UNIVERSITY OF CAPE TOWN

Attractant properties of chemical constituents of the green macroalga *Ulva* and their response effects on the commercially important sea urchin *Tripneustes gratilla.*

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Abstract

Interest in commercial sea urchin aquaculture is growing worldwide. This is because sea urchins have good quality roe which is a delicacy in many countries. Since the quality of sea urchin roe is dependent on what the sea urchin eats, increased research is being carried out to understand sea urchin feeding preferences. Feeding preference is related to the attractiveness/palatability of the feed, which is thought to be based on its chemical composition. Since an unpalatable feed will result in the poor quality of the organism, evaluating the palatability of sea urchin artificial feeds is important. In South Africa, the sea urchin *Tripneustes gratilla* has been selected for aquaculture due to its fast growth and high quality roe. Although it is a generalist herbivore, various studies have shown that *T. gratilla* has a significant preference for the macroalgae *Ulva*. The purpose of this study is to assess what chemicals contained in *Ulva* cause this preference. Chemosensory trials involving *Ulva* and its chemical constituents, as well as wounded *Ulva* and an artificial feed, were carried out using a Y-shaped maze. Results indicate that *T. gratilla* are not deterred by DMS or acrylic acid as literature suggests. Since previous studies showed that these two chemicals deter urchins, this study demonstrates that there are species-specific relationships to chemicals found in algal material. Results also indicate that *T. gratilla* are deterred by wounded *Ulva*, ulvan and ethanol, but cannot differentiate between these constituents when the constituents are compared against each other or ethanolic extract, fresh *Ulva* and feed. Since the sea urchins are not consistently deterred by wounded *Ulva*, ulvan and ethanol when compared with other constituents, this indicates that *T. gratilla* are not very selective in what they are attracted to or deterred from.

Introduction

Sea urchin aquaculture

In the global food industry, aquaculture is one of the fastest growing sectors (Smit *et al.,* 2007). One reason for this is because delicacies such as abalones and sea urchin gonads are highly sought after in the Far East (Naidoo *et al.,* 2006; Cyrus *et al., in press*). Humans have been consuming sea urchins roe since ancient times (Fernandez and Pergent, 1998). Due to a high economic value, the sea urchin harvesting industry reached its peak in 1995 and has since been in continual decline due to over-exploitation (Juinio-Meñez *et al.,* 2008). To confront this problem, sea urchin aquaculture has become an extremely lucrative industry, resulting in an increase of the annual world production of sea urchins (Fernandez and Pergent, 1998; Juinio-Meñez *et al.,* 2008).

In order for sea urchin production to be successful, growth rates need to be fast enough to reach a marketable size and condition within an economically viable time frame (Fleming, 1995). Commercially viable gonads need to be large, have a firm texture, have few/no gametes and be of a bright yellow/orange colour (Robinson *et al.,* 2002; Cyrus *et al., in press*). To achieve optimum growth and gonad index, a variety of artificial feeds which incorporate different key components have been produced (Lawrence *et al.,* 1997; Shpigel *et al.,* 2005; Dworjanyn *et al.,* 2007; Cyrus *et al., in press*). In general, artificial feeds incorporate a large amount of protein in the form of fish meal. Protein is often the most costly component of aquaculture feeds and contributes significantly to the overall production costs (McBride *et al.,* 1998; Fernandez and Boudouresque, 2000; Pearce *et al.,* 2004; Dworjanyn *et al.,* 2007).

A cheaper alternative method for achieving optimum growth and gonad index is to alter the amount of feed consumed by the animal. This is known as increasing the palatability of the feed (Dworjanyn *et al.,* 2007) and is done by adding feeding stimulants to the feed or creating desirable chemosensory characteristics of the feed (Kasumyan and Doving, 2003). Palatability is a key concern when developing artificial aquaculture diets (Dworjanyn *et al.,* 2007). The palatability indirectly effects consumption and digestibility by making the food more or less attractive to the grazer. If a feed is unpalatable it will result in a large amount of waste from uneaten/undigested food as well as poor performance (growth and quality) of the organism. Alternatively, a more palatable feed will be more readily consumed and thus lower concentrations of expensive dietary components, such as protein, will be required.

The effects of feeding stimulants in aquaculture have mainly been investigated using finfish, where feeding stimulants incorporated into plant-based diets have been shown to increase the chemosensory characteristics of the feed, increasing consumption and digestibility (Kasumyan and Doving, 2003; Dworjanyn *et al.*, 2007). Amino acids (e.g. L-alanine, L-serine) acted as feeding stimulants for red seabream *Chrysophyrys major* (Goh and Tamura, 1980) and Tilapia (Johnsen and Adams, 1986), as well as nucleotides and nucleosides, sugars and other hydrocarbons. Unpalatable diets caused the fish to become satiated more quickly and therefore eat less food, decreasing their growth in the allotted time frame. Determining which chemosensory characteristics or feeding stimulants to use, however, depends on the aquacultured organism.

The food choices as well as the algal traits which herbivores such as sea urchins use to choose their food are poorly documented, but seem to be species-specific (Granado and Caballero, 2001). Marine herbivores are known to choose algae based on a number of factors, including edibility based on morphological (Hay, 1981) and chemical attributes (Paul and Hay, 1986; Meyer *et al.*, 1994; Granado and Caballero, 2001). Many authors have shown it is the chemical composition of plants which inhibits or stimulates grazing by herbivores (Hay and Fenical, 1988; Cronin and Hay, 1996; Hay, 1996; Van Alstyne *et al.*, 2001). Some chemicals which inhibit grazing are induced by herbivore grazing.

When herbivores graze on macroalgae they cause mechanical damage to the plant. In certain macroalgae, this mechanical damage activates an enzymatic conversion of stored precursor compounds into predator-deterrent compounds to inhibit further grazing (Paul and Van Alstyne, 1992; Van Alstyne and Houser, 2003). A variety of secondary metabolites is released and is known as the activated chemical defence system (Van Alstyne *et al.*, 2001; Van Alstyne and Houser, 2003; Weisemeyer *et al.*, 2007). The seaweed *Ulva* is one such alga that has an activated defence system (Paul and Hay, 1986; Van Alstyne *et al.*, 2001).

When *Ulva* is wounded (by grazing) the enzyme DMSP-lyase is activated and converts the precursor compound dimethyl sulfoniopropionate (DMSP) into the deterrent compounds dimethyl sulfide (DMS) and acrylic acid or acrylate (Fig. 1) (Paul and Van Alstyne, 1992; Wolfe and Steinke, 1996; Erickson *et al.*, 2006). Either acrylic acid or acrylate is produced depending on the pH of the working medium (Kiene, 1990; Van Alstyne *et al.*, 2001). These

compounds only act as deterrents to certain marine organisms, deterring some fish and not invertebrates, or vice versa (Hay and Fenical, 1988).

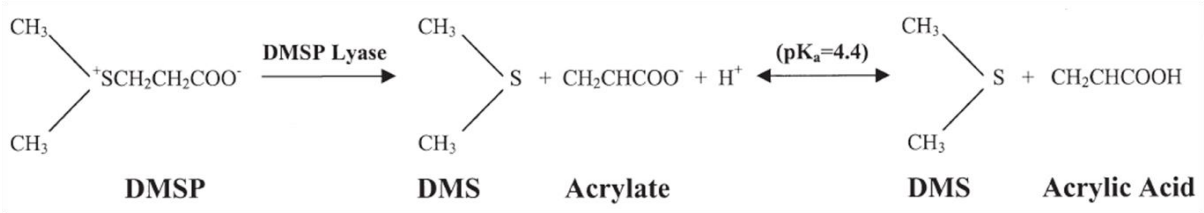

Figure 1: Cleavage pathway of DMSP into DMS and acrylate/acrylic acid (Van Alstyne *et al.*, 2001).

In spite of these chemicals, *Ulva* has been shown to be a preferred feed for *T. gratilla* (Scholtz, 2008; Cyrus *et al.*, *in press*). Unlike eastern countries, where *Ulva* is cultured for human consumption, in South Africa it is grown for feed (Bolton *et al.*, 2008). South Africa produces large amounts of this macroalga as it is easily grown (Fig. 2, DAFF, 2012). In 2006, the cultivation of *Ulva* on abalone farms was advanced by developing an integrated abalone/*Ulva* system. This system uses *Ulva* to filter the abalone effluent of nutrients such as nitrogen, enabling the reuse of the cleaned effluent for abalone tanks once again. *Ulva* has higher growth rates in abalone effluent than natural sea water (Bolton *et al.*, 2008). Most *Ulva* grown in experimental and economic aquaculture sectors in South Africa has until now been known as *Ulva lactuca* (Troell *et al.*, 2006), but the species grown at I&J (West Coast Abalone farm in Gansbaai) has been shown by molecular sequencing methods to be closest to *Ulva armoricana*, but yet has been given no valid name (L. Kandjengo and JJ Bolton, *pers. comm.*).

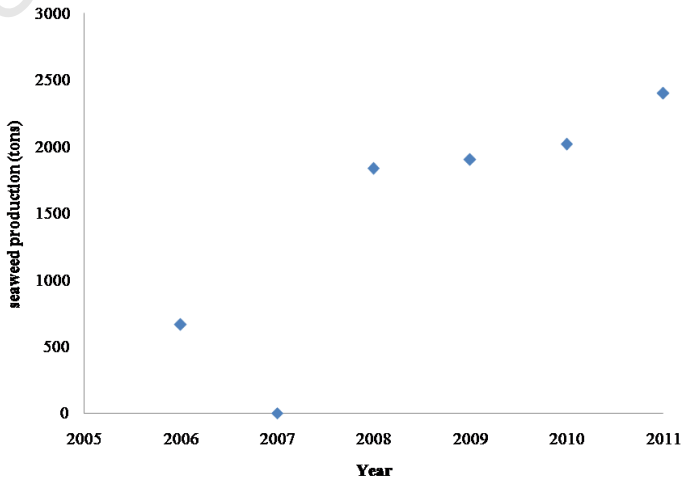

Figure 2: South Africa's seaweed production (*Ulva* spp. and *Gracilaria* spp. in tons) from 2006 to 2011. *Seaweed production at year 201 = 2400t (Roger Krone, *pers. comm*).

Potential attractant or deterrent compounds in *Ulva*

Dimethyl sulfide (DMS) is one of the most studied chemicals regarding chemical defence reactions (Van Alstyne *et al.,* 2001, 2006; Van Alstyne and Houser, 2003; Weisemeyer *et al.,* 2007) and is well known for its highly noticeable and unpleasant smell (Smit *et al.,* 2007). It is a volatile compound that is one of the most dominant contributors to atmospheric sulphur (Andreae and Barnard, 1984). Acrylic acid is bioactive and prominently acts as an antibiotic affecting gut microbes of large herbivores such as urchins and fish, and high order predators such as penguins (Sieburth, 1961, Van Alstyne *et al.,* 2001). It is extremely water soluble and, above a pH of 4.4, is deprotonated to acrylate (Van Alstyne *et al.,* 2001).

Erickson *et al*. (2006) examined DMSP related defence systems of the subtropical sea urchin *Echinometra lucunter*. DMS and acrylic acid were incorporated into artificial diets at calculated natural (5µg) and elevated (20µg) concentrations. These had no effect on grazing by the sea urchin. However, Van Alstyne and Houser (2003) showed that DMS functioned as a potent deterrent against the sea urchin *Strongylocentroutus droebachiensis* at a wide range of concentrations (0.04% - 2% FM) by offering the urchins artificial seaweed-based diets of *Laminaria saccharina,* with and without DMS. Similarly, Van Alstyne *et al.,* (2001) showed that acrylic acid placed in artificial feeds incorporating *Laminaria saccharina* deterred feeding by the sea urchin *Strongylocentroutus droebachiensis* and artificial feeds incorporating *Porphyra* sp. deterred feeding by *Strongylocentroutus purpuratus* at concentration ranges of 0.1-2% FM and 0.25 – 2% FM respectively. DMS and acrylic acid taken from *Ulva* sp. in New Zealand, Australia and Tasmania showed no effect on the snail *Littorina sitkama* when incorporated into agar based diets (Van Alstyne *et al.,* 2009; Paul *et al.,* 2011). Therefore, although many studies indicate that the DMS activated defence system deters herbivores, (Wolfe *et al.,* 1997; Kiene, 1990; Van Alstyne *et al.,* 2001; Van Alstyne and Houser 2003) secondary metabolites have a variety of effects on different herbivores, indicating a species-specific relationship. Understanding these species-specific relationships is integral to developing artificial feeds for aquaculture as it defines the preference, and thus performance, of the highly valued marine herbivore (Angell *et al.,* 2012). Fewer studies have been done on other volatile compounds released by *Ulva*. Van Alstyne *et al.* (2006) found echinoderms, molluscs and arthropods to be deterred by an amine compound called dopamine.

Ulvan, a sulphated hetero-polysaccharide found in the cell walls of green algae such as *Ulva*, is gaining increasing attention due to its unique physical and chemical properties (Paradossi, 1999; Robic *et al.,* 2008; Robic *et al.,* 2009a). It has a wide list of beneficial biological properties such as being an antioxidant (Qi *et al.,* 2006), anticoagulant (Zhang *et al.,* 2008) and immune-regulator (Leiro *et al.,* 2007). Since it is water soluble, hot water extraction methods are usually used and yield about 8-29% of the algae dry weight (Paradossi, 1999; Robic *et al.,* 2009a). These methods vary in literature as they are continually modified to try and get the optimum yield of ulvan.

Ethanolic extracts of *Ulva* (extractions using ethanol) are known to consist of carbohydrates, steroids and glycosides (Alang *et al.,* 2009) as well as phenolic compounds such as vanillin, ferulic acid and salicyclic acid (Hassan and Ghareib, 2009). This extraction method is similar to that of ulvan.

All these compounds are found within *Ulva* and may affect the palatability of the seaweed. By acting as attractants or deterrents to sea urchins, they may play a role in food preference and thus affect the choice of fresh macroalgae that should be incorporated into artificial feeds for optimum sea urchin growth and quality.

Chemosensory trials

A variety of methods exist for evaluating marine herbivore preference based on attractiveness or deterrence. The tested chemicals can be incorporated into artificial feed or agar based diets or released directly into a tank/maze at a distance to supply olfactory cues to the herbivore (Fleming, 1995). Chemosensory trials using the latter method have been used for a multitude of herbivores, such as echinoderms, due to the presence of olfactory receptors which they use to find food. This is known as chemoreception. A common method of testing chemoreception is the use of a Y-shaped maze (Castilla and Crisp, 1970; Castilla, 1972; Vadas, 1977; Prince *et al.,* 1992). The tested chemical is placed in a separate compartment at the top of one arm of the maze, while the other arm is left blank. As the water flows through it creates a chemosensory cue down one arm of the maze (Fig. 3). The tested organism is then placed at the bottom of the maze and moves up the maze following the chemical cue it is attracted to.

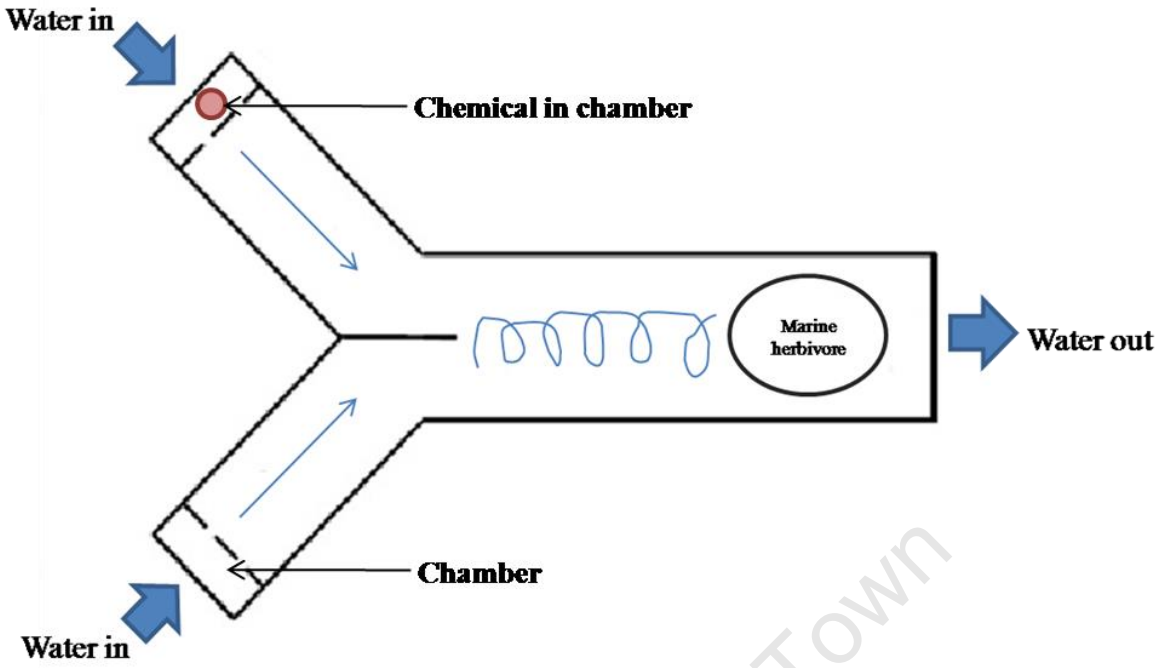

Figure 3: Chemosensory trial using the very common method of a Y-shaped maze (Castilla and Crisp, 1970; Castilla, 1972; Vadas, 1977; Prince *et al.*, 1992; Cyrus *et al.*, *in press*).

Sea urchins are often used in these chemosensory trials. Although they are generalist herbivores, they show preferences when offered choices of different seaweeds (Cyrus *et al.*, *in press*). These preferences are thought to be based upon the chemical cues the urchins receive from the constituent's chemical characteristics (presence/absence of attractants/deterrents) of sea weeds or the physical properties of the seaweed (Dworjanyn *et al.*, 2007). Many studies have investigated the presence/absence of attractants/deterrents on sea urchins. McConnell *et al.* (1982) provided evidence that the sea urchin *Lytechinus variegatus* is affected by secondary metabolites from green marine algae *Caulerpa prolifera* and *Cymopolia barbata*. Caulerpenyne, an oxygenated sesquiterpene found in *C. prolifera* and cymopol, a monoterpene-bromohydroquinone found in *C. barbata* inhibited feeding by the sea urchin. Long chain aldehydes (found in green alga *Ulva pertusa*) are also volatile compounds known to act in algal chemical defence (Akakabe and Kajiwara, 2008). As suggested by Granado and Caballero (1991, 2001) however, further work is needed on the specific compounds that inhibit or stimulate feeding by marine herbivores.

The current study follows results of Cyrus *et al.* (*in press*) where sea urchin *Tripneustes gratilla* was shown to have a significant preference for *Ulva* when offered a choice of

Ecklonia maxima, *Porphyra capensis*, *Ulva rigida* and *Gigartina polycarpa*. Results also showed that *T. gratilla* fed on artificial diets containing *Ulva* produced the most commercially acceptable gonads with respect to size, texture and colour, indicating the potential of incorporating *Ulva* into artificial feed for successful sea urchin aquaculture. Since this subtropical sea urchin is the only edible species on South African shores, it has great export potential for South Africa by supplying to growing markets in countries such as Japan where natural stocks have been exploited (Dy *et al.,* 2002; Rahman *et al.,* 2009; Cyrus *et al., in press*).

Although some literature suggests that *Tripneustes gratilla* consumes other algae, the foundations of this study are based on Cyrus *et al.* (*in press*) where *Ulva* was seen as the preferred feed. The aim of this study was to assess the chemosensory response of *T. gratilla* to three constituents, as well as four compounds found within cultured *Ulva,* to establish what it is in this seaweed that generates the preference seen in Cyrus *et al.,* (*in press*). In this study we used fresh *Ulva*, wounded *Ulva*, artificial feed, and the compounds DMS, acrylic acid, ulvan and an ethanolic extract in a Y-shaped maze to identify which compounds attract/deter the sea urchin. We hypothesize that the sea urchin will be deterred by wounded *Ulva* and DMS and acrylic acid at natural concentrations, but will be attracted to ulvan and the ethanolic extract respectively. We also hypothesize that the urchin will not be attracted or deterred by ethanol and the artificial feed. This will provide information on the specific compounds in *Ulva* that inhibit or stimulate herbivore feeding, and will thus inform which feeding stimulants should be incorporated into future artificial feeds for *T. gratilla*.

Material and Methods

Collection

A total of 40 adult *Tripneustes gratilla* (50-100mm) were collected from shallow rock pools near Haga Haga, Eastern Cape, South Africa (32°45′4.23″S, 28°16′41.30″E) on 15-17 September 2008. Following collection they were kept at the Two Oceans Aquarium in Cape Town until being transferred to a holding tank at the Department of Agriculture, Forestry and Fisheries (DAFF) Aquaculture Research Facility in Sea Point for the experiment. Specimens were maintained in a glass holding tank (150 (l) × 60 (w) × 60 (h)) cm supplied with heated and aerated flowing seawater at 24-26°C temperature and salinity of 34-36. This temperature range was selected because this is the temperature range that cultured adult *T. gratilla* are typically kept at (Dworjanyn *et al.,* 2007). Conditions were monitored using a hand-held WTW LF 340 salinity and temperature meter (Wissens-chaftlich-Technische Werkstätten, Weilheim, Germany). The urchins were fed a diet of *Ulva* between trials. Three days before each trial *Ulva* was removed and the tank was cleaned for a three day starvation period to take place.

Preparation of Constituents

Damaged and undamaged *Ulva* sp.

Ulva grown in seaweed raceways at I&J Cape Abalone farm (Gansbaai) was grown at the DAFF Research Aquarium in a large cylindrical tank 101 (h) x 120 (d) cm supplied with natural seawater from Sea Point at a temperature of 17°C. Using a scale (Radwag, 2008, PS510/C/2), 20g of fresh *Ulva* was taken from this tank and thoroughly cleaned and rinsed with filtered seawater to remove any epiphytes or organisms present on the alga. 20g of damaged *Ulva* was prepared in the same way except it was cut into 0.5 x 0.5 cm pieces using scissors, placed in cheesecloth, and tied with a rubber band to prevent pieces from escaping and flowing through the chamber into the branch of the maze.

Artificial feed

20g of artificial compound feed containing no *Ulva* from Cyrus *et al.* (*in press*) was used. The nutritional components of this feed are represented in Table 2.

Table 2: Nutrient analysis of the artificial feed with no added *Ulva* prepared by Cyrus *et al.* (*in press*) per g dry matter.

	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

DMS and acrylic acid

Existing literature was used to determine relative concentrations of DMS and acrylic acid in *Ulva* sp. Unpublished results from Michael Joubert (*pers. comm*.) found that the *Ulva* sp. used in this experiment contained 2250 $ug.g^{-1}$ DMS. Since most literature tests similar concentrations of DMS and acrylic acid, the value used for DMS (2250 $ug.g^{-1}$) was also used to determine the concentration of acrylic acid. Commercially obtained DMS and acrylic acid were used at identical concentrations of 20 µL in 150 ml sea water (see appendix for calculations). This concentration was chosen as it represents the amount that would be present in 20g of wet *Ulva*. Acrylate was not available for this experiment.

Ulvan extraction (modified from Paradossi, 1999)

38.5g fresh *Ulva* was dried at a constant temperature of 50°C for 24 hours before being ground to pieces of approximately 0.5cm using an electric grinder (32BL80 8011, USA). The coarse powder was suspended in 700ml distilled H_20 in a 1000ml glass beaker and submerged in a hot water bath (Jubalo Labortechnik GMBH, EM/U, West Germany) at ±80°C for 3 hours. The heated *Ulva* suspension was filtered through a strainer ± 1mm and the remaining liquid was spun at 8000 rpm for 10 minutes using a centrifuge (Rotina 380R, Hettich Zentrifugen 1705, Germany). The pellets were discarded and the supernatant was placed on a heating stone (Fried Electric, MH4, Israel) at 75°C for a further 3 hours to concentrate the sample to 30% of its initial volume. Two volumes (500ml) of 70% ethanol were added once the liquid cooled in order to precipitate out the ulvan. A dough-like globule formed and was removed and washed with 96.9% ethanol before being centrifuged for one minute at 8000rpm. Equal quantities of the substance were added to pre-weighed centrifuge

tubes and cleaned with 96.9% ethanol. The cleaned substance was dried under a vacuum while being centrifuged at 1200rpm for 45 minutes at a temperature of 45°C and the resultant dried powder was stored at -20°C until used. The extraction yielded 8.87% ulvan (3.416g ± 0.0172) of dry weight (38.5g) *Ulva* (Table 1) and 2.72g of this was made up to 1L with sea water and used for the experiment.

Table 1: Total ulvan yield extracted from 38.5g dry *Ulva*. *Initial Wt (g) = Initial weight of tubes before being spun in a vacuum, Final Wt (g) = final weight of tubes after being spun in a vacuum.

Tube	**Initial Wt (g)**	**Final Wt (g)**	**Ulvan (g)**
1	0.934	1.201	0.267
2	0.942	1.377	0.435
3	0.93	1.234	0.304
4	0.952	1.235	0.283
5	0.934	1.326	0.392
6	0.937	1.243	0.306
7	0.941	1.282	0.341
8	0.939	1.267	0.328
9	0.946	1.308	0.362
10	0.935	1.333	0.398
		Total	**3.416**

Ethanolic extraction (modified from Alang *et al*., 2009)

15g of dry *Ulva* pieces was suspended in 120ml of ethanol in a conical flask and placed on a heating stone (Fried Electric, MH4, Israel) at 35-40°C with a magnetic stirrer for 1.5 hours. An air hose pipette was attached to the opening of the flask directed at the surface of the solution to increase the evaporation rate of the ethanol. The heated solution was then filtered through a strainer ±0.5mm and the remaining liquid was centrifuged for 10 minutes at 8000rpm using centrifuge (Rotina 380R, Hettich Zentrifugen 1705, Germany) to 6% (20ml) of its initial volume. This was then spun under a vacuum (Mivac, MST-23050-L00, England) centrifuge (Eppendorf AG concentrator plus, Germany) for 40 minutes at 40°C to 10ml ethanolic extract. 450 µL of this extract was placed in 150 ml sea water as it represents the amount of ethanolic extract that would be present in 1g of dry *Ulva*. To test that ethanol did not affect the attraction or deterrence of *T. gratilla* to the ethanolic extract, ethanol was also

incorporated as a testable constituent in this study and used at a concentration of 450 µL in 150 ml sea water.

Chemosensory experiments

Chemosensory experiments were performed using a Y-shaped maze 71 (l) × 30 (w)× 15 (h) cm constructed by Cyrus *et al.* (*in press*) out of clear Perspex modelled after designs of Castilla and Crisp (1970) and Vadas (1977). Before chemosensory trials began, plume dynamic tests were conducted to determine whether equal mixing of water/chemical cues between the two arms took place. Green and red food colouring was used for this purpose and indicated that negligible mixing beyond the partition between the two arms took place (Fig. 2).

Figure 4: Plume dynamic tests conducted with red and green food colouring between the two arms of the Y shaped maze.

Treatments (*Ulva*, Wounded *Ulva*, DMS, acrylic acid, ulvan, ethanolic extract and artificial feed) were placed in the chamber in one arm of the Y-shaped maze. Each chamber was separated from the arm by a piece of Perspex glass that contained a row of 4mm holes. Each branch was supplied with seawater (24-26°C) at a flow rate of 8L.min^{-1} using a water pump (Dolphin Canisterfilter, C1000, China). All treatments except the *Ulva*, wounded *Ulva* and artificial feed were added using a dropper system with a flow through rate of 6ml.min^{-1} (Fig. 3). This flow rate was regularly checked between trials to ensure that each urchin received the same concentration of the tested constituent at all times.

Figure 5: Liquid dropper releasing compound into left arm of the Y shaped maze at a flow rate of 6ml.min^{-1}

12 pair-wise chemosensory trials were carried out using the method of Prince *et al.* (1992). Individual sea urchins (starved 3 days prior to the experiment) were placed at the start/bottom of the Y-shaped maze and their movements observed over a 20 min period. Preliminary experiments (Cyrus *et al., in press*) showed that urchins were capable of moving from the start of the maze to an arm within 15 minutes. A preference/choice was recorded if an urchin moved 10cm or more up one of the arms within 20 minutes, otherwise 'no choice' was recorded. After 6 trials the tested constituent was changed to the opposite arm. To prevent urchins from following chemosensory signals left by previous trials, the Y-shaped maze was emptied, washed out with seawater and refilled at this time. Treatments were tested in pairs or individually against a blank. This method was used to ensure urchins did not have a preference for a particular arm. The experiments were carried out under continuous light conditions of two 58 watt white fluorescent tubes (Light intensity = 16.5 W.m^{-2}). The following treatments were tested: fresh *Ulva* (damaged and undamaged), dimetheyl sulfide, acrylic acid, artificial feed containing 0% *Ulva* (Cyrus *et al.*, 2012), ulvan, ethanolic extract and ethanol as a control.

Statistical analysis

Each trial was treated as a Bernoulli trial having one of only two possible outcomes. The exact probability of the outcome of a series of trials was then calculated using the equation:

$$P(k) = \binom{n}{k} \times p^k \times q^{n-k}$$

where n is the number of trials, k is the number of successes, p is the probability of success and q=1-p (Prince *et al.*, 1991; Cyrus *et al., in press*). The significant level was chosen to be $p<0.054$ and not the conventional $p<0.05$. This was done to reduce type two errors created by the large number of external variables in this study.

Results

T. gratilla showed no preference for a particular arm of the Y-shaped maze when both arms were left blank. Sea urchins were attracted to *Ulva* (p=0.0002) when tested against a blank, and were deterred by wounded *Ulva* (p=0.0537) when tested against a blank. They were not attracted or deterred to the artificial feed (p=0.2256). The same result was true when the feed was compared to *Ulva* (p=0.1934) and wounded *Ulva* (p=0.1934) respectively. Sea urchins were not deterred by DMS (p=0.1934). The same was true for acrylic acid (p=0.2256). A significant deterrence was seen for ulvan (p=0.0537), however they were not attracted or deterred by the ethanolic extract (p=0.2256). The test between the ethanolic extract and *Ulva* (p=0.2256) also showed this same result as urchins did not prefer one constituent over the other. Ethanol however, acted as a deterrent (p=0.0537).

Table 3: Results of chemosensory trials testing sea urchin preference/deterrence to *Ulva*, wounded *Ulva*, DMS, acrylic acid, artificial feed containing 0% *Ulva*, ulvan, ethanolic extract ethanol (*DMS = Dimethyl sulfide, blank = natural sea water). Expected distribution in each paired test = 50:50.

Paired Test	(n)	No. of sea urchins choosing	One-tailed Probability
Ulva	12	12	**P=0.0002**
Blank		0	
Wounded *Ulva*	12	3	**P=0.0537**
Blank		9	
Ulva	12	6	P=0.2256
Wounded *Ulva*		6	
Ulva	12	5	P=0.1934
Feed		7	
Blank	12	6	P=0.2256
Feed		6	
Wounded *Ulva*	12	7	P=0.1934
Feed		5	
DMS	12	5	P=0.1934
Feed		7	
Acrylic Acid	12	6	P=0.2256
Feed		6	
Ulvan	12	3	**P=0.0537**
Blank		9	
Ethanolic extract	12	8	P=0.1208
Blank		4	
Ethanolic extract	12	6	P=0.2256
Ulva		6	
Ethanol	12	3	**P=0.0537**
Blank		9	

Discussion

Urchins showed a significant preference for fresh *Ulva* and were significantly deterred by wounded *Ulva*, ulvan and ethanol. Similar to *E. lucunter* urchins (Erickson *et al.*, 2006), *T. gratilla* were not deterred by DMS or acrylic acid. However, since other species of urchin are deterred by DMS and acrylic acid (Van Alstyne and House, 2003), these results indicate that the effects of secondary metabolites released by algae are species-specific. Since urchins were not deterred by wounded *Ulva*, ulvan and ethanol when these constituents were compared with each other or with the ethanolic extract, fresh *Ulva*, feed, results indicate that *T. gratilla* is not very selective in what it likes or dislikes.

The sea urchin *T. gratilla* showed a significant preference for fresh *Ulva*. This result was expected due to results of Cyrus *et al*. (*in press*). Since *Ulva* aquaculture is very successful in South Africa and urchins fed on *Ulva*-based diets produce the most marketable roe, this result is important as it shows the great potential that *T. gratilla* urchins and *Ulva* seaweed have for sea urchin aquaculture in South Africa.

Another expected result was that urchins were significantly deterred by wounded *Ulva*. This is because we know that when an alga is wounded it will release a mixture of deterrent compounds to defend itself from being grazed further (Van Alstyne *et al*., 2001). Thus we know that *Ulva* contains chemicals that can deter *T. gratilla*, but we have not yet identified what these chemicals are.

When fresh *Ulva* and wounded *Ulva* were placed together in the Y-shaped maze, urchins were indiscriminate in which arm of the maze they chose. This indicates that the chemosensory signal of wounded *Ulva* was not strong enough to deter urchins and the chemosensory signal of fresh *Ulva* was not strong enough to attract urchins. This was not expected since we know that *Ulva* has potential to release large amounts of deterrent compounds (Van Alstyne *et al.*, 2001). Again, this indicates that *T. gratilla* may not be very selective on what they are attracted to and thus are very general herbivores.

What was also not expected in this study was that *T. gratilla* showed no deterrence to DMS or acrylic acid. Since these chemicals are known to be deterrent compounds to some species of urchin, these results indicate that *T. gratilla* specifically is not affected by DMS and

acrylic acid or the concentrations were too low to have an effect. The major threat to chemosensory studies such as this one is the limited data regarding the speed and actual amount of DMS and acrylic acid released by wounded macroalgae. This is confounded by the fact that the concentration of secondary metabolites can even vary within a single species of alga (Cronin and Hay, 1996). Thus it is difficult to choose the true concentrations of chemicals that will be encountered by the herbivores (Geiselman and McConell, 1981; Steinberg, 1986). Only at unnatural concentrations (100x natural concentration) did Van Alstyne and Houser (2003) find that sea urchins were deterred by DMS. In the current study however, *T. gratilla* showed no deterrence at unnaturally high concentrations of either chemical.

Urchins were deterred by ulvan with only three urchins choosing ulvan and nine choosing the blank. Although not significant, there seemed to be a preference for the ethanolic extract when compared to the blank as eight urchins chose the extract and only four chose the blank. Since urchins were deterred by ethanol itself, this could have prevented urchins from choosing the extract, indicating that the extract may actually act as an attractant.

Urchins did not discriminate between the artificial feed and the blank. This indicated that they are not attracted or deterred by it, suggesting that the artificial feed may be a good feed to use in aquaculture. Interestingly there was no preference for fresh *Ulva* when compared to the feed, and no deterrence to wounded *Ulva* when compared to the feed. These results confound earlier results where urchins are shown to be attracted to *Ulva* and deterred by wounded *Ulva*. Again, this suggests that the chemosensory signal of *Ulva* is not very strong.

Literature has shown how marine herbivore responses to volatile chemicals such as DMS and acrylic acid are species specific. This study indicates that *T. gratilla* is not deterred by DMS or acrylic acid individually. Rather, we postulate that *T. gratilla* may be deterred by a mixture of DMS, acrylic acid and other chemicals found in *Ulva*. This has also been suggested by Weisemeyer *et al.* (2007) whereby behavioral assays on artificial diets did not reveal any repellant role for single isolated metabolites of DMS, trimethyl amine and acrylate on the amphipod *Amphithoe longimona*. Rather a mixture of the chemicals significantly reduced feeding, indicating that mixtures of chemicals are recognized and influence food selection. Future chemosensory studies involving *T. gratilla* could test whether the urchins are deterred by a mixture of the above chemicals. Since acrylate was not available for this study and

should have been tested instead of acrylic acid, further investigations should use acrylate instead of acrylic acid.

As this study contains contradictory results to literature, where DMS and acrylic acid are seen as deterrents to urchins, it highlights the importance of repetition of experiments in science. Few experiments in this study were conclusive, also highlighting the need for repetition and suggesting that *T. gratilla* are not very selective.

The major challenge in this, and most chemosensory studies, is the concentration of the volatile compound that will be tested. This is because there is no sound evidence of the amount, duration and location of the chemicals released. Another challenge in this study was the chosen starvation period the urchins should be subjected to before each experiment. Cronin and Hay (1996) tested feeding discrimination based on the commonly used method of starving subjects before a feeding or chemosensory trial. Results showed that starved urchins fed less discriminately than those that were not starved, indicating that hunger stress influences the outcome of herbivore preference. A variety of starvation periods have been used in past literature. Prince *et al.* (1992) starved *S. droebachiensis* for five weeks before each experiment, while Vadas (1977) starved the same species for two weeks. As this affects the discriminatory potential of urchins, wrong conclusions could be made, implicating results. This is why in this experiment a shortened period of three days was chosen.

Environmental factors such as temperature and salinity could also have affected herbivore behavior. Light intensity is not an environmental factor in this study as *T. gratilla* is a continuous feeder and thus should continuously feed under all light conditions. Seasonal changes have also been postulated to affect the food seeking behavior, and therefore preference, of urchins (Castilla, 1972). This is because there is a seasonal change in the production of attractants and deterrents by macroalgae during the year (Robic *et al.*, 2009b).

In summary, this study indicates that *T. gratilla* is more tolerant to DMSP-related defenses compared to other urchins. This is consistent with literature, indicating that the effects of metabolites in macroalgae are species specific (Hay *et al.*, 1986; Paul *et al.*, 2001; Van Alstyne *et al.*, 2001). Isolated volatile compounds (DMS and acrylic acid) showed no deterrent affects, indicating that a mixture of the compounds should be tested in future studies. Since the majority of literature has investigated chemical deterrents found in algae,

future chemosensory studies should focus on chemical attractants as these will be valuable feeding stimulants for artificial feeds. Future studies should also combine both methods of using the isolated chemical (as done in this study) and incorporating the chemical into agar. This way the chemosensory preference of the urchin, and whether this preference changes upon consumption, will be assessed. The physiological effects that these secondary metabolites have on urchins could also be tested in the future. Literature has shown that marine herbivores fed artificial feeds which incorporate fresh algal material containing high amounts of DMSP (such as *Ulva*) have been found to accumulate large amounts of DMS in their tissues, affecting their taste (Smit *et al.*, 2007, 2010). This relationship could be true for urchins as they too are fed artificial feed containing algal material, affecting their gonads and thus commercial value.

This study highlights the importance of identifying attractant and deterrent compounds for each species of animal. The identification of these compounds will provide important information for the production of successful artificial foods and will thus increase the commercial viability of the species of animal in question.

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Appendix

Calculations for DMS concentration

Using Michael Joubert (*pers. comm*) the amount of DMS in 2g dry *Ulva* must be calculated.

(1) 20g wet *Ulva* = 2g dry *Ulva*. Therefore amount of DMS released for 2g dry *Ulva* = 2250 x 2 = 4500µg.

(2) $\mathrm{n} = \frac{\mathrm{m}}{\mathrm{M}} = \frac{4.5 \times 10^{-3}}{62.13} = 7.24 \times 10^{-5}$ mol in 4500µg of DMS

(3) Volume of tank = 31.95dm^{-3} and flow rate in tank = 0.667 $dm^{-3}.s^{-1}$

(4) Volume of dropper 0.15dm^{-3} and dropper rate = $1x10^{-4}$ $dm^{-3}.s^{-1}$

Concentration must be uniform throughout the tank at all times (*Q(t) = amount of substance dissolved at time t).

(5) $\mathrm{C_{tank}} = \frac{\mathrm{n}}{\mathrm{v}}$, $\mathrm{n} = \frac{\mathrm{n}}{\mathrm{m}} = \frac{4.5\times10^{-3}}{62.13\ \mathrm{g.mol^{-1}}} = 7.2428 \times 10^{-5}$ mol, therefore,

$\mathrm{C_{tank}} = \frac{7.2428\times10^{-5}}{31.95\mathrm{dm^{-3}}} = 2.2669 \times 10^{-6}\mathrm{mol.dm^{-3}}$

(6) $\mathrm{Rate\ at\ which\ Q(t) enters} = \mathrm{Rate\ at\ which\ Q(t) exits}$

$(\mathrm{flow} \times \mathrm{c})_{\mathrm{dropper}} = (\mathrm{flow} \times \mathrm{c})_{\mathrm{tank}}$

$(1 \times 10^{-4}\mathrm{dm^{-3}.s^{-1}}) \times \mathrm{C_{dropper}} = (0.0667\mathrm{dm^{-3}.s^{-1}}) \times 2.2669 \times 10^{-6}\mathrm{mol.dm^{-3}}$

$\mathrm{C_{dropper}} = \frac{1.51204\times10^{-7}\mathrm{mols.s^{-1}}}{1\times10^{-4}\mathrm{dm^{-3}.s^{-1}}} = 1.512 \times 10^{-3}\mathrm{mol.dm^{-3}}$

(7) $\mathrm{C_{dropper}} = \frac{\mathrm{n}}{\mathrm{v}}$, therefore $\mathrm{n} = \mathrm{C} \times \mathrm{V} = 1.512 \times 10^{-3} \times 0.15 = 2.268 \times 10^{-4}\mathrm{mol}$

(8) $\mathrm{m} = \mathrm{n} \times \mathrm{M} = 2.268 \times 10^{-4}\mathrm{mol} \times 62.13\mathrm{g.mol^{-1}} = 0.01409\mathrm{g} = 1.409 \times 10^{-5}\mathrm{kg}$

(9) $p = \frac{\mathrm{m}}{\mathrm{V}}$, therefore $\mathrm{V} = \frac{\mathrm{m}}{p} = \frac{1.409\times10^{-5}\mathrm{kg}}{840840\mathrm{kg.m^{-3}}} = 1.67756 \times 10^{-8}\mathrm{m^3}$

(10) $\mathrm{V} = 1.67756 \times 10^{-3} \times 1000 = 1.67756 \times 10^{-5}\mathrm{litres} = 0.01677565\mathrm{ml}$,

Therefore $\mathrm{V} = 17\mu\mathrm{l} \approx 20\mu\mathrm{l}$ DMS. This volume was also used for acrylic acid.