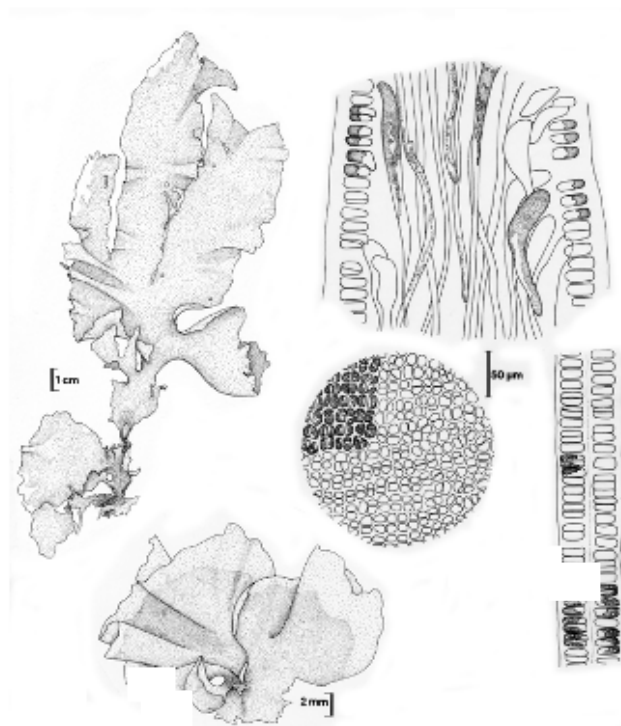




MSc Aquaculture Thesis

Domestication of the green seaweed *Ulva ohnoi* aka 'sea lettuce' for Biofloc effluent bioremediation



Student: Malachy McGovern a63326, University of Algarve, Portugal

Supervisor: Dr. Leila Hayashi, Federal University of Santa Catarina (UFSC), Brazil
Co-Supervisor: Dr. Leonardo Mata, University of Algarve (UALG), Portugal

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Supervisor: Dr. Leila Hayashi, Universidade Federal de Santa Catarina (UFSC), Brazil
leila.hayashi@ufsc.br

Co-Supervisor: Dr. Leonardo Mata, Centre of Marine Sciences of Algarve (CCMAR),
Portugal
lmata@ualg.pt

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Malachy Edward McGovern

Universidade do Algarve, 7th October 2020

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Abstract

The green macro algae *Ulva* spp. (Chlorophyta) is being increasingly used in Integrated Multitrophic Aquaculture (IMTA) due to its rapid growth rate and efficiency in the sequestration of excess nutrients. This study tested and evaluated the growth performance of *Ulva ohnoi* in various concentrations of marine shrimp biofloc effluent (*Litopenaeus vannamei*). In the first growth experiment, 29mm *Ulva* discs were cultivated in 1L flasks containing biofloc effluent at 25% [BFT25], 50% [BFT50], 75% [BFT75] and 100% [BFT100] and compared to a seawater control containing 8mL von Stosch standard solution per litre [VS8]. The mean specific growth rates (SGR) after 3 weeks cultivation were [VS8]: 13.14% per day⁻¹, [BFT25]: 13.63% per day⁻¹, [BFT50]: 14.08% per day⁻¹, [BFT75]: 13.85% per day⁻¹ and [BFT100]: 12.96% per day. From week 3 to week 4, the treatments [BFT25] and [BFT50] showed no growth whilst plants in [BFT75] and [BFT100] treatments continued to grow at a reduced rate of 2.45% per day⁻¹ and 3.35% per day⁻¹ respectively, which was not significantly different from the [VS8] control at 1.71 % per day⁻¹ (p>0.05). The results indicate that the higher nutrient budgets of [BFT75] and [BFT100] continued to support algal growth at higher stocking densities by attenuating the effects of self-shading and stress, demonstrating similar performance to a formulated high-nutrient solution (von Stosch).

In the second growth experiment, 19mm discs were cultivated in 1L flasks containing 25% [BF25], 50% [BF50], 75% [BF75] and 100% [BF100] biofloc effluent and compared to a seawater only control [FSW] and a second seawater control containing 10mL von Stosch standard solution per litre [VS10]. During week 1, the [BF75] demonstrated the highest mean specific growth rate (SGR_{max}) of 25.49 ± 3.23 % per day⁻¹ which was significantly higher than the [FSW] control (p<0.05) and the highest observed growth rate of the study. At the end of the 4 week experiment no significant difference in growth rates was observed between plants in the biofloc treatment groups (p>0.05). During experiment 2, [BF50] reduced levels of TAN by 71.3% in week 1 and by 24.83% in week 2. Nitrite was reduced by 42.73% in week 1 and 28.38% in week 2. Nitrate was reduced by 13.98% in week 1 and 33.3% in week 2. Phosphate was reduced by 13.98% in week 1 and 52.63% in week 2. The results indicate the species' preference for TAN over nitrate however the study found that sporulation events can release TAN into the medium which has implications for biofilter efficiency and bioremediation.

In the sporulation experiment, 5mm *Ulva ohnoi* discs were excised from a plant acclimatized in FSW enriched with 10mL/L von Stosch (VS10). The discs were placed in petri dishes containing 15 mL of FSW and then subjected to 4°C cold shock for [10 mins], [20 min], [1 hour] and [2 hours]. A control treatment was given no cold shock and placed in FSW [FSW control] and a second control

was given no cold shock and placed in fresh VS10 medium [VS10 control]. Combined 4°C cold shock [2 hours] and low nutrient shock proved highly effective at inducing a mean rate of sporulation of 90% after 5 days. However, this was not significantly different from low nutrient shock alone (i.e. transfer from VS10 to standard FSW [FSW control]) which caused a mean reproductive output of 76.66% ($p > 0.05$). The results suggest that cold shock treatment is not financially viable however further experiments with plants taken from cultivation in [BF50] or [BF75] are recommended.

Resumo

A macroalga verde *Ulva* spp. (Chlorophyta) está sendo cada vez mais usada em Aquicultura Multitrófica Integrada (IMTA) devido à sua rápida taxa de crescimento e eficácia no sequestro de nutrientes em excesso. Este estudo testou e avaliou o desempenho de crescimento de *Ulva ohnoi* em várias concentrações de efluente de bioflocos de camarão marinho (*Litopenaeus vannamei*). No primeiro experimento de crescimento, discos de *Ulva* de 29 mm foram cultivados em frascos de 1 L contendo efluente de biofoco a 25% [BFT25], 50% [BFT50], 75% [BFT75] e 100% [BFT100] e comparados a um controle de água do mar enriquecida com solução von Stosch na concentração de 8 mL de solução padrão por litro [VS8]. As taxas médias de crescimento específico (SGR) após 3 semanas de cultivo foram [VS8]: 13,14% por dia⁻¹, [BFT25]: 13,63% por dia⁻¹, [BFT50]: 14,08% por dia⁻¹, [BFT75]: 13,85% por dia⁻¹ e [BFT100] : 12,96% dia⁻¹. Da semana 3 à semana 4, as algas dos tratamentos [BFT25] e [BFT50] não apresentaram crescimento, enquanto as plantas nos tratamentos [BFT75] e [BFT100] continuaram a crescer a uma taxa reduzida de 2,45% por dia⁻¹ e 3,35% por dia⁻¹ respectivamente, que não foi significativamente diferente do controle [VS8] a 1,71% por dia⁻¹ ($p > 0,05$). Os resultados indicam que em condições mais elevadas de nutrientes ([BFT75] e [BFT100]) o crescimento de algas em densidades de estocagem mais altas foi possível, atenuando os efeitos de autossombreamento e estresse, e demonstrando desempenho semelhante a uma solução de nutrientes formulada (von Stosch).

No segundo experimento de crescimento, discos de 19 mm foram cultivados em frascos de 1 L contendo 25% [BF25], 50% [BF50], 75% [BF75] e 100% [BF100] de efluente de biofoco. Como controle, foram utilizadas plantas cultivadas apenas de água do mar [FSW] e em água do mar enriquecida com solução von Stosch, na concentração de 10mL de solução padrão por litro [VS10]. Durante a semana 1, o [BF75] demonstrou taxa de crescimento média de $25,49 \pm 3,23$ % por dia⁻¹ que foi significativamente maior que o controle [FSW] ($p < 0,05$) e a maior taxa de crescimento observada no estudo. No final de 4 semanas, nenhuma diferença significativa nas taxas de crescimento foi observada entre as plantas nos grupos de tratamento com biofoco ($p > 0,05$). Durante o experimento 2, o [BF50] reduziu os níveis de TAN em 71,3% na semana 1 e em 24,83% na semana 2. O nitrito foi

reduzido em 42,73% na semana 1 e 28,38% na semana 2. O nitrato foi reduzido em 13,98% na semana 1 e 33,3% na semana 2. O fosfato foi reduzido em 13,98% na semana 1 e 52,63% na semana 2. Os resultados indicam uma preferência por TAN em vez de nitrato, no entanto, o estudo descobriu que os eventos de esporulação podem liberar TAN no meio, o que tem implicações para a eficiência do biofiltro e biorremediação .

No experimento de esporulação, discos de *Ulva ohnoi* de 5 mm foram excisados de uma planta aclimatada em água do mar com 10mL/L von Stosch (VS10). Os discos foram colocados em placas de petri contendo 15 mL de FSW e então submetidos a choque frio de 4 °C por 10 e 20 minutos, 1 e 2 horas. Como controle, foi utilizado algas que não receberam choque frio e foram colocadas em FSW e algas que não receberam choque frio e foram colocadas em meio VS10. O choque frio combinado de 4 ° C (2 horas) e choque de baixo nutriente provou ser eficaz na indução de uma taxa média de esporulação de 90% após 5 dias. Porém, não foi significativamente diferente do choque apenas em baixa concentração de nutrientes (ou seja, transferência de VS10 para FSW), que causou uma produção reprodutiva média de 76,66% ($p > 0,05$). Os resultados sugerem que o tratamento por choque frio não é financeiramente viável, no entanto, outros experimentos com plantas retiradas do cultivo em [BF50] ou [BF75] são recomendados.

List of Abbreviations

ABEEólica	Associação Brasileira de Energia Eólica
ACE-inhibitory	Angiotensin-Converting Enzyme
ADP	Adenosine Diphosphate
ATP	Adenosine Triphosphate
AGMPFs	Algal Growth & Morphogenesis Promoting Factors
ANOVA	Analysis of Variance
APHA	American Public Health Association
BFT	Biofloc Technology
BF	Biofloc
[BFT25]	Biofloc 25% Treatment Group (Experiment 1)
[BFT50]	Biofloc 50% Treatment Group (Experiment 1)
[BFT75]	Biofloc 75% Treatment Group (Experiment 1)
[BFT100]	Biofloc 100% Treatment Group (Experiment 1)
[BF25]	Biofloc 25% Treatment Group (Experiment 2)
[BF50]	Biofloc 50% Treatment Group (Experiment 2)
[BF75]	Biofloc 75% Treatment Group (Experiment 2)
[BF100]	Biofloc 100% Treatment Group (Experiment 2)
°C	Degree Celsius
C	Carbon
Ca	Calcium
CAZymes	Carbohydrate Active Enzymes
CBN	Chemoautotrophic Bacterial Nitrification
CCMAR	Centre of Marine Sciences of Algarve
CDOM	Chromatic Dissolved Organic Matter
CO₂	Carbon Dioxide
DHA	Docosahexaenoic Acid
DO	Dissolved Oxygen
EAA	Essential Amino Acid
EPA	Eicosapentaenoic Acid
EU	European Union
FAO	Food & Agriculture Organisation
Fe	Iron
FSW	Filtered Seawater
[FSW control]	Filtered Seawater Treatment (Control) Group
g	Grams
GI	Gastrointestinal Tract
h	Hours
HBA	Heterotrophic Bacterial Assimilation
HBOI-FAU	Harbor Branch Oceanographic Institute at Florida Atlantic University
HSD	Honest Significant Difference
IMTA	Integrated Multi-Trophic Aquaculture
K	Potassium
L	Litres
LCM	Laboratório de Camarões Marinhos
m₂	Square Metre
mg	Milligram
min	Minute
mL	Millilitre
mm	Millimetre

n	Number, Sample Size
N	Nitrogen
NH₄⁺	Ammonia, ionized type
NH₃	Ammonia, unionized type
NO₂⁻	Nitrite
NO₃⁻	Nitrate
NADP⁺	Nicotinamide Adenine Dinucleotide Phosphate
O	Oxygen
P	p-value, Probability Value
PAR	Photosynthetically Active Radiation
ppm	Parts Per Million
P	Phosphorous
PLA	Polylactic Acid
RAS	Recirculating Aquaculture System
SEABIOPLAS	Seaweed from Sustainable Aquaculture as Feedstock for Biodegradable Bioplastics
SGR	Specific Growth Rate
SGR_{max}	Maximum Specific Growth Rate
SI-1	Sporulation Inhibitor 1
SI-2	Sporulation Inhibitor 2
SWI	Swarming Inhibitor
TAN	Total Ammonia Nitrogen
TSS	Total Suspended Solids
UALG	Universidade do Algarve
UFSC	Universidade Federal de Santa Catarina
UK	United Kingdom
UN	United Nations
UNEP	United Nations Environment Program
UNESCO	The United Nations Educational, Scientific and Cultural Organization
US	United States of America
VS	Von Stosch Nutrient Solution
VS10	FSW enriched with 10mL/L von Stosch
[VS8]	8mL/L von Stosch Treatment Group
[VS10]	10mL/L von Stosch Treatment Group
[VS10 control]	10mL/L von Stosch Treatment (Control) Group
WW	Wet Weight
[10 min]	10 Minute Cold Shock Treatment Group
[20 min]	20 Minute Cold Shock Treatment Group
[1 hour]	1 Hour Cold Shock Treatment Group
[2 hour]	2 Hour Cold Shock Treatment Group
μmol	Micromoles

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1.0 Introduction

The reader may be forgiven for thinking that the humble *Ulva* spp. exhibit basic biology and are therefore ‘unexciting’ in terms of aquaculture. These species have been studied widely as model organisms due to their simple morphology, yet their reproductive biology is remarkably complex. The genus is one of the fastest growing and has the highest carbon capture capacity when compared to any of the green, brown and red seaweeds. Known as “sea lettuce”, these green seaweeds are of considerable importance in aquaculture as a food source and are gaining increasing attention for their bioremediation potential in Integrated Multi-Trophic Aquaculture. They are increasingly studied by the pharmaceutical industry due to their array of valuable bioactive compounds. The aim of this thesis was to evaluate the use of biofloc effluent as a high nutrient medium for *Ulva* tank cultivation and also the efficacy of using *Ulva* for water bioremediation. Methods of inducing sporulation in wild-harvested *Ulva* sp. by using low temperature shock, low nutrient shock and a combination of both were evaluated. Samples cultivated in various biofloc treatments were compared, and gains in biomass and nutrient removal were evaluated. The results obtained in the present work could help establish new protocols for sporulation and domestication of the species in Santa Catarina, Brazil and offer insights into the potential of *Ulva* sp. as a functional food or protein source, as well as for biofloc effluent bioremediation. This may help advance the commercial viability of the study species and lead to more sustainable practices in aquaculture.

2.0 Literature Review

The first part of this project literature review will focus on the green macroalgae, genus *Ulva* by reviewing its importance in aquaculture. It will discuss the plant’s nutritional value as human food source, as dietary feed in traditional aquaculture and for bioremediation within integrated multi-trophic aquaculture (IMTA) systems. It will also explore the novel and emerging technologies such as biofuels, biopolymer (plastics) and carbon capture which are of significant interest to the author and of critical importance in the current ‘climate emergency’. Aquaculture is ultimately a commercial enterprise and so an understanding of the market and the commercial viability of various applications provides essential context for this thesis. The review will then focus more closely on the thesis experimental work, by discussing the species morphology, life cycle and reproductive strategies. It will critically review the available works regarding the efficacy of using temperature and nutrient (abiotic) shock to induce sporulation with some reference to microbial and epiphytic (biotic) factors. Optimisation of sporulation is of significant commercial importance for seedling production and an essential part of domestication of this species in aquaculture. Finally, the review will briefly discuss the merits of the biofloc, which is cultivation medium of interest to this thesis.

2.1 Importance in Aquaculture

Seaweeds are marine, photosynthetic macroalgae which are abundant in every ocean. There are three main classes, or phyla: Phaeophyceae (brown algae), Rhodophyta (red algae), and Chlorophyta (green algae) (Shannon, 2019). In 2018, farmed seaweeds represented 97.1 percent by volume of the total of 32.4 million tonnes of wild-collected and cultivated aquatic algae combined (FAO, 2020). Seaweed farming is practised in a relatively small numbers of countries, dominated by countries in East and Southeast Asia. The world production of marine macroalgae, or seaweed, has more than tripled, up from 10.6 million tonnes in 2000 to 32.4 million tonnes in 2018 with a market value of US \$12 billion (FAO, 2020). There are approximately 10,500 known species of seaweeds and around 220 are cultivated. Nine genera (6 groups) provide 97.9% of the production: *Kappaphycus/Eucheuma*: 40.3%, *Saccharina/Laminaria*: 28%, *Gracilaria*: 13.7%, *Undaria*: 8.5%, *Porphyra/Pyropia*: 6.6%, and *Sargassum*: 0.7% (Chopin, 2018). Seaweed aquaculture differs significantly from traditional finfish and shellfish aquaculture in that much of the recent growth and research can be accrued to its potential for economic exploitation for non-food applications (**Figure 1** below).

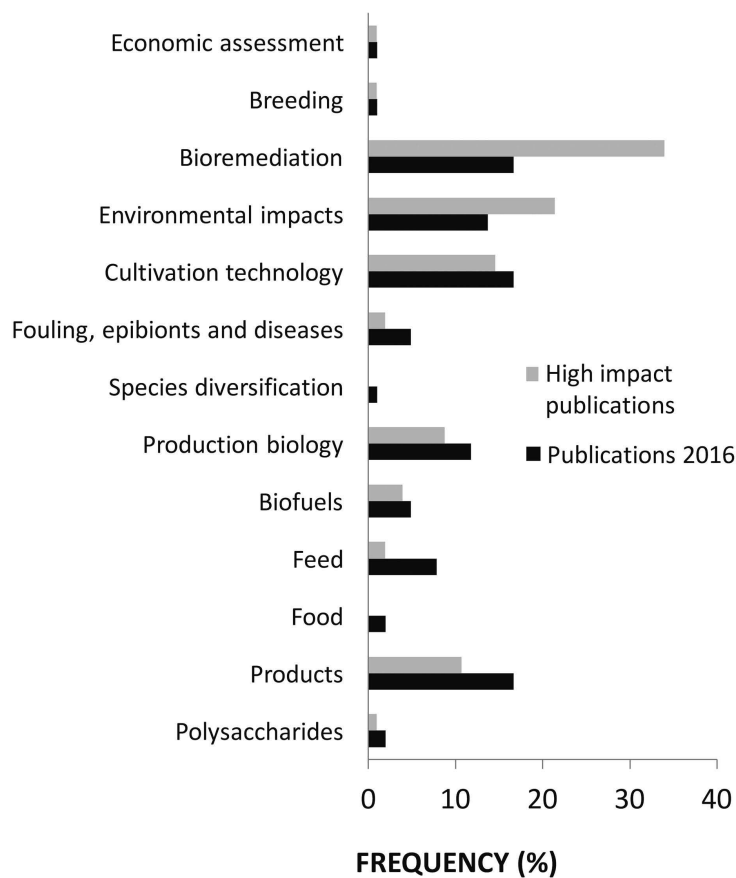


Figure 1: Research tendencies in seaweed farming. The 100 most cited papers and the 100 most recent papers published in 2016. Source: Buschmann et al (2017)

2.1.1 *Ulva* spp.

Ulva (Phylum: Chlorophyta, Class: Ulvophyceae, Order: Ulvales, Family: Ulvaceae) is a broad cosmopolitan genus of green seaweed that can be found from tropical to polar climates and from fresh to fully saline environments (Rautenberger and Bischof, 2006; Kirkendale et al., 2013). *Ulva* species are difficult to morphologically identify due to their phenotypic plasticity (Favot et al, 2019). The species of the present study, known as the “sea lettuce” *Ulva* sp., is typically found in colder temperate climates (such as the South of Brazil), has a distinctly different morphology from the ‘filamentous’ species and may differ from species found in warmer tropical regions of Brazil. The more distantly related species *Ulva mutabilis* was collected first by B. Føyn at the Southern Atlantic coast of Portugal in warmer waters (Føyn, 1958). *Ulva* biomass has become infamous due to increased eutrophication-driven green tide events especially along the tropical coasts. The first *Ulva* bloom to be described was in Belfast (in the north of Ireland) at the end of the nineteenth century (Letts, 1911) however more worryingly, the largest breakouts to date have been the green tides observed in China for ten consecutive years from 2007 and covering 10% of the Yellow Sea (Li et al, 2018).

Although still produced on a modest scale, when compared to other commercially farmed seaweed, *Ulva* spp. have been perceived as high-value aquaculture product in several Southeast Asian countries. The existing commercial production is focused on niche, high-value, edible products such as ‘aonori’ and ‘aosa’ in Japan with about 1,500 tonnes dry weight *per annum* (Carl et al. 2014). It is of particular interest that newly established companies are producing consistent, reliable high-grade food and products of bio-functional properties (AlgaPlus, Portugal), (M/s. Venus Shell Systems, Australia), nutritional supplements (M/s. Seakura, Israel) and (M/s. La Huerta Marina, Spain) from wet as well as dried *Ulva* biomass.

The production involves photo-bioreactor cultivation (Chemodanov et al. 2017), land based, and open sea farming (Castelar et al. 2014; Chemodanov et al. 2019). Unlike other commercially farmed red seaweed where clonal propagation is practiced, species of this genus requires motile reproductive cells (zoospores or gametes) for seeding or seedling production which are then transferred onto nets for outdoor cultivation or may be left ‘free flowing’ in raceways, Integrated Multi-Trophic Aquaculture (IMTA) systems, closed Recirculating Aquaculture Systems (RAS) and or Biofloc Technology (BFT) systems. *Ulva* spp. are opportunistic and have similar reproductive characteristics compared to ‘r selected’ species: a large number of spores released by the reproductive cycle, small spores (approximately 10 µm), a wide range of dispersive potential (flagellates), rapid growth, and short life cycle (Schories, 1995). In general, two aspects favour the exploitation of *Ulva* spp.: its potential to provide valuable products and its high productivity (Dominguez & Loret, 2019).

2.1.2 *Ulva* as Food & Nutraceuticals

Ulva can be a source of essential amino acids and some of them, such as histidine, are found in levels comparable to those found in vegetables and eggs (Lordan et al, 2011). The protein content is typically 11% although some research have shown this can be increased to around 40% (Shpigel et al. 2018) under IMTA systems, where high levels of nitrogen are assimilated into protein with a N to P conversion factor of 5 (Angell et al, 2016). Apart from protein, *Ulva* species are a good source of minerals, particularly calcium, magnesium, iron and zinc (MacArtain et al. 2007). Tabarsa et al. (2012) showed that *Ulva* contained high levels of K, and the amounts of Ca, Na and Fe were higher than those reported for land plants. The balanced content of Na and K (a ratio near 1 to 1) is nutritionally beneficial (Lordan, 2011).

In considering the elemental composition in terms of nutritional value of green seaweeds such as *Ulva*, caution must be applied and it must be considered that the absorbable quantity of an element in the gastrointestinal (GI) tract is not accurately predicted by its total content in the seaweed. To facilitate saccharolytic fermentation in the human colon, the gut microbiota must express functional carbohydrate active enzymes (CAZymes) to catabolise seaweed glycans as carbon sources within the colon (Cherry et al., 2019). Bioaccessibility is expressed by the share of the initial content that is available for intestinal absorption (Afonso et al., 2015). Some reports indicate that *Ulva lactuca* and ulvan polysaccharides are poorly fermented by the human gut microbiota (O'Sullivan et al, 2010, Jiao et al. (2011) however a recent in vitro faecal fermentation work indicated that ulvan stimulated the growth of *Bifidobacterium* and *Lactobacillus* populations (Seong et al, 2019). Ripol et al. (2017) assessed the bioaccessibility of bioactive compounds of the green seaweeds (*Chaetomorpha linum*, *Ulva intestinalis*, *Ulva lactuca* and *Ulva prolifera*) grown in multi-trophic aquaculture environments and concluded that the absence of bioaccessible phenolic compounds suggests that human digestion is unable to release these compounds and therefore extraction is probably necessary. Whilst many bioactive compounds are not readily available from human digestion alone, seaweeds are a low-calorie food that add protein without saturated fat, and an excellent dietary lipid alternative to EPA and DHA derived from fish (Shannon, 2019).

2.1.3 *Ulva* as Aquafeed and Bioremediation in IMTA

Lobo et al. (2016) noted that seaweed supplementation (*Gracilaria* spp., *Ulva* spp., and *Fucus* spp.) in practical diets for European seabass juveniles have no impact on growth performance, up to 7.5% supplementation level. Moreover, their work found that a dietary 2.5% incorporation level improved innate immune system indicators suggesting a prebiotic/ probiotic benefit. Similarly, Natify et al (2015) found that Nile tilapia fed on 5% seaweed diet tended to have higher growth performance than

those fed on the control (and 10% seaweed diet). That work concluded that *Ulva lactuca* meal can be included as supplement ingredient in the diet without impairing growth performance, feed utilization and body composition of Nile Tilapia.

Ulva has also been used successfully as a feed additive for reared shrimp (Cruz-Suarez et al. 2010) and has successfully replaced commercial feed without compromising shrimp growth or its essential amino acid (EAA) composition, particularly in *Litopenaeus vannamei* (Boone) (Pallaoro et al., 2016) and sea urchins (Cyrus et al. 2014, 2015a, b), with the additional benefit that the pigment content of the *Ulva*, particularly β -carotene, provides essential colour to the product. Cyrus et al. (2015b) working with the urchin *Tripneustes gratilla* have shown that fresh *Ulva* can achieve equivalent somatic growth rates to a formulated feed. Similarly, Mulvaney et al. (2013) demonstrated that the specific growth rate of juvenile Australian abalone fed with fresh *Ulva* was significantly higher compared to a formulated feed, and that *Ulva*-fed abalone had an improved condition index and tolerance to stress. Calheiros et al. (2019) have studied the benefits of *Ulva*'s abundant essential amino acids, such as tryptophan and phenylalanine and their value in fish farms as anxiolytic agents.

The rapid development of fed aquaculture (e.g. finfish or shrimp) brings with it considerable environmental concern regarding the discharge of nutrients to coastal areas, potentially leading to deterioration of local marine environments (Buschmann et al. 2006). Integrated multitrophic aquaculture (IMTA) systems represent a practical solution for mitigating the negative effects of fish farming wastes by utilising excess nutrients as a valuable resource for marine plant production (Chopin et al. 2008). IMTA systems also increase profitability and add value to the investment in finfish aquaculture by increasing the yield of total biomass produced on a single site (Chopin et al. 2012). *Ulva lactuca* shows high potential for use in integrated multitrophic systems with recirculation (Favot et al, 2019). Shpigel et al. (2018) have shown that biomass produced in maricultural effluents contained 2–4 times more protein (up to 40% in dry weight) than *Ulva lactuca* from the wild ().

2.1.4 Bioactive compounds

As stated above, caution must be applied when reviewing bioactive compounds and their pharmaceutical uses. A review of Marine Drugs publications however reveals that *Ulva* protein hydrolysates show antioxidant (Kazir et al, 2019), angiotensin-converting enzyme (ACE)-inhibitory activity (Paiva et al, 2016) and immunomodulatory effects (Cian et al, 2018). Furthermore, *Ulva* has water-soluble and insoluble cellulose (38–52%) corresponding to structural polysaccharides with a major component called Ulvan. Ulvan is up to 30% *Ulva* dry weight and contains mainly sulfonic acids, sulphated l-rhamnose, xylose and glucose. The genus also contains phenolic, chlorophyll and

carotenoids, which can be regarded as active free-radical scavengers (Cesario et al, 2018). The available literature advises of exciting developments where identified polysaccharide and oligosaccharides have anti-viral, anti-tumour, anti-coagulant, anti-lipidique, hepato-protective, immuno-stimulating, anti-depressant and anti-anxiolytic activities (Cardoso et al, 2014 and Sari-Chmayssem, 2018) and are increasingly requested for pharmaceutical and food applications. The global seaweed market was valued at \$10.4 billion in 2015 and is projected to reach \$14.7 billion by 2021. It is driven mainly by the growing use of seaweed for medicinal applications (Shannon & Ghannam, 2019).

2.1.5 Biofuel

Ulva carbohydrates can also be a carbon source for microbial production of biomaterials and building blocks to produce a range of chemicals and intermediates, such as organic acids, alcohols and bioplastics. As lipid levels in *Ulva* are low, biofuel involves the production of bioethanol (Korzen et al, 2015). After harvest, the biomass requires pre-treatment and/or saccharification and the hydrolysis to form easily fermentable compounds in anaerobic digestion, and their extraction makes possible a rapid degradation (Dominguez & Loret, 2019) and fermentation to be converted in bioethanol. *Ulva* derived biofuels have been subject to a considerable number of works (Dominguez & Loret, 2019) however the literature states that bioethanol can also be obtained from sugar cane or other agricultural productions at a much lower cost compared to *Ulva* biorefinery, regardless of the process. It is therefore probable that efforts to have a competitive source of bioethanol from green seaweed may not be financially viable in the future when electricity and hydrogen will replace organic fuels (Dominguez & Loret, 2019).

2.1.6 Biopolymers/ Bioplastic

The annual production of plastic has increased from 1.7 million metric tons in 1950 to 322 million metric tons in 2015 and now more than eight billion tons of plastic have accumulated on earth. Plastic has become so ubiquitous that some have characterized the current stage of human history as the “Plastics Age” (UN, 2019). Much of the plastic waste has ended up in the oceans, with estimates ranging from 93 to 236 thousand metric tons floating in the sea (UN, 2019). Plastic is increasingly invading the food chain, with birds, fishes and other animals directly and indirectly consuming vast amounts of harmful waste.

Terrestrial crops, such as corn, wheat, sugar beets and sugar cane are currently the main feedstock for the production of bioplastics (Belbloom & Leonard, 2016). One type of bioplastic is polylactic acid (PLA), a highly versatile, biodegradable, aliphatic polyester. Seaweed’s growth rate, productivity and

carbohydrate yield are all higher than traditional feedstocks (Hughes et al, 2012). In particular, green seaweeds such as *Ulva* contain comparatively higher levels of accessible sugars, including starch and cellulose (Wei et al, 2013). When grown on land in ponds, *Ulva* can be induced to produce even more carbohydrates through manipulation of their production factors (Chopin, 2012, Favot et al, 2019). Helmes et al. (2018) recently studied the feasibility of using *Ulva* spp. for production of lactic acid as a precursor of polylactic acid. This can be achieved by (1) processing the biomass for solubilization of sugars; (2) the fermentation of the sugars to lactic acid (using *Lactobacillus plantarum*); (3) the isolation of lactic acid from fermentation broth; and (4) extraction of lactic acid produced in the seaweed-derived growth medium using standard basic precipitation with calcium sulfate (gypsum) as a co-product. This method is currently being championed by SEABIOPLAS (Seaweed from sustainable aquaculture as feedstock for biodegradable bioplastics) which is an EU Seventh Framework Programme (FP 7) funded project which aims to introduce sustainably cultivated seaweeds as feedstock for biodegradable bioplastics.

A review of the literature suggests that seaweed-derived bioplastics are gaining momentum, and companies such as Evoware (Indonesia), Notpla (UK), Algapack (France) have made considerable progress in delivering marketable products. Novel uses such as commercial food wrapping, clothing, drinks pouches, and even babies' nappies must be embraced. In terms of commercial viability, the energy required for drying of raw *Ulva* product, hydrolysis, fermentation and subsequent purification must also be considered. It is the authors view that synergies with existing coastal industries by utilising surplus (heat) energy and ways to shorten the production supply chain are required as the viability of seaweed-based bioplastic develops further.

2.1.7 Carbon Capture & Climate Change

Carbon sequestered in both living and non-living biomass in the ocean and coastal habitats has been termed 'Blue Carbon' by the United Nations Environment Programme (UNEP) (Nellemann et al., 2009; Vierros, 2013). Although they account for 2% of the sea surface, macro-vegetated marine habitats contribute 210–244 Tg C year or 50% of all carbon sequestered in the global coastal oceans (Duarte et al., 2005). Marine algae and plants (e.g. eel grass) have been estimated to be responsible for more than 70% of the world's carbon storage (Chung et al. 2011). One direct effect of this carbon sequestration is that it mitigates ocean acidification (Nellemann et al. 2009). Chung et al. (2011) showed that *Ulva* species had the highest carbon capture capacity compared to seaweeds belonging to the Chlorophyta, Phaeophyta and Rhodophyta. The rapid growth of *Ulva* spp. is attributed to its high photosynthetic rates and high C and N nutrient uptake capacity (Naldi & Wheeler, 2002). *Ulva lactuca* has also been found to reduce ruminal fermentation parameters and methane production by

55% when incubated *in vitro* with a substrate (meadow hay) for 24 h. Interestingly, the genus *Asparagopsis* has been proven to reduce methane production by approximately 99% under similar controls (Maia et al, 2016). It is the author's view that significant future investment in seaweed production might come in response to tighter environmental and legislative controls. Polluting petrochemical companies (Petrobras, Petroleo Brasileiro SA) might seek to 'offset' their impact by funding large-scale carbon capturing seaweed farms. These could be married with existing infrastructure for example, the fast-growing offshore wind sector Associação Brasileira de Energia Eólica (ABEEólica).

2.2 Domestication and Control of Sporulation

2.2.1 *Ulva* Morphology & Life Cycle

Ulva spp. fronds are soft, distromatic sheet structures that are two cells thick and this morphology is the reason for its common name "sea lettuce". Amongst the various life history patterns, species of the green seaweed genus *Ulva* are represented by a homologous diplohaplontic isomorphic life history (**Figure 2** below).

Both sporophytic and gametophytic plants are morphologically similar, yet equally abundant and distinct as free-living individuals, which differ only in their ploidy and functions (Balar & Mantri, 2019). The sporophytic plant reproduces asexually using zoospores while gametophytes reproduce sexually via gamete fusion (Wichard et al. 2015). Reproduction involves a direct transformation of vegetative cells into generative divisions, thereby producing either bi-flagellated haploid gametes through mitosis or quadriflagellated haploid zoospores through meiosis. Haploid gametophytes are derived either from the haploid zoospores of sporophytes, from unmated biflagellated gametes, or from zoospores of parthenosporophytes (Lovlie, 1968).

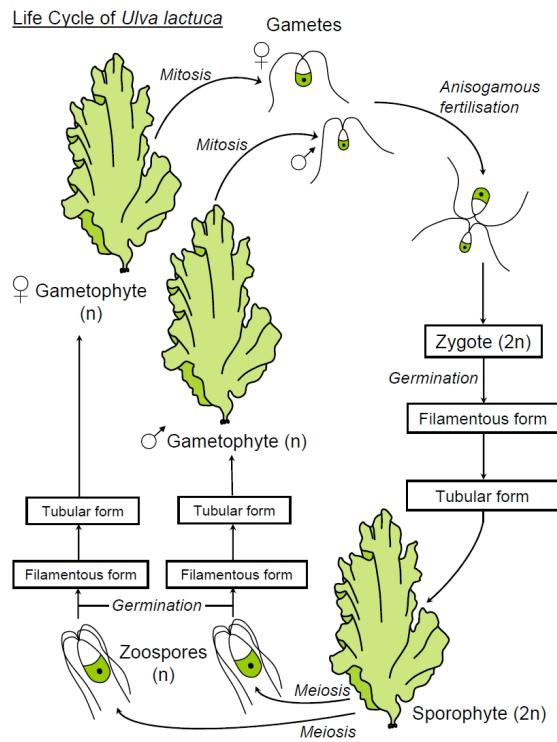


Figure 2 above: *Ulva* life stages from Cronodon (2013)

2.2.2 Reproduction & Sporulation

As vegetative cells are transformed into generative cells, zoospore formation can occur at any location within a thallus, but gametes are formed near the frond margin (Luning & Kadel, 2008). Balar & Mantri (2019) noted that the giant medullar cells or rhizoidal cells (at the base) never seen to form reproductive cells. The induction signals are therefore perceived at the thallus margin where the distromatic thallus do not adhere closely to each other (Stratmann et al. 1996; Luning and Kadel 2008). Much of the available literature suggests that the process of induction of gametogenesis has been divided into three phases, determination phase, differentiation phase and swarming phase. The last phase is the time period involving gamete release and can be induced by various physiological stimuli (Wichard & Oertel, 2010; Kessler et al. 2017).

As described by Balar (2019), the thallus cells enter into either sporogenesis or gametogenesis based on their degree of maturity and the level of sporulation inhibitors, which presumably form an internal vertical concentration gradient between the two blade cell layers (Wichard & Oertel, 2010). Vegetative thalli release a high molecular mass cell wall glycoprotein (SI-1) into the surrounding medium while containing a second low molecular weight inhibitor (SI-2) in the space between the two cell layers of the thallus. The sporulation inhibitor (SI-1) suppresses the differentiation of blade cells into gametangia until the plants become fertile. During thallus maturation, the production of this factor gradually ceases with age, and finally its concentration in the medium of the cultivation recipient drops below the inhibitory level (Wichard & Oertel, 2010). Sporulation inhibitor 2 (SI-2) keeps the

more basal sectors of the blade in the vegetative state, because here the SI-2 concentration is at its highest (Stratmann et al., 1996). These inhibitors act independently of photoperiod or any other physico-chemical factors. The transformation from a blade cell into a gametangium occurs only if SI-1 levels drop and the constantly-present SI-2 is no longer perceived by the alga, as discussed by Stratmann et al. (1996).

After initiation, the blade cells start excreting a third substance into the environment, a “Swarming Inhibitor” (SWI) which after completion of gametangium differentiation (2-3 days) inhibits gamete release despite the light signal required for swarming. Reproduction can be induced in laboratory conditions by cutting the thallus into single-layer fragments exposing the inner space of the bilayer and placing them in fresh medium to facilitate reduction of sporulation inhibitor concentration (Stratmann et al. 1996; Vesty et al. 2015). A second wash (or media change) is then required to remove the swarming inhibitor and trigger sporulation. In theory, this mechanism should allow for a very precise timing of preparation of gametes for experimental purposes (Wichard & Ortel, 2010) or for inoculation of ropes and nets in commercial aquaculture. The laboratory technique has been successfully practised in *U. mutabilis*; *U. lactuca*; *U. linza* and *U. rigida* (Stratmann et al. 1996; Wichard and Oertel 2010; Vesty et al. 2015) however little information is available regarding commercial inoculation. In nature, Stratmann et al. (1996) and Wichard & Oertel (2010) suggested that sporulation events are correlated with spring tides, as observed by Smith (1947) due to the dilution of sporulation and swarming inhibitors by incoming spring tides. The lab processes involving segmentation and media change are highly time consuming however and so novel ways to induce large scale sporulation of mature sporophytes are required.

Following initiation, the gametes are formed by repeated bipartition of the cell protoplast. The first cleavage takes place parallel to the thallus surface while the second one is vertical to the first. The conical projections are developed known as papilla at the exterior end. The microtubule apparatus is then formed. The nucleus enters into division to form nuclei of subsequent gametes (Balar, 2019). The cytokinesis gives rise of 16 oval-shaped gametes, which are ready to release (Katsaros et al. 2017). The available literature suggests that sporogenesis is similar however the divisions continue to take place and zoospore mother cells divides to form zoospores.

The final stage of reproductive induction, prior to shedding of gametes or zoospores (known collectively as ‘swarmers’) is marked by change in thallus colour, caused by the repositioning of the chlorophyll containing chloroplasts, from yellow-green (wholly vegetative state) to dark olive. A white colour is evident after the release of reproductive cells (Stratmann, et al. 1996). Some works have found that synchronization with the moon phases would occur once a month by continuous light

during full-moon nights (Lüning and Kadel 2008). That said, unsystematic reproductive rhythm has also been observed in *U. pseudocurvata* under controlled laboratory conditions where peaks occurring at every 7 days (Lüning and Kadel 2008).

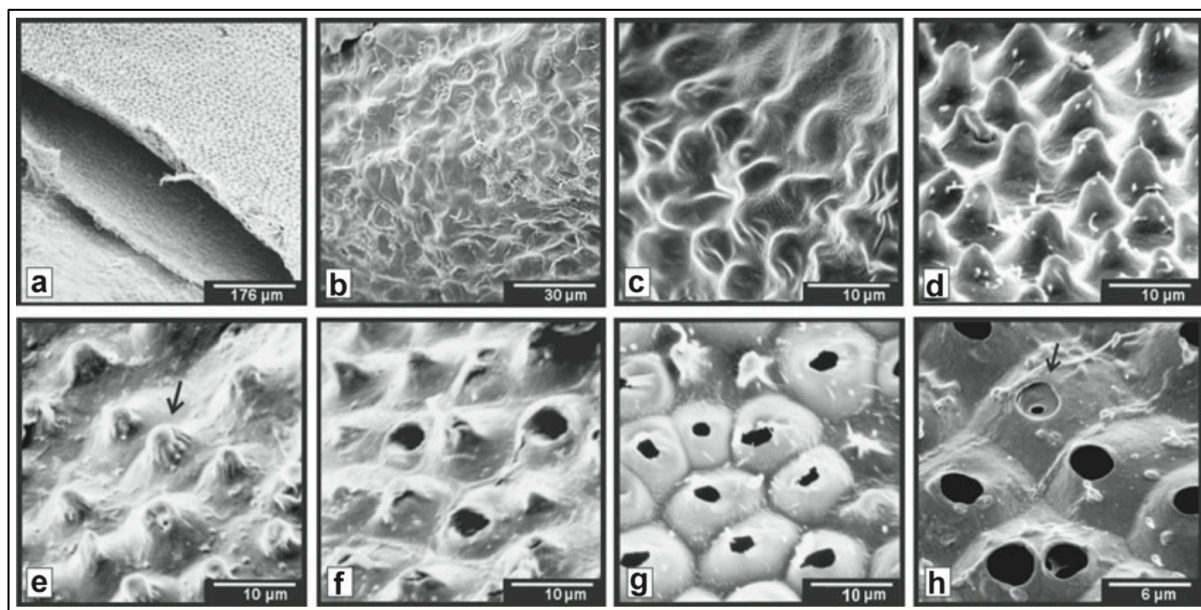


Figure 3 above: Scanning electron micrographs of developing gametangia of *Ulva mutabilis*. *U. mutabilis* (a) Blade fragment second day after induction. (b) Inner surface of this fragment. (c) Outer surface of this fragment with developing papillae. (d) Gametangia day after induction. (e) Gametangia third day after induction. The pore caps are fully developed (arrow). (f) Gametangia later on third day when the pore caps have dropped. (g) Gametangia 15 min after removal of the SWI by medium change and complete discharge on the third day after induction. (h) Gametangia discharged incompletely at noon (1200 h) of the third day after induction Arrow: presumably not discharged gametangium with inner (perforated) seal (Source: Wichard, 2010).

2.2.3 Bacterial Interactions

The algal microbiome also plays a crucial role in development, growth and reproduction through various mechanisms such as production of growth-promoting substances, quorum sensing, bioactive compounds mediated signalling, etc (Singh & Reddy, 2014). As bacteria are able to utilize algal nutrients selectively, they play a key role in biotransformation and nutrient recycling (Chesters et al. 1956). Epiphytic bacteria also offer a defensive function either through their antibiotic activity or by forcing pathogenic bacteria to compete for space within the biofilm (Goecke et al., 2010). Cross-kingdom cross-talk between macroalgae and bacteria controls algal settlement, growth and development (Joint et al., 2002, 2007). Several works have shown that whilst induction of reproduction and sporulation of *Ulva* can be achieved under axenic laboratory conditions, *Ulva* fails to form its typical morphology in the absence of the appropriate bacteria and simply proliferates as an undifferentiated clump of callus cells (Marshall et al., 2006; Spoerner et al., 2012).

Spoerner et al (2012) cultivated *Ulva* in axenic conditions and recorded that the complete morphogenesis was recovered either by the combination of the two bacterial strains *Roseobacter* sp. and *Cytophaga* sp., or by morphogenetic compounds extracted from the bacterial supernatant (Spoerner et al., 2012). *Roseobacter* sp. and *Cytophaga* sp. fulfill complementary tasks: *Roseobacter* sp. (MS2) induces cell division similar to a plant hormone cytokinin whereas the *Cytophaga* sp. (MS6) promotes, similar to plant hormone auxin, a viable basal undifferentiated cell and primary rhizoid cells in addition to the induction of the proper cell wall formation. Moreover, the *Roseobacter* species exhibits a specific chemotactic affinity to the rhizoid cells of *U. mutabilis* and seems to cooperate with the *Cytophaga* strain and the alga by chemical communication, forming a symbiotic unique tripartite community.

About 1500 different *Ulva* associated bacterial strains have been reported in the literature belonging to the α -Proteobacteria or γ -Proteobacteria and several of them have been thought to be responsible for morphogenesis (Hagström et al. 2000; Tujula et al. 2010). Whilst the specific regulatory factors resembling cytokinin and auxin were identified from *Ulva mutabilis* (Spoerner et al., 2012) these factors could not be replaced by any known phytohormones. They always work in tandem for inducing normal development. Additionally, laboratory studies have demonstrated that different bacterial communities having similar functional attributes can facilitate successful algal morphogenesis in *U. mutabilis* and *U. intestinalis* (Ghaderiardakani et al., 2017). Morphogenetic activity for induction of *Ulva*'s development has been recently determined in lagoon water and land-based aquacultures (Ghaderiardakani et al. 2019) and are sometimes known as algal growth and morphogenesis-promoting factors or AGMPFs.

Wichard et al (2010) suggests that the swarming inhibitor (SWI), besides its role in regulation of gamete release in *Ulva mutabilis*, may have additional regulatory intra-specific and/or interspecific functions particularly in *U. lactuca*. For instance, this factor may participate in the chemical communication of *Ulva* with other accompanying organisms, such as, for example, their essential bacterial symbionts or other symbiotic and competing inhabitants of the ecosystem (Marshall et al. 2006). As demonstrated by Stratmann et al. (1996), gamete release, however, seems not to be influenced by the symbiotic bacteria. One possible allelopathic function of SWI might be the inhibition of gamete release from competing *Ulva* gametangia (Wichard et al., 2010).

2.2.4 Temperature Shock

Temperature impacts photosynthesis resulting in altered productivity and hence supplies carbohydrate to the tissue (Hatfield & Prueger, 2015). The response to temperature is exhibited

through phenological attributes mediated by complex metabolic pathways (Balar, 2019). Temperature in combination with light (day length) is the most important factor determining reproductive success in seaweeds. *Ulva* spp. have cosmopolitan distribution along the intertidal zone and hence tolerate a wide variation in temperature by special physiological adaptations. The species cope up with elevated temperature by exhibiting higher content of nitrate reductase, amylase, invertase, and peroxidase, and reduced growth rate (Kakinuma et al, 2006). Temperature also significantly affects the rates of respiration, via adenylate control i.e. ADP/ATP ratios (Balar & Mintri, 2019). The optimal temperature for growth is 15- 30°C for tropical species (Hiraoka & Oka 2008; Nordby, 1974) and the optimal temperature for gamete/ spore release was found to be 15 °C – 20 °C in *U. prolifera*, *U. pertusa*, *U. mutabilis*, *U. fenestrata*, *U. spinulosa*, *U. linza*, *U. rigida*, *U. faciata*, while few species have been reported a higher range of temperature for successful reproduction namely 25 °C in *Ulva* spp. (Carl et al. 2014); and 21°C in *U. mutabilis* (Nordby, 1974).

Ulva spp. are non-perennial and so there is no strict seasonality in the onset of reproduction. Abiotic shocks are the most efficient manner to induce sporulation and obtain recruits of *Ulva* spp. in vitro (Ohno, 2006). Quality and quantity of light, photoperiod, and temperature are the main factors that induce spore release in seaweeds (Lobban & Harrison, 1994). As identified above, *Ulva* species tend to have complicated life cycles, generally involving an alternation of isomorphic diploid sporophyte and haploid gametophyte phases as well as occasional parthenogenetic development of gametes. In all cases, reproductive cells are directly transformed from vegetative cells and the transition is initiated by extrinsic factors, with environmental temperature known to be particularly important (Gao et al., 2017). For example, *Ulva lactuca* from Groton, USA, only reproduce in warmer months (Niesenbaum, 1988), whereas North Sea populations of *Ulva pseudocurvata* have approximate weekly reproductive peaks during the summer and biweekly peaks during colder seasons (Lüning et al., 2008). Kalita & Titlyanov (2011) shed further light on temperature regulation of reproductive rhythmicity by showing that the reproductive period of *Ulva fenestrata* decreased from 30 to five days when temperature increased from 10 to 20 °C, and ceased at 5 °C. Furthermore, rapid temperature changes have been found to induce gamete release over periods of hours to days (Niesenbaum, 1988; Carl et al., 2014).

Niesenbaum (1988) used temperature shock to initiate sporulation in *Ulva lactuca* in laboratory conditions successfully. When no reproduction had occurred amongst samples after 30 days, reproduction was stimulated by temperature shocking with 2°C seawater. Plants were removed from their containers which were at 22°C and washed thoroughly in filtered seawater at 2°C, and returned to clean culture chambers with fresh medium at 22°C. In this experiment two reproductive treatments, and one control were used. The design was restricted to this because 18 h after the 2°C

wash, all of the samples had become reproductive, so no vegetative algae were available. This author observed that the conversion of vegetative tissue to a reproductive state was almost immediate after the seaweed were washed in water at 2°C noting further that swarmer accumulation within the 12 hours was rapid and linear.

Working with a tropical filamentous species of *Ulva* spp., Carl et al (2014) successfully applied controlled timing of release of swarmers (motile reproductive bodies) by exposing the thalli to a temperature shock (4 °C) for 10 min and subsequently placing them into autoclaved filtered seawater under a 12 h photoperiod at 25 °C. The release of swarmers then peaked two days after initiation. Sporulation was increased by approximately 10% when thalli were exposed to chilled (4 °C) seawater for 10 min. This effect is consistent (albeit less pronounced) with Niesenbaum's study in temperate waters identifying temperature shock as a trigger to induce *Ulva lactuca*.

Gao et al (2017) has suggested that the shorter time required for fertility of *Ulva* species experiencing temperature shock reported in previous studies (Niesenbaum, 1988; Carl et al., 2014) could be down to the pre-fertile status of the plants, as gamete discharge was also found in the control group; temperature shock merely accelerated the release process. Wichard and Oertel (2010) suggested that a minimum of two days is required for the transition from vegetative to reproductive status . The enhanced reproduction under temperature shock may be a survival strategy under unfavourable conditions (Li & Brawley, 2004).

The most recent work of Gao et al. (2017) demonstrated that a 20-min temperature shock at 4 °C induced a mean reproductive response of 94.7% in blade tissue of *Ulva rigida* by day five. It is not known whether temperature shock stopped the excretion of sporulation inhibitors or triggered another reproductive pathway. Gao has suggested that the process appears to involve a two-step mechanism. The first step is from high temperature to low temperature and the second is returning to a high temperature. According to him, the results demonstrated that neither step is dispensable as continuous low temperature induction for three weeks, without returning to high temperature, did not trigger reproduction. Indeed, this finding is supported by previous studies wherein it was shown that *U. fenestrata* cannot form reproductive cells at 5 °C (Kalita & Titlyanov, 2011) and gamete release in *U. mutabilis* only occurs within a narrow temperature range between 15 and 25 °C (Wichard & Oertel, 2010).

The literature suggests that the efficacy of temperature shock treatment varies significantly between *Ulva* species, and if they are from temperate and tropical climates. This latitudinal relationship between temperature shock and reproduction in *Ulva* has not been investigated (Gao et al., 2017).

2.2.5 Nutrients

Nutrients and light are key environmental factors that determine biomass yield and productivity in an aquaculture system and can also be manipulated to control epiphytes and the number of compounds of interest formed (Harrison & Hurd, 2001). The nutrients required by seaweeds can be divided into three categories, macronutrients (e.g. N, P, C etc.), micronutrients or trace elements (e.g. Fe, Zn, Cu, Mn, etc.) and vitamins (B12, thiamine and biotin). These essential elements for growth can be found in seawater however are often concentrated within the plant tissue, for example, N and P are concentrated about 100,000 times by seaweeds (Harrison & Hurd, 2001). Nitrogen is an essential co-factor in cell anabolic reactions including nicotinamide adenine dinucleotide phosphate (NADP⁺), for synthesis of amino acids. Phosphorous is of similar importance in nucleic acid formation, and functions as one of the main promoters of photosynthesis, nutrient transport, and energy transfer via energy-rich linkages adenosine triphosphate (ATP). The ratio of nutrients required for maximal plant growth is called the optimal ratio. The average ratio for seaweeds is 30N:1P with a range of 10:1 to 80:1 (Atkinson & Smith, 1983). Where an excess of one nutrient is provided, the corresponding nutrient will become a 'limiting factor'.

Chemical factors such as nutrient concentration and the form of inorganic nutrient (e.g. unionised ammonia nitrate NO₃⁻ or ionised ammonium NH₄⁺) may influence uptake rates. Whilst some seaweeds such as kelps can uptake both NO₃⁻ and NH₄⁺ simultaneously and in equal proportions, most seaweeds including *Ulva lactuca* have a preference for assimilation of NH₄⁺. When nitrate is taken up by seaweeds its can be stored intracellularly in the vacuole and cytoplasm or reduced to nitrite via the enzyme nitrate reductase. Nitrite is transported from the cytoplasm to the chloroplasts where it is reduced to ammonium via the enzyme urease. Ammonium is taken up or converted into amino acids via glutamine synthetase in the chloroplasts (Hurd et al., 2014). NO₃⁻ uptake and assimilation require energy from the light reactions of photosynthesis (Pritchard et al., 2015; Raven et al., 1992). NO₃⁻ is assimilated to NH₄⁺, which requires the synthesis and maintenance of two enzymes (nitrate and nitrite reductase) and energy from eight electrons (Boyd & Hurd, 2009). From an energy perspective, it is therefore theoretically more efficient to supply ammonium in aquaculture rather than nitrate.

A wide variety of biological factors such as inter-species variability, nutritional history, type of tissue, life history stages and surface area: volume ratio of the thallus and morphological changes such as blade shape or the production of hyaline hairs may also influence uptake rates. Nutritional history is also a highly important consideration. Pedersen (1994) observed three phases of NH₄⁺ uptake (first observed by Conway et al., 1976) in *Ulva lactuca*. These stages are surge, internally controlled and externally controlled. When the uptake rate was several times higher than the growth rate, they

termed this phenomenon ‘surge’ uptake. This enhanced uptake rate allows the cell to overcome its previous nutrient deficiency, since it is able to ‘catch up’ by taking up the limiting nutrient much faster than is required for growth (Harrison & Hurd, 2001). For these reasons, acclimation of wild samples is important in order to homogenise any variations in uptake or nutrient requirement where possible. It should be noted that some nutrient-limited seaweeds also show a decrease in the nutrient uptake over time after it has been added to the seawater. For this reason, it is important to record the time period over which nutrient measurements are made when conducting experiments.

Physical factors such as light, temperature, desiccation and water motion influence uptake kinetics. *Ulva* spp. NH_4^+ uptake rates have been shown to decrease by 50% in the dark compared to light (Hurd, 1990). Relatively little is known about how temperature influences uptake however in general a doubling of temperature doubles uptake rates (i.e. $Q_{10} = 2$). Temperature affects all aspects of seaweed physiology through its regulation of enzyme activity, rate constants of chemical reactions, and the rate of diffusion of nutrients across boundary layers. For nutrients that are taken up by active transport, temperature is likely to affect these rates because it will affect the activity of membrane transporters; whereas, there may be less effect on uptake via passive diffusion. Higher temperatures can accelerate growth and reproduction by increasing enzyme activity, and increased nitrogen availability can support an accelerated synthesis of nucleotides and proteins (Kalita & Titlyanov, 2011).

As far as the author is aware, to date, no comparative study has looked into the nutrient physiology of different life history stages (e.g. spores, gametophytes, sporophytes) of *Ulva* spp. however some interesting experiments have been conducted using nutrient to stimulate sporulation. Castelar et al (2014) found that seeding in vitro with a high nutrient concentration was the most efficient method to induce spore release in *Ulva lactuca* and *Ulva flexuosa*, producing twice as many recruits compared to other shock treatments.

2.2.6 Biofloc Technology Systems (BFT)

Closed aquaculture systems have very low rates of water exchange, tightly controlled inputs, and are typically contained in smaller spaces than more traditional open ponds (Ray et al, 2017). These systems are increasingly being examined as a means of enhancing biosecurity, reducing water use, and producing marine animals away from coastal zones (Browdy & Moss, 2005). Conventional recirculation aquaculture systems (RAS) usually have more filtration components, leading to relatively higher start-up costs, and potentially greater operational costs (Luo et al., 2014) compared to biofloc technology (BFT). RAS systems are often problematic in that slow-growing autotrophic

bacteria require significant time to assimilate and work effectively within the biofilter. This delay in maturation means that they are often unable to adequately decompose Total Ammonia Nitrogen (TAN) effectively.

Biofloc technology (BFT) first developed in Israel, is now recognised as a promising alternative to conventional nursery systems particularly with the rearing of white shrimp *Litopenaeus vannamei*. RAS bio-filtration normally utilises plastic spheres inside the biofilter and these oxidising bacteria convert TAN to nitrate by ammonia and nitrite oxidizing bacteria. The BFT achieves ammonia control through fast growing heterotrophic bacteria that are dominant in the biofloc (Avnimelech, 2012). The biofloc is essentially a protein rich aggregate of organic material and micro-organisms including diatoms, bacteria, protozoa, algae, faecal pellets, remains of dead organisms. This aggregate can vary depending on the reared species and other biotic and abiotic factors. Typical bioflocs have irregular shapes and a broad distribution of particle sizes (50 µm- 1000 µm), are highly porous (up to more than 99% porosity) and are permeable to fluids (Chu & Lee, 2004). The underlying principle of ammonia control in the BFT is the manipulation of the carbon:nitrogen ratio to promote the heterotrophic bacteria that will then assimilate TAN into their microbial biomass. Depending on the volume of feed and the percentage of protein within that feed, an extra amount of carbohydrates must be added to the water in order to supply the heterotrophic bacteria with sufficient organic carbon to assimilate the excreted ammonia nitrogen. This microbial process, described by Avnimelech (2012) takes place inside the rearing water, and is therefore independent of external tanks and pumping systems, significantly reducing application costs. Additionally, the heterotrophic bacterial biomass forms a biofloc, which can be an additional source of protein for the reared species and so feed is recycled. It has also been observed that biofloc can improve feed utilization and protein digestion, thus, it further enhances shrimp growth performances, while reducing costs for additional dry feed (Xu & Pan. 2012). Biofloc, in general, has a positive effect on the immunity and antioxidant capacity of aquatic animals (Long et al., 2015; Truong Thy et al., 2016). For example, the total haemocyte counts, the phagocytic activity and the total antioxidant capacity were significantly higher in *L. vannamei* reared with biofloc than those organisms cultivated in clear water (Xu & Pan 2013). Overall, the BFT can contribute to the sustainability of an aquaculture operation by reducing nutrient rich effluents and needed production space, while also decreasing the dependence on marine derived fishmeal.

Researchers at the Harbor Branch Oceanographic Institute at Florida Atlantic University (HBOI-FAU) used *Ulva lactuca* to manage the nitrogenous waste produced in a biofloc IMTA system with Florida pompano, *Trachinotus carolinus*, and Pacific white shrimp, *Litopenaeus vannamei*. Shrimp were observed grazing on small pieces of the *Ulva lactuca* as well as the biofloc. However, shrimp

consuming the *Ulva* would abandon the macroalgae when feed pellets were added to the tank, indicating a preference for this diet. The productivity of *Ulva lactuca* cultivated in the HBOI-FAU system is 20 g dry weight/m² /day and had a 34% protein on a dry-weight basis (Laramore et al., 2018). A further study was conducted utilizing *Ulva* as supplement or partial replacement of the pelleted diet, thereby adding economic value to the system by decreasing feed costs. The study found that there was no significant difference in growth between the control and the *Ulva* supplemental treatments at 81 to 82%, but growth was significantly lower in both groups in which a portion of the diet was replaced with *Ulva*. It was noted that shrimp in the 50:50 treatment did not consume all of the fed macroalgae, while shrimp in the supplemental and 75:25 treatment group did, indicating a threshold for *U. lactuca* addition.



Figure 4: Biofloc Tank at Marine Shrimp Laboratory UFSC

3.0 Aim and Objectives of the Study

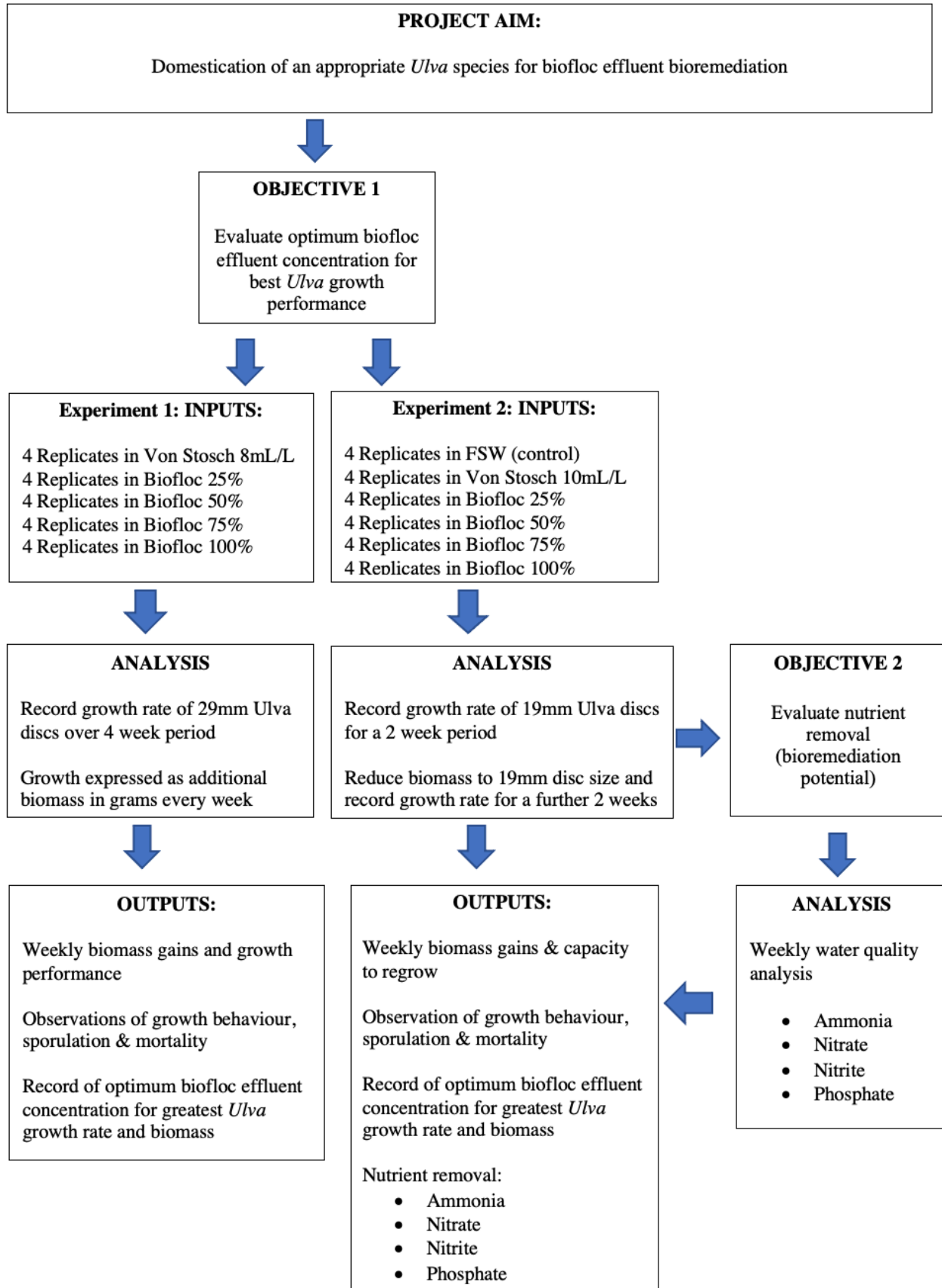
Having identified the potential commercial and environmental benefits of *Ulva* species in aquaculture, the general aim of the thesis is domestication of the *Ulva sp.* for biofloc effluent remediation in Florianopolis, Santa Catarina, Southern Brazil. The objectives are set out below:

OBJECTIVE 1: Establish the optimum biofloc effluent concentration for best *Ulva* growth performance.

OBJECTIVE 2: Evaluate nutrient removal (bioremediation potential).

OBJECTIVE 3: Establish the optimal temperature and nutrient shock for induction of sporulation in best performing plants.

4.0 Methodology



PROJECT AIM:

Domestication of an appropriate *Ulva* species for biofloc effluent bioremediation



OBJECTIVE 3

Evaluate optimal temperature and nutrient shock for induction of sporulation in best performing plants



Experiment 3: INPUTS:

3 Replicates at 4°C for 10 mins in FSW
3 Replicates at 4°C for 20 mins in FSW
3 Replicates at 4°C for 1 hour in FSW
3 Replicates at 4°C for 2 hours in FSW
3 Replicates No Shock FSW (Control 1)
3 Replicates No Shock VS10 (Control 2)



ANALYSIS

Record percentage of sporulated discs (out of a total of ten discs) per treatment type

Record rate of sporulation and patterns of change

Observe changes in cells – chloroplast position change, gamete formation, gamete release



OUTPUTS:

DATA of optimal temperature & nutrient conditions to be applied to sporulation PROTOCOL

4.1 Materials & Methods

Species Identification

Because of the broad intraspecific variation and taxonomic difficulties associated with the *Ulva* genus, the species used in this study was analysed using molecular barcoding. Barcoding compares short DNA sequences from a standardised region of the genome - the 'barcode' - to a library of reference sequences derived from individuals of known identity. The species was confirmed to be *Ulva ohnoi*.

Sample Collection

Ulva ohnoi (hereafter *Ulva*) was collected by hand from a stabilization pond within the Laboratory of Marine Molluscs of UFSC located in Barra da Lagoa, Flórianopolis (**Figure 5**).



Figure 5 Stabilization Pond at Marine Shrimp Laboratory UFSC

Samples were placed in a 10 L container filled with filtered sea water (FSW) and then transported within 10 minutes to the Seaweed Section of the Marine Shrimp Laboratory of UFSC which is located within the same grounds. Subsequently, the samples were gently washed three times with filtered seawater (FSW; 0.2 mm and UV sterilised) to remove debris, epiphytes and invertebrates. Samples remained in 50 L acclimation containers in the lab with 12 h photoperiod, $80 \pm 10 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ irradiance, and aerated with a flow rate of 100 L min^{-1} for 1 week to acclimation and homogenisation to minimise site-specific variations in nutrient levels (**Figure 6**).



Figure 6: Acclimation Tanks at Algae Laboratory UFSC

4.1.1 Select Sporophytes for Experimentation

In order to select for sporophytes only, sporulation was induced by leaving a section of thallus from 4 different plants in FSW within a 1 litre Erlenmeyer flask for 1 week. Sporulation and release of swarmers with a negative phototaxis was observed. The attachment of seedlings on the bottom of the flasks was observed and these plants were identified as sporophytes.

4.1.2 Experiment 1: Cultivation of 29 mm *Ulva* discs in Biofloc effluent

Experimental Design

An *Ulva ohnoi* plant was taken from acclimation tank containing FSW and placed in a small tray with approximately 1 litre of FSW. The plant was checked for signs of sporulation and only healthy thallus tissue in a vegetative state was chosen. One hundred twenty discs were excised from a single plant using a scalpel and a 29 mm diameter specimen container. Six discs were used for each treatment (total weight was approximately 0.25 grams \pm 0.02 grams). The 6 discs were placed in a conical 1 Litre Erlenmeyer flask and placed on the cultivation chambre at a constant 25 °C with 12 h photoperiod, $80 \pm 10 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ irradiance, and aerated with a flow rate of 100 L min⁻¹. Four replicates for each treatment were used. The flasks were organised in random blocks (see **figure 7** below). The FSW enriched with von Stosch or BFT was changed every week and at the same time discs were blotted dry with tissue paper and measured using a Mante BL3200H digital measuring scale.

Treatment 1: 4 Replicates in FSW enriched with von Stosch nutrient solution (8mL/L) – [VS8]

Treatment 2: 4 Replicates in FSW enriched with 25% Biofloc Effluent – [BFT25]

Treatment 3: 4 Replicates in FSW enriched with 50% Biofloc Effluent – [BFT50]

Treatment 4: 4 Replicates in FSW enriched with 75% Biofloc Effluent – [BFT75]

Treatment 5: 4 Replicates in enriched 100% Biofloc Effluent – [BFT100]

Substance	Final concentration
NaNO ₃	0,50 mM
Na ₂ HPO ₄ .12H ₂ O	30 µM
FeSO ₄ .7H ₂ O	1µM
MnCl ₂ .4H ₂ O	0,1 µM
Na ₂ EDTA.2H ₂ O	10 µM
Thiamine.HCl	0,59 µM *
Biotin	4,10 nM *
Cyanocobalamin	1,0 nM *

*50% reduction in vitamin concentrations

Table 1: Chemical composition of von Stosch solution according to Edwards (1970)

The Biofloc effluent was taken from the large shrimp cultivation tanks in the outdoor greenhouses within the UFSC Marine Shrimp Laboratory. The effluent was removed using a 1L plastic container and conical funnel and decanted for 30 minutes and then, filtered in a 20 microns bag filter to remove organic matter and TSS excess. The water quality parameters such as pH, salinity and nutrient loads were not adjusted at any time.

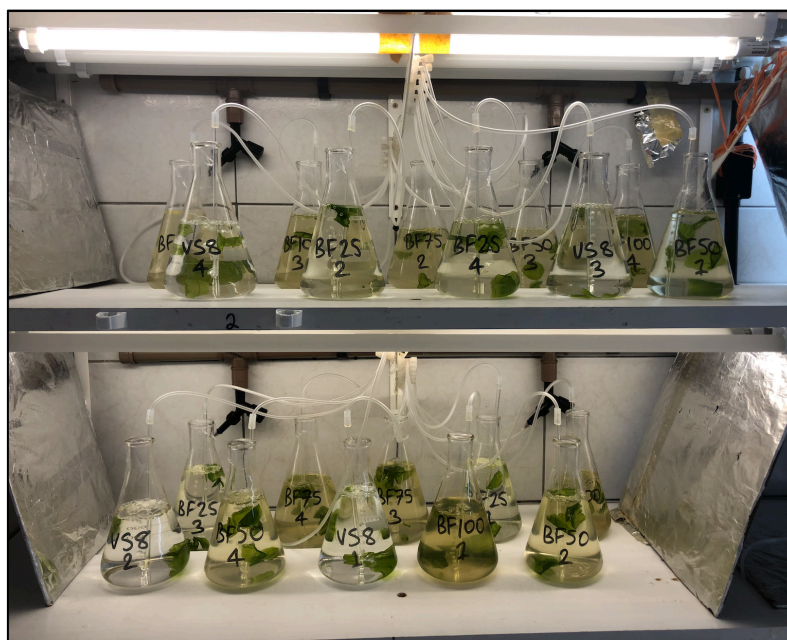


Figure 7: Cultivation chamber at Seaweed Section (LCM- UFSC) (experiment 1)

Growth Rate

Biomass of each treatment type was determined each week by weighing fresh thalli. The selected species *Ulva ohnoi* fronds are only two cell layers thick making them vulnerable to damage. Cautious handling of the disks during measurement is essential. *Ulva* thalli were blotted gently with tissue paper to remove surface water before weighing.

Growth rate determination is a basic measurement to determine the growth performance and response of a target culture. Seaweeds exhibit a characteristic growth pattern which is marked by a rapid initial phase followed by decreased growth during later stages. This is due to self-shading as the inner parts of tissue are often shaded from exposure to light, which then reduces the photosynthesis rate. Thus, in order to determine growth rate more accurately, the time interval between data was decided to be as short as weekly.

Specific growth rate (SGR) was calculated by the formula for best accuracy (Yong et al, 2013):

$$[(W_t/W_0)^{1/t} - 1] \times 100\%$$

where W_t is the weight after t days, W_0 is the initial weight and t is the number of cultivation days. The mean SGR over 6 weeks of cultivation will be based on the initial weight and the final weight at the end of the cultivation period.

4.1.3 Experiment 2: Cultivation of 19 mm *Ulva* discs in Biofloc effluent

Experimental Design

An *Ulva ohnoi* plant was taken from the acclimation tank containing FSW and 50% Biofloc Effluent and placed in a small tray containing approximately 1 litre of the same culture medium. The plant was checked for signs of sporulation and only healthy thallus tissue in a vegetative state was chosen.

Eighty discs were excised from a single plant using a scalpel and a 19 mm diameter pipette tip. Four discs were used for each treatment (total weight was approximately 0.1 grams \pm 0.02 grams). The 4 discs were placed in a conical 1 Litre Erlenmeyer flask and placed on the cultivation chambre at the same conditions described above. Four replicates were used and were organised in random blocks (see **figure 8** below). The water quality parameters such as pH, salinity and nutrient loads were not adjusted at any time.

Treatment 1: 4 Replicates in FSW – [FSW]

Treatment 2: 4 Replicates in FSW enriched with Von Stosch nutrient solution (10mL/L) – [VS10]

Treatment 3: 4 Replicates in FSW enriched with 25% Biofloc Effluent – [BF25]

Treatment 4: 4 Replicates in FSW enriched with 50% Biofloc Effluent – [BF50]

Treatment 5: 4 Replicates in FSW enriched with 75% Biofloc Effluent – [BF75]

Treatment 6: 4 Replicates in 100% Biofloc Effluent – [BF100]

*Note the “BFT” abbreviation was used in Experiment 1 and later changed to “BF” in Experiment 2

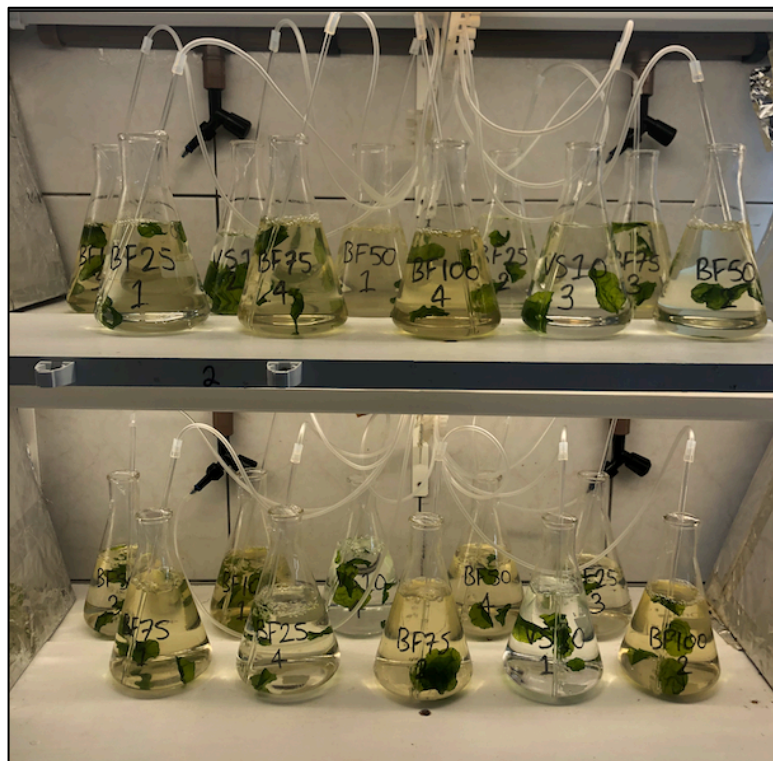


Figure 8: Cultivation chamber at Seaweed Section (LCM – UFSC) (experiment 2)

Growth Rate

As per experiment 1 above, biomass of each treatment was determined each week by weighing fresh thalli. *Ulva* thalli were blotted gently with tissue paper to remove surface water before weighing.

Specific growth rate (SGR) was calculated by the formula for best accuracy (Yong et al, 2013):

$$[(W_t/W_0)^{1/t} - 1] \times 100\%$$

where W_t is the weight after t days, W_0 is the initial weight and t is the number of cultivation days. The mean SGR over 6 weeks of cultivation will be based on the initial weight and the final weight at the end of the cultivation period.

4.1.4 Nutrient Removal

The Biofloc 50% treatment was analysed for nutrient removal in the second experiment. A sample of media comprising 50% Biofloc / 50% FSW was taken at the beginning of the experiment and sent to the water quality lab for analysis. Ammonia (TAN), Nitrate, Nitrite and Phosphate levels were measured at the beginning of the experiment and from 4 additional samples from the Biofloc 50 [BF50] treatment every week just before the media change. This procedure was repeated for the first 2 weeks of the experiment.

- TAN analysis was carried out according to the protocol by UNESCO (1983);
- Nitrite analysis were carried out following a protocol from Strickland and Parsons (1972) and the guidelines of APHA (2005);
- Nitrate analysis was undertaken using Kit da HACH NITRAVER 5 Reagent Pillow (Cat 21061-69); and
- Phosphate analysis was done according to APHA (2005).

All water analysis procedures were carried out by professional technical staff in a specialized laboratory of the LCM.

Thallus regrowth and Rejuvenation

After 2 weeks of cultivation, the biomass from each flask in each treatment was reduced to 0.09 grams (± 0.03 grams). This was done by cutting the thalli tissue in half with a scalpel. The biomass was cultivated for a further two weeks, and growth rates were calculated as outlined above.

4.1.3 Experiment 3: SPORULATION: Temperature & Nutrient shock

The initial objective of this study was to evaluate the optimal temperature and nutrient shock for induction of sporulation in best performing plants. The biomass from the 'best performing' discs cultured in experiment 1 and 2 was too limited for use in this experiment however, so a healthy sporophyte plant taken from the UFSC Shrimp laboratory grounds was used. This plant was kept in an acclimation tank containing FSW enriched with 50% biofloc effluent for 3 weeks and was further acclimated in FSW enriched with 10mL/L von Stosch for 1 week prior to the experiment.

The identified sporophyte plant was taken from a 5 L recipient containing FSW with 10mg/L of von Stosch nutrient solution (FSW+VS10mL/L) at 16:00 h. The plant was carefully removed and placed in a plastic tray containing approximately 1 Litre of FSW so as to dilute / diminish the effect of the

sporulation inhibitors. One hundred twenty *Ulva* discs measuring 5 mm diameter (Gao et al. 2017) were excised from the outer 75% region of the vegetative thalli using a stainless steel biopsy punch. Ten *Ulva* discs were placed in a 90 mm Petri dish for each treatment group and filled with 20 mL FSW at 25 °C. The nitrate and phosphate concentrations in the FSW were approximately 100 and 5 $\mu\text{mol L}^{-1}$ respectively and significantly lower than the enriched culture media.

Twelve petri dishes (4 treatment groups in triplicate) were placed in a refrigerator (4°C) at approximately 17:00 h and stored in the refrigerator for 10 mins, 20 mins, 1 hour and 2 hours. A control was retained in constant 25 °C in FSW and a second control was retained in FSW with von Stosch 10mg/L enriched medium. The control groups were also done in triplicate. The dishes were removed after their corresponding cold shock time lapsed and were placed with the two control groups on the cultivation chamber at 25 °C at an irradiance of $80 \pm 10 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$ under a 12 h photoperiod. The dishes were arranged on the shelf in randomised blocks (see **figure 9 & 10** below).

Treatment 1: 3 Replicates at 4°C for 10 mins in FSW – [10 min]

Treatment 2: 3 Replicates at 4°C for 20 mins in FSW – [20 min]

Treatment 3: 3 Replicates at 4°C for 1 hour in FSW – [1 hour]

Treatment 4: 3 Replicates at 4°C for 2 hours in FSW – [2 hour]

Treatment 5: 3 Replicates with no temp shock in FSW- [FSW Control]

Treatment 6: 3 Replicates with no temp or nutrient shock in VS10 - [VS Control]



Figure 9, 10 : Cultivation chamber at Accommodation next to UFSC Marine Shrimp Laboratory

Sporulation & Reproductive Output

Vegetative cells are green in colour, containing several granular chloroplasts and thalli become pale green when becoming fertile due to a change in the chloroplast position. Cells which have fully released swarmers appeared clear or white in colour.

Three Stages of Reproduction

The observation of sporulation in *Ulva sp.* is difficult and requires an understanding of the three main stages of transformation from vegetative state to reproductive state i.e. determination, differentiation (swarmer formation) and post-sporulation. Reproductive *Ulva* discs are recognized by a colour change which was verified by microscopy (see **Figures 11, 12 & 13**). The vegetative cells are green and contain several chloroplasts (**Figure 11**). The thalli become pale green or yellow in a reproductive state due to the shifting position of the chloroplasts. The cells appear half-full by the end of spore formation (**Figure 12**). Finally, the cells appear white after swarmers have been discharged (**Figure 13**).

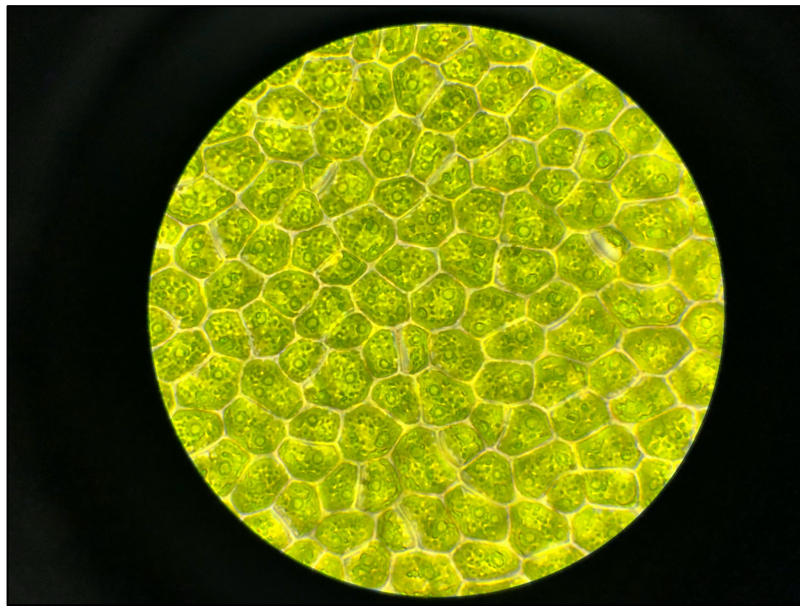


Figure 11 *Ulva ohnoi*. cells in vegetative state with numerous visible chloroplasts. Captured at X100 with Olympus CX23 light microscope and Apple iPhone 8

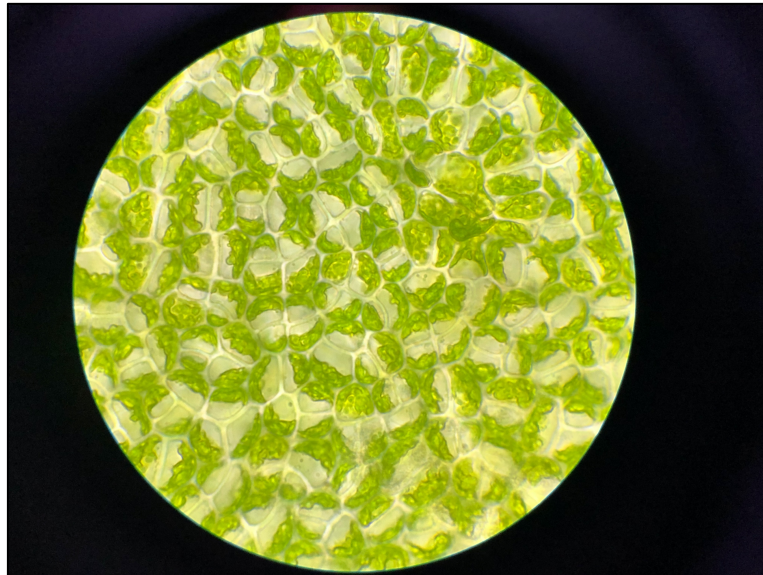


Figure 12: *Ulva ohnoi* cells in differentiation state with numerous visible swimmers developing. Captured at X100 with Olympus CX23 light microscope and Apple iPhone 8

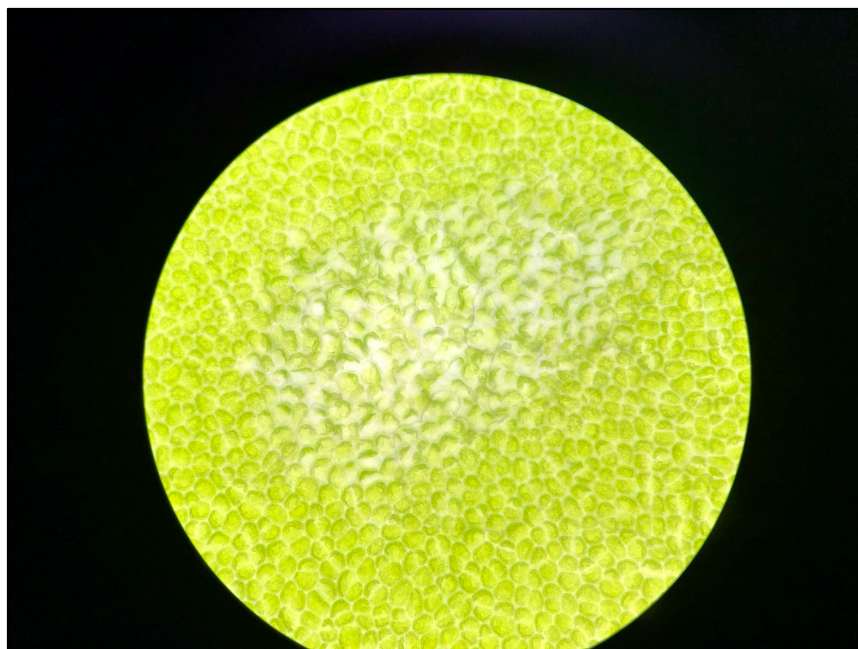


Figure 13: *Ulva ohnoi* cells in post sporulation state with numerous empty cells in the centre. Captured at X10 with Olympus CX23 light microscope and Apple iPhone 8

The dishes were regularly observed throughout the day for colour change. At 16:00 h every day the 5 mm discs were very carefully removed from each Petri dish using rubber tweezers and placed on a glass slide with a glass cover slip. The glass slides were examined under a light microscope (model Olympus CX23). The process of examination took approximately 3-4 hours and care was taken to complete the examination before the end of the 12 h light photoperiod at 20:00 h. All discs were

examined at 4, 10, 40 and then 100 magnification. At 4 magnification, the 9 mm discs almost filled the viewing range of the objective lens. Photographs of each disc were captured at 4 magnification. After examination, the discs were gently removed from the slides, placed into the same petri dishes with fresh FSW media and returned to the cultivation chamber. The period of time taken to induce swarmer release was recorded. Sporulation in more than half of the disc area was considered as equivalent to complete sporulation (Gao, 2017). The reproduction rate was recorded from the day when reproduction occurred to the end of the experiments when the *Ulva* discs yielded a reproductive success of over 90%. The reproduction rate is expressed as the number of sporulated discs out of the total number of discs in each petri dish for e.g. 8 sporulated discs will equate to 80%.

4.1.4 Statistical Analysis

All data was analysed using the statistical software program RStudio (Version 1.3.1073 – © 2009-2020 RStudio, PBC). The Shapiro-Wilk test was used to test the obtained data for normal distribution (Royston, 1995) and the Levene's test determined the homogeneity of variance (Fox, 2016). When the data was found to be normally distributed and with homogeneous variance, a one-way analysis of variance (ANOVA) (Chambers & Hastie, 1992), followed by Tukey's honest significant differences (HSD) post-hoc test (Yandell, 1997) was performed. Any data set that was found to be not normally distributed and/or without homogenous variance, was analysed using the non- parametric Kruskal-Wallis Rank Sum test (Hollander et al., 2015), followed by Dunn's test (Dunn, 1964). All significant differences were determined at a confidence level of $\alpha = 0.05$.

5.0 Results:

5.1 Experiment 1 – Growth of 29 mm *Ulva* discs in Biofloc Effluent

After the first 7 days in cultivation, plants in all treatments had increased in mass with [BFT25], demonstrating a mean biomass of 0.87 grams compared to a mean across all treatment groups of 0.77 grams (see **figure 14** and **figure 15**). During week 2, [BFT50] displayed a mean biomass value of 2.1 grams when compared to a treatment mean of 1.72 grams. During week 3, the discs in treatment [BFT50] had a mean biomass value of 4.17 grams per litre when compared to a mean of 3.45 among the treatments. Statistical analysis revealed no significant difference between the [BFT50] and the mean biomass gains at week 3 ($p=0.0527$) or between any treatments however. By the end of the experiment at 4 weeks, plants in [BFT75] displayed a mean biomass value of 4.7 grams when compared to a mean of 3.87 grams across all the treatments however no statistical difference was found between groups ($p=0.3416$).

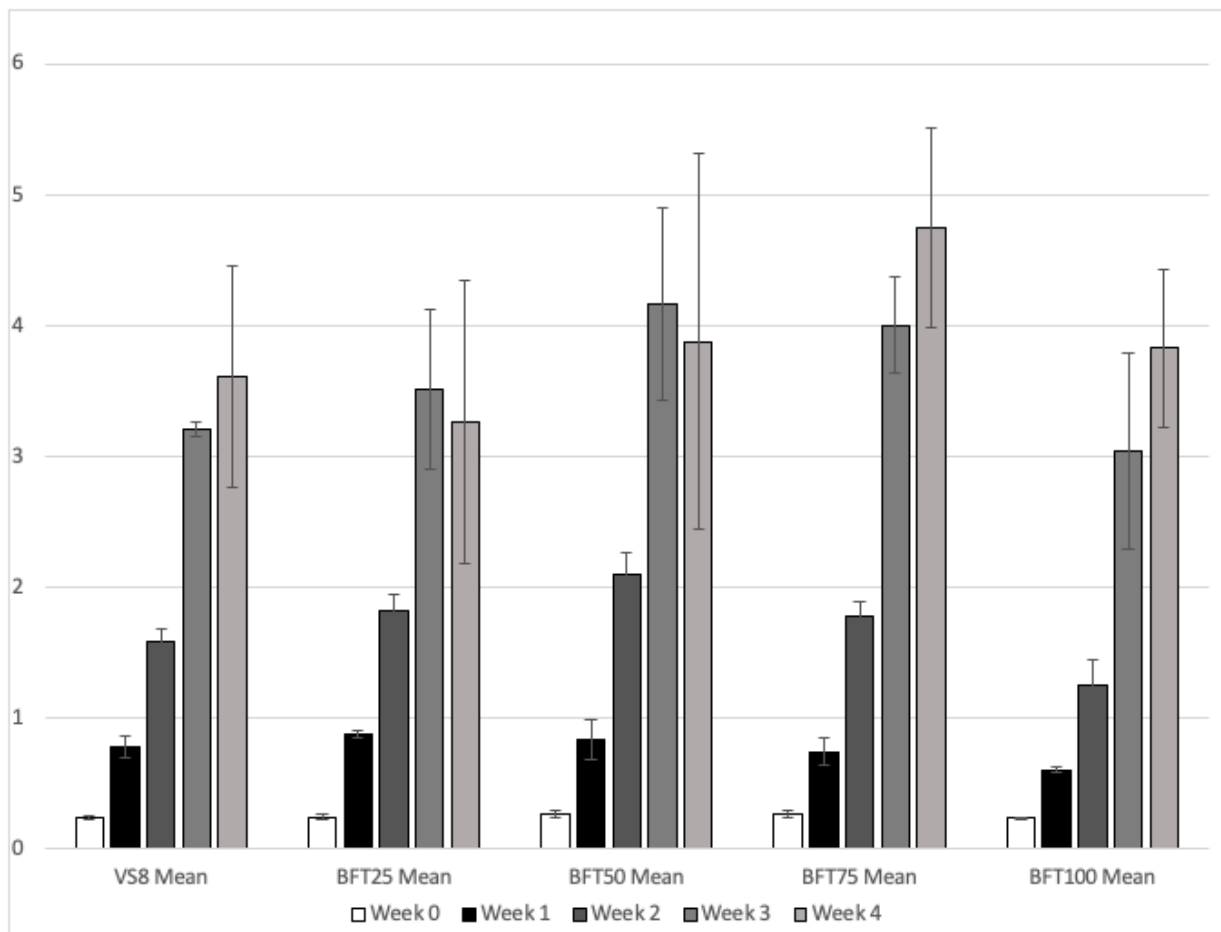


Figure 14: Weekly increase in biomass per treatment (y axis shows grams). Data are expressed as a mean \pm standard error of 4 replicates for each treatment (n = 4).

Interestingly, the gains in biomass among treatment groups [BFT25] and [BFT50] started to decline after 3 weeks in cultivation. At this point the individual discs had reached up to 90 – 110 mm in diameter which represents a dramatic increase in size when compared to the 29 mm initial size. Despite moderate aeration allowing the discs to initially tumble and roll within the flasks, the accumulation and crowding caused significant self-shading and this would have limited photosynthetic ability and growth. This indicates a maximum stocking density of 3.5 to 4 grams/L for this treatment.

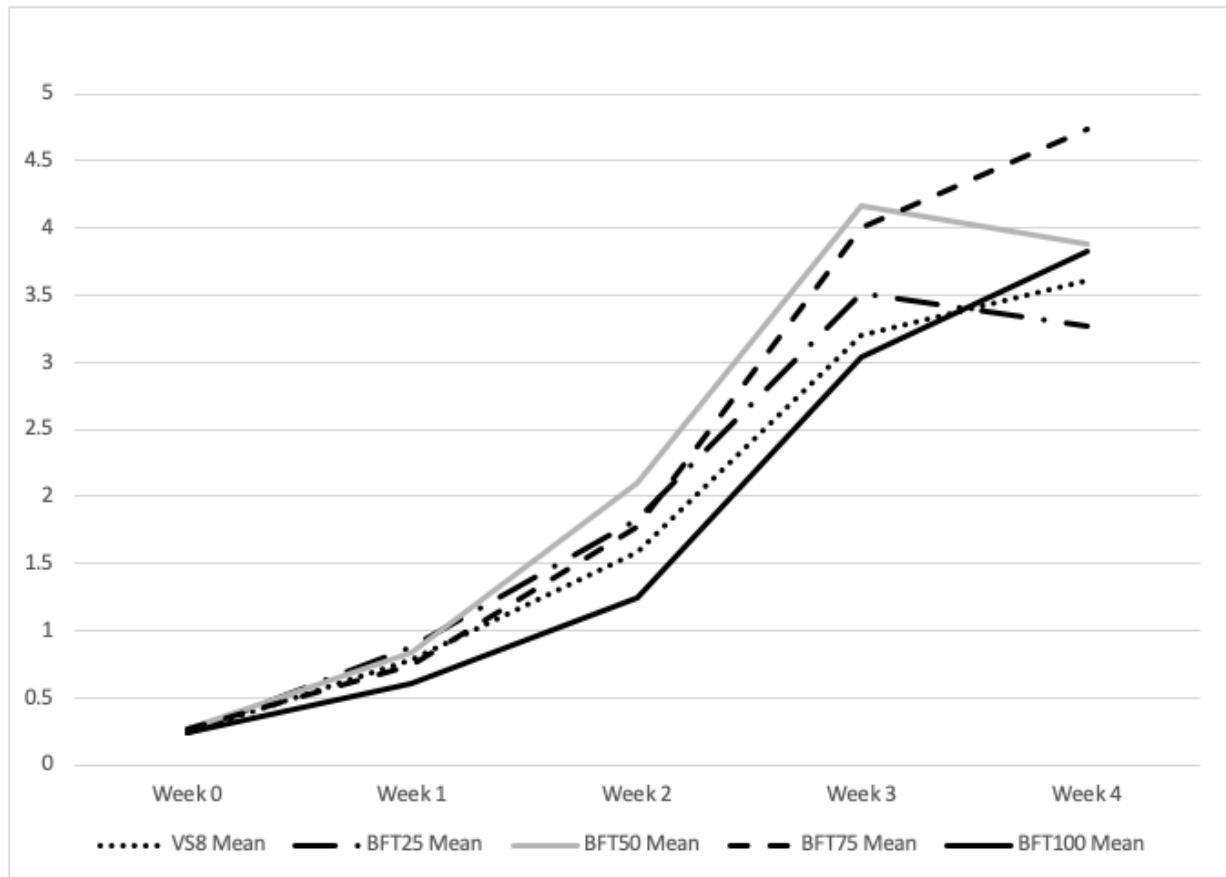


Figure 15: Weekly increase in biomass per treatment (y axis shows grams). Data are expressed as mean values of 4 replicates per treatment (n=4)

When weekly growth rates are looked at in isolation (**Figure 16**), plants in treatment [BFT25] demonstrated a mean growth rate during week 1 of 20.25 ± 3.73 % per day⁻¹ compared to plants in treatment [BFT100] which had a mean growth rate of 14.4% day⁻¹ however no statistically significant difference was found (p=0.2588). During week 2 [BFT50] had a growth at 14.08% per day⁻¹ compared to the control group [VS8] at 10.71% day⁻¹ however again the difference between treatment groups was not statistically significant (p=0.0561). During week 3, the mean growth rate of [BFT100] was 13.56% day⁻¹ and the mean growth rate observed in [BFT25] was 9.82% day⁻¹ and again the difference between treatment groups was not statistically significant (p=0.0915). During week 4,

plants in the treatment groups with the highest nutrient loads [VS8], [BFT75] and [BFT100] continued to increase in mass whilst plants in lower nutrient treatments [BFT25] and [BFT50] began to decline demonstrating 0% per day⁻¹ growth rates. Observations and photo records confirm that after 2 weeks cultivation in 1 L Erlenmeyers, the algal discs had become crowded. Most of the discs in all treatment groups had begun to accumulate near the water surface and become entangled. The degree of self-shading and abiotic stress is likely a contributing factor to the decreased growth rates. The treatments with the highest nutrient loads i.e. [VS8] and [BFT75] and [BFT100] displayed the greatest resistance to this stress and continued to grow albeit at a much reduced rate.

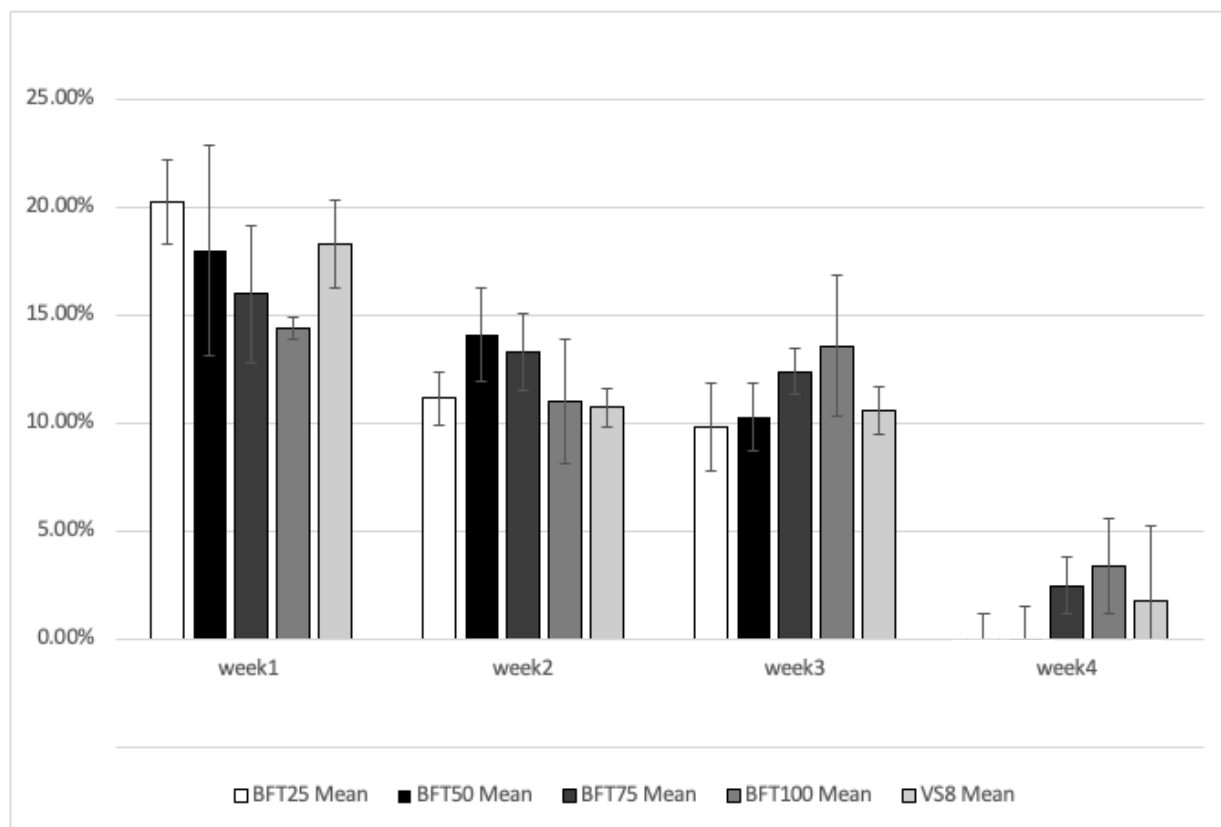


Figure 16: Specific Growth Rate (y axis shows % per day⁻¹) per treatment for each week. Data are expressed as a mean \pm standard error of 4 replicates for each treatment (n = 4).

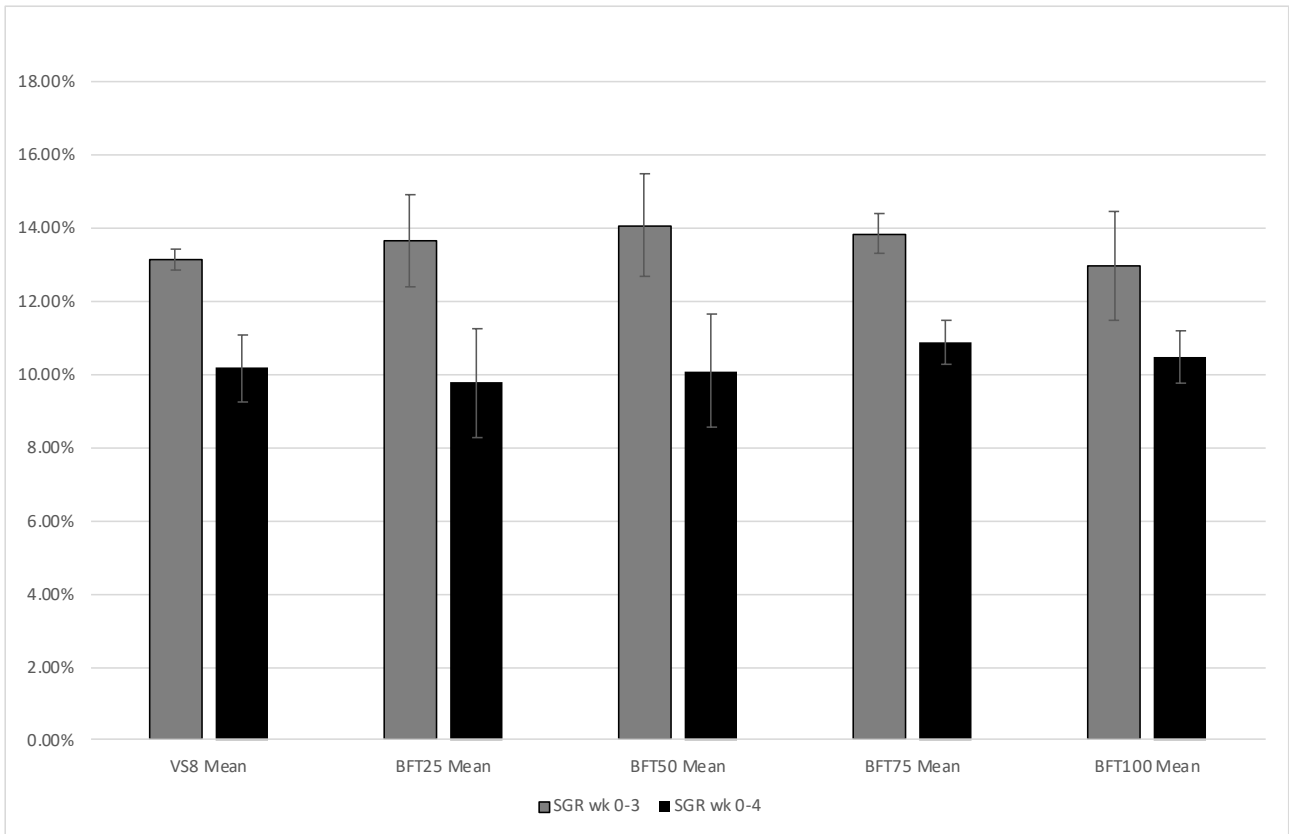


Figure 17: Specific Growth Rate (y axis shows % per day⁻¹) per treatment at the end of 3 weeks and 4 weeks. Data are expressed as a mean \pm standard error of 4 replicates for each treatment (n = 4).

The overall Specific Growth Rates (SGR) (**Figure 17**) show that after 3 weeks in cultivation, plants in [BFT50] had a SGR of 14.08% per day⁻¹ compared to a mean of 13.53% per day⁻¹ across all of the treatment groups which was not found to be statistically significant (p=0.5228) or when compared to treatment [BFT100] (p=0.5417). After 4 weeks of cultivation plantlets within [BFT75] continued to grow during week 4 when groups [BFT25] and [BFT50] declined. [BFT25], [BFT50], [BFT75] and [BFT100] performed similar to the control group [VS8] after 2 weeks or 3 weeks culture before stocking density and crowding caused a reduced rate growth rates in all treatment groups. There was no significant difference in daily SGR among groups when compared to the mean (p=0.6024). The results demonstrate that the biofloc treatments demonstrate similar high growth rates and yields similar to the [VS8] high-nutrient control group.

5.2 Experiment 2 – Growth of 19 mm Ulva discs in Biofloc Effluent

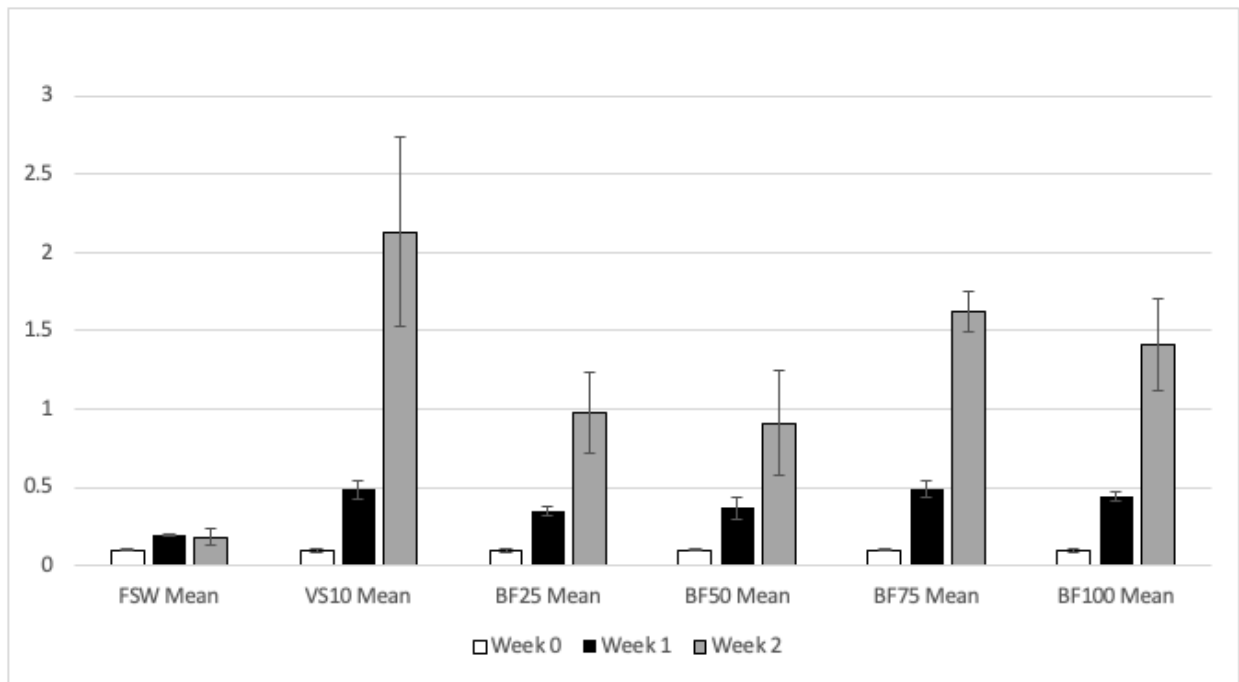


Figure 18: Weekly gains in biomass (y axis shows grams) per treatment (first 14 days culture). Data are expressed as a mean \pm standard error of 4 replicates for each treatment (n = 4).

An initial stocking density of 0.1 grams/L (± 0.003 grams) was used per treatment. After the first 7 days in culture, all treatments had increased mass with the control [VS10] and [BF75] demonstrated the largest increase in biomass with mean values of 0.4875 grams and 0.49 grams respectively compared to a mean across all treatment groups of 0.386 grams (**Figure 18**). There was a statistically significant difference between groups ($p=3.425e-06$) with the greatest difference between [FSW] and [VS10] ($p=0.0000$) and between [FSW] and [BF75] ($p=0.0001$). During week 2, [BF75] demonstrated a mean biomass of 1.62 grams and again this was not statistically different from the high nutrient control [VS10] ($p=0.2841$). It should be noted that the [FSW] control decreased in biomass by 0.0125 grams. The discs in the [FSW] control group were notably fragmented and had lost much of depth of green colour. Both control groups [FSW] and [VS10] were discontinued after this initial 2 week period.

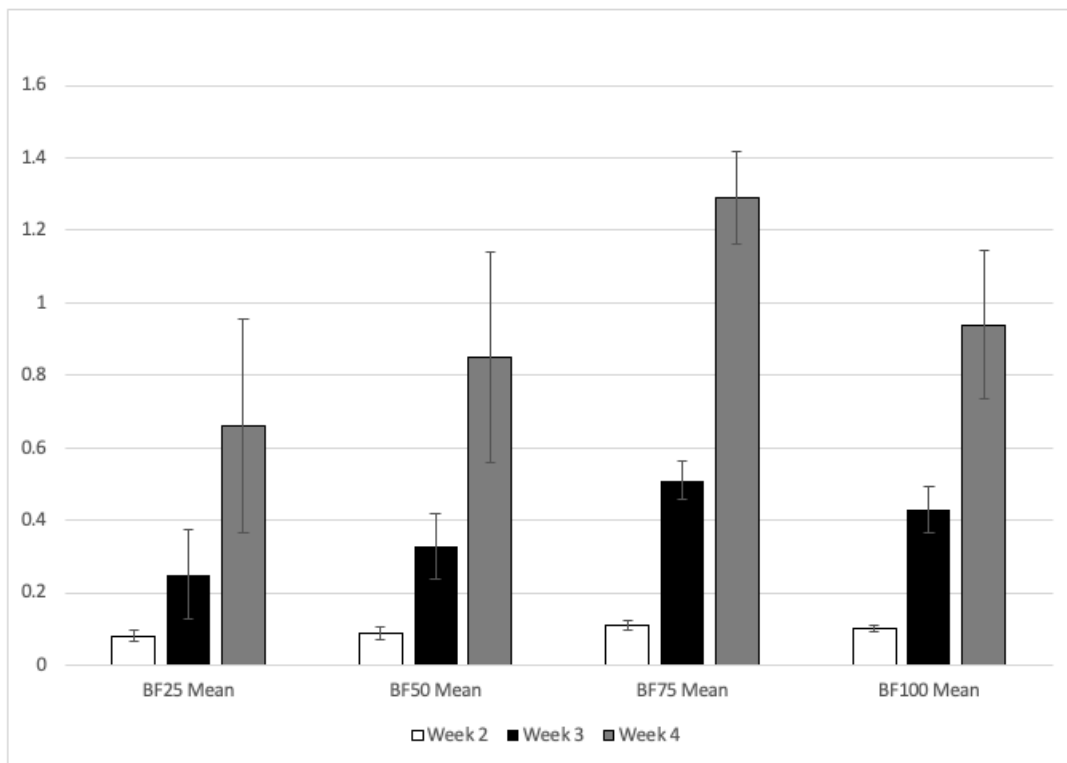


Figure 19: Weekly gains in biomass (y axis shows grams) per treatment (14 days culture after biomass reduction). Data are expressed as a mean \pm standard error of 4 replicates for each treatment (n = 4).

During regrowth period at week 3, the discs in treatment [BF75] had the greatest biomass increase with a mean mass of 0.51 grams per litre when compared to a mean of 0.377 grams among the treatments (**Figure 19**). Even with the absence of the controls, there was a statistically significant difference between the biofloc treatments ($p= 0.0059$). The greatest statistically significant difference was found between the worst performing [BF25] and best performing [BF75] treatment ($p= 0.0051$). By the end of the experiment at 4 weeks, treatment [BF75] displayed a mean biomass of 1.29 grams when compared to a mean of 0.934 grams across all the treatments although this was not statistically significant ($p=0.0192$). Interestingly, the mean values for biomass among treatment groups [BF25] and [BF50] were lower than both [BF75] and [BF100] however there was no statistically significant difference between the [BF25] and [BF75] treatment ($p=0.0133$). There were no visible problems with crowding and self-shading within the flask given the lower stocking density and shorter cultivation period.

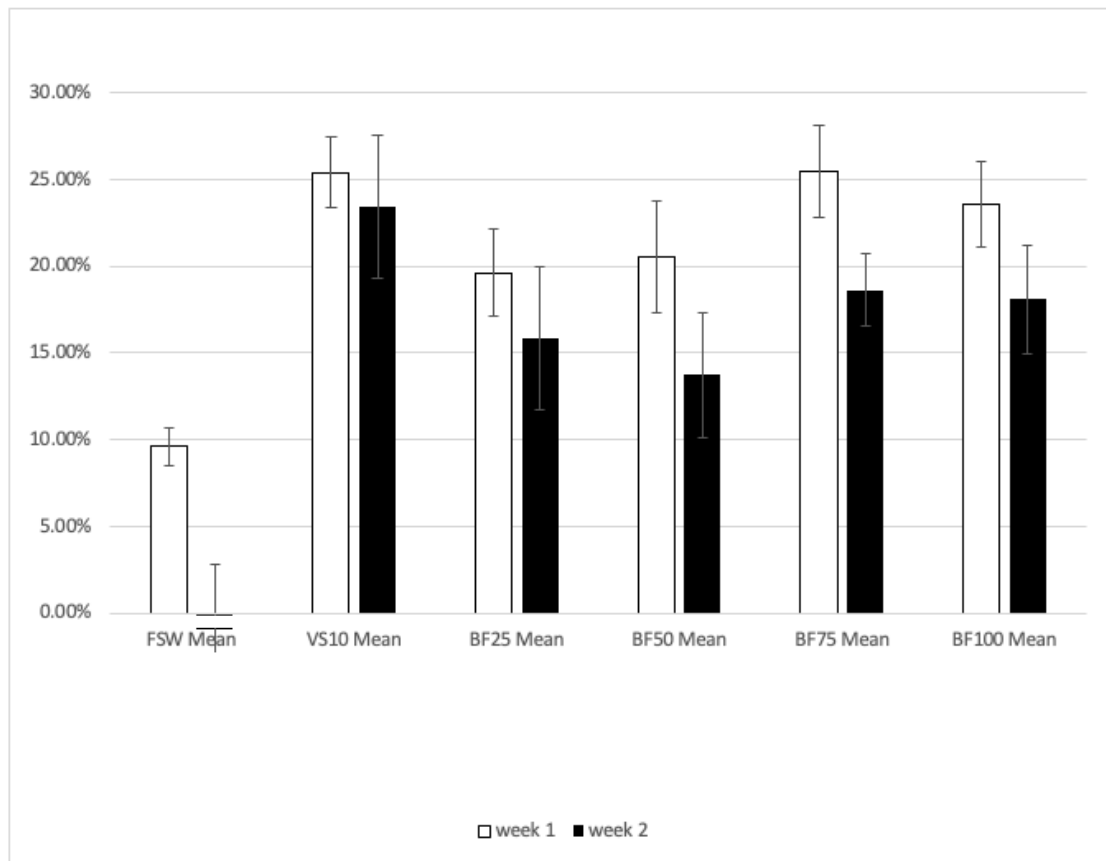


Figure 20: Specific Growth Rate for each treatment during week 1 and week 2 (y axis shows % per day⁻¹). Data are expressed as a mean ± standard error of 4 replicates for each treatment (n = 4).

When weekly growth rates are looked at in isolation (see figures 20 & 22), the [VS10] and [BF75] treatment had the highest mean values during week 1 at 25.40% per day⁻¹ and 25.49% per day⁻¹ respectively compared to treatment [FSW] which had a significantly lower growth rate of just 9.62% (p=0.0000 and p= 0.0000 respectively). There was no statistically significant difference between [BF50] and [BF75] (p=0.0747). During week 2 there was no statistically significant difference treatments [BF50] and [BF75] (p=0.2975) or between groups (p= 0.5439). The [FSW] control group demonstrated serious fragmentation of discs, and was removed from the experiment (see Figure 21).



Figure 21: [FSW] treatment displayed fragmentation and colour loss after 2 weeks of cultivation

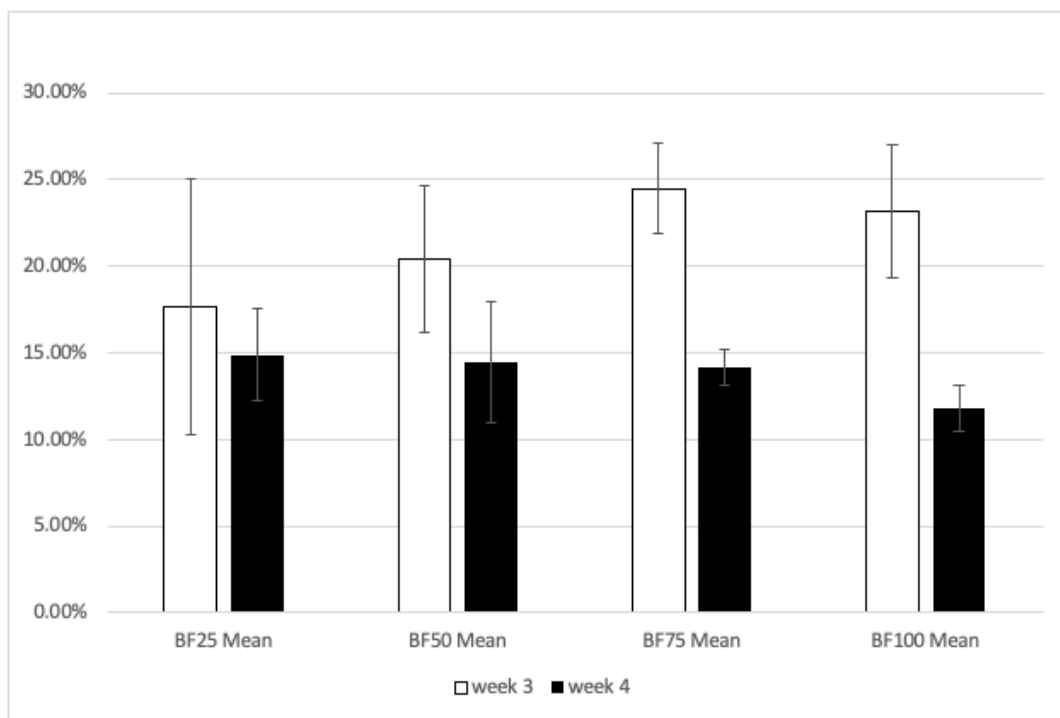


Figure 22: Specific Growth Rate (y axis shows % per day⁻¹) for each treatment during week 3 and week 4 after biomass reduction. Data are expressed as a mean \pm standard error of 4 replicates for each treatment (n = 4).

During the re-growth period at week 3, the mean growth rate of [BF75] was 24.50% per day⁻¹ and the mean growth rate observed in [BF25] was 17.68% per day⁻¹ (**Figure 22**). This was not statistically significant ($p= 0.1831$). During week 4, all treatments demonstrated a similar growth rate with a mean of 14.04% throughout all four biofloc treatments ($p= 0.1767$). The results demonstrate that the biofloc treatment [BF75] demonstrates similar high growth and yields similar to the [VS10] high

nutrient control group over short-term 2 week culture periods. These two week periods are discussed further below.

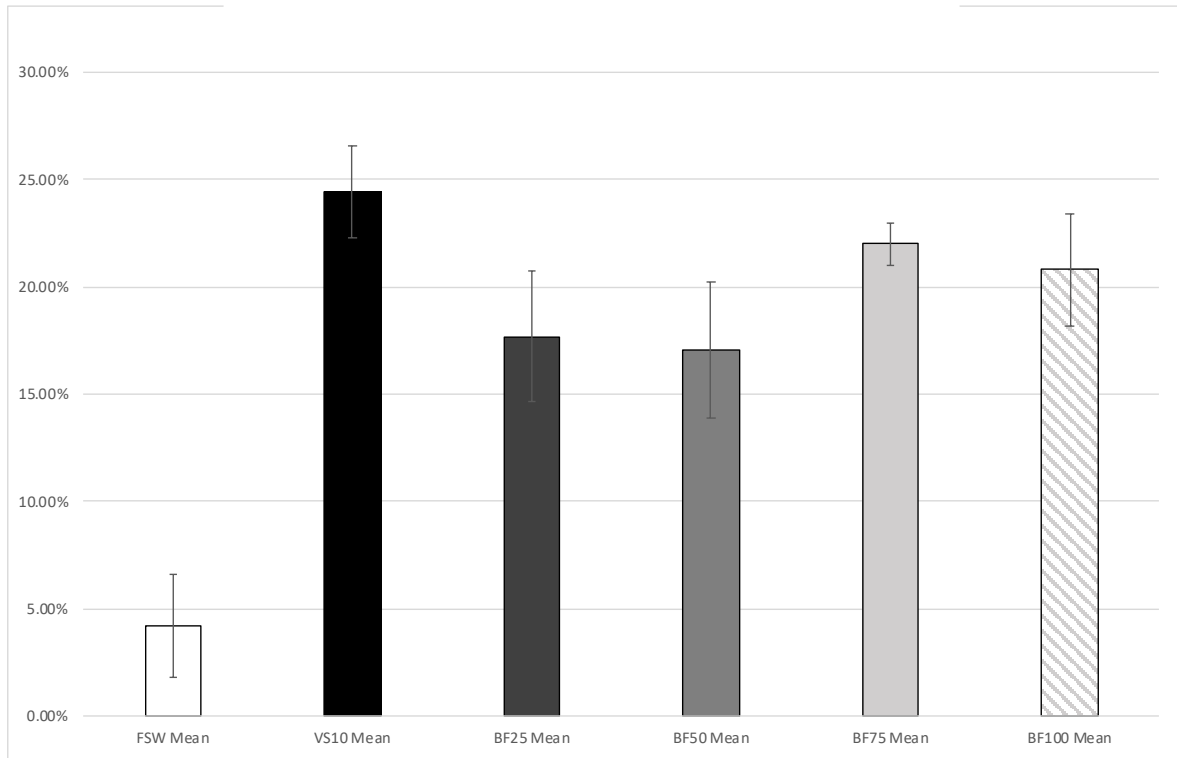


Figure 23: Mean Specific Growth Rate (y axis shows % per day⁻¹) per treatment (first 14 days culture). Data are expressed as a mean \pm standard error of 4 replicates for each treatment (n = 4).

As with experiment 1, the overall Specific Growth Rates (SGR) after the first 2 weeks in cultivation demonstrate the average daily growth during that time period. The high-nutrient control group [VS10] had the fastest mean growth rate at 24.42% per day⁻¹ whilst the filtered seawater [FSW] control group demonstrated the significantly lowest at 4.21% per day⁻¹ (p=0.0000) (**Figure 23**). [BF75] demonstrated a mean of 22.01% per day⁻¹ which compares to a mean of 17.27% per day⁻¹ across all treatment groups and which was not significant from [VS10] (p=0.6719). There was a significant difference between [VS10] and [BF50] however (p=0.0034). [BF100] also performed well with no significant difference between it and the high nutrient control [VS10] (p=0.3323) and with [BF75] (p=0.9896).

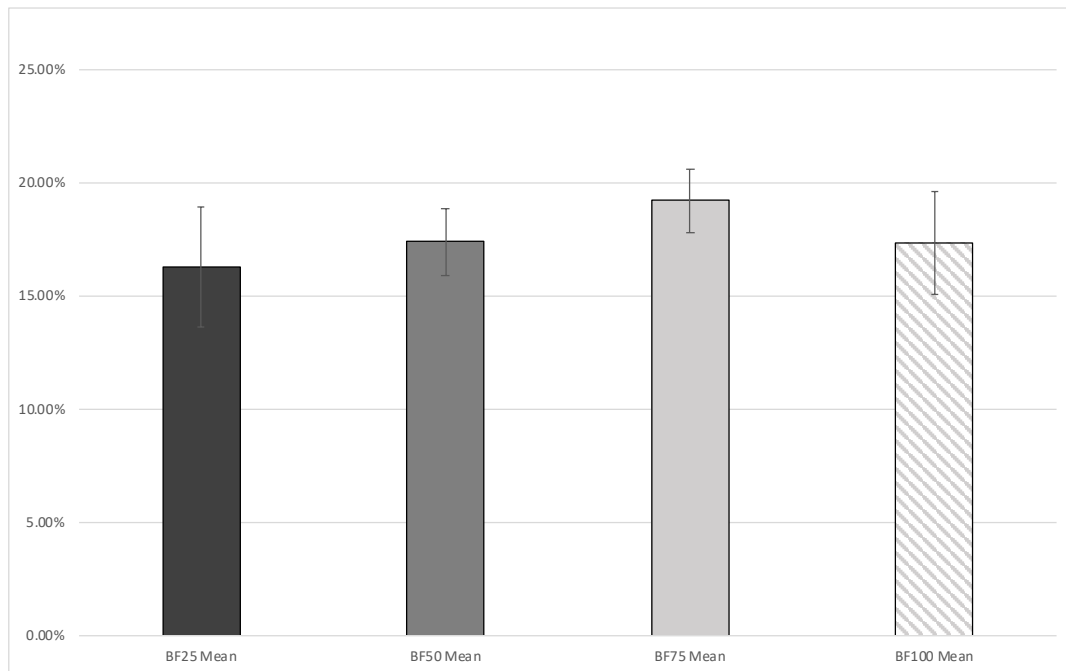


Figure 24: Mean Specific Growth Rate (y axis shows % per day⁻¹) per treatment (14 days of culture after biomass reduction). Data are expressed as a mean \pm standard error of 4 replicates for each treatment (n = 4).

After the second 2 weeks in cultivation, samples from [BF75] demonstrated the highest mean SGR at 19.23% per day⁻¹ (**Figure 24**). Overall, [BF25], [BF50] and [BF100] demonstrated similar growth rates of 16.27% per day⁻¹, 17.40% per day⁻¹ and 17.49% per day⁻¹. There was no statistically significant difference between any of the biofloc treatments over this 2 week period (p=0.2618).

5.3 Nutrient Removal:

Total Ammonia (TAN)

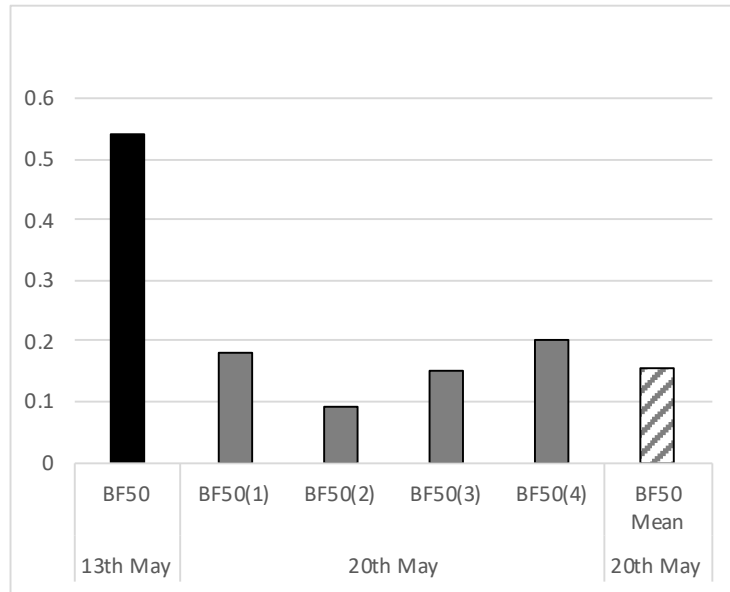


Figure 25: Total Ammonia Nitrogen (TAN) removal (y axis shows mg/L) for week 1

In week 1, Total Ammonia Nitrogen (TAN) reduced from 0.54mg/L to a mean of 0.155 mg/L representing a 71.3% reduction (**Figure 25**). In week 2 TAN reduced from 0.29mg/L to 0.218mg/L which represented a reduction of 24.83% (**Figure 26**). The X-axis presents the replicates in ().

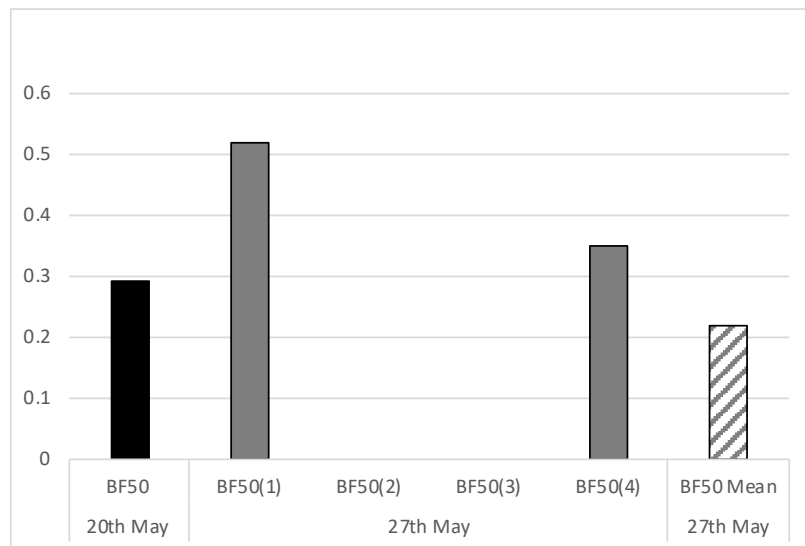


Figure 26: Total Ammonia Nitrogen (TAN) removal (y axis shows mg/L) for week 2

Interestingly, of the four replicates tested from the [BF50] treatment, the TAN levels of 2 samples actually increased from 0.29mg/L to 0.52mg/L and 0.35mg/L whereas levels in the other two replicates decreased to trace levels. A visible analysis of the discs within these treatments revealed that a greater degree of fragmentation (and perhaps sporulation) had occurred within replicate no. 1 and no. 4 with higher TAN levels (see **Figures 27** and **28** below). *Ulva* sp. have been known to release nutrient into the water medium during sporulation events and whilst appearing anomalous, these findings would be consistent with the research of Gao et al. (2018).



Figures 27 and 28 (left, right) : [BF50] replicate 1 & 4 showing signs of sporulation / fragmentation.

Nitrite

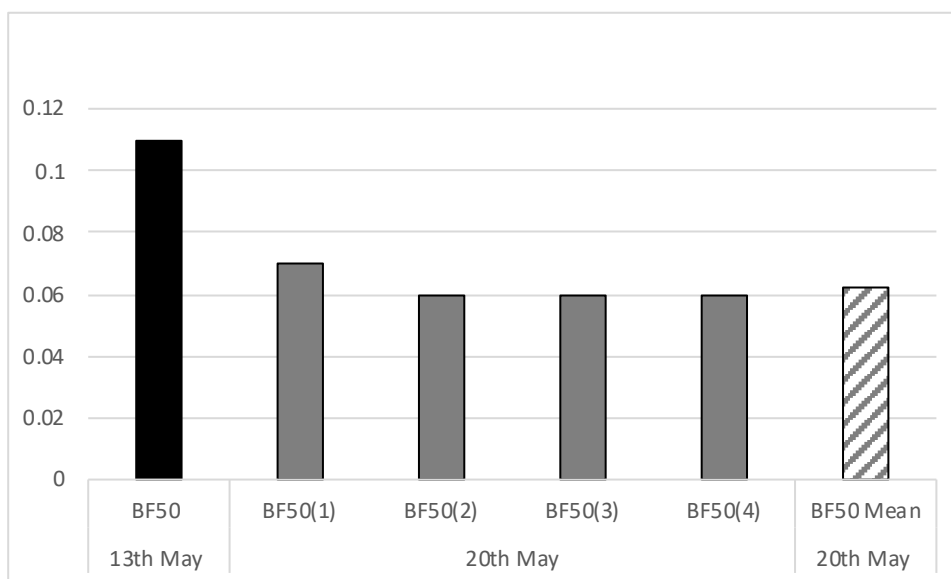


Figure 29: Nitrite removal (y axis shows mg/L) for week 1

In week 1, Nitrite reduced from 0.11 mg/L to a mean of 0.0625 mg/L representing a 42.73% reduction (**Figure 29**). In week 2 Nitrite within the flasks reduced from 0.37 mg/L to 0.265 mg/L which represented a reduction of 28.38% (**Figure 30**).

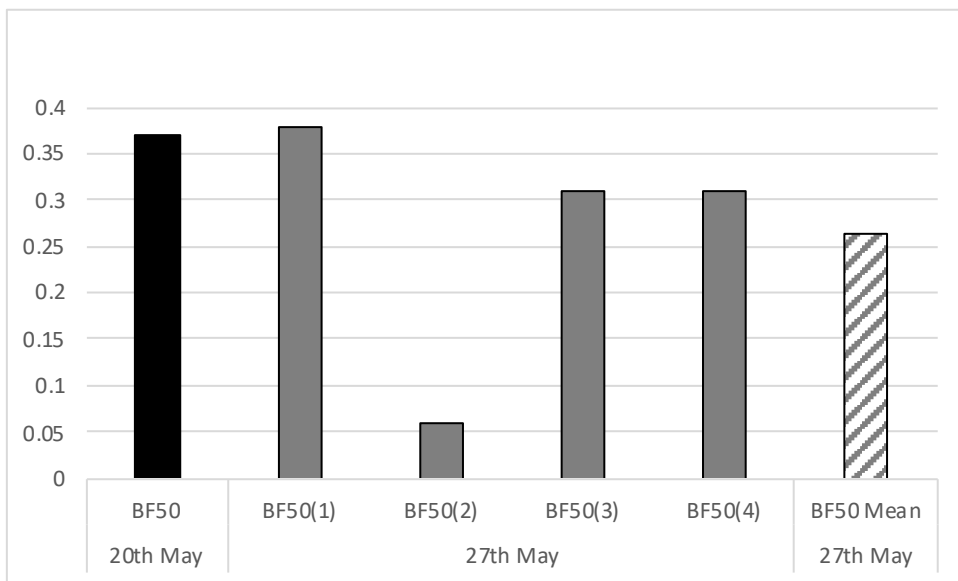


Figure 30: Nitrite removal (y axis shows mg/L) for week 2

Nitrate

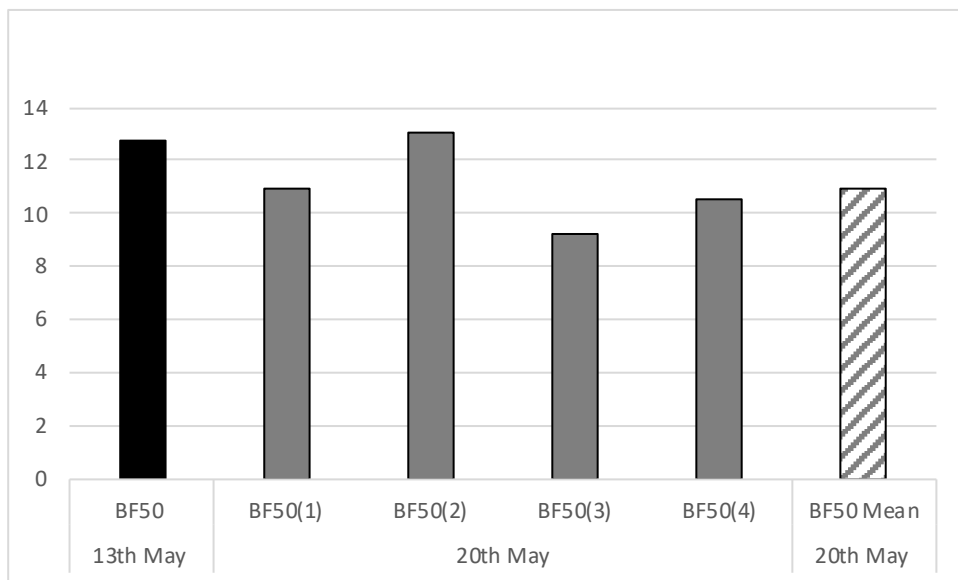


Figure 31: Nitrate removal (y axis shows mg/L) for week 1

In week 1, Nitrate reduced from 12.7mg/L to a mean of 10.93 mg/L representing a 13.98% reduction (**Figure 31**). In week 2 Nitrate within the flasks reduced from 27.1mg/L to 18.075 mg/L which represented a reduction of 33.30% (**Figure 32**).

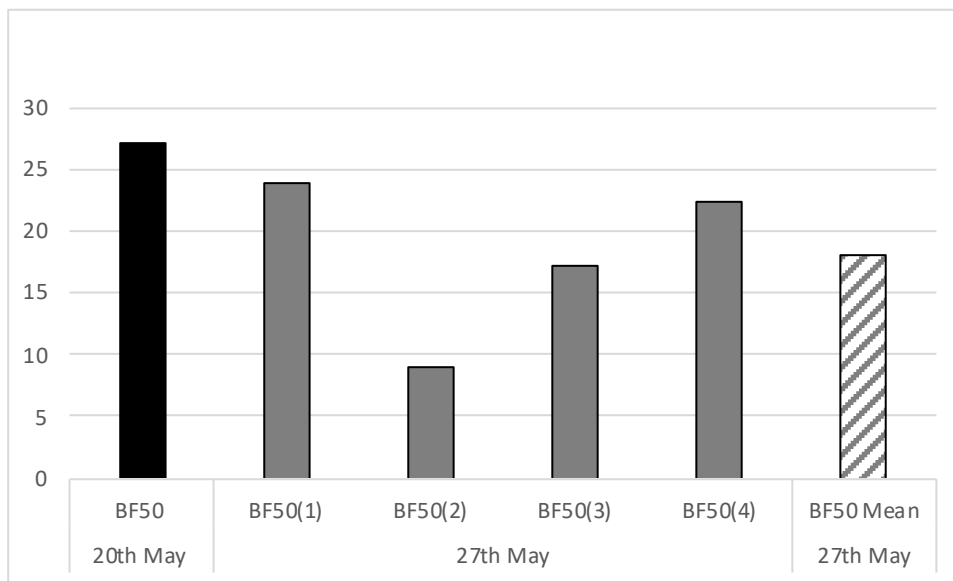


Figure 32: Nitrate removal (y axis shows mg/L) for week 2

Phosphate

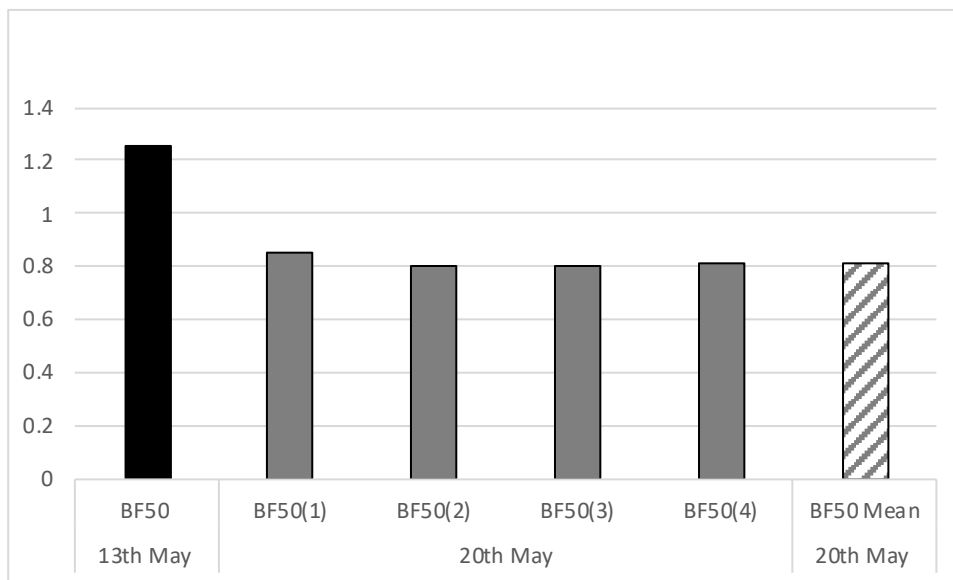


Figure 33: Phosphate removal (y axis shows mg/L) for week 1

In week 1, Phosphate reduced from 1.25mg/L to a mean of 0.815 mg/L representing a 13.98% reduction (**Figure 33**). In week 2 Phosphate within the flasks reduced from 1.9mg/L to 0.9 mg/L which represented a reduction of 52.63% (**Figure 34**).

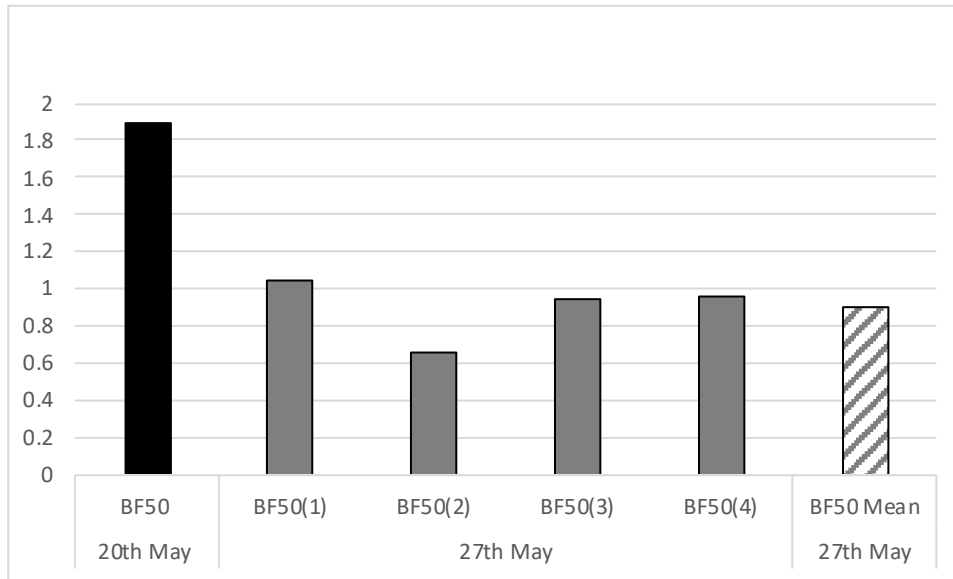


Figure 34: Phosphate removal (y axis shows mg/L) for week 2

5.4 Experiment 3: Sporulation with Nutrient and Cold Temperature Shock

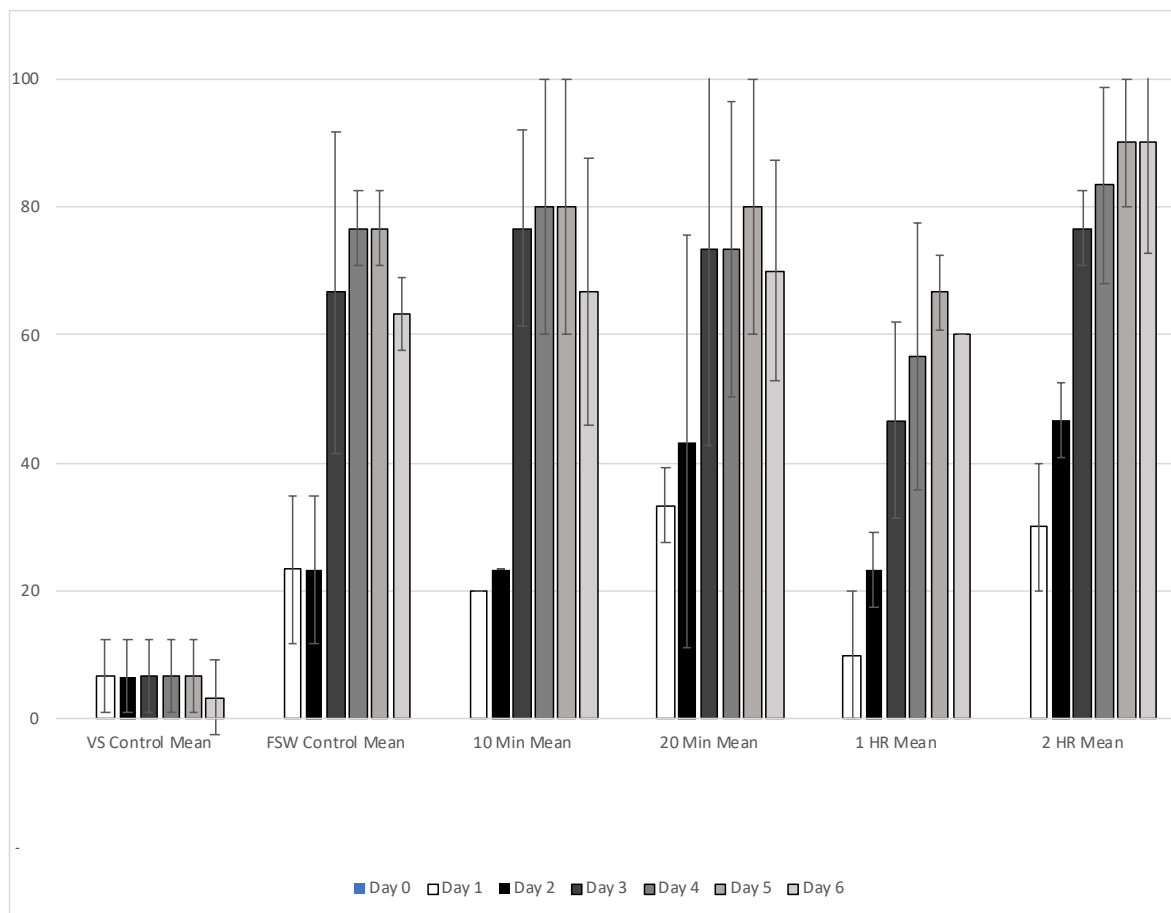


Figure 35: Percentage of Sporulated Discs per Treatment (y axis shows %). Data are expressed as a mean \pm standard error of 3 replicates for each treatment (n = 3).

In the late afternoon of day 1, 24 hours after the shock treatment, the majority of discs in all treatments demonstrated some ‘early-stage’ reproductive activity (**Figure 35, 36**). The determination phase was observed just 24 hours after shock treatment in all groups including the [VS Control], probably due to the effect of fragmentation i.e. the cutting into discs and the subsequent loss of sporulation inhibitors from within the thallus tissue into the surrounding medium.

The examination on day 1 revealed that mean value 6.66% of discs in the [VS Control] group were in the swarming phase. This was characterised by cells which are visibly clear due to the discharge of gametes. This stage is regarded as a positive result in terms of sporulation (**Figure 12**). Care was taken to ensure that discs which had predominantly cells in determination stage and which were visibly half-filled or partially clear in appearance were not counted as a positive result (**Figure 11**). Cells which were in the intermediate stage i.e. differentiation stage and which were full of partially or fully-formed gametes were easily recognised as such and also discounted. The [FSW Control] had a mean value of 23.33% reproductive output which was similar to the [10 min] treatment which had

20%. The [20 min] and [2 hour] treatment had 33.33% and 30% sporulation respectively whilst the [1 hour] treatment had the lowest of all the treatments with 10%. The difference between the treatments was statistically significant ($p=0.0099$). The [20 min] treatment demonstrated a significantly higher output at 33.33% when compared to the [VS control] at 6.66% ($p=0.0169$).

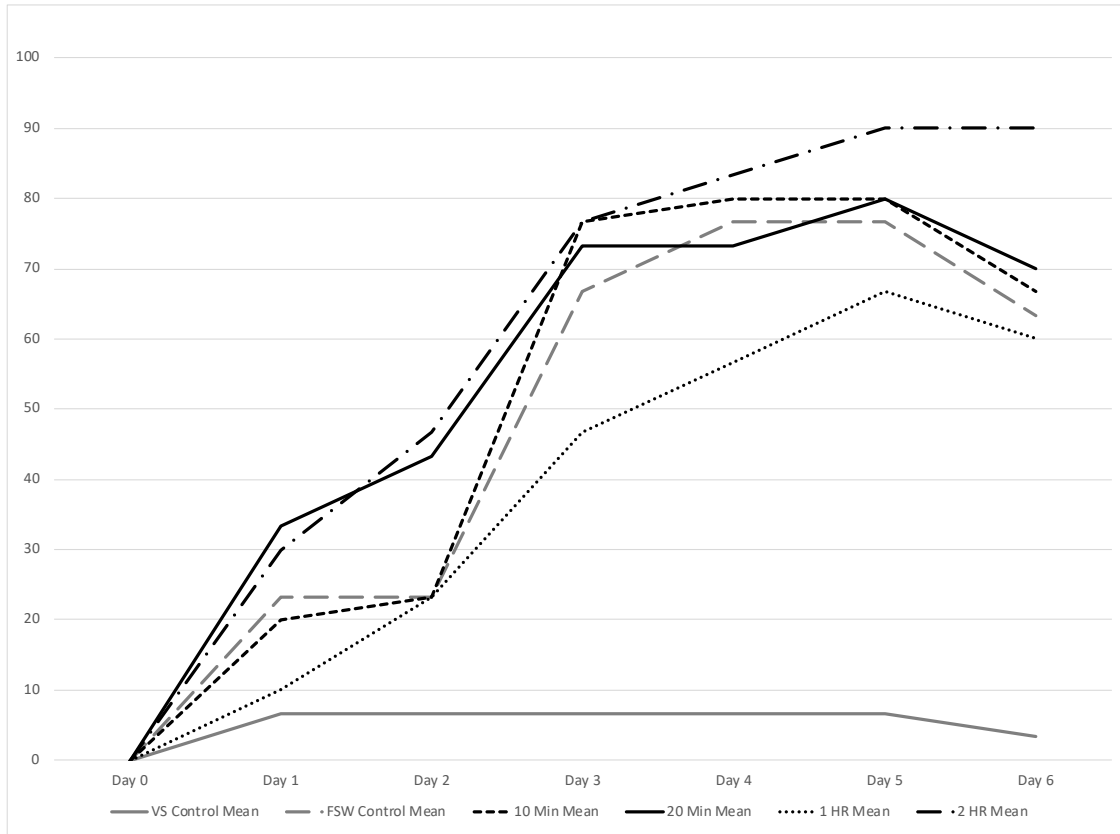


Figure 36: Percentage of sporulated discs per treatment (y axis shows %).

On the evening of day 2, the [VS control] and [FSW control] reproductive output remained unchanged with 6.66% and 23.33% respectively. The [10 min] treatment increased to 23.33%. More pronounced increases in sporulation were observed in the [20 min], [1 hour] and [2 hour] treatments with mean values of 43.33%, 23.33% and 46.66% respectively. The ANOVA confirmed the differences in sporulation amongst treatments were significantly different ($p=0.0439$) with the greatest difference between the [VS control] and the [2 hour] treatment ($p=0.0489$). On the evening of day 3, a rapid increase in sporulation was observed in the [FSW control] and the [20 min] treatment with 66.66% and 76.66% respectively with more gradual increases within the longer shock treatment groups. The treatments were significantly different from the mean ($p=0.0039$) with the greatest difference observed between [VS control] and the most reproductive [20 min] and [2 hour] treatments ($p=0.0064$). Day 4 showed similar statistically significant differences ($p=0.0009$) with the greatest difference again found between the [VS Control] and [2 hour] treatment ($p=0.0012$) with 6.66% and 83.33% respectively.

Five days after the shock treatment, a significant difference again was found between the [VS control] and [2 hour] treatment ($p= 0.0001$) with 6.66% and 90% respectively. The ANOVA analysis found no statistically significant difference between the [FSW control] and the [10 min], [20 min] or [1 hour] treatment ($p=0.9861$ in all cases) or indeed with the [FSW Control] and [2 hour] treatment ($p=0.6243$). This suggests that the nutrient shock derived from transfer from the high nutrient acclimation conditions (FSW+VS10mL/L) to standard FSW during the experiment is sufficient to give a 76.66% reproductive output on average and that this is not significantly different from the 90% output in the 2 hour cold shock treatment.

By day 6, some unusual observations were recorded whereby reproductive output appeared to have reduced. The [VS control] had reduced from 6.66% to 3.33% and indeed reductions in all other groups were witnessed with the exception of the [2 hour] treatment which remained at 90%. During the examination, it was obvious that the original 9 mm discs had grown in size over the course of the 6 days of observations despite the cutting and shock treatment. The new areas of cells with vegetative growth reduced the relative percentage of sporulated cells to the extent that discs with marginally more than 50% sporulation (counted as a positive result) experienced new growth meaning that the relative area of sporulation had become less than 50%. By the end of the experiment at day 6, the greatest difference in reproductive output was found between the [VS control] and [2 hour] treatment with 3.33% and 90% respectively ($p= 0.0001$). However as above, the ANOVA analysis of results at day 6 suggest that the nutrient shock derived from transfer from high nutrient acclimation conditions (FSW+VS10mL//L) to standard FSW is sufficient to give a 63.33% reproductive output on average and that this is not significantly different from the 90% output in the 2 hour cold shock treatment. ($p= 0.2260$).

The results demonstrate that fragmentation alone caused by cutting the tissue into 9 mm discs caused 6.66% sporulation in the [VS control]. The impact of nutrient shock from FSW+VS10mL/L acclimation culture medium to standard FSW with no cold shock caused a mean of 76.66% sporulation after 5 days. The impact of temperature shock and nutrient shock from FSW+VS10mL/L acclimation to FSW with cold shock varied also, with shorter temperature shocks causing an initially slow reproductive response which peaked on day 4 or 5 and a more gradual daily increase response experienced in the treatments with a longer temperature shock. Overall, the [2 hour] treatment demonstrated the highest mean value for reproductive output at 90% on day 5 however this was not significantly higher than the 'nutrient shock-only' [FSW control] treatment ($p=0.2260$).

6.0 Discussion

6.1 Experiment 1 – Growth of 29mm *Ulva* discs in Biofloc Effluent

Analysis of the growth rates during the first experiment demonstrate that biofloc effluent supported rapid *Ulva ohnoi* growth over the 4 week cultivation period. The maximum specific growth rate (SGR_{max}) among [BF25] was 20.25 ± 3.73 % per day⁻¹ during week 1, and the recorded mean SGR for [BFT50] after 3 weeks was 14.08% per day⁻¹ which is at the higher end of the results spectrum when compared to other studies. Favot et al., (2019) cultivated *Ulva sp.* in outdoor ponds for a shorter cultivation period of 6 – 15 days and reported an SGR of 17 % per day⁻¹ (see **Table 2 below**).

6.1.1 Biomass Increase

The initial 0.26 grams of *Ulva sp.* in [BFT75] treatment produced 4.74 grams of dry biomass after 4 weeks cultivation which represents an increase in biomass by 1,723% or 4.48 grams. This is a remarkable yield from just 4 Litres of medium (comprising 750 mL of Biofloc effluent and 250 mL FSW per L). Commercially prosperous *Ulva* production rates have been estimated to be 20–26 g dry m⁻² day⁻¹ over a full year from pond raceway systems in South Africa (Bolton et al, 2009, 2016). Whilst the results demonstrate good growth potential, further experimentation with longer term grow out period would be necessary in order to evaluate the commercial viability of the species and make more meaningful comparison with other studies and commercial productions.

Species	System	Stocking Density (WW)	SGR (% per day)	Reference
<i>Ulva sp.</i>	Flask	0.25 g/L	20.25 (BFT25 during week 1)	This study (experiment 1)
<i>Ulva sp.</i>	Flask	0.1 g/L	25.49 (BF75 during week 1)	This study (experiment 2)
<i>Ulva sp.</i>	Earth Pond	0.006 – 0.015 kg/m ²	17	(Favot et al, 2019)
<i>Ulva sp.</i>	Tank	1-8 kg/m ²	10-1	(Bruhn et al, 2011)
<i>Ulva lactuca</i>	Ropes, sea	0.0005* kg/m ²	11.95	(Castelar et al, 2014)
<i>Ulva sp.</i>	Tank	0.5* kg/m ²	22.8	(Castelar et al, 2014)
<i>Ulva clathrata</i>	Tank	0.2-0.5 kg/m ²	7	(Copertino et al, 2009)
<i>Ulva lactuca</i>	Tank (continuous aeration)	0.8 kg/m ²	13.3	(Ben-Ari et al, 2014)
<i>Ulva lactuca</i>	Tank (25% aeration)	0.9 kg/m ²	8.1	(Ben-Ari et al, 2014)

Table 2: above adapted from Favot (2019) ‘Production of *Ulva sp.* in Multitrophic Aquaculture in Earth Ponds’

6.1.2 Optimum Growth & Crowding, Plant Age & Morphology

The observed growth characteristics and flotation behaviour *in vitro* reasonably correspond with the species' behaviour and spatiotemporal growth patterns observed in their natural pond habitat next to the UFSC shrimp laboratory. Due to the confined space at the water surface within the Erlenmeyer, and the limited surface area for the discs to settle, it is likely that an alternative culture vessel with a larger surface area to volume ratio would support significantly greater stocking density and perhaps increased growth rates. Further experimentation with 30 L tanks at the UFSC laboratory is therefore recommended. Notably, many authors (Favot et al., 2019; Bruhn et al., 2011; Castelar et al., 2014; Ben-Ari et al., 2014) report stocking density in terms of grams or kilograms per square metre (m²) and not per cubic metre (m³) (see **Table 2**).

Whilst the results indicate that the treatments with the highest nutrient loads i.e. [VS8] and [BFT75] and [BFT100] displayed the greatest resistance to crowding/ stress, the high degree of variance between replicates (**Figure 14**) suggests that other factors had influenced growth and development. Genetic factors should be excluded because all discs within all treatments were excised from the same sporophyte (2*N*) plant. It is probable that the older areas of thallus tissue closer to the holdfast had an increased concentration of Sporulation Inhibitors (SI), which form an internal vertical concentration gradient between the two blade cell layers (Wichard & Ortel, 2010). Therefore excised discs with a greater proportion of 'older' tissue may have had growth performance advantage due to the SI's role in minimising the degree of transformation from vegetative to reproductive state and energy-intensive sporulation events. On the other hand, vegetative cells within younger parts of thallus tissue may have a metabolic advantage and therefore better growth performance in the presence of high nutrient. Excised discs with a greater quantity of younger thallus cells might benefit from higher cellular (and photosynthetic) efficiency and ability to construct amino acids and proteins essential for plant growth. This would be consistent with *Ulva*'s highly opportunistic nature as an 'r-selected' species, which grows rapidly during its relatively short life cycle (**Figure 2**). Whilst care was taken to randomise the selection of discs for each treatment and replicate, an uneven distribution of discs (according to tissue age) may have occurred. The 'older' areas of *Ulva ohnoi* tissue are extremely difficult to identify due to the species' non-linear morphology. The broad, 'lettuce shaped' blades of plants taken from natural habitats (or acclimation conditions) are typically torn/ fragmented and do not always present identifiable, differentiated holdfast tissue which would help indicate the oldest areas of thallus tissue. Observations during this study revealed that *U. ohnoi* tissue does not grow in either a single linear dimension, as is the case with the 'ribbon-shaped' blade species such as *Ulva intestinalis* which presents with clear distinction, allowing easy identification of older basal and younger apical parts. Nor does the thallus grow in a predictably even, outwardly concentric pattern.

In practice, it would not be feasible for a commercial aquaculture operation to select for younger (or older) parts of sea lettuce thallus tissue in any event, however further experimentation with identified older and younger plant tissue might reveal growth performance biases and inform strategies for optimum production cycles and harvesting. As far as the author is aware, no such studies exist in the literature for the study species. It is recommended that future experiments should use a greater number of replicates in order to improve the experimental design.

6.1.3 Limiting Factors:

Dissolved Oxygen (DO) concentration and pH were not measured, ruling out the possibility to evaluate if oxygen expulsion and CO₂ supply (or carbon limitation) were important contributions of aeration to the growth performance of *Ulva* sp. in the flasks.

The surprising lack of significant differences found in the SGR of plants between treatments [BF25] and [BF75] or [BF100] (which have significantly higher nutrient budgets) suggests that light may have been a limiting factor, especially in the higher nutrient treatments. Whilst Rosenberg and Ramus (1982) found no photosynthetic limitation of photosynthesis in *Ulva* between 35 and 80 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ the relatively high nutrient loads and dissolved organic matter properties of the biofloc require further enquiry. The UFSC laboratory irradiances used in the present study of 80 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ are considered to have been sufficient for good *Ulva* growth, however further experimentation with higher irradiances and nutrient analysis of all treatments over a longer time frame would be useful. It is noted that the author Xiao et al (2016) cultivated *Ulva prolifera* at 100 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ and did not report adverse irradiance-induced effects.

Whilst no spectrophotometry data is available, it is considered that the darker coloration of the [BFT75] and [BFT100] medium was caused by higher levels of Chromatic Dissolved Organic Matter (CDOM) (**Figure 37**). CDOM, also known as ‘yellow substance’ refers to a complex group of soluble organic compounds (Nima et al., 2019) which give water a darker coloration due to tannins released from organic matter. Studies have shown that CDOM absorption in the visible spectral region reduces the availability of photosynthetically active radiation (PAR) for photosynthesis and limits primary production in the ocean (Arrigo & Brown, 1996). Whilst the author has found no comparative studies for macroalgae *in vitro*, it is probable that the combined effect of plant self-shading due to crowding within the flask, as well as reduced PAR due to high levels of CDOM more affected growth performance in the higher nutrient groups adding a further dynamic to the light limitation mentioned above.

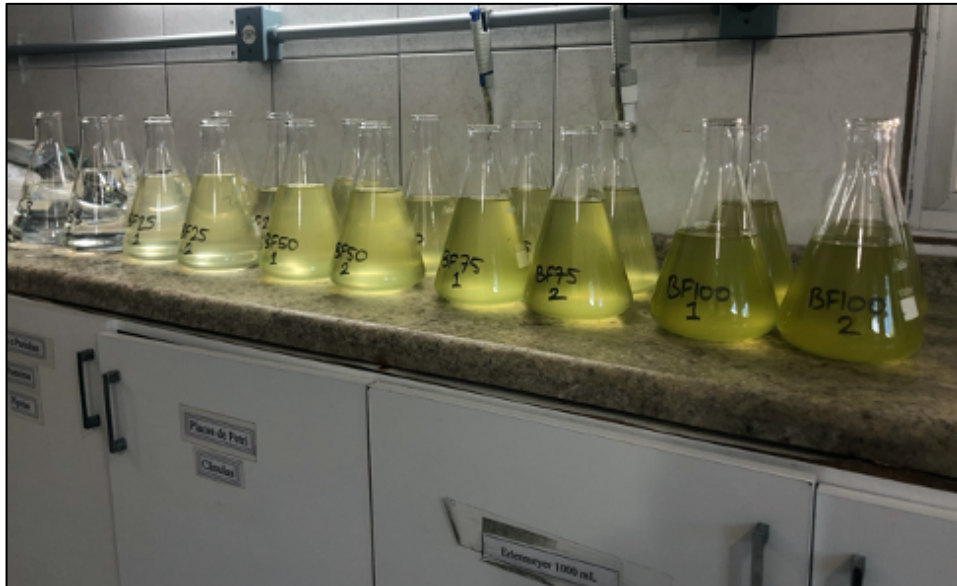


Figure 37: Photo of Treatments and varying coloration. Pure water absorbs longer wavelengths red light, whereas CDOM absorbs short wavelength light ranging from blue to ultraviolet (Nima et al. 2019). As such, water with little or no CDOM such as standard FSW or the [VS8] control appeared clear or blue whilst biofloc treatments with higher levels of CDOM were presented as green or brown.

6.1.4 High Salinity Tolerance

In general, the species performed well despite the significantly higher salinity of the Biofloc treatments (from 35 ppm for the [BFT25] to 45 ppm for the [BF100] group) when compared to the salinity of 28 ppm which was measured in the natural habitat of the laboratory pond, and in comparison to the salinity of 26 ppm in the [VS8] control. During the experiment, none of the typical water quality parameters such as pH, salinity and nutrient loads were adjusted (by adding water for example) as might be expected in the culture of fish or shrimp. The initial salinity was dictated by the initial Biofloc concentration with [BFT100] having the highest salinity and [BFT25] and the [VS8] control having lowest. Approximately 80 mL of medium evaporated from the culture flasks every week and as such salinity in each flask increased marginally by 2-3 ppm by the end of each 7 days in cultivation, by the time growth measurements were taken and the media was changed.

The results demonstrate that the biofloc treatments [BFT50] and [BFT75] produce high growth rates and yields similar to the [VS8] high-nutrient control group. Biofloc effluent can support prolonged vegetative growth in very high nutrient (0.54 mg/L TAN, 27.1 mg/L Nitrate) and high salinity (45 ppm) without significantly disruptive sporulation and reproduction events. Space (adequate surface area) for discs to settle on the water surface is a highly limiting factor. From a production point of view, it appears that *Ulva ohnoi* could be cultivated directly in high salinity (40 ppm) within a shrimp or fish culture tank containing 100% Biofloc effluent. However, better growth performance and yield would be expected from cultivating at a stocking density below 3g/L in separate tanks at 50-75%

concentrations as part of an Integrated Multi Trophic Aquaculture (IMTA) system or within separate bioreactors for water bioremediation.

6.2 Experiment 2 – Growth of 19mm Ulva Discs in Biofloc Effluent

6.2.1 Colour Loss & Fragmentation

The main interest in modelling opportunistic macroalgae such as sea lettuce lies in the possibility to predict and quantify their rapid growth capacities and the potential impacts on their surrounding environment (Lavaud et al, 2020). Analysis of the growth rates of the 19mm discs demonstrated rapid growth within all Biofloc treatments over both the initial 2 weeks and the subsequent ‘post-harvest’ 2 week culture period. Interestingly, the SGR in the [FSW] control treatment declined after the initial 2 weeks and the contents were discarded due to the fragmentation and colour loss (**Figure 21**). It is assumed that the low nutrient shock accrued from transfer of the study plants from high nutrient (FSW+50% Biofloc effluent) in the acclimation tanks, to the low nutrient FSW used in the [FSW] experimental control caused a degree of sporulation and fragmentation. The loss of colour might be attributed to the loss of chlorophyll. Nitrogen enrichment increases N-based tissue compounds (e.g. amino acids, phycobiliprotein and chlorophyll), thereby increasing photosynthetic activity and growth (Roleda & Hurd, 2019) and so the reverse was observed in this experiment. In general, diminished photosynthetic activity due to diminished N in the FSW media and depletion of carbohydrate reserves for cellular respiration were likely to be responsible for the low growth observed. No spores were observed in the culture medium however, some areas of reproductive tissue were visibly distinguishable from the disintegrated biomass. In the later part of this experiment the [VS10] control was discontinued due to human error meaning both controls were lost.

6.2.2 Somatic Cellular Division

Increased algal growth and biomass is a product of somatic cell expansion and /or cellular division. The somatic cells of *Ulva* have been reported to divide synchronously under standardized conditions once a day (Wichard et al, 2015). The maximum mean standard growth rate observed in the [BF75] treatment during its first week of cultivation was 25.49% per day suggesting that approximately 1 in 4 somatic cells divided every day (or alternatively, that all cells divided on average every four days). This rate is comparative to the studies in **Table 2**, however considerably slower than observed by Hiraoka et al, (2020) who when studying the thin ‘ribbon’ blade *Ulva meridionalis* reported a 4.1-fold daily increase, suggesting that all the cells divided at least twice a day. The known bloom-forming species *Ulva prolifera* is known to grow at an approximately two-fold growth rate per day in optimum conditions (Liu et al, 2013). Similarly, two consecutive cell divisions per day have been reported in microscopic *Ulva* germlings for the early developmental stage during a single dark phase

under ordinary light: dark cycle conditions (Kuwano et al, 2014). From an industrial and commercial point of view, the very high growth rate of *Ulva ohnoi* are consistent with other studies suggesting its value for both algal bioremediation and biomass production (Bolton et al., 2016).

6.2.3 Nutrient Levels

Within biofloc systems, there are three common methods for ammonia removal in aquaculture water: photoautotrophic algal assimilation, chemoautotrophic bacterial nitrification (CBN), and heterotrophic bacterial assimilation (HBA) (Ebeling et al., 2006). Heterotrophic bacteria convert ammonia directly and quickly to bacterial protein without producing NO_3^- (Ebeling et al., 2006). Biofloc technology (BFT) acts by manipulating the carbon/nitrogen ratio (C/N, w/w) to obtain values of 10 - 20 to stimulate the dominant growth of heterotrophic bacteria and assimilate ammonia, aerate to form bioflocs and suspend them in the water column (Hargreaves, 2013). At the UFSC shrimp laboratory, this is achieved by adding dextrose to the culture tanks. In traditional aquaculture, the deamination of metabolic proteins produces ammonia, which represents 70–95% of the total excreted nitrogen (Bartoli et al. 2008), thus constituting the main nitrogen waste however this is not the case in biofloc systems where nitrifying bacterial proliferate and nitrate accumulates. The water analysis undertaken in this study confirmed values of 0.29 and 0.54 mg/L of TAN and significantly higher values of 12.7 mg/L and 27.1 mg/L of nitrate. No bacterial analysis of the biofloc was possible however and further studies to identify Algal Growth & Morphogenesis Promoting Factors (AGMPFs) within the cultivation media are recommended, particularly where studies of spore development are concerned.

6.2.4 Nutrient Release from Sporulation

A visible analysis of the discs within these treatments revealed that a greater degree of fragmentation (and perhaps sporulation) had occurred within replicate no. 1 and no. 4 with higher TAN levels (see **Figures 31** and **32**). *Ulva* sp. have been known to release nutrient into the water medium during sporulation events and whilst appearing unusual, these findings would be consistent with the research of Gao et al. (2018). Gao observed that a higher cultivation temperature led to a negative nutrient uptake when reproductive events occurred, indicating that thalli of *Ulva rigida* were releasing rather than absorbing nutrients from the seawater. The reasons for this may be twofold. When thalli release spores, the nutrients in the cell could also be discharged to the seawater. Meanwhile, the decomposition of debris after spore release also contributes to the increase in nutrients in the seawater. This is supported by studies in which the seawater was enriched with nitrate and phosphate when macrophytes were decomposing (Hanisak, 1993, Gao et al., 2013). Gao suggested furthermore, that the higher temperature could increase the decomposition rate and thus the nutrient release rate

(Hanisak, 1993; Da et al., 2014), which may explain the recorded increase in TAN levels identified in the present study. There is of course the possibility of inaccuracy in the water analysis due to the small testing sample size of 1 sample per replicate ($n=1$), and the possibility of uneven distribution of nutrient within the medium. Notwithstanding, the findings suggest that *Ulva* species can actually be a source of nutrients when they are reproducing and disintegrating, which has implications for the biofilter efficiency of *Ulva* in any proposed bioremediation system. Maintaining a long-term vegetative state is therefore critical for promoting the biofiltering efficiency of *Ulva* species.

6.2.5 High TAN Preference

With regard to the correlation between disc growth rate and specific TAN or nitrate nutrient uptake, the data is too limited to draw solid conclusions and perform any statistical tests. During week 1 when TAN was highest (0.54 mg/L) and nitrate was lowest (12.7 mg/L) in the culture medium, a higher growth rate of 20.55% per day⁻¹ was observed amongst plants in the [BF50] treatment group. During week 2 when TAN was lowest (0.29mg/L) and nitrate levels were highest (27.1 mg/L), the daily growth rate of 13.72% per day⁻¹ was observed. It should be noted that the [VS8] and [VS10] von Stosch nutrient-enriched media are solely nitrate based (**Table 1**).

The above TAN preference would be consistent with the observations of Ale (2010) who noted that *Ulva lactuca* grew faster with ammonium than with nitrate as the nitrogen source, with the additional finding that the uptake of ammonium was faster than the nitrate uptake. This difference is probably due to the ammonium being presented in a reduced state which can easily be assimilated and directly utilized by algae for the synthesis of amino acids and protein resulting in cell and tissue growth (Doran, 1995). Nitrate, however, must first be reduced to nitrite and then to ammonium in order for the algae to utilize this nitrogen source. According to Lara et al. (1987) the reduction of nitrate to nitrite is catalyzed by the nitrate reductase enzyme that usually uses two ferredoxins as electrons donors while reduction from nitrite to ammonium is catalyzed by the nitrite reductase, a process that needs six ferredoxins. This reduction mechanism as a consequence requires energy for the bioconversion process. Thus, *Ulva lactuca* growth responses are different given the same concentration of nitrate nitrogen as they would on ammonium nitrogen. The present study with *Ulva ohnoi* suggests similarities with the species *Ulva lactuca*.

Although nitrate is much less toxic than either ammonia or nitrite, concentrations of up to 75 mg/L of NO₃⁻ will compromise the welfare and growth of the farmed animals (Kuhn et al. 2010; Furtado et al., 2015). In week 1, nitrate reduced from 12.7mg/L to a mean of 10.93 mg/L representing a 13.98% reduction. In week 2 Nitrate within the flasks reduced from 27.1mg/L to 18.075 mg/L which

represented a reduction of 33.30%. Interestingly, nitrate uptake was significantly faster in week 2 when initial TAN levels were lower in the media however the data is too limited to draw further conclusions.

6.2.6 Influence of TAN & Nitrate on Phosphate Uptake

In week 1, phosphate reduced from 1.25mg/L to a mean of 0.815 mg/L representing a 13.98% reduction. In week 2 phosphate within the flasks reduced from 1.9mg/L to 0.9 mg/L which represented a significantly greater reduction of 52.63%. This uptake was much greater when Nitrate levels were at their highest (27.1 mg/L) and TAN levels were at their lowest (0.29 mg/L) suggesting phosphate uptake is faster in a high nitrate rather than high TAN medium. These findings are consistent with the recent work of Shahar et al. (2020) who whilst studying *Ulva fasciata*, found that in contrast to N uptake, P uptake by *Ulva* when cultivated in NO₃ as sole N source was up to six-fold faster than in cultures with TAN. The relatively slower uptake of P in cultures with TAN is also consistent with previous results from studies of *Ulva fasciata* or *Ulva clathrada* in TAN-rich fishpond effluent (Guttmann et al., 2018, Copertino et al., 2009). Moreover, the faster P uptake of *Ulva* in higher NO₃-N biofloc, as measured in the current study, is consistent with measured P uptake rates of both *U. prolifera* and *U. linza* that were cultivated with NO₃ as sole N source (Luo et al., 2012).

Shahar et al. (2020) suggested that, although being taken up rapidly, storage of P by *Ulva* in NO₃-N-enriched seawater is doubtful, as the polyphosphate pools are considered lower in *Ulva* when fed with NO₃-N than with TAN (Lundberg, 1989). It is more likely that *Ulva* utilizes P for the production of phosphorylated, high energy cofactors that are required for the reduction of NO₃-N to NH₄ prior to assimilation.

In general however, there was a weak relation between specific nutrient uptake rates and growth rates, as seen for example in the results for the ammonia uptake which demonstrate highly variable values. Further studies of nutrient removal, with greater samples sizes, over a longer period would be necessary in order to draw more meaningful conclusions.

6.2.7 Disc Size & Re-growth Performance

Nutrient budgets within the biofloc effluent vary from day to day and week to week (due to feeding regimes, variances in bacterial assimilation and nitrification etc). As such, there is limited scope for comparison between the growth performance of the 29 mm discs in experiment one and the growth performance of the 19 mm discs in experiment two. However, one interesting observation was made with regard to the degree of sporulation (and subsequent breakdown of thallus tissue) when the two

disc sizes 29 mm and 19 mm are compared. A visual analysis of the photo records taken during disc drying and measurement revealed a significantly greater degree of thallus disintegration in the smaller 19 mm discs than was accounted for in experiment one with 29 mm discs. Indeed after week 2 of experiment 2, some treatments had only 3 complete discs per flask in comparison to the original 4 at the beginning of the experiment. Gao et al. (2017) has proposed two hypotheses relating to fragmentation-induced reproduction. The first is that wounding (such as initial cutting of the discs in this study) triggers the expression of genes coding for sex-inducing pheromones, such as in the green alga *Volvox* (Amon et al., 1998). Smaller discs (used in experiment 2) have a higher ratio of wounded cells to total cells, which would translate into a stronger reproductive stimulation. The second hypothesis is that *Ulva* blade cells produce two reproduction suppressors; one (SI-1) is excreted into cell walls and the other (SI-2) is located in the inner space between the two cell layers (Stratmann et al., 1996). Cutting of discs breaks the cell wall and extracellular matrix structure making it easier for small discs to leach inhibitors, thereby removing the regulatory block to reproduction (Hiraoka and Enomoto, 1998). This hypothesis is supported by differences in reproduction between *Ulva* grown in old and renewed media, i.e. fragmentation due to sporulation was more likely to happen after a media change. More wounding sites could have resulted in greater release of inhibitors into the media which were removed following the weekly media exchange. This would explain the greater degree of disintegration observed in the presents study with smaller 19 mm discs.

In general, the growth rates of all experiments demonstrate that *Ulva ohnoi* grows well after fragmentation and cutting from the original thallus. Indeed, experiment two demonstrated a second period of rapid regrowth after the discs were cut back to 19 mm after week 2. This suggests that harvesting of *Ulva ohnoi* from the same plant could be performed at least two times without significantly affecting biomass yields. Further experimental work on regrowth is necessary in order to inform the best cultivation practice i.e. if continuous gradual harvesting and regrowth of the same stock is feasible. Alternatively, the occasional addition of either 'wild' harvested *Ulva sp.* or newly cultivated *Ulva sp.* grown from spores (of the best performing plants) may be necessary to support an ongoing production cycle. All of these considerations ultimately inform the type and extent of nursery facilities, equipment and expertise necessary for a commercial aquaculture operation.

6.3 Experiment 3: Sporulation of 5mm Ulva Discs using Low Temperature and Low Nutrient Shock

6.3.1 Rapid Reproductive Response

Temperature shock is an established method to induce gamete release, but the efficacy of the approach varies with species and ecotype (Gao et al., 2017). Assuming that the degree of deviation from the environmental norm is a telling factor in the effectiveness of temperature shock, then by extrapolation, *Ulva* from more polar environments would be expected to experience even lower reproductive rates as a drop to 4 °C would equate to the weakest temperature shock relative to its ecological norm. As far as the author is aware, the latitudinal relationship between temperature shock and reproduction in *Ulva* has not been investigated therefore experimentation with the study species helps fill an identified knowledge gap. The sub-tropical - tropical species *Ulva ohnoi* found in the UFSC shrimp laboratory grounds would be expected to have a high response to 4 °C temperature shock. This was indeed found to be the case in the present study, where swarmerers were released just 24 hours after shock treatment.

The [20 min] treatment demonstrated a significantly higher reproductive output at 33.33% when compared to the [VS control] at 6.66% ($p=0.0169$) on day 1. This rapid response may have been due in part to the two-stage acclimation period where vegetative cells had already been exposed to FSW enriched with 50% biofloc effluent after being taken from the wild, then subsequently to FSW with 10mL/L von Stosch medium prior to having been ‘shocked’ in standard FSW. However, the findings are similar to Niesenbaum (1988) who reported that *U. lactuca* thalli became reproductive 18 h after a 2°C shock and Carl et al. (2014) who reported a peak in release after just two days. It is possible that the rapid response could be down to the pre-fertile status of at least some cells within the plants, as swarmer discharge was also found in the [VS control] group; fragmentation, temperature and nutrient shock inevitably accelerated the release process. Moreover, the daily media change after microscopic analysis of the discs would have reduced the effect of the sporulation and swarming inhibitors, thereby speeding up the process. Gao et al. (2017) reported to have also changed the media daily. In general, this study found that 2 hour shock at 4 °C can produce a 90% reproductive response by day five which is similar to the most recent work of Gao et al. (2017) who demonstrated that a 20-min temperature shock at 4 °C induced a mean reproductive response of 94.7% in blade tissue by day five.

6.3.2 Viability of the Cold Shock Method

The results from the sporulation experiments demonstrate a rapid response to the cold shock treatments and also to the low nutrient shock [FSW control] treatment. By day 5, the ANOVA analysis found no statistically significant difference between the [FSW control] and the [10 min], [20

min] or [1 hour] treatment ($p=0.9861$ in all cases) or indeed with the [FSW control] and [2 hour] treatment ($p=0.6243$). This suggests that the nutrient shock caused by transfer from high nutrient FSW with 10mL/L von Stosch to standard FSW is sufficient to give a 76.66% reproductive output on average and that this is not significantly different from the 90% output in the 2 hour cold shock treatment (due to the high degree of variance among the three replicates). The no-significant difference finding between treatments was surprising however provides useful guidance for sporulation and seeding protocols. Assuming commercial aquaculture enterprises must minimise costs and labour, the use of cold shock may not be financially viable as a simple high to low nutrient shock provides a similar reproductive response. If the typical cultivation conditions involve a high nutrient waste water or effluent (IMTA) then a simple transfer to standard seawater would yield a high reproductive response. Further experimentation with plants acclimated in biofloc 50% or 75% (as opposed to FSW+10mL/L von Stosch) is recommended however as the selection of plants from this medium would be more akin to the type of bioremediation or IMTA system that could be operational at UFSC as per the results of experiments 1 and 2.

It is noted that even a small degree of sporulation (6.66%) was recorded in the [VS10] control which received neither a cold shock, nor a nutrient shock as the fresh media used in the experiment had the same nutrient budget as that used in acclimation conditions. This suggests that fragmentation of the plant into a 5 mm disc and media change (thereby diluting the sporulation inhibitors) is sufficient to trigger at least some sporulation, although in general the discs largely maintained vegetative state and continued to grow. There is an incalculable number of different combinations of clusters and individual cells which might be either vegetative and/ or in any of the three stages of reproductive state simultaneously, making quantitative analysis in the absence of sophisticated computer software very challenging. Indeed, this study has found some observational evidence that discs with vegetative cells which begin to differentiate into a reproductive state can return to the vegetative state before a critical timeframe passes, as evidenced by the colour change from green to yellow and then back to green again. The microscopic observations are limited however and as such the dynamics of this 'cellular switch' back to vegetative state is not meaningfully understood. Care should be taken to appreciate this phenomenon in any future sporulation trials.

7.0 Summary of Findings & Final Conclusions

1. *Ulva ohnoi* performs well in marine shrimp biofloc effluent with the highest recorded daily mean SGR_{max} of 25.49 ± 3.23 % per day⁻¹ in [BF75] treatment.
2. Biofloc effluent supports growth rates similar to a formulated high-nutrient medium (von Stosch enrichment at 8mL/L) demonstrating its potential as an enrichment source.
3. The biofloc can support continued algal growth over 3 weeks with minimal fragmentation and sporulation. Water surface area (m²) is a highly limiting growth factor and stocking densities for future trials should be calculated with this in mind.
4. The species tolerates high salinity of up to 45 ppm ([BFT100] / [BF100] treatment) and a maximum stocking density of up to 4 g/ L in flask cultivation despite its natural habitat having a salinity of 28 ppm.
5. The higher nutrient budgets of [VS8] control or [BFT75], [BFT100] support continued growth at higher stocking densities by mitigating effects of self-shading and stress.
6. The species is effective at removing TAN, nitrite, nitrate and phosphate from the biofloc. A preference for TAN is implied however more phosphate is removed in a high-nitrate rather than a high-TAN medium.
7. Sporulation can occur and may in fact release TAN into the medium which has implications for biofilter efficiency and bioremediation.
8. Combined 4 °C cold shock and low nutrient shock [2 hours] is effective at inducing a mean rate of sporulation of 90% after 5 days. This is not significantly different from [FSW control] low nutrient shock alone which caused a mean of 76.66% sporulation. This has implications for the financial viability of cold shock treatment however further experiments with plants taken from culture in FSW enriched with 50% or 75% biofloc effluent should be carried out.
9. Next steps should involve chemical analysis of BF cultivated plants to estimate tissue-Nitrogen (Protein) content, and potential contaminants in order to determine if the biomass can be safely used as a feed additive or functional food.

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