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Improved Pre-treatment Protocol for Scanning Electron Microscopy Coupled with Energy Dispersive X-ray Spectroscopy Analysis of Selected Tropical Microalgae

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ABSTRACT

Suitable protocol for identification and classification of microalgae using scanning electron microscopy, coupled with energy dispersive X-ray spectroscopy (SEM-EDX), is important to obtain accurate information of their ultrastructure description. The objective of this study was to modify microalgae pre-treatments for reliable SEM-EDX analysis. Sixteen cultured tropical microalgae were subjected to two-step chemical fixation of glutaraldehyde and osmium tetroxide, sample washing in sodium cacodylate, ethanol and acetone dehydration, critical point-drying, mounting and gold sputter-coating prior to SEM visualisation and elemental characterisation. In this study, short period of chemical fixation and optimum separation forces, at 3213 x *g* for 3 min during every chemical solution change, were successfully established with high quality SEM images. Ultrastructure, particularly clear and useful images of cell wall ornamentation in *Scenedesmus* spp. and *Desmodesmus* sp.; areola patterns in *Biddulphia sinensis* and *Thalassiosira* sp. and morphological appearances such as interconnecting structures in *Coelastrum* sp. and *Crucigenia* sp., were obtained. Twelve elements of Y, Nb, Fe, Ca, Cl, K, Cu, F, Ir, P, Mg and Si were detected within the 16 investigated microalgae species. This study illustrated that microalgae identification and classification, as well as their elemental characterisation, could be simultaneously and effectively analysed by SEM-EDX using a modified pre-treatment protocol.

Keywords: Microalgae, morphology, ultrastructure, elemental profiles, modified SEM-EDX pre-treatment protocol

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INTRODUCTION

Microalgae are photosynthetic microorganisms that are ubiquitous in aquatic ecosystems regardless of freshwater, brackish or marine environment, either drifting in lotic or lentic water columns (planktonic) or attaching on substrates (periphytic). Many research studies have revealed that bioactive compounds derived from microalgae possess several important pharmacological activities such as antioxidant (Natrah et al., 2007; Goh et al., 2010), antimicrobial (Suresh et al., 2014), anti-inflammatory (Jo et al., 2010) and anti-cancer (Ebrahimi Nigjeh et al., 2013; Goh et al., 2014). Large untapped resources of microalgae provide great opportunities to explore novel energy and bioactive compounds such as hydrocarbons of *Botryococcus* spp. and carotenoids i.e. astaxanthin of *Haematococcus* spp., beta-carotene of *Dunaliella* spp. and lutein of *Scenedesmus* spp. (Sawayama et al., 1994; Boussiba, 2000; Ben-Amotz, 2004; Sánchez et al., 2008). However, species identification and classification remain the priority before further studies on their biological characterisation and application can be made. Besides, species identification and classification are also important in ecological studies especially on interaction between environmental factors and microalgae biodiversity (Van Staden et al., 2010; Renuka et al., 2014).

Essentially, researchers identify and classify microalgae using the guidance of dichotomous keys based on phenotypic characteristics of morphological appearances such as unicellular or multicellular, cell shape, cell arrangement and specialised cell parts like spines and flagella. Compared to light microscopy, scanning electron microscopy (SEM) offers even higher resolution, magnification, contrast and large depth of focus, and it becomes an important tool for the study of cellular and molecular components. Thus, SEM overcomes the limitation of light microscopy by displaying the ultrastructure characteristics in more detail which facilitates the microalgae identification and classification tasks. In addition, SEM coupled with energy dispersive X-ray spectroscopy (SEM-EDX) allows ultrastructure/ morphological visualisation and elemental characterisation of samples that can be conducted simultaneously. However, the quality and accuracy of the phytoplankton ultrastructure images are highly dependent on the modification of the pre-treatment protocol.

In pre-treatment protocol of SEM, all volatile substances from the sample must be removed and its strength of radiation damage resistance and electrical conductivity must be increased before submitting to the high vacuum chamber. Additionally, pre-treatment protocol is established according to the sample types which could well-preserve their native morphology without cell deterioration and eventually produce high quality images. Chen (2001) provided specific SEM pre-treatment steps for *Scenedesmus quadricauda*; however, the separation of delicate and fragile cells from fluids was not emphasised in his study. On the other hand, long fixation period was reported to be 12 hours in *Botryococcus* sp. and 24 hours in *Coelastrum* sp and

Scenedesmus obliquus (Dayananda et al., 2010; Liu et al., 2013; Basu et al., 2013; Tanoi et al., 2014). As a result, it is essential to improve SEM pre-treatments for various genera of microalgae which can be effectively used in future studies.

Elemental characterisation is one of the crucial studies to evaluate the nutritional properties of microalgae. Previous studies have shown that marine microalgae were good element sources for fish nutrition (Fabregas & Herrero, 1986; Hussein et al., 2013). Moreover, essential elements from algae have good nutritional benefits for human, but many studies provided information on edible seaweeds instead of microalgae (Ortega-Calvo et al., 1993; Tokusoglu & Ünal, 2003; Krishnaiah et al., 2008; Rohani-Ghadikolaei et al., 2012). Hence, this study aimed to develop the modified pre-treatment protocol for SEM-EDX analysis in order to produce high quality images for identification and classification, as well as to characterise the elemental compositions of 16 selected tropical microalgae.

MATERIALS AND METHODS

Chemicals

All chemicals and solvents used were of analytical grade. Chemicals such as glutaraldehyde, sodium cacodylate and osmium tetroxide were obtained from Agar Scientific (Agar Scientific, Elektron Technology, UK). Ethanol and acetone were purchased from Merck, (Merck, Darmstadt, Germany).

Microalgae Cultivation and Maintenance

Microalgae strains were obtained from the Microalgae Culture Collection of the Laboratory of Marine Biotechnology, Institute of Bioscience, Universiti Putra Malaysia (Table 1). Purified freshwater microalgae were maintained in Bold Basal medium (Bold & Wynne, 1978), pH 6.8 ± 0.2, whereas marine diatoms in f/2 medium (Guillard, 1975), pH 8.3 ± 0.2 and salinity 30 ± 1‰. All microalgae (100 mL in 250 mL conical flasks) were batch-cultured in an environmental chamber (Versatile Environmental Test Chamber, Sanyo, Japan) under constant 120 µmol photons $m^{-2}sec^{-1}$ light intensity at 12 h light: 12 h dark at 25°C for 14 days. Purity of unialgal culture was examined routinely via light microscopy. Fourteen freshwater green microalgae and two marine diatoms were used in this study for SEM-EDX analyses (Table 1).

Sample Pre-treatment Steps

Generally, the flow of pre-treatment steps included monoalgal sample preparation, pre- and post-fixation, first and second washing, dehydration, drying, mounting and sputter-coating (Figure 1) before the samples were subjected to image viewing and elemental characterisation. One millilitre of monoalgal culture (1 x 10^6 of cell density) was sampled during its late exponential growth phase and centrifuged to separate cells from liquid medium. Green microalgae and diatoms (without acid washing step) were pre-fixed in 4% glutaraldehyde ($C_5H_8O_2$) that was prepared in 0.2M sodium cacodylate buffer ($C_2H_6AsNaO_2$) at pH 7.2 for 3 hours

at 4°C. After pre-fixation, samples were washed three times with 0.1M sodium cacodylate buffer for 10 min to remove excessive fixative. Cells were post-fixed in 1% osmium tetroxide (OsO_4) for 2 hours at 4°C. The washing step was repeated for the same purpose. Samples were dehydrated gradually using a series of ethanol (30%, 40%, 50%, 60%, 70%, 80% and 90%) for 10 min each, followed by 100% ethanol and 100% acetone, twice for 15 min each. They were centrifuged for 3 min at 3213 x *g* at each changing of buffer or chemical solution.

Samples inside small handmade baskets made from aluminium foil were critical-point dried at 42ºC under pressure of 85 bar using a critical point dryer (Leica EM CPD030). The samples were mounted on stubs using double-sticky tapes and then sputter-coated with gold at 20 mA for 180 s using sputter coater (BAL-TEC SCD005). The pre-treated samples were inserted into working chamber of scanning electron microscope prior to SEM-EDX analyses. Images of the samples were visualised and their elemental profiles were analysed using variable pressure scanning electron

Table 1
Microalgae used in scanning electron microscopy coupled with energy dispersive X-ray spectroscopy (SEM-EDX) analysis

Collection code	Species	Freshwater (F)[1] / Marine (M)[2]
UPMC-A0002	*Scenedesmus* sp. A	F
UPMC-A0003	*Scenedesmus* sp. B	F
UPMC-A0004	*Scenedesmus* sp. C	F
UPMC-A0005	*Scenedesmus* sp. D	F
UPMC-A00049	*Scenedesmus* sp. E	F
UPMC-A0043	*Botryococcus* sp. A	F
UPMC-A0044	*Botryococcus* sp. B	F
UPMC-A0045	*Coelastrum* sp. A	F
UPMC-A0046	*Coelastrum* sp. B	F
UPMC-A0008	*Ankistrodesmus* sp.	F
UPMC-A0048	*Crucigenia* sp.	F
UPMC-A0042	*Desmodesmus* sp.	F
UPMC-A0006	*Kirchneriella* sp.	F
UPMC-A0009	*Selenastrum* sp.	F
UPMC-A0050	*Biddulphia sinensis*	M
UPMC-A0051	*Thalassiosira* sp.	M

[1]Freshwater species were maintained in Bold Basal Medium (BBM): chemical compositions of $NaNO_3$, $CaCl_2.2H_2O$, $MgSO_4.7H_2O$, K_2HPO_4, KH_2PO_4, NaCl, EDTA, KOH, $FeSO_4.7H_2O$, H_3BO_3, $ZnSO_4.7H_2O$, $MnCl_2.4H_2O$, MoO_3, $CuSO_4.5H_2O$ and $Co(NO_3)_2.6H_2O$ (Bold & Wynne, 1978).
[2]Marine species were maintained in f/2 medium: chemical compositions of $NaNO_3$, $NaH_2PO_4.H_2O$, $Na_2SiO_3.9H_2O$, $FeCl_3.6H_2O$, $Na_2MoO_4.2H_2O$, $CuSO_4.5H_2O$, $Na_2MoO_4.2H_2O$, $ZnSO_4.7H_2O$, $CoCl_2.6H_2O$ and $MnCl_2.4H_2O$ (Guillard, 1975).

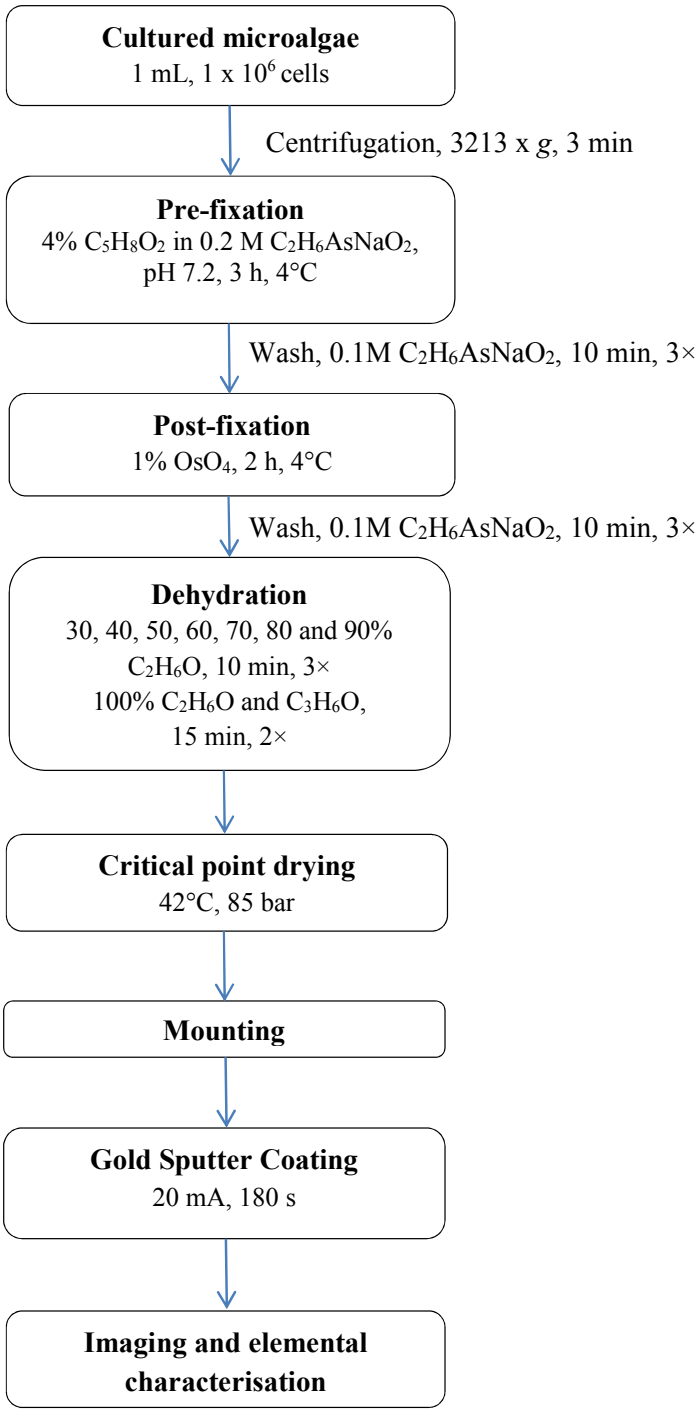

Figure 1. A modified scanning electron microscopy coupled with energy dispersive X-ray spectroscopy (SEM-EDX) pre-treatment protocol for the analyses of microalgae.

microscopy (LEO 1455 VPSEM), coupled with energy dispersive X-ray spectroscopy (Oxford Inca EDX), at an accelerating voltage of 15-20 kV and working distance of 7-15 mm.

Statistical Analysis

All data were presented as mean ± standard deviation (n=3). One-way ANOVA (SPSS 21.0, USA) was applied to test the significant differences ($p<0.05$) for atomic % of microalgae elemental characterisation.

RESULTS AND DISCUSSION

Modification of Microalgae Pre-treatment Protocol for SEM-EDX Analysis

Environmental scanning electron microscopy (ESEM) is an alternative method to eliminate sample pre-treatments due to its capability to analyse hydrated samples. However, images of the hydrated microalgae obtained directly from both freshwater and marine culture media using the ESEM (Philips XL30 ESEM) equipped with water vapour were sub-standard. In our previous trials, ESEM failed to provide high quality images of *Scenedesmus* spp. that were not subjected to pre-treatments (Figure 2A-H). Cotton-like fine strands were widely spread over *Scenedesmus* sp. A, UPMC-A0002 (Figure 2A-B), indicating their instability in a high vacuum environment. A similar phenomenon was also reported for *Anabaena flos-aquae* due to improper dehydration step during sample pre-treatments (Bellinger & Sigee, 2010). Furthermore, cell shrinkage was also observed in *Scenedesmus* sp. B, UPMC-A0003 (Figure 2C-D) and *Scenedesmus* sp. D, UPMC-A0005 (Figure 2G-H), whereas blur images were observed in *Scenedesmus* sp. C, UPMC-A0004 (Figure 2E-F). On the other hand, crystallised salt from seawater culture medium appeared in the ESEM images of marine microalgae, which could affect their electron microscopy visualisation (Figure 2I). Hence, not all hydrated samples, especially microalgae, could be viewed and imaged as clear and distinctive cell structures under ESEM. Thus, the pre-treatment protocol prior to SEM analysis was modified for microalgae in order to preserve and stabilise their native structures in a high vacuum SEM chamber.

The major pre-treatment steps of fixation, dehydration, critical point drying and coating prior to SEM imaging were commonly applied and modified according to the biological samples. In microalgae, varieties of the pre-treatment steps have been published; these ranged from freeze fixation, single-step or two-step chemical fixation; freeze, air, chemical or critical point drying and carbon, gold, or ion coating. The present study was designed to modify, develop and describe complete series of pre-treatment steps for microalgae samples obtained directly from the culture medium prior to SEM analyses.

Chemical Fixation and Washing

Chemical fixatives are used in order to preserve cell shapes and structures by crosslinking their macromolecules (e.g., polysaccharides, proteins, lipids and nuclei acids) into a rigid form. Furthermore, fixatives induce the insolubility of

macromolecules so that they will not be extracted out during the dehydration step (Lee, 1993). According to some previous reports, fixation techniques such as single-step and two-step fixation have been mostly applied on microalgae. In this study, the samples were preserved and fixed using two-step fixation technique which involved a pre-fixation step in glutaraldehyde and a post-fixation step in osmium tetroxide. In order to achieve optimal fixation, two type-specific functional fixatives were utilised in this study for targeting and fixing the different macromolecules in microalgae (i.e. glutaraldehyde) for protein and polysaccharides while osmium tetroxide for lipids. In this study, silica was evidently absent in all the examined green microalgae species. In addition, previous studies revealed that silica of acid washed diatoms was stable under extreme vacuum SEM working environment. Therefore, we concentrated on the preservations of major and imperative compounds found in microalgae such as polysaccharides, proteins and lipids. It is similar to the two-step fixation technique of Chen (2001), where *Scenedesmus quadricauda* was pre-fixed in glutaraldehyde and post-fixed in osmium tetroxide. Additionally, *Coelastrum* sp. was pre-fixed in glutaraldehyde and post-fixed in GTGO solution, which is a combination of glutaraldehyde, tannic acid, guanidine hydrochloride and osmium tetroxide (Tanoi et al., 2014). However, Han et al. (2006) and Dayananda et al. (2010) demonstrated the single step fixation on *Chlorella miniata* and *Botryococcus* sp., respectively. Besides, *Chlorella* sp. and *Chlamydomonas* sp. were also reported to be fixed in McDowell-Trump's fixative consisting of formaldehyde and glutaraldehyde in phosphate buffer (Wan Maznah et al., 2012), whereas *Dictyosphaerium chlorelloides* was fixed in the combination of paraformaldehyde and glutaraldehyde in phosphate buffer saline (PBS) (Pereira et al., 2013). In comparison, the current study showed that glutaraldehyde and osmium tetroxide (only two fixatives) substantially fixed the 16 microalgae species with promising SEM-EDX outputs.

Fixation time of microalgae in this study was modified to 3 hours for 4% glutaraldehyde fixation and 2 hours for 1% osmium tetroxide fixation, respectively. Dayananda et al. (2010) subjected the samples of *Botryococcus* sp. to 12 hours fixation with 2% glutaraldehyde before viewing the images with SEM. Comparatively, *Botryococcus* sp. in this study was fixed in significantly shorter period (3 hours fixation instead of 12 hours) by increasing the concentration of glutaraldehyde to 4% instead of 2%. Liu et al. (2013) also reported long period of glutaraldehyde fixation (24 hours) for his sample of *Coelastrum* sp. By using the present modified pre-treatment protocol, the fixation time was successfully reduced in 8 folds to 3 hours, but with comparable SEM images of *Coelastrum* sp. (Figure 5). Besides, five hours of fixation period had effectively preserved five species of genus *Scenedesmus*. In comparison, the study of Chen (2001) chemically fixed *Scenedesmus quadricauda* in a shorter period (3 hours),

i.e. pre-fixed in 4% glutaraldehyde for 2 hours and post-fixed in 2% osmium tetroxide for 1 hour, which produced reliable images. Likewise, Han et al. (2006), Wan Maznah et al. (2012) and Pereira et al. (2013) reported short fixation period on *Chlorella miniata* (2 hours in 4% glutaraldehyde), *Chlorella* sp. and *Chlamydomonas* sp. (at least 2 hours in McDowell-Trump's fixative) and *Dictyosphaerium chlorelloides* (1 hour in a combination of 0.5% paraformaldehyde and 3% glutaraldehyde).

Fixation conditions such as fixative concentration, buffer types, pH and temperature mainly determined the effectiveness of fixatives. Improper fixation conditions create osmotic pressure between fixatives and cell cytoplasm and would destroy the samples. In this study, microalgae were optimally preserved in 4% glutaraldehyde prepared in 0.2M sodium cacodylate (pH 7.2) and 1% osmium tetroxide at 4°C. Colour changes of fixative and samples mixture from colourless to brown indicated that the fixation process had taken place. In comparison, Chen (2001) also fixed *Scenedesmus quadricauda* at 4°C with 4% glutaraldehyde prepared in 0.1M sucrose and 0.1M sodium cacodylate (pH 7.0) and 2% osmium tetroxide. The cold condition could decelerate the cellular metabolism processes, particularly enzymatic activity. Previous findings of Gündisch et al. (2015) established that the cold fixation technique enhanced the protein preservation in biological samples of breast cancer tissues. However, fixations of *Chlorella miniata* and *Dictyosphaerium chlorelloides* were conducted at room temperature instead of 4°C (Han et al., 2006; Pereira et al., 2013). After the fixation step, the samples were washed three times using sodium cacodylate for 10 min each to remove excess fixative. In this study, sodium cacodylate was used for both preparation of 4% glutaraldehyde and also extraction of excess glutaraldehyde at the end of the fixation step. It is similar to the study of Burgos et al. (2012), where Millonig's buffer was used to prepare 2.5% glutaraldehyde and also for sample washing of *Chroococcus* sp., *Spirulina* sp. and *Microcoleus* sp.

Solvent Dehydration

Solvent dehydration is the most critical step among other pre-treatments in SEM that could damage samples easily due to severe osmotic changes that will interrupt water interactions which hold and maintain the cellular shape of macromolecules or membranes. This step is to remove water content of the samples and substitutes it with organic solvent which is easily removed via critical point drying. In the present study, microalgal samples were gradually dehydrated with a series of ethanol (30%, 40%, 50%, 60%, 70%, 80%, 90% and 100%) and 100% acetone to prevent any sudden osmotic shock. Microalgal-solvent was incubated in a series of ethanol dilution (30%-90%) for 10 minutes each and 15 minutes for 100% of ethanol and acetone. At this time, microalgae would clump together and its supernatants had to be discarded carefully. Both ethanol and acetone solvents were preferred to replace

water content in microalgae. However, ethanol was mostly utilised throughout the whole dehydration process because of its higher relative polarity than acetone. Thus, single solvent of ethanol was formerly and widely used in microalgae dehydration step (Burgos et al., 2012; Basu et al., 2013; Ho et al., 2013; Ponnuswamy et al., 2013). On the contrary, Pereira et al. (2013) reported that *Dictyosphaerium chlorelloides* was dehydrated using a series of acetone (25%, 50%, 75%, 85%, 95% and 100%) instead of ethanol. In order to obtain superb SEM images, 100% ethanol and acetone were used in the final stage of dehydration step for entirely replacing the water content in microalgae with organic solvent.

Critical Point Drying and Sputter Coating

After solvent dehydration step, solvents in microalgae samples were removed using critical point drying to ensure that liquid was passed to gas phase at critical point without creating surface tension forces which would damage the samples. Critical-point dried samples were mounted onto aluminium studs and then gold sputter-coated prior to SEM viewing and imaging. The samples were coated with a thin layer of gold in order to reduce or eliminate charging effects and further increase the effectiveness of electron beam activities. In this failed ESEM analysis, ultrastructure of fresh *Scenedesmus* spp. UPMC-A0002, UPMC-A0003, UPMC-A0004 and UPMC-A0005 with high water content was adversely destroyed. However, Dayananda et al. (2010) skipped the critical point drying step for their *Botryococcus* sp. and directly subjected the wet samples to gold sputter coating after alcohol dehydration. Despite of alcohol in *Botryococcus* sp., they still produced the SEM images in good feature. This was most probably due to the natural hardy attribute of *Botryococcus* sp. In contrast, Han et al. (2006) and Ponnuswamy et al. (2013) air-dried and sputter-coated *Chlorella miniata* and *Chlorella vulgaris* with carbon, whereby Chen (2001) critical point-dried and coated *Scenedesmus quadricauda* with ion beams. In addition, acid washed diatoms were air-dried and metal sputter-coated prior to SEM analyses (Govindasamy & Anantharaj, 2012; Lang et al., 2013). Nevertheless, in this present study, the acid washing step of diatoms was replaced by chemical fixation and organic solvent dehydration, and thus avoided the tedious and time-consuming procedures of repeated acid boiling and settling of samples in order to completely digest their organic matter. Furthermore, acid washed diatoms would interrupt their elemental profiles in the SEM-EDX analysis. On the other hand, chemical drying agent of hexamethyldisilazane (HMDS) is considered an alternative method to replace critical point drying which was applied on *Chlorella* sp., *Chlamydomonas* sp., *Verrucophora farcimen* and *Coolia* spp., but the extra process of removing excess HMDS needs to be included before sputter-coating (Edvardsen et al., 2007; Wan Maznah et al., 2012; Momigliano et al., 2013).

Mechanical Disturbances

Inputs of mechanical forces were unavoidable and yet constantly subjected onto samples during the mixing and separation procedures of fixative, buffer and solvent using vortex and centrifuge. Delicate and fragile structures of microalgae, especially their spines, cannot withstand strong forces and will get damaged easily. In this study, cells in 1.5 mL eppendorf tube centrifuged at 3213 x *g* for 3 min (Eppendorf Centrifuge 5810R, Germany) were separated from the chemical solutions with no induced damages (Figure 3A-B). In one of our failed trials, centrifugation of samples at 6797 x *g* for 10 min resulted in damaged spines of *Scenedesmus* sp. A, UPMC-A0002 (Figure 2J-K). Artefact images such as those in Figure 2J-K were not valid for scientific interpretation of morphological structures especially species-specific unique structures for taxonomic classification and identification of microalgae. Up to now, no study has highlighted or reported the optimum centrifugation speed, which is important to ensure the cellular structures of samples remain intact throughout the whole separation process.

Ultrastructure and Morphological Visualisation

In this study, SEM pre-treatment protocol was modified for microalgae samples obtained directly from the culture medium prior to the ultrastructure and element analyses. During the SEM pre-treatments, high preservation of ultrastructure and physical morphology, especially in fragile and delicate structures, is critical for accurate characterisation, description and identification of microalgae species. Species distinctive ultrastructure and morphological structures of 16 selected microalgae, particularly in cell ornamentation and interconnecting structures, are presented in Figure 3 to Figure 7, and the descriptions of their morphological characteristics are presented in Table 2. Size is one of the principal physical traits for phenotypic identification and classification of microalgae. Fundamentally, length is the longest distance of cell, width is the measurement gap from side to side and diameter is a straight line passing through the cell circle centre. On average, microalgae size regardless of marine or freshwater ranged from 3.42 to 80.00 µm in length, 0.42 to 11.38 µm in width and 3.5 to 13.92 µm in diameter. *Scenedesmus* sp. A, UPMC-A0002 in Figure 3A-B (10.42 µm in length and 9.21 µm in width) and *Scenedesmus* sp. D, UPMC-A00005 in Figure 3G-H (10.29 µm in length and 11.38 µm in width) formed the biggest colony, among others in the same genus. Among the spherical cells, *Botryococcus* sp. B, UPMC-A0044 (Figure 4C-D) was the smallest individual cell with the diameter of 3.50 µm whereby *Coelastrum* sp. A, UPMC-A0045 (Figure 5A-B) exhibited the largest colony with a diameter of 13.92 µm. Compared to non-spherical cells, the shortest length (3.42 µm) was observed in *Selenastrum* sp. UPMC-A0009 (Figure 6I-

J) with the width of 1.17 µm, whereby the diatom *Biddulphia sinensis* UPMC-A0050 (Figure 7A-B) has the longest length, 80.00 µm and second in width, 11.34 µm.

Scanning Electron Microscopy (SEM) images provide information on photogrammetric surfaces instead of volumetric (Friedrichs et al., 2012). Morphological characteristics such as fusiform or pointed cells of *Scenedesmus* spp., with or without curved spines and colony forming cells of *Scenedesmus* spp., *Botryococcus* spp., *Coelastrum* spp. and *Crucigenia* sp., can be observed in both light and SEM microscopy. Early developmental stage of *Scenedesmus* spp. were coccoid cells but in the mature stage they started to form colonies of 2 or 4 roughly cylindrical or pointed cells with or without curved spines (Figure 3A-J). Besides that, cells of *Botryococcus* spp. UPMC-A0043 and UPMC-A0044 (Figure 4A-D) were clustered together to form a dense colony. On the other hand, protuberance in *Coelastrum* spp. UPMC-A0045 and UPMC-A0046 (Figure 5A-D) and interconnecting structures in *Scenedesmus* sp. B, UPMC-A0003 (Figure 3F) and *Crucigenia* sp. UPMC-A0048 (Figure 6D) were minute structures and could only be observed clearly in SEM images. Additionally, the ultrastructure of cell ornamentations such as rosette structure (Figure 3A) and rib-like pattern (Figure 3J) in *Scenedesmus* spp.; two different areola patterns in *Biddulphia sinensis* UPMC-A0050 (Figure 7B) and marginal small spines of *Thalassiosira* sp. UPMC-A0051 (Figure 7C) were the results of appropriate pre-treatments prior to SEM analyses. It was observed that ornamental patterns on cell surface were unique and species-specific among the 16 selected microalgae.

Unicellular cells of *Ankistrodesmus* sp., *Kirchneriella* sp. and *Selenastrum* sp. were usually presented in clusters but they sometimes occurred in solitary. *Biddulphia sinensis* cells were normally found in solitary but sometimes they formed chains. *Thalassiosira* sp. cells usually joined to form a loose chain but would mostly be solitary in culture conditions. These images demonstrated the superiority of electron microscopy over light microscopy in the observation of unique cellular topography and minuscule structures of microalgae for their characterisations, discrimination and taxonomic classifications.

Elemental Characterisation

A total of 12 elements were detected and distributed within the 16 selected microalgae (Table 3). Yttrium (Y), a type of rare earth element (REE) was the most abundant element found in all microalgae. *Scenedesmus* sp. E, UPMC-A0049 had the highest Y content (57.56 ± 4.85% atom), while *Thalassiosira* sp. UPMC-A0051 had the lowest (7.39 ± 1.49% atom). Niobium (Nb) was another REE that was only found in green microalgae. The highest Nb content was observed in *Scenedesmus* sp. A, UPMC-A0002 (45.53 ± 3.08% atom) while the lowest in *Coelastrum* sp. A, UPMC-A0045 (15.22 ± 3.11% atom). Iridium (Ir), an element of the platinum

Table 2

Morphological characteristics of tropical microalgae as observed using scanning electron microscopy coupled with energy dispersive X-ray spectroscopy (SEM-EDX) analysis. Mean cell sizes (µm) in column with different superscript letters are significantly different by Duncan post-hoc test ($P < 0.05$).

Species	Collection code	Average cell size[3] (µm)			Morphological characteristics (Prescott, 1978; Bellinger & Sigee, 2010)
		Length	Width	Diameter	
Scenedesmus spp. Meyen 1829	UPMC-A0002	10.42±4.36^{ab}	9.21±0.76^{e}	nd[4]	Cells are more or less oval, ellipsoidal or fusiform in shape (Figure 3A-J). Colonies of cells are arranged side by side in one row. Some cells have a curved spine that is projected out from each corner (Figure 3A-D) with rosette structure (Figure 3A-B). Some cells are pointed and narrowed at the end without spines (Figure 3G-J) with apparent cell wall ornamentation of rib-like pattern (Figure 3J).
	UPMC-A0003	6.79±0.06^{a}	5.04±0.30^{d}	nd	
	UPMC-A0004	7.13±0.06^{a}	3.38±0.53^{cd}	nd	
	UPMC-A0005	10.29±1.36^{ab}	11.38±1.94^{f}	nd	
	UPMC-A0049	7.13±1.59^{a}	8.09±0.83^{e}	nd	
Botryococcus spp. Kützing 1849	UPMC-A0043	nd	nd	5.80±0.28^{a}	Cells are ovoid or spherical in shape aggregated together to form a dense colony. UPMC-A0043 has larger cells compared with UPMC-A0044 (Fig.4A-D).
	UPMC-A0044	nd	nd	3.50±0.71^{a}	
Coelastrum spp. Nägeli 1849	UPMC-A0045	nd	nd	13.92±0.59^{b}	Cells are ovoid or polygonal in shape arranged to form a hollow, spherical and many-sided colony like a football. Cells are joined together by protuberances (Figure 5A-D).
	UPMC-A0046	nd	nd	13.17±0.23^{b}	
Ankistrodesmus sp. Corda 1838	UPMC-A0008	9.34±1.89^{a}	0.42±0.12^{a}	nd	Cells are sickle-shaped, fusiform and crescent-shaped. Cells twisted about one another and usually in clusters but can be solitary (Fig.6A-B).
Crucigenia sp. Morren 1850	UPMC-A0048	5.42±0.83^{a}	3.00±0.47^{bc}	nd	Cells are trapezoidal-shaped and arranged in four cells to form quadrate plates or in multiple of four (Figure 6C-D).
Desmodesmus sp. (Chodat) An, Friedl and Hegewald 1999	UPMC-A0042	17.25±7.42^{c}	11.25±1.06^{f}	nd	Physical structure is similar as *Scenedesmus* sp. A colony composed of 2, 4, or 8 cells with curved spines and a hollow structure in between the cells (Figure 6E-F).
Kirchneriella sp. Schmidle 1893	UPMC-A0006	3.92±0.12^{a}	2.34±0.47^{abc}	nd	Cells are sharply curved and lunate in shape. They are enclosed in mucilage and usually in clusters but can be solitary (Figure 6G-H).
Selenastrum sp. Reinsch 1867	UPMC-A0009	3.42±0.83^{a}	1.17±0.23^{ab}	nd	Cells are strongly crescent-shaped but not entangled. They are not or rarely enclosed in mucilage and usually in clusters but can be solitary (Figure 6I-J).
Biddulphia sinensis Greville 1866	UPMC-A0050	80.00±5.66^{d}	11.34±0.94^{f}	nd	Cells are rectangular-shaped in girdle view and oval-shaped in valve view. A long spine is projected out from each corner (labiate process) and a short protrude is projected out from both ends (ocellus) (Figure 7A-B).
Thalassiosira sp. Cleve 1873	UPMC-A0051	nd	nd	12.17±2.60^{b}	Cells are circular and gently undulating valve face with mesh like radiated punctae. Small spines found at valve margin. Cells usually joined to form a loose chain but can be solitary (Figure 7C-D).

[3]Average cell size was measured according to length, width and diameter of cells. [4]Not detected.

Table 3
Elemental characterisation of 16 microalgae species by scanning electron microscopy coupled with energy dispersive X-ray spectroscopy (SEM-EDX) analysis. Data in column marked with different superscript letters are significantly different by LSD post-hoc test (P < 0.05).

Species	Elements (% atom)											
	Y	Nb	Fe	Ca	Cl	K	Cu	F	Ir	P	Mg	Si
UPMC-A0002	28.47 ±4.10^{d}	45.53 ±3.08^{e}	2.12 ±0.55^{ab}	2.47 ±2.33^{ab}	11.55 ±1.38^{c}	7.05 ±0.73^{bc}	1.58 ±1.43^{a}	nd	nd	nd	nd	nd
UPMC-A0003	46.59 ±16.40^{c}	27.89 ±4.38^{b}	2.93 ±0.47^{a}	2.58 ±0.26^{a}	nd^{d}	nd	2.98 ±0.71^{a}	nd	2.86 ±2.49^{a}	nd	nd	nd
UPMC-A0004	41.22 ±12.96^{c}	21.57 ±5.41^{b}	4.91 ±1.50^{a}	2.06 ±1.79^{a}	7.86 ±2.10^{a}	3.60 ±3.16^{a}	2.27 ±1.97^{a}	nd	nd	nd	nd	nd
UPMC-A0005	40.00 ±5.26^{e}	17.07 ±1.92^{d}	1.38 ±1.27^{a}	6.54 ±2.00^{b}	13.19 ±2.10^{c}	8.69 ±0.81^{b}	nd	nd	1.76 ±1.53^{a}	nd	nd	nd
UPMC-A0049	57.56 ±4.85^{c}	24.83 ±2.79^{b}	3.14 ±0.62^{a}	5.89 ±1.72^{a}	5.45 ±4.80^{a}	3.13 ±0.24^{a}	nd	nd	nd	nd	nd	nd
UPMC-A0043	46.15 ±6.34^{d}	16.41 ±4.92^{b}	1.33 ±0.35^{a}	nd	nd	nd	1.43 ±1.41^{a}	26.89 ±2.98^{c}	nd	nd	nd	nd
UPMC-A0044	45.35 ±5.79^{d}	18.12 ±4.00^{b}	nd	28.47 ±6.16^{c}	nd	nd	2.56 ±0.66^{a}	nd	2.91 ±0.54^{a}	nd	nd	nd
UPMC-A0045	35.54 ±3.86^{d}	15.22 ±3.11^{b}	nd	5.46 ±0.95^{a}	nd	nd	nd	nd	nd	30.74 ±4.38^{c}	11.32 ±4.34^{b}	nd
UPMC-A0046	15.08 ±3.33^{c}	27.20 ±2.29^{e}	nd	3.48 ±0.25^{a}	19.90 ±1.86	16.03 ±1.36^{c}	1.23 ±1.08^{a}	nd	3.32 ±3.38^{a}	nd	10.08 ±0.66^{b}	nd
UPMC-A0008	39.32 ±1.98^{f}	24.18 ±0.44^{e}	4.86 ±2.01^{ab}	2.87 ±2.56^{a}	9.1 ±0.31^{c}	13.03 ±0.66^{d}	2.36 ±2.08^{a}	nd	4.28 ±1.57^{a}	nd	nd	nd
UPMC-A0048	44.98 ±2.29^{d}	15.33 ±2.12^{b}	0.88 ±0.76^{a}	14.13 ±1.33^{b}	nd	nd	nd	nd	nd	21.11 ±2.45^{c}	nd	nd
UPMC-A0042	42.37 ±1.08^{e}	24.14 ±1.06^{d}	3.23 ±1.29^{a}	6.34 ±0.62^{b}	9.13 ±0.49^{c}	5.40 ±0.53^{b}	3.29 ±0.69^{a}	nd	6.10 ±1.38^{b}	nd	nd	nd
UPMC-A0006	44.99 ±6.14^{e}	20.78 ±1.01^{d}	3.24 ±0.86^{a}	3.88 ±0.15^{ab}	10.74 ±2.09^{c}	11.74 ±2.16^{c}	3.09 ±0.85^{a}	nd	nd	nd	nd	nd
UPMC-A0009	43.02 ±10.54^{c}	17.75 ±1.49^{b}	4.20 ±2.05^{a}	3.87 ±1.75^{a}	12.62 ±4.42^{b}	7.18 ±2.06^{ab}	nd	nd	nd	nd	nd	nd
UPMC-A0050	7.48 ±3.71^{ab}	nd	1.87 ±0.73^{a}	nd	nd	nd	nd	21.64 ±5.81^{c}	nd	nd	nd	65.95 ±12.5^{d}
UPMC-A0051	7.39 ±1.49^{ab}	nd	2.99 ±0.91^{a}	nd	nd	nd	0.73 ±0.63^{a}	nd	nd	nd	nd	76.10 ±8.89^{c}

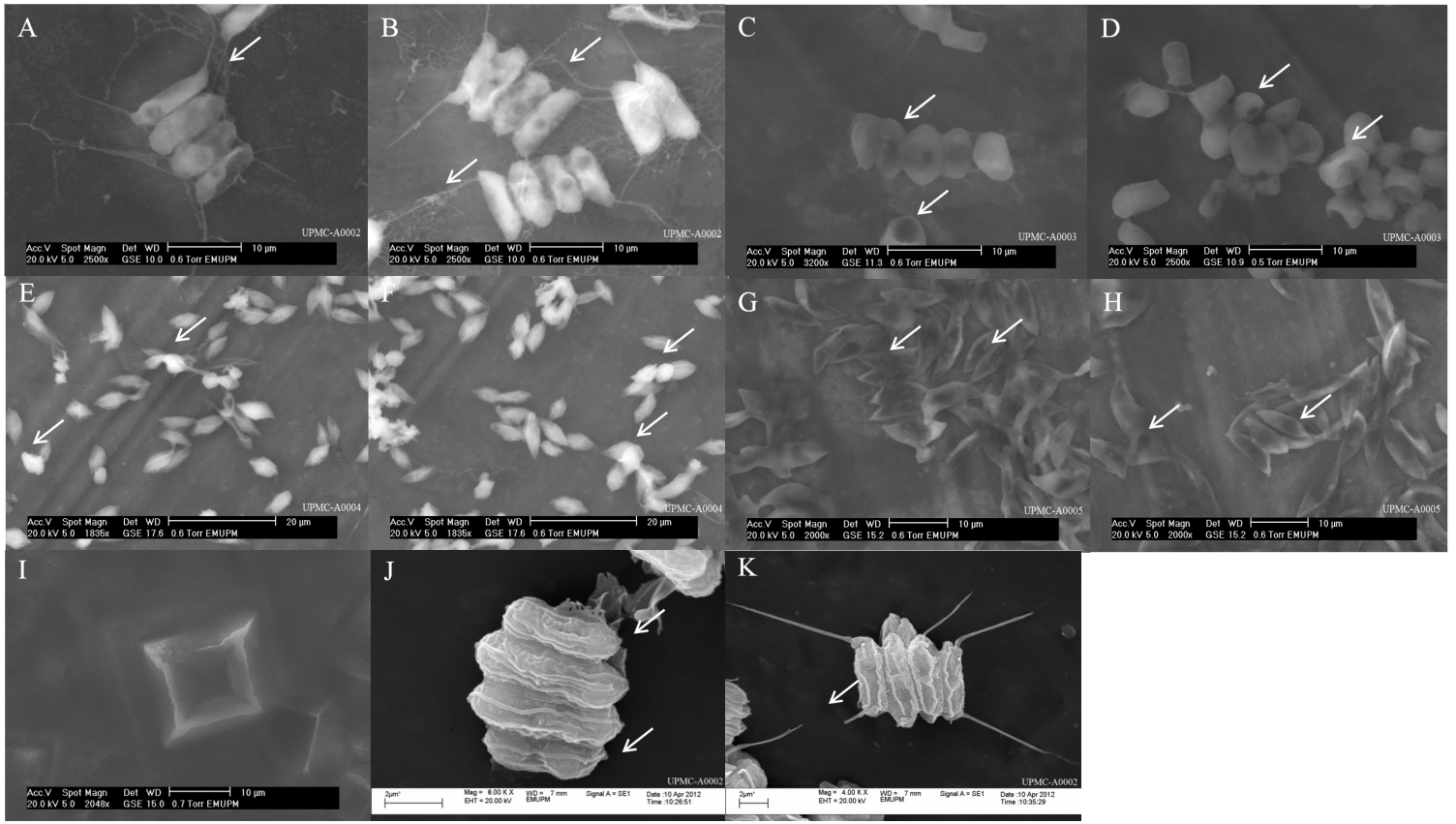

Figure 2. (A-H); Environmental scanning electron microscopy (ESEM) images of *Scenedesmus* spp. UPMC-A0002, UPMC-A0003, UPMC-A0004 and UPMC-A0005 without any pre-treatments show poor images with low resolution. Arrows in A and B elucidate cotton-like fiber strands. Arrows in C, D, G and H show cell shrinkage and damage of cell integrity that could affect the study of cell topography. Arrows in E and F show blur cells due to hydrated samples and their weak interaction with electron beams. (I); Environmental scanning electron microscopy (ESEM) image demonstrates the crystal salt derived from culture medium of marine microalgae. (J-K); Scanning electron microscopy (SEM) images of *Scenedesmus* sp. A, UPMC-A0002 which was centrifuged at high speed during pre-treatments. Arrows in J and K elucidate the absence of delicate spines that would deviate the interpretation.

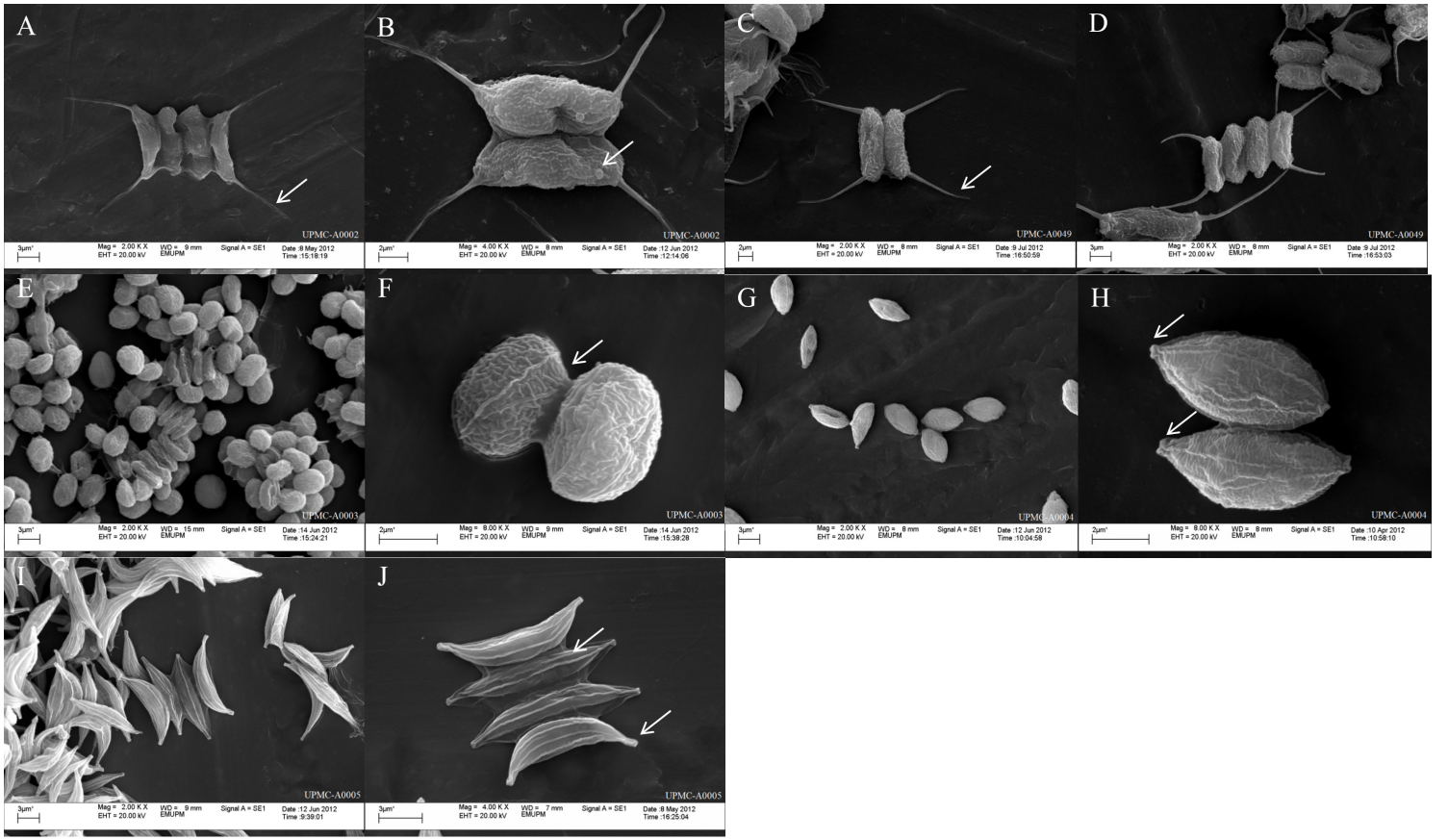

Figure 3. (A-B and C-D); *Scenedesmus* sp. A, UPMC-A0002 and *Scenedesmus* sp. E, UPMC-A0049. Arrows in A and C depict curved spine which is delicate to electron microscopy pre-treatments. Arrow in B depicts rosettes. (E-F); *Scenedesmus* sp. B, UPMC-A0003. Arrow in F depicts connecting structure. (G-H and I-J); *Scenedesmus* sp. C, UPMC-A0004 and *Scenedesmus* sp. D, UPMC-A0005. Arrows in H and J depict pointed cells and cell wall ornamentation of rib-like pattern

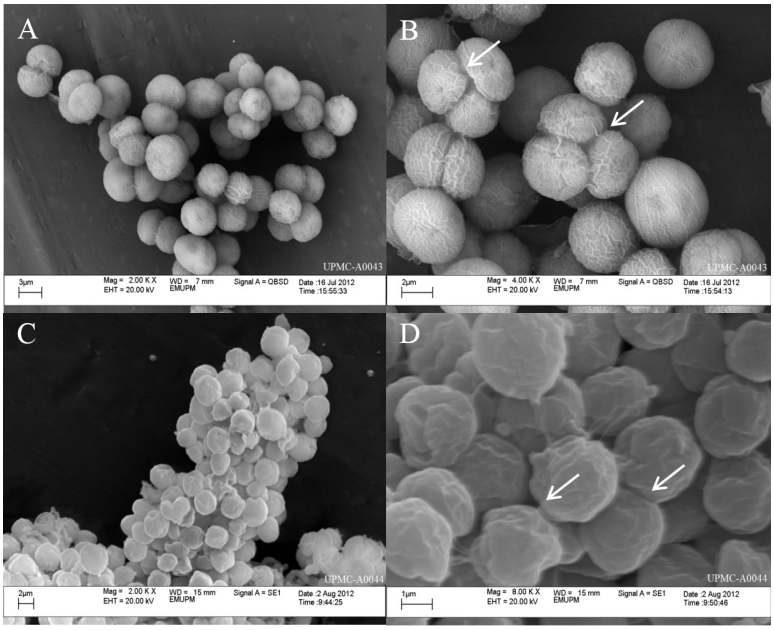

Figure 4. (A-B and C-D); *Botryococcus* spp. UPMC-A0043 and UPMC-A0044. Arrows in B and D show spherical cells are stuck together

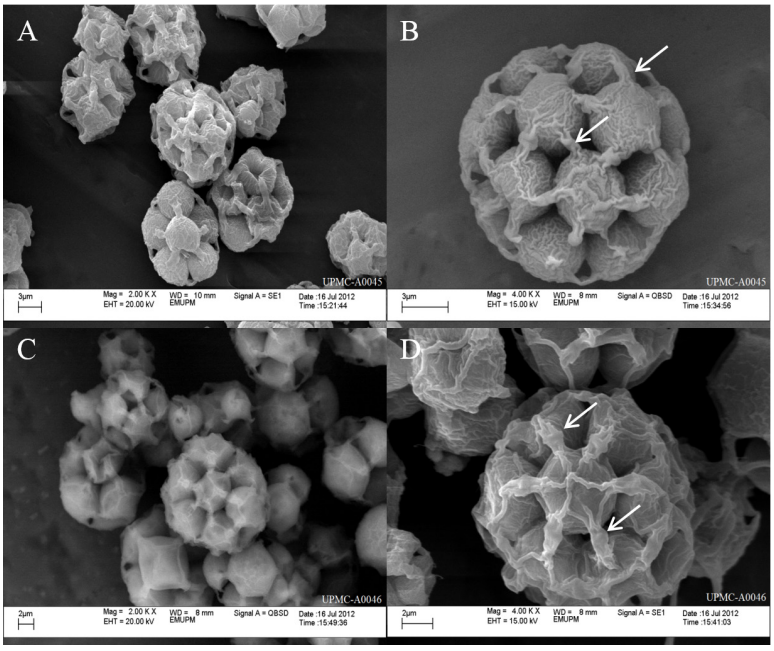

Figure 5. (A-B and C-D); *Coelastrum* spp. UPMC-A0045 and UPMC-A0046. Arrows in B and D demonstrate ovoid and hexagonal cells and their interconnecting structures of protuberances

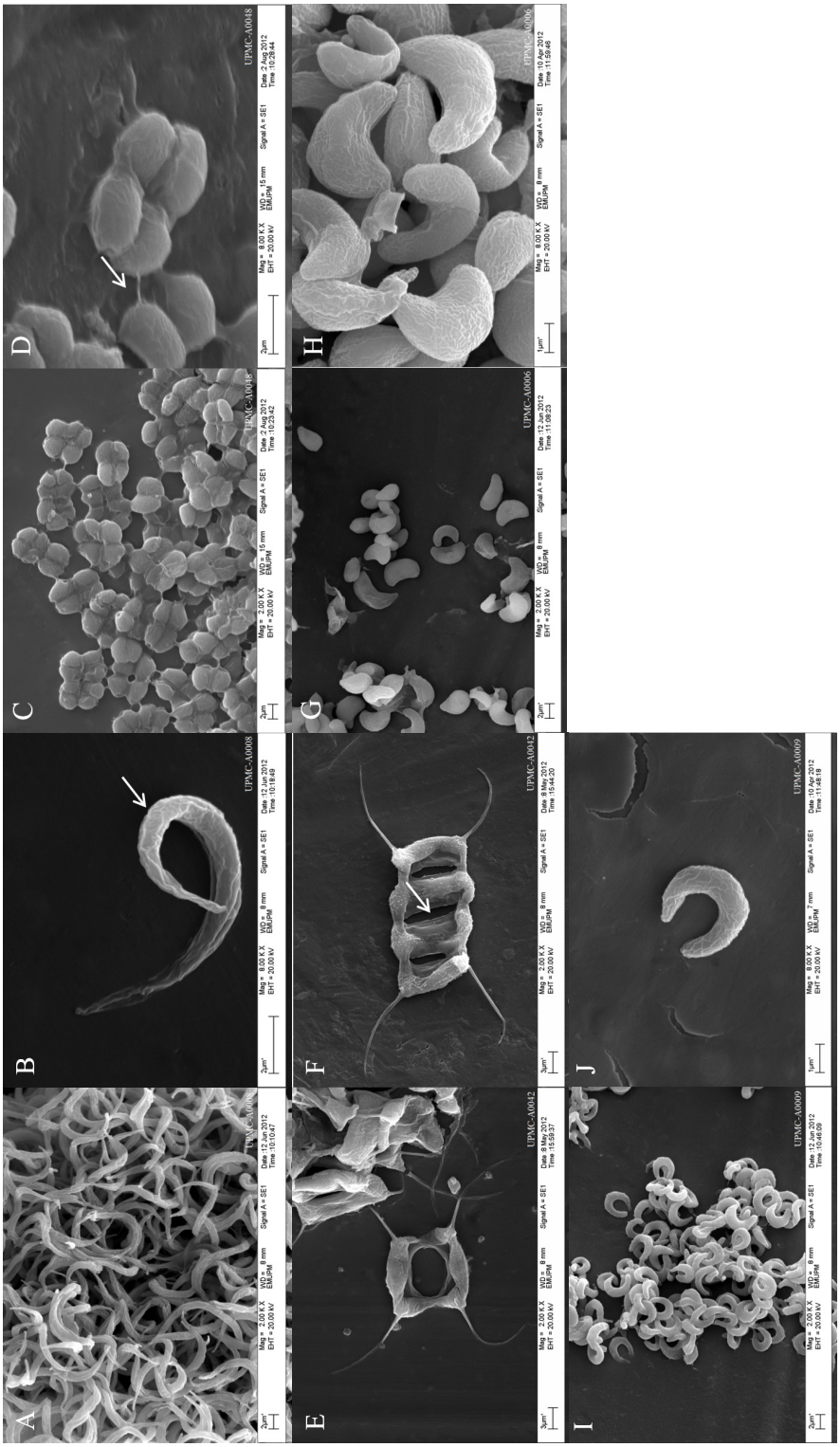

Figure 6. (A-B); *Ankistrodesmus* sp. UPMC-A0008. Arow in B shows twisted cell. (C-D); *Crucigenia* sp. UPMC-A0048. Arrow in D shows interconnecting structure. (E-F); *Desmodesmus* sp. UPMC-A0042. Arrow in F shows hollow structure. (G-H and I-J); *Kirchneriella* sp. UPMC-A0006 and *Selenastrum* sp. UPMC-A0009

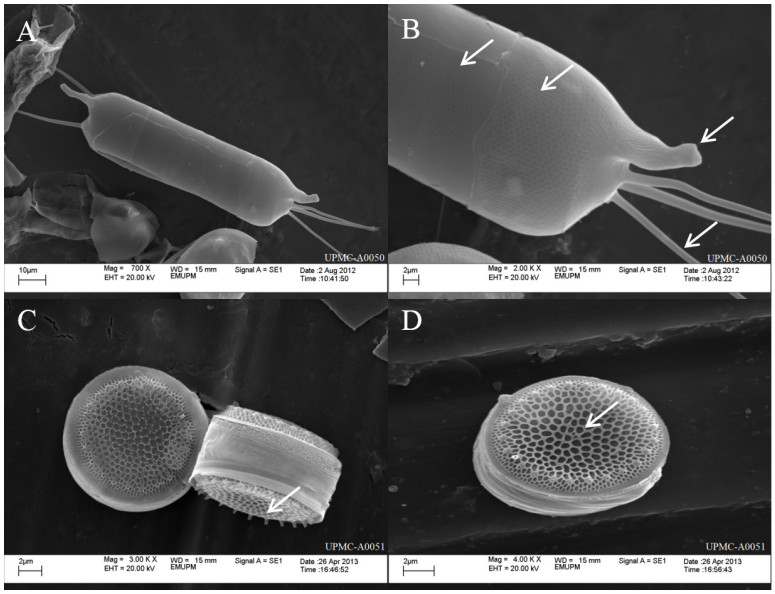

Figure 7. (A-B); *Biddulphia sinensis* UPMC-A0050. Arrows in B illustrate two different cellular areolae patterns with special parts of ocellus and long spine. (C-D); *Thalassiosira* sp. UPMC-A0051. Arrow in C illustrates marginal small spines. Arrow in D illustrates mesh-like punctae

group element (PGE), was found in six microalgal samples, namely *Desmodesmus* sp. UPMC-A0042 (6.10 ± 1.38% atom), *Ankistrodesmus* sp. UPMC-A0008 (4.28 ± 1.57% atom), *Coelastrum* sp. B, UPMC-A0046 (3.32 ± 3.38% atom), *Botryococcus* sp. B, UPMC-A0044 (2.91 ± 0.54% atom), *Scenedesmus* sp. B, UPMC-A0003 (2.86 ± 2.49% atom) and *Scenedesmus* sp. D, UPMC-A0005 (1.76 ± 1.53% atom). Meanwhile, silicon (Si) was only found in diatoms i.e. *Thalassiosira* sp. (76.10 ± 8.89% atom) and *Biddulphia sinensis* (65.95 ± 12.53% atom).

Twelve elements i.e. Y, Nb, Fe, Ca, Cl, K, Cu, F, Ir, P, Mg and Si were discovered in this microalgae SEM-EDX study. Similarly, Priyadarshani and Rath (2012) reported that microalgae were rich in elements such as Fe, Ca, K, and Mg, and these additional nutrients made them suitable for nutraceutical products such as *Chlorella* spp. and *Spirulina* spp. On the other hand, Fabregas and Herrero (1986) reported that Ca, Cl, Cu, Co, Fe, K, Mg, Mn, Na, P and Zn were found in *Tetraselmis suecica*, *Isochrysis galbana*, *Dunaliella tertiolecta* and *Chlorella stigmatophora*. In this study, three elements of Y, Nb and Ir are classified as metallic transition elements, whereby Y and Nb are rare earth elements, and Ir is platinum group element which has been detected in microalgae species being studied. Previous studies reported that the rare earth elements of lanthanum (La), gadolinium (Gd) and yttrium (Y) were found in green microalgae of *Chlorella vulgaris* and neodymium (Nd) in blue green algae of *Phormidium* sp. respectively (Sun et al., 1997; Kim et al., 2011). In comparison,

several studies of rare earth elements were intensively conducted on plants and their optimum quantities in fertilizers would enhance the plants' growth, development, chlorophyll content and photosynthetic rate (Emmanuel et al., 2010; Kastori et al., 2010; Zhang et al., 2013). However, such information on rare earth elements in microalgae is still lacking. Platinum group elements of rhodium (Rh), palladium (Pd) and platinum (Pt) were detected in *Chlorella stigmatophora* and *Ulva lactuca* (Turner et al., 2007; Shams et al., 2014). In this study, high amounts of Si were found in only diatoms, *Biddulphia sinensis* and *Thalassiosira* sp. Silicon is an essential element to diatoms and it is required in the construction of their disc-like frustules.

CONCLUSION

A modified SEM-EDX pre-treatment protocol was developed for 16 selected tropical microalgae, which could be applied on other microalgae species especially green algae and diatoms. The current protocol with shorter fixation time and optimum separation forces successfully preserved ultrastructure and morphological structures of samples especially in cell ornamentation and interconnecting structures for 10 genera of microalgae inclusive of both marine and freshwater species. Elemental characterisation of microalgae was simultaneously evaluated, whereby the rare earth elements of Y and Nb were found abundantly in most the microalgae species tested. In conclusion, this SEM-EDX study illustrates that microalgae can be characterised, classified and identified based on ultrastructure and morphological description, whereas their potential of certain element sources has also been evaluated.

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