Extraction and quantification of pigments from a marine microalga: a simple and reproducible method

M. Henriques[1], **A. Silva**[2] and **J. Rocha**[*, 2]

[1]Escola Superior Agrária de Coimbra, Polytechnic Institute of Coimbra, 3040 – 316 Coimbra, Portugal
[2]Chemical Engineering Department, University of Coimbra, 3030-790 Coimbra, Portugal

The pigment content in microalgae is a specific feature of each species. Its evaluation is essential as an indirect measure of cell growth, as well as a parameter to check the trophic level of waters. Several methods can be found in literature, most of them based on spectrophotometric analyses, referred as a good and practical tool for chlorophyll evaluation. A comparison of different methods to quantify pigments was done, using as selection criteria the simplicity, the amount of pigments identified and the time required for the analysis. Several factors were tested to maximize the yield of pigments extraction, such as: i) the solvent to be used, ii) the cell wall disruption technique, iii) the extraction time and iv) the use of different empirical correlations. The marine microalga *Nannochloropsis gaditana* was the biological material used for this study because chlorophyll is the most abundant pigment in this strain.
Methanol was shown to be the most suitable solvent to extract chlorophyll from this strain, using the Lichtenthaler correlation [1], after 24 hours of extraction. Furthermore the lysis ability of the solvent and the use of additional cell wall disruption techniques favour the extraction yield. The freezing/unfreezing technique with liquid N_2 was found to be more efficient than the use of ultrasounds for 15 min. To extract more polar pigments, such as carotenoids, hydrophilic solvents like methanol play the main role in extraction, which is not so influenced by the use of physical or mechanical disruption methods.

Keywords chlorophyll, extractions methods, *Nannochloropsis gaditana*, pigments

1. Introduction

Most methods for chlorophyll evaluation found in literature were developed to evaluate the trophic level of waters. Chlorophyll *a* is usually the parameter used as the trophic indicator, mainly because the relationship between the content of this pigment and the amount of algal biomass is quite direct. Algal biomass is strongly linked to visible evidences of eutrofication, usually on the basis of this phenomenon. The quantification of chlorophyll *a* is easier than the algal biomass itself [2-3], and can be used as an indirect method of biomass quantification. The weakness of this evaluation is the variability of the chlorophyll *a* content in each species (from 0.1 to 9.7% of cell dry weight), as well as the suitable selection of the quantification method, taking into account the differences on cell wall resistance [4]. In fact, the source of the biological material determines the selection of the best method.

Pepe et al. [5] refer two different extraction methods for the quantification of chlorophyll *a*, one involving ethanol and the other making use of cold methanol. The differences observed on the extraction yield were explained by the phytoplankton algal composition, according to the predominant microalga strains. The ethanol extraction method gave better results with *Chlorophyceae* and *Dinophyceae* as dominant strains, while the methanol extraction method showed to be advantageous for others strains like *Bacillariophyceae*. This means that the potential of a solvent extractability depends on the hydration and permeability of the microalga cell wall. Apart from the influence of the cell wall constitution in chlorophyll evaluation, pigments modification during the extraction process [1, 6], as well as the influence of pigments other than chlorophyll, may complicate its determination and may lead to errors in the estimations.

[*] Corresponding author: e-mail: jrocha@eq.uc.pt, Phone: +351 239 798 731; Fax +351 239 798 703

Louda and Monghkonsri [7] compared spectrophotometric estimates of chlorophyll contents with those obtained by high performance liquid chromatography (HPLC). They concluded that spectrophotometric evaluation of chlorophyll, using UNESCO [8] and Jeffrey and Humphrey [9] equations, gave excellent results. Those authors support that in the absence of significant differences between the two referred methods, the spectrophotometric analyses are much less expensive and much faster than HPLC analyses, making them a good tool for routine chlorophyll evaluation.

Nannochloropsys gaditana only have chlorophyll *a*; therefore the evaluation of the total amount of chlorophylls is equivalent to the evaluation of that particular type of pigment.

There are several available methods to quantify chlorophylls, making the choice random or not easy to justify. Common steps to all protocols, based on spectrophotometry, are the following ones: 1- separation of microalga cells from the supernatant; 2- extraction of pigments with an organic solvent and; 3- spectrophotometric determination of the concentration of chlorophyll in the extract.

The great diversity of methods justifies a comparative and systematic study to identify the precision and the reproducibility of each one, enabling the selection of a suitable and practical method to be used with one or a group of microbial species. The items that were identified and compared to each tested method, were the following ones: solvent type; empirical correlation for pigments quantification; extraction time and cell wall disruption method.

The extraction techniques of cell components usually make use of chemical, mechanical and/or enzymatic procedures. In this work only the chemical and mechanical procedures were used, alone or simultaneously, with the aim of maximizing the extraction process efficiency. Figure 1 shows a diagram where the different items that were used to identify and quantify pigments in the microalgae, are presented under a structured sequence.

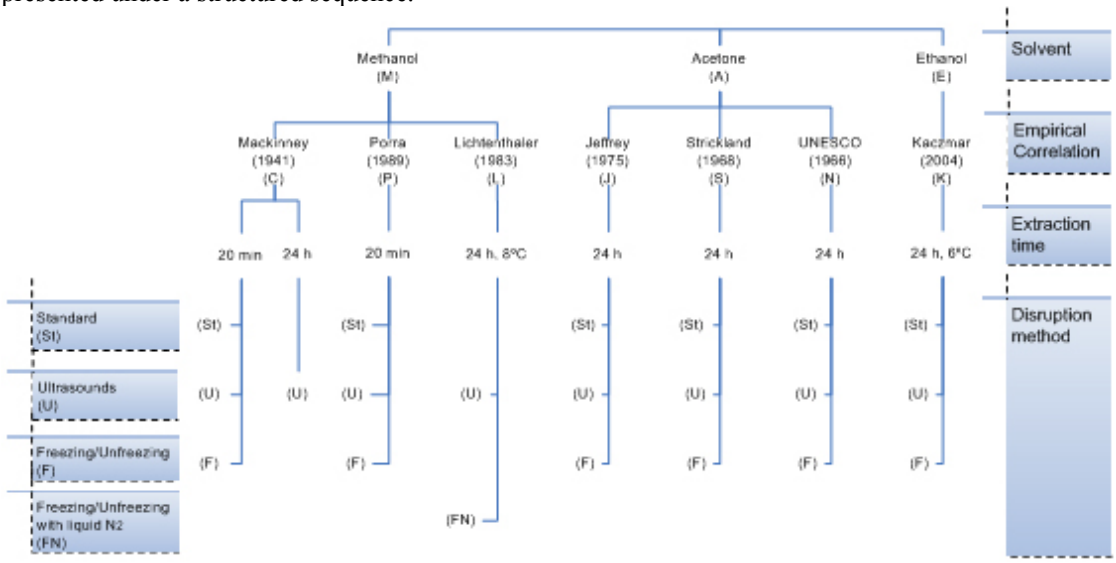

Fig. 1 Items tested to compare different methods of pigments evaluation.

The selection of the solvent to promote the extraction is a very important issue since it determines the degree of affinity to the chemical composition of the substances to be extracted. Apart from the dissolution ability towards the compounds to be extracted and quantified, the solvent also plays an important role in cell lysis. More aggressive solvents can increase the extraction yield in cells with strong walls. Methanol was the first solvent to be used to extract chlorophylls, but due to its toxicity it has been replaced by other ones. According to Edler recommendations [10], till 1995 the evaluation of the chlorophyll content was performed by using an extraction method with acetone. Since then, the use of ethanol as the extraction solvent has been suggested.

In cell wall disruption techniques, two main groups can be employed: mechanical and non-mechanical ones. Within the first group, the ultrasounds were used in some of the tested methods. Among the non-mechanical techniques of cell lysis, there are chemical processes, related to the solvent used, and physical methods, such as the freezing and unfreezing sequence [11]. This last process can be performed slowly or fastly (by using liquid nitrogen) giving rise to a greater thermal shock in the biological material, inducing a much more efficient rupture of cell walls.

The contact time between the cellular compounds to be extracted and the solvent can be determinant to the amount of extracted products. When the cell lysis power of the solvent is not too high, which can be intentional to avoid damage of the compounds to be extracted, a longer period of extraction may increase the yield of this process.

In order to simplify the comparison of different methods, and the identification of the influence of each step within an extraction method, the following definitions are proposed:

- An analytic method is made by a set of different items or procedures that are necessary to completely evaluate the pigments under study.
- Each item or procedure is described by a specific shortening. The method is designated by assembly of abbreviations corresponding to each step, in the order presented on the tree diagram of Figure 1.

The nomenclature used in the diagram, for the different steps of each method, has the following meaning:

- type of solvent and empirical correlations (Table 1);
- extraction time: 20 min and 24 h;
- cell wall disruption technique: the standard lysis ability of the solvent (St), ultrasounds (U), the freezing and unfreezing sequence (F), and the use of liquid nitrogen (FN).

As an example, and according to the nomenclature presented above, the method M-C-24h-U means the use of methanol as solvent, the empirical correlation of Mackinney, an extraction time of 24 h and the use of ultrasounds as the additional cell wall disruption technique.

Table 1 Empirical correlations to evaluate the concentration of pigments using methanol, acetone and ethanol as extraction solvents, and respective nomenclature of identification.

Methanol		**(M)**
Mackinney [12]	$\mu g_{chlorophyll} / mL_{medium} = 13.43\, A_{665}\, v/(lV)$	(C)
Porra et al. [13]	$\mu g_{chlorophyll} / mL_{medium} = (16.29\, A_{665} - 8.54\, A_{652})\, v/(lV)$	(P)
Lichtenthaler [1, 14]	$\mu g_{chlorophyll} / mL_{medium} = 15.65\, A_{666}$ $\mu g_{total\ carotenoids} / mL_{medium} = \left(\frac{1000 A_{470} - 44.76 A_{666}}{221} \right)$	(L)
Acetone		**(A)**
Jeffrey and Humphrey [9]	$\mu g_{chlorophyll} / mL_{medium} = (11.85 A_{664} - 1.54 A_{647} - 0.08 A_{630})\, v/(lV)$	(J)
Strickland and Parsons [15]	$\mu g_{chlorophyll} / mL_{medium} = (11.66 A_{665} - 1.31 A_{645} - 0.14 A_{630})\, v/(lV)$	(S)
UNESCO [8]	$\mu g_{chlorophyll} / mL_{medium} = (11.64 A_{663} - 2.16 A_{645} - 0.10 A_{630})\, v/(lV)$	(N)
Ethanol		**(E)**
Kaczmar [16]	$\mu g_{chlorophyll} / mL_{medium} = (11.64 A_{663} - 2.16 A_{645} - 0.10 A_{630})\, v/(lV)$	(K)

A_{xxx} is the absorbance at *xxx* nm, after removing the sample absorbance at 750 nm, against a blank of the solvent used. v means the volume of solvent used (mL), l is the spectrophotometric cell length (cm) and V is the sample volume (mL).

The first part of the studies presented in this work concerns the evaluation and comparison of the efficiency of different methods to quantify the chlorophyll content in the microalgae *Nannochloropsis*

gaditana, namely the effect of the solvent and the cell disruption techniques tested. The second part focus on the use of methanol as solvent and compares the efficiency of extraction, for different extraction times, when the freezing and unfreezing of samples with liquid nitrogen is used instead of ultrasounds.

2. Material and methods

2.1 Strain and culture conditions

The strain *Nannochloropsis gaditana* was kindly donated by Necton, Olhão, Portugal. It was grown up in a self made culture medium, in Erlenmeyer flasks, at room temperature and under natural light cycles. The composition of the synthetic culture medium was (per liter): 35 g NaCl and 1 mL of ALGAL solution. The composition of the ALGAL solution (per liter) was:

- 100 mL of iron solution (5.3 g of ferric citrate monohydrate, $FeC_6H_5O_7.H_2O$; 5.4 g of iron oxide, Fe_2O_3 ; 2.54 g of iron chloride hexahydrate, $FeCl_3.6H_2O$; 7.4 g of EDTA);
- 100 mL of micronutrients solution (136.4 mg $ZnCl_2$; 161.4 mg $ZnSO_4.7H_2O$; 197.8 mg $MnCl_2.4H_2O$; 242 mg $NaMoO_4.2H_2O$; 16.5 mg $CoCl_3$: 23.8 mg of Cobalt(II) chloride hexahydrate, $CoCl_2.6H_2O$; 24.9 mg $CuSO_4.5H_2O$; 2.4 g EDTA; 492 mg $MgSO_4.7H_2O$);
- 800 mL of macronutrients solution (170 g $NaNO_3$ and 13.6 g KH_2PO_4).

The pH was adjusted to 7.4 with NaOH or HCl 1M. Solutions were previously sterilized before use. All the assays to check the different methods of quantifying chlorophylls were performed with biomas from the same cell growth phase.

The cell concentration of the microalgae was evaluated by reading the optical density at 540 nm in a Beckman Du Series 600 spectrophotometer. The absorbance at 540 nm was correlated to cell dry weight and to the number of cells per culture volume. Cell counting was done by using a Neubauer hemocytometer.

2.2 Extraction methods

Among the chemical processes, only the type of solvent used was changed. For each solvent, different cell wall disruption methods were tested. In the standard assay (Fig. 1) no mechanical or physical cell wall disruption methods were used, other than the lysis power of the solvent itself. The physical extraction processes included the ultrasounds and the sequence of freezing and unfreezing, slowly in the freezer and fastly using liquid nitrogen. Each of the methods used to evaluate the chlorophyll *a* content included a previous phase of sample preparation, an extraction phase and the pigments quantification by using different empirical correlations (Table 1).

2.2.1 Sample preparation

A volume of 2 mL culture sample was withdrawn. Cells were centrifuged at 3000 rpm for 10 min (or at 4000 rpm for 5 min). The supernatant was removed and cells were then ressuspended in 2 mL of distilled water to remove any salts that could have been retained with biomass, and submitted again to centrifugation. This washing process was repeated twice.

2.2.2 Extraction and quantification

Three organic solvents were used in pigment extraction: absolute methanol (99.8%, Panreac), acetone (90% v/v) and ethanol (technical grade). Two times of extraction were tested with methanol: a short one (20 min) and a longer one (24 h). However, with acetone and ethanol only the longer extraction time was used, at room temperature with acetone and in the fridge (6ºC) with ethanol.

After the above described sample preparation, cells were suspended in 2 mL of solvent with strong vortex mixing for 15 s. In the absence of other cell wall disruption methods, the cells were centrifuged at 4000 rpm for 5 min (after the resting period mentioned in the methods schedule, i.e. 20 min or 24 h) and the supernatant absorbance was read at the wavelengths corresponding to the empirical correlation to be used. When ultrasounds were applied as additional technique of cell disruption, the suspension of cells in the solvent was kept in the ultrasonic water bath for 15 min, at 250 Hz. With the use of the freezing/unfreezing process as cell disruption technique, the cell suspension was put in the freezer until solidification, and thereafter removed for slow unfreezing at room temperature. When liquid nitrogen was used for a fast freezing process, the sample was plunged into nitrogen for 3 min, followed by an equivalent slow unfreezing.

3. Results

3.1 The effect of the extraction solvent

Using methanol as the extraction solvent to quantify the concentration of chlorophyll *a*, better results were obtained with the Mackinney correlation than with the Porra et al. correlation, independently of the cell wall disruption process applied: either chemical (standard), mechanical (ultrasounds) or physical (freezing/unfreezing) (Fig. 2). According to both correlations, an increase in the chlorophyll *a* extraction yield was observed when mechanical or physical processes were used to help cell disruption; the freezing/unfreezing process gave the highest results and ultrasounds led to spread results.

When acetone was used, the extraction yields achieved for chlorophyll *a* were much lower than the ones obtained with methanol. With this solvent, only the samples submitted to the freezing/unfreezing process gave results close to the ones obtained with methanol, but with the less favourable correlation of Porra et al. It was confirmed that a cell disruption step led to spreader results. When the samples were submitted to the same treatment, the three used correlations gave close results, which were expected taking into account the similarity of the associated empirical equations. On the other hand, the extraction power of acetone for this biological material is not as high as the extraction power of methanol. Figure 2 shows that by using the same extraction method, the chlorophyll content achieved with acetone is c.a. three times lower than the concentration obtained with methanol, particularly when using the standard and the ultrasounds disruption methods.

Ethanol has been the less used solvent for extraction and quantification of pigments. Here, only the empirical correlation of Kaczmar was tested. Results obtained with this method are quite better than the ones obtained with acetone, but they are of the same order of magnitude as the ones obtained with methanol. In this case, the disruption technique didn't significantly favour the chlorophyll extraction, giving similar values for its concentration. The standard procedure showed dispersed results, probably due to a lack of homogenization of the samples.

According to the above referred experiments, the best methods for the evaluation of chlorophyll *a* content in this marine microalgae cells, giving high values for the pigments concentration, make use of methanol as extraction solvent with the Mackinney correlation, or ethanol with the Kaczmar correlation. In practice, the first method has the advantage of the short extraction time (20 min), allowing the assessment of chlorophyll content at the same day. With ethanol the required time for complete extraction is c.a. 24 h. Acetone did not show to be efficient to extract chlorophyll in this microalgae, with very low values achieved. This evidence was not expected because methods using acetone are the most recommended in literature, for chlorophyll quantification in waters as a trophic parameter.

Another important aspect to be taken into account is the great cell wall resistance of *Nannochloropsis gaditana*. It was confirmed that this microalgae strain has a solid structure difficult to break, since the use of additional rupture methods revealed favourable to chlorophyll extraction. Perhaps due to its hydrophylicity, only a solvent with a great extraction power, such as methanol, allows an effective and fast chlorophyll recuperation.

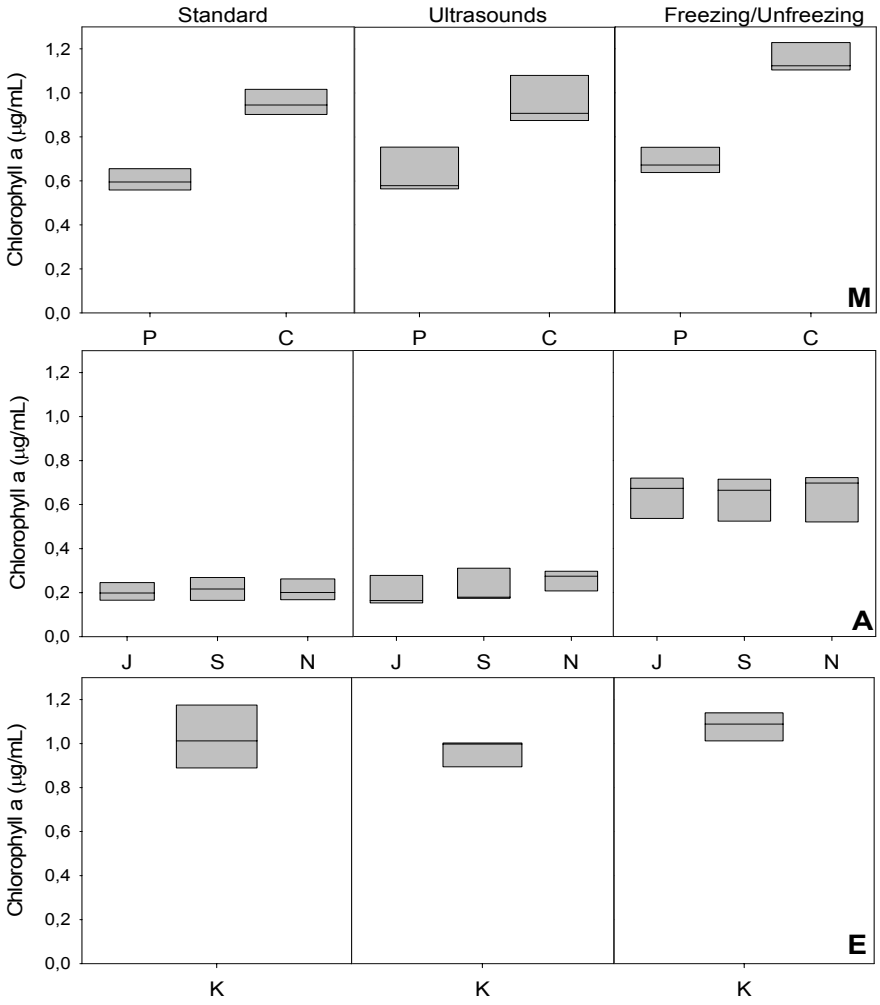

Fig. 2 Quantification of Chlorophyll *a* using as extraction solvent: methanol **(M)** during 20 min at room temperature [P - Porra et al. correlation, C - Mackinney correlation]; acetone 90% (v/v) **(A)** during 24 h at room temperature [J - Jeffrey and Humphrey correlation, S - Strickland and Parsons correlation, N - UNESCO correlation]; ethanol **(E)** during 24 h at 6ºC in the freezer [K - Kaczmar correlation]. The height of the bars means the results dispersion, and the line gives the median.

3.2 Disruption techniques and extraction time effect

Different cell rupture techniques were already tested in the previous section, although the main goal so far was to evaluate the effect of different solvents on pigments extraction from the microalgae *Nannochloropsis gaditana*. Here, more attention is placed on the effect of the mechanical and physical cell rupture techniques, as well as the effect of the duration of the treatment on the extraction yield.

Methanol was the extraction solvent used, since it was the solvent that led to the highest contents of chlorophyll from the microalgae *Nannochloropsis gaditana*. The empirical correlations and the rupture techniques tested are indicated in Table 2 (since the speed of the freezing process was suggested as beneficial).

Table 2. Identification of methods used to quantify chlorophyll and total carotenoids, using methanol (M) as solvent, according to the extraction conditions.

Disruption method	Extraction time	Empirical correlation	Method
Ultrasounds (U)	≅ 20 min	Mackinney (C)	M-C-20min-U
	≅ 24 h, 8ºC	Mackinney (C)	M-C-24h-U
		Lichtenthaler (L)	M-L-24h-U
Freezing/Unfreezing (with liquid N_2) (FN)	≅ 24 h, 8ºC	Lichtenthaler (L)	M-L-24h-FN

The data presented in Fig. 3 were normalized using as reference the method M-L-24h-U (100% of extraction) that permits the evaluation of both types of pigments, and allows the comparison of the ultrasound technique with the freezing/unfreezing technique with liquid nitrogen. The cell rupture technique that led to the best results was the freezing/unfreezing process with liquid nitrogen (with a slow unfreezing stage in the fridge). This methodology, together to Lichtenthaler correlation, gave an increase of c.a. 9% in chlorophyll content compared to the reference method.

The first two methods showed that an increase of the extraction time reveals a clear advantage. A greater contact time between pigments and solvent favoured the extraction process, allowing the phase equilibrium to be established. Comparing the second and the third methods (Fig 3a), it can also be observed that the Lichtenthaler correlation gives much better results than the Mackinney one.

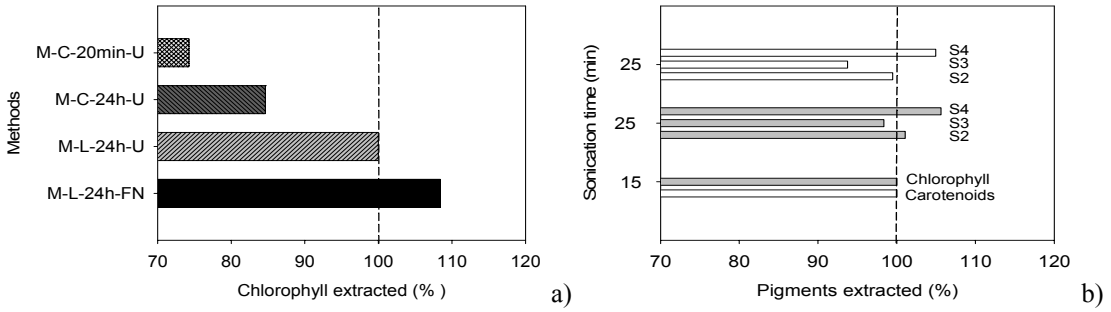

Fig. 3 a) Relative concentration of chlorophyll extracted with methanol, by using different empirical correlations, rupture techniques, and extraction times. b) Effect of sonication time on pigments extraction yield, in three different samples (S2, S3 and S4).

Empirical correlations for total carotenoids evaluation by spectrophotometry are less common in the literature. An example is the Lichtenthaler correlation which enables the comparison of the two last methods presented in Table 2. The influence of two rupture techniques on two distinct microalgal samples was evaluate to assess the ability of extracting carotenoids (data not shown). In this case, none of the methods tested (ultrasounds and freezing/unfreezing with liquid nitrogen) was shown to be more efficient on extraction. This similarity of results could be explained by the fact that carotenoids are more polar than chlorophyll, take advantage of using hydrophilic solvents like methanol; thus the effect of the solvent on extraction yield is, in this case, greater than the cell disruption technique used.

While the use of ultrasounds as a cell disruption technique is easy to carry out in the lab, the effect of the radiation time on the content of pigments extracted was also evaluated. Again, the chosen reference method was the M-L-24h-U, with ultrasounds applied for 15 min. Figure 3b shows the results obtained both for total chlorophylls and total carotenoids concentrations, concerning three different microalgae cells samples (S2, S3 and S4) submitted to ultrasounds for a longer period (25 min). An increase of the sonication time did not lead to significant variations on the concentrations of both types of pigments. A lack of uniformity of ultrasounds propagation could have influenced the results obtained. However, these

results confirm that 15 min of sonication is enough to achieve the expected maximum pigments extracted.

4. Conclusions

Methanol and ethanol compare well as extraction solvents used in chlorophyll evaluation from microalga biomass. Both of them showed to be better than acetone, which is not very efficient in the extraction and quantification of pigments from autotrophic cell cultures. In the literature most of the methods use acetone as the extraction solvent, and they are pointed out as good methods to evaluate the trophic level of waters [3]. However, this work confirmed Pepe et al. [5] and Dere et al. [17] data, suggesting that for the quantification of chlorophyll *a* in *Nannochloropsis gaditana* (*Eustigmatophyceae*), the best solvent is methanol. A spectrophotometric method to quantify chlorophyll, that uses methanol and the Mackinney correlation, even in the absence of other disruption processes, has as main advantage over the use of ethanol with the Kaczmar correlation, the short evaluation time (20 min). However it was confirmed that chlorophyll extraction yield with methanol can be improved: (i) by increasing the extraction time, (ii) using the Lichtenthaler correlation instead of Mackinney one, (iii) by applying 15 min of ultrasounds or (iv) by using the freezing/unfreezing method with liquid nitrogen.

In pigment quantification and their correlation to the amount of biomass present in the sample, it is important to take into account the structure of the cells, because cell wall sensibility differs from species to species. For *Nannochloropsis gaditana*, due to its robust cell wall, the use of additional rupture techniques was shown to be beneficial in chlorophyll extraction. However, this effect is reduced when more polar pigments, such as carotenoids, are extracted with very hydrophilic solvents like methanol, where the main role is played by the solvent itself. Sonication, after a strong mixing with the solvent, is helpful and very easy to do in practice, but a resting period in a closed vessel under refrigeration is essential to achieve a high extraction yield.

Acknowledgements To Necton company for the kind gift of the *Nannochloropsis gaditana* strain.

References

[1] H. Lichtenthaler and A. Wellburn, Biochemical Society Transactions, London, **11**, 591 (1983).
[2] S. Dere, T. Günes, R. Sivaci, Trends Journal of Botany, **22**, 13 (1998).
[3] A. Gitelson, Y. Grits, D. Etzion Optimal properties of Nannochloropsis sp and application to remote estimation of cell mass. Biotechnology Bioengineering **69**, 516 (2000)
[4] R. Vollenweider and J. Kerekes, OECD, Paris, (1982) http://lakes.chebucto.org/DATA/PARAMETERS/CHA/cha.html, Cited 15 Jan 2007, pp. 154.
[5] M. Pepe, C. Giardino, G. Borsani, The Science of the Total Environment **268**, 31 (2001).
[6] R. Livingstone, R. Parisier, L. Thompson, Journal Am Chemistry Society **75**, 3025 (1953).
[7] J. Louda, P. Monghkonsri, Florida Atlantic University, (2006), http://www.aoml.noaa.gov/ocd/sferpm/louda/louda_chl_compare.html, Cited 20 Out 2006
[8] UNESCO, Rep. SCOR/UNESCO WG 17, UNESCO, Paris, Monogr Oceanogr Methodol, **1**, 11 (1966).
[9] S. Jeffrey, G. Humphrey, Biochem Physiol Pflanzen, **167**, 194 (1975).
[10] L. Edler, Baltic Marine Biology **5(38)**, (1979)
[11] Y. Chisti, M. Moo-Young, Enzyme Microb Technol, **8**, 194 (1986).
[12] G. Mackinney, Journal Biol Chem, **140**, 315 (1941).
[13] R. Porra, W. Thompson, P. Kriedemann, Bioch Biophysica Acta, **975**, 384 (1989).
[14] A. Wellburn, Journal Plant Physiology, **144(3)**, 307 (1994).
[15] J Strickland, T. Parsons, Bull Fish Res Bd Canada, **167**, 31 (1968)
[16] Kaczmar, Phytoplankton pigments, (2004), http://water.iopan.gda.pl/~kaczmar/bdo/pigments.htm Cited 15 Nov 2004
[17] EBME (Environmental Biophysics and Molecular Ecology), (2004) http://marine.rutgers.edu/ebme/html_docs/ChlorophyllAnalysis.htm. Cited 10 Jan 2004