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Molecular Investigation of Candidate Genes for the Biosynthetic Pathway for Dimethylsulfoniopropionate (DMSP) in the Diatom *Thalassiosira pseudonana*

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ABSTRACT

Dimethylsulfoniopropionate (DMSP) is a sulfuric zwitterionic compound produced primarily by marine phytoplankton. DMSP is the main biogenic precursor for the climatically active gas dimethyl sulfide (DMS), and is involved in the assimilation of sulfur in marine food webs. In marine algae, DMSP is a multifunctional molecule involved in stress adaptations, as it may function as an osmolyte, cryoprotectant, antioxidant and as a chemical defense molecule against grazing zooplankton. Five candidate genes have been proposed to be involved in the poorly understood biosynthesis of DMSP in marine algae, featuring the four enzymatic reactions transamination, reduction, *S*-methylation and oxidative decarboxylation. In this thesis, the candidate genes *DiDECARB*, *SAMmt*, *REDOX* and *AT* were investigated for their role in the DMSP biosynthetic pathway in the diatom *Thalassiosira pseudonana*. As studies have shown that DMSP accumulates in marine algae under nutrient limitations, the gene expression of these candidate genes was examined using quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR), and the hypothesized DMSP accumulation was measured by gas chromatography-mass spectrometry (GC-MS), when diatom cultures were grown under the limitations of the nutrient silicate and nitrate. The candidate genes were also similarly investigated under complete silicate starvation. Attempts to optimize total RNA isolation of *T. pseudonana* for RT-qPCR were made. The results indicated that the QIAGEN Protocol B was the most suitable total RNA purification protocol tested, and that the Durapore® DVPP membrane filter (Merck Millipore) for cell harvesting by vacuum filtration resulted in the highest RNA yield.

The results obtained in this thesis, indicated that DMSP failed to accumulate under nutrient limitations of silicate and nitrate, but was significantly accumulated during complete silicate starvation. The candidate gene *REDOX* was found to be significantly up-regulated after 5 days of nitrate limitation, and the *DiDECARB* gene was significantly up-regulated after 72 hours of complete silicate starvation, although not strongly statistically supported. The results obtained in this thesis were not able to verify that the candidate genes are involved in the biosynthesis of DMSP. Although a link between the proposed candidate genes and the biosynthesis of DMSP cannot be ruled out, further research is needed to reveal the role of the candidate genes and/or find new candidate genes for the biosynthetic pathway for DMSP in marine algae.

NORWEGIAN ABSTRACT

Dimetylsulfoniopropionat (DMSP) er en zwitterionisk svovelforbindelse som produseres primært av marint planteplankton. DMSP er hovedforløperen for den aktive klimagassen dimetylsulfid (DMS), og er involvert i assimileringen av svovel i marine næringskjeder. I marine alger, er DMSP et flerfunksjonelt molekyl som er involvert i stresstilpasninger, ettersom det kan fungere som en osmolytt, antifrysemiddel, antioksidant og som et kjemisk forsvarsmolekyl mot beitende dyreplankton. Fem kandidatgener har blitt foreslått til å være involvert i den nokså ukjente biosyntesen av DMSP i marine alger, bestående av de fire enzymatiske reaksjonene transaminering, reduksjon, *S*-metylering og oksidativ dekarboksylering. I denne tesen ble kandidatgenene *DiDECARB*, *SAMmt*, *REDOX* og *AT* undersøkt for deres roller i DMSP biosyntesesporet i kiselalgen *Thalassiosira pseudonana*. Ettersom studier har vist at DMSP akkumuleres i marine alger under næringsbegrensing, ble utrykket av disse kandidatgenene undersøkt ved hjelp av "quantitative real-time reverse transcription polymerase chain reaction" (RT-qPCR), og den antatte DMSP akkumuleringen ble målt ved hjelp av gasskromatografi-massespektrometri (GC-MS), i kiselalgekulturer dyrket under begrensinger av næringsstoffene silikat og nitrat. Kandidatgenene ble på lignende måte også undersøkt under komplett silikatsulting. Forsøk ble gjort på å optimalisere total RNA isolering av *T. pseudonana* for RT-qPCR. Resultatene tydet på at QIAGEN Protocol B var den mest passende total RNA isoleringsprotokollen som ble testet og at cellehøsting ved vakuumfiltrering ved bruk av Durapore® DVPP membranfilter (Merck Millipore) gav de høyeste konsentrasjonene av RNA.

Resultatene som ble funnet i denne tesen, tydet på at DMSP ikke ble akkumulert under næringsbegrensing av silikat eller nitrat, men at DMSP ble signifikant akkumulert under komplett silikatsulting. Kandidatgenet *REDOX* var signifikant oppregulert etter 5 dagers nitratbegrensing, og *DiDECARB* genet ble signifikant oppregulert etter 72 timer med komplett silikatsulting, selv om resultatene ikke står sterkt statistisk sett. Resultatene som ble funnet i denne tesen kunne ikke verifisere at kandidatgenene var involvert i biosyntesesporet av DMSP. Selv om en kobling mellom de foreslåtte kandidatgenene og biosyntese av DMSP ikke kan utelukkes, kreves det videre forskning til for å avdekke kandidatgenenes rolle og/eller finne nye kandidatgener for biosyntesesporet for DMSP i marine alger.

TABLE OF CONTENTS

ABBERIVATIONS

2-ME	2-mercaptoethanol
A	Adenine
AT	Aminotransferase
bp	Base pairs
c	Concentration
C	Cytosine
Car	Carboxen
Car-PDMS	Carboxen and polydimethylsiloxane
CCAP	Culture Collection of Algae and Protozoa
CCN	Cloud condensation nuclei
cDNA	Complementary deoxyribonucleic acid
$C_6H_8O_7 * H_2O$	Citric acid monohydrate
CLAW	Charlson, Lovelock, Andreae and Warren
CO_2	Carbon dioxide
Ct	Threshold cycle
DECARB	Pyridoxyl dependent decarboxylase
DiDECARB	Diaminopimelate decarboxylase
dH_2O	Distilled water
DMS	Dimethyl sulfide
DMSHB	4-dimethylsulfonio-2-hydroxybutyrate
DMSP	Dimethylsulfoniopropionate
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
EDTA	Ethylenediaminetetraacetic acid
eV	Electron-volt
FA	Formaldehyde
FUGE	Norwegian Functional Genomics Initiative
g	The Earth's gravitational acceleration
G	Guanine
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
gDNA	Genomic deoxyribonucleic acid
h	Height of the cell
H4	Histone H4
HS-SPME	Headspace solid-phase microextraction
Hz	Hertz
IKI	Iodine-potassium iodide
In vivo	Process acting in a cell
JGI	DOE Joint Genome Institute
LOD	Limit of detection

Met	Methionine
MilliQ water	Ultra pure water purified by the MilliQ Purification system (Millipore)
MIQE	Minimum information for publication of quantitative real-time polymerase chain reaction experiments
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
MTHB	4-methylthio-2-hydroxybutyrate
MTOB	4-methylthio-2-oxybutyrate
m/z	Mass-to-charge ratio
n	Amount of substance or Number of replicates
NADPH	Nicotinamide adenine dinucleotide phosphate
Na_2HPO_4	Dibasic sodium phosphate
$NaNO_3$	Sodium nitrate
Na_2SiO_3	Sodium silicate
NCMA	National Center for Marine Algae and Microbiota
NaOH	Sodium hydroxide
NRT	No reverse transcription
NTC	No template control
PCR	Polymerase chain reaction
PCTE	Polycarbonate
PDMS	Polydimethylsiloxane
psi	Pounds per square inch
qPCR	Quantitative real-time polymerase chain reaction
r	Radius
rpm	Revolutions per minute
REST	Relative expression software tool
RNA	Ribonucleic acid
RNase	Ribonuclease
RNase H	Ribonuclease H
rRNA	Ribosomal ribonuclease
RT	Reverse transcription
RT-qPCR	Quantitative real-time reverse transcription polymerase chain reaction
SAM	*S*-adenosyl methionine
SAMmt	*S*-adenosyl methionine-dependent methyltransferase
SD	Standard deviation
SDV	Silica deposition vesicles
SEM	Scanning electron microscopy
SIM	Selected ion monitoring
$Si(OH)_4$	Silicic acid
SPME	Solid-phase microextraction
T	Thymine
TBP	TATA box binding protein
Tm	Melting temperature
U	Units

UV	Ultraviolet
V	Volume
v/v	Volume/volume

1. INTRODUCTION

1.1 Diatoms

Diatoms are a diverse group of unicellular chromophyte algae found in freshwater and marine environments. They belong to the phylum Bacillariophyta within the eukaryotic division Stramenopiles and are considered to be the most important group of eukaryotic phytoplankton, responsible for about 40 % of marine primary productivity (Falciatore and Bowler, 2002). Diatoms consist of more than 250 genera, including possibly as many as 100 000 species, ranging in sizes from 5 to 5000 µm, existing either as single cells or as chains of connected cells (Falciatore and Bowler, 2002; Armbrust, 2009). Their most distinctive feature is the ability to generate a highly patterned cell wall composed of amorphous silica [$(SiO_2)n(H_2O)$], called frustules (Falciatore and Bowler, 2002). The frustules have species-specific patterns of pores and projections, which are thought to protect against grazing zooplanktons (Armbrust et al., 2004; Falciatore and Bowler, 2002). The symmetry of the frustule is used to classify diatoms into two major groups; centric and pennate diatoms. Centric diatoms are radially symmetrical and tend to be planktonic, whereas pennate diatoms are elongated and bilaterally symmetrical and are benthic on sediments or other surfaces (Falciatore and Bowler, 2002). Examples of centric and pennate diatoms are shown in Figure 1.1.1.

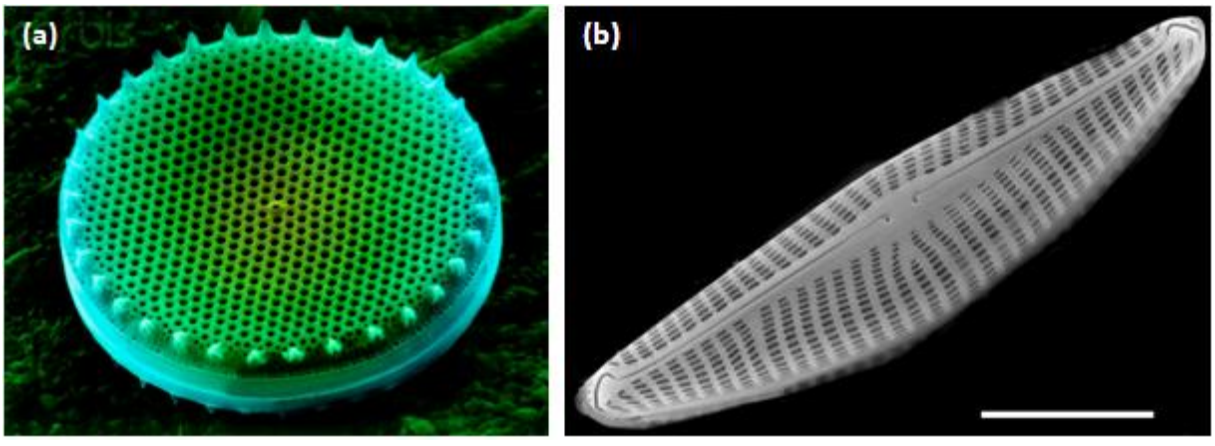

Figure 1.1.1 Diatom frustules. (A): The frustule of a centric diatom (Dennis Kunkel Microscopy, Inc., 2004). **(B):** The pennate diatom *Navicymbula pusilla* (Potapova, 2011).

1.1.1 Life cycle

The frustule of a diatom cell consists of two overlapping halves (thecae) fitting together as a Petri dish (Chepurnov et al., 2008). The smallest half, the hypotheca, fits into the larger epitheca. Each theca is composed of two parts: a valve and girdle bands. The valves form the larger outer surfaces of the diatom, and the girdle bands, which consist of circular bands of silica, attach the valves together (Falciatore and Bowler, 2002). In mitotic division, each daughter cell inherits a parental theca, which forms the epitheca of its new frustule. The daughter cells will generate a new inner hypotheca. As a result, one of the daughter cells will become smaller than the parent cell (Chepurnov et al., 2008). Therefore, populations of successive mitotically dividing cells decrease in size over time. Regeneration of the original size generally occurs through sexual reproduction, involving auxospore formation. Cells that have decreased in size to about 30-40 % of the maximum diameter generate male and female gametes through gametogenesis (Falciatore and Bowler, 2002). The old thecae are sloughed off and the resulting male and female gametes combine to form a diploid zygote that matures into a specialized cell called an auxospore. The auxospore expands until it reaches its maximum size, and a new initial cell is formed inside the auxospore envelope. The resulting new cell is about two to three times larger than either parental cells, and starts a new round of vegetative multiplication (Chepurnov et al., 2008). The life cycle of centric diatoms are illustrated in Figure 1.1.2. Although the majority of diatoms reproduce through this cycle of cell size reduction and restitution, some diatom species are able to avoid size reduction. It is not clear how this is achieved, but it is thought to involve unusual flexibility of the girdle region (Chepurnov et al., 2008).

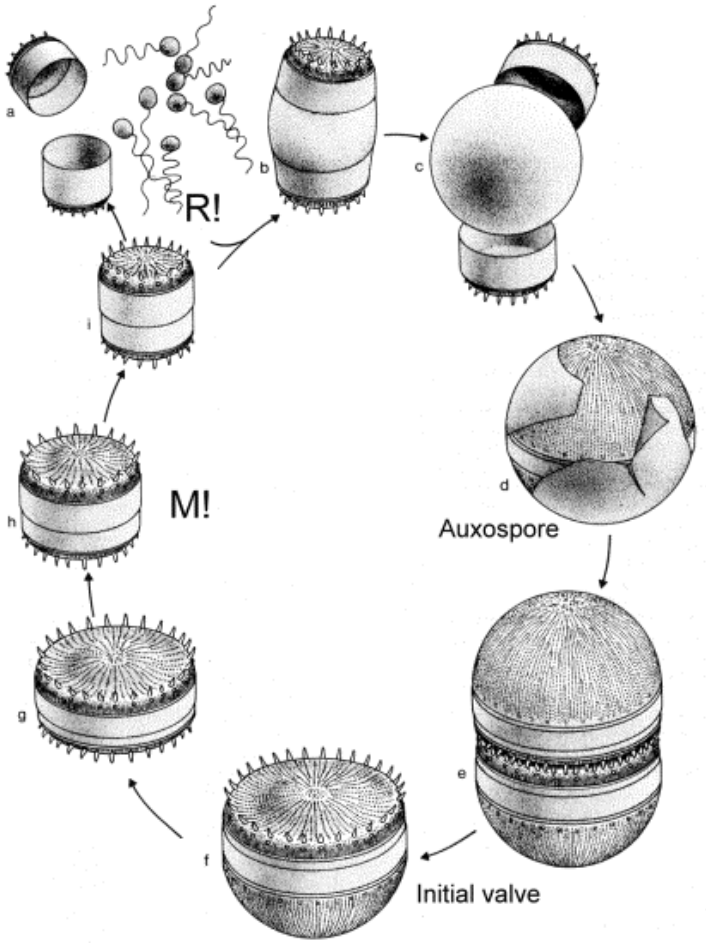

Figure 1.1.2 The life cycle of a centric diatom. Diatoms increase in cell number due to mitotic divisions. The resulting daughter cells inherit one theca each of the mother cell. The daughter cell that inherited the hypotecha becomes smaller than the parental cell. When cells have reached a critical size, sexual reproduction is initiated by gametogenesis. Male and female gametes form a diploid cell called an auxospore, which regenerates a cell of the initial cell size (Chepurnov et al., 2008; Falciatore and Bowler, 2002; Kociolek, 2010).

1.1.2 Nutrient cycling

Since silicon is required for the biogenesis of the diatom cell wall, diatoms play a key role in the biogeochemical cycling of the mineral silica in the world's oceans. In sea water, silicon is available to marine diatoms as silicic acid ($Si(OH)_4$). This is transported into the cells via novel membrane-localized silica transporters, and deposited into new siliceous valves during frustule biogenesis (Falciatore and Bowler, 2002). Cell walls from dead diatoms accumulate on the ocean floor as large deposits of silica, completing the cycle (Armbust, 2009).

In addition to its role in the silicon cycle, diatoms are also involved in the recycling of carbon and nitrogen (Armbrust, 2009; Falciatore and Bowler, 2002). About 20 % of the Earth's

photosynthesis is carried out by diatoms, producing annually more organic carbon than all terrestrial rainforests combined. The generated organic carbon is rapidly consumed and serves as a base for marine food webs, supporting fisheries in coastal waters and deep-water organisms in the open oceans. The small amount of non-consumed organic matter sinks and settles on the sea floor, where it contributes to the ocean biochemistry over geologically signifiant timescales (Armbrust, 2009). In warm oligotrophic seas, diatoms are also involved in the fixation of nitrogen. Here, symbiosis between nitrogen-fixing bacteria, cyanobacteria and diatoms contributes significantly to the amount of nitrogen in the local ecosystem (Falciatore and Bowler, 2002). Diatoms are therefore considered to be important parts of the global carbon and nitrogen cycles (Armbrust, 2009; Falciatore and Bowler, 2002).

1.1.3 Distribution of diatoms

The abundance of phytoplankton in aquatic habitats is limited to various environmental factors. Although diatoms often dominate phytoplankton communities in coastal and upwelling regions as well as in polar environments, diatoms are reliant of sufficient light, inorganic nitrogen, phosphor, silicon and trace elements to sustain their growth. In order to adapt to the variability of the light conditions, larger species of diatoms are able to move up and down through the water column by controlling their buoyancy (Armbrust, 2009). Although some pennate diatoms are able to glide along surfaces due to mucilage secretion, planktonic diatoms do not have flagella and rely heavily on passive movements like sinking and water turbulence. The small-celled species (5-50 µm) are most abundant at the beginning of spring and autumn, when nutrients are not limiting and light conditions are optimal for photosynthesis. During nutrient limitation, they tend to aggregate and sink quickly out of the photic zone (Falciatore and Bowler, 2002). Large diatoms (>50 µm in diameter or length) show less seasonal variability and are ubiquitous in all oceans (Kemp et al., 2000; Falciatore and Bowler, 2002).

1.1.4 Diatom evolution

It is estimated that diatoms arose in the Triassic period, as early as 250 million years ago (Armbrust, 2009). All photosynthetic eukaryotes are believed to have evolved from a primary endosymbiotic event, where a unicellular nonphotosynthetic eukaryote engulfed a prokaryotic cyanobacterium (Armbrust et al., 2004). Over time, this initial event gave rise to the two major plastid lineages known today: chloroplasts and rhodoplasts. Chromophyte algae, such

as diatoms, are fundamentally different from other photosynthetic eukaryotes. Whereas red and green algae and plants normally have plastids surrounded by two membranes, diatom plastids are surrounded by four. They are therefore believed to have evolved from a secondary endosymbiotic event, where a eukaryotic red alga was engulfed by a second eukaryotic heterotroph (Falciatore and Bowler, 2002).

1.1.5 Diatom research

The genome of two diatoms, the centric *Thalassiosira pseudonana* (NCMA 1335) and the pennate *Phaeodactylum tricornutum* (NCMA 632), were fully sequenced in 2004 and 2008 respectively, making them promising model organisms for biotechnological and molecular studies (Armbrust et al., 2004; Bowler et al., 2008; Armbrust, 2009; Chepurnov et al., 2008). A scanning electron microscopy (SEM) picture of *T. pseudonana* is shown in figure 1.1.3. These diatoms were selected because of their small genome sizes, ease of cultivation, rapid growth (>1 division per day) and their inability to exhibit the size reduction - restitution cycle that is unique for diatoms (Chepurnov et al., 2008). In spite of their ecological importance and industrial versatility, there is still a lot to uncover about the basic biology of the diatoms (Falciatore and Bowler, 2002; Franklin et al., 2012).

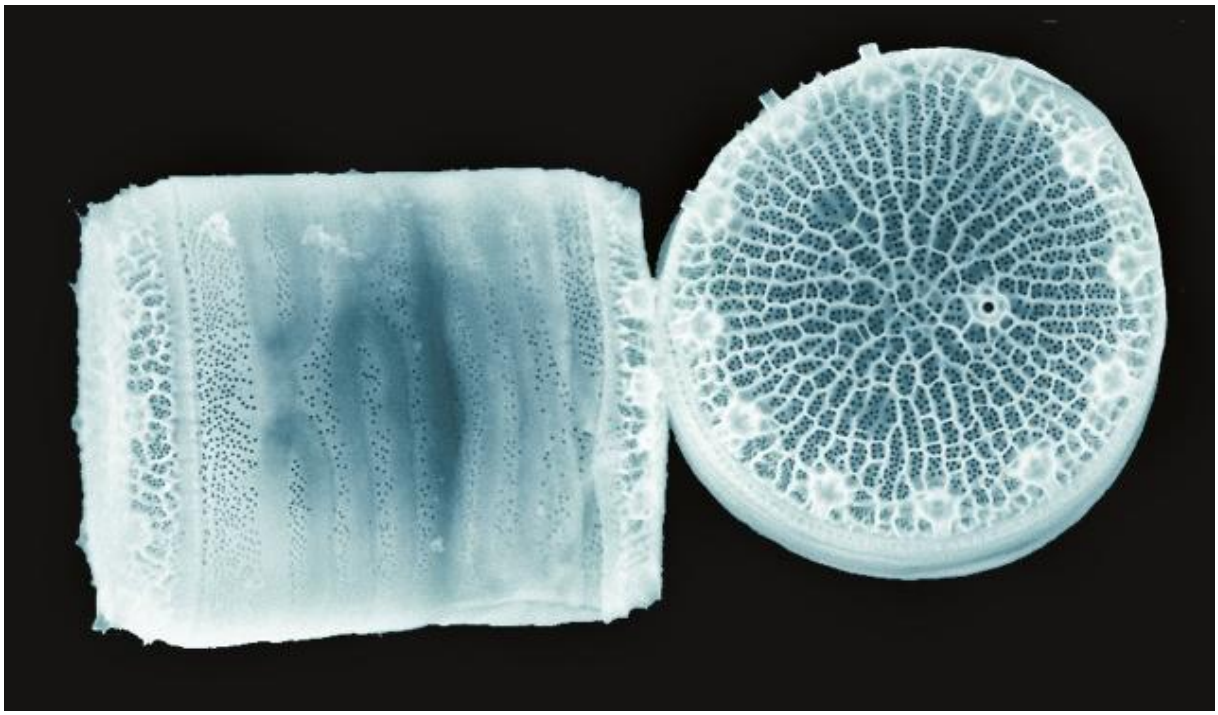

Figure 1.1.3 *Thalassiosira pseudonana*. A scanning electron microscopy (SEM) picture showing the side and front view of the diatom *T. pseudonana* (Kröger, 2012). This diatom is a promising model organism, because of its sequenced small sized genome size and are easily cultivated (Armbrust, 2009).

1.2 Dimethylsulfoniopropionate (DMSP)

Dimethylsulfoniopropionate (DMSP) is a sulfuric zwitterionic compound produced primarily by marine phytoplankton. In marine surface waters, DMSP concentrations can range from less than 1 nanomolar in the open oceans to several micromolar in phytoplankton blooms (Reisch et al., 2011). Although production of DMSP is known in higher plants, DMSP in marine surface water is mainly produced by microalgae and macroalgae, and is released during cellular lysis caused by zooplankton grazing, senescence or viral infections (Reisch et al., 2011; Lyon et al., 2011). In marine algae, DMSP can act as a compatible solute, cryoprotectant, antioxidant and a chemical defense compound against grazing zooplankton, and is therefore important in stress adaptations (Bucciarelli and Sunda, 2003; Lyon et al., 2011). The structural formula of DMSP is presented in Figure 1.2.1.

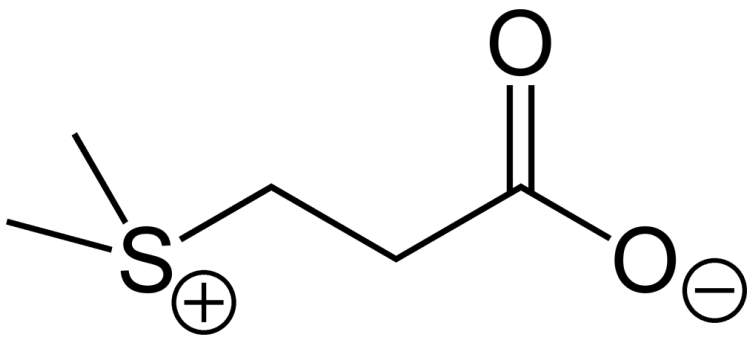

Figure 1.2.1 Dimethylsulfoniopropionate (DMSP). The structural formula of the sulfuric zwitterion DMSP (Modified from Gage et al., 1997).

1.2.1 Assimilation in marine phytoplankton

In marine microbial food webs, DMSP accounts for most of the organic sulfur fluxes from primary to secondary producers (Vila-Costa et al., 2006). DMSP is taken up and assimilated in the dominant marine phytoplankton groups, diatoms and the unicellular cyanobacteria *Prochlorococcus* and *Synechococcus*, in addition to heterotrophic bacteria. Although, the major phytoplankton groups can take up and utilize DMSP, the production of DMSP is taxon dependent, and to some extent, size dependent. Small haptophytes and dinoflagellates are often high DMSP producers, whereas diatoms (with the exception of sea ice diatoms) are generally low- or nonproducers (Vila-Costa et al., 2006). Diatoms and cyanobacteria that produce low or no levels of DMSP, have been hypothesized to consume DMSP released by

nearby high DMSP-producing phytoplankton, by transporting the DMSP into the cells in ways similar to that of heterotrophic bacteria. The algal produced DMSP supply sulfur and carbon to heterotrophic bacteria and, to a lesser extent, microzooplankton herbivores. Thereby, a proportion of the plankton produced organic sulfur is diverted from emissions into the atmosphere. Hence, DMSP can act as a carrier for sulfur and carbon through multiple levels of the marine microbial food webs and as a regulator of sulfur emissions into the atmosphere (Vila-Costa et al., 2006).

DMSP is the main biogenic precursor for the volatile sulfur compound dimethyl sulfide (DMS), which is a climatically active gas (Reisch et al., 2011; Bucciarelli and Sunda, 2003). It is estimated that <50 % of the produced DMSP are enzymatically cleaved to yield DMS (Vila-Costa et al., 2006). DMS is the primary natural source of sulfur in the atmosphere, and contributes about $1{,}5 * 10^{13}$ g of atmospheric sulfur annually (Reisch et al., 2011; Gage et al., 1997). Marine atmospheric DMS is oxidized to products, such as sulfate, sulfur dioxide and methanesulfonic acid, which form aerosols that act as cloud condensation nuclei (CNN) (Reisch et al., 2011; Bucciarelli and Sunda, 2003). Thereby, production of DMSP and DMS has been hypothesized to influence the Earth's reflective effect and the global climate (Bucciarelli and Sunda, 2003; Lyon et al., 2011).

1.2.2 The CLAW hypothesis

In 1987, the CLAW hypothesis (named after its authors Charlson, Lovelock, Andreae and Warren) postulated that DMS produced by marine phytoplankton were involved in biological regulating the global climate (Bucciarelli and Sunda, 2003; Charlson et al., 1987). At the time, DMS-derived sulphate aerosols were thought of as the major source of CCN (Charlson et al., 1987). Increase in DMS emissions from the ocean would therefore result in increasing CCN, cloud droplet concentrations and the albedo of clouds, resulting in changes in temperature and radiation (Quinn and Bates, 2011). This enabled a feedback loop, where DMS-derived CCN were able to change cloud albedo, which altered DMS production (Charlson et al., 1987; Quinn and Bates, 2011). It seems, however, that a dimethyl sulfide-based biological control over CCN may not exist and that the sources of CNN and the response of clouds to changes in aerosol levels are more complex than was recognized in the 1980s. Although a link between ocean-derived CCN and climate might exist, the evidence gained over the past 20 years suggest that the CLAW hypothesis is not entirely correct and that phytoplankton have a less

major role in climate regulation than previously assumed (Quinn and Bates, 2011). Mechanisms for CNN formation in the marine atmosphere is shown in Figure 1.2.2.

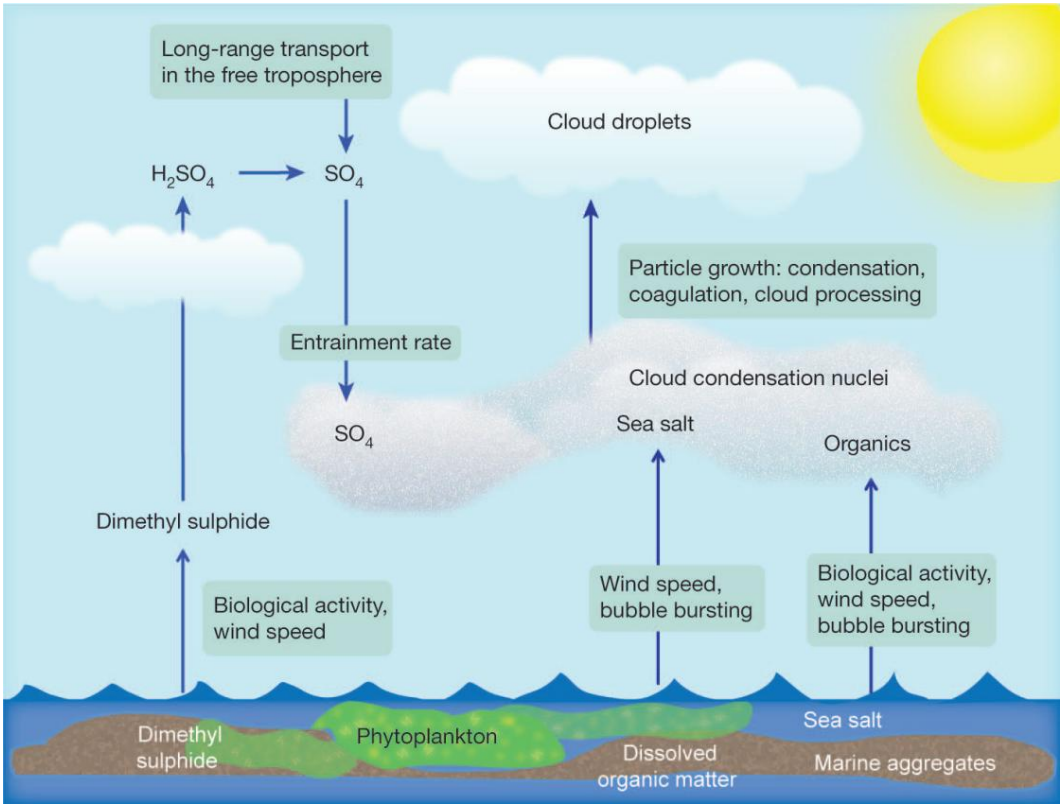

Figure 1.2.2 Production of CCN in the marine atmosphere. Cloud condensation nuclei (CCN) in the marine atmosphere are formed from aerosols derived from phytoplankton emitted DMS, sea salt particles and organic species as major sources. In cloud outflow regions in the troposphere, DMS contributes to CCN formation through particle nucleation, whereas sea salt and organic species derives from wind-driven bubble bursting (Quinn and Bates, 2011).

1.2.3 Biosynthesis of DMSP

The biosynthesis of DMSP and its regulatory pathways are yet poorly understood. Methionine (Met) is used as an amino acid precursor, although studies have shown that plants and algae have developed divergent pathways from here. Higher plants use *S*-methyl-methionine as an intermediate, whereas marine algae convert methionine into DMSP through an alternative methionine transaminase pathway, involving steps of transamination, reduction, *S*-methylation and oxidative decarboxylation (Gage et al., 1997; Lyon et al., 2011). The proposed DMSP biosynthetic pathway in marine algae, as shown in Figure 1.2.3, is initiated by a transamination of methionine to yield an unstable 2-oxo acid; 4-methylthio-2-oxobutyrate (MTOB). MTOB is reduced to 4-methylthio-2-hydroxybutyrate (MTHB), which

is *S*-methylated to its derivative 4-dimethylsulfonio-2-hydroxy-butyrate (DMSHB). The conversion of Met to MTHB is reversible. In the final reaction, DMSHB is oxidatively decarboxylated to DMSP (Gage et al., 1997).

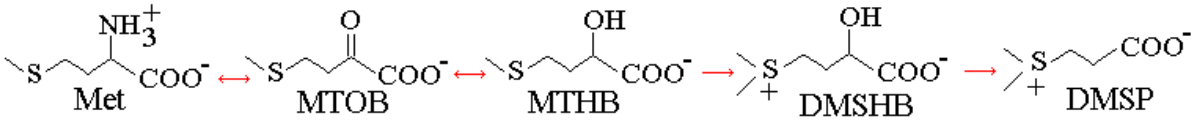

Figure 1.2.3 The proposed DMSP biosynthetic pathway. A hypothetical pathway for the biosynthesis of dimethylsulfoniopropionate (DMSP) in marine algae. Methionine (Met) is converted to 4-methylthio-2-oxobutyrate (MTOB) and 4-methylthio-2-hydroxybutyrate (MTHB) in two reversible steps, involving transamination and reduction. MTHB is *S*-methylated to 4-dimethylsulfonio-2-hydroxy-butyrate (DMSHB), which in the final reaction is converted to DMSP by oxidative decarboxylation (Gage et al., 1997; Lyon et al., 2011; Hanson et al., 2003).

1.2.4 Influence of nutrient limitations

In 2003, Bucciarelli and Sunda published a study on the influence of nutrient limitation on DMSP production in the diatom *T. pseudonana*. Under limitation of nitrate, phosphate, silicate or carbon dioxide (CO_2), the intracellular DMSP concentrations increased during the stationary phase of growth. The increase was highest in cells limited on nitrate. Silicate and CO_2 had intermediate effects, and the lowest increase was shown under phosphate limitation. During nutrient limitation intracellular oxidative stress increases. Therefore, the increase in DMSP concentrations may be linked to DMSP's role as an antioxidant. Also, it might be favorable for nitrogen limited cells to replace nitrogen-containing osmolytes such as proline with DMSP, which is a sulfur-containing osmolyte (Bucciarelli and Sunda, 2003). Since DMSP biosynthesis is initiated by a transaminase, it has also been suggested that depletion of cellular amino acids would favor the transamination reaction, thereby promoting DMSP production when nitrogen is limiting (Gage et al, 1997).

1.2.5 Candidate genes for DMSP biosynthesis

Using radiolabeling, studies have shown that algal DMSP biosynthesis uses four classes of enzymes; 2-oxoglutarate-dependent aminotransferase, NADPH-dependent reductase, *S*-adenosyl Met (SAM)-dependent methyltransferase (SAMmt) and oxidative decarboxylase (Lyon et al., 2011; Gage et al., 1997). Although the specific genes involved have not yet been

identified, five candidate genes have been proposed by Lyon et al. (2011). These encoded an unknown aminotransferase (AT; *Fc273803*), a NADPH-dependent flavinoid reductase (REDOX; *Fc173405*), a putative *S*-adenosyl methionine-dependent methyltransferase (SAMmt; *Fc207357*) and two putative decarboxylases, pyridoxyl dependent decarboxylase (DECARB; *Fc238865*) and diaminopimelate decarboxylase (DiDECARB; *Fc263016*) (Lyon et al., 2011).

In order to identify the candidate genes involved in the DMSP biosynthesis pathway, Lyon et al. investigated proteins from the proposed enzyme classes of the hypothetical biosynthetic pathway for DMSP in the polar diatom *Fragilariopsis cylindrus* (Lyon et al., 2011; Gage et al., 1997). It was hypothesized that the abundance of proteins from these enzyme classes, along with proteins associated with methionine synthesis, were to increase in abundance during hypersaline-induced DMSP production. Salinity was gradually manipulated over a period of 22 hours, where pH and carbonate alkalinity were allowed to correlate with salinity, imitating natural salinity changes during sea ice formation. Intracellular DMSP were quantified and two-dimensional gel electrophoresis was performed to identify protein changes. Elevated proteins from the 2D gel associated with increases in DMSP levels, were classified as candidate genes for the DMSP biosynthesis. Of these included the unknown aminotransferase, the NADPH reductase, putative SAMmt and the two putative decarboxylases, pyridoxyl dependent decarboxylase and diaminopimelate decarboxylase, which were elevated 1.4-, 1.5-, 2.8-, 2.4- and 1.7-fold, respectively. Homologs of the AT, REDOX, SAMmt and DiDECARB were found in the low DMSP-producing diatom *Thalassiosira pseudonana* and the high DMSP-producing haptophyte *Emiliania huxleyi*. In addition, *E. huxleyi* displayed three different proteins with similarity to SAMmt and also showed a homolog to DECARB, which may provide the genetic explanation for the high production of DMSP in this haptophyte. The nonproducing diatom *P. tricornutum*, however, had only homologs to AT, REDOX and DiDECARB, but not to SAMmt or DECARB, which may explain the inability to synthesize DMSP.

1.3 Aims of the study

The overall purpose of this study was to gain further knowledge of the poorly understood biosynthetic pathway for DMSP in marine algae. Since candidate genes have been proposed for the four metabolic enzymes involved in the biosynthesis of DMSP (Gage et al., 1997; Lyon et al., 2011), the main aim of this study is to investigate the candidate genes in order to verify whether the encoding enzymes are directly involved in the DMSP biosynthesis pathway in marine algae. For this study, the centric diatom *T. pseudonana* were used as model organism, because the genome of this diatom has been sequenced (Armbrust et al., 2004) and it is known to produce DMSP (Lyon et al., 2011). Homologs of four of the candidate genes were found in *T. pseudonana*, including the *DiDECARB, SAMmt, REDOX* and *AT* genes. Although *T. pseudonana* produces relatively low levels of DMSP in comparison to other marine algae such as the coccolithophore *E. huxleyi*, *T. pseudonana* do not degrade DMSP in order to produce DMS, making DMSP quantification easier (Lyon et al., 2011). Since diatoms are a relatively new area of research (Franklin et al., 2012), efficient protocols for RNA isolation needed to be optimized.

Therefore, the aims of this study can be summarized as:

1. Develope a procedure for efficient isolation of high quality total RNA in *T. pseudonana*, in order to perform accurate gene expression analysis using quantitative real-time reverse transcriptase polymerase chain reaction (RT-qPCR), by:

i) Optimizing the procedure for harvesting samples for RNA isolation
ii) Establishing an efficient protocol for total RNA purification

2. Measure the gene expression of candidate genes by RT-qPCR in diatom cultures grown under DMSP biosynthesis-inducing conditions, in form of nitrate or silicate nutrient limitations.

3. Quantify the total and intracellular DMSP levels in diatom cultures by headspace solid-phase microextraction (HS-SPME) and gas chromatography-mass spectrometry (GC-MS), from cultures grown under DMSP biosynthesis-inducing conditions. Show, from the quantified DMSP, whether the DMSP levels increases as the cell cultures enters stationary phase of growth, indicating an induction of the DMSP biosynthesis due to nutrient limitations.

4. Attempt to verify the candidate genes, by correlating the quantified DMSP with the measured gene expression data. If the candidate genes are significantly up-regulated under DMSP biosynthesis-inducing growth conditions and the quantified DMSP increases as the cells enter the stationary phase of growth, these results will participate in the verification of the candidate genes.

2. MATERIAL AND METHODS

In this study Reverse Transcriptase quantitative Polymerase Chain Reaction (RT-qPCR) was used to measure gene expression of the candidate genes proposed by Lyon et al in 2011. Gene expression data were supplemented by dimethylsulfoniopropionate (DMSP) measurements by Gas Chromatography-Mass Spectrometry (GC-MS). In this section the general principles of these methods are presented together with the relevant background information. The recipes for the buffer solutions and media used are listed in Appendix 1. An outline of the work performed is presented in Figure 2.1 and an experimental overview is presented in Table 2.1.

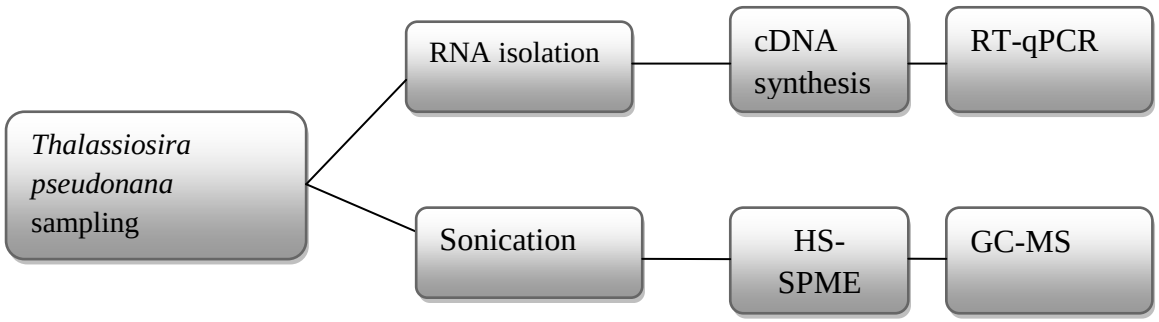

Figure 2.1 Work outline. The techniques performed in this master included quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR), headspace solid-phase microextraction (HS-SPME) and gas chromatography-mass spectrometry (GC-MS). RT-qPCR was used for Experiment 1 and 3, and GC-MS was used for Experiment 2 and 3.

Table 2.1 Experimental overview. The type of nutrient treatment and analysis of the three major experiments in this thesis.

Experiment	Nutrient treatment	Analysis
1	Nitrate and silicate limitations	Gene expression of candidate genes by RT-qPCR
2	Nitrate and silicate limitations	DMSP quantification by HS-SPME and GC-MS
3	Complete silicate starvation	Gene expression of candidate genes by RT-qPCR and DMSP quantification by HS-SPME and GC-MS

2.1 Experimental work

All experiments described in this thesis were performed using cultures of *Thalassiosira pseudonana* (Hustedt) Hasle and Heimdal (1970).

Axenic cultures of *T. pseudonana* were obtained from the Culture Collection of Algae and Protozoa (CCAP), using the CCAP strain 1085/12. This marine strain originates from Moriches Bay of the Forge River (Long Island, New York, USA), and is also known as the National Centre of Marine Algae and Microbiota (NCMA) strain 1335.

2.1.1 Culture conditions

Cultures of *T. pseudonana* were cultivated in f/2 medium in sterile BD Falcon™ tissue culture flasks (with 0.2 µm vented blue plug seal caps) of various sizes. Cells were grown under continuous cool white light (115 µmol photons m^{-2} s^{-1}) at 18 ºC in a climate controlled growth room. The cultures were maintained by subcultivation once a week.

Experiment 1:

Gene expression analysis of the candidate genes under DMSP production conditions

In the first and second experiment, cell cultures of *T. pseudonana* were grown in a VB 1514 growth cabinet (Vötsch Industrietechnik) under continuous white light at a scalar irradiance (E_{PAR}) of 210 µmol photons m^{-2} s^{-1} and 40 % humidity at 18 ºC. The experimental cultures were cultivated in sterile 250 mL BD Falcon™ tissue culture flasks (with 0.2 µm vented blue plug seal caps) under continuous shaking at 100 rpm in an orbital shaker (Heidoph Rotamax 120). Nitrate limitation was generated using f/2 media not supplemented with sodium nitrate ($NaNO_3$). Similarly, silicate limitation was generated by excluding the addition of sodium silicate (Na_2SiO_3) when preparing the f/2 media. Hence, nitrate and silicate are only available as traces from the filtrated sea water. Control samples were grown in complete f/2 medium.

Batch cultures of *T. pseudonana* were grown in 150 mL f/2 media for five days. The cell density at the starting point (t0) was 100 300 cells mL^{-1} for each experimental culture. For each treatment including controls, four biological replicates were made. To prevent uncontrolled nutrient starvation and to keep the cultures in the exponential phase for a longer

period, the cultures were fed the second day (t2), by adding 150 µL of each of the f/2 nutrients $NaNO_3$, Na_2SiO_3, dibasic sodium phosphate (NaH_2PO_4) and f/2 trace metals, in addition to 75 µL f/2 vitamin solution. Nitrate limited and silicate limited cultures were fed all nutrients except $NaNO_3$ and Na_2SiO_3, respectively. The volumes of added nutrients were equivalent to the volumes added when preparing f/2 media. Cell density was measured once daily (at the same time of day) and the cultures were harvested for gene expression analysis the fifth and final day (t5).

Experiment 2:

Quantification of DMSP levels in* T. pseudonana *under DMSP production conditions

Batch cultures of *T. pseudonana* were cultivated in 200 mL f/2 media for five days. At starting point (t0) each experimental culture had a cell density of about 102 300 cells mL^{-1}. Three biological replicates were used for each treatment of nutrient limitation, including control cultures. The experimental cultures were fed during the second day (t2) by adding 125 µL of each f/2 nutrient ($NaNO_3$, Na_2SiO_3, NaH_2PO4 and f/2 trace metals) and 62.5 µL vitamin solution, except $NaNO_3$ and Na_2SiO_3 for nitrate and siliate limited cultures respectively. Samples for cell counting and DMSP quantification were harvested once daily at the same time of day.

Experiment 3:

***The influence of complete silicate starvation on DMSP production in* T. pseudonana**

Axenic cultures of exponentially growing *T. pseudonana* were grown in continuous white light (150 µmol photons m^{-2} s^{-1}) illuminated by fluorescent tubes (Philips Master TL-D 36 W/840) at 20 ºC. The cultures were grown in f/2 media at a total volume of 2 L in 2000 mL Nalgene optically clear flasks and aerated with air to prevent settling of diatom cells. Three biological replicates were used.

At Start, cell cultures were dispersed in 250 mL centrifuge cups and centrifuged at 8000 x g for 10 minutes. The supernatant was discarded and the cell pellet were resuspended in a completely silicate-free f/2 medium. To achieve the silicate-free medium, conditioned natural sea water was used to prepare the f/2 medium. Samples for gene expression analysis using RT-qPCR, DMSP quantification, cell counting and cell volume estimation were harvested 5 times during the experiment at the following time points:

Time point	Time (hours) after Start
t0	- 20 minutes before Start
t1	7.5
t2	24
t3	48
t4	72

2.1.2 Harvesting diatom cells

Experimental cultures of the *T. pseudonana* were harvested through vacuum filtration with a Heto Master Jet SUE 300Q suction pump. The volume harvested at each sampling varied between the different experiments, as shown below:

Experiment	Harvest volume (mL)	
	For gene expression analysis	For DMSP quantification
1	≤ 150 (once)	-
2	-	25 (once daily)
3	50	25

Procedure:

1. Samples of different culture volume were decanted into a 25 mm filter funnel in polysulfone (PALL).
2. The samples were vacuum filtrated through a hydrophilic 0.65 µm DVPP Durapore® membrane filter (Merck Millipore).

From here the procedure among the different experiments varied:

Experiment 1:
Samples for gene expression analysis:

3. The filter was transferred to a 2 mL safe seal microtube (Sarstedt) containing 1 mL RNA*later*® RNA Stabilization Reagent (QIAGEN), in order to inhibit RNA degradation during the harvesting procedure. The cells were resuspended in the RNA*later* solution by vortexing.
4. The filter was removed and the tubes were centrifuged at full speed (≥ 16 000 x g).

5. The supernatant was discarded and the remaining cell pellet was flash frozen in liquid nitrogen.
6. Cell pellets were stored at -80 ºC.

Experiment 2 and 3

Samples for DMSP quantification:

3. The filter was transferred to a 13 mL tube (Sarstedt) containing 2 mL citrate-phosphate buffer (60 mM) and diatom cells were removed from the filter by vortexing.
4. The filter was removed and the tubes were stored on ice for sonification.

Experiment 3

Samples for gene expression analysis:

3. Filters were transformed to 2 mL safe seal microtubes (Sarstedt) containing 1 mL f/2 media. Samples from time point t0 were added complete f/2 media, whereas samples from t1-t4 were added f/2 media without supplemented Na_2SiO_3. Cells were removed from the filter by vortexing.
4. The filter was discarded and the tubes were centrifuged at full speed (≥ 16 000 x g).
5. The supernatant was removed by pipetting and the remaining cell pellet was flash frozen in liquid nitrogen.
7. Cell pellets were stored at -80 ºC and total RNA was isolated by scientific staff member Torfinn Sparstad at the Norwegian Functional Genomics initiative (FUGE) lab (NTNU) using the QIAGEN protocol B, described in section 2.2.1.

2.1.3 Cell counting

Cell counting of *T. pseudonana* was performed using a Bürker counting chamber (Marienfeld Superior). 1 droplet of Lugol's solution (iodine-potassium iodide (IKI)) was added to 1 mL culture, in order to stain and kill the cells. Cells were counted using a Nikon Eclipse E800 light microscope, and the cell density was calculated by multiplying the mean cell number for 6 diagonal squares from the Bürker counting chamber with 10^4.

2.1.4 Axenity testing

The axenity of the batch cultures was tested using f/2 with added peptone (1 g/L). 5 mL f/2 with peptone was added to a sample of 0.5 mL culture in a 15 mL centrifuge tube (Corning®). The tubes were wrapped in aluminum foil and incubated in room temperature. After one week, the tubes were checked for bacterial growth.

2.1.5 Estimating cell volume

Cell volume of *T. pseudonana* samples was estimated using a Nikon Eclipse E800 light microscope and the software programs NIS elements and ImageJ. For calibration, a S21 micrometer scale (0.01 - 5 mm) from Pyser-SGI Limited was utilized.

15 µL of each sample were prepared for light microscopy. Pictures were taken using a Nikon DS-Ri1 camera connected to the light microscope through a Nikon Photo Head V-TP multi-point sensor system. The pictures were captured using NIS Elements from Nikon. The height and diameter of 15 cells per sample were measured using ImageJ. Since *T. pseudonana* cells have a cylindrical shape, the cell volumes were estimated using the formula for calculating the volume of a cylinder:

$$\mathbf{V = \pi r^2 h}$$

where **V** is the cell volume, **r** is radius and **h** equals the height of the cell.

2.2 Gene expression analysis

2.2.1 RNA purification

In order to study gene expression total RNA was isolated and purified, using column based RNA isolation kits. Column based kits allow isolation of total RNA without the use of hazardous chemicals or time consuming steps. Guanidine salts and 2-mercaptoethanol (2-ME) are used to inhibit RNA degradation by denaturing any degrading enzymes present. Total RNA are bound and eluted using a charged column matrix. The isolated total RNA are pure enough for studying the expression level of a specific messenger RNA (mRNA), using sensitive techniques such as microarray analysis, cDNA synthesis, RT-PCR or Northern blot analysis (Morse et al., 2006).

Procedure

Two procedures for RNA isolation were used during this study. The first was based on the user guide of the Spectrum™ Plant Total RNA kit from Sigma Life Science (Sigma-Aldrich, 2010). The second procedure was based on the "Purification of Total RNA from Plant Cells and Tissues and Filamentous Fungi" protocol in the RNeasy® Mini Handbook using the RNeasy® Plant Mini Kit from QIAGEN (QIAGEN, 2010). This protocol included a step for reducing the viscosity of the samples, using a QIAshredder spin column (QIAGEN, 2010). In this study, the QIAshredder spin column was not always used. Therefore, the second protocol can be subdivided into "QIAGEN Protocol A" and QIAGEN Protocol B", as shown in Table 2.2.1.

Table 2.2.1 Protocols for RNA purification. RNA was purified using the Sigma Life Science " Spectrum™ Plant Total RNA" protocol and the QIAGEN protocol for " Purification of total RNA from Plant cells and tissues and filamentous fungi". The latter is subdivided into protocol A and B, depending on whether the QIAshredder spin column step was performed. QIAGEN protocol A includes the QIAshredder spin column and protocol B does not.

	Protocols	**Kit**
1	Sigma Life Science Protocol: Spectrum™ Plant Total RNA	Sigma Life Science Spectrum™ Plant Total RNA kit
2A	QIAGEN Protocol A: Purification of total RNA from Plant cells and tissues and filamentous fungi - Including the QIAshredder spin column step	QIAGEN RNeasy® Plant Mini Kit
2B	QIAGEN Protocol B: Purification of total RNA from Plant cells and tissues and filamentous fungi - Not including the QIAshredder spin column	QIAGEN RNeasy® Plant Mini Kit

Pre-treatment

Pellets of *Thalassiosira pseudonana*, stored at -80 ºC, were added a frozen, 5 mm stainless steel bead. The algal cells were homogenized in a QIAGEN TissueLyser (Retsch) for 2 minutes at 25 Hz.

RNA isolation using the SIGMA Spectrum™ Plant Total RNA kit:

1. Samples of homogenized algal cells were treated with 500 µL Lysis Solution, mixed briefly and homogenized further in the QIAGEN TissueLyser at 25 Hz for 2 minutes. The added lysis solution was supplemented with 10 µL 2-ME per 1 mL Lysis Solution.
2. Lysed cells were incubated at 56 ºC for 3 minutes and centrifuged at maximum speed (≥16 060 x g) for 3 minutes. The supernatant, including some of the floating layer, was transferred to a Filtration Column inserted in a 2 mL Collection Tube and centrifuged at maximum speed for 1 minute. The Filtration Column was thrown after centrifugation.
3. To bind RNA in the sample, 750 µL Binding Solution was added to the flow-through lysate and mixed by pipetting. The lysate was transferred to a Binding Column inserted in a 2 mL Collection Tube. Samples were centrifuged at full speed for 1 minute. The flow-through was decanted and the Collection Tube was tapped on a clean absorbent paper to drain off residual liquids.
4. To remove traces of genomic DNA (gDNA), samples were treated with on-column DNase digestion using the RNase-free DNase set from QIAGEN. 300 µL Wash Solution 1 was added to the Binding Column, and the samples were centrifuged for 1 minute at maximum speed, discarding the flow-through. A mixture of 10 µL DNase I stock solution (1500 Kunitz units dissolved in 550 µL RNase-free water) and 70 µL Buffer RDD was added directly on the center of the Binding Column membrane. Samples were incubated at room temperature for 15 minutes under closed caps. To remove the digested DNA, 500 µL Wash Solution 1 was added and the samples were centrifuged for 1 minute and the flow-through was discarded.
5. 500 µL diluted Wash Solution 2 was added to the samples (for 250 preparations, concentrated Wash Solution 2 was diluted with 300 mL ethanol prior to first time use). Samples were centrifuged at full speed for 30 seconds and the residual flow-through was discarded. This washing step was repeated, reusing the Binding Column.
6. The Binding Column was dried by centrifuging the Collection Tube containing the Binding Column at maximum speed for 2 minutes. The Binding Column was carefully transferred to a new, clean 2 mL Collection Tube.
7. RNA was eluted by adding 50 µL Elution Solution directly onto the center of the Binding Column matrix and incubating the samples in room temperature for 1 minute

with caps closed. The RNA was eluted from the Binding Column by centrifuging the Collection Tube for 1 minute.

8. Purified RNA was stored at -80 ºC.

RNA isolation using the QIAGEN RNeasy® Plant Mini Kit:

1. Pre-treated algal cells were briefly mixed with 450 µL Buffer RLT (which was added 10 µL 2-ME per mL buffer RLT).
2. The samples were homogenized in a QIAGEN TissueLyser at 25 Hz for 2 minutes and incubated at 56 ºC for 3 minutes.
3. **Protocol A:** In an additional step a QIAshredder spin column was used. This column is designed to reduce the viscosity of the lysate, by removing cell debris and simultaneously homogenize the lysate (QIAGEN, 2010). The lysate was transferred to the QIAshredder spin column placed in a 2 mL collection tube and centrifuged at maximum speed (≥ 16 000 x g) for 2 minutes.
3. **Protocol B:** Samples not treated with the QIAshredder spin column step was centrifuged at maximum speed for 2 minutes.
4. The supernatant was transferred to a microcentrifuge tube (2 mL Collection Tube from the Sigma Spectrum™ Plant Total RNA kit) and mixed with 500 µL ethanol (96 %) by pipetting. 5. The sample (usually 650 µL) was transferred to an RNeasy spin column placed in a 2 mL collection tube. The sample was centrifuged for 30 seconds at full speed, and the flow-through was discarded.
5. An on-column DNase digestion was performed on the samples with the QIAGEN RNase-Free DNase Set. 350 µL Buffer RW1 was added to the RNeasy spin column. The samples were centrifuged for 3 minutes at maximum speed and the flow-through was discarded. A mixture of 10 µL DNase I stock solution and 70 µL Buffer RDD was added directly to the membrane of the RNeasy spin column. Samples were incubated for 15 minutes at room temperature. 350 µL Buffer RW1 was added to the RNeasy spin column after incubation. The samples were centrifuged at maximum speed for 30 seconds and the flow-through was discarded.
6. 500 µL Buffer RPE (concentrated Buffer RPE is added 4 volumes of ethanol (96 %) prior to first time use) was added to the RNeasy spin column to wash the spin column membrane. The samples were centrifuged at full speed for 30 seconds and the flow-

through was discarded. This step was repeated with a centrifugation at maximum speed for 2 minutes.

7. To eliminate any traces of Buffer RPE and flow-through the RNeasy spin column was placed in a new 2 mL collection tube and centrifuged at full speed for 1 minute.
8. The RNeasy spin column was placed in a 1.5 mL collection tube. For eliminating the RNA from the RNeasy spin column, 50 µL RNase-free water was added directly to the spin column membrane and the sample was centrifuged at full speed for 1 minute (QIAGEN, 2010).

RNAsin treatment

Eluted RNA was treated with Recombinant RNAsin® RNase Ribonuclease Inhibitor from Promega. RNAsin® is a noncompetitive RNase inhibitor that inactivates RNase by nonconvalent binding and thereby inhibits degradation of RNA (Promega, 2008). 1 unit (U) RNAsin® was added per µL RNA to each sample of purified RNA. Purified RNA treated with RNAsin was stored at -80 ºC.

RNA Cleanup

RNA cleanup was carried out as according to the RNeasy® Mini Handbook using the RNeasy® Plant Mini Kit from QIAGEN (QIAGEN, 2010). This was only performed in Experiment 1.

1. RNase-free water was added to adjust the RNA sample to a total volume of 100 µL.
2. The diluted RNA was added 350 µL Buffer RLT and the sample was mixed by pipetting, along with 250 µL ethanol (96 %).
3. The sample was transferred to a RNeasy spin column, centrifuged at full speed (≥16 000 x g) for 30 seconds and the flow-through was discarded.
4. 350 µL Buffer RW1 was added, and the sample was centrifuged at 30 seconds and the flow-through was discarded.
5. The sample was treated with on-column DNase digestion from the QIAGEN RNase-Free DNase Set. A mixture of 10 µL DNase I stock solution and 70 µL Buffer RDD was added directly to the membrane of the RNeasy spin column, and incubated at room temperature for 15 minutes. 350 µL Buffer RW1 was added, the sample was centrifuged at full speed for 30 seconds and the flow-through was discarded.

6. 500 µL RPE was added, the samples were centrifuged at full speed for 30 seconds and the flow-through was discarded. This step was repeated with a centrifugation at maximum speed for 2 minutes. The RNeasy spin column was placed in a new 2 mL collection tube and was centrifuged at full speed for 1 minute.
7. The spin column was placed in a 1.5 mL collection tube and the RNA was eluted by adding 30 µL RNase-free water directly to the center of the membrane of the spin column and centrifuged at maximum speed for 1 minute. The elute was transferred to the same spin column and centrifuged at full speed for 1 minute for concentrating the RNA.

2.2.2 Optimizing RNA isolation

Sample harvesting and RNA extraction are the first potential sources of experimental variability in using sensitive methods for quantification of RNA transcripts, such as RT-qPCR (Bustin et al., 2009; QIAGEN, 2009). For reliable results, RT-qPCR requires RNA of high quality and quantity (Bustin et al., 2009). Therefore, optimizing the method for RNA isolation was needed.

Protocols for RNA purification:

The protocols; Sigma Life Science Protocol and QIAGEN protocol A and B, shown in Table 2.2.1 and described in section 2.2.1 were tested and the resulting RNA yield was compared. Nonaxenic batch cultures of *T. pseudonana* were harvested using centrifugation and RNA*later*. The cultures had been growing in a Photobioreactor TMT 150 from Photon Systems Instruments for 4 weeks in continuous light at 20 ºC.

Procedure for harvesting:

1. 450 mL of *T. pseudonana* culture were dispersed into 50 mL centrifuge tubes (Corning) and centrifuged at 4495 x g for 10 minutes at 18 ºC.
2. The supernatant was poured off and the cell pellet was resuspended in 1 mL RNA*later*.
3. Samples were transferred to 2 mL safe seal microtubes (Sarstedt) and centrifuged at ≥ 16 000 x g for 1 minute at 4 ºC.

4. The supernatant was removed by pipetting and the remaining cell pellet was flash frozen in liquid nitrogen.
5. Cell pellets were stored at -80 ºC.

In this study harvesting by centrifugation was later replaced by vacuum filtration, as described in section 2.1.2.

Membrane filters:

In addition to testing protocols for RNA purification, three different membrane filters for vacuum filtration were tested. Axenic batch cultures of *Thalassiosira pseudonana* were harvested using the following filters:

Filter	**Pore size (µm)**	**Diameter (mm)**	**Producer**
Durapore® DVPP Membrane filter	0.65	25	Merck Millipore
Supor 800 membrane disc filter	0.8	25	Pall Corporation
Polycarbonate (PCTE) membrane	1.0	25	GE Osmonics

The batch culture used was estimated to have a cell density of 2 613 300 cells mL^{-1}, and was obtained from Matilde Skogen Chauton from the Department of Biotechnology at NTNU. The batch culture was grown in f/2 without silicate supplementation at a total volume of 1800 mL in a 2000 mL Nalgene optically clear flask. Two replicas of 75 mL were harvested for each membrane filter tested. Cells were removed from the filters using 1 mL f/2 without supplemented silicate. RNA*later* was not used. The harvesting was based on the procedure described in section 2.1.2 for Experiment 3. Total RNA was isolated by QIAGEN protocol B, by FUGE scientific staff member Torfinn Sparstad. The RNA yield was used to compare the membrane filters.

2.2.3 Quantification of RNA

In this study, quantification of RNA was measured by Nanodrop and Qubit.

RNA quantification by Nanodrop

RNA was quantified by using a NanoDrop™1000 spectrophotometer. The quantification was performed using the NanoDrop™1000 spectrophotometer manual. 1.5 µL Elution Solution (Sigma-Aldrich) was used as blank.

RNA quantification by Qubit

RNA was quantified by Qubit® (1.0) fluorometer from Invitrogen, using the Quant-iT™ RNA Assay Kit from Invitrogen. The quantification was performed according to the Qubit™ RNA Assay Kit protocol (Invitrogen, 2010).

1. The Qubit™ Working solution was prepared using 199 µL Qubit® RNA Buffer and 1 µL Qubit® RNA Reagent per sample.
2. Two standards were made from Qubit® RNA Standard # 1 (0 ng/µL in TE buffer) and Qubit® RNA Standard # 2 (10 ng/µL in TE buffer), using 10 µL of each standard solution and 190 µL working solution.
3. 2 µL RNA sample was mixed with 198 µL working solution.
4. Standards and samples were vortexed and incubated for 2 minutes.
5. Quantification was performed using the Qubit® (1.0) fluorometer.

2.2.4 FA gel electrophoresis

To check the integrity and size distribution of the purified total RNA, formaldehyde agarose (FA) gel electrophoresis was performed. FA gel electrophoresis is a type of denaturing agarose gel electrophoresis that visualizes ribosomal RNA using ethidium bromide staining.
RNA of high quality should show sharp bands of ribosomal RNA on the gel and the ratio of 28S rRNA to 18S RNA should be about 2:1. Bands that appear as a smear towards smaller sized RNAs are likely to have suffered major degradation during the harvesting or RNA purification procedure (QIAGEN, 2010).

Procedure:

A FA gel (1.2 % agarose) of size 5 x 7 x 0.35 cm was prepared and FA gel electrophoresis was performed according to the RNeasy® Mini Handbook from QIAGEN (QIAGEN, 2010).

1. 0.6 g UltraPure Agarose from Invitrogen was mixed with 5.0 mL 10 x FA gel buffer, and diluted by autoclaved MilliQ water to a volume of 50 mL.
2. The gel mixture was heated in a microwave oven to melt the agarose.
3. The mixture was cooled in water bath to 65 ºC, and added 0.9 mL 37 % (12.3 M) formaldehyde and 0.5 µL of 10 mg/mL ethidium bromide stock solution.
4. The gel mixture was allowed to solidify in a gel support from BioRad.
5. A 1 x FA gel running buffer was made by mixing 50 mL of 10 x FA gel buffer with 10 mL of 37 % (12.3 M) formaldehyde. The mixture was diluted with autoclaved MilliQ water to 500 mL.
6. The molded gel was placed in a Bio-Rad Mini-Sub® Cell GT, and was left to equilibrate in the running buffer for at least 30 minutes prior to gel electrophoresis.
7. RNA samples were prepared using 1 µg RNA, 2-3 µL 5 x loading buffer and the remaining volume of autoclaved MilliQ water. Each RNA sample had a volume of 10 µL in total.
8. Samples were incubated at 65 ºC for 4 minutes and immediately chilled on ice.
9. The samples were loaded on the FA gel and the gel was run at 90 V in the 1 x FA gel running buffer, allowing the RNA bands to split.
10. The RNA was visualized using Bio-Rad Gel Doc 2002 and Quantity One version 4.2.1 by Bio-Rad.

2.2.5 cDNA synthesis

A complementary DNA molecule (cDNA) was synthesized from the RNA template by reverse transcription (RT). In reverse transcription, cDNA is synthesized by a reverse transcriptase enzyme. Reverse transcriptases originate from retroviruses and are RNA-dependent DNA polymerases that catalyses the addition of new nucleotides to the growing DNA chain in a 5' to 3' direction using a single stranded RNA-molecule as template. The RNA in the resulting RNA-DNA hybrid is rapidly degraded by the enzyme's ribonuclease (RNase H) activity (Reece, 2004). cDNA synthesis by reverse transcriptase is illustrated in

Figure 2.2.5. For this study, cDNA was synthesized prior to quantitative real-time Reverse Transcription Polymerase Chain Reaction (RT-qPCR).

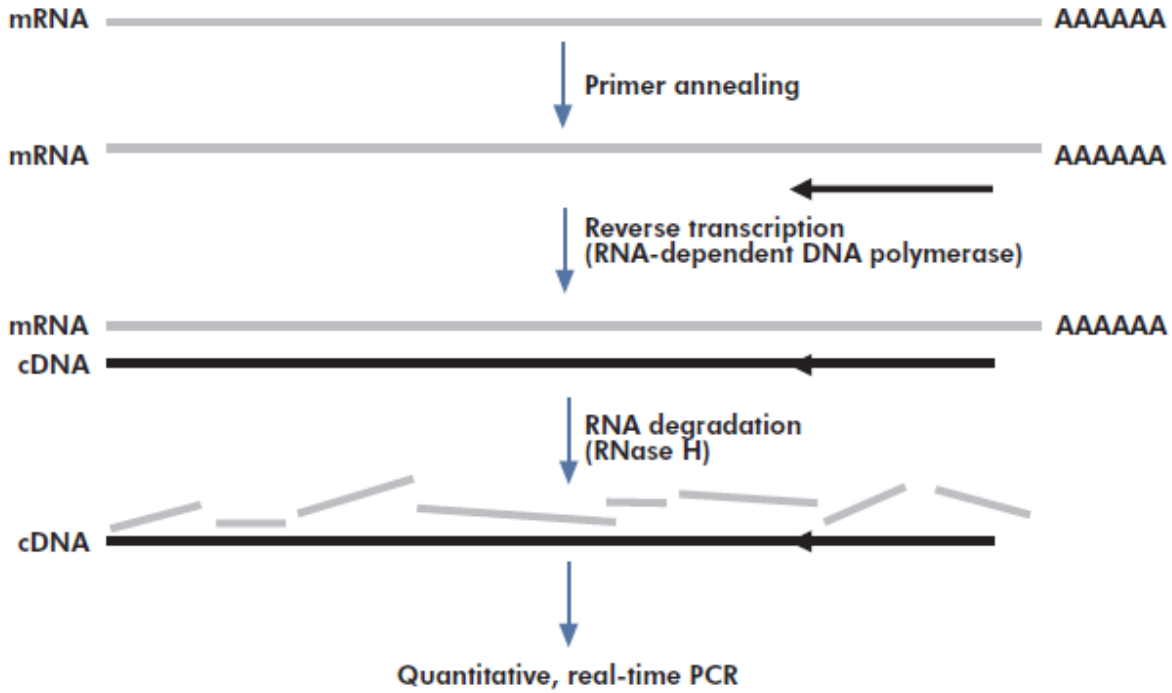

Figure 2.2.5 cDNA synthesis. A complementary DNA molecule (cDNA) is synthesized from a RNA molecule by reverse transcription. PCR primers are annealed to the mRNA at 42 ºC. A reverse transcriptase enzyme transcribes a cDNA molecule to the RNA template, and degrades the RNA in the RNA-DNA hybrid using the enzymes hybrid-dependent exoribonuclease (RNase H) activity. The enzyme is inactivated using high temperatures (95 ºC) (QIAGEN, 2009).

Procedure:

cDNA was synthesized using the QIAGEN QuantiTect® Reverse Transcription Kit, according to the "Reverse Transcription with Elimination of Genomic DNA for Quantitative, Real-Time PCR" protocol in the QuantiTect® Reverse Transcription Handbook from QIAGEN (QIAGEN, 2009).

1. Template RNA was thawed on ice.
2. gDNA Wipeout Buffer, Quantiscript Reverse Transcriptase, Quantiscript RT Buffer, RT Primer mix and RNase-free water was thawed at room temperature.
3. To eliminate any traces of genomic DNA, the RNA samples were treated with gDNA Wipeout Buffer. The following components were mixed in 0.2 mL PCR tubes on ice:

Component	Volume per Reaction
gDNA Wipeout Buffer, 7x	2 µL
Template RNA	Variable (1 µg)
RNase-free water	Variable
Total Volume	14 µL

4. Samples were incubated at 42 ºC for 2 minutes and immediately placed on ice.
5. The reverse-transcription master mix was prepared on ice in 1.5 mL autoclaved Eppendorf tubes as follows:

Component	Volume per Reaction (µL)
Quantiscript Reverse Transcriptase	1
Quantiscript RT Buffer, 5 x	4
RT Primer Mix	1
Total Volume	6

6. 6 µL of the mastermix was added to each RNA sample, now containing a total volume of 20 µL.
7. RNA samples were incubated at 42 ºC for 15 minutes for cDNA synthesis to occur. Incubation was performed in a GeneAmp® PCR System 9700 (Version 3.01) from Applied Biosystems.
8. The Quantiscript Reverse Transcriptase was inactivated by incubating the samples at 95 ºC for 3 minutes.
9. cDNA was stored at -20 ºC.
10. No reverse transcription (NRT) controls were made using half of the volumes of the genomic DNA elimination reaction and mastermix, replacing the Quantiscript Reverse Transcriptase with MilliQ water.

2.2.6 Qualitative real-time Reverse Transcription PCR (RT-qPCR)

The resulting cDNA molecules from the RT-reaction were used in gene expression analysis by quantitative real time PCR (qPCR). RT-qPCR is a fluorescence-based method for detecting and quantifying mRNA targets of low copy number *in vivo* (Bustin et al., 2009; Huggett et al., 2005). The cDNA, converted from mRNA in the RT-reaction, is amplified by PCR and the resulting reaction product is measured in real-time in every cycle. The detection and

quantitation rely upon a fluorescent reporter, like SYBR® Green, that binds to double stranded DNA in the minor groove and emits light upon excitation. The signal increases in direct proportion to the amount of PCR products in the reaction, so that when PCR products accumulates the fluorescence increases (Reece, 2004).

Crossing thresholds (Ct) are the number of cycles required to enter a defined fluorescence intensity measured in real time, marking the visible, exponential phase of the PCR. These values depend on the initial amount of target DNA in a sample at the beginning of the PCR reaction. The fluorescence intensity originates from the excitation of the non-sequence-specific dye (SYBR® Green) bound to a double-stranded DNA molecule. Samples containing high copy numbers of a target sequence will generate low Ct-values (Guénin et al., 2009). The quantification of mRNA can be used to investigate physiological changes in gene expression levels, by calculating the relative expression based on the differences in concentrations of a target gene between samples, normalized by reference genes (Pfaffl et al., 2002; Bustin et al., 2009).

Procedure:

RT-qPCR was performed using the LightCycler® 480 SYBR Green I Master kit from Roche Applied Science, according to the manufacturer's protocol (Roche Applied Science, 2011). The reactions were run in a LightCycler® 480 Multiwell Plate 96 in a LightCycler® 480 instrument from Roche Applied Science. All work on cDNA was performed on ice.

1. Samples of cDNA (20 µL) and NRT (10 µL) was diluted 1:10 using MilliQ water.
2. PCR master mixes were prepared in 1.5 mL autoclaved Eppendorf tubes as follows:

Component	Volume per Reaction (µL)
PCR grade H_2O	3
PCR primers (10 µM)	2
LightCycler® 480 SYBR Green I Master, 2 x	10
Total Volume	15

3. 15 µL master mix and 5 µL of the cDNA template were added each well on a LightCycler® 480 Multiwell Plate 96 from Roche-Applied-Science. The same volumes were applied for the NTR controls. No template control (NTC) samples

were made using MilliQ water instead of cDNA solution, in order to detect any contaminations which can cause false positive results.

4. The multiwell plate was sealed with a LightCycler® 480 Multiwell Sealing Foil.
5. The sealed multiwell plate was centrifuged at 1500 x g for 2 minutes.
6. The plate was run in a LightCycler® 480 instrument (Roche Applied Science), programmed to the following PCR parameters:

Program	Temperature (°C)	Time	Cycles
Pre-incubation	95	5 min	1
Amplification	95	10 sec	45
	55	10 sec	
	72	10 sec	
Melting curve	95	5 sec	1
	65	1 min	
	65-97	-	
Cooling	40	10 sec	1

The RT-qPCR primers used are listed in Appendix 2, together with the ID accession numbers of the candidate genes. The reference genes Histone H4 and TBP (TATA box binding protein) were chosen, based on an experiment on the diatom *Phaeodactylum tricornutum* (Siaut et al., 2007).

2.2.7 Analyses of RT-qPCR data

Raw data were obtained from the LightCycler® 480 software (v.1.5.1.62) and melting curves were generated using the Tm Calling Analysis Module. Melting curves were inspected for primer-dimers and non-specific products, and NRT controls and NTCs were checked for genomic DNA contaminations. Raw data were further analyzed using LinRegPCR (v.11.1) and REST 2009 (v.2.0.13) software.

1. LinRegPCR (v.11.1) software was used to perform linear regression. Ct values were calculated, baseline estimation was performed and the mean PCR efficiency per amplicon group (primer set) was determined.
2. Ct-values and mean PCR efficiencies per amplicon calculated by LinRegPCR were imported to the REST 2009 (v.2.0.13) software. In Experiment 1, samples from cultures grown in complete f/2 media were defined as controls and compared to samples from silicate and nitrate limited cultures. In Experiment 3, samples from the

first harvesting point, t0, were defined as controls and samples from the remaining time points, t1-4, were defined as treated samples.

3. Relative expression ratios of the target genes normalized to the chosen reference genes were obtained by REST 2009. A pair-wise fixed reallocation randomization test was performed using REST 2009 to calculate the significance of the expression ratios. Expression ratios with p-values less than 0.05 were considered as statistically significant.

2.3 Quantification of DMSP levels

Total and intracellular dimethylsulfoniopropionate (DMSP) levels were determined using solid-phase microextraction (SPME) and gas chromatography-mass spectrometry (GC-MS).

2.3.1 Sonication

Cell samples harvested for DMSP quantification were lysed by sonication. Sonication is an acoustic method for single cell lysis that uses ultrasonic waves. The high pressure generated by the ultrasonic waves leads to cavitation that shears the cells apart (Brown and Audet, 2008).

Procedure:

Sonication was performed with a Branson Sonifier 250, using a Branson double stepped microtip. The sonication was performed using the following setting on the sonifier:

Duty cycle %: 40
Output control: 3.5
Timer: off

The microtip was cleaned with MilliQ water and ethanol before and between each sonication.

1. Samples of cells solved in 2 ml citrate-phosphate buffer (60 mM) were sonicated 2 x 10 seconds, with 20 seconds break to avoid overheating. The samples were kept on ice during the sonication procedure.
2. Sonicated samples were stored at -80 ºC.

2.3.2 SPME and GC-MS

DMSP levels were quantified by using solid-phase microextraction (SPME) and gas chromatography-mass spectrometry (GC-MS). SPME is a method for extracting volatile compounds (such as DMS) using a syringe-like unit. This unit is composed of a holder and a retractable fused-silica fiber (coated with absorbents or adsorbents or both) in a septum-piercing needle. SPME used to extract volatile compounds from the headspace gas of a vial is called headspace SPME (HS-SPME). Target compounds are extracted and concentrated on the SPME fiber and can be further analyzed by GC-MS, by introducing the fiber directly into the GC injection port (Niki et al., 2004).

GC-MS is an analytical technique for the identification and quantification of the molecular components of a sample. The technique combines gas chromatography (GC) to separate the different components and mass spectrometry (MS) for analyzing each individual component. In the heated GC injection port, the injected sample vaporizes and is transported by a flow of inert gas into a capillary column. The inside of the GC column is coated with a stationary phase of low polarity, to which the components of the sample are able to bind as they pass the column. Due to different boiling points and degrees of affinity for the stationary phase, the components in the sample travel at different rates through the column. The time required for each component to travel through the column is called the retention time. As the components exit the GC column, they enter the mass spectrometer. Here, the molecules are bombarded with electrons by an electron beam source. The resulting ions and molecule fragments are detected and results in a mass spectrum for each component of the original mixture. A mass spectrum is a graph showing the abundance of each detected ion as a function of the mass-to-charge ratio. The mass spectra are able to provide structural information on each component of a sample as well as quantitative data, when used with standards of known concentrations (Solomons and Fryhle, 2008).

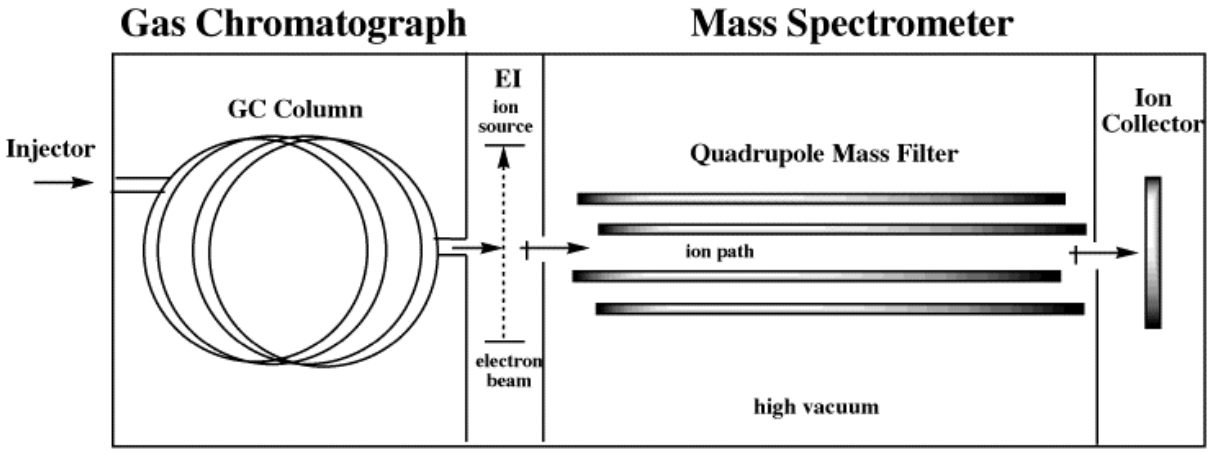

Figure 2.3.1 Gas chromatography-mass spectrometry (GC-MS). A schematic diagram of GC-MS. The gas chromatograph separates components of a mixture and the mass spectrometer provides structural information of each component (University of Colorado, 2011).

Procedure:

DMSP was quantified based on the procedure described in Niki et al. (2004). The SPME fiber was coated in a combination of carboxen (Car) and polydimethylsiloxane (PDMS). The glass bottles and screw caps used in this study belonged to the 2in1 kit (VWR, Catalog number 548-0521), which contains ND13 clear glass bottles (4 mL) and PP-screw caps (8.5 mm) with septum ND13.

1. Samples (2 mL) were thawed and transferred to a 4 mL glass bottles and added 0.2 mL 5 M sodium hydroxide (NaOH) to hydrolyze DMSP to DMS. The bottles were immediately closed with screw caps.
2. Standards were made using 200 µL DMSP of different concentrations and 2 mL NaOH (0.5 M). The preparation of standards is shown in Appendix 3.
3. Samples and standards were incubated for 30 minutes at 25 ºC in a thermostatic water bath shaker.
4. The SPME fiber was preheated in the injection port of the GC for one minute at 250 ºC.
5. DMS in the vial headspace gas of the samples was extracted by HS-SPME for 10 minutes, using the Car-PDMS fiber under static conditions at room temperature.
6. The DMS on the Car-PDMS fiber was immediately desorbed thermally in the injection port (250 ºC) of the GC in splitless mode for 2 minutes, by manual injection.

7. DMS was detected by a mass spectrometer with an electron impact (EI) ion source running in selecting ion monitoring (SIM) mode. Monitored *mass-to-charge ratios (m/z)* were 62.15 and 47.0.

GC-MS analysis was performed on a Hewlett-Packard GC 6890N linked to a 5975 inert Mass Selective Detector (Agilent Technologies). The GC column utilized was a HP-5MS 5 % phenylmethylsiloxane (30 m x 0.25 mm x 0.25 µm) from Agilent technologies and helium (He) (7.58 psi) was used as the inert carrier gas, with a flow rate at 1.0 mL min^{-1}. The GC oven temperature was programmed to rise from 50 to 150 ºC at 15 ºC/min, using a run time of 6.67 minutes. The injection temperature was 250 ºC and the interface was set to 200 ºC. The ion source temperature was at 230 ºC and the quadrupole mass analyzer at 150 ºC. The MS spectra was produced in SIM mode at 70 eV and the peak areas of the mass spectra were determined by the analysis software MSD Chemstation (Agilent Technologies).

Statistical analysis

The significance of the accumulation of DMSP during the experiments were tested using a paired student's t-test with a one-tailed distribution in Microsoft Office Excel 2007.

3. RESULTS

The overall purpose of this thesis was to gain more knowledge about the poorly understood biosynthesis pathway of the sulfur compound dimethylsulfoniopropionate (DMSP) in marine algae. The proposed candidate genes, *DiDECARB, SAMmt, REDOX* and *AT,* were investigated through various methods for their involvement in the biosynthesis of DMSP in the centric diatom *Thalassiosira pseudonana*. To induce the DMSP biosynthesis pathway, experimental cultures of *T. pseudonana* were grown under nutrient limitations, in order to trigger stress responses previously shown to induce DMSP production (Bucciarelli and Sunda, 2003). The expression of the candidate genes was analyzed using quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR). Several protocols for total RNA isolation were tested in order to find the optimal isolation procedure, yielding purified RNA of high quality and quantity. DMSP production was monitored in the experimental cultures, by quantifying DMSP using headspace solid-phase microextraction (HS-SPME) and gas chromatography-mass spectrometry (GC-MS). Axenity was tested in all the experimental cultures utilized in this study. Diatom cultures used for the first and second experiments were non-axenic, as the cultures tested positive for bacterial contaminants. The third experiment, however, was performed using axenic cultures of *T. pseudonana.*

3.1 Optimization of RNA isolation

3.1.1 Protocols for RNA purification

The three total RNA purification protocols described in Section 2.2.1 were tested in order to find the optimal procedure for RNA purification in *Thalassiosira pseudonana* for the experimental conditions used in this study. The quality of the protocols was determined by comparison of the resulting RNA yields and purities of each protocol. The resulting absorbance ratios and total RNA yields quantified by Nanodrop are shown in Table 3.1.1. Original data are presented in Appendix 4.

Table 3.1.1 Test of RNA purification protocols. Average RNA concentrations and absorbance ratios data from RNA isolation using the Sigma Life Science Protocol, QIAGEN Protocol A and QIAGEN Protocol B. The highest total RNA yield was measured in the RNA sample purified by the QIAGEN Protocol B, which also displayed the highest absorbance ratios.

Protocols	RNA concentration (ng/µL)	Absorbance ratio 260/280	Absorbance ratio 260/230
Sigma Life Science Protocol	37.6	1.84	1.36
QIAGEN Protocol A	192.6	2.06	0.53
QIAGEN Protocol B	293.6	2.17	2.07

In general, total RNA is accepted as pure if the absorbance ratio of $A_{260/280}$ lies between 2.0-2.1 and the $A_{260/230}$ ratio is close to 2.0 (Johnson et al., 2012). $A_{260/280}$ absorbance ratios under 2.0 indicate low pH or the presence of impurities such as proteins or phenols in the eluted total RNA (Johnson et al., 2012; Wilfinger et al., 1997). Low $A_{260/230}$ absorbance ratios (less than 2.0) indicate acidic pH or contaminations of polysaccharides and/or residuals reagents (phenol or guanidine) from the RNA purification, which absorb light around 230 nm (Johnson et al., 2012; Pico de Coaña et al., 2010; Wilfinger et al., 1997).

As shown in Table 3.1.1, the highest concentration of total RNA was measured in the RNA sample purified by the QIAGEN protocol B, at 293.6 ng/µL. Total RNA from the Sigma Life Science protocol was measured at 37.6 ng/µL, and total RNA from the QIAGEN Protocol A were concentrated at 192.6 ng/µL. The lowest absorbance ratios were measured in the RNA of the Sigma protocol, at 1.84 ($A_{260/280}$) and 1.36 ($A_{260/230}$). The QIAGEN protocols yielded RNA of high absorbance ratios at $A_{260/280}$, which in both cases were measured over a value of 2. The $A_{260/230}$ absorbance ratio of the QIAGEN Protocol A was low (0.53), whereas the same absorbance ratio was measured at 2.07 in the RNA sample of the QIAGEN Protocol B. The QIAGEN Protocol B was utilized in further experiments requiring purified RNA.

3.1.2 Choice of membrane filters

Three membrane filters were tested for harvesting by vacuum filtration. As described in Section 2.2.2, the membrane filters were used to harvest 75 mL samples of an axenic *T. pseudonana* culture, with a density of about 2 600 000 million cells mL^{-1}. Total RNA was

isolated from the cell pellets by using the QIAGEN Protocol B. Since objective parameters for the usability of the filters tested were difficult to define, it was decided to compare the quality of each membrane filter using Nanodrop data, indicating RNA quantify and purity. The concentrations and absorbance ratios of the isolated total RNA are shown in Table 3.1.2. Original data are presented in Appendix 4.

Table 3.1.2 Test of membrane filters. Average RNA concentrations and absorbance ratios of the purified RNA for the three membrane filters tested. Total RNA was isolated from cell pellets harvested from 75 mL samples of *T. pseudonana* culture by vacuum filtration. Two biological replicates were used per membrane filter tested.

Membrane filters	RNA concentration (ng/µL)	Absorbance ratio 260/280	Absorbance ratio 260/230
Durapore® DVPP Membrane filter (0.65 µm)	1622.40	2.19	2.03
Supor 800 membrane disc filter (0.80 µm)	1526.06	2.20	2.13
Polycarbonate (PCTE) membrane (1.0 µm)	1136.70	2.21	1.97

All of the membrane filters tested resulted in RNA of high concentrations and $A_{260/280}$ absorbance ratios. The variance in $A_{260/230}$ absorbance ratios was high between biological replicates for all the membrane filters, as shown in Table A4.2 in Appendix 4. The highest RNA yield was measured in the samples derived from cells harvested by the Durapore® DVPP Membrane filter (0.65 µm). High concentrations were also measured for the Supor 800 membrane disc filter (0.80 µm), whereas the polycarbonate (PCTE) membrane (1.0 µm) resulted in total RNA of lower concentrations. The Durapore® filter was utilized in further experiments, for reasons described in Section 4.1.2.

3.2 Gene expression analyses

Since DMSP is assumed to be involved in stress adaptations in marine algae, Experiment 1 was designed to induce DMSP biosynthesis in *T. pseudonana* by growing experimental cultures under stress-related conditions, in form of nutrient limitations (Bucciarelli and Sunda, 2003). Silicate-limited cultures were grown in f/2 medium without the amendment of the silicate f/2 nutrient Na_2SiO_3 x $9H_2O$, nitrate-limited cultures were grown in f/2 medium without supplemented $NaNO_3$, whereas controls were grown in fully enriched f/2 growth medium. Recipes of the growth media used are available in Appendix 1.

Cell cultures grown under nitrate limitations and silicate limitations were harvested to measure the gene expression of the candidate genes *DiDECARB, SAMmt*, *REDOX* and *AT*, using RT-qPCR (Lyon et al., 2011). The only available nitrate and silicate in the medium were the relatively low amounts present in the filtrated seawater. In a dense and growing culture, these traces were expected to be quickly depleted. The increase in DMSP production is shown to be highest when cells are grown in the stationary growth phase, when the influence of the nutrient limitations is strongest (Bucciarelli and Sunda, 2003). Based on the study by Bucciarelli and Sunda (2003) and early trial experiments, the experimental cultures were decided to be grown for a period of 5 days.

For this experiment, four biological replicates were used in order to increase the statistical significance of differences in the RT-qPCR results between the different nutrient treatments. To ensure that the cells in the experimental cultures were harvested in the stationary growth phase, samples were harvested daily for cell counting. The growth of the nutrient-limited cultures was compared with the growth of the control cultures, which were grown in fully enriched f/2 medium. The growth curves, showing average cell densities (cells mL^{-1}) over time (days) for each nutrient treatment, are presented in Figure 3.2.1. The original growth data of each biological replicate for the different nutrient treatments are presented in Appendix 5.

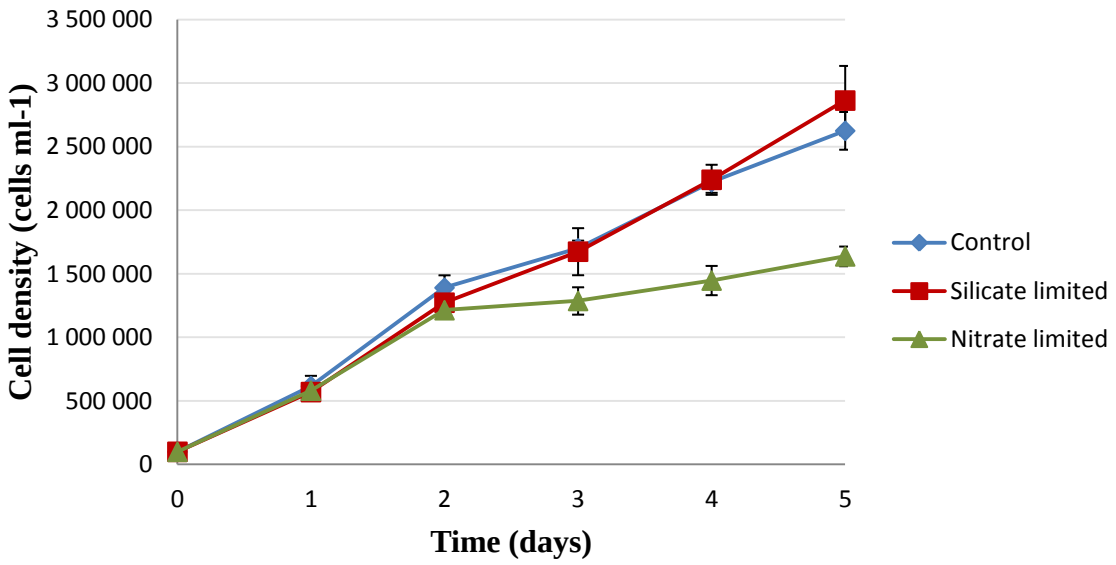

Figure 3.2.1 Growth curves from Experiment 1. Cell growth, presented as cell density (cells mL^{-1}) over time (days) of experimental cultures grown in nutrient limited media compared with control cultures grown in fully enriched medium. Standard derivations (SD) of each nutrient treatment are shown as error bars. n=4.

Each experimental culture were seeded to a density of about 100 000 cells mL^{-1} at starting point. The control cultures grew to an average cell density of about 2 600 000 cells mL^{-1} at harvest point, whereas the silicate-limited and the nitrate-limited cultures reached average cell densities of approximately 2 900 000 cells mL^{-1} and 1 600 000 cells mL^{-1}, respectively. Control and silicate-limited cultures exhibited similar growth patterns, and showed no indication of entering the stationary phase at day 5. The nitrate-limited cultures exhibited growth limitations at lower cell densities than the controls and silicate-limited cultures, and entered the stationary phase after the second day. To keep the cells in exponential phase for longer and to avoid additional nutrient limitations of unknown origin, the cell cultures were supplemented with f/2 nutrients at day 2. The silicate- and nitrate-limited cultures were not supplemented with silicate and nitrate, respectively. The feeding at day 2 showed no significant effects on the growth of the experimental cultures. Since the whole cultures were harvested at day 5, monitoring growth after the point of harvesting was not possible.

Total RNA was isolated from the cell pellets derived from the harvesting at day 5 (t5), using the QIAGEN Protocol B. The isolated RNA was further purified using the RNA cleanup procedure, as low 260/230 absorbance ratios indicated the presence of impurities. After the RNA cleanup procedure, the general quality of the RNA improved, although several samples of RNA had low $A_{260/230}$ ratios. One of the four biological replicates of the nitrate-limited

cultures (Tp 4-N) yielded RNA of poor quantity, with an average concentration of 31.21 ng/µL. This replica was not used for further analysis, because the concentration of RNA was too low for cDNA synthesis. The quantification data and integrity validations, including Nanodrop data, Qubit measurements and the formaldehyde (FA) gel, are shown in Appendix 4.

Equal amounts of isolated total RNA were converted to cDNA and amplified using two-step RT-qPCR. In order to investigate the involvement of the proposed candidate genes in the biosynthesis of DMSP, the relative gene expression of the candidate genes was compared between control and nutrient-limited cultures, using RT-qPCR analysis. The relative gene expression at harvest point, t5, is presented in Figure 3.2.2. Original data and analysis data from LinRegPCR and REST 2009 are shown in Appendix 6 and 7, respectively. The melting curves are presented in Appendix 8.

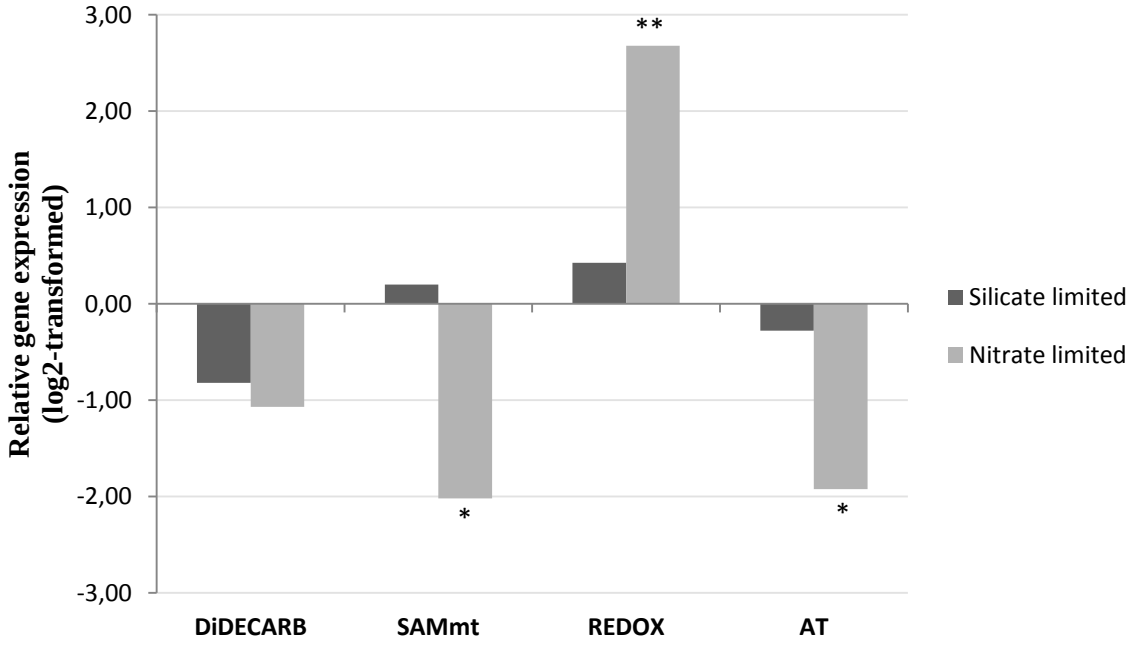

Figure 3.2.2 Relative gene expression of candidate genes. Expression ratios (log_2-transformed) for the candidate genes *DiDECARB*, *SAMmt*, *REDOX* and *AT* in silicate-limited and nitrate-limited cultures compared to control cultures. Genes that were found to be significantly differentially expressed compare to controls are indicated with an asterisk (*, P-value < 0.05; **, P-value < 0.01).

As shown in Figure 3.2.2 and Table A7.1 and A7.2 in Appendix 7, none of the candidate genes in the silicate-limited cultures were found to be significantly differentially regulated in

comparison to control cultures. In the nitrate-limited cultures, the *SAMmt* and *AT* genes were found to be significantly down-regulated (P-value <0.05) about 4 times compared to controls. The *REDOX* gene was found to be significantly up-regulated (P-value < 0.01) with a 6.4-fold change (arithmetic) compared to controls.

3.3 Quantification of DMSP

The diatom experimental cultures in Experiment 2 were grown at the same time and under the same environmental conditions as the experimental cultures in Experiment 1, to reduce the chance of variance derived from environmental sources. Total DMSP concentrations in the experimental cultures, including controls, silicate-limited and nitrate-limited cultures were quantified using HS-SPME and GC-MS analysis. The total DMSP concentration is the concentration of DMSP in the glass vial (2.2 mL) from the GC-MS analysis, containing cells harvested from 25 mL culture that have been resuspended in 2 mL phosphate-citrate buffer (60 mM), sonicated and added 0.2 mL NaOH (0.5 M) prior to GC-MS analysis. To monitor the growth of the cell cultures, samples were harvested daily for cell counting. The growth is shown in Figure 3.3.1, presented as cell density (cell mL^{-1}) over time (days). Original data are presented in Appendix 5.

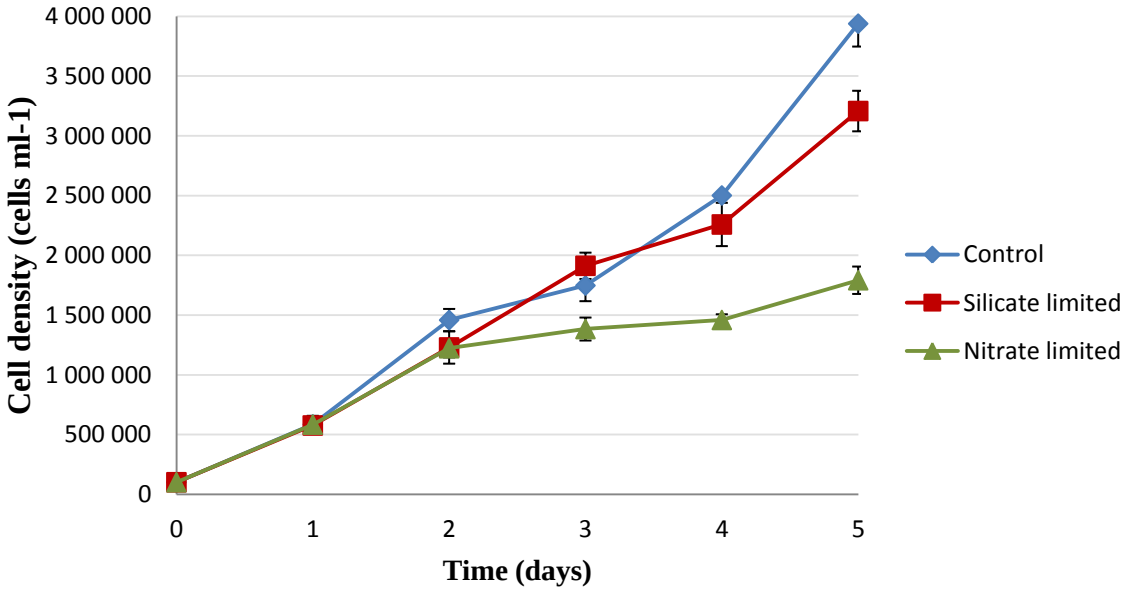

Figure 3.3.1 Growth curves from Experiment 2. Growth of the experimental cultures, including control, silicate-limited and nitrate-limited cultures, showing cell density (cells mL^{-1}) over time (days). Error bars indicate the standard deviation between the biological replicates. n=3.

The growth of the cultures, as presented in Figure 3.3.1, showed patterns similar to Experiment 1 (Figure 3.2.1). The initial cell density was calculated from the diluted inoculum culture, which had a cell density of approximately 1 700 000 cells mL^{-1}. Each experimental culture was cultivated with an initial cell density of about 102 000 cells mL^{-1}. The cell density of the control cultures increased to about 3 900 000 cells mL^{-1}, the silicate-limited cultures to about 3 200 000 cells mL^{-1} and the nitrate-limited cultures to 1 800 000 cells mL^{-1}. Similar to the first experiment, the cell cultures were added nutrients at day 2 to keep the cultures in exponential growth phase for a longer period and avoid uncontrolled nutrient limitations. From t2, the nitrate-limited cultures exhibited growth limitation, likely due to depletion of nitrate. The nitrate-limited cell cultures appeared to have entered stationary phase from day 2, regardless of the feeding at the second day. From day 2, it appears that the control and silicate-limited cultures experienced growth limitation, possibly due to nutrient limitations, and the increase in growth after day 3 might be a result of the supplementations of nutrients at t2. At the end of the experiment, the cell densities of the silicate-limited cultures were lower than in the controls, indicating that the silicate-limited cultures might exhibit growth limitation due to silicate depletion. Regardless, the growth data cannot verify that the cells of the control or silicate-limited cultures had entered stationary phase at day 5.

In addition to monitoring the growth of the experimental cultures, samples of 25 mL culture were harvested daily for DMSP quantification. Total DMSP levels were measured in each sample by HS-SPME and GC-MS, and quantified using DMSP standards of known concentrations. The original data of the standards utilized, including the calibration curve and calculations, are shown in Appendix 3, whereas the original data of the GC-MS analysis of the samples and calculations are presented in Appendix 9 and 10. The average total DMSP concentrations of each nutrient treatment over time are given in Figure 3.3.2.

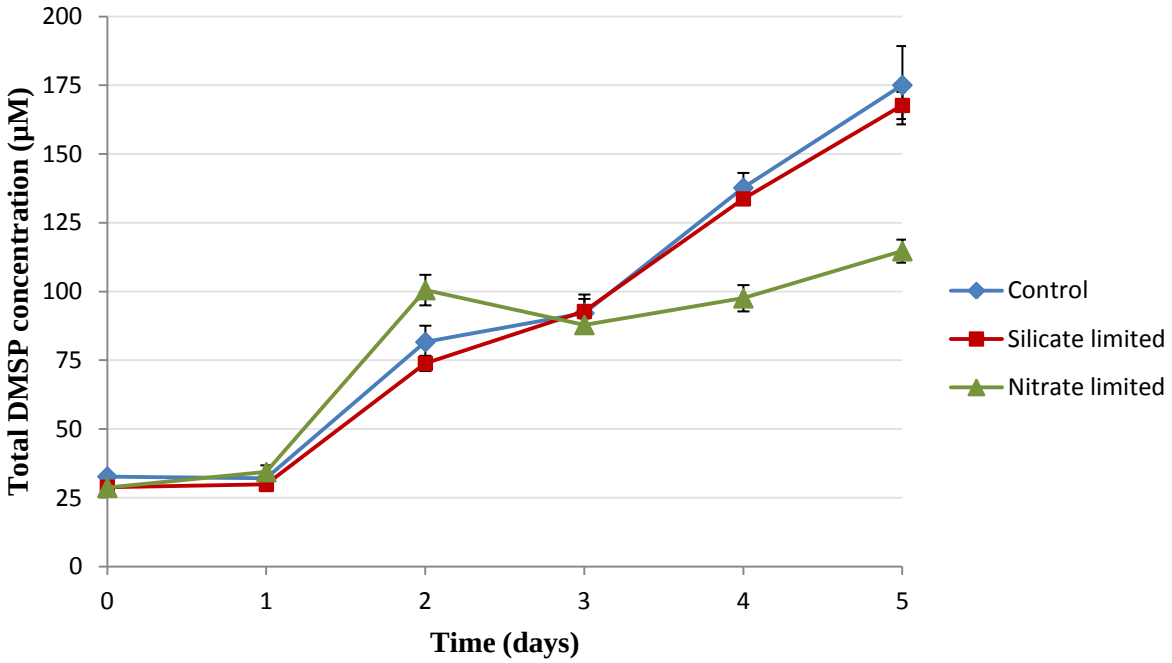

Figure 3.3.2 Total DMSP concentrations. Total DMSP concentrations, measured in micromolar (µM), in the GC-MS samples (2.2 mL) containing cells from 25 mL harvested culture, for each nutrient treatment over time (days). Standard deviations of each nutrient treatment are indicated by error bars. n=3.

The total DMSP concentrations, as presented in Figure 3.3.2, indicated an increase in the total DMSP in each of the nutrient treatments. The average total DMSP concentrations increased from 32.7 µM to 175.1 µM in the control cultures, from 28.9 to 167.7 µM in the silicate-limited cultures and from 28.7 µM to 114.7 µM in the nitrate-limited cultures. The total DMSP concentrations increased similarly in the control and silicate-limited cultures, whereas the nitrate-limited cultures displayed lower concentrations of total DMSP. The amount of intracellular DMSP was calculated from the total DMSP concentrations and the cell number of the sample harvested. The amounts of cellular DMSP are shown in Figure 3.3.3. Calculations and original data are shown in Appendix 11.

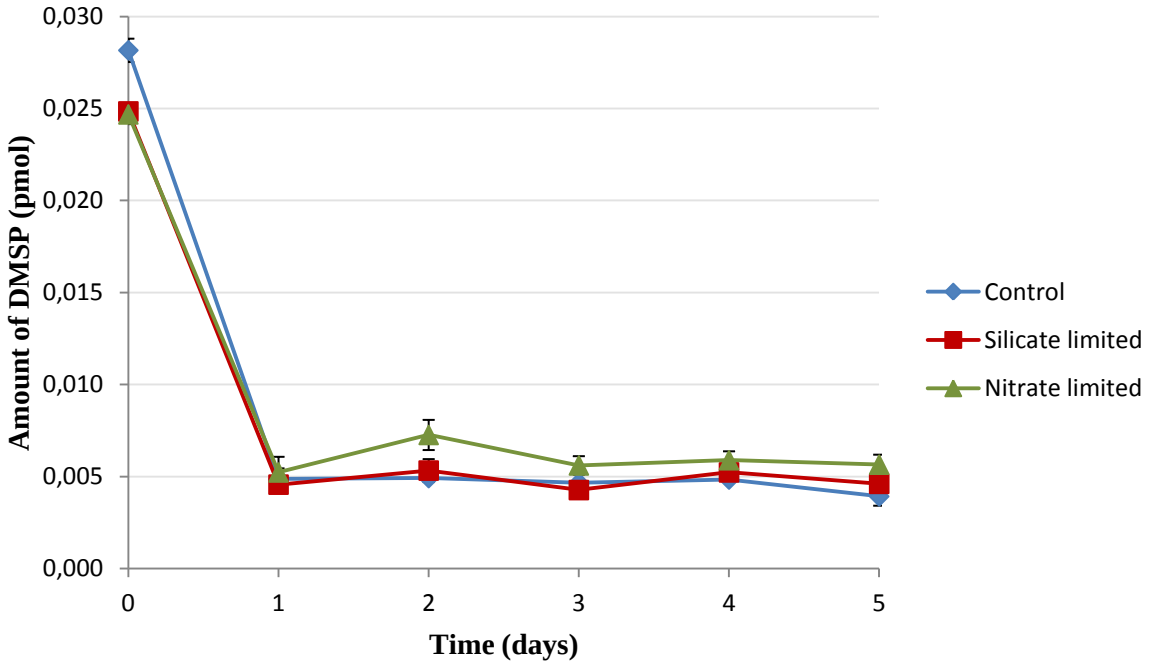

Figure 3.3.3 Amount of intracellular DMSP. Amount of DMSP per cell, measured in picomoles, over time (days) in each nutrient treatment. The error bars indicate the standard deviations of each nutrient treatment. n=3.

The amount of intracellular DMSP, as presented in Figure 3.3.3., decreased significantly during the first day in every nutrient treatment. The average amounts of intracellular DMSP decreased from 0.028 to 0.004 pmol in the controls, from 0.028 to 0.005 pmol in the silicate-limited cultures and decreased from 0.025 to 0.006 pmol in the nitrate-limited cultures. From day 1 to day 5, the cellular amounts of DMSP stabilized at around 0.005 pmol in average for the control and silicate-limited cultures, and about 0.006 pmol for the nitrate-limited cultures. The cellular amounts of DMSP were not significantly accumulated after 5 days of nutrient limitations of nitrate or silicate, compared to the starting point or day 1. The cellular amounts of DMSP in the silicate-limited cultures were not significantly higher than in the control cultures. However, significantly higher amounts of intracellular DMSP were measured in the nitrate-limited cultures at day 2 (P-value = 0.019), 4 (P-value = 0.002) and 5 (P-value = 0.0059) in compared to controls for the same time point.

3.4 Response of complete silicate starvation

In Experiment 1 and 2, non-axenic experimental cultures of *T. pseudonana* were grown under nutrient limitation of silicate or nitrate for 5 days. The experimental cultures of Experiment 1 were harvested at day 5 for gene expression analysis and samples of the experimental cultures of Experiment 2 were harvested daily for DMSP quantification. In the third experiment, however, axenic, exponentially growing cell cultures of *T. pseudonana* were grown over a time period of 72 hours in complete silicate-free f/2 media. Samples were harvested 20 minutes before Start and 7.5, 24, 48 and 72 hours after start, for gene expression analysis and DMSP quantification. The response of the silicate starvation was measured by gene expression analysis of candidate genes and DMSP quantification, similar to Experiment 1 and 2. Samples of 50 mL were harvested at different time points for gene expression analysis, and 25 mL samples were harvested for DMSP quantification. For this experiment, three biological replicates of experimental cultures were used.

The growth of the three experimental cultures was monitored by cell counting at each time point of harvesting, and the resulting growth curve is presented in Figure 3.4.1. Original data are shown in Appendix 5.

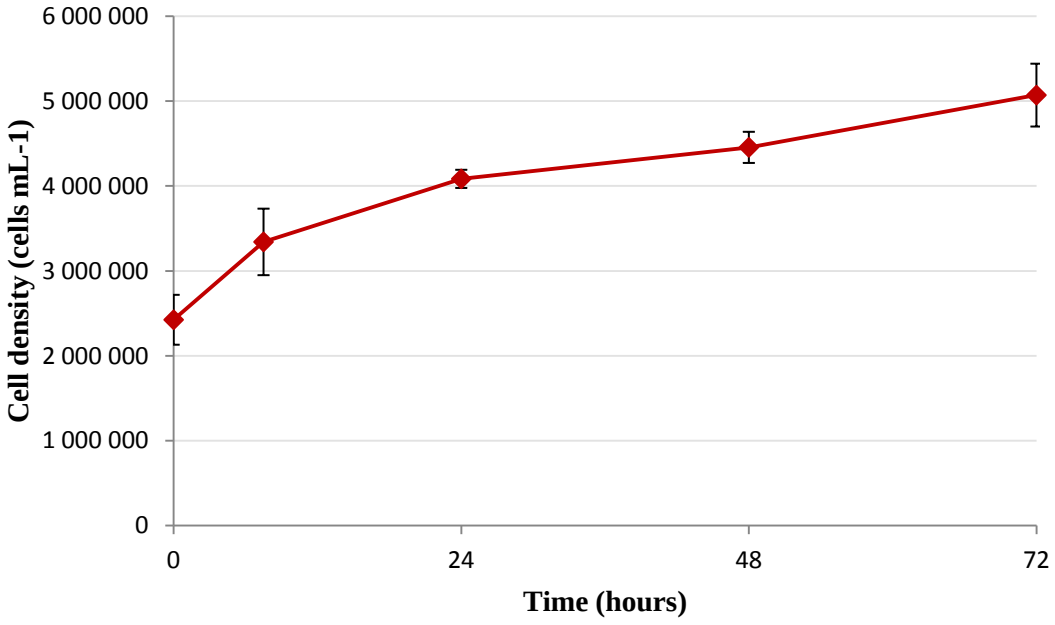

Figure 3.4.1 Growth of completely silicate-starved diatoms. Growth curve from Experiment 3, showing cell density (cells mL^{-1}) over time (days). Axenic, exponentially growing cultures of *T. pseudonana* were grown in complete silicate-free f/2 media over a period of 72 hours. Standard deviations for the three biological replicates are indicated by error bars (n=3).

The average cell density, as displayed in Figure 3.4.1, increased from about 2 400 000 to 5 100 000 cells mL^{-1} during the experiment. The cultures exhibited growth limitations after 7.5 hours, and most likely entered the stationary phase of growth somewhere between 7.5 to 24 hours after start.

At the same time points, samples were also harvested for cell volume calculations, as described in Section 2.1.5. The resulting cell volume changes are presented in Figure 3.4.2. Original data is presented in Appendix 12. Images were also taken to document any morphology changes in the cell cultures over time, as shown in Figure 3.4.3.

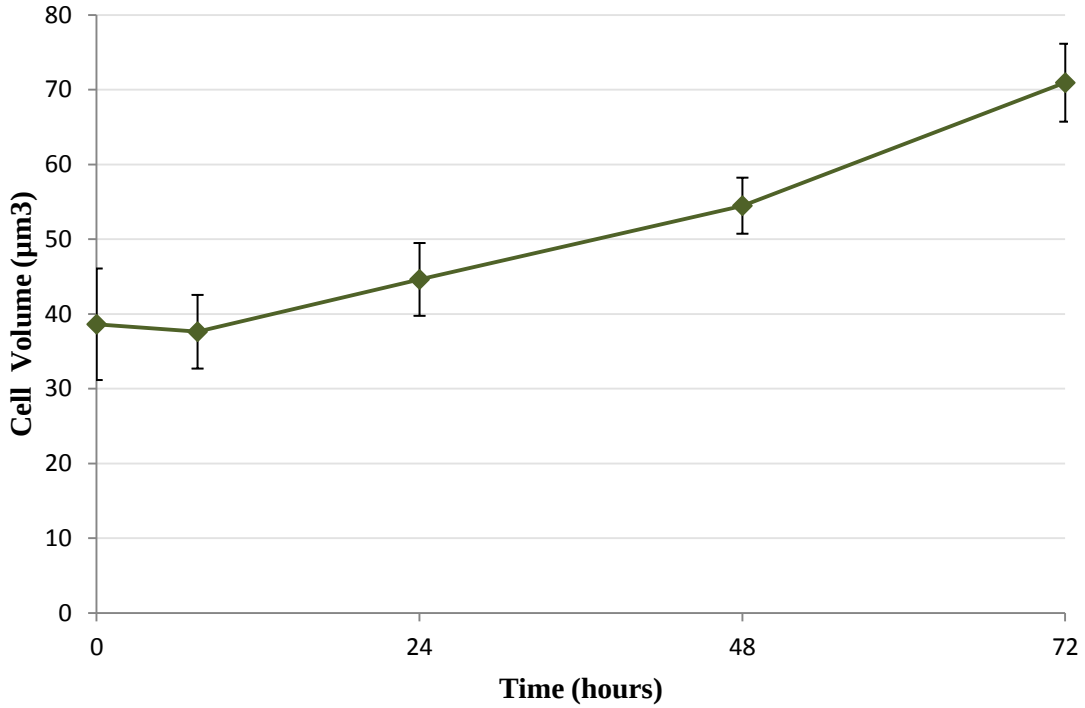

Figure 3.4.2 Changes in cell volume to complete silicate starvation. Average cell volume of the *T. pseudonana* cells from Experiment 3, calculated from the average cell volume of 15 cells per sample. Standard deviations are indicated by the error bars. n=45.

As presented in Figure 3.4.2, the average cell volume, estimated by the cell volume of 15 cells per sample, increased 84 per cent from approximately 38.6 to 70.9 μm^3 during the 72 hour experiment.

In addition to the increase in cell volume, the cell cultures changed appearance during the experiment, as displayed in Figure 3.4.3. At 7.5 hours cells started to gather in pairs and the

frequency of this phenomenon appeared to increase over time. At 48 hours, the cells had formed small clusters; these clusters, had increased in size after 72 hours. Single cells were still observed, but their frequency decreased over time.

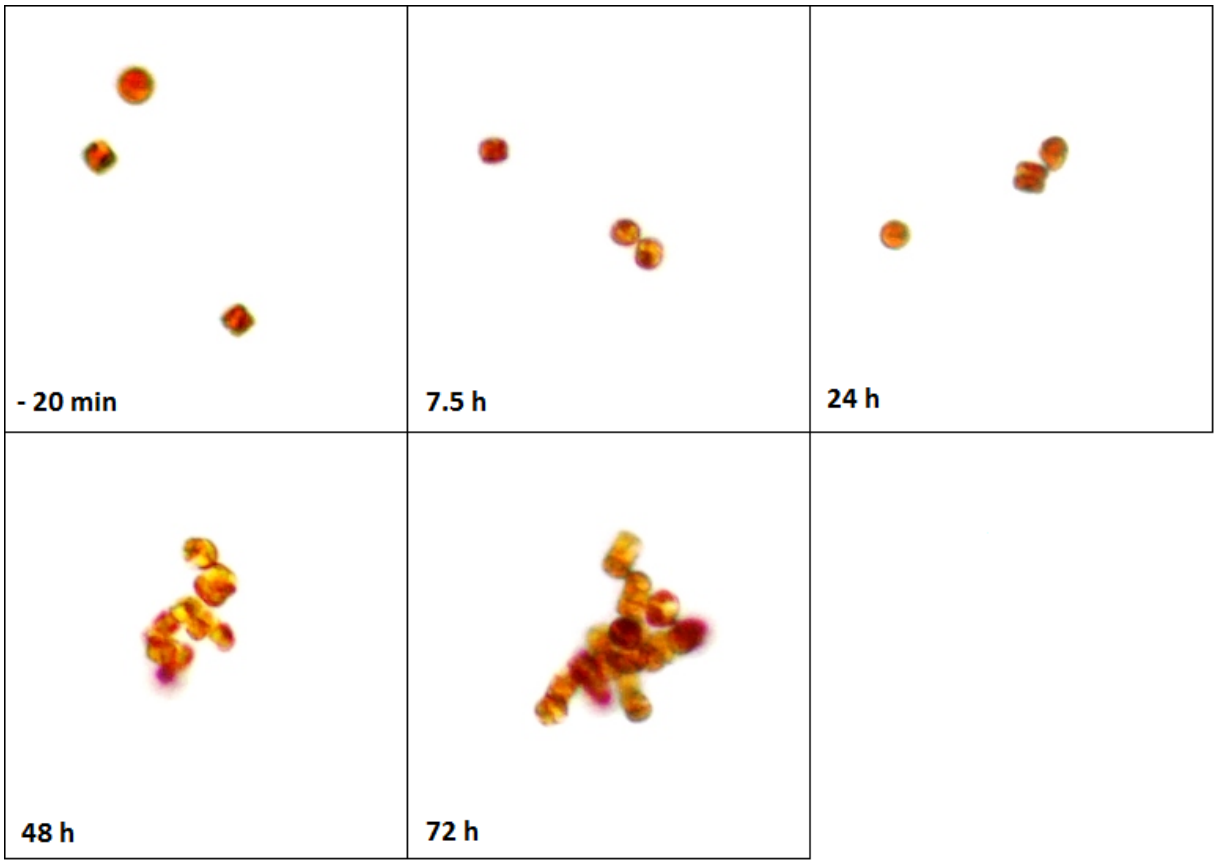

Figure 3.4.3 Morphological changes of *T. pseudonana* cultures during complete silicate-starvation. Representative light microscopy images (40 x) for each harvesting points. At the start point of the experiment the cells existed as single cells, and over time the cells formed clusters.

Cell pellets of the samples harvested for gene expression analysis were used to isolate total RNA, using the QIAGEN Protocol B. Data for RNA yield and quality is presented in Appendix 4. The total RNA served as starting material for the RT-qPCR analysis, in order to study the gene expression of the candidate genes *DiDECARB*, *SAMmt*, *REDOX* and *AT*. The relative gene expressions of the candidate genes, *DiDECARB*, *REDOX* and *AT*, are shown in Figure 3.4.4. Original data of the LinRegPCR and REST analysis are located in Appendix 6 and 7. Melting curves are presented in Appendix 8.

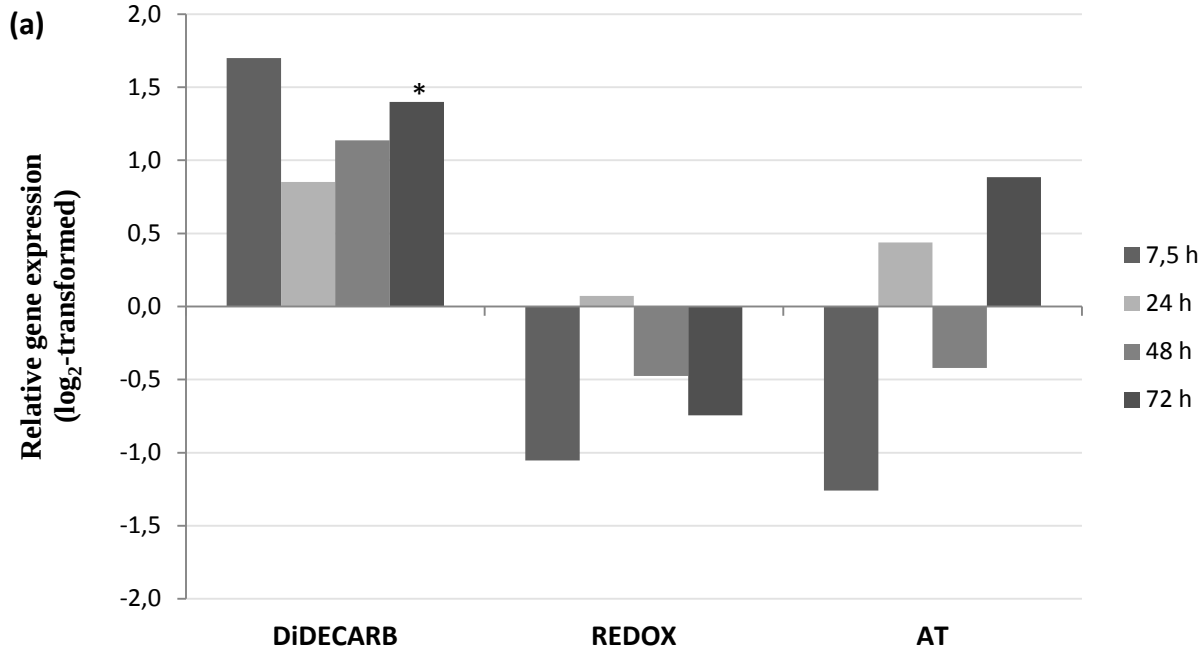

Figure 3.4.4 Relative gene expression of the candidate genes in *T. pseudonana* during complete silicate-starvation. The expression ratios (log_2-transformed) of the candidate genes *DiDECARB, REDOX* and *AT* of samples harvested at 7.5, 24, 48 and 72 hours after Start, compared to samples harvested 20 minutes before start (controls). Samples from the 7.5, 24 and 48 hour time points were normalized using the reference genes *H4* and *TBP*. The 72 hour time point was normalized using only *TBP*. Significantly regulated genes are marked with an asterisk (*, P-value < 0.01).

As presented in Figure 3.4.4, the relative gene expression data indicated that the *DiDECARB* gene was up-regulated at the last time point (72 hours) compared with the control samples harvested 20 minutes before start. Here, the fold change was estimated to be 2.6. Generally, the *DiDECARB* gene was expressed at a higher level at all the time points than in compared to the control. The *REDOX* gene was slightly down-regulated after 7.5, 48 and 72 hours, whereas the expression of the *AT* gene was highly variable throughout the experiment. For the 72 hour time point, the genes analyzed by REST were normalized with only one reference gene, the *TBP* gene. The other reference gene, encoding the Histone H4, was heavily influenced by the silicate starvation, yielding Ct-values from 0 to 45. Because of the large variance in the Ct-values of the *H4*, the *H4* reference gene was excluded from the REST analysis of the samples harvested at the last time point. The *DiDECARB* gene was the only gene showing significant difference in gene expression, at 72 hours. As presented in Appendix 6 and 7, large variances in Ct-values were also detected in the expression of the

SAMmt gene. For reasons discussed further in Section 4.4, the *SAMmt* gene was excluded from this gene expression analysis.

DMSP levels were quantified using HS-SPME and GC-MS analysis. The total DMSP concentrations are shown in Figure 3.4.5. Original data and calculations are presented in Appendix 9 and 10. Intracellular DMSP concentrations were calculated using the total DMSP concentrations and the cell volumes. The resulting concentrations of cellular DMSP are shown in Figure 3.4.6. Original data and calculations are shown in Appendix 11.

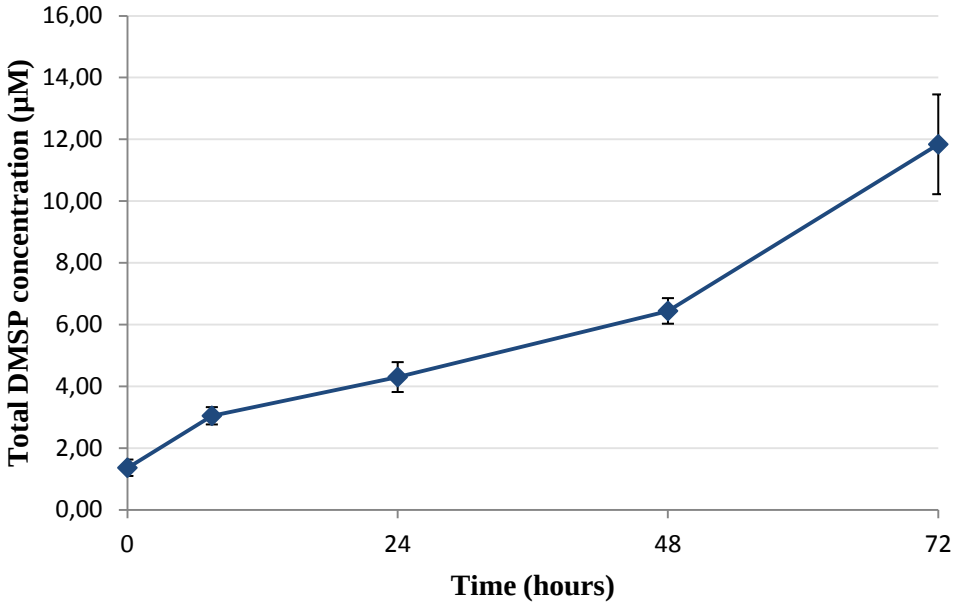

Figure 3.4.5 Total DMSP concentrations in *T. pseudonana* during complete silicate-starvation. The total DMSP concentrations, measured in micromolar (µM), for completely silicate-starved cultures over time (hours). The standard deviations are marked with error bars. n=3.

The average concentrations of total DMSP, as presented in Figure 3.4.5, increased from 1.36 to 11.83 µM during the experiment. At t0, the average total DMSP was measured at 1.36 µM, which increased to 3.04 µM at 7.5 hours (t1). The average $DMSP_{TOTAL}$ at 24 hours (t2) was 4.30 µM, increasing to 6.44 µM at 48 hours (t3) and to 11.83 µM at 72 hours (t4). The highest increase between time points were measured from 48 to 72 hours. Here, the average total DMSP concentration increased with 5.40 µM.

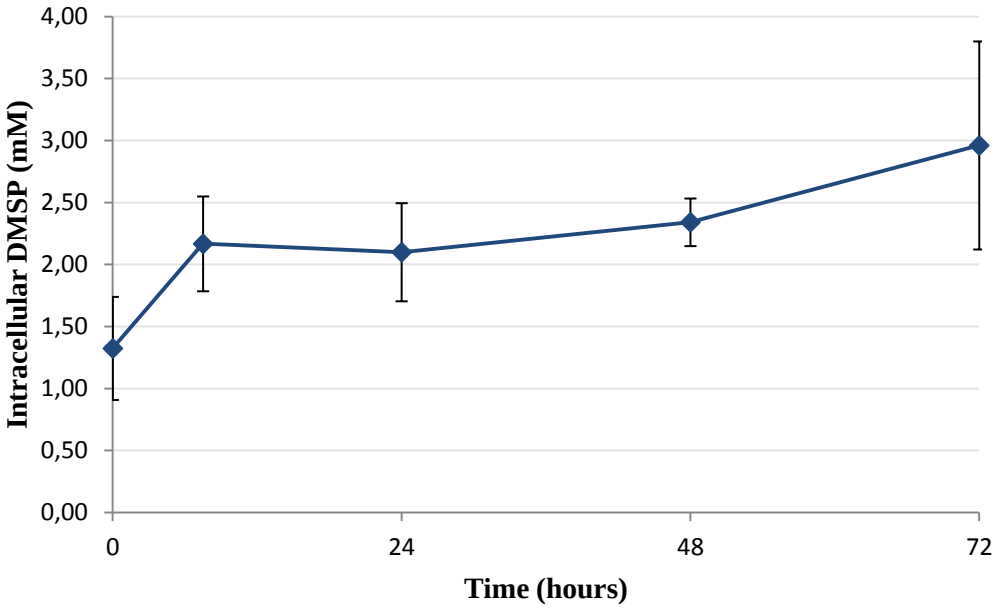

Figure 3.4.6 Intracellular DMSP concentrations in *T. pseudonana* during silicate-starvation. The average intracellular DMSP concentrations, measured in millimolar (mM), for silicate-starved cultures over time (hours). The standard deviations are marked with error bars. n=3.

The concentrations of DMSP per cell were calculated from the total DMSP concentrations, the cell number and the cell volumes. The average intracellular DMSP concentrations, as presented in Figure 3.4.6, increased from 1.32 mM before start to 2.96 mM at 72 hours after start. Thereby, the cellular concentrations of DMSP increased 2.2-fold during the course of the experiment.

4. DISCUSSION

In order to gain further knowledge about the biosynthesis of dimethylsulfoniopropionate (DMSP) in marine algae, the four proposed candidate genes *DiDECARB*, *SAMmt*, *REDOX* and *AT* were investigated using the diatom *Thalassiosira pseudonana* as model organism (Lyon et al., 2011). Three experiments were designed to induce the DMSP biosynthesis pathway through nutrient limitations, in order to measure the gene expression of the candidate genes and correlate the response in mRNA levels to quantified levels of DMSP production. It was hypothesized that the expression of candidate genes and the levels of DMSP would be elevated in diatom cultures grown under nutrient limitation-induced DMSP biosynthesis. Gene expression analyses were performed using RT-qPCR and DMSP was quantified by HS-SPME and GC-MS.

4.1 Optimization of RNA isolation

To enable accurate RT-qPCR analysis of the gene expression of the candidate genes studied, different procedures for RNA isolation, including sample harvesting, were tested to maximize the yield and quality of total RNA for the experimental conditions of this study. The three protocols described in Section 2.2.1, were compared using the obtained NanoDrop data, displayed in Table 3.1.1. Membrane filters for vacuum filtration harvesting were also tested, as described in Section 2.2.2, and compared using obtained NanoDrop data, as shown in Table 3.1.2. As usability is difficult to define as objective parameters for the quality of the protocols and membrane filters tested, the quality was compared using NanoDrop data of the resulting total RNA.

4.1.1 Choice of RNA purification protocol

The results of the protocol testing obtained in this study indicated that the Sigma Life Science Protocol and/or the Sigma Spectrum™ Plant Total RNA kit were not suitable for total RNA purification of *T. pseudonana*. It is possible that some of the components of the buffer solutions of the Sigma kit were not compatible with *T. pseudonana*, although more research is needed in order to verify this. The Sigma Protocol yielded RNA of the poorest concentration, at 37.6 ng/µL. The low absorbance ratios at 260/280 (1.84) and 260/230 (1.36) reflected the

low RNA concentration, but could also indicate the presence of contaminations such as proteins, polysaccharides and/or phenols in the isolated total RNA (Johnson et al., 2012; Wilfinger et al., 1997; Pico de Coaña et al., 2010). The QIAGEN Protocol A yielded RNA of moderate concentration, at 192.6 ng/µL, and an acceptable $A_{260/280}$ ratio at 2.06, indicating the absence of protein contaminations (Johnson et al., 2012; Wilfinger et al., 1997). The low $A_{260/230}$ ratio at 0.53, however, indicates the presence of impurities such as polysaccharides or residual reagents from the RNA isolation, like phenols and/or guanidine salts (Johnson et al, 2012; Wilfinger et al., 1997; Pico de Coaña et al., 2010). The QIAGEN Protocol B yielded the highest concentration of 293.6 ng/µL, and had acceptable 260/280 and 260/230 absorbance ratios of 2.17 and 2.07 respectively, which indicate absence of protein, phenol, guanidine salts and polysaccharide contaminations (Johnson et al., 2012; Wilfinger et al., 1997; Pico de Coaña et al., 2010). The results of the RNA yield and purity obtained in this protocol-testing experiment indicated that the QIAGEN Protocol B was the most suitable of the protocols tested for the isolation of total RNA from the diatom *T. pseudonana*.

4.1.2 Choice of membrane filter for cell harvesting

The total RNA samples isolated in the membrane filter test, using the QIAGEN Protocol B, were all of high yield RNA, with concentrations ranging from 992.75 to 1713.06 ng/µL, as presented in Appendix 4. The highest RNA concentrations were measured in the RNA samples derived by the Durapore® DVPP membrane filter with a pore size of 0.65 µm. The Supor 800 membrane filter (0.8 µm) resulted in the second highest RNA concentrations, whereas the PCTE membrane filter (1.0 µm) resulted in the poorest RNA concentrations. The high 260/280 absorbance ratio of the RNA samples indicated that protein contaminations were absent (Wilfinger et al., 1997). The low 260/230 ratios of some of the samples indicated that there might be impurities present in form of polysaccharides or reagent residuals from the RNA extraction (Johnson et al., 2012; Pico de Coaña et al., 2010). Due to the high yield of total RNA derived from the Durapore® membrane filter, this filter was utilized in further experiments.

4.1.3 Concluding remarks

The results obtained during these experiments for optimizing RNA isolation indicated that cell harvesting on the Durapore® membrane filters followed by RNA isolation using the QIAGEN RNeasy kit with Protocol B resulted in the highest RNA purification. Given the low use of biological and technical replicates, the results are not strongly supported statistically. The cultures harvested for the testing of RNA purification protocols were grown over a long period of time, 4 weeks, without changing the growth media, indicating that the physiological status of the cells in that culture were poor. In addition, the cell density of the culture was not monitored, and consequently the cell number of the culture harvested was not known. The culture harvesting for the protocol testing was also performed by centrifugation, instead of vacuum filtration as utilized in the future experiments. Therefore, the results of the protocol testing are not statistically significant and cannot be used for publication, and should be considered as a trial experiment. Although the cell number and the conditions of the culture used for testing membrane filters were known, the low number of samples limits the statistical support for the obtained results. Further testing should be performed in order to evaluate the optimal method for isolation total RNA from *T. pseudonana*.

4.2 Choice of reference genes

The genes *H4* and *TBP* were chosen as reference genes, based on a study by Siaut et al. (2007) performed on the diatom *Phaeodactylum tricornutum.* Genes involved in basic cellular processes, often termed housekeeping genes, were studied in order to find suitable stably expressed reference genes for diatom research. Among the selected genes were the histone *H4* gene and the *TBP* gene, encoding the TATA box binding protein (TBP), which appeared to be among the most suitable candidate housekeeping genes. The *H4* gene showed the highest mRNA levels, whereas the *TBP* gene were one of the two most stably expressed housekeeping genes (Siaut et al., 2007). Homologs of the genes were found in *T. pseudonana* and primers were ordered for RT-qPCR analysis.

The expression of the *H4* gene, as shown in Appendix 6, appeared to be heavily influenced by the silicate limitation and starvation treatments, resulting in higher Ct-values. Histones are positively charged proteins found in eukaryotic chromosomes, that package and order the

DNA making up structural particles called nucleosomes (Clark, 2010). Expression of histone-encoding genes is replication-dependent, and is activated at the onset of the S phase and further suppressed at the end of S phase (Ideue et al., 2012). Silicate limitations influences the diatom cell cycle by arresting the cell cycle predominantly at the G2 phase and subsequently the completion of cell division (Bucciarelli and Sunda, 2003), thereby suppressing the expression of histone genes such as *H4* (Ideue et al., 2012). As silicate starvation leads to arrest of the cell cycle, the gene expression of the histone proteins will be suppressed. Thus, the choice of the *H4* gene as a reference gene in the RT-qPCR analysis was a rather poor choice in the silicate limitation experiments.

4.3 Response of nutrient limitations

In Experiment 1 and 2, diatom cultures of *T. pseudonana* were grown under nutrient limitations to induce the biosynthesis of DMSP, in order to measure the response in gene expression and DMSP accumulation, respectively. The nutrient limitations were made using f/2 growth media without silicate supplementation for the silicate-limited cultures and without nitrate supplementation for the nitrate-limited cultures. Silicate and nitrate were only available in low amounts present in the filtrated natural seawater. The experimental cultures of the two experiments were grown at the same time and under the same environmental growth conditions.

4.3.1 Changes in expression of the candidate genes

In Experiment 1, non-axenic cultures of *T. pseudonana* were grown in different nutrient treatments for five days, in order to induce the DMSP biosynthesis pathway. Since metabolic enzymes are often regulated at transcription level (Persson et al., 2005; Nelson and Cox, 2008), it was hypothesized that the candidate genes *DiDECARB, SAMmt, REDOX* and *AT* were to be up-regulated when the diatom cultures were grown under nutrient-limiting conditions inducing DMSP accumulation. For this experiment, four biological replicates were used in order to increase the statistical significance of differences in the RT-qPCR results between the different nutrient treatments. As recommended by the minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines, using a sufficient

number of biological replicates can minimize smaller experimental differences caused by the inherent variability of biological systems (Bustin et al., 2009).

Growth of experimental cultures

The growth of the cells, as displayed in Figure 3.2.1, indicated that only the nitrate-limited cultures had entered stationary phase at harvest point. The nitrate-limited cultures exhibited growth limitations from day 2, at a cell density of about 1 200 000 cells mL^{-1}, and reached a final density of about 1 600 000 cells mL^{-1} (day 5). The controls and the silicate-limited cultures exhibited no significant indications of either growth limitation or entering stationary phase at harvest point. The silicate-limited cultures grew continuously throughout the experiment, reaching a cell density of approximately 2 900 000 cells mL^{-1} at harvest point. The silicate-limited cultures also exceeded the control cultures at day five, which had an average cell density of about 2 600 000 cells mL^{-1}. These data indicated that nitrate limitation had the strongest effect on growth of the tested nutrient treatments, and may thereby cause the highest level of stress.

The similarities in the growth pattern of the silicate-limited and control cultures were not expected. According to a similar study of Bucciarelli and Sunda (2003), which this experiment was based on, the silicate-limited cultures should display similar growth patterns as the nitrate-limited cultures. In their experiment, the silicate-limited cultures entered stationary phase as early as at day two, even though they did not feed the cell cultures during the experiment as performed here. Although the experiment performed in this thesis is not identical to that of Bucciarelli and Sunda (2003), the patterns of growth were expected to look approximately similar. A possible explanation for the unaffected growth of the silicate-limited cultures is that the concentration of silicate present in the natural sea water was higher than expected, providing the diatom cells with enough silicate to avoid growth limitations due to limited amounts of available silicate within the time frame of the experiment. Another possible explanation is that the duration of this experiment was not long enough for the cells to run out of their inner storage of silica. Diatoms have silica deposition vesicles (SDV), which are inner storage organelles for silica intended for the synthesis of new valves and silica structures (Tesson and Hildebrand, 2013; Vrieling et al., 2007). If the experiment were continued for a longer period of time, the cells would have used up the inner storage of silica, and the effect of silicate-limitations would have been more visible on culture growth rate and possibly DMSP production.

Expression of candidate genes related to growth

The silicate limitation performed in this experiment was not strong enough to cause growth limitations in experimental cultures. In the study of Bucciarelli and Sunda (2003), silicate limitations had an intermediate effect on intracellular DMSP accumulation, increasing exponentially with decreasing growth rate. As the cells did not experience stress caused by silicate limitations, the cells did not require synthesizing more DMSP compared to control cultures. According to this explanation, there was no need for the cells of the silicate-limited cultures to up-regulate the genes involved in DMSP biosynthesis in comparison to control cultures.

The nitrate-limited cultures, however, did experience growth limitation from day 3, and the cells were in the stationary growth phase when they were harvested at day 5. The *REDOX* gene was up regulated 6.4 times more (P-value < 0.01) under nitrate limitation compared to controls, whereas the *SAMmt* and *AT* gene were down regulated about 4 times (P-value < 0.05) for both genes. These results indicate that the *REDOX* gene possibly is involved in the biosynthesis of DMSP in *T. pseudonana*. Since the *DiDECARB* gene was not significantly regulated and the *SAMmt* and *AT* genes were down regulated, these results do not support the hypothesis that these genes are involved in the synthesis of DMSP.

Characterizing outliers in the REST analysis

The control cultures were analyzed using three of the four biological replicates. The last replica (Tp 1C) was discarded because it displayed Ct values that were much higher than of the other biological replicates (indicating a lower copy number of the transcripts), characterizing this replica as an outlier. The Ct values obtained from the LinRegPCR analysis is shown in Table A6.1 in Appendix 6. It is generally accepted that at least three biological replicates should be used in RT-qPCR analyses in order to mitigate the effect of biological variability (Taylor et al., 2010). The MIQE guidelines suggest that Ct values of over 40 should not be reported, due to generally implied low efficiency, although MIQE do not recommend arbitrary cutoffs of Ct-values, as that might lead to eliminating valid results or increasing false-positive results (Bustin et al., 2009). As the Ct-values of the "Tp 1C" replica were considerably higher for all amplicons compared to other replicates, this replica was characterized as an outlier and eliminated in further REST analysis.

The replica "Tp 1-Si" was also displaying Ct-values that were generally lower than the other biological replicates of the silicate limited cultures, as shown in Table A6.2 in Appendix 6. Including this replicate, could lead to false positive up regulation of the candidate genes. However, the Ct values of the *REDOX* genes for this replica were similar to the replica "Tp 3-Si". Because of this, the "Tp 1-Si" replicate was not excluded from the REST analysis. As none of the candidate genes were significantly up-regulated in the silicate-limiting treatment, a false positive up-regulation was not observed. Results of REST analyses including the "Tp 1-Si" replicate are shown in Appendix 7.

4.3.2 Changes in DMSP levels during nutrient limitations

In Experiment 2, the total and cellular levels of DMSP were quantified by GC-MS. The nutrient-depleted cultures were grown over a time period of 5 days, under the same conditions as the cultures of Experiment 1. Samples of 25 mL were harvested daily to quantify the levels of DMSP using HS-SPME and GC-MS. It was hypothesized that DMSP would accumulate in the diatom cells as the cell cultures exhibited growth limitation due to the limited amounts of available nutrients.

Growth of experimental cultures

The growth of the experimental cultures, as shown in Figure 3.3.1, indicated that only the nitrate-limited cultures had entered stationary phase during the experiment. The nitrate-limited cultures exhibited growth limitations from the third day, regardless of the addition of f/2 nutrients at the second day. The silicate-limited cultures exhibited lower cell densities than control cultures at the end of the experiment, which might indicate that they were growth-limited due to silicate depletion. However, the growth data could not verify that the control and silicate-limited cultures had entered stationary growth phase during the experiment. Consequently, elevations in the level of intracellular DMSP were only expected in the nitrate-limited cultures, as they had entered stationary phase early in the experiment.

Total DMSP concentrations

The total DMSP concentrations, the concentrations of DMSP in the glass vials from the GC-MS analysis, containing cells harvested from 25 mL culture, increased over time in all cultures as shown in Figure 3.3.2. As the total DMSP concentrations were not dependent on cell number, the increase in total DMSP concentrations were due to the increase in cell

densities of the experimental cultures. The total DMSP concentrations were used to calculate the cellular amounts of DMSP. Unfortunately, the cell volumes were not estimated for the cells in the experimental cultures in Experiment 2. Therefore, the intracellular concentrations of DMSP were not possible to estimate accurately. Thus, only the intracellular amounts were calculated.

Intracellular amounts of DMSP

The cellular amounts of DMSP decreased during the nutrient limitation treatments in Experiment 2, as seen in Figure 3.3.3. The high levels of cellular DMSP at the start point (t0) reflected the physiological condition of the inocula, which were taken from a presumably nutrient-stressed culture at a relative high cell density of about 1 700 000 cells mL^{-1}, that had been grown for six days after subcultivation and was most likely in the stationary growth phase. As the cells were transferred to fresh media, the intracellular amounts of DMSP decreased; the effect of the transfer was apparent at day 1. After the first day, the amount of cellular DMSP was approximately stabilized for each of the different nutrient treatments and no significantly accumulation of DMSP were measured after five days of nutrient limitations.

In the study of Bucciarelli and Sunda (2003), high levels of intracellular DMSP were also reported at the beginning of the batch culture experiments and followed by decreasing cellular DMSP concentrations during the exponential growth phase. This phenomenon has also been experienced in other studies (Matrai and Keller, 1994; Keller et al., 1999). Bucciarelli and Sunda (2003) suggested that these patterns supported a hypothesis stating that there might be a direct link between intracellular DMSP levels and the physiological state of the cell, related to the level and type of nutrient limitation. The growth data, displayed in Figure 3.3.1, indicated that the nitrate-limited cultures had entered the stationary phase from day three. Significantly higher amounts of intracellular DMSP were measured in the nitrate-limited cultures in compared to control cultures at day 2, 4 and 5, although the differences are small. However, since no significantly accumulation of cellular DMSP were shown over time, the nutrient limitation treatments performed in this experiment appeared to have low or no effect on the production of DMSP, indicating that induction of the DMSP biosynthesis pathway may not have occurred.

4.3.3 Differences in growth patterns in Experiment 1 and 2

Although the inocula of the experimental cultures of Experiment 1 and 2 originated from the same non-axenic batch culture, and the experimental cultures were grown at the same time under the same environmental conditions, the growth curves, shown in Figure 3.2.1 and Figure 3.3.1, displayed different growth patterns throughout the experiments. The overall cell density was generally higher in the experimental culture of Experiment 2. The nitrate-limited cultures displayed similar growth patterns in both experiments, whereas the greatest differences in growth were measured in the control cultures at the end of the experiments. At day 5, the density of the control culture of the first experiment was approximately 2.6 million cells mL^{-1}, whereas the controls in the second experiment reached a cell density of about 3.9 million cells mL^{-1}, a cell density that is 1.5 times higher than in the first experiment. In the first experiment, the growth of the silicate-limited cultures exceeded the controls in cell density the fourth and fifth day of the experiment. This, however, was not observed in the second experiment.

Despite the similarities of the first and second experiment, in terms of environmental growth conditions, important differences in cultivations and sample harvesting need to be considered. First, the cultivation volume in both experiments varied, starting with 150 mL in Experiment 1 and 200 mL in Experiment 2. Second, the cultures of the first experiment were only harvested once as opposed to the second experiments, where the cultures were sampled daily. As 25 mL samples were removed daily from the experimental cultures of Experiment 2, the cultivation volume was continuously changing during the experiment, whereas the volume remained constant in the first experiment, besides from the 1 mL samples harvested daily for cell counting. These differences in cultivation and sampling may provide an explanation of the differences in growth patterns between the first and second experiments. Consequently, the results of the gene expression analysis of the first experiment are not directly comparable with the quantification of DMSP in the second experiment.

4.3.4 Comparison of the results of Experiment 1 and 2

The results from the first and second experiment cannot be compared directly as the samples analyzed were derived from different biological samples and for the reasons described in Section 4.3.3. Hence, the obtained results should be viewed tentatively.

In Experiment 1, it was hypothesized that the DMSP biosynthesis pathway was not induced under the silicate-limitations experienced in the experiment, based on the growth data where the silicate-limited culture showed no indication of entering the stationary phase at harvest point. This hypothesis is supported by the quantitative levels of intracellular DMSP obtained in the second experiment, which did not indicate any significantly differences in the amount of cellular DMSP of the silicate-limited culture compared to the control culture. Hence, if the DMSP biosynthesis pathway was not induced, the genes encoding the metabolic enzymes of the DMSP biosynthesis pathway are not expected to be up-regulated, providing an explanation of the unregulated candidate genes in Experiment 1.

However, in case of the nitrate-limited cultures, the growth data of both Experiment 1 and 2 indicated that the cells had entered the stationary phase of growth from day 3. Unexpectedly, the amount of DMSP was not elevated over time in the nitrate-limited cultures in Experiment 2, as was hypothesized under stress caused by nutrient limitations. No measurements of intracellular DMSP were taken in the first experiment, which showed an up-regulation of the *REDOX* gene in the nitrate-limited cultures. Therefore, it is difficult to know whether the DMSP biosynthesis pathway really was induced as a result of limited availability of nitrate. Hence, the up-regulation of the *REDOX* gene cannot be related to induced DMSP biosynthesis and the results are therefore not enough to validate the *REDOX* gene as being involved in the DMSP biosynthesis pathway.

The fact that the experimental cultures were non-axenic may have influenced the gene expression analysis and the quantification of DMSP. As bacteria are known to catabolize DMSP (Reisch et al. 2011), the presence of bacterial contamination could lead to lower levels of quantified DMSP than if axenic batch cultures were used. Bacterial growth may also have influenced the growth of the diatom cultures, competing with algal cells for nutrients or producing toxic or growth-inhibiting compounds. Results of experiments using contaminated cell cultures are often heavily compromised, and therefore it is recommended to replace the contaminated cultures with new axenic ones (Freshney, 2010). Consequently, the results from Experiment 1 and 2, should therefore be carefully evaluated and are not validated for publishing.

4.4 Response of complete silicate starvation

In Experiment 3, three axenic diatom cultures of *T. pseudonana* were resuspended in complete silicate-free f/2 medium in order to induce the DMSP biosynthesis pathway. The expression of the candidate genes was measured by RT-qPCR and total and intracellular DMSP were estimated using HS-SPME and GC-MS. The cultures were grown for 72 hours after Start, sampling at five different time points, 20 minutes before Start (t0) and 7.5 (t1), 24 (t2), 48 (t3) and 72 (t4) hours after Start.

Growth and cell volume

The growth data of the experimental cultures, as shown in Figure 3.4.1, indicated that the cultures entered the stationary growth phase around 24 hours, exhibiting growth limitations at a cell density of about 4 million cells mL^{-1}. The exact point of entering stationary phase cannot be estimated, due to the limited amount of time points for sampling. The increased formations of cell clusters from 48 hours, as shown in Figure 3.4.3, also indicated that the cells suffered from nutrient limitations, as diatom cells in nature tend to cluster when nutrients are scarce (Falciatore and Bowler, 2002). It was therefore expected that the production of DMSP was to be elevated and that the genes involved in the DMSP biosynthesis pathway were up-regulated.

As displayed in Figure 3.4.2., the average cell volume increased 1.8 times during the experiment, from 38.6 to 70.9 μm^3. Increasing volume of silicate-limited cells was also observed in the study of Bucciarelli and Sunda (2003). There, the volume per cell remained constant at 52 ± 3 μm^3 during the exponential phase of growth and increased continuously under silica limitations to a value of 137 μm^3 at the end of the experiment, at day 26. Elevated cell volume during silicate limitations can be linked to the cell division cycle. Under silicate limitation, the diatom cell cycle predominantly arrests at the G2 phase, before cell division is completed. As the cells are dependent on available silica for frustule formation, the inhibition of cell division linked to the inability to synthesize new cell wall material could provide an explanation for the observed increase in volume per cell reported in their study and also in this study (Bucciarelli and Sunda, 2003).

Total and intracellular DMSP concentrations

During Experiment 3, the total DMSP increased from 1.36 to 11.83 µM, as shown in Figure 3.4.5, meaning that the total DMSP concentration was elevated 8.7 times. As the total DMSP concentrations do not account for the cell number, the intracellular DMSP concentrations were calculated. The measured cell volumes enabled accurate calculations of the intracellular DMSP concentrations, as displayed in Figure 3.4.6. The cellular concentrations of DMSP were elevated from 1.32 to 2.96 mM during the experiment. At the last time point, 72 hours, the cellular DMSP varied between 2.44 and 3.93 mM between the three cultures, implying high biological variability between the three experimental cultures. The intracellular DMSP levels were elevated with a fold change of 2.2 during the experiment. The results obtained here shows that DMSP have accumulated during the experiment, which further implies that the DMSP biosynthesis pathway was successfully, though moderately, induced by the silicate starvation.

Expression of candidate genes

As the cellular DMSP concentrations increased, it was expected that the expression of the candidate genes for the DMSP biosynthesis pathway were to be elevated. The results obtained from the RT-qPCR analysis (Figure 3.4.4) were, however, not as expected. The only gene that was significantly up-regulated compared to the t0 time point, was the *DiDECARB* gene at 72 hours. At 72 hours, however, the genes were normalized with only one of the two reference genes, due to the impact of nutrient starvation of the *H4* reference gene. The fact that only one reference gene was used for the normalization process in REST, could lead to false positive up-regulation of the candidate gene.

In the RT-qPCR analysis, the *SAMmt* gene was showing late and highly variable Ct-values, despite the fact that the gene had a relatively high mean PCR amplification efficiency at 1.855. A possible explanation for this, is that the *SAMmt* gene hit the critical limit of detection (LOD), which is described in the MIQE guidelines for RT-qPCR as the lowest concentration of cDNA that can be detected with a 95 % probability (Bustin et al., 2009). Assuming a Poisson distribution, the most sensitive LOD theoretically possible is 3 copies per PCR, yielding a 95 % chance of including at least 1 copy in the PCR. Low copy-transcripts are therefore stochastically limited, and performing RT-qPCR using LODs of less than three copies per PCR is not possible (Bustin et al., 2009). The late and highly variable Ct-values indicated that the *SAMmt* gene was expressed at a low level, and that the amplicon template

consequently had a low copy number. Therefore, the cDNA was diluted too much before the RT-qPCR, reaching the limit of detection (Bustin et al., 2009). Because the concentrations of the target copies of the *SAMmt* gene had presumably reached the LOD sensitivity, resulting in highly variable Ct-values, the *SAMmt* gene was excluded from the gene expression analysis.

Primers and cDNA are diluted before performing RT-qPCR in order to create optimal PCR conditions, generating low Ct-values and sharp peaks in the melting curves without amplification of non-specific PCR products or primer-dimer artifacts (Siaut et al., 2007). Therefore, a serial dilution experiment should be conducted for further RT-qPCR analysis of the samples, in order to find the optimal cDNA dilution, as 1:10 was too much (as indicated by the results of the *SAMmt* gene) (Bustin et al., 2009). By not diluting the cDNA as much as performed in this experiment, the chances of pipetting enough copies of the target transcript increases and the Ct-values will become less variable (Bustin et al., 2009).

The fact that several of the candidate genes displayed high Ct-values and showed low or no up-regulation when the cellular DMSP concentration increased indicate that the proposed candidate genes may not be involved in the DMSP biosynthesis pathway after all. The candidate genes were proposed in a proteomics study by Lyon et al. (2011), based on elevated protein levels belonging to the enzyme classes of the hypothetical DMSP biosynthesis pathway in marine algae by Gage et al. (1997). In the study of Lyon et al. (2011) a fifth candidate gene, *DECARB* was proposed. Because the *T. pseudonana* genome does not contain homologs for this gene, the *DECARB* gene was not tested. It could, however, be interesting to study this candidate gene as well, in for example the high DMSP-producing haptophyte *Emiliania huxleyi*, which contains a homolog gene for *DECARB* as well as for the other candidate enzymes (Lyon et al., 2011).

In Experiment 3, the samples for gene expression analysis were intended for microarray analysis. The study of Bucciarelli and Sunda (2003) showed that nitrate limitation had the largest impact on cellular DMSP accumulation. As nitrate starvation may lead to unstable nucleic acids, due to the mobilization of nitrogen-containing molecules (Hockin et al., 2012), silicate was chosen as the depleted nutrient in the third experiment. However, because of delivery difficulties, the microarray analysis was not performed in time to be included in this thesis; instead, RT-qPCR analyses were performed. Although none of the candidate genes proposed to be involved in the DMSP pathway were verified by the RT-qPCR analyses during

this thesis, the results of the upcoming microarray analysis may provide new information about the expression of the currently proposed candidate genes and/or reveal new candidate genes of the DMSP biosynthesis pathway. The method development performed in this thesis can be utilized for further research to uncover the mysteries about the marine phytoplankton production of DMSP.

5. CONCLUSION

This thesis was designed to investigate four proposed candidate genes, *DiDECARB, SAMmt, REDOX* and *AT*, for the dimethylsulfoniopropionate (DMSP) biosynthesis pathway in the diatom *Thalassiosira pseudonana*. Trial experiments to optimize RNA purification indicated that the QIAGEN Protocol B was the most suitable protocol and that the Durapore® DVPP membrane filter from Merck Millipore resulted in the highest concentrations of total RNA. DMSP did not accumulate significantly over time in cultures grown under nutrient limitations of silicate or nitrate, in Experiment 2. The only gene found to be significantly up-regulated under nutrient limitations of Experiment 1, was the *REDOX* gene in nitrate limited cultures. Here, the *SAMmt* and *AT* gene was found to be significantly down-regulated. The fact that non-axenic *T. pseudonana* cultures were used for both experiments makes the obtained results heavily compromised. In axenic batch cultures of *T. pseudonana* under complete silicate starvation, DMSP were shown to accumulate as the culture entered the stationary phase. Expression of the *DiDECARB* gene was shown to be significantly up-regulated, although not strongly statistically supported, at 72 hours after the culture were transferred to a complete silicate-free f/2 media. The results of this study were not able to provide any definitive proof of the involvement of the candidate genes *DiDECARB, SAMmt, REDOX* and *AT* in the DMSP biosynthesis pathway in *T. pseudonana*. Although a link between the candidate genes and the biosynthesis of DMSP cannot be ruled out, further research is required to verify the role of the currently proposed candidate genes and/or reveal new candidate genes for the biosynthesis pathway of DMSP in marine algae.

6. RECOMMENDATIONS FOR FURTHER WORK

For further investigation of the candidate genes for the DMSP biosynthesis pathway in marine algae, it might be beneficial to analyze the gene expression of the currently proposed candidate genes using microarray. The samples of the complete silicate starvation experiment were originally harvested for microarray analysis, which will be performed during this summer. As the candidate genes were proposed based on a proteomics study, microarray data could provide new information about the gene expression of the proposed candidate genes or reveal new candidate genes for the DMSP biosynthesis pathway in marine algae.

As homologs of the candidate genes were also found in the high DMSP-producing coccolithophore *Emiliania huxleyi*, it would be interesting to study the gene expression of the currently proposed candidate genes and also compare it to *Thalassiosira pseudonana*. The *DECARB* gene was not studied in this thesis, due to the fact that homologs for this gene have not been found in *T. pseudonana*. As homologs of the *DECARB* gene have been found in *E. huxleyi*, it could be interesting to study *DECARB* expression under DMSP-inducing conditions and correlate the gene expression of all five candidate genes with cellular DMSP accumulation. Differences in gene expression between *T. pseudonana* and *E. huxleyi* and/or the presence of the *DECARB* gene in *E. huxleyi* could provide an explanation for the high DMSP production in *E. huxleyi* compared to *T. pseudonana* (Bucciarelli and Sunda, 2003).

Another possible experiment would be to generate transgenic over-expression lines of *T. pseudonana*, expressing the candidate genes under the control of an active promoter. The effect on DMSP production could be analyzed by using gas chromatography-mass spectrometry (GC-MS). Subsequently, a localization study could be performed, where *T. pseudonana* is transformed with the candidate genes fused with fluorescence proteins that can be visualized by confocal microscopy. Furthermore, the candidate genes could be cloned and expressed recombinantly in *Escherichia coli*. The recombinant proteins could be purified and used for biochemical characterization, in order to find the enzymatic properties of the gene products. Biochemical studies could indicate whether the gene product could catalyze one of the reactions of the DMSP biosynthetic pathway. The structure of the purified enzymes would provide information about catalytically and regulatory (sub)domains. The activity of the purified enzymes could also be monitored under increasing substrate concentrations, in order

to investigate the enzyme kinetics. The purified protein could also be used to study the substrate specificity of the enzyme or whether it is subjected to allosteric feedback regulation, possibly by the end product DMSP.

REFERENCES

Armbrust E.V., Berges J. B., Bowler C., Green B. R., Martinez D., et al., (2004), *The genome of the diatom* Thalassiosira pseudonana*: ecology, evolution, and metabolism.* Science 306: 79–86.

Armbrust E. V., (2009), *The life of diatoms in the world's oceans*. Nature 459: 185-191.

Brown R. B. and Audet J., (2008), *Current techniques for single-cell lysis.* Journal of the Royal Society Interface 5: 131-138.

Bucciarelli E., Sunda W. G., (2003), *Influence of CO_2, nitrate, phosphate, and silicate limitation on intracellular dimethylsulfoniopropionate in batch cultures of the coastal diatom* Thalassiosira pseudonana. Limonology and Oceanography 48: 2256-2265.

Bustin S. A., Benes V., Garson J. A., Hellmans J., Huggett J., Kubista M., Mueller R., Nolan T., Pfaffi M. W., Shipley G. L., Vandesompele J. and Wittwer C. T., (2009), *The MIQE Guidelines:* M*inimum* I*nformation for publication of* Q*uantitative real-time PCR* E*xperiments*. Clinical Chemistry 55: 611-622.

Charlson R. J., Lovelock J. E., Andreae M. O. and Warren S. G., (1987), *Oceanic phytoplankton, atmospheric sulphur, cloud albedo and climate.* Nature 326: 655-661.

Chepurnov V. A., Mann D. G., von Dassow P., Vanormelingen P., Gillard J., Inzé D., Sabbe K. and Vyverman W., (2008), *In search of new tractable diatoms for experimental biology*. BioEssays 30: 692-702.

Clark D. P., (2010), *Molecular biology,* Academic cell update, Amsterdam; Boston: Elsevier Inc., pp. 95-96.

Dennis Kunkel Microscopy, Inc., (2004), *Saltwater Centric Diatom Frustule or Skeleton*, Source: Corbis Images. Retrieved 22.03.13, from: http://www.corbisimages.com/stock-photo/rights-managed/42-19878886/saltwater-centric-diatom-frustule-or-skeleton

Falciatore A. and Bowler C., (2002), *Revealing the molecular secrets of marine diatoms*. Annual Review of Plant Biology 53: 109-130.

Franklin D. J., Airs R. L., Fernandes M., Bell T. G., Bongaerts R. J., Berges J. A. and Malin G., (2012), *Identification of senescence and death in* Emiliania huxleyi *and* Thalassiosira pseudonana*: cell staining, chlorophyll alterations, and dimethylsulfoniopropionate (DMSP) metabolism*. Limnology and Oceanography 57:305-317.

Freshney R. I., (2010) *Culture of animal cells: a manual of basic technique and specialized applications*, 6. edition, Hoboken: John Wiley & Sons, Inc., pp. 57, 88, 299, 301-315.

Gage D. A., Rhodes D., Nolte K. D., Hicks W. A., Leustek T., Cooper A. J. L. and Hanson A. D., (1997), *A new route for synthesis of dimethylsulphoniopropionate in marine algae.* Nature 387: 891-894.

Guénin S., Mauriat M., Pelloux J., Van Wuytswinkel O., Bellini C. and Gutierrez L., (2009), *Normalization of qRT-PCR data: the necessity of adopting a systematic, experimental conditions-specific, validation of references*. Journal of Experimental Botany 60: 487-493.

Guillard R.R. L., (1975), *Culture of phytoplankton for feeding marine invertebrates,* In culture of marine invertebrate animals, edited by: Smith W. L. and Chanley M. H., New York: Plenum Press, pp. 26-60.

Hanson A. D., Gage D. A., Nolte K. D., Hicks W. A., Leustek T., Cooper A. J. and Rhodes D., (2003), *Simulation of The Pathway of DMSP Biosynthesis in* Enteromorpha intestinalis, Source: Purdue University. Retrieved 16.08.12, Modified 28.01.13, from: http://www.hort.purdue.edu/cfpesp/models/mo00006.htm

Hockin N. L., Mock T., Mulholland F., Kopriva S. and Malin G., (2012), *The response of diatom central carbon metabolism to nitrogen starvation is different from that of green algae and higher plants*. Plant Physiology 158: 299-312.

Huggett J., Dheda K., Bustin S. and Zumla A., (2005), *Real-time RT-PCR normalisation; strategies and considerations.* Genes and Immunity 6: 279-284.

Ideue T., Adachi S., Naganuma T., Tanigawa A., Natsume T. and Hirose T., (2012), *U7 small nuclear ribonucleoprotein represses histone gene transcription in cell cycle-arrested cells*. Proceeding of the National Academy of Sciences of the United States of America 109: 5693-5698.

Invitrogen, (2010), *Qubit™ RNA Assay Kits For use with the QubitR 2.0 Fluorometer*. Retrieved 13.02.12, from: http://tools.invitrogen.com/content/sfs/manuals/Qubit_RNA_Assay_QR.pdf

Johnson M. T. J., Carpenter E. J., Tian A., Bruskiewich R., Burris J. N. et al., (2012), *Evaluating methods for isolating total RNA and predicting the success of sequencing phylogenetically diverse plant transcriptomes*. PLOS ONE 7: e50226.

Keller M. D., Kiene R. P., Matrai P. A. and Bellows W. K., (1999), *Production of glycine betaine and dimethylsulfoniopropionate in marine phytoplankton. I. Batch cultures*. Marine Biology 135: 237-248.

Kemp A.E.S, Pike J., Pearce R. B. and Lange C. B., (2000), *The "Fall dump" - a new perspective on the role of a "shade flora" in the annual cycle of diatom production and export flux*. Deep-Sea Research 47: 2129-2154.

Kociolek J. P., (2010), *Glossary of Terms Relating to Diatom Valve Morphology with an Illustrated Glossary*, Source: Diatoms of the Southern California Bight. Retrieved 23.03.13, from: http://dbmuseblade.colorado.edu/DiatomTwo/dscb_site/glossary2.html
Kröger N., (2012), *Diatom Biosensor Could Shine Light On Future Nanomaterials,* Source: Science Daily. Retrieved 23.03.13, from: http://www.sciencedaily.com/releases/2012/03/120323094036.htm

Lyon B. R., Lee P. A., Bennett J. M., DiTullio G. R. and Janech M. G., (2011), *Proteomic analysis of a sea-ice diatom: salinity acclimation provides new insight into the dimethylsulfoniopropionate production pathway*. Plant Physiology 157: 1926-1941.

Matrai P. A. and Keller M. D., (1994), *Total organic sulfur and dimethylsulfoniopropionate in marine phytoplankton: intracellular variations*. Marine biology 119: 61-68.

Merck Millipore, (2013), *109261 Lugol's solution (diluted iodine-potassium iodide solution) for the Gram staining method*. Retrieved 16.04.13, from: http://www.merckmillipore.com/is-bin/INTERSHOP.enfinity/WFS/Merck-SE-Site/en_US/-/SEK/ViewPDF-Print.pdf?RenderPageType=ProductDetail&CatalogCategoryID=1_Cb.s1Lym0AAAEWZ.EfVhTl&ProductUUID=f4Cb.s1O6WIAAAEaeq9qKZJn&PortalCatalogUUID=t02b.s1LX0MAAAEWc9UfVhTl

Morse S. M. J., Shaw G. and Larner S. F., (2006), *Concurrent mRNA and protein extraction from the same experimental sample using a commercially available column-based RNA preparation kit.* BioTechniques 40: 54-58.

Nelson D. L. and Cox M. M., (2008) *Lehninger principles of biochemistry*, 5. edition, New York: Freeman and company, pp. 571-572.

Niki T., Fujinaga T., Watanabe M. F. and Kinoshjita J., (2004), *Simple determination of dimethylsulfide (DMS) and dimethylsulfoniopropionate (DMSP) using solid-phase microextraction and gas chromatography-mass spectrometry*. Journal of Oceanography 60: 913-917.

Persson S., Wei H., Milne J., Page G. P. and Somerville C. R., (2005), *Identification of genes required for cellulose synthesis by regression analysis of public microarray data sets.* Proceeding of the National Academy of Sciences of the United States of America 102: 8633-8638.

Pfaffl M. W., Horgan G. W. and Dempfle L., (2002), *Relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real-time PCR*. Nucleic Acids Research 30: e36.

Pico de Coaña Y., Parody N., Fernández-Caldas E. and Alonso C., (2010), *A modified protocol for RNA isolation from high polysaccharide containing* Cupressus arizonica *pollen. Application for RT-PCR and phage display library construction*. Molecular Biotechnology 44: 127-132.

Potapova M., (2011), *Navicymbula pusilla*, Source: In Diatoms of the United States. Retrieved 22.03.13, from: http://westerndiatoms.colorado.edu/taxa/species/navicymbula_pusilla

Promega, (2008), *Recombinant RNasin® Ribonuclease Inhibitor*. Retrieved 27.01.12, from: http://no.promega.com/~/media/Files/Resources/Protocols/Product%20Information%20Sheets/N/RNasin%20Ribonuclease%20Inhibitor%20Recombinant%20Protocol.pdf

QIAGEN, (2009), *QuantiTect® Reverse Transcription Handbook*. Retrieved 13.02.12, from: http://www.qiagen.com/resources/Download.aspx?id={F0DE5533-3DD1-4835-8820-1F5C088DD800}&lang=en&ver=1

QIAGEN, (2010), *RNeasy® Mini Handbook*. Retrieved 27.03.12, from: http://www.qiagen.com/resources/Download.aspx?id={14E7CF6E-521A-4CF7-8CBC-BF9F6FA33E24}&lang=en&ver=1

Quinn P. K. and Bates T. S., (2011), *The case against climate regulation via oceanic phytoplankton sulphur emissions*. Nature 480: 51-56.

Reece R. J., (2004), *Analysis of genes and genomes*. Reprinted 2009, England: West Sussex, pp. 177-181, 192-193.

Reisch C. R., Moran M. A., Whitman W. B., (2011), *Bacterial catabolism of dimethylsulfoniopropionate (DMSP)*. Frontiers in Microbiology 2: 172.

Roche Applied Science, (2011), *LightCycler® 480 SYBR Green I Master*. Retrieved 24.02.12, from: https://cssportal.roche.com/LFR_PublicDocs/ras/04887352001_en_12.pdf

Ruzin S. E., (1999), *Plant Microtechnique and Microscopy*. Source: University of California, Berkeley. Retrieved 28.08.12, from: http://microscopy.berkeley.edu/Resources/instruction/buffers.html

Siaut M., Heijde M., Mangogna M., Montsant A., Coesel S., Allen A., Manfredonia A., Falciatore A. and Bowler C., (2007), *Molecular toolbox for studying diatom biology in* Phaeodactylum tricornutum. Gene 406: 23-35.

Sigma-Aldrich, (2010), *Spectrum Plant Total RNA kit*. Retrieved 27.01.12, from: http://www.sigmaaldrich.com/etc/medialib/docs/Sigma/Bulletin/strn250bul.Par.0001.File.tmp/strn250bul.pdf

Solomons T. W. G. and Fryhle C. B., (2008), *Organic chemistry*, 9. edition, New York: John Wiley & Sons, Inc, pp. 399-400, 415.

Taylor S., Wakem M., Dijkman G., Alsarraj M. and Nguyen M., (2010), *A practical approach to RT-qPCR-publishing data that conform to the MIQE guidelines*. Methods 50: S1-S5.

Tesson B. and Hildebrand M., (2013), *Characterization and localization of insoluble organic matrices associated with diatom cell walls: insight into their roles during cell wall formation*. PLOS ONE 8: e61675.

University of Colorado, (2011), *Intrumentation: the inlet system*. Retrieved 12.05.13, from: http://orgchem.colorado.edu/Spectroscopy/MS/inletsys.html

Vila-Costa M., Simó R., Harada H., Gasol J. M., Slezak D. and Kiene R. P., (2006), *Dimethylsulfoniopropionate uptake by marine phytoplankton*. Science 314: 652-654.

Vrieling E. G., Sun Q., Tian M., Kooyman P. J., Gieskes W. W. C., van Santen R. A. and Sommerdijk N. A. J. M., (2007), *Salinity-dependent diatom biosilicification implies an important role of external ionic strength*. Proceeding the National Academy of Sciences of the United States of America 104: 10441-10446.

Wilfinger W. W., Mackey K. and Chomczynski P., (1997), *Effect of pH and ionic strength on the spectrophotometric assessment of nucleic acid purity*. BioTechniques 22: 474-481.

APPENDIX

APPENDIX 1: Recipes: media and solutions

f/2 growth medium with vitamins and inorganic nutrients:

The f/2 media were modified after Guillard (1975), which is a commonly used enriched sea water medium for cultivating coastal marine algae, such as diatoms.

Natural seawater provided by the Department of Biotechnology was sterile filtered (0,2 µm) before autoclaving at 121 ºC for 20 minutes. Sterile filtered (0,2 µm) nutritional salts, trace elements and vitamin stock solutions were added after autoclaving to 1 L sea water as following:

f/2 medium (1 L)			
Component	Stock solution (g/ L dH_2O)	Volume (mL)	Molecular concentrations in final medium (M)
$NaNO_3$	75	1	8.82×10^{-4}
$NaH_2PO_4 \times H_2O$	5	1	3.62×10^{-5}
$Na_2SiO_3 \times 9H_2O$	45	1	3.18×10^{-4}
Trace metal solution	*	0.5	-
Vitamin solution	**	1	-

***** see trace metal solution

****** see vitamin solution

f/2 - Si medium (1 L)			
Component	Stock solution (g/ L dH_2O)	Volume (mL)	Molecular concentrations in final medium (M)
$NaNO_3$	75	1	8.82×10^{-4}
$NaH_2PO_4 \times H_2O$	5	1	3.62×10^{-5}
$Na_2SiO_3 \times 9H_2O$	45	0	0
Trace metal solution	*	0.5	-
Vitamin solution	**	1	-

***** see trace metal solution

****** see vitamin solution

f/2 - N medium (1 L)			
Component	Stock solution (g/ L dH_2O)	Volume (mL)	Molecular concentrations in final medium (M)
$NaNO_3$	75	0	0
$NaH_2PO_4 \times H_2O$	5	1	3.62×10^{-5}
$Na_2SiO_3 \times 9H_2O$	45	1	3.18×10^{-4}
Trace metal solution	*	0.5	-
Vitamin solution	**	1	-

***** see f/2 trace metal solution

****** see f/2 vitamin solution

The f/2 growth media were stored at room temperature.

f/2 trace metal solution:

The following components were added 950 mL MilliQ water. 5 g Fe-EDTA was added to the solution and the final volume were brought to 1 L using MilliQ water. The solution was sterile filtrated (0,2 µm) and stored at 4 ºC.

Trace metal solution			
Component	Primary stock solution (g/ L dH_2O)	Volume (mL)	Molecular concentrations in final medium (M)
$CuSO_4$ x $5H_2O$	10.0	1	4.20×10^{-8}
$ZnSO_4$ x $7H_2O$	22.0	1	7.65×10^{-8}
$CoCl_2$ x $6H_2O$	10.0	1	4.20×10^{-8}
$MnCl_2$ x $4H_2O$	180.0	1	9.10×10^{-7}
Na_2MoO_4 x $2H_2O$	6.3	1	2.60×10^{-8}

f/2 vitamin solution:

200 mg thiamine HCl was dissolved in 950 mL MilliQ water. The following components were added the solution and the final volume were brought to 1 L by using MilliQ water. The solution was sterile filtrated (0,2 µm) and stored at 4 ºC.

Vitamin solution			
Component	Primary stock solution (g/ L dH_2O)	Quantity	Molecular concentrations in final medium (M)
Thiamine HCl (Vitamin B_1)	-	200 mg	5.92×10^{-7}
Biotin (Vitamin H)	0.1	10 mL	4.10×10^{-9}
Cyanocobalamin (Vitamin B_{12})	1.0	1 mL	7.38×10^{-10}

Lugol's solution:

The Lugol's solution used in this study was fabricated by Merck Millipore, consisting of diluted iodine-potassium iodide solution (Merck Millipore, 2013). 1 liter solution contained the following compounds:

Lugol's solution	
Component	Concentration (g/L)
I_2	3.4
KI	6.8

Formaldehyde (FA) gels and buffers for electrophoresis:

The FA gel and buffers for FA gel electrophoresis were prepared according to the QIAGEN RNeasy® Mini Handbook, in order to check the integrity of the purified total RNA (QIAGEN, 2010).

FA gel:

FA gels (1,2 % agarose) of size 5 x 7 x 0,35 cm were prepared as described in Section 2.2.4, containing the following components:

FA gel (1.2 % agarose)	
Agarose	0.6 g
10x FA gel buffer	5 mL
MilliQ water*	<50 mL
37 % Formaldehyde (12.3 M)	900 µL
Ethidium bromide (10 mg/mL)	0.5 µL

* MilliQ water was added to adjust the total volume of the agarose and 10x FA gel buffer to 50 mL.

FA gel buffers:

The composition of the FA gel buffers were as following, prepared after the QIAGEN RNeasy® Mini Handbook (QIAGEN, 2010):

10x FA gel buffer	
3-[N-morpholino]propanesulfonic acid (MOPS) (free acid)	200 mM
Sodium acetate	50 mM
EDTA	10 mM
pH adjusted to 7.0 with NaOH	

1x FA gel running buffer	
10x FA gel buffer	50 mL
37 % Formaldehyde (12.3 M)	10 mL
MilliQ water*	<500 mL

***** MilliQ water was added to adjust the total volume of 500 mL.

5 x RNA loading buffer	
Saturated aqueous bromophenol blue solution*	16 µL
EDTA (500 mM, pH 8.0)	80 µL
37 % Formaldehyde (12.3 M)	720 µL
87 % glycerol	2 mL
Formamide	3.084 mL
10x FA gel buffer	4 mL
MilliQ-water to a total volume of 10 mL	

***** The saturated solution was made by adding solid bromophenol blue to autoclaved MilliQ water, until no more would dissolve. The mixture was centrifuged and the saturated supernatant were utilized.

Phosphate-citrate buffer:

A phosphate-citrate (60 mM, pH 6) buffer was prepared for the quantification of DMSP according to the buffer table of Ruzin (1999). 100 mL contained the following sterile filtrated (0.2 µm) components:

Phosphate-citrate Buffer	
Na_2HPO_4 (0.2 M)	32.1 mL
Citric acid (0.1 M)	17.9 mL
MilliQ-water	50 mL

Na_2HO_4 (0.2 M)	
Na_2HPO_4	1.4196 g
MilliQ-water*	<50 mL

* MilliQ-water were added to adjust the volume to 50 mL

Citrate (0.1 M)	
Citric acid monohydrate	1.0507 g
MilliQ-water*	<50 mL

* MilliQ-water were added to adjust the volume to 50 mL

APPENDIX 2: RT-qPCR primers

The primers utilized for the quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR) are listed in Table A2.1. Two of the primer pairs were designed to span an intron, in order to eliminate any genomic DNA contaminations of the PCR products. The product length and the melting points of the RT-qPCR primers are shown in Table A2.2. The *H4* and *TBP* genes were chosen as reference genes for normalization of the RT-qPCR data. The ID accession numbers in the JGI portal and GenBank of the candidate genes are presented in Table A2.3, for the diatoms *T. pseudonana*, *F. cylindrus* and *P. tricornutum*.

Table A2.1 RT-qPCR primers. Forward (F) and reverse (R) primer sequences of the target genes and the transcript ID numbers in the JGI portal (http://genome.jgi-psf.org/Thaps3/Thaps3.home.html). Primer pairs marked with an asterisk span an intron. The *H4* and *TBP* genes were references.

Target Gene	Transcript ID (Thaps3)	Primer	Sequence (5'-3')
DiDECARB	20613	qTpDiDECARB2F2	AGTTTGAGTTCGCAGGAGTTGC
		qTpDiDECARB2R2	CTTTGCCGATGCGTTCGAGTTG
SAMmt	11247	qTpSAMmtF1	TCATCATCGTCGACAACATCCA
		qTpSAMmt R1	GACGTCAAAGCATCCTTTAGTG
REDOX	21067	qTpREDOXF1*	CATGCTGGAAGCTGATGGTACT
		qTpREDOXR1	CAGCTGGTTCAACATCCTATGC
AT	31394	qTpATF1	AGTTGTTACCGCTGTTCAGGAT
		qTpATR1	GAGTTGATATCTCCAGTTTGAC
H4	766	qTpH4F1*	GACTCTGTTACCTACACTGAAC
		qTpH4R1	TTGAGGGCATAAACGACATCCA
TBP	9662	qTpTBPF1*	TCTTCAGCTCAGGTCGTATGGT
		qTpTBPR1	AGTGGCCAGACTGCAATTGTGA

Table A2.2 Product length and melting points. The length of the RT-qPCR products of each primer, measured in base pairs, and the melting temperatures (Tm) in degrees Centigrade (ºC).

Primer	Product length (bp)	Tm (ºC)
qTpDiDECARB2F2	102	54.8
qTpDiDECARB2R2		56.7
qTpSAMmtF1	110	53.0
qTpSAMmt R1		53.0
qTpREDOXF1	57	54.8
qTpREDOXR1		54.8
qTpATF1	83	53.0
qTpATR1		51.1
qTpH4F1	70	53.0
qTpH4R1		53.0
QTpTBPF1	64	54.8
QTpTBPR1		54.8

Table A2.3 ID accession numbers of the candidate genes. The JGI ID numbers for the diatoms *F. cylindrus*, *P. tricornutum* and *T. pseudonana* and the GenBank ID for *T. pseudonana*.

Name	**Putative activity**	***F. cylindrus* JGI ID**	***P. tricornutum* JGI ID**	***T. pseudonana* JGI ID**	**T. pseudonana GenBank ID**
AT	Aminotransferase	Fc273803	Pt22909	Tp31394	XM_002285956
REDOX	NADPD-dependent flavinoid reductase	Fc173405	Pt37671	Tp21067	XM_002287028
SAMmt	*S*-adenosyl methionine-dependent methyltransferase	Fc207357	-	Tp11247	XM_002296942
DECARB	Pyridoxyl dependent decarboxylase	Fc238865	-	-	-
DiDECARB	Diaminopimelate decarboxylase	Fc263016	Pt21592	Tp20613	XM_002286548

APPENDIX 3: Standards for GC-MS

To quantify dimethylsulfoniopropionate (DMSP) using gas chromatography-mass spectrometry (GC-MS), standard solutions of known concentrations of DMSP were made. Three technical replicates were used per standard solution.

Standards for Experiment 2:

Diluted DMSP (0.04 M) solution was made by diluting the original DMSP (1.2 M) solution 30 times with MilliQ water. 43.5 µL MilliQ water were added 1.5 µL DMSP (1.2 M) and mixed by pipetting. The resulting DMSP (0.04 M) solution was used to prepare the standard solutions used in Experiment nr.2. Phosphate-citrate (60 mM) buffer were added the DMSP solution and 2 mL of NaOH (0.5 M) to hydrolyze the DMSP into the volatile DMS compound. The standards were incubation at 25 ºC for 30 minutes in a thermal shake incubator prior to GC-MS analysis, as described in Section 2.3.2. To make the standard solutions the following quantities of sterile filtrated (0.2 µm) reagents were mixed:

Concentration (µM)	DMSP (µL) (0.04 M)	Buffer (µL)	NaOH (0.5 M) (mL)
10	0.55	199.45	2
25	1.38	198.62	2
50	2.75	197.25	2
75	4.13	195.87	2
100	5.50	194.50	2

Standards for Experiment 3:

Standards for the third experiment, were made using DMSP (0.12 mM) for the lowest concentrated standard solutions of 0.01 and 0.1 µM, and DMSP (0.012 M) for the standard solutions at 1, 2, 5, 7 and 10 µM. Phosphate-citrate (60 mM) buffer were added to the DMSP solutions and NaOH (0.5 M), and were incubated prior to GC-MS analysis as described in Section 2.3.2. The standards were made by the following volumes of sterile filtrated (0.2 µm) reagents:

Concentration (µM)	DMSP (0.12 mM) (µL)	Buffer (µL)	NaOH (0.5 M) (mL)
0.01	0.18	199.82	2
0.1	1.80	198.20	2

Concentration (µM)	DMSP (0.012 M) (µL)	Buffer (µL)	NaOH (0.5 M) (mL)
1	0.18	199.82	2
2	0.36	199.64	2
5	0.90	199.10	2
7	1.26	198.74	2
10	1.80	198.20	2

To make the DMSP (0.012 M) solution, 0.5 µL DMSP (1.2 M) were added to 49.5 µL MilliQ water. 0.5 µL of the solution were further diluted in 49.5 µL MilliQ water, in order to make the solution of DMSP (0.12 mM).

Calculations of standard concentrations:

The concentration of the DMSP standard solutions were calculated from the total volume of the standard, made up by the DMSP solution, phosphate-citrate (60 mM) buffer and NaOH (0.5 M), as illustrated in Figure A3.1.

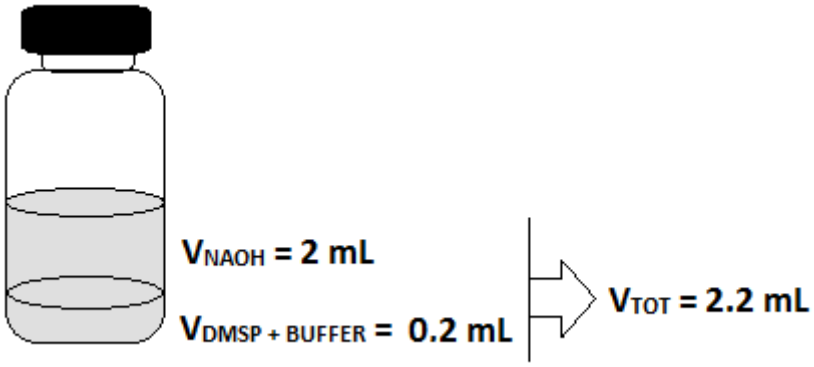

Figure A3.1 Total volume and components of the DMSP standard solutions. The total volume of the standard solutions (2.2 mL) included 0.2 mL of the DMSP solution and phosphate-citrate (60 mM) buffer and 2 mL NaOH (0.5 M).

Examples of calculation:

<u>10 µM standard (Experiment 2):</u>
10 µM = 0.000010 M
Original DMSP solution = 0.04 M

Dilution factor = 0.04 M / 0.000010 M = 4000

Total volume of the standard: V_{TOT} = 2.2 mL = 2200 µL
Volume of DMSP and buffer: $V_{DMSP+BUFFER}$ = 0.2 mL = 200 µL

$Volume_{DMSP}$ = 2200 µL / 4000 = **0.55 µL**
$Volume_{BUFFER}$ = 200 µL - 0.55 µL = **199.45 µL**

Therefore, to prepare a standard solution of 10 µM DMSP, 0,6 µL DMSP (0.04 M) were added 199.4 µL phosphate-citrate (60 mM) buffer. All standards included a constant volume of 2 mL NaOH (0.5 M) in order to hydrolyze the DMSP into the volatile compound DMS.

APPENDIX 4: RNA yield and quality

Total RNA from the RNA optimization:

Table A4.1 Test of total RNA purification protocols. The RNA concentrations and 260/280 and 260/230 absorbance ratios of the obtained from the Nanodrop. Two technical replicates were measured for each protocol tested.

Protocols	RNA concentration (ng/µL)	Absorbance ratio 260/280	Absorbance ratio 260/230
Sigma Life Science	38.03	1.84	1.35
Protocol	37.20	1.83	1.37
QIAGEN	191.40	2.06	0.52
Protocol A	193.71	2.06	0.53
QIAGEN	292.93	2.17	2.07
Protocol B	294.27	2.17	2.06

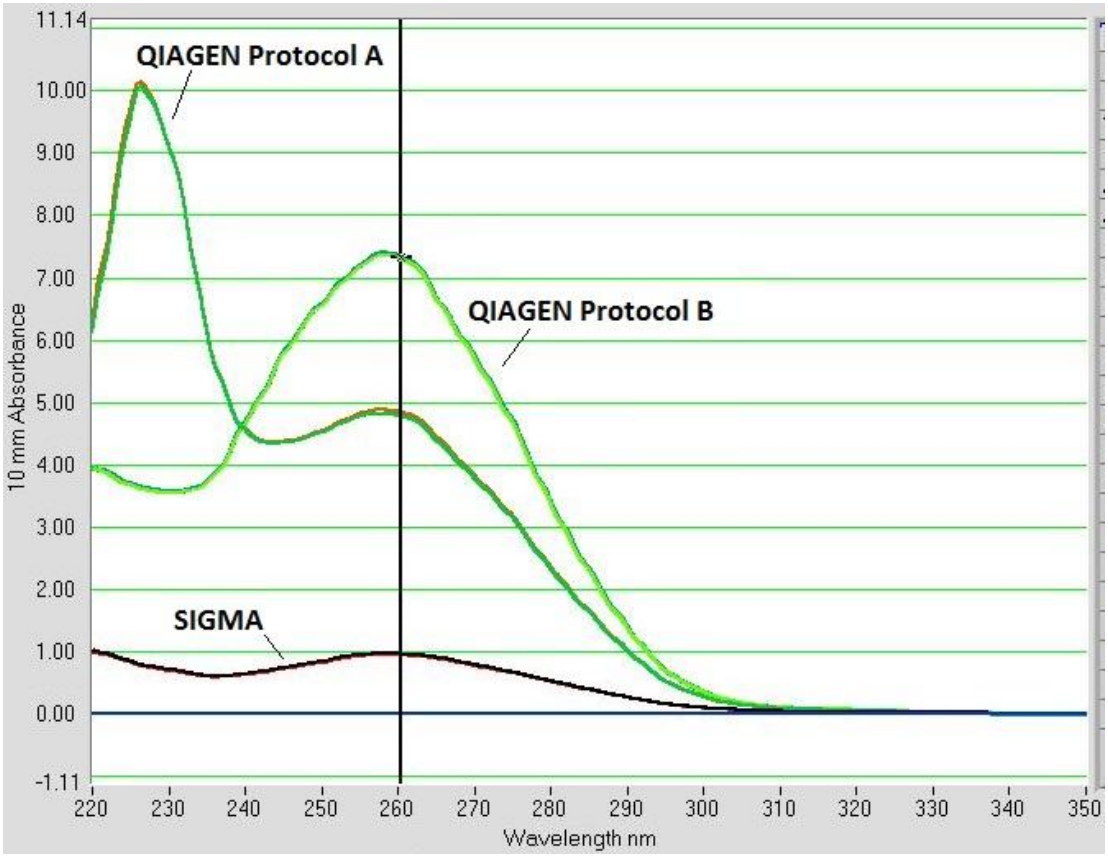

Figure A4.1 Spectral profiles of total RNA. The absorbance spectra of total RNA isolated by the Sigma Life Science protocol, QIAGEN Protocol A and QIAGEN Protocol B, obtained from Nanodrop spectrophotometer. The sharpest peak at 260 nm was obtained in total RNA purified by the QIAGEN Protocol B, indicating the purest RNA (Wilfinger et al., 1997).

Table A4.2 Test of membrane filters. The RNA concentrations and 260/280 and 260/230 absorbance ratios obtained from the Nanodrop for each of the two biological replicates tested per membrane filter. The membrane filters were used to harvest 75 mL samples of axenic batch cultures of *T. pseudonana* by vacuum filtration.

Membrane filters	RNA concentration (ng/µL)	Absorbance ratio 260/280	Absorbance ratio 260/230
Durapore® DVPP Membrane filter (0.65 µm)	1713.06	2.19	2.47
	1531.73	2.19	1.59
Supor 800 membrane disc filter (0.80 µm)	1492.50	2.20	2.40
	1559.61	2.19	1.86
Polycarbonate (PCTE) membrane (1.0 µm)	992.75	2.21	1.53
	1280.64	2.21	2.41

Total RNA from Experiment 1:

Total RNA was isolated from experimental cultures of nutrient limited *T. pseudonana* cultures. The whole cultures (<150 mL) were harvested by vaccum filtration and total RNA was isolated using the QIAGEN Protocol B. The concentrations and absorbance ratios of the total RNA is shown in Table A4.3. As the 260/230 absorbance ratios of several samples were low, RNA cleanup was performed. The resulting RNA data obtained from Nanodrop and Qubit is shown in Table A4.4. Samples derived from control cultures grown in enriched f/2 medium are called "Tp 1C" to "Tp 4C", where "Tp" stands for *Thalassiosira pseudonana*, the number indicates the number of the biological replicate and the "C" stands for control. Similarly, the samples derived from the silicate limited cultures are named "Tp 1-Si" to "Tp 4-Si" and the nitrate limited cultures are called "Tp 1-N" to "Tp 4-N".

Table A4.3 Total RNA pre RNA cleanup. The total RNA concentrations (ng/µL) and absorbance ratios before RNA cleanup was performed. Total RNA was isolated from experimental cultures grown under different nutrient treatments using QIAGEN Protocol B. Four biological replicates were used per treatment and two technical replicates were used for the Nanodrop analysis. Control samples are marked with "C", silicate limited samples are marked with "-Si" and nitrate limited with "-N".

Samples	RNA concentration (ng/µL)	Absorbance ratio 260/280	Absorbance ratio 260/230
Tp 1C	111.28	2.12	1.46
	110.89	2.09	1.49
Tp 2C	236.29	2.17	1.25
	237.84	2.17	1.26
Tp 3C	233.83	2.16	1.37
	237.46	2.17	1.40
Tp 4C	261.15	2.16	2.22
	251.85	2.18	2.15
Tp 1-Si	469.58	2.09	2.04
	469.65	2.08	2.01
Tp 2-Si	88.52	2.09	1.22
	88.07	2.07	1.32
Tp 3-Si	334.25	2.12	1.65
	334.10	2.13	1.62
Tp 4-Si	211.80	2.11	1.68
	208.38	2.14	1.75
Tp 1-N	158.44	2.17	1.49
	157.02	2.18	1.46
Tp 2-N	119.52	2.12	1.52
	119.62	2.10	1.52
Tp 3-N	152.95	2.17	0.87
	153.92	2.16	0.87
Tp 4-N	35.60	1.86	0.30
	35.43	1.96	0.30

Table A4.4 Total RNA after RNA cleanup. The total RNA concentrations from Qubit and Nanodrop, along with the absorbance ratios from Nanodrop, after RNA cleanup was performed on the total RNA of Experiment 1. Samples marked with a "C" are control samples, "-Si" are from silicate limited cultures and "-N" are samples derived from the nitrate limited cultures.

	Qubit	**Nanodrop**		
Samples	RNA concentration (ng/µL)	RNA concentration (ng/µL)	Absorbance ratio 260/280	Absorbance ratio 260/230
Tp 1C	55.00	81.12	2.05	0.90
	51.00	69.55	2.11	0.91
Tp 2C	95.00	176.45	2.17	2.00
	90.00	177.38	2.14	2.01
Tp 3C	155.50	232.14	2.17	2.05
	166.00	229.30	2.16	2.04
Tp 4C	154.00	215.08	2.13	0.97
	136.00	213.45	2.17	0.96
Tp 1-Si	290.00	512.23	2.18	2.45
	335.00	514.42	2.18	2.47
Tp 2-Si	80.00	124.39	2.20	2.15
	76.00	122.77	2.21	2.28
Tp 3-Si	108.00	254.91	2.14	2.07
	193.50	260.10	2.13	2.03
Tp 4-Si	92.00	167.36	2.19	1.25
	89.00	162.59	2.13	1.21
Tp 1-N	77.00	120.60	2.12	1.05
	71.00	121.52	2.11	1.04
Tp 2-N	54.00	78.35	2.07	1.49
	55.00	79.43	2.06	1.46
Tp 3-N	81.00	142.59	2.16	1.78
	76.00	142.16	2.17	1.75
Tp 4-N	24.90	31.08	1.95	0.11
	24.60	31.34	1.91	0.11

Total RNA from Experiment 3:

Total RNA was isolated by Torfinn Sparstad, from samples of three experimental cultures of *T. pseudonana* grown in complete silicate free f/2 media. The resulting total RNA data from the Nanodrop analysis is shown in Table A4.5. Samples were harvested from 50 mL volume of *T. pseudonana* by vacuum filtration.

Table A4.5 Total RNA from Experiment 3. The total RNA concentrations from Nanodrop, along with the absorbance ratios, of samples derived from three *T. pseudonana* cultures grown in complete silicate free f/2 media. Samples were harvested 20 minutes before Start, and 7.5, 24, 48 and 72 hours after Start. Two technical replicates were measured per sample in the Nanodrop analysis.

Samples	RNA concentration (ng/µL)	Absorbance ratio 260/280	Absorbance ratio 260/230
Tp -20i	545.25	2.29	2.50
	553.01	2.17	2.47
Tp -20ii	904.33	2.18	2.54
	918.71	2.18	2.53
Tp -20iii	995.98	2.19	2.53
	998.03	2.19	2.54
Tp 7.5i	504.72	2.13	2.45
	492.32	2.15	2.47
Tp 7.5ii	516.59	2.14	2.49
	521.18	2.16	2.49
Tp 7.5iii	620.51	2.16	2.27
	620.58	2.15	2.22
Tp 24i	1300.62	2.18	2.54
	1292.55	2.18	2.54
Tp 24ii	1381.70	2.18	2.55
	1370.43	2.18	2.55
Tp 24iii	1667.92	2.17	2.52
	1663.22	2.18	2.50
Tp 48i	1447.86	2.19	2.52
	1418.05	2.19	2.54
Tp 48ii	1192.16	2.18	2.55
	1178.09	2.19	2.55
Tp 48iii	1082.35	2.18	2.55
	1091.58	2.18	2.54
Tp 72i	1243.49	2.18	2.55
	1232.84	2.18	2.55
Tp 72ii	794.49	2.17	2.50
	807.21	2.17	2.51
Tp 72iii	724.16	2.17	2.30
	729.54	2.16	2.28

Formaldehyde (FA) gels:

FA gels from the gene expression analyses of nutrient limited cultures (Experiment 1) and the complete silicate starved cultures (Experiment 3) are displayed in Figure A4.2 and Figure A4.3, respectively. The RNA bands obtained in both experiments indicated that the total RNA was not degraded, as the 28S rRNA bands were sharper than the 18S rRNA bands and there were no bands that appeared as smears towards smaller rRNA (QIAGEN, 2010).

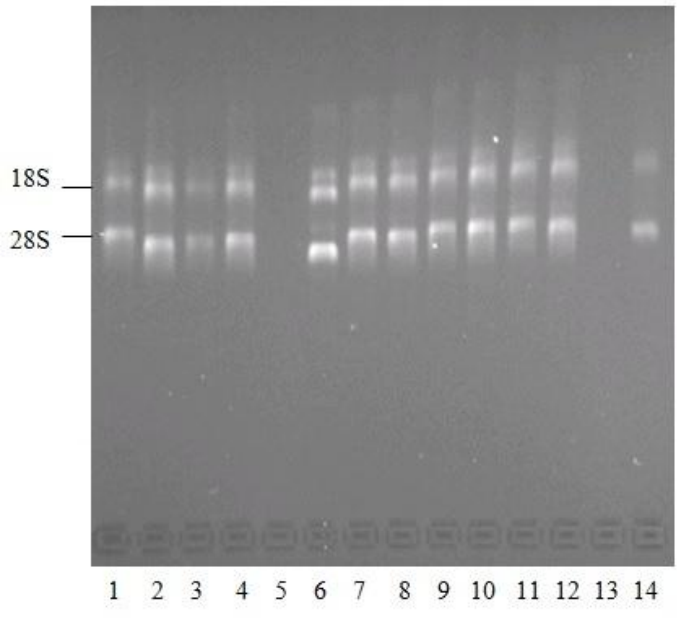

Figure A4.2 FA gel from Experiment 1. Total RNA profiles for the samples of the first experiment. Lane 1-4: Control samples, lane 5: space, lane 6-9: biological replicates of silicate-limited cultures, lane 10-12: biological replicates of nitrate-limited cultures, lane 13: space and lane 14: *Arabidopsis thaliana* control.

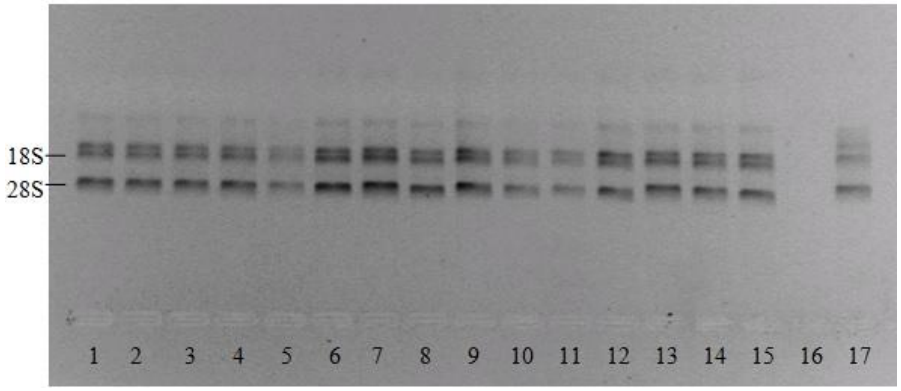

Figure A4.3 FA gel from Experiment 3. The total RNA profiles from the samples derived from the third experiment. Samples from three biological replicates of complete silicate starved *T. pseudonana* cultures were harvest at the time points 20 minutes before Start and 7.5, 24, 48 and 72 hours after Start. Lane 1-3: The samples "Tp -20i-iii", lane 4-6: "Tp 7.5i-iii", lane 7-9: "Tp 24i-iii", lane 10-12: "Tp 48i-iii", lane 13-15: "Tp 72i-iii", lane 16: space and lane 17: *Arabidopsis thaliana* control.

Appendix 5: Growth data

Growth data from Experiment 1:

Table A5.1 Cell densities of control cultures (Experiment 1). Original growth data from the cell counting, measuring cell densities (cells mL^{-1}) over time for the control cultures. Four biological replicates "Tp 1C" - "Tp 4C" were used, and the average cell density and standard deviation between replicates are given.

	Cell density (cells mL^{-1})				Average cell density (cells mL^{-1})	Standard deviation (SD)
	Tp 1C	Tp 2C	Tp 3C	Tp 4 C		
START	100 300	100 300	100 300	100 300	100 300	0
Day 1	663 300	558 300	538 300	706 700	616 700	81 300
Day 2	1 405 000	1 370 000	1 512 500	1 275 000	1 390 600	98 100
Day 3	1 785 000	1 682 500	1 640 000	1 695 000	1 700 600	61 000
Day 4	2 195 000	2 347 500	2 170 000	2 175 000	2 221 900	84 400
Day 5	2 730 000	2 600 000	2 745 000	2 425 000	2 625 000	148 400

Table A5.2 Cell densities of the silicate-limited cultures (Experiment 1). The original growth data from the cell counting, measuring cell densities (cells mL^{-1}) over time for the silicate limited cultures. Four biological replicates "Tp 1-Si" - "Tp 4-Si" were used, and the average cell density and standard deviation between replicates are given.

	Cell density (cells mL^{-1})				Average cell density (cells mL^{-1})	Standard deviation (SD)
	Tp 1-Si	Tp 2-Si	Tp 3-Si	Tp 4-Si		
START	100 300	100 300	100 300	100 300	100 300	0
Day 1	570 000	576 700	601 700	531 700	570 000	29 000
Day 2	1 322 500	1 242 500	1 325 000	1 202 500	1 273 100	60 700
Day 3	1 647 500	1 927 500	1 482 500	1 640 000	1 674 400	185 100
Day 4	2 365 000	2 210 000	2 295 000	2 090 000	2 240 000	118 400
Day 5	3 252 500	2 770 000	2 812 500	2 617 500	2 863 100	272 800

Table A5.3 Cell densities of the nitrate-limited cultures (Experiment 1). The original growth data from the cell counting, measuring cell densities (cells mL^{-1}) over time for the nitrate limited cultures. Four biological replicates "Tp 1-N" - "Tp 4-N" were used, and the average cell density and standard deviation between replicates are given.

	Cell density (cells mL^{-1})				Average cell density (cells mL^{-1})	Standard deviation (SD)
	Tp 1-N	Tp 2-N	Tp 3-N	Tp 4-N		
START	100 300	100 300	100 300	100 300	100 300	0
Day 1	595 000	493 300	595 000	640 000	580 800	62 100
Day 2	1 275 000	1 215 000	1 155 000	1 212 500	1 214 400	49 000
Day 3	1 217 500	1 345 000	1 407 500	1 177 500	1 286 900	107 600
Day 4	1 402 500	1 617 500	1 362 500	1 407 500	1 447 500	115 100
Day 5	1 537 500	1 655 000	1 632 500	1 725 000	1 637 500	77 400

Growth data from Experiment 2:

Table A5.4 Cell densities of control cultures (Experiment 2). Original growth data from the cell counting, measuring cell densities (cells mL^{-1}) over time for the control cultures. Three biological replicates "Tp 1C" - "Tp 3C" were used, and the average cell density and standard deviation between replicates are given.

	Cell density (cells mL^{-1})			**Average cell density (cells mL^{-1})**	**Standard deviation (SD)**
	Tp 1C	Tp 2C	Tp 3C		
START	102 300	102 300	102 300	102 300	0
Day 1	595 000	653 300	510 000	586 100	72 100
Day 2	1 545 000	1 472 500	1 360 000	1 459 200	93 200
Day 3	1 627 500	1 887 500	1 730 000	1 748 300	131 000
Day 4	2 487 500	2 507 500	2 510 000	2 501 700	12 300
Day 5	3 930 000	3 752 500	4 135 000	3 939 200	191 400

Table A5.5 Cell densities of silicate-limited cultures (Experiment 2). Original growth data from the cell counting, shown as cell densities (cells mL^{-1}) over time for the silicate limited cultures. Four biological replicates "Tp 1-Si" - "Tp 3-Si" were used, and the average cell density and standard deviation between replicates are given.

	Cell density (cells mL^{-1})			**Average cell density (cells mL^{-1})**	**Standard deviation (SD)**
	Tp 1-Si	Tp 2-Si	Tp 3-Si		
START	102 300	102 300	102 300	102 300	0
Day 1	538 300	601 700	591 700	577 200	34 100
Day 2	1 370 000	1 100 000	1 220 000	1 230 000	135 300
Day 3	1 915 000	1 802 500	2 022 500	1 913 300	110 000
Day 4	2 362 500	2 050 000	2 365 000	2 259 200	181 100
Day 5	3 310 000	3 012 500	3 302 500	3 208 300	169 600

Table A5.6 Cell densities of nitrate-limited cultures (Experiment 2). Original growth data from the cell counting, displayed as cell densities (cells mL^{-1}) over time for the nitrate limited cultures. Four biological replicates "Tp 1-N" - "Tp 3-N" were used, and the average cell density and standard deviation between replicates are given.

	Cell density (cells mL^{-1})			**Average cell density (cells mL^{-1})**	**Standard deviation (SD)**
	Tp 1-N	Tp 2-N	Tp 3-N		
START	102 300	102 300	102 300	102 300	0
Day 1	621 700	525 000	606 700	584 500	52 000
Day 2	1 262 500	1 135 000	1 272 500	1 223 300	76 700
Day 3	1 495 000	1 332 500	1 325 000	1 384 200	96 100
Day 4	1 490 000	1 405 000	1 485 000	1 460 000	47 700
Day 5	1 817 500	1 667 500	1 892 500	1 792 500	114 600

Growth data from Experiment 3:

Table A5.7 Cell densities of the silicate starved cultures (Experiment 2). Cell densities (cells mL^{-1}) over time for the silicate starved cultures. Three biological replicates (i, ii and iii) were used, and the average cell density and standard deviation between replicates are given.

	Cell density (cells mL^{-1})			**Average cell density (cells mL^{-1})**	**Standard deviation (SD)**
	i	ii	iii		
- 20 min	2 490 000	2 680 000	2 103 300	2 424 400	293 900
7.5 hours	3 710 000	2 930 000	3 385 000	3 341 700	391 800
24 hours	4 093 300	3 973 300	4 186 700	4 084 400	107 000
48 hours	4 273 300	4 640 000	4 453 300	4 455 500	183 400
72 hours	4 706 700	5 446 700	5 060 000	5 071 100	370 100

Appendix 6: LinReg results

The results from the LinRegPCR analysis in RT-qPCR are displayed in Table A6.1-3 for Experiment 1 and Table A6.4-8 for Experiment 3.

Table A6.1 LinReg results for the control samples in RT-qPCR (Experiment 1). Individual PCR efficiency, grouping, mean PCR efficiency and Ct values of the control samples of Experiment 1.

Sample	Individual PCR efficiency	Group	Mean PCR efficiency	Ct value
Tp 1C DiDECARB	1.000	1	1.916	0.000
Tp 2C DiDECARB	1.895	1	1.916	31.019
Tp 3C DiDECARB	1.908	1	1.916	32.545
Tp 4C DiDECARB	1.922	1	1.916	28.491
Tp 1C SAMmt	1.882	3	1.883	37.125
Tp 2C SAMmt	1.903	3	1.883	33.642
Tp 3C SAMmt	1.871	3	1.883	34.996
Tp 4C SAMmt	1.879	3	1.883	31.223
Tp 1C REDOX	1.933	4	1.937	27.353
Tp 2C REDOX	1.973	4	1.937	23.796
Tp 3C REDOX	1.933	4	1.937	24.351
Tp 4C REDOX	1.916	4	1.937	24.068
Tp 1C AT	1.857	5	1.864	36.342
Tp 2C AT	1.878	5	1.864	31.636
Tp 3C AT	1.856	5	1.864	32.921
Tp 4C AT	1.865	5	1.864	29.726
Tp 1C H4	1.923	6	1.920	33.546
Tp 2C H4	1.917	6	1.920	27.616
Tp 3C H4	1.899	6	1.920	29.279
Tp 4C H4	1.944	6	1.920	27.563
Tp 1C TBP	1.944	7	1.945	33.361
Tp 2C TBP	1.937	7	1.945	30.025
Tp 3C TBP	1.961	7	1.945	30.819
Tp 4C TBP	1.963	7	1.945	27.864

Table A6.2 LinReg results for the silicate-limited cultures (Experiment 1). Individual PCR efficiency, grouping, mean PCR efficiency and Ct values of the samples from the silicate-limited cultures of Experiment 1.

Sample	Individual PCR efficiency	Group	Mean PCR efficiency	Ct value
Tp 1-Si DiDECARB	1.921	1	1.916	30.345
Tp 2-Si DiDECARB	1.929	1	1.916	35.440
Tp 3-Si DiDECARB	1.920	1	1.916	38.464
Tp 4-Si DiDECARB	1.918	1	1.916	37.521
Tp 1-Si SAMmt	1.872	3	1.883	32.996
Tp 2-Si SAMmt	1.897	3	1.883	38.413
Tp 3-Si SAMmt	1.887	3	1.883	38.714
Tp 4-Si SAMmt	1.891	3	1.883	38.107
Tp 1-Si REDOX	1.914	4	1.937	24.001
Tp 2-Si REDOX	1.953	4	1.937	28.419
Tp 3-Si REDOX	1.951	4	1.937	24.722
Tp 4-Si REDOX	1.912	4	1.937	32.620
Tp 1-Si AT	1.856	5	1.864	31.137
Tp 2-Si AT	1.879	5	1.864	35.782
Tp 3-Si AT	1.873	5	1.864	36.682
Tp 4-Si AT	1.842	5	1.864	39.557
Tp 1-Si H4	1.941	6	1.920	27.634
Tp 2-Si H4	1.930	6	1.920	33.772
Tp 3-Si H4	1.915	6	1.920	31.109
Tp 4-Si H4	1.887	6	1.920	37.497
Tp 1-Si TBP	1.932	7	1.945	29.376
Tp 2-Si TBP	1.955	7	1.945	33.326
Tp 3-Si TBP	1.942	7	1.945	32.692
Tp 4-Si TBP	1.921	7	1.945	36.171

Table A6.3 LinReg results for the nitrate-limited cultures (Experiment 1). Individual PCR efficiency, grouping, mean PCR efficiency and Ct values of the samples from the nitrate-limited cultures of Experiment 1.

Sample	Individual PCR efficiency	Group	Mean PCR efficiency	Ct value
Tp 1-N DiDECARB	1.931	1	1.916	30.710
Tp 2-N DiDECARB	1.900	1	1.916	33.162
Tp 3-N DiDECARB	1.916	1	1.916	33.935
Tp 1-N SAMmt	1.870	3	1.883	34.474
Tp 2-N SAMmt	1.865	3	1.883	36.002
Tp 3-N SAMmt	1.899	3	1.883	38.410
Tp 1-N REDOX	1.920	4	1.937	21.200
Tp 2-N REDOX	1.959	4	1.937	23.085
Tp 3-N REDOX	2.008	4	1.937	21.802
Tp 1-N AT	1.861	5	1.864	32.601
Tp 2-N AT	1.860	5	1.864	35.282
Tp 3-N AT	1.851	5	1.864	35.259
Tp 1-N H4	1.946	6	1.920	26.852
Tp 2-N H4	1.903	6	1.920	29.684
Tp 3-N H4	1.922	6	1.920	28.595
Tp 1-N TBP	1.960	7	1.945	29.610
Tp 2-N TBP	1.931	7	1.945	31.435
Tp 3-N TBP	1.946	7	1.945	31.565

Table A6.4 LinReg results for the control samples (Experiment 3). Individual PCR efficiency, grouping, mean PCR efficiency and Ct values of the control (t0) samples of Experiment 3. The controls were harvested 20 minutes before Start. Three biological replicates (i-iii) were used.

Sample	Individual PCR efficiency	Group	Mean PCR efficiency	Ct value
Tp -20i DiDECARB	1.928	1	1.917	26.385
Tp -20ii DiDECARB	1.917	1	1.917	27.835
Tp -20iii DiDECARB	1.901	1	1.917	28.002
Tp -20i SAMmt	1.000	2	1.855	0.000
Tp -20ii SAMmt	1.844	2	1.855	33.314
Tp -20iii SAMmt	1.840	2	1.855	32.144
Tp -20i REDOX	1.883	3	1.894	23.193
Tp -20ii REDOX	1.898	3	1.894	23.635
Tp -20iii REDOX	1.916	3	1.894	24.423
Tp -20i AT	1.617	4	1.630	35.543
Tp -20ii AT	1.632	4	1.630	36.349
Tp -20iii AT	1.633	4	1.630	37.871
Tp -20i H4	1.836	5	1.886	31.997
Tp -20ii H4	1.917	5	1.886	29.825
Tp -20iii H4	1.894	5	1.886	32.656
Tp -20i TBP	1.923	6	1.928	27.147
Tp -20ii TBP	1.918	6	1.928	28.254
Tp -20iii TBP	1.937	6	1.928	28.660

Table A6.5 LinReg results for the 7.5 hour samples (Experiment 3). Individual PCR efficiency, grouping, mean PCR efficiency and Ct values of the t1 samples of Experiment 3, harvested 7.5 hours after Start. Three biological replicates (i-iii) were used.

Sample	Individual PCR efficiency	Group	Mean PCR efficiency	Ct value
Tp 7.5i DiDECARB	1.927	1	1.917	25.669
Tp 7.5ii DiDECARB	1.906	1	1.917	26.437
Tp 7.5iii DiDECARB	1.923	1	1.917	22.749
Tp 7.5i SAMmt	1.000	2	1.855	0.000
Tp 7.5ii SAMmt	1.869	2	1.855	32.757
Tp 7.5iii SAMmt	1.613	2	1.855	38.757
Tp 7.5i REDOX	1.890	3	1.894	24.360
Tp 7.5ii REDOX	1.888	3	1.894	24.753
Tp 7.5iii REDOX	1.892	3	1.894	23.594
Tp 7.5i AT	1.625	4	1.630	37.714
Tp 7.5ii AT	1.630	4	1.630	38.845
Tp 7.5iii AT	1.630	4	1.630	35.977
Tp 7.5i H4	1.894	5	1.886	30.219
Tp 7.5ii H4	1.911	5	1.886	30.706
Tp 7.5iii H4	1.877	5	1.886	30.519
Tp 7.5i TBP	1.927	6	1.928	28.281
Tp 7.5ii TBP	1.926	6	1.928	29.487
Tp 7.5iii TBP	1.921	6	1.928	25.386

Table A6.6 LinReg results for the 24 hour samples (Experiment 3). Individual PCR efficiency, grouping, mean PCR efficiency and Ct values of the t2 samples of Experiment 3, harvested 24 hours after Start. Three biological replicates (i-iii) were used.

Sample	Individual PCR efficiency	Group	Mean PCR efficiency	Ct value
Tp 24i DiDECARB	1.895	1	1.917	25.920
Tp 24ii DiDECARB	1.911	1	1.917	27.433
Tp 24iii DiDECARB	1.940	1	1.917	25.549
Tp 24i SAMmt	1.000	2	1.855	0.000
Tp 24ii SAMmt	1.852	2	1.855	32.399
Tp 24iii SAMmt	1.566	2	1.855	47.519
Tp 24i REDOX	1.890	3	1.894	23.652
Tp 24ii REDOX	1.884	3	1.894	23.548
Tp 24iii REDOX	1.896	3	1.894	23.201
Tp 24i AT	1.627	4	1.630	35.245
Tp 24ii AT	1.631	4	1.630	36.867
Tp 24iii AT	1.633	4	1.630	34.992
Tp 24i H4	1.880	5	1.886	32.669
Tp 24ii H4	1.896	5	1.886	30.282
Tp 24iii H4	1.896	5	1.886	31.742
Tp 24i TBP	1.910	6	1.928	27.218
Tp 24ii TBP	1.927	6	1.928	28.413
Tp 24iii TBP	1.913	6	1.928	27.035

Table A6.7 LinReg results for the 48 hour samples (Experiment 3). Individual PCR efficiency, grouping, mean PCR efficiency and Ct values of the t3 samples of Experiment 3, harvested 48 hours after Start. Three biological replicates (i-iii) were used.

Sample	Individual PCR efficiency	Group	Mean PCR efficiency	Ct value
Tp 48i DiDECARB	1.932	1	1.917	26.570
Tp 48ii DiDECARB	1.908	1	1.917	26.072
Tp 48iii DiDECARB	1.903	1	1.917	26.013
Tp 48i SAMmt	1.873	2	1.855	29.736
Tp 48ii SAMmt	1.839	2	1.855	32.623
Tp 48iii SAMmt	1.873	2	1.855	29.697
Tp 48i REDOX	1.911	3	1.894	23.744
Tp 48ii REDOX	1.884	3	1.894	23.912
Tp 48iii REDOX	1.879	3	1.894	25.211
Tp 48i AT	1.629	4	1.630	37.530
Tp 48ii AT	1.640	4	1.630	36.897
Tp 48iii AT	1.634	4	1.630	37.216
Tp 48i H4	1.882	5	1.886	31.920
Tp 48ii H4	1.864	5	1.886	33.021
Tp 48iii H4	1.883	5	1.886	31.996
Tp 48i TBP	1.940	6	1.928	27.531
Tp 48ii TBP	1.941	6	1.928	26.845
Tp 48iii TBP	1.935	6	1.928	27.438

Table A6.8 LinReg results for the 72 hour samples (Experiment 3). Individual PCR efficiency, grouping, mean PCR efficiency and Ct values of the t4 samples of Experiment 3, harvested 72 hours after Start. Three biological replicates (i-iii) were used.

Sample	Individual PCR efficiency	Group	Mean PCR efficiency	Ct value
Tp 72i DiDECARB	1.903	1	1.917	25.903
Tp 72ii DiDECARB	1.912	1	1.917	27.037
Tp 72iii DiDECARB	1.947	1	1.917	26.475
Tp 72i SAMmt	1.793	2	1.855	33.786
Tp 72ii SAMmt	1.849	2	1.855	32.187
Tp 72iii SAMmt	1.714	2	1.855	37.466
Tp 72i REDOX	1.909	3	1.894	24.772
Tp 72ii REDOX	1.894	3	1.894	24.945
Tp 72iii REDOX	1.892	3	1.894	25.652
Tp 72i AT	1.637	4	1.630	36.085
Tp 72ii AT	1.635	4	1.630	36.600
Tp 72iii AT	1.615	4	1.630	35.530
Tp 72i H4	1.789	5	1.886	37.223
Tp 72ii H4	1.667	5	1.886	45.888
Tp 72iii H4	1.000	5	1.886	0.000
Tp 72i TBP	1.930	6	1.928	28.462
Tp 72ii TBP	1.934	6	1.928	28.429
Tp 72iii TBP	1.939	6	1.928	28.819

Appendix 7: REST analyses

The statistical data from the REST2009 analysis of the RT-qPCR data are presented here. The data from Experiment 1 is shown in Table A7.1-2 and Figure A7.1-2, and the data from the third experiment is displayed in Table A7.3-6 and Figure A7.3-10.

EXPERIMENT 1:
Silicate limited cultures:

Table A7.1 REST2009 results for the silicate limited cultures. Reaction efficiency, expression, standard error, 95 % confidence interval, P-value and result of the target (TRG) genes *DiDECARB, REDOX, SAMmt, AT* and the reference genes (REF) *H4* and *TBP*.

Gene	Type	Reaction efficiency	Expression	Std. Error	95 % C.I.	P (H1)	Result
DiDECARB	TRG	0.916	0.566	0.069 - 1.846	0.034 - 3.246	0.829	
SAMmt	TRG	0.883	1.148	0.318 - 6.771	0.184 - 11.920	0.802	
REDOX	TRG	0.937	1.345	0.539 - 3.017	0.390 - 8.584	0.635	
AT	TRG	0.864	0.825	0.327 - 1.468	0.240 - 1.686	0.837	
H4	REF	0.920	0.731				
TBP	REF	0.945	1.369				

Interpretation:

DiDECARB sample group is not different to control group. P (H1) = 0.829

SAMmt sample group is not different to control group. P (H1) = 0.802

REDOX sample group is not different to control group. P (H1) = 0.635

AT sample group is not different to control group. P (H1) = 0.837

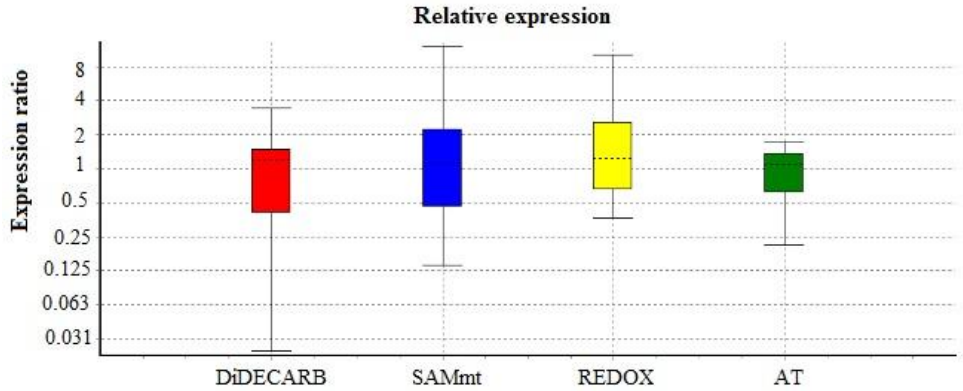

Figure A7.1 Relative expression of candidate genes during silicate limitation. Expression ratios of the candidate genes from the silicate limited cultures, compared to controls and normalized to the reference genes *H4* and *TBP*.

Nitrate limited cultures:

Table A7.2 REST2009 results for the nitrate limited cultures. Reaction efficiency, expression, standard error, 95 % confidence interval, P-value and result of the target (TRG) genes *DiDECARB*, *REDOX*, *SAMmt*, *AT* and the reference genes (REF) *H4* and *TBP*.

Gene	Type	Reaction efficiency	Expression	Std. Error	95 % C.I.	P (H1)	Result
DiDECARB	TRG	0.916	0.477	0.320 - 0.908	0.174 - 0.987	0.070	
SAMmt	TRG	0.883	0.247	0.114 - 0.628	0.063 - 0.734	0.031	DOWN
REDOX	TRG	0.937	6.400	3.383-11.702	2.594 - 19.671	0.000	UP
AT	TRG	0.864	0.264	0.214 - 0.357	0.164 - 0.372	0.037	DOWN
H4	REF	0.920	1.433				
TBP	REF	0.945	0.698				

Interpretation:

DiDECARB sample group is not different to control group. P (H1) = 0.070.
SAMmt is DOWN-regulated in sample group (in comparison to control group) by a mean factor of 0.247 (S.E. range is 0.114 - 0.628). SAMmt sample group is different to control group. P (H1) = 0.031
REDOX is UP-regulated in sample group (in comparison to control group) by a mean factor of 6.400 (S.E. range is 3.383 - 11.702). REDOX sample group is different to control group.
P (H1) = 0.000
AT is DOWN-regulated in sample group (in comparison to control group) by a mean factor of 0.264 (S.E. range is 0.214 - 0.357). AT sample group is different to control group.
P (H1) = 0.037

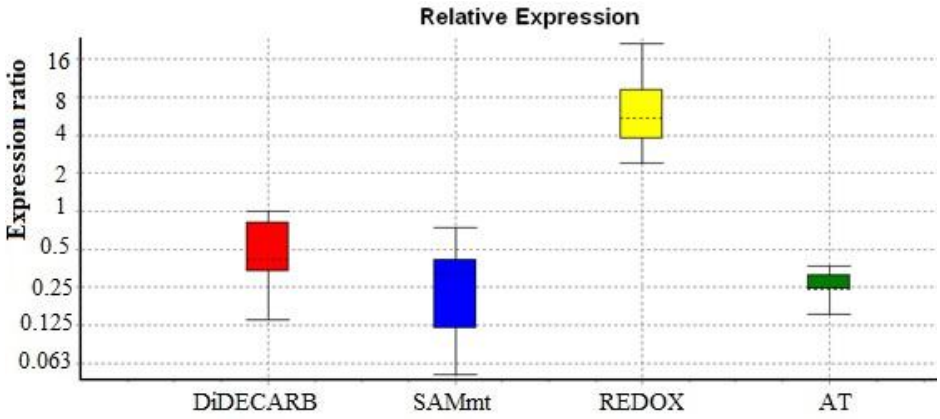

Figure A7.2 Relative expression of candidate genes during nitrate limitation. Expression ratios of the candidate genes from the nitrate limited cultures, compared to controls and normalized to the reference genes *H4* and *TBP*.

EXPERIMENT 3:

Samples harvested at 7.5 hours (t1):

Table A7.3 REST2009 results for the 7.5 hour samples. Reaction efficiency, expression, standard error, 95 % confidence interval, P-value and result of the target (TRG) genes *DiDECARB*, *REDOX* and *AT*, and the reference genes (REF) *H4* and *TBP*. The *SAMmt* gene was separately analyzed.

Gene	Type	Reaction efficiency	Expression	Std. Error	95 % C.I.	P (H1)	Result
DiDECARB	TRG	0.917	3.248	1.513 - 5.190	1.329 - 11.706	0.077	
REDOX	TRG	0.894	0.482	0.321 - 0.679	0.276 - 0.920	0.058	
AT	TRG	0.630	0.418	0.285 - 0.585	0.284 - 0.587	0.058	
H4	REF	0.886	1.248				
TBP	REF	0.928	0.801				
SAMmt	TRG	0.855	0.090	0.010 - 1.693	0.004 - 2.623	0.333	
H4	REF	0.886	0.873				
TBP	REF	0.928	1.145				

Interpretation:

DiDECARB sample group is not different to control group. P (H1) = 0.077
REDOX sample group is not different to control group. P (H1) = 0.058
AT sample group is not different to control group. P (H1) = 0.058
SAMmt sample group is not different to control group. P (H1) = 0.333

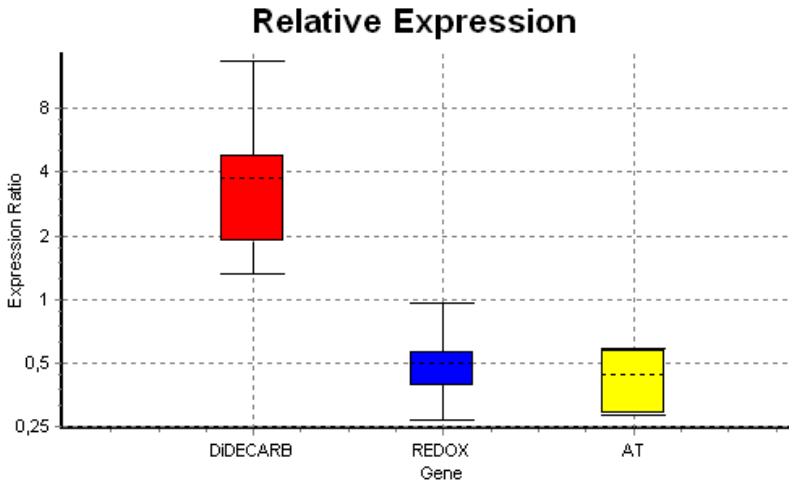

Figure A7.3 Relative expression of candidate genes at 7.5 hours. Expression ratios of the candidate genes from the 7.5 hour samples, compared to controls (t0) and normalized with the reference genes *H4* and *TBP*.

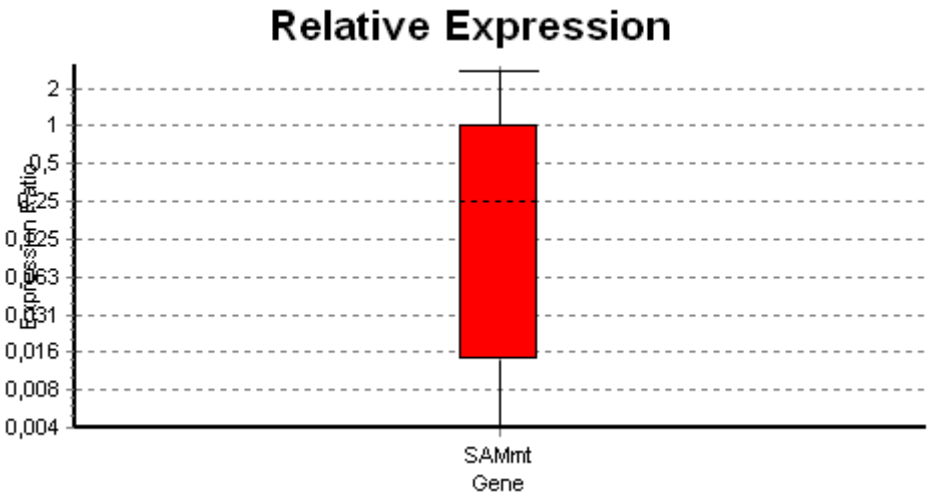

Figure A7.4 Relative expression of the *SAMmt* gene at 7.5 hours. Expression ratio of the *SAMmt* gene at the 7.5 hours, compared to controls (t0) and normalized with the reference genes *H4* and *TBP*.

Samples harvested at 24 hours (t2):

Table A7.4 REST2009 results for the 24 hour samples. Reaction efficiency, expression, standard error, 95 % confidence interval, P-value and result of the target (TRG) genes *DiDECARB*, *REDOX* and *AT*, and the reference genes (REF) *H4* and *TBP*. The *SAMmt* gene was separately analyzed.

Gene	Type	Reaction efficiency	Expression	Std. Error	95 % C.I.	P (H1)	Result
DiDECARB	TRG	0.917	1.804	0.874 - 4.627	0.481 - 5.970	0.363	
REDOX	TRG	0.894	1.052	0.793 - 1.533	0.713 - 1.713	0.875	
AT	TRG	0.630	1.354	0.773 - 2.353	0.510 - 2.885	0.419	
H4	REF	0.886	0.839				
TBP	REF	0.928	1.192				
SAMmt	TRG	0.855	0.892	0.653 - 1.862	0.415 - 2.099	1.000	
H4	REF	0.886	1.336				
TBP	REF	0.928	0.749				

Interpretation:

DiDECARB sample group is not different to control group. P (H1) = 0.363
REDOX sample group is not different to control group. P (H1) = 0.875
AT sample group is not different to control group. P (H1) = 0.419
SAMmt sample group is not different to control group. P (H1) = 1.000

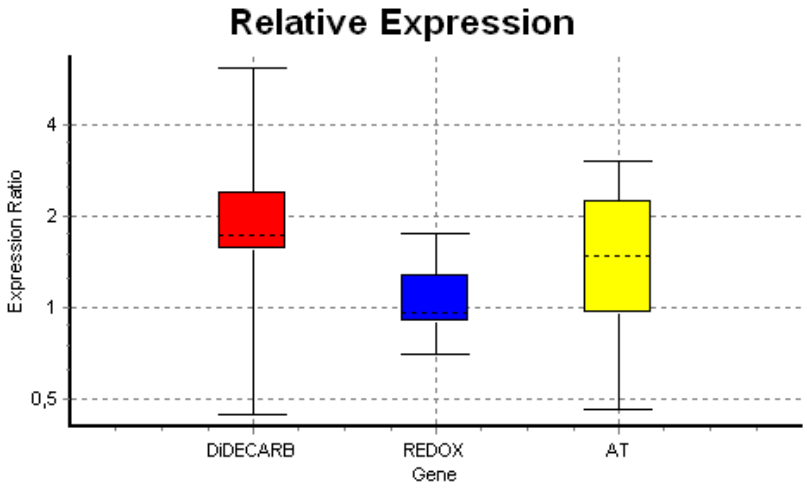

Figure A7.5 Relative expression of candidate genes at 24 hours. Expression ratios of the candidate genes from the 24 hour samples, compared to controls (t0) and normalized with the reference genes *H4* and *TBP*.

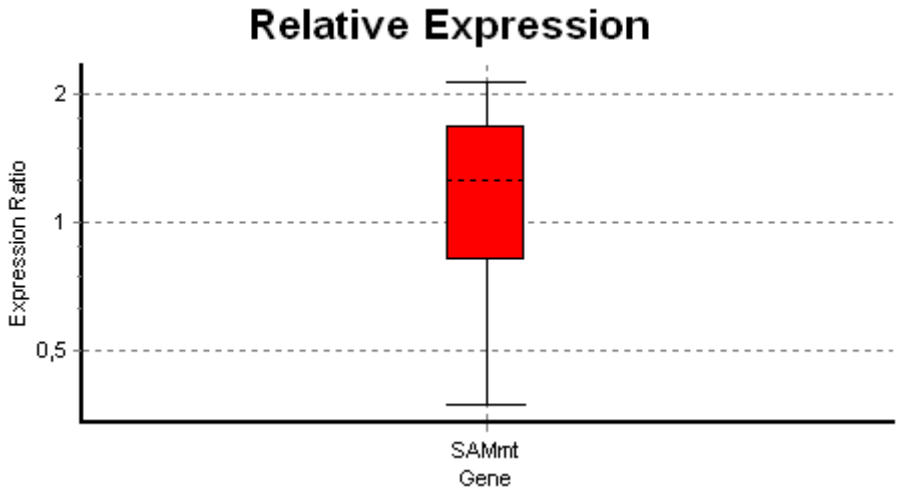

Figure A7.6 Relative expression of the *SAMmt* gene at 24 hours. Expression ratio of the *SAMmt* gene from the 24 hour samples, compared to control (t0) and normalized with the reference genes *H4* and *TBP*.

Samples harvested at 48 hours (t3):

Table A7.5 REST2009 results for the 48 hour samples. Reaction efficiency, expression, standard error, 95 % confidence interval, P-value and result of the target (TRG) genes *DiDECARB*, *REDOX* and *AT*, and the reference genes (REF) *H4* and *TBP*. The *SAMmt* gene was separately analyzed.

Gene	Type	Reaction efficiency	Expression	Std. Error	95 % C.I.	P (H1)	Result
DiDECARB	TRG	0.917	2.199	1.392 - 4.579	1.063 - 5.370	0.181	
REDOX	TRG	0.894	0.719	0.390 - 1.274	0.308 - 1.449	0.504	
AT	TRG	0.630	0.747	0.529 - 0.997	0.433 - 1.262	0.169	
H4	REF	0.886	0.603				
TBP	REF	0.928	1.658				
SAMmt	TRG	0.855	3.366	2.018-14.037	0.705 - 14.204	0.305	
H4	REF	0.886	0.482				
TBP	REF	0.928	2.073				

Interpretation:

DiDECARB sample group is not different to control group. P (H1) = 0.181
REDOX sample group is not different to control group. P (H1) = 0.504
AT sample group is not different to control group. P (H1) = 0.169
SAMmt sample group is not different to control group. P (H1) = 0.305

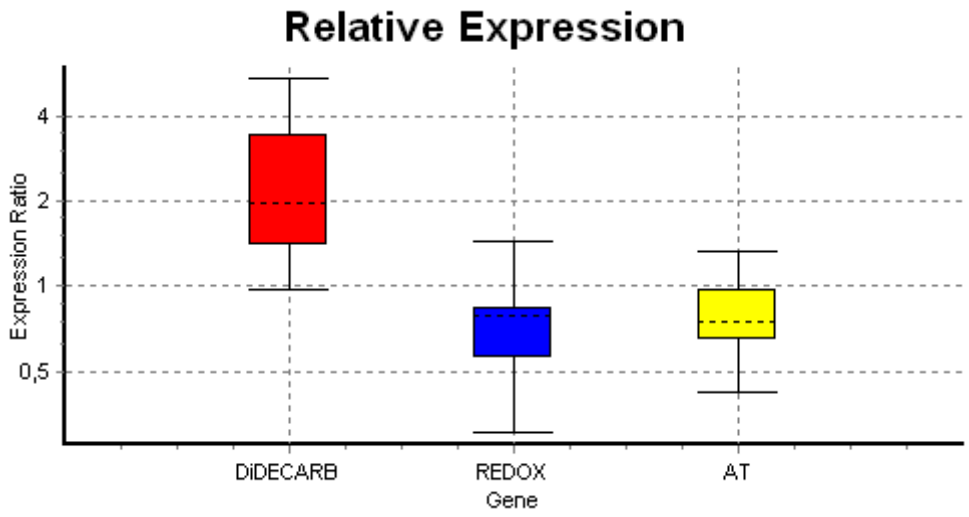

Figure A7.7 Relative expression of candidate genes at 48 hours. Expression ratios of the candidate genes from the 48 hour samples, compared to controls (t0) and normalized with the reference genes *H4* and *TBP*.

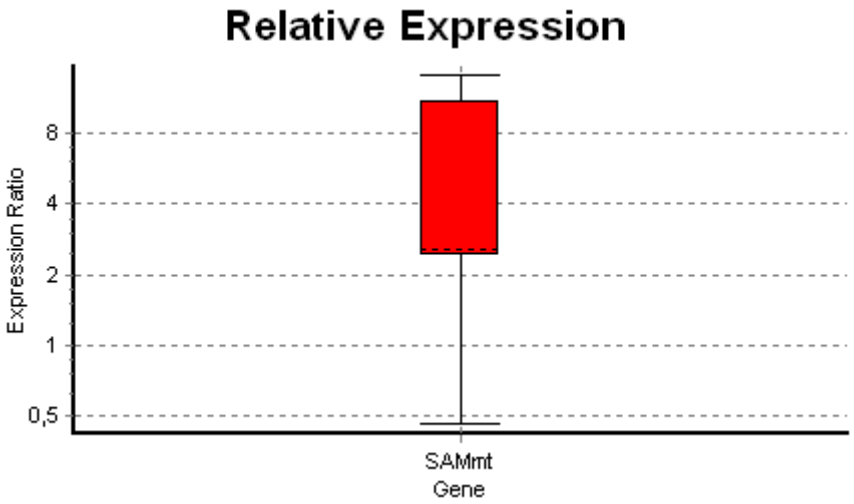

Figure A7.8 Relative expression of the *SAMmt* gene at 48 hours. Expression ratio of the *SAMmt* gene from the 48 hour samples, compared to control (t0) and normalized with the reference genes *H4* and *TBP*.

Samples harvested at 72 hours (t4):

Table A7.6 REST2009 results for the 72 hour samples. Reaction efficiency, expression, standard error, 95 % confidence interval, P-value and result of the target (TRG) genes *DiDECARB, REDOX* and *AT*, and the reference genes (REF) *H4* and *TBP*. The *SAMmt* gene was separately analyzed.

Gene	Type	Reaction efficiency	Expression	Std. Error	95 % C.I.	P (H1)	Result
DiDECARB	TRG	0.917	2.637	1.685 - 3.492	1.536 - 3.926	0.000	UP
REDOX	TRG	0.894	0.597	0.491 - 0.743	0.417 - 0.843	0.066	
AT	TRG	0.630	1.846	1.326 - 2.784	1.055 - 3.390	0.103	
TBP	REF	0.928	1.000				
SAMmt	TRG	0.855	0.365	0.097 - 1.142	0.050 - 2.076	0.296	
TBP	REF	0.928	1.000				

Interpretation:

DiDECARB is UP-regulated in sample group (in comparison to control group) by a mean factor of 2.637 (S. E. range is 1.685 - 3.492). DiDECARB sample group is not different to control group. P (H1) = 0.000

REDOX sample group is not different to control group. P (H1) = 0.066

AT sample group is not different to control group. P (H1) = 0.103

SAMmt sample group is not different to control group. P (H1) = 0.296

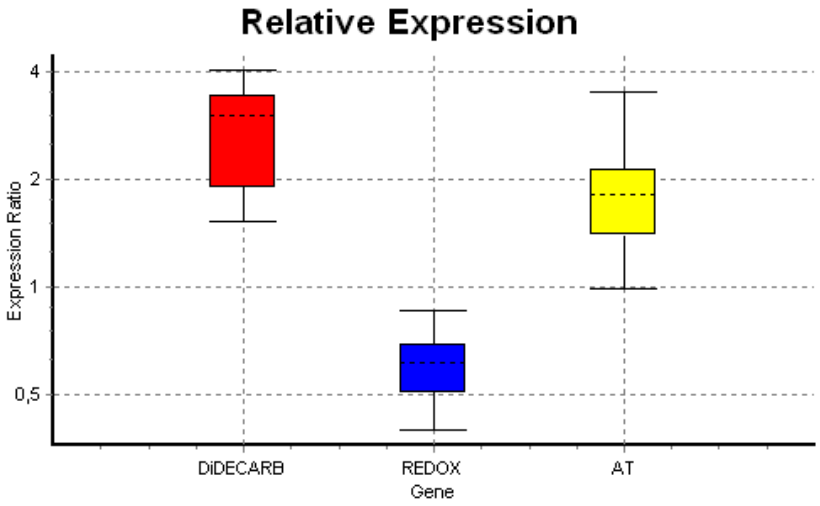

Figure A7.9 Relative expression of candidate genes at 72 hours. Expression ratios of the candidate genes from the 72 hour samples, compared to controls (t0) and normalized with the reference genes *H4* and *TBP*.

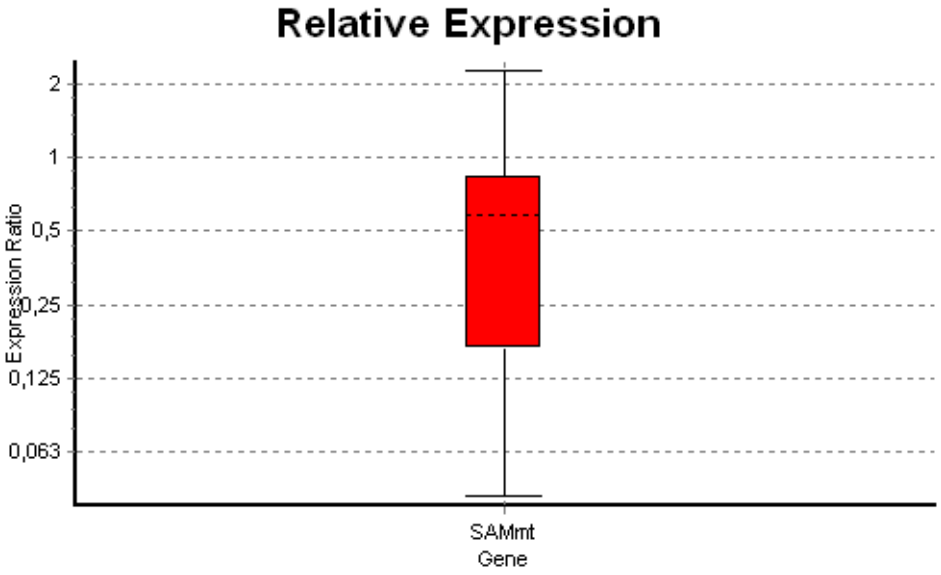

Figure A7.10 Relative expression of the *SAMmt* gene at 72 hours. Expression ratio of the *SAMmt* gene from the 72 hour samples, compared to control (t0) and normalized with the reference genes *H4* and *TBP*.

Appendix 8: Melting curves

The melting curves of the RT-qPCR analyses are shown in Figure A8.1-6 for the first experiment and in Figure A8.7-12 for the third experiment. The melting curves from the RT-qPCR analyses indicated that the samples of the third experiment contained genomic DNA contaminations from the RT reaction, indicated by positive NRT controls. The melting curves of the first experiment indicated no presence of gDNA from the RT reaction, as the NRT controls were negative.

EXPERIMENT 1:

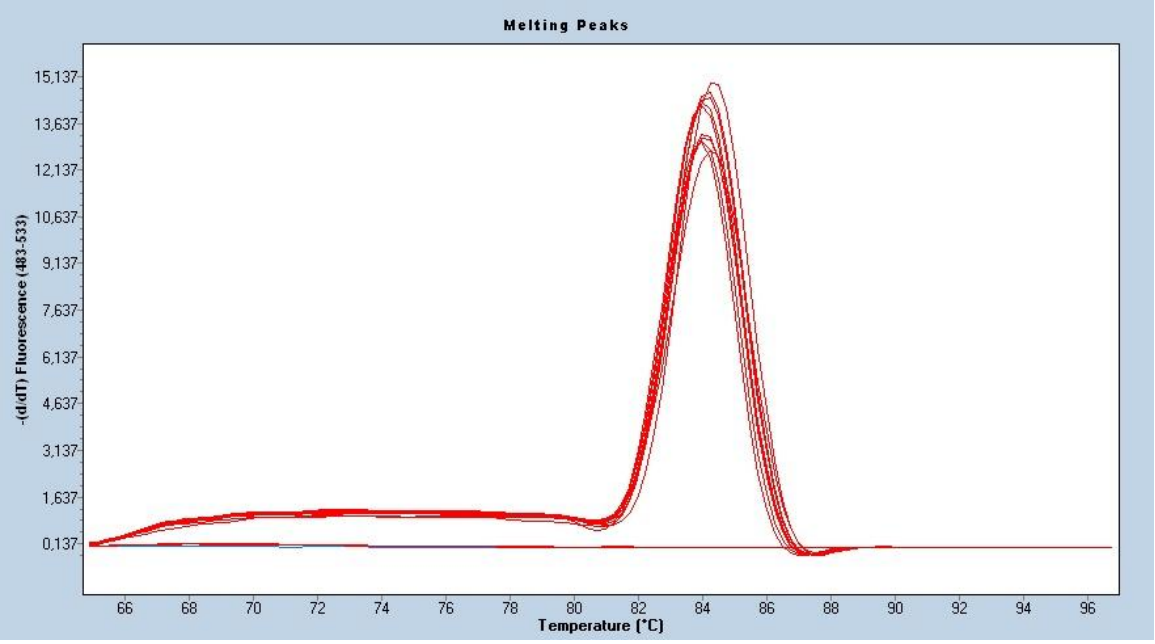

Figure A8.1 *DiDECARB* (Experiment 1). Melting curve of *DiDECARB* amplified cDNA from the first experiment, from control, silicate-limited and nitrate-limited cultures. The horizontal axis shows the temperature in degrees Celsius and the vertical axis gives the negative first derivative of fluorescence data (483-533 nm). The upper red lines represents treated and control samples and the lower lines are negative no template controls (NTC).

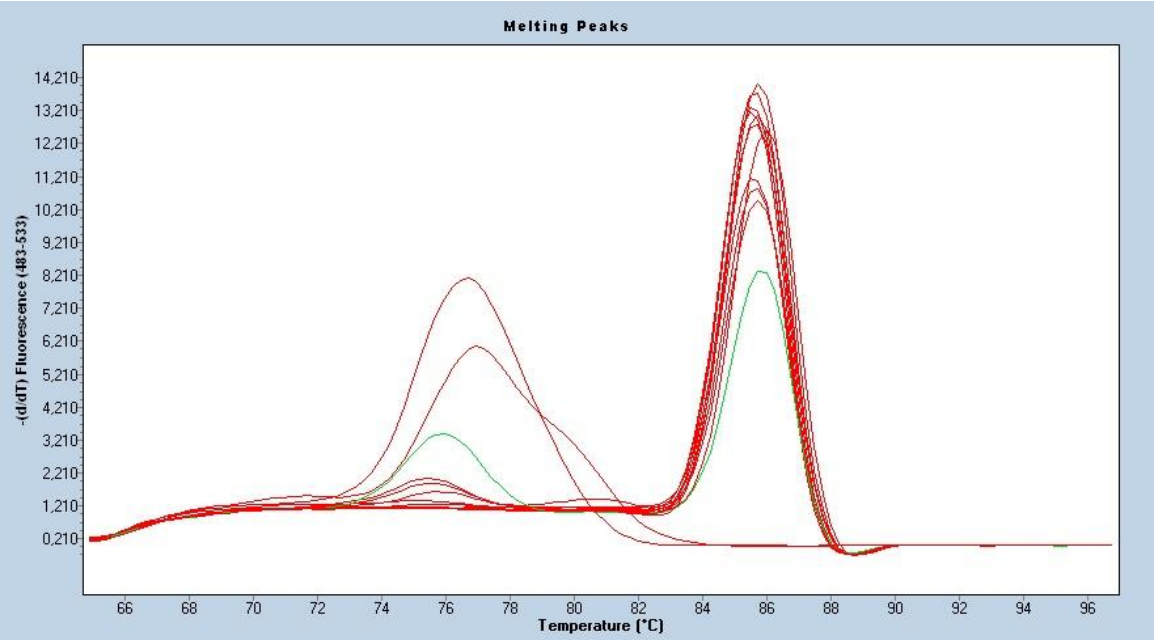

Figure A8.2 *SAMmt* (Experiment 1). Melting curve of *SAMmt* amplified cDNA from the first experiment, from control, silicate-limited and nitrate-limited cultures. The horizontal axis shows the temperature in degrees Celsius and the vertical axis gives the negative first derivative of fluorescence data (483-533 nm). The upper red and green lines to the represents treated and control samples and the upper red peaks to the left are no template controls (NTC).

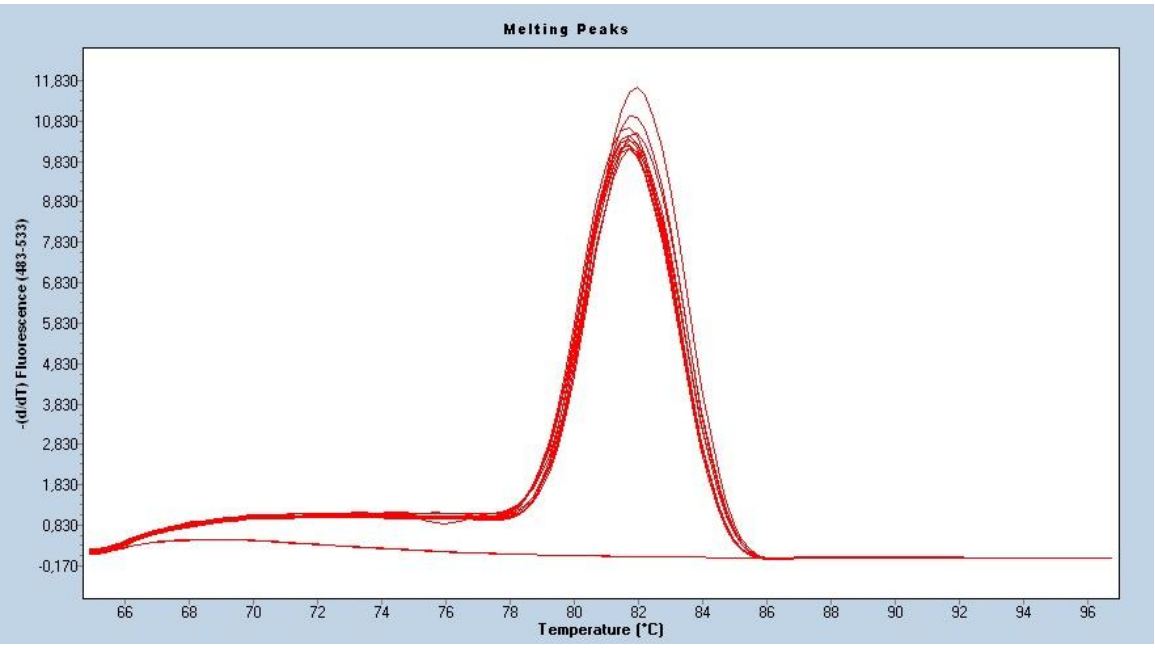

Figure A8.3 *REDOX* (Experiment 1). Melting curve of *REDOX* amplified cDNA from the first experiment, from control, silicate-limited and nitrate-limited cultures. The horizontal axis shows the temperature in degrees Celsius and the vertical axis gives the negative first derivative of fluorescence data (483-533 nm). The upper red lines represents treated and control samples and the bottom red lines represent negative no template controls (NTC).

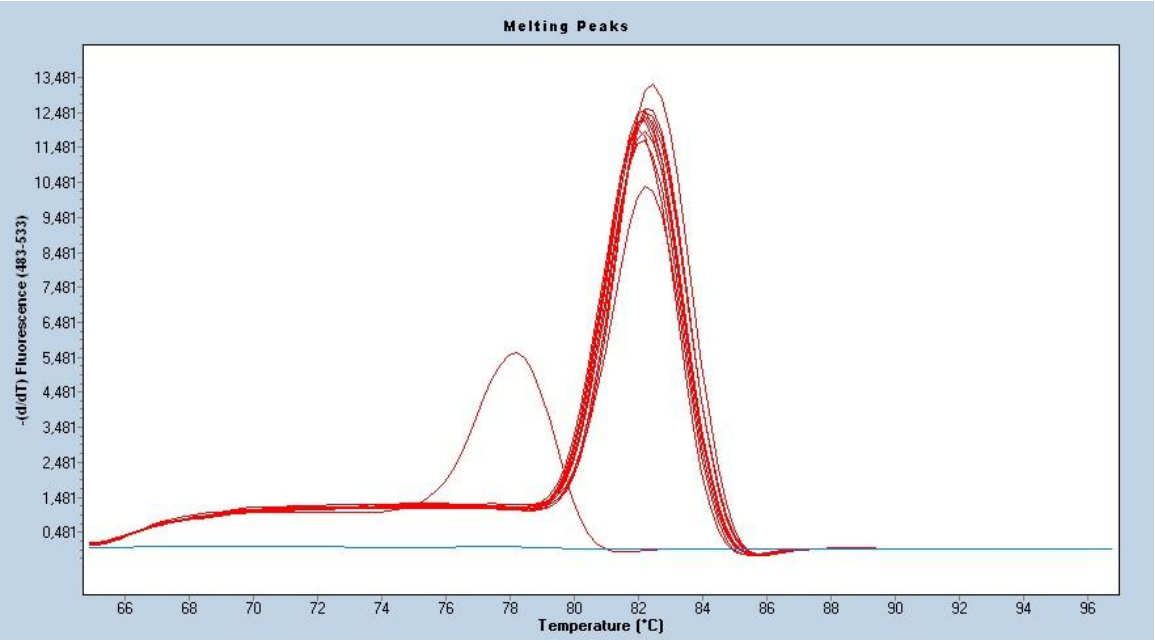

Figure A8.4 *AT* (Experiment 1). Melting curve of *AT* amplified cDNA from the first experiment, from control, silicate-limited and nitrate-limited cultures. The horizontal axis shows the temperature in degrees Celsius and the vertical axis gives the negative first derivative of fluorescence data (483-533 nm). The upper red lines represents treated and control samples and the small peak to the left and the lower blue line is no template controls (NTC).

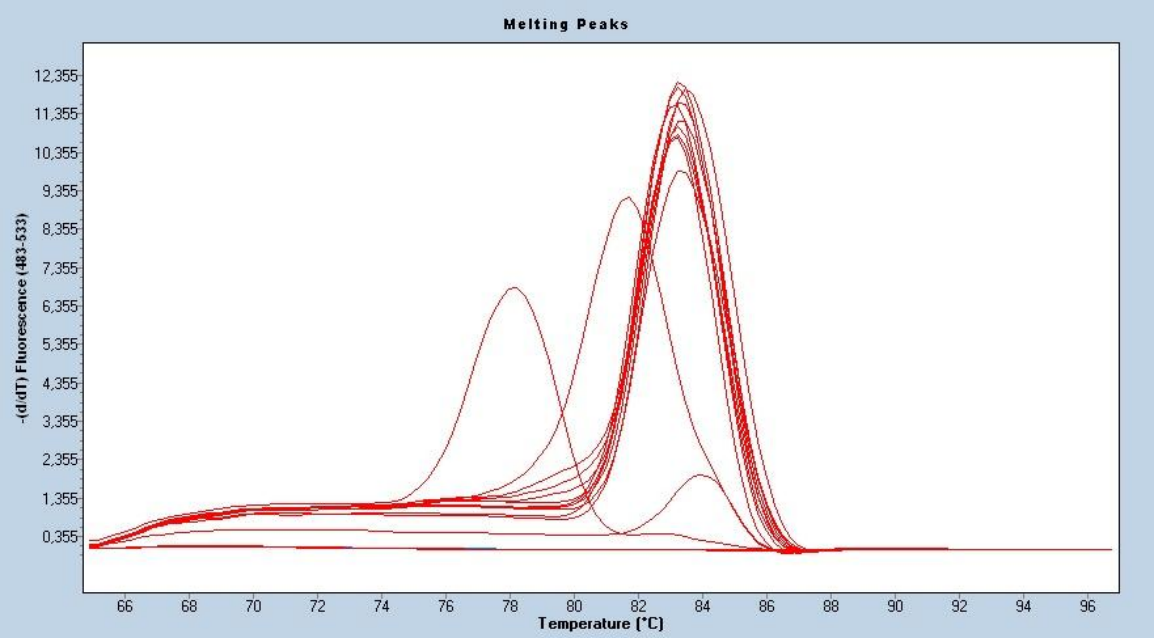

Figure A8.5 *H4* (Experiment 1). Melting curve of *H4* amplified cDNA from the first experiment, from control, silicate-limited and nitrate-limited cultures. The horizontal axis shows the temperature in degrees Celsius and the vertical axis gives the negative first derivative of fluorescence data (483-533 nm). The upper red lines represents treated and control samples and the small peak to the left is a no template control (NTC). The lower lines are NTC and no reverse transcription (NRT) controls.

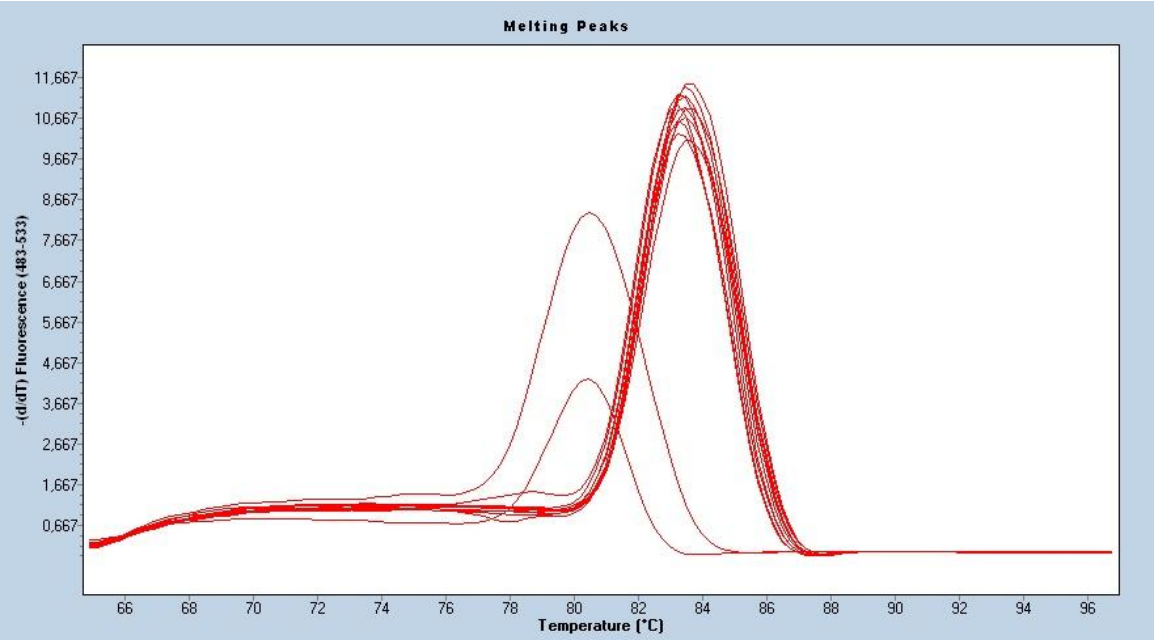

Figure A8.6 *TBP* (Experiment 1). Melting curve of *TBP* amplified cDNA from the first experiment, from control, silicate-limited and nitrate-limited cultures. The horizontal axis shows the temperature in degrees Celsius and the vertical axis gives the negative first derivative of fluorescence data (483-533 nm). The upper red lines represents treated and control samples and the two peaks to the left are no template controls (NTC).

EXPERIMENT 3:

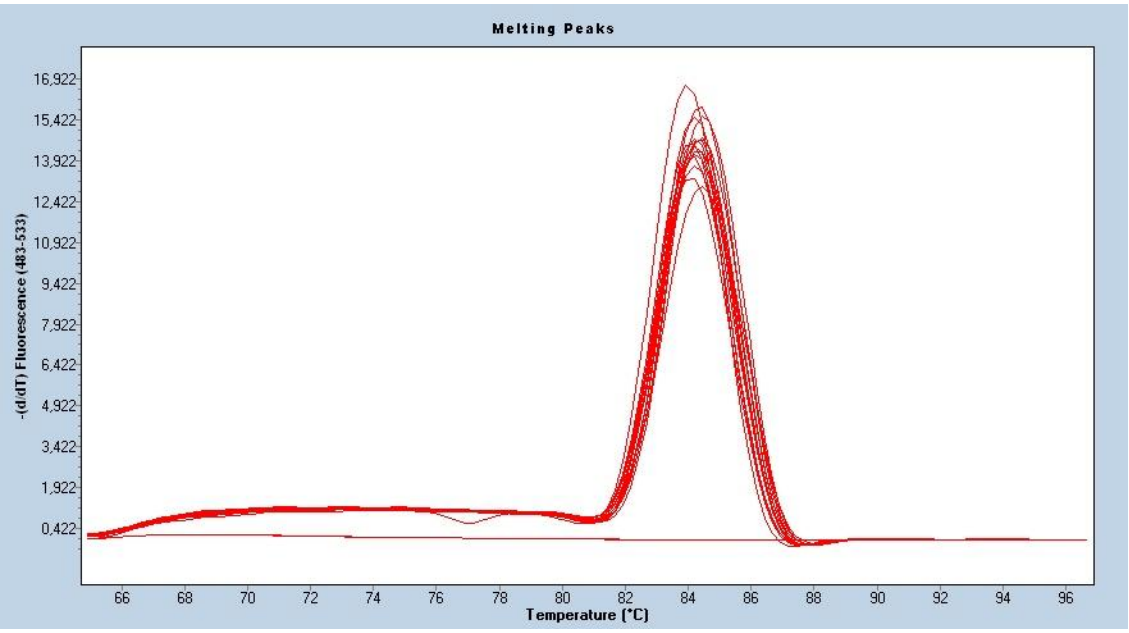

Figure A8.7 *DiDECARB* (Experiment 3). Melting curve of *DiDECARB* amplified cDNA from the third experiment at time points t0-t4. The horizontal axis shows the temperature in degrees Celsius and the vertical axis gives the negative first derivative of fluorescence data (483-533 nm). The upper red lines represents treated and control samples and the bottom red lines represent no template controls (NTC).

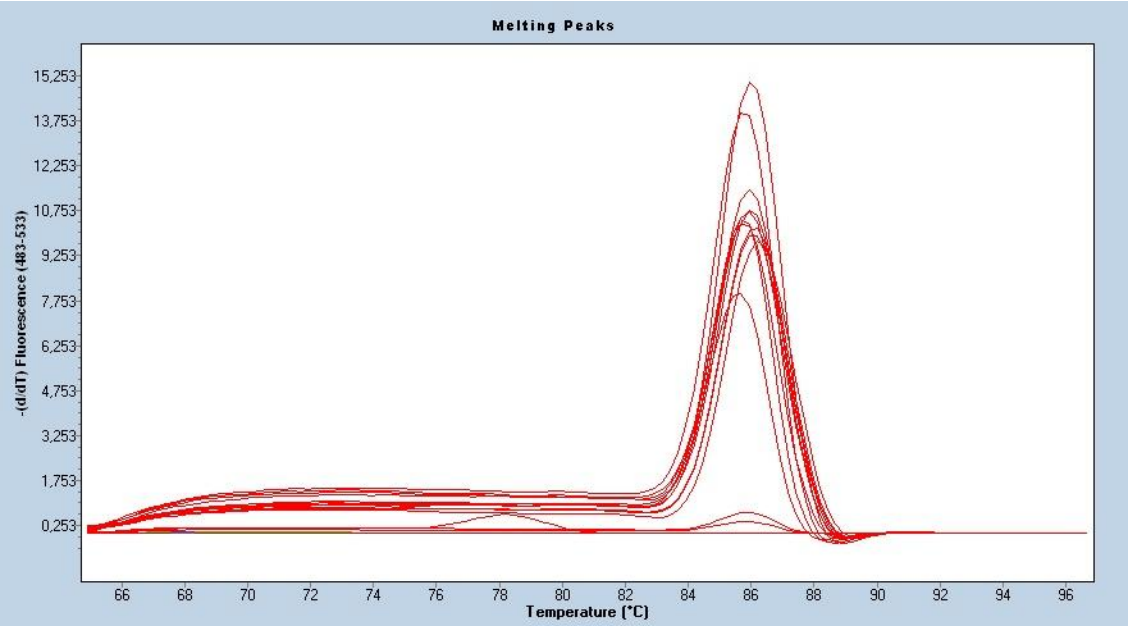

Figure A8.8 *SAMmt* (Experiment 3). Melting curve of *SAMmt* amplified cDNA from the third experiment at time points t0-t4. The horizontal axis shows the temperature in degrees Celsius and the vertical axis gives the negative first derivative of fluorescence data (483-533 nm). The upper red lines represents treated and control samples and the bottom horizontal red lines represent negative no template controls (NTC).

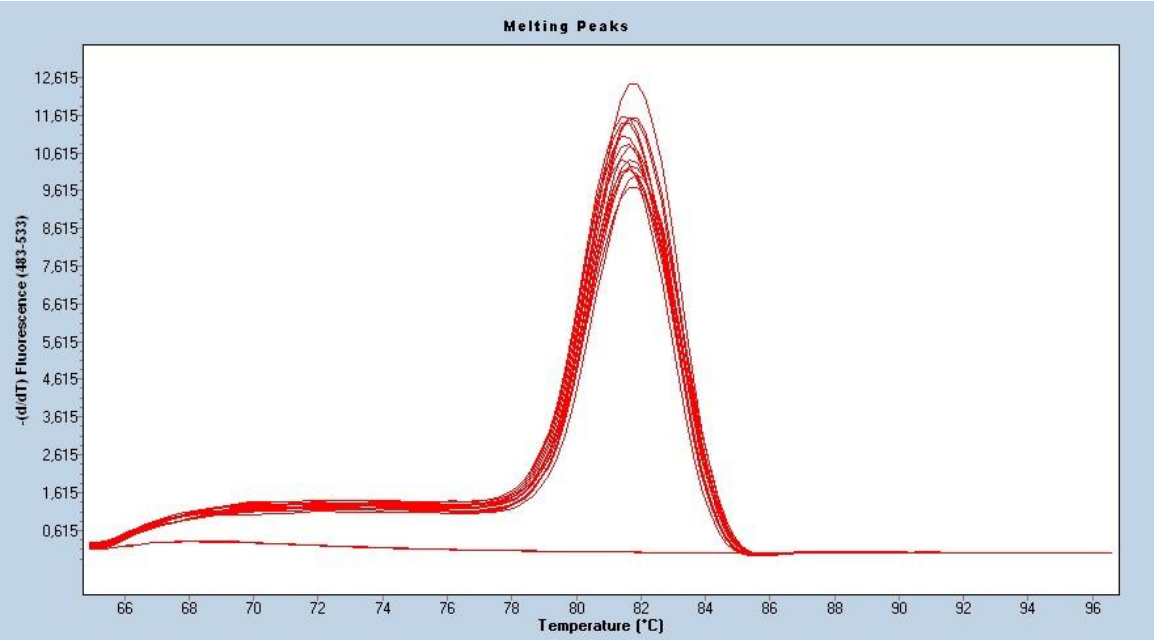

Figure A8.9 REDOX (Experiment 3). Melting curve of REDOX amplified cDNA from the third experiment at time points t0-t4. The horizontal axis shows the temperature in degrees Celsius and the vertical axis gives the negative first derivative of fluorescence data (483-533 nm). The upper red lines represents treated and control samples and the bottom red lines represent negative no template controls (NTC).

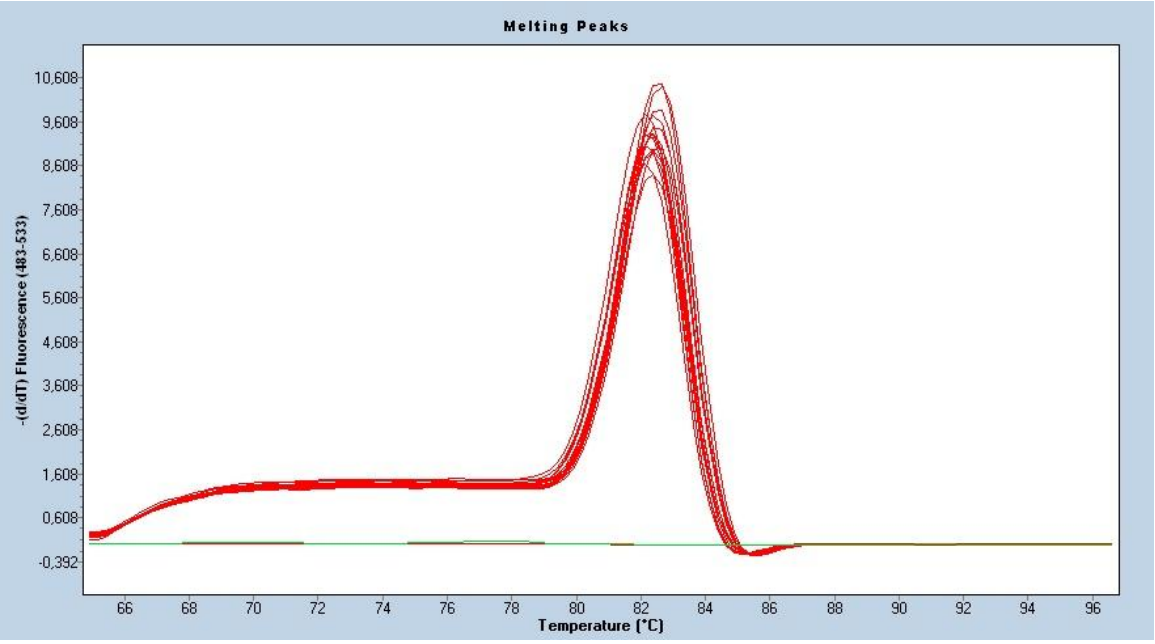

Figure A8.10 *AT* (Experiment 3). Melting curve of *AT* amplified cDNA from the third experiment at time points t0-t4. The horizontal axis shows the temperature in degrees Celsius and the vertical axis gives the negative first derivative of fluorescence data (483-533 nm). The upper red lines represents treated and control samples and the bottom red and green line represent negative no template controls (NTC).

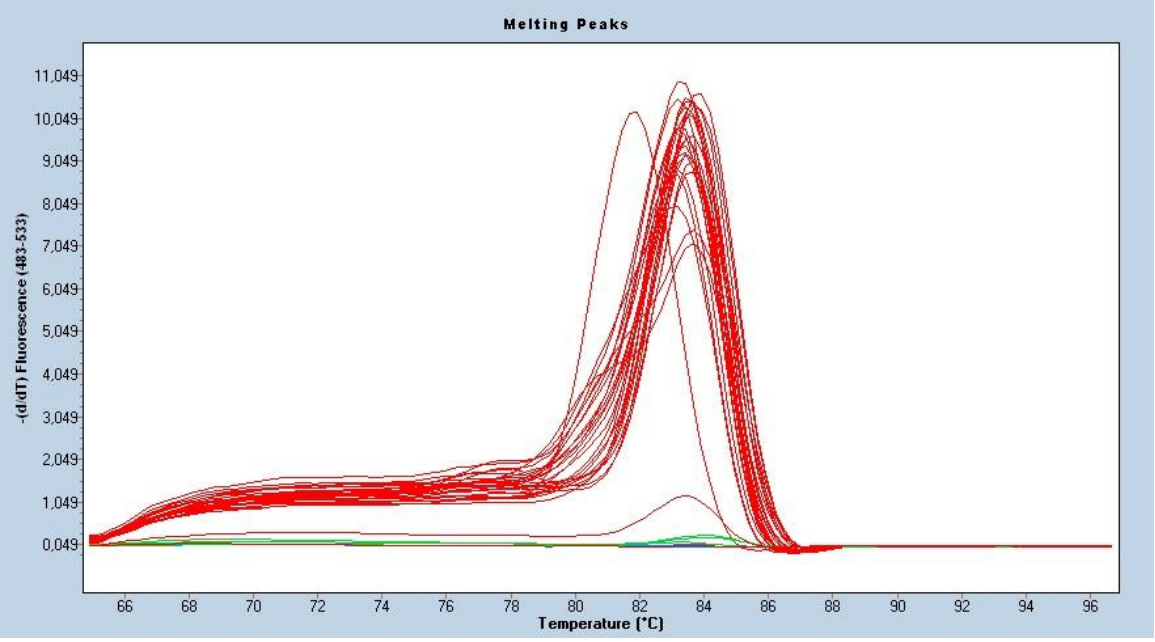

Figure A8.11 *H4* (Experiment 3). Melting curve of *H4* amplified cDNA from the third experiment at time points t0-t4. The horizontal axis shows the temperature in degrees Celsius and the vertical axis gives the negative first derivative of fluorescence data (483-533 nm). The upper red lines represents treated and control samples and the bottom horizontal red lines represent negative no template controls (NTC). Several of the red upper lines, the peak to the left and the green lower lines represent no reverse transcription controls (NRT).

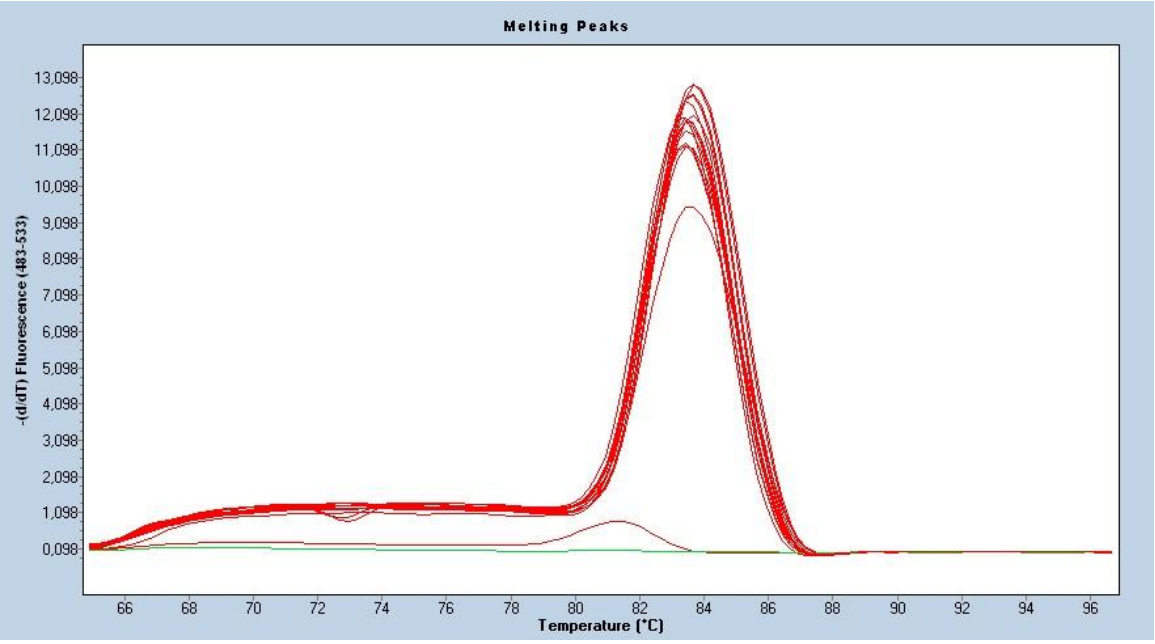

Figure A8.12 *TBP* (Experiment 3). Melting curve of *TBP* amplified cDNA from the third experiment at time points t0-t4. The horizontal axis shows the temperature in degrees Celsius and the vertical axis gives the negative first derivative of fluorescence data (483-533 nm). The upper red lines represents treated and control samples and the bottom red and green lines represent negative no template controls (NTC).

Appendix 9: GC-MS analyses

The original GC-MS data are displayed in Table A9.1-4, showing the detector responses of the samples and standards from Experiment 2 and 3. The calibration curves are displayed in Figure A9.1-4. Calibration curves were made using three technical replicates of each standard solution. The chromatogram of DMS obtained in the GC-MS analysis is shown in Figure A9.5.

EXPERIMENT 2:

Table A9.1 Detector response of samples (Experiment 2). Raw data from the GC-MS analysis, presented as detector response (peak area) of the samples from START to day 5. Samples of control cultures are marked with "C", and samples of silicate and nitrate limited cultures are marked with "Si" and "N", respectively. 3 biological replicates (i-iii) were used. Average detector response and standard deviation are also given, calculated in Microsoft Excel 2007.

Sample	Detector response			Average response	Standard deviation
	i	ii	iii		
Tp-START-C	12 251 000	11 498 617	12 034 176	11 927 931	387280
Tp-START-Si	9 672 179	9 885 619	10 173 638	9 910 479	251652
Tp-START-N	9 563 167	10 173 406	9 702 250	9 812 941	319824
Tp-Day 1-C	11 515 960	11 808 102	11 506 776	11 610 279	171381
Tp-Day 1-Si	10 398 078	10 528 043	10 385 878	10 437 333	78794
Tp-Day 1-N	12 589 091	14 194 909	11 632 815	12 805 605	1294697
Tp-Day 2-C	42 591 383	39 436 705	35 567 293	39 198 460	3518100
Tp-Day 2-Si	33 531 727	33 877 420	36 474 749	34 627 965	1608674
Tp-Day 2-N	51 769 468	53 535 974	46 838 593	50 714 678	3471047
Tp-Day 3-C	50 103 893	44 507 017	41 976 018	45 528 976	4159193
Tp-Day 3-Si	48 465 164	42 948 743	46 286 796	45 900 234	2778452
Tp-Day 3-N	41 567 073	42 691 993	44 449 261	42 902 776	1452609
Tp-Day 4-C	71 905 768	75 199 931	79 659 472	75 588 390	3891421
Tp-Day 4-Si	72 201 285	72 906 941	72 986 442	72 698 223	432193
Tp-Day 4-N	47 816 292	52 222 191	46 535 323	48 857 935	2983099
Tp-Day 5-C	107 196 091	116 030 039	92 010 008	105 078 713	12149195
Tp-Day 5-Si	93 909 645	101 117 021	100 696 665	98 574 444	4045298
Tp-Day 5-N	57 113 093	62 615 075	59 654 658	59 794 275	2753647

Table A9.2 Detector response of standards (Experiment 2). The raw data from the GC-MS analysis, presented as detector response (peak area) of the standards used for calibration. Three technical replicates (i-iii) were used, with concentrations of 10, 25, 50, 75, 100, 150 and 200 µM. Average detector response and standard deviation are also given, calculated in Microsoft Excel 2007.

Standard	Detector response			Average response	Standard deviation
	i	ii	iii		
200 µM	205 237 280	179 230 154	164 228 136	182 898 523	20 749 221
150 µM	188 008 335	94 821 631	86 137 281	122 989 082	56 475 498
100 µM	51 957 359	51 548 570	50 898 648	51 468 192	533 913
75 µM	38 451 652	39 575 772	38 190 430	38 739 285	736 100
50 µM	22 106 015	20 780 518	24 531 737	22 472 757	1 902 311
25 µM	10 890 104	10 637 157	10 826 771	10 784 677	131 622
10 µM	5 252 260	4 427 902	4 081 708	4 587 290	601 333

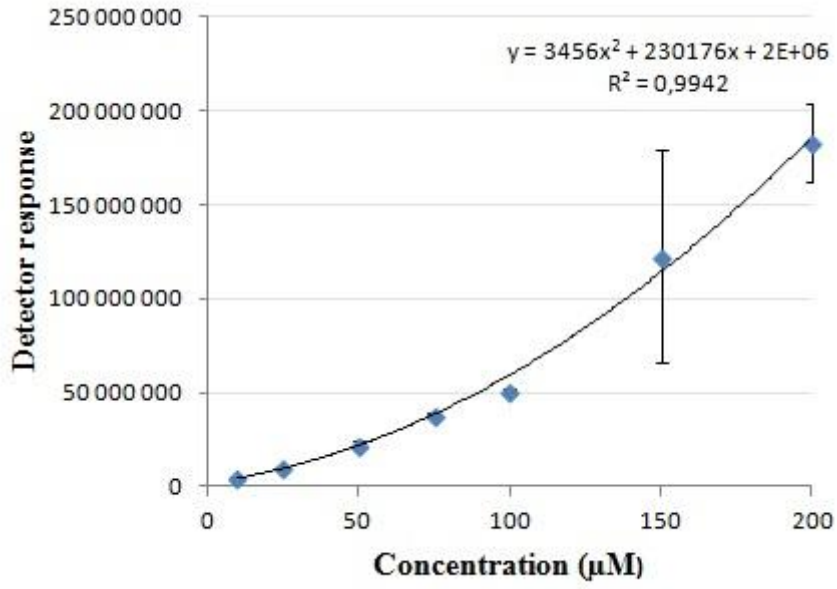

Figure A9.1 Calibration curve (Experiment 2). The calibration curve of the standard solution ranging from 10 µM to 200 µM. The error bars represent the standard deviation of the three technical replicates. The curve had a quadratic equation of "y = 3456 x^2 + 230 176x + 2E+06" (R^2 = 0.9942). This was not used to calculate the total DMSP concentrations of the sample.

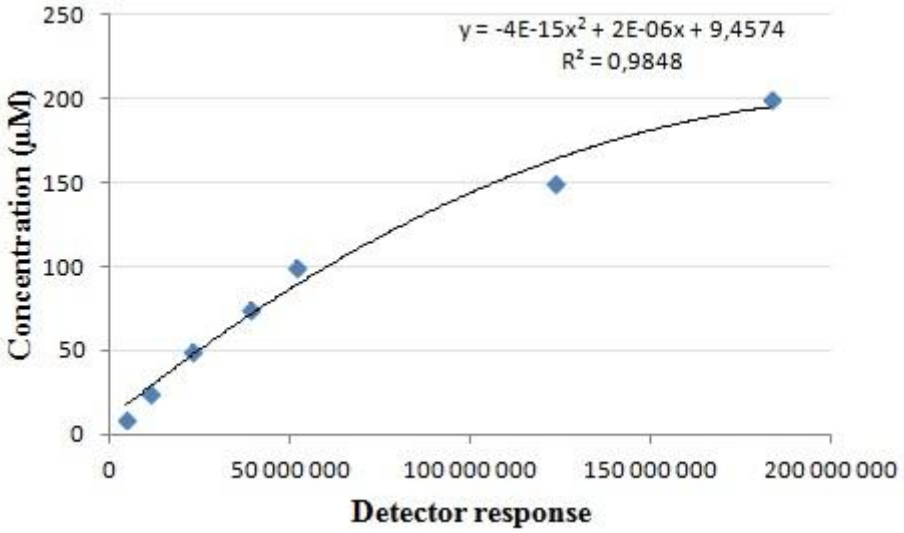

Figure A9.2 Inverted calibration curve (Experiment 2). The inverted calibration curve of the standard solution ranging from 10 µM to 200 µM, for easier calculations of DMSP concentrations. The quadratic equation "y = -4E-15 x^2 + 2E+06x + 9.4574" (R^2 = 0.9848), was used to calculate the total DMSP concentrations.

EXPERIMENT 3:

Table A9.3 Detector response of the samples (Experiment 3). The raw data from the GC-MS analysis, presented as detector response (peak area) of the samples harvested 20 minutes before Start and 7.5, 24, 48 and 72 hours after Start. Three biological replicates were used, and the average detector response and standard deviation are also given, calculated in Microsoft Excel 2007.

Sample	Detector response			Average response	Standard deviation
	i	ii	iii		
Tp -20 min	5 950 578	4 540 115	4 233 850	4 908 181	915 638
Tp 7.5 h	11 949 237	10 541 236	9 993 066	10 827 846	1 009 089
Tp 24 h	16 136 412	13 375 483	16 733 297	15 415 064	1 791 364
Tp 48 h	25 295 299	23 547 702	22 018 840	23 620 614	1 639 446
Tp 72 h	56 404 355	44 548 075	41 133 852	47 362 094	8 014 742

Table A9.4 Detector response of standards (Experiment 3). The raw data from the GC-MS analysis, presented as detector response (peak area) of the standards used for calibration. Three technical replicates (i-iii) were used, with concentrations of 0.01, 0.1, 1, 2, 5, 7 and 10 µM. Average detector response and standard deviation are also given, calculated in Microsoft Excel 2007.

Standard	Detector response			Average response	Standard deviation
	i	ii	iii		
0.01 µM	838 502	424 433	663 378	642 104	207 853
0.1 µM	443 124	267 915	864 648	525 229	306 722
1 µM	3 629 426	3 502 231	3 193 738	3 441 798	224 043
2 µM	8 601 603	8 474 766	8 522 280	8 532 883	64 080
5 µM	18 999 975	20 067 805	20 747 706	19 938 495	881 012
7 µM	32 189 783	31 305 722	25 438 120	29 644 542	3 669 588
10 µM	46 228 708	46 563 617	45 619 185	46 137 170	881 012

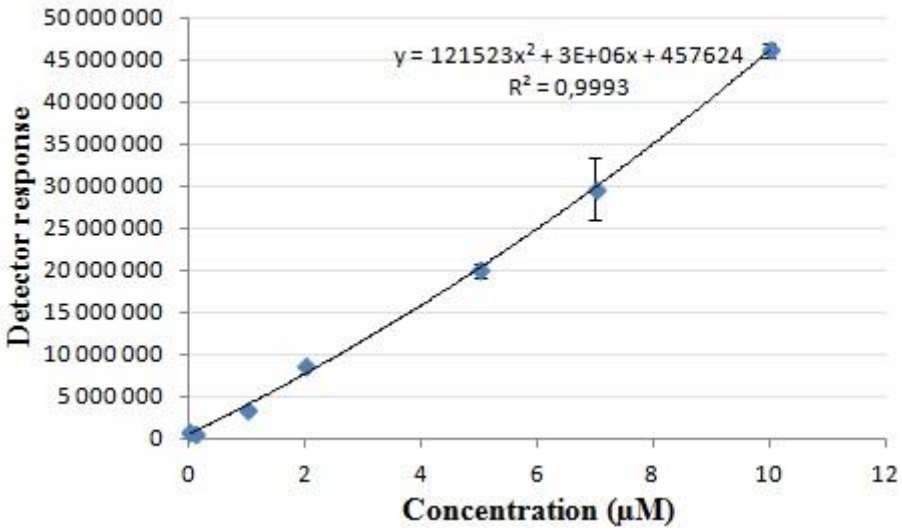

Figure A9.3 Calibration curve (Experiment 3). The calibration curve of the standard solution ranging from 0.01 µM to 10 µM. The error bars represent the standard deviation of the three technical replicates. The curve had a quadratic equation of "y = 121 523 x^2 + 3E+06x + 457 624" (R^2 = 0.9993). This was not used to calculate the total DMSP concentrations of the sample.

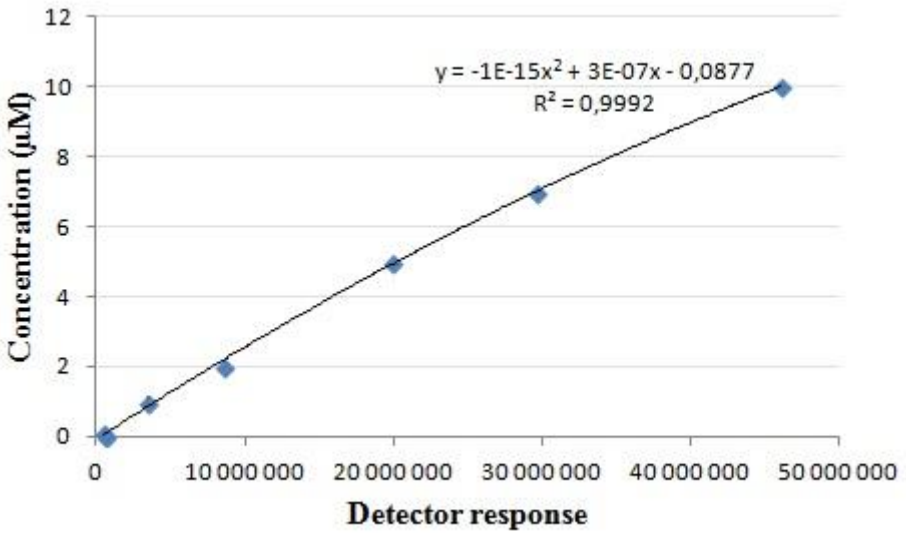

Figure A9.4 Inverted calibration curve (Experiment 3). The inverted calibration curve of the standard solution ranging from 0.01 µM to 10 µM, for easier calculations of DMSP concentrations. The quadratic equation "y = -1E-15 x^2 + 3E+07x - 0.0877" (R^2 = 0.9992), was used to calculate the total DMSP concentrations.

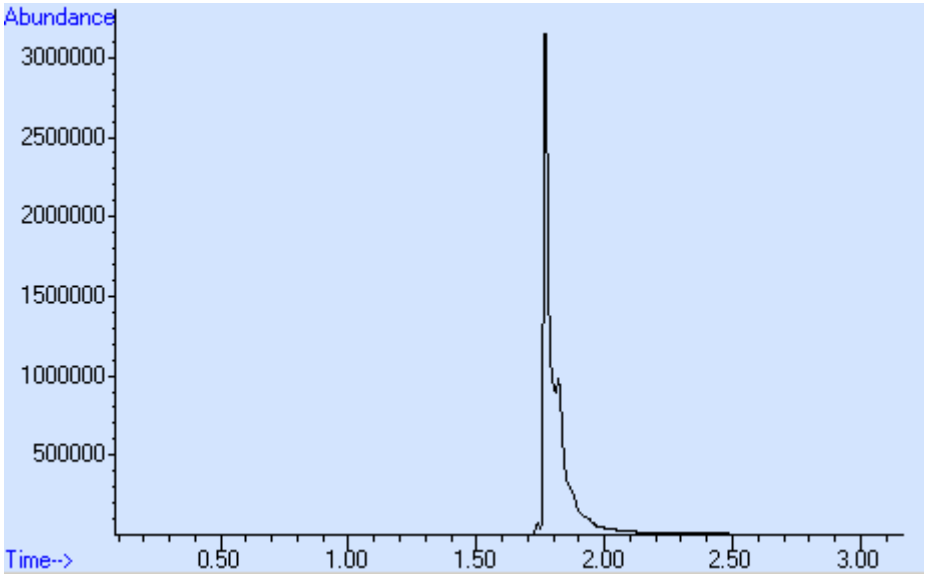

Figure A9.5 Chromatogram of DMS. Chromatogram of DMS from the GC-MS analysis, showing abundance over time (min).

Appendix 10: Calculations of total DMSP

The calculated total DMSP concentrations are shown in Table A10.1-2, provided with an example of calculation.

Table A10.1 Total DMSP concentrations (Experiment 2). Total DMSP concentrations of the samples from the second experiment, along with average concentrations and standard deviations. The total concentrations were calculated using the quadratic equation "y = -4E-15 x^2 + 2E+06x + 9.4574" (R^2 = 0.9848) from the calibration curve.

Sample	Total DMSP concentration (µM)			Average (µM)	Standard deviation
	i	ii	iii		
Tp-START-C	33.4	31.9	32.9	32.7	0,7
Tp-START-Si	28.4	28.8	29.4	28.9	0,5
Tp-START-N	28.2	29.4	28.5	28.7	0,6
Tp-Day 1-C	32.0	32.5	31.9	32.1	0.3
Tp-Day 1-Si	29.8	30.1	29.8	29.9	0.2
Tp-Day 1-N	34.0	37.0	32.2	34.4	2.5
Tp-Day 2-C	87.4	82.1	75.5	81.7	5.9
Tp-Day 2-Si	72.0	72.6	77.1	73.9	2.8
Tp-Day 2-N	102.3	105.1	94.4	100.6	5.6
Tp-Day 3-C	99.6	90.5	86.4	92.2	6.8
Tp-Day 3-Si	97.0	88.0	93.5	92.8	4.5
Tp-Day 3-N	85.7	87.6	90.5	87.9	2.4
Tp-Day 4-C	132.6	137.2	143.4	137.7	5.4
Tp-Day 4-Si	133.0	134.0	134.1	133.7	0.6
Tp-Day 4-N	95.9	103.0	93.9	97.6	4.8
Tp-Day 5-C	177.9	187.7	159.6	175.1	14.2
Tp-Day 5-Si	162.0	170.8	170.3	167.7	4.9
Tp-Day 5-N	110.6	119.0	114.5	114.7	4.2

Table A10.2 Total DMSP concentrations (Experiment 3). Total DMSP concentrations of the samples from the second experiment, along with average concentrations and standard deviations. The total concentrations were calculated using the quadratic equation "y = -1E-15 x^2 + 3E+07x - 0.0877" (R^2 = 0.9992) from the calibration curve.

Sample	Total DMSP concentration (µM)			Average (µM)	Standard deviation
	i	ii	iii		
Tp -20 min	1.66	1.25	1.16	1.36	0.27
Tp 7.5 h	3.35	2.96	2.81	3.04	0.28
Tp 24 h	4.49	3.75	4.65	4.30	0.48
Tp 48 h	6.86	6.42	6.03	6.44	0.41
Tp 72 h	13.65	11.29	10.56	11.83	1.62

Example of total DMSP concentration calculation:
The sample "Tp -20 min" replica "i" from Experiment 3:

The total DMSP concentration of this sample was calculated from the detector response and the equation of the calibration curve, shown in Table A9.3 and Figure A9.4.

Equation from calibration curve: $y = -1E\text{-}15\ x^2 + 3E+07x - 0.0877$
Detector response: x = 5 950 578

Hence, the total DMSP concentration was:
c ($DMSP_{TOTAL}$) : $y = -1E\text{-}15\ x^2 + 3E+07x - 0.0877$
$= -1E\text{-}15\ (5\ 950\ 578)^2 + 3E+07(5\ 950\ 578) - 0.0877$
= <u>1.66 µM</u>

Appendix 11: Intracellular DMSP levels

As presented in Table A11.1, the intracellular amount of DMSP from Experiment 2 was calculated from the total DMSP concentrations and cell number. The intracellular concentrations of DMSP from Experiment 3 were calculated from the total DMSP concentrations, cell number and cell volume, and are displayed in Table A11.2. An example of the calculations is provided.

Table A11.1 Intracellular amounts of DMSP (Experiment 2). The amount of DMSP per cell of the samples from the second experiment, along with average concentrations and standard deviations.

Sample	Cellular amounts of DMSP (pmol)			Average (pmol)	Standard deviation
	i	ii	iii		
Tp-START-C	2.87E-02	2.75E-02	2.83E-02	2.82E-02	6.35E-04
Tp-START-Si	2.45E-02	2.48E-02	2.53E-02	2.48E-02	4.16E-04
Tp-START-N	2.43E-02	2.53E-02	2.45E-02	2.47E-02	5.28E-04
Tp-Day 1-C	4.73E-03	4.38E-03	5.51E-03	4.87E-03	5.80E-04
Tp-Day 1-Si	4.88E-03	4.40E-03	4.43E-03	4.57E-03	2.66E-04
Tp-Day 1-N	4.81E-03	6.21E-03	4.67E-03	5.23E-03	8.51E-04
Tp-Day 2-C	4.98E-03	4.91E-03	4.89E-03	4.92E-03	4.72E-05
Tp-Day 2-Si	4.63E-03	5.81E-03	5.56E-03	5.33E-03	6.24E-04
Tp-Day 2-N	7.13E-03	8.15E-03	6.53E-03	7.27E-03	8.19E-04
Tp-Day 3-C	5.39E-03	4.22E-03	4.39E-03	4.67E-03	6.29E-04
Tp-Day 3-Si	4.46E-03	4.30E-03	4.07E-03	4.27E-03	1.96E-04
Tp-Day 3-N	5.04E-03	5.78E-03	6.01E-03	5.61E-03	5.04E-04
Tp-Day 4-C	4.69E-03	4.82E-03	5.03E-03	4.84E-03	1.70E-04
Tp-Day 4-Si	4.95E-03	5.75E-03	4.99E-03	5.23E-03	4.51E-04
Tp-Day 4-N	5.67E-03	6.45E-03	5.56E-03	5.89E-03	4.86E-04
Tp-Day 5-C	3.98E-03	4.40E-03	3.40E-03	3.93E-03	5.04E-04
Tp-Day 5-Si	4.31E-03	4.99E-03	4.54E-03	4.61E-03	3.47E-04
Tp-Day 5-N	5.36E-03	6.28E-03	5.33E-03	5.65E-03	5.42E-04

Table A11.2 Intracellular DMSP concentrations (Experiment 3). The cellular concentrations of DMSP of the samples from the third experiment, along with average concentrations and standard deviations.

Sample	Intracellular DMSP concentration (mM)			Average (mM)	Standard deviation
	i	ii	iii		
Tp -20 min	1.80	1.14	1.04	1.32	0.42
Tp 7.5 h	1.84	2.59	2.08	2.17	0.38
Tp 24 h	2.12	1.69	2.48	2.10	0.40
Tp 48 h	2.51	2.13	2.38	2.34	0.19
Tp 72 h	3.93	2.44	2.51	2.96	0.84

Example of intracellular DMSP concentration calculation:
The sample "Tp -20 min" replica "i" from Experiment 3:

The intracellular DMSP concentrations were calculated from the total DMSP concentrations and the cell number of the samples. Here, the intracellular DMSP concentration of this sample was calculated as following:

Total DMSP concentration: 1.66 µM

The total amount of DMSP per sample was:
n ($DMSP_{TOTAL}$) = **c** * **V** = 1.66 µmol/L * 0.0022 L = 0.0037 µmol

where **n** is the amount of DMSP, **c** is the total DMSP concentration and **V** is volume of the GC-MS sample.

The cellular amount of DMSP was:
Cell density: 2 490 000 cells mL^{-1}
Cell number = 2 490 000 cells mL^{-1} * 25 mL (harvesting volume) = 62 250 000 cells

n ($DMSP_{CELL}$) = **n** ($DMSP_{TOTAL}$) / cell number
= 0.0037 µmol * 62 250 000 cells = **0.00000000006 µmol/cell**
= 0.06 fmol/cell

Intracellular DMSP concentration:
c ($DMSP_{CELL}$) = n ($DMSP_{CELL}$) / V_{CELL}
= 0.00000000006 µmol/cell / 3.262 E-14 L/cell
= 1800.72 µmol/L
= 1.80 mmol/L

Appendix 12: Cell volume

The estimated cell volumes from Experiment 3 are displayed in Table A12.1. The cell volumes were estimated as described in Section 2.1.5.

Table A12.1 Cell volume. The average cell volume of 15 cells per sample of the three biological replicates harvested at five different time points, 20 minutes before Start and 7.5, 24, 48 and 72 hours after Start. Average cell volume for the three biological replicates and standard deviation is given. Cell volume was estimated by using the formula for the volume of a cylinder.

Sample	**Cell volume (μm^3)**			**Average (μm^3)**	**Standard deviation**
	i	**ii**	**iii**		
Tp -20 min	32.62	36.27	46.97	38.62	7.46
Tp 7.5 h	43.29	34.41	35.16	37.62	4.92
Tp 24 h	45.5	48.99	39.37	44.62	4.87
Tp 48 h	56.2	57.06	50.18	54.48	3.75
Tp 72 h	64.97	74.62	73.2	70.93	5.21