Growth of two red algae species (*Palmaria palmata* and *Vertebrata lanosa*) in lab culture

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Abstract

Macroalgae and their products have many applications including their use in cosmetics, agriculture, health and bio-energy industries. The most common and oldest application of macroalgae is their use as a source of food and nutrition, aging back to the fourth century AD.

Palmaria palmata is a red alga with flattened fronds and length up to 50 cm and a diplohaplontic life cycle. It is found in the cold waters of North Atlantic and North Pacific in the littoral and sublittoral zones, usually as an epiphyte on *Laminaria* species. *Palmaria palmata* is used in human diet because of its high protein content and it is harvested from natural populations since the 12th century, but in the past decades it is also cultivated in open sea to a potentially commercial scale.

Vertebrata lanosa is a small filamentous red alga that is commonly associated with the fucoid brown alga *Ascophyllum nodosum*, on which is an obligate epiphyte. Its distribution, northern parts of North Atlantic, is limited to sites that its host is present. Though, *Vertebrata lanosa* is able to photosynthesize independently, but to a lower degree. The species received culinary interest in the recent years due to its truffle-like taste and it is referred to as “the truffle of the sea” by Nordic chefs. *Vertebrata lanosa* is currently only harvested by natural populations.

This study has focused on the growth of the two species in lab cultures and investigated the temperature and salinity leading to higher specific growth rate. For *Palmaria palmata*, nutrient conditions (3 levels) were also investigated in terms of growth rate and protein content. *Palmaria palmata* had higher growth rate in 12 ^{o}C, 30 ‰ and the medium with highest nutrient compassion tested. The protein content of the species was higher in intermediate nutrient conditions, though the differences were not significant. *Vertebrata lanosa* showed higher growth rate in 10 ^{o}C and 30 ‰. Overall, the study determined the best temperature and salinity conditions for indoor controlled cultivation of the two species and proved that *Vertebrata lanosa* can be cultivated in absence of its host. Though, to move from experimental culture to a larger scale production, further research is needed both on the cultivation of the species and the biochemical interactions with its host.

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1. Introduction

1.1 Uses of macroalgae

The major algal products produced by "the seaweed industry", which is currently based on harvest and use of macroalgae, are mainly brown and red algae (Radmer 1996). Macroalgae and their products have many applications including their use as food for humans and other animals, cosmetics and agriculture (Table 1-1).

Table 1-1: *Products from macroalgae and their market value. (according to McHugh 2003; Radmer 1996)*

Product	**Use**
Nori	Food
Wakame	Food
Kombu	Food
Hydrocolloids (alginate, agar, carrageenans)	Food products Paper products Cosmetics Pharmaceutical products Biomedical applications
Seaweed meal	Animal feed
Manure ("Mearl")	Agriculture
Liquid Fertilizer	Agriculture
Phycobiliproteins	Biomedical use

1.1.1 Food industry and Nutrition

At least 145 species of macroalgae are used as food worldwide (Zemke-White & Ohno, 1999). Macroalgae have been part of human diet for centuries, while the earliest traces of this habit lead to China during the fourth century AD (Yang & Brodie, 2017). Much later, consumption of algae begun to increase in western countries, mainly because Asian cuisine, especially sushi, has been imported (Rioux et al., 2017), but still the use of macroalgae in diet is much lower in Europe. In fact, according to Darcy-Vrillon (1993), the use of edible seaweed in Europe was close to 70 tonnes of dry products, while in Japan it was $97x10^3$ tonnes per year (through Fleurence et al., 2012).

The nutrition value of macroalgae differs with species and family, but it is similar to that of terrestrial vegetation, as it is characterized by high amounts of indigestible carbohydrates which support a low-calorie diet and helps to maintain the gut microbiota in a healthy state (Rupérez, 2002; Hehemann et al., 2012; Fleurence et al., 2012). Also, macroalgae have a high amount of trace elements, minerals and vitamins. Furthermore, the concentration of proteins in red algae is relatively high compared to green and brown algae and terrestrial vegetables (Morrissey et al., 2001; MacArtain, et al., 2007). Interestingly, some species of red algae can reach a very high protein content, up to 47 % of dry weight for *Porphyra yezoensis* (Ueda) M.S.Hwang & H.G.Choi 2011 and 35 % for *Palmaria palmata* (Linnaeus) F.Weber & D.Mohr 1805. In contrast, green and brown algae show a much lower protein concentration of 10-25 % for *Ulva lactuca* Linnaeus 1753 (green alga) and 5-10 % for *Laminaria japonica* Areschoug 1851 *(brown alga)* (Fleurence et al., 2012).

In Europe, particularly in Norway and France, macroalgae are mainly used for production of hydrocolloids like alginates (alginic acid: E400, sodium alginate: E401), agar (E406) and carrageens (E407) (Kaas, 1998 through Denis et al., 2010). These substances are polysaccharides and are used as food additives due to their thickening properties (McHugh 2003).

1.1.2 Health

Macroalgal organisms produce a large number of active biomolecules and secondary metabolites as a response to their exigent, competitive and aggressive surroundings compared to terrestrial environments (Kim & Wijesekara, 2010). In fact, more than 15 000 primary and secondary metabolites from different metabolic pathways have been reported for macroalgae and different applications were assigned to them (Grosso et al., 2011 through Andrade et al. 2012).

When it comes to human health, both types of metabolites are important and can potentially have remarkable positive effects on organism. Macroalgal metabolites have been described to have antioxidant, cytotoxic and antitumoral activities (Zubia et al., 2009a) and antidiabetic properties (Zhang et al. 2007). Furthermore, it has been found that such metabolites can have neuroprotective effects (Pangestuti & Kim, 2011) as well as positive effects on heart health (Fitzgerald et al., 2011). The antioxidant properties of macroalgal compounds have numerous applications in human health and are well summed by Cornish and Garbary (2010) (Table 1-2).

Table 1-2: *Examples of health benefits of specific antioxidant compounds from macroalgae (from Cornish & Garbary 2010)*

Antioxidant compound	**Perceived health benefit**
β-carotene, lutein	Antimutagenic Protective against breast cancer
Bromophenol	α-Glucosidase inhibition
Carrageenan, oligosaccharide	Anti-tumor
Fucoidan	Anti-HIV Ameliorates hyperoxaluria Anticancer Protection against neurodegenerative disorder
Fucophlorethols	Chemopreventive
Fucoxanthin	Antiangiogenic Protective effects against retinol deficiency
Galactan sulfate	Anti-viral
Phenolic functional groups and MAAs	Antiproliferative
Phlorotannins	Anti-inflammatory Bactericide Inhibits H_2O_2 mediated DNA damage Hypertension Photochemopreventive
Phycoerythrin	Amelioration of diabetic complications
Polyphenols	Vascular chemoprotection Antimicrobial
Porphyran, shinorine	Delays aging process

1.1.3 Biofuels

In the recent years, there is a lot of research that emphasizes to the environmental effects of the use of fossil fuels. Currently, the fossil fuel resources are not regarded as sustainable and are questionable in economic, ecological and environmental aspects (Kamm et al., 2006). Thus, a possible solution, perhaps the only one, is to turn to a sustainable, renewable and economically feasible alternative source of energy. This alternative energy source can be energy derived from a variety of bio-feedstocks – all the vegetable matter that is obtained by photosynthesis –, which is what we call "Biofuels" (Yusuf et al., 2011).

Algal biomass as bio-feedstock for fuel production, is a very competitive candidate because algae are easy to cultivate, they can grow with slight or even no attention, using water unsuitable for human consumption (Mata, Martins, & Caetano, 2010). Diverse types of biofuels, such as bio-oil, -diesel, -ethanol, -methane, -hydrogen, syngas and charcoal can be produced from algal biomass (Suganya et al., 2016) (Figure 1-1).

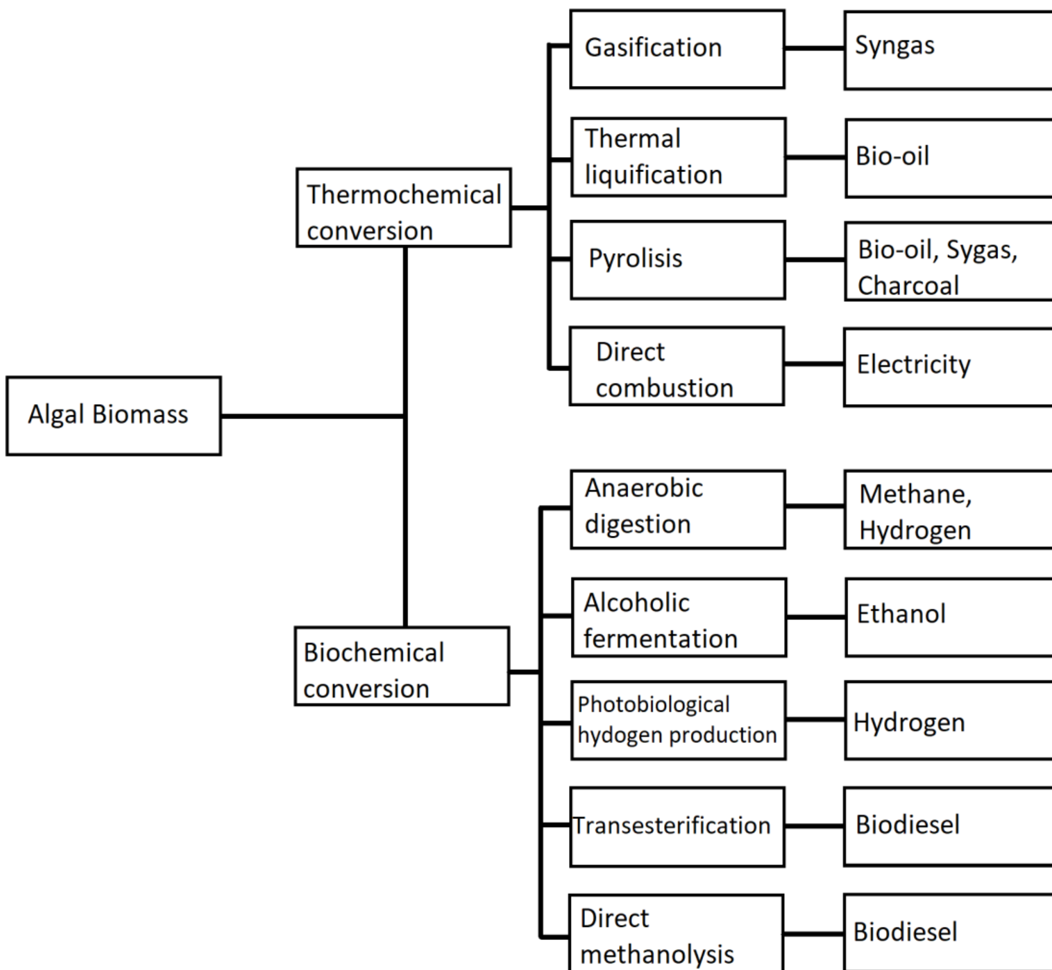


Figure 1-1: *Potential algal biofuel products and conversion processes (Suganya et al., 2016; edited)*

Although there is currently huge research investment into the microalgae massive production for biofuel, these algae are unlikely to be economically competitive for bioenergy production due to the

extremely high cost of the photo-bioreactors that are used in this process. Also, their culture in outdoor ponds is only suited to regions with relatively long sunlight periods and even then, they may still be uncompetitive in the biofuels market (Wilcox, 1977).

Macroalgae as a source of bioenergy first received intensive scrutiny as part of the US Ocean Food and Energy Farm project as proposed by Wilcox in 1973 and lasted over a decade (Bird & Benson, 1987). This resulted the construction of ocean farms for cultivation of *Macrocystis* (Leese, 1976). Macroalgae are considered to be suitable for biofuel production due to a variety of benefits when it comes to cultivation: (a) requiring no arable land, fertilizer, or fresh water resources, (b) cultivation outwit economic concerns associated with land management and (c) avoids unfavorable impacts on food supplies (Wargacki et al., 2012).

1.2 *Palmaria palmata* (Linnaeus) F. Weber & D. Mohr

Palmaria palmata, previously known as *Rhodymenia palmata,* is a red alga (Table 1-3) with flattened fronds (Figure 1-2) and its length varies from 20 to 50 cm, but sometimes it can reach 1 m. It grows from a small discoid base and it is gradually widening and subdividing. The stipe is not clearly visible as it rarely reaches 5mm in length. Small leaflet like structures grow sometimes on older parts of the algae along the margin, especially on damaged areas of the frond (Pereira, 2015; Pereira, 2016).

Table 1-3: *Taxonomy-Classification of* Palmaria palmata *(according to WoRMS, http://www.marinespecies.org).*

Kingdom	Plantae
Division	Rhodophyta
Class	Florideophyceae
Order	Palmariales
Family	Palmariaceae
Genus	*Palmaria*
Species	*palmata*

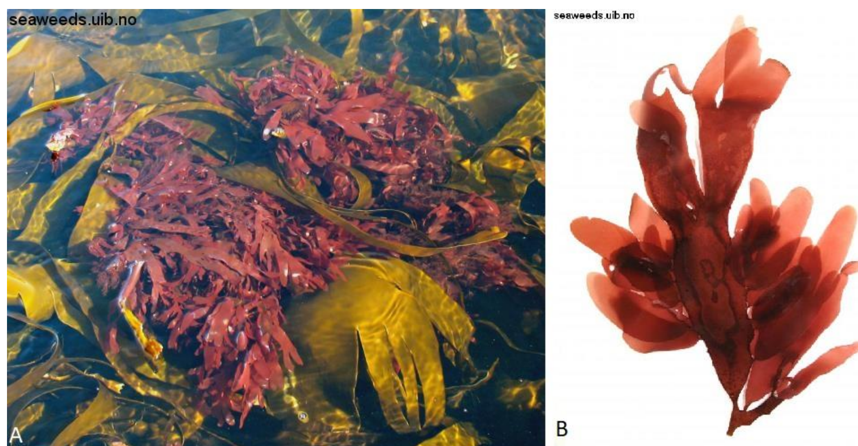


Figure 1-2: Palmaria palmata. *Many individuals growing on* Laminaria *stipes (A) and Tetrasporophytic specimen (B) (Kjersti Sjøtun 2008, Verified by Bergen Seaweed Group)*

Palmaria palmata is found in cold waters of the North Atlantic and North Pacific (Morgan, Shacklock, & Simpson, 1980a). The alga is often growing in the littoral (Hill, 2008) and sublittoral zones (Lüning 1990), under partially shaded conditions, as an epiphyte on the stems of different kelp species of the genus *Laminaria* (Figure 1-2A) (Morgan et al., 1980b). According to OBIC (Ocean Biogeographic Information System) *P. palmata* can be found along both sides of the North Atlantic Ocean, from the Arctic to cold-temperate regions.

The life cycle of *P. palmata* is diplohaplontic and with one diploid sporophyte stage and a haploid gametophytic generation. Tetrasporophytic fronds and male gametophytic fronds are macroscopic and demonstrate a similar morphology. In contrast, female gametophytes are microscopic and tetrasporangial plants are developing directly from the fertilized carpogonium by overgrowing the female plant, meaning that the carposporophyte phase is lacking (van der Meer &Todd, 1980). In this diphasic life cycle, only the tetrasporophyte stage allows recruitment, giving rise to gametophytic fronds directly (Le Gall et al., 2004) (Figure 1-3).

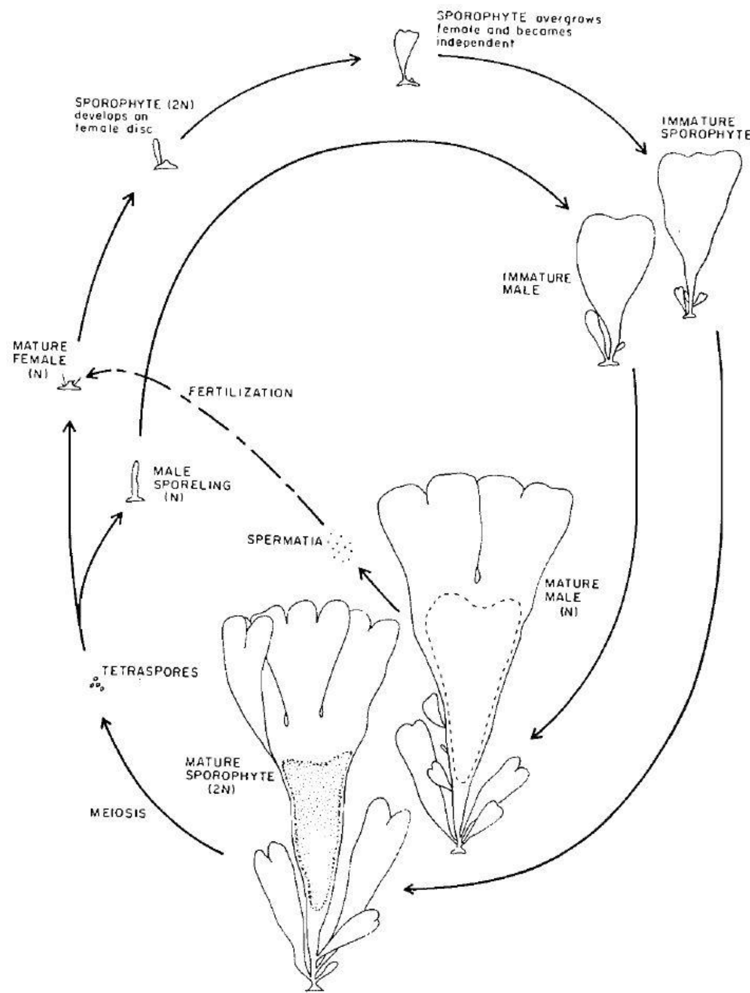


Figure 1-3: *Life cycle of* Palmaria palmata *(from van der Meer & Todd 1980)*

P. palmata can be used in human diet as a protein source as it has a very high protein content of 8-35 % and can be compared to other high protein foods like soybean (30 %), beef (25 %) and canned salmon (20 %) (Morgan et al. 1980a; Galland-Irmouli et al. 1999). This species is rich in potassium, iron, iodine and other trace elements and vitamins, such as B6 and B12 (Pereira 2011). There are also indications that some extracts from *P. palmata* are effective antioxidants and inhibitors of cell proliferation (Yuan et al., 2005). Hence, this algal species is a great addition to human diet, and it is already a very popular savory snack in Northern and West Ireland, where its hand-harvested from wild populations and dried before being eaten (Edwards & Dring, 2011).

P. palmata has been harvested from the coasts of northern Europe and America for centuries (Mouritsen et al., 2013) with the earliest recorded harvest of the species for food aging back to the 12th century as described in an Irish poem (Guiry & Guiry, 2020). The first attempt of cultivation of this species happened when the pressure on wild populations increased as a result of commercial harvesting (Edwards & Dring, 2011; Pang & Lüning, 2006). The dominant method used for *P. palmata* cultivation in the open sea, is the use of vegetative fragments that act as procreate units to acquire new plants (Le Gall et al. 2004). The species can also be cultivated from isolated spores, but this method has only been practiced for fundamental research, like investigations of the biological life cycle (van der Meer & Chen 1979). Cultivation of *P. palmata* in the open sea has been successfully performed in Northern Ireland and Northern Spain (Pang & Lüning, 2006; Martínez et al. 2006). In fact, in Asturias (Spain) the cultivation of the species in open sea ropes is developed and practiced at commercial scale (Sosa et al., 2006 through Pereira, 2016).

1.3 *Vertebrata lanosa* (Linnaeus) T.A. Christensen

Vertebrata lanosa is also a red alga (Rhodophyta) (Table 1-4) and was previously known as *Polysiphonia lanosa.* It's a small reddish-brown colored filamentous alga and the thallus is forming dense spherical tufts 3-7.5 cm in diameter. The structure of the filaments is made up by a ring of 12 -24 elongated cells, or periaxial cells, which surround a central axial cell. The erect axis is repeatedly branching pseudo-dichotomously (Maggs & Hommersand, 1993).

Table 1-1-4: *Taxonomy-Classification of* Vertebrata lanosa *(according to WoRMS, http://www.marinespecies.org)*

Kingdom	Plantae
Division	Rhodophyta
Class	Florideophyceae
Order	Ceramiales
Family	Rhodomelaceae
Genus	*Vertebrata*
Species	*lanosa*

V. lanosa is an intertidal alga and often grows on the fucoid brown alga *Ascophyllum nodosum* (Linnaeus) Le Jolis 1863 (Figure 1-4) and so the two species are commonly associated, but it has occasionally been recorded as an epiphyte on other fucoid algae, like *Fucus vesiculosus* Linnaeus 1753 (Rindi & Guiry, 2004), and, more rarely, on rocky substrata (Pizzolla, 2008). In the relationship between *A. nodosum* and *V. lanosa*, there is an additional member, the fungi *Mycophycias ascophylli* (Cotton) Kohlmeyer & Volkmann-Kohlmeyer 1998, and three-way interactions are taking place (Garabary, Deckert, & Hubbard, 2005). However, *V. lanosa* also appears to be able to photosynthesize independently, but it

has been proved that photosynthetical performance of the species is better when it grows on *A. nodosum* (Garbary et al., 2014). *V. lanosa* is also associated with the host-specific, red algal parasite *Choreocolax polysiphoniae* (Salomaki et al., 2015).

Figure 1-4: Vertebrata lanosa *growing on* Ascophyllum nodosum *(Bárbara Ignacio 2007 on WoRMS)*

V. lanosa is found mainly in the northern parts of North Atlantic Ocean, but not in Skagerrak and Greenland (Pedersen, 2011 through Bjordal, 2018). The fact that *V. lanosa* is limited to places that it's host, *A. nodosum,* is present but the distributions on the two species are not the same indicates that other environmental factors affect and limit the epiphyte's occurrence (Fralick & Mathieson, 1975; Garbary & Deckert, 2001). The absence of the species in Skagerrak, where its host is very common, is thought to be because of the low salinity levels in the area due to the Baltic Current (Åberg, 1992). This is also supported by a study indicating that *V. lanosa* demands a salinity rage between 25 and 40 ‰ to photosynthesize (Fralick & Mathieson, 1975).

V. lanosa has recently received gastronomical attention due to its truffle-like taste and Nordic chefs refer to *V. lanosa* as "the truffle of the sea". As the common truffle mushroom, this alga has a strong taste and aroma which make it perfect for flavoring (Bjordal et al., 2019).

1.4 Aim of study

For all the points mentioned in the first part of this introduction (1.1 Uses of Macroalgae), it is becoming clear that researching the growth and culturing conditions for different algal species is very important in order to cultivate them at commercial scale; and this is the driving the motivation of studying this topic.

The main goal of this project is to investigate the maximum growth rate and dietary value (proteins) and determine which conditions are the most suitable for each of the species introduced in 1.2 and 1.3 (*Palmaria palmata* and *Vertebrata lanosa* respectively). To achieve that, the growth rate in culture was tested with a variety of abiotic factors, such as salinity, nutrients and temperature.

1.4.1 Central research questions

The first question this study will attempt to answer, is whether the temperature affects the growth of the two species and which temperatures give the highest growth rate.

The second question that will be addressed, is whether the salinity levels of the growth medium is going to affect the growth rate of the species and which salinity levels are better suited.

Finally, the third question is whether the nutrient levels of the medium will have any effect on the growth rate and dietary value (protein content) of *Palmaria palmata* and which nutrient conditions result in the higher growth rate and the higher protein content. This experiment and analysis were only performed for *Palmaria palmata* since this species is consumed partly due to its promising nutritional value which brought additional scientific interest on the species. Similar work has not been done for *Vertebrata lanosa* as the gastronomical interest of this species is based only on its appealing taste.

2. Materials and Methods

2.1 Starting material

For *Palmaria palmata* the starting material used was frond-fragments of male gametophytes that were collected and isolated (clean from any other epiphytic species) prior to this study, from Frøya, an island outside the Trondheimsfjord. The fragments were taken from the upper part of each frond in a way that allowed only one apical area (at the tip of the frond) to be present per fragment.

For *Vertebrata lanosa* the cultures were initiated by small branched fragments of tetrasporophytes that were also collected and isolated prior to this study from Runde, an island south of Ålesund. The fragments were again taken from the upper parts of the alga and had the same number of branches, meaning the same number of apical areas (approximately 5 per fragment).

Stock cultures of both species were kept in 10 ^{o}C in a medium with a salinity of 30‰. Every 15-20 days the culture flasks were changed, and the nutrients were renewed by providing new medium. This way, the initial algal material kept growing and providing new starting material for the experimental cultures.

2.2 Medium

The growth medium used was IMR ½ (Eppley et al. 1967) which is a half defined medium, based on natural seawater and the addition of nutrients, trace elements and vitamins. The sea water used was collected in Drøbak from about 40 meters depth with a salinity of 34‰. This water was filtered in the lab through GF/F filters that were placed on a Millipore disk-base, as shown in Figure 2-1, with the help of a water-tub acting as a pump. The filtered seawater was collected in a conical filtering flask with a built-in outlet that was connected to the water-tub with a silicone tube.

After filtration, the salinity was adjusted (Table 2-1) by adding distilled water and the new salinity was measured with a refractometer. If the salinity was not at the desired level further adjustments were made, by adding small volumes of filtered seawater and/or distilled water and measured again.

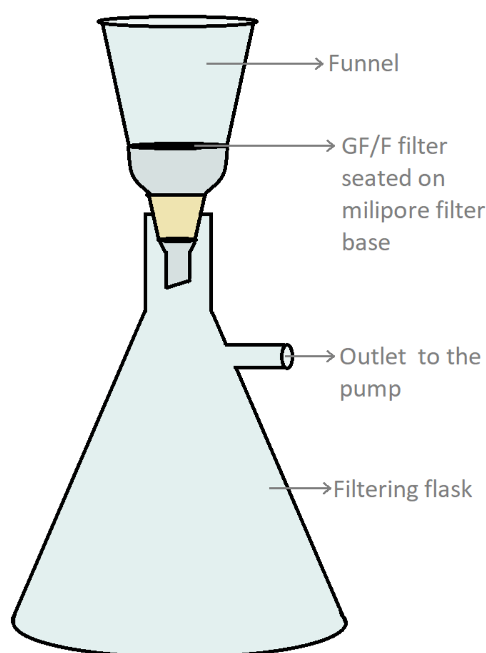


Figure 2-1: *Schematic of the filtration system.*

Table 2-1: *Composition of medium with respect to salinity level.*

Salinity of medium (‰)	Percentage (%)	Seawater - 34 ‰ (mL)	Distilled H_2O (mL)
~30	90	900	100
~20	59	590	410
~10	25	250	750

When the salinity reached the desired level, macronutrients, trace elements and vitamins were added according to Table 2-2. In addition to these, some droplets of germanium oxide (GeO_2) were added to the medium to prevent contamination with diatoms. After this step, the medium was placed in a water bath, with a thermometer inside the flask, until it reached 80 °C and stayed in this temperature for 15 minutes to be pasteurized. Then, to avoid precipitation, the medium was cooled down rapidly in a cold-water bath and transferred to a climate room, with a temperature of 6-10 °C, until used.

Table 2-2: *Composition of medium with respect to nutrients, trace elements and vitamins.*

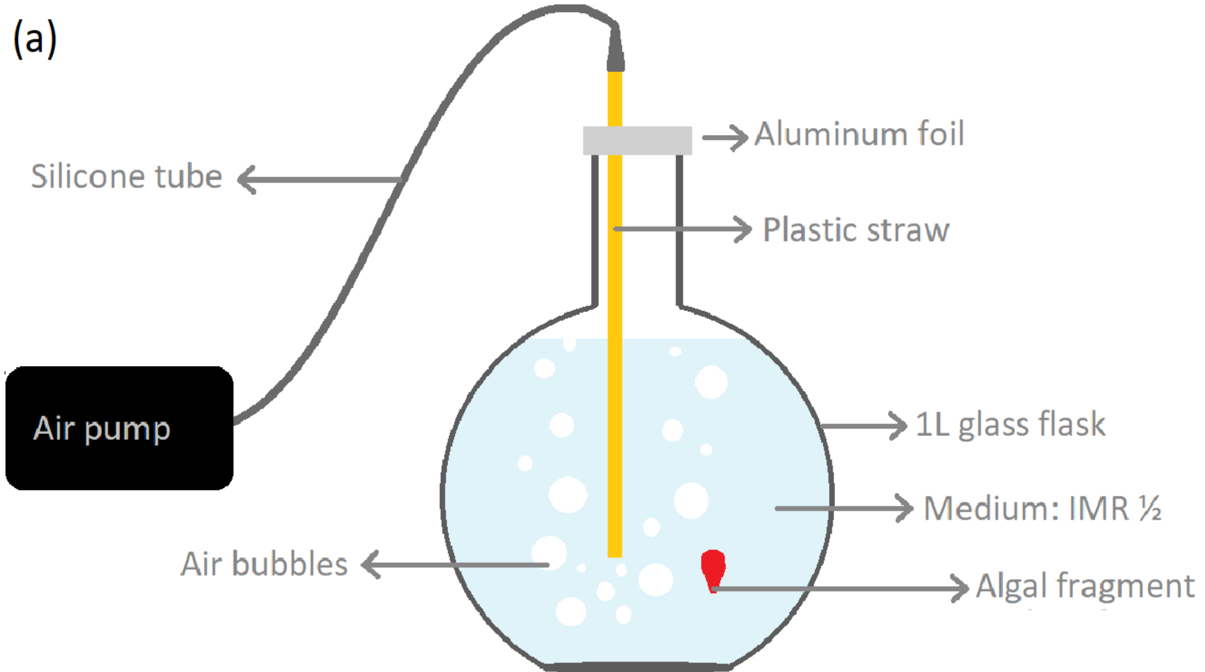
Solution	**Amount for 1 liter of medium (mL)**	**Concentration (µM/1000mL)**	**Amount for 5 liters of medium (mL)**
Nitrate stock solution (KNO_3)	0,5	250	2,5
Phosphate stock solution (KH_2PO_4)	0,5	25	2,5
Trace Metal stock solution (Na_2EDTA, $MnSO_4 \bullet 1 H_2O$, $ZnSO_4 \bullet 7 H_2O$, $Na_2MoO_4 \bullet 2 H_2O$, $CoCl_2$+$CuSO_4$)	0,5		2,5
Vitamin solution (B_1, B_6, B_{12})	0,5		2,5
Selenite solution ($Na_2O_3Se \bullet 5 H_2O$)	1	0,01	5

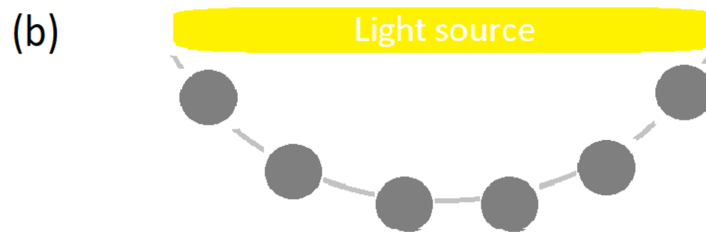
2.3 Cultures and Measurements.

Fragments of the two species were cut from the plants of the stock culture, as described earlier, and weighed with a 4 decimal places weight. Then the fragments were added into glass spherical flasks (on fragment per flask) with 1 L of medium. Small aquarium air pumps were providing air bubbles, through silicone tubes and plastic straws, to keep the cultures constantly rotating. The opening of the flasks was closed with aluminum foil with a small hole for the straw (Figure 2-2a). The flasks were placed in a crescent in front of a light source in such a distance that the light intensity reaching each flask was around 60 µmol photons $m^{-2}s^{-1}$ (measured with a photometer) (Figure 2-2b&c).

Every second week, circa 15 days, the algal fragments were weighed and put into clean flasks with new medium to ensure that the cultures will not become nutrient depleted. The weighing was done by gently patting each fragment with paper towels to remove excess water from the surface and weighed 3 times with an electronic scale. The average of the 3 measurements was calculated on spot and was used for further calculations and analysis. Also, a small amount of the used medium was collected for biochemical analysis of total nitrogen and total phosphorus concentration, to reveal if the nutrients were depleted. The analysis was performed on a selection of samples, mainly, corresponding to the ones with higher growth rates for each condition and species. This analysis was done by Berit Kaasa (Senior Engineer - Section for Aquatic Biology and Toxicology) on autoanalyzer instruments (SEAL AA3 HR AutoAnalyser for P and Flash EA 1112 NC Analyzers for TN/TOC).

For each condition or combination of conditions tested, the experiments lasted for approximately 45 days. Even though only the first and last weight measurements were needed, the algal fragments were weighed every time the medium was changed to provide a backup system in case the fragments die before the final measurement.





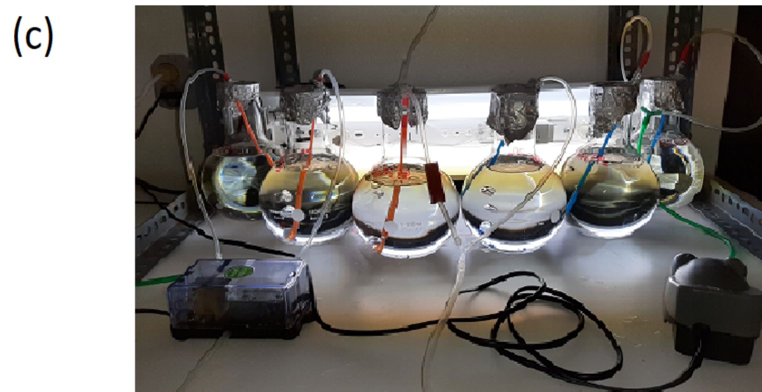


Figure 2-2: *Culturing system: schematic illustration of (a) in-flask culturing system, (b) placement of culture-flasks in front of the light source and (c) photo of culturing system.*

2.4 Growth Conditions

2.4.1 Light

As mentioned earlier, the light intensity was kept around 60 μmol photons $m^{-2}s^{-1}$ and followed a light and dark period of 14 hours light and 10 hours darkness.

2.4.2 Temperature, Salinity and Nutrients

For both species, growth experiments were conducted in culture rooms with temperature conditions of 6 °C, 10 °C, 12 °C, 16 °C and 19 °C. Initially, for all the five temperature conditions, the salinity level was 30 ‰ and full nutrients, meaning nutrients were added according to the IMR ½ recipe as described above (see paragraph 2.2 Medium; Table 2-2). The results from this experiment were used to highlight the temperatures that gave the higher growth rates in order to narrow down the temperature range for the next experiments. Thus, other salinity levels were tested in only three different temperature levels. In more detail, salinity levels of 20 ‰ and 10 ‰ were tested in temperature levels of 10 °C, 12 °C, 16 °C in full nutrients medium.

In addition to these temperature-salinity combinations, for *Palmaria palmata*, experiments were also performed with different nutrient levels in the temperature-salinity combinations that gave the higher growth rates. In this part, three nutrient levels, no added nutrients (N_0), half added nutrients (N_1) and full nutrients (N_2), were tested in two temperature levels, 10 °C and 12 °C, and with two salinity levels, 20 ‰ and 30 ‰. For the three nutrient levels, only the added macronutrients were manipulated while the vitamins and the trace metals were kept at the concentrations suggested by the medium recipe (Table 2-2). All the combinations described in these two paragraphs are summarized in Table 2-3.

Table 2-3: *Summary of the growth conditions combinations for experiments with* Palmaria palmata. *The first row shows the temperature levels and the following rows show which salinity (‰) or salinity x nutrient levels were tested in each temperature level.* N_0*: no added nutrients,* N_1*: half added nutrients and* N_2*: full nutrients.*

6 °C	**10 °C**	**12 °C**	**16 °C**	**19 °C**
x30‰	x30‰	x30‰	x30‰	x30‰
	x20‰	x20‰	x20‰	
	x10‰	x10‰	x10‰	
	x30‰ xN_0 x30‰ xN_1 x30‰ xN_2	x30‰ xN_0 x30‰ xN_1 x30‰ xN_2		
	x20‰ xN_0 x20‰ xN_1 x20‰ xN_2	x20‰ xN_0 x20‰ xN_1 x20‰ xN_2		

2.5 Calculation of Growth Rate

Using the biomass (weight) measurements of each fragment at the beginning (W_0) and the end (W_1) of the experiments and the time interval between the measurements (t) the specific growth rate (SGR) was calculated according to the following equation, as described by Kim et al. (2007):

$$SGR = \frac{lnW_1 - ln\,W_0}{t} * 100$$

The weight was given in grams (g) and the time in days. The resulting SGR is expressing the percentage of biomass increase per day (% FW d^{-1}).

2.6 Protein content analysis and preparation of samples.

After cultivation in the condition combinations involving nutrient level variability, the fragments of *Palmaria palmata* were placed in Eppendorf vials and frozen at -20 °C until used (Harnedy & FitzGerald, 2013) for the protein content analysis. When it was time for the analysis the samples were dried in a low temperature and pressure process for about 24 hours. This process is called freeze drying, lyophilization or cryodesiccation (Ratti, 2008). For this process, the Eppendorf vials containing the frozen samples were placed open in the Freeze drier. The user manual for the Freeze Drier can be found in Appendix I.

The main principle of this method is that by freezing the material and then lowering the pressure in a low temperature environment, the ice is removed in a process called sublimation (Fellows, 2000). This means that the water in the samples transitions directly from the solid phase (ice) to the gas phase, without passing through the intermediate liquid phase. In contrast to the most conventional dehydration methods, that evaporate water using heat (Prosapio et al., 2017), freeze drying allows for the product to maintain a high quality without the cellular contents, like nutrients, being deformed or deteriorated (Ratti, 2008).

After the samples were completely dry, they were pulverized using both a hand mortar and micro-pestles suited for microcentrifuge tubes (small Eppendorf vials) and a small vortex (Figure 2-3). Then for each sample 3-6 mg of the powder like product were weighed and packed in individual aluminum capsules. The aluminum capsules containing the samples were folded carefully to form small spheres without being ripped off (Figure 2-4). If the capsule was ripped off and the sample was exposed the packing was done again either by weighing new sample or by re-packing the sample in a second capsule.

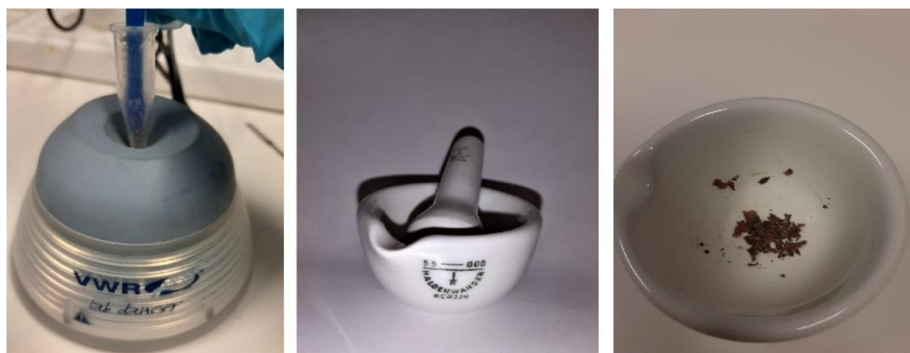


Figure 2-3: *Materials used to pulverize the samples, micro-pestles and vortex (left) and hand mortar (middle), and the final product (right).*

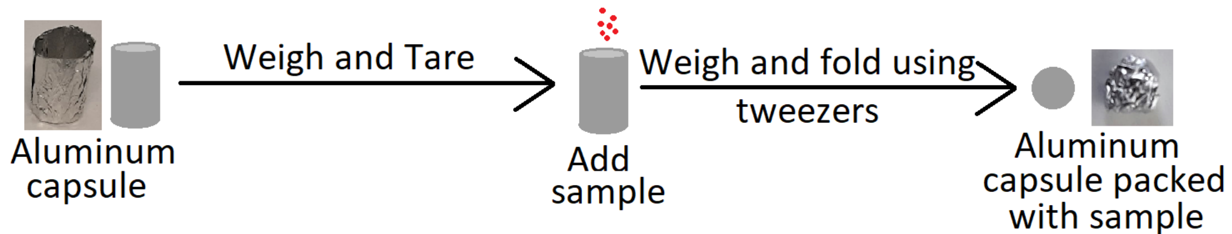


Figure 2-4: Packing the samples in aluminum capsules.

The packed samples were loaded in the gas chromatography instrument to be analyzed. The main principle of gas chromatography is that the sample is injected into the instrument and enters a gas stream which acts as a carrier and transports the sample into a separation tube known as the "column." The carrier gas is usually helium, hydrogen or nitrogen. The different components of the sample are separated in the column and the detector measures the quantity of the components that exit the column (Figure 2-5). To measure a sample with an unknown concentration, a standard sample with known concentration is injected into the instrument. The standard sample peak retention time (appearance time) and area are compared to the test sample to calculate the concentration (Evers, 2014). In this study, the standard sample used was nicotinamide, which contains known amount of nitrogen, and the carrier gas was helium.

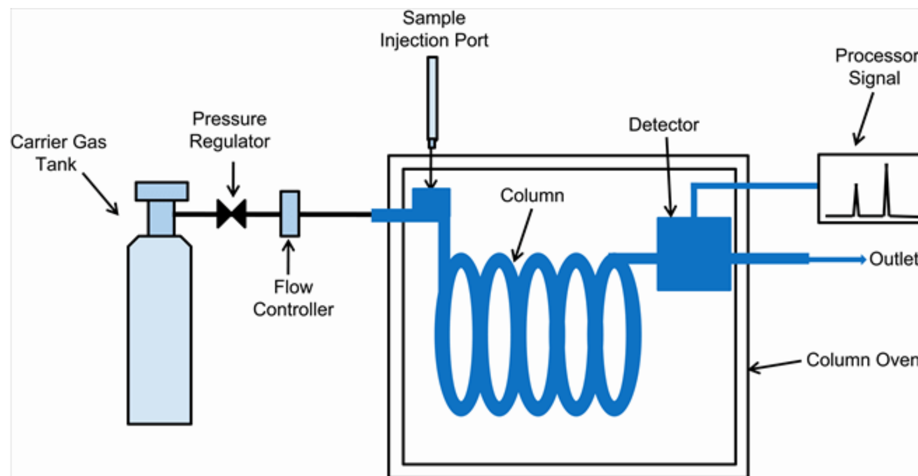


Figure 2-5: Schematic diagram of the main components of a gas chromatography system (from Evers, 2014)

The gas chromatograph was connected to a computer equipped with an analysis program that outputs the results in total nitrogen percentage of the sample. To convert the total nitrogen content to protein content, the following equation (Charrier et al., 2018) was used:

$$Nxfactor = \frac{\%\ (w/w)\ total\ protein}{\%\ (w/w)\ total\ nitrogen}$$

With a mathematical transformation this equation becomes a simple multiplication:

$$\%\ (w/w) total\ protein = Nxfactor\ *\ \%\ (w/w)\ total\ nitrogen$$

Note: $Nxfactor$ is the nitrogen-to-protein conversion factor.

The total protein content was calculated using a nitrogen-to-protein conversion factor equal to 4.7 as it's suggested for *Palmaria palmata* specifically (Bjarnadóttir et al., 2018).

2.7 Handling of data

Microsoft Excel was used for the storage of raw data and the calculation of SGR, mean values and standard errors. To produce illustrations of the data, the excel files were imported to R Studio (readxl library) and by using the R programing language (R Core, ggplot2 from tidyverse library) the data were transformed into graphs. R was also used to perform Welch two sample t-tests (Alfa: 0.05) between the maximum growth rate and the rest growth rates calculated for each experiment.

3 Results

Before presenting the results of the growth (and protein content), it is worth mentioning that for all samples where the growth medium was analysed the nutrients left where not depleted, though for some samples the total phosphorus was quite low (<2 μg/L). The full table with the results of this analysis can be found in Table A 1 in Appendix II.

3.1 *Palmaria palmata*

3.1.1 Temperature

In the temperature experiment, the highest SGR was found in 12 °C where the three replicate cultures had a mean SGR of 9,64 % FW d^{-1} (±1.24). The lowest mean SGR was 0.67 % FW d^{-1} (±0.13) when the algae was growing in 19 °C. In 16 °C the mean SGR was 1.68 % FW d^{-1} (±0.42). In temperatures of 6 °C and 10 °C the performance of the algae, in terms of growth were similar, with mean SGR of 4.16 % FW d^{-1} (±0.28) and 3.67 % FW d^{-1} (±0.05) respectively (Figure 3-1). The differences between the higher SGR and each of the rest were significant (Table3-1). Worthy of mentioning is also the fact that the specimens growing in 19 °C were found dead during the 5th week of the experiment.

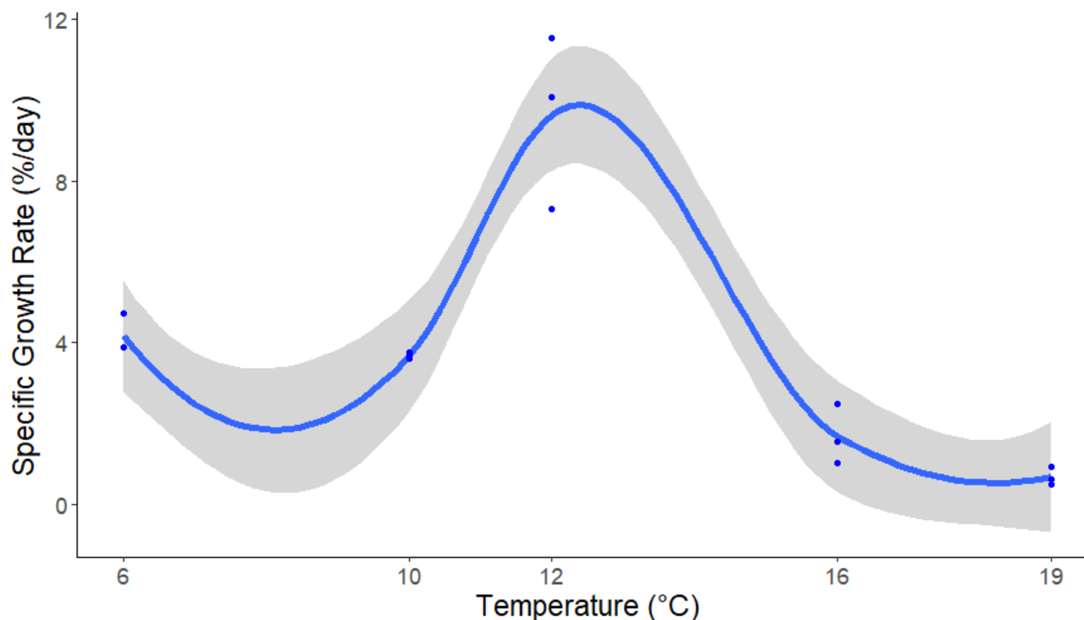


Figure 3-1: Palmaria palmata. *Specific growth rate – Temperature: illustration of the resulting SGR for the algae when growing in different temperatures and with a salinity of 30‰. Marked with grey shading is the standard deviation.*

Table 3-1: Palmaria palmata. *Specific growth rate – Temperature: mean values of SGR (meanSGR), standard errors (±SE) and p-values of the t-test between each group and the group with maximum SGR (12 °C).*

Temperature (°C)	Salinity (‰)	meanSGR (% FW d^{-1})	±SE	p-values
6	30	4.16	0.28	0.0419
10	30	3.67	0.05	0.0405
12	30	9.64	1.24	-
16	30	1.68	0.42	0.0156
19	30	0.67	0.13	0.0178

3.1.2 Salinity

In the salinity testing experiment, the mean SGR for *Palmaria palmata* was higher at 30 ‰ and in a temperature of 12 °C and it was 9.64 % FW d^{-1} (±1.24). In this salinity level, the algae had a much lower growth rate in 10 °C (3.67 % FW d^{-1}, ±0.05) and even lower in 16 °C (1.68 % FW d^{-1}, ±0.42). Overall, in a salinity of 10‰, for all three of the tested temperatures, the algae had the lower growth rates with mean SGR equal to 1.63 % FW d^{-1} (±0.07) for 10 °C, 1.80 % FW d^{-1} (±0.10) for 12 °C and 1.41 % FW d^{-1} (±0.25) for 16 °C (Figure 3-2). The differences between the maximum SGR and each of the rest were significant in most cases (Table 3-2).

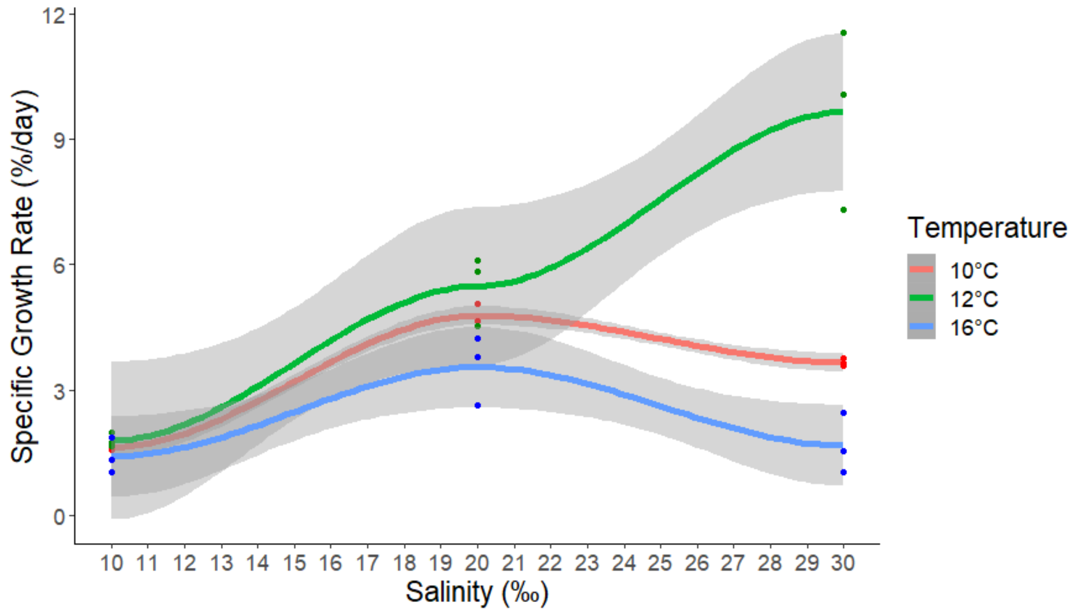


Figure 3-2: Palmaria palmata. *Specific growth rate – Salinity: illustration of the resulting SGR for the algae when growing in different salinity levels, 10‰, 20‰ and 30‰, all tested in three different temperatures (10 °C, 12 °C and 16 °C). Marked with grey shading is the standard deviation.*

Table 3-2: Palmaria palmata. *Specific growth rate – Salinity: mean values of SGR (meanSGR), standard errors (±SE) and p-values of the t-test between each group and the group with maximum SGR (12 °C, 30 ‰) (non-significant values marked with *).*

Salinity (‰)	Temperature (°C)	meanSGR (% FW d^{-1})	±SE	p-values
10	10	1.63	0.07	0.0230
	12	1.80	0.10	0.0236
	16	1.41	0.25	0.0188
20	10	4.78	0.14	0.0581*
	12	5.49	0.48	0.0640*
	16	3.55	0.48	0.0270
30	10	3.67	0.05	0.0405
	12	9.64	1.24	-
	16	1.68	0.42	0.0156

3.1.3 Nutrients

Overall, the algae had higher growth rates when growing in medium with higher nutrient levels (N_2) and when this co-occurred with a temperature of 12 °C. In more detail, when these conditions where provided with a salinity of 30‰, the mean SGR was 8.33 % FW d^{-1} (±1.07) and for 20‰ the mean SGR was 6.50 % FW d^{-1} (±0.71). When there were no added nutrients in the medium (N_0), the mean SGR was low, varying between 1.49 % FW d^{-1} (±0.35) for 10 °C and 20‰, and 2.13 % FW d^{-1} (±0.17) for 10 °C and 30‰. For intermediate nutrient conditions (N_1), the mean SGR was also low varying between 1.88 % FW d^{-1} (±0.15) for 12 °C and 20‰, and 3.31 % FW d^{-1} (±0.50) for 12 °C and 30‰ (Figure 3-3). Again, the differences between the higher SGR and each of the rest were significant in most cases (Table 3-3).

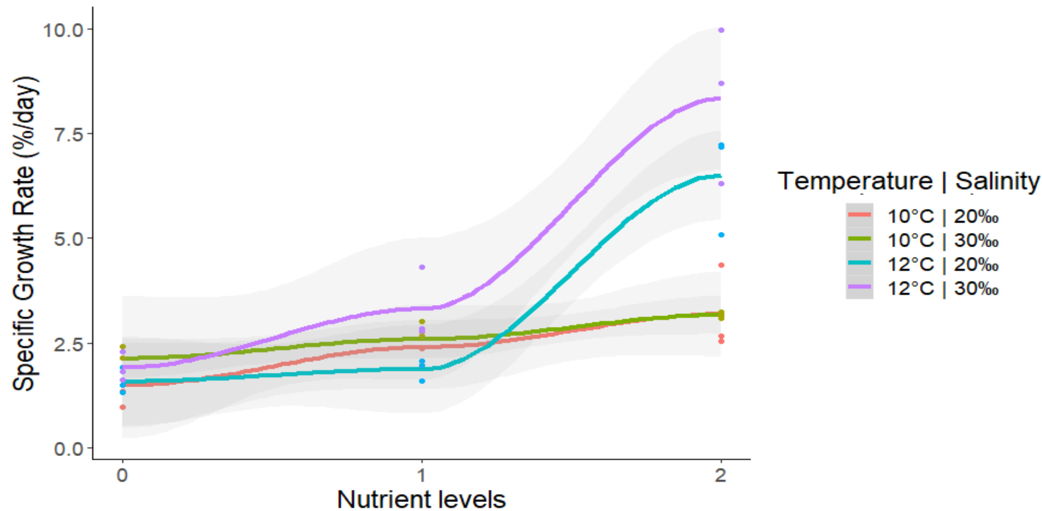


Figure 3-3: Palmaria palmata. *Specific growth rate – Nutrients: illustration of the resulting SGR for growth in different nutrient levels (0: no added nutrients, 1: half added nutrients, 2: full nutrients). Experiments were performed in a variety of salinity and temperature combinations as noted in the legend. Marked with grey shading is the standard deviation.*

Table 3-3: Palmaria palmata. *Specific growth rate: Nutrients: mean values of SGR (meanSGR), standard errors (±SE) and p-values of the t-test (non-significant values marked with *) between each group and the group with maximum SGR (12 °C, 30 ‰, N_2). [N_0 (0, no added nutrients), N_1 (1, half added nutrients) and N_2 (2, full nutrients)]*

Nutrients	Temperature (°C)	Salinity (‰)	meanSGR (% FW d^{-1})	±SE	p-values
N_0	10	20	1.49	0.35	0.0164
		30	2.13	0.17	0.0264
	12	20	1.58	0.18	0.0219
		30	1.92	0.20	0.0237
N_1	10	20	2.40	0.20	0.0279
		30	2.59	0.27	0.0272
	12	20	1.88	0.15	0.0248
		30	3.31	0.50	0.0272
N_2	10	20	3.19	0.59	0.0231
		30	3.17	0.04	0.0405
	12	20	6.50	0.71	0.2382*
		30	8.33	1.07	-

3.1.4 Protein Content

In general, as calculated with both factors, the highest protein content was a result of growth in intermediate nutrient level (N1). In this condition, the protein content was quite similar between the different temperature and salinity combinations (Figure 3-4). The protein content was slightly higher for 12 °C and 20 ‰ where the proteins consisted 34.83 % of the dry weight (DW) of the fragments, though the general similarity is also evident from the t-test results between each group and the group with the higher protein content which indicated significant difference only for three of the combinations(Table 3-4).

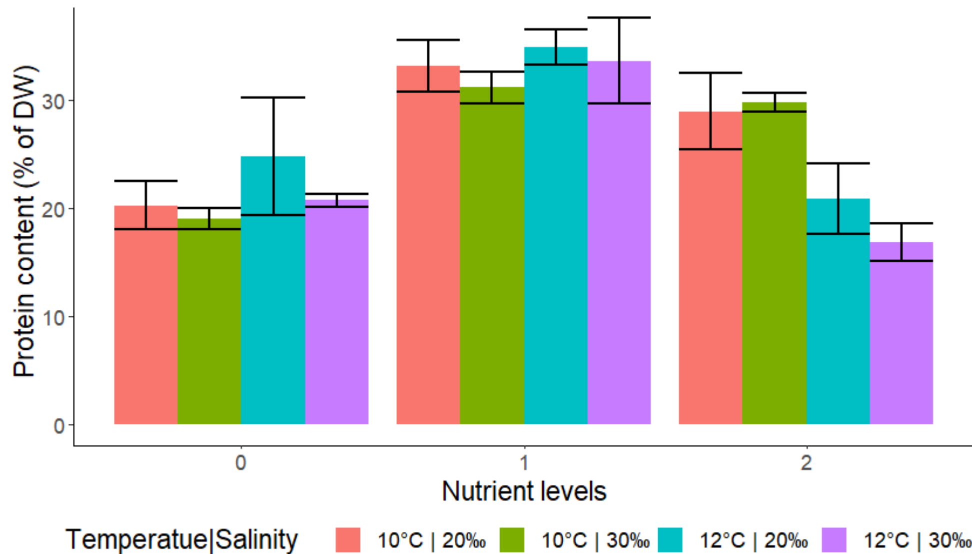


Figure 3-4: Palmaria palmata. *Mean protein content resulting from growth in different nutrient levels (0: no added nutrients, 1: half added nutrients, 2: full nutrients) and in a variety of salinity and temperature combinations. The protein content shown as calculated with the Nitrogen-to-Protein factors of* P. palmata *(N x 4.7).*

Table 3-4: Palmaria palmata. *Mean protein content (% of DW) calculated with the Nitrogen-to-Protein factor (N-to-P) of* P. palmata *(N x 4.7), standard error (±SE) and p-values of the t-test (non-significant values marked with *) between each group and the group with higher protein content (12 °C, 20 ‰, N_0). [N_0 (0, no added nutrients), N_1 (1, half added nutrients) and N_2 (2, full nutrients)].*

Nutrients	Temperature (°C)	Salinity (‰)	Protein content (N-to-P of *P. palmata*) (% of DW)	±SE	p-values
N_0	10	20	20.26	± 2.25	0.0081
		30	18.95	± 0.98	0.0025
	12	20	24.76	± 5.39	0.1959*
		30	20.73	± 0.58	0.0071
N_1	10	20	33.13	± 2.34	0.5862*
		30	31.13	± 1.46	0.1657*
	12	20	34.83	± 1.62	-
		30	33.59	± 3.93	0.7915*
N_2	10	20	28.92	± 3.52	0.2307*
		30	29.81	± 0.87	0.0702*
	12	20	20.83	± 3.26	0.0323
		30	16.84	± 1.76	0.0017

3.2 *Vertebrata lanosa*

3.2.1 Temperature

For *Vertebrata lanosa*, in relation to temperature, the highest mean SGR was found at 10 °C and it was 3.10 % FW d^{-1} (±0.05). In 12 °C the mean SGR was 2.35 % FW d^{-1} (±0.29). For the rest of the temperatures tested this species showed very similar responses in terms of growth rate as the mean SGR found for 6 °C, 16 °C and 19 °C were 1.76 % FW d^{-1} (±0.47), 1.87 % FW d^{-1} (±0.33) and 1.82 % FW d^{-1} (±0.11) respectively (Figure 3-5). The difference of SGR between the temperature conditions was significant only between the higher SGR (at 10 °C) and the lower SGR (at 19 °C) (Table3-5).

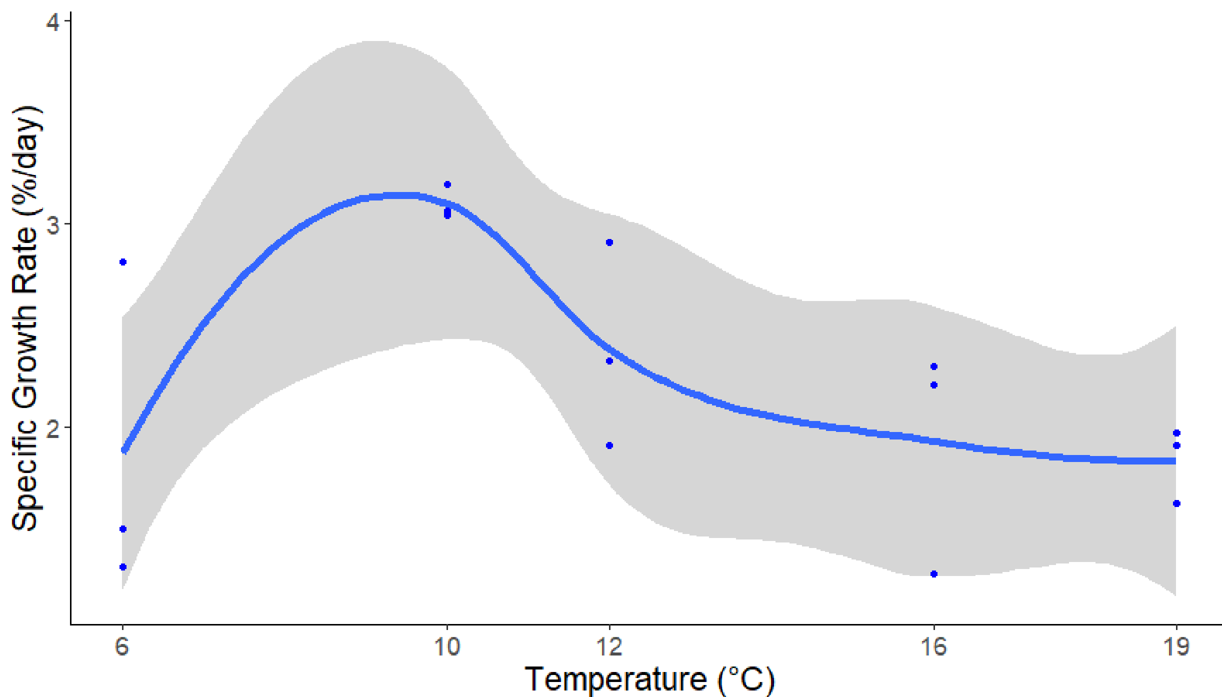


Figure 3-5: Vertebrata lanosa. *Specific growth rate – Temperature: illustration of the resulting SGR for the algae when growing in different temperatures (6 °C, 10 °C, 12 °C, 16 °C and 19 °C) with a salinity of 30‰. Marked with grey shading is the standard deviation.*

Table 3-5: *Vertebrata lanosa. Specific growth rate – Temperature: mean values of SGR (meanSGR), standard errors (±SE) and p-values of the t-test between each group and the group with maximum SGR (non-significant values marked with *).*

Temperature (°C)	Salinity (‰)	meanSGR (% FW d^{-1})	±SE	p-values
6	30	1.76	0.47	0.1199*
10	30	3.10	0.05	-
12	30	2.35	0.29	0.1292*
16	30	1.87	0.33	0.0667*
19	30	1.82	0.11	0.0026

3.2.2 Salinity

In the salinity experiment, for this species, the higher mean SGR was found to be 3.10 % FW d^{-1} (±0.05) at 10 °C and with 30‰ salinity. Though, in all temperatures the species had similar SGR when it was growing in medium with close to full-strength salinity (12 °C: meanSGR=2.90 % FW d^{-1}, ±0.15 – 16 °C: meanSGR=2.47 % FW d^{-1}, ±0.49). In the lowest salinity tested, 10‰, the species performed better when growing in 12 °C giving a mean SGR equal to 1.28 % FW d^{-1} (±0.16) while in 10 °C and 16 °C the mean SGR was 0.83 % FW d^{-1} (±0.05) and 0.71 % FW d^{-1} (±0.07) respectivly (Figure 3-6). The difference between the higher SGR and each of the rest was significant in most cases (Table 3-6).

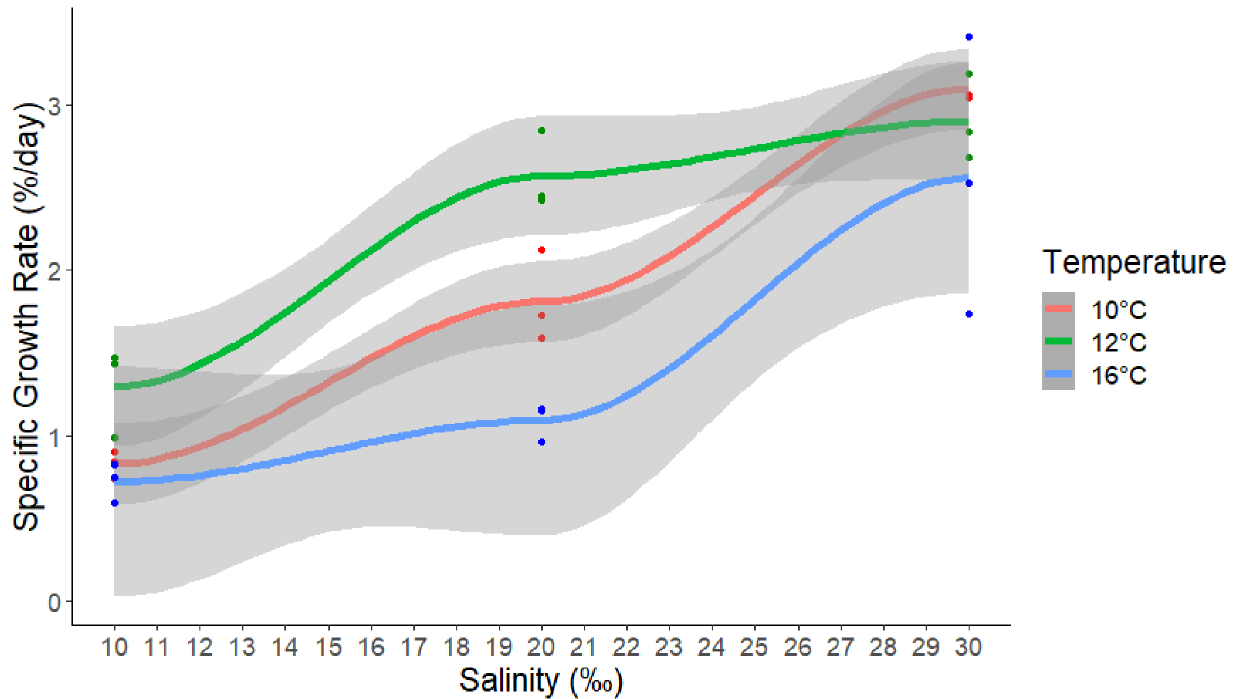


Figure 3-6: Vertebrata lanosa. *Specific growth rate – Salinity: illustration of the resulting SGR for the algae when growing in different salinity levels, 10‰, 20‰ and 30‰, all tested in three different temperatures (10 °C, 12 °C and 16 °C). Marked with grey shading is the standard deviation.*

Table 3-6: Vertebrata lanosa. *Specific growth rate – Salinity: mean values of SGR (meanSGR), standard errors (±SE) and p-values of the t-test between each group and the group with maximum SGR (non-significant values marked with *).*

Salinity (‰)	Temperature (°C)	meanSGR (% FW d^{-1})	±SE	p-values
10	10	0.83	0.05	p<<0.05
	12	1.28	0.16	0.0044
	16	0.71	0.07	p<<0.05
20	10	1.80	0.16	0.0104
	12	2.57	0.14	0.0497
	16	1.09	0.07	p<<0.05
30	10	3.10	0.05	-
	12	2.90	0.15	0.3192*
	16	2.47	0.49	0.3829*

3.2.3 A pleasant surprise: Tetraspore formation

During the year-long cumulative time of the experiments, some of the stock culture individuals, kept in 10 ºC, as well as some of the individuals growing in 12 ºC and in a medium with a salinity of 30 ‰, were observed to form tetraspores (Figure 3-7). In some individuals the tetraspores germinated while still inside the mother plants. Unfortunately, no photo was taken to document this event.

(1) 101.47μm

Figure 3-7: *Specimen of* Vertebrata lanosa *from the stock culture with tetraspores (shown in the red circle). Photo taken from Light microscope.*

4 Discussion

4.1 *Palmaria palmata*

Palmaria palmata is currently cultivated in land-based tanks and on rope longlines in sea (Werner & Dring, 2011). The present study, in an attempt to investigate the growth conditions leading to higher growth rates in lab cultures, found that the species is growing faster in 12 °C (9.64 % FW d^{-1}, SE=1.24). In this temperature, the growth rate is significantly higher (p=0.0419 or lower) than in any other temperature tested. This finding is in agreement with Morgan and Simpson (1981), who identify temperatures between 10 and 12 °C to be the optimal for the growth of *P. palmata*. The same study also claims that the alga is growing poorly in 18 °C, which again, agrees with the findings of the present study where the individuals growing in 19 °C died during the experiment and the individuals growing in 16 °C had very low growth rate (0.67 % FW d^{-1}, SE=0.13). In general, the optimal temperature range for *Palmaria palmata* was found between 6 and 14 °C by several studies (Bak, 2014; Edwards & Dring, 2011; Werner & Dring, 2011; Morgan & Simpson, 1981; Morgan et al., 1980a; Robbins, 1978) These are also consistent with the species natural distribution in northern temperate and arctic waters (Hill, 2008).

With regard to the temperature – salinity combinations, the present study determined that 30 ‰ is the optimal salinity for the growth of the species. This salinity level combined with the optimal temperature (12 °C) gave a growth rate (9.64 % FW d^{-1}, SE=1.24) significantly higher than almost any other combination of salinity and temperature levels (p=0.04047 or lower with 2 exceptions with p>0.05). The findings of the present study agree the general guidelines for cultivation of the species, which advise salinity to be close to full strength (~34‰) (Robbins, 1978; Werner & Dring, 2011). The optimum salinity level determined here is also explained by the fact that the species is characterized as stenohaline (Karsten et al., 2003; Beauchamp, 2012) with optimal salinity range between 23 and 34‰ (Robbins, 1978).

Apart from the combination of 12 °C with 30 ‰, the growth rates are quite similar for the rest of the conditions combinations. This can be explained with the fact that *P. palmata* is found in the littoral zone and thus it is regularly exposed to precipitation and evaporation that can result in lower or higher salinity. However, the species has not been recorder in areas with permanently low salinities and so it is considered to have intermediate intolerance to reduced salinity (Hill, 2008). The fact that the growth rate was distinctively higher when the optimal temperature and the optimal salinity co-occurred, indicates that these environmental factors may have similarly strong effects on the growth of the species, though this cannot be confirmed with the knowledge and data acquired during this study.

Kartsen et al. (2003) and Beauchamp (2012) identify 15 ‰ as the lethal lower level for *P. palmata*. Interestingly, the individuals grown in 10 ‰, despite the poor growth (1.41 - 1.80 % FW d^{-1}), did not die during the salinity experiments of the present study. Perhaps, if the exposure period was longer, lethal effects would be observed. From another point of view, temperature and salinity can have combined effects. For example, Druehl (1967) found that *Saccharina latissima* (Linnaeus) C.E.Lane, C.Mayes, Druehl & G.W.Saunders 2006 (Phaeophyceae) can tolerate non-optimum salinities if grown in close to optimum temperatures. Lobban and Harrison (1994) suggested that this example may be general and applied to all macroalgae. Therefore, another possible explanation for the absence of lethal effects of such low salinity in this study, is that this salinity was tested in all cases with temperatures close to optimum. Thus, the temperature being within optimal range may helped the algae overcome the stress caused by low salinity. Though, to extract conclusions about multiple stressors effects the experiment has to be designed in such a way that the two stressors overlap but are not applied simultaneously for long periods (Gunderson et

al., 2016). As the experimental design in this study is not as described, a more in-depth investigation of the combined effects of multi-stress caused by temperature and salinity is needed to supply sound conclusions on this matter.

Regarding the nutrient levels, in combination with temperature and salinity, the highest growth rate (8.33 % FW d^{-1}, SE=1.07) is, as expected, a result of growth in the most nutrient rich medium and, again, with this condition co-occurring with the optimal temperature and salinity. This growth rate was significantly higher than the rest of the combinations (p=0.04047 or lower) with one exception. This exception is the case where the alga grew in the same nutrient and temperature conditions as the maximum growth rate case, but in lower salinity (20 ‰). Here, the growth rate (6.50 % FW d^{-1}, SE=0.71) was lower but not to a significant level (p=0.2382). The reason behind this non-significant difference is the high variance of the growth rates of the individuals' growth in both conditions. Moreover, regardless of temperature and salinity, the algae had much lower and similar growth rates (1.49 - 3.31 % FW d^{-1}) in media with both intermediate and no added nutrients.

As *P. palmata* shows enhanced growth when grown waters enriched by nutrients due to fish farms (Sanderson et al., 2012), it is no surprise that in this study as well, the species had a higher growth rate when grown in the most nutrient rich medium. Furthermore, the species' preference to high nutrient content of the ambient medium can again be explained by its natural distribution, northern temperate and arctic waters (Hill, 2008), as these regions are usually characterized as eutrophic and highly productive due to the strong seasonal variation and the somewhat common short-term disturbances that redistribute nutrients, enriching the euphotic zone and enhancing the primary production (Denny, 2008).

The relatively high maximum growth rates determined in this study can be explained by several factors. First, cultures of free-floating fronds are kept in constant motion by aeration of the medium, which results in more efficient use of nutrients by reducing the boundary layers and preventing diffusion rates from limiting the growth (Hafting, 1999a, b). Second, again due to aeration of the medium, the fronds receive equivalent illumination and avoid self-shading (Hafting, 1999a). Third, by using filtered and sterilized seawater, as base for the medium, and by isolated culture epiphytes are excluded from the system preventing negative effects like shading or competition for nutrients. Fourth, new medium is supplied (before depletion of nutrients) in order to avoid negative effects on the growth rate caused by nutrient limitation.

However, the highest growth rate observed in this study (9.64 % FW d^{-1}, SE=1.24) is lower than the highest growth rate observed in *in situ* cultivations, which, as summarized by Grote (2019), is around 14 % FW d^{-1}. A reasonable explanation for this difference is that in the present study the algal fragments used for each experiment consisted of fronts with one apical area (growth region) each, while in *in situ* cultivation the fronts measured have usually more than one apical region. This means that in the case of *in situ* cultivation the algae can grow through multiple growth regions at the same time, hence achieving higher collective growth rate.

In respect of proteins, *P. palmata* is a species that contains nitrogen in non-protein compounds, like ammonium salts, amines and nitrates (Morgan et al., 1980b). This can lead to overestimation of the protein content when the nitrogen is converted into protein using a more general conversion factor, like the one used for all seaweeds, 6.25 (Galland-Irmouli et al., 1999; Kadam et al., 2017) or even the one used for all red algae, 5.1 (Angell et al., 2016). The protein content considered closer to the actual value, is the

one calculated with the nitrogen-to-protein conversion factor that is specific for *P. palmata* and is 4.7 (Bjarnadóttir et al., 2018).

Overall the protein content found here ranged from 16.84 to 34.83 % of DW. These values are consistent with values found in the literature which range from 8 to 35 % (Morgan et al., 1980b; Galland-Irmouli et al., 1999; Bjarnadóttir et al., 2018) with the most typical values being around 20 % (Mouritsen et al., 2013). Interestingly, the highest protein content in this study was found in intermediate nutrient levels which does not comply with the general narrative for other red seaweeds where maximum protein content is found in grown conditions of maximum available nutrients (Mathieson & Tveter, 1975; 1976). Though, the results of these studies concern red algae species collected from natural populations during winter and spring, where nutrients are at the highest concentrations in seawater. In the case of the present study, the alga was grown in a growth medium that was based on water from a nutrient rich depth and extra nutrients were added. For example, the nitrate solution used for IMR ½ medium has a concentration of 250 μM, which is already more than 10 times higher than that of natural seawater. Thus, the disagreement between the mentioned narrative and the findings of this study, could be explained as that the nutrients in the most nutrient rich medium were exceptionally high, at levels not found in natural seawater, to allow higher protein production and storage.

Moreover, the protein content was on average lower in the less nutrient rich medium, were no extra nutrients were added. This is also supported by Morgan et al. (1980b) were in tank cultures of *P. palmata,* the protein content showed a rapid decline when the water was depleted of nitrogenous nutrients. Though, the values found in the present study for this condition, ranging from 18.95 to 24.76 %, are still among the typically reported values mentioned above (around 20 %) and the lower value (8 %) reported by Morgan et al. (1980b). This can again be explained by the fact that the water on which the medium was based came from a nutrient rich depth, and hence the nutrient concentration of the medium even without the addition of any nutrients (Total N: 1767 μg/L, Total P: 13 μg/L, see Table A1 in Appendix II), is higher than that of nutrient depleted seawater.

The method used for the protein content determination can be true under two assumptions: (a) carbohydrates and fats do not contain N and (b) nearly all N is present incorporated into amino acids in proteins (MacLean & Warwick, 2003). Since these assumptions are not entirely true, the results of the present study, regarding this matter, provide only an indication of the potential protein content of *P. palmata* under the different growth conditions tested, and should be used with precaution.

4.2 *Vertebrata lanosa*

Vertebrata lanosa received attentions in the recent years as a new local product with an appealing taste (Viestad, 2016). The alga is almost always an epiphyte of *Ascophyllum nodosum,* and so it has been thought that it is difficult to cultivate it (Bjordal et al. 2019). As the species is getting more and more gastronomical attention, the demand for it grows. Currently, the species is harvested from natural populations putting a potential pressure to these populations. At the same time, the main host of the species, *A. nodosum*, is also harvested further reducing the abundance of *V. lanosa* (Garbary, 2017). Therefore, exploring the cultivation potentials of the species is crucial. This study, as one of the first attempts of culturing *V. lanosa* alone, shows that the cultivation of the species is possible.

The highest growth rate of the species recorder in this study (3.10 % FW d^{-1}, SE=0.05) was found in 10 °C. Though, this growth rate is not significantly different (p=0.06 or higher) than most of the growth rates found in other temperatures (1.76 – 2.35 % FW d^{-1}). The general similarity of the growth rates in the different temperatures is not a surprise as the species is naturally found in the lower intertidal zone, with its host *A. nodosum*, where it is exposed and tolerates different temperatures.

Lüning (1990) suggested that *V. lanosa*, shows an optimal temperature range of 22-24 °C for photosynthetic production (through Bjordal, 2018). Even though experiments were not conducted in such high temperatures, the findings of the present study disagree with this suggestion, as the growth rate (1.82 % FW d^{-1}, SE=0.11 in 19 °C) recorded in the temperature closer to the suggested range, was significantly lower (p= 0.002548) than the maximum observed.

In respect of salinity, the species performed better in 30 ‰ regardless of temperature. The highest growth rate (3.10 % FW d^{-1}, SE=0.05) was recorded from the combination of this salinity with a temperature of 10 °C. This growth rate was significantly higher (p=0.0497 or lower) than the growth rates observed in other salinities. This is also supported by the findings of Fralick and Mathieson (1975) who showed that *V. lanosa* has higher photosynthetic rates (oxygen production) in salinities between 25 and 40 ‰. Though, in the present study, no significant difference (p=0.3192 or higher) was found between the highest growth rate and the growth rates found in the other temperatures tested with the same salinity (30 ‰). This might suggest that salinity plays a stronger role in the growth of the species than temperature.

Moreover, in salinities lower than 30 ‰, the species showed better growth when growing in 12 °C rather than 10 °C (where highest growth rate was found for all temperatures in 30‰). But still, as explained earlier, during the experiments of this study, temperature has not been found to strongly determine the maximum growth of the species.

Despite the achievement of cultivating *V. lanosa* separately from its host, the growth rates measured during this study are relatively low. Even though it has been suggested that long term photosynthetic activity of *V. lanosa* requires its attachment to *A. nodosum* (Garbary et al., 2014), here the species was successfully maintained in stock cultures for more than a year and continued to be able to supply starting material for the experiments. Thus, it is evident that the species can be cultivated with starting material harvested from natural populations. Additionally, during this time, individuals were observed producing tetraspores, therefore with further investigation, cultivated plants can supply tetraspores for culture completely independent from the natural populations. Though it would be interesting for further studies to investigate the exact biochemical interactions of the species and provide an insight as to which *Ascophyllum* metabolites, if any, result in higher growth rates of *V. lanosa*, and how they can be used in tank and indoor cultures of the species.

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Appendix I

Freeze drier user manual

1. Put the "Sample Plate" and the "Vacuum cap" in the freezer before you are going to use the freeze-dryer.

2. Empty the waste bottle for condensate (under drain valve for condensate)

3. Check the oil level on the vacuum pump. The recommended level should be halfway between max and min on the glass.

4. Close the air valve

5. Close the drain valve for condensate (Do not turn it hard!)

6. Close the pressure valve

7. Check the "Gas Ballast Valve " on the vacuum pump is open (in position 2)

8. Set the program switch to the " KONDENSATOR VERKÛLEN "

9. Turn on Vacuum pump (in the back)

10. Allow equipment to stand for 30 min. before starting.

*Remember that you should only use frozen samples. *

11. Put frozen samples quickly on the "Sample plate". Samples are placed with the largest possible distance from each other.

12. Make sure that the edges of the " vacuum cap " (Fig. F) is clean and set it centered on top of the sample plate.

13. Open the pressure valve

14. Set the program switch to the position " TROCKNER "

15. Check for vacuum - meter drops. If so, everything is okay. If not, check valves and other opportunities reasons.

To determine if the freeze-drying process is finished do as follow: Closing the Pressure valve for 1 minute. If the pressure rises, the moisture is present, and the freeze-drying process must continue. When the pressure is constant means that the samples are dry and ready.

16. Turn off Vacuum pump

17. Turn the Program switch to " AUS " via " ABTAUEN "

18. The Air valve is carefully open until you hear a “hissing” sound. Opens the air valve fully after the hissing sound has stop.

19. Then you open the Condensate drain valve completely.

20. Wait 10-15 minutes and remove the " vacuum cap " Be careful. DO NOT USE SHARP TOOLS!

21. Turn Program switch (Fig. E) gently counterclockwise until " ABTAUEN ". When the ice is gone turn the Program switch to "AUS". If you are not going to use the freeze dryer more, set the Program switch directly to "AUS".

22. Rinse / wash vacuum cap " and sample plate with Distilled Water and dry clean with paper towels. Keep the instrument clean! (Also avoid anything that disappears into the freeze dryer through the hole in the middle of the instrument.

Appendix II

***Table A 1:** Nitrogen (Total N) and Phosphorus (Total P) analysis of used medium to a selection of samples (samples 1-24) and samples of unused medium of the three different nutrient levels (samples 25-27). In the Code column: nx→nutrients level, sx→ salinity, tx→temperature, ppx→* Palmaria palmata *sample x, vlx→* Vertebrata lanosa *sample x.*

Sample	Code	Total N (μg/L)	Total P (μg/L)
1	n2s30t12pp2	3360	248
2	n2s20t12pp2	4176	498
3	n2s30y10pp2	4114,5	485
4	n2s20t10pp2	3300	284
5	n1s30t12pp3	2776	205
6	n1s20t12pp2	3040	201
7	n1s30t10pp3	2788	203
8	n1s20t10pp3	2868	208
9	n0s30t12pp3	1826	15
10	n0s20t12pp2	1782	<2
11	n0s30t10pp3	1741	<2
12	n0s20t10pp3	1735,5	<2
13	t12pp1	4329	473
14	t19pp2	5262	652
15	t16s10pp1	4562	533
16	t12s30pp1	4422	473
17	t10vl3	4410	415
18	t6vl1	4128	548
19	t10s30vl3	N/A	390
20	t12s30vl1	4149	399
21	t16s30vl2	4235	388
22	t10s10vl1	4536	460
23	t12s10vl1	4218	428
24	t16s10vl1	2911,5	98
25	n0	1767	13
26	n1	2904	330
27	n2	3606	650

Appendix III

R script

Palmaria palmata

```
library(tidyverse)

library(readxl)

#loading data for temperature
t<-read_excel("Palmaria palmata Temperature.xlsx")


#ploting data
ggplot(data=t)+
 geom_smooth(mapping=aes(x=Temperature, y=SGRtotal), size=2)+
 geom_point(mapping=aes(x=Temperature, y=SGRtotal), color="blue")+
 scale_x_continuous(breaks=c(6,10,12,16,19))+
  theme(
   panel.grid.major = element_blank(),
   panel.grid.minor = element_blank(),
   panel.background = element_blank(),
   axis.line = element_line(colour = "black"),
   text=element_text(size=17, family="Segoe UI"))+
 labs(
  x="Temperature (°C)",
  y="Specific Growth Rate ( %/day)")

#loading data of salinity - temp
st<-read_excel("salinity-group by temp.xlsx")

#plotting data
ggplot(data=st)+
 geom_smooth(mapping=aes(x=Salinity, y=SGR10,color="10°C"), size=2)+
 geom_point(mapping=aes(x=Salinity, y=SGR10),color="Red")+
 geom_smooth(mapping=aes(x=Salinity, y=SGR12, color="12°C"), size=2)+
 geom_point(mapping=aes(x=Salinity, y=SGR12),color="green4")+
 geom_smooth(mapping=aes(x=Salinity, y=SGR16, color="16°C"), size=2)+
 geom_point(mapping=aes(x=Salinity, y=SGR16), color="Blue")+
 theme(
  panel.grid.major = element_blank(),
  panel.grid.minor = element_blank(),
  panel.background = element_blank(),
  axis.line = element_line(colour = "black"),
  text=element_text(size=17, family="Segoe UI"))+
 scale_x_continuous(breaks=10:30)+
 labs(
  x="Salinity (‰)",
  y="Specific Growth Rate ( %/day)",
  colour="Temperature")

#loading data for nutrients
n<- read_excel("nutrients x sal x temp.xlsx")

# Boxplot for Nutrient levels at Temperature=10°C, Salinity=30psu
ggplot(data=n)+
```

```
 geom_boxplot(
  mapping=aes(x=nutrients, y=SGR1030, group=cut_width(nutrients, 1), fill=nutrients,
alpha=0.5))+
 geom_point(mapping=aes(x=nutrients, y=SGR1030), size=3, alpha=0.75)+
 scale_x_continuous(breaks=c(0,1,2))+
 ylim(0,5)+
 theme(
  panel.grid.major = element_blank(),
  panel.grid.minor = element_blank(),
  panel.background = element_blank(),
  axis.line = element_line(colour = "black"),
  legend.position = "none",
  text=element_text(size=17, family="Segoe UI"))+
 labs(
  x="Nutrient levels",
  y="Specific Growth Rate ( %/day)")

# Boxplot for Nutrient levels at Temperature=10°C, Salinity=20psu
ggplot(data=n)+
 geom_boxplot(
  mapping=aes(x=nutrients, y=SGR1020, group=cut_width(nutrients, 1), fill=nutrients,
alpha=0.5))+
 geom_point(mapping=aes(x=nutrients, y=SGR1020), size=3, alpha=0.75)+
 scale_x_continuous(breaks=c(0,1,2))+
 ylim(0,5)+
 theme(
  panel.grid.major = element_blank(),
  panel.grid.minor = element_blank(),
  panel.background = element_blank(),
  axis.line = element_line(colour = "black"),
  legend.position = "none",
  text=element_text(size=17, family="Segoe UI"))+
 labs(
  x="Nutrient levels",
  y="Specific Growth Rate ( %/day)")+
 ggtitle(c(expression(paste(italic("Palmaria palmata")," - Nutrients - Salinity 20, T
emperature 10"))))

# Boxplot for Nutrient levels at Temperature=12°C, Salinity=30psu
ggplot(data=n)+
 geom_boxplot(
  mapping=aes(x=nutrients, y=SGR1230, group=cut_width(nutrients, 1), fill=nutrients,
alpha=0.5))+
 geom_point(mapping=aes(x=nutrients, y=SGR1230), size=3, alpha=0.75)+
 scale_x_continuous(breaks=c(0,1,2))+
 ylim(0,10)+
 theme(
  panel.grid.major = element_blank(),
  panel.grid.minor = element_blank(),
  panel.background = element_blank(),
  axis.line = element_line(colour = "black"),
  legend.position = "none",
  text=element_text(size=17, family="Segoe UI"))+
 labs(
```

```
  x="Nutrient levels",
  y="Specific Growth Rate ( %/day)")

# Boxplot for Nutrient levels at Temperature=12°C, Salinity=20psu
ggplot(data=n)+
 geom_boxplot(
  mapping=aes(x=nutrients, y=SGR1220, group=cut_width(nutrients, 1), fill=nutrients,
alpha=0.5))+
 geom_point(mapping=aes(x=nutrients, y=SGR1220), size=3, alpha=0.75)+
 scale_x_continuous(breaks=c(0, 1, 2))+
 ylim(0,10)+
 theme(
  panel.grid.major = element_blank(),
  panel.grid.minor = element_blank(),
  panel.background = element_blank(),
  axis.line = element_line(colour = "black"),
  legend.position = "none",
  text=element_text(size=17, family="Segoe UI"))+
 labs(
  x="Nutrient levels",
  y="Specific Growth Rate ( %/day)")

#Line plot including all nutrient data
ggplot(data=n)+
 geom_smooth(mapping=aes(x=nutrients, y=SGR1020, color="10°C | 20‰"), size=1.5, alpha
=0.1)+
 geom_point(mapping=aes(x=nutrients, y=SGR1020), color="#F8766D")+
 geom_smooth(mapping=aes(x=nutrients, y=SGR1030, color="10°C | 30‰"), size=1.5, alpha
=0.1)+
 geom_point(mapping=aes(x=nutrients, y=SGR1030), color="#A3A500")+
 geom_smooth(mapping=aes(x=nutrients, y=SGR1220, color="12°C | 20‰"), size=1.5, alpha
=0.1)+
 geom_point(mapping=aes(x=nutrients, y=SGR1220), color="#00B0F6")+
 geom_smooth(mapping=aes(x=nutrients, y=SGR1230, color="12°C | 30‰"), size=1.5, alpha
=0.1)+
 geom_point(mapping=aes(x=nutrients, y=SGR1230), color="#B983FF")+
 scale_x_continuous(breaks=c(0,1,2))+
 theme(
  panel.grid.major = element_blank(),
  panel.grid.minor = element_blank(),
  panel.background = element_blank(),
  axis.line = element_line(colour = "black"),
  legend.position = "none",
  text=element_text(size=17, family="Segoe UI"))+
 labs(
  x="Nutrient levels",
  y="Specific Growth Rate ( %/day)",
  color="Temperature | Salinity")

#loading data for protein content
p <- read_xlsx("protein analysis.xlsx")

#groupping my data in the temperature-salinity combinations
p$tempsal <- paste0("T", d$Temperature, "S", d$Salinity)
View(p)
```

```
#plotting the protein content calculated with the nitrogen-to-protein factor
#for Palmaria palmata specifically
ggplot(data=p, aes(x=Nutrients,y=Protein_PP, fill=tempsal))+
 geom_bar(stat = "identity", position=position_dodge())+
 geom_errorbar(aes(ymin=Protein_PP-SE_PP, ymax=Protein_PP+SE_PP), position = position
_dodge(), size=1)+
 theme(
  panel.grid.major = element_blank(),
  panel.grid.minor = element_blank(),
  panel.background = element_blank(),
  legend.position = "bottom",
  axis.line = element_line(colour = "black"),
  text=element_text(size=17, family="Segoe UI"))+
 labs(
  x="Nutrient levels",
  y="Protein content ( % of DW)",
  fill="Temperatue|Salinity")

#loading t-test data

tt<-read_excel("t-testPP.xlsx")

pp<-read_excel("t-testPP-protein.xlsx")

#t-test for temperature conditions
t.test(tt$t12, tt$t6)

t.test(tt$t12, tt$t10)

t.test(tt$t12, tt$t16)

t.test(tt$t12, tt$t19)

#t.test for salinity-temperature conditions
t.test(tt$s30t12, tt$s10t10)

t.test(tt$s30t12, tt$s10t12)

t.test(tt$s30t12, tt$s10t16)

t.test(tt$s30t12, tt$s20t10)

t.test(tt$s30t12, tt$s20t12)

t.test(tt$s30t12, tt$s20t16)

t.test(tt$s30t12, tt$s30t10)

t.test(tt$s30t12, tt$s30t16)

#t.test for nuttrients-salinity-temperature conditions
t.test(tt$n2s30t12, tt$n0s20t10)

t.test(tt$n2s30t12, tt$n0s20t12)

t.test(tt$n2s30t12, tt$n0s30t10)
```

```
t.test(tt$n2s30t12, tt$n0s30t12)
t.test(tt$n2s30t12, tt$n1s20t10)
t.test(tt$n2s30t12, tt$n1s20t12)
t.test(tt$n2s30t12, tt$n1s30t10)
t.test(tt$n2s30t12, tt$n1s30t12)
t.test(tt$n2s30t12, tt$n2s20t10)
t.test(tt$n2s30t12, tt$n2s20t12)
t.test(tt$n2s30t12, tt$n2s30t10)

#t.test for proteins
t.test(pp$n1s20t12, pp$n0s20t10)
t.test(pp$n1s20t12, pp$n0s20t12)
t.test(pp$n1s20t12, pp$n0s30t10)
t.test(pp$n1s20t12, pp$n0s30t12)
t.test(pp$n1s20t12, pp$n1s20t10)
t.test(pp$n1s20t12, pp$n1s30t10)
t.test(pp$n1s20t12, pp$n1s30t12)
t.test(pp$n1s20t12, pp$n2s20t10)
t.test(pp$n1s20t12, pp$n2s20t12)
t.test(pp$n1s20t12, pp$n2s30t10)
t.test(pp$n1s20t12, pp$n2s30t12)
```

Vertebrata lanosa

```
library(tidyverse)

library(readxl)

#loading data for temperature
t<-read_excel("Vertebrata lanosa Temperature.xlsx")

#plotting data
ggplot(data=t)+
 geom_smooth(mapping=aes(x=Temperature, y=SGRtotal), size=2)+
 geom_point(mapping=aes(x=Temperature, y=SGRtotal), color="blue")+
 scale_x_continuous(breaks=c(6,10,12,16,19))+
 theme(
  panel.grid.major = element_blank(),
  panel.grid.minor = element_blank(),
  panel.background = element_blank(),
  axis.line = element_line(colour = "black"),
  text=element_text(size=17, family="Segoe UI"))+
 labs(
  x="Temperature (°C)",
  y="Specific Growth Rate ( %/day)")

# loading data for salinity-temperature
st<-read_excel("salinity- by temp.xlsx")

#plotting data
ggplot(data=st)+
 geom_smooth(mapping=aes(x=Salinity, y=SGR10, color="10°C"), size=2)+
 geom_point(mapping=aes(x=Salinity, y=SGR10), color="red")+
 geom_smooth(mapping=aes(x=Salinity, y=SGR12, color="12°C"), size=2)+
 geom_point(mapping=aes(x=Salinity, y=SGR12),color="green4")+
 geom_smooth(mapping=aes(x=Salinity, y=SGR16, color="16°C"), size=2)+
 geom_point(mapping=aes(x=Salinity, y=SGR16),color="blue")+
 theme(
  panel.grid.major = element_blank(),
  panel.grid.minor = element_blank(),
  panel.background = element_blank(),
  axis.line = element_line(colour = "black"),
  text=element_text(size=17, family="Segoe UI"))+
 scale_x_continuous(breaks=10:30)+
 labs(
  x="Salinity (‰)",
  y="Specific Growth Rate ( %/day)",
  colour="Temperature")

#loading data

tt<-read_excel("t-testVL.xlsx")

#t-test for temperature conditions
t.test(tt$t10, tt$t6)

t.test(tt$t10, tt$t12)

t.test(tt$t10, tt$t16)
```

```r
t.test(tt$t10, tt$t19)

#t-test for salinity conditions
t.test(tt$s30t10, tt$s10t10)

t.test(tt$s30t10, tt$s10t12)

t.test(tt$s30t10, tt$s10t16)

t.test(tt$s30t10, tt$s20t10)

t.test(tt$s30t10, tt$s20t12)

t.test(tt$s30t10, tt$s20t16)

t.test(tt$s30t10, tt$s30t12)

t.test(tt$s30t10, tt$s30t16)
```

Appendix IV

Detailed t-test results

Palmaria palmata

Temperature

An independent-samples t-test comparing the specific growth rate in 12 °C and **6 °C** temperature conditions. The difference is significant.

Welch two sample t-test for *Palmaria palmata* Temperature		
t=4.3024	df=2.2056	p-value=0.0419
95 % confidence interval:	lower=0.4617258	upper=10.5094316
Mean of x (SGR@12°C)	9.643049	
Mean of y (SGR@6°C)	4.157471	

An independent-samples t-test comparing the specific growth rate in 12 °C and **10 °C** temperature conditions. The difference is significant.

Welch two sample t-test for *Palmaria palmata* Temperature		
t=4.8041	df=2.0058	p-value=0.04047
95 % confidence interval:	lower=0.6389185	upper=11.3164827
Mean of x (SGR@12°C)	9.643049	
Mean of y (SGR@10°C)	3.665349	

An independent-samples t-test comparing the specific growth rate in 12 °C and **16 °C** temperature conditions. The difference is significant.

Welch two sample t-test for *Palmaria palmata* Temperature		
t=6.0659	df=2.454	p-value=0.01564
95 % confidence interval:	lower=3.207292	upper=12.720790
Mean of x (SGR@12°C)	9.643049	
Mean of y (SGR@16°C)	1.679008	

An independent-samples t-test comparing the specific growth rate in 12 °C and **19 °C** temperature conditions. The difference is significant.

Welch two sample t-test for *Palmaria palmata* Temperature		
t=7.1735	df=2.0459	p-value=0.01776
95 % confidence interval:	lower=3.704159	upper=14.236785
Mean of x (SGR@12°C)	9.643049	
Mean of y (SGR@19°C)	0.6725768	

Salinity

An independent-samples t-test comparing the specific growth rate in 12°C – 30‰ and **10°C – 10‰** temperature – salinity conditions. The difference is significant.

Welch two sample t-test for *Palmaria palmata* Temperature – Salinity		
t=6.4357	df=2.0113	p-value=0.02298
95 % confidence interval:	lower=2.684783	upper=13.341894
Mean of x (SGR@12°C,30‰)	9.643049	
Mean of y (SGR@10°C,10‰)	1.629711	

An independent-samples t-test comparing the specific growth rate in 12°C – 30‰ and **12°C – 10‰** temperature – salinity conditions. The difference is significant.

Welch two sample t-test for *Palmaria palmata* Temperature – Salinity		
t=6.2935	df=2.0234	p-value=0.02364
95 % confidence interval:	lower=2.541627	upper=13.154493
Mean of x (SGR@12°C,30‰)	9.643049	
Mean of y (SGR@12°C,10‰)	1.794989	

An independent-samples t-test comparing the specific growth rate in 12°C – 30‰ and **16°C – 10‰** temperature – salinity conditions. The difference is significant.

Welch two sample t-test for *Palmaria palmata* Temperature – Salinity		
t=6.4927	df=2.1611	p-value=0.01879
95 % confidence interval:	lower=3.149187	upper=13.318815
Mean of x (SGR@12°C,30‰)	9.643049	
Mean of y (SGR@16°C,10‰)	1.409048	

An independent-samples t-test comparing the specific growth rate in 12°C – 30‰ and **10°C – 20‰** temperature – salinity conditions. The difference is not significant.

Welch two sample t-test for *Palmaria palmata* Temperature – Salinity		
t=3.8865	df=2.0468	p-value=0.05807
95 % confidence interval:	lower=-0.4041547	upper=10.1253597
Mean of x (SGR@12°C,30‰)	9.643049	
Mean of y (SGR@10°C,20‰)	4.782447	

An independent-samples t-test comparing the specific growth rate in 12°C – 30‰ and **12°C – 20‰** temperature – salinity conditions. The difference is not significant.

Welch two sample t-test for *Palmaria palmata* Temperature – Salinity		
t=3.1182	df=2.5881	p-value=0.06398
95 % confidence interval:	lower=-0.4945893	upper=8.8112199
Mean of x (SGR@12°C,30‰)	9.643049	
Mean of y (SGR@12°C,20‰)	5.484734	

An independent-samples t-test comparing the specific growth rate in 12°C – 30‰ and **16°C – 20‰** temperature – salinity conditions. The difference is significant.

Welch two sample t-test for *Palmaria palmata* Temperature – Salinity		
t=4.5739	df=2.5796	p-value=0.027
95 % confidence interval:	lower=1.434778	upper=10.752462
Mean of x (SGR@12°C,30‰)	9.643049	
Mean of y (SGR@16°C,20‰)	3.549429	

An independent-samples t-test comparing the specific growth rate in 12°C – 30‰ and **10°C – 30‰** temperature – salinity conditions. The difference is significant.

Welch two sample t-test for *Palmaria palmata* Temperature – Salinity		
t=4.8041	df=2.0058	p-value=0.04047
95 % confidence interval:	lower=0.6389185	upper=11.3164827
Mean of x (SGR@12°C,30‰)	9.643049	
Mean of y (SGR@10°C,30‰)	3.665349	

An independent-samples t-test comparing the specific growth rate in 12°C – 30‰ and **16°C – 30‰** temperature – salinity conditions. The difference is significant.

Welch two sample t-test for *Palmaria palmata* Temperature – Salinity		
t=6.0659	df=2.454	p-value=0.01564
95 % confidence interval:	lower=3.207292	upper=12.720790
Mean of x (SGR@12°C,30‰)	9.643049	
Mean of y (SGR@16°C,30‰)	1.679008	

Nutrients

An independent-samples t-test comparing the specific growth rate in 12°C – 30‰ – N_2 and **10°C – 20‰ – N_0** temperature – salinity conditions. The difference is significant.

Welch two sample t-test for *Palmaria palmata* Temperature – Salinity – Nutrients		
t=6.0606	df=2.4135	p-value=0.01637
95 % confidence interval:	lower=2.700115	upper=10.979101
Mean of x (SGR@12°C,30‰,N_2)	8.328088	
Mean of y (SGR@10°C,20‰,N_0)	1.488480	

An independent-samples t-test comparing the specific growth rate in 12°C – 30‰ – N_2 and **10°C – 20‰ – N_0** temperature – salinity conditions. The difference is significant.

Welch two sample t-test for *Palmaria palmata* Temperature – Salinity – Nutrients		
t=6.1955	df=2.1135	p-value=0.0219
95 % confidence interval:	lower=2.294276	upper=11.199041
Mean of x (SGR@12°C,30‰,N_2)	8.328088	
Mean of y (SGR@12°C,20‰,N_0)	1.581429	

An independent-samples t-test comparing the specific growth rate in 12°C – 30‰ – N_2 and **10°C – 30‰ – N_0** temperature – salinity conditions. The difference is significant.

Welch two sample t-test for *Palmaria palmata* Temperature – Salinity – Nutrients		
t=5.7026	df=2.0979	p-value=0.02635
95 % confidence interval:	lower=1.724582	upper=10.671615
Mean of x (SGR@12°C,30‰,N_2)	8.328088	
Mean of y (SGR@10°C,30‰,N_0)	2.129989	

An independent-samples t-test comparing the specific growth rate in 12°C – 30‰ – N_2 and **12°C – 30‰ – N_0** temperature – salinity conditions. The difference is significant.

Welch two sample t-test for *Palmaria palmata* Temperature – Salinity – Nutrients		
t=5.8718	df=2.1382	p-value=0.02374
95 % confidence interval:	lower=1.993303	upper=10.833556
Mean of x (SGR@12°C,30‰,N_2)	8.328088	
Mean of y (SGR@12°C,30‰,N_0)	1.914659	

An independent-samples t-test comparing the specific growth rate in 12°C – 30‰ – N_2 and **10°C – 20‰ – N_1** temperature – salinity conditions. The difference is significant.

Welch two sample t-test for *Palmaria palmata* Temperature – Salinity – Nutrients		
t=5.4293	df=2.1378	p-value=0.02786
95 % confidence interval:	lower=1.509202	upper=10.350433
Mean of x (SGR@12°C,30‰,N_2)	8.328088	
Mean of y (SGR@10°C,20‰,N_1)	2.398270	

An independent-samples t-test comparing the specific growth rate in 12°C – 30‰ – N_2 and **12°C – 20‰ – N_1** temperature – salinity conditions. The difference is significant.

Welch two sample t-test for *Palmaria palmata* Temperature – Salinity – Nutrients		
t=5.9515	df=2.0765	p-value=0.02478
95 % confidence interval:	lower=1.948335	upper=10.955057
Mean of x (SGR@12°C,30‰,N_2)	8.328088	
Mean of y (SGR@12°C,20‰,N_1)	1.876392	

An independent-samples t-test comparing the specific growth rate in 12°C – 30‰ – N_2 and **10°C – 30‰ – N_1** temperature – salinity conditions. The difference is significant.

Welch two sample t-test for *Palmaria palmata* Temperature – Salinity – Nutrients		
t=5.1801	df=2.2574	p-value=0.0272
95 % confidence interval:	lower=1.456972	upper=10.021854
Mean of x (SGR@12°C,30‰,N_2)	8.328088	
Mean of y (SGR@10°C,30‰,N_1)	2.588675	

An independent-samples t-test comparing the specific growth rate in 12°C – 30‰ – N_2 and **12°C – 30‰ – N_1** temperature – salinity conditions. The difference is significant.

Welch two sample t-test for *Palmaria palmata* Temperature – Salinity – Nutrients		
t=4.2378	df=2.8202	p-value=0.02718
95 % confidence interval:	lower=1.109369	upper=8.920620
Mean of x (SGR@12°C,30‰,N_2)	8.328088	
Mean of y (SGR@12°C,30‰,N_1)	3.313094	

An independent-samples t-test comparing the specific growth rate in 12°C – 30‰ – N_2 and **10°C – 20‰ – N_2** temperature – salinity conditions. The difference is significant.

Welch two sample t-test for *Palmaria palmata* Temperature – Salinity – Nutrients		
t=4.1954	df=3.1063	p-value=0.02306
95 % confidence interval:	lower=1.315459	upper=8.964745
Mean of x (SGR@12°C,30‰,N_2)	8.328088	
Mean of y (SGR@10°C,20‰,N_2)	3.187986	

An independent-samples t-test comparing the specific growth rate in 12°C – 30‰ – N_2 and **12°C – 20‰ – N_2** temperature – salinity conditions. The difference is not significant.

Welch two sample t-test for *Palmaria palmata* Temperature – Salinity – Nutrients		
t=1.4225	df=3.4702	p-value=0.2382
95 % confidence interval:	lower=-1.969358	upper=5.633110
Mean of x (SGR@12°C,30‰,N_2)	8.328088	
Mean of y (SGR@12°C,20‰,N_2)	6.496212	

An independent-samples t-test comparing the specific growth rate in 12°C – 30‰ – N_2 and **10°C – 30‰ – N_2** temperature – salinity conditions. The difference is significant.

Welch two sample t-test for *Palmaria palmata* Temperature – Salinity – Nutrients		
t=4.8041	df=2.0058	p-value=0.04047
95 % confidence interval:	lower=0.5517933	upper=9.7733259
Mean of x (SGR@12°C,30‰,N_2)	8.328088	
Mean of y (SGR@10°C,30‰,N_2)	3.165528	

Protein Content

An independent-samples t-test comparing the protein content in 12°C – 20‰ – N_1 and **10°C – 20‰ – N_0** temperature – salinity conditions. The difference is significant.

Welch two sample t-test for *Palmaria palmata* Protein Content		
t=5.2553	df=3.6348	p-value=0.008148
95 % confidence interval:	lower=6.558364	upper=22.584769
Mean of x (SGR@12°C,20‰,N_1)	34.83327	
Mean of x (SGR@10°C,20‰,N_0)	2026170	

An independent-samples t-test comparing the protein content in 12°C – 20‰ – N_1 and **12°C – 20‰ – N_0** temperature – salinity conditions. The difference is not significant.

Welch two sample t-test for *Palmaria palmata* Protein Content		
t=1.7894	df=2.3587	p-value=0.1959
95 % confidence interval:	lower=-10.93502	upper=31.07295
Mean of x (SGR@12°C,20‰,N_1)	34.83327	
Mean of y (SGR@12°C,20‰,N_0)	24.76430	

An independent-samples t-test comparing the protein content in 12°C – 20‰ – N_1 and **10°C – 30‰ – N_0** temperature – salinity conditions. The difference is significant.

Welch two sample t-test for *Palmaria palmata* Protein Content		
t=8.3704	df=3.2896	p-value=0.002508
95 % confidence interval:	lower=10.1120	upper=21.5848
Mean of x (SGR@12°C,20‰,N_1)	34.83327	
Mean of y (SGR@10°C,30‰,N_0)	18.98487	

An independent-samples t-test comparing the protein content in 12°C – 20‰ – N_1 and **12°C – 30‰ – N_0** temperature – salinity conditions. The difference is significant.

Welch two sample t-test for *Palmaria palmata* Protein Content		
t=8.1877	df=2.5116	p-value=0.007126
95 % confidence interval:	lower=7.966166	upper=20.240101
Mean of x (SGR@12°C,20‰,N_1)	34.83327	
Mean of y (SGR@12°C,30‰,N_0)	20.73013	

An independent-samples t-test comparing the protein content in 12°C – 20‰ – N_1 and **10°C – 20‰ – N_1** temperature – salinity conditions. The difference is not significant.

Welch two sample t-test for *Palmaria palmata* Protein Content		
t=0.59729	df=3.5571	p-value=0.5862
95 % confidence interval:	lower=-6.611243	upper=10.014043
Mean of x (SGR@12°C,20‰,N_1)	34.83327	
Mean of y (SGR@10°C,20‰,N_1)	33.13187	

An independent-samples t-test comparing the protein content in 12°C – 20‰ – N_1 and **10°C – 30‰ – N_1** temperature – salinity conditions. The difference is not significant.

Welch two sample t-test for *Palmaria palmata* Protein Content		
t=1.6968	df=3.9595	p-value=0.1657
95 % confidence interval:	lower=-2.382233	upper=9.792567
Mean of x (SGR@12°C,20‰,N_1)	34.83327	
Mean of y (SGR@10°C,30‰,N_1)	31.12810	

An independent-samples t-test comparing the protein content in 12°C – 20‰ – N_1 and **12°C – 30‰ – N_1** temperature – salinity conditions. The difference is not significant.

Welch two sample t-test for *Palmaria palmata* Protein Content		
t=0.29202	df=2.6597	p-value=0.7915
95 % confidence interval:	lower=-13.33180	upper=15.81653
Mean of x (SGR@12°C,20‰,N_1)	34.83327	
Mean of y (SGR@12°C,30‰,N_1)	33.59090	

An independent-samples t-test comparing the protein content in 12°C – 20‰ – N_1 and **10°C – 20‰ – N_2** temperature – salinity conditions. The difference is not significant.

Welch two sample t-test for *Palmaria palmata* Protein Content		
t=1.5245	df=2.8105	p-value=0.2307
95 % confidence interval:	lower=-6.910983	upper=18.729916
Mean of x (SGR@12°C,20‰,N_1)	34.83327	
Mean of y (SGR@10°C,20‰,N_2)	28.92380	

An independent-samples t-test comparing the protein content in 12°C – 20‰ – N_1 and **12°C – 20‰ – N_2** temperature – salinity conditions. The difference is significant.

Welch two sample t-test for *Palmaria palmata* Protein Content		
t=3.8485	df=2.9324	p-value=0.03225
95 % confidence interval:	lower=2.271107	upper=25.734626
Mean of x (SGR@12°C,20‰,N_1)	34.83327	
Mean of y (SGR@12°C,20‰,N_2)	20.83040	

An independent-samples t-test comparing the protein content in 12°C – 20‰ – N_1 and **10°C – 30‰ – N_2** temperature – salinity conditions. The difference is not significant.

Welch two sample t-test for *Palmaria palmata* Protein Content		
t=2.7328	df=3.0621	p-value=0.07017
95 % confidence interval:	lower=-0.7601737	upper=10.8087737
Mean of x (SGR@12°C,20‰,N_1)	34.83327	
Mean of y (SGR@10°C,30‰,N_2)	29.80897	

An independent-samples t-test comparing the protein content in 12°C – 20‰ – N_1 and **12°C – 30‰ – N_2** temperature – salinity conditions. The difference is significant.

Welch two sample t-test for *Palmaria palmata* Protein Content		
t=7.5266	df=3.9737	p-value=0.001713
95 % confidence interval:	lower=11.33941	upper=24.65006
Mean of x (SGR@12°C,20‰,N_1)	34.83327	
Mean of y (SGR@12°C,30‰,N_2)	16.83853	

Vertebrata lanosa

Temperature

An independent-samples t-test comparing the specific growth rate in 10ºC and **6ºC** temperature conditions. The difference is not significant.

Welch two sample t-test for *Vertebrata lanosa* Temperature		
t=2.5912	df=2.0389	p-value=0.1199
95 % confidence interval:	lower=-0.7744269	upper=3.2309465
Mean of x (SGR@10ºC)	3.098454	
Mean of y (SGR@6ºC)	1.870195	

An independent-samples t-test comparing the specific growth rate in 10ºC and **12ºC** temperature conditions. The difference is not significant.

Welch two sample t-test for *Vertebrata lanosa* Temperature		
t=2.4351	df=2.1021	p-value=0.1292
95 % confidence interval:	lower=-0.4934999	upper=1.9297486
Mean of x (SGR@10ºC)	3.098454	
Mean of y (SGR@12ºC)	2.380330	

An independent-samples t-test comparing the specific growth rate in 10ºC and **16ºC** temperature conditions. The difference is not significant.

Welch two sample t-test for *Vertebrata lanosa* Temperature		
t=3.5561	df=2.0815	p-value=0.06674
95 % confidence interval:	lower=-0.1939141	upper=2.5352768
Mean of x (SGR@10ºC)	3.098454	
Mean of y (SGR@16ºC)	1.927773	

An independent-samples t-test comparing the specific growth rate in 10ºC and **19ºC** temperature conditions. The difference is significant.

Welch two sample t-test for *Vertebrata lanosa* Temperature		
t=10.84	df=2.7266	p-value=0.002548
95 % confidence interval:	lower=0.8735428	upper=1.6620004
Mean of x (SGR@10ºC)	3.098454	
Mean of y (SGR@19ºC)	1.830683	

Salinity

An independent-samples t-test comparing the specific growth rate in 10ºC – 30‰ and **10ºC – 10‰** temperature conditions. The difference is significant.

Welch two sample t-test for *Vertebrata lanosa* Temperature – Salinity		
t=34.209	df=3.999	p-value=0.000004367
95 % confidence interval:	lower=2.084908	upper=2.453268
Mean of x (SGR@10ºC,30‰)	3.0984543	
Mean of y (SGR@10ºC,10‰)	0.8293663	

An independent-samples t-test comparing the specific growth rate in 10ºC – 30‰ and **12ºC – 10‰** temperature conditions. The difference is significant.

Welch two sample t-test for *Vertebrata lanosa* Temperature – Salinity		
t=11.114	df=2.3568	p-value=0.004338
95 % confidence interval:	lower=1.195428	upper=2.405598
Mean of x (SGR@10ºC,30‰)	3.098454	
Mean of y (SGR@12ºC,10‰)	1.297942	

An independent-samples t-test comparing the specific growth rate in 10ºC – 30‰ and **16ºC – 10‰** temperature conditions. The difference is significant.

Welch two sample t-test for *Vertebrata lanosa* Temperature – Salinity		
t=29.203	df=3.5702	p-value=0.00002227
95 % confidence interval:	lower=2.140843	upper=2.615330
Mean of x (SGR@10ºC,30‰)	3.0984543	
Mean of y (SGR@16ºC,10‰)	0.7203679	

An independent-samples t-test comparing the specific growth rate in 10ºC – 30‰ and **10ºC – 20‰** temperature conditions. The difference is significant.

Welch two sample t-test for *Vertebrata lanosa* Temperature – Salinity		
t=7.7139	df=2.335	p-value=0.01037
95 % confidence interval:	lower=0.6593418	upper=1.9146975
Mean of x (SGR@10ºC,30‰)	3.0984543	
Mean of y (SGR@10ºC,20‰)	1.811435	

An independent-samples t-test comparing the specific growth rate in 10ºC – 30‰ and **12ºC – 20‰** temperature conditions. The difference is significant.

Welch two sample t-test for *Vertebrata lanosa* Temperature – Salinity		
t=3.6334	df=2.4538	p-value=0.0497
95 % confidence interval:	lower=0.001469319	upper=1.051640124
Mean of x (SGR@10ºC,30‰)	3.098454	
Mean of y (SGR@12ºC,20‰)	2.571900	

An independent-samples t-test comparing the specific growth rate in 10ºC – 30‰ and **16ºC – 20‰** temperature conditions. The difference is significant.

Welch two sample t-test for *Vertebrata lanosa* Temperature – Salinity		
t=25.035	df=3.6162	p-value=0.00003478
95 % confidence interval:	lower=1.774192	upper=2.238469
Mean of x (SGR@10ºC,30‰)	3.098454	
Mean of y (SGR@16ºC,20‰)	1.092124	

An independent-samples t-test comparing the specific growth rate in 10ºC – 30‰ and **12ºC – 30‰** temperature conditions. The difference is not significant.

Welch two sample t-test for *Vertebrata lanosa* Temperature – Salinity		
t=1.2522	df=2.3859	p-value=0.3192
95 % confidence interval:	lower=-0.3823290	upper=0.7735081
Mean of x (SGR@10ºC,30‰)	3.098454	
Mean of y (SGR@12ºC,30‰)	2.902865	

An independent-samples t-test comparing the specific growth rate in 10ºC – 30‰ and **16ºC – 30‰** temperature conditions. The difference is not significant.

Welch two sample t-test for *Vertebrata lanosa* Temperature – Salinity		
t=1.1039	df=2.0368	p-value=0.3829
95 % confidence interval:	lower=-1.522797	upper=2.598556
Mean of x (SGR@10ºC,30‰)	3.098454	
Mean of y (SGR@16ºC,3t0‰)	2.560575	