Analysis of protein content of two kelps, *Ecklonia maxima* and *Laminaria pallida*, for feed in abalone aquaculture

By Cherie J. Forbes[1],
Supervisor: Prof. John Bolton[1,2]

[1]Department of Botany, University of Cape Town, South Africa

[2]Marine Reseach Institute (MA-RE), University of Cape Town, South Africa

A dissertation submitted to the University of Cape Town, in partial fulfilment of the requirements for the reward of an honours degree in Botany.

October 2009

Abstract:

Kelp is widely used as feed in the abalone aquaculture industry in South Africa, and farmers have reported different feed quality between the two dominant kelp species occurring along the west and southwest coasts with *Ecklonia maxima* reported as having a better Food Conversion Ratio (FCR) than *Laminaria pallida*. Total protein content and other nutritional components (Carbon, Nitrogen and moisture content) of two kelp species, *E. maxima* and *L. pallida* were investigated. The kelps were collected from Kommetjie (borderline of west and southwest coasts). Seasonal total protein content of *E.maxima* from a commercially-harvested kelp bed in Jacobsbaai (west coast) was compared with crude protein levels (measured as %N x 6.25). Total protein was extracted and quantified using the Bradford method. No significant difference in carbon content was found between seasons for *E. maxima* but there was a significant difference in average carbon content between *E. maxima* (30.79 %) and *L. pallida* (27.79 %). No seasonal pattern was observed in the nitrogen content, and hence crude protein content, of *E. maxima* (seasonal crude protein averaged 7.8% of dry weight) and there was no significant difference found between *E. maxima* and *L. pallida* (crude protein averaged 9.05% of dry weight). There was no significant seasonal difference in total protein (averaged 0.99% of dry weight) and no significance found between species. The reported better FCR in abalone feed comprised of *E. maxima* rather than *L. pallida*, if correct, is likely to be related to higher carbohydrate content (measured here as carbon) and not to higher protein content. *E.maxima* differs from *L. pallida* with regards to carbon storage, but not protein content.

Keywords: Bradford method, crude protein, total protein, kelp, *Ecklonia, Laminaria*

Introduction:

Kelp

Seaweeds distributed along rocky shores play a fundamental role in the high productivity of coastal ecosystems. In temperate regions brown algae in the order Laminariales (commonly known as kelps) dominate the coastal regions and contribute significantly to the marine ecosystem by providing shelter and food for other marine organisms especially invertebrates and fishes (Gevaert et al. 2008). In South Africa, currently more than 7000 tons of fresh kelp fronds are harvested annually to feed cultured abalone and this figure is expected to increase as the demand for kelp by local abalone farmers also increases (Anderson et al. 2006). *Ecklonia maxima* is the most abundant algal species occurring along the cool temperate west coast of South Africa. It forms large kelp beds between Cape Agulhas (its southern limit) and Cape Columbine but it extends far north near Lüderitz (Namibia). *E.maxima* extends further east into warmer waters than *Laminaria pallida* but *L.pallida* dominates the rocky subtidal even further north than *E.maxima,* as it reaches as far as between Rocky Point and Cape Fria in northern Namibia (Stegenga et al. 1997). Abalone farms developing along the southwest coast (in Gansbaai or Hermanus) utilize *E.maxima* more than *L. pallida*, since *L.pallida* is rare in this region. However, along the northern west coast of South Africa and in Namibia abalone farms are found to replace the use of *E. maxima* with *L. pallida* because most of the kelp found there is *L. pallida* (Stegenga et al. 1997). North of Yzerfontein on the west coast, *Laminaria* dominates inshore, such that the area where the west coast abalone farms occur, there is much more *Laminaria* available than *Ecklonia* (Bolton, pers.comm). Since the early 1950's, kelp has been collected as beach-cast for alginate production (Anderson et al 1989), especially during the winter months, when storms cause kelp to wash ashore and thus beach-cast kelp is abundant (Rothman et al. 2006) or harvested since the 1970's for the production of plant growth stimulant. But since the 1990's kelp has been harvested all year round (with very little kelp collected as beach-cast) and delivered fresh to land-based abalone farms as feed.

Carbon, Nitrogen and Protein

Lapointe and Duke (1984) performed a study on the growth of the red seaweed *Gracilaria tikvahiae* limited by light and Nitrogen and as a result, a parabolic function of the C:N ratio ?

was established. Light and N were found to have opposite effects on the relationship between the C:N ratio and the growth rate (Lapointe and Duke (1984). The C:N ratio is low when light is limited since nitrogen uptake takes place at a relatively faster rate compared to that of carbon fixation and this results in the accumulation of N reserves. Alternatively, when N is more limited than light the C:N ratio is high since carbon fixation is relatively faster than nitrogen uptake (Lobban and Harrison. 1994; Henley and Dunton, 1997). Previous studies have investigated the carbon and nitrogen contents of *Laminaria* (Diekmann. 1978,1980; Gevaert et al. 2008) and *Ecklonia* (Von Holdt et al. 1955; Smith, 2008) but no direct investigations have been carried out on the total protein content of these species. Generally, protein is measured in the nutrition industry by nitrogen-to-protein conversion factors (N-Prot factors), more commonly known as "crude protein", and this value is obtained by multiplying the nitrogen content (%N) by 6.25. The N-Prot factor of 6.25 is based on the assumption that that the samples contain protein with 16% nitrogen and an insignificant amount of non-protein nitrogen. Due to these assumptions, the conversion factor 6.25 is a general industry figure which faces high scrutiny since algae commonly has high concentrations of non-protein nitrogenous substances such as pigments (chlorophyll and phycoerythrin), nucleic acids, free amino acids and inorganic nitrogen (nitrate, nitrite and ammonia) and if these non-protein substances are present, it makes the factor 6.25 unsuitable since it overestimates the true total protein content (Lourenco et al. 2002; Barbarino and Lourenco. 2005). Recent research done by Lourenco et al. (2002) has established the specific N-Prot factors of 19 seaweed species and these conversion factors differed for reds, greens and browns.

The protein content of seaweeds has been found to differ according to the species and seasonal conditions (Fleurence. 1999) and the crude protein content of kelp, specifically, is low (i.e. 10% protein on the basis of dry weight reported by Troell et al. 2006 and 11.13% reported by Smith et al. submitted 2009). Protein is an essential but expensive component in a diet. If abalone are to be cultured intensively using formulated feeds, this may result in increased production costs. Therefore, the expensive protein fraction should be used most favourably for growth rather than be used for energy by the abalone. Knowledge of the optimum level of protein and the effects of non-protein nutrients (such as carbohydrates) is necessary for the formulation of cost-effective feeds (Bautista-Teruel and Millamena. 1999).

Abalone and Feed

Since the 1990's there has been a rapid development in the cultivation of abalone in South Africa (Troell et al.2006), driven by a decline in yields from wild fisheries as a result of over-exploitation of wild abalone stocks, South Africa has become the largest abalone producer outside Asia (FAO, 2004). South African abalone, *Haliotis midae* Linn., is highly sought-after in traditional cuisine in the Far East, and as a result about 90% of the product from our local fishery is exported there (Britz et al., 1994). Abalone growth is slow and varies with size and age (Britz, 1996a). In the wild, at an age of over 30 years, *H. midae* can reach a maximum size of approximately 200mm shell length. On the other hand, farm production is aimed towards an average size of only 100 mm, which is currently achieved after 5 years (Sales & Britz, 2001). This is because there is a high demand for small, cocktail size abalone of 40-70mm shell length in the international market (Jarayabhand and Paphavasit 1996; Najmudeen and Victor 2004). Immediately after larval settlement, abalone begin to feed on benthic diatoms and as they grow, they begin to feed on macroalgae (Stepto & Cook, 1993). *E. maxima* and *L.pallida* are the most common natural diet of the commercially exploited South African abalone, and since kelp is a major component of feed in local abalone farms, the expansion of the industry has led to a great dependence on wild kelp stocks (Anderson et al. 2006).

Studies reviewed by Uki and Watanabe (1992) investigated the protein, fatty-acids and mineral requirements of abalone. Later some work was published about the digestibility of artificial diets (Dixon. 1992) but not much information existed on what feed components were most suitable for the incorporation in abalone diets (Britz, 1996b). Seaweed as a feed is either not readily available (due to seasonality) or insufficient (since kelp is low in protein) to sustain the current commercial production of abalone *H. midae* in South Africa (Viera *et al.* 2005; Anderson *et al.* 2006; Troell *et al.* 2006). These two aspects have encouraged research into the manufacturing and use of various commercial formulated feeds (Dlaza et al. 2008; Francis et al. 2008). The most widely used formulated feed in South Africa is called Abfeed® (Marifeed Pty Ltd, South Africa) (Troell *et al.* 2006) and it nearly meets all the nutritional requirements of *H. midae*. Britz (1996a, 1996b) showed that Abfeed® (34.6%protein), significantly improves growth over other feeds but Naidoo et al (2006) investigated the

effects various seaweed-based diets and formulated feed had on the growth rate of abalone in a land-based aquaculture system. It was found that abalone fed Abfeed grew better than those that were fed the dried seaweed feeds, but poorer than the mixed fresh seaweed (*Ulva lactuca*, *Gracilaria gracilis* and kelp) diet. In general, wild abalone feed on a broad selection of algae and at least two species are found in the gut at any given time (Barkai & Griffiths, 1986). This implies that abalone typically select more than just a single species and preferentially choose a mixture of algae. Naidoo *et al.* (2006) concluded that supplementing fresh seaweed combinations (particularly if the seaweeds in the diet are grown in animal aquaculture effluent and are therefore protein-enriched) into the diet of *H. midae* may drastically improve growth even more than formulated feeds on their own. For a number of reasons, abalone farmers may choose to feed abalone, >50mm in shell length either kelp, a combination of kelp and Abfeed or only the traditional Abfeed formulation (Troell et al. 2006). The relative motives for this are as follows: Although Abfeed has high protein (34%) which increases growth rates, whereas kelp has a higher Food Conversion Ratio (FCR) which means more kelp is required to produce comparable growth, kelp is cheaper than Abfeed (Francis et al. 2008) (ca. R1000 per ton wet). High-protein formulated feed (such as Abfeed) is often associated with a higher incidence of sabellid worm infestations, mostly on farms with poor water quality and tank hygiene, since the worms feed on the nutrient rich abalone faeces (Simon *et al.* 2004, Troell *et al.* 2006). Also, at higher temperatures and relatively lower water flow rates, the negative impacts of Abfeed on water quality are greater than those of kelp (Jones and Britz 2006). Lastly, kelp is relatively high in ash content (25% on a dry-weight basis) (Smith et al. 2009) and thus rich in minerals. This often results in higher shell growth rates in larger abalone (Troell *et al.* 2006). Recently, Francis et al. (2008) compared the growth of market size abalone fed either a diet of fresh kelp (*E. maxima*) with that of those fed a low-protein commercial formulated feed ('Feed A'). It was found that both feeds produced similar gains in mean shell length (45.220 μm day^{-1} for kelp, 46.839 μm day^{-1} for 'Feed A'), but in terms of weight gain, abalone fed 'Feed A' significantly outperformed those fed the kelp diet (0.266 % body weight day^{-1} for 'Feed-A' abalone; 0.257 % body weight day^{-1} for kelp-fed abalone) (Francis et al. 2008).

Even without scientific evidence, a few abalone farmers are said to prefer using *Ecklonia* species as a part of the abalone's diet as it is speculated to have a low FCR (kelp wet weight/abalone weight gain) compared to *Laminaria* kelp species (Troell et al. 2006), thus suggesting that *Ecklonia* has a high efficiency of conversion to body weight. Abalone farmers have also reported that abalone feeding on *E. maxima* on the southwest coast have a FCR of 1:12-15, which is lower than the FRC of *E. maxima* on the west coast, 1:15-19 (Robertson-Andersson pers. comm., 2007). As a result, and regardless that this has not yet been experimentally confirmed, the southwest coast abalone farmers argue that kelp growing in this area is superior for abalone feed compared to the kelp growing along the west coast.

Methodology used for protein analysis

The methods which are most commonly used to quantify protein are as follows: (1) The Bradford method (Coomassie plus protein assay dye method) (2) the Lowry method and (3) the determination of crude protein (percentage nitrogen content x 6.25). In the Bradford method, the Coomassie Brilliant Blue dye is bound to protein mainly by arginine residues and to a lower degree by histidine, lysine, tyrosine, tryptophan and phenylalanine residues. The binding between the dye and amino acids is attributed to van derWaals forces and hydrophobic interactions (Compton& Jones 1985). The Lowry method detects protein by a reaction which is catalyzed by copper, a component of the Folin phenol reactions. The chemical reaction detects peptide bonds and is also sensitive to some amino acids such as tyrosine and tryptophan (Legler *et al.* 1985). Both the Bradford and the Lowry method are carried out by spectrophotometry. Protein extraction is a difficult process due to the presence of large amounts of polyanionic cell wall mucilages and phenolic compounds in seaweeds. Thus, in order to obtain the highest yield of seaweed protein, the use of NaOH and β - mercaptoethonal after an initial aqueous extraction is very important (Wong and Cheung 2000). The calculation of crude protein content by %N x 6.25 requires some caution since it often leads to an overestimation of the total protein content. The accuracy of this method depends on the establishment of N-Prot factors and these are specific to individual species (Lourenco et al. 2002). Lourenco et al. (2002) do not mention conversion factors about kelps specifically but reported 5.38 as an average N-Prot factor for brown seaweeds.

The aims of this study are: (1) to measure and compare the total protein content and other nutritional constituents of two kelp species, *Ecklonia maxima* and *Laminaria pallida* ; (2) to investigate and compare, seasonally, the total protein content and nutritional constituents of *Ecklonia maxima* collected for abalone feed on the west coast of South Africa and (3) to determine whether "crude protein" levels correlate with extracted total protein levels of kelp. Since abalone aquaculture continues to expand in South Africa, and with it the demand for high quality and effective feeds, thus the research question which I will be investigating is as follows: Does *Laminaria* have a lower protein level than *Ecklonia,* and will this result have implications in the abalone aquaculture industry? The primary hypothesis is that there is a significant difference in total protein content between the kelp species *E.maxima* and *L.pallida*. Predictions relating to the present study include: If the reported FCR of *E. maxima* is higher than that of and *L. pallida* then consequently *E. maxima* should have a higher total protein content than *L. pallida*. If there is a significant difference in the total protein content of the two kelp species, then the farmers using the kelp that has a lower protein content for abalone feed may be at a disadvantage in the industry. The fact that the local abalone aquaculture industry is expanding, and the low availability of knowledge based on nutritional requirements for feed are the main motivations for performing this study. If the economic demand for abalone increases, abalone farms need to increase their production levels, and as a result it is necessary for biologists to conduct studies such as this, in order to gather information that will assist in creating a diet that maximises abalone growth and production.

Materials and Methods:

Sample collection and study site

E. maxima and *L. pallida* plants were harvested at one time of the year, 22 July 2009, from a kelp bed at Kommetjie on the borderline of the west coast and south-west coast of the Cape Peninsula, South Africa. The two kelp species were co-occurring such that they occupied the same habitat with similar physical and chemical hydrological conditions. The habitat formed part of the shallow sublittoral that consisted of rocky substrata. Large hollow-stiped *E. maxima* plants formed kelp beds to the depth of 8 m while smaller solid-stiped *L. pallida* plants were dominant from about 8 to 14m, but also occur together with *E. maxima* in shallow

water in habitats somewhat sheltered from wave action. Five replicate plants of both *Ecklonia* and *Laminaria* were collected at low tide. The samples were washed in the field with seawater to remove any epiphytes, sediment and organic matter. Samples consisted of fronds that were of an intermediate maturity level and therefore lacked the presence of reproductive tissue. Eight fronds per replicate were collected from *E. maxima* plants and the frond was collected (thus excluding the meristem region and split-frond ends) from each *L. pallida* plant. Kelp samples were packed in plastic bags and kept in a cooler box until returned to the laboratory. In the laboratory, plants were gently rinsed with distilled water to remove the seawater and slime, dabbed dry with paper towel and weighed in order to obtain the wet weight. The samples were then dried in an oven for 48 hours at 60°C, after which they were weighed to get the dry weight and milled to a fine homogenous powder through a 0.5mm sieve. Milled dry kelp samples were then analysed for their N, C, H and protein content. From the 5th April 2004 to the 20th June 2005 replicate samples of healthy secondary fronds of *Ecklonia maxima* were collected once every two weeks or once every month, depending on weather and ocean conditions, and frozen. These samples were collected by abalone farm workers and few samples were collected over the summer holiday period when farms are short-staffed (December-January. 16 samples (four replicates) were collected from Omdraai (OMD) by JSP (Jacobsbaai Sea Products Abalone farm) on the west coast of the Cape Peninsula. After samples were dried and milled they were analysed for N, C, H and protein content. The data are presented in by calendar months combined into seasonal categories and are defined as follows: summer comprises of December, January and February; autumn comprises of March, April and May; winter comprises of June, July and August and spring comprises of September October and November.

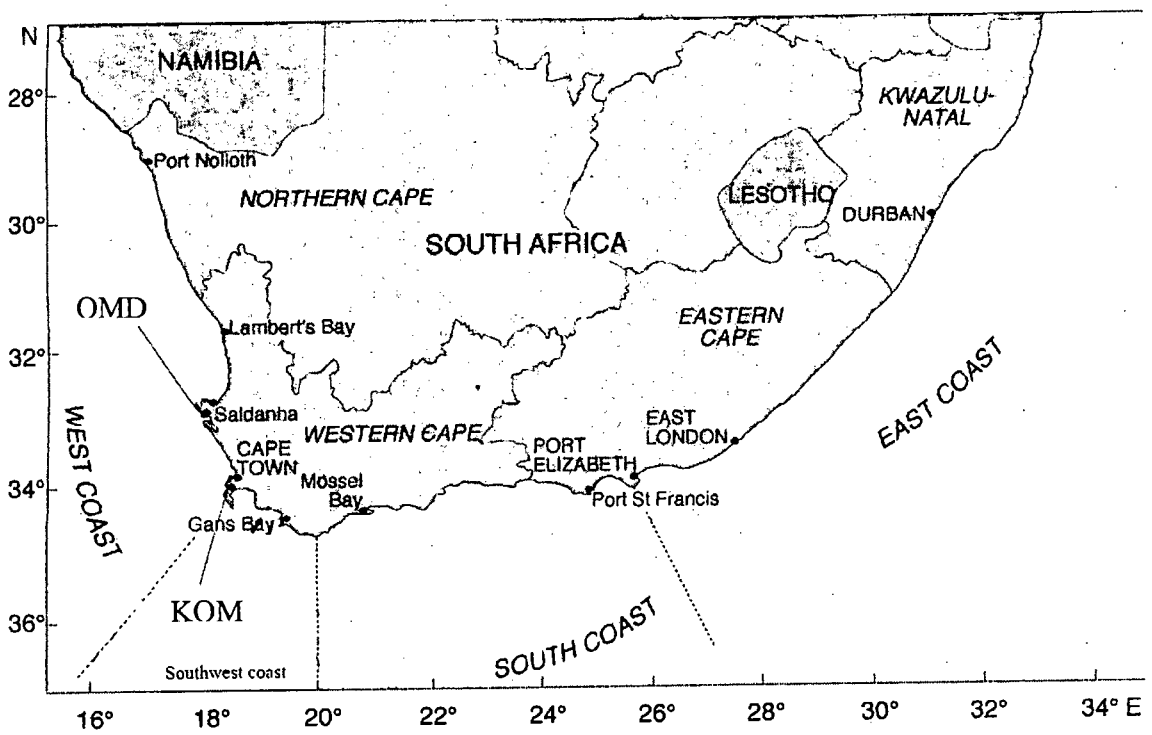

Figure 1: Map of southern Africa showing location of kelp samples used in the present study. KOM stands for Kommetjie (borderline west and southwest coasts) and OMD stands for Omdraai (west coast).

Extraction of protein

The following method of extraction follows the basic *Procedure I* by Barbarino and Lourenco (2005). An amount of 50mg of dried kelp samples were weighed out into a glass vial (30 ml volume) and suspended in de-ionized water (2 ml) to induce cell lysis by osmotic shock that facilitated subsequent protein extraction. Then the suspension was gently stirred overnight at 25°C. After the incubation period, suspensions were centrifuged at 4°C, 3000 rpm for 20 minutes. Supernatants were collected for protein assay and the pellets re-extracted with 3ml of 0.1M NaOH with 0.5% β-mercaptoethonal (v/v). The mixture of NaOH and pellets were kept at room temperature for 20min and vigorously mixed 3 times using a vortex mixer. They were then centrifuged at 4°C, 3000 rpm for 20 minutes. The second supernatants were combined with the first ones and the extraction process was repeated another six times until a total of 23ml of the extraction solution was collected.

Protein quantification

The Bradford assay was used whereby the Coomassie plus protein assay dye binds to the protein (Matto et al. 1987; Barbarino and Lourenco. 2005) . The binding of the protein with the dye is very quick and the protein-dye complex remains soluble for one hour. 0.01ml of the extracted solution is combined with 0.3ml of the Coomassie protein assay and absorbance was measured at 595nm at room temperature. In order to plot a protein standard curve, a set of Diluted Albumin (BSA) Standards were prepared. The contents of one Albumin Standard (BSA) ampule was diluted into several clean vials, the same diluent as the samples. Each 1 ml ampule of Albumin Standard was sufficient to prepare a set of diluted standards for the working range suggested in Supplementary Table 1 (Anonymous, 2000) . The standard Microplate Protocol (Working Range = 100-1,500 µg/ml) was as follows: 10 µl of each standard or unknown sample was pipetted into the appropriate microplate wells. 300 µl of the Coomassie Plus Reagent was added to each well and mixed with a plate shaker for 30 seconds. For the most consistent results, the plate was incubated for 10 minutes at room temperature. The absorbance was measured at 595 nm with a plate reader. A standard curve was prepared by plotting the average Blank-corrected 595 nm measurement for each BSA standard vs. its concentration in µg/ml. The standard curve was then used to determine the protein concentration of each unknown sample.

Elemental analysis

The milled dry kelp samples were sent to the Chemistry Department of the University of Cape Town where they were analysed for their N, C, H. A CHN elemental analyser (Thermo 1112 CHN) was used to determine the amounts of the various elements in each sample. The CHN analysis was accomplished by combustion analysis. In this technique, a sample is burned in an excess of oxygen, and various traps collect the combustion products (carbon dioxide, water, and nitric oxide). The weights of the combustion products are used to calculate the composition of the unknown sample.

Statistical analysis

Each elemental and protein percentage was treated statistically by a one-way analysis of variance (ANOVA) for assessing the effect of one variable (seasonality). Comparisons after

test for diffs btwn means!

ANOVA were made using the post hoc Tukey test to individualise specific differences. (ANOVA; Zar 1999) Prior to the statistical analysis, equality of variance was checked using the Levene's test and normality was checked using the Kolmogorov Smirnov test. A Student's t-Test was performed in order to determine the difference between the protein content of *E.maxima and L.pallida*. Prior to the statistical analysis, normality was checked using the Lilifor's test and homoscedasticity was checked using the Levene's test. When data were found not to be normal and variances were unequal then a non-parametric Mann Whitney U test was applied. Differences between seasons and species protein content were considered statistically significant at $p<0.05$. Each variable is presented as a mean with a standard error.

Results:

Moisture content

There was a significant difference in moisture content between *E.maxima and L.pallida* as *L.pallida* had about a 1.9% higher moisture content (Student's T-Test, t=-3.35589; df=8; p=0.009993) (Fig 2). The remaining dry content of *E.maxima* was approximately 13% compared with that of *L.pallida* which had approximately 11% dry content.

do you mean dry : wet weight ratios?

Carbon

The carbon content of *E. maxima* from both Omdraai (West coast) (mean of 31.73%) and Kommetjie (borderline west and southwest coast) (mean of 29.29%) varied from about 27.79% to 32.29 % of dry weight (Fig 3(a) and (b)). No significant difference was found among seasons for *E. maxima* from the West coast site (ANOVA, F=0.459; df=3; p >0.05) (Fig 3(a)). As shown in Fig 3(b) there was a significant difference in carbon content between the two kelp species, *E. maxima* (30.79 %) had 3% more carbon (% of dry weight) than *L. pallida* (27.79 %) (Mann Whitney U Test, U=0.00 ,n1=5,n2=5 p<0.01). ~~The remaining dry content of *E.maxima* was approximately 13% and it was higher than that of *L.pallida* which had approximately 11% dry content.~~ This implies that *E.maxima* comprises of 130g/kg nutritional content where as *L.pallida* only comprises of 110g/kg and thus *E.maxima* and *L.pallida* consists of 40.027g/kg and 30.569g/kg respectively.

Which is it?

30.79% looks like an avg of both sites — you should only use Kommetjie

g Carbon / kg wet weight.

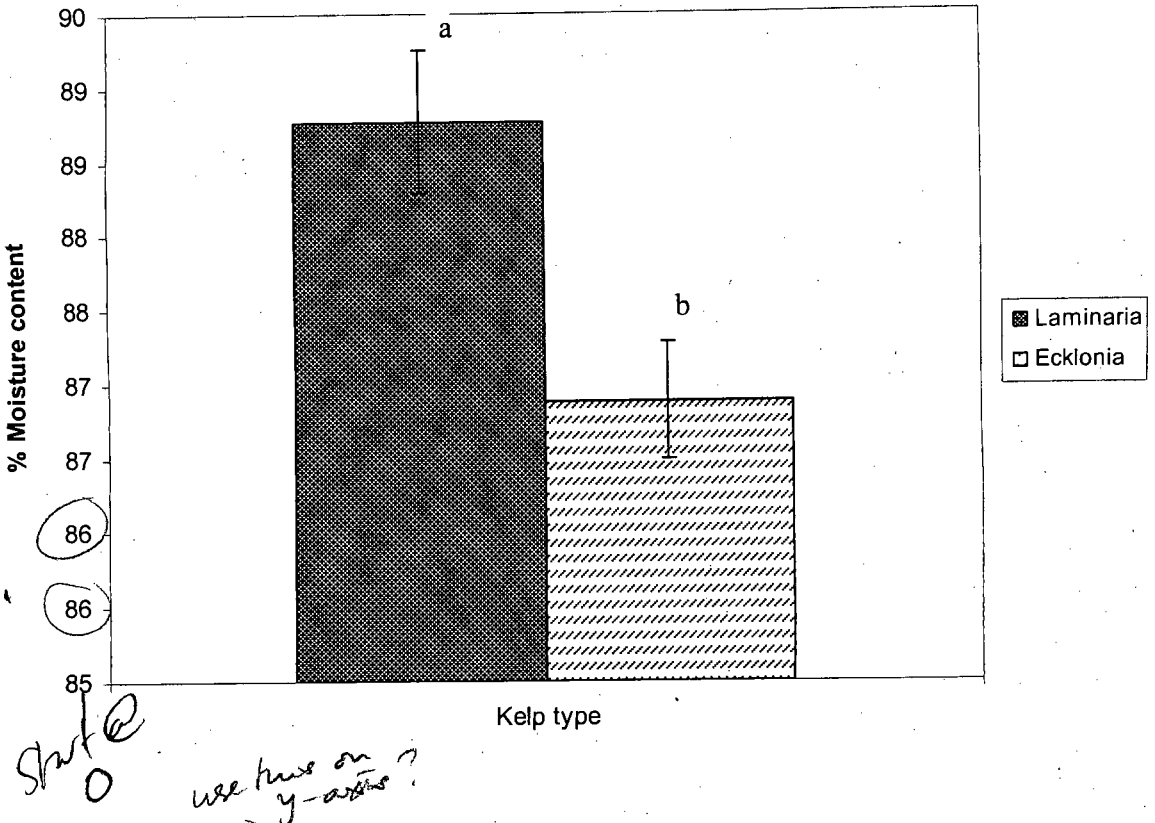

Figure 2: Water content (% of wet weight ±SE) of Ecklonia maxima and Laminaria pallida at the Kommetjie site (borderline west and southwest coasts), South Africa. Different letters (i.e.'a' and 'b') indicate statistical significance at the 95% level and the same letter (i.e. 'a' and 'a') indicate no significant differnce.

Nitrogen

There was no seasonal pattern observed in the nitrogen content (% of dry weight) of *E. maxima* at the west coast site and the nitrogen content ranged from 1.13-1.38 % of dry weight, with no significant difference between the seasons (ANOVA, F=1.5682; df= 3; p>0.05) (Fig 4(a)). There was no significant seasonal difference in crude protein (ANOVA, F= 1.5682; df= 3; p>0.05) content of *Ecklonia maxima* at the west coast site, Crude protein ranged from 7.11-8.65% of dry weight (Fig 4(b)). There was no significant difference in %N found between *E. maxima* (averaged 1.46% of dry weight) and *L. pallida* (averaged 1.44% of dry weight) (Student's t-Test, t=0.166127; df=8; p>0.05) (Fig.4(b)) and consequently no significant difference was found in crude protein either (Student's t-Test; t= 0.166127; df=8; p>0.05) (Fig.4(d)).

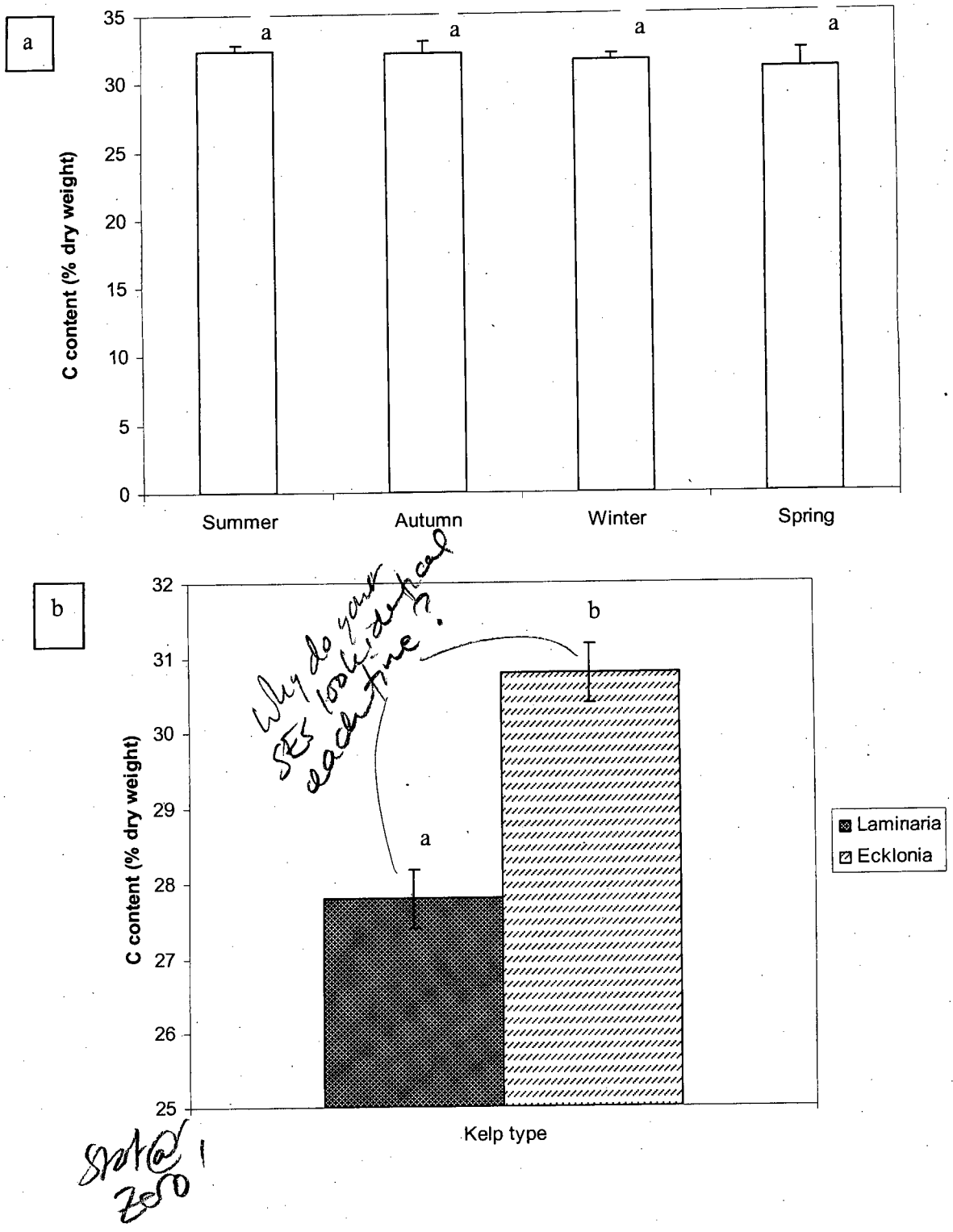

Figure 3: Average Carbon (C) content (% dry weight ±SE) (a) measured seasonally for Ecklonia maxima at Omdraai (west coast) , and (b) measured during July for Ecklonia maxima and Laminaria pallida at Kommetjie (borderline west and southwest coasts), South Africa. Different letters (i.e.'a' and 'b') indicate statistical significance at the 95% level and the same letter (i.e. 'a' and 'a') indicate no significant difference.

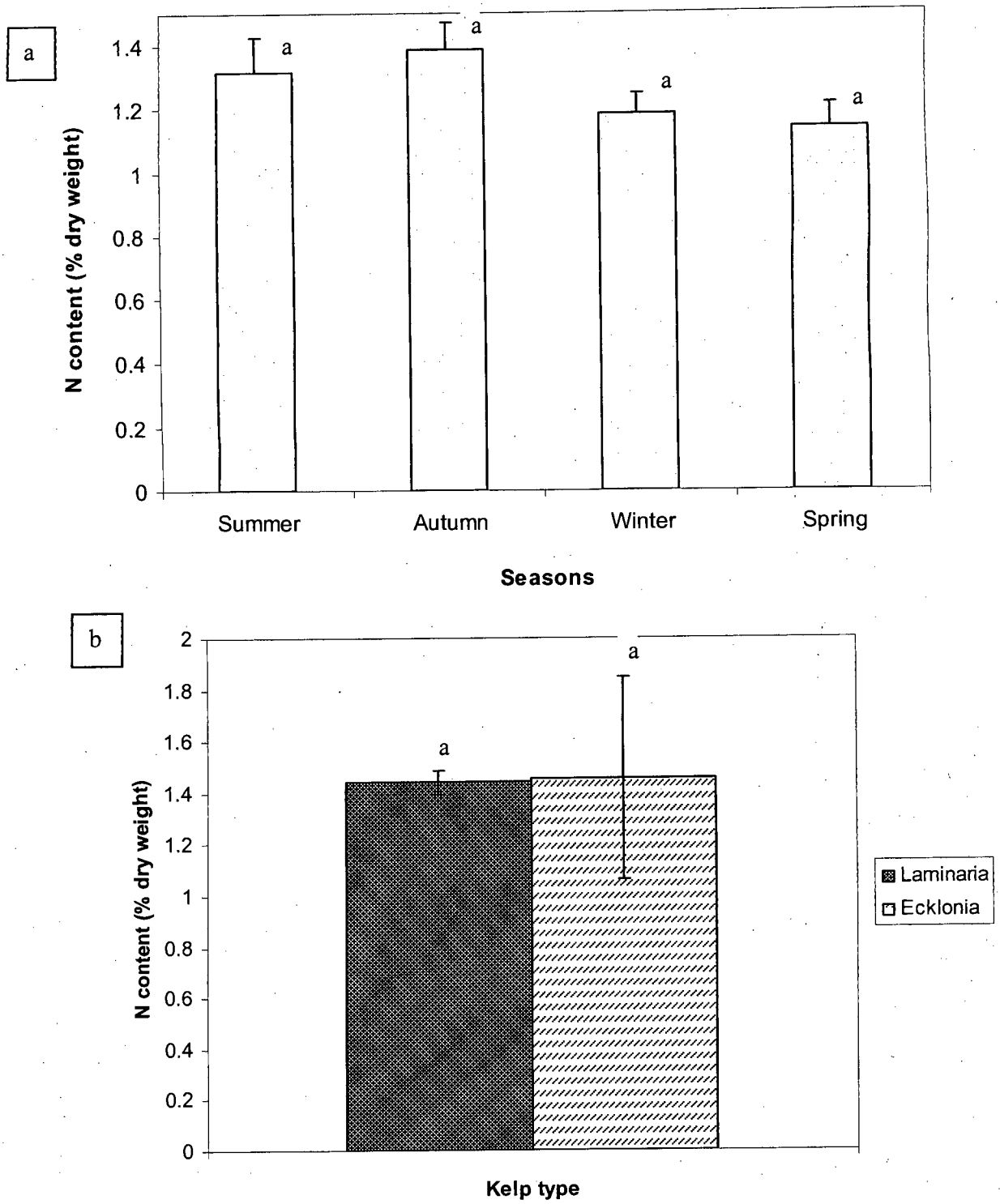

Figure 4: Average Nitrogen (N) content (% dry weight ±SE) (a) measured seasonally for Ecklonia maxima at Omdraai (west coast) and (b) measured during July for Ecklonia maxima and Laminaria pallida at Kommetjie (borderline west and southwest coasts), South Africa. Different letters (i.e.'a' and 'b') indicate statistical significance at the 95% level and the same letter (i.e. 'a' and 'a') indicate no significant difference.

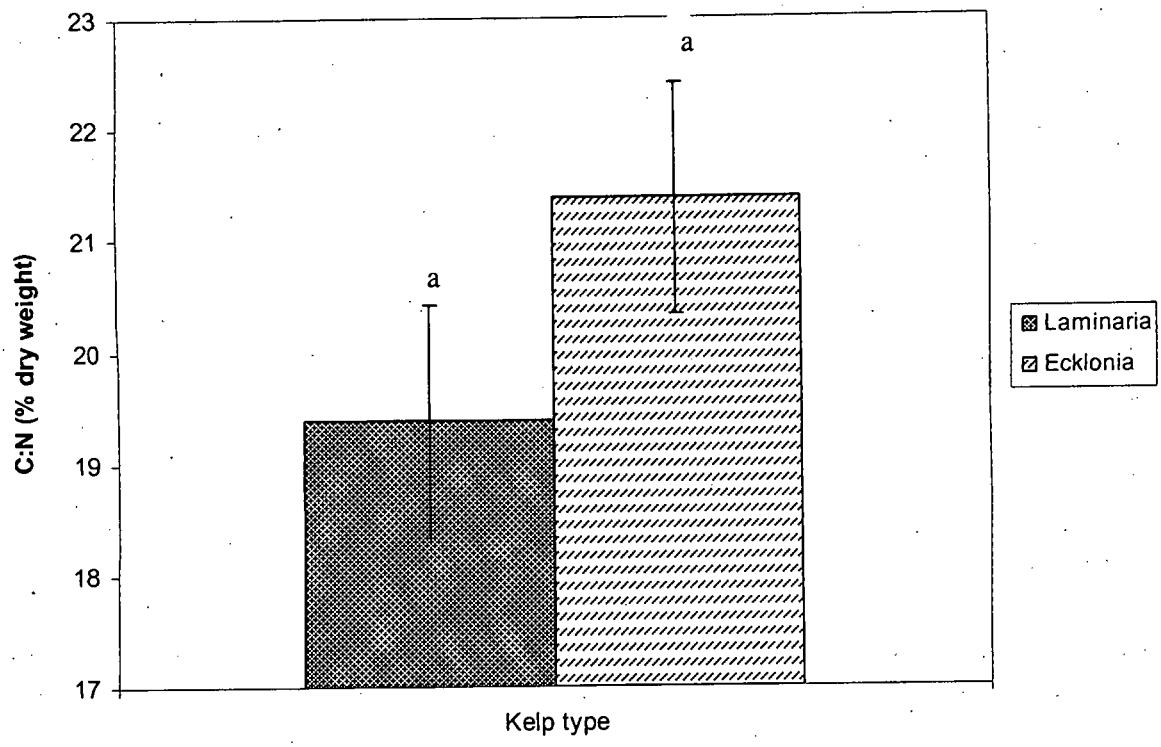

Figure 5: C:N ratio (% of dry weight ±SE) of Ecklonia maxima and Laminaria pallida at Kommetjie (borderline west and southwest coasts), South Africa. Different letters (i.e.'a' and 'b') indicate statistical significance at the 95% level and the same letter (i.e. 'a' and 'a') indicate no significant difference.

C:N Ratio

No significant difference was found among seasons for *E. maxima* from the West coast site (ANOVA, F=1.1452; df=3; p >0.05), although *E. maxima* had a higher C:N ratio than *L. pallida* (Fig 5) there was no significant difference between the two kelp species at the Kommetjie borderline site (Student's t-Test, t=1.617141; df=8; p>0.05)

Total protein

There was no significant seasonal difference in total measured protein (ANOVA, F= 0.4622; df= 3; p>0.05) content of *Ecklonia maxima* at the west coast site, total protein content ranged from 0.96-1.09% with an average of 0.99% of dry weight (Fig 6(a)). Although the total protein content seems higher for *E.maxima*, no significant difference was found between *L. pallida* and *E. maxima* in this regard (Student's T-Test ;t= 1.010357; df=8; p>0.05) (Fig 6(b)). During each of the four seasons, there was a significant difference between the crude protein

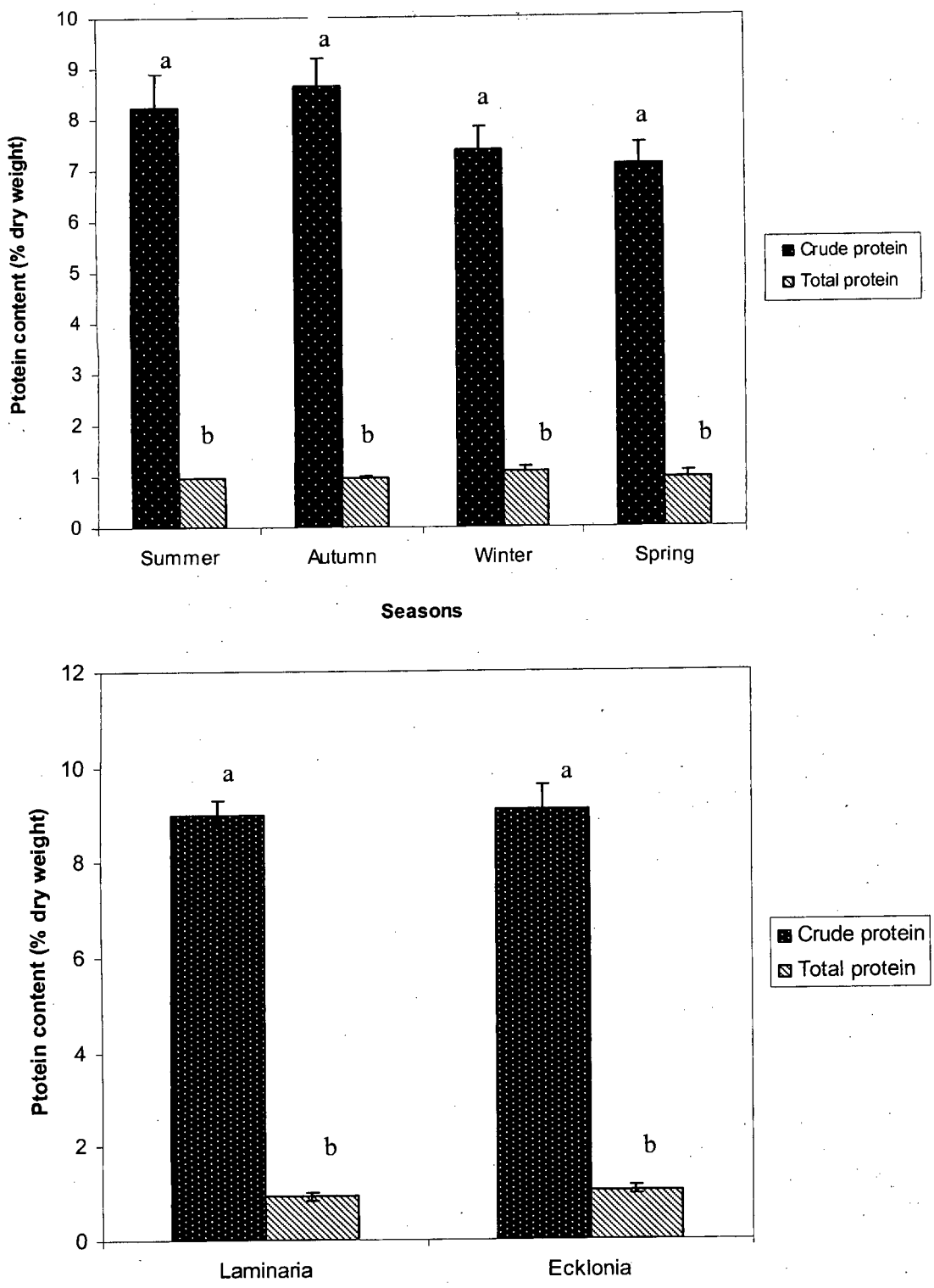

Figure 6: (a) Seasonal average protein content (% dry weight ±SE) of Ecklonia maxima from Omdraai (west coast) and (b) Protein content (% dry weight ±SE) of Laminaria pallida and Ecklonia maxima from Kommetjie (borderline of west and south west coast), South Africa. Different letters (i.e.'a' and 'b') indicate statistical significance at the 95% level and the same letter (i.e. 'a' and 'a') indicate no significant difference.

content and total protein content measured (Mann Whitney U; U=0; n1=4;n2=4; $p<0.05$). As shown in Figure 6(a and b) the total protein value was approximately eight times lower than the crude protein value in all four seasons. There was a significant difference between crude protein and total protein content for *E.maxima* (T-TEST; t= 15.16120; df=8; $p<0.01$) and *L. pallida* (Student's T-Test; t= 25.83751; df=8; $p<0.01$) (Fig. 6(b)).

Discussion:

~~Considering the fact that~~ abalone farmers along the southwest coast prefer using *Ecklonia* as a part of abalone feed, because *Ecklonia* is the dominant kelp species in the area (thus relatively more cost-effective with regards to transport, labour and time) and *Laminaria* is very scarce. From available information on the importance of protein content as a component in a diet and the possibility that it may have a significant effect on the FCR, it was hypothesized that there is a significant difference in total protein content between the kelp species *Ecklonia maxima* and *Laminaria pallida*. Based on the data collected in the present study, the primary hypothesis has not been supported. No significant difference was found between *L. pallida* (0.92% dry weight) and *E. maxima* (1.05% dry weight) with regards to the true protein measured (Fig 6(b)). No seasonal variation was found in true protein content of *E. maxima* (range 0.96-1.09% dry weight) (Fig.6(a)). The prediction that the higher reported FCR of *E. maxima* (compared to *L. pallida*) would result in *E. maxima* having a significantly higher true protein content than *L. pallida* was not justified by the present data. It was also predicted that if there was a significant difference in the protein content of the two kelp species, then farmers using the kelp (for abalone feed) with a lower protein content may be at a disadvantage in the industry. But based on current speculation of the differing food conversion ratios (FCR) of kelp growing along the west and southwest coasts, and the data represented in figure 6(b) it is impossible to demonstrate that kelp from one site (i.e *E.maxima* dominant and most available to farms along the southwest coast) has a higher nutritional quality compared to another site (*L.pallida* dominant and most available to farms along northwest coast).

In kelps, majority of the carbon content is made up of alginic acid (also known as algin or alginate) which is a viscous gum that is abundant in the cell walls of brown algae (Lobban

and Harrison 1994).Von Holdt et al. (1955) found alginate to vary seasonally from 22-30% of dry weight in *E. maxima* fronds. In the present study, although the carbon content of *E. maxima* seemed to be slightly higher during summer, there was no significant seasonal variation in carbon content at the Omdraai (west coast) site (Fig 3(a)). On the other hand, Smith et al (submitted. 2009) also tested the carbon content of *E.maxima* along the west and southwest coasts and found that the carbon content increased during the summer months and decreased during winter months. However the annual average carbon % of dry weight in the present study (31.73%) was very similar to that found by Smith at al (submitted. 2009) (31.17%). Similarly, Von Holdt et al (1955) found that there were higher values of alginate in summer than in winter. These findings can be explained by the fact that *E. maxima* species store carbon to enable growth during seasons when light is most limiting (the plant would store carbon during summer months when light is less limiting in order to be used during winter months) (Smith et al.submitted.2009). Studies performed by Chapman and Lindley (1980) and Sjøtun et al. (1996) found similar results in *Laminaria solidungula* and *Laminaria hyperborea* respectively. The findings by Smith et al. (2009) indicate that the kelp species *E. maxima* stores carbon seasonally and contrast with the findings by Dieckmann (1978, 1980) who gave evidence that *L. pallida* does not store carbon to allow growth during periods of the year when light is limiting.

Smith et al.(submitted.2009) suggested that the reported difference in FCR in abalone feed containing kelp from the different locations (west versus southwest coast) might be related to carbon content (and hence energy content) rather than protein content. The results presented in the present study imply a similar explanation for the speculation that *E.maxima* has a lower FCR compared to *L.pallida.* Since there was no significant difference between the protein content of the two kelp species, the significant difference in carbon (Fig.3(b)) indicates that carbon energy budgets may play a more important role, than previously expected, in the growth of abalone.

Henley and Dunton (1997) found that elemental composition of blades of arctic kelp *Laminaria solidungula* was more strongly influenced by light than by nitrogen. Experimentally, they found that *L. solidungula* grown in the light contained a higher %carbon

and much lower %nitrogen, and as a result a much higher C:N ratio compared to those grown in continuous darkness. Although there was a 2% difference in the C:N ratio of *E. maxima* and *L. pallida* (Fig. 5) the difference was not significant at the 95% level but perhaps the sample size was insufficient to show a clear pattern of *E. maxima* having a significantly higher C:N ratio. If this pattern is true, this would not necessarily mean that *E. maxima* is N limited and not light limited. Although it is found that plants that possess more %carbon have a higher rate of carbon fixation and thus a higher C:N ratio (Lapointe and Duke, 1987; Henley and Dunton (1997), this comparison can only be drawn if the species studied are the same or of the same genera. Since *E.maxima* and *L.pallida* in the present study were co-occurring in shallow waters of the border of the west and southwest coasts, neither one of them could have been light limited.

Why? couldn't ecklonia shade out laminaria. or laminaria is deepe?

There was a significant difference in ~~moisture~~ water content between *E.maxima* and *L.pallida* as *L.pallida* had about a 1.9% higher moisture content (Fig 2). It is expected that plants would have a higher water content when they are growing rapidly and thus rapid cell elongation and reduction in storage materials (Diekmann. 1978). This explains why *L.pallida*, with the higher moisture content (Fig.2), has a lower %C (Fig.3(b)) and is reported not to store carbon as *E.maxima* does. Given that *E.maxima* has a lower moisture content and thus comprises of 40.027g/kg of carbon where as *L.pallida* only comprises of 30.569g/kg of carbon, abalone that is fed one kilogram of *E.maxima* will consume more carbon and therefore capitalise on the energy provided in the form of carbon.

Although no significant difference in nitrogen content was obtained between the two kelp species in the present study (Fig 4(b)), the large error bar which exists for the *Ecklonia* result needs to be taken into account whereby perhaps a bigger sample size needs to be analysed to eliminate error. Von Holdt et al. (1955) found no seasonal pattern for Kjeldahl Nitrogen for *Ecklonia maxima* fronds from Kommetjie and values ranged from 1.5–1.8 % of dry weight. Smith et al (submitted.2009) also found that %N (determined by the elemental analyser method) of *E. maxima* (averaged 1.7% of dry weight) did not differ significantly among months along the west coast. Similar findings were obtained in the present study, N content showed no seasonal variation and values ranged from 1.13-1.38 % with an annual average of

1.3% of dry weight (Fig 4(a)). Since crude protein is calculated by simply multiplying the N value by 6.25, there was no seasonal variation found in crude protein of *E.maxima* either (Fig.6(a)). Smith et al. (in press 2009) obtained a crude protein range of 9-12%, similarly Von Holdt et al (1955) obtained a range of 9.4-11.25% and Probyn and McQuaid (1985) found that crude protein ranged from 11.2-13.3%. In the present study, average crude protein of *E. maxima* fronds from the west coast Omdraai site ranged from 7.11-8.65% of dry weight. The average in this study is lower than those found in previous studies, since the N content in this study was also very low but the reason for this variation between studies is not clearly understood but is alleged to be a result of a very small sample size (i.e.df= 3). One of the aims of this study was to determine whether crude protein levels correlated with true extracted protein levels of kelp. The data presented in figure 6(a) and (b), indicated that crude protein differed significantly from true (total) protein in all seasons and in both kelp species. Crude protein was found to be about eight times higher than true protein and this validates the reason why the nitrogen-to-protein method to quantify protein requires some caution. Lourenco et al (2008) established the N-Prot factors based on the ratio of amino acid residues to total nitrogen and found values ranging from 3.75 (*Cryptonemia seminervis*, red algae) to 5.72 (*Padina gymnospora*, brown algae). It was recognized that the greater the amount of non-protein nitrogen in the algae, consequently the lower the N-Prot factors are calculated to be. Green and brown algae tend to have lower amounts of non-protein nitrogen and thus averaged the N-Prot factors to be 5.13 and 5.38 respectively. The study carried out by Lourenco et al (2002) verifies that the traditional conversion factor 6.25 is unsuitable for seaweeds and that the N-Prot factors proposed by their study are recommended. In the case of the present study, using N-Prot factors proposed by Lourenco et al (2002) for brown algae, requires some concern since Lourenco et al (2002) proposed N-Prot factors for Brazilian seaweeds and the nutrients status of the Brazilian marine environments differs from ours, such that they are predominantly oligotrophic. This means that less nitrogen is available to algal populations in the marine environments of Brazil and that could explain the low concentrations of protein found in their samples and thus could affect the N-Prot factors established in their study.

An early study done by Britz (1996) found that abalone had the following feed consumption rates when various sources of protein were incorporated in their diets: feed consumption rates ranged from 0.5% body weight per day on the casein-based diet (91% crude protein) to 0.8% body weight per day on the fishmeal (70% crude protein) and *Spirulina spp.* (44% crude protein) diet, 2.8% body weight per day on the *Ecklonia maxima* (10% crude protein) based diet and 1.3% body weight per day on the *Plocamium corallorhiza* (20% crude protein) based diet. It was found that abalone consumed significantly greater amounts of the seaweed based diets compared to the formulated diets, suggesting that the more protein that was in the diet, the less the abalone had to consume. Based on this knowledge, it would be beneficial for farmers to feed abalone higher protein diets that would increase their growth performance and be a positive influence on production, since a smaller amount of high-protein feed is required to produce comparable growth. The fact that faster abalone growth rates are reported when they are fed artificial feeds as opposed to natural feeds, serves as a potential advantage of using formulated diets in abalone aquaculture (Britz. 1996b). Britz (1996b) found that although the abalone which were fed the casein-based diet experienced an efficient FRC, they had a lower growth rate in comparison with those fed the fishmeal-based diet. The reasoning proposed for this was that the casein diet had marked energy/protein imbalance present and abalone fed the casein diet had a lower feed consumption rate, perhaps as a result of the casein not being as palatable or attractive as the fishmeal (Britz. 1996). Thus, it is valuable to take many factors into consideration, because if a certain feed has a high protein content, it does not necessarily imply that it is optimal. Consequently, other factors need to be considered, for example the energy/protein imbalance as well as additional animal feeding trials to determine animal preference.

In addition, Britz (1996b) found that abalone that were fed the natural diets consisting of only seaweed (*E. maxima* or *P. corallorhiza*) had poor growth rates and FRC values possibly as a result of a deficiency of essential nutrients or a low protein to energy ratio, since marine algae are generally rich in stored carbohydrates and poor in protein (Francis et al. 2008). In light of this, the macroalgae that were fed to the abalone may have satisfied their energetic requirement but not necessarily their protein requirement which is necessary for tissue deposition (Britz. 1996b). On the other hand, Naidoo et al (2006) found that a mixed seaweed

diet was as good, if not better, quality as the high-protein artificial feed since abalone had a higher growth rate when fed the mixed seaweed diet. One must keep in mind that abalone in the wild do not only consume one type of algae, but rather a variety of algae so that any nutrients that are deficient in a particular macroalgae species can be obtained by consuming other algal types that have those nutrients needed by the abalone. Mercer et al. (1993) gave recommendations for the following balanced levels of nutritional content of natural algae necessary for optimal abalone growth performance: more than 15% protein, lipids ranging between 3-5 % and carbohydrates ranging between 20-30%, and excluding any toxic substances. Francis et al. (2008) concluded that low-protein artificial feed (26% protein of dry weight) can be seen as an alternative feed for future abalone aquaculture, since kelp is not only low in protein content (10 % of dry weight) but is also becoming hard to obtain because it is approaching its sustainable harvesting limit. Thus commercial 'Feed A' had all the benefits of both kelp (i.e. feeding stimulant) and a high-protein formulated feed (i.e. for producing meat weight gain) but none of their apparent disadvantages (i.e. potentially limited availability and low protein content of kelp; potentially higher incidence of sabellid worm infestation under poor tank hygiene and with high-protein feeds).

Sampling and experimental limitations

A possible explanation for the results on average carbon (Fig. 3(a)) and N=nitrogen (Fig.4(a)) content measured seasonally for *Ecklonia maxima* at Omdraai (west coast) differing to those from previous studies could be due to sampling limitation, since samples chosen to represent each season did not consist of every month within the seasonal category, for example samples were only collected during January (and not December and February) as a representation of the summer category. Thus a limitation in data for each month (representing variation within each seasonal category) could have an effect on the accuracy of the results found in Fig. 3(a). In addition I had fewer samples (15) to analyse and this may also play a role. One of the limitations of the Bradford's method is the trend of obtaining lower concentrations of protein using and this may be related to the binding of the dye Coomassie Brilliant Blue-G250 to both basic and aromatic amino acid residues (Compton & Jones, 1985). Most algae have relatively low concentrations of the two amino acids, tyrosine and tryptophan, as well as the two basic amino acids, lysine and histidine. As a result, the binding of the dye with protein occurs

mainly with the two amino acids, arginine and phenylalanine, and this occurrence seems to contribute to lower protein measurements.

Future research

Although there was no significant difference between the protein content of the two kelp species, the results found in the current study showed a slight indication that *E.maxima* had higher protein content than *L. pallida*. However there was a significant difference in carbon content, indicating that *E.maxima* may have a higher energy content and thus an indication of nutritional superiority. It may therefore be beneficial to scientists and more specifically abalone farmers to do animal feeding trials in future studies as they may be useful to compare the nutritional value of the two kelp species from a point of view which is directly based on animal preference. In addition, in order to challenge the significant difference in carbon content and to further investigate the slight difference in true protein content of *E.maxima* and *L. pallida*, samples should be collected from multiple sites (along the west and southwest coasts) where the two kelp species co-occur. At this point it is uncertain to say how much of the %N is allocated to protein- and non-protein nitrogen and thus N-Prot factors are not able to be established for the *E.maxima* and *L.pallida* samples collected in this study. But perhaps the specific conversion factors of *E.maxima* and *L.pallida* could be determined in the future, whereby both the nitrogen content and amino acid composition will be required to do so.

Over the years, there has been a progression in what has been regarded as the most suitable feed for commercially cultured abalone, namely the high protein formulated feed proposed by Britz (1996b), to the mixed fresh seaweed diet proposed by Naidoo et al. (2006) and most recently the low protein formulated feed (having the benefits of both kelp and high-protein formulated feed) proposed by Francis et al. (2008). I believe that the search is not yet over since the present study has revealed the overestimation of true protein content in the feed used in abalone aquaculture, as well as the realisation that % carbon (thus energy levels) may play a significant role in determining the most appropriate feed. A thorough investigation needs to be conducted whereby various diets (Abfeed (high-protein formulated feed); 'Feed A' (low-protein formulated feed); a mixed fresh seaweed diet-1 (*Gracilaria gracilis, Ulva lactuca* and *E.maxima*); and a mixed fresh seaweed diet-2 (*Gracilaria gracilis, Ulva lactuca* and

L.pallida)) are compared on a nutritional basis (nitrogen, carbon, total protein, carbohydrates, fat, fibre, ash and moisture) and compared on an abalone preference basis investigated through feeding trials of both. Different feed combinations need to be tested on the growth of not only grow-out juveniles (abalone with a shell length >20 mm) but also post-weaning juvenile abalone of a shell length ranging from >6 mm to <20 mm because different results may be obtained (Dlaza et al. 2008; Francis et al.2008).

? you have totally ignored the herbivore defense compounds Why? ~~Is this not~~ Are chemical defenses ~~chemicals~~ not NB in marine ecosystems?

Concluding remarks

The present study proves to be of importance because it contributes to the knowledge necessary (precise information on nutritional requirements of abalone and nutritional composition of seaweeds) for finding the most appropriate diet that farmers can use on abalone farms. The reported better FCR in abalone feed comprised of *E. maxima* rather than *L. pallida*, if correct, is likely to be related to higher carbohydrate content (measured here as carbon) and not to higher protein content. *E.maxima* differs from *L. pallida* with regards to carbon storage, but not protein content. The calculation of protein content by %nitrogen x 6.25 proves to be an outrageous overestimation of the true protein content found in the kelp species researched here. Based on existing knowledge from this study, it is impossible to accurately demonstrate that *E.maxima* is more superior nutritionally as a part of abalone feed than *L.pallida*, and thus conclusions as to which one would be more advantageous in abalone aquaculture can not be made.

Acknowledgments

To Prof. John Bolton (University of Cape Town), many thanks for being a fantastic project supervisor and for assistance in project design and result interpretation. To Pierro Benicassa for elemental analysis of seaweed samples. To Diina Shuuluka (PhD student, University of Cape Town), thank you for not only insight but for all the help and support with regards to all aspects of this research project.

References:

Anonymous. 2000. Instructions: Coomassie Plus- The better Bradford Assay Kit. Pierce Biotechnology, Inc. USA. 7pp

Anderson, R.J., Rothman, M.D., Share, A., Drummond, H. 2006. Harvesting of the Kelp *Ecklonia maxima* in South Africa affects its three obligate, red algal epiphytes. *J Appl Phycol* **18**:343–349

Barbarino, E. and Lourenço, S.O. 2005. An evaluation of methods for extraction and quantification of protein from marine macro- and macroalgae. *Journal of Applied Phycology* **17**: 447-460

Barkai, R., Griffths, C.L. 1986. Diet of the South African abalone *Haliotis midae. S Afr J MarSci* **4**:37–44

Bautista-Teruel, M.N., Millamena, O.M. 1999. Diet development and evaluation for juvenile abalone, *Haliotis asinine*: protein/energy levels. *Aquaculture*. **178**: 117-126

Britz, P.J., Hecht, T., Knauer, J., Dixon, M.G. 1994. The development of an artificial feed for abalone farming. *South African Journal of Science.* **90**: 6-7

Britz, P.J. 1996a. Effects of dietary protein level on growth performance of South African abalone, *Haliotis midae,* fed on fishmeal based semi-purified diets. *Aquaculture* **140**: 55–61.

Britz, P.J. 1996b. The suitability of selected protein sources for inclusion in formulated diets for the South African abalone, *Haliotis midae. Aquaculture.* **140:** 63-73

Chapman, A.R.O., Lindley, J.E. 1980. Seasonal growth of *Laminaria solidungula* in the Canadian High Arctic in response to irradiance and dissolved nutrient concentrations. *Marine Biology* **57**: 1-5

Compton, S.J., Jones, C.G. 1985. Mechanism of dye response and interferencein the Bradford protein assay. *Anal. Biochem.* **151**: 369–374.

Conklin-Brittain, N.L., Dierenfeld, E.S., Wrangham, R.W., Norconk, M., Silver, S.C. 1999. Chemical protein analysis: A comparison of Kjeldahl crude protein and total ninhydrin protein from wild, tropical vegetation. *J. Chem. Ecol.* **25**: 2601–2622.

Diekmann, G.S. 1978. Aspects of growth and production of *Laminaria pallida* (Grev.) J. Ag. off the Cape Peninsula. Msc Thesis. University of Cape Town. 98pp.

Dieckmann, G.S. 1980. Aspects of the Ecology of *Laminaria pallida* (Grev.) J. Ag. off the Cape Peninsula (South Africa) I: Seasonal Growth. *Botanica Marina.* **23**: 579 – 585

Dixon, M.G., 1992. The effect of temperature and photoperiod on the digestive physiology of the South African abalone, *Haliotis midae.* M.Sc. Rhodes University. 85pp

Dlaza, T.S., Maneveldt, G.W., Viljoen, C. 2008. Growth of post-weaning abalone *Haliotis midae* fed commercially available formulated feeds supplemented with fresh wild seaweed. *African Journal of Marine Science.* **30(1):** 199–203

FAO. 2004. The state of the world fisheries and Aquaculture 2004. FAO, Rome.

Fleurence, J., 1999. Seaweed proteins: biochemical, nutritional aspects and potential uses. *Trends in food science and technology.* **10:** 25-28

Francis, T.L., Maneveldt, G.W., Venter.J. 2008. Determining the most appropriatefeeding regime for the South African abalone Haliotis midae Linnaeus grown on kelp. *J Appl Phycol.* **20**:597–602

Francis, T.L., Maneveldt, G.W. and Venter,J. 2008. Growth of market-size abalone (*Haliotis midae*) fed kelp (*Ecklonia maxima*) versus a low-protein commercial feed. *African Journal of Aquatic Science.* **33(3):** 279–282

Fujihara, S., Kasuga, A., Aoyagi, Y. 2001. Nitrogen-to-protein conversion factors for common vegetables in Japan. *J. Food Sci.* **66**: 412–415.

Gevaert, F., Janquin, M.-A., Davoult, D. 2008. Biometrics in *Laminaria digitata* : A usefool tool to assess biomass, carbon and nitrogen contents. *Journal of Sea Research.* **60:** 215-219

Jarayabhand, P., Paphavasit, N.1996. A review of the culture of tropical abalone with special reference to Thailand. *Aquaculture.***140**:159–168

Jones, C.L.W and Britz, P.J. 2006. Development of a low-protein, water-stable kelp diet forthe South African abalone culture industry. Paper O46, 6th International AbaloneSymposium,19–24 February 2006, Puerto Varas, Chile

Lapointe, B.E. and Duke, C.S. 1984. Biochemical strategies for growth of *Gracilaria tikvahiae* (Rhodophyta) in relation to light intensity and nitrogen availability. *J. Phycol.* **20**:488-495

Legler, G., Muller-Platz, C.M., Mentges-Hettkamp, M., Pflieger, G., Julich, E. 1985. On the chemical basis of the Lowry protein determination. *Anal. Biochem.* **150**: 278-287.

Lobban, C.S., Harrison, P.J. 1994. Seaweed ecology and physiology. *Caimbridge University*

press, Caimbridge. 366.

Mercer, J.P., Mai, K.S., Donlon, J.1993. Comparative studies on the nutrition of two species of abalone *Haliotis turberculata* Linnaeus and *Haliotis discus hannai* Ino. I. Effects of algal diets on growth and biochemical composition. *Invertebrate. Reprodtion and Development* **23**: 2-3

Mattoo, R.L., Ishaq, M., Saleemuddin, M. 1987. Protein assay by Coomassie Brilliant Blue G-250-binding method is unsuitable for plant tissues rich in phenols and phenolases. *Anal. Biochem.* **163**: 376–384.

Naidoo, K., Maneveldt, G., Ruck,K. & Bolton, J.J. 2006. A comparison of variousseaweed based diets and formulated feed on growth rate of abalone in a landbased aquaculture system. *Journal of Applied Phycology.* **18**: 437–443

Najmudeen, T.M., Victor, A.C. 2004. Seed production and juvenile rearing of the tropical abalone Haliotis varia Linnaeus 1758. *Aquaculture* **234**:277–292

Probyn, T.A., McQuaid.1985. In situ measurements of nitrogenous nutrient uptake by Ecklonia maxima) and phytoplankton in a nitrate-rich upwelling environment. *Marine Biology* **88**: 149-154

Rothman, M.D., Anderson, R.J., Smit, A.J. 2006.The effects of harvesting of the South African kelp (*Ecklonia maxima*) on kelp population structures, growth rate and recruitment. *Journal of Applied Phycology.* **18**: 335-341

Sales, J., Britz, P.J. 2001. Research on abalone (*Haliotis midae* L.) cultivation in South Africa. *Aquaculture Research* **32**: 863–875.

Smith, M.J., Bolton, J.J. and Robertson-Andersson, D.V. 2009(submitted)

Simon, C.A., Kaiser,H. and Britz, P.J. 2004. Infestation of the abalone, *Haliotis midae*, by the sabellid, *Terebrasabella heterouncinata*, under intensive culture conditions, and the influence of infestation on abalone growth. *Aquaculture* **232**: 29–40

Stegenga, H., Bolton, J.J., Anderson, R.J. 1997. Seaweeds of the South African West coast. Creda Press, Cape Town. 655pp.

Stepto, N.K., Cook, P.A. 1993. Feeding preferences of the juvenile South African abalone *Haliotis midae* (Linnaeus, 1758). *Journal of Shellfish Research* **15**: 653–657

Sjøtun, K., Fredriksen, S., Rueness, J. 1996. Seasonal growth and carbon and nitrogen content in canopy and first year plants of *Laminaria hyperborea* (Laminariales, Phaeophyceae).

Phycologia **35**: 1-8

Troell, M., Robertson-Andersson, D., Anderson, R.J., Bolton,J.J., Manaveldt, G., Halling,C and Probyn,T. 2006. Abalone farming in South Africa: An overview with perspectives on kelp resources, abalone feed, potential for on farm seaweed production and socio-economic importance. *Aquaculture* **257:** 266-281

Uki, N., Watanabe, T. 1992. Review of nutritional requirements of abalone (*Haliotis* spp) and development of more efficient artificial diets. In Sheppard SA. Tregner MJ, Gruzmandel Prco (Eds.) *Abalone of the world. Biology Fisheries and Culture.* Fishing news books, Cambridge: 504-517

Viera, M.P., Pinchetti, J.L.G., de Vicose, G.C., Bilbao, A., Suárez, S., Haroun, R.J., Izquierdo M.S. 2005. Suitability of three macroalgae as a feed for the abalone *Haliotis tuberculata coccinea* Reeve. *Aquaculture* **248**: 75–82

Von Holdt, M.M., Ligtheim, S.P., Nunn, J.R. 1955. South African seaweeds: Seasonal variations in the chemical composition of some Phaeophyceae. *Journal of Science of Food in Agriculture.* **6**: 193-197.

Wong, K.H., Cheung, P.C.K. 2000. Nutritional evaluation of some subtropical red and green seaweeds Part II. In vitro protein digestibility and amino acid profiles of protein concentrates. *Food Chemistry* **72:** 11-17

Supplementary Table 1: Preparation of Diluted Albumin (BSA) Standards used in the protein analysis of the Bradford Method

Dilution Scheme for Standard Test Tube and Microplate Protocols (Working Range = 100–1,500 µg/ml)

Vial	Volume of Diluent	Volume and Source of BSA	Final BSA Concentration
A	0	300 µl of Stock	2,000 µg/ml
B	125 µl	375 µl of Stock	1,500 µg/ml
C	325 µl	325 µl of Stock	1,000 µg/ml
D	175 µl	175 µl of vial B dilution	750 µg/ml
E	325 µl	325 µl of vial C dilution	500 µg/ml
F	325 µl	325 µl of vial E dilution	250 µg/ml
G	325 µl	325 µl of vial F dilution	125 µg/ml
H	400 µl	100 µl of vial G dilution	25 µg/ml
I	400 µl	0	0 µg/ml = Blank