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Lipid content, composition and dynamics in northern marine diatoms

An experimental study using different analytical methods

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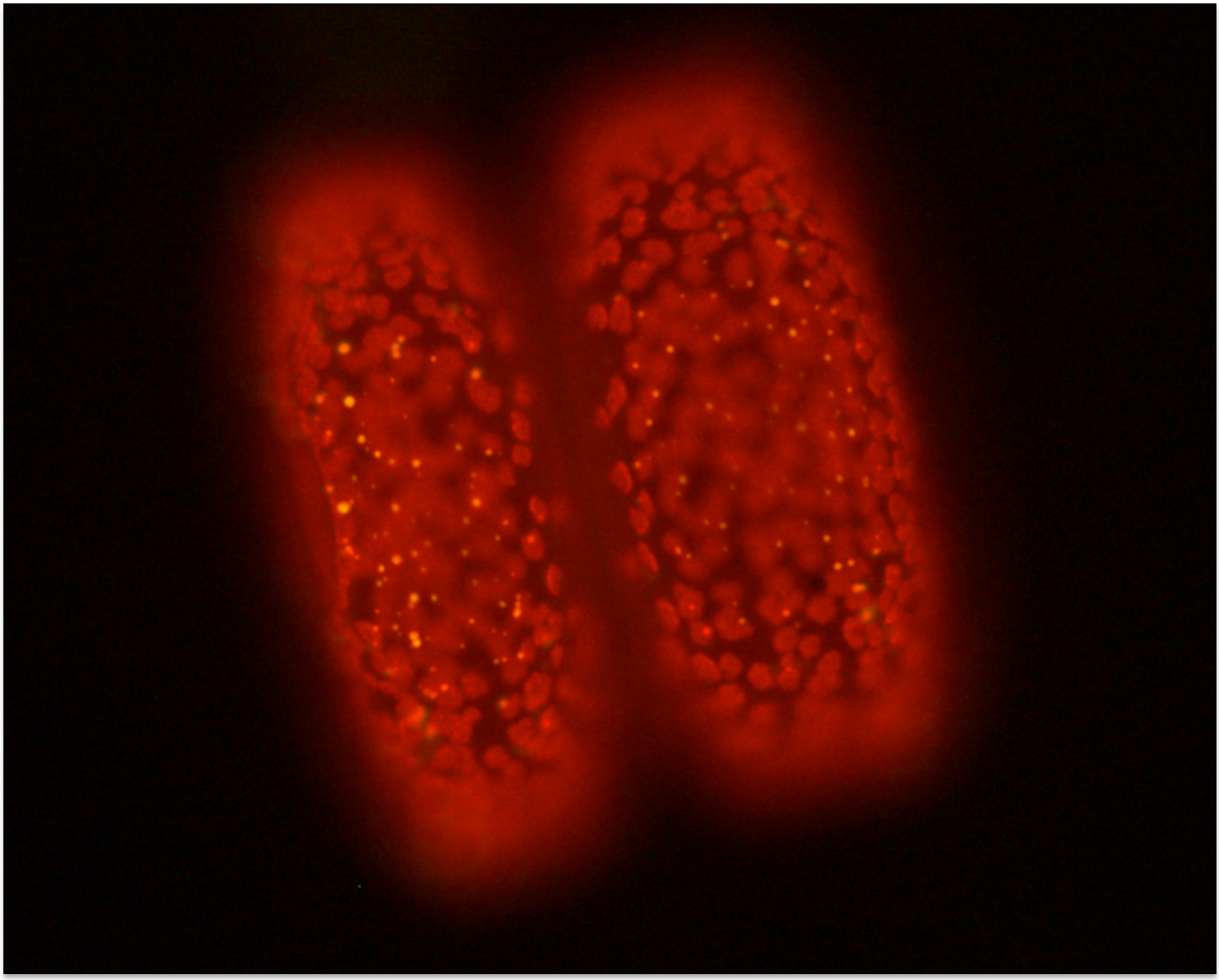

UNIVERSITY OF TROMSØ

Front page: *Coscinodiscus concinnus* stained with Nile Red

Abstract

Knowledge of the lipid content in specific species of northern/arctic microalgae and its dependency on growth conditions is vital in choosing a suitable species for mass production. The traditional methods of lipid quantification that utilize solvent extraction are time consuming and require large amounts of biomass. In this study, the fluorophore Nile Red was used as a probe for lipid accumulation during nutrient stress in the diatom *Coscinodiscus concinnus,* and to quantify lipid concentration fluorometrically in 5 monocultures of northern diatoms. The results from the fluorometrical quantification were validated by solvent extraction and HPLC-MS analysis in Bergen. In addition, 17 monocultures of 7 species of arctic diatoms were analyzed for lipid class and fatty acid composition by HPLC-MS in Prague.

Nile Red was shown to be an effective probe for visualizing the physiological state of *C. concinnus.* The quantification procedure using Nile Red yielded high correlation coefficients in the lipid standards cod liver oil and α-phosphatidylcholine (R^2 = 0.995 and 0.985, respectively), but was in all likelihood unable to quantify the true polar and neutral lipid concentration of the diatom samples, possibly due to fluorescence from an unknown source. Solvent extraction in Bergen determined a total lipid concentration of 2.15-7.32% per dry weight, but unidentified contaminations probably caused an over-estimation of the lipid content. The HPLC-MS performed in Bergen produced results that indicated hydrolysis of the sample or unknown contaminations and were not included in the study. The HPLC-MS analysis in Prague revealed large variations in the composition of the main lipid classes (TAG, PC, PG and PS) both within and between species. Large contributions of LPG and LPC were also observed. The composition of fatty acids showed less variation within species, and was similar for all species with the exception of *T. gravida*. The main fatty acids were C16:0, C16:1, C18:0, C18:1 (n-9), C20:5 (n-3) and C22:6 (n-3).

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Abbreviations

Lipids

ALA:	Alpha-linolenic acid
ARA:	Arachidonic acid
CER:	Ceramide
DAG:	Diacylglycerol
DGDG:	Digalactosyldiacylglycerol
DHA:	Docosahexaenoic acid
DPG:	Disphosphatidylglycerol
EPA:	Eicosapentaenoic acid
FA:	Fatty acid
FFA:	Free fatty acid
GLA:	Gamma-linolenic acid
LA:	Linoleic acid
LC:	Long chain
LC-PUFA:	Long chain-polyunsaturated fatty acid
LPC:	Lysophosphatidylcholine
LPE:	Lysophosphatidylethanolamine
LPI:	Lysophosphatidylinositol
LPG:	Lysophosphatidylglycerol
LPS:	Lysophosphatidylserine
MAG:	Monoacylglycerol
MGDG:	Monogalactosyldiacylglycerol
PC:	Phosphatidylcholine
PE:	Phosphatidylethanolamine
PG:	Phosphatidylglycerol
PI:	Phosphatidylinositol
PS:	Phosphatidylserine
PUFA:	Polyunsaturated fatty acid
SM:	Sphingomyelin
SQDG:	Sulfoquinovosyldiacylglycerol
TAG:	Triacylglycerol

Others

Chl *a*	Chlorophyll *a*
DPH	1,6-diphenylhexatriene
HCl	Hydrochloric acid
HNLC	High nutrient – low chorophyll
HPLC	High performance liquid chromatography
MS	Mass spectrometry
NR	Nile Red
NRWS	Nile Red working solution
NRSS	Nile Red stock solution
PTD	*Phaeodactylum tricornutum* desaturase
TCA	Tricarboxylic acid cycle
TLC	Thin layer chromatography

1 Introduction

1.1 Microalgae and Diatoms in general

Microalgae, or phytoplankton, are photosynthetic, unicellular organisms that constitute the main primary producers of the world's oceans. The largest group of microalgae are the diatoms (class *Bacillariophyceae)*. Diatoms live in a range of environments, from oceans and freshwater bodies to deserts and even clouds (Yadugiri, 2009). Diatoms are members of the supergroup *Choromophyta* and contain an estimated 20,000-200,000 different species (Mann and Droop, 1996) (Guiry, 2012), but according to recent estimations by Mann and Vanormelingen (2013), the true number is at least 30,000 and probably ca. 100,000. Of these, 12,000 have been described (Mann and Vanormelingen, 2013).

"Identifying Marine Phytoplankton" by Tomas et al. (1997) describes diatoms as autotrophic, single-celled organisms with a cell wall (frustule) of two silica valves (theca). The shape of the frustule varies, but it is almost always heterovalvate, i.e. one valve is smaller than the other, allowing them to fit together like a shoebox. The largest of the silica valves is called the epitheca, and the smallest half is called the hypotheca. In vegetative reproduction (binary fission), two daughter cells are formed within the parent frustule. Each daughter cell receives one parent cell theca each as epitheca, and a new hypotheca is formed. This leads to a reduction in cell size in one of the daughter cells, and binary fission may typically only continue until the cell is about one third of the initial cell size. The original cell size is restored by auxospore formation, in which a cell sheds its silica shell and becomes a large sphere surrounded by an organic membrane. A new frustule of maximum size is formed within this sphere. Auxospore formation may occur both vegetatively and as a result of sexual reproduction by gametogenesis and subsequent zygote development (Tomas et al., 1997). Diatom cells vary in size from 2 µm – 2 mm, and exist in two shapes: Pennate diatoms, which are bilaterally symmetrical, and centric diatoms, which are radially symmetrical. Diatoms are predominantly organized as single cells, but many species are colonial, where cells associate with each other in chains. Their growth is limited by the availability of light, inorganic nutrients (N, P and Si), dissolved CO_2 as well as trace metals such as Fe, Mn and Zn (De Baar, 1994).

In areas with pronounced seasons, e.g. north and south of the equator, algal growth occurs as spring blooms in which a major part of the annual production takes place (Sverdrup, 1953). The blooms are initiated by the increasing availability of light during spring, in the euphotic water zone where nutrients have regenerated during winter. Once initiated, the spring blooms continue exponentially until the nutrients in the water have been consumed; in the case of diatoms & other phytoplankton, the limiting nutrient is usually nitrogen. Nitrate is the most important source of nitrogen for arctic phytoplankton (Kristiansen and Farbrot, 1991), and the concentration of nitrate is approximately 1 µmol when the spring bloom ends (Kristiansen and Lund, 1989). As spring and summer progresses, the species composition of microalgal communities changes in a successional pattern (Degerlund and Eilertsen, 2010) with diatoms re-emerging whenever conditions become favorable. The ecological importance of diatoms and microalgae as a whole is unrivalled. Marine microalgae make up a fraction of the global plant biomass, yet as primary producers they bind 50% of the global CO_2 (Field et al., 1998), and create the primary nutritional basis for marine life.

This study focused on species of diatoms that are 1) Primary producers in the northern and arctic seas, which are the most productive seas in the world, and thereby very important to the global ecosystem and 2) representatives of all size classes, from very small, rapidly multiplying cells to very large, slowly multiplying cells. The species were *Chaetoceros socialis, Chaetoceros furcellatus, Coscinodiscus concinnus, Attheya longicornis, Skeletonema marinoi, Porosira glacialis* and *Thalassiosira gravida.* All of these species belong to the centric diatoms, but are not monophyletic: In a phylogenetic tree, the genera *Thalassiosira, Skeletonema* and *Porosira* are in one group, *Chaetoceros* in another, and *Attheya* is in a separate group close to the pennates (Huseby et al., 2013). The position of *Coscinodiscus* is not revealed, but it is listed in *Algaebase* as belonging to the order of *Coscinodiscales,* which does not include any of the other families. In terms of size classification, the most commonly used system (Sieburth et al., 1978) lists *C. socialis, A. longicornis S. marinoi* and *C. furcellatus* as nanoplankton (0-20 µm), while *C. concinnus, P. glacialis* and *T. gravida* are microplankton (20-200 µm).

In spite of their frequent appearance as dominant species during the arctic spring bloom and throughout the summer months (Degerlund and Eilertsen, 2010), only very few lipid analyses have previously been performed on these species. There are several arguments for why lipid analyses are important, in respect to selecting suitable species for industrial production:

Firstly, the global awareness and demand of long-chain (LC) omega-3 fatty acids as food-additives for human consumption is on the rise. Secondly, the mismatch between supply and demand of fish oils for aquaculture is expected to reach 40 million tonnes in 2030 (Miller et al., 2008), and microalgae have the potential to replace the traditional sources of fish oil. Thirdly, diatoms, like other microalgae, may contain large concentrations of neutral lipid and carbohydrates that can be converted to biodiesel or bioethanol, gradually replacing fossil fuels. Finally, diatoms are important candidates for human food production, as they have high growth rates, beneficial nutrient content and do not compete with terrestrial crops.

1.2 Lipids

Lipids are a very large group of molecules, as far as the literature goes; "Biology, 8th edition" (Campbell and Reece, 2008) defines lipids as a large group of molecules characterized by their hydrophobic nature, mainly consisting of energy rich hydrocarbons with different side groups attached to hydroxyl molecules. Garret and Grisham (2012) defines lipids as naturally occurring molecules that have limited water solubility (Garret and Grisham, 2012), while T. E. Thompson defines lipids in *Encyclopaedia Britannica* as compounds that readily dissolve in non-polar organic solvents, while also having a region that is intrinsically hydrophilic (Thompson, 2014). These definitions are vague and means that lipids are not defined by the presence of a functional group or structure, but rather by common solubility properties, namely the solubility in non-polar organic solvents. This feature separates lipids from other biomolecules such as amino acids, proteins, carbohydrates and nucleic acids. The lipid group include a wide range of compounds, such as wax esters, carotenoids, terpenes, steroids, bile acids, acylglycerols, a range of structural lipids, fatty acids and a multitude of lipid-derived biomolecules that have thousands of functions

in the cell. Many of these have little in common in terms of structure and function and the fact that they are grouped under one definition creates a false sense of affiliation. It is therefore very important that when talking about lipids, one should establish a framework that defines what kinds of lipids are being discussed.

Firstly, it is common to separate between simple, or nonpolar, and complex, or polar lipids. The simple lipids are defined by the inability to yield more than two products when hydrolyzed (Stewart, 1974), and include acylglycerols, sterols and steryl esters, nonesterified (free) fatty acids and wax esters. Fatty acids (see Figure 1) consist of a hydrophilic carboxyl group bound to a hydrophobic hydrocarbon tail. Most fatty acids in nature are straight chained with an even number of carbon atoms, as a consequence of their synthesis by lipogenesis, where the two-carbon acetyl group of acetyl CoA is elongated two carbons at a time. They are defined by the length of the carbon chain and the degree of unsaturation, for example 20:5, which translates to 20 carbons in length and 5 carbon-carbon double bonds. It is also common to denote where the terminal double bond ends, by adding n-x or ω-x at the end. For example, docosahexaenoic acid (DHA), which is a fatty acid with 22 carbons, 6 double bonds and the terminal double bond from carbon 3 to 4 counting backwards from the terminal carbon, would with this system be named 22:6(n-3) or 22:6(ω-3) (Stewart, 1974). Because fatty acids like DHA have more than two double bonds in their backbone, they are called polyunsaturated fatty acids or simply PUFAs. Acylglycerols (mono-, di- and triglycerides) are the main energy storage in cells; they consist of one to three fatty acids bound to glycerol and are usually sequestered as droplets in the cytoplasm. The fatty acids in acylglycerols are usually short-chained (16-18C) and contain few double bonds. Wax esters are not commonly found in diatoms, and free fatty acids are usually only found in small concentrations. Sterols are molecules made up of characteristic ringed structures with hydroxyl side groups.

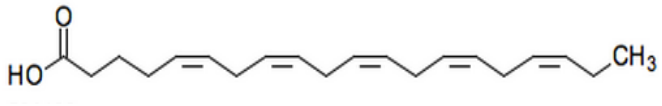
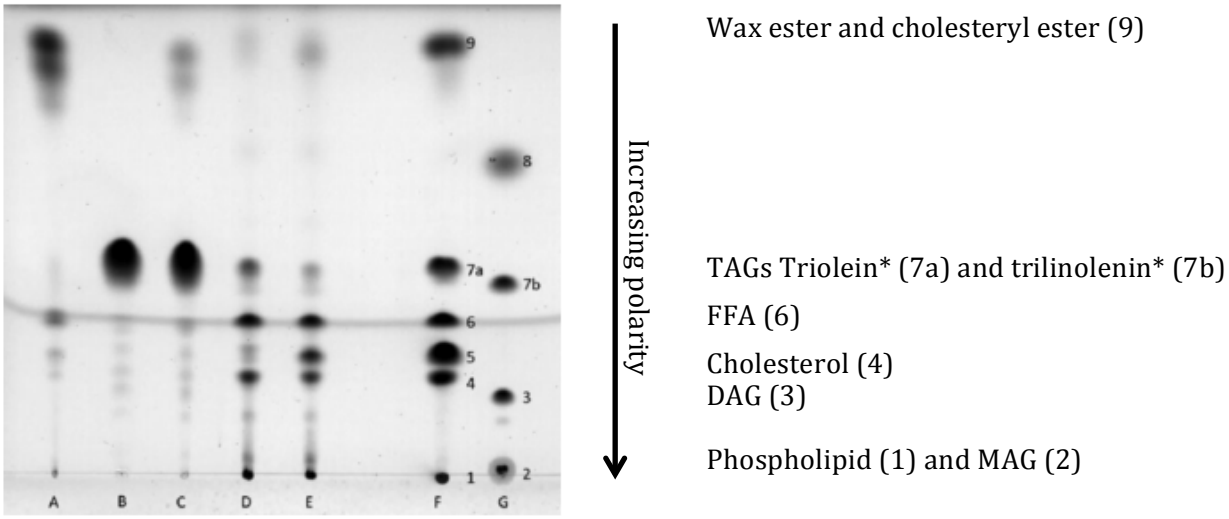


Figure 1: A fatty acid with 20 carbons, 5 double bonds and the terminal double bond at carbon 3 counting backwards from the terminal carbon. This omega-3 fatty acid is named EPA or eicosapentaenoic acid – eicosa for 20 carbons , pentaen for 5 double bonds, oic for fat.

Sterols mainly act as structural components in cell walls. In animals, cholesterol is the most important sterol. It is the major structural component in membranes, brain synapses and myelin sheaths. It provides the cell with both integrity and fluidity, removing the need of a cell wall and by consequence allows animal cells to move. Also, it acts as a precursor to vitamin D, bile, and a range of hormones. Cholesterol was for a long time thought to be absent or rare in plants, but improvements in analytical methods have shown that cholesterol is present in most plant membranes and surface lipids, but in small concentrations (Behrman and Gopalan, 2005). When one of the hydroxyl side groups of a sterol becomes esterified to a fatty acid, the sterol is called a steryl ester. Steryl esters are 'free' sterols and only occur in small concentrations in plant cells.

The complex lipids yield 3 or more products when hydrolyzed and are divided into two main classes: Lipids with a sphingosine backbone, named sphingolipids, and lipids with a glycerol backbone, named glycerolipids. Within these groups, the lipids are further defined by the structure of the polar head group: *Phospholipids* have a head group of phosphate bound to an alcohol, while *glycolipids* have a single head group of sugar. Phosphoglycerolipids and phosphosphingolipids, especially sphingomyelin, are characteristic of animal cells, where they are located in the cell- and organelle membranes, while glycoglycerolipids are characteristic of plant cells, where they are located in the photosynthetic membranes.

The simple and complex lipids are also called neutral and polar lipids, respectively. In truth, all lipids are negatively charged, but in varying degrees, the "neutral" lipids (fatty acids, sterols, acyl-esters) are simply much less polar than the polar lipids (phospho-, glyco- and sphingolipids). The polarity of lipid classes relative to each other can be illustrated by thin-layer chromatography (TLC), in which lipids are dissolved in a mobile phase. The partitioning behaviour of the lipid on the stationary phase determines how far it travels in the mobile phase: the less polar a lipid is, the further it travels. Figure 2 shows a TLC performed by Pedersen et al. (2014), in which the polarity-dependency is clearly displayed:



*Triglycerides composed of glycerol and three oleic acids (triolein) or three alpha-linoleic acids (trilinolenin)

Figure 2: TLC of *Calanus* oil (A), mouse diets (B and C), mouse feces (D and E), and lipid standards (F and G) containing PL (1), MAG (2), DAG (3), Cholesterol (4), FAOH (5), FFA (6), Triolenin (7a) and trilinolenin (7b), Fatty acid methyl ester (FAME, 8), Wax ester and Cholesteryl ester (9) (Pedersen et al., 2014).

1.3 Extraction and quantification of lipids

Historically, lipid extraction and quantification has mainly been performed by Folch's method from 1957 and Bligh and Dyer's method from 1959, where a system of chloroform, methanol and water is used to separate lipids from non-lipids in a tissue or cell. The lipids are sequestered in the non-polar chloroform phase, which can be isolated and removed by vaporization in order to find the mass of the lipids (Bligh and Dyer, 1959, Folch et al., 1957). Further separation of lipid classes can be achieved by using chloroform and methanol to collect the neutral and polar lipids respectively. The two methods use different ratios of solvents, and while the extraction step in Folch's method may take several hours or even days depending on the extraction material, the Bligh and Dyer method is possible to complete from start to finish in a mere ten minutes, according to the authors. After isolating the lipids, both the Bligh and Dyer and the Folch method require lipid fractioning by chromatographic methods in order to separate different lipid classes and quantify them individually. This makes the major

drawbacks of the conventional methods the time requirement of the Folch method and the possible inaccuracy of the Bligh and Dyer method, as well as the labour intensity required to separate polar and neutral lipids. Alternate methods have been developed that use dyes to stain lipids in order to quantify or identify them, rather than a procedure dependent on lipid extraction. There are a multitude of commercially available lipid soluble dyes, examples include Sudan Black B and Nile Red (Cooksey et al., 1987), Fluorol Yellow 088 and Sudan Red 7B (Brundrett et al., 1991), 1,6-diphenyl hexatriene, shortened DPH (Ranall et al., 2011), and the many BODIPY-dyes (Boldyrev et al., 2007). Of these, the fluorescent dyes DPH, Nile Red and BODIPY have become the most important alternative to lipid extraction. Neither Sudan Black B, Sudan Red 7B or Fluoral Yellow are fluorescent, and while BODIPY is an effective lipid stain with a high quantum yield, it has mainly been used in membrane studies of lipid trafficking, where it may for example be attached to acyl groups on phospholipids (Boldyrev et al., 2007). DPH has been shown to be a sensitive and effective reagent for analysis of lipid content, with similar performance to BODIPY and Nile Red (Ranall et al., 2011), but there are no records of this stain being used in microalgae, and its excitation/emission spectrum of 350/420 mμ may overlap with several autofluorescent biomolecules found in plants, see (Rost, 1995) for a list of autofluorescent molecules. Nile Red, however, has frequently been used to quantify the lipid content in animals, plants, bacteria, yeast, zooplankton and microalgae (Chen et al., 2009).

1.4 Fluorescence

Fluorescence is the ability of some molecules to absorb photons at a certain wavelength and as a result emit photons at a higher wavelength. The wavelength at which light is absorbed and emitted is called the excitation and emission wavelength respectively (Williams and Bridges, 1964), and the difference between the excitation and emission maxima is called the stokes shift. The *excitation maximum* of a fluorescent molecule is the wavelength of light that results in the highest level of excitation. The excitation maximum always matches the maximum absorption wavelength. The *emission maximum* of a

fluorescent molecule is the wavelength at which most photons are emitted when the molecule is being excited. Quantum yield is a term that is usually applied to fluorescent molecules or compounds (fluorophores) and describes the ratio of emitted photons per photon absorbed. A high quantum yield is a beneficial attribute in fluorophores that are used in experiments, because the fluorescence is easier to measure. Some biomolecules are intrinsically fluorescent, for example chlorophyll *a* (Chl *a*), which in a solution of acetone will have an excitation/emission spectrum of 430/670 nm (Lorenzen, 1966). When a biomolecule displays fluorescence in its natural state, it is called autofluorescence. Fluorescence as an analytical tool can be used to measure concentrations as low as 10^{-12} g/ml (Rye et al., 1993) , while colometrical approaches seldom allow for substances below 10^{-7} g/ml (Williams and Bridges, 1964). Fluorescent molecules are heavily utilized in a range of methods that are too numerous to list. They are most commonly used in microscopy techniques to stain tissues or molecular groups, or in fluorometric spectroscopy where the fluorescence is measured and related to amounts. The most well known examples are probably ethidium bromide for its use in visualising the DNA in a gel electrophoresis (Meyers et al., 1976), or GFP as a marker of gene expression (Tsien, 1998).

1.5 Nile Red

Nile Red (9-diethylamino-5H-benzo[α]phenoxazine-5-one) is a lipophilic, fluorescent dye with characteristics advantageous for *in situ* visualisation and measurement of lipids in a range of organisms: It is strongly fluorescent in hydrophobic environments (Figure 3) (Greenspan and Fowler, 1985, Greenspan et al., 1985) and organic solvents with high quantum yields, and only weakly fluorescent in water with a very low quantum yield (Fowler et al., 1987). It is photostable and its fluorescence is unaffected by pH between 4.5 and 8.5 (Sackett and Wolff, 1987). It can be used to distinguish between different types of hydrophobic material, as its excitation/emission spectrum is dependent on the polarity of the solvent (Ranall et al., 2011). Since it was first proposed as a probe for lipid measurement for microalgae by Cooksey et al. in 1987, it has been

widely used to screen microalgae, mainly of the *Bacillariophyceae, Xanthophyceae, Phaeophyceae* and *Chrysophyceae* classes (Chen et al., 2009).

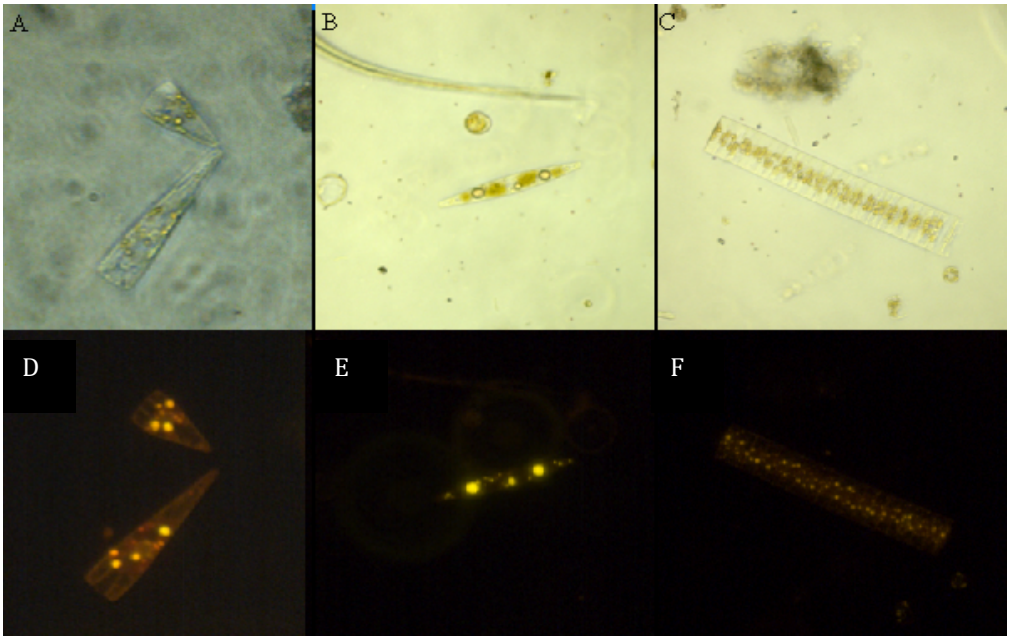


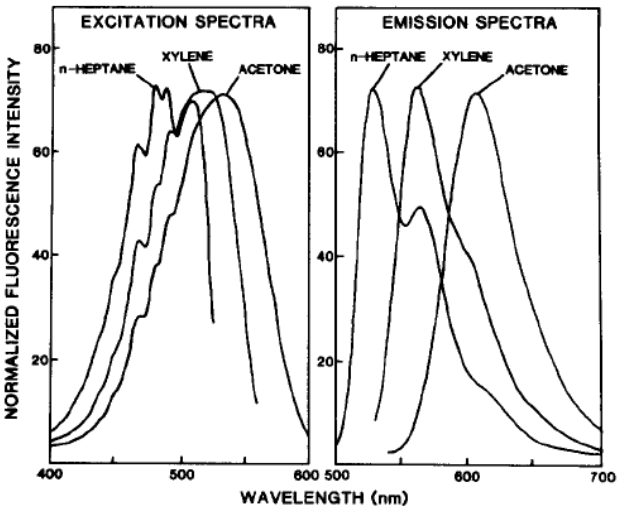
Figure 3: Cells stained with Nile Red seen with and without fluorescence. Neutral lipid droplets are observed as yellow fluorescence in D, E and F. A: *Lichmophora* sp. B: Pennate (indet). C: *Fragilariopsis* sp. Scale: 1:400 (A & B) and 1:200 (C). Pictures were taken onboard the R/V Helmer Hanssen during the Mabcent cruise in may 2013.

The excitation & emission maxima of Nile Red is blue-shifted with decreasing polarity of the solvent. Several articles (Boldrini et al., 2002, Elsey et al., 2007, Greenspan et al., 1985, Sackett and Wolff, 1987) have demonstrated this effect, by measuring the emission maxima in solvents of different polarity (Figure 4).

NILE RED EMISSION MAXIMA (nm)
IN VARIOUS SOLVENTS

Solvent	Em. max	Z[a]	D[b]
Water	665	94.6	80
30% Ethanol[c]	657	91.6	64
50% Methanol	655	90.9	58
40% Dioxane	653	88.4	44
Ethylene glycol	652	85.1	37
60% Dioxane	644	85.0	27
Methanol	642	83.6	34
Dimethyl sulfoxide	635	71.1	44
Ethanol	635	79.6	24
Butanol	633	77.7	18
80% Dioxane	633	80.2	11
2-Propanol	628	76.3	18
Acetonitrile	627	71.3	38
Dimethyl formamide	625	68.5	37
Acetone	615	65.7	21

[a] Kosower's Z index of polarity (21, 22).
[b] Dielectric constant.
[c] All solvent mixtures are in water.



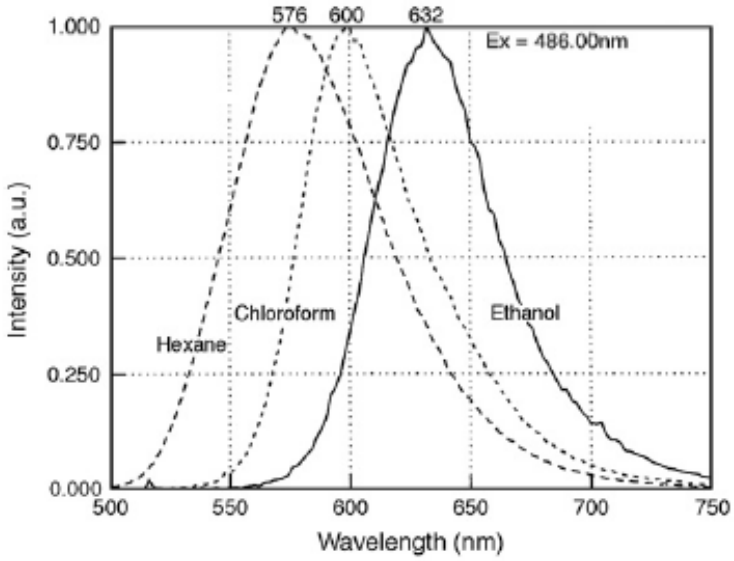


Figure 4: The spectral properties of Nile Red in different solvents. Top left: The emission of Nile Red in 15 solvents of differing polarity, when excited at 550 nm. The table was retrieved from Sackett and Wolff, (1987). Top right: The excitation and emission spectra of Nile Red dissolved in n-heptane, xylene and acetone. The figure was retrieved from Greenspan et al. (1985). Bottom: The emission spectra of Nile Red in hexane, chloroform and ethanol. The figure was retrieved from Elsey et al. (2007).

In microalgal studies, Nile Red has primarily been used in quantification of triglycerides (in articles commonly referred to as *neutral lipids*), and many studies have explored the fluorescent properties of Nile Red in triglycerides. The

emission maximum in triglyceride is 575 nm, when excited at 480-535 nm (Priscu et al., 1990, Chen et al., 2009, Cooksey et al., 1987). Nile red has also successfully been used to measure polar lipids in zooplankton (Alonzo and Mayzaud, 1999). The excitation/emission wavelengths used were the traditional wavelengths of 549/628 nm, but also a new pair of wavelengths that had been determined from scans of lipid standards and blanks to 560/610 nm. Both of these emission wavelengths are below the autofluorescence maximum from Chl *a*, which at room temperature peaks at 685 nm with a broad shoulder at about 740 nm (Mainly from photosystem II; photosystem I contributes less and peaks at longer wavelengths) (Krause and Weis, 1991). Chen et al. (2009), however, argues that the quantification of polar lipids in microalgae may be near impossible because of the fluorescence background caused by the high concentrations of chlorophyll (1-4% of dry weight). There is also a large chance that the fluorescence from neutral lipids will overlap with the fluorescence from polar lipids up to 630 nm. Depending on the type of cell measured and the staining temperature, the usual staining time is reportedly anywhere between 30 seconds and 10 minutes or longer (Chen et al., 2009, Cooksey et al., 1987). Chen et al. (2009) found that the emission maximum was reached after 10 minutes in *Chlorella vulgaris*, and did not recede for the duration of the experiment when measured every 10 minutes up to 100 minutes. Elsey et al. (2007) measured *Nannochloropsis* sp. and found that the maximum was independent of time, suspecting that the lack of reproducibility was due to varying diffusion rates through intracellular regions. Based on this, the authors suggested that a viable assay should not measure the intensity at a predetermined time point, but rather track the emission for a period of at least 40 minutes and use the maximum value recorded. It should be noted that unicellular green algae (such as *Nannochloropsis)* are exceptionally resistant to chemicals due to the presence of sporopollenin in their cell walls (Atkinson et al., 1972), and that this may effectively prolong the staining time required. Diatoms, on the other hand, have cell walls of silicate with large numbers of surface pores that should allow chemicals to enter the cell more easily. The effect of Nile Red concentration has been thoroughly tested by Chen et al. (2009); the optimum concentration was determined to be 0.5 µg/ml or 0.00157 mM.

1.6 Quantification of diatom biomass

The biomass of a diatom is a relative term and has been the source of much debate over the decades. The confusion arises in what we define as biomass and how we measure it. For example, the wet weight of an isolated diatom sample is probably the least accurate of all biomass measurements because it is highly dependent on the amount of interstitial water trapped between cells in the finished sample. Strickland (1960) comments that two "wet weights" should be recognized in phytoplankton. Firstly, the true weight of the cells themselves with no extraneous water, and secondly, the experimental wet weight that is obtained after collecting the cells in some manner. The experimental wet weight varies considerably depending on the harvesting technique and is greatly affected by interstitial water, and is therefore seldom less than twice the true algal weight (Strickland, 1960). The capability of holding interstitial water is highest in those algae that form colonies or have long setae, such as *C. socialis, C. furcellatus, S. marinoi* and *T. gravida*. Another common measurement is the dry weight, where biomass is dried at elevated temperature, but the method does not discriminate between organic matter and the silica cell walls of diatoms. As silica is an inorganic compound, it is debatable whether it should be accounted as biomass. In addition, the silica remains of dead cells (such as the one partly exposed on the right side of Figure 16D) may add significant weight to a sample's dry weight and thereby decrease the true ratio between dry and wet weight. For these reasons, the use of wet and dry weight in relation to other biomolecules should be avoided. A more applicable method is to measure the chlorophyll *a* (Chl *a*) content of a culture. Fluorescence and chlorophyll *a* correlates strongly in natural systems and laboratory studies (Matorin et al., 2004, Vedernikov et al., 1990). There are several benefits to measuring Chl *a* as opposed to other biomolecules; the method is quick, the volume requirement is small, it is detritus free and it is specific to autotrophic organisms. Holm-Hansen & Riemann (1978) developed the most commonly used method, where Chl *a* is extracted in methanol from microalgae collected on a filter, although methanol has gradually been replaced by ethanol in many laboratories, as it is safer and less expensive (Ritchie, 2006). The Chl *a* is subsequently quantified by measuring the intensity

of its autofluorescence on a fluorometer, and can be related to organic carbon content by using previously published ratios from diatom studies.

1.7 HPLC-MS of lipids

HPLC-MS is a combination of (high performance) liquid chromatography and mass spectroscopy. It is an ideal method for analyzing lipid molecular species, because it allows the user to combine separation by polarity with molecular structure. It can be used to analyze lipids across a large range of polarities without prior separation into neutral and polar lipids (Christie, 1985). Liquid chromatography functions by separating the molecules in a mobile phase based on the components' interaction with the stationary phase. Mass spectroscopy enables the characterization of these molecules. When the two methods are combined, the HPLC separate the molecules and elute them to the MS, where they are ionized as positive or negative ions by an ion source. The ions are separated based on their mass-to-charge ratio by a mass analyzer, and identified according to the mass spectrum they produce in the detector.

1.8 Diatom lipochemistry

The structural lipochemistry (e.g. polar membrane lipids & sterols) of diatoms is similar to that of green algae and higher plants. The chloroplasts are the site of lipid synthesis (Muhlroth et al., 2013), and the major phospholipids in algae are phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylglycerol (PG). There may also be substantial contributions of phosphatidylserine (PS), phosphatidylinositol (PI) and disphosphatidylglycerol (DPG). All of these are characteristic of extra-chloroplastic membranes, apart from PG, which is associated with the glycolipids in thylakoide membranes. The plastid (chloroplast) membranes associated with photosynthesis are mainly composed of glycolipids, where the main constituents are monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG) and sometimes also sulfoquinovosyldiacylglycerol (SQDG) (Arts et al., 2009). A special group of lipids called betaine lipids are also frequently found in algae, but not in higher plants, where they are thought to have been replaced by PC because of their similar structure and function. Three different types have been identified in algae so far: 1,2-diacylglyceryl-3-*O*-4′-(N,N,N-trimethyl)-

homoserine, 1,2-diacylglyceryl-3-*O*-2´(hydroxymethyl)-(N,N,N-trimethyl)-β-alanine and 1,2-diacylglyceryl-3-*O*-carboxy-(hydroxymethyl)-choline (Arts et al., 2009). The composition of sterols is similar for the entire plant kingdom and is characterized by a large set of different phytosterols (sterols that are similar to cholesterol) (Arts et al., 2009, Behrman and Gopalan, 2005). About a dozen sterols, including cholesterol, β-sisterol (24-ethyl cholesterol) and brassicasterol (24-methyl cholest-5,22-dien-3β-ol) are commonly reported as major sterols among diatom groups (Patterson and Nes, 1991, Gillan et al., 1981).

The composition of fatty acids is the most noticeable difference between terrestrial and marine plants. Higher plants mainly produce short chain unsaturates of palmitic- (C16:0) or stearic acid (C18:0), like palmitoleic acid (C16:1, n-7), linoleic acid (C18:2, n-6, shortened LA), oleic acid (C18:1, n-9) and alpha-linolenic acid (C18:3, n-3, shortened ALA), but lack the requisite enzymes to synthesize polyunsaturated fatty acids (PUFAs) of more than 18C (Spolaore et al., 2006). Marine and freshwater microalgae as well as marine bacteria have the ability to produce long-chain, unsaturated fatty acids (LC-PUFAs) from eicosanoic (C20) and docosanoic (C22) acid, such as docosahexaenoic acid (C22:6, n-3, shortened DHA), eicosapentaeonic acid (C20:5, n-3, shortened EPA) and arachidonic acid (C20:4, n-6, shortened ARA). Heterotrophic eukaryotes lack the ability to produce PUFAs in their entirety (with the exception of certain fungi), but can convert ingested ALA and LA to a range of other PUFAs including gamma-linolenic acid (C18:3, n-6, shortened GLA), ARA, EPA and DHA (Yehuda et al., 2002). In humans, the capacity to synthesize EPA and DHA is insufficient to provide ample amounts for maintenance of mental and cardiovascular health (Muhlroth et al., 2013); see section 1.7.

The knowledge of how LC-PUFAs are synthesized and regulated at the molecular level is fragmentary. Mulroth et al. (2013) exemplified the genetic pathways that control lipid metabolism and synthesis in the sequenced diatom *Phaeodactylum tricornutum.* A cluster of 106 genes was identified that encode enzymes coupled to the FA metabolism, the tricarboxylic acid cycle (TCA) and lipid synthesis. The cluster acts as a versatile machinery that, during dark periods, activate β-

oxidation of lipids in order to provide the TCA with Acetyl-CoA, producing energy in the form of ATP, FADH and NADPH, which then runs cell division. During light periods, cell division is inactive; instead many of the same enzymes involved in the β-oxidation provide Acetyl-CoA from TCA-intermediates, which act as a precursor for FA synthesis, replenishing the alga's energy stores (Muhlroth et al., 2013). EPA and DHA are synthesized from C18 saturated or unsaturated acyl-CoA intermediates, or acyl-lipids (FAs linked to the glycerol-backbone of PC). These go through several desaturation and elongation steps by a range of desaturases and elongases located in the different cellular compartments, where acyl-CoA pools are maintained by acyl-coenzyme A synthetases that esterify fatty acids into acyl-CoA. Each elongation and desaturation step adds two carbons and a carbon-carbon double bond (removing two hydrogens), respectively. The elongase- and desaturase-enzymes may be specific for each species and the compartments of the cells where they are localized; for example, in *P. tricornutum*, stearic acid (C18:0) is desaturated to oleic acid (C18:1, n-9) either by a Δ9 desaturase in the chloroplast or the cytosol, by stearyol-ACP Δ9 desaturase in the plastid, or by PTD9 (PTD for *Phaeodactylum tricornutum* desaturase*)* Δ9 desaturase in the ER-membrane (Muhlroth et al., 2013). In the case of acyl-lipids, the choice of enzyme is dependent on the *sn*-position of the fatty acid on the hydrocarbon backbone of the lipid. For example, PTD5 and PTD6 desaturases has highest desaturation activity at the *sn*-2 position of PC (Muhlroth et al., 2013).

1.9 The role and importance of PUFAs

PUFAs have two main functions (Certik and Shimizu, 1999): The first function is to act as structural components of biological membranes in phospho- or glycolipids, where they (i) regulate the phase transition, dynamics and permeability of the membranes, and (ii) modulate the behaviour of membrane-bound proteins such as receptors, ATPases, transport proteins and ion channels. The second function is to act as precursors of a multitude of metabolites (for example prostaglandins, eicosanoids and hydroxyl-fatty acids) that regulate critical biological functions. In animals, PUFA deficiencies have negative effects on the function of the nervous system, immune and inflammatory systems,

cardiovascular system, endocrine system, respiratory system, and reproductive systems (Certik and Shimizu, 1999). It is therefore not surprising that PUFAs like EPA and DHA are in high demand by animals and studies on zooplankton (Wichard et al., 2007) and cod larvae (Klungsoyr et al., 1989) have shown that these fatty acids are preserved as they are transferred upwards in the food chain from microalgae. Numerous studies have demonstrated the positive health effects of n-3 LC-PUFAS characteristic of marine lipids, namely EPA and DHA, on humans:

1) Regular consumption of LC n-3 FAs can reduce the risk of hypertension, thrombosis, myocardial infarction and cardiac arrhythmias (Kris-Etherton et al., 2002).

2) Adequate intake of EPA and DHA is crucial for development of the foetal brain (Innis, 2007, Horrocks and Yeo, 1999), and ARA and DHA are required for normal growth and development in infants (Adarme-Vega et al., 2012)

3) DHA deficiencies are associated with cognitive decline during aging and connected to the onset of Alzheimer disease (Horrocks and Yeo, 1999).

4) Increased consumption of DHA may decrease the severity of depression (Horrocks and Yeo, 1999).

5) Increasing the levels of EPA and DHA in patients with rheumatoid arthritis, asthma and ulcerative colitis has been found to reduce pain and improve conditions (Simopoulos, 2002).

1.10 Effects of light, temperature and nutrient limitation on lipid composition

A multitude of papers have demonstrated the effect of nutrient limitation on lipid allocation in microalgae: Richardson et al. (1969) found that the amount of fatty acid increased in *Chlorella sorokiniana* and *Oocystis polymorpha* when cellular nitrogen levels was reduced to 3% or less (Richardson et al., 1969). Xin et al. (2010) observed that *Scenedesmus* sp. could accumulate lipids to as high as 30% and 53% of dry weight when exposed to nitrogen or phosphorous limitation, respectively, and Klok et al. (2013) found that levels of triacylglycerol (TAG or triglyceride) were increased 8-fold in *eochloris oleabundans* grown in a nitrogen-limited system (while still maintaining cell replication). Diatoms and other microalgae are dependent on iron (Fe) to fixate nitrogen, as iron is a

structural component of nitrate-reductase (De Baar, 1994), and the limitation of Fe on growth has been linked to reduced activity of nitrate-reductase (Allen et al., 2008). In the ocean regions called the high nutrient – low chlorophyll (HNLC) waters, iron has been shown to be a limiting factor of phytoplankton growth (McKay et al., 1997). Liu et al. (2008) looked at the effects of Fe-concentration on neutral lipid accumulation. 5 cultures of *C. vulgaris* were grown at Fe-concentrations ranging from 0 to $1.2x10^{-5}$ M. The highest iron concentration used, $1.2x10^{-5}$ M (120000 nM), is very high compared to naturally occurring concentrations of 80-500 nM (De Baar, 1994), and resulted in slower growth and earlier arrival of the stationary phase compared to Fe-concentrations of $1.2x10^{-8}$ to $1.2x10^{-6}$. It was also the only Fe-concentration at which the lipid accumulation increased, by 7-fold. The authors hypothesized that the high iron concentrations probably modified some metabolic pathways related to lipid accumulation, and that the earlier onset of the stationary phase would be beneficial in an industrial setting where high lipid yield is important (Liu et al., 2008).

Temperature has been shown to be positively related to intracellular TAG in natural systems, however, the authors suggested that temperature is merely an indicator of stratification, causing nutrient limitation, which is the primary cause of increased storage lipid allocation (Wainman and Smith, 1997). The balance between storage lipids (acylglycerols) and membrane lipids seems largely dependent on environmental parameters, and while most algae accumulate short chain saturates and monounsaturates in the storage lipids under growth limitation, *T. pseudomonas* has been found to increase the incorporation of EPA in acylglycerols during its transition to the stationary growth phase (Mulroth et al., 2013). Experiments by Harrison et al. have demonstrated the complicated effects that nutrients, light and temperature have on the biochemical compositions of microalgae (Harrison, 1990). Harrison underlines that as species are inherently different in their natural composition of macronutrients, differing concentrations of these in the growth environment have species-specific effects that must be explored for each species independently. Harrison also notes that the shifts in lipid composition appear to be an inverse relationship between TAGs and polar lipids, but the relationship is clearly more

complex in some cases. The shift toward storage lipids has two purposes; Firstly, it acts as an energy sink when absorbed light no longer can be used to produce biomolecules containing the limited nutrients (Klok et al., 2013). Secondly, as an alga shifts from exponential growth to a stagnant phase, the photosynthetic machinery (with mainly polar membrane lipids) is converted to storage lipids so that the alga can reserve its energy for periods when nutrients again become available and binary fission can continue.

It should be noted that lipid *accumulation* and lipid *production* are separate from each other. Xin et al. (2010) and Klok et al. (2013) both stressed that even though TAG levels were increased under nitrogen and phosphorous limitation, the lipid product per unit volume and the overall biomass productivity of light was reduced. In other words, even though the lipid accumulation was increased, the lipid production was decreased. Many papers do not make this distinction and seem to suggest that increasing the lipid accumulation is the same as increasing the lipid production, which isn't true. This has obvious industrial implications; an industry based on bulk microalgal lipids cannot increase the yield of lipids by nutrient limitation, but should rather focus on selective breeding and genetic engineering to optimize lipid content and concentration.

1.11 Commercial use of microalgae

Large-scale production of microalgae for commercial use started in the early 1960's in Japan (Borowitzka, 1999), and by 2004 the annual global production exceeded 5000 tonnes of dry matter/year (Pulz and Gross, 2004). The main bulk of this production is for human/animal consumption and cosmetic ingredients, and the main production sites are in China, India and USA (Spolaore et al., 2006). Historically, microalgal mass-culturing systems have generally been composed of land based raceway systems in warm regions with large fresh-water deposits (Radmer, 1996), where small flagellates and cyanobacteria dominate the microalgal communities. Diatoms have therefore never caught on as targets for industrial mass production in scales comparable to those of fresh-water green algae and cyanobacteria. The commonly used strains are *Arthrospira spp., Aphanizomenon flos-aquae* (both cyanobacteria)*, Chlorella spp.* and *Dunaliella*

salina (both green algae), but some diatoms (*Phaeodactylum tricornutum, Chaetoceros* spp*., Skeletonema* spp. *and Thalassiosira* spp*.*) are grown as live feed for shrimp and bivavle larvae in aquaculture (Spolaore et al., 2006).

In the northern hemisphere diatoms dominate the cold-water microalgal communities (Degerlund and Eilertsen, 2010), but have yet to be exploited commercially. However, their derivatives (namely the marine omega-3 fatty acids DHA and EPA) are extensively harvested as fish oil from herring, anchovies, mackerel and sardines caught in the oceans of South America and Morocco, and capelin caught in the Atlantic and Arctic oceans. The fish oil is used as dietary supplements and feed for aquaculture in a global market that is expected to reach US $7.32 billions by 2020 (Grand-view-research, 2014).

1.12 Goals

This study had three main purposes: Firstly, the study aimed to analyse and compare the lipid content at two different growth temperatures, both quantitatively and qualitatively, of several species of northern/arctic diatoms by HPLC-MS. Secondly, the study would evaluate a rapid method of lipid quantification where the fluorophore Nile Red is used to stain lipids, as an alternative to lipid extraction. Thirdly, the study would visualize the effect of nutrient limitation on lipid allocation in *Coscinodiscus concinnus*.

2. Experimental

2.1 Description of species

All species were obtained from the stock collection kept in the plankton lab, Department of Arctic and Marine Biology (AMB), UiT The Arctic University of Norway. The species used were *Chaetoceros socialis, Chaetoceros furcellatus, Coscinodiscus concinnus, Porosira glacialis, Skeletonema marinoi, Attheya longicornis and Thalassiosira gravida*. They were originally collected on cruises following the Northern-Norwegian coast and the Norwegian and Barents Sea, and later identified by Huseby (2012b) by the following means: *A. longicornis, C. socialis* (AMB 33.2), *P. glacialis* (49.2D) and *S. marinoi* were identified using morphological and molecular methods, namely small subunit (SSU) 18S rDNA and large subunit (LSU) 28S rDNA gene sequencing. *C. concinnus, T. gravida* and *C. furcellatus* were identified from cleaned frustules and spores using light microscopy (Huseby, 2012b). The second strains of *C. socialis* (AMB 200) and *P. glacialis* (AMB 201) were identified on-board the R/V Helmer Hanssen using light microscopy. Table 1 below lists the species, their origin and average cell size.

Table 1: The latitude of origin, date of isolation, species identity and size of the diatoms that were studied. The size measurement is the length of the apical axis.

Species	*Origin*	*Isolated*	*ID*	*Size (µm)*
C. concinnus	74.2 °N	09.03.07	AMB 186	150-200
A. longicornis	69.5 °N	28.02.07	AMB 20.2	4-6
S. Marinoi	69.5 °N	9.3.07	AMB 39.2	2-20
*P. glacialis (7° C)**	69.4 °N	28.2.07	AMB 49.2D	30-40
*P. glacialis (1 & 7° C)**	76.3 °N	19.5.14	AMB 201	60-70
C. furcellatus	77.8 °N	8.6.07	AMB 61	8-20
*C. socialis (7° C)**	74.5 °N	2.3.07	AMB 33.2	2-14
*C. socialis (1° C)**	76.3 °N	19.5.14	AMB 200	2-14
T. gravida	69.4 °N	23.9.09	AMB 129	17-62

**Two strains of P. glacialis and C. socialis were used, isolated at two different cruises in 2007 and 2014*

2.2 list of chemicals

All chemicals that were used in the experiments are listed below (Table 2).

Table 2: All chemicals that were used in the master project.

Chemicals	*Supplier*
DMSO (99,9%)	Sigma Aldrich
Nile Red technical grade (N3013-100MG)	Sigma Aldrich
Ethanol (96%)	Sigma Aldrich
Guillard's F2 Marine water enrichment	Sigma Aldrich
Sodium metasilicate nonahydrate - ($Na_2O_3Si \times 9H_2O$)	Sigma Aldrich
Cod liver oil	Nycoplus

2.3 Preparation of silicate solution and growth medium (Guillard's F10)

Silicate solution was prepared by dissolving 3.5 g sodium metasilicate nonahydrate in 1 litre of miliQ water and adding 20 ml hydrochloric acid (37%). Guillards F/10 growth medium (Guillard and Ryther, 1962) was prepared in 10-litre volumes by filtering seawater from the Tromsø sound through a filter system (Millidisc ® 40-layer, 0,22 µm cartridge filter, Millipore corp., Billerica, USA) into a 10l beaker and heating the water to >90° C in a water bath. After cooling, the water was added 40 ml Guillard's F/2 and 10 ml silicate solution.

2.4 Cultivation procedure, harvesting procedure, Chl *a* measurements, biomass estimations and cell counts

Cultivation procedure:

Diatom monocultures were grown in temperature and light controlled rooms, or incubators, with 14 hours of light each day at a scalar irradiance of 66 mW m^{-2} nm^{-1}. The experimental temperature was constant for the entire growth period. Cultivation was initiated by diluting 5 ml of stock culture in 35 ml of growth medium in 50 ml Nunc culture flasks (Nunc A/S, Roskilde, Denmark). At one to three week intervals, depending on the density of the cultures, the cultures were transferred to larger containers and diluted with fresh medium in an approximate 1:3 ratio (one part culture and two parts F/10 medium). In a typical cultivation process, an initial 40 ml culture was diluted to a final volume of 5 liters in the following order (Figure 5):

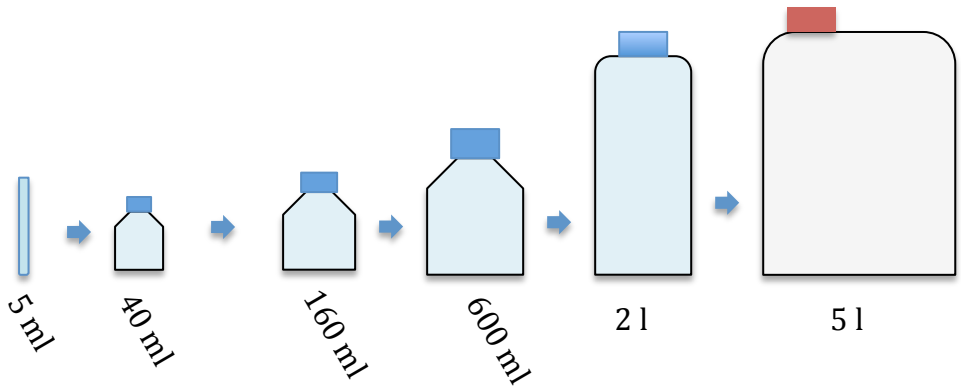


Figure 5: A typical cultivation procedure to a final volume of 5 litres, using 40, 160 and 600 ml Nunc culture flasks, 2 litre culture flasks (Falcon®) and 5 litre plastic jars (high-density polyethylene, purchased from Biltema, Tromsø).

The example shown above was the primary method of cultivation used in this thesis. Some samples were also cultivated further in 20 litre jars, and one culture was cultivated to a volume of 300 litres in a 600 litre cylinder before being harvested. The cultivation procedure was the same for these cultures.

A visual inspection of each culture was performed before each dilution, in order to make sure that no contaminating species had entered the cultures. This was done by transferring 2 ml of the culture into a lidded Nunc counting chamber (Nunc A/S, Roskilde, Denmark) and allowing the sample to sediment for at least 1 hour, depending on the species: The large species like *C. concinnus* and *P. glacialis* were fully sedimented in about 1 hour, while the smaller species were left for 3-4 hours. The visual inspection was performed in a light microscope, using 400X magnification. Contaminating species would have been recognized as cells of other, known species, but more often as small (~2 µm) flagellates that move in the medium. Contaminated cultures are usually discarded, but no contaminations were found in any of the cultures used in this study.

A total of 17 monocultures (4 of *A. longicornis,* 4 of *C. concinnus*, 3 of *S. marinoi,* 3 of *P. glacialis*, 2 of *C. socialis* and 1 of *T. gravida*) were grown at 7° C during the winter of 2013/2014. A total of 12 monocultures (3 each of *P. glacialis, C. furcellatus, C. socialis* and *A. longicornis)* were grown at 1° C, as well as one culture of *P. glacialis* grown at 7° C, during the summer and fall of 2014. A dense laboratory culture of diatoms usually contain between 0.1-0.2 grams of algae (wet weight) per litre medium. The 12 cultures that were grown at 1° C were therefore mixed according to species before being harvested, leaving 4 monocultures of 15-20 litres each. This was necessary to meet the high biomass requirement (>1 gram wet weight) for lipid quantification by solvent extraction at BioLab, Bergen. Each culture was given a unique NAPIS ID that defined the establishment where it was grown, the species, the light- and temperature-conditions for cultivation and the culture number in chronological order. For example, A NAPIS ID of 11674469 translates to: The algae lab at AMB (11), *Attheya longicornis* (67), high irradiance & high temperature (4) and the culture is number 469 to be cultivated in our records.

Harvesting procedure:

The cultures were harvested by vacuum filtration through a filter system that collected the algae onto filters (surface area: 17.35 cm^2). The filter pore size used was dependent on the size of the algal cells: *A. longicornis, C. furcellatus* and *C.*

socialis were collected onto 5 µm pore size polycarbonate filters (Nucleopore®, Costar corporation), while *P. glacialis, C. concinnus* and *T. gravida* were collected onto 20 µm pore size plankton mesh (Sefar Nytal®). The filtration set up used two Erlenmeyer flasks, where the first flask was connected to the second, and the second was connected to a vacuum pump by rubber tubes (Figure 6). The first flask captured the filtrated culture medium, while the second flask acted to prevent the culture medium from entering the vacuum pump should the first flask fill up.

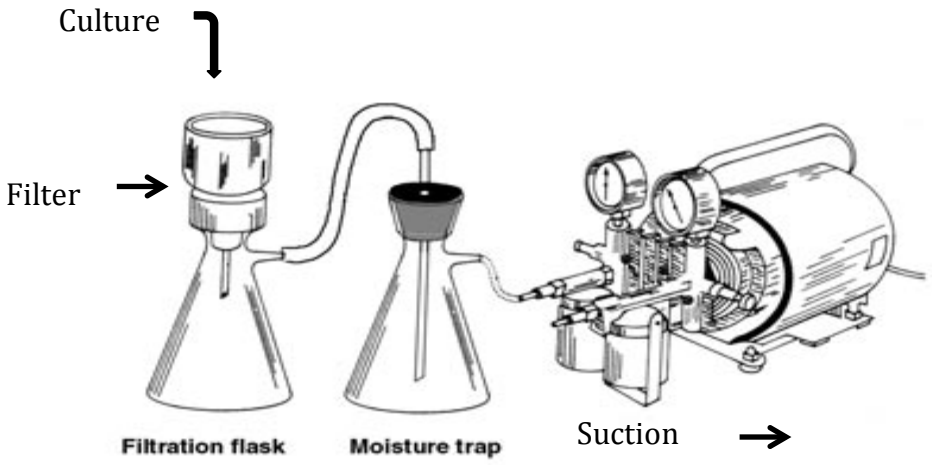


Figure 6: An illustration of the experimental set up used to filtrate cultures. Retrieved and edited from: http://www.nclabs.com/Technical%20Tips/Vacuum%20Pumps.html on October 15th, 2014

Depending on the cell density of the culture, the filter was cleaned regularly to prevent it from clogging, usually after filtering 0.5-1 litres of culture. The biomass collected on the filter was gently scraped off using a plastic spoon and collected in a beaker containing a small amount (10-20 ml) of F/10 medium. The beaker was placed in an ice bath. When all of the culture had been collected in the beaker, the resulting algal soup was transferred to a pre-weighed 50 ml Falcon tube and centrifuged for 5 minutes at 2500 RPM. After centrifugation, the supernatant was discarded using a 5 ml pipette, and the tube was weighed again. The weight of the algae was then determined by subtracting the weight of the empty tube from the weight of the tube after addition of algae.

Two of the cultures (See Table 7) were harvested at volumes of 300 and 90 litres, respectively. Harvesting these cultures with the set up described above would have taken too much time; instead the cultures were passed through a cone-shaped plankton net (KC Denmark A/S) with pore size 10 µm (for *A. longicornis*) and 20 µm (for *P. glacialis*). The diameter and height of the net was 25 and 40 cm, respectively, providing a total surface area of 2136.6 cm^2. The tips of the nets were equipped with valves. When the total culture volume had been passed through the net, the valve was opened and the algal soup collected directly in 50 ml Falcon tubes or beakers. While the filtration itself was much quicker with this method, there was a considerable loss of biomass due to cells passing through the net (mainly in the case of *A. longicornis)*, and cells prevented from entering the valve by sticking to the net wall.

Chl *a* measurements, biomass estimations and cell counts:

In vitro Chl *a* and phaeophytin content was determined using the method described in 1978 by Holm-Hansen and Riemann, using ethanol instead of methanol as the extraction medium (Holm-hansen and Riemann, 1978). The extraction volumes (volume of algal sample to be extracted for chl *a*) were at least 5 ml for all samples. The algal samples were filtered through 1 µm pore size GF/C filters (Whatman int., Maidstone, England), using a 12-channel sampling manifold (Millipore, Darmstadt, Germany) connected to a vacuum pump set to 0,3 ATM pressure. The filters were then transferred to glass sample tubes and added 5 ml ethanol (96%). The tubes were sealed with parafilm and covered in aluminium foil, and incubated at 7° C for 24 hours in the dark.

After incubation, the extracted samples were poured from the sample tubes to 10 ml quartz cuvettes, to be measured for fluorescence on a TD700 fluorometer (Turner Designs, Sunnyvale, California). The maximum fluorescence reading was recorded as the raw Chl *a* reading. The samples were then added 2 drops of HCl (10%) and recorded again; the maximum fluorescence reading was recorded as the raw phaeophytin reading. A blank containing 5 ml 96% ethanol was also measured and subtracted from the raw measurements.

The final measurements were converted to µg/l Chl *a* and µg/l phaeophytin using the equations:

$$\mu g\ Chl\ a\ l^{-1} = 0{,}003439 * (Rb - Ra)/V_{(l)} \quad (1)$$

$$\mu g\ Phaeophytin\ l^{-1} = (0{,}003439 * (2{,}11 * Ra) - Rb)/V_{(l)} \quad (2)$$

Where: Ra is the raw Chl *a* measurement and Rb is the raw phaeophytin measurement after subtracting the blank, and V is the volume in litres.

To obtain biomass measures in carbon (C) content, unpublished C:Chl *a* ratios obtained from experiments performed at the plankton lab in 2005 were used. The species were *S. marinoi*, *Thalassiosira hyalina*, *C. socialis*, *Thalassiosira nordenskioeldii* and *Bacteriosira bathyomphala*. Separate means from the two cultivation temperatures 1 and 7° C were used, the obtained values were 55.85 for algae grown at 7° C (with a standard deviation of 19.26), and 60.7 for algae grown at 1° C (std. dev. = 19.97). The dry weight was obtained by multiplying the calculated carbon content by 3.0 (Hans Christian Eilertsen, personal communication). Cell counts were performed by transferring 2 ml of algal culture into a lidded Nunc counting chamber, and counting the algal cells in a light microscope after they had sedimented. Depending on the density of the culture, cells/l were counted either as the average of three visual fields and multiplied by a factor determined by the magnification used (listed on each microscope), or as the total number in one counting chamber multiplied by 500. As a general rule, if a visual field did not contain at least 100 cells, the magnification was decreased.

2.5 Preparation of Nile Red stock- and working solution

One mM Nile Red stock solution (NRSS) was prepared by dissolving 15.92 mg technical grade Nile Red (molecular weight: 318.37) in 50 ml DMSO (>99.9% pure). The NRSS was then divided as 1 ml aliquots in Eppendorf tubes, and stored at -20° C. Nile Red working solution (NRWS) was prepared by thawing and dissolving NRSS to a concentration of 0.08 mM in 96% ethanol, in glass medicine bottles covered in aluminium foil and duct tape. NRWS was always prepared fresh at the day of experimentation, and never prepared in volumes

less than 5 ml in order to reduce the percentage variation caused by pipette inaccuracies. Whenever NRWS was used, care was taken to minimize its exposure to light. The exact volumes used in the preparation of both NRWS and NRSS are listed below (Table 3).

Table 3: The exact amounts of ingredients used to make NRSS from DMSO and Nile Red powder, and NRWS from NRSS and ethanol. The concentration of NRSS and NRWS was 1 and 0.08 mM, respectively.

	DMSO (>99,9%)	*spectral grade Nile Red*	*NRSS*	*Ethanol (96%)*
NRSS (50 ml)	50 ml	15.92 mg		
NRWS (10 ml)			800 µl	9,2 ml

The concentration of NRWS was 0.08 mM. A final NRWS concentration of 0.0016 mM (Chen et al., 2009) was achieved by dissolving 8 µl of NRWS in the total sample volume of 400 µl.

2.6 Determination of Nile Red spectral properties

The excitation/emission maxima for Nile Red in neutral and polar lipids were determined using cod liver oil ("Tran", 99.8% pure cod liver oil, Nycoplus) and L-a-phosphatidylcholine (Sigma Aldrich, P3556-25MG), respectively.

Nile Red in cod liver oil:

The emission maximum of Nile Red in cod liver oil was determined at a range of different wavelengths using a Stellarnet Black-Comet fluorescence spectrophotometer (Tampa, Florida) with a standard solution of 1 ml F/10 medium, 5 µl cod liver oil and 5 µl NRWS in a clear plastic cuvette. Before each measurement, the sample was thoroughly mixed. The excitation wavelengths used were white light, 390 µm (UV light), 470 µm (Blue light), 502 µm (green light), 590 µm (yellow light) and 660 µm (red light).

The excitation maximum at 575 nm emission was determined using 60 µl NLS (See section 2.7: Neutral lipid standard) dissolved in 332 µl F/10 medium and

added 8 µl NRWS in a black well plate. The solution was measured with the scanning function on a SpectraMax GeminiEM plate reader (Molecular Devices, serial no. EMO1102). This function measured the emission at 575 nm for all excitation wavelengths within a pre-defined range (475-535 nm), and created a spectrum of the relative emission for each excitation wavelength.

Nile Red in phosphatidylcholine:

The emission and excitation maximum of Nile Red in phosphatidylcholine was determined using 40 µl PLS (See section 2.7: Polar lipid standard) dissolved in 352 µl F/10 medium and added 8 µl NRWS in a black well plate. The solution was measured using the emission scan and excitation scan functions on a SpectraMax i3 spectrofluorometer (Molecular Devices, serial no. 35 370-1273). A blank containing 40 µl isopropanol:chloroform (10:1), 352 µl F/10 medium and 8 µl NRWS was measured simultaneously and included in the emission scan plot.

Optimum staining time:

Note: As the algae used in this thesis were 1) northern in origin and therefore experimented on at low temperatures and 2) larger than commonly studied species (with the exception of *A. longicornis* and *C. socialis*), the staining time should be *at least* 10 minutes to ensure that the Nile Red has sufficiently entered the cells.

The optimum staining time was tracked over time for four species (*A. longicornis, S. marinoi, P. glacialis and C. concinnus).* The experimental set up was as follows: Triplicates of 1 mg algae per 400 µl F/10 medium were stained with 8 µl NRWS in a black well plate. Fluorescence was measured every 10 minutes for a total of 60 minutes on a FilterMax F5 multimode microplate reader (Molecular Devices, serial no. 26 270-1233). The excitation/emission filter used was 518/574 nm (See section 3.2: Maximum emission & Maximum excitation wavelength).
The samples were incubated in the dark at 7° C for 10 minutes before and between each measurement.

As the fluorescence of Nile Red is reduced when it is exposed to light, there was a possibility that the fluorescence readings would be reduced by consecutive readings. In order to test this, two more algal suspensions of *C. concinnus* and *A. longicornis* were stained and incubated for 60 minutes before being measured and compared to the previous 60-minute measurements.

2.7 Lipid standards

Neutral lipid standard:

A neutral lipid standard (NLS) was prepared using cod liver oil. One gram (1.08 ml) of cod liver oil was weighed in a 50 ml Falcon tube and added chloroform in a 1:1 ratio, creating a stock solution with an oil concentration of 0.46 g/ml. The oil was weighed to ensure correct volume, as its high viscosity makes it unsuitable for pipetting. The stock solution was diluted 20 times with isopropanol to a concentration of 23 mg/ml. Isopropanol was used as the primary solvent because it has a low background fluorescence, and the initial addition of chloroform was required for the oil to dissolve in isopropanol (Priscu et al., 1990). The neutral lipid solution was then measured on a FilterMax F5 multimode microplate reader as triplicates of 0, 20, 40 and 60 µl in a total sample volume of 400 µl with 8 µl NRWS. The samples containing less than 60 µl of the neutral lipid standard were added isopropanol:chloroform (20:1) so that each well contained the same amount of F/10, NRWS and isopropanol/chloroform. The excitation/emission wavelengths used were 518/574 nm (See section *3.2: Maximum excitation wavelength)*. Table 4 on the next page lists the volumes used, and the concentrations of cod liver oil in mg/ml measured for Nile Red fluorescence.

Table 4: The table lists the volumes and concentration of neutral lipid standard, and the volumes of F/10 growth medium, isopropanol:chloroform (20:1 solution) and NRWS that was used to create the neutral lipid standard plot.

Volume NLS (µl)	*Conc. (mg/ml)*	*F/10 (µl)*	*Iso:chloro (µl)*	*NRWS (µl)*
0	0	332	60	8
20	0.115	332	40	8
40	0.23	332	20	8
60	0.345	332	0	8

Polar lipid standard:

The polar lipid standard (PLS) was prepared by weighing 10 mg L-a-phosphatidylcholine (Sigma Aldrich, P3556-25MG) in a 1.5 ml Eppendorf tube and adding 100 µl chloroform. The solution was then diluted with 900 µl isopropanol, resulting in a final concentration of 10 mg/ml. The standard solution was measured on a SpectraMax i3 spectrofluorometer as triplicates of 0, 2, 5 and 10 µl in total sample volumes of 400 µl, with 0.0016 mM NRWS. The samples that contained less than 20 µl of the polar lipid standard were added isopropanol:chloroform (10:1) so that each well contained the same amount of F/10 and isopropanol/chloroform. The excitation/emission wavelengths used were 550/630 nm (see section 3.2: *Maximum excitation wavelength*. The exact volumes used, and the concentrations of PLS are listed in Table 5 below:

Table 5: The table lists the volumes of F/10, isopropanol:chloroform (10:1 solution), NRWS and the volume and concentration of the polar lipid standard that was used to create the polar lipid standard plot.

Volume PLS (µl)	*Conc. (mg/ml)*	*F/10 (µl)*	*Iso:chloro (µl)*	*NRWS (µl)*
0	0	372	20	8
2	0.05	372	18	8
5	0.125	372	15	8
10	0.25	372	10	8

2.8 Visualization of lipid allocation

Two cultures of *Coscinodiscus concinnus* (AMB 186) were grown at 7° C. The cultivation procedure was as follows: 5 ml stock culture was diluted with 35 ml F/10 medium in a 40 ml Nunc culture flask and held at a photoperiod of 14 h light (irradiance: 66 mW m^{-2} nm^{-1}): 10 h darkness, and labelled "Resting phase". The "resting phase" culture was not added nutrients or growth medium for 8 weeks. At the 7th week of cultivation, another 5 ml stock culture was diluted in the same way and labelled "exponential phase". After a total of 8 weeks, 5 ml of each culture was transferred to sample tubes and stained with NRWS (5 µl/ml). The cultures were incubated in the dark for 60 minutes and then mixed thoroughly. A volume of 100 µl of each culture was transferred to glass microscope slides (VWR International) and covered with microscope cover glasses (Thermo Scientific). The cultures were observed in a fluorescence microscope (Leica Leitz DM RBE) and photographed using an attached camera (Leica DFC 420). The images were processed using the software LAS Suite v4.0.

2.9 Quantification of lipid concentration using Nile Red fluorescence

The quantification of lipid concentration using Nile Red was performed on 5 species of algae (Table 6) that were later sent to BioLab (part of Nofima) in Bergen for a full lipid analysis including lipid quantification.

Table 6: The species, growth temperature and amounts of microalgae, medium and NRWS used in the lipid quantification.

Species	*Weight (mg) in 1 mL*	*Volume (µL) needed for 1 mg*	*Medium (µL)*	*NRWS (µL)*	*Sample volume (µL)*
P. glac. (7° C)	34.3	29.15	362.85	8	400
A. longicornis	88.2	11.34	380.66	8	400
C. socialis	27.7	36.10	355.90	8	400
P. glac. (1° C)	57.2	17.48	374.52	8	400
C. furcellatus	15	66.66	325.34	8	400

The experimental procedure was as follows: A small portion of the frozen diatom samples were scooped out with a steel spatula and resuspended in 1 ml F/10 medium in 1.5 ml Eppendorf tubes. The weight of the portions was determined

by weighing the Eppendorf tubes before and after addition of algae. Of these resuspensions, triplicate proportions were transferred to black well plates so that each well had a total of 1 mg of algae, including an identical set of blank triplicates. The sample wells were then added F/10 medium and 8 µl NRWS to a total sample volume of 400 µl and incubated for 10 minutes. Neutral lipid fluorescence was measured using the FilterMax F5 multimode microplate reader at an excitation wavelength of 518 nm and an emission wavelength of 574 nm. Polar lipid fluorescence was measured on the FilterMax F5 multimode microplate reader at an excitation wavelength of 550 nm and an emission wavelength of 630 nm. The readings from the blank wells were subtracted from the sample readings, and the final readings were compared to the lipid standards to determine the lipid concentration.

2.10 HPLC-MS

High-performance lipid chromatography and mass spectroscopy (HPLC-MS) was performed at two different labs: The algae grown at 7° C were analysed at the Department of Food Analysis and Nutrition at the Institute of Chemical Technology (ICT) in Prague. The algae grown at 1° C were analysed at BioLab, which is a part of the Norwegian Institute of Food, Fisheries and Aquaculture Research (Nofima) in Bergen. An additional culture of *P. glacialis* grown at 7° C was also sent to BioLab. Table 7 lists all cultures that were sent for HPLC analysis in Bergen and Prague. The cultures sent to BioLab were crushed in a pestle and mortar in liquid nitrogen before being sent, to facilitate the lipid extraction performed there. These cultures were also measured for Nile Red fluorescence; see section 2.9: Quantification of lipid concentration using Nile Red.

Extraction procedure, ICT, Prague:

Samples of 50 mg diatom of each NAPIS ID were homogenized, added 500 µL of water and vortexed with glass bubbles in the ratio 5:1 (glass bubbles:sample) for 1 minute. 3 ml of dichlormethane/methanol (1:1 v/v) was added, and the mixture was sonicated on an ultrasonic bath for 1 hour. Finally, the samples were centrifuged for 7 minutes at 10,000 RPM and the extract transferred to a vial.

Extraction procedure, BioLab, Bergen:

Lipids were extracted according to Bligh and Dyer (1959), modified by reducing all weights and volumes by a factor of 1:5. The lipid content was determined gravimetrically from a subsample of the chloroform phase. No additional description of the method was provided.

HPLC-MS method, ICT, Prague:

Lipids were separated using a Dionex UltiMate 3000 RS UHPLC system (Thermo Fished Scientific, Waltham, USA), coupled with a AB SCIEX TripleTOF® 5600 quadrapole-time-of-flight mass spectrometer (AB SCIEX, Concord, ON, Canada). The following lipid species were searched for:

Glycerophospholipids:

- PA – Phosphatidic Acid
- PC – Phosphatidylcholine
- LPC – Lysophosphatidylcholine
- PE – Phosphatidyletanolamine
- LPE – Lysophosphatidyletanolamine
- PG – Phosphatidylglycerol
- LPG - Lysophosphatidylglycerol
- PI – Phosphatidylinositol
- LPI – Lysophosphatidylinositol
- PS – Phosphatidylserine
- LPS – Lysophosphatidylserine

Sphingolipids

- SM – Sphingomyelin
- Cer – Ceramide

Glycerolipids

- DAG – Diacylglyceride
- TAG – Triacylglyceride
- MAG – Monoacylglyceride

Fatty acyls

- FFA – Free Fatty Acid

HPLC-MS method, BioLab, Bergen:

Lipid class analyses were performed by a HPLC system (PerkinElmer, Waltham, USA). The dominant lipid classes were separated using a mobile phase (tertiary gradient), with a LiChrosphere® 100, 5 µm diol column (4 x 125 mm, Merck KGaA, Germany) slightly less polar than the standard Si-columns normally utilized. The mobile phase was evaporated using a nebulizer, ionized and measured for voltage using an ESA Corona® Plus Charged Aerosol Detector (ESA Biosciences, Inc., Chelmsford, U.S.A.). See (Homan and Anderson, 1998, Moreau, 2006) for more information on the method.

3. Results

3.1 List of cultures grown

All cultures used in this study are listed in Table 7 below:

Table 7: A list of the species used in this study. The table includes each culture's NAPIS ID, the volume at the time of harvest, the growth temperature, the location of HPLC-MS analysis and biomass measurements in wet weight, chl a content, cell number, estimated carbon content and estimated dry weight.

NAPIS	*Species*	*V. harvested (l)*	*G. T. (°C)*	*µg Chl a/L*	*Harvested (mg)*	*Cells/l (*10^6)*	*Total C (mg)*	*Dry weight (mg)*	*HPLC*
11674469	*A. longicornis*	4	7	197.40	1183.50	80.582	44.10	132.30	Prague
11674476	*A. longicornis*	3	7	391.36	647.10	334.986	65.57	196.72	Prague
11674512	*A. longicornis*	45	7	431.59	7380.00	-	1084.70	3254.11	Prague
11674492	*A. longicornis*	300	7	54.20	-	65.731	908.10	2724.29	Prague
11034470	*C. socialis*	4	7	250.36	821.70	154.347	55.93	167.79	Prague
11034477	*C. socialis*	3	7	267.55	602.60	100.301	44.83	134.49	Prague
11034486	*C. socialis*	3	7	383.79	525.00	200.255	64.30	192.91	Prague
11414473	*C. concinnus*	4	7	42.37	784.50	0.035	9.47	28.40	Prague
11414472	*C. concinnus*	4	7	98.87	1090.90	0.054	22.09	66.26	Prague
11414487	*C. concinnus*	20	7	49.35	1214.40	0.067	55.12	165.37	Prague
11414494	*C. concinnus*	40	7	16.37	829.60	0.023	36.57	109.71	Prague
11664474	*P. glacialis*	4	7	185.71	576.60	6.861	41.49	124.46	Prague
11664485	*P. glacialis*	4	7	159.23	309.20	5.616	35.57	106.71	Prague
11664497	*P. glacialis*	55	7	36.45	848.80	0.499	111.98	335.93	Prague
11684488	*S.marinoi*	2	7	282.00	508.40	253.488	31.50	94.50	Prague
11684510	*S.marinoi*	20	7	199.46	557.30	318.918	222.80	668.40	Prague
11684511	*S.marinoi*	20	7	174.70	188.70	185.021	195.14	585.42	Prague
11094478	*T.gravida*	2	7	13.96	165.50	0.823	1.56	4.68	Prague
1167520	*A. longicornis*	16	1	567.89	4250.00	146.188	551.54	1654.61	Bergen
1132521	*C. socialis*	20	1	274.43	4930.00	247.097	333.16	999.48	Bergen
11662523	*P. glacialis*	15	1	265.26	2240.00	5.860	241.52	724.56	Bergen
1182522	*C. furcellatus*	15	1	276.95	6750.00	9.340	252.17	756.50	Bergen
1166519	*P. glacialis*	90	7	458.76	6380.00	1.206	2305.97	6917.91	Bergen

3.2 Maximum emission wavelength, maximum excitation wavelength and staining time

Maximum emission and excitation wavelength of Nile Red in fish-oil:

The maximum emission wavelength of Nile Red in cod liver oil was measured at several excitation wavelengths. The results showed that the maximum emission wavelength is constant for a range of excitation wavelengths, i.e. there is a range of excitation wavelengths that will always produce the same maximum emission wavelength (Figure 7):

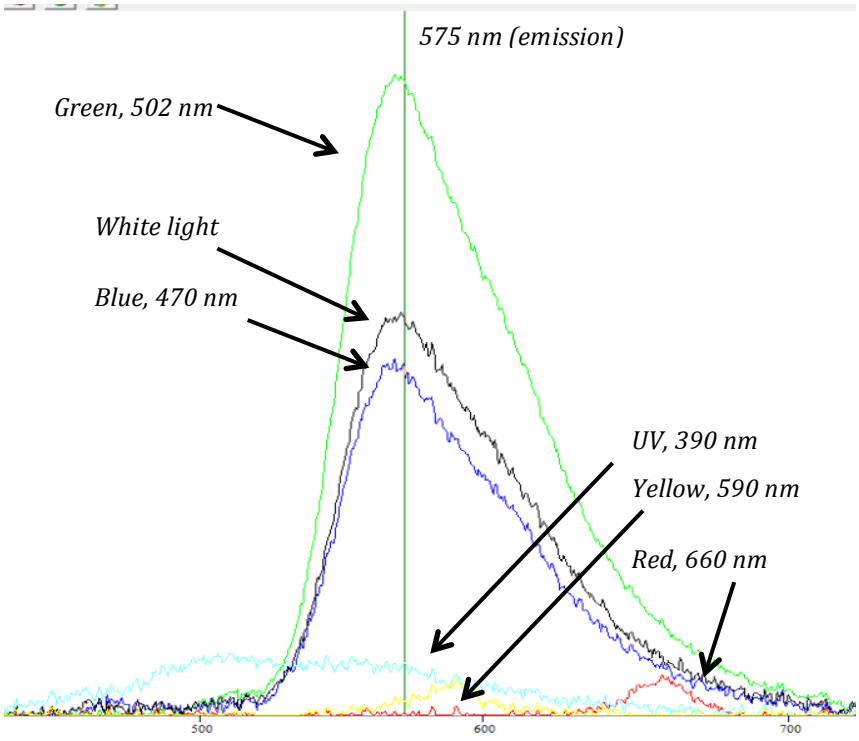


Figure 7: The maximum emission spectra for Nile Red in cod liver oil when excited at different wavelengths. The green middle line marks 575 nm.

The emission maximum was at 575 for white light, blue light (470 nm) and green light (502 nm). The intensity was highest for green light. UV light (390 nm) produced a broad shoulder of fluorescence with a peak at approximately 510 nm, but the intensity was low. Yellow (590 nm) and red (660 nm) light did not produce fluorescence; the peaks seen in the figure are from the light sources themselves. The maximum emission wavelength for Nile Red in neutral lipids

was determined to be 575 nm. The maximum excitation wavelength was determined by excitation scanning (Figure 8):

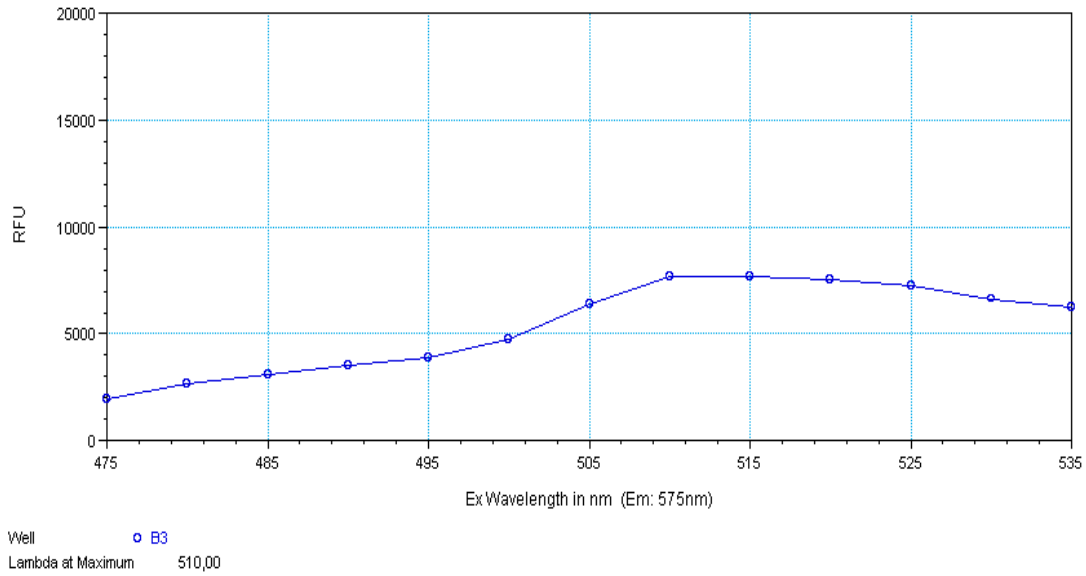


Figure 8: The excitation wavelength spectrum for Nile Red in cod liver oil, F/10 and isopropanol:chloroform with emission at 575 nm.

The emission was at its highest when Nile Red was excited at 510-520 nm. The excitation of Nile Red should therefore occur within these wavelengths.

An emission scan of Nile Red in cod liver oil confirmed that the maximum emission wavelength was 575 when excited at 515 nm (Figure 9):

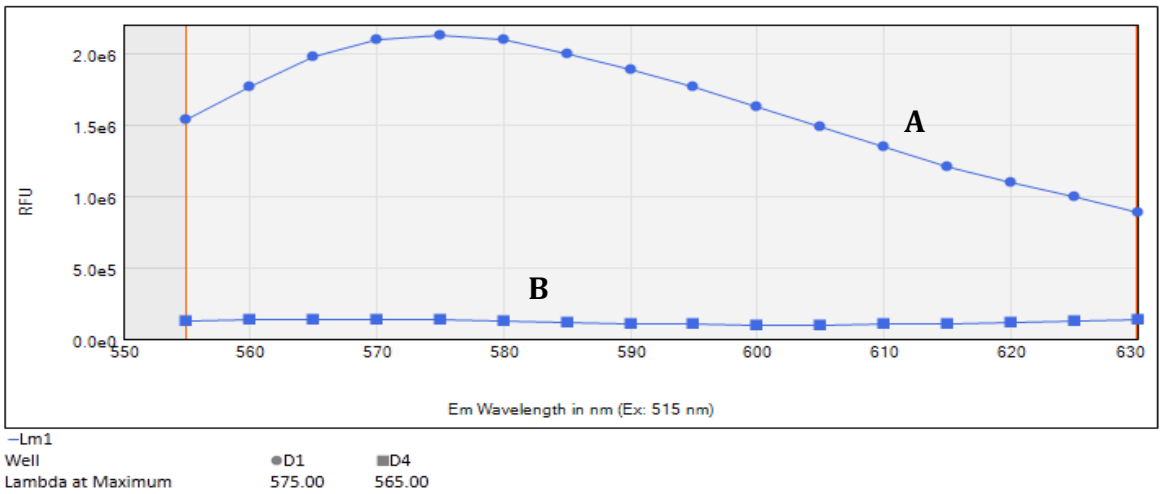


Figure 9: The relative emission spectrum of Nile Red in cod liver oil, F/10 and isopropanol:chloroform (A), and Nile Red in F/10 and isopropanol:chloroform (B), when excited at 515 nm.

Maximum emission and excitation wavelength of Nile Red in PC:

The maximum emission wavelength of Nile Red in PC was found by plotting the emission spectra of Nile Red in PC when excited at 560 nm, shown in figure 10 below. The maximum emission wavelength was 630 nm. The emission at 610 nm (reported as the maximum emission wavelength for PC by Chen et. al (2009)) was lower (25 million RFU) than the emission at 630 nm (30 million RFU).

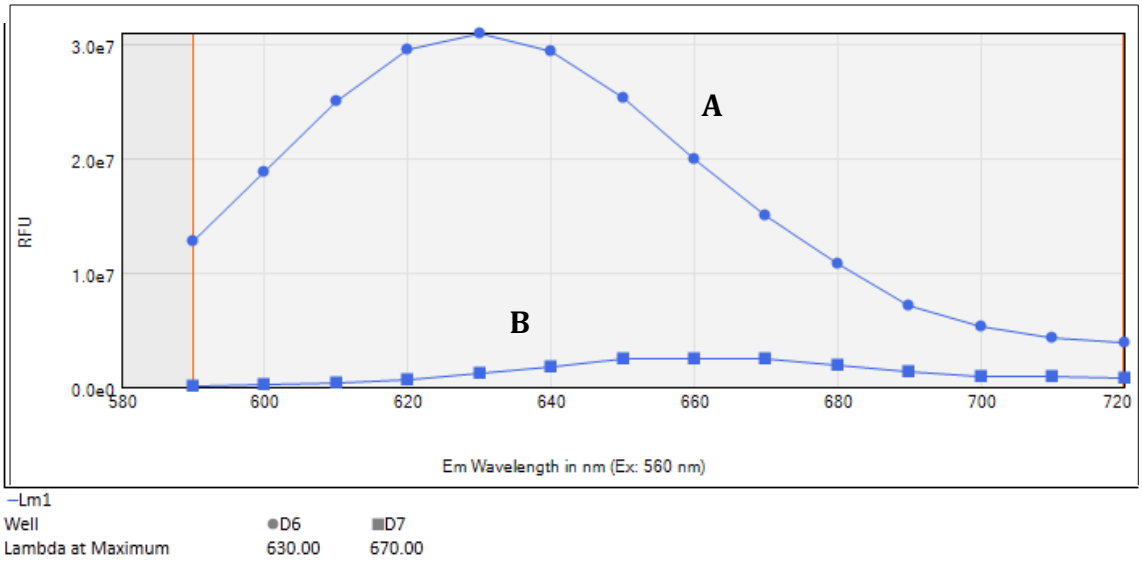


Figure 10: The relative emission spectrum (or excitation spectrum) of Nile Red in PC, F/10 and isopropanol:chloroform (A) and Nile Red in F/10 and isopropanol:chloroform (B).

The excitation spectrum of Nile Red in PC was measured at 630 nm emission and showed that the maximum excitation wavelength for 630 nm emission was 550 nm (Figure 11):

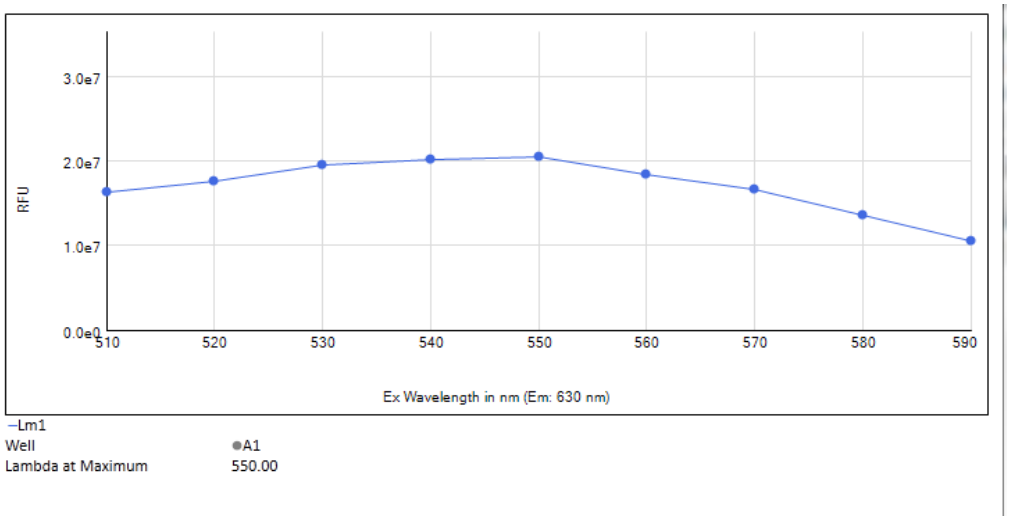


Figure 11: The excitation spectrum at 630 nm emission of Nile Red in PC and Isopropanol:chloroform.

Staining time:

Note: All raw fluorescence measurements are listed in Appendix 1 for both time and standard measurements.

Triplicate samples of *C. concinnus, P. glacialis, C. furcellatus* and *A. longicornis* were stained with Nile Red and measured for fluorescence every 10 minutes for 60 minutes. The average for each species is given in Figure 12 below:

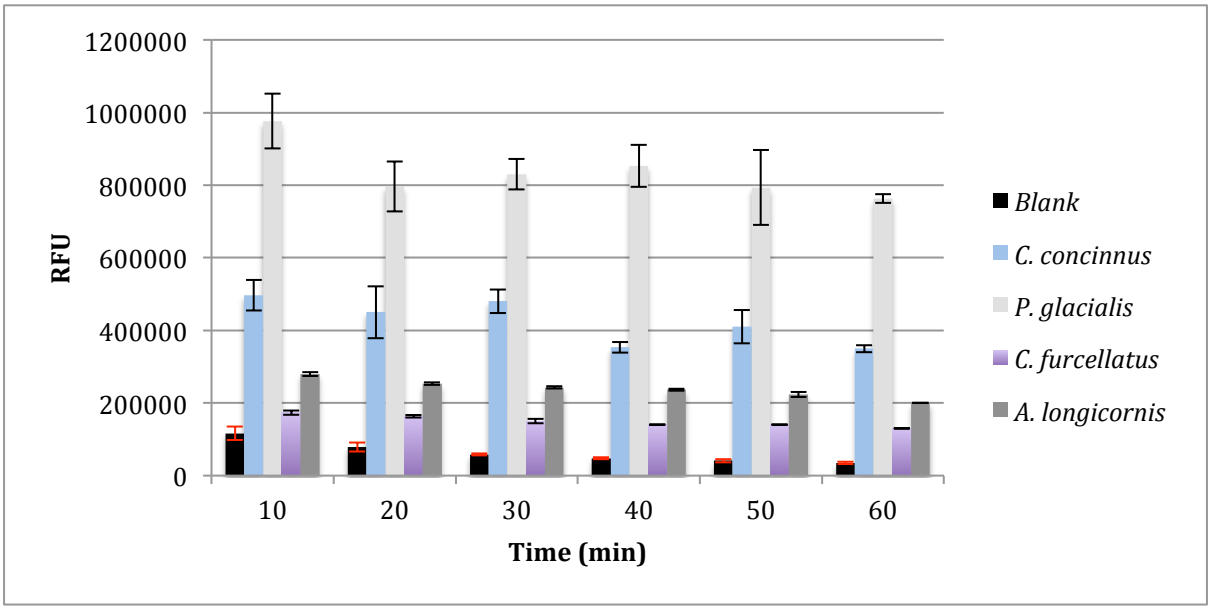


Figure 12: The fluorescence intensity of Nile Red neutral lipid fluorescence (em/ex 515/575 nm), measured every 10 minutes for 60 minutes in 4 species of microalgae and 1 blank. The columns are averages of triplicates and the error bars are the standard errors of the means.

The fluorescence intensity was at its highest after 10 minutes of staining, and the difference between measurements was significant for all species ($p<0.05$) apart from *P. glacialis* ($p=0.46$, Kruskal-Wallis).

Two more suspensions of *A. longicornis* and *C. concinnus* were stained for 60 minutes before being measured, in order to test if the fluorescence of Nile Red was reduced as a result of multiple excitations. Figure 13 shows the average measurements after 60 minutes of staining compared to the average measurements at 60 minutes measured every 10 minutes.

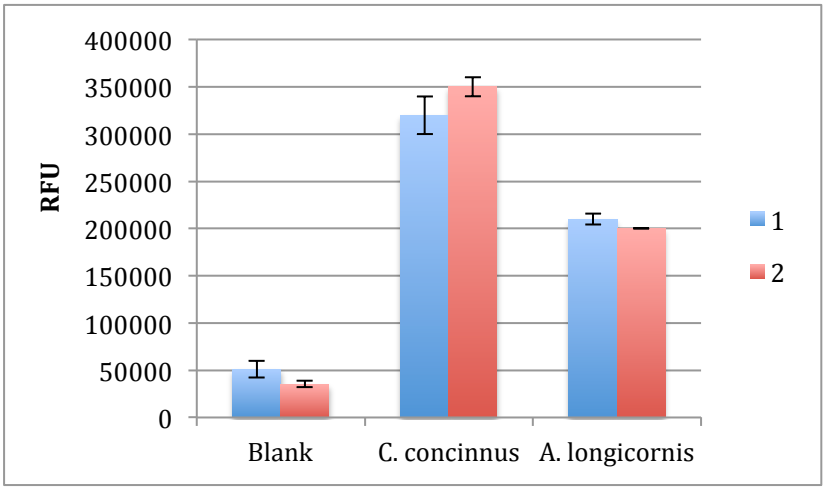


Figure 13: The average fluorescence intensity of Nile Red in neutral lipid standard, measured at 515/575 nm after 60 minutes of staining (1) compared to the average fluorescence intensity after 60 minutes of staining measured every 10 minutes (2). The error bars represent the standard error of the mean. The observed difference was not significant for either of the species ($p<0.05$, Kruskal-Wallis).

The fluorescence intensity was highest after 10 minutes of staining for all species (Figure 12), and the relative fluorescence was not reduced by multiple measurements (Figure 13).

3.3 Lipid standards

The lipid standards of cod liver oil and PC were linear and reproducible, with linear trend lines following the equations:

Neutral lipid standard: $y = 0.0755x + 0.0059$

Polar lipid standard: $y = 0.0226x - 0.0137$

Figure 14 and 15 below shows the neutral and polar lipid standard plot, respectively.

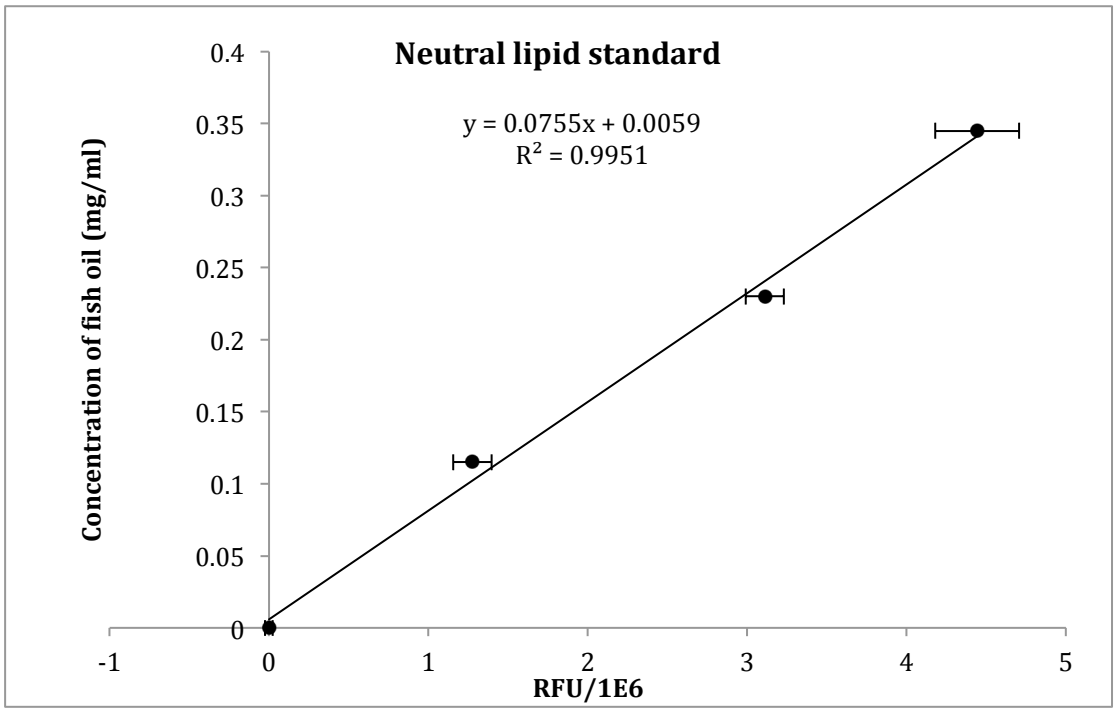


Figure 14: The neutral lipid standard measurements of NRWS in cod liver oil of increasing concentrations, with excitation/emission at 515/575 nm. The x-axis lists the raw fluorescence in RFU divided by 1 million. The background fluorescence (456,666.6 RFU) was measured at 0 mg/ml and subtracted from the measurements displayed in the plot. The horizontal error bars are standard errors of the mean (+ and -). A linear trend line, its equation and the R^2 value were included in the plot.

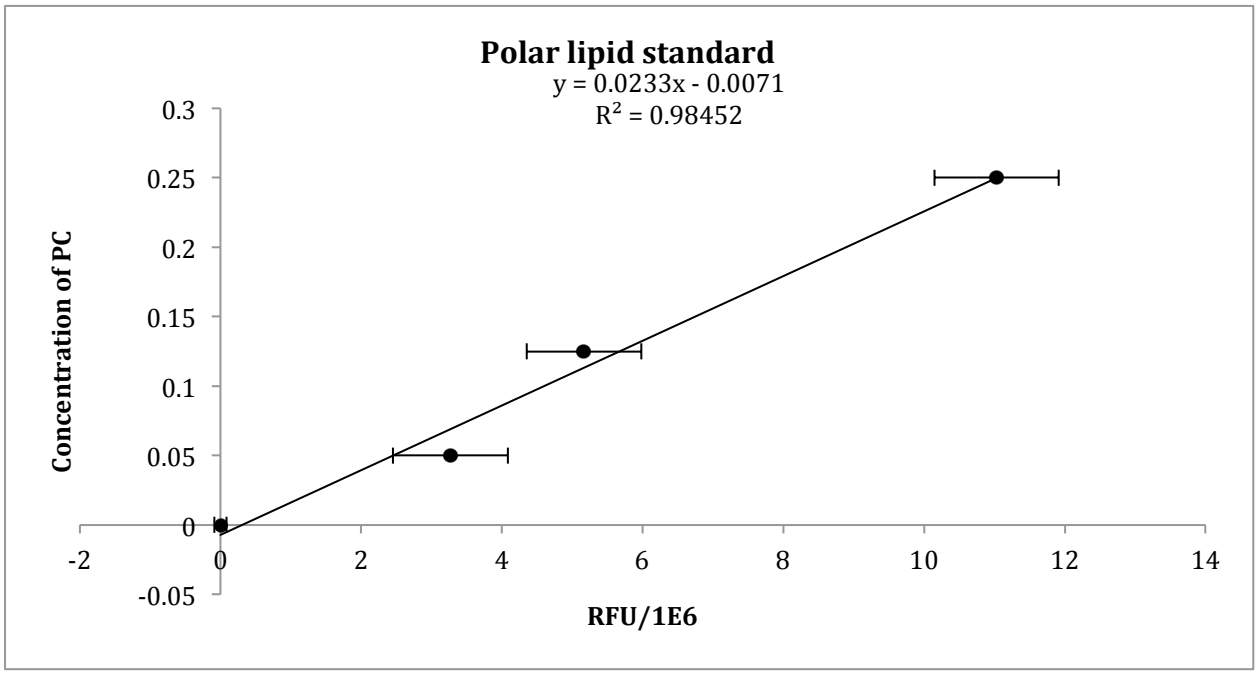


Figure 15: The polar lipid standard measurements of NRWS in PC of increasing concentrations, with excitation/emission at 550/630 nm. The x-axis lists the raw fluorescence in RFU divided by 1 million. The background fluorescence (3,633,333.3 RFU) was measured at 0 mg/ml and subtracted from the measurements displayed in the plot. One outlier was removed from the plot because its fluorescence at 0.125 mg/ml PC was extremely high (RFU = $1.1*10^7$). The well in question probably received a double dosage of PC standard. The horizontal error bars are standard errors of the mean (+ and -). A linear trend line, its equation and the R^2 value were included in the plot.

3.4 Visualization of changes in lipid allocation using Nile Red fluorescence

The changes in lipid allocation in cells of *C. concinnus* was visualized using a fluorescence microscope and is presented as a set of 6 images on the next page (Figure 16). The three images on the left shows algae grown in a culture with no nutrient or light limitation (Figure 16, A, B & C). The three images on the right shows algae grown in a culture that was not added nutrients for 8 weeks (Figure 16, D, E & F). Nile Red fluorescence is yellow in neutral lipids and red in polar lipids, but the red autofluorescence from chlorophyll renders the polar lipids impossible to distinguish when observed in a microscope.

No nutrient limitation: **Nutrient limitation:**

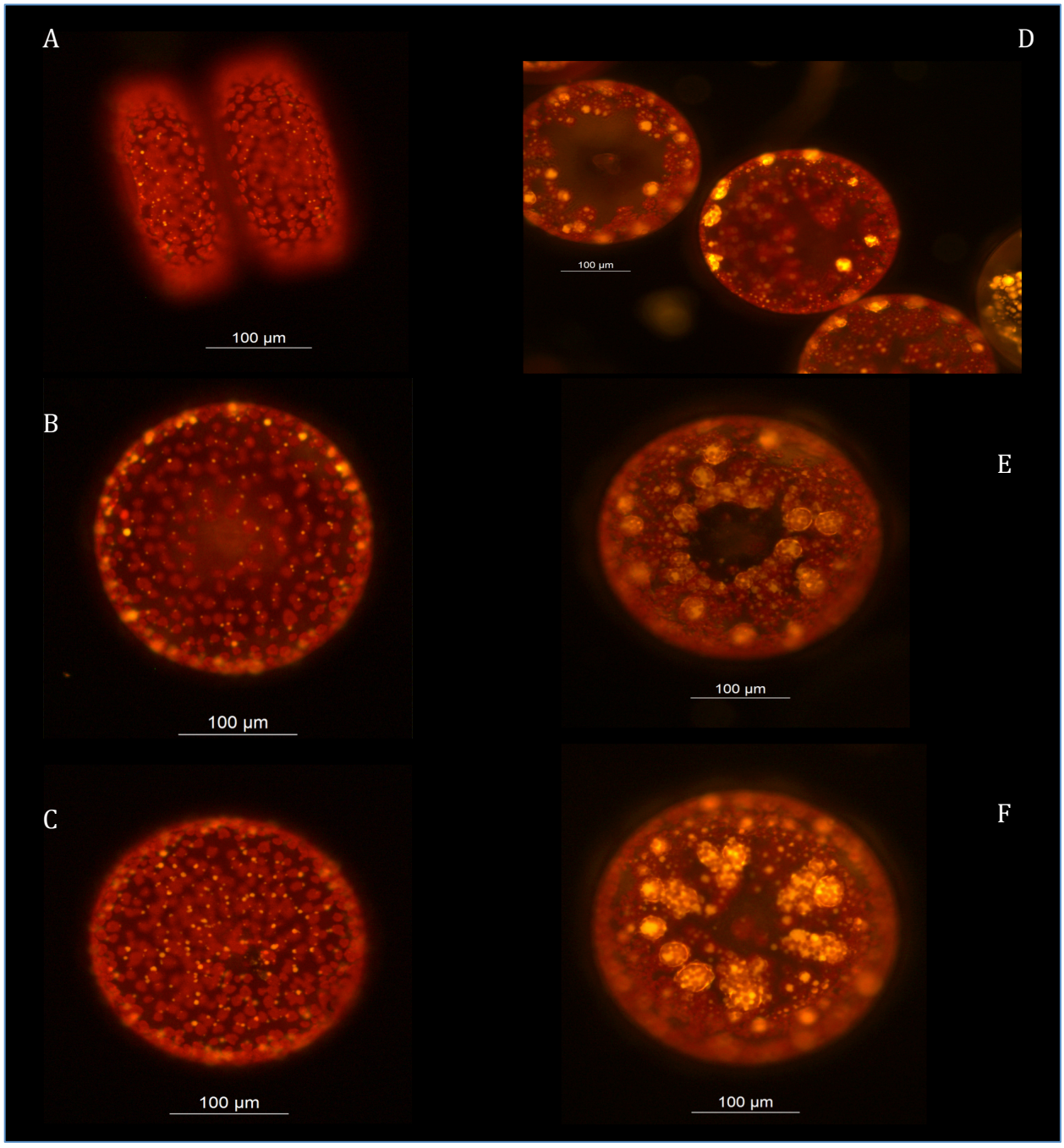


Figure 16: Fluorescence from *Coscinodiscus concinnus* stained with Nile Red, visualized on a Leica Leitz DM RBE fluorescence microscope with fluorescence filter set 43 (Zeiss Int.). The cells on the left (A, B and C) were grown for 1 week in fresh medium. The cells on the right (D, E and F) were grown for 8 weeks without addition of nutrients beyond the initial dilution from 5 ml stock culture.

The two cultures were distinctly different in both lipid- and chloroplast content. The culture without nutrient limitation was characterized by evenly distributed chloroplasts throughout the cell and low concentrations of neutral lipids, while the nutrient limited cells had uneven distributions of chloroplasts and the vacuole had started to retract in some. The neutral lipid droplets were larger and the total neutral lipid content higher. Cell size was similar in both cultures.

3.5 Lipid quantification using Nile Red fluorescence

Polar and neutral lipids were quantified fluorometrically in two different plate readers by Nile Red staining in *P. glacialis, C. socialis, C. furcellatus* and *A. longicornis*. Figure 17 below shows the fluorescence emission at 550 nm excitation (polar lipids) and 515 nm excitation (neutral lipids):

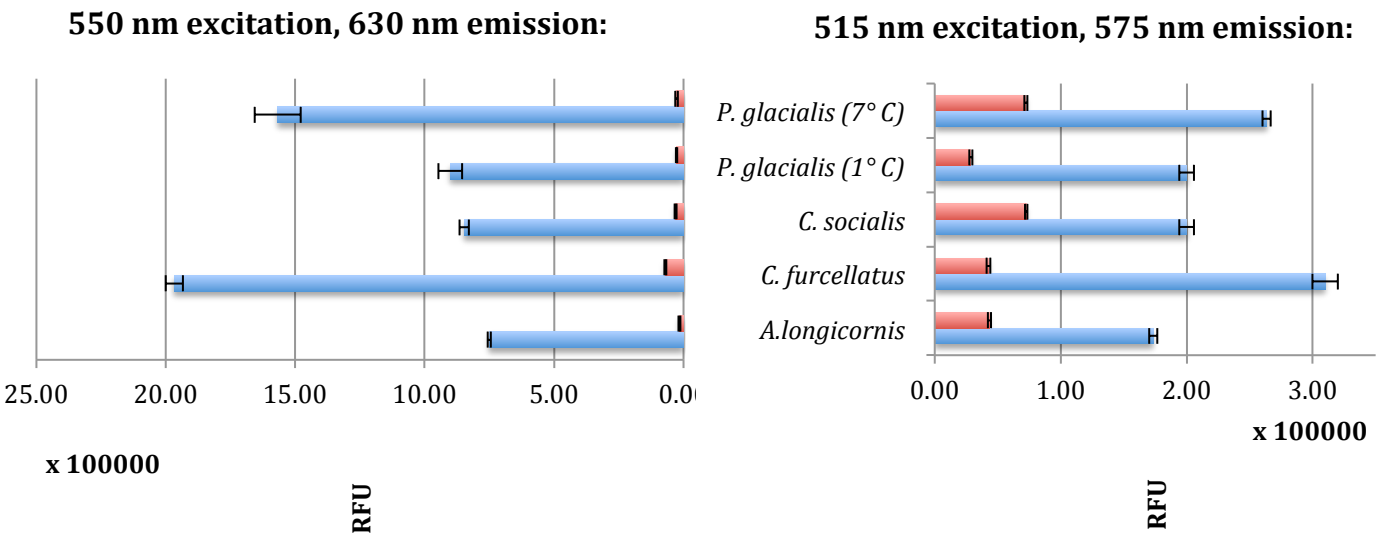


Figure 17: The emission of Nile Red in 1 mg/400 µl microalgal samples (blue bars), excited at 550 and 515 nm. The background fluorescence (red bars) was measured as the fluorescence of algal samples with isopropanol:chloroform and without NRWS. The vertical error bars represent the standard error of the mean.

The Nile Red fluorescence was highest for *C. furcellatus* and *P. glacialis* (7° C) at both excitation wavelengths. *A. longicornis, C. socialis* and *P. glacialis* (1° C) produced lower and relatively equal fluorescence. The standard errors were low,

as were the readings of the blank measurements. The most noticeable feature of the measurements is the fact that the fluorescence of each sample relative to the others is very similar at both excitation wavelengths. The readings were converted to lipid concentration by subtracting the blanks from each measurement, and inserting the resulting measurement divided by 10^6 into the equation for the trend line observed in the lipid standards. The resulting lipid concentrations were multiplied by 0.4 to find the concentration in 1 mg. The dry weight (DW) lipid concentration was estimated by multiplying the wet weight (WW) lipid concentration with the average WW:DW ratio for each temperature (See table 7 for all WW measurements and estimated DWs). Three cultures were excluded from the calculation of the average WW:DW ratio: NAPIS ID 11674492 and 1166519 because they were harvested by an alternative technique with a high loss of biomass (see section 2.4), and the single culture of *T. gravida* (NAPIS ID 11094478) because no culture of this species was measured for total lipid concentration. The average WW:DW ratios were 6.39 for the cultures grown at 7° C and 4.88 for the cultures grown at 1° C. The final values are listed in Table 8 below:

Table 8: The raw fluorescence (subtracted blanks and divided by 10^6) of Nile Red in 1 mg/ml microalgae and the neutral lipid (NL) and polar lipid (PL) content in percentage of wet weight (WW) and dry weight (DW).

Species	*FL(515/575)*	*FL(550/630)*	*NL (%, WW)*	*PL (%, WW)*	*NL (%, DW)*	*PL (%, DW)*
A. longicornis	0.130	0.734	0.629	0.116	3.067	0.564
C. furcellatus	0.267	1.897	1.043	1.167	5.090	5.692
C. socialis	0.127	0.817	0.621	0.190	3.028	0.928
P. glacialis (1° C)	0.171	0.873	0.753	0.241	3.676	1.178
P. glacialis (7° C)	0.191	1.540	0.813	0.844	5.191	5.392

The neutral lipid concentrations ranged from 0.621-1.043% of wet weight and 3.028-5.191% of dry weight. The polar lipid concentrations ranged from 0.116-1.167% of wet weight and 0.564-5.692% of dry weight. In order to investigate if the fluorescence measured in the samples actually originated from Nile Red in

lipids, emission scans of each sample (with and without Nile Red) at 515 and 550 nm excitation were performed. (Figure 18).

515 nm excitation: **550 nm excitation:**

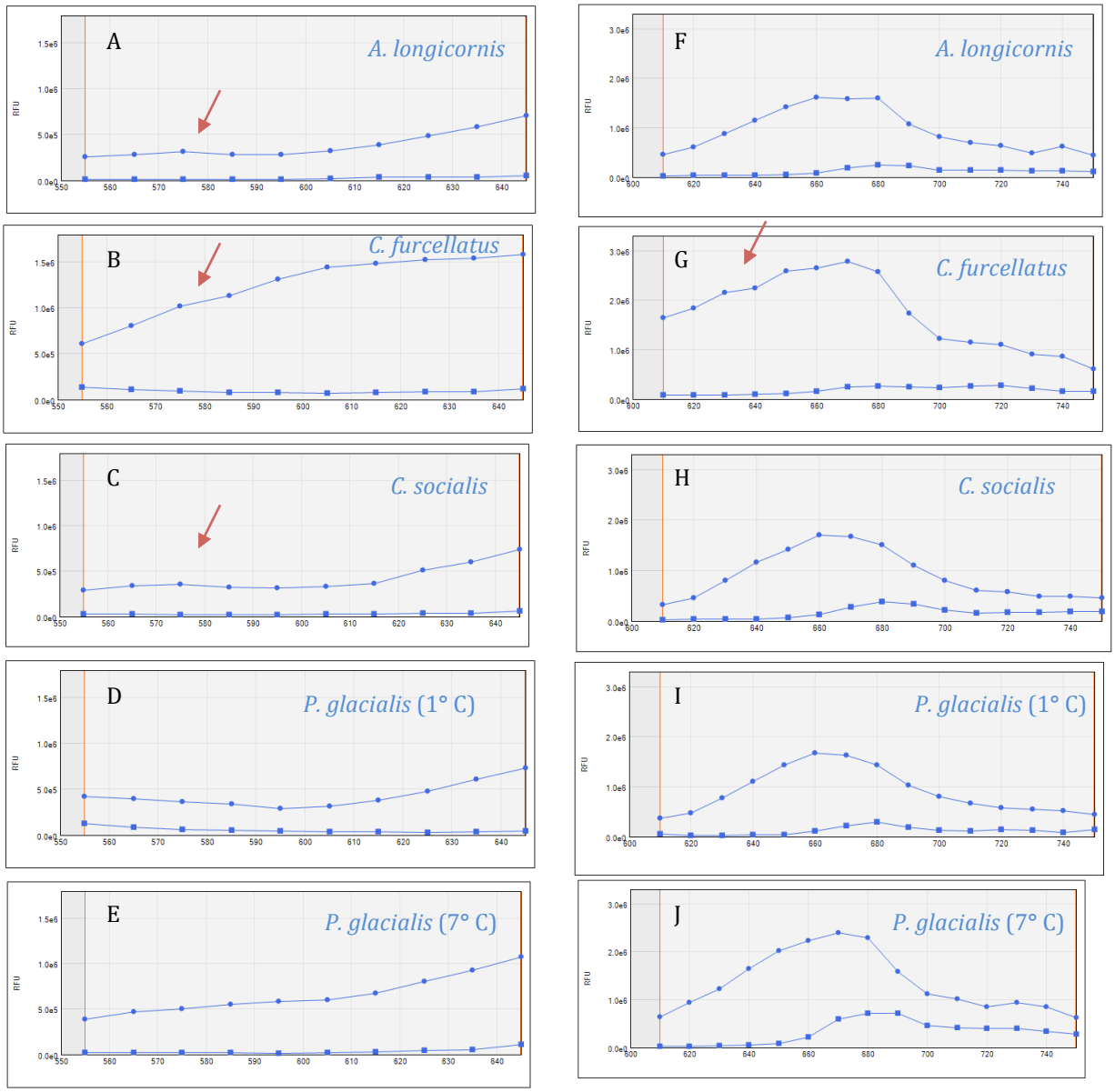


Figure 18: Emission scans of all 5 samples that were measured for neutral and polar lipid concentration by Nile Red staining. The red arrows mark where small peaks at either 575 (A, B and C) or 630 (G) nm are visible.

No clear peaks at either 575 or 630 nm were observed, but it is possible to distinguish what may be small peaks at 575 nm for *A. longicornis, C. furcellatus* and *C. socialis*, and at 630 nm for *C. furcellatus.*

3.6 Results from Bergen – lipid quantification by solvent extraction and HPLC-MS

Lipid quantification by extraction:

The results from the total lipid quantification, performed by solvent extraction at BioLab in Bergen, are presented together with the total lipid concentration (neutral + polar lipid) determined by Nile Red fluorometry in table 9 below:

Table 9. The lipid content in percentage of wet weight and dry weight, determined by Bligh and Dyer lipid extraction in Bergen. For comparison, the total lipid concentrations determined by NR fluorometry are included in parentheses.

Species	*Lipid conc., WW*	*Lipid conc., DW*
A. longicornis	0.94 (0.74)	4.59 (3.63)
C. furcellatus	0.48 (2.21)	2.34 (10.78)
C. socialis	0.44 (0.81)	2.15 (3.96)
P. glacialis (1° C)	1.5 (1.0)	7.32 (4.85)
P. glacialis (7° C)	1 (1.66)	6.39 (10.58)

The total lipid concentration determined by Bligh and Dyer was 0.44-1.5 % of WW and 2.15-7.32% of DW. There was no observable correspondence between the total lipid concentration determined by Bligh and Dyer and the total lipid concentration determined by Nile Red fluorometry.

HPLC-MS analysis:

The samples were analysed by HPLC-MS, but the constituents of the samples did not produce the expected mass spectrums (BioLab, personal comminucation): Very few phospholipids were observed; in some samples the phospholipids were completely absent. Some triglycerides were observed, but the levels were low. The amounts of free fatty acid was “unusually high”, but no absolute numbers were given. Many of the peaks in the mass spectrum were not lipids at all, and were determined to be unknown contaminations. BioLab concluded that the results were not reliable and should not be included in the study, and hypothesized that the error might be caused by hydrolysis of the lipids in the samples.

3.7 HPLC MS results – ICT, Prague

Before performing the analysis, each sample was given a number in the following order by the analytical lab at ICT (Table 10):

Table 10: The sample identification of the different algal samples used in the HPLC-MS analysis from ICT, Prague.

Number	NAPIS	Species
531	11034477	*C. socialis*
532	11034470	*C. socialis*
533	11674469	*A. longicornis*
534	11674492	*A. longicornis*
535	11674512	*A. longicornis*
536	11674476	*A. longicornis*
537	11094478	*T. gravida*
538	11664474	*P. glacialis*
539	11414473	*C. concinnus*
540	11414487	*C. concinnus*
541	11414494	*C. concinnus*
542	11664497	*P. glacialis*
543	11034486	*C. socialis*
544	11664485	*P. glacialis*
545	11414472	*C. concinnus*
546	11684511	*S. marinoi*
547	11684510	*S. marinoi*
548	11684488	*S. marinoi*

Profile of lipid classes:

The specific ions of lipid classes were identified based on 1) their accurate mass in MS mode with a maximum tolerable mass detection error of 5 ppm and 2) their isotopic pattern, with a difference of less than 20% between the experimental and theoretical isotopic patterns. Triacylglycerols, diacylglycerols and monoacylglycerols ionized most effectively as the ammonium adducts $[M+NH_4]+$, while the phospholipids, lysophospholipids and sphingolipids created the [M+H]+ ions. Signals from the negative ionization mode [M-H]- were used to detect free fatty acids. Glycolipids were not measured.

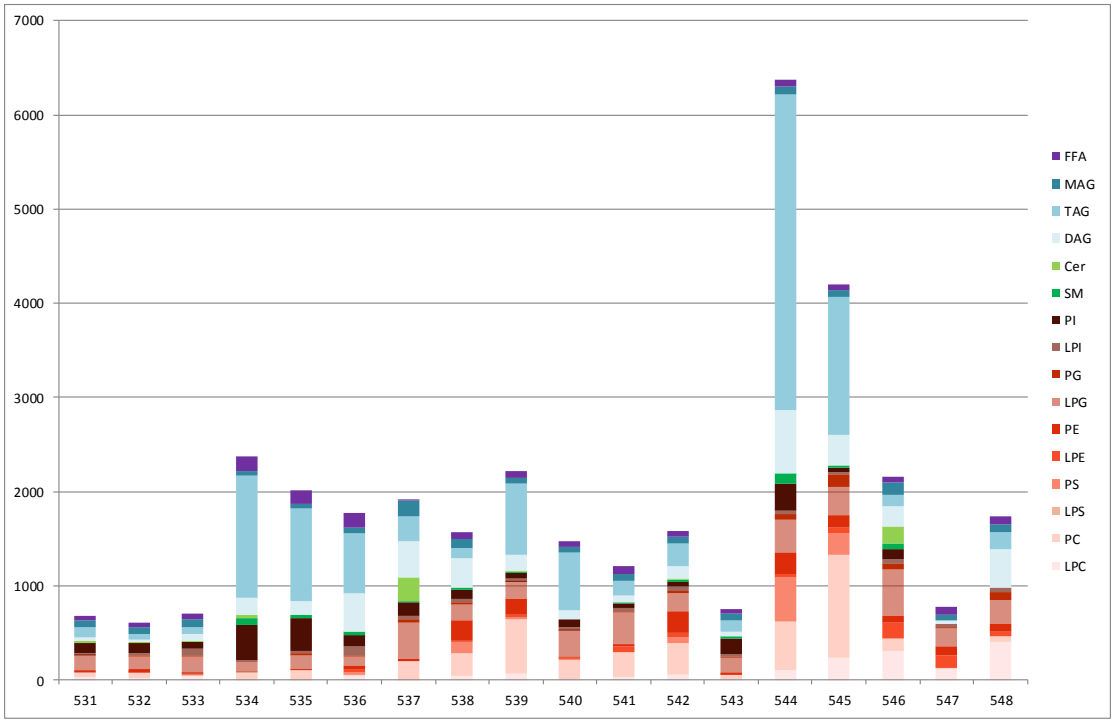


Figure 19: The distribution of lipid classes and their relative amount in each sample as determined by HPLC MS.

The distribution of lipid classes (Figure 19) was highly variable, also within the same species, but some main features of the set as a whole can be seen:

1) TAG was the dominant neutral lipid and FFAs were only present in very small amounts, with the exception of *A. longicornis* samples 534-536.
2) The polar lipids were dominated by PC, PG, and LPG, (lysophosphatidylglycerol), but some species also had large contributions of LPC (Lysophosphatidylcholine) and PS.

3) Cer (ceramides) and SM (sphingomyelin) were only observed in very low concentrations in all samples apart from 537 (*T. gravida*), 544 (*P. glacialis*) and 546 (*S. marinoi*).

Profiles of free fatty acids:

Free fatty acids were analysed using signals from the negative ionization mode [M-H]- (Figure 20):

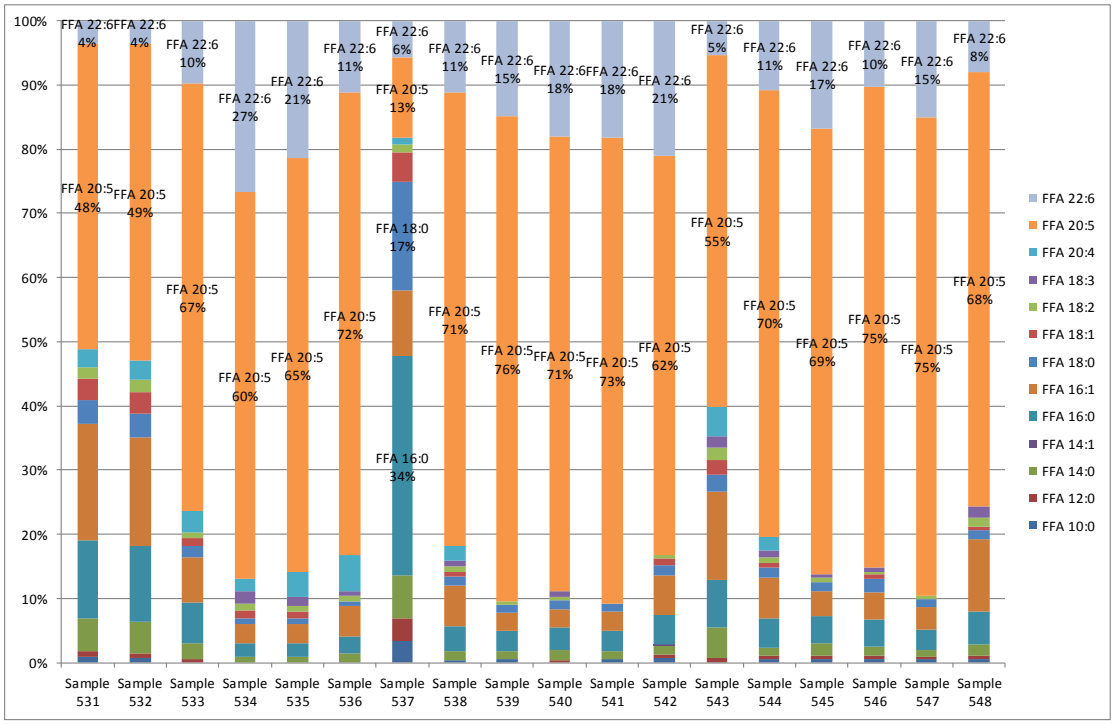


Figure 20: The distribution of specific fatty acids in the free fatty acid fraction.

The analysis of free fatty acids showed very similar results for most species, and low variance within each species; the dominant fatty acids were EPA (C20:5, n-3), DHA (C22:6, n-3) and palmitoleic acid (C16:1, n-7) in *A. longicornis, C. concinnus, S. marinoi* and *P. glacialis*. *C. socialis* (531, 532 and 543) contained large amounts of EPA and DHA, but also significant amounts of the shorter-chained palmitic acid (C16:0) and palmitoleic acid. *T. gravida* was the only algae to contain mostly short chained fatty acids, predominantly stearic acid (C18:0), palmitoleic and palmitic acid.

Analysis of fatty acids in glycerides (MAG, DAG and TAG) and phospholipids:

Identification of bound fatty acid profiles from glycerides and phospholipids was achieved through investigation of MS/MS spectra, using the mass spectral catalogue available in the LipidView software. The measured fragmentation ion patterns were compared with the theoretical fragmentation pathways, and only results matching the criterion of >50% were accounted.

The following fatty acids were profiled: C16:0, C18:0, C18:1 (oleic acid), C18:2 (linoleic acid), C18:3 (linolenic acid), C20:4 (ARA), C20:5 (EPA) and C22:6 (DHA). Figure 21 and 22 below shows the profiles in glycerides and phospholipids, respectively:

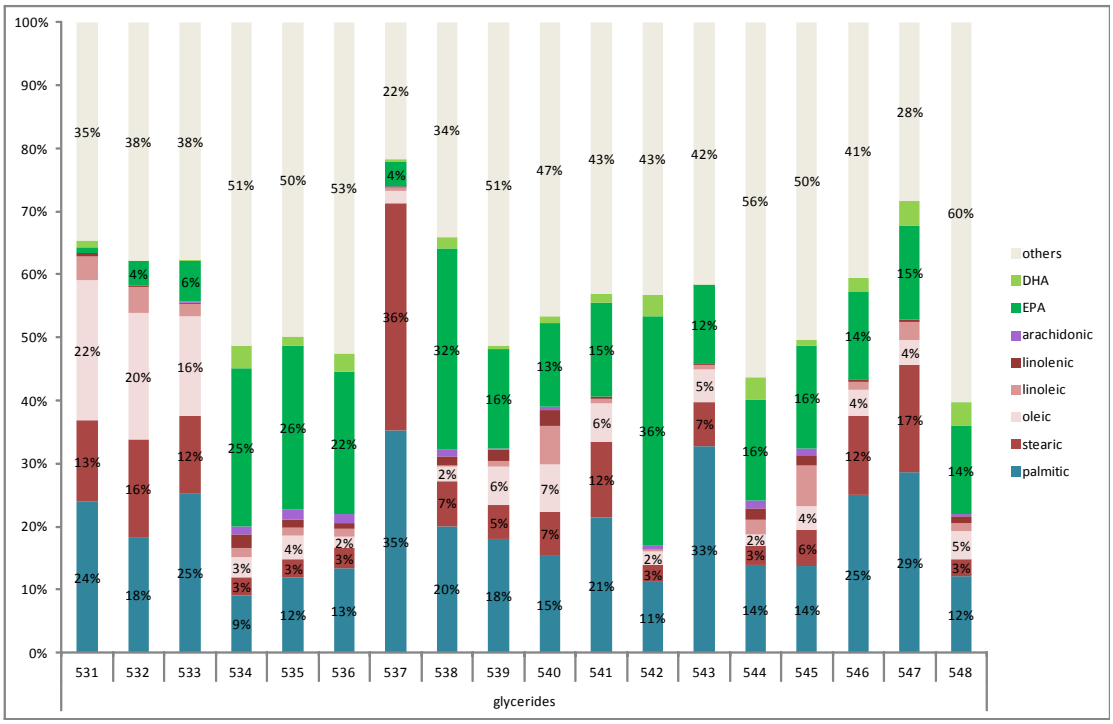


Figure 21: The fatty acid profiles of glycerides.

The fatty acids found in glycerides partly resembled those found in the free fatty acids, but there was more variation within species; Glycerides of *A. longicornis, C. concinnus, P. glacialis* and *S. marinoi* mainly contained EPA and palmitic acid, but sample 533 of *A. longicornis* deviated from the other *Attheya* samples by containing mainly palmitic, stearic and oleic acid. *C. socialis* samples 531 and 532 mainly contained oleic, palmitic and stearic acid, while sample 543 had more

palmitic acid and EPA, and less oleic and stearic acid. The single *T. gravida* sample contained very large concentrations of palmitic and stearic acid, and not much else. The C16:1 (palmitoleic acid) fatty acid was not detected by the analysis.

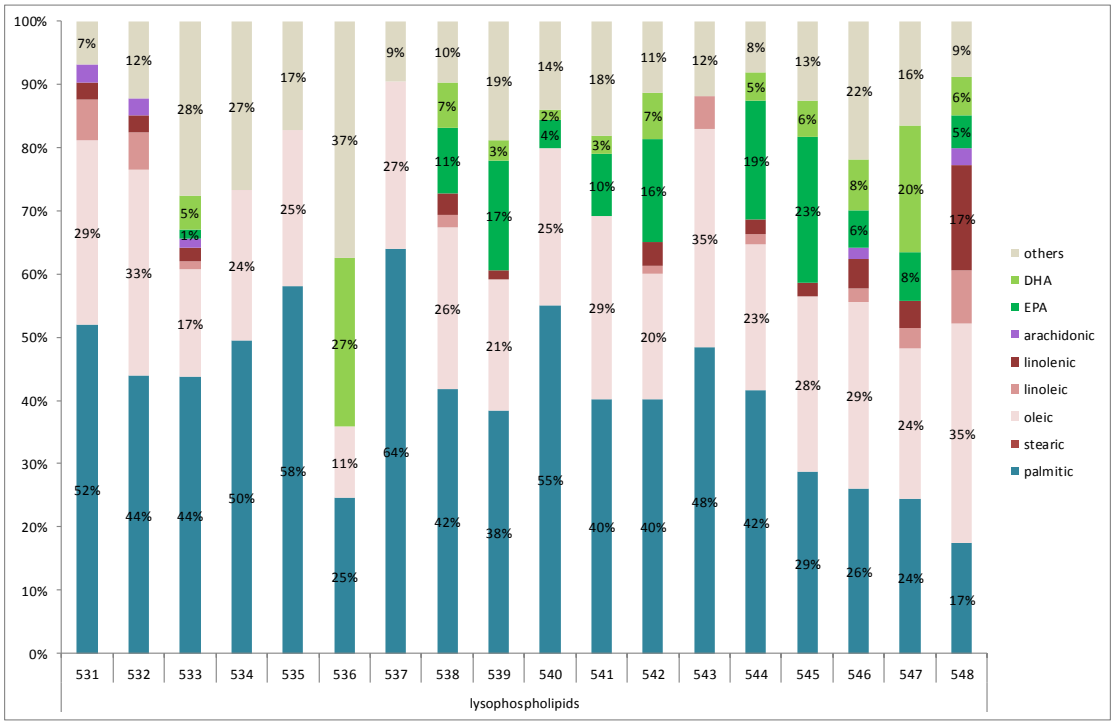


Figure 22: The fatty acid profiles of phospholipids.

The phospholipids showed less variation in the fatty acids than the glycerides. Oleic and palmitic acid dominated all samples apart from one *A. longicornis* sample (536), where DHA was the most dominant fatty acid. *C. socialis* and samples 533-535 of *A. longicornis* contained very little EPA and DHA. The fatty acids of the phospholipids in *T. gravida* contained a total of 91% oleic and palmitic acid, and 0% detected EPA and DHA. *P. glacialis* showed little variation between samples, with 11-19% EPA and 5-7% DHA. The fatty acids of *C. concinnus* was more varied; sample 540 contained 4% EPA, 2% DHA and 55% palmitic acid, while sample 545 contained 23% EPA, 6% DHA and 29% palmitic acid.

A summary of the lipid content (Figure 19-22) for each species:

C. socialis (#531, 532 and 543): The distribution of lipid classes was similar for all samples, with low concentrations of all lipid classes compared to other species. The free fatty acids were dominated by EPA, but, unlike the other species, there were also large contributions of palmitic acid (C16:0) and palitoleic acid (C16:1). The glycerides of *C. socialis* showed more variation between samples; samples 531 and 532 contained mostly oleic and stearic acid, with very little EPA (4% or less) and DHA, while sample 543 contained more EPA (12%) and a third (12%) of the total oleic and stearic acid observed in samples 531 and 532. The concentrations of palmitic acid in the glycerides were high in all samples, but sample 543 contained more (33%) than the other two (24 and 18%). The fatty acid compositions of the phospholipids in *C. socialis* were similar within the species; palmitic and oleic acid accounted for 80-90% of the total fatty acids, and none of the samples contained EPA or DHA.

A. longicornis (#533, 534, 535 and 536): Sample #533 was similar to *C. socialis* in having low concentrations of all lipid classes. The other *A. longicornis* samples contained fairly large amounts of neutral lipids (TAG, DAG and FFA), and sample 534 and 535 contained high concentrations of PI (phosphatidylinositol). The FFAs are similar for all four samples; LC PUFAs EPA and DHA dominate the FFA fraction (70-90%), while the short chained (C<20) fatty acids contributed very little. In the glycerides, samples 534-536 contained large concentrations of EPA and DHA compared to other samples, while sample 533 contained very little EPA and no DHA, but large concentrations of palmitic, stearic and oleic acid. The phospholipids were similar to *C. socialis*, with large amounts of palmitic and oleic acid, with one exception: sample 536 contained a surprisingly large amount of DHA (27%) in its phospholipids, constituting about half of the palmitic and oleic acid seen in the other samples.

T. gravida (#537): Contained a relatively even distribution of polar and neutral lipids, high concentration of Cer (ceramides) compared to other samples and very low concentrations of FFAs (Free fatty acids). The dominant neutral lipid

was DAG. The FFA fraction was clearly separated from the other species; *T. gravida* was the only species to contain mostly stearic and palmitic acid instead of EPA, which only accounted for 13%. The glycerides and phospholipids were also dominated by the short-chained fatty acids, with palmitic and stearic acid accounting for 71% of the Fas in glycerides and oleic and palmitic acid accounting for 91% of the Fas in phospholipids.

P. glacialis (#538, 542, 544): This species showed a high degree of variation between samples. The polar lipids were relatively evenly distributed between the samples, with significant contributions of PC, PS, PE and LPG, but sample 544 contained more polar lipid of all classes. Of the neutral lipids, sample 538 and 542 contained low amounts of TAG and DAG, while sample 544 contained more TAG than any other sample measured. The free fatty acids did not show the same variation; EPA and DHA represented 70-80% of the free fatty acids in all three samples. The glycerides were especially rich in EPA in samples 538 and 542, representing 32 and 36% of the fatty acids respectively, while sample 544 only contained 16% EPA in the glycerides. The phospholipid Fas were evenly distributed between samples; palmitic and oleic acid contributed 60-70%, while EPA and DHA contributed 18-25% in all samples.

C. concinnus (#539, 540, 541, 545): Lipid compositions varied significantly between each sample: Samples 540 and 541 contained low quantities of neutral and polar lipid. Sample 539 contained relatively large concentrations of both neutral and polar lipid, and sample 545 contained very large concentrations of polar lipids, especially PC, and high quantities of neutral lipids. EPA and DHA, contributing 80-90% in all samples, dominated the free fatty acids. The glycerides contained mainly EPA and palmitic acid, with noticeable contributions of stearic and oleic acid. The phospholipids showed more variance; the dominating fatty acids were oleic and palmitic acid, but samples 539 and 545 contained more EPA and DHA (20 and 29%) respectively, and less palmitic acid.

S. marinoi (#546-548): The lipid compositions of each sample varied greatly; sample 546 was rich in polar lipids, especially LPC and LPG, and contained

relatively high amounts of Cer. Sample 547 contained low concentrations of all lipid classes apart from LPG, which was the dominant polar lipid. Sample 548 contained large concentrations of LPG, LPC and neutral lipids. The neutral lipids in both sample 546 and 548 were composed mainly of DAG, while TAG was the main neutral lipid in sample 547. EPA and DHA dominated the free fatty acids, with minor contributions of palmitic and palitoleic acid. The glycerides showed even distributions of fatty acids in samples 546 and 547, while sample 548 contained less of the short-chained fatty acids. The amount of unknown fatty acid was also very large in sample 548 compared to the other two samples. The fatty acid composition of the phospholipids varied between samples; sample 546 contained mostly oleic and stearic acid, and relatively low concentrations of EPA 6%) and DHA (8%). Sample 547 contained less of the short-chained fatty acids, but more EPA (8%) and a lot more DHA (20%). Sample 548 contained mostly oleic acid, and was distinguished from the other *Skeletonema* samples by containing 17% linolenic acid and more linoleic acid.

4. Discussion

4.1 Lipid allocation in response to nutrient limitation

The images in Figure 16 illustrates visually the effect of nutrient limitation on the physiological state the diatoms. The results are in agreement with previous studies on lipid allocation in microalgae (Richardson et al., 1969, Xin et al., 2010, Klok et al., 2013), and show that the algal biomass is allocated as storage lipids during nutrient stress. The culture that was grown without nutrient limitation (Figure 16, A, B & C) had a healthy look, with evenly distributed chloroplasts and low concentrations of neutral lipids. These characteristics are typical of microalgal cells in the exponential growth phase where the energy from photosynthesis is spent on binary fission. The culture that was kept in a nutrient-limited environment, however, had a completely different physiology: The chloroplasts had accumulated in clumps and been partially or completely broken down, the vacuoles had started to retract in some cells, and the neutral lipid content was higher (Figure 16, D, E & F). These characteristics are typical of cells in the late resting phase, where the photosynthetic machinery is recycled as storage lipids until nutrients again become available. Nile Red staining is in this way an effective screening method that can rapidly reveal the physiological state of microalgal cells.

4.2 Lipid quantification using Nile Red fluorescence and solvent extraction (Bligh and Dyer, 1959)

The determination of Nile Red spectral properties revealed some differences from previous publications. Firstly, the excitation maximum of NR in cod liver oil was 510-520 nm (Figure 8), while previous publications have used other wavelengths, i.e. 480 nm (Priscu et al., 1990) and 530 nm (Chen et al., 2009). The difference may have been caused by the differing lipid composition in the two standards: Both cod liver oil and triolein are 100% triglycerides, but while triolein has a fatty acid composition of 100% oleic acid, the main fatty acids in cod liver oil are palmitic (C16:0), palmitoleic (C16:1), oleic (C18:1), EPA (C20:5) and DHA (C22:6) (Gruger et al., 1964, Lambertsen and Brækkan, 1965). As this

composition mirrors that of the northern diatoms in this study, the spectral properties of NR in cod liver oil should mimic those of NR in northern diatom lipids more closely than NR in triolein. In addition, commercial cod liver oil is inexpensive and easily available. The spectral properties of NR in phosphatidylcholine were identical to those reported by Alonzo and Mayzaud (1999), with excitation/emission maxima of 550/630 nm (See Figures 10 and 11). The proposed wavelengths of 560/610 nm were not optimal; when excited at 560 nm, emission readings were about 17% lower at 610 nm compared to 630 nm (25 million RFU and 30 million RFU, respectively). The optimal staining time was determined to be 10 minutes, although the readings were not compared to fluorescence of samples incubated less than 10 minutes. It is possible that the fluorescence measurements prior to t=10 would have been higher. Nevertheless, the observed differences in fluorescence between measurements were low, and the potential decrease from time 0 to time 10 is not likely to have affected the results as the standards were also incubated for 10 minutes before being measured. Nile Red in lipid standards produced strong linear correlations (regression lines in figure 14 and 15), and high coefficients of determination (R^2 = 0.995 in cod liver oil and 0.985 in PC).

The fluorometrical quantification of lipid concentration produced values ranging from 3.028-5.191% neutral lipid and 0.564-5.692% polar lipid on a dry weight basis. The low standard errors within samples (Figure 17) testifies to the reproducibility of the method, and the low background fluorescence (Figure 17, red bars) removes the possibility that autofluorescence from Chl *a* exceeded the fluorescence from Nile Red. The measured values are within the range listed in "Ecosystems of the Barents Sea", where the total lipid concentration of arctic phytoplankton is reported to be less than 10% of the organic matter, while proteins make up 30-50% (Sakshaug et al., 2009). A field study of the phytoplankton at two depths in Ullsfjorden and Balsfjorden (spring bloom of 1983) found lipid concentrations ranging from 8.8-28.1% of the total carbon (Sargent et al., 1985). The measured values found with the Nile Red quantification are therefore arguably within the expected ranges. However, the measured values were neither confirmed nor rejected based on the results from

the Bligh and Dyer quantification, as the lipid content in the samples was reported to be too low for quantification by lipid class. BioLab was able to measure the *total* lipid concentration of the samples, i.e. the total concentration of both polar and neutral lipids. The reported total lipid concentration determined by Bligh and Dyer in Bergen did not correspond to the total lipid concentration determined from Nile Red fluorescence (Table 9). The emission scans performed on the samples (Figure 18, A-F) may help to explain why the results from the two methods deviated: The emission scans were unable to detect clear fluorescence peaks at 575 and 630 nm in any of the samples, therefore, the lipid concentrations were calculated from fluorescence that did not originate from Nile Red in lipids. Based on these scans, it is unlikely that the fluorometrical lipid quantifications were correct, and that some other biomolecule(s) produced Nile Red fluorescence in large enough amounts to surpass the fluorescence peaks from Nile Red in lipids.

These molecules may be the same as those reported from the HPLC-MS in Bergen: Unidentified molecules that were not lipids but still sequestered in the hydrophobic lipid fraction of the Bligh and Dyer extraction. This signifies that the molecules were hydrophobic in nature and should therefore induce fluorescence of Nile Red. It is unlikely that these molecules were created as a consequence of hydrolysis during the cultivation or harvesting procedure, as the algal cultures were not exposed to conditions that would warrant lipid degradation. Considering the low lipid concentrations of the samples, it is possible that an unknown amount of the lipid content has escaped the final measurements, either as a result of the centrifugation during harvesting or by incomplete extraction during the Bligh and Dyer procedure, or both: Firstly, the supernatant that is produced during centrifugation (see section 2.2, harvesting procedure) generally has a deep green colour, but has never been analysed for chemical content. We suspect that this supernatant contains not only lipids that have escaped the cells, but we have also observed whole cells that are sufficiently buoyant (due to, for example, high lipid concentration) to remain suspended during centrifugation. During the harvesting procedure, these free-floating lipids and cells are discarded along with the rest of the supernatant. Secondly, according to the

Director of Research at BioLab (Jarle Wang-Andersen, personal communication), lipids are not the only molecules that are sequestered in the chloroform in a Bligh and Dyer extraction. Lipid-like pigments, oxidized lipids, incomplete lipids as well as detritus may also be dissolved. Usually, the amount of these compounds is negligible as they only represent fractions of the total mass of the dissolved lipids, but when the lipid concentration of the samples is as low as those reported in this study, the compounds could cause an overestimation of the lipid concentration. According to BioLab, the measured lipid concentrations are likely to have been affected by this error source. Finally, BioLab used a modified (only in terms of volumes and weights used) version of the Bligh and Dyer method on wet biomass to extract lipids, presumably because it is much quicker than the Folch method. The two methods have been compared by Iverson et. al. (2001), who found that the Bligh and Dyer method significantly underestimated the lipid content in tissues containing more than 2% lipid, and that the underestimation increased with increasing lipid content. In tissues containing less than 2% lipid, the results did not differ between the two methods (Iverson et al., 2001). In a study of 11 extraction methods on marine lipids from a clam (*Anadara inaequivalvis*), the Folch method was able to extract more lipids than 9 of the other methods, including Bligh and Dyer (Fiorini et al., 2013). The use of wet and dry biomass has also been compared: The Bligh and Dyer method determined a total concentration of 27.6% lipids, when wet biomass was analysed, and 41.4% when dry biomass was analysed, in the fungus *Mortierella alpine* (Zhu et al., 2002). This is in spite of the fact that the Bligh and Dyer method is "known to be effective" on wet material (Mercer and Armenta, 2011).

In any case, much work remains to be done in order to investigate these discrepancies. The remainder of the samples that are left from the Nile Red quantification will be studied further by lipid extraction and HPLC-MS here in Tromsø, to look for conclusive results. The questions important to answer are: Is the lipid concentration of the algal samples really as low as reported by the Bligh and Dyer method? If they are, is the low lipid concentration a result of lipids escaping the cells? And if not, why are the Bligh and Dyer and Nile Red method unable to correctly quantify the lipids?

4.3 HPLC-MS – ICT, Prague

The HPLC-MS results showed large variations in the composition of the polar and neutral lipids within species, and as a consequence illustrate the importance of controlling the growth conditions of cultures. As irradiance and temperature were constant during cultivation, fluctuating nutrient concentrations or varying cell densities that dictated the growth phase of the cultures probably caused the variations. It is known that the growth phase may influence the chemical composition of algae (Barofsky et al., 2010). The other possibility is that the lipid extraction method used in Prague was performed with varying degrees of success, and that the lipid classes would have varied less within species with a more optimal method.

The main lipid classes were the same as reported by the literature (Arts et al., 2009); PC, PG, PS were the main polar lipids and TAG was the main neutral lipid. Interestingly, the composition of FFAs (Figure 20), and the fatty acid composition of glycerides (Figure 21) and phospholipids (Figure 22) were very similar within each species, despite that the same species showed large differences in the distribution of lipid classes (Figure 19). This suggests that while each species may be very flexible in balancing the main lipid classes according to its culture conditions, the composition of free and bound fatty acids is not subjected to the same flexibility. The main fatty acids in all species apart from *T. gravida* were EPA, palmitoleic acid (C16:1, n-7) and palmitic acid (C16:0) in the FFAs and glycerides. The phospholipids also contained large amounts of oleic acid (C18:1, n-9). These results are similar to other findings performed on algae from cold water areas: Fahl and Kattner (1993) found that the main fatty acids in a diatom-dominated community in the Weddell Sea (Antarctica) were palmitoleic acid, palmitic acid, EPA and oleic acid. Apart from oleic acid, these fatty acids are typical of diatoms (Fahl and Kattner, 1993). Among the polyunsaturated fatty acids, EPA is commonly reported as a major fatty acid in diatoms (Volkman et al., 1989), while DHA is common but much less abundant. This was also observed in my analysis. The fatty acid composition of the arctic

strains studied here are therefore comparable to diatoms from southern, temperate regions, with the exception of oleic acid and myristic acid (C14:0): Zhukova and Aizdaicher (1994) studied 4 species of temperate diatoms and found the main fatty acid classes to be C14:0, C16:0, C16:1 and C20:5. Of these, C14:0 constituted between 7.4 and 15% of the fatty acids, while EPA made up 12.8-28.4% of the fatty acids in the four species. In our results, C14:0 was negligible (about 2% of FFAs) in all species apart from *T. gravida* and *C. socialis,* where it constituted about 6% to the FFA fraction. These results therefore demonstrate what seems to be an important difference between cold and warm water species; the amount of LC-PUFAs is higher, and the amount of short chained saturates is lower, in cold water diatoms compared to warm water diatoms. A study of the antarctic diatom *tauroneis amphioxys* gave similar results; the relative concentrations of C16:4, C18:4, C20:5 and C22:6 were higher when the diatom was grown at lower temperatures (Gillan et al., 1981). The same is also seen in macroalgae; cold-water, canadian algae contained predominantly C18:2, C24:0, C18:3 and C18:4, while temperate, chinese algae contained mostly C16:0, C24:0 and C18:1 (De Angelis et al., 2005). The results are not surprising since most northern and arctic species are in reality temperate species that have migrated northwards (Degerlund and Eilertsen, 2010).

The fatty acids were also specific for the main lipid classes. For example, *C. socialis* had a FFA composition of primarily EPA and palmilotelic acid; a glyceride FA composition of primarily palmitic, stearic and oleic acid and a phospholipid FA composition of primarily palmitic, oleic and linoleic acid. *C. concinnus* had a FFA composition of (almost exclusively) EPA and DHA: a glyceride FA composition of primarily palmitic acid and EPA and a phospholipid FA composition of primarily palmitic acid, oleic acid and EPA. Glycolipids were not analysed by the HPLC-MS, presumably because the method in Prague was developed for non-photosynthetic tissue. If glycolipids had been analysed, they would probably have been enriched with LC-PUFAs such as EPA and DHA, as these FAs are important contributors to the galactosylglycerides (SQDG and M/DGDG) that are abundant in the thylakoid membranes (Arts, 2009). LC-PUFAs

are assumed to be important in the photosynthetic function of algae, as studies have demonstrated the importance of SQDG for the functionality of photosystem II in the green algae *Chlamydomondas reinhardtii* (Muhlroth et al., 2013). Future studies should therefore focus on including glycolipids in analysis of diatom lipid composition.

T. gravida was the only species where EPA was present in low concentrations in all lipid classes, and also the only species in this study that is not normally associated with northern/arctic regions. It is listed in algaebase as a cosmopolitan, with references placing it in the arctic and antarctic regions, but according to our experience it is more abundant in the warmer, sub-arctic seas. The fatty acid composition support these observations, as its lipid composition more closely resemble that of algae belonging to the class *Chlorophyta* (green algae) where long-chained (20 or more C) FAs are less common (Zhukova and Aizdaicher, 1995). It should be noted that none of the 7 species that were used in this study are endemic to the northern or arctic regions. There are reports of these algae appearing in most oceans where algal studies are performed (also in tropical waters). This suggests that the species are either extremely tolerant to a wide range of temperatures (eurythermic), or, more likely, that different ecotypes have adapted to specific regions. Metabolic fingerprinting has revealed genetic differences between northern and southern strains of *C. socialis* (Huseby et al., 2012a), and observations from our lab showed that our strain of *C. concinnus* (AMB 186) was only able to continue cell division for about one week when grown at room temperature. None of this is surprising, but it is important to remember that the lipid composition in a sample of *C. concinnus* isolated from the Adriatic Sea is likely to differ from the lipid composition described here (cf. see discussion on temperature influence of fatty acid composition above).

It is also possible that many of the strains that are considered part of the same species today, are actually different species (Lakeman et al., 2009). The emergence of gene sequencing tools for species identification is likely not only to reveal new species, but also be instrumental in confirming the identity of laboratory cultures. For example, the single species of *Skeletonema* kept in our

stock cultures was until 2009 identified as *Skeletonema costatum*, until gene sequencing revealed it to be *Skeletonema marinoi* (Huseby, 2012b).

The HPLC-MS also revealed significant amounts of the lysophospholipid lysophosphatidylglycerol (LPG) in all samples, and lysophosphatidylcholine (LPC) in all samples of *S. marinoi* and sample 545 of *C. concinnus*. Lysophospholipids are intermediates in phospholipid metabolism and turnover, and are usually only found in small concentrations in biological membranes (Fuller and Rand, 2001). It was therefore surprising to find them in large concentrations in our samples. Lysolipids have been shown to inhibit sea-urchin cortical granule exocytosis (reproduction) (Chernomordik et al., 1993). Their abundance in these samples may be a product of a self-defence mechanism, where the mechanical stress associated with the harvesting procedure initiates the conversion of phospholipids to lysophospholipid, so as to ultimately reduce the fertility of grazers. Similar mechanisms have been shown before, for example the harmful effects of lipid-derived aldehydes on copepods (Miralto et al., 1999) and echinoderms (Caldwell et al., 2002).

Conclusion

This study aimed to analyse and compare the lipid composition of northern diatoms cultivated at 1 and 7° C, evaluate Nile Red staining as a rapid method of lipid quantification, and visualize the effect of nutrient limitation in *C. concinnus* using Nile Red. The lipid composition of the algae grown at 7° C was comparable to those of other cold-water diatoms, with the exception of *T. gravida*. The results suggested that fatty acid composition is not affected by changes in nutrient availability (as observed by large variations in the main lipid classes), and that they are specific for both lipid class and species. Unfortunately, neither the HPLC-MS nor the Bligh and Dyer quantification of algae grown at 1° C produced results that were considered reliable, possibly due to hydrolysis of the sample lipids or unidentified contaminations. Measurements of Nile Red in lipid standards produced strong correlations of relative fluorescence (RFU) and lipid concentration, and Nile Red staining of *C. concinnus* was shown to be an effective method for visualizing the accumulation of neutral lipids during nutrient stress. Fluorometry of Nile Red in diatoms, however, was in all likelihood unable to quantify the true concentration of the sample lipids. Several potential sources of error were identified; future work should focus on investigating these sources before a reliable method for lipid quantification can be established. De-watering of the samples, determination of total lipid content and including glycolipids in HPLC-MS analysis should be prioritized. In order to fully control the methods that are applied, future analyses should take place in a single laboratory, where the methods are fully adapted towards diatom analysis.

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Appendix 1 – raw fluorescence measurements

Nile Red optimal staining time, consecutive readings:

Table x: The raw measurements of Nile Red fluorescence tracked over time in *C. concinnus, P. glacialis, C. furcellatus* and *A. longicornis*. The samples (1 mg diatom in 400 µl F/10 medium) were stained with 8 µl NRWS and measured every 10 minutes for 60 minutes.

Time (m)	*10*	*20*	*30*	*40*	*50*	*60*
Blank	130000	82000	58000	43000	33000	29000
	79000	55000	53000	46000	42000	36000
	140000	97000	62000	54000	48000	41000
C. concinnus	580000	410000	540000	330000	500000	370000
	450000	590000	470000	350000	380000	340000
	460000	350000	430000	380000	350000	340000
P. glacialis	840000	660000	910000	950000	700000	780000
	990000	850000	770000	860000	1000000	740000
	1100000	880000	810000	750000	680000	770000
C. furcellatus	180000	160000	160000	140000	140000	130000
	170000	170000	150000	140000	140000	130000
	170000	160000	140000	140000	140000	130000
A. longicornis	280000	250000	240000	230000	210000	200000
	290000	260000	250000	240000	230000	200000
	270000	250000	240000	240000	230000	200000

Nile Red optimal staining time, single (60 minute) readings:

Table x: The raw measurements of Nile Red fluorescence after 60 minutes of staining without prior measurements in *A. longicornis* and *C. concinnus.* The samples (1 mg diatom in 400 µl F/10 medium) were stained with 8 µl NRWS.

Blank	*A. Longicornis*	*C. concinnus*
39000	220000	340000
46000	200000	340000
68000	210000	280000

Neutral lipid standard

Table x: The raw fluorescence readings of Nile Red in neutral lipid standard (cod liver oil). The readings were performed on samples containing 400 µl F/10 medium and neutral lipid standard (0, 20, 40 & 60 µl), stained with 8 µl NRWS. The samples were added isopropanol:chloroform (19:1) to a total volume of 60 µl neutral lipid standard + isopropanol:chloroform.

0	0.115 mg/ml	0.23 mg/ml	0.345 mg/ml
410000	1500000	3500000	5300000
480000	1800000	3400000	5000000
480000	1900000	3800000	4400000

Polar lipid standard

Table x: The raw fluorescence readings of Nile Red in polar lipid standard (a-phosphatidylcholine). The readings were performed on samples containing 400 µl F/10 medium and polar lipid standard (0, 2, 5 & 10 µl), stained with 8 µl NRWS. The samples were added isopropanol:chloroform (1:10) to a total volume of 10 µl polar lipid standard + isopropanol:chloroform.

0 mg/ml	0.05 mg/ml	0.125 mg/ml	0.25 mg/ml
3600000	8500000	14000000*	13000000
3500000	6400000	9800000	16000000
3800000	5800000	7800000	15000000

**This reading was removed from the final plot, as it was suspected to have received a double dosage of polar lipid standard*

Fluorometrical lipid quantification, with Nile Red

Table x: The raw fluorescence measurements of *A. longicornis, C. furcellatus, C. socialis, P. glacialis* (1° C) and *P. glacialis* (7° C) (1 mg/400 µl F/10) stained with 8 µl NRWS and measured after 10 minutes incubation at 7° C in the dark.

A.longicornis	*C. furcellatus*	*C. socialis*	*P. glacialis (1° C)*	*P. glacialis (7° C)*
Fluorescence measured at 550/630 ex/em:				
740000	1900000	880000	870000	1400000
760000	2000000	840000	840000	1600000
750000	2000000	820000	990000	1700000
Fluorescence measured at 515/575 ex/em:				
180000	290000	200000	210000	270000
170000	320000	210000	190000	260000
170000	320000	190000	200000	260000

Fluorometrical lipid quantification, blanks

Table x: The raw fluorescence measurements of *A. longicornis, C. furcellatus, C. socialis, P. glacialis* (1° C) and *P. glacialis* (7° C) (1 mg/400 µl F/10), measured after 10 minutes incubation at 7° C in the dark.

A.longicornis	*C. furcellatus*	*C. socialis*	*P. glacialis (1° C)*	*P. glacialis (7° C)*
Fluorescence measured at 550/630 ex/em:				
23000	66000	25000	25000	37000
8741	77000	36000	22000	23000
16000	67000	29000	33000	20000
Fluorescence measured at 515/575 ex/em:				
46000	45000	73000	31000	73000
42000	43000	74000	28000	74000
42000	40000	71000	27000	70000