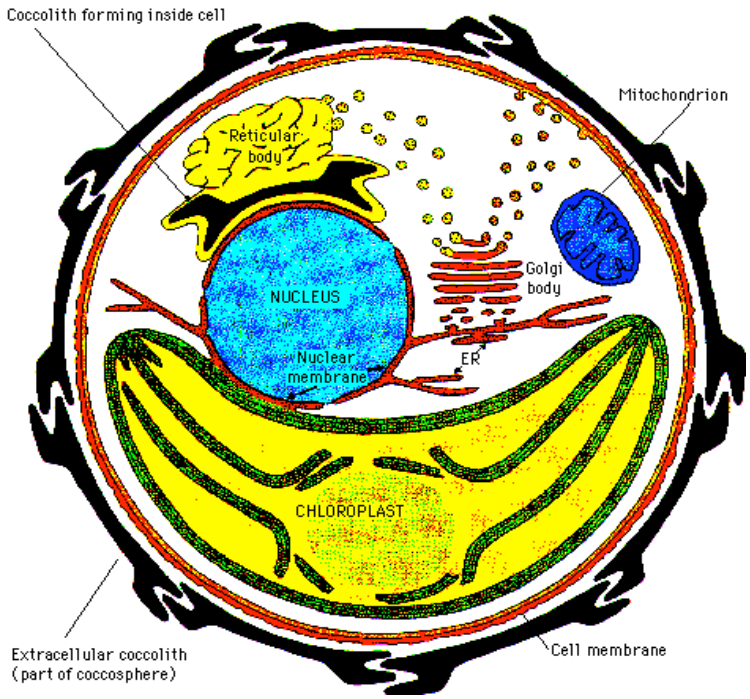
Alfred-Wegener-Institute

For Polar and Marine Research Bremerhaven

Photosynthetic Gene Expression in *Emiliania huxleyi*

By quantitative- RT PCR analysis



Master Thesis Work by Umesh Gangishetti

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Declaration

This is in accordance with the Masters Thesis test order for the course of studies Molecular biology and biochemistry at the University of Bremen. I hereby certify that the facts available in the present thesis was independently written and used no different than the sources and aids indicated by me. The used sources literally or content-wise inferred places are marked accordingly.

Bremerhaven, the 18.7.2005

Umesh Gangishetti

Dedicated to my loving Mother and my Family Members

The real study say the Upanishads, is not the study of themselves but study of that" by which we realize the changeless".

Upanishads

Abbreviation Listing

bp	Base pair
cDNA	Complementary deoxyribonucleic acid
CHP	Conservative hypothetical protein
DEPC	Diethylpyrocarbonate
DMS	Dimethylsulfide
DMSP	Dimethylsufoniopropionate
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylendiamin-tetra acetic acid
kb	kilo-basepair
mRNA	Messenger RNA
OD	Optical Density
oligo-dT	oligo- deoxythymidine
PCR	Polymerase chain reaction
PL-tubes	Phase Lock tubes
PS I	Photosystem I
PSII	Photosystem II
qRT	quantitative reverse transcription
RNA	Ribonucleic acid
RNAse	Rib nuclease
Rpm	revolutions per minute
RT	Reverse Transcription
SYBG	Sober green dye
TAE	Tries-Acetate-EDTA-buffer; Electrophoresis buffer

Table of contents

Introduction

1.1 Phytoplankton

Phytoplankton are tiny single celled organisms that live in the euphoric zone of oceans. Phytoplankton are equivalent to a terrestrial plant and have the capability of nourishing the entire food web through photosynthesis (http://footsteps.ucsd.edu/elibrary_support/lab_journals). Depending on size and shape, cell wall composition and photosynthetic pigments (fig 1) phytoplankton can be grouped into Haptophyta, Bacillariophyta, Dinophyta, Cryptophyta, and Chlorophyta. Phytoplankton constitute the major part of the pelagic biomass (Redfield et al. 1963), hence is regarded as the major primary producer in the pelagic environment (Field et al. 1998), and forms the base of the marine aquatic food web.

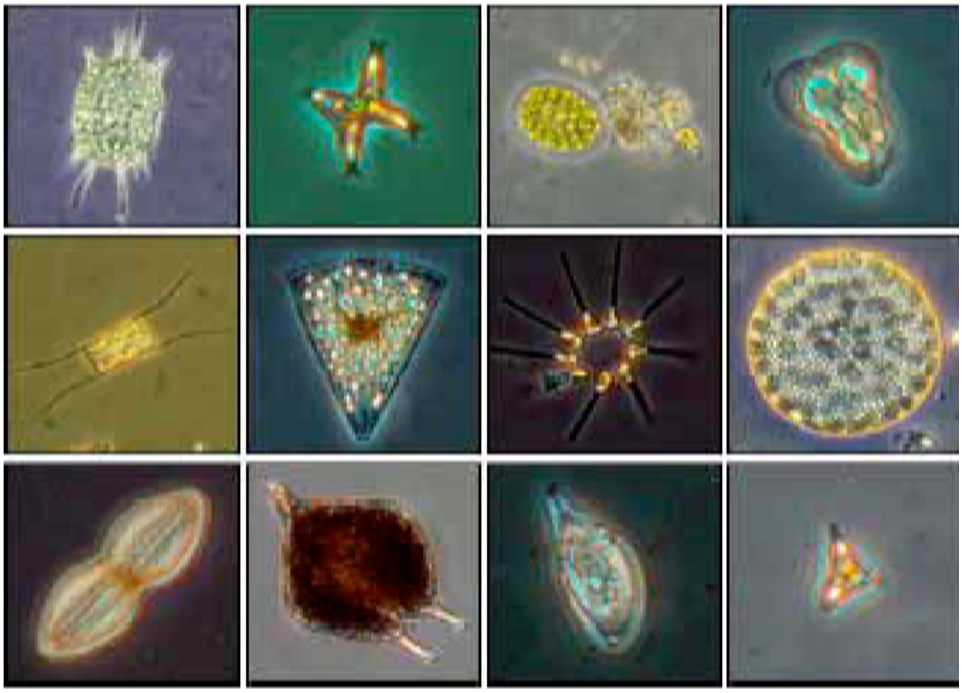


Fig 1: Phytoplankton with different size, shape, colour and photosynthetic pigments.
Source: (http://footsteps.ucsd.edu/elibrary_support/lab_journals)

Some phytoplankton's like dianoflagellates may form blooms. A phytoplankton bloom is formed during certain climatic conditions like water currents, nutrient availability, temperature, density, water salinity, and hydrography of the region, what types of zooplankton are grazing on the phytoplankton and light condition. A phytoplankton bloom is defined as high concentration of phytoplankton in an area caused by increased reproduction (http://serc.carleton.edu/eet/phytoplankton/primer.html), which often causes discolouration in water (fig 2).

Fig 2: Satellite image a harmful algal bloom of the dianoflagellates *Noctiluca scintillans,* known as a red tide. (Source: http://www.cinms.nos.noaa.gov/pcw2/images/noctiluca.jp)

A bloom can be terminated by environmental stress. In marine ecosystems it is observed that, when an algal growth escapes grazing control and reaches bloom proportions, then the physical environment is inevitably changed. In fresh water systems a bloom can causes an increase in pH to 10, which drastically reduces the bioavailability of dissolved CO_2 (Vardi et al. 1999). Consequently, cells synthesize increased quantities of carbonic anhydrase which catalyses the release of CO_2 from hydrogen carbonate (Berman-Frank et al. 1994, 1998). Thereby intracellular H_2O_2 increases and can lead to oxidative damage. Apart from these biochemical changes inside the cell, there are also nutrient depletion, decreased CO_2 levels and temperature as the major factors that can lead to bloom termination.
The other major possibility in bloom termination is viral activity.

Some marine phytoplankton can produce dimethylsulphoniopropionate, the precursor of DMS (dimethyl sulphide) (Challenger et al. 1957, Ackman & Ishida 1968). After apoptosis, viral lysis or grazing by primary consumers DMSP is released into the seawater and converted to DMS. When DMS is released to the atmosphere, it is oxidised to sulphuric acid and methane–sulphonate (Plane et al. 1989). Hence wet and dry depositions of DMS oxidation products play an important role in global sulphur cycle and significantly affect acidity in certain areas remote from anthropogenic influence (Liss et al. 1991). Dianoflagellates and haptophytes are the major DMSP producer of marine phytoplankton (Keller et al. 1989a, 1989b, Malin et al.

1992). DMSP contributes to osmotic balance (Kirst et al. 1996) and protection against cold stress in algal cells in seawater (Malin et al. 1992).

1.2 Coccolithophorids

Coccolithophorids are abundant and the most widely distributed marine phytoplankton (Okada & McIntyre 1997) and belongs to the class haptophytes. Coccolithophorids have a calcium carbonate shell called coccoliths (fig 3), covering their cell surface. (http://www.tiscali.co.uk/reference/encyclopaedia). Sinking of coccolithophorids to the ocean floor has a great influence on the marine and global carbon cycle (Westbroek et al. 1989). They contribute substantially to the limestone sediments of the ocean floor, and constitute a long-term sinking of inorganic carbon (Westbroek et al. 1993). Coccolithophorid are the main calcifying phytoplankton in the ocean. Their plastids are golden or brown in colour containing chlorophyll a, c1 and c2, as well as beta-carotene, diatoxanthin, diadinoxanthin and fucoxanthin. The coccolithophorid *Emiliania huxleyi* is among the largest producer of calcite on earth, and is studied intensively in the context of biogeochemistry, planktonic ecology, and biomineralisation.

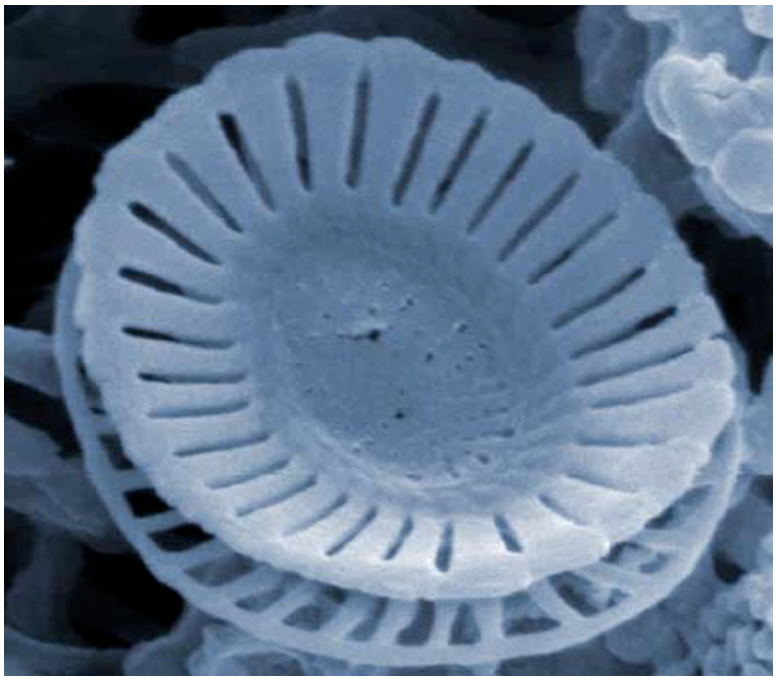


Fig 3 Structure of coccolith, the calcium carbonate shell covering in coccolithophorid (Source: www.g-o.de/ index.php?cmd=focus_detail2&f_id=2...)

1.3 *Emiliania huxleyi*

Fossil records indicate that *E. huxleyi* (fig 4) separated from the genus Gephyrocapsa oceanic Kamptner approx 270,000 years ago (Thierstein et al. 1997). It became a dominant coccolithophorid around 70,000 years ago (Bijma et al. 2001) and is now the most important coccolithophorid in the ocean. *E. huxleyi* has a worldwide distribution and is an important

member of the marine phytoplankton. It is well known for its immense coastal and open ocean blooms ranging from sub-polar to tropical latitudes (Brown & Yoder 1994) that can cover 10,000 km^2 or more areas in the ocean and counts a million cells in a liter seawater (Berge et al. 1962, Holligan et al. 1986, 1993, Ackleson et al. 1988, Aiken et al. 1992, Brown & Yoder 1994, Sukhanova & Flint 1998).

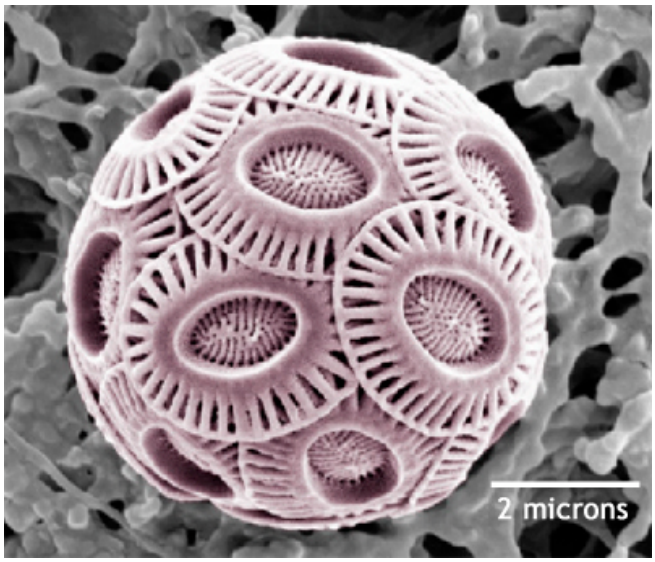


Fig 4: SEM image of coccolithophorid *Emiliania huxleyi*
Source: (http://images.google.com/imgres?imgurl=http://earthguide.ucsd.edu1)

During bloom formation the number of *E. huxleyi* cells out-numbers all other species in the sea constituting 80-90% of the phytoplankton population (http://www.soes.soton.ac.uk/staff/tt/). Due to their reflective coccolith, they can be viewed by satellite images (fig 5). Several studies have shown that natural blooms of the coccolithophorid *E. huxleyi* occur in highly stratified waters where the mixed layer depth is usually 10–20 m, and is always less than 30 m in depth (Balch et al. 1991, Robertson et al. 1994, Nanninga & Tyrrell 1996, Ziveri et al. 2000). Blooms occur often in mid-summer (Balch et al. 1991, Fernandez et al. 1993) when surface irradiances are high, suggesting that these algae preferably grow at high light intensities (Baumann et al. 2000, Ziveri & Thunell. 2000).

The ecological importance of *Emiliania huxleyi* is not only due to its ability to form enormous blooms and wide distribution, but also due to their emission of DMS (Malin et al. 1994). DMS $(CH_3)_2S$ is the dominant factor in cloud condensation nuclei (Bates et al. 1992). When DMS is released to the atmosphere, it is oxidized to sulphuric acid and methane-sulphonate forming sub-micrometer particles, and these acidic particles stimulate cloud formation and increase the earths reflectivity (Albedos) and hence affects global climate (Bates et al. 1987, Charlson et al. 1987, Ayers & Grass 1991)

Fig 5: Satellite image of *E. huxleyi* bloom in south of Cornwall (UK)
(Source: http://www.answers.com/main/ntquery;jsessionid=339hdhuas0krl?tname=cwall99-lg-jpg&sbid=lc01a)

1.4 Viruses

Viruses are the most abundant biological agents in marine aquatic environments (Bergh et al. 1989, Wagner et al. 1999, Suttle et al. 2000, Wommack & Colwell 2000). Since 1970 viruses and virus-like particles (VLP) are reported to infect systematically a broad range of aquatic algae (Van Etten et al. 1991, Van Etten & Meints 1999) including bloom forming marine phytoplankton (Nagasaki et al. 1994a, 1994b, Jacobsen et al. 1996, Nagasaki & Yamaguchi 1997, Sandaa et al. 2001). Over the last decade, significant advances have been made in understanding the dynamics of viruses and their effects on marine eukaryotic phytoplankton communities.

Several studies have investigated the role of viruses in controlling *E. huxleyi* bloom formation (Bratbak et al. 1993, 1995, 1996, Brussaard et al. 1996, Castberg et al. 2001, Jaquet et al. 2002, Wilson et al. 1998, 2002a, 2002b). From these investigations it has become evident that viruses are substantially involved in *E. huxleyi* bloom termination.

A wide range of different viruses that infect *E. huxleyi* (EhV) was isolated from the English Channel and off the coast of Bergen, Norway (Castberg et al. 2002, Schroeder et al. 2002, Wilson et al. 2002 b). Characterization of these viruses has revealed that they are large double-stranded DNA viruses with genomes approximately 410 kbp in size and that they belong to a new virus genus termed *Coccolithovirus* based on the phylogeny of their DNA polymerase gene (Schroeder et al. 2002). *Coccolithoviruses* belong to the *Phycodnaviridae* (Wilson et al. 2005), a diverse family of large icosahedral viruses that infect marine or freshwater eukaryotic algae, and all contain dsDNA genomes ranging from 180 – 560 kb (Van Etten et al. 2002).

It has also been observed that some strains of *Emiliania huxleyi* having high DMSP lyase activity are resistant to viral infection (Schroeder et al. 2002). But still the mechanism how the virus is terminating the algal bloom and why some strains with specific lyase activity are resistant to viral infection is still not clear.

1.5 Photosynthesis

The mechanism of conversion of inorganic molecules into organic energy producing carbon derivatives is called photosynthesis. It takes place in the presence of solar energy by certain photosynthetic organisms like plants, algae and cyanobacteria. These photosynthetic organisms trap solar energy with the help of chloroplasts and antennae proteins present inside the cell. Photosynthesis is mainly divided into two different kinds: a light reaction and a dark reaction or the Calvin cycle (fig 6).

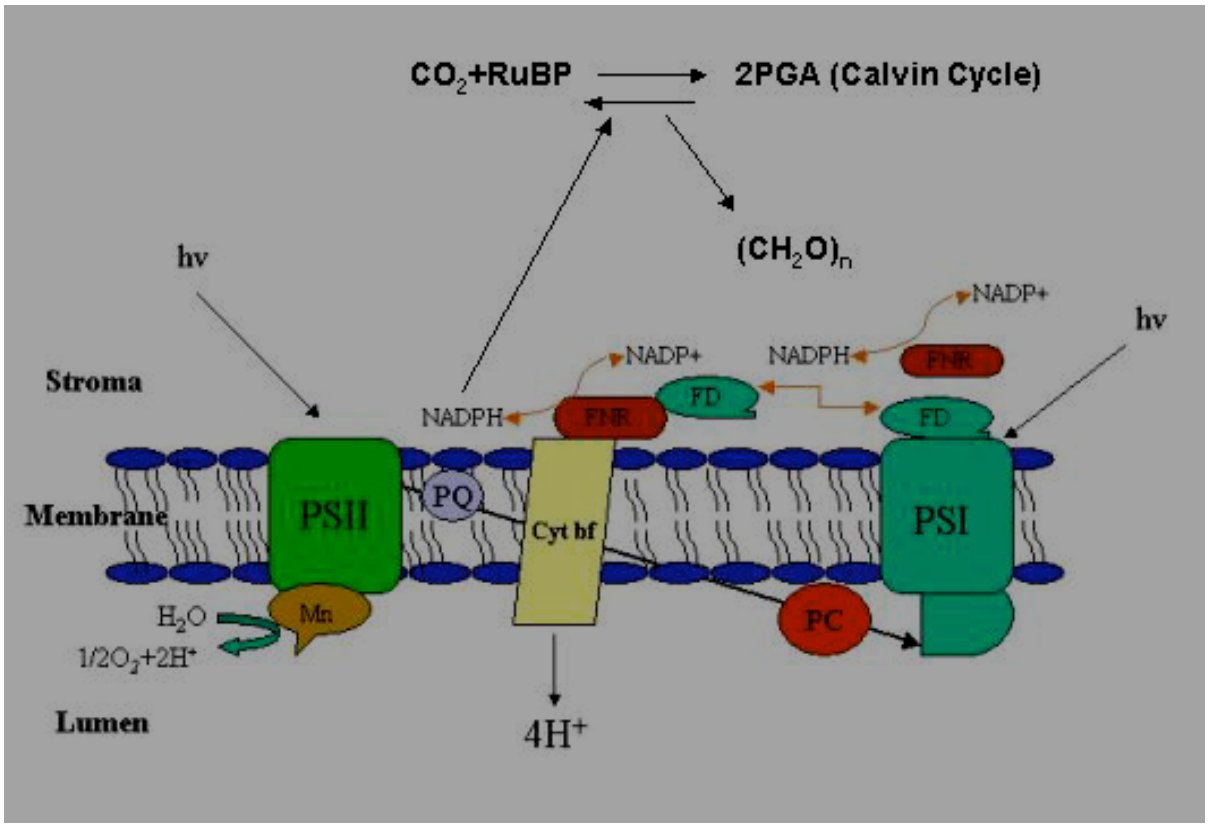


Fig 6: Schematic cross-section through a photosynthetic (i.e. thylakoid) membrane showing the orientation and some of the major components of the photosynthetic apparatus. PSII: photosystem II, PSI: photosystem I, PQ: plastoquinone, FD: ferridoxin, FNR: Ferredoxin /NADP oxidoreductase, PC: plastocyanin. (Source: Umesh Gangishetti)

1.5.1 Light Reaction

The light reaction occurs in the presence of light. Chlorophyll within the thylakoid membranes absorbs light of particular wavelengths and energizes electrons. Downstream these energized electrons are used in the production of ATP as well as taken up by $NADP^+$ to become NADPH.

1.5.2 Dark Reaction or Calvin Cycle

The Calvin cycle is a series of reactions, which ends in the formation of carbohydrate. It involves carbon dioxide fixation to 1,5 bisphosphate (RuBP) through RuBP carboxylase (rubisco), subsequent reduction and regeneration of RuBP. The primary reduction product is glycerine aldehyde-3-phosphate. From the latter and through fructose phosphate glucose is finally formed.

1.5.3 Ferredoxin-$NADP^+$oxido-reductase

Ferredoxin-$NADP^+$oxido reductase is an FAD containing enzyme that catalyzes the reversible electron transport between NADP (H) and electron carrier proteins such as ferredoxin. Isoforms of this protein are present in mitochondria, chloroplasts, and bacteria where they are involved in variety of redox metabolic pathways (Arakaki et.al 2000). Like many-other plastid proteins, chloroplast ferredoxin is encoded by genomic DNA and translated by polysomes in the cytoplasm. This protein has an amino terminal of 5 kDa, which allows the protein to be targeted and translocated across the chloroplast membrane.

1.5.4 Fucoxanthin chlorophyll a/c binding protein

Photosynthetic organisms posses protein complexes that harvest light energy and transfer it to the reaction centres (Marcus et al. 2001). Fucoxanthin is a carotene with the formula C_{40} $H_{60}O_6$. It is found as an accessory pigment in the chloroplast of brown algae as well as in most other heterokonts, giving them a brown or olive- green colour.

1.5.5 NADH-Nicotinamide adenine dinucleotide dehydrogenase

Nicotinamide dinucleotide dehydrogenase (NADHase) is one of the important co-enzymes present inside the cells. NADH is the reduced coenzyme form of vitamin B3. NAD and NADH are inter-converted into each other in numerous different metabolic activities. NADH is mainly involved in three basic respiratory reactions namely glycolysis, the Krebs cycle and electron transport. Each NADH moiety is capable of producing 3 units of ATP molecules. NADH is a relatively large and complex molecule (figure 7). It is a vitamin B3 combined with a ribose sugar and an adenine nucleotide.

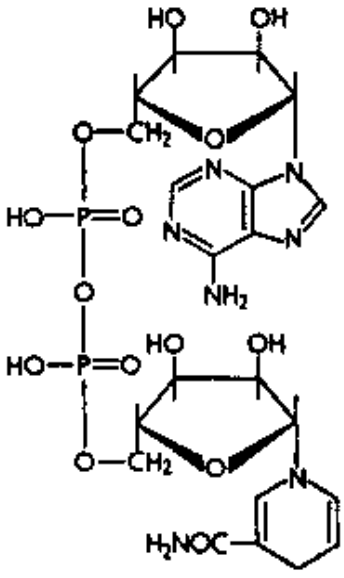


Fig: 7 NADH structure

1.5.6 Light harvesting complex (LHC)

The light harvesting complex protein (LHC) belong to the family of chlorophyll binding proteins present in all photosynthetic eukaryotes. The LHC genes are nuclear encoded pigment protein complexes that are localised to the thylakoid membranes. LHCs are divided into two different kinds of binding proteins, chlorophyll a/b binding protein in green algae and higher plants and

chlorophyll a/c binding proteins in various algal taxa (Durnford et al. 1999). PSI and PSII are the major reaction centres in light harvesting complexes, which are organised separately in the thylakoid membrane (fig 6). Light energy causes removal of an electron from a molecule of P680, which is part of the photosystem II. The P680 requires an electron, which is taken from a water molecule, breaking H_2O into H^+ and O^{2-} ions. These O^{-2} ions combine to form diatomic O_2 that is released outside (http://www.emc.maricopa.edu/faculty/farabee/BIOBK/BioBookPS.html). The electron then acquires a higher energy state and attached to a primary electron acceptor, which begins a series of redox reaction and in the end the electron is passed on to PSI. The electron is then passed onto $NADP^+$ to form NADPH, an energy carrier light reaction.

1.5.7 Photosynthesis in the marine environment

The average depth of ocean is 4000m and rarely 1% of the incident light photosynthetically active irradiance penetrates below 100m. Thus net photosynthesis is restricted to relatively thin upper layer of the ocean. Light enters the ocean from the surface, but phytoplankton are distributed throughout the water column and not fixed in space, because of turbulence in the upper layers of the ocean, resulting from wind stress and tidal energy and hence cells are constantly transported vertically (Falkowki & Wirick 1981, Falkowski et al. 1983, Denman & Mara 1986). The thermocline is a relatively quiescent layer of water several meters thick separating deep, cold, nutrient-rich water from the overlying warm, frequently nutrient limited, waters. Most of the phytoplankton are present immediately above the thermocline, where nutrient fluxes are high but light intensities are low. In the upper mixed layer of the upper ocean, the concentration of inorganic nutrients is vanishingly small. Phytoplankton in the ocean is generally limited by nutrients both in terms of biomass and growth rates (Falkowski et al. 1992). In most of the open oceans, the distribution of phytoplankton is most strongly related to vertical flux of nutrients rather than gradient irradiance (Ketchum et al. 1958, Dugdale et al. 1967, Walsh et al.1978, Yentsch et al. 1980, Laws et al. 1987, Lewis et al. 1988, Platt & Sathyendranath 1988, Lewis et al. 1992, Geider et al. 1993). Phytoplankton in the ocean are exposed to high light irradiances, which are beyond optimum and decreases the maximum rate of photosynthesis (Ryther et al. 1956, Neal et al. 1987), this decrease in photosynthesis is referred as photoinhibition. This photo inhibitory effect has been shown to be dependent on time and light intensity (Takahashi et al. 1971).

In chlorophyll c containing algae, photosystems II and I are both located in the stacked and unstacked thylakoid membranes unlike higher plants and the chlorophyta, where photosystem I is present in the stacked membrane domain (Anderson et al.1999, fig 8). Fucoxanthin a/c binding

proteins are the major light harvesting photosynthetic antennae in chlorophyll c containing algae (Green & Durnford et al.1996). The chlorophyll c and fucoxanthins are non-covalently attached to certain light harvesting proteins so as to extend spectral absorption to green regions (fucoxanthin) of the spectrum and effectively transfer to chlorophyll a in the reaction centres. The fucoxanthin-chlorophyll a/c proteins are the major thalakayoid membrane proteins in haptophytes (Anderson et al. 2000).

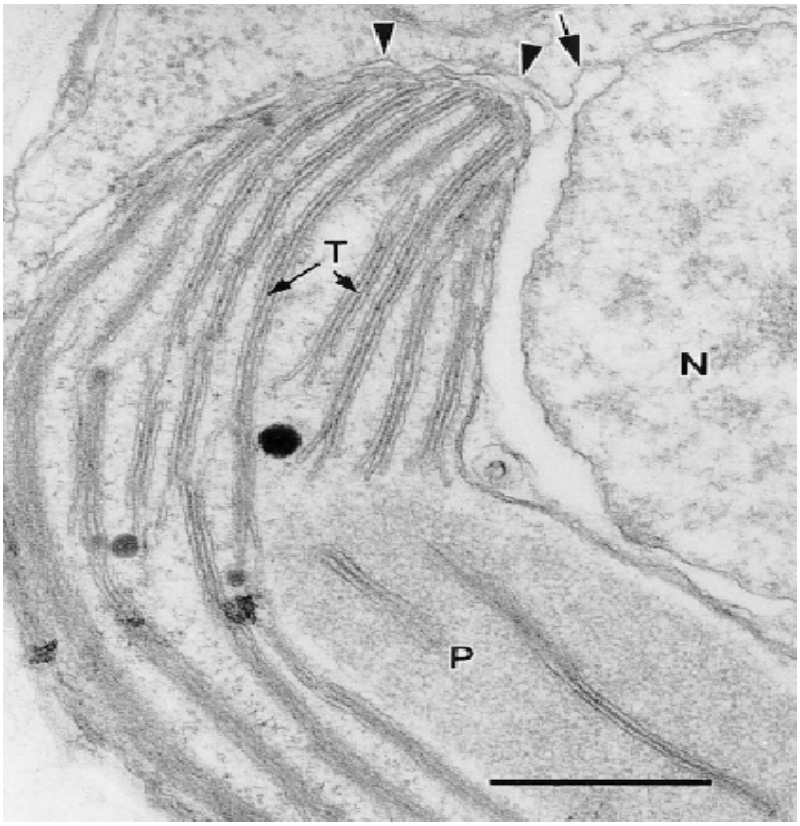


Fig: 8 A typical haptophyte chloroplast *(Isochrysis galbana*), showing the pyrenoid (P) and thylakoid (T) mainly in bands of three. The nucleus is adjacent to the chloroplast. The scale bar =1µm in length.
(Source: Anderson et al. 2000)

1.6 Aim

The coccolithophorid *Emiliania huxleyi* has a worldwide distribution and is well known for its immense blooms (Holligan et al. 1993). The abundance and wide distribution of *E. huxleyi* and its production of calcium carbonate coccoliths and DMSP emission makes it an important species with respect to sediment formation and ocean climate. (Charlson et al. 1987, Westbroek et al. 1993, Malin et al. 1994. Furthermore it is a key species for current studies on global biogeochemical cycles (Westbroek et al. 1994). Investigations on the role of viruses in controlling the bloom formation of *E. huxleyi* resulted in that viruses were involved in bloom termination (Bratback et al. 1993, 1996, Brussaard et al. 1996, Wommack & Colwell 2000, Castberg et al. 2001, Wilson et al. 2002a).

Recent work on *Emiliania huxleyi* and the mechanism how the virus is terminating the bloom has been investigated by Jessica Kegel and Klaus Valentin at the Alfred-Wegener-Institute. In her diploma work Jessica Kegel obtained four different EST libraries i.e. before and 6, 12 and 24 hrs after virus infection, respectively. From these libraries it was evident that virus was taking control of the host cell (*E. huxleyi*) transcription within one day in order to produce its own progeny, during which host cellular processes like photosynthesis was halted. EST library analysis mainly depends on clone abundances, which could only provide preliminary gene expression data. So as to obtain more detailed and corroborated information about photosynthetic gene regulation before and after virus infection in *E. huxleyi,* semi-quantitative and quantitative RT reactions should be performed.

The present master thesis covered the investigation of photosynthetic gene expression in *Emiliania huxleyi* (CCMP1516) before and after virus (Ehv 86) infection at molecular level. As it was not practically feasible to analyse all the genes obtained from EST libraries due to fixed time frame of the masters work, only few photosynthetic genes were chosen for qRT reactions i.e. 3-fucoxanthin (FCP 1, FCP 2 & FCP 3) genes, a conservative hypothetical protein, a ribosomal protein, glutaredoxin, 2-light harvesting genes (LHC 1 & LHC 2), ferredoxin NADP oxidoreductase (FNR) and 2 house keeping genes i.e. Nicotinamide adenine dinucleotide dehydrogenase (NADHase) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). House keeping genes were selected to obtain internal controls to normalise qRT-PCR reactions. The project should be started with amplification of genes using DNA as the template to obtain standards (larger PCR products) for quantitative analysis. In parallel photosynthetic gene expression in normal cultures should be performed to observe gene expression in different phases of growth curve and also between day and night. This information is necessary to verify if the change in expression in viral experiments is due to viral activity. The present experiments were divided into two sets, Experiment II and I were performed with semi quantitative method to get initial expression data from normal and viral infected cultures and Experiment IV and III were carried on to obtain quantitative expression data from viral infected and normal cultures

2 Materials and Methods

2.1 Chemicals and Reagents

- Reaction tubes: - 0. 5, 1.5 and 2.0ml, Eppendorf Germany
- Syringes: - 10, 30 and 50ml, Norm-Ject, Henk Sass Wolf GmbH, Germany
- Centrifuges: - 15 and 50ml, Sarsted corporation, Germany
- Pipet tips: - 10, 20, 100, 200 and 1000, Eppendorf Germany/Gilson, Frankfurt
- Pipet-tips: - epT.I.P.S. PCR clean, Eppendorf, Germany
- Chemicals: - All chemicals used for the respective experiments were from Sigma Aldrich GmbH, Germany
- Ethanol 100%: - Merck, Germany
-

2.2 Culturing Equipments

- Filter Midisart ® 2000 (sterile filter) 0.20 µm mesh size: - Sartorius, Germany
- Vacuum pump: - Masterflex ® Easy Load®, Cole Parmer®, Novodirect, GmbH, Germany
- Pistons: - 1 and 5l
- Cell culture bottles (250 ml): - CORNING ®, Corning Inc, USA
- Light thermostat: - RUMED, Rubarth of apparatuses GmbH, Germany
- LI-COR Radiation sensor, Typ LI-1000, LI-COR :- ® Biosciences, USA
- Vacuum pump: - KNF Neuberger, type NO35 AT 18, Pmax 4.0 bar, Germany
- Isopore TM membrane filters 25 mm in diameter, 1.2 µm mesh size: - Milli pore, Ireland
- Cryo tubes: - Nalgene ® system, Nalgene® fire Products, Nalge company, USA
- RNaqueous ®: - Ambion, Inc, USA
- RNeasy plant mini kit: - Qiagen, Germany
- RNAse Free DNase enzyme: - Qiagen, Germany
- Mercaptoethanol: - Merck, Germany
- Biospec products: - Biospec products Inc, USA
- Photometers: - Eppendorf bio-photometer, Eppendorf Netheler Hinz GmbH, Germany
- Thermo shaker: - Schutron, CLF Laboratories, Germany
- Agilent 2100 Bioanalyzer, RNA nano chip: - Agilent technologies, Germany
- Lithium chloride solution: - RNaqueous kit, Ambion Inc, USA

2.3 Sequencing Reagents and Equipments

- BigDye® terminator v3.1: - Applied of bio system, USA
- Big Dye®Terminator v1.1/3, 1 Sequencing Buffer (5x): - Applied of bio system, USA
- Mastercycler: - Eppendorf, Germany
- ABI Prism 3130xl Genetic Analyzer: - Applied Biosystems, USA

2.4 Cell sampling

Emiliania huxleyi (CCMP 1516) strain was isolated from a bloom in English Channel by the Plymouth Marine Laboratory (PML) UK. Cultures were inoculated in seven separate 1L flasks with 5000 cell/ml for seven different harvests and were grown in f/2 media at 15°C in culture rooms at Alfered-Wegner-Institute (AWI). F/2 medium contains filter sterilized in-organic nutrients, trace metals and vitamins (thiamine, biotin and vitamin B12) dissolved in seawater (Appendix II). For normal culture experiments two different RNA's were obtained from two different culturing experiments. The first culturing was performed by Jessica Kegel in 2005 and RNA was extracted in the middle of the day and in the middle of the exponential phase till initial stationary phase and stored at – 80°C. All these separately harvested RNAs were then pooled in one tube and EST libraries were obtained, this RNA was used as the calibrator for viral quantitative experiments in the present masters thesis work, it was named as Jessis cultures (see chapter 3.2). For growth curve experiments RNA was obtained from culturing experiments performed in the present masters work. Samples were harvested at different stages of cell cycle i.e. at lag phase; log phase and stationary phase (fig 9, see chapter 3.1). Virus-infected cultures were performed in Plymouth (UK) and RNA was extracted after 6hrs, 12hrs, and 24hrs of virus infection

2.5 Cell counting

Cells were counted by the coulter counter multisizer 3, which uses the electrical sensing zone method. The Coulter counter method of sizing and counting particles is based on measurable changes in electrical resistance produced by nonconductive particles suspended in an electrolyte. A small opening between electrodes is the sensing zone through which suspended particles pass. In the sensing zone each particle displaces its own volume of electrolyte. Volume displaced is measured as a voltage pulse, the height of each pulse being proportional to the volume of the particle. The quantity of suspension drawn through the aperture is precisely controlled to allow the system to count and size particles for an exact reproducible volume.

2.6 Sequencer ABI prism 3130 genetic analyzer

In sequencing reaction either forward or reverse primer is used. In addition, small amounts of fluorescently labelled dideoxynucleotides (A, T, G, or C) are added. 3' deoxy nucleotides terminate the chain and hence gene fragments with different lengths were obtained. In this way sequence of different length and different 3' ends are obtained. These sequences are then allowed to run through a capillary. Upon reaching the laser these sequences radiates light of particular wavelength and this fluorescence data is transformed to a computer. The smallest 5' end base forms the first base of the sequence. In the present masters work I used Big Dye sequencing RR100 (Appendix I)

2.7 Dye Ex kit 2.0

After the sequencing reaction, the PCR product was purified by the Dye Ex 2.0 kit to remove any unincorporated dye terminators from the sequencing reaction. Dye Ex modules contain prehydrated gel filtration resin and can cleanup-sequencing reactions containing dRhodamine, DYEnamic ET, and particularly BIG Dye terminators.

These columns contain prehydrated gel and resin. Prior to use the resin present in the columns has to be discarded. For this first the bottom clouser tab was snapped off of the spin columns and the columns were centrifuged at the calculated speed (2700 rpm with Eppendorf Centrifuge 5417 C) for 3min. Columns were transferred to new 1.5ml tubes and 10-20µl of the sequencing reaction was added to the gel bed. The tubes were then centrifuged for 3min at 2700 rpm.

2.8 RNA extraction

Gene expression study require isolation of high quality of RNA. So as to obtain good quality RNA from coccolithophorids, four different kinds of RNA extraction protocols were used.

2.8.1 CTAB+DTT RNA extraction protocol

This is an inexpensive and rapid RNA extraction protocol, which needs only CTAB buffer. The principle is selective precipitation of RNA using lithium chloride. This method avoids the use of toxic chaotrophic agents or phenol. Here dithiothreitol is used to inhibit RNAse activity and to prevent oxidative cross-linking of nucleic acid by phenolics compounds.

Extraction buffer was calculated and prepared accordingly and the stock solutions were stored at –20°C.

(Appendix II, Note: Dithiothreitol was added to the extraction buffer immediately prior to use)

2.8.2 Extraction Procedure

1) 1ml of the extraction buffer was pipetted to cell samples of approx. 50 million *E. huxleyi* cells, which had been harvested by vacuum filtration and frozen immediately in liquid N_2. Cells were lysed by vigorous vortexing and incubated for 10-15 minutes at room

temperature. During the vortexing and incubation periods, the reagents in the extraction buffer came in vigorous contact with the cell membrane, thereby achieving cell lysis.

2) Cell debris were removed by the addition of 1ml of chloroform: isoamyl alcohol (24:1 v/v). Addition of chloroform: isoamyl alcohol was followed by centrifugation at 12,000 g for 20 minutes at 20°C, separates the solution into an upper aqueous phase and a lower organic phase containing cell debris.

3) The upper aqueous phase containing polysaccharides and nucleic acids were transferred to a new tube and 0.3 volume absolute alcohol (100%EtOH) was added. The addition of alcohol resulted in the precipitation of polysaccharides.

4) The chloroform: isoamyl alcohol step (i.e. 2nd & 3rd step) was repeated under the same conditions. And the aqueous phase containing nucleic acid was transferred to a new tube.

5) RNA was precipitated with 650 µl of 12M LiCl in the presence of 1% ß-mercaptoethanol as antioxidant. Precipitation was performed over night at – 20°C. The RNA was collected by centrifugation at 14,000 g for 30 min at 4°C. A small white pellet was observed on the wall of the tube, however sometimes the pellet was transparent and care had to be taken not to loose the pellet.

6) The supernatant was discarded carefully without touching the RNA pellet and was washed with 70-80% EtOH and centrifuged at 14,000g for 10 min at room temperature.

7) The supernatant was discarded again and the pellet was dried at room temperature for 10-20 minutes. The dried pellet was resuspendended in (30-50 µl) DEPC treated RNAse free water or 10mM Tris-HCl.

2.8.3 Trizol Method

The trizol-reagent is a mono-phasic solution of phenol and guanidine isothiocyanate, which is an improvement to the single-step RNA isolation method.

Extraction protocol

1. 1ml of trizol reagent was added to approx 4 million *E. huxleyi* cells, which were previously fixed to filter paper and stored at – 80°C. The cells were destroyed by quick vortex and incubated at 60°C for 10 min in a thermoshaker with full speed. During vortexing and incubation periods the reagents in the trizol reagent came in contact with *E.huxleyi* cell walls and therefore cell lysis was achieved. Due to cell lysis, the cell components and nucleic acids were released into solution.
2. In order to separate nucleic acids from the cell contaminants, 200µl chloroform was added and vortexed immediately. The solution was kept under the fume hood for 5 min at room temperature. The separation into organic and aqueous phase was due to addition of

chloroform. This solution was then added to phase lock tubes and was kept at room temperature for 15 min. The phase lock tubes contain a phase lock seal, which forms a tight seal between the two phases

3. The phase lock tubes were centrifuged for 15 minutes at 12,000 g at 4 °C. During this centrifugation step the organic and interphase materials were effectively trapped in or below the seal, thus enabling easy transfer of the upper aqueous phase to a new tube.
4. The aqueous phase from step 3 was transferred to a new tube and 0.5 volume of isoproponol was added (calculated according to the amount of aqueous solution transferred) and vortexed for 15 sec. Tubes were placed at –20°C for at least 10 minutes and then centrifuged for 10 min at 12000 g at 4°C. This centrifugation step resulted in precipitation of nucleic acids.
5. The supernatant was discarded and 1ml of ethanol (75%) was added and vortexed immediately to redissolve the pellet. The solution was then centrifuged for 10 min at 12,000 g at 4°C. The supernatant was discarded and tubes were kept under the hood, until the pellet was completely dry (Sometimes the pellet was transparent)
6. RNA was eluted with 100µl DEPC treated RNAse free water, and immediately vortexed. A short centrifugation was performed to collect all the RNA in the solution.

2.8.4 PeQLab

Total RNA Extraction with PeQLAB protocol

Precaution*s*: All appropriate measures were taken to avoid RNAse activity throughout the protocol. Protective gloves were worn, only sterile UV treated filter tips were used, and only certified RNAse free tubes were used in all wash/elution steps. All solutions not provided by the kit, i.e. extra RNAse free water, were treated with DEPC to inhibit RNAse activity.

Prior to starting the protocol*:* 20µl ß-Mercaptoethanol was added as an RNAse inhibitor to 1ml lysis buffer (RPL) and RB buffer. 20ml of ethanol was added to 5ml of wash buffer II. It is also important when working with micro algal cells not to use too much starting material. Plant cells in particular contain many secondary metabolites such as polysaccharides that can clog the column, and prevent a satisfactory final extraction of RNA.

RNA extraction protocol

1.Flash frozen samples were thawed ‘on ice’. To open the cells 600µl of lysis buffer RPL buffer were added to the cell samples and vortexed vigorously. The cell lysate was pippeted directly into 2ml shedder columns placed in collection tubes. A centrifugation for 13,000g for 5 minutes at room temperature was performed. Centrifugation through the shredder column functions to remove cell debris, as well as homogenize the lysate. A small pellet formed at the bottom of the

collection tube. The supernatant was very carefully removed and placed in a new centrifuge tube, without disturbing the pellet at the bottom of the tube, as this contained cell debris.

2.To remove polysaccharides 140µl of SP buffer were added to the cell lysate and centrifuged for 10min at 10,000g. The lysate was transferred to a new tube and 600µl of the isopropanol was added and centrifuged for 2 minutes at 10,000g to pellet RNA.

3.100µl of pre-heated (65°C) DEPEC- H_2O was added to RNA pellet and vortexed to resuspend the pellet. To increase the binding properties of the HiBind RNA spin-columns first add 350µl RB buffer/2-ß-mercaptoethanol followed by 250µl absolute ethanol and then vortexed thoroughly to mix the solution. The entire sample was loaded onto a new HiBind column/collection tube, and was spun at 10,000g for 30 seconds.

4.The flow through was discarded. 700 µl RNA washbuffer I was added to the column, and column was spun again at 10,000 g for 30 seconds. RNA wash buffer I has a high guanidine salt concentration that functions to wash the bound RNA on the membrane. The flow through was again discarded.

5.The column was transferred to a new collection tube. 500 µl RNA washbuffer containing ethanol were added to the column, and the column was spun again at 10,000g for 30 seconds. The flow through was discarded. The column was then spun at 10,000 for 1 minute to remove all traces of ethanol from the silica membrane before continuing. Any remaining ethanol could interfere with downstream applications of the RNA, i.e. cDNA synthesis. Remaining ethanol also lowers the final yield of eluted nucleic acid.

6. The column was placed next in a new centrifuge tube, 50 µl of DEPEC treated water was pipetted directly to the center of the membrane in order to elute the RNA, a centrifugation step for 10,000g for 1 minute was performed.

2.8.5 Qiagen (RNeasy Mini kit)

The RNeasy procedure utilizes the selective binding properties of a silica-based membrane microspin technology with RNA. A specialized high salt buffer system allows RNA to bind to the RNeasy silica gel membrane. First the biological sample is lysed and homogenized in the presence of highly denaturing guanidine isothiocyanate (GITC)-containing buffer, which immediately inactivates RNases to ensure isolation of intact RNA. Ethanol is then added to provide the appropriate binding conditions, and the sample is then applied to an RNeasy mini column where the total RNA binds to the membrane, and contaminants are efficiently washed away. High quality RNA is then eluted with RNAse free water.

Extraction protocol by Qiagen

1. Cells were destroyed by addition of 450µl of a chaotrophic buffer like RLT buffer containing ß-mercatoethanol to approx. 4 million *E.huxleyi* cells. The RLT buffer contains gaunidium isothiocyanate, which has cell disruption and denaturation properties. Cells were destroyed completely by vigorous vortexing for 1min. Vortexing causes intense contact between lysis buffer and cell samples. Lysis buffer contains chaotrophic agents, which causes cell lysis and simultaneously stabilises RNA by inhibition of endogenous RNases. Non-dissolved cell debris were removed from the samples by centrifugation for 5 min at full speed. The cell lysate was transferred to a new 1.5ml eppendorf tube and care was taken not to disturb the pellet. 225 µl of ethanol (96-100%) was added to the cleared lysate. Addition of ethanol to the solution decreases polarity and thus increases the tendency of RNA to precipitate or rather bind to the polar solid phase.
2. 650µl of the extraction solution was added to a new RNeasy pink column for adsorption of RNA to the membrane and centrifuged for 15 sec at 10,000 rpm. This step allows the RNA to bind to the membrane inside the column
3. The column was washed with 700µl RW1 washing buffer and centrifuged for 15 sec at 10,000 rpm. This washing step was performed twice. Second washing step with 500µl RPE buffer was performed and centrifuged for 2min at 10,000 rpm. This washing step was also repeated twice to make sure that only pure RNA was bound to the column.
4. Prior to the elution step the column was dried by an additional centrifugation step of 1min with full speed. Immediately add 30-50µl of the RNAse-free water directly onto the membrane. Close the tube gently and centrifuge at 10,000 rpm for 1minute to elute the RNA.

2.8.6 RNeasy Mini Protocol for RNA Cleanup with DNAse digestion

1) This clean up protocol was used to purify the RNA already extracted from the cells. In this clean up protocol DNase digestion was also performed. It follows the same procedure as that of the normal RNA extraction protocol but here the RNA amount was adjusted to 100µl volume with RNAse-free water

2) 350µl RLT buffer was added to 100µl of RNA solution and mixed thoroughly by pippeting. 250µl of ethanol was added, so as to facilitate binding of RNA on to the RNeasy mini column. 700µl of the sample was loaded onto RNeasy mini-column (pink colour) and centrifuged at 10,000rpm for 15 sec.

3) For DNase digestion, the washing step with RW1 washing buffer was divided into two 350-µl steps. The column was first washed with 350µl RW1 washing buffer. In the meantime DNAase1 digestion mix was prepared by addition of 10µl DNAse enzyme to 70µl of RDD

buffer. Then 80µl of DNAse digestion mix was added onto the column and incubated at 25°C for 15 min without shaking, (DNAse I is sensitive to physical denaturation). Tubes were closed gently and centrifuged for 15 sec at 10,000 rpm. Flow through was discarded.

4) The RNA was washed with 500µl RPE buffer by centrifuging twice at 10,000 rpm for 2min. The collection tube and the flow through were discarded after the second washing step. The membrane was dried by an additional centrifugation step of 1min with full speed.

5) Elution of RNA was performed by addition of 35-40 µl of DEPEC treated RNAse-free H_2O and centrifuged at 10,000 rpm for 1minute. Tris-HCl; pH 7.5 was also used for elution when the samples had low photometric readings at A260/280 value

2.9 Semi Quantitative PCR

Gene expression analysis requires cDNA. cDNAs are complementary copies of the RNA molecules. This mechanism was first observed in retroviruses, which produce an enzyme called reverse transcriptase. It is RNA dependant DNA polymerase that copies the viral RNA into DNA (http://www.vivo.colostate.edu/hbooks/genetics/biotech/ enzymes/rt.html.). cDNA synthesis was performed in a single step by oligo dT primer and reverse transcriptase enzyme. This oligo dT primer is used to bind the poly (A) tail present at the 3'ends of the mRNA and the reverse transcriptase copies the RNA. (Appendix I)

2.10 Primers and probes for semi-quantitative analysis

Sequences were obtained from recent work at AWI, where they established 2500 EST sequences from pre and post viral cultures. Primers were designed using DNA STAR software from Lasergene Company and ordered from MWG Germany. On arrival the primers were diluted to 10 pmol/µl and stored at –80°C, 1µl of this concentration was used for 1x PCR reaction.(Appendix I)

2.11 Quantitative-Real Time PCR

Polymerase chain reaction is one of the basic tools to analyze RNA or DNA amplicons in the cell. In a well-optimised PCR reaction the amplification should approximately double during each amplification cycle. In real time PCR reaction this amplification is coupled with an increase in fluorescence intensity using a fluorescent molecule. There are two different possibility of observing this amplified PCR product 1) endpoint analysis (semi-quantitative) and 2) Real time analysis i.e. monitoring the PCR reaction while the reaction is in progress (real time quantitative analysis). In the end point analysis the reaction is run for 30-40 cycles and then analysed on an agarose gel. This method mostly depends on the size discrimination that is

not as sensitive enough as real-time PCR reaction, where the reaction is monitored after completion of each reaction. The resolution of the agarose gel is poor and we can't make a difference from 10 to 50 fold changes in expression in the same sample.

SYBR green dye: SYBR green dye non-specifically binds to minor groove of the double stranded DNA molecule. So as the reaction proceeds more and more double stranded PCR products are formed and accordingly fluorescence increases. As SYBR green was an easy and cost effective method, I choose to use SYBR green dye in our current real time PCR reactions. The main constituent of real-time PCR was pure and good quality RNA. After extraction of RNA from *E huxleyi* it was adjusted to 441ng for the cDNA synthesis by Omniscript cDNA synthesis.

2.12 Primers and probes for quantitative analysis

Different sets of primers were designed exclusively for real-time PCR reactions using DNA STAR software from Laser gene and ordered from MWG the genomic company Germany. All the primers have calculated 60°C of annealing temperature (Appendix I)

2.13 cDNA Synthesis Reagents

For quantitative PCR reaction we need exactly same amount of RNA for all the samples and in my case this amount was set to 441ng/µl (amount was calculated with respect to sample with least amount of RNA) of RNA from each harvest, so a different cDNA synthesis from Omniscript was followed for the quantitative analysis. The protocol was added unto 20µl in total for all the reagents and was flexible to prepare the master mix. Afterwards this cDNA was diluted to 1:5 with RNAse free water and aliquoted in 5 different tubes each with 20µl volume (Appendix II). Before going directly to the real time reaction a normal test PCR was run to verify the primers functioning (reagents and PCR programme in Appendix I)

2.14 Gene expression by real time qPCR

In a PCR reaction the template amount doubles after each cycle and this happens in a logarithmic pattern until the PCR reaction is complete. This logarithmic increase in PCR amount can be detected in real time with a real-time PCR machine. Real-time polymerase chain reaction is a very sensitive and power-full technique used for analysing gene expression. Real time measures the template amount as the reaction proceeds. It actually detects the fluorescence signals emitted by the PCR product formed after each cycle and a threshold is manually or automatically set based on the standard fluorescence values. From the fluorescence readings the software plots a logarithmic graph corresponding to the amount of template present at a

particular cycle number. There are two different methods for analysing data obtained after a real-time pcr reaction i e. Absolute Quantification and Relative Quantification

2.15 Absolute Quantification

This method is very precise and a direct way of analysing quantitative data, which makes use of standard curves. Standard curves are prepared from dilution series of control template of known concentration. Absolute quantification is performed when the objective of the experiment is to know exact levels of template concentration in the samples.

2.16 Relative Quantification

Most of the gene expression experiments demands for the relative quantification method. In relative quantification the quantitative data is calculated with reference to an endogenous control. This endogenous control is a housekeeping gene that is expressed approximately to same degree in all the samples. An endogenous control is also used to test the quality of RNA in absolute quantification.

2.17 Experimental Design

In absolute quantification method the most important criteria are the standards that are used to construct the standard curve. Standards are templates of known concentration which could be either as plasmid -containing the gene of interest (GOI), a PCR product, a genomic DNA, a cDNA, synthetic oligos, or an *invitro* transcript. Dilutions of standards are prepared in such a way that the samples to be analysed fall in the same concentration range. For most of the reactions triplicates and five dilution series are used for every run (96 well plate). The standard curve is generated by plotting the log of the initial template copy number against the Ct generated for each dilution. If the aliquoting was accurate and the efficiency of the template does not change over the range of template concentration being used, then the plot of these points should generate a straight line. This line is the standard curve. By comparing the Ct values of the samples with standards gives us the quantification data for the unknown samples. The linearity of the standard curve is determined as Rsq value or R^2 and should be close to 1. A linear curve also implies that the efficiency of the amplification is consistent with varying amounts of the template concentration. If the standard curve becomes non-linear at very low concentrations, then it means that it is reaching the limit of detection. Unknown samples falling in the non-linear region cannot be taken into consideration.

For the present photosynthetic gene expression analysis, absolute quantification method was performed with PCR products as the standard templates. The experiments were divided into

two different sets with the same photosynthetic genes in consideration i.e. experiment III with normal cultures and experiment IV with virus infected cultures.

2.18 Calculation for absolute quantification

In absolute quantification method exact number of amplicon molecules is calculated for different genes at a threshold, set by the software based on the standards used. By adding the numbers of A, T, G, and C molecules in an amplicon we could calculate the molecular weight of the respective amplicon. The weight (ng/µl) of the amplicon at the threshold value is obtained from the software. By dividing this weight by the molecular weight gives us the number of moles for that particular gene at the threshold. This value can be converted into number of molecules by multiplying the factor $6.023 * 10^{23}$.

Molecular weight of the amplicon = X* molecular weight of A + Y*molecular weight of T + Z* molecular weight of G +M* molecular weight of C

Where X, Y, Z and M represent the number of adenine (A), thymine (T), guanine (G) and cytosine(C) present in the amplicon.

Number of moles = Weight /Molecular weight of the Amplicon.

Number of Molecules = Number of moles $*6.023*10^{23}$

After calculating the number of molecules for different samples a calibrator and an endogenous control is set. Calibrator is the sample used as the basis for comparative results and an endogenous control is an internal active reference gene that stands the same in all samples. Once the number of molecules for the target gene and the reference gene has been calculated then division of target amplicon molecules by reference gene gives us an idea about the sensitivity of the PCR run. The purpose of using an endogenous gene is to make sure that the expression is due to differences in number of molecules of RNA and not due to certain other PCR or RNA harvesting artefacts. Once the endogenous gene is successfully standardised then expression of other target genes in different samples can be obtained as a comparison to the calibrator. In our case normal pooled RNA (from different cell stages) without any virus infection was considered as the calibrator and the gene expression of other samples was calculated as a factor value by assigning the calibrator as- 1 (fig 24).

3 Results

3.1 Algal Cell Growth

E. huxleyi. (CCMP 1516) cultures were grown in seven separate pseudo batch cultures with a light intensity of 150-160µm. Cell concentrations were determined with a Beckman coulter counter (Appendix 2 Table 1, fig 9). A test culture was performed to understand the *E.huxleyi* growth pattern. The cells demonstrated a small lag phase of three days. From the fourth day onwards cultures exhibited doubling of cells logarithmically with 349,900 cells/ml entering the exponential phase. After 9 days of exponentional phase, cultures entered into stationary phase with 40,77000 cells/ml. The cells started to loose their coccoliths after the 13th day and were settling at the bottom of the flask, which was an indication of cell-death.

The harvesting stages included an initial exponential stage, a middle exponential stage and initial stationary phase.

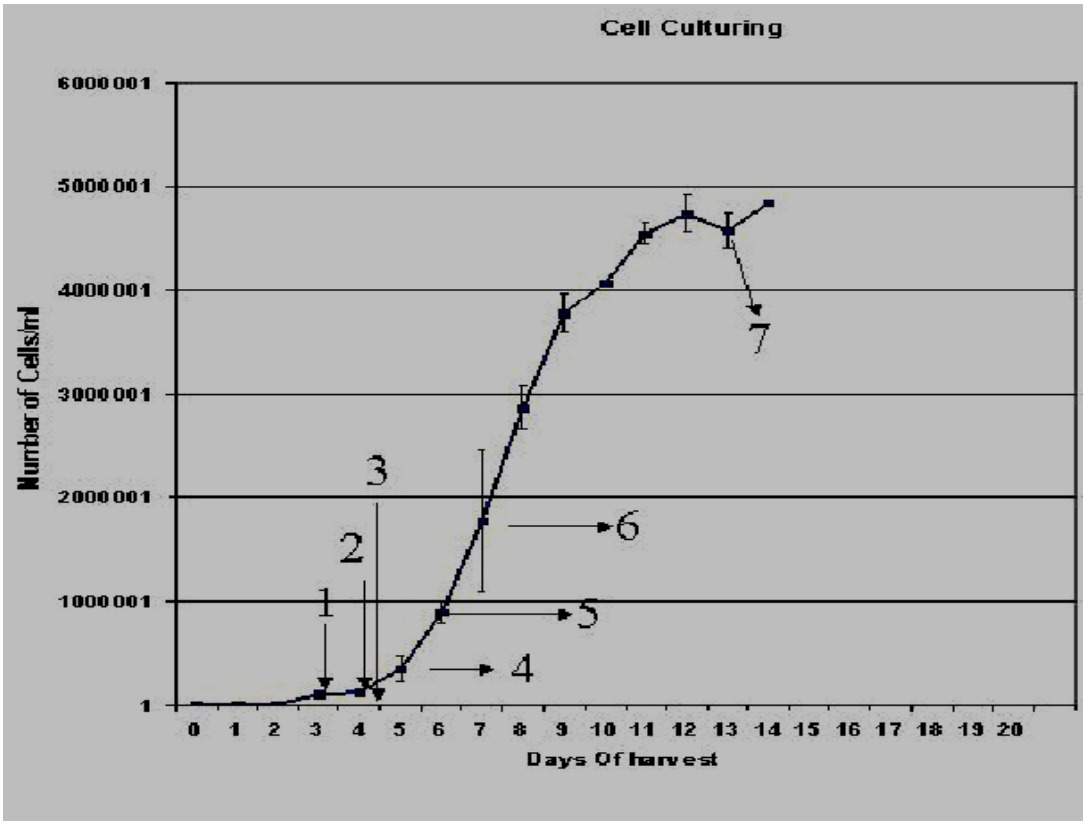


Fig 9. *E. huxleyi.* Growth-curve, numbers from 1-7 represents the harvest number. The value on the Y-axis represents cell numbers when cells were harvested.

All cell harvestings were performed in middle of the day, except for 3rd and 6th harvest, which were performed late in the night and late in the evening respectively. These two extractions were performed to verify photosynthetic gene expression throughout the day, and to compare gene expression between day and night cultures.

3.2 RNA Extraction

Four different extraction protocols were performed for RNA extraction. RNA obtained using a Qiagen protocol is shown in figure 10, which was stable and undegraded, whereas the other extraction protocols did provide good amounts of RNA which, however, was of degraded quality. The chromatograms shown in figs 10 and 11 were obtained from a biochip run. The RNA in figure 10 represents undegraded RNA that includes all the cellular RNA present inside the cell. A peak at 40 seconds represents 18s RNA and at 45 seconds shows 28s RNA. All other small peaks at 25, 30, and 35-40 sec represents smaller RNA present inside the cell. The peak at 20 sec is the marker peak, based on which the software determines the baseline for the chromatogram as well as determines the amount of RNA in the samples loaded. The height of the 28S ribosomal peak should be twice that of 18S ribosomal peak. Variability in this ratio indicates RNA degradation and in the case of complete degradation these bands disappear (figure 11).

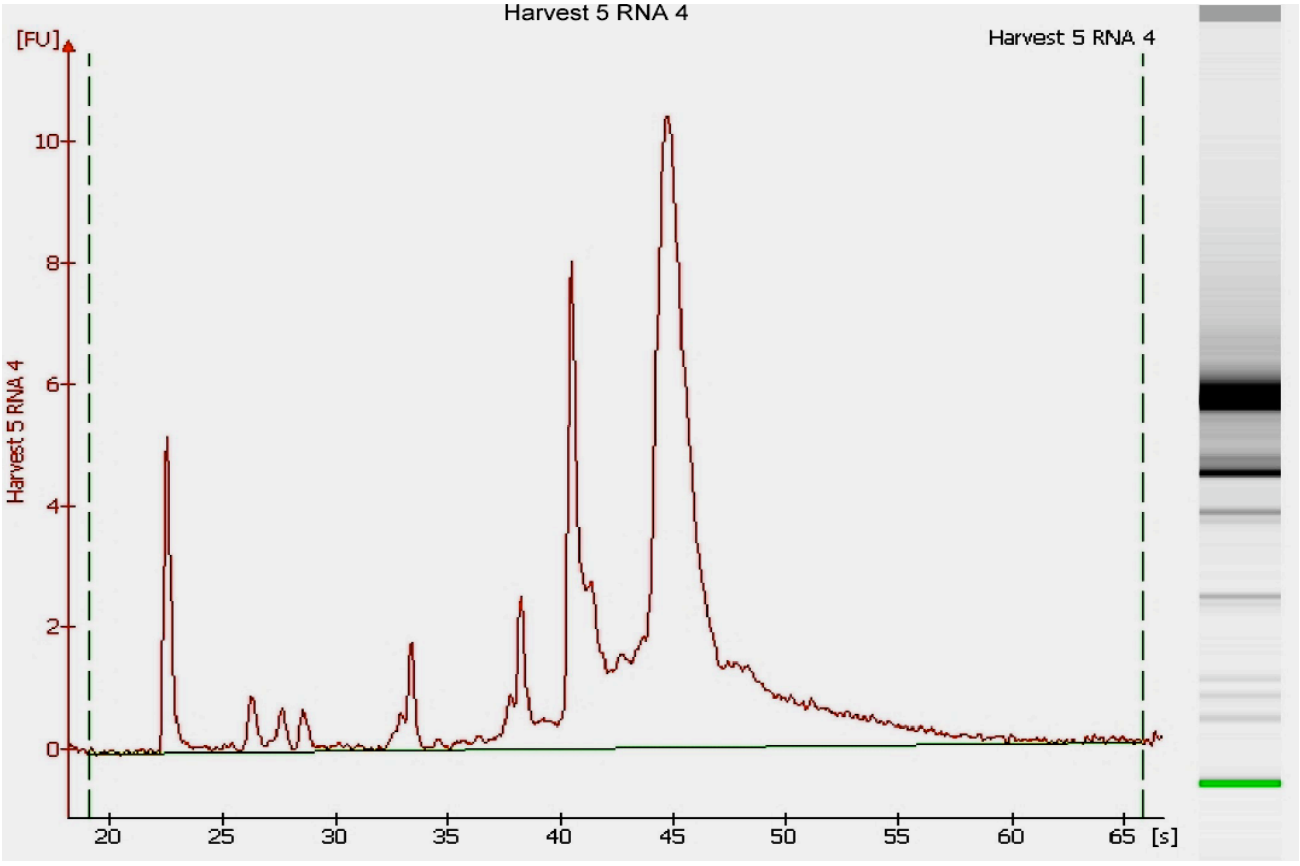


Fig 10 RNA extracted using Qiagen protocol. The value on X-axis represents time in sec. The first peak represents the ladder. Peaks at 25 sec till 30 sec represent the smaller RNA. Peak at 40 sec shows the 18s ribosomal RNA and a peak at 45 sec represents 28s ribosomal RNA.

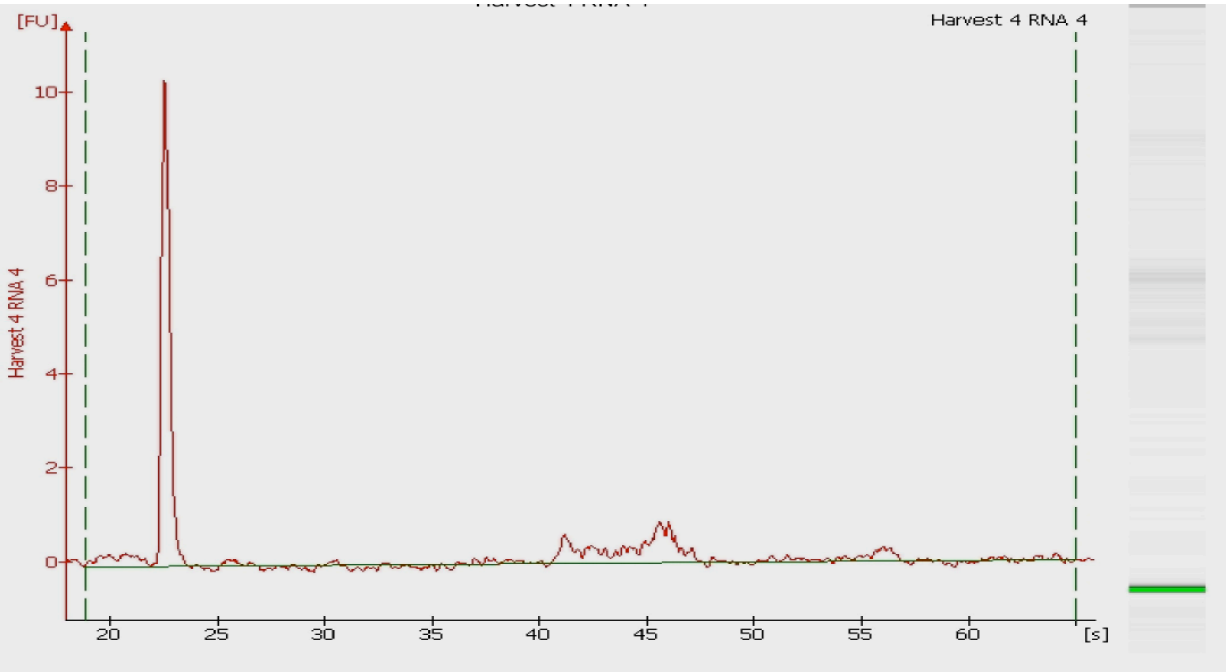


Fig 11 RNA extracted using Peq-lab protocol. The value on x-axis represents the time in sec and on Y-axis shows the size of the RNA.The first peak represents the ladder

3.3 Semi-Quantitative RT-PCR, Experiment I and II

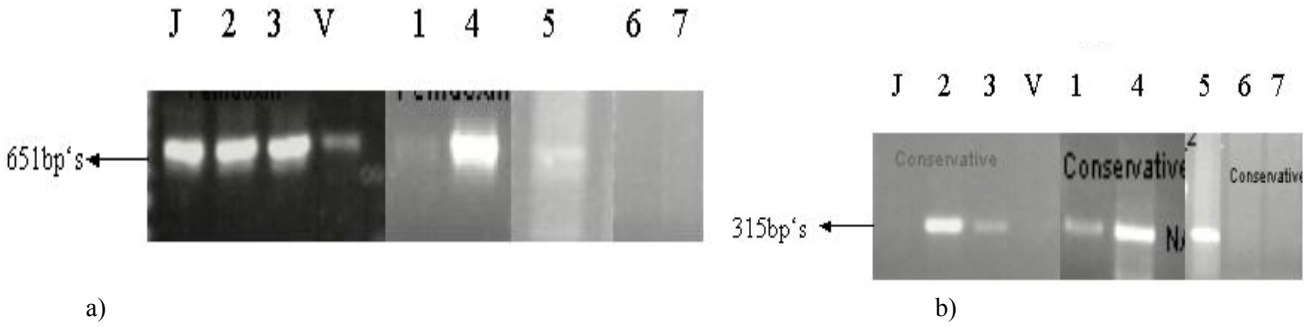
In order to observe initial gene expression data and to obtain larger PCR products using DNA as template, primers were designed based on EST sequence knowledge (Appendix III). Unfortunately we could not obtain PCR products from DNA (data not shown) and had to use cDNA as the template for PCR based amplification. The Semi-quantitative experiment were started with 12 different genes, out of which only four genes i.e. NADHase (Nicotinamide dinucleotide dehydrogenase), Ferredoxin $NADP^{+}$oxidoreductase (FNR), fucoxanthin chlorophyll a/c binding protein (Fcp) and conservative hypothetical protein (CHP known from cyanobacteria) were successfully amplified and sequenced.

Expression of successfully sequenced genes was then determined by semi quantitative analysis. Only CHP genes demonstrated a significant down regulation at night (fig 12 b). Ferridoxin and CHP both the genes showed no expression in harvest 6,7. In virus infected cultures ferredoxin showed significant down regulation, where as CHP genes demonstrated no expression. Both genes demonstrated a significant down regulation in 1^{st} and 4^{th} harvest representing a significant difference in expression between short lag phase and log phase.

In these experiments NADHase was used as a housekeeping gene. House keeping genes are used, as internal controls in such experiments to make sure that measured differences in expression are not due to culturing or cell harvesting artefacts but were based on differences in numbers of RNA molecules inside the cell. NADHase turned out to be a good reference gene, as it did not show

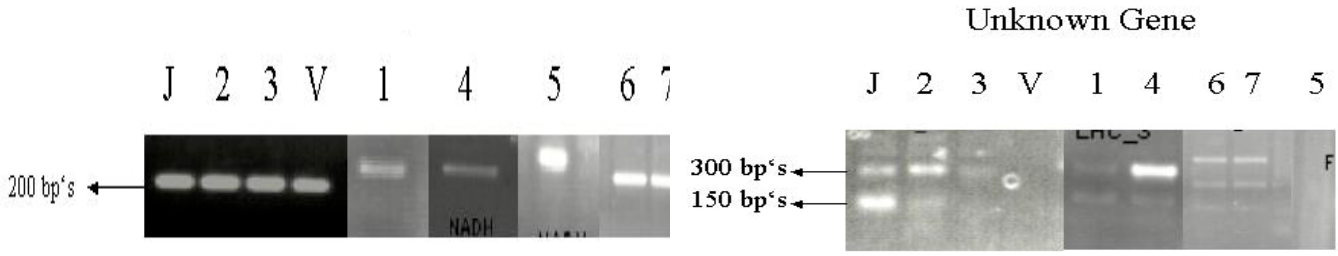
any difference in expression (fig 13). The Fcp gene demonstrated up regulation in night cultures and in viral cultures (data not shown) too. One of the primers that was designed to amplify a light harvesting gene yielded a sequence of 160 bp's of unknown function (Appendix III) which matches a sequence from *E. huxleyi* genome database and was regulated the same way as other photosynthetic genes (Fig 13 b). The unknown gene showed expression in all the harvests except for viral and 5th harvest and was down regulated in the night.

Two different experiments i.e. **experiment II** with viral infected cultures (after 12hrs) and **experiment I** with RNA harvested over a growth curve were carried out in parallel. (Fig: 12 a, b). By semi-quantitative PCR reactions, we observed a variation depending on the status of the culture and the phase of the day.



a) b)

Fig: 12 a) Ferredoxin gene and b) conservative hypothetical protein gene demonstrating gene expression in *Emiliania huxleyi*. The numbers represent the corresponding harvesting number. "J" represents pooled RNA from *Emiliania huxleyi* culture over growth curve and V represents RNA from post-virus infected cultures.



a) b)

Fig: 13 a) NADHase gene and b) Unknown gene expression over growth curve and viral cultures. The numbers represent the corresponding harvesting number."J" represents pooled RNA from *Emiliania huxleyi* cultures over growth curve and V represents RNA from post virus infected cultures

3.4 qRT PCR over-growth curve Experiment III

Based on knowledge from semi-quantitative results, a few photosynthetic genes were selected for gene expression over growth-curve i.e. Fucoxanthin chlorophyll a/c binding protein (FCP_1, fig 17), Nicotinamide adenine dinucleotide dehydrogenase (NADHase, fig 16), Light harvesting complex (LHC, fig 15), glyceraldehyde-3-phosphate dehydrogenase (GAPDH data not shown) and ferredoxin $NADP^+$ oxidoreductase (FNR, fig 14) were selected for photosynthetic gene expression analysis using quantitative RT PCR reactions. These experiments showed that photosynthetic genes were highly regulated throughout the day and also over the growth curve. In these experiments the value on y-axis is the amount of PCR product (ng/µl) at the threshold, which is obtained from the software and is relative to known standard concentrations. The standard curves, RSQ values and PCR efficiencies are calculated and attached in Appendix IV.

Photosynthetic gene expression in *Emiliania huxleyi*

In *Emiliania huxleyi* the photosynthetic genes exhibited a pattern of decrease in gene expression in night (i.e. harvest 3) except for fucoxanthin (fig 17). This decrease correlates to light and dark phases in photosynthesis.

All the photosynthetic genes exhibited an increase after first harvest, which indicates the genes were more active as the cells were growing logarithmically. Photosynthetic genes also exhibited a significant pattern of increase in expression after 4^{th} harvest and showed a high peak at 5^{th} harvest (figs 14,15, & 17). A steep decrease in gene expression with the 6^{th} harvest represents that photosynthesis was slowing down late in the evening times (see chapter 3.1). An up regulation in gene expression with the 7^{th} harvest was unexpected. This suggests that photosynthesis was still active in the initial stationary phase and the genes were active during daytime (harvest 7 was performed in middle of the day, see chapter 3.1).

The internal control NADHase showed some fluctuation during the growth curve, which correlates to changes in *E. huxleyi* cell physiology during growth curve experiments. The qRT PCR reactions are very sensitive reactions and can detect very small changes in the cell, where as these small changes could not be detected in semi quantitative PCR reactions end product analysis and are not as sensitive as quantitative PCR reactions (see chapter 4.2).

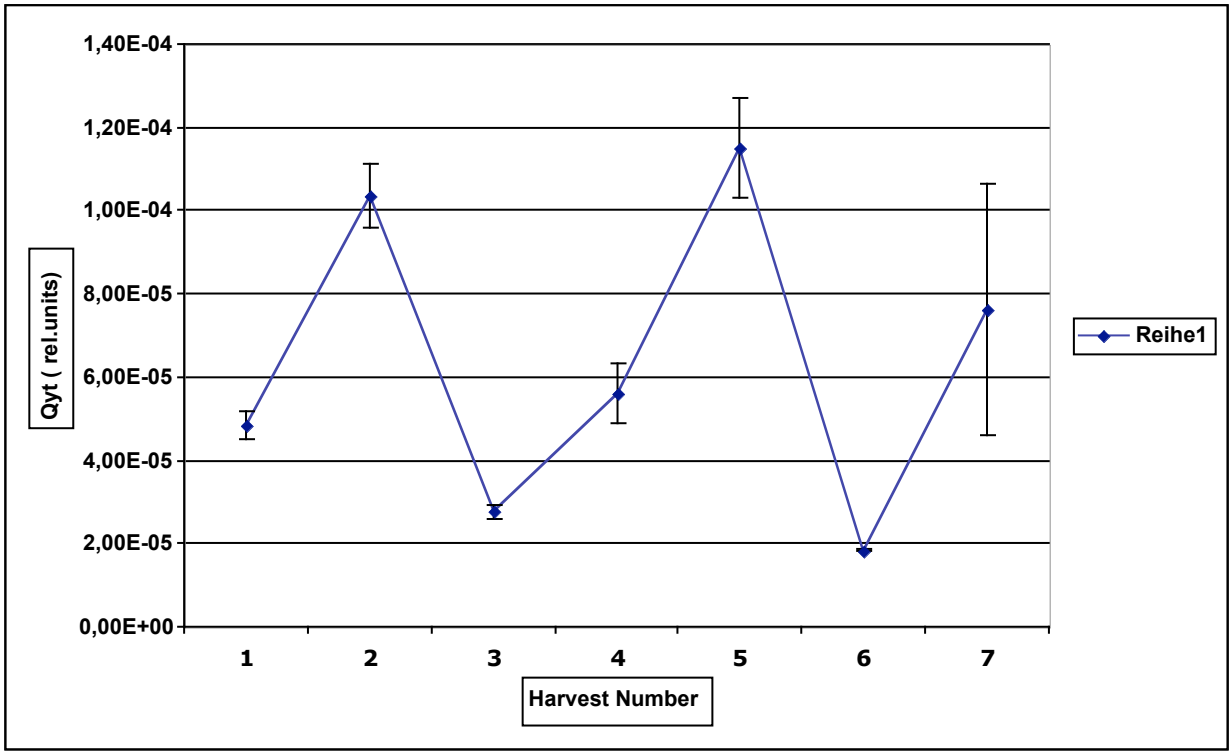


Fig.14 Ferredoxin gene expression throughout the *Emiliania huxleyi* growth curve. The value on y-axis is amount (ng/µl) of pcr product at the threshold; these values are relative to standards. The number on x-axis represents harvesting number.

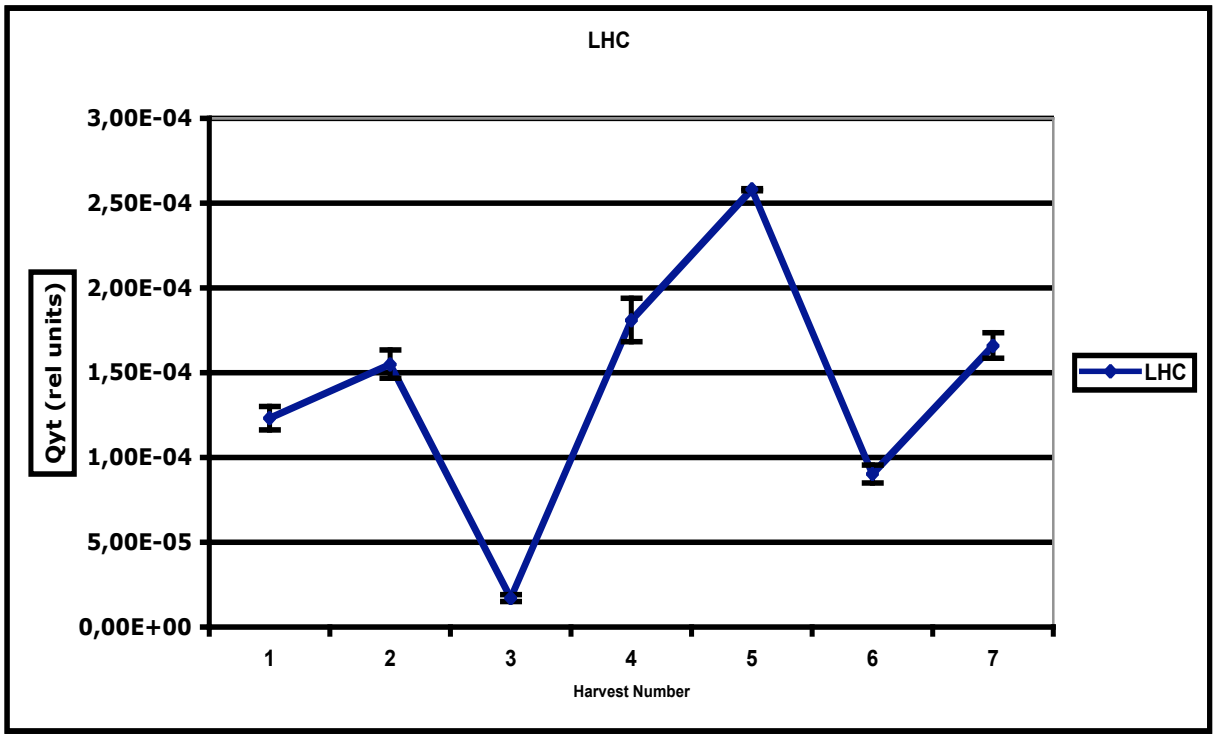


Fig.15 Light harvesting complex gene expression throughout the *Emiliania huxleyi growth* curve. The value on y-axis is the amount (ng/µl) of PCR product at the threshold.

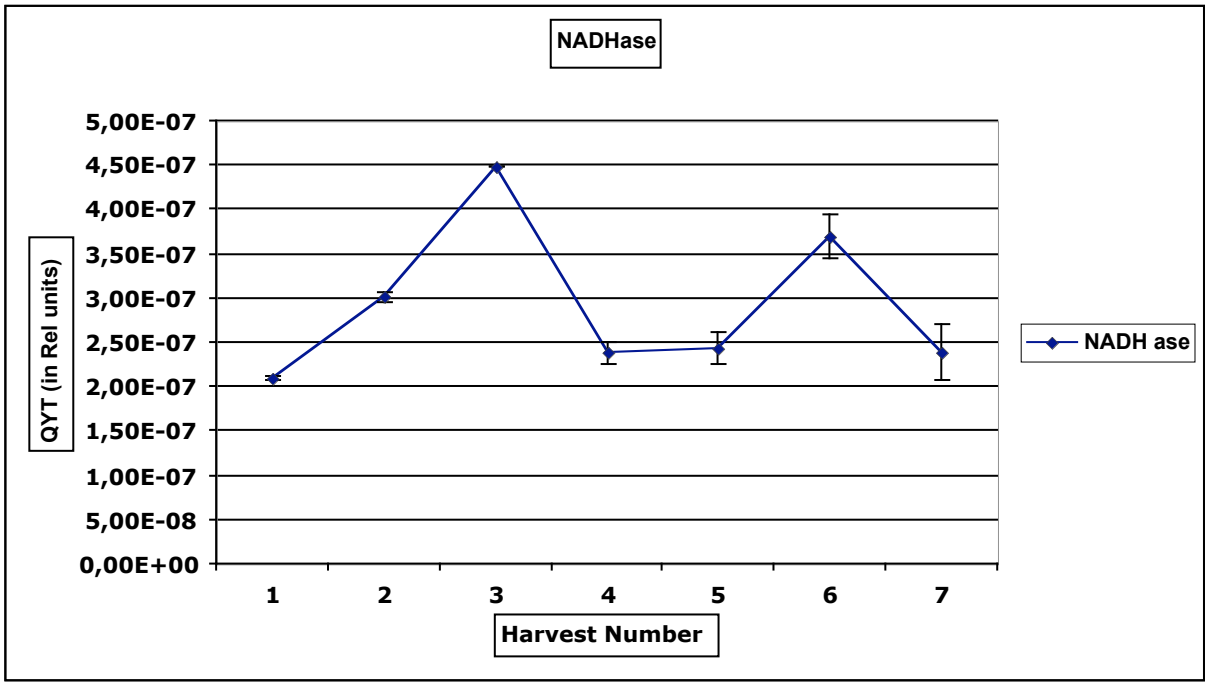


Fig.16 Nicotinamide adenine dinucleotide dehydrogenase gene expression through out the *Emiliania huxleyi* growth curve. The value on y-axis is amount (ng/µl) of pcr product present at the threshold; these values are relative to standard concentrations. The numbers on X-axis refers to the corresponding harvesting day.

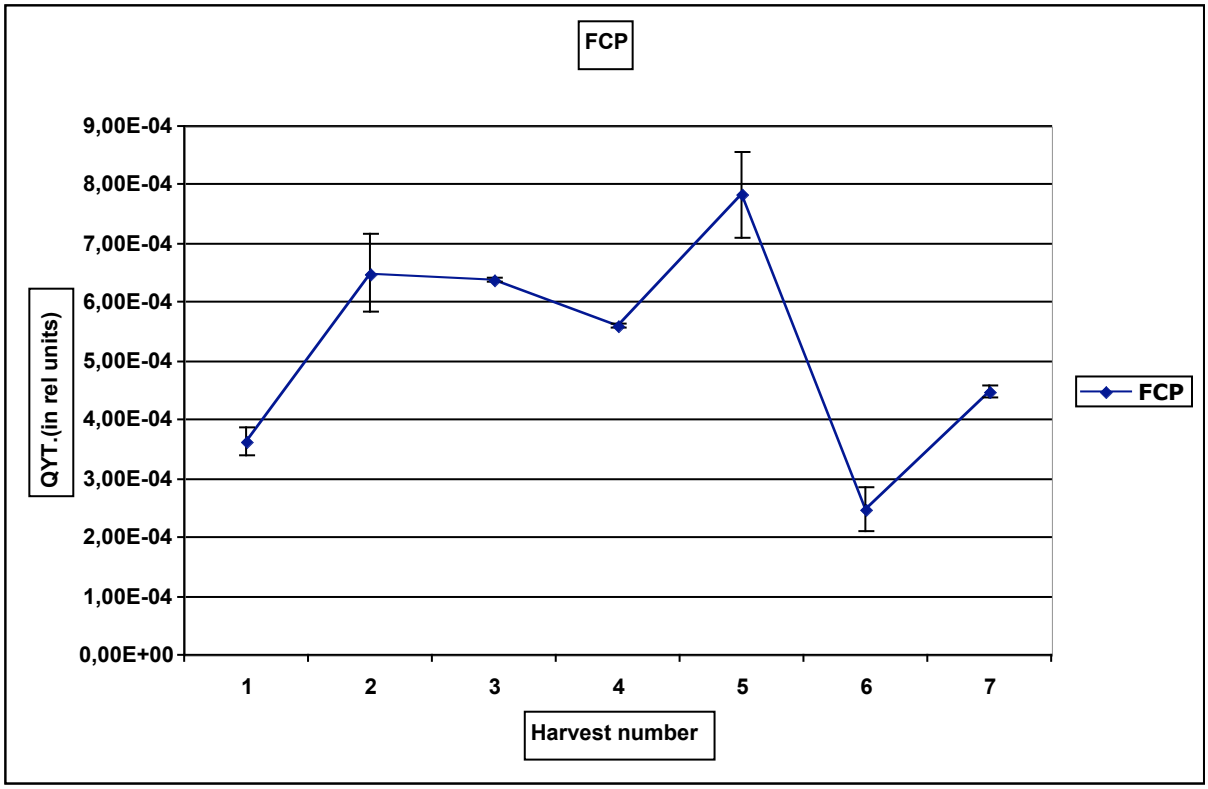


Fig.17: Fcp gene expression in *Emiliania huxleyi* through out the growth curve. The value on y-axis is amount of pcr product (ng/µl), these values are relative to standard concentrations and the numbers on X-axis refers to the corresponding harvesting day.

3.5 qRT-PCR reactions after virus infection Experiment IV

To obtain quantitative gene expression data after virus infection in *E. huxleyi,* three different photosynthetic genes i.e. light harvesting gene (LHC fig. 19), Fucoxanthin chlorophyll a/c binding protein (FCP_1 and FCP_3 figs. 22 & 23), Ferredoxin NAD^+oxidoreductase (fig 18) and two housekeeping genes i.e. Nicotinamide adenine dinucleotide dehydrogenase (NADHase fig 20), and two house keeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH fig 21) were selected.

All the photosynthetic genes showed a down regulation within 6 hrs, which correlates with night phase. An upregulation in GAPDH gene was also observed (fig 21)

LHC/FCP showed an up regulation after 12hrs of viral infection, which correlates with the light phase. This upregulation was observed only in the photosynthetic genes except FNR gene. GAPDH did not demonstrate any down regulation.

All the genes showed complete down regulation of genes after 24hrs of viral infection

In figs 18 to 23 the value on the y-axis represents the amount (ng/µl) of PCR product present at the threshold (See chapter 2.10.4). These amounts need to be normalised with the internal control NADHase (fig 24), for this the amount of the PCR product obtained from the software is converted into number of mRNA molecules (materials & method 2.18) and then plotted on the y-axis as a factor increase or decrease in expression in comparison to the calibrator (Jessica Kegel pooled RNA from uninfected cultures, Appendix III). The standard curves, RSQ values, PCR efficiencies and the number of amplicon molecules in the cell are calculated and attached in Appendix IV.

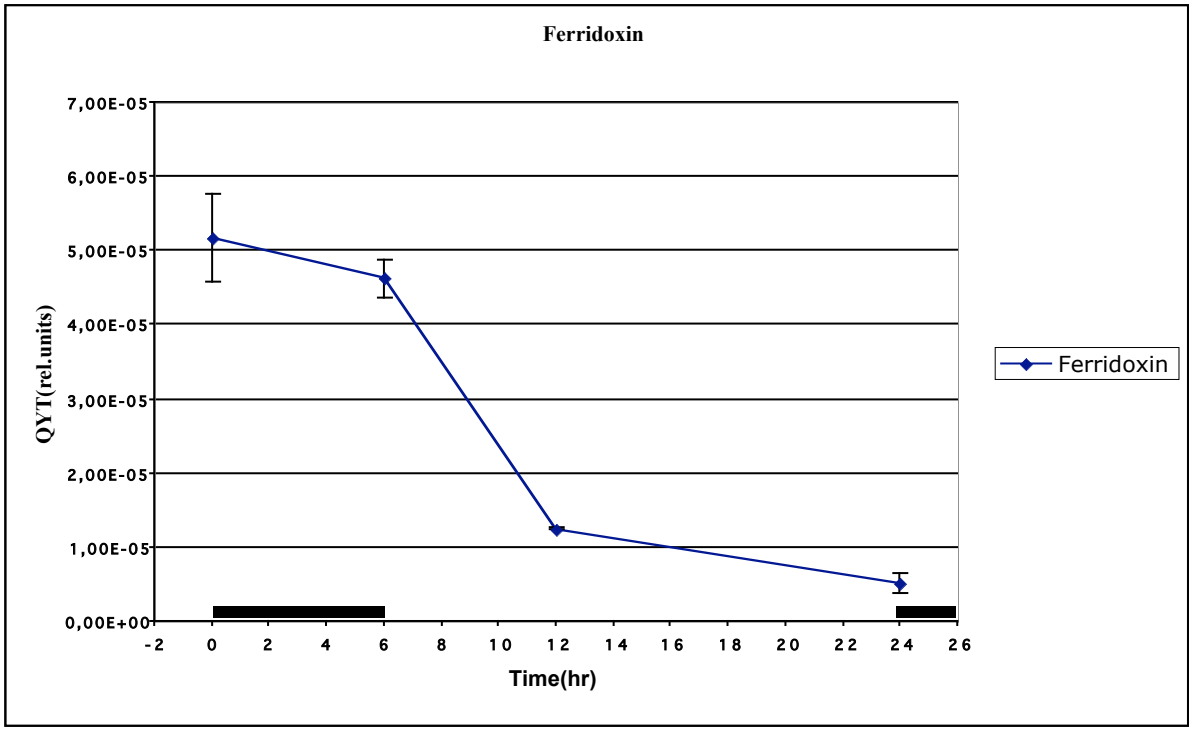


Fig: 18 Ferredoxin gene expressions after 6, 12 and 24 hrs of viral infection. The value on Y-.axis represents the amount (ng/µl) at the threshold level. The x-axis represents the time in hours. No Significant increase in ferridoxin expression was observed after 12 hrs of virus infection. The black bars represent the dark phase of 6hrs.

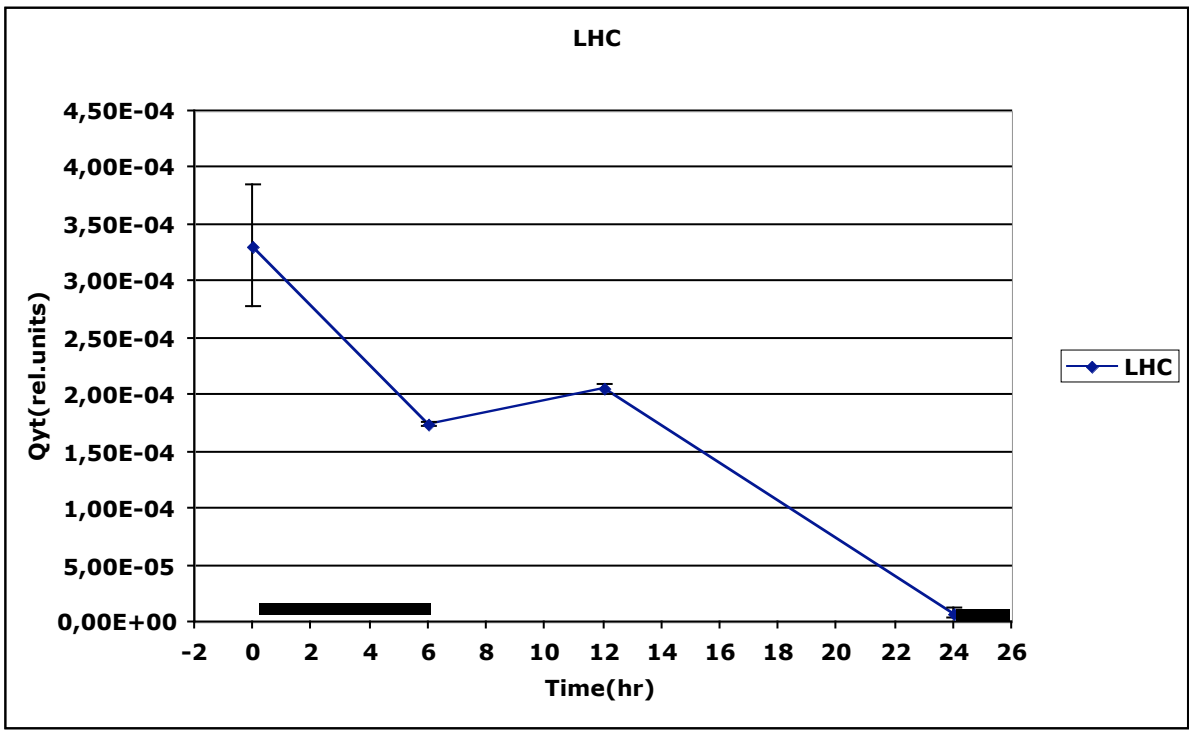


Fig: 19 Light harvesting complex gene expression after 6, 12 and 24 hrs of viral infection. The value on Y-.axis represents the amount (ng/µl) at the threshold level. The x-axis represents the time in hours. Significant increase in LHC gene expression after 12 hrs of virus infection was observed. The black bars represent the dark phase of 6hrs.

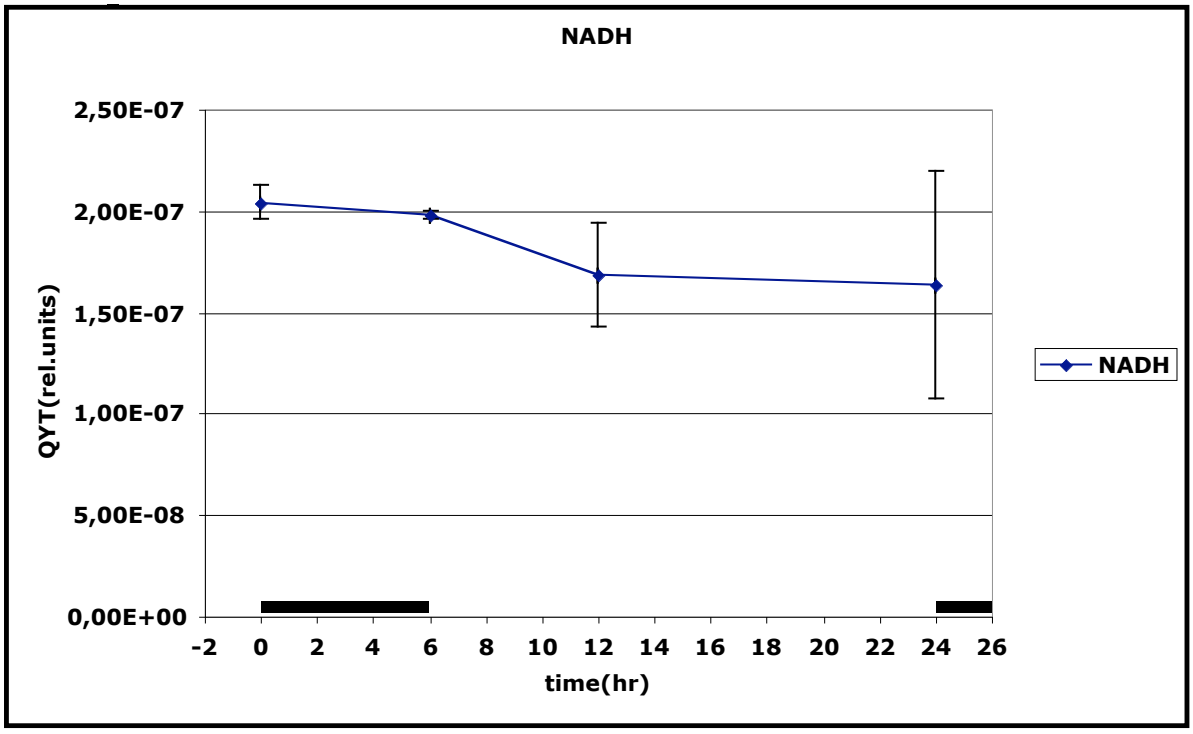


Fig: 20 NADHase gene expressions after 6, 12 and 24 hrs of viral infection. The value on Y-axis represents the amount (ng/µl) at the threshold level. The x-axis represents the time in hours. NADHase gene expression remains almost constant even after virus infection. The black bars represent the dark phase of 6hrs.

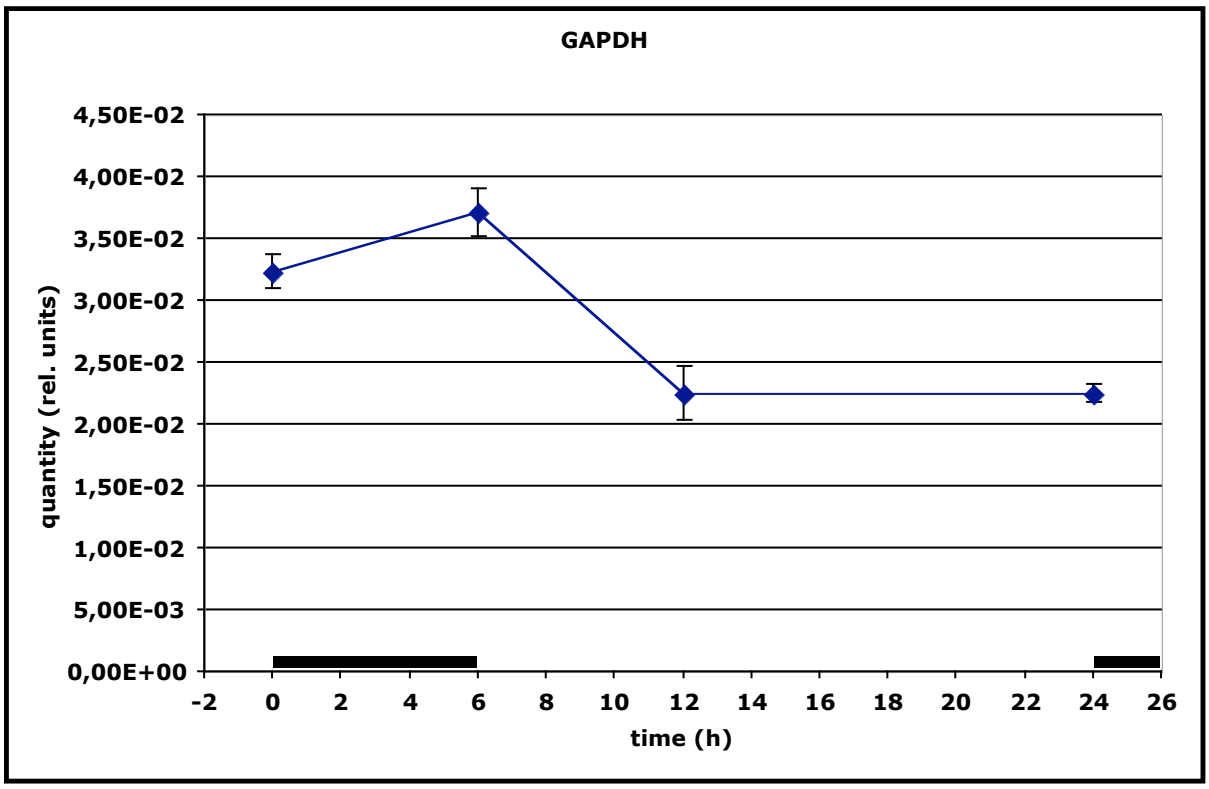


Fig: 21 GAPDH gene expressions after 6, 12 and 24 hrs of viral infection. The value on Y-axis represents the amount (ng/µl) at the threshold level. The x-axis represents the time in hours. GAPDH gene expression showed a slight increase in gene expression after 6hrs of viral infection. The black bars represent the dark phase of 6hrs.

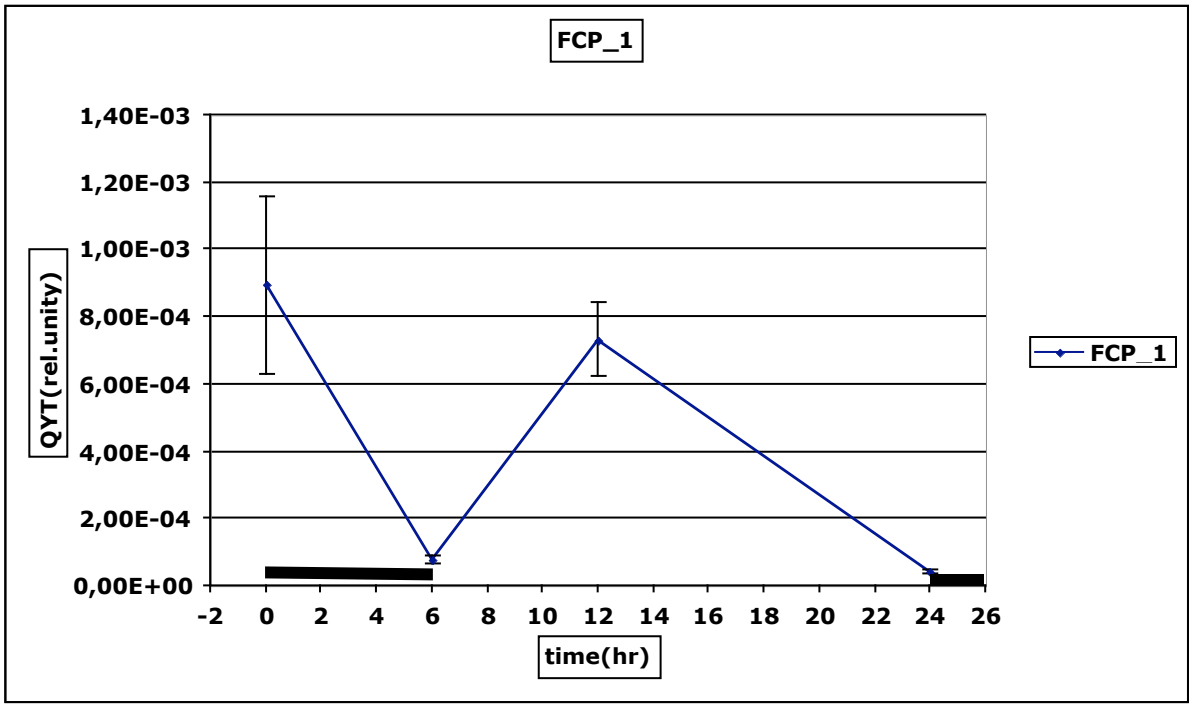


Fig: 22 FCP_1 gene expressions after 6, 12 and 24 hrs of viral infection. The value on Y-axis represents the amount (ng/µl) at the threshold level. The x-axis represents the time in hours. A significant increase in FCP_1 gene expression was observed after 12hr of virus infection. The black bars represent the dark phase of 6hrs.

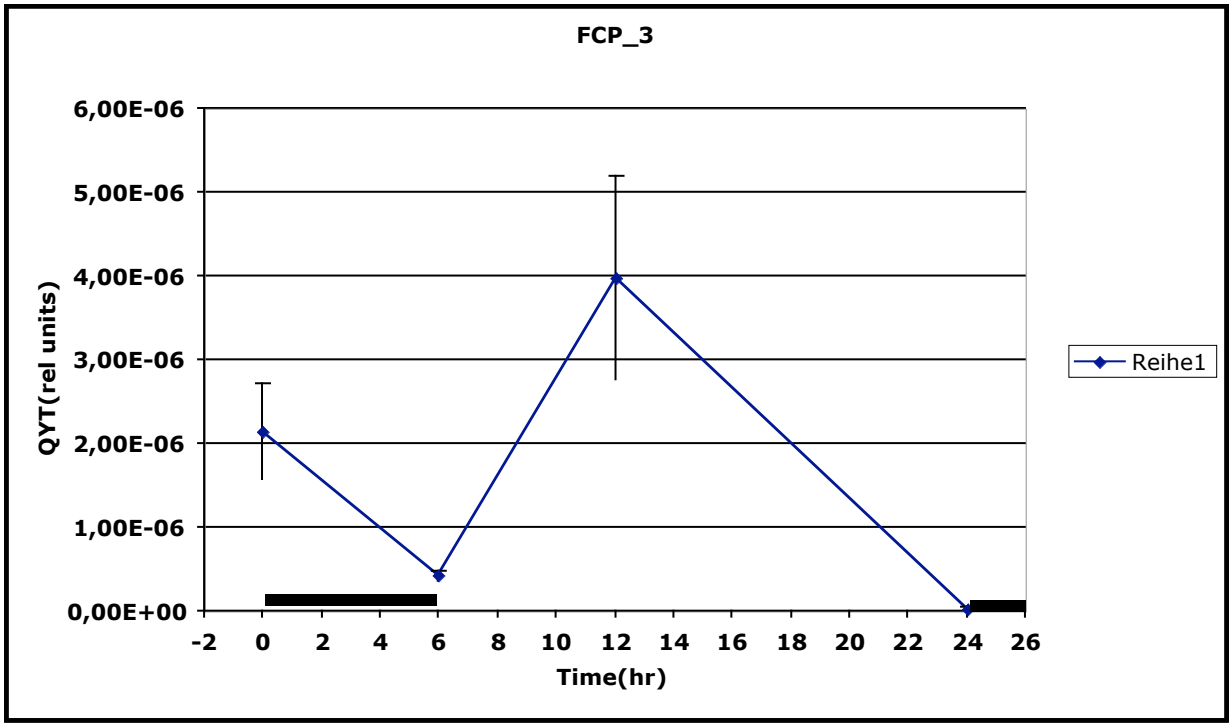


Fig: 23 FCP_3 gene expressions after 6, 12 and 24 hrs of viral infection. The value on Y-axis represents the amount (ng/µl) at the threshold level. The x-axis represents the time in hours. A significant increase in FCP_3 gene expression was observed after 12hr of virus infection. The black bars represent the dark phase of 6hrs.

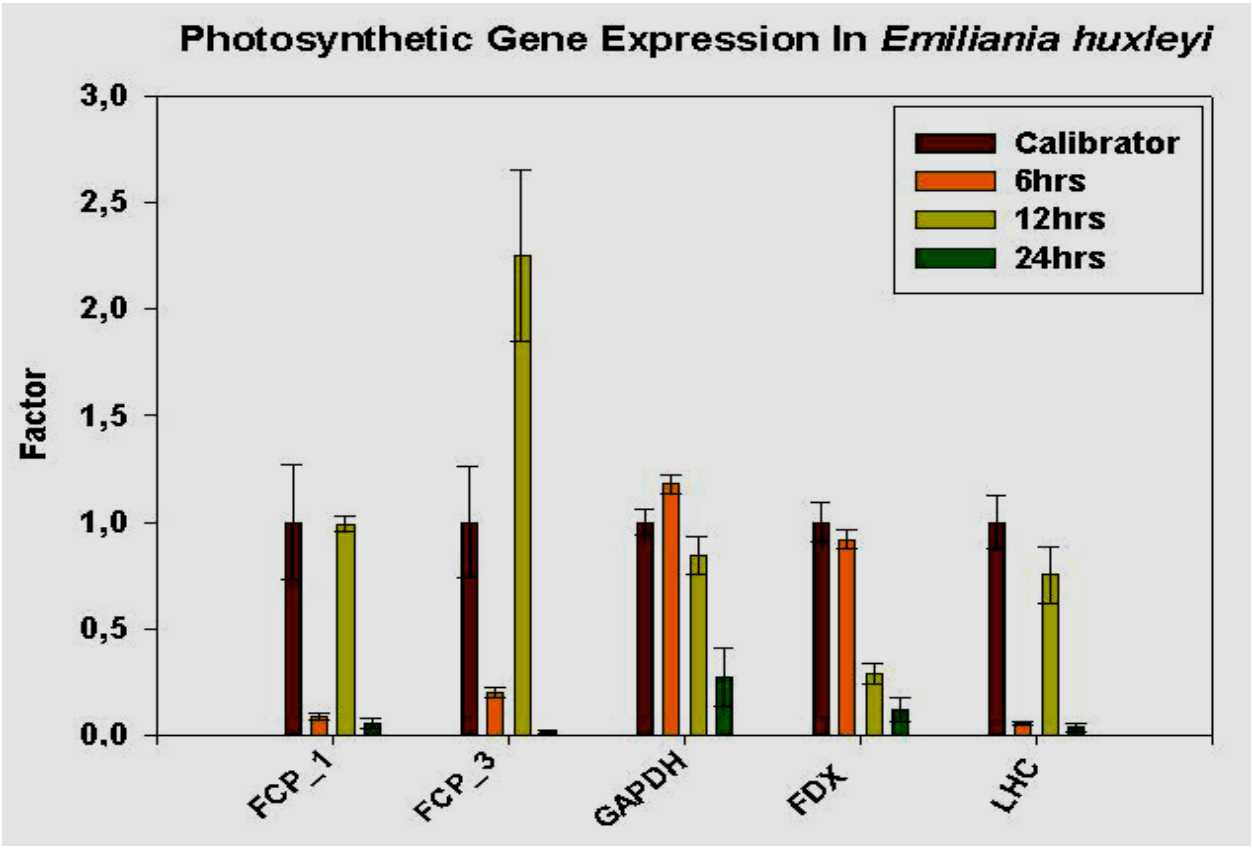


Fig: 24. The graph represents the photosynthetic gene expression after viral infection. The value on y-axis represents the factor by which the genes are up or down regulated when compared to the calibrator (pooled RNA from different phases throughout the growth curve culturing performed by Jessica, Appendix III).

4. Discussion

Photosynthesis transforms solar energy into energy rich compounds like carbohydrates and is the major energy source in the food chain. Organic molecules built by photosynthesis provide building block and energy for the cells. So it could be inferred that photosynthesis is one of the main process in the food web and plays a major role in marine ecology. *Emiliania huxleyi* is a marine phytoplankton that has the capability of forming massive blooms, which can cover huge areas in the ocean (Holligan et al. 1993, Winter et al. 1994). Its ability to produce inorganic coccoliths made up of calcium carbonate, its role in the global carbon cycle and its emission of DMS and active role in sediment formation makes it an important marine phytoplankton (Charlson et al. 1987, Westbroek et al. 1993, Malin et al. 1994). *E. huxleyi* plays a key role in global biogeochemical cycles (Westbroek et al. 1994).

Recently it has been accepted that viruses are important contributors to marine microbial food webs (Bratbak et al. 1993, 1995, 1996, Brussaard et al. 1996, Castberg et al. 2001, Jaquet et al. 2002, Wilson et al. 1998, 2002a, 2002b) that can control these algal blooms and have the capability of terminating the blooms. Recent work at AWI has provided evidence for a decrease in expression of photosynthetic genes in post viral cultures. In the present project a detailed study of expression of photosynthetic genes both in normal and in viral infected cultures has been conducted using semi quantative and real-time quantitative PCR.

4.1 Cell harvesting and RNA isolation

CCMP 1516 strain of *Emiliania huxleyi* cells were grown as pseudo batch cultures in f/2 medium at 15°C culture rooms. In order to understand the growth pattern of *Emiliania huxleyi* test growth attempts were accomplished. From fourth day onwards cultures exhibited doubling of cells logarithmically entering the exponential phase. After 9 days of exponentional phase, cultures entered into stationary phase (fig 9). To obtain DNA and RNA for expression analysis cells were harvested in seven different harvesting stages. These harvesting stages included initial exponential stage, middle exponential stage and initial stationary phase. A night harvest in initial exponential stage was performed to observe gene expression difference between day and night cultures.

To extract RNA from e. hux cells four different extraction protocols were performed (chapter 2.8). Only Qiagen plant RNeasy kit proved fruitful and provided RNA of undegraded quality (fig 10), where as all the other protocols gave good amounts of RNA but were either contaminated with polysaccharides or of degraded quality (fig 11). The Qiagen extraction protocol was followed by an RNA purification method; this additional step removes all the polysaccharides

and other cell contaminants that were eluted along with RNA after extraction procedure. The purification protocol also includes a DNAse digestion step to make sure that the entire DNA was degraded.

4.2 Semi-quantitative Gene expression analysis in *Emiliania huxleyi*

Changes in gene expression of photosynthetic genes were initially determined by semi-quantitative analysis. The semi quantitative results were end point quantitative analysis, where the expression data are collected after the amplification run has been completed. This method of quantification produced some-what inconsistent results (fig 25), but also could give us an idea about the pattern of expression in samples. In the end it and was helpful in continuing with quantification experiments with real time PCR analysis.

In the present project an attempt was made to study 12 different photosynthetic genes by semi-quantitative analysis. Only four genes were successfully sequenced (Appendix III). By semi-quantitative PCR we observed gene expression variation depending on the status of the culture and phase of the day. The semi-quantitative experiments gave us initial expression data to use the NADHase as an internal control in real-time experiments. In the present project I successfully amplified and sequenced an unknown gene, which showed similar gene expression regulation pattern as other photosynthetic genes.

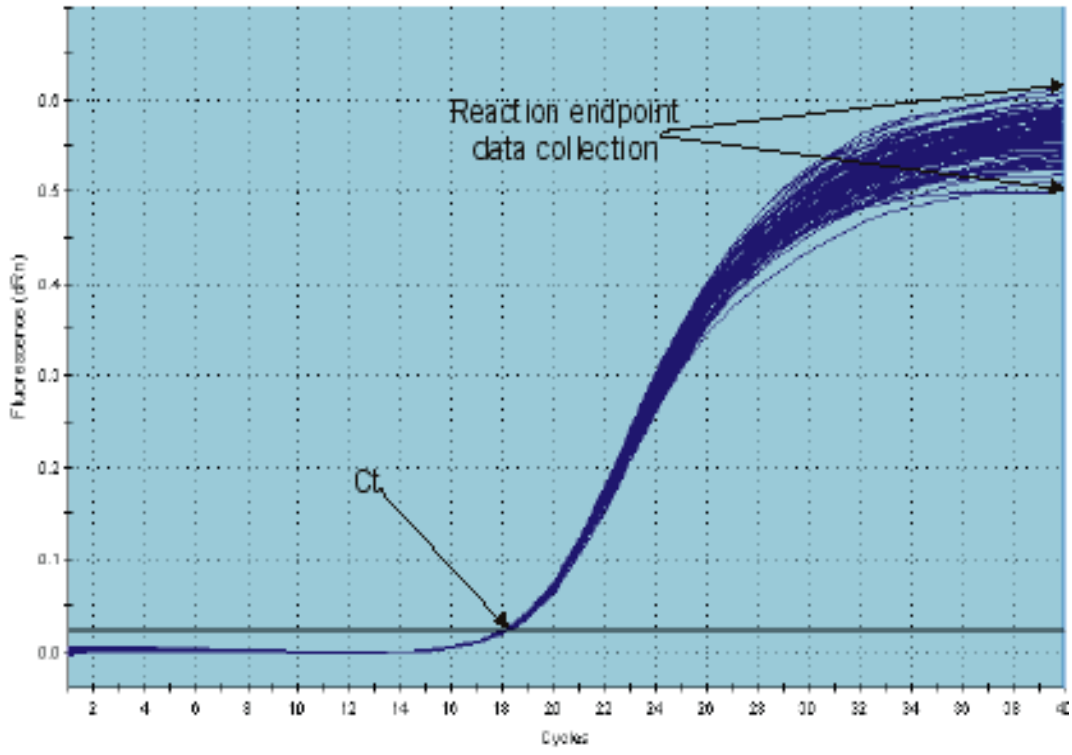


Fig 25 Difference in end point semi quantitative analysis and real -time quantitative analysis (source: qPCR manual Stratagene)

4.3 Gene expression in *Emiliania huxleyi* growth curve using qRT-PCR

By QRT-PCR reactions we could see photosynthetic gene expression in *E. huxleyi* in three different cell stages (see chapter 3.4). All photosynthetic genes demonstrated a decrease in photosynthesis in night, which is a direct indication that photosynthesis was not active during night. Additionally from these data (figs 14,15 and 17) it was understood that the genes were highly active only during the exponential phase as compared to other two phases.

4.4 Gene expression after virus infection

Virus infected cultures (CCMP1516) were grown at Plymouth Marine Laboratory (PML) UK. These cultures were then infected with virus *Ehv*86. RNA was extracted after 6hrs, 12hrs and 24hrs. The cultures were grown in 18 hrs of light cycle and 6hrs of dark cycle. The 6^{th} hour cell harvesting was performed just after the night phase, 12 hrs was in the middle of the day and 24hrs was also in day but just before the night phase (figs 18-23). So the gene regulation observed in the current gene expression experiments was also partly due to light and dark phases in photosynthesis.

All the photosynthetic genes demonstrated down regulation of gene expression after 6hrs (fig 18-23) of virus infection except GAPDH, which was not a surprise as GAPDH was involved in glycolysis a dark reaction. This indicates that the virus was active with-in hours of infection and was preparing the host cell to produce its own proteins like capsid and other metabolic enzymes required for viral DNA assemblage and viral release.

The increase in photosynthetic genes after 12 hours (fig 18-23) of virus infection correlates to dark and light phases in photosynthesis. This increase was mostly observed in LHC and FCP genes that are light harvesting proteins embedded in the cell membrane and were actively involved in harvesting light during the daytime. Hence this upregulation correlates with the day phase of photosynthesis. GAPDH did not show any down regulation suggesting that other energy producing reactions like glycolysis were still functioning normally.

After 24 hrs (fig 18-23) of virus infection the photosynthetic genes were completely shut down. A decrease in GAPDH gene expression indicates complete take over of the host cell by the virus. GAPDH is an house keeping gene which is involved in various different kinds of cellular processes.

In these experiments two different internal controls i.e. NADHase and GAPDH were considered. Only NADHase turned out to be a good reference gene to be used as internal control and did not

show any expression within the day of viral infection, where as GAPDH (fig 18 & 21) showed significant down regulation after viral infection (fig 24).

Previously with analytical flow cytometry (Wilson et al. 2002 a, b) it was shown that viral infection caused *E. huxleyi* cell death, which resulted in shedding of coccoliths and release of the lytic virus into the water due to which the virus and coccolith population increased in the bloom concentrated areas in the ocean. In the same paper they also mentioned about two viruses *Ehv84* and *Ehv86* their size and shape with electron microscopy, which were isolated from the high reflectance area that lysed *E.huxleyi* cultures CCMP 1516.
This viral demise of *E. huxleyi* bloom was also confirmed with previous EST clone library experiments (Jessica Kegel & Klaus valentine unpublished data) and current qRT-PC reactions where it is seen that most of the host cell transcription was halted after 24 hrs of viral infection.

5 Summary

- The present master thesis covers the investigation of photosynthetic gene expression in *Emiliania huxleyi* (CCMP1516) before and after viral (*Ehv 86*) infection at the molecular level. The project was started with amplification of genes using DNA as the template to obtain standards for quantitative analysis. However this attempt was unsuccessful and genes were amplified using cDNA as template. Later on all the genes were successfully sequenced and PCR products were used for building standard curves in quantitative analysis
- To conduct molecular experiments on *E. huxleyi* four different RNA extraction protocols were followed. Only the Quiagen RNeasy protocol was fruitful and provided RNA of undegraded quality.
- Semi-quantative data provided initial photosynthetic gene expression data before and after virus infection. A new gene of unknown function was amplified and sequenced. This unknown gene showed similar expression pattern as photosynthetic genes.
- In the present masters work we established NADHase as a good internal control in unicellular eukaryotic organisms to be used for qRT PCR reactions.
- Gene expression over the growth curve showed a significant pattern of regulation throughout the day. All the photosynthetic genes were down regulated at night, which indicates that photosynthesis was active only during the daytime. In these experiments it was also observed that photosynthetic gene expression was more active in the exponential phase than in lag and stationary phases.
- Using qRT PCR reactions we have shown that virus was involved in the *E.huxleyi* bloom termination. The data presented in the present master thesis work demonstrates that after 6hrs of virus infection the photosynthetic genes were down regulated and were completely shut down after 24hrs of virus infection. This phenomenon was observed only in photosynthetic genes. The housekeeping genes like GAPDH showed a significant down regulation only after 24hrs, which correlates to complete take over of the cell by the virus.
- Hence we hypothesise that bloom termination in *E.huxleyi* (CCMP1516) was due to viral infection (*Ehv86*) and occurred mainly due to a viral activity in order to produce its own progeny and other lytic enzymes required for viral release. In this process most of the host cellular process were ceased within a day.
- To understand the mechanism in much more detail more genes should be considered for gene expression with micro array analysis.
- The data shown in the present masters work correlates with the Est. library analysis and the proposed viral mechanism of bloom termination.

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Appendix I

Primers and probes for PCR

1.1 Semi quantitative PCR programme

Initial Denaturation: 94°C 5min

PCR Reaction

1. Denaturation: 94°C 1 min
2. Annealing: 50°C 1 min
3. Elongation: 72°C 1 min

Repeat step 1 to 3 for 40 cycles

Final PCR reaction at 72°C for 5min

Stop reaction at 4°C

1.2 PCR reagent concentration (50µl reaction volume)

Reagents	Amount
10x PCR Buffer	5µl
dNTPs	1µl
Water	38.8µl
Primer (forward + reverse)	2µl
Enzyme	0.2µl
Template	3µl
Total volume	50µl

1.3 cDNA synthesis for semi quantitative PCR

Two Master mixes, Mix 1 and Mix 2, were prepared for RT reaction. If several RT reactions were carried out simultaneously, a master mix of Mix 2 was prepared to minimize volume errors.

Master Mix 1 (RNA primer Mix)

Reagents		Amount
Up to 5µg total RNA		nµl
oligo dT		1µl
10mM dNTP Mix		1µl
DEPC-treated H_2O	upto	10µl

1.Incubate at 65°C for 5min,then place on ice for at least 1min

2.Prepare the following cDNA synthesis as provided below.

Master Mix 2 (cDNA Synthesis Mix)

Component	Amount
10X PCR buffer	2µl
25mM $MgCl_2$	4µl
0.1M DTT	2µl
RNAse OUT^{TM,} 40U/µl)	1µl
Superscript TM III^{RT} (200U/µl)	1µl
Total Volume	10µl

3 Add 10µl of cDNA synthesis mix to the RNA primer mix.

4 Incubate the whole reaction mix in the following pattern.

5 Oligo $(dT)_{20}$ or GSP 50min at 50°C

6 Random hexamerprimed: 10min at 25°C,
Followed by 50min at 50 °C

7 Terminate the reaction at 85°C for 5min.Chill on ice.

8 Add 1µl of RNAse-H to each tube and incubate for 20min at 37°C.

1.4 Real-Time quantitative PCR primers and probes.

Oligonucleotide sequence	**Primer name**
5' GTGAGCGCCTGTGGTCTGT 3'	LHC_1 Forward
5' GAGCGCCAAAACTCCACACT 3	LHC_1 Reverse
5' AGGCGGTCCCCACCTT 3'	FCP_1 Forward
5' TCTCCTGGAAGAGGCAGAAGA 3'	FCP_1 Reverse
5' AGGCGGTCCCCACCTT 3	FCP_2 Forward
5'CG CCG GTC GGG TAG AGC 3	FCP_2 reverse
5' ACGAGTTCACGTTTGACTCGAA 3'	FCP_3 Forward
5' CCGGCCTTGTCCTTGATCT 3'	FCP_3 Reverse
5' CAAGTCTGCCAACAGCAGCTT 3'	NADHase Forward
5' TTGGCCTGGATGCCTTGT 3'	NADHase Reverse
5' GACGACTACGACTCCAAGTCGAT 3’	GADPHase Forward
5' CTTGATGCCGAACTTCTCGTT 3'	GADPHase Reverse
5' GACGGCGGCAAGATGTACA 3’	Ferredoxin Forward
5' TTGAGGCCGCAAAAGTACATG 3'	Ferredoxin Reverse

Table 1 Primer name and sequence for quantitative analysis.

2.2 cDNA synthesis for quantitative polymerase chain reaction.

Reagents	Amount
10x buffer	2µl
dNTPs (5mM each dNTP)	2µl
oligo dT primer (10uM)	2µl
RNase Inhibitor (10units/µl)	1µl
Omniscript reverse Transcriptase	1µl
RNAse-free water	xµl
Template RNA	yµl (441ng/µl)
Total Volume	20µl

2.3 Quantitative PCR Programme

Before going to real time PCR reaction first a test PCR was run. Then only successful primers were used for the corresponding real time PCR reaction.

2.4 Test Quantitative PCR programme

Initial Denaturation: 94°C 2min

PCR Reaction

1 Denaturation: 94°C 20sec

2. Annealing: 60°C 1 min

Repeat step 1 to 2 for 40 cycles

Final PCR reaction at 70°C for 3min

Stop reaction at 4°C

2.5 Test quantitative PCR reagents (50µl reaction volume)

Reagents	Amount
10x PCR buffer	5µl
dNTPs	1µl
Water	38.8µl
Primer (forward + reverse)	2µl
Enzyme	0.2µl
Template	3µl
Total volume	50µl

2.6 Quantitative Real Time PCR reagents

Reagents	Amount
Water	7.5 µl
Primer_forward (10pmol)	0.25 µl
Primer _rev (10pmol)	0.25 µl
SYBR green mix	10µl
Template (1:5 diluted)	2µl
Total volume	20µl

2.7 Quantitative real-time PCR programme

1) Initial denaturation 95°C 10 min
2) Denaturation 95°C 15 sec
3) Annealing 60°C 1min

Repeat 2-3 for 40 cycles

Dissociation Stage for SYBG green dye technology

1) 95°C 15sec
2) 60°C 20 sec
3) 95°C 15sec

2.8 Sequencing reaction mixture

Reagents	Amount
5 x Sequencing Buffer	1.5µl
Big dye pre mix (RR100)	1.0µl
Primer (forward or reverse; 1pmol/µl)	1.0µl
Water + PCR purified product (Template)	6.5µl (Adjust Accordingly)
Total volume	10µl

2.9 Sequencing Reaction PCR programme

Initial denaturation	96°C for 1min
Denaturation	96°C for 10 sec.
Annealing	50°C for 5 sec
Extension	60°C for 4 min

Stop the reaction at 4°C. Programme Duration 90 minutes

Appendix II

2.1 Culture medium

Emiliania huxleyi was cultured in f/2 medium according to Guillard & Ryther1962.Antarctic water was stored in dark at 10°C and sterile filtered before preparation of the medium.

A) Nutrient salt stock solutions (solutions were prepared with aqua bidest.)

1) $NaNO_3$ 75.00 g/l
2) $Na_2HPO_4 \times 2\ H_2O$ 6.25 g/l
3) $Na_2SiO_3 \times 9\ H_2O$ 15.00 g/l
4) Metal mixture stock solutions

a) $ZnSO_4 \times H_2O$ 0.15 g
$CuSO_4 \times 5\ H_2O$ 0.10 g
$Co\ SO_4 \times 7\ H_2O$ 0.12 g
$MnSO_4 \times H_2O$ 2.00 g

Dissolve in 100 ml of aqua bidest.

b) $FeCl_3 \times 6\ H_2O$ 5 g / 100 ml aqua bidest.

c) $Na_2MoO_4 \times 2\ H_2O$ 0.065g / 100 ml aqua bidest.

d) Na_2EDTA (Titriplex III) 5 g / 100 ml aqua bidest.

Composition of metal mixture:

10 ml of stock solution a)

10 ml of stock solution b)

10 ml of stock solution c)

100 ml of stock solution d)

Fill up to 1000 ml with aqua bidest.

Stock solutions 1 to 4 were autoclaved.

5) Vitamin mixture

Stock solutions:

a) Biotin 1 mg / 10 ml aqua bidest.
b) Vitamin B12 1 mg / 10 ml aqua bidest.

Composition of vitamin mixture:

1 ml of stock solution a)

1 ml of stock solution b)

20 mg Thiamine-HCl

The vitamin mixture was filtered through an acetate filter with 0,2 µm pore size and stored at -20°C.

Composition of f/2-medium

Following reagents were added to 1L Antarctic seawater

- 1 ml of $NaNO_3$
- 1 ml of Na_2HPO_4
- 1 ml of Na_2SiO_3
- 1 ml of Metal mixture
- 1 ml of Vitamin mixture

After vigorous shaking pH was adjusted to 7.5 – 7.8 with 2N HCl

2.2 50x TAE (Electrophoresis buffer)

242 g Tris-Base

57,1 ml Acetic acid

100 ml 0,5 mM EDTA, pH 8,0

Fill with1000 ml H_2O

2.3 CTAB extraction buffer

1) Tris–EDTA (100mM Tris, 50mM EDTA, pH 7.5)
2) 2M NaCl
3) 2% CTAB

Store the buffer in aliquots at –20°C.

Dithioreitol

Stock-solution of 2 M was dissolved in DEPC treated water.

Immediately prior to use add DTT with a final concentration of 50mM.

LiCl

12M +1% (v/v) beta -mercatoethanol

2.4 Culture Counting

Days	Culture 1	Culture 2	Harvesting Days
0	5315	4971	Inoculation
1	12937	12463	
2	98171	96845	
3	122600	125200	Harvest 1

4	322300	349900	Harvest 2
5	718400	890900	Harvest 4
6	1620000	1776000	Harvest 5
7	1892000	2867000	Harvest 6
8	3480000	4077000	
9	3815000	4077000	
10	4575000	4550000	
11	4888000	4740000	
12	4328000	4574000	
13	4612000	4848000	Harvest 7

Table 2: Cell concentrations [ml^{-1}] and corresponding harvesting day. Culture 1 represents test culturing and culture 2 represent the cell concentrations while cells were harvested for RNA extraction. Harvest 3 was performed on 4th day in the night and cell concentration was 776400

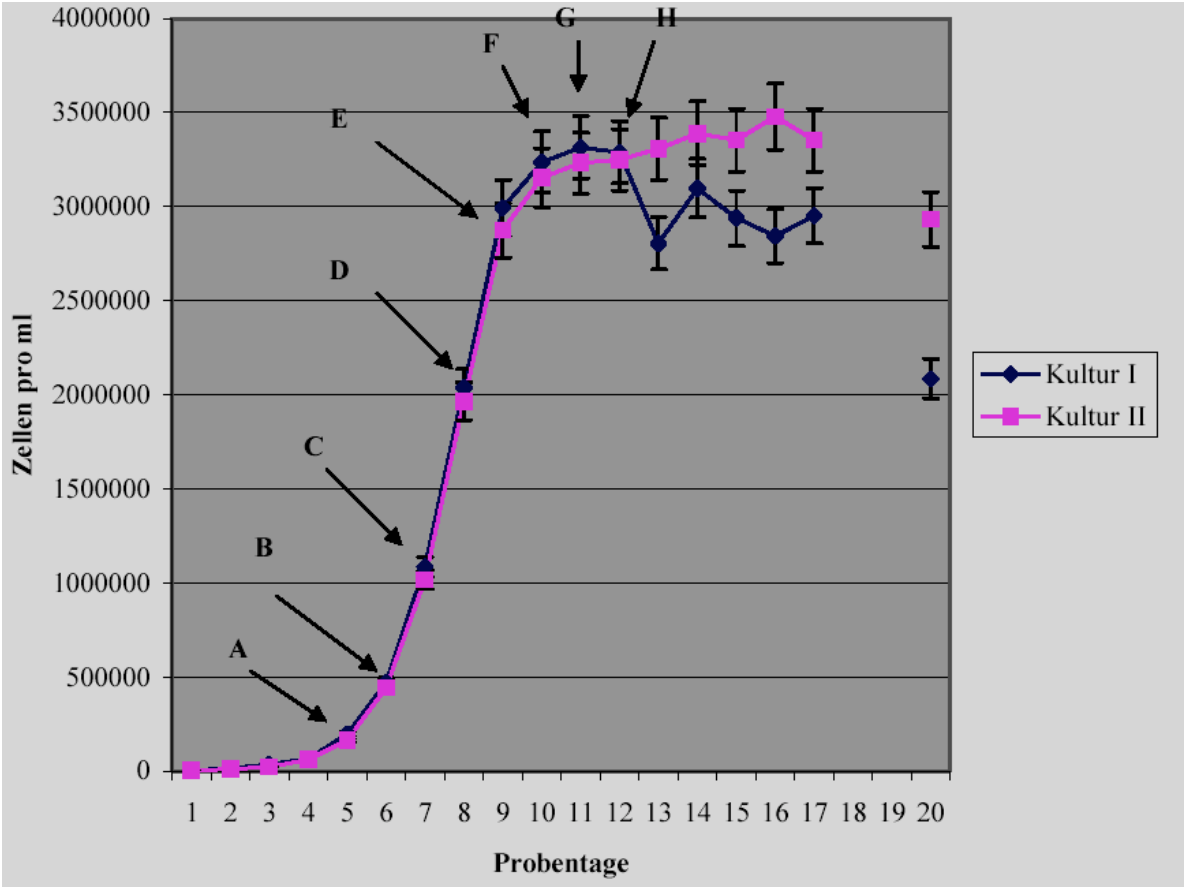

Fig: 25 *Emiliania huxleyi* cell culturing by Jessica. The cells were harvested in the middle of the day, from the beginning of initial exponential phase till initial stationary phase. The pooled RNA was then stored at –80°C.

Appendix III

3.1.1 Ferredoxin NADP$^+$ Oxidoreductase (529 bp's)

Forward primer Sequencing (5' CGC CGT ACC GCT CCT TCA 3')

5'CCTTCGGCGAGGCGTACACCAAGGGGAGCGGGCTCGCCTGGCTCTTCCTCGGCGT
CGCCAACAGCGACGCGCTGCTGTACGACGACGAGTGGCAGTCCGTCCTCAAGGCGC
ACCCCAAGAACTTCCGGCTGGACTACGCCCTCTCGCGCGAGCAGAAGAACAAGGAC
GGCGGCAAGATGTACATCCAGGACAAGGTCGCGGAGTACTCGGACGAGATCTTCAC
GCGGATGGAAACGGCGCGCACATGTACTTTTGCGGCCTCAAGGGCATGATGCCCGG
CATCACCGAGATGCTCGAGGGCGTGTGCAAGGAGAAGGGGCTCAAGTGGGAGGAC
AAGCTGACGCAGTGGAAGAAGGCGGGGCAGTGGCACGTCGAGGTCTACTAGGCGG
ACGAGGCGACCCCCCTCACCCCCTGGACAGACCGCCGACCCCCGTACACTCTCCGCC
CCCTCTCCGCCGCCTTTTCTCCGCCCCCCCATCTCCGACGGGAGAGCGTCTCGCATTT
GTGCGGCAGTTTATCTCCGGGTCT 3'

3.1.2 Ferredoxin NADP$^+$ Oxidoreductase (464 bp's)

Reverse Primer Sequencing (5' GC AGA GAC CCG GAG ATA AAC 3')

5'ATGGGGGGGCGGAGAAAAGGCGGCGGAGAGGGGGCGGAGAGTGTACGGGGGTC
GGCGGTCTGTCCAGGGGGTGAGGGGGGTCGCCTCGTCCGCCTAGTAGACCTCGACG
TGCCACTGCCCCGCCTTCTTCCACTGCGTCAGCTTGTCCTCCCACTTGAGCCCCTTCT
CCTTGCACACGCCCTCGAGCATCTCGGTGATGCCGGGCATCATGCCCTTGAGGCCGC
AAAAGTACATGTGCGCGCCGTTGTCCATCCGCGTGAAGATCTCGTCCGAGTACTCCG
CGACCTTGTCCTGGATGTACATCTTGCCGCCGTCCTTGTTCTTCTGCTCGCGCGAGAG
GGCGTAGTCCAGCCGGAAGTTCTTGGGGTGCGCCTTGAGGACGGACTGCCACTCGT
CGTCGTACAGCAGCGCGTCGCTGTTGGCGACGCCGAGGAAGAGTCAGGCGAGCCCG
CTCCCCTTGGTGT 3'

3.2.1 NADHase (153 bp's)

Forward primer Sequencing (5' CGA AGG GCA TCA GGG AC 3')

5'TCAGTCTGCCACAGCAGCTTGCCCATCCTGCTCCGCGAGTCACAAGGCATCCAGG
CCAAGCTGACGGCGGCATACCAGGGAGGAAAGGAGACCGTCGTCGTCGTCGACGG
GCTCAGCGAGGCGGAGTTTGGGGCCAAACTCGAATCCCTCAGA 3'

3.2.2 NADHase (124 bp's)

Reverse primer sequencing (5' GAG GGA TTC GAG TTT GGC 3')

5'ACGACGACGGTCTCCTTTCCTCCCTGGTATGCCGCCGTCAGCTTGGCCTGGATGC
CTTGTGACTCGCGGAGCAGGATGGGCAAGCTGCTGTTGGCAGACTTGATCGCGCTG
TACTGCTTCTTCC 3'

3.3.1Unknown Gene (189 bps)

Forward primer sequencing (5' CCT CGC TCC CGC CTT CAA 3')

5'GAGGTGGCGCCCGACGAGGGGTCGGGCTGCGTGTGCCCGCTCGACGTCAAGGCCT
TCACGCACAAGACCGCTGCCGCCGTCGCCCTCGTCAAGATGATGGTCGATGCCGAG
GGAGAGCTGCCGCCGGCTGCCGAGCCGCGCATCGTCCGTGTCGAGAAGAAGTTCTA
CTACGACGACATCTGAGTGTGC 3'

3.3.2 Unknown Gene (168 bps)

Reverse primer sequencing (5' GTC ACG GCT CAC GCA CAC 3')

5'ATGACGAGGGCGACGGCGGCAGCGGTCTGCTGCGTGAAGGCCTTGACGTCGAGCG
GGCACACGCAGCCCGGCCGCTCGTCGGGCGCCACCTCCGGGAAGGGGTCAAAGTCG
GTCTTGTCAAAGGCTTGCTGGCAGACGTCCGAGACCGCGTTGAAGGCGGGAGCGAG
G 3'

3.4.1 Conservative hypothetical protein

Reverse primer sequencing (5' GGA TGC GTG GTG GGA GTG 3')

5'TTAGGAGGCCCTGCGGCCCGAGCAGGATCTCTGAGAAGAAGGAGATCACAGCCA
CCCACACGATCGGGGTGATGTCGACGCCGGCGACGGGCGGGATGAGGCGGCGCGTG
GCTCGCAGCAGCGGCTCCGTCGGCCAGGCGGCGACGTTGTACGGGAAGGCGTTGAG
GTTGTACTTTGGGAACCAGGAGAGGACGGTGCGGAAGAGGAAGAGGAGCGTGTAC
ACGGCGAAGAGGGGCGACAGCACTGCCTTTGTCGGACCCACCCACGCCACGTCTGC
CGCACCCGCCGGCAGCGCCACCATCATAGCTGTCGCCG 3'

3.5.1 FCP-2 (374 bp's)

Forward primer sequencing (5' CAC TGT CGC CCT CGC TGT C 3')

5'GGCGTCGTCCTCCCGGGCGCGCTCACCCTGGCGGGCACCAAGTTCTCCGACATCC
CGACCAGCTGCTTCGGCTCGCTCGAGGCGGTCCCCACCTTCGGCTGGCTGCAGATC
ATGCTCTTCTGCCTCTTCCAGGAGACGGGCTACGGCCTCGGCCTGGCCAAGGGCCA
GACCGGCGGCTACGCCTCCAACCCGGACAACAACGAGGACGACGGCGAGGTGGGC

GACATCGCGGGCATCCCGTGGGTGCGGTACGACGACCCCGAGACCAAGACCTTCA
AGCTGAACGCCGAGCGCCAGAACGGCCGCGCCGCGATGCTCGGCATCACCGGCTG
CCTCGTGCACGAGATCCTTGGCGTCGACGCGCTCTACCCGAC 3’

3.5.2 FCP-2 (403 bp’s)

Reverse primer sequencing (5' CG CCG GTC GGG TAG AGC 3')

5’TGCCGAGCATCGCGGCGCGGCCGTTCTGGCGCTCGGCGTTCAGCTTGAAGGTCTT
GGTCTCGGGGTCGTCGTACCGCACCCACGGGATGCCCGCGATGTCGCCCACCTCGC
CGTCGTCCTCGTTGTTGTCCGGGTTGGAGGCGTAGCCGCCGGTCTGGCCCTTGGCC
AGGCCGAGGCCGTAGCCCGTCTCCTGGAAGAGGCAGAAGAGCATGATCTGCAGCC
AGCCGAAGGTGGGGACCGCCTCGAGCGAGCCGAAGCAGCTGGTCGGGATGTCGGA
GAACTTGGTGCCCGACAGGGTGAGCGCGCCCGGGAGGACGACGCCCGCCTTGAGC
GCGATGACGTGGAGGAAGCCCAGCATGGCGGCGCGGAAAAGGCCTGGGCCGAGTC
GAGGACAGCGAGGGCG 3’

3.6.1 FCP-3 (636 bp’s)

Forward primer sequencing (5' CCAC CGT CGC CCT CTG AA 3')

5’CGCTCGCCCCCACGGCCGTCATGGGCCGCGCGTCGGCGCCCAAGATGTCCTTCGC
GGACGAGTTCACGTTTGACTCGAAGCCGTGGTCCTCGTCCGAGATCAAGGACAAGG
CCGGCCTCGAGGCGCTGGCCAAGAAGCTGAACCCGACGGTGGGCCTCTGGGACCCG
CTCGGCATCGCGGAGACGTCGCCCGAGACGATCGGCTGGTTCCGCCACGCCGAGAT
CAAGCACGGCCGCGTCGCCATGGCCGCCTTCGTCGGCTACTGCGTTCAGTCCAACGG
CATCCACTTCCCGTGGAACATCCAGGGCTGGCAGGGCACGCCGGTCGTCAGCTTCGC
TGACATTGCCGCGGCGGGCGGCCCTGCGGACCAGTGGGATGCGCTCTCGACGCCGG
CCAAGCTGCAGATCCTTGGCGTGATCGGCTTCCTCGAGATGTGGAGCGAGACGAGC
GTCGTGCTCAAGGCGGACGGCCAGGAGCACTACGTGCGCGGCGGCAAGCCCGGCTA
CTTCCCCAAGCTGTCGCGCTCGGACGAGATGGCGTTCCCCCACCCGGTGCCGCTCAA
CCTGTGGGACCCCTTCGGGTTCACGTCCAAGATGACGCCGGAGCGCAAGGAGAAGG
CGCTCCTCGCGGAGGTCA 3’

3.6.2 FCP-3 (621 bp’s)

Reverse primer sequencing (5'GC GGC CCG TTG TTG ACC 3')

5’GGCGTCATCTTGGACGTGAACCCGAAGGGGTCCCACAGGTTGAGCGGCACCGGGT
GGGGGAACGCCATCTCGTCCGAGCGCGACAGCTTGGGGAAGTAGCCGGGCTTGCCG
CCGCGCACGTAGTGCTCCTGGCCGTCCGCCTTGAGCACGACGCTCGTCTCGCTCCAC

ATCTCGAGGAAGCCGATCACGCCAAGGATCTGCAGCTTGGCCGGCGTCGAGAGCGC
ATCCCACTGGTCCGCAGGGCCGCCCGCCGCGGCAATGTCAGCGAAGCTGACGACCG
GCGTGCCCTGCCAGCCCTGGATGTTCCACGGGAAGTGGATGCCGTTGGACTGAACG
CAGTAGCCGACGAAGGCGGCCATGGCGACGCGGCCGTGCTTGATCTCGGCGTGGCG
GAACCAGCCGATCGTCTCGGGCGACGTCTCCGCGATGCCGAGCGGGTCCCAGAGGC
CCACCGTCGGGTTCAGCTTCTTGGCCAGCGCCTCGAGGCCGGCCTTGTCCTTGATCT
CGGACGAGGACCACGGCTTCGAGTCAAACGTGAACTCGTCCGCGAAGGACATCTTG
GGCGCCGACGCGCGGCCCATGACGGCCGTGGGGGCGAGCGACAGCGGCGCGGATG
TGAGC 3’

Appendix IV

Data tables for quantitative analysis

Gene	Slope	R2 or Rsq	PCR effiencies
GAPDH	-3. 086918	0, 9971	90%
Ferredoxin	-3.029113	0.999015	86%
NADHase	-3.428617	0.99618	100%
FCP1	-2.845636	0.997565	79.8 %
LHC	-3.873212	0.991688	126%

Table 3 Slope, PCR effiencies and R^2 value for gene expression in growth curve quantitative (experiment. III) PCR reactions.

Gene	Slope	R2 or Rsq	PCR effiencies
GADPH	-5.149811	0.909	212%
LHC	-3.62954	0.974	117%
FCP_3	-3.070895	0.988	89.2%
FCP_1	-3.070895	0.992	92.46%
NADHase	-3.160847	0.997	88%
Ferredoxin	-3.047306	0.998	87%

Table 4 Slope, PCR effiencies and R^2 values for gene expression in post viral cultures PCR reactions (experiment. IV).

Gene	Calibrator	Number of transcripts after 6hrs of viral infection	Number of transcripts after 12 hrs of viral infection	Number of transcripts after 24 hrs of viral infection
FCP1	1.56692 E + 7	1.37237 E + 6	1.2824 E + 7	7.51327 E + 5
FCP3	3.68369 E + 4	7.2354 E + 3	6.83949 E + 4	5.76 E + 2
LHC	5.50319 E + 6	2.90138 E + 6	3.42 E + 6	1.71 E + 4

Ferridoxin	5.09398 E + 5	4.55466 E + 5	1.22334 E + 6	5.01177 E + 4
GAPDH	3.0686 E + 8	3.52461 E + 8	2.13123 E + 8	6.7107 E + 7
NADHase	3.38957 E + 3	3.29526 E + 3	2.79598 E + 3	2.71 E + 3

Table 5: Average number of amplicon molecules present inside the cell before and after viral infection.
(Note: The calibrator was pooled RNA from Jessica's culturing experiments)