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Bright field, dark field, phase-contrast and confocal laser scanning microscopic studies of morphologically diverse selected fresh water microalgae: a comparative report

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Abstract:

The microalgae and cyanobacteria are morphologically diverse in the eco-system. Their features play a role for conventional identification and eventually establishment of taxonomy. The aim of the study is to investigate morphological features of selected freshwater microalgae through bright field, dark field, phase-contrast and confocal laser scanning microscopy. A total number of ten morphologically diverse fresh water microalgae namely, *Chroococcus* sp., *Myxosarcina* sp., *Spirulina sp., Nostoc* sp*., Westiellopsis sp. Chlorella* sp., *Chlorococcum* sp., *Scenedesmus* sp. *Pediastrum* sp., *Cymbella* sp*.,* were obtained from National Repository for Microalgae and Cyanobacteria – Freshwater (*NRMC-F*), Department of Microbiology, Bharathidasan University, India for this study. This study paves a way to understand the genus, species and strain level clearly, authenticate the proper identification and also prepare a key for identification.

Keywords: Microalgae, Cyanobacteria, Freshwater, Morpho-taxonomy, Microscopy

Introduction:

Microalgae are photoautotrophic oxygen evolving microorganisms which includes (Chlorophyceae, Cyanophyceaeand Bacillariophyceae). They are morphologicallydiverse and ofextensive distribution. Microalgae reflect a wide range of physiological properties and are more tolerant to environmental stress (Tandeau-de-Marsac and Houmard, 1993). In the aquatic ecosystems microalgae are the primary producers at the base of the food web. Some of the algal forms can be useful indicator in major water management practice, pollution studies and water quality analysis (MubarakAli *et al.,* 2012; Pandey *et al.,* 1998).

Microalgae are unicellular or large colonies or mats quite conspicuous. They are coccoid, colonies of various shapes wherein cells are arranged in rows resulting in a flat plate, or are arranged radially in spherical colonies. Filamentous forms produce a row of cells referred to as trichome. Trichomes may be single, straight or spirally coiled and also as aggregated bundles. Some filamentous species are characterized by cell differentiation and form heterocysts. These cells are considered as the sites of nitrogen fixation. Many heterocystous cyanobacteria also form a second cell type, an akinete, which can germinate when conditions are suitable for growth. The filaments are either unbranched or may be branched with uni-seriate or multi-seriate arrangement of cells. Besides presence of true branching, false branches may occur in some forms (Thajuddin and Subramanian, 2005). Benthic algae are believed to be the good indicators of water quality (Cascaller *et al.,* 2002) while epiphytic and epizoic algae are the indicators of turbidity and mixing of water in the habitats (Cattaeneo, 1978). Diatom is single-celled eukaryote belonging to the division of Bacillariophyta. The cellwalls are composed of silica(Patrick &Reimer, 1966). Diversity, Scanning Electron Microscopic studies and Molecular systematics of cyanobacteria and diatoms have been reported (MubarakAli, *et al*., 2010; 2012a; 2012b).

The nature of production, distribution and relationship of the phytoplankton and zooplankton vary with the prevailing environmental conditions.The classification of microalgae is based on morphological characters such as trichome, width, cell size, division planes, shape and arrangement, pigmentation and the presence of characters such as gas vacuoles and a sheath (Baker, 1991). In order to overcome some problems and to permit identification of cyanobacteria at the species level, different microscopic studies would be recommended (Baldev*et al.,* 2015). In this investigation, taxonomy of morphologically diverse freshwater microalgae was studied based on the various microscopic techniques.

Materials and methods:

Culture and growth conditions:

A total of ten morphologically diverse freshwater green microalgae and cyanobacteria were obtained from National Repository for Microalgae and Cyanobacteria – Freshwater (DBT, Govt. of India), Department of Microbiology, Bharathidasan University, Tiruchirappalli – 620 024, Tamil Nadu, India for this study. All the taxa were maintained in the medium based on the nutrient requirements. All the cyanophyceae with non-heterocystous forms grows in $BG11^{+}$ medium (presence of $NaNO_3$) and heterocystous forms grows in $BG11^{-}$ medium (Rippka *et al.,* 1979). The chlorophyceae grows in Chu10 medium (Chu 1942) and bacillariophyceae in F/2 medium (Guillard and Ryther, 1962).

Microscopic investigation and identification of selected freshwater microalgae:

Microphotographs of obtained cyanophyceae, chlorophyceae and bacillariophyceae were taken using microscopes with different fields such as Light microscopy with camera lucida (*MCX500,Micros, Austria*); Bright field microscopy (BF) (*MCX500,Micros, Austria*); Dark field microscopy (DF), (*MCX500,Micros, Austria*); Phase-contrast microscopy (PC), (*TS100F*, *Nikon, Japan,* and *MCX500,Micros, Austria*); Confocal Laser Scanning Microscopy (CLSM) (CLSM 710, Carl Zeiss, Germany). The strains were taxonomically determined with the help of standard monogrphs (Desikachary, 1959; Desikachary, 1987; Philipose, 1969). The sizes were measured and salient features of the microalgae were noted like shape, size, cell arrangement, color, presence and absence of heterocyst, sheath, septum, akinetes, spikes, views (girdle or valve view in case of diatom) and microphotography is also documented.

Results and Discussion:

The morphological taxonomy of the selected microalgae was clearly studied in all the mentioned microscopesin which each microscopy has its own features to enlighten the microstructures of microalgae. Based on the morphological features, all the taxa were identified as *Chroococcus minor*, *Myxosarcina spectabilis*, *Spirulina gigantea, Nostoc calcicola, Westiellopsis prolifica*, *Chlorella vulgaris*, *Chloroococcum humicola*, *Scenedesmus quadrispina*, *Pediastrum tetras*, and *Cymbella tumida* were maintained in the suitable media are mentioned in the Table 1. The proper documentation, and taxonomic identification of microalgal flora are difficult to describe due to phenotypic plasticity (Shubert and Wozniak, 2003); Cell wall structures, sheath, spikes, special structures like heterocysts and akinetes, cell arrangements are the essential to identify the algal strains. The basic microscopy is not sufficient to study details of the cellular structures. There is a advanced microscopes like Confocal Laser Scanning Microscopy and Scanning Electron Microscopy (SEM) are to be required to study the detailed structures and other microscopy like phase contrast and dark field microscopy help to study the flagella, spines and sheath of the microalgae. Recently, Roy and Pal (2015) reported that the morpho-taxonomy and diversity if plankotonic chlorophytes in Indian Ramsar sites using various microscopy identification such as light microscopy and SEM. They pointed out that *Stauridium tetras* showed detailed cell wall morphology and lobes formed from cuneate incision end up with distinct nodules by SEM studies. A new method of using Confocal Laser Scanning Microscopy (CLSM) and Scanning Electron Microscopy (SEM) for exact three-dimensional reconstructions of diatom frustules has been reported (Friedrichs *et al*., 2012). It is agreed that the advanced microscopic studies reveals the detailed structures of the microalgae. Camera lucida diagram were drawn with light microscopy for all the selected taxa, they showed actual structures of the microalgae with distinct structures (Fig 1). It is very difficult to draw all the fine structures and laborious when compare with other microscopy, in this study it fail to record sheaths and internal cellular structures. Unlike, the larger cells could be a ideal to document in the camera lucida diagrams. The cyanobacterial diversity in saltpans was documented using camera lucida drawing, where distinguished unicellular and filamentous cyanobacteria (Thajuddin *et al.,* 2002).

Table 1Characteristics and microscopy recommendation for the selected fresh water microalgae

S.No	Strain Name	Characteristics	Medium
1.	*Chroococcus minor* (Kuetz) Naeg.	Colour-Dark blue green Cells spherical, seldom 2 to 4, sheath colourless. Size – width-3.83µm and length- 5.67 µm Desikachary-1958-Pl.24,Fig.1.	BG 11^{+}
2.	*Myxosarcina spectabilis* Geitler	Colour-Dark green Cells spherical, seldom 4 to 8, sheath colourless. Size – width-4.76µm and length- 7 µm Desikachary-1958-Pl.30,Fig.1-5.	BG 11^{+}
3.	*Spirulina gigantea* Schmidle	Colour-Thick green Regularly Spirally coiled, at the end attenuated, size- width 15 µm and spirals length- 8 µm Desikachary-1958-Pl.36,Fig.12, 14-17.	BG 11^{+}
4.	*Nostoc calcicola* Brebisson ex Born. et flah	Colour – Pale green Thallus mucilaginous, slightly diffluent, Pale green, filament, loosely entangled, sheath mostly indistinct, heterocyst light brown colour. Size –Sub spherical Heterocyst width – 6.4 µm ; barrel shaped vegetative cell size – 8.8 µm Desikachary-1958-Pl.68,Fig.1.	BG 11^{-}
5.	*Westiellopsis prolifica* Janet	Colour – Light green Main filaments are swollen and pinched along its length, short barrel shaped cells. Not constricted at the cross walls, elongated cylindrical cells. Size – Heterocyst size – 2.85µm ;Segment – 2.75 µm.Desikachary-1958-Pl.131, Figs.1-12.	BG 11^{-}
6.	*Chlorella vulgaris* Beijerinck	Colour : light green Cells usually solitary and small colonies. Cells are spherical and thin cell membrane. Chloroplast is parietal , cup shaped and with a pyrenoid. cell size - 6.8 µm. Chlorococcales – Philipose –1967-Fig. 82 d.	Chu 10
7.	*Chloroococcum humicola* Naegeli	Colour – Dark green. Size – width – 14.8 µm; Length – 10.84 µm Cells are solitary and ellipsoidal to spherical with smooth cell walls and variable size.	Chu 10
8.	*Scenedesmus quadrispina (Chodat) G.M. Smith*	Colour- Green Size – width - 6 µm ; Spine length: 4.8 µm Colonies usually 2 – 4 celled, cells broadly ovoid and about twice as long as broad. Poles of terminal cells with a single short curved spine. Chlorococcales – Philipose –1967-Fig. 187- d.	Chu 10
9.	*Pediastrum tetras (Her.) Ralfs*	Colonies rectangular, oval and round shaped Cells without intercellular spaces. Size is different- width 8 to 14 µm Length 14 to 16 µm Chlorococcales – Philipose –1967-Fig. 45 d.	Chu 10
10.	*Cymbella tumida* (Brebisson) Van Heurck	A larger, chunkier diatom; Size – width – 10.8 µm; Length – 14.2 µm; slightly bulbous ends; striae radiating from a round central area with a single stigma. Cymbellales – Biggs and Kilroy (2000); Fig. 1, Page No. 165.	F/2

Bright field images of selected microalgae showed actual color of the microalgae, heterocyst in case of *Nostoc* sp. and septum in *Chroococcus* sp., are shown clearly (Fig 2). In dark field microscopy, septum in *Chroococcus* sp. differentiation of heterocyst and vegetative cells are not seen. Branches in *Westiellopsis* sp. and cell attachment in *Cymbella* sp. were clearly shown (Fig 3). Previously, *Dicothrix spiralis*, a new report of marine cyanobacteria from south East coast of India was reported based on the morphological structures(Thajuddin and Subramanian, 1991). They studied false branching and similar structures using various monographs and found new report apart from the

monograph of Desikachary(1959). Hence, studying of branching or false branching structures is important to report them absolutely. In phase contrast microscopic study showed the detailed structures of outer sheath and septum between the vegetative cells (Fig 4). In *Chroococcus* sp., *Myxosarcina* sp., *Nostoc* sp., and *Pediastrum* sp., outer sheath very clearly visible than other strains which could help to measure the actual cell size and to differentiate single or multi layered sheaths.

The CLSM study shows the fine structures of the microalgae. CLSM emit the light based on the fluorescent pigments in the cells. In this study, *Chroococcus* sp., *Myxosarcina* sp., *Scenedesmus* sp., *Pediastrum* sp., showed their structural integrity and pigment contents and septum; cell arrangements very clearly (Fig 5). However, *Spirulina* sp., *Chloroococcum* sp., and *Cymbella* sp., did not show their structural integrity and reflect their actual structures. This preliminary and essential studyis required to understand the microscopic features of microalgae for the better identification of microalgae taxonomically. With this comparative statement, this study is recommending the microscopy for the special structural features of the microalgae (Table 2).

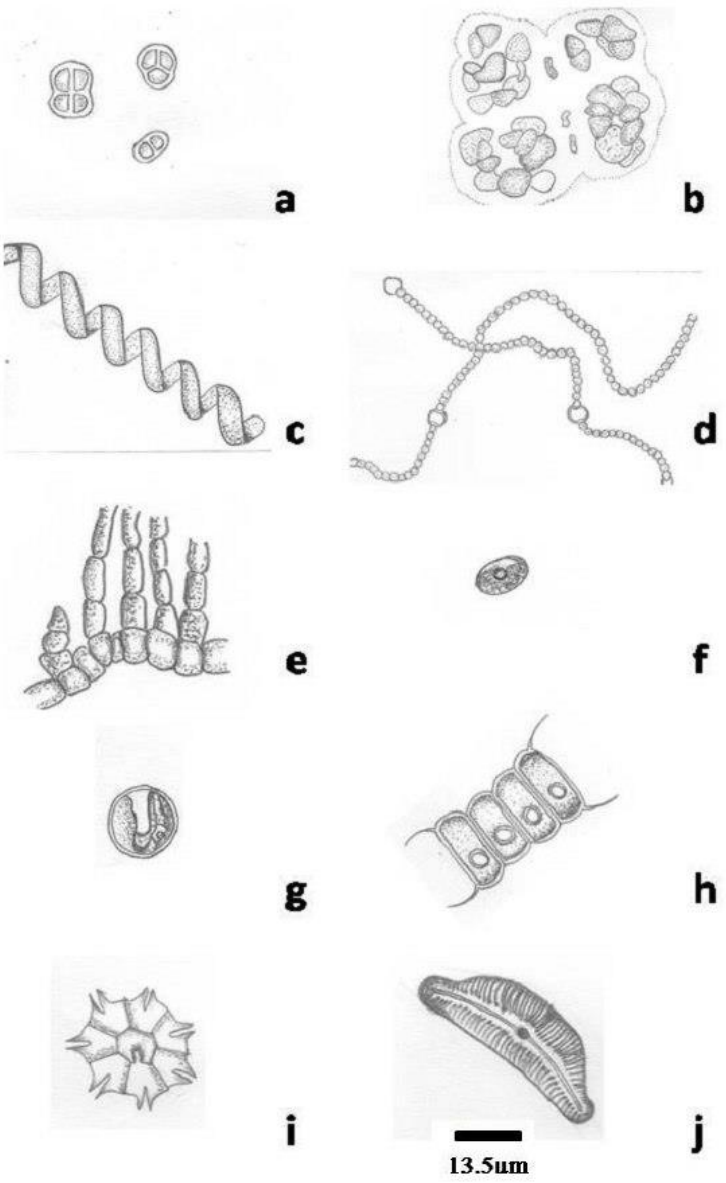

Fig 1. Light microscopic images of selected fresh water cyanobacteria and green microalge *C. minor* (a); *M. spectabilis* (b);*S. gigantea* (c); *N. calcicola* (d); *W. prolific* (e); *C. humicola* (f);*C. vulgaris* (g); *S. quadrispina* (h); *P. tetras* (i); *C. tumida* (j)

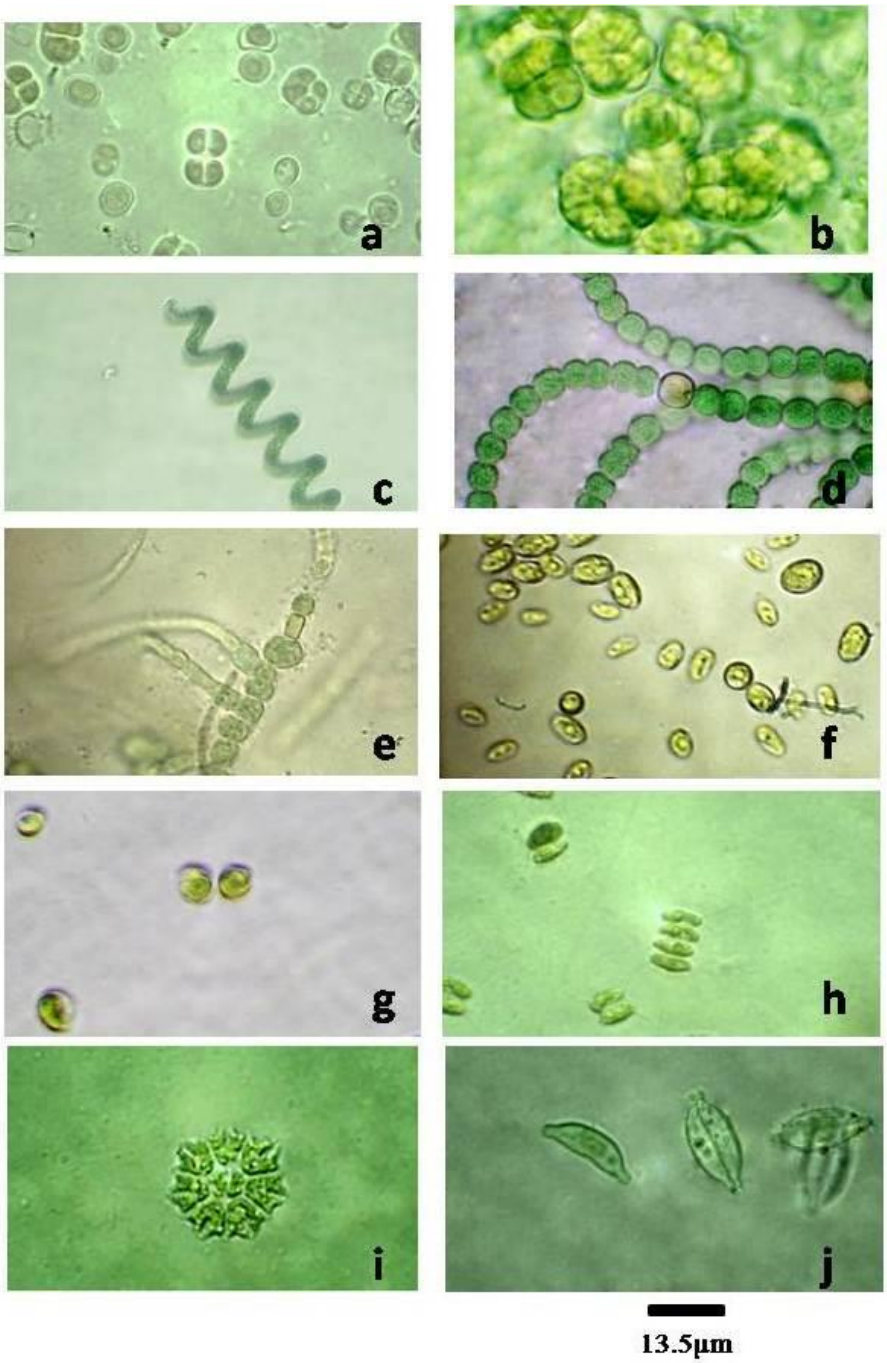

Fig 2. Bright field light microscopic images of selected fresh water cyanobacteria and green microalge: *C. minor* (a); *M. spectabilis* (b); *S. gigantea* (c); *N. calcicola* (d); *W. prolifica* (e); *C. humicola* (f);*C. vulgaris* (g); *S. quadrispina* (h); *P. tetras* (i); *C. tumida* (j)

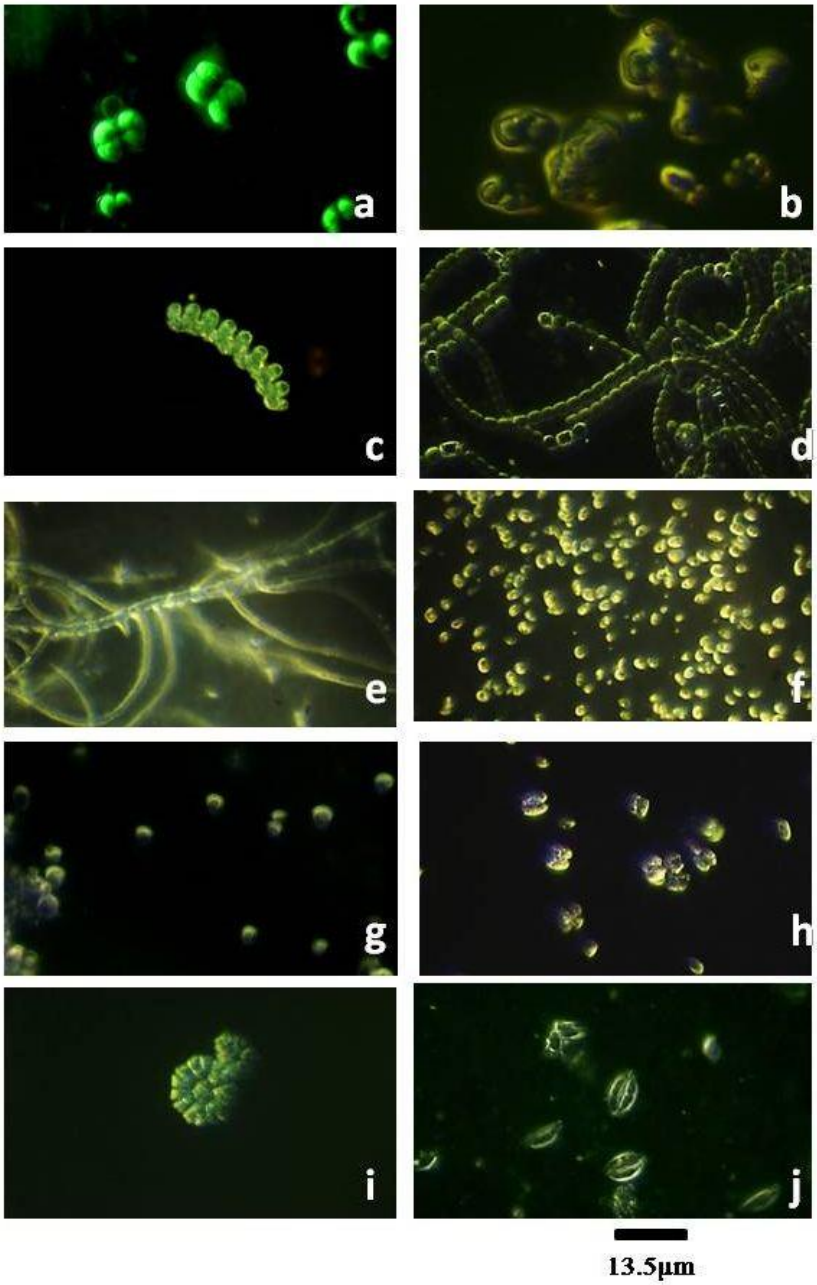

Fig 3.Dark field microscopic images of selected fresh water cyanobacteria and green microalge: *C. minor* (a); *M. spectabilis* (b); *S. gigantea* (c); *N. calcicola* (d); *W. prolifica* (e); *C. humicola* (f);*C. vulgaris* (g); *S. quadrispina* (h); *P. tetras* (i); *C. tumida* (j).

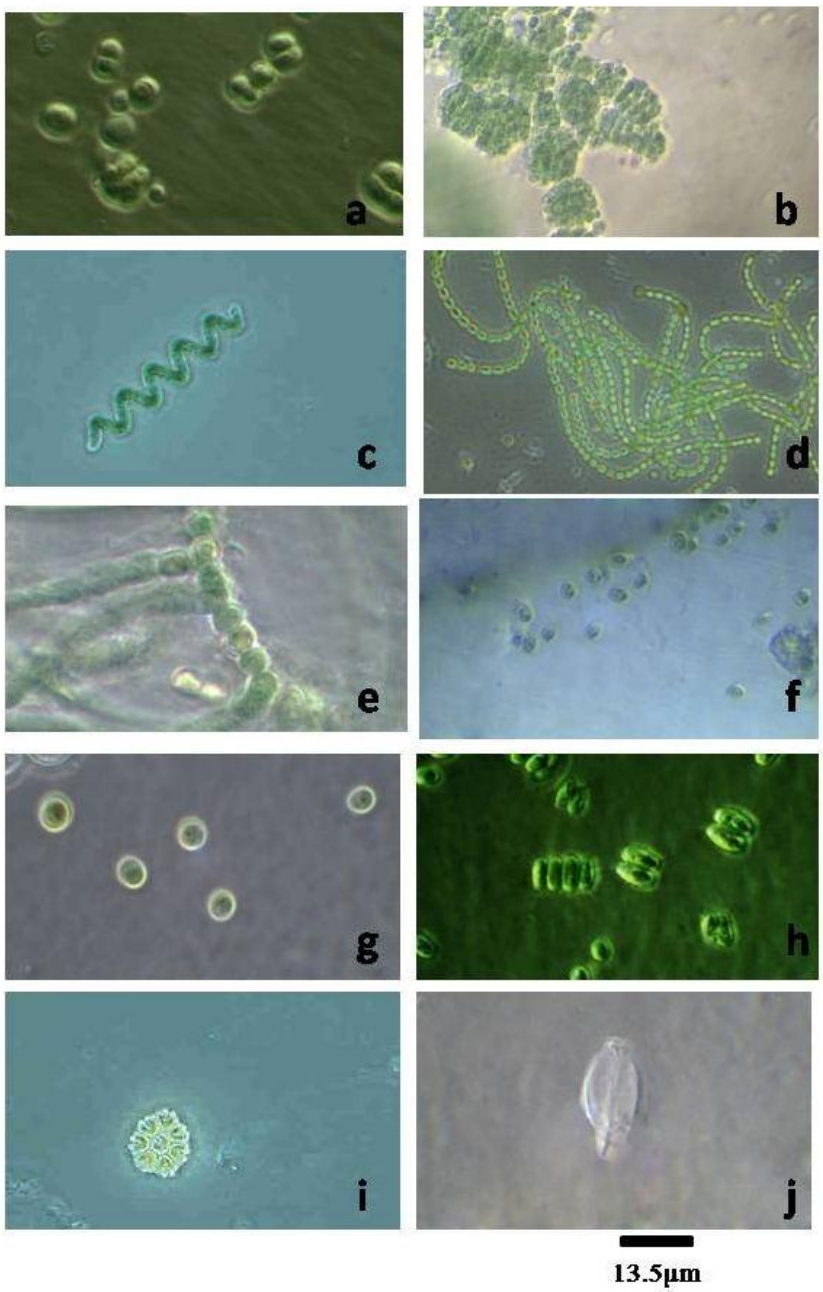

Fig 4.Phase contrast microscopic images of selected fresh water cyanobacteria and green microalge: *C. minor* (a); *M. spectabilis* (b); *S. gigantean* (c); *N. calcicola* (d); *W. prolific* (e); *C. humicola* (f);*C. vulgaris* (g); *S. quadrispina* (h); *P. tetras* (i); *C. tumida* (j).

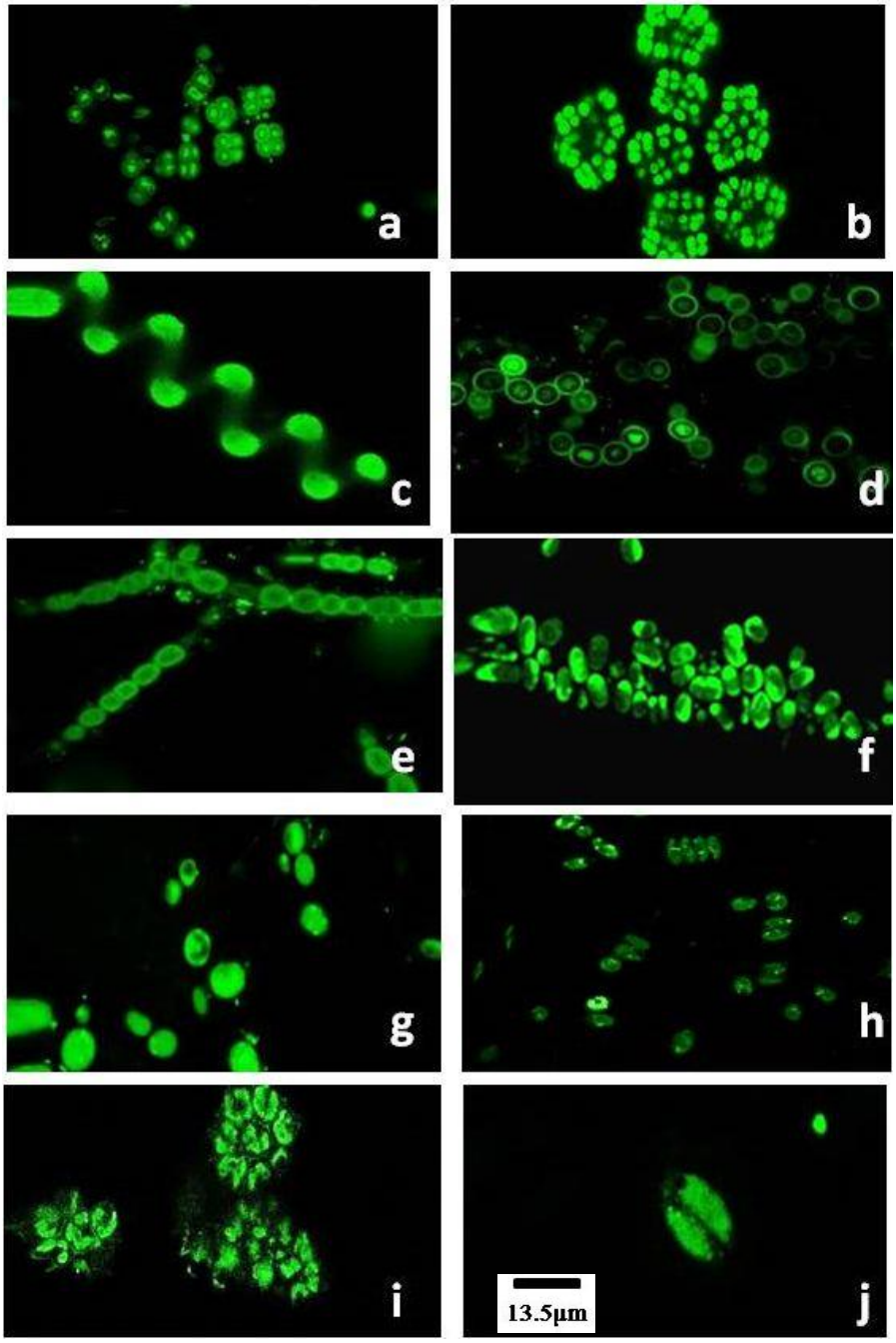

Fig 5.Confocal microscopic images of selected fresh water cyanobacteria and green microalge: *C. minor* (a); *M. spectabilis* (b); *S. gigantea* (c); *N. calcicola* (d); *W. prolifica* (e); *C. humicola* (f);*C. vulgaris* (g); *S. quadrispina* (h); *P.*

Table 2 Characteristics and microscopy recommendation for the selected fresh water microalgae

S.No	Strain Name	Salient features of different microscopy					Recommendation
		LM	BF	DF	PC	CLSM	
a.	***Cyanophyceae***						
1.	C. minor	Granules are not visible	Sheath and Septa not visible	Septum not visible	Sheath clearly visible	Sheath clearly visible	**CLSM, PC**
2.	*M. spectabilis*	Outer sheath arrangement visible	Colorful &sarcina arrangement visible	Septa arrangement not visible	Septa not visible	Cellular arrangement not visible	**CLSM, BF**
3.	*S. gigantea*	Spiral coil visible	Colorful and spiral visible	Spiral not visible	Spiral is visible	Spiral not clearly visible	**BF, PC**
4.	*N. calcicola*	Heterocyst polar bodies are visible	Colorful, and heterocyst polar bodies are visible	Heterocyst is visible	Heterocyst is not visible	Heterocyst is not visible	**BF, DF**
5.	*W. prolifica*	Branch and granules are visible	Heterocyst is visible	Heterocyst and vegetative cells not visible	Sheath Visible	Sheath not visible	**LM, BF**
b.	***Chlorophyceae***						
6.	*C. vulgaris*	Globular sheath are visible	Colored, pyrenoids are visible	Granules not visible	Spherical sheath are not visible	Spherical sheath is visible	**LM, CLSM**
7.	*C. humicola*	Chloroplast are visible	Colourful, Pyrenoids are visible	Pyrenoids not visible	Sheath observed background not visible	Sheath observed background not visible	**LM, BF**
8.	*S. quadrispina*	Spines, septum are visible	Cell arrangements are visible	Spines not observed	Sheath are visible	Spines, Sheath, granules not visible	**LM, BF**
9.	*P. tetras*	Spines, cellular granules are visible	Rectangular	Spines not visible	Septa and Sheath are visible	Tetrats structures are visible	**CLSM, PC**
c.	***Bacillariophyceae***						
10.	*C. tumida*	Structure are clearly visible	Shapes are clearly visible	Bubble ends visible clearly	Chlorophyll not visible	Frustules not clearly visible	**DF, BF**

LM: Light Microscopy; **BF**: Bright field; **DF**: Dark field; **PC**: Phase-contrast; **CLSM**: Confocal Laser Scanning Microscopy

This study was aimed to elaborate the structural features of the all the microalgae available in the repository for the development of exclusive freshwater microalgae monograph and user friendly hand out for fast and reliable identification of microalgae. This study helps to detailed investigation on the proper identification of microalgae and cyanobacteria without missing any key features. This study paves a way to understand the microalgae very clearly and also be authenticated for the proper identification.

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