Universität Bremen

Polar Night eco-physiology, and eco-evolutionary dynamics of the kelp *Saccharina latissima*

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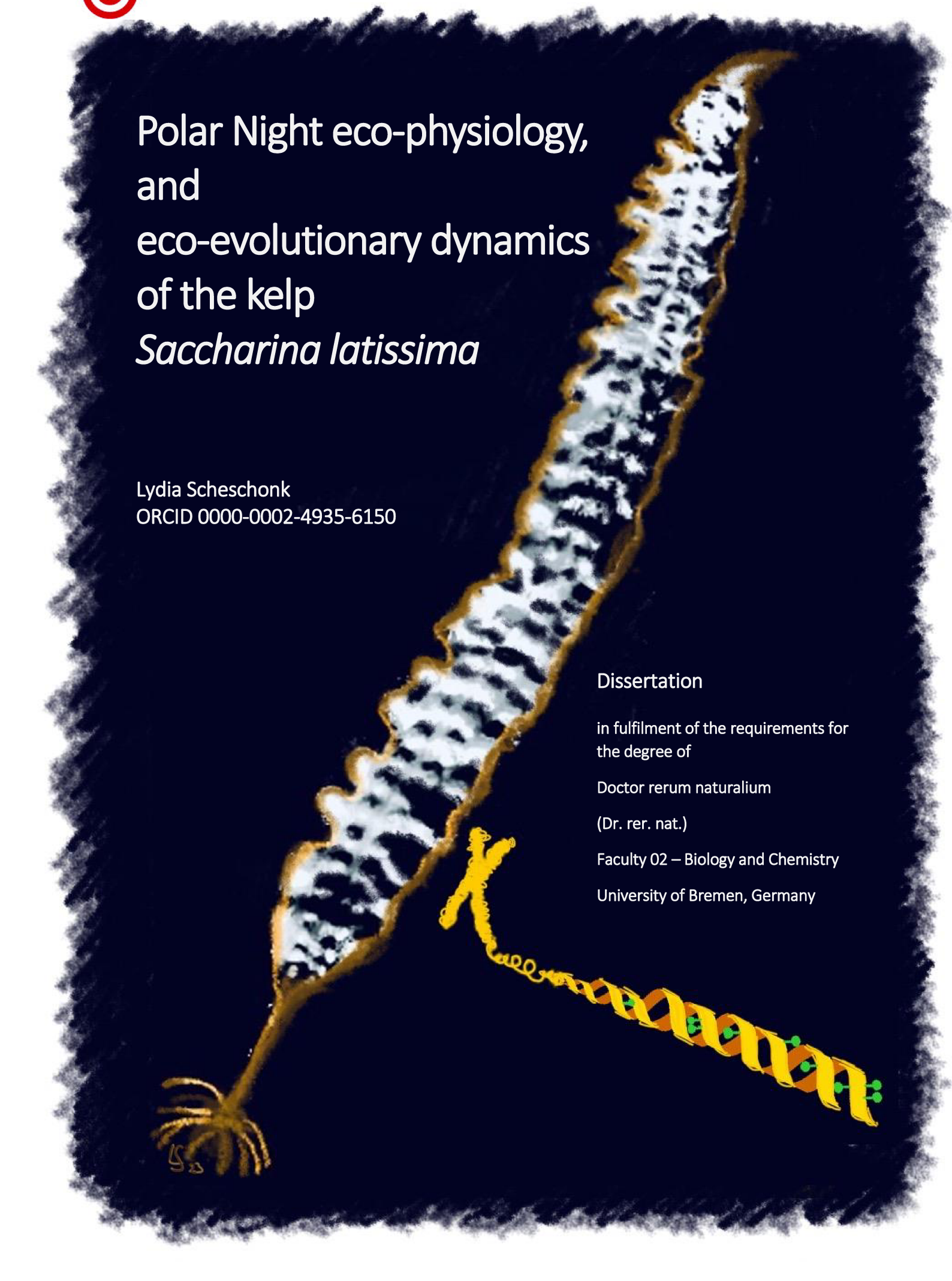

Dissertation

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1st examiner:

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Picture: own

“Look deep, deep into nature, and you will understand everything better.”

– Albert Einstein, 1951 –

Content

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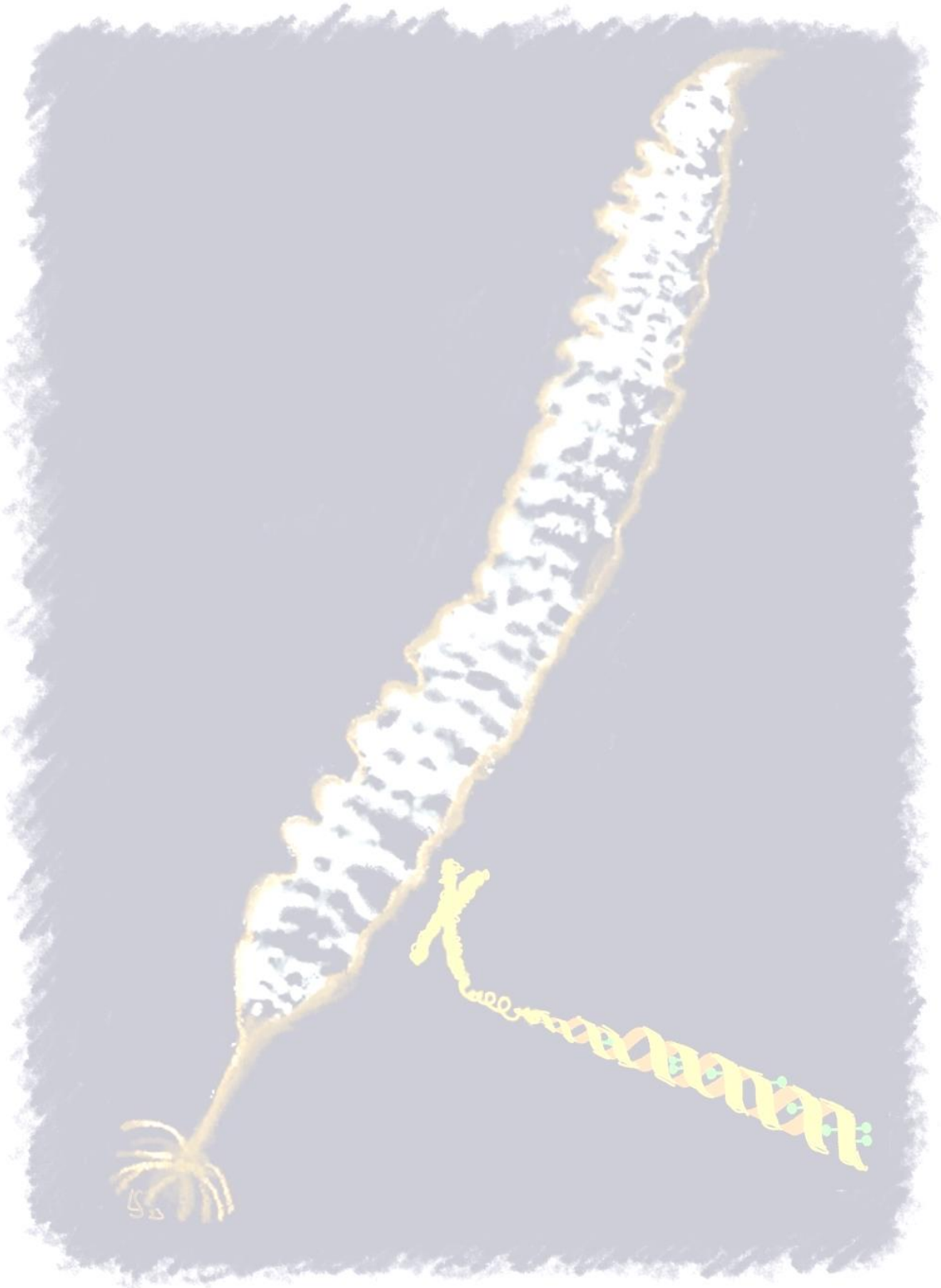

List of Publications

Publication I

Title:

Arctic kelp eco-physiology during the Polar Night in the face of global warming: a crucial role for laminarin

Authors:

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Transcriptomic Responses to Darkness and the Survival Strategy of the Kelp *Saccharina latissima* in the Early Polar Night

Authors:

Huiru Li, **Lydia Scheschonk**, Sandra Heinrich, Klaus Valentin, Lars Harms, Gernot Glöckner[ɬ], Erwan Corre, Kai Bischof

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Authors:

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Title:

Priming of Marine Macrophytes for Enhanced Restoration Success and Food Security in Future Oceans

Authors:

Alexander Jueterbock, Antoine J. P. Minne, J. Mark Cock, Melinda A. Coleman, Thomas Wernberg, **Lydia Scheschonk**, Ralf Rautenberger, Jie Zhang and Zi-Min Hu

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Authors:

Nora Diehl, Huiru Li, **Lydia Scheschonk,** Bertille Bugunter-Delamare, Sarina Niedzwiedz, Silje Forbord, Maren Sather, Kai Bischof, Catia Monteiro

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The sugar kelp *Saccharina latissima* II: Recent advances in farming and applications

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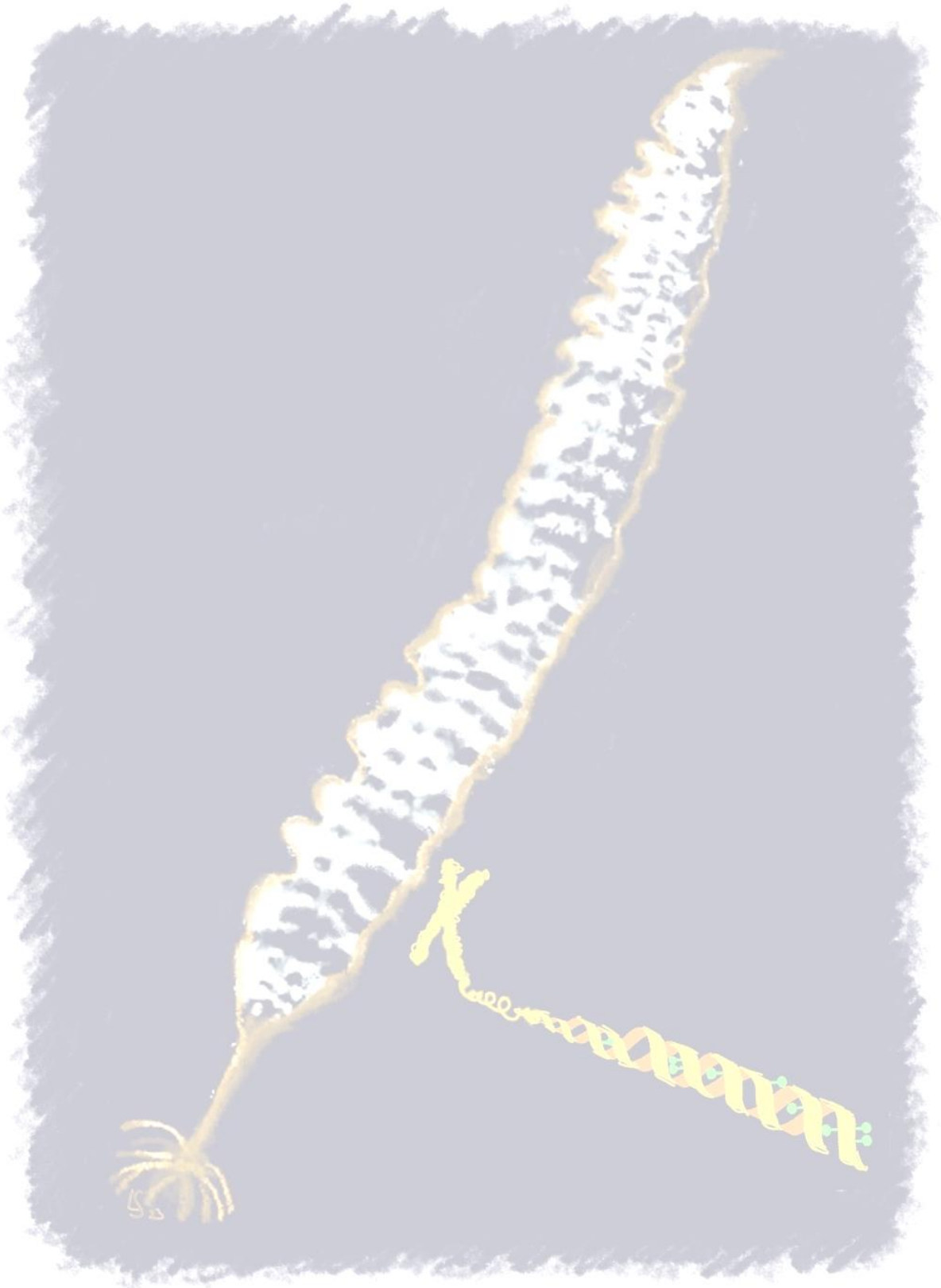


Summary

For primary producers, the suitability of the High Arctic (~ 80 °N) as habitat depends on their capacity to survive the long-term absence of light during the Polar Night (see **Chapter 1**). During the Climate Crisis of the Anthropocene, survival or demise of primary producers in the High Arctic now strongly depends on their capacity to adapt to the combined threat of rise in temperature and prolonged darkness during the Polar Night.

However, fundamental knowledge regarding Polar Night eco-physiology and epigenetic mechanisms in wild perennial (marine) primary producers has been lacking. This dissertation addresses, mainly for the kelp ***Saccharina latissima***, **eco-physiological** as well as **transcriptomic aspects** of **rising temperature** during **Polar Night**.

To gain a more holistic impression of the adaptive capacity, it further provides a **comparative epigenetic assessment** of the **nuclear** and **chloroplast genome** regarding **dynamics of eco-evolution** in this kelp.

Eco-physiological parameters measured during the High Artic Polar Night (**Chapter 3**) clearly differed from observations reported from more southern locations during winter, and from previous studies carried out in the High Arctic during early spring. While it did not affect the pigment contingent, temperature rise was found to severely impact internal carbohydrate storage during Polar Night. An adaptive transcriptomic response to prolonged darkness was further enhanced by elevated temperature during darkness (**Chapter 4**). The observed impact on internal carbohydrate storage might compromise the species' survival during warmer winters. Declines in population in turn would enhance further regime shifts in Arctic coastal eco-systems.

The swift response in gene expression (**Chapter 4**), in combination with the observed transgenerational epigenetic memory and likely capacity for temperature priming (**Chapters 5, 6**) suggests a strong capacity for rapid adaptation to Polar Night temperatures above pre-industrial levels. As long as summer hydro-optics will allow for sufficient build-up of internal storage, the eco-evolutionary dynamics of the kelp *Saccharina latissima* can likely ensure the species' survival in their High Arctic habitat, and hence, aid the stability of the ecosystem.

The significant impact of cultivation observed at the molecular level suggests that temperature priming could be a valid option to influence the genome/ gene expression to boost resilience, which is likely of interest for restoration projects, and aquaculture processess.

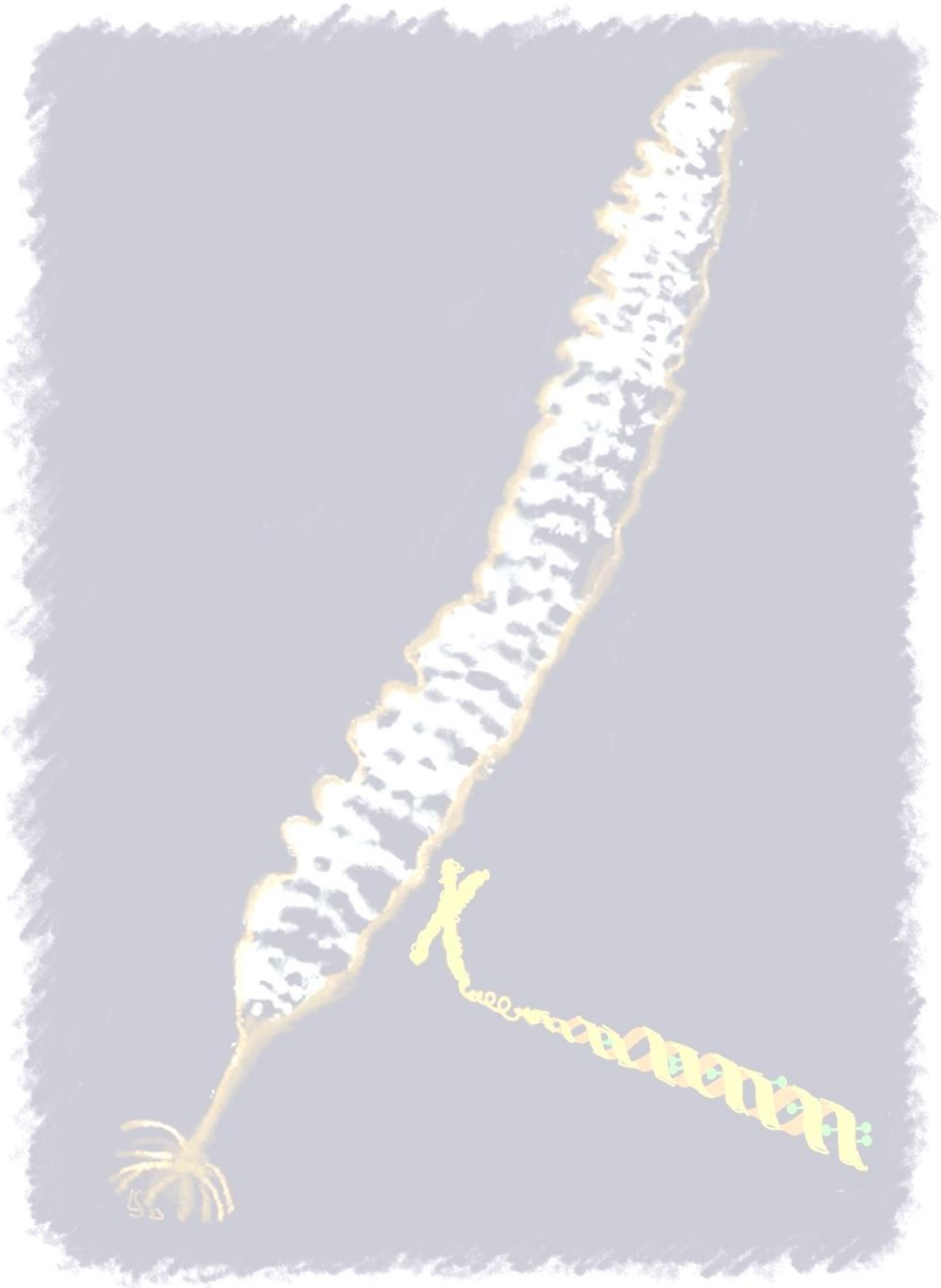
Results obtained during this dissertation provide a solid interdisciplinary data complex on two topics where data have been severely missing for (High Arctic) kelp. They have already been incorporated into a scientific book (Berge et al 2020), and have laid the foundation for a completely new branch of research on kelp (priming). In conclusion, this dissertation contributes important knowledge gains for a more holistic understanding of (High Arctic) eco-evolutionary processes during the current biodiversity crisis in the wake of this Climate Crisis, and a possible counteraction method.

Zusammenfassung

In hocharktischen Gebieten (~ 80 °N) sind Primärproduzenten an die lange Abwesenheit des Sonnenlichts während der Polarnacht angepasst (Kapitel 1). Fortbestand oder Niedergang einer Art hängt während der aktuellen Klimakrise des Anthropozens stark von der arteigenen Fähigkeit zur rapiden Anpassung ab. Im Falle von hocharktischen Photoautotrophen ist der wahrscheinlich entschiedenste Faktor die jeweilige Anpassungsfähigkeit an deutlich ansteigende Temperaturen während der monatelangen Dunkelheit der Polarnacht.

Grundlagenwissen für die Zeit der **Polarnacht** fehlte bisher für mehrjährige (marine) Primärproduzenten. Die vorliegende Dissertation befasst sich mit möglichen Auswirkungen steigender Temperaturen auf **Ökophysiologie** und **Genexpression** des Kelps ***Saccharina latissima*** (Zuckertang) während der Polarnacht. Um zudem einen vollständigeren Eindruck bezüglich der **öko-evolutiven Dynamiken** dieses Kelps zu bekommen werden sowohl das **Nuklear-**, als auch das **Chloroplastengenom** in einer vergleichenden **epigenetischen Studie auf DNA Methylierungen** untersucht.

Ökophysiologische Parameter, die während der Polarnacht gemessen wurden (**Kapitel 3**), wiesen klare Unterschiede gegenüber Ergebnissen früherer Studien aus dem Frühling auf. Die wichtigsten Erkenntnisse betrafen die internen Kohlenhydratreserven, sowie die Beobachtung, dass Photosynthesepigmente über die Dauer der Polarnacht in ihrer Funktion erhalten blieben. Anhand der differenziellen Transkriptomik **(Kapitel 4)** konnte beobachtet werden, dass die adaptive Antwort der Genexpression auf verlängerte Dunkelheit durch Temperaturen über dem historischen Niveau deutlich verschärft wird. Dennoch indiziert das festgestellte trans-generationelle epigenetische Gedächtnis, sowie die Rezeptivität gegenüber Temperaturpriming **(Kapitel 5 & 6)** ein starkes Vermögen zur rapiden Adaptation an Temperaturen oberhalb des vorindustriellen Niveaus. Eine weitere wichtige Erkenntniss dieser Dissertation ist die Beobachtung, dass ein Kultivierungsprozess einen signifikanten Einfluss auf molekularem Niveau zu haben scheint.
Unter der Voraussetzung, dass während der Sommermonate die Unterwasser- Lichtbedingungen eine ausreichende Photosyntheseaktivität und daraus resultierende Füllung des Kohlenhydratspeichers ermöglichen, deuten die Ergebnisse dieser Dissertation darauf hin, dass die öko-evolutive Dynamik des Kelps *Saccharina latissima* das Überleben der Art im hocharktischen Habitat gewährleisten kann. Dies wiederum würde die Stabilität des Ökosystems stark unterstützen. Die vermutete Möglichkeit des Temperaturpriming zur Beeinflussung des Genoms/ Genexpression zur Erhöhung der Resilienz ist von Interesse für Renaturierungsmaßnahmen und Aquakultur - Prozesse.
Im Verlauf dieser Dissertation konnte ein solider interdisziplinärer Datenkomplex in zwei für die Kelp-Forschung überaus wichtigen Themengebieten erarbeitet werden. Ergebnisse wurden bereits in ein wissenschaftliches Buch implementiert (Berge et al 2020), und haben die Grundlage für ein komplett neues Forschungsfeld innerhalb der Kelpforschung eröffnet (priming). Zusammengefasst hat diese Dissertation einen immensen Wissenszuwachs erarbeitet, der unter anderem ein besseres Verständnis öko-evolutionärer Prozesse während der aktuellen Biodiversitätskrise ermöglicht, und darüber hinaus einen potentiellen natürlichen sowie anthropogen initiierbaren Anpassungsmechanismus aufgezeigt.



Chapter 1

Introduction

1.1 Global Warming, the Climate Crisis, and its Impact on the Arctic

Models based on sediment core data suggest a mean temperature difference of ~ 5 °C between the last glacial maximum (~ 20.000 - 15.000 years ago) and pre-industrial times up to ~ 1860 (Schneider von Deimling et al. 2006). This rise in temperature occurred over a period of several thousand years. Compared to conditions found during the maximal glaciation period of the last Ice Age, the estimated ~ 5 °C temperature increase entailed enormous changes in sea level, mass extinctions, strong shifts in ecosystems and plant regimes, and severe alterations of local systems as well as the global climate. Currently, a much faster shift of the temperature regime (decades instead of millennia), known as global warming, initiated the current anthropogenic Climate Crisis. Global warming has been initiated by a man-made rise in greenhouse gas emissions (IPCC AR5). Levels of CO_2, one of the main greenhouse gases, used to fluctuate between 180 ppm to 300 ppm in the cryosphere during the last > 800,000 years, changing between warm and glacial periods (Lüthi et al. 2008 , see Fig 1 A). Over the course of the industrial age, global mean temperatures have rapidly and constantly increased, and are predicted to continue rising (Fig. 2 B; Field et al. 2014; Rogelj et al. 2018).

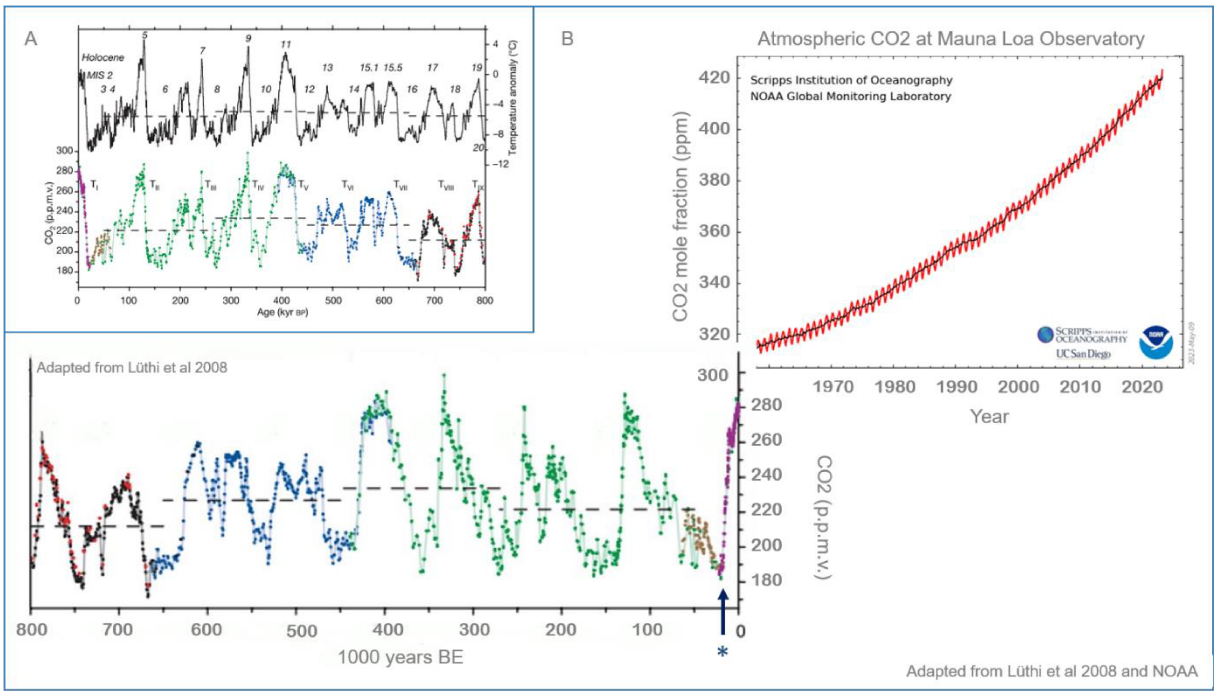


Figure 1: A) Relation of temperature (in °C, black line) and atmospheric CO_2 concentration (p.p.m.v., coloured line) during the last 800 k years. From Lüthi et al. 2008. B) Historic (year - 800 k to 0, coloured line) and current (1960 – 2020; red: seasonal fluctuations. Black: mean value) concentrations in atmospheric CO_2 (ppm). The arrow with the * depicts the last glacial maximum during the Weichselian glaciation. Adapted from Lüthi et al 2008 and NOAA Meteorological Laboratory 2023. Mind the switch in x-axis.

With a monthly mean CO_2 content of 422.23 ppm measured at the Mauna Loa Observatory, Hawaii, in April 2023 (Global Monitoring Laboratory, NOAA; see Fig 1 B) the atmosphere now contains more greenhouse gases than during the past 3 to 5 million years (that is, more than ever before in the history of *Homo spp*), and still continues to accumulate greenhouse gases. By the year 2000, global average

temperature had already increased by 0.6 °C compared to pre-industrial levels, which in turn caused a northward shift of the 15 °C summer isotherm by ~ 330 km in the North Atlantic between the years 1985 and 2000 (McMahon and Hays 2006). Between the years 2000 and 2018, global average temperature further increased to approx. 1.0 °C above pre-industrial levels (IPCC 2018). In early 2023, the World Meteorological Organization (WMO) for the first time estimated the upcoming 5 - year period to likely overshoot the Intergovernmental Panel on Climate Change's (IPCC) 'target of 1.5 °C of maximum global warming above pre-industrial level by the year 2100' (World Metereological Organization 2023). The resulting northward shift of isotherms has been modelled to be in the range of ~ 1000 km northward of those for pre-industrial levels by the year 2050 (Bastin et al. 2019a). The Arctic sea ice extent in 2018 had been the lowest in the 42-years-old satellite record (National Snow and Ice Data Center, NSIDC). Models on the ongoing decrease in Arctic sea ice during this century predict an Arctic Ocean free of summer sea ice predicted to be occurring regularly from mid-century onwards (IPCC AR5, Jahn et al. 2016). However, the Arctic sea ice likely will decrease faster than modelled until now, owing to properties of the melting ice itself that previously had not been taken into account (Hutter and Losch 2019). On land, the volume of the Greenland Ice Sheet as the second largest body of ice following the Antarctic Ice Sheet, decreases in a speed surpassing all model predictions due to a nonlinear rise in the volume of runoff meltwater (meltwater originating from land based ice; Trusel et al. 2018). This rise in runoff volume only recently went beyond natural fluctuations. And, given the warming continues, will from now on increase rapidly due to its nonlinear character (Trusel et al. 2018). This recent rapid increase in the rate of melting of the Greenland Ice Sheet is a strong indication that already present day average temperatures are having a greater impact than had been modelled for the temperatures currently observed, e.g. in the models referred to by the IPCC.

1.2 High Arctic Seasonal Light and Temperature

The shift in isotherms not only influences the melt velocity, but has direct consequences for ecosystems, as biological processes are strongly linked to temperature. In organic systems, the most basic link is described by the temperature coefficient Q_{10} (see Chapter 11.4). Due to global warming, most species are forced to follow the shift in isotherms to stabilise their metabolism, which results in a shift of species distributional ranges to more northern territories, often causing regime shifts (Grebmeier et al. 2006; Kortsch et al. 2012). Regarding primary producers, the 'Greening of the Arctic' (Bhatt et al. 2017) is a good first-grasp indicator for these northward range shifts, which occur in both terrestrial and marine habitats (Christie et al. 2019; Hansen et al. 2019). However, polar regions are extreme habitats, both in temperature, and light regime. In the High Arctic, temperatures used to range from – 40 °C (winter, Labrador/Siberia) to + 10 °C (summer, Spitsbergen). But due to global warming, temperatures especially during Arctic winter have strongly increased, locally at about 4x the pace of the global average (Maturilli et al. 2015).

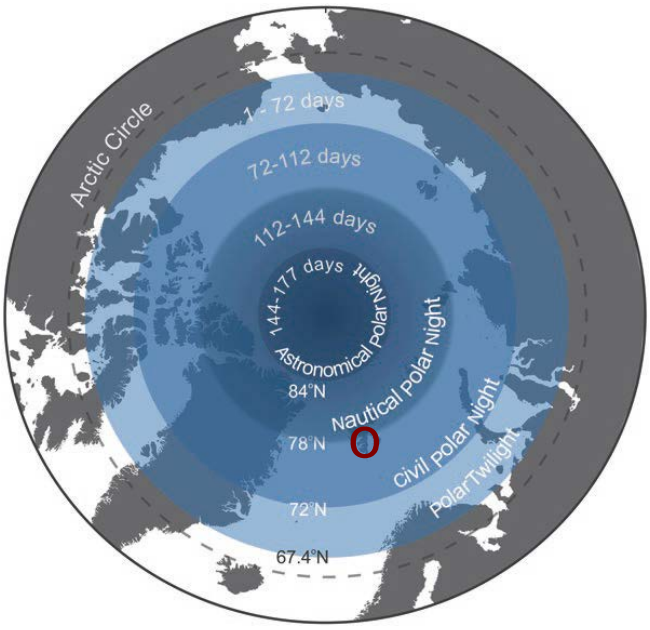


Figure 2: Duration and optical properties of the Polar Night relative to latitude. The red circle marks Spitsbergen, Svalbard. Adapted from Berge et al 2022.

What remains unchangeable is the duration of the seasonal light. Within the Arctic circle (~ 66.3 °N), the circannual light rhythm oscillates between polar day (24h of daylight, summer) and Polar Night (24h of darkness, winter), with rapidly changing day-length in the changing 'quarters' of the year (spring and autumn). The higher the latitude, the longer are the seasons of summer and winter (~ 116 days at 80° northern latitude; see Fig 2). For photoautotrophs dependent on photosynthesis, the dark season used to be prolonged by weeks. Light availability was delayed due to snow cover for perennial terrestrial primary producers, and even further delayed due to sea ice and snow cover on sea ice for marine perennial primary producers (Gerland et al. 1999; Kühl et al. 2001; Svendsen et al. 2002). The ongoing raise in temperatures during Polar Night poses a severe threat to High Arctic photoautotrophs. During recent years, winter water temperatures in Kongsfjorden at the western coast of Spitsbergen, Svalbard (79°N, red circle in Fig 2) repeatedly have been high enough to prevent sea ice formation during winter (Hop et al. 2002; Cottier et al. 2007; Berge et al. 2020), during which the sun does not rise above the horizon from October 25th to February 17th (116 days). Perennial primary producers solely rely on their internal storage compounds for survival during this time (Chapman and Lindley 1980a; Dunton and Schell 1986; Wiencke et al. 2009), while the speed of their metabolism and enzymatic reactions is tightly linked to (habitat) temperature, which is rapidly increasing (temperature coefficient Q_{10}, see Subchapter 11.4; Davison and Davison 1987). This is likely to result in a mismatch between energy demand and supply, with the potential to ultimately imperil the survival of a species.

Polar Night eco-physiological parameters in a photoautotroph, in addition to comparative data on eco-evolutionary dynamics of the same species are the subject of this dissertation. The focus here is on a key perennial marine primary producer, the kelp *Saccharina latissima* (sugar kelp).

1.3 Primary Production, Marine Primary Producers, Macroalgae and Kelp

Primary production, by which photoautotrophic organisms convert oxidised inorganic carbon (HCO_3^-/ CO_2) into organic carbon (sucrose, $C_6H_{12}O_6$) via photosynthesis, is the one crucial process that ensures the existence of nearly all food webs. Terrestrial and marine primary producers are considered to contribute about roughly the same amount to global primary production (Field et al. 1998, and form the base within each ecosystem. In case of kelp forests, the species/ community of kelp species not only constitutes the initial trophic stage, but furthermore defines the ecosystem, comparable to trees in terrestrial forests. In these ecosystems, kelp are perennial primary producers similar to e.g. conifer trees in boreal forests, in contrast to the strongly seasonal contribution of phytoplankton to primary production that in this analogy is more comparable to the herbal layer. However, due to light conditions High Arctic kelp are more comparable to broadleaved trees of boreal-temperate forests, which still provide habitat, but do not contribute to primary production during winter. The ecological relevance of marine forests equals that of terrestrial forests (Filbee-Dexter 2020). Kelp forests are among the most productive global primary producers. Brown algae account for a large proportion (an estimated 0.92 Mega tonne (Mt)) of the annual macroalgal net primary production (NPP) of about 1.32 Mt C m^{-2} yr^{-1} (Duarte et al. 2022; see 11.4). While nearly exclusively restricted to the (rocky) shorelines of temperate to polar latitude land masses, they provide extensive and diverse ecological value (Bartsch et al. 2008; Teagle et al. 2017; Duffy et al. 2019). Kelp forests contribute to carbon capture and sequestration (Queirós et al. 2019; Pedersen et al. 2020; Pedersen et al. 2021), provide constant habitat and nursery grounds, and are an all-season food source for a huge variety of marine, but also terrestrial organisms (e.g. sheep (Smale et al. 2013), or even polar bears (Lone et al. 2018), resulting in marine ecosystems of very high diversity (Smale et al. 2013; Schoenrock et al. 2018). However, kelps not only provide the base of the food web, but define the three-dimensional structure of the habitat itself. This includes the habitats' physical properties like, amongst others, light/ shade, wave damping/ exposure, current velocity, as well as its chemical properties, like decrease in local acidity via pCO_2/HCO_3^- reduction in combination with oxygen supply during light availability, exudation of saccharides etc., or decrease/ reduction in nutrients. In the High Arctic, some of those properties change during Polar Night and winter. While diversity of kelp-associated fauna significantly increases during Polar Night, likely due to their role as perennial food source (Berge et al. 2015), kelp respiration adds pressure on / competition to the ecosystem while simultaneously not providing oxygen to the local environment.

1.4 Economic Value of Kelp

The worldwide amount of kelp harvest continues to increase, and recently contributed ~ 34% of total harvested seaweed biomass (wild harvest and aquaculture, FAO 2018 (Cai et al. 2021)). For *Saccharina japonica* (kombu), e.g. it tripled from ~ 5 million tonnes in 2010 (FAO 2010) to 12 million tonnes by 2019 FAO (2021), and the seaweed sector is the fastest rising sector in western aquaculture economy. The broad spectrum of compounds offered by kelp are utilised in a broad variety of goods. This ranges from animal feed, human consumption, cosmetics or pharmaceutics, to bio-fuel, bio-plastics, and even immaterial values like shoreline protection and other ecosystem services, as well as possibly the Climate Change mitigation generally tagged 'blue carbon' (Pessarrodona et al. 2018; Hilmi et al. 2021). The macroalgal carbon sequestration mainly constitutes marine sediments, transport of macroalgal carbon into the deep sea (Krumhansl and Scheibling 2012; Smale et al. 2013), and storage in sea water in the form of recalcitrant dissolved organic carbon (RDOM, hundreds to thousands of years; Cai and Jiao 2023).

1.5 Taxonomy

Marine forests typically consist of brown macroalgae (*Phaeophyceae*) of the order Laminariales (kelp forests), which in colloquial terminology also include larger species of the order Fucales, and of the order Tilopteridales. In a strictly taxonomical sense, the kelp family comprises the perennial brown macroalgae of the order Laminariales (see taxonomy below; Guiry & Guiry 2023).

Empire: Eukaryota

Kingdom: Chromista

Phylum: Ochrophyta

Class: Phaeophyceae

Subclass: Fucophycidae

Order: Laminariales

1.6 Life Cycle of Kelp

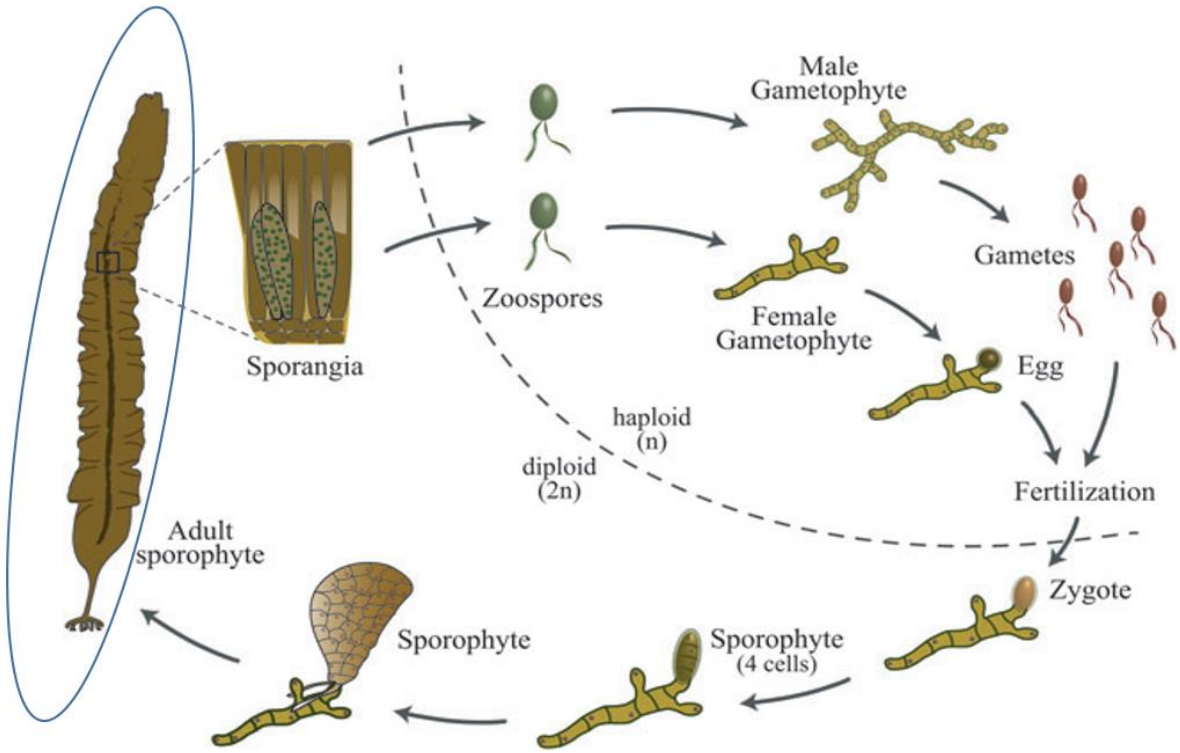


Figure 3: The haplodiplontic, heteromorph life cycle of kelp, at the example of *Saccharina latissima* with alternation between the microscopic gametophyte stages (divided in male and female) and the macroscopic sporophyte (blue ellipse) stage. Adapted from Visch et al. 2019, with permission of W. Visch.

Kelps comprise a haplodiplontic life cycle with heteromorphic stages (Picture 3; Visch et al 2019, Bringloe et al. 2020). Located along the midsection of the blade of the macroscopic sporophyte (2n, blue ellipse Fig. 1), sporangia accumulate to sori (which can appear like one large sorus) that release motile zoospores after meiosis.

The antheridia of the mature, oogamous, microscopic gametophytes (n) will release motile gametes (♂), or produce an egg (♀). After fertilization, the zygote develops into the sporophyte. Selfing (gametes and eggs from the same parent form a zygote) is common (Guzinski et al. 2016; Møller Nielsen et al. 2016), but inbreeding depression typically does not occur on a significant level in wild populations (Guzinski et al. 2016; Guzinski et al. 2020), whereas it poses a problem in Asian aquaculture in the congener species *S. japonica* (Li et al. 2017).

Both life cycle stages have been shown to react to similar factors with different susceptibility, with the gametophyte stage typically, but not e.g. regarding temperature, being the more vulnerable one (Dring et al. 1996). The perennial sporophyte is sessile once it attaches to hard substrate shortly after fertilization. Even though the zoospores and gametes are motile, dispersal rates depend on surrounding water current, but typically are limited to short distances of a few meters (Graham 2003; Gaylord et al. 2006). However, they tend to attach to substrate shortly after dispersal, but gametogenesis is only induced under the right environmental conditions, and gametophytes can grow vegetative in suboptimal conditions, stalling gametogenesis (maturation; Ebbing et al. 2021).

1.7 The Kelp Habitus

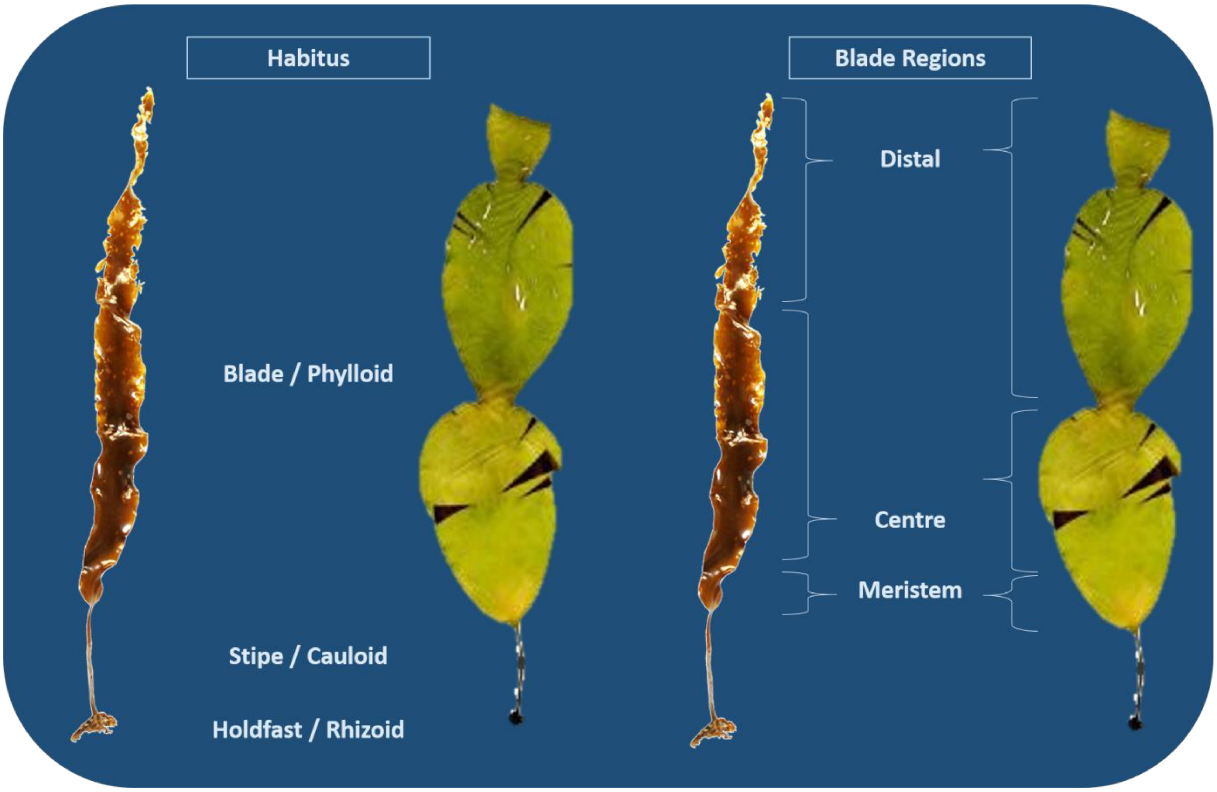


Figure 4: Kelp habitus (left) and blade regions (right) at the examples of *Saccharina latissima* (brown) and *Laminaria solidungula* (green). Pictures: *S. latissima* © S. Niedzwiedz; *L. solidungula* © S. Saupe

In contrast to filamentous marine macroalgae, kelp feature different tissues. The kelp sporophyte habitus (blue elipse in Fig 3) diverts from that of vascular plants, and typically comprises the holdfast (rhizoid) for attachment purpose only, the stipe (cauloid) to connect the holdfast to the blade (phylloid), and the phylloid with differentiated tissues of diverse functions (see picture 4). Kelp blades grow from the meristem at the base of the blade (picture 4), which typically is devoid of reproductive tissue.

1.8 Kelp Species in this Dissertation

Arctic marine macroalgae are generally regarded as impoverished cold-temperate flora, with few endemic species found in the Arctic (Wulff et al. 2009). Most arctic macroalgae therefore have a wide, cold-temperate distribution (Lüning 1990; Wilce 1990), with optimal growth temperatures of around 8 °C - 10 °C, which historically used to be well above temperatures prevalent in polar regions (Bolton and Lüning 1982; Lüning 1984; Lüning 1990; Bartsch et al. 2016). In consequence, polar algae usually grow very slowly, and become very large in comparison to their more temperate relatives (Lüning 1990).

1.8.1 *Saccharina latissima*

The main focus of this study is on the kelp *Saccharina latissima* (Linnaeus) C. E. Lane, C. Mayes, Druehl & G. W. Saunders 2006 (*Laminariaceae*, 'sugar kelp', formerly *Laminaria saccharina*). It is a key species that inhabits rocky shores between the 40th and 80th degree Northern latitude, mainly in the North Atlantic. The habitus of the blade is always without midrib, but is strongly influenced by wave exposure at the respective growth location (Coppin et al. 2020). Blade habitus ranges from sword-like, long, and narrow, with slight riffle, to very broad habitus with strong riffle (see Fig. 5).

Figure 5: Examples of *S. latissima* blade habitus. Pictures by S. Niedzwiedz

In southern populations the blade typically degrades during winter, while in High Arctic regions it usually endures for several years (even though tips have been observed to degrade after especially cold winters when light only returned after snow melt and sea ice break-up), growing to several meters. The holdfast has root-like extensions (see Fig 4, 5).

The economic value of *Saccharina latissima* has strongly increased over the last decade, predominantly owing to increasing aquaculture in Europe and North America, while harvests remained low on the traditional Asian market due to *S. japonica* being the commonly harvested species. Like with other kelp, applications entail biofuel, purification of compounds such as laminarin of high pharmaceutical (induces apoptosis in human colon cancer cells (Ji and Ji 2014), or cosmetic value, and attempts at carbon sequestration in the context of the blue carbon approach (Zhang and Thomsen 2019; Zhang et al. 2022).

1.8.2 *Laminaria solidungula*

The focus of this study is on S*accharina latissima*. However, as data on Polar Night ecophysiology is extremely scarce, *Laminaria solidungula,* as Arctic endemic kelp species, was added for comparison purposes. *Laminaria solidungula* J. Agardh 1868 is an Arctic endemic species that can only be found between 63 and 80 degrees Northern latitude (Lüning 1990). The blade of a single year is disc-like and flat, without midrib. The single discs are connected to the former and following year by a narrowed section, allowing to derive the minimum age of the alga by counting the number of blade discs (see Fig. 4, 6). The holdfast is disc-like, without rhizoid-like appendages (see Fig. 4, 6). This endemic species uncouples reproduction from photosynthesis, as it produces sori during early winter, while spores are only released in early spring (Wiencke et al. 2007).

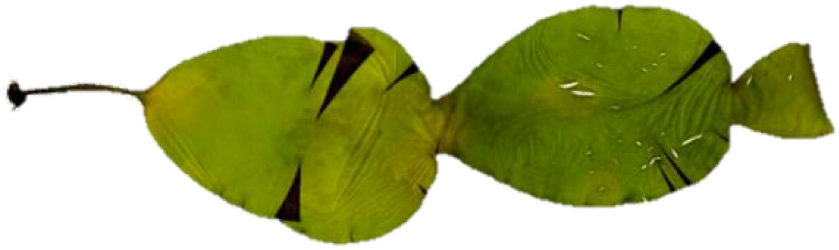


Figure 6: An example of *L. solidungula* habitus of at least 3 years of age. Picture by S. Saupe

Laminaria solidungula currently is not grown in aquaculture, hence does not have any economic importance. As part of High Arctic kelp forests, its ecological value is as described for High Arctic kelp in general, encompassing perennial food source, shelter, nursery and habitat.

1.9 Physio-Chemical Kelp Specifics

1.9.1 Chloroplasts and Pigments in Kelp

Photosynthetic processes take place in the chloroplast. Chloroplasts feature their own, albeit reduced, genome, an indicator of the endosymbiontic evolution of this organelle (Nishimura 2010). Furthermore, pigments relevant to photosynthesis (see 11.4) are found in the chloroplast.

With photosynthesis above compensation point already at 5 µmol Photons m^{-2} s^{-1} (*Laminariaceae,* Lüning 1981), species that typically inhabit the deeper sublittoral in cold-temperate regions are adapted to low light availability (Kirst and Wiencke 1995). Acclimation of pigment content to prolonged changes in light climate can be observed after one week of incubation at different radiation conditions (Ramus et al. 1976; Ramus et al. 1977; Huovinen and Gómez 2011), in addition to diurnal variation in pigment composition caused by daily changes in light intensities (Oquist and Chow 1992; Figueroa et al. 1997; Flores-Moya et al. 1998; Gevaert et al. 2002). The lower the light condition, the higher typically the gain in photosynthetically active pigments, with proportionally higher increase in accessory pigments than in Chl *a*, depending on condition and species (Ramus et al. 1976; Ramus et al. 1977). The light compensation point (E_c; Lüning 1990) as an emerging factor of respiration rate and rate of photosynthesis (both of which are temperature dependent -> Q_{10} (see Chapter 11.4)), will increase with increase in temperature, reducing photosynthetic efficiency (α). Historically, E_c in seaweed has been observed to be lower in polar than in temperate species (Lüning et al 1990). With increase in water temperatures in the Arctic, E_c likely will increase in polar species.

1.9.2 Storage Carbohydrates and Dark Metabolism in Kelp

The carbon (C) metabolism of *Phaeophyceae* is distinct from most other (green) primary producers, which usually store C as α-(1,4)-glucans from sucrose (Michel et al. 2010). The product of photosynthesis in *Phaeophyceae* is D-mannitol (Fig. 7 A), derived from the Calvin-cycle photo-assimilate D-fructose-6-phosphate (F6P; Yamaguchi et al. 1966; Michel et al. 2010, and the long-term storage carbohydrate is the polysaccharide laminarin (or laminaran; β-(1,3)-glucan; Fig7 B).

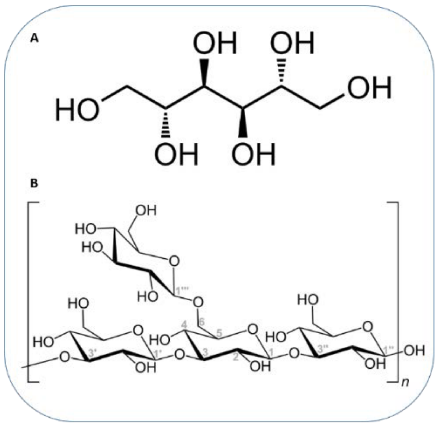


Figure 7: Structure of (A) D-mannitol and (B) laminarin. Three laminarin polymers are linked β-(1,3), while the middle one is also linked β-(1,6). Chemical structures: Wikicommons CC-SA 4.0.

The biosynthetic pathways so far remain largely unknown (Michel et al 2010), but it is known that laminarin is biochemically interchangeable with mannitol (Yamaguchi et al. 1966), and concentrations of both change with season (Nisizawa 1940; Craigie 1974; Dethier and Williams 2009). The degree of polymerisation of laminarin usually is 25 (up to 50), and its glucose residues are linked β-(1,3). Depending on season, algal species, life cycle stage, and location within the alga, laminarin can be found unbranched (linear), and with intra-chain branches (β-(1,6); Percival and Ross 1951; Craigie 1974; Chizhov et al. 1998). In addition, laminarin is polydisperse: The G-series is a polymer entirely build of glucose monomers, thus terminated by a reducing 3-linked glucose residue, whereas the M-series is terminated by a non-reducing 1-linked D-mannitol residue (Read et al. 1996; Chizhov et al. 1998). The M:G ratio varies with algal species, and the M-series usually only constitutes a small fraction, or can be entirely absent (Chizhov *et al*. 1998). Overall, 15 sub-types of laminarin have currently been identified, all distinct in molecular weight (Read et al. 1996; Rioux et al. 2010; Graiff et al. 2015). The anatomy of kelp enables C - translocation across the plant (see Fig. 8): mannitol is converted to laminarin during the light season, and stored in the blade cells (Lüning 1969; Schmitz et al. 1972; Chapman and Craigie 1978), but when mannitol is required in the meristematic regions for growth, it is remobilised from laminarin, and transported via special sieve tube cells that serve for long-distant transport between different tissues (Schmitz et al. 1972; Lüning et al. 1973; Raven et al. 2002; Wiencke and Bischof 2012).

Despite mannitol content being high from mid/ late spring to late winter (Chapman and Craigie 1978), growth rates rapidly decline with the onset of mannitol accumulation for C - storage build-up in spring (Chapman and Craigie 1977), and are only resumed during Polar Night, or towards the end of it, depending on the algal species. Several polar brown macroalgae have been shown to exhibit the greatest increase in growth during the darkness of Polar Night, or shortly before light returns, (Chapman and Craigie 1978; Chapman and Lindley 1980a; Dunton 1985), presumably to maximise photosynthetically utilisable tissue area when light returns (Lüning 1969; Wiencke et al. 2009).

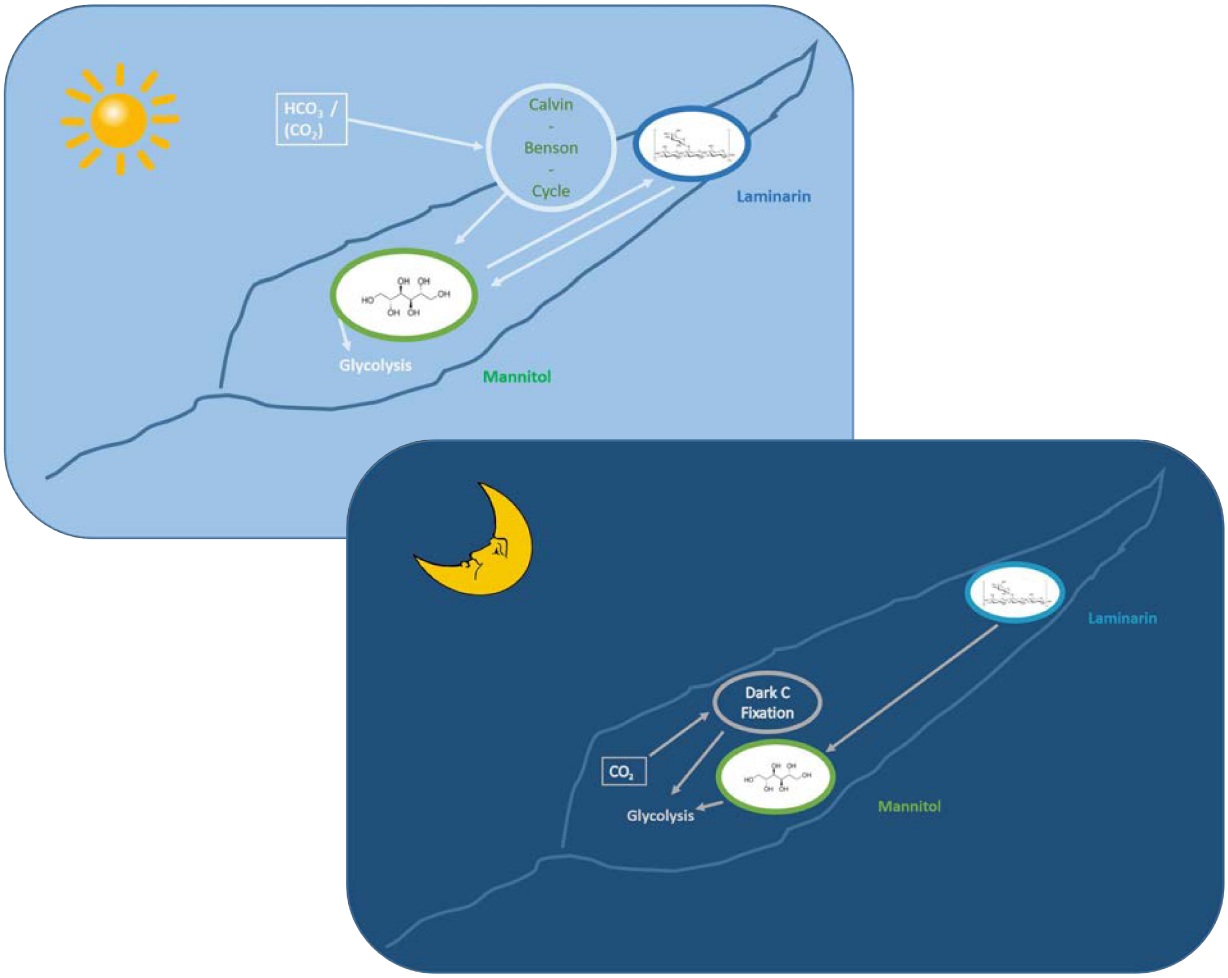


Figure 8: The difference in kelp carbohydrate metabolism between light availability (above, light blue/sun), and during prolonged darkness (below, dark blue/moon). During the light season, mannitol is stored as laminarin. During the dark season, laminarin is re-mobilised to mannitol. Dark carbon fixation, which plays a role for cell metabolism, is light-independent. Chemical structures, sun, moon: Wikicommons CC-SA 4.0.

This process is reflected in the concentrations of mannitol and laminarin. Following the C - storage build-up in the period of light availability, laminarin content is highest in late summer and autumn, and gets successively reduced during winter. Overall concentrations of both, laminarin and mannitol content, are lowest in early spring, after the onset of rapid growth (Craigie 1974).

Light-independent carbon fixation (also: dark carbon fixation, or ß-carboxylation) is another pathway to fixate carbon for cell metabolism. Dark carbon fixation can account for up to ~ 17 % relative to photosynthetic carbon fixation in the meristem of *Saccharina latissima* (Kremer 1981).

1.10 Physiological Properties of Kelp to Survive Polar Night Conditions

The prolonged darkness is an immense challenge to photoautotrophs, especially sessile ones like macroalgal sporophytes that do not form intra-generational resting stages. Some kelp uncouple photosynthesis from growth to circumvent the nutrient scarcity due to phytoplankton blooms during the summer months, instead taking advantage of high nutrient availability during winter (Johnsen et al 2020, see Fig. 9).

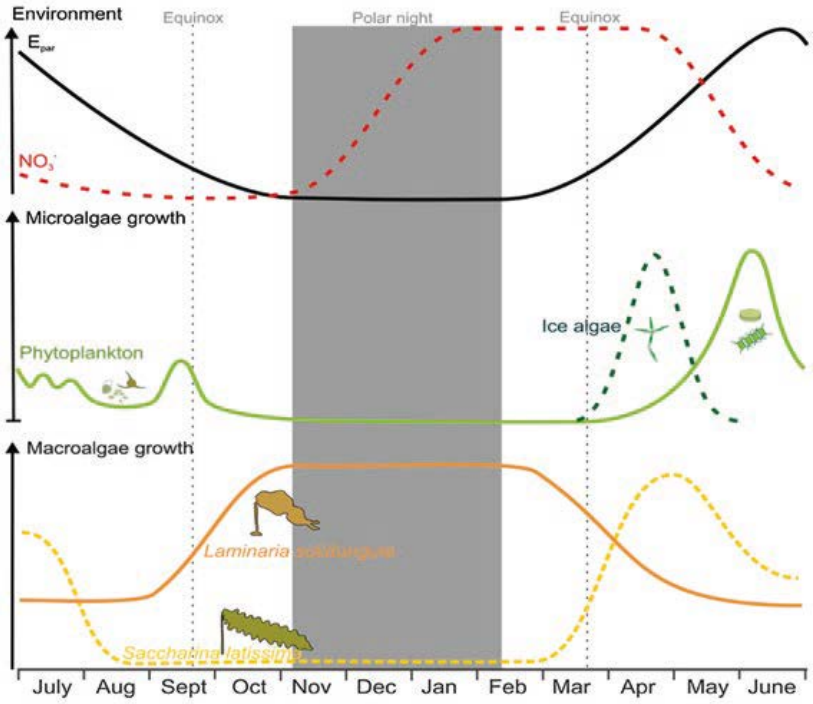


Figure 9: Annual dynamics for the environment (light availability (E_{par}) and nitrogen (NO_3); upper panel), microalgal growth (phytoplankton and ice algae; middle panel), and macroalgal growth (*Laminaria solidungula* and *Saccharina latissima*; bottom panel). Adapted from Johnsen et al 2020; picture by Malin Daase, with permission of M. Daase.

Polar macroalgae commonly are grouped into so-called 'season anticipators' and 'season responders', according to their strategy in growth and reproduction. The grouping originally regarded the algal vegetation of Antarctica (Kirst and Wiencke 1995), where nutrient availability, or shifts in temperature, are negligible, and seasonality is triggered by day length alone. Still, patterns as described for season anticipation/ response can be observed in the Arctic, but are not solely triggered by day length, but by nutrient availability (summer low, winter high), and temperature (winter low, summer high) as well (Chapman and Craigie 1977; Borum et al. 2002). Both strategies can be reflected in the physiology, for example in the usage of storage compounds, the seasonal uncoupling of photosynthesis from growth, or of photosynthesis from reproduction in kelp. Pigment synthesis or maintenance during the Polar Night, as opposed to pigment disintegration and re-build at return of light availability, by now have only been reported in red algae (Lüning 1990; Lüder et al. 2002).

Several polar brown macroalgae have been shown to exhibit the greatest increase in growth shortly before light returns towards the end of the Polar Night (Chapman and Craigie 1978; Chapman and Lindley 1980a; Dunton 1985). This ensures a maximum of photosynthetically utilisable tissue area is available when light returns (Lüning 1969; Wiencke et al. 2009). Seasonal concentrations of mannitol and laminarin reflect this: Overall concentrations of both become nearly depleted in late winter, and laminarin and mannitol content is lowest in early spring, coinciding with the onset of rapid growth (Craigie 1974).

Following the C - storage build-up in the period of light availability, laminarin content is highest in late summer and autumn, and gets successively reduced during winter. In contrast, mannitol content is high from spring to late winter (Chapman and Craigie 1978), but growth rates decline rapidly with the onset of mannitol accumulation for C - storage build-up (Chapman and Craigie 1977), and are resumed only during Polar Night, or towards the end of it, depending on the algal species.

Even though the predicted increase in sea surface temperatures might allow for a northward shift of a photoautotroph species' distributional range in the wake of global warming, the light regime of the High Arctic might counteract that. The pressure is even more severe on perennial marine primary producers, as in addition to the prolonged darkness of the Polar Night, their Arctic habitats are further impeded by changes in circannual light availability, which locally is increasingly limited due to the high turbidity caused by heightened circannual glacier meltwater runoff, and raise in precipitation (Borum et al. 2002; Niedzwiedz and Bischof 2023). With this, even species that store large amounts of excess carbohydrates during summer might not be able to stock sufficient amounts to survive the heightened metabolic demand during warmer winters.

To which extent pigments and internal storage compounds are prevalent/ utilised during Polar Night in High Arctic *Saccharina latissima* and *Laminaria solidungula* so far have only been deducted from late summer/early spring measurements. This dissertation presents first-time measurements obtained during the dark season.

1.11 Marine Epigenetics

While many genetic processes have been rigorously investigated in terrestrial and marine primary producers, epigenetic mechanisms only recently gained focus in marine photoautotrophs. Epigenetic modulation is tissue-specific (Anastasiadi et al. 2021), and is induced in reaction to local, abiotic, and biotic factors (Bossdorf et al. 2008; Richards et al. 2010; Lämke and Bäurle 2017). Epigenetic variation is common especially across latitudes (Vaughn et al. 2007; Johannes et al. 2008). It has been shown to play a role in establishing, maintenance and control of gene expression without changes to the DNA sequence (Anastasiadi et al 2021), hence is key in determining eco-evolutionary dynamics of a species (Calosi et al. 2016; Anastasiadi et al. 2021). Known epigenetic mechanisms are non-coding RNA (ncRNA), histone modification, and DNA cytosine methylation (Boquete et al. 2021). While all mechanisms become effective within a single generation, some are more stable across generations. The late germline segregation in plants is hypothesised to enhance the potential for epigenetic inheritance (Anastasiadi et al 2021). However, very few epigenetic markers have been found that do not get transmitted to F1 and F2 generations in plants and hence, likely algae (Anastasiadi et al 2021). DNA cytosine methylation, one of the most-studied modulation mechanisms in plant epigenetics (Boquete et al. 2021), is considered to be stable across mitotic cell division and meiotic inheritance (Jablonka and Raz 2009; Kumar and Mohapatra 2021), and plays a role in transgenerational inheritance (Johannes et al. 2009). Regarding processes of (rapid) adaptation and eco-evolutionary dynamics, DNA methylation hence likely is a key mechanism.
Despite being one of the most important human food sources in Asia, research on kelp epigenetics is just starting to gain momentum, evident in the lack of literature on this topic,with presently only a handful of studies (*Phaeophycea*; Cock et al. 2010; Liu et al. 2019; Fan et al. 2020b; Fan et al. 2020a; Teng et al. 2021).

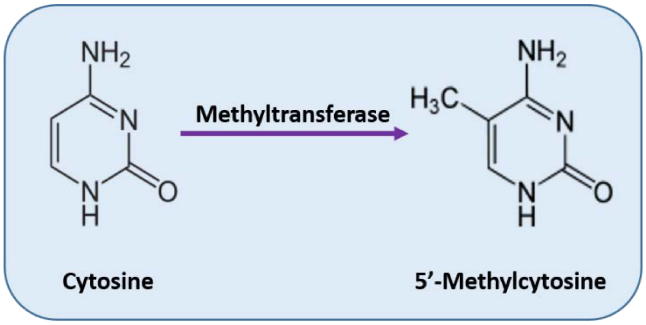


Figure 10: Difference in chemical structure between an un-methylated Cytosine (left), and a methylated Cytosine (5'-Methylcytosine, 5-mC; right). The methyl-group is added and maintained by different groups of methyltransferase enzymes.

'DNA methylation' in plants and algae describes the methylation of a cytosine in the DNA (5'-methylcytosine, 5-mC, see Fig. 10). While in animals only DNA sequence contexts of CG (cytosine guanine) are found to be methylated, DNA methylations in plants have been found in the sequence context of CG, CHG or CHH ('H' any base except G; Bewick et al. 2017). Cytosine methylations can occur within and outside of genes. Genes are usually methylated in the CG context only (Schmitz et al. 2019), and methylation of the CG context in gene bodies of nuclear DNA has been found to be between 2 % and 86 % across *Viridiplantae* (Bewick et al. 2017).

Furthermore, CG, CHG, and CHH contexts were found to act in silencing transposable elements in and outside of genes (Zhou et al. 2020), or act in regulation of transcript expression (Dubin et al. 2015; Zhang et al. 2018; Boquete et al. 2021). In addition, histone modification, specifically H3 K9, seems to be linked to CG cytosine methylation (Bender 2004). Moreover, it has been proposed that CG methylation regulates the inheritance of other types of epigenetic information in plants (Mathieu et al. 2007). One of these concerns the chloroplast genome of kelp. In terrestrial plants, DNA methylation of the chloroplast genome is uncommon in general (Fojtova et al. 2001). With this, the chloroplast genome is typically regarded as un-methylated (Ahlert et al. 2009), and therefore has been utilised as negative control e.g. during methylome mapping. However, there are known examples of chloroplast DNA methylation in few cultivated species (rice, tomato), and the unicellular green alga (*Chlamydomonas spp.*; Royer and Sager 1979; Kobayashi et al. 1990; Muniandy et al. 2019). In the kelp *S. japonica,* evidence for DNA methylation of the chloroplast genome has recently been published (Teng et al. 2021).

DNA methylation is maintained by DNA methyltransferases. Different methyltransferases are involved in the maintenance or removal of the different methylation contexts (Gehring and Henikoff 2007; Kumar and Mohapatra 2021). *De novo* methyltransferases have been found to establish differential methylation during oogenesis or spermatogenesis, and maintenance methyltransferases somatically sustain methylations (Li 2002; see Fig 11), while different demethylases are associated with re-activating previously silenced genes by removing methylations (Gehring and Henikoff 2007).

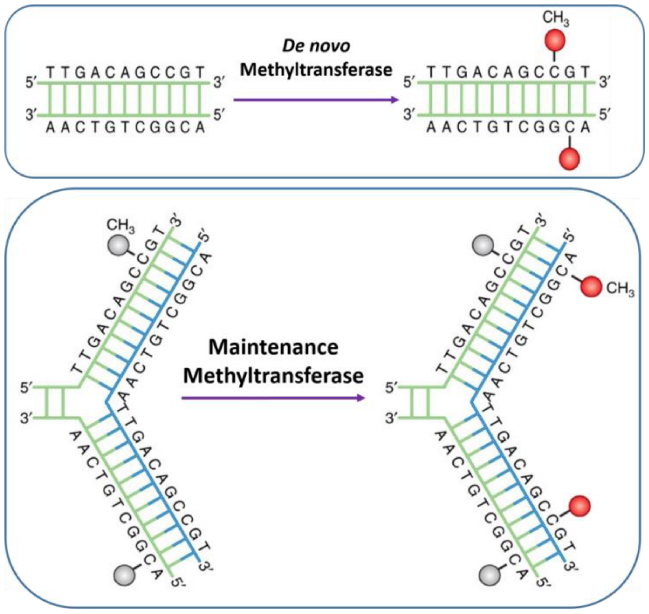


Figure 11: De novo methyltransferases (above) provide new methylation in reaction to external triggers, while maintenance methyltransferases (below) secure the transfer to the newly synthesised DNA strand during DNA replication. Adapted from Moore et al. 2013.

Not all methyltransferases seem to be encoded in every species. While an enzyme of the DMT1 (formerly CrMET1 (*C. reinhardtii* methyltransferase 1)) family has been found to be coded in the chloroplast in terrestrial plants (Nishiyama et al. 2004), only a nuclear-based methyltransferase that resembles enzymes of the DNA (cytosine-5-)-methyltransferase 2 (DNMT2) family has been observed to likely be the source of kelp chloroplast genome methylation (Teng et al. 2021). This is the same enzyme family that has been found for methylation of the nuclear genome, where only enzymes of the DMNT2 family are coded *in S. japonica* (Fan et al. 2020a). Hence, even though chloroplast methylation seems to be at least partially independent from the nuclear genome processes in plants, it is not clear whether this likewise applies to kelp (or brown algae in general). According to their phylogenetic tree topology (Teng et al. 2021), it is very likely that an enzyme of the DNMT2 family is responsible for the chloroplast DNA methylation in *S. latissima* as well.

Due to the putative direct influence on photosynthesis, evidence of methylation in the chloroplast genome is of particular interest regarding adaptation processes to rising temperatures. Photosynthesis-related genes have been shown to be one of the first processes to be affected by rising temperature in temperature and heat stress experiments (Heinrich et al. 2012; Heinrich et al. 2016; Monteiro 2020). To understand eco-evolutionary dynamics involved in rapid adaptation of photoautotrophic organisms to rising ambient temperatures, this again highlights the importance to investigate epigenetic mechanisms not only in the nuclear genome, but in the chloroplast genome as well. This has been neglected in kelp so far, but will be addressed further in this dissertation.

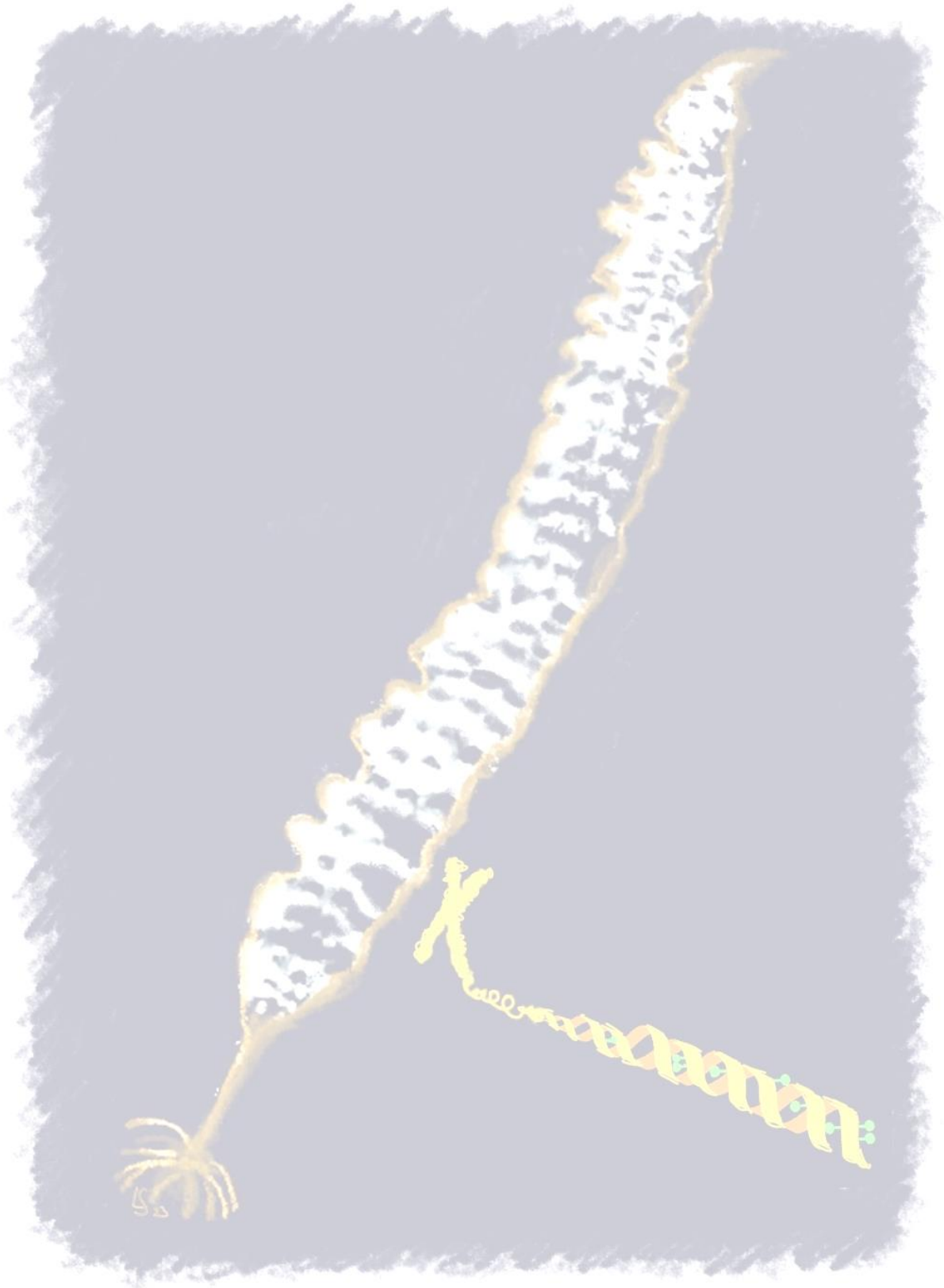
1.12 Marine Transcriptomics

Transcriptomics investigate the presence or change in mRNA expression relative to external factors, and bridge genotype to phenotype. Results can highlight changes in gene expression in reaction to a given factor, while they do not yield direct information on what controlled the genes to be differentially expressed on a molecular level. An increasing amount of data is available on marine transcriptomics (Heinrich et al. 2016; Jueterbock et al. 2020; Monteiro et al. 2019a).

Until now the assessment of how changes in abiotic factors related to Climate Change impact differential transcriptomics in kelp have mainly been focused on heat stress, or high light (Heinrich et al. 2015; Liesner et al. 2022). Like with most physiological or bio-chemical studies on High Arctic kelp, these experiments have been carried out during light availability, while abiotic aspects of Polar Night remained disregarded despite being a key factor in Polar photoautotrophs.
In the congener *S. japonica*, it has been shown that in both the nuclear and chloroplast genome, gene expression is inversely related to methylation level in gene bodies (Fan et al. 2020a; Teng et al. 2021). Hence, comparative kelp transcriptomics can indicate in which functional gene groups (GOterms) epigenetic mechanisms might be differentially expressed. During this dissertation a joint study assessed transcriptomics aspects of early Polar Night physiology at different temperatures in *Saccharina latissima.*

1.13 Knowledge Gaps regarding Polar Night Eco-Physiology, and Eco-Evolutionary Dynamics of *Saccharina latissima*

The survival of primary producers in a High Arctic habitat is primarily dependent on their capacity to persist the absence of light during the months of Polar Night. However, data on Polar Night eco-physiology of the kelp *Saccharina latissima* have been missing nearly completely, with only very scarce research on kelp, or seasonal and perennial High Arctic primary producers during Polar Night in general. These studies were mostly conducted prior to, or at the onset of global warming (commonly acknowledged year of reference 1990; Chapman and Craigie 1977; Chapman and Craigie 1978; Dunton 1985; Henley and Dunton 1995), and these studies collected data under Polar Night conditions at water temperatures below 0 °C (REF as above). Throughout the last two decades, it has become unmistakably clear that due to the Climate Crisis, temperatures during arctic winter have strongly increased, locally at about 4x the pace of the global average (Maturilli et al. 2015). The scarce data on parameters of Polar Night physiology hence are likely no longer accurate, or need to be considered with caution, as temperature, especially above 0 °C, is a crucial factor influencing metabolic processes in the High Arctic (Mikan et al. 2002). Considering that a large variety of data exists for the (short) High Arctic light season, nearly 100% of the data collected for High Arctic primary producers covers only half (or actually only 1/3) of the annual cycle. In addition, processes of rapid adaptation are likely not solely under genetic control, but strongly influenced by epigenetic mechanisms, as these play a vital role in the control of gene expression (Richards et al. 2017; Anastasiadi et al. 2021). Recent data suggest a combination of both, genetic and epigenetic mechanisms, to be important for eco-evolutionary dynamics, and hence (local) adaptation in *S. latissima:* While e.g. the sporophyte habitus in wild populations is assumed to be mainly determined by wave exposure (Coppin et al. 2020), the habitus of young lab grown sporophytes is clearly distinguishable by origin (Heinrich et al. 2016). This suggests that differences in habitus are transgenerationally stable, but then can adapt to local conditions during maturation. Strong differences in eco-physiology and transcriptomics from different European latitudinal locations (Monteiro et al. 2019b; Liesner et al. 2020a; Diehl et al. 2021) indicate that there is a more fundamental process to local adaptation than phenotypic plasticity in kelp as suggested previously (Bolton and Lüning 1982). In order to gain a more holistic assessment of the eco-evolutionary processes and the capacity for rapid adaptation in *S. latissima*, epigenetic mechanisms need to be considered in addition to baseline field data/ the genome approach. As with Polar Night data, all data collected regarding adaptation/ speciation processes in High Arctic kelp, or High Arctic primary producers in general, so far have been gathered for genetic mechanisms (SNP, COI, microsatellites etc.; Møller Nielsen et al. 2016; Assis et al. 2018; Neiva et al. 2018; Guzinski et al. 2020), hitherto omitting epigenetic processes which are presumably crucial for rapid adaptation.



Chapter 2

Aim of Study – Hypotheses - Research Questions - Outline

2.1 Aim of Study

The capacity for rapid adaptation has become key to survival during the increasingly drastic changes in ecosystems due to anthropogenic Climate Change. In addition, the already extreme condition of continuous dark exposure during the Polar Night dictating primary production in the High Arctic has become even more challenging due to the increase in metabolic turnover at rising winter temperatures as a result of global warming. This study aims to assess fundamental aspects of Polar Night eco-physiology in the perennial primary producer *Saccharina latissima* in an already warmed High Arctic environment. Further, it aims to investigate the presence of a possible epigenetic mechanism of (rapid, local) adaptation in this kelp species. Both aspects are crucial for future research and modelling approaches regarding the High Arctic survival capacity of *S. latissima*, and possibly High Arctic primary producers in general.

2.2 Hypotheses and Research Questions (RQ)

The major Hypotheses addressed in this dissertation entailed two interconnected, but distinct themes focussed on the perennial marine photoautotroph *Saccharina latissima,* concerning its Polar Night eco-physiology (Hypothesis I), and comparative epigenetics (Hypothesis II).

Hypothesis I:

> **Rising winter water temperatures due to global warming impair or compromise the capacity for survival in High Arctic kelp.**

The research questions (RQ) testing Hypothesis I entailed current Polar Night eco-physiology in High Arctic perennial kelp:

RQ 1a)

> **What are current eco-physiological parameters (storage compounds, respiration, pigments of photosynthesis) in the perennial marine primary producer *Saccharina latissima* (sugar kelp, season responder) and *Laminaria solidungula* (season anticipator) during the High Arctic Polar Night?**

The amount of the storage carbohydrate laminarin to fuel metabolism likely is a crucial factor regarding survival capacity during Polar Night. Furthermore, mannitol and C/N ratio give indications on the metabolism, as especially energy consuming processes like growth or reproduction are prone to shift the ratio.

Data on pigments during the Polar Night in High Arctic kelp so far have been collected from April onwards, or prior to 1990. In addition, difference between *S. latissima* (season responder) and *L. solidungula* (season anticipator) are expected. A higher turnover rate is be expected from *L. solidungula* (completes its reproductive cycle in winter). Furthermore, as elevated temperatures initiate higher metabolic turnover, respiration rates in darkness are likely to increase in both species.

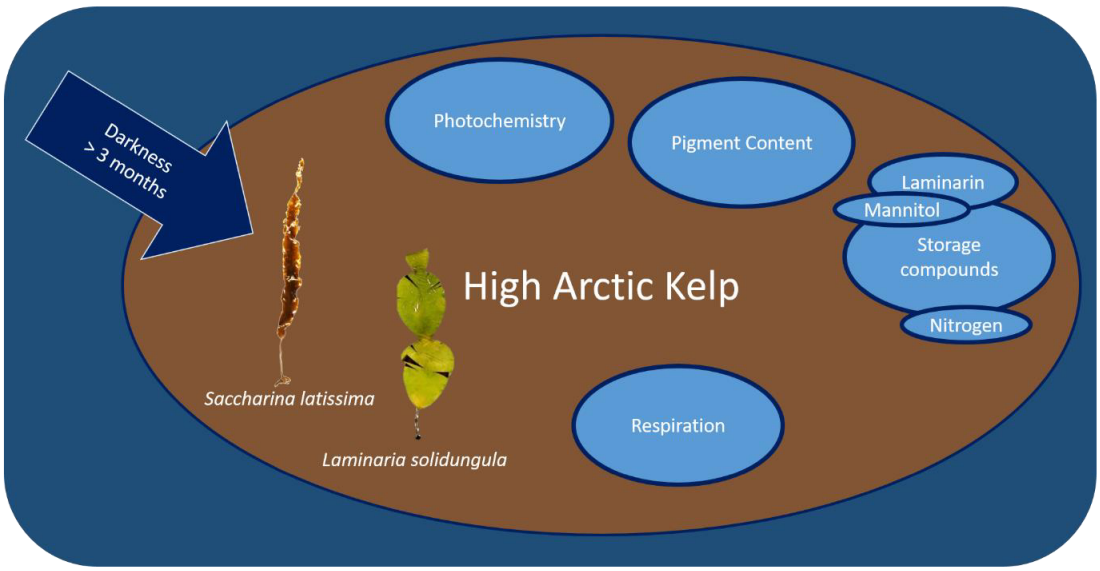


Figure 12: Visualisation of RQ 1a: 'What are current eco-physiological parameters (storage compounds, respiration, pigments of photosynthesis) in the perennial marine primary producer *Saccharina latissima* (sugar kelp, season responder) vs *Laminaria solidungula* (season anticipator) during the High Arctic Polar Night?' Pictures of kelp: S. Niedzwiedz (*S. latissima*) und S. Saupe (*L. solidungula*).

RQ 1b)

How do presumed future temperatures (~ 4 °C – 8 °C) affect High Arctic *Saccharina latissima* during prolonged darkness?

The scope of instant changes in eco-physiological parameters at elevated temperatures during continuous darkness can aid to grasp the long-term impact of global warming, and be assessed via transcriptomics. Presuming there is a difference in eco-physiological parameters at elevated temperatures, adaptive gene regulation should be observable in mRNA expression.

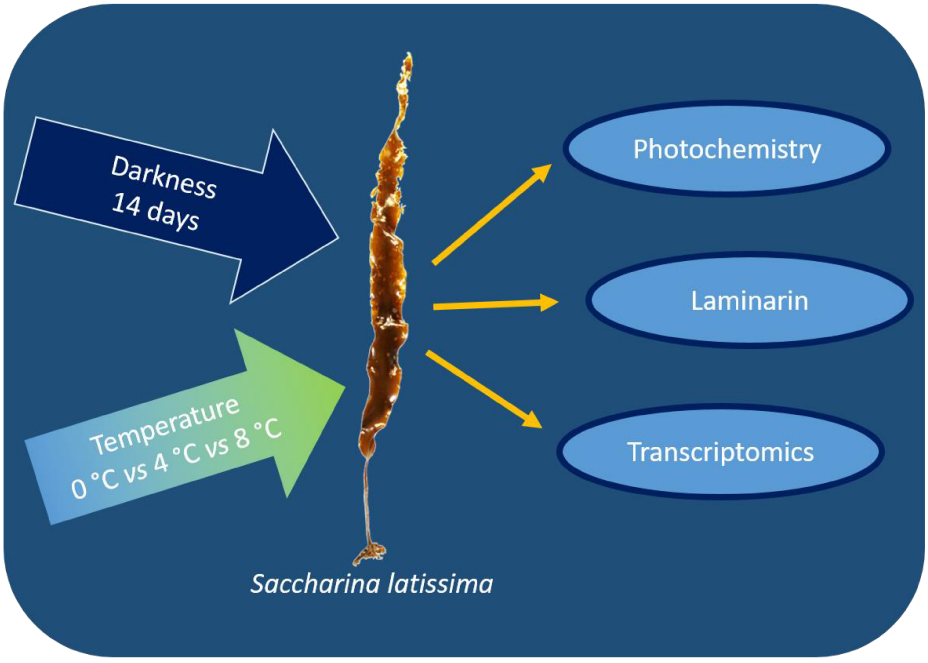


Figure 13: Visualisation of RQ 1b: 'How do presumed future temperatures (~ 4 °C – 8 °C) affect *Saccharina latissima* during prolonged darkness?' Picture: S. Niedzwiedz *(S. latissima*)

Hypothesis II:

Saccharina latissima has a heritable origin-specific methylome that reacts to divergence from origin-specific temperature as a means of rapid adaptation.

To test Hypothesis II, research questions addressed the presumed epigenetic mechanisms of DNA methylation in *S. latissima* in a comparative approach:

RQ 2a)

Is there a nuclear and/ or chloroplast methylome in _Saccharina latissima_?

As there seems to be a strong discrepancy in data even within *Phaeophyceae* regarding the occurrence of epigenetic mechanisms, specifications of epigenetic modifications of importance in *S. latissima* can only be assessed by testing for the respective mechanism in this species, or possibly the closely related *S. japonica*. Chloroplast feature their own (reduced) genome, and are the essential organelle for primary production. Epigenetically controlled genomes might likewise be found in chloroplasts of *S. latissima* in case their nuclear genomes are methylated.

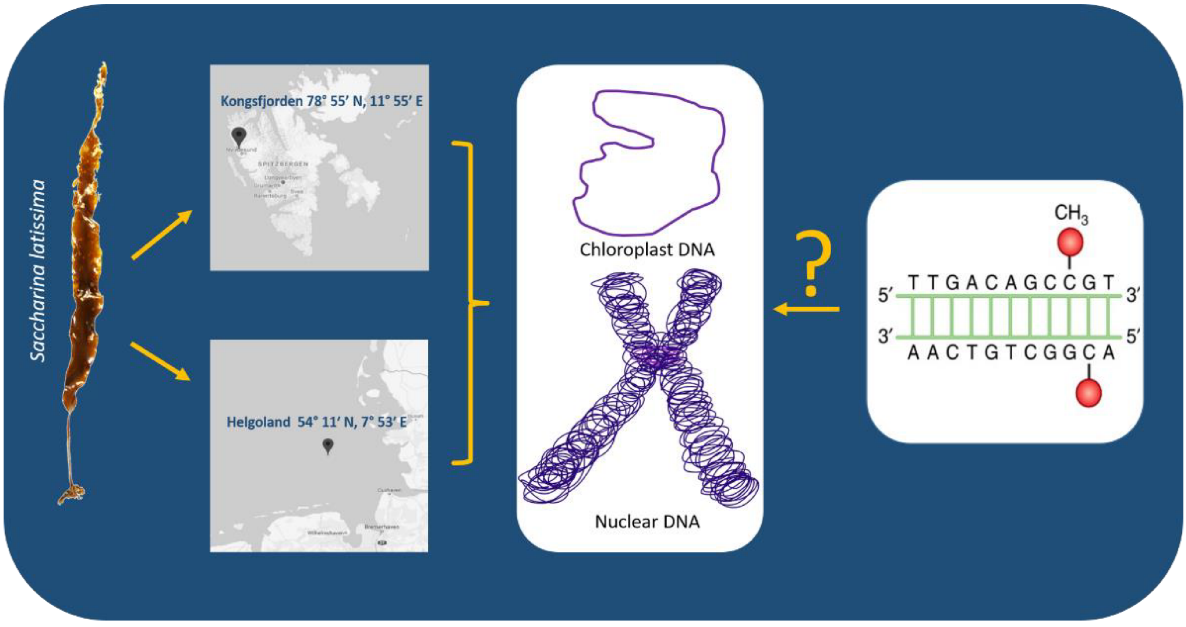


Figure 14: Visualisation of RQ 2a: 'Is there a nuclear and/ or chloroplast methylome in *S. latissima*?' Pictures: S. Niedzwiedz (*S. latissima*), Google Maps 2023a, b (Helgoland, Spitzbergen), Moore et al 2013 (methylation on DNA strand).

Note: When the experiment and sequencing on *S. latissima* for this study were carried out in 2019, there were no published data on kelp methylomes. Since then, at least for cultivars of the congener species *Saccharina japonica,* data on nuclear and chloroplast methylation have been published (see Subchapter 1.11).

RQ 2b)

Are there differences in the nuclear and/or chloroplast methylomes in response to identical abiotic factors between High Arctic and temperate origins of *Saccharina latissima*, indicating inheritance/ epigenetic memory?

As epigenetic mechanisms regulate gene expression, and are initiated specifically in response to local habitat conditions, it can be presumed that an epigenetic signature should differ between origins, as strong differences in eco-physiology have been reported between habitats, and variance across latitudes is to be expected due to the natural occurrence of epigenetic variation. Cytosine methylation in CG and CHG context is considered to be potentially heritable. If these methylations occur in the epigenome of *S. latissima*, cultivars grown from gametophytes of temperate and High Arctic origin should exhibit epigenetic signatures that are to an extent similar among their origin regardless of growth condition (different cultivation temperature), but different between origins even within the same growth condition (discrete cultivation temperature).

RQ 2c)

Are there within-origin differences in the nuclear and/or chloroplast methylomes between cultivars and wild populations, or within-origin differences between cultivars at different temperatures indicating signs of 'rapid adaptation'?

Laboratory conditions, with the reduced amount of stressors, and the only variable being temperatures well below stress levels should provide a 'non-threatening' environment. In case that cytosine methylation plays a major role in rapid temperature adaptation in *S. latissima* (within a generation), differences within the origins should be evident when grown at different temperatures, being the only variable to account for differences in epigenetic signature.

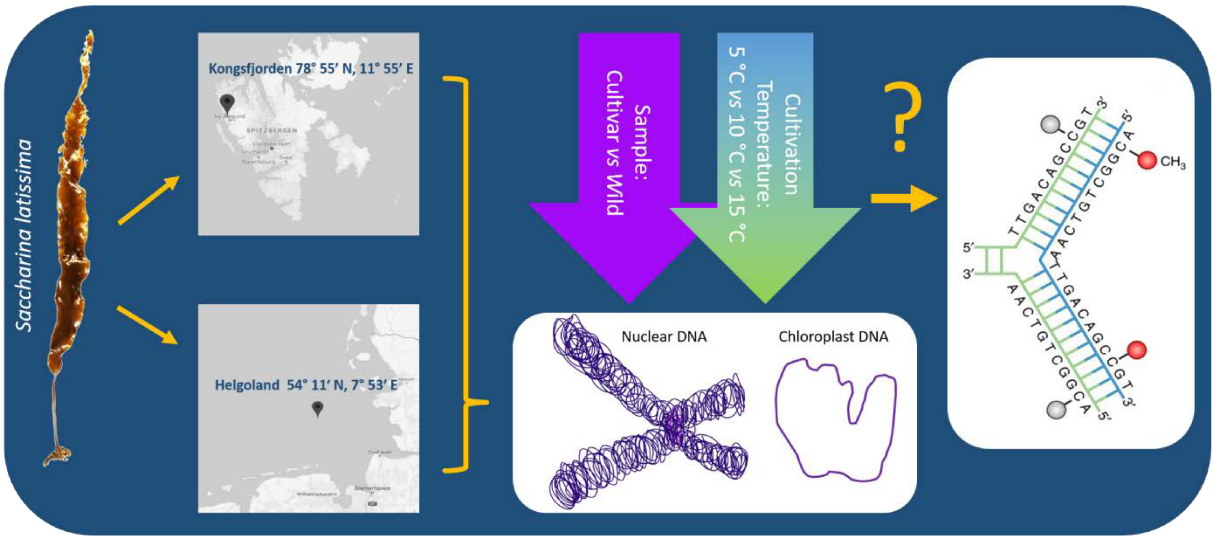


Figure 15: Visualisation of RQ 2b + c. 'Are there differences in the nuclear and/or chloroplast methylomes in response to identical abiotic factors between High Arctic and temperate origins of *Saccharina latissima*, indicating inheritance/ epigenetic memory?', and 'Are there within-origin differences in the nuclear and/or chloroplast methylomes between cultivars and wild populations, or within-origin differences between cultivars at different temperatures indicating signs of 'rapid adaptation'?'. Pictures: S. Niedzwiedz (*S. latissima*), Google Maps 2023a, b (Helgoland, Spitzbergen), Moore et al 2013 (transfer of methylation during DNA replication).

2.3 Outline / Scientific Approach

The **multidisciplinary approach** of this dissertation combined **physiological** and **molecular** assessments. The following studies were carried out to determine fundamental aspects of adaptation to the Polar Night in the perennial marine primary producer *Saccharina latissima* in the High Arctic, and investigate the presence and function of a possible epigenetic mechanism of (rapid local) adaptation in this kelp species:

Chapter 3: ***In situ*** assessment of **eco- physiology** in *S. latissima* and *Laminaria solidungula* from Kongsfjorden, Spitsbergen during the **Polar Night** of 2016/17 (RQ 1a, Fig. 12). The *in-situ* assessment was carried out in late September/ early October 2016 and late February, early March 2017, and comprised amounts of laminarin and mannitol, total carbon (C), nitrogen (N), respiration rates (oxygen consumption), pigment content (Chl *a* and antenna pigments), and photosynthesis. In addition, identical on-site experiments were conducted in both seasons. Experimental conditions entailed light withdrawal at different temperatures (0, 4, 8 °C, Fig. 13). These parameters together provid a good proxy of the Polar Night eco-physiology in High Arctic kelp: Assessing the amount of storage carbohydrates at the onset and towards the end of the Polar Night yielded a baseline understanding of the species´ capacity to survive up to 116 days of darkness in winter water temperatures of ≤ 5°C. Comparing photosynthetic pigment content and photochemistry at the onset and towards the end of Polar Night allowed to determine the capacity to resume photosynthesis after dark exposure despite presumably depleted storage. Simultaneously assessing these parameters in the Arctic endemic kelp species *L. solidungula* provided insight into the degree of local adaptation to High Arctic conditions in *S. latissima*, as the Arctic endemic *L. solidungula* is presumed to show a higher degree of adaptation to the High Arctic environment.

Chapter 4: ***In vitro*** Polar Night/ **dark adaptation transcriptomics** and photosynthesis in *S. latissima* (RQ 1 b, Fig. 13). Sporophytes were cultivated from gametophyte cultures, and kept at 0 °C and 4 °C for 2 weeks. Longitudinal growth (cm), photosynthesis (imaging PAM), and RNA (sequencing) was regularly assessed over the course of the experiment. Investigating RNA expression after prolonged dark exposure in *S. latissima* indicated most processes of dark adaptation at the onset of the Polar Night. Furthermore, differences in gene expression between current (~ 4 °C) and historical (0 °C) winter water temperatures were assessed to detect processes of dark adaptation.

Chapter 5: The **nuclear methylome** of *S. latissima* (RQ 2 a, Fig. 14) has been assessed via next-generation sequencing following the MethylRAD protocol (Wang et al 2015), which allows to investigate DNA cytosine methylations in non-model organisms. Furthermore, this chapter details ***in situ* and *in vitro* comparison** of nuclear methylomes from a **temperate** and a **High Arctic** population (RQ 2 b, c, Fig 15). Uni-parentally fertilized sporophytes of *S. latissima* from a temperate (Helgoland, 54 °N), and a High Arctic (Kongsfjorden, Spitsbergen, 79 °N) origin were cultivated at 3 different temperatures (5°, 10°, 15° C) from the zygote state onwards to prevent early life-stage effects on the methylomes. In parallel, wild samples were collected during spring 2019 from the same locations as cultivar origins.

Chapter 6: The **same parameters and settings** as described in Chapter 5 were assessed, but for the **chloroplast methylome** of *Saccharina latissima* (RQ 2 b + c, Fig. 15).
Assessing nuclear and chloroplast methylomes for the same parameters simultaneously in two populations across climate zones, with samples from both, wild and culture, was carried out for the first time in a primary producer.

Chapter 3 - Publication I

Arctic kelp eco-physiology during the Polar Night in the face of global warming: a crucial role for laminarin

Authors:

Lydia Scheschonk, Stefan Becker, Jan-Hendrik Hehemann, Nora Diehl, Ulf Karsten, Kai Bischof

Journal:

Marine Ecology Progress Series, Vol 611: 59-74 (published Feb 2019)

DOI:

10.3354/meps12860

Data archiving:

10.1594/PANGAEA.903529, 10.1594/PANGAEA.903524, 10.1594/PANGAEA.903526, 10.1594/PANGAEA.903525, 10.1594/PANGAEA.903133, 10.1594/PANGAEA.903836, 10.1594/PANGAEA.903523, 10.1594/PANGAEA.903131

See Appendix 11.5.1

Abstract:

Kelps, perennial brown seaweeds of the order Laminariales, are foundation species in Arctic coastal ecosystems. Presently, their ability to persist under polar night conditions might be significantly affected by increasing winter temperatures. We assessed physiological parame-ters (photosynthesis, pigment content, respiration, carbohydrate storage) in 2 species of Arctic kelp, the boreal-temperate *Saccharina latissima* and the Arctic-endemic *Laminaria solidungula*, during the polar night 2016/17. Algae were sampled from Kongsfjorden, Svalbard, shortly before the onset of the dark period in October, and at the end of the polar night in early February. Analyses were conducted for different tissue sections along the phylloid (meristem, centre region, distal region). Data suggest that kelp maintain their photosynthetic competence throughout the entire winter period, as indicated by photosynthesis vs. energy (PE) curve parameters and photosyn-thetic pigment contents. Overall laminarin content was reduced by 96% in *S. latissima* and by 90% in *L. solidungula* during winter, indicating that this storage glucan fuelled metabolic function during the polar night. Marked differences in laminarin content between the phylloid regions and across species indicated specific adaptive mechanisms between boreal-temperate and Arctic-endemic kelp. We suggest that laminarin turnover represents a sensitive parameter for assessing kelp physiology under a changing temperature regime.

Keywords:

Arctic, Laminarin, Climate Change, Life strategy, Kelps, Polar Night, Global warming

Chapter 4 - Publication II

Transcriptomic Responses to Darkness and the Survival Strategy of the Kelp *Saccharina latissima* in the Early Polar Night

Authors:

Huiru Li, **Lydia Scheschonk**, Sandra Heinrich, Klaus Valentin, Lars Harms, Gernot Glöckner[t], Erwan Corre, Kai Bischof

Journal:

Frontiers in Marine Science, Vol 7, Article 592033 (published Dec 2020)

DOI:

10.3389/fmars.2020.592033

Data Archiving:

NCBI SRA Bio Project PRJNA564197

see Appendix 11.5.2

Abstract:

Kelps in the Arctic region are facing challenging natural conditions. They experience over 120 days of darkness during the polar night surviving on storage compounds without conducting photosynthesis. Furthermore, the Arctic is experiencing continuous warming as a consequence of climate change. Such temperature increase may enhance the metabolic activity of kelps, using up storage compounds faster. As the survival strategy of kelps during darkness in the warming Arctic is poorly understood, we studied the physiological and transcriptomic responses of *Saccharina latissima*, one of the most common kelp species in the Arctic, after a 2-week dark exposure at two temperatures (0 and 4°C) versus the same temperatures under low light conditions. Growth rates were decreased in darkness but remained stable at two temperatures. Pigments had higher values in darkness and at 4°C. Darkness had a greater impact on the transcriptomic performance of *S. latissima* than increased temperature according to the high numbers of differentially expressed genes between dark and light treatments. Darkness generally repressed the expression of genes coding for glycolysis and metabolite biosynthesis, as well as some energy-demanding processes, such as synthesis of photosynthetic components and transporters. Moreover, increased temperature enhanced these repressions, while the expression of some genes encoding components of the lipid and laminaran catabolism, glyoxylate cycle and signaling were enhanced in darkness. Our study helps to understand the survival strategy of kelp in the early polar night and its potential resilience to the warming Arctic.

Keywords:

kelp, *Saccharina latissima*, growth rates, transcriptomic responses, dark exposure, Arctic

Chapter 5 - Publication III

Differences by Origin in Methylome Suggest Eco-phenotypes in the Kelp *Saccharina latissima*

Authors:

Lydia Scheschonk, Kai Bischof, Martina Elisabeth Luise Kopp, Alexander Jüterbock

Journal:

Evolutionary Applications, Vol 00, 1-17 (Vol 16, 262-278 print)

Special Issue: Marine Evolutionary Biology (published May 2022 online, 2023 print)

DOI:
10.1111/eva.13382

Data archiving:

NCBI SRA Bio Project PRJNA809008
10.6084/m9.figshare.19411460
10.6084/m9.figshare.19411574

see Appendix 11.5.3

Abstract:
Most kelp species are of high ecological and economic importance worldwide, but are highly susceptible to rising ocean temperatures due to their sessile lifestyle. Due to interference with reproduction, development and growth, natural kelp forests have vanished in multiple regions after extreme summer heat waves. Furthermore, increasing temperatures are likely to decrease biomass production and, thus, reduce production security of farmed kelp. Epigenetic variation, and cytosine methylation as a heritable epigenetic trait, is a rapid means of acclimation and adaptation to en-vironmental conditions, including temperature. While the first methylome of brown macroalgae has been recently described in the kelp *Saccharina japonica*, its functional relevance and contribution to environmental acclimation is currently unknown. The main objective of our study was to identify the importance of the methylome in the congener kelp species *Saccharina latissima* for temperature acclimation. Our study is the first to compare DNA methylation in kelp between wild populations of different latitudinal origin, and the first to investigate the effect of cultivation and rearing tem-perature on genome- wide cytosine methylation. Origin appears to determine many traits in kelp, but it is unknown to what extent the effects of thermal acclimation may be overruled by lab- related acclimation. Our results suggest that seaweed hatchery conditions have strong effects on the methylome and, thus, putatively on the epi-genetically controlled characteristics of young kelp sporophytes. However, culture origin could best explain epigenetic differences in our samples suggesting that epi-genetic mechanisms contribute to local adaptation of eco-phenotypes. Our study is a first step to understand whether DNA methylation marks (via their effect on gene regulation) may be used as biological regulators to enhance production security and kelp restoration success under rising temperatures, and highlights the importance to match hatchery conditions to origin.

Keywords:
aquaculture, DNA methylation, eco-phenotype, epigenetics, kelp, priming, temperature adaptation

Chapter 6 - Publication IV

Chloroplast DNA methylation in the kelp *Saccharina latissima* is determined by origin and influenced by cultivation conditions

Authors:

Lydia Scheschonk,* Anne Margrete Leiros Nilsen*, Kai Bischof, Alexander Jüterbock

*shared first author-ship

(submitted)

DOI:

10.1101/2022.12.02.518695

Data archiving:

NCBI SRA Bio Project PRJNA809008 see

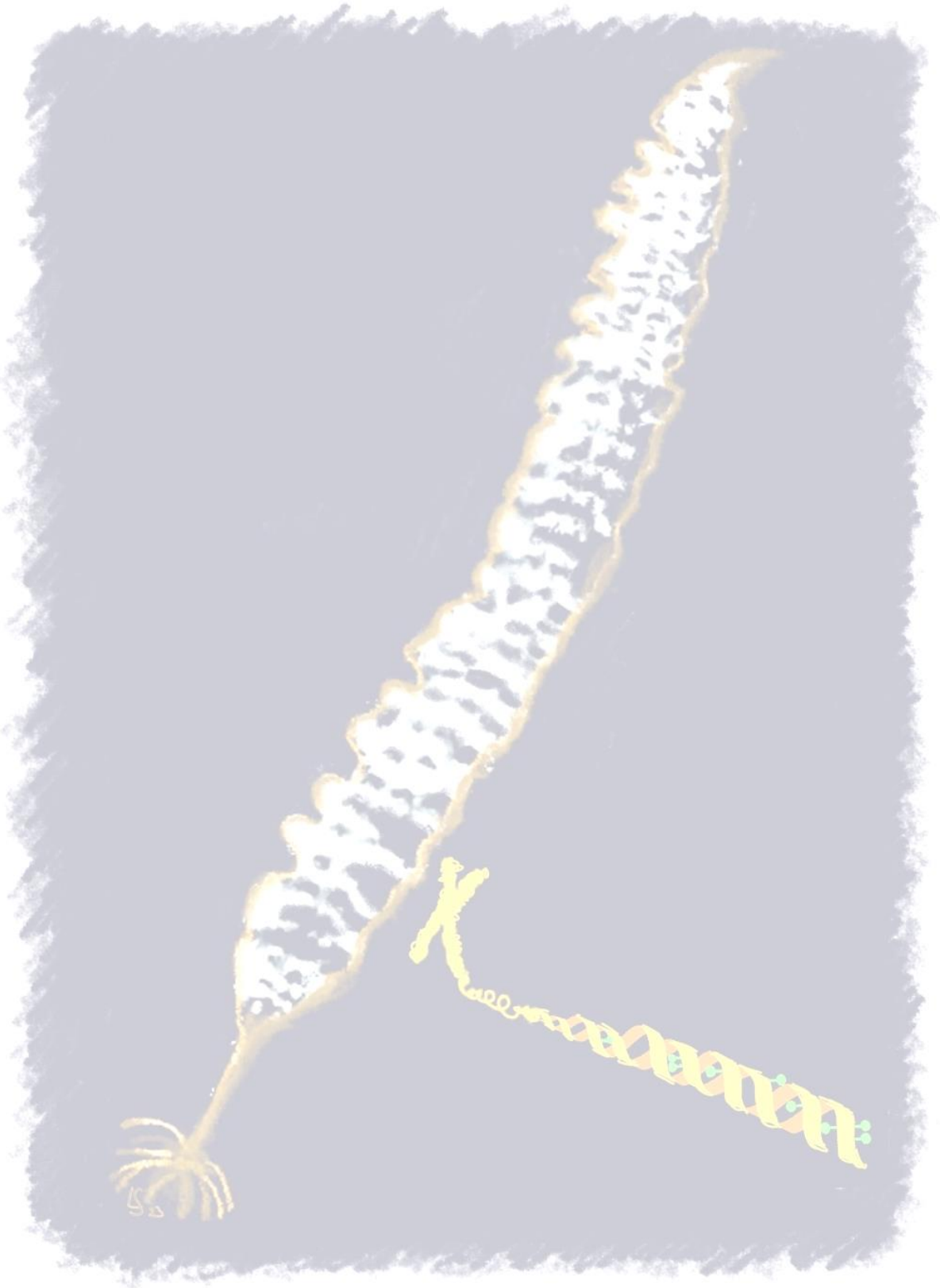
Appendix 11.5.4

Abstract:

DNA cytosine methylation is an important epigenetic mechanism in genomic DNA, but absent in the chloroplast DNA of most land plants. We detected methylation in the chloroplast DNA of the kelp *Saccharina latissima*, a non-model macroalgal species of high ecological (wild populations) and economic importance. Since the functional role of the chloroplast methylome is yet largely unknown, we compared for the first time the chloroplast DNA cytosine methylation between wild and cultured kelp from different climatic origins (High-Arctic (79 °N) and temperate (54 °N), laboratory samples at 5 °C, 10 °C and 15 °C). Our results suggest genome-wide differences in methylated sites, and methylation level, between the origins, and a strong effect of cultivation. Functions related to photosynthesis showed differential methylation only between origins. Significant differences in methylation between cultivated and wild samples of genes related to transcription and translation were unique to the high-arctic samples. Both findings indicate that origin and cultivation strongly, but differently affect the chloroplast methylome. Similar methylomes for samples from the same origin – independent from whether they grow in the wild or in the lab – suggest that origin-specific methylation marks on the chloroplast genome are inherited. Given that DNA-methylation affects gene expression, our study suggests that lab-cultivation alters epigenetically determined kelp chloroplast characteristics at least to the same degree as ecotypic differentiation does, but likely on a much shorter timescale. This indicates the capacity for rapid non-genetic adaptation in the kelp Saccharina latissima.

Keywords:

Chloroplast, organelle genome methylation, epigenetics, non-model organism, cultivation, aquaculture, marine macrophyte, marine algae



Chapter 7

Synoptic Discussion

7.1 Climate Change and Arctic Temperature Rise During Polar Night

Data obtained during this study showed the increased winter water temperatures to be one of the greatest threats compromising High Arctic kelp survival. The main findings in eco-physiological Polar Night parameters regarded the complete dependence of storage carbohydrate sufficiency on external temperature via respiration rate and metabolic rate, and the maintenance of pigments despite continuous darkness. Furthermore, early Polar Night transcriptomics showed a downregulation of photosynthetic components (Chapter 4), while simultaneously an upregulation of energy metabolism was observed.

As indicated by the results presented in Chapter 3, survival of marine primary producers in the High Arctic will strongly depend on the factor 'storage carbohydrates', and the species' respective efficiency in (dark) respiration. However, while transcriptomics showed rapid adaption to continuous darkness in *Saccharina latissima*, the severity of the reaction was enhanced by elevated temperature (4 °C, Chapter 4). The field data obtained during the Polar Night of 2016/17 with water temperatures of about 4 °C (see supplementary of Chapter 3) can be presumed as good proxy for future winter scenarios. The negative effect of elevated temperature during prolonged darkness compromising the survival of perennial kelp, as indicated by eco-physiology and transcriptomics (Chapters 3 + 4), has since been supported by results of physiological measurements in High Arctic origins during and after 4-months of artificial Polar Night (Gordillo et al. 2022).

According to results of Chapters 3 + 4, survival likely will mainly depend on available storage volume during the dark season, and tissue area still available for photosynthesis in spring and the light season. Blades of previous years significantly contribute to photosynthesis and internal storage, and thus carbon balance, for *S. latissima* in polar, but not in temperate water conditions. Perennial blade maintenance was observed for *S. latissima* in the High Arctic (Borum et al. 2002). However, severe carbon loss (40 – 50 %) due to tissue decay during autumn and winter has been reported for *S. latissima* from temperate regions in combination with a low amount of storage compounds found during both seasons (Johnston and Jones 1977). Even if a decay in blade tissue was solely dependent on temperature without major influence of the microbiome that likely is more active in warmer waters (see Q_{10}; Mikan et al. 2002), overall data shown in Chapters 3 and 4 suggest that with a decay of substantial parts of the blade neither laminarin nor pigments would likely be sufficient to secure survival during prolonged darkness. A decay of perennial blade tissue would initiate a negative spiral: the stronger the decay during Polar Night, the less tissue is available for photosynthesis in early spring. The less tissue available for photosynthesis, the lower the net primary production. The lower the mass gain via photosynthesis, the lower the internal storage compounds during winter. The lower the internal storage, the higher the decayed area. Any possibility of survival hence would depend on either sufficiently low winter temperatures, or sufficiently clear water conditions during the light season. To maximise the amount of storage carbohydrates, perennial primary producers strongly depend on light availability throughout the summer time, as storage carbohydrates can only be accumulated if net photosynthetic rate is positive (Kirk 2011). However, as a result from increased glacier runoff, internal storage build-up has been reported

to be impaired due to local decrease in light availability during summer (Niedzwiedz and Bischof 2023).

Another aspect of Climate Change impact on kelp is the connection between temperature, respiration, and photosynthesis. Elevated temperatures during Polar Night have been shown to significantly raise respiration rates above 'winter normal' (Gordillo et al 2022, own unpublished data). This rise in winter respiration is likely to impair, or imperil, survival in two ways. First, elevated respiration has a higher demand for energy, hence depletes reserves more quickly. The increase in energy demand could be observed by the upregulation of laminarin/lipid-associated metabolic processes during early polar night, as shown in Chapter 4. Second, the elevated respiration rate affects the whole photosynthetic process via its impact on the light compensation point (E_c, respiration rate equals rate of photosynthesis). E_c naturally is lower in polar than in temperate seaweeds (Lüning 1990) due to the direct physical, chemical and bio-chemical effects of low temperature. Still, values can be presumed to strongly vary by blade region and season (indirectly *via* O_2 consumption, Chapter 3). However, in response to rising water temperatures, an overall increase in E_c and, in consequence, possibly a decline in the photosynthetic efficiency (α) is to be expected. α, derived from the initial slope of the 'Photosynthesis *vs* Irradiance Curve' and hence, closely connected to E_c, has been shown to be strongly affected by elevated temperature, and ocean acidification in High Arctic kelp (Gordillo et al. 2016). Warmer waters take up less CO_2 from the atmosphere than cold waters. As higher concentrations of CO_2/HCO_3^- favour photosynthesis, while lower concentrations favour (photo-) respiration, the physical properties alone would already shift the equilibrium. Enzymatic processes of marine photosynthesis (e.g. Rubisco, external carbonic anhydrase (eCA)), as well as of respiration, in addition are dictated by temperature (link described via Q_{10}, see Chapter 11.4), adding another factor elevating E_c. Depending on the magnitude of this shift, an elevation of E_c could compromise build-up of internal storage during the light season, and in addition prolong the duration this storage would have to provide for metabolic energy demand, as a higher irradiance would be needed to surpass E_c in comparison to historical values. Even though light saturation (E_k) for growth is lower than for photosynthesis (Lüning 1990), a rise in E_c would likely force an upward shift in depth distribution in the respective species, which in turn would lead to a change in selective pressures via higher threat of desiccation during low tide (Lüning 1990), exposure to higher UV radiation during spring (Bischof et al. 2000), and ice scraping during cold winters (Lüning 1990).

Even though considered unlikely, a possibility for *S. latissima* (or High Arctic perennial photoautotrophs in general) to survive Polar Night in warmer winter waters to a minor degree might indirectly arise from the elevated temperatures. Due to temperature-induced lack of sea ice, moonlight might aid to relief some pressure off the internal storage, thus preventing excessive decay of blade tissue. Even though the intensity of moonlight certainly is too low to allow for primary production (Cohen et al 2020, Johnsen et al 2020), in combination with dark fixation it might still be enough to provide a sustained energy source via photosynthesis even when remaining below the light compensation point (see Subchapter 11.4). Sea ice free waters in winter are very clear remote of glacier/river discharge areas due to the absence of phytoplankton during Polar Night. The wavelength available from moonlight fits into the Chl *a* absorption spectrum (see Subchapter 11.4), and as shown in Chapter 3, pigments of photosynthesis/ photosynthetic capacity were maintained in *S. latissima* during Polar Night. Irradiance below E_c thus might aid to support metabolism by providing minimal steady baseline products of photosynthesis, which in turn would not have to be provided by internal storage, hence would allow to reduce drain from the internal storage by a fraction, which might be just enough to aid sustenance until the return of sunlight. However, to verify this presumption, PAR intensity of underwater moonlight would have to be monitored *in situ*, as until now, all data for High Arctic Polar Night are only derived from models (Cohen et al 2020, adapted from Cronin et al. 2016).

7.2 Ecotype - Eco-phenotype

Both regions of origin sampled in this study (Helgoland and Spitsbergen) are isolated from the 'continental' populations. This allows to compare them across latitudes, but simultaneously might mask bottle-neck effects caused by the constant isolation from new genetic 'input'. During the last glacial period, so-called *refugia* are hypothesised for the northern hemisphere (Assis et al. 2018), from which the current populations are presumed to have re-established their locations (Guzinski et al. 2020). The continental populations (Portugal to Norway) are presumed to descend from one *refugium*, while the Spitsbergen populations are presumed to stem from another *refugium* (Assis et al 2018, and therein). Hence, evolutionary processes have possibly manifested in the (epi-) genomes due to long periods of genetic isolation. Earlier studies on intraspecific variation in *S. latissima* offer phenotypic plasticity (Schlichting 1986) and local adaptation of genetically distinct ecotypes (Gerard 1988; Gerard and Du Bois 1988) as explanation for the broad differences observed e.g. in the habitus or biochemical composition of this species. The results presented in Chapters 5 and 6 (differential methylation regarding origin and cultivation) suggest that the expression of the phenotype from the blueprint of the genotype is strongly controlled by the epigenome. Phenotypic changes, e.g. due to changes in the methylome, can also give rise to genetic changes at a later stage through genetic assimilation, a process where phenotypic changes in response to the environmental factors result in new genotypes through selection (Schneider and Meyer 2017; Nishikawa and Kinjo 2018). In addition, hypomethylations are associated with higher rates of mutations (Chen et al 1998). This highlights the importance to include the epigenome into considerations regarding phenotypic plasticity/ variance, possibly as the crucial factor facilitating the expression of the genotype in accordance to local parameters. Neither of the terms 'ecotype' nor 'phenotype' fully acknowledge the important role of heritable epigenetic mechanisms for local adaptation. 'Eco-phenotype' (Chapter 5) was suggested as a term encompassing the combination and interaction of both genetic (DNA sequence-related) and epigenetic mechanisms that lead up to a specific "adaptation" of single individuals to their respective environment, which can be passed on to following generations, and typically prevails for at least one generation when this generation encounters factors differing from those of the parental generation. With this, the definition of 'adaptation' would deviate from the classical one ('heritable, but DNA sequence-based') towards 'heritable, but transcription-based', as it then explicitly includes epigenetic mechanisms influencing gene expression.

7.3 Season Anticipator vs Season Responder – Possibly Neither

For *Saccharina latissima*, the results of this study indicate traits of season response to light by switch of bio-chemical energy source (laminarin (internal storage) to mannitol (photosynthesis) in spring, mannitol to laminarin in autumn), but season anticipation in the pigments (year-round maintenance). Storage of large amounts of carbohydrates during the summer has already been known (Dunton and Schell 1986; Bartsch et al. 2008; Spurkland and Iken 2012).

Respiration in this context needs to be viewed uncoupled from season, which has been confirmed by further research (Gordillo et al 2022), as it was shown to be strongly linked to changes in temperature. Temperature as factor in turn used to be directly tied to season, but has started to uncouple due to anthropogenic global warming. Considering 'Polar Night' as synonym for 'dark season' or 'winter', respiration rate should not be counted as eco-physiological parameter linked to season, despite its tight link to all metabolic processes. Instead, the results of this study have shown that it should be taken into account when assessing a 'snap-shot' of impact of global warming on a given species, with simultaneous evaluation of directly related parameters like carbon source, or in case of primary producers, light compensation point. The inherent overall low light adaptation of most boreal-temperate kelp species (Kirst and Wiencke 1995; Raven et al. 2002; Wiencke et al. 2009; Huovinen and Gómez 2011) likely enabled them to inhabit polar regions (Wiencke et al. 2009). Following observations of different approaches to cope with the long period of darkness, the initial separation into 'season responder' and 'season anticipator' had been suggested for species of strongly isolated, temperature-constant Antarctica (Wiencke et al. 2009), and during recent decades had been adapted to seaweeds of the Northern Hemisphere (Spurkland and Iken 2012; Graiff et al. 2015). The initial reference had been made regarding *sensu* Kain (1989), and referred to a grouping according to growth and reproductive patterns (Wiencke et al 2009). However, the terms have become misleading. When referring to biochemistry and/or physiology, current data from recent studies, this one included, strongly indicate that *S. latissima* fits into neither category (Monteiro et al. 2019b; Diehl et al. 2021). Its physiology, bio-chemistry, and epigenetic shows characteristics of both, season anticipation (like maintenance of pigments during Polar Night, storage of huge amounts of carbohydrates anticipating winter) and season response (e.g. complete switch of metabolic energy source, from laminarin to mannitol, as soon as photosynthesis resumes). This does not seem to be a reaction to anthropogenic Climate Change, but inherent.

All this suggests grouping *Saccharina latissima* as neither season responder nor season anticipator, and indicates the inaccuracy of the grouping, or at least the need to indicate that that a grouping of *S.latissima* as season responder solely refers to it's growth and reproductive patterns (*sensu* Kain (1989)).

7.4 Kelp Epigenetics

Most epigenetic mechanisms have been studied on only very few model or agricultural species, typically under artificial conditions (Richards and Pigliucci 2020). This repeatedly has initiated a call to expand efforts into ecologically relevant approaches (Bossdorf et al. 2008; Johannes et al. 2008; Richards and Pigliucci 2020). This study is the first to apply eco - comparative epigenetics in seaweed.

A generally valuable finding is that the sum of epigenetic modifications in a species should only be assessed via testing for the respective mechanism in the species, or possibly a congener species (*S. japonica* in the case of *S. latissima*), but cannot *per se* be implied by findings from other genera within e.g. in the present case the group of *Phaeophyceae*. Within brown algae, there seem to be taxon-specific differences even regarding the types of epigenetic mechanisms, and absence or presence within one species does not imply its general absence or presence within *Phaeophyceae*. Histone modification has been observed in *Ectocarpus silculosus* (Bourdareau et al. 2021), while DNA cytosine methylation was found to be negligible (Cock et al. 2010), which had let to the assumption of DNA methylation to be negligible in brown algae in general. However, in the kelps *Saccharina latissima* and *Saccharina japonica* the occurrence of

methylation on a functional level has now been established, both for the nuclear and chloroplast genome (Chapter 5, Chapter 6; Liu et al. 2019; Fan et al. 2020; Teng et al. 2021; Yang et al. 2021). Despite not directly comparable due to different assays (see Chapters 5, 6), it can be inferred that both kelps feature a similar level in methylation, implying similar functions in the control of gene expression. For *Saccharina spp.,* current observations entail the impact of cytosine methylation on both life cycle stages at transcriptomic level (*S. japonica*; Liu et al. 2019; Fan et al. 2020a; Teng et al. 2021), and differences (possibly heritable traits) in cytosine methylation due to cultivation and latitudinal location observable on sporophyte stage (*S. latissima*; Chapter 5, 6). Cytosine methylation was shown to influence gene expression in both life cycle stages (Yang et al. 2021), with higher methylations found in the gametophyte than the sporophyte stage for both nuclear and chloroplast genome (Fan et al. 2020a; Teng et al. 2021). In both life cycle stages and genomes (nuclear and chloroplast), high levels of cytosine DNA methylation led to silencing of the respective DNA sequence, acting as additional control mechanism in gene expression (Fan et al. 2020a). On population level, the amount and location of DNA methylations were observed to differ between European Arctic and European temperate populations regardless of cultivation status (lab and wild; Chapter 5, 6). As DNA mutations have been found to more frequently appear at genome locations with high methylation levels (relative to un-methylated; Hare and Taylor 1989; Chen et al. 1998), the epigenetic mechanism of 'DNA cytosine methylation' seems to be important in adaptation processes as well as in reaction to rapid changes to the environment. Furthermore, some sequence regions only became methylated during the cultivation process in both origins, indicating reactions to changes in habitat within the sampled generation. This might be an explanation for the differences in gene expression observed between wild samples and cultivars in an earlier comparative study (Heinrich et al. 2016). However, the results obtained here only indicate patterns related to the sequence contexts CHG and CG, while the major part of DNA methylations in kelp (sequence context CHH; Fan et al. 2020; Teng et al. 2021) remains outside the scope of the present comparative epigenetics studies (Chapter 5, 6). Right now it remains unclear whether and how the CHH sequence contexts reacts to cultivation processes. It can only be presumed to be affected in a similar manner as the other two contexts, magnifying the epigenetic hold on gene expression.

Especially regarding food security, this study could show that temperature priming (Holeski et al. 2012) might be a valuable option in *Saccharina spp.* (see Chapter 5, Subchapter 11.1). In temperature priming, a method that is used for land plants, young individuals are exposed to temperature treatments that stimulate them to form an epigenetic stress memory that may be passed on to offspring. This stress memory can allow for a stronger and more rapid response to temperature at later life stages. Priming performed as, for example, cold temperature treatment of *Laminaria digitata* gametophytes at 5 °C increased the growth of the resulting sporophytes (Liesner et al. 2020b), at both extreme warm (20 °C) and extreme cold (0 °C) temperatures (Liesner 2020). The technique can be used in kelp aquaculture to produce crops with improved resilience to heatwaves, or for restoration purposes (see Subchapter 11.1).

Differences in kelp physiology and biochemistry have been observed between origins (Borum et al. 2002; Bartsch et al. 2008; Diehl et al. 2021), which are strongly reflected in the epigenetic signature (Chapter 5, 6). Even though differences on genome level have been reported between some of these origins (Assis et al. 2018, microsatellites, SNP), this study showed that epigenetic control likely has a major influence on phenotypes in kelp. As in plants, cytosine methylation in kelp appears to be involved in the regulation of metabolism and life-cycle (Fan et al. 2020a). Comparing the results from Chapters 5 to 6 (nuclear to chloroplast methylome), both methylomes showed significant differential methylation in regard to 'temperature', 'cultivation' in itself (which part of the process still needs to be determined), and 'origin'. The observation of 'cultivation impact on the methylome' highlighted the relevance of sample source for epigenetic analyses (wild vs cultivar). Methylome analyses in *S. japonica*, for example, have been solely made on cultivars. Deducing from the results of Chapters 5 + 6, it might lead to over-representation of epigenetic mechanisms in presumed functions of transcriptomic control. In combination with the observance of inbreeding depression in cultivars of *S. japonica* (Li et al. 2017), results of this dissertation highlight the need to assess in situ samples to investigate ecological or eco-evolutionary hypothesis, as the representativeness of cultivars might be impaired on the molecular level.

In contrast to the nuclear genome, the chloroplast genome has been shown to be only maternally inherited. In the unicellular fresh water green alga *Chlamydomonas reinhardtii*, DNA cytosine methylation only occurs in female gametes (mating type positive, mt^+), while the cells otherwise are morphologically indistinguishable (Nishiyama et al. 2002; Nishiyama et al. 2004). Cytosine methylation has been suggested to be the epigenetic mechanism that ensures reduction of the paternal and the endurance of the maternal DNA during the zygotic maturation (Nishimura et al. 1999; Nishimura et al. 2002). This results in the maternal chloroplast DNA being uniparentally inherited, hence the epigenetic mechanism of cytosine methylation seems to be crucial for chloroplast reproduction/ inheritance. Therefore, chloroplast genome inheritance is identical in uniparentally and bi-parentally fertilised individuals. This circumstance enables a direct comparison between the chloroplast methylomes of the cultured and wild samples (Chapter 6), reinforcing the observations made for the nuclear genome while simultaneously contradicting concerns regarding the differential methylation between cultivars and wild samples in the nuclear genome to be a result of inbreeding. Instead, it is likely that the difference between cultivation and wild origin can be assigned to the cultivation process, which stabilises the hypothesis that cultivation in itself has a major impact on the methylome of *S. latissima*.

Considering the results that indicated the important role of DNA methylation in epigenetic variation and the eco-evolutionary dynamics of *S. latissima*, the epigenetic mechanism of cytosine methylation likely will be key for processes of rapid adaptation to rising water temperatures during Polar Night.

7.5 Summary of Discussion

The major hypotheses addressed in this study entailed two interconnected, but distinct complexes focussed on the perennial marine photoautotroph *Saccharina latissima,* concerning its Polar Night eco-physiology (Hypothesis I), and comparative epigenetics (Hypothesis II). The combination of both complexes aids to gain a better understanding of the eco-evolutionary dynamics and likely adaptation capacity of *Saccharina latissima* to consequences of the current Climate Crisis.

Answer to RQ 1a)

> **'What are current eco-physiological parameters (storage compounds, respiration, pigments of photosynthesis) in the perennial marine primary producer *Saccharina latissima* (sugar kelp, season responder) and *Laminaria solidungula* (season anticipator) during the High Arctic Polar Night?'**

The investigated, current eco-physiological parameters showed that survival of the Polar Night is already a challenge, especially in *S. latissima* (Chapter 3). In *Saccharina latissima* and *Laminaria solidungula*, internal storage was virtually depleted towards the end of the Polar Night. Carbohydrate metabolism was shown to be the most crucial parameter regarding survival during the Polar Night in both kelp species, being assessed via the parameters 'total C', 'C/N', 'mannitol content', 'laminarin content'. Respiration, as a measure of the most prominent factor draining internal storage, as expected indicated differences between species, and between blade regions and season within species. The observed maintenance of photosynthetic capacity via sustenance of pigments in both kelps throughout Polar Night was another key finding of this study, as until now, only pigment disintegration (in red algae, Lüder et al. 2002) had been reported.

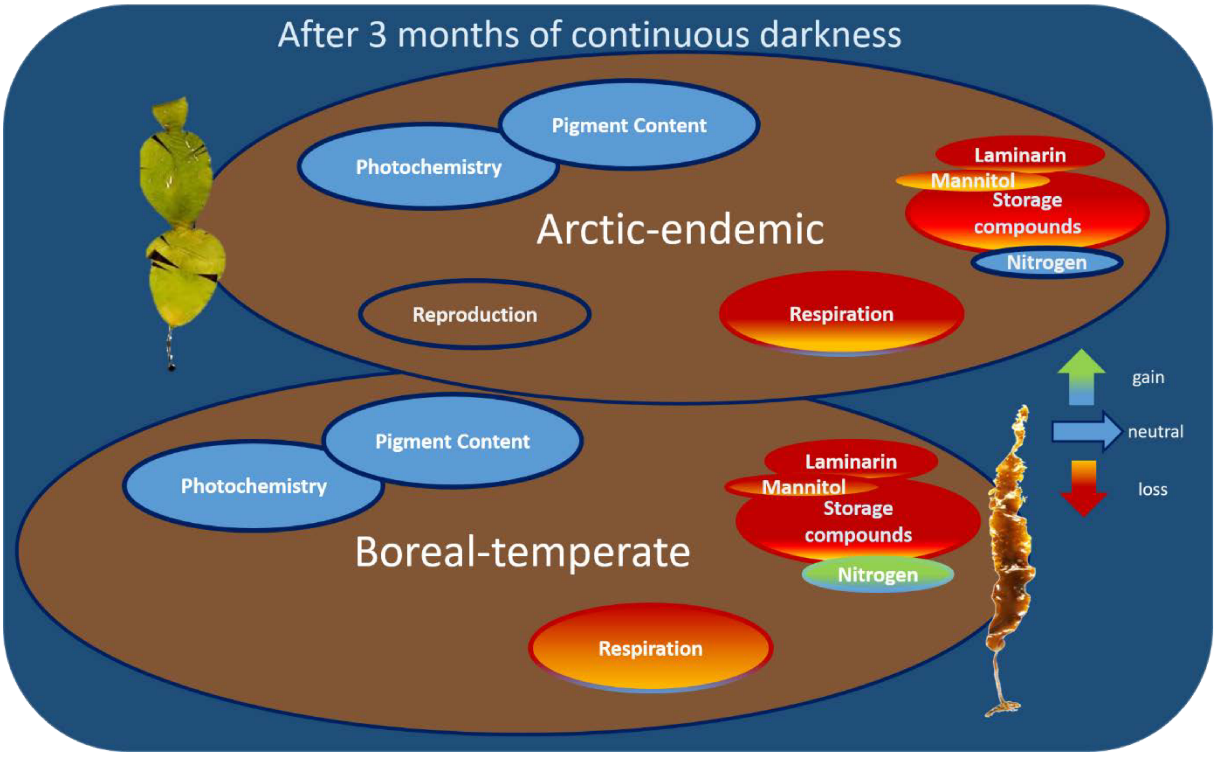


Figure 16: Visual summary of answer to RQ 1a. Differences in eco-physiological parameters observed between Arctic-endemic and Boreal-temperate kelp after 3 months of continuous darkness. Colours of the ellipses, as depicted by the arrow legend, indicate positive (green), neutral (light blue), and negative (yellow to red) effects. Gradients indicate degree of effect. Reproduction (blank) was added as a factor in Arctic-endemic due to its impact on respiration and storage compounds, but was not directly assessed. Picture *S. latissima*: S. Niedzwiedz. Picture *L. solidungula*: S. Saupe.

Answer to RQ 1b)

'How do presumed future temperatures (~ 4 °C – 8 °C) affect High Arctic *Saccharina latissima* during prolonged darkness?'

Temperature treatment during continuous darkness showed a devastating effect on the internal carbon reserves (Chapter 3). As winter water temperatures of 2016/17 remained at about 4 °C throughout the Polar Night, the obtained field data can be considered as a good proxy for future eco-physiological parameters. The transcriptomics presented in Chapter 4 clearly showed the strong temperature impact on gene regulation of presumed future (4°C) vs historical (0°C) winter water temperatures. Merging results from Chapters 3 and 4 indicated that temperatures above 4 °C have the potential to prove fatal during prolonged darkness.

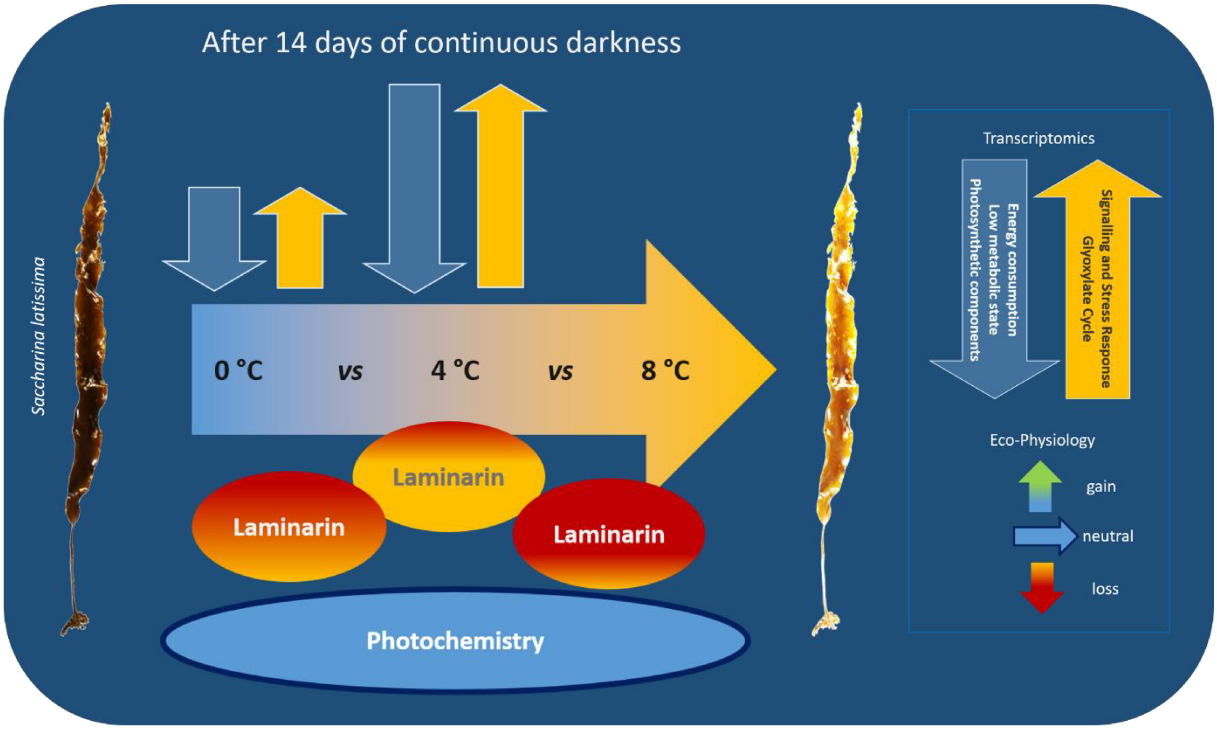


Figure 17: Visual summary of answer to RQ 1b. Reaction in gene regulation and eco-physiological parameters to different temperatures during the early Polar Night. Colours of the ellipses indicate neutral (light blue), and negative (yellow to red) effects. Gradients indicate degree of effect. Vertical arrows indicate observed upregulation (yellow) and downregulation (blue). Picture *S. latissima*: S. Niedzwiedz.

Answer to RQ 2a)

‘Is there a nuclear and/ or chloroplast methylome in *Saccharina latissima*?’

Methylomes of both, the nuclear and the chloroplast genome, of *S. latissima* were confirmed. Like in the congener *S. japonica* these DNA cytosine methylations can be presumed to play a vital role in the control of gene expression and modification.

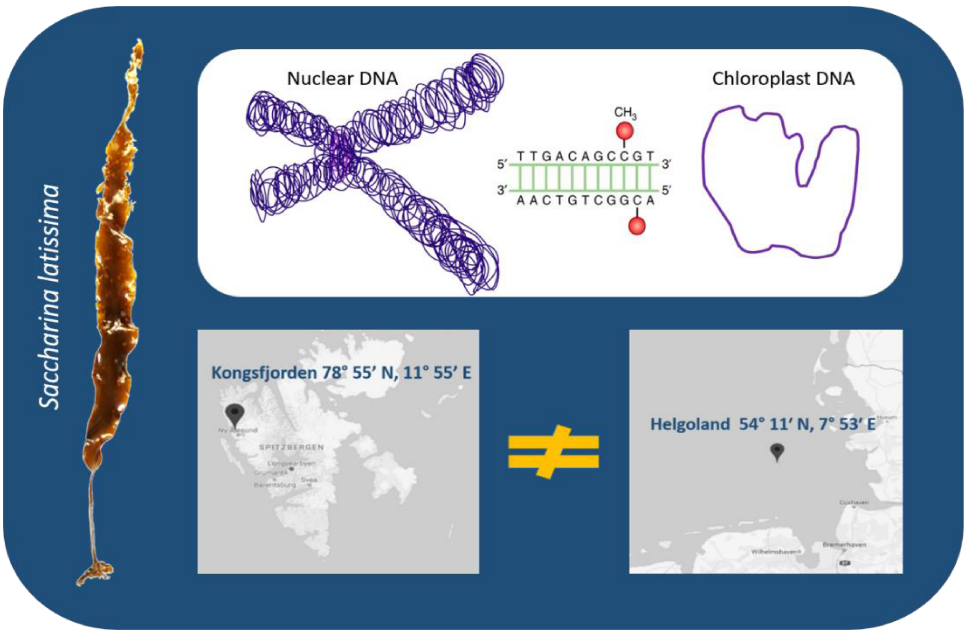


Figure 18: Visual summary of answer to RQ 2a. There is a functional DNA methylome in *Saccharina latissima* that differs between origins. Pictures: S. Niedzwiedz (*S. latissima*), Google Maps ©2023 (Helgoland, Spitzbergen), Moore et al 2013 (methylation on DNA strand).

Answer to RQ 2b)

‘Are there differences in the nuclear and/or chloroplast methylomes in response to identical abiotic factors between High Arctic and temperate origins of *Saccharina latissima*, indicating inheritance/ epigenetic memory?’

As presented in Chapters 5 and 6, this study found origin-specific signatures between the methylomes of both, chloroplast and nuclear, genomes in response to the identical abiotic factor ‘temperature’, indicating heritable traits despite the induced (externally initiated) nature of epigenetic mechanisms. Furthermore, cultivation temperature affected the origins similarly, but on different levels. Hence, DNA cytosine methylation seems to play an important role in epigenetic memory and rapid adaptation to major shifts in habitat conditions, as well as in within generation, short term temperature acclimation in sporophytes of *Saccharina latissima*.

Answer to RQ 2c)

'Are there within-origin differences in the nuclear and/or chloroplast methylomes between cultivars and wild populations, or within-origin differences between cultivars at different temperatures indicating signs of 'rapid adaptation'?'

In addition to the observed origin-specific signatures in response to cultivation temperature (RQ 2b), this study found chloroplast and nuclear methylomes to significantly be influenced by ambient temperature, as well as by the process of cultivation (significant within-origin differences between cultivation temperatures, and between cultivars and wild samples). This again highlights the heritable character of DNA cytosine methylations, as the comparative eco-epigenetic data presented in Chapters 5 and 6 indicate that ambient temperature (that is, the temperature that individuals were cultivated in from hatching to sampling) elicits an epigenetic response that is directly linked to origin.

Even though laboratory conditions were presumed to provide a 'non-threatening' environment due to the absence of stressors, and temperatures well below stress levels, the observed significant within-generation differences within the origins suggest that the epigenetic mechanism of DNA cytosine methylation plays a major role in rapid adaptation.

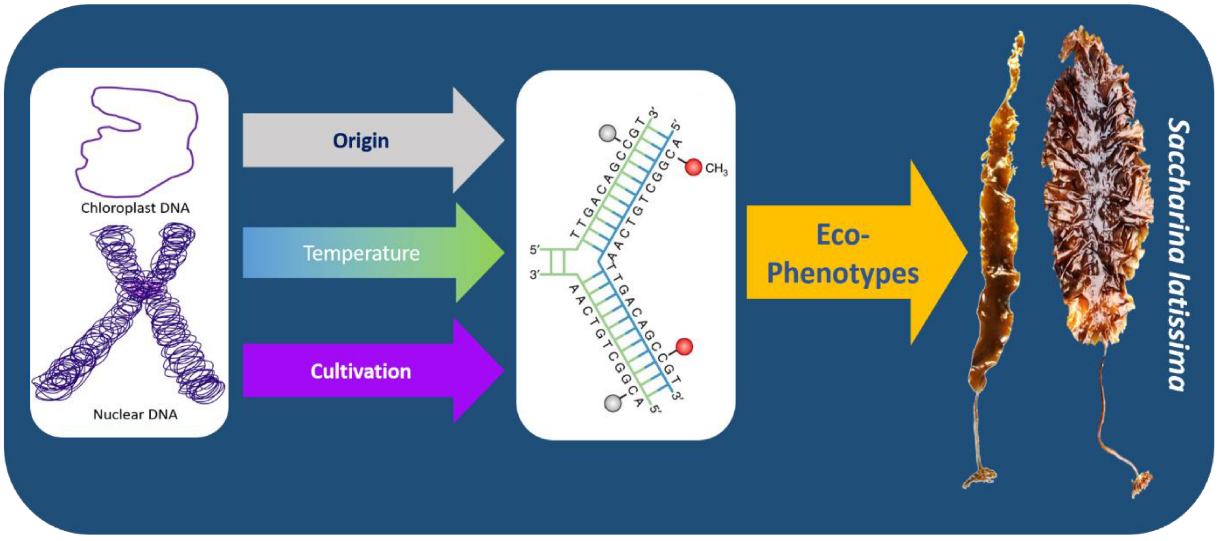


Figure 19: Visual summary of answer to RQs 2b, c. In nuclear and chloroplast DNA, methylations were affected by origin, Temperature priming, and the process of cultivation. This indicates that DNA methylation, as part of the eco-evolutionary dynamics in *Saccharina latissima*, influences the formation of eco-phenotypes. Graph of DNA methylation adapted from Moore et al 2013. Picture of *S. latissima*: S. Niedzwiedz

Answer to Hypothesis I

'Rising winter water temperatures due to global warming impair or compromise the capacity for survival in High Arctic kelp':

Results presented in Chapters 3 and 4 clearly showed a strong impact by warmer winter waters that is likely to impair or compromise sustenance, especially in a holistic approach considering changes and impacts during all seasons. During the time of light absence or severe light limitation, winter water temperatures above pre-industrial levels were shown to pose a challenge to this species' thermal tolerance due to the impact on the internal storage.

Answer to Hypothesis II

'*Saccharina latissima* has a heritable origin-specific methylome that reacts to divergence from origin-specific temperature as a means of rapid adaptation':

The nuclear and chloroplast methylomes of *Saccharina latissima* could be shown to be a likely means of rapid adaptation in response to changes in ambient temperature. Furthermore, an epigenetic memory related to origin was found in this kelp in response to divergence from temperature. However, this response was not elicited due to divergence from origin-specific temperature, but an origin-specific response to temperature was observed. The observed transgenerational epigenetic memory regarding local temperature indicated a strong capacity towards temperature priming, while the methylomes' reactions to cultivation showed within-generation rapid adaptation. DNA cytosine methylation thus was shown to be part of the eco-evolutionary dynamics in *Saccharina latissima*, and a valuable mechanism of rapid adaptation in kelp.

Conclusion:

Both hypotheses could be supported.

7.6 Ecological Implications

Sessile organisms such as kelp can only react to environmental changes in four ways: move, adapt, cope, or die (Gienapp et al. 2008). Due to their perennial life expectation but short dispersal phases and distances, High Arctic kelp are especially vulnerable to rapid changes of external conditions. Furthermore, High Arctic kelp grow slower than at more southern shores, but become larger and presumably older, and it remains unclear how the long generation time might impact their observed capacity for rapid local adaptation. High Arctic populations of *Saccharina latissima* do not face the direct threat of heat waves like populations at most more southern latitudes do. Current High Arctic Polar Night water temperatures are well within the species' general optimum to pejus temperature (Fortes and Lüning 1980; Bolton and Lüning 1982; Davison and Davison 1987). Still, results presented during this dissertation showed that these temperatures are only harmless during the light season, and might have devastating effects for the Arctic region during Polar Night. In reaction to the rapid changes in the temperature regime due to Climate Change, species already established in the Arctic can only shift to recently (newly) ice-free regions, while simultaneously facing more competition from Arctic or Sub-Arctic neobiota. As Kelp forests define their ecosystem, factors that influence or compromise a kelp species' survival have direct consequences on the whole ecosystem, and in addition, on any community or economy that directly or indirectly might benefit from the ecosystem. In the High Arctic, this entails highly specialised indigenous peoples, coastal communities, as well as (possibly foreign) fisheries industry, or even tourism.

Chapter 8

Conclusion

Knowledge acquisition in this dissertation concerned the importance of assessment of eco-physiological parameters during all seasons especially in Polar regions, as well as the key role of epigenetic mechanisms in eco-evolutionary processes in the kelp *Saccharina latissima*. DNA methylation could be shown to likely play a significant role in the formation of an origin-specific phenotype in kelp, while maintaining their capacity for within-generation adaptation. Especially for rocky shore restoration attempts, as well as cultivar selection in aquaculture, this gain in knowledge will likely prove helpful. For restoration efforts, it highlights the potential that arises from matching the origin of the seedlings to the abiotic conditions of the respective location. In areas where restoration is e.g. necessary after die-offs, origins more tolerant regarding the factor that led to the die-off might prove to be a solution for successful restoration of the ecosystem. While this may certainly lead to unnatural gene flow, in some places it could simultaneously be the most pragmatic and easiest solution to ensure fast recovery of an ecosystem without introducing neobiota. Without introducing unnatural gene flow, another improvement in restoration success can arise from the finding that temperature priming might be a suitable approach to harden the seedlings to a respective factor that is expected to be the most challenging at a given location. The gain in knowledge regarding the capacity for temperature priming is not only of relevance for restoration, but probably even more interesting for aquaculture. It indicates further improvement capacities for yield, but also improved resilience towards global warming in cultivars. For aquaculture, the discovery of an origin-specific epigenetic memory in addition highlights the capacity to optimise yield via strain selection relative to intended location of cultivation.

Regarding assessments to determine the capacity of survival in future temperature conditions, data from earlier late summer / autumn and spring measurements were found insufficient for extrapolation of implications for the season of Polar Night. This holds true for physiological as well as epigenetic experiments/ assessments. In addition, the epigenetic data, partly in combination with earlier transcriptomic assessments (Heinrich et al. 2016), have shown that molecular aspects are likely not adequately represented when assessed solely in kelp cultivars. Hence, this study highlights the importance to assess year-round parameters *in situ* to gain truly representative data on eco-evolutionary aspects for one of the key foundation species of High Arctic kelp ecosystems.

Results obtained during this study provide a solid interdisciplinary data complex on two topics where data have been severely lacking for (High Arctic) kelp. They have already been incorporated into a scientific book (Chapter 3; Berge et al 2020), and have laid the foundation for an entirely new branch of research in kelp (priming). In conlcusion, this dissertation contributes important knowledge gains for a more holistic understanding of (High Arctic) eco-evolutionary processes during the ongoing biodiversity crisis in the wake of this Climate Crisis, and a possible counteraction method to mitigate impacts on marine ecosystems or food resources.

Chapter 9

Perspectives

Given that High Arctic populations of *Saccharina latissima* already show a strong adaptation to the polar environment compared to more southern locations, it is likely that the expanding-edge populations will be capable to survive the Polar Night in warmer waters. However, as populations from Svalbard were found to be genetically distinct from mainland Norway populations (arctic *refugia*, Assis et al. 2018), the adaptation to the High Arctic environment might only be the case in the island populations, and not High Arctic populations in general. A resumption of this study (epigenetics and eco-physiology) should be carried out on circumpolar High Arctic populations, in addition to SNP / microsatellite assessments. Furthermore, kelp winter survival will only be possible with gain of sufficient reserves during summer. This, however, can become a problem in some Arctic regions due to summer water turbidity impacting metabolism (Niedzwiedz and Bischof 2023). Directly interconnected with this is the necessity to further assess the dependence of light compensation points on ambient temperature, as this is likely to be a crucial factor for High Arctic primary production. Hence, it will not be sufficient to assess either season separately. Worst case scenarios will only adequately be reflected as emerging properties of data collected across all seasons.

Especially in the face of the soaring changes in coastal ecosystems due to the anthropogenic Climate Crisis, further knowledge about epigenetic mechanisms is crucial, as these hold a key to rapid local adaptation, and hence to the capacity for species survival and ecosystem stability. This work was the first attempt to understand DNA methylation in *Saccharina latissima*, and the first to apply comparative epigenetics in an ecological context. Differences in both methylomes between origins likely are a consequence of respective habitat. Due to these contrasting methylomes within a single species, it will be of high importance to further explore the role of genome methylation, and epigenetic mechanisms in general, in kelp and marine primary producers. Only very few epigenetic markers have been found that do not get transmitted to F1 and F2 generations in plants and hence, likely algae (Anastasiadi et al. 2021). Consequently, it can be presumed that the inheritable epigenetic mechanism of DNA methylation will likely play an important role in the eco-evolutionary dynamics of *S. latissima* in reaction to the ongoing Climate Crisis. Further, it will likely be integral to help secure stability in farming processes in the economically valued species. Methylomes of 'fresh' wild spores exposed to warm winter water temperature during long periods of light absence would indicate feasibility of priming in temperate and High Arctic (Spitsbergen and Mainland) populations. But even in species that are not economically exploited, their crucial role in costal rocky shore ecosystems indicates the necessity to critically assess the role of epigenetic inheritance for conservation purposes. In the studies presented in the framework of this dissertation, transcriptomics could not be combined with epigenetic analysis, hence the regulation of gene expression via methylation level at the exact loci that showed differences in transcription is still hypothetical. Initially, the experimental setup was designed to assess whether there were methylations in *Saccharina latissima* at all, and whether those might differ between different latitudinal origins in the same setting. However, any presumption on priming processes right now are based on the observed differences between the origins (in both wild and cultivated samples), but have not been experimentally confirmed by e.g. examination of consecutive

generations of different origins in cultivation, by assessing both life cycle stages, or by reverse genetics (e.g. CRISPR). Still, in contrast to invasive and controversial methods (CRISPR), utilising epigenetic, fast adaptive mechanisms like priming offers a strong possibility for aquaculture and restoration/ conservation. Furthermore, the MethylRAD protocol (Chapters 5 + 6) does not account for the majority of the within generation methylations, as it does not detect the transgenerationally instable sequence context CHH (Richards et al. 2017; Boquete et al. 2021), which has been found to be the predominant form of DNA methylation in kelp (Fan et al. 2020a; Teng et al. 2021). Hence, the results of this study can only deduct implications for within generation processes from the modulations observed for the CG and CHG contexts.

The predominant approach concerning carbon capture and storage (to draw already emitted CO_2 out of the atmosphere) implies innovation of new technologies (Figueres et al. 2017; Rockström et al. 2017; Rogelj et al. 2018). Regarding the storage of greenhouse gas CO_2, the easiest, least dangerous, currently fastest, and cheapest way would be to boost primary production (Bastin et al. 2019b). The greening of the Arctic (Winkler et al. 2019) is a good proxy for the potential of this mitigation pathway. Despite the feasibility to achieve a substantial drop in CO_2 concentrations by relying on terrestrial plants, the main concern here regards its interference with human land use, despite sufficient areas being available for tree restoration excluding existing trees, urban areas, and agricultural areas (Bastin et al 2019a). The carbon capture capacity of global primary producers can further be boosted by enhancing, and strongly expanding marine aquaculture (mariculture, e.g. offshore) featuring fast growing macroalgae. Suggestions on suitable structures already exist to set the plant-based mitigation opportunity into action (Jansen et al. 2016; Buck et al. 2017; Fredriksen et al. 2020). In combination with epigenetic knowledge such as how to utilise priming (Chapter 11.1) to enhance resilience, this might be one of the keys to achieve the necessary CO_2 draw down. Even though the contribution of kelp to natural carbon sequestration is still debated, artificial sequestration, e.g. via forced/ aided sinking below the carbon re-cycling zone in deep sea areas could be an option. Another alternative is likely to be found in the research aiming to convert kelp biomass into construction materials, effectively disrupting natural (short term) decomposition.

Chapter 10

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Chapter 11

Appendix

QR Codes generated at qr1.at

11.1 Publication V

'Priming of Marine Macrophytes for Enhanced Restoration Success and Food Security in Future Oceans'

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Marine macrophytes, including seagrasses and macroalgae, form the basis of diverse and productive coastal ecosystems that deliver important ecosystem services. Moreover, western countries increasingly recognize macroalgae, traditionally cultivated in Asia, as targets for a new bio-economy that can be both economically profitable and environmentally sustainable. However, seagrass meadows and macroalgal forests are threatened by a variety of anthropogenic stressors. Most notably, rising temperatures and marine heatwaves are already devastating these ecosystems around the globe, and are likely to compromise profitability and production security of macroalgal farming in the near future. Recent studies show that seagrass and macroalgae can become less susceptible to heat events once they have been primed with heat stress. Priming is a common technique in crop agriculture in which plants acquire a stress memory that enhances performance under a second stress exposure. Molecular mechanisms underlying thermal priming are likely to include epigenetic mechanisms that switch state and permanently trigger stress-preventive genes after the first stress exposure. Priming may have considerable potential for both ecosystem restoration and macroalgae farming to immediately improve performance and stress resistance and, thus, to enhance restoration success and production security under environmental challenges. However, priming methodology cannot be simply transferred from terrestrial crops to marine macrophytes. We present first insights into the formation of stress memories in both seagrasses and macroalgae, and research gaps that need to be filled before priming can be established as new bio-engineering technique in these ecologically and economically important marine primary producers.

Keywords: DNA methylation, plasticity, stress memory, bio-engineering, seagrass, macroalgae farming, kelp restoration, heat hardening

INTRODUCTION

Marine macrophytes, including seagrasses and macroalgae, form the foundational basis of some of the most productive and diverse coastal marine ecosystems on the planet (Larkum et al., 2006; Costanza et al., 2014; Klinger, 2015; Teagle et al., 2017) that provide ecosystem services worth US$ 28.9 ha^{-1} $year^{-1}$ (Costanza et al., 2014). Moreover, macroalgae, traditionally cultivated in Asia (Chopin, 2017; Hu et al., 2021) at an annual value of US$ 13.3 billion (FAO, 2020), are increasingly recognized in Europe and America as a target for a new, highly profitable, and environmentally sustainable bioeconomy (Skjermo et al., 2014; Stévant et al., 2017; Grebe et al., 2019; Araújo et al., 2021).

Marine macrophytes are increasingly threatened by a variety of anthropogenic stressors, including coastal development, invasive species, agricultural run-offs, dredging, aquaculture, and rising sea levels (Orth et al., 2006; Krumhansl et al., 2016; Chefaoui et al., 2018; Filbee-Dexter and Wernberg, 2018). Nearly one-third of global seagrass areas have disappeared over the last 100 years (Waycott et al., 2009) and 60% of macroalgal forests have been in decline over the past 2–5 decades (Wernberg et al., 2019).

Above all, temperature is the most important range-limiting factor for marine macrophytes (Jueterbock et al., 2013; Repolho et al., 2017; Assis et al., 2018; Duarte et al., 2018; Martínez et al., 2018). Rising ocean temperatures, interfering with reproduction, development, and growth (Breeman, 1990; Short and Neckles, 1999), are fundamentally altering genetic diversity and adaptability (Coleman et al., 2020; Gurgel et al., 2020), and devastating macroalgal forests and seagrass meadows around the globe (Arias-Ortiz et al., 2018; Filbee-Dexter et al., 2020; Smale, 2020). In response, large-scale restoration efforts aim to avert severe ecological and economic consequences (Eger et al., 2020; Fredriksen et al., 2020; Layton et al., 2020; Tan et al., 2020; Vergés et al., 2020). Modeling studies, based on projected carbon emission scenarios, predict that poleward range shifts will intensify (Jueterbock et al., 2013; Valle et al., 2014; Assis et al., 2016, 2017; Chefaoui et al., 2018; Wilson and Lotze, 2019). Even if rising sea temperatures remain below lethal limits, they reduce macroalgal growth and performance (Nepper-Davidsen et al., 2019; Hereward et al., 2020; Smale et al., 2020), increase disease outbreaks and biofouling (Harley et al., 2012; Nepper-Davidsen et al., 2019; Qiu et al., 2019; Smale et al., 2020), and radically alter ecological interactions that determine persistence (Provost et al., 2017; Vergés et al., 2019)–thus compromising future sustainability of natural habitats, and production security of associated industries.

PRIMING POTENTIAL IN MARINE MACROPHYTES

Priming, a Common Technique for Crop Enhancement

In agriculture, priming (**Box 1**) is a commonly employed technique to enhance crop resistance to environmental challenges, including pathogen infections, hot, cold, dry, or saline conditions (Ibrahim, 2016; Pawar and Laware, 2018; Wojtyla et al., 2020); in some cases even across generations (transgenerational priming, **Box 1**) (Herman and Sultan, 2011; Lämke and Bäurle, 2017; Benson et al., 2020). For example, reproductive output of F3 *Arabidopsis* progeny increased five-fold under heat stress (30°C) if the F0 and F1 generations had previously experienced the same stress (Whittle et al., 2009). Seed priming also synchronizes germination and improves vigor, leading to improved crop establishment and yield (Pawar and Laware, 2018). Priming is now considered a promising strategy for crop production in response to future climate (Wang et al., 2017; Mercé et al., 2020), and may have large potential to alleviate negative climate change impacts on marine macrophytes as well as to enhance yield in macroalgae production.

Mechanisms Underlying Priming

Priming relies on the formation of a molecular stress memory (**Box 1**), a process that can include epigenetic mechanisms such as microRNAs (miRNAs), histone modifications, and DNA methylation (Iwasaki and Paszkowski, 2014; Balmer et al., 2015; Crisp et al., 2016; Hilker et al., 2016; Wojtyla et al., 2016; Gallusci et al., 2017; Lämke and Bäurle, 2017; Bäurle, 2018; **Figure 2**). Epigenetic mechanisms do not alter the DNA sequence but have the potential to change gene expression (Bossdorf et al., 2008). Stress memory based on non-coding RNA and histone modifications generally lasts no longer than several hours or days (Mathieu et al., 2007; Cedar and Bergman, 2009; Lämke and Bäurle, 2017; Kumar, 2018), with some exceptions (Huang et al., 2013; Bilichak et al., 2015; Morgado et al., 2017). In contrast, DNA methylation is more stable, and can even be heritable across generations (Boyko et al., 2010; Ou et al., 2012; Verhoeven and van Gurp, 2012; Bilichak and Kovalchuk, 2016; González et al., 2017). For example, mediation of transgenerational priming via inherited DNA methylation has been demonstrated in the plant *Polygonum persicaria*, in which demethylation of offspring with zebularine removed the adaptive effect of parental drought exposure in the form of longer root systems and greater biomass (Herman and Sultan, 2016).

Indications of Priming in Macrophytes

Recent studies show that seagrass can become less susceptible to heat events if it has been primed to stressful temperatures (**Figure 1**). For example, primed individuals (6 days at 29°C,

BOX 1 | Glossary of priming-related terms.

Priming–A plant's ability to acquire a stress memory, enhancing its performance when exposed to a second stress by allowing it to respond faster, stronger, or in response to a lower threshold compared to a naïve plant (**Figure 1A**). Priming is often used synonymously with hardening, conditioning, or acclimation.

Stress memory–A stress-induced alteration in epigenetic state that may last under mitotic cell divisions and results in priming.

Transgenerational priming–Stability of a stress memory under meiotic cell divisions across at least one generation that benefits the progeny of primed parental plants.

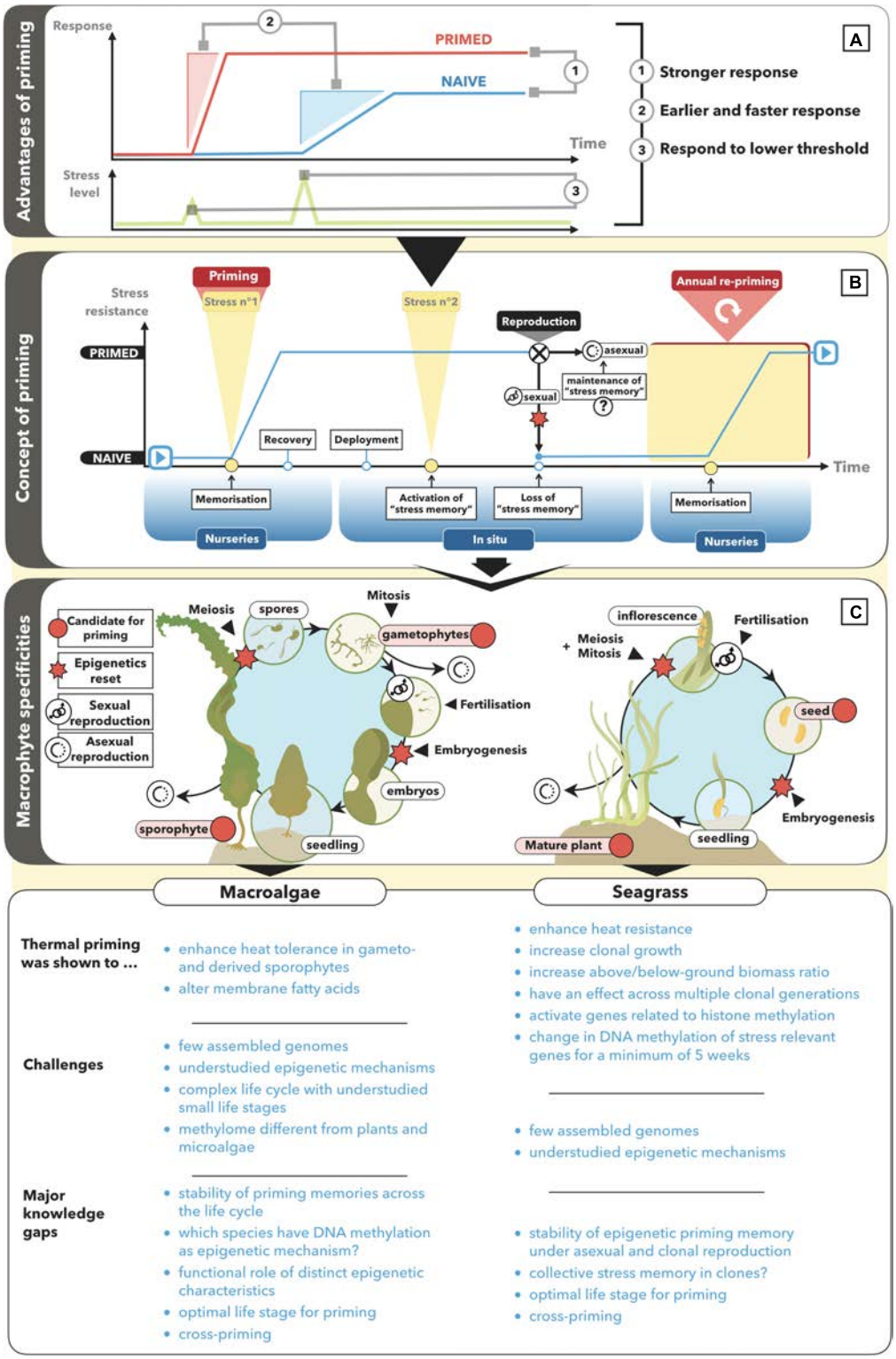

FIGURE 1 | Concept of priming and potential for its application in macroalgae and seagrass. **(A)** A primed organism responds faster, earlier, stronger or to a lower threshold of a stressful triggering stimulus as compared with a naïve organism. **(B)** A naïve organism is primed by building up a memory of a certain stress stimulus. The stress memory is more likely to be heritable across asexually produced generations than across sexually produced generations because of epigenetic reprogramming under gametogenesis (meiosis) and embryogenesis. **(C)** At which stage priming is best applied depends on the stability/transfer of a priming memory across the life cycle stages of kelp and seagrass. The state of the art, challenges and knowledge gaps to establish priming as a novel bio-engineering technique in marine macrophytes are listed for macroalgae and seagrass, respectively.

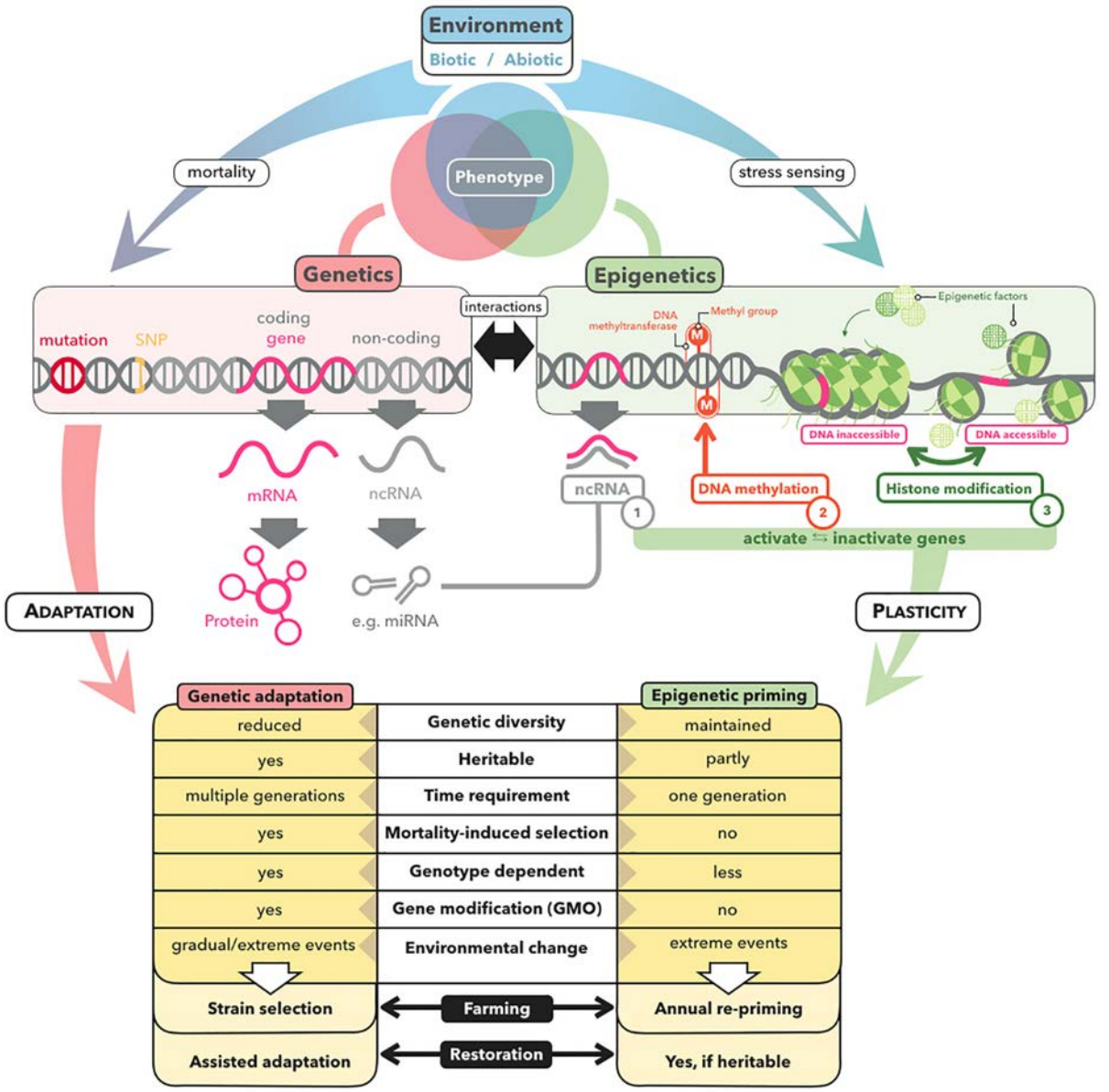

FIGURE 2 | Genetic versus epigenetic mechanisms underlying stress adaptation and their relevance for restoration and farming of marine macrophytes. Environmental stress can alter the phenotype of marine macrophytes by positive selection of pre-adapted genotypes or of new beneficial mutations, resulting in genetic adaptation of the population within several generations. In contrast, epigenetic mechanisms, comprising ncRNAs, DNA methylation, and histone modifications, contribute to phenotypic plasticity by altering the expression patterns of genes within a single generation. The key characteristics with respect to the application potential of genetic and epigenetic mechanisms for farming and restoration of marine macrophytes, are listed at the bottom.

4°C above natural conditions) of the seagrass species *Zostera muelleri* and *Posidonia australis* showed significantly enhanced photosynthetic capacity, leaf growth, and chlorophyll *a* content after exposure to heat stress (32°C for 9 days) compared with naïve plants (Nguyen et al., 2020). Moreover, previous exposure of *Zostera marina* to simulated warming (15°C for 45 days, 2°C above control temperature) resulted in an increase in clonal shoot production and shoot length, as well as a decrease in leaf growth rates and in the ratio of below to above ground biomass (DuBois et al., 2020). Vegetatively grown shoots of primed parental plants could maintain biomass production under a second warming event (ca. 16°C for 40 days) but not shoots of naïve parental plants. The changes, which lasted for several years across multiple clonal generations after the stress was removed, would likely be adaptive in a warmer environment by reducing the respiratory burden of non-photosynthetic tissues. As discussed in Nguyen et al. (2020), heat priming may explain why the Mediterranean seagrass *Posidonia oceanica* did not suffer high mortality rates after intense and long-lasting heat-waves in 2012, 2015, and 2017 (Darmaraki et al., 2019),

in contrast to an extensive die-off after the 2006 heatwave (Marbà and Duarte, 2010).

Evidence that epigenetic modifications contribute to form a thermal stress memory in seagrass is suggested by significant stress-induced regulation of methylation-related genes, in particular histone methyltransferases (Nguyen et al., 2020), and a change in DNA-methylation patterns that lasted for at least 5 weeks following exposure to heat stress (Jueterbock et al., 2020). A 5-week heat-stress memory is potentially long enough to heat-harden the same generation of previously exposed shoots. This methylation memory involved CG hyper-methylation and, thus, potentially constitutive upregulation (Zhang et al., 2006; Yang et al., 2014; Dubin et al., 2015; Niederhuth and Schmitz, 2017) of genes involved in the breakdown of heat-denatured proteins (Feder and Hofmann, 2002); which would be expected to provide a faster or stronger protective response upon exposure to a second heat stress.

In fucoid macroalgae, priming was shown to enhance resistance to dry and cold conditions (Schonbeck and Norton, 1979; Collén and Davison, 2001). Moreover, in the kelp *Laminaria digitata*, gametophyte exposure to low temperatures (5°C versus 15°C) significantly enhanced growth of the derived sporophytes under benign conditions (5 and 15°C) (Liesner et al., 2020). A small number of studies suggest that macroalgal performance under heat stress may be bio-engineered by thermal priming (**Figure 1**). First, priming the gametophyte generation of the kelp *Alaria esculenta* for 3 days at 22°C (compared with 12°C) enhanced their survival under increased temperatures, and the growth of the derived sporophyte generation (Quigley et al., 2018). Second, cultivation of *Saccharina japonica* gametophytes at 22–24°C increased the heat-tolerance of the derived sporophytes by 2°C (Wu and Pang, 1998) in Bricknell et al. (2021). Third, in the fucoid brown alga *Fucus vesiculosus,* storage of parental tissue at a higher temperature (14°C versus 4°C), or acclimation of embryos to 29°C significantly increased their survival by 30–50% under 33°C (Li and Brawley, 2004). Fourth, individuals of the red alga *Bangia fuscopurpurea* primed for 3 days at 28°C could survive 1 week at 32°C significantly better than naïve individuals (Kishimoto et al., 2019). The priming stress caused an increase in the saturation level of membrane fatty acids, suggesting that altered membrane fluidity is part of the species' heat stress memory. However, this memory lasted for only 5 days after the primed individuals were returned to benign conditions (15°C).

DISCUSSION – PROSPECTS AND CHALLENGES OF PRIMING IN MARINE MACROPHYTES

Distinguishing Priming From Selection

Just as thermal stress in natural settings can cause mortality and selection (Coleman and Wernberg, 2020; Coleman et al., 2020; Gurgel et al., 2020), priming induced mortality could inadvertently result in selection of pre-adapted genotypes–which may explain the observed transfer of positive effects from the primed gametophyte to the derived sporophyte generation of kelp (Quigley et al., 2018; Liesner et al., 2020). In order not to falsely ascribe improved stress tolerance to the formation of a molecular stress memory, it is critical to distinguish between priming and selection. This could be achieved through establishing correlations between positive priming effects and priming-induced epigenetic shifts that are independent from priming-induced genetic shifts by using partial mantel tests and multivariate redundancy analysis (Foust et al., 2016; Gugger et al., 2016; Herrera et al., 2016; Oksanen et al., 2016; Jueterbock et al., 2020). Moreover, tests for outlier loci that have become dominant allelic variants under positive selection (Narum and Hess, 2011; Günther and Coop, 2013; Ahrens et al., 2018) should be carried out in order to prove that positive priming effects cannot be explained by the survival of adapted genotypes.

Specificities of the Brown Algal Methylome

While the presence of cytosine methylation has been reported for green algae, red algae, dinoflagellates, and diatoms (Maumus et al., 2011; Tirichine and Bowler, 2011; Veluchamy et al., 2013; Bräutigam and Cronk, 2018; Lee J. M. et al., 2018), it is still not clear which brown algae share a lack of DNA methylation with the filamentous brown alga *Ectocarpus* sp. (Cock et al., 2010), in which epigenetic variation may be instead mediated at the chromatin level by histone modifications (Bourdareau et al., 2020) or via stress responsive miRNAs (Cock et al., 2017). Recently, DNA methylation was detected in the kelp *S. japonica* (Fan et al., 2019). The kelp methylome has been shown to change between life-cycle stages, to correlate with gene expression, and to differ from that of plants and microalgae. For example, methylation occurs predominately in CHH sequence contexts, which transfer methylation less reliably across mitotic cell divisions than CG sites (Law and Jacobsen, 2010). Moreover, DNA methylation appears to rely on a DNA methyltransferase (DNMT2) that is of low efficiency compared with other DNMTs, belonging to a class that mainly catalyzes tRNA methylation in plants and animals (Fan et al., 2019). How these specificities affect the functional role of the kelp methylome with respect to molecular stress memory remains unexplored.

Integrative Analyses

Parallel recording of epigenetic and transcriptomic priming responses can allow the identification of priming-induced epialleles that correlate with gene expression patterns and therefore potentially explain enhanced stress resistance (e.g., heat shock proteins). For example, that heat-induced methylation changes could be involved in stress acclimation of the red alga *Pyropia haitanensis,* was suggested by their correlation with the expression of stress-responsive genes (Yu et al., 2018). Penalized regression methods present promising integrative multi-locus models to test for statistical relationships between different "omics" data sets as they can overcome the challenge of having a small number of individuals (n) relative to the number of parameters (p) (Pineda et al., 2015; Lien et al., 2018; Zhong et al., 2019). Causal effects of DNA methylation on improved

phenotypes may be possible to model with structural equation modeling (SEM), an established multivariate method that is relatively new to the field of molecular biology (Igolkina and Samsonova, 2018; Fatima et al., 2020). To demonstrate a causative relationship between priming memories and adaptive phenotypic changes requires experimental removal of DNA methylation, e.g., using Zebularine or 5-Azacytidine (Griffin et al., 2016), or targeted modifications of epigenetic marks, e.g., using the CRISPR-Cas system (Xu et al., 2016).

Inferences about the functional effect of molecular priming memories rely on the availability of annotated genomes, which are still scarce for marine macrophytes. Genomes have been published for six brown macroalgae: *Ectocarpus* sp. (Cock et al., 2010), *S. japonica* (Ye et al., 2015), *Undaria pinnatifida* (Shan et al., 2020), *Cladosiphon okamuranus* (Nishitsuji et al., 2016), *Nemacystus decipiens* (Nishitsuji et al., 2019), and *Sargassum fusiforme* (Wang et al., 2020). Published seagrass genomes include *Z. marina* (Olsen et al., 2016), *Z. muelleri* (Lee et al., 2016), and *Halophila ovalis* (Lee H. et al., 2018). Thus, the assembly and annotation of genomes, particularly of species of high ecological or commercial relevance, is a key priority in priming-related research.

Stability and Transfer of the Priming Memory

Multi-generational stability of the priming memory (transgenerational priming) is more important for the application of priming in restoration than in cultivation. Macroalgal cultivation naturally allows for annual re-priming during the few weeks the macroalgae are cultivated under controlled laboratory conditions. For example, for kelps, priming could be annually applied to either the haploid gametophyte cultures or to the young diploid sporophytes before being deployed at sea until harvest. While priming of the gametophytes would require the least resources (i.e., space and water), it is not clear to what extent epigenetic reprogramming during fertilization would affect transmission of a priming memory to the sporophyte generation. To characterize the transfer of priming memories via small life-cycle stages such as meiospores, gametes, and zygotes may become possible with new single-cell 'omics technologies (Wang and Bodovitz, 2010; Zhu et al., 2020) that allow to sequence at DNA quantities which are too low for more traditional high-throughput sequencing technologies.

For restoration of kelp forests and macroalgae beds, thermal priming could be applied to the newly developed restoration tool "Green gravel," where macroalgae are seeded on rocks and reared in the laboratory until reaching a size of 2–3 cm (Fredriksen et al., 2020). Specifically, priming could be used to enhance initial survival of gametophytes and juvenile sporophytes to the generally harsher conditions in degraded areas where an adult canopy is lacking. However, if not transferred across generations, any positive priming effect will last, at most, until the primed individuals have died, and will not provide long-term protection against recurrent stress. Some macroalgae grow vegetatively (e.g., *Ecklonia brevipes*; Coleman and Wernberg, 2018), allowing to compare the longevity of priming effects under different modes of reproduction in macroalgae being applied in a restoration context.

In seagrass meadows, priming memories are likely to be more stable across vegetatively/mitotically grown generations than across sexually produced generations because epigenetic marks are often reset during meiosis and embryogenesis (**Figure 1C**; Hirsch et al., 2012; Douhovnikoff and Dodd, 2014; Dodd and Douhovnikoff, 2016; González et al., 2017). While direct tests for predicted sexual-asexual differences in the transgenerational stability of epigenetic marks are virtually lacking (Verhoeven and Preite, 2014), a unique system to test these differences is provided by the dramatic range in clonal diversity and life history strategies of seagrasses, ranging from predominantly vegetative to predominantly sexual reproduction (Kilminster et al., 2015).

Clonal seagrass meadows further provide a unique potential to study whether communication of epigenetic information across physically connected shoots allows the acquisition of a collective stress memory to prepare interconnected ramets for a range of future environmental challenges (Latzel et al., 2016). The transport of epigenetic information from somatic tissue to the germline via miRNAs (small ncRNAs that can cross cell barriers) has been demonstrated in humans and the nematode *Caenorhabditis elegans* (Creemers et al., 2012; Devanapally et al., 2015; Sharma, 2015; Szyf, 2015). Whether such communication may extend across interconnected ramets of the same clone has never been tested.

Prospects to Explore Priming as Biotechnological Tool in Marine Macrophytes

Priming has a large potential to enhance restoration success of macroalgal forests and seagrass meadows, and to ensure production security of macroalgal biomass under environmental challenges. Because primed organisms are not considered genetically modified, they can be grown in countries where GMO restrictions apply. Moreover, priming would likely be a less controversial and more socially acceptable way to boost resilience in macrophytes relative to the proposed gene editing approaches (Coleman and Goold, 2019). However, priming cannot be simply transferred from terrestrial plants to marine macrophytes. In particular, brown and red macroalgae are distantly related to terrestrial plants, and kelps have complex heteromorphic life cycles with free-living gametophyte generations. Thus, in order to identify whether priming can be established as a novel bio-engineering technique for marine macrophytes, we need ambitious fundamental research that uses complex experimental setups combined with multivariate analyses that can integrate multiple high-throughput sequencing datasets to test at which intensity, duration, and life-cycle stage priming has a positive and long-term effect without inducing selection or high mortality. For priming to be of commercial value to the macroalgae farming industry, we must further assess whether the cost factor added to the cultivation process pays off by enhancing yield even in years where the macroalgae are not exposed to stress or by providing cross-protection to other relevant stressors (Hilker et al., 2016). Despite these knowledge gaps, priming should be explored as

a tool to boost resilience of both seagrass and macroalgae to secure their ecological and economic values in future oceans.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

AJ wrote the first draft of the manuscript. AM created the figures. JC, MC, TW, LS, RR, JZ, and Z-MH contributed to the article by commenting and re-writing sections, and approved the submitted version. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

11.2 Abbreviations

C	carbon
CG	DNA sequence Cytosine-Guanine
CHG	Cytosine – C/T/A - Guanine
H = C or T or A	H = Cytosine or Thymine or Adenine
CHH	Cytosine - C / T / A - C / T / A
Chl *a, b*	Chlorophyll *a, b*
CO_2	Carbon dioxide
COI (gene marker)	Cytochrome c oxidase subunit 1
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats – used for gene manipulation (CRISPR/Cas)
DNA	deoxyribonucleic acid
DMT1 (formerly CrMET1)	*C. reinhardtii* methyltransferase 1
DNMT2	DNA (cytosine-5-) -methyltransferase 2
E_c	light compensation point
E_k	light saturation point
E_{par}	light availability
eCA	external carbonic anhydrase
F1 (2)	Filial Generation one (two)
F6P	D-fructose-6-phosphate
GOterms	Gene-Ontology terms, groups genes by function

g / mg / µg	gram / milligram / microgram
h	hour
H3 K9	a type of histone modification
k	thousand
km	kilometre
l / ml / µl	litre / millilitre / microliter
L. solidungula	*Laminaria solidungula*
m / cm / nm	meter / centimetre / nanometre
mol / µmol	mole / micromole;
	1 mol = 6,02214076 · 10^{23} particles
mRNA	Messenger DNA
Mt	Mega tonne
mt+	mating type positive, female gametes
N	nitrogen
n	haploid
2n	diploid
ncRNA	non-coding RNA
NO_3	nitrogen
NPP	Net Primary Production
p	Partial pressure
PAM	Pulse amplitude modulated Fluorometry
PAR	Photosynthetically active radiation
ppm	Parts per million

Q_{10}	Temperature Coefficient
RNA	Ribonucleic acid
RQ	research question
s	second
S. japonica	*Saccharina japonica*
S. latissima	*Saccharina latissima*, sugar kelp
SNP (gene marker)	single nucleotide polymorphism
Spp.	Species
T	Temperature (in °C)
UV	Ultra-violet
yr	year
°C	Degree Celsius
°N	Degrees North
α	photosynthetic efficiency
β-(1,3)-glucan	laminarin
♂	male
♀	female
5-mC	5'- Methylcytosine

11.3 Definitions / Terms

Physiology – in an living organism, it answers the question 'how does it function'?

Eco-physiology – in an ecosystem, it answers the question 'how do environmental factors such as temperature, light etc affect the individual function'?

Polar Night vs **Winter -** Polar Night defines the time in polar regions where the sun does not rise above the horizon, where the duration varies with latitude (see Picture in Polar Night, page 43 A; page 11), while winter typically defines the months of December to February in the northern hemisphere, which does not vary with latitude.

Primary Producers - photoautotrophic organisms that utilise PAR to convert water + CO_2 to sugar + O_2

Ecology -

Definitions regarding the umbrella topic of (local) **adaptation** and **acclimation** repeatedly appear inconsistent across literature, hence it is necessary to state the definitions used in this dissertation. Most terms were defined prior to the discovery of epigenetic mechanisms, and lack the additional layer these findings can introduce to a term. Even the term 'adaptation' typically omits epigenetic mechanisms, despite the heritable character of some, and their important role in adaptation processes. **Phenotypic plasticity**, the variance in a species without apparent variance in their **genotype**, for example, had been defined prior to epigenetics. With this, it lacks an aspect that is uncoupled from genetics/ genotype (hence should appear in **phenotype**), but still is connected to the genome. It is debatable whether epigenetic mechanisms fall under phenotypic plasticity, as it is not covered by the definition, but influences the phenotype (that is, the status of an individual or population in their respective habitat). As observed with the carbohydrate contents in this dissertation, fundamental metabolic processes typically are species-dependent, and regulation pre-exists in the genome, regardless of population. Adjustments within the range of the genomes' pre-print have been referred to as **phenotypic variation**, as the phenotype is the actual shape the genome template has been expressed to in a given environment. This has led to the need to define **eco-phenotype:** the combination and interaction of both genetic (DNA sequence-related) and epigenetic mechanisms that lead up to a specific "adaptation" of single individuals or origins to their respective environment, which can be passed on to following generations, and typically prevails for at least one generation when this generation encounters factors differing from those of the parental generation. With this, it encompasses ecotype (genetic + phenotypic variability in ecological niche, see above), and adaptation ("heritable, but DNA sequence-based, and evolved over generations"). Features of an eco-phenotype do not have to accumulate over many generations to meet the definition, and fall between acclimation and adaptation ('rapid adaptation', or 'heritable acclimation').

Epigenetics -

Epigenetics entails regulatory processes affecting gene expression that are not genome-based, but controlled by the epigenome. Epigenetic mechanisms in primary producers entail histone modification, non-coding RNA, and DNA cytosine methylation.

Priming: (artificial) exposure to a given stressor in order to create a stress memory to improve resilience during future encounters with the stressor.

Transgenerational priming: a stress memory which is heritable, and can improve resilience across generations.

Tags: 32-33 bp sequences around *in-silico* predicted methylation sites in CG or CHG sequence contexts.

Reads: DNA sequences obtained from the Ilumina sequencer.

Mapped reads: reads that mapped back to any site within the target genome. One read can map back to several sites.

Uniquely mapped reads: reads that only mapped back to one place in the target genome.

Coverage: number of uniquely mapped reads across all samples for a certain site of mapping.

Contig: assembly of sequenced reads of the same genetic origin.

origin: here is used in the latitudinal context of origin of samples. 'Helgoland origin' are those samples originating from 54°11'18.9"N 7°54'14.1"E, while 'Spitsbergen origin' are those originating from 78°59'26.0"N 11°58'42.3"E.

11.4 Textbook Knowledge

The temperature dependence of enzymatic processes (Q_{10})

Due to the abundance of water, processes of photosynthesis in marine primary producers are mainly limited/ inhibited by high light (Dau 1994; Bruhn and Gerard 1996; Aguilera et al. 1999). The enzymatic reactions comprised in aerobic respiration, like all chemical reactions, are directly linked to temperature increase, regardless of starting point. The velocity of an enzymatic process varies with temperature, and is described by the temperature coefficient (Q_{10}). Q_{10} is given for a defined temperature range, and indicates the increase in reaction velocity when temperature is increased by 10 ° (Celsius or Kelvin) in a respective temperature range. 'Q_{10} = 2 between 5 and 15 ° C' hence indicates that the given enzymatic process doubles in velocity for temperatures between 5 °C and 15 °C. The temperature coefficient can vary between temperature ranges for the same biological/ enzymatic process in the same species. Enzymatic activity of RubisCO, the carbon capturing enzyme in photosynthesis, can increase by a factor of 7 between 5°C and 15°C [$Q_{10}RubisCO_{5°\text{-}15°}$ = 7], but has been shown to change with temperature range (Cen and Sage 2005). RubisCO is especially vulnerable to (high) temperatures (Tabita 2007; Galmés et al. 2013). Like for the enzyme RubisCO, many biochemical and physiological processes are affected by temperature increase. Photosynthetic activity, for example, is highly sensitive to low temperatures, as the enzymatic (secondary) reactions are temperature dependent due to Q10, while the primary reactions are not (Fortes and Lüning 1980; Becker et al. 2009). Hence primary reactions were found to be regulated depending on the capacity of the secondary reactions (i.e. Calvin Cycle; Becker et al. 2009). For Laminariales, Q10 is known to change with growth temperature (Davison 1987), and is directly influenced by ambient temperature (Q10=2 for Phaeophyceae between 0 °C and 15 °C (Kanwisher 1966).

Pigments

For the process of light harvesting, most terrestrial plants, and green algae, only require chlorophyll a (Chl a; universal, light harvest and reaction centre) and Chl b in their antenna complex (Kirk 1971). To account for the spectral shifts in underwater light, especially in coastal waters (Kirk 2011), the'green gap' in light absorption of Chl a and b is narrowed down to a minimum in the antenna complex of marine brown algae (Phaeophyceae) by several accessory pigments (see Fig. App. 1).

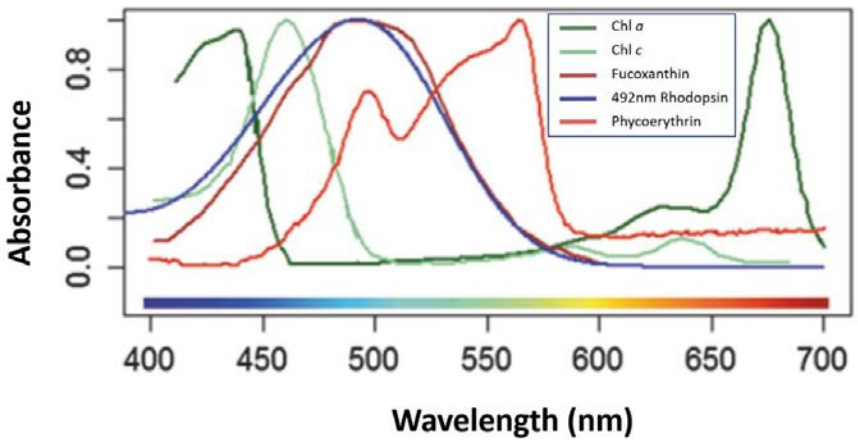

Figure Appendix 1: Absorbance of different pigments relative to PAR wavelength. Adapted from Cohen et al 2020.

These are not found in the chloroplasts of terrestrial plants, nor in marine green algae. Pigment content per thallus-area is typically higher in low light plants, causing a higher proportion of synthetic capacity needed to synthesise and support light-harvesting pigment content (Lüning 1990).

Net Primary Production - NPP

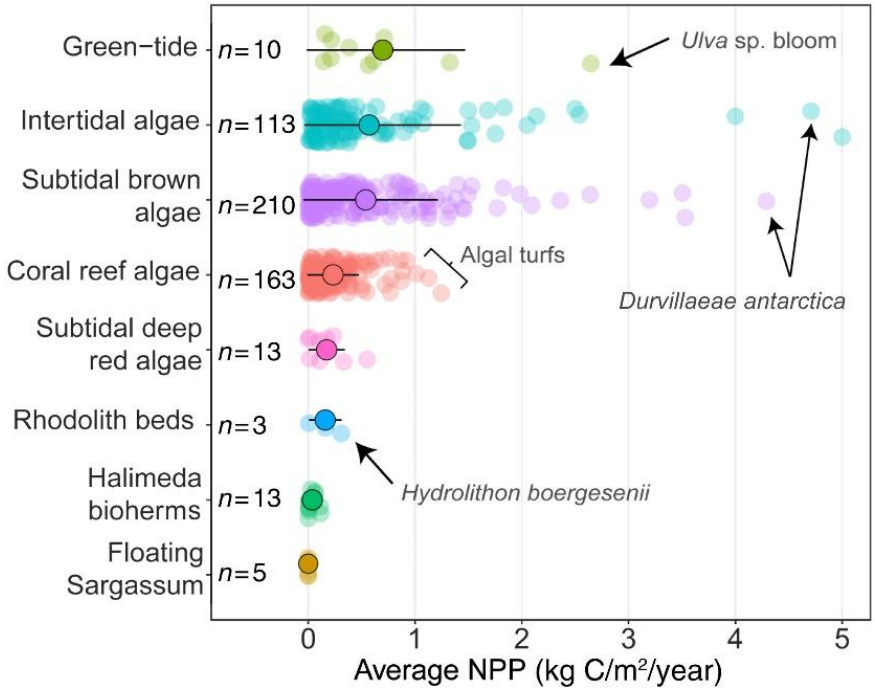

Figure Appendix 2: Estimated annual marine net primary production (NPP) split by type (from Duarte et al 2022).

Terrestrial and marine primary producers by now are known to contribute equally to global net primary production (NPP). Fig. App.2 shows the amount of different macroalgae, which in total account for approximately half of the marine NPP.

Moonlight

Considerations regarding moonlight and photosynthesis until now have focussed on the comparison to photosynthesis in sunlight, which generally only considers the parts of photosynthesis between light compensation (E_c) and light saturation point (E_k), securing primary production. However, photosynthetic processes already occur below E_c, even if the section below E_c will not contribute to primary production in the common sense (gain of biomass). Moonlight has been shown to effect terrestrial plants (Breitler et al. 2020), and corals have been shown to be able to detect the blue wavelength of the moonlight at a threshold of photoreception sensitivity of ~1.2 x 10^{15} quanta m^{-2} s^{-1} (Gorbunov and Falkowski 2002).
Even though irradiance is very low compared to the light season, phases of the moon, especially full moon, are still detectable (see Fig. App. 3) . Terrestrial moonlight is strongest in the purple wavelengths (~ 400nm) of the light spectrum (Breitler et al 2020). Marine moonlight, however, is presumably shifted to predominantly the blue part with a peak between 400 and 550nm, where irradiance has been modelled to be about 10-7 W m-2 nm-1 at depth of - 10 m (average *S. latissima* habitat) in the High Arctic during Polar Night (see Fig. App. 4; Cohen et al. 2020).

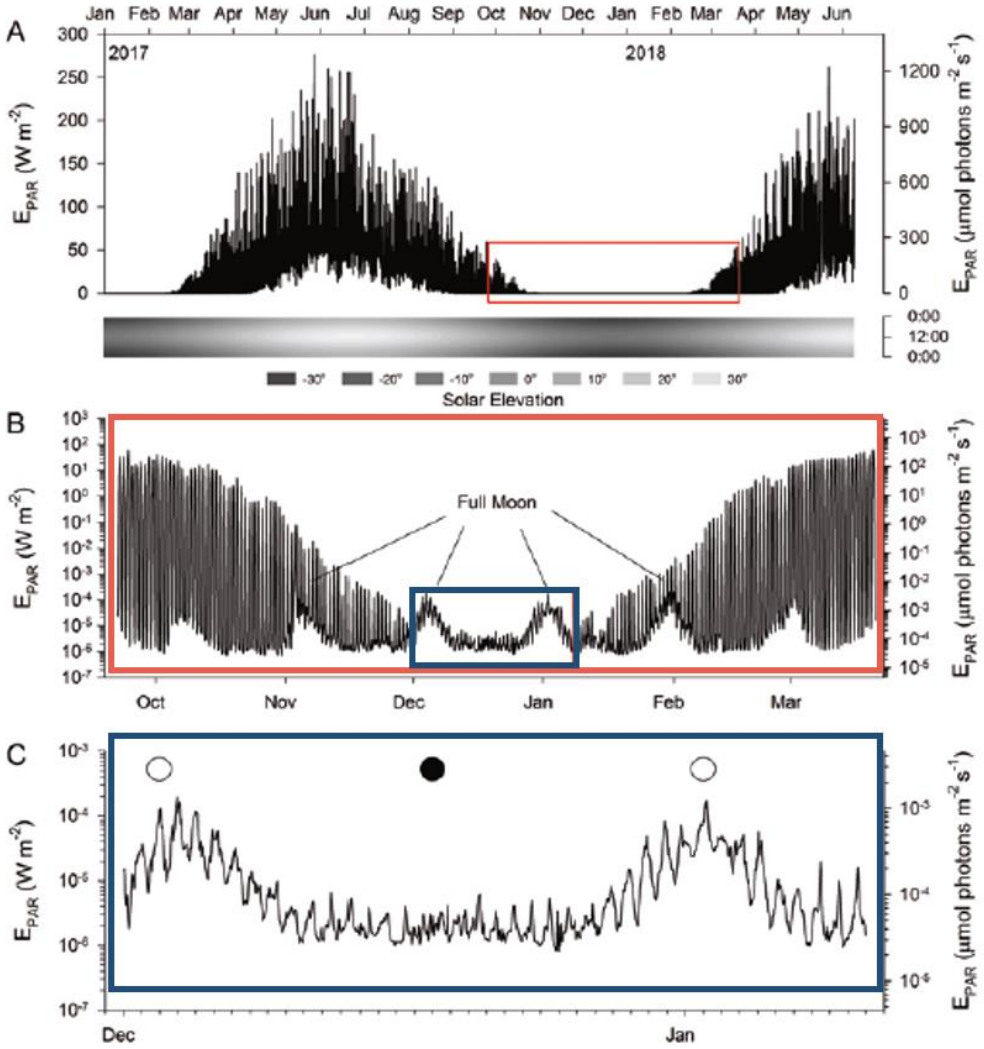

Figure Appendix 3: Intensity of Moonlight during winter in Kongsfjorden, Svalbard. Adapted from Cohen et al 2020.

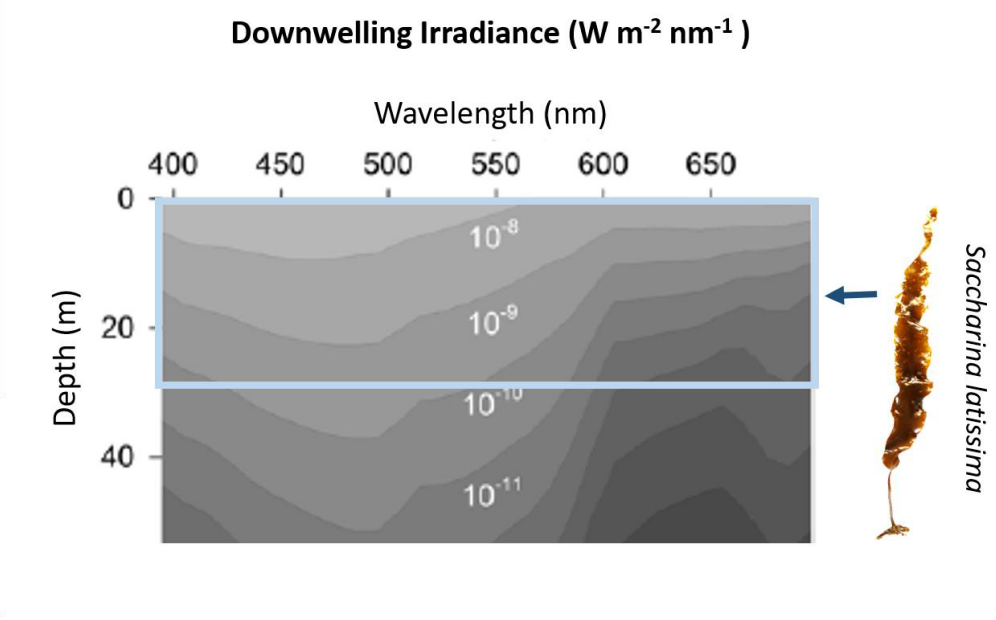

Figure Appendix 4: Modelled downwelling moonlight irradiance in Kongsfjorden, Svalbard, at 'noon' during Polar Night. Adapted from Cronin et al 2016. Picture *S.latissima*: S. Niedzwiedz

11.5 Bioinformatics, Statistics, Raw Data

11.5.1 Appendix Publication I

'Arctic kelp eco-physiology during the Polar Night in the face of global warming: a crucial role for laminarin'

DOI:

10.3354/meps12860

Data archiving publication I:

10.1594/PANGAEA.903529, 10.1594/PANGAEA.903524, 10.1594/PANGAEA.903526, 10.1594/PANGAEA.903525, 10.1594/PANGAEA.903133, 10.1594/PANGAEA.903836, 10.1594/PANGAEA.903523, 10.1594/PANGAEA.903131

Statistics Publication I

Statistical analyses of the eco-physiological parameters are archived under DOI:

10.6084/m9.figshare.23268617

11.5.2 Appendix Publication II

'Transcriptomic Responses to Darkness and the Survival Strategy of the Kelp *Saccharina latissima* in the Early Polar Night'

DOI:

10.3389/fmars.2020.592033

Data archiving Publication II:

NCBI SRA Bio Project PRJNA564197

11.5.3 Appendix Publication III

'Differences by Origin in Methylome Suggest Eco-phenotypes in the Kelp *Saccharina latissima*'

DOI:

10.1111/eva.13382

Data archiving Publication III:

NCBI SRA Bio Project PRJNA809008.

10.6084/m9.figshare.19411460, 10.6084/m9.figshare.19411574

Statistics Publication III

see Pipeline Bioinformatics (Identical for Publications III and IV)

11.5.4 Appendix Publication IV

'Chloroplast DNA methylation in the kelp *Saccharina latissima* is determined by origin and influenced by cultivation'

DOI:

doi.org/10.1101/2022.12.02.518695

Data archiving publication IV:

NCBI SRA Bio Project PRJNA809008.

Supplementary publication IV:

bioRxiv doi 10.1101/2022.12.02.518695

11.6 Pipeline Bioinformatics (Includes Statistics Publication III and IV)

Identical for Publications III and IV

Legend:

section header
command line
command line chloroplast (when different from above)
subheader
- programs/files etc.
weblinks
Output
comments, text
please mind

Sequencer: NextSeq 500 machine (Software NextSeq Control 2.2.0); 4 lanes

main folder names in working directory: 'Sequencing', 'cleanup', 'mapping'; sequencer creates own folder for raw reads

Convert sequencer output to fastq

In Sequencing folder:

###programs

- bcl2fastq v2.17.1.14 (programm running on linux, provided by illumina)

  https://support.illumina.com/sequencing/sequencing_software/bcl2fastq-conversion-software.html

- trim_galore v 0.4.1 (20 07 2015)
- FastQC v 0.11.8 (2018) (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/)
- MultiQC v 1.4

check and re-name sequencing files

Samples

Raw data file: **SequencingFiles.tar.gz** (like .zip compressed)

tar zxyf is archiving utility in linux

in folder created by sequencer (190429_XXX)

tar zxvf # extract/ 'unzip' data

This folder contains the sequencing files that need to be converted

For bcl2fastq, a file '**SampleSheet.csv**' is needed (comma separated values) -> imported data from exelSheet: is already located in the folder 190429_XXX -> get it from there in the new folder via

cp ../190429_XXX/SampleSheet . **#** '.' is important!!! means 'here'

nano SampleSheet # -> change according to current conditions and sample names and INDEX!!! (adapt date, phix control etc)

! change sampleID, SampleName, Index ID, Index ! (open excel spreadsheet, and transfer data **manually**) !

'SampleID_Concentration_Barcodes.xlsx' # from library preparation in the lab! In the sample names, S stands for Spitsbergen, H for Helgoland, and 5, 10, and 15, stands for the temperatures in degrees Celsius (°C) they were grown at (lab samples). F stands for field samples, no temperature resolution here.

Ctrl + O -> write changes

operate **bcl2fastq** in the folder where the extracted sequencing files are stored (190429_XXX)

In case indexes are too similar, 'number of allowed mismatches per barcode' needs to be adapted to 0 (instead of 1)

bcl2fastq –barcode-mismatches 0

result should look like:

-rw-r--r-- 1 root root 75M mai 6 09:41 S3_5_S1_L001_R1_001.fastq.gz

result should NOT look like:

-rw-r--r-- 1 root root 125K mai 6 09:51 H1_15_S21_L004_R1_001.fastq.gz

Note: Files that contain almost no data (e.g. 125K instead of 75M) **need to be sequenced again**.

Labsamples (general example)

The files from the 4 lanes are combined (for each sample) with:

mkdir /*locationwhereitneedstobe*/fastqfiles

for f in S3_5 S3_10 S3_15 H4_5 H4_10 H4_15 H3_5 H3_10 H3_15_2 S2_5 S2_10 S2_15 H2_5 H2_10 S1_5 S1_10 S4_5 S4_10 H1_5 H1_10 H1_15

do

cat "$f"_*_L001_R1_001.fastq.gz "$f"_*_L002_R1_001.fastq.gz "$f"_*_L003_R1_001.fastq.gz "$f"_*_L004_R1_001.fastq.gz > /*locationwhereitWAS*/fastqfiles/"$f".fastq.gz

done

cd /home/*locationwhereitneedstobe*/fastqfiles

gunzip *.gz

Fieldsamples (and repeated lab sample H1_15) (explicit example)

In folder 'sequencing':

mkdir fastqfilesFieldsamples

From the 191111_XXX folder (created by sequencer), copy the baseline-calls to folder 'fastqfilesFieldsamples' (for this, go to lowest level where both folders are)

cp 191111_XXX/Data/Intensities/BaseCalls/*fastq.gz fastqfilesFieldsamples/

The files from the 4 lanes are combined (for each sample) with:

for f in FS1 FS2 FS3 FS4 FS5 FS6 FS7 FS9 FS10 FS8a FH1 FH2 FH3 FH4 FH5 FH6 FH7 FH8a FH9 FH10 H1_15

do

cat "$f"_*_L001_R1_001.fastq.gz "$f"_*_L002_R1_001.fastq.gz "$f"_*_L003_R1_001.fastq.gz "$f"_*_L004_R1_001.fastq.gz > "$f".fastq.gz

done

With this, there will be the original 4 lanes, and the combined file. Only the combined one is needed in this folder -> delete the others with

rm *R1*.gz

Quality check

In folder 'sequencing':

In case of non-compressed files (not .gz; run1):

../FastQC/fastqc fastqfilesFieldsamples/*.fastq

In case of compressed files (.gz; run2)

../FastQC/fastqc fastqfilesFieldsamples/*fastq.gz

this results in files ending on .**fastqc.html** and **fastqc.zip**

un-zip:

for z in *fastqc.zip

do

unzip $z;

done

MultiQC in folder where fastq's are unzipped

multiqc . # '.' Means 'here'

file **multiqc_report_Sequencing.html ->** open in filezilla (download to windows)

Cleanup

 # create folder 'cleanup'

programs in folder cleanup:

- trim_galore v 0.4.1 (20 07 2015) https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/

- FastQC v 0.11.8 (2018)

- MultiQC v 1.4

- nohub

Trimming

- Bases with Phred-score <20 eliminated

- remove adapter sequences

- removal of term-2bp to eliminate artifacts that might have arisen at the ligation position

trim_galore **LabSamples**

```
for f in S3_5 S3_10 S3_15 H4_5 H4_10 H4_15 H3_5 H3_10 H3_15_2 S2_5 S2_10 S2_15 H2_5 H2_10 S1_5 S1_10 S4_5 S4_10 H1_5 H1_10 H1_15
do
nohup \
trim_galore \
--illumina \
--stringency 3 \
--quality 20 \
--clip_R1 2 \
--three_prime_clip_R1 2 \
--output_dir . \  # . depends on where it's started (see trim_galore FieldSamples)
$(ls ../sequencing/fastqfiles/"$f".fastq) \
&
done
```

trim_galore **FieldSamples**

for f in FH1 FH2 FH3 FH4 FH5 FH6 FH7 FH8a FH9 FH10 FS1 FS2 FS3 FS4 FS5 FS6 FS7 FS8a FS9 FS10 H1_15

do

**nohup **

**trim_galore **

**--illumina **

**--stringency 3 **

**--quality 20 **

**--clip_R1 2 **

**--three_prime_clip_R1 2 **

**--output_dir cleanup/cleanupFieldsamples **

$(ls sequencing/fastqfilesFieldsamples/"$f".fastq)

&

done

Quality Check

check quality before and after cleanup -> checks for fragment(over-)representation, Base AT CG content, actuall fragment length/ length of reads

Starts with FastQC

FastQC

FastQC/fastqc *.fq

this gives files ending on **.fastqc.html** and **fastqc.zip**

-> un-zip:

for z in *fastqc.zip

do

unzip $z;

done

####MultiQC

multiqc .

-> file **multiqc_report.html** # doublecheck via filezilla!

conclusion

after adapter removal, we expected 32bp (fragment length FSPE1-Cut) -2bp3'end -2bp5'end = 28bp in total, ~26bp was observed (as before for this method with zostera and latissima)

Info about the restriction enzyme FspEI

Based on this publication (Cohen-Karni et al (2011) DOI 10.1073/pnas.1018448108)

http://www.pnas.org/content/108/27/11040.full.pdf?with-ds=yes

FspEI can cut:

- The ones that were best represented in the original MethylRAD paper were CCGG and CCWGG

- All: CCGT, CCGA, CCGC, CCGG, ACAGG, CCTGT, CCAGT, CCGGT (in CCGG), CCCGT (in CCGT), CCAGA, CCGGA (in CCGG), CCCGA (in CCGA), CCAGC, CCTGC, CCCGC (in CCGC), CCAGG, CCTGG, CCGGG (in CCGG), CCCGG (in CCGG)

- Reduced/non-redundant set: CCGT, CCGA, CCGC, CCGG, ACAGG, CCTGT, CCAGT, CCAGA, CCAGC, CCTGC, CCAGG, CCTGG

- But to cut out on both sites, CC and GG need to be in a recognition site that fits to its reverse complement: CCGG, ACAGG (because CCTGT also cuts), CCAGG, CCTGG

Mapping nuclear genome

 # create folder 'mapping'

programs

- the *S. japonica* genome and annotation is on ORCAE -> 'SJ6' https://nph.onlinelibrary.wiley.com/doi/full/10.1111/nph.16125
- InSilicoTypeIIbDigestion.py (https://github.com/alj1983/BioinformaticsScripts/blob/master/InSilicoTypeIIbDigestion_corrected.py)
- RTR.py (https://github.com/alj1983/BioinformaticsScripts/blob/master/RTR.py)
- Protocol that marks which of the fragments fit to the reduced tag
- representation approach
- adapters with the ending NNNT and NNNC (20161115Oligonucleotides.pdf)
- SOAP aligner to in-silico digested genomes, file version 1.11(2008)
- HTseq count htseq-count v 0.7.2
- faidx: 0.5.5.2
- bioawk version 20110810 samtools version 1.9 (using htslib 1.9)
- CounttableOverview.r

Genomes

For new genomes to be digestet *in silico* do the following, otherwise skip until **---------**

- *S. japonica* genome and annotation from ORCAE
- File **SJ.v6.2.chromosome.block.fasta**
- **SJaponicaChloroplast_digested_RTRMarked.fasta** #chloroplast genome, only *in silico* digestion shown here. Rest of follow-up procedure (mapping etc) identical to SJ6, but needs to be carried out in own folder ('chloroplast')

*In silico* digestion

 # *Saccharina japonica* annotated genome (SJ6)

python InSilicoTypeIIbDigestion_corrected.py -f SJ.v6.2.chromosome.block.fasta -r CCGG,CCTGT,CCAGG,CCTGG -d5 14,13,13,13 -l 32,31,31,31

 # Total bases in fasta file: 548536073

#CCGG recognition sites: 1793114, density in fasta file: 305

#CCTGG recognition sites: 527987, density in fasta file: 1038

#CCTGT recognition sites: 496183, density in fasta file: 1105

#CCAGG recognition sites: 527729, density in fasta file: 1039

file: **SJ.v6.2.chromosome.block_digested.fasta**

python RTR.py -f SJ.v6.2.chromosome.block_digested.fasta -b1 'T' -b2 'C' -l1 4 -l2 4

 # 399280 of 3345013 (11.9365754333%) tags fit to these adaptor endings

files: **SJ.v6.2.chromosome.block_digested_RTRMarked.fasta, SJ.v6.2.chromosome.block_digested_RTRExtracted.fasta**

 # Chloroplast genome *S. Japonica*

python ../mapping/InSilicoTypeIIbDigestion_corrected.py -f SJaponicaChloroplast.fasta -r CCGG,CCTGT,CCAGG,CCTGG -d5 14,13,13,13 -l 32,31,31,31

#Total bases in fasta file: 130584

#CCGG recognition sites: 68, density in fasta file: 1920

#CCTGG recognition sites: 56, density in fasta file: 2331

#CCTGT recognition sites: 80, density in fasta file: 1632

#CCAGG recognition sites: 65, density in fasta file: 2008

file: **SJaponicaChloroplast_digested.fasta**

python ../mapping/RTR.py -f SJaponicaChloroplast_digested.fasta -b1 'T' -b2 'C' -l1 4 -l2 4

 # 21 of 269 (7.80669144981%) tags fit to these adaptor endings

files: **SJaponicaChloroplast_digested_RTRMarked.fasta, SJaponicaChloroplast_digested_RTRExtracted.fasta**

When using an already digested genome, start here:

alignment with SOAP aligner to in-silico digested genomes

file version 1.11(2008) works; later versions did not work as well

create parameter files **SoapAlignment.param** (e.g. **SoapShortAlignmentSJ6.param** (in folder mapping)) containing the conversion command for each sample from **trimmed.fq** (field samples: **trimmed.fq.gz**) to **.sop**, as different libraries are mapped to same reference site

specifications in the .param file:

-v 2 # two missmatches allowed

-z ! # sanger quality +33 (Starts at ;/!..123...ABC)

-f 1 # filter low quality reads containing >1 N

-s 8 # seed size

-r 0 # r reports repeated hits -> Do not report repeated (0), so only unique hits are reported

 # -r 1 # to check how many did actually map (1) - no matter if uniquely or duplicated **alternative to –r 0, see below**

Example .param file uniquely mapped:

content of **SoapShortAlignmentSJ6.param** should look as follows (this is an example for listing *uniquely mapped*! [-r 0]):

-a ../cleanup/cleanupLabsamples/S3_15_trimmed.fq -o S3_15.sop -v 2 -z ! -f 1 -s 8 -r 0

-a ../cleanup/cleanupLabsamples/H1_10_trimmed.fq -o H1_10.sop -v 2 -z ! -f 1 -s 8 -r 0

Example Fieldsamples, all hits:

content of **SoapShortAlignmentFieldsamplesLatissimaAllReads.param** should look as follows (this is an example for listing *AllReads*! [-r 1])

-a ../cleanup/cleanupFieldsamples/FH1_trimmed.fq.gz -o FH1LatissimaAllreads.sop -v 2 -z ! -f 1 -s 8 -r 1

-a ../cleanup/cleanupFieldsamples/FS5_trimmed.fq.gz -o FS5LatissimaAllreads.sop -v 2 -z ! -f 1 -s 8 -r 1

then:

Mapping

**nohup **

**./soap_1.11/soap.short ** # get the program in folder

-d SJ.v6.2.chromosome.block_digested_RTRMarked.fasta **** # take the genome

**SoapShortAlignmentSJ6.param ** # take the 'uniquely hits' parameter file

**> SoapShortAlignmentAllSamplesSJ6.out ** # write error messages in .out file

& # end

- Now mapping with **.param** files with the specifications **-r 1** to check how many reads mapped, not only how many reads mapped uniquely (see 'Specifications'; file e.g. **SoapShortAlignmentFieldsamplesLatissimaAllReads**)

Mapping AllReads to the *S.japonica* (in-silico digested, SJ6) genome

**nohup **

**./soap_1.11/soap.short **

**-d SJ.v6.2.chromosome.block_digested_RTRMarked.fasta **

**SoapShortAlignmentAllSamplesSJ6AllReads.param ** # all reads .param file

**> SoapShortAlignmentAllSamplesSJ6AllReads.out **

&

Checking mapping ratios

ratio checking

Against the SJ6 Genome (All Samples)

Labsamples

```
echo "Sample,RawReads,TrimmedReads,AllMappedReads,UniquelyMappedReads,TrimmedReadsThatMappedUniquely,MappedReadsThatMappedUniquely" > LabsamplesSJ6MappingOverview.txt

for f in H1_5 H1_10 H1_15 H2_5 H2_10 H3_5 H3_10 H3_15 H4_5 H4_10 H4_15 S1_5 S1_10 S2_5 S2_10 S2_15 S3_5 S3_10 S3_15 S4_5 S4_10

do

 beforetrimming=$(grep '+' -c ../sequencing/fastqfiles/"$f".fastq)

 reads=$(grep '+' -c  ../cleanup/"$f"*trimmed.fq)

 uniquemapped=$(wc -l "$f"LabsamplesSJ6.sop |sed 's/ .*//')
```

```
 mapped=$(wc -l "$f"LabsamplesSJ6AllReads.sop |sed 's/ .*//')

 proportion=$((100 * $mapped / $reads))

 proportion2=$((100 * $uniquemapped / $mapped))

 echo "$f: All reads $beforetrimming "

 echo "$f: Reads after trimming $reads "

 echo "$f: mapped reads $mapped "

 echo "$f: trimmed reads that mapped $proportion %"

 echo "$f: uniquely mapped reads $uniquemapped"

 echo "$f: mapped reads that mapped uniquely: $proportion2 %"

echo "$f,$beforetrimming,$reads,$mapped,$uniquemapped,$proportion,$proportion2" >>
LabsamplesSJ6MappingOverview.txt

 done
```

Fieldsamples

```
echo
"Sample,RawReads,TrimmedReads,AllMappedReads,UniquelyMappedReads,TrimmedRead
sThatMappedUniquely,MappedReadsThatMappedUniquely"                    >
FieldsamplesSJ6MappingOverview.txt

for f in FH1 FH2 FH3 FH4 FH5 FH6 FH7 FH8a FH9 FH10 FS1 FS2 FS3 FS4 FS5 FS6 FS7 FS8a FS9
FS10

do

 beforetrimming=$(grep '+' -c ../sequencing/fastqfilesFieldsamples/"$f".fastq)

 reads=$(grep '+' -c  ../cleanup/cleanupFieldsamples/"$f"_trimmed.fq)

 uniquemapped=$(wc -l "$f"FieldsamplesSJ6.sop |sed 's/ .*//')

 mapped=$(wc -l "$f"FieldsamplesSJ6AllReads.sop |sed 's/ .*//')

 proportion=$((100 * $mapped / $reads))

 proportion2=$((100 * $uniquemapped / $mapped))

 echo "$f: All reads $beforetrimming "

 echo "$f: Reads after trimming $reads "

 echo "$f: mapped reads $mapped "

 echo "$f: trimmed reads that mapped $proportion %"

 echo "$f: uniquely mapped reads $uniquemapped"

 echo "$f: mapped reads that mapped uniquely: $proportion2 %"
```

```
echo "$f,$beforetrimming,$reads,$mapped,$uniquemapped,$proportion,$proportion2" >> FieldsamplesSJ6MappingOverview.txt

done
```

H1_15 in folder fieldsamples, but labled Labsamples:

```
echo "Sample,RawReads,TrimmedReads,AllMappedReads,UniquelyMappedReads,TrimmedReadsThatMappedUniquely,MappedReadsThatMappedUniquely" >> FieldsamplesSJ6MappingOverview.txt

for f in H1_15

do

 beforetrimming=$(grep '+' -c ../sequencing/fastqfilesFieldsamples/"$f".fastq)

 reads=$(grep '+' -c  ../cleanup/cleanupFieldsamples/"$f"_trimmed.fq)

 uniquemapped=$(wc -l "$f"LabsamplesSJ6.sop |sed 's/ .*//')

 mapped=$(wc -l "$f"LabsamplesSJ6AllReads.sop |sed 's/ .*//')

 proportion=$((100 * $mapped / $reads))

 proportion2=$((100 * $uniquemapped / $mapped))

 echo "$f: All reads $beforetrimming "

 echo "$f: Reads after trimming $reads "

 echo "$f: mapped reads $mapped "

 echo "$f: trimmed reads that mapped $proportion %"

 echo "$f: uniquely mapped reads $uniquemapped"

 echo "$f: mapped reads that mapped uniquely: $proportion2 %"

echo "$f,$beforetrimming,$reads,$mapped,$uniquemapped,$proportion,$proportion2" >> FieldsamplesSJ6MappingOverview.txt

done
```

Conversion of sop files to sam format

In folder mapping:

Labsamples:

```
 for i in *sop; # sop2sam.pl (not with the fieldsamples) gives an error that can be ignored

 do

 perl soap2sam.pl -p "$i" > $(echo $i | sed 's/sop/sam/');

 done
```

Fieldsamples:

```
for i in F*sop;
do
perl soap2sam.pl -p "$i" > $(echo $i | sed 's/sop/sam/');
done
```

in case a file does not start with F like Fieldsample:

```
for i in H1_15*sop;
do
perl soap2sam.pl -p "$i" > $(echo $i | sed 's/sop/sam/');
done
```

Count reads with HTseq count

Following steps only done once for all following samples:

```
sudo pip install pyfaidx
```

version of faidx: 0.5.5.2

```
faidx --transform bed SJ.v6.2.chromosome.block_digested_RTRMarked.fasta > SJ.v6.2.chromosome.block_digested_RTRMarked.bed
```

The bed file starts counting from 0 but should start counting from 1. The old version of pyfaidx did start from 1. And **htseq-count** only works in this way. Thus, replace all 0's in the bed files in the second column with 1's.

```
awk -v 'OFS=\t' '{print $1, "1", $3}' SJ.v6.2.chromosome.block_digested_RTRMarked.bed > SJ.v6.2.chromosome.block_digested_RTRMarked_corrected.bed
```

Then using the following python script Bed2Gff3.py for file conversion

```
python Bed2Gff3.py -b SJ.v6.2.chromosome.block_digested_RTRMarked_corrected.bed
python Bed2Gff3.py -b SJaponicaChloroplast_digested_RTRMarked_corrected.bed
pip install 'HTSeq' # this installs htseq-count v 0.7.2
```

Adding a header:

htseq-count doesn't like the star * in the data after the cigar # string. The problem is that the soap mapper says that the mapped data are paired end data in the flags, but they are not, so flags need to be recalibrated as shown here:

https://www.biostars.org/p/219886/

now separate code again for lab and field

Fieldsamples:

```
for f in FH1 FH2 FH3 FH4 FH5 FH6 FH7 FH8a FH9 FH10 FS1 FS2 FS3 FS4 FS5 FS6 FS7 FS8a FS9 FS10

do

samtools view -bT SJ.v6.2.chromosome.block_digested_RTRMarked.fasta "$f"FieldsamplesSJ6.sam > "$f"FieldsamplesSJ6.bam

done
```

Labsamples:

```
for f in S3_5 S3_10 S3_15 H4_5 H4_10 H4_15 H3_5 H3_10 H3_15 S2_5 S2_10 S2_15 H2_5 H2_10 S1_5 S1_10 S4_5 S4_10 H1_5 H1_10 H1_15

do

samtools view -bT SJ.v6.2.chromosome.block_digested_RTRMarked.fasta "$f"LabsamplesSJ6.sam > "$f"LabsamplesSJ6.bam

done
```

The following uses bioawk version 20110810

samtools version 1.9 (using htslib 1.9).

Labsamples SJ6

```
for f in S3_5 S3_10 S3_15 H4_5 H4_10 H4_15 H3_5 H3_10 H3_15 S2_5 S2_10 S2_15 H2_5 H2_10 S1_5 S1_10 S4_5 S4_10 H1_5 H1_10 H1_15

do

samtools sort -n -f "$f"LabsamplesSJ6.bam "$f"LabsamplesSJ6.sorted.bam

done

for f in S3_5 S3_10 S3_15 H4_5 H4_10 H4_15 H3_5 H3_10 H3_15 S2_5 S2_10 S2_15 H2_5 H2_10 S1_5 S1_10 S4_5 S4_10 H1_5 H1_10 H1_15

do

samtools view -h -o "$f"LabsamplesSJ6.sorted.sam "$f"LabsamplesSJ6.sorted.bam

done

for f in S3_5 S3_10 S3_15 H4_5 H4_10 H4_15 H3_5 H3_10 H3_15 S2_5 S2_10 S2_15 H2_5 H2_10 S1_5 S1_10 S4_5 S4_10 H1_5 H1_10 H1_15

do

samtools view -H "$f"LabsamplesSJ6.sorted.bam > "$f"LabsamplesSJ6.sorted.header
```

```
done

for f in S3_5 S3_10 S3_15 H4_5 H4_10 H4_15 H3_5 H3_10 H3_15 S2_5 S2_10 S2_15 H2_5 H2_10 S1_5 S1_10 S4_5 S4_10 H1_5 H1_10 H1_15

do

bioawk  -c sam  '{ $flag = and($flag , 3860 ) ; print $0 }' "$f"LabsamplesSJ6.sorted.sam |    \

cat  "$f"LabsamplesSJ6.sorted.header  -    > "$f"LabsamplesSJ6.sorted2.sam

done

for f in S3_5 S3_10 S3_15 H4_5 H4_10 H4_15 H3_5 H3_10 H3_15 S2_5 S2_10 S2_15 H2_5 H2_10 S1_5 S1_10 S4_5 S4_10 H1_5 H1_10 H1_15

do

 htseq-count -f sam -s no "$f"LabsamplesSJ6.sorted2.sam SJ.v6.2.chromosome.block_digested_RTRMarked_corrected.gff3 > "$f"LabsamplesSJ6.counts

done

for f in S3_5 S3_10 S3_15 H4_5 H4_10 H4_15 H3_5 H3_10 H3_15 S2_5 S2_10 S2_15 H2_5 H2_10 S1_5 S1_10 S4_5 S4_10 H1_5 H1_10 H1_15

do

grep -v "__" "$f"LabsamplesSJ6.counts > "$f"LabsamplesSJ6.counttable

done
```

Fieldsamples

Step by step!

```
for f in FH1 FH2 FH3 FH4 FH5 FH6 FH7 FH8a FH9 FH10 FS1 FS2 FS3 FS4 FS5 FS6 FS7 FS8a FS9 FS10

do

samtools sort -n -f "$f"FieldsamplesSJ6.bam "$f"FieldsamplesSJ6.sorted.bam

done

for f in FH1 FH2 FH3 FH4 FH5 FH6 FH7 FH8a FH9 FH10 FS1 FS2 FS3 FS4 FS5 FS6 FS7 FS8a FS9 FS10

do

samtools view -h -o "$f"FieldsamplesSJ6.sorted.sam "$f"FieldsamplesSJ6.sorted.bam

done

for f in FH1 FH2 FH3 FH4 FH5 FH6 FH7 FH8a FH9 FH10 FS1 FS2 FS3 FS4 FS5 FS6 FS7 FS8a FS9 FS10

do
```

```
samtools view -H "$f"FieldsamplesSJ6.sorted.bam > "$f"FieldsamplesSJ6.sorted.header

done

for f in FH1 FH2 FH3 FH4 FH5 FH6 FH7 FH8a FH9 FH10 FS1 FS2 FS3 FS4 FS5 FS6 FS7 FS8a FS9 FS10

do

bioawk  -c sam  '{ $flag = and($flag , 3860 ) ; print $0 }' "$f"FieldsamplesSJ6.sorted.sam |    \

cat "$f"FieldsamplesSJ6.sorted.header  -    > "$f"FieldsamplesSJ6.sorted2.sam

done

for f in FH1 FH2 FH3 FH4 FH5 FH6 FH7 FH8a FH9 FH10 FS1 FS2 FS3 FS4 FS5 FS6 FS7 FS8a FS9 FS10

do

htseq-count -f sam -s no "$f"FieldsamplesSJ6.sorted2.sam SJ.v6.2.chromosome.block_digested_RTRMarked_corrected.gff3 > "$f"FieldsamplesSJ6.counts

done

for f in FH1 FH2 FH3 FH4 FH5 FH6 FH7 FH8a FH9 FH10 FS1 FS2 FS3 FS4 FS5 FS6 FS7 FS8a FS9 FS10

do

grep -v "__" "$f"FieldsamplesSJ6.counts > "$f"FieldsamplesSJ6.counttable

done
```

The results that can be used for *differential methylation analysis* are in the *.counttable files.

For this analysis it is important that lab and field samples are in separate files, hence the H1_15 (re-sequenced with the fieldsamples) now has to be run with the labsamples again.

Combining the counttables in a single file:

Lab samples

```
paste S3_5LabsamplesSJ6.counttable S3_10LabsamplesSJ6.counttable S3_15LabsamplesSJ6.counttable H4_5LabsamplesSJ6.counttable H4_10LabsamplesSJ6.counttable H4_15LabsamplesSJ6.counttable \
H3_5LabsamplesSJ6.counttable H3_10LabsamplesSJ6.counttable H3_15LabsamplesSJ6.counttable S2_5LabsamplesSJ6.counttable S2_10LabsamplesSJ6.counttable S2_15LabsamplesSJ6.counttable \
H2_5LabsamplesSJ6.counttable H2_10LabsamplesSJ6.counttable S1_5LabsamplesSJ6.counttable S1_10LabsamplesSJ6.counttable S4_5LabsamplesSJ6.counttable S4_10LabsamplesSJ6.counttable H1_5LabsamplesSJ6.counttable \
H1_10LabsamplesSJ6.counttable H1_15LabsamplesSJ6.counttable > testSJ6.txt
```

```
echo -e "Tag\tS3_5\tS3_10\tS3_15\tH4_5\tH4_10\tH4_15\tH3_5\tH3_10\tH3_15\tS2_5\tS2_10\tS2_15\tH2_5\tH2_10\tS1_5\tS1_10\tS4_5\tS4_10\tH1_5\tH1_10\tH1_15" > AllCountTablesLabsamplesSJ6.txt
cut -f 1,2,4,6,8,10,12,14,16,18,20,22,24,26,28,30,32,34,36,38,40,42 testSJ6.txt >> AllCountTablesLabsamplesSJ6.txt
cut -f 2,3,4,5,6,7,8,9,10,11,12,13,14,15,16,17,18,19,20,21,22 AllCountTablesLabsamplesSJ6.txt > AllCountTablesLabsamplesSJ6Reduced.txt

cut -f 2,3,4,5,6,7,8,9,10,11,12,13,14,15,16,17,18,19,20,21,22 AllCountTablesLabsamplesChloroplast.txt > AllCountTablesLabsamplesChloroplastReduced.txt
```

Field samples

```
paste FH10FieldsamplesSJ6.counttable FH1FieldsamplesSJ6.counttable FH2FieldsamplesSJ6.counttable FH3FieldsamplesSJ6.counttable FH4FieldsamplesSJ6.counttable FH5FieldsamplesSJ6.counttable \
FH6FieldsamplesSJ6.counttable FH7FieldsamplesSJ6.counttable FH8aFieldsamplesSJ6.counttable FH9FieldsamplesSJ6.counttable FS10FieldsamplesSJ6.counttable FS1FieldsamplesSJ6.counttable \
FS2FieldsamplesSJ6.counttable FS3FieldsamplesSJ6.counttable FS4FieldsamplesSJ6.counttable FS5FieldsamplesSJ6.counttable FS6FieldsamplesSJ6.counttable FS7FieldsamplesSJ6.counttable \
FS8aFieldsamplesSJ6.counttable FS9FieldsamplesSJ6.counttable > testFieldsamplesSJ6.txt
echo -e "Tag\tFH10\tFH1\tFH2\tFH3\tFH4\tFH5\tFH6\tFH7\tFH8a\tFH9\tFS10\tFS1\tFS2\tFS3\tFS4\tFS5\tFS6\tFS7\tFS8a\tFS9" > AllCountTablesFieldsamplesSJ6.txt
cut -f 1,2,4,6,8,10,12,14,16,18,20,22,24,26,28,30,32,34,36,38,40 testFieldsamplesSJ6.txt >> AllCountTablesFieldsamplesSJ6.txt
cut -f 2,3,4,5,6,7,8,9,10,11,12,13,14,15,16,17,18,19,20,21 AllCountTablesFieldsamplesSJ6.txt > AllCountTablesFieldsamplesSJ6Reduced.txt
```

Counttable overview

R script CounttableOverview.r to create a table and a figure for each sample that give an overview of coverages

The script is located in the mapping folder, and can be run in two ways, in R or tmux:

Tmux: look at script via '**more CounttablesOverview.r'**

Labsamples:

```
 # reading all Counttables
library(tidyr)
library(ggplot2)

cat
("Sample","Reference","N.Potential.Sites","N.Covered.Sites","N.CoveredSites>2","N.Cover
```

```
edSites>3","N.CoveredSites>4","N.CoveredSites>5","N.CoveredSites>10","Average.Covera
ge","Minimum.Coverage","Maximum.Coverage","\n",file="CounttableOverviewLabsample
sSJ6.csv",sep=",",append=FALSE)

for (r in c("LabsamplesSJ6")){

  Counttables                                                                    <-
read.csv(("AllCountTablesLabsamplesSJ6Reduced.txt"),header=TRUE,sep="\t")
                        # Get the number of sites covered in at least one samples
  n <- length(
    Counttables[
      rowSums(Counttables)>0,
1]
  )

  print (paste(r," sites covered across all samples: ", n,sep=""))

  CounttablesExcludingZeros <-      Counttables[
    rowSums(Counttables)>0,
    ]

      #  png(filename = "CounttableHistogramsStartingWithZeroCounts.png",
      #                  width = 1000, height = 1000, units = "px", pointsize = 12,
      #                  bg = "white")
      #  print(
      #  ggplot(gather(CounttablesExcludingZeros), aes(value)) +
      #    geom_histogram() +
      #  facet_wrap(~key,scales="free")
      #  )
      #  dev.off()#

      #  png(filename = "CounttableHistogramsStartingWithOneCounts.png",
      #    width = 1000, height = 1000, units = "px", pointsize = 12,
      #    bg = "white")
      #  print(
      #    ggplot(gather(CounttablesExcludingZeros), aes(value)) +
      #    geom_histogram() +
      #    facet_wrap(~key,scales="free")+
      #    xlim(1,1000)
      #  )
      #    dev.off()

for                                    (s                                    in
c("S3_5","S3_10","S3_15","H4_5","H4_10","H4_15","H3_5","H3_10","H3_15","S2_5","S2_
10","S2_15","H2_5","H2_10","S1_5","S1_10","S4_5","S4_10","H1_5","H1_10","H1_15")) {

sample <- Counttables[,which(colnames(Counttables)==s)]
PotentialSites <- length(sample)
```

```
SitesCovered <- length(sample[sample>0])
SitesCoveredAbove2 <- length(sample[sample>2])
SitesCoveredAbove3 <- length(sample[sample>3])
SitesCoveredAbove4 <- length(sample[sample>4])
SitesCoveredAbove5 <- length(sample[sample>5])
SitesCoveredAbove10 <- length(sample[sample>10])
MinimumCoverage <- min(sample[sample>0])
MaximumCoverage <- max(sample[sample>0])
MeanCoverage <- mean(sample[sample>0])
cat
(s,r,PotentialSites,SitesCovered,SitesCoveredAbove2,SitesCoveredAbove3,SitesCoveredAbo
ve4,SitesCoveredAbove5,SitesCoveredAbove10,MeanCoverage,MinimumCoverage,Maxim
umCoverage,"\n",file="CounttableOverviewLabsamplesSJ6.csv",sep=",",append=TRUE)
  }
}

### Fieldsamples
 # reading all Counttables
library(tidyr)
library(ggplot2)

cat
("Sample","Reference","N.Potential.Sites","N.Covered.Sites","N.CoveredSites>2","N.Cover
edSites>3","N.CoveredSites>4","N.CoveredSites>5","N.CoveredSites>10","Average.Covera
ge","Minimum.Coverage","Maximum.Coverage","\n",file="CounttableOverviewFieldsampl
esSJ6.csv",sep=",",append=FALSE)

for (r in c("FieldsamplesSJ6")){

Counttables                                                                    <-
read.csv(("AllCountTablesFieldsamplesSJ6Reduced.txt"),header=TRUE,sep="\t")
# Get the number of sites covered in at least one samples
n <- length(Counttables[
rowSums(Counttables)>0,
1]
)

print (paste(r," sites covered across all samples: ", n,sep=""))

CounttablesExcludingZeros <- Counttables[
rowSums(Counttables)>0,
]

#  png(filename = "CounttableHistogramsStartingWithZeroCounts.png",
#                          width = 1000, height = 1000, units = "px", pointsize = 12,
#                          bg = "white")
#  print(
#  ggplot(gather(CounttablesExcludingZeros), aes(value)) +
```

```
#     geom_histogram() +
#  facet_wrap(~key,scales="free")
#  )
#  dev.off()#

#  png(filename = "CounttableHistogramsStartingWithOneCounts.png",
#     width = 1000, height = 1000, units = "px", pointsize = 12,
#     bg = "white")
#  print(
#     ggplot(gather(CounttablesExcludingZeros), aes(value)) +
#     geom_histogram() +
#     facet_wrap(~key,scales="free")+
#     xlim(1,1000)
#  )
#     dev.off()
```

```
for (s in c("FH10","FH1","FH2","FH3","FH4","FH5","FH6","FH7","FH8a","FH9","FS10","FS1","FS2","FS3","FS4","FS5","FS6","FS7","FS8a","FS9")) {

sample <- Counttables[,which(colnames(Counttables)==s)]
PotentialSites <- length(sample)
SitesCovered <- length(sample[sample>0])
SitesCoveredAbove2 <- length(sample[sample>2])
SitesCoveredAbove3 <- length(sample[sample>3])
SitesCoveredAbove4 <- length(sample[sample>4])
SitesCoveredAbove5 <- length(sample[sample>5])
SitesCoveredAbove10 <- length(sample[sample>10])
MinimumCoverage <- min(sample[sample>0],na.rm=TRUE)
MaximumCoverage <- max(sample[sample>0],na.rm=TRUE)
MeanCoverage <- mean(sample[sample>0],na.rm=TRUE)
cat
(s,r,PotentialSites,SitesCovered,SitesCoveredAbove2,SitesCoveredAbove3,SitesCoveredAbove4,SitesCoveredAbove5,SitesCoveredAbove10,MeanCoverage,MinimumCoverage,MaximumCoverage,"\n",file="CounttableOverviewFieldsamplesSJ6.csv",sep=",",append=TRUE)
}
}
```

Counttableoverview Lab-And Fieldsamples combined:

```
# The commands can be entered (one after another) in R console

# Or the whole script can be run in tmux via (remove #)

#R CMD BATCH CounttablesOverview.r

# reading all Counttables
library(tidyr)
library(ggplot2)
```

```
cat
("Sample","Reference","N.Potential.Sites","N.Covered.Sites","N.CoveredSites>2","N.Cover
edSites>3","N.CoveredSites>4","N.CoveredSites>=5","N.CoveredSites>5","N.CoveredSites>
10","Average.Coverage","Minimum.Coverage","Maximum.Coverage","\n",file="Counttabl
eOverviewLabAndFieldsamplesSJ6.csv",sep=",an",append=FALSE)

for (r in c("LabsamplesSJ6","FieldsamplesSJ6")){

Counttables <-
read.csv(paste("AllCountTables",r,"Reduced.txt",sep=""),header=TRUE,sep="\t")
# Get the number of sites covered in at least one samples
n <- length(
Counttables[
rowSums(Counttables)>0,
1]
)

print (paste(r," sites covered across all samples: ", n,sep=""))

CounttablesExcludingZeros <- Counttables[
rowSums(Counttables)>0,
]

#  png(filename = "CounttableHistogramsStartingWithZeroCounts.png",
#                          width = 1000, height = 1000, units = "px", pointsize = 12,
#                          bg = "white")
#  print(
#  ggplot(gather(CounttablesExcludingZeros), aes(value)) +
#    geom_histogram() +
#  facet_wrap(~key,scales="free")
#  )
#  dev.off()#

#  png(filename = "CounttableHistogramsStartingWithOneCounts.png",
#    width = 1000, height = 1000, units = "px", pointsize = 12,
#    bg = "white")
#  print(
#    ggplot(gather(CounttablesExcludingZeros), aes(value)) +
#    geom_histogram() +
#    facet_wrap(~key,scales="free")+
#    xlim(1,1000)
#  )
#    dev.off()

for (s in
c("S3_5","S3_10","S3_15","H4_5","H4_10","H4_15","H3_5","H3_10","H3_15","S2_5","S2_
10","S2_15","H2_5","H2_10","S1_5","S1_10","S4_5","S4_10","H1_5","H1_10","H1_15","F
```

```
H10","FH1","FH2","FH3","FH4","FH5","FH6","FH7","FH8a","FH9","FS10","FS1","FS2","FS3"
,"FS4","FS5","FS6","FS7","FS8a","FS9")) {

sample <- Counttables[,which(colnames(Counttables)==s)]
PotentialSites <- length(sample)
SitesCovered <- length(sample[sample>0])
SitesCoveredAbove5 <- length(sample[sample>5])
SitesCoveredAbove10 <- length(sample[sample>10])
MinimumCoverage <- min(sample[sample>0])
MaximumCoverage <- max(sample[sample>0])
MeanCoverage <- mean(sample[sample>0])
cat
(s,r,PotentialSites,SitesCovered,SitesCoveredAbove5,SitesCoveredAbove10,MeanCoverage,
MinimumCoverage,MaximumCoverage,"\n",file="CounttableOverviewLabAndFieldsamples
SJ6.csv",sep=",",append=TRUE)
}
}
```

The findings are summarized in

CounttableOverview.csv

two further graphs summarize the findings in

CounttableOverview.xlsx

It shows that an increase in the raw number of reads does not necessarily increase the number of sites covered > 5 times

Converting raw reads to reads per million

Prior to statistical analysis with edgeR (or DeSeq) the reads need to be standardised.

This is done using the following script:

```
### Labsamples

Table=read.table("AllCountTablesLabsamplesSJ6.txt",header=TRUE)

Rawdata <- Table[,2:length(Table[1,])]

# Set coverages <3 to 0

Rawdata[Rawdata<3]=0

Rawdata2 <- cbind(Table[,1],Rawdata)

colnames(Rawdata2) <- colnames(Table)

write.table(Rawdata2,file="AllCountTablesLabsamplesSJ6CoverageAbove2.txt", append =
FALSE, quote = FALSE, sep = "\t",

eol = "\n", na = "NA", dec = ".", row.names = FALSE,

col.names = TRUE)
```

Calculate reads per million

```
Divided <- t(t(Rawdata)/colSums(Rawdata))

RPM=1000000*Divided

RPM <- as.data.frame(RPM)

RPM <- cbind(Table[,1],RPM)

colnames(RPM)=colnames(Table)

write.table(RPM,file="AllCountTablesLabsamplesSJ6CoverageAbove2_RPM.txt", append = FALSE, quote = FALSE, sep = "\t",

eol = "\n", na = "NA", dec = ".", row.names = FALSE,

col.names = TRUE)
```

restrict to those tags that are covered at least in one of the libraries

```
RPMdata.reduced=RPM

Rawdata.reduced=Rawdata2

RPMdata.reduced <- RPMdata.reduced[rowSums(RPMdata.reduced[, -1])>0, ]

colnames(RPMdata.reduced) <- c("Scaffold",colnames(RPMdata.reduced)[2:length(colnames(RPMdata.reduced))])

Rawdata.reduced <- Rawdata.reduced[rowSums(Rawdata.reduced[, -1])>0, ]

colnames(Rawdata.reduced) <- c("Scaffold",colnames(Rawdata.reduced)[2:length(colnames(Rawdata.reduced))])

write.table(RPMdata.reduced,file="AllCountTablesLabsamplesSJ6CoverageAbove2_RPM_CoveredInAtLeast1Library.txt", append = FALSE, quote = FALSE, sep = "\t",

eol = "\n", na = "NA", dec = ".", row.names = FALSE,

col.names = TRUE)
```

Fieldsamples

```
Table=read.table("AllCountTablesFieldsamplesSJ6.txt",header=TRUE)

Rawdata <- Table[,2:length(Table[1,])]
```

Set coverages <3 to 0

```
Rawdata[Rawdata<3]=0

Rawdata2 <- cbind(Table[,1],Rawdata)

colnames(Rawdata2) <- colnames(Table)

write.table(Rawdata2,file="AllCountTablesFieldsamplesSJ6CoverageAbove2.txt", append = FALSE, quote = FALSE, sep = "\t",
```

```
eol = "\n", na = "NA", dec = ".", row.names = FALSE,

col.names = TRUE)

# Calculate reads per million

Divided <- t(t(Rawdata)/colSums(Rawdata))

RPM=1000000*Divided

RPM <- as.data.frame(RPM)

RPM <- cbind(Table[,1],RPM)

colnames(RPM)=colnames(Table)

write.table(RPM,file="AllCountTablesFieldsamplesSJ6CoverageAbove2_RPM.txt", append = FALSE, quote = FALSE, sep = "\t",

eol = "\n", na = "NA", dec = ".", row.names = FALSE,

col.names = TRUE)

# restrict to those tags that are covered at least in one of the libraries

RPMdata.reduced=RPM

Rawdata.reduced=Rawdata2

RPMdata.reduced <- RPMdata.reduced[rowSums(RPMdata.reduced[, -1])>0, ]

colnames(RPMdata.reduced) <- c("Scaffold",colnames(RPMdata.reduced)[2:length(colnames(RPMdata.reduced))])

Rawdata.reduced <- Rawdata.reduced[rowSums(Rawdata.reduced[, -1])>0, ]

colnames(Rawdata.reduced) <- c("Scaffold",colnames(Rawdata.reduced)[2:length(colnames(Rawdata.reduced))])

write.table(RPMdata.reduced,file="AllCountTablesFieldsamplesSJ6CoverageAbove2_RPM_CoveredInAtLeast1Library.txt", append = FALSE, quote = FALSE, sep = "\t",

eol = "\n", na = "NA", dec = ".", row.names = FALSE,

col.names = TRUE)
```

Splitting between CG and CHG sites

```
### Labsamples

Table <- read.csv("AllCountTablesLabsamplesSJ6CoverageAbove2_RPM_CoveredInAtLeast1Library.txt",header=TRUE,sep="\t")

Scaffold.splitted <- strsplit(as.character(Table$Scaffold),",")

SequenceContexts <- unlist(lapply(Scaffold.splitted, '[[', 2))

SequenceContexts.converted <- SequenceContexts
```

```
SequenceContexts.converted[SequenceContexts.converted=="CCGG"]="CG"

SequenceContexts.converted[SequenceContexts.converted=="CCAGG"]="CHG"

SequenceContexts.converted[SequenceContexts.converted=="CCCGG"]="CHG"

SequenceContexts.converted[SequenceContexts.converted=="CCTGG"]="CHG"

SequenceContexts.converted[SequenceContexts.converted=="CCTGT"]="CHG"

TableWithSequenceContexts <-cbind(Table,SequenceContexts=SequenceContexts.converted)

# Counting total number of methylated CGs and CHGs

length(TableWithSequenceContexts$SequenceContexts[TableWithSequenceContexts$SequenceContexts=="CG"])

length(TableWithSequenceContexts$SequenceContexts[TableWithSequenceContexts$SequenceContexts=="CHG"])

write.table(TableWithSequenceContexts, file = "AllCountTablesLabsamplesSJ6CoverageAbove2_RPM_CoveredInAtLeast1Library_CGAnnotated.txt", append = FALSE, quote = FALSE, sep = "\t",

eol = "\n", na = "NA", dec = ".", row.names = FALSE,  col.names = TRUE)


### Fieldsamples

Table <-read.csv("AllCountTablesFieldsamplesSJ6CoverageAbove2_RPM_CoveredInAtLeast1Library.txt",header=TRUE,sep="\t")

Scaffold.splitted <- strsplit(as.character(Table$Scaffold),",")

SequenceContexts <- unlist(lapply(Scaffold.splitted, '[[', 2))

SequenceContexts.converted <- SequenceContexts

SequenceContexts.converted[SequenceContexts.converted=="CCGG"]="CG"

SequenceContexts.converted[SequenceContexts.converted=="CCAGG"]="CHG"

SequenceContexts.converted[SequenceContexts.converted=="CCCGG"]="CHG"

SequenceContexts.converted[SequenceContexts.converted=="CCTGG"]="CHG"

SequenceContexts.converted[SequenceContexts.converted=="CCTGT"]="CHG"

TableWithSequenceContexts <-cbind(Table,SequenceContexts=SequenceContexts.converted)

# Counting total number of methylated CGs and CHGs

length(TableWithSequenceContexts$SequenceContexts[TableWithSequenceContexts$SequenceContexts=="CG"])
```

```
length(TableWithSequenceContexts$SequenceContexts[TableWithSequenceContexts$SequenceContexts=="CHG"])

write.table(TableWithSequenceContexts, file = "AllCountTablesFieldsamplesSJ6CoverageAbove2_RPM_CoveredInAtLeast1Library_CGAnnotated.txt", append = FALSE, quote = FALSE, sep = "\t",

eol = "\n", na = "NA", dec = ".", row.names = FALSE,  col.names = TRUE)
```

edgeR

```
LabSJ6 <- read.table("AllCountTablesLabsamplesSJ6.txt",header=TRUE)

FieldSJ6 <- read.table("AllCountTablesFieldsamplesSJ6.txt",header=TRUE)

AllSJ6Tables <- merge(LabSJ6,FieldSJ6,by="Tag",all=TRUE)

Tables=c("AllSJ6Tables")

for (t in Tables){

Table <- get(t)

Rawdata <- Table[,2:length(Table[1,])]

# Set coverages <3 to 0

Rawdata[Rawdata<3]=0

# Calculate reads per million

Divided <- t(t(Rawdata)/colSums(Rawdata))

RPM=1000000*Divided

RPM <- as.data.frame(RPM)

RPM <- cbind(Table[,1],RPM)

colnames(RPM)=c("Scaffold",colnames(Rawdata))

# Annotate CG and CHG regions

Scaffold.splitted <- strsplit(as.character(RPM$Scaffold),",")

SequenceContexts <- unlist(lapply(Scaffold.splitted, '[[', 2))

SequenceContexts.converted <- SequenceContexts

SequenceContexts.converted[SequenceContexts.converted=="CCGG"]="CG"

SequenceContexts.converted[SequenceContexts.converted=="CCAGG"]="CHG"

SequenceContexts.converted[SequenceContexts.converted=="CCCGG"]="CHG"

SequenceContexts.converted[SequenceContexts.converted=="CCTGG"]="CHG"

SequenceContexts.converted[SequenceContexts.converted=="CCTGT"]="CHG"
```

```
TableWithSequenceContexts <-
cbind(RPM$Scaffold,SequenceContexts=SequenceContexts.converted,RPM[,2:length(RPM[
1,])])
```

Counting total number of methylated CGs and CHGs

```
length(TableWithSequenceContexts$SequenceContexts[TableWithSequenceContexts$Sequ
enceContexts=="CG"])
```

```
length(TableWithSequenceContexts$SequenceContexts[TableWithSequenceContexts$Sequ
enceContexts=="CHG"])
```

restrict to those tags that are covered at least in one of the libraries

```
RPMdata.reduced <- TableWithSequenceContexts[rowSums(TableWithSequenceContexts[,
c(-1,-2)])>0, ]
```

```
write.table(RPMdata.reduced, file =
paste(t,"CoverageAbove2_RPM_CGAnnotated_CoveredInAtLeastOneLibrary.txt",sep="."),
append = FALSE, quote = FALSE, sep = "\t",
```

```
eol = "\n", na = "NA", dec = ".", row.names = FALSE,  col.names = TRUE)
```

```
}
```

To separate field from LabSamples

```
AllSamplesSJ6=read.table("AllSJ6Tables.CoverageAbove2_RPM_CGAnnotated_CoveredInA
tLeastOneLibrary.txt",header=TRUE)
```

```
AllLabSamplesSJ6=AllSamplesSJ6[,c(1,2,grep("_",colnames(AllSamplesSJ6)))]
```

```
AllFieldSamplesSJ6=AllSamplesSJ6[,c(1,2,grep("_",colnames(AllSamplesSJ6),invert=TRUE))]
```

Principal component analysis (PCA) (R)

```
library(ggplot2)
library("FactoMineR")
library("factoextra")
library(devtools)
#Load latest version of heatmap.3 function (is an R script that has to be in working directory)
source("heatmap3.R") # heatmap.3 based on https://www.biostars.org/p/18211/
add_legend <- function(...) {
opar <- par(fig=c(0, 1, 0, 1), oma=c(0, 0, 0, 0),
      mar=c(0, 0, 0, 0), new=TRUE)
on.exit(par(opar))
plot(0, 0, type='n', bty='n', xaxt='n', yaxt='n')
legend(...)
}
# defining colors
# http://www.stat.columbia.edu/~tzheng/files/Rcolor.pdf
Spitsbergen <- "cyan1"
Helgoland <- "darkorange1"
Five <- "blue1"
Ten <- "yellow"
Fifteen <- "red2"
```

```
LabSample <- "green"
FieldSample <- "purple3"
#read in data to make counts table; row names are in the first column -> row.names=1
for (species in c("SJ6")){
TableID <-
read.delim(paste("All",species,"Tables.CoverageAbove2_RPM_CGAnnotated_CoveredInAtL
eastOneLibrary.txt",sep=""), header=TRUE, stringsAsFactors=TRUE,row.names=1)
All=TableID[,-1]
Labsamples=All[,grep("_",colnames(All))]
Fieldsamples=All[,-grep("_",colnames(All))]
# Running PCA
for (labfield in c("All","Labsamples","Fieldsamples")){
Transposed <- as.data.frame(t(get(labfield)))
res.pcatall <- PCA(Transposed, scale.unit=FALSE, ncp=5, graph = FALSE)

save.image("SJ6Image.Rdata")
# Define Differences
if (labfield=="All"){
Location <- c(rep("Spitsbergen",3),rep("Helgoland",6),
              rep("Spitsbergen",3),rep("Helgoland",2),
              rep("Spitsbergen",4),rep("Helgoland",13),
              rep("Spitsbergen",10))
LabField <- c(rep("LabSample",21),rep("FieldSample",20))
Temperature <-
c(rep(c("5","10","15"),4),rep(c("5","10"),3),"5","10","15",rep("10",10),rep("5",10))

Location.levels <- factor(Location,levels=c("Spitsbergen","Helgoland"))
LabField.levels <- factor(LabField,levels=c("LabSample","FieldSample"))
Temperature.levels <- factor(Temperature,levels=c("5","10","15"))

Diffs <- paste(Location,LabField,Temperature,sep=".")
Diffs.levels <- factor(Diffs,levels=c("Spitsbergen.LabSample.5",
                                      "Spitsbergen.LabSample.10",
                                      "Spitsbergen.LabSample.15",
                                      "Helgoland.LabSample.5",
                                      "Helgoland.LabSample.10",
                                      "Helgoland.LabSample.15",
                                      "Spitsbergen.FieldSample.5",
                                      "Helgoland.FieldSample.10"
))

png(filename=paste("PCA_",labfield,"_",species,"_LabField.png",sep=""),width=180,height
=180,units='mm',res=700,pointsize=10)
print(fviz_pca_ind(res.pcatall,
       habillage=LabField.levels,
                  repel=TRUE,
             addEllipses = TRUE, # Concentration ellipses
     ellipse.type = "confidence",
    legend.title = "Lab or Field Sample",
                               title="",
                          pointsize=2.7,
                          labelsize=2)+
```

```
scale_color_brewer(palette="Dark2")+

theme(axis.title=element_text(size=17),
          axis.text=element_text(size=15),
      legend.title=element_text(size=17),
      legend.text=element_text(size=15)
      )
  )
dev.off()

}

      if (labfield=="Labsamples"){
        Location <- c(rep("Spitsbergen",3),rep("Helgoland",6),
                      rep("Spitsbergen",3),rep("Helgoland",2),
                      rep("Spitsbergen",4),rep("Helgoland",3))
        Temperature <- c(rep(c("5","10","15"),4),rep(c("5","10"),3),"5","10","15")
        Location.levels <- factor(Location,levels=c("Spitsbergen","Helgoland"))
        Temperature.levels <- factor(Temperature,levels=c("5","10","15"))

        Diffs <- paste(Location,Temperature,sep=".")
        Diffs.levels <- factor(Diffs,levels=c("Spitsbergen.5",
                                              "Spitsbergen.10",
                                              "Spitsbergen.15",
                                              "Helgoland.5",
                                              "Helgoland.10",
                                              "Helgoland.15"
                                              ))
}

      if (labfield=="Fieldsamples"){
        Location <- c(rep("Helgoland",10),rep("Spitsbergen",10))
        Temperature <- c(rep("10",10),rep("5",10))
        Location.levels <- factor(Location,levels=c("Helgoland","Spitsbergen"))
        Temperature.levels <- factor(Temperature,levels=c("10","5"))

        Diffs <- paste(Location,Temperature,sep=".")
        Diffs.levels <- factor(Diffs,levels=c("Spitsbergen.5",
                                              "Helgoland.10"
                                              ))
}


png(filename=paste("PCA_",labfield,"_",species,"_Location.png",sep=""),width=180,height
=180,units='mm',res=700,pointsize=10)
      print(fviz_pca_ind(res.pcatall,
                         habillage=Location.levels,
                         repel=TRUE,
                         addEllipses = TRUE, # Concentration ellipses
                         ellipse.type = "confidence",
                         legend.title = "Location",
                         title="",
```

```
                pointsize=2.7,
                labelsize=2)+
        scale_color_brewer(palette="Dark2")+

        theme(axis.title=element_text(size=17),
            axis.text=element_text(size=15),
            legend.title=element_text(size=17),
            legend.text=element_text(size=15)
      ))
    dev.off()


### PCA plots
png(filename=paste("PCA_",labfield,"_",species,"_Temperature.png",sep=""),width=180,h
eight=180,units='mm',res=700,pointsize=10)
    print(fviz_pca_ind(res.pcatall,
                habillage=Temperature.levels,
                repel=TRUE,
                addEllipses = TRUE, # Concentration ellipses
                ellipse.type = "confidence",
                legend.title = "Temperature",
                title="",
                pointsize=2.7,
                labelsize=2)+
        scale_color_brewer(palette="Dark2")+

        theme(axis.title=element_text(size=17),
            axis.text=element_text(size=15),
            legend.title=element_text(size=17),
            legend.text=element_text(size=15)
            )
        )
    dev.off()


png(filename=paste("PCA_",labfield,"_",species,"_Diffs.png",sep=""),width=180,height=18
0,units='mm',res=700,pointsize=10)
    print(fviz_pca_ind(res.pcatall,
                habillage=Diffs.levels,
                repel=TRUE,
                addEllipses = TRUE, # Concentration ellipses
                ellipse.type = "confidence",
                legend.title = "All",
                title="",
                pointsize=2.7,
                labelsize=2)+
        scale_color_brewer(palette="Dark2")+

        theme(axis.title=element_text(size=17),
            axis.text=element_text(size=15),
            legend.title=element_text(size=17),
            legend.text=element_text(size=15)
```

```
    )
    )
     dev.off()
```

###Create tree plots

```
     res.hcpctall <- HCPC(res.pcatall, nb.clust=3,min=1,graph = FALSE,order=TRUE)

png(filename=paste("Cluster_",labfield,".png",sep=""),width=250,height=120,units='mm',r
es=700,pointsize=10)
par(xpd=NA,mar=c(1,4,3,1),oma=c(0,0,0,0),font=7,ps=10,bg="white")
plot.HCPC(res.hcpctall,choice="tree",tree.barplot=FALSE,main="tall")
dev.off()
```

Creating dendrogram that can be recognized by the heatmap.2 function

```
h.d <- as.dendrogram(res.hcpctall$call$t$tree)

if (labfield=="All"){
   Location <- c(rep(Spitsbergen,3),rep(Helgoland, 6),
                 rep(Spitsbergen,3),rep(Helgoland,2),
                 rep(Spitsbergen,4),rep(Helgoland,13),
                 rep(Spitsbergen,10))[match(labels(h.d),colnames(All))]
   LabField <- c(rep(LabSample,21),rep(FieldSample,20))[match(labels(h.d),colnames(All))]
   Temperature <- c(rep(c(Five,Ten,Fifteen),4),
                    rep(c(Five,Ten),3),Five,Ten,Fifteen,
                    rep(Ten,10),rep(Five,10))[match(labels(h.d),colnames(All))]
CategoryColors <- cbind(LabField,Location,Temperature)
                  }

if (labfield=="Labsamples"){
   Location                    <-                    c(rep(Spitsbergen,3),rep(Helgoland,6),
rep(Spitsbergen,3),rep(Helgoland,2),
rep(Spitsbergen,4),rep(Helgoland,3))[match(labels(h.d),colnames(Labsamples))]
Temperature                                                                                    <-
c(rep(c(Five,Ten,Fifteen),4),rep(c(Five,Ten),3),Five,Ten,Fifteen)[match(labels(h.d),colnames
(Labsamples))]
 CategoryColors <- cbind(Location,Temperature)
      }

if (labfield=="Fieldsamples"){
    Location                                                                                   <-
c(rep(Helgoland,10),rep(Spitsbergen,10))[match(labels(h.d),colnames(Fieldsamples))]
    Temperature <- c(rep(Ten,10),rep(Five,10))[match(labels(h.d),colnames(Fieldsamples))]
    CategoryColors <- cbind(Location,Temperature)
         }
```

Use this as orientation https://www.datanovia.com/en/lessons/heatmap-in-r-static-and-interactive-visualization/

```
     n <-  as.matrix(get(labfield))[,match(labels(h.d),colnames(get(labfield)))]

png(filename=paste("Hierclust_",labfield,"SJ6.png",sep=""),width=160,height=190,units='
mm',res=700,pointsize=9)
par(oma=c(15,0,0,15),mar=c(1,1,1,1))
```

```
heatmap.3(n[1:200,],
dendrogram="column",
scale="none",
margins=c(5,3),
key=FALSE,
trace="none",
density.info="none",
Colv=FALSE,
labRow="",
ColSideColors = as.matrix(CategoryColors),
cexCol=1
)
if(labfield=="All"){
legend("topright",legend=c("Labsamples","Fieldsamples","","Spitsbergen","Helgoland","",
"5","10","15"),
fill=c(LabSample,FieldSample,"white",Spitsbergen,Helgoland,"white","blue","yellow","red
"), border=FALSE, bty="n", y.intersp = 0.7, cex=0.7)
}
if(labfield=="Labsamples"){
legend("topright",legend=c("Spitsbergen","Helgoland","","5","10","15"),
fill=c(Spitsbergen,Helgoland,"white",Five,Ten,Fifteen), border=FALSE, bty="n", y.intersp =
0.7, cex=0.7)
}
if(labfield=="Fieldsamples"){
legend(x=0.5,y=0.8,legend=c("Spitsbergen","Helgoland","","5","10"),
fill=c(Spitsbergen, Helgoland,"white",Five,Ten), border=FALSE, bty="n", y.intersp = 0.7,
cex=0.7)
}
dev.off()

png(filename=paste("Hierclust_",labfield,"SJ6_2.png",sep=""),width=160,height=190,units
='mm',res=700,pointsize=9)
par(oma=c(15,0,0,15),mar=c(1,1,1,1))
heatmap.3(n[1:200,],
dendrogram="column",
scale="none",
margins=c(5,3),
key=FALSE,
trace="none",
density.info="none",
Colv=h.d,
labRow="",
ColSideColors = as.matrix(CategoryColors),
cexCol=1
)
dev.off()

save.image("SJ6Image.Rdata")

}
save.image("SJ6Image.Rdata")
}
```

Hierclusts: in GIMP or similar, take the dendrogram from hierclust_xxx_2 and put it in the hierclust_xxx (where everything else is correct, but the dendro needs to be cast out), then safe as .png or else)

Chloroplast (alternative coding to above, hence shown)

Packages
```
library(FactoMineR)
library(ggplot2)
library(factoextra)
library(devtools)
library(FactoInvestigate)
library(reshape2)
library(ggpubr)
library(ggrepel)
```

load RPM normalized table from working directory
```
rpm <- read.table("CountTable.CoverageAbove2_RPM_CGAnnotated_CoveredInAtLeastOneLibrary.txt",sep = "\t",dec = ".",row.names = 1, header = T )
```

remove SequenceContexts column and outliers FH1 and FH9
```
rpm <- subset(rpm, select = c(-SequenceContexts,-FH1, -FH9))
```

Making subsets
```
Labsamples=rpm[,grep("_",colnames(rpm))]
Fieldsamples=rpm[,-grep("_",colnames(rpm))]
# Transposing tables
T.rpm<-as.data.frame(t(rpm))
T.Fieldsamples <- as.data.frame(t(Fieldsamples))
T.Labsamples <- as.data.frame(t(Labsamples))
```

PCA
```
res.pca <- PCA(T.rpm, scale.unit=FALSE, ncp=5, graph = F)
res.pca.field <- PCA(T.Fieldsamples, scale.unit=FALSE, ncp=5, graph = F)
res.pca.lab <- PCA(T.Labsamples, scale.unit=FALSE, ncp=5, graph = F)
get_eigenvalue(res.pca)
get_eigenvalue(res.pca.field)
get_eigenvalue(res.pca.lab)
```

Using Factoinvestigate for outlier check
This gave 2 outliers FH1 and FH9 when they were included in the PCA at first
```
res <- res.pca
#Investigate(res, cex = 0.7, ellipse = T)
outliers(res, file = "out.Rmd", graph = TRUE, cex = 0.7, options = NULL)
```

scree plots
```
#sc1 <- fviz_screeplot(res.pca,addlabels=T, barfill= "grey", barcolor = "grey", title = "Scree plot, outliers included")+ ylim (0, 60) +
  theme_classic()
```

```
#sc2 <- fviz_screeplot(res.pca,addlabels=T, barfill= "grey", barcolor = "grey", title = "Scree plot, outliers excluded") + ylim(0, 60) +
  theme_classic()
#sc2
#scree <- ggarrange(sc1, sc2, ncol = 2, nrow = 1)
#png(filename = "screegrid.png",res = 100,width = 800,height = 300)
#scree
#dev.off()

#### Making levels for sampling location/lab or field/temperature ####
LabField <- c(rep("Lab",21),rep("Field",18))
LabField.levels <- factor(LabField,levels=c("Lab","Field"))
Location <- c(rep("Spitsbergen",3),rep("Helgoland",6),
              rep("Spitsbergen",3),rep("Helgoland",2),
              rep("Spitsbergen",4),rep("Helgoland",11),
              rep("Spitsbergen",10))
Location.levels <- factor(Location,levels=c("Spitsbergen","Helgoland"))
LocationF <- c(rep("Helgoland",8),rep("Spitsbergen",10))
LocationF.levels <- factor(LocationF, levels = c("Spitsbergen", "Helgoland"))
LocationL <- c(rep("Spitsbergen",3),rep("Helgoland",6),
               rep("Spitsbergen",3),rep("Helgoland",2),
               rep("Spitsbergen",4), rep("Spitsbergen",3))
LocationL.levels <- factor(LocationL, levels = c("Spitsbergen", "Helgoland"))
Temperature <- c(rep(c("5","10","15"),4),rep(c("5","10"),3),"5","10","15")
Temperature.levels <- factor(Temperature,levels=c("5","10","15"))


### PCA plots

# lab/field
PCA_labfield <- fviz_pca_ind(res.pca, habillage=LabField.levels, repel = T, addEllipses = T, ellipse.type = "confidence", legend.title = "Sample type") +
  scale_color_manual(values =c("#E69F00", "#0072B2")) + theme(axis.title = element_text(size = 14))+
  theme_classic()
# Location both lab and field samples
PCA_location <- fviz_pca_ind(res.pca, habillage=Location.levels, repel = T, addEllipses = T, ellipse.type = "confidence", legend.title = "Population") +
  theme(axis.title = element_text(size = 14)) + scale_color_manual(values =c("#009E73", "#F0E442"))+
  theme_classic()
# Temperature Labsamples
PCA_temp <- fviz_pca_ind(res.pca.lab, habillage=Temperature.levels, repel = T, addEllipses = T, ellipse.type = "confidence", legend.title = "Temperature")+
  theme(axis.title = element_text(size = 14)) + scale_color_manual(values =c("#999999", "#56B4E9","#CC79A7" ))+
  theme_classic()
# Sampling location labsamples
PCA_location_lab <- fviz_pca_ind(res.pca.lab, habillage=LocationL.levels, repel = T, addEllipses = T, ellipse.type = "confidence", legend.title = "Population")+
  theme(axis.title = element_text(size = 14)) + scale_color_manual(values =c("#009E73", "#F0E442"))+
```

```
  theme_classic()
PCA_location_lab
```
Sampling location fieldsamples
```
PCA_fieldlocation <- fviz_pca_ind(res.pca.field, habillage=LocationF.levels, repel = T, addEllipses = T, ellipse.type = "confidence", legend.title= "Population") +
  theme(axis.title = element_text(size = 14)) + scale_color_manual(values =c("#009E73", "#F0E442"))+
  theme_classic()
```

#Combine into grid and make png file
```
a<-ggarrange(PCA_labfield, PCA_location_lab, PCA_location, PCA_fieldlocation, PCA_temp, ncol = 2, nrow = 3, labels = c("A", "B", "C", "D", "E"), common.legend = F)
png(filename = "pcagrid2.png",res = 100,width = 1000,height = 800)
a
dev.off()
```

Plots showing methylation levels:

number of methyalated sites per sample

```
d <- rpm

n_sites <- colSums(d != 0)
n_sites <- as.data.frame(n_sites)
n_sites$sample <- row.names(n_sites)
n <- n_sites
n$location <- Location.levels
n$labfield <- LabField.levels
n$sample<-as.factor(n$sample)
rownames(n)<-NULL

nsitesplot <- ggplot(n,aes(sample, n_sites, fill=labfield))+ geom_bar(stat = "identity") +
  facet_wrap(.~location, scales = "free_x", ncol = 1) + scale_fill_manual(values =c("#E69F00", "#0072B2")) +
  ylab("Methylated sites") + xlab(NULL) +
  theme(axis.title = element_text(size = 18), legend.text = element_text(size=18), legend.title = element_text(size=18)) +
  labs(fill='Sample type')+
  theme_classic()+
  theme(axis.text.x = element_text(angle = 90, vjust = 0.5, hjust=1))
```

RPM boxplot without 2 outliers
```
d3 <- as.data.frame(t(Transposed.rmo))
d3 <- melt(d3)
d3$labfield <- d3$variable
d3$labfield<-as.character(d3$labfield)
d3$labfield[grep("_",d3$labfield)]<-"lab"
d3$labfield[grep("F",d3$labfield)]<-"field"
d3$location <- d3$variable
d3$location<-as.character(d3$location)
```

```
d3$location[grep("S",d3$location)]<-"Spitsbergen"
d3$location[grep("H",d3$location)]<-"Helgoland"
ggplot(d3, aes(x=variable, y=value, fill = labfield)) + geom_boxplot() +
 xlab("sample") + ylab("RPM") +
 facet_wrap(.~location, scales = "free_x", ncol = 1) +
 scale_fill_manual(values =c("#0072B2", "#E69F00")) +
 theme(axis.title = element_text(size = 18), legend.text = element_text(size=18), legend.title
= element_text(size=18)) +
 labs(fill='Sample type')+
 theme_classic()
```

differential methylation expression

```
### SJ6
library(DESeq2) # Version 1.24.0
library(ggplot2) # Version 3.0.1.1
library("IHW") # To correct p-values
library("VennDiagram")
# read in data to make counts table - MAKE SURE THE DATA IS IN RAW READS (integers)
for (species in c("SJ6")){
countsTable <-
read.delim(paste("All",species,"Tables.CoverageAbove2_Counts_CGAnnotated_CoveredIn
AtLeastOneLibrary.txt",sep=""), header=TRUE, stringsAsFactors=TRUE, row.names=1)
countsTable=countsTable[,-1]
head(countsTable)
# Categories
Location <-
c(rep("Spitsbergen",3),rep("Helgoland",6),rep("Spitsbergen",3),rep("Helgoland",2),rep("Sp
itsbergen",4),rep("Helgoland",13),rep("Spitsbergen",10))
LabField <- c(rep("LabSample",21),rep("FieldSample",20))
Temperature <-
c(rep(c("5","10","15"),4),rep(c("5","10"),3),"5","10","15",rep("10",10),rep("5",10))
# The decision what comparisons to be done is based on the hierarchical cluster
# compare lab - field independent of location, independent of temperature
replicatesLabField <- LabField
# Within Lab and Field we compare locations, independent of temperature
replicatesLabField.Location <- paste(LabField,Location,sep="")
# Within lab and location, we compare temperatures. Here, we don't
# need the field samples. The temperature within the fieldsamples is
# completely reflected by the location.
replicatesLabField.Location.Temperature <- paste(LabField,Location,Temperature,sep="")
# need to remove the fieldsamples at a later step
colDataLabField <-
as.data.frame(cbind(LabField=LabField,Location=Location,Temperature=Temperature,repli
cate=replicatesLabField))
rownames(colDataLabField) <- colnames(countsTable)

colDataLabField.Location <-
as.data.frame(cbind(LabField=LabField,Location=Location,replicate=replicatesLabField.Loca
tion))  # ,Temperature=Temperature out
rownames(colDataLabField.Location) <- colnames(countsTable)
```

```
colDataLabField.Location.Temperature <-
as.data.frame(cbind(LabField=LabField,Location=Location,replicate=replicatesLabField.Loca
tion.Temperature))  # ,Temperature=Temperature out
rownames(colDataLabField.Location.Temperature) <- colnames(countsTable)

DataAll.LabField <- DESeqDataSetFromMatrix(countData = countsTable,colData =
colDataLabField, design = ~replicate)
Data2All.LabField <- DESeq(DataAll.LabField,minReplicatesForReplace=Inf)

DataAll.LabField.Location <- DESeqDataSetFromMatrix(countData = countsTable,colData =
colDataLabField.Location, design = ~replicate)
Data2All.LabField.Location <-
DESeq(DataAll.LabField.Location,minReplicatesForReplace=Inf)

DataAll.LabField.Location.Temperature <- DESeqDataSetFromMatrix(countData =
countsTable[,LabField=="LabSample"],colData =
colDataLabField.Location.Temperature[LabField=="LabSample",], design = ~replicate)
Data2All.LabField.Location.Temperature <-
DESeq(DataAll.LabField.Location.Temperature,minReplicatesForReplace=Inf)
```

Comparisons

```
comparisons.LabField <- list(c("LabSample","FieldSample"))
comparisons.LabField.Location <- list(c("LabSampleSpitsbergen","LabSampleHelgoland"),
                                      c("FieldSampleSpitsbergen","FieldSampleHelgoland")
                                     )
comparisons.LabField.Location.Temperature <-
list(c("LabSampleSpitsbergen5","LabSampleSpitsbergen10"),
                                      c("LabSampleSpitsbergen5","LabSampleSpitsbergen15"),
                                      c("LabSampleSpitsbergen10","LabSampleSpitsbergen15"),
                                      c("LabSampleHelgoland5","LabSampleHelgoland10"),
                                      c("LabSampleHelgoland5","LabSampleHelgoland15"),
                                      c("LabSampleHelgoland10","LabSampleHelgoland15")
                                      )

for (fn in comparisons.LabField){
  actual.contrast <- c("replicate",fn)
  resIHW <- results(Data2All.LabField, contrast=actual.contrast, filterFun=ihw)
  resSig <- subset(resIHW,padj < 0.05)
  resSigLFC.positive <- resSig[resSig$log2FoldChange>0,]
  resSigLFC.negative <- resSig[resSig$log2FoldChange<0,]

  filename1 <- paste(species,fn[1],".vs.",fn[2],".",fn[1],".up.csv",sep="")
  filename2 <- paste(species,fn[1],".vs.",fn[2],".",fn[2],".up.csv",sep="")

  write.csv(as.data.frame(resSigLFC.positive), file=filename1)
  write.csv(as.data.frame(resSigLFC.negative), file=filename2)
}

for (fn in comparisons.LabField.Location){
  actual.contrast <- c("replicate",fn)
```

```
  resIHW <- results(Data2All.LabField.Location, contrast=actual.contrast, filterFun=ihw)
  resSig <- subset(resIHW,padj < 0.05)
  resSigLFC.positive <- resSig[resSig$log2FoldChange>0,]
  resSigLFC.negative <- resSig[resSig$log2FoldChange<0,]

  filename1 <- paste(species,fn[1],".vs.",fn[2],".",fn[1],".up.csv",sep="")
  filename2 <- paste(species,fn[1],".vs.",fn[2],".",fn[2],".up.csv",sep="")

  write.csv(as.data.frame(resSigLFC.positive), file=filename1)
  write.csv(as.data.frame(resSigLFC.negative), file=filename2)
}

for (fn in comparisons.LabField.Location.Temperature){
  actual.contrast <- c("replicate",fn)
  resIHW <- results(Data2All.LabField.Location.Temperature, contrast=actual.contrast,
filterFun=ihw)
  resSig <- subset(resIHW,padj < 0.05)
  resSigLFC.positive <- resSig[resSig$log2FoldChange>0,]
  resSigLFC.negative <- resSig[resSig$log2FoldChange<0,]

  filename1 <- paste(species,fn[1],".vs.",fn[2],".",fn[1],".up.csv",sep="")
  filename2 <- paste(species,fn[1],".vs.",fn[2],".",fn[2],".up.csv",sep="")

  write.csv(as.data.frame(resSigLFC.positive), file=filename1)
  write.csv(as.data.frame(resSigLFC.negative), file=filename2)
}
}

save.image("DifferentialExpression.RData")

### Chloroplast
# Lab vs field for ALL
# Lab vs field for only He
# Lab vs field for only Sp
library(Rcmdr) # version 2.8-0
library(DESeq2) # Version 1.36.0
library(ggplot2)
library("VennDiagram")  # Version 1.7.3
# read in data, either by the following command, or via 'import' in R commander
s <-
read.table("directory/CountTable.CoverageAbove2_Counts_CGAnnotated_CoveredInAtLea
stOneLibrary.txt", header=TRUE, stringsAsFactors=TRUE, sep="", row.names=1, dec=".",
strip.white=TRUE)
#remove tag
s <- s[,-1]
#make sure no "NA" is in the data
s[is.na(s)] <- 0
# Removing outliers FH1 and FH9
s <- s[,-22]
s <- s[,-29] # NOT -30!!!
head(s)  # returns the first 6 rows to check everything was deleted correctly
colnames(s)
```

Categories
lab - field overall and in each location, independent of temperature
this will be the command: **DESeqDataSetFromMatrix(countData=s, colData=meta, design=~0+lf)**
it takes one data frame to extract the values (countDATA), one data frame to define the comparison (colData), and specifies the column on colData that sets the comparison (design).
Hence, prior to DESeq2, categorise and prepare sub-data frames

Helgoland Spitsbergen subsets with Lab and Field combined
```
H1 <- s[,4:9]
H2 <- s[,13:14]
H3 <- s[,19:29]
H <-cbind(H1,H2,H3)  # Helgoland Subset H
head(H)
colnames(H)
S1 <- s[,1:3]
S2 <- s[,10:12]
S3 <- s[,15:18]
S4 <- s[,30:39]
S <-cbind(S1,S2,S3,S4) # Spitsbergen Subset S
head(S)
colnames(S)
```

specifying samples + groups ALL
lab and field subsets
```
s_lab <- s[,1:21]
s_field <- s[,22:39]
sample<-  colnames(s)
lf<- colnames(s)
loc <- colnames(s)
sample_lab <- colnames(s_lab)
loc_lab <- colnames(s_lab)
sample_field <- colnames(s_field)
loc_field <- colnames(s_field)
```
making groups for ALL
```
lf[grep("_",colnames(s))] <- "lab"
lf[-grep("_",colnames(s))] <- "field"
loc[grep("H",colnames(s))] <- "Helgoland"
loc[grep("S",colnames(s))] <- "Spitsbergen"
loc_lab[grep("H",colnames(s_lab))] <- "Helgoland"
loc_lab[grep("S",colnames(s_lab))] <- "Spitsbergen"
loc_field[grep("H",colnames(s_field))] <- "Helgoland"
loc_field[grep("S",colnames(s_field))] <- "Spitsbergen"
```
making metadata sets for lab/field ALL
```
meta<-data.frame(sample, lf)
meta$lf<-as.factor(meta$lf)
```
for locations ALL
```
meta2 <- data.frame(sample, loc)
meta2$loc <- as.factor(meta2$loc)
```
lab subset ALL
```
meta_lab <- data.frame(sample_lab, loc_lab)
meta_lab$loc_lab <- as.factor(meta_lab$loc_lab)
```

field subset ALL
```
meta_field <- data.frame(sample_field, loc_field)
meta_field$loc_field <- as.factor(meta_field$loc_field)
### DESeq2 for lab/field ALL
dds <- DESeqDataSetFromMatrix(countData=s, colData=meta, design=~0+lf)
dds<- DESeq(dds)
res <- results(dds)
head(results(dds, tidy=TRUE))
```
#This file with the results for lab/field analysis ALL (change directory when working on other Station)
```
write.table(res, file=" directory/deseq2_labfieldALL.csv", col.names=NA,sep = ";")
```

```
### specifying samples + groups Helgoland
sampleH<-  colnames(H)
lfH<- colnames(H)
lfH[grep("_",colnames(H))] <- "lab"
lfH[-grep("_",colnames(H))] <- "field"
metaH<-data.frame(sampleH, lfH)
metaH$lfH<-as.factor(metaH$lfH)
### DESeq2 for lab/field Helgoland
ddsH <- DESeqDataSetFromMatrix(countData=H, colData=metaH, design=~0+lfH)
ddsH<- DESeq(ddsH)
resH <- results(ddsH)
head(results(ddsH, tidy=TRUE))
```
#This file with the results for lab/field analysis Helgoland (change directory when working on other Station)
```
write.table(resH
,file="directory/DESeq2_Chloroplast/deseq2_labfieldHelgoland.csv",col.names=NA,sep      =
";")
```

```
### specifying samples + groups Spitsbergen
sampleS<-  colnames(S)
lfS<- colnames(S)
lfS[grep("_",colnames(S))] <- "lab"
lfS[-grep("_",colnames(S))] <- "field"
metaS<-data.frame(sampleS, lfS)
metaS$lfS<-as.factor(metaS$lfS)
###  DESeq2 for lab/field Spitsbergen
ddsS <- DESeqDataSetFromMatrix(countData=S, colData=metaS, design=~0+lfS)
ddsS<- DESeq(ddsS)
resS <- results(ddsS)
head(results(ddsS, tidy=TRUE))
```
#This file with the results for lab/field analysis Spitsbergen (change directory when working on other Station)
```
write.table(resS ,file=" directory/deseq2_labfieldSpitsbergen.csv",col.names=NA,sep = ";")
```

```
### VennDiagram
rm(list=ls())
library(ggplot2)
        library(VennDiagram)
```

```
Both_Locations <-
c("Tag1","Tag2","Tag3","Tag4","Tag5","Tag6","Tag7","Tag8","Tag9","Tag10","Tag11","Tag
12","Tag13","Tag14","Tag15","Tag16")
Helgoland <-
c("none","none","none","none","none","none","none","none","none","none","none","no
ne","none","none","none","none","none")
Spitsbergen <-
c("Tag1","Tag2","Tag3","Tag4","Tag5","Tag6","Tag7","Tag8","Tag9","Tag10","Tag11","Tag
17")
Helgolandcolor="orange"
Spitsbergencolor="blue"
allcolor= "grey"
mycol <- c(allcolor, Helgolandcolor, Spitsbergencolor)
V <- list(Both_Locations, Helgoland, Spitsbergen)
a <-venn.diagram(x = list(Both_Locations, Helgoland, Spitsbergen), category.names =
c("Both Origins" , "Helgoland" , "Spitsbergen"), filename = NULL, col=mycol, fill=mycol,
cat.default.pos = "outer", cat.pos = c(-27, 20, 125), cat.dist = c(0.055, 0.025, 0.055),
cat.fontfamily = "sans", cat.col = mycol, rotation = 1)
#see plot in RStudio
grid.draw(a)
#save as png
png(filename = "venndiagram.png",res = 100,width = 500,height = 500)
grid.newpage()
grid.draw(a)
dev.off()
 # alternative graphic
b <- draw.triple.venn(area1=17, area2=13, area3=0, n12=12, n23=0, n13=0, n123=0,
category=c("Both Locations", "Spitsbergen", "Helgoland"), col="purple",
fill=c("Green","Blue","Yellow"))

grid.draw(b)
```

Linux Tmux R Cheat Sheet

Tmux

tmux new –s nameofsession	# starts completely new session
tmux attach	# will add new session window to existing session
str+b c	# creates new window
tmux attach –t nameofsession	# re-open original session
strg+b n	# will switch between sessions
ctrl+b c	# create new window; in case new window is needed while operating in current window
ctrl+b p	# get back to previous window
ctrl+c or ctrl+q	# in case cursor does not get back to enter mode

kill session that is stuck in a process:

ctrl+b & (strg+b, dann hochpfeil+&) # is supposed to close current window; if this does not work:

ctrl+b c # creates new window, then

tmux kill-session –t nameofsession

Linux

cd # change directory

cp filename directory # copy file filename to folder or directory

more filename # look at file content -> to exit, just press 'q'

nano filename # write in file content

mkdir # make directory – creates new folder

cat > filename.txt # creates textfile; content then is written/copied into the next rows

ctrl+c # in case file does not return to entercursor (>)

ls –lhcrt # to check whether changes occur during a process (files being generated in a folder)

ls filename* # will list all file types with name filename

ls *filetype # lists all files of the inquired type

gunzip files*gz # unzip gz folders

mv currentname newname # rename folder

mv folder/file new directory # moves folder or file into new directory

rm filename # delete file filename

rm –r directoryname # delete directory/folder

tail filename # moves to the end of a (count)table -> to check whether a table ended abruptly before containing all data ()

R

Setwd() # creates directory of session/ script

Getwd() # check in which working directory R is currently operating

[,-22] # will remove the 22th column of a matrix

[-22,] # will remove the 22th row of a matrix

rm(list=ls()) # empty workspace to make sure no old variables/ data frames remain

library(Rcmdr) # opens r commander

FEHLER: <text> # in case of copy-paste of script from word file: possibly “”, as in .docx different than in ‘R’ -> substitute all “ in console, are needed to be more vertical

https://rpubs.com/IamAfshin/bestNormalize_in_r # using the bestNormalize package

https://clayford.github.io/dwir/dwr_05_combine_merge_rehsape_data.html # Merge and re-shape columns

http://www.sthda.com/english/wiki/colors-in-r # how to code the colours

excel

https://www.youtube.com/watch?v=ob6rihE0FZA # several columns re-arranged into one