Cell- and genome size responses to different temperatures in haptophytes

A long-term experiment study on the algal species
Prymnesium kappa and *Calyptrosphaera sp*

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ABSTRACT

In recent years, environmental concerns and global temperature increase has attracted a wide interest. Global temperature has increased in the recent years, and will continue to do so in the near future. How organisms, especially those that are important for the ecosystem, i.e. primary producers, such as algae, react to this, is of interest to get a better understanding of. The relationship between cell size and temperature has been an interesting field of study since the Bergmann-rule was introduced. Though, this was meant to describe endotherm organisms, the rule has often been applied to ectotherms, also giving rise to the Temperature-Size rule (TSR). The cell size and temperature has been shown to often correlate negatively. It has also been shown to exist a relationship between cell size and genome size, being positively correlated. The direct relationship between genome size and temperature is therefore an interesting study.

To study this, two different species, *Prymnesium kappa* and *Calyptrosphaera sp*, was used as study organisms and cultivated on different temperatures, 11°C and 19°C, for over a year. Genome size was estimated using flow cytometry (FCM). Other parameters, such as cell size, RNA amount, protein amount and stoichiometry (C:N:P-ratios) was also analysed. The algal cells were also examined using electron microscopy, and DNA sequencing and k-mer analysis was used for an additional genome size estimation.

The genome sizes estimated using FCM indicated that a change in genome size had occurred for both species, but the 11°C treated cultures of *Prymnesium kappa* showed a strong increase in genome size. The other independent analysis also suggested that a change had occurred between the two temperature treatments, indicating that temperature change can induce visible changes, not only in the genome, but in other parameters, as those mentioned above, as well.

From all the acquired results from this study, temperature has shown to have a great impact on the cells and can, and have shown to, induce a change in genome size, where the genome size is negatively correlated with temperature.

Key words: Genome, Flow Cytometry, Temperature, Algae, DNA

1   INTRODUCTION

1.1. Temperature and organism size

In the recent years, environmental concerns and global warming are, and have, becoming more and more popular topics. Not only within the scientific environment, but also among the commoners. And this is not without any reason. In the recent years, we have seen an overall increase in global temperature.
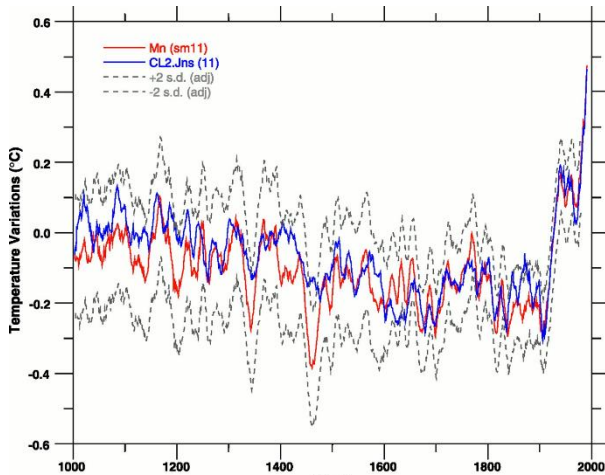
Figure 1: A comparison of different mean annual temperature variations records (Northern Hemisphere) reconstruction of Mn (from and named after Mann et al.1999) and CL2 (originally from and named after Crowley et al. 2000), which is a new splice of CL with a slightly better fit. Figure adopted from "Causes of Climate Change Over the Past 1000 Years" by Thomas J. Crowley (2010).

Throughout the last millennia, there have always been fluctuations in annual average temperature, especially when including environmental disasters such as the small ice age (Tkachuck, 1983), but in general, there have not been too large variation in temperature. In the last 100 years on the other hand, one can see an evidential increase in average temperature (in the Northern Hemisphere), as figure 1 demonstrates, and natural variations can only 25% ca be attributed to natural variations (Crowley, 2010, Mann and Jones, 2003). And for the last 30 years, the global surface average temperature has risen with 0.2°C per decade (Hansen et al. 2006). Compared to that of 60 years ago, measured surface temperature has especially increased around the poles. With increased temperature, large amounts of the energy are stored in the oceans. In the upper 700-meter ocean layer, have the heat content increased with 14×10^{22} Joule (J) and ocean temperature has increased with 0.6°C over the past 100 years (Hoegh-Guldberg & Bruno, 2010). This is an ongoing trend, and from May to January, 2016, an all-time high global ocean temperature was measured to be 0.77°C over the average for the 20th century (National Oceanic and Atmospheric Administration), but the last two years we see a slight decrease. The warmer water gives arise to more stratification of the water currents and reduces mixing. Since 1998, have the size of "ocean deserts" (low on nutrient) expanded on average between 0.8% and 4.3% each year (Polovina et al. 2008). It is also expected that the warming of the upper oceans will lead to reduced nutrient recycling and the subsequent increase in nutrient

limitation (Ayo et al. 2017). There is a lot of statistics showing that large, important glaciers (especially those of Greenland) and the melted ice-water is flowing into the oceans (Rignot et al. 2008; Rignot et al. 2010; Abdalati and Steffem, 2001; Nghiem et al. 2012) which makes it more difficult to get good and accurate measurement of the ocean temperature. The melting of ice caps, also lowers the globes overall albedo, which leads to less light reflection and more light/energy absorption (Box et al. 2012).

Temperature is a key factor for organisms and a lot of their life traits is either directly or indirectly linked to temperature. Organism size is one of the key traits that is linked to temperature and one of the pioneers within this topic is Carl Bergmann discovered that, for endotherm organisms, those who lived on a higher latitude, tended to be of a bigger size (Bergmann, 1847) and thus had the Bergmann's Rule named after him. In all generality, the rule claim that if two organisms of the same species lived on different latitudes, the one of the highest latitudes would be the largest. Endotherm (from Greek *endon* – "within" and *thermē* – "heat") organisms maintains a catalytic preferable internal temperature without the need of external heat/energy (to a certain extent). The American zoologist J. A. Allen described a related phenomenon (Allen, 1907) and gave his name to the Allen's rule. This rule state that endotherm animals reared in colder environments, tend to give arise to shorter limbs compared to animals of the same species reared in warmer environments. The more the exposed surface area, the greater is the heat and energy loss for the animal. A higher volume to surface ratio is therefore beneficial in colder areas. But this explanation, and rule, can only be used to explain the increased size of endotherm organisms, and not ectotherm organisms.

Ectotherm (from Greek *ektós* – "outside" and *thermós* – "hot") organisms have none, or highly limited, control over their internal heat and must entrust heat production to external heat sources, such as hot rocks, sunlight, etc. (Davenport 1992). The internal temperature of an ectotherm is more or less the same as the external environment temperature. The vast majority of the species on earth are ectotherms, making up 93 % (Bar-On, Phillips and Milo, 2018) of the total animal biomass. According to Atkinson and Simbly (1997) as much as 99.9% of the species on Earth are ectotherm, in the way that they rely on external heat sources, including everything from bacteria to ants. Although there are a few exceptions to the rule (Atkinson, 1995), most ectotherms respond to temperature change in the same way – body size is negatively correlated with temperature. This phenomenon is called Temperature-Size Rule (TSR) and states that organisms reared in colder temperatures reaches majority with

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larger size bodies compared to those of warmer climates (Atkinson, 1994; Atkinson et al. 2006; Forster and Hirst 2012; Forster et al. 2013). Cell size is often associated with growth rate – in warmer conditions does organisms grow faster, but to a smaller adult size, while in colder conditions, organisms grow slower, but becomes larger at mature stage (Atkinson, 1994; Angilletta Jr. et al. 2004). There is hypothesised that temperature affect the allometries of anabolism and catabolism differently (Strong and Daborn, 1980), resulting in a decrease thermal optimum for growth rate throughout development. So, the majority of ectotherm organisms reared at low temperatures, started with a slower growth rate earlier in development, but ended up with a maximal growth rate. Their growth rate accelerates with age. (Most) Ectotherm organisms raised at higher temperatures are facing opposite trends by starting with a maximal growth rate, before ending up with a sub-maximal growth rate (Angilletta Jr. et al. 2004). Under higher temperature circumstances, there will generally be a higher cellular metabolism. This leads to more rapid development and facilitating prematurely development reproduction, which would be favoured by natural selection (Atkinson, 1994).

Although, a true general explanation behind the general physiological mechanism of TSR may not be to likely, as TSR in unicellular and multicellular organisms seem to operate differently (Forster, Hirst and Atkinson, 2011; Forster, Hirst and Estaban, 2012). So even though most ectotherm (>80 % of all ectotherms (Atkinson, 1994)) reacts similarly in respect to cell size and growth rate, one cannot expect same underlying TSR mechanisms, as all ectotherm does not exhibit the same behaviour and physiology (Angilletta Jr. and Dumnham, 2003).

1.2. The basics of the algae

Algae are a highly diverse, in morphological, genetically and physiological, group of organisms. The can be both unicellular and multicellular, both photosynthetic and heterotrophic, and, they can live freely, in symbiosis or as parasites (Graham and Wilcox, 2000). Most algae produce oxygen, and sugar, which is crucial for life on earth, making algal cells important primary producer. Thus, they are an important food source for many small organisms (zooplankton, crustacean, etc.), as well as producing oxygen, the gas of life. Algae can be found all over the world, both in freshwater and the oceans (also brackish waters), but also found on ice covered mountains, in deserts soils and hot springs (Lee, 1999). As

mentioned, algae are a diverse group, and can vary a great deal in size. They can be either haploid or diploid, or the can even alternate between the different ploidy levels (Lee, 1999). Some are small, single-celled organisms, while other form large multicellular seaweed, which may grow to become several meters (or over 30 meters, like the giant kelp (Park and Allaby. 2013).
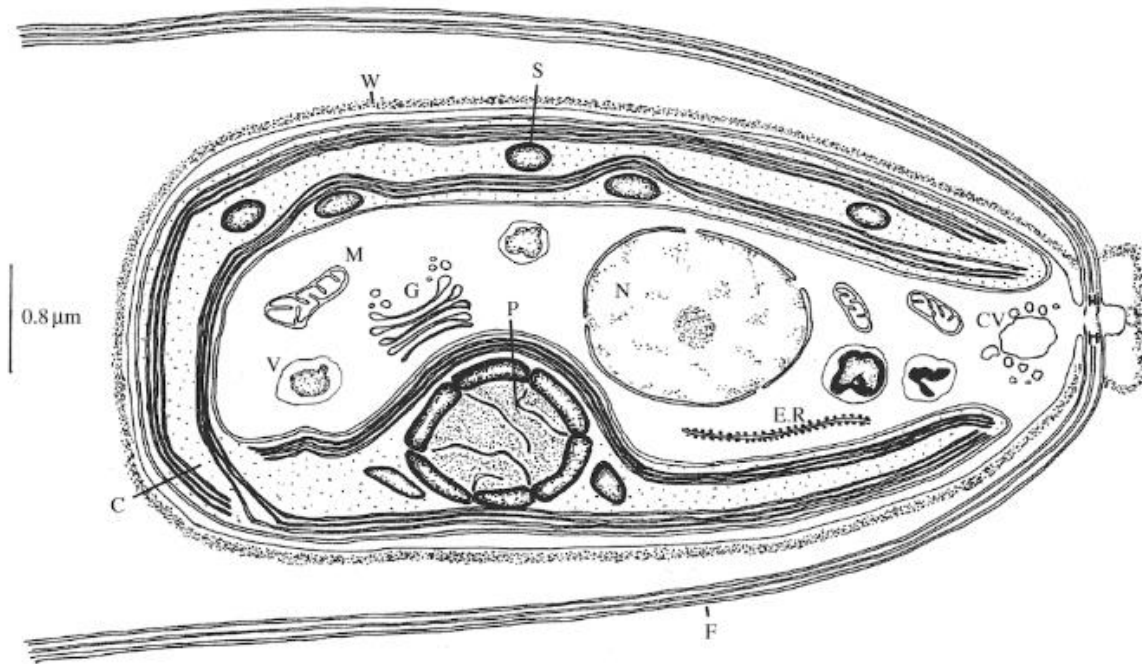
Figure 2: A drawn representation of a green algae cell showing its cellular structure with all components: Nucleus (N), Endoplasmic Reticulum (ER), Contractile vacuole (CV); Mitochondrion (M), Vacuole (V), Wall (W), Chloroplast (C), Golgi apparatus (G), Starch (S), Pyrenoid (P), Flagella (F). Figure adopted from Lee, R. (2018).

The oceans, and then specifically algae, are important drivers of the photosynthesis and accounts for 46.2 % of the global photosynthetic carbon fixed, producing both oxygen and sugar (Field at al. 1998; Moroney and Ynalvez, 2009; Sommer et al. 2016). Algae are therefore important as a primary producer, but there are reports suggesting that, since oceanographic measurements began at late 1800s, total Chlorophyll pigment (Chl) concentration has declined in the world oceans. This negative effect is most evident in tropical regions and it is believed that sea surface temperature increase is the major cause of the decline in Chl concentration (Boyce, Lewis and Worm. 2010).

When the nutrient concentrations are high, algae, which grows proportionally with nutrient availability, may form algal blooms. Not only can it reduce water quality (Park and Allaby. 2013), toxic blooms can be produced, which in 1988, was responsible for the killing of benthic and pelagic species of many phyla as well as several hundred tons of fish in the

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Skagerrak and Kattegat areas, during the bloom of *Prymnesium polylepis* (previously named *Chrysochromulina polylepis*) (Underdahl et al. 1989). Coccolithophorids is an algae family that produces calcite ($CaCO_3$) plates (coccoliths) and contribute to the carbon cycle, and carbon pump, by transporting carbon down to the ocean floor. Thigh, it is believed that in the largest habitat for coccolithophorid in the world, the North Atlantic, the areal extent of coccolithophorid algal blooms will decline by 50% by around 2050 (Iglesias-Rodríguez, 2002).

1.3. Genome size, in relationship with cell size, in eukaryote organisms

Even before the discovery of the DNA structure by Watson and Crick (1953) (with the good help of the famous "Photograph 51" captured by Rosalind Franklin and her student Raymond Gosling in 1952), the DNA content of cells was investigated. Even as early in 1893, did Eduard Strasburger discover that the ratio between diameter of a plant meristematic cell size was constant with that of its nucleus (Price, Sparrow and Nauman. 1973). In the following years, several similar discoveries were being made: "*What can be said, however, is that when DNA per cell increases, whether due to an increase in number of chromosomes or to an increase in the number of strands per chromosome, an increase in cell size follows.*" (Mirsky and Ris. 1951). Holm-Hansen (1969) discovered that DNA quantity correlates almost perfectly with cell size (or carbon/cell). It is also argued that an increase in cell size or nuclear size, could be directly caused by an increase of nucleoskeletal DNA (or S-DNA), giving arise to the nucleoskeletal theory (Cavalier-Smith, 1978). This theory asserts that genome size contributes to much more than just encoding genetic information, it controls the cell volume and the size of the nuclear envelope, which is dependent of the genome size (Cavalier-Smith 2005). As today, the correlation between cell size and genome size has been investigated, and observed, in several different families and species (Price, Sparrow and Nauman. 1973; LaJeunesse et al. 2005; Connolly et al. 2008; Hessen et al. 2013). It may seem like genome size and cell size is a universal phenomenon. Although, the correlation is not always positive, it may be negative as well, but in more or less all cases, there is a clear relationship between genome and cell size (Beaulieu et al. 2008).

The genome size, or C-value (the amount of DNA, in picogram (pg), contained within a haploid nucleus), has been discovered to differ a lot, not only between distant related species,

but also a great deal within the same family, or kingdom, of species. The term C-value was first used by Swift (1950) when describing DNA amount compared to haploid (i.e. 2C was measured to be twice as large as a haploid genome) where the C just was a constant. Veldhuis et al. (1997), in their investigation, discovered that between the 90 tested algal species (or 121 strains), their genome sizes could differ with a factor of 20.000 (measured using PicoGreen: Prochlorococcus – between 0.0056 and 0.0083 CRBC (Chicken Red Blood Cell) 2.33 units*cell^{-1} and *Prorocentrum micans* – 119.34 CRBC 2.33 units*cell^{-1}). Since then, several new records holders of largest genome have been reported. First was Pedersen's marbled lungfish Protopterus (*Protopterus aethiopicus*) measured to 132.83 pg (Pedersen. 1971) and then was the *Melanthiaceae* (family), *Paris japonica* measured to be 152.23 pg (Pellicer et al. 2010). But, there is a genome estimated to be several times larger than this again, the amoeba, *Polychaos dubium*, which is measured to 700 pg (Fritz 1968; McGrath and Katz, 2003). Although, the authors suggest that these results should be taken with caution, as present day, advanced molecular techniques, have yet to be used. Anyways, it became quickly obvious that humans (Homo sapiens) did not have the largest genome, as one could (and previously did) expect based on organism complexity, and that there was not necessarily any direct link between genome size and complexity (e.g. Gregory, 2004). This was referred to the C-value paradox (first dubbed by Thomas, 1971), but later referred to as the C-value "enigma" (Gregory, 2000).

When Ohno (1972) compared the human genome to the *E. coli* (*Escherichia coli*), which have a much smaller genome, he made a simple assumption that the human genome should contain roughly three million genes, something, as he stated, is far from the truth. It has later been suggested that the human genome only contains around 19,000 protein-coding genes (Ezkurdia et al. 2014). The fact that genes in the genome, not only the human, only accounted for a few percentage of the total genome, led to the term "junk DNA", formalized by Onho (1972). Although the term "junk" is somewhat debated, "junk DNA" is described as essential excessive DNA, functional or not, which have the capability change its concentration and location, without a change in actual gene number (Freeling et al. 2015). In the same article they propose that "*Organisms with junk survive as a spandrel of the potential to have survived because the junk existed as raw material for novel adaptive mutations*", meaning that the "junk" (or maybe more concrete, non-protein coding regions) is a by-product of the few, proven essential, items (Freeling et al. 2015). Although it is currently estimated that less than 10% of the human genome is conserved through purifying selection, ENCODE

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(ENCyclopedia Of DNA Elements) suggests that more than 80% of the human genome is functional. But, this is heavily criticized by e.g. Doolittle (2013) and Graur et al. (2013).

The idea of selfish DNA became popular in the late 1970s. Richard Dawkins, in his book, The Selfish Gene (1976), described selfish genes shortly, but accurate, before Doolittle and Sapienza (1980) again mentioned it a few years later. Selfish DNA was described as a DNA sequence which was replicated (and in some cases, transcribed) as any other genes, without contributing to the phenotype of the organism, except for acting as a burden for the host (Orgel and Crick, 1980). The selfish DNA was therefor compared with (not-too-harmful) parasites. Both Selfish DNA and "junk" DNA consist of repetitive, non-coding sequences, such as satellite DNAs, simple sequences, tandem repeats and transposable elements, were especially transposable elements (TEs) play a major role in determination of genome size (Kidwell, 2002).

Transposable elements (originally discovered by Barbara McClintock (Ravindran. 2012)) appear in most cases to not have any significant function in the biology for the host and have the capability to "move around" in the genome through the process of transposition (the process whereby these sequences copied, or cut, out of the donor DNA and inserted a new genome site). It has been suggested that TEs are important, and major, contributors to genomic restructuring that facilitates macroevolution (McDonald, 1998). There are two major classes of TEs; DNA transposons and retrotransposons. Where DNA transposons move via a DNA intermediate which is excised for the donor, retrotransposons are first transcribed, using RNA polymerase, and the RNA intermediate is revere-transcribed into the DNA, which is inserted into the target DNA (Lodish, 2013). TEs moves to new locations with little sequence selectivity, meaning that they can be inserted within genes, and completely disrupt the gene function (McDonald, 1998. Watson, 2014). TEs carry their own genes and terminal repeats – long terminal repeats (LTR) in retrotransposons and shorter, inverted terminal repeats, which carry the recombinase recognition sequence, in DNA transposons. DNA transposons carry a transposase (the recombinase responsible for transposition) gene and sometimes additional genes, such as encoding proteins that regulate transposition, or even producing useful elements for itself or its host. Retrotransposons carry two enzyme genes for reverse transcriptase and integrase (Watson, 2014). Transposons can be both autonomous (as those recently mentioned) and nonautonomous. The latter are simple DNA segments that only carry the terminal inverted repeats.

The TEs found in most genomes, especially of those of higher organisms, are mostly reversible inactive, or dormant, called "epigenetically silenced". As epigenetics study became more and more popular in the late 20th century, epigenetic silencing was proposed to have evolved to control the proliferation of TEs and their destructive potential (Fedreoff, 2012). Epigenetics (first described/defined by Waddington, 1939) can be defined as heritable changes in gene expression, and to the phenotype, that does not change the primary nucleotide sequence (Richards, 2006; Bossdorf et al. 2007; Slatkin, 2009). Epigenetics involves a broad variety of regulatory mechanism, from single nucleotide (e.g. methylation of Cytosine (Griffith & Mahler, 1969; Holliday, 2006)) to chromosome modification (e.g. HP1 (heterochromatin protein 1) which binds to the 3-methylated Lysin 9, Histone 3 (H3K9me3) (Lodish, 2013)). In contrast to regular DNA sequence mutation were evolutionary changes needs several generations to take place, will epigenetic evolution work much more rapidly (Bossdorf et al. 2007; Slatkin, 2009). Many factors can have an influence on epigenetic expression, such as temperature, and maybe traumas and torture (ref. children from Holocaust survivors – Kellermann, 2013). Plants have a more complex epigenetic system than animals and make use of several mechanisms (Federoff, 2012). Prolonged treatment to cold temperatures for plant can induce chromatin and DNA methylation changes at specific genomic loci (Steward et al. 2002; Bastow et al. 2004; Richards, 2006). Chromatin-modifying enzymes are sensitive to environmental changes, such as temperature (Turner, 2009). Cold stress regulates several downstream transcription factors and de-repression (facilitated by a decrease in H3K27me3) of cold responsive genes which ensures acclimatization to low temperatures (Banerjee, Wani & Roychoudhury, 2017).

1.4. Flow cytometry

Flow Cytometry (FCM) is a quick and rather simple method to estimate genome sizes, and was heavily used throughout this thesis. According to Animal Genome Size Database, of the total 6,222 recorded genomes, are 2,302 genome sizes estimated using Flow Cytometry (Gregory, 2018). In all generality, cytometry is the process of measuring chemical of physical properties of a cell or other biological (or even non-biological) particles. As the name imply, flow cytometry measures particles, one by one, as they flow in a fluid stream, past the measure apparatus (Shapiro, 2003). The main components of the FCM is a fluid system, optics (excitation and collection), electronic network/detectors and computer. The fluidics is

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responsible for transporting the particles from the sample and organising the particles to make them pass though the laser(s) one and one. As particles rapidly flows past the 488 nm, blue laser (measure apparatus), each particle absorbs and emits light, either scattered or fluorescent light, at different wavelengths which again is collected by the numerus detectors, see figure 3. Fluorochromes (the electrons) as PI or chlorophyll is excited and emits light at a higher wavelength, while scattered light is absorbed without being excised and sent out at the same wavelength. The emitted signal is, after detection, sent to a computer for analysis.

As the particle moves past the light, or laser, source (flow cell in figure 3), the particle emits/scatter light in different directions, collected by different detectors. The flow cytometer
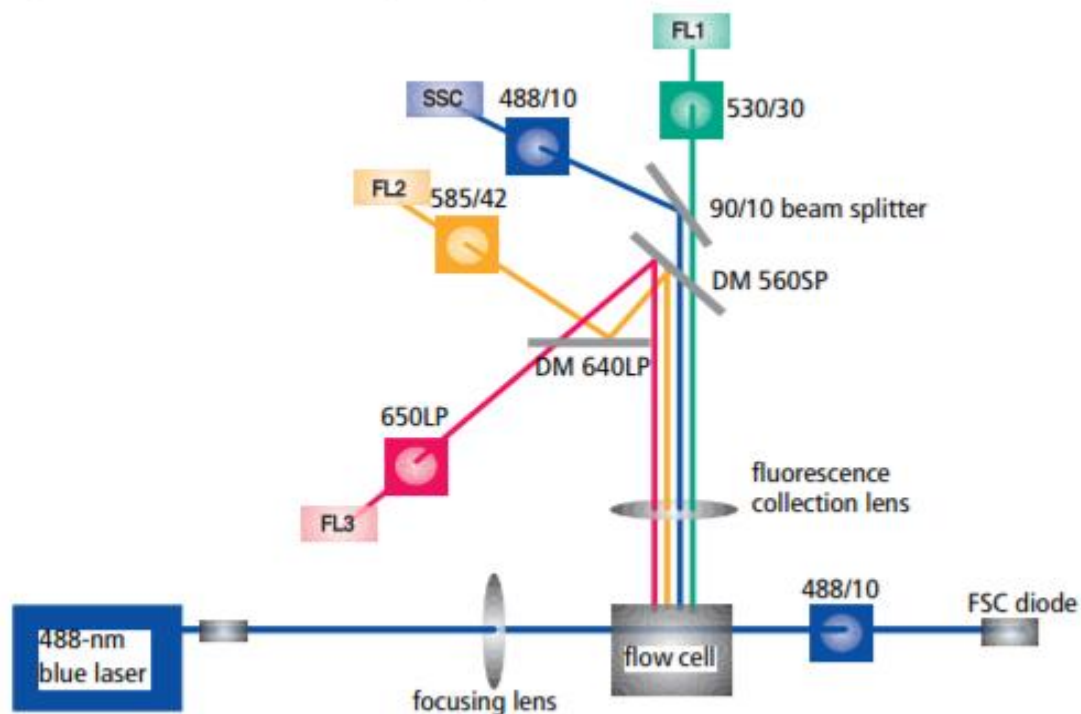
Figure 3: BD FACSCalibur optics system overview. Picture adopted from the manufacture's instruction manual: BD FACSCalibur Instructions For Use.

used during this thesis, BD FACSCalibur™ (BD Biosciences (Becton, Dickson and company), 2009, New Jersey, USA) was fitted with three fluorescent detectors as well as forward scatter (FSC) and side scatter (SSC) detector. FSC diode detects particle size, while SSC measure the particle's granularity, structure and complexity. In addition to be able to measure the genome size, FCM can be used to measure various cell components such as; membrane, cytoplasmic and nuclear antigens, whole cells, RNA, chromosomes, cytokines, hormones and protein as well as cell proliferation and cell cycle (Adan et al. 2016). To enable the fluorescent detectors to detect particles, a fluorochrome, which binds specifically to the

particle of interest, must be used. To be able to get an estimation of the sample genome size, a standard, with known DNA content (known C-value), must be used (usually CRBC – Chicken Red Blood Cell) and can be calculated using this formula (Galbraith et al. 1997):

sample 2C DNA content (pg DNA) $= \frac{sample\ G_1\ mean\ peak}{standard\ G_1\ mean\ peak} *$ standard 2C DNA content

1.5. Goals and hypothesis

This experiment departs from two basic hypotheses:

1: Cell size is negatively correlated with temperature.

2: Genome size is positively correlated with cell size.

1. As mentioned earlier, empirical studies and well asserted rules, have proposed that cells and organisms at higher latitude and lower temperature, reach a larger adult size compared to those of lower latitude and warmer temperature. It is proposed that cells grown at lower temperature have a lower cell division rate (or growth rate) and thus grows slower, but becomes larger at adult age. Though, this effect, and correlation, is not as well explored, but there is experimental evidence that this trend exists for algae as well (Winder et al. 2009; Finkel et al. 2005; Daufresne et al. 2009)

2. The genome size (C-value) have been investigated in a number of studies, across a large range of organisms. Although the genome size has been shown to vary a great deal from specie to specie, it is also shown to a great degree, to correlate well with cell size (reviewed by Hessen et al. 2013), usually showing a positive correlation. Although there are only a few studies on algae, the same, positive correlation between cell size and genome size within phyla, seem to apply here as well (Price, Sparrow and Nauman. 1973; LaJeunesse et al. 2005; Connolly et al. 2008; Hessen et al. 2013).

Both of these hypotheses have limited experimental support among phytoplankton (or algae). Therefore, the objective of this study is to test, a possibly gain support for this, both at the intraspecific level and in the two selected algal species, and at the intraspecific level by testing genome- and cell size correlation across different algal species. Thus, the main goal om this thesis is to study the direct relationship, and correlation, between genome- and cell

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size in two different algal species, using two different (natural occurring) temperatures, and thereby linking the two hypotheses, making a third hypothesis:

3: Genome size is negatively correlated with temperature.

2. MATERIALS AND METHODS

2.1. Model organisms

To get a better understanding on how cells and organisms react to a change in temperature and if or how their genome will change, a model organism with a short lifespan and cell cycle is important to use. Algae, a diverse group of heterogenous organisms that are spread over different "supergroups" of eukaryotic organisms, such as Archaeplastida, Hacrobia, Stramenopila and Alveolata (Edvardsen, 2018). Algae can be multicellular, heterotrophic and symbiotically (Graham and Wilcox, 2000), but the algae used in this experiment are a free floating, single cell organism, with photosynthesis, that are mostly found in sea water. These organisms are highly important for the Earth's climate as these organisms account for a large amount, roughly 50 % (Field at al. 1998; Moroney and Ynalvez, 2009; Sommer et al. 2016), of the primary production, producing both Oxygen and sugar. Algal species are small and have a short lifespan, which makes it an ideal model organism. This is because one can get through a high number of generations on a relatively short time span and therefore have a larger chance of a seeing long-term effects on the organisms. There is also a well-known body size trend for phytoplankton (and algae) (e.g. Barton et al. 2013).

In this, or these, experiments, several different algae species were studied, where two of them, *Prymnesium kappa* and *Calyptrosphaera sp*, made up the temperature experiment. All the algae used throughout this thesis were provided by supervisor Bente Edvardsen.

Prymnesium kappa was first described in 1955 as *Chrysochromulina kappa* by Parke & Manton (Parke et al. 1955), but later transferred to the genus *Prymnesium* by Edvardsen, Eikrem & Probert (Edvardsen et al. 2011). The strain used in this experiment was UIO 032, which was isolated by Wenche Eikrem from Oslofjorden, Norway. The ploidy level is not yet confirmed, but unpublished results by B. Edvardsen indicates that these are most likely haploid, but were also found to be diploid.

Calyptrosphaera sp was isolated by Wenche Eikrem and the strain used in this experiment was UIO 309. This might be a new specie, but it might also be a special strain of *Calyptrosphaera sphaeroidea*. It is a coccolithophorid with two different phases, a motile holococcolith phase (and as all holococcolthophorids are assumed to be haploid, *Calyptrosphaera sp* is also assumed to be haploid) and a nonmotile heterococcolith phase.

Both phases can also be reversed to its alternate phase (Noël, Kawachi and Inouye, 2004). It was first described in 1913 (Schiller. 1913).

The two previous algae species were included in the temperature experiment. The following species, including *Calyptrosphaera sp*, were selected to investigate the correlation between cell- and genome size.

Isochrysis galbana was first described by Parke, M (1949). It is a haptophyte and is mostly used to feed juvenile fish and crustaceans, as well as bivalve larvae (Godet et al. 2010). The strain used in this experiment was UIO 140 which was isolated by Sergio Seoane.

Diacronema lutheri was first described as *Monochrysis lutheri* by Droop, M.R (1953) and was later named *Phaeaster lutheri* (Droop) Bourrelly, P. (1957) and *Pavlova lutheri* (Droop) Green, J.C. (1975), before it got its final, taxonomically accepted name, *Diacronema lutheri* by Bendif & Véron in Bendif et al. (2011). This algal specie is able to produce large amount of polyunsaturated fatty acids and is therefore often used to feed bivalve crustaceans and fish. The strain used in this experiment was UIO 090.

Hymenomonas carterae (NIVA-2/92 strain) is a heterococcolith algae strain, but might just be a homotypic synonym for *Chrysotila carterae*.

Prymnesium nemamethecum was first, and only to date, described by Pienaar, R.N. & Birkhead, M. (1994). The strain used in this experiment was K-0394 and was isolated by Marianne Ødegaard Jensen from Ballen Havn Samsø Denmark.

Phaeocystis globosa was first described by Scherffel, A. (1899). The specie is associated with blooming in nutrient-rich areas, in both temperate and tropical waters (Lancelot, C. et al. 1998). The strain used in this experiment was K-1321 and was isolated by Gert Hansen from Horta, Faial, Portugal.

Pavlova gyrans was first described by Butcher, R.W. (1952). The strain used in this experiment was K-1310, which was isolated by Gert Hansen from San Sebastián, La Gomera, Canary Islands, Spain.

Chrysotila carterae was first described as *Syracosphaera carterae* by Braarud & Fargerland (1946), then changed to *Pleurochrysis carterae* before it was transferred to the genus *Chrysotila* by Andersen, R.A., Kim, J.I., Tittley, I. & Yoon, H.S. (2014). The strain used in

this experiment was UIO 095 and was isolated by von Stosch, H.A. *Chrysotila carterae* is a heterococcolith (and as all other heterococcolith, assumed to be diploid).

Prymnesium polylepis was first described as *Chrysochromulina polylepis* by Manton, I. & Parke, M. (1962) before it was moved to the genus *Prymnesium* by Edvardsen, Eikrem & Probert (Edvardsen et al. 2011). This algal specie is known for producing toxic bloom which was responsible for the killing of benthic and pelagic species of many phyla as well as several hundred tons of fish in the Skagerrak and Kattegat areas (Underdahl et al., 1989). *P. polylepis* have two alternate ploidy levels, haploid and diploid (Edvardsen and Valout, 1996). The strain used in this experiment was UIO 041 which was isolated by Lars Edler.

2.2. Experimental setup

In this experiment, temperature, both high and low temperature, was chosen as the main variable to test for cell- and genome size responses over a multigenerational time-span. The algal cultures were cultivated in two different temperature-controlled climate rooms with one ranging between 10°C – 12°C (from now designated 11°C) and the other ranging between 18°C and 20°C (from now designated 19°C). To maximize the possible effect of the temperature-treatment, the experiment was designed as a long-term study. Experiments with the aim of studying the genotypic changes should be truly long-lasting, but for practical reasons (the time constraints of a master study), the time period was confined to one year.

Two different algae species, *Prymnesium kappa* (UIO 032) and *Calyptrosphaera sp* (UIO 309) was used in the temperature experiment. For each specie, at both temperatures, three replicates were made, ending up with a total of 12 separate cultures. A simple overview of the setup is provided in table 1, and how each culture is designated (e.g. 309.11.2 and 032.19.1, where the first number is the specie strain code, the second number is the temperature the cultures were cultivated in, and the third number is the replicate number).

Calyptrosphaera sp (UIO 309)						*Prymnesium kappa* (UIO 032)					
11ºC			19ºC			11ºC			19ºC		
1	2	3	1	2	3	1	2	3	1	2	3

The cultures were cultivated in 40 ml nunclon filtercap flasks (Thermo Scientific) using an algal culture medium (described in the next section, 2.3. Medium). To maintain a high growth rate, the cultures was run as semi-continuous chemostats and were diluted 3 times a week with fresh medium. The amount of medium used to dilute the cultures usually differed from the 11ºC cultures and the 19ºC cultures. The 19ºC cultures had the highest growth-rate and thus was diluted the most, to avoid resource limitations. Independent of the amount of medium used, all cultures were diluted down to ca. 50 000 cells ml^{-1}, to ensure that the algae cultures were kept at a high growth rate. The nunclon filtercap flasks were changed from time to time, usually each month, except for two replicas, 032.19.2 and 309.19.2, which was
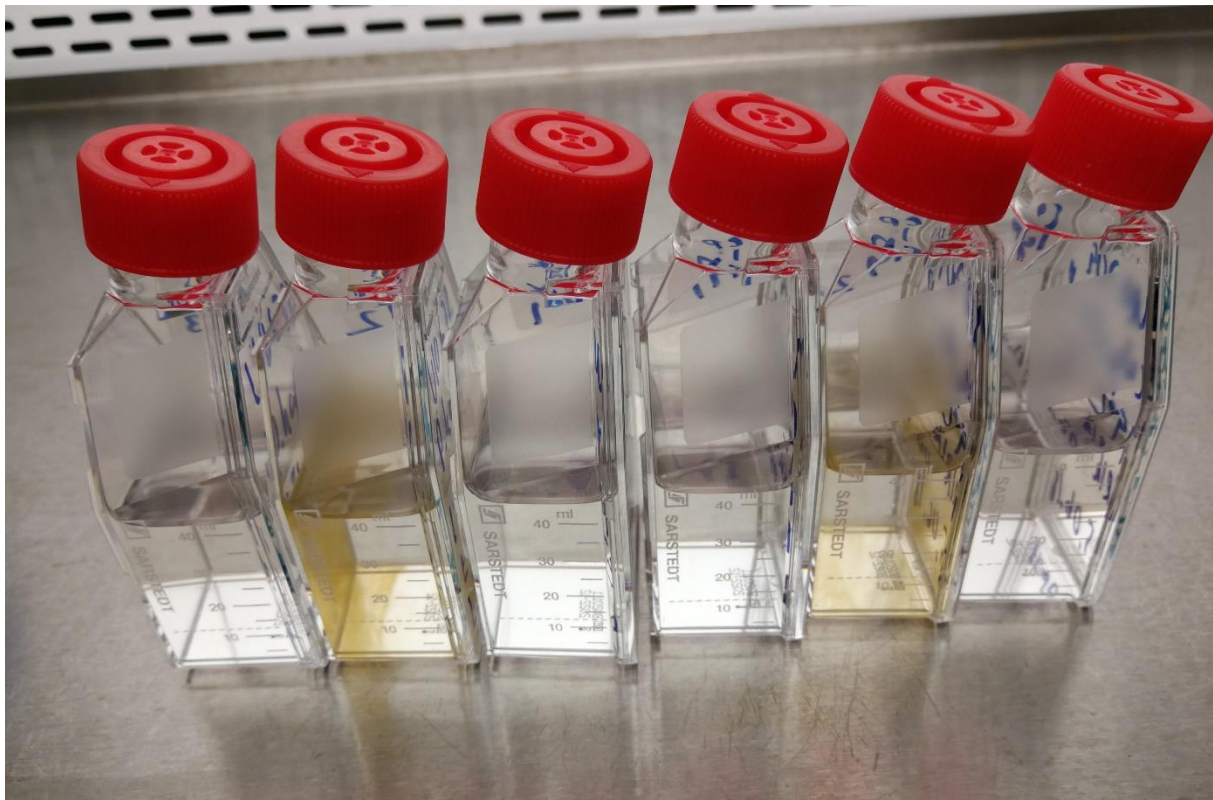
Figure 4: Six cultures from 19ºC. Cultures are recently diluted and figure shows how Prymnesium kappa, 19ºC, replicate 2 (032.19.2) as second leftmost culture and Calyptrosphaera, 19ºC, replicate 2 (309.19.2) as the second rightmost culture, have visible amount of algal growth on flask walls.

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transferred to new flasks more frequently due to cell growth on flask walls. These two had much algal growth on the walls of the flask, as figure 4 shows. The figure depicts how two of the 19°C treated cultures shows distinct algal growth on the flask walls, recently after dilution (of all cultures). The more growth one wall, the less light is available for free floating cells. Getting a precise cell concentration estimation is also a problem when facing cells that not only grow on the flask walls, but also sticks to them.

All the algae for the temperature experiment received the same light intensity – ca 50 µmol photons m^{-2}s^{-1}, measured using a light meter (LI-COR LI-1000 DataLogger, LI-COR Biosciences). Initially, different light intensities were tested, ranging from ca 20 to 100 µmol photons m^{-2}s^{-1}, but 50 µmol was chosen, judged from the growth rates. Although, as some of the cultures showed sign of cell growth on the flask walls, they were moved further away from the light (to around 40 µmol) which somewhat resolved the problem, at least reduced the growth on the walls. In the start of the experiment, both cool and warm white light lamps was used, but after one month, two cool, white light sources were chosen instead. Fluorescent lamps with a power of 36 watts (2x 18W, PHILLIPS TL-D 90 De Luxe) were used throughout the experiment.

In addition to the in the temperature experiment, an additional experiment was carried out using all the alga species, except for *P. kappa*, described in section 2.1. The algal cultures were kept and maintained for a period of ca. 10 months. These cultures were cultivated in a climate room with a temperature of ca 16°C and had a light/dark-cycle which was set to 14:10h L:D cycle. One cool and one warm fluorescent lamp (same type as in the temperature experiment) was used with a power of 2x28 watts emitting a light intensity at 20-25 µmol photons m^{-2}s^{-1}. These cultures were kept at a lower dilution rate, where ca 1 ml culture were transferred to new 40 ml flasks (same used here as in the temperature experiment) every third week and ca 40 ml medium were added to the flasks. The purpose of this comparative experiment was to test the correlation between cell-and genome size at the interspecific level across alga species.

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2.3. Medium

In this experiment, a 30 PSU IMR ½ medium was used for all the species. The IMR medium was first described by Eppley et al. (1967) and is sea water enriched with natural occurring minerals and molecules that's important for the cell's survival. The IMR ½ medium used in this experiment is of half concentration of stock solutions compared to Eppley et al.'s initial recipe. Sea water, ca 34 ‰ salinity and collected from 40-meter depth (Drøbak, Akershus, Norway), was filtrated trough a Bottle Top Vacuum Filter (millipore centre disc) and a 0.2 µm pore GF/C filter. For each 1 litre 30 PSU IMR ½ medium made, 900 ml filtrated sea water, as well as 100 ml distilled water, was added to a 1 litre flask. 0.5 ml KNO_3 solution, 0.5 ml phosphate (KH_2PO_4) solution, 0.5 ml vitamin solution, 0.5 ml Trace Metal solution and 1 ml selenite ($Na_2SO_4 \cdot 5H_2O$) solution was added to the 1 litre medium (See appendix I for a more detailed recipe). The flasks (usually 4 litres were made each time) was then placed in the autoclave chamber (Getinge HS 6610 EC-1 (2012)) and the medium was autoclaved for ca. 1.5 hour (this is the total time for the whole cycle, including heating and cooling) before the medium flasks was then placed in a $13^{o}C$ climate room.

2.4. Cell number and cell size measurement

To make sure that all the algae in the cultures grew exponentially, and as healthy and quickly as possible, the cell number, or density, was closely monitored. Measuring the size, or volume, of the cells were also performed to see if the temperature treatment had an effect of the size of the cells. To measure this, two different methods that could be used to measure both cell number and cell size was used. In the start of the experiment, the cells were measured using a light microscope. After some time, the Casy Cell Counter was used (sometimes together with microscopy). In the start of the experiment, is was also tested to measure the culture density, cells/ml, by measuring the absorbance, using a spectrophotometer. The idea was to first count the cell manually, then measure the absorbance. This was done with several dilutions of lower density, to be able to plot a curve and determine a regression line so that it would be possible to just measure the absorbance and find the concentration from the regression line. However, the spectrophotometer (UV-160A, UV-VIS Recording Spectrophotometer, Shimadzu) that was available, was not

calibrated for such low concentrations and the results acquired was not trustworthy, and this method was therefore quickly abandoned.

2.4.1. Light microscopy measurement

Around 4 millilitres of algal culture (which is inverted a few times to ensure that the cells are evenly distributed) was transferred to a small glass container/flask with lid and was added one drop (50-100µl) of Lugol's Iodide was added. After a quick mixing, just by inverting the flask, and a short incubation, the culture sample was ready for measurement. A sample aliquot was added onto a Fuchs-Rosenthal Cell Counting Chamber, holding 3.2 µl per grid, (Preciss Europa, Auxilabs S.L.) and a special cover slide was used to cover the sample. The cells were allowed to sink for around a minute or more to ensure that the cells were at the same level of depth. The counting chamber was inserted into the light microscope (Leica DMLS, Leica microsystems) and a minimum of 200 cells was counted each time (and for each culture). The Fuchs-Rosenthal Chamber Slides have a Grid system where you count all the cells within a 0.25 mm^2, 1 mm^2 or 4 mm^2 square (as figure 5 shows) and from this number, you can calculate cell/ml.
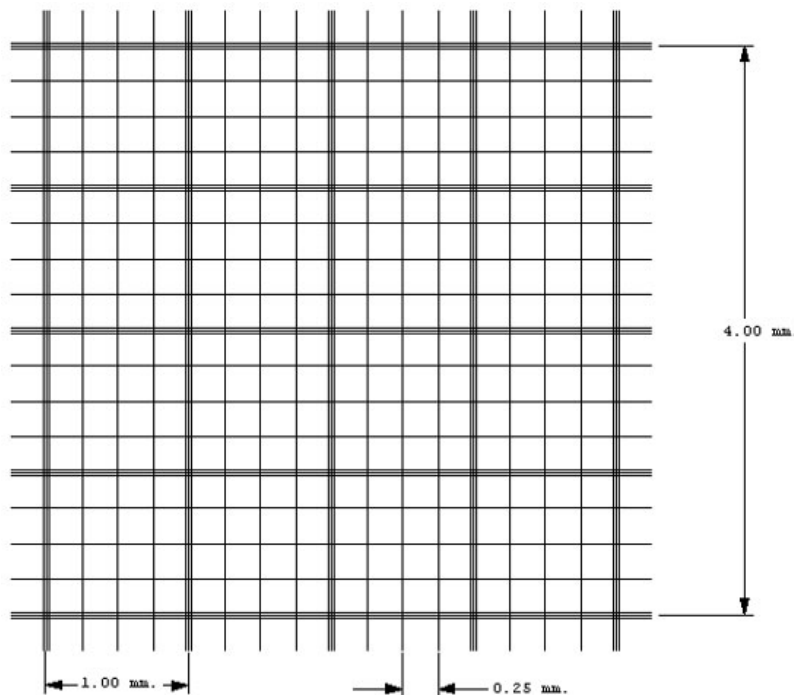
Figure 5: Fuchs-Rosenthal Counting Chamber Grid

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To measure cell size using microscope, a Fuchs-Rosenthal Counting Chamber can be used, but an ordinary microscope slide works just as well. Most microscopes have a built-in "ruler" and based on the magnification (objective used) used, one can calculate/estimate the size of the cells. To get a completely and a more precise measurement, close to 50 cells should be measured. This way, possible outliers will not have such a great impact on the result.

2.4.2. Casy cell counter measurement

Cell concentration and cell size can also be estimated using the Casy Cell Counter (Scharfe Systems). The Casy can only count up to 100 000 particles pr. ml, hence, the algal culture has to be diluted to get an accurate number. Using a syringe, 5 ml CasyTon was filtrated through a 0.45 µm filter into an empty, clean Casy Cup and 500 µl algal culture was added to the same cup. But before addition of the algae, the culture (ca 700-800 µl to make sure that 500 µl was left) was filtrated trough a 35 µl mesh filter (35 µl BD Falcon Filter cap) to remove any larger debris which may clough the tubes. The 500 µl algae and 5 ml CasyTon (1:10 diluted) was gently mixed before the cup is installed into the Casy Counter. Two other containers are needed. One, which is empty, for waste and the other for clean, pre-filtrated CasyTon. Several counts/runs are necessary when measuring more than one culture, so several washing cycles and some measure cycles (using only filtrated CasyTon in the Casy cup), to make sure that there are not large amounts of debris that disrupt the signal.

The Casy Cell Counter settings was set to 200 µl sampling volume (3 cycles) and 60 µm capillary used. To make sure that all small debris is not counted (Casy counts not only living cells, but also dead as well as bacteria etc.), a minimum (and maximum) thresholds were set to 3.75 – 10.58 µm. When the (3-cycle) measurement is finished, an information output window (like figure 6 shows) is shown on the screen. From this, information, as cell concentration (if a diluted sample (as in this example) is used, calculation is necessary), estimated cell size (assuming spherical particles) and (size) distribution, is shown.
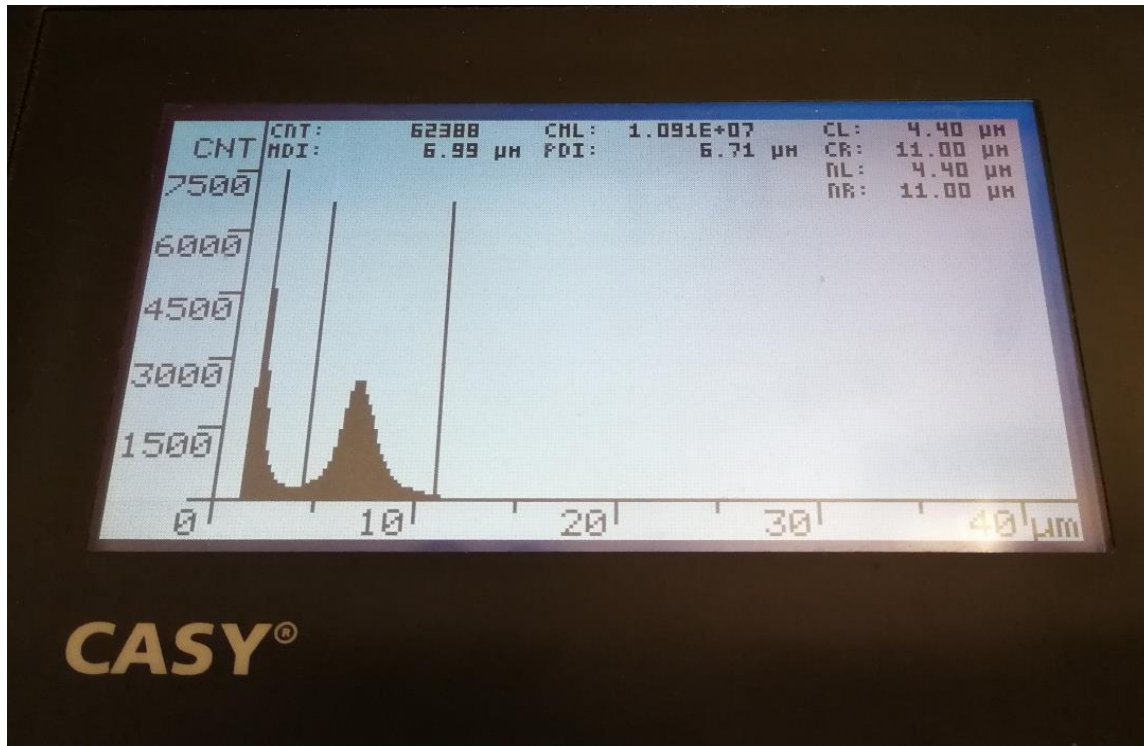
Figure 6 Example of a Casy Cell Counter output after the measurement cycles are completed. The output window shows counts (CNT), distribution of cells, size (MDI) of cells and counts pr. ml (CML).

2.5. Genome size estimation using Flow cytometry

The DNA have a great impact on the organism and, for most ectotherm organisms, it is presumed that the genome size is correlated with cell size. Also, as the genome may be altered as a consequence of random mutation, external forces and selective evolution, studying the genome size is of great interest. All the genome sizes in this experiment was estimated using FACS Calibur Flow Cytometer (FCM) (some cultures were also sequenced, section 2.6).

2.5.1 Cell lysis

The first step of FCM is to lyse the cell, isolating the nuclei and making the DNA available for staining. The recipe used is a modified version Dominique Marie's protocol from Marie et al. (2001). A Nuclei Isolation Buffer (NIB) is necessary to isolate the nuclei from the cell and the recipe used is a somehow modified version of Marie's (Marie et al. 2001). $MgCl_2$ (30 mM), Sodium Citrate (20 mM), D-Sorbitol (120 mM), HEPES (55 mM), EDTA disodium salt (5 mM) (for a more details, see Appendix I, supplementary table 4).

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Around 1.5 ml of algae culture was transferred to an Eppendorf tube and centrifuged for ca. 11 minutes at 1300 rpm and room temperature (RT). The IMR ½ medium supernatant was removed and only the cell pellet was left. The salt water medium will disturb the colouring and give false results, hence centrifugation and removal of excess medium. The centrifugation time differed a bit from algal to algal species. Other than that, this step was the same for all cultures.

The cell lysis step is an important step in FCM and here there were some bigger differences from species to species. For simplicity reason, only two of the formulas, *Calyptrosphaera sp* (UIO 309) and *Prymnesium kappa* (UIO 032) from the temperature experiment, are mentioned in this section (the different formulas used for the other algal species are listed in Appendix I, supplementary table 5). For *Prymnesium kappa* – 260 µl NIB, 740 µl PBS EDTA and 6 µl Triton X-100 (Diluted 1:10 using MQ H_2O). For *Calyptrosphaera sp* – 200 µl NIB, 800 µl PBS EDTA and 4 µl Triton X-100 (1:10 diluted). All work was done on ice. When several samples were prepared at the same time, a mastermix was usually prepared (and 1 ml of this was transferred to each of the Eppendorf tubes). The cell pellet (after centrifugation) was resuspended both by pipetting up and down, and vortexing. After mixing was the sample incubated in a fridge (dark and 4°C) for minimum 20 minutes, but even longer was sometimes necessary (in section 2.5.3. there will be another incubation step). During incubation, vortexing was conducted a few times to make sure that all the cells was completely lysed and the nuclei was isolated.

2.5.2. Percoll filtration

Percoll filtration is a method to filtrate out isolated nuclei (or any other particle or organelle) from a sample that contains bacteria, debris or other unwanted particles. This method is not only used for isolation of nuclei which than can be stored, percoll filtration is also used to filter out other unwanted organismal nuclei (DNA) which can affect the DNA sequencing. Percoll filtration of the lysed cell sample (from 2.5.1.) can be an additional step before genome size estimation using FCM, to make sure that your nuclei sample does not contain a lot of debris, not fully lysed cells or other particles that may disrupt the signal. For FCM, percoll filtration is not necessary, but for some samples, it may improve the result. In this experiment, Percoll filtration was especially used for algae, from the temperature experiment,

that were to be (DNA) sequenced. The main reason for why percoll filtration was conducted before DNA isolation (and sequencing) was to make sure that there only was algae DNA that was isolated and not large amounts of bacteria.

A 2 ml Eppendorf tube (a larger tube was used when a higher sample volume was used) was placed on ice to cool. The volumes used, differed, but for a 2 ml tube, ca. 400 µl 1.08 density Percoll (Sigma) solution was added to the tube and left for a few minutes to cool (the densities used varied (between 1.05 and 1.09 as the bottom layer) a little based on which algal specie used). Carefully, 1.06 density Percoll (this upper layer were usually 0.02 lower) was added onto the 1.08 layer and again left a few minutes to cool. The two different Percoll densities must not mix and to make sure that the stay apart, the 1.06 density Percoll was slowly pipetted on the side of the tube (which was held as sideways as possible). The amount used differed a bit based on how large algae sample volume that was going to be filtrated, but in this instance where a 2 ml Eppendorf tube was used, 400 µl of each density Percoll was used. On top of the Percoll layers, 1 ml of the algae nuclei sample was carefully added (using the same technique as when adding the second Percoll layer onto the first), making a total volume of 1.8 ml. A refrigerated centrifuge (Eppendorf microcentrifuge 5415R) was pre-cooled to 4°C and the 2 ml Eppendorf tube(s) was centrifuged for 50 minutes, 1000 rpm and 4°C.

After centrifugation, was the 1 ml algae layer removed. The nuclei should now be between the two Percoll densities layers, so ca. 250-300 µl was carefully removed from the top without dipping the pipette tip too deep. Around 200-300 µl of what was left in the tube, was transferred to a clean Eppendorf tube (a 1.5 will work well). The isolated nuclei should now be in the new, clean Eppendorf tube. To wash the nuclei and remove Percoll, 500 µl – 1 ml PBS EDTA was added and the sample was mixed by inverting and vortexing, before another centrifugation. This time at 4°C, 1000 rpm and 30 minutes. After centrifugation, was 0.5-1 ml (based on how much PBS EDTA was added) of the upper part of the sample, removed. A second wash cycle was conducted, but this time not more than 500 µl PBS EDTA was added. After vortexing and centrifugation, 500 µl was removed and washed and isolated nuclei should still be in the Eppendorf tube. The whole percoll filtration cycle was repeated two more times (for all algae in temperature experiment) to make sure that enough DNA would be isolated for DNA sequencing.

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2.5.3. Flow cytometry – FCM

Flow cytometry is a method to quickly estimate the genome size, as well as relative cell size, internal structure and ploidy level. In this experiment is genome size more or less the only factor of interest and to study this, BD FACS Calibur Flow Cytometry (Becton Dickson, San Jose, USA). In order to analyse the result acquired, the computer software Cellquest Pro (BD, San Jose, USA) was used.

Although several (slightly) different formulas was used to lyse the different algal species cells, the recipe for staining and preparing the samples for FCM, were all more or less the same. First of all, some difficulties were encountered and a lot of tests was performed, but more of this in the discussion. Samples from 2.5.1 (or 2.5.2.) was transferred to a BD Falcon 5 ml round-bottom tubes (12x75 mm). To these, 10 µl Chicken Red Blood Cells (CRBCs) was added. The CRBC works as an internal reference which is used to calculate the unknown genome size for the algal cells. 25 µl Propidium Iodide (PI) (0.1 mg/ml) was added to the sample. PI colour the DNA by binding to the major groove in dsDNA or dsRNA (RNase A testing in respect to PI colouring will be discussed later). The samples, after a quick vortex, was left to incubate in the fridge (dark and 4ºC). Several tests were conducted on PI, and in some results runs (at a later stage) was 50 µl used, but the amount of PI (until a certain amount) does not affect the final result in any significant way.
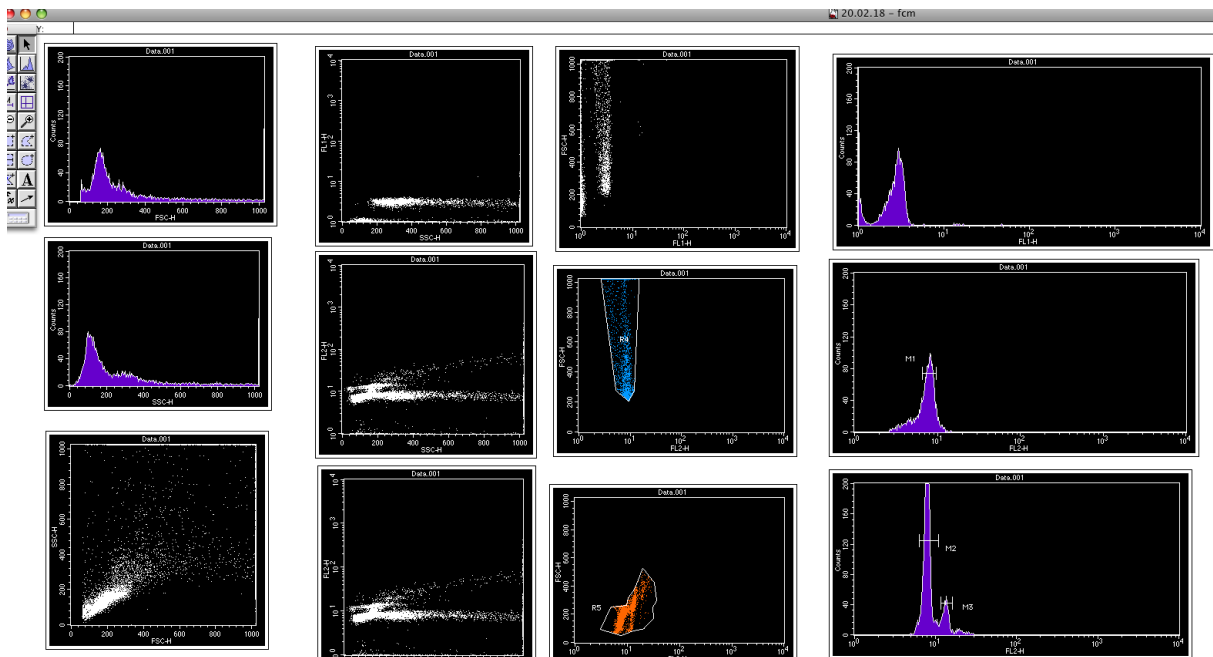
Figure 7: An example of a Flow Cytometer result output. The three to the left (from top to bottom): FSC histogram plot, SSC histogram plot, FSC/SSC dot plot. Middle left: FL1/SSC dot plot, 2x FL2/SSC dot plot. Middle right: FL1/FSC dot plot, 2x FL2/FSC dot plot. Right: FL1 histogram plot, 2x FL2 histogram plot. The top FL2/FSC dot plot is gated with respect to CRBCs and the bottom is gated with respect to algae nuclei.

After incubation was the BD Falcon tube inserted into the sample injection port (SIP) and ran with a flow rate between 100 and 200 events/sec on low flow rate. The settings used can be of great importance and especially FSC (Forward scatter) must set the voltage to E01 (for algal cells) or else you may not get any results (E00 was used for some time during the start of the experiment, and yielded no results for the algal nuclei). SSC (Side scatter) was set to 505 voltage and linear (lin) scale (logarithmic scale was sometimes used). Propidium Iodide has a broad emission spectrum from 535-617 nm and FL2 detects wavelengths at 585 nm. Detector FL1 does not detect PI stained particles, but can detect CRBC, as FL1 detects wavelengths at 530 nm. Therefore, FL2 fluorescent detector was used as it detects all particles stained with PI. Both high and low voltage on logarithmic (log) scale, as well as linear (lin) scale, was tested. For the actual results, both lin and log scale was set to 705 voltage. No extra amp gain was used for linear scale run. For many algal species, there was a clear overlap between algal nuclei and CRBCs. Gating was there for necessary. Figure 7 shows an example on how the FCM result screen could look like (this example is for 350 voltage (log)) using active gating to separate CRBCs from algae cells. FL1 histogram plot is used to easily see which pattern belongs to which CRBC.

2.6. DNA isolation and DNA sequencing

To ensure if the acquired Flow Cytometer results were correct or not, the DNA were sequenced. This way, not only can one get an (another independent) estimation of the genome size, one can determine the DNA sequence and arrangement (repetitive elements, etc.).

DNA isolation was carried out using DNeasy Blood and Tissue Kit (QIAGEN Group) and following the accompanied protocol (DNeasy® Blood & Tissue Handbook. 2006).

Before the DNA isolation process, the nuclei from the 12 culture samples (from the temperature experiment) were isolated using percoll filtration (section 2.5.2), after cell lysis (section 2.5.1). To ensure that enough nuclei were isolated, the following procedure was repeated two more times. The acquired nuclei samples (for each culture) from these filtrations was mixed together (making 12 instead of 36 samples), centrifuged and the (or most of the) supernatant was removed.

As mentioned, the protocol followed is from DNeasy® Blood & Tissue Handbook. 2006 and the protocol used was: Purification of Total DNA from Animal Blood or Cells (Spin-Column Protocol). This protocol was followed and the full description can be found in detail in Appendix I. Protocol step 1, 1c, was followed and 250 µl PBS was added to all samples (with pellets), as well as 20 µl Proteinase K and 8 µl RNase A (0.1 mg/ml). In step 7/8, two cycles of DNA filter extraction, using100 µl Buffer AE was performed, giving 200 µl with isolated DNA which is now in the 1.5 ml Eppendorf tubes.

Except for these two steps (were something is optional), the protocol was followed completely.

To determine the DNA concentration, Qubit 3.0 Fluorometer (Invitrogen, Thermo Fisher Scientific) was used. First of all was a mastermix prepared by mixing 15 µl Qubit reagent and 2985 µl Qubit buffer from Qubit dsHS set (this set measures samples with concentration between 0.2 and 100 ng/µl). 198 µl of the mastermix was added to the 12 different 0.5 ml Qubit tubes and 190 µl of the mastermix was added to two other 0.5 ml Qubit tubes. In these two, 10 µl standard #1 was added to one, while 10 µl standard #2 was added to the other. In the 12 tubes with 198 µl mastermix, was 2 µl from the 12 samples of the isolated DNA added. All 14 tubes, all with 200 µl, was vortexed before measuring, simply by placing the Qubit tubes one by one into the fluorometer. The two standards were measured first.

Sequence analysis was conducted by Jon Bråte and Øyvind Gulbrandsen.

2.7. Electron microscopy

To determine if there were any visible, structural differences that had occurred in the different temperature treated algal cultures, electron microscopy was used.

2.7.1. Transmission electron microscopy (TEM)

The procedure described by Eikrem and Moestrup (1998) was followed throughout this section. Transmission electron microscopy (TEM) grids was used and a droplet of algae culture was added onto the grid. Three drops of Osmium tetra-oxide acid (with gas heavier

than air) was dripped onto a Petri dish which was quickly turned up-side-down over the prepared TEM grids – gassing the samples for ca 30 min (although, in the procedure from Eikrem and Moestrup, 1998, 2 minutes was used). After this, was the Osmium removed and the samples left to dry for 40-50 minutes. When dry, was the grid samples washed in distilled water to remove the salts. They were then left to dry over the night. The grids were then placed on a droplet (two grids on each) of 4 % Uranyl Acetate (in H_2O) to stain the samples and left to incubate for ca. 20 minutes. After incubation, the samples were quickly washed in distilled water and left to dry before the grid samples were analysed using a Transmission electron microscope (JEM-1400 Electron Microscope, JEOL, USA).

2.7.2. Scanning electron microscopy (SEM)

Algal samples (around three drops) was added onto a Nuclepore Track-Etch Membrane (polycarbonate 13 mm diameter, 0.8 µm pores) and the medium was removed by filtration, using a vacuum suction pump. Salts were removed by adding two drops of PBS (pH 7.6) directly on the membrane (with algal cells on it). The membranes were left to incubate for 30 minutes in a 50°C incubation cabinet. The algae-membrane samples were covered by a 4 nm thick layer of gold and palladium in a sputter coater (308R-ER, Desktop Modular Coating System, Cressington Scientific Instruments, UK), before they could be visualised using a Scanning electron microscope (S-4800 Scanning Electron Microscope, HITACHI).

2.8. RNA, protein and C:N:P analysis

Temperature affects several basic parameters in organisms, especially growth rate. Thus, I included RNA, protein and Carbon:Nitrogen:Phosphorous (C:N:P) analysis as additional parameters. RNA scales with growth rate, and so does generally cellular P (of which RNA often constitute the major pool, cf. Skau et al. 2017; Hessen et al. 2017). Also, if more RNA is necessary to maintain the protein synthesis rate at low temperatures, this could require more cytoplasm, and therefore causing elevated cell volume.

For each of the 12 cultures from the temperature experiment, eight samples were prepared (four analyses and two replicas), making a total of 96 prepared samples. For RNA and protein

analyses was 1 ml algae culture added onto a nuclear pore filter. For CN (Carbon, Nitrogen) and P (Phosphorus) analyses was 3 ml algae culture added onto a GF/C filter. A water/vacuum pump was used to suck medium and smaller debris/bacteria trough, while the algal cells sticks to the membrane. Before samples for RNA analyses was acquired, all equipment was washed using RNase Erase (RNase Away). Membrane filters for RNA and protein was stored in Eppendorf tubes and GF/C filters was wrapped in tight using aluminium foil. All samples were stored in -80ºC freezer.

2.8.1 RNA quantifying analysis

To measure RNA amount in the cells, a protocol by Francisco Bullejos, derived from Skau et al. (2017) and Hessen et al (2017), which again is based on a protocol by Gorokhova and Kyle (2002), was used. The protocol can be divided into to five steps: nucleic acid extraction, RiboGreen dying, fluorescence measurement (RNA + DNA), RNA digestion, second fluorescence measurement (DNA).

As this protocol is derived (and also described there) from other protocol, the full, detailed protocol is presented in Appendix I.

To all 26 (24 samples + two controls) samples 1000 µl Extraction Buffer 1 were added. The sample membrane was grinded using Kontak Pestle and was sonicated for 3 repetitions of ca. 40 seconds sonication and 1-minute resting in the ice bath. After incubation for ca 2 hours (while shaking), DNA and RNA standards "supplementary table 6 and 7) was already prepared by Francesco Bullejos and further preparation of these was accomplished following supplementary table 8, Appendix I. A RiboGreen working solution was prepared by adding 50 µl commercial stock Quant-iT™ RiboGreen®RNA reagent (Thermo Fisher Scientific) to 15 ml tube and diluting it with 9950 µl TE buffer making a total of 10 ml.

70 µl of the DNA and RNA standards (and their blanks) was added to a 96 well plate. Two of each. In 26 x 2 (total 52) wells in the 96 well plate, was 68 µl TE buffer added as well as 2 µl of the experimental algal samples (two of each) added to the wells (making a total volume o 70 µl). Then, 70 µl of the working RiboGreen was added to all the wells, before it was placed inside the Plate Reader (SYNERGI Mx (BioTek)). Before the actual measurement, the well plate was left to shake for 5 minutes. The software, Gen 5 1.10. was used, with the settings;

480 nm Excitation and 528 nm Emission Wavelength. After the first measurement was completed, 5 µl RNase A (0.1 mg/ml) was added to each well and then left to incubate for 30 minutes in dark. The well plate was then shaken for 5 min before measurement using the same settings as the previous measurement. In the first measurement, DNA+RNA amount was measured, and in the second, DNA only was measured. Therefore, to get RNA amount, DNA measured amount value was subtracted from DNA+RNA value.

2.8.2. Protein Quantifying Analysis

Just as for the RNA analysis, a protocol prepared by PhD. Francisco Bullejos (which is based on a protocol by Barbarino and Lourenço (2005)) was used. Therefore, a more detailed protocol is presented in Appendix I, as it is already thoroughly described.

The protocol can be divided into two steps – protein extraction (1) and Fluorescence measurement (2). All samples were grinded and added 1000 µl of before the was left to incubate for 24 hours at 4ºC (in a fridge). The samples then underwent a sonication treatment of a 3-cycle, 40 sec sonication and 1-minute resting (in the ice water). All were centrifuged for 20 minutes at 4ºC and 16189 rpm (or 15000 xG) and after this was (around) 1 ml supernatant removed and transferred to new 2 ml Eppendorf. Then, 1 ml EM2 were added to all pellet samples and were again centrifuged before 1 ml supernatant was removed and added to the 2 ml Eppendorf tubes (now with a total of 2 ml).

Protein standard stocks were prepared (see Appendix I) and 100 µl of each was added to its own well in a 96-well plate. The protein samples were quickly vortexed before 100 µl of each (including the blanks) were added to its own well. Then, 100 µl WQPR* were added to the wells, making a total of 200 µl in each well (with samples in them). The microplate was then left to incubate for 15 minutes in the dark while shaking, before the fluorescence scan using the plate reader (Synergy Mx Microplate Reader, BioTek Industries). Using the software Gen5 1.10, and 485 nm excitation and 580 nm emission wavelengths, was the microplate scanned.

2.8.3. C:N:P ratio analysis

All 50 C:N:P membrane samples (24 CN + 24 P + 2 blanks) was (removed from -80°C freezer and) transferred to small Petri dishes. All membranes were placed leaning against the side so that as much as possible of the membrane was is contact with the air. This way they will dry evenly and without risking them getting stuck to the Petri dish. The frozen membranes were to thaw and dry for around 2-3 hours, all the time without the lid on. All 50 Petri dishes, with the membrane sample, was moved to a heat incubator where they were left over the night (almost 20 hours) at 50-55°C.

The samples were analysed by Berit Kaasa.

3. RESULTS

This thesis consists of two parts – the temperature experiment is the major part of this thesis, while a small part where addressed to investigate the interspecific differences and cell- and genome size correlation. In this part, only genome size and cell size were examined, with the main goal to find out how large, or if any, of a correlation there is between the two. In the first paragraph of this result section, I will present the interspecific study, while the following paragraphs will be devoted to the results from the major part – the temperature experiment.

3.1. Cell- and genome size correlation across different algal species

All algal cell sizes were measured using a light microspore at 40x objective. Figure 8 shows the size of all algal species, *Isochrysis Galbana* (UIO 140), *Diacronema lutheri* (UIO 090), *Hymenomonas carterae* (NIVA-2/92), *Prymnseium nemamethecum* (K-0394), *Calyptrosphaera sp* (UIO 309), *Phaeocystis globosa* (K-1321), *Pavlova gyrans* (K-1310), *Chrysotila carterae* (UIO 095) and *Prymnesium polylepis* (UIO 041). The algal species do differ a lot in cell sizes, ranging from 3.4 µm for *Diacronema lutheri* to 12.6 µm for

	UIO 140	UIO 090	NIVA-2/92	K-0394	UIO 309	K-1321	K-1310	UIO 095	UIO 041
Min	5	2,75	6,25	5,75	4,75	10	3	7,5	7,5
Avarage	5,4	3,4	9,6	7,2	5,0	12,6	4,0	9,2	10,8
Max	6,5	4,75	12,5	8	5,25	14,75	5,25	12,25	13
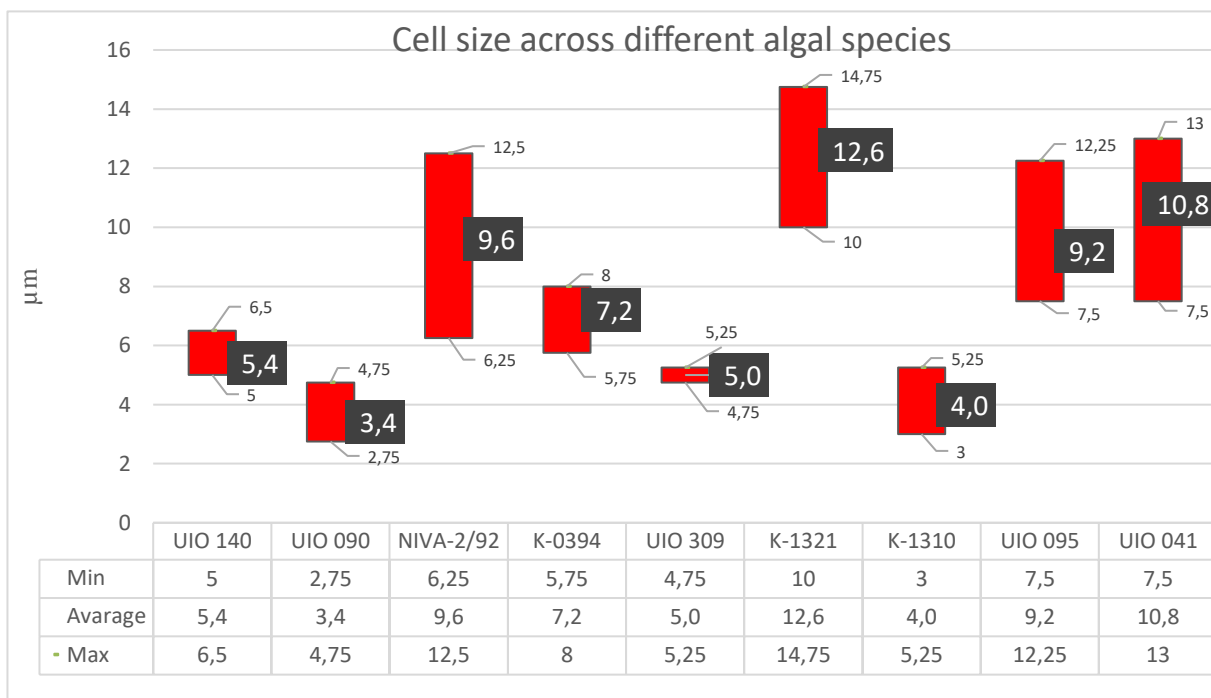
Figure 8: An overview showing the different cell sizes (in um) of nine different algae species, using light microscope. Both figure overview and adjacent table on bottom shows average size measured (with black background) as well as largest and smallest estimated size. From left to right: Isochrysis Galbana (UIO 140), Diacronema lutheri (UIO 090), Hymenomonas carterae (NIVA-2/92), Prymnseium nemamethecum (K-0394), Calyptrosphaera sp (UIO 309), Phaeocystis globosa (K-1321), Pavlova gyrans (K-1310), Chrysotila carterae (UIO 095) and Prymnesium polylepis (UIO 041)

Phaeocystis globosa. Each algal specie also has an internal variety and for some, the difference in size differ a lot, such as in *Hymenomonas Carterae* which was measured to be both 12.5 µm and 6.25 µm. When cells divide, the cell volume reduces with (almost) 50% of the total volume, so some "internal" differences are expected. Although all cultures were harvested when they were fairly dense, to reduce the number of dividing or just divided cells, we still see a great variety in sizes. The variety within a specie is also bigger in the larger species than in the smaller ones, with *Calyptrosphaera sp* measured to 4.75 µm and 5.25 µm, with a size difference at 0.5 µm. The four species (NIVA-2/92, K-1321, UIO 095 and UIO 041) with the highest measured difference between the largest and the smallest measured cell, have in average difference at 5.3 µm. The average difference between the largest and smallest algal cells for those species (UIO 140, UIO 090, K-0394, UIO 309 and K-1310) that showed the smallest "internal" difference, was estimated to be 1.7 µm.
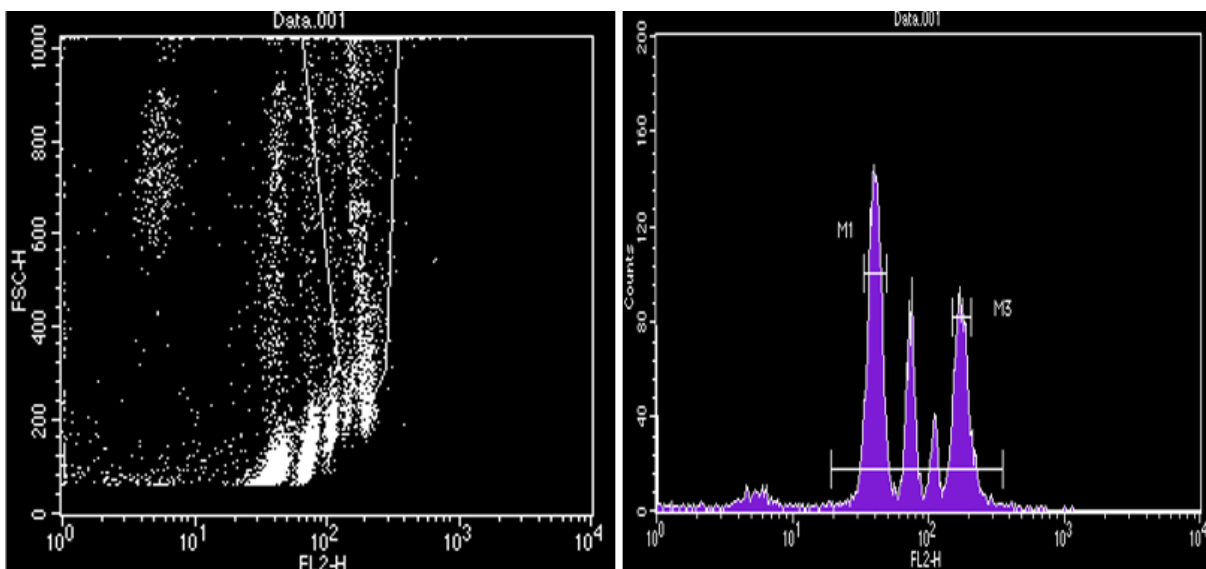
Figure 9: FCM result for Isochrysis galbana, strain UIO 140. Left picture shows FSC/FL2 dot-plot and picture to right shows FL2 histogram-plot. **Right:** *First peak (designated M1) belongs to UIO 041 nuclei C1 peak. The two next peaks is most likely C2 and C4 while the rightmost peak (designated M3) belongs to CRBC.* **Left:** *The same peaks can be found in the Dot-plot as well, together with a (white) gate, but this is not active.*

The genome size of all the algal species was measured using the flow cytometer (FCM). Figure 9 shows the result for *Isochrysis galbana* (UIO 140) with a FL2/FSC Dot-plot to the left and a FL2 Histogram-plot to the right (see supplementary figure 1 for more plots. All peaks are clearly visible and there is no overlap with CRBC. Therefore, the gate seen on left picture is not activated. Some debris is visible, but the majority of counts (of 10,000 total counts) is cells. The C1 value was measured to be 40.4 (mean channel) and CRBC was measured to be 173.5 (mean channel) giving a ratio 0.20 (genome size results are summarized in table 3).

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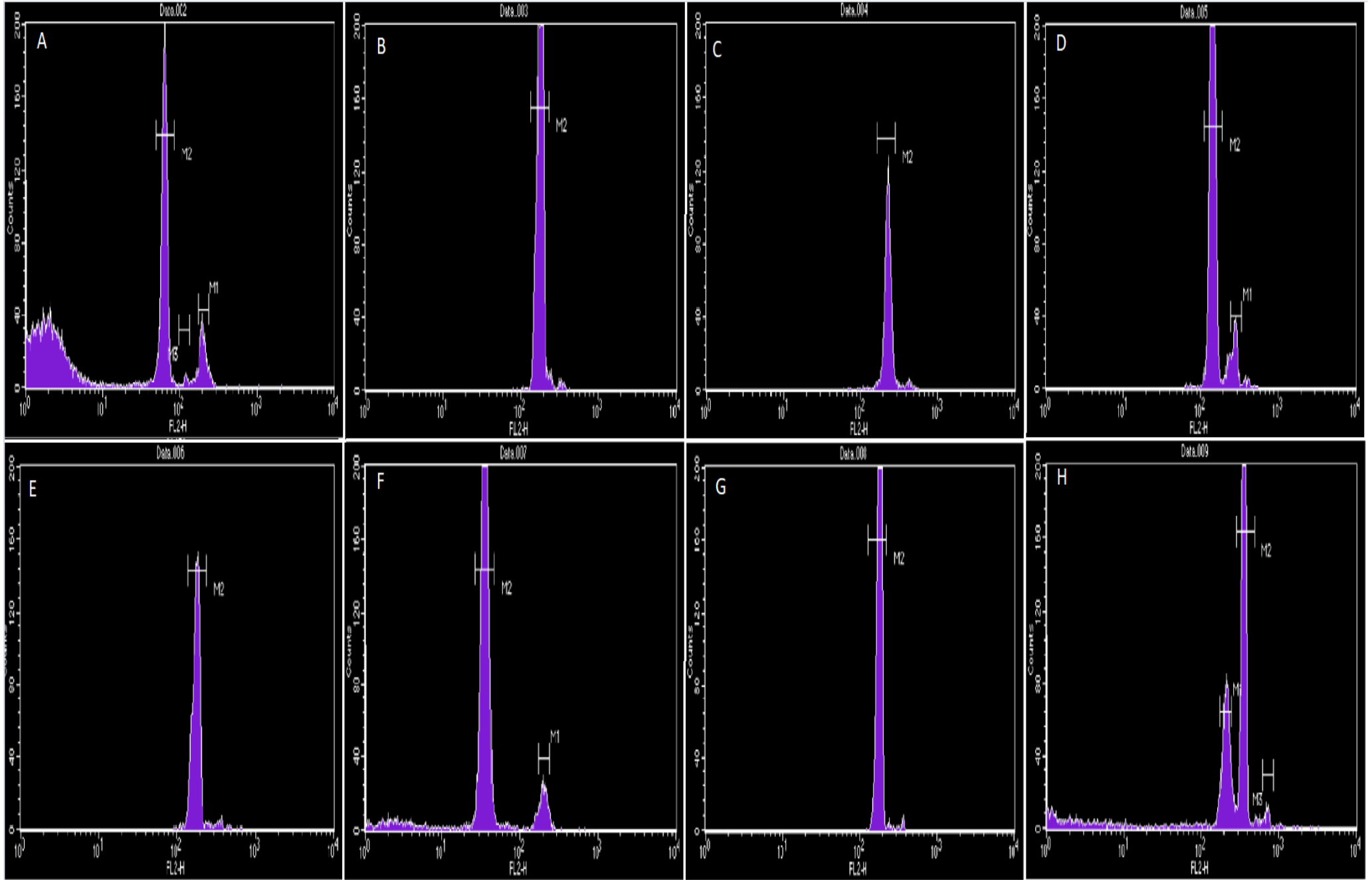
Figure 10: FCM results for the following eight algal species, respectively A to H: Diacronema lutheri (UIO 090), Hymenomonas carterae (NIVA-2/92), Prymnseium nemamethecum (K-0394), Calyptrosphaera sp (UIO 309), Phaeocystis globosa (K-1321), Pavlova gyrans (K-1310), Chrysotila carterae (UIO 095) and Prymnesium polylepis (UIO 041). A FL2 histogram plot is used for all of the different species and results. Also, the first algae nuclei peak, designated M2 for all, belongs to C1 (haploid cells), unless anything else is mentioned. For A, F and H, no gating is used and the plots therefore includes CRBC (designated M1). The plots B, C, D, E and G is acquired using gating, as CRBC overlapped, so the plots does not include CRBC. For plot D, Calyptrosphaera sp., the smaller, second peak (designated M1, but must not be mistaken for CRBC), belongs to the algae nuclei C2 (diploid form). Diacronema lutheri (UIO 090) and Prymnesium polylepis (UIO 041), A and H respectively, shows signs of a second (to regards to the algal nuclei) smaller peak (designated M3) which belongs to the algae nuclei C2 (presuming that P. polylepis are in haploid form). But, these second algal nuclei peaks are all small. This regards all species, except Calyptrosphaera sp. (plot D). NIVA-2/92 and UIO 095 are heterococcolith and therefore diploid. For the rest, as the diploid level is not certain, they are presumed to be haploid.

The FCM results for the rest of the algal species are presented in figure 10. Except for *Diacronema lutheri* (UIO 090) (A in figure 10), *Pavlova gyrans* (K-1310) (F in figure 10) and *Prymnesium polylepis* (UIO 041) (H in figure 10), are all the plots acquired by gating out CRBC. This was necessary as there was a clear overlap between the algal nuclei and the CRBCs. See Appendix II, supplementary figure 2 – 9 (for plot/specie A – H, respectively), for more FCM plots. Plot A shows a large amount of what is presumed to be debris in the lefteside of the plot. Plot A, together with plot C and E shows small, short peaks, indicating that a much smaller amount of algal nuclei were detected compared to the other plots/species. Nonetheless, the quality of all the peaks looks fairly good, (were most) looking tall and slender.

The lower peaks, indicating fewer algal nuclei events is supported in table 2, showing that for*Diacronema lutheri* (UIO 090, plot A), *Prymnseium nemamethecum* (K-0394, plot C) and *Phaeocystis globosa* (K-1321, plot E), roughly 3000 algal nuclei events were detected for each. Especially K-0394 and K-1310 shows large amounts of possible debris. Also included in the table is the average, measured mean channel for each specie. The point of interest here, is to see how stable CRBC is throughout the results.

Table 2: Summary of counted events and measured mean channel from FCM results for the different algal species, showing the counts for the algal nuclei, CRBC and possible debris, and the measured mean channel for algal nuclei and CRBC.

	Counted events			Measured mean channel	
	Algal nuclei	CRBC	Possible debris	Algal nuclei	CRBC
UIO 090	3000	5000	2000	62	223
NIVA-2/92	6000	1500	2500	175	196
K-0394	2600	1100	6300	222	166
UIO 309	8200	1000	800	141	193
K-1321	3800	2000	4200	173	196
K-1310	8500	500	1000	35	199
UIO 095	6000	2000	2000	176	205
UIO 041	6000	2000	2000	345	205

Table 3: A simple overview over the different algae species and their genome size presented in algae nuclei:CRBC ratio, size in pg and size in Gb (Giga base pairs). Propidium Iodide (PI) was used to dye the samples. NIVA-2/92 and UIO 095 are heterococcolith and therefore diploid. For the rest, as the diploid level is not certain, they are presumed to be haploid.

Algae Species	Genome size Ratio between algae and CRBCs	Genome size in pg	Genome size in Gb (Giga base pairs)
UIO 140	0.20	0.578	0.565
UIO 090	0.28	0.697	0.682
NIVA-2/92	0.88	2.207	2.158
K-0394	1.34	3.343	3.270
UIO 309	0.73	1.823	1.783
K-1321	0.88	2.205	2.157
K-1310	0.18	0.448	0.438
UIO 095	0.86	2.157	2.109
UIO 041	1.69	4.228	4.135

In table 3, one can see an overview over the different genome sizes. There is a fairly large difference in genome sizes with *Isochrysis galbana* (UIO 140), *Diacronema lutheri* (UIO 090) and *Pavlova gyrans* (K-1310) as the smallest and *Prymnesium polylepis* (UIO 041) as the largest, with *Prymnseium nemamethecum* (K-0394) as the second largest. To get the genome size in pg, the ration found was multiplied with 2.5 pg (the genome size of CRBC). To find genome size in Gb, the genome size (in pg) is multiplied with 0.978 (Doležel et. al (2003)). The interesting part of this study was to investigate if there is any significant correlation between genome size and cell size across the different algal species.

This correlation, between cell size and genome size, can be seen in figure 11. All dots/points are derivatives from table 3 and figure 8 (the mean cell size). From the figure, one can see that there seems to be a correlation between cell size and genome size, e.g. *Prymnesium polylepis* (UIO 041) with a cell size at 10.8 µm have a genome size at 4.2 pg, while *Diacronema lutheri* (UIO 090) have a cell size at 3.4 µm with a genome size at 0.70 pg. As one can see, there is not a perfect correlation, but there is some spread present. A regression line is included in the figure, showing a positive correlation with $r^2 = 0.48$ (where 1.0 is equal to 100% correlation and 0.0 is 0% correlation).
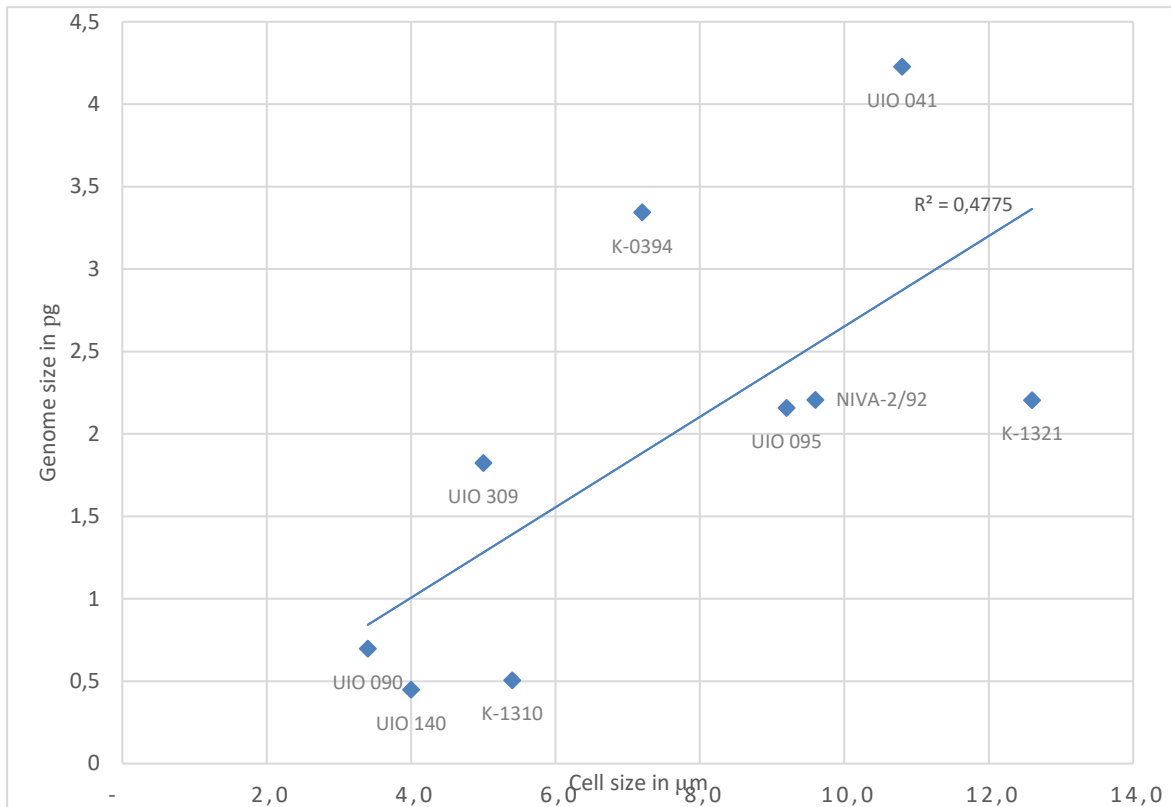
Figure 11 A plot showing the correlation between cell size (in μm on x-axis) and genome size (in pg) on y-axis. A regression line is plotted included, with a correlation value r`²=0.4775(≈0.5). All nine algae species are marked with their strain code-name.

3.2. Long-term temperature experiment

All further sections will contain results from the temperature experiment where *Caloptrysphaera sp.* (UIO 309) and *Prymnesium kappa* (UIO 032) were cultivated under two different temperatures, 11°C and 19°C. The main goal was to see if any significant changes to the genome size had occurred after one year in semi-continuous cultures. Other parameters have also been tested to get a more complete understanding on how different temperatures can induce changes in algae cells.

3.2.1. Cell sizes and cell concentration

For the first 10 to 11 months, the algae were cultures diluted three times a week, usually Monday, Wednesday and Friday. The exceptions were for a month in the summer, some weeks around yule and towards the end (after the first FCM result run were the cultures again diluted three times a week for around a month before the second FCM result run). To figure

out how much the cultures needed to be diluted each time, the concentration (cell/ml) had to be estimated. This was mostly carried out using light microscopy, but also the Casy Cell Counter. Light microscopy was mostly used in the start of the experiment before Casy was more and more used after around three to four months. After each measurement was the cultures diluted down to ca. 50,000 cells/ml.

Table 4 shows the measured average cell concentration. Throughout the whole experiment, the concentration was noted down and these data was used to calculate the average for each temperature and algae specie. As one can see, there is a clear difference between the two temperatures where the 19^oC algae cultures grew (more rapid cell division) faster than the 11^oC algae cultures. Another interesting about these data, is that the Casy Cell Counter seem to measure a higher concentration than light microscopy (e.g. 426,898 versus 244,669). This was also a bit of a concern when measuring, so a control test was carried out. In this, simple, control, the algae culture (032.11.1) was measured first in the Casy Counter before measured using light microscope. 141,023 cells/ml was measured using light microscope and 251,800 cell/ml was measured using the Casy Cell Counter. This is a difference at 110,768 cells/ml or a 78.5 % increased concentration measured in the Casy compared to light microscopy.

Table 4: Table showing average cell concentration for the four different algae species and temperature measured throughout the experiment using Light Microscope and the Casy Cell Counter.

	Light Microscope	Casy Cell Counter
032.11	191023	271176
032.19	244669	426898
309.11	138307	218787
309.19	376931	413796

Cell concentration was not the only aspect that was measured using light microscopy and the Casy Cell Counter. Also, cell size was measured. Even though the cell sizes were measured from time to time, measurement was not consistent and was somewhat faded out as other parts (especially the FCM) of the experiment took up large portion of the time. Therefore, the cell size development throughout the experiment period, is not included. Although, a more general cell size measurement was performed around 11 months after the experiment started, as one can see in figure 12 (Casy Cell Counter) and 13 (light microscope).
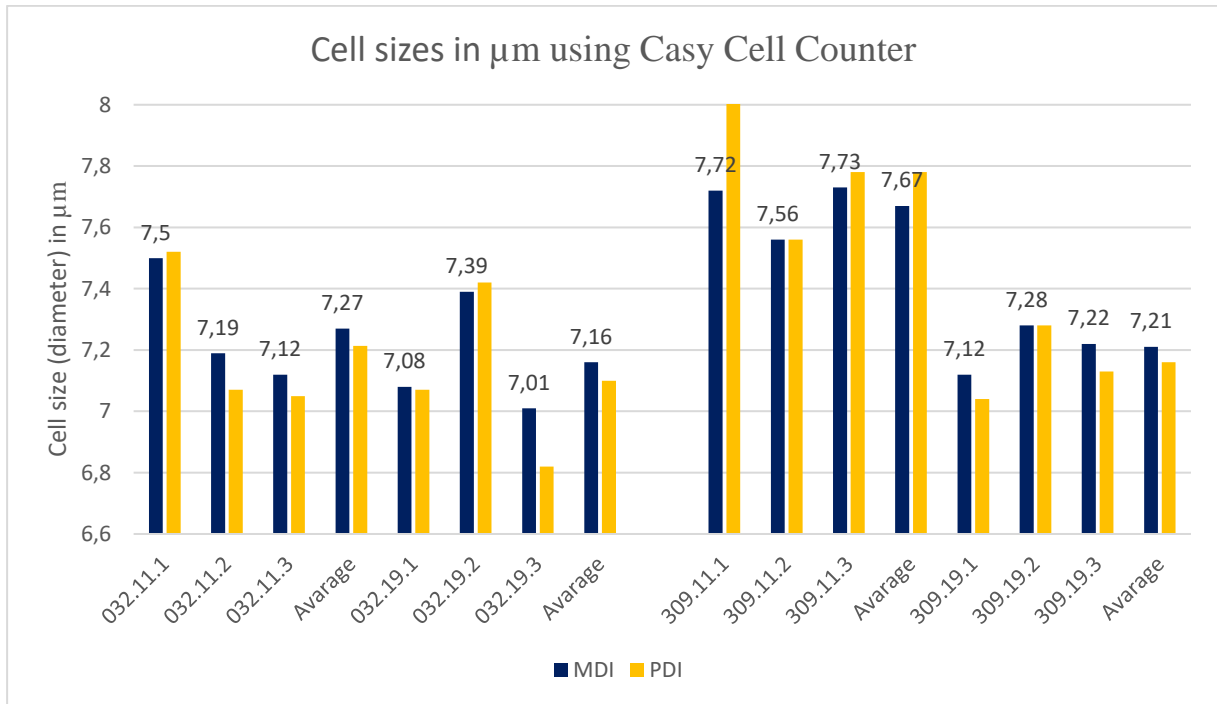
Figure 12: Overview of the 12 different algal cultures and their cell sizes, measured in both MDI (Mean Diameter) in dark blue and PDI (Peak diameter) in yellow, using the Casy Cell Counter. The four averages of each algal specie and temperature (e.g. 309.11) is also included. All sizes are measured in µm (y-axis). Y-axis is converted and values 0-6.59 is cropped out.

Figure 12 shows the measured cell sizes for all 12 algal cultures (e.g. 032.19.3) as well as the four averages for each two species and temperature treatments (e.g. 309.19). In the figure, each "average" is the average of the three previous replicates. From the figure, one can see that there is a small difference between MDI (mean diameter) and PDI (peak diameter) in all of the replicates, and especially for 309.19.1, measured PDI is 0.4 larger than MDI. All further values are in respect to MDI. In respect to *Prymnesium kappa* (UIO 032), both 032.11.1 and 032.19.2 seem to be somewhat larger than the other replicates with around 0.30 µm for 032.11.1 and 20 to 40 µm for 032.19.2 and this will of course affect the average. Average 032.11 was measured/calculated to 7.27 µm and average 032.19 was measured/calculated to 7.16 µm. In respect to *Calyptrosphaera sp.* (UIO 309), there seem to be stable measurements and not too much variation in the results. Although, PDI for 309.11.1 is 0.30 µm larger than its MDI counterpart, but MDI looks to be more stable (and is therefore focused on). 309.11.2 is a bit smaller than its two counterparts, but there is not a strong impact on the average due to this. Compared to the two other replicates, is 309.19.1 a bit smaller, but again, this does not impact the average too much. The 309.11 average, at 7.67 µm is around 0.45 µm larger than 309.19 average at 7.21 µm.
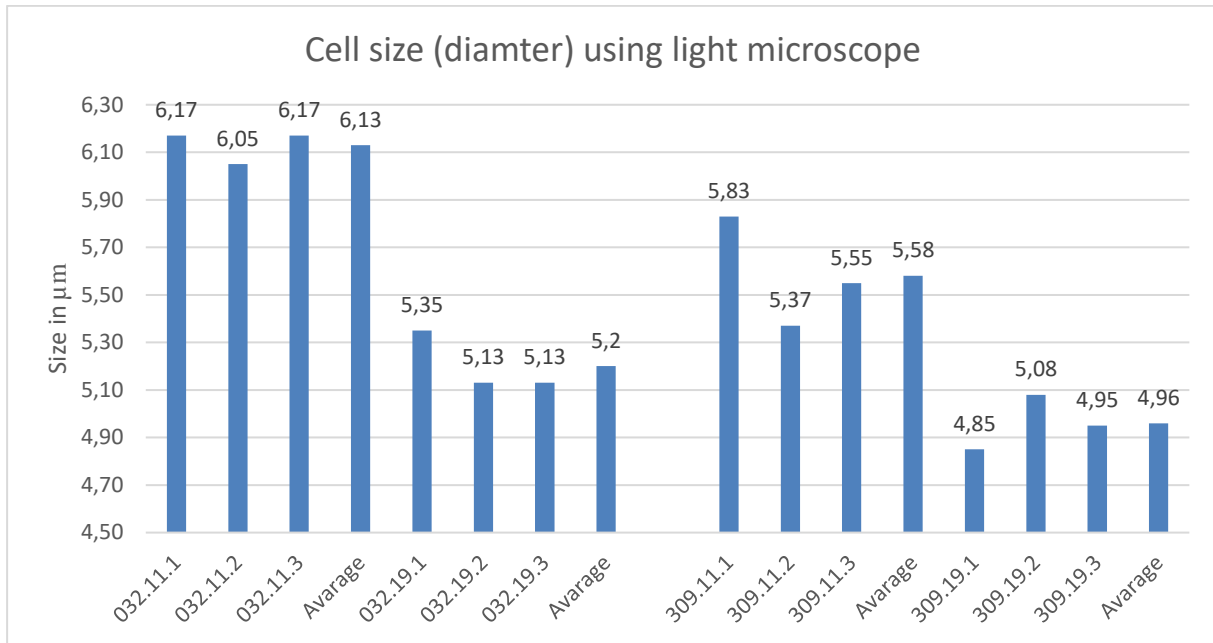
Figure 13: Overview of the 12 different algae replicas and their cell sizes, measured using a light microscope. The four averages of each algae specie and temperature (e.g. 309.19) is also included. All sizes are measured in µm (y-axis). Y-axis is slightly converted and doesn't include values 0-3.99.

Figure 13 shows the measured cell sizes for all 12 algal cultures (e.g. 309.19.2) as well as the average for the four temperatures and algal species (e.g. 032.11). In the figure each "average" is the average of the three previous replicates. For all cultures, several algae cells were measured and the average of these are the values for each given replicate. In respect to *Prymnesium kappa* (UIO 032), there does not seem to be any significant outliers. Average 032.11 was measured/calculated to 6.13 µm and average 032.19 was measured/calculated to 5.2 µm, giving almost a 1.0 µm difference between the two averages. In respect to *Calyptrosphaera sp* (UIO 309), there may be some outliers in 309.11.2 and 309.11.1 with around 0.45 µm difference, but it is difficult to say as 309.11.3 lies in between. As does the average, at 5.58 µm. For 309.11 there does not seem to be any big outliers, even though there is a difference at 0.23 µm between 309.19.1 and 309.19.2, but again, the average is in between these two, as is 309.19.3. The 309.19 average, at 4.96 µm is around 0.62 µm smaller than 309.11 average.

Even though both figures show that there is a difference between the two temperatures, Casy Counter claims that the cell are 1-2 µm bigger than that of light microscopy. Furthermore, while microscopy measurement suggest that the biggest difference is between 11ºC and 19ºC *Prymnesium kappa*, Casy Cell Counter claims that the biggest difference is between 11ºC and 19ºC *Calyptrosphaera sp*.

3.2.2. Flow Cytometry

The following results have been acquired using the Flow Cytometer. Although results were acquired on three different settings (high logarithmic (log), low log, and linear (lin) scale), only the results using high log scale is included (table 7 summarises results for *Calyptrosphaera sp* and table 8 summarises the results for *Prymnesium kappa* acquired using all three scales). Figure 14 shows all the acquired FCM results for *Calyptrosphaera sp* and figure 15 shows all the acquired results for *P. kappa.* See Appendix II for more plots (respectively supplementary figure 10 – 21).

From figure 14, one can see the FCM results for the six *Calyptrosphaera sp.*, both 11$^{\mathrm{o}}$C- and 19$^{\mathrm{o}}$C treated cultures. All are assumed to be haploid, 1C. Plot A – F is respectively 309.11.1, 309.11.2, 309.11.3, 309.19.1, 309.19.2 and 309.19.3. In all of the FL2 histogram plots, a FL2/SSC dot plot depicting both the algae nuclei and CRBCs (marked with red outlining). The algae nuclei's first peak (designated M2 for all plots), is in all of the plots tall and slender. Although, for 309.19.2 (plot E), the first peak is a bit shorter and the gap between the first and second peak, is not as deep, indicating more counts of particles at this size, in this plot, compared to the others. For all the plots, a second peak, most likely the algae nuclei C2 (diploid) (designated M3) is visible. Also, there are signs of a third, much smaller, peak, that may be the algae nuclei C4, or possibly debris.
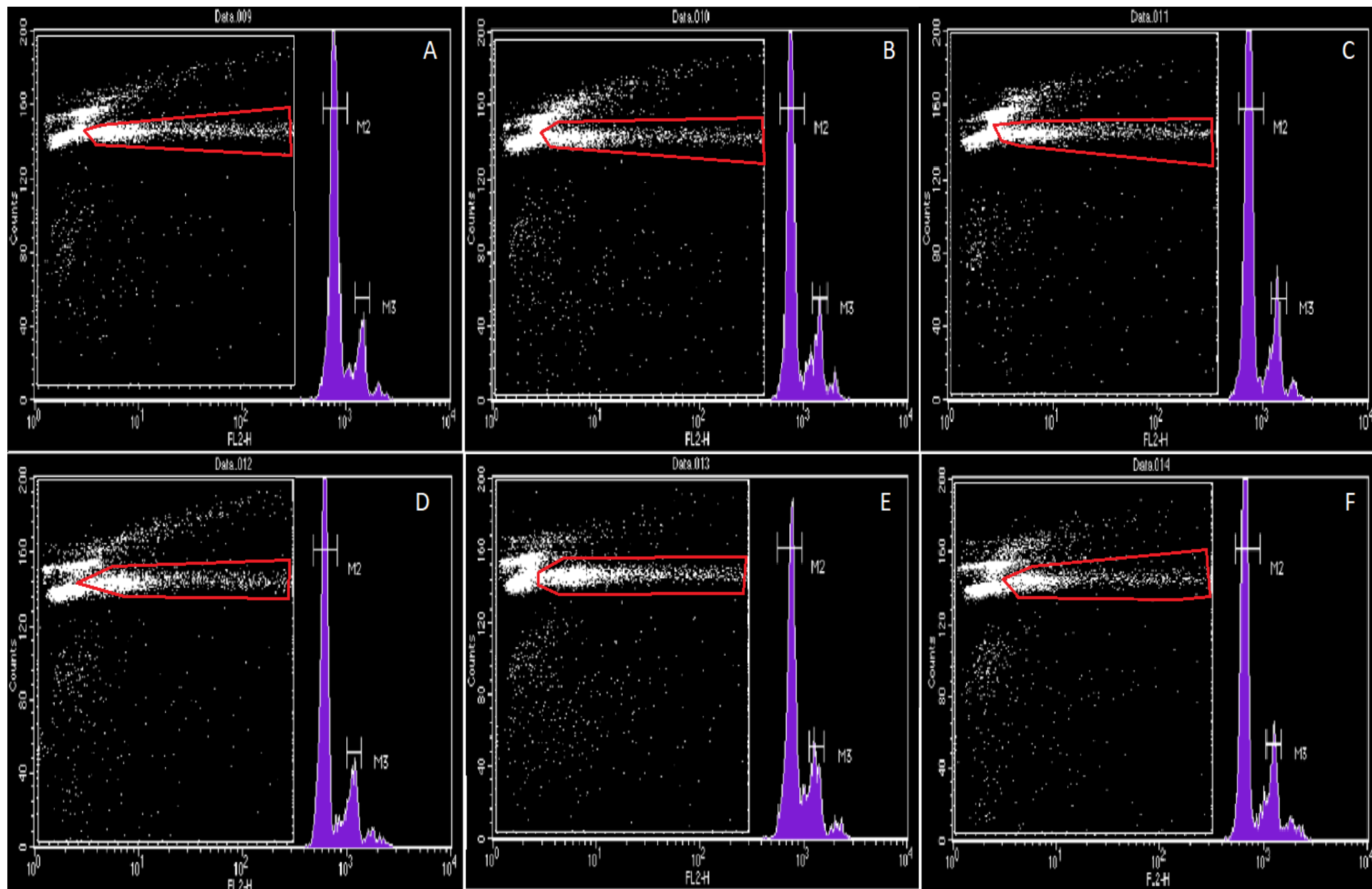
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Figure 14: FCM results for Calyptrosphaera sp., both 11ºC- and 19ºC temperature treated culture, where. A, B and C represent respectively replicate 1, 2 and 3 from the 11ºC treated cultures, and D, E and F represent respectively replicate 1, 2 and 3 for the 19ºC treated cultures. All plots depicted in this figure, are FL2-histogram plots using logarithmic scale. In all the plots, a (cropped) FL2-SSC dot plot (the SSC is in linear scale), is included within the histogram window, without overlapping. Since the dot plots as only added onto the histogram plot, the pattern is only relative fitted, as the y-axis values belongs to the histogram plot. For all plots, CRBC is gated out as it overlapped with the algae nuclei, but is shown in the dot plot (marked with red outlining). The first peak belongs to algal nuclei C1 is designated M2 and the second peak belongs to algal nuclei C2 is designated M3. A third pattern, or peak, is visible after the second, which may belong to algal nuclei C4, or it might be debris. The random scattered dots on the dot plot, is debris.

The summarised counted events and the measured mean channel for both the algal nuclei and CRBC, can be seen in table 5. From figure 14, there is not any strong evidences for any outliers, and this is more or less backed up by table 4. Although, 309.19.1, do show a larger amount of possible debris. 309.19.2 is shown to have the smallest amount of counted algal cells, something that is backed up by figure 14, even though the difference is not large.

Table 5: Summary of counted events and measured mean channel from FCM results for Calyptrosphaera sp, showing the counts for the algal nuclei, CRBC and possible debris, and the measured mean channel for algal nuclei, CRBC and the ratio between them.

	Counted events, of 10,000			Measured Mean channel		
	Algal nuclei	CRBC	Possible debris	Algal nuclei	CRBC	Ratio
309.11.1	5900	3900	200	754	836	0.9
309.11.2	5800	3800	400	734	828	0.89
309.11.3	6100	3400	500	743	828	0.88
309.19.1	5700	3000	1300	598	754	0.79
309.19.2	5300	4100	600	741	837	0.88
309.19.3	7200	2200	600	637	786	0.81

The FCM results for *Prymnesium kappa* (UIO 032) is presented in figure 15. All the six cultures are represented, with the 11ºC treated cultures depicted as plot A-C, 032.11.1, 032.11.2 and 032.11.3, respectively, and the 19ºC treaded cultures depicted as plot D-F, 032.19.1, 032.19.2 and 032.19.3 respectively. All cultures are represented by their own FL2 histogram plot with a (cropped) SSC/FL2 dot plot included without overlapping with any datapoints. As the CRBC overlapped with the algal nuclei, CRBC is gated out from the histogram plot, but is present in the dot plot (red outlining). For all the (histogram) plots, the first peak (designated M2) belongs to the algal nuclei (presumed to be) C1, while the second peak (designated M3) belongs to the algal nuclei C2 (diploid form). Both of these peaks can also be seen in in the dot plots, here as strong patterns.

The first algal nuclei peak looks both tall and slender in all of the plots, except for 032.19.2 (plot E). For this culture, the peak is much wider and shorter and the second peak, with a short gap between, is not as clear as for the other cultures. In contrast to the 11°C treated cultures, a third peak is present in (two of) the 19°C treated cultures, maybe except for 032.19.2. This may just be debris, or it may belong to the algal nuclei C4.

A summary of the counted events and the measured mean channel from the FCM results for *P. kappa* is presented id table 6. Here, one can see that there is an overall low amount of debris. There seem to be an outlier in 032.19.2 (which is supported by figure 15) as it has a much lower counted algal nuclei events and is measured to be somewhat larger compared to its replicates.

Table 6: Summary of counted events and measured mean channel from FCM results for Prymnesium kappa, showing the counts for the algal nuclei, CRBC and possible debris, and the measured mean channel for algal nuclei, CRBC and the ratio between them.

	Counted events			Measured mean channel		
	Algal nuclei	CRBC	Possible debris	Algal nuclei	CRBC	Ratio
032.11.1	6600	2700	700	824	826	0.99
032.11.2	6500	2400	1100	797	808	0.98
032.11.3	6200	2400	1400	823	819	1.01
032.19.1	7800	1500	700	636	773	0.82
032.19.2	4100	5500	400	812	842	0.96
032.19.3	7600	1500	900	627	767	0.82

Results from the *Calyptrosphaera sp* FCM results are collected and presented in table 7. Although only results using high log (logarithmic) settings are represented as figures in the result section, low log and lin (linear) settings was also used when measuring the genome size. First of all, one can see that there is a slight difference in acquired data using the three different settings, where High log and lin are closest together (e.g. 2.243 pg and 2.298 pg000 versus 2.423 pg and 2.243 pg). The genome sizes seem to be stable throughout each replicate for 309.11. (e.g. 2.284 pg, 2.292 pg and 2.268 pg with 0.025 pg in difference), but for 309.19.

there seem to be one deviation in 309.19.2 being close to 0.2 pg larger than the others of the 19ºC replicates. There is a clear difference between the two temperature treated algal cells, where the largest is between 309.11.1 and 309.19.1. The biggest difference between the averages is between 2.433 and 2.239 (giving 0.194 pg). There is a size difference at 0.163 pg between the two averages of the averages, and it could have been bigger if not for the possible outlier.

Table 7: Results from FCM of Calyptrosphaera cultures/replicates using three different settings, Low logarithmic, high logarithmic and linear scale. Average in yellow and average of the average in green. All data (except averages) stem from results earlier in this section and the ratios has been multiplied with 2.5 pg to get these numbers.

Genome size for *Calyptrosphaera sp* in pg			
	Low Log	High Log	Lin	
309.11.1	2.456	2.255	2.284	
309.11.2	2.423	2.243	2.298	
309.11.3	2.419	2.221	2.268	
Average	2.433	2.240	2.283	2.319
309.19.1	2.111	1.983	2.111	
309.19.2	2.392	2.213	2.284	
309.19.3	2.214	2.026	2.067	
Average	2.239	2.074	2.154	2.156

FCM results for *Prymnesium kappa* are collected and presented it table 8. Earlier in this section, FCM results figures collected using high log settings, is the only results represented so far, but low log and lin settings was also used to measure the genome sizes and they are all represented in this table. First of all, one can see that there are some differences in measured genome size between the three settings, where they all seem not too different (in respect to the average) for 032.11, but for 032.19, there is a small difference between high log and lin, compared to low log (in respect to average). For 309.11.2, there seem to be a small deviation compared to 032.11.1 and 032.11.3 especially for low log where the difference is around 0.3pg (032.11.2 is not that much smaller when looking at high log and lin scale). There also seems to be a deviation in 032.19, where 032.19.2 appear to be larger than the others, where the difference is the largest for high log with around 0.361 pg. In contrast to 032.11.2, the 032.19.2 outlier is indeed a deviation in regards to all the different scale settings (see figure 15, plot E). There is a clear difference between the two temperature cultivated algae cells

where the largest is between 032.11.3 and 032.19.3 at 0.468 pg. The biggest differences between the two averages is between 2.513 and 2.161 (lin scale) giving 0.346 pg in genome size difference. There is a size difference at 0.294 pg between the two averages of the averages, and it could have been even bigger if not for the two possible outliers.

Table 8: Results from FCM of Prymnesium kappa cultures/replicas using three different settings, Low logarithmic, high logarithmic and linear scale. Average in yellow and average of the average in green. All data (except averages) stem from results earlier in this section and the ratios has been multiplied with 2.5 pg (the size of CRBC genome) to get these numbers.

Genome size for *Prymnesium Kappa* in pg.			
	Low Log	High Log	Lin	
032.11.1	2.625	2.494	2.540	
032.11.2	2.324	2.466	2.473	
032.11.3	2.628	2.512	2.527	
Average	2.526	2.491	2.513	2.510
032.19.1	2.265	2.057	2.122	
032.19.2	2.420	2.411	2.258	
032.19.3	2.243	2.044	2.122	
Average	2.309	2.171	2.167	2.216

In addition to these results, another FCM run was performed a few days earlier, but because CRBC was not in the algae samples, but on its own, the results are not included in the main article. Though, it is included in the Appendix II (see supplementary table 8). The interesting is those results is to see the difference between peak, mean peak and median peak, and how, especially for low log settings, reading the histogram statistic and using peak to measure genome size can give some unnecessary inaccuracies.

3.2.3. Second FCM results for *Prymnesium kappa*

At a later point, around two months later, another FCM result run was performed with brand new CRBC. The intention was to test if there were any differences between the two CRBCs, but the acquired results was not as expected. There seemed to be a slight difference between the two CRBC (the old one had expired) where one was larger than the other, but the interesting part was to see what had happened to the 032.11 algal cultures. To make sure that

the result was not a fluke or something else, the algal cultures was diluted three times a week (as before the first FCM results) for around a month. After the diluting month, the algal cultures were again tested and the following results are from this run.

From figure 16 (see Appendix II, supplementary figure 22-27 for more plots), one can see all the FCM results for *P. kappa*, both 11°C and 19°C treated cultures. In the first row, A, B and C, is respectively 032.11.1, 032.11.2 and 032.11.3. In the second row, D, E and F, is respectively 032.19.1, 032.19.2 and 032.19.3. For all the cultures, a FL2 histogram plot is used, with an included (cropped) SSC-FL2 dot plot. First of all, one can see a huge difference in the 11°C treated cultures. Where there earlier was one larger peak, followed by a shorter, second peak, there is now two peaks with more or less the same height. For 032.11.2 (plot B), the second peak (C2) is actually higher than C1, the first peak. In contrast, the 19°C treated cultures looks good, with tall and slender first peaks, followed by a clear, second peak. Also, especially for 032.19.2 (plot E) and 032.19.3 (plot F), there seem to be a pattern after the second peak (visible in both dot plot and histogram plot) which are not seen in any of the 11°C treated cultures. When comparing the two different treatments, one can see a distinct difference between them in respect to the algal nuclei pattern in the dot plot (as different settings was used to acquire these results compared to the earlier *P. kappa*, the plots are not identical).

The unexpected results for the 11°C treated cultures is supported by the low amounts of counted algal nuclei, which is presented in table 9. Here, one can see how much lower the counted events are for the 11°C, with an average at ca. 2500 compared to the 19°C treated with an average at ca. 6600 counted events. This is a dramatic difference. The estimated genome size seems also to have been affected. There is still a difference, but now, only a slight difference.

Table 9: Summary of counted events and measured mean channel from FCM (second) results for Prymnesium kappa, showing the counts for the algal nuclei, CRBC and possible debris, and the measured mean channel for algal nuclei, CRBC and the ratio between them.

	Counted events			Measured mean channel		
	Alagl nuclei	CRBC	Possible debris	Algal nuclei	CRBC	Ratio
032.11.1	3000	5300	1700	202	190	1.06
032.11.2	2200	6000	1800	194	181	1.07
032.11.3	2000	5800	2200	207	185	1.12
032.19.1	6400	2500	1100	158	162	0.98
032.19.2	6000	2900	1100	168	175	0.96
032.19.3	7000	1700	1300	172	172	1.00

As one can see, there seem to be a large difference from 032.11. and 032.19. This difference was not there earlier, as figure 15 shows. Flow rate had decreased in all 032.11. samples from the first test to the second. The algal cell:CRBC ratio is decreased in all three samples and the algae nuclei C1 and C2 counts are more or less the same. A change has happened in the 032.11. algal culture, but not 032.19. And as figure 17 shows, there does not seem to happened anything to the 309.11. algal cultures (as well as the 309.19 cultures (not shown)).
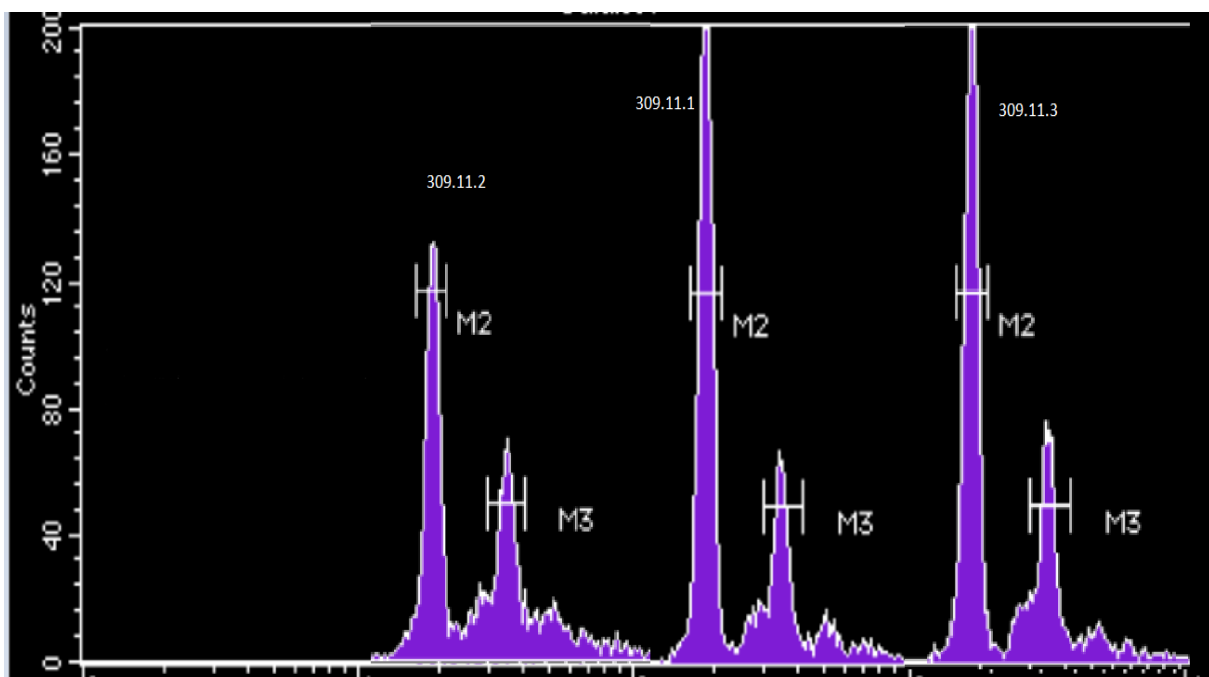
Figure 17: An FCM result picture of the three replicates of 309.11. with replicate 1 in the middle, 3 to the right and 2 to the left. Replicate 2 and 3 is cut and pasted into the same Histogram plot as 309.11.1 and the x-axis is therefore cut out. (Number of counts are still correct). M2 corresponds to nuclei C1 and M3 corresponds to C2, for all replicates.

Also, from figure 17, one can see that following the second peak, possibly belonging to alagl nuclei C2 (designated M3), there is a pattern that may belong to algal nuclei C4. This pattern is not visible for the 11ºC treated *P. kappa* cultures from figure 16.

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To try to get a better understanding of what had happened to the 032.11 algal replicates, 032.11.1 and 032.19.1 was studied in a fluorescent microscope. The flow rate had vastly declined from the first FCM results to the second (in respected to 032.11.), but number of cells/ml had not declined in the cultures (as seen using a light microscope). The hypothesis was therefore that the algal cell nuclei did not colour as it should and that the Flow Cytometer did not pick up the fluorescent signal from PI. Figure 18 shows the results from the fluorescent microscopy of 032.11.1 (which was prepared using the standard FCM lysis preparation) and from it, one can see that the nuclei indeed are coloured. Though, one thing
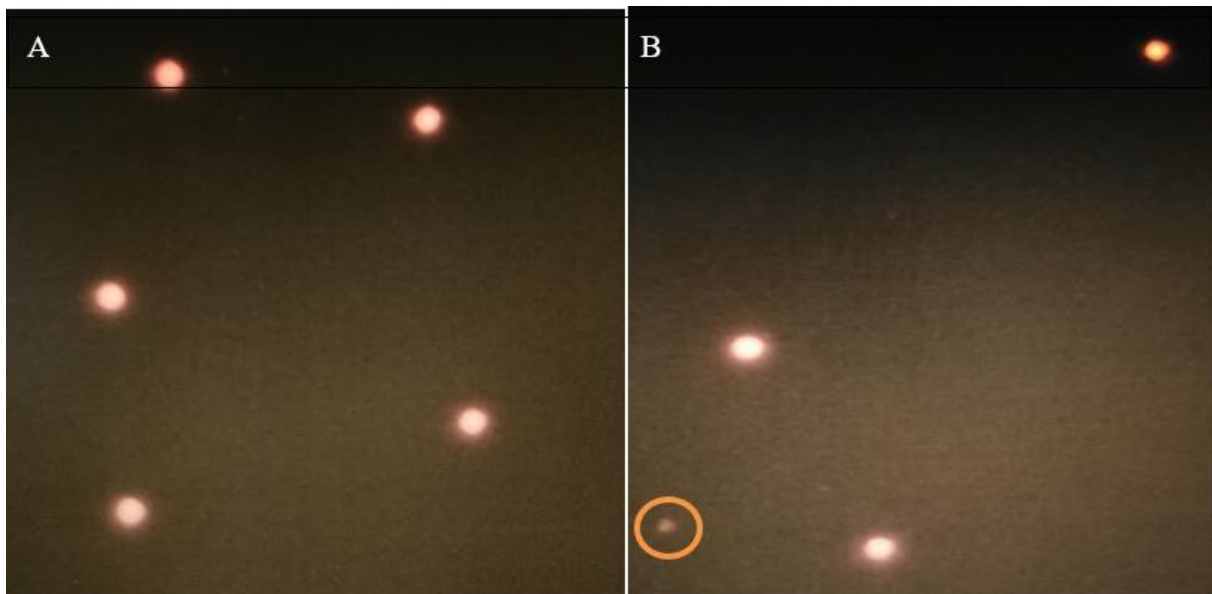
Figure 18: Fluorescent microscopy pictures of 032.11.1 algal nuclei. Picture A shows 5 clear, red dyed, cell nuclei. B shows three clear and strong dyed nuclei, as well as a smaller, not as bright nuclei (orange ring). Nuclei is coloured with PI.

that was discovered, was that there seemed to be a bit few cells when compared to light microscopy tests. Cell nuclei was spread throughout the microscopy glass plate, but there were not many condensed areas where as many as 5 nuclei was present in the same picture frame. In B, one can see a nuclei fluorescent signal that is both smaller and of weaker intensity compared to the others. If this is the 1C and the others are 2C, or if smaller, newly
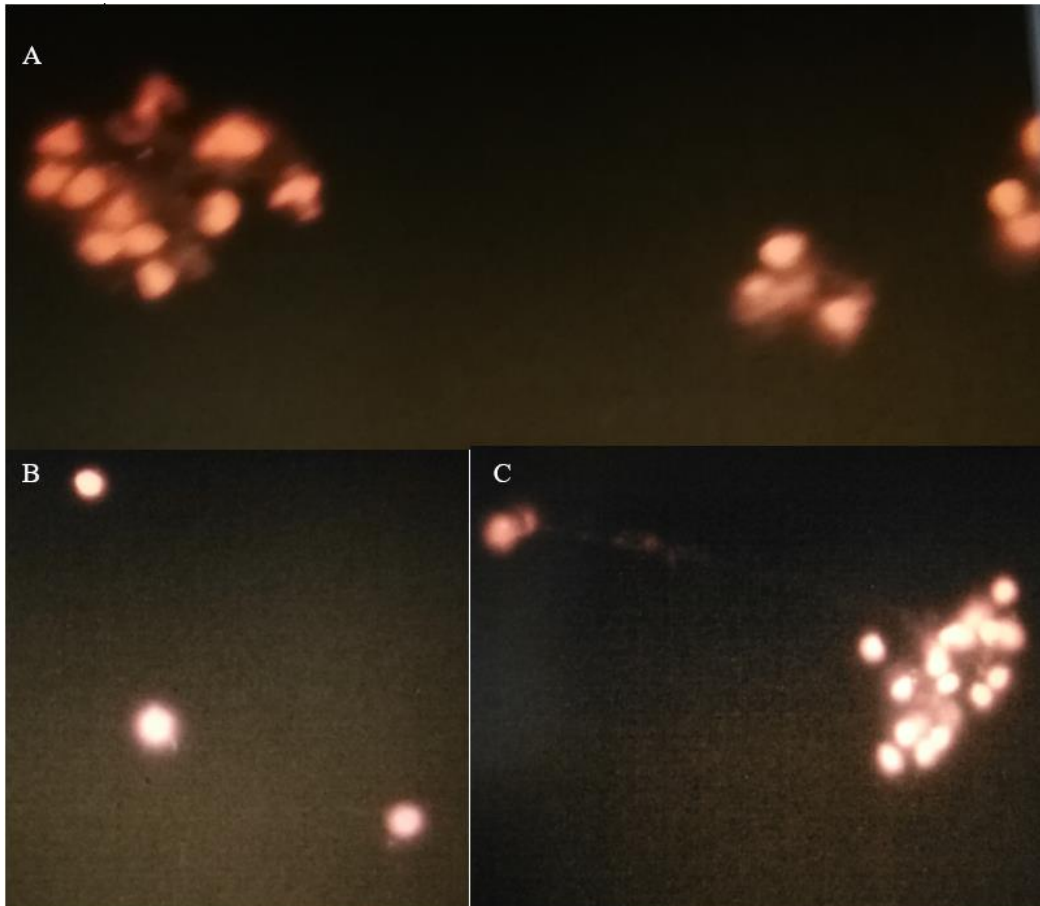
Figure 19: Fluorescent microscopy pictures of 032.19.1 algal nuclei dyed with PI. A and C shows clusters of nuclei while B shows three single algae nuclei.

synthetized cell, is difficult to say. But, there were not many of these smaller, weaker nuclei throughout the sample.

Figure 19 shows a fluorescent microscopy picture(s) of 032.19.1 algae nuclei. Unlike 032.11.1, here, the nuclei are much more often compiled in clusters and there was in general many more nuclei compared to 032.11.1. This is maybe not too surprising, but there seemed to be much more than twice the nuclei in 032.19.1 compared to 032.11.1. There also seemed to be just as many single nuclei, that appeared to be larger, as there were nuclei compiled into clusters.

3.2.4. Genome size and cell size correlation

Based on the results acquired from cell size measurement (figure 13) and the FCM results (in table 7), a correlation between cell size and genome size can be calculated and the result for

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Calyptrosphaera sp can be viewed in figure 20. The correlation between genome size (results from table 8) and cell size (from figure 13) *for Prymnesium kappa* can be viewed in figure 21.
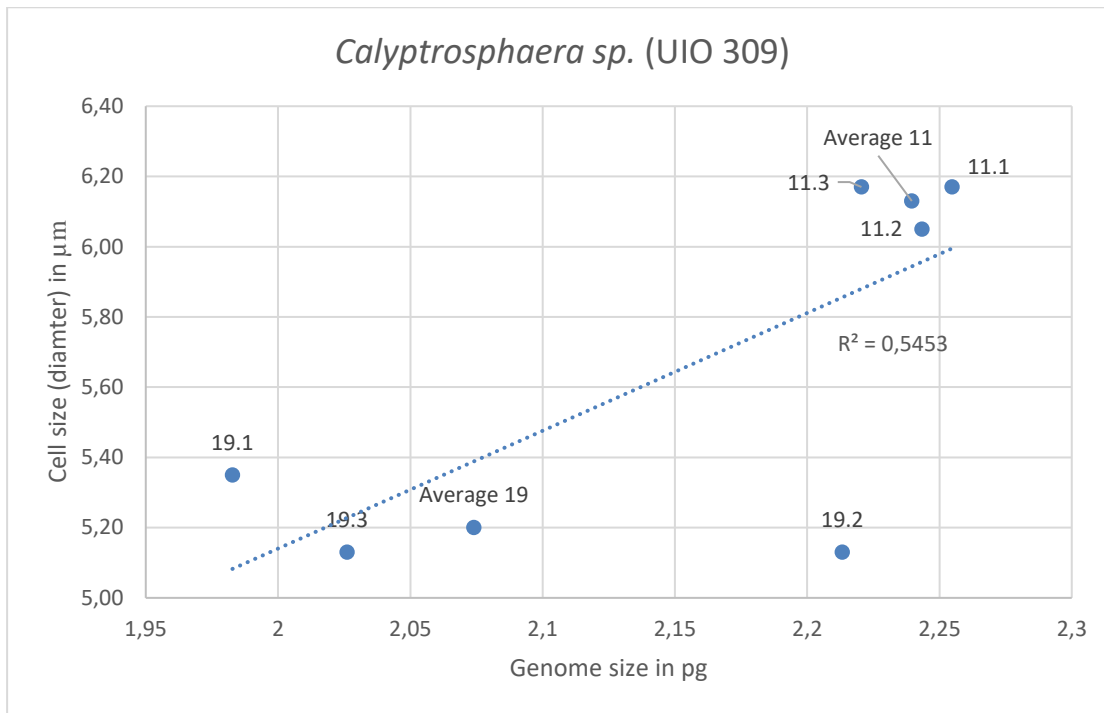
Figure 20: A plot showing the correlation between cell size (in μm on y-axis) and genome size (in pg) on x-axis. A regression line is plotted down, with the correlation value r²=0.5453. All six replicas have been named (without the 309-part), as has both averages.

As one can see in figure 20, the 11ºC replicates are nicely organized more or less together while the 19ºC replicates have a bit more space between them, where especially 309.19.2 (19.2 in the figure) looks like an outlier. Nevertheless, there seem to be a positive correlation (R^2) at 0.5453, but the included averages may affect the correlation.

For *Prymnesium kappa* (figure 21), there also seem to be one outlier in 032.19.2 (19.2 in figure) which will have an impact on the result. The 11ºC treated *P. kappa* replicates does not seem to be as close together as the 309.11 ones, but there does not seem to be any significant outliers. The is a positive correlation at 0.7491, but again, the included averages may affect the actual result.

In both figures, one can see that the lower temperature replicas seem to have both larger genome and bigger cells.
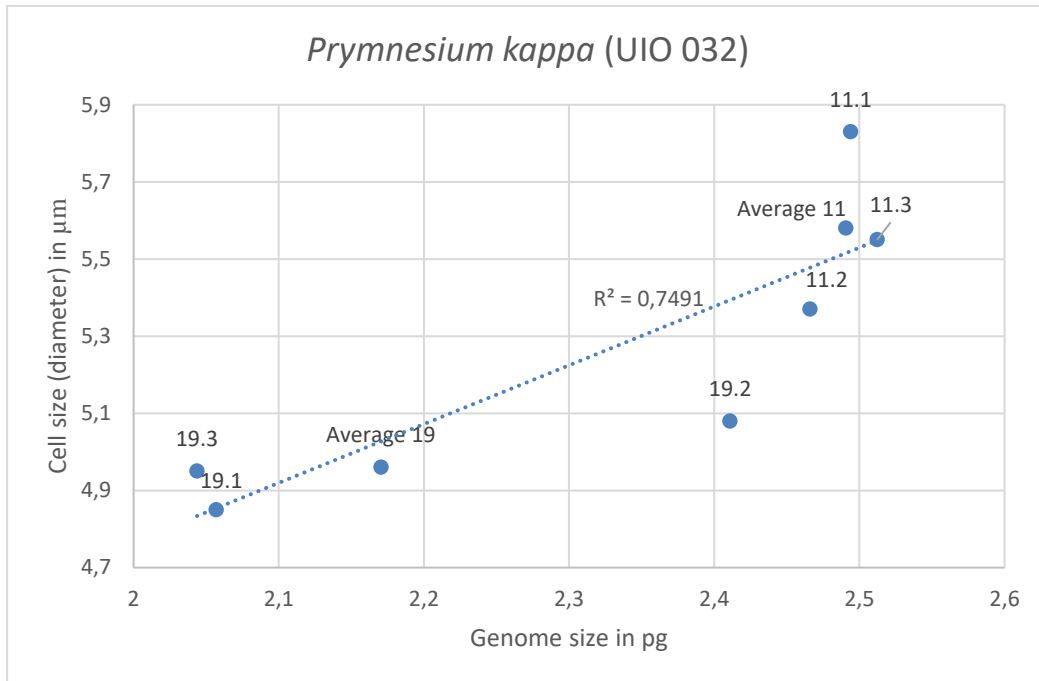
Figure 21: A plot showing the correlation between cell size (in µm on y-axis) and genome size (in pg) on x-axis. A regression line is plotted down, with the correlation value r^2=0.7491. All six replicas have been named (without the 032-part), as has both averages.

3.3. Electron Microscopy

3.3.1. Transmission Electron Microscopy

The previous results gave reason to believe that a change has occurred in the algae cells, and especially in *Prymnesium kappa*. All the UIO 032 cultures were therefore tested, but only 032.11.2 and 032.19.3 is showed here (as many other was destroyed or damaged). In figure 22 one can see the organic body scales and a clearly visible, dark rim. In picture A, typical flat scales, with a radial pattern, is shown. Picture B and C, in contrast, shows scales which seems somewhat deformed.

Figure 23 shows TEM pictures of 032.19.3. Picture A is a close up of one single body scale, while B and C shows a cluster of scales (C is an enlarged part of B). In great contrast to 032.11.2 in figure 22, here one cannot easily see the strong, dark rim of the scale. Also, the

straight, weak radial pattern of the scale is lacking. Overall, the 032.19.3 scale does not seem as structured as 023.11.2.
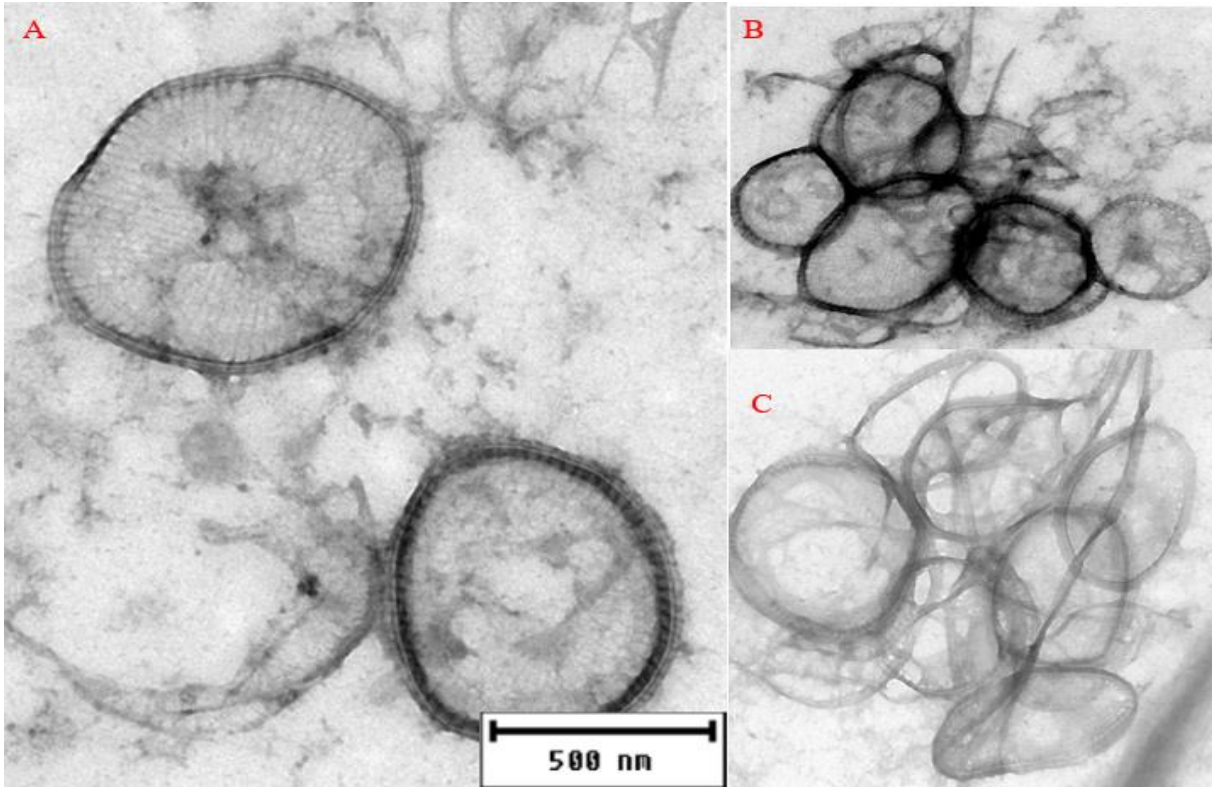
Figure 22: Transmission Electron Microscopy (TEM) of algae Prymnesium kappa, 11ºC, replica 2. All pictures are from grid position R4. A shows two body scales with a 500 nm ruler and both B and C shows a small cluster of body scales.
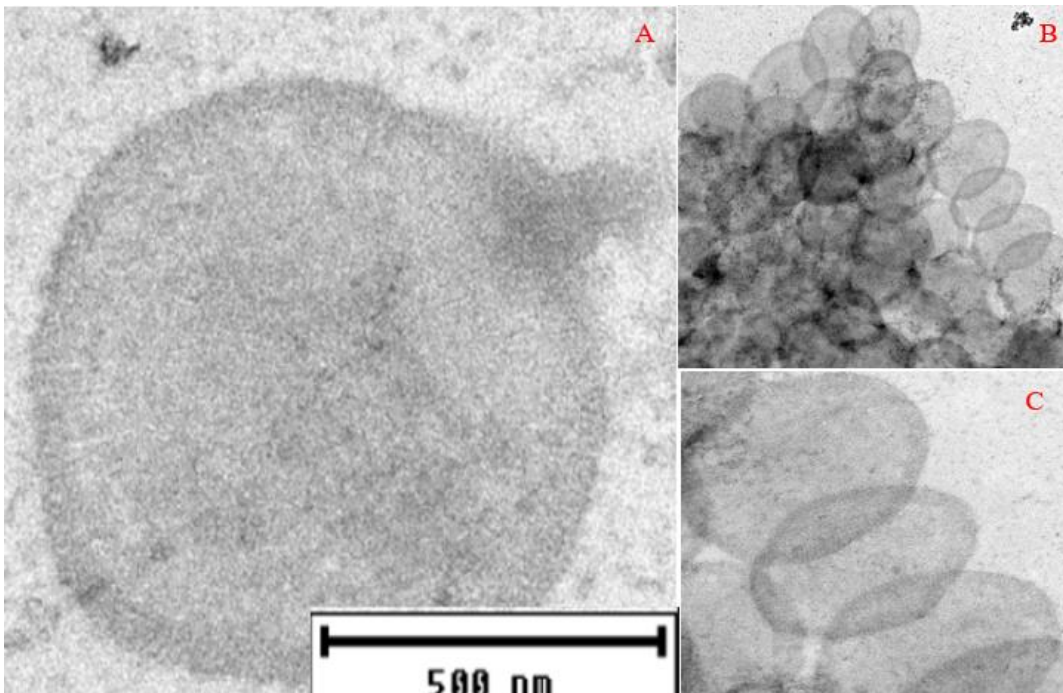
Figure 23 Transmission Electron Microscopy (TEM) of algae Prymnesium kappa, 19ºC, replica 3. All pictures are from grid position Q5. A show a body scale with a 500 nm ruler and B shows a large cluster of body scales where C shows an enlarged part of B.

3.3.2. Scanning Electron Microscopy

To see if a change had occurred in *Calyptrosphaera sp*, 309.11.3 and 309.19.1 was examined in a Scanning Electron Microscopy. As mentioned earlier, *Calyptrosphaera sp* can exist in two forms, a holococcolith stage and heterococcolith stage. There is a possibility that the 11°C treated cultures have attained a change in the coccolith scales due to the low temperature. Figure 24 shows a SEM micrograph of 309.11.1. As seen, there are many, small coccoliths, almost like crystals, covering the cell in a somewhat random fashion. Heterococcolith plates tend to be larger and more structured. Culture 309.19.1 was also analysed, but because an error occurred during the test, that result is not included here.
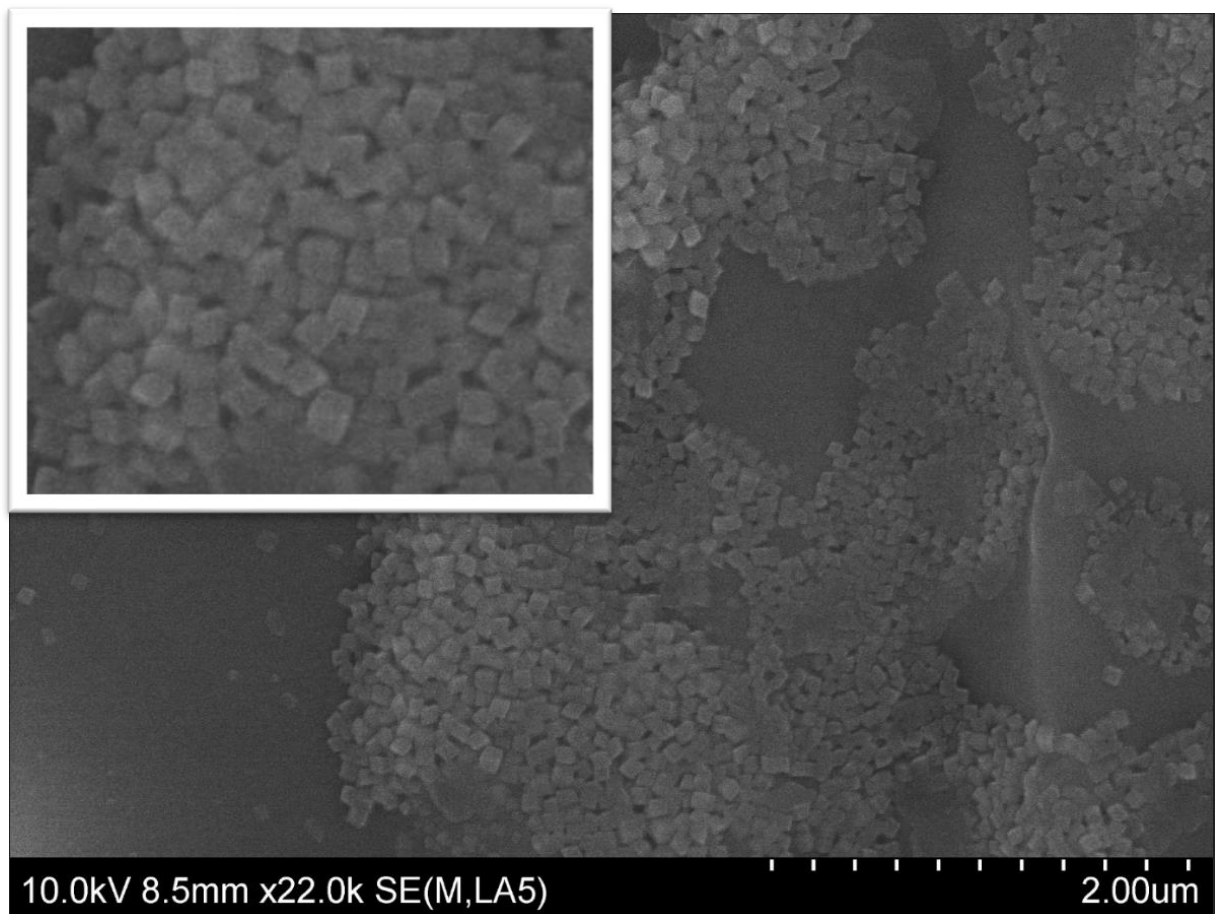
Figure 24: A Scanning Electron Microscopy picture of Calyptrosphaera sp, 11°C, replicate 3. Upper left picture is an enlarged version of a smaller part of the main picture. SEM shows holococcolith layer as the cell cover.

3.4. C:N:P, RNA and Protein Analysis

3.4.1. RNA quantifying analysis

The RNA quantity was carried out by first measure the total nucleic acid fluorescence before RNase A was added and DNA fluorescence was measured, using a Fluorometric Plate Reader (480 nm excitation and 528 nm emission wavelength). From these results, [RNA] (in µg ml^{-1}) could be calculated and is presented in table 10. To be able to examine [RNA] (µg mL^{-1}) pr. cells ml^{-1}(or just µg RNA per cell), the algal cells was counted after all the filter with algal cells was prepared and frozen down. The algal cells were counted using the Casy Cell Counter so the number of cells may seem high.

As one can see in table 10, each algal species and temperature (e.g. 032.19) have six values instead of just three. This is because each replicate was duplicated. Number of cells/ml seems to vary a bit throughout the replicates for each algal species and temperature (e.g. 032.11). In 032.19, 032.19.2 have much lower cell concentration than the two others. In 309.19 there is also some variation where 309.19.2 and 309.19.3 differ a lot. Both 032.11 and 309.11 is quite stable in comparison. As the averages shows (the non-coloured parts of the table), the cell concentration is higher in the 19°C samples than the 11°C samples. The difference could (and should) be bigger between 309.11 and 309.19 (see table 4) if it was not for the two possible outliers (309.11.2 and 309.19.2).

Table 10:Table of number of cells ml^{-1} in the first 4 columns and [RNA] (µg ml^{-1}) in the last four columns. Columns from left to right: P. kappa 11°C and 19°C, Calyptrosphaera sp 11°C and 19°C (032.11, 032.19, 309.11 and 309.19 respectively). The bottom numbers (uncoloured background) are the average of the different replicates for the different algal species and temperatures. Each replicate is duplicated, with the duplicate number in parenthesis, i.e. replicate 1(1) means duplicate 1 of replicate 1.

	Number of cells ml^{-1}				[RNA] (µg ml^{-1})			
	032.11	032.19	309.11	309.19	032.11	032.19	309.11	309.19
Replicate 1(1)	2533800	5504850	3995800	4565150	1.16	1.00	1.20	1.18
Replicate 1(2)	2533800	5504850	3995800	4565150	1.02	0.99	1.09	0.95
Replicate 2(1)	1861600	3267600	4307400	3367650	1.10	0.89	0.61	0.82
Replicate 2(2)	1861600	3267600	4307400	3367650	1.02	0.49	0.88	0.86
Replicate 3(1)	1975850	5783600	3691600	5631150	1.10	1.00	1.30	1.12
Replicate 3(2)	1975850	5783600	3691600	5631150	1.03	1.27	1.28	1.13
Average	2123750	4852016.7	3998266.7	4521316.7	1.07	0.94	1.06	1.01

Table 10 also includes the measured (and calculated) values for [RNA] (in µg mL^{-1}) for all four temperatures and species, as well as the averages. And again, each replicate is duplicated.

For 032.11, the measured RNA concentration seems to be more or less stable throughout the replicates (and duplicates) where 032.11.1 (1) at 1.16 [RNA] is furthest away from the average with 0.09 µg ml^{-1}. For 032.19 on the other hand, there seem to be an aberration in 032.19.2, duplicate 2 at 0.49 [RNA], being 0.45 µg ml^{-1} lower than the 032.19 average, and 0.40 µg ml^{-1} lower than its duplicate, 032.19.2(1). Replicate 309.11.2 duplicate 1 is also around 0.4 smaller than the 309.11 average. For 309.19, there may be a small outlier in 309.19.2 where both duplicates are around 0.2 smaller than the 309.19 average. In respect to the averages, there is not such a large difference, but one can see that both 11°C treated cultures have a higher concentration value compared to their 19°C counterpart. The possible outliers can affect the result, but interesting enough, the 309.11 replicates do have the highest value in 1.30 and 1.28 (in 309.11.3). The average for 032.11 is around 0.13 µg ml^{-1} higher than 032.19, and the average fir 309.11 is around 0.05 µg ml^{-1} higher than that of 309.11 average.

Figure 25: RNA (in µg) per cell for the 12 (plus their duplicates) different algal cultures. From top to bottom: 309.19, 309.11, 032.19 and 032.11. For all: The first column is the average, which is marked with its values. Respectively column 2 – 7: replicate 3, duplicate 2 (in parenthesis from here), replicate 3(1), replicate 2(2), replicate 2(1), replicate 1(2), replicate 1(1). A regression line (based on the averages) is included with the formula: y=-0.783x + 4.977. (R^2=0.493)

From table 10, RNA pr. cell (in µg) can be calculated and the result can be seen in supplementary table 14, Appendix II. From these values, figure 25 is created, and all values in figure 25 is multiplied with 10^{7}. Since the values of supplementary table 14, and therefore also figure 25 is based on table 10, the possible outliers from table 10 may also have an

impact on figurer 25, but as one can see, most replicates have values close to the replicates average (all values mentioned are obtained from supplementary table 14). Although, for 032.11, 032.11.1(2) is around 1.1E-07 lower than the average, bringing it down. Duplicate 1 of 032.19.2 is around 0.77E-07 higher than the average, bringing it up. The largest, possible, outlier is 309.11.2(1) being around 1.20E-07 lower than the average, and bringing it down. For 309.19, there are not one clear outlier, where around three are around 0.30 µg over the average, and three are around 25 µg under. In respect to the averages, one can clearly see that the 11°C replicate averages have higher values (higher RNA amount per cell) than their 19°C counterpart. 032.11 is around 3.1E-07 µg higher than 032.19 and 309.11 is around 0.43E-07 µg higher than 309.19 (all are average values). These results indicate that the 11°C treated cultures contain for RNA than the 19°C treated cultures. The regression line of figure 25 also indicates this, but would have been even greater (or smaller as the value is negative) if the 19°C *P. kappa*, which has the lowest average, had been on the top.

3.4.2. Protein quantifying analysis

The Protein quantity was carried out by measured the protein fluorochrome fluorescence, using a Fluorometric Plate Reader (480 nm excitation and 528 nm emission wavelength). From these results, [Protein] (in µg mL^{-1}) could be calculated and is presented in table 11. To be able to examine [Protein] (µg mL^{-1}) pr. cells ml^{-1}(or just µg Protein per cell), the algal cells were counted after all the filter with algal cells was prepared and frozen down. The algal cells were counted using the Casy Cell Counter so the number of cells may seem high.

As one can see in table 11, each algal species and temperature treatment (e.g. 032.19) have six values instead of just three. This is because each replicate was duplicated. The number of cells values are the same here as in the [RNA] analysis. See that section, section 3.4.1, for more information.

As well as number of cells ml^{-1}, table 11 includes the measured (and calculated) values for [Protein] (in µg mL^{-1}) for all four temperatures and species, as well as the averages. And again, each replicate is duplicated (duplicate number in parenthesis). For the last three columns, 032.19, 309.11 and 309.19, the measured concentration seems to be more or less stable. It is some fluctuation between

	[Protein] (µg ml⁻¹)				Number of cells ml⁻¹			
	032.11	032.19	309.11	309.19	032.11	032.19	309.11	309.19
Replicate 1(1)	15.75	14.62	15.82	10.83	2533800	5504850	3995800	4565150
Replicate 1(2)	20.39	12.53	11.54	12.98	2533800	5504850	3995800	4565150
Replicate 2(1)	21.97	14.23	15.28	7.17	1861600	3267600	4307400	3367650
Replicate 2(2)	12.36	13.44	17.34	8.53	1861600	3267600	4307400	3367650
Replicate 3(1)	16.64	13.24	13.23	11.04	1975850	5783600	3691600	5631150
Replicate 3(2)	13.64	16.38	16.05	10.21	1975850	5783600	3691600	5631150
Average	16.79	14.08	14.88	10.13	2123750	4852016.7	3998266.7	4521316.7

the replicates, and between the different duplicates, e.g. between 309.19.2(1) and 309.19.1(2) with a difference at around 6 (µg ml⁻¹), and between 309.11.1(2) and 309.11.2(2), with around the same. Between the different duplicates (from same replicate) the differences are not as big, but still visible. For 032.11, the largest difference between two duplicates from the same replicate, among all species and temperatures, can be found. Between 032.11.2(1) and 032.11.2(2) it is a difference at around 9.5 (µg ml⁻¹).

Nonetheless, as one can see from the different averages, there is a clear, visible difference between the cold treatment and the warm treatment. Between the two different temperature treatments of *P. kappa*, it is a measured a [protein] difference at 2.71 µg ml⁻¹. Between the two *Calyptrosphaera sp's*, the measured [protein] difference is at 4.75 µg ml⁻¹, showing a clear trend that the 11ºC treated cells seem to contain more protein.

From table 11, protein (in µg) pr. cell can be calculated and the result can be seen in supplementary table 17, Appendix II. From these values, figure 26, is created, and all the values in figure 26 is multiplied with 10⁶. Since the values of supplementary table 17, and therefore also figure 26, is based on table 11, the possible outliers from table 11 may also have an impact on figurer 26 (all values mentioned further are obtained from supplementary table 17). Both *Calyptrosphaera sp* temperature treatments seem to be somewhat stable. Although, 309.11.1(2) is measured/calculated to be smaller than the other replicates, and around 0.80E-06 smaller than the average, bringing down the average. For 19ºC *Prymnesium kappa*, both duplicates of 032.19.2 is measured/calculated to be somewhat larger than the two other replicates (with duplicates), being around 1.15E-06 µg higher than the average and even
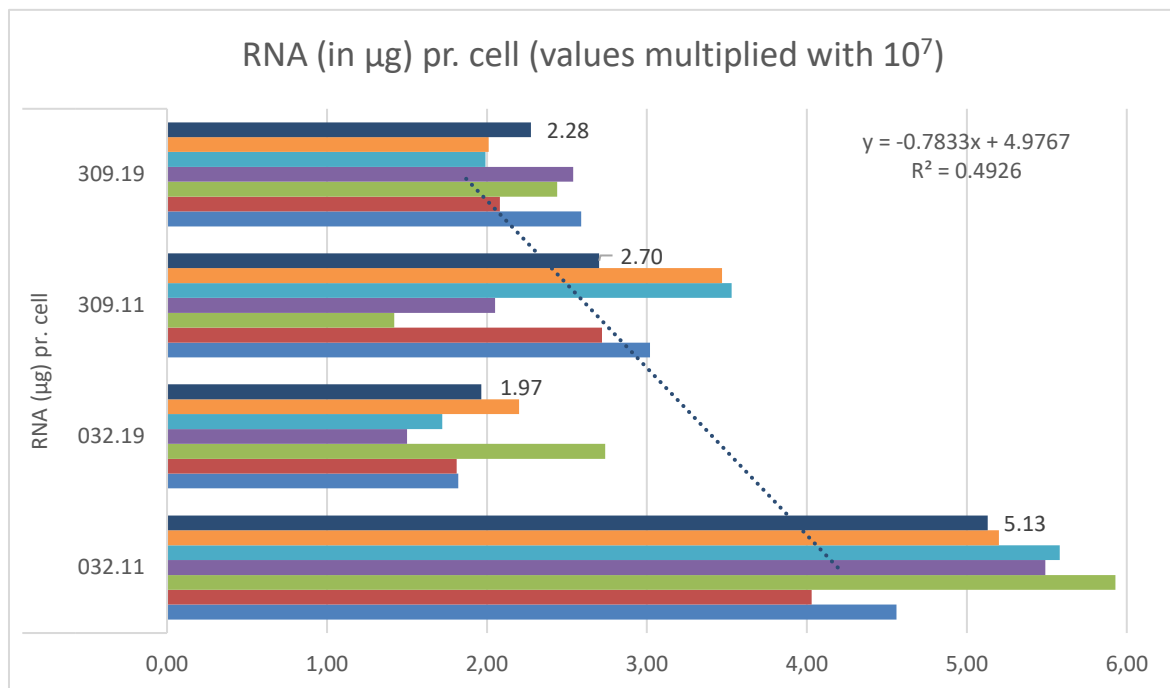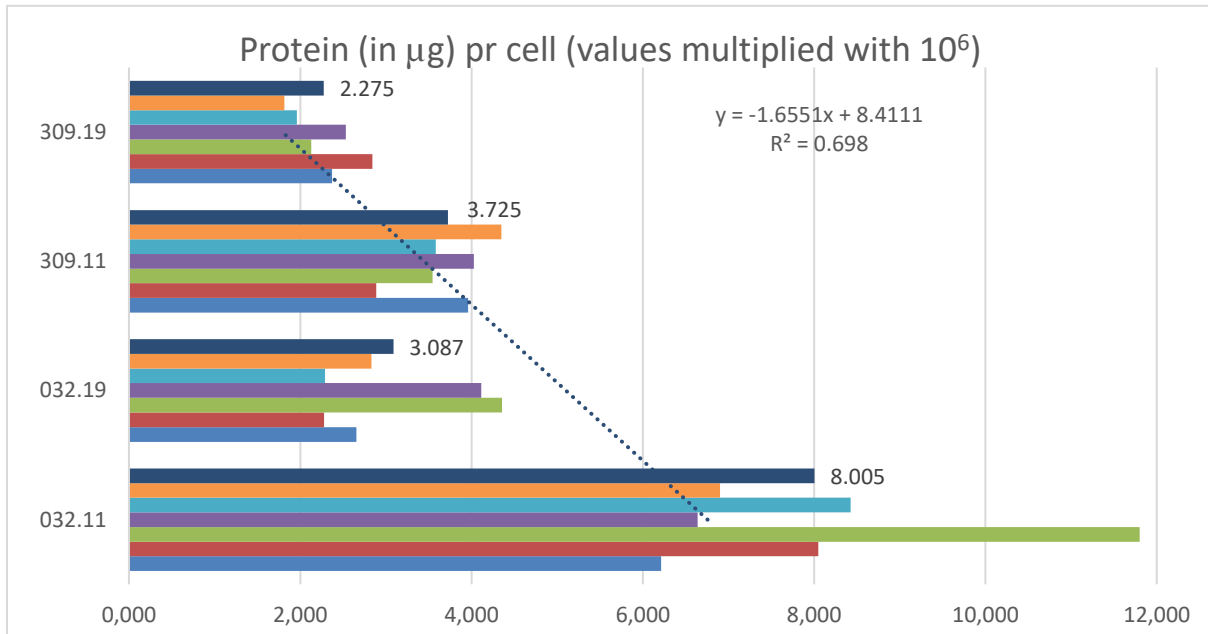
Figure 26: Protein (in µg) per cell for the 12 (plus their duplicates) different algal cultures. From top to bottom: 309.19, 309.11, 032.19 and 032.11. For all: The first column is the average, which is marked with its values. Respectively column 2 – 7: replicate 3, duplicate 2 (in parenthesis from here), replicate 3(1), replicate 2(2), replicate 2(1), replicate 1(2), replicate 1(1). A regression line (based on the averages) is included with the formula: y=-1.655x + 8.411. (R^2=0.698)

higher compared to the other replicates, bringing the average up. The largest aberration can be found for 11ºC *P. kappa*, between 032.11.2(1) and 032.11.1(1) (from the same replicate, 032.11.2(2) is also much measured to a much lower value) at around 5.59E-06 µg protein per cell. In respect to the averages, there seem to be a clear trend, as seen in the last table, with a higher protein amount for the 11ºC treated cells, compared to the 19ºC treated cells. The difference between the two *P. kappa* averages is 4.92E-06 protein in µg pr. cell and the measured/calculated difference between the two *Calyptrosphaera sp* averages is 1.45E-06 protein in µg pr. cell, indicating that the 11ºC treated algal cultures contain more protein. The regression line of figure 26 also indicates this.

3.4.3. C:N:P ratio analysis

Both sample for P and CN analysis was carried out by Berit Kaasa.

From the analysis, [Carbon], [Nitrogen] and [Phosphorus] in µg ml^{-1} for all the replicates, and duplicates, was estimated. From these, the molar ratio between, C:N, C:P and N:P, for the four specie and temperature treatments was calculated and presented in table 12. As one can see, there seem to be a difference between the two temperature treatments, for both species. In respect to the C:N ratio, the 032.11 average is estimated to be 28.96, while the 032.19 average

is estimated to be 40.33. This is a difference at around 11. The 309.19 average is estimated to be roughly the same as 032.19, while the 309.11 average is estimated to be 33.41, giving a difference at around 7.

For the C:P molar ratio, the 032.11 average is estimated to be around 76 smaller than the 032.19 average. The 309.11 average is estimated to be around 37 smaller than the 309.19 average. The overall C:P ratio is around 8 times larger than the C:N molar ratio. The smallest molar ratio is N:P being between three to four times smaller than the C:N ratio. The 032.11 (here there was a clear outlier. This duplicate was replaced by the other duplicate from the same replicate) average is around 1.4 lower than the N:P ratio for the 032.19 average. The 309.11 average is around 0.61 larger than the 309.19 average. This is in contrast to the other ratios where the 11ºC treated cultures was estimated to be smaller.

Table 12: Overview of the molar ratio between Carbon, Nitrogen and Phosphorus, C:N, C:P and N:P between the averages for the different temperature treatments and species. From the top is respectively P. kappa 11ºc and 19ºC averages, and Calyptrosphaera sp 11ºC and 19ºC averages. The values are colour-coded based in size (in respect to each ratio)

	C:N (molar ratio)	C:P (molar ratio)	N:P (molar ratio)
032.11 average	28,96	195,47	6,83
032.19 average	40,33	322,87	8,20
309.11 average	33,41	313,79	9,51
309.19 average	40,28	350,99	8,90

3.5. DNA sequencing

Each culture replicate was lysed and the nuclei was isolated using Percoll filtration. Before the DNA extraction from the nuclei samples, were some tested using the FCM to make sure that there indeed was acquired nuclei after percoll filtration. In most of the *P. kappa* culture replicates did it seem to be reasonable amounts of nuclei, but not for the *Calyptrosphaera sp* cultures. Two extra percoll filtration cycles was therefore performed. The samples from the three different cycles was put together and centrifuged, to reduce the volume. In more or less all *P. kappa* culture samples did pellets form, but not in any of the *Calyptrosphaera sp* culture samples. After the DNA extraction preparation was finalized, were all DNA samples measured to get an estimation on how much DNA there were in each sample, and the result can be found in table 13. As one can see, there is a clear difference in DNA concentration between the two species, even though 032.19.3 is fairly lower than the rest (032.19.2 is not too high either). All samples of *Calyptrosphaera sp* is estimated to have under 2 ng/µl DNA

concentration. Nevertheless, since the samples had a volume of 198 µl (200 µl initially, but 2 µl was used for concentration measurement), the total DNA amount was of an acceptable level.

Table 13: Isolated DNA for all 12 culture samples, both P. kappa and Calyptrosphaera sp for both temperatures. Both DNA (in ng/µl) and DNA amount (in ng) in each culture samples (of 198 µl) is included.

Culture sample	DNA (ng/µl)	ng DNA in 198 µl	Culture sample	DNA (ng/µl)	ng DNA in 198 µl
032.11.1	4.52	904	309.11.1	1.95	390
032.11.2	4.00	800	309.11.2	1.69	338
032.11.3	2.92	584	309.11.3	1.58	316
032.19.1	5.43	1086	309.19.1	1.62	324
032.19.2	2.22	444	309.19.2	1.63	326
032.19.3	1.94	388	309.19.3	1.81	362

3.5.1. DNA sequencing – K-mer analysis
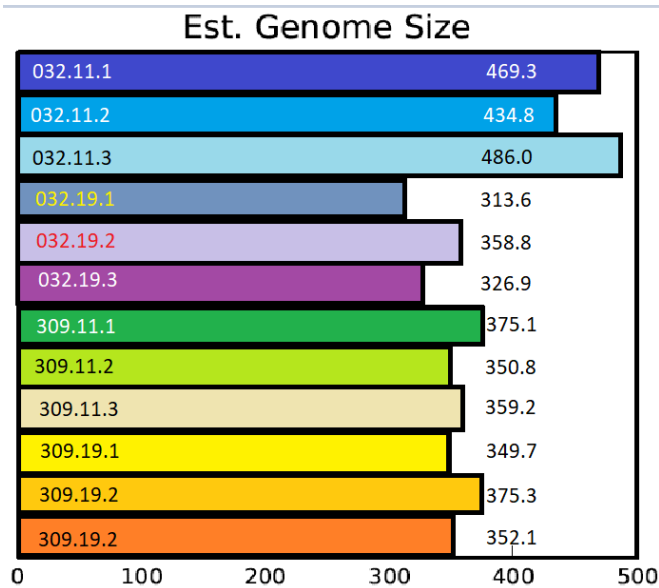
Figure 27: Estimated genome sizes for all the different culture samples using a K-mer analysis. Genome sizes are given in Mbp. The replicate/culture name is to the lest, and the sizes, in Mbp, is to the right in the figure.

As the time was running out, more advanced sequencing analysis just were not possible, but, with the help of (especially) Øyvind Gulbrandsen and Jon Bråte, a simple K-mer analysis was conducted. In a K-mer test, the DNA is divided into certain lengths, k, and from a DNA sequence of the length L, the amount of acquired k-mers is L – k + 1. Figure 27 shows the estimated genome sizes (measured in Mbp) for all the 12 culture samples. First of all, the estimated sizes are not 100%

consistence between the culture samples from same species and temperature (e.g. 032.11), where especially 032.11.2 is a bit smaller than its replicates, where the estimated genome size is around 51 Mbp smaller than 032.11.3. The 032.19.2 genome size is estimated to be around 45 Mbp larger than 032.19.1. The 309.11.1 and 309.19.2 also differ not as much compared to their replicates, where 309.11.1 is around 25 Mbp larger than 309.11.2, and 309.19.2 is also

around 25 Mbp larger than 309.11.1. Cultures from 309.11 seem to be somewhat more stable, or consistent between the replicates, compared to the *P. kappa* replicates. One can see that all the 032.11 replicates are measured to be larger than those of 032.19 replicates, but the two *Calyptrosphaera sp* treatments do not show the same difference in estimated genome size.

The averages from all the replicates from figure 27, can be seen in table 14. From this, one can easily and clearly see the difference between 032.11 and 032.19, with an estimated difference in genome size is 130.4 Mbp. The difference between the two *Calyptrosphaera sp* is estimated/calculated at 2.7 Mbp, which is not a significant difference, though, the possible outliers can affect the result.

Table 14: Averages in Mbp calculated from each replicate in figure 27.

Specie-Treatment	Mbp
032.11	463.4
032.19	333
309.11	361.7
309.19	359
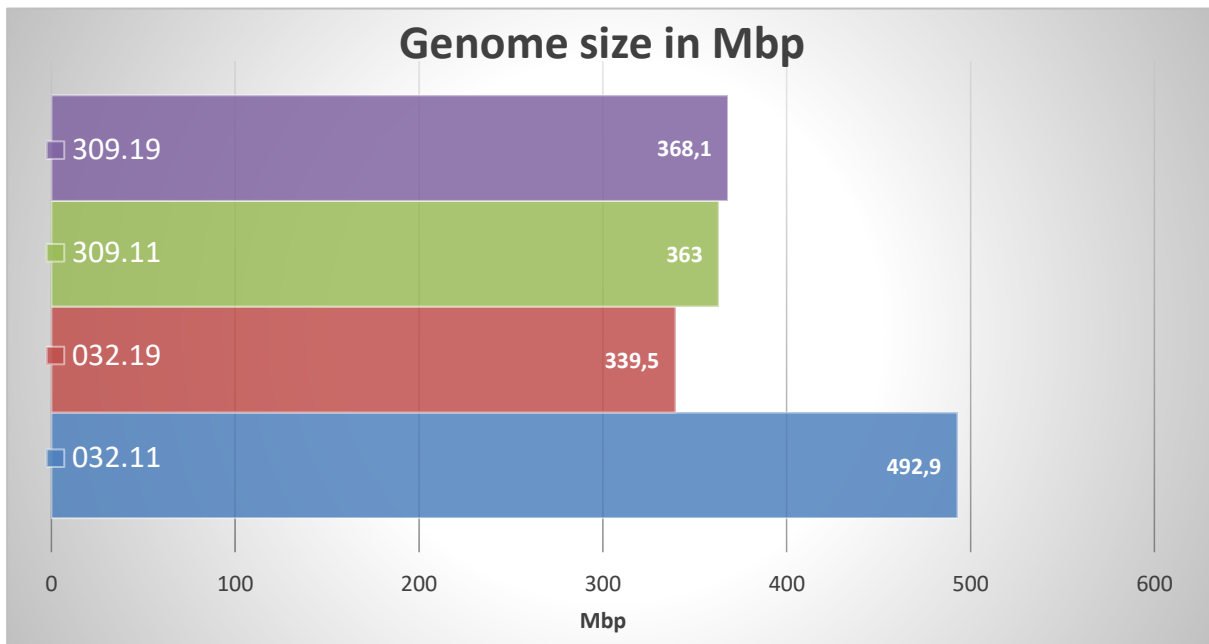
Figure 28: Estimated genome sizes using k-mer analysis. Figure shows the averages for the three replicates from each specie and temperature treatment. Sizes measured in Mbp. For 032.11, 032.19 and 309.19 a comma (,) is used instead of a full stop (.) in the Mbp values. This is because a Norwegian Excel was used.

Figure 27 (as well as table 15) is based on analysis where each culture sample has been treated independently. Figure 28, in contrast t0 figure 27, is based on analysis where the three replicates (from each temperature and specie) are treated together, as 032.11, 032.19, 309.11 and 309.19. First of all, as one can see, the averages in figure 28 is slightly different from the averages in table 13. Actually, all values are estimated to be bigger in figure 28. Especially 032.11 is estimated to be much larger, with a difference at 29.5 Mbp. Secondly, in figure 28, 309.11 is not estimated to be higher than 032.19. Actually, 309.19 is estimated to be 5.1 Mbp larger than 309.11. Also, 309.19 has increased more from table 13 to figure 28, compared to

309.11. Where 309.11 is estimated to be 5.1 Mbp smaller than 309.19, is 032.11 is estimated to be 153.4 Mbp larger than 032.19.

Figure 29 shows the frequency of variant branches in k-de Bruijn graph. The larger degree of variance is often interpreted as heterozygosity. Although, both species are most likely haploid, and therefore not homozygote/heterozygote. Though, in unpublished data, B. Edvardsen discovered that *P. kappa* do exist in a diploid form, but from her data, that form was easily recognisable and the results acquired so far did not look like her diploid results. As one can see, 032.11 (yellow line) has the highest frequency of variant branches, while 309.19 (red line) has the lowest, across the whole x-axis (k-mer). The green line, 032.19 and 309.11, the blue line, are estimated to have roughly the same frequency of variant branches, throughout the x-axis. Both 309.11 and 032.11 are measured to be higher than their 19°C counterparts, suggesting that both 11°C treated species have a higher frequency of variant branches.

Figure 29: Frequency of variant branches in k-de Bruijn graph. Both x-axis and y-axis are cropped respectively between 0 and 20, and 0 and 10^{-4}. Yellow: 032.11, Green: 032.19, Blue: 309.11, Red: 309.19.

4. DISCUSSION

4.1. Experimental set-up and cell counting

The main hypothesis of this thesis was to see whether or not one can see changes in genome size if the algal cells was cultivated on different temperatures. Maybe the most important premise to see any possible changes is to make sure that one gets through a certain amount of cell division/ generations. To ensure this, the temperature experiment was begun as early as possible and the cell cultures was diluted more or less three times a week. When selecting temperatures, there are especially two, maybe three, important parameters that need to be considered; large enough difference in temperature to make sure that the given temperature will have an impact on the cells (1), but not too large of a difference which makes one, or both, temperatures inhabitable for the algae cells (2). Also, too make sure that this experiment had at least some connection to the real world, the temperatures used should be possible to see in nature (3). The temperatures 11°C and 19°C was therefore selected. The crucial part of any experiments like this is to see if the algal cultures will thrive, or even just survive. Both algal species adapted to the new temperatures and they seemed to have a high growth rate (rapid cell division), especially both species on 19°C.

As table 4 shows, the average cell concentration before dilution showed that the cells grown at 19°C, grew faster that those at 11°C. After each dilution, the cultures were diluted to approximately 50 000 cells ml^{-1}. Since the cultures were diluted 3 times a week, it took the cultures, on average, 2.33 days to reach the concentration stated in table 4. From all this, there is possible to calculate how much each culture grew each day, in average, and this growth rate is presented in table 15. The formula used: $(t_2/t_1)^{1/d} - 1$ = Growth rate, where t_2 is measurement (before dilution), t_1 is first cell concentration after dilution to roughly 50,000 cell ml^{-1} and d is number of days between dilution and measurement.

Table 15: Culture/population growth rate each day, in average, for the different cultures, using both Casy Counter and microscopy.

Specie and temperature	Start Concentration (Cells/ml)	Light microscope – Population growth rate in each culture	Casy Cell Counter – Population growth rate in each culture
032.11	50000	0.778	1.066
032.19	50000	0.977	1.510
309.11	50000	0.548	0.884
309.19	50000	1.380	1.477

As one can see from table 15, the 11°C cultures performed cell division between every other day and once a say, while the 19°C cultures grew in general faster, close to twice as fast. The reason for the faster growth, is most likely the higher temperature. The 19°C algal cultures were added a larger amount of fresh medium each time, but since they grow faster, and get denser much quicker, this should not be the cause. All 12 cultures also were exposed to the same amount of light (I will come back to this later), so this should neither be the major cause of the quicker growth. That increased temperature may correlate to increased culture population growth is a common suggestion, e.g. by Orcutt and Porter (1984). Under higher temperature circumstances, will there generally be a higher cellular process. This leads to more rapid development and facilitating prematurely development reproduction, would be favoured by natural selection (Atkinson, 1994).

About the different measured cell concentration between light microscope and Casy Counter, as seen in e.g. table 4, it is important to keep in mind that the Casy Cell Counter counts more than just live, whole algae cells. It is possible to set certain parameters restrictions on how large or small diameters the Casy will detect. This will exclude small bacteria, or large chucks of debris, but "debris" (or not whole, live cells) that is within the selected parameters, will be counted. In figure 6, one can see an overview of the acquired information after a Casy cell count run. Here, the counts/ml is estimated to be 10,910,000 for a 10x diluted algal culture sample. The number of counted particles (counts) was 62,338. There is not any obvious way to reach this concentration. There were therefore some uncertainties about what the correct concentration (for both the diluted sample and the actual cultures), and there is possible that mistakes were done when calculating the final culture concentration.

A few different light intensities were tested out before it was settled to around 50 µmol photons $m^{-2}s^{-1}$. In the start of the experiment, a higher light intensity was used, but, mostly in the 19°C cultures, were there after sometime detected somewhat large amount of cell growth on the culture flask walls. I quickly found out that the 19°C cultures was moved closer to the light (closer than the starting point). This happened around three months into the experiment, about the same time as the 19°C cultures was moved to a different room. For the 11°C cultures, around the same time, was it detected that they did not grow too well, and some also got some growth on the flask wall. They were therefore moved a little further away from the light, ending up with 50 µmol photons $m^{-2}s^{-1}$, which was used to the end, without any extra problems. Most of the 19°C cultures grew without any problem, but as figure 4 shows, there

was two cultures that suffered more than the others, with growth on flask walls. As the replicas was turned 180° each time they were diluted, it was not too easy to find out if they were to close or too far away. Although, all 19°C cultures was moved a bit closer (to 55-60 µmol) to the light, but almost immediately did it start growing cells on the rest of the flask walls. Therefore, they were moved further away from the light (to around 40 µmol) which somewhat resolved the problem (for most cultures), at least reduced the growth on the walls for the two that suffered the most.

4.2. Cell and genome size correlation across different species

The temperature experiment was not the only one in this thesis, it was also an idea to look at several different algal species and measure their genome- and cell size, and to see if there is a correlation between them cell- and genome size. These algae cultures did not cultivate as long as the algae cultures in the temperature experiment. Neither did they have the same light intensity, nor was they diluted as often. In contrast to the cultures in the temperature experiment, these cultures were cultivated under a 14:10 day-night cycle. They were diluted around every fourth week where around 1 ml of algal culture was diluted with ca 40 ml IMR ½. There were not many problems related to the caretaking for these cultures, except for a few things. First of all, one of the cultures died, or maybe more correct, it never really settled and was quickly discarded. All the other cultures survived, and even quite well, but it did not take many days, after dilution, that the algae cells sank to the bottom, even though the cultures were gently shaken each day. But in contrast with some of the cultures in the temperature experiment, the cells did not stick to the flask walls/bottom, and some gently mixing (by inverting the flask) usually resolved the problem. All cultures were gently mixed on a regular basis to ensure that the cells were free floating with large (or larger) amount of medium around each of them.

All the different algal species in this test (*Calyptrosphaera sp* was in this test as well) was all of different sizes. Some had similar cell sizes (diameter). When these sizes were acquired, all cultures were a little dense, except for *Prymnesium polylepis* (UIO 041) which was closer to the exponential growth phase. Since most of the cultures were closer to the stationary phase (the population growth curve started to flatten out), there should not be as many dividing cells. Although, as the cultures are getting denser, and is further away from the exponentially

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growth, the algal cell might become somewhat smaller compared if they all were in the exponentially phase.

Supplementary table 5 (Appendix I) shows the FCM cell lysis formulas used for the different algal species from result section "3.1. Cell- and genome size correlation across different algal species". As stated, and as one can see the FCM results (see figure 9 and 10) in section 3.1, some formulas did not work all too well – giving to much debris (see table 2), not good enough peaks (wide instead of slender) or a small amount of counted algal nuclei events (giving shorter peaks). *Prymnseium Nemamethecum* (K-0394), *Phaeocystis globosa* (K-1321) and somewhat *Diacronema lutheri* (UIO 090), showed large amount of debris. Still, acceptable results were acquired. All the different algal species was tested several times, both before and after the results from section 3.1. For K-0394 and K-1321, and also *Hymenomonas carterae* (NIVA-2/92), using the exact same formulas as used earlier, resulted in more debris and much smaller algae nuclei peaks. When testing the different species, to find their optimal formula, I had troubles with some of them where a good formula proved difficult to obtain. Such was the case for all the species mentioned so far, as well as *Pavlova Gyrans* (K-1310). Although, stable formula was finally acquired for K-1310. About NIVA-2/92, good results were difficult to obtain, but in a test to see if time of day had anything to say, the results improved throughout the day, and around 16:00, the result looked more or less like the results acquired in figure 10. As already mentioned, these algal cultures were cultivated with a 14:10 day night cycle, so there is a chance that time of day actually had something to say. Overall, though there were some difficulties obtaining the different formulas, most of the results looks good and for those of a somewhat poorer quality, there is still possible to get a genome size estimation.

The final results from the flow cytometry (FCM) runs is summarized in table 3. Genome size (ratio) compared to CRBCs, genome size in pg and genome size in base pairs have all been included. As well as cell size, the genome size between the different species differ – some al lot. *Prymnesium polylepis* has been analysed before, and according to unpublished results acquired by Bente Edvardsen, it has a genome size (C-value) at 4.14 pg. In this experiment, I found the genome size of *P. polylepis* to be 4.2 pg DNA cell[-1], which is in accordance with her results. There is not any data for the other species, but based on the results form *P. polylepis* (mine compared to B. Edvardsen's), there are reason to believe that the methods for acquiring genome sizes by FCM are correct. Although, Veldhuis et al. (1997) found *P.*

polylepis (older name used in the article) to be (using both PicoGreen and SYTOX Green) 6.5 or 5.9 pg (multiplied Veldhuis' values with CRBC units = 2.33 pg DNA cell^{-1}), which is around 2 pg larger than mine (and Edvardsen's) results.

The main objective of this part of the thesis was to see if there is any clear correlation between cell size and cell diameter. This correlation can be seen in figure 11. The first thing one can see is that *Hymenomonas carterae* (Niva-2/92) and *Chrysotila carterae* (UIO 095) have both similar cell size (diameter) and genome size. As stated in section 2.1, *H. carterae* might be a homotypic synonym (same species, but wrong name) of *C. carterae*. When one think of this, while looking at figure 11, where they are shown to have similar cell- and genome size, there is a possibility that this is the case. The formula (for FCM analysis) used for both of the them, are also quite similar.

Not only was there an interest to get an estimation of the cell- and genome size, the most interesting part was to see if there was a correlation between them. This genome- and cell size correlation is presented in figure 11, and from this, one can see that there is a positive correlation. The r^2-value is calculated to be 0.4775 (≈0.5). As there are only nine datapoints, or nine tested species, in this test, some possible outliers will have a greater impact compared if there were more datapoints. The overall result is therefore affected. Also, there are some inaccuracies in respect to cell size measurement. Nevertheless, there appear to be a clear correlation between cell size and genome size, something that e.g. Shuter et al. (1983), LaJeunesse et al. (2005) and Connolly et al. (2008) also have suggested.

4.3 The temperature experiment – FCM, genome- and cell size correlation

4.3.1. Cell sizes

The alga cell sizes were measured using both Casy Cell Counter and light microscope. Figure 12 shows the measured sizes (diameter) using Casy and figure 13 shows the measured sizes using light microscopy. First of all, for the size estimate using Casy, both MDI (median diameter) and PDI (peak diameter) is included in figure 12. The PDI estimates size based on the tallest peak (in which channel (the dimeter size) most counts are detected), and is not affected by any other counts (in other channels), such as outliers. But since the gating parameters excludes counts that is either too small or too large, outliers will not affect the

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mean too much and therefore, the MDI is focused on as it gives a size estimate based on all counted cells.

The y-axis in both figure 12 and 13 have been cropped, so the fluctuations between each culture seems bigger than if the scale went from 0.0. Nevertheless, some cultures are measured to be much larger (e.g. 309.11.1 (figure 13), and 032.11.1 and 032.19.1 (figure 12)). Some size variation between the different replicates (from same species and temperature) is visible, but that is not unexpected, as all the different algal cultures (and replicates) have been cultivated independently, so there is natural to see that some varied reactions to the treatments.

When comparing the two figures and the two methods, one can quickly see that Casy generally estimated the algal cells to be bigger than that of light microscopy. Also, where light microscopy estimates that *P. kappa* have the largest cells and the biggest difference between 11ºC and 19ºC, while Casy estimates that *Calyptrosphaera sp* have both the biggest cells and the biggest difference between 11ºC and 19ºC. One possible reason for why Casy estimates *Calyptrosphaera sp* to be bigger compared to the microscopy-measurement, may be the coccolith scales of *Calyptrosphaera sp*. As the Casy measure electrolyte resistance in the particle, algae with coccoliths (calcium carbonate scales) may be measured as larger than those without these scales, even though they are of the same size. The reason for why the cells is measured to be smaller when using light microscope may be that acidic Lugol, which is used to fix the algae cells, have caused a shrinkage off the cells, which is something that is reported by e.g. Choi and Stocker (1989). When the cell sizes were estimated, 40x objective was used, which was the maximum for the microscope used. The edges of the cells are not always a 100% clear which gives some inaccuracies when estimating the sizes. In general, as all the sizes are estimated by eye sight alone, the results may be somewhat inaccurate, as the human senses are flawed compared to accurate machinery.

Both measure methods suggest that there is a difference between the 11ºC and 19ºC algal species (in respect to the average), but light microscopy estimations suggest that both 11ºC *Calyptrosphaera sp* and *P. kappa* are larger than their 19ºC counterparts, while Casy suggests that 11ºC *Calyptrosphaera sp* are much bigger with *P. kappa* only slightly bigger than their 19ºC counterparts. As the sizes seemed to fluctuate more from time to time when measuring the sizes with the Casy compared to when measuring with the light microscope, the sizes estimated using microscopy was focused on. Also, as earlier mentioned, it is believed that the

coccoliths can affect the electrolyte signal, and therefore also the estimated cell size, when measuring with the Casy Cell Counter.

Nevertheless, an increase in body size has been measured, using both methods. That colder climate tends to give an arise of larger cells (or organisms), is an old principle first described by Carl Bergamnn (1847) – the Bergmann Rule. In the following years, several papers have been published on this subject. Ectotherms organisms, such as algae (somewhat based on which definition one use for ectotherm (see introduction)), grows slower in a colder environment and thus gives arise to larger cells (Angilletta et al. 2004; Atkinson et al. 1994; Sommer et al. 2017). That my algal cultures that been cultivated on lower temperatures, has showed an increased cell size therefore follows the TSR (temperature-size rule).

A T-test (Student's T-test) was performed, using the formula: where ED: Sum of the differences (x-y), ED2: Sum of the squared differences ((x-y)^2). The difference acquired by subtracting the values for three replicates from 19°C from the values from the 11°C treated.

$$t = \frac{ED/N}{\sqrt{\frac{ED2 - (\frac{(ED)^{\wedge}2}{N})}{(N-1)(N)}}}$$

This T-test was performed for all the different cell size (and most other parameters) estimation (see supplementary table 18, Appendix II). As figure 12 shows, there are some fluctuation in the estimated cell size for *Prymnesium kappa*, and the average difference is not large. According with the T-test, with a calculated T-value at 0.461 (for PDI) and 0.615 (MDI), which is below the t-table value at 4.303. The values are higher for *Calyptrosphaera sp*, with a T-value at 3.149 (PDI) and 4.863 (MDI). The MDI value is more than 0.05 higher than the t-table value, but the PDI is not.

For light microscopy (figure 13), *P. kappa* have a calculated T-value at 14.571 and *Calyptrosphaera* with 3.124. Here, the *P. kappa* difference is significant, while *Calyptrosphaera sp* is not. Still, the differences from figure 13 and the T-values show some difference between the treatments, but as there are only three replicates, the t-table value is high and outliers will have a greater impact on the calculated t-value.

4.3.2 FCM Testing

The main objective with the temperature experiment was to find out if any visible changes have occurred on the genome level. To figure this out, Flow cytometry (FCM) was used. There were some troubleshooting and lack of results in the start of the experiment. A NIB (more or less the same that was mentioned in section 2.5.1.), but with 0.5% (v/v) β-mercaptoethanol, was used. This was not added directly to the NIB, but added to a smaller amount NIB (usually around 10 ml), before use, as β-mercaptoethanol is not stable over time. Also, not enough EDTA was added to the NIB. The same protocol (Marie et al. 2001) was used here as well, but with some differences. 50 µl non-centrifuged algal culture was added to 1 ml NIB, with 10 µl RNase A and incubated for 30 min at 37°C. More algae culture and more diluted NIB was tested, but without any results what so ever. It was not before both the 37°C incubation and β-mercaptoethanol was removed from the protocol used that actual results were acquired. A new NIB was made, with sodium bisulfite instead of β-mercaptoethanol, but since the sulphite was delayed, a test without bisulfite or β-mercaptoethanol, was performed. This was the first time a result was acquired. From this, several modifications to the protocol used, was made and a lot of different tests was performed.

Amount of NIB, Trion X-100, sodium bisulfite and other possible reagents was all tested for all algal species. The amount of NIB was heavily reduced compared to the protocol first used and e.g. a test with 700 µl NIB versus 200 µl NIB yielded significant more debris. To get the total volume up to 1 ml, some different mixing solutions was tested. MQ (Milli-Q) H$_2$O was used in the start, but as it has low salinity, the result might be disrupted as algae cells might be affected by osmotic power (therefore, PBS EDTA or FACS Flow (both with a pH around 7.5) was used).

As the algae species used was cultivated in IMR ½, there is a lot of saltwater in the culture samples. A few tests were executed and it was quickly clear that IMR ½ have an undoubtable impact on the results. The medium turned out to disrupt the PI (propidium iodide) signalling and in general, the more IMR ½ that was in the sample, the weaker was the signal and therefore gave an inaccurate result. Centrifugation of, especially diffuse, culture samples, appear to be highly beneficial.

With good formulas used, when the amount of NIB and strong reagent is close to perfect, the samples seemed to be stable over several hours. So, incubating 30 minutes versus 90 minutes, did not seem to make such a large difference. Only a minimum incubation time seemed to matter. Although, after incubation for several hours (3+ hours) the cell nuclei started to disintegrate and yield more debris, and in samples with sodium bisulfite (not all algae species needed it), the cell nuclei seemed to disintegrate quicker.

As mentioned earlier, the CRBC used was about to expire and a new CRBC was ordered. At first sight the new one did not look any different from the old, but when testing the New one up against the old, there turned out to be a difference between them – the old CRBC was measured to be 1.45 times as large as the new. As *P. polylepis* was measured to be close to the same size as what Bente Edvardsen also had found, the results using the old CRBC was used. Although, as mentioned earlier, Veldhuis et al. (1997) found *P. polylepis* (*Chrysochromulina polylepis* (as it was called earlier) used in their results), using both PicoGreen and SYTOX green, to be respectively 2.777 and 2.532 CRBC units (= 2.33 pg DNA cell^{-1}).

RNase A was not used for the acquired results, but it was tested a lot. There is RNA (e.g. messenger RNA) in the nuclei, and PI colours not only DNA, but RNA as well (Riccardi and Nicoletti, 2006). It was there for some reason to believe that there would be a difference between RNase A treated samples and samples without RNase A, but no clear difference was discovered. According to Hare and Johnstone (2011), PI binds unspecific to the major groove of the DNA (or (double stranded) DsRNA), and single stranded RNA (ssRNA) will not be stained by PI.

As barely mentioned earlier, both 50 µl and 25 µl PI was used during the result section. When
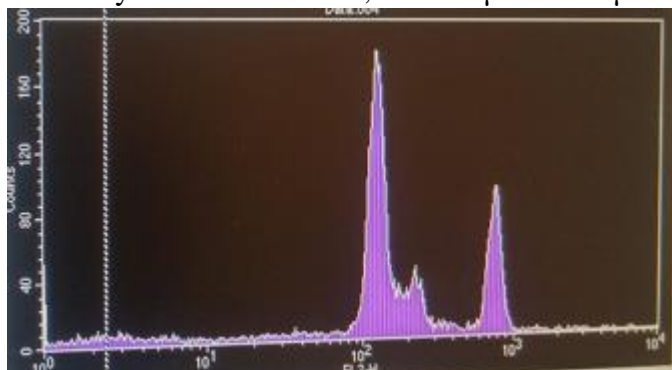
Figure 30: FCM result for 19ºC treated Calyptrosphaera sp culture. The first and tallest peak is C1, the small coherent peak, or bump, is C2 and the third is CRBC.

testing, many different concentrations and amounts of PI was used and there were some differences between e.g. a sample with 20 µl and 60 µl, but not any too significant. And, as long as every sample that is compared to its other have the same amount of PI, absolutely no significant differences between 25 and 50 µl PI was detected. If one is most interested in the ratio/difference between two samples, but the exact size is not that important, the PI amount should not make such a

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difference. When exact genome size is more important, the PI amount may have a greater impact. Figure 30 shows how a low concentration PI can give false results. The first peak belongs to *Calyptrosphaera sp* and the second clear peak belongs to CRBC. In the results, *Calyptrosphaera sp* was measured to be much closer in size with CRBC (2.5 pg) than what this figure implies. A much lower PI concentration (instead of 0.1 mg/ml, 5.0 µg/ml was used) was used in this example, but it is important to note that the algae nuclei and CRBC not always moves parallelly.

As Skjelbred et al. (2012) discovered, the estimated size difference between nucleus (*Pseudochattonella verruculosa*, strain JG8) stained with PI (0.77 pg) and SYBR green I (1.42 pg) was 0.65 pg. This is a significant difference and shows that the genome size estimation through the use of FCM (and PI) might not always be accurate. But, as this (or these) experiment(s) mainly focuses on the difference between different species and treatments, the absolute values are not the focus-point compared to the ratios, measured using the same dyeing (which at least will result in the same errors for all). Although, using inaccurate materials, methods or equipment, are never ideal.

4.3.3. FCM results and genome sizes

As already mentioned, there were some difficulties getting results at all from the FCM-tests in the start of the experiment. Earlier result runs for the temperature experiment was performed, but as the wrong concentration of PI was used, the result was therefore unnecessary inaccurate. But still, it was already then estimated that there was a difference between the two temperature treatments. When the correct PI concentration was used, the difference became even clearer.

As one can see from section "3.2.2. Flow Cytometry", most of the acquired histogram plots looks good. Since there was an overlap between the algae nuclei and CRBCs, gating was necessary to be able to distinct the two peaks from each other. Because of the gating, no, or little, debris or "noise" is included in the algae nuclei, nor CRBC, histogram plots. Nevertheless, most peaks look tall and slender, and by analysing the histogram statistics, both

for algae nuclei and CRBC, amount of debris could be estimated, and as table 5 and table 6 shows, the amount of debris were relatively small compared to the algal nuclei counted events. Although most of the cultures looked good, culture 309.19.2 (figure 14, plot E) had a bit shorter and maybe a bit wide (especially in the bottom) main peak than the others. This becomes especially clear when one looks at the linear scale result, seen in figure 31. Here, one can see that the main peak is shorter (compared to figure 32) and the C2 peak (designated M3) is of low quality, and does not look like a peak. But, this result, and other lin scales FCM results) should be compared to other lin scale results (such as figure 32), and not the log scales. Lin scale results tend to look wider and not as slender as log scale FCM results. This is because log scale histograms have 10 times more channels (compared to lin) which the counted events is fitted into, and the acquired peaks are "pressed together" in log scale and widened when using lin scale (as the channels are wider).

Figure 31: FCM result of 309.19.2 using lin scales. FL2 histogram plot is cropped.

The debris amount is low in all culture samples, but 032.19.2 (figure 15) is the one that looks the worst. First of all, there are a much smaller amount of counted algal nuclei compared to the rest of the *P. kappa* cultures. The first peak of 032.19.2 is really short and wide. The second peak, the C2, does also look almost more like a bump than a peak. That it is exactly these two that looks the worst (032.19.2 is worse than 309.19.2) is not too surprising when looking at figure 4, it is exactly the same two replicates, 309.19.2 and 032.19.2, that shows extensively cell growth on the flask walls. As all cultures (of the same species) are treated the same (in respect to cell lyses (FCM)), there is a chance that these cells, especially those of 032.19.2, have reacted to either the light or temperature, differently than the others. There is a possibility that the cells in these cultures have a slight change in cell structure which also altered its reaction to the same lysis formula. But as figure 16 shows, the 032.19.2 culture now looks like the other replicates (from 19°C). Prior to the result of figure 16, all the 19°C treated cultures was moved further away from the light (to ca 40 instead of 50 µmol light intensity). After 2-3 months after this, the 032.19.2 culture still had some cell growth on the flask wall, but the FCM results looks better, suggesting that this culture reacted differently to the light intensity.

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By first looking at the FCM results for the six *Calyptrosphaera sp* cultures (figure 14) and then the six *Prymnesium kappa* cultures (figure 15), one can see that all the UIO 309 cultures shows signs of a 4C peak (a third algae nuclei peak), while only the 19°C UIO 032 shows signs of this peak (032.19.2 does not look good and have no visible third algae nuclei peak, but in figure 16, 032.19.2 shows sign of a third peak) and not 11°C, UIO 032 cultures. And this is not because of some gating error where the third nuclei peak is gated out. As one can see from figure 16, when comparing the two temperature treatments, there seems to be a pattern after C2 (the second peak) in the 19°C treated cultures, but not in the 11°C treated cultures. For the first *P. kappa* FCM results (figure 15), the results are the same. One cannot exclude the possibility that it is just debris, but as it is only the 032.11 cultures that did not show this pattern, it does raise some questions.

By looking at table 7, where all the estimated genome sizes (in pg) for *Calyptrosphaera sp* are included, one can see that there is a difference in measured genome sizes between the three different settings used (low log, high log and lin). Though, the differences are not too large, with a difference at ca. 0.2 pg between the largest and smallest measured average. When using low voltage setting (this includes both when using log and lin settings), the end result might not be as strong to withstand measurement errors e.g., measuring the nuclei to channel 8 instead of 6 can give a big difference in genome size (if the CRBC is measured to the same channel) compared to a channel measurement difference of 256 and 258. Figure 32 shows the FCM result for 309.19.1 using lin scales. Two other plots (FL2 histogram plot for CRBC and an ungated dot plot) are pasted into the same histogram plot, so this is not an accurate representation of the actual result where several plots were acquired. Here one can see that the second algal nuclei peak is wide, especially compared to the earlier FCM results. The same applies to the CRBC. When the peaks are this wide, getting accurate results gets more troublesome. Although, one should keep in mind that even though a peak using logarithmic scale might look thinner, it can still be spread out over hundreds of channels even though it does not look like it. And that we see a not so steep curve from C1 to C2, is not too illogical as there might be some cells that have just started the replication of DNA (interface – end of G_1 and start of S phase), but not yet finished and are "arrested" with a half-finished set of DNA.
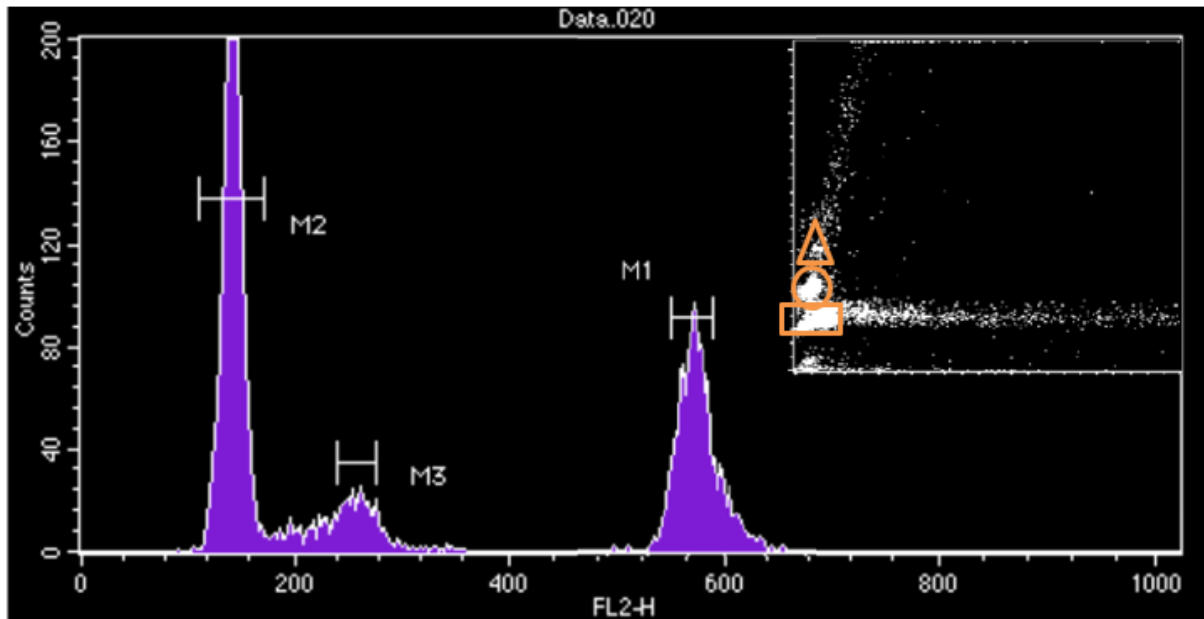
Figure 32: A FCM result of, Calyptrosphaera sp, 19ºC treated, replicate 1, 309.19.1, using linear (lin) scales. This is not one single histogram plot, but two histogram plots plus one dot plot, in the same picture. The CRBC (designed M1) is pasted into the same histogram plot as the algal nuclei (designated M2 and M3) and is not measured to 550-600 channel, but more or less to the same channel as the algal nuclei. A dot plot is also included and shows algae nuclei C1 (square), C2 (circle) and possible C4 (triangle). The non-marked pattern, that overlays with the nuclei inside the square, belongs to CRBCs. The possible C4 is gated out and not included in the algae nuclei histogram plot.

If one looks at the averages in table 7, one can see that the low log scale estimated the genomes to be larger than the other. 309.19.2 is larger than its two replicates, which makes sense when looking at the earlier results and figures including 309.19.2. The 19ºC average is therefore somewhat increased, but there is still a clear size-difference between the 11ºC and 19ºC treated cultures. When comparing the results for *Calyptrosphaera sp* from table 7 to *Calyptrosphaera sp* from table 3, one can see that they are not estimates to be of the same size. Those cultures have been exposed to different environments, in respect to growth rate, culture density and light intensity, and the two results was acquired with a few months between them. As mentioned, the CRBC used was past its expiration date, and this might have had an impact on the result. Also, the flow cytometer used was not the newest and most advanced, so there is a possibility that an, somewhat, outdated flow cytometer has affected the result.

When looking at the results for *P. Kappa* in table 8, more or less the same applies to this as already mentioned for the *Calyptrosphaera sp* results from table 7. Here, culture 032.19.2 much larger compared to the other replicates from the same temperature. Especially for high log settings, are 032.19.2 much larger, estimated to be almost 0.4 pg larger than the two other replicates. But unlike *Calyptrosphaera sp*, where all the 11ºC replicates was measured to be

pretty much the same size, is 032.11.2 somewhat smaller, but not by much for high log, with around 0.05 pg difference. In contrast, for low log, there is a genome size difference at ca. 0.3 pg. Where there was a clear difference in genome size between the 11ºC and the 19ºC *Calyptrosphaera sp* cultures, there is an even bigger size difference between the two temperatures of *P. Kappa*, even though there was at least one possible outlier, suggesting than the temperature had an impact on genome size.

Figures 20 and 21, showing the correlation between cell- and genome size, also reinforce the statement that temperature may induce larger genome size (and cell size). As one can see, in both figures, 032.19.2 (figure 21) and 309.19.2 (figure 20), are possible outliers in respect to both cell size (volume) and genome size. This affect the averages, as well as the overall correlation.

4.3.4. Second FCM results for *Prymnesium kappa*

The results from table 7 and 8 was not meant to be the final result to be used in this thesis. A too high log settings used for the included FCM results. After these results was acquired, the time forward was used for the different algae species from result section 3.1. When this was finished, the final result for the temperature experiment was meant to be acquired, but then it was discovered that something unexpected had happened to *P. kappa*, as can be seen in section "3.2.3. A second FCM results for *Prymnesium Kappa*" (figure 16).

When one look at the earlier results for *P. kappa*, one quickly sees a difference in figure 16, plot A, B and C. No longer is there a tall first peak, with a shorter following peak, but there are now two algal nuclei peaks of more or less the same height. Also, there are large difference in the total amount of counted algae nuclei events (see table 9 compared to 6), where the counts have decreased by 3000-4000 events. This change has only happened to 11ºC *P. kappa* and not in any other cultures. The first idea was that the cultures had been switched or mixed, but as only the 11ºC *P. kappa* showed any signs of changes, this hypothesis was quickly abandoned. The next hypothesis was that the algal cells had been infected by some kind of virus or bacteria, but again, that only 11ºC *P. kappa* was infected and not 11ºC *Calyptrosphaera sp*, even though they are cultivated next to each other. Although, one could have imagined that since they have been cultivated next to each other,

one should expect both to show sign of being infected, but that is maybe not always the case. There are in fact viruses that been shown to infect *P. kappa* (Johannessen et al. 2015) and as these viruses har a narrow host range, there is a chance that this virus only infects *P. kappa*. Still, one cannot exclude the possibility that it is the temperature treatment which is responsible for the change. This possibility is strengthened by the fact that the cultures did not seem to have been affected, in respect to growth rate. The cells seemed to grow just as normal. The cell concentration should therefore be the same as always. The reason for the smaller amount of detected algae nuclei compared the first *P. kappa* FCM results, gives reason to believe that some nuclei are not stained. The reason for this may be some kind of structural change in the cell, which makes the cells not react as expected to either the dye or the lysis solution.

As for the cell size measurement, a Students T-test was performed (using the same formula) and the whole table is presented in Appendix II (supplementary table 18). As for the cell size T-test, there are only three replicates, which will have an impact on the result. Not only as the t-table values (which the calculated t-value is compared to), will be higher, but also as outliers will have a greater impact on the calculated t-value. The calculated t-value for *P. kappa* using low log gave the lowest t-value at 1.385 and lin scale (also for *P. kappa*) gave the highest value, at 5.265. From all the values, only *P. kappa* at lin settings gave a significant difference in measured genome size. The other t-values were roughly calculated to 2.3 (when comparing to the t-table value 4.303), which makes them non-significant. For *Calyptrosphaera sp*, the FCM estimated genome sizes, according to table 7, did differ, but not as much as *P. kappa*, and the reason for why (most) calculated t-values for *P. kappa* were non-significant, may be because of the possible outlier, 032.19.2, which was measured to be larger the its two replicates.

4.4. Electron Microscopy – TEM and SEM

Since there seemed to have happened some kind of change between the 11ºC and 19ºC algae cultures, it was decided to take a look at the different cultures in an electron microscope to see if there was a change in the algae structure as well. Klaveness (1973) (and further by Noël, Kawachi and Inouye 2004) claimed that *Calyptrosphaera sphaeroidea Schiller* can exist in two phases (with different coccolith layers) – a haploid and motile holococcolithophorids into

a diploid and non-motile heterococcolithophorids. Because *Calyptrosphaera sp* in the temperature experiment had shown increased cell- and genome size, there was a possibility that a change had occurred. To find out if this was the case, the coccolith layer scales of the algae cells was examined using a Scanning Electron Microscope (SEM). The result of this test can be seen in figure 24. But because of a preparation error (wrong side up during the sputter coating of gold and palladium) of the 19°C sample, only the 11°C sample, which was where the possible change would have occurred, was examined. The SEM picture (figure 24) clearly shows several, random ordered, holococcolith scales, which is what one could expect to find in the holococcolith phase. From this result, there are no reason to believe that such a change, of coccolith stage, has occurred.

A larger genome size difference was discovered between the two temperatures of *Prymnesium kappa*, and there was therefore interesting to see if anything had happened to the structure as well. All replicates of *P. kappa* were prepared to be examined using Transmission Electron Microscopy (TEM) and figure 22 shows TEM for 11°C and 23 shows TEM for 19°C treated *P. kappa*. Due to contamination, most samples were destroyed. The algal cells in figure 22 shows a clear darker rim of the cell, with a flat scale with a visible radial pattern. These two distinct patterns are not visible for the 19°C cell in figure 23. From these figures, one can see a clear difference in the cell structure between the two temperature treatments (although only two out of six cultures were tested).

4.5. RNA, protein and C:N:P-ratio nnalysis

4.5.1. RNA quantifying analysis

Till now, we have seen results that gives reason to believe that the algae cells cultivated on 11°C have increased in both cell- and genome size. There is also natural to assume that changes had occurred to other parameters. In table 10 one can see the measured [RNA] (in µg ml^{-1}) and cell concentration (ml^{-1}). And in figure 25, which is based on the values of supplementary table 14 (Appendix II), one can see the estimated RNA pr. cell. First of all, the cell cultures were counted the following day (using the Casy Cell Counter). After the first procedure step was finalized and the samples was stored in the -80°C freezer, the rest of the algal culture samples were stored in 15 ml tubes in a fridge. The cultures (which there was 20

ml culture left in) in the Nunclon Filtercap Cell Flask were diluted up to 40 ml and stood over the night. Early the next day were the culture samples (from the 15 ml tubes) counted. But, since the cultures were dense and where closer to the stationary phase and not the exponentially growth phase, the result might not be too inaccurate. The cultures from the nunclon filtercap flasks (which were diluted with 20 ml IMR ½ the day before) was also counted and the alternative results can be found in Appendix II (supplementary table 12 and 13), and gives more or less the same result.

From table 10, when looking at the algal cell concentration, it is pretty much as expected, with higher concentration in the 19°C culture samples. Although (as mentioned earlier), there is a possibility that there were some inaccuracies in the calculations of cells ml^{-1}, resulting in higher a higher concentration that there actually was, but since every acquired value was multiplied with the same number, the results should still be valid (in respect to each other). As seen in earlier sections, there seem to be a possible outlier in 032.19.2 and somewhat 309.19.2. Nonetheless, the averages for each temperature and specie, is more or less just as expected. Also, in table 10, is the RNA concentration (μg ml^{-1}), and here as well there appear to be some outliers. Not only in 032.19.2 (both duplicates, but especially duplicate 2) and 309.19.2 (somewhat both duplicates), but there appear to be in 309.11.2 (both duplicates, but especially duplicate 1). This may affect the result, nonetheless, the [RNA] are somewhat similar across the different temperatures and species, but with slightly higher values for the 11°C culture samples.

In figure 25 (see supplementary table 14 for more accurate values for all duplicates), which is based on table 10, one can see that 032.11 average is notably higher than its 19°C counterpart. Also, the 309.11 average is shown to have a higher RNA concentration (μg pr. cell) than 309.19 average. The outliers from table 10 is more or less equalized, except for 309.11.2 which still shown to be a possible outlier and it is lowering the 309.11 average. Nevertheless, from figure one can see that the 11°C cultures are measured to have a higher RNA concentration that those of the 19°C treatment. It is believed that the increased RNA is a compensating mechanism to maintain increased protein synthesis, by increasing the abundance of ribosomes (Toseland et al. 2013), and similar results has also been acquired by e.g. Woods et al. (2003) and Hessen et al. (2017).

The T-values were calculated for both species (see Appendix II, supplementary table 18), using the same formula and method as first mentioned for the cell size measurement

discussion. The *P. kappa* difference was calculated to be 11.606, which is a significant difference when comparing to the t-table value at 2.571. *Calyptrosphaera sp* have a calculated T-value at 1.016, but had one clear outlier, and this t-value without the outlier was calculated to be 2.429, which is significant higher, but still lower than the t-table value. From the values of table 11, the difference between the two UIO 032 treatments is visible greater than those of UIO 309 treatments, so the calculated t-value seem to make sense. At least for *P. kappa* it is likely that reduction in temperature indices an increase in RNA amount.

4.5.2. Protein quantifying analysis

As the cell numbers counted using the Cays Cell Counter is the same in this test ([protein]) as the [RNA] analysis, the cell number section in table 11 is not discussed in this section (see first paragraph in section 4.5.1. for discussion). Again, in Appendix II (supplementary table 15 and 16) is the alternative results using the counted values from the diluted cultures. These alternative values also give more or less the same result as those of table 11 and figure 26 (and supplementary table 17).

First of all, when looking at table 11, as mentioned in the results section, there seem to be some possible outliers. At least, there is some fluctuation between the different replicates and duplicates. Overall, most of the acquired values are close to the average, and even if we remove the two possible outliers, 032.11.2(1) and 032.11.2(2), the average only changes from 16.79 to 16.61 [protein] in $\mu g\ ml^{-1}$. One would expect the duplicates of the same culture sample to me more or less the same, and except for the 032.11.2 duplicates, that is more or less the case. When comparing the averages, one quickly notices that the [protein] values is measured to be higher in the 11°C treated cultures compared to those of 19°C.

Based on the results in table 11, the values presented in figure 26 (which is based on the values from supplementary table 17, Appendix II. See this table for more accurate values), is just as expected. The calculated/measured values are all over stable, except for 032.11.2(1) which is somewhat larger than the rest, and maybe the two duplicates of 032.19.2, which is a bit larger than the other replicates. As one can read from the averages, the difference between the two *P. kappa* treatments is larger than that of *Calyptrosphaera sp*. Nonetheless, the trend is the same – lower temperature treatment seems to give higher protein amount. Even though

it is stated that protein synthesis decreases with lower temperature (Toseland et al. 2013; Hessen et al. 2013), my results suggest that the total protein amount increases with lower temperatures. Similar results have also been acquired by Woods et al. (2003), but Sönmez and Gülel (2008) got some contradicting results where they discovered that the Bean Beetle got increased Protein amount with increased temperature. But, this is a completely different specie and did not undergo as many generations as my algae. Siminovitch and Briggs proposed in 1949 that the increase of soluble proteins is a mechanism to prevent intracellular freezing. Also, it is an idea that the amount of protein (and RNA) need to be higher in organisms at lower temperature to counter slower biochemical and metabolic enzyme reactions (Hazel and Prosser, 1974; Guy, 1990; Woods et al. 2003).

By using the same formula and method mentioned in the earlier section, 4.5.1 RNA quantity analyses, a t-value was calculated for the two species to test for a significant difference between the two treatments (see Appendix II, supplementary table 18). *Prymnesium kappa* was calculated to (calculated t-value=) 6.558 and *Calyptrosphaera sp* have calculated t-value at 4.431. Both values are more than 0.05 (the p-value) higher compared to the t-table value, at 2.571, suggesting that both differences in protein quantity, between the two temperature treatments, are significant, i.e. lower temperature induces an increase in protein amount.

4.5.3 C:N:P-ratio analysis

The different ratios; C:N, C:P and N:P, is presented in table 12. There, one can see that there is a difference between the two treatments. For both C:N and C:P, for both species, the molar ratios are visible smaller in the 11°C treated cultures compared to the 19°C treated cultures. The N:P ratio as well is higher is the 19°C treated *P. kappa.* In contrast, in *Calyptrosphaera sp*, the N:P ratio is higher for the 11°C treated culture average compared to the 19°C treated cultures. As a e.g. C:N ratio at 40 means that for each Nitrogen atoms, there are 40 Carbons in the cells. Therefore, in the 11°C treated cells, which shows a lower C:N and C:P, there appear to be more P and N, compared to C, than for the 19°C treated cultures.

Actually, although the C amount is estimated to be lower in the 11°C treated, the P amount is more or less exactly the same throughout all the averages. There is therefore possible to

assume that temperature may affect the stoichiometry, and that the P ratios (especially compared to C) is higher in the 11°C treated cultures.

A students t-test, as the one used earlier, is used to analyse the difference between the 11°C and 19°C treated cultures to see if the measured difference is significant (see supplementary table 18, Appendix II for the full table). As in the other t-test, the experimental value is affected by possible outliers and that there is only six values (duplicates). Only two is estimated to be significant, C:P (at 3.777) and C:N (2.635) for *P. kappa*, compared to the t-table value at 2.57. The calculated t-value for *Calyptrosphaera sp*, C:N and *P. kappa* N:P, is respectively 2.385 and 2.024, being slightly under the t-table value (making them non-significant). For *Calyptrosphaera sp*, C:P and N:P ratio, the t-value is calculated to be roughly 1.1, being much smaller than the t-table value, and making them non-significant.

Anyways, as table 12 shows, there appear to have happened a change between the two temperature treatments and the interaction between temperature and stoichiometry have already been investigated by other. For some species, no clear, or no significant, correlation between temperature increase/decrease and C:N:P ratios, is discovered (Hessen et al. 2017; Skau et al. 2017). Other studies have discovered a positive trend between C:P and N:P ratios, and temperature (Yvon-Durocher et al. 2003), as well as a positive correlation (in higher plant leafs) between C:P, N:P and C:N, and temperature (Reich and Oleksyn. 2004). The two latter shows more or less the same results as mine, except for the N:P ratios between the two temperature treatments from *Calyptrosphaera sp*, where N:P ratio decreased with temperature (although, this was not a true significant value). As mentioned earlier, the two 11°C treated cultures may have larger amount of both RNA and protein compared to the 19°C treated cultures. As proteins are nitrogen-rich and rRNA is phosphorus-rich, is higher N and P amounts, i.e. lower C:N and C:P ratios, these results seem somewhat logical.

This is supported by figure 33 (especially for *P. kappa*), where RNA (multiplies with 10^6) in µg is plotted against C:P ratio (multiplied with 10^{-3}). From the figure, one can clearly see how the RNA is higher than the C:P ratio for 032.11, but lower for 032.19 and 309.19. For 11°C *Calyptrosphaera sp*, the values are fluctuating somewhat more.
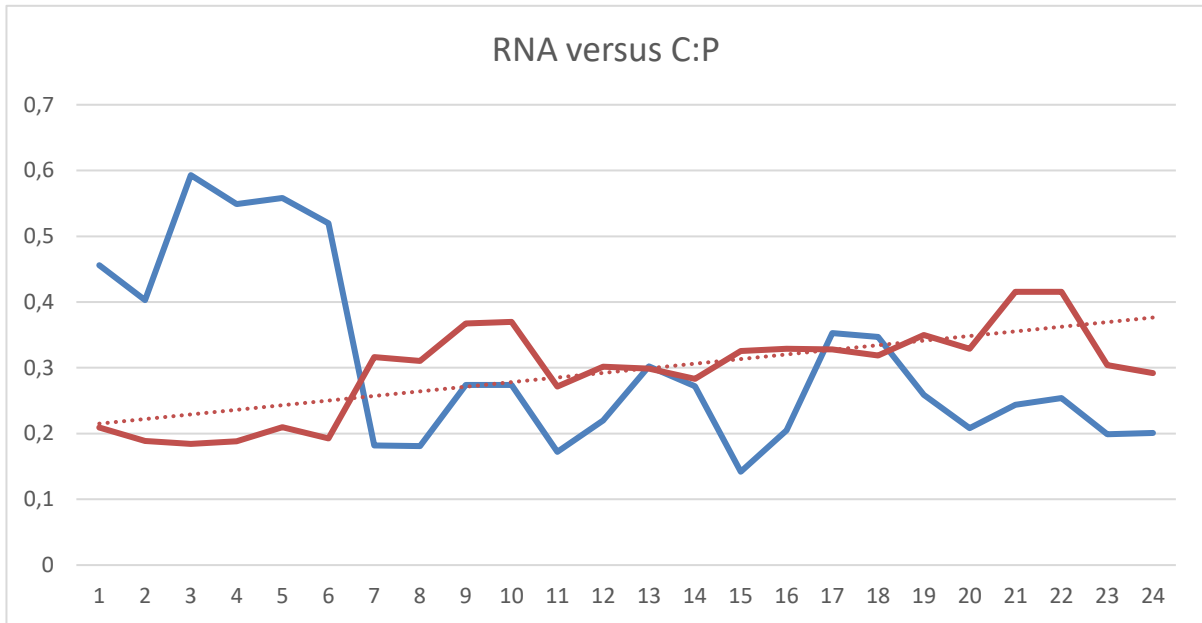
Figure 33: Plot with RNA plotted against C:P ratios for all 12 cultures (Calyptrosphaera sp (UIO 309) and P. kappa (UIO 032)) with their duplicate. X-axis: 1-6 is 032.11, respectively 1(1), 1(2), 2(1), 2(2), 3(1) and 3(2), where first number is replicate number and parenthesis is duplicate number. 7-12, 13-18 and 19-24 is respectively 032.19, 309.11 and 309.19, with the same duplicate values as for 032.11. Blue line is RNA (in µg) amount per cell (multiplied with 10^6) and red line is C:P ratio (multiplied with 10^{-3}). Red dotted line is regression line for C:P ratio. Sample 10 value is removed as it was measured to be more than 10 times larger, and replaced by the other duplicate from the same replicate. (Y-axis uses comma (,) instead of dot (full stop (.)) because Norwegian Excel was used.

4.6. DNA Sequencing

First of all, in respect to table 13 and the amount of extracted DNA from each culture sample, some samples, especially the *Calyptrosphaera sp* cultures, are shown to contain a smaller amount of DNA (an average difference at around 350 ng) compared to *P. kappa* (with an average at ca. 700 ng DNA). As mentioned earlier, the *Calyptrosphaera sp* cultures was, after the first percoll filtration, measured (using FCM) to contain a much lower amount of cell nuclei. This became also clear after centrifugation of the samples (the combined sample from the three percoll filtration cycles), did not yield any visible pellet (something the *P. kappa* samples did). The reason for the lower amount of extracted DNA, may be the fact that wrong percoll density gradients was used. A percoll filtration test showed that while 1.06 and 1.08 (nuclei "arrested" at this layer) worked well for *P. kappa*, 1.05 and 1.07 (nuclei "arrested" on top of this layer) was the preferred density gradients for *Calyptrosphaera sp*. Indeed, both the result in table 13, and the test mentioned, proves that 1.06 and 1.08 density gradients works, but it is not the most beneficial and some nuclei might have gotten lost throughout the percoll filtration cycle.

4.6.1. DNA sequencing – K-mer analysis

From figure 27, one can see the estimated genome sizes (in Mbp) for all the 12 culture samples using analysis where each individual culture samples were treated independently. There is some variation between the different replicates (from the same specie and treatment), but as all the cultures have been cultivated independently for such a long time, changes due mutation, and therefore also variation, is expected. For some, the difference between the replicates is greater, such as 032.11.2, 032.19.2, and 309.19.2, but for all of these, the exact same variation can be found for the Flow Cytometry results as well. This presumable variation is most likely due to natural differences in the cultures.

The averages of the replicates can be found in table 14. The possible outliers from figure 27 will have an impact on the average. Even though 032.11.2 is smaller than the other 032.11 replicates and 032.19.2 is larger than the other 032.19 replicates, the average still results in a big difference (at ca 130 Mbp) between the two temperature treatments. For *Calyptrosphaera sp* on the other hand, the difference between the two temperature treatments is minimal (with only a difference at ca. 3.0 Mbp). Although, 309.11.2 is measured to be somewhat smaller than the other 309.11 replicates and 309.19.2 is larger than the other 309.19 replicates. This will have an impact on the result, but anyways, the difference between the two temperature treatments is not as large as for *P. kappa*.

As in the earlier sections, e.g. protein and RNA quantity analysis' sections, a T-value was calculated (see Appendix II, supplementary figure 18) from the values from figure 27. The *P. kappa* T-value difference was calculated to be 4.788, while *Calyptrosphaera sp* have a calculated T-value at 0.183, where *P. kappa* is higher (<0.05) than the t-table value at 4.303, while *Calyptrosphaera sp* is much lower. In accordance with these values, as also figure 28 summarises, the two *P. kappa* temperature treaded cultures are significant different, while *Calyptrosphaera sp* is not. Actually, the two different temperature treatments for the *Calyptrosphaera sp* cultures, are shown to be more or less similar, as both figure 26 and 28 also shows.

Figure 28 shows the estimated genome sizes (in Mbp) for the averages using analysis where all the replicates was combined together. As one can see, this method yields higher estimated values for all the replicate averages. Though, some have increased more than other. The 309.19 average is for instance now ca. 5 Mbp larger the the 11ºC treated. The 032.11 average,

on the other hand, is still lager (by ca 150 Mbp) The reason for the increase may be that when the replicates is treated independent, there is a lower amount of available sequences. There may be some sequenced regions that is covered in some samples and not the others. Therefore, all the unique regions which is only completely covered by some replicates, but not others, will affect the independently replicate analysis more than the combined replicate analysis. Anyways, from this (and these) result(s), there are evidences that temperature have induced changes to the genome, where lower temperature induces an increase in genome size.

Figure 29 shows the frequency of variant branches using the k-de Bruijn graph, which is often interpreted as heterozygosity. The graph is baes on the combined replicate analysis. From this figure, one can see that both species from the 11°C treatment have a higher frequency of variant branches than the 19°C treated. Also, *P. kappa* have in general higher values compared to *Calyptrosphaera sp*. But, for a cell to be heterozygote (or homozygote) it has to be diploid (or at least not haploid). As mentioned earlier, there is not too much reason to believe that *Calyptrosphaera sp*, both 11°C and 19°C treated, are diploid. One can assume the same for *P. kappa*, based on the unpublished data from Bente Edvardsen. Though, in other unpublished data, B. Edvardsen discovered that *P. kappa* do exist in a diploid form, but from her data, that form was easily recognisable and the results acquired so far did not look like her diploid results. It is unlikely that the frequency of variant branches is due to heterozygosity, but as the replicates do show different DNA amount, this is something that can affect the result, giving higher values of variant branches.

Table 16: Results from k-mer analysis compared to those from Flow Cytometry (FCM). Genome size presented in both pg and in base pairs (Bp) (Mega base pairs Mbp and Giga base paris, Gb).

Specie-Treatment	K-mer		FCM	
	Mbp	pg	pg	Gb
032.11	463.4	0.47	2.49	2.44
032.19	333	0.34	2.17	2.12
309.11	361.7	0.37	2.24	2.19
309.19	359	0.37	2.07	2.03

As one can see from table 16 (K-mer values are the same as presented in table 14. The values from figure 28 is maybe more accurate, but that is not as relevant in this part), the results from this test differ a lot compared to the FCM results. There is some uncertainty regarding the FCM results. First of all, the Flow Cytometer used is not the newest or the most advanced and

I did have some problems with the CRBC used. Towards the end of the experiment had the CRBC expired. How this affect the result is not clear. A new CRBC was tested, but it seemed to be much smaller than the earlier CRBC and the estimated genome sizes were measured to be much larger than earlier predictions. So, an expired CRBC does not seem to be a good enough explanation.

A k-mer test counts the number of unique events. This means that in heavily repeatable sequences, such as telomeres (for Green Algae: TTTAGGG (Olsson et al. 2018)), it is a chance that some DNA material just is not accounted for when estimating using a simple K-mer test. Although, as table 16 shows, it is unlikely that the k-mer test have failed to account for 1.5-2 pg of the genome. Exactly what is the cause, is difficult to say. The most important thing in this test, which also was the point of the whole experiment, is that there seem to be a clear difference between the two temperature treatments (especially for *P. kappa*). For *Calyptrosphaera sp*, repeatable elements that have not been accounted for using the k-mer test may explain why the FCM result show a difference (although not a significant) between the two temperature treatments. There is also possible that the temperature decrease has induced epigenetic changes to *Calyptrosphaera sp* (e.g. induces a change in the chromatin structure (e.g. heterochromatin $\rightarrow$ euchromatin) which makes it easier for PI to bind to the DNA), which can affect the other parameters tested (RNA, protein etc.) and the FCM results where the genome size is estimated to be larger than it actually is. Either way, it is a clear possibility that the temperature decrease has induced a change to the cell and its genome (DNA structure).

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5. CONCLUSION

In the temperature experiment, all the cultures were diluted frequently, ensuring that there always was enough fresh medium for the algal cells. All the cultures were exposed to the same light intensity and the cultures, with 3 replicates for each temperature and specie, were cultivated independently in their own nuclon filtercap flasks. Thus, ensuring that there was no interaction, nor gene flow, between the cultures, making true replicates. The only tested variable was therefore temperature.

In addition to the temperature experiment, a second part of this thesis, "Cell- and genome size correlation across different algal species", was also conducted. Both cell size and genome size were measured. Although, to get an estimation of the genome size, the results were only acquired using logarithmic scale and not linear. Still, results were acquired and from the cell size (diameter) measurement, using Casy Cell Counter, and genome size measurement, using flow cytometry (FCM), the correlation was estimated. Even though only nine algae species were tested, where two possibly were the same (but with different names and strains), and the correlation (r^2) was estimated to be roughly 0.5, a positive correlation was acquired. Thus, suggesting that there indeed is a relationship, a positive correlation, between cell- and genome size across algal species. Algae genome and size correlation is pretty much confirmed, but there are still more algal species, and strains, which have yet to have their genome analysed. Algal genome and size correlation have been tested, and confirmed, earlier, for many other (algal) species, but there are still more algal species, and strains, which have yet to have their genome analysed.

For the temperature experiment, the Flow cytometry results suggested that there indeed was a difference between the two temperature treatments. As both temperature-treated cultures were treated with the same cell lysis formula (different between the species), the only parameter was the temperature. The observed change was therefore, most likely, due to the different temperatures. A change in genome size was estimated for both *Calyptrosphaera sp* (UIO 309) and *Prymnesium kappa* (UIO 032), but especially for *P. kappa*. Both in the first FCM results, but especially in the second FCM results. Though, for the second, as the results had changed a lot for the 11°C treated cultures, one cannot exclude contamination or virus infection, even though it may seem unlikely. Even though a difference in genome size was discovered (although, the experimental t-test did not suggest that the changes for UIO 309 were

significant), only two algal species were tested in this experiment. And, though there were changes in both, only *P. kappa* showed a strong evidence in respect to a change in its genome. More algae species and other (ectotherm) organisms should therefore be tested to control and maybe confirm the results acquired in this experiment. Also, all tests should be controlled using a more modern Flow cytometer, and other methods, especially more advanced molecular methods should be used to get a better understanding of the mechanisms behind the possible changes that had occurred.

The RNA, Protein and C:N:P analysis was only conducted one time. Though, each culture had its own duplicate. The protein and RNA quantity analysis do show strong evidences that there is a different amount of RNA and protein it the two temperature treatments, with increased values, for both species, in the 11°C treated cultures. For the C:N:P-ratio analysis, it was discovered that there appeared to be a clear connection between the ratios (C:N, C:P and N:P), especially for *P. kappa*, where some differences between the two temperature treatments were truly significant.

A DNA sequencing was conducted, but as there was little time left (of my master thesis), only a (simple) K-mer test was conducted. Using the K-mer analysis, a genome size estimation was acquired. Even though the test resulted in a much lower C-value (genome size) for all cultures, it was estimated that the 11°C treated *P. kappa* cultures was clearly larger than the 19°C treated cultures. In contrast, *Calyptrosphaera sp* showed no significant difference between the two cultures. Actually, they were close to identical. Although, as this K-mer test estimates the genome size based on "unique" sequences, there is a chance that heavily repeatable elements were excluded and not accounted for when estimating the size. Thus, the 11°C treated *Calyptrosphaera sp* cultures may still have a larger genome size, as the FCM results estimated.

The results acquired in this study suggests that temperature have a direct impact on genome size and that lower temperature induces a larger genome (more DNA). Further sequencing analysis, finishing the already sequenced genomes from this experiment, should be a priority. Here, I also suggest to do similar testing, both on the same algal species, but also on different species. To get a better understanding on the epigenetics of algae (and other organisms), and how it is related to temperature change (and other possible climate change effects), more investigation should be conducted on this topic.

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APPENDIX I

Recipes:

IMR ½ medium:

Supplementary table 1: IMR ½ medium recipe:

SUBSTANCE	Amount used	Concentration
KNO_3 solution	0.5 ml	0.5 g/ml
KH_2PO_4 solution	0.5 ml	6.8 mg/ml
Vitamin solution	0.5 ml	(See Trace Metal Solutiuon)
Trace metal solution	0.5 ml	(See Vitamin Solution)
$Na_2O_3Se \cdot 5H_2O$	1 ml	2.63 mg/l

Supplementary table 2: Trace Metal Solution:

SUBSTANCE	Amount
Na_2EDTA	6 grams
NaFeEDTA	1360 milligrams
$MnSO_4 \cdot 1H_2O$	620 mg
$ZnSO_4 \cdot 7H_2O$	250 mg
$Na_2MgO_4 \cdot 2H_2O$	130 mg
$CoCl_2 + CuSO_4$ [4 mg/ml]	1 ml

MilliQ H_2O up to 1000 ml added and pH adjusted to 7.8-8.

SUBSTANCE	Amount used
Tiamin B_1	100 mg
Cyanokobalamin B_{12}	1 mg
Biotin	1 mg

1000 ml MilliQ H_2O added

Flow Cytometry recipes:

Supplementary table 4: Nuclei Isolation Buffer

SUBSTANCE	MOLARITY	Molar Mass (M)	Amount for 100 ml buffer
$MgCl_2$	30 mM	95.22 g/mol	285.7 mg
Sodium Citrate	20 mM	340.08 g/mol	588.2 mg
D-Sorbitol	120 mM	182.17 g/mol	2.186 g
HEPES	55 mM	238.30 g/mol	1.310 g
EDTA disodium salt (Titriplex III)	5 mM	372.24 g/mol	186.1 mg

pH adjusted to 7.5

Supplementary table 5: FCM-lysis formulas for different algae species.

	UIO 140	UIO 090	NIVA-2/92	K-0394	K-1321	K-1310	UIO 095	UIO 041
NIB	220µl	250 µl	350 µl	200 µl	310 µl	250 µl	275 µl	125 µl
PBS EDTA	-	750 µl	-	800 µl	690 µl	750 µl	-	875 µl
FACS Flow	-	-	650 µl	-	-	-	725 µl	-
50/50 Flow/MQ H_2O	780 µl	-	-	-	-	-	-	-
Triton x-100 (1:10 diluted)	8 µ-l	4 µl	8 µl	7.5 µl	12 µl	4 µl	14 µl	6 µl
Hydrogen Sulfite	-	6.5 µl	2 µl	2 µl	2 µl	6.5 µl	2 µl	-
Glutaralaldehyde 2.5%	-	5 µl	-	-	-	5 µl	-	-
96% Ethanol	-	5 µl	-	-	-	5 µl	-	-

OBS! Some algae species turned out to be difficult to work with and these formulas might not be 100 % optimal.

Standard stock for RNA (and DNA) quantification, section 2.8.1 (stocks prepared by F. Bullejos):

Supplementary table 6: RNA standards preparation.

x	[RNA] (µg mL^{-1}) (x)	Volume (mL) (x)	Mass (µg RNA) (x)	Volume$_{x-1}$ (mL)	Volume of TE Buffer (mL)	
Commercial stock	100,00	1,0	100,00			
Stock R1	8,00	0,5	4,00	0,04	0,46	Pay attention to this
Stock R2	4,00	0,5	2,00	0,25	0,25	
Stock R3	2,00	0,5	1,00	0,25	0,25	
Stock R4	1,00	0,5	0,50	0,25	0,25	
Stock R5	0,50	0,5	0,25	0,25	0,25	
Stock R6	0,25	0,5	0,13	0,25	0,25	
Stock R7	0,13	0,5	0,06	0,25	0,25	
Stock R8	0,06	0,5	0,03	0,25	0,25	

Volume$_{x-1}$ is volume taken from previous stock, e.g. 0.04 ml is taken from the commercial stock to make stock R1.

Supplementary table 7:DNA standards preparation

x	[DNA] (µg mL^{-1}) (x)	Volume (mL) (x)	Mass (µg DNA) (x)	Volume$_{x-1}$ (mL)	Volume of TE Buffer (mL)	
Commercial stock	10500,00	1,0	10500,00			
Stock D1	58,33	1,8	105,00	0,01	1,79	Pay attention to this
Stock D2	29,17	0,5	14,58	0,25	0,25	
Stock D3	14,58	0,5	7,29	0,25	0,25	
Stock D4	7,29	0,5	3,65	0,25	0,25	
Stock D5	3,65	0,5	1,82	0,25	0,25	
Stock D6	1,82	0,5	0,91	0,25	0,25	
Stock D7	0,91	0,5	0,46	0,25	0,25	
Stock D8	0,46	0,5	0,23	0,25	0,25	

RNA standards (June 2018)

Standard	Standard stock		Volume of Extraction Buffer (µL)	Volume of TE Buffer (µL)	[RNA] (µg mL^{-1})
	[RNA] (µg mL^{-1})	Volume (µL)			
R1	8,000	11	6	67	1,048
R2	4,000	11	6	67	0,524
R3	2,000	11	6	67	0,262
R4	1,000	11	6	67	0,131
R5	0,500	11	6	67	0,065
R6	0,250	11	6	67	0,033
R7	0,125	11	6	67	0,016
R8	0,063	11	6	67	0,008
Blank	0,000	0	5	65	0,000

DNA standards (June 2018)

Standard	Standard stock		Volume of Extraction Buffer (µL)	Volume of TE Buffer (µL)	[DNA] (µg mL^{-1})
	[DNA] (µg mL^{-1})	Volume (µL)			
D1	58,333	11	24	290	1,974
D2	29,167	11	24	290	0,987
D3	14,583	11	24	290	0,494
D4	7,292	11	24	290	0,247
D5	3,646	11	24	290	0,123
D6	1,823	11	24	290	0,062
D7	0,911	11	24	290	0,031
D8	0,456	11	24	290	0,015
Blank	0,000	0	5	65	0,000

Standard stock for RNA (and DNA) quantification, section 2.8.1 (stocks prepared by F. Bullejos):

Protein standard stocks (August 2018)

x	[Protein] (µg mL^{-1})	Volume (mL)	Mass (µg Protein)
Stock P1	500	0,5	250,00
Stock P2	400	0,5	200,00
Stock P3	300	0,5	150,00
Stock P4	200	0,5	100,00
Stock P5	100	0,5	50,00
Stock P6	50	0,5	25,00
Stock P7	25	0,5	12,50
Blank	0	0,5	0,00

Protein standard stocks (August 2018)

	Commercial tubes							
	Stocks							
x	[Protein] (µg mL^{-1})	Volume (µL)	Mass (µg Protein)	Quant-itTM Protein Buffer (µL)	Extraction reagent: Water (µL)	Extraction reagent: NaOH 0,1 N (µL)	Extraction reagent: *β*-Mercaptoethanol (µL)	Extraction reagent: Protease inhibitor cocktail (µL)
P1	500	10	5,00	90,00	49,75	49,75	0,5	0
P2	400	10	4,00	90,00	49,75	49,75	0,5	0
P3	300	10	3,00	90,00	49,75	49,75	0,5	0
P4	200	10	2,00	90,00	49,75	49,75	0,5	0
P5	100	10	1,00	90,00	49,75	49,75	0,5	0
P6	50	10	0,50	90,00	49,75	49,75	0,5	0
P7	25	10	0,25	90,00	49,75	49,75	0,5	0
Blank	0	10	0,00	90,00	49,75	49,75	0,5	0

				Plate Well; Standard points in the curve				
x	Extraction reagents added (µL)	Reagents added (µL)	Total volume (µL)	[Protein] (µg mL-1)	Volume (µL)	Mass (µg Protein)	NaOH (µg mL-1)	β-Mercaptoethanol (µg mL-1)
P1	100	190	200	25	100	2,5	995	0,2287875
P2	100	190	200	20	100	2	995	0,2287875
P3	100	190	200	15	100	1,5	995	0,2287875
P4	100	190	200	10	100	1	995	0,2287875
P5	100	190	200	5	100	0,5	995	0,2287875
P6	100	190	200	2,5	100	0,25	995	0,2287875
P7	100	190	200	1,25	100	0,125	995	0,2287875
Blank	100	190	200	0	100	0	995	0,2287875

Protocol: Purification of Total DNA from Animal Blood or Cells (Spin-Column Protocol)

First, 250 µl PBS was added to all samples (with pellets), as well as 20 µl Proteinase K and 8 µl RNase A (0.1 mg/ml). Samples incubated for 2 min and vortexed for ca. 10 seconds (all vortexing performed is pulse-vortexing).

Then, 200 µl Buffer AL was added to all samples and vortexed before incubation at 56ºC for 10 minutes. The samples were vortexed a few times throughout this 10-minute period.

Next, 200 µl 96 % Ethanol was added to all samples and vortexed before transferred to mini spin columns placed in 2 ml collection tubes and centrifuged at 8000 rpm for 1 min (all centrifugation in RT). Flow trough was discarded and samples was centrifuged again, to make sure that the ethanol was completely removed.

Then, 500 µl Buffer AW1 was added to the mini spin columns and centrifuged for 1 min at 8000 rpm. Mini spin columns were placed in new collection tubes and 500 µl AW2 was added and centrifuged for 3 min at 14000 rpm. Flow trough was removed and centrifugation was repeated, but this time for 1 minute.

Mini spin columns were placed in new 1.5 ml Eppendorf tubes and 100 µl Buffer AE was added directly onto the membrane and left to incubate for 1 min before centrifugation at 8000 rpm for 1 minute. New 100 µl Buffer AE was added to the membrane and centrifugation was repeated.

Giving 200 µl with isolated DNA which is now in the 1.5 ml Eppendorf tubes.

RNA quantity analysis from section 2.8.1, detailed protocol.

To measure RNA amount in the cells, a protocol by Francisco Bullejos, derived from Skau et al. (2017) and Hessen et al (2017), which again is based on a protocol by Gorokhova and Kyle (2002), was used. The protocol can be divided into to five steps: nucleic acid extraction, RiboGreen dying, fluorescence measurement (RNA + DNA), RNA digestion, second fluorescence measurement (DNA).

To all 24 samples of RNA analysis samples plus two controls (one with membrane and one without), 1000 µl Extraction Buffer 1% (1% sarcosyl) (Ext buffer: 100 ml TRIS EDTA (TE) buffer + 1 g N-Lauroylsarcosine) was added and the sample membrane was grinded using Kontak Pestle, which was washed with 96% ethanol, distilled water and RNase Erase, before use. To induce physical disruption of the cells, the samples was placed in a VWR ultrasonic

cleaner, filled with ice cold water. After 3 repetitions of ca. 40 seconds sonication and 1-minute resting in the ice bath, the samples were left to incubate for ca 2 hours while shaking in a Vortex agitator (Vortex Genie 2, Scientific Industries) at 490 speed.

DNA and RNA standards (D1-D8 and R1-R8 stock) was already prepared by PhD. Francesco Bullejos using a commercial stock (deoxyribonucleic acid solution from calf thymus, Sigma-Aldrich; 9-12 mg/ml) (see supplementary table 6 and 7 for stock standard preparation). To all 8 RNA standards (R1-R8), 67 µl TE buffer and 6 µl Extraction buffer was added directly to an Eppendorf tube containing 11 µl of the standard stock. A blank containing 65 µl TE buffer and 5 µl Extraction, was also prepared. The 8 DNA standards (D1-D8) was prepared by adding 290 µl ET buffer and 24 µl Extraction buffer directly to Eppendorf tubes containing 11 µl DNA stock. Another blank, just like the last one, was prepared. A RiboGreen working solution was prepared by adding 50 µl commercial stock Quant-iT™ RiboGreen®RNA reagent (Thermo Fisher Scientific) to 15 ml tube and diluting it with 9950 µl TE buffer making a total of 10 ml.

70 µl of the DNA and RNA standards (and their blanks) was added to a 96 well plate. Two of each. In 26 x 2 (total 52) wells in the 96 well plate, was 68 µl TE buffer added. After shaking-incubation, the samples were centrifuged for 5 min at 3000 rpm. After centrifugation was 2 µl of the samples (two of each) added to the wells. Each well now have a total volume of 70 µl. Then, 70 µl of the working RiboGreen was added to all the wells, so that the total volume is 140 µl per well. The plate was covered with aluminium foil and incubated for only a few minutes before it was placed inside the Plate Reader (SYNERGI Mx (BioTek)). Before the actual measurement, the well plate was left to shake for 5 minutes. The software, Gen 5 1.10. was used, with the settings; 480 nm Excitation and 528 nm Emission Wavelength. After the first measurement was completed, 5 µl RNase A was added to each well and then left to incubate for 30 minutes in dark (inside the Plate reader). The well plate was then shaken for 5 min before measurement using the same settings as the previous measurement. In the first measurement, DNA+RNA amount was measured, and in the second, DNA only was measured. Therefore, to get RNA amount, DNA measured amount value was subtracted from DNA+RNA value.

Protein quantity analysis, section 2.8.2, detailed protocol:

This protocol is prepared by Francisco Bullejos (which is based on a protocol by Barbarino and Lourenço (2005)). The protocol can be divided into two steps – protein extraction (1) and Fluorescence measurement (2). All 24 protein filter samples, plus blanks, (from section 2.8) was removed from freezer and grinded. This was done by adding 300 µl EM1 (Extraction mixture 1; (99ml) protease-free water with (1ml) protease inhibitor cocktail (Sigma-Aldrich)) to the sample tubes before grinding the filters with a pestle. Then, 700 µl of EM1 was added to all samples before the was left to incubate for 24 hours at 4ºC (in a fridge).

All samples were vortexed and then placed in an ice water filled sonication chamber (VWR ultrasonic cleaner) were the samples underwent a sonication treatment of 40 sec sonication and 1-minute resting (in the ice water). This was repeated two more times (3 sonication steps in total). The samples were quickly centrifuged (ca. 5-6 seconds) before 1 ml of supernatant (without filter residue) was transferred to ultra-microcentrifuge tubes. All the samples (now in the ultra-microcentrifuge tubes) were centrifuged for 20 minutes at 4ºC and 16189 rpm (or 15000 xG) using a Micro-Ultracentrifuge (Sorvall™ MTX 150 Micro-Ultracentrifuge (2009), Thermo Scientific, ThermoFisher Scientific) and a S110-AT rotor. After centrifugation was 1 ml of (around) supernatant removed and transferred to new 2 ml Eppendorf tubes (which was stored at 4ºC), without touching the pellet (no pellets visible in any. Therefore, not all supernatant was removed and care was taken not to touch any tube walls). After the supernatant was removed, 1 ml EM2 (Extraction Mixture 2; 500 ml EM2 = 497.5 ml 0.1 M NaOH, + 2.5 ml 7.15 M β-Mercaptoethanol) were added to all pellet samples and pellets were resuspended by pipetting up and down. After ca 30 minutes (or more) of incubation (in 4ºC fridge), were all samples again centrifuged, using the same centrifuge and same settings. After centrifugation were 1 ml removed and added to the 2 ml Eppendorf tubes (now with a total of (almost) 2 ml).

Protein standard stocks were prepared (see Appendix I) and 100 µl of each was added to its own well in a 96-well plate. The protein samples were quickly vortexed before 100 µl of each (including the blanks) were added to its own well. Then, 100 µl WQPR* (Working solution of Quant-iT^{TM} Protein Reagent* (QPR: component A of the Quant-iT^{TM} Protein Asssay Kit; ThermoFisher Scientific); 10 ml (1:100) WQPR = 100 µl QPR + 9.9 ml QPB (Quant-iT^{TM} Protein Buffer; component B of the Quant-iT^{TM} Protein Assay Kit; ThermoFisher Scientific)) were added to the wells, making a total of 200 µl in each well (with samples in them). The

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microplate was then left to incubate for 15 minutes in the dark while shaking, before the fluorescence scan using the plate reader (Synergy Mx Microplate Reader, BioTek Industries). Using the software Gen5 1.10, and 485 nm excitation and 580 nm emission wavelengths, was the microplate scanned.
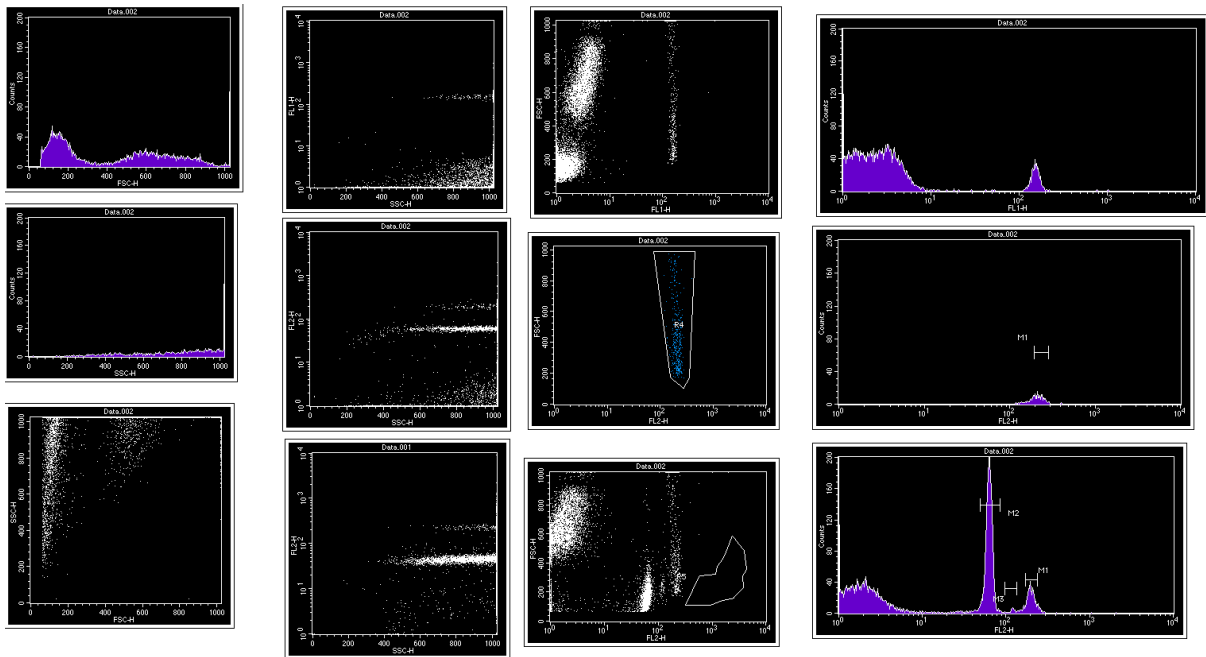
APPENDIX II

Additional figures from Result section 3.1.:

Isochrysis Galbana (UIO 140):
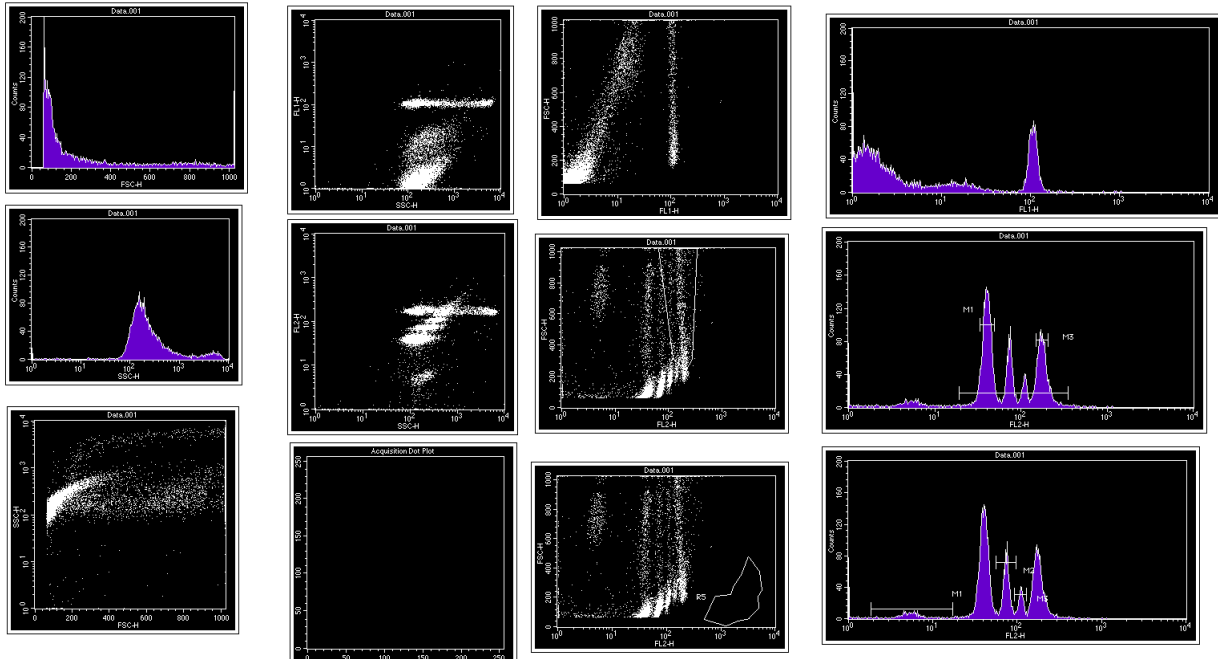
Supplementary Figure 1: A picture overview over all different plots acquired using Flow Cytometry for Isochrysis Galbana (UIO 140)

Diacronema lutheri (UIO 090):

Supplementary Figure 2: A picture overview over all different plots acquired using Flow Cytometry for Diacronema lutheri (UIO 090)

Hymenomonas carterae (NIVA-2/92):
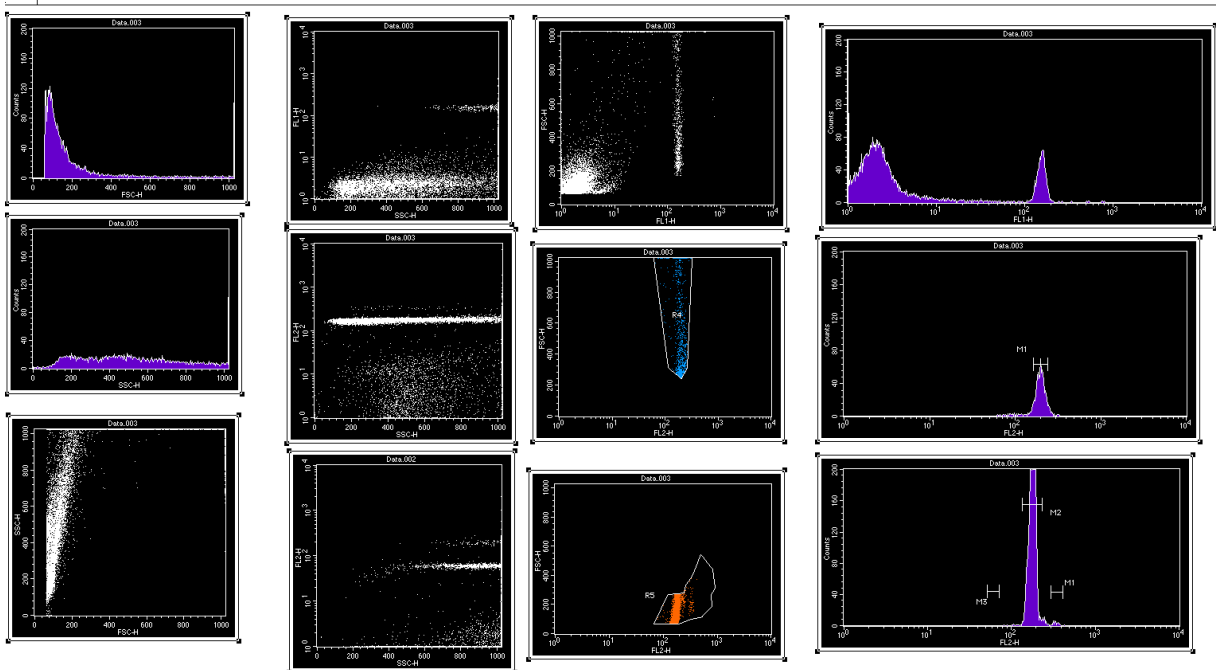
Supplementary Figure 3: A picture overview over all different plots acquired using Flow Cytometry for Hymenomonas carterae (NIVA-2/92).

Prymnseium Nemamethecum (K-0394):
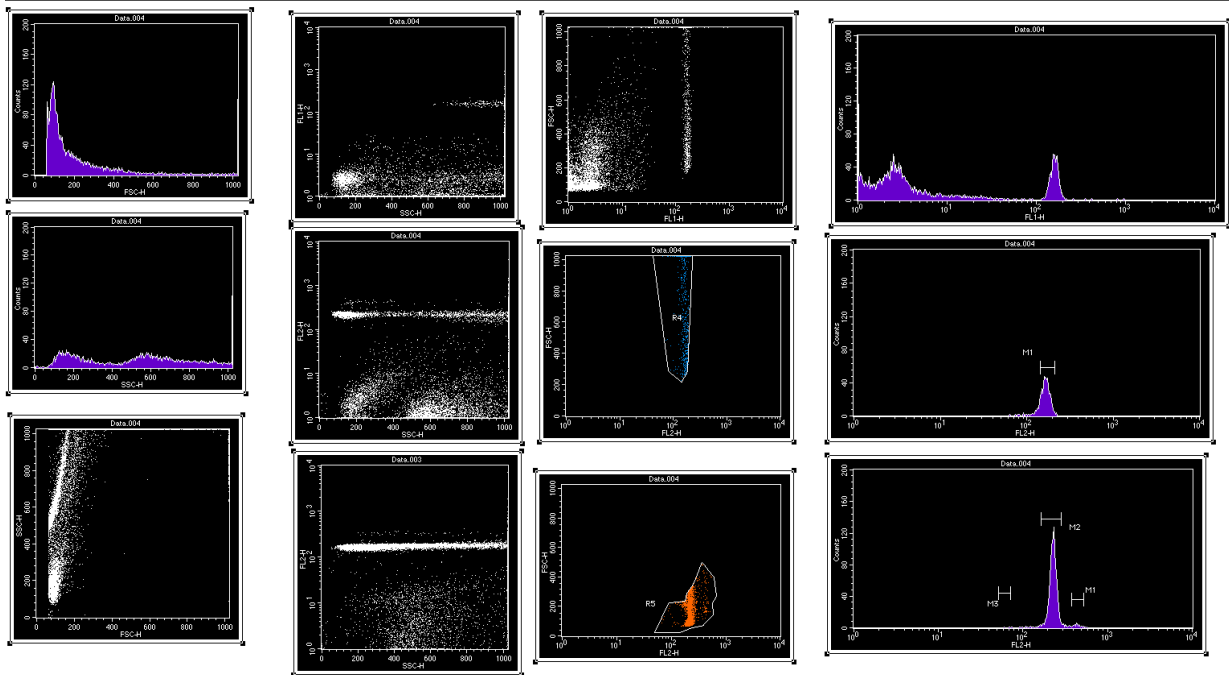
Supplementary Figure 4: A picture overview over all the plots acquired using Flow Cytometry for Prymnseium Nemamethecum (K-0394).

Calyptrosphaera sp (UIO 309):
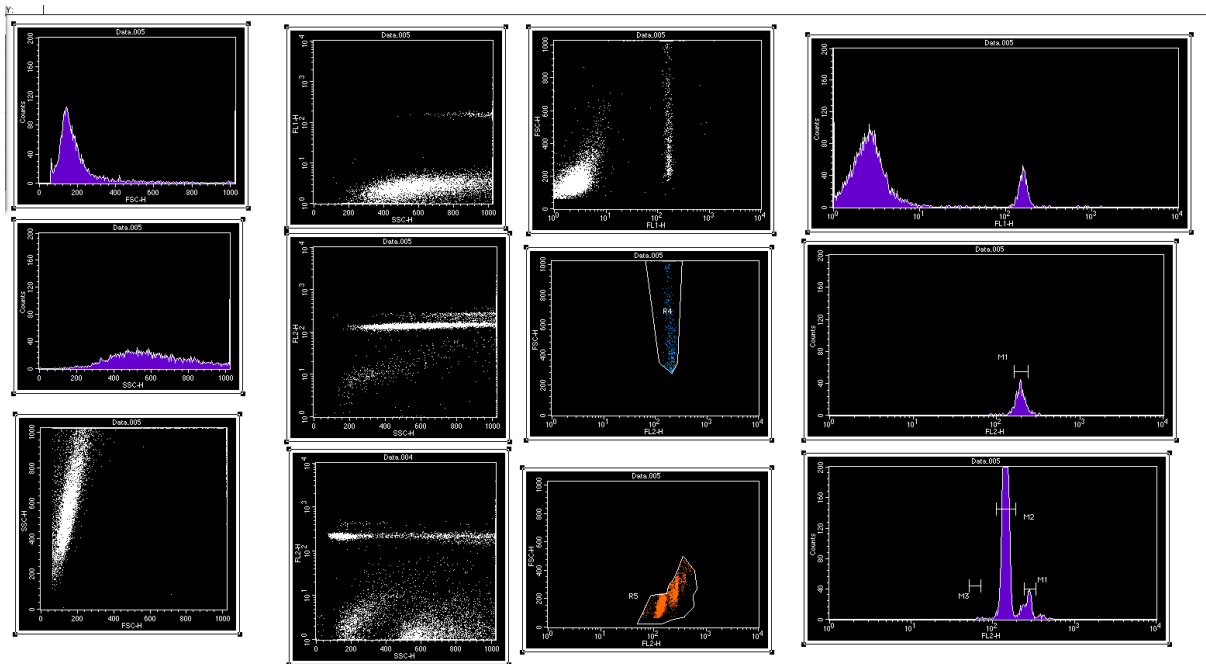
Supplementary Figure 5: A picture overview over all the plots acquired using Flow Cytometry for Calyptrosphaera sp (UIO 309)

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Phaeocystis globosa (K-1321):
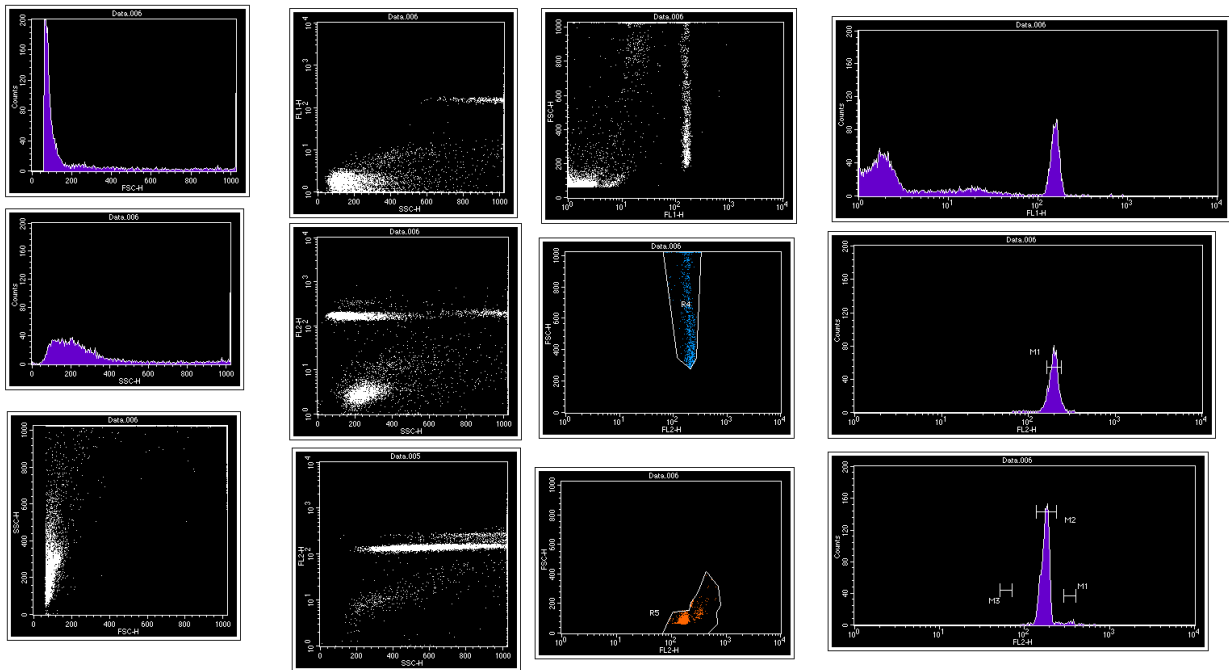
Supplementary Figure 6: A picture overview over all the plots acquired using Flow Cytometry for Phaeocystis globosa (K-1321)

Pavlova Gyrans (K-1310):
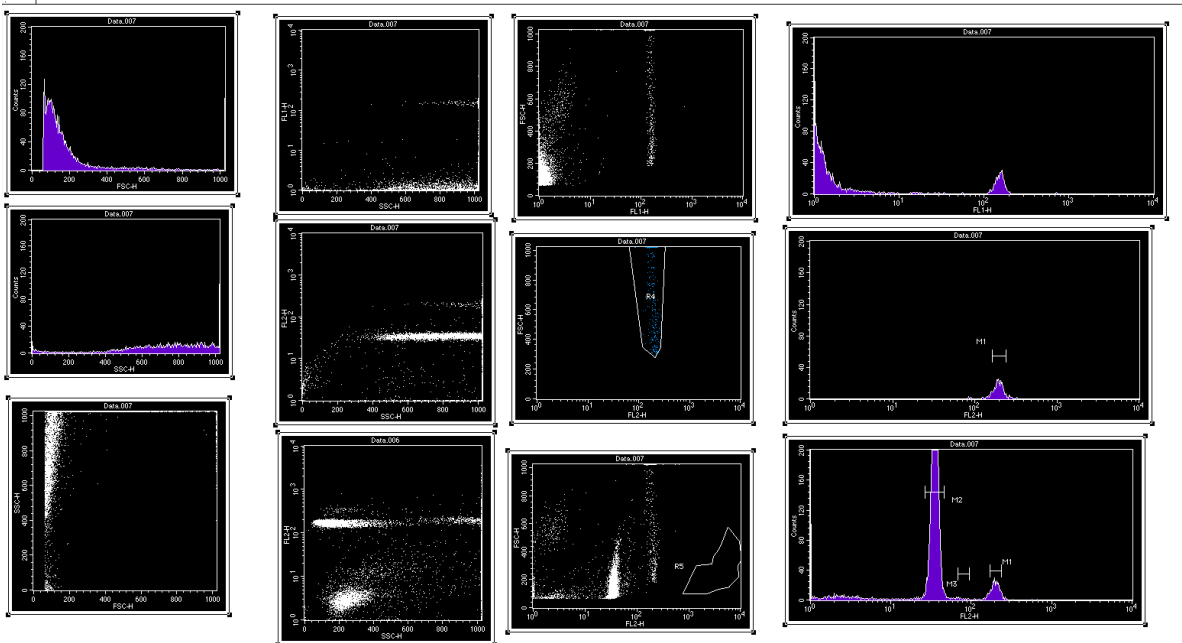
Supplementary Figure 7: A picture overview over all the plots acquired using Flow Cytometry for Pavlova Gyrans (K-1310).

Chrysotila Carterae (UIO 095):
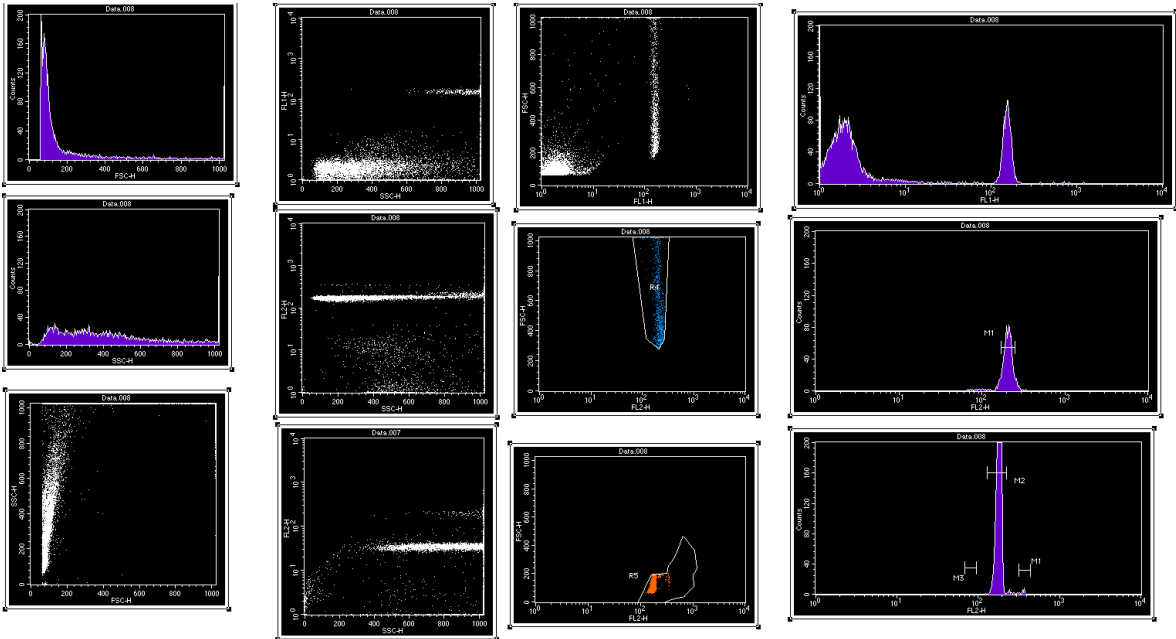
Supplementary Figure 8: A picture overview over all the plots acquired using Flow Cytometry for Chrysotila Carterae (UIO 095).

Prymnesium Polylepis (UIO 041):
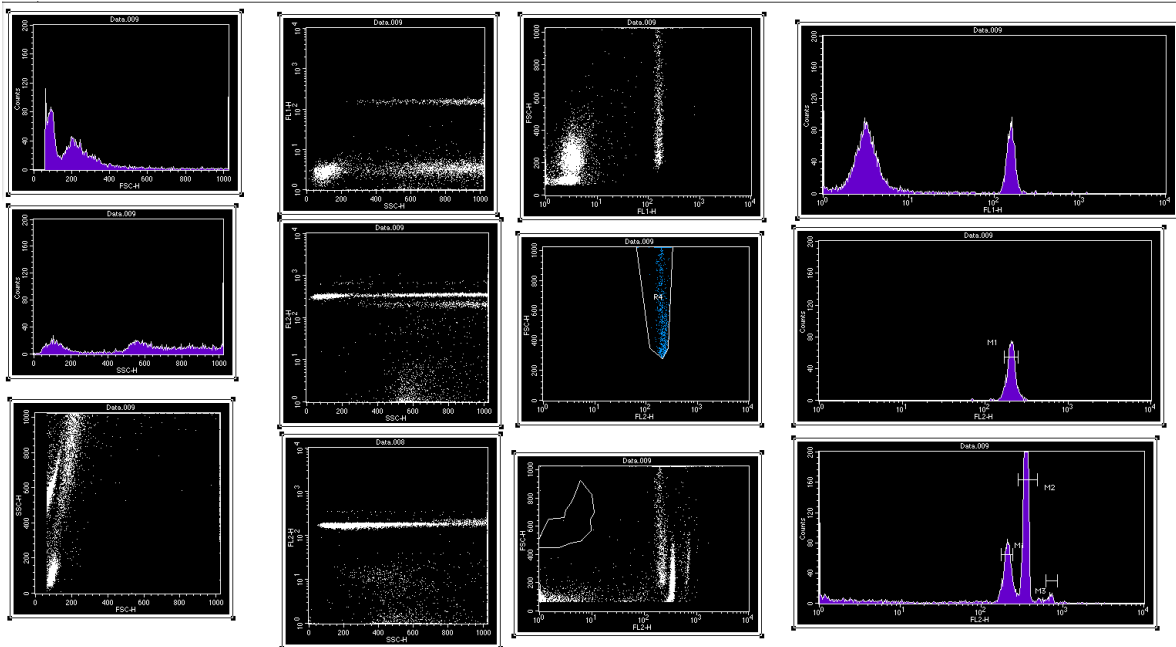
Supplementary Figure 9: A picture overview over all the plots acquired using Flow Cytometry for Prymnesium Polylepis (UIO 041).
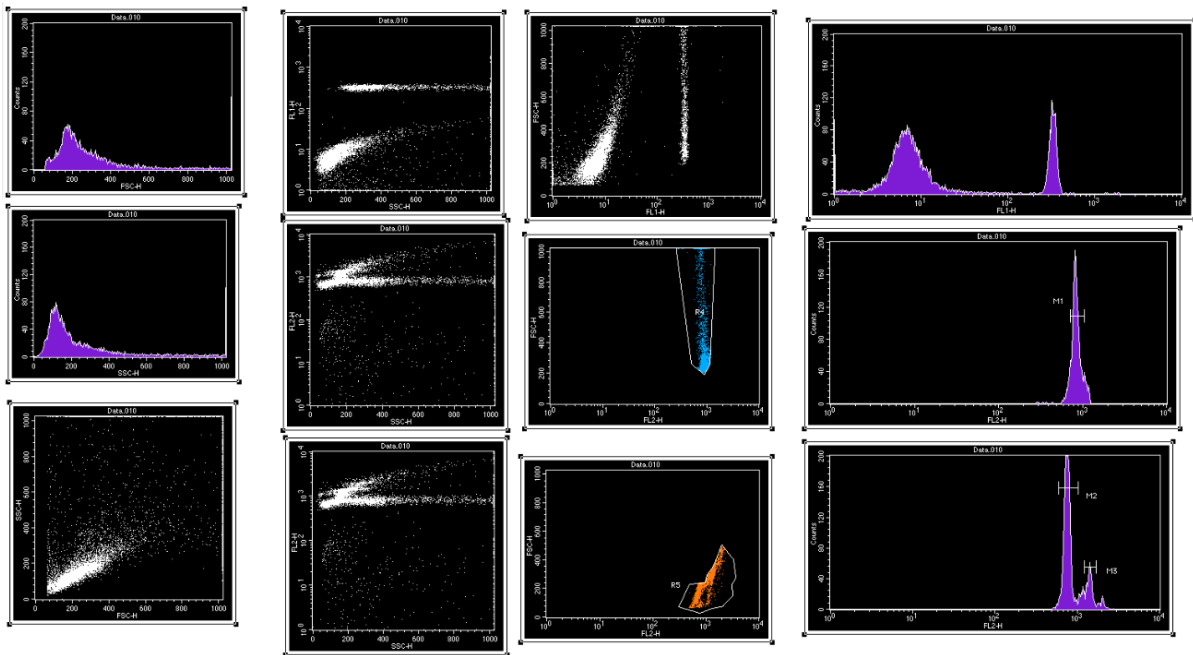
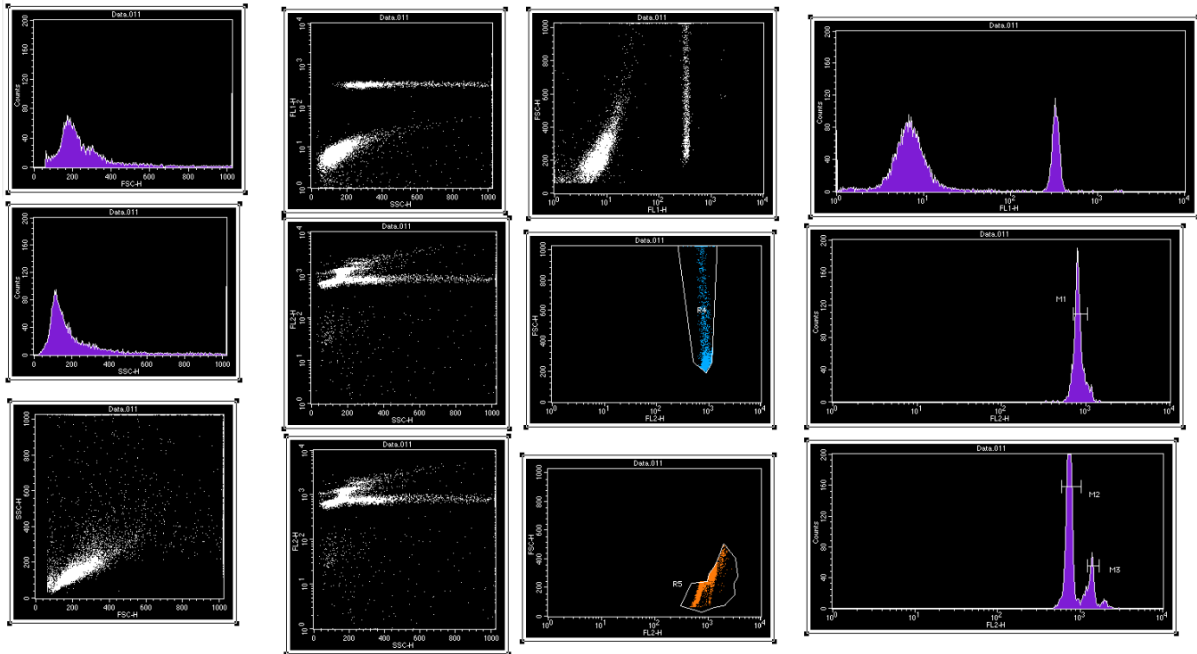Additional figures from result section 3.2.1.:

309.11.1:
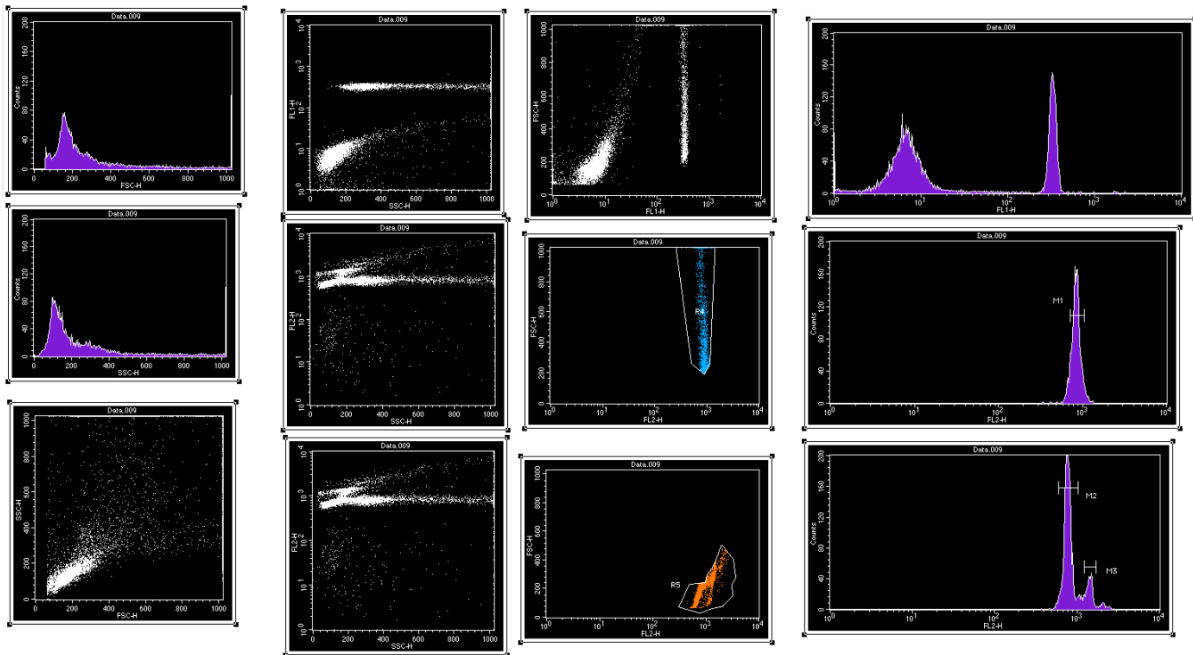
Supplementary Figure 10: A picture overview over all the plots acquired using Flow Cytometry for Calyptrosphaera sp (UIO 309), 11ºC, replica 1.
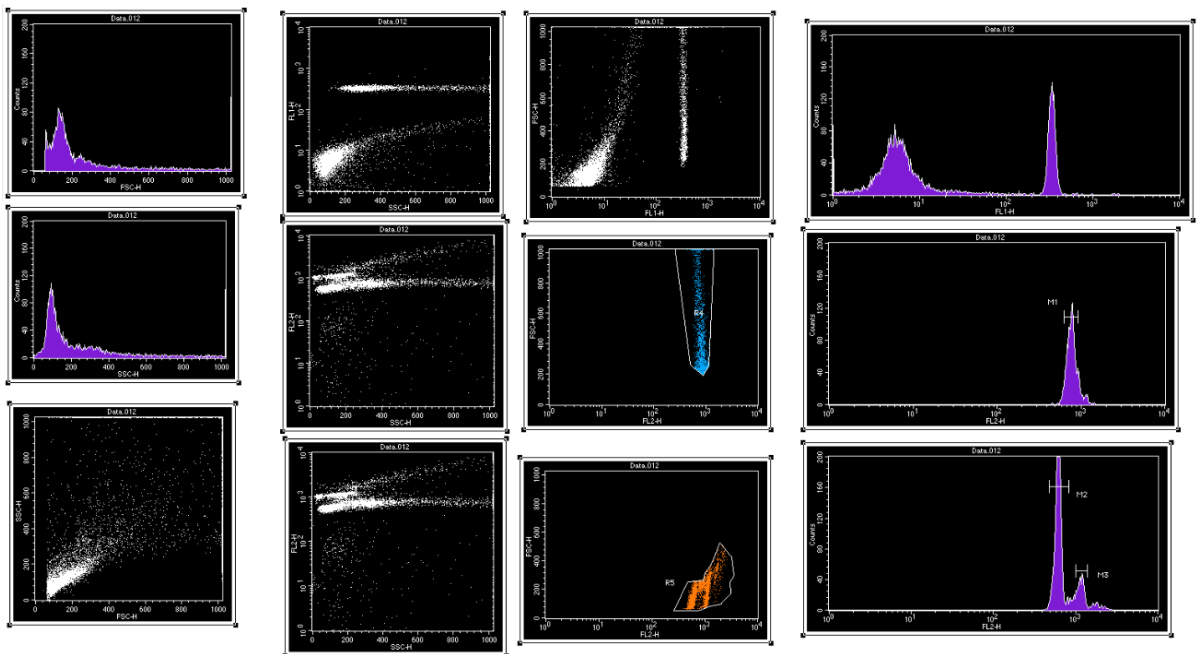
309.11.2:

Supplementary Figure 11: A picture overview over all the plots acquired using Flow Cytometry for Calyptrosphaera sp (UIO 309), 11ºC, replica 2.
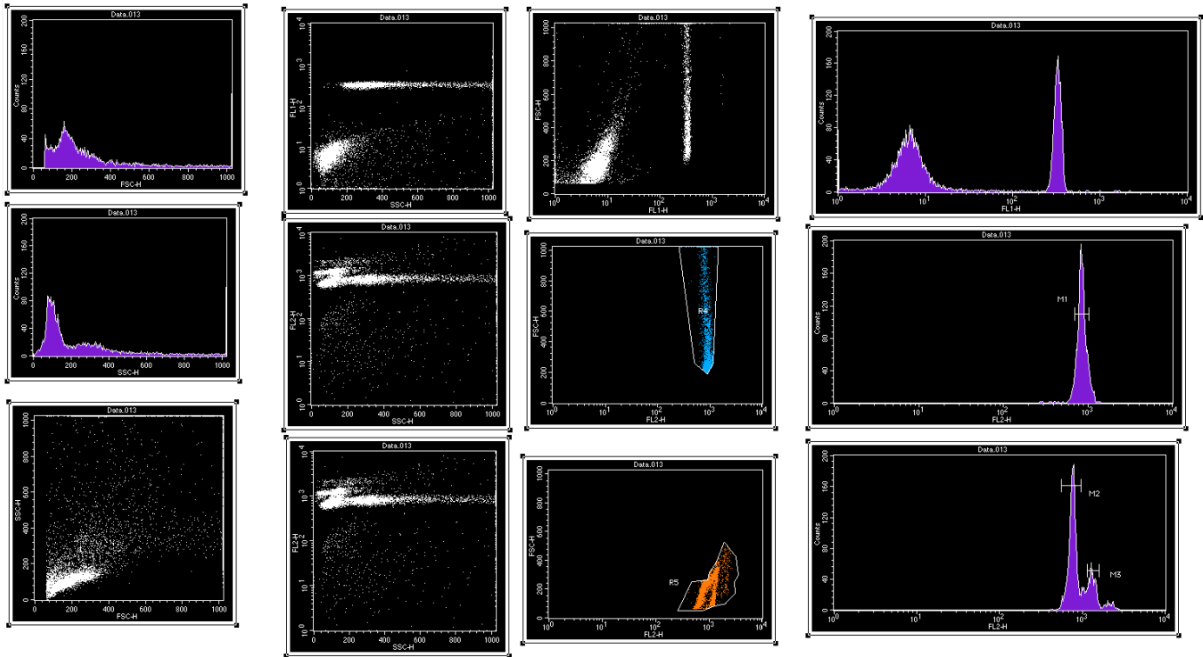
309.11.3:

Supplementary Figure 12: A picture overview over all the plots acquired using Flow Cytometry for Calyptrosphaera sp (UIO 309), 11ºC, replica 3.

309.19.1:

Supplementary Figure 13: A picture overview over all the plots acquired using Flow Cytometry for Calyptrosphaera sp (UIO 309), 19ºC, replica 1.

309.19.2:

Supplementary Figure 14: A picture overview over all the plots acquired using Flow Cytometry for Calyptrosphaera sp (UIO 309), 19ºC, replica 2.
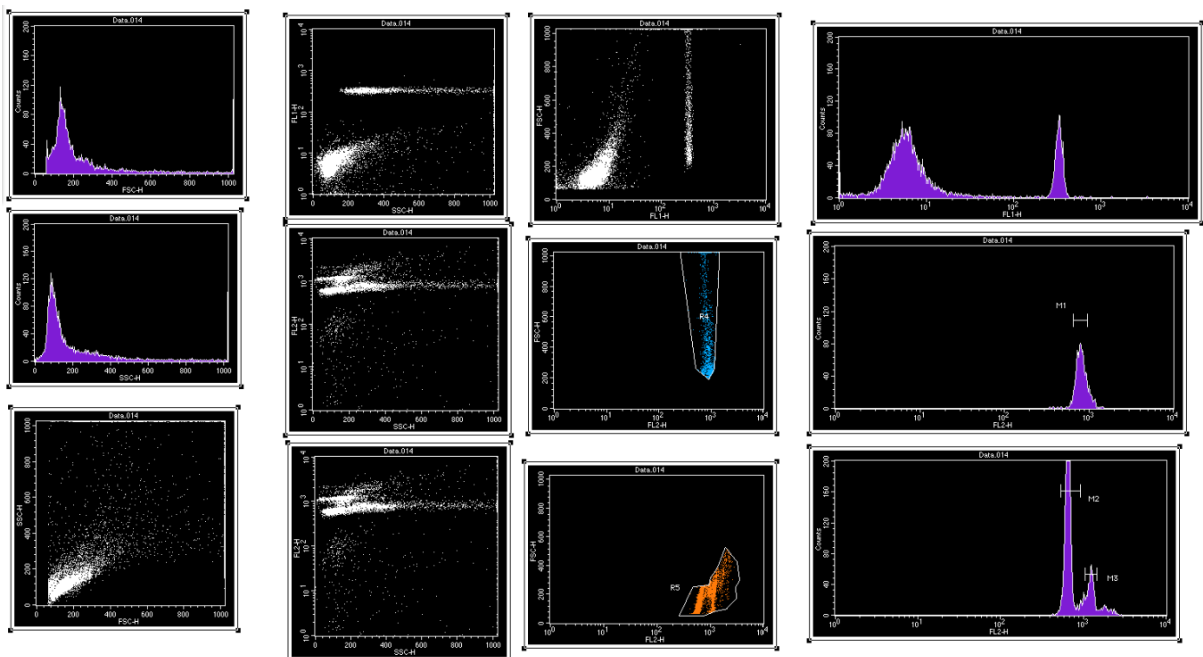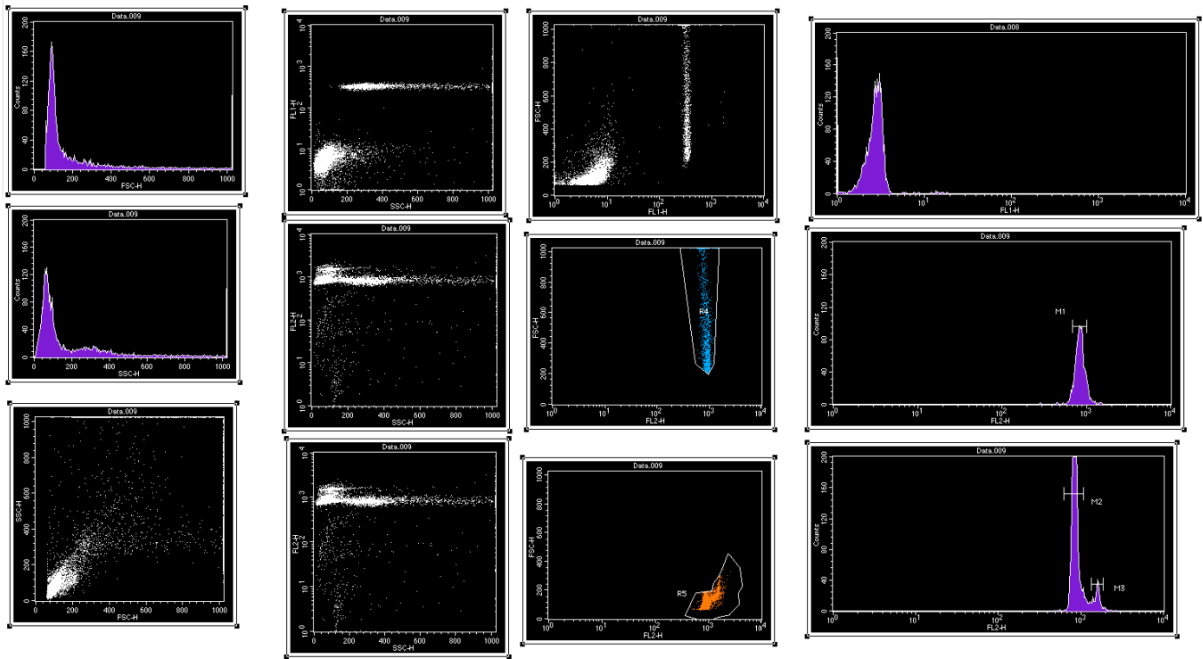
309.19.3:

Supplementary Figure 15: A picture overview over all the plots acquired using Flow Cytometry for Calyptrosphaera sp (UIO 309), 19ºC, replica 3.

032.11.1:

Supplementary Figure 16: A picture overview over all the plots acquired using Flow Cytometry for Prymnesium Kappa (UIO 032), 11ºC, replica 1.

032.11.2:

Supplementary Figure 17: A picture overview over all the plots acquired using Flow Cytometry for Prymnesium Kappa (UIO 032), 11ºC, replica 2.

032.11.3:

Supplementary Figure 18: A picture overview over all the plots acquired using Flow Cytometry for Prymnesium Kappa (UIO 032), 11ºC, replica 3.
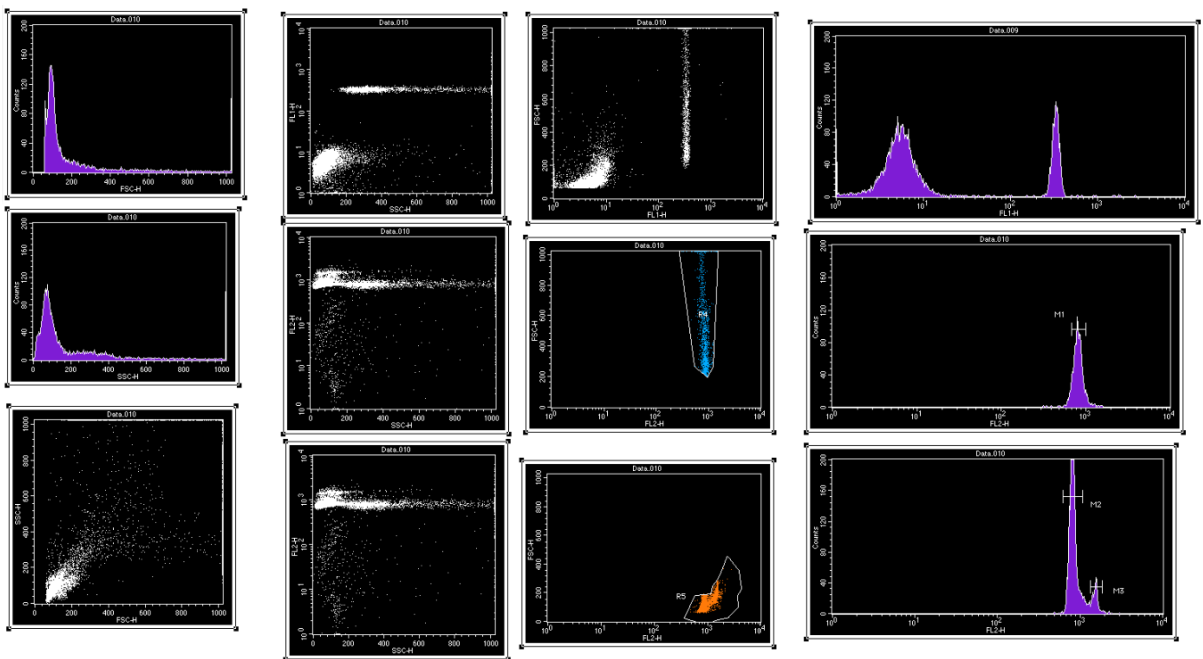
032.19.1:

Supplementary Figure 19: A picture overview over all the plots acquired using Flow Cytometry for Prymnesium Kappa (UIO 032), 19ºC, replica 1.
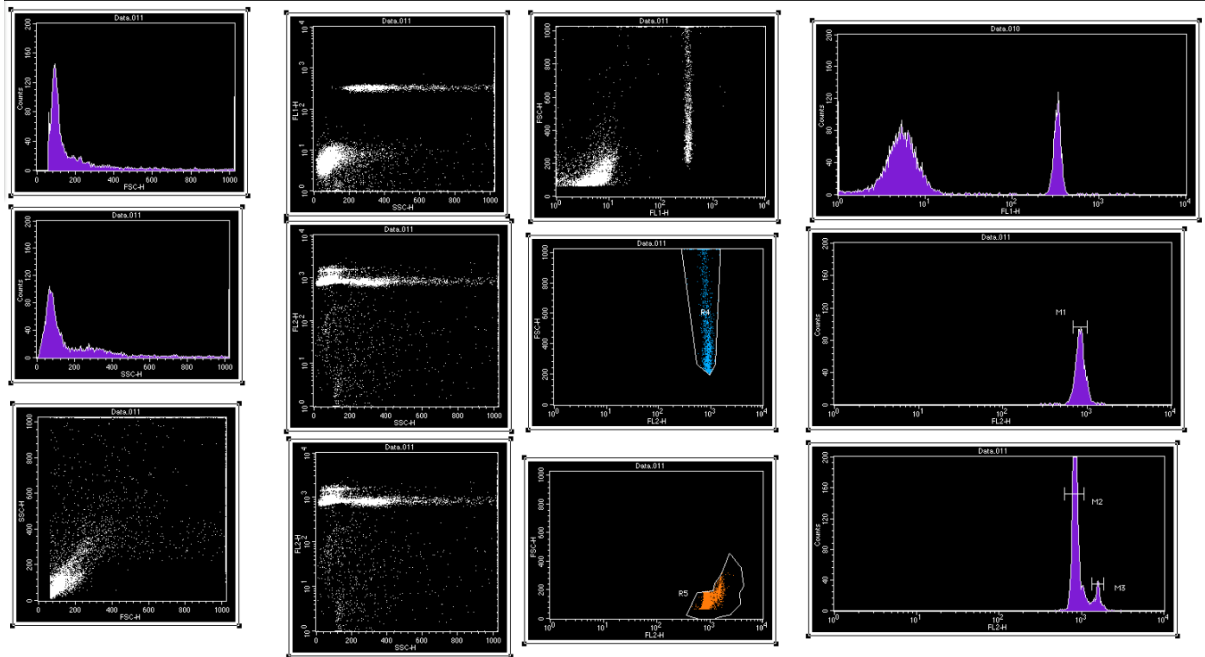
032.19.2:

Supplementary Figure 20: A picture overview over all the plots acquired using Flow Cytometry for Prymnesium Kappa (UIO 032), 19ºC, replica 2.

032.19.3:

Supplementary Figure 21: A picture overview over all the plots acquired using Flow Cytometry for Prymnesium Kappa (UIO 032), 19ºC, replica 3.

Second Prymnesium Kappa FCM results (also from section 3.2.2.):

032.11.1:

Supplementary Figure 22: A picture overview over all the plots acquired using Flow Cytometry for Prymnesium Kappa (UIO 032), 11ºC, replica 1.
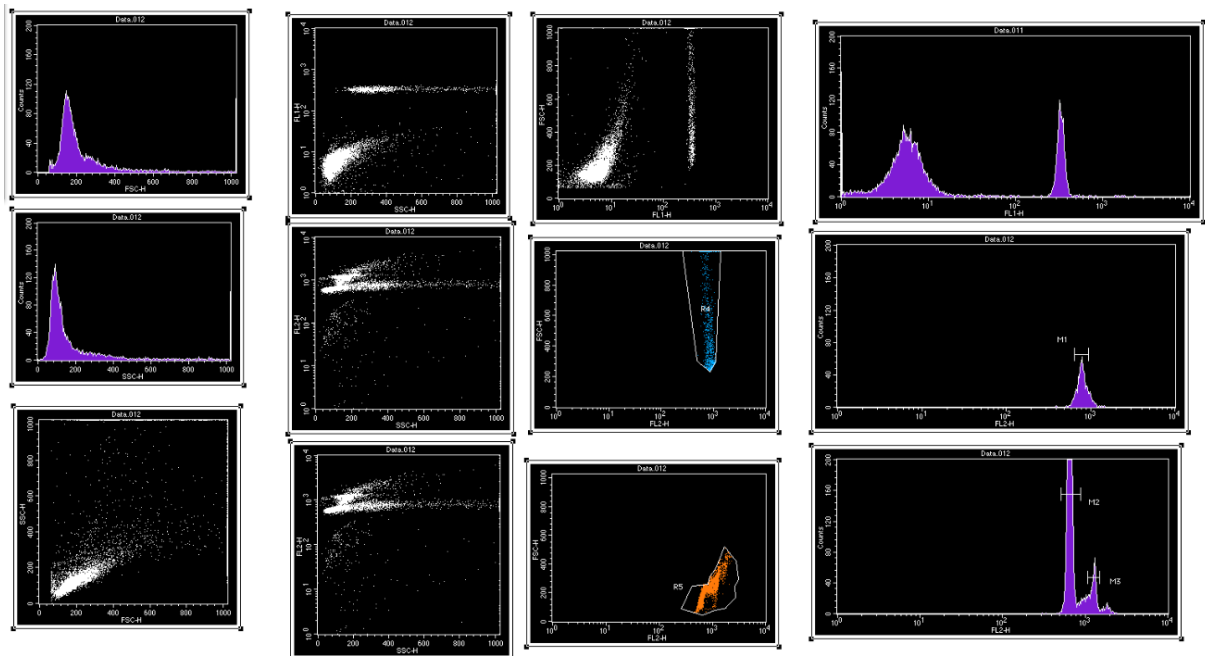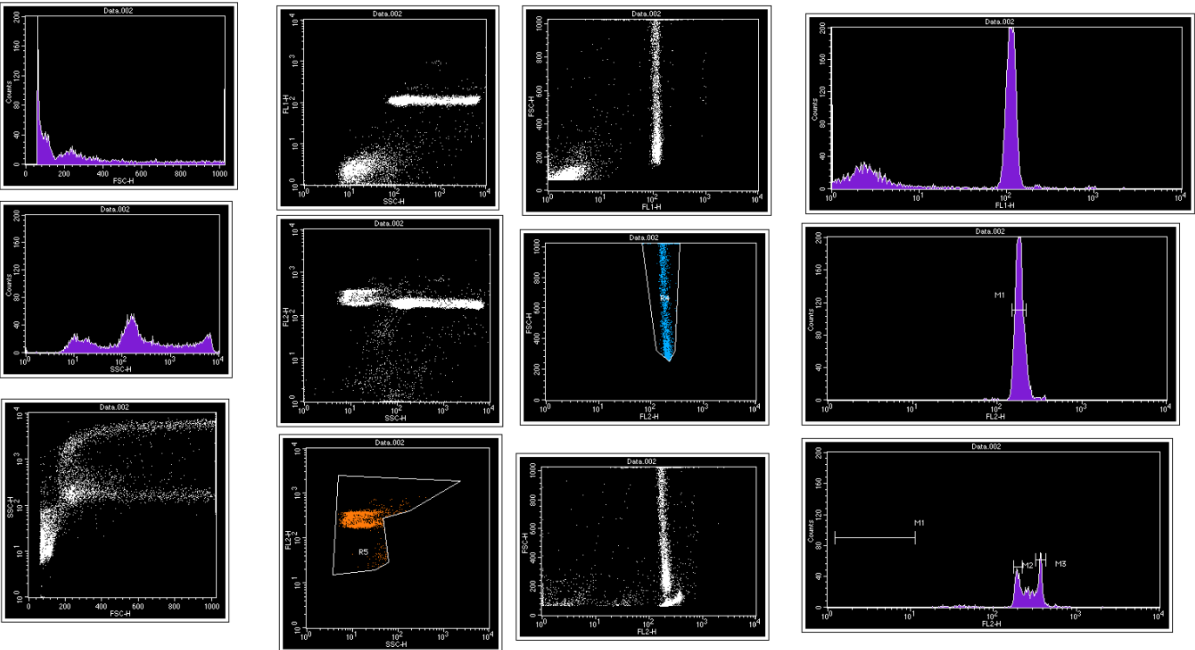
032.11.2:

Supplementary Figure 23: A picture overview over all the plots acquired using Flow Cytometry for Prymnesium Kappa (UIO 032), 11ºC, replica 2.
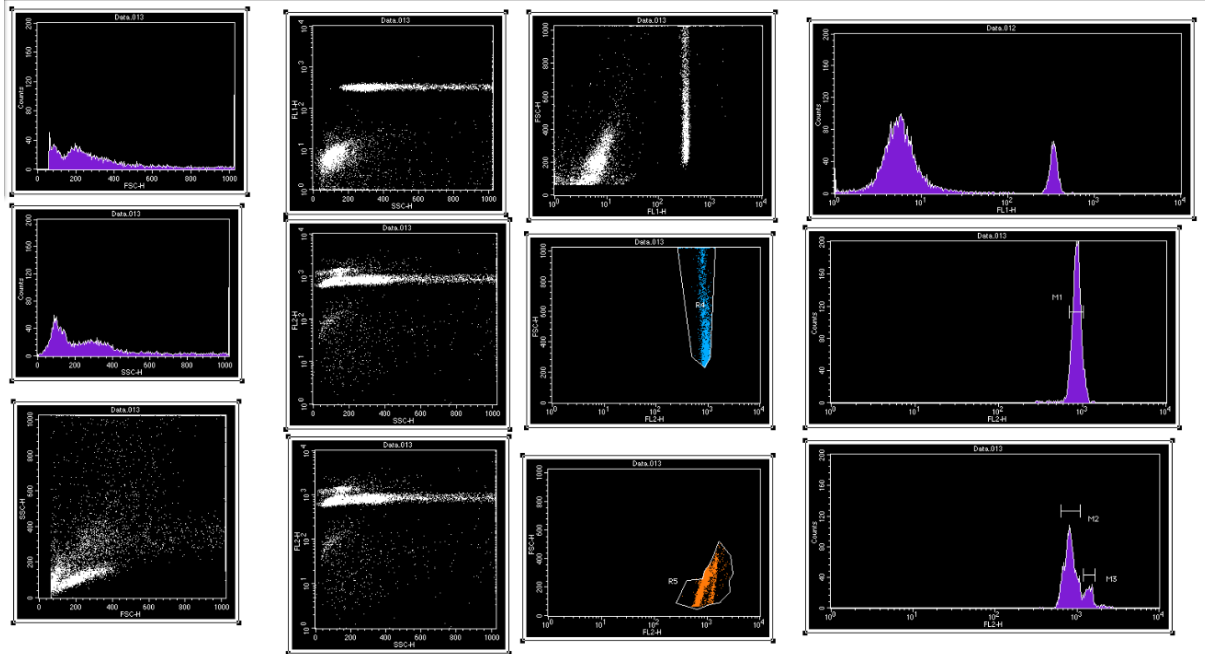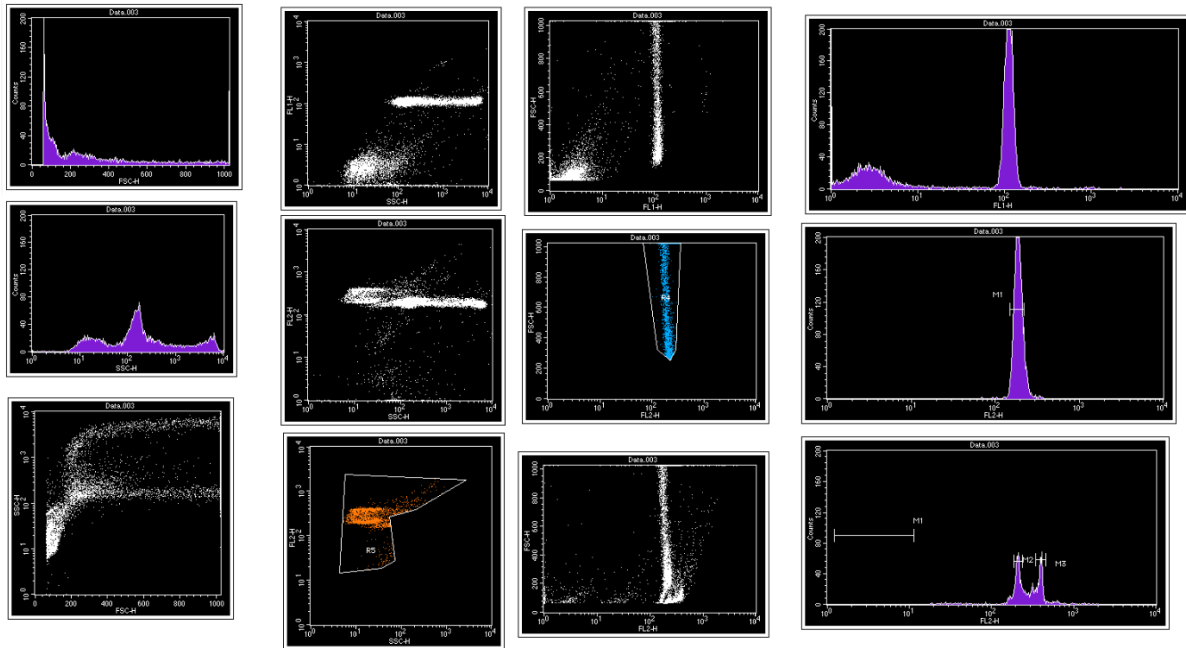
032.11.3:

Supplementary Figure 24: A picture overview over all the plots acquired using Flow Cytometry for Prymnesium Kappa (UIO 032), 11ºC, replica 3.

032.19.1:

Supplementary Figure 25: A picture overview over all the plots acquired using Flow Cytometry for Prymnesium Kappa (UIO 032), 19ºC, replica 1.
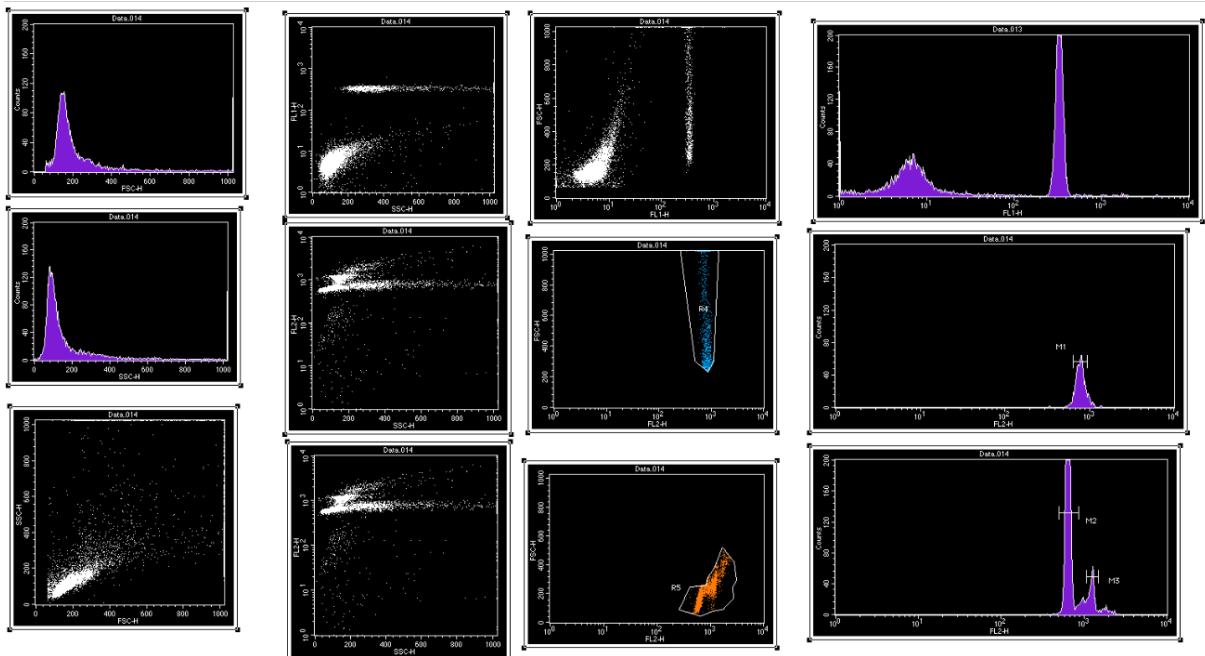
130

032.19.2:

Supplementary Figure 26: A picture overview over all the plots acquired using Flow Cytometry for Prymnesium Kappa (UIO 032), 19ºC, replica 2.
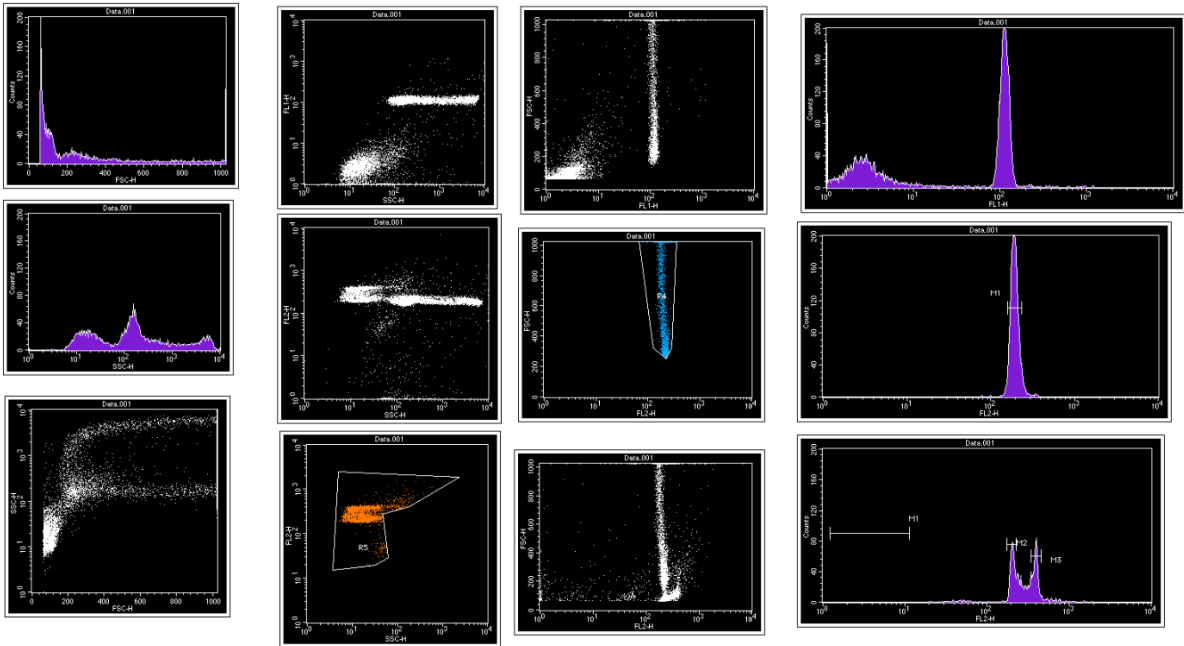
032.19.3:

Supplementary Figure 27: A picture overview over all the plots acquired using Flow Cytometry for Prymnesium Kappa (UIO 032), 19ºC, replica 3.
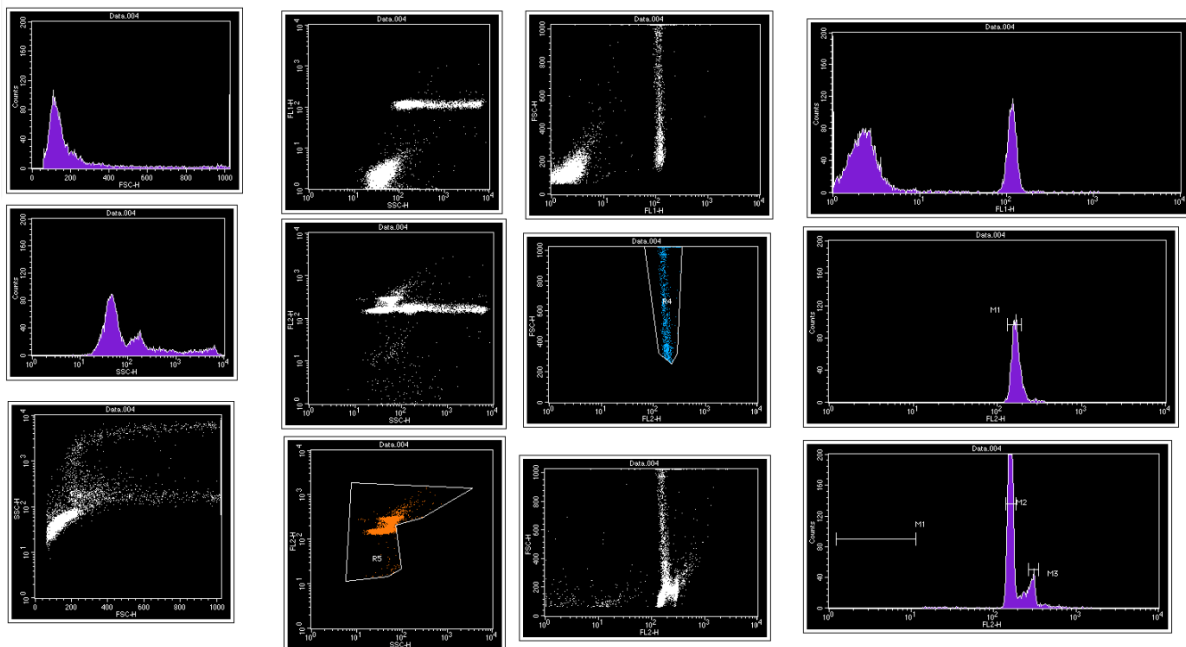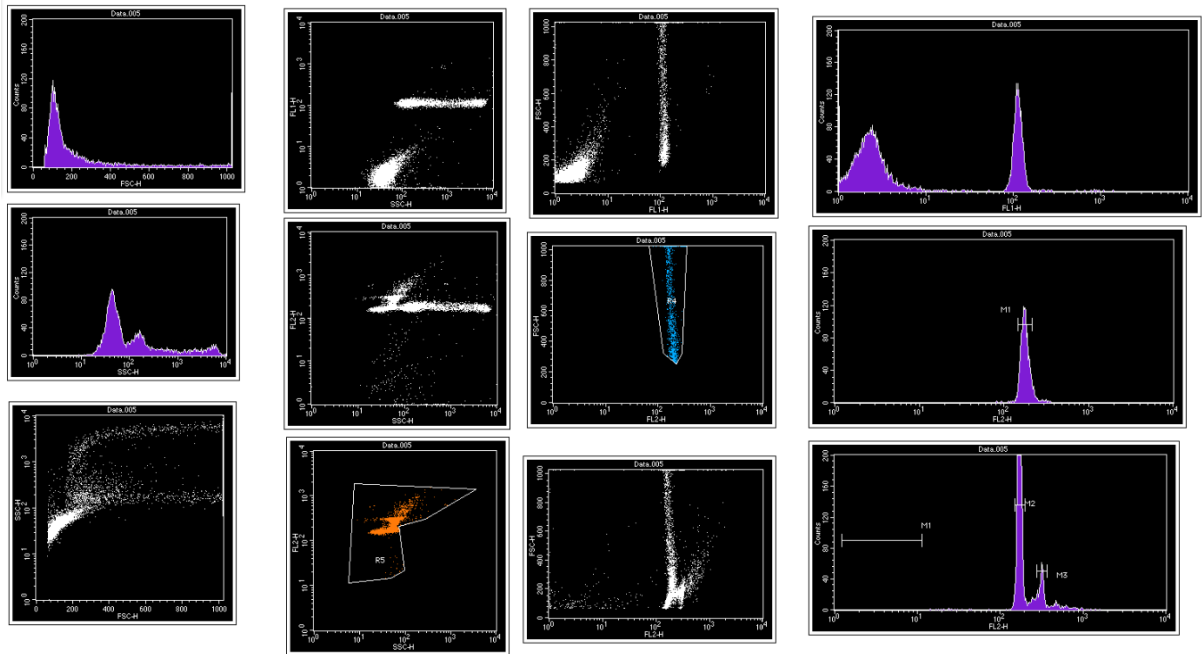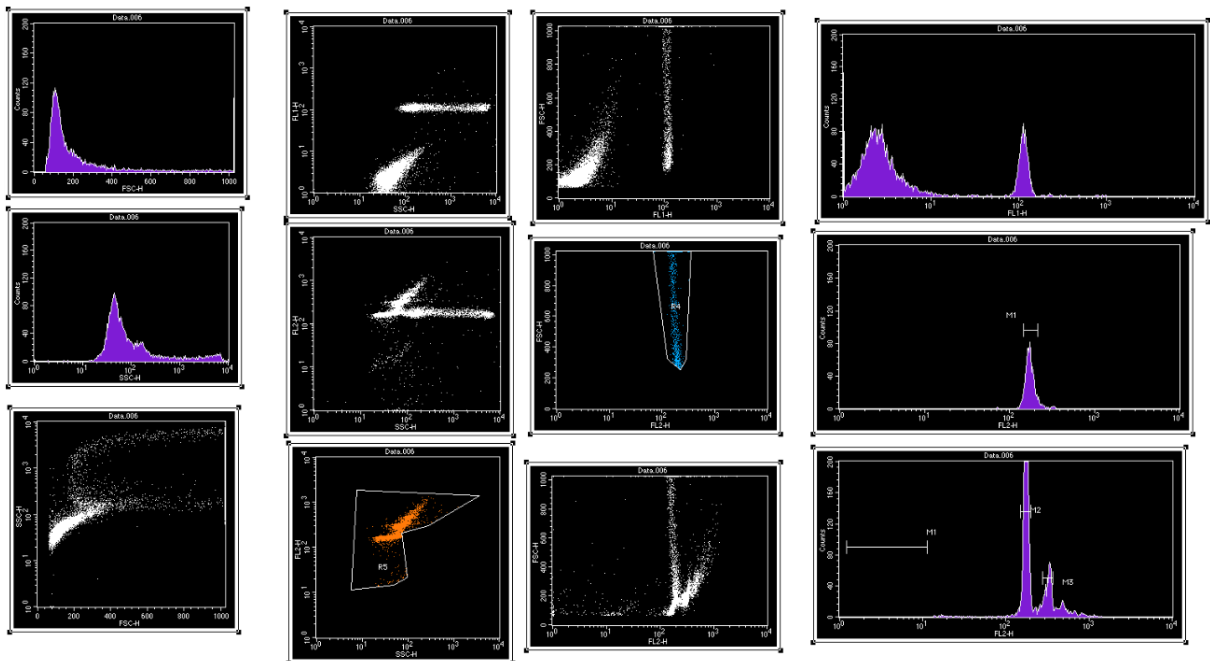
Supplementary Table 11: Alternative genome sizes for Prymnesium Kappa and Calyptrosphaera sp. Commas (,) asre used instead of full stop, dot (.), as Norwegian Excel was used.

Genome size for Prymnesium Kappa in pg				Genome size for Calyptrosphaera in pg			
Low log	peak	mean	median	Low log	Peak	mean	median
632.11.1	2,50	2,51	2,52	309.11.1	2,22	2,30	2,31
632.11.2	2,50	2,43	2,45	309.11.2	1,94	2,28	2,29
632.11.3	2,50	2,41	2,43	309.11.3	1,94	2,19	2,19
Average	2,50	2,45	2,47	Average	2,04	2,26	2,26
632.19.1	1,88	1,98	2,00	309.19.1	1,94	1,99	2,00
632.19.2	2,19	2,22	2,24	309.11.2	1,94	2,18	2,19
632.19.3	2,19	2,00	2,01	309.19.3	1,67	1,90	2,00
Average	2,08	2,07	2,08	Average	1,85	2,03	2,06
High log	peak	mean	median	High Log	Peak	Mean	median
632.11.1	2,41	2,43	2,52	309.11.1	2,24	2,22	2,22
632.11.2	2,33	2,34	2,35	309.11.2	2,20	2,19	2,18
632.11.3	2,31	2,34	2,35	309.11.3	2,18	2,12	2,13
Average	2,35	2,37	2,40	Average	2,21	2,18	2,18
632.19.1	1,84	1,90	1,91	309.19.1	1,91	1,92	1,94
632.19.2	2,16	2,15	2,16	309.11.2	2,16	2,13	2,13
632.19.3	1,84	1,90	1,89	309.19.3	1,82	1,83	1,84
Average	1,95	1,99	1,99	Average	1,96	1,96	1,97
Lin	peak	mean	median	Lin	Peak	Mean	Median
632.11.1	2,54	2,50	2,49	309.11.1	2,25	2,25	2,24
632.11.2	2,50	2,44	2,43	309.11.2	2,23	2,23	2,23
632.11.3	2,50	2,45	2,43	309.11.3	2,11	2,14	2,14
Average	2,51	2,46	2,45	Average	2,20	2,21	2,21
632.19.1	1,97	1,92	1,91	309.19.1	1,96	1,96	1,96
632.19.2	2,31	2,20	2,21	309.11.2	2,19	2,16	2,17
632.19.3	1,99	1,95	1,94	309.19.3	1,85	1,85	1,85
Average	2,09	2,02	2,02	Average	2,00	1,99	1,99

Additional result tables from section 3.4.1.:

Supplementary Table 12: Alternative results for [RNA] analysis. Table of number of cells ml^{-1} in the first 4 columns and [RNA] (μg mL-1) in the last four columns. Columns from left to right: 032.11,032.19, 309.11 and 309.19. The bottom numbers (uncoloured background) are the average of the different replicates for the different algae species and temperatures

Number of cells ml^{-1}				[RNA] (μg mL^{-1})			
1690000	3597600	2535800	3308350	1.16	1.00	1.20	1.18
1690000	3597600	2535800	3308350	1.02	0.99	1.09	0.95
1638000	2454600	2491300	1632450	1.10	0.89	0.61	0.82
1638000	2454600	2491300	1632450	1.02	0.49	0.88	0.86
1540350	3168700	2544200	3119400	1.10	1.00	1.30	1.12
1540350	3168700	2544200	3119400	1.03	1.27	1.28	1.13
1622783	3073633	2523767	2686733	1.07	0.94	1.06	1.01

Supplementary Table 13: Alternative results for [RNA] pr. cell. : [RNA] (μg mL^{-1}) pr. cells ml^{-1} for the 12 (plus their duplicates) different algae replicates. From left to right: 032.11, 032.19, 309.11, 309.19. Averages in bottom with non-coloured background.

[RNA] (μg mL^{-1}) pr. cells ml^{-1}			
2.59E-07	2.79E-07	4.75E-07	3.57E-07
6.04E-07	2.76E-07	4.29E-07	2.87E-07
6.74E-07	3.65E-07	2.46E-07	5.04E-07
6.24E-07	2E-07	3.54E-07	5.25E-07
7.16E-07	3.14E-07	5.12E-07	3.59E-07
6.68E-07	4.02E-07	5.04E-07	3.62E-07
6.61E-07	3.06E-07	4.20E-07	3.99E-07

Supplementary table 14: RNA (in μg) per cell for the 12 (plus their duplicates) different algal cultures. Columns from left to right: P. kappa 11ºC and 19ºC, Calyptrosphaera sp 11ºC and 19ºC (032.11, 032.19, 309.11 and 309.19 respectively). Averages in bottom with non-coloured background. Each replicate is duplicated, with the duplicate number in parenthesis, i.e. replicate 1(1) means duplicate 1 of replicate 1.

	RNA (in μg) pr. cell			
	032.11	032.19	309.11	309.19
Replicate 1(1)	4.56E-07	1.82E-07	3.02E-07	2.59E-07
Replicate 1(2)	4.03E-07	1.81E-07	2.72E-07	2.08E-07
Replicate 2(1)	5.93E-07	2.74E-07	1.42E-07	2.44E-07
Replicate 2(2)	5.49E-07	1.50E-07	2.05E-07	2.54E-07
Replicate 3(1)	5.58E-07	1.72E-07	3.53E-07	1.99E-07
Replicate 3(2)	5.20E-07	2.20E-07	3.47E-07	2.01E-07
Average	5.13E-07	1.97E-07	2.70E-07	2.27E-07

Additional results from section 3.4.2:

Supplementary table 15: Alternative; Table [Protein] (µg ml⁻¹) in the first 4 columns and of number of cells ml⁻¹ in the last four columns. Columns from left to right: 032.11,032.19, 309.11 and 309.19. The bottom numbers (uncoloured background) are the average of the different replicates for the different algae species and temperatures.

	[Protein] (µg mL⁻¹)				Number of cells ml⁻¹			
	032.11	032.19	309.11	309.19	032.11	032.19	309.11	309.19
replicate 1	15.75	14.62	15.82	10.83	1690000	3597600	2535800	3308350
replicate 1	20.39	12.53	11.54	12.98	1690000	3597600	2535800	3308350
replicate 2	21.97	14.23	15.28	7.17	1638000	2454600	2491300	1632450
replicate 2	12.36	13.44	17.34	8.53	1638000	2454600	2491300	1632450
replicate 3	16.64	13.24	13.23	11.04	1540350	3168700	2544200	3119400
replicate 3	13.64	16.38	16.05	10.21	1540350	3168700	2544200	3119400
Average	16.79	14.08	14.88	10.13	1622783	3073633	2523767	2686733

Supplementary table 16: Alternative; [Protein] (µg mL⁻¹) pr. cells ml⁻¹ (or just [Protein] pr cell) for the 12 (plus their duplicates) different algae replicates. From left to right: 032.11, 032.19, 309.11, 309.19. Averages in bottom with non-coloured background.

	Protein pr cell			
	032.11	032.19	309.11	309.19
replicate 1	9.3173E-06	4.0641E-06	6.239E-06	3.2739E-06
replicate 1	1.2067E-05	3.4837E-06	4.5495E-06	3.9222E-06
replicate 2	1.3411E-05	5.7985E-06	6.1318E-06	4.3946E-06
replicate 2	7.5468E-06	5.4748E-06	6.9604E-06	5.2262E-06
replicate 3	1.0806E-05	4.1796E-06	5.2E-06	3.5385E-06
replicate 3	8.854E-06	5.1707E-06	6.3084E-06	3.2737E-06
Average	1.0334E-05	4.6952E-06	5.8982E-06	3.9382E-06

Supplementary table 17:[Protein] (µg mL⁻¹) per cells ml⁻¹ (or just Protein per cell) for the 12 (plus their duplicates) different algae cultures. Columns from left to right: P. kappa 11ºC and 19ºC, Calyptrosphaera sp 11ºC and 19ºC (032.11, 032.19, 309.11 and 309.19 respectively). Averages in bottom with non-coloured background. Each replica is duplicated, with the duplicate number in parenthesis, i.e. replicate 1(1) means duplicate 1 of replicate 1.

	Protein in µg pr cell			
	032.11	032.19	309.11	309.19
Replicate 1(1)	6.21E-06	2.66E-06	3.96E-06	2.37E-06
Replicate 1(2)	8.05E-06	2.28E-06	2.89E-06	2.84E-06
Replicate 2(1)	1.18E-05	4.36E-06	3.55E-06	2.13E-06
Replicate 2(2)	6.64E-06	4.11E-06	4.03E-06	2.53E-06
Replicate 3(1)	8.42E-06	2.29E-06	3.58E-06	1.96E-06
Replicate 3(2)	6.90E-06	2.83E-06	4.35E-06	1.81E-06
Average	8.01E-06	3.09E-06	3.73E-06	2.28E-06

Supplementary table 18: A T-Test summarises the acquired results and differences between the two temperature treatments, 11ºC and 19ºC. Colour coded, where rede means lower values and green are higher calculated t-values.

	T-test summary					
Specie	Analysis	Calculated t-value	T-table value	Alpha level	Degree of freedom (DF)	
P. kappa	Cell size CASY PDI	0.461	4.303	0.05	2	
Calyptrosphaera sp	Cell size CASY PDI	3.149	4.303	0.05	2	
P. kappa	Cell size Casy MDI	0.615	4.303	0.05	2	
Calyptrosphaera sp	Cell size Casy MDI	4.863	4.303	0.05	2	
P. kappa	Cell size microscope	14.571	4.303	0.05	2	
Calyptrosphaera sp	Cell size microscope	3.124	4.303	0.05	2	
P. kappa	Genome size FCM low log	1.385	4.303	0.05	2	
Calyptrosphaera sp	Genome size FCM low log	2.130	4.303	0.05	2	
P. kappa	Genome size FCM high log	2.409	4.303	0.05	2	
Calyptrosphaera sp	Genome size FCM high log	2.321	4.303	0.05	2	
P. kappa	Genome size FCM LIN	5.265	4.303	0.05	2	
Calyptrosphaera sp	Genome size FCM LIN	2.233	4.303	0.05	2	
P. kappa	RNA	11.606	2.571	0.05	5	
Calyptrosphaera sp	RNA with outlier	1.016	2.571	0.05	5	
Calyptrosphaera sp	RNA without outlier	2.429	2.571	0.05	5	
P. kappa	Protein	6.559	2.571	0.05	5	
Calyptrosphaera sp	Protein	4.431	2.571	0.05	5	
P. kappa	K-mer	4.789	4.303	0.05	2	
Calyptrosphaera sp	K-mer	0.183	4.303	0.05	2	
P. kappa	C:N ratio	2.635	2.571	0.05	5	
Calyptrosphaera sp	C:N ratio	2.385	2.571	0.05	5	
P. kappa	C:P ratio	3.777	2.571	0.05	5	
Calyptrosphaera sp	C:P ratio	1.185	2.571	0.05	5	
P. kappa	N:P ratio	2.024	2.571	0.05	5	
Calyptrosphaera sp	N:P ratio	1.038	2.571	0.05	5	

T-table values from "Statistic How To"

(https://www.statisticshowto.datasciencecentral.com/tables/t-distribution-table/#two)

28.09.2018