Hydrobiologia (2013) 707:81–95
DOI 10.1007/s10750-012-1412-6

PRIMARY RESEARCH PAPER

An improved method for determining phytoplankton chlorophyll *a* concentration without filtration

Hongjie Qin · Shuangshuang Li · Dunhai Li

Received: 26 April 2012 / Revised: 29 October 2012 / Accepted: 24 November 2012 / Published online: 8 December 2012

Abstract The common and routine procedure for the quantification of chlorophyll *a* (chl *a*) in aquatic studies has a series of steps. Here, we sought to find optimal conditions for phytoplankton cell harvesting, chl *a* extraction, and chl *a* measurement and calculation, to find an effective, cost-saving, safe, and environment-friendly procedure for determining phytoplankton chl *a* concentration. We replaced the traditional GF/C filters with inorganic polymer flocculants (IPFs) and clay for phytoplankton harvesting, and then various solvents (acetone, ethanol, DMF, and DMSO), IPFs (PAC, PFS, and PAFS) and clay were tested for their suitability for chl *a* extraction, with or without homogenization at different temperatures for different extraction durations. About 0.3–1.0 g l^{-1} of PAC or PFSA combined with 1.0–2.5 g l^{-1} clay were found to provide optimal conditions in terms of yield and cost for phytoplankton cell harvesting from water samples. Based on our results, we recommend flocculation and centrifugation instead of glass-fiber membrane filtration for harvesting phytoplankton cells from environmental water samples, 95% ethanol for chl *a* extraction without homogenization and heating, and spectrophotometry to determine chl *a* concentration.

Keywords Chlorophyll *a* · Clay · Cyanobacteria · Extraction solvent · Flocculation · Spectrophotometry

Handling editor: Boping Han

H. Qin · S. Li · D. Li (✉)
Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, People's Republic of China
e-mail: lidh@ihb.ac.cn

H. Qin
e-mail: hongjieqin111@126.com

S. Li
e-mail: lishuangs2010@163.com

H. Qin · S. Li
Graduate University of Chinese Academy of Sciences, Beijing 100049, People's Republic of China

Introduction

The concentration of phytoplankton chlorophyll *a* (chl *a*) is widely used as an indicator of the quality of freshwater bodies (Pápista et al., 2002). Its determination usually includes three steps: algal cell harvesting, chl *a* extraction, and chl *a* measurement and calculation. Each of these steps introduces several sources of error and inaccuracy (Wasmund et al., 2006).

Algal cells are usually separated from the water by filtration. Glass-fiber filters are widely used because of large filtration capacity and flow rate (Aminot & Rey, 2001). Whatman GF/C and GF/F glass-fiber filters are used by most researchers. These filters are available with different pore sizes (0.7 or 0.45 μm) and diameters (25 or 47 mm). An appropriate filter pore size and a moderate suction pressure are required to obtain the desired, undisrupted fraction (Wasmund et al., 2006). Higher filtration pressures or excessively

long filtration times (>10 min) may damage the cells and result in loss of chl *a* (Arar, 1997). Humphrey & Jeffrey (1997) suggested that the residual pressure under the filter must be higher than 0.5 bar, whereas Aminot & Rey (2001) applied vacuum to maintain the pressure under 0.2 bar. Other researchers have recommended that residual pressure should be kept higher than 0.7 bar (Arar, 1997). However, no standard procedure for filtration has been designed to date. Filtration occasionally takes 2 h or much longer for environmental sample, especially for samples containing more particulate matter but less phytoplankton. In turbid coastal regions, filtration of one sample may take a long time and may require more than one filter (Aminot & Rey, 2001). In addition, ideal storage temperature and storage time of the sampled filtrates exist disputes (Wasmund et al., 2006).

Flocculation is an efficient and cost-effective method that is commonly used in various solid–liquid separation processes, such as chemical and biological wastewater treatment, water purification, and as a tailing treatment in the mining and oil industries (Farid et al., 2011; Piazza et al., 2011). A flocculant is a substance that causes the suspended particles in a fluid to aggregate and form discrete flocs (Piazza et al., 2011). Most flocculants are polymeric and are used to accelerate or improve the settling of suspended solids in various types of wastewater (Patil et al., 2011), such as during wastewater clarification (Maximova & Dahl, 2006). These flocculants are also used as filtration and centrifugation aids (Lewellyn & Avotins, 1988).

Several solvents have previously been explored for the extraction of pigments from phototrophic organisms (Castle et al., 2011). The most commonly used solvent, 80% acetone, was proposed by Arnon (1949) to determine the concentration of chl *a* in plant materials. Richards & Thompson (1952) applied acetone to phytoplankton and provided the first equation for spectroscopic quantification of chl *a* from algae. However, acetone can sometimes be a poor extractant for chl *a* in many vascular plants, algae, particularly green algae (Schumann et al., 2005), and cyanobacteria (Porra, 1991). Aside from acetone, other solvents that can be used in chl *a* extraction are ethanol, DMF (*N,N′*-dimethylformamide) and DMSO (dimethylsulfoxide) (Porra, 2002). However, the use of DMF and DMSO as extractants is not encouraged for safety reasons. The extraction efficiencies of different solvents have been shown to vary with water samples (Schumann et al., 2005), and even with algal taxa (Pápista et al., 2002). However, the efficiencies of these extraction solvents (acetone, ethanol, DMF, and DMSO) for the spectrophotometric analysis of chl *a* in bloom-forming cyanobacteria have not been formally assessed.

In this study, we used flocculation and centrifugation instead of glass-fiber membrane filtration to harvest algal cells. The first step for chl *a* determination should be effective, safe for the environment, cost-efficient, and relatively quick. We estimated the chl *a* extraction efficiencies of acetone, ethanol, DMF, and DMSO using both water samples from the field and axenic cultures. Chl *a* concentrations were determined by spectrophotometry and were calculated using various equations. The aim of our study was to develop a standard, efficient, economical, and time-saving protocol for the determination of chl *a* concentration in phytoplankton, especially in samples from eutrophic waters.

Materials and methods

Cyanobacterial cultures and water samples

Both water samples from the field and axenic cultures were used for chl *a* determination. Axenic cyanobacterial cultures included unicellular cyanobacterium *Microcystis aeruginosa* 905 and filamentous cyanobacterium *Anabaena* sp. PCC 7120. The two strains were provided by the Freshwater Algae Culture Collection of the Chinese Academy of Sciences. They were axenically cultured in BG11 liquid medium (Rippka et al., 1979). The cultures were subjected to a 12 h light:12 h dark cycle with a photosynthetically active radiation (PAR) intensity of 30 μmol photons s^{-1} m^{-2} provided by cool white fluorescent lamps at 25 ± 1°C. The cultured cyanobacteria were harvested during the exponential growth phase.

The water samples were collected from the eutrophic Lake Chaohu (in Anhui Province, China), where a clear succession in the phytoplankton community was observed. The phytoplankton was dominated by *Anabaena* in the spring, which was rapidly replaced by *Microcystis* in early summer. Two samples were collected from the surface of the lake in pelagic water in April 2011 and June 2011, and they were dominated by *Anabaena* (>92% total biomass) and *Microcystis*

(>95% total biomass), respectively. The phytoplankton in each sample was counted and identified according to the method described by Eker et al. (1999).

Clay, flocculants, and extraction solvents

Clay was collected from the shore of Lake Chaohu, air-dried to constant weight, and ground to fine powder using a mortar and pestle. The powder was then sieved using an 80-mesh screen and kept in a dry place until use. The three flocculants used for the flocculation experiment were polyaluminum chloride (PAC), polymeric ferric sulfate (PFS), and polyaluminum ferric silicate (PSFA) (Chucheng Biological Co. Ltd. Wuhan, China). Acetone, ethanol, DMF, and DMSO (Sinopharm Chemical Reagent Co. Ltd., Shanghai, China) were used as the extractants. The physical and chemical properties of the four extractants are enumerated in Table 1.

Cell harvest: filtration

The water samples were passed through Whatman GF/C filters (47 mm in diameter). For each subsample, the volume was first measured with a measuring cylinder, and then it was passed through a vacuum filtration apparatus with a suction pressure of less than 0.3 bar. The filter was removed from its placeholder with tweezers and folded once with the particulate matter inside (Arar, 1997), and then immediately used for the chl *a* extraction step. Otherwise, the container was wrapped with aluminum foil to protect the phytoplankton from light and stored at −80°C.

Cell harvest: flocculation

Flocculation was performed using the commercial inorganic polymer flocculants (IPFs) PAC, PFS, and PSFA. Water samples were placed into screw-capped centrifuge tubes (50 ml). Each of the IPFs was added to the respective tubes to final concentrations of 0, 0.05, 0.1, 0.3, 0.5, 1.0, 1.5, and 2.0 g l^{-1} (three tubes for each concentration). The floc in the tubes was manually mixed by shaking and was then left to stand for 30 and 60 min. Turbidity (NTU) of the supernatant was measured around 1 cm to 3 cm below the water surface using a UV–Visible spectrophotometer (TU-1810, Purkinje General Instrument Co. Ltd, Beijing, China) as described by Zou (2002).

We designed the "Flocculation and sedimentation" experiment based on the procedures outlined above. Screw-capped centrifuge tubes (50 ml) were filled with lake water samples, and then PAC or PSFA were added to a final concentration of 0.5 g l^{-1}. Clay was immediately added to these tubes at eight concentrations: 0, 0.2, 0.5, 1.0, 1.5, 2.5, 3.0, and 3.5 g l^{-1}. The tubes were quickly mixed by manually shaking and were left to stand for 30 min. The contents of tubes were then centrifuged for 10 min at either 2,000 or 4,000 rpm (4,800 or 9,600×*g*) with a Hitachi centrifuge (CR22GII, Tokyo, Japan). The NTU was measured as described in the previous subsection. Three replicates were analyzed for each treatment group.

Cell harvest: centrifugation

The laboratory cultures were harvested via centrifugation for 10 min at 10,000 rpm (24,000×*g*) with a Hitachi centrifuge. The pellets were resuspended in high-quality ultra-pure Millipore water, then centrifuged again. The supernatant was discarded and the pellets were used for chl *a* extraction with different solvents (acetone, ethanol, DMF, and DMSO).

Effect of homogenization on the extraction efficiency

Chl *a* was extracted with 90% acetone and 95% ethanol with or without grinding as follows: (1) the frozen GF/C filters (with cyanobacterial cells) were ground with a glass mortar and pestle filled with about

Table 1 The physical and chemical properties of solvents acetone, ethanol, DMF, and DMSO

Solvents	Melting point (°C)	Boiling point (°C)	Volatile	Flammable	Toxicity	Form
Acetone	−96.4	56.5	Easily	Highly	Lower	Water-like
Ethanol	−114.3	78.4	Easily	Highly	Lower	Water-like
DMF	−61	153	Hardly	No	Higher	Oil-like
DMSO	18.5	189	Hardly	No	Higher	Oil-like

5 ml of 90% acetone or 95% ethanol for several minutes in subdued lighting conditions. After grinding, the slurry was carefully transferred into a 15 ml stoppered and graduated centrifuge tube. The rinsing volumes were added to the centrifuge tube containing the filter slurry. The extract in the centrifuge tube was then added with the corresponding extractant to achieve a volume of exactly 10 ml. (2) Alternatively, the frozen GF/C filters (with cyanobacterial cells) were directly soaked in centrifuge tubes filled with about 10 ml of the respective solvents. The centrifuge tubes were then kept in the dark for 24 h at 4°C prior to extraction by grinding. Grinding of the filters by the second method was conducted in a fume hood as acetone and ethanol are both volatile (Arar, 1997).

Effect of heating on the extraction efficiency

Heating procedures were carried out with DMSO and DMF because these solvents are suitable for heating but not for grinding. Hot ethanol is an effective extractant; however, the heat treatment needs to be done in the dark and requires ethanol neutralized with $MgCO_3$. The procedures were divided into two sets with four treatments each as follows: for DMSO, the lake water samples on frozen GF/C filters were placed in screw-capped centrifuge tubes filled with 10 ml of DMSO and incubated in the dark at either 25 or 60°C for both of the two sets. Extraction times for DMSO were set at 15, 30, 60, 90, 120, 150, 180, and 240 min. For DMF, the lake water samples on frozen GF/C filters were placed in screw-capped centrifuge tubes filled with 10 ml of DMF and incubated in the dark at either 25 or 100°C for both of the two sets. The extraction times for DMF were set at 1, 2, 4, 6, 8, 15, and 30 min for the samples at 100°C, and at 10, 20, 30, 60, and 90 min for the samples at 25°C. The temperature was controlled using a water bath. After the extraction step, the heated samples were left to stand in the dark at room temperature for 30 min before further analysis.

Effect of flocculants and clay on extraction efficiency

Tests of the flocculants and clay were carried out using lake water samples and laboratory cultures. For lake water samples, 50 ml screw-capped centrifuge tubes were filled with 40 ml of lake water. For laboratory cultures, 15 ml centrifuge tubes were filled with 10 ml of the cultures. PAC or PSFA was added to each tube at a final concentration of 0.5 and 1.0 g l^{-1} clay was added immediately. The tubes were quickly mixed by manually shaking, allowed to stand for 30 min, then centrifuged for 10 min at 2,000 rpm (4,800×*g*). Lake samples without "Flocculation and sedimentation" treatment were used as controls and were filtered using Whatman GF/C filters. Similarly, 10 ml of cultures that were directly centrifuged for 10 min at 2,000 rpm (4,800×*g*) without any additional chemicals were also used as controls. For all harvested cells, chl *a* was extracted using four solvents (90% acetone, 95% ethanol, 100% DMF, and 100% DMSO). Three replicate samples were prepared for each treatment group.

Chlorophyll *a* measurement and calculation

Spectrophotometer with a 1-cm light path cuvette was used to measure and calculate chl *a* content, according to the methods described in Table 2. All extracts were centrifuged for 10 min at 10,000 rpm (24,000×*g*), and their respective supernatants were decanted into clean centrifuge tubes (to a constant volume of 10 ml), and transferred into the measuring cuvette of a UV–Visible spectrophotometer (TU-1810, Purkinje General Instrument Co. Ltd, Beijing, China). If the absorbance at 750 nm exceeded 0.005 AU for any sample, the sample was re-centrifuged prior to analysis (Arar, 1997). If the sample extract needed to be acidified, four drops of 0.1 mol l^{-1} HCl were added to the centrifuge tubes with 10 ml of the supernatant. The respective wavelengths of the extracts were measured again 3 min after acidification (Arar, 1997; Wasmund et al., 2006). To obtain scanning spectra, the absorption spectrum of the supernatant was obtained between 750 and 600 nm with a Beijing Purkinje TU-1810 UV–Visible spectrophotometer using a quartz cuvette in spectral scanning mode. The baseline of the spectrum had previously been set to zero. The parameters were set to medium-speed scanning in single-scanning mode with a 0.1 nm bandwidth interval (Jeffrey et al., 1999; Ritchie, 2008). Equations for chl *a*, *b*, *c*1, *c*2, and *d* are available for acetone and ethanol (Humphrey & Jeffrey, 1997; Ritchie, 2006), but equations are only available for chl *a* and *b* for DMF and DMSO. Hence, DMF and DMSO are therefore unsuitable in many situations.

Table 2 Chl *a* extraction conditions, calculation equations and references for four solvents

Extractants	Calculation and references	Temp. (°C)	Duration (h)	Acidification	Homogenization
90% Acetone					
Acetone-1	11.4062*A664* V_1/V_2 Ritchie (2006)	4	24	No	Yes
Acetone-2	(11.6*A665–1.31*A645–0.14*A630)*V_1/V_2 Parsons & Strickland (1963)	4	24	No	Yes
Acetone-3	[11.64*(A663–A750)–2.16*(A645–A750) + 0.10*(A630–A750)]*V_1/V_2 SEPB (2002)	4	24	No	Yes
Acetone-4	[27.3*(A_b665–A_b750)–(A_a665–A_a750)]*V_1/V_2 Yang et al. (2007)	4	24	Yes	Yes
95% Ethanol					
Ethanol-1	(13.7*A665–5.76*A649)*V_1/V_2 Wintermans & de Mots (1965)	4	24	No	No
Ethanol-2	[27.9*(A_b665–A_b750)–(A_a665–A_a750)]*V_1/V_2 Lan et al. (2009)	4	24	Yes	No
Ethanol-3	11.9035*A665*V_1/V_2 Ritchie (2006)	4	24	No	No
100% DMF					
DMF-1	(12.7*A664–2.79*A647)*V_1/V_2 William & Paul (1985)	25	1	No	No
DMF-2	(12.7*A663–2.35*A645)*V_1/V_2 Zhang (1990)	25	1	No	No
DMF-3	(12.00A664–3.11A647)*V_1/V_2 Porra (2002)	25	1	No	No
DMF-4	(11.85*A664–1.54*A647–0.08A*630)*V_1/V_2 Yu et al. (1995)	25	1	No	No
DMF-5	(12.65*A664–2.99*A647–0.04*A625)*V_1/V_2 Yang et al. (2007)	25	1	No	No
100% DMSO					
	(12.19*A665–3.45*A649)*V_1/V_2 Alan (1994)	25	3	No	No

For each equation, V_1, volume of solvent (ml); V_2, volume of sample (ml). *Subscripts* indicate absorbance before acid addition (A_b) and after acidification (A_a), respectively

Statistical analysis

All treatments were performed in triplicates. Data were statistically analyzed by performing one-way ANOVA followed by the least significant difference (LSD) post hoc test (SPSS, Chicago, IL, USA), at the 95% confidence level. All data are presented as mean ± standard deviation (SD).

Results

Flocculation efficiency of the three IPFs

PAC and PSFA have significantly better flocculation abilities than PFS (Table 3). The phytoplankton quickly flocculated from the water column after the IPFs were added. The optimal concentration ranges of PAC and PSFA were 0.1–0.5 and 0.1–1.0 g l^{-1}, respectively, for April *Anabaena*-dominated water sample, whereas the optimal ranges were 0.1–1.0 and 0.3–1.0 g l^{-1}, respectively, for June *Microcystis*-dominated water sample.

Flocculation and sedimentation method for cell harvesting

A relatively stable sedimentation effect of clay was observed between 1.0 and 2.5 g l^{-1} (Table 4). In addition, the sedimentation effect of centrifugation at 2,000 rpm was much better than that at 4,000 rpm. However, no significant difference in the sedimentation efficiency was observed when PAC or PFSA was used as the flocculant.

Table 3 The changes of NTU of water samples collected from Lake Chaohu after the three IPFs (PAC, PSFA, and PFS) were added

Sample	Flocculant	Time (min)	0 g l^{-1}	0.05 g l^{-1}	0.1 g l^{-1}	0.3 g l^{-1}	0.5 g l^{-1}	1.0 g l^{-1}	1.5 g l^{-1}	2.0 g l^{-1}
April	PAC	0	218 ± 15.3	218 ± 15.3	218 ± 15.3	218 ± 15.3	218 ± 15.3	218 ± 15.3	218 ± 15.3	218 ± 15.3
		30	169 ± 11.2	19 ± 1.3	18 ± 0.7	18 ± 1.2	17 ± 1.6	37 ± 0.9	224 ± 16.4	341 ± 32.9
		60	143 ± 15.3	12 ± 0.7	5 ± 0.2	5 ± 0.5	5 ± 0.7	25 ± 3.0	247 ± 11.3	329 ± 18.6
	PSFA	30	178 ± 12.4	16 ± 0.9	16 ± 1.4	15 ± 0.7	16 ± 1.1	17 ± 1.5	252 ± 18.2	462 ± 32.9
		60	139 ± 10.9	9 ± 0.5	4 ± 0.3	5 ± 0.5	5 ± 0.2	4 ± 0.3	290 ± 19.8	429 ± 35.7
	PFS	30	175 ± 13.2	158 ± 16.1	68 ± 7.2	62 ± 3.6	79 ± 5.9	137 ± 12.4	271 ± 23.5	364 ± 37.8
		60	148 ± 11.6	169 ± 13.5	43 ± 2.5	45 ± 3.3	65 ± 4.9	159 ± 12.7	216 ± 21.6	347 ± 35.7
June	PAC	0	144 ± 9.2	144 ± 9.2	144 ± 9.2	144 ± 9.2	144 ± 9.2	144 ± 9.2	144 ± 9.2	144 ± 9.2
		30	110 ± 8.7	52 ± 4.8	32 ± 4.1	14 ± 1.7	9 ± 0.7	63 ± 8.1	313 ± 15.3	342 ± 23.5
		60	97 ± 7.5	37 ± 4.2	28 ± 2.9	9 ± 1.0	3 ± 0.1	22 ± 1.1	284 ± 11.9	367 ± 19.8
	PSFA	30	118 ± 10.0	84 ± 7.1	53 ± 3.4	11 ± 0.9	10 ± 0.7	4 ± 0.3	512 ± 29.8	599 ± 33.6
		60	99 ± 8.5	65 ± 6.9	39 ± 2.3	7 ± 0.5	3 ± 0.4	2 ± 0.1	473 ± 29.7	568 ± 36.1
	PFS	30	115 ± 9.9	113 ± 6.7	95 ± 5.8	87 ± 8.9	98 ± 5.8	139 ± 9.4	382 ± 21.7	425 ± 28.6
		60	90 ± 5.9	87 ± 6.9	68 ± 5.7	48 ± 3.1	53 ± 3.3	125 ± 7.9	369 ± 21.8	467 ± 42.1

Effect of homogenization on extraction efficiency

Ninety percent acetone without homogenization had the lowest extraction efficiency (Fig. 1). For the April *Anabaena*-dominated sample, only 76.2% of the pigment (relative to homogenized extraction) was extracted with 90% acetone, whereas for the June *Microcystis*-dominated sample, 76.3% of the pigment was extracted. Homogenization with 90% acetone significantly ($P < 0.05$) improved the extraction efficiency from both samples, but not to the levels obtained with 95% ethanol. The extraction efficiencies of 95% ethanol with or without homogenization on both the April *Anabaena*-dominated and June *Microcystis*-dominated samples did not differ significantly ($P > 0.05$).

Effect of heating on extraction efficiency

The effect of heating on chl *a* extraction efficiency was tested using the April and June samples, which were extracted with 100% DMSO (Fig. 2). For the April *Anabaena*-dominated sample, the chl *a* extraction efficiency reached its maximum (1.12 mg l^{-1}) with 30 min incubation at 25°C. No significant differences in the extraction times were observed between 30 min and 180 min incubations. Meanwhile the extracted amount at 60°C incubation reached its maximum (1.10 mg l^{-1}) after 90 min, before a slightly declining trend was observed from 150 to 180 min. For the June *Microcystis*-dominated sample, the extraction yield reached its maximum after 180 min (0.86 mg l^{-1}) and after 120 min (0.86 mg l^{-1}) when incubated at 25 and

Table 4 The changes of NTU of water samples collected from Lake Chaohu when 0.5 g l^{-1} flocculants (PAC and PFSA) were added combining with different concentrations of clays

	0.5 g l^{-1}	Clay (rpm)	0 g l^{-1}	0.2 g l^{-1}	0.5 g l^{-1}	1.0 g l^{-1}	1.5 g l^{-1}	2.5 g l^{-1}	3.0 g l^{-1}	3.5 g l^{-1}
April	PAC	4,000	92 ± 7.4	21 ± 1.5	9 ± 0.6	5 ± 0.6	4 ± 0.1	5 ± 0.3	11 ± 0.4	15 ± 0.9
		2,000	87 ± 6.5	11 ± 0.6	4 ± 0.1	1 ± 0.0	1 ± 0.1	1 ± 0.1	10 ± 0.8	17 ± 0.7
	PFSA	4,000	85 ± 6.5	17 ± 1.1	6 ± 0.1	3 ± 0.3	3 ± 0.1	3 ± 0.0	9 ± 0.4	13 ± 0.5
		2,000	87 ± 5.9	7 ± 0.3	2 ± 0.1	1 ± 0.1	1 ± 0.0	1 ± 0.0	8 ± 0.2	15 ± 0.7
June	PAC	4,000	79 ± 6.7	26 ± 2.3	11 ± 0.8	9 ± 0.3	10 ± 1.1	11 ± 0.5	17 ± 0.8	23 ± 1.5
		2,000	92 ± 10.2	15 ± 0.8	7 ± 0.3	2 ± 0.0	1 ± 0.0	1 ± 0.1	11 ± 1.2	28 ± 2.1
	PFSA	4,000	84 ± 7.8	21 ± 1.6	8 ± 0.3	9 ± 0.6	11 ± 0.5	10 ± 0.7	18 ± 0.5	27 ± 1.8
		2,000	89 ± 6.8	9 ± 0.3	4 ± 0.1	1 ± 0.1	1 ± 0.0	1 ± 0.1	16 ± 1.7	31 ± 2.4

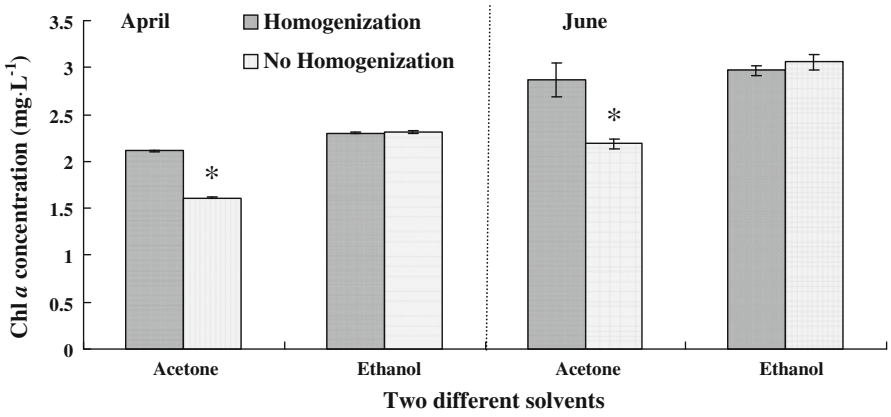

Fig. 1 The effect of homogenization on chl *a* extraction efficiency in cell harvesting from April and June samples using 90% acetone and 95% ethanol as solvents, respectively. Data are presented in mean ± SD with $n = 3$. The levels of significant differences between homogenized and non-homogenized samples were indicated by $^*P < 0.05$ (LSD)

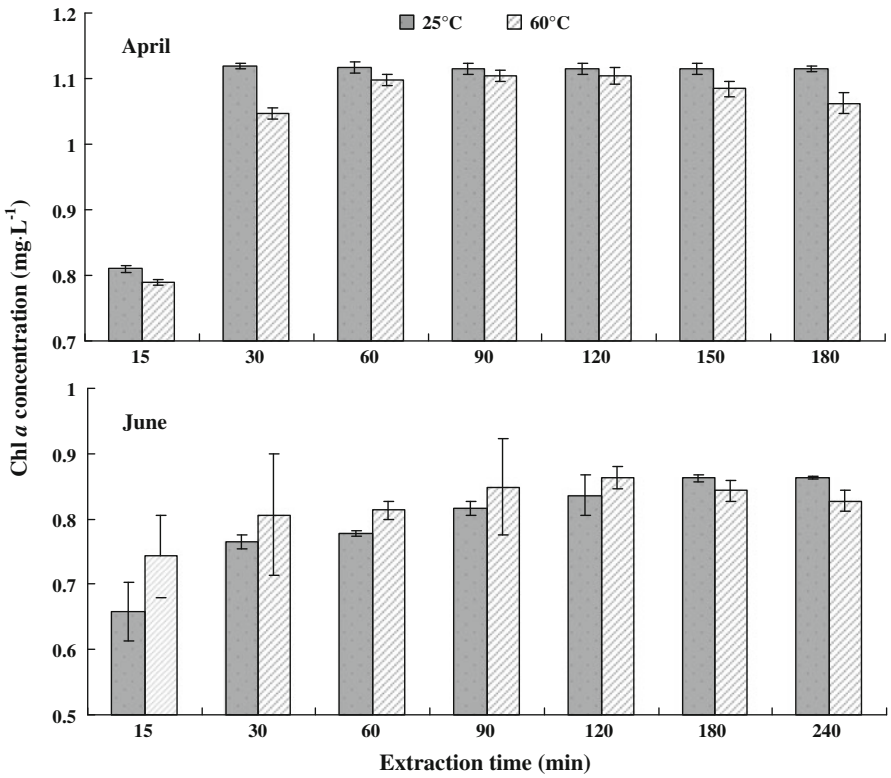

Fig. 2 The effect of heating on chl *a* extraction efficiency in cell harvesting from April and June samples using 100% DMSO as solvent. Data are presented in mean ± SD with $n = 3$

60°C, respectively. A similar trend was observed at 60°C, where the extraction yield decreased slightly from 80 to 240 min. These results clearly showed that incubation at 60°C was less preferable to that at 25°C when using 100% DMSO.

Efficiencies of chl *a* extraction in 100% DMF with incubation at 100 and 25°C were compared using April and June samples from Lake Chaohu (Fig. 3). For the April *Anabaena*-dominated sample, the chl *a* concentration reached its maximum (0.76 mg l^{-1}) after extraction for 2 min before it gradually decreased to 0.71 mg l^{-1} 30 min later. The extraction yield reached its maximum (0.76 mg l^{-1}) after 30 min of extraction at 25°C using DMF, and remained stable thereafter. For the June *Microcystis*-dominated sample, the optimum extraction time was 6 min at 100°C using DMF, and the yield declined when the extraction time was more than 6 min. The highest yield (0.79 mg l^{-1} chl *a*) was achieved after extraction for 30 min in 25°C using DMF, which was significantly

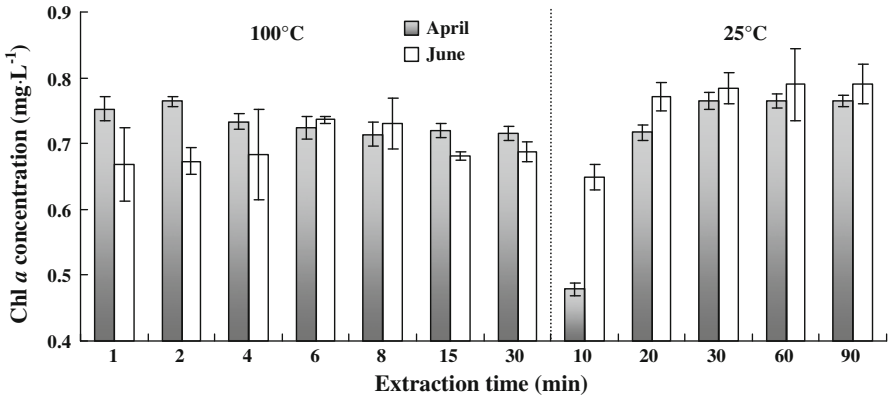

Fig. 3 The effect of heating on chl *a* extraction efficiency in cell harvesting from April and June samples using 100% DMF as solvent. Data are presented in mean ± SD with $n = 3$

higher than the maximum amount (0.73 mg l^{-1}) extracted at 100°C using DMF ($P < 0.05$). In addition, the chl *a* concentration after extraction at 25°C using DMF was constant for extraction times ranging between 30 and 90 min. These results indicated that extraction at 25°C was more suitable than that at 100°C in 100% DMF for either sample.

Chlorophyll *a* measurement and calculation using different methods and equations

For the *Anabaena* sp. PCC 7120 cultures, the average chl *a* concentration as measured by 13 methods was 2.34 mg l^{-1} (Fig. 4). 90% acetone produced the lowest concentration with an average value of only 1.874 mg l^{-1}, whereas 100% DMF produced the highest yield with an average value of 2.66 mg l^{-1}. The measured values of 95% ethanol extraction were very close to the average chl *a* concentration in all the 13 methods. For the *M. aeruginosa* 905 cultures, similar results as those of the *Anabaena* sp. PCC 7120 cultures were observed. However, the extracts with DMSO showed the largest variation between the two cultures. Moreover, our results indicated that the monochromatic equation overestimated chl *a* extracted from *M. aeruginosa* 905 in 90% acetone as compared with the trichromatic equation (Fig. 4; Table 2). The trichromatic equation (DMF-4) significantly underestimated

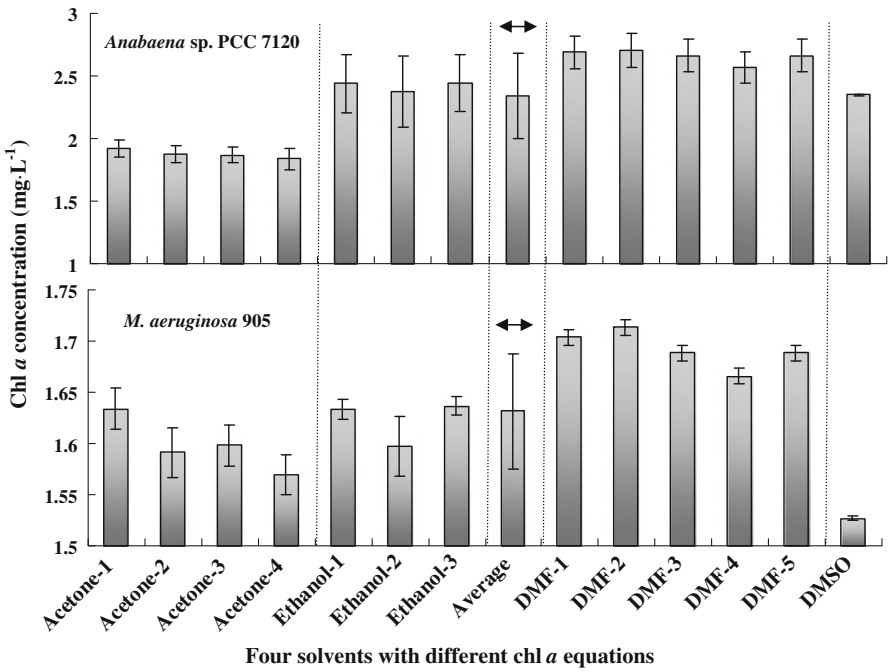

Fig. 4 Chl *a* extracted with laboratory cultures *Anabaena* sp. PCC 7120 and *M. aeruginosa* 905 using four different solvents. The extraction and calculation of chl *a* were based on the methods and equations presented in Table 2. Data are presented in mean ± SD with $n = 3$. *Long left right arrow* the average chl *a* concentration of the thirteen methods

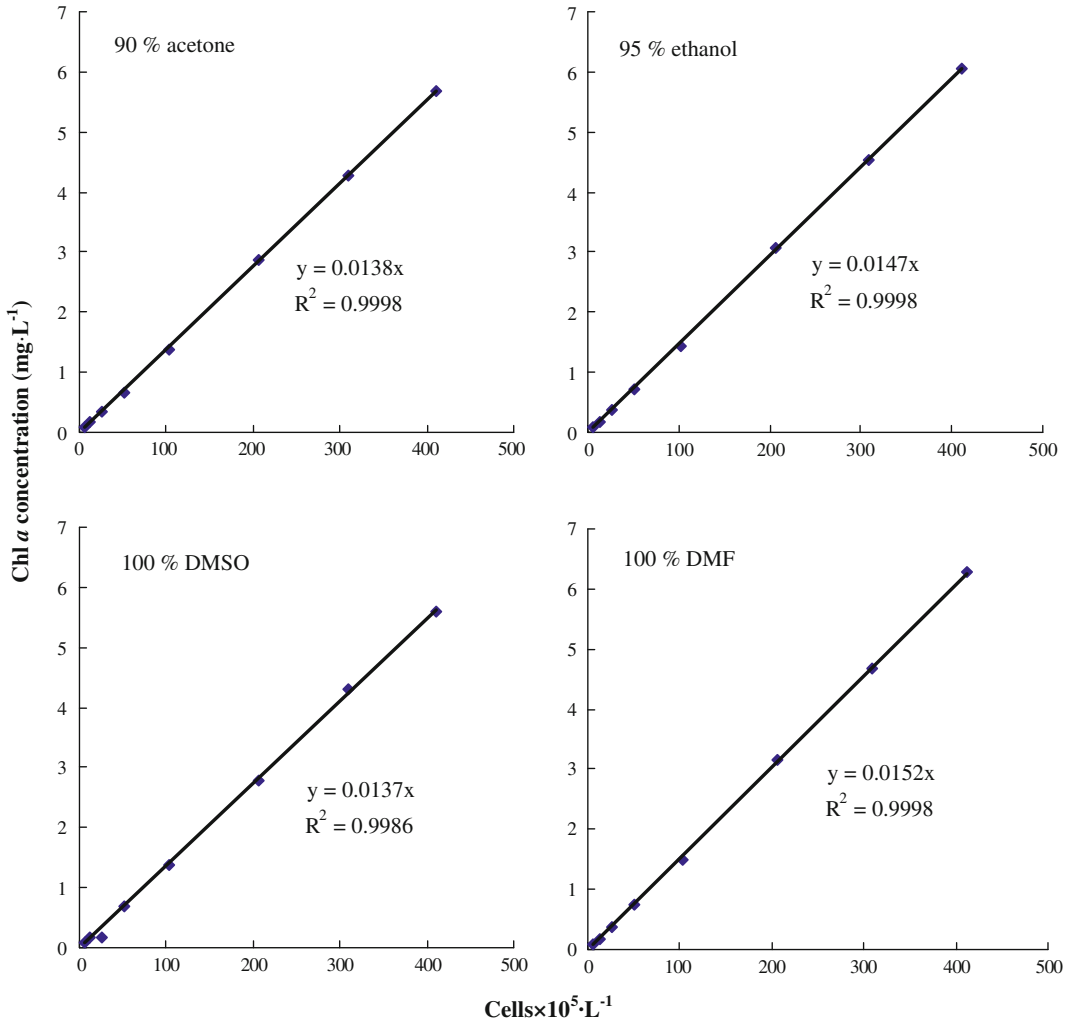

Fig. 5 Correlation analysis of chl *a* concentration with cell density. Chl *a* was extracted with *M. aeruginosa* 905 using four different solvents

the chl *a* content extracted from *M. aeruginosa* 905 in 90% acetone compared with the dichromatic equations (DMF-1 and DMF-2).

A significant positive correlation was observed between chl *a* concentration and cell density (Fig. 5). This correlation was relatively less significant for the extracts in 100% DMSO when compared with extracts using the other three solvents. According to the mathematical principle of the straight-fitting line equation $Y = kX$ ($k > 0$), a greater k value produces greater Y. The order of chl *a* yield using the four solvents was 100% DMF > 95% ethanol > 90% acetone > 100% DMSO.

The four solvents showed different chl *a* extraction efficiencies (Fig. 6). No statistically significant differences were observed between the flocculants with clay and the controls for the four solvents. Similarly, as shown in Fig. 4, the extraction efficiencies of chl *a* in 90% acetone and 100% DMSO were lower than those of the other two solvents.

The chl *a* extracts obtained by using the four solvents were scanned with a spectrophotometer. Chl *a* peaks were at 663.4, 664.6, 664.1, and 665.6 nm in 90% acetone, 95% ethanol, 100% DMF, and 100% DMSO, respectively, and their respective absorbance values were 0.560, 0.525, 0.549, and 0.511 (Fig. 7).

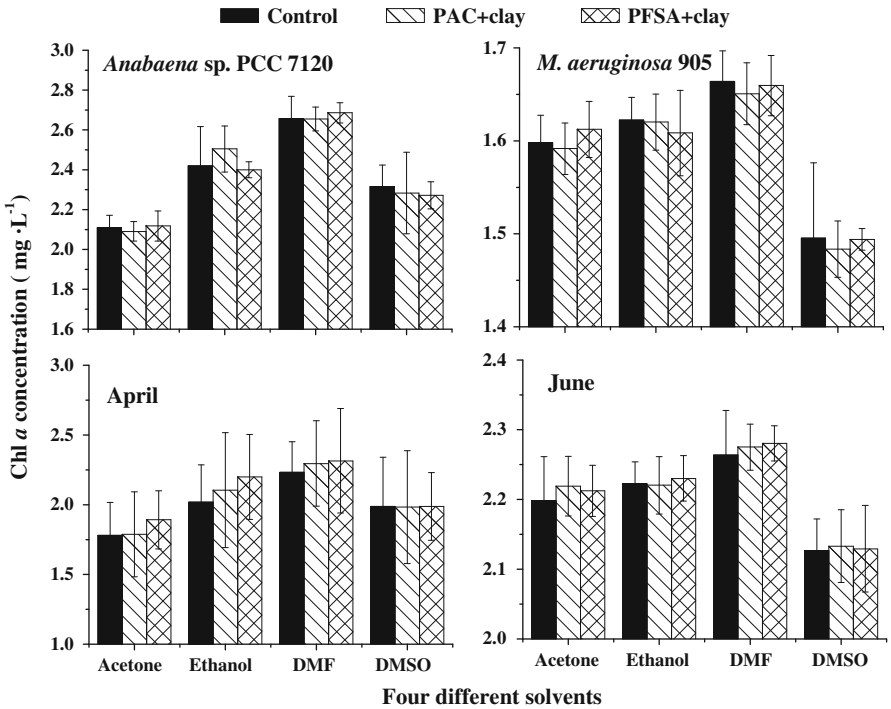

Fig. 6 The effect of flocculants (PAC and PFSA) and clay on extraction efficiency of chl *a* in laboratory cultures (*Anabaena* sp. PCC 7120 and *M. aeruginosa* 905) and water samples from Lake Chaohu (April-*Anabaena* sp. and June-*Microcystis* sp.) using four different solvents. Data are presented in mean ± SD with $n = 3$

Thus, the peaks had shifted conspicuously in the four solvents. The scanning spectra of the PAC + clay and PFSA + clay groups were very similar to that of their controls, as shown in Fig. 8, thus indicating that these additives had no significant effects on the waveform and peak of chl *a* extracts in the four solvents.

Discussion

In this study, we examined whether flocculation with IPFs can substitute filtration with glass-fiber filters for efficient water-algae separation. Among the IPFs, PAC, PSFA, and PFS are the most efficient and widely applied flocculants (Lu et al., 2009; Wang et al., 2011). PAC and PFSA exhibited perfect separation efficiency, while PFS was not found to be suitable for flocculation in our studies.

Flocculant dosage is an important parameter for the optimal destabilization of colloidal suspensions as excess use of the polymer represents extra costs (Daniel et al., 2011). If the flocculant dosage is quite low, it would not be able to induce rapid flocculation (Ferretti et al., 2003). In contrast, an excess of flocculant could disperse the colloidal suspension by charge inversion or steric stabilization (Ferretti et al., 2003; Daniel et al., 2011). The flocculants PAC and PFSA used in our study had lower flocculation efficiency when their concentrations were either lower than 0.3 g l^{-1} or exceeded 1.5 g l^{-1}. After flocculation, the floc that floated on the water column was in a bulk state and floated to the water surface even after centrifugation. This can be attributed to the biological characteristics of planktonic cyanobacterial cells (Liu et al., 2010), which possess gas vesicles that cause them to float to the water surface in calm water at the appropriate temperature (Ingrid & Jamie, 1999). Therefore, it is difficult to completely separate cyanobacterial cells from the water column by relying only on PAC or PFSA.

Clay has been shown to act as useful ballast that causes deposition of the flocculation complex (Lee et al., 2008; Wang et al., 2012). In our study, clay exhibited robust effects on sedimentation of flocs within the optimal range from 1.0 to 2.5 g l^{-1} (Table 4). An insufficient amount of clay cannot provide enough ballast to hold down the floc. However, excess clay may affect the chl *a* extraction volume at later stages of extraction. For efficiency and cost-saving purposes, 0.3–1.0 g l^{-1} of the flocculant (PAC or PFSA) and 1.0 g l^{-1} clay were found to be

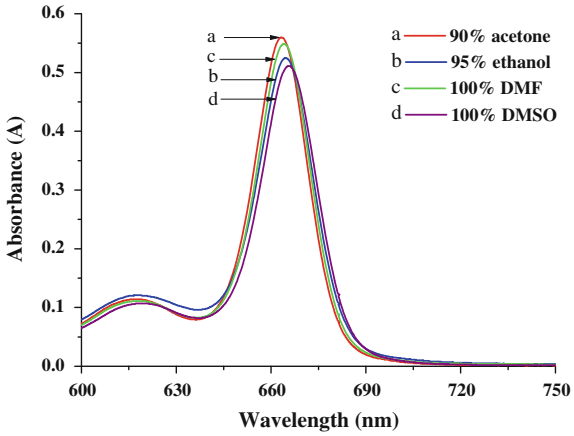

Fig. 7 Absorption spectra of chl *a* extracted from *M. aeruginosa* 905 using solvents 90% acetone, 95% ethanol, 100% DMF, and 100% DMSO, respectively

the optimum concentration levels to achieve robust flocculation and sedimentation efficiency for cells harvested from water samples.

Homogenization leads to improved extraction efficiency as a result of cell wall disruption (Wasmund et al., 2006). Some algal groups, especially cyanobacteria and chlorophyceae, are rather resistant to extraction and may require mechanical disruption (Holm-Hansen & Riemann, 1978). In our study, we found that homogenization (grinding) did significantly improve chl *a* extraction efficiency in 90% acetone ($P < 0.05$). However, there was no significant difference between homogenized and non-homogenized samples if they were extracted in 95% ethanol ($P > 0.05$). Sartory & Grobbelaar (1984) reported that homogenization significantly improved extraction efficiency from *Selenastrum capricornutum* but not to the extent achieved with 95% ethanol. Lorenzen (1967) strongly recommended grinding the filters if acetone was used as a solvent. In addition, homogenization introduces additional errors and variability because of the additional exposure of the extracts to light, heat, and irreproducible manipulation (Sartory & Grobbelaar, 1984; Wasmund et al., 2006). At the very least, homogenization can lead to evaporation, which needs to be compensated by replenishing the solvent or some other form of correction (Arar, 1997). Therefore, ethanol is a better extractant than acetone for most purposes.

Heating may increase the extraction efficiency of chl *a*, but heat treatment destroys the enzyme chlorophyllase (Bacon & Holden, 1967), which converts chlorophyll to chlorophyllide (Sartory & Grobbelaar, 1984). Pan et al. (2001) proved that boiling at 100°C was optimal for chl *a* extraction with DMF from *Chlorella ellipsoidea* and *Scenesmus obliquus*. Wang et al. (2009) extracted chl *a* from several plants with DMSO at 60°C. The hot ethanol extraction method has been used for chl *a* extraction in eutrophic water (Chen et al., 2006), but Sartory & Grobbelaar (1984) concluded that hot ethanol had poor extraction efficiency. In this study, the extraction efficiencies of

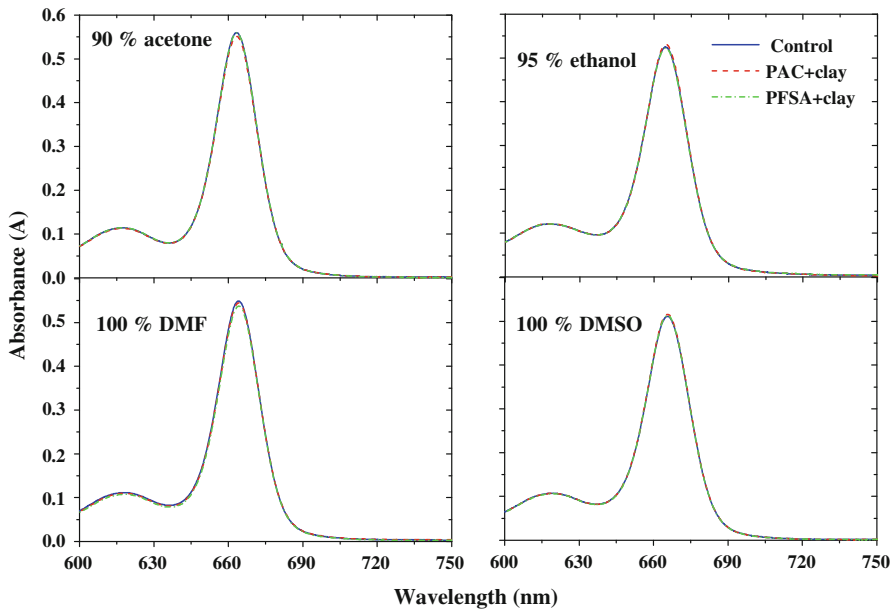

Fig. 8 The effect of PAC + clay and PFSA + clay on the scanning spectra of chl *a* extracted from laboratory culture *M. aeruginosa* 905 using four different solvents

DMSO and DMF were compared at incubation temperatures ranging between 25 and 60°C (Fig. 3) and between 25 and 100°C (Fig. 4), respectively. Chl *a* concentration showed a slightly decreasing trend with increase in temperature. Our results indicated that higher extraction efficiency and a more stable chl *a* concentration could be obtained at 25°C compared to those obtained by extraction with heating. The main reason for this trend could be the accelerated degradation of chl *a* at high temperatures (Wasmund, 1984). Sartory & Grobbelaar (1984) also pointed out that boiling in water bath at 100°C eventually resulted in the formation of phaeophytin (within 30 s in 95% ethanol and within 2 min in absolute methanol). Riemann (1976) also reported observing degradation of chlorophyll when methanolic extracts were boiled at 100°C for more than 3 min.

A large variety of methods for determining chl *a* concentrations are available in hydrobiology (Pápista et al., 2002). The main difference among these methods is the choice of the extracting solvent (Castle et al., 2011). In this study, we compared the efficiency of multiple extraction solvents (acetone, ethanol, DMF, and DMSO) for the spectrophotometric analysis of chl *a* extracted from different forms of cyanobacteria.

Since acetone has very sharp chl *a* absorption peaks, it is the solvent of choice for chl *a* assays (Arnon, 1949; Porra, 2002). However, it is also a poor extractant for chl *a* from many vascular plants and several algae, particularly cyanobacteria and green algae (Schumann et al., 2005). Although 90% acetone is most widely used as an extractant, it can cause underestimation of chl *a* concentration (Furuya et al., 1998). This is an important point for consideration in extraction by soaking, as even the mechanical treatments of samples, such as homogenization, contributes to loss of materials (Wright et al., 1997). The data presented in this study clearly indicate that acetone is an unsuitable solvent for cyanobacteria because of its low efficiency of chl *a* extraction and its homogenization requirement. In addition, acetone is very volatile and highly flammable, and it can cause headaches, acts as a narcotic in high concentrations, and is a skin irritant (erythema) (Ritchie, 2008). The widespread use of plastic laboratory-ware also leads to difficulties because acetone attacks polystyrene and polymethacrylates (Ritchie, 2006).

Previous studies have noted that ethanol is an efficient extractant (Wright et al., 1997). We found extraction efficiency to be much better in 95% ethanol than in 90% acetone. Moreover, extraction in the latter solvent requires the filters to be homogenized. Moreover, although ethanol is flammable, it is not very toxic and is suitable for use in a laboratory (Castle et al., 2011). Ethanol does not attack polystyrene; thus polystyrene plastic cuvettes and laboratory plastic wares can be used during extraction (Ritchie, 2006). Ethanol is also much easier to transport and easier to handle in the field compared with acetone. Thus, use of ethanol as solvent for chl *a* extraction and assay is not only safe but also considerable practical, safe, and economic advantages.

Among the solvents used for chl *a* extraction, DMF is known for its extraction ability and efficiency across various taxonomic groups (Furuya et al., 1998; Schumann et al., 2005). The main advantages of using DMF include its non-volatility, higher extraction efficiency, rapid extraction time (tens of minutes), and the lack of heating or grinding steps (Suzuki & Ishimaru, 1990). Among the methods we tested for chl *a* extraction from cyanobacteria, use of DMF resulted in the highest concentration of chl *a* from all samples and only required 30 min of incubation at room temperature. The same conclusion was reported by Suzuki & Ishimaru (1990) and Schumann et al. (2005). The only disadvantage is that DMF may cause liver problems when it is absorbed through the skin. Thus, disposable latex gloves should always be used for protection while handling DMF (Suzuki & Ishimaru, 1990).

DMSO has been used previously for extracting chl *a* from biological soil crusts (Castle et al., 2011), lichens (Ronen & Galun, 1984), and tree leaves (Alan, 1994). In our tests, among the four solvents tested for chl *a* extraction from *M. aeruginosa* 905, DMSO gave the lowest yield in all tested extraction methods. In addition, DMSO showed the poorest correlation between chl *a* concentration and cell density among the four solvents. Moreover, DMSO solidified into a crystal at lower temperatures because of its high melting point of 18.5°C. Therefore, it was not suitable for use at temperatures below 18.5°C. In addition, DMSO is more toxic than acetone and ethanol (Porra, 2002). Significant health concerns that are associated with routine use of DMSO exist and should be taken into consideration (Castle et al., 2011).

We used three kinds of equations for spectrophotometric analysis of chl *a* in this study: trichromatic, dichromatic, and monochromatic equations. It has been reported that a trichromatic equation

overestimates chl *a* concentration compared with a monochromatic equation due to the presence of pheopigments (Aminot & Rey, 2001). However, our results indicate that no significant differences exist between the three kinds of equations when computing chl *a* concentration in the *Anabaena* sp. PCC 7120 cultures. Several differences in *M. aeruginosa* 905 cultures were seen, but no consistent trend was observed for the trichromatic, dichromatic, and monochromatic equations.

Conclusion

We used IPFs as flocculants and clay as a precipitant to replace glass-fiber filters for algal cell harvesting. The results indicated that different combinations of the flocculant and clay concentrations had no negative impacts on chl *a* concentration and their scanning spectra. Another advantage of using IPFs and clay is that they are cheaper. PAC and PFSA cost 2 RMB and 3.2 RMB per kilogram, respectively, whereas Whatman GF/C filters (47 mm in diameter) cost 2 RMB per piece. The extraction procedure requires only about 1 g PAC or PFSA but at least one piece of GF/C filter is needed for one sub-sample. For solvents, 95% ethanol was found to be optimal for chl *a* assay for its low safety risk and convenience, whereas 100% DMF was deemed unsuitable owing to potential hazards to human health and the environment even though it is a highly efficient extractant. DMSO was the worst extractant and gave the worst overall results. Therefore, we recommend a combination of flocculation and centrifugation instead of glass-fiber membrane filtration to harvest phytoplankton cells from water samples, using 95% ethanol as solvent for chl *a* extraction without homogenization and heating, and spectrophotometric determination of chl *a* concentration in the extracts.

Acknowledgments We thank anonymous reviewers for manuscript revising and good suggestions. We thank Jason Moore from the EasyStar Company for their excellent English language editing. This research was financially supported by National Basic Research and Development Plan of China: "Occurrence mechanism of cyanobacterial blooms in large shallow lakes" (2008CB418002).

References

Alan, R. W., 1994. The spectral determination of chlorophyll-*a* and chlorophyll-*b*, as well as total carotenoids, using various solvents with spectrophotometers of different resolution. Journal of Plant Physiology 144: 307–313.

Aminot, A. & F. Rey, 2001. Chlorophyll *a*: determination by spectroscopic methods. ICES Techniques in Marine Environmental Sciences: 18 pp.

Arar, E. J., 1997. In vitro determination of chlorophylls *a*, *b*, c_1+c_2 and pheopigments in marine and freshwater algae by visible spectrophotometry. EPA (EPA.metod 446.0).

Arnon, D. I., 1949. Copper enzymes in isolated chloroplasts—polyphenoloxidase in Beta-vulgaris. Plant Physiology 24: 1–15.

Bacon, M. F. & M. Holden, 1967. Changes in chlorophylls resulting from various chemical and physical treatments of leaves and leaf extracts. Phytochemistry 6: 193–210.

Castle, S. C., C. D. Morrison & N. N. Barger, 2011. Extraction of chlorophyll a from biological soil crusts: a comparison of solvents for spectrophotometric determination. Soil Biology & Biochemistry 43: 853–856.

Chen, Y. W., K. N. Chen & Y. H. Hu, 2006. Discussion on possible error for phytoplankton chlorophyll-*a* concentration analysis using hot-ethanol extraction method. Journal of Lake Sciences 18: 550–552 (in Chinese).

Daniel, P., D. Hunkeler & S. Stoll, 2011. Comparison of two cationic polymeric flocculant architectures on the destabilization of negatively charged latex suspensions. Polymer 52: 1019–1026.

Eker, E., L. Georgieva, L. Senichkina & A. E. Kideys, 1999. Phytoplankton distribution in the western and eastern Black sea in spring and autumn 1995. ICES Journal of Marine Science 56: 15–22.

Farid, V. G., R. S. Sanders & J. H. Masliyah, 2011. Flocculation kinetics and aggregate structure of kaolinite mixtures in laminar tube flow. Journal of Colloid and Interface Science 355: 96–105.

Ferretti, R., S. Stoll, J. Zhang & J. Buffle, 2003. Flocculation of hematite particles by a comparatively large rigid polysaccharide: schizophyllan. Journal of Colloid and Interface Science 266: 328–338.

Furuya, K., M. Hayashi & Y. Yabushita, 1998. HPLC determination of phytoplankton pigments using *N,N*-dimethylformamide. Journal of Oceanography 54: 199–203.

Holm-Hansen, O. & B. Riemann, 1978. Chlorophyll *a* determination: improvements in methodology. Oikos 30: 438–447.

Humphrey, G. F. & S. W. Jeffrey, 1997. Test of accuracy of spectrophotometric equations for the simultaneous determination of chlorophylls *a*, *b*, *c*1 and *c*2. In Jeffrey, S. W., R. F. C. Mantoura & S. W. Wright (eds), Phytoplankton pigments in oceanography: guidelines to modern methods. UNESCO Publ., Paris: 616–621.

Ingrid, C. & B. Jamie, 1999. Toxic cyanobacteria in water: a guide to their public health consequences monitoring and management. WHO, London.

Jeffrey, S. W., H. S. MacTavish, W. C. Dunlap, M. Vesk & K. Groenewpud, 1999. Occurrence of UVA- and UVB-absorbing compounds in 152 species (206 strains) of marine microalgae. Marine Ecology Progress Series 89: 35–51.

Lan, S. B., Y. D. Liu & C. X. Hu, 2009. Comparison of contents of chlorophyll-*a* extracted by different organic solvents in biological crusts. Journal of Desert Research 29: 524–528 (in Chinese).

Lee, Y. J., J. K. Choi, E. K. Kim, S. H. Youn & E. J. Yang, 2008. Field experiments on mitigation of harmful algal blooms using a sophorolipidd—yellow clay mixture and effects on marine plankton. Harmful Algae 7: 154–162.

Lewellyn, M. E. & P. V. Avotins, 1988. Dewatering/filtering aids. In Somasundaran, P. & B. M. Moudgil (eds), Surfactant Science Series, Reagents in Mineral Technology, Vol. 27. Marcel Dekker, Inc., New York and Basel: 559–578.

Liu, G. F., C. X. Fan, J. C. Zhong, L. Zhang, S. M. Ding, S. H. Yan & S. Q. Han, 2010. Using hexadecyl trimethyl ammonium bromide (CTAB) modified clays to clean the *Microcystis aeruginosa* blooms in Lake Taihu, China. Harmful Algae 9: 413–418.

Lorenzen, C. J., 1967. Determination of chlorophyll and pheopigments: spectrophotometric equations. Limnology and Oceanography 12: 343–346.

Lu, X. J., M. J. Zhang & L. Han, 2009. An application of composite coagulants to the treatment of reactive dyes wastewater. Shanghai Environmental Sciences 28: 87–89 (in Chinese).

Maximova, N. & O. Dahl, 2006. Environmental implications of aggregation phenomena: current understanding. Current Opinion in Colloid & Interface Science 11: 246–266.

Pan, X., J. H. Li, Y. T. Hao, L. H. Min & Y. X. Ma, 2001. An improved method for chlorophyll *a* extraction in microalgal with DMF. Biotechnology 11: 39–41 (in Chinese).

Pápista, E., E. Ács & B. Böddi, 2002. Chlorophyll-*a* determination with ethanol—a critical test. Hydrobiologia 485: 191–198.

Parsons, T. R. & J. D. H. Strickland, 1963. Discussion of spectrophotometric determination of marine-plant pigments, with revised equations for ascertaining chlorophylls and carotenoids. Journal of Marine Research 21: 155–163.

Patil, S. V., C. D. Patil, B. K. Salunke, R. B. Salunkhe, G. A. Bathe & D. M. Patil, 2011. Studies on characterization of bioflocculant exopolysaccharide of *Azotobacter indicus* and its potential for wastewater treatment. Applied Biochemistry and Biotechnology 63: 463–472.

Piazza, G. J., A. J. McAloon & R. A. Garcia, 2011. A renewable flocculant from a poultry slaughterhouse waste and preliminary estimate of production costs. Resources, Conservation and Recycling 55: 842–848.

Porra, R. J., 1991. Recent advances and re-assessments in chlorophyll extraction and assay procedures for terrestrial, aquatic, and marine organisms, including recalcitrant algae. In Scheer, H. (ed.), Chlorophylls. CRC Press, Boca Raton: 31–57.

Porra, R. J., 2002. The chequered history of the development and use of simultaneous equations for the accurate determination of chlorophylls *a* and *b*. Photosynthesis Research 73: 149–156.

Richards, F. A. & T. G. Thompson, 1952. The estimation and characterisation of plankton populations by pigment analysis. II. A spectrophotometric method for the estimation of plankton pigments. Journal of Marine Research 11: 156–172.

Riemann, B., 1976. Studies on the biomass of the phytoplankton. Ph.D. Thesis. Reports from the Botanical Institute, University of Aarhus, Aarhus: 186 pp.

Rippka, R., J. Desrulles, J. B. Waterbury, M. Herdman & R. Y. Stanier, 1979. Generic assignment, strain histories and properties of pure cultures of cyanobacteria. Journal of General Microbiology 11: 1–61.

Ritchie, R. J., 2006. Consistent sets of spectrophotometric chlorophyll equations for acetone, methanol and ethanol solvents. Photosynthesis Research 89: 27–41.

Ritchie, R. J., 2008. Universal chlorophyll equations for estimating chlorophylls *a*, *b*, *c*, and *d* and total chlorophylls in natural assemblages of photosynthetic organisms using acetone, methanol, or ethanol solvents. Photosynthetica 46: 115–126.

Ronen, R. & M. Galun, 1984. Pigment extraction from lichens with dimethyl sulfoxide (DMSO) and estimation of chlorophyll degradation. Environmental and Experimental Botany 24: 239–245.

Sartory, D. P. & U. G. Grobbelaar, 1984. Extraction of chlorophyll *a* from freshwater phytoplankton for spectrophotometric analysis. Hydrobiologia 114: 177–187.

Schumann, R., N. Häubner, S. Klausch & U. Karsten, 2005. Chlorophyll extraction methods for the quantification of green microalgae colonizing building facades. International Biodeterioration and Biodegradation 55: 213–222.

State Environmental Protection Bureau (SEPB), 2002. Methods of monitoring and analysis for water and wastewater, 4th edn. China Environmental Science Press, Beijing.

Suzuki, R. & T. Ishimaru, 1990. An Improved method for the determination of phytoplankton chlorophyll using *N,N*-dimethylformamide. Journal of Oceanography Society of Japan 46: 190–194.

Wang, D. S., S. F. Wang, C. P. Huang & C. W. K. Chow, 2011. Hydrolyzed Al(III) clusters: speciation stability of nano-Al_{13}. Journal of Environmental Sciences 23: 705–710.

Wang, W. J., H. S. He, Y. Guan, W. X. Li, Z. H. Zhang & Y. H. Zu, 2009. Methodological comparison of chlorophyll and carotenoids contents of plant species measured by DMSO and acetone-extraction methods. Bulletin of Botanical Research 29: 224–229 (in Chinese).

Wang, Z. C., D. H. Li, H. J. Qin & Y. X. Li, 2012. An integrated method for removal of harmful cyanobacterial blooms in eutrophic lakes. Environmental Pollution 160: 34–41.

Wasmund, N., 1984. Probleme der spektrophotometrischen Chlorophyllbestimmung. Acta Hydrochimica et Hydrobiologia 12: 255–272.

Wasmund, N., I. Topp & D. Schories, 2006. Optimising the storage and extraction of chlorophyll samples. Oceanologia 48: 125–144.

William, P. I. & R. B. Paul, 1985. Extinction coefficients of Chlorophyll *a* and *b* in *N,N*-dimethylformamide and 80% acetone. Plant Physiology 77: 483–485.

Wintermans, J. F. G. M. & A. de Mots, 1965. Spectrophotometric characteristics of chlorophylls *a* and *b* and their pheophytins in ethanol. Biochimica et Biophysica Acta 109: 448–453.

Wright, S. W., S. W. Jeffrey & F. R. C. Mantoura, 1997. Evaluation of methods and solvents for pigment analysis. In Jeffrey, S. W., R. F. C. Mantoura & S. W. Wright (eds), Phytoplankton pigments in oceanography: guidelines to modern methods. UNESCO Publ., Paris: 261–282.

Yang, C., X. Song & B. Sun, 2007. Comparison of simple methods for extraction and measurement of phytoplanktonic chlorophyll-*a*. Marine Sciences 31: 6–9.

Yu, Z. G., J. Zhang, Y. H. Zhang & S. M. Liu, 1995. A new method for determination of chlorophyll *a* concentration in macroalgae. Experiments and Techniques 5: 1–2.

Zhang, Z. L., 1990. Guidance of plant physiology experiments guidance of plant physiology experiments, 2nd ed. Higher Education Press, Beijing.

Zou, Y. H., 2002. Algae removal from eurtrophic waters. Wuhan University of Science and Technology. Master of Science text paper, p 25 (in Chinese).