Culturing *Undaria pinnatifida* gametophytes

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A thesis submitted to the Faculty of Health and Environmental Sciences of Auckland University of Technology in partial fulfilment of the requirements for the Degree of Master of Science (MSc)

2018

School of Science

Auckland University of Technology

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ATTESTATION OF AUTHORSHIP

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in the acknowledgements), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning.

Signed _____ Date_____

ACKNOWLEDGEMENTS

I would like to thank and acknowledge the following people who have been patient with me and sacrificed their time to make this study possible.

I would like to sincerely acknowledge my supervisor, Associate Professor Dr. Lindsey White for his help and assistance in this study. His knowledge and information on the subject is what got my interest piqued in this topic and this study was completed with his ongoing input and support.

National Institute of Water and Atmospheric Research (NIWA), for letting me use their facility at Ruakaka to conduct my experiments. I would also like to thank the staff there, who helped me with the logistics and with any small issues when stationed there.

Dr. Sonya Popoff, for ordering all chemicals and supplies and all the technical assistants in the laboratory at Auckland University of Technology who helped and assisted me when needed with equipment and facilities in the lab.

Brid Lorigan, who helped me order and document all supplies needed for this study.

All the staff in the Applied Sciences department, who helped when I approached them for their expertise and knowledge.

My colleagues and friends at the university who kept me motivated and helped me on this journey.

I would like to specially thank my wife, Tanvi for helping me and providing me with mental and financial support throughout this endeavor. I appreciate it fully.

ABSTRACT

Undaria pinnatifida is a species of brown laminarian seaweed, primarily found in its native habitat, in the temperate waters of East Asia. It is also farmed extensively in Japan, South Korea and China, where it has huge economic importance. It is primarily used for human consumption and some other products are made from it as well. The seaweed was accidentally introduced to New Zealand in the late 1980's and since then has spread uncontrollably around the country's extensive coastline. Because of its rapid spread and failed attempts of eradication, Ministry of Primary Industries (MPI) classified the seaweed as a pest, leading to a total ban on harvesting or cultivating this species in NZ waters.

Because of its global commercial importance, there was an interest in farming *U. pinnatifida* in NZ waters. To that effect, Cawthron Institute was awarded funding in 1994 to research and streamline Asian cultivation procedures by mass culturing gametophytes in the laboratory and planting out seedlings to marine farms. In 2010, MPI altered their policy regarding the seaweed and allowed for harvesting and cultivation in heavily infested areas of the country. This change in policy opened avenues for expanding on the research started by Cawthron. The first part of this study tested existing methods of growing vegetative gametophytes in a controlled laboratory environment. This was followed by growing gametophytes in aquaculture effluent. Crude fucoidan yield from *U. pinnatifida* gametophytes was also researched in this study.

U. pinnatifida sporophytes were harvested from mussel farms from Port Underwood in the Marlborough Sounds region of NZ. The plants were brought back to the AUT laboratory in Auckland, where spores released from the sporophylls were cultured in 'French' seaweed media. *U. pinnatifida* gametophytes were successfully cultured vegetatively in a laboratory environment under 24 hr. LD irradiance cycle at 40 $\mu mol.m^{-2}s^{-1}$ (~2000 lux) and a constant temperature of 22°C in the environmental chamber. They displayed rapid culture growth and male

and female gametophytes were easily sexed in a sample under a microscope. Some cultures got contaminated despite practicing and maintaining sterile techniques.

The gametophytes were also successfully cultured in aquaculture effluent on-site of an abalone farm. Effluent was collected from 4 different sites on the farm to test gametophyte growth and nutrient uptake. Growth and nutrient uptake parameters were also tested in a control of seaweed 'French' media. Gametophyte growth varied between the various effluent categories in this study. The best growth was seen in Settling Pond Overflow (SPO) followed by Settling Pond Inflow (SPIF) under 24 hr. LD irradiance cycle at 40 $\mu mol.m^{-2}s^{-1}$ (~2000 lux) and a temperature range of 20-25°C. Measured dry weight of *U. pinnatifida* gametophytes in SPO increased from 4.32mg to 40.8mg and in SPIF from 4.91mg to 23.4mg over the experimental duration. Other categories of effluent tested showed no growth. The effluent was also tested for nutrients in a commercial laboratory over the duration of the study. Total ammonia (NH_3) exhibited the most uptake in the effluent. SPIF had $0.33g/m^3$ and SPO had $0.98g/m^3$ total ammonia at the start of culturing. Over the first week of the experiment, total ammonia uptake by the gametophytes brought the level down to $0.25 g/m^3$ in SPIF and $0.26 g/m^3$ in SPO. The other nutrients tested were Nitrate (No_3), Nitrite (NO_2) and dissolved reactive phosphate (PO_4), which displayed negligible uptake below the minimum detection limit of $0.10g/m^3$, $0.0010g/m^3$ and $0.004g/m^3$ respectively. There was a 10% w/w yield of crude fucoidan when extracted from freeze-dried vegetative gametophytes using the deionized water extraction method.

INTRODUCTION

Background

Undaria pinnatifida (henceforth known as *U.pinnatifida*) (Phaeophyta, Laminariales) is a species of laminarian brown seaweed native to the cold and temperate regional waters of Japan, Korea and parts of China (Tseng 1984; Akiyama & Kurogi 1982). As this seaweed is of considerable commercial importance in these parts of Asia, it has been successfully grown and farmed for several decades. In recent decades, *U.pinnatifida* has spread around the world, mostly through international shipping and anthropogenic methods like aquaculture of native north-east Asian shellfish (Floc'h et al., 1991). It has increased its range to around 12 countries on 4 continents including Argentina, Australia (and Tasmania), Britain, Chile, France, Italy, the Netherlands, New Zealand, Spain, Mexico and California (USA). (Floc'h et al., 1991; Sanderson, 1990; Casas & Piriz, 1996; Farrell & Fletcher, 2006; Silva et al., 2002; Casas et al., 2004; Russell et al., 2008; Fletcher & Farrell, 1998).

Since its introduction in New Zealand waters in 1987 (Hay & Luckens, 1987), it has spread extensively around the country (Hay, 1990) mostly around the east coast by coastal shipping and anthropological activities, which prompted the Ministry of Primary Industries (MPI) to classify the seaweed as a pest species in New Zealand. As it is classified as a pest species, much research hasn't been done over the years on its potential as an aquaculture species in New Zealand. In 2010, MPI relaxed regulations regarding harvesting the seaweed from man-made structures, like mussel farm ropes or jetties. These change in regulations were mostly influenced due to the high commercial value of this species globally and its importance in its native region and habitat in east Asian waters as a food source. It is used in a variety of preparations and consumed dried or in soups or salads (Yamanaka & Akiyama, 1993). *U.pinnatifida* is also a good source for fucose containing polysaccharides, commonly referred to as fucoidan which possess antioxidant, anti-inflammatory, anticoagulant and antitumour properties to name a few.

Purpose of the study

There is high potential for aquaculture of *U.pinnatifida* in the waters around New Zealand. The introduced seaweed has been growing and spreading unchecked around the country for the past two decades, demonstrating that the local waters have ideal physical parameters required for its growth. Investigating the feasibility for aquaculture is also commercially important, given the high demand for *U.pinnatifida* in the Asian markets.

Research on *U.pinnatifida* in New Zealand has recently gained pace owing to the aforementioned factors and various studies have been conducted to study physiology, spread and growth factors, impact on the environment and fucoidan content (Russell et al., 2008; Hay & Villouta, 1993; Mak et al., 2013; Stuart, 2004). Because of this existing research material and the loosening of regulations from MPI, there is further merit in subsequent research on the potential of aquaculture and hatcheries research of *U.pinnatifida* in New Zealand. Currently it is unknown if *U.pinnatifida* farmed in New Zealand will be of equal or greater value in the global market when compared to the Asian varieties. The research on this seaweed will also facilitate in the understanding of maintaining a viable *U. pinnatifida* gametophyte library in New Zealand. A gametophyte library can be used to seed the aquaculture farms throughout the year (Gibbs et al., 1998). Because of the growing stresses by aquaculture on the environment, mainly due to the waste-water released from land-based farms, an avenue was investigated to grow *U.pinnatifida* gametophytes in effluent from an aquaculture plant.

Hence, in the present study, the following aspects of *U.pinnatifida* were investigated: (1) growing vegetative *U.pinnatifida* gametophytes in ideal temperature, nutrient and irradiance parameters; (2) hybridizing *U.pinnatifida* gametophytes under ideal parameters to check the viability of hybridised gametophytes; (3) growing vegetative *U.pinnatifida* gametophytes in aquaculture effluent from a land-based paua farm to check feasibility and examine nutrient

uptake; (4) comparison of fucoidan content in *U.pinnatifida* gametophytes against that from mature sporophyte from *U.pinnatifida* and other brown algae species.

Literature Review

Seaweed is a collective term used to classify several species of macroscopic, multicellular marine algae. This term includes all types of brown, red and green algae. There are two basic environmental factors required for growth, one being seawater or brackish water and the other being sufficient light for photosynthesis. The different types of red, brown and green seaweeds are grouped, primarily on the basis of the thallus colour. But besides pigmentation, they differ considerably in many biochemical and ultrastructural features, including storage compounds, composition of cells walls, fine structure of chloroplasts, size, structure, photosynthetic pigments, ecology and habitat. Seaweeds most commonly inhabit the intertidal or littoral zone in the ecosystem and occupy a wide range of ecological habitats.

Undaria pinnatifida, also known as *'wakame'* is a species of brown seaweed which is native to east Asian temperate waters and is widely harvested and farmed in these regions. Japan, China and South Korea are the main growers and consumers of large quantities of *U. pinnatifida*. The seaweed is used as a popular food source and is consumed either fresh or in a dried variety (Yamanaka & Akiyama, 1993). Because of its popularity, very soon, the wild harvesting of the seaweed outgrew the rate at which it grew naturally in its habitat, therefore rope cultivation was introduced in 1955 (Tseng, 1981).

Biology and Life-cycle

U. pinnatifida is a brown seaweed and can grow to a maximum length of 1-3 meters. Physiologically, it has a midrib which makes the stipe and the blade of the adult plant. It also consists of a spiral cone shaped sporophyll, which is the reproductive organ of the seaweed. *U. pinnatifida* can be found growing on varying types of substrates and has shown to be a very adaptive seaweed, looking at the extent of its global distribution. It has been observed growing equally well on natural and artificial substrates like rocky reefs to aquaculture farm ropes and jetties/boat hulls (Hay, 1990).

The life history of *U. pinnatifida* is representative of a laminarian kelp, with an annual life cycle. In Asia, in their native habitat, the adult sporophyte exhibits its main growth in spring. In the wild, the spores are hybridized and settle on a substrate at the end of winter. Once settled, the plantlets grow rapidly through late winter and spring, transforming into mature plants in summer. These mature plants release zoospores from the sporophyll and degenerate in late summer and autumn. In New Zealand, where *U. pinnatifida* is a non-native species, the adult sporophytes are present in winter as well, owing to the cooler water temperatures. as the temperatures ae not cold enough for the plants to completely degenerate (Parsons, 1995). The annual seasonality displayed by *U.pinnatifida* in its native region is not always evident throughout the introduced range of this seaweed. In New Zealand, the sporophytes of *U.pinnatifida* are found throughout the year in some places, displaying overlapping generations. This might be attributed to the fact that the water temperatures fluctuations are less extreme than the native regions of *U.pinnatifida* (Russell et. al 2008).

U.pinnatifida, in its native habitat displays an annual heteromorphic life cycle comprising of two separate phases or alternating generations. The macroscopic large plant (diploid, 2n), which is farmed and consumed is the spore-producing sporophyte. The other phase encompasses the microscopic zoospores (haploid, n) which are released by the sporophyll (reproductive part of the sporophyte) and the microscopic male/female gametophytes which develop from the zoospores. Upon releasing zoospores, the adult sporophyte undergoes senescence by mid-summer (Stuart et. al 1999, Stuart, 2004). The spores drift with the water current and upon settling on a suitable substrate, germinate into filamentous male and female gametophytes. A mature sporophyll usually releases up to 10,000,000 spores that germinate into dioecious gametophytes. The dioecious gametophytes of *U.pinnatifida* are perennial and can remain viable for up to 24 months (Stuart 2000). The gametophytes persist on a suitable substratum until the next winter (Hewitt et. al 2005).

U.pinnatifida sporophytes show favorable growth in cold temperate waters, with ideal temperatures being around 12-15 °C. The sporophytes begin to disintegrate

and die-off at temperatures above 23 °C. *U.pinnatifida* gametophytes require an ideal temperature of around 17-20 °C for growth and maturation. Zoospore release ideally occurs in this temperature range with germination occurring around 20 °C. However, with warmer waters and temperatures above 22 °C, there is less longevity of zoospores and a decrease in the rates of germination (http://www.thefishsite.com/articles/2095/how-to-farm-wakame-undaria-pinnatifida-seaweed/)
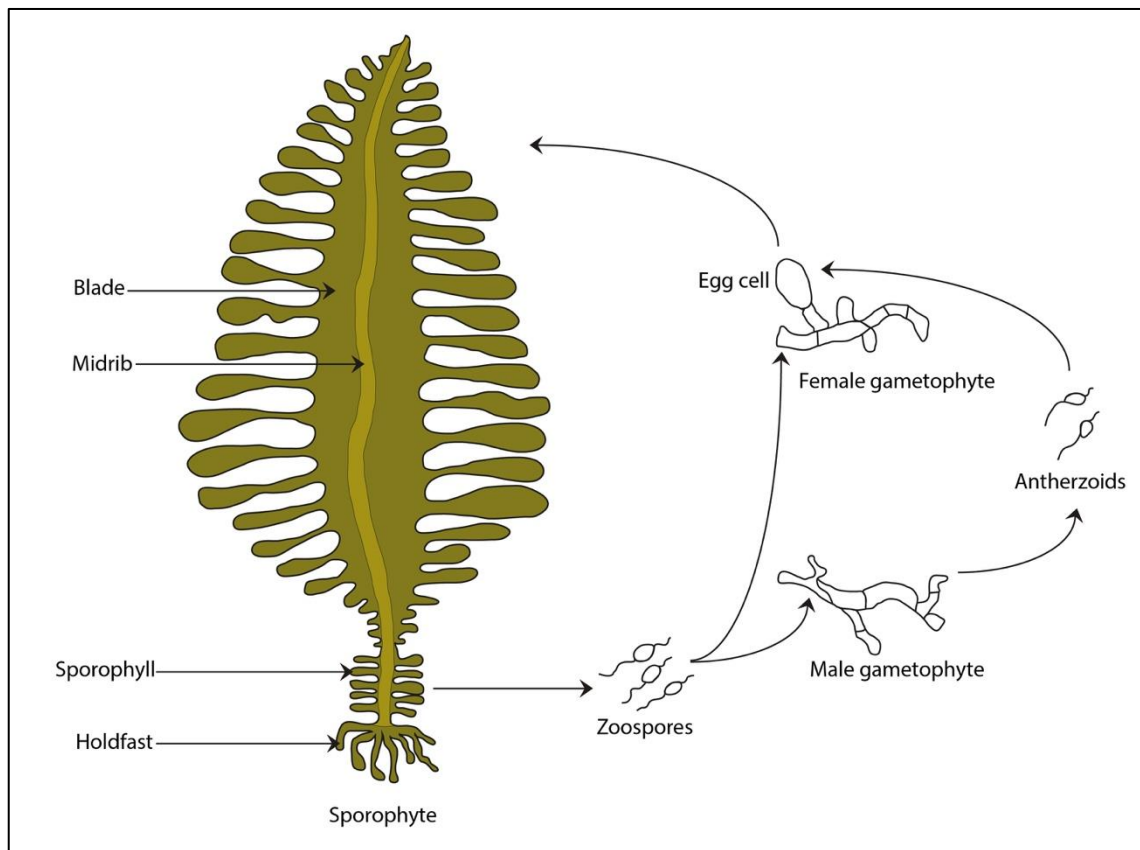
Figure 1. *Lifecycle of Undaria pinnatifida*

Distribution of U. pinnatifida

U. pinnatifida is native to the temperate waters of Japan, China and South Korea. It has spread globally, originating from its native habitat to places such as New

Zealand, Australia, Chile, California, France, the Mediterranean coast of Europe. It can now be found in around 12 countries on 4 continents. In New Zealand, *U.pinnatifida* was first discovered growing sub-tidally along the shoreline of Wellington harbour in 1987. As it was found in the main commercial region of Wellington harbour, which is devoid of any aquaculture activity, and thus circumstantial evidence pointed to the introduction of *U.pinnatifida* by Japanese and Korean fishing vessels over the previous decade (Hay & Luckens, 1987; Hay, 1990). Since its introduction to New Zealand, *U.pinnatifida* has rapidly spread around the country, primarily on the east coast of the country. The natural and anthropogenic spread of *U.pinnatifida* around the country prompted the New Zealand government to classify it as an unwanted organism under the Biosecurity Act 1993 and led to its inclusion as a pest species in several regional pest management strategies (Stuart 2004). Once the seaweed was introduced in these areas by anthropogenic activities, natural dispersal facilitated further spread to the surrounding areas, primarily through dislodged fragments of sporophytes which were carried over distances of hundreds of meters to kilometers (Forrest et. al 2000). One of the main reasons for the rapid spread and invasion of *U.pinnatifida* in differing habitats around the world has been because of the seaweeds ability to grow on many different natural and artificial substrates. *U.pinnatifida* can grow by attaching itself to almost any natural substrates like rocks, shells, other macro algae or artificial substrates like boat hulls, jetties, wood, aquaculture farm equipment etc.
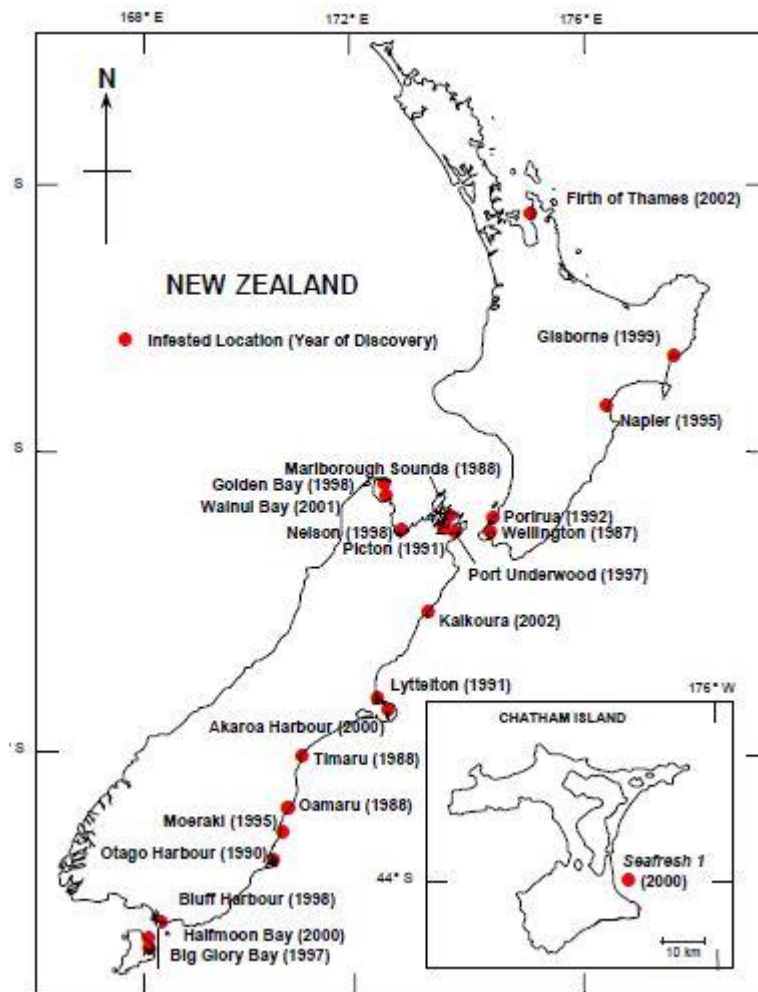
Figure 2. Known dispersal of Undaria pinnatifida around New Zealand with year of discovery. (Source: Stuart, 2004)

Historical background and commercial importance of U.pinnatifida cultivation

U.pinnatifida is a commercially important seaweed in its native region. It has been harvested and farmed in Asia for several decades. Farming and cultivation of *U.pinnatifida* was first studied in the 1940's in the Dalian region of northeastern China by Youshiro Ohtsuki who subsequently patented the cultivation techniques (Shao et al., 2015). In 1953, the first commercial cultivation began in Japan and it has been extensively cultivated since the 1960's on a commercial level. Initially, the hanging technique was used to cultivate and farm the seaweed which later shifted to the horizontal longline method. Preservation and salting technique

advances gradually led to the increase of annual production for *U.pinnatifida*. In South Korea, cultivation of *U.pinnatifida* began in 1964 and has increased in total volume since the process was industrialized. This resulted in increased annual production amounting up to 400,000 tonnes (wet weight) by 1995. The 2013 harvest figures show an annual production of 327,380 tonnes which constitutes an estimated 29% of the total seaweed cultivation in South Korea (Shao et al., 2015). FAO records from 2012 for the worldwide production of seaweed, show that around 2 million tonnes of *U.pinnatifida* was cultivated in China, South Korea and Japan, with the biggest contributor being China with 1.7 million tonnes (White and Wilson, in press).

Besides northeast Asia, there are a few other places in the world where *U.pinnatifida* is farmed or harvested. The annual production is minimal compared to the Asian countries and in some places *U.pinnatifida* is only harvested from wild growing populations because of its classification as a pest species in those regions. *U.pinnatifida* was introduced in the North Atlantic region off the coast of Brittany in France with commercial interests and exploitation and since then different experimental methods have been successfully tried to cultivate high quality of *U. pinnatifida* in that region. It is also grown on a small commercial-scale off the Galician coast of Spain. In 2010, New Zealand government amended the Biosecurity Act, which subsequently permitted harvesting of the seaweed from heavily infested areas, under the conditions that only the seaweed growing on artificial surfaces (including marine farm structures) and any cast ashore can be harvested. In 2012, three areas were identified for farming subject to MPI approval, in the waters of Wellington, Marlborough and Banks Peninsula.

U.pinnatifida is mostly used in Asian countries in a variety of preparations and is either consumed dried or in various soup and food dishes. Upon harvesting, the seaweed is washed with seawater followed by freshwater. The midrib is removed and the remainder is dried in the sun or a hot drier. The resultant product is called *suboshi wakame.* This product has a disadvantage of fading and losing colour over time when stored for a longer duration, because of the still active enzymes in the seaweed. A different method to inhibit the fading is to mix the fresh seaweed with

ash from wood or straw and spread on the ground for a couple of days before placing it in the dark in a plastic bag. The alkaline ash inactivates the enzymes, upon which the earlier process of drying the seaweed is repeated. This product is called *haiboshi wakame* and it retains the desired deep green colour for a longer period (Watanabe & Nisizawa, 1984) A major *U.pinnatifida* product is made by blanching and salting the freshly harvested seaweed. Fresh seaweed is boiled in 80 °C water for one minute and then cooled quickly in cold water. Approximately one-third amount of salt by weight is added and mixed in the seaweed before storing it for 24 hours. The salt dehydrates the seaweed and once the excess water is removed, it is stored at -10°C. Before packing, the midrib is removed and the pieces are placed in bags convenient for cooking and eating (Yamanaka & Akiyama 1993, Shao et al., 2015).

Impact and Management of U. pinnatifida in New Zealand

U. pinnatifida has spread majorly around the east coast of the country since its introduction and subsequent finding in 1987, in Wellington harbour. It is classified as an unwanted organism under the Biosecurity Act 1993. Because of its invasive nature, unwanted organism status and extensive distribution around the country, MPI established several eradication programs to curb the spread of the seaweed. Complete eradication wasn't achieved in the highly affected areas, but there was some success in restraining along the coast of Fiordland in Sunday Cove in Breaksea Sound. Regular dive surveys to check for any signs of *U. pinnatifida* growth in the area have managed to keep the total population of mature plants to less than ten individual plants from 2010 to 2015 (MPI Biosecurity, 2015). MPI subsequently implemented a policy for harvesting the seaweed as bycatch during other activities like mussel farming or during as part of a control programme.

This policy was later revised by MPI in May 2010, focusing on the commercial use of *U. pinnatifida*. This updated policy allows i) farming in selected heavily infested areas; ii) allows harvest when *U. pinnatifida* is growing on artificial surfaces (including marine farms); iii) allows harvest when *U. pinnatifida* is cast ashore in areas not vulnerable or sensitive to commercial harvest processes and iv) prohibits

harvest when *U. pinnatifida* is growing on natural surfaces, except when part of a programme specifically designed to control *U. pinnatifida*. Harvesting of *U. pinnatifida* was allowed in infested areas because there is minimal risk of additional spread as the seaweed was already heavily present in the five main marine farming allocated areas. In 2012, MPI announced assigned areas where farming of *U. pinnatifida* is allowed, subject to approval, in the waters of Wellington, Banks Peninsula and Marlborough Sounds.

Table 1. Comparison of MPI policies for U. pinnatifida management in New Zealand

Activity	2004 Policy	2010 Policy
Harvesting as bycatch or other activities	Yes	Yes
Harvesting from natural surfaces	No	No
Harvesting from artificial surfaces	No	Yes
Harvesting when part of a control programme	Yes	Yes
Farming in heavily infested areas	No	Yes

Previous research on U. pinnatifida in New Zealand

Since its arrival in 1987 and subsequent spread around New Zealand, there have been many research studies undertaken to know more about the physiology and biology of *U. pinnatifida* growing in the local waters. Much is known about the seaweed from their native habitat, but there are some subtle differences between *U. pinnatifida* growing in NZ and in Asia. Research studies were also focused on establishing the impacts on local ecosystems by the invasive seaweed and to establish crucial factors important to the growth of the seaweed. Parsons (1995) compiled a detailed report for the Department of Conservation (DoC), detailing the

spread and growth of *U. pinnatifida* in New Zealand algal communities while also studying the various factors influencing growth of the seaweed. In 1997, the DoC also ran a monitoring and removal programme for *U. pinnatifida* in Fiordland, New Zealand. This included regular research dives for manually removing any mature sporophytes found in the harbour.

Cawthron, a Crown Research Institute, based in Nelson, New Zealand did some preliminary research on culturing *U. pinnatifida* gametophytes and plantlets, eventually culminating in a small trial in Marlborough for farming on seeded ropes. They based this method on a similar French trial. (Hay & Gibbs, 1996; Gibbs et al., 1998; Gibbs et al., 2000). Cawthron Institute also ran several research programmes to discern links between transport and establishment and also determining the different pathways for spreading by marine farm equipment and vessels (Sinner et al., 2000). Over the years, there has been considerable research been undertaken by universities around New Zealand on the physiology and ecology of *U. pinnatifida*. The University of Otago has investigated the dispersal characteristics of the algae in conjunction with the Cawthron Institute. Victoria University of Wellington has also studied spread on the seaweed in Wellington harbour, while Canterbury University did studies to ascertain impact of *U. pinnatifida* on native flora in shallow waters (Stuart, 2004).

Methods of culturing *U.pinnatifida*

U.pinnatifida has been cultured in the temperate waters off the coast of Japan, South Korea and China for the past half century in increasingly commercial quantities. The methods used in these regions have been refined over time to cultivate seaweed with the ideal qualities for human consumption. Initial methods of cultivating *U.pinnatifida* were by using the hanging lines method and subsequently it was shifted to the horizontal longline method. Cultivating and farming *U.pinnatifida* in Asia is based on the longline method. The farms for mariculture are usually based in a sheltered bay in areas with strong water current. The production is also heavily dependent on the seasonality of the *U.pinnatifida* life cycle.

The seed supply for longline farming is sourced from the previously farmed population. The plants used for spore stock are usually left on long-lines after the annual harvest. These plants are left in deeper water to lengthen the process of growth for sustenance till the start of the seedling process. The seedling process starts at the end of June, when the water temperatures have risen to approximately 18-19 °C. With the ideal conditions met, mature sporophylls are separated from the adult sporophytes, brought to shore and dried in a shaded place. The drying helps in the release of zoospores once the sporophylls are reintroduced in filtered seawater for spore release. The filtered seawater is maintained at an ambient temperature of approximately 16-18 °C. Once the density of zoospores reaches 100,000-150, 00/mL, and PVC pipes covered with thick nylon strings are inserted into the zoospore solution to work as collectors. These collectors are then removed from the seedling tank once sufficient zoospores have attached to the nylon strings to be transferred to another tank with seawater for the next step in the cultivation process. Here, the spores evolve into male and female gametophytes, hybridizing into *U.pinnatifida* plantlets. Once the water temperature at the farm site drops to approximately 22 °C towards the end of September in the Northern hemisphere, the collectors are transferred to the site in the open sea at a water depth where the light intensity is around 200-300 µmol photons $m^{-2}s^{-1}$. The strings with plantlets are then transferred onto the horizontal longlines at a distance of about 35-40 cms and the lines are spaced out by 2 m from each other. The lines are kept afloat by buoys and the strings hang down from these lines, usually within 1m from the surface. The plantlets are about 1 cm in length when they are transplanted in the sea on the horizontal lines and they can grow up to 2-3 m by the harvest season in February to April. Further to using this technique of cultivation, advances have taken place in the mariculture of *U.pinnatifida* with research being undertaken on the effects of various environmental and physiological factors affecting the growth of the seaweed. Seawater temperature, nutrient availability and light intensity are some of the environmental factors that were studied to enhance the capability of the farms producing *U.pinnatifida* and to maximize production by setting up farms in ideal locations (Nanba et al., 2011; Park et al., 2008; Peterio, 2011; Li et al., 2013; Choi et al., 2009; Gao et al., 2013; Gao et al., 2013; Gao et al., 2013; Gao et al., 2013).

Experiments were also performed to modify the physiological aspects of the seaweed to better suit the demands for increased commercial success. Further research was carried out by hybridizing different *U.pinnatifida* species to achieve ideal length, frond width and colour of the seaweed (Hwang et al., 2014; Hwang et al., 2012; Shan & Pang, 2009; Shan et al., 2013; Shibneva et al., 2013; Sato et al., 2014; Watanabe et al., 2014; Yoshikawa et al., 2001). Owing to the long duration of cultivation in Asia and continual research carried out over the past half century on maximizing annual production, *U.pinnatifida* farming has been very successful and is able to meet the rising demand for this seaweed.

U.pinnatifida was deliberately introduced in the north Atlantic region near Brittany, France with commercial interests. It was introduced in 1983 by the French Research Institute for the Exploitation of the Sea (IFREMER) and was initially cultivated at three sites. Driven by the huge commercial importance and market for *U.pinnatifida*, in 1986 ANVAR (National Agency for Promotion of research) provided a large grant to develop a new and original method of cultivation by artificial production of seedlings (gametophytes) in the laboratory. This method was mainly developed to compete with the production cost per ton of the farms in Asia and to also take advantage of the local environmental factors for optimal *U.pinnatifida* growth in the commercial farms (Perez et al., 1984).

Culturing *U.pinnatifida* off the coast of Brittany, France required application of different techniques in order to take advantage of the varying environmental factors. Initial tests were carried out using the existing longline technique used in Asia and on confirmation that the seaweed could grow in the waters off the coast of Brittany, an innovative method was developed for *U.pinnatifida* mariculture. The waters off the coast of France generally stay below 17 °C, so cultivation could be carried out throughout the year once a reliable source of seedling was developed to constantly seed the strings. This new innovative technique was developed based on the observations that in a laboratory environment, the gametophytes of *U.pinnatifida* grow rapidly when maintained at a stable temperature of 21-22 °C. Additionally, vegetative growth is accelerated and gametogenesis suspended until the temperature is lowered to 17 °C (reference). This process involves gathering

several mature sporophylls and rinsing them in sterilized seawater with intermittent cleaning using 0.5% vol/vol bleach solution. Once cleaned, the sporophylls are dried and stored in a cool dark place before spore release. The partially dehydrated sporophylls are reintroduced to sterilized seawater for zoospore release. This zoospore solution is then filtered through a very fine 20µ mesh cloth which filters out the mucilaginous substances exuded by the seaweed. The resultant spore solution is used for culturing vegetative gametophytes. This spore solution is then mixed into a synthetic seaweed media first devised by Provasoli et. al in 1957. The media is called the French media and is a nutrient solution consisting of sterilized seawater mixed with four different stock solutions. The quantities for the stock solution are described in Tables 1-4

Aquaculture environmental impacts, bioremediation and IMTA systems

Aquaculture is a term used to describe the farming of aquatic organisms like fish, molluscs, and seaweeds. Aquaculture entails culturing freshwater or seawater species in controlled environments where each step of the farming process is monitored and maintained to achieve commercially desired organisms and results (FAO Global Aquaculture Production, 2016). The demand for aquaculture has been steadily growing over the past several decades in part due to overfishing and the resulting dwindling stocks of naturally harvested fish and aquatic plant stocks Recent figures from 2013 published by the Fisheries and Aquaculture Organization (FAO) show global aquaculture production increasing and reaching 97.2 million tonnes valued at an estimated USD157 billion. These figures include a total of 575 aquatic species farmed in freshwater, seawater and brackish water combined. A further breakdown of the above figures shows farmed food fish to be 70.2 million tonnes and farmed aquatic plants to be 27 million tonnes in 2013 (FAO 2014). Global aquaculture can be broadly broken down into marine (seawater) aquaculture systems and land-based aquaculture systems. Marine or open-water aquaculture systems are generally described as growing or farming the fish or aquatic plant species out in the open sea in enclosed and sheltered spaces. The farming at sea can be either on ropes, in cages or as free moving stock in defined spaces. Land-based aquaculture systems are purpose built facilities solely for the

farming of aquatic plants or fishes in controlled environments where all aspects and stages of the organism's' life cycle are carefully monitored to enhance commercial productivity of the farmed species.

Land-based aquaculture consists mainly of inland finfish and molluscs aquaculture in facilities built to optimize production of commercially important species. These facilities can be either used to farm marine or freshwater fishes. Globally, the most commercially important species farmed are, in order, carp, salmon, tilapia and catfish and in the case of molluscs, oysters and abalone (FAO 2016). For finfish and molluscs farming, there are different practices employed in a land-based system. In case of finfish, some facilities capture wild fish as broodstock, which are then used to maintain hatcheries of the species. Juvenile fish from these hatcheries are then transferred to an outgrow tank using recirculating water technology, where they are subjected to a variety of methods to aid them in their maturation till ideal length and weight for commercial use are achieved. Some facilities use the land-based tanks to grow fingerlings from broodstock and then outgrow the fingerlings to marketable size in operational sea cages. For molluscs like paua, there are different techniques used around the world, but in New Zealand they are traditionally grown in land-based facilities and are configured to operate on flow-through water supply systems. In this method, seawater is pumped from the sea, over the paua and then returned to the sea. However, one of the largest paua farms in the country relies on water recirculation technology which is better for the species farmed due to the ability to maintain constant water temperature, avoiding fluctuating environmental conditions and improving biosecurity for the farm.

U.pinnatifida gametophytes can be grown vegetatively in ideal temperature, irradiance and nutrient parameters (Hay & Gibbs, 1996). The gametophytes are grown in a synthetic seaweed media which is made up by adding four different stock solutions and an antibiotic to deter parasitic growth to sterilized seawater (Provasoli et al., 1957). This media has been shown to be effective to culture *U.pinnatifida* gametophytes and has been adapted from a French experimental setup for *U.pinnatifida* aquaculture (Perez et al., 1984). Culturing *U.pinnatifida* in

synthetic seaweed media has its advantages as the growth results are a known parameter and thus can be depended upon in the instance of an aquaculture or a research setup. While growing them in seaweed media is ideal, there is merit to further test the ability of growing *U.pinnatifida* gametophytes in aquaculture effluent. Testing growth of the gametophytes in aquaculture effluent can be a good precursor in removing dependency on preparing synthetic seaweed media and also be of interest to an industry where the effluent is wasted and has to be treated before being discarded. This experimental setup can also be used to calculate the level of nutrient uptake by *U.pinnatifida* gametophytes from aquaculture effluent. Testing nutrient uptake from aquaculture effluent will be helpful in aiding the treatment process of effluent before being discarded back in the local ecosystem.

Aquaculture has become a huge global industry in the past several decades. The ratio of wild caught fisheries to farmed fisheries has only gone down over the years. This is in part to lesser wild stock available of many species due to over fishing over the years and subsequently due to the restrictions being placed upon the catch to maintain wild stock. Aquaculture today is practiced around the world in both freshwater and marine environments. According to FAO statistics from 2014, fish harvested from aquaculture amounted to 73.8 million tonnes, with an estimated value of US$160.2 billion. Global aquaculture production of fish accounted for 44.1 percent of total production from capture fisheries and aquaculture in 2014. This figure was up from 42.1 percent in 2012 and 31.1 percent in 2004. In addition to fish, molluscs and crustaceans, aquaculture produces considerable quantities of aquatic plants. Globally, cultured fish and plants production reached 101.1 million tonnes in live weight out of which 27.3 million tonnes was aquatic plants. In terms of global production volume of farmed fish and aquatic plants, it surpassed that of capture fisheries in 2013. Also, in terms of food supply, aquaculture provided more fish than capture fisheries for the first time in 2014 (FAO 2016).
 These numbers represent a significant growth for aquaculture in ratio to total capture fisheries and also the total fish production. Aquaculture as an industry has been steadily increasing in its output, whereas most of the fishing areas globally, have reached their maximum potential for wild caught fisheries. At the same time, global demand for seafood is steadily increasing as well. Owing to these

pressures, onus in the past decades has been on increasing aquaculture production through advancements in biotechnology (Hardy, 1999; Hew & Fletcher, 2001; Melamed et al., 2002).

Table 2. *Production of farmed aquatic plants globally spanning a decade from 2005-2014. (Source: FAO 2016)*

Production of Farmed Aquatic Plants in the World (Thousand tonnes)				
	2005	2010	2013	2014
Kappaphycus alvarezii and *Eucheuma* spp.	2444	5629	10394	10992
Laminaria japonica	4371	5147	5942	7655
Gracilaria spp.	936	1696	3463	3752
Undaria pinnatifida	2440	1537	2079	2359
Porphyra spp.	1287	1637	1861	1806
Sargasum fusiforme	86	78	152	175
Spirulia spp.	48	97	82	86
Other aquatic plants	1892	3172	2895	482
Total	13504	18993	26868	27307
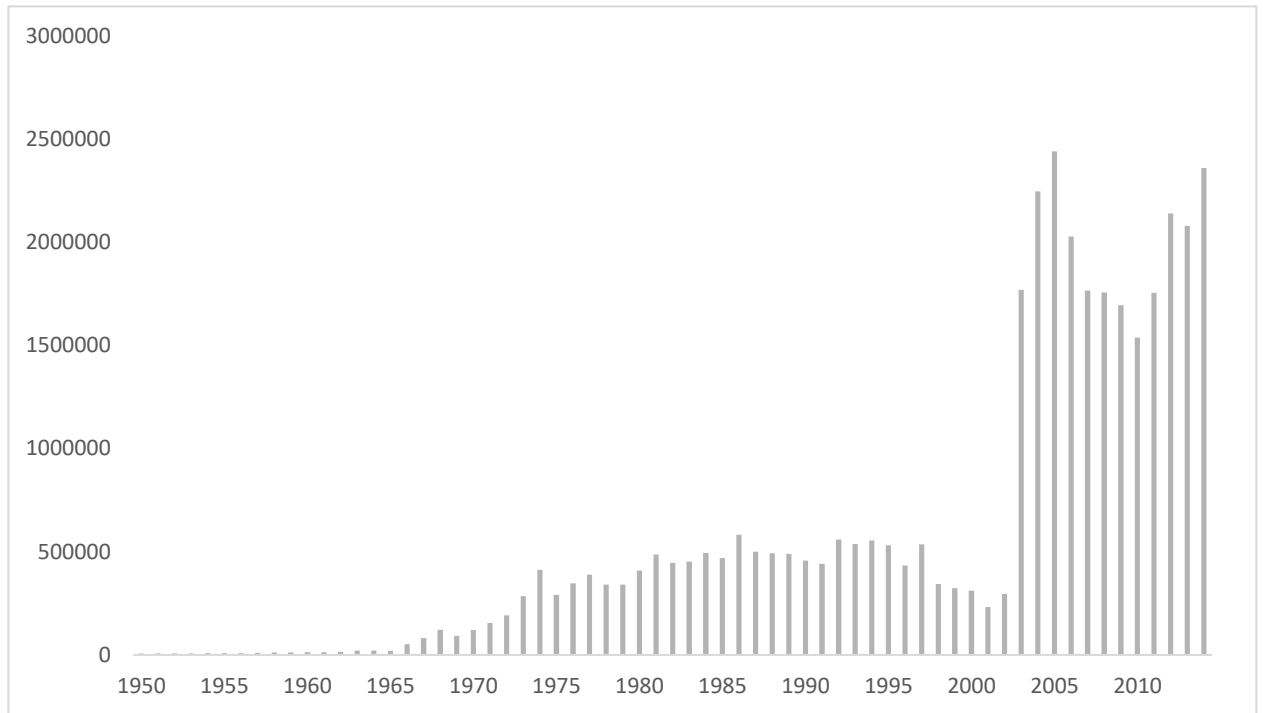
*Figure 3.*Global aquaculture production (in tonnes) for U. pinnatifida. (Source: FAO 2016)

This rapid development of marine and inland aquaculture throughout the world has placed enormous demands on the raised concerns over the environmental impacts on the local ecosystem. Modern aquaculture practices require high levels of water input, fertilizers, feeds and chemicals which inevitably leads to a release of a high number contaminants (Chung et al., 2002). One of the leading causes of environmental concern because of this development is the release of significant nutrient loads into coastal ecosystems by marine based aquaculture farms and the effluents released into the local waterbodies by land-based aquaculture farms. (Chopin et al., 2001). Particularly, specific concerns have been raised regarding the discharge of solid and dissolved organic matter along with nutrients and chemicals, which have a damaging impact on the ecosystem. (Mente et al., 2006) Previous studies have shown that aquaculture practices, primarily used on fish and shrimp marine farms, which are dependent on extensive feeding have been shown to negatively impact the environment (Beveridge, 1996). In spite of these advancements, there is a demand for developing aquaculture practices pertinent to

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developing countries, which are relevant and can be implemented in developing countries to maintain the current levels of demand and supply for global fisheries (Naylor et al., 2000; Williams et al., 2000; Hambrey et al., 2001). These practices should ideally incorporate technologies with environmental and economical sustainability. Taking these requirements into consideration, integrated aquaculture has been suggested as a possible alternative to develop environmentally sound aquaculture techniques and to better manage the available resources with a balanced ecosystem of cultured species (Chopin et al., 2001). IMTA systems could play an integral part in reduction of long term negative environmental impacts from aquacultural activities which will in turn ensure long term sustainability of the aquaculture industry. Reducing negative impacts of the aquaculture industry is a key factor in ensuring long-term sustainability of the environment and the industry. In reference to the above challenges faced by the industry, integrated aquaculture has been tried as an alternative to develop environmentally friendly farms and practices, and to balance the available resources to reduce and negate any major biological changes to the local ecosystem. This method has also been proposed to try and reduce the burden of nutrient loading from fish farm effluents (Chopin et al., 2001).

Integrated multi-trophic aquaculture (IMTA) system is defined as a concurrent or sequential method of farming two or more species of commercially important fish, mollusc or aquatic plants (Chopin et al., 2001; Chopin, 2006). The main objective behind using this method of aquaculture is to increase productivity of the resources used in the system and to contribute to an increase in production of the farmed aquaculture species. Integrated aquaculture systems can be setup to achieve sustainability by integrating waste generating and cleaning organisms in the same farm.

In IMTA, by integrating fishes from a higher trophic level with inorganic and organic extractive species like seaweeds or shellfish, the byproducts or wastes from one species are recycled to become food or fertilizer for another concurrently farmed species. Ideally, the concurrently cultured species each yield a commercially viable crop. A multi-trophic system is so named because it involves species of different

trophic or nutritional levels in the same system. To achieve the objective of a commercially successful yield, it is imperative to select appropriate species for the multi trophic system. Ideally, selected species should be sized and paired accordingly so as to provide the necessary biological and chemical processes required for a balanced ecosystem, benefitting the organism's health (Chopin et al., 2001; Chopin, 2006; Neori et al., 2004). A balanced working IMTA system results in higher gross output for the co-cultured species owing to the mutual benefits and the increased ecosystem health. In some cases, it can be higher than the respective individually cultured species.

Currently, the aquaculture industry including marine and freshwater or brackish and marine-based or land-based systems have a major problem of producing waste in the form of untreated water or inorganic nutrients released into the local ecosystem and the waterways. Commercial scale marine and brackish water aquaculture are currently operated as expansive monocultures. These large systems generate a tremendous amount of waste in the form of excreta and uneaten feed. The waste generated from these facilities often results in a negative effect on the local ecosystem by modifying the biodiversity, pollution and eutrophication (Buschmann et al., 1996; Wang et al., 2012; Farmaki et al., 2014; León-Cañedo et al., 2017). These monocultures have been the major cause of pollution of the surrounding waters. The extent of the pollution and its quality and quantity depends on the characteristics of the system used in conjunction with the characteristics of the species used for aquaculture at that site. The environmental impacts by farms on the ecosystem can be categorized into internal, local and regional. The internal impacts constitute the effects the farm has on its immediate environment and upon itself. Local impacts due to fouling from the farm can affect nearby farms and the existing native wild marine population. Regional impacts can cover a much larger area and encompass a whole body of water with effects displaying through multiple seasons (Silvert, 1992). Previous studies conducted on the pollution of coastal areas have indicated that high inputs of feeds rich in organic and inorganic matter contribute to the nutrient loading in coastal areas (Chopin et al., 2010; Herath & Satoh, 2015) and the nutrient enrichment or eutrophication of the local ecosystem. A main source for nutrient enrichment is

non-consumed feed, fish excretion and decomposition of dead organisms (Chislock et al., 2013; Bureau and Hua, 2010). Based on eutrophication research, it has been studied that out of the total nitrogen supplied to a farm system, only 20 to 50% is utilized and retained by the farm organisms with the rest getting dissolved in the water column or the sediment (Troell et al., 2013).

Integrated aquaculture is not a new concept and has been practiced at differing levels since centuries, particularly in Asian countries (Li, 1987; Tian et al., 1987; Wei, 1990; Liao, 1992; Edwards, 1992; Edwards, 1993; Chan, 1993; Chiang, 1993; Qian et al., 1996). The main reason for using integrated aquaculture has been to maximize the use of limited resources and to also use the waste generated as a valuable resource. Traditionally, marine, brackish or freshwater aquaculture systems have always been setup as a big monoculture. Having a monoculture setup of a profitable species like fish or shrimps, guarantees profits over a short term. Once these systems are setup, other farmers in the region tend to emulate the setup and establish similar monocultures. The existence of similar species in a local environment can eventually affect the balance of the ecosystem due to the high amounts of nutrient loading and waste generation resulting from uneaten food and excreta. It can lead to possible disease outbreaks for those species, thus decimating production and resulting in a loss. The amount of waste generation depends and varies per the species, but most of the nutrients that are added to the system as feed, get passed on as waste (Troell et al., 1996; Troell et al., 2003).

Over the years, as technology has improved, there have been various efforts and advances in reducing the waste generated from monoculture systems. These improvements range from a better quality feed, to better water circulation on the farm and also advancements in better effluent treatment before releasing the waste water in the ecosystem. In spite of these efforts to reduce the waste footprint of these farms, even today, untreated water is released from mariculture farms globally because of cost cutting practices and economic factors in farm operations (Troell et al., 2003; Amirkolaie, 2011). Over the course of advancements in aquaculture techniques, various physical, biological and chemical methods used in conventional waste-water treatment systems have been tried and applied to

aquaculture systems. The biological processes often use nitrification to bacteriologically transform ammonia to nitrates (Poxton, 1981). Other biological processes include submerged bio filters, trickling filters, rotating biological contractors and fluidized bed reactors. These processes mentioned are used in either oxidation of organic matter, nitrification or denitrification (van Rijn, 1996). Chemical filters used for waste treatment include different types of activated carbon filters and ion-exchange filters. Other types of chemical filters that have been tried on land-based farms are chemical coagulation-flocculation for the removal of suspended solids and orthophosphate for recirculating effluent discharge (Ebeling et al., 2003). Sieving has also been tested as a possible effective design of effluent treatment of aquaculture, where all suspended solids greater than the sieve size would be removed from the effluent (Makinen, 1988). Another type of method trialed by researchers is the wetland system, which can significantly remove suspended solids, nitrogen, phosphorous, organic matter, trace elements and microorganisms from the wastewater (Lin Ying-Feng et al. 2002).

Mariculture Nutrient Wastes

Bivalves	⟶ 75%
Abalone	⟶ 60-75%
100%	
Salmon	⟶ 70-75%
Shrimp	⟶ 77-94%
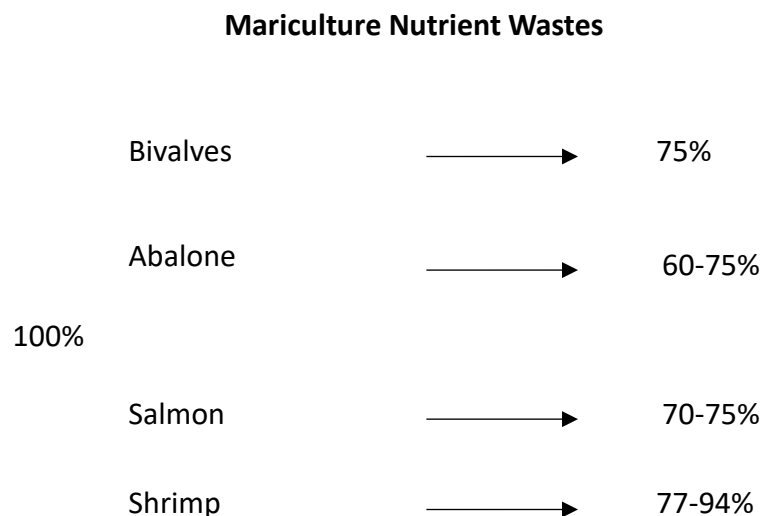
Figure 4. Range of Nutrient discharge (total) for aquaculture species. For abalone and bivalves, the percentage are only for nitrogen. For salmon and shrimp, the

All the techniques for aquaculture effluent treatment, while effective, can be quite cost prohibitive for large scale land-based farm setups. In addition to the various physical, biological and chemical waste treatments used for aquaculture effluent, another researched and frequently used method is of an integrated land-based system where two different species, fed and extractive, are used in conjunction to form an integrated culture (Chopin et al. 2001, Rawson et al. 2002). The main characteristics of an approach like this are the environmental and economic advantages of this system when applied to a land-based farm. This system can also be better understood when the cultured species can be distinctively classified into fed and extractive species. Fed species are defined as organisms which are fed with synthetic or biological feed, while extractive species, as the name implies, extract their nourishment and nutrients needed for growth from their environment (Neori et al. 2004).

Extractive species like filter feeding shellfish and seaweed (FAO, Troell et al. 2003) have been used in monocultures for a long time, owing to their low setup cost and economic input. Seaweed has always been in global demand and has been farmed extensively in Asian countries (FAO 2014). According to the FAO updated aquaculture production statistics (2013), China has been leading in seaweed mariculture, constituting 50.1% of the global production, followed by Indonesia and the Philippines. China farms about 13,479,355 tons of seaweed alone and globally this figure rises to 26,896,004 tons of seaweed mariculture. Of all the farmed seaweed species, *Kappaphycus alvarezii* and *Eucheuma* spp. are the most farmed ones (FAO)(FAO table). Seaweeds have great value as a monoculture because of the natural resource value that they provide in terms of human consumption and their applications. Seaweeds can be eaten raw or cooked and many species are also used in dietary supplements and or cosmetic or pharmaceutical products. Many commercially sold products contain seaweed polysaccharides like agar, alginates or carrageenan (Neori et al., 2004).

The advances in cultivation techniques and the focus on ecological sustainability fueled the development of integrated systems in marine and inland/brackish environments. Using seaweeds as a means for waste water treatment and nutrient unloading originated from research on treating sewage outlets. Originally, research on treating sewage from land-based aquaculture farms was initiated in the 1970's (Haines, 1975; Ryther et al., 1975; Langton et al., 1977; Harlin et al., 1978). Over the years, rapid proliferation of intensive maricultural systems and consecutively, a rise in environmental awareness resulted in the seaweed method for waste-water research being researched on a larger scale from the 1990's onwards (Vandermeulen and Godin, 1990; Cohen and Neori, 1991; Neori, 1996; Buschmann et al., 1996; Jimenez del Rio et al., 1996). All these research studies have further shown that different types of seaweeds (macroalgae) can be used for nutrient unloading and wastewater treatment from land-based aquaculture farms. Integrated maricultures with seaweed as wastewater treatment have been tried with multiple pilot scale systems using effluent from land-based systems. These studies have proved that the seaweeds grown in these systems have had equal or better growth over the experimental duration than in seawater or synthetic media. The research has also highlighted some species of seaweeds significantly reducing concentrations of ammonia, nitrate and phosphates from the effluent.

Research done until now has primarily shown the potential of culturing macroalgae in effluent of land-based aquaculture systems. The benefits of culturing macroalgae in a polyculture setup is that the seaweed isolates the nutrients from the waste water, thus cleaning the effluent which can be discharged back into the environment or recirculated to the fishponds (Neori et al., 2004). Seaweed biofilters can also be advantageous because they can also provide oxygenated water back to the fishponds in a recirculation system (Hirata et al., 1993). Seaweed mariculture with land-based systems has been extensively researched throughout the years as further developments in seaweed biofilter R&D has progressed. (Macdonald et al., 2011; Mata et al., 2010; Lawton et al., 2013; Chopin et al., 2012; Nobre et al., 2010; Barrington et al., 2010; Al-Hafedh et al., 2015; Samocha et al., 2015; Trang and Brix, 2014; Ju et al., 2015).

While integrated mariculture on land based systems with macroalgae have been extensively studied, there has been little to no research on using microalgae to limit nutrient loading by uptake of various nutrients in aquaculture effluent. Also, along with nutrient uptake, studies showing growth of microalgae in aquaculture effluents has not been researched extensively as of yet. This research experiment aimed to gauge the nutrient uptake for *U.pinnatifida* gametophytes in paua (abalone) aquaculture effluent while also measuring their growth over the experimental duration.

Fucoidan Overview and Uses

Fucoidan is a class of sulphated, fucose rich polysaccharides found in the cell walls and intercellular spaces of brown seaweeds of the class Phaeophycae and some marine invertebrates (Ale et al., 2011). In the seaweeds, it is found in wakame (*U.pinnatifida*), mozuku, hijiki, kombu and bladderwrack, while in the marine invertebrates it can be found in some sea urchins and sea cucumbers. Fucoidan is found in cell walls of the seaweeds and marine algae. The main function of fucoidan is to impart a slippery texture to the seaweed and protect the seaweed from some of the harsher environmental conditions (Li et al., 2008). Studies conducted on fucoidan isolated from different species over the decades have displayed a wide range of biological activities. These include anticoagulant, antithrombotic, antivirus, antitumour, anti-inflammatory, antioxidant and anticomplementary properties against hepatopathy, uropathy, gastric protective effects and therapeutic potential in surgery (Jin et al., 2013; Hwang et al., 2016; Yuan & Macquarrie, 2015; Rabanal et al., 2015; Zorofchian et al., 2014). Fucoidan is usually extracted from the mature alga/seaweed. A part of the sporophyll of the adult sporophyte is used in extraction methods. There are several methods which have been employed and researched over the years for extraction of fucoidan. The extraction and isolation of fucoidan is influenced by the presence of interfering substances, the chemical nature of the components and the extraction method used (Wijesinghe and Jeon, 2012). Different seaweed species have differing levels of polysaccharide content in their cell walls. Early extraction techniques involved the use of dilute acid treatments with either hydrochloric acid

or acetic acid used as a primary extraction step to hydrolyze the non fucose containing polysaccharides, and isolating the desired fucose containing polysaccharides (Ale et al., 2011).

Table 3.Percentage yield of Fucoidan extracted from different species of seaweed

Species	Percentage Yield
Pelvetia canaliculate [a]	61.2
Fucus vesiculosus [a]	52.2
Sargassum muticuma [a]	51.8
Laminaria digitate [a]	41.0
Laminaria japonica [b]	1.9
Alaria fistulosa (blade) [c]	13.2
A. fistulosa (sporophyll) [c]	58.7
Sargassum swartzii [d]	6.7
Adenocystis utricularis [f]	2.9
U. pinnatifida f typica [g]	1.1
U. pinnatifida f. distans [g]	2.1
U. pinnatifida (Samcheok) [g]	3.8
U. pinnatifida (sporophyll) [h]	8.8
Sophora wightii [j]	71.5
Dictyota dichotoma [j]	67.2
Turbinaria decurrens [j]	57.2

[a] (Mabeau et al., 1990); [b] (Wang et al., 2007); [c] (Usov et al., 2005); [d] (Ly et al., 2005);

[f] (Ponce et al., 2003); [g] (Lee et al., 2006); [h] (Yang, Chung, & You, 2008), [j] (Eluvakkal et al., 2010).

Subsequent research and studies has helped modify the methodologies employed to isolate fucoidan/ fucose containing polysaccharides from brown seaweed

biomass. These polysaccharides are also extracted using methods involving water or aqueous organic solvent (Albuquerque et al., 2004). But due to the cell walls consisting complex polymers, organic solvent extractions are not wholly feasible. An alternate, more effective method used for extraction includes using an enzyme assisted extraction technique which proves more useful for the extraction of bioactive polysaccharides from the seaweed (Athukorala et al., 2009, Kang et al., 2011).

Fucoidan has been researched extensively and has been extracted from different species of seaweed. The amount of crude fucoidan extracted varies according to the differing levels of polysaccharide content in the cell walls and thus different species have vastly varying percentage yields. Also, the amount of fucoidan can be different depending on whether it was extracted from the blade or the sporophyll of the seaweed. The below table lists the percentage yield of fucoidan based off of existing research conducted on different species of seaweed.

To summarize, most fucoidan isolation and extraction techniques/protocols studied over the past decades on different species of brown seaweed employ treatment with dilute acids at higher ambient temperatures.

Fucose containing polysaccharides have been extracted primarily from mature adult seaweed or macroalgae. The sporophyll or the stipe/blade is used as the biomass for extraction and isolation but there is merit as well to researching fucoidan extraction from gametophytes. Gametophytes can be grown easily in large quantities under ideal nutrient, temperature and irradiance parameters. Large quantities of gametophytes are also easier to cultivate than macroalgae which require extensive setups with large pens/tanks or open ocean rope cultivation in sheltered bays.

U.pinnatifida gametophytes were used as biomass in the extraction of fucose containing polysaccharides (fucoidan). The gametophytes used have been grown in laboratory conditions under ideal physical parameters.

MATERIALS & METHODS

Collection of sporophytes and maintaining vegetative gametophytes for hybridization

U.pinnatifida was harvested in November from Port Underwood in the Marlborough Sounds, New Zealand (-41.308488, 174.159376). The seaweed was harvested individually by hand, on a barge, from selected mussel lines on the farms, ensuring the mussels did not get damaged in the process. Mature and healthy-looking plants were collected. Once the seaweed was on the barge deck, it was flattened and the length and width of the frond were recorded. The length was recorded from the tip of the stipe to the bottom of the sporophyll and the width was recorded by calculating the distance between the widest extremities of the frond. Once the dimensions of the entire plant were recorded, the sporophyll was cut off from the main plant using a sterilized scalpel and separate measurements were taken of the sporophyll. The whole plant and the sporophyll were photographically documented for a structured record keeping.
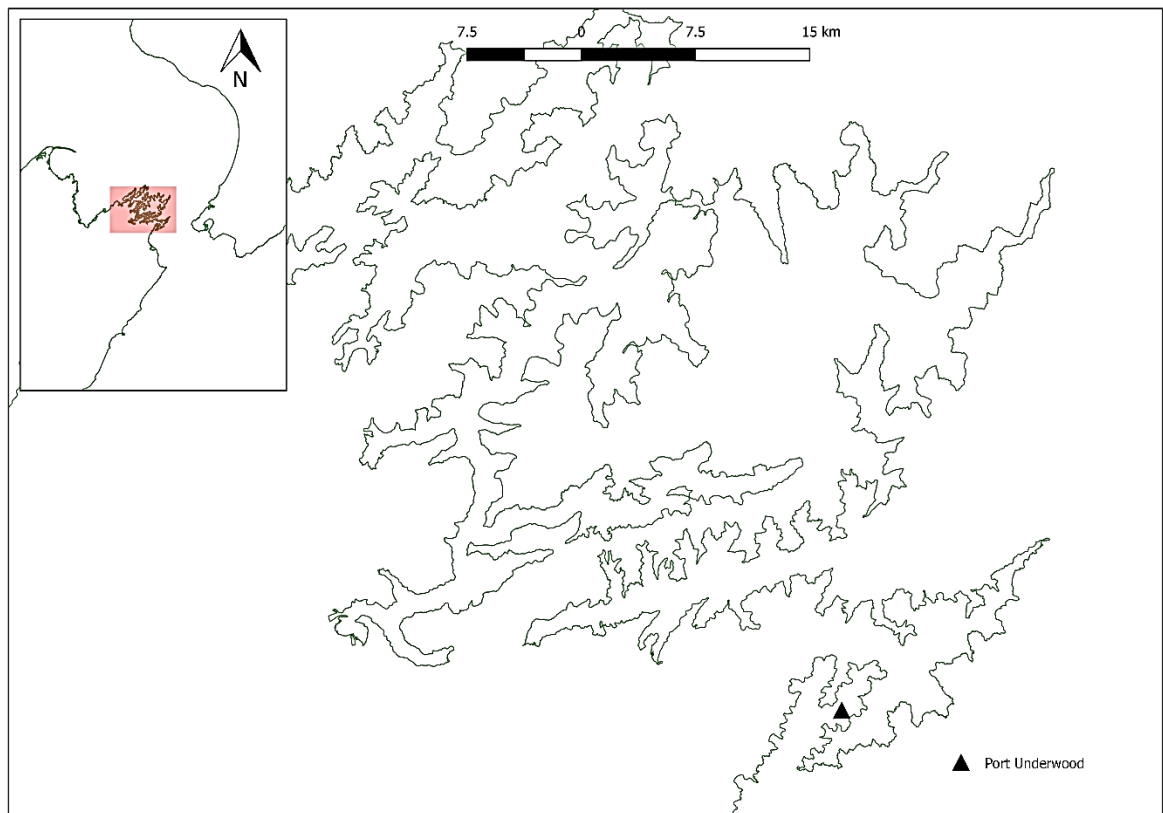
Figure 6. Harvesting U. pinnatifida at Port Underwood from long-lines on mussel farms.

Once the physical dimensions were recorded, the sporophylls were halved by slitting the stipe longitudinally and further cutting the half segments into approximate semi-circular segments. The segments were then washed thoroughly in sterilized seawater using a soft nail-brush. The brush was used to remove epibionts and other debris that might be attached to the sporophyll. After rinsing in the seawater, the sporophylls were subsequently surface sterilised in a diluted solution of 0.5% vol/vol 3.4% sodium hypochlorite (commercial bleach). These segments were then rinsed again in a bath of sterilized seawater to rid them of any lingering smell of chlorine. Once washed, the sporophyll segments were blotted dry using paper towels. The segments were arranged vertically on strips of paper and

were subsequently blotted with another sheet of paper on top. After drying, the segments were rolled up in dry papers and stored in resealable plastic bags, which were then placed in the dark in a chilly bin. The chilly bin had ice packs in it to maintain an approximate temperature of around 15°C. The dried segments were stored for 5-6 hours in the chilly-bin, till they could be transported back to the lab at the university later that evening. This slight induced dehydration of the chlorophyll segments results in a favorable outcome for zoospore release when rehydrated later in the laboratory.

Once back at the lab, the dried sporophyll segments were removed from the chilly-bin to start the process of rehydration and spore release. Spore release was done using a synthetic seaweed media, called the "*French*" media. This media was prepared by referring to Cawthrons "A practical manual for culturing the Asian sea vegetable 'wakame' *(Undaria pinnatifida)*. This media is a mixture of five stock solutions derived from those described by Provasoli et al. 1957.

i)   Miguel A
ii)   Miguel B
iii)   Provasoli P6
iv)   Germanium dioxide
v)   Kanamycine

Table 4. Miguel A stock solution composition

Chemical		$g.l^{-1}$
Magnesium sulphate	$MgSO_4$	100
Sodium chloride	$NaCl$	100
Sodium sulphate	$NaSO_4$	50
Ammonium nitrate	NH_4NO_3	10
Potassium nitrate	KNO_3	20
Sodium nitrate	$NaNO_3$	20
Potassium bromide	KBr	2

Potassium iodide	KI	2

All chemicals are made up to 1 litre solution in distilled water.

Dilution rate: 2ml per litre of sterilised seawater.

Table 5. Miguel B stock solution composition

Chemical		$g.l^{-1}$
Sodium hyposulphite	Na_2HPO_4	24.83
Calcium chloride	$CaCl_2$	33.55
Hydrochloric acid (conc)	HCl	25ml
Ferric chloride	$FeCl_3$	25

All salts used are anhydrous salts. Ferric chloride was mixed in 250ml distilled water, more 500ml water was added after which sodium hyposulphite and calcium chloride were added. Lastly, conc. HCl was added and the volume was brought up to 1ltr with distilled water. The solution was left on a magnetic stirrer overnight. It was filtered the next day with a 0.45µ filter to remove any undissolved ferric chloride to obtain a clear, straw coloured solution.

Dilution rate: 1 ml per litre of sterilised seawater

Table 6. Provasoli P6 stock solution composition

Chemical		$g.l^{-1}$
Zinc chloride	$ZnCl_2$	0.03129
Cobalt chloride hexahydrate	$CoCl_2.6H_2O$	0.01213
Copper sulphate pentahydrate	$CuSO_4.5H_2O$	0.0041
Sodium EDTA	Na EDTA	3.0
Ferric chloride hexahydrate	$FeCl_3.6H_2O$	0.3892

Manganese chloride tetrahydrate	$MnCl_2.4H_2O$	0.4224
Boric acid	H_3BO_3	3.432
Sodium molybdate	Na_2MoO_4	0.10935

The first three chemicals were dissolved in 100ml of distilled water and the rest were dissolved in 990ml of distilled water. 10ml of the above concentrated solution was added to the main solution to bring the volume up to 1 litre.

Dilution rate: 2.5 ml per litre of sterilised seawater

Table 7. Germanium dioxide stock solution composition

Chemical		$g.l^{-1}$
Germanium dioxide	GeO_2	1.0

The solution was heated gently to achieve complete dissolution, as GeO_2 is not highly soluble in water. It was added to the media to control the growth of diatoms.

Dilution rate: 1 ml per litre of sterilised seawater

Table 8. Kanamycine stock solution composition

Chemical		$g.100\ ml^{-1}$
Kanamycine acid sulphate		10

Kanamycine was used as an antibiotic, and was added to the media in initial stages to control growth of blue-green algae which compete with *Undaria* for nutrients.

Dilution rate: 0.5 ml per litre of media

The 'French' medium was prepared a day in advance of going for collection of sporophylls. This was done to ensure the media was maintained at ~15 deg C

overnight, before used to release zoospores from the sporophylls. Once the sporophylls were brought back to the lab, the dried sporophyll segments were placed in 500ml beakers and ~200ml sterilized seawater was added. Sporophyll segments from different plants were suspended in separate beakers to avoid mixing the zoospores. The water was sloshed around on the segments and once rehydrated, they immediately released zoospores as a cloudy, translucency in the water. When the water became translucent, a sample was taken and checked under a microscope at 40X magnification which had 100's of actively swimming spores. The sporophylls weren't kept for a long duration in the seawater, to minimize the risk ok contamination. The sporophylls were then removed from the beakers with sterile forceps and the resultant 'spore soup' was drained through a 20μ mesh filter into labelled sterile 500ml flasks. This was done to further reduce the transfer of any contaminants and debris like mucilage exuded from the sporophyll sections, from interfering with the growth of the zoospores.

Once the filtrate was transferred to the final labelled flasks, they were topped up with culture media and sealed with bungs made from cotton wool. These beakers were then arranged in a Panasonic MLR 352 series environmental chamber which was pre-set to a light intensity of ~1000 lux and 15 deg C. They were all set up over 3 levels of the environmental chamber while ensuring that similar levels of irradiation will be reaching all the experimental beakers. This was done using a light meter to gauge the light intensity in lux units reaching the various sections of the environmental chamber. The flasks were arranged in banks of 3 and rubber tubing was used to facilitate airflow through them, to ensure the gametophytes remained 'free living' and in suspension. An air-pump mounted on top of the environmental chamber was used for air-flow. Using a splitter, the main pipe coming from the air-pump was split to individual flasks and to further ensure no contamination, 20μ air filters were attached to the tubing before the air entered the flasks.

Figure 7. Panasonic MLR 352 environmental chamber used for culturing gametophytes

Figure 8. Setup for U. pinnatifida cultures in the environmental chamber.

U. pinnatifida gametophytes growing in the culture media were monitored under a Leica DM2500 microscope. 10X, 20X and 40X magnifications were used to monitor growth and differentiate between the sexes. It was also used to check for any contamination of the culture by blue-green algae or other microbes. A Leica image capture software was used in conjunction with a camera mounted on the microscope to capture gametophyte images for data and keeping a record of their growth and development.

Experimental separation for male and female gametophytes was trialed with the gametophytes. This was done by taking a very dilute spore solution and pouring it into a 96 well plate. The spores settled separately and they were then allowed to

grow into clumps. These clumps were then individually transferred by sterile forceps to another 16 well plate and teased to separate the gametophytes. Once the gametophytes clumped again, that mass was transferred to another sterile flask and set up in the environmental chamber to be cultured in the normal way.

Culturing in aquaculture effluent and nutrient uptake

This experiment was carried out at the National Institute of Water and Atmospheric Research (henceforth known as NIWA) site located at Bream Bay, Northland, New Zealand, 35°52'41.6"S 174°28'02.9"E.

The effluent used in this research experiment was sourced from the wastewater ponds on the NIWA site. The effluent is from the Oceanz paua (abalone) land-based aquaculture farm located on the site. The effluent discarded from the farm ends up in the settling pond initially, from where it is drained into the digestion pond after the suspended solids have settled in the pond. The overflow from the settling pond is diverted away to mix with the overflow from the digestion pond, resulting in a mixture which is eventually released back into the ecosystem. The effluent was sourced from four different extraction points across the existing pond setup by NIWA. The choice of four different extraction points was to gauge the level of microalgae growth across the varying levels of wastewater treatments and to gauge the amount of nutrient uptake from each extraction point.

The four effluent extraction points were Settling Pond Inflow (SPIF), Settling Pond Overflow (SPO), Digestion Pond Overflow (DPO) and Combination Outfall Drain (COD) (a combination of Settling Pond Overflow and Digestion Pond Overflow with a ratio of 30%-70% in that order).
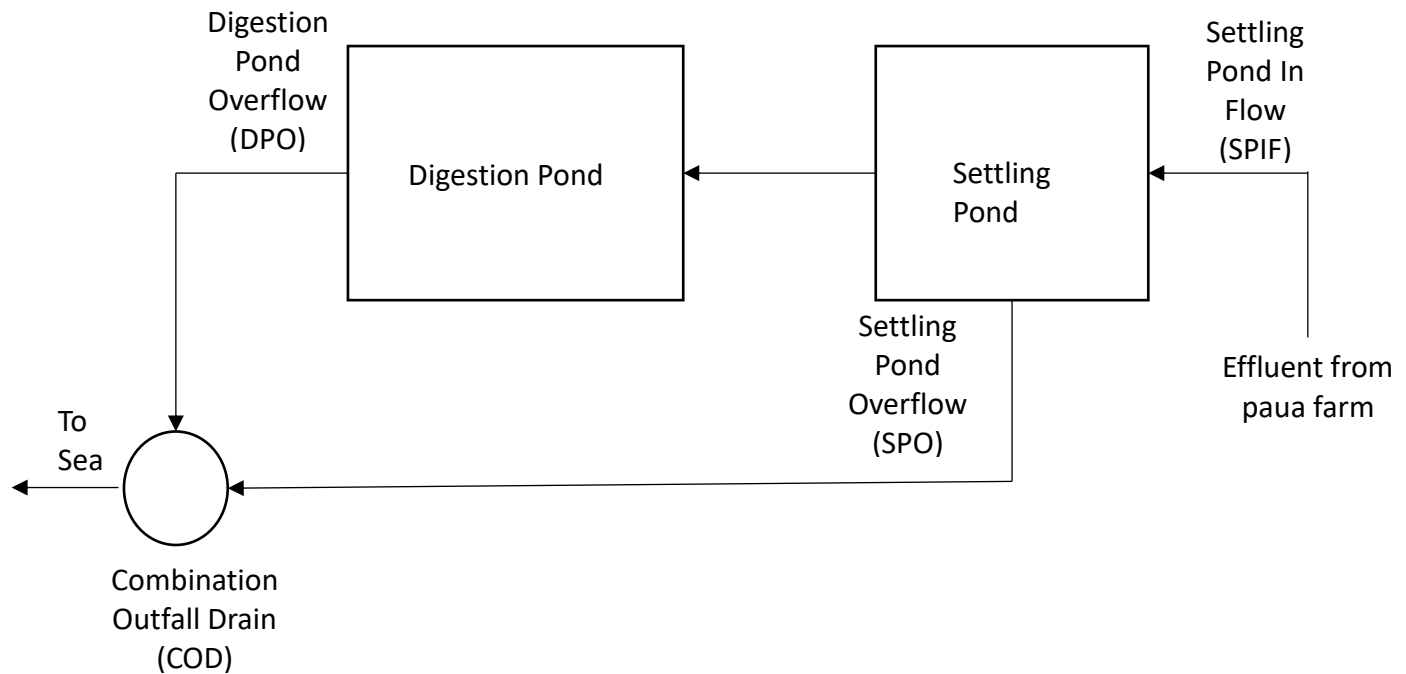
Figure 9. Layout for effluent ponds on NIWA Bream Bay site and effluent extraction points.

A water pump was used to extract and collect the wastewater from the ponds in clear 15L plastic carboys. Upon collection, the wastewater was filtered through a 5μm mesh bag and measured up to 9.5L per carboy. Subsequently they were autoclaved along with the rubber stoppers and air tubes to ensure sterilization of the whole setup. After sterilization, the carboys were arranged in two rows on a metal shelf unit in a temperature-controlled room. A space heater was used to maintain the temperature in the room within a preset range of 21 deg-25 deg. Philips TLD 58W/840 cool white lights were used in four banks (two tubes in each bank) attached to the roof of the metal shelf and were arranged along both rows of carboys, providing light intensity in the range of 2000-3500 lux (mention in Einstein units) to all the carboys. Air was supplied to each of the 15 individual carboys by connecting tubing from the existing air-line in the experiment room.

Figure 10. Experimental setup at NIWA Bream Bay Aquaculture Park at Ruakaka, Northland, NZ.

Once the carboys were setup with all the essential pre-determined physical parameters, *U.pinnatifida* gametophyte solution was added to each carboy. This stock solution had been cultured and was growing in a laboratory environment under ideal irradiance and temperature parameters. It was transported to the research facility at NIWA Bream Bay after requesting and being granted the appropriate travel and research permissions by the Ministry of Primary Industries (MPI), NZ. Permission was also obtained from MPI to undertake *U.pinnatifida* research at the NIWA facility, as this seaweed is still classified as a pest species in NZ.

The experiment was setup as triplicates for each wastewater treatment to ensure accuracy of data and results. Samples for data analysis were taken at three time points during the experimental duration. Samples for *U.pinnatifida* gametophyte growth were taken at 3, 9 and 15 days of the experiment. Falcon tubes were used to store 10ml samples taken from each treatment using disposable plastic pipettes and a pipette controller. These samples were then centrifuged and the supernatant was discarded leaving behind a mass of *U.pinnatifida* gametophytes, which was dried overnight in a freeze drier and weighed to calculate dry weight of the gametophytes. 60ml samples for nutrient analysis were taken at T0, T1 (9th day) and T2 (15th day). These samples were frozen immediately and were dispatched in a frozen state to Hills Laboratory in Hamilton for testing. The nutrient tests

performed by the lab included tests for Total Ammoniacal-N, Nitrate-N, Nitrite-N Screen and Dissolved Reactive Phosphorous.

Table 9. Description for nutrients tested from samples and summary of methods used to conduct analysis.

Test	Method Description	Default Detection Limit
Total Ammoniacal-N	Filtered sample. Phenol/hypochlorite colorimetry. Discrete Analyzer. (NH4-N = NH4+-N + NH3-N). APHA 4500-NH3 F (modified from manual analysis) 22nd ed. 2012.	0.010 g/m^3
Nitrite-N Screen	Automated Azo dye colorimetry, Flow injection analyzer. APHA 4500-NO3- I 22nd ed. 2012 (modified).	0.10 g/m^3
Nitrate-N	Calculation: (Nitrate-N + Nitrite-N) - NO2N. In-House.	0.0010 g/m^3
Dissolved Reactive Phosphorous (DRP)	Filtered sample. Molybdenum blue colorimetry. Discrete Analyser. APHA 4500-P E (modified from manual analysis) 22nd ed. 2012.	0.004 g/m^3
Phosphate from DRP	Calculation: from Dissolved Reactive Phosphorus * 3.065.	0.004 g/m^3

Over the duration of the experiment, pH fluctuation was measured for all the treatments at T0, T1 (9th day) and T2 (15th day). The pH of the treatments was measured by a Mettler Toledo Seven2Go pro pH meter, which was calibrated using pH buffer solutions of ph 4, 7, and 10 before every use. The temperature in the room was monitored every six hours from the start of the experiment using a Hoboware tidbit device and an accompanying software program to download the readings.

Figure 11. Equipment used to determine pH (Mettler Toledo Seven2go pro) and temperature (Hoboware tidbit) over the experimental duration.

Fucoidan Extraction

Fucoidan extraction and isolation from *U.pinnatifida* gametophytes following an extraction protocol of using deionized water, Calcium chloride ($CaCl_2$) salt and absolute ethanol (C_2H_5OH). *U. pinnatifida* gametophytes used for extracting fucoidan were obtained from the stock solution of vegetative gametophytes

growing in the environmental chamber. The gametophyte solution was transferred to two pre-weighed 50ml falcon tubes. These tubes were centrifuged at 4000rpm for 30 mins (Eppendorf Centrifuge 5810R V3.1), after which the supernatant was discarded to leave behind a wet mass of gametophytes. These tubes were then placed in a 50°C oven overnight to dry the gametophyte mass to be subsequently used in fucoidan extraction.

The extraction protocol followed the water extraction method, by heating about ~50ml deionized water to 80°C in a water bath and adding 20ml each to both tubes. Upon addition of the water, both the falcon tubes containing dried gametophytes were placed in a tube rack in the same water bath for ~4 hours. Over the course of the 4hrs, the gametophytes in the tubes were continuously flicked and stirred every 30 mins to ensure all the mass remained submerged in the deionized water. After 4hrs, the gametophyte solution was removed from the water bath and the supernatant was poured out into 2 separate measuring cylinders. In two separate falcon tubes, 2% w/v $CaCl_2$ was measured and added to the supernatants when still warm. This solution was reintroduced into the water bath for ~15 mins till a clear precipitate was observed. After precipitation, the tubes were taken out of the water bath and centrifuged again at 4000rpm at 30^0 C for 20 mins.
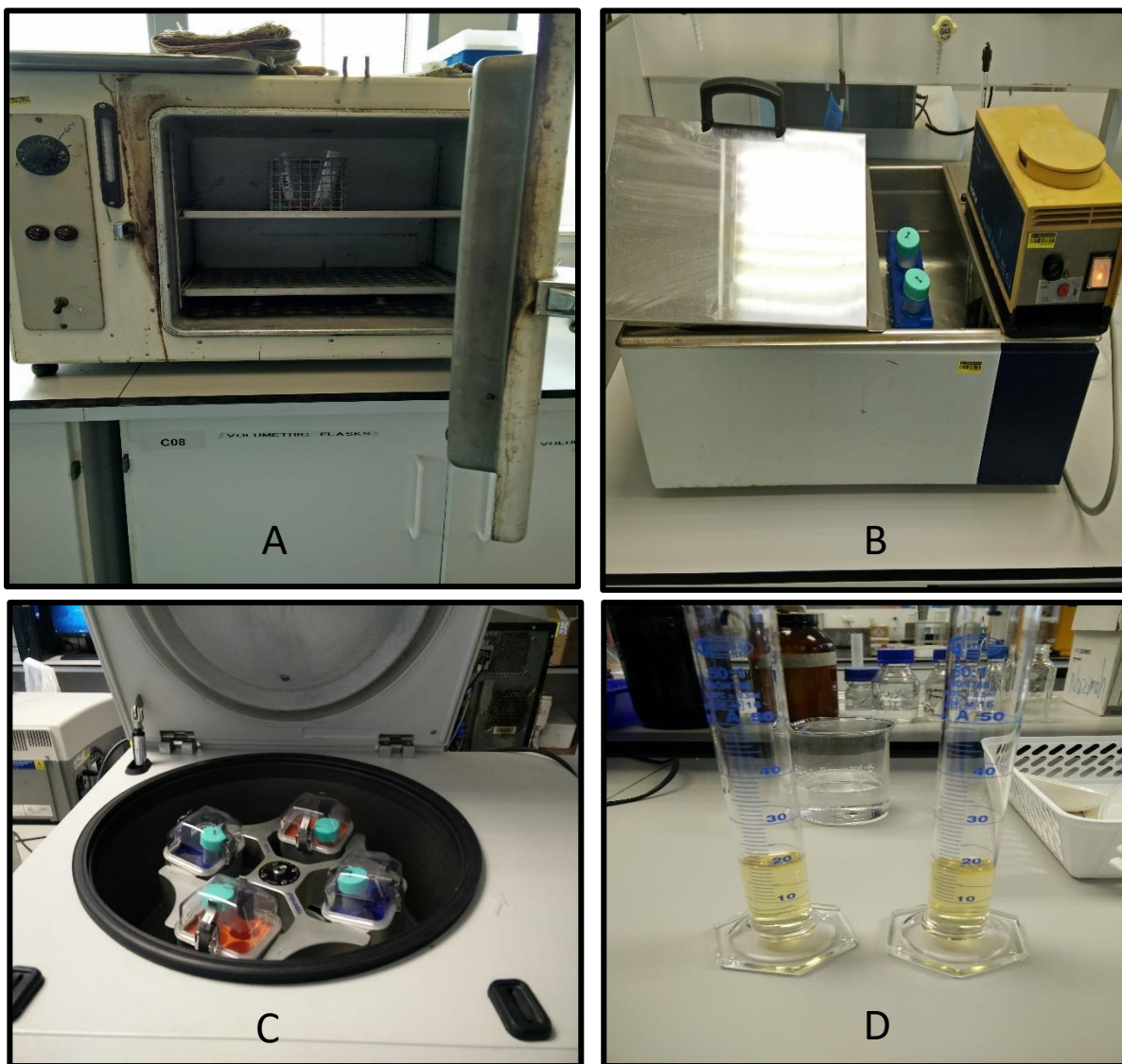
Figure 12. Equipment used for extraction of the polysaccharide Fucoidan from U. pinnatifida *gametophytes. Clockwise from left: (a) Oven drying the gametophytes in falcon tubes at 50^0 C. (b) Water bath at 80^0C. (c) Centrifuging the tubes to precipitate alginate. (d) Supernatant before adding absolute alcohol to precipitate Fucoidan.*

Centrifuging the tubes precipitated alginate, which was discarded and the supernatant was poured into 2 separate measuring cylinders again to measure the exact volume. Absolute ethanol was added to this supernatant to achieve a 70% ethanol solution. This solution was then transferred to two separate pre-weighed falcon tubes and refrigerated overnight (at 4°C) to precipitate crude fucoidan. The next morning, the cold tubes were centrifuged at 4000rpm at 18^0 C for 30 mins, to separate the fucoidan, which had a cottony appearance. The supernatant was

discarded and the precipitated fucoidan was dried in a freeze drier overnight. The falcon tubes with dried fucoidan was then weighed on the same balance to measure the dry weight of the final product. The percentage yield of fucoidan was calculated as a percentage of the original dried biomass of *U. pinnatifida* gametophytes.

RESULTS

Growing and maintaining vegetative *U. pinnatifida* gametophytes

U. pinnatifida gametophytes were cultured in a laboratory environment using an
environmental chamber to maintain ideal irradiation and temperature for growth.
The gametophytes were cultured vegetatively based on previous research
undertaken by the Cawthron Institute. The zoospores released by the sporophyll
segments were used as a starting point for these cultures. The spores were
released in a sterile beaker by rehydrating the field collected sporophylls. This
solution was further strained through a 20μ mesh filter into sterile 500ml flasks to
remove any debris or impurities. Once in the flasks, a small sample of this solution
was taken on a microscope slide, covered by a coverslip and examined under a
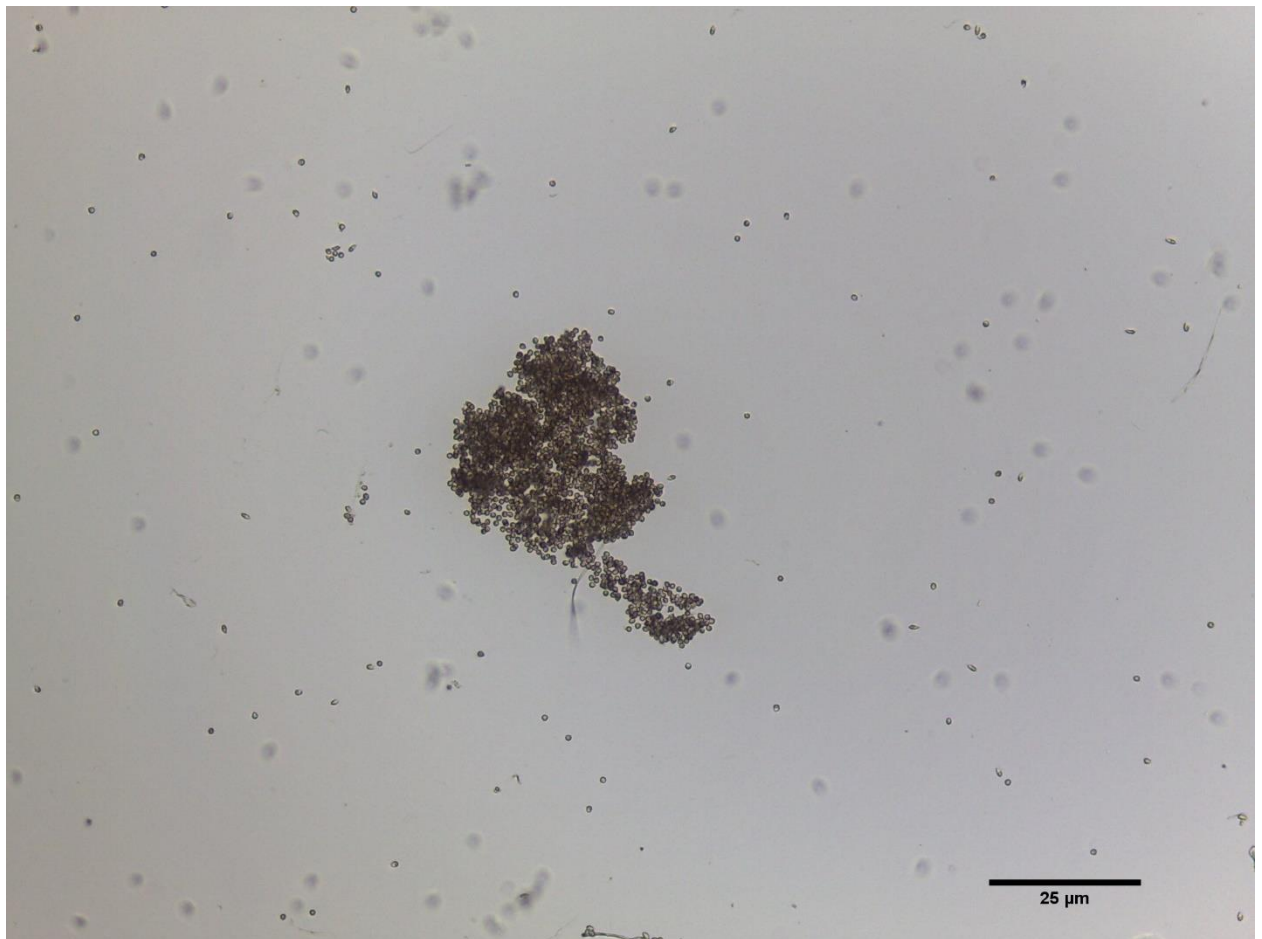microscope at 10X and 40X magnification.

Figure 13. U. pinnatifida zoospores

Initially, zoospores were identified under a microscope and after 3-4 days of culture, a sample was obtained and studied under the microscope. This sample displayed the first appearance of gametophyte cells. They had a long germination tube with the initial stages of cell division. These initial cells adhered to the phosphate precipitate crystals in 'French' media.

25 µm

Figure 14. Germinating zoospore of u. pinnatifida

Once there was a good percentage of germinated gametophytes observable in the samples, the cultures were vigorously bubbled with filtered air via a pasteur pipette connected to PTFE air filters and an air pump. If any zoospores attached to the surface of the flasks, they were scraped off every day to maintain a homogenous culture. When the cultures started to grow and thicken, the gametophytes started clumping. The clumps were broken up with an 'Ultra turrax' homogenizer to keep the cultures homogenous.
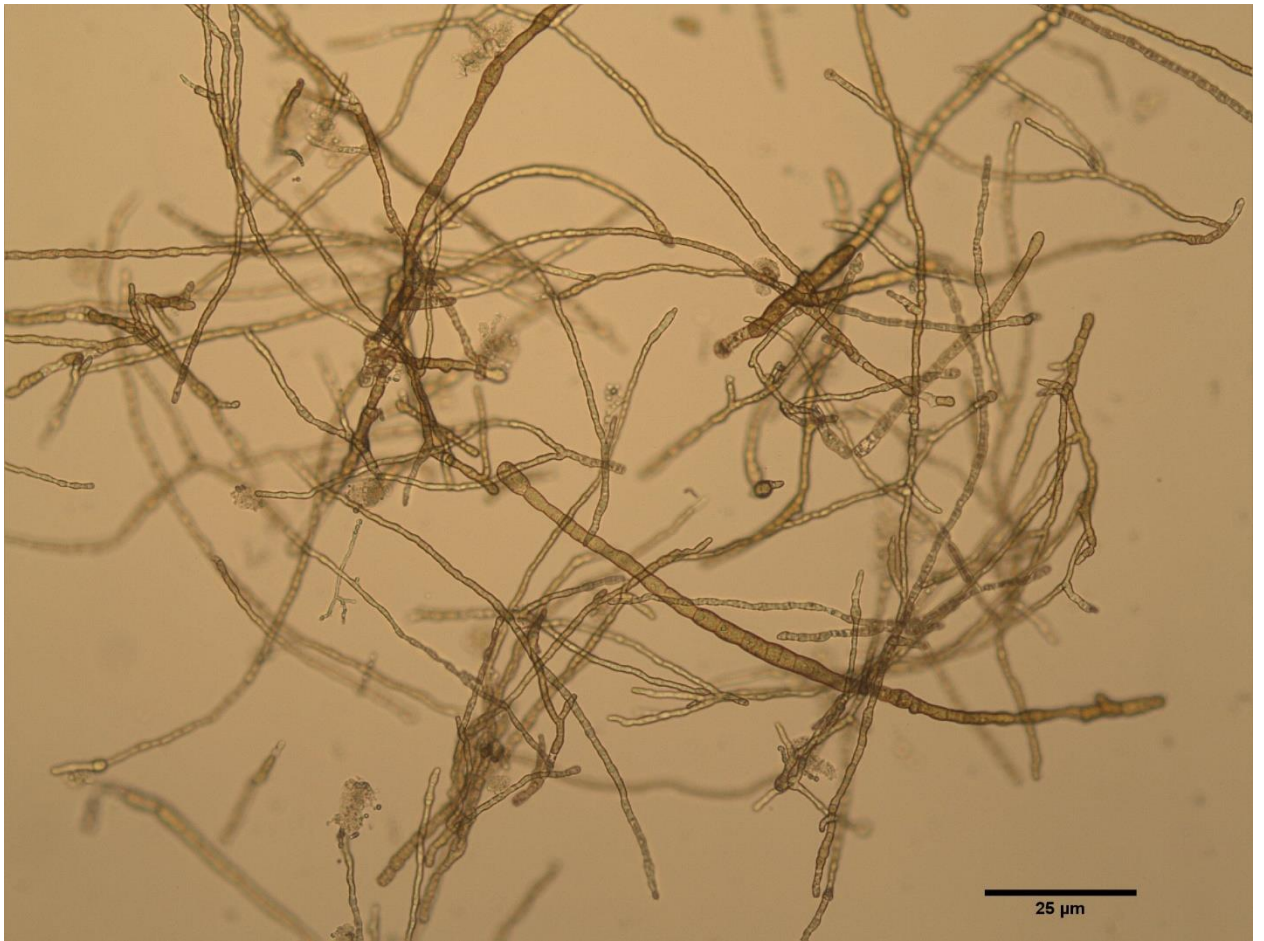
Figure 15. Clump of gametophytes after bubbling the cultures.

The cultures were then left to grow at 40μmol.cm^2 (~2000 lux) at 22°C. These parameters helped the gametophytes multiply and grow without going reproductive. Hence, they were vegetative free-living gametophytes. When the free-living *U. pinnatifida* gametophytes were observed under a microscope, they could be sexed under magnification and were distinguishable as male and female.
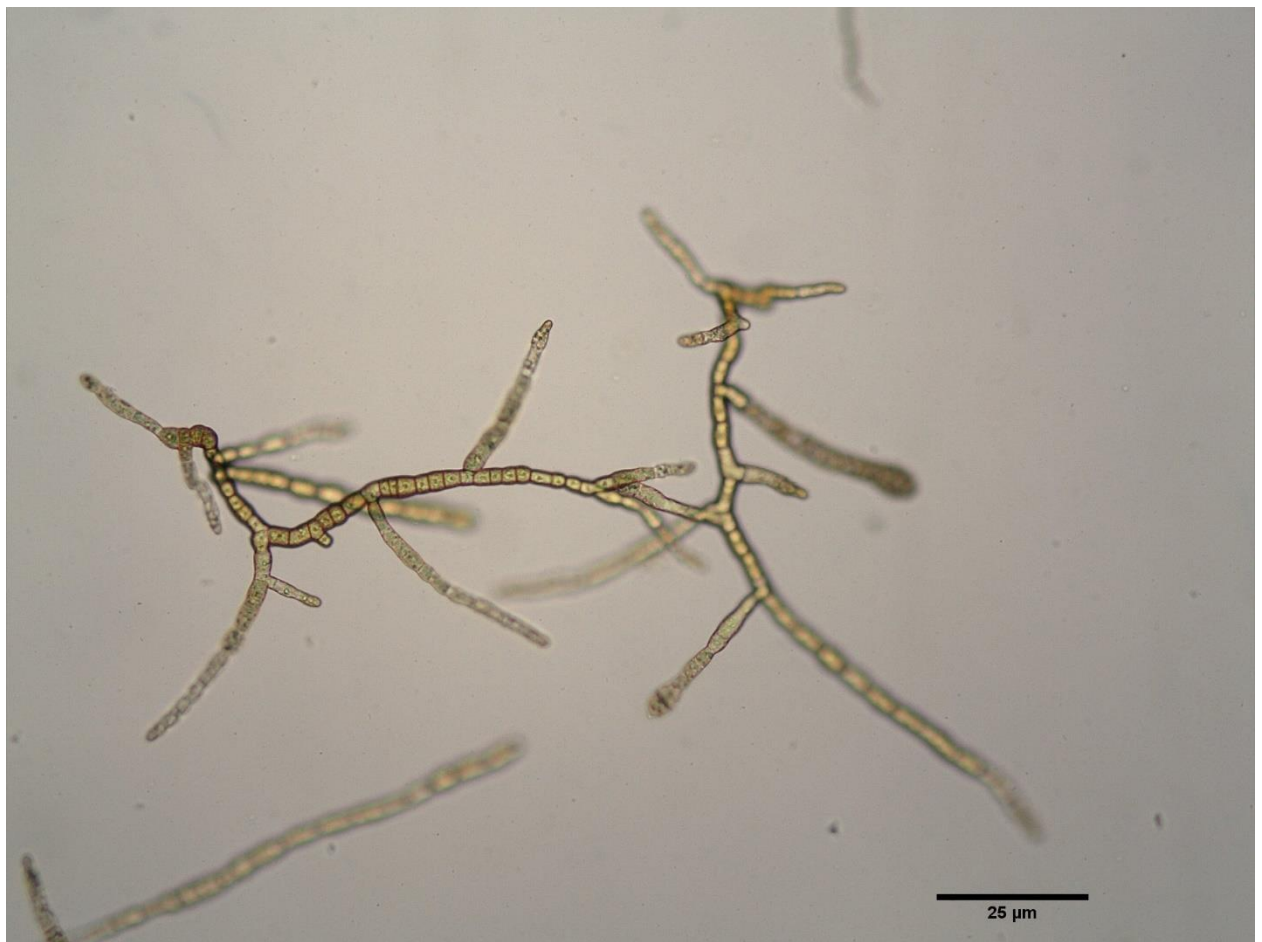
Figure 16. Male U. pinnatifida gametophytes.

Gametophyte growth in effluent

U. pinnatifida gametophytes were cultured in effluent of a land-based abalone farm. The effluent was sourced from four different extraction points from the settling and digestion ponds on-site of the abalone farm. There were significant differences in the measured growth of gametophytes over the experimental duration when grown in effluent at different stages in its treatment cycle. The growth measured for *U. pinnatifida* gametophytes cultured in effluent is represented in Figure 12. Along with growing gametophytes in effluent, they were also grown in a previously established seaweed growth medium, called the French media. This was done to assess and compare the results for growth in effluent to currently used media.
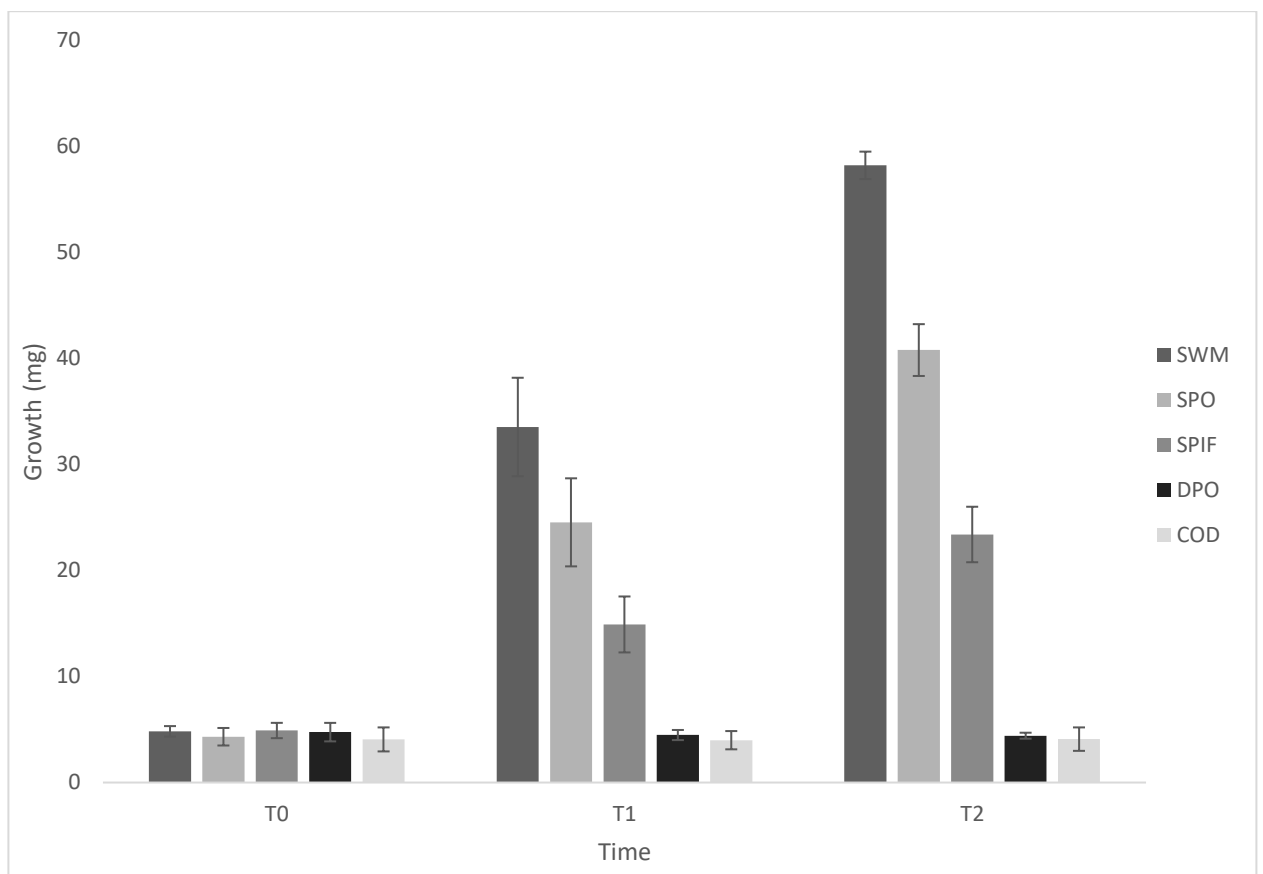
*Figure 17. G*rowth in dry weight (mg) of U. pinnatifida gametophytes when cultured in aquaculture effluent and compared to synthetic seaweed media as standard.

In the graph below, growth of *U. pinnatifida* gametophytes has been charted in relation to the percent increase over the experimental duration.
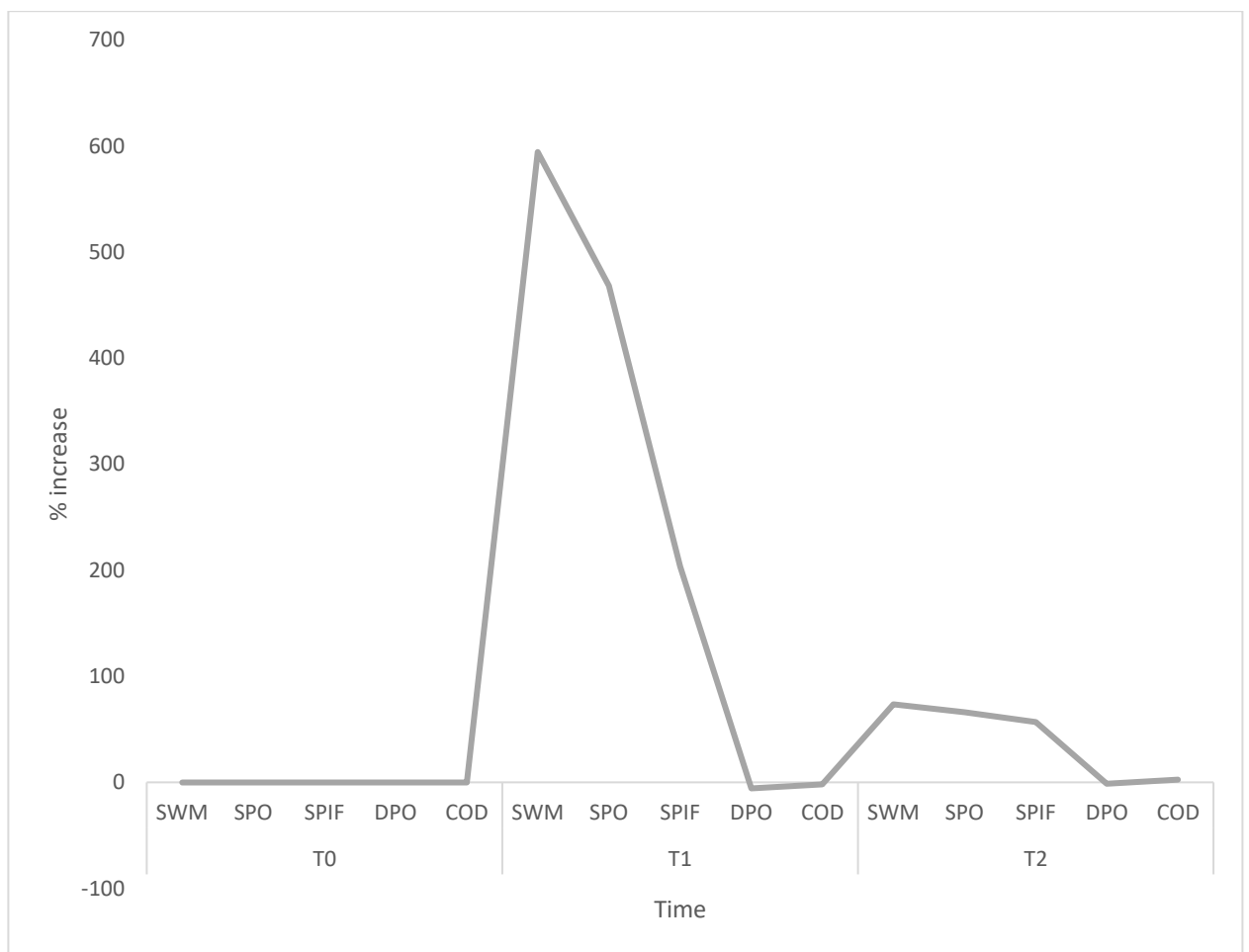
Figure 18. % increase in dry weight growth of U. pinnatifida gametophytes when cultured in aquaculture effluent and seaweed media. SWM – Seaweed media. SPO – Settling Pond Overflow. SPIF – Settling Pond Inflow. DPO – Digestion Pond Overflow. COD – Combination Outfall Drain.

There was notable growth of U. pinnatifida gametophytes in the first week of the experiment. In standard synthetic seaweed media, there was an increase in the calculated gametophyte dry weight from 4.83mg to 33.54mg in the first week, with a 594.4% increase in the growth of *U. pinnatifida* gametophytes. Over the second week of the experiment, the observed and calculated growth of the gametophytes slowed, going from 33.54mg to 58.22mg, resulting in a 73.6% increase in dry weight for the gametophytes. When comparing the growth in standard seaweed media to growth in effluent, the best growth and percentage increase is observed when the gametophytes were cultured in effluent collected from the settling pond. Specifically, the settling pond overflow (SPO) sample, taken from when the solids have settled at the bottom showed the best growth out of all effluent samples. The

dry weight of the gametophytes in this sample increased from 4.32mg to 24.55mg during the first week, showing a 468.3% growth percentage and increased to a final dry weight of 40.8mg, increasing 66.2% during the second week. Following on, the sample with the next best possible growth was the one taken from the Settling Pond again, but this time, it was the untreated effluent direct from the aquaculture farm. This was the Settling Pond In-Flow (SPIF) and the dry weight for the samples taken from here increased from 4.91mg to 14.91mg in the first week of testing, which corresponds to a 203.7% percentage increase in the dry weight for *U. pinnatifida* gametophytes. In the second week, this sample showed an increase from 14.91mg to 23.4mg, showing a 56.9% increase in dry weight. As depicted in the graph, while these three samples showed a positive increase in dry weight of the sampled gametophytes and an overall percentage increase in gametophytes, the other two effluent samples, namely the Digestion Pond Overflow (DPO) and Combination Outfall Drain (COD) exhibited no discernible growth for *U. pinnatifida* gametophytes. Observing the graphs and the results, notable differences were found in the percentage increase and dry weight increase of U. pinnatifida gametophytes when grown in effluent at different stages of treatment from a land based aquaculture farm.

Nutrient uptake of gametophytes from effluent

When *U. pinnatifida* gametophytes were being cultured in aquaculture effluent, nutrient uptake of those gametophytes was also investigated to gauge possible bioremediation properties. Nutrient uptake from the effluent was analyzed by testing initial and subsequent concentrations of NH_3, NO_3, NO_2 and dissolved reactive PO_4. The testing was all done by sending frozen samples to Hills Laboratory in Hamilton, New Zealand, who have a comprehensive facility for testing wastewater and inorganic nutrients. The tests used to analyze nutrient concentrations by the laboratory are mentioned in Table 4.

Out of all the nutrients tested, there was visible uptake of NH_3 from the samples where gametophyte growth was observed, namely Seaweed media, Settling Pond In-Flow and Settling Pond Overflow.
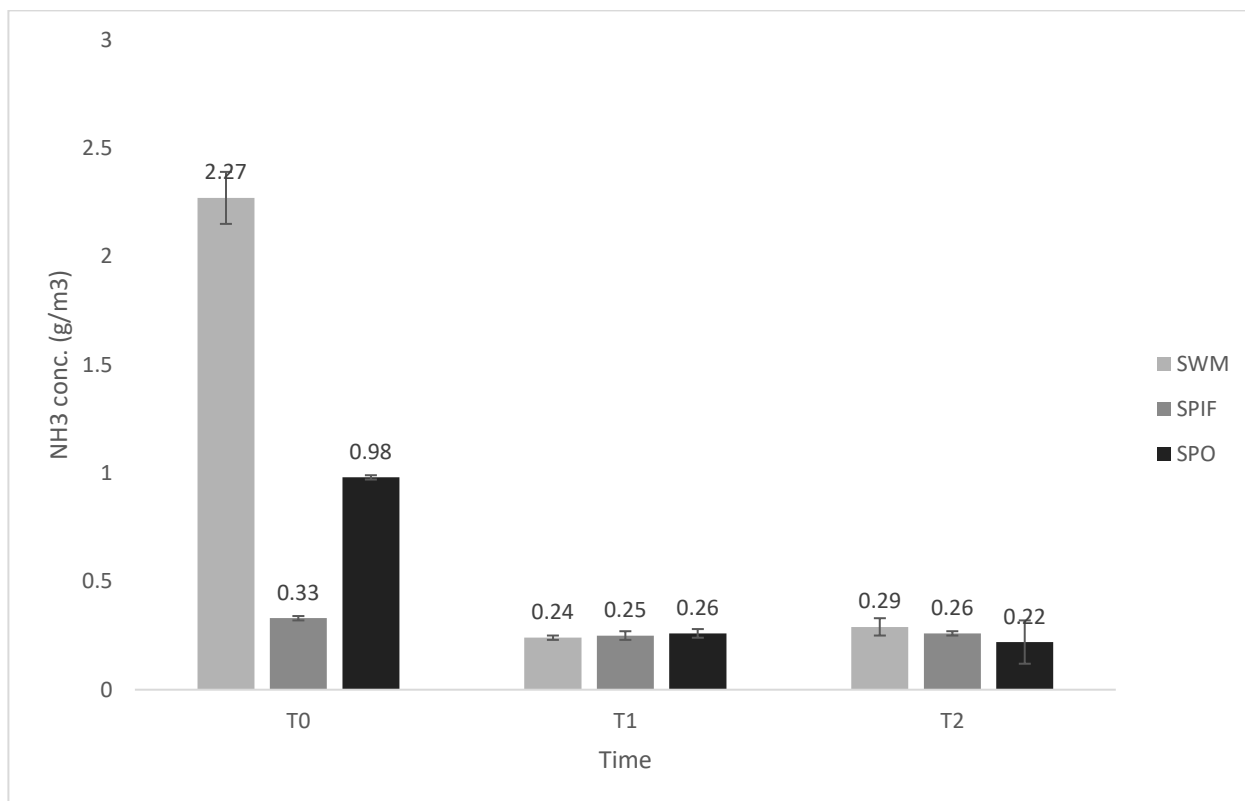
Figure 19. NH₃ uptake from effluent observed from seaweed media, and settling pond samples which exhibit growth in U. pinnatifida gametophytes. SWM – Seaweed media. SPO – Settling Pond Overflow. SPIF – Settling Pond Inflow. Default detection limit 0.010 g/m³

Total ammonia (NH_3) concentration in the effluent, calculated from the samples sent down to the lab is depicted in g/m^3. At the start of the experiment, Seaweed media (SWM) had a NH_3 concentration of 2.27g/m^3 while Settling Pond In-Flow (SPIF) had 0.33g/m^3 and Settling Pond Overflow (SPO) had 0.98g/m^3. Subsequently over the first week of the experiment, concentration in SWM had dropped down to 0.24 g/m^3, SPIF to 0.25 g/m^3 and SPO to 0.26 g/m^3. During the second week of the experiment, NH_3 concentration stayed almost the same or dropped by very little. This depicts a lesser uptake of ammonia by U. pinnatifida gametophytes by the second week of the experiment which is also reflected in a lesser percentage increase of gametophytes in the respective media solutions. The lesser uptake might also be due to a reduced concentration of ammonia in the media and effluent samples. When compared to ammonia, uptake for nitrite (NO_2), nitrate (NO_3) and dissolved reactive phosphate (PO_4) from all effluent samples

during the experimental duration was insignificant and was below the minimum detection limit of 0.10g/m^3, 0.0010g/m^3 and 0.004g/m^3 respectively.

pH and Temperature range for gametophyte growth in effluent

Along-with the growth of *U. pinnatifida* gametophytes and nutrient uptake from the effluent, pH of all effluent samples and media was recorded over the experimental duration to measure changes to the acidity/alkalinity of the media used. Per existing literature (Cawthron), seaweed media (SWM) has a pH of ~7.4 when made with Provasoli's stock solutions and allowed to stand for 24 hours. That pH goes up to 8.2 or 8.3 after about a week of gametophyte growth. In this experiment, as depicted graphically in Fig: 15, the pH for SWM was 7.34 at the start and increased to 7.65 after the first week and 7.75 at the end of the experiment. When compared to the effluent samples, pH for SPIF was 8.65 at the start and dropped down to pH 7.5 at the end of the first week where it stayed till the end of the experiment. SPO began with pH 8.65 as well and dropped to 7.65 and 7.6 at the first and second week respectively. The effluent samples which didn't show any gametophyte growth, namely DPO and COD had a starting pH of 8.4 and 8.87 respectively which changed to 7.87 and 7.7 at the end of the second week of the experiment.
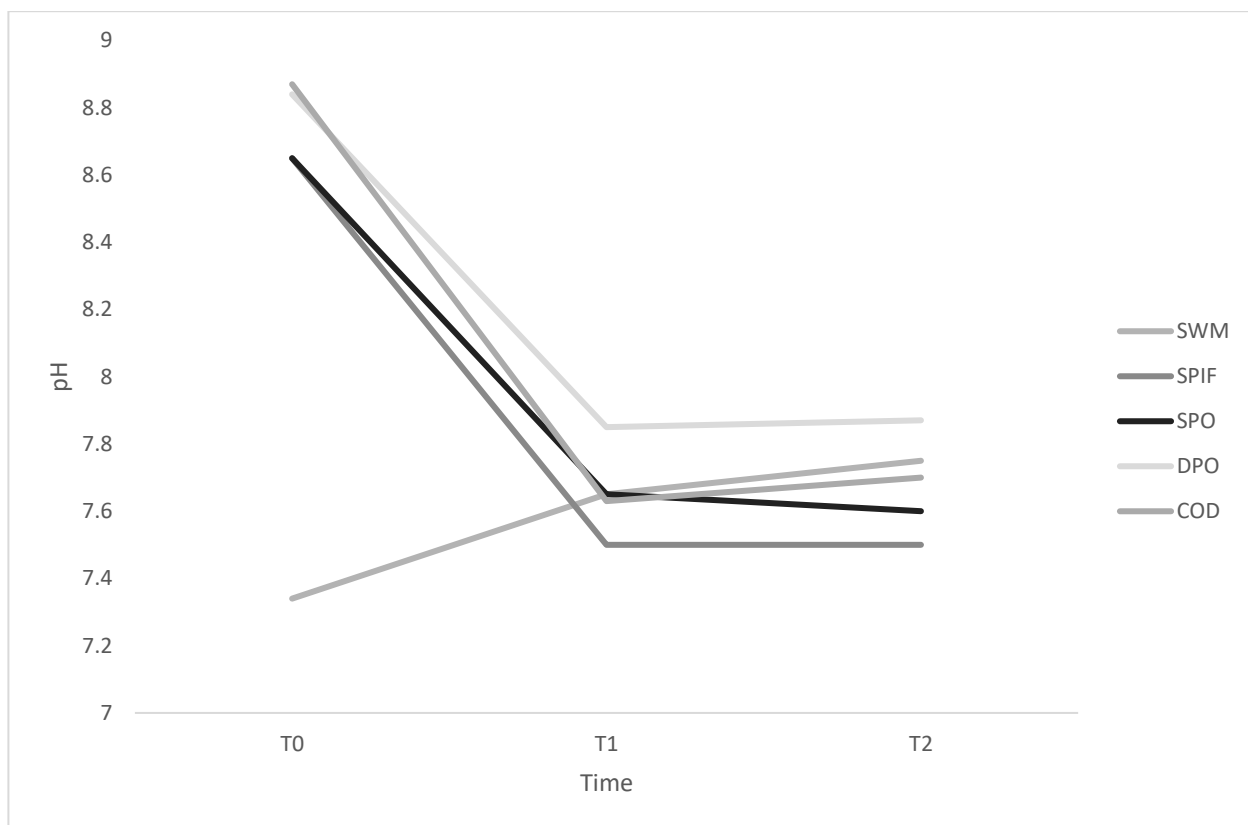
Figure 20. pH fluctuation for cultures throughout the experimental duration.

Temperature was also recorded (Fig: 16) throughout the experiment to ensure that an accurate temperature range was maintained for growth of U. pinnatifida gametophytes as per existing literature. U. pinnatifida gametophytes display ideal growth in a temperature range of ~18°C - 25°C, which was maintained throughout the experiment.
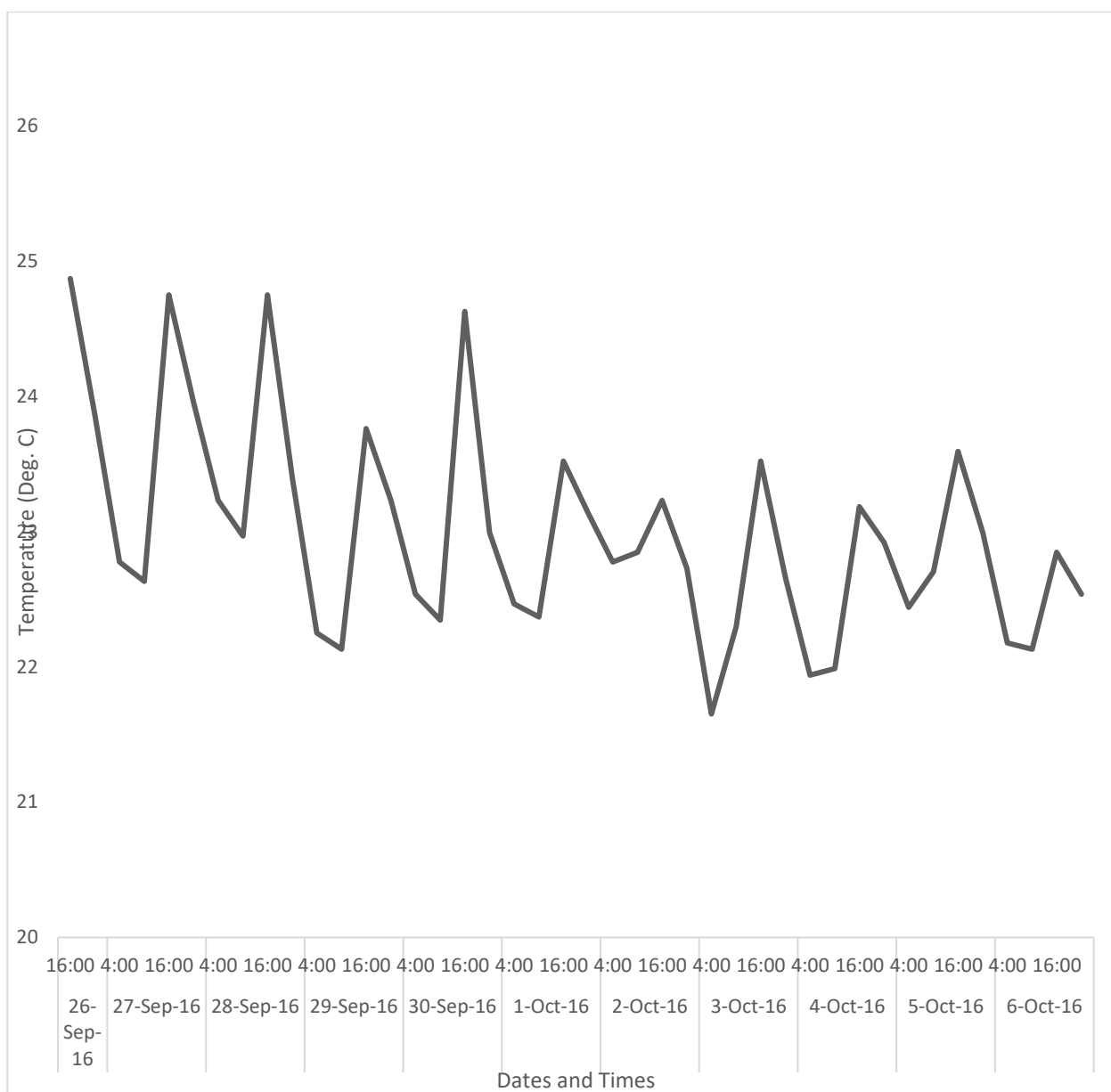
Figure 21. Temperature variations recorded over the experimental duration.

Fucoidan extraction from vegetative gametophytes

In this research study component, *U. pinnatifida* gametophytes were used to test the extraction yield of crude fucoidan. In earlier studies pertaining to *U. pinnatifida* and fucoidan extraction, crude fucoidan was extracted from the blade and sporophyll of adult mature plants. The water extraction method from Yang et al.,

2008 was used to extract fucoidan from a dried biomass of *U. pinnatifida* gametophytes.
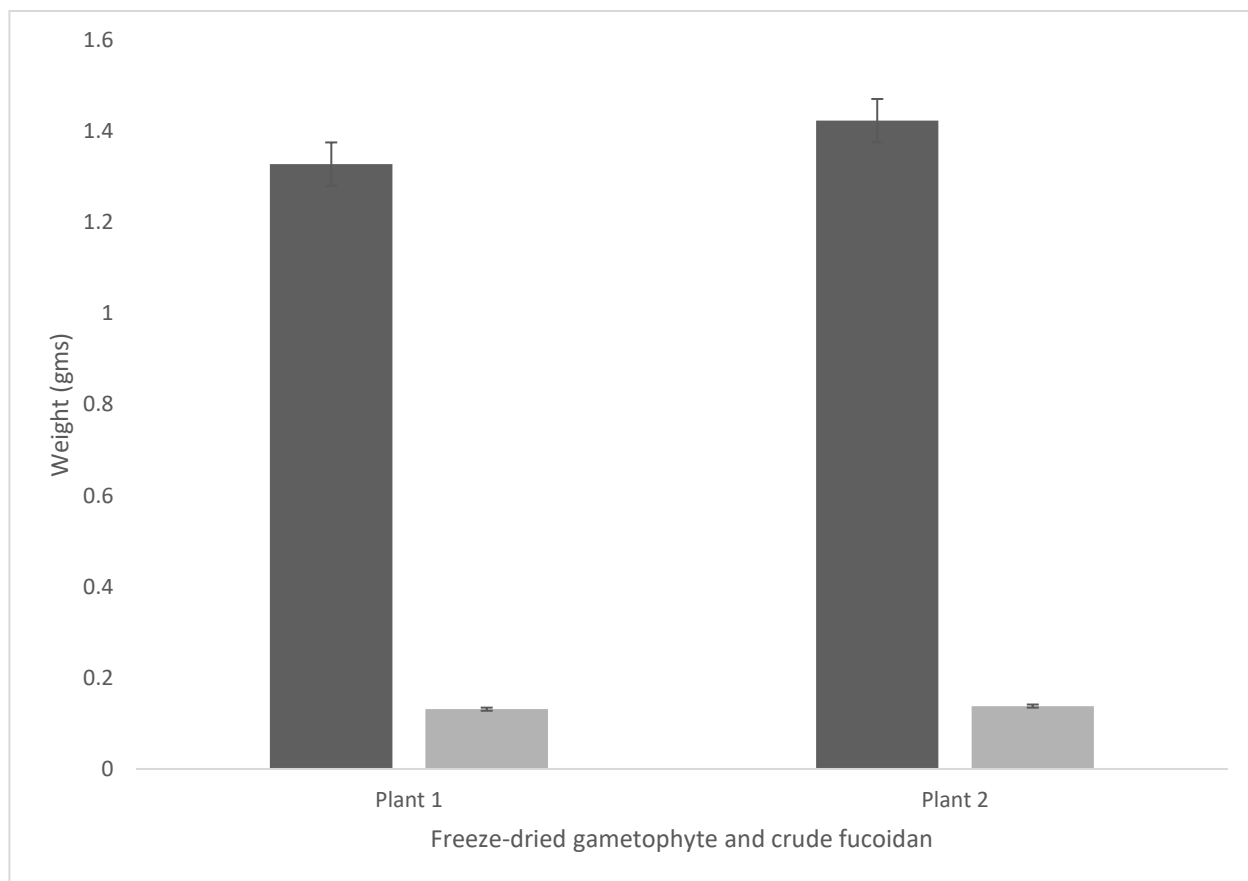
Figure 22. Crude fucoidan extracted (w/w) from freeze-dried U. pinnatifida gametophytes using deionized water extraction technique.

There were no significant differences in the fucoidan content extracted from gametophytes of two different plants. The initial weight of the dried tissue from U. pinnatifida gametophytes was approximately 1.5gms. From this dried tissue, the final dried weight of crude fucoidan extracted was 0.13gms. The percentage yield of fucoidan from *U. pinnatifida* gametophytes when treated with deionized water as the extraction solvent, was 9.88% and 9.70% from the two different plants respectively.
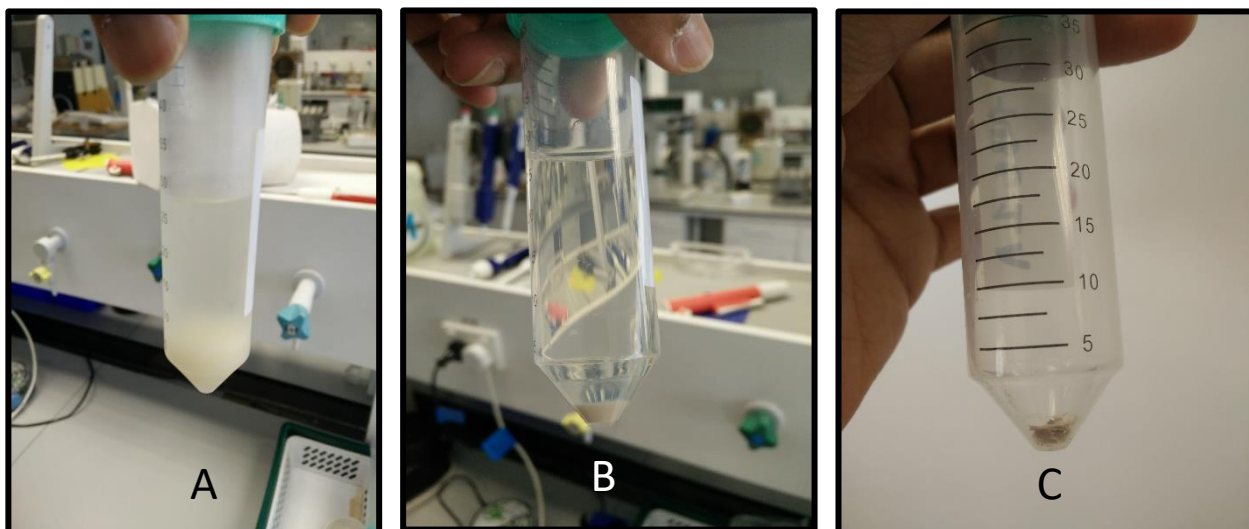
Figure 23. From left: (a) Test tube with ethanol and fucoidan. (b) Centrifuged test tube with fucoidan extract settled at the bottom. (c) Freeze-dried fucoidan extract from U. pinnatifida gametophytes.

DISCUSSION

In this study, we found that *U. pinnatifida* gametophytes could be vegetatively cultured indefinitely by renewing the culture media every 10 days and sub-culturing. The gametophytes can also be grown in aquaculture effluent as a possible replacement for seaweed media. Also, fucoidan can be successfully extracted from the vegetative gametophytes.

Vegetative gametophyte growth

U. pinnatifida gametophytes were vegetatively grown under controlled laboratory conditions of 40µmol.cm^2 (~2000 lux) (24hr irradiation) at 22°C. Zoospores were released from mature sporophylls and cultured into gametophytes to determine their survival rates and growth potential. Subsequently, single sex cultures were attempted to test future possible hybridization potential for the gametophytes. Single sex cultures are vital for hybridization because most cultures don't have an even distribution of male and female gametophytes. In cultures that are started from zoospores taken from several different sporophylls, there might be a chance of 50:50 ratio for male and female gametophytes. If a culture is predominantly consisting of one sex, then it is imperative to add single sex cultures to them during hybridization to increase the chances of inducing gametogenesis (Hay & Gibbs 1996).

Results showed that *U. pinnatifida* gametophyte cultures can be started very easily using zoospores and maintained indefinitely under ideal conditions by regular changes of 'French' media. A media change every 10 days was sufficient to maintain the growth levels of the cultures and to avoid any contamination by paramecia or fungi. The cultures grew quite quickly as well, showing good growth and getting dark in the first fortnight. Even after splitting the dark cultures, the resultant sub-cultures grew equally dark and at a similar rate.

Choi et al (2005) in their study, demonstrated that growth of *U. pinnatifida* gametophytes increased when the daylength was increased progressively from 8h to 16h. Vegetative growth was better for gametophytes at 60µmol.m^{-2}s^{-1} when

compared to 30µmol.m^{-2}s^{-1}, under a 8:16LD (Light: Dark) cycle. The responses by *Undaria* gametophytes in this study was closely related to their natural environmental daylength in their native habitat in South Korean coastal waters. Both, the daylength and the temperature of 16°, which displayed optimal gametophyte growth were similar to the natural exposure in the environment. Similar studies have shown that lower irradiance of 8.52µmol.m^{-2}s^{-1} with a 12:12LD cycle displays considerably lower growth of *Undaria* gametophytes. At this low irradiance, even higher levels of nutrients were not capable to offset the stunt in growth after zoospore settlement. In the same study, when irradiance was increased to 27.57µmol.m^{-2}s^{-1}, gametophyte growth showed improvement relative to low irradiance treatments (Morelissen et al.). Research by Akiyama (1965) and subsequent studies has demonstrated that low irradiance of ~10µmol.m^{-2}s^{-1} stunts and inhibits the growth of laminarian gametophytes. Wu et al. demonstrated that a higher irradiance level of 80 µmol.m^{-2}s^{-1} with a 12:12 LD cycle and favorable temperatures of 22-25°C showed a high growth of about 37% when compared to other low irradiance treatments in their experimental setup. This irradiance and temperature theory is supported by research from Pang and Wu (1996), who cultured *U. pinnatifida* gametophytes at 25°C and at 80µmol.m^{-2}s^{-1} light intensity with a light regimen of 12:12 LD. These parameters showed the best growth for male and female *U. pinnatifida* gametophytes with a daily fresh weight increase of about 20%. Kim and Nam (1997), noted in their study that maximum growth of the gametophytes was observed at 12:12 LD, 17°C and 60µmol.m^{-2}s^{-1}. All these studies have exhibited that a higher irradiance is crucial to the ideal growth and multiplication of *U. pinnatifida* gametophytes. Along with high irradiance, most of these studies employed a 12:12 LD light cycle to get optimum results. Temperature and light intensity are important factors for determining growth of *U. pinnatifida* gametophytes. In our study, light intensity was kept constant at 40 µmol.m^{-2}s^{-1} (~2000 lux) for 24hrs. This intensity was primarily maintained to ensure the gametophytes don't go reproductive and prevent them from becoming fertile. Once fertile, the cell division stops and the cultures won't grow anymore (Hay and Gibbs 1996). Temperature range is equally crucial when aiming for good cultures and an ideal range for growing good cultures is between 16°C-25°C. This temperature range mimics the environmental range for *U. pinnatifida* in its habitat and thus

gives the best results for growth. *U. pinnatifida* gametophytes can remain viable for up to 24 months (Stuart 2003), which enables them to delay growth and development of the gametophyte stage until physical and nutrient conditions are suitable for growth.

Gametophyte growth in aquaculture effluent

In this study, gametophytes of the brown seaweed *U. pinnatifida* were cultured in aquaculture effluent. This study was undertaken with the help of NIWA, using their facilities at the aquaculture park located at Bream Bay in Northland, New Zealand. The effluent was obtained from a functional land-based abalone aquaculture plant located on-site

Obvious differences were found in the relative growth of gametophytes in the different stages of aquaculture effluent collected from the waste-water ponds. Furthermore, better gametophyte growth was observed in the control synthetic seaweed media when compared to the best growth in aquaculture effluent. Visual inspection of the experimental carboys showed that Settling Pond Overflow had the best and densest gametophyte growth, followed by Settling Pond Inflow. The effluent extraction points of Digestion Pond Overflow and Combination Outfall Drain had no growth at all. Off all the effluent experimental samples, none had as dense growth as the control culture in 'French' media.

Previous studies have shown promising results for growing commercial quantity seaweed in aquaculture effluent. Effluents were used to grow different types of seaweed for their rich nutrient source, while also calculating nutrient availability for growth and subsequent nutrient removal. Research conducted by Corey et al. studied the growth of *palmaria palmata* integrated with Atlantic halibut in a land-based aquaculture system. Their results concluded that stocking density of the seaweed farmed in effluent with a 16:8 LD cycle and between 8.0 to 9.0°C, increased from 2.95 kg m^{-3} to 9.85 kg m^{-3} in the year of research. Seaweed grown in ambient seawater had relatively lesser stocking density when farmed under the same physical parameters. *Palmaria palmata* also displayed an uptake in nitrogen from the effluent as evidenced by 4.2 to 4.4% DW tissue nitrogen in the seaweed

when compared to 3.0 to 3.6% DW in the control ambient seawater. Experimental research on integrated aquaculture of three potentially valuable red seaweeds (*Chondrus crispus, Gracilaria bursa pastoris and Palmaria palmata*), in effluent rich farms of local turbot and sea bass exhibited good growth and increases in stocking densities across all three seaweeds. All treatments displayed varying levels of nitrogen uptake efficiency, ranging from 40.2% to as high as 83.5% with a cascade system. The growth and nitrogen uptake efficiency of the seaweeds was heavily influenced by the environmental factors and thus were highly seasonal (Matos et al.). In yet another study undertaken to gauge nutrient uptake and growth, *Ulva lactuca* was cultivated in an integrated aquaculture system, to serve as abalone feed. Effluent from abalone farms was supplemented with fertilizers to increase efficacy of seaweed growth. This experiment showcased a high rate of ammonium bioremediation at the experimental farms, with about 60-90% ammonium being removed from the effluents during day and night (Robertson-Andersson, 2003).

This study shows similar results in culturing *U. pinnatifida* gametophytes in aquaculture effluent. Gametophytes show growth in effluent and also display ammonium uptake during the growth phase.

Fucoidan extraction from vegetative gametophytes

Fucoidan is a sulphated polysaccharide, found predominantly in the intercellular spaces and cell walls in seaweeds. Fucoidan extraction from seaweeds is of much importance because of its potential uses in pharmaceutical or nutraceutical products (Ly et al. 2005). Due to its commercial importance, extensive research has been conducted on fucoidan extraction from various seaweeds. Up until now, studies have shown ways and methods to extract fucoidan from mature sporophylls or fronds of mature plants. In this study, fucoidan was extracted from vegetative *U. pinnatifida* gametophytes. The yields of crude fucoidan from mature plants and sporophylls growing in New Zealand waters served as an interesting comparison to yield from vegetative gametophytes. In a study conducted by Mak et al. (2013), crude fucoidan was extracted using $CaCl_2$ method on a monthly basis from July to October 2011 from *U. pinnatifida* harvested from mussel farms in the

Marlborough Sounds, New Zealand. Fucoidan yield from the mature sporophyll showed significant increase from July (25.4-26.3%) to September (57.3-69.9%). Compared to the above research, extraction from vegetative gametophytes using deionized water method, yielded 10% w/w crude fucoidan. Cumashi et al., conducted a study on nine species of brown algae and evaluated fucoidan content in them. The fucoidan content in all nine species studied ranged from 24.8% to 58.7% w/w.

In summary, from our study, crude fucoidan content in vegetative gametophytes was quite low when compared to extracted crude fucoidan from mature sporophylls and plants. Previous studies for *U. pinnatifida* and other brown seaweed display a much higher w/w ratio of crude fucoidan. But, when extracting from vegetative gametophytes, because of proven continuous lab culturing, there is an almost never-ending supply of material to extract from which can make up for the deficit in actual fucoidan content. Crude fucoidan extraction doesn't have to depend on environmental factors for a supply of mature plants.

CONCLUSION

This study exhibited that vegetative *U. pinnatifida* gametophytes can be cultured in a conducive laboratory environment and thus can be used as a springboard for further hybridization studies leading to eventual farm trials. The gametophytes can be cultured indefinitely in their vegetative state by sub-culturing the dark, well grown cultures.

These gametophytes can also be cultured in nutrient rich aquaculture effluent without needing any other additives. This can possibly lead to further trials in bioremediation of aquaculture effluent and integrated systems with land-based aquaculture farms. Finally, fucoidan can also be extracted and refined from the gametophytes, leading to another revenue stream down the line for any interested farmers. This research proved that there is merit to further experiments on *U. pinnatifida* farming which are economically viable as there are various environmental advantages to growing Undaria in NZ waters.

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