Chemosensory properties of different *Ulva* extracts and their effects on *Tripneustes gratilla*

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Abstract:

Identifying the chemical compounds that attract urchins to seaweeds will increase sea urchin aquaculture efficiency as these compounds can be utilized in the production of artificial feeds enhancing their attractiveness and palatability. This study investigated the chemosensory preferences of the tropical sea urchin *Tripneustes gratilla* for four crude extracts of *Ulva* as well as Fresh Ulva and an artificial feed. The four crude extracts of *Ulva* (Methanolic, Ethanolic, Chloroform and Chloroform-Methanol extracts) were tested by pair-wise chemosensory trials in a Y shaped maze. Urchins were significantly deterred by both the Chloroform and Chloroform-Methanol extracts as well as the solvent controls and artificial feed, however *T. gratilla* was significantly attracted to both the Methanolic and Ethanolic extracts. These two extracts were tested individually against *Ulva* and the urchins could not distinguish between *Ulva* and the extracts. When these two extracts were tested against one another it was found that the urchins were significantly attracted to the Methanolic extract. The Methanolic extract was then added to the artificial feed and significantly improved the feeds attractability. This indicates that these extracts, or the compounds which are responsible for this effect, can be incorporated into artificial feeds thereby making them more attractive to the sea urchin. The percentage yield of *Ulva* that each solvent extracted was different, although only the Ethanolic extract was found to be significantly different from the Chloroform and Chloroform-Methanol extracts. Future studies are needed to examine the chemical composition of these specific chemosensory compounds. By incorporating these attractive extracts into artificial feeds, South Africa can potentially improve the cultivation success of *T. gratilla* therefore expanding the economy of the local aquaculture industry through the export of this highly valued seafood product.

Introduction:

There has recently been an over-exploitation of natural populations of many sea urchin species due to the high demand for their roe (gonads) which is consumed in East Asian and European countries as it is a highly valued seafood product (Kelly *et al*., 2000; Scholtz, 2013; Onitsuka *et al., in press*). This decline in the natural stocks of echinoid species has sparked an interest in research to improve cultivation methods of edible sea urchin species for commercial use (Kelly *et al*., 2000; Scholtz, 2013). Aquaculture has been practiced for over a thousand years, however, the recent increase in the demand for sea urchin gonads, as well as the high price associated with this product, has attracted entrepreneurs and researchers to the echinoculture industry, thus, leading to the increase in the annual world production of sea urchins (Fernandez and Pergent, 1998; Juinio-Meñez *et al.*, 2008; Scholtz, 2011).

In order for the cultivation of edible echinoid species to be financially rewarding, one needs to develop an efficient method to enhance the growth of the juvenile sea urchins (Fleming, 1995). This will however require sufficient knowledge of the chemosensory characteristics of particular chemicals which may attract the sea urchins to specific feed as well as make this feed more palatable.

There are some known echinoid species that have the suitable characteristics to be used in aquaculture today. These characteristics are a large gonadal production, rapid growth and a relatively low mortality rate (Lawrence, 2007; Scholtz, 2011). *Tripneustes gratilla* is one such South African species that possesses all of the characteristics needed for culture success. This species also has a high market acceptance in Japan, where the gonads are known as Uni (Scholtz, 2011). Recently there has been a push by the South African government to develop new high value aquaculture species for export (DAFF, 2013). *Tripneustes gratilla* is one of these recognized species and shows real potential to improve the economy through international trade (Cyrus *et al*., *in press*; Scholtz, 2013; Cyrus *et al*., 2014). This species is advantageous for use in aquaculture due to having a circumtropical distribution, a fast growth rate, the ability to feed continuously, early maturation and the ability to produce large gonads of excellent quality and a high market standard (Lawrence, 2007; Rahman et al., 2009; Scholtz, 2011; Cyrus *et al*., *in press*; Cyrus *et al*., 2014). However, in order for gonads to be suitable they need to be large enough, have a firm texture, have a dark orange or bright yellow colour and have no or a small amount of gametes inside (Robinson *et al*., 2002; Cyrus *et al*., 2014).

To improve both growth rate of cultured sea urchins and their gonad quality, high protein artificial feed needs to be used as the natural diet of seagrasses and macroalgae are not sufficient to make their gonads commercially viable (Cyrus *et al*., 2014). This is primarily due to the fact that a large amount of macroalgae would have to be consumed by the sea urchins in order to meet their protein requirements as these seaweeds have low protein content (Hammer *et al.,* 2006; Cook and Kelly, 2007; Shuuluka *et* al., 2012; Cyrus *et al*., 2014). Therefore the consumption of a small amount of artificial feed, which is high in protein and energy, would have the same effect as a large amount of macroalgae. Artificial feeds have high protein and energy content due to the incorporation of fish meal, which is expensive, potentially unsustainable and contributes a significant amount to the overall farm production costs (Fleming *et al.*, 1996). However this artificial feed can often be wasted if the artificial feed is not palatable to the organism being fed (McBride *et al.,* 1998; Dworjanyn *et al.,* 2007). The amount of energy and protein delivered to the organism that consumes the feed can be manipulated by changing the concentration or digestibility of certain feed ingredients or by changing how much of the feed the organism consumes (Jobling *et al.*, 2001; Cyrus *et al., in press*).

Artificial feeds are often made palatable by the addition of some type of attractant or feeding stimulant which encourages consumption and can thereby reduce the amount of wastage and enhance the organisms' growth (Kasumyan and DÖving, 2003; Dworjanyn *et al.,* 2007). The palatability of artificial feed is important, as shown in a previous study by Kasumyan and DÖving (2003). In this study they fed Rainbow trout fingerlings earthworms, which contained unpalatable compounds in the coelomic fluid, thus causing the fish to become satiated and consume less. Once these compounds were removed from the earthworm and replaced with a feeding stimulant (squid extract) there was an improvement in feed intake and therefore an improvement in their growth.

Ulva has become a very important macroalga, especially for use in feed for a variety of different organisms and has been identified as an ideal dietary ingredient especially for certain fish species (Ergün *et al.*, 2008). Ergün *et al.* (2008) investigated the effects that meal supplemented with *Ulva* and different levels of dietary lipids had on the body composition, growth performance, utilization of nutrients and feed efficiency of juvenile *Oreochromis niloticus,* the Nile tilapia, through nutrition trials. When they added 5% *Ulva* meal to the diet that were fed to the tilapia they found that these fish had a significantly greater growth performance compared to the tilapia fed the 0% *Ulva* diet. This 5% *Ulva* diet was also found to improve the protein efficiency ratio and feed conversion ratio. Thus the use of *Ulva* in feed has a wide application in the aquafeed industry as it improves growth rate, resistance to disease and feed digestibility (Jobling *et al.*, 2001; Ergün *et al.*, 2008; Cyrus *et al., in press*).

When working with the sea urchin *Tripneustes gratilla*, Cyrus *et al*. (*in press*) tested their preference for four species of South African macroalgae (*Ecklonia maxima, Gigartina polycarpa*, *Ulva rigida* and *Porphyra capensis*) and found that these urchins were significantly attracted to *Ulva*. When they added dry *Ulva* to artificial feeds that were fed to *T. gratilla*, they determined that the feed that contained the highest inclusion of *U. rigida* (20%) significantly improved the feeds palatability compared to a feed that did not contain *U. rigida*. During this study they also determined that consumption and protein intake were increased due to increased protein digestibility which was contributed to the inclusion of *Ulva*. Similarly a study by Akakabe and Kajiwara (2008) tested the preference of the herbivorous gastropod, *Lunella coronata coreensis*, for 7 algae species (*Ulva pertusa, Carpopeltis affinis, Codium fragile, Undaria pinnatifida, Gelidium amansii*, *Caulerpa okamurae* and *Dictyopteris prolifera*) and found that the green algae *Ulva pertusa* was significantly preferred by the gastropods. In order to determine what role the chemical compounds have on the snails, an essential oil was prepared of *U. pertusa* through using a "simultaneous distillation extraction apparatus" and incorporated into artificial food (Akakabe and Kajiwara, 2008). They found that this artificial feed containing essential oils of *U. pertusa* induced a strong positive response similar to that of fresh *U. pertusa*. This suggests that the feeding behaviors observed in this study are controlled by the volatile compounds of the algae extracted in the essential oil as feeding attractants.

These organisms are likely to be attracted to these marine algae because the algae are known to produce volatile secondary metabolites. These compounds are biosynthesized by marine algae and other organisms such as sponges in order to remain in a state of homeostasis within their environment and to protect themselves against grazers (Selvin and Lipton, 2004). The volatile secondary metabolites have been reported to show a variety of different biological activities that have ecological significance such as biofouling and controlling settlement in organisms as well as provide insight into the development of new drugs such as antivirals and antioxidants (Kim *et al.*, 1997; Selvin and Lipton, 2002; Selvin and Lipton, 2004; Wang *et al.*, 2010; Babu *et al.*, 2014). However there have been very few studies that have focused on looking at what volatile secondary metabolites of algae cause herbivores such as sea urchins to search for the chemical cue source and which of these secondary metabolites enhance attraction and consumption by herbivores (Hay and Fenical, 1988; Hay, 2009).

The volatile compounds produced by marine algae can act as either allelochemicals, which protect the algae against grazers, or pheromones that direct grazer social behavior or act as cues for grazers (Hay *et al.*, 1998; Pohnert and Boland, 2002; Akakabe and Kajiwara, 2008; Rowan, 2011). Interestingly, some species of algae can change the concentration and type of defenses when grazed upon (Van Alstyne *et al.*, 2001). One such volatile compound produced by algae is dimethylsulfoniopropionate (DMSP), which is regarded as a potent feeding attractant (Van Alstyne *et al.*, 2001; Garren *et al.*, 2014). *Ulva lactuca*, an alga that is consumed readily by the gastropod *Haliotis midae*, has been found to contain high levels of DMSP (Smit *et al.*, 2007). Furthermore, DMSP has been shown by Van Alstyne *et al.* (2001) to be a pheromone that is a significant attractant for the sea urchins *Strongylocentrotus purpuratus* and *Strongylocentrotus droebachiensis*. However, Van Alstyne *et al.* (2001) further found that an enzyme called DMSP-lyase was found in two algal species under study, *Ulva fenestrata* and *Polysiphonia hendryi*. This enzyme is able to catalyze the reaction that cleaves DMSP, an attractive compound, to dimethylsulfide (DMS) and acrylic acid when the plant is damaged by grazing herbivores. Van Alstyne (2001) further found that acrylic acid significantly deterred the urchins, *Strongylocentrotus purpuratus* and *Strongylocentrotus droebachiensis,* but not at the lowest concentration of this chemical. Furthermore, Van Alstyne and Houser (2003) found that when the urchin, *Strongylocentrotus droebachiensis*, were offered a choice between a diet with DMS and one without, they avoided diets containing DMS therefore concluding that DMS is a potent deterrent against this urchin species. These findings suggest that acrylic acid and DMS function as a chemical defense of the plant to grazing. However, when a diet containing acrylic acid was fed to the isopod *Idotea wosnesenskii*, it was not deterred at all but was instead attracted to the feed at low concentrations (Van Alstyne, 2001). A study by Bowker (2013) did pair-wise chemosensory trials in order to test whether DMS and acrylic acid deterred the tropical sea urchin, *Tripneustes gratilla*. The results suggested that DMS and acrylic acid did not deter or attract the urchins. This suggests that volatile chemical compounds produced by algae are species specific in that it may deter one herbivore, but it can significantly attract another (Hay *et al.*, 1987; Duffy and Hay, 1990).

Following on from the work of Cyrus *et al*. (*in press*) and Bowker (2013), this study shall focus on the volatile secondary metabolite extracts of *Ulva armoricana*. This species was chosen as it has been shown to attract *T. gratilla*, is easy to grow and is naturally occurring in South Africa (Dworjanyn *et al.*, 2007; Cyrus *et al*., *in press*). Extraction of the various chemical constituents can be done by using different solvents such as methanol, ethanol and chloroform. Each of these solvents has a polarity and their molecular structures differ. This means that these solvents will have a variety of different affinities for the chemicals found within *Ulva*. A polar solvent will dissolve polar secondary metabolites and likewise for non-polar solvents. Each extraction will provide a 'crude' product that can be used in chemosensory trials to establish whether they attract or deter *T. gratilla*.

This study investigated the Methanolic, Ethanolic and Chloroform extracts of *Ulva*. Methanolic extracts of seaweeds have been found to contain polyphenolic compounds, pigments, polyunsaturated fatty acids, glycerolipids and minerals (Sakata *et al*., 1988; Sakata *et al.*, 1989; Takahashi *et al.*, 2002; Santoso, 2004). A study by Kitamura *et al.* (1993) found that polyunsaturated fatty acids extracted from coralline red algae caused settlement and metamorphoses in two commercially important sea urchins. Sakata *et al.* (1988) and Sakata *et al*. (1989) showed that glycerolipids extracted from *Ulva pertusa* and the brown kelp *Eisenia bicyclis* were found to be a significant feeding stimulant for gastropods, *Turbo cornutus*, *Omphalius pfeifferi* and abalone *Haliotis discus* as well as the sea urchin *Strongylocentrotus intermedius*. Thus it would be interesting to test whether this extract that contains polyunsaturated fatty acids and glycerolipids attracts adult *T. gratilla*. Ethanolic extracts of seaweeds have been found to contain phenolic compounds, carbohydrates and glycosides (Alang *et al.*, 2009; Hassan and Ghareib, 2009). A study by Bowker (2013) did a preliminary chemosensory trial testing whether the adult urchin *T. gratilla* was attracted to an Ethanolic extract of *Ulva*. She found that this extract was not significant in attracting or deterring the urchin, although the urchins preferred this extract over the blank. Hence it will be interesting to do more trials with this. A study by Alang *et al.* (2009) showed that Chloroform extracts of *Ulva* consisted of carbohydrates, steroids and glycosides. Chloroform is relatively non-polar compared to Methanol and Ethanol and therefore should extract non-polar chemical compounds. A study by Díaz *et al.* (2006) found that agar-based artificial pellets containing non-polar *Gracilaria capensis* extracts were not preferred by grazers, however they found that when these pellets contained non-polar *Hypnea spicifera* extracts it caused the snail *Tricolia capensis* to increase its consumption of these pellets. In addition, Steinberg and Van Altena (1992) found that different concentrations of non-polar compounds extracted from seaweeds can either attract or deter *T. gratilla.* As a result, it would be interesting to see whether these non-polar compounds extracted from *Ulva* by chloroform would attract or deter *T. gratilla* compared to the effect of the polar extracts. Due to Chloroform being heavier than water as well as immiscible in water, there is a chance that the urchins might not pick up the extracts that are dissolved in the chloroform solvent. To ensure that the fraction of *Ulva* that Chloroform extracts is tested, the solvent will be evaporated so that only the extract remains, and this extract will be resuspended in methanol, a solvent which is miscible in water and by which certain non-polar chemicals can be re-suspended in methanol.

Table 1: Table showing solvents and their relative polarities and common chemicals extracted by each. Relative polarities from Reichardt (2003)

Solvents	Relative Polarities	Common Extracts
Methanol	0.762	polyphenolic compounds, pigments, polyunsaturated fatty acids, glycerolipids and minerals[1,2,3,4]
Ethanol	0.654	phenolic compounds, carbohydrates and glycosides[5,6]
Chloroform	0.259	carbohydrates, steroids and glycosides[5]

1= Sakata *et al*., 1988; 2= Sakata *et al.*, 1989; 3=Takahashi *et al.*, 2002; 4= Santoso, 2004; 5= Alang *et al.*, 2009; 6= Hassan and Ghareib, 2009.

The aim of this study was to test the chemosensory preferences of the tropical sea urchin *Tripneustes gratilla* for four crude extracts of *Ulva* as well as Fresh *Ulva* and an artificial feed (0U). In the future, this information may help us determine the secondary metabolites of *Ulva* that causes the sea urchins to significantly prefer this seaweed to others. In order to test this, Ethanolic, Methanolic, Chloroform and Chloroform-Methanol extracts of *Ulva* were prepared, and tested in a Y-shaped maze to determine whether the extracts acted as attractants or deterrents to *T. gratilla*. The results that will be attained from this study will provide valuable information on which extracts of *Ulva* attract or deter the urchin. The extract that is found to be the most attractive can then be combined with artificial feed in order to stimulate feeding and therefore enhance growth of these commercially important sea urchins, as the current suggestion is to add a feeding stimulant in the form of powdered seaweed to the feed (Sakata *et al*., 1988; Cyrus *et al.*, *in press*).

Materials and Methods:

Sea urchin collection and spawning

Twenty four adult *T. gratilla* with an average weight of 41±2g, width of 48±1mm and height of 29±1mm were collected by Dr. Brett Macey from shallow rock pools near Haga-Haga in the Eastern Cape, South Africa (32°45'4.23"S, 28°16'41.30"E) during low tide and transported to the Department of Agriculture, Forestry and Fisheries (DAFF) Aquaculture Research Facility in Sea Point, Cape Town, South Africa. They were held in a large glass tank (150cm (L) x 60cm (W) x 61cm (H)) supplied with heated recirculating filtered seawater with a salinity of 34-36 PSU and maintained at 24-26°C under natural light conditions as previously described by Cyrus *et al*. (*in press*). Heated water was used as this species of urchin has a mostly tropical distribution; however they do occur as far south as Haga-Haga near East London where the minimum mean monthly temperature of the ocean is 19°C (Bolton, 1986). Between each trial the urchins were fed *Ulva armoricana* and starved for 2 days before the start of the next trial to ensure the urchins were not satiated.

Chemosensory experiments

The chemosensory experiments were performed using a Y-shaped Plexiglas (LxWxH: 71x30x15cm) tank constructed by Cyrus *et al*. (2014) following the designs of Castilla and Crisp (1970) and Vadas (1977) (Fig. 1). Heated water (25±1°C) was supplied to each arm of the Y-shaped maze at a rate of $8L.min^{-1}$ using a Dolphin Canister filter water pump (C1000, China). The water entering each of these arms first flowed into a chamber at the start of each arm, and this chamber was separated from the rest of the Y-maze tank by Perspex that had a line of 4mm holes drilled into it.

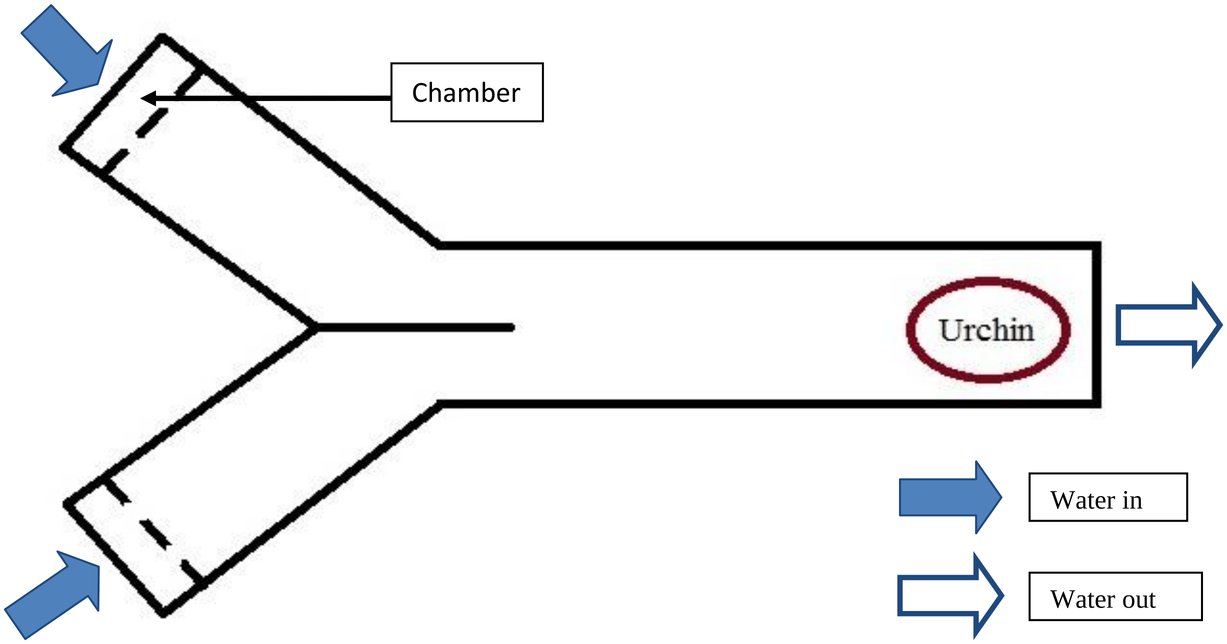

Figure 1: Y-maze used in chemosensory trials following the designs of Castilla and Crisp (1970) and Vadas (1977).

Before the chemosensory trials took place, the plume dynamics of this maze was assessed by dripping blue and red food colouring into each arm of the Y-maze as to determine whether equal mixing of the water and chemicals involved would occur between the two arms. This showed that there is equal mixing of the food colouring a few centimetres away from the split (Fig. 2).

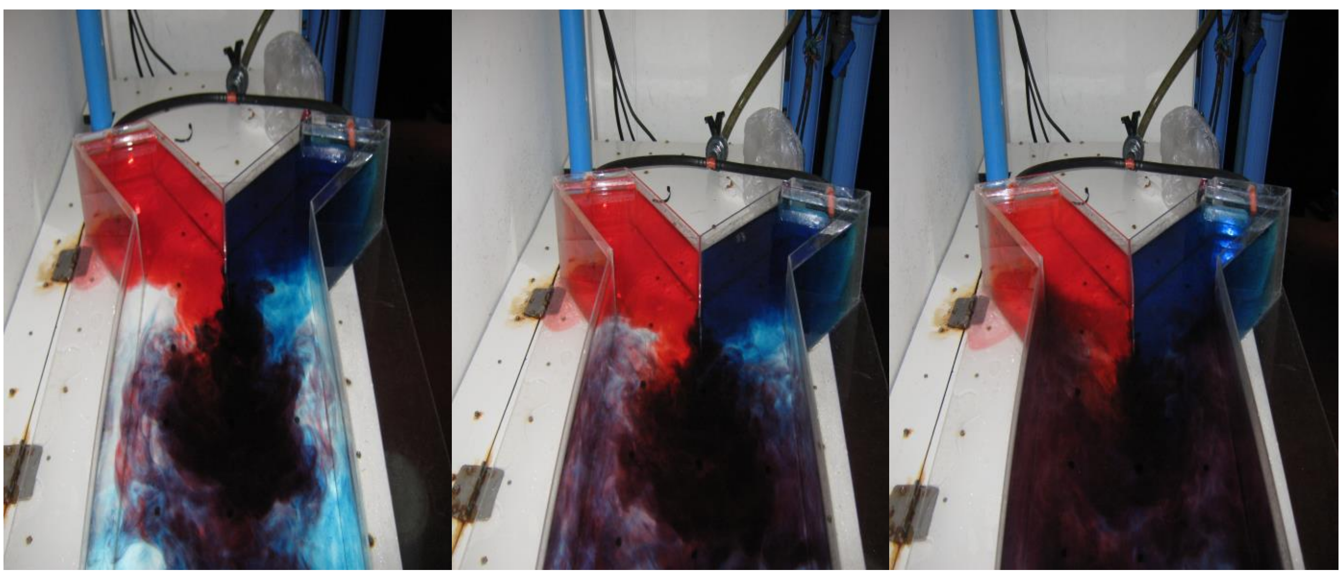

Figure 2: Maze plume dynamic tests assessed by using blue and red food colouring dropped in each arm (Photo: DS du Plessis)

The treatments tested were *Ulva*, artificial feed (0U: the 0% *Ulva* feed of Cyrus *et al.*, *in press*), Ethanolic, Methanolic, Chloroform, Chloroform-Methanol extracts and artificial feed with added Methanolic extract. The artificial feed was placed in a 120µm mesh bag prior to being placed into the Y-maze chamber as to allow the chemical cues to be released by this feed and flow into the Y-maze but prevent any feed particles flowing down the maze (Cyrus *et al*., 2014). Chemical extracts were dropped into the chambers using a dripper system that had a flow rate of 6ml.min^{-1} as to insure that each trial received the same concentration of the tested constituent each time (Fig. 3).

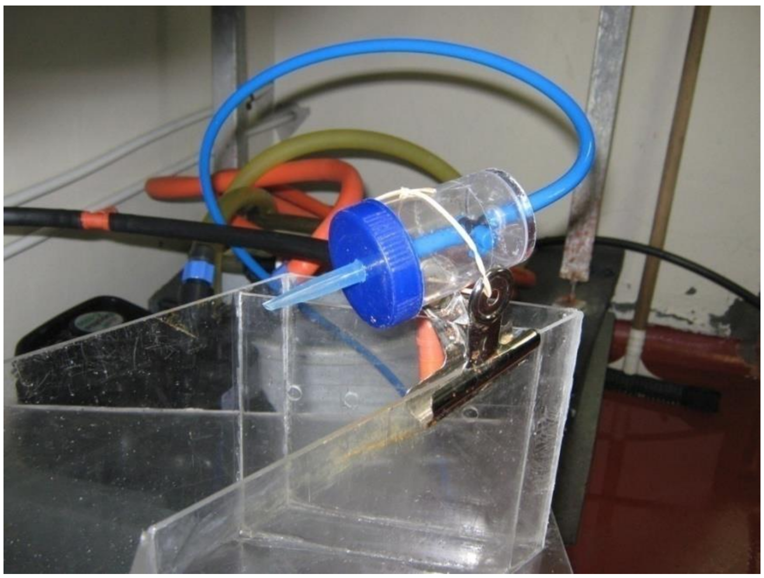

Figure 3: Dripper system used to drop extracts into each arm of the Y-shaped maze (Photo: DS du Plessis)

Pair-wise chemosensory trials (n= 12-15) were conducted over several days following the methods used by Prince and LeBlanc. (1992). During each trial an individual sea urchin was placed at the drainage end of the Y-shaped maze where their movements were then monitored over a 15 minute period. Preliminary experiments done by Cyrus *et al.* (2014) found that sea urchins are able to move from the start of the maze to one of the arms within 5 minutes. A chemical preference choice was recorded if the sea urchin moved from the drain end of the Y-shaped maze to one of the arms and was located 10 cm or more up this arm within 15 minutes. If the sea urchin could not complete this task within the allocated time-frame then a ‘no choice’ was recorded. The treatments tested were either tested in pairs or on an individual basis against a blank. The treatments were tested against blank arms to eliminate any Y-arm preference. After 3 trials the chemical constituent being tested was swapped to the other arm. To prevent the sea urchins from following a trail, and making the same choices as the previous urchin, the Y-maze was drained of all water, washed with fresh water and refilled in between each trial. For each trial, each individual sea urchin was tested only once in that trial day. The Ethanolic, Methanolic and Chloroform extracts were all tested against a control of ethanol, methanol and chloroform respectively. These controls were tested on their own at the same concentration as the extracts, against a blank, in order to determine whether the responses observed were a result of the chemical solvents themselves or to the extract.

Preparation of tested Constituents:

Ulva

20g of fresh *Ulva* was used in each trial and was collected from a cylindrical tank (101cm (H) x 120cm (D)) at the DAFF Research Aquarium. *Ulva* was contained in a flow through system and kept at an average temperature of 17°C. *Ulva* stock cultures were previously acquired and transported from Irvine & Johnson (I&J) Cape Abalone farm (Gansbaai) on the 1st of July 2014 and used to culture *Ulva* on site at the aquarium.

Artificial feeds

5g of the 0U artificial feed, as used by Cyrus *et* al. (2014), was used in each trial. This is a "semi-purified" feed, containing natural ingredients in as pure a form as is available, and it contains no dried *Ulva* (Cyrus *et al.*, 2014). As a final treatment, the chemical extract that was found to be the most attractive to the urchins will be added to 5g of artificial feed (560µL of extract; equivalent to the constituents contained in 1g of *Ulva*) and allowed to dry, to test whether this causes the urchins to be attracted to the feed. The nutrient analysis of this feed is shown in Table 2.

Table 2: The proximate nutrient analysis of 0% *Ulva* artificial feed prepared by Cyrus *et al.* (2014) (per g dry matter)

	0% Artificial feed
Protein (%)	26.48
Fat (%)	2.72
Moisture (%)	8.55
Ash (%)	7.57
Fibre (%)	6.07
Carbohydrates (%)	48.61
Gross Energy (MJ kg^{-1})	17.18

Crude extracts

Adapted from the methods of Tan *et al.* (2011), 50 g of dry *Ulva* was suspended in 450ml of each solvent within a 500 ml beaker. This beaker was then placed on a hot plate stirrer at 40°C and stirred for 3 hours at maximum speed. The resultant solution was then filtered through a mesh net of 0.5mm and strainer of approximately 60 micron and put into 50ml centrifuge tubes. These tubes were then put into a Rotina 380R centrifuge and centrifuged for 10 minutes at 10°C and at 6000 rpm to remove particulates. The supernatant in the centrifuged tubes was then poured into a 500ml laboratory bottle ensuring that the pellet of particulates was not disturbed. The bottle was covered in foil and placed in a freezer to be used later. The extract was placed in 24 Eppendorf tubes where each Eppendorf contained 1ml of the extract, and these were placed in a vacuum (Mivac, MST-23050-L00) centrifuge (Eppendorf AG concentrator plus) to be spun for 1 hour at 30°C. The resultant Eppendorfs were then consolidated into 6 Eppendorfs (4 Eppendorfs put into 1) each containing approximately 0.25ml of extract. 560µL of the resultant extract was then placed into 150ml of sea water as this represents the amount of the extract to be tested that would be in 1g of dry *Ulva* (see Appendix 1). To test that the solvent did not impact the choices made by each urchin, each solvent (methanol, ethanol, chloroform) was used as a control in this study where 560µL of the solvent was placed in 150ml of sea water.

The Chloroform-Methanol extract was initially made using the Chloroform extract procedure above except that all the chloroform solvent was evaporated in the vacuum centrifuge until only the fraction that chloroform extracted out of *Ulva* remained in each of the 24 Eppendorfs. This fraction was then resuspended with Methanol by placing 0.05ml of this solvent in each of the 24 Eppendorfs where they were then consolidated into 6 Eppendorfs ready to be used for the Chloroform-Methanol trials.

Yield of extracts

In order to calculate the percentage yield extracted from *Ulva* by each solvent, 16 Eppendorfs were initially weighed and then had 1ml of each extract (Methanolic, Ethanolic and Chloroform extract) placed inside four of these Eppendorfs. The remainder 4 Eppendorfs were left empty as a control to see if their mass changes. These Eppendorfs were placed in a vacuum centrifuge to be spun for 1 hr 30 minutes at 30°C or until the solvent had been completely evaporated. These Eppendorfs, now containing dry fractions of *Ulva* that the solvents extracted, were weighed once again. The difference between the initial mass of the Eppendorfs and this new mass was calculated to give the mass of the *Ulva* constituents extracted by the solvents in an individual Eppendorf. The yield was then calculated as follows using the mass of this extract:

(1) 0.001g in 1ml of Ethanolic extract was extracted out of $0.11\,\frac{g\ of\ \text{Ulva}}{ml\ Solvent}$ (calculated below: See Appendix 1)

(2) Therefore the yield is $\frac{0.001}{0.11} \times 100 = 0.9\%$ extracted in the first Ethanolic extract Eppendorf

The values (mean ± SE) shown in the results are an average of the 4 Eppendorfs analysed individually for each extract. For the Chloroform-Methanol extracts, once the Chloroform solvent was evaporated from the Chloroform extract 0.05ml of methanol was placed into each of the 4 Eppendorfs and vacuum centrifuged until all the methanol solvent was evaporated. This was then weighed and the difference between the empty Eppendorfs and these final Eppendorfs were used as to calculate the percentage yield.

Statistics:

A Bernoulli trial was used on each of the chemosensory trials where there was one of two possible outcomes, either a ‘choice’ or ‘no choice’ where the exact probability of a series of trials was calculated in Microsoft Excel (Prince and LeBlanc, 1992; Microsoft, 2007). Where in each paired test there was an expected distribution of 50:50. A two-tailed non-parametric Mann-Whitney U test was conducted using the statistical program R (R Core Team, 2013) to test whether the yields that each solvent extracted from *Ulva* were significantly different from one another.

Results:

When *T. gratilla* was put into the Y-maze with both arms left blank, the urchin showed no preference for either arm (Table 3). *T. gratilla* showed a significant preference for *Ulva*, Methanolic extract and Ethanolic extract when tested against a blank. No preference was recorded when Methanolic and Ethanolic extracts were each tested against *Ulva*. The ethanol, methanol and chloroform controls were shown to strongly deter the sea urchins when tested against blanks as well as the artificial feed, Chloroform extract and Chloroform-Methanol extracts. When the Methanolic extract was tested against the Ethanolic extract it was found that *T. gratilla* significantly preferred the Methanolic extract. Thus Methanolic extract was added to the artificial feed, and shown to significantly attract the urchins to the feed.

Table 3: Chemosensory trial results testing sea urchin attraction/deterrence to 0% *Ulva* Artificial feed, *Ulva*, ethanol, methanol, chloroform and the Ethanolic, Methanolic, Chloroform, Chloroform-Methanol extracts and the Artificial feed containing Methanolic extracts.

Paired Test	(n)	No. of sea urchins choosing	One-tailed Probability
Left Blank: Right Blank	12	7:5	P=0.1934
Artificial feed: Blank	13	3:10	p=0.0349
***Ulva*: Blank**	12	11: 1	p=0.0029
Ethanolic extract: Blank	15	11: 4	p=0.0417
ethanol: Blank	13	3: 9	p=0.0349
Methanolic extract: Blank	12	11:1	p=0.0029
methanol: Blank	13	3: 10	p=0.0349
Methanolic extract: *Ulva*	15	9: 6	p=0.1527
Ethanolic extract: *Ulva*	15	7: 8	p=0.1964
Chloroform extract: Blank	12	1: 11	p=0.0029
chloroform: Blank	12	2: 10	p=0.0161
Chloroform-Methanol extract: Blank	12	2: 10	p=0.0161
Ethanolic extract: Methanolic extract	12	1: 11	p=0.0029
Artificial feed + Methanolic extract: Blank	12	12: 0	p=0.0002

Each solvent extracted a different percentage of *Ulva* constituents. The ethanol solvent was found to extract the lowest percentage of chemical compounds whereas the chloroform solvent was shown to extract the highest percentage of chemical constituents compared to the rest of the solvents.

Table 4: Yield of *Ulva* each solvent extracts

Extract	Yield of extracts (%)
Ethanolic extract	1.1±0.23
Methanolic extract	2.1±0.57
Chloroform extract	2.7±0.37
Chloroform-Methanol extract	2.4±0.23

The yield of *Ulva* that ethanol extracts was found to be significantly different to that of which chloroform and chloroform-methanol can extract but not significantly different to that of which methanol extracts. The methanol, chloroform and chloroform-methanol solvents were not significantly different in terms of the yield each was able to extract from *Ulva*.

Table 5: Non-parametric Mann-Whitney U test, testing the difference between the yield each solvent extracts from *Ulva*

Mann-Whitney U test	U statistic	Degrees of freedom	Two-tailed probability
Ethanolic extract: Methanolic extract	3.5	6	p=0.2059
Ethanolic extract: Chloroform extract	0.5	6	p=0.0359
Ethanolic extract: Chloroform-Methanol extract	0.5	6	p=0.0325
Methanolic extract: Chloroform extract	4.5	6	p=0.3688
Methanolic extract: Chloroform-Methanol extract	5	6	p=0.4480
Chloroform extract: Chloroform-Methanol extract	9.5	6	p=0.7389

Discussion:

Herbivores such as sea urchins are known to be attracted or deterred by algae depending on their chemical characteristics and how attractive the algae are to the organism (Hay and Fenical, 1988; Meyer *et al.*, 1994; Dworjanyn *et al.*, 2007). In this study, several pair-wise chemosensory trials were carried out to test the chemical preference of *T. gratilla* for a number of 'crude' extracts of *Ulva*. The extract that is found to be the most attractive to the urchins could then be incorporated into the artificial feeds, in order to increase the attractiveness of the feed to the urchins thereby increasing the delivery of nutrients to an individual (Jobling *et al.*, 2001; Cyrus *et al.*, *in press*). These chemosensory trials were carried out using 10 treatments namely *Ulva*, artificial feed (0U feed of Cyrus *et al.*, *in press*), Ethanolic, Methanolic, Chloroform and Chloroform-Methanol extracts, artificial feed containing 0.560µL Methanolic extract as well as the methanol, ethanol and chloroform solvent controls. The urchins were found to be significantly attracted to fresh *Ulva*, Methanolic and Ethanolic extracts as well as the artificial feed with the added Methanolic extract, and were significantly deterred by the artificial feed, Chloroform and Chloroform-Methanol extracts as well as the solvent controls. The percentage yield of *Ulva* extracted by each solvent was found to differ, where the chloroform solvent had the highest yield and the ethanol solvent extracted the lowest yield. The Chloroform-Methanol extraction procedure seemed to extract a lower yield compared to the Chloroform extraction procedure, possibly due to methanol not being able to take up all the non-polar compounds extracted by chloroform. The Chloroform-Methanol extract had a higher yield than the Methanol extract, and although not significantly different, this suggests that chloroform extracts more chemical constituents from *Ulva* than methanol. It is possible that chloroform can extract chemicals from *Ulva* that are soluble in methanol which methanol alone cannot remove from *Ulva*.

Studies by Cyrus *et al.* (2014) and Dworjanyn *et al.* (2007) support our findings that fresh *Ulva* is attractive to the sea urchin *T. gratilla*. However their results as well as the results of Bowker (2013) were conflicting when we looked at the attractiveness of the artificial feed which was shown to significantly deter urchins, compared to the previous studies where it was found that the urchin either ignored the artificial feed or showed no discrimination between the blank and the feed (note: Bowker (2013) and Cyrus *et al.* (2014) used the same artificial feed as in the current study). However, the result obtained in the present study could have occurred due to the feed being created in 2011 and having undergone prolonged storage in a freezer where it may have deteriorated and perhaps changed its chemical composition thereby deterring the urchins (Karatarakis, 2005).

The Methanolic extract of *Ulva* was found to significantly attract the sea urchins. These results support the findings by Sakata *et al.* (1988) and Sakata *et al.* (1989) where Methanolic extracts of algae (*Ulva pertusa* and the kelp *Eisenia bicyclis* respectively) were found to contain several glycerolipids that were demonstrated to be a powerful feeding stimulant for the marine gastropods, *Turbo cornutus, Omphalius pfeifferi* and abalone *Haliotis discus* as well as for the sea urchin *Strongylocentrotus intermedius*. Interestingly, in a study done by Kitamura *et al.* (1993), polyunsaturated fatty acids extracted from *Corallina pilulifera*, a geniculate coralline red alga, was found to cause the larvae of two commercially important sea urchins, *Pseudocentrotus depressus* and *Anthocidaris crassispina*, to settle and metamorphose. Furthermore, Santoso (2004) found that the Methanolic extract of seaweeds contain polyunsaturated fatty acids, polyphenolic compounds, pigment and minerals. A study by Garren *et al.* (2014) used methanol to extract DMSP from homogenized mucus of the corals *Acropora millepora* and *Pocillopora damicornis*. DMSP has been shown by Van Alstyne *et al.* (2001) to be a potent attractant for sea urchins. This suggests that methanol is able to extract compounds from seaweeds that are attractants for certain marine herbivores. The methanol control was found to significantly deter the urchins, therefore further supporting that the attractive properties are in the fraction of *Ulva* that this solvent extracts rather than the solvent itself. It also indicates that the extracted constituents are strong enough to overcome the deterrent properties of the solvent. When the Methanolic extract was tested against *Ulva*, it was found that there was no significant differentiation between the treatments, with the urchins choosing the Methanolic extract nine times compared to choosing *Ulva* six times. These findings suggest that *T. gratilla* respond to the chemosensory characteristics of *Ulva*, and that this Methanolic extract could be used to make artificial feed more appealing and palatable to urchins.

T. gratilla significantly preferred the Ethanolic extract when given a choice between a blank and the extract. Even though 4 out of 15 urchins selected the blank, the results found in this study contradict the findings of Bowker (2013) where the Ethanolic extract of *Ulva* seemed to neither attract nor deter the sea urchins significantly. In that study, 8 out of 12 urchins chose the extract whereas 4 out of 12 chose the blank, therefore favoring the extract over the blank. The urchins were significantly deterred by the ethanol control, suggesting that the ethanol solvent also extracts a fraction of *Ulva* that the urchins are attracted to and that this attraction is not caused by the solvent itself. As with the Methanolic extract, the urchins could not differentiate between *Ulva* and the Ethanolic extract which was also found by Bowker (2013). This further supports the above suggestion that *T. gratilla* responds to the chemosensory characteristics of *Ulva*. However, *T.* gratilla was found to be significantly attracted to the Methanolic extract when given a choice between the Methanolic extract and the Ethanolic extract. Even though the urchins are significantly more attracted to the Methanolic extract compared to the Ethanolic extract, this Ethanolic extract could too be used in combination with artificial feed to make feeds more attractive to urchins.

The Chloroform extract was found to significantly deter the urchins. However, the chloroform solvent likewise deterred the urchins. Therefore the solvent could be causing the deterrence observed because chloroform does not appeal to the urchins. Since chloroform extracts relatively the same yield percentage as a Chloroform-Methanol extract from *Ulva*, a Chloroform-Methanol extract was made to test whether it was the fractions in *Ulva* that chloroform extracted or the chloroform solvent itself that caused this avoidance. However, we found that the Chloroform-Methanol extract also significantly deterred the urchins. This suggests that the fraction of *Ulva* that chloroform extracts could contain non-polar compounds or secondary metabolites that deter the urchins. These results support the finding by Diaz *et al.* (2006) where the grazers (isopods *Paridotea rubra*) that were fed agar-based artificial feed containing non-polar extracts of *Gracilaria capensis* showed no preference for this feed but rather preferred the algae itself. Which is similar to this study where the *Ulva* was found to significantly attract the sea urchins, but the Chloroform extract of *Ulva* was found to deter the sea urchins. However, the non-polar compounds extracted here might deter *T. gratilla* but it could be an attractant for another organism. Hay *et al.* (1987) found that the brown algae, *Dictyota dichotoma*, produces diterpenoid alcohols that deter both urchins and tropical fish but was found to have no effect on or was an attractant for tubicolous amphipods that reside and feed on this alga. Therefore chemical defenses of algae are species specific, where one compound might deter one herbivore; the same compound might attract another. The present study used a constant concentration of the Chloroform extract. Perhaps changing the concentration of this extract will elicit a different result as was found by Steinberg and Van Altena (1992). They found that depending on the concentration of secondary non-polar compounds, these compounds can either attract or deter the urchin *T. gratilla*. At low concentrations of non-polar compounds the urchin was found to be deterred, while at high concentrations they were attracted. However, Steinberg and Van Altena (1992) tested the effects of different concentrations of phlorotannins, a non-polar compound from brown algae, on the preference of the urchins. Therefore the results shown in their study might not elicit the same response when using non-polar extracts of the green algae, *Ulva*. Future studies should look at the effect different concentrations of the *Ulva* Chloroform extract has on *T. gratilla* to determine whether different concentrations of this extract will elicit a different response.

Since the Methanolic extract of *Ulva* was found to be the most attractive treatment out of the extracts and was found to be as attractive as the fresh *Ulva*, this was applied to the artificial feed. The artificial feed, which was specifically manufactured for the production of local *T. gratilla* (Cyrus *et al.*, *in press*), was successfully made more appealing to the urchins by dripping approximately 0.560µL of Methanolic extract onto it and allowing this to dry. This artificial feed that contained the Methanolic extract of *Ulva* was found to significantly attract the urchins whereas previously the same artificial feed without extract was found to significantly deter the urchins. This finding suggests that artificial feeds attractiveness can be improved to the same extent by the incorporation of an *Ulva* Methanol extract rather than having to incorporate dry *Ulva* into the feed (Cyrus *et al.*, *in press*).

Future studies should look into varying the concentration of the chemical extracts of *Ulva* used as different concentrations of each extract could elicit a different result. This was shown by Cyrus *et al.* (*in press*), where they used varying amounts of *Ulva* in artificial feed and found that the consumption of this feed by *T. gratilla* was lower for the 0% and 5% *Ulva* artificial feed compared to the 15% and 20% *Ulva* feed. The effect that seasonal changes have on this urchin's food preferences should be tested as with different seasons come changes in productivity. This change in productivity is due to changes in salinity, temperature, light and nutrient availability and as a result can change the chemical composition of *Ulva* thereby changing the attractiveness of this alga to the urchin (Abdel-Fattah and Sary, 1987; Shuuluka *et al*, 2012). As found by Steinberg and Van Altena (1992), the absolute consumption of algae (*Sargassum linearifolium, Ecklonia radiata*, *Phyllospora comosa*, *Sargassum verstitum*, *Homoeostrichus sinclairii* and *Delisea pulchra*) by *T. gratilla* was less in winter. This could have occurred due to two reasons. The cool seawater temperatures during winter could have resulted in a slower metabolism of the sea urchins and therefore lead to a reduced consumption of algae or this reduced consumption occurred due to changes in the chemical composition of the algae. Therefore as a future study, it might be interesting to find out why the consumption changed, thereby potentially providing insight into what properties of algae are needed in order to attract urchins.

This study shows that *T. gratilla* is attracted to the more polar extracts compared to the non-polar extracts of *Ulva*. This suggests that there might be a similar chemical fraction in the Methanolic and Ethanolic extracts that causes the attraction observed. Future studies should investigate how these three extractions differ in their chemical constituents and what is in the Methanol and Ethanol extracts that isn't in the Chloroform extract. By doing chemosensory trials on the different chemicals found in these extracts one can identify which chemical is causing the attraction. This can be done using similar methods to that of Takahashi *et al.* (2002), where the extract can be separated through bioassay in combination with thin-layer and column chromatography which isolates active compounds where these compounds chemical structures can be determined through chemical and spectral methods. Another method to identify the chemical constituents present in extracts of *Ulva* is by using Gas chromatography-mass spectrometry (GC-MS) analysis as done by Babu *et al.* (2014). This is an accurate and simple method that can be used to predict the number of chemical constituents present in extracts. By using this method Babu *et al*. (2014) found 17 different compounds in an Ethanolic extract of *Ulva lactuca*. However, it is quite possible that there is a suite of chemicals, not just one, in *Ulva* that causes the urchins to be attracted to it.

This study has provided useful insight into the properties of *Ulva* that attract *T. gratilla* and helped us to significantly improve the appeal of the artificial feed to the urchins. Through future studies that assess what chemicals in these extracts attract or deter the urchins to the feed we could enhance the palatability of this feed thereby promoting the consumption of the feed and consequently boosting the growth of the urchin and its gonads. South Africa is currently looking to expand its export of highly valued marine products and in order to do this South Africa will need a reliable feed that is readily consumed. Thus these findings will help South African aquaculture industries in furthering the development of artificial feed by incorporating attractants into feed allowing aquafarmers to produce these commercially important urchins locally and at a more efficient rate thereby potentially benefitting the local economy.

References:

Abdel-Fattah, A., Sary, H. H. (1987). Selective isolation of glycoprotein materials from the green seaweed *Ulva lactuca. Pakistan Journal of Biochemistry*, 20, 61–65.

Akakabe, Y., Kajiwara, T. (2008). Bioactive volatile compounds from marine algae: feeding attractants. *Journal of Applied Phycology*, 20(5), 661–664.

Alang, G., Kaur, R., Singh, A., Singla, P., Pradesh, U. (2009). Antimicrobial activity of *Ulva lactuca* extracts and its fractions. *Pharmacology online*, 3, 107–117.

Babu, M., Johnson, M., Raja, D., Arockiaraj, A., Vinnarasi, J. (2014). Chemical Constituents and their Biological Activity of *Ulva Lactuca Linn*. *International Journal of Pharmaceutics and Drug Analysis*, 2(7), 595–600.

Bolton, J. J. (1986). Marine phytogeography of the Benguela upwelling region on the west coast of southern Africa - A temperature-dependent approach. *Botanica Marina*, 29, 251-256.

Bowker, J. (2013). Honours Thesis - Attractant properties of chemical constituents of the green macroalga *Ulva* and their response effects on the commercially important sea urchin *Tripneustes gratilla.* (Zoology Department, University of Cape Town). Record number: 000065758

Castilla, J.C., Crisp, D.J. (1970). Responses of *Asterias rubens* to olfactory stimuli. *Journal of the Marine Biological Association of the United Kingdom*, 59, 829-847.

Reichardt, C. (2003). Solvents and Solvent Effects in Organic Chemistry, Wiley-VCH Publishers, 3rd edition.

Cook, E.J., Kelly, M.S. (2007). Enhanced production of sea urchin *Paracentrotus lividus* in integrated open-water cultivation with Atlantic salmon *Salmo salar*. *Aquaculture*, 273, 573-585.

Cyrus, M.D., Bolton, J.J., De Wet, L., Macey, B.M. (2014). The development of a formulated feed containing *Ulva* (Chlorophyta) to promote rapid growth and enhanced production of high quality roe in the sea urchin *Tripneustes gratilla* (Linnaeus). *Aquaculture Research*, 45(1), 159-176.

Cyrus, M.D., Bolton, J.J., De Wet, L., Macey, B.M. (*in press*). The advantages of *Ulva* (Chlorophyta) as an additive in sea urchin formulated feeds: Effects on palatability, consumption and digestibility. *Aquaculture Nutrition*.

Department of Agriculture, Forestry and Fisheries (DAFF). (2013). South Africa’s Aquaculture Yearbook 2013. South Africa, Cape Town.

Díaz, E., Güldenzoph, C., Molis, M., McQuaid, C., Wahl, M. (2006). Variability in grazer-mediated defensive responses of green and red macroalgae on the south coast of South Africa. *Marine Biology*, 149(6), 1301–1311.

Duffy, J., Hay, M. (1990). Seaweed adaptations to herbivory. *Bioscience*, 40(5), 368–375.

Dworjanyn, S., Pirozzi, I., Liu, W. (2007).The effect of the addition of algae feeding stimulants to artificial diets for the sea urchin *Tripneustes gratilla*. *Aquaculture*, 273, 624–633.

Ergün, S., Soyutürk, M., Güroy, B., Güroy, D., Merrifield, D. (2008). Influence of *Ulva* meal on growth, feed utilization, and body composition of juvenile Nile tilapia (*Oreochromis niloticus*) at two levels of dietary lipid. *Aquaculture International*, 17(4), 355–361.

Fernandez, C., Pergent, G. (1998). Effect of different formulated diets and rearing conditions on growth parameters in the sea urchin *Paracentrotus lividus*. *Journal of Shellfish research*, 17, 1571–1581.

Fleming, A. (1995). Growth, intake, feed conversion efficiency and chemosensory preference of the Australian abalone, *Haliotis rubra*. *Aquaculture*, 132, 297–311.

Fleming, A. E., Van Barneveld, R. J., Hone, P. W. (1996). The development of artificial diets for abalone: a review and future directions. *Aquaculture*, 140, 5-53.

Garren, M., Son, K., Raina, J.-B., Rusconi, R., Menolascina, F., Shapiro, O.H., Tout, J., Bourne, D.G., Seymour, J.R., Stocker, R. (2014). A bacterial pathogen uses dimethylsulfoniopropionate as a cue to target heat-stressed corals. *The ISME Journal*, 8(5), 999–1007.

Hammer, B.W., Hammer H.S., Watts, S.A., Lawrence, J.M., Lawrence A.L. (2006) The effect of dietary protein and carbohydrate concentration on the biochemical composition and gametogenic condition of the sea urchin *Lytechinus variegatus*. *Journal of Experimental Marine Biology and Ecology*, 344, 109–121.

Hassan, S., Ghareib, H. (2009). Bioactivity of *Ulva lactuca* L. acetone extract on germination and growth of lettuce and tomato plants. *African Journal of Biotechnology*, 8, 3832–3838.

Hay, M. E. (2009). Marine chemical ecology: chemical signals and cues structure marine populations, communities, and ecosystems. *Annual Review of Marine Science*, 1, 193–212.

Hay, M., Duffy, J., Pfister, C., Fenical, W. (1987). Chemical defense against different marine herbivores: are amphipods insect equivalents? *Ecology*, 68(6), 1567–1580.

Hay, M.E., Fenical, W. (1988). Marine plant-herbivore interactions: the ecology of chemical defence. *Annual review of ecology and systematic*, 19, 111–145.

Hay, M.E., Piel, J., Boland, W., Schnitzler, I. (1998). Seaweed sex pheromones and their degradation products frequently suppress amphipod feeding but rarely suppress sea urchin feeding. *Chemoecology*, 8, 91–98.

Jobling, M., Gomes, E., Dias, J. (2001). Feed types, manufacture and ingredients. In: Food Intake in Fish. (ed. by Houlihan, D., Boujard, T., Jobling, M.), *Blackwell Science*, Oxford, 25–48.

Juinio-Meñez, M.A., Bangi, H.G., Malay, M.C., Pastor, D. (2008). Enhancing the Recovery of Depleted *Tripneustes gratilla* Stocks Through Grow-Out Culture and Restocking. *Reviews in Fisheries Science,* 16, 35–43.

Karatarakis, N.J. (2005). Common problems with aquafeeds and feeding in marine farms in Greece. In :Montero D. (ed.), Basurco B. (ed.), Nengas I. (ed.), Alexis M. (ed.), Izquierdo M. (ed.). Mediterranean fish nutrition. Zaragoza : CIHEAM. 155-158 (Cahiers Options Méditerranéennes; n. 63)

Kasumyan, A., DÖving, K. (2003). Taste preferences in fishes. *Fish and Fisheries*, 4, 289–347.

Kelly, M.S., Hunter, A.J., Scholfield, C.L., McKenzie, J.D. (2000). Morphology and survivorship of larval *Psammechinus miliaris* (Gmelin) (Echinodermata: Echinoidea) in response to varying food quantity and quality. *Aquaculture*, 183, 223-240.

Kim, J.H., Hudson, J.B., Huang, A.M., Bannistes, K., Jin, H., Choi, T.J., Towers, G.H.N., Hong, Y.K., De Wreede, R.E. (1997). Biological Activities of Seaweed Extracts from British Columbia, Canada, and Korea. I. Antiviral Activity. *Canadian Journal of Botany,* 75(10), 1656-1660.

Kitamura, H., Kitahara, S., Koh, H.B. (1993). The induction of larval settlement and metamorphosis of two sea urchins, *Pseudo centrotus depressus* and *Anthocidaris crassispina*, by free fatty acids extracted from the coralline red alga *Corallina pilulifera*. *Marine Biology,* 115, 387–392

Lawrence, J. M. (2007). Edible Sea Urchins: Biology and Ecology. Second edition. *Developments in Aquaculture and Fisheries Science,* 37:i-xxv, 1-529.

Meyer, K.D., Paul, V.J., Sanger, H.R., Nelson, S.G. (1994). Effects of seaweed extracts and secondary metabolites on feeding by the herbivorous surgeonfish *Naso lituratus*. *Coral Reefs*, 13, 105-112.

McBride, S.C., Lawrence, J.M., Lawrence, A.L., Mulligan, T.J. (1998). The effect of protein concentration in prepared feeds on growth, feeding rate, total organic absorption, and gross assimilation efficiency of the sea urchin *Strongylocentrotus franciscanus*. *Journal of Shellfish Research*, 17, 1563–1570.

Microsoft. (2007). Microsoft Excel [computer software]. Redmond, Washington: Microsoft.

Onitsuka, T., Unuma, T., Kawamura, T. (*In press*). Effects of diet and temperature on post-settlement growth and survival of the short-spined sea urchin *Stronglyocentrotus intermedius*. *Aquaculture Research*.

Pohnert, G., Boland, W. (2002). The oxylipin chemistry of attraction and defense in brown algae and diatoms. *Natural Product Reports*, 19, 108–122.

Prince, J.S., LeBlanc, W.G. (1992). Comparative feeding preference of *Strongylocentrotus droebachiensis* (Echinoidea) for the invasive seaweed Codium fragile spp. tomentosoides (chlorophyceae) and four other sea weeds. *Marine Biology*, 113, 159-163.

Rahman, S., Tsuchiya, M., Uehara, T. (2009). Effects of temperature on hatching rate, embryonic development and early larval survival of the edible sea urchin, *Tripneustes gratilla*. *Biologia*, 64, 768–775.

R Core Team (2013). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Robinson, S.M.C., Castell J.D., Kennedy E.J. (2002). Developing suitable colour in the gonads of cultured green sea urchins (*Strongylocentrotus droebachiensis*).*Aquaculture*, 206, 289–303.

Rowan, D. D. (2011). Volatile metabolites. *Metabolites*, 1(1), 41–63.

Sakata, K.N., Sakura, T., Ina, K. (1988). Algal phagostimulants for marine herbivorous gastropods. *Journal of Chemical Ecology*, 14, 1405–1416

Sakata, K., Kato, K., In, K., Machiguchi, Y. (1989). Glycerolipids as potent feeding stimulants for the sea urchin, *Strongylocentrotus intermedius*. *Agricultural and Biological Chemistry*, 53, 1457–1459.

Santoso, J., Yoshie-Stark, Y., Suzuki, T. (2004). Anti-oxidant activity of methanol extracts from Indonesian seaweeds in an oil emulsion model. *Fisheries Science*, 70(1), 183–188.

Selvin, J., Lipton, A.P. (2002). Development of a Rapid ‘Mollusc Foot Adherence Bioassay’ for Detecting Potent Antifouling Bioactive Compounds, *Current Science*, 83, 735-737.

Selvin, J., Lipton, A. (2004). Biopotentials of *Ulva fasciata* and *Hypnea musciformis* collected from the peninsular coast of India. *Journal of Marine Science and Technology*, 12(1), 1–6.

Scholtz, R., Bolton, J., Macey, B. (2011). Effects of various microalgae on development and settlement of larvae of *Tripneustes gratilla* (Linnaeus) (Echinodermata : Echinoidae) and prospects of echinoculture in South Africa.

Scholtz, R., Bolton, J., Macey, B. (2013). Effects of different microalgal feeds and their influence on larval development in the white-spined sea urchin *Tripneustes gratilla*. *African Journal of Marine Science*, (May 2013), 37–41.

Shuuluka, D., Bolton, J. J., Anderson, R. J. (2012). Protein content, amino acid composition and nitrogen-to-protein conversion factors of *Ulva rigida* and *Ulva capensis* from natural populations and *Ulva lactuca* from an aquaculture system, in South Africa. *Journal of Applied Phycology*, 25(2), 677–685.

Smit, A., Robertson-Andersson, D., Peall, S., Bolton, J. (2007). Dimethylsulfoniopropionate (DMSP) accumulation in abalone *Haliotis midae* (Mollusca: Prosobranchia) after consumption of various diets, and consequences. *Aquaculture,* 269, 377-389.

Steinberg, P.D., Van Altena, I. (1992). Tolerance of marine invertebrate herbivores to brown algal phlorotannins in temperate Australasia. *Ecological Monographs*, 62, 189–222.

Takahashi, Y., Itoh, K., Ishii, M., Suzuki, M., Itabashi, Y. (2002). Induction of larval settlement and metamorphosis of the sea urchin *Strongylocentrotus intermedius* by glycoglycerolipids from the green alga *Ulvella lens*. *Marine Biology,* 75.

Tan, S. P., O'Sullivan, L., Prieto, M. L., Gardiner, G. E., Lawlor, P. G., Leonard, F., Duggan, P., McLoughlin, P., Hughes, H. (2011). Extraction and bioautographic-guided separation of antibacterial compounds from *Ulva lactuca*. *Journal of Applied Phycology*, 24(3), 513–523.

Vadas, R. (1977). Preferential feeding: an optimization strategy in sea urchins. *Ecological Monographs,* 47, 337–371.

Van Alstyne, K., Houser, L. (2003). Dimethylsulfide release during macroinvertebrate grazing and its role as an activated chemical defence. *Marine Ecology Progress Series,* 250, 175–181.

Van Alstyne, K.L., Wolfe, G.V., Freidenburg, T.L, Neill, A., Hicken, C. (2001). Activated defense systems in marine macroalgae: evidence for an ecological role for DMSP cleavage. *Maine. Ecology Progress Series*, 213, 53-65

Wang, C., Han, L., Kang, K., Shao, C. (2010). Secondary metabolites from green algae *Ulva pertusa*. *Chemistry of Natural Compounds.*

Appendix 1:

Calculations for extract concentration:

The amount of extract in 1g dry *Ulva* calculated:

(1) 50g dry *Ulva* suspended in 450ml solvent= $\frac{50g}{450ml} = 0.11\,\frac{g\ of\ \text{Ulva}}{ml\ Solvent}$

(2) Twenty four 1ml Eppendorfs can fit into the vacuum centrifuge at one time.

Therefore $24ml \times 0.11\,\frac{g\ of\ \text{Ulva}}{ml\ Solvent} = 2.67g\ of\ Ulva$

(3) 24ml of extract vacuum centrifuged at 30°C for 60 minutes

(4) The resultant Eppendorfs were consolidated into 6 Eppendorfs (4 into 1)

(5) 6x0.25ml=1.5ml

(6) $\frac{2.67\ g\ Ulva}{1.5ml\ solvent} = 1.78\,\frac{g\ Ulva}{ml\ Solvent}$

(7) The amount of extract in 1g dry Ulva = $\frac{1g\ Ulva}{1.78\,\frac{g\ Ulva}{ml\ Solvent}} = 0.56ml$ of extract needed to resemble 1g of *Ulva* in 150 ml of seawater