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Cultivation of *Saccharina latissima* (Sugar kelp) using sea urchins crush as a nutrient source

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Acknowledgments

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Abstract

Saccharina latissima is a species of brown seaweed suitable for cultivation and has different usage such as food, biofuel, and feed. Further, the fisheries industry produces tons of byproducts while processing seafood, which will be discarded as waste. In the sea urchin processing industry, for instance, most parts of a sea urchin, such as shell, spines, and viscera, will be discarded after removal of the commercially used gonads (roe). The thesis aims to investigate the potential of a method of utilizing sea urchin byproducts as fertilizer to enrich the water for the cultivation of *S. latissima* sporophytes. The young sporophytes were cultivated in (a) 10 (low) versus 50 (high) ml of nutrient concentrations of urchins rough crush solution supplied for a repeated short period (pulse) and (b) 10 (low) ml of nutrient concentration of the urchins rough crush solution supplied pulsed versus continuously. The addition of urchins rough crush solution did not increase the nitrate concentration but considerably increased the phosphate concentration in the water. Results reveal that sporophytes exposed to a different volume of urchins rough crush solution have less length growth than the control (unenriched) treatment. Sporophytes in high concentration of urchins rough crush solution supplied pulsed have the poorest length growth. However, the nutrient exposure period (pulse or continuous), was of negligible impact on the length growth of young sporophytes. Both, the concentration of urchins rough crush solution and exposure duration had a negative impact on the length of a color bleached part of lamina of *S. latissima*. The sporophytes exposed to high concentration of urchins rough crush as pulsed and low concentration of urchins rough crush supplied continuously have longer part of lamina color bleached than the control. From this study byproducts from the fisheries industry are recognized as potential fertilizer in the cultivation of *S. latissima*. However, the use of urchins rough crush is less suitable for the length growth of *S. latissima* and increased the bleaching of *S. latissima* lamina. Further studies should be done in the analysis of urchins rough crush, to better understand the cause of less length growth and enhanced color bleaching in *S. latissima*.

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Abbreviations

ANOVA	Analysis of variance
C	Carbon
DM	Dry matter
N	Nitrogen
NO3 -	Nitrate
Nofima	Norwegian Institute of Food Fisheries and Aquaculture Research
PO_4^{-1}	Phosphate
P	Phosphorus
WW	Wet weight

1. Introduction

1.1 Seaweeds and their global production

Seaweeds or marine macroalgae are found in coastal regions from warm tropics to cold polar regions all over the world. They are divided into three categories: red seaweed, brown seaweed, and green seaweed based on their color pigmentation (Mouritsen, 2013). Seaweeds play a crucial role in the marine ecosystem as a primary producer, they provide food, habitat, and breeding area to many marine organisms including fish and crustaceans (Moy & Christie, 2012; Wiencke & Bischof, 2012). Seaweeds are used as food, feed for livestock, and bioenergy by humans (Van den Burg et al., 2016). Hence, the cultivation and harvest of wild seaweeds have been increasing with time. In 2019, global seaweeds production was 34.7 million tons and 1.1 million tons from culture and capture, respectively (Cai et al., 2021). Global seaweed cultivation is largely dominated by Asian countries. For instance, China cultivated 20 million tons of wet weight (WW) of seaweed followed by Indonesia which cultivated 9 million tons (WW) in 2019 (Cai et al., 2021). In European countries, wild kelp has been harvested for a long time, while the cultivation process is still in its preliminary phase (Forbord, 2020). In 2019, Europe contributed 0.8 percent to the global seaweed production with 2,87,033 tons (WW), of which 11,125 tons (WW) originate from cultivation (Cai et al., 2021).

1.2 Application of seaweeds

Seaweeds have an over 2000 year long history of production and consumption as food in Asian counties like China, Japan, and Korea (Tiwari & Troy, 2015). In Europe, the consumption of seaweed as food has been limited to coastal communities, and over the past centuries (Stévant et al., 2017). However, at present, seaweeds are widely consumed as food throughout the world. The seaweeds that have been already used in Asian cuisine for decades can be now seen as a promising ingredient in Nordic cuisine, main species like *Saccharina latissima* and *Plamania palmata* because of their umami taste (Mouritsen et al., 2012). Seaweeds are rich sources of polysaccharides, vitamins, and minerals. Apart from that, they contain bioactive substances like proteins, lipids, and polyphenols with anti-inflammatory, anti-microbial, and antioxidant properties (Holdt & Kraan, 2011). Thus, they are used by pharmaceuticals for their medical and clinical purposes. Seaweeds are used as feed to farm animals and organic fertilizers in agriculture.

Seaweeds of genera *Laminaria*, *Ascophyllum*, and *Sargassum* are used as organic fertilizers in agriculture (Pati et al., 2016). The industrial use of seaweeds to extract hydrocolloids (alginate, carrageenan, and agar), is growing at a rate of 1 to 3% per year (Bixler & Porse, 2011). Besides their commercial value, seaweeds can also act to bioremediate excess nutrients in the marine environment originating from agriculture and/or aquaculture practices. A wide range of seaweed genera; green seaweed (*Ulva*), red seaweed (*Gracilara*), and brown seaweed (*Saccharina*, *Undaria*, and *Sargassum*) are used for bioremediation (Neveux et al., 2018). Intensive open water aquaculture releases a considerable amount of waste in the marine environment which results in eutrophication and has a negative impact on the marine ecosystem. One of the main challenges that open water aquaculture is facing is to minimize the waste impact released from the farm. In integrated multi-tropic aquaculture (IMTA), seaweeds (non-feed) are cultivated with feed species (fish or shrimp), where seaweeds utilize the excess nutrients from fish farms. Seaweeds in IMTA are a sustainable method to remove nutrient waste as well as increase the commercial production from a farm (Stévant et al., 2017).

1.3 Study species

1.3.1 Sugar kelp (*Saccharina latissima)*

Saccharina latissima (Linnaeus) (Lane et al., 2006) known as sugar kelp, is found in temperate to the polar rocky coastal ecosystems and its growing season ranges from late winter to early spring (Forbord et al., 2021). The species is present in the northern hemisphere on both sides of the Atlantic Ocean, along the North American Pacific coast, and in some regions in Japan and Arctic Russia (Forbord, 2020; Matsson, 2021). *Saccharina latissima* is a long brown seaweed that can reach up to 4 meters in length. It is characterized by a wavy frond (lamina). The lamina is flat and frilly with dark brown to yellow color (Skaar, 2019). *Saccharina latissima* is one of the best suited species for cultivation due to its high growth rate of 0.75 cm per day from March to May (Nielsen et al., 2014), rich in mineral and bioactive components (Holdt & Kraan,2011), and a well described life cycle (Matsson, 2021).

Approximately half of Europe's natural beds of *S. latissima* are found along the coast of Norway, suggesting the habitat suitability may here also be high for its cultivation (Bekkby & Moy, 2011). Despite that, the cultivation of *S. latissima* is a relatively new industry in Norway (Wang et al., 2021). In 2018, the Norwegian production of *S. latissima* was 174 metric tons, which reduced to 66 metric tons in 2019 (Jevne et al., 2020). It has been suggested that by 2050, the Norwegian production of seaweed may reach 20 million tons per year with a value of the seaweed industry of 4 billion euros per year in Norway (Olafsen et al., 2012).

Saccharina latissima has a diplo-haplontic, heteromorphic life cycle with alternation between a microscopic haploid (*n*) gametophyte generation and a macroscopic diploid (2*n*) sporophyte generation (Forbord, 2020; Kain, 1979). *Saccharina latissima* is fertile during autumn and sori (singular = sorus = cluster of sporangia) develop in the center of the lamina (Skaar, 2019). The sorus formation is triggered by short day length and usually takes 3-4 weeks (Bartsch et al., 2008). Each sporangium produces 32 zoospores by meiosis that are released into the surrounding water. The zoospores are haploid and flagellated and grow into either male or female gametophytes. The male gametophyte contains a cluster of colorless one-celled antheridia producing single spermatozoid while female gametophyte cell develops into one-celled oogonium producing a single egg (Baweja et al., 2016). Female gametophytes produce lamoxirene to attract the sperm from male gametophytes to fertilize the eggs. After the fertilization, a new juvenile sporophyte will grow (Figure 1), normally it takes two weeks for gametophytes to fertilize (Forbord, 2020; Matsson, 2021)

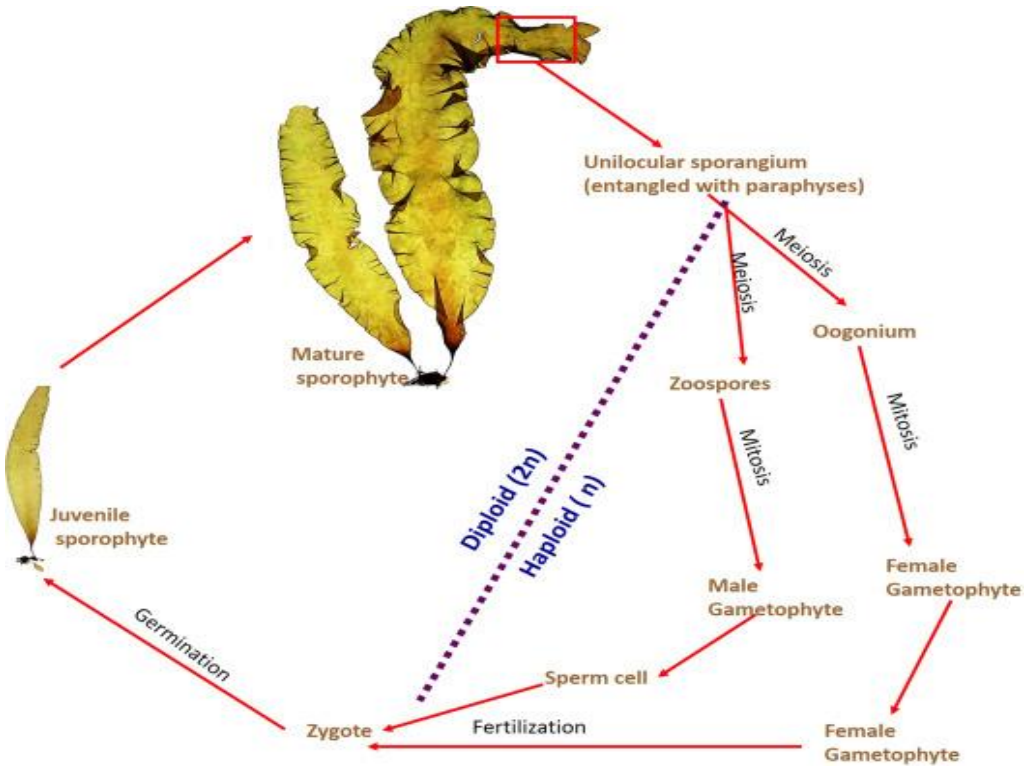

Figure 1: The life cycle of *Saccharina latissima (Baweja et al., 2016)*

The abiotic factors mainly affecting the growth of *S. latissima* are light intensity, temperature, salinity, and available nutrient concentrations. The optimal temperature required for *S. latissima* growth is between 10 and 17 °C and salinities of 30–35 PSU (Kerrison et al., 2015). *Saccharina latissima* grown in temperature at 20 °C or above have high mortality, tissue deterioration, and reduced pigmentation (bleaching), which result in reduced photosynthesis (Andersen et al., 2013; Fortes & Lüning, 1980). The optimum light intensity for the growth of *S. latissima* is at 110 µmol m^{-2} s^{-1}, while a 50 % reduction in growth was observed at a high light intensity of 250 µmol m^{-2} s^{-1} (Fortes & Lüning, 1980).

Inorganic nutrients are essential for the growth and physiological function of seaweeds. In summer, the concentration of dissolved inorganic nitrogen (DIN) depletes to almost zero in coastal habitats, because of phytoplankton blooms. This occurs both globally and along the Norwegian coast (Forbord, 2020). As a result, nitrogen is considered a limiting nutrient for the growth of seaweeds, especially during the summer month. The growth limiting nutrient can be defined as nutrient available in the smallest quantity concerning nutrient required for growth (Harrison & Hurd, 2001). Thus, the growth of seaweeds varies with the seasons due to fluctuation in nutrient concentrations

in the marine water. Many seaweed species are well adapted to such change in their surrounding by the ability to take and store nutrients when excess nutrients are available in seawater and utilize it when limited for growth (Fujita, 1985). Similarly, *S. latissima* are able to take, store nutrient at high nutrient availability, and utilize it for growth when it is limited in surrounding water. The main sources of inorganic nitrogen in the marine environment are nitrate ($NO3^{-}$), ammonium ($NH4^{+}$), and urea (Forbord et al., 2021). When nutrient concentration is high in the surrounding environment, the sporophyte of *S. latissima* can uptake and store nitrate in intracellular pools (I-DIN) in the vacuole and cytoplasm or convert it into nitrite. In chloroplasts, nitrite is further reduced to ammonium and converted to amino acids (Forbord, 2020). Thus, the reserved nitrogen helps *S. latissima* to grow even in areas where nitrogen concentration varies seasonally. The high tissue nitrogen concentration in *S. latissima* suggests high environment nitrogen concentration (Bruhn et al., 2016). The nitrogen uptake is indirectly affected by light intensity as the energy demand for the active uptake and fixation of N to molecules and proteins comes from photosynthesis (Jevne et al., 2020).

The tissue N:P ratio observed in kelp was 9-25:1 (Atkinson & Smith, 1983). This ratio indicates kelp requires a higher N than P concentration for growth. *Saccharina latissima* cultivated in low nitrogen concentration results in reduced color pigmentation (bleaching). In comparison to *S. latissima* cultivated in high nitrate concentration up to 20 µmol/L, those cultivated in low nitrate concentration of 0 to 3 µmol/L appeared to be extremely light pigmented (Chapman et al., 1978). In a study about the initial short nitrate uptake kinetics in *S. latissima* juvenile sporophytes, sporophytes were starved for eight days which result in bleaching and decomposition of tissue, suggesting the nitrogen limitation and was most likely due to loss of pigment-protein complex (Forbord et al.,2021).

1.3.2 Sea Urchin (*Strongylocentrotus droebachiensis)*

The global production and harvest of sea urchins are specifically to supply roe (gonad) to markets where they are consumed as a high-end seafood product. Sea urchin roe is a culinary delicacy in several parts of the world.

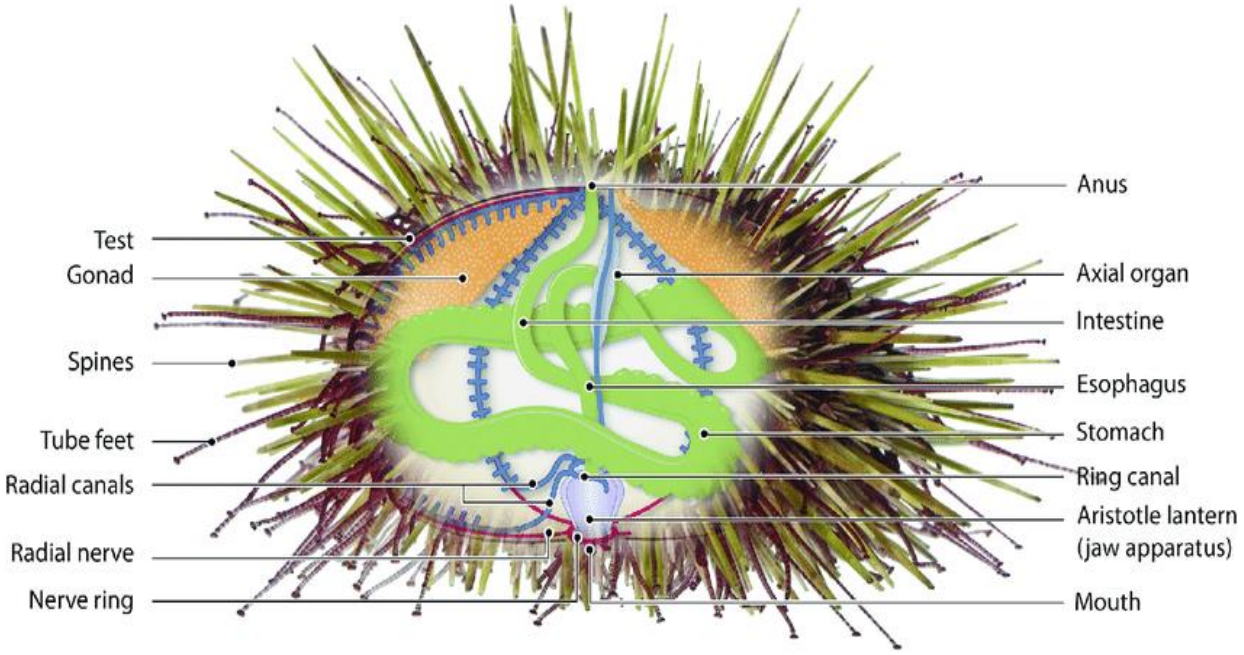

Figure 2: The anatomy of sea urchin (James et al., 2018)

Sea urchin gonad size is measured by the gonad index (GI). The GI of captured sea urchins varies from less than 1% to 20%, while the relative share of the gonads of cultured, or enhanced sea urchins may be as high as 35% of the total animal (James et al., 2018). In the sea urchin processing industry, large inedible parts of sea urchins such as shells, spines, and viscera are discarded as waste upon gonad removal (Figure 2). After the gonad is removed there is potential for the byproduct to be reused and converted into a new product. This will maximize the value of the product and contribute to a circular economy.

By 2050, it is estimated that the human population will reach 9 billion (Hamam et al., 2021). With the increasing population, the demand for natural resources will be increasing, and our natural resources are limited. Thus, several problems in terms of food security, resources depletion, and economic issues will increase. The fish industry produces a vast amount of waste such as fin, scale, and head depending upon the fish processing, and between 30-70% of the original fish wet weight will be discarded as waste (Olsen et al., 2014; Toppe et al., 2018). This byproduct released from the industry might have low commercial value but contains high-quality protein, lipids, vitamins, and minerals. Recycling and reusing these nutrients will minimize the waste for sustainable

fisheries as well as offer alternatives for the economic sustainability of the fish industry. One traditional way of utilizing the fish waste in the coastal community is the production of fertilizer for crops to supply nutrients such as nitrogen, or a combination of nitrogen and phosphorous (Ahuja et al., 2020). For example, in Northern Norway, fish waste such as backbones and heads of cod is used directly or after composting to fertilizer both leys and row crops (Ahuja et al., 2020).

To our best of knowledge, this is the first study on the potential of using sea urchin's byproduct as a nutrient fertilizer for cultivating *S. latissima*. Hence, there is no previous literature on *S. latissima* being cultivated with sea urchins as their nutrient source.

Saccharina latissima takes up nutrients for growth and development and, like other autotrophs in the marine environment, they derive nutrients directly from the water column (Worm et al., 2000). Thus, it is necessary to enhance seawater with nutrients especially nitrogen (N) and phosphorus (P) to fertilize the young sporophytes to obtain optimal growth (Elser et al., 2007). In this study, the waste product from sea urchins was used as a source of nutrients (N and P) for the cultivation of *S. latissima* sporophytes. The sea urchins were crushed and soaked in water to release the nutrients. The *S. latissima* sporophytes were then supplied with 10 ml and 50 ml of sea urchins rough crush solutions, i.e., low and high nutrient concentration, respectively. The latter treatment was either applied continuously or repeatedly over the short-term (= pulsed).

The main objective of the study is to investigate the feasibility of using sea urchins crush as a nutrient source for *S. latissima*. The secondary objective was to observe the length growth performance and length of the color bleached part of *S. latissima* lamina enriched to different concentrations (10 or 50 ml) of sea urchins rough crush solutions supplied to the nutrients the urchins rough crush either continuously or as a pulse.

1.4 Hypothesis of the study

i. Nutrient (N and P) concentrations vary among experimental units that were treated with different volumes (10 and 50 ml) of urchins rough crush solution.
ii. The growth performance of *S. latissima* sporophytes is not affected by the exposure time (pulsed or continuously) of urchins rough crush solution.

iii. There is an increase in the growth performance of *S. latissima* when exposed to pulses of low versus high nutrient concentrations (from urchins rough crush solution).

iv. The length of color bleaching of part of *S. latissima* lamina is not affected by the exposure time (pulsed or continuously) of urchins rough crush solution.

v. There are variations in the length of color bleaching of the lamina of *S. latissima* when exposed to pulses of low versus high nutrient concentrations of urchins rough crush solution.

2. Materials and methods

2.1 Sporeling production

Saccharina latissima sporophytes were collected from Kraknes, on the island of Kvaløya (69°45.259 N / 019°02.176 E) near Tromsø, in December 2020 and were brought back to the seaweed hatchery in Nofima, Tromsø. Ripe sorus tissue from ca. 10 sporophytes was cut out and used for the production of sporelings. Sporelings lines were produced in the seaweed hatchery in Havbrukstasjonen i Tromsø (HiT), with minor revision of Forbord et al. (2018). A solution of about 20.000 spores mL^{-1} seawater was brushed onto 1.2-mm-diameter twine coiled around PVC spools.

The sporeling spools were then incubated for 8 weeks in seawater from 50 m depth in a flowthrough (120 L h^{-1}) cylinder (150 cm high and 50 cm diameter) in a temperature-controlled room at 10°C. LED lights were placed both in and outside the cylinders, the light intensity was set at 20 µmol photons m^{-2} s^{-1} at the start and increased to 70 µmol photons m^{-2} s^{-1} to the end of the incubation period. Seawater temperature was kept at about 10 °C in the first 4 weeks and then gradually decreased to ambient seawater temperature of 4-5 °C.

The PVC spool containing the twine with attached sporeling of an average length of (±SE) 1.48 (±0.03) cm was packed in a polystyrene box and transported to the Nofima building, Brevika, Tromso where the experiment was conducted. The PVC spool was stored in a seawater tank at 1°C temperature with aeration until they were used for the experiment.

2.2 Preparation of sea urchins crush

Sea urchins were collected from Kvalsund in Tromsø and stored at -30°C. In order to release nutrients in seawater, the sea urchins were crushed. For this purpose, frozen sea urchins were first defrosted over 24 hours. The defrosted sea urchins were split in half to increase exposure for complete drying. Then they were spread on an aluminum tray and placed in a drying oven at 60 °C temperature for four days. Oven-dried sea urchins were crushed into both powder and rough crush form. For the powder form, sea urchins were ground in a centrifugal grinder into fine particles of 1 mm size. For the rough crush, the sea urchins were placed in a bucket and crushed using a flat-ended piece of wood. Then, they were filtered using a metal sieve with a mesh size of 2.5 mm to refine the size of the rough crush (Figure 3: left). The large shell fragments that remain after being filtered were discarded (Figure 3: right).

Figure 3: Sea urchins rough crush; (left) The rough crush of sea urchins relatively small and homogenous particles after being filtered through a metal sieve of mesh size 2.5 mm; (right) large and uncrushed particles that were discarded

2.3 Nutrient content in sea urchins crush solutions

Two pilot studies were run to test the nutrient release in seawater by analyzing the nitrate released from sea urchins crush in the seawater.

2.3.1 First nutrient pilot study

In the first nutrient pilot study, both rough crush and powdered sea urchins crush was used to study the efficacy of dissolution and nutrient release into seawater. Five grams and 15 g of both rough crush and powder crush were packed in an individual teabag, respectively. Afterward, the teabags containing rough or powdered crush were soaked in beakers filled with 500 ml seawater for 360 minutes. During that period, 2 ml of water samples from each beaker were taken at 120, 240, and 360 minutes after the start of the trial, to analyze the amount of nitrate released into seawater. Before a water sample was taken, the water in the beaker was stirred with a spatula for a few seconds.

2.3.2 Second nutrient pilot study

In the second pilot study, the rough crush of sea urchins was used to study hourly the nutrient release for six hours. The rough crush of sea urchins was used as the powder sea urchins have the poor release of nutrients relative to rough crush. Henceforward, the oven-dried rough crush of sea urchins was used in the experiment and termed as urchins rough crush. Twenty grams of urchins rough crush was packed in a teabag and soaked in 800 ml seawater in a beaker for 360 minutes with two replicates (n=2). A water sample of four ml was taken every 60 minutes to analyze nitrate release in water. Before a water sample was taken, the water in the beaker was stirred with a spatula for a few seconds.

2.4 Experimental set-up

The experiments were performed in a temperature-controlled room (5 °C) at Nofima, Breivika, Tromsø. A rectangular table of 79cm ×175cm dimension was divided into two halves by a wooden divider. Two rows of 1000 ml beakers were placed in each half of the table (Figure 4: a). The LED lights were attached horizontally beside the beakers (Figure 4: b). The aquarium air pump (EHEIM-200) was hooked to air stones, which were submerged in each beaker to increase the amount of oxygen in the water. Light intensity was measured using a digital lux meter. The digital lux meter sensor was placed at one end of the table and the light intensity was read in lux meter.

The switch (Figure 4: c) to adjust the intensity of light. The seawater for the cultivation of *S. latissima* in the experimental beakers was replaced by clean seawater and treatment nutrient urchins rough crush every week during the experiment.

Figure 4: The experimental set-up in the laboratory in Nofima, Breivika, Tromsø with (a) two-row beakers on each side. (b) LED light is attached beside the beakers. (c) The intensity of light could be adjusted through the switch.

2.5 *Saccharina latissima* sporophyte preparation

In the first and second pilot studies, each experimental beaker contained a 15 cm long string with three *S. latissima* sporophytes attached. If applicable, surplus sporophytes were removed with tweezers and a scalpel. In the main experiment, the number of sporophytes increased to five with an average length of (±SE) 1.64 (±0.04) cm as sporophytes in both pilot studies were broken from string due to attached suspend particles in sporophytes. The distance between each sporophyte in the string was maintained at a minimum distance of 20 mm. This was to prevent one sporophyte from shading another. Also, a minimum gap of 30 mm between the water surface and top sporophyte was maintained to accommodate changes in the water level due to water sampling,

and/or evaporation, without sporophytes being exposed to air. One end of the string was attached to a thin wooden stick that sat across the top of the beaker (Figure 5: a). A metal nut was fixed to the opposite end of each string as a sinker to keep the string vertically suspended in a beaker (Figure 5: b). The length of all sporophytes was quantified (see a detailed description of the length measurement below) before they were transferred to the experimental beaker, i.e., at the beginning of the experiment, and subsequently each week during the study period.

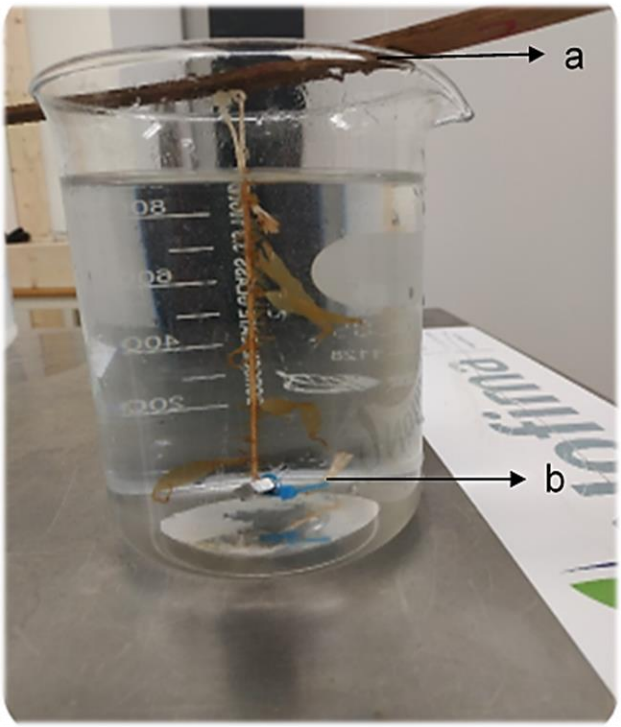

Figure 5: Experimental beaker with a single string suspended vertically; (a) one end of a string was attached to a thin wooden stick; (b) a metal nut was fixed at the bottom end of the string

2.6 Cultivation of *Saccharina latissima*

In the study, the use of urchins rough crush as fertilizer for *S. latissima* growth was a new approach for which no literature or methodology was available. Hence, the experiment begins with two pilot studies to observe the responses of *S. latissima* to the exposure in urchins rough crush and whether any problems arise in order to restructure the method for the main experiment.

2.6.1 First pilot study

In the first pilot study, the *S. latissima* sporophytes were exposed to four different amounts of urchins rough crush which was soaked three different times to observe the condition for the cultivation of sporophytes. Urchins rough crush of weight 8 g, 18 g, 28 g, and 38 g was packed in teabags. The opening of a teabag was closed tightly to prevent urchins rough crush directly falling into the seawater. The prepared string attached with sporophytes was kept in a beaker filled with 800 ml seawater. Then, the prepared urchins rough crush teabags were soaked for 150, 300, or 450 minutes after the start of the study to enrich nutrients before it was removed from beakers. Each treatment was replicated thrice i.e., nine beakers for each weight of the urchins rough crush treatment levels. The control treatment consisted of a three beaker filled with seawater to which no urchins rough crush was added. The sporophytes were exposed to light for 24 hours per day.

On the third day of the study, turbidity was observed in the water column due to the presence of small particles of urchins rough crush. Those small particles of urchins rough crush had been released into the water when the teabags were soaking in the beakers and then settled to the bottom. However, the air stone turned back on to aerate the water continuously in the beakers, resulting in turbidity in water. The suspended particles were observed to attach to the sporophytes. As a consequence, the study was ended in three days of the experiment.

2.6.2 Second pilot study

In the second pilot study, an attempt to prevent water turbidity was made. Instead of soaking urchins rough crush in each experimental beaker, separately prepared urchins rough crush solution was used. To prepare the urchins rough crush solution, 800 ml of seawater was warmed for two minutes in a microwave oven to increase the temperature to 25 °C for better nutrient dissipation in water. Then, 40 g urchins rough crush was packed in a teabag, which had its opening tightly closed. The urchins rough crush teabag was soaked in the warmed seawater for 180 minutes. Subsequently, the urchins rough crush solution was filtered through coffee filter paper to remove particles. For this purpose, a coffee filter paper was placed on a metal sieve and the filtered urchins rough crush solution was collected upon its filter passage. The filtered urchins rough crush solution was then autoclaved for sterilization. In one beaker, an 800 ml urchins rough crush nutrient solution was prepared, and the remaining volume of urchins rough crush solution required for the

experiment was prepared following the same procedure. The seawater used for the cultivation of sporophytes in the experiment was boiled for 5 minutes then stored in the chill room at -1 ℃ to cool down before it was used in the experiment. For the cultivation of *S. latissima*, a total of 45 beakers were arranged in the experiment table. For the control treatment, 800 ml seawater was added to the beakers. The urchins rough crush solution treatments beakers were filled with seawater subtracting the volume of urchins rough crush solution treatment in 800 ml. Then, the prepared string attached with sporophytes was arranged in each beaker. The experimental treatments were as follows: 20 ml, 40 ml, 60 ml, 80 ml, 100 ml, 120 ml, 140 ml, and 160 ml of urchin rough crush solution and control with five replicates of each treatment (n=5) was added in beakers. Sporophytes were exposed to continuous light.

On the third day of the study, turbidity still occurred in the water column of the experimental beakers. In comparison to the turbidity in the first pilot study, it was visually less in the second pilot study. Along with turbidity, bleaching of sporophytes was identified as another problem. Thus, the study was concluded on the fourth day of the experiment.

2.6.3 Set-up of the main experiment

Regardless of using the filtered urchins rough crush solution, the turbidity problem was observed in the water column. So, in the main method, *S. latissima* sporophytes were cultivated in a way where the exposure of urchins rough crush was reduced either with volume or exposure time. The sporophytes were supplied with low and high concentrations of urchins rough crush solution for a short period (pulse). Other ways sporophytes were supplied with low concentration of urchins rough crush for short and a long period (continuous).

To generate a low and high concentration treatment 10 or 50 ml of urchins rough crush solution were added to experimental beakers. The seawater for the cultivation of sporophytes and urchins rough crush solution in beakers exchange weekly for six experimental weeks. As for the pulsed supplied of urchins rough crush solution, in a week (seven days) the sporophytes were exposed to urchins rough crush solution for three days (pulse), and the following day the water was exchanged with clean seawater. As for sporophytes exposed to urchins crush solution supplied continuously were exposed to the urchins rough crush solution continuously for a week. After a week (seven

days) every beaker was refreshed with clean seawater and enrichment of urchins rough crush solution repeating the same procedure to expose urchins rough crush solution to sporophytes.

The seawater used for cultivation and urchins rough crush solution was prepared as described in the second pilot study. The treatments were as follows: control, low continuous receive 10 ml of urchins rough crush solution continuously, low pulse receive 10 ml of urchins rough crush solution for a repeated short period, and high pulse receives 50 ml of urchins rough crush solution for a repeated short period with each treatment replicated nine times. The number of sporophytes in each beaker was increased to five to increase the survival of sporophytes. This increased the sporophytes covered area on the string and to maintain the gap between sporophytes, the volume of water in the beaker has increased to 900 ml. For the cultivation of *S. latissima*, a total of 36 beakers were arranged in the experiment table. For the control treatment, 900 ml seawater was added to beakers. For urchins rough crush treatments, beakers were filled with seawater subtracting the volume of urchins rough crush solution treatment in 900 ml. Then, the prepared string attached with sporophytes was arranged in each beaker. Then, the urchins rough crush solution was added to respective beakers. Sporophytes were exposed to 8 hours of light and 16 hours of darkness per day with a light intensity of 23 µmol photons m^{-2} s^{-1}. The continuous exposure to light in pilot studies has been reduced to eight hours and intensity was controlled as it was assumed the light was most likely to cause bleaching in *S. latissima*.

2.7 Water sample analysis for Nitrate (NO_3^{-1}) and Phosphate (PO_4^{-1})

About 50 ml of seawater samples were taken for nutrient analyses from each beaker at the first and last week of the main experiment on the day of urchins rough crush solution enrichment in beakers. Before collection of seawater samples for nutrient analysis, the water in the beaker was stirred with a spatula for a few seconds. Seawater samples were filtered through a 0.22 µm Whatman GF/C filter to remove particles and were stored at -35 °C until further analyses. Seawater samples were analyzed for the nitrate and phosphate concentration with an autoanalyzer (Flow Solution IV System, I.O. Analytical) according to the Norwegian Standard 4745.

2.8 Length measurement of *Saccharina latissima*

The length of *S. latissima* sporophytes was measured using graph paper (1 mm graph lines). The graph paper was placed in a tray. Then each string with attached sporophytes was carefully removed from its experimental beaker and placed in graph paper. Blunt tweezers were used to

straighten each sporophyte before a picture was taken. The length of the sporophyte was measured by counting lines covered by the sporophyte from the vertical lines in the image (Figure 6). The length of each sporophyte from every individual beaker was measured. Then, the average length of five sporophytes was calculated for the beaker. The length of sporophytes was measured every 7 days to estimate the length growth of sporophytes in the respective urchins rough crush and control treatments. In this study average length growth of sporophytes in each beaker was calculated with the change in length of sporophytes per week using the formula:

Average length growth per week = $L_t - L_i$

Where L_i is the average initial length of the five sporophytes of a beaker of a given week and L_t is the average length of the five sporophytes of a beaker at the end of this week.

Figure 6: *Saccharina latissima* sporophyte length measured on graph paper. Each smallest box is equal to 1mm in length

The bleaching starts from the tip (section described Figure 7) of the lamina; in beginning, the brown color of the lamina became light-colored and slowly turn into transparent before it broke down. To quantify the length of the bleached part of the lamina of the experimental sporophytes,

the lamina part of the sporophyte which has less color pigment was measured excluding the healthy part (bright brown color part) in each sporophyte of the individual beaker was measured each week during the experiment. The average length of the color bleached part of the lamina in each beaker was calculated by measuring and calculating the average color bleach part of the lamina in the five sporophytes in each beaker. The change of length of the bleached part of the lamina was calculated every week of the experiment period by using the formula:

Average length color bleach per week = $B_t - B_i$

Where B_i is the average initial length of the bleached part of the lamina of five sporophytes at the beginning of a given week and B_t is the average bleached part of the lamina of five sporophytes at the end of this week.

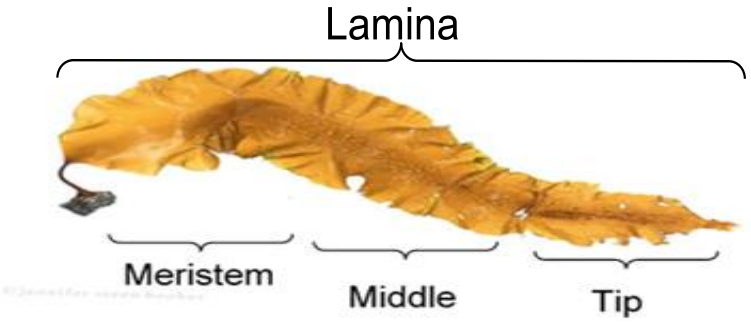

Figure 7*: Saccharina latissima* divided into three sections in the study of color bleaching (Booher, 2017)

The treatment effect size on average sporophyte length growth and change in the length of the bleached part of lamina of sporophytes was calculated using the percentage and ratio, respectively to compare effect among the urchins rough crush treatments with relative to control treatment.

Effect size for length growth = (Ls/Lc)* 100

Where, Ls is the average length growth of the response variable in urchins rough crush enrichment treatment in a week and Lc is the average length growth in the unenriched control treatment

Effect size for color bleaching= Bs/Bc

Where, Bs is the average length of the color bleached part of lamina of the response variable in urchins rough crush enrichment treatment in a week and Bc is the average length of the color bleached part of the lamina in the unenriched control treatment

The effect size for nutrient concentration in water sample analysis was not calculated as the phosphate concentration in the control treatment was zero.

2.9 Statistical analyses

Data for the average length growth, color bleaching, and nutrient concentrations in water were tested for normality using the Shapiro test and for homogeneity of variance using Levene's test. Data were log-transformed if they violated the normal distribution and homogeneity of variance assumption. The comparison of average weekly length growth and change in the length part of lamina bleaching in sporophytes in response to urchins rough crush treatment was analyzed by one-way ANOVA. However, the nutrient concentration in water samples in Week 1 and Week 6 was analyzed by the Kruskal-Wallis test because the water analyses data violate the normality assumption even after the data were log transformed. The significance level was set to 0.05. All the data are reported as mean ± SE. If the test results showed a significant difference in the treatment, then a post hoc test (Student-Newman Keuls =SNK) was used to test for significant differences among treatments. The overall difference between urchins rough crush and control treatment over the entire study period as well as interactive effects between treatments and time on average growth, nutrient content, and color bleaching of sporophytes during the experiment, were analyzed with repeated measures ANOVA using the R packages rstatix (Kassambara, 2021), datarium (Kassambara, 2019), tidyverse (Wickham et al., 2019), and ggpubr (Kassambara, 2020). Mauchly's test of sphericity was used to test for equality of the variance of urchin rough crush treatment differences between times. If the assumption of sphericity was violated, the degrees of freedom and F-ratio were corrected by Greenhouse-Geisser, following the recommendations of Quinn & Keough (2002, p. 284). The statistical analyses and plots were made in R, using version

4.0.2 and R studio using version 1.3.1073. The cumulative length growth and color bleaching in sporophytes was calculated and plotted in line graph using Microsoft Excel.

3. Results

3.1 Nutrient concentrations

3.1.1 Nitrate (NO_3^{-1}) concentration

The nitrate concentration averaged over the entire study period showed no significant variation between the three urchins rough crush treatments and control (Repeated measure ANOVA: $F_{3,32}$=1.929, p=0.145). When pooled overall treatments, the nitrate concentration was not significantly different between the first and last week of the experiment (Repeated measure ANOVA: $F_{1,32}$=0.766, p=0.390). Moreover, there was no significant interaction between treatments and time in the nitrate concentration (Repeated measure ANOVA: $F_{3,32}$=0.363, p=0.780).

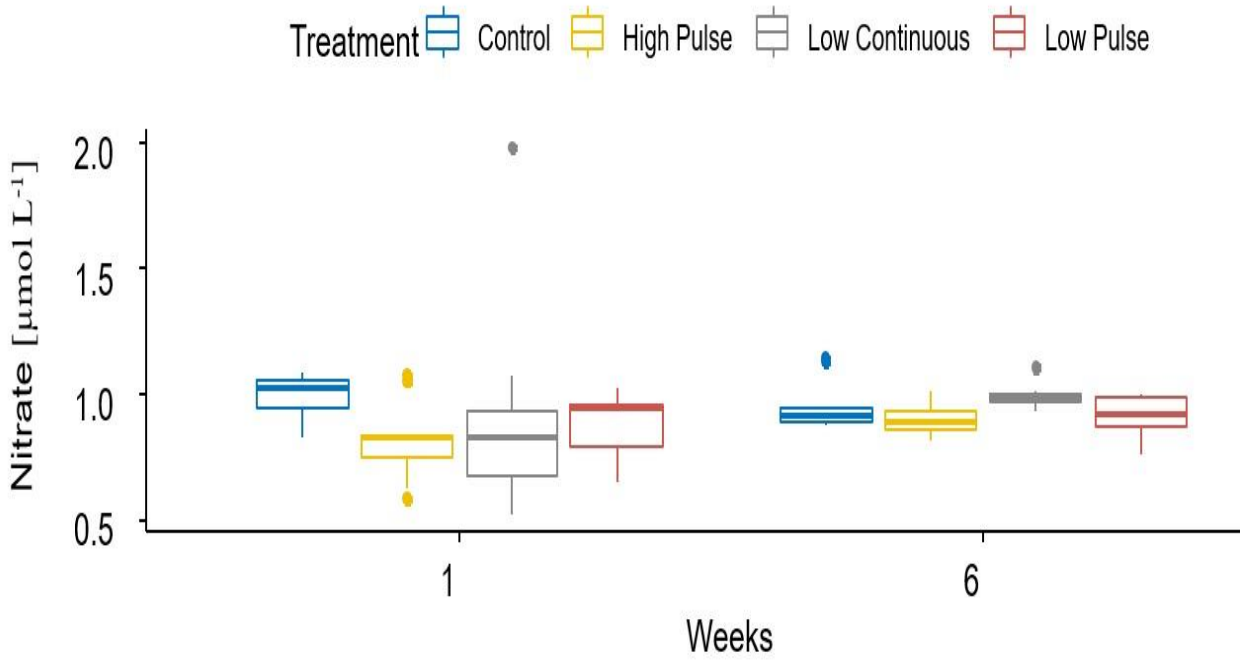

Figure 8: Average nitrate concentration in water samples of the treatments in Week 1 and Week 6. The bottom and top horizontal lines of a box show the first quartile and third quartile. The thick horizontal line in a box shows the median. Whiskers show a 95 % confidence interval. The single points are outliers.

3.1.2 Phosphate (PO_4^{-1}) concentration

The phosphate concentration averaged over the entire study period showed significant variation between the three urchins rough crush treatments and control (Repeated measure ANOVA: $F_{3,32}$=2032.39, p=0.0001). When pooled overall treatments, phosphate concentration was showed significantly different between the first and last week of the experiment (Repeated measure ANOVA: $F_{1,32}$=381.439, p=0.0001). Also, there was a significant interaction between treatments and time in phosphate concentration (Repeated measure ANOVA: $F_{3,32}$=43.53, p=0.0001). The variation in phosphate concentration among treatments in first and last was explained below.

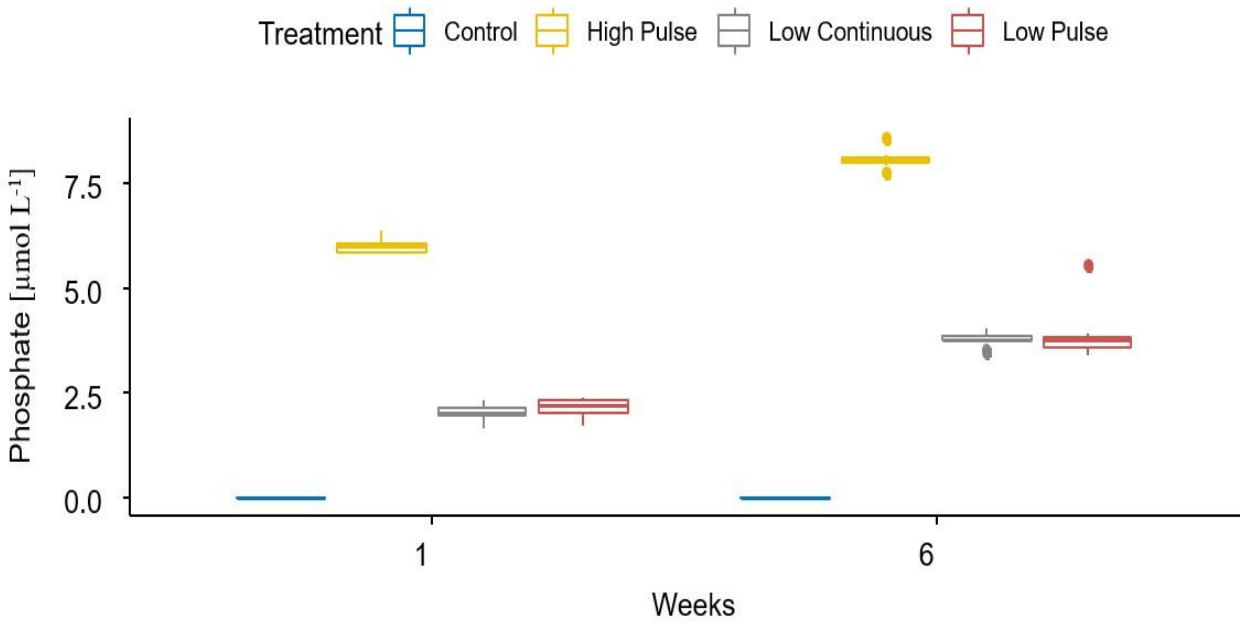

Figure 9: Average phosphate concentration in water samples of treatments in Week 1 and Week 6. The bottom and top horizontal lines of a box show the first quartile and third quartile. The thick horizontal line in the box shows the median. Whiskers show a 95% confidence interval. The single points are outliers.

When analyzing phosphate concentrations for each week separately, there was a statistically significant difference in average phosphate concentration between treatments in Week 1 (Kruskal-Wallis test: χ^2=30.506, p = 0.0001). The phosphate concentration was zero in the control treatment

and distinctly increase in high pulse treatment to 5.99 ± 0.06 µmol/L. The phosphate concentration in the low concentration of urchins rough crush treatment was 2.08 ± 0.05 µmol/L. In the last week of the experiment, the phosphate concentration in the water sample with urchins rough crush solution has increased. In Week 6, there was a statistically significant difference in average phosphate concentration between treatments (Kruskal-Wallis test: χ^2=30.165, p = 0.0001). In the last week of the experiment, the phosphate concentration in a high and low concentration of urchins rough crush solution was 8.06 ± 0.07 µmol/L and 3.82 ± 0.11 µmol/L respectively.

3.2 *Saccharina latissima* cultivation

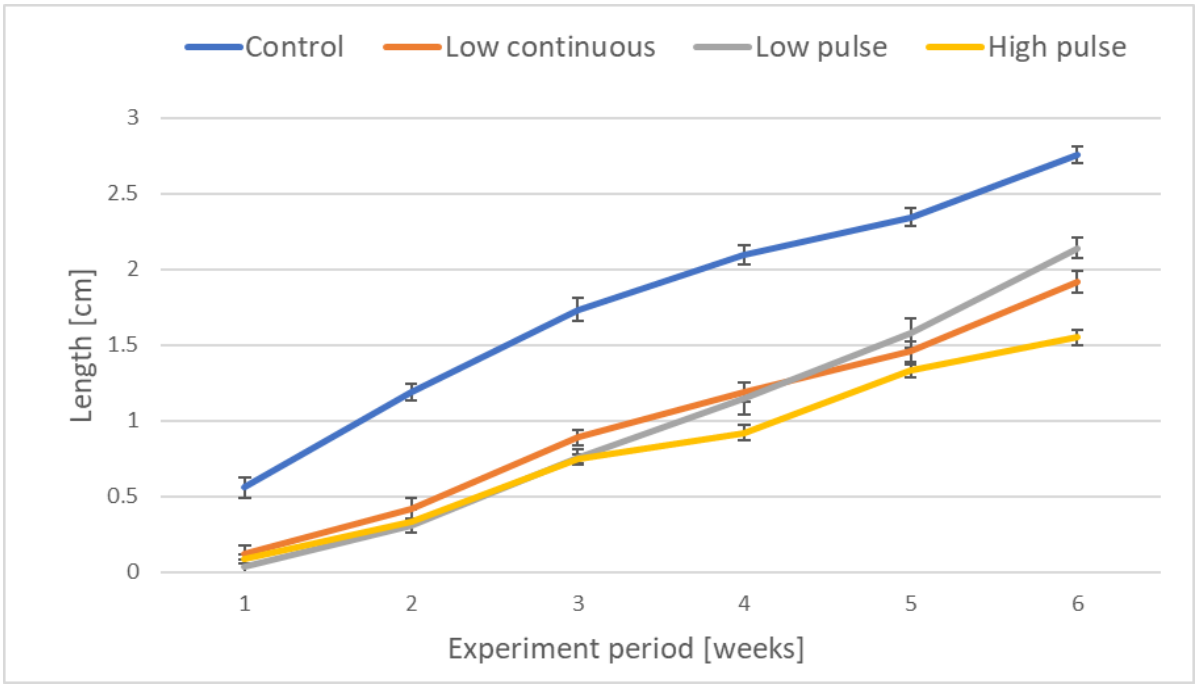

Figure 10: The average length growth of *S. latissima* at each treatment in the individual week during the experiment. The error bars indicate the standard errors.

The cumulative length growth of sporophytes was calculated by summing all average weekly length growth in each treatment in the individual week of the experiment.

3.2.1 Exposure time effect of urchins rough crush on the growth of *Saccharina latissima*

On average over the entire study period, the length growth of *S. latissima* showed significant variation between the treatments of different exposure times (Repeated measure ANOVA: $F_{(2,24)} = 4.07$, $p = 0.031$). The average length growth in sporophytes exposed to pulses of low concentration of urchins rough crush solution was 77.56 % of the control treatment. Similarly, the average length growth of sporophytes continuously exposed to the low concentration of urchins rough crush solution was 69.47% times of the control treatment. On average overall treatments, length growth of *S. latissima* was significantly different over the six weeks of the experiment (Repeated measure ANOVA: $F_{(5,120)} = 9.64$, $p = 0.0003$). Moreover, there was a significant interaction between treatments and time in the growth of sporophyte (Repeated measure ANOVA: $F_{(10,120)} = 6.38$, $p = 0.0003$) (Figure 10). The average length growth over each week was different depending on treatments which were further explained below.

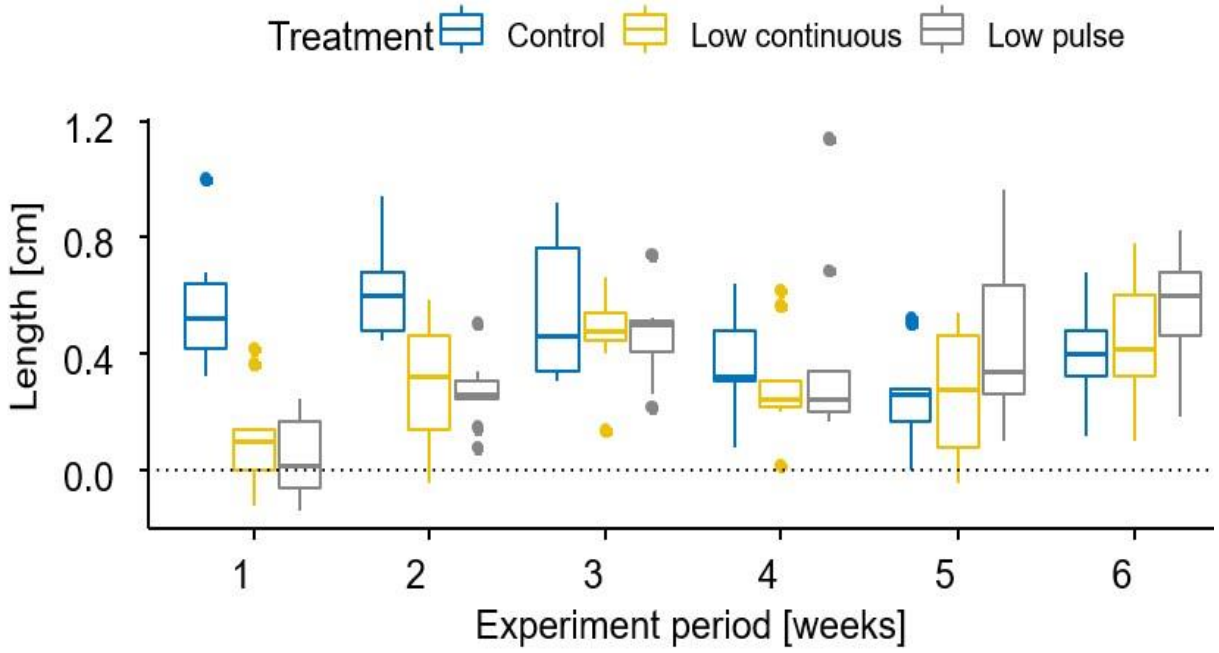

Figure 11: Average length growth of *S. latissima* sporophytes (n=9) in response to a pulsed or continuous exposure to a low concentration of urchins rough crush solution throughout the experiment period (six weeks). Value (weekly change in length) is the mean of five sporophytes across nine replicates within each treatment. The first and last horizontal line of the box shows the first quartile and third quartile. The thick horizontal line in the box shows the median. Whiskers show a 95% confidence interval. The single points are outliers.

In the Week 1 and Week 2 sampling, the length growth of *Saccharina latissima* was significantly different exposed as pulsed or continuously to low concentration of urchins rough crush solution and control treatment (Week 1: $F_{(2,24)} = 23.29$, $p=0.0002$; Week 2: $F_{(2,24)} = 12.58$, $p=0.0018$). In Week 1, the length growth of sporophytes with pulsed or continuous exposure to a low concentration of urchins rough crush solution was 6.25 % and 20.89 % of the control, respectively. In Week 2, it length growth of sporophytes with pulsed or continuous exposure to low concentration was 42.51 % and 48.08 % of the control treatment respectively. However, post-hoc tests revealed that there was no significant difference in *Saccharina latissima* length growth between the treatments in Week 3 ($F_{(2,24)} = 0.61$, $p=0.552$), Week 4 ($F_{(2,24)} = 0.439$, $p=0.265$), Week 5 ($F_{(2,24)} = 1.712$, $p=0.202$), and Week 6 ($F_{(2,24)} = 1.369$, $p=0.274$) of the experiment.

3.2.2 Concentration effects of urchins rough crush on the growth of *Saccharina latissima*

On average over the study period, the length growth of *S. latissima* showed significant variation between treatments; control, low pulse, and high pulse (Repeated measures ANOVA: $F_{(2,24)} = 11.057$, $p=0.00001$). The average length growth in sporophytes exposed to a low and a high pulsed concentration of rough crush solution was 77.56 % and 56.11%, respectively of the control treatment over the study period. On average overall treatments, *S. latissima* length growth was significantly different over time (Repeated measures ANOVA: $F_{(5,120)} = 8.025$, $p=0.003$). Also, the relationship between length growth response in treatment and time showed a significant interaction (Repeated measures $F_{(10,120)} = 8.167$, $p=0.0002$) (Figure 10). There was a difference in length growth of sporophytes between treatments over each week of the experiment which was further examined below.

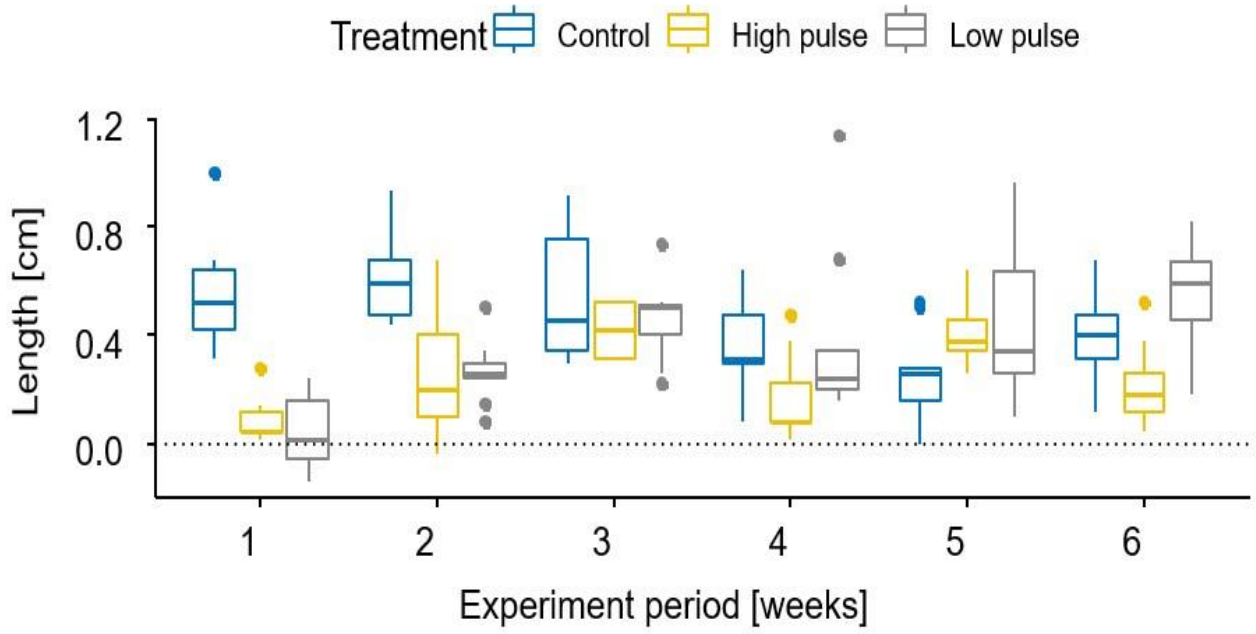

Figure 12: Average length growth of *S. latissima* throughout the experiment period (six weeks) exposed to pulses of low and high concentered crush solution. Value (weekly change in length) is measured as the average of the five sporophytes per beaker across nine replicates within each treatment. The first and last horizontal line of the box shows the first quartile and third quartile. The thick horizontal line in the box shows the median. Whiskers show a 95 % confidence interval. The single points are outliers.

In Week 1 and Week 2 the length growth of *Saccharina latissima* was significantly different between treatments in Week 1 ($F_{(2,24)} = 32.91$, p=0.0003), Week 2 ($F_{(2,24)} = 13.26$, p=0.0001), Week 4 ($F_{(2,24)} = 4.546$, p=0.0212), and Week 6 ($F_{(2,24)} = 9.179$, p=0.001). In Week 1, length growth of sporophytes exposed to low and high pulses of urchins rough crush solution was 6.25 % and 15 % of in the control treatment, respectively, and in Week 2 to the length growth of sporophytes exposed to low and high pulses of urchins rough crush solution was 42.51 % and 39.96 % of control, respectively. In Weeks 4 and 6, however, only the length growth of sporophytes exposed to pulses of high concentrated urchins rough crush solution was 46.67 % and 52.54 % respectively of controls. However, there was no significant difference in the length growth of sporophytes exposed to low pulse and control treatments in Weeks 4 and 6. In Weeks 3 and 5,

there was no significant difference in the length growth of sporophytes between the treatments (Week 3; $F_{(2,24)} = 1.406$, $p=0.265$ and Week 5: $F_{(2,24)} = 2.084$, $p=0.146$).

3.3 Color bleaching on *Saccharina latissima*

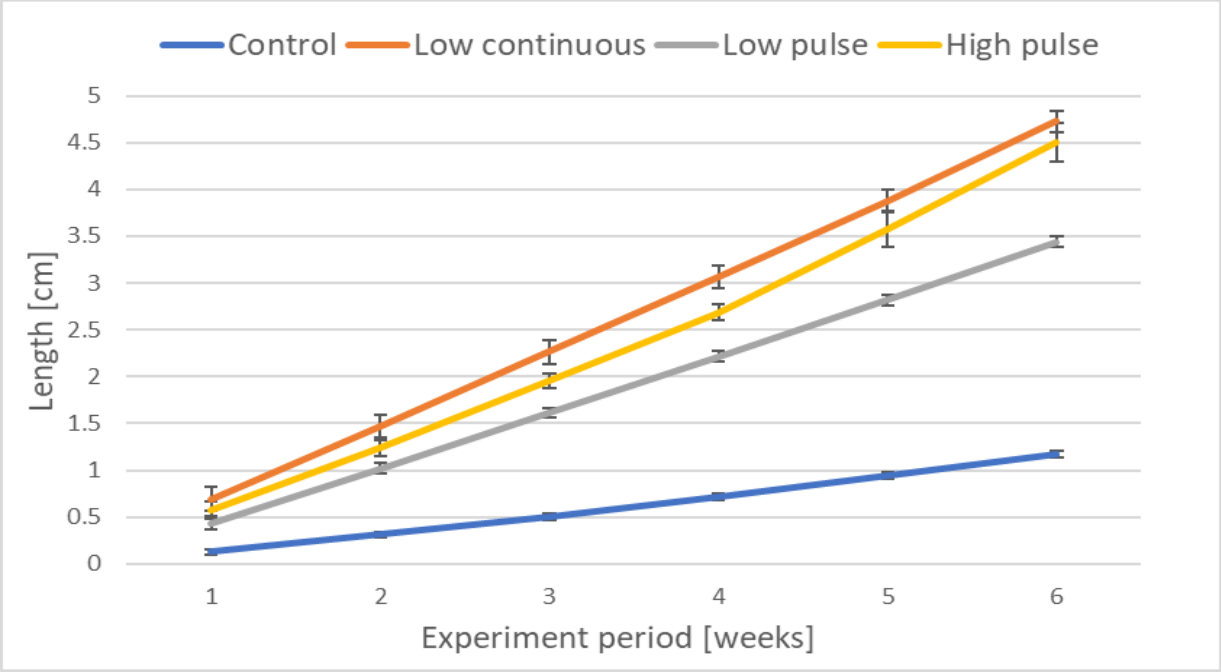

Figure 13: The average length of color bleached parts of *S. latissima* lamina in each treatment in the individual week during the experiment. Error bars indicate the standard error.

The cumulative length of the lamina part to color bleached was calculated by summing all average weekly lengths of the color bleached part of the lamina in each treatment in the individual week of the experiment. *S. latissima* exposed continuously to low concentration of urchins rough crush have a longer part of the lamina to be color bleached in other treatments.

3.3.1 Exposure time effect of urchins rough crush on the color bleaching of *Saccharina latissima*

On average over the entire study period, the length of the color bleached part of *S. latissima* sporophytes showed significant differences between treatments (Repeated measures ANOVA: $F_{2,24}$=13.812, p=0.0001). The average length of the color bleached part of *S. latissima* exposed to a low concentration of urchins rough crush solution supplied continuous and pulsed treatment was 4.05 and 2.94 times longer, respectively than in the control treatment. On average overall treatments, the length of the color bleached part of *S. latissima* lamina was significantly different during the experiment (Repeated measures ANOVA: $F_{2.4,57.68}$=69.187, p=0.0001). Moreover, there was a significant interaction between treatments and time in the length of the color bleached part of sporophytes (Repeated measures ANOVA $F_{4.82,57.68}$=3.304, p=0.012) (Figure 13). The variation in the length of the color bleached part of sporophytes lamina in treatments in each week was further explained below.

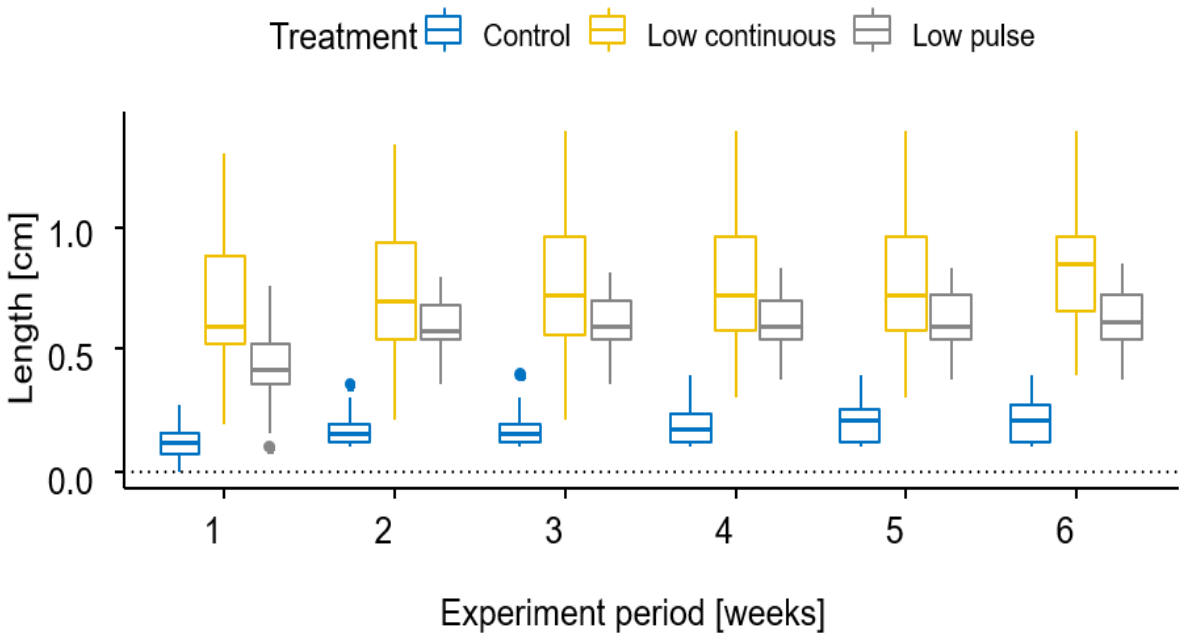

Figure 14: Average length of the color bleached part of *S. latissima* lamina showing the effect of low concentration of urchins rough crush solution available for a pulse versus continuous periods. Values (length) are an average of five sporophytes across nine replicates within each treatment. The first and last horizontal line of the box shows the first quartile and third quartile. The thick horizontal line in the box shows the median. Whiskers show a 95% confidence interval. The Single points are outliers

There was significant variation in the average length of the color bleached part of *S. latissima* lamina between treatments in each week during the experiment (Table 1). In Week 1, the length of the color bleached part of lamina was, on average, 5.36 times longer in low continuous and 3.37 times longer in low pulse than in the control treatment. In Week 2, on average the length of the color bleached part of the lamina was 4.26 times longer in low continuous and 3.20 times longer in low pulse than in the control. In Week 3, the length of the color bleached part of sporophytes lamina was 4.15 and 3.10 times longer in low continuous and low pulse treatments, respectively than in the controls. In Week 4, the length of the color bleached part of lamina was 3.85 and 2.87 times longer in low continuous and in low pulsed treatments, respectively, than in control. In Week 5, the color bleached length part of lamina was 3.56 times longer in low continuous and 2.67 times longer in low pulse than in the control treatment. In Week 6, the color bleached length part of lamina was 3.71 times longer in low continuous and 2.68 times longer in low pulse than in the control treatment.

Table 1: Results of one-way ANOVA analyzing the variation in the average length color bleached part of *S. latissima* lamina among treatments the control, low continuous, and low pulse each week during the experiment. The df indicates the calculated degree of freedom of treatments, the F value is the variance, and the p-value (≤ 0.05) is the statistical significance.

Experiment period [Weeks]	Df	F value	p-value
1	2	4.506	0.0212
2	2	25.76	0.0001
3	2	24.26	0.0001
4	2	24.11	0.0001
5	2	20.63	0.0006
6	2	24.61	0.0001

3.3.2 Concentration effect of urchins rough crush on the color bleaching of *Saccharina latissima*

On average over the entire study period, the length of the color bleached part of *S. latissima* lamina showed variation between treatments (Repeated measures ANOVA: $F_{2,24}$=20.11, p=0.018). The average length of the color bleached parts of *S. latissima* lamina exposed to a low and high concentration of rough crush solution supplied pulsed was 2.94 and 3.86 times longer, respectively than in the control treatment. On average overall treatments, *S. latissima* lamina length color bleached was significantly different during the experiment (Repeated measures ANOVA: $F_{1.3,31.13}$=13.45, p=0.0003) (Figure 13). Moreover, there was no significant interaction between treatments and time in sporophytes color bleaching (Repeated measures ANOVA: $F_{2.6,31.13}$=1.19, p=0.324).

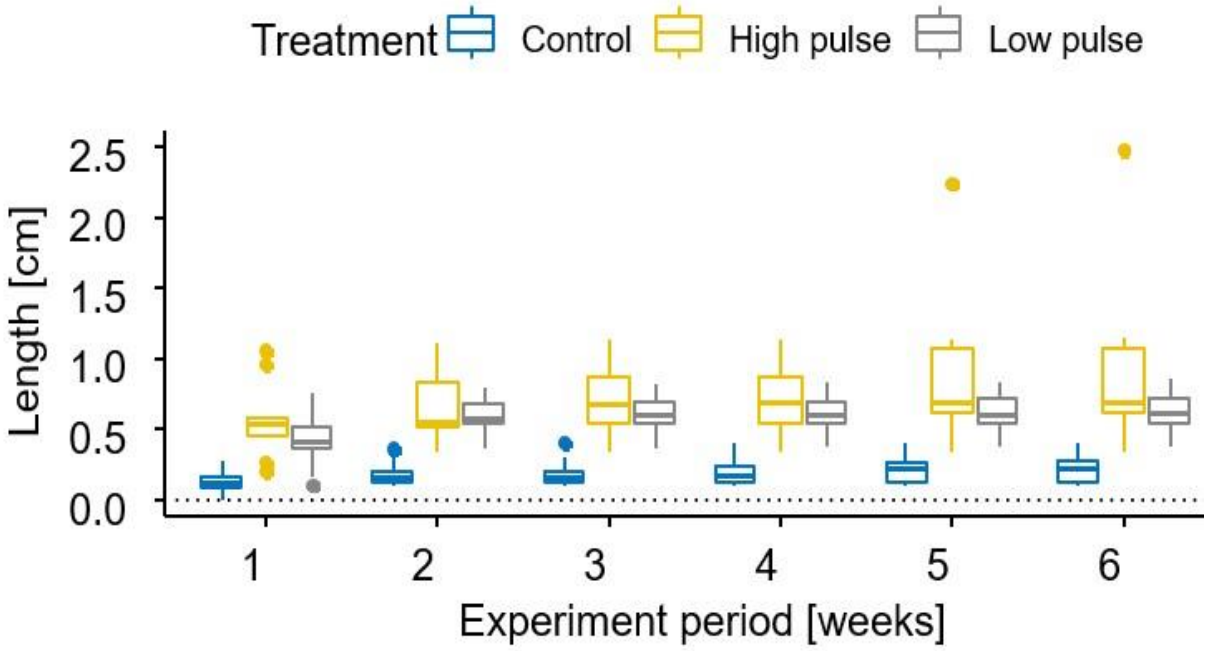

Figure 15: Average length of the color bleached part of *S. latissima* showing the effect when exposed to the low and high concentration of rough crush solution in pulse period. Values (length) are an average of five sporophytes across nine replicates within each treatment. The first and last horizontal line of the box shows the first quartile and third quartile. The thick horizontal line in the box shows the median. Whiskers show a 95 % confidence interval. The single points are outliers.

There was statistically significant variation in the average length color bleached parts of *S. latissima* lamina exposed to urchins rough crush treatments and control treatment in each week of the experiment (Table 2). In Week 1, *S. latissima* exposed to pulses of low and high concentrations of urchin rough crush solution had, on average, 3.37 and 4.41 times, respectively, longer parts of their lamina color bleached than sporophytes in the control treatment. In Week 2, on average the length of the color bleached part of lamina exposed low pulse and high pulse was 3.20 and 3.69 times, respectively longer than in the control. In Week 3, S. latissima exposed to a pulse of low and high concentration of urchins rough crush solution had, on average, 3.10 and 3.74 times, respectively, longer part of their lamina color bleached than in the control treatment. In Week 4, *S. latissima* exposed to pulses of low and high concentrations of urchin rough crush solution had, on average, 2.87 and 3.47 times, respectively, longer parts of their lamina color bleached than sporophytes in the control treatment. In Week 5, *S. latissima* exposed to pulses of low and high concentrations of urchin rough crush solution had, on average, 2.67 and 3.95 times, respectively, longer parts of their lamina color bleached than sporophytes in the control treatment. In Week 6, the average length of the color bleached part of *S. latissima* lamina was 2.68 and 4.04 times longer in low and high pulse treatments, respectively than the control treatment.

Table 2: Results of one-way ANOVA analyzing the variation in the length color bleached part of *S. latissima* lamina exposed to control, low pulse, and high pulse treatments each week during the experiment. The df indicates the calculated degree of freedom of treatments, the F value is the variance, and the p-value (≤ 0.05) is the statistical significance.

Experiment period [Weeks]	Df	F value	p-value
1	2, 24	10.11	0.0006
2	2, 24	18.74	0.0001
3	2, 24	21.03	0.0005
4	2, 24	19.13	0.0001
5	2, 24	21.33	0.0004
6	2, 24	20.66	0.0006

4. Discussion

4. 1 Growth response to urchins rough crush solution

In the present study, the addition of different concentrations of urchins rough crush solution supplied either pulsed or continuously to fertilize the water for *Saccharina latissima* sporophytes length growth does not seem to have benefits over six weeks (Figure 10). The average length growth of *S. latissima* in urchins rough crush treatments is significantly higher than zero indicating the positive response but the length growth in *S. latissima* enriched with urchins rough crush solution is less relative to the control treatment. In a meta-analysis on nutrient enrichment studies done in marine environments, it was observed that N and P have positive effects on seaweeds growth (Elser et al., 2007). However, N enrichment showed in that study have significantly stronger effects than P enrichment. Surprisingly, the nitrate concentrations in all treatments containing urchins rough crush solution are similar to the control treatment (Figure 8). Despite the addition of low (10 ml) and five times higher (50 ml) concentrations of urchins rough crush solution; there is no variation in nitrate concentration in urchins rough crush enriched and the control treatment. This suggests that the addition of urchins rough crush solution did not enrich the nitrate concentration in water. On the other hand, the addition of urchins rough crush solution tremendously increased the phosphate (PO_4^{-1}) concentration of water relative to control treatment with zero phosphate concentration (Figure 9). The phosphate concentration extremely increased in addition to the high concentration of urchins rough crush solution and moderately increase in low concentration of urchins rough crush solution enrichment. The phosphate concentration in urchins rough crush solution treatments was higher in Week 6 in comparison to Week 1 even though the volume of urchins rough crush solution in treatments was the same. The possible reason for the difference in phosphate concentration in the first and last week of the experiment even after receiving the same volume urchins rough crush solution might be the crush was heterogeneous, it contains different organs like viscera and shells which might have been unequally distributed while packing in a teabag. Therefore, the phosphate concentration was slightly different between Week 1 and 6.

In this study, a significant variation in the length growth of *S. latissima* was found among the treatments. The similar concentration in the nitrate but the difference in phosphate concentration among treatments suggest that the phosphate concentration available in water appears to constrain the growth of *S. latissima.* Although, the growth of *Saccharina latissima* is dependent on inorganic

nitrogen (Wheeler & Weidner, 1983). The tissue N:P ratio observed in kelp was 9-25:1(M. Atkinson & S. Smith, 1983). This ratio indicates kelp requires a higher N than P concentration for growth. The suggested optimal concentration of N and P for growth of brown seaweed is given as 48µmol g^{-1} DM d^{-1} (Pedersen & Borum, 1997) and 0.4 (Pedersen et al., 2010). This implies that the N concentration in seawater is more important for *S. latissima* growth, but the nitrate concentration was low in our study. However, the seawater analysis in the current study shows considerably higher dissolved phosphate concentration in the treatments enriched with urchins rough crush solution.

The length growth of *S. latissima* in a low concentration of urchins rough crush solution exposed for a continuous period or repeated short period (pulse) has a variation in the first two weeks of the experiment but remains similar for the rest of four weeks (Figure 11). When the observe the effect of low urchins rough crush solution treatment over six weeks of the experiment seems that the difference in the growth of *S. latissima* exposure pulse and continuous did not appear to have much impact except the first two weeks. Because the *S. latissima* exposed in low concentration of rough crush solution in pulse and continuously have a most likely negligible difference in the length growth. In support of the study hypothesis, *S. latissima* stores nutrients when available for growth, there is no variation when exposed to low (10ml) concentration of urchins rough crush solution for short (pulse) or continuous period. The study suggests that a repeated short supply of nutrients can support the growth of *Saccharina latissima*. In natural marine water, nitrogen is considered a limiting nutrient for seaweed growth during the summer season after the phytoplankton bloom (Hanisak, 1983). *Saccharina latissima*, like other *Laminaria* species stores, dissolved nutrients when nutrients are available in excess in the environment and utilized when ambient nutrients concentration is limited for the growth (Bartsch et al., 2008; Harrison & Hurd, 2001).

The length growth of *S. latissima* in both low and high concentrations of urchins rough crush solution supplied as pulse was lesser than in the control. The length growth of *S. latissima* was poorest in treatment with a high concentration of urchins rough crush solution than control except in Week 3 and 5, there is no variation in the length growth between treatments (Figure 13). The high concentration of urchins rough crush solution supplied pulse has the highest phosphate concentration which might have causes negative impact on average length growth than in the

control treatment which has zero phosphate concentration and high length growth. It was supported by a study that showed high dissolved inorganic phosphate (DIP) concentrations of 6µmol/L have a negative effect on the growth of *Laminaria digitata* (Lubsch & Timmermans, 2019). Similarly, daily exposure to DIP of 6µmol/l has a fatal effect such as texture loss and disintegration in juvenile *Saccharina latissima* sporophytes within three weeks (Lubsch & Timmermans, 2019). Sporophytes treated with a low, pulsed concentration of urchins rough crush solution exposure increased the phosphate concentration in experimental beakers to less than 6µmol/L. Thus, the low pulsed treatment has a moderate effect on the length growth of *S. latissima*.

4.2 Color bleaching

The unexpected issue of color bleaching in *S. latissima* sporophytes was observed from the first week of the main experiment. Our study findings suggest *S. latissima* with urchins rough crush solution treatments have longer part of lamina color bleached than the control treatment. The *S. latissima* exposed to a low concentration of urchins crush solution continuously has four times the longer part of their color bleached than in the control. Likewise, the *S. latissima* exposed to a low concentration of urchins rough crush for a repeated short time have approximately double length of color bleached part of their lamina than in the control. The result did not support the study hypothesis, there is no variation in the length of the color bleached part of *S. latissima* lamina in low concentration of urchins rough crush solution as exposed short (pulse) or continuously. Instead, it appears that the longer *S. latissima* exposed to urchins rough crush has a higher negative impact on its coloration. Meanwhile, the *S. latissima* exposed to a high concentration of urchins rough crush solution shows approximately three times longer parts of their lamina color bleached than sporophytes in the control treatment.

A study in *S. latissima* short-term nitrate uptake, where sporophytes were nitrogen starved for eight days showed bleaching and decomposition afterward due to loss of protein pigmentation (Forbord et al., 2021). Similarly, *S. latissima* sporophytes grown in low nitrate concentration of 0 to 3 µmol/L appeared to be extremely light pigmented than in high nitrate concentration up to 20 µmol/L (Chapman et al., 1978). In the current study, there was low nitrate concentration among treatments (Figure 8). This indicates that a low concentration of nitrate in water might be one of the possible factors for the color bleaching of *S. latissima* but there was no variation in nitrate

concentration among the experimental treatments. However, the average length of the color bleached part of S. *latissima* lamina varies among treatment control, and different concentrations of urchin rough crush solutions depending on how concentration is supplied and exposed to seawater solutions (continuously or as a pulse). Then again, on average *S. latissima* exposed to urchins rough crush solution has a longer length color bleached of their lamina than the control implies that the concentration of urchins rough crush and their exposure have a strong negative effect on the coloration of *S. latissima*. It suggests there is another component in urchins rough crush solution which encourages the bleaching. In water sample analysis, P and N were the only nutrient component to be analyzed but there might be other components present in urchins rough crush which encourage the color bleaching of *S. latissima* lamina.

Another factor that may have affected the coloration of *S. latissima* is the light intensity. In a study by Fortes and Luning (1980), ten individuals of *S. latissima* (1-2 years) attached in 3-5 cm stripes were shown to have decreased chlorophyll content and pigmentation when exposed to light intensity higher than 30 µE $m^{-2}s^{-1}$. In this study, five young juvenile sporophytes of S. *latissima* (eight weeks) attached in approximately 15 cm string were exposed to light intensity 23 µmol $m^{-2}s^{-1}$. However, there were still bleaching of color in *S. latissima* when exposed to control and different concentration of urchins rough crush solution supplied as pulse or continuously. This was presumably an effect of the light source in the experimental set-up as LED light was horizontally attached near beakers with directly exposed to *S. latissima* (see Figure 3 Experimental set-up for clarity) which might have caused the bleaching in *S. latissima*.

5. Conclusion

This is the first study on the cultivation of *Saccharina latissima* by utilizing the byproduct of sea urchins as fertilizer. The use of sea urchins byproducts is applicable for sustainable fisheries and cost-effective fertilizer. However, the urchins rough crush enriched seawater appeared to be less suitable for the cultivation of *S. latissima*, as it appears it has less length growth and longer part of their lamina color bleached with the urchins rough crush enrichment. *S. latissima* cultivated with of urchins rough crush nutrient enrichment has less length growth, most likely due to high phosphate concentration and low nitrate concentration. Despite that the nutrient (urchins rough crush) exposure time pulse or continuous have a negligible effect on the length growth of *S.*

latissima, suggesting *S. latissima* can equally grow even if the nutrient is supplied repeatedly for short time. Moreover, the *S. latissima* exposed to urchins rough crush has a longer length part of lamina color bleached than the unenriched control treatment. An important area for further research should be to study the other content of urchins rough crush and whether one of the other components presents in the urchins rough crush could have resulted in less length growth and encourage the color bleaching in *S. latissima*.

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