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Development of an Optimal Methodology for the Extraction of Microphytobenthic Chlorophyll

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Abstract: Benthic microalgae are important primary producers in intertidal shallow systems. Their biomass can be estimated by the assessment of chlorophyll *a* concentration. A rapid and reliable method of measuring chlorophyll a is by spectrophotometer. There is however, no standard protocol for the analysis of benthic chlorophyll *a*. Although the most common solvent generally used is 90% acetone, some authors showed better results with methanol and ethanol. Some pre-treatments, such as the addition of fine inert granules or ultrasound bath, have also been suggested as factors that improve the extraction efficiency. Sediment samples were collected from two sites, muddy and sandy, located within Ria Formosa (Portugal). The aim of this work was to test the effectiveness of different pre-treatments in the extraction and to develop an optimal method for chlorophyll *a* extraction and analysis. Pre-treating samples did not yield any significant differences in chlorophyll *a* extracted. Treating sediments with acetone was found to yield higher concentrations of chlorophyll *a*, both for muddy and sandy sediments. Acetone was therefore found to be the best solvent for both sediment types, with 90% being the best strength for sandy and 80% the best for muddy sediments. These differences may be related to differences in the structure of the algal communities. Six hours of extraction was found to be sufficient, since after a six hour period the extraction efficiency did not improve.

Keywords: *chlorophyll* a*; extraction efficiency; microphytobenthos; spectrophotometry; Ria Formosa.*

Introduction

Microphytobenthos (MPB) communities are important primary producers in coastal areas, especially in intertidal and shallow systems (Schreiber & Pennock, 1995; Kromkamp *et al*., 1998; Blackford, 2002; Easley *et al*., 2005; Jesus *et al*., 2005). They live and photosynthesize on the surficial sediment, but can be easily suspended into the water column (De Jonge & Van Beusekom, 1992; Koh *et al*,. 2007). MPB may represent up to 50% of the total microalgae chlorophyll present in the water column (De Jonge & Van Beusekom, 1992). They are an important source of food not only to deposit feeders but also to suspension feeders. These benthic algae also exhibit a high spatial heterogeneity (Brotas & Plante-Cuny, 1998; Underwood & Kromkamp, 1999; Seuront & Spilmont, 2002) and some authors have suggested higher chlorophyll *a* content in muddy sediments, when compared with sand (Riaux-Gobin & Bourgoin, 2002; Perkins *et al*., 2003). Different characteristics of sediment may also result in differences in the structure of the microalgae (and cyanobacteria) communities, as reported by Cartaxana *et al*. (2006).

Several techniques can be used to estimate the microphytobenthos biomass, such as: High Performance Liquid Chromatography (HPLC) pigment analysis (Brotas & Plante-Cuny, 2003; Cartaxana *et al*,. 2006), Pulse Amplitude Modulated (PAM) fluorometry (Komkamp *et al*., 1998; Consalvey *et al*., 2005; Jesus *et al*., 2005; Serôdio *et al*., 2005) as well as chlorophyll *a* extraction and analysis by spectrophotometry (Migné *et al*., 2004; Koh *et al*., 2007) or fluorometry (Riaux-Gobin & Bourgoin, 2002). The first techniques (HPLC and PAM fluorometry) seem very promising but require specific knowledge and equipment. Besides, HPLC is probably the only method that really allows the measurement of the pure pigment chlorophyll *a* (Jeffrey *et al*., 1997). Other methods may have contamination in the measurements caused by other pigments. In this paper the term chlorophyll *a*,

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will be used although it is acknowledge that this does not represent the pure pigment. Spectrophotometry and fluorometry are very useful for rapid and reliable measurements. Nevertheless, a standard method for benthic chlorophyll *a* does not exist and extraction is a crucial step. This step has been widely discussed in the literature for phytoplankton, however for benthic algae less reports are available (*e.g.* Tett *et al*., 1978; Hagerthey *et al*., 2006; Devesa *et al*., 2007). No solvent can provide complete extraction efficiency, although 90% acetone has been cited as providing a reasonable value (90%, Van Leeuwe *et al*., 2002) and has been used in the majority of algal studies (Strickland & Parsons, 1972; Garrigue, 1998; Wiltshire *et al*,. 2000; Van Leeuwe *et al*., 2002; Tada *et al*., 2004; Grinham *et al*., 2007). In addition, the use of acetone also allows the use of accurate spectrophotometric equations (Jeffrey *et al*., 1997; Ritchie, 2006). Nevertheless, some authors have suggested other solvents for benthic algae such as: methanol (Tett *et al*., 1975; Tett *et al*., 1977; Hagerthey *et al*., 2006; Cibic *et al*., 2007; Devesa *et al*., 2007) and ethanol (Sartory & Grobbelaar, 1984; Rowan, 1989; Ritchie, 2006). The equations developed for spectrophotometry using ethanol and methanol as solvents are not widely accepted and used. The efficiency of extraction varies with species composition (Wasmund *et al*., 2006; Ritchie, 2008); therefore the methodology should be tested and adjusted for each system (Jeffrey *et al*., 1997). Another important aspect to take into consideration is practicality and safety. Acetone is highly flammable, narcotic in large concentrations and attacks polystyrene. This could be an issue if the cuvettes are made of this material. Ethanol is flammable as well, but safer than acetone. Furthermore, it does not attack polystyrene. Methanol is extremely toxic by inhalation or skin contact and attacks polystyrene. In addition, some authors (Wiltshire *et al*., 2000; Van Leeuwe *et al*., 2002) have indicated that certain treatments can improve the efficiency of the extraction, such as the addition of fine inert granules of quartz to the samples or an ultrasound bath treatment.

The aim of this study was to develop an optimal methodology for chlorophyll *a* analysis of the microphytobenthos of Ria Formosa, so that it could be applied to MPB ecological investigations. A reliable and feasible (in terms of time consumption) method is essential for ecological studies, such as the assessment of spatial patchiness or seasonal cycles. The optimization was done by assessing the effectiveness of pre-treatments and testing of different solvents, concentrations and extraction times in two sediment types, mud and sand.

Material and Methods

Study Site

This study was carried out in Ria Formosa, a shallow mesotidal lagoon located in the south of Portugal, extending along the eastern part (36º58'N, 8º02'W to 37º03'N, 7º32'W, Newton & Mudge, 2003). It has an extension of 55 km (E-W, from Ancão to Cacela) and a maximum width of 6 km (N-S, Newton & Mudge, 2003). The lagoon covers an area of 100 km^2 (Asmus *et al.*, 2000) with a mean depth of 1.5 m (Nobre *et al.,* 2005). During low water, the exposed area may be half the total area of the lagoon. The tidal range varies from 1.3 on neap tides to 3 m on spring tides (Instituto Hidrográfico, 1986).

Standard Method

In July 2006 several sediment samples (between 600–800 g total wet weight) were collected during low water in two intertidal areas: Ramalhete and Ponte. Ramalhete is a flat consolidated area with medium/fine sand (Table 1; following the classification of Holme & McIntyre, 1984). Ponte is a soft, dynamic area with ripples composed by muddy sand (following the same classification as before). Both are intercalibration sites for the implementation of the Water Framework Directive. The samples were collected with a Petri dish of 47 mm diameter and 13 mm height to ensure that only the top layer, with higher chlorophyll *a* content, was taken. A plastic card was used to manoeuvre underneath the sample. The samples were placed in 1 dm^3 plastic bottles wrapped in aluminium foil and transported in a cool box to the laboratory, always protected from light and high temperatures (and thus protecting chlorophyll *a* from being degraded).

Table 1 - Grain sizes distribution (%) and organic matter (%) of samples obtained at Ramalhete (A), Ponte (mud; B) and Ponte (sand; C).

Sediment size fractions (%)	Ramalhete	Ponte - mud	Ponte - sand
> 1000 µm	2.21	2.49	2.04
1000 - 710 µm	6.09	1.25	2.38
710 - 500 µm	13.34	2.13	3.07
500 - 355 µm	18.79	2.54	3.67
355 - 250 µm	8.99	2.20	5.56
250 - 180 µm	3.11	3.51	12.15
180 - 125 µm	1.34	23.17	26.17
125 - 90 µm	0.53	8.39	3.65
90 - 63 µm	0.48	4.19	1.63
< 63 µm	45.12	50.13	39.68
Organic matter (%)	1.54	2.27	1.62

As soon as possible, the plastic bottles were hand-stirred thoroughly to ensure homogeneous chlorophyll *a* content in each of them. Then, the sediment within each bottle was divided in as many homogeneous samples as necessary for the analysis (Table 2). Each sample was placed in 50 cm^3 plastic tubes, covered with aluminium foil. All samples were freeze-dried for 30 hours to avoid any potential errors arising from the water content within the sediment (Buffan-Dubau & Carman, 2000). Freeze-drying importance was tested by Buffan-Dubau & Carman (2000) and by Van Leeuwe *et al*. (2006), using methanol and acetone. Both studies clearly yielded and increased extraction efficiency after freeze-drying. The weight of the sediment was determined after freeze-drying. The solvent (90% acetone stored over sodium bicarbonate) was added to each sample, keeping a constant proportion of solvent volume to sediment weight and the tubes were agitated in the vortex. The samples were placed in the freezer at -20 ºC for 30 hours. Afterwards, the tubes were centrifuged for 10 min at 3000 *rpm*. This method was adapted from Parsons *et al.* (1984) to measure chlorophyll *a* and phaeopigments using Lorenzen's equations (Lorenzen, 1967). The samples were measured in 1 cm path length spectrophotometer glass cells at 663 and 750 nm wavelengths against a 90% acetone blank. Two drops of 1.2 M HCl were added and the samples were measured again. In August 2007, additional samples were collected following the same procedure to repeat the methodological approach. This time, 'sand' samples were collected from an area with fine sand (Table 1; following the classification of Holme & McIntyre, 1984) next to the area used to collect muddy sand at Ponte. The new site is similar to Ramalhete in morphological terms. This modification was carried out to ensure that any potential differences obtained in the analysis were not due to the fact that the sites were different, but was instead related to the different types of sediment.

Methodological experiments

Pre-treatments

The effectiveness of treating the samples in the ultrasound bath was tested by submitting eight of the sixteen muddy samples to an ultrasound bath (following Wiltshire *et al.,* 2000) for 1.5 hours after the addition of the solvent as described below. The effectiveness of fine granules in the extraction was tested by adding fine inert granules (63 µm to 250 µm) to five of ten muddy samples. The granules added were collected from sediment samples from Ponte by sieving. In the laboratory, the sieved samples were placed in the muffle at 475 ºC for 4 hours to remove the organic matter, were treated with a strong acid bath (concentrated HCl) and subsequently washed and dried. In the test of both pre-treatments 90% acetone buffered with sodium bicarbonate was used.

Table 2 - Description of the methodological experiments: test of the effectiveness of pre-treatments and test of the optimal methodology

		control	Treatment 1	Treatment 2	Treatment 3	Source of material	Subsamples
Pre-treatments		standard	inert granules	N/A	N/A	Ponte, Jul-06, MS	5, 5
		standard	ultrasound bath 1.5 h	N/A	N/A	Ponte, Jul-06, MS	8, 8
Optimal Methodology	Type of solvent	standard	extract in 90% ethanol/water	extract in 95% methanol/water	N/A	Ponte, Jul-06, MS	5, 5, 5
		"	"	"	N/A	Ramalhete, Jul-06, S	5, 5, 5
		"	"	"	N/A	Ponte, Aug-07, MS	5, 5, 5
		"	"	"	N/A	Ponte, Aug-07, S	5, 5, 5
	Solvent concentration	standard	extract in 95% acetone/water	extract in 80% acetone/water	extract in 70% acetone/water	Ponte, Jul-06, MS	5, 5, 5, 5
		"	"	"	"	Ramalhette, Jul-06, S	5, 5, 5, 5
		"	"	"	"	Ponte, Aug-07, MS	5, 5, 5, 5
		"	"	"	"	Ponte, Aug-07, S	5, 5, 5, 5
	Time of extraction	standard, except extract 48 hr	extract 24 hr	extract 12 hr	extract 1 hr	Ponte, Jul-06, MS	5, 5, 5, 5
		"	"	"	"	Ramalhette, Jul-06, S	5, 5, 5, 5
		"	"	"	"	Ponte, Aug-07, MS	5, 5, 5, 5
		"	"	"	"	Ponte, Aug-07, S	5, 5, 5, 5

Obs.- Treatment as standard except if stated
MS - Muddy Sand S- Sand

Optimal methodology

In order to ascertain the best solvent, three solvents were tested at the strengths recommended in the literature, namely 90% acetone (Garrigue, 1998; Miles & Sundbäck, 2000; Riaux-Gobin & Bourgoin, 2002), 90% ethanol (Sartory & Grobbelaar, 1984; Papista *et al*., 2002) and 95% methanol (Marker, 1972). The conditions used for testing the best solvent concentrations and the best time of extraction were chosen based on an extensive review of the literature. This study was performed following several steps, using the most appropriate options in the process. For example, to test the optimal concentrations of the solvent, only the solvent that yielded the largest extracts of chlorophyll *a* was used. Fifteen samples were used for the solvent test (5 per solvent), twenty samples were used for the solvent concentration test and twenty samples were used in the assessment of the time of extraction.

For samples with solvent other than 90% acetone, a 10% dilution was carried out in 90% acetone, so that the spectrophotometric equations for 90% acetone could be used. To obtain the chlorophyll *a* content (μg/g), the weight of the freeze-dried sediment was used in the calculations instead of the usual volume of filtered water when studying pelagic algae.

Statistical Analysis

Data were tested for normality and homoscedasticity of variance and parametric tests conducted, as possible. Otherwise, data were transformed and re-checked. All the statistical tests and numerical

analyses were carried out using Minitab 14. To test the effectiveness of the use of fine granules and the ultrasound bath during the extraction a two sample T-test was carried out. To assess any differences between solvents, solvent concentrations and extraction times, one-way ANOVA tests (significance level of 0.05) were used both for sand and for mud. Multiple comparisons among pairs of means were performed using the Tukey test, when a significant difference was found with ANOVA. The Mann-Whitney non parametric test was used to compare phaeopigment contents obtained in this study, using a significance level of 0.05.

Results

Pre-treatments

The chlorophyll *a* contents obtained when assessing the effectiveness of the ultrasound bath were similar (Figure 1-A), for samples with treatment (11.10 µg/g ± 0.35 SE) and with no treatment (10.72 µg/g ± 0.13 SE). A T-test was used after a transformation of data [cosine(x)] and no significant differences were found ($p = 0.379$) between the two treatments.

The chlorophyll *a* contents obtained when assessing the effectiveness of the addition of fine granules were also very similar (Figure 1-B) both for samples with treatment (with fine granules) and with no treatment. The means obtained were 12.29 (± 0.16 SE) µg/g of chlorophyll *a* for samples with treatment and 12.47 (± 0.16 SE) µg/g of chlorophyll *a* for samples without treatment. Data were found to be normally distributed; therefore a T-test was conducted. No significant differences ($p = 0.438$) were found between the two treatments.

Tests for the best method for extraction

Solvent

Ethanol was the solvent which yielded the lower values of chlorophyll *a* in 2006 (mud - 13.49 µg/g, sand - 9.28 µg/g) and acetone was the solvent which yielded the highest, both for mud and sand (mud – 14.55 µg/g, sand – 11.56 µg/g; Figure 2-A). Nevertheless no significant differences were found (ANOVA) between the chlorophyll *a* contents obtained with different solvents for each sediment type ($p = 0.173$ (mud); $p = 0.069$ (sand)). In 2007, acetone was again the solvent that yielded larger chlorophyll *a* contents (Figure 3–A). The smallest values were obtained using ethanol in sand (5.76 µg/g) and methanol in mud (8.29 µg/g). No significant differences were found (ANOVA) between the chlorophyll *a* contents obtained with different solvents for each sediment type [$p = 0.600$ (mud); $p = 0.935$ (sand)].

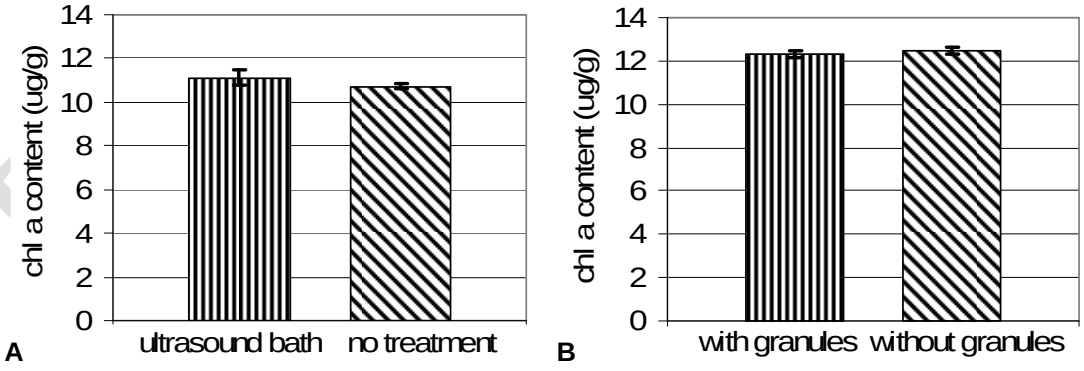

Figure 1- Chlorophyll *a* contends (µg/g, ± SE) obtained in two tests: **A-** test of the effectiveness of the ultrasound bath in the chlorophyll *a* extraction; **B-** test of the effectiveness of the addition of fine granules in the chlorophyll *a* extraction.

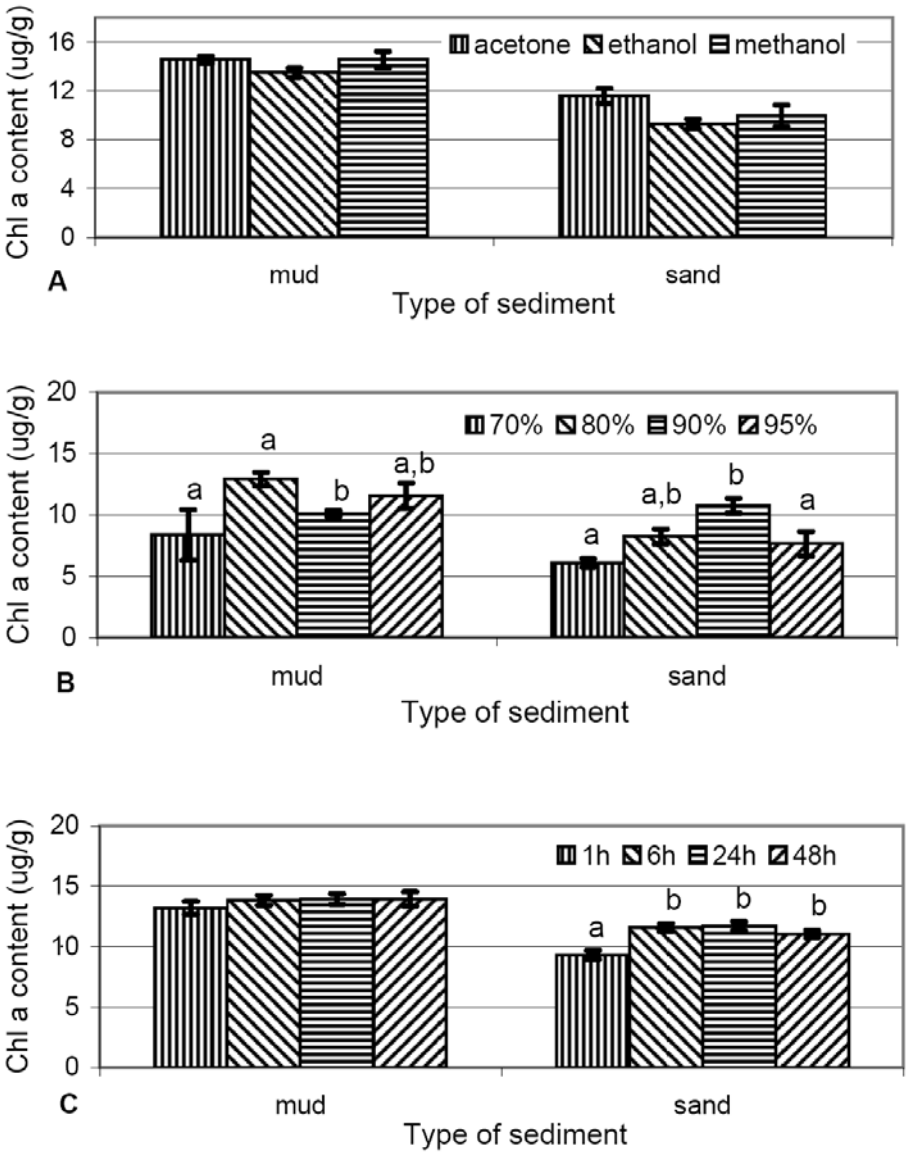

Figure 2- Chlorophyll *a* contents (µg/g, ± SE) obtained in 2006 studies: A- three solvents (90% acetone, 95% methanol and 90% ethanol); B- four concentrations of the solvent, acetone at 70%, 80%, 90% and 95%; C- four extraction times (1h, 6h, 24h and 48h). The symbols a, b, c and d represent significant different groups from Tukey's test.

Solvent concentration

For the optimization of the method, it is also important to know the appropriate concentration of acetone. For the samples collected in 2006, an ANOVA showed significant differences between chlorophyll *a* values obtained using different acetone concentrations ($p < 0.01$ (mud); $p < 0.005$ (sand)). The chlorophyll *a* content obtained using different acetone concentration was lower for 70% acetone, both for mud and sand (Figure 2 - B). The statistical analysis for mud was carried out on transformed data (cosine(x)). A Tukey test was then used and significant differences were found as follows: 90% > 70%, 95% (sand) and 80% > 90% > 70% (mud). Therefore, the values of chlorophyll *a* extracted were larger using 90% acetone for sand and 80% acetone for mud. From the test performed in 2007 (Figure 3 – B), significant differences were found between treatments ($p < 0.001$ for mud and sand; ANOVA). For both sediment types, a Tukey test showed significant differences between the results obtained using 70% acetone (lower extractions) and the other concentrations. The chlorophyll *a* content means were larger using 90% acetone for sand and 80% acetone for mud, as obtained in 2006.

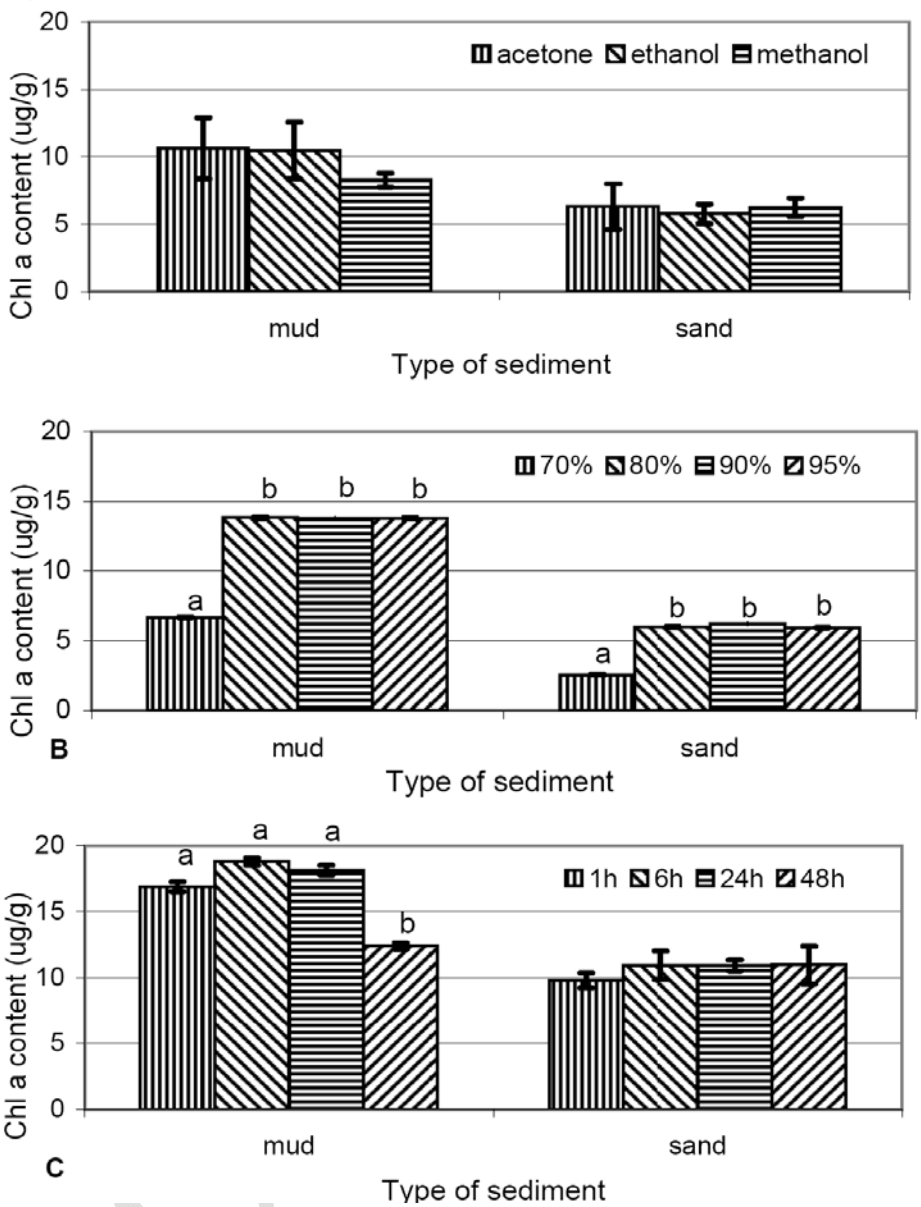

Figure 3- Chlorophyll *a* contents (µg/g, ± SE) obtained in 2007 studies: A- three solvents (90% acetone, 95% methanol and 90% ethanol); B- four concentrations of the solvent, acetone at 70%, 80%, 90% and 95%; C- four extraction times (1h, 6h, 24h and 48h). The symbols "a" and "b" represent significant different groups from Tukey's test.

Extraction time

The test of the best extraction time performed on the samples collected in 2006 showed a similar pattern for mud and sand (Figure 2-C). The lower values were obtained after only one hour of extraction with the values similar for larger times of extraction. Significant differences were found between the chlorophyll *a* values obtained with the four extraction times for sand (p = 0.001). No significant differences were found between extraction times for mud (p = 0.678) after a cosine (1-x) transformation. A Tukey test was used to check the temporal differences for sand and significant differences were found between 1 hour and all the other levels of treatment. No significant differences were found between 6 h, 24 h and 48 h. During the 2007 test, a different pattern for mud and sand was found (Figure 3-C). The results for sand were similar to the ones obtained in 2006 (sand), but no significant differences were found. A smaller mean was obtained with 1 hour of extraction. An ANOVA showed significant differences between the extraction times for mud (p = 0.001). A Tukey test showed significant differences between the results obtained 48 hours after the extraction (smaller) and the other levels of treatment.

Table 3– Phaeopigment concentrations (µg/g) and Phaeopigment / Chl. *a* ratios observed in all tests carried out. Best option indicated in the table corresponds to the treatment that yielded the highest chl *a* concentration or simply the one recommended for future use.

		Minimum		Maximum		Mean		
		conc. (µg/g)	phaeop / chl ratio	conc. (µg/g)	phaeop/ chl ratio	conc. (µg/g)	phaeop/ chl ratio	phaeop/chl best option
Pre-treatments								
	Fine inerts	9.76	0.76	13.16	1.12	11.69	**0.95**	
	Ultrasound bath	10.63	0.86	15.53	1.49	14.5	**1.34**	
Optimal methodology (06)								
Mud	Solvent	0.26	0.2	6.79	0.48	3.9	0.23	**0.42 (acet)**
	Solvent concentration	1.21	0.17	11.13	1.17	6.93	0.62	**0.52 (80%)**
	Extraction time	3.89	0.29	8.43	0.718	5.85	0.43	**0.43 (6 h)**
Sand	Solvent	0.05	0.01	2.89	0.32	1.2	0.12	**0.16 (acet)**
	Solvent concentration	0.08	0.01	4.6	0.78	1.71	0.23	**0.14 (90%)**
	Extraction time	0.03	0.02	3.02	0.38	0.14	0.15	**0.14 (6h)**
Optimal methodology (07)								
Mud	Solvent	0.24	0.02	15.6	3.62	4.54	0.73	**0.6 (acet)**
	Solvent concentration	1.25	0.19	7.75	0.55	4.59	0.37	**0.31 (80%)**
	Extraction time	0.46	0.03	9.2	0.61	3.47	0.21	**0.15 (6h)**
Sand	Solvent	0.11	0.02	5.43	1.1	2.14	0.64	**0.5 (acet)**
	Solvent concentration	0.3	0.05	3.6	0.7	1.07	0.24	**0.23 (90%)**
	Extraction time	0.13	0.01	3.18	0.34	0.99	0.1	**0.10 (6h)**

Phaeopigments

The phaeopigment contents found in the pre-treatments were high, around twice the values obtained during the other tests (Table 3). The ratio between phaeopigments and chlorophyll *a* was around 1, which means that contents were similar. Smaller phaeopigment contents were obtained for sandy sediments when compared with mud. Significant differences were found between phaeopigment contents from pre-treatments and other tests ($p < 0.001$). Considering solely the tests for the optimal methodology, significant differences were also found between phaeopigment contents found in sand and mud ($p<0.001$).

Mud vs Sand

Six two-sample T-tests were carried out to compare the chlorophyll *a* contents of muddy and sandy samples using all the samples of each of the 6 tests. Given that data were used for another statistical test, the Bonferroni correction was used in this analysis and a significance level of 0.025 was considered. The results showed larger values for mud with a p-value ≤ 0.005 for each test.

Discussion

Pre-treatments

Both pre-treatments, addition of fine granules and ultrasound bath, did not show any significant differences between treated and non treated samples. The use of these methodologies was a consequence of the need to achieve a more efficient extraction of pigments, mainly by breaking down the algal cell walls. These two pre-treatments were tested by Wiltshire *et al*. (2000) in *Scenedesmus* sp. which is a 'difficult to extract' alga and yielded significant larger results. Although some authors, such as Schagerl & Künzl (2007), have considered that cell wall disruption by pre-treatment is

essential, others like us, have found no differences with or without the treatments (Sartory & Grobbelaar, 1984; Schumann *et al*., 2005; Hagerthey *et al*., 2006). This may be the result of the non-existence of 'difficult to extract' species in Ria Formosa. Another aspect that may have improved the extraction efficiency is the freeze-drying that was performed to eliminate water dilution problems. This procedure may help with the breakdown of the protein matrix of membranes and thus facilities the penetration of the solvent (Buffan-Dubau & Carman, 2000), as well as decreasing the chlorophyllase enzyme activity by reducing the water content (Van Leeuwe *et al*., 2006). Nevertheless, the phaeopigment content found after performing these pre-treatments suggests that no benefit comes from this approach. Besides not improving the extraction efficiency, these pre-treatments also provided much higher contents of chlorophyll degradation products. The comparison with the other tests provides indication that these degradation products were a consequence of the method itself due to material handling.

Tests for the best method for extraction

Solvent

In the tests of the samples collected in 2006 and 2007, acetone yielded the largest mean value, both for mud and sand, as indicated previously by Conde *et al*. (1999), Miles & Sundbäck (2000) and Migné *et al*. (2004). Van Leeuwe *et al*. (2006) discussed how the efficiency of the extraction may be species dependent. For example, they observed an efficiency 50% higher using acetone to extract chlorophyll *a* from the diatom *Thalassiosira weisfloggi* than using methanol (Van Leeuwe *et al*., 2006). A natural algal community is a mixture of different species that will most likely have different individual proportions through the year. This may affect extraction efficiency. In addition, ethanol and methanol are well known to produce chlorophyll *a* artifacts (Ritchie, 2006; Schagerl & Künzl, 2007). These artefacts are modifications of the original chlorophyll *a* pigment and have different spectral characteristics. One problem concerns the enzyme chlorophyllase that releases the phytol group of chlorophyll *a*. This enzyme is inhibited in large concentrations of acetone, while in methanol and ethanol it is still active (Ritchie 2006). Moreover, the accepted and widely used spectrophotometric equations are a relevant point of favour to acetone (Jeffrey *et al*., 1997). The problems coming from the fact that acetone is highly flammable and does attack polystyrene are solved if acetone is handled in a fume cupboard and glass cuvettes are used. So, as discussed by Wasmund *et al.* (2006), the correct solvent to use depends on several aspects, one being the taxonomic composition of the algal community.

Solvent concentration

Ninety percent acetone showed the largest means in 2006 (significant differences) and 2007 for sand. These results are in agreement with Van Leeuwe *et al.* (2006), working on microphytobenthos, as well as many others using phytoplankton. It is also a useful solvent since it has the most used spectrophotometric equations. For muddy sediment, a concentration of 80% of acetone yielded the largest contents of chlorophyll *a* for the samples of 2006 (significant differences) and 2007. This strength was initially used by Mackinney (1941). It was then used for several years and was a reference for many researchers working with algae (Margulies, 1970; Porra, 2002). Its importance decreased with new findings on extraction efficiency using different concentrations and other solvents. Our results suggest that the extraction methodology should always be adapted and optimised for each location. As stated before, communities composition may be a major factor in this procedure.

Extraction time

The tests performed during 2006 and 2007, for both sandy and muddy sediments, showed that 6 hours was sufficient for an efficient extraction. The goal is to do the extraction as quickly as possible, in order to get the maximum chlorophyll *a* possible and the minimum value of chlorophyll *a* artifacts (Hagerthey *et al*., 2006; Van Leeuwe *et al*., 2006). Lengthy extraction periods may increase the degradation products (Buffan-Dubau & Carman, 2000; Hagerthey *et al*., 2006). The period needed for the chlorophyll *a* extraction also depends on the species compositon, as indicated by Hagerthey *et al*. (2006). For example, using 100% acetone, Cartaxana & Brotas (2003) found a 2% difference in chlorophyll *a* results from 6 hours to a 24 hours extraction period. Buffan-Dubau & Carman (2000)

found a difference of 18% for the same conditions. A difference in the communities between 2006 and 2007 might be the reason why we observed a significant decrease in the chlorophyll content in 2007 for mud. Several paths have been suggested to explain the production and degradation cycles of the chlorophyll pigments, which are commonly complex and interdependent (see for example Porra & Sheer, 2000 and Van Leeuwe *et al*., 2006 about chlorophyll degradation). Thus, it is not possible to identify exactly what was the difference in the communities or the degradation pathway which took place in these instances. As before, taxonomic studies of algal community would be key component to understand these processes.

Phaeopigments

The evaluation of phaeopigment contents is especially important in sediments and particularly in mud, as they generally have a larger contribution of detritus and therefore detrital chlorophyll. If the ratio between phaeopigments and chlorophyll is high, it is likely that the main contributors of chl *a* are not living cells. The overall content of phaeopigments in these sediments is considered to be relatively small. They are mainly present in muddy sediments, which was expected. Collos *et al*. (2005) indicated that all non-degraded plant systems have a phaeopigment percentage of around 4%. In their study they reported phaeopigment/chlorophyll *a* ratios from 0.17 to 1.86 (autumnal decay) for phytoplankton. Several other authors, such as Sun *et al*. (1994), Rabalais *et al*. (2004) and Reuss *et al*. (2005) reported phaeopigment/chlorophyll *a* ratios larger than 1 in sediments. This ratio can express an indication of the functional state of the algae community, being high when the community is decaying (Collos *et al*., 2005). A ratio of 1, which means that phaeopigment and chlorophyll contents were similar, suggests that part of the chlorophyll measured was extracted from non living cells. However, it is important to keep in mind that some methods, as ours, do not take into account the content of other types of chlorophyll, which may lead to an increased estimate of phaeopigments (Jeffrey *et al*., 1997). For a deeper understanding, the oxygen conditions of the sediments should be known, as they might indicate if the chlorophyll *a* is accumulating in a stable form and therefore estimates being biased (Sun *et al*., 1993a; Sun *et al*., 1993b). Moreover, according to Sun *et al*. (1993b), chlorophyll *a* degradation is temperature dependent under oxic conditions, being higher for high temperatures. This suggests that for sites such as Ria Formosa, where the sediments can reach very high temperatures, the chlorophyll associated with detritus should be rapidly degraded. Therefore, we do think that the ratios obtained during this study are within reasonable ranges and that most of the chlorophyll contribution is actually coming from living MPB cells.

Mud vs Sand

Significant differences were consistently found between the values of chlorophyll *a* in muddy and sandy sediments. Muddy sediment samples always had a larger content. These samples were taken from the top (1cm) of the sediment and this result is in accordance with the literature (Cartaxana *et al*., 2006). These authors suggested that both mud and sand have similar chlorophyll *a* concentrations, however, in muddy sediments, cells are mainly at the top, while in sandy sediments, chlorophyll *a* is present deeper, with the concentration at the top tending to be smaller.

Comments and Recommendations

Since chlorophyll *a* content has been widely used as an indicator of water quality and the trophic status of several systems (e.g. Tett *et al*., 2003; Nobre *et al*., 2005; Yoshiyama & Sharp, 2006), the need to obtain accurate results is extremely important. It is most likely that different algal taxa may yield different extraction efficiency (Papista *et al*., 2002). It is worth to investigate the biotic and abiotic characteristics of studied sites before adjust and establish the methodology.

Finally, for future studies on the same conditions, our recommendations are to use 90% acetone for sand and 80% acetone for mud with no pre-treatments. The extraction should be performed during 6 hours or between 6 and 24 hours.

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