Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biology

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Effect of environmental factors on growth and development of *Phaeodactylum tricornutum*

Master's thesis in Cell and Molecular Biology
Supervisor: Atle M. Bones
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Abstract

Diatoms are eukaryotic photosynthetic organisms found in marine and freshwater environments as primary producers accounting for ~20% of total world photosynthetic output and ~50% of oceanic biomass. Diatoms are of industrial interest as they are lipid accumulating, which could be used in the production of animal feed or biofuels without occupying agricultural space. An important model diatom is *P. tricornutum* which is a resilient, fast-growing organism. Our knowledge of diatoms is not proportional to their importance and lags behind that of plants and green algae. It is important to better understand them so that we might predict how they will adapt to a changing world climate.

Through this thesis we have looked at how three environmental factors, salinity, pH and presence of herbicides (glyphosate derived from roundup) impact the growth of *P. tricornutum*. Salinity treated cells were RNA sequenced, underwent lipid extraction with fatty acid analysis. Our findings indicate that high salinity led to a decrease in growth rate, in contrast to low salinity conditions which led to an increase in growth rate. Analysis of fatty acid composition showed that the low salinity treatment had a higher percentage of polyunsaturated fatty acids (PUFAs) with high salinity treatment showing the opposite response. RNA sequencing showed many genes to be differentially expressed, several of which were associated with sodium and chloride. However, a large portion of the differentially expressed genes coded for proteins of unknown function.

The pH treatments showed a decrease in *P. tricornutum* growth as the pH decreased from normal (pH 8.1), with growth ceasing entirely at pH 5.1. Cell death staining using propidium iodide was performed on the glyphosate treatments. From the glyphosate treatments we saw rapid cell death staining at the highest concentrations (0.8 and 0.4 g/L) while the lower concentration treatments showed a slowly increasing cell death stain percentage, reaching ~20% following 96 hours.

In conclusion, our results show that alterations in environmental conditions can dramatically impact the growth of *P. tricornutum* depending on the degree of environmental change. Additionally, we saw several opportunities to further develop the knowledge of *P. tricornutum* and diatoms in general.

Preface

The Master Thesis has been carried out in connection with the completion of a Master of Science in Biology at the institute of biology (IBI) at the Norwegian University of Science and Technology (NTNU). The theme of the thesis was given by our supervisor Atle M. Bones and the laboratory work was done at the laboratories within realfagbygget and NTNU SeaLab. We would like to thank the various staff and faculty members who facilitated our master, as well as the professors from our various courses.

We would like to extend our gratitude towards our supervisor professor Atle M. Bones for guiding us onto the right path and helping us during the writing process. You have been an incredible supervisor and a huge help with your large amount of knowledge and experience, helping us from getting stuck when things have been going slow. Special thanks to professor Per Winge for helping us get through and understand the RNA sequencing and for letting us use your vast knowledge of the *P. tricornutum* genome. Thank you to senior engineer Ralf Kissen for helping teach us how to extract and quality control RNA and the variety of small lab things. Thank you to senior engineer Zdenka Bartsova for helping teach us lipid extraction and analysis and for helping with the GC-MS despite the busy schedule. Finally thank you to the various people in the CMBG group and the rest of NV faculty who have helped us with all the little things; Amit K. Sharma, Manuel Serif, Tore Brembu, Sigrid Lindmo and Grethe S. Eggen.

We would also give a special thanks to our fellow master's student Simen F. Pettersen for helping with laboratory work and all the others at the lab for being able to discuss and improve routines and methods as well as giving a good environment to work in.

Lastly we would like to thank our friends and family for being with us and helping us stay sane when not working on the thesis.

Trondheim, June 5th, 2023

Kristoffer E. Andreassen

Atle Indergård

1 Abbreviations

BAM file: binary alignment file

CA: carbonic anhydrase

CCM: carbon concentrating mechanism

cDNA: coding DNA

CT: cycle threshold

ddNTP: dideoxynucleotide

DHA: cervonic acid

DIC: dissolved inorganic carbon

dNTP: deoxynucleotide

dsDNA: double stranded DNA

EPA: Eicosapenthenoic acid

EPSP: 5-enolpyruvylshikimate-3-phosphate

EPSPs: 5-enolpyruvylshikimate-3-phosphate synthase

FAME: Fatty Acid Methyl Ester

FDR: false discovery rate

FSC-H: forward scatter height

FSW: filtered salt water

GC-MS: gas chromatography-mass spectrometry

logCPM: log counts per million

logFC: log fold change

MUFA: monounsaturated fatty acid

mRNA: messenger RNA

NGS: Next Generation Sequencing

NRT control: non reverse transcriptase control

PCR: Polymerase Chain Reaction

PEP: phosphoenolpyurvate

PI: propidium iodide

ppm: parts per million

ppt: parts per thousand

PSII: Photosystem II

PUFA: polyunsaturated fatty acid

P. tricornutum: *Phaeodactylum tricornutum*

QY: Quantum yield

RIN: RNA integrity number

rRNA: ribosomal RNA

RT-qPCR: Real time quantitative PCR

SAM file: sequence alignment file

SFA: saturated fatty acid

SNPs: single nucleotide polymorphisms

SSC-H: side scatter height

Contents

1 Introduction:

1.1 Diatoms and *Phaeodactylum tricornutum*

Diatoms are photosynthetic eukaryotes found in both marine and freshwater environments where they are found both as plankton (for example *Phaeodactylum tricornutum*) and in biofilms within benthic habitats (for example *Seminavis robusta*) (Osuna-Cruz et al., 2020). They are primary producers, accounting for around 20% of total world photosynthetic output (Osuna-Cruz et al., 2020; Field et al., 1998). This photosynthetic importance gives diatoms a high potential for use within biotechnology, for example in the production of fish feed (Nymark et al., 2021; Sharma et al., 2021). However, this potential is not matched by the knowledge we have of diatoms and their photosynthetic processes, which significantly lags behind our knowledge of plant and green algae systems (Nymark et al., 2021).

Currently the modern world is reliant upon fossil fuels for the majority of our energy, both for electricity and transportation. This reliance is one of the most major problems faced by our world today as not only are fossil fuels finite, they also contribute to CO_2 emissions and are the driving force behind climate change (Salih, 2011). Additionally recent geopolitical events have highlighted vulnerabilities of the fossil fuel supply chain and led to drastic increases in oil and gas prices, which have led to increases in power costs throughout Europe. With all of this in mind the need for a transition to renewable and carbon neutral energy sources is more important than ever and only grows more important by the day. One potential alternative power source is biofuels, fuel originating from organic material (typically high lipid plants) used in place of fossil fuels (Chisti, 2007; Pulz & Gross, 2004). While high lipid plants have typically been used for this purpose, this takes up valuable agricultural space. Thus an alternative source of lipids for biofuel production is microalgae as they are capable of being grown in large batches and do not rely on the use of fertile soil which could be used to grow food crops (Chelf et al., 1993).

Diatoms are from a lineage of photosynthetic eukaryotes which evolved around 150 – 200 million years ago that acquired their chloroplasts from a secondary endosymbiotic event (Kooistra et al., 2007; Medlin, 2016). The evolution of a silica frustule made them resilient and was a major factor in their ecological success (Kooistra et al., 2007; Raven & Waite, 2004)

P. tricornutum is a species often used as a model diatom. It has a weakly silicified shell and can be found in three morphotypes; Pennate fusiform, oval and triradiate. It has a short life cycle and replicates fast and has shown resilience in the lab. It has a unique ability to continue growth despite an absence of silicic acid making it easier to cultivate than other diatoms (Martino et al., 2007). In nature it's often found in coastal environments having the ability to adapt to unstable conditions such as estuaries and rock pools (Martino et al., 2007).

P. tricornutum's genome has been sequenced and is relatively small (~13 000 genes and is easily transformable through conjugation, which is in part why it has become a model diatom (Huang et al., 2011). *P. tricornutum* has also been reported to be rich in polyunsaturated fatty acids (PUFA), with eicosapentaenoic acid (EPA) being the most abundant. EPA is an important omega-3 fatty acid for both humans and marine life, which makes *P. tricornutum* an appealing candidate as a food organism. (Dyerberg et al., 1978; Huang et al., 2011; Yongmanitchai & Ward, 1991)

1.2 Aim of study and hypothesis

As part of this thesis, we performed three major experiments, each testing a different factor that might impact diatom growth and development. These factors were salinity, pH and the presence of glyphosate (an herbicide) and the thesis is therefore split into three parts.

For all of the experiments our hypothesis is that changes in conditions will lead to a negative impact on growth, intensifying as they deviate further from the norm.

1.3 Introduction salinity

1.3.1 Salinity history

Due to living on the water surface, changes in surface water conditions caused by climate change will likely impact *P. tricornutum* growth and development, with one important factor being salinity (Nikitashina et al., 2022). Regular seawater salinity lies between 3.4-3.6% (w/v). However, in more extreme regions it can vary from around 1.0% (w/v) in the Baltic Sea to 3.8% (w/v) in the Mediterranean Sea. This salinity fluctuates more depending on several factors, such as vicinity to rivers, evaporations and precipitation (Durack & Wijffels, 2010). Additionally, anthropocene activities seem to cause more saline conditions in subtropical conditions, while higher latitude environments become less saline (Durack & Wijffels, 2010). Changes in salinity away from the regular 3.5% (w/v) will result in osmotic stress (Nikitashina et al., 2022). While a recent paper by Nikitashina, et. al provides a view into the metabolic change of diatoms, changes in gene expression (transcription) have not been investigated. Additionally, as *P. tricornutum* is of industrial interest in lipid production, seeing how lipid content is impacted both in quantity and types of lipid would also be of interest.

An early experiment by Larry E. Brand tested the salinity tolerances of 46 marine phytoplankton (Brand, 1984) whereas one of them was *P. tricornutum*. The salinities tested were from 0-4.5% (w/v). They found that *P. tricornutum* grew in all the salinities with an optimum at 2.5% (w/v) salinity. From testing these diatoms, patterns emerged. Estuarine diatoms were shown to generally be the most euryhaline (being able to tolerate a wide range of salinity) and oceanic diatoms being the most stenohaline (being able to tolerate only a narrow range of salinity). Coastal species were shown to tolerate lower salinities better than the oceanic ones, despite living in almost as saline waters as the oceanic ones. The article explained this phenomenon with two points: Firstly, in the past these species may have lived in less saline waters during the last ice age, either due to runoff as the ice melted or due to low ocean levels meaning they lived in low salinity estuaries. Secondly due to this first factor they suggest that the majority of their genetic populations live in estuaries or other low salinity environments (Brand, 1984).

In a newer study, the biomass of a selection of marine algae including *P. tricornutum* was conducted with salinities ranging from 2-4% (w/v) (Isik et al., 2021). From their experiment they

found the optimal salinity for increasing biomass to be 4% (w/v). However, this optimum salinity has been reported in other studies to be in the range from 2.8% (w/v) (Qiao et al., 2016) to 4.5% (w/v) (Ishika et al., 2017).

From early studies diatoms were known to show a preference for specific salinities. The general understanding of the preferences for marine diatoms was that the individual strains were acclimated to their local habitat and had their salinity optimum in that range. Diatoms acclimated to brackish waters usually had a wider range salinities with high growth (Braarud, 1951; Brand, 1984; McLachlan, 1961). Similarly as was observed in (Brand, 1984) different strains of the same species have been shown to have different salinity optimums (e.g. *Asterionella glacialis* with 1.5% (w/v) (Brand, 1984) and 3.0% (w/v) (Kain & Fogg, 1958)).

Photosynthetic activity has also been researched in relation to salinity. Liang et al. (2014) found that the maximal photochemical efficiency (Fv/Fm) of photosystem II (PSII) was highest when grown between 2-4% (w/v) salinity. They recorded the specific growth rate to be highest in the range 2-3% (w/v) salinity. (Liang et al., 2014)

From a newer study by Nikitashina et al. (2022) researched the metabolic adaptation of *P. tricornutum* in hypersaline conditions of 5% (w/v) salinity. Examples of such adaptations are an up-regulation of amino acids and increased production of saccharides and inositols. Additionally, 4-hydroxyproline, pipecolinic acid, myoinositol, threonic acid, and acylcarnitines were affected by the treatment, despite previously not having been associated with osmotic stress. (Nikitashina et al., 2022)

1.3.2 RNA extraction

RNA extraction can be done in several ways. One of which is through column binding and often sold as kits. We will be describing a kit by “Sigma-Aldrich” (Sigma spectrum Plant Total RNA kit).

This kind of extraction method works by grinding the tissue to a fine powder when frozen, and adding a lysis buffer that releases the RNA from the sample as well as inactivating ribonucleases and interfering metabolites. The lysate is filtered through a filter column to remove remaining cellular debris. Binding solution is added to the lysate that makes RNA bind to the binding

column and prevents polysaccharides and most genomic DNA from binding. Through the use of DNase and several wash steps with wash solutions, most residual impurities are removed, and pure RNA is left in the binding column which can be eluted and stored. (Sigma-Aldrich, 2023)

1.3.3 NanoDrop

The “Thermo Fisher NanoDrop” is a simple spectrophotometer that analyzes small volumes of liquid. It accepts 1-2 µL of liquid and can from this volume quantify DNA, RNA and proteins in the sample. Nucleic acids absorb light at 260 nm, which allows for this wavelength to be used in finding nucleic acid concentrations and purity. Purity ratios are often used to check if the samples are pure. Absorbance ratio A260/A280 giving an indication of contaminations of residual phenol, guanidine salts or proteins that absorb strongly at 280 nm. For DNA an A260/A280 ratio is considered pure when it’s over 1.8 and for RNA 2.0 The ratio A260/A230 is another ratio used to indicate contaminants that absorb at 230 nm, for example carbohydrates. This ratio is often a bit higher than A260/A280 and is normally in the range of 2.0-2.2. (Loughrey & Matlock, 2017; Matlock, 2015)

Some common contaminants in samples are phenols, which also absorb light at 260 nm and can cause the calculation of concentrations of nucleic acids to be overshot. NanoDrop uses a full absorbance spectrum and computer algorithms to identify such contaminants and subtract the contaminants absorbance to get a corrected value. (Loughrey & Matlock, 2017; Matlock, 2015)

While NanoDrop is used to test nucleic acid purity, it says nothing about nucleic acid quality. Thus, to test for nucleic acid degradation other methods must be employed, such as the bioanalyzer. The bioanalyzer determines RNA integrity by looking at the ribosomal RNA (rRNA) within a sample, as this is typically the most abundant RNA in any given cell. The principle behind this is that if any RNA is degraded, then at least some of the ribosomal RNA will be degraded as well. Since rRNA varies between organisms, software must be programmed to specifically look at the peaks belonging to the type of organism one is looking at (such as “plants” or “animal”). Degradation of RNA is expressed with RNA integrity number (RIN) as a value between 1 - 10 with 10 being not degraded. For our experiments we use plant RNA as a basis since they are the organisms available with the most similar ribosomal RNA profile. (Kiewe et al., 2009)

1.3.4 RNA sequencing

Using high throughput sequencing/Next Generation Sequencing (NGS) it is possible to sequence the whole transcriptome to identify and quantify the number of expressed genes. The Illumina platform is widely used for this due to its high throughput and low costs. The Illumina platform utilizes a bridge amplification system in a flow cell to sequence by synthesis. Sequencing platforms cannot directly sequence RNA, so part of the library construction is converting the RNA to coding DNA (cDNA). This system is not typically able to sequence long sequences and is limited to a couple of hundred base pairs (bp), meaning fragmentation is required before sequencing (Pease & Sooknanan, 2012; L. Wang et al., 2011).

1.3.5 Real-Time quantitative PCR

Real-time quantitative polymerase chain reaction (RT-qPCR) is based on the same principles as regular polymerase chain reaction (PCR) with the addition of fluorescent markers that can be measured after every cycle of PCR. Fluorescence will correlate with the amount of double stranded DNA (dsDNA) present in the samples and can thus be used to quantify the amount of dsDNA following each step. Highly expressed genes will consist of a larger portion of the cDNA content in the samples and will therefore more rapidly see a change in fluorescence patterns. This allows RT-qPCR to be used as a method to find (relative) gene expression. (Taylor et al., 2010)

To do an RT-qPCR one needs primers for the gene/transcript in question. A good primer must be specific for the product produced by the primer pair. To find areas in the genome/transcript that are unique and together with another primer only produces one product, BLAST tools can be used. The NCBI Primer-BLAST is a tool that combines “Primer3” (Untergasser et al., 2012) and “NCBI BLAST” (Ye et al., 2012) to specify criteria for the primers, what template it should match to and if there are any off target products. The suggested primers can then be checked if they are in a spot with common single nucleotide polymorphisms (SNPs).

1.3.6 Lipids

Lipid extraction in marine algae is notably different from plants, with oilseed crops simply requiring a press or something similar (Ranjith Kumar et al., 2015). In contrast, microalgae such as *P. tricornutum* are single celled and have a rigid cell wall, creating the need for other extraction methods (Ranjith Kumar et al., 2015). Thus lipid extraction from *P. tricornutum* involves lysing the cells before using an extraction method, such as Bligh and Dyer's method of lipid extraction (described below in chapter 2.1.15: Lipid extraction and analysis) (Bligh & Dyer, 1959).

Several methods have been described to extract lipids from cells, with the Bligh and Dyer method having been shown to work with several different organisms. (Bligh & Dyer, 1959; Iverson et al., 2001). Bligh and Dyer's method separates the organic (nonpolar) soluble and aqueous (polar) soluble compounds into separate phases, allowing for the retrieval of the organic phase which contains the lipids.

1.3.7 GC-MS

Analysis of fatty acids can be done with gas chromatography–mass spectrometry (GC-MS). This analysis uses capillaries with a stationary phase to change the flow-through rate of gases that pass through it. Based on the retention time compared to internal control and specific weight for each peak measured by the mass spectrometer. A "fingerprint" for each compound is created in a chromatogram and mass spectrum. This can be looked up in a database to get the ID of the compound as well as the amount present. (Karasek & Clement, 2012)

To make lipids suitable for GC-MS the compound needs to be volatile enough to become a gas in the instrument. To improve the volatility the fatty acids are converted to fatty acid methyl esters (FAMEs). When using solvents to extract lipids, leachables from plastic consumables as well as some glassware must be taken into consideration. Common leachables are oleamides and palmitamides which when esterified, will produce the same FAMEs as corresponding fatty acids. Presence of such leachables therefore lead to skewed results during GC-MS. (Jug et al., 2020; Tumanov et al., 2015)

1.3.8 Aim of study

In this experiment we wanted to grow *P. tricornutum* in media of different salinities, ranging from 1.5% (w/v) to 5.5% (w/v). Through the use of flow cytometry over a seven day growth period both short term (transferring algae from normal media to test media) and long term (transferring algae from normal media to test media) and long term (algae kept in exponential phase while grown in the test media for four weeks before starting the measurements) growth could be determined. We aimed to perform RNA sequencing and lipid analysis on *P. tricornutum* grown over a three-day period.

1.4 Introduction pH

1.4.1 History pH

In aquatic environments, carbon can take several forms, collectively referred to as dissolved inorganic carbon (DIC). DIC comes in the form of aqueous CO_2, carbonic acid (H_2CO_3), hydrogencarbonate (HCO_3^-, also known as bicarbonate) and carbonate (CO_3^{2-}), existing in the following equilibrium (Equation 1.4.1) (Doney et al., 2009)

$$CO_2(aq) + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^- \rightleftharpoons 2H^+ + CO_3^{2-} \qquad \textbf{Equation 1.4.1}$$

Most DIC in the ocean takes the form of hydrogencarbonate (~90%), with dissolved CO_2 present in low concentrations (~1%) with little diffusion (Doney et al., 2009; Raven et al., 2020). This poses a problem for photosynthetic organisms, as Rubisco, the enzyme responsible for carbon binding, is dependent on CO_2. Resulting in photosynthetic algae having a need to convert hydrogencarbonate into CO_2 and to concentrate CO_2 to be used by Rubisco. This is referred to as carbon-concentrating mechanisms (CCM) and allows for much higher photosynthetic levels than what would otherwise be possible. (Badger et al., 1998; Raven et al., 2020; Shen et al., 2017; Tachibana et al., 2011)

P. tricornutum has been found to have several genes similar to mammalian *SLC4* and *SLC6* (sharing 27-29% homology), which are hydrogencarbonate transporters (hydrogencarbonate regulates blood pH). Research shows that these genes are more actively expressed under low CO_2 conditions, while they are repressed when CO_2 is in higher concentrations. These findings imply that not only do *P. tricornutum* have hydrogencarbonate transporters (as expected), but they are also something that evolved early in eukaryotic evolution. This might indicate an evolutionary advantage in being capable of transporting hydrogencarbonate, possibly related to their modern function of pH regulation. (Badger et al., 1998; Shen et al., 2017)

Conversion of hydrogencarbonate to CO_2 occurs through a dehydration by carbonic anhydrases (CAs), producing CO_2 and OH^- in a reversible process (Tachibana et al., 2011; Tripp et al., 2001). *P. tricornutum* has several CAs localized in various parts of the cell, with multiple present in the chloroplast membrane, allowing for rehydration of CO_2 into hydrogencarbonate that enters and exits the chloroplast. Additionally, *P. tricornutum* lacks CAs in the chloroplast stromas, allowing

for accumulation of hydrogencarbonate. Finally *P. tricornutum* has two CAs present with Rubisco in the pyrenoid, allowing Rubisco to access the CO_2 (Shen et al., 2017).

DIC plays an important role as a buffer in seawater, as the reactions in equation 1.4.1. involves the release (and therefore uptake) of H^+. This leads to algae having an impact on the local ocean pH as consumption of DIC involves the dehydration of hydrogencarbonate, which releases OH^-, causing depletion of H^+. (Nixon et al., 2015)

Despite this photosynthetic push towards alkalinity, growth for phototrophs is limited by essential nutrients such as phosphorus, nitrogen and trace metals (notably iron) (D'Elia et al., 1977). Combined with regulation and relative stability of carbon in the atmosphere, ocean pH typically being considered stable and having low variance. Most ocean waters have a pH of 8.1, with human carbon emissions having lowered this pH by roughly 0.1 compared to pre-industrial levels (Doney et al., 2009). pH fluctuates a lot more on a local scale, such as in algal blooms, where high growth and photosynthetic activity due to a lack of nutrient limitations leads to the waters becoming more alkaline (Nixon et al., 2015). This would lead to a selection pressure for an alkaline tolerance among photosynthetic algae, such as *P. tricornutum* (Goldman, Azov, et al., 1982; Goldman, Riley, et al., 1982).

While this suggests that *Pheaodactylum* may have a high tolerance to alkaline environments, acidic tolerance is also of great interest as this relates to industrial flue gas cleaning potential (Goldman, Azov, et al., 1982; Goldman, Riley, et al., 1982). This in part being due to, in part, how *Phaeodactylum* is considered to be used in carbon capture from industrial waste, which would create a high CO_2 environment and thus an acidic environment. Therefore, acidic tolerance is an area of interest for *Phaeodactylum*, as understanding how it grows in different pH conditions could have industrial impact (Pawlowski et al., 2014).

1.4.2 AquaPen

AquaPen-C AP-C 100 is a handheld instrument and works on the same principles as PAM fluorometry and functions by measuring the chlorophyll fluorescence of a sample. Fv is measured as the difference between the saturating pulse and the maximum fluorescence (Fm), with Fv/Fm being minimum fluorescence divided by maximum fluorescence. The principles behind Fv/Fm measurements (the measurement types we used, also referred to as quantum yield (QY)) is to first induce minimal chlorophyll fluorescence using weak flash pulses, then saturating the chlorophyll using a saturating pulse. A value from 0-1 indicates the efficiency of photosystem II (PSII) where 0 is no conversion of light in photosynthesis, and 1 is all light is converted by photosynthesis. Healthy phytoplankton not limited by nutrient availability typically have a Fv/Fm value of 0.6 to 0.7. (Hartig et al., 1998; White et al., 2011)

1.4.3 Aim of study

Our aim with this experiment was to see the effect of pH lower than normal seawater conditions (8.1) using buffers on the growth of *P. tricornutum*. Additionally, we wanted to see the effect of *P. tricornutum* growth on the pH of unbuffered and buffered media.

1.5 Introduction glyphosate

1.5.1 Glyphosate history

Glyphosate was initially synthesized in 1950, by Dr. Henri Martin, however no pharmaceutical applications were found and thus nothing was published. Glyphosate's re-discovery started with Dr. Phil Hamm who tested compounds from Monsanto's Inorganic Division for herbicidal activity. During this testing Dr. Hamm found some herbicidal activity and recruited a Monsanto chemist, Dr. John Franz, to discover stronger derivatives. Through Dr. Franz's efforts Monsanto first synthesized Glyphosate in May 1970, which rapidly went through testing and the herbicide Roundup® was released 1971. (Dill et al., 2010)

However, despite entering commercial use 1971, mode of action was not fully understood until 1980 (Cerdeira & Duke, 2006; Steinrücken & Amrhein, 1980). Glyphosate's inhibition of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPs), comes as a function of its binding site overlapping with that of phosphoenolpyurvate (PEP), leading to an inability for EPSPs to bind to PEP and thus inhibiting the pathway (Dill et al., 2010). As the active site of EPSPs is highly conserved and plants do not metabolize or transport glyphosate, the evolution of glyphosate resistance has traditionally been considered unlikely (Bradshaw et al., 1997; Cerdeira & Duke, 2006; Dill et al., 2010). However, more recent evolution of glyphosate resistant plant species has called this view into question (Bradshaw et al., 1997; Cerdeira & Duke, 2006; Powles, 2008).

Today glyphosate is the most used herbicide in the world, owing its success to its efficiency and in part to the development of genetically modified glyphosate resistant crops (Dill et al., 2010; Funke et al., 2006; Peng et al., 2012; Steinmann et al., 2012; Sylwestrzak et al., 2021). Marketed as "roundup ready" crops by Monsanto, these plants use an EPSPs derived from *Agrobacterium* sp. strain CP4, rendering them resistant to the effects of glyphosate (Funke et al., 2006; Peng et al., 2012). This variation of EPSPs has a different 3D structure and thus does not bind EPSPs while still binding PEP (Funke et al., 2006). With this increased usage, coupled with the glyphosate used in roundup being very water soluble, comes the concern that runoff into natural water supplies (rivers, estuaries, the ocean, etc.) might harm the natural algal populations (Sylwestrzak et al., 2021).

1.5.2 Glyphosate chemistry

Glyphosate's efficiency comes from its main mode of action, which is inhibiting the activity of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPs) (also known as 3-phosphoshikimate 1-carboxyvinyltransferase), an important enzyme in the shikimate pathway which is responsible for the synthesis of phenylalanyl, tyrosine and tryptophan (Funke et al., 2006; Peng et al., 2012; C. Wang et al., 2016). EPSPs catalyzes the conversion of shikimate-3-phosphate (S3P) to 5-enolpyruvylshikimate-3-phosphate (EPSP) by catalyzing the transfer of PEP's enolpyruvyl moiety (Bromke, 2013; C. Wang et al., 2016). This is the penultimate step of the shikimate pathway, with the final product, Chorismate, being synthesized from EPSP by Chorismate synthase, which is then further processed to produce the aforementioned amino acids (Bromke, 2013; C. Wang et al., 2016). Glyphosate activity leads to cell death as it is incapable of further processing substrate (leading to substrate accumulation) and producing new amino acids (Agostini et al., 2020; Bai & Ogbourne, 2016). A summary of the full shikimate pathway is shown in figure 1.5.1 made by Nunes et al. (2020).

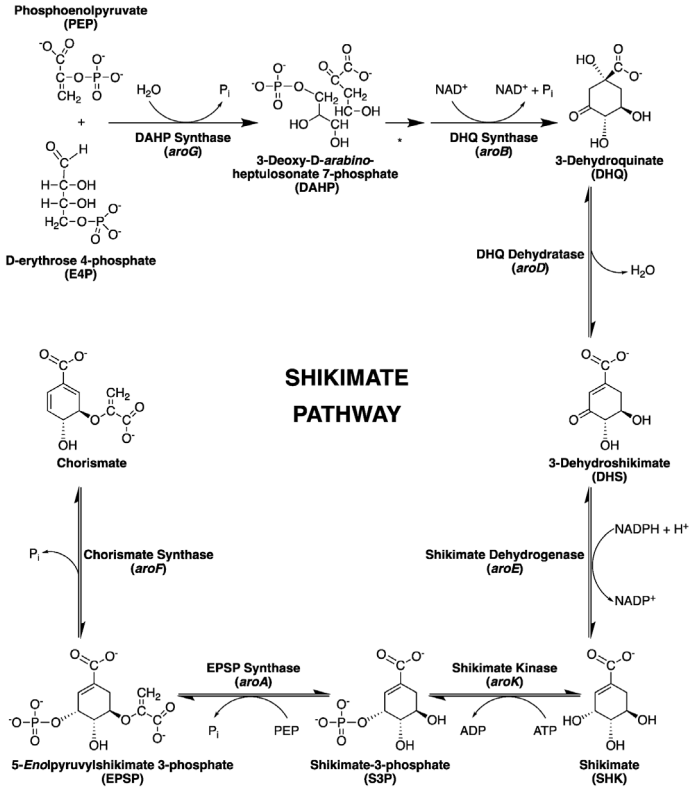

Figure 1.5.1: A summary of the Shikimate pathway, the production of chorismate from phosphoenolpyruvate (PEP) and D-erythrose 4-phosphate (E4P). Glyphosate inhibits EPSP synthase in the penultimate step. Figure retrieved from (Nunes et al., 2020).

EPSPs is present in *Phaeodactylum* (going by 3-phosphoshikimate 1-carboxyvinyltransferase (uniprot ID: B7FSS5)) and thus they should be susceptible for glyphosate, meaning effect on algae may be a concern near agricultural areas (C. Wang et al., 2016). Glyphosate also presents an opportunity to allow for the study of *P. tricornutum* cell death. This is due to *P. tricornutum* being a resilient species, making ways of causing cell death without causing mechanical damage (such as heat) a useful tool for research on cell death and cell death signaling.

Pure glyphosate has a solubility of 12 g/L, however when in salts, such as an isoproponyammonium salt as used in roundup, its solubility greatly increases (PubChem, 2023). This highlights an issue with comparing roundup with "glyphosate", as it contains several unknown compounds to aid in its function as an herbicide.

1.5.3 Propidium Iodide

Propidium iodide (PI) is a stain often used to determine cell viability as PI is unable to permeate a functional cell membrane but enters through a compromised one. PI normally has an excitation/emission max at 493 nm and 638 nm respectively, meaning wavelengths of light of 493 nm excites it and causes it to emit light at a wavelength of 638 nm. When PI attaches to DNA its spectrum shifts to 535 nm and 617 nm respectively and quantum yield increases. Dead cells, which have compromised cell membranes will therefore be stained by PI and can be detected using various methods, such as flow cytometry. PI stains intracellular DNA and is unable to permeate through a healthy membrane and functions to stain for cell viability. (Hudson et al., 1969; Waring, 1965)

An assay used for viability staining of *P. tricornutum* is "Annexin-FITC Apoptosis Detection Kit" (BD Biosciences, San Jose, CA, USA) (Liu et al., 2019). This kit uses the Annexin-FITC to counterstain for alive cells and PI for cell death. If the autofluorescence from chlorophyll A is intact, this autofluorescence can be used as a counterstain instead, as found by fellow master student Simen F. Pettersen. This means *P. tricornutum* only needs to be stained by PI to assess viability. We continued the development of this method, optimizing it to be used with lower volumes and non-normalized cell counts.

The flow cytometer had detection filters for 530 nm, 572 nm and 675 nm and could not detect PI in the optimal range of 617 nm. The 572 nm filter for detection of PI-stained cells was the closest to the optimum and had sufficient signal intensity from stained cells.

1.5.4 Aim of study

Our aim with this experiment was to see the effect of different concentrations of glyphosate on *P. tricornutum* growth and establish a method for staining to measure cell death that is compatible with flow cytometry.

2 Salinity, methods & results

2.1 Materials and methods

2.1.1 Growing algae and experiments

Axenic *P. tricornutum* stock (strain CCMP2561) was always kept in exponential phase and grown in F/2 media in Falcon Tissue Culture Flasks. When the tissue flasks were considered overgrown (substantial amount of algae attached to the flask walls), flasks were replaced. Larger flasks were used when a higher number of algae was required. Algae were kept under the same conditions as experiments, except for the tested condition. If any significant changes occurred (changes to light conditions) the algae were given a two-week acclimation time. Growth conditions were 100 ± 10 µmol·m $^{-2}$·s^{-1} of continuous light, temperature set to 15 °C, containers being either tissue flasks, erlenmeyer flasks (for large volumes) or 24 well plates (for smaller volumes/dilution series). Algae were refreshed every 3-4 days to ensure they maintained exponential growth. Whenever handled, the algae were in a laminar flow bench to ensure an aseptic environment and reduce contamination.

During experiments algae from the stock were taken 2-3 days after last passage to fresh medium. Starting concentration for growth experiments was set to 50 000 cells/mL. Parallels of algae culture flasks were kept in randomly distributed areas under the same light conditions to account for small variations in growth conditions. Measurements were taken at the same time of day during each of the experiments, 10 AM unless specified otherwise. The algae were out of the growth room as little as possible, returning them immediately after sampling.

The growth medium used was F/2 derived from filtered seawater. Local seawater collected at "NTNU SeaLab" was supplied in large tanks. This was vacuum-filtered with 0.2 µm filters for the removal of debris giving filtered salt water (FSW) which was autoclaved. Nutrients were added to the autoclaved FSW as shown in table 2.1 (Guillard, 1975).

Table 2.1: Recipe for F/2 medium using filtered seawater as a base, which is the growth medium used throughout our experiments.

Component	stock (g/L dH2O)	Add pr. liter of medium
$NaNO_3$	75 g/L	1 mL
$NaH_2PO_4 \bullet H_2O$	5 g/L	1 mL
Trace metals stock (1 L)	Na_2 EDTA: 4.36 g $FeCl_2 \bullet 6H_2O$: 3.15 g $CuSO_4 \bullet 5H_2O$: 0.01 g $ZnSO_4 \bullet 7H_2O$: 0.022 g $CoCl_2 \bullet 6H_2O$: 0.01 g $MnCl_2 \bullet 4H_2O$: 0.18 g $Na_2Mo_4 \bullet 2H_2O$: 0.006 g	1 mL
Vitamins stock (1 L)	Cyanocobalamin (Vitamin B12): 0.0005 g Thiamine HCl (Vitamin B1): 0.1 g Biotin: 0.0005 g	0.5 mL

Light conditions were measured and adjusted using a “LI-COR LI-250A Light Meter” with a “LI-COR QUANTUM” light sensor to measure the intensity of light. Light intensity set for our experiments was set to 100 ± 10 µmol·m $^{-2}$·s^{-1}. The light meter was moved around to mark a perimeter where the range of light intensity was acceptable.

2.1.2 Flow cytometry

In this thesis the main way of counting cells has been through flow cytometry. The base principle of flow cytometry is that you measure the scattering of light when a single cell passes a laser. Forward scattering of light is used as a proxy for size and side scatter is for “cell complexity”. When cells pass the laser, pigments that get excited by the laser will in turn emit a wavelength of light, which is read by the flowcytometer’s sensors. We used the NovoCyte 2000 Flow cytometer, which has two excitation lasers of 488 nm and 640 nm and detectors of 530 nm, 572 nm and 675 nm. The flow cytometer itself is operated through the novoexpress software, which creates plots based on readings from the sensors and allows for creation of filters to filter the data. For

measuring growth, we filtered out particles based on forward scatter height (FSC-H) and side scatter height (SSC-H), creating a population of “p2”. “p2” was filtered based on FSC-H and forward scatter area (FSC-A), creating population “p2/p3”. To get the final cell count “p2/p3” was filtered based on chlorophyll content, creating the “M1” population, which was what we considered live cells.

For flow cytometry measurements, 200-1000 µL of sample was placed in a flow cytometer tube or 24 well plate. The tube was then vortexed and placed in a rack within the flow cytometer, then measurements were initiated by selecting “start single well” in the novoexpress program. When the measurement, taking 1-3 minutes (depending on sample concentration), was finished selecting “next sample” in the software would make the instrument measure the next tube, which could then be measured as mentioned above. A feature of the flow cytometer is that it records all the measurements from all samples run, allowing for filters to be applied retroactively.

2.1.3 Salinity setup

In these experiments we created several media with different salinities, with the methods listed being used to make 200 mL of altered media:

F/2 media with reduced salinity was made by mixing regular F/2 medium (made as stated above) with F/2 medium created with Milli-Q H_2O instead of FSW. These were mixed to produce solutions with (assumed) 2.5% (w/v) salinity by mixing 143 mL standard F/2 and 57 mL distilled F/2 and solutions with (assumed) 1.5% (w/v) salinity by mixing 86 mL standard F/2 with 114 mL distilled F/2.

F/2 media with increased salinity was made by mixing additional salt (NaCl) with regular F/2 medium. Salt being dissolved in a portion of the medium before being run through a 0.20 µm syringe filter from Sarstedt to ensure sterility. For 4.5% (w/v) salinity solutions 2 of salt was used, while 5.5% (w/v) salinity solutions used 4 g of salt per 200 mL. The salt was weighed down to a thousandth of a gram.

For RNA extraction, larger volumes of media were prepared (1 L). Media was prepared in the same way as described above, with different amounts of media/salt.

1.5% (w/v) salinity used 430 mL standard F/2 medium and 570 mL distilled F/2 medium.
2.5% (w/v) salinity used 715 mL standard F/2 medium and 285 mL distilled F/2 medium.
4.5% (w/v) salinity used 10g of salt.
5.5% (w/v) salinity used 20g of salt.

2.1.4 Short term exposure

We prepared 100 mL media for each salinity as described above and added the volume required (776 µL) to get a start cell count of 50 000 cells/mL of wild type *P. tricornutum* to each flask. We took three 50 mL Falcon Tissue Culture Flask for each salinity and added 33 mL of the corresponding media to each flask. These algae were then grown under SL3500 RGB LED panels from PSI with a cool white light produced by mixing LED panel gains of red: 7%, green: 8%, blue: 6%. Lamp height was adjusted to get a light intensity of 100 ± 10 µmol·m $^{-2}$·s^{-1} and algae growth were measured daily at 15:30 for a period of 7 days.

2.1.5 Long term exposure

Three parallel flasks of each salinity were prepared in the same way as in the short term exposure, adding 1804 µL wild type algae to the media before spreading the algae solution between the three 100 mL flasks. The cultures were grown for 4 weeks under standard conditions, except SL3500 RGB LED panel gain values were adjusted to Red: 10%, green: 11%, blue: 8% to get an increased growth area. Height was adjusted to compensate for increased light intensity to achieve 100 ± 10 µmol·m $^{-2}$·s^{-1} in the larger area. During the acclimation period excess cells were discarded and the media were replenished to roughly 30 mL every 4 days. Following these four weeks a specified volume from each flask (shown for each flask in table 2.2) was added to 30 mL of fresh media in 50 mL flasks to get a start concentration of 50 000 cells/mL. These cultures were then grown under the same conditions and growth was measured daily at 15:00 for 8 days.

Table 2.2: Flow cytometry measurements showing cell concentration (cells/mL) for each parallel at the end of 4 weeks of acclimation to different salinities. Also displays the desired cell total with the volume required to gain a cell count of 50 000 cells/mL in 30mL.

Sample	Cell concentration (Cells/mL)	Desired total cell count	Required volume (mL)
1A	1 329 155	1 500 000	1.129
1B	1 432 902	1 500 000	1.047
1C	1 204 596	1 500 000	1.245
2A	2 789 827	1 500 000	0.538
2B	2 205 509	1 500 000	0.680
2C	2 578 573	1 500 000	0.582
3A	2 641 645	1 500 000	0.568
3B	2 415 416	1 500 000	0.621
3C	1 868 442	1 500 000	0.803
4A	2 082 134	1 500 000	0.720
4B	2 009 588	1 500 000	0.746
4C	1 933 864	1 500 000	0.776
5A	765 544	1 500 000	1.959
5B	930 083	1 500 000	1.613
5C	848 788	1 500 000	1.767

For both the short term and the long-term experiment divisions per day were calculated by dividing the “cell count” by the “cell count the previous day”, then taking $\log_2$ of this value. Maximum divisions per day for each treatment in exponential phase was picked from these values.

2.1.6 Microscopy

The microscope used was an “Invitrogen EVOS M5000 microscope”. This was an inverted microscope. The samples were transferred to a 24 well plate (Thermo Fisher Scientific) and imaged with a 20x objective. Pictures were saved as a “tif” file with a measuring scale in the bottom right corner.

Analysis of the pictures was done using the image analysis software “ImageJ”. ImageJ was used to do measurements of the pictures for statistical analysis. When measuring *P. tricornutum* length, the “freehand” line tool was used to draw through the center of the cell, end to end. The Width was measured across the cell at its center.

2.1.7 RNA extraction

Extraction of RNA from the different saline concentration was done using samples taken from four points in time (day 1, 2, 3 and 4).

The experiment was set up with three replicates for each salinity concentration plus an additional three for the control concentration (3.5% (w/v)). The three control flasks had a starting volume of 100 mL and were harvested day 1 of the experiment to provide a day 1 control RNA sample. The rest of the samples were grown in 500 mL flasks with a starting volume of 350 mL. All flasks had a start cell count of 50 000 cells/mL. Cell growth and Fv/Fm were also measured. Harvesting and measurements were done at 12:00 PM every day. Cell counting was performed following the standard protocol.

All 100 mL of the day 1 control samples were harvested day 1. For the five test salinity concentrations the following volumes were harvested for each day:

Day 2: 150 mL, Day 3: 100 mL and Day 4: 50 mL

Cell harvesting was done by vacuum filtering onto a filter paper. The cells left on the paper were put into a 2 mL Sarstedt tube with 1 mL of the growth medium (from the respective flask) and vortexed into the solution. The tube was centrifuged for approximately 20 seconds and the

supernatant was pipetted off leaving a pellet of *P. tricornutum*. The tube was put into liquid nitrogen and transferred to a -80 °C freezer for storage.

A sample from each replicate was pipetted off for cell counting and Fv/Fm measurements before harvesting. Photon Systems Instrument (PSI) AquaPen-C AP-C 100 was used to measure Fv/Fm. Measuring Fv/Fm was done by placing a sample in the instrument and waiting for 3 minutes after closing the lid to measure.

The RNA extraction was done using the Sigma spectrum Plant Total RNA kit, following the protocol for RNA extraction (Sigma-Aldrich, 2023).

The extraction process derived from the Sigma-Aldrich protocol. All centrifugations were done at room temperature:

1. Frozen samples in 2 mL Sarstedt tubes had a frozen TissueLyser steel ball added and were put in a chilled TissueLyser-module to keep the samples cold. TissueLyser was set to run for 2x1 minutes at 25 hz.
2. Lysis solution mix (500 µL) was added to each sample and run in the TissueLyser once more for 2x1 minutes at 25 hz. The purpose of the lysis solution was to help lyse the cells and ensure that as much RNA as possible was available for extraction.
   Lysis solution/sample mix was incubated at 56 °C for 3-5 minutes and centrifuged at 13 000 rpm for 1 minute.
3. Supernatant was transferred to the filtration columns and centrifuged (13 000 rpm) for 1 minute. Filtration columns themselves served as filters to separate nucleic acids from other cell material.
4. Filtration column was discarded and 500 µL of Binding Solution was added to the filtrate and mixed with a pipette. Binding solution served as a reagent to help the nucleic acids bind to the binding column used in the next steps.
   700 µL of mixed filtrate was added to the Binding column and centrifuged (13 000 rpm) for 1 minute. The flow-through was discarded, and the rest of mixed filtrate (approx. 300 µL) was added to the column and centrifuged for one minute. Flow through was discarded.

5. Wash solution 1 (300 µL) was added to the column and centrifuged (13 000 rpm) for 1 minute and flow through was discarded. With wash solutions (both 1 and 2) serving to remove non nucleic acid material from the column.
6. Prepared fresh DNAse mix: mix 70 µL of Buffer RDD and 10 µL DNAse I (1 unit*/µL) for each sample and mixed carefully. Created enough DNAse mix for one more sample than required for pipetting. *One unit being defined as: "One unit completely digests 1 µg of plasmid DNA to oligonucleotides in 10 min at 37 °C."
7. Added 80 µL DNase mix to the center of the column and incubated at room temperature for 15 minutes.
8. Added 500 µL Wash Solution 1 to the column and centrifuged (13 000 rpm) for 1 minute. Discarded the flow through.
9. Added 500 µL Wash Solution 2 to the column and centrifuged (13 000 rpm) for 30 seconds. Discarded the flow through.
10. Added 500 µL Wash Solution 2 to the column and centrifuged (13 000 rpm) for 30 seconds. Discarded the flow through.
11. Centrifuged at 13 000 rpm for 1 minute to dry the column.
12. Moved the Binding column to the last collection tube. 50 µL Elution Solution was added to the center of the column. Waited for 1 minute, then centrifuged (13 000 rpm) for 1 minute.
13. Added 50 µL Elution Solution to the center of the column. Waited for 1 minute, then centrifuged (13 000 rpm) for 1 minute. The column was discarded, and the extraction process was finished.

For the high salinity samples, we expected a lower yield of RNA (as these contained less cells) and therefore we used a lower volume of elution buffer (2x40 µL) to gain a higher RNA concentration.

Extracted samples were analyzed with the Thermo Fisher NanoDrop One to get the concentration of RNA in the samples. For analysis using the NanoDrop the RNA program was chosen and blanked with the elution buffer used in the extraction. Small amount of sample (1 µL) was loaded onto the measurement pedestal when prompted, the lid was carefully brought down and the NanoDrop measured absorbance. Following measurements, the pedestal was cleaned with Milli-

Q H_2O and the next sample was loaded. Following measurement of all the samples they were transferred to -80 °C freezer for storage.

2.1.8 BioAnalyzer

To check if the samples were of suitable quality for RNA sequencing, they were run on "Agilent 2100 BioAnalyzer" with the "Agilent RNA 6000 Nano" kit. The procedure was based on technical documentation from the supplier (Agilent Technologies, 2016).

The quantitative range of the assay was 25-500 ng/µL total RNA. All samples tested were inside of the range and were run without any dilution. As the BioAnalyzer assays were time sensitive, it was important to prepare the instrument ahead of time so that the samples could be analyzed as soon as they were ready. Before analysis, the electrode of the instrument had to be decontaminated from potential RNases. To ensure the RNase free instrument environment, the electrode was cleaned with RNaseZAP. This was done by adding 350 µL of RNaseZAP to an electrode cleaner chip. Pipetting was done using non-filtered, non-autoclaved pipette tips that were RNase-free. The lid of the instrument was closed for 1 minute with the cleaning chip inserted for the RNaseZAP to take effect. The electrodes were then cleaned with 350 µL RNase-free water with another cleaning chip for 10 seconds and dried for another 10 seconds by leaving the lid open. After the electrodes were dry, the lid was closed again.

Bioanalyzer analysis was done using the "2100 Bioanalyzer Expert Software" on the connected computer and with the assay "plant RNA" selected. Sample information could then be filled in, leaving the instrument ready for analysis.

The chip had 12 sample wells, 1 ladder well and 3 gel wells, where 1 of the gel wells were made to be pressurized. Reagents used were equilibrated to room temperature for 30 minutes.

"Agilent RNA 6000 Nano gel matrix" (550 µL) was placed in the top receptacle of a spin filter and centrifuged at 1500 g for 10 minutes. Filtered gel was aliquoted to 65 µL aliquots and stored at 4 °C. These aliquots could be used for up to one month after preparation.

The dyes used were light sensitive, so whenever a dye was being used, it was put in the dark as soon as possible to limit the decomposition. The dye mixture used was made by vortexing the

RNA 6000 Nano dye concentrate for 10 seconds and spun down. Dye concentrate (1 µL) was added to a 65 µL aliquot of the filtered gel. The mixture was vortexed thoroughly and centrifuged for 10 minutes at 13 000 g at room temperature. This mixture degrades over time, so it was important to use it on the day of preparation.

The base plate of the chip priming station was set to position C and the adjustable clip was set to the topmost position to fit RNA 6000 Nano chips.

Gel-dye mix was equilibrated to room temperature for 30 minutes in the dark before use. A new chip was placed in the priming station and 9 µL of the gel mixture was pipetted in the indent at the bottom of the well marked with an infilled "G". The lid of the priming station was closed, and the plunger of the attached syringe was pressed down until held by the syringe clip. The pressurized well was allowed to disperse the gel for exactly 30 seconds before the plunger was released by the clip. The plunger was slowly returned to its starting position and the lid of the loading station was taken off. Gel-dye mixture (9 µL) was pipetted in the indent at the bottom of the two other wells marked with "G".

Green-capped RNA 6000 Nano marker (5 µL) was added into the ladder well and all the 12 sample wells. If there were unused wells, they also had to be filled with the RNA Nano marker.

The ladder and samples were thawed on ice, then samples were heat-treated at 70 °C for 2 minutes to minimize secondary structures, preparing them to be added to the wells. Following sample preparation, 1 µL of the ladder was added to the ladder well and 1 µL of each sample was added to respective sample wells. If any well were unused, 1 µL of the elution buffer was added to those to not run the well dry.

The finished chip was placed in the Vortexer (IKA -Model MS3) set to 2400 rpm for 60 seconds. The loaded chip was used within 5 minutes, to prevent reagents from evaporating. Vortexed chip was then put into the BioAnalyzer for analysis. RNA Integrity Number (RIN) for each sample was acquired from the assay.

2.1.9 Primer design

Primers for use in RT-qPCR were designed using several tools. NCBI Primer-BLAST was used with most of the recommended settings left as is. Altered parameters were the template of the transcript, product size maximum of 200 base pairs (bp) and target organism (*P. tricornutum*) to get suitable options for primers. Returned primers were checked and chosen using some of the best practices for designing primers. The parameters we looked for were GC% within 40-60%, GC clamp at the 3' to promote binding, low chance of dimerization, balanced distribution of the bases and avoiding 4 or more consecutive base or dinucleotide repeats. If the targeted gene is larger (~1000 bp) it's also preferable to have the primers in the 3' of the gene. Not all criteria had to be fulfilled, but we tried to get as many of the points in as possible. Candidates for primers were checked again using BLAST against a dataset of polymorphisms (internally curated on the NTNU Cell and Molecular Biology groups servers) to check if there were any common SNPs that could stop the primers from annealing. When all the primes were checked they were ordered from Sigma-Aldrich.

Received primers were shipped as dry oligos and were ready to be used after resuspension. Primers were resuspended in autoclaved Milli-Q H_2O to a concentration of 100 µM. A new utility solution of 5 µM with both primers was made (15 µL forward primer + 15 µL reverse primer + 270 µL Milli-Q H_2O) and stored at -20 °C until further use.

2.1.10 cDNA synthesis

cDNA synthesis was performed using a "QuantiTect reverse transcription kit" and our method is based on the kit manual. RNA samples were thawed on ice while gDNA wipeout Buffer, RT, QRT Buffer, RT primer Mix and RNase-free water were thawed at room temperature. When samples were thawed, they were flicked and vortexed to mix their contents, then placed on ice. RNA samples were diluted to a concentration of 200 µg/µL in a 10 µL volume using RNAse free water. 2 µL gDNA wipeout buffer 7x, 5 µL RNA sample and 7 µL RNAse free water were mixed together and incubated for 2 minutes at 42 °C and moved back to ice. 1 µL Quantiscript reverse transcriptase, 4 µL Quantiscript RT buffer 5x and 1 µL RT primer mix was added to each sample before being placed in a BIO RAD T100 Thermal cycler. The heat cycler program used was 30

minutes of 42°C, followed by 3 minutes of 92 °C reverse transcriptase deactivation. cDNA samples were stored at -20 °C.

Non reverse transcriptase control (NRT control) was made with the same procedure, but instead of adding RT, H_2O was added instead. This was to check for DNA contamination in the samples.

2.1.11 RT-qPCR

The RT-qPCR was done using the ROCHE LightCycler 96.

LightCycler 480 SYBR Green 1 Master is a reaction mix containing all components needed for RT-qPCR except for the template DNA and primers. cDNA was diluted 1:5 and 5 µL was added, 1 µL of 5 µM the primer mix (with both forward and reverse prime) was added to 10 µL of LightCycler 480 SYBR Green 1 mix in an opaque 96 well plate. H_2O was added so that the total volume in each well was 20 µL. For each plate, negative controls with water instead of sample was added. First runs also had replicas of NRT controls to check for DNA contamination in the cDNA samples.

To determine the relative expression of the tested genes, a reference gene was used to normalize the expression data. Gene used was Phatr3_J10566 (also known as Phatr2_10566), coding for "Splicing factor 3B subunit 5".

The program started with preincubation at 95 °C for 10 minutes. The 3-step amplification was 95 °C for 10 seconds, 60 °C for 10 seconds and 72 °C for 10 seconds for 40 cycles. Fluorescence was measured after the elongation at 72 °C for each cycle. After all cycles were finished a melting curve was made. Holding 65 °C for 1 minute before targeting 97 °C with a ramp of 0.2 °C/s with a continuous fluorescence reading. Normalized melting curves derived from the fluorescence readings were used to see if there were any off-target products.

From the qPCR analysis the quantitative measures and melting curves were analyzed. The cycle threshold (CT) value from the replicates, both technical and biological, were averaged together for each gene. As the reference gene should be expressed similarly in all samples, this was used to normalize the values. Normalized ΔCT values were derived by taking the mean of the CT values from the gene of interest and subtracting the mean CT value for the reference gene.

Normalized ΔCT values from the experimental salt concentrations were then compared to the control to get a ΔΔCT value, showing the relative expression from the different treatments. This can further be used to get fold change through the equation $FC=2^{-\Delta\Delta CT}$. Additionally this shows how one can use the relationship logFC=-ΔΔCT.

2.1.12 RNA sequencing and data analysis

Isolated RNA from samples 1.5% (w/v), 3.5% (w/v) and 5.5% (w/v) from day three were sent to Novogene for "mRNA sequencing". Samples were shipped with an ample supply of dry ice to be kept cold during shipment. Sequencing ordered was 20 million 150 bp pair-end reads per sample. Novogene first checked the samples using their own quality control. After the quality had been confirmed the library construction could start, which is the process of preparing the samples for sequencing (L. Wang et al., 2011).

To capture messenger RNA (mRNA) from a sample of total RNA the poly-A tail of all mRNA-transcripts are exploited for capture (Z. Wang et al., 2009). When the mRNA is captured, they can be fragmented to the appropriate size. This can be done using enzymatic or mechanical means. Reverse transcription RNA to cDNA forms a DNA-RNA hybrid. Second strand cDNA is synthesized from the first strand cDNA. To attach adaptors to the cDNA fragments they have to be primed. Priming of the ends starts with cleaving any jagged ends so that all ends are blunt using T7 and Klenow DNA polymerases. Next, phosphate is added at the 5' end and an adenine-overhang at the 3' end. Adaptors are ligated to these ends with T4 DNA ligase (L. Wang et al., 2011).

Adaptors serve a couple of functions, with the sequence of the adaptors being complementary to oligos on the surface of the flow cell. Adaptors also have an indexing sequence so that when multiplexing, one can see which read comes from which sample. As well as the binding site for the sequencing primer.

Libraries were quantified with the "Qubit" Fluorometer and rt-qPCR. Bioanalyzer was used to check the size and distribution of the fragments. Finished libraries were sequenced on the Illumina platform.

Sequencing in the Illumina is done using flow cells with bridge amplification PCR. This will produce clusters of the same fragment (sense and antisense). The antisense product is cleaved and washed off with the 3' end blocked to prevent unwanted priming. Different reagents are flushed over the flow cell in steps. The first reagent contains different dideoxynucleotides (ddNTPs) which differ from normal deoxynucleotides (dNTPs) by not allowing further synthesis. A consequence of this is that only one ddNTP is attached when synthesizing. ddNTPs also have different fluorophores so that when excited, they can be differentiated. A picture of the flow cell is taken identifying which nucleotide has been attached to each of the clusters. A new reagent mix flushes over the flow cell which cleaves off the fluorophore as well as the 3' OH group of the ddNTP, regenerating the ability to further elongate the strand. The process of flushing over ddNTPs, taking a picture and regenerating its function to elongate is done for a set number of times. Pictures taken are added in a sequence to acquire the sequence of the clustered fragments. When the forward strand is finished sequencing, the synthesized strand bends over to bind to the reverse strand oligo and becomes synthesized. The forward strand is cleaved off and the reverse strand is sequenced in the same manner as the forward strand (L. Wang et al., 2011).

Information produced by the Illumina platform produces large (several hundred Gigabytes) Fastq files, which contains information of the coordinates of the clusters in the flow cell, indexing information to identify which reads come from the same sample, the read sequence for the cluster as well as quality information about the read. One example of this quality information is the phred value (Q) which has the negative logarithmic relationship for error probability of base calling (e.g. Phred value 20 = 99%. Phred value 30 = 99.9%). Notably the quality score can be lowered if there are several clusters near each other and the output signal is distorted.

Output files could then be mapped to a reference sequence or be used to produce an assembly. Reference can be from the cDNA transcriptome or the whole genome. Reads from the instrument will correspond and map to the reference sequence. The greater the number of reads that corresponds to a single position, the greater the depth of the read it is. The process of mapping the reads to the sequences is done with specialized software designed for the task. Some of the most predominant softwares are "Bowtie2" and "Tophat 2" (Kim et al., 2013; Langdon, 2015)

Output from such softwares are sequence alignment files (SAM) or the compressed version binary alignment file (BAM). SAM files are a text file format where the alignment of reads

compared to a reference sequence is stored. Both can be used to generate count tables where the number of reads of each transcript is counted and annotated.

We used a Phatr3 based cDNA file with additional curation from the EMBL database at NTNU's cell and molecular biology group, which is not freely available.

Raw data from the sequencing were filtered by removing all reads containing adapters, >10% of undefined bases/nucleotides and reads of low quality (Qscore<=5). Results from the sequencing were delivered in the form of FASTQ files. The delivered FASTQ files were processed by the program "Bowtie2" with the augments "-x -1 -2 –very-sensitive-local" against the Phatr3 assembly to produce SAM files for each sample. By using a script to extract the number of reads per gene, along with its quality information from the SAM-files, count-tables were generated. The count tables were analyzed with the statistical program "R" with the bioinformatic extension edgeR designed for this kind of data (Robinson et al., 2010).

2.1.13 R analysis

Following is a short summary of the R analysis, the full code can be found in the appendix under "R code". For this analysis we made use of the following R packages: BioManager, EdgeR, ggplot2 and Melter (for creating the maps).

Analysis starts by establishing the correct targets for analysis, using these to make a group for the design matrix (grouping together the different parallels) and then reading the count tables. We proceeded by calculating the counts per million and removing any samples with less than 20 reads (filter out genes with low expression), then selecting what data to keep by requiring at least 2 samples being above ~20 reads, finishing by making a list with low expression genes having been removed. This list is then used to make factor "y_1", a DGElist based on the groupings made earlier which then has its raw library size converted into effective library size using calcNormFactors. Using the previously mentioned groups again, a model matrix was made. "y_2" together with the model matrix was used to calculate mean dispersion across all genes, creating "y_2'" in the process, then this new "y_2'" was used to calculate mean dispersion across all genes with similar abundance, producing "y_3" in the process, then this next "y_3" was used to calculate gene-specific dispersions, creating the final "y_4". Finally, "y_4" and the design matrix were used to

fit a quasi-likelihood negative binomial generalized log-linear model to count data using "glmQLFit". Design matrix was used to make contrasts between the separate samples (control vs low salt and high salt). These contrasts together with the fitted model were used to calculate the log2 ratio and false discovery rate (FDR) using a glm likelihood test with the code "glmQlFTest". New tables were then made for each tested contrast with the most differentially expressed genes using "topTags", with these tables being further processed to only have genes with an FDR of <=0.0001 and abs(logFC) = ± 1 show up. Tables were then written out, manually merged and annotated. These final tables contain information on logFC (log fold change)-values, logCPM (log counts per million), p-values andFDR. The annotation process was helped along by professor Per Winge, who provided detailed annotations, as well as comparisons with earlier analyses done in the department. The annotation process was helped along by professor Per Winge, who provided detailed annotations, as well as comparisons with earlier analyses done in the department.

Additionally, R was used to create heat maps, the code (with explanations) can be found in the appendix under "R-code".

2.1.14 Gene filtering

When looking at the count tables, there were 3020 differentially expressed genes for low salt and 387 differentially expressed genes for high salt when compared to day 1 control, filtered on a FDR of <0.0001 and absolute logFC>1. To narrow down what to look at, we started looking at specific subsets of genes/transcripts, the first of which being genes with annotations related to Na^+ or Cl^- (for NaCl related genes) where the logFC of either low or high salinity treatment or the contrast between them was ± 1 and used R to make a heat map. Next, we picked the top five and bottom five most differentially expressed genes, as well as the top/bottom 5 most contrasted genes (when comparing low salinity-high salinity). Lastly we found genes annotated to be connected to metabolites that were found to be upregulated in hypersaline conditions (Nikitashina et al., 2022). From there we looked, which of these genes were part of a biosynthesis pathway as described in Bromke (2013).

2.1.15 Lipid extraction and analysis

For gravimetric lipid analysis, much larger volumes of algae were needed to get desired amount of dry weight, thus we grew three parallels of 1.5 L of F/2 medium for high (5.5% (w/v)), medium/control (3.5% (w/v)) and low (1.5% (w/v)) salt concentrations. Otherwise, the starting setup was the same as before, with a starting algae concentration of 50 000 cells/mL growing in 100 ± 10 µmol·m $^{-2}$·s^{-1} light. Following three days of growth cells were harvested by transferring them to 50 mL falcon tubes, then centrifuging them at 4000 g for 5 minutes (Thermo Fisher Scientific SL plus series centrifuge), supernatant was discarded, and new cells were added until all material was harvested. Cells were washed with Milli-Q H_2O and centrifuged again, with supernatant being discarded. Cells were frozen with liquid nitrogen and set to dry overnight (18 hours) using a Leybold Heraeus GT2 freeze dryer. By closing the valve between the freeze dryer and the vacuum pump we could check if the drying was complete if the vacuum stayed unchanged. Pressure readouts from the monometer did not change, confirming the samples had dried. Dried samples were weighed on a "Mettler Toledo sartorius" weight and dry samples were transferred to glass tubes.

Lipid extraction was done using a modified Bligh and Dyer's lipid extraction (Bligh & Dyer, 1959). Drops of Milli-Q H_2O were added to the samples to allow them to reconstitute (amount needed varying between samples). 3 mL of methanol was added, and the samples were homogenized on ice using a Branson-Sonifier W-250-probe at 40% power output for 2 minutes each. Chloroform (6 mL) was added, and the methanol/chloroform/sample mixture was split into two tubes due to being full, one with a total volume of 6 mL and another with a volume of 3 mL (split with as few pipetting steps as possible to avoid loss of material). Following the split, 1500 µL and 750 µL 0.88% (w/v) KCl dissolved in water was added to lower and higher volume tubes respectively, corresponding to 1/4th of the total volume. Samples were vortexed thoroughly until they were a homogenous solution, then centrifuged for 4 minutes at 30 g and organic phase was collected and transferred to a new collection glass tube. New chloroform (1 mL for low volume and 2 mL for high volume) was added, and the extraction step repeated, with new extract being added to the tube from the previous extraction step. Samples were transported to an N_2 evaporator and dried in a stream of nitrogen until most of the solvent evaporated. Following drying, the samples were transferred to pre weighed glass vials (weighed 3 times at the weight mentioned earlier to account for instrument error), as the collection tubes we were using gave

inconsistent weight. Evaporation then continued in these vials until completely dry, at which point they were transferred to a desiccator where they stood for 30 minutes in a vacuum. When the samples were completely dry, we weighed them on the same weight as before three times (to account for instrument error) to determine “vial+lipid” weight, which was used to determine total lipid weight and lipid percentage.

After determining the lipid contents the samples were reconstituted and normalized in chloroform to a concentration of 5 mg/mL and stored in a -20 °C freezer.

2.1.16GC-MS

The extracted lipids had to be methylated before further analysis, which was done via the following method.

Chloroform with internal standard 23:0 (C23:0) was prepared using an internal standard stock solution. The amount of internal standard per sample should be around 10% of the lipid weight, and 14 µg was chosen and a solution of 14 µg/mL was prepared. Additionally, 30 mL 1% H_2SO_4 Methanol (MeOH) mix was prepared using 0.1 mL 95% H_2SO_4 stock and 29.9 mL MeOH. Next 40 µL sample was added to new kimax glass tubes and. 1.0 mL of Chloroform w/ISTD 23:0 (14 µg/mL) and 2.0 mL 1% H_2SO_4:MeOH (methylation reagent) was added in the given order. The tubes were flushed with N_2-gas and incubated at 50 °C on a heating block with the caps screwed on overnight (16-18 hours). Screw caps were tightened after 10 minutes.

After methylation samples were cooled down to room temperature. To flush the samples and be able to transfer the lipids the following procedure was done.

Saturated NaCl (5 mL) and 2 mL of isooctane were added to the samples. Samples were vortexed and centrifuged at 4000 rpm for 3 minutes at 4 °C. Lipids would now be solved in the top layer. The top layer was transferred to a new glass tube to avoid getting any of the bottom aqueous layer. Adding 2 mL isooctane, vortexing, centrifuging, and transferral of the top layer was done 2 additional times. Lipid phase was then dried under N_2-gas until completely dry and redissolved in 200 µL isooctane. The solution was transferred to a GC glass including an insert. The instrument used for analysis was the “Agilent 7890B GC”

2.1.17 Statistics

All values are expressed as mean ± SE to make 95% confidence intervals (CI). All ANOVA with post-hoc Tukey HSD Test was performed using an online calculator (Vasavada, 2016), while other statistical analysis was performed in excel spreadsheets unless specified otherwise. All samples with a p-value of $p<0.05$ were considered statistically significant.

2.2 Results

2.2.1 Short term exposure

Mean growth from the daily flow cytometry measurements can be seen figure 2.2.1 with all values in appendix table 1.

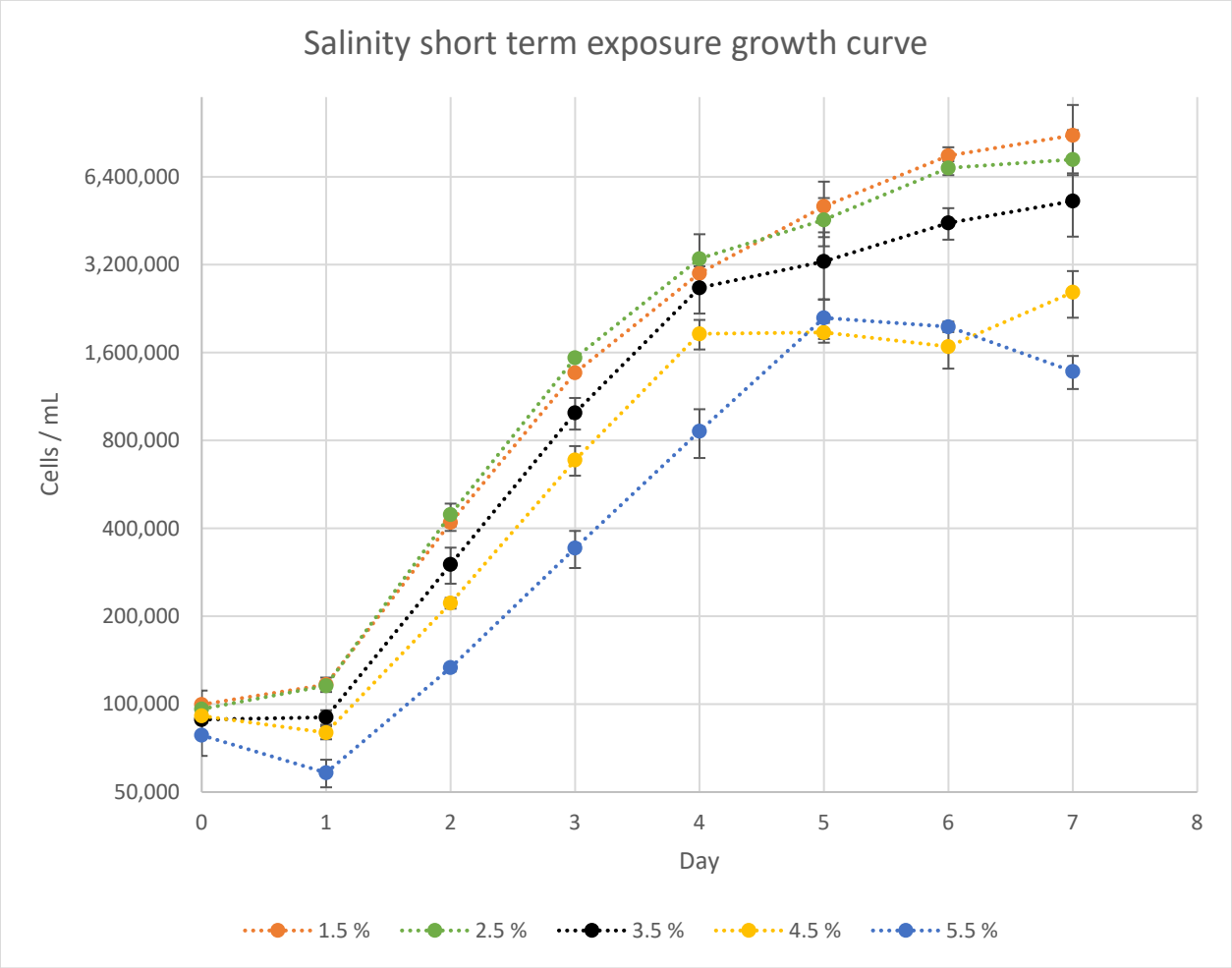

Figure 2.2.1: Plot showing the mean cell counts each day for triplicates of *P. tricornutum* grown in different salinity media (1.5%, 2.5%, 3.5%, 4.5% and 5.5% (w/v). Y-axis (cells/mL) is $\log_2$. N=3, error bars represent 95% confidence intervals.

Figure 2.2.1 shows how the two lowest salinities (4.5% and 5.5% (w/v)) have significantly lower growth compared to the other samples (and each other), with the cell count increasing with a decreasing salinity. Contrary to this the two lowest salinities (1.5% and 2.5% (w/v)) show very similar growth with overlapping error bars, while control (3.5% (w/v)) is “in the middle”.

The maximum divisions per day for the short-term exposure experiment is shown in table 2.3 alongside the confidence interval and day of maximum divisions.

Table 2.3: Maximum divisions per day for the short term experiment performed with several salinity treatments (1.5%, 2.5%, 3.5%, 4.5% and 5.5% (w/v)) of *P. tricornutum*, as well as the confidence interval and the day of maximum growth. N=3

Salinity treatment	Maximum divisions/day	CI	Day
1.5%	1.84	0.12	2
2.5%	1.94	0.13	2
3.5%	1.73	0.25	2
4.5%	1.62	0.11	3
5.5%	1.35	0.19	3

The results from the Fv/Fm measurements are shown in figure 2.2.2, with the raw data in appendix table 2.

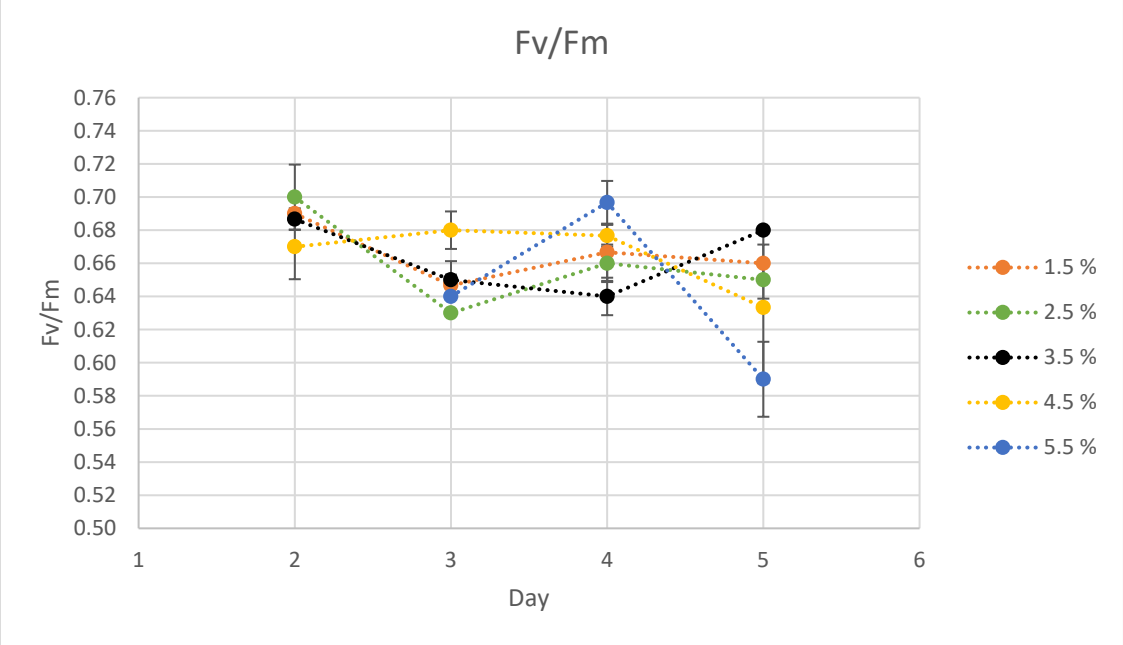

Figure 2.2.2: Mean Fv/Fm measurements for triplicates of *P. tricornutum* grown in different salinity media (1.5%, 2.5%, 3.5%, 4.5% and 5.5% (w/v)). N=3, error bars represent 95% confidence intervals.

Using ANOVA statistical test we found the p-value to be 0.5298 > 0.05 indicating no significant differences between the samples (whole dataset). From the Tukey HSD test we found the samples that were significantly different from another and summarized in table 2.4. On day 2 no significance between the samples (p=0.1076 > 0.05) On day 3 there was a significant difference (5.5% were not included because of only one reading) (p=0.0003 < 0.05). Tukey HSD Test showed 4.5% were significantly different from the rest, while also 2.5% and 3.5% were significantly different from each other. On day 4 ANOVA showed it to be a significant difference (p=0.0011 < 0.05). Tukey HSD test further showed 4.5% to be significantly different from 3.5% and 5.5% was significantly different from 1.5% (w/v), 2.5% (w/v) and 3.5% (w/v). On day 5 they were still different: (p=0.0041 < 0.05). Tukey HSD test showed 5.5% were significantly different from 1.5% (w/v), 2.5% (w/v) and 3.5% (w/v).

Table 2.4: Significantly different results from Tukey HSD analysis of Fv/Fm values from *P. tricornutum* treated with five different salinity treatments (1.5%, 2.5%, 3.5%, 4.5% and 5.5% (w/v)). N=3.

	treatments pair	Tukey HSD Q statistic	Tukey HSD p-value
Day 3	1.5% vs 4.5%	7.5593	0.0030623
	2.5% vs 3.5%	4.5356	0.0496863
	2.5% vs 4.5%	11.3389	0.0010053
	3.5% vs 4.5%	6.8034	0.0058391
Day 4	1.5% vs 5.5%	4.7434	0.0453874
	2.5% vs 5.5%	5.7975	0.0143754
	3.5% vs 4.5%	5.7975	0.0143754
	3.5% vs 5.5%	8.9598	0.0010053
Day 5	1.5% vs 5.5%	5.7368	0.0153449
	2.5% vs 5.5%	4.9172	0.0375018
	3.5% vs 5.5%	7.3758	0.0028053

2.2.2 Long term exposure

Mean results of daily measurements following 4 week acclimation were plotted in figure 2.2.3 , with the raw data available in appendix table 3.

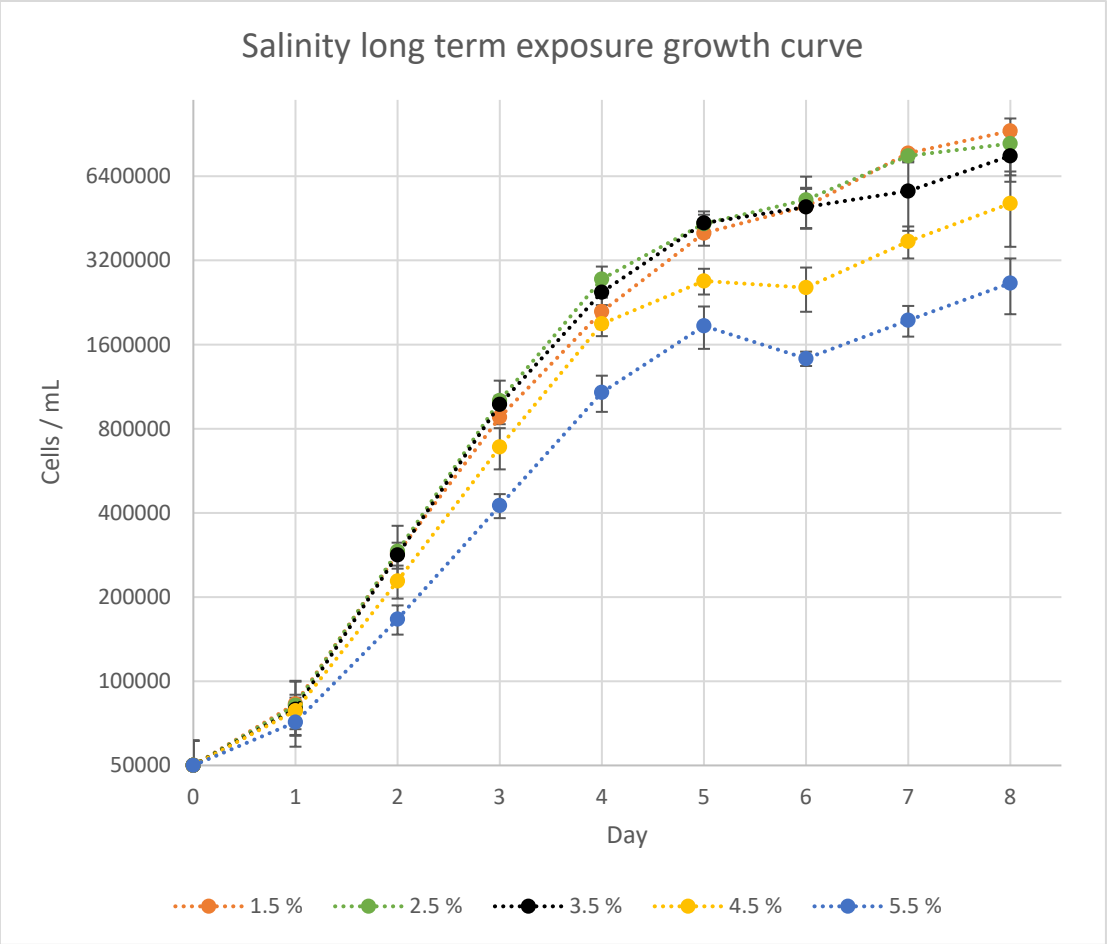

Figure 2.2.3: Plot showing the mean cell counts each day for triplicates of *P. tricornutum* grown in different salinity media (1.5%, 2.5%, 3.5%, 4.5% and 5.5% (w/v)) after having been acclimated to their salinities. Y-axis (cells/mL) is $\log_2$. N=3, error bars represent 95% confidence intervals.

Figure 2.2.3 shows how the lowest salinity (5.5% (w/v)) have significantly lower growth compared to the other samples, with the cell count increasing with a decreasing salinity. While the 4.5% (w/v) treatment also shows lower growth, it has an overlapping confidence interval with control (3.5% (w/v)), which has an overlapping confidence interval with the two lower salinities (1.5% and 2.5% (w/v)) throughout the experiment.

We saw a dip in the 4.5% (w/v) and 5.5% (w/v) salinity treatments at day 6, caused by a shift in the chlorophyll peak, making some cells fall out of the M1 filter on the flow cytometer.

The maximum divisions per day for the long-term exposure experiment is shown in table 2.5 alongside the confidence interval and day of maximum divisions.

Table 2.5: Maximum divisions per day for long term experiment performed with several salinity treatments (1.5%, 2.5%, 3.5%, 4.5% and 5.5% (w/v)) of *P. tricornutum*, as well as the confidence interval and the day of maximum growth. N=3.

Salinity treatment	Maximum divisions/day	CI	Day
1.5%	1.79	0.04	2
2.5%	1.83	0.04	2
3.5%	1.86	0.26	2
4.5%	1.59	0.12	3
5.5%	1.35	0.05	3

2.2.3 Transmission electron microscopy

Fellow master student, Simen Femanger Pettersen, did work on confocal and transmission electron microscopy (TEM). The same experimental setup was used to harvest *P. tricornutum* in different salt concentrations to analyze them. A small sample of pictures taken with TEM can be seen below in figure 2.2.4.

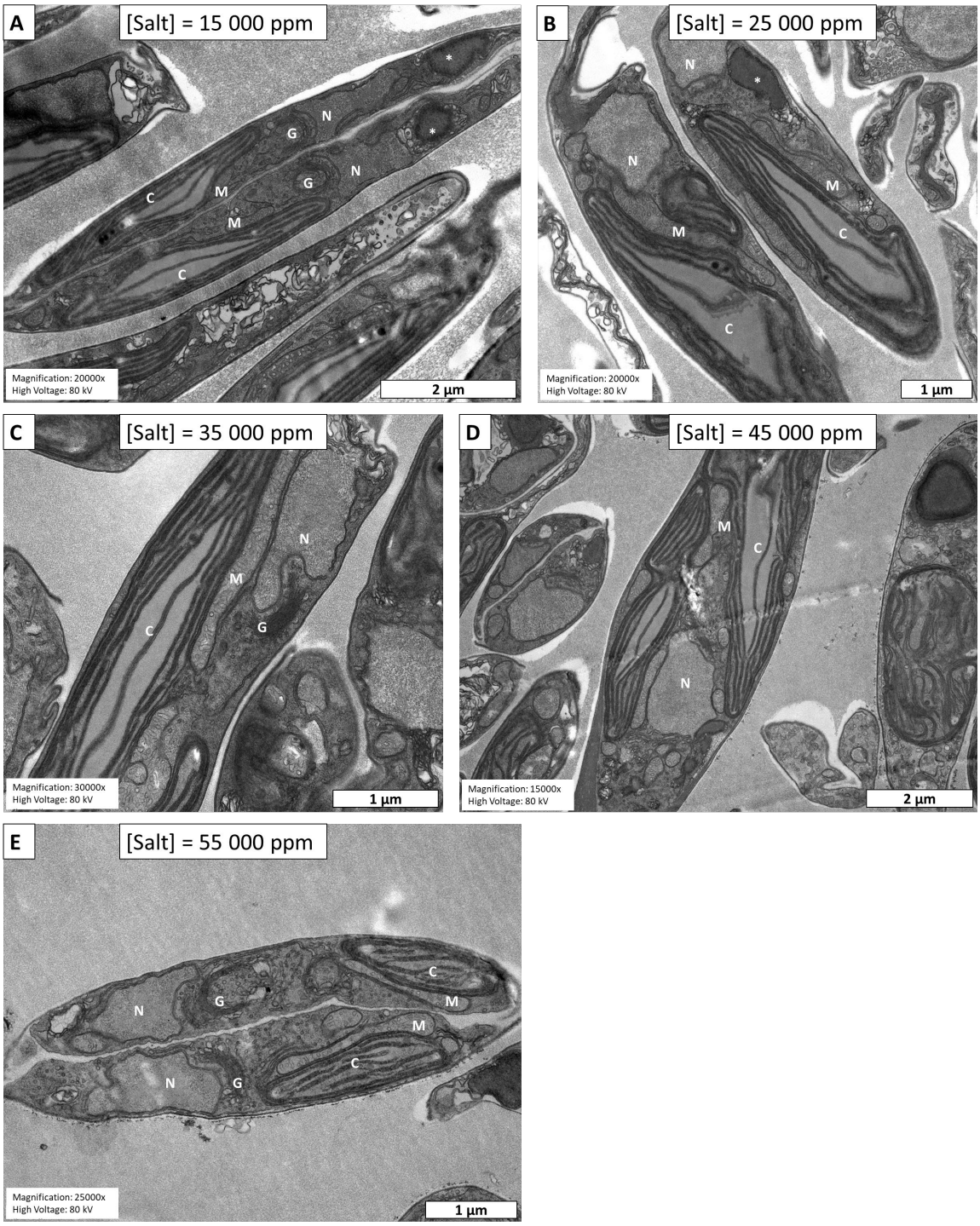

Figure 2.2.4: TEM images by Simen F. Pettersen of *P. tricornutum* in different salt concentrations as shown in parts per million (ppm). Letters on the pictures mark C: Chloroplast, G: Golgi apparatus, M: Mitochondria, N: Nucleus, *:Unknown vesicle

From these pictures no notable changes in the structure of the cells between the different salt concentrations were observed.

2.2.4 RNA extraction

Table 2.6 shows the measurements of the RNA following extraction for the day 3 samples as well as the control samples, which were grown in regular F/2 media and harvested after one day of growth.

Table 2.6: Measured concentrations of RNA following RNA extraction for day 3 samples grown in different salinities (1.5%, 2.5%, 3.5%, 4.5% and 5.5% (w/v)) and control harvested on day 1. ***** Due to a low RNA concentration from the initial extraction, the experiment was repeated for the 5.5% (w/v) salinity solutions with extra care to maximize RNA output.

D3		ng/µL	A260/A280	A260/A230
Control	1	112.7	2.09	2.21
	2	74.6	2.06	2.15
	3	95.2	2.1	2.28
1.5 %	A	70.1	2.15	2.47
	B	68.4	2.14	2.32
	C	256.1	2.15	2.4
2.5 %	A	275.2	2.09	2.23
	B	129.2	2.11	2.33
	C	240.5	2.12	2.28
3.5 %	A	417.1	2.11	2.27
	B	361.4	2.12	2.4
	C	568.8	2.15	2.38
4.5 %	A	218.8	2.11	2.39
	B	466.8	2.13	2.4
	C	154.6	2.08	2.34
5.5 %*	A	320.4	2.12	2.31
	B	288.6	2.12	2.4
	C	340.5	2.11	2.34

Absorbance measurements A260/A280 and A260/A230, shown in table 2.6 are an indication if there were any contaminants in the samples. The Thermo Fisher user manual defines pure RNA samples as having an A260/A280 of ~2.0, with our samples falling within that range (2.06-2.15). For A260/A230 the manual states that values of 2.0-2.2 are typical for pure solutions with our samples falling slightly above that (2.15-2.40).

2.2.5 Bioanalyzer

Plots from the summary of the bioanalyzer run can be seen in figure 2.2.5 with a closer look at an example of the typical bioanalyzer run plot can be seen in figure 2.2.6. The RIN values from our samples were in the range 6.50-7.80. RIN values for each sample can be seen in appendix table **4**.

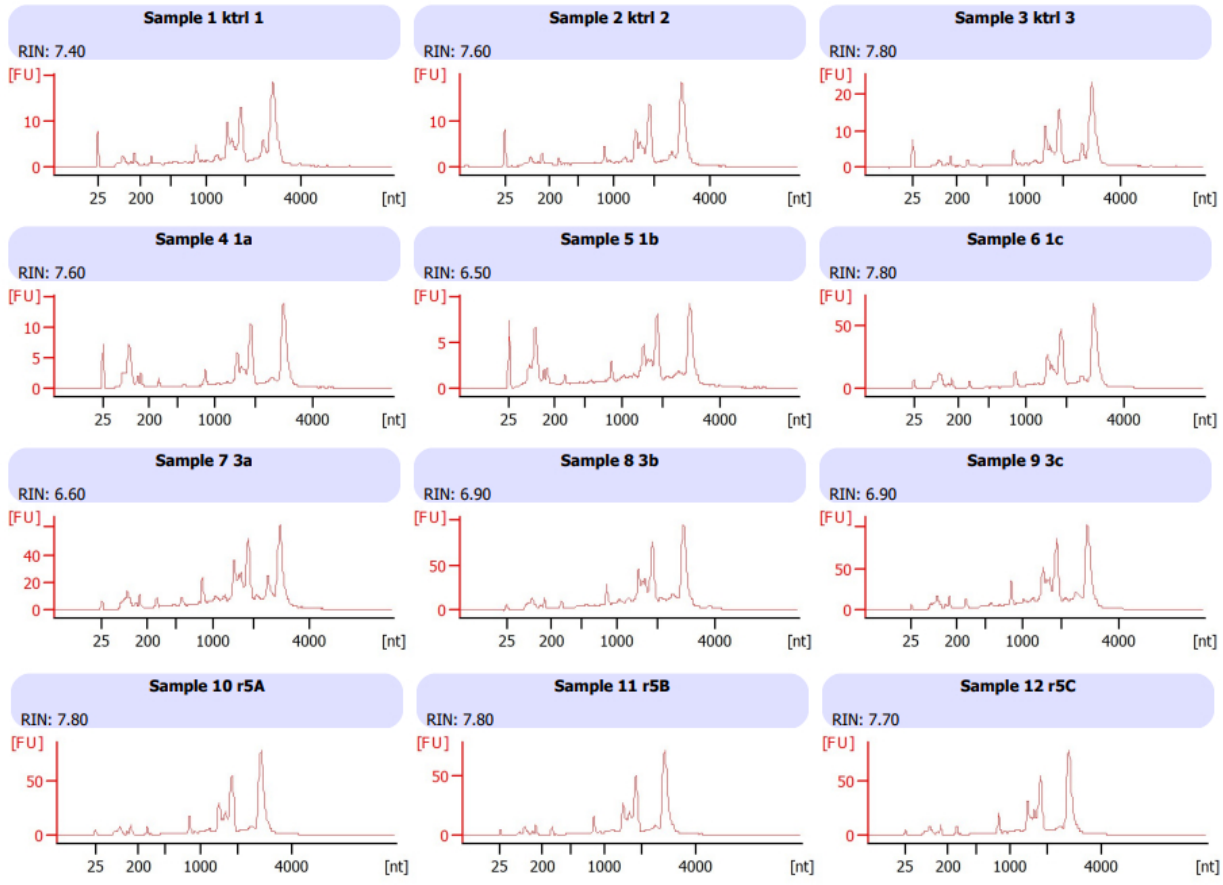

Figure 2.2.5: Summary of bioanalyzer results run on samples grown in various salinity treatments; low (1.5% (w/v), 1a,b,c), medium (3.5% (w/v), 3a,b,c) and high (5.5% (w/v), r5a,b,c), as well as control (ktrl 1, 2, 3). RIN values shown in blue box over each of the graphs.

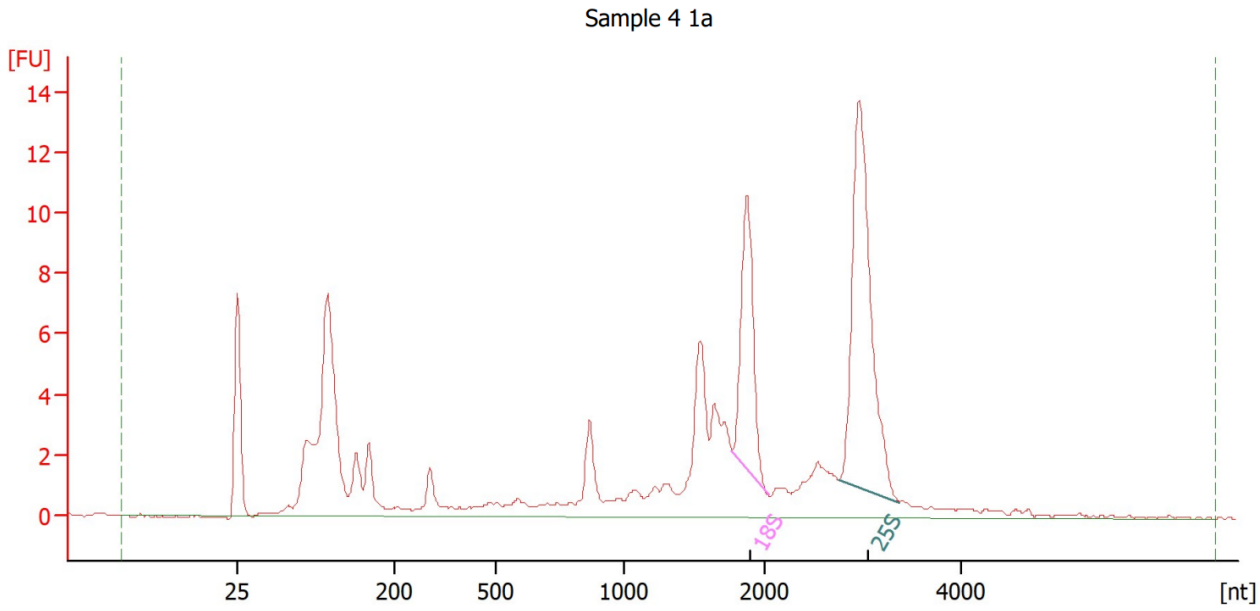

Figure 2.2.6: Bioanalyzer plot example of sample 1a (parallel A, 1.5% salinity (w/v)), with 18S and 25S rRNA peaks marked.

Figure 2.2.5 shows RIN values of a sufficient quality for RNA sequencing (Novogene request RIN >4).

In these electropherograms the big peaks correspond to ribosomal RNA (18s and 25s, figure 2.2.6) If the sample is degraded these larger peaks are broken down and several smaller peaks appear, with these peaks being the dominating peaks in all the samples (figure 2.2.5). If the rRNA is fragmented, so is the rest of the RNA. An ideal run should be a flat line with a couple of sharp peaks corresponding to rRNA.

2.2.6 RNA sequencing

QC analysis from Novogene

Following RNA sequencing, Novogene sent a quality control report containing RNAseq quality statistics, shown in table 2.7.

Table 2.7: Summary of RNAseq quality report produced by Novogene. Contains information of RNA extracted from *P. tricornutum* treated with low salinity (1.5% (w/v)) and high salinity (5.5% (w/v)), as well as day 1 control.

Sample	Raw reads	Raw data	Effective(%)	Error(%)	Q20(%)	Q30(%)	GC(%)
Low salt A	63165958	9.5	98.23	0.03	97.21	92.51	51.43
Low salt B	43967530	6.6	98.61	0.03	97.02	92.06	51.44
Low salt C	44233758	6.6	98.26	0.03	97.14	92.36	51.38
Day 1 control A	53859444	8.1	98.64	0.03	97.21	92.47	52.19
Day 1 control B	54806784	8.2	97.84	0.03	96.82	91.63	52.36
Day 1 control C	49617234	7.4	98.36	0.03	97.23	92.48	52.53
High salt A	63416724	9.5	98.67	0.03	97.22	92.44	52.37
High salt B	51809120	7.8	98.55	0.03	97.15	92.28	52.37
High salt C	51739158	7.8	98.42	0.03	97.37	92.78	52.26

Table 2.7 shows the total number of reads produced (raw reads), as well as the raw data, which is the raw reads times the sequence length. Effective % is the number of clean reads over usable reads (the effectively usable reads) and error % shows the base error rate. Next Q20 and Q30 are the base count percentages with Phred value 20 or 30 respectively. Lastly GC % is the percentage of cysteine and guanine in the reads (amount of G/C in reads/total nucleotides in reads).

From the results QC analysis of the RNAseq data, we saw a high effective percentage with a low error %, indicating most data was of good quality. Further, the Q20 and Q30 showed that the base calls were certain with ~97% of base calls being 99% certain and ~92% being 99.9% (Q20: 96.82 - 97.37 %, Q30: 91.63 - 92.78 %). GC% was within expected levels of approximately 50%. From

all this combined we saw that the data was of good quality. From a list of genes with logFC ≥1 compared to control, or ≥1 between High and Low salt. Genes annotated to have anything to do with Na+ or "salt" were compared in a heatmap seen in figure 2.2.7.

Heat maps created in R showing genes associated with Na^+/Cl^-/NaCl and showing the five most up/down regulated genes (compared to day 1 control) for each treatment (as well as the most different genes between treatments) are shown in figure 2.2.7 and figure 2.2.8. The values shown in the colored bars are the logFC values for that gene. The most differentially expressed (among the annotated) gene from the low salinity treatment is Phatr3_EG02360, which is "possibly a Cl^-/HCO_3^- exchanger". In general, HCO_3^- transporters form a group within the NaCl related genes, with Hydrogen based transporters forming another group. Another notable gene is Phatr3_EG01952, which codes for a chloride channel and is downregulated in both high and low salinity conditions when compared to day 1 control. The low salinity treatment induced a stronger transcriptional change than high salinity treatment.

Figure 2.2.7: Heatmap showing differential expression *P. tricornutum* grown for 3 days in high (5.5% (w/v)) and low (1.5% (w/v)) salinities compared to day 1 control. Genes annotated to be connected to Na^+, Cl^- or "salt" were analyzed by taking the expression of day 3 treatment (low or high salinity) subtracted by the expression of day 1 control. LogFC values for the genes are shown within the bars.

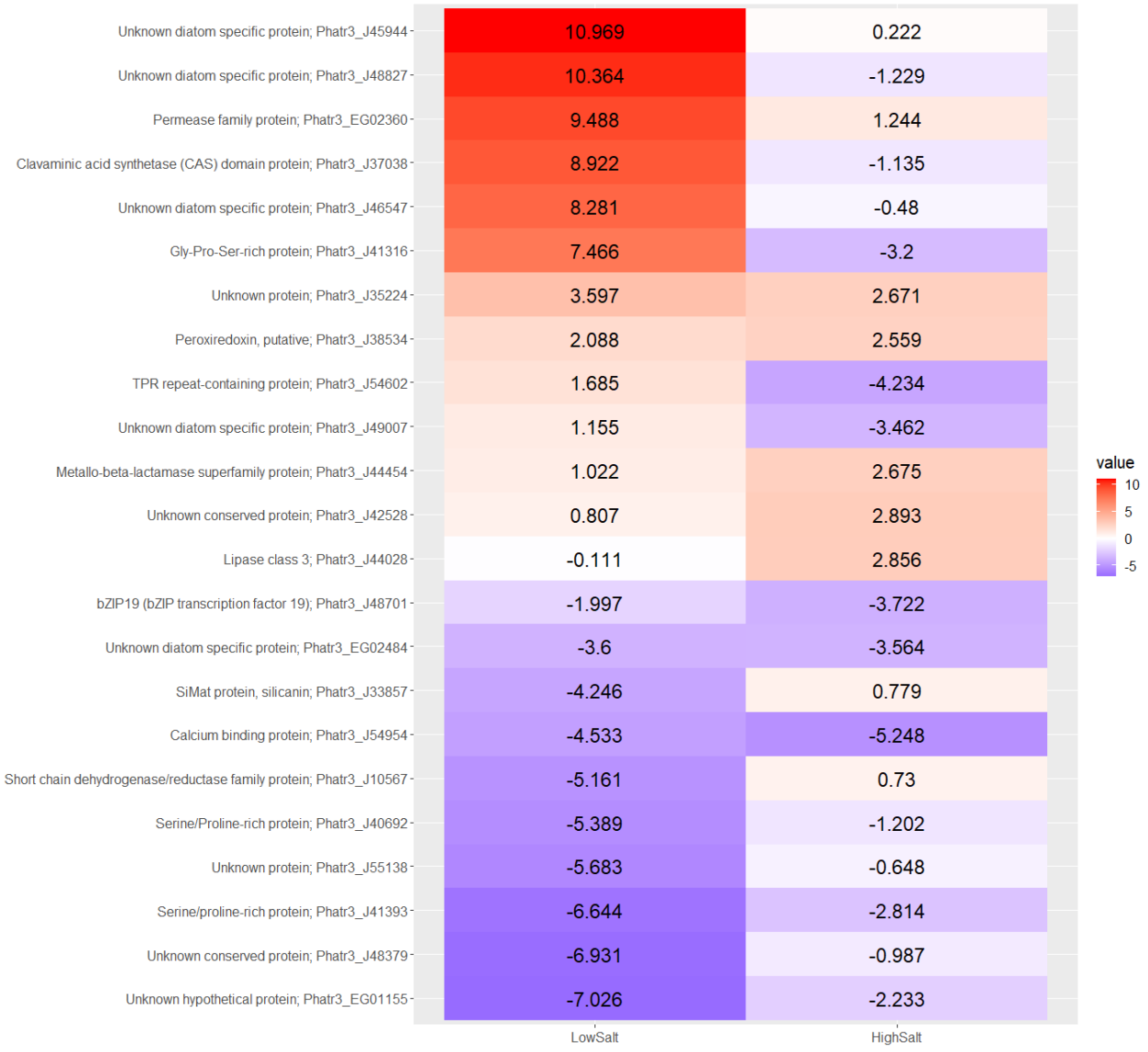

Figure 2.2.8: Heatmap showing differential expression *P. tricornutum* grown for 3 days in high (5.5% (w/v)) and low (1.5% (w/v)) salinities compared to day 1 control. The heatmap shows the top 5 and bottom 5 most differentially expressed genes for both high salinity and low salinity treatment when compared to day 1 control, as well as the three non-overlapping genes with the highest difference between the two (low salinity expression - high salinity expression). Differential expressions were obtained by taking the expression of day 3 treatment (low or high salinity) subtracted by the expression of day 1 control. LogFC values for the genes are shown within the bars.

The differentially expressed genes related to metabolites found by Nikitashina et al. were compiled and presented in table 2.8.

For the high salinity samples, we saw that the genes for Methionine synthase and Methylenetetrahydrofolate reductase were upregulated by a factor of logFC ~1 compared to day 1 control. These are part of the Asparagine, Threonine, Methionine and Lysine Biosynthesis pathways, and it is therefore possible that high salinity impacts the synthesis of these amino acids. In addition, the high salinity samples showed an upregulation of glycine decarboxylase, part of the glycine and serine biosynthesis pathways, by a factor of logFC ~1.

As for the low salinity treatment, more genes in general were different from the standard, which also includes transcripts from biosynthesis pathways. Asparagine, Threonine, Methionine pathways had 8 genes associated with this pathway. Of these genes 7 of them were downregulated from a factor of logFC of -1.71 to -3.75. One of these, Phatr3_J23399 (Methionine synthase (METH)), was found to be upregulated in high salinity and downregulated in low salinity. The Phatr3_J28056 (5-methyltetrahydropteroyltriglutamate--homocysteine S-methyltransferase) gene was found to be upregulated logFC 1.41. In the glycine and serine pathway for the low salinity no genes were found to be differentially expressed. In the Arginine and Proline Biosynthesis pathway two genes were upregulated in low salinity. In the Phenylalanine, Tyrosine and Tryptophan Biosynthesis pathway one gene was downregulated and one was upregulated. In the Valine, Leucine and Isoleucine Biosynthesis pathway three genes were found. Two of these, Phatr3_J30967 (Ketol-acid reductoisomerase) and Phatr3_Jdraft348 (Ketol-acid reductoisomerase), are paralogous genes and were both downregulated. The last gene, Phatr3_J45141 (3-hydroxyisobutyrate dehydrogenase) was found to be upregulated. In the Cysteine biosynthesis pathway two genes were found to be downregulated.

Table 2.8: Differential expression (compared to day 1 control) for a selection of genes associated with various metabolic pathways from *P. tricornutum* cultivated in high (5.5% (w/v)) and low (1.5% (w/v)) salinity for 3 days. The selection of genes is based on findings from Nikitashina et al., who found altered amounts of metabolites associated with the genes listed here. Note that these are not all the metabolites and only a selection which also showed significant differences in expression when compared to day 1 control. The Asparagine, Threonine, Methionine and Lysine Biosynthesis Pathway related genes are colored blue. The Glycine and serine pathway related genes are colored orange. The Arginine and Proline Biosynthesis pathway related genes are colored purple. The Phenylalanine, Tyrosine and Tryptophan Biosynthesis pathway related genes are colored pink. The Valine, Leucine and Isoleucine Biosynthesis pathway related genes are colored red. The Cysteine biosynthesis pathway related genes are colored yellow. Differential expressions were obtained by taking the expression of day 3 treatment (low or high salinity) subtracted by the expression of day 1 control. * Methionine synthase (Phatr3_J23399) was impacted by both treatments.

Salt	Gene	Description	logFC	logCPM	PValue	FDR
High	Phatr3_J23399*	Methionine synthase (METH)	0.94	10.26	3.03E-06	2.43E-04
High	Phatr3_J30471	Methylenetetrahydrofolate reductase	1.03	7.68	4.35E-07	8.41E-05
High	Phatr3_J22187	Glycine decarboxylase, P-protein (GDCP)	1.19	8.66	9.62E-06	5.44E-04
Low	Phatr3_J23399*	Methionine synthase (METH)	-1.72	10.26	3.89E-09	7.53E-08
Low	Phatr3_J4025	Dihydrodipicolinate reductase (EC 1.3.1.26)	-2.27	6.34	1.12E-09	3.30E-08
Low	Phatr3_J10757	Aspartate-semialdehyde dehydrogenase	-1.72	8.41	1.25E-07	9.49E-07
Low	Phatr3_J34582	Diaminopimelate epimerase, chloroplastic type	-1.84	6.24	8.06E-09	1.28E-07
Low	Phatr3_J28056	5-methyltetrahydropteroyltriglutamate--homocysteine S-methyltransferase	1.41	5.58	2.12E-07	1.41E-06
Low	Phatr3_J21592	Diaminopimelate decarboxylase	-1.75	7.61	6.73E-10	2.38E-08
Low	Phatr3_J22909	LL-diaminopimelate aminotransferase, aspartate aminotransferase	-2.80	7.75	2.59E-11	2.82E-09
Low	Phatr3_Jdraft913	S-adenosylmethionine synthetase	-3.75	10.81	7.80E-10	2.63E-08
Low	Phatr3_J843	Methylcrotonoyl-CoA carboxylase alpha subunit	2.63	6.71	6.29E-08	5.68E-07
Low	Phatr3_J51245	Propionyl-CoA carboxylase, alpha subunit	1.12	6.96	9.34E-05	2.28E-04
Low	Phatr3_J41702	Tryptophan synthase (alpha / beta chains)	-1.40	8.52	7.92E-07	4.04E-06
Low	Phatr3_J13175	Phenylalanine 4-hydroxylase	2.25	5.80	4.52E-10	1.85E-08
Low	Phatr3_J45141	3-hydroxyisobutyrate dehydrogenase	2.60	4.04	2.14E-11	2.65E-09
Low	Phatr3_J30967	Ketol-acid reductoisomerase	-2.13	7.72	2.83E-09	6.10E-08
Low	Phatr3_Jdraft348	Ketol-acid reductoisomerase	-1.89	8.37	8.52E-10	2.73E-08
Low	Phatr3_J42282	ATP-sulfurylase	-2.97	8.69	1.18E-11	1.84E-09
Low	Phatr3_J51242	Adenosine kinase	-3.21	8.34	2.63E-12	7.73E-10

2.2.7 qPCR

Table 2.9 shows the calculated logFC values from the qPCR run, compared to the logFC values from the RNA sequencing. Raw relative expression data shown as CT values can be found in appendix table 6.

Table 2.9: Comparison between logFC values of the same genes with qPCR and RNA sequencing from day 3 of high and low salinity treatments using biologically independent samples. The samples were compared to day 3 control for qPCR and day 1 control for RNA sequencing. Differential expression was obtained by taking the expression of day 3 treatment (low or high salinity) subtracted by the expression of their respective control (day 1 for RNA sequencing and day 3 for qPCR).

Gene	Phatr3_J45944[1]		Phatr3_J48827[2]		Phatr3_J37038[3]		Phatr3_J54987[4]	
Treatment	qPCR	RNAseq	qPCR	RNAseq	qPCR	RNAseq	qPCR	RNAseq
Low salinity	7.00	10.97	6.37	10.38	7.62	8.93	-1.54	3.31
High salinity	-0.07	0.19	-0.88	-1.24	-1.55	-1.14	-0.26	-1.29

[1]Phatr3_J45944: Unknown diatom specific protein.
[2]Phatr3_J48827: Unknown diatom specific protein.
[3]Phatr3_J37038: Clavaminic acid snythetase (CAS) domain protein.
[4]Phatr3_J54987: Iron starvation induced protein (ISIP2B).

From table 2.9 we saw how the low salinity treatment had a strong upregulation for three of tested genes, while the fourth one shows a relatively weak downregulation. Contrary to this the high salinity treatment had a downregulation in all four genes, with only Phatr3_J37038 (Clavaminic acid synthesase (CAS) domain protein) having a relative expression lower than -1 when compared to day 3 control.

Table 2.9 also showed that the RNA sequencing and qPCR data follow similar trends, but the RNA sequencing showed stronger responses in general. The biggest difference between the two is how the low salinity Phatr3_J54987 (iron starvation induced protein (ISIP2B)) was negatively regulated under the qPCR experiment, while it was positive in the RNA sequencing data.

Normalized melting peaks of the tested genes can be seen in figure 2.2.9. The melting curves were automatically grouped together by the software (LightCycler® 96 SW 1.1) to identify different melting curves.

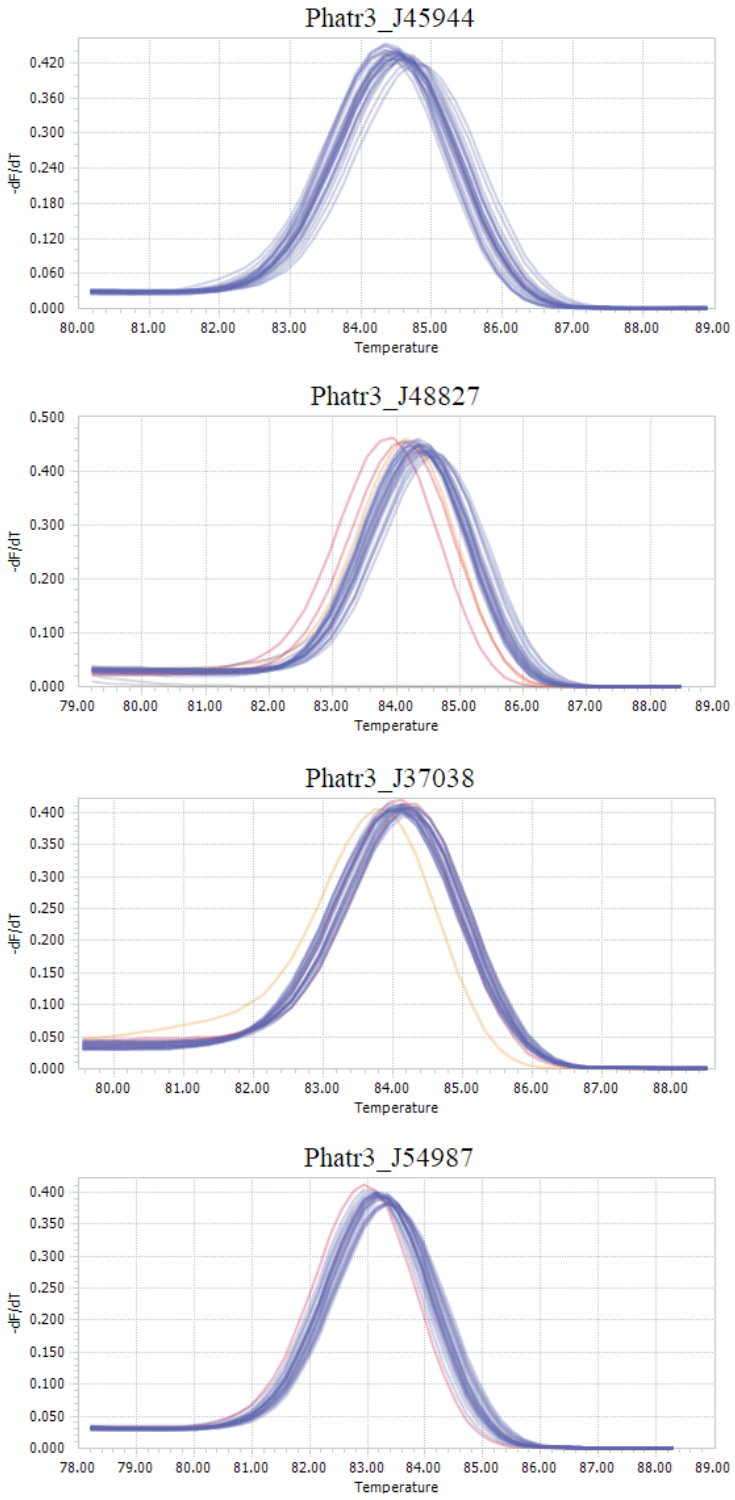

Figure 2.2.9: Melting curves following qPCR experiment using four genes for samples grown in different salinity media (1.5%, 3.5% and 5.5% (w/v)) harvested following 3 days of growth. There is only one peak (per curve) and therefore only one product. With the curves themselves being grouped based on the characteristics of the curve, with all equal curves having the same color. The genes shown in the melting curve are the following: Phatr3_J45944: Unknown diatom specific protein. Phatr3_J48827: Unknown diatom specific protein. Phatr3_J37038: Clavaminic acid snythetase (CAS) domain protein. Phatr3_J54987: Iron starvation induced protein (ISIP2B).

The normalized melting curve peaks showed largely one grouping with only one product. In all curves except Phatr3_J45944 there were a couple of ungrouped curves with only one peak.

2.2.8 Lipid analysis

During harvesting the low salt concentration gave very small, compact pellets which were very difficult to disturb. Medium salt concentration gave big, less compact pellets where the upper layers were easily solubilized while the lower levels were more resistant. Finally, the high salt concentration gave small, loose pellets which were very easily disturbed. It should be noted that these images contain roughly half the amount of the total biomass and that the final "merged" pellets were twice the size however they had the same characteristics and consistencies. Examples of the different pellets are shown in figure 2.2.10.

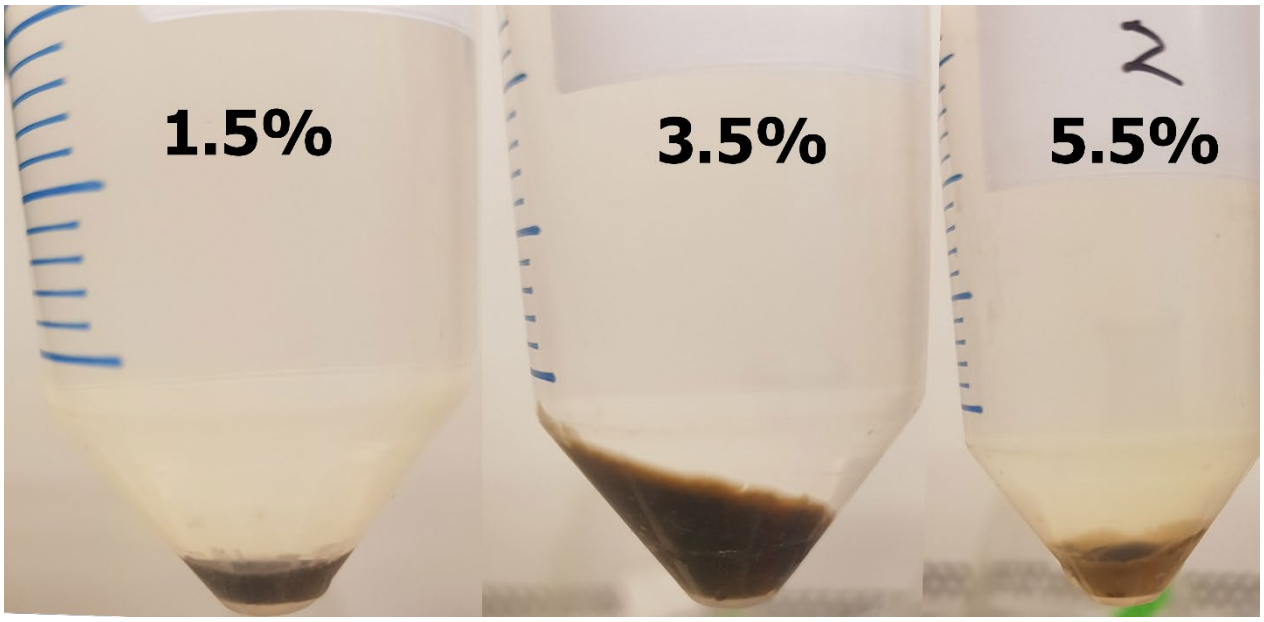

Figure 2.2.10: Picture example of pellets produced by harvesting pellets from the salt experiments. The leftmost is an example of low salt (1.5% (w/v)) parallels and contains roughly half the total amount of biomass harvested. These pellets were very difficult to disturb. The middle part is an example of control (3.5% (w/v)) parallels and contains roughly half the total amount of biomass harvested. The upper layers of these pellets were easily disturbed, while the lower levels were more resistant to disturbances. The rightmost part is an example of high salt (5.5% (w/v)) parallels, containing roughly half the total amount of biomass harvested. Pellet was very "loose" and was easily disturbed.

Total dry weight, lipid weight and lipid percent can be seen in figure 2.2.11 and figure 2.2.12. The raw data for the figures can be seen in appendix table 7.

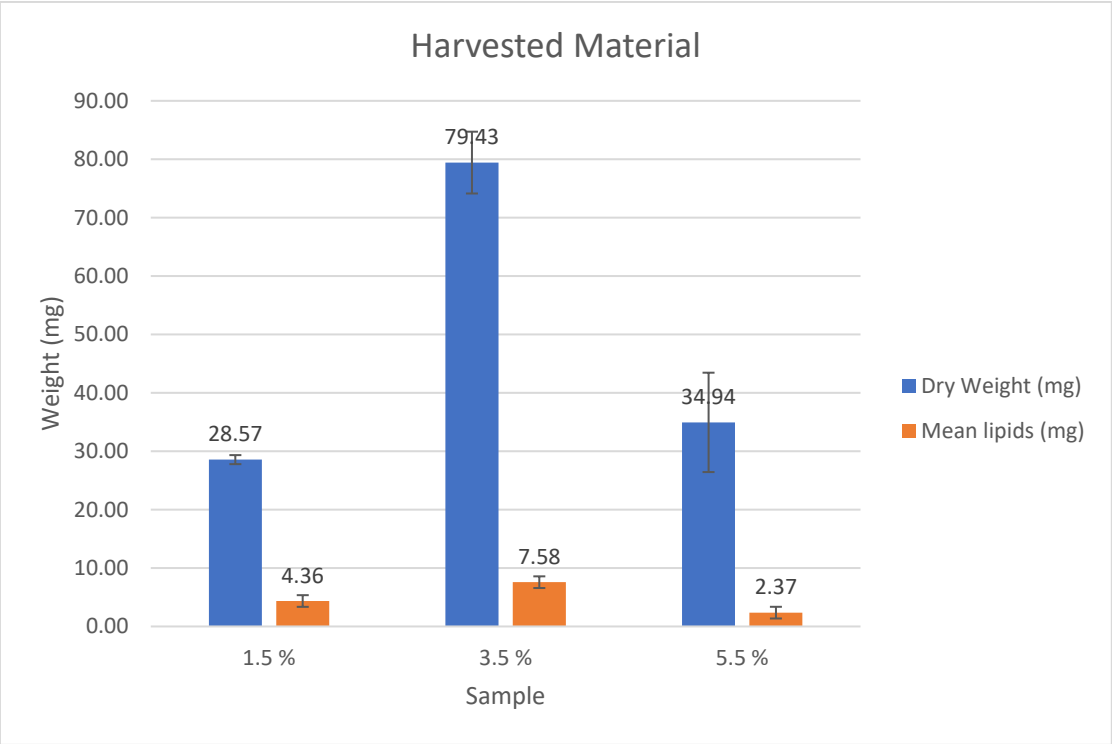

Figure 2.2.11: Measured dry weight for triplicates of *P. tricornutum* grown in different salinity media (1.5%, 3.5% and 5.5% (w/v)) harvested from 1.5 L of media through centrifugation, following 3 days of growth, as well as the mean lipid weight following lipid extraction. Lipids were extracted using the Bligh and Dyer method. N=3, error bars represent 95% confidence intervals.

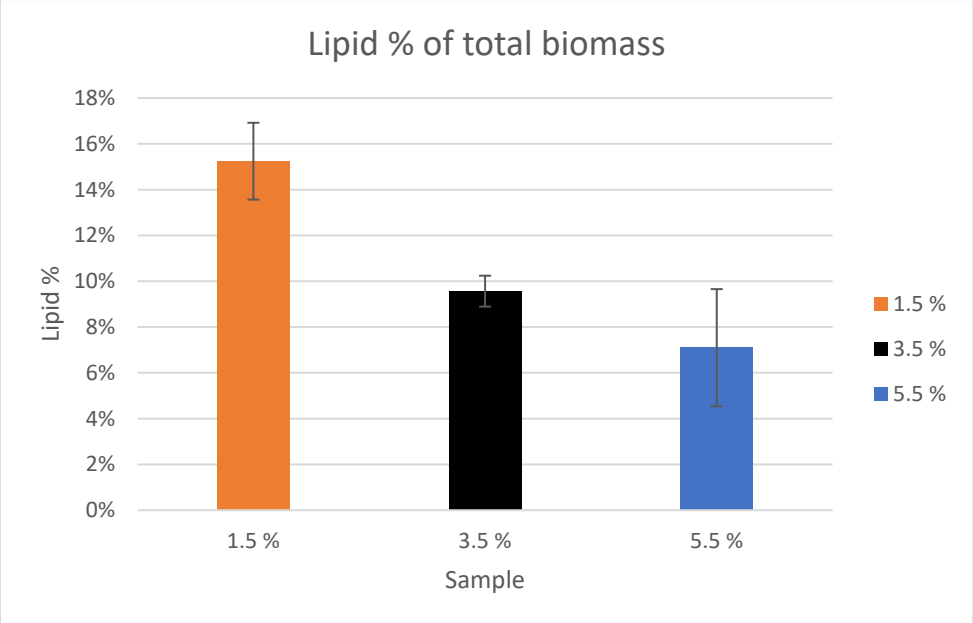

Figure 2.2.12: Calculated lipid percentage of total biomass for *P. tricornutum* grown in media of three different salinities (1.5%, 3.5% and 5.5% (w/v)) harvested following three days of growth. Lipids were extracted using the Bligh and Dyer method. N=3, error bars represent 95% confidence intervals.

Figure 2.2.11 shows that the medium salinity treatment (3.5% (w/v)) clearly had the most biomass (mean 79.43 mg), followed by high salinity (34,94 mg) then low salinity with the least (28.57 mg). Figure 2.2.12 shows that low salinity had the highest lipid percentage of biomass, followed by medium salinity then high salinity.

Cell size measurements done on imaged cells measured with "imageJ" are graphically plotted for each salinity treatment in figure 2.2.13.

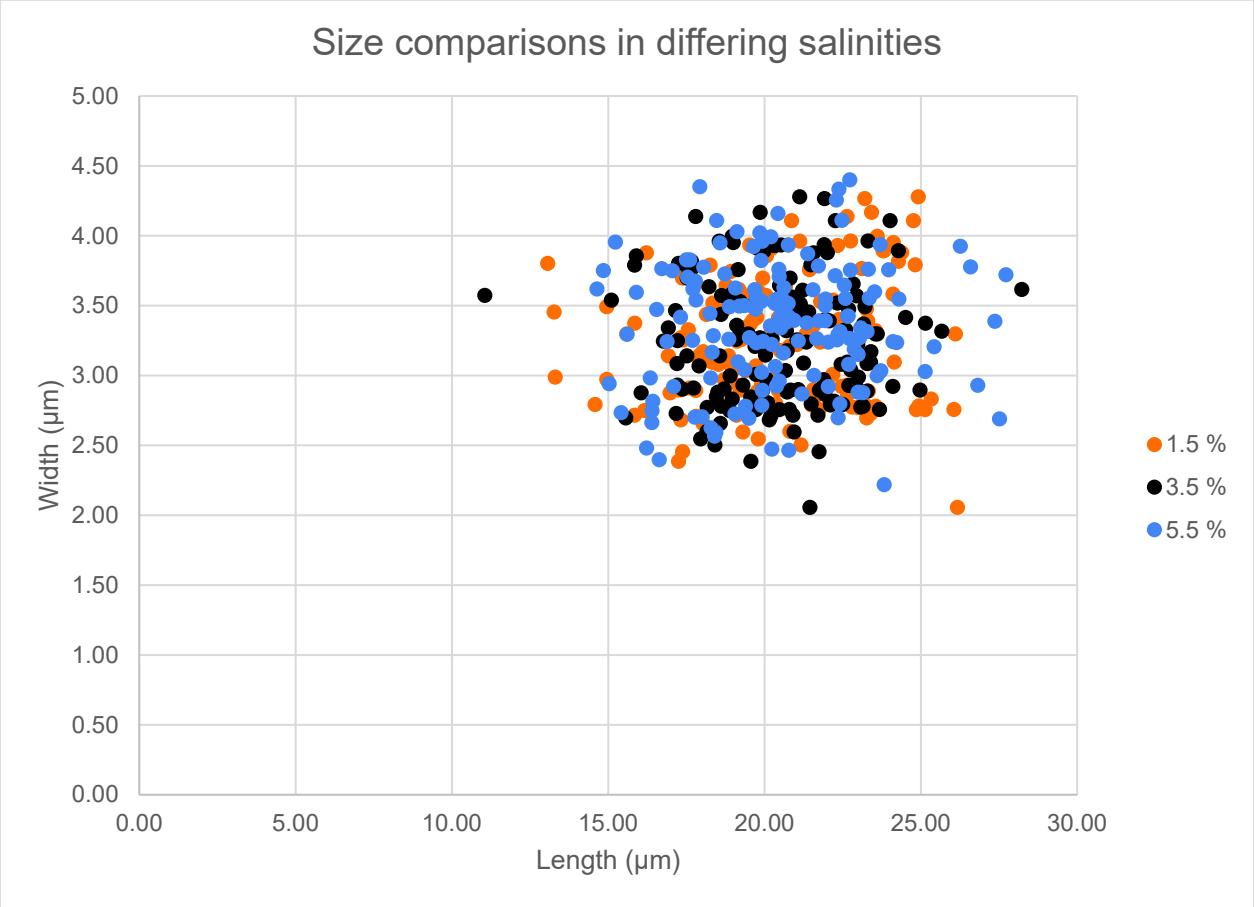

Figure 2.2.13: Length and width of cells grown in different salinities (1.5%, 3.5% and 5.5% (w/v)) measured from microscope pictures using "imageJ". N=150.

Figure 2.2.13 shows that salinity treatment has no clear correlation with diatom cell size when observed through microscopy.

2.2.9 GC-MS

Fatty acid composition of *P. tricornutum* in low (1.5% (w/v)), medium (3.5% (w/v)) and high (5.5% (w/v)) salinity was analyzed and presented in figure 2.2.14. The most prominent fatty acid in all samples were eicosapentaenoic acid (EPA) (C20:5ω3, 26.67-32.21%) followed by palmitoleic acid (C16:1ω7, 22.16-25.60%), palmitic acid (C16:0, 11.09-18.27%), then cervonic acid (DHA) (C22:6ω3, 6.91-9.23%).

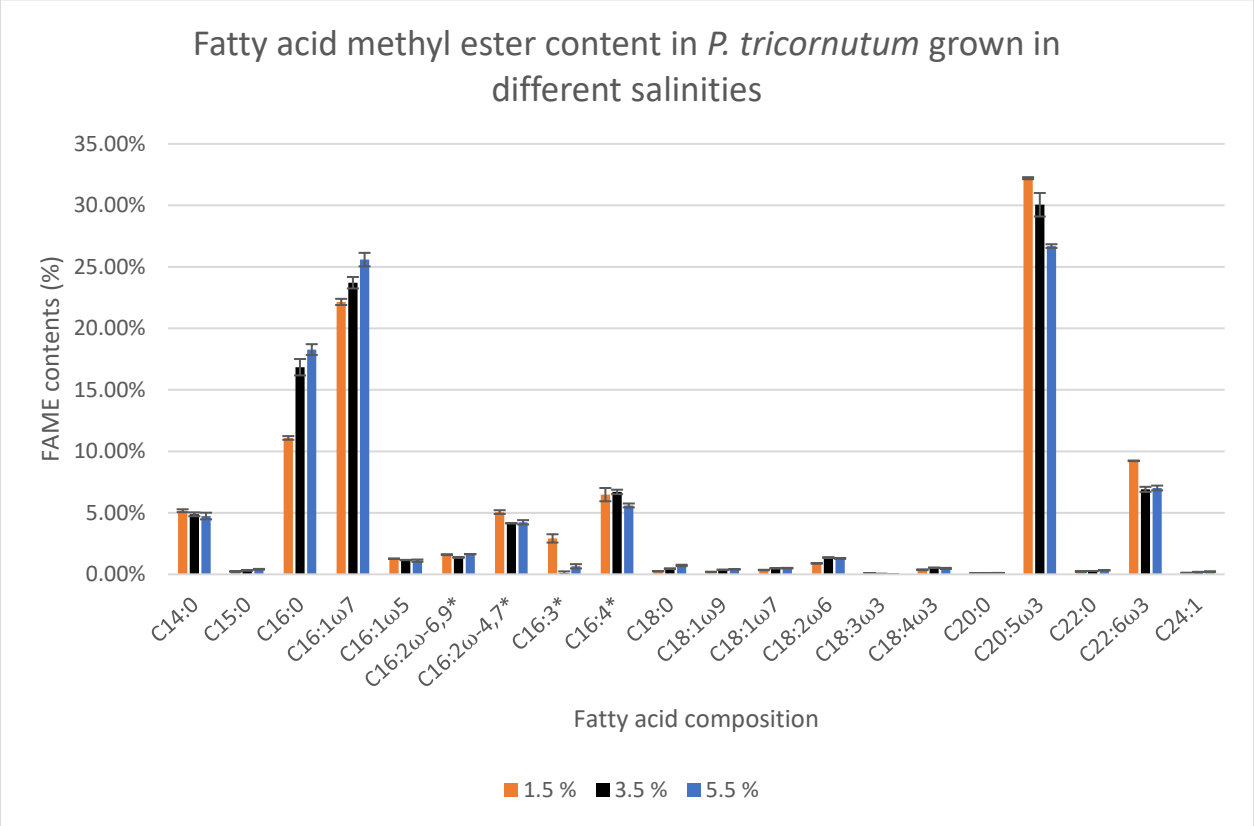

Figure 2.2.14: Results from fatty acid methyl ester (FAME) analysis of lipids from *P. tricornutum* grown in low salinity (1.5% (w/v)), control (3.5% (w/v)) and high salinity (5.5% (w/v)). Fatty acid compositions marked with "*" has a tentative ID. The tallest peaks in descending order: eicosapentaenoic acid (EPA) (C20:5ω3, 26.67-32.21%), y palmitoleic acid (C16:1ω7, 22.16-25.60%), palmitic acid (C16:0, 11.09-18.27%) and cervonic acid (DHA) (C22:6ω3, 6.91-9.23%). N=3, error bars represent 95% confidence intervals.

The summarized findings of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) is presented in figure 2.2.15.

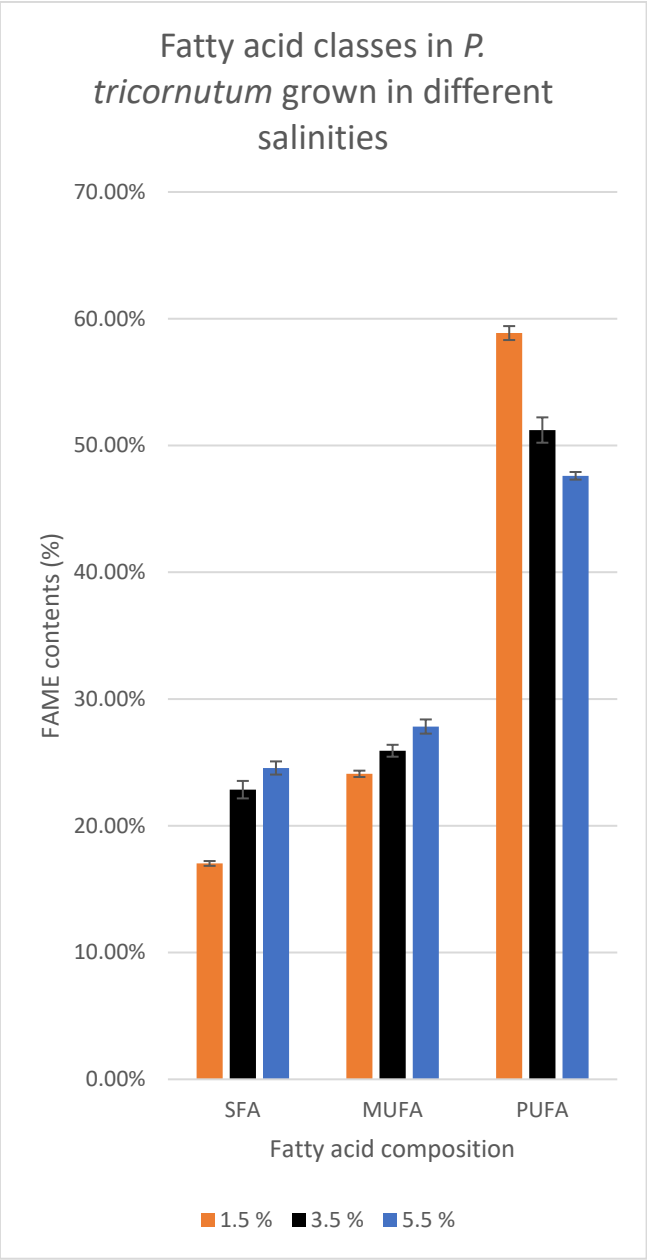

Figure 2.2.15: Results from fatty acid methyl ester (FAME) analysis of lipids from *P. tricornutum* grown in low salinity (1.5% (w/v)), control (3.5% (w/v)) and high salinity (5.5% (w/v)). Summary of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA). N=3, error bars represent 95% confidence intervals.

Figure 2.2.15 shows that most fatty acids in low and medium (control) salinity treatments consist of PUFAs, with low salinity treatments producing the most PUFAs of the treatments. High salinity treatment produced the most SFAs and MUFAs, which together make up a majority of its lipid profile.

3 pH methods & results

3.1 Methods

3.1.1 Experimental setup

Algae growth conditions were set up the same way as in the salt experiment chapter 2.1.1: Growing algae and experiments.

Following preliminary experiments, the following buffers and pH values were decided upon (summarized in table 3.1); HEPES 8.1, HEPES 7.1, MES 6.1 and Citrate 5.1. Buffer solutions were made by diluting the buffers in 1:3, diluting with F/2 media in 160 mL (120 mL F/2, 40 mL buffer) stocks. F/2 nutrients for 40 mL media was added, then the pHs of the solutions were adjusted with HCl and NaOH, pH measured with a "Sension+ PH31 pH-meter" by HACH. Mock was made by replacing the buffer with Milli-Q H_2O. Upon repeating the experiment, stocks of MES and Citrate with their pH set to 8.1 were made to test for buffer toxicity (to see if the buffer had toxic effects in alkaline conditions).

Table 3.1: Buffer solutions used for pH experiment. The Citrate and MES buffers set to 8.1 were used to test for buffer toxicity at "normal" pH levels.

Treatment	Buffer	F/2 amount (mL)	Buffer volume (mL)	Final stock volume (mL)	Final buffer concentration (M)
pH 8.1	HEPES, 0.1M	120	40	160	0.025
pH 7.1	HEPES, 0.1M	120	40	160	0.025
pH 6.1	MES, 0.1M	120	40	160	0.025
pH 5.1	Citrate, 0.1M	120	40	160	0.025
Ctrl	-	160	0	160	-
Mock	Milli-Q H_2O	120	40	160	-
Citrate 8.1	Citrate, 0.1M	120	40	160	0.025
MES 8.1	MES, 0.1M	120	40	160	0.025

From these stocks desired volume (33 mL for the initial experiment and 40 mL for the repeat) were transferred to three separate 50 mL Falcon Tissue Culture Flasks and algae in exponential phase was transferred to get a start concentration of 50 000 cells/mL (496 µL for the initial experiment and 893 µL for the repeat). The algae were grown under OSRAM HQI-BT 400 W/D PRO lights with a light intensity of 100 100 ± 10 $\mu mol \cdot m^{-2} \cdot s^{-1}$.
Cell growth and, for the repeat experiment, Fv/Fm for these samples was measured daily for 5 (for the repeat) and 6 (for the initial experiment) days, using the established flow cytometry methods and AquaPen (for the repeat experiment). Measurements of media pH were done by transferring the sample to a falcon tube, centrifuging it down using 4695 g, then measuring the supernatant with a "Sension+ PH31 pH-meter" by HACH. This measurement was done daily for the initial pH experiment, and once at the end for the repeat pH experiment.
For improved reproducibility, the AquaPen method was altered compared to the salinity experiment. AquaPen measurements were done using an AquaPen-C AP-C 100, by adding 2 mL of samples to cuvettes, then leaving these cuvettes in a box in a dark room for 15 minutes. Following 15 minutes the cuvettes were added to the AquaPen which was turned 2-3 times to mix the sample, before measuring Fv/Fm.

To check if the decrease in cell count was due to hampered growth or cell death, a staining with PI was performed during the long-term pH experiment. While this was initially not planned to be part of the experiment, we found that we had the time, ability and curiosity to perform it on day 2, 3 and 5 for the two lowest pH values (the ones most likely to have a stress response and therefore die), with time preventing us from doing measurements day 1 and 4.

3.2 Results

For the daily pH measurements, cell growth as measured in the flow cytometer is shown in figure 3.2.1 and pH measurements shown graphically in figure 3.2.2. Raw data for these plots can be found in appendix table 8 and appendix table 9.

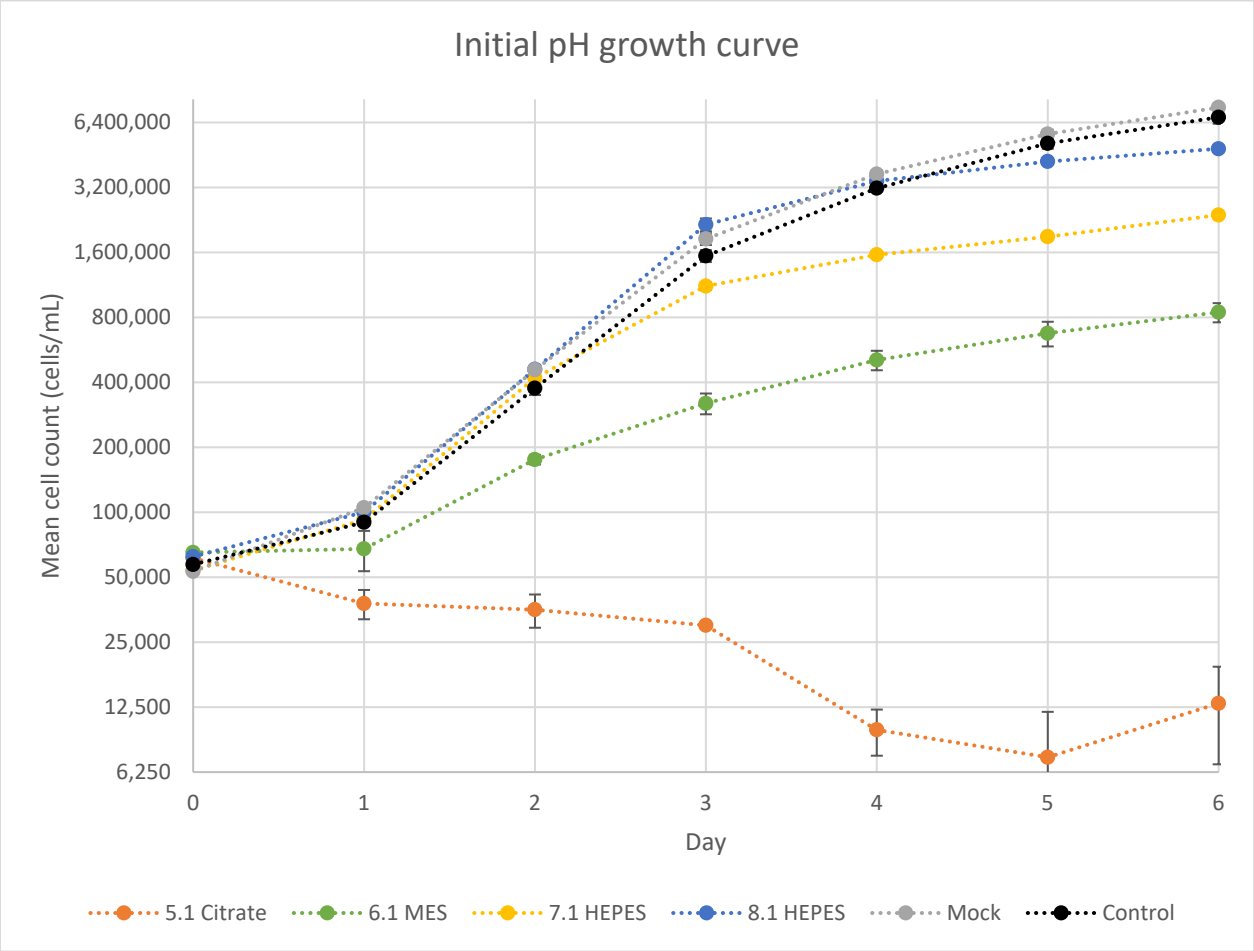

Figure 3.2.1: Cell count for pH measurement experiment with *P. tricornutum* grown in buffered media with pH ranging from 5.1-8.1 (75% media, 25% buffer dissolved in Milli-Q H_2O with added F/2 nutrients, buffer concentration 0.025M), with additional control (regular F/2 media) and mock (75% F/2 media, 25% Milli-Q H_2O with added F/2 nutrients). Y-axis (cells/mL) is $\log_2$. N=3, error bars represent 95% confidence intervals.

From the initial pH growth experiment, the flow cytometer growth measurements we got figure 3.2.1, which gave the following observations. Control and mock had a seemingly similar growth, with the mock growing a bit faster. pH 8 followed a similar growth to control but seemed to plateau at a lower cell count. Cells grown in HEPES pH 7 had similar growth to control/mock/pH

8.1 up until day 3 where it started to lag behind. Cells grown in pH 6 had a hampered growth response, not reaching 1 million cells at the end of the experiment, however the samples still showed some growth. Samples grown in pH 5 showed no growth and a decrease in cell count.

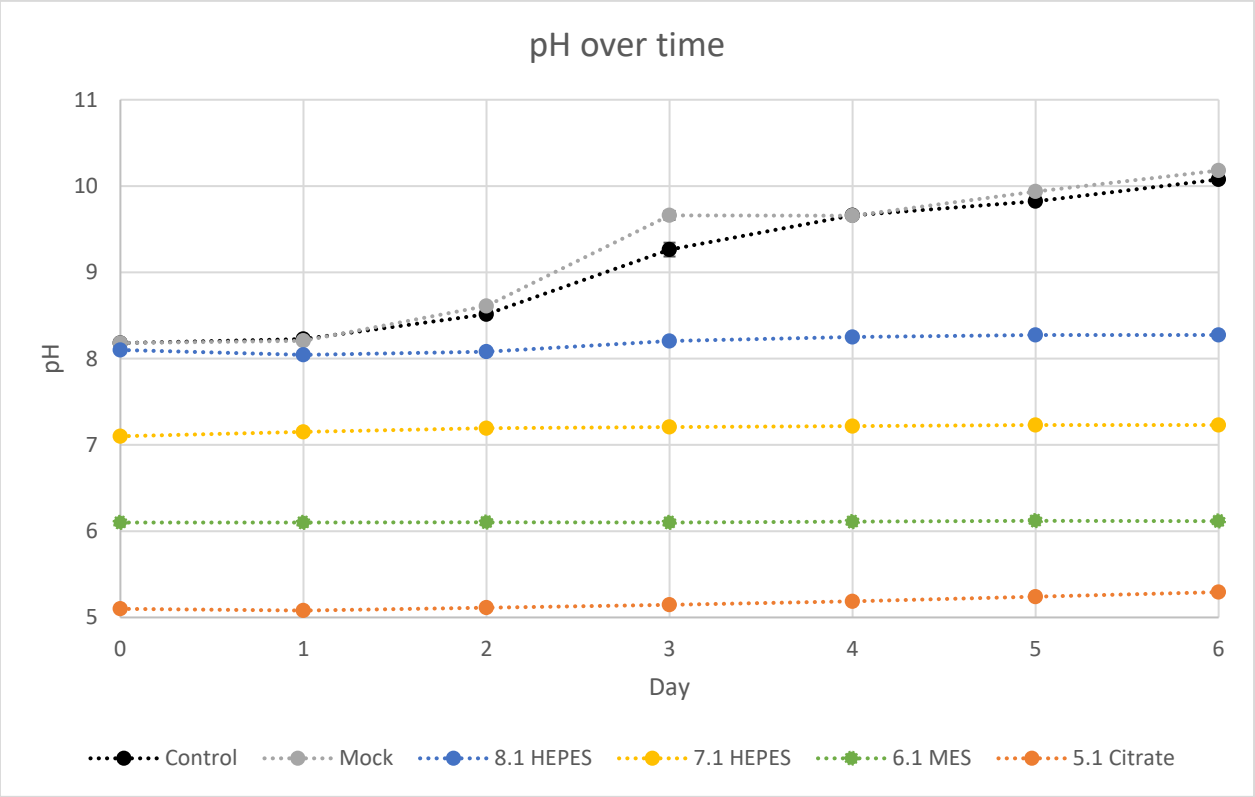

Figure 3.2.2: Measured pH over 6 days of growth in *P. tricornutum* cultures grown in differently buffered media with pH ranging from 5.1-8.1 (75% media, 25% buffer dissolved in Milli-Q H_2O with added F/2 nutrients, buffer concentration 0.025M), with additional control (regular F/2 media) and mock (75% F/2 media, 25% Milli-Q H_2O with added F/2 nutrients). N=3, error bars represent 95% confidence intervals.

Figure 3.2.2 showed that the unbuffered samples have a large change in pH, while the buffered samples showed no significant change.

Results from the repeat pH experiment are shown in figure 3.2.3 with all values listed in appendix table 10.

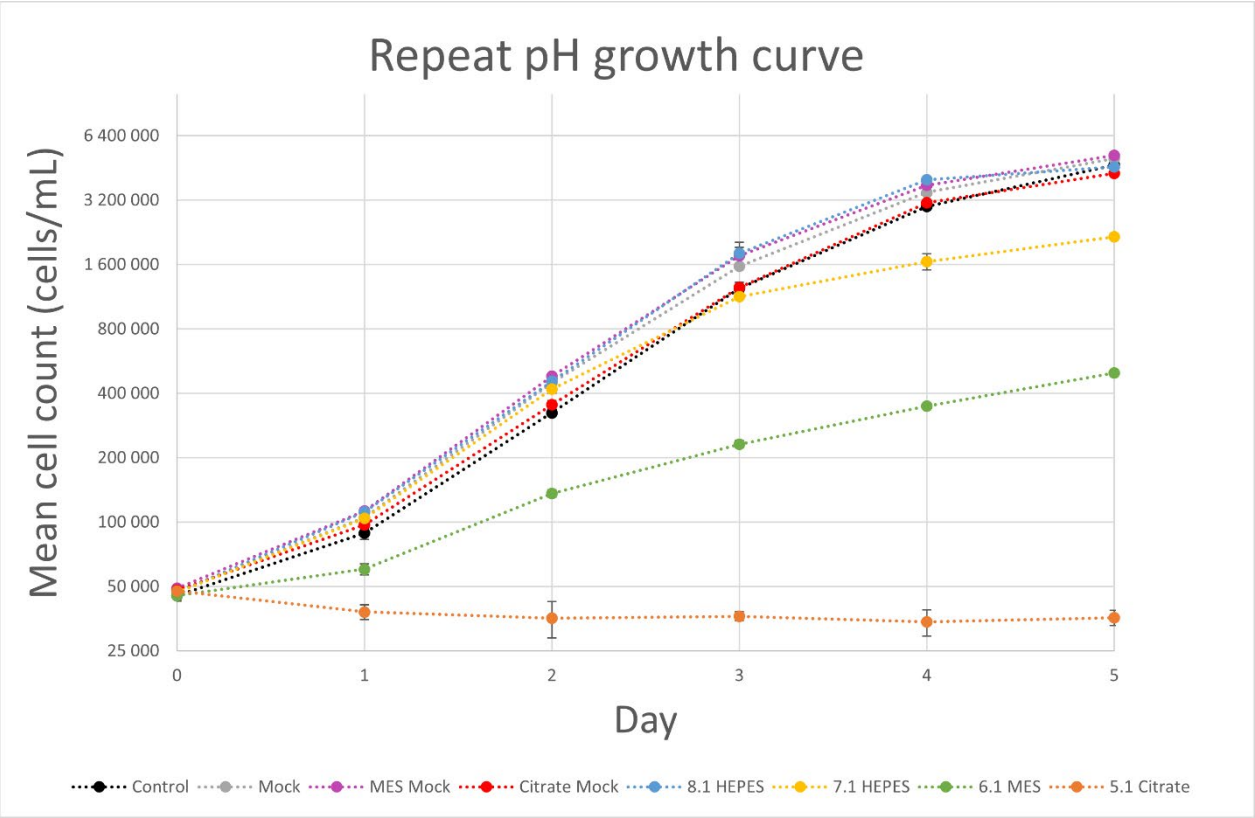

Figure 3.2.3: Cell count for repeated pH experiment, with *P. tricornutum* grown in media buffered at a pH of 5.1, 6.1, 7.1 and 8.1 as well as control, mock, MES and Citrate buffers with their buffer capacity "ruined" to test for buffer toxicity. The buffered media was made using 75% media and 25% buffer dissolved in Milli-Q H_2O with added F/2 nutrients (buffer concentration 0.025M). Y-axis (cells/mL) is $\log_2$. N=3, error bars represent 95% confidence intervals.

From the repeated pH experiment shown in figure 3.2.3 we saw similar results to the initial pH experiment. However, the cells grown at pH 5.1 did not have as drastic a decrease in cell count as in the initial experiment. Additionally, the "broken" buffer solutions (pH 8.1 MES mock and pH 8.1 Citrate mock) showed growth similar to control/mock.

Cell death stain measurements from the repeated pH experiment are shown in figure 3.2.4.

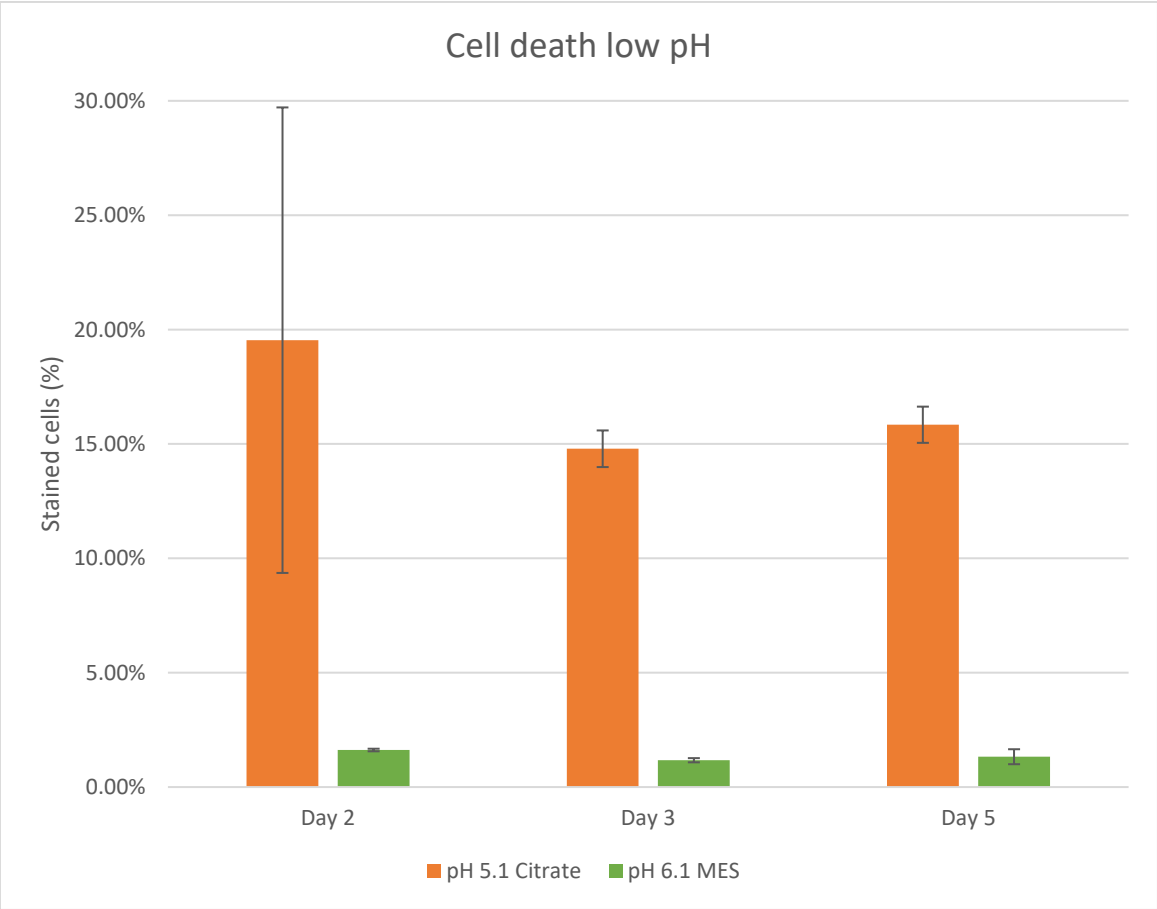

Figure 3.2.4: Cell death stain percentage measured using flow cytometry with 5 µL PI (1 mg/mL) in 200 µL of sample for *P. tricornutum* grown in buffered solutions of pH 5.1 and 6.1 (consisting of 75% F/2 media and 25% Citrate/MES buffer respectively with added F/2 nutrient) for day 2, 3 and 5 of growth. N=3, error bars represent 95% confidence intervals.

From the cell death stain shown in figure 3.2.4, we saw how the pH 5.1 samples had a stained cell percentage of ~20% for day 2 and ~15% for day 3 and 5. Additionally, we saw that the pH 6.1 samples had a stained cell percentage of ~1.5%.

For the repeat pH experiment measured pH at start and end are shown in table 3.2. There was no confidence interval for day 1 as the pH was adjusted before splitting the medium. The AquaPen Fv/Fm measurements are shown in figure 3.2.5 with raw values in appendix table 11.

Table 3.2: pH measurements at start and end of the five day experiment with Citrate and MES buffers over buffer range. There was no confidence interval for day 0 as the respective media had their pH measured from a common stock. All measurements were taken at 23°C.

Treatment	pH day 0	pH day 5	CI day 5	ΔpH
Control	8.18	9.98	0.01	1.80
Mock	8.18	10.05	0.03	1.87
MES 8.1	8.11	9.52	0.01	1.41
Citrate 8.1	8.10	9.53	0.01	1.43
pH 8.1 HEPES	8.10	8.41	0.01	0.31
pH 7.1 HEPES	7.10	7.27	0.00	0.17
pH 6.1 MES	6.10	6.18	0.01	0.08
pH 5.1 Citrate	5.10	5.19	0.01	0.09

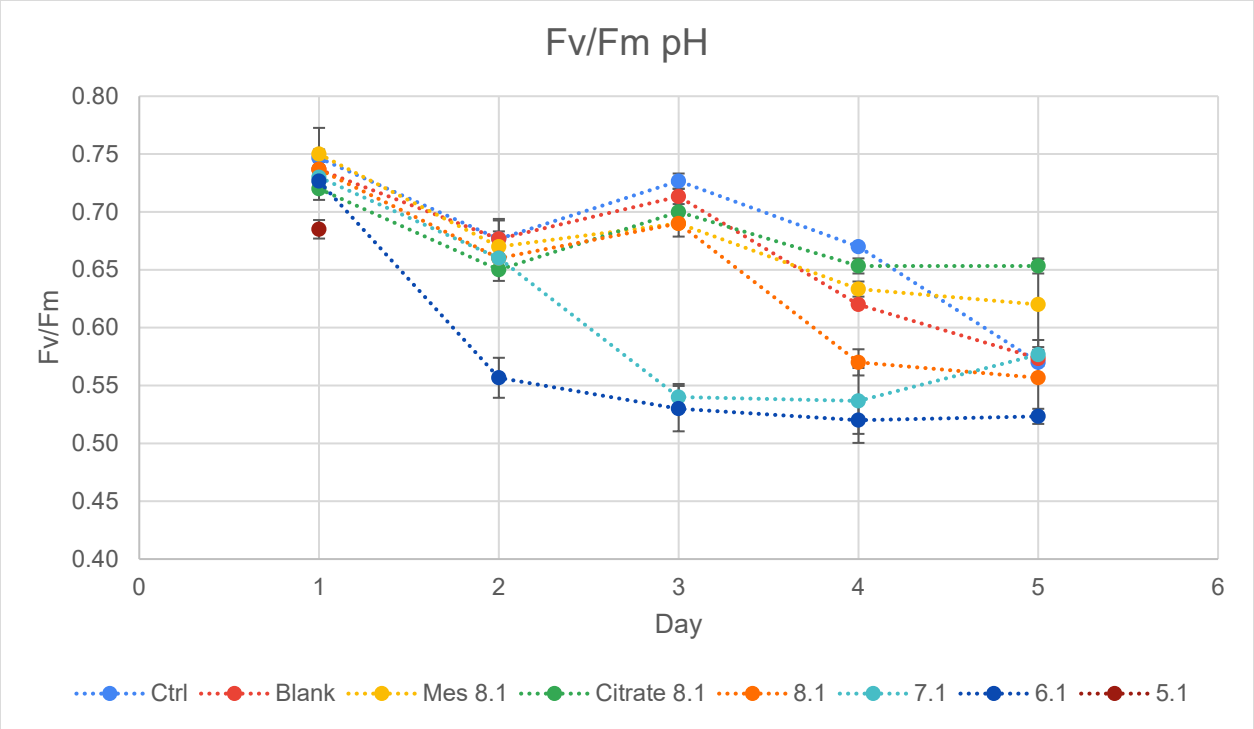

Figure 3.2.5: Fv/Fm measurements from the five day experiment with *P. tricornutum* grown in buffered media at pH 8.1, 7.1, 6.1 and 5.1 (using HEPES/HEPES/MES/Citrate buffers respectively) as well as Citrate/MES buffer brought up to pH 8.1, control and blank. All the media except control consisted of 75% F/2 media and 25% 0.1M of their respective buffer (Milli-Q H_2O in blank), with a final buffer concentration of 0.025M. N=3, error bars represent 95% confidence intervals.

As seen in figure 3.2.5 there was a general downshift in Fv/Fm over the course of 5 days. pH 5.1 had a reading the first day, but on subsequent days we were unable to get a reading from those samples.

ANOVA and Tukey HSD analysis of the Fv/Fm values gave the following (significant) results, which are also shown in appendix table 12.

ANOVA and Tukey HSD analysis of the Fv/Fm values day 2, 3, 4 and 5 gave the following (significant) results shown in table 3.3, with a text explanation and detailed data being found in the appendix (appendix table 12). Due to low cell count the pH 5.1 treatment gave no Fv/Fm values and is therefore excluded.

Table 3.3: Overview over significantly different interaction in Fv/Fm measurements for *P. tricornutum* cultivated in different buffers set to the specified pH values (8.1, 7.1 and 6.1 using HEPES, MES and citrate buffers), as well as control and mock for day 2, 3, 4 and 5 of growth. The MES and citrate 8.1 buffers were intentionally "set" outside their pH range to test for buffer toxicity. Green cells indicate one day of difference, blue cells indicate two days of difference, yellow cells indicate three days of difference and red cells indicate four days of difference.

	Ctrl	Mock	MES 8.1	Citrate 8.1	HEPES 8.1	HEPES 7.1	MES 6.1
Ctrl	-	Day: 4	Days: 3, 4, 5	Days: 3, 5	Days: 3, 4	Days: 3, 4	Days: 2, 3, 4
Mock	Day: 4	-	Days: none	Days: 4, 5	Day: 4	Days: 3, 4	Days: 2, 3, 4, 5
MES 8.1	Days: 3, 4, 5	Days: none	-	Days: none	Days: 4, 5	Days: 3, 4	Days: 2, 3, 4, 5
Citrate 8.1	Days: 3, 5	Days: 4, 5	Days: none	-	Days: 4, 5	Days: 3, 4, 5	Days: 2, 3, 4, 5
HEPES 8.1	Days: 3, 4	Day: 4	Days: 4, 5	Days: 4, 5	-	Days: 3, 4	Days: 2, 3, 4
HEPES 7.1	Days: 3, 4	Days: 3, 4	Days: 3, 4	Days: 3, 4, 5	Days: 3, 4	-	Days: 2, 5
MES 6.1	Days: 2, 3, 4	Days: 2, 3, 4, 5	Days: 2, 3, 4, 5	Days: 2, 3, 4, 5	Days: 2, 3, 4	Days: 2, 5	-

4 Glyphosate methods & results

4.1 Material and methods

The starting conditions were as described in chapter 2.1.1: Growing algae and experiments, with some alterations. Starting concentration of 300 000 cells/mL for cell death experiments/staining. The cell cycle of the diatoms was synchronized, which was done by keeping them in darkness for 48 hours before the experiment. 24 well plates were used to grow the algae in the experiments.

First a "usage concentration" of glyphosate was determined by using the concentration and dilution of "regular" roundup (roundup derived glyphosate, henceforth referred to as glyphosate^{r}) and was set at 4.8 g/L. This concentration was diluted in 1/3rds in a 24 well plate as shown in table 4.1. The dilution was performed by preparing 1 mL of algae stocks of 300 000 cells/mL in the wells of a 24 well plate, except for well A2 and C2 which were prepared with a concentration of 450 000 cells/mL. A stock dilution of use concentration glyphosate^{r} was prepared and 500 µL was added to well A2 and C2, the well was mixed and 500 µL was transferred to the next well, where the process was repeated until reaching well B6 and D6. This was done on two separate 24 well plates. Growth was measured on both plates using flow cytometry after 24 hours.

Table 4.1: Overview of 24 well plate dilution series of roundup used in preliminary experiments to determine general effects of glyphosate^{r}. The concentrations noted are the concentrations of glyphosate^{r} calculated from concentration given on the roundup bottle, with a standard use concentration being 4.8 g/L.

Row/ column	1	2	3	4	5	6
A	Ctrl	1.6 g/L	0.53 g/L	0.18 g/L	0.059 g/L	0.020 g/L
B	0.0066 g/L	0.0022 g/L	0.00073 g/L	0.00024 g/L	8.1E-05 g/L	2.7E-05 g/L
C	Ctrl	1.6 g/L	0.53 g/L	0.18 g/L	0.059 g/L	0.020 g/L
D	0.0066 g/L	0.0022 g/L	0.00073 g/L	0.00024 g/L	8.1E-05 g/L	2.7E-05 g/L

Using information gained from this dilution series, five dilutions of glyphosate^{r} were selected to test growth and cell death: 0.8 g/L, 0.4 g/L, 0.2 g/L, 0.1 g/L and 0.05 g/L (~100x dilution from usage concentration). Using these concentrations, another 24 well plate was prepared as shown in figure 4.1.1. An algae stock was prepared to get 300 000 cells/mL, then glyphosate^{r} (8.33 g/L) was added as shown in table 4.2. For the mock solutions Milli-Q H_2O (19.2 µL) was added in place of glyphosate^{r}. Dead control was prepared through heat shock treatment at 65 °C for 15 minutes using a heat block. These plates were then measured for growth (standard protocol) and cell death at start (hour 0) and after 6 and 12 hours. Cell death was measured using propidium iodide staining (elaborated on below).

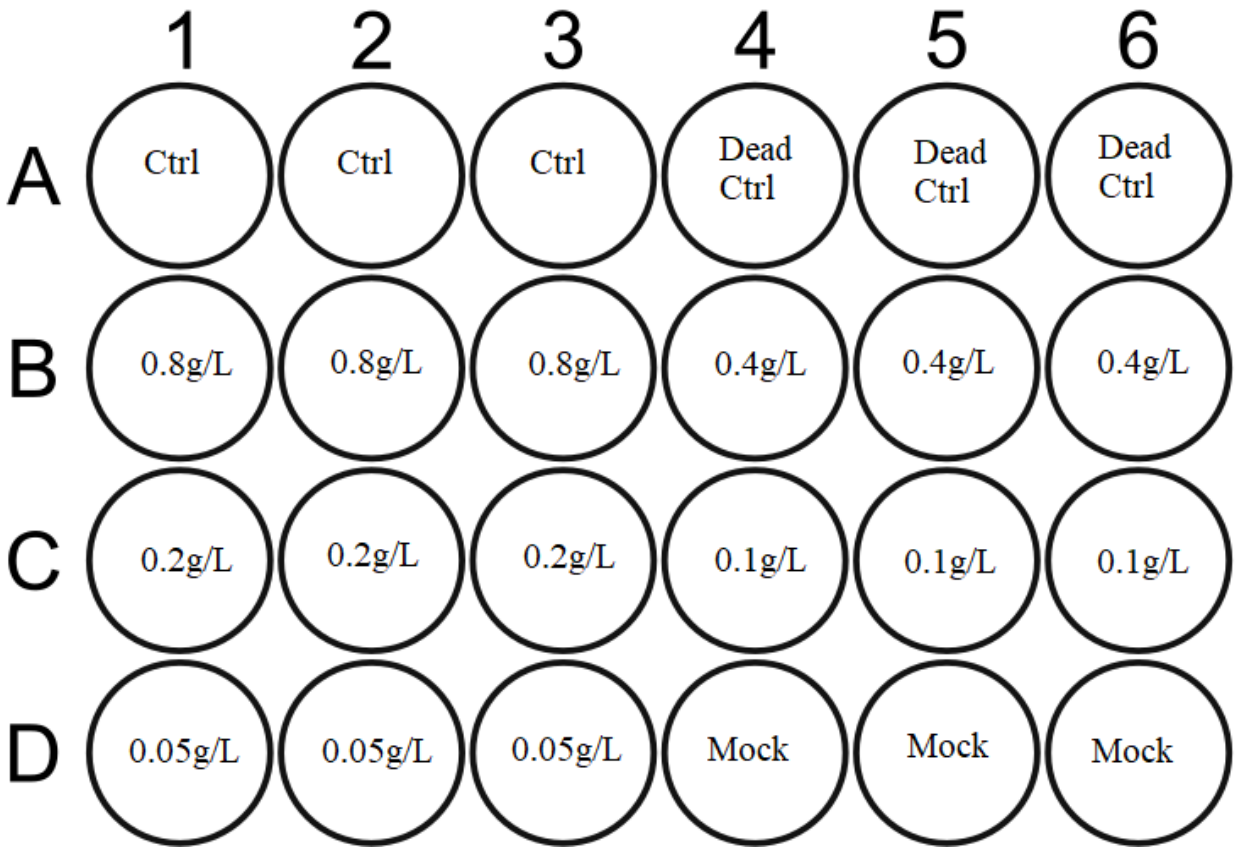

Figure 4.1.1: Experimental setup for 12 hour glyphosate^{r} experiment. Each well contained either control, dead control (Cells treated with heat shock at 65 °C for 15 minutes), mock or a concentration of glyphosate^{r} (0.8, 0.4, 0.2, 0.1 or 0.05 g/L), derived from the stock solution of roundup (8.33 g/L glyphosate^{r}).

Table 4.2: Amount of roundup stock (8.33 g/L glyphosate^r) added to each well to achieve desired glyphosate^r concentrations during both 12 hour and 96 hour glyphosate^r experiments. For the mocks Milli-Q H_2O was added in place of roundup stock. Dead control was prepared through heat shock treatment at 65 ℃ for 15 minutes. * Milli-Q H_2O

Sample	Glyphosate^r stock added (µL)
Ctrl	0
dCtrl	0
0.8 g/L glyphosate^r	19.2
0.4 g/L glyphosate^r	9.60
0.2 g/L glyphosate^r	4.80
0.1 g/L glyphosate^r	2.40
0.05 g/L glyphosate^r	1.20
0.025 g/L glyphosate^r	0.60
0.0125 g/L glyphosate^r	0.30
0.00675 g/L glyphosate^r	0.16
Mock (12 hour)	19.2*
Mock (96 hour)	9.60*

Following the 12 hour experiment, another similar experiment was set up, however this one utilized the lower concentrations of 0.4 g/L, 0.2 g/L, 0.1 g/L, 0.05 g/L, 0.025 g/L, 0.0125 g/L and 0.00675 g/L, as well as control, dead control and mock. These samples were measured for cell death/growth every 24 hours for five days. Volumes of glyphosate^r stock (8.33 g/L) added to each well is also shown in table 4.2.

Cell death staining was done using a modified propidium iodide stain protocol originally developed by fellow master student Simen F. Pettersen. His protocol used 25 µL propidium iodide (1.0 mg/mL) to stain a normalized 1 mL 1 000 000 cells/mL algae solution. The algae solution was normalized by first measuring algae concentration, then taking an appropriate volume of algae then centrifuging them, removing supernatant then resuspending the pellet in 1

mL F/2 media. For this experiment, this protocol was modified to not normalize the algae and instead use 5 µL propidium iodide (1.0 mg/mL), adding it to 200 µL of algae solution. After adding the propidium iodide, the samples were left to stain in darkness for 15-20 minutes. Following staining the samples are measured using flow cytometry, using a filter to differentiate stained (dead) cells from unstained (alive) cells.

To test these stains, we killed *P. tricornutum* through heat treatment for 15 minutes at 65 °C. These cells were mixed with living cells to create a 50% alive/50% dead cell mixture, which was stained alongside 100% alive (non heat treated, regular wild type at day 3 of growth) and 100% dead (heat treated) cell samples. The staining procedures were 5 µL stain in an undiluted 200 µL sample/mix of cells ("adapted method, high cell count"), 5 µL stain in a 200 µL cell sample/mix diluted to 300 000 cells/mL ("adapted method") and 25 µL stain in a sample/mix normalized to be 1 000 000 cells/mL ("standard method").

4.2 Results

4.2.1 Stain confirmation

Results from propidium iodide stain experiments are shown in figure 4.2.1.

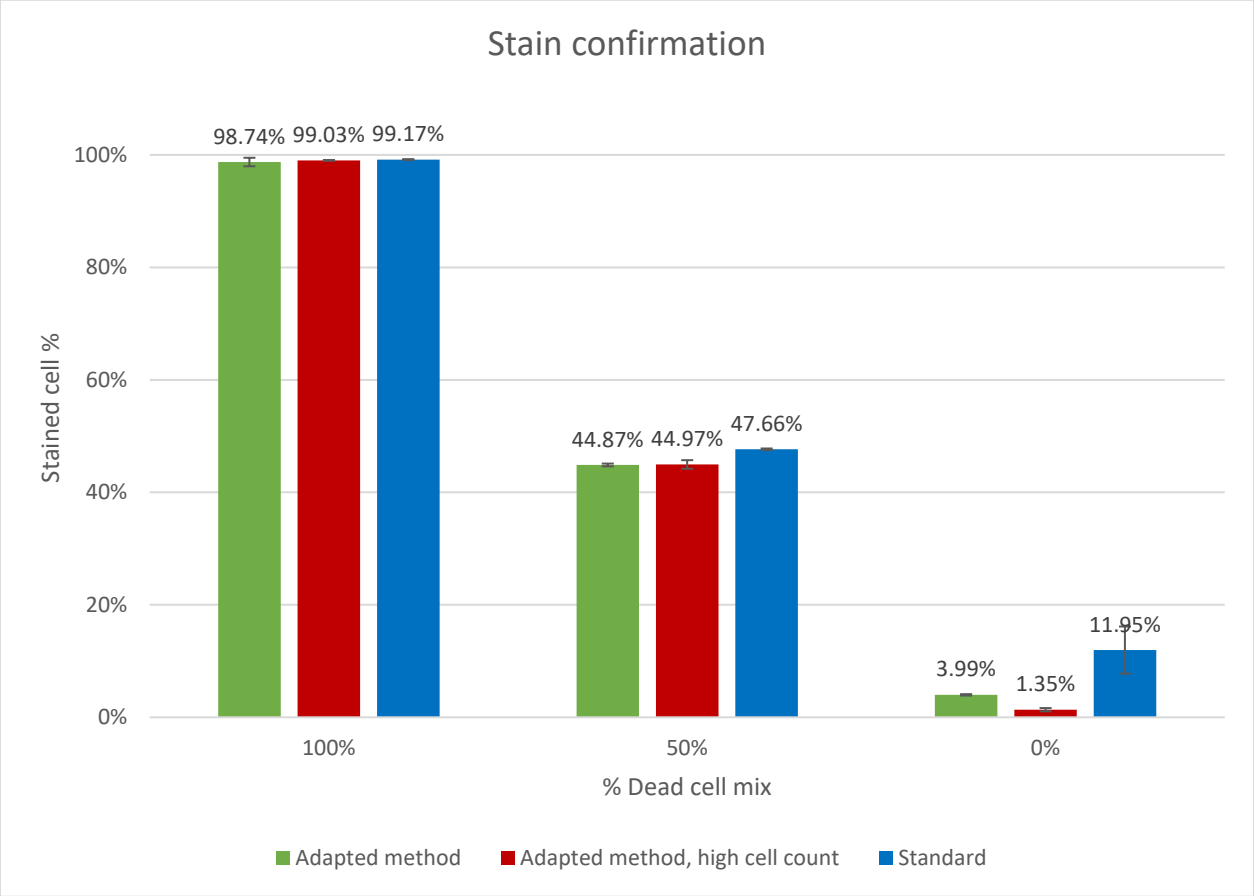

Figure 4.2.1: Results from different staining methods utilizing propidium iodide on mixes of living and dead (heat shock treated at 65 °C for 15 minutes) cells. The cells were filtered to only be cells with chlorophyll A autofluorescence. With "standard" method being a normalization to 1 million cells per mL before 15 minute staining using 25 µL PI (1 mg/mL). "Adapted method" being diluted down to 300 000 cells/mL before 15 minute staining with 5 µL PI (1 mg/mL). Finally, "adapted method, high cell count" stained the full cell concentration of ~2.5 million cells. N=3 Error bars represent 95% Confidence intervals.

Figure 4.2.1 showed the three staining methods compared to each other and shows how the adapted method had very similar stain percentage in both the normal and "Adapted method, high cell count" treatment. In contrast the normalized standard staining had a higher stain percentage in all the solutions, including the "living cell" mix.

4.2.2 Glyphosate experiments

Percentage of stained cells for the 12 hour glyphosate^{r} experiment are shown in figure 4.2.2, with dose response curves being shown in figure 4.2.3.

Mean cell count and stained cell percentage for the 12 hour glyphosate^{r} experiment different time points are shown in table 4.3 and appendix table 13 respectively.

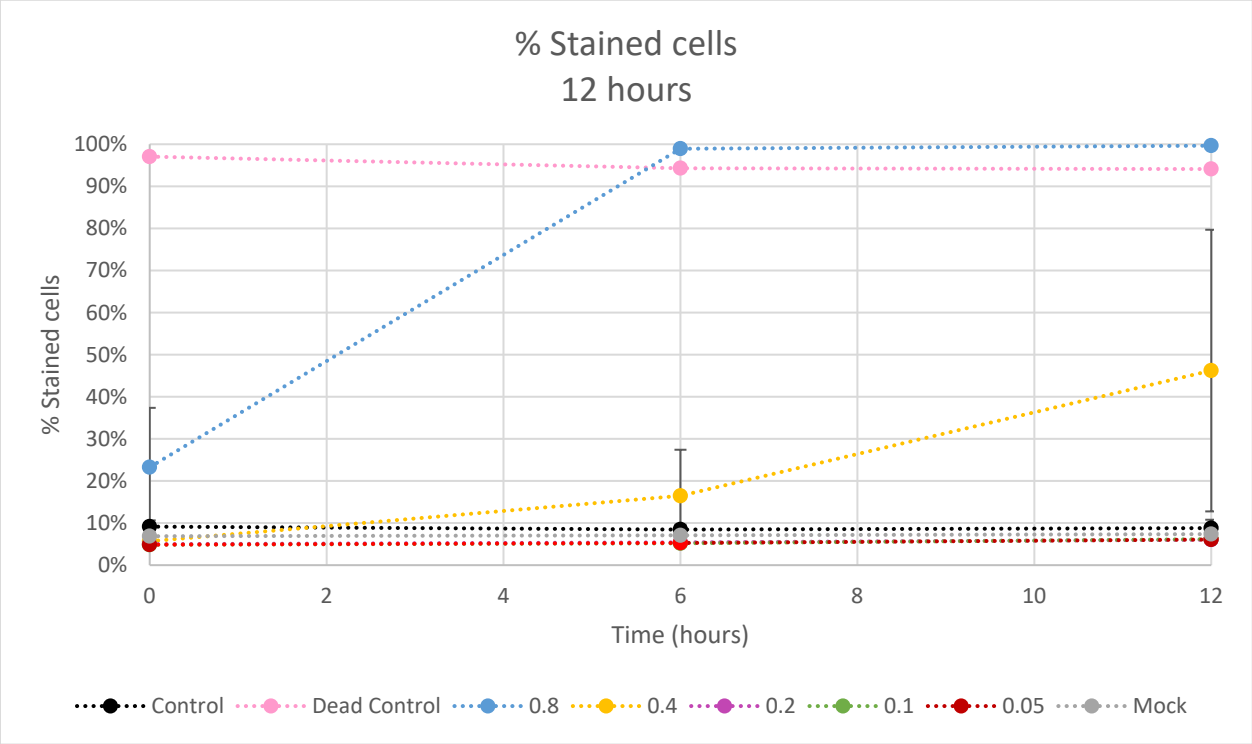

Figure 4.2.2: Percentage of stained cells at 0, 6 and 12 hours for several concentrations of glyphosate^{r} (0.8 g/L, 0.4 g/L, 0.2 g/L, 0.1 g/L and 0.05 g/L), as well as control, dead control (killed by heat shock at 65 °C for 15 minutes) and mock. The cells were stained by using 5 µL of PI (1 mg/mL) in 200 µL of sample then keeping them in the dark for 15 minutes. N=3, error bars representing 95% confidence intervals.

Figure 4.2.2 showed how the dead control treated with heat shock had an above 90% stain (~94%-97%) throughout the experiment. *P. tricornutum* treated with 0.8 g/L glyphosate^{r} started with a stain percentage of ~23%, increasing to ~99% following 6 hours, increasing to ~99.7% after 12 hours. Meanwhile the 0.4 g/L treatment started with a stained cell percent below control/mock (6.88% mock vs 5.68% 0.4 g/L), but higher than the other treatments (except the 0.8 g/L treatment). However, this increased to ~16% after 6 hours and 46% after 12 hours. The rest of the treatments show no remarkable difference from each other, mock or control.

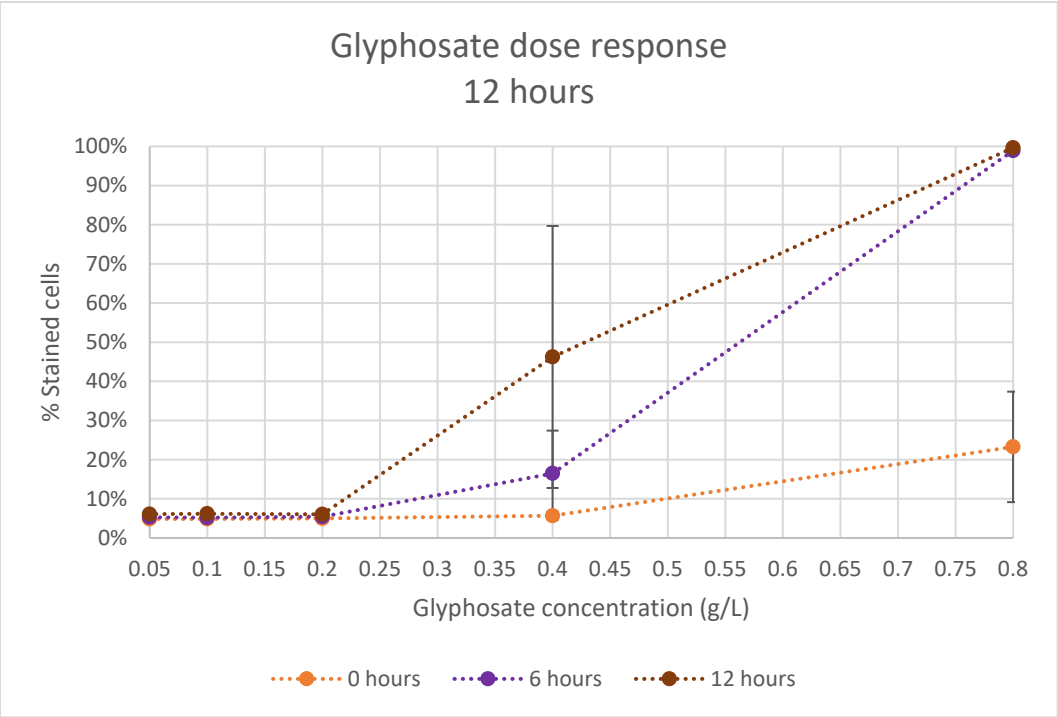

Figure 4.2.3: Dose-response curve after 0-12 hours of growth in different glyphosate^{r} concentrations (0.8 g/L, 0.4 g/L, 0.2 g/L, 0.1 g/L and 0.05 g/L), measured in 6 hour intervals. The cells were stained by using 5 µL of PI (1 mg/mL) in 200 µL of sample then keeping them in the dark for 15 minutes. N=3, error bars represent 95% confidence intervals.

Figure 4.2.3 showed similar results to figure 4.2.2, with increasing response with increasing dosage and the response in the 0.8 g/L and 0.4 g/L treatments increased over time.

Table 4.3: Mean cell count for 12 hour glyphosate^{r} experiment grown in different concentrations of glyphosate^{r} (0.8 g/L, 0.4 g/L, 0.2 g/L, 0.1 g/L and 0.05 g/L), as well as control, dead control (dControl) (killed by heat shock treatment at 65 ℃ for 15 minutes) and mock.

M1 Count	0 Hours	CI	6 Hours	CI	12 Hours	CI
Control	197 990	22 196	197 990	16 823	185 847	34 390
dControl	140 123	23 871	91 483	6 614	80 237	10 252
0.8 g/L	398 463	8 397	396 820	21 381	409 220	3 150
0.4 g/L	372 863	12 336	339 920	11 937	271 760	39 700
0.2 g/L	369 787	6 102	357 823	1 945	318 873	5 408
0.1 g/L	368 990	4 521	364 710	5 279	321 220	6 110
0.05 g/L	361 400	5 018	347 130	9 602	331 230	8 359
Mock	204 890	27 696	217 403	13 057	196 987	21 592

Table 4.3 showed how during the 12 hour measurement period there was no growth and the dead control lost cells. The glyphosate^{r} treatments had twice the start cell count compared to control/mock.

Dose response curves and cell count for the 96 hour glyphosate^{r} experiment are shown in figure 4.2.2, with dose response curves being shown in figure 4.2.3.

Mean stained cell percentage and cell count of the 96 hour glyphosate^{r} experiment at different time points are shown appendix table 14 and appendix table 15 respectively.

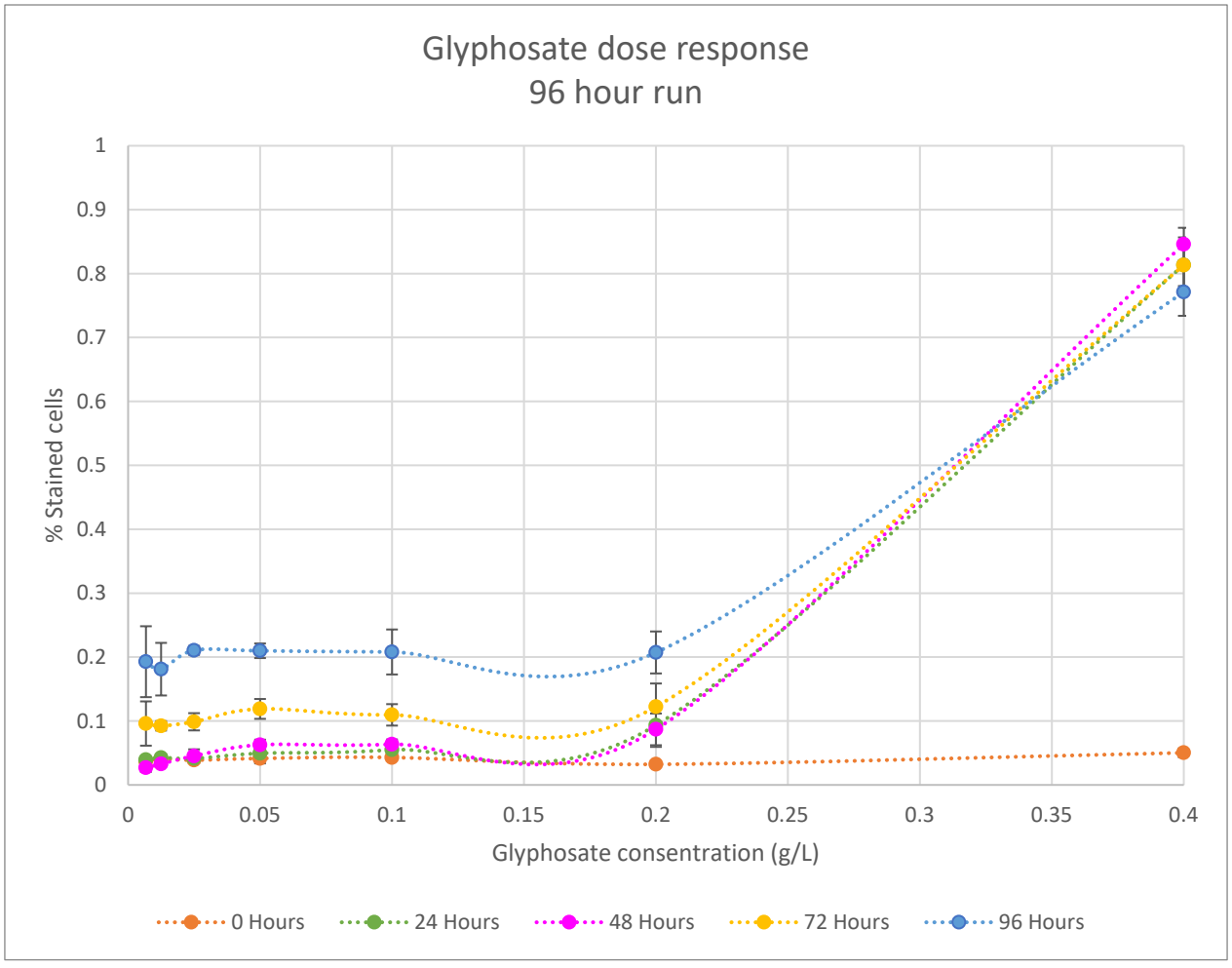

Figure 4.2.4: Dose-response curve after 0-96 hours of growth in different glyphosate^{r} concentrations (0.4 g/L, 0.2 g/L, 0.1 g/L, 0.05 g/L, 0.025 g/L, 0.0125 g/L and 0.00675 g/L), measured in 24 hours intervals. The cells were stained by using 5 µL of PI (1 mg/mL) in 200 µL of sample then keeping them in the dark for 15 minutes. N=3, error bars represent 95% confidence intervals.

Dose response curve shown in figure 4.2.4 shows similar results to figure 4.2.3, with an increasing dose and increasing time leading to increasing responses from the algae. It also showed that in the following 24 hours the 0.4 g/L glyphosate^{r} treatment had ~80% stained cells. Additionally, the other glyphosate^{r} treatments showed a very similar response to each other over time, ending at ~20% stained cell count following 96 hours.

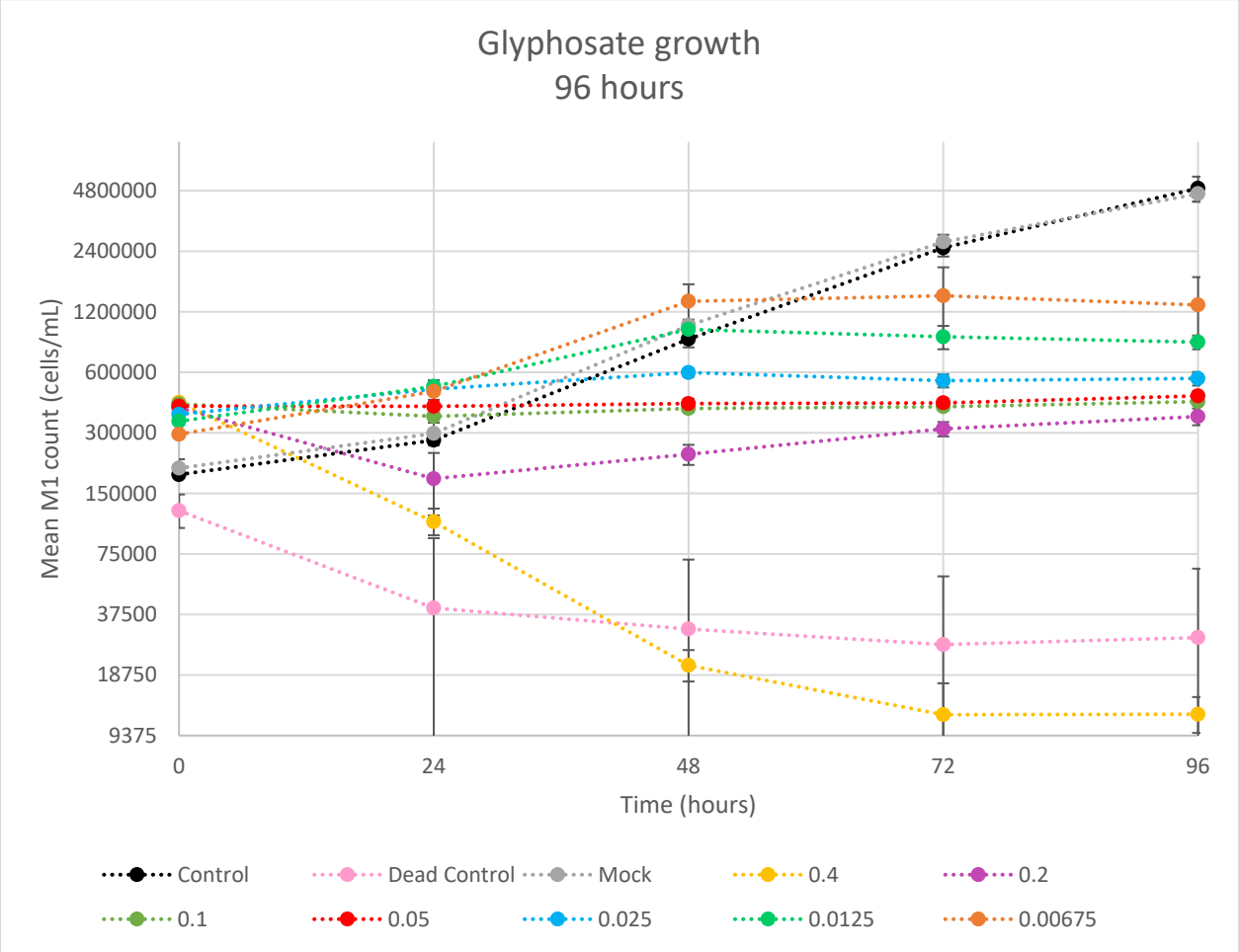

Figure 4.2.5: Cell count from *P. tricornutum* cultures in several concentrations of glyphosate^{r} (0.4 g/L, 0.2 g/L, 0.1 g/L, 0.05 g/L, 0.025 g/L, 0.0125 g/L and 0.00675 g/L), as well as control, dead control (killed by heat shock treatment at 65°C for 15 minutes) and mock over 96 hours. Y-axis (cells/mL) is $\log_2$. N=3, error bars represent 95% confidence intervals.

Figure 4.2.5 showed how all the glyphosate^{r} treatments had a higher cell count at the start compared to control and mock (~320 000-450 000 cells/L in treatments, ~210 000 cells/mL in mock/control). Following 24 hours 0.00675-0.025 g/L treatments had similar growth rates compared to control/mock. However, 0.025 and 0.0125 g/L treatment started to lag behind

control/mock in the 24 hour-48 hour period, then remaining stable. The 0.00675 g/L had greater growth up to 48 hours, then did not show any more growth for the rest of the experiment. Treatments with 0.05 g/L and 0.1 g/L did not show growth throughout the experiment, remaining very close to start cell counts. The 0.2 g/L treatment had a decrease in cell count following 24 hours, then slowly recovering to past cell count levels after 96 hours. Lastly, the 0.4 g/L glyphosate treatment led to a drastic decrease in cell count over the 96 hours, going from ~450 000 (at 0 hours) to ~20 000 (at 96 hours). Additionally, the 0.4 g/L treatment had a greater decrease in cell count compared to dead control, which had a big decrease following 24 hours then remained relatively stable.

5 Discussion & Conclusion

5.1 Salinity

5.1.1 Initial short term exposure growth experiment

Figure 2.2.1 showed a clear negative effect of high salinity, with the 4.5% (w/v) and 5.5% (w/v) treatments having a general reduced growth. The treatments with a lower than standard salinity (1.5% (w/v) and 2.5% (w/v)) grow better than 3.5% (w/v), however their confidence intervals are mostly overlapping so nothing concrete can be said about this. This is also seen in the divisions per day values (table 2.3) which showed the same observations, with 2.5% (w/v) showing the highest (1.94± 0.13) and 5.5% (w/v) showing the lowest (1.35±0.19), with control showing (1.73±0.25).

The growth of the standard cells in unmodified F/2 media with 3.5% (w/v) salinity follows an s-curve that exits its exponential growth at day 4. The other salinities follow a similar pattern except for 5.5% (w/v) where it seems to exit exponential growth at day 5.

From Brand (1984) it was shown to grow the slowest at 0% (0.80 divisions/day) and 4.5% (w/v) (1.42 divisions/day) salinity and had the highest growth at 2.5% (w/v) salinity (1.72 divisions/day) with the other salinities showing comparable growth. (Brand, 1984). This optimum salinity has been reported in other studies to be in the range from 2.8% (Qiao et al., 2016) to 4.5% (w/v) (Ishika et al., 2017). Comparing this with our results, there seems to be an agreement, with our short term 2.5% (w/v) salinity treatment having the highest growth. We saw similar patterns to Brand (1984) with the highest salinity having the lowest growth, however we also saw a difference in that our lowest salinity did not impede growth. With that it is worth noting we did not test salinity down to 0% and might have seen the same patterns if we did.

Our results seem to indicate that our strain of *P. tricornutum* grew optimally at a lower salinity than regular seawater. However, it is worth noting that for our experiments this could be an effect arising from the dilution of seawater, as the way we made our less saline media was to dilute it with Milli-Q H_2O. This might be an issue as this would impact other aspects of the seawater as it is essentially a “seawater dilution” and not just a salinity dilution.

These results might indicate that using a lower salinity seawater could be advantageous for industrial production, as this could potentially boost growth. This would be extra relevant when regular seawater is unavailable and artificial seawater must be made on site. However, while our results might indicate this, we have not tested this extensively and we only know the effects on a batch culture and seeing the effects when grown in a bioreactor could further enhance our knowledge.

5.1.2 Fv/Fm

From figure 1.5.1 we saw no significant difference between the measured samples, but from day three and onwards we saw significant differences between the samples. Even though there was significant statistical difference between the samples at different times during the experiment, all samples (except 5.5% (w/v) on the last day) fell between the normal range (0.60-0.70). This low biological variance indicates a lack of biological significance.

The Fv/Fm measurements gave results that indicated the PSII efficiency was unaffected by the salinity treatments, with the treated cells seeming healthy in general with a low variation in their measurements. However, the low growth of the diatoms in the 5.5% (w/v) salinity samples led to them having too few cells to measure for most of the experiment which might explain some of this as we expect these cells to be the least healthy.

5.1.3 Long term exposure experiment

Figure 2.2.3 showed that the low salinities had a more similar growth pattern compared to control, in contrast to the short term test, with overlapping confidence intervals during the whole experiment. Additionally, while the 4.5% (w/v) salinity treatment had a lower mean, it showed an improved growth and had an overlapping confidence interval with the lower salinities during the exponential growth phase (days 1-4). When looking at maximum cell divisions per day from table 2.5, we saw control had the highest (1.86±0.26), however it also had the biggest confidence interval. In contrast, 2.5% (w/v) had a slightly lower maximum cell divisions per day (1.83±0.04) and 5.5% (w/v) still had the lowest (1.35±0.05). In comparison to the short term experiment, the lower salt concentrations had a decreased growth rate/maximum cell divisions per day.

These results point to the possibility that the diatoms acclimate themselves to their conditions. We saw that the confidence interval for the long term experiment is smaller than in the short term experiment for the altered salinity solutions, while control had an unchanged confidence interval. This might indicate that there is a selection pressure forcing the growth to be more similar in the treatments due to a lowered cell diversity (cells more tolerant to altered conditions would be favored). In contrast this diversity would still exist in the control, leading to an increased variety in growth rates.

A hypothesis for this behavior is that the lowered salinity is not stressful enough to warrant a severe stress response. To the contrary, these results might indicate that a lowered salinity leads to less energy being spent maintaining osmotic balance, leaving more energy to grow. Additionally, the slightly lowered growth following acclimation might indicate more energy spent on building up energy reserves (lipids), however we cannot say anything for certain without testing this specifically.

5.1.4 TEM

From the pictures taken in figure 2.2.4 after acclimatization for 4 weeks, few differences were seen. In all salinities, cell structures were comparable to each other, with the exception of some unknown vesicle-like structures. These were observed mainly in lowered salinity (2.5% (w/v) and 1.5% (w/v)) and were thought to be lipid bodies. Following further testing this idea was called into question and we are not sure what these vesicle-like structures actually are. However, in general no significant differences in cell morphology between treatments could be seen.

5.1.5 RNA extraction

While performing the RNA extraction the 5.5% (w/v) salinity solutions showed reduced growth compared to previous results. We are uncertain as to what might have caused this as this was the case for all three parallels, which might seem to indicate something went wrong during the making of the F/2 solution. The diatoms seemed to adhere particularly well to the surface of the flask compared to previous experiments. This might be the cause for why we measured low growth, though we do not know why they showed this behavior. However, the other RNA

extractions were successful and the repeat of the 5.5% (w/v) salinity samples gave enough RNA for further analysis.

Following the guidelines of best practices both the A260/A280 and A260/A230 were above their recommended minimum values for all samples as seen in table 2.6. A260/A230 Were recommended to be in the range of 2.0-2.2, whereas all except one of our samples were above this range. A lower value than recommended indicates contamination with absorbance in the 230 nm wavelength. A higher would then indicate a lower amount of light absorbing particles in these wavelengths or a pollution of compounds with absorbance in the 260 nm wavelength. The cause of this increase in A260/A230 is uncertain, with the manual either suggesting the use of a blank different from RNA solute, or use of a dirty sample pedestal when blanking. Both seemed unlikely as the pedestal was thoroughly cleaned both before and after the experiment and got blanked using the elution buffer. Either way a value above the expected does not indicate pollution. If something went wrong during the blanking, there is a possibility of some pollution as the A260/A280 would be higher than expected.

Conferring with our supervisor we learned that these kinds of values are not uncommon and therefore concluded that these RNA samples were of sufficient quality to be used for further analysis.

5.1.6 Bioanalyzer

While the bioanalyzer provided sufficient RIN values (appendix table **4**) for what Novogene requested for mRNA sequencing (>4), they were not as high as we wanted/expected. This is exemplified by how our plots (figure 2.2.5 and figure 2.2.6) showed several minor peaks which the software considers degraded RNA. An explanation for this is that the Bioanalyzer kit and software was made for plant RNA, leading to the ribosomal RNAs not matching up perfectly, thus causing the software to interpret the RNA as lower quality than in reality. However, while this explains some of the lowered quality, we could still see peaks outside the ribosomal RNA “areas”. Additionally, they had baselines that were not completely flat indicating we had some breakdown of RNA. Ultimately, we concluded the RNA quality was good enough to be sent to sequencing, as they were well above the levels Novogene requested (>4).

5.1.7 RNA sequencing

Following data processing we ended up with one large spreadsheet giving an overview of genes differentially expressed when comparing treatments (lowsalt/highsalt) to control (day 1). To more easily interpret what these results actually said, we pulled out a selection of genes based on the annotations, related to NaCl and the top 5/bottom 5 most differentially expressed genes for both treatments when compared to day 1 control. This is due to these being relevant to our current study and is a lot more manageable for us to process. While this does not give the full picture of this sequencing data, it is within the scope of this thesis, which cannot take into account the entirety of the data.

In general, we saw the low salinity treatment had a lot more of an effect on the gene expression of *P. tricornutum*. This was surprising, as we expected a stronger effect on the high salinity treatment, however it is possible that it might be related to the altered growth patterns. The fact that the high salinity treatment grew slower might have meant that it was under less nutrient stress compared to control and high salinity treatment, which may have made its gene expression seem more similar to day 1 control. However, we are uncertain if that is the case and further research/experiments on later days under this treatment/experiments following long term exposure could help clarify.

As noted in the results section, the heatmap of NaCl related genes in figure 2.2.7 showed altered regulation of several genes influencing HCO_3^- and hydrogen related transporters (when compared to day 1 control), with most expression differences occurring as upregulation in low salinity. The simple explanation for this pattern is that most of these are co-transporters with Na^+, which would be in short supply in the low salinity treatment. A lowered concentration of Na^+ would therefore lead to the need for more exchangers/transporters for them to efficiently perform their tasks. Additionally, both HCO_3^- and hydrogen are related to photosynthesis (as explained in the pH introduction), meaning that an increasing amount of photosynthesizing cells would lead to a decrease in HCO_3^- and an increase in H^+. This effect could compound with the lowered Na^+ availability and lead to an increased need for these transporters. Also, the gene Phatr3_EG01952 coding for a chloride channel in the ClC family, has been downregulated for both high and low

salt concentrations. To keep homeostasis, it makes sense for passive channels to be downregulated as the external concentration of Cl^- ions would be different from normal.

Looking at the second heatmap, figure 2.2.8, of the top/bottom 5 most differentially expressed genes compared to day 1 control, we saw a lot of (10) genes transcribing unknown proteins. This presence of unknown proteins is something which permeates the whole data set and makes analysis somewhat difficult and highlights our knowledge gap when it comes to diatoms. We also saw several (3) Serine/proline-rich proteins, however their actual functions are unknown, further exemplifying this issue. In general, this lack of knowledge makes it difficult to say anything about these genes, despite their greatly altered expression.

As a recent paper by Nikitashina et al. (2022) described the metabolites found in *P. tricornutum* in hypersaline (5.0%) conditions, we looked at genes related to the production of those metabolites. They found an amino acid upregulation to be one of the common adaptations in diatoms. Pathways linked to production of amino acids as described in Bromke (2013) were cross referenced to our dataset to find differentially regulated genes seen in table 2.8. Nikitashina et al. (2022) also found production of saccharides and inositols were also found to be increased. However, we did not see these patterns in our RNA sequencing data set, implying this altered production might come from another response.

We found little upregulation of the genes involved in the synthesis of the amino acids (found by Nikitashina et al. (2022)) in high salinity treatments when compared to day 1 control. This is likely in part due to the high salinity treatment having less altered expression in general, as explained above. However, we did find some upregulation (logFC=~1) in two asparagine/threonine/methionine/lysine related genes and one glycine/serine related gene (compared to day 1 control). In the high salinity treatment, we saw a lot more genes related to these pathways having their expression altered (when compared to day 1 control) and some of the pathway related genes showed patterns. With most of the glycine/serine related genes being downregulated (except one), both affected arginine/proline related genes being upregulated and both cysteine related genes being downregulated, when compared to day 1 control.

It should be noted that as gene expression and protein production do not correlate perfectly, our results do not necessarily contradict Nikitashina et al. (2022). Additionally, their way of inducing

salt stress in the algae was different from ours which, coupled with harvesting at different time points, leaves room for the expression to change.

5.1.8 RNA analysis potential errors

We should note we did not use day 3 - 3.5% (w/v) salinity treatment as the control for the RNA sequencing analysis, which is because it had an odd expression pattern markedly different from day 1 control. We were unsure as to what caused this and have noted some potential errors which might help explain this oddity.

When it comes to RNA sequencing there are a few potential sources of error. First, the high salt concentration (5.5% (w/v)) needed to be redone by itself due to too little RNA being extracted during the first extraction. While this might be a potential source of error, it was done under the same conditions as the original experiment and thus there should not be any major difference due to this. Next, during the actual harvesting of the samples there was a decent bit of time delay between each parallel, as we harvested over filters which took time. This time spent waiting for harvesting and the time spent being harvested might have altered the gene expression. This is an especially big potential source for error as the harvesting conditions were different from the growth conditions. When it comes to RNA quality there is the possibility for RNA degradation during the RNA extraction, however as we followed protocol and added 2-mercaptoethanol, which should prevent degradation, this is unlikely to be the case. Additionally, this potential degradation was controlled by checking RIN values with the BioAnalyzer.

During the handling of the samples an exchange of samples could have occurred, though we consider this to be very unlikely. All tubes and containers were clearly marked and when transferring to a new tube a double check to confirm the labeling was done to avoid mix-up.

5.1.9 qPCR comparison

The purpose of our qPCR was to validate our findings from the RNA sequencing and to confirm that none of the samples had been mixed during the experiment. New biological replicates were used for confirmation. By looking at the results in table 2.9 we saw the same trends, albeit different values, however this does not say much as the values are not necessarily numerically

comparable. We saw a large upregulation of the first three genes, Phatr3_J45944 (Unknown diatom specific protein), Phatr3_J48827 (Unknown diatom specific protein) and Phatr3_J37038 (Clavaminic acid snythetase (CAS) domain protein), in low salinity treatment, and some low downregulation in high salinity treatment compared to control (day 1 for RNA sequencing, day 3 for qPCR). However, for the final gene, Phatr3_J54987 (Iron starvation induced protein (ISIP2B)), we observed a downregulation where we expect an upregulation in the low salinity treatment, when compared to control (day 1 for RNA sequencing, day 3 for qPCR) though a similar response was observed in the high salinity treatment. We believe this could be a consequence of different batches of seawater having been used for the qPCR and RNA sequencing. This is because this major difference in relative expression comes from an iron starvation induced protein, so we consider it likely that the different batches of seawater might have had different iron contents.

Looking at the melting curves from figure 2.2.9 we could see a couple of outliers colored differently than the majority. These had a temperature shift of up to 1 °C, most of these being near the edge of the 96 well plate. It is common for samples on the edges of 96 well plates to show differences from those “inside” the plates and we believe that is the effect we are observing here. Additionally, we find it highly unlikely for two different products to be produced in only some samples while still showing such a similar melting curve. Therefore, we conclude that the qPCR validates our RNA sequencing data, despite some minor differences in expression values.

5.1.10Lipids

Figure 2.2.12 and appendix table 7 shows that our lipid extraction resulted in ~6-9% lipid for high salt, ~9-10% for medium salt and ~14-17% for low salt treatments. The literature states that *P. tricornutum* has a lipid content of ~20-30%, however this varies by strain and growth conditions. All our samples had lipid contents below this value. Determining why we got these lower values is difficult, in part because finding the original source that states these values is difficult. In addition, we do not know a lot of the conditions these experiments were performed in. For example, many experiments looking at triacylglycerol (TAG) state that they grow the algae normally before exchanging the media for media without nitrogen, thus inducing nitrogen starvation. Additionally, when seeking to optimize lipid output, which studies seeking to study

lipids tend to do, it is beneficial to leave the cells in stationary phase for a while as this will grant them time to accumulate lipids. In contrast our study seeks to look at the effects during growth and compare while the algae are still in an exponential growing phase, meaning our findings are not entirely comparable. (Fajardo et al., 2007; Remmers et al., 2018; Vandamme et al., 2018)

During lipid extraction and cell harvesting there are a few sources where material loss could potentially have occurred. During harvesting, the cells were centrifuged many times (15+) which may have resulted in rupturing/other damage which could have caused loss of material. Additionally, removal of media was done by decanting the assumed cell-less supernatant, which again could have led to material loss, especially if cells had ruptured. However, this is mostly just relevant for the cell/volume to dry material relationships, as for the actual lipid analysis the dry weight was used, which is independent of any loss during cell harvesting.

The freeze drying went without problems and after 18 hours in the freeze dryer, the manometer showed no increase in pressure when the vacuum pump was disconnected, and samples were confirmed completely dry.

The next potential source of material loss is during the extraction process itself, where the material transference between tubes went smoothly. This is except for the medium salt samples, where a large amount of solid biomass accumulated between the organic and aqueous phases, making it difficult to remove all the organic phase. Due to this there is a possibility that some lipids were lost as we do not know if some was trapped within this accumulation of biomass, however we managed to remove the vast majority of organic phase despite this. To counteract this an additional extraction step could have been implemented to further extract and dilute the remaining lipids in the organic phase. However, from a preliminary test there were negligible amounts left after the second extraction (appendix table 16).

When adding chloroform to the samples that called for a specific volume at first and when normalizing the samples, plastic pipette tips were used. Plastics are dissolved by chloroform, meaning that any steps where chloroform came into contact with plastics had the potential to cause contamination with leachables. Thus, the number of steps where plastics were used to "transport" chloroform was minimized.

Lastly during the weighing procedure, the extracted lipids were transferred from their glass tubes to vials following drying (and re-dissolving in chloroform). This might have led to some material loss, however due to thorough rinsing we are certain all lipids were successfully transferred.

During this extraction process, other lipophilic compounds are also extracted, most notably chlorophyll, giving the lipid extracts a characteristic green color. What effect this presence of chlorophyll in the samples had on the measurements we cannot account for. However, it is assumed to be negligible.

5.1.11 GC-MS results

The GCMS results in figure 2.2.14 and figure 2.2.15 shows how the three salinity treatments had somewhat different lipid profiles, with the lipids EPA and DFA at high and medium levels respectively. Additionally, the low salinity treatment had a higher percentage of these lipids compared to the other two treatments. This comes with low salinity having a comparatively much lower percentage of palmitic acid and a somewhat lower percentage of palmitoleic acid. However, similar to earlier findings by Yongmanitchai & Ward (1991) EPA, palmitic acid and palmitoleic acid have a combined percentage of around 60%.

The general trend we could derive from the data was that salt concentration was inversely proportional to the amount of PUFAs. There was both a significant increase in PUFAS and decrease in SFA in the low salt concentrations.

There is of course the question of if this has any industrial relevance given that the medium salinity (control) treatment had by far the most biomass/lipids. However, due to the volumes of algae needed for sufficient biomass, we only tested the two extreme salinities (as well as control), with salinities around 2.5% (w/v) being of particular interest. Investigating yields specifically around this growth optimum could help inform what salinity is optimal for lipid production.

From the lipid extraction blank there were some small peaks, indicating some contamination. We assume this to be leachables from the small use of plastic consumables during the extraction process. As the blank was handled in the same way as the samples these peaks were subtracted from the samples with all values listed in appendix table 17. The methyl esterification blank showed no peaks indicating no contaminants from this part of the process.

5.1.12 Cell size vs dry weight

Figure 2.2.11 showed that the cells grown in “regular” (3.5% (w/v)) salinities produce by far the most biomass, whereas 1.5% (w/v) and 5.5% (w/v) produced relatively similar amounts of biomass, despite large differences in cell counts. Initially we thought this could be due to cell size, however measurements of cell dimensions from microscopy pictures indicate no correlation between salinity treatment and cell size as seen in figure 2.2.13. This difference in biomass is also reflected in the pellets formed during cell harvesting as seen in figure 2.2.10, with the “normal” salinity having by far the largest pellet. However, we do not think any significant difference in material loss occurred during this process, as the supernatant of the treatments looked identical. The different consistencies of the pellets, with low salinity being hard and insoluble and high salinity being light and easily soluble is also notable. We also do not know if the more intermediate treatments (notably 2.5% (w/v)) would lead to the same effect, or if this treatment would retain the lipid production of control while having a slightly boosted growth. While we believe these patterns are in some way caused by the harvesting process, we are uncertain how. Further research into this could be of interest as this would be of industrial interest provided this could increase lipid production. (Hamilton et al., 2014; Qiao et al., 2016; Yongmanitchai & Ward, 1991)

A potential answer to this difference might be found in the long term salinity experiment, where we saw that the lower salt concentrations slowed their growth rate. With the possibility that an initial “faster” growth rate leads to the individual cells producing less biomass before adaptation. Following adaptation this might have changed, however we cannot tell without harvesting the biomass from an acclimated culture. This is another area that could benefit from further research.

5.1.13 Conclusion salinity

Our findings indicate that high salinity led to a decrease in growth rate, in contrast to low salinity conditions which led to an increase in growth rate. Analyzing the lipid contents on day three we saw a significantly lower amount of dry weight in low and high salinity treatments compared to control. The low salinity treatment produced a lower amount of dry weight than control despite

higher cell count. Comparing mg lipid per mg biomass we saw that low salinity treatment had a significantly higher amount compared to control and high salinity treatment, which were comparable. Control had the highest lipid amount on account of the higher total biomass. Further researching the cause behind this difference in biomass and investigating lipid yield following extraction would be interesting areas of further research.

Analysis of fatty acid composition showed that the low salinity treatment had a lower percentage of SFA and MUFA with a higher percentage PUFAs when compared to control, with high salinity treatment showing the opposite response.

RNA sequencing showed high salinity conditions had a lot less impact on expression compared to low salinity conditions, which had a lot more drastic changes in gene expression, when compared to day 1 control. Of the differentially expressed genes we saw several proteins associated with sodium and chloride, with most of these being transporters, with these differences being strongest in the low salinity treatment.

Many differentially expressed genes were coding for proteins with unknown function, such as Phatr3_J45944 which was the highest upregulated gene in low salinity, highlighting the need for further research. We have very clear knowledge gaps, despite the importance and interest in these algae.

5.2 pH

Figure 3.2.2 shows that the buffers we selected were functional at the chosen pH values, however we also saw a distinct increase in pH in the control and mock from ~8 to ~10. This pH increase was expected from a photosynthetic organism as most dissolved CO_2 takes the form of hydrogencarbonate (HCO_3^-). As explained in the introduction, hydrogencarbonate consumption results in the release of OH^-, which would cause the solution to become more alkaline. Therefore, we saw that as the cells grow, more hydrogencarbonate is consumed and thus we saw an increase in pH. Interestingly their growth does not seem to be hampered by these high pH conditions, which may be related to how *Phaeodactylum* seems to be unusually resistant to alkaline conditions compared to other diatoms (Goldman, Azov, et al., 1982). This alkaline resistance seems to confer a competitive advantage over other diatoms which would be especially useful in bloom conditions where pH would rise significantly (Goldman, Riley, et al., 1982) However, it is worth noting we did not test for any buffered alkaline solutions due to mainly wanting to investigate acidic conditions, difficulty finding buffers, not anticipating these results and a lack of time. However, given research by Goldman, Riley, et al. (1982) *P. tricornutum* seemed to grow fine in pH up until 10.3 which they state as their threshold, findings that may help supplement what we found.

Both growth experiments (figure 3.2.1 and figure 3.2.3) showed a decrease in growth with decreasing pH, with HEPES buffered pH 8 growing similarly to mock and control. Meanwhile both pH 5.1 and 6.1 had a lowered growth from day 1, with pH 6.1 not reaching a million cells and pH 5.1 appearing to lose cells. These results were as expected, with a pH below normal conditions (which are assumed to be optimum) having an adverse effect on growth, an effect that intensifies as the pH decreases. Meanwhile pH 8.1 having a normal growth (normal being similar to mock and control) was expected due to this being the pH of the ocean which *P. tricornutum* should be adapted to.

From the PI cell death staining (figure 3.2.4) we saw that citrate buffered pH 5.1 had a lethal effect on the cells, resulting in ~15%-~20% cell stain. However, these results should be taken with a pinch of salt as we lacked a control and did not have the time to test regular pH citrate buffer to see if it had any baseline lethal effects. Nevertheless, we consider these to be interesting observations. Also note that usage of PI stain in low pH has not been validated.

5.2.1 Fv/Fm

When measuring the Fv/Fm from the samples they were not diluted. When the concentration of cells became too high, some of the emitted light from the cells got reabsorbed, giving a lower Fv/Fm than reality. When using a PAM fluorometer the recommended number of cells is 1 million cells per mL or below. This will correspond to day 4 being well above the recommended amount and will thus lower the Fv/Fm of the samples that grew well. The general downtrend from the control and blanks might thus be explained by their cell concentration.

From the Tukey HSD analysis of the individual day Fv/Fm measurements from figure 3.2.5 (findings in table 3.3) we saw no significant difference between the samples on day one. Day two we saw a difference between all the samples and the pH 6.1 treatment, however following this the relationships become a lot more complicated, with every sample having various significant differences with a variety of others. It is also worth noting that while there might be some statistical significance here, most of the samples were within healthy Fv/Fm levels (0.6-0.7) indicating that there is a lack of biological significance. However, the pH 6.1 treatment did dip below this range on day two and stayed there throughout the rest of the experiment, implying that the treatment had some effect on the PSII. We also saw this same response for the pH 7.1 treatment day three onwards. Several samples dipped below this point from day 4 onwards as well, however at this point they were out of exponential phase, opening the possibility for nutrient deficiencies to have an impact as well as a too high cell concentration. From the second day onwards the pH 5.1 treatment showed “low value” likely due to the low cell count. Therefore, we cannot really do any statistical analysis with the pH 5.1 treatment.

5.2.2 Buffers

We were uncertain of the chemical effects that the buffers would have on the diatoms, other than pH. The buffers chosen (Citrate, MES, HEPES) were picked based on their pH range and being considered to be unharmful to biological tissue (Good et al., 1966). To see if the buffers themselves had any adverse effect on the diatoms, we tested the growth of the diatoms with the buffers added to solution and brought back to original pH (8.1).

For HEPES buffer this is the same as the experiment sample HEPES 8.1 and has the limitation that in this sample HEPES will buffer the solution and the pH would not rise in the same way that the control and mock did.

For both Citrate and MES, the buffer was out of the buffer range when the pH was brought up to pH 8.1. As such the pH in these samples were raised the same way as the control. This however meant that only the weak base of the buffer was present in solution and by extension, none of the weak acid, meaning any effects from the weak acid would be overlooked.

From the growth curves in figure 3.2.3 the HEPES pH 8.1, Citrate pH 8.1 and MES 8.1 followed the same general growth as control and mock. By the use of an ANOVA test we saw that at day 0 the samples were not different to each other (p value 0.2391). Day 1, 2 and 3 Citrate had a bit lower growth than the rest, while the others were equal. Onwards from day 3 the Citrate were still lagging a bit behind. The HEPES pH 8.1 outgrew mock in day 4, but plateaued and lagged behind at day 5. We concluded that the HEPES and MES buffers had the same general trend of growth as the control, even though there were some individual differences. Citrate buffer had a somewhat negative effect on the growth of *P. tricornutum* from day 1. However, due to the extreme response from the cells grown at pH 5.1, we believe that this effect has no major impact on the growth patterns.

As we observed in our salt experiment, diluting the sample with Milli-Q H_2O we saw an increased growth in our mock compared to our control. As all the buffers essentially diluted the salt concentration it is possible that this is one of the contributing factors for increased growth in some of the samples.

5.2.3 Conclusion pH

Our findings indicate that the lower the pH from pH 8.1 the worse *P. tricornutum* grows, with our lowest pH treatment of 5.1 having a lethal effect. *P. tricornutum* will increase the pH of its media as photosynthesis consumes hydrogencarbonate, up to a PH of ~10. We observed lowered Fv/Fm values for pH 6.1,indicating that this lowered pH had a negative impact on the photosynthetic capability of the algae. pH 5.1 gave no Fv/Fm readings at all due to low cell count. HEPES and MES buffers seem to have a biologically insignificant effect on *P. tricornutum* growth. Citrate buffer had a mild negative impact on *P. tricornutum* growth, however this effect is biologically insignificant compared to the full effect of the pH 5.1 treatment. It would be interesting for future research to investigate pH effects on lipids and gene expression, especially if this investigated higher pH values as well.

5.3 Glyphosate

Figure 4.2.2 and figure 4.2.3 showed that cells were rapidly stained by PI when grown in 0.8 g/L glyphosate^{r}. Additionally figure 4.2.4 and figure 4.2.5 showed cells grown in 0.4 g/L glyphosate^{r} start staining in increasing amounts over time. Figure 4.2.4 showed that at lower concentrations of glyphosate^{r} cells do not stain nearly as quickly, however figure 4.2.5 showed that even at the lowest concentration (0.0675 g/L) growth is significantly impeded. This might result from the glyphosate^{r} levels not being high enough to kill all the cells, however it serves as a sufficient stressor to impede cell growth.

Figure 4.2.4 showed a glyphosate^{r} concentration of 0.2 g/L was not enough to result in total cell death stain, even following the full 96 hours, while the 0.4 g/L concentration resulted in almost complete cell death stain following 24 hours. This could indicate that more intermediate responses exist in the concentrations between these two (such as 0.3 g/L) which would be an interesting area to explore for future experiments. The same can be said for concentrations below 0.0675 g/L, where finding the exact area roundup stops adversely affecting the cells would be interesting.

With this we could see that, as expected, *Phaeodactylum tricornutum* is susceptible to glyphosate^{r} and that it likely has a lethal effect at higher concentrations while impeding growth even at lower concentrations. We do not know at what concentrations this growth inhibiting effect ends and looking further into this would be interesting.

Another result of interest is how the samples with added glyphosate^{r} had an increased number of cells at the start of the experiment compared to controls and mock, with the higher concentrations having over twice as many starting cells. The cause of this is unclear, as this increase was only seen in the roundup treatments across both experiments, with parallels of the same treatment being internally consistent. It is difficult to determine the cause of this as the increase in cells is not proportional to roundup concentration added and it is unlikely to be a pipetting error due to the internal consistency. One possible explanation is that the cells being synchronized may indicate that it induces cells to divide, however this is only speculation and we have seen no definite proof of this. This odd interaction is something that could be of interest in future research, as inducing cell division like that could have interesting applications.

Figure 4.2.1 showed that the propidium iodide staining protocol developed by fellow master student Simen F. Pettersen for detecting dead *P. tricornutum* cells was effective. We say this because the vast majority of cells in the 100% dead sample were stained (lowest percentage stain being 98.74%) with the dead/living cell mix having roughly 45% stained cells. It is worth noting that the "alive" sample also had some stained cells, however this is likely due to a certain amount of dead cells present in all populations, in addition to the stain having a lethal effect, figure 4.2.1 also showed how our modification of the method had no major impact on the results, and was deemed suitable for use. Therefore, we assume that any stained cells are dead cells.

5.3.1 Limitations

With these results in mind, it is worth noting some limitations of our experiments and what we might have done differently given more time. At first our intent was to find the concentrations of glyphosate^{r} where cells would immediately die and where it would have no effect. However, we did not have the time to find the exact concentration where immediate death takes place, which is likely between 0.2 and 0.4 g/L. In addition, while we found this "threshold" point where complete cell death stopped, cells still died at some level even at the lowest concentration we tested. While this is interesting, we did not have the time to test even lower concentrations where this low level of cell death would cease. Additionally, we only tested these concentrations over the short term and seeing how *P. tricornutum* reacts or adapts in the long term would have been interesting.

It is worth mentioning we did not test the effect of glyphosate but that of roundup. This means that cells exposed to pure glyphosate may have different reactions, especially considering other additions to the roundup would enhance herbicidal properties. With these additional compounds probably influencing the cell response to the roundup and we cannot be entirely sure on what exactly is in it and what effects that causes. However, one could argue that testing pure glyphosate would be somewhat unrealistic, as pure glyphosate is unlikely to ever be used as an herbicide. Other glyphosate-based herbicides other than roundup are likely to have a similar effect, as they would still use glyphosate as the active ingredient.

5.3.2 Glyphosate conclusion

Our adaptation of the propidium iodide cell staining was successful and provided a suitable method for identifying cell death in *P. tricornutum*.

Cell death occurred rapidly when *P. tricornutum* was treated with a concentration of 0.8 g/L glyphosate, seeing ~23% of cells stained during 0 hour measurements and >90% cells stained during 6 hour measurements. We also saw the 0.4 g/L glyphosate^{r} treatment resulting in ~16% stained cells during 6 hour measurements, increasing to ~46% after 12 hours and peaking at >80% following 24 hours. For the rest of the glyphosate^{r} treatments we saw the same stained cell percentage increase over time, towards a final stained cell percentage after 96 hours at the end of the experiment of ~20%. Further research would be useful as it could help reveal at what level glyphosate loses its lethal effect. Additionally, it would be interesting to see changes in gene expression as this could give indications into what genes are involved in herbicide response.

5.5 Conclusions

From our experiments we saw negative impacts on growth from an increase in salinity, decreases in pH and the presence of glyphosate. Decreased salinity led to an increase in cell count, however it also led to a decrease in biomass at the most extreme low salinity treatment (1.5% (w/v)). In the salinity experiment we saw how low salinity produced significantly more PUFAs than control and high salinity treatment, with the latter having the lowest PUFA levels. From RNA sequencing we saw a lot more expression changes in the low salinity treatment, with several genes related to salt, however a large portion of the transcripts translated into products with unknown functions. These unknown transcripts highlight the diatomic knowledge gap and need for further research to fully understand this organism. In the lowest pH treatment (pH 5.1) some cell death was measured through propidium iodide staining. We also saw a steady increase in pH in the unbuffered control and mock treatment during the pH experiment. When treating the cells with roundup derived glyphosate, the two highest concentrations (0.8 and 0.4 g/L) died rapidly, with the rest of the treatments slowly reaching ~20% cell death after 96 hours. In general, our experiments provide a basis which can be used to perform further research that can help bridge the knowledge gap between diatoms and plants.

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Appendix

R-analysis:

```
#Install package BioManager and edgeR, if already installed skip to
"library(edgeR)" (line 6)

if (!require("BiocManager", quietly = TRUE))

  install.packages("BiocManager")

BiocManager::install("edgeR")

library(edgeR);

library(ggplot2)

setwd("Drive:/RNAseq");

ROoT <- ("Drive:/RNAseq");      #Give a name to the main folder for ease of
access "Drive" can be any available disk (C, D, etc)

setwd (RoOT);

CountTAB <- ("Drive:/RNAseq/CountTAB");       # Folder with count tables

Results <- ("Drive:/RNAseq/Results");         # Folder for results to be
output

#Defining your samples, a way to give names to the lines in your count table
(for example naming A1 control, etc)

targets <- read.delim("Samples.txt", row.names="Samples");

setwd(CountTAB);

x <- read.delim("countTable.txt", row.names="Phatr3_ID");      # The count
table is "named" x and can thus be edited and easily accessed by R

countsPerMillion <- cpm(x);                                   # Calculate counts
per million reads

countCheck <- countsPerMillion > 1                            # TRUE FALSE
matrix, remove genes with too low expression

keep <- which(rowSums(countCheck) >= 2);                      # Must have at
least 2 samples above the threshold (~ 20 reads)

List_OkExp <- x[keep,];                                       # A list which
keeps only the genes that fullfill the criteria above.

Group <- factor(paste(targets$group));                        # make group used
for design matrix

y1 <- DGEList(counts=List_OkExp, group=Group);                 # y contain
DGEList data, here using only genes above threshold

y2 <- calcNormFactors(y1);                                     # Trimmed Mean of
M-values

design <- model.matrix(~0+Group);                             # Create a model
matrix
```

```
colnames(design) <- levels(Group);

y2’ <- estimateGLMCommonDisp(y2,design);                          # mean
dispersion across all genes

y3 <- estimateGLMTrendedDisp(y2’,design);                         # mean
dispersion across all genes with similar abundance

y4 <- estimateGLMTagwiseDisp(y3,design);                         # calculates
gene-specific dispersions

qfit <- glmQLFit(y4, design)                                    # Fit a quasi-
likelihood negative binomial generalized log-linear model to count data

Low.contrasts <- makeContrasts(Ctrl_vs_LowNA=LowNa-Ctrl, levels=design);
#Differences between low salinity treatment and control

High.contrasts <- makeContrasts(Ctrl_vs_HighNA=HighNa-Ctrl, levels=design);
#Differences between high salinity treatment and control

LowHigh.contrasts <- makeContrasts(LowNa_vs_HighNA=LowNa-HighNa,
levels=design);  #Differences between low salinity treatment and high salinity
treatment

setwd(Results);

# Calculate log2 ratio and False discovery rate (FDR) using GLM likelihood
ratio test

lrtCtrl_vs_LowNA <- glmQLFTest(qfit,
contrast=Low.contrasts[,"Ctrl_vs_LowNA"]);

lrtCtrl_vs_HighNA <- glmQLFTest(qfit,
contrast=High.contrasts[,"Ctrl_vs_HighNA"]);

lrtLowNa_vs_HighNA <- glmQLFTest(qfit,
contrast=LowHigh.contrasts[,"LowNa_vs_HighNA"]);

TopScoreLow <- topTags(lrtCtrl_vs_LowNA, n=nrow(x));
# include all genes without filtering on FDR or logFC value

TopScoreHigh <- topTags(lrtCtrl_vs_HighNA, n=nrow(x));

TopScoreLowHigh <- topTags(lrtLowNa_vs_HighNA, n=nrow(x));

# Create a txt file containing a table with all genes, not filtering on FDR or
logFC

write.table(TopScoreLow, file="Lowsalt_vs_Ctrl.txt", sep="\t", quote=FALSE);

write.table(TopScoreHigh, file="Highsalt_vs_Ctrl.txt", sep="\t", quote=FALSE);

write.table(TopScoreLowHigh, file="LowSalt_vs_HighSalt.txt", sep="\t",
quote=FALSE);

# Filter genes to only include those with FDR <= 0.0001 and abs(log2)= +1

keepLow <- TopScoreLow$table$FDR <=0.0001 & abs(TopScoreLow$table$logFC) >=1;

keepIgh <- TopScoreHigh$table$FDR <=0.0001 & abs(TopScoreHigh$table$logFC)
>=1;

keepLowHigh <- TopScoreLowHigh$table$FDR <=0.0001 &
abs(TopScoreLowHigh$table$logFC) >=1;
```

```
# Create a txt file containing only genes which fulfill the criteria above

write.table(TopScoreLow[keepLow,], file="Lowsalt_vs_Ctrl_Filtered.txt",
sep="\t", quote=FALSE);

write.table(TopScoreHigh[keepIgh,], file="Highsalt_vs_Ctrl_Filtered.txt",
sep="\t", quote=FALSE);

write.table(TopScoreLowHigh[keepLowHigh,],
file="LowSalt_vs_HighSalt_Filtered.txt", sep="\t", quote=FALSE);
```

HeatMaps:

```
#Make R read the file and name it "x". Row names are what determines the
labels (in our case the gene names). "FileEncoding" is there to solve an error
and is not strictly neccesary (depends on encoding of data file)

x <- read.delim("HeatMapData.txt", row.names="Names", fileEncoding =
"UTF16LE");

library(ggplot2)

#Create a data matrix using the data

dMatrix=data.matrix(x)

#A way to edit the names of the columns, not essential but helpful.

colnames(dMatrix) <- c("LowSalt", "HighSalt")

#Package used for the melt function to allow for ggplot to create a heatmap

library("reshape")

#Create a new matrix that is transposed, used here since it had the X and Y
axis flipped from what we wanted, not neccesary but useful

tMatrix = t(dMatrix)

#Enables the data to be used by ggplot to make a heatmap

tmelt = melt(tMatrix)

#Create a ggplot which is then used to create the heatmap

tggp = ggplot(tmelt, aes(X1, X2)) + geom_tile(aes(fill=value))

#Creating the actual heatmap object, scale_fill_gradient2 determines the
colour range, geom_text is what inserts the logFC values "inside" the heatmap
cells

HeatMap = tggp + scale_fill_gradient2(low = "blue", mid="white", high="red")+
geom_text(aes(label = value), color = "black", size = 4)

#A way to view the heatmap

HeatMap

#A way to view the heatmap and adjust the label font size

HeatMap + theme(text = element_text(size = 15))
```

Appendix table 1: Mean cell count (cells/mL) of *P. tricornutum* grown in different salinity media (1.5%, 2.5%, 3.5%, 4.5% and 5.5% (w/v)) for 7 days, alongside their confidence interval (CI). N=3.

Day/ sample	1.5 %	CI	2.5 %	CI	3.5 %	CI	4.5 %	CI	5.5 %	CI
0	99 697	11 615	96 223	4 243	88 545	2 554	91 183	2 217	78 267	11 771
1	116 693	6 612	115 600	2 460	90 172	5 145	79 877	4 081	58 213	6 311
2	419 237	27 253	446 258	40 021	301 081	42 508	221 840	9 360	133 453	4 123
3	1 364 257	23 622	1 535 139	25 607	995 268	123 413	685 739	79 652	342 353	49 723
4	2 989 153	304 025	3 355 651	713 001	2 667 630	490 909	1 855 049	215 194	860 161	163 385
5	5 068 628	1 091 955	4 556 649	856 175	3 283 704	846 340	1 876 213	146 462	2 104 425	322 460
6	7 559 557	519 578	6 860 409	375 951	4 447 705	549 828	1 677 796	267 650	1 961 700	80 191
7	8 880 378	2 397 439	7 336 689	1 907 141	5 285 249	1 292 573	2 574 745	468 123	1 379 399	178 808

Appendix table 2: Fv/Fm measurements from the salinity treatment experiment using five salinity concentrations (1.5%, 2.5%, 3.5%, 4.5% and 5.5% (w/v)). Low val given when the samples did not have a sufficient cell concentration.

Sample	Day 2	Day 3	Day 4	Day 5
1a	0.69	0.64	0.65	0.65
1b	0.69	0.65	0.67	0.66
1c	0.69	0.65	0.68	0.67
2a	0.68	0.63	0.65	0.64
2b	0.71	0.63	0.66	0.65
2c	0.71	0.63	0.67	0.66
3a	0.69	0.64	0.63	0.68
3b	0.68	0.66	0.64	0.68
3c	0.69	0.65	0.65	0.68
4a	Low val	0.67	0.68	0.59
4b	0.66	0.68	0.68	0.64
4c	0.68	0.69	0.67	0.67
5a	Low val	0.64	0.69	0.59
5b	Low val	low val	0.69	0.61
5c	Low val	low val	0.71	0.57

Appendix table 3: Mean cell count (cells/mL) of *P. tricornutum* grown in different salinity media (1.5%, 2.5%, 3.5%, 4.5% and 5.5% (w/v)) for 8 days following acclimation to said salinities for 4 weeks, alongside their 95% confidence interval (CI). N=3.

Day/sample	1.5 %	CI	2.5 %	CI	3.5 %	CI	4.5 %	CI	5.5 %	CI
0	50 000	11 316	50 000	11 316	50 000	11 316	50 000	11 316	50 000	11 316
1	82 987	4 386	81 970	17 702	79 360	21 020	78 447	11 061	71 400	7 577
2	287 173	7 995	291 485	68 294	282 864	30 146	228 347	30 840	166 790	19 960
3	879 535	7 649	1 009 635	179 154	977 285	72 198	688 123	115 666	425 031	41 919
4	2 099 251	255 688	2 743 445	297 731	2 462 341	247 242	1 898 952	184 184	1 078 860	159 549
5	4 004 884	393 574	4 329 267	460 128	4 353 900	308 247	2 701 101	285 171	1 866 842	322 230
6	4 994 046	824 721	5 266 444	1 110 484	4 967 853	795 645	2 556 378	460 311	1 425 357	84 137
7	7 737 446	224 516	7 566 340	393 766	5 663 458	1 581 690	3 740 875	489 910	1 953 689	245 590
8	9 289 044	214 765	8 372 317	1 924 199	7 567 064	1 452 459	5 116 312	1 537 463	2 652 900	598 760

Appendix table 4: RNA integrity numbers (RIN) for each sample sent to RNA sequencing, retrieved from bioanalyzer sequencing. The samples were taken from *P. tricornutum* grown in different salinity media (1.5%, 3.5% and 5.5% (w/v)) at day 3 of growth, with control being samples grown in normal salinity media (3.5% (w/v)) and harvested at day 1.

Sample	RNA integrity number (RIN)
Control A	7.4
Control B	7.6
Control C	7.8
1 A	7.6
1 B	6.5
1 C	7.8
3 A	6.6
3 B	6.9
3 C	6.9
5 A	7.8
5 B	7.8
5 C	7.7

Appendix table 5: Table of primers used for rt-qPCR, based on *P. tricornutum* genes that were differentially regulated in salt treatments when compared to day 1 control. Phatr3_J10566 was a reference gene. *Was not used, produced side products

Gene name	Protein name	Forward	Reverse
Phatr3_J45944	Unknown diatom specific protein	GGCCTCCACGAATGGATGTT	AAGCCGTCTAGGGAGTCGTC
Phatr3_J48827	Unknown diatom specific protein	CGAACGGATGTTTGCTCGAC	TCGCTGGGTGAATCACTGTC
Phatr3_J37038	TauD domain-containing protein	TCGGATAGTGTCCAGGCTCA	CGGTCCAACATGTGCTTGTC
Phatr3_J54987	Iron starvation induced protein	CGCCTGTCAGTTCTCCTGTT	AATGCTCCTGAGCCTCCATC
Phatr3_J10566	Splicing factor 3B subunit 5	GAGTGGATGACGAACCAGCA	TCCACACGGCTGTACCATCT
Phatr3_J40433*	Sodium-dependent phosphate transport protein 2A	ATGTCTGCCCTTGTCTCTGC	ACTCAACGGAACTTGACGCA

Appendix table 6: CT values from qPCR of RNA isolated from *P. tricornutum* grown in different salinity media (1.5%, 3.5% and 5.5% (w/v)) at day 3 of growth. The primers used were; Phatr3_J45944: Unknown diatom specific protein, Phatr3_J48827: Unknown diatom specific protein, Phatr3_J37038: Clavaminic acid snythetase (CAS) domain protein and Phatr3_J54987: Iron starvation induced protein (ISIP2B). Phatr3_J10566 (Splicing factor 3B subunit 5) was a reference gene.

Gene	Sample/Replicate	1	2	3
Phatr3_J45944 (Unknown diatom specific protein)	1A	21.72	22.02	21.91
	1B	22.32	22.33	22.33
	1C	23.77	23.6	22.86
	3A	29.32	29.18	29.64
	3B	28.77	28.71	28.77
	3C	29.43	29.71	29.76
	5A	29.66	29.64	29.18
	5B	28.9	29.05	28.69
	5C	29.2	29.62	29.42
Phatr3_J48827 (Unknown diatom specific protein)	1A	24.13	24.06	24.9
	1B	24.77	25.02	24.9
	1C	25.52	25.59	25.49
	3A	32.67	31.28	30.68
	3B	30.57	30.07	30.23
	3C	31.3	30.9	31.45
	5A	31.86	32.36	32.34
	5B	31	31.2	30.33
	5C	32.73	32.79	31.89
Phatr3_J37038 (Clavaminic acid snythetase (CAS) domain protein)	1A	24.29	24.32	24.25
	1B	24.41	24.35	24.65
	1C	25.6	25.58	25.51
	3A	32.02	32.2	33.22
	3B	30.93	31.63	31.63
	3C	33.86	31.54	31.96
	5A	32.59	32.17	33.96
	5B	33.32	32.54	34.47
	5C	34.73	33.63	34.91

Phatr3_J54987 (Iron starvation induced protein (ISIP2B))	1A	28.87	28.6	28.68
	1B	28.49	28.42	28.5
	1C	28.34	28.76	28.42
	3A	26.54	27.06	26.59
	3B	25.33	25.67	25.37
	3C	27.7	28.28	28.15
	5A	26.61	26.92	26.9
	5B	25.95	25.97	25.91
	5C	28.06	28.09	27.98
Phatr3_J10566 (Splicing factor 3B subunit 5)	1A	23.4	22.71	22.26
	1B	23.46	23.2	22.65
	1C	24.1	23.75	22.81
	3A	23.63	23.08	22.93
	3B	23.52	22.73	21.52
	3C	23.47	22.82	22.09
	5A	24.17	23.83	23.55
	5B	22.5	22.09	21.66
	5C	23.07	22.45	21.87

Appendix table 7: Measured dry weight for P. tricornutum grown in different salinities (1.5%, 3.5% and 5.5% (w/v)) as well as the mean lipid weight (mean of the three weightings) and their confidence intervals following lipid extraction plus calculated lipid percentage.

sample	Dry Weight (mg)	Mean lipids (mg)	CI (mg)	Lipid %
1.5% A	28.92	4.14	0.05	14.33%
1.5% B	29.00	4.93	0.06	16.95%
1.5% C	27.78	4.01	0.03	14.45%
3.5% A	75.90	7.58	0.03	9.98%
3.5% B	77.65	7.64	0.10	9.83%
3.5% C	84.73	7.53	0.11	8.88%
5.5% A	26.28	2.55	0.03	9.69%
5.5% B	38.79	2.35	0.04	6.06%
5.5% C	39.76	2.20	0.02	5.54%

Appendix table 8: Mean cell count for initial pH experiment for *P. tricornutum* grown in buffered media at a pH of 5.1, 6.1, 7.1 and 8.1, as well as mock and control. The buffered media consisted of 75% F/2 media and 25% 0.1 M buffer dissolved in Milli-Q H_2O with added F/2 nutrients, resulting in a final buffer concentration of 0.025 M, with mock using Milli-Q H_2O in place of buffer. There was no standard error for day 0 as all the samples were taken from a common stock solution which gave the measurement for that day. N=3.

Days	5.1 Citrate	CI	6.1 MES	CI	7.1 HEPES	CI	8.1 HEPES	CI	Mock	CI	Control	CI
0	61 270	0	65 110	0	53 830	0	62 250	0	53 230	0	57 260	0
1	37 780	5 860	67 645	14 337	92 340	5 253	100 100	1 176	104 965	1 127	89 820	3 214
2	35 373	6 210	175 583	8 869	418 852	38 108	459 946	9 248	457 206	22 593	375 704	26 414
3	29 910	686	319 733	35 335	1 117 674	46 362	2 151 541	147 325	1 846 998	115 568	1 541 330	97 885
4	9 813	2 369	507 519	52 550	1 560 423	24 161	3 427 112	71 673	3 692 063	151 654	3 172 398	47 631
5	7 323	4 565	675 022	88 294	1 891 047	47 421	4 221 002	27 394	5 656 363	267 948	5 124 475	290 913
6	13 017	6 225	844 975	86 198	2 384 054	57 045	4 835 913	55 154	7 512 942	281 222	6 769 202	455 977

Appendix table 9: Mean pH measured over 6 days in various buffered media used to grow *P. tricornutum*, as well as control and mocks. The buffers used were HEPES for pH 8.1 and 7.1, MES for 6.1 and Citrate for 5.1. N=3. *There is no standard deviation for day 0 as this was measured from a common stock.

Day	5.1 Citrate	CI	6.1 MES	CI	7.1 HEPES	CI	8.1 HEPES	CI	Mock	CI	Control	CI
0	5.10	0.00	6.10	0.00	7.10	0.00	8.10	0.00	8.18	0.00	8.18	0.00
1	5.08	0.00	6.10	0.00	7.15	0.00	8.04	0.01	8.21	0.01	8.23	0.01
2	5.11	0.01	6.10	0.01	7.19	0.01	8.08	0.00	8.61	0.01	8.51	0.03
3	5.15	0.01	6.10	0.00	7.21	0.01	8.20	0.01	9.66	0.05	9.26	0.08
4	5.19	0.01	6.11	0.00	7.22	0.01	8.25	0.00	9.66	0.03	9.66	0.00
5	5.24	0.01	6.12	0.00	7.23	0.00	8.27	0.01	9.94	0.02	9.82	0.02
6	5.29	0.02	6.12	0.01	7.23	0.00	8.27	0.01	10.18	0.01	10.08	0.02

Appendix table 10: Mean cell count for *P. tricornutum* grown in buffered media of pH 5.1, 6.1, 7.1 and 8.1 for 5 days, as well as bank and control plus Citrate and MES buffers put outside their buffer range to test for toxicity. The buffered media was made using 75% media and 25% buffer dissolved in Milli-Q H_2O with added F/2 nutrients (buffer concentration 0.025M). N=3, 95% confidence interval can be seen in the lower half of the table.

Day	5.1 Citrate	6.1 MES	7.1 HEPES	8.1 HEPES	Mock	Citrate Mock	Control	MES Mock
0	47 660	45 427	47 060	47 043	47 150	48 023	45 257	49 117
1	38 050	60 293	104 240	111 790	105 063	97 310	88 883	112 833
2	35 633	136 427	418 207	456 197	449 430	354 620	324 686	482 176
3	36 360	231 760	1 132 180	1 808 634	1 569 869	1 252 175	1 239 142	1 764 773
4	34 147	349 400	1 653 517	3 992 549	3 479 224	3 111 650	2 987 874	3 758 368
5	35 827	498 682	2 162 996	4 586 836	5 013 247	4 270 716	4 680 902	5 182 460
	5.1 CI	6.1 CI	7.1 CI	8.1 CI	Blank CI	Citrate Mock CI	Control CI	MES Mock CI
0	1 379	1 959	843	782	1 277	2 032	2 480	1 368
1	3 000	3 485	3 798	3 895	5 496	3 535	5 712	3 731
2	6 862	5 220	17 040	30 151	3 164	15 812	11 055	6 125
3	1 838	8 469	23 807	220 601	5 771	71 715	48 672	160 617
4	4 778	5 556	142 373	117 350	26 086	91 184	109 489	259 871
5	2 969	11 329	73 826	71 635	219 984	106 643	365 137	186 252

Appendix table 11: Mean Fv/Fm for *P. tricornutum* grown in buffered media of pH 5.1, 6.1, 7.1 and 8.1 for 5 days, as well as bank and control plus Citrate and MES buffers put outside their buffer range to test for toxicity. The buffered media was made using 75% media and 25% buffer dissolved in Milli-Q H_2O with added F/2 nutrients (buffer concentration 0.025M). Low val given when the samples did not have a sufficient cell concentration. N=3.

	Day 1	CI	Day 2	CI	Day 3	CI	Day 4	CI	Day 5	CI
Ctrl	0.75	0.01	0.68	0.02	0.73	0.01	0.67	0.00	0.57	0.00
Mock	0.74	0.01	0.68	0.01	0.71	0.01	0.62	0.00	0.57	0.01
MES 8.1	0.75	0.02	0.67	0.02	0.69	0.00	0.63	0.01	0.62	0.03
Citrate 8.1	0.72	0.00	0.65	0.00	0.70	0.00	0.65	0.01	0.65	0.01
pH 8.1 HEPES	0.74	0.02	0.66	0.00	0.69	0.01	0.57	0.01	0.56	0.03
pH 7.1 HEPES	0.73	0.02	0.66	0.02	0.54	0.01	0.54	0.03	0.58	0.01
pH 6.1 MES	0.73	0.01	0.56	0.02	0.53	0.02	0.52	0.01	0.52	0.01
pH 5.1 Citrate	0.69	0.01	Low value	-	Low value	-	Low value	-	Low value	-

Appendix table 12: Significant results from Tukey HSD test performed on repeat pH experiment testing buffered pH between 5.1-8.1 (using HEPES buffer for pH 8.1 and 7.1, MES buffer for pH 6.1 and citrate buffer for pH 5.1) with citrate and MES buffers brought up to pH 8.1 to test for buffer toxicity, control and blank

Day	Treatments pair	Tukey HSD Q statistic	Tukey HSD p-value	Day	Treatments pair	Tukey HSD Q statistic	Tukey HSD p-value
2	Ctrl vs 6.1	15.8745	0.0010053	4	Ctrl vs 6.1	19.7484	0.0010053
	Mock vs 6.1	15.8745	0.0010053		Mock vs Citrate 8.1	4.9065	0.0487002
	MES 8.1 vs 6.1	14.9926	0.0010053		Mock vs 8.1	7.3598	0.0024414
	Citrate 8.1 vs 6.1	12.3468	0.0010053		Mock vs 7.1	12.2663	0.0010053
	8.1 vs 6.1	13.6697	0.0010053		Mock vs 6.1	13.1656	0.0010053
	7.1 vs 6.1	13.6697	0.0010053		MES 8.1 vs 8.1	9.3224	0.0010053
3	Ctrl vs MES 8.1	7.0586	0.0028911		MES 8.1 vs 7.1	14.2289	0.0010053
	Ctrl vs Citrate 8.1	6.1335	0.0340226		MES 8.1 vs 6.1	14.921	0.0010053
	Ctrl vs 8.1	7.0586	0.0028911		Citrate 8.1 vs 8.1	12.2663	0.0010053
	Ctrl vs 7.1	35.9346	0.0010053		Citrate 8.1 vs 7.1	17.1729	0.0010053
	Ctrl vs 6.1	37.8596	0.0010053		Citrate 8.1 vs 6.1	17.5541	0.0010053
	Mock vs 7.1	33.3678	0.0010053		8.1 vs 7.1	4.9065	0.0487002
	Mock vs 6.1	35.2929	0.0010053		8.1 vs 6.1	6.5828	0.0062031
	MES 8.1 vs 7.1	28.876	0.0010053	5	Ctrl vs MES 8.1	4.9225	0.0444758
	MES 8.1 vs 6.1	30.8011	0.0010053		Ctrl vs Citrate 8.1	8.2041	0.0010053
	Citrate 8.1 vs 7.1	30.8011	0.0010053		Mock vs Citrate 8.1	7.876	0.0010505
	Citrate 8.1 vs 6.1	32.7261	0.0010053		Mock vs 6.1	4.9225	0.0444758
	8.1 vs 7.1	28.876	0.0010053		MES 8.1 vs 8.1	6.2351	0.0082472
	8.1 vs 6.1	30.8011	0.0010053		MES 8.1 vs 6.1	9.5168	0.0010053
4	Ctrl vs Mock	7.3598	0.0024414		Citrate 8.1 vs 8.1	9.5168	0.0010053
	Ctrl vs MES 8.1	5.3972	0.0266762		Citrate 8.1 vs 7.1	7.5478	0.001569
	Ctrl vs 8.1	14.7196	0.0010053		Citrate 8.1 vs 6.1	12.7984	0.0010053
	Ctrl vs 7.1	19.6261	0.0010053		7.1 vs 6.1	5.2506	0.0292878

Appendix table 13: Mean stained cell percentage for 12 hour glyphosate^{r} experiment grown in different concentrations of glyphosate^{r} (0.8 g/L, 0.4 g/L, 0.2 g/L, 0.1 g/L and 0.05 g/L), as well as control, dead control (killed by heat shock treatment at 65 °C for 15 minutes) and mock. The cells were stained by using 5 µL of PI (1 mg/mL) in 200 µL of sample then keeping them in the dark for 15 minutes. N=3.

M5 %	0 Hours	CI	6 Hours	CI	12 Hours	CI
Ctrl	9.16%	1.48%	8.46%	1.16%	8.80%	1.99%
dCtrl	97.06%	0.16%	94.30%	0.51%	94.11%	0.31%
0.8 g/L	23.27%	14.11%	98.93%	0.46%	99.66%	0.11%
0.4 g/L	5.68%	0.99%	16.48%	10.94%	46.23%	33.46%
0.2 g/L	4.99%	0.32%	5.42%	0.31%	6.04%	0.63%
0.1 g/L	4.85%	0.14%	5.19%	0.15%	6.18%	0.29%
0.05 g/L	4.87%	0.17%	5.28%	0.53%	6.08%	0.39%
Mock	6.88%	0.61%	7.08%	0.86%	7.37%	0.77%

Appendix table 14: Mean stained cell count for 96 hour glyphosate^{r} experiment grown in different concentrations of glyphosate^{r} (0.4 g/L, 0.2 g/L, 0.1 g/L, 0.05 g/L, 0.025 g/L, 0.0125 g/L and 0.00675 g/L), as well as control, dead control (killed by heat shock treatment at 65 °C for 15 minutes) and mock. The cells were stained by using 5 µL of PI (1 mg/mL) in 200 µL of sample then keeping them in the dark for 15 minutes. N=3.

Treatment	0 Hours	CI	24 Hours	CI	48 Hours	CI	72 Hours	CI	96 Hours	CI
0,00675 g/L	3,71%	0,20%	3,94%	0,37%	2,69%	0,64%	9,61%	3,46%	19,28%	5,55%
0,0125 g/L	4,07%	0,48%	4,26%	0,53%	3,28%	0,29%	9,25%	0,73%	18,11%	4,12%
0,025 g/L	3,88%	0,43%	4,14%	0,60%	4,52%	1,05%	9,88%	1,35%	21,05%	0,69%
0,05 g/L	4,15%	0,78%	4,94%	0,40%	6,27%	0,81%	11,89%	1,55%	20,99%	1,14%
0,1 g/L	4,30%	0,11%	5,51%	0,52%	6,36%	0,75%	10,96%	1,68%	20,80%	3,52%
0,2 g/L	3,23%	0,21%	9,35%	3,40%	8,69%	2,48%	12,24%	3,63%	20,73%	3,28%
0,4 g/L	5,03%	0,68%	81,35%	3,30%	84,59%	2,57%	81,31%	4,35%	77,11%	3,72%
Mock	5,30%	0,65%	4,61%	1,04%	2,89%	0,36%	1,18%	0,40%	0,67%	0,26%
Control	5,94%	0,82%	4,82%	0,35%	2,29%	0,15%	1,08%	0,22%	0,54%	0,11%
Dead Ctrl	91,03%	0,84%	89,80%	3,24%	88,05%	4,36%	87,34%	5,57%	86,74%	6,76%

Appendix table 15: Mean cell count and 95% confidence interval for 96 hour glyphosate^{r} experiment grown in different concentrations of glyphosate^{r} (0.4 g/L, 0.2 g/L, 0.1 g/L, 0.05 g/L, 0.025 g/L, 0.0125 g/L and 0.00675 g/L), as well as control, dead control (killed by heat shock treatment at 65 °C for 15 minutes) and mock. N=3.

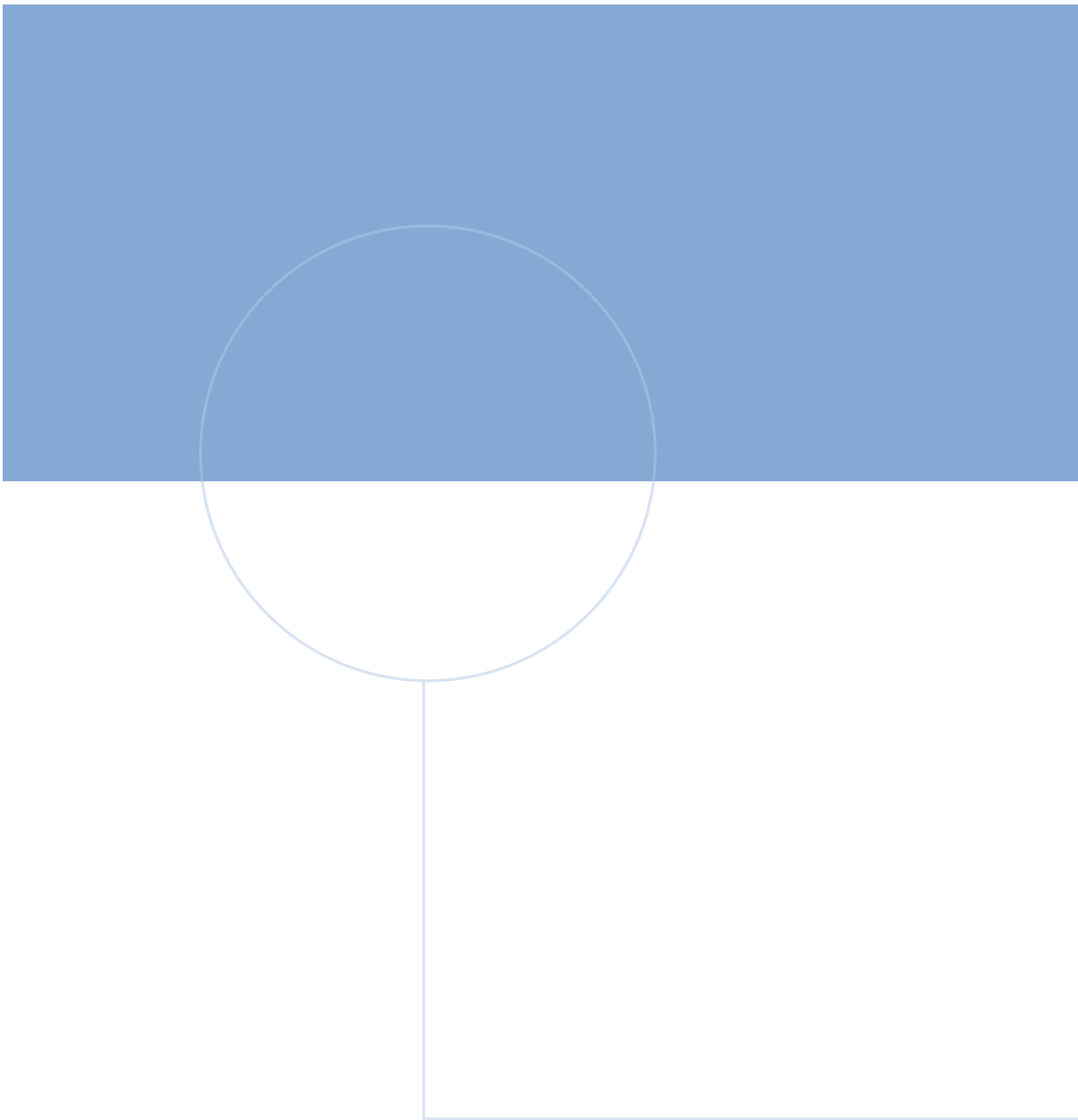
Treatment	0 Hours	CI	24 Hours	CI	48 Hours	CI	72 Hours	CI	96 Hours	CI
0.00675 g/L	295 087	10 920	482 405	44 699	1 530 936	257 486	1 441 618	555 170	1 296 911	487 460
0.0125 g/L	342 650	12 704	509 927	38 071	1 109 421	34 920	901 119	119 719	847 830	66 870
0.025 g/L	370 777	12 011	494 508	24 466	677 340	25 842	545 028	40 440	558 693	43 252
0.05 g/L	407 200	8 676	406 430	17 145	474 490	20 675	422 457	20 820	457 817	26 130
0,1 g/L	417 230	4 254	362 320	16 172	448 723	16 311	404 527	17 896	429 043	8 943
0,2 g/L	400 260	32 913	177 497	60 845	265 547	23 757	313 790	25 642	361 720	34 439
0,4 g/L	425 487	6 009	108 397	17 607	23 703	3 540	11 903	5 159	11 963	2 602
Mock	200 163	21 506	298 197	38 564	1 163 126	61 050	2 669 547	228 378	4 638 323	396 094
Control	186 123	2 026	275 830	13 236	993 150	70 209	2 496 260	238 085	4 932 355	705 400
Dead Control	123 107	25 071	40 397	49 429	35 921	34 067	26 570	31 451	28 783	34 591

Appendix table 16: Small scale lipid extraction test results with *P. tricornutum* grown in 3.5 (w/v) and 4.5% (w/v) salinity media, showing low yield from the third extraction step.

Extracted lipids (mg)	First extraction	Second extraction	Third extraction
3.5% A	0.298	0.111	-0.015
3.5% B	0.454	0.072	0.089
4.5% A	0.254	0.065	0.037
4.5% B	0.517	0.037	-0.017
Blank	-0.027	-0.055	-0.035

Appendix table 17: Concentrations (ng/µL) from GC-MS with blank subtracted from their concentration. The most prominent fatty acid in all samples were eicosapentaenoic acid (EPA) (C20:5ω3, 26.67-32.21%) followed by palmitoleic acid (C16:1ω7, 22.16-25.60%), palmitic acid (C16:0, 11.09-18.27%), then cervonic acid (DHA) (C22:6ω3, 6.91-9.23%) and are highlighted in bold. Names ending in "*" has tentative ID

Name	1A	1B	1C	3A	3B	3C	5A	5B	5C	Blank
C14:0	45.13	39.28	47.39	42.87	43.38	37.51	37.64	36.64	52.04	10.91
C15:0	2.03	1.72	2.13	2.80	2.84	2.52	3.13	3.28	4.44	0.55
C16:0	98.76	85.88	98.08	150.18	146.74	130.19	156.20	140.37	189.77	13.21
C16:1n7	**195.48**	**172.61**	**196.65**	**207.44**	**206.88**	**186.89**	**209.64**	**203.95**	**267.75**	**-**
C16:1n5	11.12	9.81	11.57	9.83	10.36	9.15	10.08	8.34	11.13	0.84
C16:2n-6,9* ID	13.84	12.56	14.52	11.58	12.30	10.97	13.59	12.82	17.16	-
C16:2n-4,7*	44.76	40.10	44.19	35.64	36.75	32.86	33.91	34.17	44.47	-
C16:3*	28.72	20.79	25.03	1.71	1.33	-0.15	6.63	5.56	5.17	16.14
C16:4*	**53.38**	**49.25**	**62.67**	**55.91**	**59.96**	**53.97**	**48.07**	**42.88**	**58.12**	**-**
C18:0	2.27	1.74	2.27	3.93	4.25	3.40	5.48	5.85	7.79	0.84
C18:1n9	1.60	1.66	1.63	3.31	3.32	2.84	3.39	3.09	4.35	-
C18:1n7	3.21	2.57	3.20	4.33	4.28	3.83	4.35	3.72	5.20	-
C18:2n6	7.66	6.97	8.12	11.39	11.98	11.08	10.75	10.04	13.59	-
C18:3n3	0.89	0.72	0.89	-	-	0.35	-	-	-	-
C18:4n3	3.25	3.10	3.07	4.79	4.55	4.20	4.39	3.55	4.82	-
C20:0	0.54	0.58	0.67	0.85	0.58	0.73	0.93	0.93	1.14	-
C20:5n3	**284.73**	**247.78**	**288.73**	**249.75**	**268.47**	**243.83**	**222.21**	**207.98**	**280.33**	**-**
C22:0	2.10	1.90	1.83	2.31	2.39	2.16	2.67	2.71	3.33	-
C22:6n3	**81.67**	**71.13**	**82.63**	**57.53**	**61.65**	**55.95**	**60.31**	**54.80**	**71.91**	**-**
C24:1	1.01	1.03	1.02	1.67	1.54	1.49	1.81	1.59	2.46	-
SUM	882.16	771.17	896.28	857.80	883.55	793.77	835.18	782.29	1,044.97	42.50



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