Thesis for the partial fulfilment of the degree of Doctor scientiarum:

Nuclear Magnetic Resonance spectroscopy of marine microalgae:

Metabolic profiling and species discrimination from High-Resolution Magic Angle Spinning NMR analysis of whole-cell samples

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Since the start of this project on ”microalgae and NMR” in 2000, I have had the pleasure to work with highly skilled people and I have learned a lot! A short version is presented below:

My basic knowledge of the intriguing world of microalgae was very much influenced by my supervisor to the Master degree, Prof. Egil Sakshaug, who also made sure that I was trained in other miscellaneous topics such as cooking and literature! I thank him for the thorough base he gave me to proceed with scientific work. The work presented here has been supervised by Prof. Geir Johnsen, and without his endless enthusiasm (and his knowledge, of course!) it would have been much less pleasant to do this work. He also brought me out into the world outside Trondheim, either as a student participating on “Svalbard courses”, or working with him on different projects and going to conferences and cruises. I am not sure if a mere “thank you” is enough to express my gratitude for all the opportunities he has have given me to learn more about microalgae! I must also mention here that the original project plan was formed by Geir Johnsen and Zsolt Volent (SINTEF Fisheries and Aquaculture), and I hereby thank them both for bringing me into the project and handing over the idea of using NMR to analyze microalgae! There were moments when I wished I had never gone into this, but looking back now I am of course very happy to have been a part of this project! I owe the Norwegian research council many thanks for the financial support.

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Trondheim, December 2004 *Matilde S. Chauton*

LIST OF PAPERS

I Matilde S. Chauton, Gavin H. Tilstone, Catherine Legrand, Geir Johnsen. 2004. Changes in pigmentation, bio-optical characteristics and photophysiology, during phytoflagellate succession in mesocosms. *Journal of Plankton Research* 26: 315-324

II Matilde S. Chauton, Odd I. Optun, Tone F. Bathen, Zsolt Volent, Ingrid S. Gribbestad, Geir Johnsen. 2003. HR MAS ^{1}H NMR spectroscopy analysis of whole cells of marine microalgae. *Marine Ecology Progress Series* 256: 57-62

III Matilde S. Chauton, Tone F. Bathen. Metabolic profiling of microalgae whole cells with HR MAS ^{1}H NMR and principal component analysis. Manuscript

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IV Matilde S. Chauton, Trond R. Størseth, Geir Johnsen. 2003. High-resolution magic angle spinning ^{1}H NMR analysis of whole cells of *Thalassiosira pseudonana* (Bacillariophyceae): Broad range analysis of metabolic composition and nutritional value. *Journal of Applied Phycology* 15 (6): 533-542

V Matilde S. Chauton, Trond R. Størseth, Jostein Krane. 2004. HR MAS DEPT ^{13}C NMR analysis of whole cells of *Chaetoceros muelleri* (Bacillariophyceae), and comparison with ^{13}C NMR and DEPT ^{13}C NMR analysis of lipophilic extracts. *Journal of Phycology* 40: 611-618

VI Matilde S. Chauton, Trond R. Størseth. HR MAS NMR spectroscopy of marine microalgae whole-cell samples, part 1: Classification and metabolite composition from HR MAS ^{1}H NMR spectra and multivariate analysis. Contribution to "*Handbook of Modern Magnetic Resonance"* (*in prep*.), section ed. M. Aursand, ed. in chief G. A. Webb. Kluwer Academic Publishers

LIST OF ABBREVIATIONS/GLOSSARY

COSY	correlation spectroscopy
CPMG	Carr-Purcell-Meiboom-Gill
DEPT	distortionless enhancement by polarisation transfer
DMSP	dimethylsulphoniopropionate
FC	fuzzy clustering
FID	free induction decay
^{1}H	proton
HPLC	high-performance liquid chromatography
HR MAS	high-resolution magic angle spinning
NMR	nuclear magnetic resonance
nuclide	atom characterized by its nucleus
PCA	principal component analysis
PR	pattern recognition
PUFA	polyunsaturated fatty acid

1 INTRODUCTION

1.1 Background

Microalgae[a] are unicellular eucaryotic organisms, found in all aquatic environments including oceans, lakes, rivers, and even on snow and under ice. Although the basic structure of the cell is the same for all, they also differ in many ways. Some are autotrophs and rely on sunlight to produce chemically bound energy for production of organic compounds, while others are heterotrophs and utilise dissolved organic carbon or ingest bacteria or small algae. Many species are planktonic, while others live attached to the surface on the bottom layer in shallow-water areas or on the surface of macroalgae in the littoral zone. Some are even photosynthetic symbionts, such as the zooxanthella found in corals. A further example of another highly productive microalgal society is that which is found attached to sea-ice in the Arctic and Antarctic regions.

The most important function of the autotrophic microalgae is to incorporate inorganic carbon through photosynthesis, and provide organic carbon to the food chain. Actually, microalgae contribute to more than 45% of the global annual net primary production[1]. Microalgae are also important as feed for secondary producers such as zooplankton and fish larvae, providing energetic carbohydrates and important fatty acids. Some microalgae are toxic, and when they occur in high numbers or 'blooms', it can be a problem to fish or humans who are intoxicated through consumption of shellfish which have ingested toxic algae. The important role as primary producers and also as nuisance organisms, are examples of why it is desirable to monitor the size of microalgal communities and the species composition. The role of microalgae as food source led to numerous studies of their chemical composition, often focussing on carbohydrates and polyunsaturated fatty acids[2,3,4]. Furthermore, the modern field of metabolomics (metabolic profiling of cells or whole tissue) has opened up for new methods to screen the cells for metabolic composition[5,6].

At the present time, cell counts, species composition and selected metabolites such as pigments are routinely studied with light microscope and high-performance liquid chromatography (HPLC)[7]. These methods do have some drawbacks: Microscopy examination is time-consuming and requires trained operators, and identification and cell counts can be biased according to the operators' experience. Also, cells in the samples can be

[a] The term 'phytoplankton' is commonly used for photosynthetic microalgae, but NMR as an analytical tool for species discrimination and metabolic profiling can be used for all microalgae including heterotrophs and so on, so not to exclude any non-photosynthetic algae the term 'microalga' is used in this text.

destroyed or changed to the unrecognisable by the added fixative. Pigment chromatography requires sample preparation and instrumental adjustments, both including organic solvents, but the advantage is increased reproducibility (no operator bias) and large sample sets can be analysed automatically. The instrumental set up in terms of column, solvents and detector has to be adjusted for e.g. pigment analysis, otherwise HPLC is an analysis that can be used for separation and detection of many different metabolites. Different groups of autotrophic algae have different pigmentation, and a software program called CHEMTAX has been developed to handle the pigment data and describe microalgae populations from the group-specific pigmentation[8].

Nuclear magnetic resonance (NMR) signals were observed for the first time in 1946 by two different groups, lead by Nobel prize winners F. Bloch and E. M. Purcell, respectively. From there, one-dimensional and later two-dimensional NMR analyses have become a very suitable tool for molecular structure studies, widely used in Physics and Chemistry. In 1991 the Nobel prize in Chemistry was awarded to R. R. Ernst, who pioneered the new era of NMR with his "….contributions to the development of the methodology of high resolution nuclear magnetic resonance spectroscopy"[b]. The application of pulse sequences to 'manipulate' the nuclear spin systems, and the introduction of 'magic angle spinning'(MAS)[9], have opened the way for NMR in other fields of Natural sciences such as Biology.

An ideal situation for NMR analysis is a suspension of low molecular weight molecules of the same sort, since low viscosity of the medium and free rotational mobility of smaller molecules favours the NMR analysis. The result is well defined spectra with high resolution and narrow peaks. This is far from the situation when analysing directly on whole cells. A cell has rigid cell walls, semi-solid membranes and organelles, and liquid compartments with suspended molecules. In the NMR analysis this means huge variation in sample viscosity, variations in molecular size and interactions, compartmentation and overall heterogeneity of magnetization. The outcome then, is a complex spectrum with broad and poorly resolved peaks.

NMR analysis of extracted samples is one possibility to overcome these problems. Extraction, however, means more time spent on sample preparation and exposure of operators to unhealthy solvents. It is also a selective process, where the given extraction method determines the metabolite composition of the sample (e.g. water vs. fat soluble compounds). Furthermore, the extraction may be incomplete, so information might be lost, or it may alter the molecular structure which in turn changes

[b] http://www.nobel.se/chemistry/laureates/1991/

the NMR properties of the molecule. Also, any information on compartmentation or cellular distribution is lost when metabolites are extracted from their original location. It is therefore desirable to analyse samples of intact material such as tissues and cells. This is possible with todays advanced state of NMR technology including higher magnetic field instruments and techniques such as MAS. Experiences from NMR spectroscopy of biofluids[10] and plants[11] contain much of the information on what is achieved until now.

For analysis of organic molecules, both ^{1}H and ^{13}C NMR is applied. A principal difference in the studies of the two nuclides lies in the natural abundance: Whereas ^{1}H has a natural abundance of nearly 100%, only about 1% of naturally occuring C is ^{13}C. ^{1}H NMR spectroscopy also benefits from the higher gyromagnetic ratio of protons, which in turn affects the sensitivity of the NMR analysis. On the other hand, the increased resolution due to the wider shift scale in ^{13}C NMR makes it possible to extract quite detailed information on e.g. lipids, but both ^{1}H and ^{13}C NMR analyses yield information on various metabolites such as carbohydrates, amino acids and lipids.

NMR has been used in different studies of microalgal extracted material, and some examples are: Structural studies of toxins by ^{1}H or ^{13}C NMR[12,13,14,15] or pigments by ^{1}H NMR[16], and reserve products by ^{13}C NMR[17]. Functional-group distribution of organic carbon in seawater net-samples (including microalgae) was determined by ^{13}C NMR[18]. The structure of the bio-polymer algaenan in *Botryococcus braunii*[19] and the storage glucan of *Chaetoceros mülleri*[20] were studied with HR MAS ^{13}C NMR. ^{31}P NMR was used to study light stress in the green alga *Dunaliella salina*[21] and phosphate metabolism in the raphidophyte *Chattonella antiqua*[22].

The combination of NMR analysis and multivariate statistics such as principal component analysis (PCA) has proven useful both in terms of data reduction and cluster analysis, and graphical presentation of data. In pharmacology and studies of biofluids PCA is used to distinguish “normal” from “treated” samples[23,24] or distinguish pathologically affected tissue samples from healthy tissue[25]. Other examples where NMR and PCA was used for classification of samples are quality assessment of orange juices[26] or phenotypic differentiation of earthworms[27]. Furthermore, several studies of genetic strain differences or consequences of genetic modification have been done on e.g. bacteria [28], mice[29] and tomatoes[30]. A study on *Arabidopsis thaliana* is one example of metabolic fingerprinting by NMR and PCA[31]. These examples are just a few of the many studies performed with NMR and PCA, given here to illustrate the wide use in classification and metabolic profiling.

This project of "Microalgae and NMR" started from an idea that HR MAS NMR could be used as a tool to identify microalgae[c]. As the first results started to come it became clear that HR MAS NMR spectroscopy analyses directly on algal cells in addition to the taxonomic purposes could be used also for metabolic profiling, and the original project plan was expanded to include this. The work presented here has been done by a biologist with no prior knowledge of NMR, and it is not meant as a contribution to new knowledge on NMR. Instead, it is meant as a contribution to biologists on how NMR can be used in analyses of microalgal cellular chemistry, and as a tool for discrimination or identification of species.

1.2 Structure of thesis and aim of this work

This thesis is based upon 4 papers and 2 manuscripts, which are divided into 4 sections based on their content (see List of papers). The first paper gives an overview of some common methods that are used to survey the pigmentation, photophysiology and size of microalgae populations: Pigment chromatography by HPLC, *in vivo* absorption and fluorescence excitation, and classical cell counts with light microscope. The results obtained from the application of these established methods formed the basis from where the results of the NMR analyses were being evaluated. It turned out, however, that pigmentation and photophysiological characters were not the most salient features when microalgal cells were analysed with NMR spectroscopy. Therefore, this thesis does not contain a direct comparison of the different methods; instead it is a presentation of NMR spectroscopy as an alternative analytical tool with great potential in other aspects of the analysis of microalgae.

The next two papers present the use of HR MAS ^{1}H NMR spectroscopy on whole cells together with multivariate statistics as a tool for species discrimination. Paper II shows the distinct separation of different species, while Paper III continues by describing the origin of differences in the signals. Further on, Papers IV and V describe the use of NMR spectroscopy in metabolic profiling, and contain results and comparison of whole-cell and extract samples. The two papers differ in that paper IV describes ^{1}H NMR spectroscopy of the diatom *Thalassiosira pseudonana*, while Paper V is about ^{13}C NMR spectroscopy of another diatom, *Chaetoceros mülleri*. Finally, in VI the topics of discrimination and metabolic profiling by ^{1}H NMR are combined.

[c] Projects no. 133618/120 and no. 128726/420 MODTEQ, funded by the Norwegian Research Council.

A short introduction to NMR is given, intended for biologist with little knowledge of this method, and a short introduction of microalgae is also given for those who are not familiar with these organisms. Both parts are written to explain the background of the discussion of HR MAS analysis of microalgae whole-cell samples, so neither part is meant to give details on other topics related to either NMR or microalgae. Topics which are considered relevant as background information or important for the final argumentation, are summarized in boxes at the end of each section.

Based on what is said about the background and structure, this thesis presents a discussion of the use of NMR spectroscopy as a tool for species discrimination and metabolic profiling of microalgae. The results of various analyses including NMR and statistics are evaluated from what is known from already established identification methods such as pigment chromatography and chemotaxonomy, and the chemical composition of microalgal cells. The main focus is on the application of HR MAS on whole cells, which means quick and easy sample preparation with no extraction involved and large information output in terms of metabolite composition in a single analysis run.

2 NMR

2.1 NMR signal acquisition and spectral parameters[d]

The principle of NMR spectroscopy is that atomic nuclei assume one of a discrete number of orientations in an applied magnetic field, and unless disturbed by some external source the population of spinning nuclei reach a state of equilibrium[e]. A radio-magnetic pulse is applied to excite the spinning nuclei and bring them out of equilibrium. As they relax and equilibrium is restored, excess energy is emitted and can be recorded (Figure 1). The signal strength decreases as equilibrium is approached, and it is called a Free Induction Decay (FID) signal. The FID signal is converted via Fourier transformation from a time domain scale to a frequency domain scale.

The result is a series of peaks along a "parts per million" (ppm) scale where the position along the scale represents a chemical shift (δ) with reference to a determined point. The spectral output is further determined by spin-spin coupling between nuclei (or weak interactions via chemical bonds), and the coupling constant (J) is related to the multiplicity of coupled peaks. In addition, the relaxation of magnetization is composed of two parts: a longitudinal (T_1, or spin-lattice relaxation) and a transverse (T_2, or spin-spin relaxation) component. The inverse of T_2 ($1/T_2$) determines the spectral line width for nuclei in freely tumbling molecules, so that a short T_2-relaxation rate leads to broad peaks.

Only nuclides with spin number $I \neq 0$ can be detected by NMR, while nuclides with $I = 0$ are termed 'NMR silent'. Common nuclides in organic compounds such as ^{1}H, ^{13}C, ^{15}N, ^{31}P all have spin $I = ½$ and can be observed by NMR. The radio-magnetic pulse is tuned for observation of the desired nuclei, so each run in the spectroscopic analysis is aiming at specific nuclides (^{1}H or ^{13}C, and so on). Yet, all nuclei of the selected type can contribute to the signal, and each particular peak in a ^{1}H NMR spectrum reflects all the ^{1}H nuclei that occur in exactly the same chemical environment, e.g. identical methyl groups or allylic ^{1}H in fatty acids (Figure 2, Tables 1-3 of Paper IV). The shift along the ppm scale reflects a change in chemical surroundings of the observed nuclei, and this means that different ^{1}H on a given molecule appear as different peaks in the NMR

[d] This chapter is meant as an "introduction to NMR for Biologists" since there are some important aspects that makes NMR spectroscopy very different from other, more commonly applied methods in studies of microalgae. For those familiar with physics and the principles of NMR, this text is absolutely superfluous.

[e] For those interested in the physics of NMR, and equations to explain this, there are several recommendable introductory books: "High-Resolution NMR Techniques in Organic Chemistry" by Timothy D. W. Claridge (1999, Elsevier), is one example.

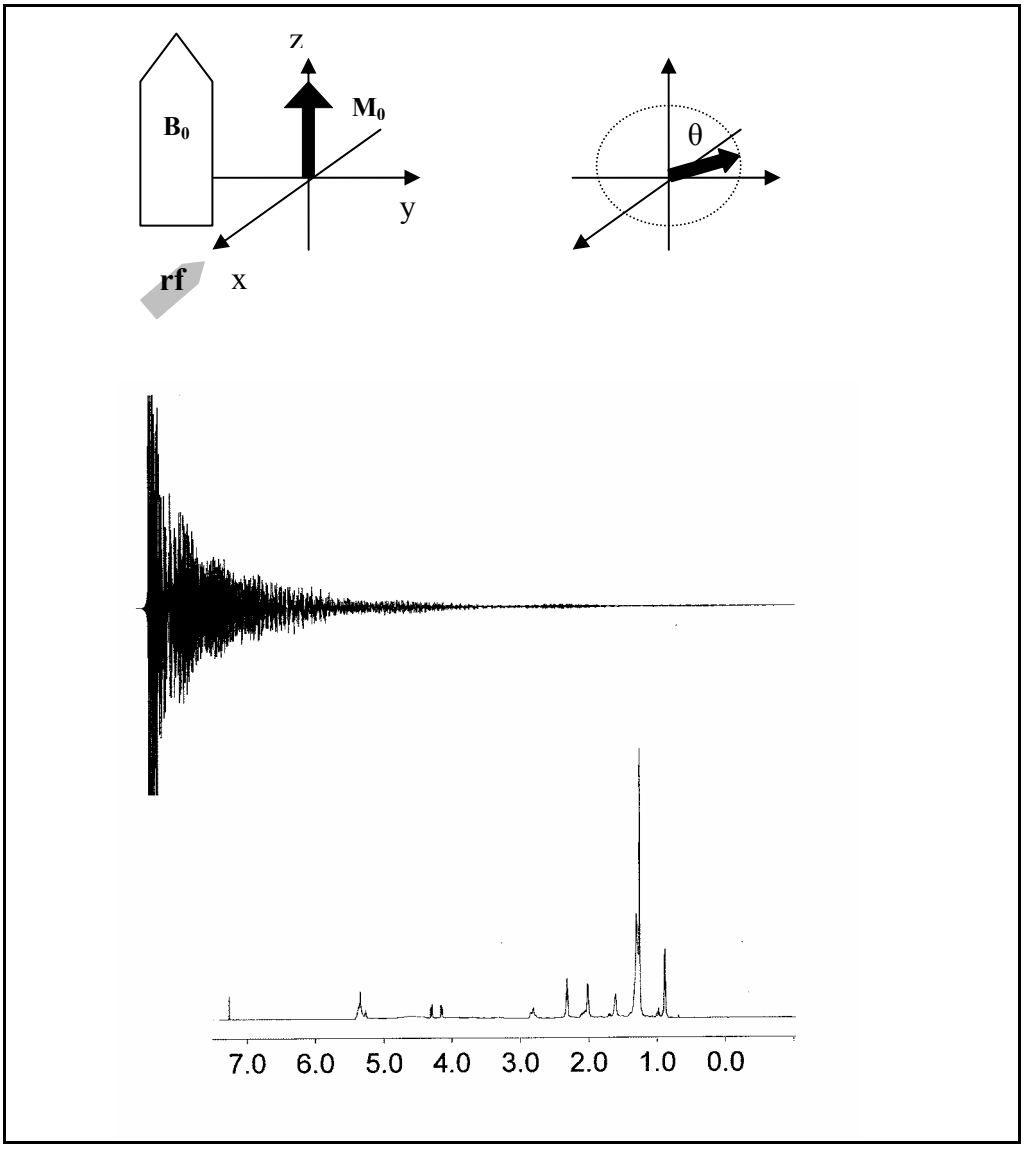

Figure 1 Top: (from left to right) Vector model of population of spinning nuclei in applied magnetic field B_0, where overall magnetization vector M_0 is directed along *z* at equilibrium. Radiofrequency pulse applied along *x* forces the population out of equilibrium and towards the x-y plane. Tip angle θ depends on radio frequency pulse, e.g. a 90° brings M_0 to the x-y plane, while a 180° pulse brings M_0 down to – z. The resonance signal is acquired along *y* before M_0 relaxes back to equilibrium. **Middle:** The acquired free induction decay (FID) signal, on a time domain scale. **Bottom:** The FID signal is transformed via Fourier transformation to a frequency domain scale (ppm). The FID and the resulting NMR spectrum shows components of a lipophilic extract of the diatom *Chaetoceros mülleri*, recorded on a Bruker Avance DRX 600 spectrometer (by courtesy of T. Størseth).

spectrum (contrary to e.g. HPLC analysis where a single peak represents an entire molecule). Since ^{1}H and ^{13}C are found in all organic compounds, the NMR analysis of either of these nuclides yields a very complex spectrum of peaks that reflect many different chemical groups on various molecules (Figure 3 of Paper IV and Figure 2 of Paper V). This means that NMR is both highly specific since it aims at selected nuclei, but also that it is a broad range analysis in terms of the massive molecular information output.

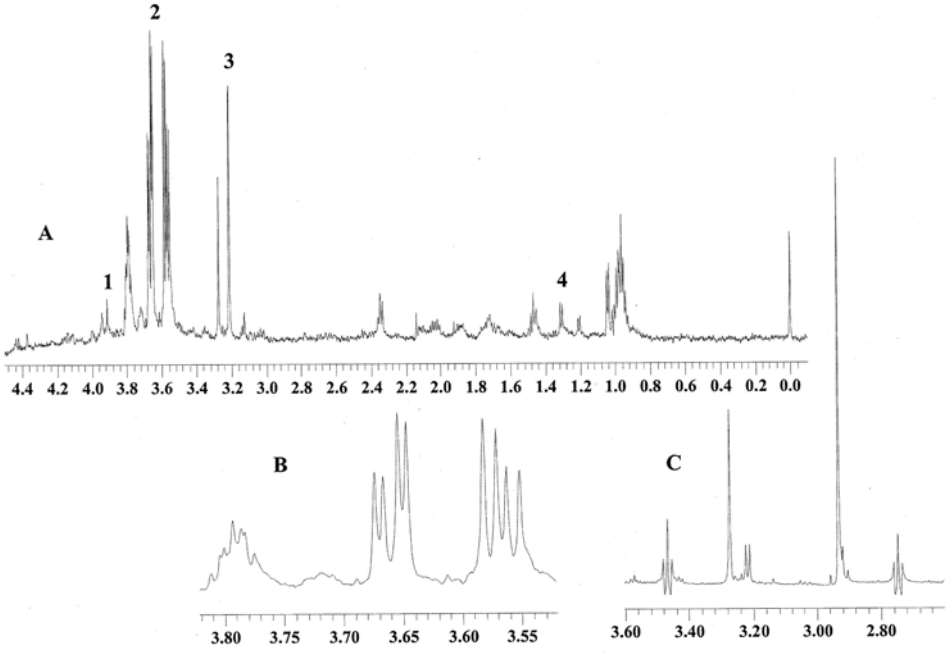

Figure 2 A: HR MAS ^{1}H NMR spectrum of whole cells of the green alga *Dunaliella* sp. Peak numbers refer to 1: Betaine CH_2, 2: Glycerol CH_2, 3: Betaine and choline, $N(CH_3)_3$, 4: Lactate CH_3. **B**: Detail showing glycerol CH_2 double doublets and CH multiplet. **C**: Detail from HR MAS ^{1}H NMR spectrum of whole cells of the dinoflagellate *Prorocentrum minimum*: Dimethylsulphoniopropionate singlet and distorted triplets. See Paper III for details.

Analytical sensitivity and spectral resolution

The relatively low inherent sensitivity of the NMR analysis (for all nuclides) is compensated by repeating and averaging of scans. There are also differences in the sensitivity between the different nuclides, related to natural abundance and gyromagnetic ratio (γ). In Paper V this is seen in the analysis of ^{13}C, which has a lower natural abundance and lower γ than ^{1}H. The ^{13}C NMR analysis, however, has the advantage that the chemical shifts are spread over a wider shift scale: While ^{1}H chemical shifts in biological molecules are spread over approx. 14 ppm, the ^{13}C shifts are spread over 200 ppm. This increases the resolution of the analysis (Figure 1 of Paper V). It is also possible to enhance the sensitivity of the ^{13}C by magnetization transfer from protons, in a process called Distortionless Enhancement by Polarisation Transfer (DEPT). In addition to the increased signal-to-noise ratio which enhances the ^{13}C signal, the DEPT analysis is also used for spectral editing: depending on the applied pulse tip angle it is possible to distinguish carbon multiplet resonances since they are phased differently in the spectra. In the analysis in Paper V a pulse tip angle of 135° (DEPT-135) was applied, and the result is that methyl and methine signals are phased upwards, while the methylene signal is phased downwards (Figure 2 of Paper V).

- Some important parameters in NMR spectroscopy are the chemical shift (δ), coupling constant (J), and longitudinal (T_1) and transverse (T_2) relaxation rates.
- ^{1}H, ^{13}C, ^{15}N and ^{31}P NMR spectroscopy is suitable for analyses of organic molecules (Figure 2).
- The NMR analysis is selectively aimed at specific nuclides, e.g. ^{1}H or ^{13}C, but the information output is broad ranged and includes signals from many different metabolites (Papers IV and V).
- Natural abundance of ^{1}H is close to 100%, while the natural abundance of ^{13}C is only about 1.1%. This affects the sensitivity of the NMR analysis and reduces the acquisition time of the ^{1}H NMR analysis compared to ^{13}C NMR.
- The chemical shift scale of ^{13}C is 200 ppm, while the chemical shifts of ^{1}H spread over 14 ppm only. The result is increased spectral resolution of ^{13}C NMR (Paper V).
- The sensitivity of the NMR signal is increased by repeating and averaging of scans, and magnetization transfer between nuclei through application of special pulse sequences.

2.2 NMR in biology

In the history of NMR, the development of cryo-magnets and specialized pulse sequences is important for the successful use of NMR in natural sciences. Increased cooling makes it possible to achieve higher magnetic field strengths, which in turn increases the sensitivity of the analysis. Today, magnets that operate at more than 18 Tesla are used, corresponding to ^{1}H resonance frequencies of 800 MHz[f]. The basis of the pulse sequences is the long lifetime of excited nuclear spins, from seconds up to minutes, which makes it possible to manipulate spin systems in several steps so that the final signal contains the desired information. Two examples from our work is the application of Carr-Purcell-Meiboom-Gill (CPMG) spin-echo sequence (Paper II and IV)[32], and the DEPT editing sequence (Paper V)[33].

In Papers II and IV the spin-echo sequence CPMG was applied to reduce lipid signals, which could otherwise give broad and overlapping resonance signals. Lipid signals are not completely removed, however, as suggested from the tentative assignments (Table 3 in Paper IV). A 'water pre-saturation sequence' was added to the CPMG pulse-sequence to diminish the water signal, which can otherwise be much larger than the signal from cellular metabolites and disturb the dynamic range of signals (Figure 2 C of Paper IV). This is the case with solvents in extraction samples and residual water in whole-cell samples, if it is not eliminated physically through lyophilization or evaporation (Figure 1 of Paper II). The DEPT sequence used in Paper V was applied to enhance the sensitivity of the ^{13}C nuclei and reduce the acquisition time, and to clearly distinguish signals from ^{13}C in methyl-, methylene- and methine-groups (Figure 2 B of Paper V).

High Resolution Magic Angle Spinning

NMR is extensively used in pharmacology for screening of biofluids such as blood and urine[10], while in medicine NMR is applied to detect e.g. cancer in breast tissue samples[34]. Organ, tissue or cell samples are complex and do not fall into the "conventional" classes of NMR spectroscopy, namely 'liquid' or 'solid' state NMR. The problem that arises with increasing solidity of the sample material is partly related to physical features of molecular motion: anisotropy (or unsymmetrical distribution around electron bonds) and dipolar couplings. The result is that nuclei in tumbling molecules experience shifts in the local magnetic properties relative to the applied static field, and the effect is spectral line broadening. Also, macromolecular (e.g. to proteins) and membrane binding will restrain the molecular motion and add to the spectral line broadening. If the molecules

[f] For comparison, the magnetic flux at the Earth's surface is about 0.3-0.5 x 10^{-4} T.

can move freely such as in liquid samples, these interactions are averaged to zero and the spectral lines are narrow.

The application of high-resolution magic angle spinning (HR MAS) counteracts the effects of molecular anisotropy in heterogeneous tissue and cell samples, and provides a solution to the problem of line broadening in biological samples. More specifically, in a HR MAS analysis the sample is spun around its own axis at an angle of 54.7° relative to the static magnetic field, in order to reduce the angular dependence due to dipolar couplings (D) to zero:

$$D \propto r^{-3} (3\cos^2\theta - 1)$$

where r is the distance between two nuclei, and θ is the angle between the static magnetic field and the nuclear vector. Spinning at 5 kHz of small amounts of sample in specialized rotors leads to increased signal-to-noise levels and improved peak resolution even in complex samples of whole cells (Figure 1 of Paper II, figure 3 of Paper IV, and figure 2 of Paper V).

- Cryo-magnets yield higher magnetic field strengths and increased sensitivity of the NMR analysis.
- Series of pulses are used to manipulate spin systems, in order to enhance the desired signal information or suppress unwanted signals e.g. from dominating metabolites or residual solvent.
- Certain pulse sequences lead to enhanced sensitivity of selected nuclei, e.g. DEPT-135 and ^{13}C analysis, thereby reducing the acquisition time. It is also used for spectral editing, since signals from e.g. methyl, methylene and methine are distinguished (Paper V).
- A solution of small molecules is ideal for NMR spectroscopy. Larger molecules and molecules embedded in membranes or organelles are subjected to motional restriction, which in turn gives poorly resolved peaks in the NMR spectrum. Also, both intra- and intermolecular interactions (anisotropy and dipolar couplings) affect the magnetic properties of nuclei, leading to spectral line broadening.
- 'Magic angle spinning' at 54.7° (rel. to the applied magnetic field) of complex biological samples reduces the spectral line broadening since the effects of molecular anisotropy and dipolar couplings are counteracted. This greatly improves the spectral resolution, even in whole cell samples (Papers II, IV and V).

2.3 NMR and multivariate analysis

The NMR analysis provides spectral profiles of a broad range of molecules in relatively short time, and the sample preparation can be minimized since it is possible to analyse whole cells. The data output, however, is a complex spectrum and most of the information within it is not extractable by simple visual examination. A very common approach is to apply Principal Component Analysis (PCA), which is a multivariate clustering analysis with no prior assumption regarding the distribution of data[23,35,36,37,38]. In PCA the variance in the data set is reduced to a smaller set of variables, or Principal Components (PC) that can explain the majority of the original variance. The PCs represents the original variables (here metabolite concentrations or ratios, Papers II, III and VI) in decreasing order of significance for sample discrimination, so that the first few PCs usually explain most of the variance.

In the analyses described in Paper II the input matrix consisted of data from the spectral region of 4-3 ppm. The three first PCs explained 90% of the total variation in the microalgae data set (Paper II) and distinct grouping of different microalgal species was seen (Figure 3). In the analysis described in Paper III, a wider spectral region from 4.5-0.5 ppm was used as input (Figure 2 of Paper III). Here the 3 first PCs explained 78% of the variance in the original data. From there, it is possible to go back into the loading plots and find the origin of the variance (usually determined peaks in the NMR spectrum), and in this way identify metabolites that differentiate the samples. This is described in detail in Paper III, were concentrations and ratios of the two osmolytes dimethylsulphoniopropionate (DMSP) and betaine largely determined the variance in the data set (Figure 3 of Paper III).

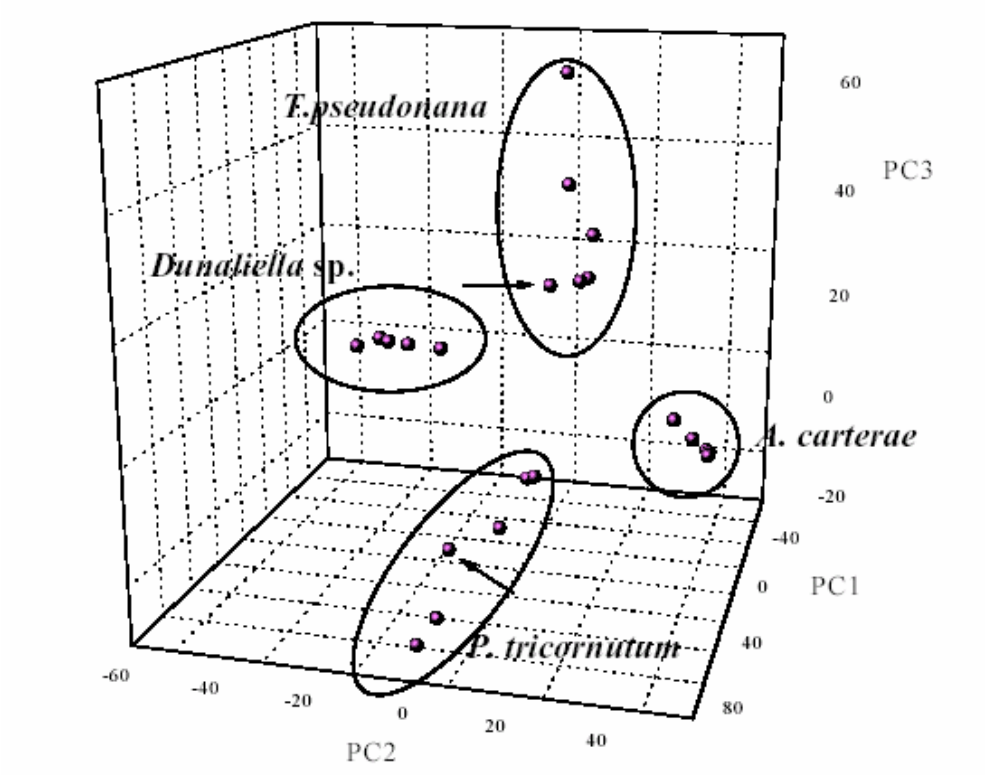

Figure 3 3D plot of Principal component analysis of 5 samples of 4 different microalgae (two diatoms *Thalassiosira pseudonana* and *Phaeodactylum tricornutum*, one chlorophyte *Dunaliella* sp. and the dinoflagellate *Amphidinium carterae*), showing distinct grouping of replicate samples. Arrows point to additional samples from a previous sampling (subjected to the same growth and harvesting conditions as the other samples) which were included to show the reproducibility of the method. PC = principal component. See Paper II for details.

The first data set (spectral region 4-3 ppm) was also subjected to another type of multivariate analysis, namely Fuzzy clustering (FC). Cluster analyses are described as classification techniques where data sets are separated into different groups based on quantitative comparison of multiple characteristics. In the FC, each group or cluster is characterized by a 'centre' which is defined mathematically, and the probability of group membership is subsequently calculated for each data point in the group. The 5 replicates from each of the four algae (and the 2 samples from a previous sampling) in our study were distinctly separated with high probability of class membership ($p>0.9$) also in the FC analysis (Table 1 of Paper II).

- Multivariate data analysis is used for data reduction and to highlight clusters within the data (Figure 3).
- In PCA the original variance in the data set is reduced to a set of significant variables that can explain most of the original variance.
- In FC a mathematically defined ‘centre’ is determined for replicate spectra, and the probability of group membership for each spectrum related to this centre is calculated.
- The origin of signals which are most significant in the clustering of the data sets in the PCA can be retraced to the NMR spectra to identify responsible metabolites and yield a biologically relevant explanation for the observed grouping. The observed grouping of microalgae in the PCA was related to differences in cellular content of various osmolytes such as DMSP and betaine (Papers II and III).

3 MICROALGAE

3.1 Species discrimination

There are many thousand identified species of microalgae and they vary morphologically in many different ways[1,39]. The classical way of identification and enumeration through light microscope is based on outer cellular characteristics: shape and size of the cell, cell wall or covering scales, and numbers of flagella (Figure 4). In some cases it is possible to determine whether the cells in question are autotrophs or heterotrophs since pigmented cells are coloured, and sometimes it is possible to see the shape and numbers of chloroplasts. This is then, analysis on a size level high above metabolites and therefore very different from the NMR spectroscopy analysis.

Chemotaxonomy based on group-specific pigmentation is now routinely used for identification and biomass measurements[40]. Paper I presents class-specific pigment markers of some flagellates and diatoms in mesocosms, together with the photophysiological effects of pigment variations. This method is, however, limited to the pigmented algae and not all the groups have distinctly different pigmentation. This is seen from the samples in Paper I: both prymnesiophytes and diatoms contain chlorophyll c's and fucoxanthins, so chemotaxonomic identification requires separation of the various chl c's and fucoxanthins. Prasinophytes on the other hand, contain the green algal marker pigment chlorophyll *b*, which is clearly distinguished. Furthermore, the cellular pigment content is variable also in time, since the cells can acclimate to changes in light through changes in their pigmentation[41]. Chemotaxonomy is comparable to NMR analysis in that it occurs on the level of metabolites, but otherwise these two methods must not be confused. In the NMR analysis of whole cells there is no separation of molecules and the molecules can not be fractionated and restored such as in a HPLC analysis.

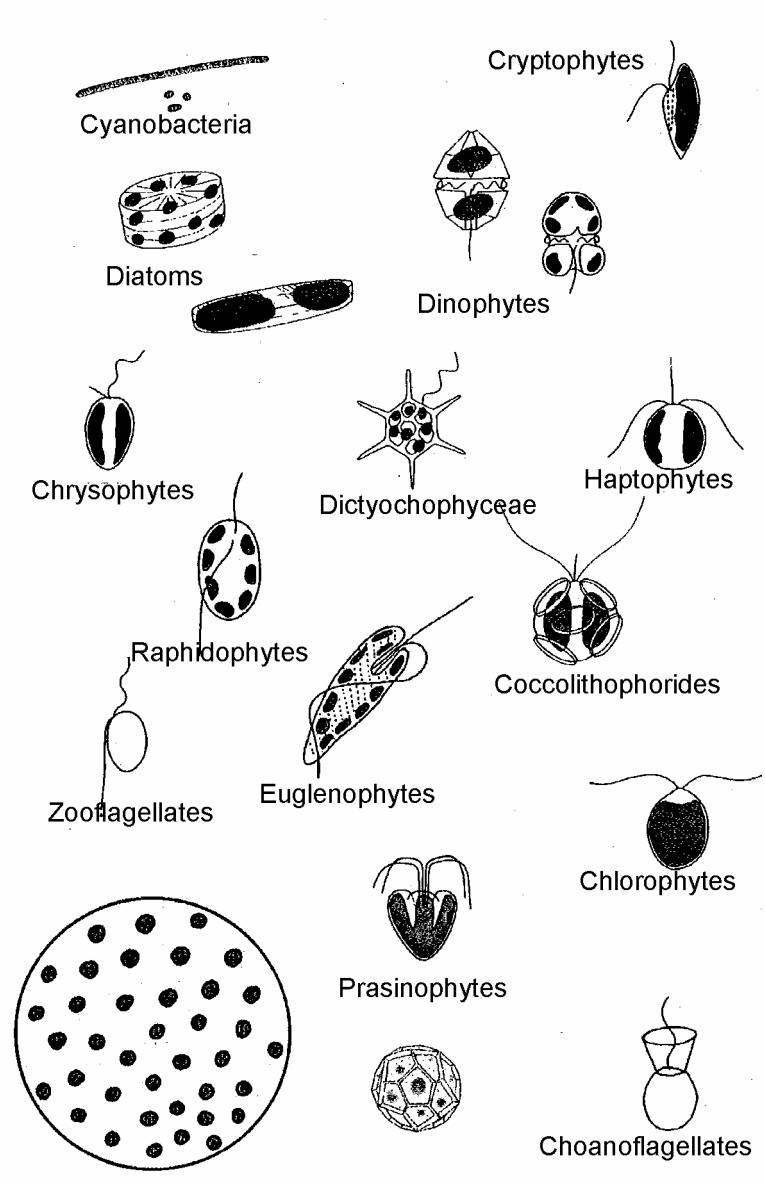

Figure 4 Morphological diversity of microalgal groups, with examples of numbers and shapes of chloroplasts (shaded areas). Reproduced (with permission) from Throndsen & Eikrem (2001)[42]

- The many thousand of identified species of microalgae show a wide variety of morphological structures (Figure 4).
- The classical way of identification is through light microscope, but the work is time consuming and the results are biased according to the operators experience (Paper I).
- In chemotaxonomy marker metabolites are used to identify groups or species. A widely applied method is pigment chemotaxonomy, where HPLC analysis and determination of group-specific pigments form the basis for identification and biomass estimation (Paper I). In the NMR analysis of whole cells there is no separation of molecules such as in chromatography.

3.2 Metabolites and cellular organisation

In Paper I the dominating groups of flagellates and diatoms were studied in mesocosm experiments, through abundance (cell numbers), cell chemistry related to organic C and N, and pigments and photophysiology (Table II of Paper I). The variables were chosen specifically for a study related to identification through species-specific pigmentation and the following differences in photosynthetic effectiveness, but the same variables are central in other microalgal studies. Any study of microalgal primary production, nutritional value or toxicity usually involves measurements of one or several of the following metabolites (Figure 5):

Amino acids: Building blocks in proteins and important in processes such as N-uptake by Glu/Gln.

Carbohydrates: Energy storage product of several microalgal groups, e.g. β-D-(1→3)-glucan chrysolaminaran.

Fatty acids: Microalgae produce important polyunsaturated fatty acids such as the ω-3 acid eicosapentaenoic acid (EPA).

Pigments: Involved in the photophysiology and can be used to identify algal groups, e.g. fucoxanthins.

Toxins: Wide variety of molecular structures and effects, e.g diarrhoea, amnesia or paralysis in man and higher animals. E.g. saxitoxin from *Alexandrium* spp.

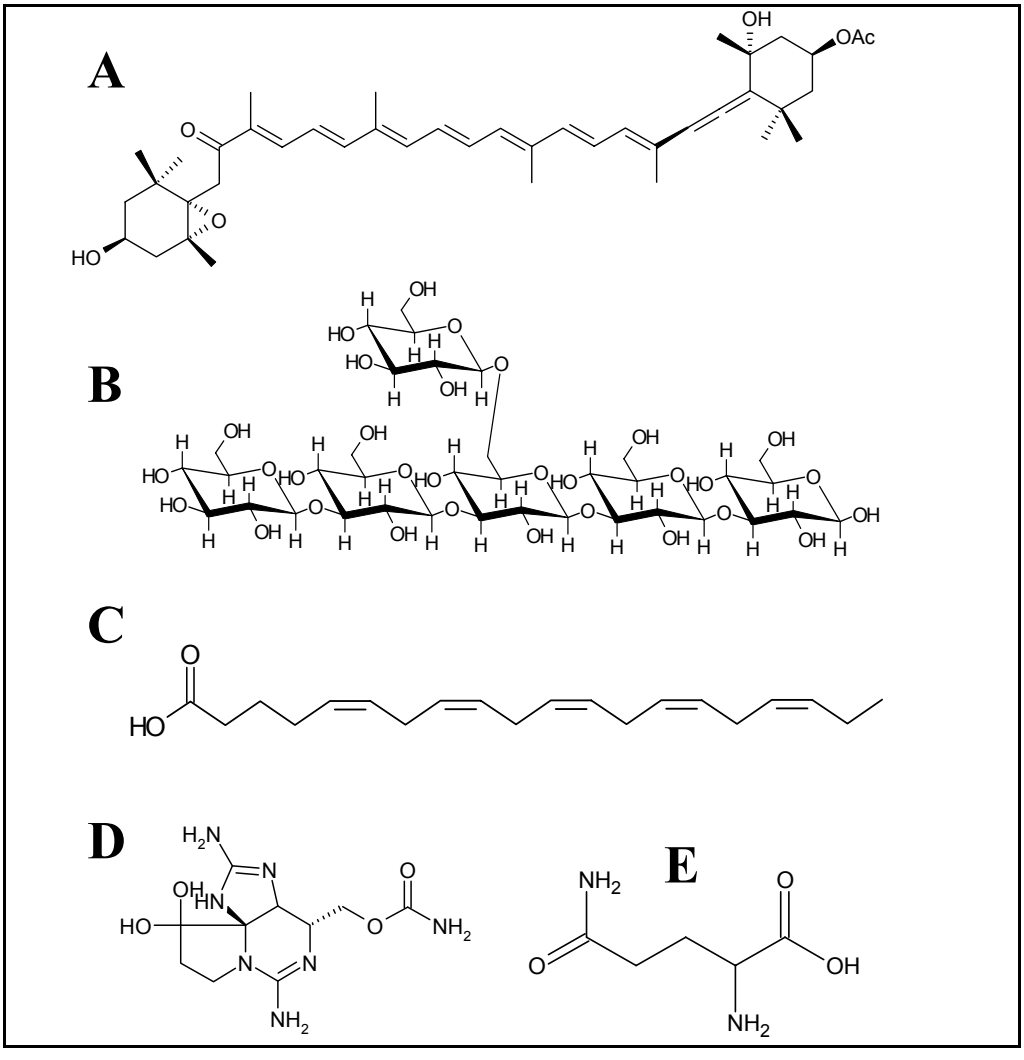

Figure 5 Molecular structures of selected microalgal compounds. **A** The pigment *Fucoxanthin*. **B** The carbohydrate *β-1,3-glucan* (1,6 branching). **C** The polyunsaturated fatty acid *Eicosapentaenoic acid*. **D** The toxin *Saxitoxin*. **E** The amino acid *Glutamine*. Molecular drawings were made using MDL ISIS Draw 2.5 and ACD/ChemSketch 5.12 (including templates), by courtesy of T. Størseth.

Most of these metabolite groups have, although to a varying extent, species-specific representatives that can function as chemotaxonomic marker metabolites. Pigments have already been mentioned (Paper I) and pigment-based chemotaxonomy is an established field[8,40]. Toxins can function as group-specific markers at least, since there are a wide variety of molecular structures involved[51,63]. Lipids and sterols have been used for chemotaxonomic purposes[43,49]. Carbohydrates does not seem to be as useful as biomarkers, since the common storage carbohydrates in e.g. diatoms and dinoflagellates are β-1,3- and α-1,4-glucans (chrysolaminarin and starch, respectively). A high variety of saccharides on the other hand, is found in the organic covering or scales on the cell walls of e.g. the chlorophyte *Tetraselmis striata*[44].

As mentioned, nuclides such as ^{1}H and ^{13}C are found in the vast majority of organic compounds, and both ^{1}H and ^{13}C NMR is successfully applied to biological samples. ^{1}H and ^{13}C HR MAS NMR spectroscopy of diatom cells lead to the tentative assignment of different molecules such as fatty acids, amino acids, and carbohydrates (Table 3 of Paper IV, Table 1 of Paper V, and Paper VI). Signals from pigments and toxins were possibly seen, but it is suggested that the signals are weak either due to low cellular concentration or inhibition due to cellular localization (see discussion in Part 4 on Metabolic profiling). In terms of molecular weight, the examples mentioned above are all relatively small molecules varying from about 100 to a few hundred Da. Polymers of β-D-glucans such as chrysolaminaran can consist of 20-60 sugar units and have a molecular weight of 3300-9800 Da[20], still within the size range that is considered feasible for NMR analysis[45].

The cellular organisation varies among microalgal classes, and an example here is taken from the diatoms (Figure 6). In *Thalassiosira weissflogii*, which is a close relative to the studied *T. pseudonana* (Paper IV), a large central vacuole occupied most of the cell volume, and the cytoplasm (including organelles) was distributed in the periphery of the cell[46]. Connections were observed between e.g. endoplasmic reticulum and various organelles, probably facilitating exchange and transport of metabolites or nutrients. Cells grown in nutrient rich medium contained material of varying density (graded “less dense” or “very dense” in electron microscopy examination) often located in the central vacuole. Lipid droplets were seen in the chloroplasts, while in the chlorophyte *Dunaliella* lipid droplets were stored in the cytoplasm.

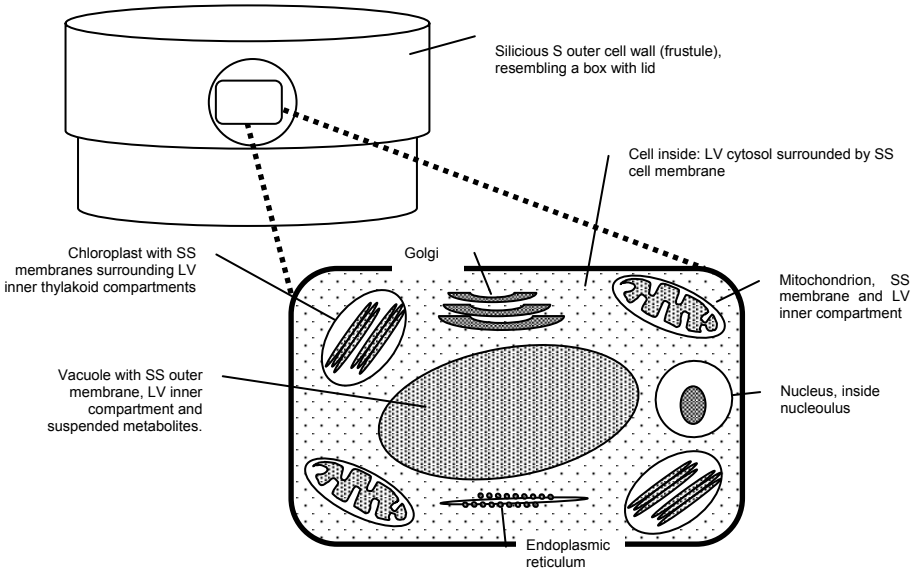

Figure 6 Schematic diatom cell showing silica outer cell covering (frustule), and eucaryote cell inside. Examples of organelles that are supposed to be significant in the NMR analysis of whole cells, particularly due to their inner compartments with low viscosity content (chloroplasts, mitochondria and vacuoles) or involvment in production or transport of metabolites (Golgi, endoplasmic reticulum and cytosol). Drawings are not to scale, and numbers in cell may vary. Comments regarding differences in viscosity or structural solidity are shortened to S = solid, SS = semi-solid, LV = low viscosity.

The cellular distribution of metabolites is important for the NMR analysis. Low viscosity areas such as cytosol and vacuolar spaces are suitable for the NMR analysis, especially of small molecules that tumble freely at high rates. In Paper III this is seen from the details of the multivariate analysis, where several osmolytes determined the outcome of the clustering analysis. As mentioned, motional restriction of molecules determines the spectral information output, but not necessarily in a negative sense. The cellulosic theca of dinoflagellates consist of long polymers of glucose (> 3500 units in each chain) and their contribution to the NMR spectrum could possibly be obscuring other signals. Since the nuclides of the cellulose theca are motionally hindered due to the rigid construction, they seem to be “NMR invisible” and contribute little to the signal. Also, large molecules with slow tumbling rate, such as proteins, contribute little to the NMR signal. Instead, low molecular weight molecules such as free amino acids or osmolytes in liquid or semi-liquid compartments contribute significantly to the NMR analysis of whole cells (Table 3 of Paper IV, Paper VI).

- Amino acids, carbohydrates, fatty acids, pigments and toxins are important metabolites in many algal studies (Figure 5).
- Group- or species specific marker molecules can be found in most of these metabolite groups, and e.g. chemotaxonomy based on pigmentation is an established field in phycology (Paper I).
- Fatty acids, amino acids, and carbohydrates were indicated in whole cell samples of diatoms analysed by ^{1}H and ^{13}C HR MAS NMR spectroscopy, and possibly signals from toxins and pigments (Papers IV and V).
- Differences in cellular concentrations of metabolites and variations in molecular mobility due to localization in areas of varying viscosity or solidity (e.g. vacuoles vs. membranes) are suggested reasons to explain the variations in signal intensities (Figure 6).
- Osmolytes determined the outcome of PCA (Paper III), in consistency with the expectation of prominent signals from small molecules in low viscosity areas such as vacuoles and cytoplasm in the NMR analysis. Large molecules with slow tumbling rates yield broad resonances, or are 'NMR invisible' due to motional restriction.

4 NMR AS A TOOL FOR SPECIES DISCRIMINATION AND METABOLIC PROFILING OF MICROALGAL SAMPLES

Species discrimination

The commonly used taxonomic identification methods for microalgal samples, microscopy and pigment based chemotaxonomy, both rely on species- or group specific traits that can distinguish the algae. In the first case it is a matter of morphological differences, while in the chemotaxonomic analysis we look for molecular biomarkers such as group specific pigments in the cells. The HR MAS ^{1}H NMR spectra however, display a multitude of molecular signals but from the tentative assignment of peaks it seems that the separating signals are related to molecules that are quite common in algal cells, e.g. amino acids or osmolytes (Table 2 of Paper II, Table 1 of Paper III). In the PCA of Paper II the spectral input region from 4-3 ppm is characterized by amino acid and carbohydrate signals (among others), while in the PCA of Paper III the input region was expanded to 4.5-0.5 ppm. Despite the wide data input region the outcome seemed to be related to variations in a only a few molecules, and these molecules are not species specific to any algae. Yet, the significant grouping of replicate samples in two PCAs and the FC support the use of NMR spectroscopy as a tool for discrimination of algae (Papers II, III and VI). Rather than distinct biomarkers for each alga, it may be that the differences are related to varying concentrations or ratios of different cellular components.

The fact that there are no clear species or group specific biomarkers involved in the PC and FC analyses is not a problem for separation into groups or clusters of a set of samples[26,47] but it may indicate that it is more difficult to analyse “anonymous” samples and determine the taxonomic origin. On the other hand, detailed studies of the whole metabolite profile of the algae may reveal proper biomarkers, while in the two Pattern recognition (PR) methods applied here only parts of the HR MAS ^{1}H NMR was used as input and other useful regions may have been excluded. An example is the low-field region of the spectral signature of the dinoflagellate *Amphidinium carterae*, where some distinct signals are seen from 6.2-5.6 ppm while no similar signals are apparent in the spectra from the other algae (Figure 1 of Paper II). This part of the spectrum contains signals from aromatic parts of molecules, and few signals from “obscuring” molecules such as carbohydrates and fatty acids[48]. Thorough investigation of the signals in this region may reveal group- or species specific biomarkers such as pigments or toxins[49,50,51], and due to the low interference of other metabolites it may be a suitable input region for PCA although the signals are sometimes of low intensity.

True biomarkers, in addition to being species specific must be relatively stable in the cells through the life cycle, and also through the sample handling and analysis. The tentative assignment of distinguishing signals in the PCA of Paper III indicated that lactate was involved. Lactate is a small molecule connected to anaerobic processes and the cellular content is rapidly changed naturally during the life cycle. The levels of lactate dehydrogenase (LDH) was very low in plants and macroalgae, and no clear explanation for its function could be found[52]. To my knowledge, there is little work done on lactate in microalgae, but some references found that the activity of lactate dehydrogenase was pH and temperature dependent in cells of *Dunaliella salina* and *Chlamydomonas reinhardtii*[53,54]. Both pH and temperature differences leads to differences in NMR properties as well. Furthermore, lactate became invisible in NMR analysis depending on binding to other compounds[55]. Therefore, although involved in the determination of the analyses here, lactate is an example of a metabolite which is not a suitable candidate for biomarker.

The spreading of replicate samples in the PCAs indicate high intraspecific variance in some molecular constituents, and the question arises whether this variance is large compared to the interspecific variance between algal species or groups (Figure 2 of Paper II and Figure 2 of Paper III). In the first PCA the third PC introduced separation even between the two diatoms *Thalassiosira pseudonana* and *Phaeodactylum tricornutum* (Figure 2 of Paper II), while in the second PCA the two first PCs explained most of the variance and further PCs did not add any significant information (Figure 3 of Paper III). The result of the FC analysis applied to the data in Paper II also emphasize the higher interspecific variance since the data set again exhibited grouping. In a study of treated vs. untreated liver tissue samples the untreated (or "normal") samples showed higher within-group variability than the treated samples in a PC analysis, yet the distinguishing grouping of the two sample sets was clear[56].

In order to distinguish a treated sample from a normal, or in the case of distinguishing a set of microalgal "normal samples" of different groups or species, the intraspecific variance in the data needs to be controlled so that methodological or regular physiological variation is not determining the output. An initial attempt to check the reproducibility of NMR analyses on microalgae was done in the first PCA analysis (Paper II), were a set of two samples from a previous experimental setup was included in the data input. These two samples, one of each of the two diatoms, were prepared under the same conditions as the other through all the stages of culturing and harvesting (albeit at different times), and then storage followed by NMR analysis. In both the PCA and the FC analysis they were correctly classified, so the reproducibility of the NMR analysis is supported also for algal

samples. In a comparative study of a split set of rat urine samples, the conclusion was that NMR and pattern recognition (PR) methods such as PCA is a robust and precise method with high reproducibility, and analytical variance was much smaller than the normal physiological variance in the samples[57]. Since there have been few NMR studies of microalgae this is something that must be evaluated specifically for these samples, so that the variance in the data is controlled or accounted for.

- The distinguishing signals in the PCA are related to common metabolites such as amino acids and osmolytes, rather than species or group-specific metabolites (Paper III). Instead, variations in concentrations or ratios can be the determining factor. For separation of data sets into groups, or clustering analysis, this is not a problem. On the other hand, to determine the taxonomic belonging of an "anonymous" sample is more difficult without specific markers.
- True biomarkers must be group or species-specific, and stable throughout the life cycle, sample preparation and analysis. Lactate gives clear signals in the NMR analysis unless it bonds to other molecules, but is an example of a compound that is common to most organisms and also quickly changed in the natural cell cycle and therefore not suitable as biomarker. Instead, low-field signals (>5.5 ppm) in the HR MAS ^{1}H NMR spectrum from *A. carterae* and other algae are suggested as a possibility to find true species-specific biomarkers such as pigments or toxins, and signals in this region also benefit from little interference from otherwise protruding signals from e.g. sugar and water.
- Variations in pH and temperature during sample handling and analysis will affect the chemical shifts, and methodological and physiological variations must be controlled so that the outcome is not determined from these factors.
- The outcome of the PCA and the FC showed clear grouping of replicate spectra, even though the within-group variability was high in some cases (Paper II). Two different species of diatoms were separated, indicating a possibility for sorting down to species level (Figure 3).
- Robustness and reproducibility of using NMR and PCA to distinguish algae was shown, since samples from different experimental setups fell into the correct groups (Papers II and III).

Metabolic profiling

NMR is established as an excellent tool for metabolic profiling of cells, or metabolomics[58,59]. Furthermore, studies of NMR analyses of biofluids are often related to metabonomics, or ‘ the quantitative measurement of the multiparametric metabolic response of living systems to patophysiological stimuli or genetic alterations’[5]. Experiences from studies of plant cells should apply to microalgae, albeit apparent differences such as the algal cell being an entire organism (contrary to the plant cell which is one of many cells in the organism)[11, 31, 58]. Many metabolites found in human biofluids are also found in microalgae, so also here it should be possible to use the extensive knowledge from e.g. pharmacological studies on blood and urine samples[10].

Both ^{1}H and ^{13}C HR MAS NMR analyses on diatom cells indicated the presence of metabolites such as amino acids, carbohydrates and fatty acids (Papers IV and V). These are very different molecules, both in size and structure. Individual amino acids are small molecules and they usually give narrow, clear peaks in the NMR spectrum. Large proteins on the other hand, have slow tumbling rates and in the NMR analysis this means that the obtained signal peak is broad and obscuring, or even that proteins can be “NMR invisible”. The carbohydrate β-1,3-glucan is used as a storage product in diatoms, and signals are found both in ^{1}H NMR (Table 3 of Paper IV) and ^{13}C NMR (Paper V). Signals from carbohydrates tend to overlap with others and cause a “crowded” appearance of certain regions in the spectra in the ^{1}H NMR analysis, so ^{13}C NMR analyses of carbohydrates benefits from the improved resolution of a wider chemical shift scale[20]. Fatty acids, which are important membrane constituents, can also be studied in detail with ^{13}C NMR (Paper V). Signals from important polyunsaturated fatty acids (PUFAs) were seen, and it is possible to study ratios and positions of substituted fatty acids from selected signal peaks[60]. Signals from parts of fatty acid molecules are indicated also in the HR MAS ^{1}H NMR spectrum from microalgae whole cells (Table 3 of Paper IV), but mainly the fatty acids signals are suppressed during acquisition since they would otherwise yield broad and unresolved signal peaks.

The other two metabolite groups which are central in studies of microalgae, namely pigments and toxins, can be used to illustrate another important point when using NMR for simultaneous metabolic profiling: the relatively large differences in cellular concentrations of NMR-detectable metabolites. In our analyses it is suggested that signals from aromatic regions of some carotenoids can be seen in the low-field end of the spectrum, but the signals are weak (Paper IV). A reason for this can be the relatively low cellular concentration of the various pigments compared to e.g. fatty acids (Paper V). The location of pigments on membranes in the chloroplast can also be

the reason for the apparent NMR invisibility, since this may restrict the molecular motion[61]. Microalgal toxins are molecules of many different types, and the cellular concentration is dependent on physiological status and growth conditions[62]. Saxitoxin is a N-rich molecule and up to 10% of the cellular N can be associated with toxins in the dinoflagellate *Alexandrium tamarense*. It is a water soluble molecule with molecular weight around 300-400, and ring structures[63]. These conditions should make NMR a suitable tool for toxin analysis, but the cellular concentration of toxins is possibly too low for detection in whole cell analysis: Cells grown at an irradiance of 150 μmol m^{-2} s^{-1} contained up to 450 femtomoles $cell^{-1}$ of saxitoxin[64]. An alternative could be to apply ^{15}N NMR, but the natural abundance of this isotope is low, only about 0.4%. Other toxins are lipophilic, such as the neurotoxin ciguatoxin, and if they are restricted in membranes or inside organelles, they may be NMR invisible due to the motional restriction.

NMR spectroscopy on cell samples does seem to favour certain metabolites, namely low molecular weight compounds in liquid compartments. In a cell, this corresponds to the cytoplasm and vacuoles, and the metabolites within. Osmolytes seems to fall into this category, and the largest variations between the algal spectra that were analysed with PCA were related to different molecules of osmolytic function (Paper III). A high score for PC3 was found to indicate increasing choline content in the microalgae (Paper III). The visible choline signals in a NMR analysis were linked to cytosolic choline in a study of NMR detectable changes during cell transfection, since membrane-bound choline give broad and poorly resolved peaks[65], and the same argument possibly applies here. If the signals are indeed from cytosolic choline it supports the statement that NMR signals from whole cell samples reflect certain cellular regions. The similarity between spectra from whole cells and hydrophilic extract seen in the ^{1}H NMR analysis here is also partly explained by the use of the pulse sequence CPMG in the MAS analysis of whole cells (which leads to suppression of signals from larger molecules such as lipids) but otherwise this similarity between spectra supports the claim that the NMR analysis of whole cells is selective (Figures 2, 3 and Tables 2, 3 in Paper IV). This selectivity of signal origin is not necessarily negative, since it can be used intentionally to study transport or compartmentation in cells as metabolites can be localized from their presence or absence in the spectra[66].

Extraction samples were used here for initial identification of metabolite signals, since extract spectra usually have better S/N ratio and less overlapping peaks. On the other hand, extraction spectra reflect the solubility of metabolites rather than real occurrence in whole tissue, since extraction is not always complete and some metabolites may be

encapsulated in an environment not affected by the given solvent (e.g. micelles)[65, 67]. A comparison of the spectra indicated some shift differences between extract and whole cell samples (Tables 1-3 of Paper IV, Table 1 of Paper VI). Shift differences are related to changes in chemical environment of the nuclides, and extracted metabolites will display different chemical shifts than metabolites in intact cells. As seen from the tables, the shift differences were small, which can indicate that the metabolites are intact also after extraction. Another factor that can affect the chemical shifts in whole cell samples is variation in pH. The thylakoid lumen of several diatoms is acidic, and if membranes are broken during the analysis this may affect the chemical shifts of other metabolites in the sample[68]. On the other hand, the suggested proton shifts at 3.77, 2.08 and 2.44 ppm for the amino acid Glutamine (Gln, Figure 5) in a whole cell sample of *Thalassiosira pseudonana* (Table 3 of Paper IV) is very close to the shift values reported for ^{1}H in Gln at neutral pH: 3.77, 2.14 and 2.45 ppm, for αCH, βCH_2 and γCH_2 respectively[69]. This indicates that the amino acid is located in some cellular compartment where the pH is neutral, at the moment of analysis at least. Closer studies of chemical shift variations due to extraction or variations in cell chemistry is a topic that will be addressed in parallel with the planned establishment of a reference library for microalgal metabolites.

- Signals from amino acids, carbohydrates and fatty acids were seen in both ^{1}H and ^{13}C HR MAS spectra (papers IV and V). Some signals were also assigned to common metabolites such as osmolytes and organic acids (Paper III).
- Broad and overlapping signals from carbohydrates and fatty acid benefit from the wider shift scale of ^{13}C analysis. Signal suppression and application of HR MAS increased the spectral resolution also on whole cell samples, both in ^{1}H and ^{13}C analysis (Papers IV and V).
- Possible signals from pigments and toxins are weak perhaps due to low cellular concentration of the actual metabolite, or motional restriction due to localization in membranes.
- Signals from low molecular weight compounds in low viscosity regions of the cell (cytosol, vacuoles, chloroplasts, etc.) do seem to be favoured in the NMR analysis of whole cells (Papers III, IV and V). This fact can be used to study transport or compartmentation in cells.
- Extraction spectra have higher S/N ratio and better resolution, but the spectra reflect metabolite solubility and extraction may be incomplete. On the other hand, shift differences were small between extract and whole cell spectra, indicating that the metabolites are intact also in extract (Papers IV and V).

NMR analysis of microalgal cells, general topics

Traditionally, NMR has been used to resolve molecular structures and identify compounds, and in a liquid sample with only one type of molecules this is a relatively straight forward procedure from comparison with known standards. In more complex samples such as whole cells the spectral assignment is more complicated. In the articles presented here the assignment was based on comparison of chemical shift values with literature references of other published works, and is therefore tentative (Papers IV-VI). The next step is to perform ”true” spectral assignment with the aid of 2D spectra and correlation spectroscopy (COSY)[61]. Once the spectral assignments have been verified also for microalgal whole cell samples and a library of chemical shift for commonly occuring metabolites is established, it should be possible to use the method of tentative assignment from comparison of shift values as a routine since this offers a rapid way of spectral analysis.

For the purpose of comparison, the spectra used in the PCAs were acquired using a standardized protocol. This may lead to some variations in signal quality between the different algae, but visual inspection of the spectra showed that the quality was high for all spectra (Paper II). Obstacles such as spectral line broadening and peak overlapping improved from the pulse manipulation of spin systems and application of HR MAS.

This work is based on results from analysis of whole cell samples, but the application of centrifugation and then spinning of samples may lead to some degree of cell rupture (Papers II and V). During harvesting and storage the microalgal cell samples were subjected to centrifugation at 5865 *g* and freezing at -20° C, and then thawing before they were inserted into the NMR tubes. Visual inspection of some selected samples after centrifugation showed that e.g. cells of *Alexandrium* were mostly intact, although the cell walls (but not the cell membranes) were disrupted in many cases. It has been argued during the work on the papers included here that since the signal from whole cell samples reflects small molecules in low viscosity surroundings, most cells were probably disrupted and the analysis performed on a “slurry” of cell content. In the MAS NMR analysis the microalgal samples were spun at max 5 kHz. For comparison, cells of the yeast *Pichia anomala* were subjected to centrifugation (force not reported) and MAS spinning at 5 kHz. In addition, some yeast samples were kept at 30° C for 20 min to simulate the temperature effect of the NMR analysis, and other yeast samples were kept at 4° C to investigate the effect of cold storage, and then checked for viability. The treatments had minimal effects on viability[70]. Viability is not an important parameter here, but based on these findings (and other works cited in Papers II and V) it is reasonable to

believe that also microalgae cells can be subjected to MAS NMR without being completely destroyed.

The are several factors that can explain why the 'whole-cell signals' show similarity with signals from extract samples or organelles in suspension. As mentioned, the cellular cytosol is an excellent location for NMR analysis due to the low viscosity, and many metabolites find their way through cytosol either during cellular uptake or exportation, or at some stage during biosynthesis or transport inside the cell. In microalgal cells from 25-80% of the cell volume can be occupied by the cytoplasm[71], and the contribution from metabolites in the cytosolic region to the NMR signal of whole cells should therefore be significant. Also, many microalgae have a large central vacuole, and the conditions here would be similar to those described above[46]. Nutrient storage vacuoles can occupy up to 40% of the diatom cell[1], so it is reasonable to argue that any low molecular weight metabolites in low viscosity surroundings here will be prominent in the NMR spectrum even though the cell is not disrupted. Furthermore, the final step in the biosynthesis of DMSP occurs in chloroplasts in the plant *Wollastonia biflora*[72]. If this is the case also for DMSP in algae, it is an indication that some signals stem from suspended molecules in the thylakoid lumen inside chloroplasts. The suggested chemical shifts of DMSP (Table 1 of Paper III) were coincident with shifts measured at acidic pH[69], which may indicate that the observed DMSP signals do actually stem from the thylakoid lumen. Whether the 'whole cell signal' really represents compartments or parts of the cell, is an important topic for further investigations.

- Spectral assignment based on comparison with literature references is tentative, and is considered a temporal solution until a library of spectral shifts of algal metabolites is established.
- All spectra were acquired using a standard protocol, for the purpose of comparison in the multivariate analyses (Papers II and III). This may not be an optimal solution considering the cellular differences of the algae, but visual inspection of the spectra showed that the quality was high in all cases.
- Visual inspection of *Alexandrium* cells after centrifugation showed that most cells were intact. Based on comparison of other published work on comparable cell types, it is argued that the cells are mainly intact ('whole cells') also during the MAS analysis (Paper II).
- The similarity between whole-cell and water extract spectra is partially explained from the large cytoplasmic content of some algae, or large vacuoles of others (Papers IV and V). In addition, signals can stem from metabolites in enclosed liquid domains such as thylakoid lumen in chloroplasts.

5 CONCLUSION AND FUTURE PERSPECTIVES

This work started out with the question "Can we use NMR to identify microalgae?". This work shows that it is possible to discriminate between different classes of microalgae based on their specific HR MAS ^{1}H NMR spectra, and also that species of the same class are separated in the multivariate analysis. It is not clear if the origins of the differentiating signals are specific enough to discriminate all groups or classes of algae. If the signals originate from molecules common to many or all species, then it is not right to claim that NMR can be used as a taxonomic tool since it will be difficult to identify different species of algae based on their NMR spectra. On the other hand, being able to discriminate between different algae even without species identification is very often what is needed, and in such cases the NMR analysis is a useful tool.

There have been relatively few studies performed with NMR on microalgae, and a lot of work remains. As shown in this work, NMR provides a non-invasive analysis of metabolites in microalgae, and the analysis can be performed in a relatively short time (from minutes up to an hour, in many cases) without any use of organic solvents or tedious sample preparations, and the result is broad range information on low molecular weight metabolites simultaneously. Yet, further studies including 2D NMR and COSY on extract and whole cell samples are necessary to properly assign peaks and establish a reference library for microalgal metabolites. Also, closer studies of chemical shift variations due to extraction or natural physiological variations in cell chemistry are necessary, and the search for proper biomarkers continues. The low-field end of the NMR spectra may be a good place to search since it displays characteristic signals without the interference of high-concentration metabolites such as fatty acids and carbohydrates. The statistical application PCA has shown to be a robust method with high reproducibility also for microalgal spectral data, and a suitable way of data reduction and graphical presentation. What remains is a thorough study of inherent biological variance in samples, and a mapping of the parts of variance which must be assigned to methodological and analytical variance.

Some ideas for future applications have come up during this first project period. The topic of metabolite transport and cellular compartmentation was briefly mentioned, and NMR could be an useful tool for such studies. An example is ^{13}C NMR study of labelled HCO^{3-}, to follow the uptake into the cell and the transport to the cytoplasm where it is converted to malate before it is transferred to the chloroplast and goes into the Calvin cycle[73]. This could possibly offer some answers to the questions around the whole cell signals, and signal origin. An example study includes ^{13}C and ^{31}P NMR to study transport and compartmentation in higher plant cells, and metabolism

of homoserine[74]. The results show distribution of homoserine between cytoplasm and vacuoles as a function of time after incubation. Another possibility is to create micelles or liposomes with a hydrophobic outer layer of lipids and a low viscosity inner compartment, to simulate a distribution of metabolites in compartments or membranes.

Also, the application of NMR to field samples would be interesting, although at the moment it would mean to collect samples in the ocean and bring them to the laboratory for analyses. Plankton net tows from five different oceanic sites were analysed by ^{13}C CP/MAS NMR, to determine the major biochemical composition in elemental ratios[75]. A typical coastal spring bloom is dominated by one or a few species, often from the same group of microalgae (e.g. diatoms) and the results of a NMR-analysis could therefore be related to the dominating species. Also, identification of proper biomarkers for different algae would make it possible to extract information from the NMR spectrum to the different algal species or groups in a mixed field sample.

Rather than a comparison of NMR spectroscopy to other relevant analytical methods for microalgae (such as the mentioned HPLC and bio-optical measurements) which is difficult due to the very different nature of the analyses, this work instead pretends a presentation of some of the clear advantages offered by NMR: It can be used non-invasively on whole cells, thereby avoiding non-healthy solvents. The massive information output from a single analysis run includes signals from different metabolites independent of solubility, and is applicable for many purposes such as species discrimination and metabolic profiling. Also, a comparison of NMR and infrared (IR) spectroscopy for authentication and composition analyses of foodstuffs concluded that NMR has some advantages in that it is non-destructive and colour-independent, requires less calibration and the signals are proportional to the analysed quantities. Furthermore, the reproducibility of the NMR analysis is higher and the analytical operation is easier to implement among non-specialists, than IR spectroscopy[76].

Based on the results obtained in this work and the planned work on further characterisation of microalgae from their NMR signatures, and the views for future applications of NMR spectroscopy in marine biological science, I am tempted to say that NMR is a promising new tool for phycologists!

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