boron content of farmed *Ulva* species apart from May 2009 when farmed *U. rigida* had a higher content.

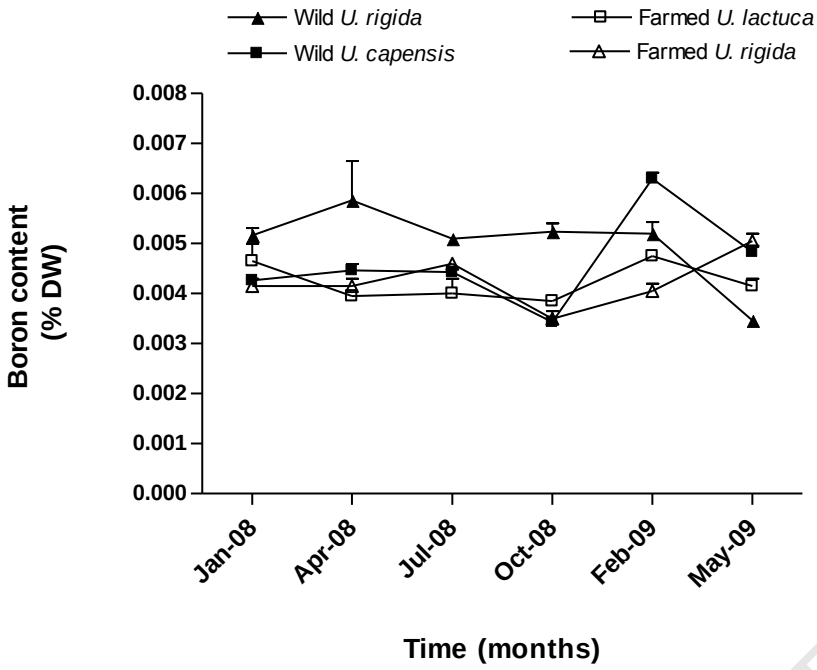

Figure 7.14: Boron content of *U. capensis* and *U. rigida* collected from Kommetjie and *U. lactuca* and *U. rigida* collected from I & J farm (growing in aquaculture effluent).

7.4 Discussion

Information on the biochemical constituents of seaweed is vital in understanding their nutritive values. The aim of this study was to investigate the seasonal variations in the chemical composition and mineral content of wild and cultivated *Ulva* with emphasis on any general or temporal differences between *U rigida* in the wild and aquaculture conditions, since it might have been expected that aquaculture conditions would produce differences. The study also aimed at investigating differences or similarities between two apparent species in the wild which have different habitats but are thought to be very closely related, if not conspecific. Many of the elements that were analysed showed significant temporal, interspecific or inter-site differences, while in other cases no differences were detected. While details of significant differences were noted in the Results section because they may be useful to abalone farmers and researchers, in many cases sudden (*e.g.* temporal) fluctuations could not be explained except in general terms (*e.g.* temporal changes in seawater nutrients, growth rates etc.). As a result, only the more important features of the results are discussed here.

The tissue water content for both wild and farmed *Ulva* species fell within the range of 80 – 90 % which is the case for many macro-algae (DeBoer, 1981). There was a slight seasonal pattern in the moisture content of *Ulva* at both locations: the monthly average was at its lowest during winter and rose during spring and summer, although farmed *U. lactuca* showed a pattern that was not clear due to rapid and random fluctuations. I would have expected *Ulva* in the farm to have higher growth rates due to fertilising which could perhaps increase the water content, however, *U. capensis* from the wild had higher water content.

Ash contains mostly magnesium, phosphorus, potassium, calcium and iron as well as trace minerals in small amounts. There are thus many other elements in the ash that were not

measured in the current study. The ash content of wild *Ulva* species showed a seasonal trend with an increase during the late-winter to spring months, reaching a maximum around September and October 2008. Similar findings on seasonal fluctuation in the ash content were observed in nine intertidal algae at Dalebrook, an exposed shore in the Cape of Good Hope. Ash values were lowest in winter and highest in spring or summer (McQuaid, 1985b). Msuya and Neori (2002) reported an ash content of 22.2% DW in *U. reticulata* growing in effluent from tidal fishponds in Tanzania and this value is close to those obtained for wild *U. capensis* in the current study. In addition, Ratana-arporn and Chirapart (2006) obtained an ash content of 17.58% DW for *U. reticulata* from Pattani Bay in Thailand that was closer to the values recorded for farmed *U. lactuca* in the present study. Generally, the ash content of seaweeds is much higher than those of terrestrial vegetables other than spinach (Sanchez-Machado *et al*., 2004. The farmed material shows more variation in the ash content than the wild material, and the farmed material generally has less ash (mineral) content than wild material and this can be a concern for abalone farmers.

The crude fibre content of wild and farmed *Ulva* species is similar to that of soy meal, which is considered to be a nutritional product (Table 7.2). Ratana-arporn and Chirapart (2006) recorded the crude fibre value of 4.84% DW in *U. reticulata* from Pattani Bay which was close to values obtained in the present study. Crude fibre content was markedly high in wild *U. capensis* compared to other *Ulva* species.

Farmed *Ulva* species had higher carotenoid contents than wild species and there seems to be an inverse relationship between nitrogen level and accumulation of carotenoids. The relation between nitrogen and carotenoids has previously been shown (e.g Orosa *et al*., 2000; Chenard *et al*., 2005). Farmed *Ulva* had lower tissue nitrogen content than *Ulva* from the wild. Under environmental stress conditions *e.g.* nitrogen deficiency, high irradiances or high salt

concentrations, green algae of the genus *Dunaliella* and *Haematococcus* over-accumulate secondary carotenoids such as β-carotene or astaxanthin (Ben-Amotz and Avron 1988; El-Baz *et al*., 2002). This implies that under the current culture conditions at I & J farm, *Ulva* is subjected to stress conditions especially low nitrogen level in the culture medium (see chapter 6) and this could result in enhanced accumulation of carotenoids. Cyrus (2009, unpublished data) is investigating the potential culturing of sea urchin *Tripneustes gratilla* in South Africa using artificial diets with varying *Ulva* composition. His preliminary results show that inclusion of *Ulva* in the artificial diet significantly enhanced the colour of gonads. In addition, a study by Cruz-Suarez *et al.* (2009) investigating the comparison of *U. clathrata* and the kelps *Macrocystis pyrifera* and *Ascophyllum nodosum* as ingredients in shrimp feeds, found that shrimps fed on *Ulva* developed a markedly darker red colour.

Nitrogen is one of the elements that can limit the growth of seaweeds in the natural environment, and seaweed growth tends to parallel nitrogen supply at a particular instant (Duke *et al*., 1989). *Ulva* in the farm is fertilised on a weekly basis hence higher nitrogen would be expected. However, contrary results were obtained: the average nitrogen content of wild *Ulva* was higher than farmed *Ulva* and the difference in N levels could be due to differences in water motion. At high levels of water motion, seaweeds take up nutrients faster; perhaps lower N in farmed *Ulva* is more likely to be due to lower water motion than lower N levels in the medium. Water flow is one of the factors that is known to affect the growth and chemical composition of *Ulva* (and other cultured seaweeds) in tank cultivation (Duke *et al.* 1989; Friedlander *et al.,* 1990). Under conditions of low water flow, seaweeds growing in the culture systems are known to be nutrient (carbon or nitrogen) limited (Lapointe *et al*., 1976).

In the present study, carbon content increased during summer and decreased during winter but was not significantly different between species and locations (wild and farm). Higher percentages of carbon content in the dry tissue of these *Ulva* species indicate higher caloric contents (Platt and Irwin, 1973) and thus *Ulva* is likely to have a similar energy value as feed whether collected from the wild or grown in aquaculture systems.

The C:N ratio is a useful indicator of the N (and therefore protein) content of food for humans and other animals. Russell-Hunter (1970) demonstrated that most animals need a C:N ratio of 17 or less in their diet. In the present study the C:N ratio for both wild and farmed *Ulva* species ranged from 7.9 to 15.7, indicating that these species contain sufficient N for an animal diet.

The average calcium concentrations did not reveal a seasonal pattern and there were no difference between species or locations. Calcium is an important component in the formation and strengthening of the abalone shell (Day, 1974; Culver *et al*., 1997) and the levels measured in the present study are nutritionally useful to the abalone and in human nutrition.

Phosphorus in seawater is available to marine algae at concentrations of 1-3 µM and can be a limiting factor for seaweed growth under high seaweed densities (DeBoer, 1981). The tissue phosphorus values in the current study ranged from 0.1 to 0.2 % DW and there were no clear temporal patterns. These values are similar to those reported by Msuya and Neori (2002) in *U. reticulata* growing in tidal fishpond effluent in Tanzania.

The trace metals (copper, manganese, iron, boron and zinc) had low values and revealed no temporal patterns. Trace metals are important co-factor of enzymes and catalyse in metabolic reactions (Gaetke and Chow, 2003). Zinc is also an essential micronutrient for the growth and

shell bio-mineralization in abalone (Liao *et al*., 2002; Mai *et al*., 2003; Liao and Ling, 2004). The levels found in *Ulva* species in the current study are very useful for animal and human nutrition.

Overall, it seems clear that in the wild material, temporal changes in the contents of the various elements are more gradual than in cultivated material, where changes are rapid and often haphazard. The latter is probably a result of how water quality is managed on the farm, for example the periodic addition of fertilizers. However, there were no clear seasonal patterns in all *Ulva* species collected from both locations.

The results of the chemical composition and mineral content analyses show that *U. rigida* and *U. capensis* from the wild have slight but significant differences in their chemical composition and mineral content. .If they are indeed the same species, there might be two possibilities: (1) They might have evolved differences in their ability to take up and store certain elements which can only be proved by genotypic differences; (2) There is a possibility that the different habitats may cause phenotypic differences in plants.

Ulva is a nutritionally rich species for abalone feed in terms of chemical composition and mineral content. Biological analysis using abalone feeding trials would be necessary to establish the absolute nutritional value of wild and cultivated *Ulva.* Finally, a lack of seasonal pattern is a benefit for using *Ulva* as feed because the *Ulva* quality is consistent throughout the year.

Chapter 8

Protein content, amino acid composition and calculation of nitrogen-to-protein conversion factors of *U. rigida* and *U. capensis* from natural populations and aquaculture

8.1 Introduction

Protein is the most critical component contributing to the nutritional value of food. Crude protein in food products was first quantified by Henneberg in 1865 who multiplied the total nitrogen content of animals by a conversion factor of 6.25 (Salo-Väänänen and Koivistoinen, 1996). For many years, quantification of crude protein content for most feed materials was determined by a conversion factor of total N x 6.25 (Dintzis *et al.,* 1988). This approach is based on two assumptions: that dietary carbohydrates and fats do not contain nitrogen, and that nearly all of the nitrogen in the diet is present as amino acids in proteins. On the basis of early determinations, the average nitrogen (N) content of proteins was found to be about 16 %, which led to the use of the calculation N x 6.25 (100/16 = 6.25) to convert nitrogen content into protein content (Jones, 1931).

In response to concerns about calculating protein content by N × 6.25, Jones (1931) suggested that N x 6.25 be abandoned and replaced by N multiplied by a factor specific for the food in question. These specific factors, now referred to as "Jones factors", have been widely adopted. Jones factors for the most commonly eaten foods range from 5.18 for nuts and seeds to 6.38 for milk. However, most foods with a high proportion of nitrogen as non-protein nitrogen (NPN) contain relatively small amounts of total N (Merrill and Watt, 1973). As a result, the range of Jones factors for major sources of protein in the diet is narrower and his disregard of non protein nitrogen was a limitation in the accuracy of his calculation. He was unable to correct it due to the lack of knowledge of non-protein constituents during his

time (Tkachuck, 1969). Therefore a correction factor is needed to account only for the nitrogen found as protein amino acid which represents the "true protein" contents (Tkachuk, 1969). Since then several authors such as Heathcote (1950) and Tkachuck (1966b) used quantitative amino acid data to calculate the nitrogen-protein factors for plants such as oats, cereals and oilseeds. The use of specific nitrogen-to-protein conversion factors (N-Prot factors) using quantitative amino acid profiles is the most practical way of determining protein content. The establishment of specific N-Prot factors is gaining attention and specific nitrogen-to-protein conversion factors for several plants species have been documented in the literature. For example, Heathcote (1950) established a conversion factor of 5.40 for oats; values of 5.68, 5.79 and 5.71 were reported by Ewart (1967) for wheat, barley and rye flours; in some tropical plant tissues, values of 5.1 to 5.8 have been established by Milton and Dintzis (1981); Khanizadeh *et al* (1992) established a conversion factor value of 5.51 for apple flower buds; Yeoh and Truong (1996 a, b) proposed factors of 3.59 for sweet potato and 4.48 for cassava roots; Levey *et al* (2000) established a factor of 5.64 for wild fruits from south-eastern United States. Conversion factors for various macroalgae and microalgae have been found to vary from 3.75 to 5.72 (Lourenço *et al.,* 2002; 2004). Aitken *et al.* (1991) determined a factor of 5.00 for two species of the edible red alga *Porphyra* from New Zealand.

Furthermore, one of the alternatives for measuring protein content of algal biomass is by colorimetric methods (Lowry *et al.,* 1951; Bradford, 1976; Smith *et al.,* 1985). Colorimetric assays rely on the binding of a dye to protein or of the protein being involved in a redox reaction. Colorimetric assays are sensitive to interferences (Peterson, 1979) and the interference is a consequence of the effects of some chemical substances on specific amino acids, since the chemical reactions that produce the quantification of protein result from the

reactivity with side groups of the amino acids (Peterson, 1983; Stoscheck, 1990). As a result the amino acid composition of each species is important in the results obtained with different methods because of their distinct reactivity (Compton and Jones, 1985). Therefore both methods of N x 6.25 and colorimetric methods require some caution to produce reliable end results.

Moreover, the protein content of seaweeds differs according to the time of the year and species (Fleurence, 1999). For example, a seasonal monitoring of protein level from *Palmaria palmata* (Dulse) collected on the French Atlantic coast showed that the crude protein content of this alga can vary between 9 and 25% dry weight (Galland-Irmouli *et al.*, 1999). Seasonal variation of the algal protein content was also reported for various species including *Ulva lactuca* (Abdel-Fattah and Sary, 1987). Generally, the crude protein fraction of brown seaweeds is low (3 - 15% of the dry weight) compared with that of the green or red seaweeds (10 - 47% of the dry weight) (Arasaki and Arasaki, 1983). In some green seaweeds such as species belonging to the genus *Ulva,* the crude protein content can range between 10 and 26% dry weight. For instance, *Ulva pertusa* was reported to have a high crude protein level of between 20 and 26% dry weight (Arasaki - Fujiwara *et al.,* 1984). A local study by Robertson-Andersson (2003) who looked at the crude protein content of *Ulva* grown on an experimental scale at Jacobs Bay (Jacobsbaai) Sea Products Abalone Farm (JSP) in Jacobs Bay obtained an average tissue crude protein values of 36.6 % from *Ulva* grown in the turbot, 33.35 % in abalone effluent and 30.03 % in seawater.

In addition, the amino acid composition of seaweeds has been frequently studied and compared to that of other foods such as eggs or soybean (Fleurence, 1999). Amino acids are divided into essential and non essential amino acids. Essential amino acids cannot be synthesized by the organism and therefore must be supplied in the diet whereas non-essential

amino acids can be synthesized in the body and do not have to be obtained from the diet. In aquaculture, the ratio of essential to non essential amino acids in the feed is as important as the protein content itself (Sales and Britz, 2001) and is often the first consideration when new processed feeds are formulated and the insufficiency of some important amino acids in seaweed could make it less suitable for abalone feed than the processed alternative (Mai *et al.* 1995).

The South African abalone aquaculture industry is expanding and there is a lack of information on the nutritional profiles of the seaweeds which are used as feed. *Ulva* cultivation on local abalone farms is a successful commercial enterprise and therefore in order to provide information on the nutritional value of this crop, this study aimed:

(i) To determine the protein and amino acid composition of wild and farmed *Ulva* in order to provide this information to abalone farmers.

(ii) To investigate whether there are temporal variation in the protein content of these *Ulva* species.

(iii) To establish the nitrogen to protein conversion factors for wild *U. capensis* and *U. rigida* as well as farmed *U. lactuca*. This is the first study in South Africa to establish such conversion factors for seaweeds.

(iv) To discuss the benefits of these findings to the local abalone aquaculture industry.

8.2 Materials and Methods

8.2.1 Sample collection

U. capensis and U. rigida used in this study were collected from the natural populations at Kommetjie (34º09′06″S, 18º19′22″E) on the borderline of the west coast and south-west coast of the Cape Peninsula, South Africa. The two *Ulva* species are found growing in the same intertidal zone where one grows in pools and the other on the rock. About 400 g wet weight of each species was collected monthly during low tide (Jan 2008 to May 2009), and three samples were collected for each species every month. In addition, *U. lactuca* and *U. rigida* were obtained every two weeks from an abalone/seaweed integrated system at I & J farm (34˚37'S, 19˚17'E) between Jan 2008 and May 2009 and samples were collected from four raceway ponds. The *Ulva* raceway ponds of this system were fertilized once a week with 1.5 kg of ammonium sulphate and supergrow (P: 203g/kg, S: 23,6g/kg, Ca: 171, 4g/kg) in a ratio of 6:1 (Luvuyo September, pers. comm.). All *Ulva* samples were briefly washed with freshwater to remove any epiphytes and weighed to determine the wet weight. The samples were then dried in an oven for 48 hours at 60°C, after which they were weighed for dry weight and milled to a fine homogenous powder through a 0.5 mm sieve. Milled dry *Ulva* samples were then analysed for total nitrogen, protein content and amino acid composition.

8.2.2 Direct protein measurement and calibration curve

The Bradford method (Bradford, 1976) was used to quantify protein in this study. This method requires comparing the spectrophotometric absorbance of the unknown sample at a wavelength of 595 nm to a calibration curve prepared using standard solutions of a protein. Bovine serum albumin (BSA) was used as a standard. Using BSA as the standard, the calibration curve was linear in the absorbance range of 0 to 1.5 corresponding to a protein concentration range from 0 to 2000 μg.mL^{-1} (Figure 8.1).

8.2.3 Extraction of protein

50 mg of *Ulva* powder was suspended in de-ionized water (1: 20 w/v) to induce cell lysis by osmotic shock that facilitated subsequent protein extraction. The suspension was gently stirred overnight at 35 ^{o}C, which has been shown to be the optimal temperature for seaweed protein solubility (Dua, Kaur and Ahluwalia, 1993). After incubation, the suspension was centrifuged at 10,000 rpm and 4 ^{o}C for 20 min. The supernatant was collected and the pellet was resuspended in de-ionized water in the presence of 0.5% (v/v) 2-mercaptoethanol (Venkataraman and Shivashankar, 1979) and pH of the mixture was adjusted to 12 with 1 M NaOH. The mixture was gently stirred at room temperature for 2 h before centrifugation under the same conditions as above. The supernatant was collected and combined with the previous supernatant. The extraction procedure mentioned above was repeated five times on the residue.

8.2.4 Nitrogen analysis

Total nitrogen analysis was done at the Department of Chemistry of the University of Cape Town using the following method: The total nitrogen content of dried *Ulva* biomass was measured by elemental analysis (CHNS elemental analyser (Thermo 1112 CHNS). Helium was used as a carrier gas. Acetanilide (C = 71.09%; N = 10.36%; H = 6.71%) was used to calibrate the instrument. Crude protein was determined from total tissue nitrogen by multiplying by a conversion factor of 6.25. For both wild and farmed *Ulva* species, nitrogen analysis was done monthly and three replicates were analysed.

8.2.5 Amino acid analysis

Amino acid analysis was done at the Department of Molecular and Cell Biology of the University of Cape Town. For all three *Ulva* species (wild *U. capensis* and *U. rigida*; farmed

U. lactuca), analysis was done for samples collected in January, April, July and October 2008), using the following methods. Five mg of each seaweed sample was weighed out and 3 mL boiling HCl was added to the seaweed and then 600 µl of 5% phenol then added as an antioxidant (final concentration of 1%). Hydrolysis was conducted at 110°C for 24 h under vacuum (purged with nitrogen). All samples were evaporated to dryness and dissolved in 100 µl NLE-100 (sample application buffer containing internal amino acid standard (norleucine at 100 nmol/ml). 10µl of each sample was injected on the cation-exchange column using pH gradient from pH 3 to pH 9.5 (column kept at 63°C). Amino acids were subjected to oxidation with sodium hypochlorite (5 ml of 6% hypochlorite in 500 ml borate buffer) in order to open up the proline ring for proline detection. Amino acids were detected using a post-column detection system with OPA (ortho-phthalaldehyde) reagent. Nitrogen in amino acids was determined by multiplying the concentration of individual amino acids by corresponding factors calculated from the percentage N of each amino acid (Sosulski and Imafidon, 1990). The ammonia content was included in the calculation of protein nitrogen retrieval, as it comes from the degradation of some amino acids during acid hydrolysis (Mossé 1990; Yeoh and Truong, 1996). The ammonia nitrogen content was calculated by the multiplication of ammonia by 0.824 (N =82.4% of NH_3) (Lourenco *et al.,* 2002). *Ulva* protein contents were calculated from the amino acid analysis and expressed as g/100 g dry weight of samples.

8.2.6 Calculation of nitrogen-to-protein conversion factors

Protein values in terms of dry weight percentage of amino acids plus NH_3 were calculated from amino acid profile data by using both hydrated and anhydrous amino acid formula weights. The assumption was made that NH_3 in the hydrolysates was derived from glutamine and asparagines (Dintzis *et al.,* 1988). The actual conversion factor for plant tissues should lie

between the hydrated- and anhydrous-based values, but should lie closer to those calculated on the basis of anhydrous amino acid residue weights (Morr, 1982).

The nitrogen to protein conversion factors were determined according to Mossé (1990) and the N-factor limits in the NF calculator were calculated from amino acid and nitrogen data.

(1) The upper limit is defined as:

$$k_A = \Sigma E_i / \Sigma D_i$$

Where:

E_i = the grams of the *i*th amino acid per100 grams of sample (dry weight basis)

D_i = the grams nitrogen of the *i*th amino acid per100 grams of sample (dry weight basis)

(2) The lower limit is defined as:

$$kp = \Sigma E_i / N$$

Where:

E_i = the grams of the *i*th amino acid per100 grams of sample (dry weight basis)

N = the grams nitrogen per100 grams of dry sample

(3) The appropriate N-Factor is average of k_A and k_P.

(4) The protein content of this sample is calculated as follows:

% protein = % nitrogen x NF

Where: NF = nitrogen factor

8.2.7 Statistical analysis

The analysis for this study was done using STATISTICA 8.0, mean values were analyzed by one- way ANOVA and Tukey-HSD at $P<0.05$ to detect significant differences among groups. 2-Way ANOVA was used to compare the protein content by the Bradford method and crude

protein values across species and seasons. Pearson correlation was used to determine the coefficient of variation of *Ulva* protein vs. *Haliotis midae* essential amino acids.

8.3 Results

8.3.1 Calibration curves

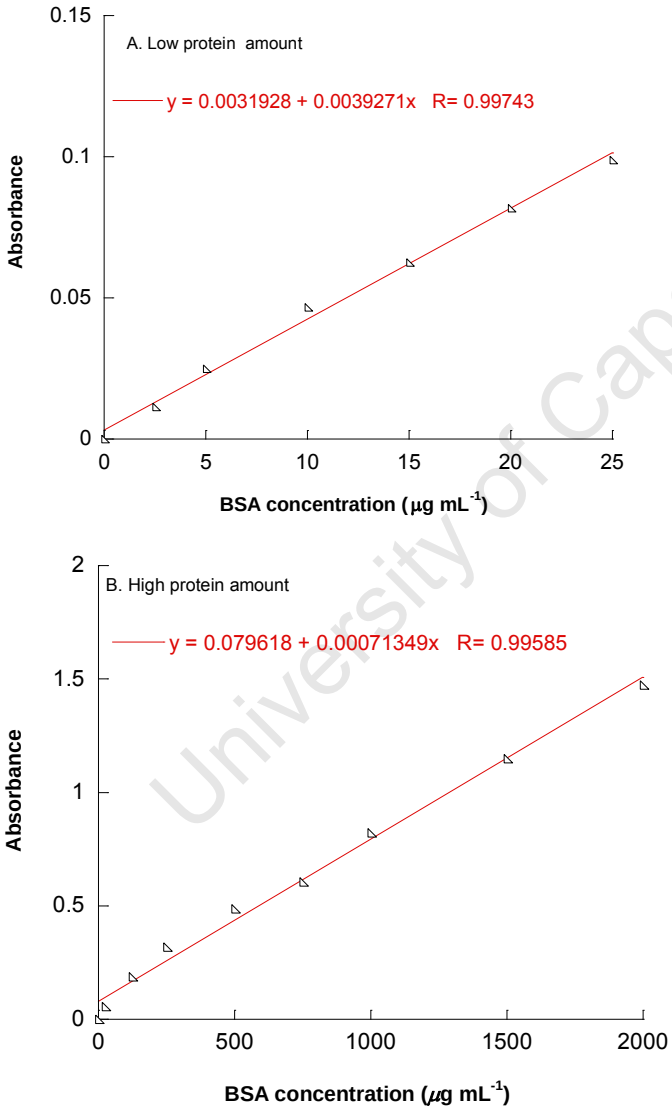

Figure 8.1: Absorbance versus protein (BSA) concentration standard curves.

8.3.2 Protein content by Bradford method and N x 6.25

The highest protein contents of wild *U. capensis* and *U. rigida* were recorded during February to June 2008 (ANOVA, $p<0.05$, Tukey test) (Fig. 8.2). During that period there seemed to be an increase in the synthesis of protein in both species. There were no temporal variations in the protein content of either species from July 2008 to May 2009. There was little temporal variation in the protein content of both species from May 2008 to May 2009. Protein content of wild *U. capensis* was always higher than that of *U. rigida* (2-way ANOVA, $P<0.05$). The average values for wild *U. capensis* were 6.31 ± 0.64 % DW and wild *U. rigida* were 4.60 ± 0.41% DW.

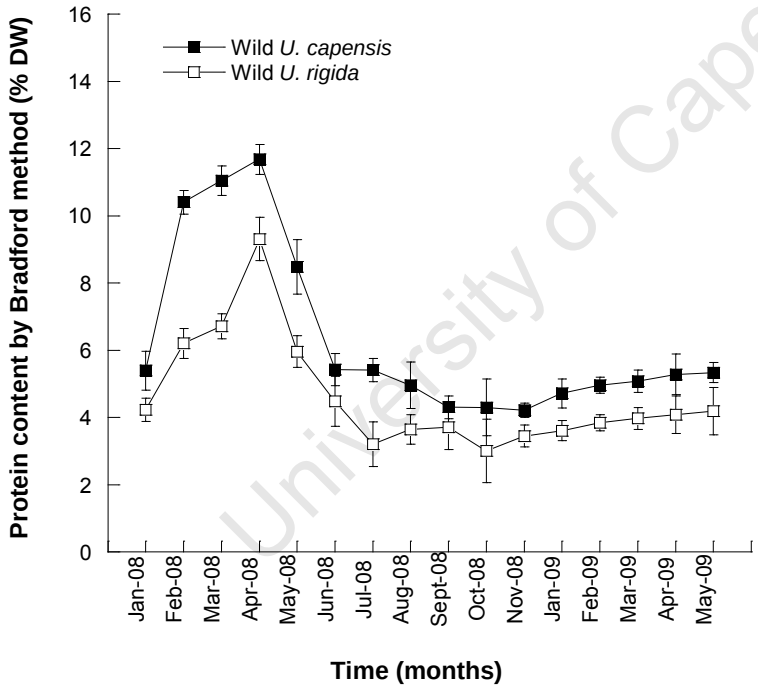

Figure 8.2: Monthly quantification of protein content by Bradford method in wild *U. capensis* and *U. rigida*. Bars denote standard error of three replicates.

There was no clear temporal variation in the protein content of farmed *Ulva* species (Figure 8.3). In addition, there was no significant difference in the protein content between the species (ANOVA, $p>0.05$). The protein content fluctuated considerably, with the protein content of farmed *U. lactuca* being between 5.23 and 9.89 % DW, whilst the contents of farmed *U. rigida* varied between 4.98 and 8.96 % DW. The average values for farmed *U. lactuca* were 7.28 ± 0.24 % DW and farmed *U. rigida* were 6.67 ± 0.22% DW.

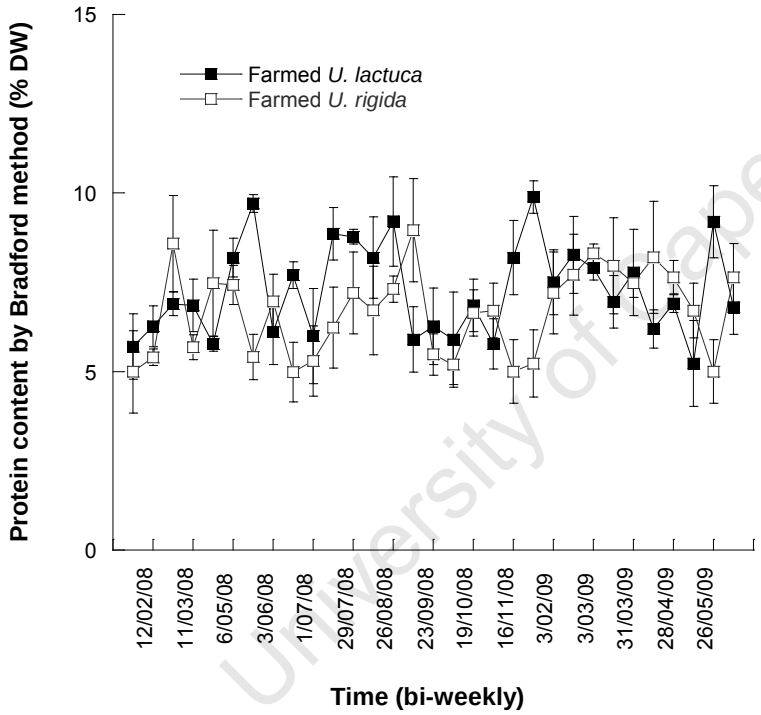

Figure 8.3: Biweekly quantification of protein content by Bradford method of both farmed *U. lactuca* and *U. rigida* over a 15-month period. Bars denote standard error of four replicates.

Figures 8.4 and 8.5 show the crude protein of wild and farmed *Ulva* species and there was no clear temporal pattern observed in the crude protein for either wild or farmed *Ulva* species. However, farmed *U. lactuca* showed higher crude protein content during May to August 2008 ($p<0.05$) whilst farmed *U. rigida* had higher values during March to April 2008 and in

Sept 2008. The results showed large differences, for all *Ulva* species, between the two protein quantification methods (Bradford and N x 6.25), values obtained with the traditional conversion factor of 6.25 were always significantly higher ($p<0.01$). For instance, for wild *U. capensis* the values obtained from N x 6.25 were 64.1% higher than the Bradford values; wild *U. rigida* values were 77.1% higher; farmed *U. lactuca* values were 58.9% higher and farmed *U. rigida* values were 66.0% higher.

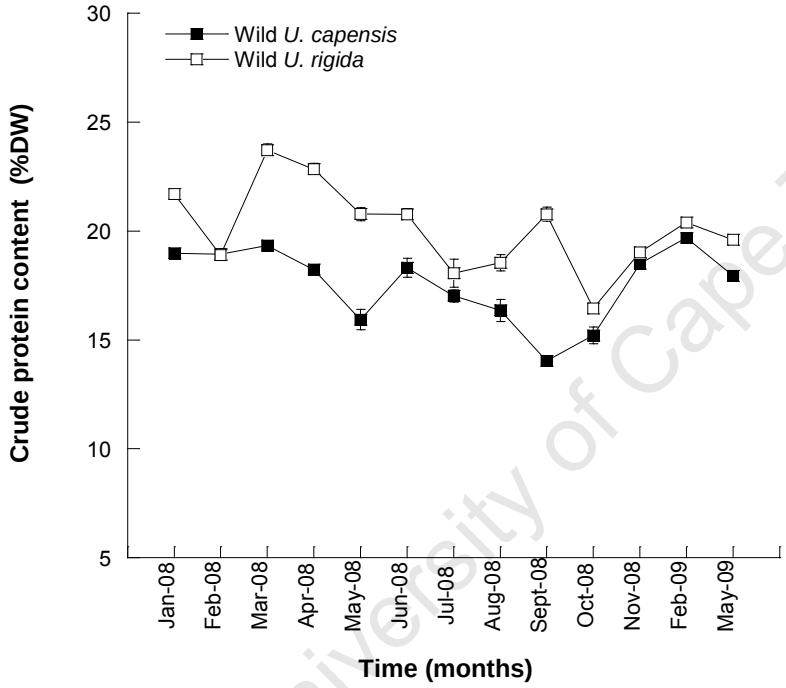

Figure 8.4: Monthly crude protein content of *U. capensis* and *U. rigida* collected from the natural populations at Kommetjie. Bars denote standard error of three replicates.

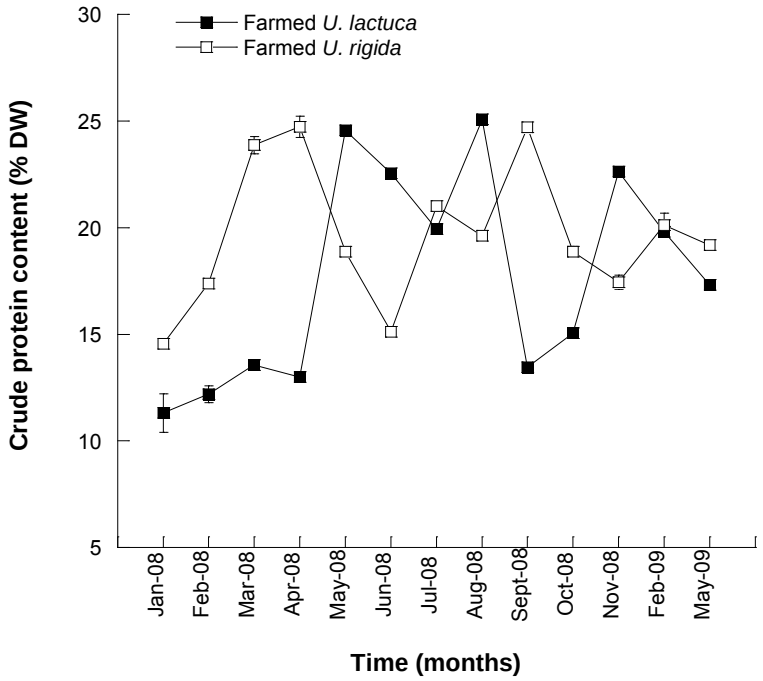

Figure 8.5: Monthly crude protein content of *U. lactuca* and *U. rigida* collected from an abalone/*Ulva* integrated system at I & J farm. Bars denote standard error of four replicates.

8.3.3 Amino acid composition

Data on amino acid composition of three *Ulva* species are shown in Table 8.1. Aspartic acid was the most abundant amino acid in all species. Moreover, these species were rich in glycine and alanine but poor in histidine, methionine and cysteine. Interestingly, all wild *U. capensis* samples analysed during different months showed no cysteine in their proteins, however cysteine was found in the proteins of wild *U. rigida* and farmed *U. lactuca*. Furthermore, the essential amino acid profiles of *U. capensis*, *U. rigida* and *U. lactuca* seem to be relatively close to those of leguminous plants and ovalbumin (eggs) with values representing 32.7%, 30.8% and 33.6% of total amino acids, respectively. Mean values for individual amino acids tended to be similar among the three species.

The N-Prot factors for three *Ulva* species are shown in Table 8.2 and two types of conversion factors were considered. These are: (1) k_A, based on the ratio of protein to total N recovered from amino acid analysis and (2) k_P, the ratio of protein to total N determined using a CHNS elemental analyzer. The k_A values ranged from 5.69 to 5.86 with a mean value of 5.76 ± 0.05 whilst the conversion factor k_P varied from 4.5 to 5.6 with a mean value of 5.13 ± 0.33. The N-prot factors established were 5.65 for farmed *U. lactuca*; 5.58 for wild *U. capensis* and 5.12 for wild *U. rigida*. Wild *U. rigida* had the lowest N-prot factor compared to other species.

Table 8.1: Total amino acid of wild and farmed *Ulva* species (in g amino acid/100 g protein)

	Wild ***U. capensis***	**Wild** ***U. rigida***	***U. lactuca*** **(I & J farm)**	**Leguminous plant** [1]	**Ovalbumin (eggs)** [2]
Isoleucine	3.5 ± 0.0	3.1 ± 0.2	3.7 ± 0.0	3.6	4.8
Leucine	6.8 ± 0.2	5.2 ± 0.2	6.7 ± 0.1	7.3	6.2
Lysine	3.7 ± 0.1	3.7 ± 0.3	4.2 ± 0.1	6.5	7.7
Methionine	1.5 ± 0.1	1.5 ± 0.2	1.6 ± 0.1	1.4	3.1
Cysteine	0.0 ± 0.0	1.1 ± 0.1	0.4 ± 0.4	1.3	0.0
Phenylalanine	4.0 ± 0.2	3.3 ± 0.2	4.0 ± 0.0	2.4	4.1
Tyrosine	2.0 ± 0.1	2.2 ± 0.2	2.1 ± 0.1	2.6	1.8
Threonine	5.0 ± 0.1	5.0 ± 0.3	4.7 ± 0.0	4.0	3.0
Valine	6.3 ± 0.0	5.6 ± 0.4	6.2 ± 0.0	4.5	5.4
Histidine	1.7 ± 0.1	1.4 ± 0.2	1.8 ± 0.1	4.0	4.1
Aspartic acid	17.2 ± 0.6	13.0 ± 1.1	12.3 ± 0.9	5.4	6.2
Glutamic acid	10.9 ± 0.2	9.4 ± 1.0	9.0 ± 0.5	6.7	9.9
Proline	3.6 ± 0.2	4.3 ± 0.4	5.3 ± 0.6	0.0	2.8
Serine	6.4 ± 0.2	6.1 ± 0.8	5.9 ± 0.0	0.0	6.8
Glycine	8.8 ± 0.2	7.8 ± 0.2	10.7 ± 0.6	0.0	3.4
Alanine	11.8 ± 0.2	12.3 ± 0.7	14.2 ± 0.4	1.3	0.0
Arginine	3.3 ± 0.3	4.6 ± 0.5	3.6 ± 0.3	14.0	11.7
Ammonia	1.5 ± 0.0	1.2 ± 0.1	1.3 ± 0.1	-	-

Results represent the mean of four replicates ± SE (n = 4). 1. Fowden, L (1954) 2. Fujiwara-Arasaki *et al*. (1984).

Table 8.2: Nitrogen content and conversion factors

	Total N	**Total AA**	**N recovered from AA analysis**	**k_A**	**k_P**	**NF**
Wild *U. capensis*	3.1 ± 0.13	16.4	2.8	5.86	5.3	5.58
Wild *U. rigida*	3.4 ± 0.24	15.2	2.7	5.73	4.5	5.12
Farmed *U. lactuca*	2.9 ± 0.30	16.3	2.9	5.69	5.6	5.65
Mean		**16.0**	**2.8**	**5.76**	**5.13**	**5.45**
± SE		**± 0.38**	**±0.06**	**±0.05**	**±0.33**	**±0.17**

*N-Nitrogen

*AA- Amino acids

*AAR- Amino acid residue

*NF- Nitrogen to protein conversion factor

*Data are expressed as g/100 g of dry tissue. N content represent replicates of 13 months (*n=13*); AA represent replicates of 4 months (*n=4*)

Table 8.3: Contents of Essential Amino Acids (g/100 g DW) in Some Vegetables* and the mean range found in *Ulva* species in the present study.

Amino acid	Cauliflower	Carrot	Potatoes	*Ulva* species
Isoleucine	8.8	2.9	7.7	3.1 - 3.7
Leucine	13.0	3.8	11.0	5.2 - 6.8
Lysine	12.0	3.5	12.0	3.7 - 4.2
Methionine	3.1	0.9	2.9	1.5 - 1.6
Cysteine	1.5	0.1	1.7	0 - 1.1
Phenylalanine	8.4	2.6	8.4	3.3 - 4.0
Tyrosine	5.2	1.4	4.0	2.0 - 2.2
Threonine	8.4	2.6	7.1	4.7 - 5.0
Valine	14.0	4.3	12.0	5.6 - 6.3

*****Møller *et al.*, 1991

Table 8.4: Nitrogen (N) contents of *Ulva* (g/100 g DW); Crude protein (CP); Nitrogen to protein factor (NP factor); Net protein (NP); Percentage difference between crude protein contents and protein content from N x specific N-Prot factor (difference %; CP - NP).

	N content	CP	NP factor	NP	difference % (CP - NP)
Wild *U. capensis*	3.1	19.4	5.58	17.3	10.8
Wild *U. rigida*	3.4	21.3	5.12	17.4	18.3
Farmed *U. lactuca*	2.9	18.1	5.65	16.4	9.4

*N content represent replicates of 13 months (*n=13*); NP factor represent replicates of 4 months (*n=4*).

As described above, NP conversion factors are very specific for each species and the use of a single factor may lead to errors in protein values. However, the mean NP conversion factor for *Ulva* analyzed in the present study was 5.45 ± 0.17. When using this factor, very good estimation of protein contents could be obtained for the main species of *Ulva*. When comparing crude protein values obtained using a conversion factor of 6.25 with net protein values, which are probably nearest to the true values, differences ranged from 9.4 to 18.3% (Table 8.4).

Ulva species showed a balance amino acid profile which yielded an r-value ranging from 0.75 to 0.83 when compared to the essential amino acid profile of *H. midae* (Table 8.5).

Table 8.5: Essential amino acid profile of abalone whole soft tissue compared with that of *Ulva* proteins used in this study.

	Abalone[1]	***Ulva* species**
Isoleucine	**4.11**	3.1 - 3.7
Leucine	**6.93**	5.2 - 6.8
Lysine	**6.21**	3.7 - 4.2
Phenylalanine + Tyrosine[2]	**7.71**	5.0 - 6.2
Threonine	**4.99**	4.7 - 5.0
Tryptophan	**0.82**	-
Valine	**4.61**	5.6 - 6.3
Arginine	**7.91**	3.3 - 4.6
Histidine	**1.82**	1.4 - 1.8
Methionine + Cysteine	**3.44**[3]	1.5 - 2.7

Correlation of *Ulva* protein vs. *H. midae* essential amino acid**, r^2 value = 0.76 - 0.83, P=<0.05**

1. The amino acid profile of *Haliotis midae* (Knauer *et al.*, 1994a)

2. Tyrosine value (3.81% of protein) for *Haliotis rufescens* (Allen and Kilgore, 1975)

3. Methionine =2.09, Cysteine =1.35 (Department of Animal Science, University of Natal)

8.4 Discussion

Previously published data on the protein content results of seaweeds is very varied, and much of this variation is caused by the analytical methods (Fleurence *et al*., 1995), different species and seasonal periods (Fleurence, 1999). Our results with Bradford's method agree with Kaehler and Kennish (1996), who found predominantly low values for some seaweed (from 1.3 to 12.6%) from Hong Kong using the Bradford method. Peters *et al.* (2005) also used the methods of Bradford to extract protein of various seaweeds from Antarctica and found the concentrations to be generally low, ranging from 1.3 to 17.3% dry weight. The trend of obtaining lower concentrations of protein using Bradford's method may be related to two main factors: 1) the binding of the dye Coomassie Brilliant Blue-G250 to both basic and aromatic amino acid residues (Compton and Jones, 1985). Most of the algae show relatively low concentrations of the two amino acids (tyrosine and tryptophan) as well as the two basic amino acids (lysine and histidine). Thus, the binding of the dye with protein occurs mainly with the two amino acids, arginine and phenylalanine, and this may contribute to lower protein measurements (Stoscheck, 1990); 2) Differences in cell wall composition of algae during extraction establish strong and negative effects on the final results (Fleurence, 1999).

Results from the present study show that wild *U. capensis* and *U. rigida* had higher protein content by Bradford method during February – June 2008. There seemed to be an increase in the protein synthesis of these species at the end of summer growth period, perhaps as a result of high nitrogen levels during summer upwelling. Furthermore, protein content by Bradford method for farmed *Ulva* species showed no seasonal pattern possibly due to the fertilization regime which was consistent.

The crude protein obtained in the present study for *Ulva* species had an average range of 17.6 – 20.1 % DW and is similar to values reported for other *Ulva*, for instance, 20 to 26% in *U. pertusa* (Fujiwara- Arasaki *et al.*, 1984); 18-24% dw in *U. amoricana* (Fleurence, 1999); 27.2 % in *U. lactuca* (Oritz *et al.*, 2006). In general, the crude protein of *Ulva* species varies between 10 - 26% DW (Fleurence, 1999). In addition, crude protein for farmed *U. lactuca* was higher during May to August perhaps due to a drop in growth rates, and luxury accumulation of nitrogen as the growth slows. The opposite trend was observed for farmed *U. rigida* suggesting that these species have different growth patterns. Furthermore, the crude protein values obtained from the present study were lower than those obtained by Robertson-Andersson (2003) from Jacobs Bay (Jacobsbaai) Sea Products Abalone Farm (JSP) culture system. This could possibly due to the difference in culture conditions, because the system at JSP was on an experimental scale where the growth conditions were controlled while *Ulva* from I & J farm is grown on a commercial scale. Moreover, the difference in the protein content between the two farms is also likely due to the poor fertilizing regime or low water velocity at I & J farm which was identified in Chapter 4 and 5. However, it should be noted that the conversion factor of total nitrogen content multiplied by a factor of 6.25 includes N not in the form of protein but intracellular reserve pools of N as well (Fleurence *et al.*, 1995). Therefore this method would tend to overestimate the actual protein content.

In the present study, the values of crude proteins were always significantly higher (between 58.9 -77.1% higher) than the protein content determined by Bradford method or from net protein estimated from the specific N-Prot factor. Wild *U. rigida* had significant amount of non-protein nitrogen with the net protein lower by up to 18.3% than the crude protein whilst wild *U. capensis* and farmed *U. lactuca* were lower by 10.8% and 9.4%, respectively. Similar observations were reported by Salo-Väänänen and Koivistoinen (1996), who found that the

net protein was always lower by up to 20%, than the crude protein content of different foods. From an abalone feed perspective, net protein of seaweed must be determined since cultured abalone have special dietary requirements.

The nutritional quality of *Ulva* species as a source of protein was investigated further by determining their amino acid composition. Various studies on the relationship between the nutritive value of feeds and culture organisms have demonstrated that the content of essential amino acids is the principal factor in their dietary value (Hidalgo *et al.*, 1987; Tibaldi and Lanari, 1991; Wong *et al.*, 2004). It is commonly accepted that the essential amino acid profile of an organism approximates the ideal balance of dietary amino acids (Benitez, 1989). For instance, good growth of abalone requires the presence of essential amino acids in their diet (Britz *et al.*, 1996). Comparisons of the essential amino acid profile of *H. midae* soft tissue (Knauer *et al*., 1994a) with the proteins of *Ulva* species obtained in the present study, show strong correspondence, suggesting that *Ulva* closely matched the abalone profile. Arginine content of abalone soft tissue is significantly higher than *Ulva* tissue and this could be the most limiting amino acid in using *Ulv*a alone as a feed for abalone. A study by Robertson-Andersson (2003) showed that cultured abalone (*H. midae*) at I & J farm achieved good growth when fed a combination of *Ulva*, kelp and artificial feed. The same trend was reported by Naidoo *et al* (2006), who showed that South African abalone grew better when fed a mixed diet of fresh seaweed, than when fed the formulated feed (Abfeed®) alone.

The amino acid profiles of various seaweeds have been studied and compared to those of other foods such as eggs or soybeans (Fleurence, 1999) as this provides an estimate of the nutritional value of the seaweed protein. In the present study, essential amino acids of *Ulva* proteins accounted for 30.8 – 33.6% of total amino acid content which was comparable to

that of eggs, soybean, other *Ulva* species and green seaweed proteins reported in earlier work: 33.6% in leguminous plants (Fowden, 1954); 36.1 % in ovalbumin (Fujiwara-Arasaki *et al.,* 1984); 37.0 - 37.9% in *U. pertusa* and *Codium fragile* (Fujiwara-Arasaki *et al.,* 1984); 37.1% in *U. lactuca* (Ochiai *et al.,* 1987) and 36.5 - 38.6% in *U. rigida* and *U. rotundata* (Fleurence *et al.,* 1995). In the present study, all three *Ulva* species' proteins exhibited similar amino acid patterns in which aspartic and glutamic acids were the predominant types. This observation is in accordance with previous reports on *Ulva* proteins, for instance in *Ulva amoricana* (Fleurence, 1999) as well as *Ulva rigida* and *Ulva rotundata* (Fleurence *et al.,* 1995).

The determination of specific N-Prot factors depends on the nitrogen recovered from amino acids after acid hydrolysis (Lourenço *et al.,* 2002). Wild *U. rigida* had the lowest N-Prot factor compared to other species and this is possibly due to its high content of arginine. Sosulski and Imafidon (1990) found that organisms that are proteins rich in highly nitrogenous amino acids (*e.g.* arginine) tend to have lower N-Prot conversion factors. Moreover, the current study established lower N-Prot factors than the traditional factor 6.25 for all *Ulva* species. The average N-prot factor for three *Ulva* species is 5.45 which is 5% higher than the average N-prot factors of 5.19 for green seaweeds from the coast of Brazil (Lourenço *et al.,* 2002). Our average N-Prot factor of 5.45 provides a more accurate estimate of the protein content of *Ulva* species studied. This is important since for feed purposes it is always necessary to determine accurately the nutrient composition of feed materials, in order to achieve optimal utilization of such ingredients in feed.

The advantage of determining the N-prot factor is that it requires no assumptions about either the non-protein nitrogen (NPN) content of seaweed or the relative proportions of specific

amino acids - thus removing the two problems with the use of total N x 6.25 as a conversion factor. Its disadvantage is that it requires more sophisticated equipment than the Kjeldahl method or CHNS elemental analyser, and thus may be beyond the capacity of many laboratories, especially those that carry out only intermittent analyses. In addition, experience with the method is important; some amino acids (*e.g.* the sulphur-containing amino acids and tryptophan) are more difficult to determine than others. However, the use of data of total amino acid for calculating protein without determination of free amino acids is a widely accepted practice, since during acid hydrolysis some sulphur-containing amino acids are destroyed (*e.g.* cystine, methionine, serine) and tryptophan is partially or totally destroyed though such effects should not significantly alter results (Spies and Chambers, 1949; Jennings, 1969; Mossé, 1990; Yeoh and Truong, 1996). The quantity of free protein amino acids is generally less than 5% of total amino acids (Yeoh and Watson, 1982). In addition, Danell and Eaker (1992) found that 15% of the ninhydrin-detectable nitrogen came from amino acids destroyed during acid hydrolysis, but up to half of it is transformed to ammonia. Thus the influence of free amino acids on the calculations of N-Prot factors is minor, considering the losses associated with the analytical procedures (Lourenço *et al.*, 2002).

In the present study the percentage of total N recovered as amino acid was less than the N content determined by an elemental analyzer. However, Yeoh and Truong (1996) found that N recovered as amino acid is usually significantly less than the total Kjeldahl N due to possible destruction of amino acids during hydrolysis, or presence of nitrogenous compounds that yield neither ammonia nor measured amino acids. Despite the complexities of amino acid analysis, in general there has been reasonably good agreement among laboratories and methods (King-Brink and Sebranek, 1993). Determination of a specific N-prot factor for

seaweeds used in aquaculture feed is essential to calculate the protein content from tissue total nitrogen content.

Chapter 9

General Discussion

This study was designed to provide information on the ecophysiology and biochemical constituents of *Ulva* species collected from natural populations at Kommetjie as well as from an integrated seaweed/abalone system at I & J farm. The *Ulva* species currently grown on abalone farms are *U. lactuca* and *U. rigida* (Bolton *et al*., 2008). Farmers needed data on the ecological growth requirements of these species and it was also necessary to compare the ecophysiology of *U. capensis* with that of *U. rigida* because they are thought to be one species according to ITS (Internal Transcribed Spacer) while TCS (a computer program to estimate gene genealogies) showed that these species could well be haplotypes of a single genetically diverse polymorphic species (Kandjengo, 2003).

The findings of this thesis showed that *U. capensis, U. rigida* and *U. lactuca* can sustain high growth rates and productivity over a range of temperatures, salinities, nutrient levels and light conditions in laboratory culture. There were differences in the response of these three *Ulva* species to various environmental factors. For instance, *U. capensis* and *U. rigida* grew significantly better under low light conditions (up to irradiance of 80 µmol photons m^{-2} s^{-1}) while *U. lactuca* grew significantly better under higher light conditions of 160 µmol photons m^{-2} s^{-1}. The finding that *U. capensis* and *U. rigida* prefer lower light environments while *U. lactuca* prefers a higher light environment explains why the latter species tends to predominate in cultivation ponds in summer while *U. rigida* predominates in winter, when they grow together. The preferred light intensity environment of each species may be an ecological niche strategy.

It was observed that *U capensis* and *U rigida* have similar temperature tolerances, although they are reported to have different biogeographical distributions. Furthermore, the south coast entities previously given these names may not be *U. rigida* or *U. capensis* because the growth rates of these two species declined towards the end of the experimental period, and plants started changing their shape and becoming crinkled, when grown at 25 ^{o}C, a common summer temperature on warmer regions of the South African coastline. This observation supports the contention of Kandjengo (pers. comm., unpublished molecular evidence) that the species growing on the south coast is not *U. rigida* and should be referred to another species, currently without a name.

U. lactuca showed high growth rates at high nitrogen and phosphorus concentrations as compared to *U. capensis* and *U. rigida*. This suggests that *U. lactuca* is adapted to more eutrophic waters, which makes it ideal as a biofilter in an integrated aquaculture system. Many species of *Ulva* grow best in eutrophic habitats (Sousa *et al*., 2007) and nutrient-enriched water (Fong *et al*., 2004).

The overall research also aimed at comparing *U. capensis* with *U. rigida* on a variety of different measures as molecular studies have suggested that they represent a single polymorphic species. This study found *U. capensis* and *U. rigida* to respond similarly to variations in irradiance, light quality, N (NH_4^+) and P enrichments but to differ somewhat with respect to reponse to salinity and N (NO_3^-) levels, and also *U rigida* grew faster than *U capensis* in all experiments. For example, *U. capensis* contained no cysteine, but *U. rigida* had cysteine present during all the months it was analysed. Also both species were found to differ not only in morphology, but also have slight but significant differences in their mineral and chemical composition. What these differences mean for the taxonomy of the two entitities will require further molecular studies.

This thesis also focused on comparing the photosynthesis of *U. capensis* and *U. rigida* which grow close together but in different habitats, investigating the seasonal patterns of their primary production**.** The different photosynthetic characteristics of these two *Ulva* species to different light intensity reflects well the acclimation of each species to their natural habitats. Both species grow in the intertidal zone, however *U. rigida* is found high on the shore on open rock whilst *U. capensis* is usually found growing in tidal pools and in the shallow subtidal. It is possible that *U. rigida* is subject to more drastic changes in light compared to *U. capensis*, and extreme varying light conditions are known to set different physiological strategies in macroalgae (Dring *et al.,* 1996). Therefore, acclimation to light in macroalgae is a habitat-conditioned phenomenon that relates to their distribution (Dring *et al.,* 1996).

The commercial *Ulva* production in raceway ponds of the integrated aquaculture system at I & J farm has been recorded since 2006. The production data and future production targets for 2006 – 2007 were reported in Bolton *et al.* (2008). The current study investigated the actual *Ulva* production in the raceways, relative to production targets from January 2008 - December 2009. Comparing our data to those obtained by Bolton *et al.* (2008), it is clear that the average monthly yield per raceway throughout 2006 – 2009 did not increase significantly, and more realistic targets are proposed. A realistic target should be based on real production per raceway and should make provision for environmental growth conditions in the raceway and the maintenance of such raceways. Based on the data obtained from this study, a more realistic summer target for summer would be 2 t per month as production methods in the system can be improved and winter target should remain at 1.5 t per month as initially proposed. The management procedures at the farm are far from optimal for growing *Ulv*a, and the production methods can be improved by enhancing the reliability and sustainability of the culture systems by improving our understanding of physiological attributes of growing *Ulva* in outdoor raceway ponds and the optima for each of the environmental factors obtained in

Chapter 2 can be applied (as far as possible) on seaweed farms. In addition, developing informative health assays for farmed *Ulva* in order to provide early warning of unfavourable conditions in the culture systems should be one of the management tools.

In years prior to this study it was noted that *Ulva* health can vary considerably throughout the year, and during late spring/summer there were outbreaks of an epiphyte infection (the brown crustose seaweed *Myrionema strangulans*) which caused severe damage to *Ulva* and reduced the production significantly. Covering of raceway ponds with shade cloth during spring/summer has been a farm management tool to prevent or reduce epiphyte infection. Despite this, the epiphyte problem persisted, leading I & J farm to request us to assess the occurrence of this epiphyte further and to determine environmental conditions which could prevent its occurrence. It was hypothesized that by late spring and summer, when *Ulva* is growing rapidly, it's internal nitrogen reserves were depleted, making it vulnerable to sustained epiphyte attack. The results supported this idea because infected *Ulva* thalli growing in natural seawater at the highest irradiance (160 µmol m^{-2} s-1) tested had no significant reduction in the epiphyte infection, indicating that high light and insufficient nutrients doesn't help *Ulva* to fight epiphyte infection. *Ulva* cultivated at I & J farm should be able to resist *Myrionema* infection provided it is grown with adequate nitrogen, irrespective of the season. However, macro-elements and trace elements that were present in our culture media may also be important, and farm managers should take note of this. These findings are important for farming operations as they should help to reduce the level of infection and diminish the direct negative effects of the epiphytes on *Ulva*. Providing *Ulva* with sufficient nutrients should help manage the epiphyte, but may also decrease the farm's reliance on doing this by shading raceway ponds. Reduced shading, perhaps together with a better fertilising regime, may greatly improve efforts to meet production targets. Our

recommendation is that 30 -50% shade cloth could be tried in both summer and winter, This could allow *U. lactuca* to dominate in summer during conditions of high irradiances, without growing too fast for the available nutrients, and at the same time minimize *Myrionema* infections and allow *U. rigida* which is suitable for low light conditions to dominate in winter.

Although seaweeds are nutritionally valuable, they may also accumulate high levels of heavy metals which may be toxic to the consumer (Almela *et al*., 2002; 2006). Therefore it is important to provide information on the concentrations of these potentially harmful heavy metals present in farmed *Ulva* species as well as *Ulva* from natural populations. The results indicated that the consumption of *Ulva* species cultivated at I & J Abalone farm should not pose a risk for abalone health at least with respect to their content of Hg, As, Cd and Pb, which were well within the allowable value for humans in France. Wild *U. capensis* and *U. rigida* collected from Kommetjie had an average Cd content of 0.5 and 0.6% dry weight, respectively, which exceeded the set limit. The large variability in metal concentrations found in all *Ulva* species at different times of the year must be taken into account when designing bio-monitoring programs, because different conclusions can be reached when using different sampling dates. In this study the cadmium levels of wild *Ulva* species have been above the set limit during all analysed months except in April 2008, when the levels were 0.1 % and 0.3% dry weight for *U. capensis* and *U. rigida*, respectively. These results also demonstrated how complicated it is to compare data from studies carried out at different times of the year or in different locations. The average metal concentrations for seaweeds from the two locations (Kommetjie and I & J farm) were different and could be differentiated by PCA. Therefore, future studies should focus not only on increasing the data for different metals and different algae but also on the processes governing metal accumulation.

It was also important to compare the chemical constituents of *Ulva* in the wild with *Ulva* grown in aquaculture effluent at I & J farm to determine any seasonal or general differences that might make one or the other nutritionally superior. The results showed that the wild material has gradually changing temporal patterns, whereas the cultivated material varies rapidly and rather randomly, presumably because of variation in water quality and nutrient content. The ash (mineral) content of wild *Ulva* species showed a seasonal trend with an increase during the late-winter to spring months. The farmed material shows more variation in the ash content than the wild material, and the farmed material generally has less ash (mineral) content than wild material. The crude fibre content of wild and farmed *Ulva* species ranged from 3.11 to 5.71 % dry weight and is similar to that of soy meal, which is considered to be a good nutritional product. Farmed *Ulva* species had higher carotenoids contents than wild species and there seems to be an inverse relationship between nitrogen level and accumulation of carotenoids. The C: N ratio for both wild and farmed *Ulva* species ranged from 7.9 to 15.7, and according to Russell-Hunter (1970) most animals need a C: N ratio of 17 or less in their diet, therefore C: N obtained from this study indicates that the three *Ulva* species (*U. capensis*, *U. rigida* and *U. lactuca*) contain sufficient N for an animal diet.

Furthermore, cultivated *Ulva* was expected to have higher levels of biochemical and mineral content than wild *Ulva* but this was not the case. It is possible that low water velocities and turnover rates at the farm during the current study may have negatively influenced the chemical composition of cultivated *Ulva*. Depending on the farm priorities an optimal flow rate should be set to grow *Ulva*. Furthermore, repair work on some of the raceway ponds during the current study meant that some of the working raceway ponds were stocked with a high biomass of *Ulva*, and the amount of nutrients added to each raceway was not based on seaweed biomass in the raceways. As a farm management tool, determination of optimal

stocking density is critical to achieve high growth rates and good quality *Ulva* and the amount of nutrients added to each raceway should depend on seaweed biomass.

Proteins are a nutritional component of particular importance to animal aquaculture, and protein is typically the most costly component of formulated feed (Miles and Chapman, 2007). This thesis provides information on the protein and amino acid composition of wild and farmed *Ulva* as well as establishing the nitrogen to protein conversion factors for wild *U. capensis* and *U. rigida* as well as farmed *U. lactuca*. The results showed large differences, for all *Ulva* species, between the two protein quantification methods used (Bradford and N x 6.25). Values obtained with the traditional conversion factor of 6.25 were always significantly higher than values obtained with Bradford method. For instance, in wild *U. capensis* the values obtained from N x 6.25 were 64.1% higher than the Bradford values; in wild *U. rigida* were 77.1% higher; in farmed *U. lactuca* were 58.9% higher and in farmed *U. rigida* were 66.0% higher. However, both methods are unreliable (Yeoh and Wee, 1994; Conklin-Brittain *et al*., 1999; Ezeagu *et al.,* 2002). The calculation of protein content by N × 6.25 may tend to over or under estimate the actual protein content in the sample due to many shortcomings of this method. Furthermore, the Bradford method may be compromised by a large amount of interference (Stoscheck, 1990). Numerous substances such as phenol and phenolases (Mattoo *et al.*, 1987) and flavonoids (Compton and Jones, 1985) interfere with the Bradford method and therefore this would have a negative effect on the final results.

For the above reasons, the use of specific nitrogen-to-protein conversion factors (N-Prot factors) using quantitative amino acid profiles is considered the most accurate way of determining protein content. Our data suggest that an average specific N-Prot factor of 5.45 provides a more accurate estimate of the protein content of the *Ulva* species studied.

The South African abalone aquaculture industry is expanding and there is a lack of information on the protein quality of *Ulva* species which are used as feed. The amino acid composition is important in determining the nutritional value of abalone feed. The amino acid profile of the three *Ulva* species in the present study appears to provide a balanced ratio of amino acids, when it is compared to the amino acid profile of abalone tissue, of species such as *Haliotis discus hannai* and *Haliotis tuberculata (*Mai *et al*., 1995), and South African *Haliotis midae* (Sales and Britz, 2003). Although tryptophan is found in the soft tissue of abalone and was not found in the *Ulva* species, Daume *et al.* (2003) and Sales and Britz (2003) showed that tryptophan is not considered essential for abalone. Nevertheless, supplementary feeding with formulated feed or other algae is normal practice on South African abalone farms (and could provide tryptophan).

Based on the results of this thesis if I were to grow *Ulva* on a farm, I would recommend *U. lactuca* and *U. rigida* for different seasons. *U. lactuca* has high growth rate and takes up more N and P as well as tolerates higher light intensity – it is the preferred species for growing during summer. *U. rigida* also has a high growth rate, takes up significant amount of N and P but is a better species to grow during conditions of low light *e.g.* during winter. Therefore by growing a combination of *U. rigida* and *U. lactuca* one can obtain high growth rates throughout the seasons, which is essential to maintain a food source for the abalone. However, it must be borne in mind that *Ulva* may perform differently in systems other than paddle-wheel raceways, and this must be considered when experimental findings are applied in commercial cultivation. Also, it is critical to correctly identify the *Ulva* species that are being cultivated, as different species behave differently under different conditions, and misidentification is a widespread problem.

This thesis provides useful information that should help *Ulva* farmers to improve their cultivation techniques and enhance productivity and nutritional quality of this lucrative crop. Furthermore, as large amounts of *Ulva* can be grown in such raceway ponds, farmers might consider opportunities to use the seaweed in other applications beside fresh feed for abalone. For example: (1) Biomass *e.g.* biofuel (ethanol) production. Bolton *et al* (2008) compared yield of *Ulva* from the South African systems, and the top yields obtained by Ryther *et al* (1984) and showed them to be very similar to figures for microalgae grown throughout the year in similar systems. (2) The amino acid profiles of the examined *Ulva* species indicates that they may be useful as partial sources of dietary protein as compared with common vegetables such as potatoes, carrots, or cauliflower as they proved to be good sources of almost all essential amino acids. Therefore they can be utilized for human consumption and health. (3) Cyrus (2009) used dry *Ulva* as a feed component of sea urchins (*Tripneustes gratilla*) in South Africa and his results showed that inclusion of *Ulva* in the artificial diet significantly enhanced the colour of gonads. There are many lucrative opportunities for using *Ulva* as a component of aquafeed for instance, Valente *et al.* (2006) suggested that the inclusion of up to 10% of *G. bursa-pastoris* and *U. rigida* in a fish-formulated diet can be considered as very useful for sea bass juveniles. Cruz-Suarez *et al.* (2009) showed that the *Ulva* intake by shrimp improved the artificial feed conversion ratio and the growth rate and the diet performance on snakehead fish (*Channa striatus*) showed that the incorporation of 5% *Ulva spp*. into the formulated diet resulted in increased growth rate, feed efficiency and feed consumption (Hashim and Mat Saat, 1992).

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