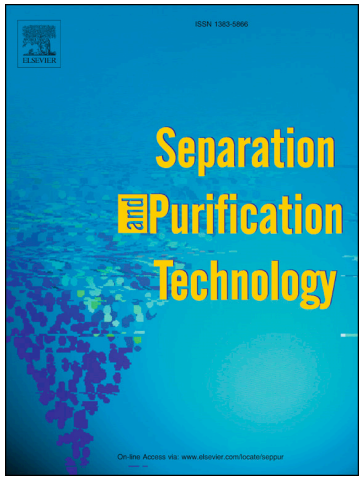

Impact of harvesting method on total lipid content and extraction efficiency for *Phaeodactylum tricornutum*

Dries Vandamme, Lore Gheysen, Koenraad Muylaert, Imogen Foubert

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Impact of harvesting method on total lipid content and extraction efficiency for *Phaeodactylum tricornutum*

Dries Vandamme[1*], Lore Gheysen[2,3], Koenraad Muylaert[1], Imogen Foubert[2,3]

[1]KU Leuven Kulak, Laboratory for Aquatic Biology, E. Sabbelaan 53, 8500 Kortrijk, Belgium

[2]KU Leuven Kulak, Research Unit Food & Lipids, Department of Molecular and Microbial Systems Kulak, Etienne Sabbelaan 53, B-8500 Kortrijk, Belgium

[3]Leuven Food Science and Nutrition Research Centre (LFoRCe), KU Leuven, Kasteelpark Arenberg 20, B-3001 Heverlee, Belgium

[*]Corresponding author: Email: Dries.Vandamme@kuleuven.be

Tel: +32 56 246041

Fax: +32 56 246999

Abstract

Flocculation is a promising low-cost alternative to centrifugation for harvesting microalgae for bulk lipid production. However, little is known about the effect of the harvesting method on the lipid content on the one hand and the lipid extraction efficiency on the other hand. In this study, both total lipid content (and fatty acid composition and free fatty acid amount) and lipid extraction efficiency of *Phaeodactylum tricornutum* biomass were compared after harvesting using either alum or alkaline flocculation, or centrifugation. Alum and alkaline flocculation did not severely impact total lipid content when expressing results on ash-free dry weight basis to account for the salts transferred to the biomass during flocculation. The fatty acid composition does not change substantially and alum nor alkaline flocculation had any effect on the extraction efficiency when using a commercial solvent system. This study demonstrates that alkaline flocculation can be an excellent primary harvesting method for *Phaeodactylum tricornutum* without impacting the lipid extraction efficiency.

Keywords: flocculation, dewatering, microalgae, biofuels, FAME, lipid extraction

Introduction

Microalgae are seen as a promising new source of biomass that can serve as a feedstock in various new production processes for food, feed, biofuels, or chemicals integrated in novel biorefinery concepts [1–3]. Amongst others, *Phaeodactylum tricornutum* is a microalgae species that accumulates valuable pigments, triacylglycerols, and omega-3 long-chain polyunsaturated fatty acids (omega-3 LC-PUFA) such as eicosapentaenoic acid (EPA; C20:5) [4–6].

Production of microalgae on a commodity scale is however still hampered by high cost and energy investment, so that solely high-value products such as food supplements, pharmaceuticals or fine chemicals can be commercialized at limited scale [7–9]. The high financial and energetic requirements of microalgal biomass production are to a large extent due to the cost of harvesting. Since biomass densities are generally very low, >90% water needs to be removed during a harvesting process [10,11]. Flocculation could reduce cost and energy inputs via a first dewatering of the biomass by simple gravity sedimentation [12,13]. Consequently, the required water removal would be significantly reduced in a secondary dewatering step using for example centrifugation [14].

Metal salts such as aluminum sulfate (alum) and ferric chloride are widely used flocculants in (waste)water treatment [15]. Dissolved aluminum or ferric ions form positively charged metal hydroxides that efficiently cause flocculation via charge neutralization or sweeping flocculation, depending on pH and dose [16]. Biomass recovered by flocculation with these metal salts can however have a certain amount of flocculant irreversibly bound to it, which can be a limitation for certain biomass application [17]. Recently, alkaline flocculation mediated by calcite and brucite precipitation at pH 9.5-11 has been described as an interesting flocculation option for several species including *Phaeodactylum tricornutum*

[18–22]. Alkaline flocculation is triggered by the coordination of those calcium and magnesium mineral precipitates to anionic algal carboxylate groups [23]. Vandamme et al. additionally demonstrated that magnesium precipitated as brucite caused flocculation of *Phaeodactylum tricornutum* by charge neutralization, while calcium precipitated as calcite caused flocculation dominantly by sweeping flocculation [21]. The only cost involved is that of the base used to increase pH as background concentrations of calcium and magnesium are high enough in most waters for flocculation to occur [12,24]. Moreover, it has been demonstrated that calcium and magnesium could be recovered to be reused [25].

The application of flocculation methods should however not interfere with downstream processes, such as the extraction, nor alter the biomass composition or stability. Harvesting is thus a crucial process step that needs to be studied and designed carefully using an integrated approach, not solely by evaluating harvesting efficiency, but also potential up- and downstream process implications [26].

Only a few studies have up to now tested the effect of harvesting methods on biomass and more specifically lipid composition and the results are clearly contradicting. Chatsungnoen and Chisti [17] reported that metal salts such as aluminum sulfate and ferric chloride irreversibly bind to the biomass of *Chlorella, Neochloris*, and *Nannochloropsis sp*. The metal coagulants did however not significantly affect total lipid content in the biomass. Also Lee et al. [27] and Anthony et al. [28] did not observe a significant effect of harvesting method (for alkaline flocculation, aluminum sulphate and a microbial flocculant in Lee et al. [27] and for cationic starch, aluminum sulphate and centrifugation in Anthony et al. [28] on the lipid content of *Botryococcus braunii* and the total mass of transesterifiable lipids in *Scenedesmus obliquus*. This is in contradiction to findings by Rwehumbiza et al. [29] who observed a different total fatty acid methyl ester (FAME) content extracted from

Nannochloropsis depending on the used flocculant dosage. Borges et al. [30] reported that the use of polyacrylamides in the flocculation process did not affect total lipid content but did affect the fatty acid composition although the effect did depend on species (*Nannochloropsis* versus *Thalassiosira*) and on the type of polyacrylamide. Rios et al. [31] showed that total lipid and total FAME content depend on the used harvesting cascade process (higher for cross flow filtration followed by centrifugation compared to alkaline flocculation followed by cross flow filtration) for both *Phaeodactylum* and *Nannochloropsis*.

Only Anthony et al. [28] studied the effect of harvesting method on the ability of the extraction process (in their case wet lipid extraction with hexane after several other biorefining steps) to isolate the algal lipids. The centrifuged samples had the highest recovery efficiency of lipids and this was statistically significant compared to the cationic starches and alum harvested microalgae. The yields for the cationic starches harvested microalgae were in turn significantly higher than alum harvested microalgae.

The goal of this study was to further (hopefully clarify the contradicting results) evaluate the impact of several harvesting methods on biomass composition in terms of lipid content and fatty acid composition, but also (for the first time) in terms of free fatty acid (FFA) content. Furthermore a potential influence on the extraction efficiency was studied. Alum and alkaline flocculation were evaluated versus centrifugation as the reference method for the marine diatiom *Phaeodactylum tricornutum*.

Materials and methods

Cultivation of Phaeodactylum tricornutum

The marine diatom *Phaeodactylum tricornutum* 1055/1 (CCAP) was used as model species and cultured in batch mode in 30-L plexiglass bubble column photobioreactors (20 cm diameter) using modified Wright's Cryptophyte medium prepared from pure salts and deionized water [32]. Additional synthetic sea salt (Homarsel, Zoutman, Belgium) was added at a final concentration of 30 g L^{-1}. The reactors were aerated with 0.2-µm-filtered air (5 L min^{-1}) and pH was maintained at 8.5 through pH-controlled addition of carbon dioxide to the air flow. The culture was irradiated from two sides with daylight fluorescent tubes (Osram Grolux Sylvania, Germany), yielding a photon flux of 60 µmol m^{-2} s^{-1} at the surface of the reactor. Microalgal biomass density was monitored by measuring absorbance at 750 nm. (ash-free) dry weight was determined using GF/F filters according to method 16.2 in Moheimani et al. [33]. Absorbance measurements were calibrated against dry weight, which was in turn determined gravimetrically on pre-weighed GF/F glass fiber filters [25]. Harvesting was carried out at stationary growth phase at day 13 of cultivation.

Harvesting: flocculation and centrifugation experiments

P. tricornutum was harvested using alum (Al), alkaline flocculation (pH), and centrifugation (C). The optimal parameters for these flocculation methods were determined in previous published studies [25,34]. Flocculation experiments were carried out in triplicate using 2-L bottles. Cultures were mixed via magnetic stirring, and centrifuged at 4,000g for 20 min at 21°C. For alum flocculation, pH was adjusted to 7 using 1 M of a hydrochloric acid

stock solution prior to addition of 20 ml of a 5 g L^{-1} stock solution of aluminum sulphate octadecahydrate (ACS gradient, Sigma Aldrich). Subsequently, the broth was gently mixed at 250 rpm for 30 min. For alkaline flocculation, 12 mL of a 0.5 M sodium hydroxide solution was added while the broth was gently mixed at 250 rpm for 30 min. Sedimentation was allowed for 30 min for both flocculation methods. The separation efficiency, or the percentage of microalgal biomass removed from suspension, was calculated based on changes in the optical density (measured at 750 nm) prior to alum or sodium hydroxide addition (OD_i) and after settling (OD_f):

$$\text{Separation efficiency (\%)} = \left(\frac{OD_i - OD_f}{OD_i}\right) \times 100 \quad \text{(Eq. 1)}$$

After settling, the supernatant was decanted and the particulate phase was poured into a volumetric cylinder to measure volume. The concentration factor (CF) was determined by dividing the total volume (2 L) by the volume of the particulate phase. This parameter provides useful information about the residual water content of the particulate phase after flocculation [34]. Subsequently, the particulate phase was centrifuged at 4,000g for 20 min at 21°C and stored at −80°C prior to lyophilisation.

Biomass composition

The total lipid fraction was extracted from the harvested lyophilized microalgal biomass with chloroform/methanol 1:1 using 100 mg of biomass and 24 mL of solvent according to the method described by Ryckebosch et al. [35]. The total lipid content was subsequently determined gravimetrically. The amount of extracted lipids was expressed on the basis of both biomass dry weight and ash-free dry weight to compensate for residual flocculant in the biomass fraction after harvesting. All extractions were performed in triplicate

for each harvesting method replicate. The experimental and analytical replicates were pooled to report mean values and standard deviations. All extracts were further analyzed in terms of FFA content and fatty acid profile.

The determination of the FFA content of the extracts was based on the selective formation of dimethyl amide derivates as described by Kangani et al. [36]. At the start of the FFA determination, an internal standard (C12:0, Nu-Chek Inc., USA) was added to the extract. The separation of the derivates was performed by gas chromatography (GC) with cold on-column injection and flame ionization detection (FID) (Trace GC Ultra, Thermo Scientific, Interscience, Louvain-la-Neuve, Belgium) using an EC Wax column (length: 30 m, ID 0.32 mm, film: 0.25 lm) (Grace, Lokeren, Belgium). The temperature-time program was: 100-160°C (10°C/min), 160–240°C (2 °C/min), 240°C (7 min). Peak areas were quantified with Chromcard for Windows software (Interscience, France). The amount of FFA was calculated by comparing the sum of the peak areas to the peak area of the internal standard (C12:0).

To determine the fatty acid composition, fatty acid methyl esters (FAMEs) were formed by methylation of the lipid extracts as described by Ryckebosch et al. [35]. The FAMEs were then separated by the same GC-FID as described above. The temperature-time program was in this case: 70–180°C (5°C/min), 180–235°C (2°C/min), 235°C (9.5 min). Fatty acid identification was performed using standards containing a total of 35 different FAMEs (Nu-Chek Inc., USA). Peak areas were quantified with Chromcard for Windows software (Interscience, France).

Lipid extraction efficiency

The extraction efficiency with hexane/isopropanol (3:2) was determined as the ratio of the extraction yield with hexane/isopropanol (HI) compared to the extraction yield with chloroform/methanol (CM). The CM extraction (described above) has previously been demonstrated to extract the total amount of lipids, while HI (3:2), although commercially applicable and even food grade, is not able to penetrate tough, intact microalgal cell walls and consequently functions less efficiently [37]. The HI extraction was conducted as described in Ryckebosch et al. [37], without the disruption step during extraction to evaluate a potential impact of the harvesting method on the extractability. The HI extraction efficiency was determined in triplicate for each harvesting method replicate (n=9).

Statistical analysis

All experimental data are reported as mean values with an experimental error calculated as 1 standard deviation of the mean ($\mu \pm 1\sigma$). All results were statistically evaluated using a one-way analysis of variance (ANOVA) test with a level of significance of 0.05, followed by a Tukey's post-hoc test to analyze pairwise differences. Normality of the data was determined with the Shapiro-Wilk normality test (Sigmaplot 11, Systat Software Inc.).

Results and discussion

Harvesting of Phaeodactylum tricornutum using alum and alkaline flocculation

Table 1 shows the flocculation parameters, separation efficiency, and concentration factor for alum and alkaline flocculation. For both methods, the separation efficiency was higher than 90 % and the concentration factor was above 10. This is comparable with previous results [12,18], which demonstrates that flocculation was conducted in optimal conditions in this study.

Table 1: Harvesting of *Phaeodactylum tricornutum* using alum and alkaline flocculation (n=3; $\mu \pm 1\sigma$)

Flocculation method	Dose (mM)	pH	Separation efficiency (%)	Concentration factor (−)
Alum	0.075	7	94±2	18±2
Alkaline	3.0	10.6	91±3	12±2

While the separation efficiency was comparable for both flocculation methods, the dose to obtain that efficiency was dramatically different (Table 1). Only 0.075 ton alum per ton microalgae biomass was needed, while for alkaline flocculation 0.18 ton sodium hydroxide ton per ton biomass was required to obtain efficient separation. The flocculant operational expenses would consequently be lower for alum compared to alkaline flocculation (Table 2: USD 23 ton^{-1} biomass versus 63 ton^{-1} biomass for sodium hydroxide). However, the usage of alum could limit the product application of the harvested biomass due to toxicological concerns [17]. Alkaline flocculation could therefore be a good alternative supposing that there is no impact on downstream processing (e.g. impact on the lipid content and extraction efficiency).

Table 2: Estimation of flocculant operational expenses for alum versus alkaline flocculation

Flocculant	Alum[a]	NaOH[b]
Dose (ton ton^{-1} biomass)	0.075	0.18
Cost (USD ton^{-1} biomass)	23	63

[a] Alum industrial grade USD 300 ton^{-1} [18]
[b] NaOH industrial grade: USD 350 ton^{-1} [25]

Impact of the harvesting method on biomass composition in terms of total lipid content, fatty acid composition and free fatty acid content

The total lipid content of *Phaeodactylum* harvested using alum, alkaline flocculation, or centrifugation was determined by extraction using chloroform:methanol (Fig 1). When expressed on dry weight basis, the total lipid content of biomass harvested using alum and alkaline flocculation was significantly lower than biomass harvested via centrifugation ($p<0.001$). However, when expressed on ash-free dry weight basis, no significant difference in lipid content was observed between biomass harvested using alum and alkaline flocculation. The centrifuged biomass however had a slightly higher amount of total lipids (47%) compared to biomass harvested by alum (42%; $p<0.001$) or alkaline flocculation (43%; $p<0.001$).

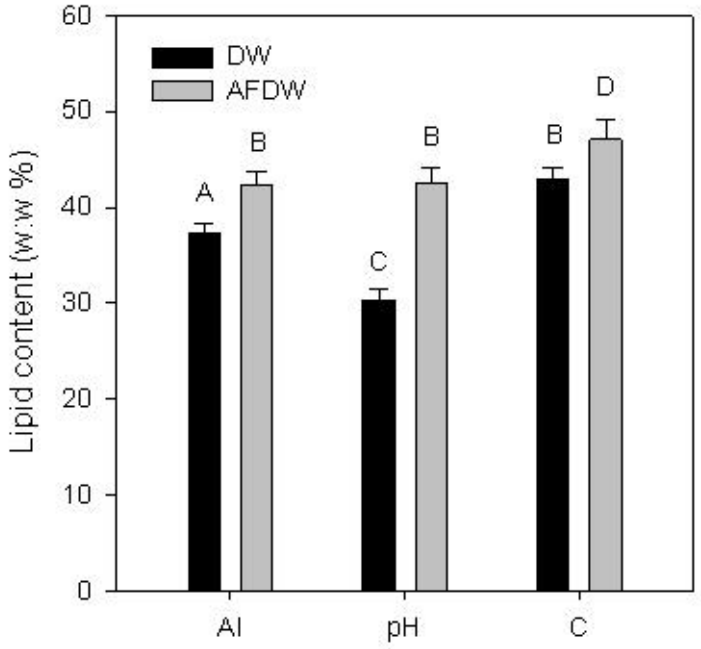

Fig 1: Lipid content expressed as percentage of total dry weight (DW) and ash-free dry weight (AFDW) basis for *Phaeodactylum tricornutum* harvested using alum (Al), alkaline flocculation (pH), and centrifugation (C). (n=9; error bar = 1σ). Different letters indicate statistical differences with a significance level of 0.05

Alum and alkaline flocculation are mediated by the precipitation of metal salts, and/or calcium carbonate and this results in a transfer of those salts to the particulate phase after separation [38,39]. This results in an increase of the ash content of the biomass and consequently, a comparison of total lipid content expressed on dry weight biomass basis is misleading. Lipid content can only be accurately compared on ash-free dry weight basis, which is in agreement with previous observations using alum as flocculant [17,31]. This can already explain some of the contradicting results obtained in literature as some authors have neglected this necessary correction [29,30]. Another cause of contradicting results can be the standard harvesting method with which the comparison is made and the method applied for total lipid extraction.

Our results correspond well with literature when only taking into account the studies which have used centrifugation as the golden standard and have applied the correction using AFDW. In Chatsungnoen and Chisti [17] and Anthony et al. [28] the slightly lower lipid content when using alum compared to centrifugation was not significant (as in our case) but numerically their results showed the same trend. Rios et al. [31] also observed the slightly but significantly lower total lipid content for alkaline flocculation compared to centrifugation (in combination with cross flow filtration).

The slightly lower lipid content of biomass obtained by flocculation (using alum or alkaline flocculation) compared to biomass obtained by centrifugation might be explainable by the extracellular algal organic matter (AOM) that is known to interfere with flocculation processes by binding to the used coagulant [40,41]. While ash-free dry weight corrects for the amount of minerals and salts present in the biomass, it does not correct for the AOM that might partially be transferred to the particulate phase. For *Chlorella,* AOM comprised up to 20 mg C L^{-1} which was nearly 15% of the total dry weight [42]. For *Phaeodactylum,* total AOM concentrations between 2 and 5% have been observed (unpublished data). AOM can increase the amount of ash-free biomass significantly for the flocculated biomass and this might lead to seemingly lower total lipid results. This hypothesis disserves further study.

An interaction between the ions and the lipids leading to interference of the flocculants with the CM extraction as suggested by Rios et al. [31] does not seem plausible to us, given the strength of this solvent system. Moreover, one would then expect an even bigger effect when a weaker solvent such as HI is used, which is completely not the case as shown in the next section.

Apart from the total lipid content, the relative fatty acid profile (as FAMEs) was also determined (Table 2). The main fatty acid was C16:1 which contributed more than 40% to the

283 total FAME content. Other fatty acids present in substantial amounts were C14:0, C16:0,
284 C18:1 and C20:5. This coincides with what has been reported earlier in literature for
285 *Phaeodactylum tricornutum*, although some variation always exists depending on cultivation
286 conditions [43]. Between the different harvesting methods, no meaningful differences in fatty
287 acid profile were observed. The statistical analysis resulted only in one case, for C18:1, in a
288 very low p value ($p<0.001$), and therefore the rejection of the null hypothesis (Table 2).
289 However, the relative increase of 0.12% for C18:1 (reported as % of total FAME) for biomass
290 harvested by alum is, although statistically significant, very small, as compared to the results
291 of Rios et al. [31].
292
293 **Table 2: FAME profile for *Phaeodactylum tricornutum* using chloroform:methanol 1:1**
294 **harvested using alum (Al), alkaline flocculation (pH), and centrifugation (C). (n=9;**
295 **$\mu \pm 1\sigma$)**

FAME (% of total FAME)	Al	pH	C	p-value
C14:0	5.19±0.05	5.23±0.03	5.16±0.02	0.003
C16:0	28.74±0.13	28.69±0.09	28.85±0.08	0.006
C16:1	43.76±0.15	43.83±0.10	43.90±0.08	0.100
C18:1	9.59±0.05	9.47±0.03	9.47±0.02	<0.001
C20:5	6.83±0.20	6.94±0.14	6.78±0.11	0.122

296
297 Finally, the FFA content was determined since it has been shown that even during
298 short storage of wet biomass, endogenous hydrolytic enzymes present in the biomass can
299 cause lipolysis, being the dissociation of FFA from the lipids [44]. These FFA are
300 disadvantageous for several applications and it is thus important that no lipolysis occurs
301 during the flocculation treatment.
302
303 In general, the FFA content was lower than 1.5 % of total lipids for all treatments (Fig
304 2). Moreover, there were no significant differences in FFA content between biomass
305 harvested using flocculation and centrifugation ($p>0.115$). This suggests that the flocculation

procedures did not significantly enhance lipolysis, at least not when after flocculation the wet biomass is immediately further processed (centrifugation and drying) as was the case in this experiment.

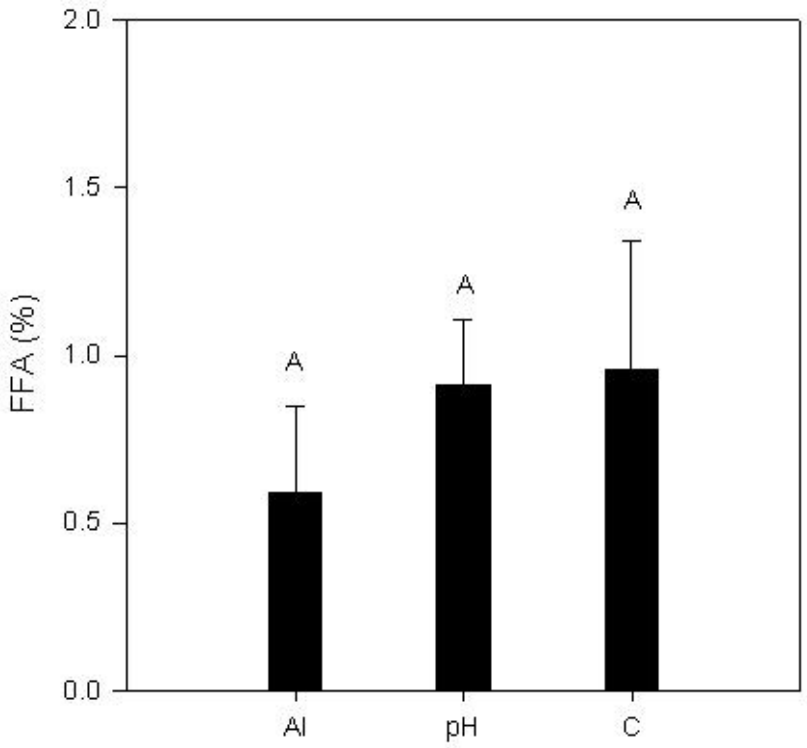

Figure 2: Free fatty acid (FFA) content as % of total lipids for *Phaeodactylum tricornutum* harvested using alum (Al), alkaline flocculation (pH), and centrifugation (C) extracted using chloroform:methanol 1:1 (CM) (n=9; error bar = 1σ)

Different letters indicate statistical differences between harvesting methods with a significance level of 0.05

316

317 ***Impact of the harvesting method on lipid extraction efficiency***

318

319 Hexane/isopropanol (HI) is one of the most efficient solvent systems for commercial
320 microalgae lipid extraction since it is a less toxic, non-halogenated system that combines
321 relatively high lipid yields with low non-lipid co-extraction. It is however not able to
322 penetrate tough, intact microalgal cell walls and consequently functions less efficiently than
323 CM. Therefore the extraction efficiency was determined as the ratio of the extraction yield
324 with HI compared to the extraction yield with CM, which has previously been demonstrated
325 to extract the total amount of lipids [37]. The harvesting method was hypothesized to have a
326 potential positive or negative effect on the extraction efficiency. A positive effect could come
327 from a possible cell (wall) disruption caused by a certain harvesting method and leading to a
328 better contact between the solvent system and the lipids. A negative effect could come from
329 an interference of the flocculants with the extraction procedure, especially when weaker
330 solvent systems such as HI are used (compared to CM).

331

332 Figure 3 shows the lipid extraction efficiencies for the three harvesting methods
333 applied in this research. It can be seen that about 70% of the total lipids obtained by CM
334 extractions were recovered using HI but that no significant differences occurred between the
335 different harvesting methods ($p>0.05$). This means that, at least for this species, none of the
336 above hypothesized influences on extraction efficiency occur. It cannot be excluded that
337 especially the first effect might be different for other microalgae species having another cell
338 (wall) structure.
339 The seemingly contradicting results of Anthony et al. [28], who did observe a lower
340 extraction efficiency when using alum compared to centrifugation, might be explainable by

the different extraction method (hexane / isopropanol on dry biomass in this study versus hexane on wet biomass in Anthony et al. [28] used. Hexane is less efficient in extracting lipids from microalgae [37] and extraction from wet biomass is less efficient than from dry biomass [45]; making that the wet extraction using hexane might be hindered more by an interference from the flocculants.

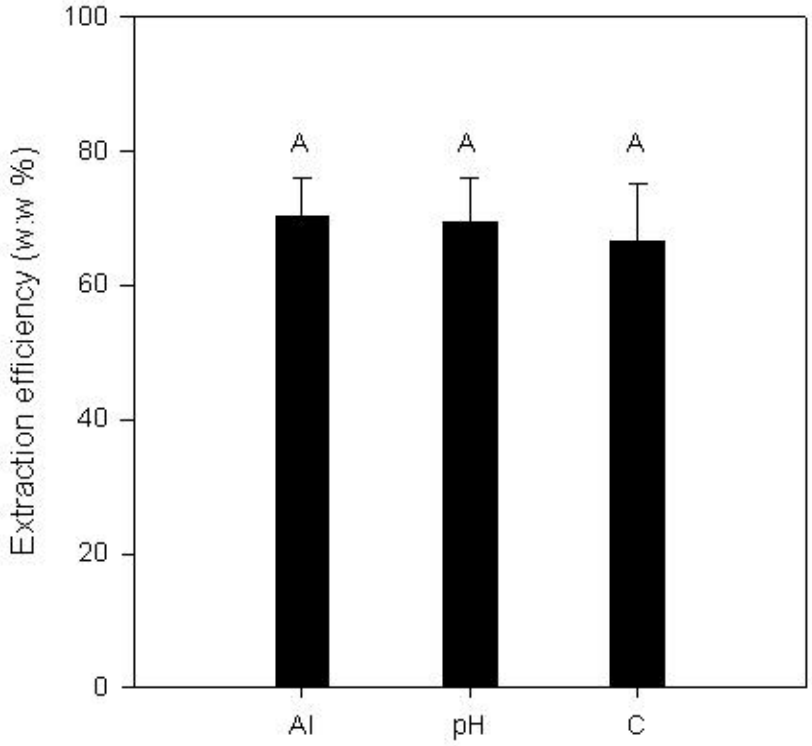

Figure 3: Lipid extraction efficiency for *Phaeodactylum tricornutum* harvested using alum (Al), alkaline flocculation (pH), and centrifugation (C). (n=9; error bar = 1σ) Different letters indicate statistical differences with a significance level of 0.05

Conclusions

Harvesting is a central process in any microalgae biomass production process. It is therefore crucial that any kind of impact on biomass composition and downstream processing is avoided. The present results show that alum and alkaline flocculation did not severely impact total lipid content of the marine diatom *Phaeodactylum tricornutum* when one correctly accounts for the salts which are transferred to the biomass when flocculation is used (by expressing results on ash-free dry weight). The slightly lower total lipid content obtained after flocculation might be explained by organic material transferred to the biomass (e.g. AOM) during flocculation, which is not accounted for by expressing on ash-free dry weight. Importantly also, the fatty acid composition does not change substantially and no detrimental free fatty acids are formed during flocculation. Neither a positive nor a negative effect of the flocculation on the extraction efficiency when using a commercial solvent system was observed, but this result may be different for other microalgae species, flocculation methods and extraction methods and thus disserves further study.

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