2014

CELLULAR AND MOLECULAR MECHANISMS OF BIOMINERALISATION IN A SILICIFYING HAPTOPHYTE PRYMNESIUM NEOLEPIS

Durak, Grazyna

http://hdl.handle.net/10026.1/3098

http://dx.doi.org/10.24382/3274
Plymouth University

All content in PEARL is protected by copyright law. Author manuscripts are made available in accordance with publisher policies. Please cite only the published version using the details provided on the item record or document. In the absence of an open licence (e.g. Creative Commons), permissions for further reuse of content should be sought from the publisher or author.
CELLULAR AND MOLECULAR MECHANISMS OF BIOMINERALISATION IN A SILICIFYING HAPTOPHYTE
PRYMNESIUM NEOLEPIS

by

GRAŻYNA MAŁGORZATA DURAK

A thesis submitted to the University of Plymouth in partial fulfilment for the degree of

DOCTOR OF PHILOSOPHY

School of Marine Science & Engineering
Faculty of Science & Technology

In collaboration with
The Marine Biological Association of the United Kingdom

-August 2013-
Copyright Statement

This copy of the thesis has been supplied on condition that anyone who consults it is understood to recognise that its copyright rests with its author and that no quotation from the thesis and no information derived from it may be published without the author's prior consent.
ABSTRACT

CELLULAR AND MOLECULAR MECHANISMS OF BIOMINERALISATION IN A SILICIFYING HAPTOPHYTE PRYMNESIUM NEOLEPIS

Haptophytes are renowned for the most prominent and biogeochemically important group of marine calcifiers: coccolithophores. The unexpected discovery of a unique, silicifying member of this clade - *Prymnesium neolepis* - prompted questions regarding mechanisms of silicification and their origin in the calcifier-dominated haptophytes. To address these questions I used cell physiology, biochemistry and molecular approaches, investigating cellular and molecular mechanisms involved in silicification in haptophytes. Comparisons of this system with calcification in coccolithophores and other silica-based systems in eukaryotes were also made. Here I report that *P. neolepis* is an obligate silicifier, producing silica scales in a process fundamentally different to that observed in coccolithophores. Scale deposition and secretion in *P. neolepis* is localized in the posterior, vacuolar part of the cell rather than in the anterior part near the flagellar roots as in calcifying coccolithophores. The organic matrix underlying silica scales in *P. neolepis* was found to be non-homologous with organic scales, which in coccolithophores serve as coccolith baseplates. This suggests, that silica scales and coccoliths arise from two distinct, most likely non-homologous processes, which is further supported by the comparative investigation of the role of cytoskeleton in silica scale production in *P. neolepis* and coccolithogenesis in a representative calcifier, *Coccolithus pelagicus*. Using cytoskeleton inhibitors I established, that the cytoskeleton components used for morphogenesis and secretion of biomineralised structures are different in these two systems. Analysis of *P. neolepis* biosilica revealed the presence of an intimately associated organic fraction consisting of a putatively chitin-containing material, potentially serving as an organic matrix underlying silica scales. Further biochemical investigation of the biosilica-associated organics confirmed the presence of long chain polyamines (LCPAs) dissimilar to those previously reported in diatoms and sponges. Additionally, a potentially novel, proline and lysine-rich protein sharing a weak homology with lipocalins was recovered, suggesting that this silicification system is unique to haptophytes. Several theories concerning acquisition of the ability to silicify in haptophytes were proposed. Overall, the findings presented in this study provide a detailed description of Si biomineralisation system in this unique, silicifying haptophyte and supply novel information on biomineralisation systems in marine haptophytes. This study contributes a basis on which the phenomenon of silicification in haptophytes can be further investigated, as well as novel information which can be further used in elucidation of origins of silicification in algae and other Eucarya.
# List of Contents

**Abstract** ............................................................................................................................................. 3

**List of Figures and Tables** .................................................................................................................. 11

**List of Abbreviations** .......................................................................................................................... 17

**Acknowledgements** ............................................................................................................................. 20

**Author's Declaration** ............................................................................................................................ 21

**Chapter I: Metabolism and Physiology of Silicon. Mechanisms and Functions of Biominalisation in Different Phyla** .................................................................................................................. 23

I.1. Introduction ........................................................................................................................................ 23

I.2. Algae: silica ........................................................................................................................................... 26

I.3. Diatoms (Heterokontophyta: Bacillariophyceae) ................................................................................. 27

  I.3.1. Cell cycle ....................................................................................................................................... 27

  I.3.2. Transport and storage .................................................................................................................... 28

  I.3.3. Si Uptake and transport models .................................................................................................... 29

  I.3.4. Valve formation: SDV .................................................................................................................. 33

  I.3.5. Components involved in silica polymerization ............................................................................. 34

  I.3.6. Organic matrix of the frustule ....................................................................................................... 36

  I.3.7. Polyanionic polysaccharides ......................................................................................................... 38

I.4. Chrysophytes ...................................................................................................................................... 38

I.5. Haptophytes (Haptophyta: Coccolithophyceae) .................................................................................. 39

  I.5.1. Coccolithales and Isochrysidales: calcification ............................................................................. 40

    I.5.1.1. Heterococcolith formation ....................................................................................................... 40

    I.5.1.2. Holococcolith formation ......................................................................................................... 43

  I.5.2. Prymnesiales: Prymnesium neolepis: silicification in haptophytes .......................................... 44

I.6. Sponges (Porifera) ................................................................................................................................. 45
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.6.1</td>
<td>Spicule formation</td>
<td>46</td>
</tr>
<tr>
<td>I.6.2</td>
<td>Silicateins</td>
<td>50</td>
</tr>
<tr>
<td>I.6.3</td>
<td>Silicase</td>
<td>52</td>
</tr>
<tr>
<td>I.6.4</td>
<td>Silintaphins</td>
<td>53</td>
</tr>
<tr>
<td>I.6.5</td>
<td>Long chain polyamines</td>
<td>54</td>
</tr>
<tr>
<td>I.7</td>
<td>Plants</td>
<td>54</td>
</tr>
<tr>
<td>I.7.1</td>
<td>Si transporters</td>
<td>55</td>
</tr>
<tr>
<td>I.8</td>
<td>Summary and Aims</td>
<td>56</td>
</tr>
<tr>
<td>II</td>
<td>Prymnesium neolepis: General Organism Description, Biology and Physiology of Silicification</td>
<td>59</td>
</tr>
<tr>
<td>II.1</td>
<td>Introduction</td>
<td>59</td>
</tr>
<tr>
<td>II.1.1</td>
<td>Putative capacity for silicification in Prymnesium sp.</td>
<td>61</td>
</tr>
<tr>
<td>II.2</td>
<td>Methods</td>
<td>63</td>
</tr>
<tr>
<td>II.2.1</td>
<td>Culture conditions</td>
<td>63</td>
</tr>
<tr>
<td>II.2.2</td>
<td>DNA extraction, gene amplification, sequencing and molecular analysis</td>
<td>64</td>
</tr>
<tr>
<td>II.2.3</td>
<td>Confocal microscopy</td>
<td>66</td>
</tr>
<tr>
<td>II.2.4</td>
<td>Differential Interference Contrast (DIC)/light microscopy imaging</td>
<td>67</td>
</tr>
<tr>
<td>II.2.5</td>
<td>NaOH etching of biosilica material</td>
<td>67</td>
</tr>
<tr>
<td>II.2.6</td>
<td>Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM) and Energy Dispersive Microanalysis (EDX)</td>
<td>68</td>
</tr>
<tr>
<td>II.2.7</td>
<td>Fourier Transform Infrared Spectroscopy (FTIR)</td>
<td>68</td>
</tr>
<tr>
<td>II.2.8</td>
<td>Light: dark experiment: flow cytometry assessment of silica scale production</td>
<td>69</td>
</tr>
<tr>
<td>II.2.9</td>
<td>High/Low Si experiment</td>
<td>69</td>
</tr>
<tr>
<td>II.2.10</td>
<td>Si concentration determination: silicomolybdate assay</td>
<td>70</td>
</tr>
</tbody>
</table>
II.2.11. Ge supplementation of P. neolepis cultures and biosilica material purification for EDX analysis .............................................................................................................. 72

II.3. RESULTS ...................................................................................................................... 73

II.3.1. Molecular identification of P. neolepis strain ......................................................... 73
II.3.2. P. neolepis: general morphology ............................................................................. 77
II.3.3. In-vivo visualization of silicification in P. neolepis: silica scale formation and exocytosis .................................................................................................................. 79
II.3.4. Morphological observations of silica scales .......................................................... 84
II.3.5. Non-biomineralised elements of P. neolepis cell covering ..................................... 86
II.3.6. FT-IR characterization of chemical structure of P. neolepis biosilica .................. 89
II.3.7. Physiology of silicification in P. neolepis ............................................................... 92
II.3.8. Dependency of silicification on light availability ................................................... 92
II.3.9. Dependency of silica scale production on silica availability ............................... 94
II.3.11. Inhibition of silica scale production in P. neolepis with Ge: CLSM observations ...................................................................................................................... 98
II.3.13. Ge/Si co-feeding of P. neolepis: Ge incorporation into silica scales ............... 101

II.4. DISCUSSION ................................................................................................................. 102

II.4.1. P. neolepis strain TMR5: morphological and biological characteristics and comparisons with closely related algae ................................................................. 102
II.4.2. Organic and biomineralised cell covering ............................................................. 103
II.4.3. Silica scale formation ........................................................................................... 105
II.4.4. Physiology of silicification in P. neolepis ............................................................. 106
II.4.5. Ge inhibition of silicification ................................................................................ 107
II.4.6. P. neolepis: the only silicifying haptophyte described to date? ....................... 108
CHAPTER III: THE ROLE OF CYTOSKELETON IN ALGAL BIOMINERALISATION .. 111

III.1. INTRODUCTION .................................................................................................................. 111

III.2. METHODS.................................................................................................................................. 115

III.2.1. Culture conditions and cytoskeleton inhibitor dosing .................................................. 115
III.2.2. Monitoring of culture health under experimental conditions ................................. 116
III.2.3. Confocal microscopy observations of P. neolepis under cytoskeleton inhibitor treatment .................................................................................................................................................. 116
III.2.4. Flow cytometry monitoring of silica scale production in P. neolepis ............. 117
III.2.5. C. pelagicus: Differential Interference Contrast (DIC) imaging of cytoskeleton inhibitor treated cells .................................................................................................................................................. 118
III.2.6. C. pelagicus: Electron Microscopy imaging of cytoskeleton inhibitor treated coccoliths .................................................................................................................................................. 119
III.2.7. Tubulin Immunofluorescence labelling ............................................................................. 120

III.3. RESULTS................................................................................................................................... 121

III.3.1. Assays to determine effects of cytoskeleton inhibition on culture health .... 121
III.3.2. Effects of cytoskeleton inhibitors on P. neolepis health ............................................. 122
III.3.3. Effects of cytoskeleton disruptors on silica scale morphogenesis in P. neolepis .................................................................................................................................................. 126
III.3.4. Quantification of cytoskeleton inhibitor effects on silica scale production in P. neolepis.................................................................................................................................................. 131
III.3.5. P. neolepis: immunofluorescence imaging of the MT cytoskeleton ............ 133
III.3.6. Effects of cytoskeleton inhibition on culture health of C. pelagicus ........ 137
III.4. Effects of cytoskeleton disruptors on biomineralisation in C. pelagicus: light microscopy imaging ................................................................................................................................. 138
III.4.1. SEM analysis of cytoskeleton inhibitor-induced coccolith malformations in C. pelagicus

III.4.2. C. pelagicus: immunofluorescence imaging of the MT cytoskeleton

III.5. DISCUSSION

III.5.1. Cytoskeleton inhibitors and culture health

III.5.2. The role of actin in biomineralisation

III.5.3. The role of tubulin in biomineralisation

III.5.4. Differential role of cytoskeleton in silicification in haptophyte and Bacillariophyte (diatom) algae

III.6. CONCLUSIONS

CHAPTER IV: BIOCHEMICAL ANALYSIS OF PRYMNESIUM NEOLEPIS SILICA SCALES

IV.1. INTRODUCTION

IV.2. METHODS

IV.2.1. Silica scale purification

IV.2.2. Glycerol cushion separation of the purified silica scales and further assessment of sample purity

IV.2.3. Ammonium fluoride extraction of the organic phase

IV.2.4. Separation of the putative polyamine fraction from the NH4F flow-through on an ion exchange column

IV.2.5. Polyacrylamide gel analysis

IV.2.6. Trypsin digestion of the NH4F soluble organic extract

IV.2.7. Amino acid analysis

IV.2.8. Mass spectrometry analysis

IV.2.9. FESEM, TEM and EDX analysis of the NH4F insoluble extract

IV.2.10. Confocal microscopy imaging of the NH4F insoluble extract
IV.3. RESULTS

IV.3.1. Amino acid analysis of purified P. neolepis biosilica

IV.3.3. Sample purity verification: Schägger-PAGE analysis of the NH₄F soluble phase generated from glycerol cushion-separated silica scales and EDTA extraction of protein from whole scales

IV.3.4. Trypsin digest of the NH₄F soluble extract: verification of presence of protein

IV.3.5. Cation-exchange liquid chromatography column samples: purification of putative LCPAs from the NH₄F extraction flow through

IV.3.6. Mass spectrometry analysis of the protein extracts

IV.3.7. Mass spectrometry analysis of the putative polyamine phase isolated on the cation exchanger

IV.3.8. FESEM and EDX and confocal analysis of the NH₄F insoluble extract, detection of polysaccharides with fluorescent markers

IV.4. DISCUSSION

IV.4.1. NH₄F soluble organic extract: silica-associated proteins

IV.4.2. LCPAs

IV.4.3. NH₄F insoluble organic extract

IV.5. SUMMARY

CHAPTER V: GENERAL DISCUSSION AND CONCLUDING REMARKS

V.1. INTRODUCTION

V.1.1. Origins of photosynthetic Eucarya

V.1.2. Evolution of silicification in Eucarya

V.1.3. Evolution of other biomineralisation systems in Eucarya

V.2. SILICIFICATION IN P. NEOLEPIS: EVOLUTIONARY CONTEXT
V.3. SUMMARY ........................................................................................................... 200
LIST OF APPENDICES ............................................................................................ 202
REFERENCES .......................................................................................................... 209
LIST OF FIGURES AND TABLES

Fig. I. 1. A tree of life with clades including silicifying representatives underlined in red ................................................................. 26
Fig. I. 2. A: silicon starvation induced arrest points in the cell cycle .......................... 28
Fig. I. 3. Reconstruction of a diatom silicon transporter........................................... 30
Fig. I. 4. Diatom Si uptake models. Gray oval represents SDV (after Thamatrakoln and Hildebrand, 2008). ................................................................. 32
Fig. I. 5. Examples of LCPAs from different diatom species with different attachment moieties .............................................................................. 35
Fig. I. 6. Chemical representation of native Silaffin 1a structure................................. 36
Fig. I. 7. A schematic of a diatom frustule ..................................................................... 37
Fig. I. 8. Coccolithus pelagicus ssp. braarudi heterococcolith bearing diploid phase ... 41
Fig. I. 9. A diagram of a coccolithophore cell ............................................................... 42
Fig. I. 10. Holococcoliths of Syracosphaera anthos .................................................... 43
Fig. I. 11. SEM of a P. neolepis cell ............................................................................. 45
Fig. I. 12. SEM cross section of a Suberites domuncula ............................................. 47
Fig. I. 13. Immunogold TEM images of primmorph cross sections ............................ 49
Fig. I. 14. Silicatein ................................................................................................. 51
Fig. I. 15. A - model of silicase .................................................................................. 53
Fig. II. 1. A - P. parvum light microscopy image ......................................................... 61
Fig. II. 2. Electron micrographs of Prymnesium sp ..................................................... 62
Table II. 1. Chemical composition of ASW ............................................................... 64
Table II. 2. Primers used for amplification of SSU, LSU and rbcL genes. ............... 65
Table II. 3. List of reagents used in the silicomolybdate assay and their composition .. 71
Table II. 4. A summary of the degree of homology between rbcL and SSU gene sequences obtained from P. neolepis strains isolated from different locations. .....74

Fig. II. 3. Unrooted maximum likelihood phylogenetic tree of selected haptophytes based on the rbcL gene sequences ...........................................................................75

Fig. II. 4. Unrooted maximum likelihood phylogenetic tree constructed for haptophytes and heterokontophyte silicifiers based on SSU rRNA sequences........................76

Fig. II. 5. P. neolepis: A - light microscopy image of a naked and silicified cells .........78

Fig. II. 6. DIC images of P. neolepis .............................................................................79

Fig. II. 7. Confocal microscopy 3D projection of a Z-stack of P. neolepis cells ............80

Fig. II. 8. 3D projections of Z-stacks of P. neolepis ......................................................81

Fig. II. 9. Time course of a silica scale secretion event ..............................................82

Fig. II. 10. Silica scales at different developmental stages ........................................83

Fig. II. 11. SEM images of a P. neolepis biosilica sample etched with 0.5 M NaOH....84

Fig. II. 12. SEM image of KMnO4/HCl cleaned P. neoelpis silica scales .................85

Fig. II. 13. P. neolepis: A - polarized light micrograph of a cell with a disturbed scale cover .........................................................................................................................86

Fig. II. 14. 3D projections of confocal microscopy Z-stacks of Concanavalin-A labelled cells of P. neolepis: A - 3D reconstruction with labelled detached scales (arrows), B - individual slice showing irregularities and partial detachment of the Con-A labelled cell coating, C - decalcified C. pelagicus cells with holes in polysaccharide covering marking spaces where coccoliths were attached, D - individual C. pelagicus coccoliths. P. neolepis was additionally labelled with PDMPO (in blue) to visualize cell body. Con-A is shown in green, chlorophyll autofluorescence in red. Scale bars=5 µm. ........................................................................................................88

Fig. II. 15. A,B-TEM images of uranyl-acetate stained organic scales .....................89
Fig. II. 16. FT-IR transmission spectra of purified *P. neolepis* (red), *Odontella sinensis* (black) biosilica................................................................. 91
Fig. II. 17. Box-whisker plots of the newly deposited to old scale ratios as measured with flow cytometry .................................................................................................................. 94
Fig. II. 18. Confocal microscopy images of PDMPO stained *P. neolepis* cells.......... 95
Fig. II. 19. Validation of the method used for cell counts in the low Si experiment..... 96
Fig. II. 20. Quantification of silica scales produced after 18 h incubation period under low Si and control conditions ................................................................. 97
Fig. II. 21. A - silicified cells in control, B - non-silicifying cells post 10 µM Ge treatment with partially formed intracellular scales........................................... 99
Fig. II. 22. A-Growth curve for 3 µM Ge supplemented and control *P. neolepis* cultures. ........................................................................................................ 100
Fig. II. 23. EDX spectrum of purified *P. neolepis* silica scales ................................. 101
Fig. III. 1. Mechanism of motor protein interaction with MTs and F-actin............. 113
Table III. 1. Cytoskeleton inhibitor and control DMSO doses.............................. 115
Table III. 2. Photosynthetic efficiency and cell viability counts recorded for *P. neolepis* 26.5 h post cytoskeleton inhibitor treatment. Measurements were taken from triplicate samples of each treatment and averaged........................................ 123
Fig. III. 2. SYTOX Green labelled *P. neolepis* cells representative of the three cell viability categories ................................................................. 124
Table III. 3. Results of χ² test between DMSO and nocodazole treatment labelled with SYTOX Green ................................................................. 126
Fig. III. 3. Confocal microscopy images representative of *P. neolepis* cells following a 24 h treatment with 1 µM latrunculin B .................................................. 127
Fig. III. 4. 3D projections of confocal microscopy generated Z-stacks of *P. neolepis* after 24 h treatment with cytoskeleton inhibitors ........................................ 128
Table III. 4. T-test comparisons of *P. neolepis* scales between different cytoskeleton inhibitor treatments ................................................................. 129

Fig. III. 5. A - frequency distribution of *P. neolepis* scales according to different size classes ........................................................................................................ 130

Fig. III. 6. Box-whisker plots of fluorescent vs. non-fluorescent scales ratios post 24h treatments........................................................................................................ 132

Table III. 5. Results of the *t*-test comparisons of the fluorescent vs. non-fluorescent scales ratio........................................................................................................ 133

Fig. III. 7. Confocal microscopy 3D projection of a Z-stack of *P. neolepis* tubulin-immunolabelled control cells ........................................................................ 134

Fig. III. 8. *P. neolepis*: 3D confocal microscopy projection of a Z-stack of anti-α-tubulin immunolabelled nocodazole treated cells ........................................................................ 135

Fig. III. 9. Confocal microscopy image of a *P. neolepis* immunofluorescence labelling control ........................................................................................................ 136

Table III. 6. Photosynthetic efficiency recorded post 25 h cytoskeleton inhibitor treatment of *C. pelagicus*. ......................................................................................... 137

Fig. III. 10. DIC of decalcified *C. pelagicus* cells at the beginning of the experiment. 138

Fig. III. 11. DIC images of cytoskeleton inhibitor treated *C. pelagicus* ...................... 140

Fig. III. 12. DIC image of *C. pelagicus* post 48 h recovery from latrunculin B treatment ........................................................................................................ 142

Fig. III. 13. FESEM images of *C. pelagicus* coccoliths used for morphological aberration ranking of coccoliths produced ......................................................... 143

Table III. 7. χ² statistic results of DMSO and nocodazole treatments ....................... 144

Fig. III. 14. A-C - malformed/incomplete coccoliths representative of latrunculin B treatment ........................................................................................................ 145
Fig. III. 15. 3D projections of confocal microscopy Z-stacks of anti-α-tubulin immunolabelled C. pelagicus control cells ......................................................... 146

Fig. III. 16. 3D projections of confocal microscopy Z-stacks of anti-α-tubulin immunolabelled C. pelagicus .................................................................................. 147

Fig. III. 17. 3D confocal microscopy projections of Z-stacks of C. pelagicus nonspecific labelling control ......................................................................................... 148

Table III. 8. Summary of the effects of cytoskeleton inhibitors on P. neolepis and C. pelagicus ........................................................................................................ 158

Fig. IV. 1. A schematic summarising processing of the P. neolepis biosilica .......... 165

Table IV. 1. Concentrations and incubation times of fluorophores used............. 170

Fig. IV. 2. HPLC chromatogram of amino acids of the whole scales ................... 172

Fig. IV. 3. NH4F soluble extract run on a 16% Schägger gel................................. 173

Fig. IV. 4. Coomassie Blue staining of the NH4F soluble extract of the silica scale fraction.................................................................................................................. 175

Fig. IV. 5. A - Coomassie stained 16% Schägger gel............................................. 176

Fig. IV. 6. Coomassie stained 16% Schägger gel with samples isolated on a cation exchanger................................................................................................................. 177

Table IV. 2. Summary of putative properties of the comp1037 peptide recovered from the transcriptome........................................................................................................ 178

Fig. IV. 7. Amino acid composition of comp1037 peptide retrieved from P. neolepis transcriptome. Abbreviations are as follows: Ala - alanine, Cys - cysteine, Asp - aspartic acid, Glu - glutamic acid, Phe - phenylalanine, Gly - glycine, His - histidine, Ile - isoleucine, Lys - lysine, Leu - leucine, Met - methionine, Asn - aspargine, Pro - proline, Gln - glutamine, Arg - arginine, Ser - serine, Thr - threonine, Val - valine, Trp - tryptophan, Tyr - tyrosine.................................................. 179
Fig. IV. 8. A,B - mass spectra of the ammonia eluate isolated from the NH4F flow through on a cation exchanger ................................................................. 180

Fig. IV. 9. Collision induced fragmentation spectrum of the m/z=574.6 peak. ........ 181

Fig. IV. 10. FESEM images of the NH4F insoluble biosilica-associated organic phase .............................................................................................................. 183

Fig. IV. 11. EDX analysis of the NH4F insoluble extract ...................................... 184

Table V. 1. Table summarizing differences between the formation of the silica scales and coccoliths................................................................. 192

Fig. V. 1. The hypothetical chain of evolutionary events leading to plastid acquisition and subsequent lineage differentiation in eukaryotes................................. 194
LIST OF ABBREVIATIONS

ASW-artificial sea water

BLAST- Basic Local Alignment Search Tool

BSA- bovine serum albumin

BYA- billion years ago

Con-A- Concanavalin A

CV- coccolith vesicle

CW- Calcofluor White

CLSM- confocal laser scanning microscopy

DF- degrees of freedom

DIC- differential interference contrast microscopy

DMSO- dimethyl sulfoxide

dNTP- deoxyribonucleotide triphosphate

DNA- deoxyribonucleic acid

EDTA- ethylene glycol tetraacetic acid

EDX- energy dispersive microanalysis

F- actin- linear polymer microfilament of actin

FESEM- field emission scanning electron microscopy

FSW- filtered sea water
FT-IR - Fourier transform infrared spectroscopy

HCK-123 - LysoTracker Yellow HCK-123

HPLC - High-performance liquid chromatography

LC-MS - Liquid chromatography–mass spectrometry

LCPAs - Long Chain Polyamines

LSU rDNA - 28S Large subunit of the rRNA gene

MAFFT - Multiple Alignment using Fast Fourier Transform

MS - mass spectrometry

MT - microtubule

MYA - million years ago

NCBI - National Centre for Biotechnology Information

NH$_4$Ac - ammonium acetate

NTP - nucleoside triphosphate

PCR - polymerase chain reaction

PDMPO - [2-(4-pyridyl)-5-[[4-(2-dimethylaminoethylaminocarbamoyl) methoxy] phenyl] oxazole]

PITC - phenylisothiocyanate

PSII - photosystem II

$rbcL$ - large subunit of RuBisCO (Ribulose-1,5-bisphosphate carboxylase oxygenase)

RNA - ribonucleic acid
SDS-sodium dodecyl sulfate

SDS-PAGE-sodium dodecyl sulfate polyacrylamide gel electrophoresis

SDV-silica deposition vesicle

SEM-scanning electron microscopy

SIT-silicon transporter

STVs-Silica Transport Vesicles

TEM-transmission electron microscopy

TPCK- Tosyl phenylalanyl chloromethyl ketone

WGA- Wheat Germ Agglutinin
ACKNOWLEDGEMENTS

This study was funded by the EU Interreg IV Marinexus project and carried out at the Marine Biological Association of the United Kingdom and the University of Plymouth.

I would like to thank Prof. Colin Brownlee for all help and support during the course of my study and for securing funding, enabling me to further broaden my expertise and form new, fruitful collaborations. I would especially like to thank Dr. Glen Wheeler, for his inexhaustible patience and his help and guidance during my Ph.D. study. I would also like to thank Prof. Jason Hall-Spencer for his supervision of the project and Dr. Ian Probert for providing transcriptome data.

Further I would like to thank Dr. Declan Schroeder for his help and advice with the molecular work and Mr. Matt Hall for assistance with the laboratory equipment. Thanks also go to Dr. Roy Moate, Mr. Glenn Harper and Mr. Peter Bond of the Electron Microscopy unit at the University of Plymouth, for all their help with various electron microscopy techniques. Special thanks also go to Dr. Rowena Stern and Dr. Andrea Highfield, for their friendship, help with phylogenetic analysis and stimulating discussions on various scientific and non-scientific subjects.

I am also grateful to Prof. Nils Kröger and Dr. Nicole Poulsen for hosting me at the B-Cube Dresden Centre for Molecular Engineering and their kind help with biochemical analysis undertaken in this study.

I would also like to thank my siblings, especially my sister Marta for their encouragement and support during my Ph.D. study.
AUTHOR'S DECLARATION

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award without prior agreement of the Graduate Committee.

This study was funded by the EU Interreg IV Marinexus project and carried out in collaboration with the Marine Biological Association of the UK.

Relevant presentations and conferences attended:

- Biomineralisation in *Hyalolithus neolepis*. Oral presentation given at the EU Marinexus Meeting, Roscoff, France, January 2010.


- Biomineralisation in *Hyalolithus neolepis*. Oral presentation given at the MBA Laboratory Group Meeting, Plymouth, UK, June 2012.


- Calcification and silicification in marine haptophyte algae. Oral presentation given at the B-Cube Centre for Molecular Engineering, Dresden, Germany, June 2012.

Word count of main body of thesis: 34,232

Signed

Date
CHAPTER I: METABOLISM AND PHYSIOLOGY OF SILICON. MECHANISMS AND FUNCTIONS OF BIOMINERALISATION IN DIFFERENT PHYLA

I.1. INTRODUCTION

Biomineralisation is a process by which living organisms deposit minerals—a phenomenon widespread in both prokaryotic and eukaryotic taxa (Weiner and Dove 2003). Mineral biodeposition serves a variety of purposes, e.g. formation of exo- and endo-skeletal components, such as mollusc shells, algal cell walls, vertebrate bones and other functional structures, such as teeth and statoliths. Biomineralisation can occur both intracellularly, as is the case in e.g. metazoan holothurians (Märkel et al. 1986) and unicellular algae such as diatoms or coccolithophores (Schmid and Schulz 1979; Westbroek et al. 1984) or extracellularly, e.g. in mollusc shells, bones and teeth (Addadi et al. 2006; Veis 2003). Some organisms, such as silicifying sponges, deposit biomineralised structures (spicules) sequentially, with an intracellular and extracellular phase to the process (Müller et al. 2006; Müller et al. 2005). Calcium based biominerals (e.g. carbonates, phosphates, oxalates) account for 50% of known types of minerals of biological origin (Weiner and Dove 2003). Iron-based minerals contribute about 40% of the total, with the remaining part comprising silicon, lead, barium, strontium, zinc, arsenic, manganese, copper and fluorine based minerals (Lowenstam and Weiner 1989; Weiner and Dove 2003).

Silicon is a metalloid element and the second most common element in the Earth's crust (Earnshaw and Greenwood 1997). Silicon is utilized by many organisms for a variety
of reasons, mainly related to its superior structural, mechanical and optical properties. Si can be taken up by organisms in aqueous solution as an uncharged orthosilicic acid, Si(OH)$_4$, (e.g. plants) or in an anionic form as silicates (e.g. as SiO(OH)$_3^-$ in diatom algae) (Del Amo and Brzezinski 1999; Earnshaw and Greenwood 1997; Ma and Yamaji 2006). As a structural component, silicon in a form of silica (SiO$_2$) is particularly attractive due to its inherent toughness, relatively high density (~1.7-2.0 g/cm$^3$), and low chemical reactivity (Hamm et al. 2003; Hurd et al. 1981; Pak and Yoo 2013). Silica is also characterised by a low refractive index (n≈1.43), making it a material highly transparent to visible light (Aizenberg et al. 2004).

Properties of silica are determined by its structure, from the organization on a molecular level, through nano-, micro-, meso- to macrostructure, dictating parameters such as surface area, porosity, interaction with light etc. (Perry 2003). Both mechanical and optical properties of silica can be further enhanced by the specific architecture, and interaction with e.g. organics via formation of biocomposites with highly modified mechanical and optical properties, tailored to a specific function (Aizenberg et al. 2004; Yamanaka et al. 2008).

As a structural component, Si is deposited in a form of amorphous, hydrated silica (SiO$_2$*nH$_2$O) in a process of biosilicification in organisms such as algae (e.g. diatoms), radiolarians, cercozoans, plants and sponges (summarized in Fig. I. 1.). Apart from the obvious structural benefit of the ability to silicify in these organisms, a number of other factors relating to metabolism and physiology have been identified. In higher animals for example, Si facilitates the formation of connective tissue, bone and calcification in avians and mammals (Carlisle 1981; Carlisle 1988). It is involved in aging processes, where the decline in Si is subject to hormonal regulation (Charnot 1971). In algae such as diatoms and in sponges, Si has also been shown to function as a regulator of gene
expression (Schröder et al. 2004b; Schröder et al. 2006; Schröder et al. 2003; Thamatrakoln and Hildebrand 2007).

In this chapter, the so far described silicification systems are summarized. Their respective physiological, structural and metabolic roles in different organisms, as well as homologies and analogies and their evolutionary connections in different taxa are also discussed. Particular attention is paid to silicon metabolism and biomineralisation in algae and sponges, as these two systems are so far best described. Also, a brief overview of the other main algal biomineralisation mode-calcification, utilized by haptophytes is provided, enabling comparisons with the only silicifying representative of this group described to date - Prymnesium neolepis.
Fig. I.1. A tree of life with clades including silicifying representatives underlined in red (modified from (Adl et al. 2012)). Haptophytes (mainly calcifiers, underlined in green) and heterokontophytes (Stramenopiles) are marked with a star.

I.2. ALGAE: SILICA

Silica is used for structural component formation in several algal groups belonging to Heterokontophyta (i.e. Chrysophyceae, Dictyochophyceae, Synurophyceae, Parmophyceae, Xanthophyceae and most importantly Bacillariophyceae) as well and one species belonging to Haptophyta, Prymnesium neolepis (Prymnesiophyceae) (Graham et al. 2009; Van den Hoek et al. 1995; Yoshida et al. 2006).
I.3. DIATOMS (HETEROKONTOPHYTA: BACILLARIOPHYCEAE)

Evolved around 250 MYA diatoms are obligatory silicifiers, producing a silica cell wall known as the frustule (Medlin 2011). Various explanations have been put forward to elucidate the role of biomineralisation in these algae. The silica frustule has been so far suggested to serve as a defensive structure due to its rigidity, as well as to provide protection against enzymatic attack, regulate sinking rate, confer UV protection, maintain cell shape and aid osmoregulation (Hamm et al.; 2003, Raven 1982; Raven; 1983; Raven, Waite 2004; Davidson et al., 1994). Using silica as a cell wall material may also be favoured due to the relatively low energetic costs of silica deposition in comparison to e.g. organic carbon material (Raven 1983). Another potential role of silicified frustules in diatoms is facilitating the functioning of carbon concentrating mechanisms via buffering the proton transfer between HCO$_3^-$, CO$_2$, and the active site of the extracellular carbonic anhydrase (Milligan and Morel 2002). Si in diatoms has also been reported to be involved in DNA synthesis, as Si is essential for thymidylate kinase and DNA polymerase synthesis (Sullivan and Volcani 1973). Si dependence of chlorophyll and protein production has also been demonstrated (Werner 1966; Werner 1967).

I.3.1. Cell cycle

The diatom cell cycle is Si dependent, as cell division requires formation of new valves (Fig. I. 2. B), and the majority of cells will arrest in the $G_1/S$ and $G_2/M$ phase if deprived of Si (Fig I. 2. A), (Hildebrand et al. 2007; Vaulot et al. 1987). These two arrest points have been linked to active silica deposition, also suggesting that the $G_1/S$
halt might serve as a check point to determine, if extracellular Si is present in sufficient quantity for cell division to proceed (Vaulot 1985). Diatoms respond to lowering of the Si concentration by producing thinner cell walls, in order to maintain the rate of cell division (Sullivan 1977). Si in diatoms also acts as a regulator for gene expression and a metabolite of various functions (Hildebrand et al. 1993).

![Fig. I. 2. A: silicon starvation induced arrest points in the cell cycle (after Martin-Jézéquel et al., 2000). B: schematic diagram of a diatom cell cycle. SDV and daughter valves are shown in red (after Sumper and Kröger, 2004).](image)

I.3.2. Transport and storage

Diatoms have been shown to be able to retain soluble, supersaturated Si pools intracellularly at 19-340 mM concentrations (Martin-Jézéquel et al. 2000). This suggests that the chemical form of Si is altered once transported into the cell, likely via complexation with a so far undescribed organic component/stabilising factor, as
otherwise orthosilicic acid undergoes auto-polymerisation at concentrations exceeding 2 mM (at pH 7.0) (Azam et al. 1974; Iler 1979). However, evidence that only ca. 5-10% of intracellular Si forms organosilicon complexes and the remaining Si exists as silicic acid have been later supplied (Kinrade et al. 2002). Sumper and Brunner (2008) therefore suggested that Si is intracellularly maintained as pre-polymerised silicic acid, forming silica sols that might be stabilised by long-chain polyamines, to enable its subsequent use for silica biodeposition. The alteration of the chemical form of Si is also supported by the lack of Si efflux from diatom cells with high internal Si pools in the absence of extracellular Si (Martin-Jézéquel et al. 2000).

I.3.3. Si Uptake and transport models

Diatom silicon transporters - SITs - are a unique family of membrane-bound proteins that mediate active transport of orthosilicic acid into the cell by acting as Na\(^+\)/Si symporters, using inwards Na\(^+\) gradient to drive the uptake of Si (Fig. I. 3.) (Hildebrand 2000). The highest peaks of SIT protein levels have been shown to be controlled internally by the requirement for active silicification with SIT gene expression peaking at S phase of the cell cycle, preceding the period of maximum uptake of Si, and subsequent frustule deposition (Thamatrakoln and Hildebrand 2007). The so far isolated SITs have been divided into two groups: SIT\(_1\) and SIT\(_2\) have been suggested to be active transporters with potential differences in their transport capacities/affinities linked to differences in their amino acid composition (Thamatrakoln and Hildebrand 2007). SIT\(_3\) has low Si affinity, and has been suggested to play a role as Si sensor in Si-replete conditions, triggering high affinity transporter expression, or to function as a
specific Si targeting component acting intracellularly (Curnow et al. 2012; Thamatrakoln and Hildebrand 2007).

![Reconstruction of a diatom silicon transporter](after Curnow et al., 2012).

Si uptake in diatoms usually conforms to the Michaelis-Menten saturable kinetics model of nutrient uptake, however, under certain conditions, a biphasic and non-saturable modes can be observed (Thamatrakoln and Hildebrand 2008). In an attempt to explain the variability of Si uptake kinetics, Thamatrakoln and Hildebrand (2008) revised information that has been already published, and proposed two main Si(OH)$_4$ uptake models for exponentially growing cultures:

1. **Biphasic mode** of Si uptake in cultures starved for a short period of time (between 5-10 min), involving a transition from unsaturable to saturable (Michaelis-Menten) uptake.
2. **Saturable, Michaelis-Menten mode** of Si uptake in cultures with an extended Si starvation period (24 h).

In both cases, Si uptake has been proposed to be controlled by three main factors: Si availability in the media, the rate of Si incorporation, and the binding capacity of internal Si pools. In the first model, the internal Si level is assumed to be low, as the Si released by intracellular binding components for incorporation into the cell wall is not replenished, increasing their Si binding capacity (Fig. I. 4. B). Therefore upon Si addition, a non-saturable surge uptake occurs (Fig. I. 4. C) until the capacity of Si binding components returns to equilibrium with the rate of Si incorporation into the cell wall (Fig. I. 4. B).

According to the second model involving a prolonged Si starvation period, the internal Si levels drop to minimum, causing a decrease in Si binding components (Fig. I. 4. E). Si uptake becomes saturable upon Si replenishment due to Si binding component level (and hence internal Si pool) reduction (Fig. I. 4. F). The amount of Si binding components increases over time to re-equilibrate the internal Si pool capacity with Si incorporation into the cell wall (Fig. I. 4. D).
Fig. I. 4. Diatom Si uptake models. Gray oval represents SDV (after Thamatrakoln and Hildebrand, 2008).

Depending on the external Si concentration, the modes of Si transportation change. At Si(OH)$_4$ concentrations exceeding 30 µM, Si transport occurs mainly via simple diffusion due to limitations in the amount of carriers available for facilitated diffusion. The presence of an inward Si gradient is suggested to be a result of a change in
chemical form of intracellular Si via complexation with organic compounds (Thamatrakoln and Hildebrand 2008). Below 30 µM the inward Si transport is likely SIT mediated (Thamatrakoln and Hildebrand 2008).

Additionally to the above, it has been proposed that the Si efflux, which only occurs if the threshold, intracellular concentration of Si is exceeded (Sullivan 1976) may play a role in equilibration of the internal Si pool capacity and the rate of Si incorporation into the frustule (Thamatrakoln and Hildebrand 2008).

I.3.4. Valve formation: SDV

The valve formation process in diatoms occurs within a specialised, membrane enclosed acidic compartment, derived from coalescence of small, putatively Golgi and endoplasmic reticulum originating vesicles and is termed the Silica Deposition Vesicle (SDV) (Lee and Li 1992; Li et al. 1989; Schmid 1987). The new valves are formed by highly coordinated interactions between cytoskeleton and cytoplasmic organelles responsible for shaping of the SDV, as well as organic matrix forming components, also involved in subsequent Si deposition (Pickett-Heaps 1990; Schmid 1994; Tesson and Hildebrand 2010). Si transport into the SDV has been proposed to occur via Silica Transport Vesicles (STVs), as fusion of a type of cytoplasmic vesicles during the SDV maturation has been observed (Schmid and Schulz 1979; Sumper 2004). However, the STVs are yet to be proven to contain Si (Sumper and Brunner 2008).
1.3.5. Components involved in silica polymerization

The most important components involved in diatom biosilicification are two phosphoprotein families: silaffins and silacidins, along with long-chain polyamines (LCPAs) (Kröger et al. 1999; Kröger and Poulsen 2008a; Sumper and Brunner 2008; Sumper and Kröger 2004; Wenzl et al. 2008).

LCPAs are linear chains of N-methyl polypropyleneimine attached mainly to a putrescine, ornithine, propylenediamine or spermidine/spermine basis (Kröger et al. 1999; Kröger and Poulsen 2008a; Sumper et al. 2005; Sumper and Kröger 2004). LCPAs are involved in silicic acid polycondensation and silica sol stabilisation (Mizutani et al. 1998; Sumper and Kröger 2004). They exhibit both variability within and between diatom species (e.g. differences in chain length, attachment moieties, degree of methylation), (Fig. I. 5.), which properties might be a factor involved in producing the unique frustule ornamentation (Sumper 2004; Sumper and Lehmann 2006). Si polymerization by LCPAs proceeds in the presence of polyanions, i.e. phosphate, which act as cross-linkers, facilitating LCPA assembly into higher order structures, significantly affecting the morphology of the resulting silica precipitates (Sumper 2004). Some diatom species (e.g. Cylindrotheca fusiformis) do not posses any free LCPAs, but only contain LCPAs attached to a peptide backbone of silaffins (Kröger et al. 2000; Otzen 2012).
Fig. I. 5. Examples of LCPAs from different diatom species with different attachment moieties: propyldiamine - red, putrescine - green, spermidine - blue; n, m - denote propyleneimine unit repeat number (Kröger and Poulsen 2008a).

Silacidins are polyanionic phosphopeptides composed mainly of serine phosphates, aspartic and glutamic acids and have been proven capable of facilitating LCPA assembly by acting as cross-linking molecules, with the LCPA/silacidin ratios controlling the size of Si nanospheres formed (Wenzl et al. 2008). Phosphorylated silacidins are highly efficient at facilitating silica precipitation and their abundance in frustule increases relative to remaining proteins under Si-replete conditions (Richthammer et al. 2011).

Another group of components involved in guiding the silica deposition via matrix formation due to zwitterionic properties are silaffins (Kröger et al. 1999). These highly post-translationally modified peptides are capable of ionic-interaction driven self-assembly. They consist of a phosphorylated peptide backbone, with repeated polyamine
modified lysine units forming side-chains (Fig. I. 6.) (Kröger et al. 1999; Kröger et al. 2002). Their role in Si deposition is similar to that of LCPAs (Sumper, Kröger, 2004). Additionally, a serine-specific group of endoplasmic reticulum-associated silaffin kinases with a capacity to phosphorylate silaffins, but not silacidins has been found to be partially responsible for silaffin phosphorylation (Otzen 2012; Sheppard et al. 2010).

![Chemical representation of native Silaffin 1a structure. Charges predicted for solution at pH=5. Polyamine chains circled in red (after Kröger et al., 1999).]

I.3.6. **Organic matrix of the frustule**

Silica frustules of diatoms are underlain by an organic matrix, containing frustule-specific proteins and polysaccharides involved in frustule morphogenesis via active silica precipitation, interaction with other silica precipitating components, or by providing a scaffolding (Kröger and Poulsen 2008a; Scheffel et al. 2011). The ability to
actively precipitate silica has so far only been documented for one type of organic matrix-forming proteins: cingulins (Scheffel et al. 2011). These water insoluble proteins along with chitin, form biotemplates for girdle bands (Fig. I. 7. gbs and arrows), with a species-specific nanopattern (Durkin et al. 2009; Scheffel et al. 2011). Another group of proteins isolated from silica frustules are frustulins. These were found to contain multiple acidic and cysteine-rich domains, and to exhibit a specific affinity for Ca\(^{2+}\) ions (Kröger et al. 1994; Kröger et al. 1997). Frustulins create a coat on the frustule once silica deposition has been completed and are suggested to prevent cell wall dissolution (Bidle and Azam 1999; Poll et al. 1999). The last group of proteins isolated to date from diatom organic matrix are pleuralins. These proteins are tightly associated with the biosilica of pleural bands of the epitheca (Fig. I. 7. e), and are suggested to play a role in hypotheca-epitheca differentiation during diatom cell division (Kröger et al. 1997; Kröger and Wetherbee 2000).

Fig. I. 7. A schematic of a diatom frustule. Epitheca (e) and hypotheca (h) are capped by valves with species-specific patterning. Thecae are composed of silicified, overlapping girdle bands (gbs and arrows). In some diatoms girdle bands are not full rings, but taper to fit the curve (the ligula-L) of the adjacent girdle band (after Hildebrand et al., 2008).
I.3.7. Polyanionic polysaccharides

Recently, a polysaccharide containing mannose as the main component has been isolated from *Stephanopyxis turris* biosilica and suggested to be involved in silicification process (Hedrich et al. 2013). Polysaccharides have been proposed to be components of the silicification system, creating a polyanionic matrix for LCPAs, and thus contributing to control over silica deposition (Chiovitti et al. 2005; Gautier et al. 2008b; Hedrich et al. 2013).

I.4. CHRYSOPHYTES

Chrysophytes (Heterokontophyta: *Chrysophyceae*) are an algal clade that evolved in the late Precambrian (600 MYA) (Preisig et al. 1991). A large number of species within this group is able to produce siliceous scales, spines, bristles and distinct stomatocysts (resting cysts) (Preisig 1994; Preisig et al. 1991). Very little is known about the formation of these structures, however, some similarities between diatoms, chrysophytes and synurophytes can be observed, i.e. formation of an SDV occurs during frustule deposition in diatoms, and the encystment process of the two latter classes (Preisig 1994; Sandgren 1983). The process of stomatocyst silicification has been divided into two phases (Sandgren 1983):

1. Rapid silicification within the SDV resulting in a deposition of a thin primary wall, a process occurring without close association between the silica structure and the SDV membrane.
2. Thickening of the wall and addition of a collar and surface patterns. This is a much slower process resembling deposition of highly complex diatom frustules, where the SDV membrane adheres very closely to the structures formed.

Unlike in diatoms though, the SDV is fully formed prior to deposition of the cyst wall (Preisig 1994; Sandgren 1983). The pore in the cyst is formed either via rupture of SDV during the early stage of encystment, or from a pre-formed aperture (Preisig 1994; Sandgren 1983). Recently, Likhoshway et al., (2006) have been able to confirm the presence of a SIT protein in a synurophyte: *Synura petersenii* and a chrysophyte: *Ochromonas ovalis*, concluding that the ability to transport Si into the cell and to biomineralise appeared long before the evolution of diatoms, which appeared only ca. 250 MYA (Medlin 2011).

I.5. HAPTOPHYTES (HAPTOHYTA: COCCOLITHOPHYCEAE)

Belonging to the haptophyte clade, coccolithophores, next to diatoms, are the most prominent group of marine phytoplankters capable of depositing biomineral structures. Unlike diatoms, coccolithophores use a calcium carbonate based biomineralisation system, producing calcitic exoskeletons surrounding the cell (Billard 2004).

Haptophytes diverged from Stramenopiles ca. 824 MYA, with the ability to calcify estimated to have evolved ca. 243 MYA or even earlier, between Carboniferous and early Triassic eras (Liu et al. 2010). Within this group, in addition to being able to form organic scales, two orders (*Coccolithales* and *Isochrysidales*) posses the ability to produce highly complex calcitic scales (coccoliths), forming a characteristic scale covering (coccosphere) around the cell. The exact function of coccoliths is still
unknown, however, a number of potential uses have been suggested, such as maintenance of the microenvironment around the cell, regulation of sinking through the water column, protection from pathogens, grazing and physiological implications (Buitenhuis et al. 1999; Raven and Waite 2004; Young 1994). Coccoliths generally comprise an organic baseplate, on which calcite crystals are deposited in an intricate, species specific pattern (Young and Henriksen 2003). Coccolithophores deposit two types of coccoliths, depending on their ploidy level: heterococcoliths (diploid phase) and holococcoliths (haploid phase) (Young and Henriksen 2003).

1.5.1. Coccolithales and Isochrysidales: calcification

1.5.1.1. Heterococcolith formation

Heterococcoliths consist of an organic base plate on which calcite crystals are radially arranged to form a structure typically consisting of two shields connected by tube elements, and interlock with neighbouring coccoliths to form a mono- or multi-layered cell covering (Fig. I. 8. A,B), (Marsh 2003; Young et al. 1997; Young and Henriksen 2003).
Fig. I. 8. *Coccolithus* *pelagicus* ssp. *braarudi* heterococcolith bearing diploid phase: A - SEM of a whole cell, B-Backscattered Electron (BSE) SEM image of an individual coccolith. Scale bars =5 and 1 µm respectively.

Heterococcoliths are produced intracellularly, within a specialised, Golgi derived compartment - coccolith vesicle (CV, Fig. I. 9.), sometimes associated with additional vesicular extensions termed coccolithosomes (Outka and Williams 1971; Parke and Adams 1960; Westbroek et al. 1984). Coccolithogenesis begins with deposition of an organic base plate (Young et al. 1999). This acts as a base on which calcite crystal nucleation proceeds, giving rise to the first mineralized structure - the protococcolith ring, which is forms around the edge of the organic scale (van der Wal et al. 1983; Young et al. 1999). The crystal growth then progresses in an upward and outward direction, with concomitant CV expansion, until the double-shielded coccolith structure is completed (Young et al. 1999). At this stage organic/polysaccharide coating is added, and the mature coccolith is secreted outside the cell and arranged in a particular manner on the plasma membrane (Taylor et al. 2007). Additionally, coccolith associated polysaccharides, putatively involved in control over the growth of individual crystal components, the cytoskeleton, actively shaping the CV and the reticular body are also

Fig. I. 9. A diagram of a coccolithophore cell combining morphological features observed in various coccolithophores. Different coccolith types are represented by silhouettes (black for heterococcoliths, dotted for holococcoliths). Coccolith vesicle (CV) types of *Pleurochrysis* and *Emiliania* are marked at the top right and bottom of the Golgi body respectively. P1 and 2 are pyrenoids. D - peculiar dilations of the Golgi vesicles, F - flagellum, H - haptonema, N - nucleus, Re - reticular body, CL - columnar deposit, SC - organic scales (unmineralized) - diagram reproduced from Billard (2004).
I.5.1.2. Holococcolith formation

Holococcoliths are composed of euhedral rhombohedral calcite crystallites assembled into species-specific shapes and patterns (Fig. I. 10. A,B) (Henriksen et al. 2004; Young and Henriksen 2003). Details of holococcolith formation to date are still lacking, however, the process is hypothesized to occur either extracellularly, within an envelope ("skin") compartment closely associated with the plasma membrane (Manton and Leedale 1963; Rowson et al. 1986), or intracellularly. In the latter scenario, the holococcolith deposition might be a rapid process localised just below the plasma membrane, succeeded by immediate exocytosis (Young and Henriksen 2003).

Fig. I. 10. Holococcoliths of Syracosphaera anthos: A - a coccosphere, B - detail of individual coccoliths. Images reproduced from Henriksen et al. (2004).
1.5.2. Prymnesiales: Prymnesium neolepis: silification in haptophytes

P. neolepis (formerly Hyalolithus neolepis) is a widely distributed marine alga belonging to the non-biomineralizing haptophyte order: Prymnesiales. It is the only haptophyte species described to date utilising silica for scale mineralization and subsequent formation of multilayered cell covering, very similar to that produced by coccolithophores (Fig. I. 11.) (Yoshida et al. 2006). The ultrastructural studies by Yoshida et al. (2006) revealed the intracellular arrangement typical of haptophyte algae, as well as the presence of typical haptophyte unmineralized, organic scales formed in Golgi body. However, the mineralized scales were not found to be produced in Golgi-associated vesicles, as it is the case in the calcifying coccolithophores. The developing silica scales were found in a compartment suggested to be a silica deposition vesicle more similar to that found in diatoms and chrysophytes, localized on the posterior end of the cell instead (see section II.3.3.). The evolutionary events leading to acquisition of the ability to silicify in this haptophyte are not known. Potential scenarios leading to development of silica biomineralisation system in P. neolepis are further discussed in chapters IV and V.
I.6. SPONGES (PORIFERA)

Poriferans evolved between Neoproterozoic and Cambrian periods and are the oldest metazoans (Xiao et al. 2005). Two out of three sponge classes, Demospongiae and Hexactinellida, utilize amorphous, hydrated silica as a structural component for formation of a skeleton, consisting of siliceous spicules, which can constitute up to 75% dry weight of a sponge (Bergquist 1978; Shimizu et al. 1998). The remaining class, Calcarea utilizes CaCO₃ for the same purpose (Bergquist 1978). Siliceous skeletal parts, spicules, apart from the inherent structural role, have many other functions, such as deterring predators, forming anchoring structures enabling soft sediment colonization and acting as light collecting fibres in an internal, light-signalling system in an Antarctic species Rosella racovitzae, suggested to possess endosymbiotic algae (Cattaneo-Vietti R. 1996; Müller et al. 2010; Simpson 1984; Wiens et al. 2010).

Fig. I. 11. SEM of a P. neolepis cell (after Yoshida et al., 2006). Scale bar=5 µm.
I.6.1. Spicule formation

It has been postulated that all silicified sponges are able to actively accumulate Si by utilizing a putative $\text{Na}^+\text{HCO}_3^-$ $\text{[Si(OH)]}_4^-$ cotransporter (Schröder et al. 2004a), and to be able to use silicatein-mediated enzymatic processes allowing silica precipitation, using silicon alcoxides as a substrate (Cha et al. 1999; Müller et al. 2007b). Sponges are also able to intracellularly accumulate silica in specialized compartments within sclerocytes, termed silicasomes, also containing silicateins, responsible for the gel-sol silica state. Silicasomes have an ability to exocytose their contents (Schröder et al. 2007; Schröder et al. 2008).

Spiculogenesis is a process regulated by growth, differentiation and Si availability factors, as well as formation of the matrix composed of structural and enzymatic components shaping the spicules (Schröder et al. 2007). The primary structural component controlling spicule formation is galectin, localized concentrically around the spicule, and in unorganized bundles in the extracellular space (mesohyl), interacting with $\text{Ca}^{2+}$ ions to provide a matrix for silicatein and silicase molecule assembly into ring-like structures around the axial filament (Schröder et al. 2007; Schröder et al. 2006; Wang et al. 2011). The secondary structural component, collagen, is involved in formation of a highly organized fibrillar network, further shaping growing spicules (Schröder et al. 2007). Finally, a pair of anabolic/catabolic enzymes, silicatein and silicase are responsible for polymerizing and depolymerising silica respectively (Schröder et al. 2006).

The spicule production process is initiated in vesicles of specialized cells, sclerocytes, where a proteinaceous, silicatein-containing axial filament positioned within the axial
canal is deposited (Fig. I. 12. "ac") (Müller et al. 2005; Simpson 1984). The spicule formation process in demosponges consists of an initial intracellular phase (1), occurring in sclerocytes, and the final phase localized in the extracellular space (2), where the spicules increase in size (appositional growth sub-phase) and acquire their final shape (Müller et al. 2005).

Fig. I. 12. SEM cross section of a *Suberites domuncula*. Spicule showing axial canal (ac) and the surrounding Si lamellae deposited during the appositional growth (image after Müller et al., 2006).

1. **Intracellular phase:**

Si substrate is actively transported into sclerocytes, and stored in specialised vesicles - silicasomes prior to deposition (Schröder et al. 2006; Wang et al. 2012). Silicatein in a form of a pro-enzyme is transported through endoplasmic reticulum and Golgi, undergoes post translational modification, mainly concerning phosphorylation and is subsequently transported to vesicles within sclerocytes, where the mature enzyme is
deposited in a form of a rod-like, axial filament, followed by deposition of the initial Si layer (Fig. I. 13. A, C, E-G) (Müller et al. 2006; Müller et al. 2005).

2. **Extracellular phase**

Immature spicules are extruded into the extracellular space, where the appositional growth commences, increasing the length and the diameter of the spicule due to silicatein presence in both extracellular space and the surface of the lamellar depositions (Fig. I. 13. B, H, I, Fig. I. 5.). Silicateins have been shown to form larger, galectin and collagen associated string-like aggregations parallel to the spicule surface, where their function is to mediate lamellar and stepwise Si deposition (Müller et al. 2006; Müller et al. 2005).

3. **Spicule hardening via water syneresis (removal)**

Water, a by-product of silica polycondensation trapped in the galectin scaffolding surrounding the growing spicule is taken up by cells that then migrate away from the silicification site to maintain water equilibrium (Brinker and Scherer 1990; Wang et al. 2012). This process was also suggested to be implicated in shaping of spicules via localised water removal (Uriz 2006).
Fig. I. 13. Immunogold TEM images of primmorph cross sections: nanogold anti-rabbit IgG visualization of the immune complexes between the polyclonal anti-silicatein antibodies. A-Sclerocytes with silicatein molecules, B-concentric rings (ri) surrounding the immunostained silicatein. C-first electron dense ring associated linear clods (> <) . D-fusion of the inner rings, formation of linear clods, E,F-Si accumulation in the centre of the multi-lamellar silicatein-containing material. G-deposition of the first Si layer onto the spicule (sp), H-further growth of the silica layer and an increase in the ring (ri) number. I-silicatein axial filament (af) with Si rings. Scale bars=2 μm.

Images reproduced from Müller et al. (2006).

Exogenous Si in demosponges and hexactinellids is also an element up-regulating a number of spicule formation-associated genes, such as those coding for silicase (Schröder et al. 2003), galectin (Schröder et al. 2006), collagen (Krasko et al. 2000), and morphogenetic protein, noggin (Schröder et al. 2004b). Overall, the spiculogenesis
process in sponges is highly energetically expensive, requiring active exogenous Si transport, followed by internal transportation to specialised storage and spicule-forming cells and production of structural and enzymatic components involved in spicule morphogenesis (Schröder et al. 2004a; Schröder et al. 2008).

I.6.2. Silicateins

Belonging to the papain-like cysteine protease superfamily and most closely related to cathepsin family of proteases, silicateins exist in three isoforms: α, β and γ (Cha et al. 1999; Schröder et al. 2008; Shimizu et al. 1998). Silicateins exhibit cathepsin L-like proteolytic ability, as well as their main silica polymerase and esterase activity (Cha et al. 1999; Müller et al. 2003; Schröder et al. 2008; Shimizu et al. 1998). As an *in vitro* catalyst for silica polymerization, silicatein acts as an esterase, sequentially hydrolysing silicon alkoxides, allowing for condensation of the produced silanol groups (Fig. I. 14 A) (Schröder et al. 2007). As silica polymerase, silicatein is involved in sequential addition of silica monomers (Schröder et al. 2008). The *in vivo* catalytic function of silicatein is interlinked with a range of other components occurring in sponges, allowing Si(OH)₄ or even SiO₂ to be used as substrate for the enzyme (Müller et al. 2003). The catalytic properties of silicatein have been proposed to result from presence of the imidazole nitrogen of the histidine and the hydroxyl group of serine localized in the active site of the enzyme (Fig. I. 14 A), (Zhou et al. 1999). The hydrolytic activity of silicateins has been shown to be unspecific, as other metal oxides, such as TiO₂ and ZnO₂ have been precipitated using this enzyme (Bansal et al. 2004; Sumerel et al. 2003).
Fig. I. 14. Silicatein: A-Active silicatein enzyme. Cc-catalytic centre, signal sequence displayed in green. Serine (Ser) cluster amino acids marked in red (Ser26, His165 Asn185), (after Schröder et al., 2007). B-silicatein mediated hydrolysis of bis(p-aminophenoxy)-dimethyLsilane. Serine oxygen localized in the silicatein catalytic centre mediates a nucleophilic attack on the silicon, displacing the p-aminophenol. This is followed by formation of a (alkoxyl)-monosilane and facilitated by formation of a hydrogen bond between the imidazole nitrogen of Histidine (His) and Serines’ hydroxyl group. (after Müller et al., 2008).

An alternative hypothesis for the role of silicateins in spicule formation has been proposed by Ehrlich et al. (2008). In their model, silicateins are suggested to exhibit collagenolytic capacity similar to that of cathepsins. It is then proposed, that silicateins are involved in restructuring of collagen, forming templates on which silica is precipitated rather than being actively involved in silica deposition. Instead, silica precipitation is suggested to be a non-enzymatic self-assembly process, promoted by
the positively charged amine groups of collagen matrix (Ehrlich et al. 2008; Heinemann et al. 2007).

I.6.3. Silicase

Located both on the surface and within the axial filament of a growing spicule, silicase is a member of carbonic anhydrase family (Fig. I. 15. A) and catalyzes silica depolymerisation (Schröder et al. 2003). The reaction of silicase-mediated silica depolymerization (Fig. I. 15. B) proposed by Schröder et al. (2007) involves a reaction between Zn ion, acting as a Lewis acid and H$_2$O (Lewis base). Zn ion binds the hydroxide group of H$_2$O, and then facilitates a nucleophilic attack on the oxygen-bound Si. Subsequently, the zinc complex binds to the Si atom of the silica polymer, cleaving the oxygen bond and releasing Si(OH)$_4$ in a reaction requiring consumption of water for regeneration of the hydroxide group to continue the catalytic cycle.
Fig. I. 15. A - model of silicase: 3 histidine residues marked in red (His181, His183 His206), cc - catalytic centre bound to a Zn2+ ion. B - the proposed mechanism of Si polymer hydrolysis (after Schröder et al., 2007).

I.6.4. Silintaphins

Silintaphins 1 and 2 do not resemble any known proteins, apart from Silintaphin 1 possessing a pleckstrin homology domain (Wiens et al. 2009). Both proteins co-localize with silicateins inside and around spicules, controlling silica deposition via interaction with silicateins (Wiens et al. 2009; Wiens et al. 2011) Silintaphin 1 is thought to interact with silicatein to promote fibre formation and assembly into organized structures (Wang et al. 2012; Wiens et al. 2009). Silintaphin 1 was also reported to significantly increase the catalytic activity of the silicatein enzyme, as well as to promote non-enzymatic silica polycondensation (Schloßmacher et al. 2011). Silintaphin
2 exhibits Ca\textsuperscript{2+} binding affinity, and is suggested to facilitate silicatein interaction with Si substrate and signal transduction during spicule morphogenesis (Wang et al. 2012; Wiens et al. 2011).

I.6.5. Long chain polyamines

An interesting discovery was also made by Matsunaga et al. (2007), namely isolation of long chain polyamines (LCPAs) similar to those controlling biosilicification in diatoms. Sponge LCPAs were identified as oligomeric, methylated chains of polypropylamines present as sulphates or phosphates, attached to a butaneamine unit. It has been suggested that LCPAs of sponges facilitate silification in association with larger molecules, as their presence in spicules as well as in cellular extract has been verified.

I.7. Plants

Silicon in plants is an important quasi-nutrient accumulated in substantial amounts, ranging from 0.1-10% of dry weight (Epstein 1994). It is essential for growth of plants from the Equisetales order (Chen and Lewin 1969). Silicon has been shown to be an inductor of defensive responses via interaction with plant signalling system and increasing synthesis of defence compounds, enhancing their resistance to various pathogens (Callot 1992; Raven 1983). Si has also been implicated in alleviation of abiotic stress, such as dehydration, or uptake of toxic metals due to Si deposition
beneath the cuticle, decreasing cuticular transpiration, chelation and decreased uptake of toxic metals due to Si deposition in roots and as phytoliths (Hinsinger et al. 1992; Sangster et al. 2001). Si in plants also serves as a structural component, promoting cell elongation, enhancing the strength of the stem and maintaining leaves erect, contributing to improvement of light interception (Epstein 1994; Ma and Takahashi 2002). Si also seems to play a very important role in enhancing growth at different developmental stages in e.g. rice, where Si deficiency during the heading stage results in severe decrease in grain yield (Yamaji and Ma 2007).

I.7.1. Si transporters

Si(OH)₄ transport in plants is an active, energy requiring process (Mitani and Ma 2005). Expressed primarily in roots, plant Si transporters belong to the aquaporin gene family and are upregulated by the level of exogenous Si, diurnal pattern, and down regulated by stress caused by dehydration and abscisic acid (Hattori et al. 2003; Ma et al. 2006). Two coupled Si transporters, influx (Lsi1) and efflux (Lsi2) exist in the root cells and are responsible for Si transport into the stele for further distribution within the plant (Ma et al. 2006; Ma et al. 2007). Subsequently, located mainly at the leaf xylem parenchyma cells, Lsi6 (Lsi1 homologue) becomes responsible for Si distribution within the shoot and its unloading from xylem (Yamaji et al. 2008). Lsi1 belongs to a subfamily of Nod26 major intrinsic protein (NIPIII) unique to plants, and is localized within the plasma membrane of both endo- and exodermis of the root cells (Ma et al. 2006). Lsi2 is a proton gradient driven, energy dependent putative anion transporter bearing no resemblance to Lsi1 (Ma et al. 2007). Si transporters belonging to the NIPIII
subfamily were previously found in e.g. rice, wheat pumpkin and barley (Chiba et al. 2009; Ma et al. 2006; Ma et al. 2007; Mitani et al. 2011; Montpetit et al. 2012).

Another group of plant Si transporters described, NIP3s, belongs to NIPII protein group responsible for transporting of small, uncharged molecules (Grégoire et al. 2012). These transporters were found to be highly expressed in roots and shoots of a horsetail _Equisetum arvense_, where Si transporters were mainly localized to roots (Chiba et al. 2009; Grégoire et al. 2012; Ma et al. 2006).

NIP selectivity for Si(OH)$_4$ has been suggested to be linked with two highly conserved aspargine-proline-alanine motifs and four aminoacid residues of the aromatic/arginine substrate selectivity filter in the pore (Grégoire et al. 2012; Wu and Beitz 2007). All previously described NIPIII plant Si influx transporters are characterised by GSGR (glycine, serine, glycine, and arginine ) pore, whereas the NIPII NIP3s possess STAR (serine, threonine, alanine, arginine) pores, (Grégoire et al. 2012; Mitani-Ueno et al. 2011).

**I.8. SUMMARY AND AIMS**

In summary, in all taxa described in this review Si serves multiple functions, from structural component to regulation of gene expression (Thamatrakoln and Hildebrand 2007). In terms of transport, in all cases Si is taken up in a soluble form of silicic acid. Apart from that, the mechanisms of silicification described in this chapter were quite divergent, the plant system being entirely unique to this taxon. The sponge and diatom silicification systems bear little resemblance to each other. Sponges posses the ability to silicify both intracellularly in sclerocytes and extracellularly, upon the extrusion of the
young spicule into the mesohyl, whereas the entire biosilicification process in diatoms or chrysophytes is confined to an intracellular SDV (Sandgren 1983; Schröder et al. 2007; Schröder et al. 2006). Also, the principal components involved in silicification in those organisms function on a fundamentally different basis: in diatoms Si deposition proceeds via Si polycondensation by silaffins and LCPAs in the presence of polyanions, whereas in sponges silicification is mediated enzymatically by silicateins, or alternatively it is facilitated by collagen (Ehrlich et al. 2008; Ehrlich and Worch 2008; Schröder et al. 2006; Sumper and Brunner 2008). However, the detection of LCPAs in sponges suggested, that there might be more similarities between the two systems than it was initially anticipated, perhaps arising from the chemistry of Si, potentially promoting convergent evolution. An interesting suggestion was made by (Likhoshway et al. 2006), namely that the ability to silicify evolved simultaneously in different taxa around 600 MYA due to the high silicate content of the sea water, which could have facilitated the incorporation of Si into the metabolic cycles of organisms such as poriferans and chrysophytes, also implying that the system present in diatoms did not evolve on its own and was acquired from chrysophytes instead. Indeed, similarities in the cellular and molecular mechanisms of biosilicification (diatom-like SITs, the presence of an SDV) have been identified (Likhoshway et al. 2006).

The discovery of a silicifier in the calcifier-dominated haptophyte clade, *P. neolepis* (Yoshida et al. 2006) has also raised questions, about whether this system evolved *de novo*, was acquired via convergent evolution, or whether a horizontal transfer of genes from other silicifiers, e.g. diatoms has occurred. Recently, diatom-like SITs have also been detected in an opisthokont choanoflagellate (Marron et al. 2013) suggesting occurrence of horizontal gene transfer from SIT-possessing algae (e.g. diatoms) to choanoflagellates, which have previously been documented to acquire other algal genes.
in this manner (Marron et al. 2013; Nedelcu et al. 2008; Sun et al. 2010). It was further suggested by Marron et al. (2013) that biomolecules with a capacity to precipitate silica could exist in various taxa, however, capacity for biosilicification could only be attained, if Si-selective transport and concentration systems are in place, allowing Si substrate acquisition. Still, very little is known about the molecular mechanisms of silicification in different taxa, hence the theories proposed by both Likhoshway et al. (2006) and Marron et al. (2013) remain to be proven with more research.

Given the above information and a unique opportunity to investigate the sole silicifying representative of the haptophyte algae the main aims of this project are to address the following questions:

1. Why and how is *P. neolepis* silicifying?

2. How does the *P. neolepis* silicification system compare to calcification in coccolithophores and silica deposition systems in other taxa?

3. What is the evolutionary origin of the silicification system in *P. neolepis*?

These questions are further elaborated on and addressed in chapters II-V.
CHAPTER II: PRYMNESIUM NEOLEPIS: GENERAL ORGANISM DESCRIPTION, BIOLOGY AND PHYSIOLOGY OF SILICIFICATION

II.1. INTRODUCTION

Haptophytes are a group of mainly marine algae characterised by the presence of a unique flagellum-like appendage, in some cases used for prey capture: the haptonema (Kawachi et al. 1991). Haptophytes were estimated to have diverged from Stramenopiles ca. 824 MYA, with extant groups appearing ca. 543 MYA (Liu et al. 2010). Haptophytes are divided into two classes: Pavlovophyceae and Prymnesiophyceae. Pavlovophytes are represented by a single order, Pavloales, characterised by asymmetrical cell shape sometimes covered with knob-shaped organic scales/modified hairs and unequal (anisokont) flagella (Cavalier-Smith 1994; Green 1980; Green and Hori 1994). Prymnesiophytes are divided into six orders, of which four (Coccolithales, Isochrysidales, Syracosphaerales and Zygodiscales) have an ability to produce calcified scales (coccoliths) and two (Phaeocystales and Prymnesiales) do not calcify.

Diverged ca. 291 MYA the order Prymnesiales is represented by widely distributed, often toxic bloom forming flagellates such as Prymnesium and Chrysochromulina and an exclusive within the entire haptophyte clade, silica scale producing species (Fig. II.1. A-C) (Lundholm and Moestrup 2006; Simonsen and Moestrup 1997; Weissbach and Legrand 2012; Yoshida et al. 2006). This unique silicifier - Prymnesium neolepis - was previously known as Hyalolithus neolepis and was then reassigned into the Prymnesium genus based on additional molecular and taxonomical analyses (Edvardsen et al. 2011;
Yoshida et al. 2006). In contrast to the remainder of *Prymnesium* genus, comprising of highly motile flagellates with a flexible, non coiling haptonema, *P. neolepis* persists in a non-motile stage in culture (Fig. II. 1. A,C) (Green et al. 1982; Yoshida et al. 2006). Non-motile *P. neolepis* cells possess two short, quiescent flagella, a long, stationary haptonema and a characteristic silica scale cover overlying organic scales (Fig. II. 1. C) (Yoshida et al. 2006). The silica scales of *P. neolepis* are non-interlocking hat-shaped, with two apices, a recurved rim and regular perforations over the central part of the scale (Fig. II. 1. C). Both the silica scales and the significantly smaller, organic scales similar to those of closely related *Prymnesium* and *Chrysochromulina* sp. are produced intracellularly and then deposited outside the plasma membrane (Yoshida et al. 2006).

A motile phase in *P. neolepis* cultures was also observed. Motile cells are characterised by the presence of two long flagella and lack of silica scales. A cell cover consisting of organic scales of variable size, similar to those of closely related *C. polylepis* was observed instead (Yoshida et al. 2006). These flagellated cells appear in cultures maintained at 10 °C under oligotrophic conditions and frequently revert back to the non-motile, silicified phase (Yoshida et al. 2006). It is currently unknown whether the non-motile stage is also dominant under environmental conditions, however, silica scales and whole silicified cells of the non-motile *P. neolepis* stage have been reported sporadically in samples from all over the world (Yoshida et al. 2006).
II.1.1. Putative capacity for silicification in Prymnesium sp.

Earlier, preliminary reports on *Prymnesium* sp. cyst formation in culture indicated, that cyst wall associated organic scales are covered with silica-containing fibrous material on their distal faces, hinting, that *Prymnesium* sp. might have a capacity to silicify (Fig. II. 2. A,B) (Green et al. 1982; Pienaar 1980; Pienaar 1981). The presence of Si-containing fibrous deposits was suggested to be linked with small, oval bodies localized immediately below the plasma membrane and occasionally associating with organic scales (Pienaar 1980). However, the presence of Si detected in the cyst wall of...
*Prymnesium* in this case could be an artefact of culturing conditions and will be discussed later in this chapter.

![Electron micrographs of Prymnesium sp.](image)

Fig. II. 2. Electron micrographs of *Prymnesium sp.*: A - organic scales with electron dense, fibrous, Si containing material (arrows), B - whole cyst (arrow: cyst wall composed of organic scales with fibrous deposits). Images modified from Pienaar (1980).

This chapter presents studies of the physiology of silica scale production as well as the sequence of events involved in the formation of individual silica scales. The original morphological description of *P. neolepis* by Yoshida et al. (2006) is also supplemented with information on some biological and biochemical properties of this alga. Comparisons in terms of cell morphology and scale formation with other members of *Prymnesium*, closely related *Chrysochromulina* and biomineralizing coccolithophores and diatoms are made. This chapter also contributes the basis for further investigations undertaken in this work.
II.2. METHODS

All chemical reagents used in this investigation were obtained from Sigma-Aldrich, unless otherwise specified.

II.2.1. Culture conditions

*Prymnesium neolepis* strains TMR5, PZ241 and VF28 were obtained from Roscoff Culture Collection and were originally isolated from the Mediterranean Sea (PZ241, VF28) and from around Japan (TMR5). A diatom *Odontella sinensis* strain PLY624 and a coccolithophore, *Coccolithus pelagicus ssp braarudii* strain PLY182g were obtained from the Plymouth Culture Collection of Marine Microalgae. Algae were maintained in aged filtered sea water (FSW) supplemented with f/2 media (Guillard and Ryther 1962) under irradiance of 80-100 µmol/s/m², at 18 °C for *P. neolepis* and 15 °C for the remaining strains. Additionally, *P. neolepis* and *O. sinensis* were supplemented with 100 µM silicate. Photoperiod used for maintenance of *P. neolepis* was set to 18:6 h light:dark and at 12:12 light:dark for other algal species. Dilute batch cultures were maintained in glass conical flasks, as cells would not grow well in plastic vessels and cultures reaching densities ≈10^5 cells/ml would rapidly collapse. Culture health and density were periodically monitored under a light microscope. Cell counts were done using a Sedgewick-Rafter cell counting chamber. Three samples per experimental repeat were aliquoted and counted.

Artificial Sea Water (ASW) used in high/low silica experiments was prepared according to a recipe by Dr. Alison Taylor (Table II. 1.). Chemicals were dissolved in distilled
water, autoclaved and 0.22 µm filtered NaHCO₃ was added after the solution has cooled.

Table II. 1. Chemical composition of ASW.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Concentration [mM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>450</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>30</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>16</td>
</tr>
<tr>
<td>KCl</td>
<td>8</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>10</td>
</tr>
<tr>
<td>HEPES</td>
<td>20</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>2</td>
</tr>
</tbody>
</table>

II.2.2. DNA extraction, gene amplification, sequencing and molecular analysis

DNA extractions were made using DNAeasy blood and tissue Kit (Qiagen) following the manufacturer's instructions, or a standard phenol/chloroform protocol. Small subunit rRNA gene (18S, SSU) and the large subunit of the ribulose-bisphosphate carboxylase (rbcL) marker genes were amplified using universal primers (Table II. 2.) and then sequenced for three P. neolepis strains: TMR5, VF28 and PZ241.

PCR conditions for gene amplification were as follows: 35 cycles, each at 95 °C for 5 min, 95 °C for 30 s, and 1 min at 56 °C for rbcL and 58 °C for 18S, followed by 72 °C for 1 min. Prior to sequencing, PCR products were treated with ExoSapit (Affymetrix) according to manufacturer's instructions to remove excess primers, dNTPs, and single
stranded DNA. Cleaned PCR products were then amplified again (PCR cycle: 96 °C for 1 min, 96 °C for 10 s, 55 °C for 5 s, individual annealing temperatures as specified in PCR conditions above for 4 min) and sent off for sequencing, performed by Source Bioscience (Cambridge).

Table II. 2. Primers used for amplification of SSU, LSU and rbcL genes.

<table>
<thead>
<tr>
<th>Loci</th>
<th>Primers</th>
<th>Primer Sequence 5’-3’</th>
<th>Amplicon size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>rbcL</td>
<td>090F</td>
<td>CCATATGC(CT)AAAATGGGATATTGG</td>
<td>673 (Yoon et al. 2002)</td>
<td></td>
</tr>
<tr>
<td>rbcL</td>
<td>770R</td>
<td>ATACATTCTTCCATAGTTGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SSU</td>
<td>A18 DIR</td>
<td>AACCTGGTGATCCTGCCAGT</td>
<td>1688 (Medlin et al. 1988)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A18 REV</td>
<td>TCCTTCTGCAGGTTTCACCTAC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

A phylogenetic tree based on the rbcL gene and including haptophyte representatives was constructed to ascertain that the strains in culture were indeed *P. neolepis*. Additionally, an SSU-based tree including *P. neolepis*, other haptophytes and representatives of heterokontophyte silicifiers was produced. In both cases trees have been constructed with a neighbour-joining method using Kimura 2-parameter model to compute evolutionary distances and with maximum likelihood method, using Kimura 2-parameter gamma distributed with invariant sites models in MEGA5 software (Kimura
1980; Tamura et al. 2011). A bootstrap of 1000 replicates was used in both cases. SSU and rbcL sequences of *P. neolepis* and relevant haptophytes and heterokontophytes were aligned with MAFFT (Multiple Alignment using Fast Fourier Transform) online utility. *P. neolepis* rbcL sequence alignment along with additional sequences obtained from GenBank and used for rbcL and SSU alignments were supplemented as electronic appendices 1-3.

### II.2.3. Confocal microscopy

Silica scale formation was tracked with a fluorescent probe, LysoTracker yellow HCK-123 (Invitrogen), visualised with argon laser at $\lambda=488$ nm excitation, and emission collected between $\lambda=500$-550 nm. Chlorophyll autofluorescence was collected simultaneously between $\lambda=650$-710 nm. Calcite was imaged with a He/Ne laser with $\lambda=633$ nm excitation and emission filter set between 650-700 nm. FITC-Concanavalin-A (Invitrogen) used for targeting polysaccharides and binding to $\beta 1,3$- and $\beta 1,4$-linkages between glucose and mannose backbone (Mandal et al. 1994) was imaged using an argon laser, with excitation and emission filters set to $\lambda=488$ nm and $\lambda=500$-550 nm respectively.

LysoSensor Yellow/Blue DND-160 (PDMPO, Invitrogen) also used for tracking the new silica scale deposition and silica scale reflectance were imaged using multiphoton excitation with a Mai Tai pulsed infra-red laser (Spectraphysics) set to $\lambda=740$ nm. Emission was collected between $\lambda=435$-485 nm and $\lambda=500$-550 nm for PDMPO and with a 560 nm long pass filter for silica scale reflectance. All imaging was carried out on a Zeiss LSM 510 meta confocal laser scanning microscope.
Time lapses: time frames were taken every 4 mins for time-lapse 1, and at 150 s intervals for time-lapse 2. Time frames for time-lapses 3, 4 and 5 were taken every 45 s. List of all time-lapses with their brief description is attached in appendix II.1. All time-lapses were supplied as an electronic appendix.

II.2.4. Differential Interference Contrast (DIC)/light microscopy imaging

DIC images were taken with a Nikon Eclipse Ti microscope equipped with a Photometric Evolve EMCCD camera. Images were taken every 45 s for all time lapses. Transmitted light imaging was done using a Nikon Diaphot 300 microscope with an attached Photometrics CoolSnapEZ CCD camera.

II.2.5. NaOH etching of biosilica material

*P. neolepis* biosilica purified with the KMnO₄ method detailed in section II.2.6. and was subsequently etched with 0.5-10 M NaOH for a period of 4-6 h to gain insights into silica deposition pattern in silica scales. Samples were then washed 3x with deionised water and kept at -20 °C until SEM analysis.
II.2.6. Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM) and Energy Dispersive Microanalysis (EDX)

Samples were filtered onto a 0.45 µm filter which was dried or placed directly onto the stub, sputter coated with gold or chromium for imaging and with carbon for EDX analysis. Imaging was carried out using a JEOL JSM-7001F Field Emission Microscope at 5-15 kV accelerating voltage. Images were acquired with JEOL JSM-6610LV and JEOL JSM 5600 Scanning Electron Microscopes at 15 kV. EDX analysis was carried out using a JEOL 6100 Scanning Electron Microscope and with JSM-7001F Field Emission Microscope at 7-15 kV, using Oxford instruments Inca and Aztec X-Ray micro-analysis suites respectively.

TEM imaging of non-mineral material on copper grids (Agar) stained with 2% uranyl acetate for 30 s was carried out using a JEOL 1400 Transmission Electron Microscope.

II.2.7. Fourier Transform Infrared Spectroscopy (FTIR)

Biosilica samples from *P. neolepis* and *O. sinensis* were purified using an SDS/EDTA treatment as detailed in section IV.2.1. and analyzed with a Bruker IFS-66 FT-IR spectrometer attached to a Bruker Hyperion 1000 microscope operating in transmission mode. Spectra were recorded between 600 cm\(^{-1}\) and 4000 cm\(^{-1}\) at 4 cm\(^{-1}\) resolution, with 32 scans/spectrum. The baseline was corrected using the "rubberband correction" method with a standard preset, included in Bruker OPUS software. Silica gel was obtained by adding 0.5 M sulphuric acid to a saturated solution of sodium metasilicate. Gel was then washed 3x with deionised water and dried in a flow hood prior to FT-IR analysis.
II.2.8. Light: dark experiment: flow cytometry assessment of silica scale production

1 ml of concentrated exponentially growing culture (density between 1-1.5x10^5 cells/ml) was added to 4 ml of fresh f/2 media in FSW at pH 8.20, with 100 µM Si supplement. Cells were then labelled with HCK-123 (1 µM) and incubated in light at 100 µmol/s/m^2 and in dark for 10 h. Cells were then harvested by centrifugation and washed 3x with 2% SDS with 0.1 M Tris/EDTA (pH 8.00), and incubated for 15 mins with intermittent vortexing to remove cellular debris. Samples were then pelleted with a bench top centrifuge at max rpm, and washed 3x with deionised water. Samples were analysed using an Accuri C6 flow cytometer set to record side scatter and green fluorescence (SSC vs. FL1A), gated for fluorescent and non-fluorescent particles. Experiments were repeated five times in triplicate. Ratios of fluorescent (new) vs. non-fluorescent (scales produced prior to the treatment), serving as a proxy of silicification in treatments were compared using a student's t-test for independent samples (Statsoft: Statistica).

II.2.9. High/Low Si experiment

Initial attempts to grow *P. neolepis* in low silica (< 3 µM, below quantification level with the silicomolybdate assay) ASW in batch cultures were unsuccessful. No growth was observed and within a few days of the start of the experiment cells could no longer be found in culture upon examination with light microscopy. Therefore, a short-term experiment aimed to investigate *P. neolepis* response to low Si conditions was performed. Cells grown in FSW f/2 media without silicate enrichment were settled in glass bottom petri dishes (5 control and 5 low Si dishes in total) and washed 5x with
low Si (< 5 µM) f/2 ASW. Media were then replaced with 100 µM Si and f/2 ASW for control dishes and low Si f/2 ASW for the low Si experiment. Dishes were then incubated for 18h with 1 µM PDMPO at 18 °C with a 16:8 h light:dark cycle at 150 µmol/s/m². After the period of incubation, 10 images/dish were taken for estimation of cell density. 1ml sample was aliquoted from each dish for subsequent determination of Si concentration in the media post incubation (refer to section II.2.10. for detailed method description). Cells were then lysed with 0.5 ml of 10% H₂O₂ and left for 20 min to allow the silica scales to settle on the bottom of the dish. 10 pictures/dish were taken for estimation of silica scale production. Imaging was carried out using a Zeiss Confocal microscope as specified in section II.2.3.

Images were then processed with Image J software by thresholding grey values to remove background fluorescence and select for values above a certain threshold, corresponding to scale fluorescence. This allowed calculation of the number of particles and the total area of particles in each image, an average number of scales per cell and average scale area/cell.

II.2.10. Si concentration determination: silicomolybdate assay

Silica concentrations were determined following a colorimetric method modified from Strickland and Parsons (1968). This assay relies on formation of a silicomolybdic acid complex via reaction of ammonium heptamolybdate with silicic acid. This complex is subsequently reduced to eliminate interference from phosphorous, resulting in formation of molybdenum blue complex, allowing absorption corresponding to silica concentrations to be measured (Coradin et al. 2004; Strickland and Parsons 1968).
Samples for the analysis were prepared as follows: 0.3 ml of the molybdate reagent was added to a 0.75 ml sample. Sample was then vortexed and incubated for 10 min at room temperature. Next, 0.45 ml of the reducing reagent was added and the sample was vortexed. The sample was then incubated for 3 h with occasional vortexing. Absorbance at 810 nm was then determined with a Varian Cary 100 Bio UV-Visible Spectrophotometer. Calibration curves were based on a series of dilutions of standard silicate solution in synthetic seawater (1/1000, 1/500, 1/100, 1/50) and distilled and synthetic seawater blanks, all set up in 4 replicates. Reagents used in the analysis are listed in Table II. 3.

Table II. 3. List of reagents used in the silicomolybdate assay and their composition.

<table>
<thead>
<tr>
<th>Solution/volume</th>
<th>Chemicals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molybdate reagent</td>
<td>4 g (NH₄)₆Mo₇O₂₄·4H₂O, 12 ml 12 M HCl</td>
</tr>
<tr>
<td>(500 ml)</td>
<td></td>
</tr>
<tr>
<td>Reducing reagent</td>
<td>10 ml of metol-sulphite solution (6 g Na₂SO₃, 10 g Metol,</td>
</tr>
<tr>
<td>(30 ml)</td>
<td>filtered), 6 ml oxalic acid at 71.44 g/l, 6 ml 0.5 M H₂SO₄</td>
</tr>
<tr>
<td>Synthetic Seawater</td>
<td>25 g NaCl, 8 g MgSO₄·7H₂O</td>
</tr>
<tr>
<td>(1 l)</td>
<td></td>
</tr>
<tr>
<td>Silicate standard</td>
<td>0.96 g Na₂SiF₆</td>
</tr>
</tbody>
</table>
II.2.11. *Ge supplementation of P. neolepis cultures and biosilica material purification for EDX analysis*

Cultures were set up in triplicate and maintained as specified in section II.2.1., in f/2 supplemented filtered sea water with 100 µM Si enrichment in the control and 50 µM Si with 3 µM Ge or 50 µM Ge without Si supplementation in test cultures. Growth was monitored during the experiment, and once cell counts were no longer possible due to cells aggregating together, cultures were harvested and biosilica material was purified for SEM analysis as specified below.

Cell culture pellets were treated with KMnO₄/HCl, following a modified method for diatom frustule cleanup by Diana Sarno, obtained during a phytoplankton identification course hosted by the MBA and SAHFOS. *P. neolepis* pellets were washed 3x with deionised water, resuspended in 500 µl of deionised water and transferred to 3 ml glass bottles. 0.5 ml of saturated KMnO₄ was then added, the bottle contents were vortexed and left to incubate at room temperature overnight. Solutions were then transferred to glass test tubes, placed in a heating block at 90 ºC and 2 ml of concentrated HCl was added. Samples were then boiled until the colour changed to clear yellow. Samples were transferred to 50 ml tubes containing 45 ml of deionised water to neutralise them and centrifuged for 20 min at 6000x g. Pellets were washed 3x with deionised water and stored at -20 ºC until analysis with SEM and EDX as specified in section II.2.6.

Additionally, *P. neolepis* responses to 10, 20 and 50 µM Ge supplementation were monitored using fluorescent dye HCK-123, and visualised with a confocal microscope as specified in section II.2.3. For this experiment, samples were set up in duplicate on glass bottom dishes, incubated for 18 h with Ge concentrations specified above and
maintained in conditions as specified in section II.2.1. Control samples were supplemented with 100 µM silicate.

II.3. RESULTS

II.3.1. Molecular identification of P. neolepis strain

To ensure, that the newly isolated strains in culture were in fact *P. neolepis*, amplification and sequencing of 2 marker genes: *rbcL* (all three strains) and of SSU gene for strain TMR5, has been carried out. Phylogenetic analysis of sequences obtained from *P. neolepis* in this study, and those downloaded from GenBank confirmed, that all three strains examined (TMR5, VF28 and PZ241) are in fact *P. neolepis* (Fig. II. 3., Fig. II. 4.). Although all strains are strongly clustered together with a strong bootstrap support, small differences between strains on both *rbcL* and SSU genes have been recorded (Table II. 4.). These are likely due to different geographical locations of sites from which the sequenced strains were collected. Strains PZ241 and VF28 were originally collected from the Mediterranean Sea, and are identical on the *rbcL* gene, whereas TMR5 and NIES1393, both collected around Japan are different from the Mediterranean strains and less dissimilar between each other (Table II. 4., Fig. II. 3.).
Table II. 4. A summary of the degree of homology between rbcL and SSU gene sequences obtained from *P. neolepis* strains isolated from different locations.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Gene</th>
<th>% Identity</th>
<th>Strain</th>
<th>Collecton Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMR5 vs. PZ241</td>
<td>rbcL</td>
<td>99.85</td>
<td>TMR5</td>
<td>Japan</td>
</tr>
<tr>
<td>TMR5 vs. VF28</td>
<td>rbcL</td>
<td>99.85</td>
<td>NIES1393</td>
<td>Japan</td>
</tr>
<tr>
<td>TMR5 vs. NIES1393</td>
<td>rbcL</td>
<td>99.26</td>
<td>PZ241</td>
<td>Mediterranean Sea</td>
</tr>
<tr>
<td>PZ241 vs. VF28</td>
<td>rbcL</td>
<td>100.00</td>
<td>VF28</td>
<td>Mediterranean Sea</td>
</tr>
<tr>
<td>PZ241 vs. NIES1393</td>
<td>rbcL</td>
<td>99.11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VF28 vs. NIES1393</td>
<td>rbcL</td>
<td>99.11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TMR5 vs. NIES1393</td>
<td>SSU</td>
<td>98.95</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The phylogenetic position of *P. neolepis* strains examined in this study is consistent with that described by Edvardsen et al. (2011) and the previous study by Yoshida et al. (2006), placing *P. neolepis* within the order *Prymnesiales* of the haptophyte clade (Fig. II. 3.). The distant relationship between *P. neolepis*, clustering within the haptophyte lineage and silicifiers of the heterokontophyte lineage is depicted in Fig. II. 4.
Fig. II. 3. Unrooted maximum likelihood phylogenetic tree of selected haptophytes based on the \textit{rbcL} gene sequences. Numbers at nodes indicate bootstrap support. Branches marked with: \textcolor{red}{\textgreater} 90\% and \textcolor{green}{\textgreater} 70\% bootstrap support from both maximum likelihood and neighbour joining methods. Isoc.=Isochrysidales. \textit{Prymnesium neolepis} grouping marked with a red bracket. Scale bar indicates a distance of 0.05 substitutions per site.
Fig. II. 4. Unrooted maximum likelihood phylogenetic tree constructed for haptophytes and heterokontophyte silicifiers based on SSU rRNA sequences. Numbers at nodes indicate bootstrap support. Branches marked with: 🔺 > 90% and 🔻 > 70% bootstrap support from both maximum likelihood and neighbour joining methods.

Bacill.=Bacillariophyceae. P. neolepis strains marked with a red bracket. Scale bar indicates a distance of 0.05 substitutions per site.
II.3.2. *P. neolepis*: general morphology

All morphological and experimental analyses were conducted on *P. neolepis* strain TMR5 as it proved to be the easiest one to maintain in culture. In order to gain general information on *P. neolepis* morphology and cell properties, various microscopy techniques, enabling immediate and continuous observations have been employed.

*P. neolepis* strain TMR5 cells are spherical to ellipsoidal, on average 11 µm in diameter (n=25, SD=1.39). Cells are strongly polarized and covered with a layered silica scale covering, which can exceed 30 µm in diameter (Fig. II. 5. A,B). The posterior, non-flagellar pole of the cell is vacuolar, devoid of pigment and characterized by a distinct, rough surface covered in "bumps" (Fig. II. 5. A,B, see Fig. II. 7.). The posterior pole also contains the developing scales (Fig. II. 8. A,B, Time-lapses 1,2). The anterior part of the cell is occupied by a large chloroplast with finger-like projections, and contains most of the main cell organelles (see Fig. II. 7., Yoshida et al., 2006). The haptonema and two short flagella are quiescent and protrude from the centre of the chloroplast (Fig. II. 7.).
Fig. II. 5. *P. neolepis*: A - light microscopy image of a naked and silicified cells (haptonema marked with an arrow), B - DIC image with marked posterior pole of the cell (small arrow) and haptonema (thick arrow), C - rhizopodial-amoeboïd cell. A - scale bar corresponds to 5 µm, B,C scale bars=10 µm.

Time-lapse DIC observations of *P. neolepis* revealed, that spheroid cells exhibit frequent metaboly, especially at the posterior, non-flagellar pole of the cell (Time-lapse 3,4). They are also able to assume amoeboid shape and move by crawling (Fig. II. 5. C). Cells can subsequently return to their usual, spheroid form (Time-lapse 3). The cues for this shape transition have not been identified, however, amoeboid cells were observed to occur more frequently in cultures in stationary to senescent phases.

Cells characterised by the presence of 2 haptonemas were also encountered (Fig. II. 6. A,B). These were found to be capable of phagocytosis via pseudopodium-like protrusion emerging from the posterior pole of the cell, preying on their conspecifics (Time-lapse 5). This cell type was found to occur more frequently in cultures reaching stationary phase. Additionally, cells can almost cease to silicify in culture due to unknown factors. Increased number of naked or poorly silicified cells was observed in cultures in stationary phase, which was probably related to a decrease in Si availability for uptake from the media. Late exponential and senescent phases in *P. neolepis*
cultures are also characterized by cells aggregating together into palmelloid assemblages lodged in a mucous-like material.

Fig. II. 6. DIC images of \( P. \text{neolepis} \): A - cell with two haptonemas. B - double haptonema cell feeding on remnants of another \( P. \text{neolepis} \) cell (Time-lapse 5). Scale bars=10 µm.

II.3.3. In-vivo visualization of silicification in \( P. \text{neolepis} \): silica scale formation and exocytosis

Intracellular silica polymerization has previously been visualized in organisms such as diatoms and sponges using two fluorescent probes: [2-(4-pyridyl)-5-[[4-(2-dimethylaminoethylaminocarbamoyl)methoxy]phenyl]-oxazole] (PDMPO) and LysoTracker Yellow HCK-123 (HCK-123) (Schröder et al. 2004a; Shimizu et al. 2001). PDMPO is an acidotropic fluorescent pH indicator with dual excitation and emission.
dependent on the pH, emitting strong green fluorescence in the presence of polysilicic acid (Diwu et al. 1999; Shimizu et al. 2001). Like PDMPO, HCK-123 is a dye selectively accumulating in acidic cellular compartments, such as the SDV, and is incorporated into the newly forming silica structure (Desclés et al. 2008). In this study, these two fluorescent probes were successfully adapted for labelling the silica in a haptophyte *P. neolepis*, enabling detailed investigation of the localization and mechanism of silica scale formation (Fig. II. 7.).

![Fig. II. 7. Confocal microscopy 3D projection of a Z-stack of *P. neolepis* cells with HCK-123 labelled scales (green) accumulated at the posterior pole of the cell. Note haptonemases (arrows) projecting from the middle of the finger-like chloroplast (red) at the cells' anterior. Scale bar=5 µm.](image-url)
Confocal microscopy investigations in conjunction with silica-labelling fluorophores revealed that cells maintain between 2-3 scales intracellularly at one time and secrete them sequentially at the posterior pole of the cell (Time-lapses 1, 2, 3, Fig II. 8. A,B). Silica scales are always maintained with the concave side of the scale directed towards the cell membrane when in the cell (Fig. II. 8. A,B, Fig. II. 9., Time-lapse 1).

Fig. II. 8. 3D projections of Z-stacks of *P. neolepis* collected with a confocal microscope, showing cell polarity relative to the site of silica scale production:
A - PDMPO-labelled cell with 2 intracellular scales (green, arrows) localized in the vacuolar, posterior region of the cell (chlorophyll autofluorescence in red). B - tubulin immunofluorescence labelled cell with visible flagella (small arrows) and the haptonema (thick arrow) at the anterior pole of the cell and HCK-123 labelled intracellular scales (green) localized on the posterior pole of the cell (see sections III.2.3. and III.2.7. for methodology pertaining to tubulin immunolabelling and imaging). Scale bars= 5 µm.
To further investigate the mechanism of scale secretion, time lapse DIC microscopy observations were made. These revealed, that scale liberation is preceded by the mature scale migrating close to the cell membrane at the posterior, vacuolar part of the cell. Then, the cytoplasm filling the concave part of the scale is moved to the side, forming an invagination, to allow the scale to break through the membrane with one side of the rim. This phase is accompanied by localized protoplast contractions. The scale is then pushed out of the cell at a slight angle and inverted, so that the concave side of the scale is again directed towards the cell membrane. Scale secretion events were captured in Time-lapse 4 and summarized in Fig. II. 9. Time between scale secretion events was highly variable (from 6-78 min) and averaged at 39 min (n=22, SD=17.99). Additionally, some cells do not silicify for prolonged periods of time. The averaged time between secretion events obtained can be potentially skewed towards longer intervals due to scales exiting the cell at different points, sometimes out of focus and therefore impossible to capture.

Fig. II. 9. Time course of a silica scale secretion event: 0 - mature scale migrating towards the cell membrane (arrow), 10 - removal of cytoplasm and forming of an invagination, 12- early stage of scale exocytosis starting from the rim of the scale, 14 - scale further pushed out at an angle relative to the plasma membrane, 15-inversion of the liberated scale, 17 - scale positioning on the cell surface bar=5 µm. Time indicated in minutes at the top of each image.
The sequence of events during silica scale formation was further examined with SEM imaging of purified *P. neolepis* biosilica, visualising scales captured at different developmental stages. The first stage of the formation appears to be deposition of a ring structure delimiting the size of newly forming scale (Fig. II. 10. A). Subsequently, thread-like structures projecting from the rim are formed, and give rise to a number of loop-like structures (Fig. II. 10. A). Deposition of loophole structures continues until the internal part delimited by the initial ring structure is densely packed (Fig. II. 10. B). This stage is also accompanied by thickening of the original ring structure, forming the rim of the scale. The last stage of the silica scale growth appears to be deposition of an additional layer of silica on top of the whole structure, decreasing pore size arising from previously deposited loops and sealing gaps between them (Fig. II. 10.).

Fig. II. 10. Silica scales at different developmental stages: A-basal rim with loops beginning to form, B-scale with secondarily thickened rim and most of the loops formed, C-complete scale. Scale bars=1 µm.
To ensure that these findings were not artefacts of abnormal scale formation, slow etching of silica scales with alkali was applied. SEM imaging of etched biosilica samples corroborated the existence of thread-like precursor structure anchored to the rim of the scale, and forming loophole pattern underlying pores present in the convex side of the scale (Fig. II. 11.).

Fig. II. 11. SEM images of a *P. neolepis* biosilica sample etched with 0.5 M NaOH for a period of 6 h, revealing loophole pattern projecting from the rim towards the centre of the scale (A) and the primary ring structure underlying the rim of the scale (B). Images were collected at different locations within the same sample.

**II.3.4. Morphological observations of silica scales**

Silica scales produced by *P. neolepis* varied in shape from elliptical to round, with two apices on the convex side and a re-curved rim (Fig. II. 12., inset). Additionally, scales were often bent around the longitudinal axis to a variable degree, much like thin potato crisps. Average scale dimensions measured 6.8 x 4.4 μm (n=100, SD=1.58 and
SD=0.88 for length and width respectively). Individual scales varied greatly in size, with scale length and width ranging from 2.8-14.6 µm and 2.1-6.7 µm respectively.

Fig. II. 12. SEM image of KMnO4/HCl cleaned P. neoelpis silica scales. Inset: TEM cross-section of an individual scale: scale rim indicated by black arrows, two apices on the convex side marked with red arrows. TEM sections of P. neolepis scales were kindly supplied by Mr. Glenn Harper (University of Plymouth).

*P. neolepis* does not appear to arrange scales in any specific pattern, and the loose, non-interlocking and non-adhering sphere of scales surrounding the cell arises most likely from random protoplast rotations in suspension (Fig. II. 13. B). If the cell is stationary, i.e. resting on a flat surface, scales accumulate at the posterior pole of the cell (Fig. II. 7.). Cells secrete scales continuously and accumulate multiple layers of scales, often thicker at the posterior, secretory pole of the cell. The loose silica scale-cell association
is easily perturbed by pipetting of media or stirring, which often results in the scale covering being partially or completely removed (Fig. II. 13. A).

![Image of P. neolepis cell covering](image)

Fig. II. 13. *P. neolepis*: A - polarized light micrograph of a cell with a disturbed scale cover. B - confocal microscopy 3D reconstruction of a cell with uniformly distributed scale cover (white: reflectance of silica scales, red: chlorophyll autofluorescence). Scale bars=5 µm.

II.3.5. *Non-biomineralised elements of P. neolepis cell covering*

Previous electron microscopy investigations on *P. patellifera* and *P. parvum* detected a layer of material deposited on top of the plasma membrane, underlying the organic scale layer, which was suggested to be composed of glycocalyx (Green et al. 1982). Similarly, coccolith bearing haptophytes possess a layer of columnar (fibrillar) material overlying the plasma membrane, suggested to play a role in securing the biomineralised elements to the cell surface (Drescher et al. 2012; Rowson et al. 1986; Taylor et al.
2007). Additionally, coccoliths and entire coccospheres have previously been shown to be coated with polysaccharide-containing organics, labelling with a fluorophore conjugated lectin - Concanavalin A (Con-A) (Fig. II. 14. C,D) (Godoi et al. 2009; Hirokawa et al. 2005; Marsh 2003). Therefore, Con-A was used as a preliminary investigative tool to probe the loosely associated silica scale cover for the presence of potentially polysaccharide-containing matrix.

Con-A labelling and subsequent imaging of *P. neolepis* with a confocal microscope revealed that a thick, on average 1.8 µm layer (n=30, SD=0.8) of potentially polysaccharide, mannose-containing material is indeed covering the cell (Fig. II. 14. A,B). As the silica scale covering does not adhere closely to the plasma membrane and is not uniform in thickness, this material is proposed to provide a matrix, in which layers of organic and silica scales are lodged, therefore maintaining the loose, non-interlocking scale covering around the cell (Fig. II. 13. B). Unlike calcified coccoliths of coccolithophores, silica scales making up the cell cover in *P. neolepis* are not covered with this material (Fig. II. 14. A-C). However, scales which have been shed often display strong fluorescence upon Con-A labelling, suggesting that they can be coated secondarily (Fig. II. 14. A, arrows).
Fig. II. 14. 3D projections of confocal microscopy Z-stacks of Concanavalin-A labelled cells of *P. neolepis*: A - 3D reconstruction with labelled detached scales (arrows), B - individual slice showing irregularities and partial detachment of the Con-A labelled cell coating. C - decalcified *C. pelagicus* cells with holes in polysaccharide covering marking spaces where coccoliths were attached, D - individual *C. pelagicus* coccoliths. *P. neolepis* was additionally labelled with PDMPO (in blue) to visualize cell body. Con-A is shown in green, chlorophyll autofluorescence in red. Scale bars=5 µm.

In addition to silica scales, *P. neolepis* also deposits much smaller organic scales, previously reported to form a layer under the silica scales (Yoshida et al. 2006). Organic scales produced by *P. neolepis* strain TMR5 were ellipsoidal, with serrated edges which
were absent from organic scales of strain NIES 1393, and radially arranged ridges (Fig. II. 15. A,B). Organic scales were on average 1.06 µm in length and 0.95 µm in width (n=10, SD=0.13, 0.1 for length and width respectively). The imaging resolution did not allow for any further investigation into additional patterning potentially marking scales, and was not pursued any further.

![Fig. II. 15. A,B-TEM images of uranyl-acetate stained organic scales with a clearly visible radial ridge pattern. B-scale with prominent serrated edges. Scale bar=1 µm.](image)

**II.3.6. FT-IR characterization of chemical structure of P. neolepis biosilica**

In order to obtain information on the structure of biosilica produced by *P. neolepis* and to enable comparisons with the dominant silicifiers, diatoms, Fourier transform infrared spectroscopy (FT-IR) has been applied. This tool was also used to obtain preliminary information on the presence of organic matter potentially occluded within the purified silica scales. FT-IR transmission spectra of biosilica produced by *P. neolepis* and a
diatom, *O. sinensis* were collected and compared against each other and a poorly condensed silica gel. As expected, all three spectra exhibited peaks characteristic of amorphous, hydrated silica.

The FT-IR spectrum of biogenic amorphous silica can be divided into two main regions: (1) > 2500 cm\(^{-1}\), with the main broad peak between 2800-3800 cm\(^{-1}\) corresponding to OH stretching vibrations of adsorbed/molecular H\(_2\)O, (2) a region < 1300 cm\(^{-1}\), representing the major vibrational modes of silica, detailed in Fig. 16. Based on the shape of the peak around 1100 cm\(^{-1}\) (peak 3, Fig. II. 16.), silica gel represented the least and *P. neolepis* biosilica represented the most ordered amorphous silica structures (very sharp peak compared to that of *O.sinensis* and silica gel) (Chukin and Malevich 1977).
Fig. II. 16. FT-IR transmission spectra of purified *P. neolepis* (red), *Odontella sinensis* (black) biosilica and a silica gel (green). Peaks are assigned as follows: 1:~800 cm$^{-1}$ symmetric Si-O bond stretching (Stolen and Walrafen 1976), 2:~960 cm$^{-1}$ Si-OH vibrations, OH vibrates as a single mass, antisymmetric Si-O stretching of O$_3$SiOH (Hartwig and Rahn 1977; Stolen and Walrafen 1976). 3,4: 1000-1400 cm$^{-1}$-a peak with a shoulder corresponding to deformation vibrations of the SiOH hydroxyl, 5-1538 cm$^{-1}$ amide II band (Wellner et al. 1996), 6:~1643 cm$^{-1}$ H-O-H bending-molecular water deformation (Davis and Tomozawa 1996; Little 1966). 7-absorption bands between 2800-3000 cm$^{-1}$ assigned to organic matter ($\delta$ CH aliphatic) (Landais et al. 1993), 8: ~2800-3800 cm$^{-1}$, OH stretching of the adsorbed/molecular water (Chukin and Malevich 1977).

No peaks in the FT-IR spectrum of *P. neolepis* biosilica at wave numbers corresponding to vibrations of groups indicative of the presence of organic fraction, as visible around...
2800-3000 cm$^{-1}$ (CH stretching bands) and the amide II band at 1538 cm$^{-1}$ in biosilica isolated from *O. sinensis* were observed (Fig. II. 16., peaks 5,7). However, subsequent dissolution of purified silica scales with acidified ammonium fluoride revealed presence of associated soluble and insoluble organics (see sections IV.3.4. and IV.3.8. for details).

**II.3.7. Physiology of silicification in *P. neolepis***

The main questions regarding the physiology of silicification in *P. neolepis* were aimed at establishing whether it is essential for this organism to silicify and what are the consequences of limiting Si availability and inhibiting the process in terms of growth and silica structure formation. The possibility of light dependency of silica biomineralisation in this species was also investigated. However, experimental verification of the above questions proved to be very difficult and in many cases impossible due to technical issues arising from poor culture growth and cells often not withstanding experimental conditions due to cell fragility.

**II.3.8. Dependency of silicification on light availability***

Biomineralisation processes in photosynthetic phytoplankton are known to be to some extent dependent on light availability, often via secondary metabolic and cellular processes or energetic requirements. In calcifying coccolithophores, biomineralisation occurs mainly during the day and has been suggested to be potentially dependent on energy derived from photosynthesis (Paasche 1999; Paasche 2001; Sekino and Shiraiwa
In diatoms, silicification is directly correlated with cell division and also tends to be more prevalent during the light phase (Chisholm et al. 1978; Martin-Jézéquel et al. 2000). To determine if similar light dependencies might be linked with silica deposition in *P. neolepis*, a simple experiment involving a 10h incubation in light and dark conditions was conducted. Silica scales deposited during the experiment were labelled with Lysotracker HCK-123, allowing their quantification with flow cytometry. Statistical analysis of the resulting ratios of labelled vs. unlabelled scales, providing a proxy for silicification rate revealed no statistical difference ($t=-0.42$, df=28, $p=0.68$, $n=15$) in the silicification rates between light and dark conditions (Fig. II. 17.). This result indicates strongly that light is not essential for silicification in *P. neolepis*, and there is no short-term dependence on light for scale production in this silicifier.
II.3.9. Dependency of silica scale production on silica availability

Although originally the impact of silica starvation on growth was to be determined, measurement of growth under Si-replete conditions proved to be impossible. Attempts to grow *P. neolepis* in ASW (artificial sea water) with Si concentrations < 3 µM were unsuccessful. Cells introduced into these cultures as an inoculum could no longer be observed with a light microscope after 2-3 days from inoculation and control cultures supplemented with 100 µM Si in ASW grew poorly and frequently collapsed. Therefore a short term response to low Si conditions was investigated instead. For this purpose, a method utilizing confocal microscopy in conjunction with silica labelling fluorescent dyes was developed. The method involved incubation of *P. neolepis* cells in high (supplemented with 100 µM Si) and low (< 3 µM) Si ASW with f/2 media in the presence of a silica-labelling fluorescent probe for a period of 18 h. Cell counts were
made at the end of the incubation period on random fields of view, directly on the glass bottom dishes (Fig. II. 18. A). Cells were then lysed in order to enable quantification of silica scales produced during the experiment, for which purpose images of random fields of view were taken (Fig. II. 18. B).

**Verification of the method:** cell counts per field of view were variable both within and between dishes (Fig. II. 19. A,B), which was compensated for by taking 10 counts per dish, and increasing the number of replicates to 5. This variability was most likely caused by washing cells with treatment media directly on the dish. Cell counts were averaged to obtain cell density per dish, which was used in further analysis.

![Confocal microscopy images of PDMPO stained *P. neolepis* cells (18 h incubation in 100 μM Si in ASW with f/2 nutrients). A-example image used for cell counts. Green channel was switched off for easier image processing. B - example image of silica scales post H₂O₂ lysis (all channels except green were switched off for clarity). Scale bars=20 and 10 μm for A and B respectively.](image-url)
Results obtained from this experiment indicated, that *P. neolepis* possess an efficient mechanism for Si extraction from the media, as average Si concentration in control samples decreased from 100 µM (plus > 3 µM of unavoidable Si contamination introduced with ASW) to 29.05 µM (n=4, SD=1.26) at the end of the incubation period. Additionally, cells were still able to produce considerable amounts of silica scales in low Si conditions (Fig. II. 20. A,B), with Si concentrations at the end of the experiment averaged at 5.87 µM (n=4, SD=1.82).

Fig. II. 19. Validation of the method used for cell counts in the low Si experiment: A - variability of cell counts between all replicates in all dishes (D=dish). B - cell count variability within 1 dish ("replicate" on x-axis refers to a replicate of a cell count carried out within one dish).

Overall, limiting Si supply resulted in a lower average number of scales/cell (51.49% decrease), scale area/cell (46.97% decrease) compared to the control (Fig. 20. A,B). The effect of limiting Si supply on the scale size (area), was quite minor and a 11.11%
decrease in area/scale in the treated vs. control samples was recorded, indicating, that limiting Si supply has little effect on the size of scales produced. Qualitative inspection of scales produced under the low Si treatment indicated no morphological departures from those produced in the control.

Fig. II. 20. Quantification of silica scales produced after 18 h incubation period under low Si and control conditions. Box-whisker plots of: A - scale area/cell, B - average number of scales/cell. Box plots are based on data averaged per dish.

II.3.10. Silicon metabolism in P. neolepis: disruption with germanium

Ge acts as a competitive inhibitor of Si metabolism, with growth and silicification inhibitory effects becoming apparent once a species-specific threshold ratio of Ge:Si in the media is approached (Azam and Chisholm 1976; Azam et al. 1974; Lewin 1966). Ge was previously shown to interfere with growth of some algae, especially silicifiers, when introduced to cultures at concentrations between 2.1-14.3 µM (Lewin 1966;
Markham and Hagmeier 1982). Diatoms were shown to be especially prone to germanium induced growth inhibition due to Ge interfering with cell wall formation and respond to ratios as low as 0.02 Ge:Si, a property that has been long used to eliminate diatom contamination of non-silicifying species in algal culture collections (Azam and Chisholm 1976; Azam et al. 1973; Lewin 1966; Markham and Hagmeier 1982). Ge uptake and co-deposition with silica frustules in diatoms was also shown to result in malformations (Azam et al. 1973).

Here Ge was used to determine, if (1) silicification in *P. neolepis* can be inhibited and with what consequences, (2) if Ge can be taken up and co-incorporated into silica structures and (3) whether it affects the morphology of silica scales. In order to answer these questions, growth experiments, confocal laser scanning microscopy in conjunction with fluorescent silica markers, SEM imaging and EDX analysis were carried out. Methodological limitations relating to Si assay development did not allow for Si concentration measurement in these early experiments, however, special care was taken to keep Si contamination in the media as low as possible.

II.3.11. Inhibition of silica scale production in P. neolepis with Ge: CLSM observations

Results of incubation of *P. neolepis* with a range of Ge concentrations revealed, that as in diatoms, Si metabolism can be blocked with Ge, resulting in inhibition of silica deposition. Multiple experiments involving cell treatment with 10, 20 and 50 µM Ge consistently indicated, that cells were unable to produce and deposit silica scales under these Ge concentrations. At 10 µM Ge partially formed internal scales were still present in some cells (Fig. II. 21. B), however at 50 µM Ge intracellular scale formation was no longer observed (Fig. II. 21. C). Incubation with 10 µM Ge also resulted in some cell
mortality as determined by visual inspection, with the effects becoming much more severe at 50 µM Ge, where very few cells remained intact after 18 h incubation period (Fig. II. 21. A-C).

<table>
<thead>
<tr>
<th>A - control</th>
<th>B – 10 µM Ge</th>
<th>C - 50 µM Ge</th>
</tr>
</thead>
</table>

Fig. II. 21. A - silicified cells in control, B - non-silicifying cells post 10 µM Ge treatment with partially formed intracellular scales marked with arrows, C - few surviving, non-silicifying cells post 50 µM Ge treatment. Scale bars=10 µm (A,C) and 20 µm (B). Images representative of 18 h incubation experiments conducted in duplicate. HCK-123 labelling of silica shown in green, chlorophyll autofluorescence in red.

II.3.12. Ge/Si co-feeding of P. neolepis: growth

Since the potency of Ge toxicity to Si metabolism depends on concentration of both Ge and Si, a much lower (3 µM) Ge concentration, supplemented with 50 µM Si was chosen for the next experiment. The aim of this experiment was to determine if Ge/Si
co-feeding could result in Ge co-incorporation into the silica structure and if so, whether it caused any adverse effects on silica scale morphology. Results of this experiment revealed, that at these Ge and Si concentrations culture growth was not inhibited nor decreased (Fig. II. 22.). In a growth experiment where cells were dosed with 50 µM Ge/Si however no growth was observed. After 2 days cells could no longer be found in these cultures when examined with a light microscope.

![Graph](image_url)

Fig. II. 22. A-Growth curve for 3 µM Ge supplemented and control P. neolepis cultures. Error bars=SD based on 3 cell counts per each replicate (n=3 replicates). Growth rates calculated for the mid-exponential phase between day 8-10 were at μcontrol=4.1 and μGe=3.9.
II.3.13. Ge/Si co-feeding of P. neolepis: Ge incorporation into silica scales

To determine if formation of silica scales was adversely affected by Ge, SEM analysis of biosilica obtained from the 3 μM Ge/50 μM Si treated culture was carried out. No morphological abnormalities were visible in the material examined (relevant images attached in the appendix II.2.), and silica scales produced in Ge treated samples were not significantly different from the controls in terms of length or width ($t=1.64$ for length and $t=-0.243$ for width, df=198, p>0.01). Ge co-incorporation into scales was confirmed with EDX analysis (Fig. II. 23.), indicating that the Si transporters and further metabolic pathways are not strictly Si specific and are not significantly impaired at low Ge concentrations.

![EDX spectrum of purified P. neolepis silica scales obtained from a culture dosed with 3 μM Ge and 50 μM Si. High C peak is caused by the nitrocellulose filter on which the sample was deposited.](image-url)
II.4. DISCUSSION

II.4.1. *P. neolepis* strain TMR5: morphological and biological characteristics and comparisons with closely related algae

Analysis of *rbcL* and SSU gene sequences indicated, that some genetic variability between *P. neolepis* strains from different geographical locations exists. In terms of morphology, *P. neolepis* exhibits traits characteristic of other members of *Prymnesium* genus. *P. neolepis* cells are polar, with the posterior, non-flagellar pole being dominated by vacuoles and vesicles, which in other *Prymnesium* and closely related *Chrysochromulina* species contain storage metabolites (Green et al. 1982; Parke et al. 1955). Additionally, some morphological variation between different strains was noted. Compared to *P. neolepis* strain NIES 1393, organic scales produced by strain TMR5 were larger (1.06 x 0.95 vs. 0.6 x 0.4 µm for TMR5 and NIES 1393 respectively), and possessed serrated edges absent in NIES 1393 (Yoshida et al. 2006). This is not unusual, as members of *Prymnesium* genus are known to produce several types of organic scales (Green et al. 1982).

As previously observed for cultures of other *Prymnesium* and *Chrysochromulina* species, approaching stationary phase and senescence in *P. neolepis* is marked by cells becoming more irregular and amoeboid in shape, increasingly forming gelatious palmelloids towards senescence (Conrad 1941; Green et al. 1982; Parke et al. 1955). In natural marine microalgal populations, aggregation of phytoplankton was suggested to be a response to limited nutrient supply, increasing sinking rates to reach deeper, higher in nutrients layers within the water column (Bernard 1963; Smetacek 1985; Thornton 2002). Senescence in *P. neolepis* is also characterised by cells becoming increasingly
phagotrophic, however, unlike in continuously mixotrophic flagellates of *Prymnesium* and *Chrysochromulina*, this behaviour appears to be related to nutrient limitation. In all the aforementioned algae, phagotrophy always occurs at the non-flagellar pole of the cell, often via pseudopodial cell protrusions (Jones et al. 1993; Legrand et al. 2001; Parke et al. 1955; Tillmann 1998). This mixotrophic behaviour was previously suggested to be a mechanism allowing for N and P supplementation in normal conditions and conferring an advantageous compensation mechanism when nutrients become limited (Legrand et al. 2001; Nygaard and Tobiesen 1993), which appears consistent with the increased incidence of phagotrophy towards culture senescence in *P. neolepis*. No cells ingesting conspecifics were observed during the exponential growth phase, however, no queries into bacterial ingestion during this growth phase were made at this time either. Thus, a possibility that the fully silicified, non-nutrient limited *P. neolepis* could be capable of feeding on bacteria remains unresolved.

II.4.2. Organic and biomineralised cell covering

The thick, irregular Concanavalin-A-staining, putatively polysaccharide containing deposit covering the plasma membrane in *P. neolepis* is suggested to act as a matrix in which silica scales are lodged, thus maintaining them in a spherical formation surrounding the cell surface. A similar role was attributed to a layer of extracellular, columnar deposits in coccolithophores, which have been suggested to act as an adhesive, directly connecting organic scales and coccoliths to the plasma membrane (Drescher et al. 2012; Manton and Leedale 1969; Rowson et al. 1986).
Biosilica scales of *P. neolepis* consist of an amorphous, although relatively well organised hydrated silica phase similar to that found in diatoms, in which FT-IR frustule signatures are species-specific (Kammer et al. 2010; Vardy and Uwins 2002). The lack of specific signatures produced by organics, of which presence in biosilica was later confirmed with EDX and SDS-PAGE analysis (see Chapter IV for details) could have been due to signal levels falling below detection limits of the machine used.

The loosely associated, delicate scale covering of *P. neolepis* does not appear to have a structural role, as has been suggested for biomineralised cell covering in diatoms or coccolithophores, however it could potentially act as grazing deterrent (Hamm et al. 2003; Young 1994). As silica scales in this species are embedded in a thick layer of the diffuse, extracellular deposit, forming a "shell" at a distance from the protoplast, it is possible that this structure plays a role in maintaining a privileged environment. This was previously suggested as one of potential functions of coccospheres forming analogical, but more compact and resilient structures in coccolithophores (Paasche 2001). The formation of a loose silica scale cover in *P. neolepis* might also serve to regulate buoyancy of cells in suspension in a manner similar to that previously suggested for coccoliths and diatom frustules, thickening in response to nutrient limitation to sink into deeper, nutrient-rich waters (De la Rocha et al. 2000; M Franck et al. 2000; Young 1987). The shape and patterning of *P. neolepis* silica scales can be speculated to have similar optical properties and functions as the nanopatterned diatom frustules. Both organisms produce silica structures characterised by a periodic nature of the pore pattern, which in diatoms was suggested to confer photonic crystal properties to the silica cell wall, potentially serving to enhance light acquisition and therefore photosynthesis (Fuhrmann et al. 2004; Yamanaka et al. 2008). However, given the loose and disorganised scale arrangement on the *P. neolepis* cell surface, such a role is unlikely.
II.4.3. Silica scale formation

Organic, unmineralized scales formed in Golgi vesicles and liberated near flagellar roots are typical of haptophytes (Green et al. 1982; Manton 1966; Manton and Leedale 1969; Parke et al. 1955). In calcifying coccolithophores, organic scales usually co-occur with mineralized coccoliths in a layered arrangement, with organic scales underlying coccoliths (Inouye and Pienaar 1984; Manton and Leedale 1969). A similar arrangement is found in *P. neolepis*. Organic scales serve as a baseplate for the mineralized coccoliths (with the exception of *Emiliania huxleyi*) (Inouye and Pienaar 1984; Klaveness 1972; Manton and Leedale 1969; Pienaar 1994). This is in contrast with biomineralised scales produced by *P. neolepis*. Organic scales produced by this species are much smaller and solid, not matching the perforated, concave silica scales morphologically. These observations are further supported by SEM imaging of silica scales at various stages of development, where no associated baseplate structure was observed under the forming loophole pattern of scales. Etching of the silica scale material further confirmed the absence of an underlying organic baseplate. Instead, *P. neolepis* biosilica is associated with a filamentous, insoluble, likely cellulose-containing organic phase, potentially serving as a matrix for sequential silica deposition processes (see sections II.3.3. and IV.3.8. for details). This suggests, that silica scales do not arise from biomineralisation of an organic scale as it is the case in most coccolithophores, but are separate structures.

The lack of homology between organic and silica scales is further supported by the formation site of silica scales being different to that of organic scales and coccoliths. Biosilica scales of *P. neolepis* are formed in the posterior, vacuolar region of the cell, in
a compartment previously proposed to be a Silica Deposition Vesicle (SDV) similar to that of diatoms, as opposed to a Golgi-derived vesicle in which organic scales and coccoliths are formed (Billard 2004; Manton 1966; Yoshida et al. 2006). Furthermore, as with unmineralized haptophyte scales, coccoliths are secreted at the anterior pole of the cell, near the flagellar root (Manton 1967; Taylor et al. 2007). In contrast, silica scale secretion in P. neolepis occurs at the posterior, non-flagellar pole of the cell, co-localized with silica scale formation site instead. The silica scale formation process in P. neolepis appears to bear some similarities to frustule formation in diatoms, where an organic, chitin-containing matrix is mineralized within a specialized SDV and then secreted (see Chapter I for a review) (Durkin et al. 2009; Kröger and Poulsen 2008a; Tesson and Hildebrand 2010). Unlike frustule formation in diatoms, which is closely related and synchronised with the cell cycle to ensure that each daughter cell receives one newly formed frustule valve and derives the other from the parent cell and then restricts silicification to girdle bands following cell division (Graham et al. 2009), silica scale formation in P. neolepis appears to be a continuous process occurring during the entire cell cycle.

II.4.4. Physiology of silicification in P. neolepis

Silicification in marine microalgae is an energetically costly process, involving specialised Si transport systems and metabolism (Hildebrand 2005; Martin-Jézéquel et al. 2000). The uniformity of silicification rates in P. neolepis under light and dark conditions indicates, that it is a continuous process not dependent on light, and therefore energy derived directly from photosynthesis. This also suggests that the energy required for silica scale formation is obtained from respiration instead, as was previously shown.
for diatoms (Martin-Jézéquel et al. 2000; Sullivan 1976). Silicification in *P. neolepis* also persists through cell division, as dividing cells were observed to usually contain 2-3 intracellular scales.

Attempts to grow *P. neolepis* at low Si concentrations (<3 µM) failed, suggesting that Si is important for normal growth of this organism. Subsequent efforts to assess the ability of *P. neolepis* to deposit silica scales under low Si concentrations suggests, that an efficient Si-selective, most likely active transport system, enabling substrate acquisition and concentration is present. The decrease in the scale area/cell and the number of scales/cell in low Si treatments without a considerable decrease in average scale area indicates that Si limitation decreases silica scale production without a concomitant decrease in size. Additionally, the slight increase in Si concentration after incubation in low Si media (from <3 µM to ≈5 µM) indicates, that the elaborate, putatively polysaccharide-containing matrix maintaining the loose association between cells and silica scales might have contributed to Si carryover and retention from pre-experimental, Si-containing culture media. This also further supports the role of the putative polysaccharide and siliceous cell covering in stabilizing the microenvironment around the cell.

**II.4.5. Ge inhibition of silicification**

Similar to diatoms, Ge was found to act as a competitive inhibitor of Si in *P. neolepis*, resulting in a complete inhibition of silicification at Ge concentrations ≥10 µM. At higher concentrations (≥10 µM), Ge becomes increasingly toxic to *P. neolepis*, resulting in cell mortality, potentially due to disruption of Si metabolism. This, combined with the lack of growth and subsequent cell death observed for growth
experiments in low Si media and those involving Ge/Si co-feeding at 50 µM Ge/Si suggests, that Si metabolism is essential for normal growth of *P. neolepis* cultures. Due to methodological inconsistencies between previous studies investigating the use of Ge for elimination of diatoms from algal cultures (i.e. insufficient information on the method of media preparation pertaining to Si concentration, dissolution of the poorly soluble GeO$_2$ directly in sea water etc.), comparisons between different algae, especially diatoms and *P. neolepis* in terms of sensitivity to Ge were not possible. Treatments at lower (3 µM) Ge concentrations resulted in Ge incorporation into silica scales without causing silica scale malformation, analogous to previous observations documented for diatom frustules produced in Ge/Si co-feeding experiments at low Ge concentrations (Jeffryes et al. 2008). This also suggests that the Si transport system is not strictly Si-specific, and facilitates transport of analogous but larger ions, such as germanic acid.

**II.4.6. *P. neolepis*: the only silicifying haptophyte described to date?**

Findings by Pienaar (1980, 1981), previously mentioned in the introduction and pertaining to the presence of silica associated with the fibrous, organic deposits in *Prymnesium sp.* could potentially be an artefact of an interaction of the aforementioned material with an excess of silicic acid in the media. Controlled, biologically mediated silica precipitation has received a great deal of attention over the past two decades due to its potential application in materials science, it is now known, that it can be facilitated by a wide range of different organic compounds. These include polycationic polysaccharides, polypeptides, highly acidic proteins or polyamine/quaternary ammonium group containing organic compounds amongst many others (see sections
I.3.5-1.3.7, I.6. for details), which are ubiquitously distributed amongst taxa (Gautier et al. 2008a; Hedrich et al. 2013; Kröger et al. 1999; Shchipunov et al. 2005; Wenzl et al. 2004; Wenzl et al. 2008). It is therefore not implausible, that the organic material coating of the cyst cover-forming scales, which themselves are often associated with polysaccharides, could facilitate precipitation of excess Si out of the growth media. This is also a likely scenario, as culture growth media are commonly prepared and autoclaved in glass bottles, significantly increasing the concentration of free silicic acid, often precipitating in a form of needle-like glass structures (Lohmiller and Lipman 1998). Additionally, silica aggregates precipitated during the autoclaving process could simply get lodged in the organic, fibrous coating of the outermost scales of a cyst, contributing to detection of silica in this material.

In the light of the above information, silica association with the organic deposits covering Prymnesium cysts is unlikely to be a product of an active silicification process occurring within cells. These preliminary observations remain highly speculative until further, more thorough inquiries are made. Thus, *P. neolepis* remains the only haptophyte described to date, capable of actively producing fully biomineralised scales composed of silica. Therefore, a unique opportunity to investigate the process of biosilicification, potentially unique to haptophytes, and comparisons of mineralized and unmineralized cell covers between haptophytes and other silicifying algae is presented.

**II.5. SUMMARY**

*P. neolepis* is the only obligate silicifier within the haptophyte clade described to date. Silicification in *P. neolepis* occurs in a compartment of origin different to that of other biomineralizing haptophytes yet putatively similar to that of diatoms. The organic
matrix underlying silica scales is non-homologous with organic scales in contrast to the case in coccolithophores. The site of silica scale exocytosis is also different to that of coccoliths, further indicating that silica scales are structures formed in a process fundamentally different to coccolithogenesis in calcifying coccolithophores.
CHAPTER III: THE ROLE OF CYTOSKELETON IN ALGAL BIONERALISATION

III.1. INTRODUCTION

Extracellular structures in haptophytes, such as organic scales and mineralized coccoliths are produced in specialised vesicles originating from the Golgi body (Graham et al. 2009; Schmid 1987). In calcifying coccolithophores, biomineralised scales are deposited in specialized coccolith vesicles (CVs) in a sequential process, involving finely controlled nucleation and growth of calcite crystals on an organic baseplate, serving as a biotemplate (see Chapter I for more details) (Young and Henriksen 2003). The CV itself has been suggested to be externally moulded during coccolithogenesis by an interaction with a fibrillar structure of putative cytoskeletal composition adjacent to the CV, although little is known about the exact processes involved (Didymus et al. 1994; Drescher et al. 2012; Langer et al. 2010; Marsh 1999; Marsh et al. 2002; Westbroek et al. 1984). Similarly, the presence of specialised vesicles and their close interaction with the cytoskeleton during silica cell wall morphogenesis has been observed and well documented in diatoms of heterokontophyte origin (Schmid 1987; Tesson and Hildebrand 2010). In both cases mineral deposition proceeds in specialised, membranous compartments and entails a complex interplay of the entire cellular machinery, where the role of cytoskeleton can be assigned to three main types of tasks: (1) intracellular transport of vesicles, membranes and other building blocks required, (2) active shaping of biomineralised structures during deposition, (3) secretion of complete structures and their localization on the cell surface. The capacity of cytoskeleton microfilaments to facilitate these processes arises from
intrinsic structural properties of actin and tubulin microfilaments, enabling dynamic interaction with each other as well as with other molecules in the cell (Aylett et al. 2011).

The cytoskeleton in Eukaryotes consists of actin microfilaments, microtubules and intermediate filaments, serving a vital role in maintenance of cell shape, movement, molecule delivery and localization, cell division, genetic material segregation or cytoplasmic streaming (Desai and Mitchison 1997; Kueh and Mitchison 2009; Verchot-Lubicz and Goldstein 2010). Actin microfilaments (F-actin) and tubulin protofilaments (building blocks of microtubules, MTs) are polar polymers of G-actin and αβ-tubulin monomers respectively (Wickstead and Gull 2011). Due to the filaments' polar structure, addition of monomer units is in general favoured at the barbed end in actin and plus end in tubulin, whereas depolymerisation processes are favoured at the pointed and minus ends for actin and tubulin filaments respectively (Fig. III. 1 A,B) (Allen and Borisy 1974; Desai and Mitchison 1997; Le Clainche and Carlier 2008; Small et al. 1978).

Both actin and tubulin can use NTP (nucleoside triphosphate) hydrolysis to generate dynamic instability to shrink/grow, initiate localized polymerization/depolymerisation, treadmilling, or bending in order to e.g. shape membranes, move molecules, organelles or vesicles (Löwe and Amos 2009). They can also create directional intracellular tracks on which a range of motor proteins, such as microtubule-associated kinesins and dyneins or F-actin-associated myosins can traffic molecules to target sites (Fig. 1A,B) (Gross et al. 2007; Löwe and Amos 2009; Vale 2003).
Historically, an array of cytoskeleton disruptive chemicals has been used to probe functions of cytoskeleton by specifically disrupting selected cytoskeletal components within a cell (Peterson and Mitchison 2002). Use of such substances will not only cause disruption of the microfilaments targeted, but will also secondarily impair intracellular transport facilitated by motor proteins associated with them. Depending on the type and dose of the drug used, cytoskeleton disruptors can cause microfilament capping and stabilization, filament shortening, dissociation of supramolecular structures or almost complete depolymerisation (Jordan et al. 1992; Margolis et al. 1980; Peterson and Mitchison 2002). This in turn depends on the inhibitor binding site in the filament or
association with its monomeric subunits, making the choice of the right inhibitor and dosage critical to interpretation of the response produced.

To date, only one investigation has endeavoured to probe the specific role of actin and tubulin microfilaments in coccolithogenesis, using cytoskeleton inhibitors in *Emiliania huxleyi* to confirm their involvement in coccolith morphogenesis (Langer et al. 2010). No investigations at all have been made concerning silicification within the haptophyte clade, represented by a single, silica scale producing species - *Prymnesium neolepis* (Yoshida et al. 2006). Therefore this study aims to interrogate and compare these two fundamentally different biomineralisation systems of the haptophyte algae. To achieve this, two inhibitors, latrunculin B and nocodazole have been employed. The former binds to actin monomers and facilitates F-actin depolymerisation, whereas the latter binds to microtubules capping them, concomitantly binding to tubulin monomers preventing their incorporation into filaments, and at micromolar concentrations used for the purpose of this study causes MT depolymerisation (Jordan et al. 1992; Margolis et al. 1980; Morton et al. 2000; Spector et al. 1999). Effects of individual system component disruption on biomineralisation in terms of morphology of the biomineralised structure, quantitative mineral deposition and secretory abilities were compared. Additionally, the haptophyte silicification system present in haptophytes has been compared with that of the most prominent and thoroughly investigated heterokontophyte silicifiers - diatoms.
III.2. METHODS

III.2.1. Culture conditions and cytoskeleton inhibitor dosing

*Coccolithus pelagicus* ssp *braarudi* (strain PLY 182g) and *Prymnesium neolepis* (strain TMR5) were cultured as described in Chapter II. Cytoskeleton inhibitor doses used are specified in Table III.1. The concentrations correspond to the lowest effective concentrations as determined in a preliminary cytoskeleton inhibitor trial on *C. pelagicus*, where latrunculin B concentrations between 0.1-100 µM and nocodazole concentrations between 0.1-10 µg/ml were tested. As DMSO was used as a solvent for cytoskeleton inhibitors, an additional control with DMSO concentration corresponding to the highest amount of DMSO introduced to treatments with cytoskeleton inhibitors was added.

Table III.1. Cytoskeleton inhibitor and control DMSO doses.

<table>
<thead>
<tr>
<th>Chemical:</th>
<th>Control DMSO Control</th>
<th>latrunculin B</th>
<th>nocodazole</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose:</td>
<td>-</td>
<td>0.25%</td>
<td>1 µM</td>
</tr>
</tbody>
</table>
III.2.2. Monitoring of culture health under experimental conditions

Fv/Fm ratios, indicating photosystem II efficiency were recorded using a Z985 Cuvette AquaPen (Qubit Systems) before and after the experimental period for both *P. neolepis* and *C. pelagicus*. Dark adaptation times prior to Fv/Fm measurement were 20 min and 30 min for *P. neolepis* and *C. pelagicus* respectively.

Additionally, 1 µM SYTOX Green (Invitrogen) was used to determine if the *P. neolepis* cell membrane integrity and health were compromised during the treatment. Cells were subjected to treatment with cytoskeleton inhibitors, transferred onto glass bottom dishes and incubated for 15 min with 1 µM SYTOX Green. Cells were imaged with a confocal laser scanning microscope as specified in section III.2.3. Images were then analyzed, and 25 cells per replicate from each treatment were grouped into three categories: unlabelled, weakly labelled and strongly labelled. The first two categories were considered viable cells. Each treatment was replicated three times and a total of 75 cells/treatment was analysed. Chi-square tests were used to determine if there were any differences between treatments.

III.2.3. Confocal microscopy observations of *P. neolepis* under cytoskeleton inhibitor treatment

5 ml of *P. neolepis* culture was aliquoted into 7 ml bijou bottles, supplemented with f/2 media and dosed with cytoskeleton inhibitors for a period of 24 h. Cells were incubated in cytoskeleton inhibitor for 2.5 h prior to addition of the fluorescent marker to ensure disruption had occurred prior to measuring silica scale formation. Once the initial inhibitor incubation period was over, 1 µM Lysotracker Yellow HCK 123 (HCK 123)
or LysoSensor DND-160 Yellow/Blue (PDMPO) were added to all treatments. Cells were then carefully transferred onto poly-L-lysine coated glass bottom dishes and imaged with a confocal microscope. Additionally, latrunculin B treated cells were imaged as Z-stacks of whole cells and then selective cell lysis with a Mai Tai pulsed multiphoton infra-red laser (Spectraphysics) set to 740 nm was administered in order to inspect the silica scale content of individual cells. Additionally, scale length, measured along the longest axis of each individual scale under DMSO (24 h incubation), nocodazole and control treatments (5 h incubation) was recorded. Variation in scale length between treatments was assessed using a two sample Kolmogorov-Smirnov two sample test and a \( t \)-test (Statsoft: Statistica).

**Imaging:** Texas Red-labelled tubulin antibodies were imaged using excitation with a \( \lambda = 543 \) nm He/Ne laser, with emission filters set to \( \lambda = 565-615 \) nm. HCK 123 and SYTOX Green were excited with \( \lambda = 488 \) nm (Argon laser) and emission filters were set to \( \lambda = 500-550 \) nm and \( \lambda = 650-710 \) nm for HCK 123 and chlorophyll autofluorescence respectively. Calcite was imaged using excitation with a He/Ne laser at \( \lambda = 633 \) nm, and emission was collected between \( \lambda = 650-700 \) nm. PDMPO excitation and emission filters were set as specified in section II.2.3. All confocal images were taken with a Zeiss LSM 510 Meta microscope.

**III.2.4. Flow cytometry monitoring of silica scale production in P. neolepis**

1ml of concentrated exponentially growing culture was added to 4 ml of fresh FSW (pH 8.20), supplemented with f/2 nutrients and 100 \( \mu \)M Si and yielding cell densities between 1-1.5x10^5 cells/ml. Samples were then dosed with 5 \( \mu \)g/ml nocodazole or 1 \( \mu \)M
latrunculin B and 0.25% DMSO, corresponding to the amount of DMSO present in the nocodazole solution. Overall, DMSO concentration in all treatments did not exceed 0.35%. Additional 0.1% DMSO treatment, only containing DMSO introduced with the fluorescent dye, was set up to confirm, that the higher DMSO concentrations used in the experiment had no adverse effects on scale production.

Samples were incubated with cytoskeleton inhibitors/DMSO for 2.5 h to allow cytoskeleton disruption. Cells were then labelled with 1µM LysoTracker Yellow HCK 123, and incubated for 24 h. Cell count and Fv/Fm ratio immediately before and 26.5 h post dosing with cytoskeleton inhibitors were recorded to monitor culture health. Cells were left to silicify over a 24 h period (18:6 light:dark cycle). Cells were then harvested by centrifugation, washed 3x with 2% SDS solution with 0.1 M Tris/EDTA (pH 8.00) to remove cellular debris and then washed again 3x with deionised water. Samples were then analysed using Accuri C6 flow cytometer set to record side scatter and green fluorescence (SSC vs. FL1A), gated for fluorescent and non-fluorescent particles. Experiments were repeated five times in triplicate in order to ensure, that consistent response was exhibited both within and between experiments. Student's t-test for independent samples was used to determine differences between treatments (Statsoft: Statistica).

III.2.5. C. pelagicus: Differential Interference Contrast (DIC) imaging of cytoskeleton inhibitor treated cells

C. pelagicus cells were decalcified by decreasing the pH with HCl down to pH 4.00 for 3 min. pH was then brought back up to 8.20 with NaOH, and the cells were inspected under a microscope to ensure complete decalcification. 1ml of cells was then aliquoted
onto poly-L-lysine coated glass bottom dishes, supplemented with 2 ml of fresh f/2 media and dosed with inhibitors as listed in table 1. Images were taken 24h after inhibitor application. Cytoskeleton inhibitors were then washed off 3x with fresh f/2 media and cells were allowed to recover to ensure, that the malformations were due to inhibition of the respective cytoskeletal components and not imminent cell death, resulting from cytoskeleton inhibitor poisoning. Images were taken again 24 and 48 h post cytoskeleton inhibitor removal. DIC images were taken with a Nikon Eclipse Ti microscope equipped with a Photometrics Evolve EMCCD camera.

III.2.6. *C. pelagicus: Electron Microscopy imaging of cytoskeleton inhibitor treated coccoliths*

*C. pelagicus* samples: 15 ml samples were supplemented with 5 ml of filtered sea water (FSW) and f/2 media, and dosed with cytoskeleton inhibitors as specified in "culture conditions and cytoskeleton inhibitor treatment" section. Cells were then incubated in inhibitros for 1 h and subsequently decalcified at pH 4.0 for 3 minutes. Samples were inspected under a light microscope to ensure complete decalcification. Cells were incubated with cytoskeleton inhibitors for 24 h following decalcification. Samples were then pelleted and 1ml of NaOCl was added to each sample. Samples were vortexed and placed on a rocking table for an hour to dissociate organic matter and prevent the sample from settling. Samples were then vortexed again, pelleted and NaOCl was removed. The remaining pellet was washed 3x with deionised water adjusted to pH 10.00 with NH₄OH. Samples were then filtered onto a 0.22 μM filter membranes, put on a stub and sputter coated with gold-palladium. Imaging was done with a JEOL JSM-7001F Field Emission Microscope and JEOL JSM-6610LV Scanning Electron
Microscope at 15 kV accelerating voltage. The images were then analysed and 100 coccoliths per replicate, giving a total n=300 per three replicates, were examined and assigned into one of three categories: complete, partially formed/slightly malformed and malformed coccoliths. Chi-square tests were then performed on data obtained to determine, whether there were differences in frequencies of occurrence of coccoliths from different categories between treatments.

**III.2.7. Tubulin Immunofluorescence labelling**

Monoclonal Anti-α-Tubulin antibody produced in mouse (Sigma-Aldrich) was used as primary anti-α-tubulin antibody, and Texas Red conjugated anti-mouse IgG raised in goat (Thermo scientific, #31660) was used as a secondary antibody.

**Pre-labelling treatment:**

*P. neolepis* cultures were placed in 7 ml bijou bottles and dosed with 1µM HCK 123 and 5 µg/ml nocodazole for 5 h to disrupt the tubulin network and to stain internal silica scales. Normal, control samples without the tubulin inhibitor were also examined.

*C. pelagicus* cells were decalcified with 25 mM EGTA solution in Ca$^{2+}$ free artificial sea water, according to a protocol modified from Taylor et al. (2007), dosed with nocodazole and incubated for 5 h. Decalcification with low pH proved to be a much quicker and more efficient method and was therefore used in subsequent experiments instead of EGTA based protocol.
Fixing and labelling: cells were settled onto a poly-L-lysine coated glass bottom dish, washed 3x with ASW (see section II.2.1. for details) and then fixed in 2% glutaraldehyde with 1.7% BSA (bovine serum albumin) in artificial sea water (ASW, recipe specified in section II.2.1.) for 10 min. Cells were then washed 3x with ASW/1.7% BSA with 0.5% glutaraldehyde and incubated for 5 min. Solution was then removed and replaced with 0.05% Triton X-100 in ASW for 10 min. Cells were then washed 3x for 5 min with ASW/1.7% BSA and incubated for 20 min. 1/50 primary ant-α-tubulin antibody was then added and cells were incubated on bench overnight. Cells were then rinsed 3x with ASW/1% BSA, and secondary, Texas Red-conjugated antibodies in 1/150 dilution. Samples were then left to incubate for 2.5 h, washed with ASW/1.7% BSA 3x and viewed under a confocal microscope.

The experiment was repeated three times with three replicates per experiment, in order to ensure the consistency of the labelling pattern. Cells were also treated with secondary antibodies alone to control for non-specific antibody staining.

III.3. RESULTS

III.3.1. Assays to determine effects of cytoskeleton inhibition on culture health

In order to determine the roles of actin and tubulin in calcification and silicification in haptophytes, cytoskeleton inhibitors latrunculin B (actin) and nocodazole (tubulin) were used. Effects of both inhibitors are rapid, and reversible, once chemicals are removed from the system (De Brabander et al. 1976; Morton et al. 2000). To determine the
involvement of different cytoskeleton components, it was crucial to ensure that cells were healthy and viable whilst under cytoskeleton inhibitor treatment. Failing that would introduce effects non-specific to cytoskeleton inhibition and resulting from poor health and death of cultures.

Photopigment degradation, especially chlorophyll $a$, is one of the first signs of cell death (Berges and Falkowski 1998; Franklin et al. 2012). Chlorophyll fluorescence is a parameter often used as a proxy for chlorophyll concentration, and arises mainly from photosystem II (PSII) fluorescence (Krause and Weis 1991; Kromkamp and Forster 2003). Photosynthetic capacity of PSII, measured as the $F_v/F_m$ ratio, has therefore been established as general health and stress indicator (Berges et al. 1996; Boyd and Abraham 2001; Kromkamp and Forster 2003; Schagerl and Möstl 2011). $F_v/F_m$ represents the maximum potential photochemical efficiency of PSII (photosynthetic quantum yield), where $F_v$ is the difference between maximum and minimum fluorescence and $F_m$ is the maximum fluorescence in a dark adapted sample (Kromkamp and Forster 2003).

This parameter has been measured for both $P. neolepis$ and $C. pelagicus$ before and after the cytoskeleton inhibitor treatment.

**III.3.2. Effects of cytoskeleton inhibitors on $P. neolepis$ health**

Results of post treatment screening of $P. neolepis$ cultures revealed, that the $F_v/F_m$ ratio was only slightly diminished in latrunculin B treated cells (Table III. 2.), thus indicating that overall cultures were in good health post treatment.
In addition to PSII monitoring, cell mortality assessed with cell counts before and after sample incubation with cytoskeleton inhibitors was also screened, and did not exceed 15% in all treatments. This was considered acceptable, as in addition to the innate fragility of *P. neolepis*, cytoskeleton inhibitors, incapacitating cell division and preventing compensation for natural cell mortality were used. Additionally, 4.5% and 6.5% cell mortality was recorded for Control + 5 µl DMSO (accounting for the amount of DMSO introduced with the dye Lysotracker HCK123 in the flow cytometry experiments) and the DMSO control (Table II. 2.), indicating a slightly detrimental effect of the solvent on cultures.

Table III. 2. Photosynthetic efficiency and cell viability counts recorded for *P. neolepis* 26.5 h post cytoskeleton inhibitor treatment. Measurements were taken from triplicate samples of each treatment and averaged.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Control with 5 µl DMSO</th>
<th>DMSO Control</th>
<th>latrunculin B [1 µM]</th>
<th>nocodazole [5 µg/ml]</th>
</tr>
</thead>
<tbody>
<tr>
<td>average F&lt;sub&gt;v&lt;/sub&gt;/F&lt;sub&gt;m&lt;/sub&gt;</td>
<td>0.73</td>
<td>0.71</td>
<td>0.71</td>
<td>0.65</td>
<td>0.71</td>
</tr>
<tr>
<td>SD</td>
<td>0.03</td>
<td>0.01</td>
<td>0.01</td>
<td>0.03</td>
<td>0.02</td>
</tr>
<tr>
<td>Mortality 26.5 h</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>post treatment (%)</td>
<td>0</td>
<td>4.5</td>
<td>6.5</td>
<td>14.6</td>
<td>13.9</td>
</tr>
</tbody>
</table>

Rescue of cytoskeleton inhibitor effects following removal of chemicals was not possible in *P. neolepis* due to cell fragility, rendering cells unable to withstand repeated
washing. Therefore another method of culture health evaluation was employed. This involved assessment of plasma membrane integrity, as it is one of the main criteria of cell viability and can be verified with a fluorescent DNA-binding probe, SYTOX Green (Darzynkiewicz et al. 1994). This fluorescent marker is only able to penetrate compromised plasma membranes, and has been previously applied in health assessment of a wide range of unicellular organisms, including phytoplankton (Reavie et al. 2010; Ribalet et al. 2007; Sato et al. 2004).

Following a prior study on phytoplankton mortality involving SYTOX Green application, three categories of cells have been distinguished based on the intensity of labelling: 1-viable cells (no staining), 2-weakly loaded cells considered to be of reduced viability, 3-strongly stained, dead cells (Fig. III. 2.) (Veldhuis et al. 2001).

Fig. III. 2. SYTOX Green labelled *P. neolepis* cells representative of the three cell viability categories: A - live cell with no labelling, B - live cell with weak, partial labelling (reduced viability), C,D - strongly labelled cells that are dead or leaky/approaching apoptosis (4 - D a partially lysed cell). Scale bar≈5 µm.
45% of cells in *P. neolepis* controls showed weak staining with SYTOX Green, with further 12% exhibiting strong labelling diagnostic of leaky and dead cells (Table III. 3.), indicating fragility of this species. The control, DMSO and latrunculin B treatment population structure upon labelling was not statistically different, and comprised of approx. 43% unlabelled cells, 35% presenting with weak labelling and strongly labelled cells contributing approx. 12% of the total cells examined (Table III. 3.). The proportion of unlabelled cells in nocodazole treatment decreased significantly (20%), whereas the number of weakly stained cells amounted to 38%, which was not statistically different from the control sample (Table III. 3.). The percentage of strongly labelled cells increased significantly (41%), indicating, that nocodazole had some detrimental effects on cells. It is worth noting however, that all weakly labelled and a high proportion of strongly labelled cells in the nocodazole treatment had intact, healthy looking chloroplasts. This indicates, that the increased labelling might be a result of cells becoming more "leaky" due to microtubule depolymerization rather than dying/approaching apoptosis, which would be in agreement with the lack of effect of nocodazole on PSII efficiency. Therefore, the effects of cytoskeleton inhibitors on culture health following treatment were considered minor and culture health was deemed satisfactory.
Table III. 3. Results of $\chi^2$ test between DMSO and nocodazole treatment labelled with SYTOX Green. Examples of cell labelling on which category assignment was based are provided in Fig. III. 2. A-C. Results based on an experiment conducted in triplicate, n=75.

<table>
<thead>
<tr>
<th>Labelling</th>
<th>$\chi^2$ DMSO vs. nocodazole</th>
<th>Df</th>
<th>p</th>
<th>Control sample [%]</th>
<th>nocodazole sample [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>9.82</td>
<td>1</td>
<td>&lt;0.05</td>
<td>42.67</td>
<td>20.00</td>
</tr>
<tr>
<td>Weak</td>
<td>0.28</td>
<td>1</td>
<td>&gt;0.05</td>
<td>45.33</td>
<td>38.67</td>
</tr>
<tr>
<td>Strong</td>
<td>53.78</td>
<td>1</td>
<td>&lt;0.05</td>
<td>12.00</td>
<td>41.33</td>
</tr>
<tr>
<td>Whole sample</td>
<td>63.88</td>
<td>2</td>
<td>&lt;0.05</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

III.3.3. Effects of cytoskeleton disruptors on silica scale morphogenesis in *P. neolepis*

Silica scales produced under experimental conditions were tracked with a fluorescent marker LysoTracker Yellow HCK 123 (HCK 123) and LysoSensor DND-160 Yellow/Blue (PDMPO), introduced into samples pre-incubated with cytoskeleton inhibitors for 2.5 h. Confocal microscopy imaging of cells post 24 h incubation with latrunculin B and fluorescent marker revealed that inhibition of actin polymerisation interfered with scale secretion. This resulted in retention of newly formed scales within cells (Fig. III. 3. A). Virtually no scales were secreted under this treatment, and cells accumulating an abnormally high number of silica scales were observed (Fig. III. 3. B). Normally, *P. neolepis* maintains 2-3 scales intracellularly at one time (see Chapter II for
However, up to 8 scales were found within cells post latrunculin B treatment, using cell lysis with a multiphoton laser at 740 nm to confirm the number of internal scales (Fig. III. 3. A,B). The percentage of cells over-accumulating scales and their exact numbers as well as scale length were not possible to determine due to scales overlapping in the cell as well as their displacement, floating and loss of fluorescence following cell lysis with a laser. Despite the inability of cells to secrete completed silica scales, no obvious visible morphological aberrations in the overall scale shape were observed. This suggests that actin plays a role in secretion of scales but not their formation.

Fig. III. 3. Confocal microscopy images representative of *P. neolepis* cells following a 24 h treatment with 1 µM latrunculin B. A - cells pre-lysis showing signs of silica scale over-accumulation and the lack of extracellular scales (individual optical slice), B - the same cells lysed to demonstrate over-accumulated scales, C-DMSO control (B,C - 3D projections of Z-stacks). Scales produced during the treatment were labelled with PDMPO (green), chlorophyll autofluorescence is shown in red. Intracellular scales marked with arrows (A). Scale bars correspond to 5 µm (A,B) and 2 µm (C). A total of 87 individual cells was examined (16-27 cells per each of the four replicates).
Microtubule disruption did not have as severe an effect on scale secretion as interference with actin polymerization. Scales produced under nocodazole treatment were significantly smaller and appeared more round compared to controls (Fig. III. 4. A-D, Fig. III. 5. B, Table III. 4.).

Fig. III. 4. 3D projections of confocal microscopy generated Z-stacks of *P. neolepis* after 24 h treatment with cytoskeleton inhibitors and a fluorescent marker HCK 123: A,B - nocodazole (scale bar=5 µm), C - DMSO (scale bar=2 µm), D - control (scale bar=5 µm). Silica scales deposited during treatment labelled with HCK 123 (green), chlorophyll autofluorescence in red.
Only scale length, measured along the longest axis of the scale could be determined due to the scales' flattened shape and consequent frequent positioning at an angle, rendering simultaneous measurement of width and length not possible.

Scales produced by cells treated with tubulin disruptor were on average 1.3 µm shorter than those produced in the DMSO treatment, measuring 3.21 µm and 4.52 µm respectively (Table III. 4.). DMSO and control scale lengths were not statistically different from each other (t=1.07, p=0.29, df=118) (Table 4).

Table III. 4. T-test comparisons of *P. neolepis* scales between different cytoskeleton inhibitor treatments (5 h for nocodazole and control, 24 h for DMSO). Experiments were conducted in triplicate, n\text{total}=60.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean scale length [µm]</th>
<th>SD</th>
<th>Difference of means</th>
<th>t</th>
<th>df</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO vs. nocodazole</td>
<td></td>
<td></td>
<td>1.31</td>
<td>-5.83</td>
<td>118</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Control</td>
<td>4.69</td>
<td>0.89</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>4.45</td>
<td>0.85</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nocodazole</td>
<td>3.21</td>
<td>1.52</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The difference in the mean scale length in nocodazole treated samples was not caused by a uniform decrease in scale length, but resulted from formation of frequently occurring, abnormally small scales between 0.5-2 µm in length (Fig. III. 5. A,B). Also, the frequency of occurrence of scales above 4µm was considerably lower than in the control and DMSO, contributing to lowering of the mean scale length produced by cells.
treated with nocodazole (Fig. III. 5. A). The overall distribution of scale length upon
in the disruption of tubulin was significantly different to that of DMSO (two sample
Kolmogorov-Smirnov test, $p < 0.001$, $n=60$). Distributions of scale length in control and
DMSO treatment were not statistically different (two sample Kolmogorov-Smirnov test,
$p > 0.1$, $n=60$)

Fig. III. 5. A - frequency distribution of *P. neolepis* scales according to different size
classes (red-nocodazole, black-control, gray-DMSO). B - confocal microscopy 3D
projection of a Z-stack of a nocodazole treated *P. neolepis* cells immunolabelled with
anti-α-tubulin antibodies. Silica scales produced under treatment were labelled with
Lysotracker HCK123, shown in green. Abnormally small, round scales typical of the
nocodazole treatment are marked with white arrows. An intracellular scale of normal
morphology is indicated with a red arrow. Such abnormally small scales are absent from
controls (see Fig. III. 4. C,D and Fig. III. 7. for images representative of control cells).
III.3.4. Quantification of cytoskeleton inhibitor effects on silica scale production in *P. neolepis*

To quantify the cytoskeleton inhibitor effects on silicification in *P. neolepis*, flow cytometry measurements in conjunction with fluorescent labelling of silica scales produced during treatment were used. As cells were lysed prior to flow cytometry analysis, both intracellular and extracellular scales deposited during incubation with cytoskeleton inhibitors were quantified. The ratio of fluorescent (new) vs. non-fluorescent (scales produced prior to labelling) scales was used as a proxy for silicification rate.

Flow cytometry analysis of HCK 123 labelled scales after treatment with cytoskeleton inhibitor for 24 h revealed that both nocodazole and latrunculin B caused a severe decrease in silicification rate (Fig. III. 6. A,B, Table III. 5.). DMSO and control samples were not significantly different, indicating, that DMSO did not have an inhibitory effect on silica scale production (Fig. III. 6. A, Table III. 5.). As the DMSO treatment corresponded to the highest amount of DMSO used as a solvent in cytoskeleton inhibitor dosed samples, it was used as a reference for analysis of data obtained from actin and tubulin inhibitor treatments (Table III. 5.).
Fig. III. 6. Box-whisker plots of fluorescent vs. non-fluorescent scales ratios post 24h treatments with: A - latrunculin B, nocodazole, DMSO and control. B - latrunculin B and nocodazole (expanded y-axis).

A 78.9% decrease in average silicification rate in latrunculin B, and a 71.5% decrease in nocodazole treated cells relative to DMSO treatment was observed. Additionally, a significant decrease in silicification rate in the latrunculin B treatment relative to nocodazole was found (Table III. 5.), consistent with confocal microscopy observations (Fig. III. 3, Fig. III. 4.).
Table III. 5. Results of the t-test comparisons of the fluorescent vs. non-fluorescent scales ratio between 24 h Control, DMSO, latrunculin B and nocodazole treatments. Experiment conducted 5 times in triplicate, n=15.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Difference of means</th>
<th>t</th>
<th>df</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO vs. nocodazole</td>
<td>0.142</td>
<td>10.754</td>
<td>28</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>DMSO vs. latrunculin B</td>
<td>0.157</td>
<td>12.387</td>
<td>28</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>nocodazole vs. latrunculin B</td>
<td>0.015</td>
<td>2.097</td>
<td>28</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

III.3.5. *P. neolepis*: immunofluorescence imaging of the MT cytoskeleton

Eukaryote microtubules (MTs) are assembled from protofilaments, arising from linear α and β tubulin heterodimer polymerization (Wickstead and Gull 2011). To investigate the MT arrangement in *P. neolepis*, secondary immunofluorescence was employed. The method relies on binding of primary anti-α-tubulin antibodies, and then their visualisation with secondary antibody-fluorophore conjugates, targeted to primary antibodies. Immunofluorescence labelled samples were examined with confocal laser scanning microscopy. Due to the small size of individual microtubules and background fluorescence due to cell fixation with glutaraldehyde, only higher order structures such as cables and networks were detectable (Tagliaferro et al. 1997). Imaging of labelled control sample cells did not show any MT- intracellular silica scale associations (Fig. III. 7.).
Fig. III. 7. Confocal microscopy 3D projection of a Z-stack of *P. neolepis* tubulin-immunolabelled control cells. 1: karyokinetical spindle between dividing cells 2-4: intracellular scales (tubulin: yellow, silica scales in green). Scale bar=5 µm.

Imaging of the nocodazole treated samples confirmed, that the tubulin polymerization inhibitor was effective and disrupted the microtubular network, as evident in Fig. III. 8., where the mitotic spindle between two dividing cells has been dissociated and only rudimentary spindle microtubules remained discernible. Virtually no MT structures apart from the haptonema and flagella persisted post nocodazole treatment. The remaining vestigial microtubular associations appeared severely disrupted (Fig. III. 8.).
Fig. III. 8. *P. neolepis*: 3D confocal microscopy projection of a Z-stack of anti-α-tubulin immunolabelled nocodazole treated cells. 1: a malformed, intracellular scale, 2-haptonema and a flagellum, 3-intracellular scale, 4-remnants of a dissociated karyokinetic spindle (tubulin: yellow, silica scales in green). Scale bar=5 µm.

A further control using secondary antibodies only (Texas-red conjugated antibodies specific to anti-mouse IgG), yielded non-specific labelling patterns in control samples (Fig. III. 9.), indicating, that patterning obtained from un-dosed and nocodazole dosed cells was a result of antibody-binding with α-tubulin and not a result of non-specific binding.
Fig. III. 9. Confocal microscopy image of a *P. neolepis* immunofluorescence labelling control: cells incubated with secondary antibodies only, exhibiting a non-specific labelling pattern (yellow). HCK123 scales labelled in green.

Imaging of the actin component of the cytoskeleton with Texas Red conjugated-Phalloidin—a fungus derived toxin binding specifically to actin was also attempted. The attempt was, however, unsuccessful, perhaps due to low F-actin content or its degradation, and was not pursued further.
III.3.6. Effects of cytoskeleton inhibition on culture health of C. pelagicus

Culture health in *C. pelagicus* was assessed in two ways: (1) measurement of photosynthetic efficiency following a 25 h cytoskeleton inhibitor treatment and (2) the ability to recalcify following removal of cytoskeleton inhibitors after the 25 h incubation period.

Photosystem II efficiency in *C. pelagicus* was not affected by cytoskeleton inhibitors, and only a small decrease in average F\textsubscript{v}/F\textsubscript{m} ratio was recorded for the latrunculin B treatment. However, given that cells were able to re-calcify normally (see below), this small decrease in F\textsubscript{v}/F\textsubscript{m} was deemed negligible (Table III. 6.).

Table III. 6. Photosynthetic efficiency recorded post 25 h cytoskeleton inhibitor treatment of *C. pelagicus*.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>DMSO Control</th>
<th>latrunculin B</th>
<th>nocodazole</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>average</strong></td>
<td>0.62</td>
<td>0.61</td>
<td>0.58</td>
<td>0.61</td>
</tr>
<tr>
<td>F\textsubscript{v}/F\textsubscript{m}</td>
<td>0.01</td>
<td>0.01</td>
<td>0.02</td>
<td>0.01</td>
</tr>
<tr>
<td><strong>SD</strong></td>
<td>0.01</td>
<td>0.01</td>
<td>0.02</td>
<td>0.01</td>
</tr>
</tbody>
</table>
III.4. Effects of cytoskeleton disruptors on biomineralisation in *C. pelagicus*: light microscopy imaging

In order to investigate the *in-vivo* effects of actin and tubulin disruption on *C. pelagicus* coccolithogenesis and its subsequent recovery from cytoskeleton inhibitors, differential imaging contrast light microscopy imaging (DIC) was employed. Cells were decalcified 1 h prior to the experiment (Fig. III. 10.), to enable tracking of coccoliths produced during the subsequent 24 h incubation with cytoskeleton inhibitors. Chemicals were then removed and replaced with fresh f/2 media in filtered sea water, and cells were imaged again at 24 and 48 h intervals to monitor recovery.

![Fig. III. 10. DIC of decalcified *C. pelagicus* cells at the beginning of the experiment. Scale bar=10 µm.](image-url)
Disruption of actin polymerization in *C. pelagicus* resulted in a complete cessation of coccolith secretion, and hence new coccolith production (Fig. III. 11. C). Coccoliths visible in some cells post treatment most likely represent those present in cells prior to dosing with latrunculin B (Fig. III. 10.). A number of cells without any intracellular coccoliths was also observed, and could be a result of actin inhibition or an intrinsic mechanism halting coccolithogenesis.

Cells treated with nocodazole were still able to produce and secrete coccoliths, however, coccoliths were malformed and often detached from cells (Fig. III. 11. D, Fig. III. 12.). These malformations were further investigated with SEM.

Both control and DMSO treated cells had re-deposited nearly full coccospsheres 24 h post treatment, indicating no adverse effects of DMSO on coccolithogenesis (Fig. III. 11. A,B).
Fig. III. 11. DIC images of cytoskeleton inhibitor treated *C. pelagicus*: A-D:24 h post treatment: A - control, B-DMSO, C-latrunculin B, D - nocodazole treatment. E-H - 48 h post cytoskeleton inhibitor removal: E - control, F - DMSO, G - latrunculin B, H - nocodazole. Each treatment was conducted in triplicate, images are representative of a response in all replicates. Intracellular coccoliths visible in latrunculin B treated cells are a pre-treatment artefact. Scale bars=10 μm.
Cells were successfully recovered post 24 h cytoskeleton inhibitor treatment and resumed calcification once the inhibitors have been removed (Fig. III. 11. E-H). Control, DMSO and most of the nocodazole treated cells reassembled a full coccosphere 48 h post experiment. The effect of latrunculin B on coccolithogenesis was more severe than that of nocodazole, however, cells resumed calcification as both coccoliths adhering to individual cells and a large amount of shed coccoliths was observed post 48 h latrunculin B removal (Fig. III. 11. G, Fig. III. 12.). The accumulation of shed coccoliths was a common feature between all treatments and consistently comprised of a high proportion of malformed coccoliths, which may have been the cause of their detachment/failure to attach to the cell surface in the first instance.
Fig. III. 12. DIC image of *C. pelagicus* post 48 h recovery from latrunculin B treatment. Image was collected from the centre of the dish, where all shed coccoliths would accumulate during sample transport. Note that the vast majority of shed coccoliths are malformed (marked with thin arrows, normal coccolith indicated with a thick arrow). Similar images were obtained from all treatments.

**III.4.1. SEM analysis of cytoskeleton inhibitor-induced coccolith malformations in *C. pelagicus***

Intracellular and extracellular coccoliths produced during treatment with cytoskeleton inhibitors were isolated and examined with SEM, to further investigate malformations visible on previously collected DIC images.

Coccoliths produced post cytoskeleton inhibitor treatments presented various states of completion and malformation and were therefore divided into three categories based on
their morphology, summarised in Fig. III. 13. A-D. Coccoliths classed as partially malformed/incomplete were characterised by a largely complete structure with some slight aberrations to the shape of crystal deposited (Fig. III. 13. B). The malformed coccoliths were characterised by completely distorted crystal structure and often resembled disorganised blocks of calcite aggregated into a larger "lump" structure (Fig. III. 13. C,D).

Fig. III. 13. FESEM images of *C. pelagicus* coccoliths used for morphological aberration ranking of coccoliths produced in controls, DMSO and nocodazole treatments: A - normal coccolith, B - partially malformed/incomplete coccolith, C, D - malformed coccoliths. Scale bars correspond to 1 µm.
Statistical analysis of frequency of occurrence of coccoliths belonging to three distinct classes between control and DMSO treatment confirmed, that these two samples were not significantly different from each other ($\chi^2$\(\text{df}=2, n=300)=0.16, p > 0.2\). The DMSO treatment data were then used as a more suitable control against the dataset obtained from the nocodazole treatment due to solvents' presence in the inhibitor-treated sample. The Chi-square comparison between the DMSO and nocodazole treatment revealed, that significantly more malformed and partially formed coccoliths were produced in nocodazole treated sample (Table III. 7.). Malformed and partially formed coccoliths accounted for 44.3% and 28% of the total number of coccoliths analysed in the nocodazole sample, whereas in the DMSO treatment, corresponding numbers were much lower at 15.6% and 18.6% respectively. Normal coccoliths in the nocodazole treatment comprised only 27.7% of the total sample, whereas the majority of coccoliths produced in the DMSO sample (65.7%) presented as normal.

<table>
<thead>
<tr>
<th>Coccolith category</th>
<th>$\chi^2$</th>
<th>Df</th>
<th>p</th>
<th>DMSO sample [%]</th>
<th>Nocodazole sample [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>66.0</td>
<td>1</td>
<td>&lt;0.05</td>
<td>65.70</td>
<td>27.70</td>
</tr>
<tr>
<td>Partially formed</td>
<td>16.7</td>
<td>1</td>
<td>&lt;0.05</td>
<td>15.60</td>
<td>28.00</td>
</tr>
<tr>
<td>Malformed</td>
<td>144.0</td>
<td>1</td>
<td>&lt;0.05</td>
<td>18.60</td>
<td>44.30</td>
</tr>
<tr>
<td>Whole sample</td>
<td>226.6</td>
<td>2</td>
<td>&lt;0.05</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Coccoliths obtained from latrunculin B treatment were not included into the statistical analysis, as virtually no coccoliths were observed to have been secreted during the DIC
imaging. Therefore, the images obtained from this treatment are either of coccoliths that were almost complete/at various stages of completion prior to dosing with latrunculin B and/or of those, that were being deposited during the treatment. It is therefore not possible to determine, if coccoliths were formed during the treatment. However, it appears that coccoliths extracted post incubation with latrunculin B, that were present in cells at various stages of formation prior to inhibitor treatment (or those that were being produced during the treatment) within cells have also been affected and severely malformed (Fig. III. 14. A-C). This indicates, some degree of calcification still occurred after latrunculin treatment had begun.

Fig. III. 14. A-C - malformed/incomplete coccoliths representative of latrunculin B treatment. As determined with DIC imaging, these coccoliths were mostly intracellular at the time of extraction. Scale bars correspond to 1 µm.

III.4.2. C. pelagicus: immunofluorescence imaging of the MT cytoskeleton

Confocal microscopy imaging of the α-tubulin-immunolabelled C. pelagicus cells revealed the presence of MT networks close to the CV, creating an ovoid structure
beneath the maturing coccolith (Fig. III. 15.:1,2). No intimate MT-CV association was observed, likely due to the resolution limitation arising from imaging of very fine structures with high background fluorescence levels.

Fig. III. 15. 3D projections of confocal microscopy Z-stacks of anti-α-tubulin immunolabelled *C. pelagicus* control cells. A: 1-3-tubulin network around the forming coccolith (tubulin: yellow, calcite reflectance in white). B-Same cells with the white channel switched off for clarity.

The extensive MT network in *C. pelagicus* cells was severely disrupted and reduced post 24 h nocodazole treatment (Fig. III. 16. A,B). Virtually no discernible organised MT features were visible post treatment, however, some remains of the MT structures were still present in some cells (Fig. III. 16. A,B).
Fig. III. 16. 3D projections of confocal microscopy Z-stacks of anti-α-tubulin immunolabelled *C. pelagicus* after 5 h treatment with nocodazole. A-1,3- coccolith vesicles with a forming coccolith. B-same cells with white channel turned off, with remnants of tubulin cables near the coccolith of cell 1 (α-tubulin: yellow, calcite reflectance in white). Scale bar=5 μm.

Incubation of *C. pelagicus* with secondary antibody only did not yield any specific labelling patterns (Fig. III. 17. A,B).
Fig. III. 17. 3D confocal microscopy projections of Z-stacks of *C. pelagicus* nonspecific labelling control (secondary antibodies only, shown in yellow). A - nonspecific staining only, forming coccoliths in white. B - same image with the white channel switched off for clarity.

**III.5. DISCUSSION**

**III.5.1. Cytoskeleton inhibitors and culture health**

Monitoring of the *P. neolepis* and *C. pelagicus* culture viability during incubation with cytoskeleton inhibitors was essential for establishing that the effects observed resulted from disruption of the target cytoskeleton components and were not an artefact of poor culture health. Screening of PSII did not indicate any significant departures in the $F_v/F_m$ ratios from the control in *C. pelagicus* and only a small decrease in PSII efficiency was found in latrunculin B treated *P. neolepis* cultures, potentially caused by higher sensitivity of *P. neolepis* to disruption of actin-dependent photosynthesis regulation or
via secondary effects arising from interruption of other vital, actin-based cellular processes (Takagi 2003).

SYTOX Green screening of *P. neolepis* control cultures indicated that high percentages of cells with reduced viability were normally present in exponentially growing cultures and were therefore considered to arise from intrinsic fragility of *P. neolepis*. High percentage of cells with reduced viability is also normally found in field phytoplankton samples (30-60% of the population) as well as in cultures of other fragile algal species (Veldhuis et al. 2001; Zetsche and Meysman 2012). The significant decrease in the share of unlabelled cells, with a concomitant significant increase in strongly labelled cells in nocodazole treatment could be an artefact resulting from an increased permeability of the plasma membrane due to the presence of DMSO, which is known to permeabilize and thin membranes, combined with effects of a potent microtubule inhibitor (Gurtovenko and Anwar 2007). This seems likely, as the efficiency of PSII was not affected by this drug.

The mortality recorded for cytoskeleton inhibitor and DMSO treatments in *P. neolepis* was considered minor and a likely result of cell loss to autolysis, which could not have been compensated by cell division, as both latrunculin B and nocodazole disrupt cytoskeleton integrity, prompting mitosis suppression (Gachet et al. 2001; Jordan et al. 1992). In the case of DMSO, it is more likely that it had a minor, negative effect on cell growth rather than mortality, potentially by secondarily altering water and ion transport in cells (Gurtovenko and Anwar 2007).

Calcification recovery assay in *C. pelagicus* showed that coccolithogenesis was re-established in all treatments after inhibitor removal. The slower recovery of cells following latrunculin B treatment might be either a result of the presence of residual inhibitor due to entrapment in cell coating or a slower recovery of actin cytoskeleton.
Overall, the combined results indicate that both *P. neolepis* and *C. pelagicus* cells remained viable and in good healthy during cytoskeleton inhibitor treatments and responses observed were specific to disruption of target elements of cytoskeletal network.

**III.5.2. The role of actin in biomineralisation**

Upon incubation with latrunculin B, *P. neolepis* cells were unable to secrete scales, indicating the presence of an actin-dependent mechanism responsible for silica scale exocytosis. However, *P. neolepis* cells were still capable of forming scales internally, where they accumulated, although there was a significant decrease in silicification rate, as determined with flow cytometry. This, along with no obvious scale malformations suggests, that the silica biodeposition process in this haptophyte is not strictly actin dependent and the main factor limiting scale formation when actin is disrupted is the cells' physical capacity to maintain multiple intracellular scales. As cells were still able to deposit scales it appears, that impairing actin cytoskeleton did not majorly interfere with intracellular transport of components required for silica scale biomineralisation to be carried out.

In contrast to the above response of *P. neolepis*, incubation of *C. pelagicus* with latrunculin B had more severe effects. In this case, the entire process of coccolithogenesis and coccolith secretion was brought to a complete halt following F-actin disruption. This indicates, that actin-dependent intracellular transport of vesicles and substrates, facilitated by motor proteins is essential for coccolithogenesis to proceed in this species. The severe intracellular coccolith malformations observed post actin
inhibitor treatment suggest, that actin may be involved in actively shaping the growing coccolith. However, it cannot be ruled out that the short term exposure to low pH during decalcification could have contributed to malformations observed. The pH shock would have caused a temporary cell acidification, coupled with a decrease in calcification rate for a short period of time, until the cells have re-adjusted pH and re-established normal calcification rate (Taylor et al. 2011). The consequence of this short-term interference with the calcification system is an increased malformation of coccoliths formed during and directly after the decalcification event, contributing to the 19% of malformed coccoliths recorded in control and DMSO samples. Additionally, a small percentage of malformed coccoliths is naturally produced in coccolithophore cultures (GMD, personal observation; Langer et al., 2010).

Compared to the previous study by Langer et al. (2010), involving actin disruption in another coccolithophore, *Emiliania huxleyi*, effects of actin disruption in *C. pelagicus* were much more severe, causing a complete inhibition of coccolithogenesis. In the study by Langer et al. (2010), treatment with cytochalasin B did not completely stop coccolith formation, but induced a 50% increase in malformed coccolith production instead. This divergence in response might arise from the fact, that unlike latrunculins, cytochalasin B does not depolymerize F-actin, but caps the actively growing barbed ends, preventing both polymerization and depolymerisation, inducing filament shortening (MacLean-Fletcher and Pollard 1980; Morton et al. 2000; Theodoropoulos et al. 1994). It also promotes isotropic F-actin network disruption, creating focal F-actin accumulations, however it does not affect filament annealing or monomer addition at the pointed end of actin microfilaments (Forscher and Smith 1988; MacLean-Fletcher and Pollard 1980; Schliwa 1982). Additionally, at the concentration used by Langer et al. (2010), cells were still able to divide, suggesting, that although severely impaired, F-
actin was still present and probably still able to facilitate motor-protein or microfilament polymerization/depolymerization driven intracellular transport to some extent. Considering the above, it is conceivable, that the severe coccolith malformations in latrunculin B treated *C. pelagicus* are at least partially due to cells actively forming coccoliths upon drug addition, resulting in a progressive F-actin inhibition and depolymerisation as the drug takes effect, causing coccolith malformation and ultimately bringing coccolithogenesis to a stop.

The observed inhibition of biomineralised scale secretion in both *P. neolepis* and *C. pelagicus* post latrunculin B treatment indicates, that this process, along with subsequent scale positioning on the cell surface is actin-based in both organisms. This is consistent with previous reports on transport of organelles, secretory vesicles and vacuoles in plant, animal and fungal cells, which have been show to be driven by myosin-facilitated trafficking along actin cables, and rapid membrane movement in plants (Hammer and Sellers 2011; Schott et al. 2002; Tabb et al. 1998; Vale 2003).

III.5.3. *The role of tubulin in biomineralisation*

Treatment of *P. neolepis* and *C. pelagicus* with nocodazole caused severe disintegration and almost complete depolymerisation of MTs in both species, as confirmed with immunofluorescence imaging. Disruption of the microtubular cytoskeleton caused a significant decrease in scale production in *P. neolepis*, indicating its heavy involvement in silica biomineralisation in this species. The observed decrease in silicification rates could result from disruption of three main mechanisms essential for biomineralisation to proceed and mediated by MTs: (1) intracellular transport, (2) active, mechanical
shaping of the growing biomineral structure and (3) secretion of mature biomineralised elements.

The observed decrease in silicification rate in *P. neolepis* could potentially arise from substrate limitation, as like F-actin, MTs form extensive and dynamic intracellular networks, serving as tracks for intracellular transport-mediated motor proteins, or direct attachment sites for the cargo (Vale 2003). MT-based motor proteins, such as kinesins and dyneins are responsible for transporting both non-membranous molecules, such as mRNA as well as membranous vacuoles, vesicles, lysosomes, organelles, or endoplasmic reticulum (ER) (Brendza et al. 2000). Therefore, disruption of microtubular network impairs both MT and motor protein mediated transport of specific substrates into the biomineralising compartments such as CV or SDV. It also affects distribution and dynamics of the ER and the Golgi body, structures that are both heavily involved in biomineralisation in haptophytes, resulting in the adverse effects of tubulin disruption on both silica scale and coccolith formation (Billard 2004; Lippincott-Schwartz et al. 1995; Wubbolts et al. 1999). Due to the profound role of MTs in intracellular transport, MT disruption could also affect the general cell health, potentially introducing secondary effects leading to reduction of cell capacity for biomineralisation and further contributing to the observed decrease in silica scale production in *P. neolepis*. The frequent manifestation of abnormally small, scales and overall shift towards production of smaller scales post nocodazole treatment in *P. neolepis* also indicates, that MTs-although not forming structures visible with immunofluorescence or TEM-are involved in maintaining the size and shape of the newly forming silica scale in this species. This might be achieved either via direct interaction with the SDV or by acting in concert with actin cytoskeleton, with scale size potentially limited by substrate availability due to disruption of the intracellular, tubulin-based transport system (Rodriguez et al. 2003; Yoshida et al. 2006).
Depolymerisation of the microtubular network in *C. pelagicus* caused a significant increase in mild to severe coccolith malformation, consistent with findings by Langer et al. (2010). In fact, only 28% of coccoliths obtained post tubulin disruption in this species were fully developed. The arrangement of calcite under nocodazole treatment became severely distorted, indicating that MTs are likely to be involved in delimiting and guiding crystal growth via mechanical control over the CV, as evident from coccoliths resembling disarrayed blocks of crystals obtained from this treatment (Fig. 13B-D). Indeed, extensive MT networks have been confirmed to be localized in close proximity to the maturing CV, and could be utilised for substrate transportation into the CV (Fig. 15A,B). Existence of fine microtubular structures directly associated with the growing coccoliths and not resolved with immunofluorescence is also plausible. Based on the severe malformation cases, it is also possible, that formation of the organic template involved in guiding nucleation of crystals during coccolithogenesis could be affected, resulting in displacement of crystal nucleation centres, accounting for the complete lack of organisation of the developing structure. In addition to the increased production of aberrant coccoliths, the frequency of occurrence of slightly malformed and incomplete coccoliths also increased post nocodazole treatment, indicating increased premature secretion to be related to MT network disruption in *C. pelagicus*. This is in contrast with the findings of Langer et al. (2010), who concluded, that this process was cytoskeleton independent and might be due to the specifics of the cytoskeleton inhibitor used for the purpose of this study.

It will be important to determine whether the calcification rate in *C. pelagicus* has also been affected by MT disruption, as over 70% of coccoliths from the nocodazole treatment were to some extent malformed, and detached from cells. Consequently, very few coccoliths were seen attached to the cell surface under this treatment, with the
majority of coccoliths accumulating at the bottom of the dish, making visual estimations impossible. As similar accumulations of shed, malformed coccoliths were seen in all treatments and after recovery experiments, it has been concluded, that aberrations in coccolith shape interfere with its adherence to the cell surface.

III.5.4. Differential role of cytoskeleton in silicification in haptophyte and Bacillariophyte (diatom) algae

Silicification in diatoms requires heavy involvement of both the actin and the tubulin cytoskeleton, actively controlling frustule morphogenesis by mechanical moulding of the SDV (Tesson and Hildebrand 2010; Van De Meene and Pickett-Heaps 2002). Actin has been identified as the major component involved in control over silica deposition during diatom valve formation at the micro and meso scale as well as the overall valve shape, and positioning of structures such as e.g. the raphe (Cohn et al. 1989; Tesson and Hildebrand 2010; Van De Meene and Pickett-Heaps 2002). In contrast, in the haptophyte, *P. neolepis*, actin appears to be involved in exocytosis rather than formation of the silica scales, as treatment with an actin inhibitor latrunculin B did not produce any visible aberrations in the overall shape of scales deposited. Microtubules in diatoms are responsible for localising the SDV formation site, and frustule formation cannot be initiated if MTs are absent (Van De Meene and Pickett-Heaps 2002). Disruption of MTs during valve morphogenesis results in severe distortions, as the microtubular cytoskeleton in diatoms is responsible for strengthening and positioning of specific structures of the frustule (Van De Meene and Pickett-Heaps 2002). In *P. neolepis*, although no SDV-MT associations could be resolved with immunofluorescence, MTs are involved in controlling the overall shape of silica scales. This is potentially achieved
by delimiting the shape of the SDV. Disruption of MTs in this species results in production of dwarf scales and alteration of the shape, producing more round morphologies. In this case scale production was severely decreased, but nucleation of new scales was not completely inhibited like initiation of frustule formation in diatoms.

III.6. CONCLUSIONS

Differential roles in biomineralisation for actin and tubulin in the silicification and calcification in haptophytes and bacillariophytes have been established. Actin inhibition in haptophytes prevents secretion of the biomineralised structure, however, the entire biomineralisation process is halted in the calcifier, whilst the silicifier is still able to proceed with siliceous scale deposition until its cell capacity is reached. Effects of cytoskeleton inhibition on formation of mineralized scales were mainly caused by direct, mechanical interaction of the cytoskeleton with the vesicle, in which the structure was being deposited and by the intracellular transport, limiting substrate delivery and capacity for structure exocytosis.

The cytoskeleton appears to be more heavily involved in morphogenesis of biomineralised scales in *C. pelagicus* than in *P. neolepis*, as cytoskeleton disruption resulted in severe coccolith malformations. In *P. neolepis* silica scale morphology was not as severely affected, although production of abnormally small silica scales was observed post microtubule disruption (see Table III.8, for a summary of the effects of cytoskeleton inhibitors on *P. neolepis* and *C. pelagicus*). The differences in the use of cytoskeletal elements for biomineralisation in these two haptophytes are likely to be related to the differences in the formation site of biomineralised structures (Golgi-
derived CV in *C. pelagicus* and an SDV in *P. neolepis*), the absence of an organic scale serving as a baseplate in *P. neolepis* and a different secretion site of the completed structure (see Chapter II for details on silica scale formation process).
Table III. 8. Summary of the effects of cytoskeleton inhibitors on *P. neolepis* and *C. pelagicus*.

<table>
<thead>
<tr>
<th>Cytoskeleton inhibitor</th>
<th>Effects</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Latrunculin B (actin inhibitor)</td>
<td>Exocytosis of the biomineralized scales inhibited</td>
<td>Silica scales formed and accumulated intracellularly until internal storage capacity exceeded.</td>
<td>Coccolith formation ceased</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Significant decrease in silica scale production.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>No visible malformations to the silica scales</td>
<td>Coccoliths malformed</td>
</tr>
<tr>
<td>Nocodazole (MT-inhibitor)</td>
<td></td>
<td>The size distribution of the silica scales shifted significantly towards smaller sizes. Scale morphology more round, frequent occurrence of abnormally small &quot;dwarf&quot; scales</td>
<td>Significant increase in production of malformed coccoliths. Frequent detachment of coccoliths from the cell surface, likely due to the malformations</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Significant decrease in silica scale production</td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER IV: BIOCHEMICAL ANALYSIS OF PRYMNESIUM NEOLEPIS SILICA SCALES

IV.1. INTRODUCTION

Silica biomineralisation mechanisms have been of great interest due to their potential for extrapolation and subsequent use in materials science, as well as their relevance from the evolutionary point of view (i.e. evolution of different biomineralisation systems in eukaryotes). Therefore, they have been investigated in a variety of organisms (see Chapter I for an extensive review). Most research efforts within this area have been focused on the silicification systems of two evolutionarily distinct groups of organisms: the diatoms (unicellular algae) and sponges (simple metazoans). The former utilise silica to deposit a rigid external cell wall, termed the frustule, whereas the latter produce silica spicules, maintaining structural support of the organism amongst other functions (Uriz et al. 2003). In both cases silica structures produced contain an organic phase directly involved in silica precipitation and organics acting as a scaffolding for the developing structures (Pickett-Heaps 1990; Schröder et al. 2007). Silicification systems employed by these two groups of organisms are, however, quite different in terms of the major organic fractions involved in silica deposition and their mode of activity, i.e. enzymes in sponges vs. polycationic polypeptides in diatoms (see sections I.3. and I.6. for detailed information). Nonetheless, they also share common traits. These include the presence of an organic matrix underlying the biosilica structures, acting as a template in conjunction with the remaining organic fraction capable of active precipitation of silica.
out of solution, such as long chain polyamines (LCPAs) (e.g. Matsunaga, Sakai et al. 2007; Sumper, Kröger et al. 2004).

In order to investigate the biosilica associated organic fraction in different taxa a variety of approaches for extraction from the silica matrix have been developed. The oldest and still popular methods for biosilica dissolution are based on the use of HF acid (Ehrlich et al. 2010). These methods however have significant drawbacks, in that HF has been found to cleave disulfide, O-glycosidic and phosphate ester bonds leading to dephosphorylation of peptides and proteins recovered after treatment (Fuchs and Gilvarg 1978; Mort and Lamport 1977). Another popular method of silica dissolution, commonly used for extraction of the organic matrix from sponge spicules is the use of alkali such as NaOH (Ehrlich et al. 2010). Alkali can be used to etch biosilica at various concentrations and with addition of other chemicals, such as sodium dodecyl sulfate and other anionic biosurfactants (Ehrlich et al. 2010; Ehrlich et al. 2006). However, despite the attempts to reduce the detrimental effects that this treatment has on the organic phase, NaOH based methods cause protein denaturation (Ehrlich et al. 2010).

Finally, a relatively mild treatment involving acidified ammonium fluoride (NH₄F) solution, which dissolves biosilica via conversion into ammonium hexafluorosilicate can also be applied (Ehrlich et al. 2010; Kröger et al. 2002). This method is often used as an alternative to the harsh HF or alkali treatments, and was successfully applied to extract relatively intact proteins and LCPAs from diatom biosilica (Kröger et al. 2002; Swift and Wheeler 1992).

Overall, as each silica dissolution method has its own constraints and only allows to retrieve biomolecules which are not specifically affected by it, the NH₄F based method was deemed to be the best choice for extraction of biosilica-associated organics from the silicifier investigated in this study - *P. neolepis*. 
*Prymnesium neolepis* is a marine haptophyte alga, producing small (≈1-1.5 μm), unmineralized organic scales as well as larger (≈2-17 μm) silica scales (see section I.3. for detailed description). To date, it is the only haptophyte alga known to deposit silica structures rather than calcite-mineral phase utilized by the dominant biomineralizing group within this clade-coccolithophores. As such, it presents a unique and interesting opportunity to investigate the biochemical composition and mechanisms of silicification within another evolutionarily distinct, calcifier-dominated group of organisms. This would enable further comparisons between the systems as well as provide an insight into the evolution of the ability to silicify within the algal-and to some extent-within the metazoan clades.

Therefore, the objectives of this chapter are to:

1. Verify the presence of silica-scale associated organics.
2. To characterize the organics, if present.
3. To draw comparisons between the silicification systems present in *P. neolepis*, diatoms and sponges.
IV.2. METHODS

IV.2.1. Silica scale purification

The silica scale purification protocol was modified from a procedure for the isolation of diatom frustules (Kröger et al. 1999). Cells were harvested by low pressure suction pump filtration, removed from filters with a pipette and pelleted by centrifugation. An excess of 2% SDS in 100 mM EDTA, 0.1 M Tris at pH 8.00 was added, the solution was vortexed and left for 30-60 min, with occasional vortexing in order to dissociate the protoplast debris from scales. Samples were then centrifuged at 6000x g for 10-15 min, supernatant was discarded and the procedure was repeated 5-7 times. Samples were then sonicated for 10-15 min, vortexed, centrifuged at 6000x g and the supernatant was discarded. Samples were then boiled in the SDS solution at 95 °C for 10 min to further remove the organic phase from silica, vortexed and centrifuged. Samples were washed with the SDS solution or sonicated again if required based on a visual assessment with a microscope. Once sample purity was deemed satisfactory (i.e. no pigments or any other visible protoplast contamination was observed), the samples were washed 5x with deionised water and sub-sampled for further sample purity verification using a FESEM and TEM. This involved further subsampling of the material onto stubs/copper grids and screening for the presence of cellular debris and organic scales. Once samples were confirmed to consist of silica scales only and the absence of protoplast debris and organic scales were confirmed, samples were frozen until further processing.
IV.2.2. Glycerol cushion separation of the purified silica scales and further assessment of sample purity

A portion of the biosilica material purified as described above was further separated on a glycerol cushion in order to verify if samples were contaminated with organic scales produced by *P. neolepis* (Yoshida et al. 2006). 176.5 mg of silica scales were resuspended in 2.5 ml of deionised water and 500 µl of the suspension was overlaid on top of 4 ml of 50% glycerol solution and spun down at 3200x g for 2 minutes. The supernatant was collected into a separate tube, and the pellet was resuspended in 500 µl of deionised water and spun down through a glycerol cushion again. The procedure was repeated 4 times. The pellet was then washed 5x with deionised water and stored in the freezer. The supernatant collected was also pelleted for 15 min at 6000x g, washed 5x with deionised water and frozen. Both the purified silica scale pellet (termed "scale fraction") and the supernatant (termed "supernatant fraction"), which should contain the organic scales (if present), were then sub-sampled for FESEM examination. The procedure yielded 103 mg of the silica scale fraction and 34 mg of the supernatant fraction, in which all organic scales (if present) should be retained along with lighter silica scale material.

The material was then treated with ammonium fluoride to extract the NH$_4$F soluble and insoluble organic fractions. The NH$_4$F soluble fractions were then run on a 16% Schägger gel (Schägger and von Jagow 1987) and compared. The insoluble material was washed 5x with deionised water and examined with a FESEM and TEM to reassess sample purity and the absence of organic scale contaminants.

In order to further ensure the purity of biosilica material, 30 mg of biosilica scales were incubated on a rocking table for a period of 20 h with 100 mM EDTA (pH 8.00) at 4 ºC, to extract any proteins that were accessible prior to silica dissolution (method modified
from Kröger et al., 1997). After the incubation period was over, the sample was centrifuged for 1 min at max rpm, the supernatant was transferred to a 3 kDa cutoff filtration column (Amicon) and concentrated to 500 µl. The sample was then washed once with 5 ml of 50 mM EDTA, lyophilized and resuspended in 100 µl of deionised water. Sample was then run on a 16% Schägger gel and stained with EZBlue gel staining reagent (Sigma-Aldrich) to confirm the absence of protein contamination.

IV.2.3. Ammonium fluoride extraction of the organic phase

In order to dissolve the silica component of scales, 2 ml of 10 M NH₄F was added to 30-100 mg biosilica sample and vortexed until the pellet was dissolved. 0.5 ml of 6 M HCl was then added to the mixture, vortexed, and the pH was adjusted to between 4-5 with 6 M HCl. The sample was incubated on bench for 30 min. Mixture was then centrifuged for 15 min at max rpm and the supernatant was transferred to a 3 kDa cutoff filtration column (Amicon) to concentrate and desalt protein. The NH₄F insoluble pellet was washed twice with 50 mM ammonium acetate and stored in the freezer. The supernatant from the insoluble phase washes was added to the amicon column with the rest of the supernatant and concentrated to 1 ml. The concentrate was washed once with 500 mM and 200 mM NH₄Ac (ammonium acetate) and 3x with 50 mM NH₄Ac. 5 ml of each solution was used per wash. The sample was then further concentrated to 150-400 µl. All flow through was collected and stored in the freezer for further analysis. Protocol was modified from (Kröger et al. 1999).

Fig. IV. 1. shows the above silica scale purification steps and the fractions obtained post NH₄F treatment of the biosilica.
Fig. IV. 1. A schematic summarising processing of the *P. neolepis* biosilica in terms of fractionation post SDS/EDTA purification of scales and the subsequent phase separation post NH$_4$F extraction.
IV.2.4. Separation of the putative polyamine fraction from the NH₄F flow-through on an ion exchange column

The flow through from the NH₄F extraction described above was diluted (4.5:100) with deionised water and passed through an 8 ml liquid chromatography column (Sigma) containing 2 ml of high S strong cation exchange resin (Bio-Rad). The column was previously washed 10x with deionised water, the resin was added and then the system was flushed with 10 ml of deionised water, 10 ml of 2 M ammonium acetate and then again washed 3x with deionised water. The diluted flow-through sample was then fed into the column, the whole process being gravity-driven. Once the sample has passed through the column, the resin was washed 3x with 1ml of 200 mM NH₄Ac and eluted 4x with 1ml of 2 M ammonia, the eluant was neutralised with acetic acid and lyophilized (sample referred to as "ammonium eluate"). Resin was then eluted again 4x with 1ml of 2 M NaCl, 100 mM NH₄OH, 50 mM NH₄Ac, neutralised with acetic acid and dialysed 1x against 5 l of 50 mM ammonium acetate in a 1 kDa dialysis tube, collected into a tube and lyophilized (sample referred to as "dialysate"). Both samples were then resuspended in 100 µl of deionised water and examined on a 16% Schägger gel.

IV.2.5. Polyacrylamide gel analysis

All NH₄F soluble organic phase extracts were run on a 16% Schägger gel (Schägger and von Jagow 1987) in order to retain both high and low molecular weight components. The gels were then stained with Coomassie Brilliant Blue (colloidal or R-250, Sigma-Aldrich), Stains-all (Sigma-Aldrich) as per manufacturer's instructions and silver
stained following a protocol modified from (Chevallet et al. 2006). Sequential staining with Coomassie Brilliant Blue, Stains-all and silver stain was also used to enhance the sensitivity of labelling (Goldberg and Warner 1997). Unstained Broad Range Page Ruler (Fermentas) and Mark 12 protein standard (Invitrogen) were used as a size reference for Coomassie and silver staining. Pre-stained Ladder (Thermo-Scientific) was used with Stains-all. Additionally, a standard 12% SDS-PAGE was used for diatom protein digest analysis.

**IV.2.6. Trypsin digestion of the NH₄F soluble organic extract**

In order to determine if the organic fraction obtained as the NH₄F soluble phase consisted of protein, a trypsin digest was conducted, where 10 µl of the NH₄F soluble extract was treated with 2 µg of TPCK (tosyl phenylalanyl chloromethyl ketone) treated trypsin in a 15 µl reaction, buffered with 100 mM Tris-Cl at pH 8.80. A control without trypsin was also included. Samples were incubated at 37 °C overnight and analysed on a 16% Schägger-PAGE.

Controls for the digest consisted of 15µl reactions containing 2 µg of TPCK treated trypsin buffered with 100 mM Tris-Cl at pH 8.80 and 2 µg of diatom proteins-silaffin rSilC and cingulin rmCinY3 (kindly provided by Prof. Kröger) and one reaction only containing trypsin. Samples were incubated at 37 °C overnight and analysed on 12% SDS-PAGE gel.
IV.2.7. Amino acid analysis

Amino acid analysis was carried out using a protocol modified from Bidlingmeyer et al. (1984). 10 mg of purified biosilica material and an equivalent amount of deionised water for a blank control were transferred into 2 ml glass tubes, covered with Parafilm and lyophilized. 100 µl of 6 M HCl (ultrapure, Pierce) was added into each sample. 100 µl of phenol (100%, ultrapure) was then added to prevent oxygen degradation of the amino acids. Samples were then transferred into hydrolysis vials, frozen with liquid nitrogen and evacuated with a vacuum pump for 3 min and nitrogen was pumped into the vials. The procedure was repeated 3x to eliminate oxygen. The samples were then thawed and incubated at 110 °C for 24 h. The hydrolyzed samples were transferred into Eppendorf tubes, vacuum dried and stored at -20 °C until analysis. The samples were then PITC (phenylisothiocyanate) derivitized and subjected to a HPLC (high-performance liquid chromatography) analysis using Dionex UltiMate® 3000 HPLC (Thermo Scientific) against appropriate amino acid standards prepared fresh before the analysis.

IV.2.8. Mass spectrometry analysis

Mass spectrometry analysis of the putative polyamine phase separated on the cation exchanger was kindly provided by Prof. Kröger. A replicate sample of the polyamine extract and the excised protein bands from the NH₄F-soluble extract visualized on a 16% Schägger gel with EZBlue (Sigma-Aldrich) (one band at≈63 kDa and two bands around 55 kDa) were sent for mass spectrometry analysis to Alta Bioscience (UK). To aid the mass spectrometry identification of the proteins, the *P. neolepis* transcriptome
Durak, G.M.  

Chapter IV: *Biochemical analysis of P. neolepis silica scales*

(kindly supplied by Dr. I. Probert) was translated into protein sequences in all 6 possible open reading frames and forwarded to Alta Bioscience.

Protein samples were analysed using an Orbitrap Mass Spectrometer (Thermo Scientific), following a standard LC-MS/MS (liquid chromatography–mass spectrometry/mass spectrometry) analysis of trypsin digested samples. Peptide sequences obtained were then compared against the transcriptome data translated into protein sequences. DNA sequences corresponding to the peptide data generated by the MS were recovered from the transcriptome, translated into protein sequences with BioEdit software and blasted against the NCBI protein database in order to look for homologous sequences in other taxa. Amino acid composition of the two protein sequences retrieved were generated with BioEdit software.

**IV.2.9. FESEM, TEM and EDX analysis of the NH₄F insoluble extract**

The NH₄F insoluble extract was washed 5x with deionised water and 5x with absolute ethanol (molecular grade) before being deposited on an aluminium stub in order to prevent appearance of drying artefacts on the organic structures. FESEM/TEM imaging and EDX analysis were conducted as described in section II.2.6.

**IV.2.10. Confocal microscopy imaging of the NH₄F insoluble extract**

Alexa-Fluor conjugated Wheat Germ Agglutinin (WGA, Invitrogen), Calcofluor White (CW, Sigma) and Concanavalin-A (Con-A, Invitrogen), targeting β 1,4 N-acetyl glucosamine linkages (WGA), β 1,3- and β 1,4- linkages (CW) and internal and non-
reducing, terminal α-D-mannosyl/glucosyl groups in polysaccharides (Con-A) (Harrington and Hageage 2003; Mandal et al. 1994; Nagata and Burger 1974) were selected to provide preliminary information on the presence of polysaccharides in the NH₄F insoluble extract. Concentrations and incubation times used to label the extract are specified in Table IV. 1.

Table IV. 1. Concentrations and incubation times of fluorophores used.

<table>
<thead>
<tr>
<th>Dye</th>
<th>Concentration</th>
<th>Incubation time [min]</th>
</tr>
</thead>
<tbody>
<tr>
<td>WGA</td>
<td>5µg/ml</td>
<td>20</td>
</tr>
<tr>
<td>CW</td>
<td>10µg/ml</td>
<td>20</td>
</tr>
<tr>
<td>Con-A</td>
<td>20µg/ml</td>
<td>60</td>
</tr>
</tbody>
</table>

Additionally, controls with whole cells and whole scale extracts were incubated with the dyes. Imaging was done on a Zeiss LSM 510 meta confocal laser scanning microscope. Alex-fluor WGA and Con-A were imaged using an argon laser, with excitation and emission filters set to λ=488 nm and λ=500-550 nm respectively. CW was imaged using a multiphoton excitation via a Mai Tai pulsed infra-red laser (Spectraphysics), set to λ=740 nm excitation and λ=435-485 nm emission.
IV.3. RESULTS

**IV.3.1. Amino acid analysis of purified P. neolepis biosilica**

In order to provide preliminary information on the presence and nature of the organic component associated with silicified scales, the amino acid content of purified biosilica was analysed using HCl/phenol digestion. This approach allows to confirm the likely presence of proteins in the silica material and indicates the gross amino acid composition of the silica scale associated organic fraction.

The amino acid analysis of the purified whole scales resulted in recovery of 13 different amino acids, indicating that an organic phase was indeed present and putatively contained protein. Biosilica was confirmed to be proline rich, relative to other amino acid residues detected (Fig. IV. 2.).
Fig. IV. 2. HPLC chromatogram of amino acids of the whole scales. Abbreviations of the amino acid residues are as follows: D - aspartic acid, E - glutamic acid, O - glutamine, S - serine, G - glycine, T - threonine, A- alanine, P - proline, V - valine, I - isoleucine, L - leucine, F - phenylalanine, K- lysine.

IV.3.2. NH₄F soluble phase extract: putative protein and polyamine phase

In order to recover whole proteins from *P. neolepis* biosilica without hydrolysing them, dissolution of silica with acidified NH₄F was applied. The resulting NH₄F soluble fraction, containing silica-associated organic phase extract was run on a 16% Schägger gel, yielding 2 bands between 50-70 kDa (one singlet and one doublet) and a broad band around 2.5 kDa (Fig. IV. 3. A-C). The broad band around 2.5 kDa has only labelled with Coomassie Blue, suggesting binding by charge in absence of protein and was predicted to likely contain polyamines as it was the case with diatoms (Fig. IV. 3. A-C) (Prof. Kröger, personal communication). The two bands between 50-70 kDa
labelled pink with Stains-all, indicating presence of potentially slightly acidic proteins (Fig. IV. 3. C).

Fig. IV. 3. NH4F soluble extract run on a 16% Schägger gel, subsequently stained with:

A - Coomassie Blue, B - silver stain, C - Stains-all. Molecular weight markers are indicated on the left hand side lane on gels.
IV.3.3. Sample purity verification: Schägger-PAGE analysis of the NH₄F soluble phase generated from glycerol cushion-separated silica scales and EDTA extraction of protein from whole scales

In addition to the silica scales, *P. neolepis* also produces non-silicified, organic scales which could potentially contaminate the biosilica fraction and contribute to the organics recovered. Separation of the biosilica material on the glycerol cushion aimed to fractionate the heavier silica scales separate from the lighter, organic scales/remnants of organic debris and shards of silica scales by density, with the lighter (organic) fraction being unable to penetrate into the cushion. Thus, the pure "silica scale" phase at the bottom of the cushion and the overlying "supernatant" phase, containing all putative organic contamination and lighter siliceous material were obtained. No organic scale/debris contamination was detected upon TEM and FESEM screening of subsamples of the above fractions (appendix IV.1.). TEM and FESEM screening of the NH₄F insoluble extract obtained from the "supernatant" fraction, in which contamination was expected to be more evident due to the "enrichment" in the putative organic contamination and subsequent demineralization did not show any evidence of contamination either.

Analysis of the NH₄F soluble extract of the "silica scale" and "supernatant" fractions yielded identical Schägger-PAGE labelling pattern (Fig. IV. 4.). This further suggested, that the "silica scale" and "supernatant" fractions were unlikely to be contaminated with organic scales produced by *P. neolepis*, as the banding pattern would differ between the two extracts if organic scales/contaminants were responsible. Thus the banding visible on the Schägger-PAGE represent an NH₄F soluble organic phase intimately associated with the silica scales.
Fig. IV. 4. Coomassie Blue staining of the NH4F soluble extract of the silica scale fraction run on a 16% Schägger gel (lane 1) and the supernatant fraction (lane 2).

Schägger-PAGE analysis of the EDTA extract from the whole scales did not detect the presence of any protein, further confirming that the protoplast and organic scale contamination was absent from the biosilica material.

IV.3.4. Trypsin digest of the NH4F soluble extract: verification of presence of protein

In order to confirm, that bands between 50-70 kDa consisted of protein, a trypsin digestion of the NH4F soluble extract was carried out. Upon trypsin digestion, the two bands between 50-70 kDa disappeared (Fig. IV. 5, A,B). The broad band below 2.5 kDa however persisted post digest, suggesting that it might not consist of protein (Fig. IV. 5, A,B). Both diatom rmCinY3 and rSilC proteins were digested by trypsin (Fig. IV. 5, C). Trypsin itself has undergone self digestion and no band was observed for controls containing trypsin only (Fig. IV. 5, A-C).
Fig. IV. 5. A - Coomassie stained 16% Schägger gel: lane 1 - trypsin digested NH4F soluble extract, lane 2 - non-digested NH4F extract control, lane 3 - non-digested rmCinY3 control, lane 4 - trypsin, B - gel A stained with silver stain on top of Coomassie Blue and Stains-all, C - Coomassie Blue stained 12% SDS-PAGE gel: lane 1: trypsin digested rSilC, lane 2 - trypsin digested rmCinY3, lane 3 - non-digested rSilC control, lane 4 - non-digested rmCinY3 control, lane 5 - trypsin.
IV.3.5. Cation-exchange liquid chromatography column samples: purification of putative LCPAs from the NH₄F extraction flow through

LCPAs purification via cation exchange was possible on account of their positive charge, after the preliminary separation from larger proteins was performed on a size seclusion column (Kröger et al. 2000). The putative LCPA material retained in the NH₄F flow through fraction, subsequently bound to the HighS cation exchange resin and eluted with ammonia yielded one broad band around 2.5 kDa upon Coomassie Blue staining, suggesting putative presence of LCPAs (Fig. IV. 6.). The purpose of the second wash with NaCl/NH₄OH/NH₄Ac solution was to elute any biomolecules that did not elute with ammonia. After dialysing the salts out of the second eluant ("dialysate" sample) however, no bands were observed.

![Coomassie stained 16% Schägger gel with samples isolated on a cation exchanger](image)

Fig. IV. 6. Coomassie stained 16% Schägger gel with samples isolated on a cation exchanger: lane 1 - ammonium elute, lane 2 - dialysate.
IV.3.6. Mass spectrometry analysis of the protein extracts

The results of mass spectrometry analysis of trypsin digested protein samples yielded sixteen unique peptide matches to a sequence from the *P. neolepis* peptide translation of the transcriptome, named comp1037 (Table IV. 2.). The corresponding nucleotide sequence was then retrieved from the transcriptome, translated into a peptide sequence and blasted against the NCBI protein database, revealing some degree of homology with lipocalin like proteins (Table IV. 2.). The amino acid sequence of comp1037, along with an alignment with NCBI-derived, partially homologous sequences are attached as appendix IV.2. and IV.3. respectively.

Table IV. 2. Summary of putative properties of the comp1037 peptide recovered from the transcriptome following MS analysis of trypsin digested protein samples extracted from *P. neolepis* biosilica.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Length (amino acids)</th>
<th>MW (kDa)</th>
<th>Identity</th>
<th>E value</th>
<th>Coverage</th>
</tr>
</thead>
<tbody>
<tr>
<td>comp1037</td>
<td>294</td>
<td>31.6</td>
<td>38%-Lipocalin like protein from <em>Karlodinium veneficus</em></td>
<td>$1 \times 10^{-8}$</td>
<td>28%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>26%-Apolipoprotein D/lipocalin from <em>Osteococcus tauri</em></td>
<td>$1 \times 10^{-5}$</td>
<td>68%</td>
</tr>
</tbody>
</table>
Putative amino acid composition of comp1037 is summarised in Fig. IV. 7. Comp1037 appears to be rich in proline and lysine, which characteristics are common in proteins with silica precipitating properties (Kauss et al. 2003; Kröger et al. 1999).

Fig. IV. 7. Amino acid composition of comp1037 peptide retrieved from P. neolepis transcriptome. Abbreviations are as follows: Ala - alanine, Cys - cysteine, Asp - aspartic acid, Glu - glutamic acid, Phe - phenylalanine, Gly - glycine, His - histidine, Ile - isoleucine, Lys - lysine, Leu - leucine, Met - methionine, Asn - aspartagine, Pro - proline, Gln - glutamine, Arg - arginine, Ser - serine, Thr - threonine, Val - valine, Trp - tryptophan, Tyr - tyrosine.

**IV.3.7. Mass spectrometry analysis of the putative polyamine phase isolated on the cation exchanger**

The ammonia eluate sample isolated on a cation exchanger contained molecules in the mass range of 500-800 Da (Fig. IV. 8. A,B). Each peak represents a molecular ion with
a single positive charge. Two main series ("triplets") of peaks with mass to charge ratio (m/z) values of 559.6 (a)/573.6 (b) /587.6 (c) in triplet 1 and 630.7 (a'/) 644.7 (b')/ 658.7 (c') in triplet 2 were recorded. The mass differences of 14 within each triplet suggested, that the three peaks are methylation isoforms of each other. The mass differences of 71 between the pairs a-a', b-b', and c-c' suggested presence of N-methyl propyleneimine units, a pattern previously found in diatom LCPAs (Kröger et al. 1999).

Fig. IV. 8. A,B - mass spectra of the ammonia eluate isolated from the NH4F flow through on a cation exchanger. A - spectrum obtained by Prof. Kröger, B - spectrum of a replicate sample obtained by Alta Bioscience.
Collision induced fragmentation (CID) of individual ions revealed the presence of multiple N-methyl propyleneimine units, as two series of peaks with mass differences of 71 were present between neighbouring peaks in each series (228.2/299.3/370/441.4 and 313.3/384.4/455.4). This confirms, that the parent molecule of m/z=573.6 contained multiple linearly linked N-methyl propyleneimine units (Fig. IV. 9.). Fragmentation analysis with other peaks yielded analogous results, confirming the presence of N-methylated oligopropyleneimines (data not shown).

Fig. IV. 9. Collision induced fragmentation spectrum of the m/z=574.6 peak.
IV.3.8. FESEM and EDX and confocal analysis of the NH₄F insoluble extract, detection of polysaccharides with fluorescent markers

The NH₄F insoluble extract was imaged in order to verify if any remnants of organic structures potentially underlying silica scales were present post demineralization, as was previously found in e.g. sponges and diatoms (Scheffel et al. 2011; Uriz et al. 2003). The FESEM images of the ethanol washed NH₄F insoluble extract revealed that the material is represented by two distinct morphological components: a fibrous one (Fig. IV. 10. A) and a globular one (Fig. IV. 10. B). The majority of the extract consisted of the fibrous component.

Successful labelling of the NH₄F insoluble extract with both WGA (Wheat germ agglutinin) and CW (Calcofluor white) (Fig. IV. 10. C,D), indicated putative presence of chitin or a similar polysaccharide. The whole cells and purified scales displayed very little and no fluorescence respectively when the above fluorescent dyes were used, indicating that non-specific labelling was unlikely (data not shown). The NH₄F insoluble extract did not label with Con-A.
Fig. IV. 10. FESEM images of the NH4F insoluble biosilica-associated organic phase:
A - fibrous component, B - globular component. Scale bars correspond to 100 nm.
C - D: confocal images of the same extract: C - labelled with WGA, D -labelled with calcofluor white. Scale bars correspond to 5 and 10 μm respectively.

The EDX analysis of the NH4F insoluble extract indicated presence of carbon, oxygen, sulphur, phosphorus and calcium in the extract, confirming presence of organic material (Fig. IV. 11.). Some trace remains of the mineral phase were also detected. Both fibrous and globular components of the extract returned similar spectra.
Fig. IV. 11. EDX analysis of the NH4F insoluble extract. The high aluminium signal is due to the stub, the copper signal is also most likely a contamination.

IV.4. DISCUSSION

The biochemical analysis approaches adopted in this investigation have been previously used to successfully identify and characterise a variety of organic, silica precipitating components in organisms such as silicifying sponges and diatoms (Ehrlich et al. 2010; Kröger et al. 1999; Kröger et al. 1997; Müller et al. 2003). Indeed, they have also yielded good results when applied to investigation of the biosilica produced by *P. neolepis*. Preliminary amino acid analysis of the *P. neolepis* silica scales indicated that the material indeed contains a proline rich organic fraction, suggesting the presence of proteins. Subsequent NH₄F extraction and SDS-PAGE analysis further confirmed the
presence of silica scale associated proteins and LCPAs. An insoluble organic fraction potentially acting as an organic scaffolding in an intact scale has also been recovered.

IV.4.1. NH₄F soluble organic extract: silica-associated proteins

The disappearance of the singlet and doublet bands between 50-70 kDa upon trypsin digestion confirmed, that these bands generated from the NH₄F soluble extract consisted of protein (Fig. IV. 5. A,B). The mass spectrometry analysis of the aforementioned bands in conjunction with transcriptome and NCBI blast analysis generated a putative biosilica associated protein candidate, exhibiting a weak homology with lipocalin-like proteins, which are ubiquitously expressed in both prokaryotes and eukaryotes and are responsible for transport of small hydrophobic molecules (Flower et al. 1993). This finding suggests a potential existence of a novel biosilica-associated type of proteins specific to silicifying haptophytes. Although based on the results obtained in this study further description of comp1037 peptide was not possible, these preliminary data constitute a valuable basis for further investigation into the nature and identity of silica scale-associated protein fraction in P. neolepis.

IV.4.2. LCPAs

The persistence of the broad band around 2.5 kDa after treatment with trypsin indicated, that this fraction either did not contain any protein or that the cleavage sites for trypsin (peptide bonds between the carboxyl group of arginine and lysine and the amino group of the adjacent amino acid), were not accessible to the enzyme (Simpson 2006).
Additionally, the broad band around 2.5 kDa corresponded to a size region similar to the previously described for silica precipitating LCPAs, isolated from diatoms and sponges (Matsunaga et al. 2007; Sumper and Kröger 2004). Indeed, subsequent separation of the NH₄F flow through on a high S cation exchanger and an MS analysis confirmed, that the material appearing on the Schägger gel around 2.5 kDa consisted of LCPAs. The MS analysis indicated the presence of linearly linked N-methylated oligopropyleneimine repeats (n=4-9 repeats) with the [m+H]⁺ ion unit value identical with the diatom oligo-propyleneimine modified lysine residues that form side chains in silaffins (Kröger et al. 1999). To date, the LCPAs found in diatoms have been found to contain putrescine, propylenediamine and spermidine as a base molecule (Kröger and Poulsen 2008b), whereas the sponge LCPAs contain butaneamine and N-linearly linked propyleneimine sulfate chains (Kröger et al. 1999; Matsunaga et al. 2007; Sumper and Kröger 2004) (Fig. IV. 12. A-C). Hence the LCPAs found in P.neolepis are the first putatively lysine-based LCPAs reported to date. It is possible however, that degradation of the protein occurred prior to extraction, and the LCPAs isolated are a degradation product of a protein similar to a diatom silaffin, which is modified with LCPA side-chains (Kröger et al. 1999). This could have happened due to the biosilica material remaining in culture solution for extended periods of time as a result of the slow growth of the species, and therefore its accessibility to proteolytic enzymes potentially present in the media. However, the former case, indicating free LCPAs seems more likely, as the amount of the polyamine material extracted per sample vastly exceeded that of protein material, and some remnants of degraded protein material would be expected to still be evident on a gel upon silver staining. Instead, the characteristic pattern of one singlet and one doublet band around 50-70 kDa along with a broad band around 2.5 kDa was consistently found post NH₄F extraction of three different biosilica samples. Also, the LCPAs in e.g. diatoms have been shown to vary both within and between species in
terms of the chain length, attachment moieties and the degree of methylation, characteristics that are thought to be responsible for morphogenesis of the species-specific frustule ornamentation (Sumper and Kröger 2004; Sumper and Lehmann 2006), hence the presence of a lysine based LCPA in *P. neolepis* would not be surprising. At this stage, further analysis is required to show categorically whether these are indeed non-protein bound LCPAs or degradation products of a larger protein, with the first step in this direction involving molecular identification and biochemical characterization of the biosilica associated proteins extracted from *P. neolepis*. In both cases it is a significant find, as the polyamine fraction found in *P. neolepis* biosilica contributes a novel type of a polyamine, putatively involved in silicification or a new type of a non-protein bound polyamine. It is the first report of a polyamine component putatively involved in biosilicification within the haptophyte clade, indicating that convergent evolution of silica biomineralisation might have occurred in poriferan, heterokontophyte and haptophyte clades.

![Diagram A](image1.png)

![Diagram B](image2.png)

![Diagram C](image3.png)

Fig. IV. 12. A - sponge LCPA structure after Matsunaga et al. (2007), B - schematic structure of a putrescine based diatom LCPA (putrescine moiety in blue), after Kröger, Poulsen (2008), C-polyamine-modified lysine from a diatom sillaffin (lysine moiety in gray), after (Kröger and Poulsen 2008b). Propyleneimine unit repeats are indicated by "n" number.
IV.4.3. \( \text{NH}_4\text{F insoluble organic extract} \)

The EDX analysis of the \( \text{NH}_4\text{F} \) insoluble material, indicating the presence of C, O, Ca, P, S and Si (Fig. IV. 11.) provided additional confirmation of the presence of an organic fraction, consisting potentially of carbohydrates with some residual mineral fraction. The presence of proteins in the extract is also likely, despite the absence of N from the spectra obtained. N is very difficult to detect by EDX analysis due to the detectors' construction, even when the N content of the sample is expected to be very high (Laskin et al. 2003). Also, an amino acid analysis of the \( \text{NH}_4\text{F} \) insoluble extract yielded similar results to the whole scale extract, indicating presence of at least peptide components (Prof. Kröger, personal communication).

The fibrous material revealed by FESEM of the \( \text{NH}_4\text{F} \) insoluble extract (Fig. IV. 10. A) is hereby hypothesised to act as an organic matrix, as it is the case with diatoms and sponges. The fibrous material is thus proposed to form a scaffolding, on which the loop structures are deposited during silica scale morphogenesis (see section II.3.3. for details). The globular fraction of the same material (Fig. IV. 10. B) consists of organics of unknown function, occluded during scale formation and then aggregated once the mineral phase was removed. The EDX spectra obtained from the extract, along with the information obtained in previous studies involving diatoms, where chitin was found to constitute a major organic matrix component within girdle bands (Durkin et al. 2009), prompted a preliminary screening for polysaccharides (chitin in particular) in the extract. The material stained with WGA, which has previously been used for detection of chitin in diatom frustules by Durkin and Mock et al. (2009) and binds specifically to \( \beta \text{ 1,4 N-acetylglucosamine polymers} \) (Nagata and Burger 1974) as well as with CW, which binds to \( \beta \text{-1,3- and } \beta\text{-1,4-linked polysaccharides} \), such as chitin and cellulose.
Thus preliminary information on the putative presence of chitin in the *P. neolepis* biosilica has been gained. The presence of chitin should be further verified i.e. using a highly chitin-specific Chitin Binding Probe (CBP). The lack of labelling with Con-A, which binds to internal and non-reducing terminal α-D-mannosyl and α-D-glucosyl groups (Mandal et al. 1994) indicates absence of mannose-backbone containing polysaccharides or their inaccessibility for binding. Further characterization of the NH$_4$F insoluble organic phase associated with *P. neolepis* silica scales should focus on providing more information on the nature of the organic matrix underlying the silica scales in terms of its structure, composition and capacity to actively precipitate silica.

**IV.5. Summary**

The biochemical investigation of the *P. neolepis* biosilica resulted in detection of closely associated organics, fractioning into a soluble and insoluble phase upon NH$_4$F demineralization of the material. The NH$_4$F soluble fraction was found to contain potentially novel, silica-associated proteins as well as a new type of lysine-based polyamine, contributing the first report of putatively silica-precipitating components present within a haptophyte alga. The fibrous fraction of the NH$_4$F insoluble organic extract was proposed to serve as a matrix underlying biosilica structure-a feature shared also by the diatom and sponge systems. It is also suggested to contain chitin. The presence of LCPAs, silica-associated proteins and an insoluble organic matrix as common components involved in silica biomineralisation in diatoms, haptophytes and sponges, provides an indication, that the silica biomineralisation systems have been
acquired via means of convergent evolution, which topic will be further addressed in Chapter V.
V.1. INTRODUCTION

In this study physiological and biochemical aspects of silicification in *P. neolepis* were successfully identified, allowing for comparisons between other biomineralizing algal taxa such as diatoms and coccolithophores. I have shown that *P. neolepis* is so far the only obligate silicifier within the haptophyte clade, in which formation of biomineralised elements of cell covering fundamentally differs from that of calcifying members of this taxon, bearing some similarities to the silicification system of heterokontophytes instead. Differences in the formation and exocytosis site, cytoskeleton involvement and structure morphogenesis between silica scales and calcitic coccoliths were revealed (see summary in Table V.1.), indicating that the biomineral structures produced in these two systems as well as the systems themselves are most likely non-homologous. Biochemical analyses of *P. neolepis* biosilica revealed that the organic matter associated with silica scales contained soluble and insoluble proteins, LCPAs and putatively organic template-forming chitin, hinting that silica biomineralisation in haptophytes, heterokontophytes and silicifying sponges share some common features and mechanisms involved in controlled silica precipitation, of which evolutionary significance is discussed further in this chapter.
Table V. 1. Table summarizing differences between the formation of the silica scales and coccoliths.

<table>
<thead>
<tr>
<th></th>
<th>Silica scale</th>
<th>Coccolith</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Material</strong></td>
<td>Silica (SiO$_2$·nH$_2$O)</td>
<td>calcite (CaCO$_3$)</td>
</tr>
<tr>
<td><strong>Basis of the</strong></td>
<td>Organic, likely chitin-containing, filamentous matrix underlying the silica scale; presence of novel, Si-associated lipocalin-like proteins and LCPAs putatively involved in silica precipitation</td>
<td>Organic baseplate homologous with an organic scale</td>
</tr>
<tr>
<td><strong>Formation site</strong></td>
<td>SDV-like compartment localized in the posterior, vacuolar area of the cell</td>
<td>Golgi-derived coccolith vesicle (CV)</td>
</tr>
<tr>
<td><strong>Secretion site</strong></td>
<td>Posterior of the cell</td>
<td>Anterior of the cell, near the flagellar root</td>
</tr>
<tr>
<td><strong>The role of actin in</strong></td>
<td>Non-essential for silica scale formation, essential for exocytosis of a complete scale</td>
<td>Essential for both coccolith formation and exocytosis</td>
</tr>
<tr>
<td><strong>The role of microtubules in</strong></td>
<td>Likely involved in transport of substrates required for silica scale formation. Also involved in defining the overall shape of the silica scales</td>
<td>Play a very important role in correct morphogenesis of the coccoliths</td>
</tr>
</tbody>
</table>
V.1.1. Origins of photosynthetic Eucarya

Photosynthetic eukaryotes are hypothesized to have originated ca. 1.5 BYA (billion years ago), acquiring a plastid via primary endosymbiosis of a cyanobacterium, however some estimates suggest that this event could have occurred even 2 BYA (Douzery et al. 2004; Yoon et al. 2004). Chromalveolates are thought to have subsequently differentiated via secondary endosymbiotic event, in which a non-photosynthetic eukaryote engulfed a photosynthetic eukaryote, estimated to have taken place ca. 1.4 BYA (Fig. V. 1.) (Bhattacharya et al. 2007; Keeling 2010; Yoon et al. 2002). It has been generally accepted that green and red algae and higher plants are a product of a primary endosymbiotic event involving a non-photosynthetic eukaryote and a cyanobacterium (Fig. V. 1.) (Graham et al. 2009). The reminder of biomineral forming algae were derived from a non-photosynthetic eukaryote engulfing a red alga (Fig. 1) (Graham et al. 2009).

However, recent studies by Moustafa et al 2009, involving analysis of whole diatom genomes indicated that diatoms were derived from two secondary endosymbiotic events, of which the first involved a green, prasinophyte-like alga. The red alga, previously accepted to be the plastid donor in the remaining algal lineages (apart from the green algae) did not undergo endosymbiosis until much later (Graham et al. 2009; Moustafa et al. 2009). This chain of endosymbiotic events was then indicated as a common sequence of events leading to differentiation of all Chromalveolates (Fig. V. 1.) (Hohmann-Marriott and Blankenship 2011; Prihoda et al. 2012). In addition to assimilation of genetic material in the course of endosymbiotic events, a number of genes in algae, e.g. in diatoms was also acquired via HGT (horizontal gene transfer) from bacteria and Archaea (Prihoda et al. 2012).
Fig. V. 1. The hypothetical chain of evolutionary events leading to plastid acquisition and subsequent lineage differentiation in eukaryotes (after Hohmann-Marriott and Blankenship 2011).
V.1.2. Evolution of silicification in Eucarya

The evolution of silicification in eukaryotes dates back to Neoproterozoic/Cambrian periods (710-680 MYA), which are characterized by supersaturation of the ocean with respect to monosilicic acid, where silica biomineralisation was first used in skeleton formation by benthic demosponges (Kooistra et al. 2007; Love et al. 2009; Maliva et al. 1989; Müller et al. 2007a). Silica biomineralisation systems were subsequently developed in radiolarians in Ordovician and then in diatoms, of which radiation period falls in Cretaceous (Kooistra et al. 2007; Maliva et al. 1989). The extensive proliferation of diatoms during Cretaceous and Tertiary resulted in a progressive depletion of Si from the euphotic zone, which in turn caused other silicifiers, such as sponges to decline due to the ensuing Si limitation (Harper and Knoll 1975; Maldonado et al. 1999). Changes in Si availability leading to its limitation due to biological uptake led to extinction of some silicifiers and generated selective pressure forcing development of active Si transport and concentration mechanisms in others (Raven and Waite 2004; Siever 1992). Therefore, silicifiers themselves constituted the driving force behind the evolution of efficient Si-specific transport systems via removal of Si from the surface ocean in a process of coevolution of organisms with their environment (Raven and Giordano 2009). To date, almost all contemporary natural waters except some silica-rich hot springs are undersaturated with silicic acid (Channing and Edwards 2004). Therefore capacity to actively transport and accumulate Si is essential for silica biomineralizing organisms.

Another theory pertaining to the evolution of Si-based systems and Si incorporation into cellular processes of algae has been put forward by Darley and Volcani (1969). In their view Si could have acted as an “inorganic enzyme”, providing bioactive surfaces within
the cell that then could be utilized to proceed with other vital reactions involved in prevention of cell aging. Due to the resulting Si accumulation in the cell, the excess was hypothesized to be sequestered in the endoplasmic reticulum and finally to be removed into acidic vacuole, where it would then autopolymerize. As solid SiO$_2$ could no longer serve as a reaction site, it was extruded from the cell that would then have to replenish Si from external source, thus developing a continuous requirement for Si supply that is evident in i.e. diatoms (Darley and Volcani 1969).

**V.1.3. Evolution of other biomineralisation systems in Eucarya**

The most common and widespread types of biomineralisation products of the extant eukaryotes are represented by calcium carbonates, calcium phosphates and silica (Knoll 2003). The ability to deposit these biominerals is widespread across the phyla, which has been hypothesised to be a consequence of two mechanisms: (1) HGT and (2) paraphyletic, independent evolution of biomineralisation systems partially based on common biochemical pathways which developed early within the eukaryotes (Knoll 2003). A large number of theories concerning the evolution of biomineralisation in Eucarya has been proposed over the years. Some of them indicated the necessity of rigorous control of the intracellular concentrations of vital elements such as Ca and Fe that were present at very high extracellular concentrations at that time, as a driving force for further development of these systems and their subsequent adaptation for biomineral deposition (Weiner and Dove 2003). This theory was further explored by Westbroek and Marin (1998) in an endeavour to explain a phenomenon of molluscan nacre and coral induced bone regeneration and their biocompatibility with human tissues (Atlan et al. 1997; Dupoirieux et al. 1994; Silve et al. 1992). In the scenario proposed by
Westbroek and Marin (1998) multiple taxa inherited biochemical components from a common, non-mineralized ancestor and subsequently independently adapted them to form biomineralised skeletal elements. Therefore, the polyphyletic character of calcareous biomineralisation systems is a result of independent development of the basic, common physiological and biochemical properties of cells, such as the ability to bind Ca$^{2+}$ ions and regulate internal inorganic carbon chemistry with carbonic anhydrase and other enzymes, enabling supply of ions required for mineral deposition, which properties are widespread in all Eucarya (Aizawa and Miyachi 1986; Sanders et al. 1999; Westbroek and Marin 1998). The authors then suggest that the common physiological and biochemical basis originated in a common, unmineralized eukaryote ancestor during the Neoproterozoic. As Neoproterozoic oceans were oversaturated with CaCO$_3$, organisms inhabiting this environment had to develop efficient systems preventing spontaneous CaCO$_3$ precipitation on the body surface, which provided the basis for subsequent evolution of mechanisms allowing for localization and precise control over biomineralisation of evolutionarily beneficial calcareous elements such as skeletons (Grotzinger and Knoll 1995; Knoll and Swett 1989; Westbroek and Marin 1998).

Additionally, in a "grand unified theory of biomineralisation" proposed by Kirschvink and Hagadorn (2000) magnetite biomineralisation is argued to represent a "protobiomineralisation" system, of which understanding could provide a template for elucidating vacuolar-based biomineralisation processes in metazoans. Magnetite biomineralisation is estimated to have evolved 2 BYA in early eukaryotic ancestors, which putatively acquired it via endosymbiosis of magnetotactic bacteria (Kirschvink and Hagadorn 2000). Kirschvink and Hagadorn's theory introduces magnetite
biomineralisation as a missing link between biomineralisation systems and a precursor on which the extant biomineralisation systems are based.

V.2. SILICIFICATION IN P. NEOLEPIS: EVOLUTIONARY CONTEXT

The peculiar capacity of *P. neolepis* to biomineralise silica could be a result of a number of different processes that occurred during the evolution of the *Prymnesiales*. Based on the available information on the evolution of biomineralisation systems in Eucarya, four likely scenarios leading to acquisition of the ability to silicify in *P. neolepis* can be proposed: the system could have been (1) inherited from diatoms or a common silicifying ancestor of haptophytes and heterokontophytes or (2) result from a *de-novo* convergent evolution. In another scenario (3), the system could have been developed following acquisition of crucial components via HGT, in a process similar to that which was suggested to have occurred in choanoflagellates after acquisition of SITs (Marron et al. 2013). Another, potential theory (4) can be proposed based on the information concerning algal evolution, which involved a series of endosymbiotic events and consequently resulted in (at least partial) assimilation of the genetic material of the endosymbiont into the host genome. This process could have contributed vital components such as Si transporters, later adapted for biomineralisation purposes (e.g. a non-silicifying green, prasinophyte alga *Platymonas* was found to have a capacity for active Si uptake, the mechanism, acquisition route and incidence of this trait in prasinophytes are however unknown) (Nelson et al. 1984). This process could be further facilitated by HGT and as previously suggested by Westbroek and Marin (1998), could involve a further adaptation and development of a pre-existing biochemical machinery, inherited or acquired via HGT, ultimately allowing for biomineralisation. In this
I propose that basic biochemical components which could potentially be developed into calcification or silicification system were present in ancestral haptophytes before the ability to biomineralise was acquired. During the course of evolution, potentially facilitated by events such as HGT, environmental pressure and other specific variables affecting different haptophyte lineages lead to development of one system and not the other (or none at all), giving rise to calcifying clades such as coccolithophores, the silicifier, *P. neolepis* (and potentially more, undescribed silicifying haptophytes) and non-biomineralizing members. However, at this stage features supporting this theory, such as e.g. the presence of Si precipitating LCPAs in *P. neolepis* as well as in a variety of organisms (e.g. sponges, diatoms) could also be applied in favour of another theory proposed. As LCPAs are found in broad distribution of prokaryotic and eukaryotic species, the existing polyamine metabolism could have been adapted for Si precipitation via convergent evolution (Cohen 1998). The fact that LCPAs retrieved from *P. neolepis* were different from those found in diatoms and sponges could therefore support either of these two theories. The presence of a novel, silica-associated protein in *P. neolepis*, which displays no homology with Si-associated proteins isolated from diatoms or sponges could in turn point towards either de-novo system evolution, convergent evolution or adaptation of a pre-existing component (the Si-associated protein isolated from *P. neolepis* shares some homology with lipocalins).

No evidence for HGT in *P. neolepis* were found during this study. Therefore, in spite of the novel and evolutionarily very important information obtained in this study, it is still not possible to determine which exact mechanisms were involved in development of silicification system in haptophytes.

The polyphyletic nature of silicification systems in different taxa combined with recurring common elements such as the aforementioned LCPAs, SITs, presence of an organic matrix (containing either chitin or collagen) etc. or the complete de-novo
evolution of the ability to deposit Si via cooptation of the pre-existing mechanisms (e.g. as in the case of plants, where acquaporins were adapted for Si transport) further indicate, that processes involved in evolution of Si biomineralisation in Eucaryya were highly complex and are therefore very difficult to resolve (Knoll 2003; Ma et al. 2006; Marron et al. 2013; Raven and Waite 2004). Ultimately all silification systems described to date are unified by the chemistry of silicic acid and consequently bear similarities in the form of e.g. active groups precipitating silica (eg. amines in collagen and LCPAs) and the presence of an organic matrix on which Si is deposited in a controlled manner (e.g. chitin, collagen, proteins such as frustulins) (Durkin et al. 2009; Ehrlich and Worch 2008; Kröger and Poulsen 2008a; Matsunaga et al. 2007; Poll et al. 1999). Paradoxically, despite the vast amount of information available on different biomineralisation systems, our knowledge on their in-vivo mechanisms and evolution is overall still very limited. However, an array of genetic and biochemical tools are now available for a slow but methodical investigation of biomineralisation systems used by extant organisms, in an effort to finally unravel their evolutionary origins and cellular basis.

V.3. SUMMARY

Overall this study provided valuable insights into the biomineralisation systems utilized by marine haptophyte algae. A potential evolutionary scenario for development of silicification in haptophytes has been proposed. This study provided important information pointing towards common physiological and biochemical aspects of biomineralisation in algae. Novel information on the physiological and biochemical mechanisms of silicification in haptophytes also provides a highly valuable basis for
further research into the evolution of biomineralisation in algae and the remaining Eucarya.
LIST OF APPENDICES

Appendix II.1.

List of time-lapses:

**Time-lapse 1:** confocal microscopy time lapse of HCK-123 labelled *P. neolepis* cells with scales developing in the posterior pole of the cells (in green). Cell secreting a scale is marked with an arrow. Chloroplast autofluorescence in red. Recording time: 76 min.

**Time-lapse 2:** confocal microscopy time lapse of PDMPO labelled *P. neolepis* cells with multiple intracellularly developing scales visible after cells are lysed (scales in green and indicated with arrows, chloroplast autofluorescence in red). Recording time: 23 min.

**Time-lapse 3:** DIC time lapse of a *P. neolepis* cell exhibiting metaboly and crawling behaviour, subsequently returning to its original spheroid shape. Recording time: 140 min.

**Time-lapse 4:** DIC time lapse of *P. neolepis* cells secreting scales. Recording time: 150 min.

**Time-lapse 5:** DIC time lapse of a *P. neolepis* cell with two haptonemas feeding on remnants of a conspecific. Recording time: 142 min.
Appendix II.2.

Fig. 1. SEM image of silica scales purified from cultures supplemented with 3 µM Ge and 50 µM Si, showing no morphological aberrations.
Appendix IV.1.

Fig. 1. FESEM image of a non-purified *P. neolepis* sample with visible organic scales (arrows).
Fig. 2. FESEM image of a purified *P. neolepis* biosilica sample with no visible organic contamination (i.e. remnants of the protoplast, organic scales).
Fig. 3. TEM image of a uranyl acetate stained NH$_4$F insoluble organic phase isolated from purified *P. neolepis* biosilica, showing no signs of contamination with organic scales.
Appendix IV.2.

Comp1037 peptide sequence.

>comp1037

CKYFMPSPPLPPFPSSPSSPPKPKPCKPPPLEEESQSSSPFHVISKTTPTAPVAK
PPLECPPVKTVPVLEFDEFAEKGWWFVQKQMPTAIETTKLSYCVYHYLLVGG
TTESGKLTLNMFADKTALKKANGEGASFTDYITTDKYRSEGGLCLFEGKKTA
KVQFGMDGEKRGAFFVIDFDSKEGWAVVSGGPQPTIKTKTPPKGCGKTGDGF
LDAGLWILTKDPLPEFWIVETAVAAASKAGFDIEVLDDVMHEGCDYFMPSPPIP
PGSPPAPPTSPLPPSSPSSPPPP

Appendix IV.3.

Alignment of comp1037 and weak homology-exhibiting NCBI-derived sequences.
Fig. 1. Alignment of Comp1037 protein isolated from *P. neolepis* biosilica with NCBI-derived sequences of a lipocalin-like protein from *Karldinium veneficum* and an apolipoprotein/lipocalin protein sequence from *Ostreococcus tauri*.


demineralized bone matrix (DBM) and coral as bone graft substitutes in
23(6), 395-398.


Heinemann.

Edvardsen, B., Eikrem, W., Thronsden, J., Sáez, A. G., Probert, I., and Medlin, L. K.
(2011). "Ribosomal DNA phylogenies and a morphological revision provide the
basis for a revised taxonomy of the Prymnesiales (Haptophyta)." *European

views on desilicification: biosilica and abiotic silica dissolution in natural and
artificial environments." *Chemical reviews*, 110(8), 4656-4689.

Ehrlich, H., Ereskovskii, A., Drozdov, A., Krylova, D., Hanke, T., Meissner, H.,
of spicules in glass sponges (Porifera: Hexactinellida) for the purpose of
extraction and examination of the protein matrix." *Russian Journal of Marine

Ehrlich, H., Heinemann, S., Heinemann, C., Simon, P., Bazhenov, V. V., Shapkin, N.
"Nanostructural organization of naturally occurring composites-part I: silica-

Spicules of the Meter-Long Hyalonema sieboldi", *Handbook of

National Academy of Sciences*, 91(1), 11-17.

Fichtinger-Scheperman, A. M. J., Kamerling, J. P., Versluis, C., and Vliegenthart, J. F.
(1981). "Structural studies of the methylated, acidic polysaccharide associated
with coccoliths of*Emiliania huxleyi*(lohmann) kamptner." *Carbohydrate Research*,
93(1), 105-123.

relationships in the lipocalins and related proteins." *Protein Science*, 2(5), 753-
761.

actin filaments and microtubules in a neuronal growth cone." *J Cell Biol*, 107(4),
1505-16.

Franklin, D. J., Airs, R. L., Fernandes, M., Bell, T. G., Bongaerts, R. J., Berges, J. A.,
huxleyi* and *Thalassiosira pseudonana*: Cell staining, chlorophyll alterations, and
dimethylsulfoniopropionate (DMSP) metabolism." *Limnology and Oceanography*,
57(1), 305.

lipid intermediates in peptidoglycan synthesis." *Analytical biochemistry*, 90(2),
465-473.


dependent actin checkpoint ensures proper spindle orientation in fission yeast." *Nature*,
412(6844), 352-355.
Durak, G.M.  

List of references


<table>
<thead>
<tr>
<th>Author(s)</th>
<th>Title</th>
<th>Journal</th>
<th>Volume</th>
<th>Issue</th>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parke, M., and Adams, I. (1960)</td>
<td>&quot;The motile (Crassitellithus hyalinus Gaarder &amp; Markali) and non-motile phases in the life history of Coccolithus pelagicus (Wallich) Schiller.&quot;</td>
<td><em>Journal of the Marine Biological Association of the United Kingdom</em></td>
<td>39(02)</td>
<td></td>
<td>263-274</td>
</tr>
<tr>
<td>Pienaar, R. N. (1981)</td>
<td>&quot;Ultrastructural studies on the cysts of Prymnesium (Prymnesiophyceae).&quot;</td>
<td><em>Phycologia</em></td>
<td>20</td>
<td></td>
<td>112</td>
</tr>
</tbody>
</table>


Formation and Maturation during Spicule Formation in Sponges. "PLoS ONE, 6(6), e20523.


