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Identification and Characterization of Some Species of Cyanobacteria, Chlorophyta and BacillariophytaUsing Fourier-Transform Infrared (FTIR) Spectroscopy

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Abstract: *In this study FTIR was used to determine and identify 12 species of freshwater microalgae. Cyanobacteria, Chlorophyta and Bacillarophyta species were cultured in Allen media in batch culture. These algae were collected from different freshwaters in Ankara, Turkey. FTIR spectroscopy is easy to use and a robust technique allowed for macromolecular analysis and discriminates different genius of microalgae. FTIR enables discrimination at the strain level. When compared with identified molecular fingerprints regions (1500-1200 cm^{-1}) in previous studies, our best separation range of spectral bands (1660-1190 cm^{-1}) were similar. The results show that the FTIR technique has the potential to become applicable in the discrimination of Cyanobacteria, Chlorophyta and Bacillarophyta species.*

Keywords – *Characterization, FTIR, Microalgae, Spectroscopy*

I. Introduction

Fourier Transform Infrared (FTIR) Spectroscopy has served as the widely used method through its wave numbers causing vibrations on the functional constituents in molecules. Concerning algae, infrared microscopy (IMS) enables spatial resolution and allows for the analysis of macromolecules. Basic cell structure is vital to understanding cellular physiological responses to their environment. There has been a progression on algal IMS research from a strictly biological focus concerning the identification and localization of cellular contents to an ecological focus of species- specific or multispecies response to environmental changes [1]. Algal IMS research has focused on several macromolecular pools, including proteins (amide I and II), lipids (methyl and methylene groups, esters), carbohydrates (starch, cellulose), nucleic acids and phosphorous groups, and silica (in diatoms and Cyanobacteria). FTIR spectroscopy has been extensively applied to Cyanobacteria species such as *Synechococcus* sp., *Microcystis aeruginosa* (two strains), *Anabaena variabilis*, *Oscillatoria* sp. [2], *Phormidium luridum* [3], *Calothrix* sp., [4][5][6], *Microcystis aeruginosa*, *Nostoc* sp., *Chroococcus minutus* [7], *Aphanizomenon flos-aquae*, *Anabaena flos-aquae* [8][9] and *Microcystis aeruginosa* [10].

FTIR spectroscopy is also being applied on species differentiation and classification. Algal species within major groups can be differentiated by their infrared absorption spectra [1]. Kansiz et al. differentiated five species of Cyanobacteria and a green alga [2]. Vardy and Uwins (2002), had compared the spectra of two similar diatom species [11]. Sigee, Dean et al. (2002) classified microalgae from natural lake assemblages [12]. Dean and Sigee (2006) also, compared two species of Cyanobacteria from different depths within a lake [8]. Dean, Martin et al. (2007) had differentiated a cyanobacterium and a dinoflagellate from natural lake populations [10]. Most FTIR studies on whole organisms (including phytoplankton) have been carried out on laboratory-cultured materials, with relatively few analyses of environmental organisms [13][9][14][15][16][17] [18][7]. FTIR can be applied successfully on the determination of molecular characteristics of species spectrum, but on comparison with species, some researchers have encountered some problematic situations because of similar spectra and lost data, especially in finger-print regions [19]. If this is compared with multiple data spectra, it would add value to solutions.

The goal of this study was to use FTIR analysis to identify and classify macromolecules in different species belonging to Chlorophyta, Cyanobacteria and Bacillariophyta obtained from different freshwater sources and cultured in the same nutrients media.

II. Methodology

1. Collection and processing of samples

Samples were collected from natural freshwaters in Ankara (Turkey), and *Arthrospira platensis* Gomont (Syn: *Spirulina platensis* (Gomont) Geitler), *Dolichospermum affine* (Lemmermann) Wacklin, L.Hoffmann & Komárek (Syn: *Anabaena affinis* Lemmermann), *Geitlerinema lemmermannii* (Woloszynska) Anagnostidis (Syn: *Oscillatoria lemmermannii* Woloszynska), *Microcoleus autumnalis* (Gomont) Strunecky, Komárek & J.R.Johansen (Syn: *Phormidium autumnale* Gomont), *Oscillatoria princeps* Vaucher ex Gomont, *Oscillatoria* sp., *Pseudanabaena mucicola* (Naumann & Huber-Pestalozzi) Schwabe (Syn: *Phormidium mucicola* Nauman & Huber-Pestalozzi), *Spirogyra* sp., *Stigeoclonium nanum* (Dillwyn) Kützing, *Stichococcus subtilis* (Kützing) Klercker, *Achnanthes* sp., *Nitzschia palea* (Kützing) W.Smith and strains isolated from them. Micromanipulation technique was used to isolate the microalgae [20] stored and maintained in 250 mL Erlenmeyer flasks containing 100 mL of Allen medium. The pH of the medium was adjusted to 6.8 with 1N NaOH [21]. All cultures were illuminated at 20 - 25°C in a 18 h-light/6 h-dark cycle with a photon flux density of 50 µmol photons m^{-2} s^{-1} under fluorescent tubes. The mean biovolume for individual algal cells was calculated from the geometrical formula according to Standard Operation for Phytoplankton Analysis (2007). For the measurement, fresh weight and determination of Chlorophyll *a*, samples were filtered through Whatman GF/C glass fiber filters. The pigments were extracted in 90% methanol. Extract absorbance was measured at 665 and 750 nm [22]. Quantitative features of the species in culture medium are given on Table 1. For spectral analysis, (and all measurements) samples were prepared during their exponential phases and from this, 0.5 mL aliquots were resuspended in deionized water and droplets of the culture suspension spread out all over 'Low-e' reflectance slides (Kevley Technologies) for monolayer preparations. Samples were then subsequently air-dried in a laminar flow at room temperature and stored in a desiccators pending analysis.

2. Infrared Analysis

Analysis of the batch cultures was performed by means of FTIR spectroscopy. Infrared analysis was carried out at the nanotechnology laboratory of Bilkent University, Ankara (Turkey), using a Vertex 70 Hyperion Scanning microscope fitted with a Bruker Tensor Model 37 FTIR spectrophotometer. Spectral resolution of mercury cadmium telluride (MCT) detector was 4 cm^{-1}, with 128 co-added scans. Spectra were collected using a 20x20 µm square aperture over the wave-number range 4000- 650 cm^{-1}. Infrared absorption spectra were then collected from a clear field (background) from algal colonies (Fig. 1), and a ratio was obtained of the sample-to-background spectrum. The specimens were examined and analyzed in the dry state, without a mounting medium or cover slip samples of the species studied, were initially examined by bright-field microscopy, and 33 individual colonies (one spectrum per colony). Spectral absorption bands were identified in relation to published information. Supporting information on band assignments was also obtained by analysis of range of pure biochemical standards (protein, nucleic acid, fatty acid and soluble carbohydrate) as detailed in [12].

3. Spectral Analysis

The manipulation of spectra was carried out using Bruker OPUS 6.5 software. The spectra were base-line corrected, using automatic baseline correction algorithm and normalized to amide I.

III. Results

Under light microscope, from living cells, air-dried preparations of specimens showed typical image of *Arthrospira platensis* , *Dolichospermum affine, Geitlerinema lemmermannii* , *Microcoleus autumnalis Oscillatoria princeps Oscillatoria* sp., *Pseudanabaena mucicola, Spirogyra* sp., *Stichococcus subtilis, Stigeoclonium nanum, Achnanthes* sp., and *Nitzschia palea* (Figure 1A-H). Spectral analysis was carried out over 25x25 µm areas of specimens in the same figures, generating infrared absorption spectra that were closely similar to each containing 11 clear bands over the wave-number range 4000-900 cm^{-1}. Table 2 shows the assignments of the bands based on literature sources.

Table 1. Quantitative features of the species in culture medium

Cultures	Number of species ($org.ml^{-1}$)	Total Biovolume (cm^3)	Fresh weight (gr.)	Chlorophyll a ($\mu g\ L^{-1}$)
Arthrospira platensis		**0.12**	**0.1**	**2.67**
Dolichospermum affine		**0.08**	**0.08**	**3.08**
Geitlerinema lemmermannii		**0.2**	**0.1**	**8.62**
Microcoleus autumnalis		**0.1**	**0.04**	**3.18**
Oscillatoria princeps		**0.15**	**0.54**	**5.20**
Oscillatoria sp.		**0.2**	**0.42**	**6.67**
Pseudanabaena mucicola	**$4.34x10^6$**		**0.07**	**4.21**

Spirogyra sp.		**0.38**	**1.34**	**7.56**
Stichococcus subtilis		**0.2**	**0.12**	**1.77**
Stigeoclonium nanum		**0.23**	**1.12**	**4.35**
Achnanthes sp.	**$0.35x10^5$**			**0.38**
Nitzschia palea	**$0.28 x10^5$**			**0.42**

Total biovolume of flamentous species and number of species of the others are given.

Table 2. Tentative assignment of bands found in FTIR spectra of air-dried preparation of divisions

Band number	Band wavenumber(cm^{-1})			Wavenumber range (cm^{-1})	[1]Tentative assignment of bands to major macromolecules
	Cyanobacteria	Chlorophyta	Bacillariophyta		
1	3568	3405	3287	3029-3639	**WATER***ν*(O-H) stretching **PROTEIN***ν*(N-H) stretching (amide A)
2	2925	2919	2920	2809-3012	**LIPID - CARBOHYDRATE** Mainly $\nu_{as}(CH_2)$ and $\nu_s(CH_2)$ stretching
3	1732	1731	1730	1763-1712	**CELLULOSE – FATTY ACIDS** *ν*(C=O) stretching of esters
4	1664	1645	1626	1583-1709	**PROTEIN** amide I band Mainly *ν*(C=O) stretching
5	1543	1524	1547	1481-1585	**PROTEIN** amide II band Mainly *δ*(N-H) bending and *ν*(C-N) stretching
6	1453	1442	1443	1425-1477	**PROTEIN** $\delta_{as}(CH_2)$ and $\delta_{as}(CH_3)$ bending of methyl **LIPID**$\delta_{as}(CH_2)$ bending of methyl
7	1380	1394	1442	1357-1423	**PROTEIN** $\delta_s(CH_2)$ and $\delta_s(CH_3)$ bending of methyl **CARBOXYLIC ACID**ν_s(C-O) of COO^- groups of carboxylates
8	1258	1226 1260	1201	1191-1356	**NUCLEIC ACID** (and other phosphate-containing compounds) $\nu_{as}(>P=O)$ stretching of phosphodiesters
9	1160	1152	1159	1134-1174	**CARBOHYDRATE***ν*(C-O-C) of polysaccharides
10	1090	-----	-----	1072-1099	**CARBOHYDRATE***ν*(C-O-C) of polysaccharides **NUCLEIC ACID** (and other phosphate-containing compounds) $\nu_s(>P_{=}O)$ stretching of phosphodiesters
11	1034	1043	1053	980-1072	**CARBOHYDRATE***ν*(C-O-C) of polysaccharides

[1]Band assignment based on [10][8][4][5][12][23][24][25][26][27][28]

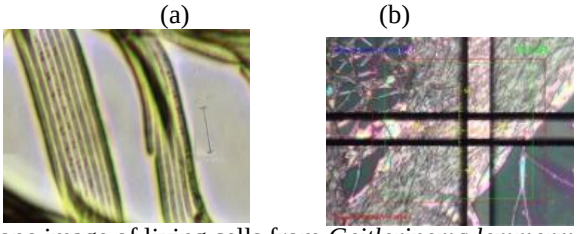

Figure 1A (a). Light microscope image of living cells from *Geitlerinema lemmermannii* culture. (b). The region of analysis of air-dried sample position taken with light microscope attached to FTIR microscope.

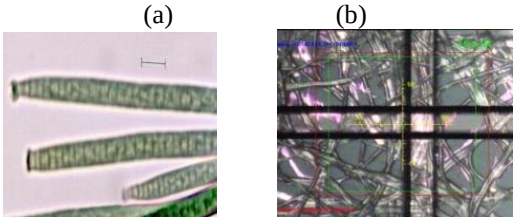

Figure 1B (a). Light microscope image of living cells from *Microcoleus autumnalis* culture. (b). The region of analysis of air-dried sample position taken with light microscope attached to FTIR microscope.

(a)

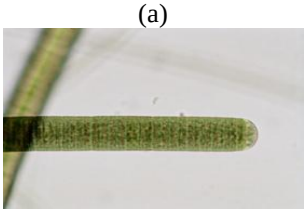

Figure 1C (a). Light microscope image of living cells from *Oscillatoria princeps* culture.

(a)

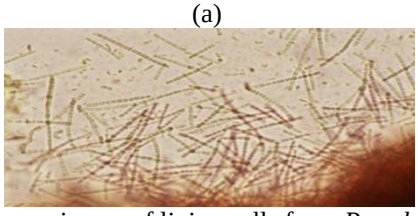

Figure 1D (a). Light microscope image of living cells from *Pseudanabaena mucicola* culture.

(a) (b)

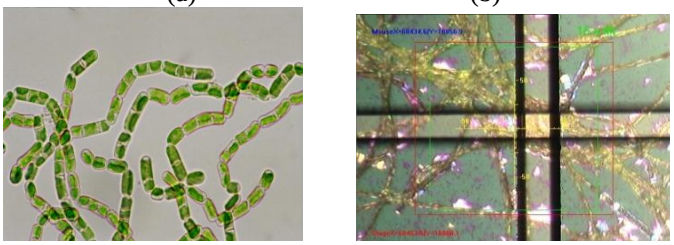

Figure 1E (a). Light microscope image of living cells from *Stichococcus subtilis* culture. (b). The region of analysis of air-dried sample position taken with light microscope attached to FTIR microscope.

(a) (b)

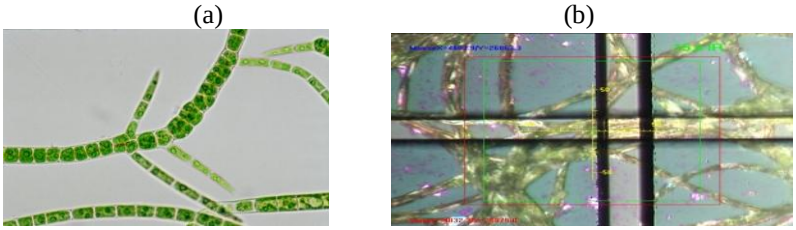

Figure 1F (a). Light microscope image of living cells from *Stigeoclonium nanum* culture. (b). The region of analysis of air-dried sample position taken with light microscope attached to FTIR microscope.

(a) (b)

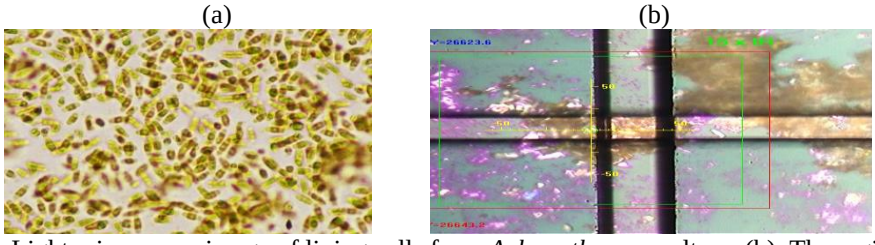

Figure 1G (a). Light microscope image of living cells from *Achnanthes* sp. culture. (b). The region of analysis of air-dried sample position taken with light microscope attached to FTIR microscope.

(a) (b)

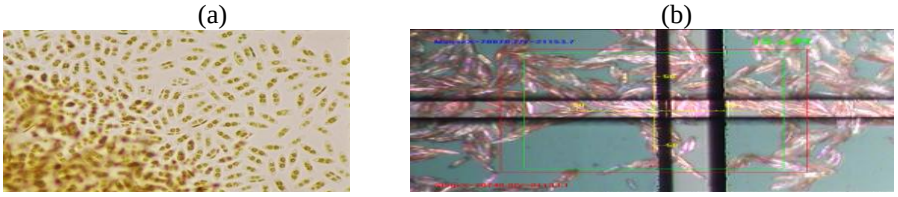

Figure 1H (a). Light microscope image of living cells from *Nitzschia palea* culture. (b). The region of analysis of air-dried sample position taken with light microscope attached to FTIR microscope.

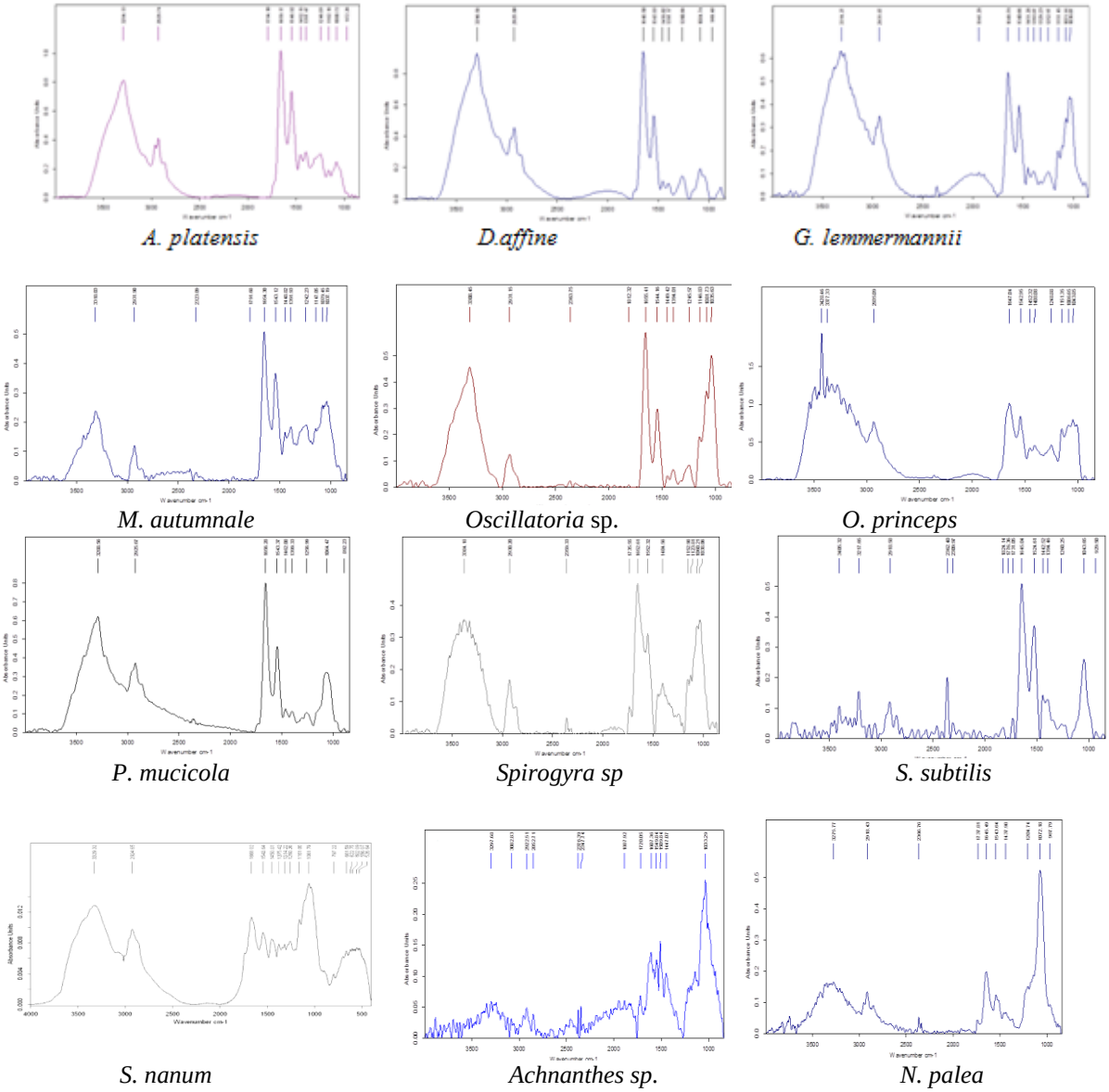

Figure 2. Infrared absorption spectra from air dried colony of studied species

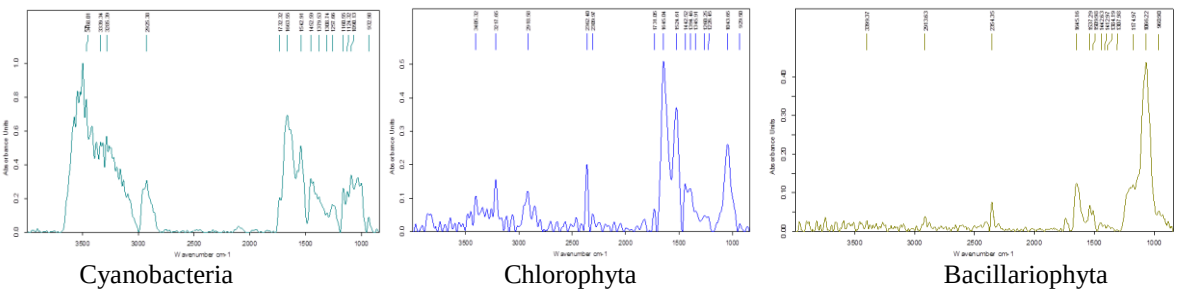

Figure 3. Average Spectra of the 3 Divisions

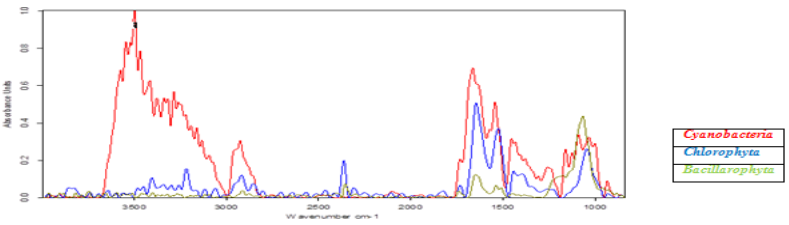

Figure 4. Differences between 3 division

IV. Discussion

Many of the infrared spectral features have been previously used in relation to purified biochemicals, including the four major groups of biological macromolecules- nucleic acids [29], proteins [25], lipids [30] and polysaccharides [31]. In recent years, it is also, being applied to more complex biological samples and whole organisms such as bacteria [26][4][17][32], fungi [33][34], higher plants [35], tissue culture cells [36], macroalgae [37][38][39] and microalgae [12][40][8][14][41][42][13]. Algal IMS research has focused on several macromolecular pools, including proteins (amide I and II), lipids (methyl and methylene groups, esters), carbohydrates (starch, cellulose), nucleic acids and phosphoryl groups, and silicate (in diatoms and cyanobacteria). Green algae, diatoms, and cyanobacteria have distinct chemical composition that can be observed in the infrared absorption spectra [1]. FTIR spectra of phytoplankton cells in the range of 4000-700 cm^{-1} are characterized by specific absorption bands of the major macromolecular compounds [42]. Typically, green algae have a relatively high starch and cellulose content (cell walls and energy storage product) form ~1100 to 900 cm^{-1} [9][1], diatoms have a distinctive silicate absorption at ~ 1100-1600 cm^{-1} and ~800 cm^{-1} due to silica frustule (cell wall) [24][11], and cyanobacteria spectra are dominated by protein and lipid with less abundant carbohydrates relative to green algae [5][4]. However, the proportions of these macromolecular pools can vary substantially among species within these groups and with nutrient availability [24][3][7].

All FTIR spectra showed a closely similar sequence of 11 distinct bands and were assigned a range of vibrationally, active chemical groups, including residual water (–OH), lipid ($–CH_2$), cellulose (–C=O), protein (amide), nucleic acid (>P=O) and starch (–C–O). Average spectra were obtained for all species in this study. The differences among the divisions of band positions in the fingerprints were observed. Particularly obvious, were the differences among the band intensities. FTIR microspectroscopy of phytoplankton results in complex absorption spectra, analysis of which provide information on, qualitative (molecular ratios, population variability, species differentiation) information in freshwater systems [8]. FTIR spectra with clear bands were obtained from *A. platensis, D. affine, G. lemmermannii, M. autumnalis, O. princeps, Oscillatoria* sp., *P. mucicola, S.* sp., *S. subtilis, S. nanum, Achnanthes* sp., and *N. palea* species (Fig.2). The molecular assignments of bands given on Table 2 are based on published data on phytoplankton, cyanobacterial, noncyanobacterial and human cells. The positions of individual bands are derived from several molecular sources.

This study was consistent among bands 9, 10 and 11, with their joint derivations from carbohydrate in all the three divisions. In Cyanobacteria, Chlorophyta and Bacillariophyta, while the average positions of band 9 were at 1160 cm^{-1}, 1152 $cm^{-1}$and 1152 cm^{-1} respectively, band 11 was at 1034 cm^{-1}, 1043cm^{-1} and 1053 cm^{-1} respectively, with the position of band 10 being detected at 1090 cm^{-1}only in Cyanobacteria (Table 2). The grouping of bands 9-11 is due to their joint derivation from υ(C–O–C) stretching of polysaccharides. The band position at 1080 cm^{-1} was attributed to the υ(P = O) stretching of nucleic acid [27][4][26]. In these algae, bands 6 and 7 suggested that they were dominated by the δas(CH_3) and δs(CH_3), bending modes of methyl groups of protein. Other researchers have stated that bands 6 and 7 may have contribution from lipid [4] and carboxylic acid [26][24], respectively. Band 8 (υas (>P=O)) at a position ~ 1248 cm^{-1} is likely to have had a strong contribution from nucleic acid, but it was likely that this band also had a contribution from the internal storage of polyphosphates, which are important in Cyanobacteria such as Microcystis [43][10]. On species spectrum level, the differences in band positions and intensities can be seen more clearly (Fig. 2). Spectra taken from *Nitzschia* and *Navicula* species are similar to the macromolecular location of diatom species in other studies (e.g. Stefano *et al.* 2008)[44].

All band intensities also showed differences between the species but with bands 5-11,showing more differences particularly, where the band intensities (2 and 5-8) in Cyanobacteria were higher than in Chlorophyta and Bacillariophyta, including when the band intensities (1-3) in Bacillariophyta, were less than others. All FTIR spectra showed a closely similar sequence of 11 distinct bands and were assigned a range of vibrationally, active chemical groups, including residual water (–OH), lipid ($–CH_2$), cellulose (–C=O), protein (amide), nucleic acid (>P=O) and starch (–C–O). Average spectra were obtained for all the species in this study. The differences among the divisions of band positions in the fingerprints were observed. And particularly obvious, were the differences among the band intensities. The use of FTIR in first derivative spectra (1750-900 cm^{-1}) is still in its (multivariate techniques) preliminary stage. It is now beginning to be recognised as a technique with great potential in phycological research. This study showed a distinct success in using multivariate techniques in separating microalgae according to their taxonomy.

V. Conclusion

When the spectra of species were examined, it was noticed that FTIR plays an important role in the identification, taxonomy and the physiology of phytoplankton species. It also helps in determining the location of some macromolecules in living cells. In general, even though some species can be distinguished through visual spectrum, plastic organisms require a larger number of spectra for a through identification due to more compositional heterogeneity. In comparison with conventional chemical analyses, FTIR spectroscopy has

important advantages due to the high reliability, sensitivity, and the speed of measurement. Nevertheless, the differences between patterns representing different species are not easy to perceive by visual inspection, even when using the first derivative spectra. So, statistical treatment is essential.

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