Determining larval settlement, post-settlement and weaning substrates and regimes for the sea urchin *Tripneustes gratilla* in intensive aquaculture.

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Abstract

Sea urchins gonads are a highly valued and priced seafood. Demand is stable and unlikely to decline in the future. Successful echinoculture is limited by the ability to produce large enough quantities of viable larvae and/or juveniles economically and efficiently due to a bottleneck in production during the settlement and post-settlement phases. In this study, larval settlement, post-settlement growth, and weaning regimes were investigated in the collector urchin, *Tripneustes gratilla*. Two cohorts of urchins were spawned for two separate growth trials. Growth trials assessed settlement, post-settlement growth, and the timing of weaning onto macroalgae (*Ulva lacinulata*). Experimental substrates tested include: *Ulvella lens,* fresh *Ulva*, dried *Ulva* and alginate, dried *Ulva* and agar, *Nitzschia* sp. (undescribed diatom), dried *Isochrysis galbana* and alginate, probiotic *Vibrio midae* SY9 and alginate, *V. midae* SY9 and *Ulva* extract F9 and alginate, an ethanol-alcohol and alginate control, and a null-alginate control (replicates=4, n=35 individuals). The highest average settlement success was achieved on fresh *Ulva* (67.14% ± 8.45) followed by *Ulvella lens* (55.71% ± 12.26) and then *Nitzschia* sp. (40.71% ± 5.88). These treatments were significantly different from all the other treatments ($p<0.05$) but not from each other ($p>0.05$). *U. lens* facilitated the greatest significant change in test diameter in *T. gratilla* post-settlement (difference of 3013µm over 4 weeks) and maintained high survival over this time (61.43% ± 10.47). Weaning was successful at 4 weeks post-settlement but was accompanied by a lag-phase in observable growth that was not observed when urchins were subjected to delayed weaning (three weeks later). Survival of urchins with delayed weaning was significantly greater than that of juveniles subjected to early weaning ($p<0.05$): 100.00% compared to 92.50%; and achieving a significantly greater size: ~1.5 mm difference in test diameter over 6 weeks. Results suggest that *U. lens* can induce settlement while maintaining high survival. When inducing settlement using *U. lens*, fresh *Ulva* should be placed in the same tank to facilitate increased settlement, *U. lens* facilitating post-settlement growth thereafter. The timing of weaning is important in facilitating optimal growth.

International sea urchin trade

Sea urchins are considered a highly prized seafood, particularly in East Asia and are sold for their gonads/roe (called '*uni*' in Japanese). The gonads are highly valued, fetching between R4000 and R9000/kg on the famous Tsukiji-market in 2016 (Hagen, 1996; Sonu, 1995; Tsukiji-market, 2016). The average price of domestic '*uni*' on the Toyosu market (Tokyo Metropolitan market) in 2022 was between 2900-4000 R/kg (HYPERLINK "http://www.seafood.media" Seafoodmedia, 2022). The demand for *'uni'* is stable and unlikely to decline in future (James *et al*., 2016; Rahman, Arshad, & Yusoff, 2014; Sun & Chiang, 2015). The ongoing demand has resulted in the harvesting of natural sea urchin populations to the extent that many are now depleted (Bertocci *et al.,* 2018). The world sea urchin industry was in decline 10 years ago and was unable to satisfy the demand for sea urchin roe (Asia, Villamor, & Faylogna, 2012). Consequently, a global interest in the development of sea urchin aquaculture (or echinoculture) has arisen.The viability of successful echinoculture is limited, to a great extent, by the ability to produce large enough quantities of viable larvae and juveniles economically and efficiently. This is due to a bottleneck in production during the settlement and post-settlement phases of the urchin life cycle (Dworjanyn & Pirozzi, 2008).

The collector urchin (*Tripneustes gratilla* Linnaeus, 1785) is being investigated for its inclusion in the South African aquaculture industry. Significant local research has been devoted to develop culture technologies for this species in the last decade (Cyrus *et al*., 2013; 2015; 2019; Cyrus, Bolton, Scholtz, & Macey, 2015; Onomu *et al*., 2020). This thesis aims to provide insight as to how to improve production during the younger stages of the *T. gratilla* life cycle, thereby facilitating a greater number of juveniles to progress to the adult stages, where their potential as high value consumables lies.

T. gratilla as a prospective aquaculture species

Echinoculture is a sustainable way of filling global demands for sea urchin products and is gaining interest across the world. The inclusion of *T. gratilla* in the local aquaculture industry would mark the first echinoderm species to be grown for commercial gain in South Africa. The commercial culture of *T. gratilla* would certainly benefit the country's economy as the species is one of the most sought-after species in international markets (like Japan and the Philippines) (Kato, 1972; Lawrence & Bazhin, 1998; Rahman, Tsuchiya, & Uehara, 2009). The development of a sustainable echinoculture industry was included as one of the goals of South Africa's marine development plan, Operation Phakisa (www.Operationphakisa.gov.za, 2017).

Despite 850 species being described (Kroh and Smith, 2010), only 19 urchin genera are collected for food across the world (Lawrence, 2007). This selectivity can be explained by: (1) accessibility – most consumed species are found in shallow waters (Cyrus, 2013) and are easy to harvest, (2) palatability – urchins can be abundant but may not be palatable (Lawrence & Bazin, 1998), (3) historical or cultural preference – it may be a longstanding tradition in some cultures to consume only a specific species (e.g., Asia, Polynesia, the Mediterranean and Chile) (Andrew *et al*., 2002).

The collector urchin *Tripneustes gratilla* has an Indo-Pacific distribution and is found along the South African coastline with the westernmost recording from Haga Haga (32°45'4.23"S, 28°16'41.30"E) in the Eastern Cape Province, South Africa (Cyrus, 2013; Marshall *et al*., 1991). The species occurs predominantly in seagrass beds and algal meadows (Klumpp *et al*., 1993; Lyimo *et al*., 2011), coral reef flats (de Loma *et al*., 2002; Byrne *et al*., 2004) as well as rubble and rock (Schumacher, 1974). The range of *T. gratilla* is primarily determined by sea temperature (Lawrence, 2007) but is also influenced by natural salinity gradients (Lessios *et al*., 2003).

T. gratilla together with its palatability and proven market acceptance (Dworjanyn *et al*., 2007), has several qualities that make it an excellent candidate for inclusion into South Africa's aquaculture industry (Cyrus, 2013). The commercial production of *T. gratilla* has been shown to be a feasible venture in Australia but the operation was halted due to the global recession in 2010 (Brown & Eddy, 2015). Sea urchins are commercially produced in China but most of the product is exported to Japanese markets (Echinoculture Handbook, 2013). In Japan, most of the seed produced by hatcheries are used for reseeding local fishery grounds (when the post-settled urchins have reached ca. 5-10 mm in test diameter) (Echinoculture Handbook, 2013).

Bottlenecks in echinoculture production

Sea urchins, like most other marine invertebrates, are broadcast spawners and follow the r-reproductive strategy (Hyman 1955; Bruce, 1988). When spawning, billions of gametes are released into the water column and this is often synchronised between individuals and associated with specific environmental cues and conditions, thereby ensuring as much fertilisation as possible and maximising reproductive success (Cyrus, 2013).

Urchin larval development is planktonic for 15 to 52 days after post-fertilisation (Shikambukuro, 1991). During this time, settlement and metamorphosis occurs when competent larvae are exposed to a suitable substrate and/or settlement cue (Dworjanyn & Pirozzi, 2008; Scholtz et al., 2013). Competent larvae have been observed to display certain behaviours such as vertical movements in the water column, "probing" prospective settlement substrates and briefly attaching to substrates with suction disks. The larvae will attach to the substrate and undergo metamorphosis if the substrate is deemed suitable. Newly settled larvae now experience a benthic habit and will continue to develop through the adult stages into sexual maturity.

The generally accepted paradigm states that urchin larvae preferentially settle on substrates they deem favourable for survival and thus likely offers a safe platform for metamorphosis (Morse, 1991; Harris *et al*. 1994; Harris and Chester 1996; Lambert and Harris 2000). A favourable substrate also provides access to a sufficient nutrition source to sustain the newly settled larvae. Marine invertebrates have thus had an evolutionary pressure to be substrate specific. Chemical cues have been shown to be important in initiating metamorphosis for a variety of marine invertebrates (Hadfield, 1984; Moss & Tong, 1992; Li, *et al*., 2006). Moreover, abalone have been observed rejecting settlement surfaces by briefly attaching, detaching, and moving elsewhere (Morse, 1991). This observation implies that a decision-making process is involved in substrate selection. It is also hypothesised that this is informed by chemical cues released from the substrate, via sensory and secretory cells on the suction pads of primary podia (Burke, 1980).

In commercial hatcheries, the number of fertilized eggs that successfully complete larval development, metamorphose, and survive post-settlement has been observed to be less than 1% (Mos *et al*., 2011). This phenomenon can be explained as a function of the r-reproductive strategy, where more energy is invested into producing greater numbers of offspring, instead of investing into parental care for a limited number of offspring. This strategy results in relatively few fertilised eggs surviving to adulthood. This problem represents one of the great bottlenecks in producing urchin products at a commercial scale and severely limits the growth of the echinoculture industry.

Despite the bottlenecks in urchin production, *Tripneustes gratilla* aquaculture and other echinoculture has progressed well beyond the available techniques used in its inception. Grow out culture of *Tripneustes gratilla* in sea cages was developed to enhance natural populations that were depleted due to over-exploitation, in Japan (Shimabukuro, 1991). This same line of research was investigated in the Philippines to enhance natural sea urchin stocks (Juinio-Menez *et al.,* 1998). The continued restocking endeavours have led to the recovery of natural populations in these areas (Juinio-Menez *et al.,* 1998*,* 2001, 2008).

The preferences of differently aged *T. gratilla* for different species of seaweed was also investigated, in Hawaii, for the potential biocontrol of alien invasive seaweeds (Westbrook *et al.,* 2015). The research findings divulge increased diet specificity at younger ages and smaller sizes, compared to urchins of older ages and larger test diameters (Westbrook *et al*., 2015) Other research was directed at overcoming the bottlenecks in sea urchin production (Mos and Dworjanyn, 2020). The research findings included water quality parameter thresholds that limit urchin larval survival, as well as the influence of variable stocking densities, food availability and diet type on larval survival during the rearing process. The research also investigated artificial diet formulations and their effects on urchin growth, production, and gonad conditioning (Mos and Dworjanyn, 2020). In South Africa, artificial formulated diets with seaweed inclusions was shown to have positive outcomes for adult sea urchin production (Cyrus *et al*., 2012, 2013, 2014, 2015, 2019). More recent research on the differential effects of diets on maternal investment in urchin progeny were investigated (Brink-Hull *et al*., 2022A) as well as broodstock conditioning (Brink-Hull *et al.,* 2022B).

Induction of settlement in sea urchins

The successful development of echinoculture requires specific, appropriate settlement substrates to maximise the production capacity of a commercial operation.

Echinoculture facilities, as well as abalone farms, typically use naturally occurring biofilms (consisting primarily of diatom communities with associated bacteria) to settle their invertebrate larvae (Abu Affan *et al*., 2015; Daume *et al*., 2004; Knauer *et al*., 1996; Leighton, 1989; Mos *et al*., 2011; Searcy-Bernal *et al*., 1992). Plates are covered with diatoms (single cell eukaryotic microalgae) and presented to competent ("settlement-ready") larvae. These plates also serve as the initial feed for the newly settled marine invertebrate larvae (Brown & Eddy, 2015; Cárcamo *et al*., 2005; McBride 2005; Shimabukuro, 1991). The disadvantages of using naturally occurring diatoms and biofilms as settlement substrates appears to be low settlement success, when compared with other settlement cues such as *U. lens* (Takahashi et al., 2002). Settlement success has the potential to be increased by using specific settlement cues rather than a combination of naturally occurring diatoms and the associated surface microbiota on the resulting biofilms.

Several studies have documented the ability of specific diatoms to induce settlement and support the growth of post-settled sea urchin larvae. For example, Zupo *et al*. (2018) explored the effects of eight species of benthic diatoms as well as mixed diatom communities, on the settlement and post-settlement response of larvae of the purple sea urchin (*Paracentrotus lividus*) (also Zupo & Messina, 2007). The latter researchers showed that the most adhesive diatom (*Cocconeis scutellum* var. *parva*) induced the greatest settlement (63%) of larvae and highest survival two months post-settlement (Zupo *et al*., 2018).

There are few studies that compare the settlement-inducing effects of diatoms that vary in adhesiveness (Cárcamo *et al*., 2005; McBride, 2005). Dworjanyn & Pirozzi (2008) and Xing *et al*. (2007) investigated the effects of diatoms, like *Amphora* and *Navicula* species, on the settlement of urchin larvae. However, Xing *et al*. (2007) found that *Nitzschia* species induced the greatest settlement (58.32 % ± 5.00; mean ± standard error (SE)) out of a range of monoculture and polycultured diatom communities Similar results have been replicated using *Nitzschia* (Mos *et al.,* 2011). The authors also suggested that this species has the potential to be used in the commercial industry (Xing *et al*., 2007). These findings have also recently been corroborated by the work of Castilla-Gavillan *et al*. (2020).

Bacteria associated with biofilms may also be significant. Zobell and Allen (1935) first observed that marine invertebrate taxa, including "barnacles, molluscs, tunicates, hydroids and hydrozoans", likely require particular bacterial biofilms (of single strain and mixed species communities) to successfully induce settlement. This phenomenon has since been observed for many species of marine invertebrates including echinoderms (Johnson *et al*., 1991b, Huggett *et al*., 2009, Mos *et al*., 2011), cnidarians (Negri *et al*., 2001), polychaetes (Unabia & Hadfield, 1999), gastropods (Rodriguez *et al*., 1995) as well as crustaceans (Neal and Yule, 1994).

Bacteria that live on the surface of macroalgae may also play a decisive role in inducing settlement, particularly for generalist herbivores like sea urchins (Dworjanyn and Pirozzi, 2008, Mos *et al*., 2011). Dworjanyn and Pirozzi (2008) demonstrated that macroalgae that have been cleaned (surface sterilised) induced significantly less settlement, due to the reduction in abundance of surface bacteria (ca. 95% reduction) normally present on the macroalgae. Though this approach did not eliminate the possible contributions of algal metabolites in inducing settlement, it implied that bacterial biofilms contribute to the ability of certain macroalgal species to induce settlement of marine invertebrates (Dworjanyn and Pirozzi, 2008). Moxley and Coyne (2020) showed that settlement success of abalone *Haliotis* *midae* could be increased when settled on an alginate matrix containing the probiotic *Vibrio midae* SY9. In fact, the settlement of several species of marine invertebrates has been shown to be stimulated by the cues of surface bacteria on the biofilms of the settlement surface (Hadfield, 2011).

A number of macroalgal species have been reported to successfully induce settlement of marine invertebrates (Dworjanyn and Pirozzi, 2008; Pearce and Scheibling, 1991, Mos *et al*., 2011). Mos *et al*., (2011) concluded that the red geniculate coralline alga *Corallina officinalis*, and the brown alga *Sargassum linearifolium* are particularly effective at inducing settlement in *Tripneustes gratilla*. Dworjanyn and Pirozzi (2008) assessed these capabilities for *T. gratilla*, as well as Mos *et al.* (2011)*l*. Dworjanyn and Piroozi (2008) found that the greatest settlement was achieved on *Corallina officinalis* (32% settlement) and found that the lowest settlement was induced by *Ulva lactuca (8%).* They also demonstrated that algal metabolites (or metabolites from surface bacteria) influence urchin settlement by demonstrating differential settlement of *T. gratilla* larvae in seawater conditioned with *C. officinalis,* which induced the greatest settlement (Dworjanyn & Pirozzi, 2008). This work was corroborated by Mos *et al.* (2011).

Ulvella lens has been extensively used in the aquaculture industry as a settlement cue, and a post-settlement feeding substrate (Daume *et al*., 1999; Krsinich *et al*., 2000; Ohshiro *et al*., 1999; Takahashi & Koganezawa, 1988). *U. lens* is a small, green macroalga that encrusts surfaces by increasing the radius of its disk-shaped thallus (Hannon *et al*., 2014). The crust is able to induce settlement in marine invertebrates such as sea urchins (Brown & Eddy, 2015; Brundu *et al*., 2016; de Vos, 2017; Hannon *et al*., 2014; Sakai *et al*., 2004; Takahashi *et al*., 2002; Williamson *et al*., 2000; Wozniak, 2016; Cyrus, unpublished data), and abalone (Daume *et al*., 2004, 2014; 2015). *U. lens* has also been shown to induce greater settlement than diatomic biofilms (Krsinich *et al*., 2000).

The presence of conspecifics has also been shown to trigger settlement of competent *T. gratilla* larvae (Dworjanyn and Pirozzi, 2008, Mos *et al*., 2011). Dworjanyn and Pirozzi (2008) reported that *T. gratilla* settled in response to both live juveniles and adult faeces. Mos *et al*. (2011) also observed successful settlement in response to bacteria associated with adult conspecifics. Settlement in response to conspecifics has also been observed for *P. lividus* (Brundu *et al*., 2016), the sea cucumber *Psolus chitonoides* (Young and Chia, 1982), two species of sand dollar (Burke, 1984; Pearce and Scheibling, 1990b) and has been shown to be common for other marine invertebrates (Crisp, 1974; Pawlik, 1992; Hadfield and Paul, 2001). The presence of conspecifics suggests that the habitat occupied by these animals supports post-larval growth (Zupo *et al*., 2018), lowers the probability of individual predation (Pawik, 1992), and increases future spawning success (Penington, 1985). *T. gratilla* has a shorter lifespan than most other sea urchins and thus has had a disjunct temporal and spatial distribution throughout history (Lawrence and Agatsuma, 2007), in part because of gregarious settlement. Gregarious settlement implies that the effectiveness of a settlement cue need only induce settlement in a few individuals, which would in turn facilitate the settlement of multiple other individuals

Post-settlement production of sea urchins

Although *T. gratilla* has a well-documented range of settlement substrates, it is necessary to monitor post-settlement survival and growth as well as settlement success in order to ensure that a sufficient number of urchins make the transition to feeding on macroalgae (Mos *et al*., 2011). Several cues may effectively induce settlement, but fail to sustain the post-larvae during the post settlement phase (Mos *et al*., 2011). This is often because the settlement substrate is either indigestible or not nutritious to the urchins. Marine invertebrate mortality rates are generally between 60-99% during this vulnerable period of development (e.g. Chao *et al*., 2010; Grosjean *et al*., 1998; Mos *et al*., 2011). It is thus important to determine the appropriate feed type (or combination of different feed types), for promoting the growth of urchins after settlement (Cyrus *et al*., 2015).

Tripneustes gratilla often graze on seagrasses in tropical regions (Dy, Uy & Corrales 2002; Vaitilingon, Rasolofonirina & Jangoux 2003) and are more likely to graze on macroalgae in temperate regions (Lawrence & Agatsuma, 2001) like South Africa, which has no seagrasses on its eastern rocky coastlines (Browne et al., 2013). Abalone farms in South Africa make use of wild harvested macroalgae as feed (e.g., *Ecklonia maxima* and/or *Laminaria pallida*) as well as aquacultured *Ulva* and/or *Gracilaria* on some farm but natural populations of macroalgae are unlikely to be used as the sole feed for urchin rearing (unviable for large scale commercial operations) (Cyrus *et al*., 2014). The kelp harvesting industry in South Africa is well regulated, the management of which controls the harvesting natural kelp resources by enforcement of quotas and limited harvesting permit allocation (Anderson *et al*. 2003; 2006; 2007; Troell et al. 2006). Artificial formulated feeds are thus used to make up the bulk of adult diets on abalone aquaculture farms in SA, despite SA abalone farms culturing *Ulva* on site as feed supplement as well (Bolton, pers. comm.).

Plastic plates covered with benthic microalgae, such as *U. lens* (Crouan rat. 1859) and diatoms, are often used to induce larval settlement in commercial operations. These substrates also serve as important first feeds post-settlement and directly influence the post-settlement phases of the urchin life-cycle (e.g. Rahim, Li & Kitamura 2004). Cyrus *et al.* (2015) demonstrated that *T. gratilla* is able to successfully settle, complete metamorphosis and transition to feeding on macroalga diets when a mixture of four benthic microalgae species (*Amphora sp., Cocconeis sp., Nitzschia closterium* and *Navicula jeffreyi*) are used for the settlement and post-settlement phases to produce juvenile urchins for full life-cycle grow-out.

Most of the success of diatoms or biofilms in inducing settlement are attributed to the specific nutritional advantages they present to larvae that settle on them. However, highly nutritious diatoms do not necessarily correlate with greater survival and settlement (Viana *et al*., 2007). Digestion efficiency, for example, has been shown to have a greater influence on juvenile abalone growth than nutrition alone (Onitsuka *et al.,* 2007). Digestion efficiency is influenced by diatom morphology, attachment strength, frustule strength as well as the post-settlement size of the grazer (Onitsuka *et al.,* 2007). It has also been suggested that the relative abundance, composition, and associated unique nutritional and digestion efficiency profiles of diatom films may be critical in ensuring higher levels of survival and fitness in marine invertebrate post-larvae (Majewska *et al.,* 2014).

The size at which urchins can successfully transition to, digest and process macroalgae is not well known and the underlying mechanisms surrounding this transition remain poorly understood (Onitsuka *et al*., 2015). It was shown that *Strongylocentrotus purpuratus* are able to switch to feeding on macroalgae at 30-50 days post-settlement (0.8-1.2 mm test diameter) (Rowley, 1990). Devin *et al.* (2004) also tested several species of macroalgae as feed for *S. droebachiensis* that had 2.5 mm test-diameter (Devin *et al.,* 2004). Sea urchins between 1-3mm in test-diameter have shown higher growth rates when feeding on a diet of diatoms than those that were fed macroalgal diets (Onitsuka *et al*., 2015). There is evidence to suggest that urchins will only switch to macroalgal diets (from detritus and microalgae) between 5-10 mm test diameter (Kawamura *et al*., 1973). Once this dietary shift has been made, macroalgae appear to be able to support high growth rates in urchins, like *S. intermedius* (e.g., Agatsuma, 2000; Machiguchi and Kitamura, 2012). The current study attempts to investigate when such a dietary shift is feasible for newly settled *T. gratilla*. It appears that the critical size for weaning onto macroalgae is different for different species of urchins.

It is possible that feeding on fleshy macroalgae can only occur once the Aristotle's lantern (mouth complex) and digestive tract have been sufficiently developed. The post-settlement feed of urchins may directly affect the development of these body parts. The Aristotle's lantern becomes larger relative to the test size of echinoids when food is limiting (Ebert, 1980; Black *et al*., 1982, 1984; Edwards and Ebert, 1991; Levitan, 1991). Urchins with larger jaws are able to more easily scrape and bite food than urchins with smaller jaws (Black *et al*., 1984). Proportionally larger feeding complexes are indicative of diets that are more difficult to masticate and ingest (Daggett *et al*., 2005). Morris & Campbell (1996) also found that juvenile *Strongylocentrotus franciscanus*, when fed eelgrass *Zostera marina*, had higher jaw length to test diameter ratios than urchins fed the kelp *Nereocystis luetkeana*, which is a higher quality food (Morris & Campbell; 1996). Hence energy has been invested into growing the feeding apparatus rather than promoting test growth (which extends the time required for '*uni'* to be market ready as a consequence). Furthermore, early urchin juveniles also have only one convolution in their digestive tract as there has not yet been any differentiation between the stomach and the intestine (De Ridder & Jangoux 1982). Energy may be invested into developing gut complexity before test growth as well. Conversely, urchin juveniles that have made the transition to feed on macroalgae develop a superior intestine in their gut which allows for the digestion of relatively tougher macroalgae (De Ridder & Jangoux 1982).

Another hypothesis for effecting the dietary shift to macroalgae is that enzymatic activity and gut flora need to develop fully for the successful digestion of ingested macroalgae. Onitsuka *et al*. (2015) suggested that this shift could be achieved with improved enzymatic activity which correlated with increased size. Urchin juveniles (*Strongylocentrotus intermedius*) over the size of ca.3 mm test-diameter were able to increase their enzyme production abilities, at the same time regulating the types and concentrations of specific enzymes in response to ingested food items. Urchin growth rates at ca. 6 mm in test-diameter were significantly larger when fed macroalgae as opposed to microalgae, further supporting the hypothesis that enzymatic activity enables efficient/complete digestion of ingested nutrients trapped in macroalgae, promoting increased growth (Onitsuka *et al*., 2015). The authors suggest that the primary nutrient source shifts form microalgae to macroalgae is at 6 mm in test-diameter. Onitsuka *et al*. (2015) suggested that the physical characteristics of diets were important to consider, as smaller juveniles had limited digestive abilities. However, as the juveniles grew larger and had developed relatively superior enzymatic activity, the nutritional composition of ingested feeds were more important to produce optimal growth (Onitsuka *et al*., 2015).

Survivorship in the initial period of development post-settlement can be critical to the successful recruitment of sea urchins in both natural habitats and for the production of juveniles on farms. There are only a limited number of studies examining growth and survival of sea urchins in early life-stages especially when smaller than 5 mm in test-diameter (Azad *et al*. 2010). The current study hopes to contribute to this sparse body of knowledge by studying *T. gratilla*, with a focus minimising mortality and stimulating growth during this vulnerable period, for the purposes of commercial aquaculture.

Strategies for improving larval settlement, metamorphosis, and post-settlement production

Metamorphosis involves the resorption of existing larval structures and the development of new structures/organs to facilitate the transition to benthic juveniles after settlement. Xing *et al*., (2007) demonstrated that juvenile urchins could only start growing optimally from nine days after settlement once the Aristotle's lantern (urchin mouth complex) was fully developed and could thus rasp the substratum. Newly settled marine invertebrates cannot digest the macroalgal diets that adults normally consume. Therefore, *T. gratilla* juveniles require a diet capable of promoting high growth to stimulate their development, and eventually facilitate their transition to feed on macroalgal diets.

A benefit of using *U. lens* as a feeding substrate is that recently settled larvae can easily change from feeding on diatom biofilms that grow on *U. lens*-coated surfaces, to feeding on the crust itself without having to be subjected to the stress of being transferred to a new substrate (Kawamura *et al*., 1983; Brundu *et al*., 2016). Such substrates serve not only as settlement inducers but also as convenient feeds for newly settled larvae (Takahashi *et al*., 2002). *Ulvella lens* is known to contain high concentrations of glycerolipids (Takahashi *et al*., 2002) and it is generally assumed that these promote urchin survival when ingested. *U. lens* is thus also used as a post-settlement food source in the invertebrate aquaculture industry (Daume *et al*., 1999; Krsinich *et al*., 2000; Ohshiro *et al*., 1999; Takahashi & Koganezawa, 1988).

Probiotics, as well as other non-specific immune stimulants, have garnered considerable attention as tools for improving systems and animal health. Probiotics are defined as "cultured products or live microbial feed supplements, which beneficially affect the host by improving the intestinal (microbial) balance" (Fuller, 1989). Probiotics have several modes of action including competitive exclusion of adhesion sites, the production of inhibitory compounds, immune stimulation, improvement of water quality, a source of nutrition and enzymatic contribution to digestion, and promoting growth of the host (Verschuere *et al*., 2000). The probiont also needs to be present in great enough quantities to effect a response and be able to reach the location where the probiotic effect is required (Verschuere *et al*., 2000).

The specific mechanisms by which probiotics operate make them uniquely effective for marine invertebrates, as well as potentially combating the proliferation of pathogens, and improving the health of the culture system. No studies have been published on the influence of probiotics on the growth of juvenile sea urchins like *T. gratilla*, but rather only their settlement response, in intensive aquaculture (Mos *et al.* 2011).

However, juvenile *H. midae*, for example, have been shown to have stimulated growth rates, by up to 30%, when fed with a probiotic-supplemented feed (Macey & Coyne, 2006; ten Doeschate & Coyne, 2008).. Probiotic supplementation is likely to increase survival rates of newly settled marine invertebrates such as *T. gratilla*, in hatcheries by stimulating their growth during their vulnerable life-stages. There is also evidence that probiotics stimulate immune function in marine invertebrates, e.g., juvenile abalone show significantly greater survival when challenged with a known pathogen, *Vibrio anguillarum* (Macey & Coyne, 2005). *Vibrio anguillarum* is dangerous for the production prospects of abalone farms in South Africa, as an infestation in the water supply could lead to the eradication of farm stock or harvest.

The green seaweed *Ulva* is also grown on South African abalone farms to supplement the diets of cultured animals on site, and is an example of Integrated multi-trophic aquaculture (IMTA). The co-culture of *Ulva* and *T. gratilla* (together with abalone) in a commercial setting is currently being investigated and assessing its viability is one of the aims of the ASTRAL IMTA lab, in South Africa. *Ulva* was shown to be an important inclusion in the diet of *T. gratilla*, for the purposes of commercial aquaculture (Cyrus, 2013; Cyrus *et al*., 2014; 2015). *Ulva* is consumed in greater proportions by smaller size classes of abalone than when the abalone are older and larger (Sales & Britz, 2001). This implies that *Ulva* aids in survival of the younger, more vulnerable abalone life-stages perhaps facilitating the animal's transition to sexual maturity. This may hold true for other marine invertebrates like *Tripneustes gratilla* as well.

Research from the same laboratory as the current study showed that *T. gratilla* was attracted to methanolic and ethanolic extracts of the seaweed *Ulva* (du Plessis, 2014; Etwarysing, pers. comm.). Etwarysing made a series of crude extract fractions from *Ulva* (using step-gradient Si- column chromatrography). The glycolipids Monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) have been isolated from two of the most polar of these extracts and have previously been recorded as being phagostimulatory agents for marine herbivores like urchins and abalone (Sakata *et al*. 1985, 1988, 1989). Chemical cues from *Ulva* that attract adult sea urchins and stimulate feeding may also signal for larval settlement and metamorphosis. Algal metabolites that are phagostimulatory may signal that a suitable food source is in the vicinity, indicating that it is available for consumption if competent larvae successfully settle in the same area. Consequently, the phagostimulatory cues may act as settlement inducing cues.

The effects of *Ulva* on the growth of juvenile urchins are unknown for newly settled post-larvae. Previously, Cyrus *et al*. (2015) tested various diet regimes on the somatic and gonadal growth of juveniles starting from 33.05 mm ± 0.77 (mean test-diameter ± SE). From their experiments, the researchers suggested that, for commercial aquaculture, fresh *Ulva* diets be used during to stimulate greater somatic growth, where the later benefits of gonad enhancement (with artificial/formulated diets) would not be hindered by an otherwise smaller test size (Cyrus *et al*., 2015). Floreto *et al*. (1996) tested the effects of three different seaweeds (including *Ulva pertusa*) on the growth and fatty acid composition of *T. gratilla* juveniles, test diameter of 16.44 mm ± 1.26 (mean ± SE). The work of the above researchers used the smallest size classes of metamorphosed *T. gratilla* found in the published scientific literature, at the time of the current study.

The use of alginate is an exciting new technology being developed for the aquaculture industry. Alginates can form gels, which are able to encapsulate cells, drugs as well other biological materials. They are also used in the biomedical industry as a delivery mechanism for pharmaceuticals (Pawar & Edgar, 2012). Alginates are polysaccharides made up of ¼ linked b-D-mannuronic acid (M) and its C-5 epimer a-L-guluronic acid (G) (Pawar & Edgar, 2012). These polysaccharides can be found in the intracellular matrix of brown algae, Phaeophyceae), in the form of gels containing the likes of sodium, calcium, magnesium, strontium and barium ions (Pawar & Edgar, 2012). The calcium cation (Ca^{+2}) is the most common ion used to produce alginate gels. Commercially available alginates are obtained from seaweeds but can also be acquired from bacterial sources (Pawar & Edgar, 2012).

Bacteria have been encapsulated in sodium alginate and used as a delivery mechanism for probiotics. The encapsulated bacteria can be ingested and delivered to the intestinal tract of an animal, where their beneficial effects may occur (Rosas-Ledesma *et al*., 2012). The laboratory of Coyne has been investigating the potential of probiotic supplementation in stimulating the growth and settlement of abalone *Haliotis midae*, using alginate-based substrates (Moxley and Coyne, Macey and Coyne, 2005; 2020; ten Doeschate & Coyne, 2008). Formulated feeds may also be encapsulated in a similar manner and combined with probiotics for the easy administration of feed with or without probiotic(s) to aquacultured fish (Cordero *et al*., 2015) and potentially to marine invertebrates. While many other gelling agents have been considered for immobilization matrices (agar, carrageenan, and chitosan), alginate has been a preferred choice due to its simplicity and fast polymerisation in making gelling agents (Kube *et al*., 2018). It is also non-toxic, has broad biocompatibility properties, and mild gelling conditions which all ensure high cell viability (survival) (Martin *et al*. 2015; Rathore *et al*. 2013). Sodium alginate was used in this study as a delivery mechanism, presenting different probiotic, seaweed extractions and microalgae to *Tripneustes gratilla* post-settlement juveniles.

Aims:

This work aims to investigate how to maximise the production of *Tripneustes gratilla* by minimising loss of urchins during settlement and post-settlement stages of the aquaculture process.

The above is to be accomplished by:

- Comparing the effects of varying artificial and natural substrates on settlement success
- Comparing the effects of varying artificial and natural substrates on post settlement survival and growth
- Investigating the optimal timing for larval weaning from benthic substrates to macroalgae (*Ulva*)

Materials and Methods

All experimentation was conducted at the Department of Forestry, Fisheries and the Environment (DFFE) Marine Research Aquarium (MRA) in Sea Point (Cape Town) (33°55’12”S 18°22’52”E). The MRA receives sea water pumped directly from the Atlantic Ocean via pipes running from the subtidal zone. The water is pumped through a drum filter and a series of sand and cone filters prior to entering sump tanks at the highest point in the MRA, whereafter seawater is gravity fed to the various systems, including those mentioned below, within the MRA.

Sea urchin spawning

Two separate cohorts of urchins were spawned from the same batch of cultured broodstock: ten adult urchins were spawned for growth experiment A (five males and five females), and eight urchins were spawned for growth experiment B (five males and three females)(Fig. 1). The protocol used for spawning *Tripneustes gratilla* broodstock at the MRA was adapted from Scholtz *et al*. (2013). Adult urchins were placed aboral-side down on top of 500 ml Erlenmeyer flasks containing 0.22 µm filter sterilized seawater (FSW; Fig. 1). Spawning was induced by injecting 0.5–1.0 ml of a sterile solution of 2 M KCl into the celomic cavity. Spawning was observed as the ejection of gametes into the Erlenmeyer flasks: male gametes were viewed as a white stringy ejection and remained suspended in the water column, whereas the female gametes were orange/yellow in colour and sunk to the bottom of the flasks (Fig. 1). The male and female gametes were pooled separately in 5 L beakers. The beakers were stirred gently to ensure homogenisation of the gametes and aliquots of the eggs were counted using a Bogorov tray under a dissection microscope and averaged to determine the number of eggs per ml. Aliquots of the male sperm were diluted and replicate 1 ml samples counted using a haemocytometer under a compound light-microscope (400 magnification). Male gamete samples were mixed with 10 % formalin- seawater solution to inhibit the sperm’s motility prior to counting. Following counting, all the eggs were fertilised at a sperm:egg ratio of 100:1. The gametes were left to fertilise for 10 minutes and checked for fertilisation, by the presence of a fertilisation membrane, under a compound light microscope (100x magnification).


Figure 1: *Tripneustes gratilla* spawning was induced by injecting 0.5-1ml 2M KCl solution into the coelomic cavity of individual urchins. Eggs are orange and sink, while sperm are white and swim in the water column.

The fertilised eggs were gently rinsed with sterile seawater, using a 63 µm sieve to remove excess sperm, before being distributed into large white troughs (L x W x H: 400 mm x 880 mm x 270 mm) containing pre-acclimated (at 25 ^{0}C) 0.22 µm filtered seawater to hatch. Hatching occurred within 48 h, after which urchin plutei were distributed into the larval rearing tanks at a density of 5 larvae per ml. This stocking density was chosen as previous work has shown this to be a suitable density for *T. gratilla* larval rearing (Byrne, *et al*., 2008; Dworjanyn & Pirozzi, 2008; Mos, *et al*., 2011). Low initial stocking densities also improve the survival rates of sea urchin larvae (Brundu *et al.,* 2016).

Larval rearing systems

Urchin larvae were reared in nine ca. 100-litre black cylindrical polyethylene tanks (Fig. 2) or in 12 white ca. 150 L conical tanks housed in the DFFE MRA sea urchin hatchery. Water temperature in the hatchery was maintained at 25 °C ± 1, as *T. gratilla* are typically cultured between 24-25 °C<< CITATION Dwo08 \l 7177 (Dworjanyn & Pirozzi, 2008)>>. The hatchery was run under a 12:12 day cycle. Fluorescent bulbs were used that emitted cool white light (T-10, 75 W, 4500 lm for black tanks at 2 m away with an incident angle of ca. 60^o, whereas the bulbs for the conical tanks were only 0.8 m with an incident angle of ca. 30^o for conical tanks).

Figure 2: Nine black polyethylene cylindrical tanks were used to rear *T. gratilla* larvae, each fitted with a banjo sieve.

Each of the nine black polyethylene cylindrical tanks had an overflow hole drilled in the side of the tank, at a height of 555 mm from the bottom of the tank (Fig. 2), and a drainage hole near the bottom. The 12 white conical tanks were also fitted with overflow holes drilled near the top of each tank, with drainage holes at their bottoms. Each larval rearing tank was equipped with its own heating rod (300 W) to ensure that the water temperature did not fluctuate and was maintained at 25 °C (this was checked each day throughout the rearing period). Each overflow outlet was fitted with a banjo sieve to prevent urchin plutei from exiting the system when the tanks were supplied with constant water flow. Banjo sieves of two different sizes (125 µm and 200 µm) were used. The 125 µm sieves were used immediately following stocking and were later replaced with 200 µm sieves once larvae were larger than 200 µm. This was done to limit clogging and the degree of algal fouling in the tanks. Each tank was supplied with constant fine aeration through air stones (black cylindrical tanks; supplied by an ACQ-007 BOYU Air Compressor at ca. 100 L per min or by aeration from the MRA’s built in air lines (supplied to the white conical tanks)).

Larval rearing and feeding

The larvae in all tanks were initially reared using a ‘static system’ approach for the first week of the larval rearing process. This was done to minimise stress on larvae and increase survival.

The tanks were monitored and cleaned/ siphoned daily to remove settled larval feeds and dead plutei at the bottom of the tanks. After the first week of larval rearing, water was provided to each of the tanks via water drippers (at 4 L/h) during the night (25 ^{o}C), and 50 % manual water exchanges were done every three days. Filtered seawater (FSW) sterilised with ultra-violet (UV) light was pumped through the system during larval rearing.

The larvae were reared in the larval rearing tanks for 23 days. During this period the larvae were fed the haptophyte *Isochrysis galbana,* the diatom *Chaetoceros muelleri* and the cryptophyte *Rhodomonas salina.* These microalgae were selected as it was shown that they are optimal for rearing *T. gratilla* larvae (Scholtz, *et al*., 2013; Castilla-Gavilan, *et al*., 2018). Algal feeds were cultivated in the algal culture laboratory in the MRA. *Isochrysis galbana (CS22), Chaetoceros muelleri (CS176)* and *Rhodomonas salina (CS174)* cultures were started in 2 L Schott bottles, supplied with constant aeration under 24 h fluorescent light (T-10, 75 W, 300 lux). Meta-silicate (40 g of sodium meta-silicate powder dissolved in 1 L filtered freshwater) was added to *C. muelleri* (1 ml/L), because it is a diatom. F2-media (1 ml/L) was added to each culture to stimulate algal growth. The Schott bottles were covered with aluminium foil caps and were cultured in this way for five days after which they were transferred to respective Perspex algal incubation tubes, for upscaling (Fig. 3). These algal tubes were cylindrical columns (300 mm diameter × 1000 mm height) with square bases and round lids. The algal tubes were filled with 40 L of 0.22 µm FSW as well as 40 mL F2-media, and 40 mL metasilicate for the *Cheatoceros muelleri* culture. The tubes were supplied with constant aeration and light (T-10, 75 W, 4500 lm, vertical long bulbs 0.5 m away from tubes with an incident angle of 0^{o} , Fig. 3).

Feed was administered 3 days after the rearing tanks were seeded with larvae and consisted of a mixture of *I. galbana* and *C. muelleri* (2000 cells per ml of each species). *R. salina* was included (in conjunction with the other two feeds) roughly one week after the first feed. Urchin larvae were fed at a ratio of 6000 algal cells to one urchin larva daily (2000 cells of each species of algae per one larva minimum). This ratio of feed was chosen to ensure that there would be sufficient feed in close proximity to each larva in the tanks. The algal concentration was increased to 10000 cells per larva per day, after two weeks (3333.3 cells of each algae per larvae).

Larval counts were recorded daily during the rearing period by taking samples from each of the larval rearing tanks (Fig. 4), before transferring 1ml aliquots (n=5 triplicates) to separate wells of a Bogorov tray for counting. The average number of larvae per ml was calculated and used to estimate the number of larvae in each tank and subsequently how many algal cells were required for feeding.

Algal counts were also completed daily throughout the rearing period. Samples of *I. galbana, C. muelleri* and *Rhodomonas sp*. were collected from their respective algal incubation tubes. Serial dilutions of each of these were prepared to find an appropriate concentration for counting (using a haemocytometer), as the original cell density of the samples varied between each algae. The number of algal cells per ml was subsequently calculated for each algal species. The number of algal cells per ml in the rearing tanks were also counted and used to estimate the number of algal cells needed per tank daily. This was done to limit the amount of algal fouling in the tanks and ensure optimal water quality. Each tank was inspected daily for cleanliness and siphoned once a week (or more if required) to remove dead larvae and settled food matter (Fig. 4).

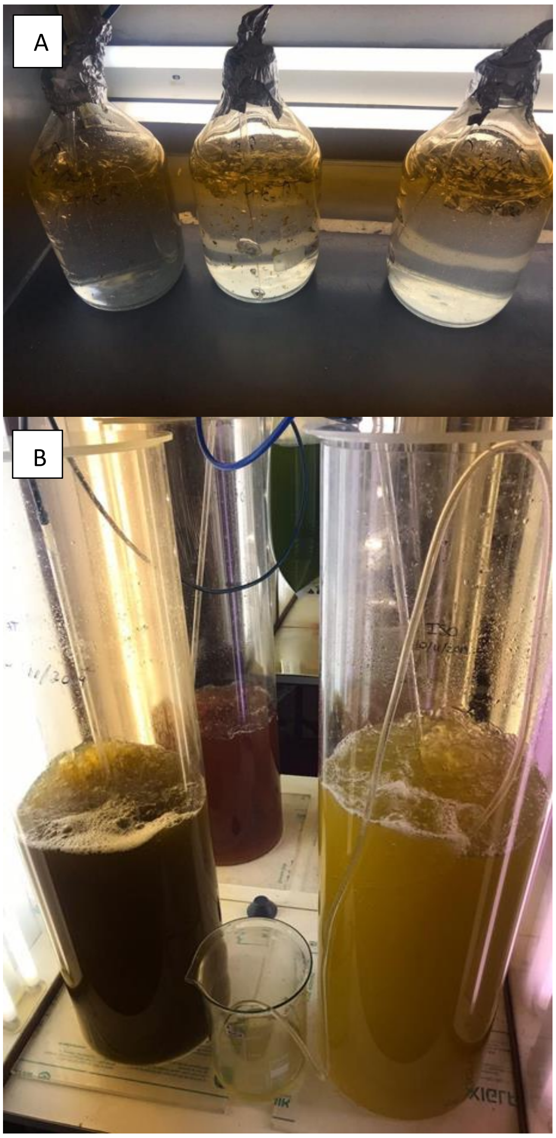

Figure 3: Larval feeds were cultured in 2l Schott bottles (A) before being transferred to larger algal tubes for upscaling (B).

Larval settlement

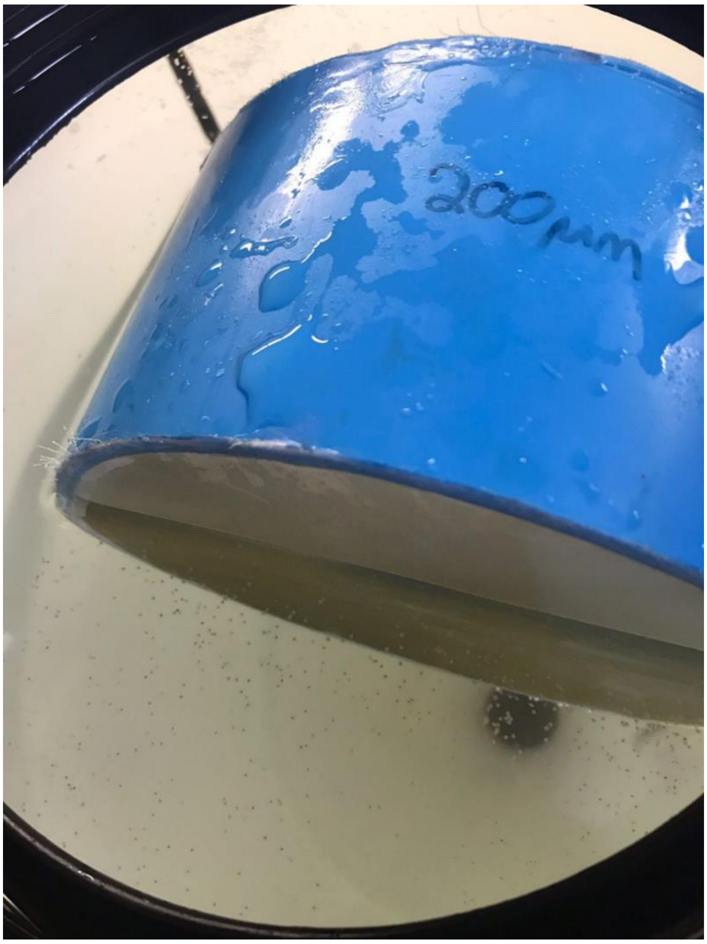

Figure 4: Samples were collected from cleaned larval rearing tanks for counting. Here, mature larvae can be seen near the surface of the water column.

Once the larvae were deemed competent to settle, they were used for trials described below. Competence was based on the presence of certain morphological (8-arm pluteus stage, rudiment larger than the gut), pedicellaria present (Pedicellariae I stage from Mos and Dworjanyn*,* 2016), and behavioural (swimming to and remaining near the bottom of the rearing tank) - characteristics. The larvae were also tested with a preliminary settlement assay, consisting of petri-dishes coated with *Ulvella lens*, which is a known settlement induction cue (Hinegardner, 1969; Carcamo, *et al*., 2005; Dworjanyn & Pirozzi, 2008) that has been shown previously in our laboratory to be very effective for settlement of competent *T. gratilla* larvae. When larvae were deemed to be competent, they were drained from rearing tanks and concentrated in a large beaker. The concentration of the larvae was calculated by taking samples and counting them under a dissection microscope. The number of larvae per ml was calculated and the appropriate volume was transferred into each larval rearing trough to achieve an average of 35 larvae per replicate trough. Troughs were stocked with thirty-five larvae to compensate for the potential loss of larvae encountered during the settlement process, and ensure enough larvae survive to complete the experiments. Settlement was deemed successful when the presence of tube feet was observed.

The juvenile rearing system for growth experiments

Post-settlement growth trials were conducted in an access- and temperature-controlled (25 ^{o}C) laboratory (2.5 m x 4.0 m) at the MRA. The laboratory was exposed to a 12:12 light cycle using cool-white fluorescent tubes (T-10, 75 W, 4500 lm, 2 m away from troughs with an incident angle of ca. 60^{o}).

The laboratory housed 18 glass fish tanks, with dimensions: 460×230×300 mm (L×W×H), which were set up as a flow-through system (Fig. 5). A 20mm hole was drilled near the top of each glass tank to serve as an overflow hole. A large sump tank (300L), equipped with an AquaDrive 390 SPECK pump (model 6452LTL-A12X), provided pre-acclimated (25 °C) 0.45 µm filtered seawater to each glass tank via a ring main at a rate of 4 L/h using dripper lines (Fig. 6). The sump received 0.45 µm filtered water from the MRA holding tanks and was fitted with a float valve to ensure that the sump remained full and supply of water to the fish tanks was uninterrupted. The sump was also fitted with rod heaters (300 W) set at 25 °C to ensure a constant supply of heated seawater (25 ^{o}C) to each trough over the trial period.

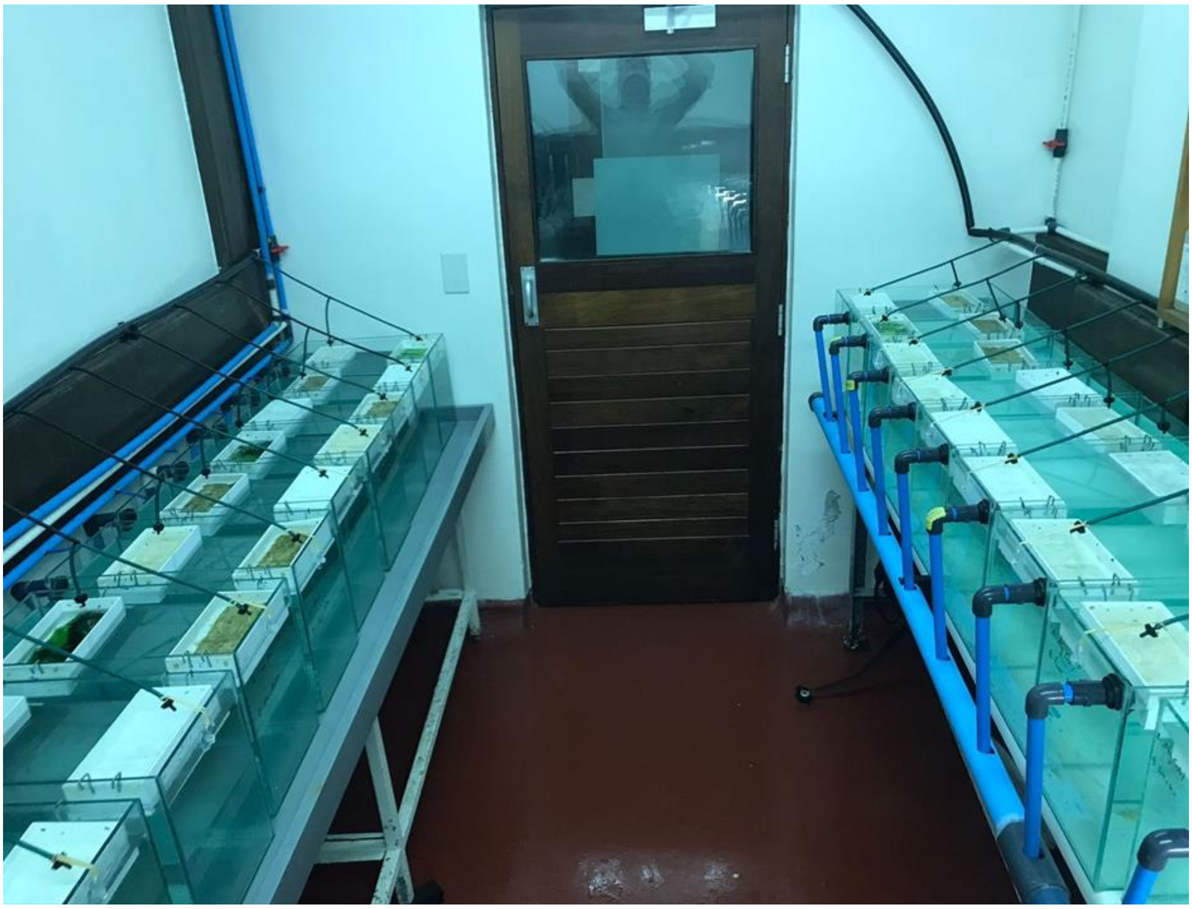

Figure 5: Fish tanks were set up in a flow-through system in a temperature-controlled laboratory, in which the experiments were conducted.

Each of the 18 glass fish tank housed two “troughs” (for a total of 36) (Fig. 5). The troughs were made of white acrylic Plexiglass (3 mm thick) and had the following dimensions: 200×100×50 mm (L×W×H). Suspending the troughs in glass tanks housing water in a temperature controlled laboratory ensured better control of the seawater temperature within each trough. Troughs were made of individual plates glued together using Magmabond C2 Acrylic Adhesive (with catalyst). These boxes were made to be watertight and were made of white perspex to be able to take a clear photograph under a stereomicroscope (for data collection purposes).

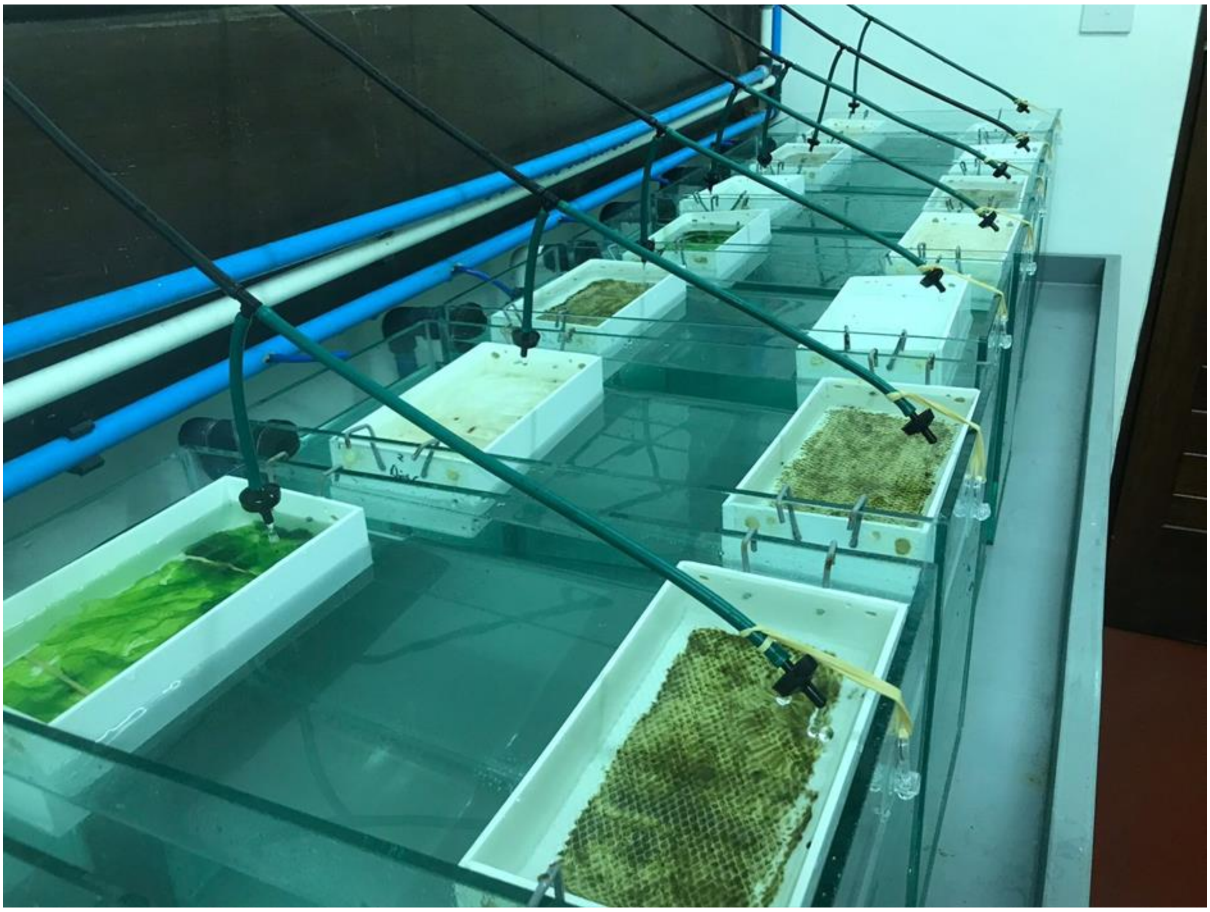

Figure 6: Each tank held two troughs suspended by PVC hooks in the water. Water drippers were held taught over each through with elastic bands.

The small troughs also had holes drilled into the short sides: this was for hooks from which the troughs were suspended in the glass tanks. Troughs were suspended in the glass tanks with PVC hooks (Fig. 6). The holes for the hooks were drilled 5 mm from the top of the boxes and 25 mm from the side. Hooks were made from thin PVC plastic rods (2 mm) bent using a heat gun. Overflow holes were drilled into the sides of each trough near the top and were covered with 200 µm mesh to prevent newly settled urchins from escaping.

Additional plexiglass plates (95×195 mm) were designed for the base of each trough and were used as removable bases for the experimental substrates. Plates were sanded with a rotary sander equipped with 100 pt grain sandpaper to create a rough surface to which algae and other substrates could easily adhere to and evenly coat. These plates were washed after sanding and hexagonal mesh (2 mm diameter) was glued onto some of these plates (for sodium alginate treatments, *see Treatments below*).

Each tank was fitted with a plastic suction cup with a hook to anchor the water lines (drippers) to each tank and keep them taut (with an elastic band) (Fig. 6). These water lines were connected to the ring main in the lab. This ring main was connected to the sump which pumped water through the system. The drippers supplied seawater from the sump to each trough at a rate of 4l/h (with a turnover rate of ca. 4 L/h).

The glass tanks were filled with seawater to act as insulation and buffer any potential temperature changes from the environment. Each glass tank was filled to its overflow hole with 0.22 µm filtered seawater (FSW), which could come to the set room temperature (before experimentation started).

Experiments

Two growth experiments were conducted (referred to as growth trial A and B). Different facets of the hatchery rearing process were assessed in the two growth experiments. Growth trial A assessed settlement success and post-settlement survival and growth on different substrates, as well as the timing for the transition from *U. lens* to feeding on the macroalgae *Ulva lacinulata*. Growth trial B assessed growth and survival of post-settled larvae that had all been settled on *Ulvella lens* on different post-settlement substrates, and the timing for the transition from these substrates to feeding on the macroalgae *U. lacinulata*.

Both growth trails were conducted in the temperature controlled laboratory (at 25 ^{0}C ± 1) at the MRA. Water temperature of the sump tank as well as the individual troughs and glass tank) was assessed daily with a temperature probe – DTK 2017SD portable pH/Conductivity/TDS/Temperature meter.

Treatments

Different experimental substrates were selected for investigating their efficacy in inducing settlement and maintaining post-settlement growth and survival for *T. gratilla*. *Ulvella lens* was selected because it is already being used for the settlement of echinoid larvae in the echinoculture industry (Daume *et al.,* 1999; Krsinich *et al.,* 2000; Ohshiro *et al.,* 1999; Takahashi & Koganezawa, 1988), and thus useful for comparing the performance of other substrates. Previous work in the same laboratory has shown that *Ulva* sp. is important for the successful grow out of *T. gratilla* (Cyrus *et al*., 2013, 2014, 2015). It is plausible that if *Ulva* is important in the later life stages of *T. gratilla*, it may be important for the early life stages (settlement and post-settlement) as well. Other work has shown that adult *T. gratilla* are attracted to the methanolic and ethanolic extracts of *Ulva* (Etwarysing, pers. comm.). The feed attractant cues from *Ulva* may also signal for settlement and is investigated in this study.

Since the alginate base supplemented *Ulva* extract fraction 9 alone was thought to have insufficient nutrients to sustain the growth of post-settled urchin larvae, a treatment consisting of a combination of the probiotic *Vibrio midae* SY9, *Ulva* extract F9 and the alginate base was included as a treatment. Probiotic supplementation (*Vibrio midae* SY9) when delivered through sodium-alginate based substrates have been shown to have stimulate the growth rates of newly settled abalone *Haliotis midae* (Moxley and Coyne, 2020), and this was tested for the settlement and post-settlment growth of *T. gratilla* in the current study. Alginate-based substrates were designed to include dried marine algae: dried *Ulva lacinulata* (green macroalgae) and dried *Isochrysis galbana* (microalgal haptophyte), as dried seaweed was thought to be more nutrient dense than their fresh counterparts (Carrier *et al.,* 2017; Lawrence, 2000; MacArtain *et al.,* 2007; Otero-Villaneuva *et al.,* 2004). A treatment containing dried algae and agar was also included with which to compare alginate-based treatments with. *Ulva* extracts were resuspended with ethanol and as such a control including sodium alginate and ethanol was tested. Another control treatment with only sodium alginate solution was also included with which to compare other alginate-based treatments with.

Treatments can be classified as either "natural substrates" (non-alginate based substrates) or "prepared non-algal substrates". Natural substrates include *Ulvella lens*, fresh *Ulva,* and *Nitzschia* sp. whereas "non-algal substrates" include all the other substrates that incorporated sodium alginate into their base, including substrates made with agar-agar. The preparation of each of the substrates used in the experiment is given below.

Ulvella lens

U. lens (CS801) was sporulated as described by Hannon *et al*. (2014) one month (4 weeks) prior to the start of the experiments. *Ulvella lens* was sporulated in a large white trough (400 mm x 880 mm x 270 mm), supplied with constant aeration and light (T-10, 75 W) in the algal laboratory, in the MRA (16 ^{0}C ± 1 FSW). F2 medium (Cell-Hi F2P from Varicon Aqua Solutions) was added to the trough to stimulate sporulation (300 g F2 medium per 1000 mL water). Several sanded plexiglass plates were placed on the bottom of the trough to be coated with spores. After being well-coated the plates were transferred to a new trough with clean seawater where they were maintained until needed for the experiment. Plates coated with *Ulvella lens* were checked two to three times a week and were lightly scrubbed to remove unwanted diatom/algal growth.

Fresh *Ulva*

Fresh *Ulva lacinulata* was collected from tanks within the MRA facility (originally stocked with *Ulva* collected from Irvin and Johnson (I&J) Cape Abalone farm Pty (Ltd)). Tanks were situated on the roof of the MRA and exposed to direct sunlight throughout the day. The tanks were supplied with FSW at the MRA. Fresh *Ulva* was rinsed with clean seawater to remove epiphytes and other foreign material before being used in the growth experiments. Fresh *Ulva* was administered *ad libitum* throughout the course of both experiments.

Nitzschia sp.

Three species of undescribed local diatoms were isolated from a local diatom community growing on the inside of an *Ulva* tank at the MRA in Sea Point, Cape Town (Wozniak, 2016). Initial work done by de Vos (2017) identified one of the three diatoms as a potential settlement substrate. This diatom was later confirmed to be an undescribed *Nitzschia* species which fits in the Nitzschia section *dissipatae* (Prof Andrzej Witkowski, University of Szczecin, Poland, pers. comm.). This was determined from morphology but the diatom has not yet been sequenced.

Cultures of this diatom were prepared by aseptically transferring cells (250 mL) from the master stock culture to two separate 2 L Schott flasks. Each flask (sterilised by autoclaving) was pre-filled with 1.75 L of 0.22 µm FSW before being inoculated with cells from the master stock. Thereafter, 2 mL of F2-media and meta-silicate solution was transferred to each flask (at 1 ml/L), under sterile conditions and mixed. The flasks were covered with foil caps, to prevent contamination, and placed under fluorescent light (T-10, 75 W, 40 cm away with an incident angle of 30^{o}) with constant aeration in the algal growth room (which was temperature controlled – set to 16 °C).

Nitzschia sp. was cultured for 1 week, after which the cultures were upscaled to larger white containers (400 mm x 880 mm x 170 mm) and supplemented with 0.22 µm FSW (16 ^{o}C). These containers were supplied with fine aeration and exposed to a 12:12 day cycle (cool white light, T-10, 75 W). Perspex plates (plexiglass) were placed within these containers to be coated. After two weeks of growth the plates were used in the experiments. Plates were taken from these larger containers to replace older plates in the experimental system once a week.

Prepared non-algal substrates

Sodium alginate was used in several of the experimental substrates evaluated in growth trial A and B. A preliminary test showed that using 5 % w/v sodium alginate salt resulted in the most stable substrates for the purposes of these experiments (this was the case for agar-agar substrates as well). The preliminary work also showed that 6-7 mL of an alginate mixture was sufficient to cover the plexiglass plates (95 × 195 mm) entirely with an even coating of the substrate. Cooled, autoclaved alginate (5 %) was used as a base in which other components were mixed. The respective mixtures were spread over the meshed plates with a laboratory spatula (Fig. 7). All treatments involving alginate were polymerised by spraying the coated plates with a $CaCl_2$-solution (0.25 M). Plates were left to polymerise for 10 min after which they were tilted at an angle (45^o) to allow any excess $CaCl_2$ solution to run off the plates. Plates were prepared one to two days before replacement and were stored in a refrigerator, before being used in the growth trials.

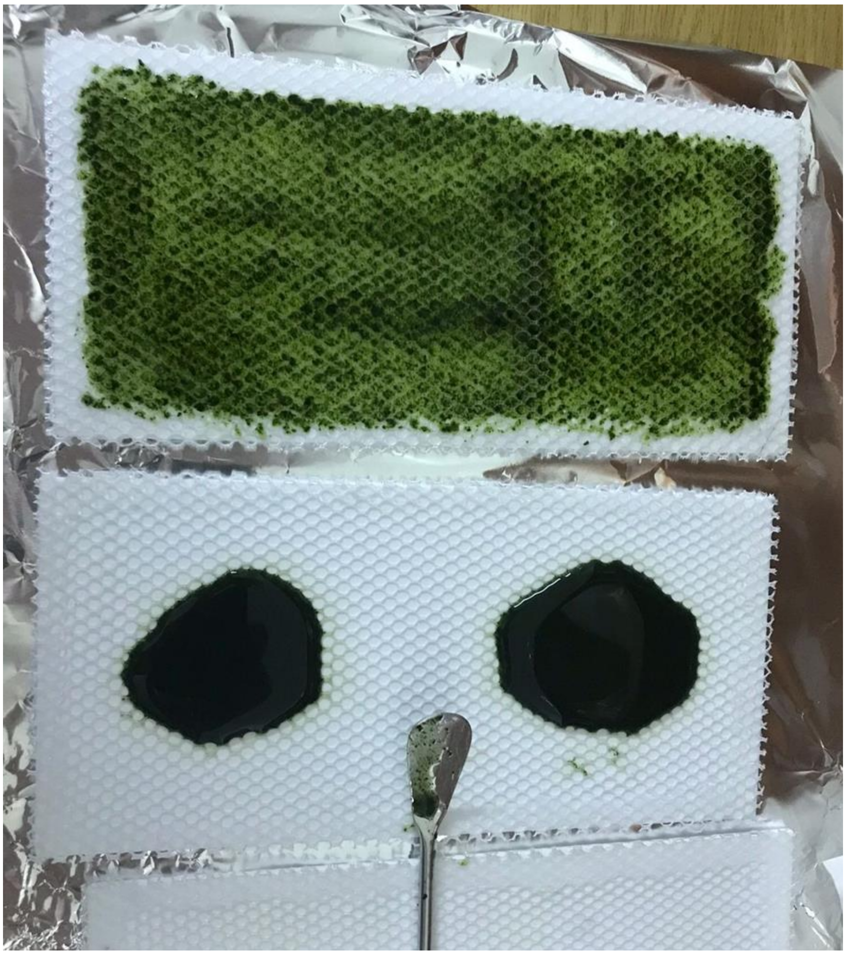

Figure 7: Plates were prepared by spreading 6-7m of each treatment combined with alginate base, over the meshed plates with a laboratory spatula.

Isochrysis galbana

The dried *I. galbana (*$1.56x10^{10}$ $cells.g^{-1}$) (PhytoBloom©) used in this study had the following proximate composition (% w/w): protein 41-46; lipids 20-23; carbohydrates 17-19. For the preparation of the *I. galbana* substrate, an aliquot (0.15 g) of the dried *I. galbana* was mixed with 30 mL of the 5 % (w/v) sodium alginate solution. This mixture was spread evenly onto the surface of the meshed coated plexiglass plates (6-7ml per plate) before polymerisation with the $CaCl_2$ solution, equating to ca.0.0325 g dried *I. galbana* per plate (approximately $5.07x10^7$ cells per plate).

Dried *Ulva*

Fresh *U. lacinulata* was dried in an oven (at 60 °C over three days). The dried *Ulva* was then placed in a blender and blended until fine before being placed in a ball bearing mill to further reduce the size of the *Ulva* pieces, resulting in an ultra-fine *Ulva* powder.

A lower concentration of dried *Ulva* was used for the respective treatments in growth trial A (0.1875 g per plate) compared with growth trial B (0.76 g dried *Ulva* per plate). These were weighed out on a balance for all replicates in the treatment and the total for 4 replicates mixed with 40 mL of alginate.

Dried *Ulva* and agar-agar

Growth experiment B included a dried *Ulva* and agarose substrate. Agar-agar (5 % w/v agar-agar) was autoclaved for the preparation of this substrate. The ultra-fine *Ulva* powder was weighed out on a scientific balance and 3.05 g was mixed with 40 mL of agar-agar. This equated to 0.76 g of dry *Ulva* on each plate.

Ulva extract F9

A series of *Ulva* extract fractions were prepared using Si-gel chromatography. These fractions are characterised by their differing polarities, with fraction 1 the least polar and fraction 9 the most polar (details provided below).

Fresh *U. lacinulata*, grown in abalone effluent water, was collected from Irvin and Johnson (I&J) Cape Abalone farm Pty (Ltd), South Africa. This *Ulva* was stored in a -20 ^{0}C freezer until needed. Prior to use, the *Ulva* was defrosted and washed 3 times with fresh water to remove sediment, epiphytes and small invertebrates, before spinning the *Ulva* in a salad spinner (ca. 20 turns) to remove excess water and patted down with paper towel.

A bulk extraction of 1200 g was done to provide sufficient *Ulva* extract for all trials in this study and to avoid possible inconsistencies from multiple extractions (differences in extract fractions arising from different Ulva growth conditions).

Three extractions were done.:

i) distilled methanol

ii) + iii) 2 distilled dichloromethane (DCM):1 distilled methanol solution (x2)

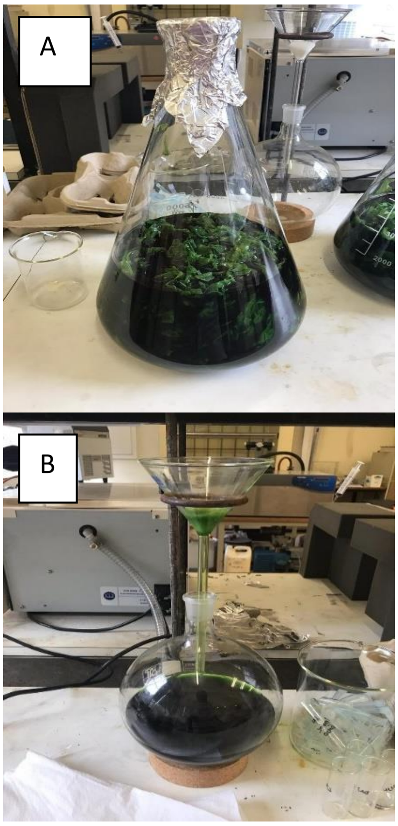

Figure 8: A) *Ulva* was submerged in solvents during bulk extraction. B) Supernatant was filtered into round bottom flasks.

The *Ulva* was placed in large Erlenmeyer flasks (5 L) and submerged in the solvents for 2 days (Fig. 8A). The supernatant was drained (filtered) into round-bottom flasks (Fig. 8B) and evaporated off with a Roto-vap (evaporator) (Fig. 9). The concentrated extracts were then resuspended in DCM and added together in one round-bottom flask and dried. A "crude extract" was thus formed [i) + ii) + iii)].

Figure 9: A Roto-Vap evaporator was used to evaporate off the solvents used during bulk extraction.

Water was found in the supernatant and needed to be removed before fractionation. This was done through water partitioning to separate the water from the solution. Supernatant was transferred to a separatory funnel and then DCM was added. The funnel was closed and shaken intermittently opening the tap to let gaseous build-up escape.

The funnel was placed in a stand and left so the fluids could separate out (some DCM was added in the top of funnel to aid in the process). Water has a lower density than the supernatant, thus it formed a layer at the top of the solution. The supernatant could then be drained out of the funnel. Then the drained supernatant was placed in the Roto-vap to evaporate off any remaining solvents. The crude extract was highly concentrated: 12.70 g of crude extract was produced from 1.2 kg of fresh *Ulva*. Celite was added to the crude extract to aid in drying it and forming a powder.

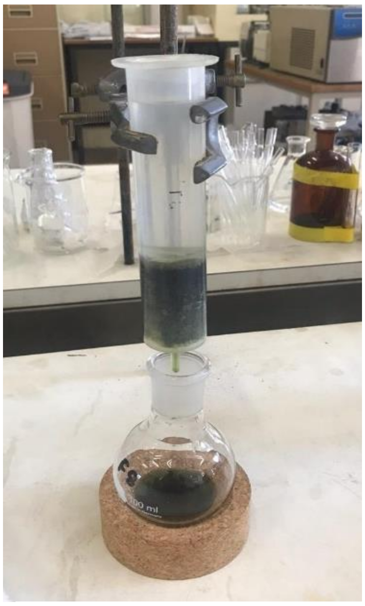

Figure 10: Si-gel column chromatography was used to fractionate the crude extract.

Fractionation was conducted using silica gel step-gradient chromatography (Fig. 10): The column was prepared by placing cotton wool at the bottom of a 50 mL syringe before loading with the solid phase that consisted of 10 g silica gel in 50 mL hexane. Following preparation of the column, 1 g of the powdered crude extract was added to the top of the Si-gel column. The different liquid phases (solvent solutions) were then added to the top of the column, starting with the solvent of the least polarity (fraction 1) and ending the fraction with the highest polarity (fraction 9), sequentially). The fractions consisted of mixtures of distilled hexane (C_6H_{14}), ethyl acetate ($CH_3COOC_2H_5$) and methanol (CH_3OH) (Table 1).

Table 1: The liquid-phase ratios of for the different *Ulva* fractions.

Fraction	**Hexane (ml)**	**Ethylacetate (ml)**	**Methanol (ml)**
1	50	0	
2	45	5	
3	40	10	
4	35	15	
5	30	20	
6	20	30	
7	10	40	
8	0	50	
9		25	25

All *Ulva* fractions were collected separately and dried off with the Roto-Vap or covered and placed in a desiccator. The dried concentrated fractions were stored in a refrigerator or freezer. Ethanol (70 % solution) was used to reconstitute the desired dried *Ulva* fractions. This was done by adding 1.5 mL ethanol was added to the vial containing dried fractions and then lightly agitating the vials until the fraction was completely dissolved.

Ulva extract fraction 9 (*Ulva* F9) was previously demonstrated to be most preferred extract for *T. gratilla* as a feed attractant (Lekraj Etwarysing, *pers. comm*.) and settlement inducer (Mark D. Cyrus, *pers. comm*.), and was subsequently chosen for both growth trials in the current study.

As with the dried *Ulva* treatments, a lower concentration of *Ulva* extract was used to assess urchin settlement and post-settlement growth and survival in growth trial A (42.86 µL in 50 mL alginate) as opposed to growth trial B (388.8 µL in 50 mL alginate).

Ulva F9 was thought to have an insufficient nutritional profile to sustain the juvenile urchins by itself and so was also tested in combination with the probiotic *Vibrio midae* SY9 and alginate mixture (50 mL), in the above concentrations.

Probiotic *Vibrio midae* SY9

V. midae SY9 has previously been used as a probiotic for the cultured abalone *Haliotis midae* and has been used successfully in alginate based substrates for supporting the growth of post-settled abalone (Moxley and Coyne, 2020), and for promoting the growth of adult *H. midae* (when added as a feed supplement to formulated feeds) (Macey, 2005):

The *V. midae* SY9 probiotic was obtained from a glycerol stock, kindly donated by A/Prof Vernon E. Coyne (from the Marine Biotech laboratory, Department of Molecular and Cell Biology, University of Cape Town). The bacterium was modified to be resistant to the antibiotics chloramphenicol and streptomycin, facilitating selective growth and re-isolation of this bacterium for laboratory based studies. For the preparation of the substrates/plates, the bacterium was inoculated onto a modified tryptone soy agar (mTSA) media (w/v 1.7 % tryptone, 0.3 % peptone, 0.25 % glucose, 3 % NaCl, 0.25 % K2HPO4, 2 % bacteriological agar), by aseptically transferring cells from the glycerol stock onto the solid media, and left to grow for 24 h at 30 °C, in a temperature controlled incubator. The plate was turned upside down to avoid condensation and possible contamination. Following incubation, cells from the overnight culture were used to aseptically inoculate three separate McCartney bottles, each containing 10 mL of modified tryptone soy broth (mTSB) (w/v 1.7 % tryptone, 0.3 % peptone, 0.25 % glucose, 3 % NaCl, 0.25 % K2HPO4) supplemented with Streptomycin (10 µl of a 120 µg/mL antibiotic stock solution) and chloramphenicol (5 µL of a 15 µg/mL stock). The inoculated bottles were incubated for 4 h on a shaking platform, set to rotate at 80-100 rpm, in a temperature controlled incubator set at 30 °C. After four hours of growth, the contents of the entire contents of the McCartney bottles (10 mL) were transferred to separate Erlenmeyer flasks (contents of 1 McCartney bottle was transferred to 1 Erlenmeyer flask), each containing 500 mL of mTSB supplemented with streptomycin (0.008 µg/mL) and chloramphenicol (0.005 µg/mL). These flasks were then returned to the shaking platform in the temperature controlled incubator and incubated overnight.

Following overnight growth, the bacterial cells were harvested by centrifugation at 6000 rpm for 15 min at 25 °C. The supernatant was discarded and the resultant pellet from each Erlenmeyer flask was re-suspended in ca. 10 ml\L of artificial sea water (ASW) (w/v 2.47 % NaCl, 0.47 % MgCl2.6H2O, 0.19 % CaCl2.6H2O, 0.63 % MgSO4.7H2O, 0,066 % KCl, 0.0018 % NaHCO3).

The pellets obtained from each Erlenmeyer flask were pooled and ASW was added until a final volume of 50 mL SY9 broth was reached. This was combined with 100 mL of sodium alginate (5 % w/v) and mixed thoroughly to obtain a homogeneous solution before applying the mixture to plexiglass plates and polymerising, as described above.

Ethanol-alginate control

The ethanol-alginate controls consisted of 5 % (w/v) alginate mixed with the same amount of solvent that was used in treatments containing *Ulva* extract F9. The control in growth trial B was made up with 1.5 mL of ethanol combined with 30 mL alginate, whereas 11 µL of ethanol was added to 25 mL of alginate for the control for growth experiment A.

Alginate control

The alginate control consisted of cooled, autoclaved sodium alginate (5 % w/v) and was administered and polymerised as previously described. This control was used in growth trial A and contained no other additives.

Growth trial A

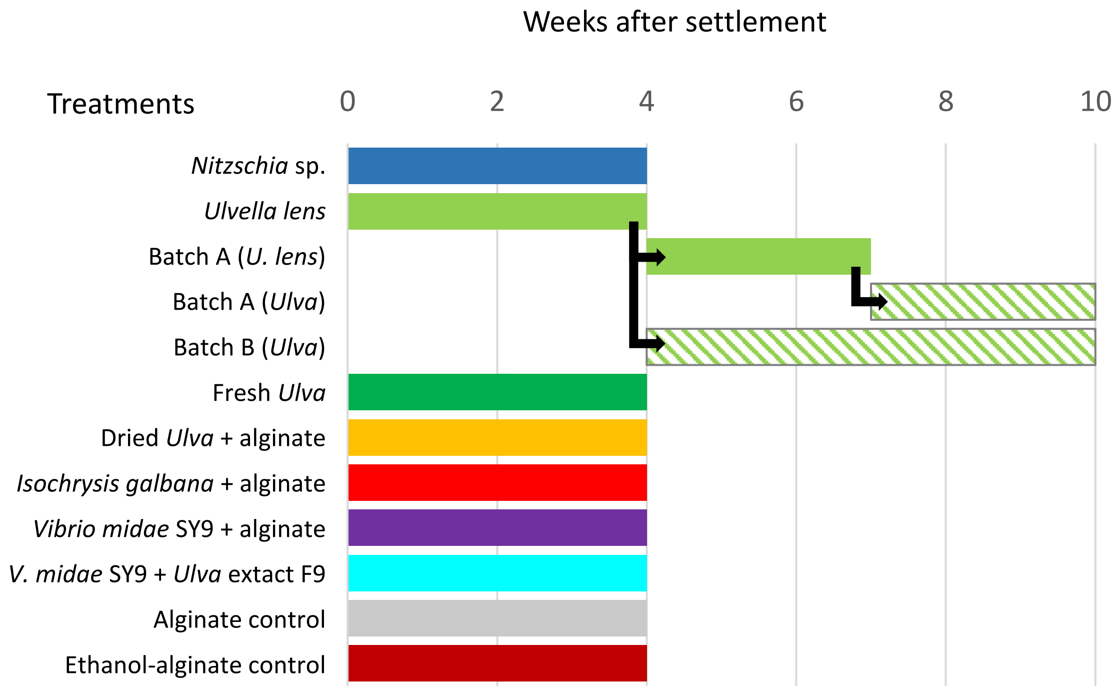

Figure 11: Timeline showing the treatments and timing of transition to feeding on *Ulva lacinulata* during growth trial A.

Growth trial A tested of nine experimental substrates: *Ulvella lens*, fresh *Ulva*, dried *Ulva* and alginate, *Nitzschia sp. (local undescribed diatom)*, dried *Isochrysis galbana* and alginate, *Vibrio midae* SY9 (probiotic) and alginate, *V. midae* SY9 with *Ulva* extract fraction F9 and alginate, an alginate control, and an ethanol-alginate control (see *Treatments* below). Each experimental substrate (treatment) was replicated four times and each treatment was inoculated with 35 competent urchin larvae at the start of the experiment. Substrates in each trough were replaced once a week. The replacement plates/substrates were prepared one to two days before the replacement. The troughs in which the urchins were housed, were also thoroughly sprayed out and scrubbed clean once a week over the course of the experiment.

Larvae were settled directly into the Perspex troughs on the varying treatments. This allowed for collection of size measurements immediately after settlement and determination of settlement success on the varying substrates. Settlement success was recorded as a percentage of larvae that successfully metamorphosed. Settlement was assessed once every 20 h from the beginning of the experiment over a period of 60 h.

Average urchin test diameter was assessed once a week, for ten weeks from settlement, to assess the efficacy of experimental substrates in stimulating growth. Ten randomly selected individuals from each trough were photographed once a week. Photographs were taken with a Nikon SMZ1500 stereomicroscope and a Nikon DS-Fi2-U3 Digital sight camera. The test diameter of the urchins was measured once a week from these photographs over the course of the experiment. Where not enough individuals were present (due to mortality or failure to settle) to make up ten, the calculation of average test diameter was adjusted to account for missing data, by dividing by the total number of measured individuals. Survival data of each trough was also recorded. Dead urchins were removed when checked daily and when old substrates were replaced with fresh ones weekly. Small urchins were gently brushed from one substrate to the other using a fine paint brush and larger urchins were carefully transferred with a pair of fine forceps.

Four weeks post-settlement, individuals from the *Ulvella lens* treatment were subdivided into two further treatments: *U. lens* (Batch A) and fresh *Ulva (Batch B)*. Individuals from the *U. lens* treatments were randomly divided into 8 troughs with ten animals in each to make up the two new treatments of four replicates each. Urchins in the Batch A treatment were fed on *U. lens* for an additional three weeks, whereas urchins in the Batch B treatment were fed with *U. lacinulata*. After three weeks, the animals still being fed with *U. lens (Batch A)* were switched onto a diet of fresh *U. lacinulata* for the remainder of the trial (another 3 weeks), whereas urchins in the Batch B treatment were continually fed with fresh *Ulva* (Fig. 11). Fresh *Ulva* was placed in the troughs (*ad libitum*), after being rinsed with FSW to remove any foreign material on the *Ulva* (like epiphytes or isopods). Test-diameter and survival data was recorded once a week (in the same manner as described above). Other treatments were terminated at the end of week four due to low urchin survival.

Growth trial B

Larvae were raised to competency (as described above) and were settled in large plastic troughs coated with *U. lens*. Post-settled urchins were left to grow and feed from *U. lens* for one month (four weeks) prior to being transferred onto the different experimental substrates (Fig. 12).

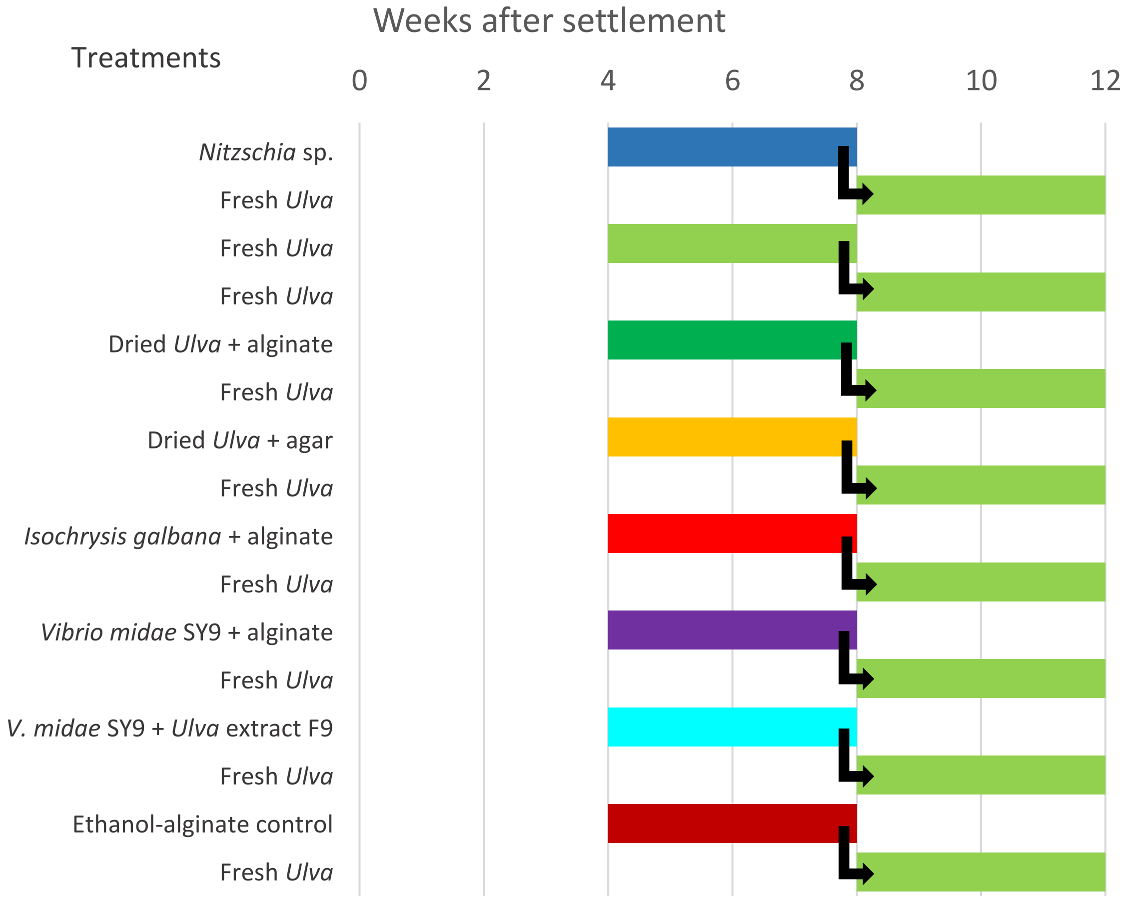

Figure 12: Timeline showing the treatments and timing of transition to feeding on *Ulva lacinulata* during growth trial B.

Ten post-settled *T. gratilla* were transferred into each of the experimental troughs. A total of eight different experimental substrates were assessed in growth trial B: fresh *Ulva*, *Nitzschia* sp. *(diatom)*, *Ulva* and alginate, *Ulva* and agar, dried *Isochrysis galbana* and alginate, *Vibrio midae* SY9 and alginate, *V. midae* SY9 and *Ulva* extract fraction F9 and alginate, and an ethanol-alginate control (see *Treatments*). Treatments were replicated four times (40 animals per treatment) and urchins were maintained on the experimental substrates for four weeks. At the end of week four, the experimental plates/substrates were removed and fresh *Ulva* was placed in each trough (*ad libitum*), as was done in growth trial A (Batch A and B). Test diameter and survival was assessed once a week (as described above). Dead urchins were removed from the troughs during this time. Troughs were sprayed with clean seawater and scrubbed clean once a week when old plates were replaced with new plates over the course of the experiment.

Statistical analyses

Data was recorded and summarised in graphs using Microsoft Excel 2016. Data are expressed as means ± standard error of the mean (SEM) (see also *appendix A*).

Statistical analyses were performed using R (v4.0.5). and R Studio. Normality of the data was determined using a Shapiro-Wilks test and the homoscedasticity of the data was determined with a Levine's test. Analysis of variance (ANOVA) and post-hoc Tukey analyses were performed on normally distributed data with equal variances. Two-tailed independent two sample t-tests were conducted where only two treatment groups were present.

Where the data showed a non-normal distribution, it was subjected to a Kruskal-Wallis analysis (when variances were equal) as well as post-hoc Dunn testing. In the case of only two independent samples or treatments, Mann-Whitney U analyses (for equal variances) and Welch testing (for unequal variances) were conducted on the data. Differences were considered significant at $p<0.05$.

Effect of ethanol on SY9

An experiment was conducted in which the effect of ethanol on the growth of the *Vibrio midae* SY9 probiotic was investigated. The same strain that was modified to be resistant to the antibiotics chloramphenicol and streptomycin was used in this experiment. The aim was to assess the differences in growth curves resulting from the addition of the same dose of ethanol alcohol which was used in the growth experiments. The experiment was conducted in triplicate.

The SY9 was streaked out onto mTSA plates and left to grow for 24 h. 500 mL mTSB was prepared and 50 mL was transferred into each of 6 × 500 mL Erlenmeyer flasks (three flasks for the ethanol treatment and three for the just mTSB null control). 5 mL of mTSB was also transferred into each of six McCartney vials. These flasks and vials were autoclaved at 121 ^{o}C for 20 min and left to cool to room temperature. Streptomycin (10 µL at 120 µg/mL) and chloramphenicol (5 µL at 15 µg/mL) were transferred to three McCartney vials aseptically. These three vials were then inoculated with the SY9 bacteria. These vials were used to get the OD_{600} reading on a spectrophotometer at the start of the experiment. Each vial would be used to inoculate two Erlenmeyer flasks (i.e., one replicate of each treatment), using the $C_1V_1=C_2V_2$ formula. The inoculated Erlenmeyer flasks were placed in a 30 ^{o}C incubator for a period of 10 hours. Three spectrophotometer readings were taken from each flask every hour, from the start of the experiment. These readings were then used to construct a growth curve for each set of replicates in the experiment. There was no significant difference in the growth curves of the null control and ethanol treatment (Kruskal-Wallis chi-squared = 11, p= 0.4433, df= 11, Kruskal-Wallis test) (appendix A, Fig. 13).

Results

Growth Trial A

Settlement

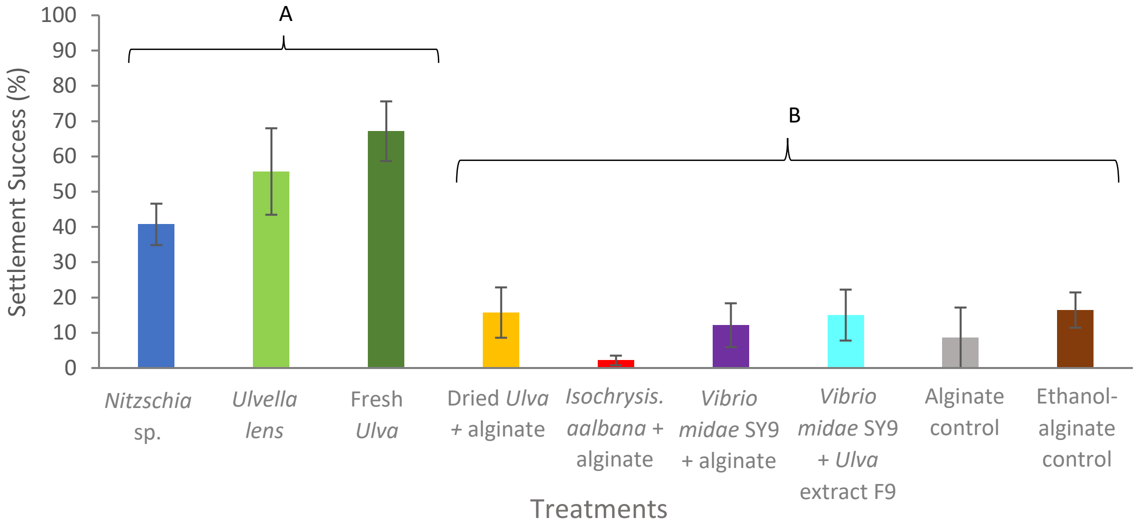

Figure 14: The ability of a range of substrates to induce successful settlement (% of total of 35 individuals) at the end of a 60h period, in competent *Tripneustes gratilla* larvae. Labels A and B refer to treatments that are not significantly different from one another.

ANOVA was performed on percentage data. The highest average settlement success was achieved when fresh *Ulva lacinulata* (67.14 % ± 8.45) was used as a settlement substrate, followed by *Ulvella lens* (55.71 % ± 12.26) and the *Nitzschia* sp. (40.71 % ± 5.88) (Fig. 14 - A) substrates. These three algal settlement substrates induced significantly higher settlement compared with the other prepared non-algal treatments ($p<0.05$, df, = 8, Dunn test, n = 4; Table 2), despite not being significantly different from each other ($p>0.05$, df = 8, Dunn test, n = 4, Table 2). The alginate-based treatments did not induce high settlement, with less than 16.43 % ± 5.00 settlement success across all these treatments, and none of these treatments were significantly different from each other ($p>0.05$, df = 8 Dunn test, n = 4, Table 2) (Fig. 14 - B). Of the prepared non-algal substrates, the treatment containing *Isochrysis galbana* exhibited the lowest average settlement success (2.14 % ± 1.37). These results are further summarised in Table 3 .

Table 3: The success (%) of a variety of substrates to induce settlement in competent *T. gratilla* larvae, at the end of 60 h.

Treatment	Mean ± Standard error (%)
Nitzschia diatom	40.71 ± 5.88
Ulvella lens	55.71 ± 12.26
Fresh *Ulva*	67.14 ± 8.45
Ulva + alginate	15.71 ± 7.14
I. galbana + alginate	2.14 ± 1.37
Vibrio midae SY9 + alginate	12.14 ± 6.21
V. midae SY9 + *Ulva* extract F9 [1/8]	15.00 ± 7.23
Alginate control	8.57 ± 8.57
Alginate + ethanol control	16.43 ± 5.00

Post-settlement growth and survival

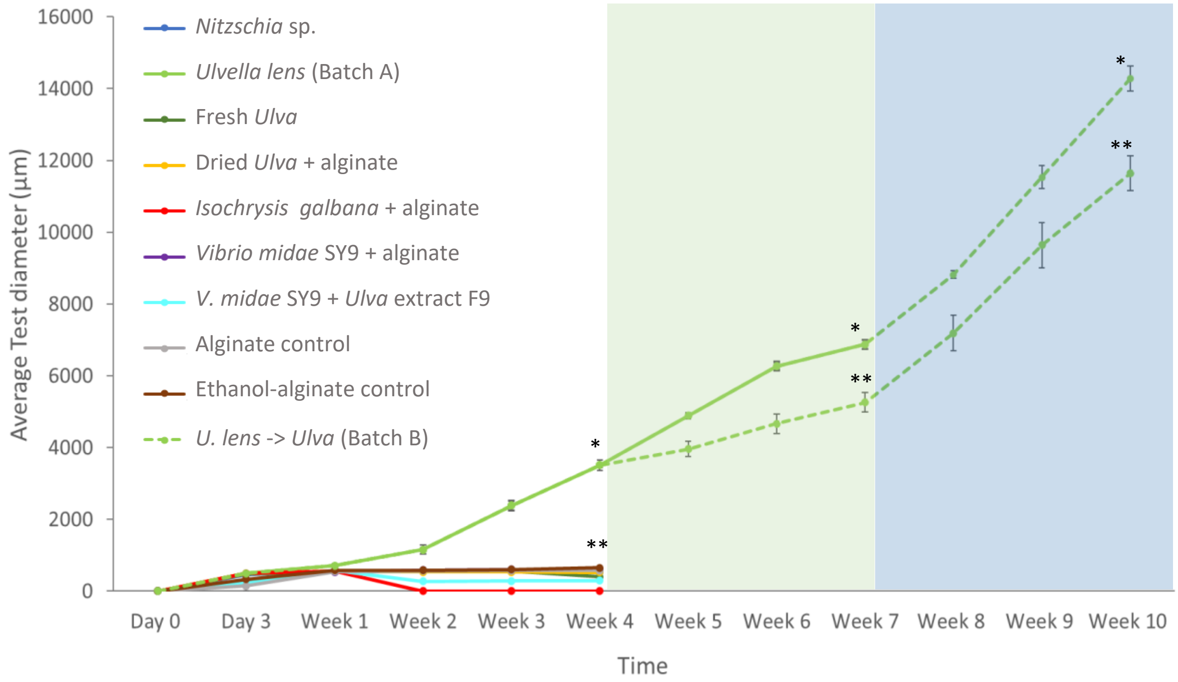

Figure 15: The change in average test diameter (µm) of post-settled *T. gratilla* larvae. Day 3 marked the end of settlement. After 4 weeks the *Ulvella lens* replicates were subdivided into two treatment groups (green background): one was maintained on *U. lens* (Batch A), whereas the other was weaned onto Ulva (Batch B). After a further 3 weeks, all urchins were provided with *Ulva* (blue background). Separate significance tests were conducted at week 4, 7 and 10 between Batch A and B, and significance levels are indicated with *.

Competent urchin larvae were transferred to their respective treatments/substrates in the settlement troughs on day 0 and allowed to settle and undergo metamorphosis. Before the first measurements of observation of urchin test-diameter were recorded on Day 3 (Fig. 15). Urchins in the *U. lens* treatment exhibited the greatest average test-diameter at the end of week 4, reaching an average test diameter of 3507 ± 147 µm (Fig. 15 and Table 4, Appendix

A). Post-settled urchins in the latter treatment had a significantly greater test diameter than urchins in any of the other treatments at the end of week 4 (p<0.05, df = 8, Dunn test, n = 4, Table 5) (Fig. 15) whereas all the other treatments were not significantly different from one other (p>0.05, df =8, Dunn test, n = 4, Table 5) (Fig. 15). By the end of week 4, the difference in test-diameter between urchins in the *U. lens* treatment and urchins in the ethanol-alginate control, the group with the smallest average test diameter (652 ± 8 µm) was ca. five-fold.

Average survival of urchins in all treatment groups decreased over the course of the first 4 weeks of the experiment (Fig. 16). Urchins in the *U. lens* treatment had the highest average survival at the end of week 4 (61.43 % ± 10.47) (Table 6, Appendix A) and was significantly higher than the survival of urchins in all the other treatments(p<0.05, df = 8, Dunn test, n = 4, Table 7) (Fig. 16). Conversely, the average survival of urchins from the other treatments in the experiment, other than the *U. lens* treatment, were not significantly different from one another and were low by the end of week 4 (ranging from 0 to 17%) (p>0.05, df = 8, Dunn test, n = 4, Table 7) (Fig. 15).

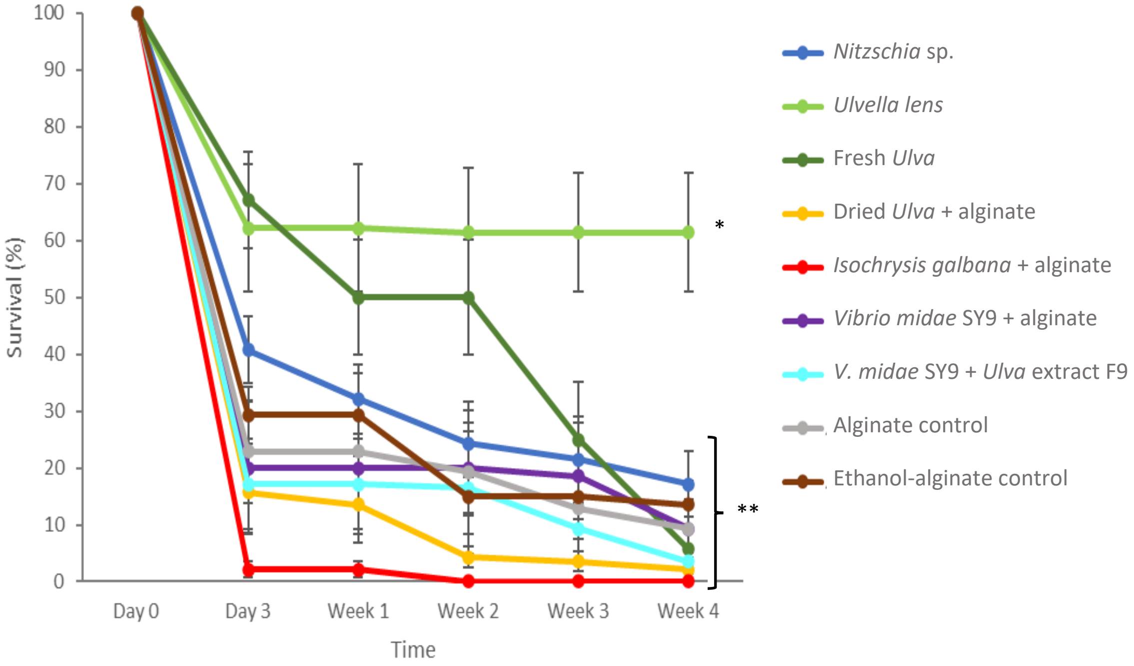

Figure 16: The average survival of *T. gratilla* post-larvae (% ± SE) over the first 4 weeks of a growth trial, on a variety of experimental substrates. Day 3 marked the end of settlement. Significance testing was conducted at the end of week 4 and significance levels are indicated with * at p< 0.05.

Urchins that settled and maintained on *U. lens* were subdivided into two treatments (Batch A and B) at the end of week 4. Urchins in Batch A were maintained on *U. lens* for an additional three weeks, before being switched over onto a diet of fresh *U. lacinulata* for the remaining three weeks of the trial (weeks 7-10) (Fig. 15). Conversely, urchins assigned to Batch B were immediately transferred to a diet of fresh *U. lacinulata* at the end of week 4, and fed this diet for the remainder of growth trial A. Urchins maintained on *U. lens* (Batch A) had significantly greater average test-diameter (W = 0, p = 0.02857, df = 6, Mann Whitney U-test, n = 4) than those fed fresh *Ulva* (6874 ± 133 µm and 5260 ± 268 µm respectively; Table 8, Appendix A), by the end of week 7 (Fig. 15). This amounts to increased growth of 1478 – 1749 µm for urchins with delayed weaning (Batch A). The average survival of urchins in these two treatments (Batch A vs Batch B) was also significantly different (Kruskal-Wallis chi-squared = 4.2, df = 1, p-value = 0.04042, Kruskal-Wallis test, n = 4), with the survival of urchins in Batch A significantly greater (100.00 % ± 0.00) than the survival of urchins in Batch B (92.50 % ± 2.50) (Fig. 17, Z = -2.04939, p = 0.04042398, Dunn test, n = 4), with percentages calculated following the switch in diets at the end of week 4 (week 7 vs. week 4).

Average test diameter of urchins in Batch A increased by 3367 µm ± 133 from week 4 to week 7. This equates to an estimated growth rate of 1122 ± µm 44 per week over this time period. Conversely, the change in average test diameter for urchins in Batch B over the same period was 1753 µm ± 268 which equates to an estimated growth rate of 584 µm ± 89 per week. The

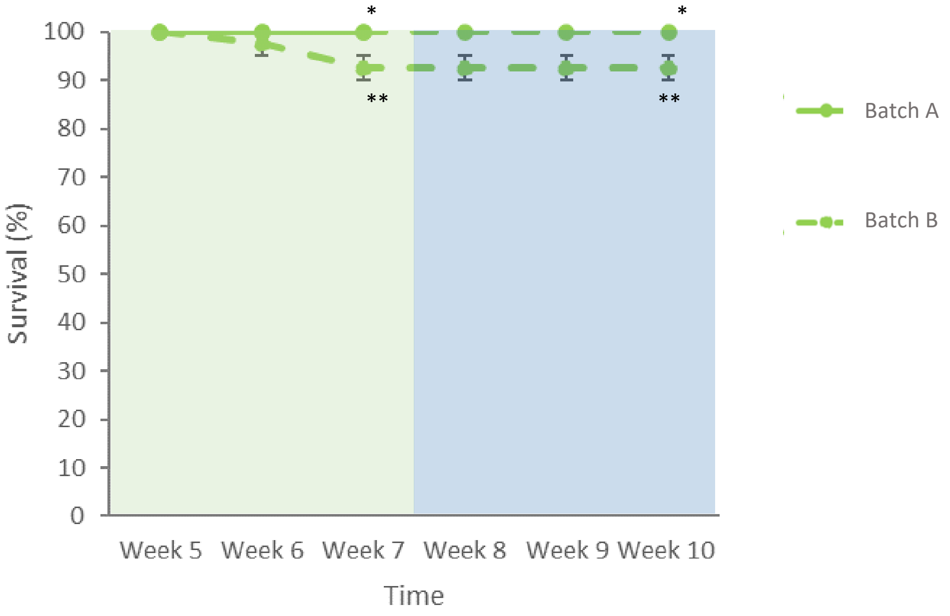

Figure 17: The average survival of *T. gratilla* post-larvae (% ± SE) over the last 6 weeks of growth trial A, where the urchins in the *U. lens* treatment was subdivided into two treatment groups (green background): Batch A were urchins were maintained on *U. lens* for three weeks and then weaned onto *Ulva (*blue background*)*, and Batch B where urchins were transitioned to fresh *Ulva* at the end of week 5. Separate significance tests were conducted at the end of weeks 7 and 10, significance levels are indicated with *.

growth rates of urchins maintained on *U. lens* (Batch A) between week 4 and week 7 was significantly greater (t = -5.3865, df = 4.3923, p = 0.004382, Welch test, n = 4) than that of urchins transferred earlier, at week 4, onto a diet of fresh *Ulva lacinulata* (Batch B).

By the end of growth trial A (week 10), the average test-diameter of urchins in Batch A was significantly greater than the test-diamater of urchins in Batch B, which were transferred earlier onto the diet of fresh *Ulva* (t = -4.4245, df = 6, p = 0.004449, two-tailed independent sample t-test, n = 4) (Fig. 15). At the end of week 10, the average test-diameter of urchins in Batch A and B was 14278 µm ± 351 and 11638 µm ± 481, respectively (Table 8, Appendix A). The survival at the end of the trial followed a similar pattern to week 7, with urchins in Batch A having significantly higher survival than urchins in Batch B (Week 10: Kruskal-Wallis chi-squared = 4.2, df = 1, p = 0.04042, Kruskal-Wallis test, n=4). No mortality was observed during the last 3 weeks of the growth trial (Fig. 17) and was the same as it was at the end of week 7: Batch A had an average survival rate of 100.00 % ± 0.00 and Batch B was 92.50 % ± 2.50 (Table 9, Appendix A).

The increase in test-diameter for urchins in Batch A from week 8 to 10 was 7404 µm ± 361 equating to a calculated increase of 2468 µm ± 120 per week, over the last three weeks of growth trial A. The increase in test-diameter for urchins in Batch B over the same time period was 6378 µm ± 241 (Fig. 15) which is a calculated increase of 2126 µm ± 80 per week. There was no significant difference between the rates of test-diameter change between urchins from these two treatments (t = -2.3588, df = 6, p = 0.05637, two-tailed independent sample t-test, n = 4).

Growth trial B

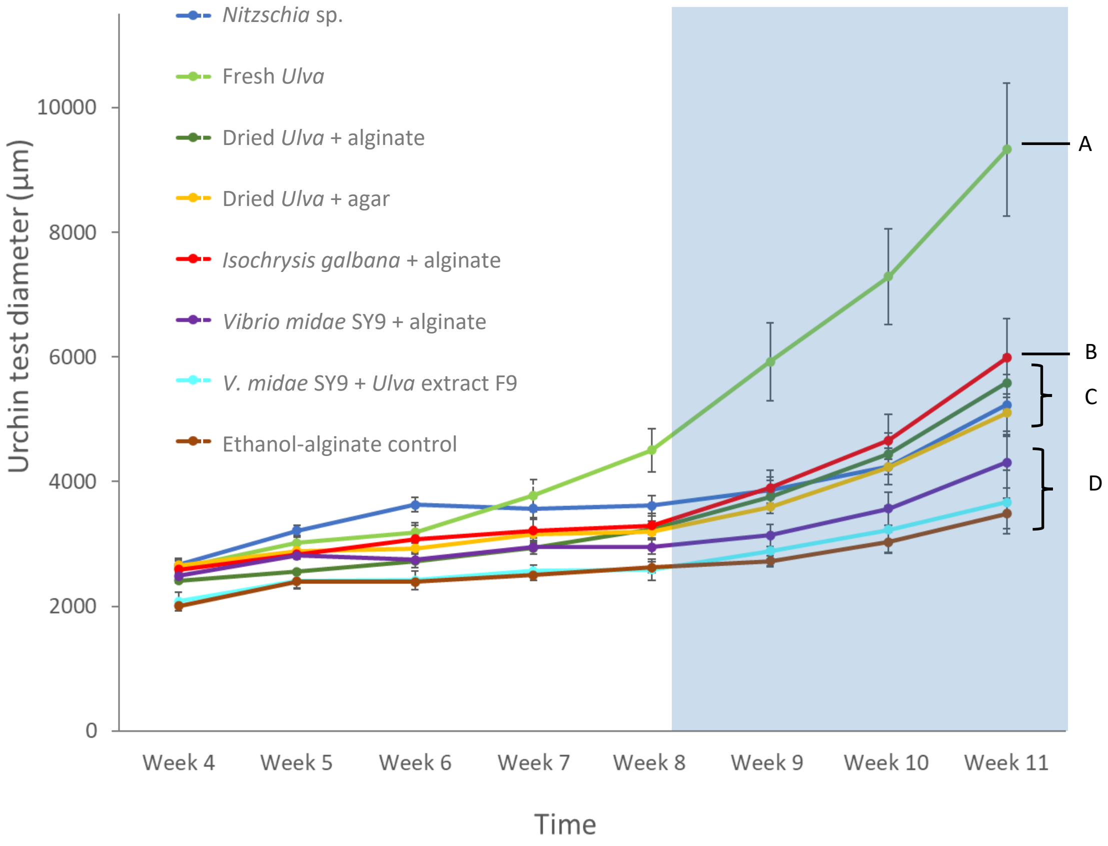

Figure 18: Growth trial B: increase in average test diameter (µm) of *T. gratilla* juveniles on different experimental substrates. "Time" was measured in weeks after settlement. After 4 weeks, all juveniles were provided with fresh *Ulva* (blue background). Significance tests were conducted at the end of week 11, different significance levels are indicated by different letters.

During growth trial B, the growth and survival of post-settled larvae that were initially all settled on *U. lens* was assessed on various post-settlement substrates for a period 4 weeks (week 4-8), before switching all urchins over onto a diet of fresh *U. lacinulata* (after week 8 post-settlement). In general, there was a net average increase in urchins test diameter over the course of the experiment (Fig. 18). Urchins maintained on the *Nitzschia* sp. exhibited the greatest average test-diameter at the end of week 2 (3216 µm ± 79), whereas individuals maintained on fresh *Ulva* throughout the trial had the greatest average test diameter (4504 µm ± 348) by the end of week 8 of the trial (Fig. 18) ($p<0.05$, df = 7 Dunn test, n = 4, Table 10).

Urchins in the latter two treatments, fed with fresh *Ulva* and the *Nitzschia* sp., also exhibited the highest average survival at the end of week 8 (85.00 % ± 6.45 and 85.00 % ± 9.75, respectively) (Table 11, Appendix A); although this was not significantly different from any of the other treatments (F = 1.004, df = 7, p= 0.471, ANOVA, n = 4).

Eight weeks after post-settlement (week 8), the individuals from all the treatments were weaned onto fresh *Ulva* for the remainder of the growth experiment (another 3 weeks) (blue background, Fig. 18). Individuals maintained on a diet of fresh *Ulva* throughout the trial had the largest average test-diameter at the end of Week 11 (9325 µm ± 1069) (Table 12, Appendix A). The test-diameter of urchins fed fresh *Ulva* was significantly different from all other treatment groups (p<0.05, df = 7, Dunn test, n = 4, Table 13), and ca. 1.5 times greater than the test-diameter of urchins fed *Isochrysis galbana*, the treatment that had the second largest test-diameter (5983 µm ± 632) by the end of week 11, and was significantly different from all other treatments (p<0.05, df = 7, Dunn test, n = 4, Table 13). Urchins from the *Ulva* and the *Nitzschia* sp. treatment had the highest survival at the end of the trial (85.00 % ± 6.45 and 82.50 % ± 9.46) (Table 14, Appendix A); and was significantly higher than the survival of any of the other treatments (Fig. 19 – A), when the survival data was “arc-sin^{-}(x)” transformed

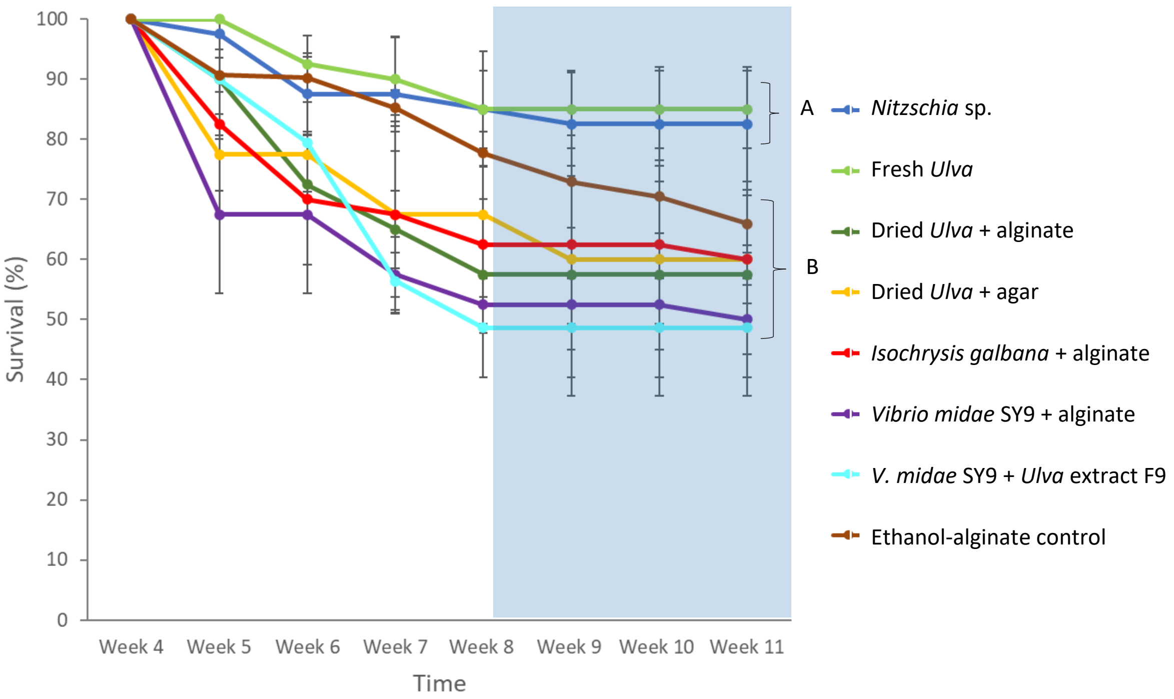

Figure 19: The average survival of *T. gratilla* post-larvae (% ± SE) over the second growth trial. After 4 weeks, all juveniles were provided with fresh *Ulva* (blue background). Significance levels are indicated on the graph (A and B) based on analysis of sin^{-}(x) transformed survival data.

($p<0.05$, Dunn test, n = 4, Table 15). *T. gratilla* with the lowest average test-diameter belonged to the ethanol-alginate control (3486 µm ± 242, Table 12, Appendix A) but this was not significantly different from urchins in the *Vibrio midae* SY9 treatments (Fig. 18 - D) ($p>0.05$, df = 7, Dunn test, n = 4, Table 13).

The average difference in test diameter of *T. gratilla* maintained on fresh *Ulva* by week 8 (4820 µm ± 727) was significantly greater than the test-diameter of urchins from all other treatments ($p<0.05$, df = 7, Tukey test, n = 4, Table 16). This amounts to an increase in test-diameter of 1606 µm ± 242 since the beginning of the trial (week 4). Over the last 3 weeks of the trial, the changes in test diameter of all treatments, with the exception of *Ulva* and *I. galbana*, were not significantly different from each other ($p>0.05$, df = 7, Tukey test, n = 4, Table 16). The average linear increase in test-diameter for these treatments was calculated to be 510 µm per week (excluding *Ulva* and *I. galbana* treatments).

Discussion

The results from the settlement experiment suggest that fresh *Ulva* results in the greatest settlement success of *Tripneustes gratilla* (followed by *Ulvella lens)*, out of the range of cues tested. None of the settlement cues containing extracts from the *Ulva* seaweed, bacteria nor alginate induced settlement success comparable to live substrates. The ensuing growth trials provide evidence to suggest that fresh *Ulva* may not be a suitable post-settlement feed. *Ulvella lens* may be a more suitable diet for post-settled *T. gratilla* as it resulted in the lowest number of mortalities throughout the growth trials. Weaning onto fresh *Ulva* from a post settlement diet of *Ulvella lens* resulted in the lowest mortality and greatest positive change in urchin test diameter, out of the substrates tested. The timing of weaning onto fresh *Ulva* may be important for hatchery management as results suggest differences may result in different rates of test diameter change thereafter.

Settlement

Fresh *Ulva lacinulata* induced the greatest settlement (67.14 % ± 8.45) of *T. gratilla* larvae, though not significantly different from *Ulvella lens* and the *Nitzschia* sp. ($p>0.05$, Fig. 14). Dworjanyn and Pirozzi (2008) found that *Ulva lactuca* induced low settlement of competent *T. gratilla* larvae (8 %), whereas Carbonara *et al*. (2018) managed to achieve 80 % settlement in *P. lividus* with a ground *Ulva* solution (GUS). The latter authors also tested varying dilutions of the GUS and found that settlement decreased markedly with declining GUS concentrations (Carbonara *et al*., 2018). The latter authors' work was comparable to results from Mos *et al*. (2011), who achieved 80% settlement success in *T. gratilla.* However, Mos *et al*. (2011) also tested combinations of other algae, including *Sargassum sp., Corallina sp, Nitzschia* diatoms, and seawater conditioned with these algae. These treatments all resulted in settlement success greater that of the *Ulva* treatment assessed in their study, for *T. gratilla* (Mos *et al.* 2011). *Ulvella lens* was also shown to successfully induced settlement of competent *T. gratilla* larvae (55.71 % ± 12.26).In comparison, Hannon *et al.* (2017) achieved 50% settlement success in *P. lividus* when using *U. lens* as a settlement substrate. Similarly, Brundu *et al.* (2016) recorded settlement success of 36 % in *P. lividus* when using cleaned *U. lens* as a settlement substrate and suggested that the natural biofilm that grows on *U. lens* should not be dismissed for its influence on the settlement of urchin larvae (Dworjanyn and Pirozzi, 2008), which was echoed by Brundu et al. (2016). The presence of surface bacteria associated with *Ulva* as well as phagostimulants and/or attractants (e.g., glycoglycerolipids, *more below*), may well be the settlement inducing cues that competent larvae are responding to.

The relative successes of *U. lens* and *Ulva* spp. in inducing settlement of competent urchin larvae may be attributed to specific polyunsaturated fatty acids (PUFA's) they generate. It has previously been shown that PUFA's such as eicosapentaenoic acid (EPA) and arachidonic acid (Kitamura *et al*., 1993), as well as dibromomethane (Taniguchi *et al*. 1994) are able to induce settlement and metamorphosis of competent urchin larvae. Takahashi *et al*. (2002) isolated a series of fatty acids from *U. lens* extracts, including sulfoquinovosyl monoacylglycerols (SQMGs), monogalactosyl monoacylglycerols (MGMGs), monogalactosyl diacylglycerols (MGDGs), and digalactosyl monoacylglycerols (DGMGs), that are capable of inducing settlement of *Strongylocentrotus intermedius* larvae (Takahashi *et al*., 2020), so it is plausible that these compounds may also induce settlement of *T. gratilla* larvae. However, given that organic compounds such as PUFAs are not readily dissolved in seawater and are often bound in cells, they may not be responsible for inducing settlement in nature as marine larvae detect settlement cues in the water column (Mos *et al.,* 2016).

There is a wealth of resources that provide evidence for the largely accepted hypothesis that macroalgal bacterial epibionts are largely responsible for high settlement induction in marine invertebrate larvae (Pawlik 1992, Hadfield and Paul 2001, Freckelton *et al*., 2017). The precise mechanism of macroalgal bacterial epibionts and macroalgae has not yet been described but there is evidence to suggest that both the bacteria and macroalgae play a role in the successful settlement of marine invertebrate larvae (Huggett *et al* 2006). Results from Mos *et al*. (2011) indicated that live algae in combination with bacterial epibionts induce greater settlement than either bacteria or cleaned macroalgae alone, in *Tipneustes gratilla*. Dworjanyn and Pirozzi (2008) found that when various species of macroalgae were cleaned to lower the abundance of surface bacteria, *T. gratilla* settlement in response to these algae were significantly reduced or completely inhibited. This provides evidence to support that *T. gratilla* settle in response to bacterial biofilms on the surfaces of live macroalgae.

The results of this study suggest that *U. lens* is able to induce high settlement of competent *T. gratilla* larvae, while promoting high survival of the post-settled juveniles thereafter. It is advised that when inducing settlement using *U. lens,* fresh *Ulva* be placed in the same settlement tank because it appeared to have specific properties that allow for otherwise increased settlement. It is also plausible that the combination of *U. lens* and *Ulva* may have allelopathic interactions that inhibit the growth of settlement-inhibiting algae/microbes, thus promoting urchin settlement, survival, and post-settlement growth. Harlin and Rice (2008) review available information on macroalgal allelopathy but direct evidence of such occurrences are limited and the authors motivate for further lab and field experiments in this area.

The *Nitzschia* sp. tested in this study induced relatively successful settlement of competent *T. gratilla* larvae as well (40.71 ± 5.88%). This differs from results of Xing *et al*. (2007) as well as Castilla-Gavillan *et al*. (2020), who also tested a species from the *Nitzschia* genus. These researchers induced successful settlement in *Strongylocentrotus intermedius* at 58.32 % ± 5.00 and 90 % respectively. Mos *et al* (2011) induced 60 % successful settlement in *Tripneustes gratilla*, using *Nitzschia*. The variation in urchin settlement response has been hypothesised to be caused by variation in the biofilms that are created when using *Nitzschia* (Xing *et al*., 2007; Castilla-Gavillan *et al*., 2020). This species secretes high levels of extracellular polymeric substances (EPS) which facilitate its strong attachment to a surface (Xing *et al*., 2007) and thus create variation in the biofilm.

Species of *Nitzschia* have been shown to have a high capacity for the production of PUFA's such as EPA (Barclay, Meager & Abril, 1994; Chen *et al*., 2007). A previous study revealed that MGDG and DGDG are produced by *Nitzschia laevis* (Chen *et al*., 2007) and may be responsible for inducing successful settlement responses of urchin larvae. Besides EPA, the fatty acids tetradecanoic acid, hexadecenoic acid and palmitoleic acid were shown to be abundant in the lipids identified from of *N. laevis* (Chen *et al*., 2007). The lipid and fatty acid profile of *N. laevis* was also shown to vary greatly under different growing conditions (Chen *et al*., 2007), so it is possible that a different settlement response may have been observed if the benthic microalgae were grown under different conditions. Despite this there is limited evidence to suggest that PUFAs may be important for inducing settlement in *Tripneustes gratilla* larvae, owing to their limited capacity to dissolve and disperse in the water column.

The non-algal based substrates tested in the present study did not induce high levels of successful settlement (Fig. 14) and many larvae died during settlement and metamorphosis (Fig. 16, day 3). These substrates included extracts from *Ulva*, bacteria and alginate. It is possible that the larvae were unable to sense the chemosensory settlement cue(s) in the different non-algal based treatments due to the cell encapsulating nature of alginate gels, despite alginate being semi-permeable (Awrey *et al* 1996). These results suggest that alginate-based substrates not be used for the purposes of inducing settlement in *T. gratilla* but rather as post-settlement feeds as an alternative to natural post-settlement feeding substrates (*see below*).

Post-settlement growth

U. lens facilitated the greatest increase in test-diameter of the *T. gratilla* juveniles during the weeks that followed settlement (increase of 3013 µm within 4 weeks from post-settlement) (Fig. 15 - A, Growth trial A). *U. lens* also promoted high survival of the post-settled urchin juveniles over this time period (61.43 % ± 10.47) (Fig. 16 - A, Growth trial A). The success of *U. lens* in supporting the growth of *T. gratilla* juveniles may be attributed to the nutritional profile of *U. lens* as well as the micro-organisms growing in the biofilm associated with the *U. lens* crust, and their associated metabolites. This data supports the ongoing application of *U. lens* in commercial echinoculture as a post-settlement feed (Brundu *et al*., 2016; Hannon *et al*., 2014 & 2015; Dworjanyn and Pirozzi, 2008; Kawamura *et al*., 1983; Mos *et al*., 2011; Takahashi *et al*., 2002)

We demonstrated that fresh *Ulva lacinulata* is not a suitable post-settlement substrate/feed for sustaining *T. gratilla* , and also showed that urchins maintained on Ulva exhibited similar growth to animals maintained on all the other substrates tested, with the exception of the *U. lens* substrate (Week 0 -week 4, Fig. 15, Growth trial A). The test-diameter of *T. gratilla* juveniles that successfully settled and metamorphosed on *Ulva* (Day 3, Fig. 15, Table 3, Appendix A) did not increase over the 4 week trial period and survival of urchins in this treatment group were low (5.71 % ± 3.09) (Fig. 16). The lack of growth and poor survival of post-settled urchins on fresh *Ulva* may be due to the physical and/or structural properties of the seaweed not being suitable for the juveniles possibly also due to the fact that *Ulva* floats and is difficult to access/grab (*Ulva* has a 3D structure unlike *Ulvella lens*, which has a flat 2D structure), not having a sufficient nutritional profile to sustain newly settled *T. gratilla*, having low nutrient density (high ratio of water to nutrient content) (Larson *et al*. 1980; Lobban and Harrison 1994), inability of *T. gratilla* recruits to digest or process fresh *Ulva* as a feed source (due to underdeveloped mouthparts and gut). The feeding mechanism present in post-settled urchins at this age features the scraping of a benthic surface and as such, substrates like *U. lens* provide an easily accessible nutrient source. This changes when urchins are weaned onto macroalgae, where the feeding mechanism involves cutting the macroalgal thallus and digesting the cut pieces (personal observation).

Even though fresh *Ulva* was shown to be an inadequate post-settlement feed when compared to *U. lens* but it exceled in inducing successful settlement. The above contradicts the idea that substrate selection should be based on its ability to induce settlement and sustain an animal throughout metamorphosis and early settlement (Dworjanyn & Pirozzi, 2008), at least in as much as it pertains to *U. lacinulata*. We have demonstrated that *U. lacinulata* is an excellent substrate for inducing settlement but should be used in combination with other substrates that will sustain the growth of post-settled larvae.

Despite the capabilities of sodium alginate to form hydrogels, results from our study suggest that alginate containing substrates (as prepared in this study: "diffusion" method), are not suitable for the rearing of early post-settled urchin larvae. The alginate-based substrates we tested all yielded low survival (less than 20 %) and promoted minimal growth of the post-settled larvae (Fig. 15 & 16, Growth trial A). We observed that the alginate matrix tends to absorb liquid once submerged in water and becomes unstable (where it detaches from the surface of the plexiglass plates and breaks apart). This instability may have contributed to the poor growth and low survival observed in our study. This is a known phenomenon for alginate-based substrates (Moxley and Coyne, 2020).

Due to the variation in the stability of the alginate matrix in this study, the effect of probiotic, *Vibrio midae* SY9, on the growth of *T. gratilla* juveniles immediately after settlement was limited. Conversely, Moxley and Coyne (2020) demonstrated benefits following probiotic supplementation with *Vibrio midae SY9* to newly settled abalone *Haliotis midae,* in terms of improved growth and survival, when compared to a non-probiotic supplemented control diet of the diatom *Cocconeis* and alginate. These authors also emphasised the benefits of artificial feeds (like alginate-based substrates) in aquaculture, stating that the use of a consistent substrate (with known nutritional value and effect) can facilitate the consistent production of juvenile marine invertebrates for the grow-out phase in aquaculture (Moxley and Coyne, 2020).

It is likely that urchins settled and maintained on the alginate-based substrates were not sufficiently developed from a physiological perspective and did not have sufficient, or the required, enzymes to digest a complex polysaccharide such as alginate (de Ridder & Jangoux, 1983; Onitsuka *et al*., 2015). Larger urchin juveniles appeared to cope better with alginate-based substrates than the younger, smaller urchins. This is evidenced by the greater growth and survival of urchins previously settled and maintained (fed) on *U. lens* until 4 weeks post-settlement (Fig. 18, Growth trial B). In fact, all urchins that were previously fed *U. lens* for 4 weeks prior to being transferred onto the new substrates (diet change at 4 weeks after settlement, Growth Trials B), showed higher growth and survival compared with urchins on the same substrates in the first growth trial (Growth trial A). Increased size and age, and thus the associated increase in the development of essential structures such as the Aristotle's lantern, gut and enzymatic profile, is hypothesised to have facilitated the observed increase in growth and survival, as shown by others (de Ridder & Jangoux, 1983; Onitsuka *et al*., 2015). This is supported by our data on urchins that were fed on fresh *Ulva*, following settlement and maintenance on *U. lens* for 4 weeks, which exhibited the greatest test-diameter increase at 8 weeks post-settlement (Fig. 18), suggesting that weaning onto macroalgae at 4 weeks after settlement is possible (Growth trial B). Once *T. gratilla* reached a certain size (ca. 2615 µm ± 134, 4 weeks after settlement, Growth trial B), settled urchins were capable of feeding and growing on fresh *Ulva* (despite the stress of a diet change). Urchins maintained on *Ulva* during this time also had the highest survival rate (85 % ± 6.45) despite not being significantly different from the *Nitzschia* sp. we tested (Fig. 19, $p>0.05$).

It may be worth considering the use of non-algal alginate-based substrates as an emergency backup feed, should unavoidable circumstances prevent the use of natural substrates such as *U. lens, Ulva and Nitzschia sp.* (e.g., contamination or disease). Alginate-based substrates (as prepared in this study) take little time to prepare and can be stored for up to a week when refrigerated. Although urchin growth and survival has been shown to be lower than natural substrates, it is better to provide the animals with a suboptimal substrate than lose an entire cohort and get no return on the resources invested (in rearing urchins) up to that point. It is advised that alginate-based substrates be used more as a measure of maintaining current urchin stock and minimising the losses endured during adverse events than trying to maximise production.

As we have demonstrated in this study, a substrate that induces settlement may fail to sustain newly settled urchins. Consequently, it may be necessary to move urchins that settled on one substrate to a new substrate, or combine species that are able to optimally settle, sustain and facilitate their growth and survival post-settlement. From a production perspective, the success of a diet change is directly related to the proportion of urchins that successfully progress to the adult stages of life. Urchins that are moved onto a new substrate before they have been allowed to develop enough, have a higher mortality risk (Daume *et al*., 2003, Zupo *et al*., 2018). On the other hand, allowing urchins to develop for too long indicates inefficient/excess use of resources and labour in maintaining the urchins and their pre-weaning substrates, despite the increased likelihood that they would survive weaning. Determining the optimal time to effect a diet change during the rearing process as well as determining a suitable weaning diet would result in the minimal loss of urchin individuals (potential production) as well as optimising resource use.

Weaning Diets

Growth data from this study implies that it is necessary to achieve a specific critical size during the early stages of juvenile (post-settled larval) development prior to the transition to feeding on macroalgae. This may be due to increased size, and associated development of the mouthparts and the digestive tract which gives access to new previously unavailable food sources like macroalgae, thus improving the individual’s chances to develop to the adult stages. Larger individuals are less susceptible to mortality during the post-settlement phases (Meidel *et al*., 1999) and it has been suggested that reaching a specific size is critical for the continued survival of urchins (Brundu *et al*., 2016) and abalone alike (Vicose *et al*., 2012), to adults. The need for an early post-settlement feed that facilitates the successful weaning of urchin juveniles, is further supported by the critical size hypothesis, which states that ‘the larger an urchin can potentially grow on a post-settlement feed, the less impact the stressors of the weaning process will have when moving animals between two diets’. The successful weaning of a post-settlement urchin onto a new diet that supports its continued growth and development may mark *(a posteriori*) that the critical size for survival has been reached.

Growth trial A showed that the successful weaning of *T. gratilla* juveniles was possible at 4 weeks after settlement, when a test diameter of approximately ca. 3.507 mm ± 0.147 is obtained (Fig. 15). This was accompanied by an observable decrease in test diameter growth (lag-phase) in the weeks that followed (Batch B, Fig. 15), when compared to juveniles that were maintained on their original post-settlement feed (*U. lens*) over the same period (Batch A, Fig. 15). Indeed, the size of urchin juveniles three weeks hence, in Batch A was significantly larger than those already weaned onto *Ulva* (Batch B): ca. 6.874 mm ± 0.133 (Batch A) compared to ca. 5.260 mm ± 0.268 (Batch B) (Fig. 15, Week 7).

Interestingly, when juvenile urchins from Batch A were maintained on a diet of *U. lens* for longer, prior to being switched over to and weaned onto fresh *Ulva* thereafter, their test growth remained constant, as opposed to urchins from batch B, between week 4 to 5, that where weaned onto fresh *Ulva* earlier and a noticeable decrease in growth rate was evident (Fig. 15). This implies that urchins maintained on *U. lens* for longer were better prepared for the new substrate. We hypothesised this is due the juveniles having reached a critical size, as discussed above, making them less vulnerable to the stressors of adapting to a new foodstuff, as suggested by Taylor *et al*. (2009). Uninterrupted growth, and the lack of a 'lag-phase', meant that juvenile urchins in growth trial A of our study achieved a greater size by the end of the experiment than those transferred to the macroalga *Ulva* earlier: ca. 14.278 mm ± 351 for Batch A compared to ca. 11.638 mm ± 481 for Batch B.

Whether ca. 1.5 mm difference in urchin test-diameter (over 3 weeks) is worth spending the additional resources required to maintain a post-settlement substrate, such as *U. lens*, for longer remains to be determined. For some aquaculture farmers, this includes some of the abalone farms in South Africa that grow large amounts of *Ulva lacinulata* in abalone effluent (Bolton, 2006; Bolton *et al*., 2009; Robertson-Andersson, 2007; Robertson-Andersson *et al*., 2008; Shuuluka, 2011), it may be logistically easier and less costly to divert those resources to maintain a macroalgal stock (weaning feed). *Ulva* has been shown to grow readily and rapidly in aquaculture tanks (Bolton *et al* 2016; Chopin *et al*., 2001) and minimal preparation is required to feed it to urchins. *Ulvella lens* requires preparation in advance and constant care and maintenance to maintain healthy (uncontaminated) crusts for urchin post-larvae to feed from (Hannon *et al*., 2014; Takahashi & Konegazawa, 1988). It also remains to be determined whether a 1.5 mm difference in test diameter early in the production cycle would dramatically affect the time it takes for those urchins to reach marketable size/quality. These aspects require further investigation to determine how this difference would affect farm productivity throughout the grow-out phase (e.g., 3 months, 6 months, and harvest).

Survival of post-settled juveniles should such also be considered when deciding on the timing for the transition to feeding on macroalgae. The survival of urchins subjected to a longer weaning period on *U. lens* (Batch A) was significantly greater than that of juveniles subjected to the shorter weaning period (Batch B)($p<0.05$, Fig. 17): 100 % for Batch A compared to ca. 93 % for Batch B. Despite this difference, urchins feeding on *Ulva* at this size (>3.507 mm ± 0.147) showed high survival. Indeed, *Ulva* appeared to maintain constant survival even when urchins that were feeding on unique post-settlement substrates were subjected to a weaning change (Growth trial B, Fig. 19).

T. gratilla juveniles also showed similar increases in test-diameter during growth trial B, when urchins were successfully weaned onto a new diet of fresh *Ulva*, irrespective of the experimental post-settlement substrates they previously fed from (Fig. 18, Appendix A). The increase in test-diameter of urchins that have been weaned onto fresh *Ulva* irrespective of their post-settlement feeding substrate were not significantly different from one another, after weaning ($p>0.05$). Fresh *Ulva lacinulata* thus has the potential to stimulate similar growth rates in post-settled *T. gratilla* juveniles (8-11 weeks after settlement), even if post-settled urchins were maintained on suboptimal post-settlement feeding substrates.

The fact that fresh *Ulva* was readily consumed during the weaning experiments, lends credence to the confirmation that *Ulva* would be a good/suitable weaning feed. This also suggests that *Ulva lacinulata* has phagostimulatory cues (Cyrus *et al*., 2013; 2015; Perez *et al*., 2016; Dworjanyn & Pirozzi, 2008) which facilitate greater consumption and maintain urchin growth. This study suggests that the use of *Ulva* is beneficial for promoting urchin growth (hence the weaning onto *Ulva*), thereby shortening the time of the vulnerable post-settlement/juvenile stages of the *T. gratilla* life history.

It has previously been shown that the successful transition of *Strongylocentrotus purpuratus* (Stimpson, 1857) from a benthic diet to fleshy macroalgae is soonest possible at 50 days old, approximately 7 weeks after settlement when a test diameter of between 0.500 – 1.200 mm is attained (Rowley, 1990). The current study shows that *T. gratilla* can be successfully weaned onto *U. lacinulata* at 4 weeks (28 days) after settlement, when a test diameter of ca. 3.507 mm± 0.147 is attained, provided the juveniles have access to a suitable post-settlement feed, such as *U. lens* (Fig. 15, 16, & 17). Rowley (1990) investigated the growth of wild *S. purpuratus* in Santa Barbara (California, water temperature of 15 ^{0}C) and not urchins raised in intensive aquaculture (25 °C in the case of the current study). The temperature difference and the intrinsic growth rates of each species may explain at which different sizes each is able to undergo a weaning diet change, suggesting that that the specific size/age for a weaning diet change is different for each species but further research would be required to confirm this.

Echinoculture practise commonly weans urchins onto macroalgae (such as brown seaweeds) which urchins consume naturally (Agatsuma, 2000). This study supports the use of *Ulva* as a suitable macroalga for urchins to be weaned onto, as urchin growth and survival was not detrimentally affected regardless of post-settlement feed type (Fig. 15 & 18). Weaning onto *Ulva* may also be beneficial as *Ulva* has been shown to be beneficial to *T. gratilla* later in their life-history (Cyrus *et al*., 2014, 2015) and may already be grown on site for bioremediation (Bolton *et al*., 2016). However, the current study is limited in that it has not investigated the effects of formulated feeds on the early stages of *T. gratilla* performance.

Considerable research effort has gone into developing and investigating different artificial feed formulations for adult sea urchins, primarily for gonad enhancement. However, diets for juvenile sea urchin performance have received far less attention. Possible reasons for this difference include the potential economic value that adult sea urchins have accrued due to possessing commercially valuable gonads (which are able to be conditioned with formulated feed diets), the sensitivity of juvenile sea urchins to disturbance (which may lead to unwanted mortalities), bottlenecks in juvenile urchin production to test potential diets with, and knowledge gaps surrounding the nutritional requirements for optimal urchin juvenile development. This study has provided support for the use of live algal feeds (namely *Ulva* and *Ulvella lens*) for *Tripneustes gratilla* production, as well as highlighting the importance of the timing of weaning (onto macroalgae, *Ulva*) and the potential subsequent effects that may have on the growth of *T. gratilla* juveniles.

Future research

This body of work was conducted at a small scale under laboratory conditions and not in a realistic production scale setting. Future research should be directed towards conducting similar experiments at farm scale to determine whether results of this study are repeatable when assessed at farm scale.

Future research efforts should be directed at isolating the chemical settlement induction cue(s) from *Ulva* (as is being undertaken by Etwaysing, in publication). The effects of enriched seaweeds on the settlement and post-settlement growth should also be investigated particularly as it pertains to the timing of weaning post-settlement. Isolating probiotic gut bacteria from adult *T. gratilla* (as has been done with *Haliotis midae* (Macey 2005)), and then testing their influence on *T. gratilla* at various life stages (through probiotic supplementation) may prove particularly useful, as it pertains to production. Designing suitable artificial post-settlement substrates and investigating the effects of formulated feeds on the post-settlement growth and the timing of weaning may also be useful in ensuring the full life-cycle grow out of *T. gratilla* becomes efficient and economically viable.

Conclusion

Settlement is one of the major bottlenecks to the production of marine invertebrates. Utilising an optimal settlement cue ensures that urchin mortality during settlement is minimized. *Ulvella lens* in combination with fresh *Ulva* is recommended for use as a cue to induce high successful settlement. There is evidence to suggest that exposing *T. gratilla* to suitable post-settlement feeds is beneficial for production in an intensive aquaculture setting. Results suggest *Ulvella lens* is the most optimal post-settlement feed (out of the substrates tested in this work) for use in this regard, promoting the necessary growth for eventual weaning onto macroalgae. Echinoculture practise commonly weans urchins onto macroalgae (which urchins consume when wild in the ocean). Fresh *Ulva* is recommended as a suitable weaning diet for *T. gratilla*, due to maintaining high survival and positive growth. The timing of a weaning diet change is also of crucial importance as the ability of juvenile urchins to cope with a diet change is directly related to their survival. This work recommends that a weaning diet change only be effected at 7- 8 weeks after settlement having previously been reared on *Ulvella lens* (as urchin growth is minimally affected at this time). This work does show that a weaning change at 4 weeks after settlement is possible (or at ca. 3.507 mm ± 0.147) with an estimated 1.5 mm reduction in potential test-diameter as compensation. The results of this work highlight the benefits of using *Ulvella lens* and fresh *Ulva* for the purposes of commercial aquaculture during the settlement, post-settlement and juvenile stages of *Tripneustes gratilla*.

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Appendix A

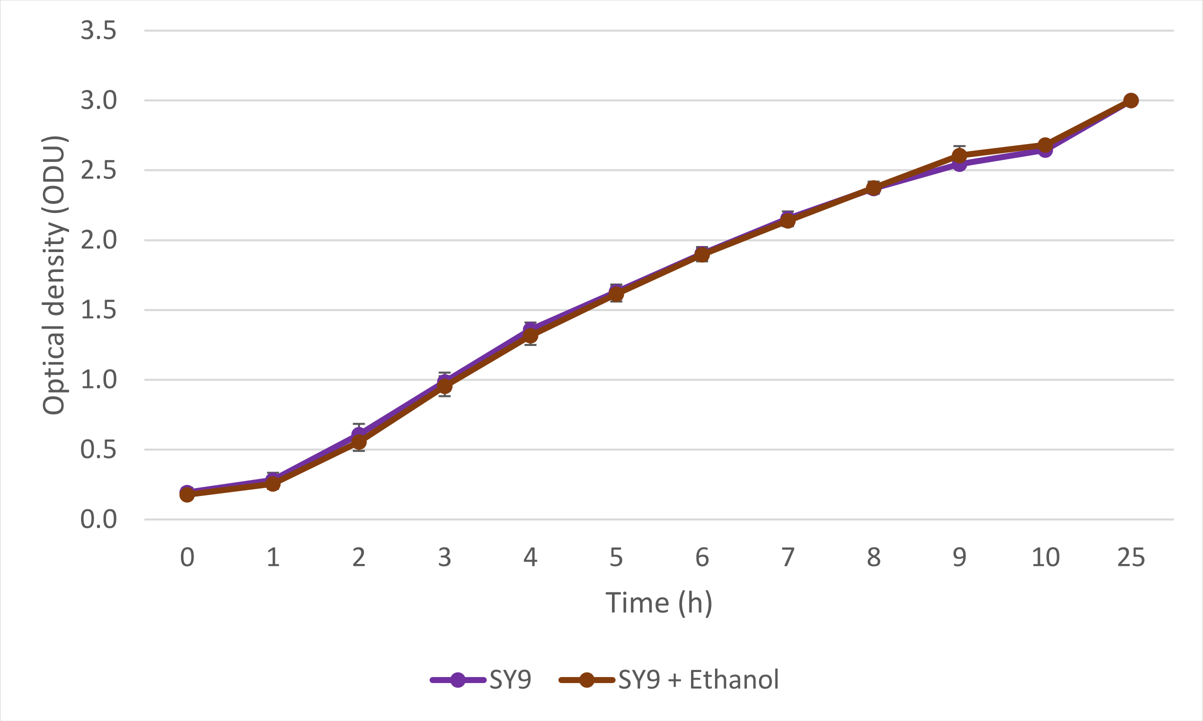

Figure 13: The effect of ethanol on the growth of Vibrio midae SY9, as prepared for growth experiments.

Tables

Table 2: The results of non-parametric Dunn test analysis on the settlement success of *Tripneustes gratilla* on a variety of settlement substrates in Growth trial A. P values in bold are below the 0.05 significance level.

Comparison		Z	P
Alginate control	X *Nitzschia* sp.	-2.32599	**0.02002**
	X Ethaniol-alginate	-0.97759	0.32828
	X Fresh *Ulva*	-3.23616	**0.00121**
	X *Isochrysis galbana* + alginate	0.26968	0.78741
	X *Vibrio midae* SY9 + alginate	-0.55621	0.57806
	X *Vibrio midae* SY9 + *Ulva* extract F9	-0.77533	0.43814
	X Dried *Ulva* + alginate	-0.79218	0.42825
	X *Ulvella lens*	-2.83164	**0.00463**
Nitzschia sp.	X Ethaniol-alginate	1.34840	0.17753
	X Fresh *Ulva*	-0.91017	0.36273
	X *Isochrysis galbana* + alginate	2.59567	**0.00944**
	X *Vibrio midae* SY9 + alginate	1.76977	0.07676
	X *Vibrio midae* SY9 + *Ulva* extract F9	1.55066	0.12098
	X Dried *Ulva* + alginate	1.53380	0.12508
	X *Ulvella lens*	-0.50565	0.61310
Ethanol-alginate control	X Fresh *Ulva*	-2.25857	**0.02391**
	X *Isochrysis galbana* + alginate	1.24727	0.21230
	X *Vibrio midae* SY9 + alginate	0.42137	0.67348
	X *Vibrio midae* SY9 + *Ulva* extract F9	0.20226	0.83971
	X Dried *Ulva* + alginate	0.18541	0.85291
	X *Ulvella lens*	-1.85405	0.06373
Fresh *Ulva*	X *Isochrysis galbana* + alginate	3.50584	**0.00046**
	X *Vibrio midae* SY9 + alginate	2.67994	**0.00736**
	X *Vibrio midae* SY9 + *Ulva* extract F9	2.46083	**0.01386**
	X Dried *Ulva* + alginate	2.44397	**0.01453**
	X *Ulvella lens*	0.40452	0.68583
Isochrysis galbana + alginate	X *Vibrio midae* SY9 + alginate	-0.82589	0.40886
	X *Vibrio midae* SY9 + *Ulva* extract F9	-1.04501	0.29602
	X Dried *Ulva* + alginate	-1.06186	0.28830
	X *Ulvella lens*	-3.10132	**0.00193**
Vibrio midae SY9 + alginate	X *Vibrio midae* SY9 + *Ulva* extract F9	-0.21912	0.82656
	X Dried *Ulva* + alginate	-0.23597	0.81346
	X *Ulvella lens*	-2.27542	**0.02288**
Vibrio midae SY9 + *Ulva* extract F9	X Dried *Ulva* + alginate	-0.01686	0.98655
	X *Ulvella lens*	-2.05631	**0.03975**
Dried *Ulva* + alginate	X *Ulvella lens*	-2.03945	**0.04140**

Table 4: The average test diameter (µm ± SE) of *T. gratilla* on a variety of substrates for the first 4 weeks of growth trial A. 0 values indicate complete mortality of all individuals in all replicates of a treatment.

Treatments	Day 3	Week 1	Week 2	Week 3	Week 4
Nitzschia diatom	470.73 ± 19.59	559.38 ± 8.00	558.39 ± 13.85	578.02 ± 17.58	523.07 ± 21.59
Ulvella lens	493.67 ± 16.36	711.44 ± 35.69	1157.6 ± 57.77	2384.06 ± 124.66	3507.20 ± 147.37
Fresh *Ulva*	474.9 ± 12.81	544.2 ± 7.75	558.84 ± 4.35	556.74 ± 11.82	403.21 ± 135.36
Ulva + alginate	498.84 ± 6.51	557.05 ± 12.61	538.16 ± 32.13	531.46 ± 21,23	523.77 ± 8.35
I. galbana + alginate	493.23 ± 9.80	551.29 ± 16.84	0	0	0
Vibrio midae SY9 + alginate	295.36 ± 147.73	534.07 ± 1.61	584.87 ± 21.85	600.12 ± 12.10	599.26 ± 15.81
V.midae SY9 + *Ulva* extract F9 [1/8]	229.27 ± 125.97	569.42 ± 4.63	275.86 ± 7.38	280.31 ± 6.73	288.83 ± 6.76
Alginate control	145.46 ± 145.46	541.24 ± 5.35	569.11 ± 8.52	572.19 ± 7.77	570.01 ± 7.80
Alginate + ethanol control	335.42 ± 114.40	586.51 ± 9.97	584.34 ± 13.30	604.87 ± 17.99	652.30 ± 8.31

Table 5: The results of non-parametric Dunn test analysis on the differences in test diameter success of *Tripneustes gratilla* at week 4 of Growth trial A. P values in bold are below the 0.05 significance level.

Comparison		Z	P
Alginate control	X *Nitzschia* sp.	0.28832	0.77310
	X Ethaniol-alginate	-1.67903	0.09315
	X Fresh *Ulva*	0.44096	0.65924
	X *Isochrysis galbana* + alginate	1.71295	0.08672
	X *Vibrio midae* SY9 + alginate	-0.33920	0.73446
	X *Vibrio midae* SY9 + *Ulva* extract F9	0.59360	0.55278
	X Dried *Ulva* + alginate	0.74624	0.45552
	X *Ulvella lens*	-2.22175	**0.02630**
Nitzschia sp.	X Ethaniol-alginate	-1.96735	**0.04914**
	X Fresh *Ulva*	0.15264	0.87868
	X *Isochrysis galbana* + alginate	1.42464	0.15426
	X *Vibrio midae* SY9 + alginate	-0.62752	0.53032
	X *Vibrio midae* SY9 + *Ulva* extract F9	0.30528	0.76015
	X Dried *Ulva* + alginate	0.45792	0.64701
	X *Ulvella lens*	-2.51007	**0.01207**
Ethanol-alginate control	X Fresh *Ulva*	2.11999	**0.03401**
	X *Isochrysis galbana* + alginate	3.39199	**0.00069**
	X *Vibrio midae* SY9 + alginate	1.33984	0.18030
	X *Vibrio midae* SY9 + *Ulva* extract F9	2.27263	**0.02305**
	X Dried *Ulva* + alginate	2.42527	**0.01530**
	X *Ulvella lens*	-0.54272	0.58732
Fresh *Ulva*	X *Isochrysis galbana* + alginate	1.27200	0.20337
	X *Vibrio midae* SY9 + alginate	-0.78016	0.43530
	X *Vibrio midae* SY9 + *Ulva* extract F9	0.15264	0.87868
	X Dried *Ulva* + alginate	0.30528	0.76015
	X *Ulvella lens*	-2.66271	**0.00775**
Isochrysis galbana + alginate	X *Vibrio midae* SY9 + alginate	-2.05215	**0.04015**
	X *Vibrio midae* SY9 + *Ulva* extract F9	-1.11936	0.26299
	X Dried *Ulva* + alginate	-0.96672	0.33369
	X *Ulvella lens*	-3.93471	**0.00008**
Vibrio midae SY9 + alginate	X *Vibrio midae* SY9 + *Ulva* extract F9	0.93280	0.35092
	X Dried *Ulva* + alginate	1.08544	0.27773
	X *Ulvella lens*	-1.88255	0.05976
Vibrio midae SY9 + *Ulva* extract F9	X Dried *Ulva* + alginate	0.15264	0.87868
	X *Ulvella lens*	-2.81535	**0.00487**
Dried *Ulva* + alginate	X *Ulvella lens*	-2.96799	**0.00300**

Table 6: The average survival (% ± SE) of *T. gratilla* over the first 4 weeks of growth trial A. 0 values indicate complete mortality of all individuals in all replicates of a treatment.

Treatments	Day 3	Week 1	Week 2	Week 3	Week 4
Nitzschia diatom	40.71 ± 5.86	32.14 ± 5.77	24.29 ± 5.77	21.43 ± 7.54	17.14 ± 5.83
Ulvella lens	62.14 ± 11.50	62.14 ± 11.15	61.43 ± 11.34	61.43 ± 10.47	61.43 ± 10.47
Fresh *Ulva*	67.14 ± 8.45	50.00 ± 10.07	50.00 ± 10.07	25.00 ± 10.19	5.71 ± 3.09
Ulva + alginate	15.71 ± 7.14	13.57 ± 6.64	4.29 ± 1.84	3.57 ± 1.80	2.14 ± 0.71
Isochrysis galbana + alginate	2.14 ± 1.37	2.14 ± 1.37	0	0	0
Vibrio midae SY9 + alginate	20.00 ± 11.58	20.00 ± 11.55	20.00 ± 11.55	18.57 ± 9.37	9.29 ± 4.27
V. midae SY9 + *Ulva* extract F9 [1/8]	17.14 ± 8.00	17.14 ± 8.00	16.43 ± 11.45	9.28 ± 6.10	3.57 ± 2.14
Alginate control	22.86 ± 9.04	22.86 ± 9.4	19.29 ± 7.13	12.86 ± 5.28	9.29 ± 5.13
Alginate + ethanol control	29.29 ± 5.00	29.29 ± 7.32	15.00 ± 3.38	15.00 ± 4.10	13.57 ± 4.10

Table 7: The results of non-parametric Dunn test analysis on the differences in survival success of *Tripneustes gratilla* at week 4 of Growth trial A. P values in bold are below the 0.05 significance level.

Comparison		Z	P
Alginate control	X *Nitzschia* sp.	-0.8845380	0.37641
	X Ethaniol-alginate	0.3231966	0.74655
	X Fresh *Ulva*	1.7180449	0.08579
	X *Isochrysis galbana* + alginate	-0.9866	0.32384
	X *Vibrio midae* SY9 + alginate	-0.1190724	0.90522
	X *Vibrio midae* SY9 + *Ulva* extract F9	0.7654655	0.44399
	X Dried *Ulva* + alginate	0.9525793	0.34080
	X *Ulvella lens*	-2.2283553	**0.02586**
Nitzschia sp.	X Ethaniol-alginate	1.2077345	0.22715
	X Fresh *Ulva*	2.6025829	**0.00925**
	X *Isochrysis galbana* + alginate	-0.1020621	0.91871
	X *Vibrio midae* SY9 + alginate	0.7654655	0.44399
	X *Vibrio midae* SY9 + *Ulva* extract F9	1.6500035	0.09894
	X Dried *Ulva* + alginate	1.8371173	0.06619
	X *Ulvella lens*	-1.3438173	0.17901
Ethanol-alginate control	X Fresh *Ulva*	1.3948483	0.16306
	X *Isochrysis galbana* + alginate	-1.3097966	0.19026
	X *Vibrio midae* SY9 + alginate	-0.442269	0.65829
	X *Vibrio midae* SY9 + *Ulva* extract F9	0.442269	0.65829
	X Dried *Ulva* + alginate	0.6293828	0.52910
	X *Ulvella lens*	-2.5515518	**0.01072**
Fresh *Ulva*	X *Isochrysis galbana* + alginate	-2.7046449	**0.00684**
	X *Vibrio midae* SY9 + alginate	-1.8371173	0.06619
	X *Vibrio midae* SY9 + *Ulva* extract F9	-0.9525793	0.34080
	X Dried *Ulva* + alginate	-0.7654655	0.44399
	X *Ulvella lens*	-3.9464001	**0.00008**
Isochrysis galbana + alginate	X *Vibrio midae* SY9 + alginate	0.8675276	0.38565
	X *Vibrio midae* SY9 + *Ulva* extract F9	1.7520656	0.07976
	X Dried *Ulva* + alginate	1.9391794	0.05248
	X *Ulvella lens*	-1.2417552	0.21433
Vibrio midae SY9 + alginate	X *Vibrio midae* SY9 + *Ulva* extract F9	0.884538	0.37641
	X Dried *Ulva* + alginate	1.0716518	0.28388
	X *Ulvella lens*	-2.1092828	**0.03492**
Vibrio midae SY9 + *Ulva* extract F9	X Dried *Ulva* + alginate	0.1871138	0.85157
	X *Ulvella lens*	-2.9938208	**0.00276**
Dried *Ulva* + alginate	X *Ulvella lens*	-3.1809346	**0.00147**

Table 8: The average test diameter (µm ± SE) of *T. gratilla* for the last 6 weeks of a growth trial. Green squares indicate that individuals previously on *Ulvella lens* were subdivided and switched onto *U. lens* and fresh *Ulva*, respectively. Blue squares indicate when all treatments were switched onto fresh *Ulva*.

Treatments	Week 5	Week 6	Week 7	Week 8	Week 9	Week 10
Ulvella lens	4887.00 ± 61.12	6267.09 ± 91.42	6874.25 ± 133.16	8820.47 ± 112.11	11535.27 ± 322.18	14278.54 ± 351.76
U. lens -> fresh *Ulva*	3956.88 ± 210.97	4661.35 ± 270.97	5260.24 ± 268.42	7184.34 ± 495.23	9641.37 ± 631.14	11638.88 ± 481.87

Table 9 The average survival (% ± SE) of *T. gratilla* over the last 6 weeks of growth trial A. Green squares indicate that individuals previously on *Ulvella lens* were subdivided and switched onto *U. lens* and fresh *Ulva* respectively. Blue squares indicate when all treatments were switched onto fresh *Ulva*.

Treatments	Week 5	Week 6	Week 7	Week 8	Week 9	Week 10
Ulvella lens	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
U. lens -> *Ulva*	100.00 ± 0.00	97.50 ± 2.50	92.50 ± 2.50	92.50 ± 2.50	92.50 ± 2.50	92.50 ± 2.50

Table 10: The average test diameter (µm ± SE) of *T. gratilla* juveniles on a variety of substrates for the first 5 weeks of a second growth trial B. The independent time variable denotes the time that has elapsed after settlement had occurred.

Treatment	Week 4	Week 5	Week 6	Week 7	Week 8
Nitzschia diatom	2666.44 ± 104.77	3216.72 ± 79.41	3631.46 ± 121.66	3564.20 ± 163.94	3613.90 ± 165.24
Fresh *Ulva*	2615.16 ± 134.32	3014.64 ± 89.52	3188.64 ± 146.25	3774.38 ± 258.26	4504.97 ± 348.97
Ulva + alginate	2414.04 ± 44.74	2557.96 ± 32.46	2724.09 ± 106.98	2933.05 ± 92.23	3235.73 ± 137.25
Ulva + agar	2658.65 ± 70.77	2890.8 ± 98.54	2931.29 ± 78.43	3162.22 ± 70.98	3192.61 ± 47.40
I. galbana + alginate	2586.07 ± 35.48	2832.81 ± 77.65	3081.43 ± 219.84	3212.93 ± 208.95	3292.09 ± 199.50
V.midae SY9 + alginate	2488.36 ± 30.97	2815.26 ± 63.22	2746.32 ± 23.22	2946.09 ± 102.29	2948.78 ± 116.80
V. midae SY9 + *Ulva* extract F9	2078.44 ± 151.57	2404.97 ± 118.25	2417.61 ± 147.69	2570.93 ±92.03	2587.90 ± 166.42
Alginate + ethanol control	2005.75 ± 80.59	2400.22 ± 110.74	2393.87 ± 40.48	2497.98 ± 83.35	2620.26 ± 88.45

Table 11: The average survival (% ± SE) of *T. gratilla* over the first 5 weeks of a second growth trial B. The independent time variable denotes the time that has elapsed after settlement had occurred.

Treatments	Week 4	Week 5	Week 6	Week 7	Week 8
Nitzschia diatom	100.00 ± 0.00	97.5 ± 2.50	87.5 ± 6.29	87.5 ± 9.46	85.00 ± 9.75
Fresh *Ulva*	100.00 ± 0.00	100 ± 0.00	92.5 ± 4.79	90.00 ± 7.07	85.00 ± 6.45
Ulva + alginate	100.00 ± 0.00	90 ± 7.07	72.5 ± 4.79	65.00 ± 6.45	57.5 ± 4.79
Ulva + agar	100.00 ± 0.00	77.5 ± 10.31	77.5 ± 10.31	67.5 ± 16.52	67.5 ± 13.77
I. galbana + alginate	100.00 ± 0.00	82.5 ± 11.09	70.00 ± 10.80	67.5 ± 13.77	62.50 ± 13.15
Vibrio midae SY9 + alginate	100.00 ± 0.00	67.5 ± 13.15	67.5 ± 13.15	57.5 ± 6.29	52.50 ± 4.79
V. midae SY9 + *Ulva* extract F9	100.00 ± 0.00	90 ± 10.00	79.44 ± 8.18	56.39 ± 4.72	48.61 ± 8.28
Alginate + ethanol control	100.00 ± 0.00	90.68 ± 6.43	90.23 ± 4.09	85.23 ± 3.02	77.73 ± 7.63

Table 12: The average test diameter (µm ± SE) of *T. gratilla* juveniles on a variety of substrates for the last 3 weeks of a second growth trial B. The independent variable denotes the time that has elapsed after settlement had occurred. All juveniles were switched onto fresh *Ulva.*

Treatment	Week 9	Week 10	Week 11
Nitzschia diatom	3862.36 ± 216.48	4242.66 ± 296.23	5231.54 ± 479.28
Fresh *Ulva*	5921.95 ± 620.00	7287.5 ± 766.43	9325.76 ± 1069.22
Ulva + alginate	3756.92 ± 265.64	4446.09 ± 326.88	5586.88 ± 445.21
Ulva + agar	3591.53 ± 34.38	4232.68 ± 122.43	5101.69 ± 301.72
I. galbana + alginate	3899.43 ± 285.92	4658.92 ± 423.65	5983.81 ± 632.04
V.midae SY9 + alginate	3140.07 ± 177.33	3565.93 ± 261.54	4309.20 ± 409.20
V. midae SY9 + *Ulva* extract F9	2888.15 ± 252.45	3230.49 ± 374.23	3666.27 ± 509.21
Alginate + ethanol control	2727.66 ± 81.26	3027.41 ± 160.47	3486.90 ± 242.02

Table 13: The results of non-parametric Dunn test analysis on the differences test diameter of *Tripneustes gratilla* at week 11 of Growth trial B. P values in bold are below the 0.05 significance level.

Comparison		Z	P
Ethanol-alginate control	X *Nitzschia* sp.	-0.1507	0.88017
	X Fresh *Ulva*	-1.9598	**0.05002**
	X *Isochrysis galbana* + alginate	1.77138	0.07650
	X *Vibrio midae* SY9 + alginate	1.54525	0.12229
	X *Vibrio midae* SY9 + *Ulva* extract F9	0.79147	0.42867
	X Dried *Ulva* + agar	-0.8668	0.38603
	X Dried *Ulva* + alginate	-0.5276	0.59775
Nitzschia sp.	X Fresh *Ulva*	-1.8090	0.07044
	X *Isochrysis galbana* + alginate	1.92213	0.05459
	X *Vibrio midae* SY9 + alginate	1.69600	0.08989
	X *Vibrio midae* SY9 + *Ulva* extract F9	0.94222	0.34608
	X Dried *Ulva* + agar	-0.7160	0.47394
	X Dried *Ulva* + alginate	-0.3768	0.70626
Fresh *Ulva*	X *Isochrysis galbana* + alginate	3.73120	**0.00019**
	X *Vibrio midae* SY9 + alginate	3.50507	**0.00046**
	X *Vibrio midae* SY9 + *Ulva* extract F9	2.75129	**0.00594**
	X Dried *Ulva* + agar	1.09298	0.27440
	X Dried *Ulva* + alginate	1.43218	0.15209
Isochrysis galbana + alginate	X *Vibrio midae* SY9 + alginate	-0.2261	0.82110
	X *Vibrio midae* SY9 + *Ulva* extract F9	-0.9799	0.32713
	X Dried *Ulva* + agar	-2.6382	**0.00833**
	X Dried *Ulva* + alginate	-2.2990	**0.02150**
Vibrio midae SY9 + alginate	X *Vibrio midae* SY9 + *Ulva* extract F9	-0.7537	0.45098
	X Dried *Ulva* + agar	-2.4120	**0.01586**
	X Dried *Ulva* + alginate	-2.0728	**0.03818**
Vibrio midae SY9 + *Ulva* extract F9	X Dried *Ulva* + agar	-1.6583	0.09725
	X Dried *Ulva* + alginate	-1.3191	0.18713
Dried *Ulva* + agar	X Dried *Ulva* + alginate	0.33920	0.73446

Table 14: The average survival (% ± SE) of *T. gratilla* over the last 3 weeks of a second growth trial B. The independent time variable denotes the time that has elapsed after settlement had occurred. All juveniles were switched onto fresh *Ulva*.

Treatments	Week 9	Week 10	Week 11
Nitzschia diatom	82.50 ± 8.54	82.50 ± 9.46	82.50 ± 9.46
Fresh *Ulva*	85.00 ± 6.45	85.00 ± 6.45	85.00 ± 6.45
Ulva + alginate	57.50 ± 4.79	57.50 ± 4.79	57.50 ± 4.79
Ulva + agar	60.00 ± 22.73	60.00 ± 22.73	60.00 ± 22.73
Isochrysis galbana + alginate	62.50 ± 13.15	62.50 ± 13.15	60.00 ± 11.55
Vibrio midae SY9 + alginate	52.50 ± 7.50	52.50 ± 7.50	50.00 ± 5.77
V. midae SY9 + *Ulva* extract F9	48.61 ± 8.28	48.61 ± 8.28	48.61 ± 8.28
Alginate + ethanol control	72.95 ± 7.66	70.45 ± 6.05	65.91 ± 4.77

Table 15: The results of non-parametric Dunn test analysis on the differences in arc-sin transformed survival data of *Tripneustes gratilla* at the end of week 11 of Growth trial B. P values in bold are below the 0.05 significance level.

Comparison		Z	P
Ethanol-alginate control	X *Nitzschia* sp.	1.62365	0.10445
	X Fresh *Ulva*	0.40114	0.68832
	X *Isochrysis galbana* + alginate	-1.85287	0.06390
	X *Vibrio midae* SY9 + alginate	-0.70677	0.47971
	X *Vibrio midae* SY9 + *Ulva* extract F9	-0.66856	0.50377
	X Dried *Ulva* + agar	1.37533	0.16903
	X Dried *Ulva* + alginate	-0.32473	0.74539
Nitzschia sp.	X Fresh *Ulva*	1.22251	0.22151
	X *Isochrysis galbana* + alginate	3.47652	**0.00051**
	X *Vibrio midae* SY9 + alginate	-2.33042	**0.01978**
	X *Vibrio midae* SY9 + *Ulva* extract F9	-2.29221	**0.02189**
	X Dried *Ulva* + agar	-0.24832	0.80388
	X Dried *Ulva* + alginate	-1.94838	0.05137
Fresh *Ulva*	X *Isochrysis galbana* + alginate	-2.25401	**0.02420**
	X *Vibrio midae* SY9 + alginate	-1.10790	0.26790
	X *Vibrio midae* SY9 + *Ulva* extract F9	-1.06970	0.28475
	X Dried *Ulva* + agar	0.97419	0.32996
	X Dried *Ulva* + alginate	-0.72587	0.46792
Isochrysis galbana + alginate	X *Vibrio midae* SY9 + alginate	1.14611	0.25175
	X *Vibrio midae* SY9 + *Ulva* extract F9	1.18431	0.23629
	X Dried *Ulva* + agar	3.22820	**0.00125**
	X Dried *Ulva* + alginate	1.52814	0.12648
Vibrio midae SY9 + alginate	X *Vibrio midae* SY9 + *Ulva* extract F9	-0.03820	0.96953
	X Dried *Ulva* + agar	-2.08209	**0.03733**
	X Dried *Ulva* + alginate	-0.38204	0.70244
Vibrio midae SY9 + *Ulva* extract F9	X Dried *Ulva* + agar	-2.04389	**0.04096**
	X Dried *Ulva* + alginate	-0.34383	0.73097
Dried *Ulva* + agar	X Dried *Ulva* + alginate	-1.70006	0.08912

Table 16: The results of a Tukey analysis on the difference in test diameter from week 8-week 11 of *Tripneustes gratilla* during growth trial B. P-values in bold are below the 0.05 significance level.

Comparison		lwr	upr	P
Ethanol-alginate control	X *Nitzschia* sp.	-1132.660	2634.661	0.882
	X Fresh *Ulva*	2070.495	5837.816	**0.000**
	X *Isochrysis galbana* + alginate	-58.582	3708.739	0.062
	X *Vibrio midae* SY9 + alginate	-1389.881	2377.440	0.986
	X *Vibrio midae* SY9 + *Ulva* extract F9	-1671.937	2095.384	1.000
	X Dried *Ulva* + agar	-841.217	2926.104	0.606
	X Dried *Ulva* + alginate	-399.153	3368.168	0.201
Nitzschia sp.	X Fresh *Ulva*	1319.494	5086.815	**0.000**
	X *Isochrysis galbana* + alginate	-809.583	2957.738	0.571
	X *Vibrio midae* SY9 + alginate	-1626.439	2140.882	1.000
	X *Vibrio midae* SY9 + *Ulva* extract F9	-1344.384	2422.937	0.978
	X Dried *Ulva* + agar	-1592.218	2175.103	0.999
	X Dried *Ulva* + alginate	-1150.153	2617.168	0.894
Fresh *Ulva*	X *Isochrysis galbana* + alginate	245.417	4012.738	**0.019**
	X *Vibrio midae* SY9 + alginate	1576.716	5344.037	**0.000**
	X *Vibrio midae* SY9 + *Ulva* extract F9	1858.771	5626.092	**0.000**
	X Dried *Ulva* + agar	1028.052	4795.372	**0.001**
	X Dried *Ulva* + alginate	585.987	4353.308	**0.005**
Isochrysis galbana + alginate	X *Vibrio midae* SY9 + alginate	-552.361	3214.959	0.313
	X *Vibrio midae* SY9 + *Ulva* extract F9	-270.306	3497.015	0.132
	X Dried *Ulva* + agar	-1101.026	2666.295	0.859
	X Dried *Ulva* + alginate	-1543.090	2224.231	0.999
Vibrio midae SY9 + alginate	X *Vibrio midae* SY9 + *Ulva* extract F9	-1601.605	2165.716	1.000
	X Dried *Ulva* + agar	-1334.996	2432.325	0.975
	X Dried *Ulva* + alginate	-892.932	2874.389	0.662
Vibrio midae SY9 + *Ulva* extract F9	X Dried *Ulva* + agar	-1052.941	2714.380	0.820
	X Dried *Ulva* + alginate	-610.876	3156.444	0.366
Dried *Ulva* + agar	X Dried *Ulva* + alginate	-1441.596	2325.725	0.993