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Development in culture, ecophysiology and nutritional content of three South African *Porphyra* (Rhodophyta, Bangiales) species

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Development in culture, ecophysiology and nutritional content of three South African Porphyra (Rhodophyta, Bangiales) species

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Abstract

Porphyra species are simple, monostromatic or distromatic, foliose red seaweeds distributed globally along the subtidal and intertidal zone of rocky shores of the Antarctic to warm-temperate waters. In Asian countries, Porphyra is of significant commercial importance since it is consumed as nori. Although there is a small market limited to sushi bars for Porphyra in South Africa currently, preliminary studies have shown Porphyra to be a potentially valuable supplementary food for the farmed abalone, Haliotis midae. As a result, interest in harvesting Porphyra as abalone feed has increased recently. However, it has been shown that the Porphyra biomass in South Africa is insufficient to support the feed demands of the rapidly increasing abalone industry. Alternative means of increasing this biomass must be found, e.g. through cultivation. However, the biology of these species was not known and cultivation techniques have not been attempted. Furthermore, the nutritional content of the various Porphyra species found in South Africa had not been investigated.

The first aim of this study was therefore to study the reproductive biology and phenology of three common Porphyra species (Porphyra aeodis Griffin, Bolton et Anderson 1999, Porphyra capensis Kützing 1843 and Porphyra saldanhae Stegenga, Bolton et Anderson 1997), which occur on the west coast of South Africa. The second aim of the study was to investigate, in culture, the ecophysiological responses of the different phases of the life histories of these threes species to various environmental variables. The third aim of this study was to quantify some important aspects of the nutritional composition of these three species and to investigate seasonal variations in these nutrients. The seasonal variation in crude protein (using the 6.25 conversion factor) and soluble protein (using the Bradford, Bovine Serum Albumin method) was also
investigated in these species. A final aim was to collect and briefly describe the previously unidentified taxa that are morphologically different along the west coast.

*Porphyra aeodis* was found to be seasonal and present only from spring until autumn, while *P. capensis* and *P. saldanhae* were found throughout the year. *Porphyra capensis* was the largest (maximum mass = 22.94±3.29g, maximum length = 326.35±19.71mm, maximum width = 367.75±12.04mm) while *P. aeodis* (maximum mass =8.89±0.31g, maximum length = 156.25±8.32mm, maximum width = 146.85±10.66mm) was heavier and broader than *P. saldanhae* (maximum mass = 6.23±0.72g, maximum length = 209.65±9.08mm, maximum width = 122.05±13.99mm). Fertility was highest in summer for all species (*P. aeodis* 19.9±0.01% of adult plant, *P. capensis* 12.7±0.01% of adult plant and *P. saldanhae* 16.6±0.02% of adult plant) although there was no correlation between the diameter and mass of fertile area and the overall size of the individual thallus for all species.

When mature gametophyte thalli of the three species were induced to release spores under different environmental conditions, increased salinity, irradiance and prolonged incubation periods resulted in more spores being released from these species. Extremely low temperatures (5ºC) resulted in fewer spores released by these species (*P. aeodis* = 107.67±15.6 spores/ g wet weight⁻¹, *P. capensis* = 69.33±9.70 spores/ g wet weight⁻¹ and *P. saldanhae* = 88±14.4 spores/ g wet weight⁻¹) while extremely high temperatures (25 ºC) only significantly reduced the number of spores released from *P. aeodis* (81±6.80 spores/ g wet weight⁻¹) and *P. saldanhae* (157±17.62 spores/ g wet weight⁻¹). Meanwhile, the highest number of spores was recorded at 20ºC for *P. aeodis* (531±47 spores/ g wet weight⁻¹), *P. capensis* (602±83 spores/ g wet weight⁻¹) and *P. saldanhae* (487±75 spores/ g wet weight⁻¹). Exposure to different desiccation periods resulted in
P. capensis releasing more spores than both P. aeodis and P. saldanhae. Longer periods (i.e. beyond 4 - 8 hours) of desiccation had no significant effects on the number of spores released from both P. capensis and P. saldanhae, while the number of spores released from P. aeodis were reduced from 255±28 spores/ g wet weight\(^{-1}\) (after 2 hours of desiccation) to 122±47 spores/ g wet weight\(^{-1}\) (after 8 hours of desiccation).

All three species released zygotospores that were spherical (diameter of P. aeodis = 9.67±0.15\(\mu\)m, diameter of P. capensis = 10.01±0.12 \(\mu\)m and diameter of P. saldanhae = 11.03±0.18 \(\mu\)m) and contained a central chromatophore. All three species also released larger spores (22 to 36\(\mu\)m diameter), which are proposed to be mega-zygotospores based on their developmental characteristics. Both the zygotospores and the mega-zygotospores germinated into filamentous conchocelis while the third type of spores, (found only in P. aeodis) and proposed to be archeozygotospores (12 - 18\(\mu\)m diameter), gave rise to both conchocelis and protothalli.

The conchocelis of P. aeodis branched in an irregular manner, while P. capensis branched in an alternate-opposite manner and P. saldanhae had secundly-branched conchocelis. Growth of the conchocelis phase of the three species was highest in long-day photoperiod (16 Light : 8 Dark) compared to short-day photoperiod (8 Light : 16 Dark). Increasing temperature resulted in increased growth rates for all species in both the short-day and long-day photoperiod. Increased irradiance resulted in increased growth rates for P. aeodis (26.37±0.24\% day\(^{-1}\)) and P. saldanhae (22.52±0.21\% day\(^{-1}\)) at 160\(\mu\)mol photon m\(^2\) s\(^{-1}\), while there were no significant differences
recorded with increased irradiance (SGR = 20.33±0.27, 20.69±0.22 and 19.45±0.21% day⁻¹ at 80, 120 and 160µmol photon m² s⁻¹ respectively) for *P. capensis*.

No conchosporangia developed at 5°C for all species under both photoperiods. No conchospores were released at 10°C, long-days, for *P. aeodis* and *P. saldanhae*, while more than 30 days were required for conchospores to be released in *P. capensis*. Conchospores release was enhanced by short-day photoperiod in *P. aeodis* and *P. saldanhae* while long-days were more suitable for *P. capensis*, at 15°C and 20°C. Stocking gametophytes of *P. capensis* at different densities resulted in different morphologies with stocking density of 10g/50ml yielding more cordate blades while 5g/50ml and 15g/50ml stocking densities were dominated by lanceolate forms.

Concentrations of the measured total macroelements (C, N, H, S, P, Ca, Mg, Na and K) were higher in winter, when sea surface water temperatures were higher, while no seasonal pattern was observed for the measured total microelements (Cu, Zn, B, Mn and Fe) and heavy metals (As, Cd, Pb and Hg). *Porphyra capensis* had higher levels of selected total macroelements (as Na+K+Ca+Mg, mean = 4352±345 mg/100g) compared to *P. aeodis* (4268±528 mg/100g) and *P. saldanhae* (3928±475 mg/100g). The levels of selected total microelements (as Fe+Zn+Mn+Cu) were higher in *P. aeodis* (18.31±2.18 mg/100g), *P. capensis* (24.73±3.10 mg/100g) and *P. saldanhae* (22.96±3.32 mg/100g) compared to other edible seaweeds such as *Undaria* and *Porphyra* species from other parts of the world. Although there was year-to-year variation in the Na/K ratio of all species, and higher values in winter 2007 compared to winter 2006, their Na/K ratio was always below the maximum recommended ratio (1.5) in human nutrition. The Cu + Zn values for all three species were below maximum toxic limits (10 mg/100g) and the As, Cd and
Pb concentrations were below the maximum allowed levels set in France for edible seaweeds (Pb < 5, Cd < 0.5, and inorganic As < 3 mg kg\(^{-1}\) dry weight). Mercury levels were too low to be detected in all three species.

The soluble protein content, measured by the Bradford method, was always much lower than the crude protein of all these species. *Porphyra saldanhae* had significantly more crude protein (average 30.85±0.49% of DW) than *P. capensis* (28.46±0.48% of DW) and *P. aeodis* (27.76±0.30% of DW). The soluble protein of *P. saldanhae* (4.16±0.16% of DW) was also significantly higher while there were no significant differences between *P. aeodis* (2.99±0.18% of DW) and *P. capensis* (3.08±0.16% DW). The crude protein of *P. capensis* and *P. saldanhae* was highest in spring and summer while no seasonal pattern was detected for the soluble protein. No seasonal pattern was observed for the crude protein and soluble protein content of *P. aeodis*. There was also no correlation between the crude protein and the soluble protein content in all species.

Collection of *Porphyra* gametophytes along the South African coastline yielded four proposed new species provisionally, but not formally, named (*P. auriculata*, *P. chameleona*, *P. gravesiae* and *P. ramiculata*). These species differed from known species in lamina habit, vegetative structure, and reproductive pattern and division formulae.
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I owe a great deal of thanks to my brothers and sisters, for their constant support and motivation. This thesis is dedicated to my late parents Katriena K. and Mandlakayise G. Dlaza.
Declaration

I hereby grant the University free license to reproduce this thesis in whole or in part, for the purpose of research.

I declare that all the experimental work discussed in this thesis was carried out under the supervision of Professor JJ Bolton of the Botany Department, University of Cape Town and Doctor RJ Anderson of the Seaweed Research Unit, Department of Agriculture, Forestry and Fisheries.

All the material presented here is original work by the author and has never been submitted in this or any other form to another university. Where use has been made of research of others, it has been duly acknowledged in the text.

I am now presenting the thesis for examination for the Degree of PhD.
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Chapter I

Introduction
1. Introduction

1.1. The genus Porphyra

The genus *Porphyra* was first established by C. Agardh (1824) and comprises simple, monostromatic or distromatic, foliose seaweeds belonging to the primitive order Bangiales of the phylum Rhodophyta (Oohusa 1993). The taxonomy of many *Porphyra* species is confusing and, as a result, there is no certainty as to how many species exists (Yoshida *et al.* 1997; Silva 1999; Sahoo and Yarish 2005; Guiry & Guiry 2006; Pereira *et al.* 2006; Brodie *et al.* 2008) although the number is believed to exceed 130 species. The major reason for such discrepancy is due to cryptic diversity and relative paucity of characters for species recognition (Stiller & Waaland 1993; Lindstrom & Fredericq 2003), which may result in the incorrect use of multiple names that may refer to a single species (Guiry & Guiry 2006) and/or the underestimation of species in other areas (Hurd *et al.* 2004; Jones *et al.* 2004). For example, Broom *et al.* (2002) conducted a molecular study of three species (*P. suborbiculata* Kjellman 1897; *P. carolinensis* Coll & J. Cox 1977 and *P. lilliputiana* W.A. Nelson, G.A. Knight & M.W. Hawkes 1998 collected from the North Pacific and South Pacific) that have similar morphologies and growth habits, and share very similar type descriptions and habitat records. Their results established that these taxa are conspecific as *P. suborbiculata* and thus reducing the number of species. While using the *rbcl* gene (i.e. chloroplast Rubisco large subunit gene), Lindstrom & Fredericq (2003) recognized *P. conwayae* SC Lindstrom & S Fredericq 2003 from New Zealand as a new species that was previously lumped under *P. fallax* SC Lindstrom & KM Cole 1992, as *P. fallax* ssp *conwayae*, based on morphological similarities.

Furthermore, some *Porphyra* species form complexes that consist of several morphologically similar but genetically distinct species (Holmes & Brodie 2005). For example, genetically
distinct *Porphyra yezoensis* f. *narawaensis* A Miura 1984 and *P. tenera* var. *tamatsuensis* A Miura 1984 from Japan have blades that are morphologically extremely similar and therefore difficult to distinguish the two species with certainty using morphology (Niwa et al. 2004; Niwa et al. 2005b). Other cryptic species that are genetically distinct but share similar morphological and habitat features are *P. tasa* (Yendo) Ueda 1932 with *P. papenfussii* V Krishnamurty 1972 (both dioecious and found in mid intertidal zone) as well as *P. fallax* with *P. perforata* JG Agardh 1883 (both found in the mid intertidal zone and being monoecious and mixed reproduction) from Alaska (Lindstrom 2008).

Stiller and Waaland (1993) suggested that the circumscription of species based on differences in morphological and ecological characters, or similarities thereof, could sometimes be artificial. For example, Stiller and Waaland (1996) found a close genetic relationship between two species from Washington, *P. rediviva* JW Stiller & JR Waaland 1996 and *P. purpurea* (Roth) C Agardh 1824, that are different morphologically, ecologically and in number of chromosomes. Morphological plasticity has also been observed in European *P. amplissima* (Kjellman) Setchell & Hus ex Hus 1900 which consists of either monostromatic or distromatic thalli even from transverse sections of the same individual specimen (Brodie et al. 1998). However, this species was also found to be genetically closely related to the strictly distromatic *P. variegata* (Kjellman) Kjellman 1900; *P. miniata* (C Agardh) C Agardh 1824; *P. occidentalis* Setchell & Hus 1900; and *P. cuneiformis* (Setchell & Hus) V Krishnamurthy (Brodie et al. 1998; Lindstrom & Fredericq 2003). Nelson et al. (2006) also reported that some species of *Porphyra* around the world have been reported to be dioecious but may in fact be monoecious with the development of distinct male and female regions occurring sequentially (see section 1.3 on reproduction).
Although all South African *Porphyra* species are monostromatic, their taxonomy is also
difficult and the diversity has been significantly underestimated (Griffin 2003). Kützing
(1843) initially described and divided the South African *Porphyra* species into *P. capensis*
Kützing 1843 and *P. augustinae* Kützing nom. illeg based on morphological differences.
Later studies by Agardh (1890) resulted in these two species being lumped together as *P.
capensis*. Since then, until recently, the South African *Porphyra* species were all recorded as
*P. capensis* Kützing, although various growth and reproductively distinct forms were later
recorded by Isaac (1957) and Graves (1969). Certain characters, such as the serrated or
smooth margins, were used to group *P. capensis* into various forms. Some forms,(the
dioecious form; the monoecious form with diffused male and female sori; the monoecious
form with sectored reproductive sori; the diploplastic form; and the monoplastic form), were
recorded on rocks and mussels, while they also mentioned that there were different epiphytic
forms of *Porphyra*, growing on kelp, from the south-west Cape.

Recently, Stegenga et al. (1997) documented one unidentified *Porphyra* species, *Porphyra*
*sp. indet*, and three other species (*P. capensis*, *P. gardneri* GM Smith et MW Hollenberg
1977, and *P. carolinensis*) from the South African west coast. They also described a new
endemic species, *P. saldanhae* Stegenga, Bolton et Anderson 1997. They further recorded
another new species that was later described by Griffin et al. (1999c), using morphometric
and molecular techniques, as *Porphyra aeodis* Griffin, Bolton et Anderson 1999.

Of the South African species, only *P. capensis* and *P. saldanhae* are always epilithic, while
*Porphyra carolinensis* has been found to be epilithic, epiphytic on other red algae and epizoic
on barnacles and mussels (Coll & Cox 1977). *Porphyra sp. indet* and *P. gardneri* are
epiphytes on kelps *Ecklonia maxima* and *Laminaria pallida* (Stegenga et al. 1997) while *P.
aeodis is an epiphyte on *Aeodis orbitosa* (Griffin *et al.* 1999c). Although *P. aeodis* has been found to be host-specific (Griffin *et al.* 1999c), it is not known whether the other species are obligatory epiphytes on their hosts, since *P. gardneri* has also been found on the fucoid *Anthophycus longifolius* (Stegenga *et al.* 1997). Furthermore, some unidentified epiphytic and epizoic forms similar to *P. gardneri* have been observed growing on other seaweeds such as *Nothogenia ovalis* and mussels (Griffin 2003).

Currently there are five *Porphyra* species, and one undescribed species, recorded in South Africa. Molecular analysis of this genus, see Jones *et al.* (2004), revealed higher diversity than previously estimated and suggested high levels of endemism. However, the nucleic rDNA SSU gene region employed by Jones *et al.* (2004) is a very conservative gene and may not always be reliable for species delineation since Brodie *et al.* (2008) found that their partial rDNA SSU dataset only represented just under a third of the gene in a region that is useful for species identification. Jones *et al.* (2004) also found the South African taxa to be distinct and belonged to the *P. capensis* complex that was different from the rest of the other species in the world. Although this study revealed these distinct taxa, the study did not incorporate all the known South African species, but numbered entities that were not clearly ascribed morphologically as species, making it impossible to relate these taxa to other local species such as *P. gardneri* and *P. carolinensis*. Furthermore, *P. capensis* has also been recorded along the middle intertidal zone along the coast of Chile (González & Santelices 2003) but no study has ever compared the local *P. capensis* with the Chilean species. González & Santelices (2003) referred to the thallus of the Chilean *P. capensis* as thin and membranous with thickness ranging from 60 - 122 µm, while the South African *P. capensis* has been referred to as thick and generally 100 - 150 µm thallus thickness (Stegenga *et al.* 1997). Lately, the use of other genes, in combination with rDNA SSU, resulted in Broom *et
al. (2002) reducing *P. carolinensis* Coll et Cox to synonymy with *P. suborbiculata* Kjellman, although no similar studies have yet been carried out using South African material of this species. The genetic relationship of *P. gardneri* to other South African species is also not known yet since, the DNA sequence for *P. gardneri* was only obtained for the first time by Lindstrom (2008), and therefore the local *P. gardneri* also requires molecular verification.

Employment of molecular techniques in combination with morphological characteristics has proven more useful than using either method singly. For example, Brodie *et al.* (1996) and later Brodie and Irvine (1997) distinguished *P. dioica* J Brodie & LM Irvine 1997 from *P. purpurea*, by using both molecular and detailed morphological descriptions. Brodie *et al.* (2008) also revealed that comprehensive collection over a small area at different times of the year may yield more species, since some species are ephemeral or have restricted times when they occur. For example, the late discovery of the *Plocamium* epiphytic *Porphyra plocamiestris* RW Ricker 1987 in Antarctica may have been due to *P. plocamiestris* only appearing in late summer, while *Antarcticosaccion applanatum* grows on the *Plocamium* in spring to early summer (Clayton *et al.* 1997). As a result, the number of *Porphyra* species is constantly fluctuating with the development of new taxonomic techniques and the improvement of characters used. Furthermore, there are likely to be more species added to the current species list although some may not necessarily be recorded for the first time, but may have previously been overlooked or lumped under existing species (Brodie *et al.* 2008). The major morphological taxonomic characters currently used in the classification of *Porphyra* are listed in Lindstrom and Cole (1993) and include the division formulae of sexual product; number of cell layers; size of vegetative cells; blade shape and geographical distribution.
1.2. The biogeographical distribution of Porphyra

The biogeographical distribution of marine benthic algae is determined by the temperature regime of their habitat and temperature requirements for growth, reproduction and survival of the various phases of their life histories (Yendo 1914; van den Hoek 1982, Hwang et al. 2004; Chung et al. 2007). *Porphyra* species are distributed globally along the intertidal zone, predominantly, of rocky shores of polar to tropical seas (Bold & Wynne 1978; Tseng & Sun 1989; Karsten 1999; Mai et al. 2004). Although the majority of the species are found in temperate waters, *Porphyra* can also be found on rocky shores from cold polar to warm tropical seas (Bold and Wynne 1978; Zhang et al. 2005). Cold water species from Polar Regions include *P. plocamiestris* and *P. endiviifolium* (A Gepp & ES Gepp) YM Chamberlain 1997 that are found in the Antarctic (Clayton et al. 1997; Beardall & Roberts 1999; Zacher et al. 2007). Such species tend to be strongly shade adapted, seasonal, opportunistic and to grow during the Antarctic summer on ice-free eulittoral rocks (Wiencke & Clayton 1998; Wiencke et al. 2007). *Porphyra* species found in warm temperate to tropical waters include *P. rosengurtii* Coli & Cox (Kapraun & Luster 1980), *P. spiralis* EC Oliveira & Coll 1975 (Kapraun & Lemus 1987), *P. columbina* Montagne 1842 (Woolcott & King 1998), *P. drewiana* Coll & EC Oliveira 2001 (Coll & Oliveira 2001) and *P. vietnamensis* Tanaka & Pham-Hoang Ho (Sahoo et al. 2006).

Species from this genus may grow to be as large as 3 meters long (Waaland et al. 1990b) and they can either be epilithic, epizoic or epiphytic. Wherever it grows epilithically, *Porphyra* tends to form conspicuous horizontal bands, on the rocky shores, that may sometimes be sandwiched between limpet zones (Yendo 1914; Zavodnik 1987). Although most species grow epilithically there are also many epiphytic species including *P. koreana* MS Hwang & IK Lee 1994, *P. woolhousiae* Harvey 1863, *P. lacerata* A Miura 1967, *P. subtumens* J
Agardh ex RM Laing 1928, *P. leucosticta* Thuret 1863 and *P. plocamiestris* (see for example Hawkes 1977; Dickson & Waaland 1985; Waaland *et al.* 1990; Sidirelli-Wolff 1992; Nelson and Knight 1996; Candia *et al.* 1999; Chopin *et al.* 1999; Griffin *et al.* 1999c; Kim & Notoya 2001; Orfanidis 2001). These species grow on brown or other red seaweeds, although some epiphytic species e.g. *P. nereocystis* CL Anderson 1892 (Dickson & Waaland 1985; Waaland *et al.* 1990a; b) and *P. koreana* (Kim & Notoya 2001) are not obligate epiphytes. However, some species are obligate epiphytes on their host species e.g. *P. gardneri* (Hawkes 1978), *P. subtumens* (Nelson & Knight 1996) and *P. moriensis* H Ohmi 1954 (Notoya & Miyashita 1999). Culture studies of the obligate species have, however, revealed that they can grow unattached to their host species although wound sites on the host may be important for conchospore establishment (see Hawkes 1977). Some species e.g. *P. lacerata* (Notoya & Nagaura 1999) and *P. katadae* A Miura 1968 (Neefus *et al.* 2008) can grow both epilithically and epiphytically while some e.g. *P. lilliputiana* (Nelson *et al.* 1998) *P. suborbiculata* and *P. yezoensis* Ueda 1932 (Neefus *et al.* 2008) can grow on rocks, other seaweeds and on shells of limpets and barnacles.

Although the distribution of *Porphyra* species tends to be confined regionally resulting in high endemism (Yoshida 1997), the genus itself is cosmopolitan (Broom *et al.* 2002). This cosmopolitan distribution prompted Lindstrom and Cole (1992a) to propose a genetic link between Pacific and Atlantic species based on their findings of clusters that consisted of species from both these regions. Furthermore, Stiller and Waaland (1993) found a putative intron, which may be conserved in species over wide geographical ranges, and reflecting that the genus is ancient and polyphyletic with *Bangia*. Broom *et al.* (1999) also suggested that the close genetic relationship of *P. lilliputiana*, from New Zealand, to the northern Pacific entities could reflect ancient species radiations. Nelson *et al.* (2006) further suggested that the
southern hemisphere could be a centre of origin for the Bangiales, thus supporting the relationship between species from the different localities. Recently, Brodie et al. (2008) observed a possible link between the Chilean *Porphyra* flora and those in the North Atlantic due to the identical Rubisco spacer region detected in *P. amplissima* and *P. cuneiformis*, which are both closely related to *P. variegata*.

### 1.3. Reproduction and life history of Porphyra

*Porphyra* species are generally either dioecious (separate distinct male and female individuals) or monoecious (both male and female sori on the same individual). Although most *Porphyra* species are either dioecious or monoecious, monoecious thalli of typically dioecious species (e.g. *P. purpurea, P. linearis* Greville 1830, *P. mumfordii* SC Lindstrom & KM Cole 1992, *P. lanceolata* (Setchell & Hus) GM Smith 1943, *P. dentata* Kjellman and *P. pseudolinearis* Ueda 1932) have been found both in the wild and in culture (see Lindstrom & Cole 1992b; 1993; Kim 1999; Valera-Álvarez 2005). Most of the monoecious species have their fertile sections composed of mixed male and female patches that are irregularly arranged along the margin of the blade. However, some species have monoecious blades that are longitudinally divided into male and female halves with a clear line of demarcation between the two sides (Mitman & van der Meer 1994) and such species are sometimes referred to as androdioecious (Neefus et al. 2008). Monoecious (androdioecious) species with male and female reproductive tissues in distinct halves of the thallus include *P. purpurea* (Kornman 1994; Mitman & van der Meer 1994; Brodie & Irvine 1997), *P. haitanensis* TJ Chang & BF Zheng Baofu 1960 (Tseng & Sun 1989), *P. variegata* (Notoya & Sugawara 1999), *P. birdiae* Neefus & Mathieson 2002 (Neefus et al. 2002), *P. linearis* (Valera-Álvarez 2005) and *P. katadae* (Tang et al. 2004; Neefus et al. 2008; Lin et al. 2009). Of the South
African species, only *P. capensis* is dioecious while the other known species are all monoecious.


The ploidy of the various phases of the life history of *Porphyra* has been studied extensively by various authors (see Zhou *et al.* 2007 for list of authors) who sometimes came up with conflicting conclusions. The major challenge in determining the ploidy in this genus was mainly the small size of *Porphyra* chromosomes, 0.5-3.5µm in length, (Mumford & Cole 1977), and somatic pairing of cells and the variation in chromosome numbers across and within a single species (Valera-Álvarez *et al.* 2005). The other challenge was the use of staining methods that yielded very weak staining of the chromosomes, making counting the chromosomes inaccurate (Wang *et al.* 2007). For example this could have led to Krishnamurthy (1959; 1972), Conway & Cole (1973), Coll & de Oliveira Filho (1977), Mumford & Cole (1977) and later Kapraun & Luster (1980) finding that *P. umbilicalis* var *laciniata* (Lightfoot) J Agardh 1883, *P. sanjuanensis* V Krishnamurthy 1972, *P. papenfusii*, *P. leucosticta*, *P. variegata* and *P. rosengurtii* respectively had life history alternation of morphological phases with no changes in their ploidy. Freshwater and Kapraun (1986) also reported that four chromosomes occurred in all stages of the life history of *P. coralinensis* (now *P. suborbiculata*).
However, it has since been established that the foliose blade phase of *Porphyra* is haploid and the conchocelis phase is diploid (Abdel-Rahman 2005; Wang *et al.* 2007; Shimizu *et al.* 2008). Burzycki and Waaland (1987) found that the conchocelis and conchosporangial cells of *P. torta* Krishnamurthy 1972 were all diploid (2n = 6) while Mitman & van der Meer (1994) found the conchocelis phase of *P. purpurea* to be diploid with up to ten chromosomes found. Valera-Álvarez *et al.* (2005) also found the conchocelis phase of *P. linearis* to be diploid although the number of chromosomes varied from eight to ten. Wang *et al.* (2007) found the conchocelis phase of *P. yezoensis* and *P. haitanensis* to be diploid with ten and six chromosomes for these species respectively. Zhou *et al.* (2007) also found that although the spores of *P. yezoensis* contain homologous chromosomes, paired at prophase and metaphase, they germinated by mitosis to produce the diploid conchocelis phase.

The first study on meiosis in *Porphyra* was done by Ishikawa in 1921 on *P. tenera* Kjellman 1897 (Tseng & Sun 1989; Wang *et al.* 2006). Since then, various authors have located different sites of meiosis in the life history of *Porphyra* (see Mitman & van der Meer 1994; Wang *et al.* 2006; Zhou *et al.* 2007). Some authors located the site of meiosis during the initial division of the fertilized carposporangia, others located it in the conchosporangial branches, while yet others located it during conchospores germination. However, there has been growing support for the occurrence of meiosis during conchospore germination compared to the other sites previously suggested. For example, it has been demonstrated that in some species of *Porphyra*, including *P. haitanensis, P. tenera, P. torta* and *P. yezoensis*, meiosis occurs in the germinating conchospores (van der Meer 1986; Burzycki & Waaland 1987; Tsang & Sun 1989; Wang *et al.* 2008). Mitman & van der Meer (1994) also demonstrated mitosis in the reproductive sori of the thallus and meiosis in the germinating conchospores of *P. purpurea*. While studying meiosis in *P. yezoensis*, Wang *et al.* (2006) and
Shimizu et al. (2008) also concluded that meiosis occurred during conchospore germination, with meiosis II occurring at the first cell division during germination of conchospores.

Sahoo et al. (2002) described a typical and simplified life history of this genus which starts with the fertilization of the female gametes by the male gametes, and results in the formation of various spores in the female gametangial sori. Although various terms have been used to refer to such spores, the terminology adapted from Nelson et al. (1999) is used here. During asexual reproduction, the vegetative cells of the blade differentiate to release the blade archeospores that germinate into the foliose phase. Alternatively, mitotic division occurs on the zygote during sexual reproduction, resulting in the release of the zygotospores which germinate into the conchocelis phase. The blade may also produce phyllospores, which are spores produced when the ploidy level and development are not known. During asexual reproduction, the blade cells may also undergo mitotic cleavage, in the absence of fertilization, to produce neutral spores that germinate into blades.

The conchocelis cell differentiates to form conchocelis archeospores and/or neutral conchospores that germinate into more conchocelis. The conchocelis may also develop into conchosporangia, with sporangia releasing conchospores. Alternatively, the conchocelis may give rise to protothalli, which are cellular masses which do not exhibit development polarity and release protoplasts that develop into foliar plants. The conchospores, except the neutral conchospores, germinate into the foliose/blade phase.

1.4. Culture techniques used to complete the life history of Porphyra

The culture of the microscopic phase (spores, conchocelis, conchosporangia and conchospores) of Porphyra is usually conducted in a controlled environment (in the
laboratory) while the newly attached blades may later be transferred to the ocean or tanks. The general field cultivation methods used in the *Porphyra* industry are described in Akatsuka (1990); Oohusa (1993); Sohn (1998) and in Sahoo and Yarish (2005). *Porphyra* cultivation began about 200 years ago with the casual collection of wild specimens resulting in ‘rock clearing’ which was believed to improve the substrate for the settlement of spores through the removal of competing organisms such as seaweeds and barnacles (Mumford & Miura 1988).

The cultivation methods have improved since then with the introduction of both the fixed-pole method and the floating method. The fixed-pole method was conventionally used, whereby wild *Porphyra* blades were collected and laid on twigs to induce spore release and subsequent blade formation (Yendo 1914; Mumford & Miura 1988). The twigs were then taken and planted in sandy shores where there was low salinity and less vigorous wave action to promote large and luxuriantly soft fronds (Yendo 1914). This restricted cultivation to only places such as the shallow waters of estuaries, bays and inlets where freshwater continuously mixed with seawater to dilute the seawater salinity (Miura 1975). Furthermore, the twigs did not have sufficient surface area for sustainable commercial cultivation and were therefore replaced with nets made of thin bamboo shoots and branches, which were eventually replaced with nets made from a mixture of polypropylene fibers for strength and Vinylon or Cremona fibers (Mumford & Miura 1988).

The use of nets, coupled with the discovery of the conchocelis phase, resulted in a technique whereby the blades were suspended horizontally, and thus made it possible to cultivate *Porphyra* in deeper waters instead of being restricted to river mouths (Miura 1975; Tseng 1981). However, this resulted in increased fouling and it was found that *Porphyra* blades did
not require permanent submersion and therefore the nets were suspended on bamboo rafts (Akatsuka 1990; Oohusa 1993). Such rafts were designed so as to expose the *Porphyra* during low tides or during harvest periods and thus control the degree of fouling (Sohn 1998; Sahoo & Yarish 2005). The use of nets on rafts resulted in the use of the various stages of the life history of *Porphyra* and thus the introduction of laboratory culture techniques.

The techniques used to culture *Porphyra* in the laboratory are generally similar throughout the world. However, there may be some modifications to the techniques depending on the species used (Sahoo and Yarish 2005). In general, the laboratory culture of *Porphyra* involves 1) the release and collection of spores 2) conchocelis growth 3) conchosporangial development and conchospore production. Depending on the size of the laboratory and the scale at which the culture is conducted, out-planting to shells and seeding of nets may also be done for blade development.

1.4.1. The release and collection of spores

*Porphyra* species release spores of various types and sizes, with different roles in the life history of that species. The released spores may be zygotospores, archesporospores, phyllospores, endospores, neutral spores and/or agamospores. For the initial collection of such spores, mature fertile thalli of *Porphyra* are usually harvested from the wild populations. While the whole blade may be used to release the spores, it is frequently pieces excised from the fertile sections that are used. The size and shape of the excised pieces varies. The pieces may either be discs {e.g. 1mm diameter - Notoya 1999, 1.2cm diameter - Korbee et al. 2005}, strips {e.g. 0.5-2mm - Nelson & Knight 1996; Knight & Nelson 2000} or quadrants {e.g. 1mm² - Candia et al. 1999, 25mm² - Tang et al. 2004; He & Yarish 2006, 2-2.5cm² - Kraemer & Yarish 1999}.  

The excised pieces are then rinsed, several times, in filtered sterile seawater (e.g. Davies et al. 1997; Dai et al. 2004). To remove any surface fouling, the surfaces of the excised pieces are either wiped with a paper towel (e.g. Niwa et al. 2005a) or scrubbed with a makeup brush (e.g. Kim & Notoya 2001), an artist’s brush (e.g. Notoya & Nagaura 1999; Monotilla & Notoya 2004) or a rolled cotton ball (e.g. He & Yarish 2006). The pieces are then placed in Petri dishes, crystallizing dishes or any other dish where the released spores could be contained.

1.4.2. The conchocelis phase

Various culture media are used to culture Porphyra conchocelis. The choice of medium depends on the Porphyra species used as well as with the experience gained by the researcher while using the various media. As a result, some of the original culture media have been modified to yield better growth rates.

Most culture media, however, involve the enrichment of seawater with trace metals and vitamins that may be present in insufficient amounts or missing from the natural seawater. For example, in their experiments, Dai et al. (2004) used cooled boiled seawater supplemented with 10 mg l\(^{-1}\) KNO\(_3\)-N and 1 mg l\(^{-1}\) KH\(_2\)PO\(_4\)-P as their culture medium. On the other hand, Hafting (1999) used filtered seawater enriched with trace metals and vitamins following the f/2 recipe of McLachlan (1973). Orfanidis (2001) and Carmona et al. (2006) used Von Stosch's solution to enrich seawater, while Uppalapati and Fujita (2000) used a modified enriched seawater medium (revised by Tompkins et al. 1995 from Schreiber 1927) containing 20 mg L\(^{-1}\) Na\(_2\)HPO\(_4\).12H\(_2\)O, 100mg L\(^{-1}\) NaNO\(_3\), and 1mL L\(^{-1}\) trace metal mix. However, the most frequently used culture medium (e.g. in Candia et al. 1999; Katz et al. 2000; Choi et al. 2002) involves the use of filtered sea water that has been sterilized and enriched with Provasoli’s enriched solution (PES Medium) (details in Provasoli 1968,
modified in McLachlan 1973). This solution has been further modified by West and McBride (1999) in Andersen (2005).

When a medium of choice is selected, the spores are then cultured in the medium until they grow into either the filamentous conchocelis phase or directly to the foliose phase, depending on the types of spores present. The frequency at which the culture medium is changed depends mainly on the stage of development. For example, Hollenberg (1958) suggested that the medium be changed every two or three days during the early days of development. As the conchocelis develop further they are maintained in vitro by vegetative fragmentation (Mumford & Miura 1988) and the medium may be changed less frequently, depending on whether the conchocelis are maintained as free-living with aeration or attached.

1.4.3. Seeding and maintenance of conchocelis

Vegetative fragments from stock cultures of free-living conchocelis are used to start the large-scale culture of conchocelis phase growing on shell substrate (Mumford & Miura 1988). The maturation of conchocelis promotes the development of conchosporangia which bear the conchospores (Lindstrom & Cole 1993; Nelson et al. 1999). The maturation of sporangia in *P. tenera* is promoted by reduced light, increased N:P ratio (3:1) and increased temperature (Mumford & Miura 1988).

Various techniques are employed to trigger the liberation of the conchospores in culture. In the farming industry, the conchospores are released by placing conchocelis-containing shells in deep tanks so that a spore suspension is created when the conchospores are released (Mumford & Miura 1988). Alternatively, the shells are placed in a floating horizontal raft and a large number of nets spread over the shells with the released conchospores floating and thus
attaching to the netting (Mumford & Miura 1988). In the laboratory the liberation of conchospores is achieved by using a household blender to chop the conchosporangia-bearing conchocelis. The chopped fragments, approximately 200 microns long, are thrown into the culture medium and covered with cardboard or black plastic to reduce photosynthesis (Melvin et al. 1986). Upon removal of the cardboard or plastic, photosynthesis produces small oxygen bubbles causing the empty conchosporangial fragments to float on top while the conchospores and conchospore-bearing fragments remain at the bottom (Melvin et al. 1986). Alternatively, the conchospores are released in the laboratory by manipulating the culture conditions (see section 1.5 below). Although release conditions are species-specific, reducing the irradiance and shortening the photoperiod generally induces the release of conchospores (Waaland et al. 1990).

To inoculate the released conchospores, a suitable substrate is needed to attach them to. However, the rate of attachment depends on the type of substrate used as well as the volume of the culture medium. Gualtieri and Barsanti (2006) suggested that the volume of the culture medium should just cover the substrate, so that the conchospores would not be suspended for long. Furthermore, Xiaolei et al. (2008) observed that the conchospores of *P. yezoensis* tended to adhere to the substratum immediately after release but their adhesive ability seemed to decrease quickly with time, eventually resulting in death of conchospores due to lack of cellwall. The frequency at which the culture medium is changed also affects the success of conchospore attachment since sufficient time is required for spore attachment (Mumford 1986).

Although *Porphyra* grows either epilithically, epiphytically or epizoic on mollusk shells, the conchocelis burrows and grows beneath the surfaces of mollusk shells in nature (Melvin et al. 1986).
1986). This may have resulted in most farmers preferring to seed the conchospore-bearing fragments on mollusk shells (such as that of oysters; scallops and clams) in order to reach the foliose stage. However, lately there have been some developments towards using alternative substrates such as those made of artificial vinyl films covered with calcite granules (Sahoo and Yarish 2005).

1.5. Factors affecting Porphyra growth

In the wild, the growth of seaweeds is affected by various chemical, physical and biological factors (Young & King 1980; Pedersen et al. 2004). The growth and development of cultured Porphyra is also affected by factors such as light quality (Figueroa et al. 1993; 1995; Aguilera et al. 1999; Lüning 2001; Korbee et al. 2005b), light quantity (irradiance); photoperiod, pH, salinity, temperature, nutrients (Young & King 1980; Hannach & Waaland 1989; Pereira et al. 2005), stocking density (Pereira et al. 2006) as well as the duration of the culture period. Although the growth of cultured species may sometimes be influenced by a direct multiplicative interaction of these conditions (Droop 1973; Brown & Button 1979; Young & King 1980), only four of these factors were directly and independently investigated in this study.

1.5.1 Irradiance

Various terms (such as light quantity; irradiance; photon flux density and light intensity) have been employed to quantify the amount of light received by seaweeds, for photosynthesis. However, some of these terms, as used by various authors, are confusing. For example, light intensity usually refers to the light emitted by the source rather than the light received by the seaweed (Lobban & Harrison 1994).
The term irradiance is used here to refer to the amount of photosynthetically active radiation (PAR) falling on a particular area and measured in micromoles of photons per square meter per second (μmol photons m$^{-2}$s$^{-1}$ or μE.m$^{-2}$s$^{-1}$). The relationship between irradiance and photosynthesis is usually reflected graphically with light-saturation, or photosynthesis – irradiance response curves for net photosynthesis versus incident irradiance (Lobban & Harrison 1997). Such curves define the maximum photosynthesis; gross photosynthesis; net photosynthesis; respiration; compensation irradiance; and saturating irradiance levels for a species under defined irradiance levels.

Irradiance varies with depth and density of cultures, as some of the terms may only refer to the amount of light hitting a specified surface without considering the effects of depth in an algal culture. Gualtieri and Barsanti (2006) suggested that the light requirements of cultured species varied with depth and stocking density. Less light is required at low stocking density and/or shallow depth since light penetration is high and therefore photoinhibition may easily occur in culture. However, at high algal density and depth more light is required since the rate of photosynthesis depends on the available light and thus the irradiance absorbed (Lobban & Harrison 1994).

Hafting (1999) found that blades of Porphyra yezoensis from Japan assimilated nitrogen differently under different irradiance levels, with more nitrate assimilated in high irradiance conditions and no difference between nitrate and ammonium assimilated under low irradiances. In 1984, Ying conducted irradiance studies on the reproductive blades of the same Porphyra species and reported a direct influence of irradiance on the formation of monospores and their adhesion to the substrate. His results showed that increased irradiance resulted in an increased number of monospores formed and also an increased percentage
adhesion of the released spores. However, his results were subject to other environmental conditions such as photoperiod. Xiaolei et al. (2008) found that the optimum irradiance for conchospore release in *P. yezoensis* was 200\(\mu\)mol photons m\(^{-2}\)s\(^{-1}\) and observed that conchospore division increased with increased irradiance and wavelength.

Generally, the light requirements of seaweed species tend to correspond to their geographical position, with lower values in Polar Regions and higher values in warmer regions (Roleda et al. 2006). As a result the species growing in Polar regions tend to be adapted to shade conditions (Wiencke et al. 2007) thus requiring less light and reaching photosynthetic saturation between 14 and 52 \(\mu\)mol photons m\(^{-2}\)s\(^{-1}\) (Hanelt et al. 2003) while subtropical species require more light. For example Stekoll et al. (1999) reported growth inhibition of Alaskan *P. abbottae* Krishnamurthy 1972 conchocelis in irradiance over 40\(\mu\)mol photons m\(^{-2}\)s\(^{-1}\) at 15ºC or higher while Waaland et al. (1990) showed good growth rates, for the same species collected in Washington, in the range 10 - 15ºC and 5 - 100\(\mu\)mol photons m\(^{-2}\)s\(^{-1}\). Furthermore, *P. endiviifolium* from Antarctica also reached photosynthetic saturation at 33\(\mu\)mol photons m\(^{-2}\)s\(^{-1}\) (Zacher et al. 2007) while species from warmer areas can only reach photosynthetic saturation at high irradiances e.g. 200\(\mu\)mol photons m\(^{-2}\)s\(^{-1}\) (Zou & Gao 2005).

However, light requirements of Porphyra may be species-specific and may differ with the different life history stages. For example, Katz et al. (2000) found that cultured blades of *P. linearis* from the Meditereranean reached photosynthetic saturation at 20\(\mu\)mol photons m\(^{-2}\)s\(^{-1}\) and associated that with the shade-adapted growth habit of this species in the wild rather than geographical location. Lin et al (2008) also found that the photosynthetic saturation (ranging from 83 - 250 \(\mu\)mol photons m\(^{-2}\)s\(^{-1}\)) for the conchocelis phase of *P. pseudolinearis, P. abbottiae* and *P. torta* was species specific and was sometimes not significantly related to temperature changes.
1.5.2 Photoperiod

Photoperiod refers to the daily ratio of hours of light : dark that the seaweeds are exposed to over a 24-hour period. The light : dark cycle is responsible for the induction of different phases of life cycle in many seaweeds (Gains and Lubchenco 1982) and therefore the effect of photoperiod on seaweeds is mainly pronounced during reproduction, as photoperiod and other factors often control reproduction in seaweeds (Hwang & Dring 2002; Pang & Lüning 2004; Kawai et al. 2005). Inappropriate photoperiod conditions may lead to reproductive stages and/or structures that may not germinate under standard culture conditions (Lorenz et al. 2005). For example, Iwasaki (1961) cultured conchocelis of P. tenera under different photoperiods and found that conchospore release was prevented by continuous light in this species. The photoperiodic response of Porphyra conchocelis was first demonstrated by Dring (1967) and this response was found to be mainly due to the ‘short-day’ photoperiods, which was a response regulated by the phytochrome pigment as in land plants, albeit that the main triggers for the life history of Porphyra are interactions of temperature, photoperiod and irradiance (Krishnamurthy 1969; Kapraun & Lemus 1987; Waaland et al. 1987; Kim 1999).

For example, Waaland et al. (1987) found that the maximum number of conchospores released from P. torta was recorded at 8L:16D, 10 – 15°C conditions. Mumford and Miura (1988) then found that the liberation of conchospores in P. tenera was triggered by reducing the temperature (26-30°C to 5-10°C), raising the irradiance to (18-27µE.m⁻².s⁻¹) and/or by shortening the photoperiod to 8L:16D. Waaland et al. (1990a) also conducted different photoperiod studies on five North East Pacific Porphyra species (P. abbottae, P. nereocystis, P. perforata, P. pseudolanceolata V Krishnamurthy 1972 and P. torta) and concluded that photoperiod was an essential environmental trigger for the release of conchospores in those species. Although short day photoperiods were generally ideal for spore release, they also
found that a combination with other environmental factors e.g. irradiance and temperature was also needed for most species. For example *P. nereocystis* required 16L:8D photoperiod to release conchospores at 8 – 10°C while it required 8L:16D to release conchospores at 12 – 15°C under the same irradiance (25 – 40 \( \mu \)mol photons m\(^{-2}\)s\(^{-1}\)). Abdel-Rahman (2005) also found that *P. leucosticta* released conchospores in neutral-day 12L:12D and long-day 16L:8D photoperiods only in warmer temperatures (15 and 20°C) while the same species only released under short-day 8L:16D photoperiod in low temperature (10°C).

1.5.3 Temperature

Since the main triggers for the various stages in life history of *Porphyra* are temperature, photoperiod and irradiance (Krishnamurthy 1969; Kapraun & Lemus 1987; Waaland *et al.* 1987; Kim 1999), temperature is important since it determines the seasonal occurrence of most *Porphyra* species. This is because some species may have evolved life histories that are synchronized to avoid occurrence of the gametophytes during unfavourable environmental conditions (Conway *et al.* 1976). For example, the gametophytic phase of *P. endiviifolium* is opportunistic and only grows on ice-free rocks during the Antarctic summer (Wiencke & Clayton 1998).

In culture, temperature is therefore an important abiotic parameter as it may determine the survival and growth rates of the species used. Localized heat may lead to mortality as a result of pigment destruction, while higher temperatures generally result in increased evaporation (Lorenz *et al.* 2005). Temperature also determines the rate at which *Porphyra* takes up nutrients (Lobban & Harrison 1994). Apart from determining the metabolic rate of seaweeds, temperature also affects the intracellular nutrient stores of seaweeds (Hawkes 1977).
Furthermore, it also influences the biochemical composition of algae (Kaehler & Kennish 1996), which is important in determining its nutritional value for herbivores and humans.

1.5.4 Nutrients

The growth rate of cultured seaweeds is a reflection of the nutrients that are available, or lacking, in the culture medium used. The mean concentrations of various nutrient elements in seawater are listed in Lobban & Harrison (1997). The primary growth-limiting nutrients for Porphyra include phosphorus and nitrogen which are usually found in relatively small concentrations in seawater (Harvey 1963; Dunstan & Menzel 1971; Ryther & Dunstan 1971; Weiss et al. 1985). Phosphorus is essential for cellular metabolism as it is involved in generating and transforming metabolic energy (Hernández et al. 1999). In the wild, seaweed growth in some temperate regions is often limited by phosphorus in spring (Howarth 1988; Flores-Moya et al. 1997) when there are lower P-concentrations in the seawater (Lobban & Harrison 1997). The nitrogen-to-phosphorus (N:P) ratio is the most important nutritional component for algal growth, as the optimum ratio varies from species to species and may therefore provide a basis for competitive elimination and co-existence of some algal species (mostly in unicellular algae) in water (Wu & Suen 1985). Although the critical ratio [where the growth rate is simultaneously limited by both nitrogen and phosphorus (Terry et al. 1985)] of Porphyra is not known, Wheeler and Björnsåter (1992) found that Porphyra spp. containing N:P ratios less than 12 were nitrogen-limited while those having a ratio greater than 17 were phosphorus-limited. Hafting (1999) thus demonstrated that P. yezoensis had to maintain an N:P ratio of 13–15 for its growth to not be limited by either nitrogen or phosphorus.
Nitrogen is by far the most common limiting nutrient in the growth of *Porphyra*, both in the wild and under culture conditions. Fujita *et al.* (1989) showed that temperate seaweeds, including *Porphyra* species, had a critical level of 1.5% dry weight for nitrogen. Below this critical level, the *Porphyra* blades tended to lose some of the photosynthetic pigments and thus become bleached.

Blade color has thus been used to reflect the concentration of nitrogen assimilated from the medium in which the *Porphyra* is growing. Amano and Noda (1987) showed that the photosynthetic pigments of *P. yezoensis* increased with increased nitrogen content, thus resulting in a darker thallus. Hafting (1999) also found that the growth of *P. yezoensis* was generally higher with NO$_3^-$ and suggested that this was a result of increased efficiency of tissue P utilization by blades when NO$_3^-$ was the N-source. Furthermore, most of the seawater nitrogen used by *Porphyra* and other seaweeds is mainly assimilated in the nitrogenous form of nitrate (NO$_3^-$), because this is the most abundant form of N in the sea, although ammonium (NH$_4^+$) and urea may be used as a nitrogen source in farms (Wu *et al.* 1984; Lobban & Harrison 1994).

Apart from nitrogen and the other macronutrients, *Porphyra* also requires trace elements (micronutrients) in order to grow properly. These elements are found in smaller quantities, and zinc is by far the most important essential trace element found in seaweeds (Liao *et al.* 2004). Noda and Horiguchi (1971) showed that *P. tenera* required zinc, at an optimum concentration of 0.5nM. They also showed that without zinc, chlorophyll and phycobilin production were hindered. Furthermore, they showed that zinc deficiency caused the disappearance of cytoplasmic ribosomes, resulting in a decrease in the content of high-molecular-weight proteins and their synthesis. Iron is also an important cellular component as
it is involved in photosynthesis, respiration and nitrogen fixation and is active in a variety of enzymatic reactions (Weiss et al. 1985).

1.6. Global overview of Porphyra mariculture

Although the mariculture of *Porphyra* dates back to the seventeenth century (South & Whittick 1987; Oohusa 1993; Ohno and Largo 1998; Sahoo and Yarish 2005) between 1624 and 1651 (Miura 1975), *Porphyra* cultivation is still mainly concentrated in Japan, Korea and China (Khan and Satam 2003, Marsham et al. 2007, Niwa and Aruga 2006). The culture of *Porphyra* in these areas was revolutionized by the findings of Drew (1949), who demonstrated for the first time that the shell-boring *Conchocelis rosea* Batters (1892) organism was actually a phase of *Porphyra umbilicalis* (L.) Kützing 1843, and not an independent organism as previously thought. This discovery made it possible to artificially seed *Porphyra* and thus predict and control the size and quality of the harvest crop (Iwasaki 1961; Lee & Fultz 1970; South & Whittick 1987; Lobban & Harrison 2004; Dring 1995; Vymazal 1995; Sahoo et al. 2002; Gualtieri & Barsanti 2006; Jiang & Gao 2008).

Conventionally, *P. yezoensis* and *P. tenera* cultivars were the most cultivated because of their economic value (Tseng 1944; Park et al. 2003). *Porphyra yezoensis* has been, by far, the most widely cultivated of the two due to its ability to withstand physical and chemical conditions in mariculture farms (Guiry 1996; Blouin et al. 2006). Furthermore, the Japanese nori consumers consider *P. yezoensis* to have a better flavor compared to *P. tenera* (Blouin et al. 2006). Although these two still remain the principal species, with *P. yezoensis* forming the bulk of the crop, the number of cultured *Porphyra* species has rapidly increased recently, mainly as a result of improved culture techniques (Uppalapati and Fujita 2000). Furthermore, the degeneration of the *P. yezoensis* cultivar quality, characterized by outbreaks of thallus
discoloration called “iroochi” of the cultivated strains, has resulted in greater loss in *Porphyra* farming in recent years (Wang *et al.* 2006; Kakinuma *et al.* 2008). Currently more than six species of *Porphyra* are frequently cultured on a commercial scale (Sahoo and Yarish 2005).

In the Asian countries *Porphyra* has been traditionally used for various human needs, primarily for human consumption (Tseng 1944). *Porphyra* is used mainly for the production of various forms of ‘nori’ in Japan (Oohsa 1993, Ohno and Largo 1998), ‘kim’ in Korea (Sahoo *et al.* 2002; Subba Rao *et al.* 2007) or ‘zicai’ in China (van der Meer 1983; Vymazal 1995; Preisig and Andersen 2005). In Korea, for example, *Porphyra* blades are usually rolled and toasted and eaten with rice (Sohn 1998). *Porphyra* is also used as sheets to prepare sushi (Rodriguez *et al.* 2003).

The consumption of nori has increased in countries, outside Asia, that have experienced growth of their Japanese and Korean populations (Mumford 1986). In parts of the world such as Chile (Alveal 1998), Argentina (Rodriguez *et al.* 2003), Wales (Aitken *et al.* 1991) and some areas of the USA (Merrill and Waaland 1998) *Porphyra* is used mainly for the production of ‘luche’ and ‘laver’. In New Zealand, *Porphyra* is consumed as karengo (Aitken *et al.* 1991). However, *Porphyra* is not the major economic seaweed produced by the latter countries, and most of this *Porphyra* is harvested from wild populations.

By contrast, in the Asian countries, *Porphyra* is regarded as the most economically valuable red seaweed (van der Meer 1983; Tseng & Fei 1987; Akatsuka 1990; Guiry 1996; Gualtieri & Barsanti 2006; Niwa *et al.* 2006). By weight, *Porphyra* production represents 12.5% of the world’s seaweed mariculture, which in turn, represents 23% of the world’s total
production of marine organisms. (Pereira et al. 2008). In 1994, the production of Porphyra reached about 270,000 tons (wet weight) in Korea (Sohn 1998; Park et al. 2003). In the Japanese economy, Porphyra production amounts to an annual average of 400,000 tons (McHugh 2003; Subba Rao et al. 2007) with a monetary contribution amounting to almost 100\times 10^9 yen/year (Niwa et al. 2006). Lately the production of Porphyra has been recorded at over 1.01 billion metric tons wet weight, worth over U.S. $1.8 billion dollars in the Asian food industry (He & Yarish 2006; Pereira et al. 2008). As a result of this high economic value of Porphyra, various means of constantly increasing its production have been devised, leading to the mass cultivation of Porphyra for commercial purposes.

1.7. South African seaweed industry

In South Africa, however, the seaweed industry only started in the early 1950’s (Anderson et al. 1989; Critchley et al. 1998) after the Second World War. The unavailability of Japanese agar to Britain, during and after the Second World War, is suggested to have been the major driving force behind the inception of the South African seaweed industry (Isaac & Molteno 1953). Although Isaac (1942) identified about 20 species with economic potential, only a few were abundant in quantities large enough to support the market. As a result, economic interest was focused mainly on the kelps (i.e. Laminaria pallida and Ecklonia maxima) and certain red seaweeds (i.e. Gracilaria and Gelidium).

Since then, the seaweed industry has grown steadily with increased demand for kelp both in the local and international market (Anderson et al. 1989). This increase was attributed mainly to the commercial production of the plant-growth stimulant (Kelpak\textsuperscript{TM}) since the 1970s (Troell et al. 2006). More recently, the rapid development of the South African abalone industry, where seaweed forms the primary diet of the farmed abalone, has increased the
seaweed demand locally. As a result, 23 Concession Areas (Anderson et al. 2003) have been allocated to specific companies and individuals as a means of regulating the harvest and collection of seaweed species of economic importance.

The South African industry relies mainly on the harvesting of wild populations as well as on collection of beach-cast seaweeds. Although kelp makes up the bulk of the harvested seaweeds in South Africa (Anderson et al. 2003; Rothman et al. 2006), wild Porphyra capensis was also harvested for export between 1965 and 1978 (Anderson 1989; Critchley et al. 1998). However, there have been no further Porphyra harvests since then, probably because there was no local or international market for South African Porphyra. More recently, the biomass of Porphyra was found to be insufficient for sustainable harvesting on a large scale, hence no concessions were allocated (Griffin et al. 1999b). Meanwhile, according to Griffin et al. (1999b), individuals in local coastal communities may have been harvesting Porphyra, in relatively small quantities for their own domestic consumption, since the last documented records.

### 1.8. Seaweed mariculture in South Africa

Although the South African export market is based on collection of beach-cast and harvesting wild populations, there are seaweeds that are cultivated. In the 1990s, attempts were made to cultivate Gracilaria at Saldanha Bay, because of the unpredictable nature of beach-cast supply of Gracilaria there. However, these were not commercially successful due to low nutrient levels in the water during summer. Lately, however, the expanding abalone mariculture industry has provided opportunities for research on the integration of seaweed mariculture with that of abalone.
Research into the potential to cultivate South African seaweeds was started in 1985, when Anderson and Bolton investigated the potential to cultivate *Gelidium vittatum* (as *Suhria vittata*) in the laboratory. The first attempts to cultivate seaweed were then initiated in the early 1990s (Anderson *et al.* 1996; 2003; 2005) when growth experiments with *Gracilaria* were conducted on rope rafts in Saldanha Bay. Aken *et al.* (1992) and Wakibia *et al.* (2001) also conducted experiments on the cultivation of *Gelidium* and *Gracilaria* respectively. However, the main factor limiting the potential for open-water cultivation of seaweeds in South Africa is the extremely wave-exposed nature of the coastline, which has only a few large sheltered bays (e.g. St Helena Bay and Saldanha Bay on the west coast). Commercial attempts to farm *Gracilaria* at Saldanha Bay failed, mainly because of low nutrient levels in the bay in summer. Although St Helena Bay is apparently a better site for cultivation (Wakibia *et al.* 2001; Anderson *et al.* 2003) no commercial cultivation has been attempted there.

Collaborative research on the mariculture of various seaweeds, in tanks, has since been conducted (Troell *et al.* 2006). The studies into the various components of *Gracilaria* cultivation include those of Fourie (1994), Smit (1994; 1998), Smit *et al.* (1997); Hampson (1998); Wilson (1999), Morgan (2000), Leitao (2001; 2005), Miller (2001) and Njobeni (2006). Such tank cultivation studies lead Wild Coast Abalone to experiment on large-scale cultivation of *Gracilaria* and *Ulva* as supplement food for the farmed abalone (Steyn 2000).

As a result, Wild Coast Abalone Ltd have become world leaders in *Ulva* cultivation, having large cultivation paddle ponds of 40 m×10 m dimensions and 0.5–0.75 m depth (Bolton *et al.* 2009). Early in 2006 a collaboration involving research projects by Kandjengo (2000); Potgieter (2005); Flodin (2005); Hansen (2005); Brandt (2006); Lindstrom (2006);
Robertson-Andersson (2003), Robertson-Andersson (2007) and Robertson-Andersson et al. (2008) led to the inception of an Ulva-abalone integrated partial recirculating culture system in I&J West Coast Abalone farm producing approximately 1,100 ton wet weight of Ulva per year (Bolton et al. 2009). These research studies, together with the success in Wild Coast Abalone Ltd, have demonstrated that Ulva and Gracilaria species can be cultured successfully in raceways on a large-scale in South Africa. Species from these two genera, mainly Ulva, are thus currently cultured to supplement the feed requirements of the local abalone farming industry and subsequently improve the water quality of the farms.

The prevalence of epiphytic growth of diatoms and filamentous brown algae on Gracilaria resulted in more attention being paid to Ulva cultivation. The seasonal occurrence of a brown epiphyte, Myrionema strangulans (Robertson-Andersson 2003), may be inconvenient to farmers since it is only controlled by addition of fertilizer or by restocking with new Ulva when fertilization fails (Bolton et al. 2008). As a result, lately there has been growing interest in the suitability of the local Porphyra as a supplement for abalone feed and for effluent bioremediation in and around mariculture farms. As a result, Griffin et al. (1999a; b) conducted research on the biomass and distribution of Porphyra along the South African coastline and found it to be relatively low to sustain the bulk nutritional demands of the abalone industry. As a recommendation, they suggested the cultivation of Porphyra as sustainable means to support the abalone feed industry. Apart from the attempts of Graves (1955) to cultivate and study the conchoelis phase of the life history of P. capensis in the laboratory, there has been no further research on the life histories or cultivation of Porphyra in South Africa.
1.9. Background to this study

Wild abalone generally feed on a broad selection of seaweeds, normally with at least two species being found in their gut content at any one time (Barkai & Griffiths 1986; 1987). However, a number of abalone species tend to favor red seaweeds, compared to the other seaweeds (e.g. Shepherd & Steinberg 1992; Stepto & Cook 1993). This is also the case with the South African abalone (*Haliotis midae*) which consumes a variety of red seaweeds in combination with kelp (Barkai & Griffiths 1986). Furthermore, *H. midae* is sedentary and is therefore largely opportunistic, feeding mainly on loose, drifting seaweeds in nature.

Since *H. midae* is relatively slow-growing, and the South African abalone mariculture industry is rapidly developing, a steady supply of feed resources is needed for sustainable productivity. Formulated feeds and other seaweeds, particularly kelp, are therefore used as abalone feed. However, formulated feeds are expensive and therefore large quantities of kelp are harvested in order to meet the feed requirements of the industry. As a result, in the year 2004, the abalone industry used approximately 5400 tons of kelp for abalone feed (Troell *et al.* 2006). This harvesting puts pressure on the natural kelp beds because only limited amount of kelp harvesting is permitted (Anderson *et al.* 2006) and harvest limits are close to being reached in certain areas (Troell *et al.* 2006), and therefore any alternative sources of abalone feed are worth investigating.

Thus far, mixed diets of kelp and other seaweeds such as the green seaweed *Ulva* and the red seaweed *Gracilaria* have been tested and found to enhance *H. midae* growth rates (e.g. Simpson & Cook 1998; Naidoo *et al.* 2006). *Gracilaria* and *Ulva* have also been successfully cultivated and used, in combination with formulated feeds, to feed abalone in farms that have
no kelp-beds nearby. Recently, it has also been shown that fortification of formulated feed with seaweeds yields improved growth rates (Naidoo et al. 2006; Dlaza et al. 2008).

*Porphyra* species have high contents of digestible protein and free amino acids (Lobban & Harrison 1997). These species are nutritious and contain relatively high contents of protein, polysaccharides, vitamins and minerals (Nisizawa et al. 1987; Noda 1993; Davies 1997). The crude protein content of *Porphyra* has been found to be as high as 56% dry weight (Noda & Horiguchi 1975 in Aitken et al. 1991) and in 1993, *P. endiviifolium* was found to constitute 73 – 100% of the biomass found in the gut content of the wild fish *Notothenia coriiceps* (Iken et al. 1999). Based on the study by Simpson (1994), it has therefore been suggested that *Porphyra* could be a valuable supplement food for the South African abalone, *H. midae*, (Stepto and Cook 1996; Griffin et al. 1999a; Gualtieri & Barsanti 2006).

The relatively high crude protein of *Porphyra* makes it suitable for use as feed for post-larval abalone. The relatively thin monostromatic thallus of the local *Porphyra* species makes them suitable candidates since *Porphyra* may be more easily digested than the other locally cultivated seaweeds that are thicker (e.g. the distromatic *Ulva*) or pseudoparenchymatous (e.g. *Gracilaria*) species. Since abalone feed by trapping their food and rasping with their radula, the flat morphology of *Porphyra* blades would increase the feeding surface area for the abalone, compared to the stringy *Gracilaria*. Furthermore, Morse and Morse (1984) found that *Porphyra* sp. contained gamma-aminobutyric acid mimetic molecules that induced metamorphosis and settlement in the larvae of abalone, *Haliotis rufescens*, making *Porphyra* a suitable candidate for cultivation in the South African abalone industry.
The export of South African *Porphyra* for human consumption (nori) was not commercially successful because of the low biomass in the wild and the tough texture of *P. capensis* (Griffin *et al.* 1999a). The positive identification of other species that were previously lumped under *P. capensis* increases the possibilities of finding a species that may be more suitable for the production of quality nori. Finding a local species suitable for cultivation could provide an international nori market to the Far East and also create a local market as well. The cultivation of the various South African species is therefore necessary to realize the economic potential of South African *Porphyra*.

### 1.10. Aims of this study

Since the main triggers for the life history of *Porphyra* are temperature, photoperiod and irradiance (Krishnamurthy 1969; Kapraun & Lemus 1987; Waaland *et al.* 1987; Kim 1999) and the seasonal occurrence of *Porphyra* species may be constrained by unfavourable environmental conditions (Conway *et al.* 1976), the first aim of this study was therefore to study the reproductive biology and phenology of three common *Porphyra* species which occur on the west coast of South Africa. Since *Porphyra* are intertidal species where there is an environmental gradient characterised by significant differences of various physical factors, including irradiance, temperature, nutrient availability, salinity and desiccation (Lüning 1990; Lobban & Harrison 1994; Davison & Pearson 1996), the second aim of the study was to investigate the ecophysiological responses of the different phases of the life histories of these three species to various environmental variables in culture. Although the nutritional effects of the strong upwelling cold Benguela current are well documented for the South African seaweed ecosystems (Bolton & Levitt 1987; Emanuel *et al.* 1992; Waldron & Probyn 1992; Stegenga *et al.* 1997; Waldron *et al.* 1997), no studies have looked into the effects of this phenomenon with special regards to the nutritional variability of *Porphyra*. The third aim of
this study was to quantify some important aspects of the nutritional composition of these three species and to investigate seasonal variations in these nutrients. The seasonal variation in crude protein (using the 6.25 conversion factor) and soluble protein (using the Bradford, Bovine Serum Albumin method) was also investigated in these species. Since Griffin (2003) recorded high morphological diversity in South African *Porphyra* and the rDNA SSU results reflected high levels of variation and possibilities of new species, the final aim of this study was to collect and briefly describe the previously unidentified taxa that are morphologically different along the west coast. This study also makes recommendations on the species most suitable for culture in South Africa as well as for incorporation in the diet of farmed abalone *Haliotis midae*. 
Chapter 2

Reproductive phenology of three wild South African 

*Porphyra* species
2.1. Introduction

Seaweeds are important primary producers in marine ecosystems (Ramirez et al. 2003), contributing 3.2% to the global aquatic primary production (Zacher et al. 2007). The biomass of various species in a community may be structured by the seasonal variations in atmospheric and biological conditions. Various studies have examined seasonal changes in seaweed populations along different coastlines (e.g. Rhodes 1970; Lebednik et al. 1971; Lubchenco & Cubit 1980; Chock & Mathieson 1983; Pacheco-Ruíz et al. 1992; 1999; Cruz Ayala et al. 1998; Scorsati 2001; Ramirez et al. 2003). Studies specifically related to the seasonality of Porphyra species include those of Waaland et al. (1990); Lindstrom and Cole (1993); Karsten (1999); Yarish et al. (1999) and Pereira et al. (2005). Santelices et al. (1981) and Martinez (1988) conducted studies that were specifically designed to observe the seasonality in Porphyra propagule availability and consequent recruitment rates.

Thus far, there have been several studies conducted on the seasonality of Porphyra in South Africa. Graves (1969) studied the seasonal occurrence and distribution of P. capensis along the South African coastline and documented various growth forms of what she considered to be this species. However, it is likely that she was dealing with a number of different species, since all South African species were lumped under P. capensis at that time (Griffin et al. 1999c; Jones et al. 2004). Stegenga et al. (1997) investigated and recorded the distribution of four Porphyra species in South Africa. Griffin et al. (1999a; b) studied the distribution, seasonal biomass and the biomass suitable for commercial harvesting of South African Porphyra. Although there have been such studies conducted on the South African Porphyra species, much of the biology remains unknown.
Most studies on seasonality of *Porphyra* have focused on the foliose stage of the life history. Since the cultivation of *Porphyra* used to be initiated with the collection of zygotospores from natural wild populations in the past, collection for experimental and commercial cultivation was traditionally conducted annually; to coincide with the necessary stage in the life cycles of the target species. In Asia, for an example, the spores were collected in spring when the *Porphyra* is most fertile (Dai *et al.* 2004).

When spores can only be collected at a particular time of the year, this may affect the commercial production in farms. As a result, various culture methods are employed to ensure continuous production, including tissue culture (Liu & Gordon 1987; Notoya 1997; 1999), enzymatic isolation of thallus spores (Dai *et al.* 2004) and growing conchocelis indefinitely.

Very few studies have investigated temporal variations in the release of *Porphyra* zygotospores. Holmes and Brodie (2004) conducted an annual reproduction study on *P. leucosticta* and found that, although spores were released throughout the year, quantities released in culture varied. This was because the fertility of the species may somehow be influenced by the annual fluctuations in ‘primary ecological factors’ (Lüning and tom Dieck 1989), such as water temperature and irradiance.

Thus far, no fertility studies have been conducted for any of the South African *Porphyra* species. The aim of this study was therefore to investigate the reproductive phenology of *P. aeodis*, *P. capensis* and *P. saldanhae* in culture and to determine the best time to collect spores to initiate cultivation.
2.2. Materials and Methods

2.2.1. Selection of Porphyra species and collection site

Three South African Porphyra species (Porphyra aeodis Griffin, Bolton et Anderson 1999, Porphyra capensis Kützing 1843 and Porphyra saldanhae Stegenga, Bolton et Anderson 1997) were selected for the experiments conducted to complete this study. These species were chosen based on their high biomass and wide range of distribution compared to the other known Porphyra species in South Africa.

Fresh mature thalli were collected from Kommetjie (34°09′06″S, 18°19′22″E) along the Cape Peninsula, South Africa. Kommetjie was chosen because it is the type locality site for P. aeodis and where the other two species (P. capensis and P. saldanhae) are also found in abundance. Furthermore, this site is a marine protected area and therefore anthropogenic effects, e.g. the removal of other organisms from the ecosystem, should be minimal to the seaweed community. This site was therefore regarded as most ideal since the community structure of the seaweed populations was relatively pristine. Water temperature was measured, with an electric temperature logger (Staroddi) recording every 30 minutes at a depth of 5m, throughout the study period, at Oudekraal (33°58′51″S, 18°21′47″E) north of Kommetjie. This is the nearest site for which continuous temperature measurements were available.

At Kommetjie, the foliose plants were visually identified based on morphological characters used by Stegenga et al. (1997) and Griffin et al. (1999a). In the laboratory, cytological identification of the collected material was checked microscopically. Collections were made in July 2006 and every second month thereafter until January 2007. The collections were then made monthly from January 2007 through to February 2008. For P. aeodis, the collection
period was extended to April 2008 based on the pattern observed in 2007, whereby this species was not found from May until August. Although collection was done during low spring tide, only thalli that were damp or found immersed in water were collected. A minimum of thirty thalli of *P. capensis*, *P. aeodis* and *P. saldanhae* was randomly collected for each species to ensure that sufficient material was available. Collected material was kept in plastic bags, filled with seawater, that were transported in a sealed bucket.

### 2.2.2. Fertility studies

After identifying each species, surface water was removed from thirty individuals of each species using a salad spinner and then the plants were blotted with paper towel to remove excess water. The wet mass (grams), the length (mm) and width (mm) of each individual was measured. The length was measured across the holdfast to the tip while the width was measured across the widest point of each individual. On rare occasions where individuals had more than one blade, only the longest and widest blade (as in Holmes and Brodie 2004) was measured.

To measure the fertility of each individual thallus, two methods were used. In the first method, the fertile area (FA), in equation 1, was determined by tracing and measuring the widest and narrowest fertile sections for each individual. In the second method, the fertile mass (FM), in equation 2, was determined by excising the whole fertile margins and weighing them.

\[
FertileArea(\%) = \left[ \frac{(F_w + F_n)/2}{(W + L)/2} \right] \times 100 \tag{1}
\]

Where \(F_w\) is the widest fertile section, \(F_n\) is the narrowest fertile section, \(W\) is the width and \(L\) is the length of the individual.

\[
FertileMass(\%) = \left[ \frac{F_{wm}}{W_{wm}} \right] \times 100 \tag{2}
\]

Where \(F_{wm}\) is the wet mass of the fertile margin and \(W_{wm}\) is the wet mass of the whole individual.
Spore release was estimated as follows: Nine fertile discs (1x 2 mm-diameter disc per randomly selected blades) were excised with a cork borer for each species, and three of these discs were incubated in three different Petri dishes containing 25ml sterile seawater each for 24 hours. Three subsamples of 1ml were then taken from each dish and the spores were counted. This was done from January 2007 until April 2008.

2.2.3. Statistical analysis

Data were presented as tables or plotted graphically as means with Standard Error ($\bar{x} \pm SE$). To assess the effects of annual variation, a single-factor Analysis of Variance (ANOVA) for the monthly means for the wet mass, length and width was performed for each species using Statistica Release 8, StatSoft Inc., Tulsa, USA. For each month, a two-tailed 2 Sample t-Test was performed to test the relationship between length and width of each species. Pearson’s linear correlation analysis was performed for each species to test the relationship between the total area of individuals and their fertile area as well as between the total wet mass of individuals and their fertile mass. All data were regarded as significantly different at $p<0.05$.

For seasonal analysis, the months were grouped into: summer (December – February), autumn (March – May), winter (June – August) and spring (September – November) to correspond with meteorological seasons.
2.3. Results

2.3.1. Field observations

Of the three species, *Porphyra capensis* was the most abundant throughout the study period. It varied in shape (Figure 1) and was epilithic and abundant higher up the intertidal zone. In the mid intertidal zone it was found growing side-by-side with *P. saldanhæ*. Although these species were normally growing as separate individuals, they were sometimes found growing with their holdfasts fused together (Figure 2). *Porphyra saldanhæ* (Figure 3), throughout the study period, was either epilithic, epizoic on black mussels or (seldom) epiphytic on *Nothogenia ovalis*. *Porphyra aeodis* (Figure 4) was restricted to the lower intertidal fringe where it was epiphytic on *Aeodes orbitosa*. Although a few vegetative individuals of this species were found in July 2006, none could be found in the winter months of 2007 and 2008. During these periods, *A. orbitosa* was sometimes heavily epiphytised by the red seaweed *Aristothamnion collabens*.

The *Porphyra* individuals were either: vegetative, had separate male gametangial and female gametangial sori (*P. capensis*); had a separate blade bearing male sori and one bearing female sori attached to the same holdfast (*P. capensis*); or had both male and female gametangial sori on the same blade (*P. aeodis* and *P. saldanhæ*). In *P. capensis*, individuals with dark red margins turned out to be fertile individuals bearing female gametangial sori while the individuals bearing male sori had a distinct yellowish-green margin. Based on these features, reproductive male and female individuals of *P. capensis* could be easily distinguished in situ. For *P. aeodis* and *P. saldanhæ*, however, the reproductive margins were distinguished by a band of dark red (female sori) and whitish-cream (male sori) patches interspersed along the margins of thalli.
Figures 1 – 4. Figure 1. The various morphological forms of P. capensis individuals: a) cordate, b) umbilicate and c) hermaphroditic with both male and female blades sharing a single holdfast (arrow). Figure 2. Individuals of both P. capensis and P. saldanhae sharing a fused holdfast. Figure 3. P. saldanhae with typical undulations along the thallus margins and various morphologies: a) linear but more cordiform morphology b) lanceolate morphology with a smooth mid-rib section and c) P. saldanhae growing epiphytic on Nothogenia ovalis. Figure 4. P. aeodis showing morphological plasticity with a) an umbilicate shape, b) cordate and c) a rosette shape. Scale: Ruler in figure 1c = 30cm, pencil in figure 3b = 13cm and solid line in other figures = 5cm.
2.3.2. Temporal variation in temperature, wet mass, length and width of fertile individuals

Seawater temperatures were higher in winter (Figure 5) and there were year-to-year variations, with winter temperatures significantly higher ($p = 0.0074$) in winter of 2007 compared to 2006.

![Figure 5. Monthly mean seawater temperatures from Oudekraal.](image)

Reproductive individuals of *P. capensis* were significantly heavier ($p = 0.0032$) and larger in diameter ($p = 0.024$) than both *P. aeodis* and *P. saldanhae* throughout the study period. Although water temperatures were highest in winter, both *P. capensis* and *P. saldanhae* had the smallest individuals during this period (Figure 6). The largest individuals were found in spring 2007 for *P. capensis*, while the t-Test revealed that there were no significant differences ($p > 0.05$) between the length and width of this species most of the time. For *P. saldanhae*, the individuals were smaller and thinner during winter (Figure 6) and the t-Test revealed that the individuals were always significantly longer than broad ($p = 0.0037$) for this species. For *P. aeodis*, the t-Test revealed that the individuals of this species were generally
broader than long (p = 0.0371), and the narrowest individuals were found in spring while the broadest in autumn (Figure 6).

![Graphs showing mass, length/width ratio, and seasonal temperature of Porphyra species](image)

**Figure 6.** Temporal variation in the mean sea surface temperature (from Oudekraal), wet mass and length-to-width ratio of three *Porphyra* species from Kommetjie. The vertical bars represent standard error between the various months: summer (December – February), autumn (March – May), winter (June – August) and spring (September – November). The gap in *P. aeodis* is the period when this species could not be found in the wild.
2.3.3. Reproductive anatomy and phenology

Fertile female sori of *P. aeodis* had zygotosporangial cells arranged in groups of 4 (2x2), with each cell being 6 - 10 µm wide and 12 - 16 µm long (Figure 7a) in surface view (SV). In transverse view (TV), each of the mature zygotosporangia divided into 4 zygotospores (Figure 7b), giving rise to division formula: a/2, b/2, c4. Actively dividing sori that were not yet mature enough to release spores had each cell divided into 2 zygotosporangia (Figure 7c), resulting in division formula: a/2, b/2, c/2. In female individuals of *P. capensis*, the zygotosporangial packets had 8 isomorphic cells (4x4) in SV (Figure 8a). In TV, each zygotosporangium divided into 8 zygotospores (Figure 8b), resulting in reproduction division formula: a/4, b/4, c/8. Immature zygotosporangia had fused cells (Figure 8c) while spores could be clearly seen when the matured zygotosporangia (Figure 8d) are ready to release spores. The female sori of *P. saldanhae* had zygotosporangial packets with 8 (4x4) cells (Figure 9a) with each cell, (TV), divided into 2 cells when immature (Figure 9b) and then eventually diving into 4 cells (Figure 9c), resulting in division formula: a/4, b/4, c/4.

![Figure 7](image1.png)

**Figure 7.** Female sori of *P. aeodis*: a) surface view, b) transverse view of mature sporangia and c) transverse view of immature sporangia.

![Figure 8](image2.png)

**Figure 8.** *P. capensis*: a) surface view of female sori, b) transverse view of zygotosporangia, c) transverse view of immature zygotosporangia and d) mature zygotosporangia ready to release spores.
While there was no correlation \((r=0.521, p=0.068)\) between the FA and FM methods in \(P.\) aeodis, there was a significant correlation for both \(P.\) capensis \((r=0.528, p=0.029)\) and \(P.\) saldanhae \((r=0.872, p<0.001)\) with FM being greater than the FA method in both species. Both methods (FA and FM) reflected that the degree of fertility was generally not significantly related \((p>0.05)\) to thallus size of the collected fertile individuals of these three species (Table 1), although there were months when both methods detected some correlations.

**Table 1.** Annual correlation \(\left( r^2 \right) \) between diameter and fertile area (FA) then total wet mass and mass of fertile tissue (FM) for individuals from each of the three Porphyra species. Rows with a dash sign (-) represent months when \(P.\) aeodis was absent or when \(P.\) capensis and \(P.\) saldanhae were not collected. All correlations significant at \(p<0.05\) are denoted by an asterisk (*).

<table>
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**Figure 9.** Surface and transverse view of the female reproductive sori of \(P.\) saldanhae: a) surface view, b) transverse view of immature zygotosporangia and c) mature zygotosporangia in transverse view.
Porphyra aeodis had significantly more fertile area \((p = 0.0294)\) and fertile mass \((p = 0.0417)\), while there were no significant differences between P. capensis and P. saldanhae. Both P. capensis and P. saldanhae were fertile throughout the study period (Figure 10), while P. aeodis was fertile mainly in summer and autumn. All three species had more fertile area, and fertile mass, percentage in the summer (Figure 10). Although both P. aeodis and P. saldanhae were fertile in summer, ANOVA reflected that P. aeodis was significantly more fertile \((p<0.05)\) than P. saldanhae during this period.

![Figure 10](image.png)

**Figure 10.** Temporal variation in the fertility of the three Porphyra species from Kommetjie. Each point is a mean percentage of 30 individuals and reflects the average percentage of the average mass of each species per month.

Porphyra capensis released more spores than both P. aeodis and P. saldanhae (Figure 11). Although P. capensis and P. saldanhae were both fertile throughout the year, there were significantly fewer \((p<0.05)\) spores released from these species in the winter season. The highest number of spores released was during end of spring to late summer for all three species.
Figure 11. The mean number of spores, (means of three sub-samples taken for each month per species), released from discs of the three species. Since \( P. \) \textit{aeodis} was absent in winter, no spore release experiments could be performed for this species during this period. Vertical bars are standard error bars.
2.4. Discussion

Seaweeds often demonstrate pronounced seasonal patterns of growth and reproduction (Lubchenco & Cubit 1980; Lüning 1993) and in *Porphyra* species such seasonality is evident in the gametophytic foliose phase (Kurogi 1961; Mitman & van der Meer 1994). Some *Porphyra* species occur in spring through summer while some occur during winter through spring (see Rhodes 1970; Yabu 1978; Dickson & Waaland 1985; Waaland et al. 1990a; Sfriso et al. 1992; Griffin et al. 1999a,b; López-Vivas & Riosmena-Rodriguez 2000; Holmes & Brodie 2004; Valera-Álvarez et al. 2007; Kim et al. 2008). Species that occur in spring through summer include *P. abbottae*; *P. endiviifolium*; *P. fucicola*; V Krisnamurthy *P. kanakaensis* TF Mumford 1973; *P. maculosa* E Conway 1976; *P. nereocystis*; *P. occidentalis*; *P. papenfussii*; *P. perforata*; *P. smithii* Hollenberg & IA Abbott 1968 and *P. variegata* while those occurring during winter through spring include *P. brumalis* TF Mumford 1976; *P. katadae*; *P. leucosticta*; *P. pseudolanceolata*, *P. torta* and *P. yezoensis*. The results of this study revealed that the foliose phase of *P. aeodis* occurred seasonally from spring through summer and this was consistent with the original description of this species (see Griffin et al. 1999c). Although this seasonality was different from that of other local species (*P. capensis* and *P. saldanhae*) its seasonal occurrence range was similar to *P. gardneri*, another epiphytic species also found in South Africa, which grows in spring through summer in the coast of America (Mumford 1986).

Although *P. aeodis* was recorded in the winter of 2006, its absence in winter of 2007 and 2008 was not surprising. Firstly its host, *Aeodis orbitosa*, is a summer annual (Levitt et al. 1995), that recruits in winter and grow bigger in summer with new juvenile *P. aeodis* thalli only in late winter (Griffin et al. 1999c). Furthermore, Dickson and Waaland (1985) suggested that the seasonal and epiphytic *P. nereocystis* might synchronize its life history.
with that of the host to ensure successful attachment. They suggested that conchospore
release and the subsequent blade development of *P. nereocystis* occurred when the stipe of
the annual *Nereocystis luetkeana* sporophyte had ceased elongation, but before the stipe was
covered by other epiphytes.

The second reason for the absence of *P. aeodis* in winters of 2007 and 2008 may be because
of *Aeodis orbitosa* was partly colonized by other epiphytes during these periods, suggesting
that *P. aeodis* may have failed to settle before these epiphytes. However, competition for
space may not have been a significant reason for the absence of *P. aeodis* since the various
epiphytes on *A. orbitosa* seemed to prefer different sites for attachment. *Porphyra aeodis* was
frequently found growing on the margins and wound sites while the other epiphytes were
always found on the smooth surface of *A. orbitosa*. However, it was observed that the other
epiphytes were absent whenever *P. aeodis* was present.

A third reason for the absence of *P. aeodis* could be that the environmental conditions were
not favourable for germination and the conchocelis phase of *P. aeodis* remained dormant
during these years. This was because the seasonal occurrence of *Porphyra* species may be
due to unfavourable environmental conditions (Conway *et al.* 1976), since the main triggers
for the life history of *Porphyra* are temperature, photoperiod and irradiance (Krishnamurthy
1969; Kapraun & Lemus 1987; Waaland *et al.* 1987; Kim 1999). For example, *P. endiviifolium*
has been found to be an opportunist that only grows on ice-free rocks during
the Antarctic summer (Wiencke & Clayton 1998) and its conchocelis may remain dormant
during periods with severe unfavourable environmental conditions. Furthermore, Griffin
(2003) found that even though there was no correlation between the South African *Porphyra*
biomass and atmospheric weather conditions, *Porphyra* recruitment seemed to respond to
environmental cues although the nature of such cues was not determined since he may have been dealing with a number of species.

Other species such as *P. dioica*, *P. miniata* and *P. schizophylla* Hollenberg may not demonstrate consistent seasonal patterns as they may be found growing from spring through summer sometimes, while they may also grow in winter (Rhodes 1970; Mumford 1986; Sahoo *et al.* 2006). This was similar to *P. saldanhae* which was found throughout the study period, but seemed to have lower densities in winter. However, the detection of *P. saldanhae* throughout the year was unexpected since this species was said to be a winter species (Griffin *et al.* 1999c), growing from June until December (Karsten 1999). *Porphyra capensis* was also found growing throughout the whole year and this was similar to reports by Holmes and Brodie (2004) and Pereira *et al.* (2004) who found *P. dioica* growing throughout the year. Although both *P. capensis* and *P. saldanhae* were found throughout the year, their individuals were smaller in winter suggesting that there were more small recruits present at that time of the year.

The recruitment patterns of seaweeds have been documented, by Shannon *et al.* (1988), to be maximal during autumn. Griffin *et al.* (1999b) also observed increased *Porphyra* recruitment rates in autumn. A similar pattern was observed for *P. capensis* and *P. saldanhae*, and this could account for the abundance of small individuals observed in autumn and winter for these species respectively. Brawley and Johnson (1991) found that the survival, in the habitat where successful recruitment for *Pelvetia fastigiata* occurred, was dependent upon the occurrence of “recruitment windows” when emersion coincided with benign (nondesiccating) climatic conditions. This explains the possible increased recruitment in winter when cloud cover and rain reduced desiccation, thus allowing survival and germination of *P. capensis*
and *P. saldanhae* germlings from spores that were released in autumn (see Chapter 5 for duration required to germinate from spores to blade). Monotila and Notoya (2004) also hypothesized that blades of *P. suborbiculata* were only visible months after the spores were released in the wild. For *P. aeodis*, the recruitment window was possibly during late winter – to – early spring, since most of the fertile individuals were found between November and February although individuals of *P. aeodis* were found from September to April. This was similar to *P. leucosticta* from Canada where the mature individuals were only found from November to February, albeit that this species was found in abundance from October to February (Yabu 1978). Although in the current data, there was no correlation found between the degree of fertility and the size of individuals of all three species, the fertile female sori of these species had zygotosporangial packets with fused spores (as shown in figures 9d for *P. capensis*) suggesting that most of the sori were not yet fully matured to form, encapsulate and liberate spores during the recruitment periods.

By contrast, the spores were readily liberated from the blades of all three species in summer resulting in more spores liberated for each species. This was because all the species had fertile individuals that had sori with zygotosporangial packets that were larger and matured to release spores (as shown for *P. capensis* e.g. in figure 8d). Cannon (1989) demonstrated that carpospore formation was initiated by rapid dividing and duplication of meristematic cells to form carpogonial cells that were thicker when ready to release carpospores in *P. abbottae*. He also found that the carpospores were released by pushing through the cell wall matrix, via escape passages, of mature carpogonia. Most bumps, and spore escape passages, on the mature zygotosporangia of *P. capensis* and *P. saldanhae* were observed in spring and summer while most of the immature sporangia, which had no distinct spores or bumps on the
surface, were observed during winter. For *P. aeodis*, the spore escape passages were more abundant during summer suggesting the peak maturity of zygotosporangia for this season.

The seasonal changes in size of fertile individuals could also explain the thicker zygotosporangial sori of these species in summer, since all these species had bigger individuals with thalli which could easily accommodate more spores in summer. The presence of bigger individuals corroborated the findings of McQuaid (1985) who reported that the growth of *Porphyra* along the Cape Peninsula was greatest during summer. Freshwater and Kapraun (1986) and later Mathieson (1989) also recorded maximal seaweed growth in summer and attributed such growth to increased daylength and temperature. Although the nutrient uptake of these species will be discussed in Chapter 6, it is worth mentioning that this region experiences annual upwelling that occurs mainly in spring (Bolton 1986; Waldron & Probyn 1992; Waldron *et al.* 1997). The observed high growth of these species in summer could therefore be attributed to the nutrient influx associated with the seasonal upwelling.

The number of spores released by *P. capensis* and *P. saldanhae* was less in winter and peaked in spring and this could also be due to the lower temperatures (see Chapter 3 for temperature effects on spore release) during this season. Holmes and Brodie (2004) also found that, although spore release occurred through out the year in *P. dioica*, there was seasonal variation in the quantity of spores released (with fewer spores in winter). Besides temperature effects, Lüning and tom Dieck (1989) suggested that the fertility of seaweed species may also be influenced by the seasonal fluctuations in irradiance. Although the irradiance was not measured in the current study, it is expected to have decreased during winter since this area experiences rainfall during winter. This, coupled with the reduced day length, would have reduced spore released from the two species. However, the photoperiodic
effects on these two species are expected to be minimal since Candia et al. (1999) stated that presence of Porphyra thalli throughout the year suggested a lack of photoperiodic control in the differentiation of their reproductive cells.

Environmental parameters not only affect seaweed reproduction but may also affect seaweed morphology (Mathieson et al. 1981) and such effects have been demonstrated in Porphyra blades in culture (eg in Freshwater & Kapraun 1986; Hannach & Waaland 1989; Monotilla & Notoya 2004). Seasonal plasticity in the characteristics of blades of P. fucicola resulted in summer blades being called a different species from the winter blades (Lindstrom & Cole 1992). Blades of P. aeodis tended to be predominantly rosette-shaped in summer, while they were more cordiform and umbilicate in spring and autumn respectively. Individuals of P. capensis were more lanceolate in summer with the majority being more ovate to umbilicate in winter and spring. The length/width ratio of collected individuals was also higher in summer compared to other seasons. In the wild, Mathieson (1989) only observed seasonal morphological changes that were due to extensive blade fragmentation resulting from extremely low temperatures. Although marginal fragmentation was observed in the local species during winter, it could not be attributed to extreme temperature changes since this area experiences minimal seasonal temperature variation (Bolton & Anderson 1997; Britz et al. 1997; Stegenga et al. 1997) compared to northern hemisphere cold temperate areas. Furthermore, temperature data also reflected increased temperatures during winter in this area. Fragmentation could have been due to a combination of wave action and the degeneration of reproductive cells that had recently released spores. Marginal fragmentation was more pronounced in P. aeodis in March – April since this species had very patchily arranged reproductive cells and became very perforated thus resulting the less spores released towards autumn.
Besides the annual morphological plasticity, there was also a gradual change in morphology perpendicular to the shore. The lanceolate to cordate individuals of *P. capensis* were mainly on the lower fringe while more umbilicate forms dominated higher up the shore. Since morphological plasticity could enhance light absorption and nutrient uptake (Hannach & Waaland 1989), the dominance of umbilicate individuals higher up the shore could be a survival response to longer desiccation periods, which result in water loses that significantly reduces productivity. Skene (2004) reported higher respiration and photosynthesis rates for seaweeds higher up the shore and suggested such habit as means to compensate for less photosynthesis time due to prolonged desiccation exposure. The umbilicate individuals therefore have a bigger surface area and may thus be more suitable for water retention, through folding and self-shading during desiccation periods (Norton *et al.* 1982) and therefore more ideal for the upper shore. Increased water motion has also been demonstrated to result in elongate blades in cultured *P. abbotiae* (Hannach & Waaland 1989) and this could explain the dominance of lanceolate individuals on the lower shore.

However, not all the lanceolate individuals found on the lower shore were of *P. capensis*. Monoecious species (resembling *P. aeodis*) and androdioecious species (see Chapter 8) were found growing on limpets and mussels during spring. Griffin (2003) detected seasonal shifts in the distribution of South African *Porphyra* within each of the sampled sites and suggested that there may be several species growing within each site. Brodie *et al.* (2008) also revealed that comprehensive collection over a small area at different times of the year may yield more species, since some species are ephemeral or have restricted times when they occur. The different morphologies, and possible species in this area, will be discussed in detail in Chapter 8.
Having conducted a seasonal study of these species, it was obvious that *P. capensis* and *P. saldanhae* are present throughout the year while *P. aeodis* can be rarely found in winter. The reproductive phenology of these three species showed that they were more fertile during summer. However, the results revealed that the presence of reproductive sori had no relation to thallus size for all three species. Seasonal variations in size and shape were evidence of also resulted in morphological plasticity in both *P. aeodis* and *P. capensis*. Throughout the study period, there were seasonal changes in the biomass and suggesting a possibility of new undescribed species in this area. Such possible species appear to be either ephemeral or have restricted times when they occur, with high biomass during spring through summer. The FM method for measuring the fertility of these species is recommended over the FA method, although the FA method could perhaps be improved with digital measuring.
Chapter 3

The effects of salinity, irradiance, temperature and incubation period on the release of spores from mature thalli of three South African Porphyra species in culture.
3.1. Introduction

Reproduction in the genus *Porphyra* may either be sexual or asexual, depending on the different stages of the genus life history (Nelson *et al.* 1998; Candia *et al.* 1999; Notoya & Nagaura 1999). Sexual reproduction, through the fertilization of the female gametes by the male gametes, results in the formation of zygotospores in the female gametangial sori (Tseng & Chang 1955; Mei *et al.* 2005). On the other hand, asexual reproduction occurs in the blade through production of archeospores, agamospores, neutral spores, endospores, while it occurs in protothalli through production of proplasts (Notoya 1997).

Mature fertile thalli of *Porphyra* release different types of spores as described by Nelson *et al.* (1999) i.e. 1. blade archeospores – results from vegetative cell differentiation and germinates directly into blade, 2. zygotospores – results from fertilization then zygote mitosis and germinate into conchocelis, 3. agamospores – from mitotic cleavage without fertilization and germinates into conchocelis, 4. neutral spores - from mitotic cleavage without fertilization and germinates into blades, 5. phyllospores – produced by blades where ploidy and development is unknown, 6. endospores – formed by mitotic division of a blade cell. This terminology is mainly used to describe their functional role in the life history of this genus, although some physical characteristics e.g. colour and size may also be used as descriptive characters. However, not all types of these spores may be simultaneously present in all species and the release of various types of spores may be influenced by environmental conditions such as temperature, irradiance and photoperiod (Pueschel & Cole 1985; Sindirelli-Wolff 1992).

The influence of environmental conditions on the release of spores has been studied in various other seaweeds (e.g. Umamaheswara Rao 1976; Ngan & Price 1983; Fonck *et al.*
1998; Azanza & Aliaza 1999; West & McBride 1999). Studies of the liberation of spores from *Porphyra* species include those of Kitade *et al.* (1998); Pacheco-Ruiz *et al.* (2005) and Bellgrove & Aoki (2006), although these studies investigated the effects of dessication, temperature, irradiance, salinity, photoperiod and diurnal rhythms on the release of conchospores instead of spores from *Porphyra* thalli. However, the diurnal rhythm effects on the discharge of monospores from *P. vietnamensis* were studied by Subba Rangaiah (1986) and later by Narasimha Rao and Subba Rangaiah (1991) who observed peak shedding between 10h00am and 14h00pm.

In the wild, the availability of spores is important in maintaining the algal community (Narasimha Rao & Subba Rangaiah 1991). Since most spores have a greater sensitivity to different abiotic and biotic factors than macro-stages of the life history (Schonbeck & Norton 1979; Brawley & Johnson 1991; Davison *et al.* 1993), they are therefore crucial for the survival and distribution of a species (Sousa *et al.* 2007). The abundance of different spores can also provide valuable information on phenological events experienced by the natural populations of seaweeds on the shore (Holmes & Brodie 2004). It is therefore important to have a sound knowledge of the characteristics of various spores released by different *Porphyra* species, as well as the environmental triggers for their release. This study thus examined the types of spores released from three South African *Porphyra* species. Since the optimal environmental conditions for the release of spores may vary between species (Pueschel & Cole 1985; Sindirelli-Wolff 1992), this study also investigated the role of different environmental conditions (i.e. temperature, irradiance, salinity, desiccation and incubation period) on the release of spores.
3.2. Materials and Methods

3.2.1. Seaweed collection
Mature, fertile thalli of three South African Porphyra species (*P. capensis*, *P. saldanhae* and *P. aeodis*) were collected from Kommetjie (34°09′06″S, 18°19′22″E) along the west coast of the Cape Peninsula, Cape Town. Although collection was done during low spring tide, on the 29th January 2007, only thalli that were damp or found immersed in water were collected. Collected material was kept in plastic bags, filled with seawater, that were transported in a sealed bucket. All experiments were initiated within two hours of collection.

3.2.2. Spore release
The surfaces of the collected thalli were washed in sterile seawater using a soft artist’s brush to scrub and remove any surface fouling. Discs (2cm diameter) from each species were excised with a cork borer and rinsed in sterile seawater. One disc from each species was then put into each of five wells of 25-well 100ml plastic repli-trays (Bibby, Sterilin®, Newport, UK). Each tray therefore had five discs from all species at once, and two trays were used for each experiment, so that there was a total of ten discs per species.

Since macroalgal species growing in the intertidal zone are subjected to changes in salinity, temperature and irradiance (Dhargalkar 2004), the effects of salinity, irradiance, temperature, desiccation and incubation period, on spore shedding were investigated for the three species. To determine the effects of salinity, excised discs were subjected to a range of five salinities (0, 10, 20, 30 and 40 PSU) for six hours (Table 1). To obtain lower salinities (0 - 20 PSU) seawater was diluted by adding distilled water while the seawater was evaporated by boiling (as in Stekoll et al. 1999) to obtain 40 PSU salinity. Five irradiance levels (0, 40, 80, 120 and 160 µmol photon m² s⁻¹) and five temperature levels (5, 10, 15, 20 and 25°C) were also tested for their effects on the release of spores from excised discs. To achieve 0 µmol photon m² s⁻¹
the trays were covered with a black plastic, while the other irradiance levels were achieved by varying the number of bulbs lit and the distance of the trays from the bulbs. The various temperatures were achieved through using a water cooler (for 5°C), water baths (for 20 and 25°C) and temperature-controlled cold rooms for 10 and 15°C. Four time intervals (3, 6, 12 and 24 hours of incubation) were used to test the effects of incubation period on spore shedding. Umamaheswara Rao (1976) and Tseng (1981) suggested that exposure of thalli to moisture, after a drying period, increased turgor pressure and ruptured the cells to release the zygotospores in Porphyra. Furthermore, eulittoral species are exposed to air for as long as 8 hours (Kim et al. 2008) and to determine the effects of desiccation, the excised pieces were therefore blotted dry and exposed to air at 15°C (as used by Waaland et al. 1990) for 0, 2, 4, 6 and 8 hours before being incubated in sterilized filtered seawater for six hours. The 0-hour experiment, where pieces were immediately incubated in seawater for six hours, was used as a control (as in Azanza & Aliaza 1999). For all experiments, the discs were incubated in a volume of 3ml of sterile seawater.

Table 1. A summery of the experiment conditions used to conduct the various experiments.

<table>
<thead>
<tr>
<th>Experiments</th>
<th>Temperature (°C)</th>
<th>Salinity (PSU)</th>
<th>Irradiance (μmol photons m⁻²s⁻¹)</th>
<th>Desiccation Period (h)</th>
<th>Incubation Period (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (°C)</td>
<td>5,10,15,20,25</td>
<td>30</td>
<td>80</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Salinity (PSU)</td>
<td>15</td>
<td>0,10,20,30,40</td>
<td>80</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Irradiance (μmol photons m⁻²s⁻¹)</td>
<td>15</td>
<td>30</td>
<td>0,40,80,120,160</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Desiccation Period (h)</td>
<td>15</td>
<td>30</td>
<td>80</td>
<td>0,2,4,6,8</td>
<td>6</td>
</tr>
<tr>
<td>Incubation Period (h)</td>
<td>15</td>
<td>30</td>
<td>80</td>
<td>0</td>
<td>3,6,12,24</td>
</tr>
</tbody>
</table>

After the incubation period, the discs were removed and the number of discs that released for each species was counted. The spore suspension from all wells for each species was then transferred to a graduated glass beaker which was subsequently diluted up to a 1liter volume.
To count the spores, an agitated subsample of 1ml of the spore suspension was taken and the number of liberated spores was counted with a counting grid in an inverted compound light microscope. All the experiments were repeated three times and an average value of three counts was used to calculate the number of spores released per experiment, resulting in a total of nine counts per treatment. The wet weight of the discs was measured and the liberated spores were expressed only as spores per gram of wet weight (g wet weight\(^{-1}\)), since they were not projected to a 24-hour daily cycle.

### 3.2.3 Statistical analysis

All data for the three repetitions were pooled and plotted graphically as means with Standard Error (\(\bar{X} \pm SE\)). Within each culture condition, a One-way ANOVA was performed to compare the number of spores released by each species. A Tukey honest significant difference (HSD) post hoc test was then performed for multiple comparisons between the three species. To compare across the various species, a Two-Way ANOVA was used. The data were analysed using the QED Statistics, Version 1.1, (Pisces Conservation Ltd, Hants, UK). All results were regarded as being significantly different at \(p<0.05\).

### 3.3 Results

#### 3.3.1. Spore characteristics

The excised discs of *P. aeodis*, *P. capensis* and *P. saldanhae* released spores of various shapes and sizes (Figures 1a–c). These spores were categorized into three types due to uncertainty on the proper terminology to apply, as discussed below.

(a) **Type I spores:** The majority of the Type I spores for all species were spherical, ranging in diameter from 9-12 \(\mu m\) with non-significantly different \((p=0.074)\) means of 9.67±0.15\(\mu m\), 10.01±0.12 \(\mu m\) and 11.03±0.18 \(\mu m\) for *P. aeodis*, *P. capensis* and *P. saldanhae* respectively.
Some of the spores looked amoeboid in shape immediately after liberation, but later became more spherical. All the spores had a single orange-reddish to dark-red central chromatophore. The spores of *P. aeodis* contained a chromatophore with two strands (Figure 1d). The strands were thick and short and were distally held together by a thin strand forming an apparently hollow ring. *Porphyra capensis* released spores with a stellate chromatophore that appeared hollow in the centre (Figure 1e). The chromatophore of *P. saldanhae* spores had two thick segments which were arranged in a ‘v’ shape originating from a circular strand at one end (Figures 1f & 1g). For all species, the rest of the spore (cytoplasm) looked pale yellowish thus emphasizing the structure of the chromatophore.

For all three species, the majority of these spores grew into germlings that germinated into conchocelis. Although the conchocelis filaments will be discussed in detail in the next chapter, it is relevant here that *P. aeodis* and *P. capensis* Type I spores gave rise to a maximum of three conchocelis filaments per spore. A maximum of five conchocelis filaments were recorded germinating from a single spore of *P. saldanhae*. However, the majority of the spores germinated into either one or two conchocelis for all species. Where two conchocelis germinated, they were always located at opposite ends of the Type I spores for *P. capensis*. There was no distinct spacing pattern observed for *P. aeodis* and *P. saldanhae* conchocelis. However, for all species, one filament was often longer than the other filament growing from the same spore.

(b) Type II spores: The diameter of these spores for both *P. aeodis* and *P. capensis* ranged from 18 – 40µm, with the majority of them mainly 20–36µm. All these spores were spherical with a diffuse cytoplasm, of a greenish-grey colour, with dark margins (Figures 2a-c). The spores of *P. aeodis* and *P. capensis* were more densely pigmented and seemed to have a
granular cytoplasm, with conspicuous darker dots throughout the entire cytoplasm. These spores either degenerated or germinated into conchocelis.

The Type II spores from *P. saldanhae* were also spherical but sometimes differed with a polygon-shaped dark brown solid pigmented portion located at the centre of the spores (Figure 2c). This pigmented portion seemed to be attached to the circumference of the spore by means of thin strands stretching from each of the polygonal angles of the pigmented portion. The diameter of these spores was two-to-three times larger than the Type I spores, ranging from 22 to 36 µm. Although some of these spores germinated into conchocelis filaments, most of them degenerated within two to three weeks, resulting in the shrinking of the cytoplasm.

(c) Type III spores: A third type of spores, (Figure 3), was recorded only in *P. aeodis*. The diameter of these spores ranged from 12 to 18 µm. These spores frequently divided in a plane that formed two halves (Figure 4a) that were either equal or unequal. Those that divided would divide further and give rise to fairly short conchocelis filaments (Figure 4d). However, the majority of these spores germinated into protothalli (Figure 4b), with some spores having both protothalli and conchocelis germinating from them (Figure 4c).
Figure 1. Various spores released from a) P. aeodis, b) P. saldanhae and c) P. capensis showing the presence of spores of different sizes and amoeboid shapes. Figures 1d - g show the chromatophore structure of the Type I spores from (d) P. aeodis, (e) P. capensis and (f & g) P. saldanhae respectively. Figure 2. The Type II spores from a) P. aeodis, b) P. capensis and c) P. saldanhae respectively. Figure 3. Type III spore from P. aeodis. Figure 4. Development of the Type I spores from P. aeodis with a) a median plane division, b) protothallus germination, c) two protothalli and conchocelis, d) conchocelis growth and development into conchosporangia.
3.3.2 Spore release

(a) Salinity effects

*Porphyra capensis* released more spores (*p*=0.0027), but Tukey’s test detected no significant differences (*p*>0.05) between *P. aeodis* and *P. saldanhae*. The amount of spores (Figure 5A) and the number of discs that released spores (Figure 5B) increased, for all species, with increased salinity. However, no significant differences (*p*>0.05) were recorded in the number of spores between 30 and 40 PSU for both *P. capensis* and *P. saldanhae*, while spore production was significantly lower (*p*=0.0097) for *P. aeodis* at 40 PSU compared to 30 PSU.

![Graphs showing the effects of salinity on spore release](image)

**Figure 5.** The effects of different salinity levels on the liberation of spores from the three *Porphyra* species. Each point is a mean of three experiments with vertical standard error bars. The number of spores released per gram of wet weight for each species is presented in figure 5A. Figure 5B shows the % of discs that released spores by each species under different salinities. The discs were incubated for 6 hours at 15°C, 80 μmol photons m⁻² s⁻¹.

(b) Temperature effects

For *P. capensis*, there were significantly more spores (*p* = 0.0039) released at 15, 20 and 25°C than at 5 and 10°C (Figure 6A). Although there was no significant difference (*p*=0.7160) between 10, 15 and 25°C, there were significantly (*p* < 0.05) more spores released at 20°C for *P. saldanhae*. For *P. aeodis*, release of spores at 10 and 15°C was significantly (*p* = 0.017) lower than at 20°C but significantly (*p* < 0.05) higher than at 5 and 25°C. All three
species had more discs releasing spores at 10, 15 and 15°C while the number of discs releasing spores was less at 5 and 25°C (Figure 6B).

Figure 6. Spores (Mean ± SE) released from the three species under different temperature conditions. (A) The number of spores released per gram of wet weight, (B) the % of discs that released spores. The discs were incubated for 6 hours at 30psu, 80 μmol photons m⁻²s⁻¹.

(c) Irradiance effects

Increased irradiance resulted in increased number of spores released from P. aeodis and P. capensis discs (Figure 7A). However, no significant difference ($p>0.05$) could be detected between 120 and 160μmol photons m⁻²s⁻¹ for P. capensis. Porphyra saldanhae needed less light for maximal spore release (Figure 7A), with no significant differences ($p=0.0735$) between 80, 120 and 160μmol photons m⁻²s⁻¹. Although there were sometimes no significant differences ($p>0.05$) between these species, the number of discs that released spores was generally more in P. aeodis compared to the other species (Figure 7B).
(d) Incubation period

Increasing the incubation period significantly ($p<0.05$) increased the number of spores released from all species (Figure 8A). Percentage of discs releasing spores in *P. aeodis* and *P. saldanhae* was significantly higher at 6 – 24 hours of incubation (Figure 8B). In *P. capensis*, maximum spore release was reached at 12 and 24 hours.

Figure 7. The spores (Means ± SE) released from the three species under different irradiance levels. The number of spores released per gram of wet weight per day for each species is presented in figure 7A, while figure 7B shows the percent of discs that released spores for each species. The discs were incubated for 6 hours at 15°C, 30 psu.

Figure 8. Spores (Means ± SE) released from the three species under different incubation periods. The number of spores released per gram of wet weight for each species is presented in figure 8A while in figure 8B is % of discs which released spores for each species under different incubation periods. Culture conditions: 15°C, 80 μmol photons m$^{-2}$s$^{-1}$ and 30psu.
(e) Desiccation effects

When subjected to different desiccation periods, there was a significant difference between the species, with *P. capensis* releasing more spores while *P. aeodis* released the least amount of spores (Figure 9A). Although increasing the desiccation period resulted in an increased number of discs releasing spores (Figure 9B), there were no significant differences after 4 hours of desiccation for both *P. capensis* (*p*=1.075) and *P. saldanhae* (*p*=0.947) while the number of spores released in *P. aeodis* decreased significantly (*p*=0.0031).

![Figure 9](image-url)  
*Figure 9*. Spore liberation from the three species under different desiccation periods. The number of spores released per gram of wet weight per day for each species is presented in figure 9A. Figure 9B shows % of discs releasing spores for each species under different desiccation periods. Each point is a mean of three experiments with vertical standard error bars. The discs were incubated at 15°C, 80 μmol photons m⁻²s⁻¹ and 30psu salinity.
3.4 Discussion

Although sexual reproduction has been recognised in Porphyra since 1892, (Nelson et al. 1999), the various terms used to refer to the types of spores released by the blades of Porphyra have been controversial. Conventionally, various authors (e.g. Drew 1949; Kurogi 1953a; Kurogi & Hirano 1956b; Hollenberg 1958; Iwasaki 1961; Kornmann 1961; Conway & Cole 1977; Hawkes 1978; Yabu 1978; Cannon 1989; Zhou et al. 2007) referred to all spores released from the foliose thalli of Porphyra as ‘carpospores’. Although Kornmann (1961) reported that the ‘carpospores’ from P. leucosticta not only germinated into conchocelis filaments, but also into dwarf thalli, most of these authors assumed that the ‘carpospores’ released from thalli grew into conchocelis and thus paid little attention to their morphological characteristics. Even when ultrastructural studies were conducted, they were performed to study the internal structure of ‘carpospores’ and not to distinguish the various spores. However, different spores have since been recorded and the application of the term ‘carpospores’ has been disputed. Various authors have since contributed to resolving the terminology applied to the reproductive structures of Porphyra (see Nelson et al. 1999 for list of authors). As a result, the terminology applied herein to refer to the spores released from the foliose stage of Porphyra, is based on Nelson et al. (1999).

The mean diameter of the Type I spores released from these three species fell within the size range observed in the zygotospores of other Porphyra species as recorded by Knight and Nelson (1999). These spores were also similar to those found by Krishnamurthy (1969) in P. nereocystis (12µm), although they were smaller than those of P. cuneiformis (15 - 20µm) and P. perforata f. patens (12 - 16µm). The colour of these spores was also consistent with the pink-to-reddish colour observed in other species. However, the red coloration was more intense in the spores of P. capensis due to the size and shape of the chromatophore. The
chromatophores of the zygotospores from four *Porphyra* species (*P. suborbiculata*, *P. tenera*, *P. umbilicalis* and *P. yezoensis*) were studied by Kurogi (1953a) who described and recorded them as parietal, band-shaped or plate-shaped. In the current study, these shapes could not be confirmed since no ultrastructural studies were conducted for these species and only a light microscope was used. However, the star-shaped chromatophores observed in *P. capensis* were similar to those reported by Lewmanomont and Chittpoolkusol (1993) for *P. vietnamensis*. Furthermore, the chromatophores of *P. aeodis* and *P. capensis* seemed to be more flattened than cylindrical while those of *P. saldanhae* looked more cylindrical. The chromatophores of both *P. aeodis* and *P. capensis* could therefore fit in the plate-shaped category while *P. saldanhae* fits more within the band-shaped chromatophore category of Kurogi (1953a).

Some of the Type I spores displayed an amoeboid shape that was also observed in *P. tenera* (Kurogi 1953a), *P. papenfussii* (Conway & Cole 1973) and *P. pulchella* Ackland, JA West, JL Scott & Zuccarello 2006 (Ackland *et al.* 2007). Since these spores became spherical after liberation, this suggested that these spores had not fully developed during liberation. This was because such spores were also observed in transverse sections of immature zygotosporangia from these species. Furthermore, when the excised discs were incubated for periods longer than 24 hours they released spores that were predominately amoeboid shaped. Spores released in this manner generally took longer to attach and germinate than the normal four day period observed for *P. aeodis*, *P. capensis* and *P. saldanhae* (see Chapter 4).

The release of a mixture of both larger and smaller spores in *Porphyra* has been reported in other species e.g. *P. perforata* (Smith 1944) and *P. dioica* (Kornmann & Sahling 1991). Sahoo *et al.* (2006) observed two types of spores released from *P. vietnamensis* and recorded
them to be zygotospores (which grew into conchocelis) and archeospores (which grew directly into blades). They recorded the archeospores as larger in size (18–20µm) compared to the zygotospores. Knight and Nelson (1999) also recorded spores of different sizes in some New Zealand species, and their characteristics were similar to those of the Type II spores reported in this study. However, some of the Type II spores of *P. saldanhae* seemed to be different sometimes from the Type II spores in *P. aeodis* and *P. capensis* due to the presence of the ‘polygonal’ concentrated chromatophore (see figure 2c) which could have been due to shrinking of the cytoplasm as the spores were dying.

The Type III spores frequently divided in a plane that formed two halves that were either equal or unequal. In other red seaweed genera e.g. *Gracilaria*, the spores that divided with a median plane are referred to as carpospores. However, the use of carpospore in this instance would be inappropriate since such spores do not exist in *Porphyra* and the Type III spores gave rise to conchocelis, protothalli or both. Furthermore, in *Gracilaria cornea* (e.g. Orduña-Rojas & Robledo 1999) the carpospores divided further into smaller planes and gave rise to carposporelings, which was not the case in the Type III spores that mainly gave rise to protothalli in this study.

The presence of protothalli in the life history of *Porphyra* was first documented by Cole and Conway (1980), in *P. variegata*, who defined protothalli as cellular masses which do not exhibit developmental polarity. They were later documented by Nelson and Knight (1996) as cellular masses that occasionally grew on the conchocelis of *P. subtumens*. Not only did protothalli germinate directly from the Type III spores of *P. aeodis*, they also developed from the conchocelis filaments of this species (the protothalli germinating from conchocelis will be discussed in detail in Chapter 4). The protothalli of *P. aeodis* were also granulated masses
with no visible segmentation (see Chapter 4), a feature that distinguished them from the distinctly segmented conchocelis and conchosporangia. However there was colour variation within any one protothallus. This sometimes gave the protothallus a false segmented appearance, with the basal parts of the protothallus being largely brownish-grey and granulated while the distal margin opposite the Type III spore had yellowish-orange colour bands. The protothalli were shorter and thicker than both the conchocelis and the conchosporangia, but varied in thickness with the thickest parts being 14µm. In the study by Cole and Conway (1980) the protothalli disintegrated to form thalloid germlings, while the fate of the protothalli was not known in the study by Nelson and Knight (1996). The protothalli of *P. aeodis* grew to a maximum width of 18µm, 28 µm long, and started losing pigmentation and eventually degenerated after six weeks in culture. In those Type III spores producing conchocelis and protothallus, the conchocelis developed into conchosporangia while the protothallus died after five weeks in culture.

It is therefore fitting to refer to the Type I spores as zygotospores based on their morphological characteristics and their role in the life history of these three species. Although an appropriate name for the Type II spores could not be found, they could be lumped into the zygotospores and referred to as mega-zygotospores since they germinated similarly to the zygotospores and gave rise to conchocelis. However, the formation of such spores in the thallus needs to be investigated in order to explain their morphology and existence. Although the Type III spores did not fully match the characteristics of blade archeospores, and their ploidy is unknown, it is proposed that they be called zygotoarcheospores. This is because they fall close to the size of the archeospores and they germinated mainly into protothalli which could be an equivalent of the foliose thalli. Furthermore, they also grow into conchocelis which makes them similar to the zygotospores. However, ultrastructural studies
are needed to elucidate the nuclear structure of these different spore types in South African species.

When subjected to various environmental conditions, *P. capensis* released more spores than the other species. This may reflect differences in the reproductive strategies of these three species. *Porphyra capensis* is dioecious, while both *P. aeodis* and *P. saldanhae* are monoecious. As a result *P. capensis* has more surface area, per disc, to release spores than the other two species.

Although all three species liberated spores at all salinity ranges, all the spores released at salinities below 20 PSU had shrunken chromatophores due to plasmolysis that occurred in the spores due to the hypotonic conditions. The osmotic shock experienced by the spores was because these three species naturally grow in relatively salty water, since the South African west coast seawater has salinities close to 35 PSU (Stegenga *et al.* 1997). This was further reflected by the high number of spores that were released at 30 and 40 PSU for all three species. Osmotic shock resulted in these three species being able to release spores even at lower salinities, since Ackland *et al.* (2007) found that exposing *P. pulchella* thalli to osmotic shock for 15 minutes resulted in the gradual breaking down of the cell wall surrounding each sporangium thus releasing the archeospores. However, the spores that were released at lower salinities died within two days, while very little mortality was observed in spores released at 30 and 40 PSU. Kapraun (1989) found that *Enteromorpha linza* was more susceptible to osmotic changes during reproduction and attributed that susceptibility to possible lack of cell walls on algal spores resulting in the high spore mortality observed at lower salinities for these three species. Spores of *Porphyra* (e.g. archeospores of *P. pulchella*, Ackland *et al.*
2007), are likely to be surrounded by only a plasma membrane immediately after their release which makes them more susceptible to lysis and subsequent mortality.

The differences in the number of spores released and the number of discs releasing spores for these three species was due to differences in their salinity tolerance, since the salinity tolerances of algae correlate with their intertidal zonation (Biebl 1952; Smith & Berry 1986). Larsen & Sand-Jensen (2006) also found that although the red seaweeds in their study had lower tolerance to low salinities compared to green and some brown seaweeds (e.g. *Fucus vesiculosus* & *F. evanescens*), *Porphyra purpurea* had similar tolerances to *Ulva lactuca* and they attributed that to the colonization of the same zonal region by these two species. Vertical zonation explains the lower number of discs releasing spores and low number of spores released at lower salinities (0 – 20 PSU) in *P. aeodis*, found lower in the interdral zone, compared to *P. capensis* and *P. saldahae* which are found higher up the shore. This was because species located in the upper littoral zone experience very low salinities during rainy periods and low tides (Larsen & Sand-Jensen 2006) making them more tolerant to very low salinities compared to species found lower down the shore. Eppley and Cyrus (1960) subjected *P. perforata* to different salinity ranges and concluded that rain reduced salinity in the intertidal and was potentially a more serious threat to survival of Porphyra higher up the shore, due in part to breakdown of ion transport through the cells. The absence of *P. aeodis* during winter, when heavy rainfalls are experienced on the west and southwest coast, may testify to the inability of this species to tolerate fluctuating interidal salinities while *P. capensis* and *P. saldahae* seem to be tolerant to such rainfalls and the fluctuating salinities.

Doty (1946) contended that the duration of exposure, i.e. submergence or emergence, was of prime importance for the survival and reproduction of intertidal seaweeds. Brawley and
Johnson (1993) found that although the 6-hour-old zygotes and embryos of *Pelvetia fastigiata* survived natural emersion, they experienced reductions in photosynthesis indicating the effects of desiccation. Vadas et al. (1992) also found that desiccation delayed rhizoid development in *Pelvetia fastigiata*, thus increasing the susceptibility of zygotes to dislodgement by water motion. However, desiccation periods did not have a significant effect on the number of spores released by both *P. capensis* and *P. saldanhae* suggesting that although desiccation temporarily reduced sporulation, spores may be released whether there are tidal cycles or continuous submergence of these species in the wild. Species growing higher in the intertidal (e.g. *P. linearis*) have been shown to have a higher level of tolerance to temporary desiccation, losing water quickly and returning quickly to normal photosynthesis after even after long periods of severe desiccation (Lipkin et al. 1993). On the other hand, in *P. aeodis*, the decreased number of spores released with increased desiccation period, suggested that prolonged low tides may stop sporulation in this lower intertidal species in the wild. Quadir et al. (1979) also found that species growing in the higher reaches of the intertidal zone tend to be more productive during emergence, whereas species growing in the lower reaches are more productive during submergence. Furthermore, the tidal cycles experienced by *P. aeodis* in the West Coast are very short, since it is normally submerged or soaked to a certain degree during low tides, thus exposing this species to very short periods of desiccation. Exposure to desiccation seems to increase the number of discs releasing spores, by breaking the zygotosporangial cell walls, but not necessarily increasing the quantity of spores released.

Prolonged incubation period resulted in more spores released in all three species. This was similar to Kitade et al. (1998) who found that archeospore release and germination in *P. yezoensis* increased with time. This suggested that these three species continuously release
spores on the shore and this continuous release increases the potential for the survival of the species in the intertidal zone.

Although increased irradiance resulted in increased spore release, for the three species, the irradiance levels were all relatively lower than the natural levels. This was because these levels only covered the range of irradiance that can be economically produced in culture (Blouin et al. 2007) and were less than 10% of the full irradiance (>600 µmol m$^{-2}$s$^{-1}$) experienced by intertidal seaweeds in the wild (Gualtieri & Barsanti 2006). Pacheco-Ruíz et al. (2005) also found that, when cultured at neutral and long-days, the number of spores released in *P. perforata* decreased as temperature increased from 15 to 27$^\circ$C and as irradiance increased from 0 to 300 µmol photons m$^{-2}$s$^{-1}$. Orduña-Rojas and Robledo (1999) also found that the number of carpospores released from cystocarps of *Gracilaria cornea* decreased with increased temperatures (25 - 31$^\circ$C) and with increased irradiances (10 – 100 µmol m$^{-2}$ s$^{-1}$). However, Narashima Rao and Rangaiah (1991) cultured *P. vietnamensis* at 30$^\circ$C, 8:16 photoperiod, and found that spore release only increased at very low irradiances (0 - 44 µmol photons m$^{-2}$s$^{-1}$) but decreased at 62 - 97 µmol photons m$^{-2}$s$^{-1}$. Direct comparison with *P. vietnamensis* was difficult since it is a warm-temperate to sub-tropical species, and was cultured at a higher temperature which none of the three species studied here could survive.

Increased temperature also resulted in more spores being released by these three species. These results did not conform with those found by Freshwater and Kapraun (1986) who found that ‘carpospores’ were only present in *P. carolinensis* during November – April, when temperatures were lower. This was expected since *P. carolinensis* has only been reported from the east of Cape Agulhas, in the warmer coast of South Africa (Jones et al. 2004), thus making it physiologically different, in temperature tolerance, to the three species studied here. Since the majority of *Porphyra* species are distributed in temperate regions (Karsten
1999; Mai et al. 2004) and, considering that seawater surface temperatures in this area (see
Chapter 2) ranged from a summer minimum of 8.19°C to a winter maximum of 16.42°C from
May 2006 to January 2009 both, the distribution range of these three species explains the
higher number of spores released at 15 and 20°C for all three species. Pueschel and Cole
(1985) as well as Pacheco-Ruiz et al. (2005) also showed peak zygotospore release at
temperatures of 15–18°C for P. variegata and P. perforata. Our results also conformed to
Kornmann (1961) who showed that it was possible to release zygotospores in P. leucosticta
at 15°C while Orfanidis (2001) also found that P. leucosticta released spores at 15°C
irrespective of photoperiod. The results from this study also conformed with Monotilla and
Notoya (2004) who found that P. suborbiculata strain from Kagoshima only released
zygotospores at 15 and 20°C while Kim (1999) found that P. dentata and P. pseudolinearis
only released zygotospores at 15°C, suggesting that extremely high and extremely low
temperatures were not suitable for spore release in these species. Such behaviour explains the
very low number of discs releasing spores and spore quantities at 5 and 25°C for all three
South African species.

The life history of these three species differed from other species such as P. angusta
Okamura & Ueda 1932, P. gardneri, P. yezoensis, P. pulchella, P. lacerata, P.
suborbuculata, P. onoi Ueda1932, P. kuniedai Kurogi, P. tenera, P. tanegashimensis I
Shinmura 1974, P. crispata (Kjellman) Kuntze 1898 and P. akasakai A Miura 1977 with the
absence of blade archeospores in the three species. Agamospores, neutral spores,
phyllospores and endospores were also not found in the South African species. These species
were similar in that they released spores of different sizes, the smaller zygotospores and
larger mega-zygotospores. Porphyra aeodis differed from P. capensis, P. saldanhae and
other Porphyra species with the presence of zygotoarcheospores and protothalli. Careful
examination of the chromatophore of the spores may be used as a valuable taxonomic
character in *Porphyra*. Until the effects of short-day and neutral-day photoperiods are tested, this study reflected that currently the best conditions for spore release are between 15 - 20°C, 120 $\mu$mol photons m$^{-2}$s$^{-1}$, long-days with continuous incubation (i.e. 24 hours of incubation) for all three species. More detailed microscopic studies may distinguish the protothallus-bearing spores from conchocelis-bearing spores.
Chapter 4

The effects of temperature, irradiance and photoperiod on the growth and development of the conchocelis phase of three South African Porphyra species in culture.
4.1 Introduction

Although the biology and ecology of *Porphyra* has been studied more thoroughly than that of many other red algal genera (Sahoo *et al.* 2006), the taxonomy of *Porphyra* is still problematic (Coll & Oliveira 2001, Brodie *et al.* 2008). This is due to high phenotypic plasticity and relative paucity of characters for species recognition (Stiller & Waaland 1993; Lindstrom & Fredericq 2003; Jones *et al.* 2004), which makes species description difficult. As a result, characters obtained from blade morphology have proven to be insufficient in distinguishing different species (Milstein & De Oliveira 2005). Beside molecular studies (e.g. Lindstrom & Cole 1992, Lindstrom 1993, Stiller & Waaland 1993; 1996, Oliveira *et al.* 1995, Brodie *et al.* 1996; 1998; 2008, Woolcott & King 1998, Broom *et al.* 1999; 2002, Lindstrom & Fredericq 2003, Milstein & de Oliveira 2005, Nelson *et al.* 2006, Niwa *et al.* 2005b, Niwa & Aruga 2006), life history studies of *Porphyra* have also been conducted to obtain more characters to distinguish species. Knight and Nelson (1996) examined the conchocelis phase of various New Zealand *Porphyra* culture strains and noted differences in their conchocelis.

The conchocelis is the microscopic filamentous sporophyte phase in the life history of the genus *Porphyra* (Pueschel & Cole 1985). Knowledge of its morphology has been suggested as a potentially helpful character that may help to distinguish different *Porphyra* species (Conway & Cole 1977, Zheng 1984, Knight & Nelson 1999). Furthermore, during mass cultivation, sufficient knowledge of the biology and culture conditions for the conchocelis phase is important to control the size of the harvest crop (Dring 1995).

In 1949 a British phycologist named Kathleen Drew demonstrated, for the first time, that the shell-boring *Conchocelis rosea* Batters (1892) was actually a phase of *Porphyra umbilicalis* (L.) Kütz [a study that was later found by Brodie *et al.* (1996) to have been done on *P.*
purpurea and P. laciniata rather than P. umbilicalis]. Since then the conchocelis phases of various Porphyra species have been studied. For example, in 1954, Drew gave a detailed description of the conchocelis phase of ‘Porphyra umbilicalis var. laciniata’ [P. dioica (Holmes & Brodie 2004)]. Iwasaki and Matsudaira (1963) described the growth and development of the conchocelis of P. tenera under different photoperiods. In 1969, Krishnamurthy studied the characteristics of the conchocelis phase of P. perforata f. patens, P. cuneiformis and P. nereocystis. In his study, Krishnamurthy gave detailed diagrammatic representations of the characteristics of the conchocelis phase of these three species. Conway and Cole (1977) compared the conchocelis of 21 species and found differences in their morphological development. Zheng (1987) studied the morphological characteristics of the conchocelis of four Porphyra species (P. katadai Miura var. hemiphylla Tseng & Chang, P. yezoensis Ueda, P. marginata Tseng & Cheng and P. oligospermatangia Tseng & Zheng) and catalogued their growth and development patterns.

Besides studies on the morphological characteristics of the conchocelis phase, cytological and ultrastructural studies have also been conducted. Lee and Fultz (1970) studied the ultrastructure of the conchocelis phase of P. leucosticta and demonstrated the presence of pit connections, an irregularly shaped nucleus, v-shaped limosomes and a chloroplast that enlarged as the conchocelis cells aged. Puschel and Cole (1985) studied the ultrastructural development of conchocelis and demonstrated the presence of septa where the conchocelis joined the spore. They also showed differences in the cell wall of the conchocelis in relation to that of the spore.

Although Griffin (2003) observed conchocelis of unidentified Porphyra species in the wild, the only study on the conchocelis phase in any South African Porphyra species was that by
Judith Graves in 1955. In her experiments she demonstrated that, when seeded on egg shells, ‘P. capensis’ (a name used in that study to refer to all South African *Porphyra* species) spores burrowed and produced branched conchocelis filaments. However, her studies gave little detail on the characteristics of these filaments. Furthermore, the species she studied could easily have been misidentified, as all South African *Porphyra* species were lumped into ‘*Porphyra capensis*’ at that time. This study therefore aimed to give a detailed description of the morphological characteristic of the conchocelis phase of three South African *Porphyra* species. Since photoperiod, temperature and irradiance are the major environmental factors affecting the growth of conchocelis (Sahoo *et al.* 2006), the growth and development of the conchocelis of these three species were also examined under different photoperiods, temperatures and irradiance levels.

4.2. Materials and Methods

4.2.1. Porphyra collection

Mature, fertile thalli of *P. capensis*, *P. saldanhae* and *P. aeodis* were collected from Kommetjie. Although collection was done during low spring tide (13 February 2007), only thalli that were damp or found immersed in water were collected and such thalli were immediately transported to the laboratory in sealed buckets.

4.2.2. Spore release

In the laboratory, five fertile individual were selected from each of the three species, and squares of thallus (1x1cm) were excised from their fertile margins. The excised strips were rinsed in sterile seawater and brushed with a soft brush to clean the surfaces. They were then soaked in fresh water for 20 minutes to remove remaining epibionts (as in Floreto & Teshima 1998). After this, the strips were incubated in 5ml 1/3Provasoli’s enriched seawater (PES) culture medium, containing 0.05ppt of Germanium dioxide (GeO₂). The GeO₂ was used to
prevent the development of diatoms (Markham & Hagmeier 1982, Polne-Fuller & Gibor 1987, Choi et al. 2002, Shea & Chopin 2007) that were observed in preliminary studies conducted prior to the beginning of these experiments. The strips were kept overnight in this solution to allow time for liberation of sufficient spores for the experiments. The strips were then removed and the concentrated spore suspensions, for each species, emptied into a 1liter glass beaker. To dilute the spore suspensions, the beakers were filled up to 1liter volume with PES solution and stirred to ensure a homogeneous suspension. For each species, a diluted spore suspension volume of 3.5ml (ca 20 spores) was added into each of ten cells of 25-celled 100ml plastic repli-trays (Bibby, Sterilin®, Newport, UK). A total of twenty-four trays was used, with each tray containing two species, in order to ensure homogeneous conditions for all three species. Preliminary experiments had shown that the spores frequently needed to settle before germination. The trays were therefore left at room temperature for 24hours, so that the spores could settle and attach to the bottom of the plastic cells.

4.2.3. Conchocelis culture
The growth and development of conchocelis was studied under long- and short-day photoperiods (16h light: 8h dark and 8h light : 16h dark cycles) respectively. Preliminary experiments had shown that at 25°C the conchocelis filaments germinated extremely slowly and died after 10 days. To measure the influence of temperature on the conchocelis filaments, the spores were therefore cultured at 5°C, 10°C, 15°C and 20°C. Three trays were placed in each of the above-mentioned culture conditions. Illumination was provided by Hellweiss Cool’white fluorescent tubes (OSRAM L30W/20) and the irradiance level for photoperiod and temperature studies was set at 75 – 85 μmol photons.m⁻².s⁻¹, using a Skye Quantum Sensor. To test for irradiance effects, four irradiance levels (40, 80, 120 and 160 μmol photons m⁻².s⁻¹) were employed, by adjusting the number of fluorescent tubes. Each of the three repli-trays was treated as a true replicate while each cell, within each tray, was treated
as a pseudoreplicate. The sample size required for each of these studies was estimated according to Zar (1999). Since conchocelis morphology has been largely ignored in research (Knight & Nelson 1999), the filaments were therefore compared instead of conchocelis colonies although the filaments of these species were also grown into colonies.

The growth of the conchocelis filaments was measured microscopically, using an inverted light microscope (LEITZ DM IL, Leica Mikroskopie, Portugal), after 24 hours of exposure to the various conditions. Thereafter, the filaments were measured every third day for 16 days. Preliminary experiments had shown that after 16 days the filaments became too tangled to track individual conchocelis. Where more than one conchocelis filament germinated, all filaments were measured and summed up to get the total length of that conchocelis. The culture medium was changed after taking the measurements and the development of the conchocelis filaments was also monitored. After the 16-day period the conchocelis had grown into turfs (mats) that were then cultured, as discussed in the next chapter, for the conchosporangial development.

The specific growth rate (SGR) of the conchocelis was measured as a percentage of increase in length per day over the culture period using the following formula, as used by Hafting (1999), Makarov (1999), Smit & Bolton (1999), and Wakibia et al. (2006):

\[
SGR(\% \text{ day}^{-1}) = \left( \frac{\ln L_f - \ln L_i}{n} \right) \times 100
\]

Where \(\ln L_i\) and \(\ln L_f\) are the natural logs of the initial and final length of conchocelis filaments and \(n\) is the duration of the experiment in days.

### 4.2.4 Statistical analysis

The data were presented as either tables or plotted graphically as means with standard errors (\(\bar{x} \pm SE\)). Statistical analysis for all data was performed using the statistical package Statistica Release 8, StatSoft Inc., Tulsa, USA. The data for all species, at different conditions, was tested for normality using the Shapiro-Wilk test. To test for the homogeneity of variance,
Bartlett’s test was used. To test for differences between the different pseudoreplicates, a T-test was performed and the results were then pooled together for each species under each culture condition. A single factor analysis of variance (One-Way ANOVA) was then used to test for differences between the means of the three species under different conditions. Where there were no statistical significant differences detected between the three species, a Tukey honest significant difference (HSD) *post hoc* test for multiple comparisons was performed. All results were regarded as being significantly different at *p*<0.05.

### 4.3 Results

#### 4.3.1 Conchocelis morphology

(a) *P. aeodis*: The zygotospores germinated and gave rise to a maximum of three conchocelis filaments (Figure 1a-c). The conchocelis filaments were narrow (mainly 6µm, but may rarely expand to 12µm) and normally narrowed towards the tip thus becoming more pointed. The mega-zygotospores always bore a single conchocelis that was thin with a pointed tip (Figure 1d). These spores lost their cytoplasmic pigmentation colour, which seemed to be transferred to the germinating conchocelis. The conchocelis from these spores sometimes decomposed and gave rise to the development of protothalli instead of conchosporangia (Figure 1e). The zygotoarcheospores had conchocelis with pointed tips (Figure 1f) that later became swollen and became more rounded than pointed (Figure 1g). The swollen tips eventually developed into conchosporangia, as described in Chapter 5. All the conchocelis of this species had a cross-wall separating the spore and the conchocelis (Figure 1h). All the conchocelis filaments branched randomly and always tended to be undulate, curving distally around the spores, and seldom grew into straight filaments.

(b) *P. capensis*: The zygotospores of *P. capensis* germinated into one, two or three conchocelis filaments (Figure 2). Where more than one filament resulted from a spore, one
would frequently be longer than the others. The conchocelis filaments of *P. capensis* were thin (6-8 µm) and multicellular. The length of the cells varied but the longest cells were 30 µm and found mainly in the middle of the filaments. Very few filaments showed clear narrowing towards the tips, but the cells became shorter at the tips. Branching was mainly alternate off the main filament (Figure 3a) and no obvious swelling was observed where the branching occurred. The presence of a slight indentation on the main filament suggested that the primary branching cell was initiated from within the cell of the main filament (Figure 3b). Furthermore, the branching was more or less specific to the centre of the cells and never occurred on the margins connecting adjacent cells of the main filament.

Although most conchocelis of *P. capensis* developed into conchosporangia (see Chapter 5), some of the filaments gave rise to secondary spores (Figures 4a-c). These spores were identified as conchocelis archeospores, [spores formed by differentiation of a vegetative conchocelis cell which releases a single cell product that germinates into the conchocelis phase (Nelson *et al.* 1999)]. The conchocelis archeospores were oval to spherical and grew up to 32 µm in diameter. In turn, the conchocelis archeospores gave rise to more conchocelis filaments that were similar to the conchocelis from which the conchocelis archeospores originated. The conchocelis archeospores differed from the zygotospores in that they did not contain any structurally distinct chromatophore and their cytoplasm did not contain any distinct contents but seemed to be divided in various random proportions (Figure 4c).

(c) *P. saldanhae*: A maximum of five conchocelis filaments was recorded germinating from a single spore of *P. saldanhae* (Figure 5). Deformation of the spores from a spherical to a more oval shape with a flat side attached to the substrate (Figures 6a & b), occurred when conchocelis began germinating. The hollow ring of the chromatophore formed the base of the conchocelis, while the conchocelis cells had a variegated pigmentation seemingly within the
entire conchocelis filament. The conchocelis were 5 to 7μm in diameter, with varying cell sizes (up to 36μm long) and narrowing towards the tip. No conchocelis archeospores were recorded in *P. saldanhae* although the conchocelis developed into conchosporangia (Chapter 5). The branching differed from *P. aeodis* and *P. capensis* in that up to three filaments were recorded branching in a secund pattern to the main filament (Figure 7).

Branching resulted in intertwined filamentous colonies (Figures 8a-c) for all species. The colonies reproduced vegetatively by fragmentation which resulted in smaller colonies that would eventually break off from the parent colony. In still culture, the colonies of *P. aeodis* started fragmenting when they were 3cm in diameter. Conchocelis mats of *P. capensis* also fragmented when they were about 3cm in diameter, but they could grow up to 5cm colonies without fragmenting. Unlike *P. aeodis* and *P. capensis*, *P. saldanhae* colonies were less dense and fragmented continuously in still culture.

![Image 1](image1)

**Figure 1.** *P. aeodis* zygotospores bearing: 1a) one, 1b) two and, 1c) three conchocelis filaments. Figure 1d shows a conchocelis filament from a ‘mega-zygotospore’, 1e) a fading conchocelis giving rise to protothallus, f & g) conchocelis development from a ‘zygotoarcheospore’ and 1h) shows a cross-wall in ‘mega-zygotospore’.

![Image 2](image2)

**Figure 2.** *P. capensis* zygotospore bearing 2a) one, 2b) two and 2c) three conchocelis.

![Image 3](image3)

**Figure 3.** *P. capensis* conchocelis showing a) the typical alternate branching and b) the initiation of branching with the branch originating from within the cell of the primary conchocelis filament.
4.3.2 Photoperiodic and temperature effects

Culturing the spores under long-day photoperiod resulted in significantly faster growth rates ($p<0.05$), compared to those in short-day photoperiod (Figure 9). However, the post hoc test revealed that growth was significantly higher ($p=0.039$) in short-day photoperiod for *P. saldanhae* at 5°C. Growth increased with increased temperatures in short-day photoperiod for all species (Figure 9), while no significant difference ($p=0.751$) was detected between 15 and 20°C for *P. capensis* under long-day photoperiod. The best growth rates were attained at 20°C for *P. aeodis* and at 15°C for *P. saldanhae* under long-day photoperiod (Figure 9).
Figure 9. Specific growth rates (SGR) of conchocelis filaments of three Porphyra species cultured at different temperatures under long-day (16L:8D) and short-day (8L:16D) photoperiods. Conchocelis were cultured for 16 days at 75 – 85 μmol photons.m$^{-2}$.s$^{-1}$.

The growth rate of *P. capensis* was significantly higher ($p=0.0415$) than that of *P. aeodis* and *P. saldanhae* under long-day photoperiod (Figure 10). Although the length of conchocelis filaments (data not shown here) of *P. capensis* were significantly longer ($p=0.0146$), the growth rates of *P. aeodis* were significantly higher ($p<0.05$) under short-day photoperiod. The growth of *P. saldanhae* was significantly lower ($p<0.05$) than that of *P. aeodis* and *P. capensis* at both 15°C and 20°C under short days. In short-day photoperiod, *P. capensis* grew significantly slower ($p<0.05$) than both *P. aeodis* and *P. saldanhae* at 5°C and 10°C.
Figure 10. The specific growth rate (SGR) of the conchocelis filaments of three *Porphyra* species at different temperatures under long-day and short-day photoperiods. Conchocelis were cultured for 16 days at 75 – 85 μmol photons.m⁻².s⁻¹.

### 4.3.3 Irradiance effects

The specific growth rate of *P. aeodis* increased with increased irradiance (Figure 11), while it decreased for *P. saldanhæ* after 80μmol photon m⁻².s⁻¹ and no significant differences \((p=0.0502)\) were detected for *P. capensis* between 120 and 160μmol photon m⁻² s⁻¹. Although *P. capensis* had the longest conchocelis filaments (data not shown here), *P. aeodis* had the highest growth rates at all irradiances while *P. saldanhæ* had the slowest growth rates (Figure 11).

Figure 11. Effects of irradiance on the growth of conchocelis filaments of three *Porphyra* species cultured at 15°C in long-day photoperiod, 30 PSU salinity for 16 days.
Distinct morphological characteristics were observed in *P. capensis* under different irradiance levels. The germination rate was lower by day four, with 10 and 30% of spores having not germinated in 40 and 160 \(\mu\)mol photon m\(^2\) s\(^{-1}\) respectively. By day seven, all spores had germinated at 40\(\mu\)mol photon m\(^2\) s\(^{-1}\), while it took up to ten days for all spores to germinate at 160\(\mu\)mol photon m\(^2\) s\(^{-1}\). Most of the bipolar-growing conchocelis were recorded at 160\(\mu\)mol photon m\(^2\) s\(^{-1}\), and the thickest filament (8\(\mu\)m) was recorded at 40\(\mu\)mol photon m\(^2\) s\(^{-1}\) germinating from the mega-zygospores. The longest unbranched filaments were 75\(\mu\)m at 40\(\mu\)mol photon m\(^2\) s\(^{-1}\), 55\(\mu\)m at 120\(\mu\)mol photon m\(^2\) s\(^{-1}\) and 120\(\mu\)m at 160\(\mu\)mol photon m\(^2\) s\(^{-1}\). The longest branched individual was 70\(\mu\)m at 120\(\mu\)mol photon m\(^2\) s\(^{-1}\), with one branch of 16\(\mu\)m long, while the shortest branched filament was 16\(\mu\)m at 120\(\mu\)mol photon m\(^2\) s\(^{-1}\).

### 4.4 Discussion

For all three species, the germination of the conchocelis was initiated by a protrusion from the spore. This was consistent with observations of Krishnamurthy (1969) in the germinating spores of *P. perforata* f. *patens*, *P. cuneiformis* and *P. nereocystis*. Puschel and Cole (1985) also observed protrusions consisting of extracellular material in *P. variegata*, and that a septation was sometimes present between the spore and germ tube. Although a septum was sometimes observed between the conchocelis and the zygospores of the three species, septation was more readily seen in the mega-zygospore of *P. aeodis*.

As the individual conchocelis filaments developed further, they became multicellular in all three species and the different cells were visible under an inverted light microscope. Although Holmes and Brodie (2005) managed to see pit connections in *P. dioica* using a light microscope, no pit connections could be seen between the cells of conchocelis for the three species studied here. However, the pit connections of *Porphyra* are very small as
demonstrated by Lee and Fultz (1970) who found that the pit connections of *P. leucosticta* conchocelis were 0.6 µm wide and 0.5 - 1 µm long. This small size makes pit connections difficult to detect without first fixing and staining the conchocelis, since the Bangiales have been found to have no pit plug membrane (Pueschel 1987) and this may have contributed to the inability to detect pit connections in these three species. As a result, Krishnamurthy (1969) used an electron microscope to find pit connections in *P. perforata f. patens* although he failed to find them in *P. cuneiformis* and *P. nereocystis*. Pit connections have also been found, using an electron microscope, by Puschel and Cole (1982) in the conchocelis of *P. kanakaensis, P. miniata, P. perforata, P. schizophylla* and *P. smithii*. They were also found by Pueschel (1987) in *P. brumalis* and *P. nereocystis*.

The width of the conchocelis filaments of the three species was similar to that found by Krishnamurthy (1969) in *P. perforata f. patens* (4 - 8 µm) and in *P. cuneiformis* (3 - 7 µm). They were also similar to *P. dioica* (7.5 ± 2.4 µm) in Pereira *et al.* (2004) as well as to the width measured by Zheng (1984) in *P. yezoensis* (6 µm), *P. marginata* CK Tseng & TJ Chang 1958 (6 µm) and *P. oligospermangia* (6 µm). However, the conchocelis of the three species were broader than those recorded by Krishnamurthy (1969) in *P. nereocystis* (2 - 3 µm), but thinner than those recorded by Zheng (1984) in *P. katadai* conchocelis (11 µm).

Although Fritsch (1945) and Krishnamurthy (1969) found that the conchocelis of *Porphyra perforata* contained a stellate and/ or sometimes a ribbon-shaped chloroplast, the chloroplast of *P. capensis* was frequently more evenly distributed within each conchocelis cell. The conchocelis of *P. aeodis* and *P. saldanhae* did not have a stellate chloroplast, but *P. aeodis* had a chloroplast running along the sides of the conchocelis, while the chloroplast was coiled and ribbon-like in *P. saldanhae*. For all species, the chloroplast was not evenly distributed...
throughout the different cells of the conchocelis. Some of the cells had less chloroplast, compared to others, irrespective of their position in relation to the spore. This could be attributed to the lack of proplastids, for generating new chloroplasts, in the various cells (Lee & Fultz 1970). Lee and Fultz (1970) also suggested that the chloroplast distributed throughout the conchocelis originated from the pinching of the original chloroplast, by the cross-wall between adjacent cells, and this could explain the uneven chloroplast distribution from cell-to-cell along the conchocelis.

Since the conchocelis colour is extremely variable (Melvin et al. 1986; Knight & Nelson 1999), the chloroplast distribution may determine the colour of the conchocelis. The conchocelis filaments of *P. saldanhae* were frequently variegated with red and light-brown patches, while *P. aeodis* and *P. capensis* were less variegated although some patches often existed. Although Holmes and Brodie (2005) suggested that the degree of pigmentation could depend on the angle at which the cells of each filament are viewed, the high degree of variegation in *P. saldanhae* could arise from the spiralling ribbon-shaped chloroplast of the conchocelis of this species. The conchocelis colonies of *P. capensis* were normally dark-red to maroon in colour, while *P. aeodis* had red conchocelis and *P. saldanhae* colonies were light-red to pink.

The color of conchocelis was also determined by irradiance levels since low irradiance (40µmol m\(^{-2}\) s\(^{-1}\)) resulted in the conchocelis being more pigmented, with *P. aeodis* and *P. capensis* colonies looking dark-red to black in color. At 160µmol m\(^{-2}\) s\(^{-1}\), the conchocelis colonies of all three species were darker in the centre and became pale towards the outer edges. Although the conchocelis of the different species could still be easily distinguished at 5ºC after five weeks in culture, the differences became less distinct at the other temperatures.
Niwa et al. (2005b) also observed that, when free-living, some conchocelis strains could not be distinguished to species level even when detailed morphological observations were employed.

The results of this study also revealed high diversity in the morphology and development of the conchocelis filaments of the three species. Although Griffin et al. (1999) stated that the gametophytes of P. aeodis and P. saldanhae were sometimes morphologically similar, for example in their reproduction, this study revealed differences in their conchocelis phase. *Porphyra saldanhae* differed from the other species with the structural modification of the spore during conchocelis development as well as with the apparent coiled, ribbon-like, chloroplast along the entire conchocelis filament. The presence of protothalli distinguished *P. aeodis* from the other species, while *P. capensis* differed from the other species mainly in the presence of conchocelis archeospores.

Knight and Nelson (1999) and Nelson et al. (1999) described conchocelis archeospores as spores originating from conchocelis and subsequently giving rise to further development of more conchocelis. Based on this description, the conchocelis archeospores were therefore first observed in *P. tenera* by Iwasaki and Matsudaira (1963) who proposed the term “concho-monospores” to distinguish them from the blade-forming monosporas. The conchocelis archeospores produced by the conchocelis of *P. capensis* appear to conform to this description and therefore the use of the term is valid in this species. The life history of *P. capensis* was also similar to *P. abbottiae* and *P. torta* with respect to having conchocelis archeospores since Lindstrom et al. (2008) also found these spores in the latter two species.
These species also differed in the manner in which their conchocelis branched, with irregular branching in *P. aeodis*, alternate-opposite in *P. capensis* and secund branching, in *P. saldanhæ* respectively. The branching in most *Porphyra* species tends to be irregular (e.g. see Krishnamurthy 1969; Melvin *et al.* 1986; Sidirelli-Wolff 1992; Holmes & Brodie 2005; Sahoo *et al.* 2006) a pattern that was also observed in *P. aeodis*. The alternately opposite branching pattern observed in *P. capensis* is similar to that observed by Zheng (1984) in *P. katadai* Miura var. *hemiphylla* as well as by Campbell and Cole (1984) in *P. variegata*. However, no other similarities are known that could relate these species since the foliose phase of *P. variegata* and related species (e.g. *P. miniata*, *P. occidentalis*, *P. amplissima* and *P. cuneiformis*) differs from *P. capensis* in that they are distromatic while *P. capensis* is monostromatic. The reproduction in *P. capensis* is dioecious, while these species tend to be monoecious. Furthermore, *P. capensis* is present year-round (see Chapter 2), while *P. variegata* and related species occur seasonally. *Porphyra* species with secund branching were not found in the literature, making *P. saldanhæ* unique for this character.

Branching has also been associated with the shape and density of conchocelis colonies, with richly branched filaments resulting in densely tangled colonies (Knight & Nelson 1999). The densely tangled colonies of *P. aeodis* and *P. capensis* could have arisen from the random branching and alternate-opposite branching in the conchocelis of these species respectively. In contrast, the secund branching could be responsible for the fluffy and delicate appearance of the *P. saldanhæ* conchocelis colonies. Although the fragmentation of conchocelis colonies is associated with asexual reproduction, the secund branching in *P. saldanhæ* could also have been the cause of the continuous fragmentation of the conchocelis colonies in still culture even when they were small. This was because the conchocelis filaments of this species were observed to take longer to tangle even when they were longer and branched,
while those of *P. aeodis* and *P. capensis* started getting tangled within sixteen days in culture. Frazer and Brown (1995) observed that the conchocelis colonies of *P. columbina* grew in a circular manner and in one plane. Although the colonies of the three species also tended to grow in all directions, they only grew laterally when attached to the glass slides or base of culture trays. In such instances growth would be two-dimensional with the filaments immediately adjacent to the slides or tray growing laterally while the rest grow vertically. However, the conchocelis would grow in three dimensions resulting in spherical colonies when free-floating in bubble culture.

Although the type of branching in these species could have been genetically derived, the degree of branching was influenced by the environmental conditions. Photoperiod, temperature and irradiance are the major environmental factors that affect the growth of conchocelis (Avila *et al.* 1986; Waaland *et al.* 1990; Sahoo *et al.* 2006) and thus the degree of branching. The quickest branching (within ten days) was recorded at 15°C for all species, although conchocelis in 20°C had the fastest growth rates, and this was not surprising since this temperature range is within the seawater temperature range in the natural environment where these species grow (Chapter 2).

Not only did the environmental conditions influence branching in these species, they also affected the growth rate of the conchocelis filaments, with the growth rates being affected by an interaction of photoperiod, temperature and irradiance. Although Notoya and Iijima (2003) found that the conchocelis colony of *Bangia atrapurpurea* achieved fastest growth rates at 20 - 30°C under short day photoperiod, growth rates from these three species were higher under long day photoperiod, higher temperatures and higher irradiances. These results were consistent with those of Pereira *et al.* (2004) who found that increased temperatures resulted
in increased conchocelis germination and growth rates in *P. dioica*, with the highest growth rates at 15°C, 25µmol m\(^{-2}\)s\(^{-1}\) neutral-day photoperiod. In warm temperate species, Kapraun and Lemus (1987) found the growth of *P. spiralis* var. *amplifolia* to be highest at temperatures between 19 and 25°C. Bird *et al.* (1972) recorded ideal growth temperature and irradiance combinations 10-15°C, at 25-100µmol m\(^{-2}\)s\(^{-1}\) long-days for *P. linearis* Greville, while Avila *et al.* (1985), recorded ideal conditions at 5-13°C, 72-144µmol m\(^{-2}\)s\(^{-1}\) for *P. columbina*. Waaland *et al.* (1987) also found the growth of *P. torta* to be fastest at 15°C at 9.5µmol m\(^{-2}\)s\(^{-1}\) while Sidirelli-Wilff (1992) recorded ideal growth temperature and irradiance combinations of *P. leucosticta* Thuret at 15°C at 45µmol m\(^{-2}\)s\(^{-1}\).

Although intertidal seaweeds require an equivalent of 10% of the full irradiance, in order to germinate properly (Barsanti & Gualtieri 2006), the growth of *P. columbina* conchocelis was recorded to occur even at 10µmol m\(^{-2}\)s\(^{-1}\) which is equivalent to 0.8% full surface irradiance in New Zealand summer (Frazer & Brown 1995). Furthermore, Porphyra conchocelis have also been found growing on shells at depths ranging from 46 to 78m (Clokie *et al.* 1981). This suggested that the initiation of growth of conchocelis can require very little light. When growing the conchocelis of *P. dentata* and *P. pseudolinearis* conchocelis under different irradiance levels Kim (1999) also did not find any significant differences in their growth and concluded that the levels of irradiance tested were not critical in conchocelis growth. The results from our study also revealed that temperature and photoperiod were more critical than irradiance, in the growth and development of the conchocelis filaments of the three species.

The results of our experiments also showed that the conchocelis arising from the different types of spores are similar in morphology, although their development differed in the distribution of their chloroplast and their branching pattern. The branching pattern of the
conchocelis of these three species distinguished them from one another. The conchocelis of
*P. aeodis* also differed from *P. capensis* and *P. saldanhae* with the presence of protothalli while the presence of conchocelis rheospores distinguished *P. capensis* from the other two species. Although the conchocelis of these three species grew in temperatures from 5 to 20ºC it must be borne in mind that the optimum temperature for growth found in laboratory can however be different from that observed in the natural environment, due to the frequent changes of temperature in the natural environment, as opposed to the usually constant temperature of the laboratory experiments (Weiss *et al.* 1985). From the results of the present study it is recommended that the conchocelis of these species be cultured at 15ºC, 80 µmol m⁻²s⁻¹, long-day photoperiod for fast growth and development
Chapter 5

Conchosporangial development, conchospore release and blade development of three South African *Porphyra* species in culture
5.1. Introduction

Since Drew (1949) demonstrated the existence of the conchocelis phase in the life history of *Porphyra*, the life history of more than thirty *Porphyra* species have been documented in culture (Mei et al. 2005). The various stages of the life history of this genus have since been studied extensively (e.g. Kurogi 1953a; 1953b; 1959; 1961; Kurogi & Hirano 1956a; 1956b; Iwasaki 1961; Iwasaki & Matsudaira 1963; Krishnamurthy 1969; Chen et al. 1970; Lee & Fultz 1970; Conway & Cole 1973; 1977; Dickson & Waaland 1985; Kapraun & Lemus 1987; Mumford & Miura 1988; Waaland et al. 1990; Lewmanomont & Chittpoolkusol 1993; Knight & Nelson 1999; Nam-Gil 1999; Notoya & Miyashita 1999; Orfanidis 2001; Pereira et al. 2004; Tang et al. 2004; Pacheco-Ruíz et al. 2005; Lindstrom et al. 2008; Xiaolei et al. 2008). All these studies demonstrated that the conchocelis phase developed into sporangia which resulted in the formation of spores that were different from spores released by the foliose blade phase.

Drew (1949) was the first researcher to record conchosporangia in *P. umbilicalis* and referred to them as ‘sporangial structures’ with no specific name since she saw no liberation of spores from such sporangia. Kurogi (1953a) then demonstrated that (in *P. suborbiculata*, *P. tenera*, *P. umbilicalis* and *P. yezoensis*) these sporangia released monospores which germinated into blades and referred to these sporangia as monosporangia. However, Hollenberg (1958) avoided the term monosporangium when culturing *P. perforata* and referred to them as “sporangia branchlets” that released fertile “conchospores” which grew into blades. Conchosporangium was then adopted and the use of monosporangium for this stage was dropped since monosporogenesis was found to be more appropriate for use in the gametophyte stage rather than in the sporophyte. For example, Hawkes (1980) referred to monosporogenesis as taking place in a 5 – 10mm band along the distal thallus margin of *P.
gardneri, with monospore release occurring at the margin. Li (1984) also stated that monospores of *P. yezoensis* are reproduced asexually by the gametophyte thalli and are larger, giving rise to larger sporelings, while conchospores are smaller and released from sporophyte conchosporangia. Furthermore, the term monosporangia was found to be inappropriate for *Porphyra* sporophytes as the term monospores was restricted to the special monosporangia in the *Erythropeltidaceae* (Magne 1991). It is now accepted that the conchocelis phase develops and matures to produce conchosporangia, which then release conchospores, and that the liberation of the conchospores is species-specific and triggered by specific environmental conditions in different species.

Iwasaki (1961) cultured conchocelis of *P. tenera* under different photoperiod regimes and concluded that maturation of sporangia and release of spores are both induced by a short photoperiod and prevented by continuous light in this species. Kurogi and Sato (1962) then cultured the conchocelis of five species (*P. angusta, P. tenera, P. kunieda, P. pseudolinearis* and *P. yezoensis*) in different photoperiods (8L:16D, 10L:14D, 12L:12D, 13L:11D, 14L:10D, 15L:9D) and observed that all species formed sporangia in all photoperiods. However, they also found that there were more spores discharged in short-days, fewer spores in neutral-day photoperiods, while no spores were released under long-day photoperiods. Waaland *et al.* (1990) conducted photoperiod studies on various North East Pacific *Porphyra* species (*P. abbottae, P. nereocystis, P. perforata, P. pseudolanceolata* and *P. torta*) and also concluded that photoperiod was an essential environmental trigger for the release of conchospores in all those species. However, Kurogi (1959) and later Waaland *et al.* (1990) found that although short day photoperiods were generally ideal for spore release, a combination with other environmental factors e.g. irradiance and temperature was also needed for most species. Abdel-Raman (2005) also found that although at higher temperatures (15 and 20°C) *P. leucosticta* released sufficient conchospores under long-day (16L:8D) and neutral-day
(12L:12D) photoperiods, it released more spores under short-day (8L:16D) photoperiods when exposed to lower temperatures (10 - 15°C).

No such studies have ever been conducted for any of the South African species. The aims of this study were to describe the morphological changes in the development of the conchosporangial phase of three local Porphyra species and to quantify the conchospore release in these species. Subsequent blade growth and development was also investigated.

5.2. Materials and Methods

5.2.1. Conchosporangia formation
To monitor conchosporangium development, conchocelis were grown from zygotospores in 30psu salinity, long-day (16L:8D) and short-day (8L:16D) photoperiods at different temperatures (5, 10, 15 and 20°C) under 80\(\mu\)mol photons m\(^{-2}\).s\(^{-1}\) irradiance for four weeks. A total of sixteen 25-welled 100ml plastic repli-trays (Bibby, Sterilin\(^\text{®}\), Newport, UK) were used to complete the experiment, with two trays used per environmental condition. Each tray had five wells containing spores from one species so that there was a total of fifteen wells, of the 25 wells, used for the three species per repli-tray. The spores were cultured in 4ml, per well, of 1/3 strength Provasoli’s Enriched Seawater (PES) medium and 2ml of the medium was changed every three days, when observations were made. The experiment was run for 28 days.

5.2.2. Conchospore release
In order to investigate the release of conchospores, these trays were subsequently all put in long-day photoperiod and cultured at 15°C, 80\(\mu\)mol photons m\(^{-2}\).s\(^{-1}\) for an extra 28 days to promote further conchosporangia growth. Thereafter, the conchosporangia from each species were combined and chopped using a household blender and filtered to fit through 145 -
125µm plankton net. Filaments with conchosporangia were then isolated, using forceps and a dissecting microscope, and transferred to new repli-trays. These conchosporangia were cultured in the same conditions as in section 5.2.1 for eight weeks.

5.2.3. Gametophyte growth
The effects of temperature and stocking density on the growth of \textit{P. capensis} blades produced from cultured conchospores were tested. Blades from \textit{P. capensis} were used because cultures of this species were the only ones which produced enough blades to complete these experiments. Gametophytes (0.03±0.004g, length 33.08±2.27mm, width 8.8±0.25mm) were cultured in long-days (16L:8D) under different temperatures (15ºC, 20 and 25ºC) at 80µmol photons m$^{-2}$s$^{-1}$ for eight weeks. Blade weight was recorded at weekly intervals, while length and width were only measured at the beginning and end of the culture period.

To test for the effects of stocking density, gametophytes (mass 0.11±0.001g, length 10.86±1.12mm, and width 9.01±1.75mm) were cultured in 16L:8D, 15ºC, 80µmol photons m$^{-2}$s$^{-1}$ for five weeks. Stocking densities of 5, 10 and 15 grams of gametophytes were put into 100ml conical flasks containing 50ml full-strength PES medium and cultured for five weeks. Each experiment, provided with aeration, was repeated and the culture medium was changed every seven days, when the blades were weighed and measured.

5.2.4. Statistical analysis
A student t-test was used to compare the two experiments before the data from the two experiments were pooled together. All growth data were presented graphically as specific growth rate over time (see chapter 4 for formula). A one-way ANOVA was used to compare the growth of gametophytes under different temperatures, as well as under different stocking densities. A Tukey honest significant difference (HSD) \textit{post hoc} test for multiple
comparisons was performed and all results were regarded as being significantly different at $p<0.05$. All statistics were performed using Statistica Release 8, StatSoft Inc., Tulsa, USA.

5.3. Results

5.3.1. Conchosporangial morphology and development

Although the conchosporangia frequently developed directly from the spores in *P. aeodis* (Figure 1a), they only developed from the vegetative conchocelis in *P. capensis* (Figures 1f & 1g) and *P. saldanhae* (Figure 1k - m). *Porphyra aeodis* had the thickest conchosporangia (maximum 28µm) while *P. capensis* and *P. saldanhae* attained a maximum thickness of 24µm. The conchosporangia of *P. aeodis* were composed of irregularly-shaped cells of varying sizes (Figures 1b) which resulted in irregular branching (Figure 1c). On the other hand, the cells were rectangular for *P. capensis* (Figure 1h) and more irregular for *P. saldanhae* (Figures 1k-m). Conchospore development caused the deformation of the conchosporangia (Figures 1d, 1i & 1n) which subsequently resulted in death of conchosporangia upon release of conchospores (Figures 1e, 1j & 1o) in *P. aeodis*, *P. capensis* and *P. saldanhae* respectively. Conchosporangial cells had a central chloroplast for all three species, and each cell eventually became more oval-to-spherical with conchospore development and maturity.
Figure 1. Conchosporangial development and conchospore release in the three *Porphyra* species.

**Figure 1a-e, *P. aeodis***: a) conchosporangium growing directly from a zygotospore and sharing a spore with a vegetative conchocelis filament, b) varying conchosporangial cell sizes and cell arrangement, c) irregular conchosporangial branching, d) conchospore development and, e) conchospore maturation and release.

**Figure 1f-j, *P. capensis***: f&g) conchosporangia arising laterally from a vegetative conchocelis, h) conchosporangial rectangular cells and branching, i) conchospores maturation and, j) conchosporangia after release of conchospores.

**Figure 1k-o, *P. saldahae***: k-m) conchosporangia growing apically and laterally from the vegetative conchocelis, n) conchospore formation and, o) conchospore liberation.

### 5.3.2. Conchospore germination and conchosporeling development

The conchosporangia of all species liberated spherical conchospores (Figure 2a) that were of similar size, 18µm average diameter, and were orange-brown in color. The chloroplast of the conchospores was irregular and large, filling two thirds of the conchospore. Before blade germination, the chloroplast of the conchospores of all three species became granulated and unevenly distributed throughout the spore (Figure 2b). The germination of conchospores into
blades was initiated by the appearance of a narrow white tail-like (rhizoidal) extension seemingly originating from the wall of *P. aeodis* conchospore (Figures 2c & 2d). For *P. capensis*, the interior of conchospores split randomly (Figure 2e) or divided into two identical daughter cells (Figure 2f), while the conchospore was frequently deformed and the chloroplast was clearly separated from the rest of the cytoplasm in *P. saldanhae* (Figures 2g & 2h). The microscopic blades of *P. aeodis* were characterized by the formation of cells that divided more horizontally than vertically, resulting in ovate-to-cordate blades (Figure 2i). For *P. capensis*, the conchospores that were randomly split resulted in lanceolate blades (Figure 2j) while conchospores that divided into daughter cells resulted in blades that had two lobes (Figure 2k). The blades of *P. saldanhae* were frequently lanceolate (Figure 2l) although ovate individuals were seen on rare occasions and the blade cells divided horizontally suggesting a possibility of apical growth.

**Figure 2.** Morphological characteristics of conchospores and their subsequent development for the three *Porphyra* species. **a)** Conchospore of *P. capensis* showing the large chloroplast, **b)** partitioning of chloroplast in conchospore of *P. aeodis*, **c&d)** the appearance of white extension during conchosporeling germination, **e&f)** splitting of *P. capensis* conchospores during germination, **g&h)** conchospore deformation during *P. saldanhae* germination, **i)** *P. aeodis* blade, **j&k)** *P. capensis* blades and, **l)** *P. saldanhae* conchosporeling.
5.3.3. Macroscopic blade development

In macroscopic *P. capensis* individuals less than 2cm long, the basal cells of gametophytes were irregularly-shaped and not pigmented, resulting in a pale appearance in surface view (Figure 3a). In cross section, cells in this region were not fully developed, with fibrous tissue extending throughout the thallus. Above this section, the surface view showed vegetative cells arranged in bean-shaped pairs (Figure 3b) in the middle while the marginal cells tended to be more rectangular. In cross section, the vegetative cells were spherical with a diffuse, granulated chloroplast extending throughout the whole cell (Figure 3c). Although thallus thickness ranged from 70 - 80µm, the diameter of these cells was 20 - 28µm.

In bigger individuals (2 - 5cm) of *P. capensis* (Figure 3d), the vegetative thallus thickness was up to 100µm, with oval to club shaped cells having two chloroplasts. The length of these cells was 32 - 40µm, and the width was 12 - 20µm. The marginal cells were elliptical to spherical, with a single diffuse chloroplast and a diameter range of 10 - 18µm. Surface view of the cells suggested that growth was through cell expansion (Figure 3e) followed by splitting (Figure 3f) leading to two closely arranged cells.

In individuals above 6cm, the cells were 2 - 3 times longer than broad, with cell length ranging from 80 - 90µm while width ranged from 30 - 40µm in transverse view. The cells were oval, but more rectangular towards the centre of the blade, and contained two distal chloroplasts (Figure 3g). Thallus thickness was 100µm in the basal areas of the blade but decreased slightly towards the margins and the apex. In surface view, the cells were arranged in groups of two, with one cell expanding before actively dividing into two irregularly-shaped cells (Figure 3h).
Porphyra capensis cells for:
(Figure 3a – c) Individuals less than 2cm long: a) surface view of irregularly-shaped rhizoidal cells, b) surface view of vegetative cells and, c) transverse view of the vegetative cells.
(Figure 3d - f) Individuals between 2 and 5cm long: d) transverse view of the vegetative cells, e) surface view of expanded vegetative cell and, f) splitting of expanded vegetative cell.
(Figure 3g & h) Individuals longer than 6cm: g) rectangular-shaped vegetative cells in transverse view and, h) actively dividing cells in surface view.

Porphyra capensis gametophytes developed into blades of diverse morphologies (Figure 4a-h), influenced sometimes by the stocking density in culture (see later). The morphologies varied from simple lanceolate (Figure 4a), cordate (Figure 4c), umbilicate (Figures 4d), two-lobed (Figure 2d), to branched (Figures 2e - h). In cross section, the cells had two chloroplasts and were typical of the wild P. capensis (Figure 4i & j). The holdfast of these individuals was frequently found to be branched with each branch shaped in a pyramid-like manner (Figure 4k) although this shape was less distinct as the blades grew larger.
Figure 4. Variation in morphology of cultured blades of *P. capensis*: a) lanceolate, b) cordate, c) umbilicate, d) split, e&h) lanceolate and branched, f) two blades sharing a holdfast with one blade split and the other branched, g) branched blades sharing a holdfast. Transverse section of i) marginal cells and j) mid-section vegetative cells and k) surface view of holdfast (white arrows) attached to plastic repli-tray surface. All blades were cultured under long-day (16L : 8D) photoperiod.

5.3.4 Conchosporangia, conchospore and blade triggers
No conchosporangia developed at 5ºC for all species under both photoperiods. For *P. saldanhae*, there were also no conchosporangia recorded at 10ºC under short-day photoperiods. Although conchosporangia developed at 10ºC in long-days, no conchospores were released for *P. aeodis* and *P. saldanhae*, while more than 30 days were required to for conchospores to be released in *P. capensis* at this temperature in long-days (Figure 5). At 15ºC and 20ºC, the release of conchospores was enhanced by short-day photoperiod in *P. aeodis* and *P. saldanhae* while long-days were more suitable for *P. capensis*. Upon release of conchospores, the period required for germination into blades was longer in short-day photoperiod for all three species. Furthermore, increased temperatures reduced the time required for the released conchospores to germinate into blades in all three species irrespective of photoperiod (Figure 5).
Figure 5. The development of the life history stages of the three Porphyra species under various culture conditions (LD = long-day photoperiod and SD = short-day photoperiod). No conchosporangia were produced at 5°C. The time on the y-axis indicates when the various stages were recorded for the first time and incomplete lines reflect the absence of the proceeding stages thereafter.
5.3.5 Temperature and stocking density effects on blade growth

The highest growth rates \( (p = 0.007) \) were recorded in blades cultured at 15°C while blades grown at 25°C never grew but lost pigmentation and died within four weeks (Figure 6). Length data also revealed that at 15°C the growth rate was higher than at 20°C while blades at 25°C did not grow at all but instead gradually degenerated from the outer margins.

![Figure 6](image)

**Figure 6.** Specific growth rate of *P. capensis* blades cultured at different temperatures in long-day photoperiods at 80µmol photons m\(^{-2}\)s\(^{-1}\). The blades were cultured at 30psu salinity.

Overall, culturing at 10g/ 50ml stocking density yielded significantly higher \( (p = 0.031) \) growth rates while growth was lowest at 15g/ 50ml density (Figure 7). Although the SGR of blades was sometimes higher in 15g/ 50ml compared to 5g/ 50ml, the *post hoc* test detected that the growth was significantly higher \( (p = 0.047) \) at 5g/ 50ml compared to 15g per 50ml.
Figure 7. Specific growth rate of *P. capensis* blades under different stocking densities (5, 10 and 15g of seaweed in 50ml full strength PES). Plants were cultured at 15°C, 80µmol photons m⁻²s⁻¹ irradiance under long-day photoperiods.

The blades cultured at 5g/50ml and 15g/50ml were much longer than broad while those cultured at 10g/50ml were much broader than long (Figure 8). As a result, the majority of blades cultured at 10g/50ml became more cordate in morphology while 5g/50ml and 15g/50ml were dominated by lanceolate forms.

Figure 8. Length/Width ratio of blade cultured at 5, 10 and 15g of seaweed per 50ml PES at 15°C, 80µmol photons m⁻²s⁻¹ irradiance under long-day (16Light : 8Dark) photoperiods.
5.4. Discussion

Conchosporangial development in *P. aeodis* differed from *P. capensis* and *P. saldanhae* in that it was the only species in which conchosporagia sometimes developed directly from the spores. This type of development of conchosporangial branches directly from spores was also reported in *P. schizophyllia* (Cole & Conway 1980), *P. kinositae* (Notoya et al. 1992) and *P. koreana* (Kim & Notoya 2001). In this respect, the life history of *P. aeodis* is different to *P. capensis* and *P. saldanhae*.

Although the conchosporangia of the three species were different, these morphological differences gradually disappeared during the culture period as the conchosporangia elongated and branched. The conchosporangia became very long, comb-like and arranged themselves in parallel rows. Although Knight and Nelson (1999) suggested that this type of growth was probably related to phototropic response, in the current study they seemed to assume this structure with maturity irrespective of culture conditions. The three species were also similar in that development of the conchosporangia led to the disappearance of the vegetative conchocelis. Furthermore, the conchosporangial color changes (from dark-brown to orange) in all species seemed to be induced by conchospore development and maturation in all species rather than species-specific characteristics.

Unlike *P. torta* and *P. nereocystis* (Dickson & Waaland 1985; Melvin et al. 1986), no capitate cells (i.e. swollen bulbous-shaped cells that form at the sporangia tip) were observed in *P. aeodis* and *P. capensis*. However, larger cells towards the tip were sometimes observed during conchospore development in *P. saldanhae* although the apical cell was always the smallest resulting in a tapering tip. The results from these three species suggest that maturity of the conchosporangia was either uniform throughout the conchosporangium (as illustrated
in figure 2e, 2i and 2o), or began at the basal cells which were the first cells formed during conchosporangial development. Furthermore, the complete emptying of the conchosporangia after conchospore release also suggested that conchospore release was uniform throughout the conchosporangium. Sidirelli-Wolff (1992) found that conchospore release occurred through rupturing of the mucilage layer of the conchosporangia in *P. leucosticta*, thus suggesting uniform conchosporangial maturity. The absence of capitate cells in these three species was consistent with findings of Iwasaki and Matsudaira (1963) who demonstrated the liberation of conchospores from *P. tenera* showed no capitate cells. The results also agreed with Hollenberg (1958) who illustrated conchosporangial development but did not show any capitate cells in *P. perforata* conchosporangia.

The results also revealed that the development of conchosporangia was promoted by both temperature increase and long-day photoperiod in all three species. The results were consistent with Kurogi and Hirano (1956b) and Chen et al. (1970) who found that increases in temperature promoted conchosporangial development for *P. tenera* and *P. miniata* respectively. These results were also similar to findings of Notoya and Iijima (2003) and Lindstrom et al. (2008) who found that long-days and increased temperatures resulted in increased conchosporangial development in both *Bangia atropurpurea* and *P. fallax* respectively. Although Kurogi and Sato (1967) also reported conchosporangial formation for *P. umbilicalis* as triggered by long-day photoperiods at 10 and 20ºC and not occurring at short-days, their data differed from the current study in that they recorded more conchosporangial development in short-days (8 hours dark) and neutral-days (12 hours dark).
The results for *P. aeodis, P. capensis* and *P. saldanhae* also conflicted with studies of Kurogi (1959); Kurogi & Sato (1962); Notoya *et al.* (1999) and Abdel-Rahman (2005) who found that conchosporangium formation was due to short-day photoperiod. Based on their results, Dickson and Waaland (1985) also concluded that conchosporangium formation in *P. nereocystis* occurred at the end of autumn into winter, during short-days and when water temperatures are lower. This conflict of results may reflect that the latter species responded more to photoperiod than to temperature, since it is a seasonal species that has to synchronize its life history with its host, *Nereocystis luetkeana*.

In contrast to *P. capensis*, the release of conchospores in *P. aeodis* and *P. saldanhae* was higher in short-day photoperiods than in long-days, although none of the three species released conchospores at 5°C irrespective of the photoperiod. The photoperiodic effect of conchospore release in the latter two species was consistent with previous studies (e.g. Iwasaki 1961; Kurogi & Sato 1962; Kapraun & Lemus 1987; Waaland *et al.* 1987; 1990a; Mumford & Miura 1988; Lindstrom *et al.*, 2008) which reflected short-day photoperiods as a primary trigger of conchospore release in most *Porphyra* species. Although the specific kinetics that commonly triggers the release of conchospores under short photoperiods is not well known, the illumination shock on the phytochrome of *Porphyra* during short-days has been suggested as a possible conchospore trigger (Dring 1967).

Conchospore release in *P. capensis* was induced more by increased temperature than by photoperiod changes and this possibly allows this species to occur all year in the wild. Candia *et al.* (1999) stated that presence of *Porphyra* thalli throughout the year suggested a lack of photoperiodic control in the differentiation of their reproductive cells. However, if occurring throughout the year was solely due to lack of photoperiod, then photoperiodic control would
also be expected to be absent in *P. saldanhae* since it was also found throughout the year in the wild. These results therefore suggest that apart from temperature and photoperiodic control, other untested factors control conchospore release from *P. capensis* and *P. saldanhae*. Since the experiments were only conducted at 80 µmol photons m\(^{-2}\)s\(^{-1}\) irradiance, it is possible that the difference in the two species was due to their reaction to this irradiance under different photoperiods. The other reason for differences in these two species could be due to possible genetic differences between these species.

Due to lack of detailed literature on the morphology of *Porphyra* conchospores, it was difficult to compare the conchospores of the three species with other species. The conchospores released from the three species (average diameter 18 µm) were of similar diameter to those found by Ogawa and Lewmanomont (1978) in *P. vietnamensis*. However, they were larger than those observed by Lin (1984) and Zheng (1984) in *P. yezoensis* (average 10-14 µm), in *P. katadai* var. *hemiphylla* (16 µm), in *P. marginata* (14 µm) and in *P. oligospermatangia* (10-11 µm) and were orange-brown in color compared to the purplish brown conchospores of *P. yezoensis*. The size and color differences could be genetic, since Jones *et al.* (2004) found the South African species to be molecularly distinct from species around the world. However, the chloroplast size of these three species was consistent with the large conchospore chloroplast size mentioned by Mumford and Cole (1977) and Shimizu *et al.* (2008) for the *Porphyra* genus.

Conchospore germination in *P. aeodis*, *P. capensis* and *P. saldanhae* was similar to the germination of blade archeospores (*sensu* Nelson *et al.* 1999), as shown by Ogawa and Lewmanomont (1978) in *P. vietnamensis* and by Abdel-Rahman (2005), in *P. leucosticta*. The non-pigmented parts of the conchospores formed the holdfast while the chloroplast
became compartmented to produce the blade vegetative cells. This was consistent with observations of Iwasaki (1961) and Krishnamurthy (1969) who stated that during normal development of a germling of an erect thallus, the basal cell forms the rhizoids, while the apical cell forms the frond. The results also revealed that although the mechanisms controlling the morphological differences of the holdfasts of these species were not clear, the dividing pattern of the pigmented part of the individual conchospore may determine the morphology of the germinating gametophyte. For example, within *P. capensis* the random conchospore splitting (Figure 2e) may have resulted in lanceolate gametophytes while the dichotomous division (Figure 2f) may have resulted in cordate-to-umbilicate individuals.

Environmental factors such as temperature and water motion have also been shown to affect the morphology of *Porphyra* blades in culture (Freshwater & Kapraun 1986; Hannach & Waaland 1989; Monotilla & Notoya 2004). Although temperature effects were not obvious in the morphology of cultured *P. capensis* gametophytes during the eight-week period, stocking density affected blade morphology. Blades with higher width SGR and lower length SGR were more abundant at the intermediate 10g/50ml stocking density while blades cultured at 5g/50ml and 15g/50ml were longer but narrower. Since Hannach and Waaland (1989) found that increased water motion resulted in elongated blades in cultured *P. abbottiae*, the observed morphological differences could be attributed to the increased agitation of blades cultured at the lowest 5g/50ml, by the bubbling water, compared to those cultured at intermediate (10g/50ml) density. However, the same logic could not explain the abundance of blades with higher length SGR compared to lower width SGR at the highest (15g/50ml) stocking density. The latter could either be due to coincidental selection and culturing of more blades that were formed through the random division of the conchospore (as mentioned above) or more likely due to competition of blades for light and nutrients. This
was because, Miura (1975), Tseng (1981), Mumford and Miura (1988) and Akatsuka (1990) also found that higher stocking density resulted in smaller and thinner *Porphyra* fronds due to competition for both nutrients and light. Furthermore, competition could also explain the texture of the blades with those cultured at 5g/ 50ml feeling more rigid and leathery while those grown at 15g/ 50ml were more membranous and fragile.

Although Hannach and Waaland (1989) found that temperature did not significantly affect the growth of *P. abbottiae* gametophytes, the current study revealed that temperature and stocking density accounted for the different growth rates recorded for *P. capensis* blades in culture. The complete death of gametophytes at 25°C was not surprising since the water temperatures along the area where the species were collected never reached 25°C and therefore this temperature was an extreme. On the other hand, the mean sea surface temperatures around this area varied from 8 to 16°C (see Chapter 6) and could explain the high growth rates recorded at 15°C.

Conchospore release in *P. aeodis* and *P. saldanhae* was promoted by short-day photoperiods while there was a lack of photoperiodic control on the release of conchospores from *P. capensis*. The lack of photoperiodic control in *P. capensis* was supported by the ability of bigger blades (84mm) from this species to become fertile at 15°C, 80 photons m\(^{-2}\)s\(^{-1}\) irradiiance, under long-day photoperiod (16L:8D) after five months in culture. It can thus be projected that in all three species, conchosporangial development might occur mainly during the autumn when the water temperatures are rising and there are still long-day photoperiods in the wild. For *P. capensis*, conchospore release might be expected to occur mostly in winter when water temperatures are highest while in *P. aeodis* and *P. saldanhae* more conchospore release is likely to occur towards the end of winter, triggered mainly by
the break of winter short-days into spring long-day photoperiods. This timing of conchospore release, in all three species, coupled with the slow growth rate recorded in *P. capensis*, would support the larger individuals of these species in summer. However, other factors such tidal cycles need investigation since Lindstrom *et al.* (2008) found that conchospore release appeared to be aided by desiccation and re-immersion of shells bearing *P. fallax* conchosphorangia.
Seasonal variation in mineral element composition of three South African *Porphyra* species collected from the wild
6.1. Introduction

*Porphyra* species are not only of economic importance, but also nutritionally important as they have traditionally been eaten in many parts of the world: as ‘limu pahée’ in Hawaii, ‘sloke’ in Scotland, ‘nori’ in Japan, ‘kim’ in Korea, ‘zicai’ in China, ‘luche’ in Chile, ‘sleachán’ in Ireland, ‘karengo’ in New Zealand and ‘laver’ in Wales (Mumford 1986; Aitken *et al.* 1991; Turner 2003; Subba Rao *et al.* 2007). These species are nutritious and contain relatively high contents of crude protein, polysaccharides, vitamins and minerals (Nisizawa *et al.* 1987; Noda 1993; Davies 1997). The dietary fibre content has been recorded as high as 50% of dry weight in *Porphyra* species from China and Japan (Dawczynski *et al.* 2007). Darcy-Vrillon (1993) reported low contents of saturated fats while Sánchez-Machado *et al.* (2004) and Blouin *et al.* (2006) recorded high levels of polyunsaturated fatty acid in different *Porphyra* species. Watanabe *et al.* (1999) recorded high percentages of vitamin B12 in *Porphyra* spp., while Rupérez and Toledano (2003) also recorded the total sugar content of *P. tenera* to be as high as 158.6±5.5 g kg⁻¹ dry weight.

*Porphyra* owes its high nutritional value to having a high surface/volume ratio which makes it more efficient in assimilating nutrients such as nitrogen and phosphorus (Neori *et al.* 2004; Pereira *et al.* 2008). Since seaweeds obtain their nutrients directly from the seawater (Anderson *et al.* 1978; Hernández *et al.* 1999) and the uptake of nutrients from seawater is influenced by various chemical, physical and biological factors (Lobban & Harrison 1994) such as changes in water temperature, salinity, turbidity, light and seawater nutrients (Riget *et al.* 1997; Dawes 1998; Vasconcelos and Leal 2001; Lozano *et al.* 2003; Marinho-Soriano *et al.* 2006; Riekie *et al.* 2006), their nutrient uptake may be influenced by the seasonal fluctuations in the seawater conditions (Lüning and tom Dieck 1989). The nutritional
composition of *Porphyra* species therefore varies seasonally due to seasonal differences in seawater nutrients (Flores-Moya et al. 1997).

Although several studies have investigated the seasonal variation in nutritional composition of other seaweed species (e.g. in Floreto et al. 1993; Nelson et al. 2002 and Rodde et al. 2004), most studies on *Porphyra* species (e.g. Rupérez 2002 on *P. tenera*; McDermid & Stuercke 2003 on *P. vietnamensis*; Subba Rao et al. 2007 on *P. vietnamensis*; Kumar et al. 2009 on *P. vietnamensis* and Tuzen et al. 2009 on *P. umbilicalis*) have been conducted specifically to quantify the average mineral composition of these species without focusing on the seasonal variation of the minerals. However, Kusumo (1993) conducted a seasonal study of the mineral contents of five *Porphyra* species (*P. fallax*, *P. pseudolanceolata*, *P. torta*, *P. perforata* and *P. mumfordii*) from the Pacific Northwest and found no seasonal pattern for carbon in all species while the nitrogen showed a distinct seasonal pattern, being higher in January and decreasing towards May in all species. Fajardo et al. (1998) also found that *P. columbina* from Argentina had significantly more sodium, potassium and magnesium in the winter season compared to the rest of the year. Pérez et al. (2007) also collected *P. columbina* from Argentina and found that the Pb content of this species was highest in summer while the Cd content was highest in winter.

Although nutrient-related effects of the strong upwelling of the cold Benguela current are well documented for the South African ecosystems e.g. nutrient cycling, harmful algal bloom occurrence, phytoplankton biomass and production (Bang 1973; Andrews & Hutchings 1980; Carter 1982; Schumann et al. 1982; Shannon 1985; Kudela et al. 2005), no studies have looked into the effects of this phenomenon on the nutritional variability of *Porphyra*. Since the west coast of South Africa experiences nutrient loading through upwelling periods in
spring, this phenomenon is likely to affect the seasonal variation in nutrient content of the local *Porphyra* species in the wild. However, there have been some few studies on the nutritional composition of South African *Porphyra* species. According to Mumford (1986), 30% porphyran was extracted from *P. capensis* by Batey (1974). However, the taxonomy of the species used could not be confirmed as it was collected in Chile and the Chilean samples have yet to be compared to the South African *P. capensis*. Zhang *et al.* (2005) studied the polysaccharide structure of *P. capensis* growing at different sites (Lüderitz, Swakopmund and Cape Town) and collected at different times [1998 (January; August and November) and 1999 (January)]. They found that the polysaccharide from this species had a typical porphyran structure, with a 1.2:1 ratio of α-L-galactose-6-sulfate to 3,6-anhydrogalactose units. Karsten (1999) compared the seasonal concentration of different carbohydrates (i.e. L-isofloridoside, D-isofloridoside and floridoside) of *P. saldanhae* (collected from Kommetjie from June to December) with that of other species (*P. dioica, P. perforata, P. columbina* and *P. umbilicalis*) from various parts of the world and found no temporal variation in *P. saldanhae* throughout, with *P. dioica* and *P. umbilicalis* having more carbohydrate in spring and summer while *P. leucosticta* had the highest carbohydrate concentration in winter.

This study was thus designed to investigate the seasonal variation in the mineral composition (i.e. certain macroelements, microelements and heavy metals) in three South African *Porphyra* species. It also aimed to quantify the nutrient content of these three species in relation to human and abalone nutrition and to compare these three species to other edible seaweed species and vegetables.
6.2. Materials and Methods

6.2.1. Sample preparation

The three *Porphyra* species (*P. aeodis*, *P. capensis* and *P. saldanhae*) were collected from Kommetjie (34°09’06″S, 18°19’22″E), near Cape Town. The collection was done from July 2006 and every second month thereafter until January 2007. Due to the seasonal occurrence of *P. aeodis*, the collection of all three species was then done monthly from January 2007 until February 2008, whereas for *P. aeodis*, the collection period was extended to April 2008 to complete the seasonal cycle observed in 2007. Water temperature was measured as outlined in Chapter 2. For seasonal analysis, the elements were grouped into: summer (December – February), autumn (March – May), winter (June – August) and spring (September – November) to correspond with meteorological seasons.

The fresh material was washed thoroughly, with freshwater, and drained with a salad spinner. Thalli were then blotted with paper towel and air-dried in an oven, at 60°C, for 72 hours. The dried samples were weighed again, hand-crushed and pulverized (using a Säizer milling machine) to pass through a screen with an aperture of 2mm. The milled seaweed samples were stored in air-tight glass vials and kept in the dark, at room temperature, until element analysis.

6.2.2. Macroelement, microelement and heavy metal analysis

For the element analysis, the ground powder (3g dry weight) of the three species for each month was sent to an independent element analyzing laboratory (BemLab, Stellenbosch, South Africa). The laboratory uses the official methods of analysis of the Association of Official Analytical Chemists (AOAC). The various elements were determined by CHNS/O element analyzer (Perkin Elmer 2400, Connecticut, USA) and flame atomic–absorption spectrometry.
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 (Combs 1996). In seaweed growth, the Redfield ratio (C:N:P) is often used as an indicator of the presence of N or P limitation, with high ratios indicative of such limitations in the environment (Atkinson & Smith 1983; Vrede et al. 2004; Peters et al. 2005; Kuznetsov et al. 2008). Macroelements in seaweeds include Ca, Mg, Na and K (Lüning et al. 1990; Matanjun et al. 2009) and therefore these macroelements, together with C, N, H, S and P were analyzed.

Microelements, on the other hand, are sometimes referred to as beneficial elements (Combs 1996) or trace elements (Goldhaber 2003; Tuzen et al. 2009). Such elements are essential for seaweed metabolism since they may limit algal growth at low external concentrations (Reed & Gadd 1990).

In human nutrition, microelements such as manganese are essential trace elements, deficiency of which may result in reduced reproductive performance and growth retardation (Goldhaber 2003). Adequate iron intake is also necessary for growth and development in humans and is also responsible for transporting oxygen in the human hemoglobin, myoglobin and enzymes in the bloodstream and for the prevention of anemia (Ikem et al. 2002). Some incidents of cardiovascular diseases have been found to be associated with the zinc-to-copper ratio in diets since copper is also important for transportation of oxygen in human blood (Goldhaber 2003) and in the hemocyanin in abalone (Viant et al. 2002).

In abalone nutrition, zinc is an essential micronutrient for growth and shell biomineralization (Liao et al. 2002, 2004; Mai et al. 2003). Copper is also an essential microelement since low concentrations may limit the growth of abalone (Skinner et al. 2004) through gill-tissue damage. Although the microelements in seaweeds include nickel, selenium and chromium
(Reed & Gadd 1990; Tuzen et al. 2009) this study was designed to only investigate the seasonal variation of Cu, Zn, B, Mn and Fe.

Besides having value in the nutrition of animals, seaweeds may also accumulate high levels of metal pollution (Almela et al. 2002; 2006) and may therefore be toxic to humans and other consumers. For example, exposure to lead may suppress mental capacity or cause retardation in children and therefore reduce their intelligence level while exposure to cadmium can lead to kidney dysfunction (Ikem et al. 2002). Four heavy metals: arsenic (As), cadmium (Cd), lead (Pb) and mercury (Hg), were therefore analyzed in the three species. It must be noted that the arsenic analysed here was total arsenic and not specific arsenic e.g. inorganic arsenic.

6.2.3. Statistical analysis
The results were presented as either percentages (%), milligrams per hundred grams (mg 100g⁻¹ d wt) or micro grams per gram dry weight (µg g⁻¹d wt). Principal Component Analysis was performed for the interaction between species, months and elements using XLSTAT Version 2008.7.01, Addinsoft™, USA. The PCA was regarded as the most appropriate test, since it could be done on absolute and relative data (Novosyolov & Satchkov 2008). Furthermore, the PCA gives a complete unbiased view of the data where one does not impose any preconceived ideas of how the data should be modeled (Esbensen et al. 2002) and is a simple, non-parametric method of extracting relevant information from confusing data sets (Shlens 2005), by finding a set of base vectors to explain the maximum variance-covariance structure of the data set through sets of linear combinations of the original variables (Jolliffe 1986; Cichocki & Amari 2002). This method is widely used in interest rate markets to describe yield curve behavior (Novosyolov & Satchkov 2008), in image representation and recognition (Murase & Nayar 1995) such as in the diagnostics of breast cancer through modeling the cancer conductivity of the breast (Stasiak et al. 2007), and in population
dynamics through production of score plots classifying data into four quadrats (Esbensen et al. 2002). Kruskal-Wallis one-way-ANOVA was used to test for year-to-year variation while a 2-way ANOVA was used to compare species-season interaction. All ANOVA tests were performed using Statistica Release 8, StatSoft Inc., Tulsa, USA.

6.3. Results

6.3.1. C:N:P ratio

Although the average C:N:P ratio for *P. aeodis*; *P. capensis* and *P. saldanhae* was 74:8:1, 54:6:1 and 90:11:1, the C:N:P ratio for all these species varied temporally and was generally higher in summer (Table 1). Although the C:N:P ratio of *P. aeodis* was lower from spring 2007 to autumn 2008 compared to the spring 2006 to autumn 2007, there was no clear year-to-year variation in C:N:P ratio of *P. capensis* and *P. saldanhae*. However, the C:N:P ratio of *P. saldanhae* was always higher than that of *P. capensis* although *P. aeodis* had ratios that were sometimes similar to those of *P. saldanhae*.

<table>
<thead>
<tr>
<th>Table 1. Seasonal variation in the dry weight atomic C:N:P ratios of three Porphyra species. Symbols used in the table: # = No collection conducted, ‡ = No individuals could be found or insufficient material available.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<tr>
<td></td>
</tr>
<tr>
<td>Winter-06</td>
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<tr>
<td>Summer-06</td>
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<tr>
<td>Autumn-07</td>
</tr>
<tr>
<td>Winter-07</td>
</tr>
<tr>
<td>Spring-07</td>
</tr>
<tr>
<td>Summer-07</td>
</tr>
<tr>
<td>Autumn-08</td>
</tr>
</tbody>
</table>

There were minor variations in the C:N ratios for *P. aeodis* (8.50–9.46), *P. capensis* (8.31–8.64) and *P. saldanhae* (8.02–8.53), with *P. saldanhae* having the lowest C:N ratio while *P. capensis* usually had the highest ratio (Figure 1). However, no seasonal pattern could be detected for all three species. Furthermore, there was no correlation between the CN ratio of
the three species with the seawater temperature. On the other hand, the N:P ratio for both *P. capensis* and *P. saldanhae* was significantly higher (*p*=0.041) in spring and summer compared to autumn and winter (Figure 2). The N:P ratio for *P. saldanhae* was usually higher than both *P. aeodis* and *P. capensis*, while the 1-Way ANOVA detected no significant difference (Tukey: *p* = 0.0703) between *P. aeodis* and *P. saldanhae* in autumn 2007 and between *P. aeodis* and *P. capensis* (Tukey: *p* = 0.859) during spring 2007 and summer of 2008.

![Graph showing C:N ratio variation across seasons for three species of Porphyra](image)

**Figure 1.** Variation in the C:N ratio in the three *Porphyra* species. It must be noted that the gap in *P. aeodis* reflects that this species was not found winter 2007 although it was found in 2006.
Figure 2. The seasonal variation in the N:P ratio in the three *Porphyra* species. *Porphyra saldanhae* and *P. capensis* were negatively correlated with seawater temperature, while no correlation was found for *P. aeodis* with temperature. Although *P. aeodis* was found in winter 2006, it could not be found in winter 2007. Furthermore there was not enough material to analyse for *P. aeodis* in spring 2006.

### 6.3.2 Macroelement content

*Porphyra capensis* had more Ca, C, H and P while *P. saldanhae* had the highest N contents ($p = 0.0297$) throughout the study period. Carbon was the most abundant element in all species while Mg and Ca were the least abundant macroelements (Table 2).

Table 2. The macroelement composition of three *Porphyra* species (% dry weight) with data presented as means and standard errors

<table>
<thead>
<tr>
<th>Macroelements (%)</th>
<th>C</th>
<th>H</th>
<th>S</th>
<th>N</th>
<th>P</th>
<th>K</th>
<th>Ca</th>
<th>Mg</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. aeodis</em></td>
<td>36.8±0.68</td>
<td>5.93±0.06</td>
<td>2.39±0.05</td>
<td>4.44±0.05</td>
<td>0.54±0.04</td>
<td>2.06±0.22</td>
<td>0.20±0.01</td>
<td>0.44±0.01</td>
</tr>
<tr>
<td><em>P. capensis</em></td>
<td>37.8±0.35</td>
<td>6.17±0.05</td>
<td>1.92±0.05</td>
<td>4.55±0.08</td>
<td>0.73±0.04</td>
<td>2.21±0.09</td>
<td>0.24±0.02</td>
<td>0.42±0.01</td>
</tr>
<tr>
<td><em>P. saldanhae</em></td>
<td>37.3±0.60</td>
<td>6.11±0.08</td>
<td>2.02±0.04</td>
<td>4.93±0.08</td>
<td>0.44±0.03</td>
<td>1.55±0.12</td>
<td>0.22±0.02</td>
<td>0.40±0.02</td>
</tr>
</tbody>
</table>

The Principal Component analysis (Figure 3) revealed that *P. saldanhae* had more N and Na while *P. capensis* had most of the other macroelements, and *P. aeodis* had less Ca, C and H. *Porphyra saldanhae* tended to have a higher N content in spring and summer while it had more Na in winter (Figure 3). There were no clear seasonal patterns observed in either *P.*
aeodis and P. capensis. The S content was not correlated to any of the other macroelements ($r = 0.041$), while Mg correlated with N ($r = 0.562$), C ($r = 0.76$), H ($r = 0.77$) and Na ($r = 0.762$) for all species. There was also a strong correlation between P and K ($r = 0.733$), C and H ($r = 0.801$) and C and Na ($r = -0.820$) for all species.

**Figure 3.** Principal Component Analysis showing interaction between months (numbers), species (color coded with symbols) and elements. Key: *P. aeodis* (●) in black text, *P. capensis* (Δ) in blue text and *P. saldanhae* (x) red text. The sampling times are represented in numbers, with the last two digits of each number representing the year.

When grouped into the various seasons, the nitrogen, hydrogen, calcium and carbon contents of all species generally decreased in autumn and winter while the other macroelements increased (Figure 4). However, these patterns were not clear and the statistical results reflected that there were no significant differences ($p > 0.05$) between the seasons for all species, except for N ($p = 0.046$) and Na ($p = 0.041$) in *P. capensis* and *P. saldanhae*. 
Figure 4. The seasonal variation in macroelements for the three species from Kommetjie in relation to the sea surface temperatures from Oudekraal.
There was year-to-year variation in the Na/K ratio of both *P. capensis* and *P. saldanhae* with higher values in winter 2007 compared to 2006 (Figure 5), while the Na/K ratio of *P. aeodis* was highest in autumn and lowest for in spring in both years (Figure 5).

![Figure 5](image)

Figure 5. The seasonal trends in mean Sodium-to-potasium, Na/K, and Calcium-to-Magnesium, Ca/Mg, ratio of the three *Porphyra* species.

### 6.3.3. Microelement content

The order of microelement concentration was Fe>Zn>Mn>B>Cu (Table 3), although the B content was higher than the Mn content and lower that the Cu content in winter for *P. capensis*. When compared to the other two *Porphyra* species, *P. capensis* had significantly more Mn and Zn ($p = 0.0394$), although there were no significant differences ($p>0.05$) in their Fe, Cu and B contents. *Porphyra capensis* also had the highest ($p<0.05$) total
microelement concentration as Fe + Zn + Mn + Cu + B (24.72±3.10 mg 100g⁻¹) while *P. aeodis* had the lowest amount (18.31±2.18 mg 100g⁻¹) of the three species. The selected total macrominerals (Na+K+Ca+Mg) of all species increased significantly in winter (*p* = 0.0375) and were lowest in summer.

**Table 3.** The mean content of microelements of the three *Porphyra* species.

<table>
<thead>
<tr>
<th>Microelements (mg 100g⁻¹)</th>
<th>Mn</th>
<th>Fe</th>
<th>Cu</th>
<th>Zn</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. aeodis</em></td>
<td>1.81±0.13</td>
<td>11.96±1.08</td>
<td>0.67±0.68</td>
<td>3.87±0.68</td>
<td>1.60±0.18</td>
</tr>
<tr>
<td><em>P. capensis</em></td>
<td>2.82±0.11</td>
<td>13.87±1.06</td>
<td>1.39±0.49</td>
<td>6.63±0.49</td>
<td>1.73±0.13</td>
</tr>
<tr>
<td><em>P. saldanhae</em></td>
<td>1.66±0.09</td>
<td>14.35±2.02</td>
<td>1.5±0.48</td>
<td>3.64±0.47</td>
<td>1.71±0.25</td>
</tr>
</tbody>
</table>

The PCA (Figure 6) showed that *P. capensis* frequently had more Mn and Fe than both *P. aeodis* and *P. saldanhae*. It also showed that *P. aeodis* had the lowest contents of all the microelements, while *P. saldanhae* sometimes had more B, Cu and Zn. The concentration of zinc was always correlated to the copper content (*r* = 0.605) for all species (Figure 6) while Fe was only correlated to Mn (*r* = 0.786) in *P. aeodis* and *P. saldanhae* (*r* = 0.566) but not in *P. capensis* (*r* = 0.358). Boron was only correlated to Cu in *P. saldanhae* (*r* = 0.506) but not in *P. aeodis* (*r* = -0.114) and *P. capensis* (*r* = 0.258). No clear seasonal patterns were observed for all species, although both *P. capensis* and *P. saldanhae* seemed to contain more of these elements in late winter months through early spring (i.e. June – November, Figure 6)
Chapter 6

Figure 6. PCA explaining 63.52% of data for the seasonal variation in the content of microelements (B, Cu, Fe, Mn and Zn) in the three Porphyra species: *P. aerdis* (●) with black text, *P. capensis* (△) with blue text and *P. saldanhae* (x) with red text. The sampling times are represented in numbers, with the last two digits of each number representing the year.

When pooled into seasons, the data showed that there was more B in all three species in autumn 2007 while the B contents were lower in winter of 2006 and 2007 for both *P. capensis* and *P. saldanhae* (Figure 7). No seasonal patterns were found for the other elements, for all species, although the Zn contents of all three species was significantly higher (*p* = 0.0416) in spring of 2007.
Figure 7. The seasonal variation in microelements, in relation to the seawater temperature, for the three Porphyra species.
6.3.4. Heavy metal content

The levels of Hg were too low to be detected and the order of concentration of all elements was As>Cd>Pb>Hg in all species (Table 4). The Kruskal-Wallis ANOVA also detected significant differences ($p<0.05$) between the various heavy metals between the different months, for all species and thus supported the observed order of element concentration. *Porphyra capensis* had significantly more ($p = 0.0438$) heavy metals than either *P. aeodis* or *P. saldanhae*.

**Table 4.** The mean heavy metal content of three *Porphyra* species. Values are expressed as means ± SE of dry weight (N=12 for *P. aeodis*, N=17 for *P. capensis* & *P. saldanhae*). Mercury was not listed since levels in all samples were too low for detection.

<table>
<thead>
<tr>
<th></th>
<th>As</th>
<th>Cd</th>
<th>Pb</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. aeodis</em></td>
<td>16.1±1.82</td>
<td>4.7±0.60</td>
<td>0.4±0.14</td>
</tr>
<tr>
<td><em>P. capensis</em></td>
<td>26.9±2.79</td>
<td>6.3±1.23</td>
<td>0.3±0.07</td>
</tr>
<tr>
<td><em>P. saldanhae</em></td>
<td>18.0±2.01</td>
<td>3.6±0.60</td>
<td>0.6±0.09</td>
</tr>
</tbody>
</table>

The PCA (Figure 8) revealed that, although the highest amount of Pb was detected in *P. aeodis* in March 2008, *P. saldanhae* frequently had a higher Pb content while *P. capensis* had more Cd. In all species, Cd was always correlated to As ($r = 0.533$) while Pb was correlated with As in *P. aeodis* ($r = 0.618$), and *P. saldanhae* ($r = 0.589$), but not in *P. capensis* ($r = 0.338$). There were no seasonal patterns in any of the species.
Figure 8. Principal Component Analysis of seasonal variation in the content of heavy metals (As, Cd and Pb) in the three Porphyra species: P. aeodis (●), P. capensis (Δ) and P. saldanhae (X). The months are represented in numbers, with the last two digits of each number representing the year. Black numbers represent P. aeodis, blue for P. capensis and red for P. saldanhae.

Although there were no definite seasonal patterns for any of the heavy metals (Figure 9), the Cd contents for both P. capensis and P. saldanhae were slightly lower in winter (between 2.06 and 3.36 µg g⁻¹), except for P. capensis in June 2007 when 13.5 µg g⁻¹ was recorded. There was year-to-year variation in Cd contents of P. saldanhae, with significantly lower (p = 0.0429) contents recorded in winter and spring of 2007. For all species, the heavy metal content was not related to the sea surface temperatures.
Figure 9. Mean seasonal variation in heavy metal contents of three *Porphyra* species in relation to sea surface temperatures.
6.4. Discussion

The ratio between carbon, nitrogen and phosphorus (C:N:P Redfield ratio) is often used as an indicator of the presence of nitrogen or phosphorus limitation, with high ratios indicative of such limitations in the environment (Vrede et al. 2004; Peters et al. 2005). Although the average C:N:P ratio of photosynthetic organisms in the sea is 106:16:1 (Atkinson & Smith 1983; Kuznetsov et al. 2008), the average C:N:P ratios for *P. aeodis* (74:8:1), *P. capensis* (54:6:1) and *P. saldanhae* (90:11:1) were much lower. The C:N:P ratios of these three species were also lower than those of *P. yezoensis* (137:23:1, Johnston 1971 in Atkinson & Smith 1983) and *P. umbilicalis* (258:20:1 in winter and 495:38:1 in spring, Hernández et al. 1993). This suggests that either the growth of these three species was N- and P- limited, or they have lower requirements for these elements since there is evidence showing that different algae (Boynton et al. 1982; Atkinson & Smith 1983) and different taxonomic groups of organisms (Cross et al. 2003; Liess & Hillebrand 2005) have different ratios. Wu et al. (1984), Hafting (1999) and Pereira et al. (2008) found critical N-values ranging from 4 to 4.7% dry weight for *P. yezoensis* and *P. dioica*, and observed that nitrogen contents above these values did not affect growth rates in these species. Fujita et al. (1989) also showed that temperate seaweeds, including *Porphyra*, had a critical level of 1.5% dry weight for nitrogen. Considering that *P. aeodis*, *P. capensis* and *P. saldanhae* all had N-values similar to those critical values of Wu et al. (1984), Hafting (1999) and Pereira et al. (2008), and significantly higher than that found by Fujita et al. (1989), it could be concluded that the growth of these three species was never N-limited throughout the study period.

Although the C:N ratio of benthic seaweeds normally ranges from 10 to 70 (Atkinson & Smith 1983) it can be lower in some seaweeds and, according to Pinchetti et al. (1998), C:N values closer to 10 have been described as optimal or normal for the nitrogen status of algae.
and those greater than 10 indicate N-limitation. Contrary to Atkinson & Smith (1983) values above 10 are interpreted as N-limiting some seaweed species (D'Elia & DeBoer 1978) and this would suggest that all three species studied herein were not N-limited as their C:N ratios were lower than, but close to, ten. The C:N ratio of these species was similar to *P. leucosticta* (Korbee *et al.* 2005b) and *P. endiviifolium* (Weykam *et al.* 1996) and based on the C:N ratio obtained by Weykam *et al.* (1996) and by Peters *et al.* (2005) for *P. endiviifolium*, the low C:N ratio (6.4 - 7.91) of *P. endiviifolium* would also suggest no N-limitation since it was lower than ten. This would be true since *P. endiviifolium* is an Antarctic species (Clayton *et al.* 1997; Wiencke *et al.* 2007) growing where the coastal waters have nutrient concentrations that are high and non-limiting to the growth of seaweeds (Drew & Hasting 1992).

Besides the C:N ratio, the nitrogen-to-phosphorus (N:P) ratio has also been used to detect whether growth was either N-limited or P-limited. This was because the growth of wild *Porphyra* spp. is often limited by phosphorus (Flores-Moya *et al.* 1997) when there are lower P-concentrations in the seawater (Lobban & Harrison 1997). The N:P ratios of the three species analyzed here were generally lower which suggested no nutrient limits, since lower N:P ratios are associated with high growth rates while higher values reflect the opposite (Vrede *et al.* 2004; Liess & Hillebrand 2005). In 1992, Wheeler and Björnsäter found that *Porphyra* spp. containing N:P ratios less than 12 were N-limited while those having a ratio greater than 17 were P-limited. This would suggest that the growth of both *P. aeodis* and *P. capensis* was never P-limited, while *P. saldanhae* may have been P-limited in January (with ratios of 20.4 and 18.8 in year 2007 and 2008 respectively).

Although Hafting (1999) demonstrated that *P. yezoensis* had to maintain an N:P ratio of 13 - 15 for its growth to not be limited by either nitrogen or phosphorus in culture, the use of C:N
and N:P ratios as indicators of nitrogen or phosphorus limitation, however, poses cause for concern. Firstly their use was designed based on data from phytoplankton (Atkinson & Smith 1983) and, secondly, they were based on the assumption that nutrient loading and recycling was constant or at a steady state in the water-column (Olsen et al. 1986; Elser et al. 1988; Kahlert 2002). In reality, the nutrients are often supplied in pulses, rendering the use of nutrient ratios alone inadequate, since these ratios are constantly altered due to both pulse and uptake rates especially when there is more than one algal group in an area (Kudoh 1987; Fong et al. 1993). Furthermore, pulses such as of strong upwelling of Benguela current water bring sudden influxes of nutrients to the South African west coast seaweed ecosystems (Emanuel et al. 1992) and are bound to significantly influence these ratios. As a result, the N:P ratio was significantly higher in summer when the temperatures were lowest and this area had just experienced frequent pulses of upwelling during the spring season. Wheeler and Björnsäter (1992) also found that upwelling resulted in more nutrients, with high but variable concentrations of NO$_3^-$ and PO$_4^{3-}$, resulting in higher N:P and C:N ratios in seaweed species from Yaquina Head, Oregon. As a result, Kuznetsov et al. (2008) proposed a variable C:N:P ratio (106 – 400): (16 – 60): 1 for cyanobacteria instead of the classical Redfield ratio of 106:16:1 as means of catering for the changing conditions. Based on these, it can be concluded that the use of such ratios in Porphyra should be used with caution and in combination with other factors that may be limiting growth and nutrient uptake in various species. Furthermore, the C:N:P ratio could also be affected by differences in the physiology and morphology of Porphyra species compared to other seaweeds, since Porphyra has a high surface/volume ratio which makes it more efficient in assimilating nutrients such as nitrogen and phosphorus compared to many other genera (Neori et al. 2004; Pereira et al. 2008). Conitz et al. (2001) therefore recommended the use of phycoerythrin as a rapid and reliable
way of assessing the nitrogen status of wild and cultivated *Porphyra*, since phycoerythrin concentrations of *P. torta* responded very quickly to nitrogen limitations.

In contrast to the results of Korbee *et al.* (2005b) and Pereira *et al.* (2008) who found that the C:N ratios of *P. leucosticta* and *P. dioica* were influenced by increased nitrogen contents, the current study revealed that the C:N changes were mainly due to higher variation in the carbon content of *P. aeodis*, *P. capensis* and *P. saldanhae* relative to their nitrogen content. The low C:N ratios in these species could therefore be due to a decrease in proteins and an increase in carbohydrates, as observed by Neish *et al.* (1977) with *Chondrus crispus*. This was true for the carbon and hydrogen contents (see chapter 6), which are primary components of carbohydrates that were strongly correlated and increased whenever the nitrogen content was slightly lower for all three species.

The correlations detected between the various elements reflected the regulation of these elements by cellular homeostasis (Blinks 1951; Scott & Hayward 1954; Eppley 1958; 1959) or their metabolic role and/or synthesis in seaweeds. Potassium is the principal cellular cation of *Porphyra* species (Eppley & Cyrus 1960) and has been found to prevent accumulation of toxic elements such as lithium in *P. perforata* (Eppley 1959) and regulate the expansion and contraction of cells for nutrient uptake during the growth in *P. leucosticta* (Escassi *et al.* 2002). The correlation recorded between K and P for these three species could therefore be related to energy requirements of K to regulate ion transport across the cells. Magnesium was found to be correlated to nitrogen and this was because they are both involved in protein and protein synthesis. For example, Kroll and Elin (1985) found magnesium concentration to be linearly related to total protein. *Porphyra* species contain at least three structural polysaccharides found mainly on the cell membrane (Miwa 1940), and this explains the
significant correlation between H and C content, since these elements form the basic structural chains of carbohydrates.

The results revealed that, except the N; H; C and Ca, most of the macro- and microelements in both *P. capensis* and *P. saldanhae* increased as the sea surface temperature increased in this area during autumn and winter months. The uptake of nutrients by these species from seawater may therefore be influenced by changes in water temperature (Martínez & Rico 2002; Dawes 1998; Marinho-Soriano *et al.* 2006) since optimum temperatures for uptake frequently coincide with optimum temperatures for growth in other seaweeds (Asare and Harlin, 1983; Duke *et al.* 1989; Kautsky 1990; Peckol *et al.* 1994; Chopin *et al.* 1995; Rivers & Peckol 1995; Davison & Pearson 1996; Gerard 1997; Kinney & Roman 1998; Ozaki *et al.* 2001). However, there is no growth data to support the relationship between growth, nutrient uptake and temperature changes in the wild for South African seaweeds. Furthermore, these species were found to have the biggest individuals during summer (see Chapter 2) when water temperatures were lower. It is therefore that the growth rate (cell elongation) of these species exceeds the uptake rate of elements thus resulting in lower contents of some elements during summer. Other factors such as photoperiod and irradiance (not measured in the wild but measured in culture for the different phases of the life history, see preceding chapters) could account for the higher contents in summer when there is more light and longer day length.

The increased temperature in winter would have increased the metabolic activity of these two species, without necessarily increasing growth, resulting in increased nutrient uptake rates (Lozano *et al.* 2003) thus explaining the high contents in winter. However, the nutrient content of these species was expected to be lower when water temperatures are higher since
Osare and Harlin (1983), Waldron & Probyn (1992) and later Whitehouse et al. (1996) found that seawater temperature was negatively linearly correlated to nutrients, with higher nutrient contents at cold temperatures. Carter (1982) also found that phytoplankton production/biomass ratio peaked in Oudekraal during spring and summer then decreased in autumn and winter, and attributed the latter to reduced light and nutrients in winter. The increased nutrient content found in South African Porphyra species during warmer periods could therefore be a reflection of increased uptake rates rather than increased seawater nutrient concentrations.

Although elevated water temperatures have been shown to increase nutrient uptake in cultured seaweeds (Jacques 1983; Priddle et al. 1986), warmer waters are always associated with lower nutrient levels on the South African west coast (Waldron & Probyn 1992; Waldron et al. 1997) and other parts of the world (Martínez & Rico 2002; Chung et al. 2004). Therefore another explanation for the high mineral content of these three species during the winter period could be the rainfall occurring in this area during winter. Amano and Noda (1987) found that increased rainfall increased surface runoff and increased nutrients while low rainfall reduced the nutrients available to the benthic flora in the intertidal zone. Sfriso et al. (1992) also found that stormy weather lifted and mixed the nutrient concentrations of the surface and resulted in increased total inorganic nitrogen and reactive phosphorus while Chung et al. (2007) found that increased in NO$_3^-$, NH$_4^+$ and NO$_2^-$ during spring and summer in Gracilaria coronopifolia were associated with typhoons, which result in water mixing and nutrient enrichment. However, there is no evidence from our study site to test these suggestions, and is likely that heavy rainfalls would be needed to induce flooding that would significantly promote nutrient accumulation in the intertidal zone. Furthermore, such rainfall could be detrimental to the Porphyra species since Eppley and Cyrus (1960) suspected that
heavy winter rains may be involved in the disappearance of *P. perforata* during the winter. They believed that heavy rains washed away the sea water film normally present between and around the blades, even during emergence, thus inducing loss of cellular cations. However, Griffin (2003) found that the biomass of *Porphyra* was higher during winter in this area although his study was not species-specific. Other unknown factors, such as road runoff and sewerage discharge from the Kommetjie residents, may therefore have contributed to the significantly higher nutrient content of *P. capensis* and *P. saldanhae* during winter.

Such factors may include the continuous supply of nutrients to this area coupled with the delayed appearance of such nutrients in these three species. For example, Bang (1973) found that the Southern Benguela was characterised by continuous nutrient pulses, due to the prevailing south easterly winds occurring over spring, summer and autumn thus resulting in nutrients rarely limiting primary production in this area. Such pulses may have resulted in increased growth which would only be pronounced during the “relaxation phase of upwelling” (Carter 1982), i.e. in winter, when the water column is more stable than during the upwelling phases. Furthermore, the winter temperatures in this region fell in the type 2 and type 3 upwelling sequential development class of Barlow (1982) resulting in nutrients in this area likely to remain high even in winter. Since there were no planktonic algal blooms (which normally deplete nutrients in the absence of upwelling) recorded in this area during the study period, such nutrients would then be available for uptake by the seaweeds in this area.

The increased C, H, N and Ca contents observed in the three species during spring probably reflected higher levels of these elements in upwelled water. Temperature data reflected that there were series of upwelling events during spring, typical of the upwelling phenomenon of
the cold Benguela Current system (Andrews & Hutchings 1980; Carter 1982; Schumann et al. 1982; Shannon 1985, Kudela et al. 2005; Emanuel et al. 1992). For example, seawater temperatures dropped in October 2006 from 13.87ºC on the 14th to 8.62ºC by the 18th, in September 2007 they dropped from 15.32ºC (22nd) to 9.52ºC (25th) and back to 14.93ºC (28th) and in November 2007 dropping from 15.25ºC (14th) to 9.75ºC (16th). This temperature fluctuation is indicative of upwelling, resulting in high nutrient loading (Andrews & Hutchings 1980; Tait & Dipper 1998; Hwang et al. 2004; Carter 1982).

The results also showed that there were no significant differences between contents of (C, H, K, Ca, Mg, B and Cu) in *P. capensis* and *P. saldanhae*, which might be explained by them occupying the same habitat level on the shore (see Chapter 2). Kim et al. (2008) found that zonation patterns affected nutrient uptake, with uptake by the sublittoral *P. yezoensis* being lower while it was significantly higher in the eulittoral *P. umbilicalis* and *P. leucosticta*. This vertical distribution may explain the lower nutrient content found in the sublittoral *P. aeodis* compared to the eulittoral *P. capensis* and *P. saldanhae*, although the actual kinetics involved are not understood.

The mean Na/K ratios of *P. aeodis* (0.85± 0.14), *P. capensis* (0.68±0.11) and *P. saldanhae* (1.09±0.18) were below 1.5 and similar to the ratio obtained by Rupérez (2002) for *P. tenera* (1.04) but higher than those found by Turner (2003) in *Porphyra* sp. (0.37). The mean Na/K ratios of *P. aeodis* and *P. capensis* were similar to those obtained by Hanif et al. (2006) in cabbage (0.67) and lettuce (0.77) while individual monthly Na/K ratios of these three species were similar to those in spinach (0.29), carrots (0.31) and cauliflower (0.39) during spring. Such low ratios are useful for human nutritional purposes since intakes of NaCl and diets with high Na/K ratios have been related to a higher incidence of hypertension (Ikem et al.
This is because potassium is crucial for regulating heartbeat while sodium maintains balance for water flow in and out of cells. Furthermore, Matanjun et al. (2009) suggested that seaweeds, due to their low Na/K ratios, can help balance high Na/K ratio in diets such as olives (Na/K ratio = 45.63) and sausages (Na/K ratios = 4.89). The maintenance of low Na/K ratios in seaweed is regulated by intracellular cation homeostasis since seaweeds such as Valonia sp. (Blinks 1951) accumulate K with increased seawater salinity, while Ulva lactuca consistently maintained its K and Na contents even in diluted seawater (Scott and Hayward 1954).

The total microelement concentrations of P. aeodis, P. capensis and P. saldananhae were lower than those recorded by Pérez et al. (2007) in P. columbina (28.03 mg 100g⁻¹). McDermid and Stuercke (2003) however, reported total microelements of 24.5 mg 100g⁻¹ in P. vietnamensis, which are lower than those in P. capensis but higher than those in P. aeodis and P. saldananhae. Selected microelement content expressed for direct comparison as Fe + Zn + Mn + Cu (as in Rupérez 2002) was low in the South African species (average for P. aeodis = 18.31±2.18 mg 100g⁻¹, for P. capensis = 24.73±3.10 mg 100g⁻¹ and for P. saldananhae = 22.96±3.32 mg 100g⁻¹) compared to those obtained by Tuzen et al. (2009) in P. umbilicalis from Turkey (maximum 84.30 mg 100g⁻¹) and by Subba Rao et al. (2007) in P. vietnamensis (45.5 – 309 mg 100g⁻¹) from India. The selected microelement content of P. saldananhae was comparable to those found by McDermid and Stuercke (2003) in P. vietnamensis (21.3 mg 100g⁻¹) from Hawaii, while P. aeodis had the lowest values that were similar to that found by Rupérez (2002) in P. tenera (15.23 mg 100g⁻¹).

When compared to other edible seaweeds, the average Fe + Zn + Mn + Cu microelement contents of P. aeodis, P. capensis and P. saldananhae were higher than those found by
Matanjun et al. (2009) in tropical seaweeds *Eucheuma cottonii* (7.53 mg 100g\(^{-1}\)), comparable to *Caulerpa lentillifera* (24.99 mg 100g\(^{-1}\)) and lower than *Sargassum polycystum* (71.53 mg 100g\(^{-1}\)). They were higher than those found by Rupérez (2002) in *Fucus vesiculosus* (13.91 mg 100g\(^{-1}\)), *Laminaria digitata* (6.06 mg 100g\(^{-1}\)), *Undaria pinnatifida* (10.67 mg/100g) and *Chondrus crispus* (12.93 mg 100g\(^{-1}\)). The microelement content of *P. aeodis* was similar to that obtained by Runcie and Riddle (2004) in *Palmaria decipiens* (19.89 mg 100g\(^{-1}\)) while *P. capensis* and *P. saldanhae* had values that were higher than this edible seaweed.

The total microelement content of *P. aeodis* (19.93±2.75) was lower than values found by Amaro López et al. (1999) in *Asparagus officinalis* (28.79 mg 100g\(^{-1}\)), while *P. capensis* (42.13±4.65 mg 100g\(^{-1}\)) and *P. saldanhae* (30.7±2.46 mg 100g\(^{-1}\)) had values that were higher than this vegetable. The microelement content of these three *Porphyra* species was lower than the non-conventional vegetables consumed in African countries (e.g. Nigeria, by Barminas et al. 1998 such as *Moringa oleifera* (63.2 mg 100g\(^{-1}\)); *Adansonia digitata* (61.2 mg 100g\(^{-1}\)); *Colocasia esculenta* (39.9 mg 100g\(^{-1}\)); *Corchorus tridens* (42.6 mg 100g\(^{-1}\)); *Cassia tora* (47.9 mg 100g\(^{-1}\)) and *Amaranthus spinosus* (53.2 mg 100g\(^{-1}\)).

Low concentrations of copper in the environment may limit the growth of abalone (Skinner et al 2004). White and Rainbow (1985) calculated a theoretical metabolic requirement for Zinc (9 µg g\(^{-1}\)) and Copper (6.5 µg g\(^{-1}\)) in mollusks. Although red seaweeds have been found to contribute less, compared to brown seaweeds, to the diet of abalone elsewhere (Shepherd 1973; Guest et al. 2008) and in the South African abalone (Barkai & Griffiths 1986) the microelement content of *P. aeodis, P. capensis* and *P. saldanhae* could help to fulfill the Zn and Cu requirements of abalone, especially in farms where there is no kelp available.
Although microelements are necessary in animal diet, some microelements such as zinc and copper may be toxic at high levels (Malea 1994). As a result, Cu + Zn limits (maximum 10 mg 100g⁻¹) allowed in edible seaweeds for human consumption have been set in France and Japan (Indegaard & Minsaas 1991). The Cu + Zn values for all three South African species were below maximum limits rendering these species suitable for human consumption in this respect. The Cu + Zn content of *P. capensis* (mean 8±1.9 mg 100g⁻¹) was occasionally higher, while *P. aeodis* (mean 4.54±0.96 mg 100g⁻¹) and *P. saldanhae* (mean 5±0.9 mg 100g⁻¹) had lower values that were similar to those found by Muse *et al.* (1999) in *P. columbina* (4.13 mg 100g⁻¹) and by Subba Rao *et al.* (2007) in *P. vietnamensis* (4.31 mg 100g⁻¹).

In abalone nutrition, the toxicity of microelements is not well documented although Martin *et al.* (1977) observed histopathological gill tissue abnormalities, such as gill swelling and excessive mucus secretion, in *Haliotis rufescens* following exposure to seawater containing >32µg l⁻¹ copper. Viant *et al.* (2002) also found that *H. rufescens* individuals exposed to high concentrations of copper in seawater (131 - 506µg l⁻¹) secreted pale blue mucus, had retracted tentacles, and died. The low copper content of *P. aeodis*, *P. capensis* and *P. saldanhae* (always below 20µg g⁻¹, except in winter) renders these three species suitable for abalone feed. Chen and Liao (2004) and Tsai *et al.* (2004) found that exposing *H. diversicolor supertexta* to Zn concentrations of 0.031 – 0.065µg ml⁻¹ stimulated growth, while levels of 0.125 – 0.5µg l⁻¹ slowed growth, and 1.0µg l⁻¹ resulted in 40% mortality. Although the Zn content of *P. aeodis*, *P. capensis* and *P. saldanhae* was relatively higher than the concentrations mentioned above, also higher than the Zn acute-to-chronic ratio of 13.59 for *H. diversicolor supertexta* (Tsai *et al.* 2004) and higher than the Zn acute-to-chronic ratio of 13.27 for other aquatic invertebrates (Länge *et al.* 1998), these three Porphyra species were regarded as non toxic to abalone since Liao *et al.* (2002) found that the depuration rate of Zn
acquired through algal ingestion was higher than that acquired through seawater in *H. diversicolor supertexta*. The toxicity in abalone nutrition would therefore more affected by the concentrations of water-borne Zn and Cu concentration in the farm than by the concentration of these elements in the three *Porphyra* species. Furthermore, Arai *et al.* (2002) found an inverse relationship between *H. discus hannai* growth and Zn and Cu concentration, with abalone developing a physiological ability to differentiate and reduce toxic elements in their bodies as they grow.

The order of heavy metal element concentration observed in this study was consistent with the order of abundance of these elements in the ocean (see Tait & Dipper 1998). De la Rocha *et al.* (2009) also observed this order of abundance in *Porphyra* spp., from Asia and Europe, and also found Hg values below the detection limit. In *P. columbina*, Pérez *et al.* (2007) detected mean dry weight contents of As ($22.9\pm4.4 - 33.8\pm4.5 \mu g \ g^{-1}$), Cd ($2.83\pm0.39 - 3.54\pm0.46 \mu g \ g^{-1}$) and Pb ($<0.05 \mu g \ g^{-1}$) from different locations in Argentina while Subba Rao *et al.* (2007) detected mean As ($1.60\pm0.18 \mu g \ g^{-1}$), Cd ($0.29\pm0.01 \mu g \ g^{-1}$), Pb ($0.07\pm0.01 \mu g \ g^{-1}$) and Hg ($0.02\pm0 \mu g \ g^{-1}$) in *P. vietnamensis* from India. However the order of abundance of these elements was not consistent with results of Almela *et al.* (2002), who analyzed the heavy metal content of processed product of *P. tenera* and found them to contain more Hg than Pb and Cd [i.e. total As ($23.7\pm0.5 - 30\pm1 \ mg \ kg^{-1}$), Cd ($0.18\pm0.02 - 0.38\pm0.01 mg \ kg^{-1}$), Pb ($0.29\pm0.02 - 0.31\pm0.06 \ mg \ kg^{-1}$) and Hg ($4\pm1 - 14\pm2 \ mg \ kg^{-1}$ dry wt)].

The As contents of *P. aeodis*, *P. capensis* and *P. saldanhae* were lower than those detected by Almela *et al.* (2002) and Pérez *et al.* (2007) in *P. tenera* and *P. columbina* respectively. However, the Cd content of all three species was found to be considerably higher than that of *P. tenera*. Although the Pb content of *P. saldanhae* was higher, the Pb content of *P. aeodis*
and *P. capensis* was similar to that found in *P. tenera*. However, the Pb content of all three species was significantly higher than that detected by Astorga-Espaňa *et al.* (2008) in *P. columbina* from Chile (maximum 8.20µg g⁻¹) and by Besada *et al.* (2009) in *P. umbilicalis* from Spain (maximum 0.270mg kg⁻¹).

According to Almela *et al.* (2002) and Besada *et al.* (2009) in most countries except Australia, France, Japan and New Zealand, there is currently no legislation specific to consider the toxicity of edible seaweeds. The maximum allowed levels set in France and Japan for edible seaweeds are (Pb<5, Cd<0.5, Hg<0.1 and inorganic As<3 mg kg⁻¹ dry weight) while in Australia and New Zealand the maximum level for Cd is 0.2 and for inorganic Arsenic 1mg kg⁻¹ dry weight. The Cd and As content of *P. aerodis*, *P. capensis* and *P. saldaneae* exceeded the limits set above while the Pb and Hg content of all these species was below the maximum allowed levels.

Although the total As content was above the maximum allowed levels for all four countries, most of it could be organic arsenic since less than 10% of total arsenic in seaweeds is inorganic arsenic (Ichikawa *et al.* 2006). As a result total arsenic content is not useful for nutritional studies (Almela *et al.* 2006) since most of it is found in non-toxic organic dimethylarsenic compounds such as arsenosugar (Ichikawa *et al.* 2006) and arsenobetaine (Nakajima *et al.* 2006). When 10% of total arsenic is assumed to be inorganic in these three species, levels fall to (1.61±0.18 mg kg⁻¹) in *P. aerodis*, (2.69±0.28 mg kg⁻¹) in *P. capensis* and (1.80±1.20 mg kg⁻¹) in *P. saldaneae*. As a result, inorganic arsenic contents would be above the maximum allowed levels set for Australia and New Zealand, but less than those set for France and Japan.
Based on the results of this study, it can be concluded that the growth of these three species in nature was not limited by either nitrogen or phosphorus. The mineral content of *P. aeodis*, *P. capensis* and *P. saldanhae* was relatively high although some of their heavy metal content was sometimes above the maximum allowed toxic levels for edible seaweeds. Since more than 95% of the abalone shell is calcium carbonate and calcium (Mai *et al.* 2003), the high Ca and C content of these three *Porphyra* species makes them suitable as feed for shell mineralization in juvenile abalone. The phosphorus content of these three species was also regarded as sufficient for dietary requirements of cultured animals, since Coote *et al.* (1996) reported that the P dietary requirements of most aquatic species ranged from 0.45 to 1.5% while Tan *et al.* (2002) recommended 0.9 to 1.1% dietary phosphorus for maximum growth in *Haliotis discus hannai*. Although the element content of *P. capensis* and *P. saldanhae* generally varied in relation to seawater temperature, this may not be a direct causal relationship. The seasonal variation in the element content of these species could also not be attributed to salinity changes, since the salinity of most ocean water is within the range 34 - 36‰ (Tait & Dipper 1998) and the salinity of West coast also falls within this range (Waldron & Probyn 1992; Waldron *et al.* 1997). However, the seawater temperature data suggested that upwelling during spring and summer contributed in increasing the element contents of all three species. Although the order of abundance of the heavy metals in the ocean (see Tait & Dipper 1998 for values) certainly affected the order of accumulation of these metals in these three species, the role of the chemical form in which these metals are found in the ocean should be investigated, as well as the kinetics of individual element uptake by these three species. Overall, the vertical distribution of these three species may be the most likely source of variation and difference in the element content between them.
Chapter 7

Seasonal variation in protein content of three wild South African *Porphyra* species
7.1. Introduction

Humans and animals require a diverse diet in order to meet their nutritional requirements (Burlingame et al. 2006). Protein is important in tissue construction (e.g. growth, reproductive success and survival) and in physiological processes involved with health and diseases of animals (Combs 1996). For example, protein has been found to be the main source of nitrogen and essential amino acids in finfish diet and therefore an important constraint to their growth (Cowey 1979).

The quality and quantity of protein consumed is very important (Jones 1931) since protein digestibility and subsequent composition, in indispensable and conditionally indispensable amino acids (AA), can modulate protein metabolism (Dangin et al. 2002). For example, Williams et al. (1987) demonstrated that when compared to casein diets with equivalent quantities of protein, soy protein diets at 12 or 24% led to higher survival, lower urinary protein excretion, less renal hypertrophy and less histological damage in the rat kidney. Mustafa et al. (1995) compared the growth of red sea bream fingerlings fed three different seaweeds (Ulva pertusa, Porphyra yezoensis and Ascophyllum nodosum) and found that P. yezoensis resulted in higher growth and energy retention due to its protein efficacy. Davis (1997) also found that although different P. purpurea protein quantities (16.5 & 33%) resulted in different growth rates of thick-lipped grey mullet, inclusion of high levels of P. purpurea resulted in decreased feed efficacy. Goñi et al. (2002) and Rupérez & Toledano (2003) compared the protein digestibility of four edible seaweeds to P. tenera and found that P. tenera had the highest digestible protein content (17.27±0.26% d wt) compared to the other seaweed species which had digestible protein contents ranging from 1.21±0.02 to 7.27±0.10% dry weight. Teixeira et al. (2003) investigated the effects of protein quality (Casein & Soy protein) and quantity (12 & 20% energy) on diabetic nephropathy in male
diabetic mice and found that the increase in urinary albumin excretion (UAE) that occurred in
the mice when fed casein was absent when soy protein was consumed.

Quantification of protein in foods was first conducted by Henneberg (1865) who multiplied
the total nitrogen content of animals by a conversion factor of 6.25 (Salo-Väänänen &
Koivistoinen 1996). This method was universally accepted until Jones (1931) pointed out its
limitations by generating new conversion factors for different food types such as grains,
oilseeds and fruits. Since then, different nitrogen-to-protein conversion factors have been
proposed (see Lourenço et al. 2004 and references therein for microalgal species) although
the 6.25 factor is still widely used for food labelling.

The quantification of protein content obtained by using the 6.25 factor was then referred to as
“crude protein” (AOAC 1995) since alternative methods of obtaining nitrogen-to-protein
factors were developed. One of the alternative methods is referred to as “net protein of a
food” and represents the true quantitative protein value for foods and is determined by the
total amount of the 20 bound and free amino acids (Salo-Väänänen & Koivistoinen 1996).
Using this method, Aitkens et al. (1991) obtained a conversion factor of 5.0 using nitrogen
values and corresponding sum of amino acids from Porphyra columbina and P. subtumens
from New Zealand and different Porphyra species from the literature. After summing the
amino acids, Lourenço et al. (2002) proposed a factor of 4.47 for P. acanthophora var.
acanthophora and cautioned that although the summing of amino acids gave a reliable factor,
this method did not cater for the influence of the free amino acids on the calculation.

The methods involve in the extraction of soluble protein using the Lowry; the Bradford and
the Smith protein assays (Lourenço et al. 2002). Berges et al. (1993) compared the Lowry;
Bradford and Smith protein assays in extracting protein from the diatom *Thalassiosira pseudonana*, and found that the Lowry and Smith assays were similar and indicated up to 60% greater protein than the Bradford assay. Barbarino and Lourenço (2005) also found that the Lowry method yielded higher protein values compared to the Bradford method, with Lowry: Bradford ratios up to 3.2 depending on the protein source used. Korbee *et al.* (2005a) used Bradford’s method to extracted soluble protein in *P. leucosticta* and *P. umbilicalis* and found their phycobiliprotein and soluble protein concentrations to be affected by ammonium concentration. Korbee *et al.* (2005b) extracted soluble protein, using the Bradford method, and found that the protein content was highest in white light and lowest in red light for cultured *P. leucosticta* discs. Peters *et al.* (2005) also used the method of Bradford to extract protein from *P. endiviifolium* and other seaweeds from Antarctica and found the concentrations to be generally low, ranging from 1.3 to 17.3% dry weight. Other researchers who used the Bradford method to extract protein from seaweed include Duffy & Hay (1991); Bolser & Hay (1996); Cronin & Hay (1996) and Cruz-Rivera & Hay (2001).

Although Simpson (1994), Stepto & Cook (1996), Griffin *et al.* (1999a), Jones *et al.* (2004) and Barsanti & Gualtieri (2006) have suggested *Porphyra* as a valuable supplement in the diet of farmed South African abalone, *Haliotis midae*, the protein content of South African *Porphyra* is not known. It is known that some *Porphyra* species are very rich in proteins, and there is thus a need to compare the South African species with those found in other parts of the world. Thus the objective of this study was to quantify and investigate the seasonal variation in the crude and soluble protein of three *Porphyra* species common and abundant in South Africa.
7.2. Materials and Methods

7.2.1. Seaweed collection

Three South African *Porphyra* species (*P. aeodis*, *P. capensis* and *P. saldanhae*) were collected from Kommetjie. The collection was initially done from July 2006 and every second month until January 2007. To study the seasonal appearance of *P. aeodis*, the collection was then done monthly from January 2007 until February 2008. For *P. aeodis*, the collection period was extended to April 2008 to complete the seasonal cycle observed in 2007. Three extra samples were collected in November 2008, when the biomass of all three species was highest, to compare with the previous November months. This was important since conditions may change every year resulting in significantly different annual conditions and thus protein concentrations.

7.2.2. Crude protein determination

The N content was determined by the Kjeldahl method (see Chapter 6) and the percentages of crude protein were calculated by multiplying Kjeldahl nitrogen content by the factor of 6.25. Although this factor has been disputed for *Porphyra* species (see Mukai *et al*. 1981; Aitken *et al*. 1991 and Lourenço *et al*. 2002), it was used because it is widely used in the nutritional industry and was approved by AOAC in 1995 (Salo-Väänänen & Koivistoinen 1996; Marsham *et al*. 2007). Since this method is mainly applicable to eggs, meat and legumes and may be an overestimation of the protein content (Jones 1931; Lourenço *et al*. 2002) the Bradford protein assay was also used to extract soluble protein. This method was chosen over the Lowry and Smith methods to allow for a direct comparison with previous studies.
7.2.3. Soluble protein extraction

To induce cell lysis by osmotic shock, 5mg of seaweed powder were suspended in 2ml de-ionized water and gently stirred for 24 hours at 25°C prior to the subsequent protein extraction. After incubation, the suspensions were centrifuged at 20 000xg and 4°C for 20 minutes. The supernatants were collected and, for protein solubility, the pellets were re-suspended in de-ionized water in the presence of sodium hydroxide and 0.5%(v/v) 2-mercaptoethanol (Venkataraman & Shivashankar 1979). The mixtures were gently stirred with a Vortex mixer for at least thirty minutes before centrifugation again. The extraction procedure was repeated seven times and each time, 10µl of supernatant was added to 300µl Coomassie Brilliant Blue assay to test for the completion of the extraction. The extraction was regarded as complete when a visible blue solution was not produced, when the supernatant was added to the assay. The protein was analyzed using a Multiskan Spectrum spectrophotometer, Version 1.2, at 595nm wavelength and the Bovine Serum Albumin (BSA) standard was used to calibrate the protein concentration.

7.2.4. Statistical Analysis

For the crude protein and soluble protein, all monthly values were pooled together according to summer, autumn, winter and spring (see Chapter 2) and analyzed using a 2-Way ANOVA, comparing across species and seasons. Pearson’s correlation was performed between protein and nitrogen contents of all species using the different monthly values. The 2-Way ANOVA was performed using Statistica Release 8, StatSoft Inc., Tulsa, USA, while correlations were performed using the QED Statistics package, Pisces Conservation Ltd, Hants, UK. The QED package was used because it gives both the correlation matrix as well as the corresponding p-values, lacking in Statistica.
7.3. Results

7.3.1. Protein content

The soluble protein content was lower than the crude protein of all these species (Figure 1). Although there were no significant differences detected across the three species \((p=0.053)\), \(P.\) saldanhae had more crude protein content than \(P.\) aeodis and \(P.\) capensis.

![Crude Protein](image1)

![Soluble Protein](image2)

**Figure 1.** Mean (± standard error bars) seasonal crude protein and soluble protein variation in the three South African Porphyra species.

The soluble protein content of \(P.\) saldanhae was significantly higher \((p=0.014)\) than that of the other species, while the post hoc test detected no significant differences \((p=0.724)\) between \(P.\) aeodis and \(P.\) capensis. The crude protein of \(P.\) capensis and \(P.\) saldanhae was lower in November 2008 \((2.68±0.14\text{ and }3.74±0.07\%)\text{ wt respectively}\) compared to the same period in 2006 \((3.93\text{ and }4.58\%)\text{ wt respectively}\) and 2007 \((3.38\text{ and }5.09\%)\text{ wt respectively}\) while \(P.\) aeodis was significantly higher \((p=0.0037)\) than 2007 although no significant difference \((p=0.836)\) was found between 2008 and 2006. The crude protein \((p=0.0024)\) and soluble protein \((p=0.047)\) of \(P.\) capensis and \(P.\) saldanhae was significantly higher in summer and spring, while no seasonal pattern could be found for \(P.\) aeodis. No
correlation was found between nitrogen and soluble protein content of \( P. \text{aeodis} \) \((r=1.423, \ p=0.169)\), \( P. \text{capensis} \) \((r=0.329, \ p=0.198)\) or \( P. \text{saldanhae} \) \((r = 0.328, \ p=0.197)\).

7.4. Discussion

The protein content of seaweed differs according to species and seasonal conditions (Fleurence 1999; Rødde \textit{et al.} 2004; Jadeja & Tewari 2008). This study also revealed that the crude protein and soluble protein content of \textit{Porphyra} varied according to species, with \( P. \text{saldanhae} \) always having a higher content than the other species. Protein in these species was highest in spring and summer, unlike in \( P. \text{columbina} \) and \( P. \text{subtumens} \), which had highest protein contents in winter (Aitken \textit{et al.} 1991). The crude protein content of \( P. \text{aeodis} \), \( P. \text{capensis} \) and \( P. \text{saldanhae} \) was within the crude protein content range (21 – 47\%d. wt) of the commercially cultivated \( P. \text{tenera} \) (Nisizawa \textit{et al.} 1987; Fleurence; Fleurence \textit{et al.} 1999; Rupérez & Saura-Calixto 2001; Goñi \textit{et al.} 2002; Arasaki & Arasaki 1983 in Marinho-Soriano \textit{et al.} 2006; Marsham \textit{et al.} 2007). They were also sometimes similar to the protein content of \textit{Porphyra} sp.\textsuperscript{A} \((31.4±8.4\%d. \text{wt})\) and \textit{Porphyra} sp.\textsuperscript{B} \((30.9±3.9\%d. \text{wt})\) from Japan, Korea and China (Dawczynski \textit{et al.} 2007) and also similar to \( P. \text{purpurea} \) \((27.37±0.09)\) from Devon, United Kingdom (Davies \textit{et al.} 1997) and \textit{Porphyra} sp. \((24.11±1.03\%d. \text{wt})\) from Spain (Sánchez-Machado \textit{et al.} 2004). When using the crude protein conversion factor \((6.25)\) the Arctic \( P. \text{endiviifolium} \) would contain higher crude protein content \((36.75\%d. \text{wt})\) - see Peters \textit{et al.} 2005 for N-content) than all three South African species while the tropical \( P. \text{vietnamensis} \) would have less \((16.5±0.6\%d. \text{wt})\) crude protein (McDermid & Stuercke 2003). This would be consistent with other literature (e.g Kaehler & Kennish 1996; Weykam \textit{et al.} 1996; Wong & Cheung 2000; 2001; Dunton 2001; Peters \textit{et al.} 2005) that showed lower nutrient content in seaweeds from tropical regions compared to temperate and Polar Regions.
Jadeja & Tewari (2008) also suggested that the crude protein content may also vary with size of seaweed, with bigger individuals always having higher protein than the smaller ones. This correlation between size of individual thalli and crude protein content might apply to the three South African species, since the crude protein content was higher in spring and summer when thalli were generally larger. However, this correlation does not apply across these species because the largest, *P. capensis*, almost always contained less crude protein than the smallest *P. saldanhae*.

When compared to vegetables, the crude protein of these three species was similar to that reported by Jain *et al.* (2007) for spinach (20.69 – 32.56) cultivated in elevated CO$_2$. The crude protein content of these three *Porphyra* species was also similar to that reported by Tee *et al.* (1988) on white Soya beans (33.8%), but far exceeding their protein contents for red chilli (2.8%) green bean sprouts (2.6%) and broccoli (4.1%). Hanif *et al.* (2006) also found lower values in cabbage (1.6%), cauliflower (1.8%), carrot (1.5%), lettuce (1.2%), potato (1.9%) and tomato (0.9%). Since the Recommended Dietary Allowance (RDA) for protein for healthy young men and women is 0.80g protein/kg$^{-1}$/day$^{-1}$, 1.0 and 1.4g protein/kg$^{-1}$/day$^{-1}$ is suggested for older sedentary person and older persons who habitually perform high-intensity exercises respectively (Campbell *et al.* 2006), consumption of fragments or powder (as a spice) of any of the three *Porphyra* species would contribute significantly in human daily protein requirements. Furthermore, consumption of these species would increase protein synthesis, since protein intake $>1.5$g protein kg$^{-1}$/day$^{-1}$ was found to induce strong hyperaminoacidemia and thus stimulate protein synthesis in humans (Pacy *et al.* 1994).
However, the crude protein content of the three *Porphyra* species studied here was insufficient for the optimum crude protein requirement (47%) for the South African farmed abalone, *Haliotis midae* (Britz 1996, Sales *et al*. 2003). The crude protein content of these three species was also lower than the formulated K34 Abfeed® (34.7% crude protein) used as feed for *H. midae*. However, in spring and summer, *P. capensis* and *P. saldanhae* had crude protein contents that were higher than the recently formulated K26 Abfeed® (26% crude protein) suggesting that these two species could be used to substitute this formulated feed in juvenile abalone. Since the K34 Abfeed® has been associated with increased sabellid infestation in grow-out abalone due to relatively high nitrogenous waste that is conducive to polychaete generation (Simon *et al*. 2004), the two *Porphyra* species could be used to partially replace the K26 or K34 Abfeed® protein in grow-out abalone.

Although the crude protein and soluble protein content of these species followed similar temporal patterns when data were pooled into seasons, there was no correlation between the nitrogen and the soluble protein content of these species. This was because crude protein differs from soluble protein in that it assumes that all the nitrogen is protein nitrogen and that all proteins contain 16% nitrogen (Jones 1931). This may be an over estimation of the protein content (Salo-Väänänen & Koivistoinen 1996; Dawczynski *et al*. 2007), especially if samples contain high levels of non-protein nitrogen (Marsham *et al*. 2007), since not all nitrogen is protein-bound nitrogen (Jones 1931) and seaweeds contain pigments and amino acids that store non-protein nitrogen (Lourenço *et al*. 2002). Most seaweeds also tend to store excess nitrogen in their tissues for growth during periods of low nitrogen concentration (Thomas & Harrison 1985; Björnsäter & Wheeler 1990; Smit *et al*. 1997; Hafting 1999) and *Porphyra* species can use nitrogen stored as organic compounds during periods of N-limitation (Conitz
et al. 2001). Such storage occurs as inorganic nitrates and ammonium as well as organic compounds, free amino acids and photosynthetic pigments besides proteins (Rosell & Srivastava 1985; Jones et al. 1996; Naldi & Wheeler 1999).

Since red seaweeds contain high contents of chlorophyll and biliproteins when there is an abundance of nitrogen available (López-Figueroa & Niell 1991), this could explain the high crude protein content and constantly low soluble protein content found in the three species. Korbee et al. (2005a) found that although the phycobiliproteins represented about 20% of soluble proteins in both *P. umbilicalis* and *P. leuchoicostta*, and were affected by ammonium concentrations, this change in ammonium concentration did not affect the amount of soluble protein extracted from these species. This suggested that the soluble protein concentration was not related to the nitrogen concentration, is the case with the three species studied here. Lourenço et al. (2002) also found that the protein nitrogen of the Brazilian *P. acanthophora* var. *acanthophora* accounted for only 78% of total nitrogen while the remaining 22% was non protein nitrogen, and this supported the idea that soluble protein was not related to nitrogen concentration. While comparing the protein content of different foods, Salo-Väänänen & Koivistoinen (1996) also found that the net protein was always lower than the crude protein.

The results of this study also revealed that the soluble protein content of the three species was within the maximum concentration (7%d wt) suggested by Peters et al. (2005) for seaweeds from areas other than Antarctica. The results further revealed that the soluble protein content of the South African species was up to 4 times greater than that found by Hernández et al. (1993) in *P. umbilicalis* (7 – 14mg protein g⁻¹ fresh weight) from the Mediterranean Sea. The soluble protein content of the three species was lower than that extracted by Peters et al.
(2005) in *P. endiviifolium* (11.8±0.3 %d wt.), but more than that of *P. plocamiestris* (3.5±2.2%d wt). Considering that both these species are from Antarctica, with sufficient nutrients, this difference in the soluble protein suggests that the protein is not influenced by nutrient availability but rather by differences in other factors such as the amino acid composition.

Lourenço *et al.* (2002) reported that the amino acid composition of each species influenced the amount of protein extracted by the Bradford method. This was because Compton and Jones (1985) found that the Coomassie brilliant blue dye was extra sensitive to aromatic amino acids and thus bound more easily with amino acids such as arginine and phenylalanine. Berges *et al.* (1993) further suggested that the Bradford assay was less effective in reacting and removing free amino acids and small peptides, and was thus likely to underestimate protein content of species rich in these amino acids and peptides. Hernández *et al.* (1993) also found that the amount of soluble protein increased with decreased mean amino acid content of *P. umbilicalis* suggesting that soluble protein was not affected by the amount of amino acids but instead by the type of amino acids present. Since the species rich in arginine and/or phenylalanine may have high protein values (Lourenço *et al.* 2004), and the amino acid composition of the three South African species studied here is not known, differences in the type of amino acids present could account for the difference observed in them as well as in *P. endiviifolium* and *P. plocamiestris*. However, no studies have been done to compare the amino acid composition of these two Antarctic *Porphyra* species as well as in the South African species therefore the idea of protein content differences due to differences in amino acid composition in *Porphyra* species is only assumed currently.
Based on the results of this study, it can be concluded that the crude protein and soluble protein content of these species was comparable to *Porphyra* species from other parts of the world. However, the amino acid composition (i.e. total amount of the bound and free amino acids) of these species needs to be analysed in order to quantify the net protein content of these species and devise an appropriate nitrogen-to-protein conversion factor for the South African species. The concentration of the various types of amino acids present in the South African *Porphyra* species also needs to be analyzed as they may explain the difference in soluble content of these species and may also help in determining the most appropriate protein extraction method for each species.
Chapter 8

Morphological diversity in South African Porphyra species
8.1. Introduction

The taxonomy of *Porphyra* is problematic since this genus has a simple morphology and includes cryptic species as well as species with phenotypic plasticity (Stiller & Waaland 1993). As a result, the major morphological characters used are listed in Lindstrom and Cole (1993) and include the division formulae of spermatia and zygotosporangia; number of cell layers; size of vegetative cells; and blade shape. Due to the limited number of morphological characters available (Wiencke & Clayton 1998), delineation of species based on morphology alone may be inadequate (Lindstrom & Cole 1992b; Brodie *et al.* 1998). However, when morphological characters are used to delineate species, they should be critically evaluated since failure to do so may have serious implications in ecological studies (Brodie *et al.* 1996).

For an example, casual evaluation of *Porphyra* along the South African coastline resulted in all taxa that were morphologically different being lumped into *P. capensis* (Isaac 1957), although they were not formally described as such, but regarded as different forms of this species (Graves 1969). However, Stegenga *et al.* (1997) employed intensive morphological studies on *P. capensis* and distinguished it from one unidentified species, *Porphyra* *sp.* indet, and two other species (*P. gardneri* G.M. Smith *et al.* Hollenberg, and *P. carolinensis* Coll *et al.* Cox; now *P. suborbiculata* Kjellman) from the South African west coast. They also described a new endemic species, *P. saldanhae* Stegenga, Bolton *et al.* Anderson. They further recorded another new species that was later described by Griffin *et al.* (1999c), using morphometric and molecular techniques, as *Porphyra aeodis* Griffin, Bolton *et al.* Anderson. While using data from Griffin (2003), Jones *et al.* (2004) also recorded high morphological diversity in South African *Porphyra* and their rDNA SSU results reflected high levels of variation and possibilities of new species.
Although the circumscription of species based on differences in morphological and ecological characters could sometimes be artificial, morphological characters are critical as a basis for species delineation. The morphological distinctions in this chapter were regarded as significant enough to delineate species and this chapter therefore aimed to contribute towards resolving the taxonomy of South African *Porphyra* through critically evaluating the major morphological characteristics as listed in Lindstrom and Cole (1993). Before formal species description, it is considered important to carry out molecular studies to demonstrate species-level differences of these entities. However, samples were collected for the molecular taxonomic study which is however beyond the scope of this thesis.

### 8.2. Materials and Methods

Specimens were collected from various sites along the South African coastline, from the south-west coast through the Cape Peninsula to the west coast. The collection sites were Arniston (34°42′32″S, 20°11′12″E), Muizenberg (34°06′38″S, 18°28′05″E), Kommetjie (34°09′06″S, 18°19′22″E) Oudekraal (33°58′51″S, 18°21′47″E), Seapoint (33°55′15″S, 18°22′44″E), Elands Bay (32°18′89″S, 18°20′20″E), Kleinzee (29°40′26″S, 17°02′45″E) and Port Nolloth (29°15′05″S, 16°52′01″E). Since Brodie *et al.* (2008) suggested that comprehensive collection over a small area at different times of the year may yield more species, extensive collection was conducted in the Kommetjie area.

The collected specimens were washed in seawater and those regarded as different or unique were pressed as voucher specimens. A portion of the vegetative tissue was excised from these specimens and dried in silica gel for molecular analysis. However, a molecular taxonomic study was beyond the scope of this thesis. Pressed specimens were deposited in the Bolus Herbarium, University of Cape Town, South Africa. Only specimens with no morphological
characters overlapping with those of existing taxa were described, while those with minor morphological differences will only be described when molecular studies have been conducted on them.

8.3. Results and Discussion

(a) *Porphyra ramiculata* Dlaza, Bolton et Anderson sp. nov.

Holotype: TSD ANTP1 (Figure 1a).

Type locality: Arniston, South Africa, Dlaza, 2007.

Etyomology: Species name based on its distinctive branching pattern.

Habitat and seasonality: At the type locality the gametophyte phase was collected in spring, epilithic in the upper littoral zone.

Distribution: A South African endemic so far collected from the south-west coast.

Habit and vegetative structure: Fronds monostromatic, multiple blades branched with secondary branches; attachment by discoid holdfast; blades linear-ovate, up to 30cm long; margins smooth; color yellowish green when alive becoming light-brown when pressed; texture membranous but firm; firmly adherent to paper when pressed. In surface view vegetative cells elliptic basally but spherical at the margins, irregularly arranged, in transverse section cells with single evenly distributed chloroplast, oblong or rectangular, up to 14 to 26µm long and 10 to 15µm broad, thallus thickness up to 40µm.

Reproduction: Dioecious, spermatangia pale yellow and indistinct, arranged in groups of eight in surface view, each spermatangium dividing into four spermatia (division formula a/4, b/2, c/4 tiers), spermatangial thallus 36 to 40µm thick, zygotosporangia pale-red to maroon, arranged in pairs, each zygotosporangium dividing into four zygotospores (division formula a/2, b/2, c/4 tiers), zygotosporangial thallus thickness 40 - 46µm.
Figure 1. *Porphyra ramiculata*: a) holotype, b) surface view of vegetative area, c) vegetative cells in cross section, d) zygotosporangial region in surface view and e) cross section of zygotosporangia.

Remarks:

The ramuliferous nature of this species distinguishes it from all known *Porphyra* species. Although the vegetative cells are similar to those of *P. saldanhae*, the reproduction division formula makes this species different from other local species. Griffin (2003) and Jones *et al.* (2004) also sequenced the nSSU rDNA region of this species, recorded as entities ZPP956 and ZGR 903 respectively, and revealed that this species was different from the other taxa within the Cape cluster of South Africa although bootstrap support was weak. As a result, this species is regarded as a new species and the name *Porphyra ramiculata* is proposed.
(b) *Porphyra aurifolia* Dlaza, Bolton et Anderson sp. nov.

Holotype: TSD SPTP8O (Figure 2a).

Type locality: Seapoint beach, Cape Town, South Africa, Dlaza, November 2007.

Etymology: Species name based on its distinctive golden-yellow color in the wild.

Habitat and seasonality: At the type locality the gametophyte phase was collected in spring through summer growing epilithically in the upper littoral zone.

Distribution: South African endemic restricted to the Cape Peninsula

Habit and vegetative structure: Fronds monostromatic, single or more blades, seldom branched but deeply lacinete to produce multiple lobes; attachment by an inconspicuous holdfast; blades lanceolate, obovate, cordate or irregular, up to 40 cm long; color shiny gold to yellow-green when alive then becoming yellowish-gold on drying; texture firm and rigid; firmly adherent to paper. Margins undulate; in surface view vegetative cells elliptic and arranged in pairs with each cell having a central chloroplast, each cell up to 20 µm long and up to 12 µm broad, in transverse view cells showing two chloroplasts and no visible pyrenoids, narrow and oblong, up to 60 µm long and 15 - 20 µm broad, thallus thickness on average 70 µm but can be up to 90 µm thick.

Reproduction: Dioecious, spermatangia pale yellow, arranged in groups of eight in surface view, in transverse view each spermatangium divides into eight spermatia (division formula a/4, b/2, c/8 tiers), zygotosporangia dark gold to orange-red, arranged in groups of eight in surface view, each zygotosporangium dividing into four zygotospores (division formula a/4, b/2, c/4 tiers), average thallus thickness 80 µm in both spermatangial and zygotosporangial areas.
This species differs from the other known species in its bright golden-yellow colour, reproduction division formulae, and the deeply laciniate nature of the thalli. Furthermore, specimens of this species were only found in Seapoint, Oudekraal and Llandudno suggesting that this species maybe restricted to the west coast of the Cape Peninsula.
(c) *Porphyra gravesiae* Dlaza, Bolton et Anderson sp. nov.

Holotype: TSD KOMP21 (Figure 3).

Type locality: Kommetjie, Cape Peninsula, South Africa, Dlaza, 2007.

Etymology: Named in honour of Judith Graves for her contribution to the study of South African *Porphyra* genus.

Habitat and seasonality: Plants abundant in summer, epilithic, in isolated clusters on rocks in the upper intertidal zone.

Distribution: A South African endemic

Habit and vegetative structure: Blades monostromatic, unbranched but laciniate; attachment by discoid holdfast; blades broadly-lanceolate, umbilicate or obovate, up to 25cm long; color pale red to reddish-brown with a haze of yellow in the wild becoming purple in color when dry; texture membranous and fragile; firmly adherent to paper. Margins entire and smooth; surface vegetative cells elliptical and arranged in pairs with each cell having a central chloroplast, in transverse section cells with one stellate chloroplast, elongate with somewhat rounded tips, cells up to four times as long as broad, 24 - 60µm long and 6 - 20µm broad, vegetative thallus thickness frequently about 60µm but may sometimes be up to 80µm thick.

Reproduction: Dioecious; reproductive cells formed by splitting of vegetative cell chloroplast into two and formation of cell-plate; spermatangia bright yellow, arranged in groups of eight in surface view, each spermatangium dividing into four spermatia (division formula a/4, b/2, c/4 tiers), thallus 40 - 70µm thick; zygotosporangia dark-red and indistinct, arranged in groups of four, each zygotosporangium dividing into four zygotes (division formula a/2, b/2, c/4 tiers), thallus thickness 60µm.
Figure 3. *Porphyra gravesiae*: a – f) Morphological variation, g) surface view of vegetative cells, h) vegetative cells in cross section, i) surface view of zygotosporangial region and, j) cross section of actively dividing zygotosporangia

Specimens of this species were first described by Graves (1969) who categorized them under the delicate form of *P. capensis* characteristic of the relatively warm waters of False Bay and of the south and east coasts. She also alluded to the thin thalli of this form resulting in them being easily torn and often deeply laciniate. Although she regarded the single chloroplast in these specimens as a reflection of immaturity in *P. capensis*, we found mature reproductive individuals of this species throughout the False Bay area. The presence of the single
chloroplast, coupled with the membranous nature, of this species distinguishes it from the other South African species.

Although this species is similar to *P. moriensis* in being membranous and having a single chloroplast, they differ in that *P. moriensis* is either monoecious or androdioecious (Ohmi 1954) while *P. gravesiae* is strictly dioecious. *Porphyra gravesiae* also differs to the delicate and membranous *P. endiviifolium* in that the chloroplast is stellate and in the centre of the cell in *P. gravesiae*, while the chloroplast is aligned along the sides of the cells in *P. endiviifolium* (Wiencke & Clayton 1998). The single chloroplast coupled with blade colour and blade margins, which make this species similar to *P. tenera*, are overshadowed by differences in reproduction between these species. *Porphyra tenera* is monoecious (Niwa et al. 2005a), with spermatangial division formula (a/4, b/4, c/4) and zygotosporangial formula (a/2, b/1, c/2). Another membranous species, *P. leucosticta* differs from *P. gravesiae* in that the vegetative cells of the latter are rectangular and 1.5 to 2 times as long as they are broad (Taylor 1962) while the cells are longer and more oval in *P. gravesiae*.

(d) *Porphyra chameleona* Dlaza, Bolton et Anderson sp. nov.

Holotype: TSD KOMPO15 (Figure 4).

Type locality: Kommetjie, Cape Peninsula, South Africa, Dlaza, 2007.

Etymology: Named for its unpredictable substrate, branching and reproduction nature; after the changeable chameleon.

Habitat and seasonality: Plants collected in spring through summer, epizoic on limpets and mussels, epilithic in mid to lower intertidal zone.

Distribution: A South African endemic
Habit and vegetative structure: Blades monostromatic, branching by tearing of single blade to produce multiple lobes; attachment by a discoid holdfast; blades linear-lanceolate, umbilicate or cordate, up to 30cm long; color olive-green basally becoming brownish-red towards apex when alive and retaining color even when pressed; texture rigid; firmly adherent to paper on drying then becoming rubbery. Margins entire and smooth; in surface view the vegetative cells randomly arranged, in transverse view cells with two opposite chloroplasts and no visible pyrenoids, elongate to oval, up to 60µm long and 24µm broad, thallus thickness 90µm in midsection.

Reproduction: Mainly androdioecious but sometimes dioecious; spermatangia yellowish and irregularly-shaped, arranged in groups of eight in surface view, each spermatangium dividing into eight spermatia (division formula a/4, b/2, c/8 tiers), thallus 60 - 70µm thick; zygotosporangia dark-red, arranged in groups of four, each zygotosporangium dividing into four zygotospores (division formula a/2, b/2 c/4 tiers), thallus thickness 60µm.

Figure 4. *Porphyra chameleona*: a – c) gametophyte morphological variation, d) surface view of vegetative cells, e) vegetative cells in cross section, f) surface view showing smooth separation of zygotosporangial region from spermatangial region and, g) spermatangia in cross section.
At first glance, *P. chameleona* can be easily confused with *P. capensis* in the wild, since the smaller vegetative plants of these species may look similar in color and shape. Furthermore, these two species occupy the same tidal zone with the dioecious reproductive female plants looking similar on first impression. As a result, Isaac (1957, Fig. 2) described blades of this species as *P. capensis* with a peculiar type of monoecious reproduction whereby the carpospores and spermatia are produced in different sectors of the thallus.


However, in all these species the male part is separated from the female part by a straight line halving the plant into two distinct halves while in *P. chameleona* the separation may sometimes be restricted to a small portion of the margins only. Furthermore, blades of *P. chameleona* have been found containing an androdioecious blade and strictly male or female branches. The branching by tearing observed in *P. chameleona* also distinguishes it from all these species.

*Porphyra chameleona* also differs from the above-mentioned species in thallus thickness, transverse cell chloroplasts, substrate, and reproduction division formula. From the
descriptions of Miura (1968) and Neefus et al. (2008) of *P. katadae*, differences between that species and *P. chameleona* are that the vegetative thallus of *P. chameleona* is almost twice as thick as that of *P. katadae* and *P. chameleona* cells have two chloroplasts while *P. katadae* cells have a single stellate chloroplast. The vegetative thallus thickness of *P. moriensis*, 20 - 53µm, is also smaller than that of *P. chameleona* and the former species has a single chloroplast per cell (Ohmi 1954). *Porphyra chameleona* differs from *P. variegata* in that the latter has a distromatic thallus (Kurogi 1977).

There are differences in substrate between *P. chameleona* and some of the species, which are epiphytic on other algae. *Porphyra moriensis* is an epiphyte on *Chorda filum* (Ohmi 1954; Notoya & Miyashita 1999), *P. katadae* is epiphytic on red and brown seaweeds (Miura 1968), and *P. yezoensis* is epiphytic on other algae (Neefus et al. 2008).

The spermatangial division formula for *P. chameleona* is 4x2x8 tiers and the zygotosporangial formula is 2x2x4 tiers, while in *P. katadae* the spermatangial formula is 4x2(or 4)x4 tiers and the zygotosporangial formula is 2x2x2 tiers). In *P. moriensis* the spermatangial division formula is 4x4x8 tiers and the zygotosporangial formula is 2x2x2 tiers: these are different from those of *P. chameleona*. *Porphyra variegata* has a different but inconsistent reproduction formula, with zygotospores in 8 to 32 tiers and spermatia in 16 to 64 tiers (Kurogi 1977). Although the spermatia division formula of *P. chameleona* is similar to that of *P. purpurea*, the presence of trichogynes and the arrangement of zygotosporangia in *P. purpurea* distinguish it from *P. chameleona* which has no visible trichogynes and fewer sori.

Of all the species, *P. chameleona* is most similar to *P. purpurea* in vegetative and spermatangial morphology. Furthermore, *P. purpurea* has a cosmopolitan distribution from
Asia, Europe, North America, to Australia and New Zealand (Yoshida et al. 1990; Silva et al. 1996; Bates et al. 2005; Bárbara et al. 2005). However, it has never been recorded in Southern Africa and Jones et al. (2004) revealed that most of the South African taxa formed distinct clusters endemic to this region. Furthermore, the name *P. purpurea* has frequently been misapplied (Brodie et al. 1996; Brodie & Irvine 1997; Guiry 1997; Lindstrom & Fredericq 2003) raising suspicion that more than one taxon has been confused under this name (Neefus et al. 2002). As a result, the name *Porphyra purpurea* may not be appropriate for the current species and this species was therefore regarded as a new species, pending molecular data, and the name *Porphyra chameleona* is proposed.
8.4. Identification key for the gametophyte phase of South African *Porphyra* species

1a. Fronds yellowish-green to yellowish-gold when fresh, pale yellow when pressed ..............2
1b. Fronds olive-green or dark-red when fresh, brownish or retaining color when pressed ......3
   2a. Multiple blades with entire margins and ramuli .................................................. *P. ramiculata*
   2b. Blades with undulated margins and laciniate ...................................................... *P. aurifolia*
3a. Texture fragile with vegetative cell having a single stellate chloroplast .............. *P. gravesiae*
3b. Firm texture with single or more vegetative cell chloroplasts .....................................4
   4a. Epiphytic thalli ........................................................................................................5
   4b. Epilithic and/or epizoic thalli ................................................................................7
5a. Epiphytic on *Aeodis orbitosa* .............................................................................. *P. aeodis*
5b. Epiphytic on kelp and other large brown algae .........................................................6
   6a. Presence of recognizable trichogyne in carpogonia .............................................. *P. gardneri*
   6b. Carpogonia without trichogyne ............................................................................ *P. sp. indet.*
7a. Strictly dioecious reproduction ................................................................................ *P. capensis*
7b. Reproduction androdioecious or monoecious .........................................................8
   8a. Androdioecious with smooth margins ................................................................. *P. chameleona*
   8b. Blades strictly monoecious .................................................................................... *P. suborbiculata*
   9a. Blades with undulated margins .............................................................................. *P. saldanhae*
   9b. Smooth margins .................................................................................................... *P. suborbiculata*
Chapter 9

General discussion
9.1. Life history of the three local *Porphyra* species

The life history of the three South African *Porphyra* species was typical of the genus, with a conspicuous foliose gametophyte phase alternating with a filamentous conchocelis phase. Although various spores were released from the thalli of these species, no *blade archeospores, agamospores, neutral spores, phyllospores* or *endospores* were recorded. The reason for the absence of these spores is not known although the absence of blade archeospores could be as a result of using mature reproductive blades instead of vegetative plants in this study. Notoya (1999) and Notoya & Iijima (2003) found that archeospores were easily released from vegetative cells of young thalli compared to mature thalli. However, the presence of protothalli distinguished *P. aeodis* from the other two South African species and most other *Porphyra* species worldwide. The simultaneous germination of both conchocelis and protothalli directly from the spores of *P. aeodis* was recorded for the first time in this genus. Although the tendency for the three species to release spores of different sizes was similar to other *Porphyra* species worldwide, the characteristics of the spore chromatophores were unique to these three species. The characteristics of the microscopic phase (*zygotospores, conchocelis, conchosporangia, conchospires and protothalli*) of these three species add valuable information on the biology of these species and contribute to resolving the taxonomy of South African *Porphyra*.

The release of spores from thalli of *P. aeodis* was affected more by prolonged desiccation period compared to both *P. capensis* and *P. saldaneae*. *Porphyra capensis* released more spores than the other two species, although the fertile area of both *P. aeodis* and *P. saldaneae* was frequently higher than that of *P. capensis*. This was due to *P. capensis* being dioecious, thus having sporangia that are closely arranged, while both *P. aeodis* and *P. saldaneae* are monoecious thus having patches of male and female sori along the reproductive margin. Long photoperiods and increased temperatures resulted in faster conchocelis growth rates for all
three species. Conchosporangial development was enhanced by long photoperiod and increased temperatures for *P. capensis*, while conchosporangial development was enhanced by increased temperatures and shorter photoperiods for *P. aeodis* and *P. saldanhae*. No distinct photoperiodic control was recorded for the release of conchospores in *P. capensis* while short days were important in the release of conchospores for both *P. aeodis* and *P. saldanhae*. No blades germinated under extremely low temperatures (5°C) irrespective of the photoperiod or species.

### 9.2. Potential for South African *Porphyra* as food for humans

In the classification scheme of Kornmann (1994), the three species fall within the type 2 life history, with thick or moderately thick thalli. These species were found to be thicker than *P. tenera* and *P. yezoensis* (Table 1), which are species currently used commercially for nori production. Although the thallus of *P. saldanhae* is thicker than both *P. tenera* and *P. yezoensis*, the cells of this species are very short with cell length similar to that of both *P. tenera* and *P. yezoensis*. Based on thallus thickness and cell length alone, *P. saldanhae* maybe a suitable candidate for nori production in South Africa.
Table 1. Comparison of three South African *Porphyra* species with species commercially cultivated for nori production.

<table>
<thead>
<tr>
<th>Species</th>
<th>Size (maximum)</th>
<th>Blade</th>
<th>Color</th>
<th>Texture</th>
<th>Thallus</th>
<th>Reproduction</th>
<th>Seasonality</th>
<th>Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. tenera</em> Kjellman (1897)</td>
<td>Length 17cm Width 11cm</td>
<td>Ovate - linear-lanceolate</td>
<td>Reddish purple – bluish green</td>
<td>Membranous &amp; delicate</td>
<td>Monostromatic 14 - 35µm cells 19 - 27 µm</td>
<td>Monoecious</td>
<td>Seasonal</td>
<td>Epilithic</td>
</tr>
<tr>
<td><em>P. yezoensis</em> Ueda (1932)</td>
<td>Length 100cm Width 13cm</td>
<td>Linear - to-oblanceolate</td>
<td>Bright purple – dark purplish</td>
<td>Membranous</td>
<td>Monostromatic 25 - 53µm cells 26 - 35 µm</td>
<td>Monoecious</td>
<td>Seasonal</td>
<td>Epilithic</td>
</tr>
<tr>
<td><em>P. aeodis</em> Griffin <em>et al.</em> (1999)</td>
<td>Length 22cm Width 28cm</td>
<td>Cordiform -to-umbilicate</td>
<td>Red brown – olive green</td>
<td>Rigid</td>
<td>Monostromatic 60 - 140µm Cells 20 - 35 µm</td>
<td>Monoecious</td>
<td>Seasonal</td>
<td>Epiphytic</td>
</tr>
<tr>
<td><em>P. capensis</em> Kützing (1843)</td>
<td>Length 50cm Width 59cm</td>
<td>Elongate -to-Umbilicate</td>
<td>Red – olive green</td>
<td>Rigid membranous</td>
<td>Monostromatic 100 - 150µm Cells 68 - 90 µm</td>
<td>Dioecious</td>
<td>All-year</td>
<td>Epilithic</td>
</tr>
<tr>
<td><em>P. saldanhae</em> Stegenga <em>et al.</em> (1997)</td>
<td>Length 41cm Width 35cm</td>
<td>Linear – to-lanceolate</td>
<td>Reddish purple - dark purple</td>
<td>Membranous</td>
<td>Monostromatic 60 – 100µm cells 25 - 40µm</td>
<td>Monoecious</td>
<td>All-year</td>
<td>Epilithic</td>
</tr>
</tbody>
</table>

Although seaweed consumption is mainly restricted to the Asian countries, the demand for seaweed as food has increased in western countries. Molloy (1990) in Namibia, and later González & Santelices (2003) in Chile, suggested *P. capensis* as a potential seaweed species to use for human consumption due to its abundance and surface area as well as the dark coloration. However, for nori production Noda and Iwata (1978) mentioned four types (black, unclassified, mixed and green) and seven grades (Superior, Select, First, Second, Third, Fourth and Fifth) based on criteria such as color, luster, softness as well as aroma and flavour. For superior quality nori the color must be black tinged with green, while the lustre must be shiny after the drying process. Superior quality nori must also be very soft and literally melt when placed in the mouth and must be of superior flavour (Amano and Noda 1987). Based on colour alone, *P. capensis* would be the most ideal species to use in nori production since it was found to the darkest of the three species. However, based on toughness this species would not be ideal since it was the thickest (Table 1). Although *P.*
saldanhae had undulated margins, which might affect its finishing since flat and smooth finishing is important (Amano and Noda 1987), it could be suitable to make the first-grade nori. Visually this species has a transparent midsection and this, coupled with the length of the cells (Table 1), makes this species soft enough to produce nori. The high nutritional value of P. saldanhae (especially the carbon, potassium and protein) makes this species more suitable for nori production, compared to the other two species.

All three species were found to contain more macro- and microelements than other edible seaweeds and would therefore contribute more to the mineral requirements of human beings. They were also found to have more protein, macro- and microelements than many vegetables and could thus be incorporated to improve the nutritional value of peoples’ diets. For small-scale nori production, Porphyra is processed by only drying in the sun (Mumford 1986) and this preserves the nutrient content compared to other forms of drying. For example, Chan et al. (1997) found that although oven-drying and freeze-drying increased the total amino acid and mineral composition of Sargassum hemiphyllum, these drying methods reduced the crude protein of this species compared to sun-drying. They also found that oven-drying and freeze-drying significantly increased the aluminium, zinc and iron contents and this could be toxic to the human body and cause illness.

Since Porphyra requires minimal processing (Almela et al. 2006), it is recommended over processed vegetables because the thermal conditions experienced during commercial processing of vegetables have been found to result in nutritional losses. Nutritional losses are incurred through leaching during blanching operations, washing, peeling and cutting of vegetables such as asparagus (Zurera & Morino 1990). For example, canning was found to cause up to 88g/kg wet weight Vitamin C (ascorbic acid) loss in carrots (Howard et al. 1999)
while Murcia et al. (2000) recorded vitamin C losses of up to 84g/kg wet weight in canned broccoli. Amaro López et al. (1999) also found that processing (i.e. washing, peeling and blanching) reduced the iron and manganese content of asparagus while increasing its copper and zinc content. Canning has also been found to decrease the protein content of asparagus, tomatoes and mushrooms due to removal of stem in mushroom and peel in asparagus and tomato (Martín-Beloso & Llanos-Barriobero 2001).

Since nori is consumed raw, Porphyra does not undergo any nutritional losses incurred by other vegetables during the cooking process, since cooking has been found to reduce the nutritional value of vegetables (Rickman et al. 2007). Wills et al. (1984) found that the green beans lost up to 60% (potassium) and up to 29% (calcium) while green peas lost up to 50(sodium), 44% (potassium) and 42% (calcium) during cooking. Yadav and Sehgal (1995) also found that cooking with open pan reduced the calcium content of vegetables by up to 36.4% (spinach) and 30% (amaranth) while cooking with a pressure cooker resulted in losses of up to 34.1% (spinach) and 33.3% (amaranth).

Besides the nutritional properties, Porphyra consumption also has other health benefits. For example, the consumption of calcium-rich diets depresses magnesium absorption, resulting in insoluble magnesium thus reducing magnesium going into the bones (Cohen 1988). Since deaths due to coronary heart disease and ischemic heart disease have been observed to correlate with the dietary calcium: magnesium ratio (Seeling & Heggtveit 1974), the recommended dietary calcium-to-magnesium ratio to lower heart diseases has been set at 2–4:1 (Kikunaga et al. 1999). The low mean Ca:Mg ratios of P. aeodis (0.46±0.04), P. capensis (0.58±0.05) and P. salданхæ (0.55±0.09) may help in balancing the Ca:Mg ratios in human diets and subsequently reduce heart-related deaths. These three species also had Na:K ratios
that were lower than 1.5 (Chapter 6) and this would further help to reduce risks of cardiovascular arrests, especially in the elderly. It therefore recommended that any of the three species be incorporated in the diet to increase the magnesium content in the body.

9.3. South African *Porphyra* as potential feed for abalone

In abalone nutrition, *P. saldanhae* would be recommended as fresh blades while *P. capensis* would be recommended as an ingredient incorporated into the formulated diets. This was because *P. capensis* had more macro- and microelements and would thus contribute more in meeting the mineral requirements of abalone. The higher calcium/phosphorus ratio of *P. saldanhae* (0.28 – 1.125), compared to *P. aeodis* (0.192–0.743) and *P. capensis* (0.16–0.75), makes this seaweed much better for juvenile abalone growth. This is because although Coote *et al.* (1996) and Tan *et al.* (2001) could not determine an optimal calcium/phosphorus ratio for *H. laevigata* and *H. discus hannai*, they respectively found that satisfactory growth occurred at calcium/phosphorus ratios of 0.1–9.0:1 for *H. laevigata* and 0.72–2.68:1 for *H. discus hannai* respectively. Furthermore, *P. saldanhae* was found to have more protein content which is essential for juvenile abalone growth.

In contrast, *P. capensis* would be more suitable for augmenting mineral contents of formulated abalone feeds because this species had the highest P content and Tan *et al.* (2001) recommended that 1.25% of total phosphorus be incorporated in formulated diets of juvenile *Haliotis discus hannai* in order to get maximum growth rates in terms of daily increment in shell length (DISL) and weight gain rate (WGR). Although most aquatic animal species have no dietary calcium requirements, since animals such as molluscs can absorb calcium directly from the surrounding water through drinking, calcium is important for shell mineralization (Coote *et al.* 1996). *Porphyra capensis* would also contribute significantly to shell formation.
of juvenile abalone since zinc is essential for growth and shell biomineralization in abalone (Liao et al. 2002, 2004; Mai et al. 2003) and a high Zn concentration has been recorded in juvenile *H. discus* and *H. discus hannai* compared to older abalone of these species (Arai et al. 2002). The significantly high Zn content of *P. capensis* (6.63±0.49 µg g⁻¹), compared to *P. aeodis* (3.87±0.68 µg g⁻¹) and *P. saldanhae* (3.64±0.47 µg g⁻¹), would contribute more in meeting the theoretical metabolic requirement for Zinc (9 µg g⁻¹) set by White and Rainbow (1985) in molluscs.

Although not tested directly, the nutritional composition of these three *Porphyra* species indicates that they would be valuable supplementary diets for the farmed abalone *Haliotis midae*. When compared to the *Ulva* genus, which is currently used on a number of farms as supplementary diet (Bolton et al. 2008), the protein of these three species was higher. For example, preliminary results (Shuuluka in prep.) showed that the crude protein content of these three *Porphyra* species (average 27% for *P. aeodis*, 30% for *P. capensis* and 32% for *P. saldanhae*) was higher than that of the *Ulva* species currently cultivated, and used for abalone feed, in South African farms. The protein content of these three *Porphyra* species was higher than the results of Fujiwara-Arasaki et al. (1984) who found 20 to 26% crude protein in cultivated *U. pertusa* while Jadeja and Tewari (2008) found that *U. fasciata* in polluted areas had low protein contents ranging from 13.65±0.19 to 18.55±0.13% dry weight.

### 9.4. Conclusions

This study demonstrated for the first time that *P. capensis* and *P. saldanhae* are reproductive and occur throughout the year, while the results for *P. aeodis* corroborated those of Griffin et al. (1999c) showing this species as occurring seasonally. Based on the abundance of larger individuals of the three species in summer, their reproductive phenology and their life
history, it can thus be hypothesized that, in all three species, conchosporangial development might occur mainly during the autumn when the water temperatures are rising and there are still long-day photoperiods in the wild. For *P. capensis*, conchospore release might be expected to occur mostly in winter when water temperatures are highest while in *P. aeodis* and *P. saldanhae* more conchospore release is likely to occur towards the end of winter, triggered mainly by the break of winter short-days into spring long-day photoperiods. This study therefore provides a good basis for the cultivation of the three *Porphyra* species.

The high mineral content of the three species makes them valuable supplementary feeds in the diet of the farmed South African abalone, *Haliotis midae*. Since the nutritional study comparing crude protein to soluble protein (Bradford method) showed that there was no correlation between the crude and soluble protein, it can also be concluded that the use of the 6.25 conversion factor is not appropriate for determining protein content of the three *Porphyra* species.

There is high morphological plasticity within each of the three South African *Porphyra* species, making it extremely difficult to delineate species based solely on morphological characters. Comprehensive collections at different times of the year revealed that there were more potential new species worth describing, and this thesis contributed to the taxonomic knowledge of South African *Porphyra* species.

**9.5. Further studies**

It would be worthwhile to study the amino acid profile of the three species (*P. aeodis*, *P. capensis* and *P. saldanhae*) so that the most appropriate protein conversion factor could be derived for each of these species. Knowing the amino acid profile of these three species
would also help in quantifying the appropriate amount of each species to be included should any of them be incorporated in formulating pellet feeds for the South African abalone.

It would also be important to analyze for biochemical constituents such as carbohydrates, energy and dietary fiber in these species. The energy content of these three species is important, since the effectiveness of any abalone feed is determined by the energy-to-protein ratio of that particular feed. Knowing the dietary fiber (i.e. polysaccharides, oligosaccharides and lignin) content of these species would help to explore the possible physiological benefits in laxation and blood cholesterol or glucose attenuation in humans. This is very important in human nutrition, since dietary fiber can bind toxic compounds and thus eliminate their mobility in the human body (Mišurcova et al. 2010)

Since there are no documented records of the amount and frequency of *Porphyra* consumption in South Africa, by humans, it would be important to determine the tolerable concentrations (TC, measured in mg/kg of that particular *Porphyra* species) through conducting trials and applying the following formula adapted from Belitz et al. (2009):

\[ TC = \frac{NOAEL \times FV}{SF} \times \frac{BW}{CA \times ASF} \]

Where NOAEL = no observed adverse effect level (mg/kg *Porphyra*), FV = daily intake of feed by test animal (kg *Porphyra*/ kg body weight), SF = safety factor (10 – 2000, but usually 100), BW = body weight of an adult (50 – 80kg), CA = amount in kg consumed per day of *Porphyra* species for which the TC is being calculated, ASF = additional safety factor for particularly sensitive persons such as children or the sick (the accepted ASF is 2.5).

It would also be important to complete the life history, as well as the nutritional analysis, of the proposed four new species so as to compare them with the other known local species. A molecular study of these proposed new species would be essential to resolve their taxonomy.
For further studies, the potential to cultivate the gametophyte phase of the three cultured species in the sea, using the methods similar to those used to cultivate nori in Asia, should be examined. This would help increase the biomass of the individual species, thus allowing for *Porphyra* species-specific feeding trials on the farmed abalone in South Africa.
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Porphyra in the world, with special reference to the type locality and bibliography.

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