

PIGMENT-BASED CHEMOTAXONOMY OF THE PHYTOPLANKTON COMMUNITY FROM A MARINE MUSSEL FARMING AREA

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ABSTRACT

Niero, H. & Tamanaha, M. S. (2018). Pigment-based chemotaxonomy of the phytoplankton community from a marine mussel farming area. *Braz. J. Aquat. Sci. Technol.* 25(1). eISSN 1983-9057. DOI: 16492/bjast.v25n1. Microscopy technique is traditionally employed for assessing phytoplankton taxonomy. However, chemical approaches, such as the identification of biomarkers, allow to elucidate microalgae groups. This study aimed to identify the pigment profile of isolated microalgae species and to determine the seasonal pigment fluctuation in water samples from a marine mussel farm located in Armação do Itapocoroy (26°47'00"S, 48°36'30"W), Brazil. For unialgal cultures, phytoplanktonic organisms were isolated and cultivated in f/2 liquid medium under controlled temperature and light cycle. Cell content extracts were obtained and the pigment content for each species analyzed by High Performance Liquid Chromatography (HPLC). Pigment profiles of 13 marine microalgae were identified (12 Diatoms and 1 Dinoflagellate). Chlorophyll *a* and *c2* were detected in all species. Fucoxanthin was not detected only in *Prorocentrum micans*, and diadinoxanthin was not detected only in *O. mobiliensis*. Chlorophyll *a* and fucoxanthin were most present in Diatoms, while peridinin was the major carotenoid in *P. micans*. For assessing the seasonal pigment fluctuation, water samples were collected periodically (within one to three weeks interval) through the years of 2014 and 2015. Along with microscopy counting, pigment present in the water samples were analyzed by HPLC. Bacillariophyceae had higher density in the summer and fall. On the other side Dinophyceae, Cryptophyceae, Euglenophyceae, Prasinophyceae, Dictyophyceae and Ebrideae, had an increase in density during winter and spring. Pigments such as fucoxanthin, zeaxanthin, diadinoxanthin, chlorophyll *c3* and *c2*, were detected in samples throughout the year. Whereas prasinoxanthin, peridinin, dinoxanthin and antheraxanthin were detected mainly in samples collected during winter and spring.

Key Words: Pigment. Community composition. Chemotaxonomy. HPLC. Phytoplankton. Unialgal cultures.

INTRODUCTION

Phytoplanktonic organisms (Protista Kingdom Chromista) present great diversity in marine environments (Graham et al., 2009). They are more represented by Diatoms (Bacillariophyta), Dinoflagellates (Dinophyta) and Euglenophytes (Euglenophyta). All these groups have chloroplasts, within which the photosynthetic pigments, such as chlorophylls and carotenoids, are found as macromolecular complexes (Jeffrey et al., 1997). Chlorophylls play major role in photosynthesis acting in the capture of active radiation, constituting the system that absorbs and transforms light energy (photons) into chemical energy (Reynolds, 2006).

Carotenoids are known as protective agents, inhibiting the formation or dissipating the energy of an excited chlorophyll that would form a damaging oxygen state to the photosynthetic apparatus (Jeffrey et al., 1997). These biomolecules have a high antioxidant power (Skibsted, 2012), anti-hypertensive effect (Rafieian-Kopaei et al., 2016), anticancer activity (Nishino, 1998; Tanaka et al., 2012) and are widely used as food supplements (Venil et al., 2013). In the environment, they also provide resistance against exposure to solar radiation (Tong & Lighthart, 1997), and they may serve as taxonomic biomarkers (Liaaen-Jensen, 1978; Sanz et al., 2015).

Marine coastal areas have nutrient input coming from the continent through natural discharge and from domestic discharge derived from population occupation. Population uses this environment to carry out different activities, such as tourism, transportation, fishing and organism cultivation. The latter, of great social and economic relevance, had its global production estimated to be around 27 million tons of food in 2014 (16% of the total captured and cultured in the world, including fishing and aquaculture activities, both inland and marine) (FAO, 2016). Phytoplankton community play an important role in marine mussel farm areas for mollusk growth and development (Brown, 2002; Hemaiswarya et al., 2011), so understanding its nutrition content and quality is important.

Studies related to microalgae pigment profiles are quite complex and employ advanced analysis and technologies for the detection of the wide range of pigment compounds (Garrido & Roy, 2015). Moreover, the chemotaxonomy of algal communities through biomarker pigments can be used as a complement in phytoplankton study, helping in the structuring, composition and estimation of abundance of classes and species (Schlüter et al., 2016). Although pigment patterns among phytoplanktonic classes are evident, it is necessary to characterize local species in order to achieve a more reliable chemotaxonomic application

(Henriksen et al., 2002; Irigoien et al., 2004; Schluter et al., 2016). This work aimed to characterize the pigment profiles of microalgae and elucidate the seasonal variation of pigments in a marine mussel farm. Chemotaxonomy of microalgae groups with pigments as biomarkers was employed, both in unialgal cultures and water samples collected throughout the year.

MATERIAL AND METHODS

Study Area and Sampling

Comprises the area where bivalve shellfish (mussel) cultivation takes place, in Armação do Itapocoroy, located in Penha, Santa Catarina, Brazil (26°47'00"S, 48°36'30"W). The cove has a maximum of 15 m depth and average of 8 m depth. Local organic and nutrient import is derived from Itajaí-Açu River plume and from organic matter biosedimentation by the shellfish culture (Schettini et al., 1999). Water samples were collected with a hose (4 m long and 4 cm diameter) with a faucet at one end. Between August 2014 and August 2015, 24 samples were collected (within one to three weeks interval). On average, 200 mL of water was fixed with a Lugol solution (2%), for further monitoring by microscopy. For microalgae strain isolation, samples were collected using a plankton net (20µm) in vertical trawls in the water column. Samples were taken to laboratory for subsequent procedures. Temperature (°C) and salinity were measured with a multiparameter sonde (YSI-30/10) and water transparency estimated with a Secchi disk and visual observation (Secchi disk depth, in meters).

Culture and Microscopy

Phytoplankton strain isolation was made employing hematocrit capillary and manual suction techniques (Lourenço, 2006). Isolated species were cultured in Erlenmeyer flasks, with Guillard (1975) f/2 as culture medium, controlled luminosity (12:12h light-dark) and temperature (25 °C). Taxonomic analysis was performed under microscopy with 200 X magnification using an Olympus IX51 Trinocular Inverted Microscope with phase contrast. For quantification of species abundance in field samples, the Utermöhl sedimentation technique was used. For cultures quantification the Sedgewick Rafter technique was used, counting the organisms present in a known volume. Organisms identification by microscopy was made with the aid of specialized bibliography, analyzing the organism morphometric characteristics to the smallest taxon. Bacillariophyta was classified according to Round et al. (1990). Other groups were classified according to Tomas (1997).

Pigment Extraction and HPLC Analysis

A known volume of each water sample collected throughout the year and of each unialgal culture was filtered through GF-5 glass fiber microfilters (0.4 µm pore size). Filters were then kept in freezer (-20 °C) until analysis. Frozen filters having the phytoplankton biomass were submitted to extraction procedure with 5mL methanol (95%), under ice bath, employing an ultrasonic high frequency probe for 5 min. The supernatant, which contained the phytoplankton cell content, was collected and filtered through GF-5 glass fiber microfilters (0.4 µm pore size) to remove debris. An aliquot (1 mL) of the methanolic extract was mixed with 0.2 mL ultrapure water, according to Zapata et al. (2000). Samples were analyzed as soon as possible or stored at -20 °C for a relatively short period of time (Mantoura et al., 1997). All the extraction procedures were carried out in low light, avoiding pigment photodegradation (Repeta & Bjørnland, 1997).

Each sample was injected into a HPLC system (Shimadzu) and the pigments were detected with a RF-10AXL fluorescence detector and a SPD-M20A PhotoDiode Array (PDA UV-VIS) detector. Analytical separation was performed in a Waters Symmetry C8 column (150 x 4.6mm, 3.5µm particle size) and the mobile phase employed according to Zapata et al. (2000). Pigments were identified by their retention times and wavelength absorptions, comparing with external standards and specialized literature. Equations obtained from pigment standard curves were used to estimate pigment concentration per number of cells and sample volume.

Data Analysis

In order to see the presence of microalgae clusters in the field samples, a regression analysis was performed between the results obtained by microscopy and the pigments identified by HPLC. Multivariate variance analysis (Permanova) was performed to test the hypothesis of difference between the factors of the samples and among the taxa. Relationships were evidenced through Multidimensional Scaling (MDS, Bray Curtis similarity index) and Canonical Correspondence Analysis (CCA) to verify the variables responsible for taxa distribution in samples. PRIMER 6 (Clarke, 1993; Clarke & Gorley, 2006) and CANOCO™ (Ter Braak & Smilauer, 1998) softwares were used.

RESULTS

Cultures

Thirteen strains were isolated, cultivated and had its cell content extract analyzed: *Lauderia annulata*, *Thalassiosira* sp., *Pseudo-nitzschia* sp.,

Odontella mobiliensis, *Skeletonema* sp.1, *Skeletonema* sp.2, *Thalassionema nitzschioides*, *Asterionellopsis glacialis*, *Chaetoceros* sp., *Rhizosolenia* sp., *Pleurosigma normanii*, *Dactyliosolen phuketensis* and *Prorocentrum micans*. The pigment profiles of the strains are shown in Figures 1 and 2.

Chlorophyll *c2* and *a* were detected in all species (Table 1), other chlorophylls found were chlorophyll *c3* and *c1*. The carotenoids peridinin, fucoxanthin, diadinoxanthin, dinoxanthin, diatoxanthin and β -carotene were detected. No chlorophyll *c1* was present in *Pseudo-nitzschia* sp. and *P. micans*. Peridinin and dinoxanthin were only detected in *P. micans*, since both pigments are characteristic of Dinoflagellates. The carotenoid fucoxanthin was not detected only in *P. micans*. All taxa showed diadinoxanthin, except for *O. mobiliensis*.

Because microalgae vary in size, pigment quantity per cells can be higher in bigger cells. To avoid this issue, chlorophyll *a* normalized pigment ratio was calculated making easier to compare different strains. Chlorophyll *c3*:chlorophyll *a* ratio was high in *Pseudo-nitzschia* sp., while *P. normanii* showed the lowest ratio (Table 2). Chlorophyll *c2*:chlorophyll *a* ratio was higher in *Skeletonema* sp., exceeding the fucoxanthin:chlorophyll *a* ratio. Fucoxanthin was the pigment with the highest pigment:chlorophyll *a* ratio, except for the species *O. mobiliensis* and *Skeletonema* sp.1. *Pseudo-nitzschia* sp. presented fucoxanthin:chlorophyll *a* ratio of 1.0, evidencing a high concentration of this carotenoid in this organism. The diadinoxanthin:chlorophyll *a* ratio was similar in almost all taxa.

Species were clustered in three groups (A, B and C) in MDS (similarity level of 85) (Figure 3). Phytoplanktonic species were grouped with respect to their similar pigment:chlorophyll *a* ratio, which means they are chemotaxonomically closer. The species *T. nitzschioides* and *Rhizosolenia* sp. were clustered in group (A), which is positively related to diatoxanthin:chlorophyll *a* axis. The second group (B) includes the species *L. annulata*, *D. phuketensis*, *A. glacialis* and *Skeletonema* sp.2, which has a weak positive relation with diadinoxanthin:chlorophyll *a* and chlorophyll *c2*:chlorophyll *a* ratios. In the third group (C) are the species *Pseudo-nitzschia* sp., *P. normanii*, *Chaetoceros* sp. and *Thalassiosira* sp., which have positive relation to higher chlorophyll *c3*:chlorophyll *a* and fucoxanthin:chlorophyll *a* ratios. *Skeletonema* sp.1 and *O. mobiliensis* remained apart from the others. The first have a strong positive relation with chlorophyll *c2*:chlorophyll *a* and diadinoxanthin:chlorophyll *a* ratios, whereas the second have strong positive relation with diatoxanthin:chlorophyll *a* ratio.

The division of the classes to which each species belongs is also shown in Figure 3. Species of the classes Fragilariophyceae and Coscinodiscophyceae were clustered in group A and B. Group C was composed by Bacillariophyceae and Coscinodiscophyceae. These Diatoms classes showed great similarity of their pigment compositions ($p > 0.05$ in Table 3).

Phytoplankton and pigments seasonal distribution

Water temperature ranged from 19 to 20 °C between the months of June to September and reached 28 °C between the months of January and March. Following the trend of warmer waters in summer and colder in winter. The average salinity during the sample period was 33, varying from 30 to 35 throughout the year. The average water transparency was 2 m, while the annual variation was 1 to 3.5 m.

Phytoplankton density ranged from 34,400 to 7,269,076 cells/L. Species richness remained in the range of 13 to 35 taxa. Diatoms were present in greater abundance in all samples. Among them, Coscinodiscophyceae had higher density throughout the year (Figure 4). In this class are classified genera such as *Thalassiosira*, *Odontella*, *Rhizosolenia*, *Dactyliosolen*, *Skeletonema* and *Chaetoceros*, from which were obtained pigment profiles from unialgal cultures. Fragilariophyceae, where genera such as *Asterionellopsis* and *Thalassionema* are classified, and Bacillariophyceae, of genera such as *Pseudo-nitzschia* and *Pleurosigma*, had higher densities in some collections between November and December 2014. Dinoflagellates (isolate strain *Prorocentrum*) had greater abundance in October. Dictyochophyceae, Euglenophyceae, Prasinophyceae and Ebridia, were present in lower densities, with abundance peaks in the months of July, August and September.

Chlorophyll *a*, *c3*, *c2* and *c1*, and the carotenoid fucoxanthin were found in all samples along the year (Figure 5 and Table 4). Other chlorophylls found were chlorophyll *b*, monovinyl chlorophyll *c3*, Mg-3,8-divinyl-pheoporphyrin *a_s* monomethyl ester (MgDVP) and chlorophyllide *a*. Other carotenoids detected were peridinin, diadinoxanthin, dinoxanthin, diatoxanthin, 19'-butanoyloxyfucoxanthin, 19'-hexanoyloxyfucoxanthin, prasinolaxanthin, violaxanthin, alloxanthin, lutein, neoxanthin, antheraxanthin, zeaxanthin and β -carotene.

Prasinolaxanthin, dinoxanthin, antheraxanthin, 19'-butanoyloxyfucoxanthin, 19'-hexanoyloxyfucoxanthin, β -carotene, violaxanthin and lutein were detected in samples from July to October (Figure 5 and Table 4). Peridinin, neoxanthin, chlorophyll *b* and diatoxanthin, were detected in samples along the year, showing a

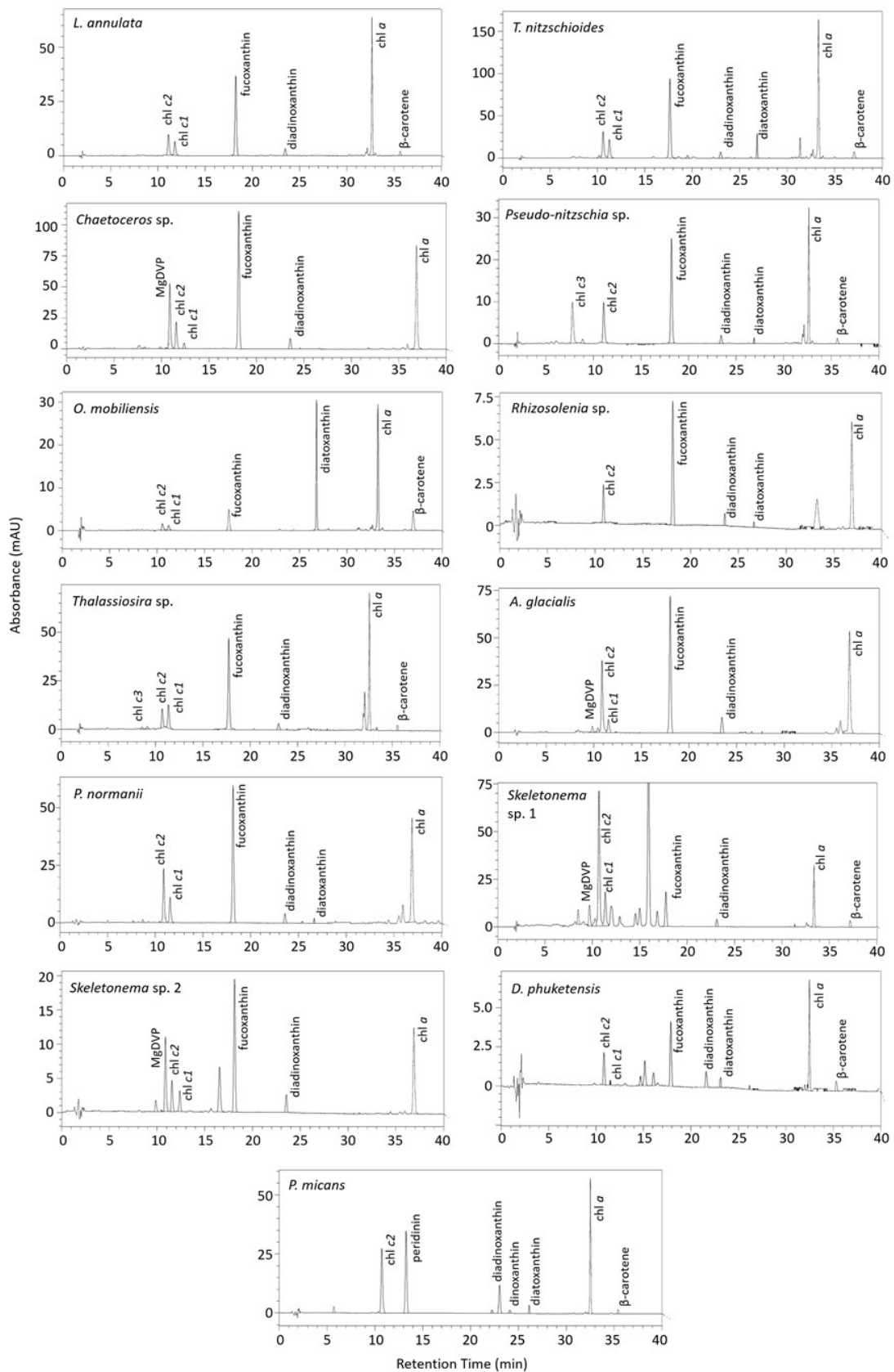


Figure 1 - HPLC-PDA chromatograms of detected pigments in unialgal cultures. Absorbance detection: 431nm – 662nm (PDA detector). Chlorophyll c3 (chl c3); chlorophyll c2 (chl c2); chlorophyll c1 (chl c1); chlorophyll a (chl a); Mg-3,8-divinyl-pheoporphyrin a₅ monomethyl ester (MgDVP).

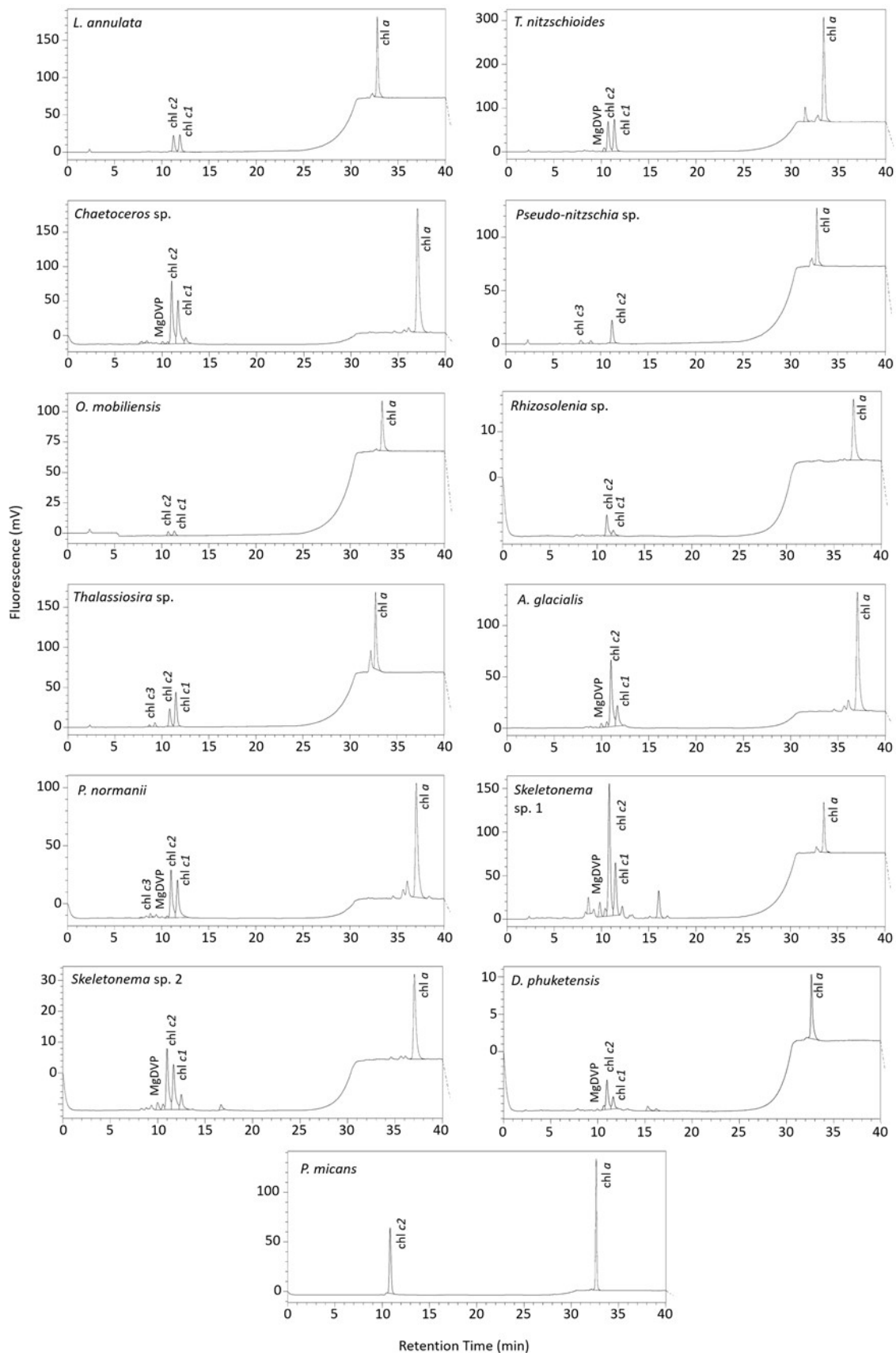


Figure 2 - HPLC-UV chromatograms of pigments in unialgal cultures. Detection by fluorescence: Ex 440/Em 650 nm (UV detector). Chlorophyll c3 (chl c3); chlorophyll c2 (chl c2); chlorophyll c1 (chl c1); chlorophyll a (chl a); Mg-3,8-divinyl-pheoporphyrin a_3 monomethyl ester (MgDVP).

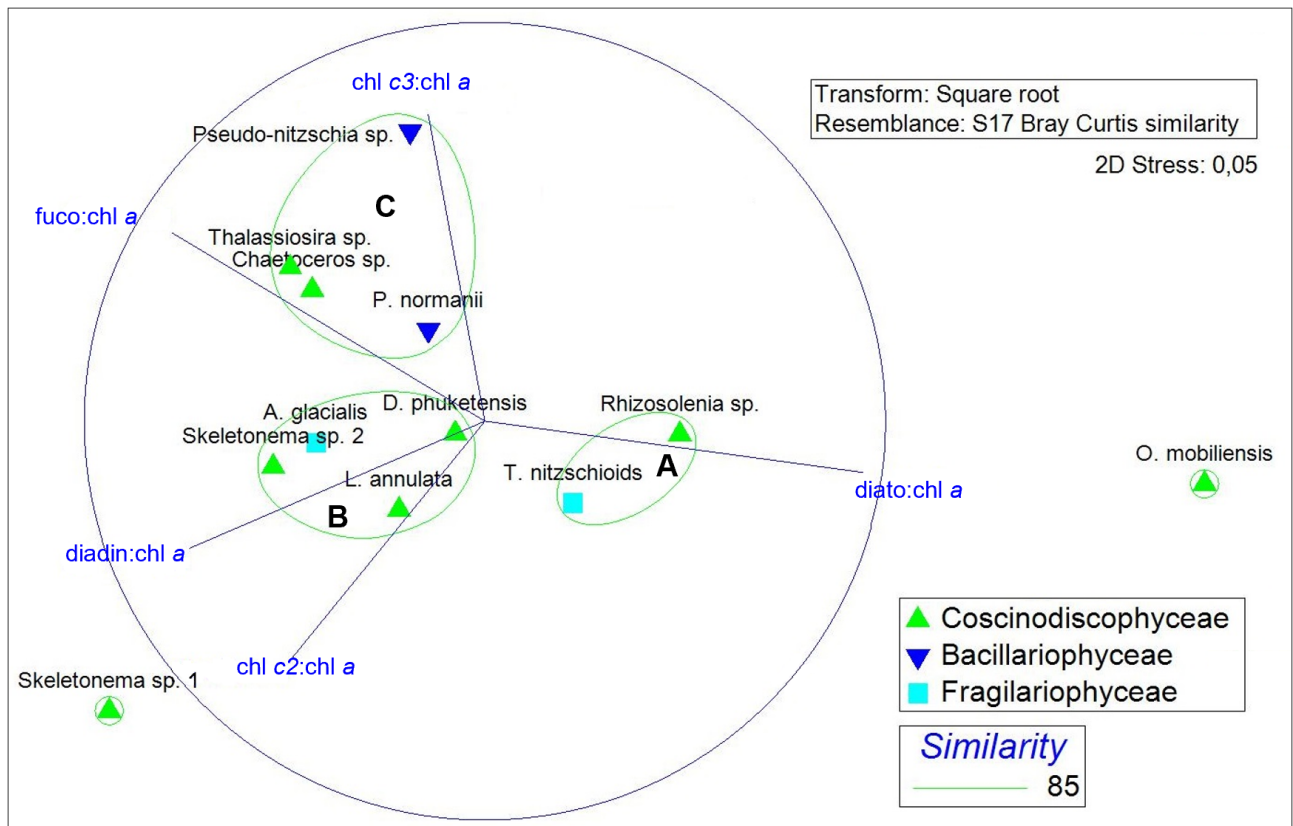


Figure 3 - Multidimensional Scaling Analysis (MDS) for cultured microalgae grouped by pigment:chlorophyll a ratio.

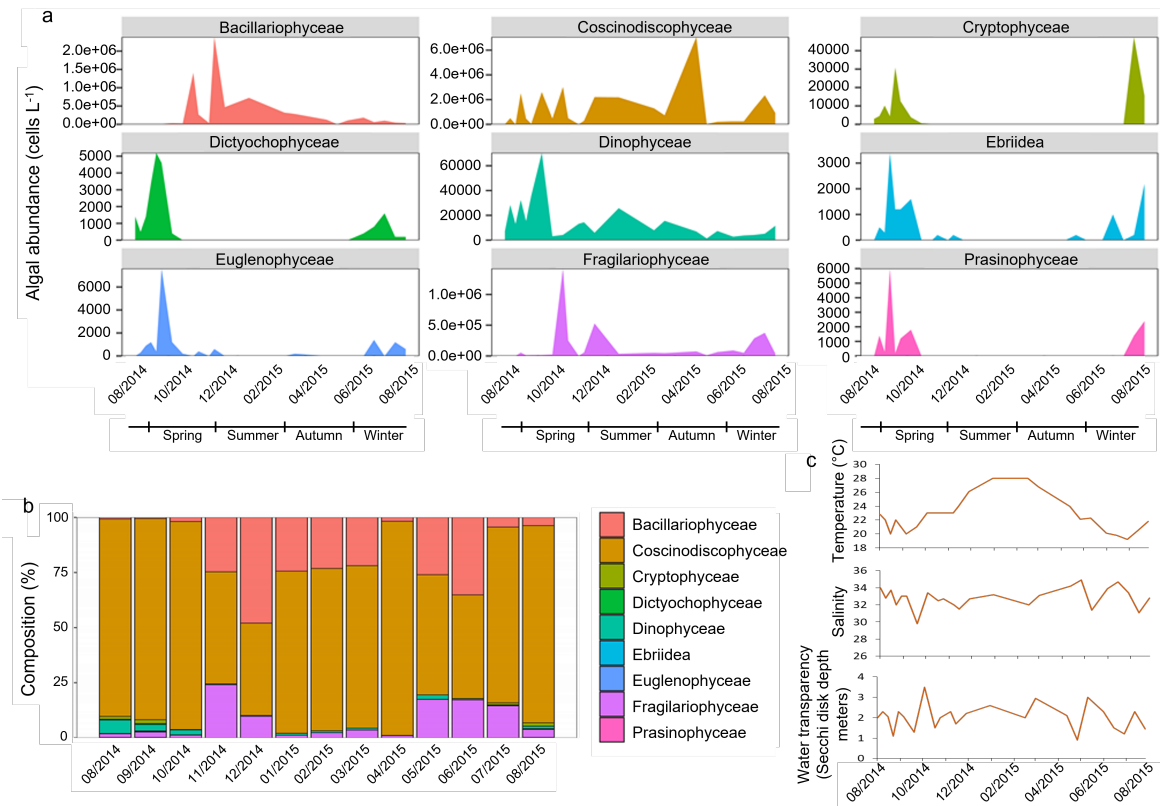


Figure 4 - Phytoplankton community seasonal variation. a: Algal abundance (cells/L). b: Class composition (%). c: Seasonal variation of Temperature (°C), Salinity and Water transparency (Secchi disk depth meters).

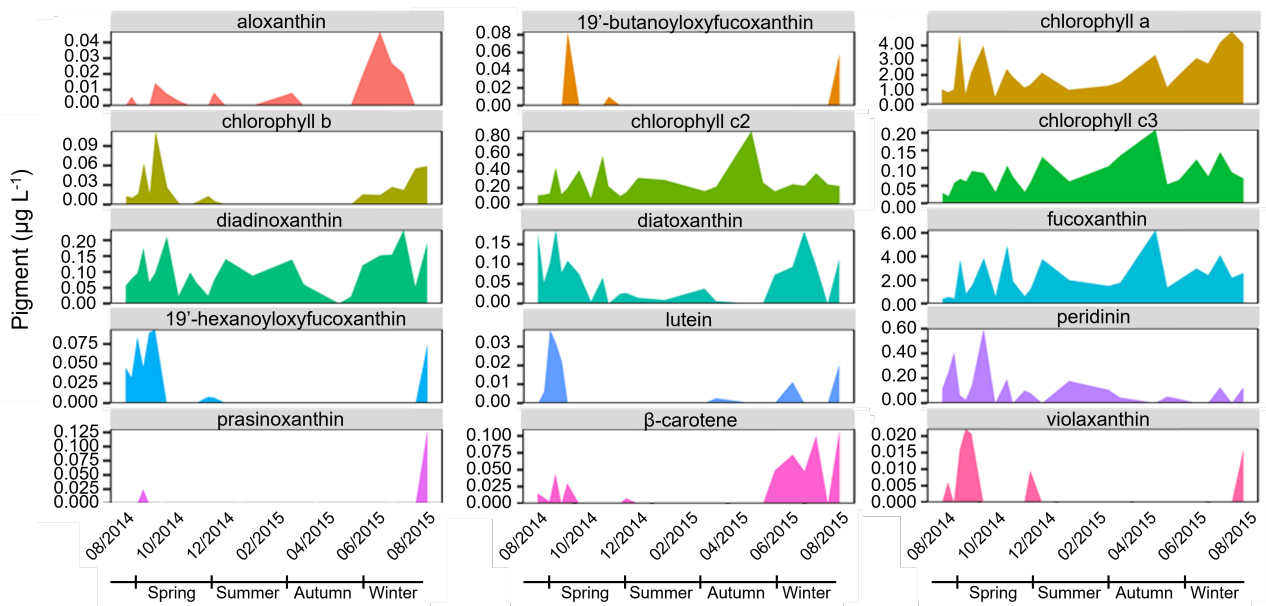


Figure 5 - Pigment seasonal variation in water samples from Armação do Itapocoroy. Exact values are shown in Table 4.

Table 1 - Pigment distribution in cultured microalgae. Chlorophyll c3 (chl c3); chlorophyll c2 (chl c2); chlorophyll c1 (chl c1); chlorophyll a (chl a); Monovinyl chlorophyll c3 (MV-chl c3); Chlorophyllide a (cld a); Mg-3,8-divinyl-pheoporphyrin a5 monomethyl ester (MgDVP); Methyl chlorophyllide a (me-cld a); peridinin (per); fucoxanthin (fuco); diadinoxanthin (diadin); dinoxanthin (dino); diatoxanthin (diato); β carotene (β-car). (+) positive for pigment; (-) negative for pigment. Values are shown in micrograms of pigment per million cells.

Species	Pigment (µg per 10 ⁶ cells)													
	chl c3	MV-chl c3	cld a	MgDVP	chl c2	chl c1	me-cld a	per	fuco	diadin	dino	diato	chl a	β-car
<i>L. annulata</i>	-	-	-	-	1.680	+	-	-	23.090	1.490	-	-	31.470	0.670
<i>O. mobilensis</i>	-	-	-	-	2.740	+	-	-	30.300	-	-	67.310	146.200	18.300
<i>T. nitzschioides</i>	-	-	-	+	0.270	+	-	-	2.940	0.150	-	0.310	4.070	0.150
<i>Skeletonema</i> sp.1	-	+	+	+	0.060	+	+	-	0.060	0.010	-	-	0.090	0.005
<i>Thalassiosira</i> sp.	2.240	+	-	-	1.940	+	-	-	32.770	1.490	-	-	34.940	0.830
<i>Pseudo-nitzschia</i> sp.	0.160	-	-	-	0.070	-	-	-	0.670	0.030	-	0.010	0.660	0.020
<i>A. glacialis</i>	-	-	+	+	0.190	+	-	-	1.360	0.090	-	-	1.540	-
<i>Skeletonema</i> sp.2	-	+	+	+	0.050	+	+	-	0.320	0.030	-	-	0.320	-
<i>Chaetoceros</i> sp.	0.270	+	+	+	0.520	+	+	-	3.870	0.160	-	-	4.750	-
<i>Rhizosolenia</i> sp.	-	-	-	-	0.140	+	-	-	1.350	0.080	-	0.430	1.800	-
<i>P. normanii</i>	3.180	+	-	+	14.250	+	-	-	136.100	5.500	-	1.960	162.800	-
<i>D. phuketensis</i>	-	-	-	+	0.460	+	-	-	3.040	0.290	-	0.080	3.510	0.320
<i>P. micans</i>	-	-	-	-	4.500	-	-	25.110	-	4.680	+	0.820	29.700	0.580

Table 2 - Chlorophyll a-normalized pigment ratio (µg/µg) for phytoplankton species. Chlorophyll c3 (chl c3); chlorophyll c2 (chl c2); chlorophyll a (chl a); peridinin (per); fucoxanthin (fuco); diadinoxanthin (diadin); diatoxanthin (diato); β-carotene (β car); n.d.: no data.

Species	Pigment:chlorophyll a ratio						
	chl c3:chl a	chl c2:chl a	per:chl a	fuco:chl a	diadin:chl a	diato:chl a	β-car:chl a
<i>L. annulata</i>	n.d.	0.05	n.d.	0.73	0.05	n.d.	0.02
<i>O. mobilensis</i>	n.d.	0.02	n.d.	0.21	n.d.	0.46	0.12
<i>T. nitzschioides</i>	n.d.	0.06	n.d.	0.72	0.04	0.08	0.04
<i>Skeletonema</i> sp.1	n.d.	0.67	n.d.	0.64	0.08	n.d.	0.06
<i>Thalassiosira</i> sp.	0.06	0.06	n.d.	0.94	0.04	n.d.	0.02
<i>Pseudo-nitzschia</i> sp.	0.24	0.11	n.d.	1.00	0.05	0.02	0.02
<i>A. glacialis</i>	n.d.	0.12	n.d.	0.89	0.06	n.d.	n.d.
<i>Skeletonema</i> sp.2	n.d.	0.16	n.d.	0.99	0.08	n.d.	n.d.
<i>Chaetoceros</i> sp.	0.06	0.11	n.d.	0.82	0.03	n.d.	n.d.
<i>Rhizosolenia</i> sp.	n.d.	0.08	n.d.	0.75	0.04	0.24	n.d.
<i>P. normanii</i>	0.02	0.09	n.d.	0.83	0.03	0.01	n.d.
<i>D. phuketensis</i>	n.d.	0.13	n.d.	0.87	0.08	0.02	0.09
<i>P. micans</i>	n.d.	0.15	0.85	n.d.	0.16	0.03	0.02

Table 3 - Diatom class pigment content Permanova hypothesis test (Permutational Multivariate Analysis of Variance). Cl: Classes; P(perm): permutation test p value; P(MC): Monte Carlo test p value.

Source	df	SS	MS	Pseudo-F	P (perm)	perms	P (MC)
Cl	2	348.86	174.43	0.40543	0.725	705	0.765
Res	9	3872.2	430.24	-	-	-	-
Total	11	4221	-	-	-	-	-

tendency to higher concentrations between the months of June and November. Chlorophyll *a*, chlorophyllide *a*, chlorophyll *c1*, monovinyl chlorophyll *c3*, MgDVP, fucoxanthin, zeaxanthin, diadinoxanthin, chlorophyll *c3*, chlorophyll *c2*, and alloxanthin were detected with a more homogeneous distribution than the other pigments along the year. Chlorophyll *a* and fucoxanthin were detected in higher concentrations throughout the sample period, often with concentration value above 1 µg/L. Other pigments were detected below this concentration. Violaxanthin and lutein were detected in lower concentrations and restricted to some samples, not exceeding 0.040 µg/L.

Microscopy data was crossed with the information of the pigments detected in each analysis, generating the Canonical Correspondence Analysis graph (Figure 6). An effort was made to put in the same analysis the classes found (circles, squares and triangles) with the detected pigments (axes) for each sample collected throughout the year. When possible, it was indicated in the graph (uppercase letters) the species which had its pigment profile generated by unialgal cultivation.

P values lower than 0.05 were found through the Monte Carlo test (0.006 in the first axis and 0.044 in all), indicating that there is significance between the two matrices. The first axis was responsible for 18.7% of species distancing, with a correlation of 0.962, while the second axis explained cumulatively 25.2%.

Most Coscinodiscophyceae and Fragilariophyceae showed positive correlation with chlorophyll *c3*, fucoxanthin and chlorophyll *c2*, grouping to the graph left side. Some species, which include *Chaetoceros* sp. (B), were grouped at the chart bottom. Some Dinophyceae showed positive relation to peridinin, which include *Prorocentrum* (G), but it was not a rule as seen by the scattered feature observed within other species of this group.

DISCUSSION

Cultures

Although many studies are using chemotaxonomy techniques to determine phytoplanktonic groups, few studies are analyzing unialgal cultures. These studies were gathered from the literature and the relevant results are summarized in Table 5. Chlorophyll *a* and *c2* were detected in 51 Diatoms strains isolated from tropical and subtropical waters from Australia, as well as β-carotene, fucoxanthin, diatoxanthin and diadinoxanthin (Stauber & Jeffrey, 1988). Chlorophyll *c1*

Table 4 - Pigment concentration (µg/L) in water samples. chlorophyll *c3* (chl *c3*); chlorophyll *c2* (chl *c2*); chlorophyll *b* (chl *b*); chlorophyll *a* (chl *a*); peridinin (per); 19'-butanoyloxyfucoxanthin (but-fuco); fucoxanthin (fuco); prasinolaxanthin (pras); violaxanthin (viola); 19'-hexanoyloxyfucoxanthin (hex-fuco); diadinoxanthin (diadin); alloxanthin (allo); diatoxanthin (diato); lutein (lut); β carotene (β-car); monovinyl chlorophyll *c3* (mv-chl *c3*); chlorophyllide *a* (clide *a*); Mg-3,8-divinyl-pheoporphyrin *a*₅ monomethyl ester (MgDVP); chlorophyll *c1* (chl *c1*); neoxanthin (neo); dinoxanthin (dino); antheraxanthin (ant); zeaxanthin (zea). Values are shown for pigments with concentration calculated using calibration curves made with external pigment standards. When external standards were not available for concentration calculation, the presence of the pigment in the sample is indicated with the symbol *.

Pigment	2014														2015											
	18/ Aug	25/ Aug	01/ Sept	08/ Sept	15/ Sept	22/ Sept	06/ Oct	20/ Oct	03/ Nov	10/ Nov	24/ Nov	01/ Dec	15/ Dec	16/ Jan	04/ Mar	18/ Mar	29/ Apr	13/ May	27/ May	17/ June	01/ July	15/ July	29/ July	12/ Aug		
chl <i>c3</i>	0.029	0.020	0.058	0.069	0.062	0.091	0.086	0.032	0.107	0.075	0.032	0.060	0.131	0.062	0.105	0.136	0.209	0.054	0.066	0.124	0.077	0.146	0.088	0.070		
chl <i>c2</i>	0.111	0.118	0.132	0.443	0.125	0.199	0.416	0.075	0.585	0.219	0.103	0.146	0.322	0.295	0.161	0.215	0.885	0.264	0.159	0.243	0.226	0.377	0.242	0.222		
chl <i>b</i>	0.129	0.107	0.165	0.635	0.166	1.131	0.260	0.020	0.000	0.040	0.133	0.057	0.000	0.000	0.000	0.000	0.000	0.000	0.157	0.147	0.272	0.225	0.557	0.591		
chl <i>a</i>	1.012	0.823	0.997	4.770	0.719	2.191	3.992	0.513	2.408	1.890	1.143	1.329	2.154	0.989	1.264	1.522	3.363	1.172	2.001	3.148	2.777	4.221	4.968	4.108		
per	0.119	0.247	0.412	0.062	0.026	0.138	0.601	0.000	0.193	0.000	0.100	0.079	0.000	0.177	0.106	0.044	0.000	0.052	0.034	0.000	0.000	0.126	0.000	0.128		
but-fuco	0.000	0.000	0.000	0.000	0.000	0.084	0.000	0.000	0.000	0.010	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.059		
fuco	0.404	0.559	0.429	3.706	0.876	1.512	3.847	0.641	4.908	1.871	0.627	1.229	3.778	1.986	1.496	1.766	6.246	1.361	1.996	2.974	2.409	4.106	2.197	2.581		
pras	0.000	0.000	0.000	0.025	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.130		
viola	0.000	0.006	0.000	0.016	0.022	0.021	0.000	0.000	0.000	0.000	0.000	0.010	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.016		
hex-fuco	0.045	0.033	0.084	0.046	0.089	0.093	0.000	0.000	0.000	0.000	0.008	0.007	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.075		
diadin	0.057	0.079	0.096	0.175	0.068	0.096	0.211	0.024	0.098	0.066	0.025	0.073	0.140	0.088	0.139	0.060	0.000	0.022	0.121	0.152	0.154	0.232	0.054	0.193		
allo	0.000	0.006	0.000	0.000	0.014	0.008	0.003	0.000	0.000	0.000	0.000	0.008	0.000	0.000	0.000	0.000	0.000	0.000	0.019	0.047	0.027	0.021	0.000	0.000		
diato	0.174	0.054	0.101	0.186	0.079	0.108	0.074	0.005	0.066	0.000	0.025	0.027	0.014	0.009	0.038	0.006	0.000	0.000	0.073	0.093	0.183	0.098	0.000	0.113		
lut	0.000	0.006	0.039	0.032	0.022	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.003	0.000	0.000	0.000	0.011	0.000	0.000	0.000	0.021		
β-car	0.015	0.009	0.003	0.044	0.000	0.030	0.000	0.000	0.000	0.000	0.000	0.008	0.000	0.000	0.000	0.000	0.000	0.000	0.049	0.072	0.048	0.100	0.000	0.110		
mv-chl <i>c3</i>	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	
clide <i>a</i>	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	
MgDVP	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	
chl <i>c1</i>	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	
neo	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	
dino	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	
ant	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	
zea	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	

Table 5 - Comparison among pigments detected in unialgal cultures. Chlorophyll c3 (chl c3); chlorophyllide a (cld a); Mg-3,8-divinyl-pheoporpyrin a₅ monomethyl ester (MgDVP); chlorophyll c2 (chl c2); chlorophyll c1 (chl c1); peridinin (per); fucoxanthin (fuco); diadinoxanthin (diadin); dinoxanthin (dino); diatoxanthin (diato); (+) positive for pigment; (-) negative for pigment; (+/-) positive for some species.

Culture	chl c3	cld a	MgDVP	chl c2	chl c1	per	fuco	diadin	dino	diato	Reference
<i>Odontella</i>				+	+		+			+	This work
				+	+		+	+		+	Seville et al., 2017
<i>Skeletonema</i>		+	+	+	+		+	+			This work
		+	+	+	+		+	+			This work
				+	+		+	+		+	Lavieale and Neveux, 2011
				+	+		+	+		+	Yao et al., 2011
				+	+		+	+		+	Seville et al., 2017
		+	+	+	+		+	+		+	Zapata et al., 2000
<i>Thalassiosira</i>	+			+	+		+	+			This work
	+	+		+	+		+	+			Suzuki et al., 2015
		+		+	+		+	+		+	Seville et al., 2017
		+		+	+		+		+	+	Yao et al., 2011
<i>Pseudo-nitzschia</i>	+			+			+	+		+	This work
	+/-		+/-	+	+/-		+	+		+	Zapata et al., 2011
<i>Chaetoceros</i>	+	+	+	+	+		+	+			This work
				+	+		+	+			Suzuki et al., 2015
				+	+/-		+	+		+	Seville et al., 2017
				+	+		+	+		+	Yao et al., 2011
<i>Prorocentrum</i>				+		+		+	+	+	This work
				+		+		+	+	+	Lavieale and Neveux, 2011
				+	+/-	+		+	+	+	Zapata et al., 2012

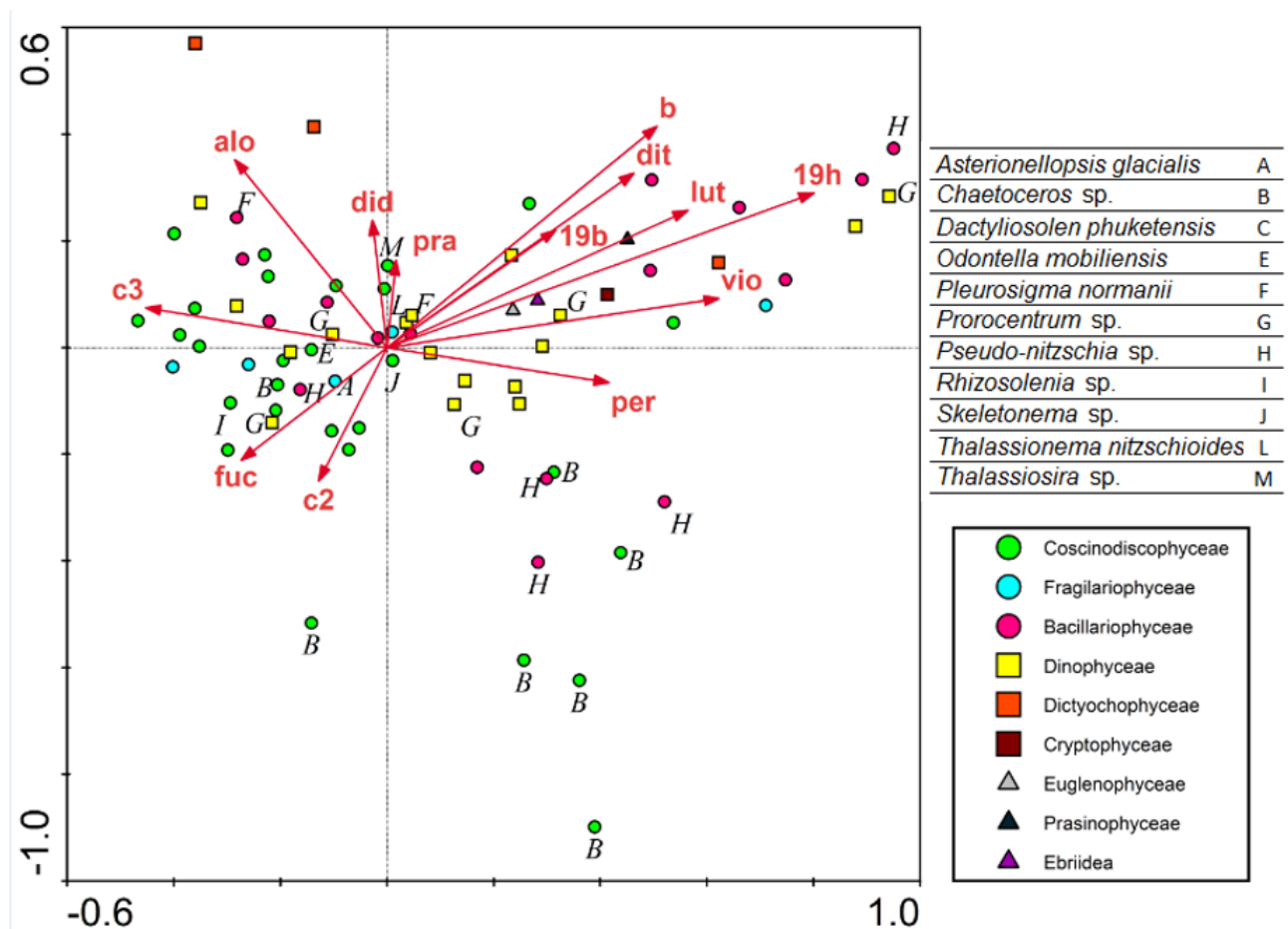


Figure 6 - Canonical Correspondence Analysis (CCA) between phytoplankton community structure and pigments found in environmental samples. Each dot refers to a species which belongs to its respective class, highlighted by the circles, squares and triangles as the legend shows. Chlorophyll b (b); chlorophyll c3 (c3); chlorophyll c2 (c2); alloxanthin (alo); diadinoxanthin (did); prasincoxanthin (pra); fucoxanthin (fuc); diatoxanthin (dit); 19'-butanoyloxyfucoxanthin (19b); 19'-hexanoyloxyfucoxanthin (19h); lutein (lut); peridinin (per); violaxanthin (vio).

occurred together with chlorophyll *c*2 in 88% of them. Pigments detected in Diatoms in this work were similar in composition, with exception to chlorophyll *c*3, which was detected in four species. In *Chaetoceros* sp. and *Thalassiosira* sp., chlorophyll *c*3 was detected in addition to chlorophyll *c*1 and *c*2. Other differences are the absence of diadinoxanthin in *O. mobilensis* and absence of diatoxanthin in *L. annulata*, *Skeletonema* sp., *Thalassiosira* sp., *Chaetoceros* sp. and *A. glacialis*. This suggests there are differences in the pigment compositions between same species from different regions. There is also the possibility that because the methods employed were different, the results varied between species.

In a culture of *Skeletonema costatum* isolated in Spain, the pigments chlorophyllide *a*, MgDVP, chlorophyll *c*2, chlorophyll *c*1, methyl chlorophyll *a*, fucoxanthin, diadinoxanthin, diatoxanthin, chlorophyll *a*, β -carotene and monovinyl chlorophyll *c*3 were detected (Zapata et al., 2000). All these were found in the *Skeletonema* sp. strain analyzed in this study, except for diatoxanthin, which may not have minimum concentration for detection. This carotenoid has been previously found in other works in *Skeletonema* species (Lavieale and Neveux, 2011; Yao et al., 2011; Serive et al., 2017).

Pseudo-nitzschia spp. pigment profile analyzed in this study is similar to *Pseudo-nitzschia* species isolated from Spain coastal waters. All *Pseudo-nitzschia* strains showed fucoxanthin, diadinoxanthin, diatoxanthin, β -carotene, chlorophyll *a* and chlorophyll *c*2 in its pigment composition (Zapata et al., 2011). Some of them (seven out of 18) had chlorophyll *c*1, and others (14 out of 18) had chlorophyll *c*3 as accessory pigments (Zapata et al., 2011). For convenience, the authors organized these species in three pigmentary types. Type 1 including chlorophyll *c*1 and *c*2, type 2 including chlorophyll *c*1, *c*2 and *c*3, and type 3 including chlorophyll *c*2 and *c*3. *Pseudo-nitzschia* species analyzed in this work fit in pigment type 3, as it has only chlorophyll *c*2 and *c*3.

Zapata et al. (2012) analyzed the composition of 64 Dinoflagellates, including the species *P. micans* analyzed in this work. Peridinin was the major carotenoid in almost all Dinoflagellates, which were divided into six chloroplast types. Most Dinoflagellates were labeled as chloroplast type 1 (71% of species), with chlorophyll *c*2 as the major accessory chlorophyll and traces of MgDVP (Zapata et al., 2012). Prorocentrum strains were also grouped in chloroplast type 1, with peridinin, chlorophyll *c*2, chlorophyll *c*1, diadinoxanthin and diatoxanthin as main pigments. Although chlorophyll *c*1 was not detected in *P. micans*, (Zapata et al., 2012). Similar pigment composition was detected in the strain of *P. micans* analyzed

in this study, with only exception to diatoxanthin. This composition was also found for *P. minimum* (Laviale & Neveux, 2011). Fucoxanthin and chlorophyll *c*3 were not detected in any Prorocentrum species (Table 5).

Phytoplankton community and pigment distribution

Until now, there were no works employing chemotaxonomy for phytoplankton in the studied area, however, the phytoplankton community structure is already known. Dell'Agnolo (2011) studied the diversity and seasonal variation of marine Diatoms in the region employing microscopy technique. A total of 82 Diatoms species were found, divided into 21 orders. Two species analyzed in this work, *Thalassiosira nitzschoides* and *Skeletonema* sp., were considered as abundant in the region. In other work Miotto & Tamanaha (2012) found 20 Dinoflagellates species in the region, ten of them of the genus Prorocentrum. The Dinoflagellates contribution to the phytoplankton community reached 65% in one sample (Miotto & Tamanaha, 2012). In this work, a similar trend occurred in the phytoplankton seasonal distribution, with abundance peaks in spring, characteristic in this area (Dell'Agnolo, 2011).

The great majority of the species belonging to the classes Coscinodiscophyceae, Bacillariophyceae and Fragilariophyceae showed a positive correlation with chlorophyll *c*2, *c*3 and fucoxanthin in the Correspondence Canonical Analysis (Figure 6). These pigments are characteristic of these classes, which belong to the Diatom group (Jeffrey et al., 1997). After chlorophyll *a*, fucoxanthin was detected as the pigment with highest concentration in the samples, as was found in oceanic water samples collected in the southeast part of Brazil (Rodrigues et al., 2014).

Most Dinoflagellates showed a positive correlation with peridinin, since this pigment is characteristic of Dinoflagellates species (Zapata et al., 2012). Some Dinoflagellates also showed positive correlation with pigments from Diatoms, because Dinoflagellates can feed on them, besides presenting endosymbiont pigments (Jeffrey et al., 1997). The broad Dinoflagellate distribution in the graph may be related to the possibility that this group of organisms have pigments characteristic of Bacillariophyta, Chlorophytes, Haptophytes or Cryptophytes (Laza-Martinez et al., 2007).

Cryptophyceae shows association with alloxanthin, which is considered the pigment representative of this class (Delizo et al., 2007; Araujo et al., 2012). Interesting, this class remained contrary to alloxanthin with X axis. Prasinophyceae did

not present a strong correlation with prasinoxanthin, the characteristic marker pigment of this group. However, it correlated positively with lutein and violaxanthin. This can be explained because not all species of this class have prasinoxanthin, but have violaxanthin and lutein instead, as well as other pigments (Latasa et al., 2004), such as the species *Pyramimonas* sp., found in great number in the samples.

Chaetoceros (B) and *Pseudo-nitzschia* (H) species showed a positive correlation with chlorophyll *c2* and fucoxanthin (Figure 6). Some *Prorocentrum* (G) species showed a positive correlation with peridinin. However, several species remained grouped near the chart center. Such disposition may be influenced by an interference of the group diversity. Due to the relationships with other classes and pigments, some classes may not have a positive relation with a specific pigment as their marker. Dominance of a particular species can cause distancing between groups, as well as groups with lower abundance may have distinguished them from the others, remaining on the graph opposite side in a more dispersed way, not necessarily indicating correlation with a particular pigment.

Stress situations due to changes in environmental conditions lead organisms to adapt, synthesizing and transforming their pigments as they need (Koyama, 1991; Alami et al., 2012; Hannoufa & Hossain, 2012). This can cause fluctuations in the concentration of chlorophylls and carotenoids in same species or group (Koyama, 1991; Alami et al., 2012; Hannoufa & Hossain, 2012), thus reflecting the pigment variation found among the same species from different regions. Under conditions of high luminous intensity, the pigment ratios between diatoxanthin, diadinoxanthin, zeaxanthin and lutein to chlorophyll *a* can increase (Schlüter et al., 2000). These results show how one species can alter its pigment composition under environmental stresses. Thus, this could cause variations in the results obtained in the comparison of phytoplankton groups at different times of the year and regions.

Implications of microalgae pigments in bivalve mollusks nutrition

The content of polyunsaturated fatty acids, proteins and vitamins is the main factor defining microalgae nutritional value. In addition, pigments and sterols are important elements, which are transferred through the trophic chain (Ronnestad, 1998; Brown, 2002; Hemaiswarya et al., 2011). In this sense, carotenoids found in marine animals can directly represent their microalgae diet, or, indirectly, be metabolic products of ingested carotenoids.

Filter feeders, such as bivalves (oysters, mussels, scallops, etc.), contain several carotenoids in their tissue, many of which are fucoxanthin, peridinin, diatoxanthin, diadinoxanthin and alloxanthin metabolic products (Liaaen-Jensen, 1991; Maoka, 2009). For example, the gonads of scallop *Nodipecten nodosus*, grown in Santa Catarina (Brazil), are rich in carotenoids (0.410 µg of carotenoids per mg of gonad), which is relevant in the nutritional and biochemical context, as well as in scallop reproduction (Suhnel et al., 2009). The tissue of oysters, scallops and other mollusks become green because of marennine assimilation through their feeding, a blue-green pigment synthesized by Diatom species belonging to the genus *Haslea* sp. (Turpin, 2001; Gastineau et al., 2014). Similarly, diet based on different algae species affected the coloration of Pacific abalone (*Haliotis discus hannai*) shell, improving shell pigmentation under culture conditions (Ju et al., 2016).

Variation in phytoplankton communities composition, either by regionality or seasonality caused by algae adaptations to environmental conditions, will reflect on the nutritional content of the organisms they serve as food, causing significant differences in bivalve carotenoid contents (Murphy et al., 2002; Pettersen et al., 2010; Borodina & Soldatov, 2016; Merdzhanova et al., 2016). Therefore, knowing the phytoplankton community of a site and its pigment composition is important to estimate not only the availability, but also the quality of the base nutrition.

CONCLUSION

Pigments detected in the isolated strains were those normally found in microalgae of the class Bacillariophyta and Dinophyta, such as the carotenoids fucoxanthin, diadinoxanthin, diatoxanthin and peridinin. Diatom species *T. nitzschioides* and *Rhizosolenia* sp. were considered chemotaxonomically closer. *Lauderia annulata*, *D. phuketensis*, *A. glacialis* and *Skeletonema* sp.2, were also chemotaxonomically closer, and so the species *Pseudo-nitzschia* sp., *P. normanii*, *Chaetoceros* sp. and *Thalassiosira* sp. Obtaining the pigment profile of these species it became possible to clarify, at least in part, the pigments present in this environment, which may be a reflection of the environmental conditions to which these phytoplankton groups are exposed.

Phytoplankton community was dominated by Diatoms throughout the year, with abundance peaks in spring and autumn. Other groups showed peaks during late winter and early spring, and low density in late spring, summer and fall. Pigments found in the environmental samples also varied according to

the seasonality, following phytoplankton composition variation. Chlorophyll c2, chlorophyll c3 and fucoxanthin had high concentration peaks in the samples with high abundance of Coscinodiscophyceae, as well as the pigment peridinin that varied similarly to the class Dinophyceae. The use of chemotaxonomy with isolated strain pigment profile elucidation can help in the determination of the main phytoplankton groups of a region, and in understanding their physiological adaptations to local environmental conditions.

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