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HR MAS ^{1}H NMR spectroscopy analysis of marine microalgal whole cells

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ABSTRACT: To study the use of nuclear magnetic resonance (NMR) spectroscopy as a method of classification, we performed high-resolution magic angle spinning proton (HR MAS ^{1}H) NMR spectroscopy analysis of whole-cell samples of *Dunaliella* sp. (Chlorophyceae), *Amphidinium carterae* (Dinophyceae), *Phaeodactylum tricornutum* and *Thalassiosira pseudonana* (Bacillariophyceae). Emphasising the potential use of NMR spectroscopy as a routine analysis of microalgae we chose a straightforward procedure for culturing and harvesting, without extraction or radioactive labelling. We obtained well-resolved HR MAS ^{1}H NMR spectra from the 4 algae, despite the fact that our samples contained whole cells and some residual sea water. Selected parts of 5 replicate spectra from each microalga were used as input in 2 multivariate pattern-recognition strategies (principal component analysis and fuzzy clustering), both analyses showing clear grouping of the different species. Two spectra from a previous sample run (cultures grown under the same conditions) were also included, and both were correctly classified. We therefore consider HR MAS ^{1}H NMR spectroscopy to be a potential method of classification for microalgae, with statistical data processing indicating replicability and robustness of the method.

KEY WORDS: HR MAS ^{1}H NMR spectroscopy · Microalgae · Whole cell sample · Classification · Principal component analysis · Fuzzy clustering

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INTRODUCTION

Microalgae are important primary producers in aquatic environments (Sakshaug et al. 1997). The majority of known species are beneficial, supplying organic carbon to the rest of the food chain. A few selected species are frequently used as feed for fish larvae and bivalves, and some algae have also become popular in the health food industry. However, a small number of microalgae are harmful, and although each individual is small, they may occur in huge numbers known as 'blooms' (Zingone & Enevoldsen 2000). Humans are exposed to microalgae toxins, e.g. through filter feeding mussels, which concentrate algae from the surrounding water. The mussel itself is unharmed by these toxins, since they affect nerve systems or intestines of vertebrates. Therefore, we need to monitor microalgae in the water to prevent harvesting of shellfish when toxic algae are present. Also, when culturing selected algae for feed or health food purposes, we want to ensure optimal quality of the desired species, also in terms of chemical composition.

Identification of microalgae is time consuming and tedious work, which traditionally has been done by microscopy. Development of modern techniques such as HPLC has provided efficient tools in this field since microalgae have group-specific pigmentation, and in many cases unique marker pigments (Jeffrey et al. 1997). However, in oceanic environments the biomass is often low, so filtration of large volumes of water is

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required, and samples must be extracted before analysis. Also, HPLC analysis requires a different set-up for the analysis of different substances, while using NMR provides us with a large amount of information on, for example, protein, lipid, and carbohydrate composition in each spectrum, or dynamics in cell constituents (Broberg et al. 1998, Kimura et al. 1999).

NMR is well known from chemistry and medicine, where important results have been obtained in areas such as molecular-structure determination or analysis of human tissue or blood samples. NMR spectroscopy of semi-solids is a relatively recent technique, but while liquid samples yield spectra with narrow lines and good separation of peaks, in solid or semi-solid tissue or cell samples (which are often highly heterogenous throughout the sample) this is not always the case. Increased line widths and spectral overlapping in semi-solid samples is known to occur due to dipolar couplings and chemical shift anisotropy which affect the chemical environment of hydrogen in the different cellular constituents (Warschawski et al. 1998). High-resolution magic angle spinning (HR MAS), where samples are spun at low frequency around their own axes at an angle of 54.7° relative to the magnetic field, has the effect on semi-solid or solid samples that it decreases spectral line broadening due to molecular anisotropic interactions (Andrew 1981). With HR MAS it is therefore possible to obtain well resolved spectra with narrow peaks, also from samples of intact biological material. It follows naturally that NMR is becoming popular outside the traditional fields as a useful tool in studies of biological systems (Krainer et al. 1994, Ohara et al. 2001, Viant et al. 2002). Application of NMR in studies of higher plants is reviewed by Ratcliffe et al. (2001), and HR MAS NMR studies have been performed on macroalgae (e.g. Broberg et al. 1998). HR MAS studies of microalgae are scarce.

Two strategies of multivariate pattern recognition were applied in this study in order to evaluate the possible classification of microalgal species from their specific ^{1}H NMR spectra. Principal component analysis (PCA) is a linear dimension reduction technique, where systematic variation in a data set is extracted to principal components (PCs) through the modelling of variance- and covariance structure (Martens & Næs 1991). Fuzzy clustering (FC) involves partitioning of the available data into fuzzy subsets that can be approximated by linear regression models locally (Babuska et al. 2001). The use of clustering techniques is motivated by the fact that structures in the data can be revealed without relying on assumptions common to conventional statistical methods, such as the underlying statistical distribution. In FC the probability of a sample being a member of a specific class is calculated based on NMR spectra and the expected number of classes. Recent studies showed that PCA and hierarcical cluster analysis of ^{1}H NMR spectra could be used to separate earthworm species (Bundy et al. 2002), and multivariate analysis of ^{1}H NMR spectra was used in metabolic fingerprinting of the plant *Arabidopsis thaliana* (Ward et al. 2003).

The green alga *Dunaliella* sp. is widely used as feed in aquaculture, and the 2 diatoms *Phaeodactylum tricornutum* and *Thalassiosira pseudonana* are both biochemically suitable as feed. The dinoflagellate *Amphidinium carterae* can produce haemolytic compounds and might be related to cases of Ciguatera fish poisoning which affects human fish consumers (Hallegraeff et al. 1995). Compared to traditional application of NMR, extensively used in chemistry and medicine, our sample preparation and analysis is quite 'rough', and this is a great advantage since we want to obtain results in a quick and efficient way. With the development of methods such as HR MAS and solid state NMR, and also the special pulse sequences that facilitate analysis of semi-solid, heterogenous biological samples, we see a great potential in the field of microalgae identification and cell composition studies.

MATERIALS AND METHODS

Five replicate cultures of 4 species of microalgae (*Dunaliella* sp. Teodoresco, *Amphidinium carterae* Hulburt, *Phaeodactylum tricornutum* Bohlin, and *Thalassiosira pseudonana* Hasle & Heimdal) (Tomas 1997) were cultured in nutrient enriched, filtered and pasteurized sea water from the Trondheim fjord (Norway). Nutrient enrichment (nitrogen, phosphorous, silicate, vitamins and trace elements) was performed according to Guillard (1975) for f/2-medium. Cultures were grown in a continuous, low light environment (35 µmol m^{-2} s^{-1}) at 15 ± 1°C. After 3 to 4 wk of growth, approximately 3.5 l was harvested from each culture by centrifugation at a relative centrifugal force of 5856 × *g* for 5 to 10 min at 5°C. All samples were frozen at –20°C until the NMR analysis was performed.

Samples for HR MAS NMR were prepared from the frozen material by adding 50 µl D_2O to a 5 mg wet weight algae sample. From this, 12 µl was withdrawn and pipetted into a rotor with a spherical insert. Proton (^{1}H) NMR spectra were recorded using a Bruker Avance DRX600 spectrometer equipped with a $^{1}H/^{13}C$ HR MAS probe. All spectra were recorded at 296 K, and samples were spun at 5 kHz. A 1D spin-echo sequence (Carr-Purcell-Meiboom-Gill) with water presaturation (CPMGpr, BRUKER) and total spin-spin relaxation delay ($2n\tau$) of 37.8 ms was used to acquire all ^{1}H MAS NMR spectra. Spin-echo sequences are applied to suppress broad resonance signals (Meiboom

& Gill 1958). A total of 64 transients over a spectral region of 7 kHz were collected. The raw data were multiplied with 1 Hz exponential line broadening before Fourier transformation. Internal standard for chemical shift referencing was 3-(trimethylsilyl)-propionate (TSP).

The chemical shift region between 4.6 and 0.8 ppm was initially chosen for multivariate analysis. After several analyses, the region between 4.0 and 3.0 ppm was found to give equally good classifications of the 4 species. In addition to the 5 replicate spectra of each microalga, we also included previously obtained spectra from *Thalassiosira pseudonana* and *Phaeodactylum tricornutum*, grown and harvested in the same way. PCA and FC results in this paper are thus based on a matrix consisting of 22 samples × 1500 variables. Variable reduction from an original 3000 points in this region to 1500 points was done by averaging. Baseline offset between spectra in the matrix was adjusted to a common value at a specific point, and mean normalisation to the area below the curve was performed. Both PCA and FC were done in an unsupervised manner, i.e. no *a priori* information was utilised in the classification. PCA was performed with mean-centering and full cross-validation. FC was performed by utilising inhouse programmed software based on the algorithms described elsewhere (Babuska 2001).

RESULTS AND DISCUSSION

The purpose of this study was to perform HR MAS ^{1}H NMR spectroscopy analyses on microalgae whole cell samples, and evaluate the use of NMR together with multivariate analysis as a possible classification method. We obtained well resolved proton HR MAS NMR spectra (Fig. 1) with a standardised acquisition protocol for the different samples. However, the algae exhibit differences in structure and chemical composition as well as cell size, and residual water also varies between samples. Signs of J-modulation due to spin echoes with an interpulse delay which is too long in some spectral regions could therefore be the result, but in this study we wanted to ensure that all spectra were acquired using the same protocol.

Two multivariate analyses, PCA and FC, were used to study possible clustering or patterns in the NMR spectra. Selected regions of 5 replicate ^{1}H-NMR spectra from each of the 4 microalgae were used as input in multivariate PCA and FC, demonstrating that replicate samples clustered in 4 distinct groups. In the PCA, the 3 first principal components account for 90% of total variation in the original 22 spectra and the score plot shows distinct grouping of different species, clearly separated from each other (Fig. 2). Two samples that were included from a previous analysis were correctly

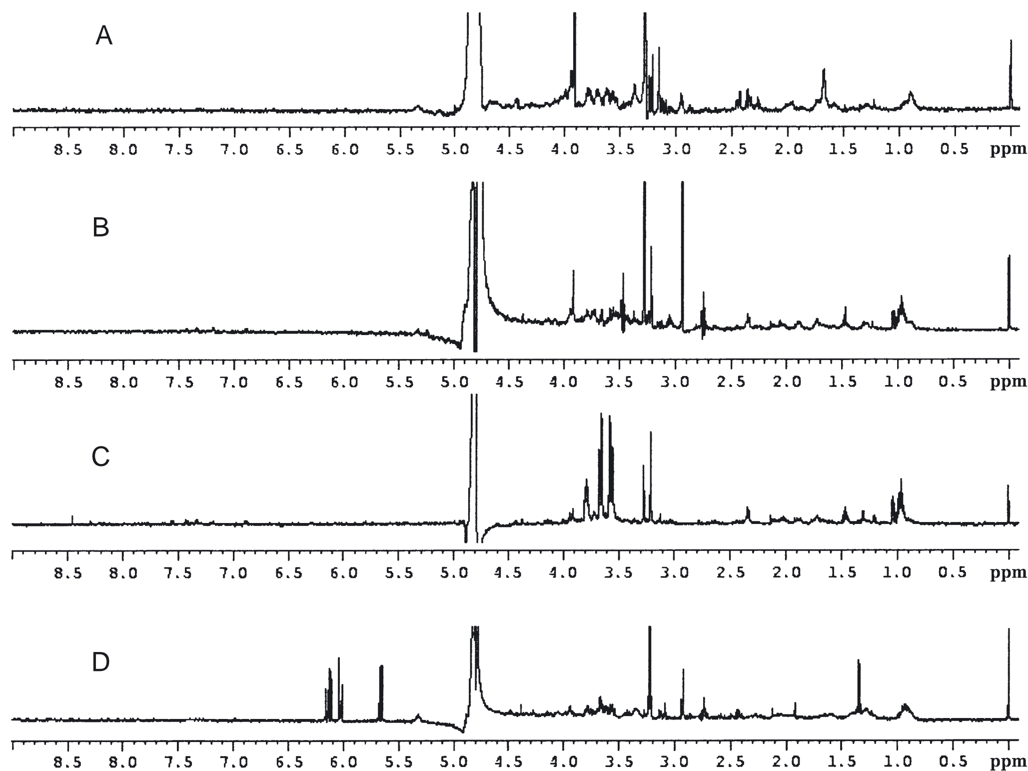

Fig. 1. The 600 MHz high-resolution magic angle spinning proton (HR MAS ^{1}H) nuclear magnetic resonance (NMR) spectra of whole-cell samples of (A) *Phaeodactylum tricornutum*, (B) *Thalassiosira pseudonana*, (C) *Dunaliella* sp. and (D) *Amphidinium carterae*

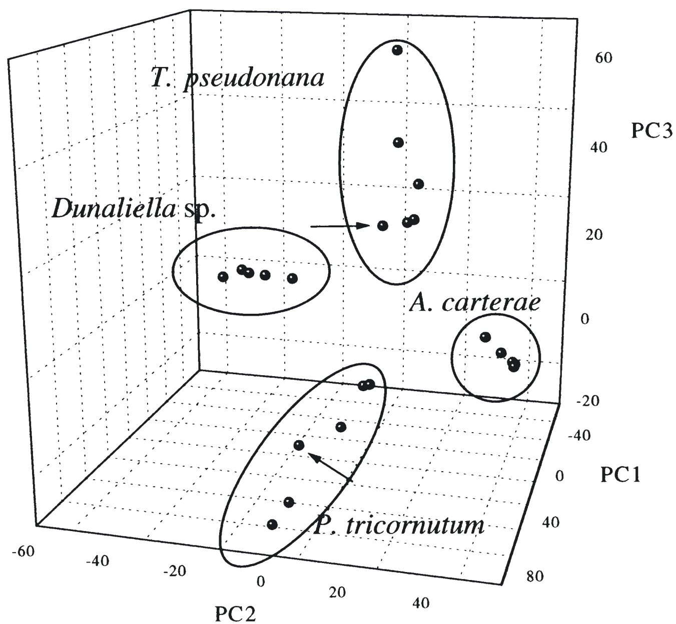

Fig. 2. Principal component analysis (PCs 1 to 3) of high-resolution magic angle spinning proton (HR MAS ^1H) nuclear magnetic resonance (NMR) spectra of 5 replicate samples of 4 microalgae. Arrows indicate samples from a separate experiment (same growth conditions and sample handling), both are grouped correctly

classified (arrows in Fig. 2). The 2 diatoms *Phaeodactylum tricornutum* and *Thalassiosira pseudonana* have similar scores along PC2 but are distinguished by the spreading in PC3, which may indicate a possibility for classification of microalgae down to species. All samples are correctly classified with $p > 0.9$ in the FC analysis (Table 1). Statistical analysis was performed as 'blindfold' classification of the NMR spectra, without any identification of resonance signals. Both PCA and FC confirm the point that intraspecific variation of the replicate proton NMR spectra is smaller than interspecific variation between groups, so it is possible to use these analyses and classify the microalgae based on their NMR signature.

We kept the work of culturing, harvesting and sample handling to a minimum, emphasising the fact that it is possible to obtain informative NMR spectra from whole cell samples without tedious procedures. NMR spectroscopy of biological samples is a relatively new technique, and the application of HR MAS has proven very efficient in removing line broadening due to incomplete averaging of anisotropic interactions. A microalgal cell is a heterogeneous system, wherein one finds many different chemical compounds in varying states of solidity (e.g. membranes, cell walls, organelles such as chloroplasts and mitochondria) or solution (e.g. soluble compounds in vacuoles, cell water, suspended free amino acids). Our NMR spectra reflect this in broadened and sometimes overlapping peaks (Fig. 1). Cell components may also occur at different concentrations, with carbohydrates and proteins being major cell constituents in microalgae (Geider & La Roche 2002). The selected region for our PCA and FC analysis was 3.0 to 4.0 ppm, and a tentative assignment of peaks in this region is based on reported shift values of metabolites in human blood plasma and breast cancer tissue (Nicholson & Foxall 1995, Sitter et al. 2002). Chemical shifts between 4–3 ppm are characteristic of carbohydrate protons (Schripsema et al. 1991), and microalgae use various carbohydrates as storage compounds (Jeffrey et al. 1997) so starch and chrysolaminarin for example are obviously important cell constituents. The amino acids glutamine and glutamic acid are involved in nitrogen assimilation (Turpin & Harrison 1978), and together with asparagine, arginine and tyrosine, make up more than 60%

Table 1. Fuzzy clustering (FC) of 4 different microalgae. Total input is the region from 4.0 to 3.0 ppm of 22 high-resolution magic angle spinning proton (HR MAS ^1H) nuclear magnetic resonance (NMR) spectra (lines 1 to 22), where lines 1 to 5 are replicates of *Phaeodactylum tricornutum*, lines 7 to 8 replicates of *Thalassiosira pseudonana*, lines 13 to 17 of *Dunaliella* sp., and lines 18 to 22 are replicates of *Amphidinium carterae*. Probability of class membership for each of microalga in 1 of 4 groups (each column) is shown, and values close to 1.000 indicate strong possibility of class membership (bold face)

Line no.	*Dunaliella* sp.	*P. tricornutum*	*T. pseudonana*	*A. carterae*
1	0.000	**1.000**	0.000	0.000
2	0.000	**1.000**	0.000	0.000
3	0.001	**0.953**	0.045	0.001
4	0.002	**0.902**	0.093	0.003
5	0.000	**0.999**	0.001	0.000
6[a]	0.000	**1.000**	0.000	0.000
7	0.000	0.000	**1.000**	0.000
8	0.000	0.000	**1.000**	0.000
9	0.001	0.015	**0.982**	0.002
10	0.000	0.000	**1.000**	0.000
11	0.000	0.000	**1.000**	0.000
12[a]	0.001	0.003	**0.994**	0.001
13	**1.000**	0.000	0.000	0.000
14	**1.000**	0.000	0.000	0.000
15	**1.000**	0.000	0.000	0.000
16	**1.000**	0.000	0.000	0.000
17	**1.000**	0.000	0.000	0.000
18	0.000	0.000	0.000	**1.000**
19	0.000	0.000	0.000	**1.000**
20	0.000	0.000	0.000	**1.000**
21	0.000	0.000	0.000	**1.000**
22	0.000	0.000	0.000	**1.000**

[a]Sample grown and analysed under equal conditions, but several months prior to other samples, included to show replicability of method

Table 2. Tentative ^{1}H chemical shift assignment in the region 4.0 to 3.0 ppm of high-resolution magic angle spinning (HR MAS) spectra of *Phaeodactylum tricornutum* (Bacillariophyceae), reference is 3-(trimethylsilyl)propionate (TSP). Based on breast cancer tissue (Sitter et al. 2002) and blood plasma (Nicholson & Foxall 1995)

δ, HR MAS ^{1}H whole cells	MAS
4.0	Asparagine[a]
3.94	Tyrosine[b]
3.92	Creatine[a]
3.91	
3.87	Glycerol[b]
3.78	Alanine[a]
3.77	Lysine[a]
3.76	Glutamine[a]/Alanine[b], β-glucose[b]
3.75	Glutamate[a]
3.72	α-glucose[b]
3.71	α-glucose[b]
3.7	Citrulline[b]
3.69	Leucine[b]
3.68	Leucine[a]
3.63	myo-Inositol[b]
3.61	myo-Inositol[a]
3.6	Threonine[b]
3.57	Valine[b]
3.56	Glycerol[b], myo-Inositol[b]
3.55	Glycine[a]
3.54	Glycine[b]
3.53	α-glucose[a]
3.38	
3.27	Myo-Inositol[a]
3.25	Taurine[a]/Taurine[b], Histidine[b]
3.24	α-glucose[a]/β-glucose[b], Arginine[b]
3.21	Phosphocoline[a]/Choline[b]
3.15	Citrulline[b]
3.14	Histidine[b]
3.12	Phenylalanine[b]
3.11	Tyrosine[a]
3.09	
3.06	Tyrosine[a]
3.05	Creatinine[a]

[a]Source is Sitter et al. (2002)
[b]Source is Nicholson & Foxall (1995)

of the total amino acid concentration in 5 species of microalgae (Derrien et al. 1998). Proton signals from all 4 could be identified in our alga spectrum (Table 2).

Lipids and pigments have proven more useful as chemotaxonomic markers for microalgae classification due to their group-specific distribution (Jeffrey et al. 1997, Volkman et al. 1998). Methyl and methylene protons in lipids which show high field resonances around 0.9 ppm, and resonance signals from the double-bond region of unsaturated lipids, appear at 5.2 to 5.3 ppm in proton NMR spectra (Nicholson & Foxall 1995). In the proton spectrum of the dinoflagellate in our study, *Amphidinium carterae*, there are distinct peaks between 6 and 6.2 ppm which might be due to the dinoflagellate marker-pigment peridinin (Fig. 1). Protons along the carbon chain in carotenoids appear as shifts around 6.06 to 6.71 ppm in another dinoflagellate pigment, gyroxanthin (Bjørnland et al. 2000). Also of great interest are the data on the cytotoxic polyketid Amphidinoketid, with ^{1}H NMR shifts up to 6.08 (Bauer et al. 1995). Our next step is to pursue the peak assignment and possibly identify such compounds in our microalgae proton NMR spectra.

In addition to natural cell components, our samples also contained some residual sea water which resonates heavily in proton NMR analysis. However, this problem was partially solved by water signal suppression by a presaturation pulse technique. Also, since we succeeded in measuring ^{1}H NMR spectra on whole-cell samples, we avoid the use of non-healthy chemicals such as carcinogenic solvents and radioactive labelling compounds. It is a non-invasive analysis, and cells might very well be viable after analysis unless samples have been frozen at some stage. Trypan blue tests of preadipocyte cells showed that only a small fraction of the cells stained after spinning, and visual inspection of these cells showed that they mainly remained intact, with only minor membrane defects and improved spectral features related to MAS conditions rather than cell lysis (Weybright et al. 1998). Viability of yeast cells after MAS spinning has been shown (Krainer et al. 1994).

Further work including new microalgae species and species grown under different conditions will amplify the 'data base' to sort and classify a great variety of microalgal species. In addition, complete assignments of all the peaks observed in the NMR spectra of different species will give valuable information concerning the metabolic state of cells and dynamics in cell physiology, and selected peaks and related cellular compounds can be used to trace microalgae for example in field samples.

The field of 'NMR as an identification tool' is developing through studies such as the one by Ohara et al. (2001) on bacteria, and Henrion et al. (1997) showed that PCA could be used to classify algae species based on their fluorescence emission spectra. HR MAS is clearly a useful tool for classification of microalgae, especially with the development in recent years of higher magnetic field instruments and increased signal resolution, and pulse techniques, which makes it possible to enhance some signals and suppress others. Our results show that although sampling and NMR analysis are done without any meticulous procedures, and at different times but always using the same aqusition protocol, the results are reproducible as long as growth conditions and sample handling are the same.

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