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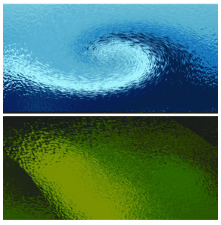

RESEARCH ARTICLE

Selecting an HPLC method for chemotaxonomic analysis of phytoplankton community in Mediterranean coastal lagoons

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Abstract

1 - Phytoplankton observations are commonly used to contribute to the assessment of aquatic ecosystem health and their trophic status. Compared to other methods, chemotaxonomic analysis based on High Pressure Liquid Chromatography (HPLC) presents many advantages (e.g., rapidity, reproducibility, and capacity to include pigments from all cell sizes), but its use in coastal lagoons studies is still not very common. The method of Wright *et al.*, (1991) recommended by the UNESCO (Jeffrey *et al.*, 1997) and most frequently used for phytoplankton analysis in coastal lagoons, so far, was selected and compared to the more novel method of Hagerthey *et al.* (2006).

2 - The two methods that differed slightly with respect to their solvent gradients during chromatography (mobile phase) and column (stationary phase), were tested using a pigment mix from DHI Water and Environment comprising 30 different pigments. Extraction methods were tested using replicates of 1l of sub-surface water from the Thau lagoon (South of France), sampled in June 2013. Optimization of the extraction was performed by testing different volumes of solvent (2 to 5 ml), different solvents based on a mix of methanol, acetone, dimethylformamide, water, compared to acetone 90% and pure methanol, as well as different extraction times (10 min to 2 h), and the addition of the ion-pairing agent tetrabutyl ammonium acetate hydroxide (TBAA).

3 - The second method of analysis allowed better separation and resolution of most of the pigments, especially of lutein and zeaxanthin. The early-eluting most polar pigments and the more hydrophobic pigments eluting in the end of the chromatogram (chlorophylls and carotenoids) showed also better separation and peak shapes. 5 mL of the mix of acetone/ methanol/ water (45:45:10) allowed the best extraction of the pigments. The use of TBAA showed negative effects.

4 - For pigment analysis in coastal lagoon, our final protocol used 1 h extraction with 5 mL of acetone/ methanol/ water, and analysis with the gradient from Hagerthey *et al.* (2006). On our analytical equipment it needed some adjustments. It uses a longer chromatography run and quantified the phytoplankton pigment markers better than the method of Wright *et al.* (1991).

Keywords: HPLC, Chemotaxonomy, Phytoplankton Pigment analysis, C18, Ternary gradient, Coastal lagoon

Introduction

Phytoplankton constitutes a basis for aquatic food webs (Pace *et al.*, 1999), and in most of the coastal lagoons it represents a major source of energy and organic matter for the other trophic levels both in the pelagic and the benthic compartments (Bec *et al.*, 2005; Grami *et al.*, 2008). Coastal lagoons are vulnerable to eutrophication (Viaroli *et al.*, 2008; Zaldivar *et al.*, 2008; Cartaxana *et al.*, 2009) to a degree that depends on the nutrient loading from their watersheds, their hydro-morphological characteristics and water residence times. Nutrient over-enrichment has been a major problem during the last fifty years, due to strong demographic and economic developments in the coastal zone, which has resulted in a strong degradation of many of these ecosystems (Cloern, 2001; Bricker *et al.*, 2003; Paerl *et al.*, 2003 ; Carlier *et al.*, 2008). These environmental conditions are reflected by phytoplankton biomass (Souchu *et al.*, 2010) and biodiversity (Ptacnik *et al.*, 2008; Palffy *et al.*, 2013). Because of their rapid turnover compared to other autotrophic compartments, phytoplankton populations respond most quickly to environmental change, by variations of their biomass and taxonomic and functional diversity (Cloern, 2001; Paerl *et al.*, 2003; Cloern and Jassby, 2008; McQuatters-Gollop, 2008). Therefore, phytoplankton studies are of paramount importance for understanding aquatic ecosystem functioning and dynamics (Duarte and Cebrian, 1996), and many phytoplankton studies have been published in this journal. For these reasons, many monitoring programs and studies of eutrophication in coastal waters have focused on the phytoplankton compartment (Coelho *et al.*, 2007; Devlin et al., 2007; Campbell *et al.*, 2008). For the case of French Mediterranean coastal lagoons, a Lagoon Monitoring Network (Réseau de Suivi Lagunaire, RSL) operated a monitoring program from 2000-2013, which included yearly monitoring during the summer periods of phytoplankton and water column chemistry of 24 lagoons in the Languedoc-Roussillon region. For this program, phytoplankton biomass was estimated by the chlorophyll *a* concentration (Souchu *et al.*, 2010), and flow-cytometry has been used for obtaining cell counts of nano- and picophytoplankton abundances (Bec *et al.*, 2011). Chemotaxonomic analysis of lipophilic pigments based on the use of High Pressure Liquid Chromatography (HPLC) has been added in 2006 to gain more insight in the taxonomic diversity of the phytoplankton.

In oceanography, chemotaxonomic analysis with HPLC has been developed since the 1980's to assess phytoplankton pigment diversity in aquatic ecosystem. Frequently used for marine waters (Wright *et al.*, 1991; Mantoura and Llewellyn, 1983; Goericke and Repeta, 1993; Zapata *et al.*, 2000; Goericke, 2002; Vidussi *et al.*, 2011), it has been adopted and sometimes modified for phytoplankton studies in coastal, estuarine (Rodriguez *et al.*, 2003; Heil *et al.*, 2007; Carreto *et al.*, 2008; Seoane *et al.*, 2011), and lacustrine systems (Descy *et al.*, 2000; Schlüter *et al.*, 2000; Greisberger and Teubner, 2007). But except a few examples (Lohrenz *et al.*, 2003; Paerl *et al.*, 2003, 2007; Cartaxana *et al.*, 2009; De Wit *et al.*, 2012), so far HPLC pigment analysis has not been often applied in coastal lagoon ecosystems, and this is the first report published in this journal.

Chemotaxonomic analysis based on HPLC has numerous advantages. While microscopic analysis of phytoplankton samples is long, requires good skills and expertise, and is prone to subjectivity depending on the operator (Schlüter *et al.*, 2000), HPLC chemotaxonomic analysis presents a good compromise. First of all, this method is rapid, reproducible, and can be used in a routine procedure. It allows the identification of pigments that are specific biomarkers for some of the major taxonomic

groups, like Dinophytes, Chlorophytes, diatoms, Prasinophytes, and Cryptophytes. Hence, it provides us a good indication of phytoplankton community structure (Jeffrey *et al.*, 1997; Paerl *et al.*, 2003). HPLC analyses encompasses all the phytoplankton cell sizes in the sample, while microscopy does not allow the identification of the smallest cells, i.e. the picophytoplankton (Breton *et al.*, 2000), and flow cytometry can not count the largest cell sizes, i.e. the larger microplankton and the mesoplankton.

The aim of this study was to select an HPLC protocol that is both practical and performant for the pigment analysis in coastal lagoons. In addition, we wanted to optimize the pigment extraction protocol. In the first place, we considered the method described by Wright *et al.* (1991) because it was recommended by the Scientific Committee on Oceanic Research (SCOR) working group 78 on the Determination of photosynthetic pigments in seawater and described in detail in a UNESCO monograph (Jeffrey *et al.*, 1997). This method has been used for phytoplankton analysis in coastal lagoon in scientific studies (Lohrenz *et al.*, 2003; Paerl *et al.*, 2002, 2007), such as the Lagoon Monitoring Network. The method of Wright *et al.* (1991) offers numerous advantages, i.e. it provides a good resolution of 40 algal carotenoids and 12 chlorophylls and their derivatives, and it is simpler than most other methods (e.g., no need for adding a ion pairing reagent or a methylation reagent, cf. Zapata *et al.*, 2000; Airs *et al.*, 2001a), is less expensive, and allows the identification of biomarkers for many of the major taxonomic groups typical for marine waters, like Haptophytes, Dinophytes, Chlorophytes, diatoms (Wright *et al.*, 1991). This method has also been used for coastal waters where it has provided good results despite their environmental variability, as e.g. covering the entire range from oligotrophy to hypertrophy (Paerl *et al.*, 2003; Barocio-Leon *et al.*, 2006; Vilicic *et al.*, 2008 ; Paerl *et al.*, 2010). However, in comparison with more recently developed HPLC methods (Garrido and Zapata, 1996; Van Heukelem and Thomas, 2001; Hagerthey *et al.*, 2006; Louda, 2008), the method of Wright *et al.* (1991) presented some disadvantages. Some markers pigments, like lutein and zeaxanthin (Chlorophytes, Cyanobacteria), or some chlorophylls pigments co-elute (Metaxatos and Ignatiades, 2002). Carotenoids and phaeopigments are also not well identified. So, on the basis of the HPLC literature since 1991, we tried to select and optimize an HPLC protocol. Without fundamentally changing the chromatographic principle of the method (reverse phase chromatography on a C-18 column) we aimed to select a closely related method, which showed a better performance for the pigment analysis. Particular care was taken to maintain the same order of elution of the pigments as in the method of Wright *et al.* (1991). In addition, we aimed at optimizing all steps of the extraction protocol. The precision and the accuracy of pigment content are ultimately regulated by the method used to extract pigments from the cell. In addition, the extraction solvent needed to be compatible with the HPLC chromatography protocol. For this optimization, the differences in the composition of cell walls and in pigment polarity were considered, as well as the toxic or flammable properties of some solvents. The aim of optimizing the extraction protocol was to use an efficient solvent, mechanical disruption and extraction time to produce a solvent matrix that minimizes the formation of degradation products and stabilize pigments, while being the least toxic for users (Hagerthey *et al.*, 2006).

Materials and methods

Equipment and Software

We used a Waters D600 equipment, including

a quaternary solvent delivery system (600 controller 600 pump), an in-line degasser AF, a 717 plus Autosampler with autoinjector. Chlorophylls and carotenoids were detected by a Waters 2996 photo-diode array detector (optic resolution 1.2 nm) from 400 to 700 nm. Chromatography software (Empower Pro3) allowed the monitoring and the extraction of chromatograms at any selected wavelength. We have chosen 440 nm as a compromise for detecting chlorophylls and xanthophylls as well as their derivatives. However, "a posteriori" the spectral signature of each peak can be obtained at each selected retention time. Each peak was checked and readjusted to minimize errors due to noise. A preliminary identification of the pigments was based on an automated comparison with a spectral library previously created from pigment standards. The HPLC system was regularly calibrated with external standards (pigment standards from DHI Water and Environment, Hørsholm. Denmark) and pigments were quantified by using pigment response factors (Mantoura and Repeta, 1997). Finally, the pigment identification was controlled by (i) checking its position in the elution order, and (ii) comparing its absorption spectra with that observed for the corresponding standard and described by Roy *et al.* (2011).

Field samples

Lagoon water was obtained from the Thau lagoon, in the South of France (N 43° 26' 412; E 003° 39' 742; 3 m depth). Three hundred liters of water were collected at sub-surface (20 cm depth) on 12 June 2013. Samples were obtained by filtration of 1l water through 47 mm diameter Whatman GF/F filter (0.7 µm nominal pore size). Filters were blotted from below to absorb water while the filtrate on top remains in place, folded with precision wipes (Kimtech Science) (Wright, 2005). They were then stored in 10 ml polypropylene tubes at -80°C prior to HPLC analysis.

Extraction protocols and tests for optimizing extraction yield

The extraction solvent was added to the polypropylene tubes with the filters (see above). The filters soaked in the extraction solvent were sonicated 5 times, by immersing the tip of a sonic probe below the surface of the liquid 10 sec (20 Watts). Each sonication was followed by 10 sec of storage on ice to avoid excessive heating of the extract. These manipulations were performed in very dim light to prevent pigment degradation. Subsequently, these extracts were stored in darkness at 4°C for the different durations listed below (Table 1. Factor 4 = extraction time).

The extracts were filtered on 0.45 µm cellulose acetate filters, and the filtrates were stored in 1.5 ml eppendorf, at 4°C before the injection. 1 ml of extract were then pipetted to HPLC vials with 125 µL of Milli-Q water, to increase the affinity of pigments for the column, resulting in sharper peaks (Wright *et al.*, 1991). 150 µl of these extracts were injected to the HPLC system. We studied how to enhance the extraction efficiency by optimizing the yield of the pigments obtained in the organic extraction solvent. Therefore, we studied the impact of four different factors (Table 1) according a factorial design. One of the factors was the volume of the extraction solvent added (factor 1). It has been argued that lower extraction volumes, as e.g. 2 ml (Zapata *et al.*, 2000), may be useful to concentrate the extract (Wright *et al.*, 1991). It was compared to 2.5 ml as used for the Lagoon Monitoring Network method, and a higher extraction volume i.e. 5 mL (Eker-Develi *et al.*, 2012). The type of solvent and the addition of the ion-pairing reagent were the two factors related to the composition of the extraction solvent. Therefore, we studied the impact of four different factors (Table 1) according a factorial design. One of the factors was the

Table 1. Factors tested for improving pigment extraction (factors 1-4) compared to the extraction treatments that have been used most often in combination with the HPLC protocol according Wright *et al.* (1991). The tested extraction treatments have been tested both in combination with the selected HPLC protocols of Wright *et al.* (1991) and Hagerthey *et al.*, (2006). In addition, the impact of addition of the ion-pairing

Factors	Parameters	Initial treatment	Tested treatments
1	Volume	2.5 ml	2 ml – 5 ml
2	Composition (mixture) of the Extraction solvent	Methanol (100%) **(solvent 1)**	Acetone / methanol / water (45:45:10) **(solvent 2)** Methanol / acetone / *N-N*-dimethylformamide / water (30:30:30:10) **(solvent 3)** Acetone 90%, water 10 % **(solvent 4)**
3	TBAA addition in extraction solvent	No	Yes – in the extract (+ 125 µl)
4	Extraction time	15 min	10 min – 2 hours
5	TBAA addition in the mobile phase	No	Addition of 28 mM final conc. of TBAA instead of Ammonium acetate

volume of the extraction solvent added (factor 1). It has been argued that lower extraction volumes, as e.g. 2 ml (Zapata *et al.*, 2000), may be useful to concentrate the extract (Wright *et al.*, 1991). It was compared to 2.5 ml as used for the Lagoon Monitoring Network method, and a higher extraction volume i.e. 5 mL (Eker-Develi *et al.*, 2012). The type of solvent and the addition of the ion-pairing reagent were the two factors related to the composition of the extraction solvent. Thus, we compared different extraction solvents (factor 2), and studied the impact of adding the ion-pairing agent tetrabutyl ammonium acetate hydroxide (TBAA) to the extraction solvent, compared to the non-amended control (factor 3). Hence, four extractions solvents were compared (Table 1, factor 2), i.e. (1) methanol, (2) acetone/ methanol/ water (45:45:10), (3) methanol/ acetone/ N-N-dimethylformamide/ water (30:30:30:10) and (4) acetone/ water (90:10) (Jeffrey *et al.*, 1997; Hagerthey *et al.*, 2006; Louda, 2008). For the TBAA treatment, the sample was prepared using 1.0 ml of filtered extract with 125 µL of the ion-pairing solution (Table 1, factor 3). The TBAA was added in the extraction solvent only, without adding it to the mobile phase used for the chromatography (Louda *et al.*, 2000; Hagerthey *et al.*, 2006; Louda, 2008). Finally, the impact of extraction duration (factor 4) was studied by comparison between 2 hours extraction time as initially proposed by Hagerthey *et al.*, (2006) with 10 min as a simplified extraction protocol.

Chromatography, comparison of two methods

We compared the performance of the HPLC protocols described by Wright *et al.* (1991)

and Hagerthey *et al.* (2006). Both are based on the same chromatographic principles (reverse phase chromatography, use of ternary gradient) but differ slightly in their stationary and mobile phases, which are specified below.

Stationary phase

For testing the protocol of Wright *et al* (1991) we used a C18 pre-column (3.9 x 20 mm, 5 µm particle size), coupled to a reversed phase ODS2 C18 Waters Spherisorb column (4.6 x 250 mm, 5 µm particle size). For testing the protocol of Hagerthey *et al.* (2006), we used a NovaPak C18 guard column 2/PK coupled to a reversed phase Waters NovaPak C18 column (3.9 x 300 mm, 4 µm particle size), according to (Louda, 2008). For both protocols, the temperature of the column was controlled at 24°C (560-CIL oven).

Mobile phases

Both methods, i.e., Wright *et al.* (1991) and Hagerthey *et al.* (2006), use ternary gradients specified in Tables 2 and 3. The latter uses a longer time-span for the gradient, and a slightly different A phase (0.5 M ammonium acetate in methanol/water 85:15), as adopted earlier by Louda *et al.* (2000). In addition, we tested the impact of addition of the ion-pairing agent TBAA in the mobile phase (Table 1). Therefore, we replaced the solvent A1 and A2 (Tables 2 and 3), by a modified solvent composed of 70:30 28 mM TBAA:Methanol as is used for the CSIRO method (Hooker *et al.*, 2009). This modification was considered as an independent factor when comparing the two different chromatography protocols and also taken into consideration in the factorial design as factor 5, together with the four factors (Table 1). The composition of the extraction solvent that is injected on the column is expected to interact strongly with the mobile phase particularly during the early phase of pigment elution.

Both methods were calibrated with pigment standards from DHI using four different masses for injections to obtain calibration curves. To compare the separation performances of the two protocols, we analyzed a mixed pigment set from DHI in triplicate for each method (mix-112, DHI, Denmark). The qualitative composition of this pigment mixture was known and described in a certificate by DHI; however, the concentrations of the different pigments were not specified.

Table 2. Solvent profile used with RP-HPLC from Wright *et al.* (1991).

Time (min)	Flow rate (ml min^{-1})	% Solvent A1	% Solvent B	% Solvent C
0	1.0	100	0	0
2	1.0	0	100	0
18	1.0	0	20	80
21	1.0	0	100	0
24	1.0	100	0	0
29	1.0	100	0	0

Table 3. Solvent profile used with RP-HPLC from Hagerthey *et al.*, (2006).

Time (min)	Flow rate (ml min^{-1})	% Solvent A2	% Solvent B	% Solvent C
0	1.0	60	40	0
5	1.0	60	40	0
10	1.0	0	100	0
40	1.0	0	30	70
45	1.0	0	30	70
46	1.0	0	0	100
47	1.0	0	100	0
48	1.0	60	40	0

The resolution of separation between two adjacent peaks (e.g., lutein and zeaxanthin was calculated using the formula below:

$$R_S = 2 . \frac{t_{R1} - t_{R2}}{\omega_1 + \omega_2}$$

Where t_{R1} and t_{R2} were the retention time of the two pigments analyzed, and ω1 and ω2 the peak width at their basis. The peak width was determined as recommended by GXP laboratories, by the Empower software, which calculated the best value, using the *V3.0.X Style Peak and Threshold Determination routine*.

The selectivity of the two methods for the lutein and the zeaxanthin was determined using the formula below:

$$\alpha = \frac{t_{R1} - t_0}{t_{R2} + t_0}$$

Where t_{R1} and t_{R2} were the retention time of the two pigments analyzed and t0 the difference in time between injection and detection of system peak, i.e. the time taken for the mobile phase to pass through the column.

The higher is the selectivity and resolution of a method for a couple of two pigments, the better is the separation and the quality of their quantification.

Statistical analysis

The software R (R Core Team, 2013) and its specific packages were used for statistical analyses. The student test was used for comparing selectivity and resolution for pigment couples in two methods i.e. Wright *et al.* (1991) and Hagerthey *et al.* (2006). To compare the analysis efficiency of the two methods, the concentrations of the pigments in the mixture obtained with the two protocols were compared, and we considered the one that gave the highest values as the best (Student test, R).

The factorial experiments for optimization of the extraction protocols were analyzed according a step-wise approach. Taking into account the whole pool of pigment concentrations measured (chl *a*, chl *b*, chl

*c*2, alloxanthin (allo), fucoxanthin (fuco), peridinin (peri), diadinoxanthin (diadino), prasinoxanthin (prasino), lutein (lut), neoxanthin (neo), zeaxanthin (zea)), we used a non-parametric multifactorial analysis of variance i.e. PERMANOVA (Anderson, 2001). This first analysis allowed us to specify those factors whose levels had a significant effect on the pigment concentrations. Secondly, we then tested the effect of these parameters separately on every pigment concentration, using a parametric multifactorial ANOVA, after checking the conditions of application of this method (normal distribution, homoscedasticity and independence of residuals). Thirdly, when some effects were significant, ANOVA were completed by Tukey posthoc tests to determine significant differences in pairwise comparisons. When the conditions of application of ANOVA were not satisfied, we first used logarithm transformation of the pigment concentration. When conditions were still not satisfied, non-parametric ANOVA were applied to test the effect of every modification of the protocol on the measured pigment concentrations (Kruskal test and posthoc Kruskalmc, R). This sequence was performed for the analysis of the results obtained both with the protocols of Wright *et al*. (1991) and Hagerthey *et al*. (2006), independently.

Results

Choice of a chromatography method for pigment analysis in coastal lagoons

We selected a method that will allow us to easily compare the chromatograms with those earlier obtained by using the method described by Wright *et al.* (1991), and that does not introduce specific problems in the laboratory. Hence, some methods have been discarded because of the use of very toxic or explosive chemicals as e.g. pyridine (Zapata *et al.*, 2000) and diazomethane (Airs *et al.*, 2001a; de Wit *et al.*, 2012), respectively. All protocols that use a column that differs from the C18 column, like a C8 column (Barlow *et al.*, 1998; Zapata *et al.*, 2000; Van Heukelem and Thomas, 2001), or employ a very long chromatography (Airs *et al.*, 2001a) were also discarded. We finally selected the protocol described by Hagerthey *et al.* (2006) as it is based on the same chromatographic principles and showed the same elution order for the pigments as the method of Wright *et al.* (1991). However, before selecting a final protocol, we compared the addition of 0.125 mL TBAA solution (in 1 ml of extract prior to analysis) with a simplified extraction protocol without adding ion-pairing agent in the extract.

The performances of both methods, i.e., Wright *et al.* (1991) and Hagerthey *et al.* (2006), were compared using the same pigment mixture from DHI (Fig. 1). Firstly, while the elution order was indeed the same for both methods (Table 4), the number of identified pigments was slightly different. Hence, we did not observe 19'Hex-fucoxanthin and dihydrolutein with the method from Wright *et al.* (1991) (Fig. 1, Table 4). Secondly, the method of Hagerthey *et al.* (2006) showed a better quality of the chromatogram for early-eluting compounds and thus improved the recognition and peak-shape of the most polar pigments (Fig. 1). Thirdly, the method of Hagerthey *et al.* (2006) also showed better results for lutein and zeaxanthin. It particularly increased the resolution (R_S = 1.34 ± 0.01) and selectivity ($\alpha = 1.02 \pm 6.7 \times 10^{-5}$) for these two pigments compared to those obtained by Wright *et al.* (1991), which suffered from partial co-elution of both pigments, showing values of RS = 1.26 ± 0.05 and $\alpha = 1.01 \pm 2.1 \times 10^{-3}$ (p-value = 0.036 and p-value = 0.049 for resolution and selectivity respectively, Wilcoxon test, R). Finally, we observed a significant difference between the measured concentrations of fucoxanthin, alloxanthin, and chl *b*, which were more concentrated with the method of Hagerthey *et al.* (2006)

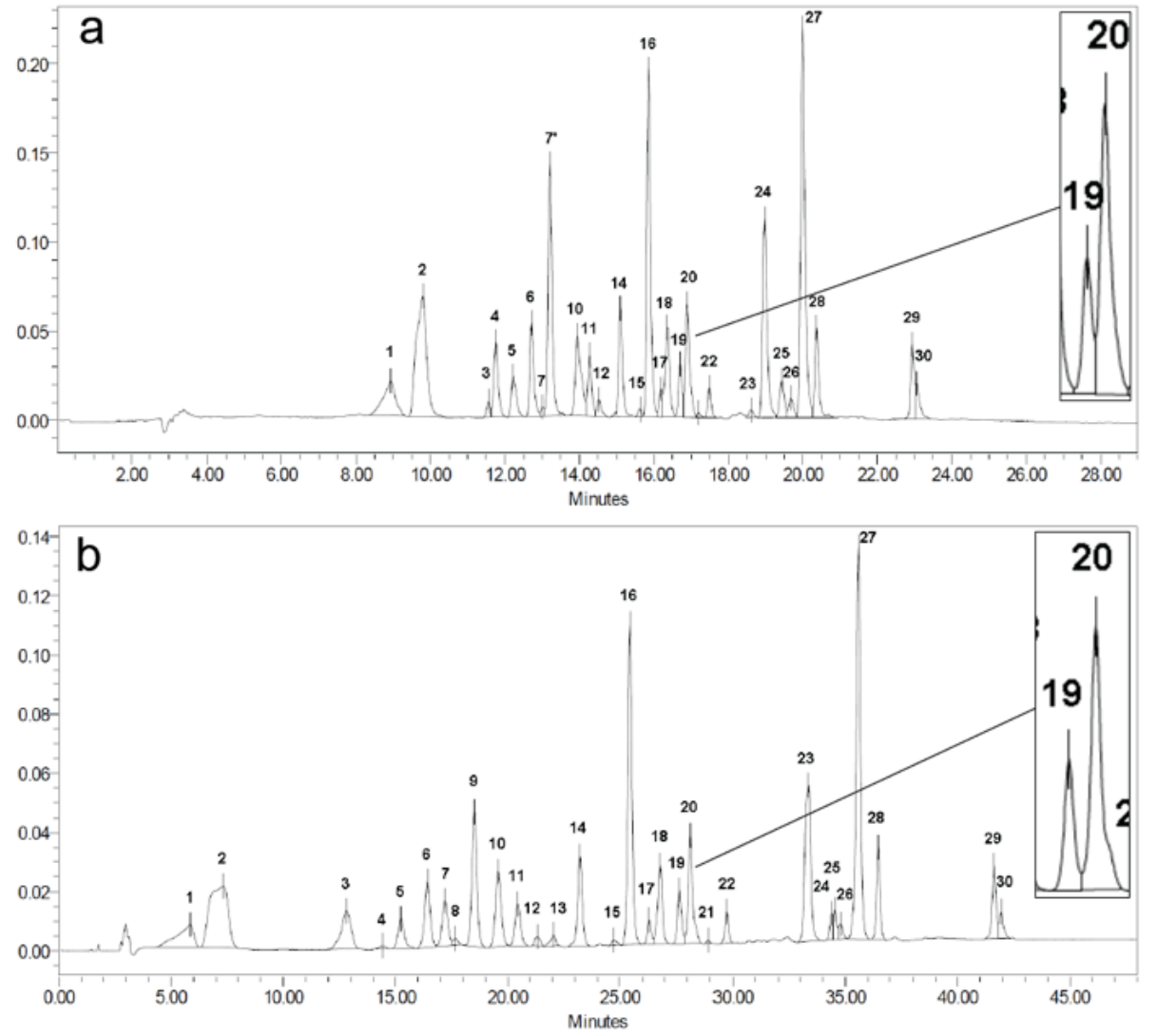

Fig. 1. HPLC chromatogram of a mixed pigment from DHI (DHI reference: mix-112. plant pigment in 90% acetone) analyzed with the Wright *et al.*, 1991 method (a) and the Hagerthey *et al.*, 2006 method (b). Each number corresponds to a pigment, detailed in Table 4.

The peak shape for alpha and beta-carotenes at the end of the chromatogram showed an improvement with the method from Hagerthey *et al.* (2006). Surprisingly, when analyzing the pigment mix, both methods showed co-elution of chl *c*2, Mg-DVP and chlorophyllid a (peak n°2, fig.1 a and b), while in most of our samples we observed a clear separation of these pigments. However, these three compounds also co-eluted with the analysis method from Van Heukeulem and Thomas (1991) used in the certificate of analysis of this mix. The addition of the TBAA in the solvent A or in the extract prior to injection had no significant effect on the chromatography of most of the pigments (e.g., chl *a*, *b*, peri, zea, Tukey posthoc p-adj. > 0.05), but it showed a clear and significant negative impact on the concentration of other pigments including allo, fuco, lut, neo and prasino (Tukey posthoc p-adj.: allo= 0.044; fuco= 0.0010; lut= 0.0025; neo= 1.18 x 10^{-5};

Table 4. Elution order (peak number) of pigments from the DHI mix (analyzed with two different methods, i.e. Wright *et al.* (1991) and Hagerthey *et al.*, (2006). Retention times (Rt) and wavelengths of the in-line absorption maxima (λmax) for the different pigments according the two methods.

Peak number	Name	Rt Wright	Rt Hagerthey	Hagerthey			Wright		
				λmax1	λmax2	λmax3	λmax1	λmax2	λmax3
1	Chlorophyll *c*3	8.933	5.856	453.5	586.1		453.5	583.7	
2	Chlorophyll *c*2*	9.796	7.336	445	581.2	632.6	443.8	580	630
3	Peridinin	11.755	12.816	476.5			472.9		
4	Siphonaxanthin		14.415	448.7	471.7				
5	19'But-fucoxanthin	12.225	15.246	446.3	471.7		446.3	471.7	
6	Fucoxanthin	12.715	16.425	448.7	467.9		448.7	469.6	
7	Cis-Neoxanthin	13.016	17.207	413.6	439	466.8	417.6	442.6	471.7
8	Trans-Neoxanthin		17.677	444.3	470.5				
9	19'Hex-fucoxanthin	n.d.	18.519	446.3	472.9				
10	Prasinoxanthin	13.943	19.578	455.9			453.5		
11	Violaxanthin	14.276	20.453	417.7	441.4	470.5	418	442.6	471.7
12	Unknown		21.313	439	463.2				
13	Unknown	14.522	22.045	421	442.6	471.7	421	442.6	471.7
14	Diadinoxanthin	15.104	23.224	425	448.7	479	426	448.7	479
15	Dinoxanthin	15.627	24.723	418.5	442.6	471.7	421	446.5	472.9
16	Alloxanthin	15.857	25.450	430.5	454.7	483.8	432	454.7	483.8
17	Monadoxanthin	16.193	26.311	426.4	447.5	477.8	427	448.7	477.8
18	Diatoxanthin	16.367	26.799	429.3	454.7	481.4		453.5	481.4
19	Lutein	16.708	27.655	425.7	448.7	475.3	425	447.5	476.5
20	Zeaxanthin	16.893	28.139	454.7	481.4	481.4	454.7	481.4	481.4
21	Dihydrolutein	n.d.	28.937	406.1	429.3	455.3	405.4	428.7	455.7
22	Canthaxanthin	17.491	29.714	475.3			477.8		
23	Chlorophyll *b*	18.612	33.360	466.8	650.9		460.8	649.8	
24	Chlorophyll *b*	18.975	34.394	458.4	646		464.4	650.9	
25	Chlorophyll *b*	19.443	34.525	466.8	650.9		464.4	650.9	
26	Chlorophyll *a*	19.680	34.782	430.5	660.7		429.3	660.7	
27	Chlorophyll *a*	19.994	35.584	431.7	660.7		431.7	660.7	
28	Chlorophyll *a*	20.372	36.467	431.7	660.7		431.7	660.7	
29	**β-ε** Caroten (**α**)	22.936	41.627	449.9	477.8		448.7	476.5	
30	**β-β** Caroten (**β**)	23.052	41.937	455.9	480.2		452.3	477.8	

* coelutes with Mg-DVP and Chlorophyllid *a*

Table 5. Statistics of mean comparison results under two hypotheses: (A) concentrations obtained with the two methods are equal; (B) concentration obtained with the new method is higher than those from the method from Wright *et al*. (1991). Student test on R were used after checking normality and homoscedasticity. Mean comparison of the number of identified pigments has been assessed with non-parametric Wilcoxon test. Stars precise significativity of P-value (*P-value < 0.01, **P-value < 0.001)

Pigments	HP	*t*	P-value
Alloxanthin	A	-9.13	0.010*
	B	9.13	0.005*
Chl *a*	A	0.70	0.554
Chl *b*	A	-9.53	6.757×10^{-4} **
	B	9.53	3.378×10^{-4} **
Fucoxanthin	A	-36.62	7.418×10^{-4} **
	B	36.62	3.709×10^{-4} **
Lutein	A	1.11	0.379
Peridinin	A	1.99	0.184
Zeaxanthin	A	-0.65	0.552
N° identified pigments	A	W = 5.5	0.814

prasino = 0.045).

Concerning the use of the protocol of Hagerthey *et al.* (2006) some problems occurred with the first samples from each injection series. We suspected this to be related to insufficient pre-conditioning and/or equilibration of the chromatography (conditioning of the column 48 min, followed by the following steps (i) equilibrate 5 min, (ii) purge of the injector 10 min, and (iii) 5 min equilibrate, before the first injection). Thus, the first sample showed some irregularities of retention time and its baseline, causing some problems of pigments identification. Therefore, we adapted the protocol, by adding in the beginning of the run the injection of 150 µl of a blank sample and eluting during 10 min, using the solvent gradient that corresponded to the first 10 min of the solvent profile, until 100% of solvent B (Table 3). This additional step solved the problems mentioned above and thus contributed to optimize the equilibration of the column prior to analysis. With this modification the method of Hagerthey *et al.* (2006) was adopted by us for further analyses.

Choice of an extraction protocol

Preliminary tests followed by chromatographic separation according Wright *et al.* (1991) had shown that a reduction of the extraction volume to 2 ml resulted in a

severe decrease of pigment extraction yield, and this lowest extraction volume was, therefore, abandoned in our tests. After the choice of the method by Hagerthey *et al.* (2006), we tested which changes with respect to the extraction protocol initially used, improved the pigment quantification with this chromatography method. On some of the pigments, including those that were the most important for our analysis and which concentrations were measured (Table 6), we observed a significant effect of the extraction solvent, extraction volume and extraction time (PERMANOVA, p-value < 0.05). There was a significant interaction between the extraction solvent and the volume (Table 7). All the other couples of tested factors showed no significant interaction terms for this pool of calculated pigment concentrations, so they were not studied further.

Table 6 shows the results of multifactorial ANOVA's and non-parametric tests (for those cases where conditions for parametric ANOVA were not fulfilled) for the different pigments. Firstly, the use of 5 ml of solvent significantly increased the mass of most of the extracted pigments compared to use of 2.5 mL (pairwise comparison of Tukey posthoc on ANOVA results, p-adj. < 0.05) (Fig. 2). Secondly, the solvent 2 resulted in a significant increase of the extracted pigment mass, except for alloxanthin, fucoxanthin and lutein for which we did not observe significant differences between the 4 solvents (pairwise comparison of Tukey posthoc p-adj. > 0.05). Extraction using acetone (solvent 4) had a negative impact on the quality of the chromatography, as it did not allow the separation of the polar pigments peak at the beginning of the analysis (chlorophyll c3, c2, chlorophyllid a) and created distortions of the baseline and peaks. Thirdly, between the simplified method (10 min of extraction, without ion-pairing agent in the extract), and the complete one, we observed some significant differences. For allo, chl a, b, and prasino, the complete protocol showed slightly higher values compared to the simplified one that were significant (pairwise comparison of Tukey posthoc on ANOVA results, p-adj.: allo = 0.04; chl *a* = 4.6 x 10^{-3}; chl *b* = 3.5 x 10^{-4}; pras = 1.37 x 10-^{5}). In contrast, for lutein we observed the opposite (p-adj. = 1.35 x 10^{-5}), while fuco, peri and zea showed no significant difference between the two protocols (p-adj. > 0.05). The yield of chl *a* and *b* increased when the volume of solvents increased from 2.5 to 5 mL, with the solvent 2 and with 2 h extraction. Chl *b* showed a lower yield with solvent 3 compared to the solvent 2 (pairwise comparison of Tukey Posthoc, p-adj. < 0.05). The measured peridinin concentration increased significantly with the use of the solvents 2 and 3 (pairwise comparison of Tukey Posthoc, p-adj. < 0.05). However, Fig. 2 (a) shows that solvent 2 seems to extract significantly more with 5 ml than solvents 1 and 3. Thus, we observed a synergy between these two factors, with the highest pigment yields observed with 5 ml of solvent 2 (pairwise comparison of Tukey posthoc, p-adj. < 0.05). Globally, the solvent 4 differed from the others by a higher variability of pigment concentrations between replicates. The other pigments have not fulfilled the conditions of validity for the multifactorial ANOVA. To conclude, the solvent that allowed the highest extraction yields (most of pigments significantly most extracted) was the second one (methanol/ acetone/ water). The best volume of solvent seems to be 5 ml.

We also tested which changes of the extraction protocol improved the pigment quantification with the protocol of chromatography from Wright *et al.* (1991), which is still widely used by the scientific community for phytoplankton chemotaxonomic studies and recommended SCOR Working group 78 (Jeffrey *et al.*, 1997). We followed a similar procedure. First, collectively the data set

Table 6. Pigment extraction yields analyzed by using the HPLC protocol of Hagerthey *et al.* (2006): Results of the multifactorial ANOVA, and non-parametric tests (for those cases where conditions for parametric ANOVA were not fulfilled), comparing yields of chlorophyll *a*, *b*, alloxanthin, fucoxanthin, lutein, peridinin, prasinoxanthin and zeaxanthin, obtained with four different solvents (90% acetone, methanol, methanol/acetone, and MAD), with two different volumes, two different solvents A, and with the classical method of extraction or an adaptation. The star (*) indicates which pigment needed a log-transformation.

	Chl *a*			Chl *b*			Allo★			Fuco★			Lut			Peri			Prasino			Zea	
Source	df	F-ratio	P-value	df	F-ratio	P-value	df	F-ratio	P-value	df	F-ratio	P-value	df	F-ratio	P-value	df	F-ratio	P-value	df	F-ratio	P-value	df	F-ratio	P-value
Volume	1	50.19	**6.8×10^{-9}**	1	40.84	**8.2×10^{-8}**	1	19.82	**5.4×10^{-5}**	1	4.63	**3.7×10^{-2}**	1	69.62	**2.2×10^{-10}**	1	0.32	5.8×10^{-1}	1	3.53	6.7×10^{-2}	1	5.42	4.2×10^{-2}
Solvent	3	4.76	**5.6×10^{-3}**	3	5.97	**1.6×10^{-3}**	1	2.00	1.3×10^{-1}	3	1.89	1.4×10^{-1}	3	3.04	**4.0×10^{-2}**	3	6.62	**8.7×10^{-4}**	3	0.84	4.8×10^{-1}	3	0.82	5.1×10^{-1}
Classic / simplifie	1	12.22	**1.1×10^{-3}**	1	20.82	**3.9×10^{-5}**	1	6.12	**1.7×10^{-2}**	1	4.11	**4.9×10^{-2}**	1	34.53	**6.5×10^{-7}**	1	0.17	6.8×10^{-1}	1	32.15	**1.2×10^{-6}**	1	0.69	4.2×10^{-1}
Solv. A + TBAA	1	14.61	**3.9×10^{-4}**	1	4.56	**3.8×10^{-2}**	3	1.57	2.2×10^{-1}	1	6.11	**1.7×10^{-2}**	1	26.41	**7.2×10^{-6}**	1	1.64	2.1×10^{-1}	1	28.86	**3.2×10^{-6}**	1	0.05	8.3×10^{-1}
Interactions																							
V x solv.	3	4.25	**9.8×10^{-3}**	3	2.50	7.2×10^{-2}	3	2.41	7.9×10^{-2}	3	2.12	1.1×10^{-1}	3	3.13	**3.6×10^{-2}**	3	0.03	9.9×10^{-1}	3	1.00	4.0×10^{-1}	2	0.65	5.4×10^{-1}
V x solv. A	1	2.75	1.0×10^{-1}	1	1.26	2.7×10^{-1}	1	3.34	7.4×10^{-2}	1	1.83	1.8×10^{-1}	1	9.63	**3.5×10^{-3}**	1	0.67	4.2×10^{-1}	1	0.08	7.8×10^{-1}	1	0.28	6.1×10^{-1}
V x c/s	1	0.12	7.3×10^{-1}	1	7.71	**8.0×10^{-3}**	1	8.24	**6.2×10^{-3}**	1	0.04	8.3×10^{-1}	1	4.76	**3.5×10^{-2}**	1	0.36	5.5×10^{-1}	1	7.62	**8.5×10^{-3}**	1	0.63	4.5×10^{-1}
Solv. x solv. A	3	1.46	2.4×10^{-1}	3	1.51	2.2×10^{-1}	3	0.37	7.7×10^{-1}	3	0.74	5.3×10^{-1}	3	0.18	9.1×10^{-1}	3	0.26	8.5×10^{-1}	3	1.09	3.6×10^{-1}			
Solvent x c/s	3	0.27	8.5×10^{-1}	3	0.79	5.1×10^{-1}	3	0.49	6.9×10^{-1}	3	0.21	8.9×10^{-1}	3	3.66	**2.0×10^{-2}**	3	0.76	5.2×10^{-1}	3	1.72	1.8×10^{-1}			

★ Log-transformation

Table 7. Pigment extraction yields analyzed by using the HPLC protocol of Hagerthey *et al.* (2006): Results of the PERMANOVA with 999 permutations comparing the pigments concentrations yields (chlorophyll *a*, *b*, alloxanthin, fucoxanthin, lutein, peridinin, prasinoxanthin, zeaxanthin), for the five factors of the factorial experiment (see method). Signification codes: 0 '***' 0.001 '**' 0.01 '*'.

Fact. Number	Factors	d*f*	*F* ratio	*p*-value
1	Extraction volume	1	37.510	0.001 **
2	Composition of the extraction solvent	3	4.94	0.001 **
3	TBAA addition in extraction solvent	1	16.864	0.001 **
4	Extraction time	1	16.710	0.001 **
5	Solvent A with TBAA	1	2.720	0.063
Interactions				
1 x 2	Extraction volume x Extraction solvent	3	2.580	0.030 *
1 x 4	Extraction volume x Time	1	0.760	0.465
1 x 5	Extraction volume x Solvent A	1	1.720	0.142
2 x 4	Extraction solvent x Time	3	0.840	0.519
2 x 5	Extraction solvent x Solvent A	3	0.760	0.589

was analyzed by PERMANOVA (Table 8) and the significant factors (the extraction solvent, it's volume and the addition of ion pairing reagent, PERMANOVA, p-value < 0.05) were further analyzed. Therefore, we used parametric and multifactorial ANOVA applied on each pigment concentration, when the conditions of application of ANOVA were fulfilled. Otherwise, non-parametric tests were chosen. As observed when analyzed in combination with the protocol by Hagerthey *et al.* (2006), the addition of the ion-pairing agent (Tetrabutyl ammonium acetate) in the solvent A had a negative impact on a large number of pigments (PERMANOVA p-value < 0.05; chlorophyll *c*2, fucoxanthin, lutein, neoxanthin, violaxanthin, zeaxanthin, ANOVA, p-values < 0.05). The extraction solvent had a clear significant impact (PERMANOVA, p-value < 0.01) as extraction volume (PERMANOVA, p-value < 0.001). As observed for pigment extractions in combination with the HPLC-protocol of Hagerthey *et al.* (2006), we observed that the two solvents allowing the highest extraction yields were solvent 2, a mix of methanol/ acetone / water (45:45:10), and the solvent 3, a mix of methanol/ acetone/ dimethylformamide / water (30:30:3010). Again, 5 ml extraction solvent often resulted in higher extraction yields than using 2 ml or 2.5 ml (all pigments except chlorophyll *b* and prasinoxanthin).

Discussion

For the purpose of our analyses of the phytoplankton community composition, we need to detect and identify most efficiently the pigments which are markers of phytoplankton groups, i.e., alloxanthin for Cryptophytes, fucoxanthin for fucoxanthin-rich diatoms, peridinin for Dinophytes (Bustillos-

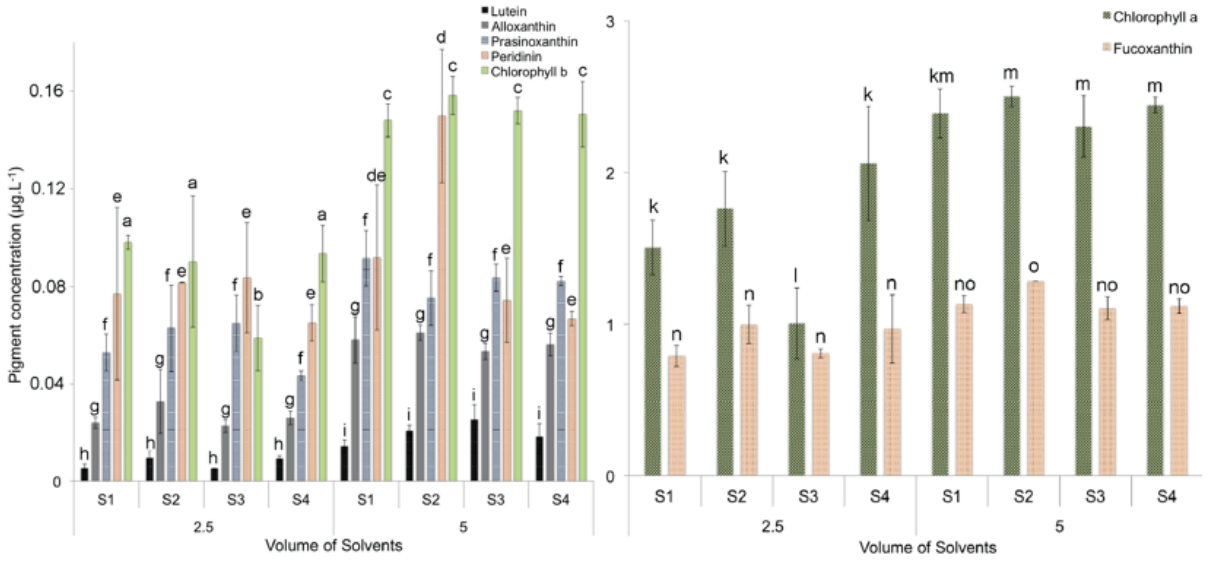

Fig. 2. Pigment yield with the four solvents (see Table 1) and their two different volumes for (a) lutein, alloxanthin, prasinoxanthin, peridinin, chlorophyll *b*, (b) chlorophyll *a* and fucoxanthin. For each pigment, the significant differences between groups are illustrated with letters (a to c for chlorophyll *b*, d to e for peridinin, f for prasinoxanthin, g for alloxanthin, h to i for lutein, k to m for chlorophyll *a*, and n to o for fucoxanthin).

Table 8. Pigment extraction yields analyzed by using the HPLC protocol of Wright *et al.* (1991). Results of the PERMANOVA with 999 permutations comparing the pigments concentrations yields (chlorophyll *a*, *b*, *c*2, alloxanthin, diadinoxanthin, fucoxanthin, 19'Hex-fucoxanthin, lutein, neoxanthin, peridinin, prasinoxanthin, violaxanthin, zeaxanthin,for the four factors of the factorial experiment (see method). Stars precise significativity of P-value (*P-value < 0.05, **P-value < 0.005).

Fact. Number	Factors	*df*	*F* ratio	*p*-value
1	Extraction volume	1	107.820	0.001 ***
2	Composition of the Extraction solvent	1	5.568	0.004 **
3	TBAA addition in the extraction solvent	1	4.518	0.011 *
4	Extraction time	1	2.698	0.055
2 x 1	Extraction solvant x Extraction volume	1	2.133	0.112
2 x 4	Solvant x Extraction time	1	2.493	0.060
1 x 4	Extraction volume x Time	1	1.187	0.283
4 x 3	Extraction time x TBAA	1	6.631	0.002 **
1 x 3	Extraction volume x TBAA	1	1.827	0.128

Guzmán *et al.*, 2004), prasinoxanthin for Prasinophytes, neoxanthin, lutein, violaxanthin, chlorophyll *b* for Green algae, zeaxanthin for Cyanobacteria and Chlorophytes, echinenone for Cyanobacteria (Wright *et al.*, 1991; Schlüter *et al.*, 2006; Cartaxana et al., 2009), 19'-But-Fucoxanthin and 19'-Hex-Fucoxanthin for Haptophytes (Zapata *et al.*, 2000; Goericke, 2002).

While the two methods of chromatography we tested showed little differences in the number of identified pigments, some supplementary pigments seem to be more efficiently observed with the protocol from Hagerthey *et al.* (2006), as 19'Hex-fucoxanthin and dihydrolutein. Globally, thanks to the longer analysis time (48 minutes instead of 29 (Table 2 and 3)), which helped to spread peaks along the gradient, the chromatograms obtained with this method visually show a better peak shape and separation than in the chromatograms obtained with Wright *et al.* (1991). This is especially the case for the early eluting most polar compounds as well as for the final eluting hydrophobic compounds. More particularly, the selectivity and the resolution of the lutein and the zeaxanthin showed better results with the protocol from Hagerthey *et al.* (2006). These two pigments need to be separated as best as possible because they can be used as markers of different phytoplankton groups (Chlorophytes versus Cyanobacteria). Finally, the longer protocol of Hagerthey *et al.* (2006) helped to improve the peak shapes for fucoxanthin, alloxanthin, and chl *b* that facilitated better quantification of their concentrations. With the modifications that we adopted to improve the equilibration prior to analysis, the protocol of Hagerthey *et al.* (2006) has yielded satisfactory results for the pigments usually used to estimate phytoplankton diversity in coastal lagoons.

We tested different extraction protocols, to take the characteristics of our samples and their variations during the monitoring period into account. Indeed, we had to consider a strong variability of pigment concentration, from low (< 0.05 µg/L) to very high values (412.6 µg/L). We first tried to increase the extraction time, because after sonication we extracted only during 15 min, while bibliography showed much longer extraction times, ranging from 1 h - 3 h (Louda, 2008; Vidussi *et al.*, 2011) to 24 h (Schlüter *et al.*, 2000). Thus after sonication, we compared an extraction time of 15 min with that of 1 h and 2 h. The extraction yield of the pigments in the sample from Thau lagoon was clearly improved by the use of extraction solvents 2 and 3 that both comprised a mixture of methanol, acetone and water, with or without N-N-dimethylformamide which showed the better results when coupled with a high-powered probe sonicator (Furuya *et al.*, 1998; Wright, 2005), unlike the use of pure or diluted acetone or methanol preconized in many references of phytoplankton chemotaxonomic analysis (Wright *et al.*, 1991; Goericke and Repeta, 1993; Schlüter *et al.*, 2000; Zapata *et al.*, 2000; Airs *et al.*, 2001a, 2001b; Van Heukelem and Thomas, 2001; Hooker *et al.*, 2009; Seoane *et al.*, 2011; Eker-Develi *et al.*, 2012). The solvent with dimethylformamide added seems to be a very efficient extraction solvent, and it does not need a too long extraction time, since Hagerthey and his colleagues (2006) showed that more than 98% of the chl *a* content was extracted after 2 hours. Moreover, dimethylformamide has been shown to decrease the degradation of chlorophyll *a* during the extraction (Furuya *et al.*, 1998). We also observed an improvement of the number of identified pigments with 5 ml. There was a synergetic effect between the factors 1 and 2 (extraction volume and composition of the solvent), resulting in the highest pigment yields with 5 ml of the solvent 2. Using a higher extraction volume results in proportionally lower concentrations in the pigment extract. With the same injection volume this results

in proportionally lower pigment masses injected on the column resulting in lower peak areas for all pigments, which may be a drawback. However, this drawback is largely compensated by the fact that some of the pigments were only detected on the HPLC when we used 5 ml extraction volume. Hence, the extraction with 5 ml of solvent 2 (Table 1), allowed the detection of alpha and beta carotenoids, and peaks shape enhancement, with the typical three peak carotenoid absorption spectra (Wright, 2005). The filter contains a small but unknown amount of water. A larger volume of extraction solvent is useful to dilute this small amount, which minimizes the proportion of water in the sample extract, and thus the dilution effects (Latasa, 2014).

The protocol initially used for the Lagoon Monitoring Network was a 5 min extraction in 2.5 ml of methanol in obscurity at 4°C, followed by 5 sonications, by immersing the tip of a sonic probe below the surface of the liquid 10 sec (20 Watts), each followed by 10 sec of storage on ice to avoid excessive heating of the extract. The increase of the extraction time, initially about 10 minutes, to at least one hour (we observed a decrease of the prasinoxanthin content after two hours of extraction time) enhances the extraction efficiency too. The length of extraction time is an important factor to define, because it impacts the diversity of pigments identified and their concentration, that increase with the optimum extraction time (Hagerthey *et al.*, 2006). But they may also decrease if the extraction time is too long, because of the formation of degradation products, depending on the composition of the extraction solvent (Latasa *et al.*, 2001). We decided that extracting 1 h was a good compromise between a better extraction of many pigments and a reasonable time-frame for the analyses.

The addition of the TBAA in the extract before the injection did not improve the separation, the peak shape, or their resolution. Moreover, the use of solid TBAA from Aldrich as ion-pairing agent in the mobile phase have already resulted in some problems, like unstable retention times, pressure increase in the column, or suboptimal pigment separation (Hooker *et al.*, 2009). Therefore, we decided to totally abandon the use of TBAA as other researchers have already adopted (Wright et al., 1991; Van Heukelem and Thomas, 2001). To conclude, the best extraction was obtained with 5 ml of methanol/ acetone/ water (45:45:10), and 1 h duration. This extraction allows a good compromise between the number of identified pigment of interest, their concentration, and a good quality of the chromatogram, without complicating or excessively increasing the time needed for these analyses. The best analysis method for our pool of pigment was the one proposed by Hagerthey and his colleagues (2006), without adding ion-pairing reagent before injection, which allow a better concentration measurement of several pigments, and a better separation of lutein/zeaxanthin, and chlorophyll *c*3/*c*2 couples. This method still does not chromatographically separate chlorophylls *a* and *b* from their divinyls forms. A specific method for separating these compounds has been proposed, using a C8 column (Barlow *et al.*, 1998). However, the presence of prochlorophytes has never been demonstrated in European coastal lagoons, so we do not consider this failure as problematic for a routine analysis of phytoplankton diversity in these ecosystems. We conclude that we have now an efficient protocol that is appropriate for performing large-scale surveys of photosynthetic lipophilic pigments in coastal lagoons. In the near future, when these pigment data can be confronted with microscopic observations of micro- and nanophytoplancton, and flow cytometry for picophytoplancton, particularly abundant in coastal lagoons, we will have the possibility to apply the CHEMTAX program (Mackey *et*

al., 1996). For efficient data interpretation CHEMTAX still needs to be calibrated specifically for coastal lagoons.

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