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Phycoerythrin productivity and diversity from five red macroalgae

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Abstract

Five red algae, *Agardhiella subulata*, *Gracilariopsis longissima*, *Gracilaria vermiculophylla*, *Polysiphonia morrowii*, and *Pyropia elongata* were sampled in winter for the extraction of phycoerythrin. The extracted phycoerythrin molecules were compared with the commercial phycoerythrin to determine the intrinsic fluorescence characteristics of the single pigments. An improved selective method for phycoerythrin extraction was set up for routinary investigation. The use of the mortar and pestle method for tissue homogenization with a freeze-thawing cycle allowed a simple and complete homogenization of the red algae. The extraction of phycoerythrin with diluted EDTA solutions (1 mM) at pH 9 enabled a selective and easy extraction of the pigment with 95–98% extraction efficiency. The way pH affected the phycoerythrin, phycocyanin, and allophycocyanin selective extraction was also evaluated. The 3D fingerprint of each pigment was recorded, and a comparison of different phycoerythrin spectra was performed by fluorescence spectroscopy highlighting differences in *A. subulata* and *P. morrowii* phycoerythrins in comparison with commercial standards purified from *Pyropia*. The productivity and the advantages of phycoerythrin that was extracted from unattached red algal species are discussed.

Introduction

The phycobiliproteins are a group of toroidal light-harvesting proteins present in red algae, cryptophytes and cyanobacteria. This family of fluorophores is subdivided into three separate groups with different absorption spectra and molecular weights (Kao et al. 1971; Glazer and Fang 1973; Kawsar et al. 2011): phycoerythrin (PE, $\lambda_{max} = 565$, M.W. = 240 kD), phycocyanin (PC, $\lambda_{max} = 620$, M.W. = 30 kD), and allophycocyanin (APC, $\lambda_{max} = 650$, M.W. = 105 kD). These proteins are part of an efficient energy transfer chain, channeling the excitation energy from the light-harvesting complex to the reaction centers containing chlorophyll-*a*. In particular, PE is widely used for biotechnological applications, as colorant in food and cosmetics, and in fluorescence techniques such as microscopy, cell sorting, and flow cytometry. Phycoerythrin has a role in immunoassay probes and cross-linking techniques, allowing to bind proteins without altering the biliprotein spectral characteristics (Oi et al. 1982).

Phycoerythrin and PC are also used as low-toxicity photosensitizers in the photodynamic therapy for the treatment of solid tumors (Pan et al. 2013; Sonania et al. 2014). Moreover, the scientific interest for phytopigments is so wide that recently a patent survey on the patent search engine "Espacenet" (European Patent Office 2017) for "phycoerythrin*; phycocyanin*; allophycocyanin*" revealed an exponential increase of patent publications. A total of 215 patents were released between 1980 and 2016, most of which (more than 70%) were released in the last 10 years. About 40% of these patents concern the extraction and purification of these proteins from algae and cyanobacteria, while the remaining 60% refers to healthcare and cosmetic applications.

The recent commercial interest for algal pigments requires a reliable and selective extraction protocol. The extraction of phycobiliproteins from macroalgae is notoriously difficult due to the presence of polysaccharides that retain these photosensitive proteins. Macroalgae are usually extremely resistant to chemico-physical and mechanical extraction techniques. Many solvents, surfactants, pH, and thermal shocks may disrupt the proteins and cause a decrease in both quality and fluorescence signal (Liu et al. 2009; González-Ramírez et al. 2014). Phycobiliprotein extraction techniques are usually complicated and time consuming because they require equipments and reagents that are not always available in all

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laboratories, such as French press, nitrogen cavitation vessels, liquid nitrogen, or hydrolytic enzymes (Table 1).

One of the main issues in PE extraction is to find an extraction solution which is good enough to separate the three phycobiliproteins PE, PC, and APC without altering the protein quality and the fluorescence emission. The quality of the extract affects directly the subsequent phase of determination or purification. The simple quantification usually is performed on clear crude extracts. Many extinction coefficients and equations have been proposed for the biliprotein estimation from aqueous extracts by spectrophotometric methods (Rowan 1989). These methods involve the use of species-specific coefficients and whenever chlorophyll-*a* is present in the extract produce an overestimation of PC and APC contents (Lauceri et al. 2017) resulting in an underestimation of the actual content of PE with common equations (Beer and Eshel 1985; Wiley and Neefus 2007). The spectrofluorimetric analysis is practical and reliable also on crude extracts, especially for PE, and allows a sharper separation of the signals of the different fluorophores (Sasim et al. 2014).

The techniques for the purification of small quantities of phycobiliproteins are quite time demanding and were developed following three steps: protein extraction, protein precipitation, and purification with chromatography (Moraes and Kalil 2009). The most common techniques following the extraction, in a PE purification protocol, usually involve consecutive ammonium sulfate precipitations at 25%, for PC removal, followed by PE precipitation at 45%. This semi-purified protein pool then undergoes column chromatography and gel filtration for pharmaceutical grade PE purification (Bermejo et al. 2003; Liu et al. 2005).

The study of phycobiliprotein extraction shows that researchers prefer to use cyanobacteria or microalgae and only recently benthic macroalgal species for pigment extraction and purification (Mensi et al. 2012; Cai et al. 2014; Nguyen et al. 2017). As far as we know, there is no literature on unattached macroalgal species, which is quite surprising because unattached macroalgae, growing in mid-water, are an abundant resource that quickly colonize the shallow bottoms in coastal transition environments (Francavilla et al. 2013).

This is why the aim of this paper was focused on developing an effective method for the extraction, purification, and quantification of phycoerythrin and other phycobiliproteins from common unattached red macroalgae, comparing them with common attached species, and evaluating by fluorimetric characterization the quality of the red pigment.

Materials and methods

Sampling

Red algae were sampled in the Venice Lagoon in winter (January 2016), when the highest production of PE occurs (Francavilla et al. 2013; Ismail and Osman 2016). The algae *Pyropia elongata* (Kylin) Neefus *et* J.Brodie, *Agardhiella subulata* (C.Agardh) Kraft *et* Wynne, *Gracilaria vermiculophylla* (Ohmi) Papenfuss, *Gracilariopsis longissima* (S.G.Gmelin) Steentoft et al., and *Polysiphonia morrowii* Harvey were kept alive in a tank filled with natural seawater for 24 h. The pigment extraction and determination followed.

Phycobiliprotein extraction

Fresh red algae were gently drained of the excess water, dabbed with a paper towel, and reduced into minute fragments (1–2 mm). They were then homogenized with a watch glass on a Petri plate. An aliquot of 50 mg was ground with 30 mg of silica beads (diameter 0.1 mm) using an agate mortar and pestle (Wiley and Neefus 2007). The pigment extraction was obtained after one freezing-thawing cycle, and the process was completed by keeping the sample overnight at 4 °C. The extracts were purified by centrifugation at 14,000×*g* for 10 min before the pigment determination.

Three different extracting solutions were tested for R-phycoerythrin (PE), C-phycocyanin (PC), and

Table 1 Common extraction techniques for PE from algae and cyanobacteria

Authors	Tissue disruption	Extracting solution	Time
Benavides and Palomares (2006)	Freeze-thawing/sonication/pestle and mortar	Distilled water	10 min
Dumay et al. (2013)	Liquid nitrogen and enzyme (xylanase)	Acetate buffer 50 mM, pH 5	–
Glazer and Fang (1973)	French press	1 mM C_2H_6OS + 50 mM $C_2H_3NaO_2$, pH 5.5	30 min
Glazer and Hixson (1975)	Freeze-thawing/tissue grinder/French press	10 mM PB, pH 7.0	–
Moreth and Yentsch (1970)	Hand drill tissue homogenizer	100 mM PB, pH 6.0	1–5 min
Niu et al. (2006)	Freeze-thawing	Distilled water, 0.02% NaN_3	24 h
Sasim et al. (2014)	Glass tissue grinder	250 mM Trizma base, 10 mM EDTA, lysozyme	Max 30 h
Wiley and Neefus (2007)	Pestle and mortar	100 mM PB, pH 6.8	24 h
Zimba (2012)	Freeze-thawing/sonication	PB, asolectin-CHAPS, pH 3.7 & pH 6.7	–

allophycocyanin (APC) extraction such as Milli-Q only, phosphate buffer (PB) at different concentrations (0.1, 1, 10, and 100 mM at pH 7.0), and ethylenediaminetetraacetic acid disodium solutions (EDTA) at different concentrations (0.1, 1, 10, and 100 mM). The extraction protocol was also tested at different pH ranges from 5 to 9.

Fluorescence determination of pigments

Fluorescence spectra of PE, PC, and APC were determined by a luminescent spectrometer (PerkinElmer LS 55) equipped with a Xenon discharge lamp equivalent to 20 kW for an 8-μs duration pulse, wide at half eight minor 10 μs with excitation slit 5 nm and emission slit 5 nm. Three-dimensional excitation emission matrix (EEM) fluorescence spectroscopy was used to characterize and distinguish molecular changes among the PEs extracted from the five red algae. Indeed, the determination of the 3D fluorescent excitation/emission spectra is very sensitive even to small molecular changes, and for this reason, it also has been used to characterize, unambigously, organic dyes with similar hues (Soltzberg et al. 2012). The EEM fluorescence matrix was obtained by scanning simultaneously excitation and emission on algal extracts and residual pellets to ascertain the extraction efficiency. The phycobiliproteins R-PE, C-PC, and APC were purchased from Sigma-Aldrich for quantification and molecular characterization. The peak chosen for the determination of PE was on the excitation/emission (E_{ex}/E_{em}) of 542/575 nm. An E_{ex}/E_{em} of 609/635 nm was monitored for PC determination, and an E_{ex}/E_{em} peak at 650/660 nm was chosen for APC.

EEM contour maps were obtained and processed by an original equipped computer and also by Spekwin32 software (Menges 2016). The excitation (E_{ex}) spectra were determined in a range from 300 to 630 nm and the emission (E_{em}) from 530 to 680 nm. All the chemical analyses were performed in triplicate and repeated until the analytical reproducibility (coefficient of variation) was within 5% in the same sample.

Data analysis

The data were processed by the programs Statistica 10 and Office Excel 2007. The Shapiro-Wilk test was used to ascertain the normal distribution, and a Student t test was performed to compare the effect of different extraction buffers. A single linkage dendrogram was plotted to compare different PE excitation profiles in the range between 430 and 600 nm.

Results

Excitation-emission matrix spectroscopy

The 3D spectra of PE, PC, and APC were determined by EEM spectroscopy. In particular by analyzing and comparing the spectral pigment fingerprints, which are sensitive to structural changes of the studied molecules, it was possible to differentiate between different PEs.

In Fig. 1, the PE fingerprint of *P. elongata* is reported. This red alga is the most commonly used species as raw material for the production of PE. This PE is also the reference molecule of this study.

The fingerprint of PE (4 μg mL^{-1}) shows that fluorochrome emits at 575 nm and shows a weak peak centered at $E_{ex} = 375$ nm and three other excitation peaks in the visible region: the first one at $E_{ex} = 496$ nm, the second one at $E_{ex} = 542$ nm (used for quantification), and the third one $E_{ex} = 567$ nm next to Rayleigh-Tyndall's scattered light lines (Fig. 1). The contour of PE is between E_{ex} 450–550 nm and E_{em} from 550 to 650 nm while PC displays a peak at $E_{ex}/E_{em} = 609/635$ nm and APC at E_{ex}/E_{em} 650/660 nm. Phycocyanin and APC fluorescence intensities were low in comparison to PE, because their concentration and specific molar fluorescence are lower.

A comparison of the PE excitation profiles from the algal extracts with the PE purchased from Sigma-Aldrich (extracted from the genus *Pyropia* as by supplier statement) is plotted in Fig. 2a and reveals significant differences between the excitation profiles, especially for *P. morrowii* which displayed a 10-nm blue shift of the first peak in the visible region. As expected, *P. elongata* displayed the highest degree of similarity with the commercial PE as it can also be seen in the single linkage dendrogram (Fig. 2b). Both *G. vermiculophylla* and *G. longissima* cluster together while *A. subulata* and *P. morrowii* show the highest relative Euclidean distance from the commercial PE.

Phycobiliprotein extraction

The phycobiliproteins from the red alga *A. subulata* were extracted by using different solutions to assess the best conditions for the retrieval of the highest concentration of PE, which is by far the most representative pigment. The extraction efficiency was improved by decreasing the molarity of the solutions (Fig. 3). A solution of 1 mM EDTA allowed to extract in 24 h the highest concentrations (2.26 mg mL^{-1}) of PE. A longer extraction time of 72 h did not significantly improve PE retrieval by EDTA 1 mM solution as confirmed by an independent-sample t test (t test: d.f. = 4, $p = 0.39$) for the comparison of the results at 24 and 72 h. The extraction efficiency was evaluated as fluorescence loss, by measuring PE fluorescence prior and after the extraction, that is to say, on the

Fig. 1 EEM spectra of phycobiliproteins from *Pyropia elongata* (ex *Porphyra* sp.); 3D spectrum of PE with cutoff at 700 RFU. Inset: 3D spectra of PC and APC with cutoff at 250 RFU. PE extracted by 1 mM EDTA at pH 9 and PC and APC by 1 mM PB at pH 7
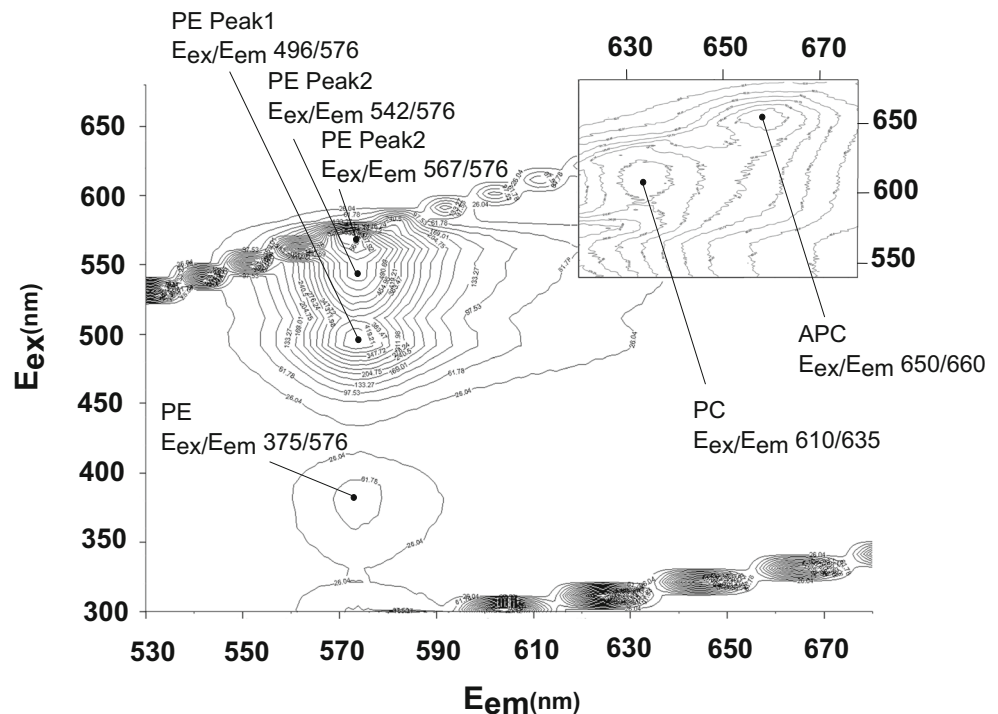
algal pulp and the residual pellets, respectively; its range was between 95 and 98%. The fluorescence emission of phycoerythrin after the extraction from *A. subulata* by EDTA and PB (1 mM) reduced from 1940 ± 95 RFU to 39.0 ± 1.9 and 79.8 ± 3.5 RFU, respectively.

The extraction efficacy of PE, PC, and APC was evaluated at different pH values from five red algae: *A. subulata*, *G. longissima*, *G. vermiculophylla*, *P. morrowii*, and *P. elongata*. The not buffered EDTA solution, originally at pH 7, was measured during the seaweed extraction revealing a pH increase up to 9 ± 0.2 in all the seaweed extracts due to the physiological buffers of the algae.

With reference to PE content of all the red algae, the box-plot diagram shown in Fig. 4 and an independent-sample *t* test (*t* test: d.f. = 8, *p* = 0.33) revealed no significant difference when the extraction with PB at pH 9 (mean value 1.23 mg g^{-1} fw, SD = 0.55 mg g^{-1} fw) and that with EDTA at pH 9 (mean value 1.73 mg g^{-1} fw, SD = 0.93) were compared. The algae responses to the extraction by EDTA 1 mM at pH 9 and PB 100 mM at pH 9 were different and species-specific. *Agardhiella subulata*, *G. vermiculophylla*, and *P. elongata*, extracted by phosphate buffer, reached from 60 to 66% of the pigment extracted by the EDTA solution; *G. longissima* and *P. morrowii* reached 92–98% instead. Moreover, the amount of EDTA 1 mM at pH 9 is the best solution to extract selectively the highest quantity of PE, from all the investigated species, without extracting the other phycobiliproteins (PC and APC), whereas PC and APC can be optimally extracted by 1 mM PB at pH 6–7 (Fig. 4).

A quick test of fluorescence loss for 5 min was performed by diluting, and then measuring, the fluorescence intensity of the phycobiliprotein standards with the extracting solutions. No change in fluorescence was recorded for PE, PC, and APC from pH 5 to pH 9, nor by EDTA solutions.
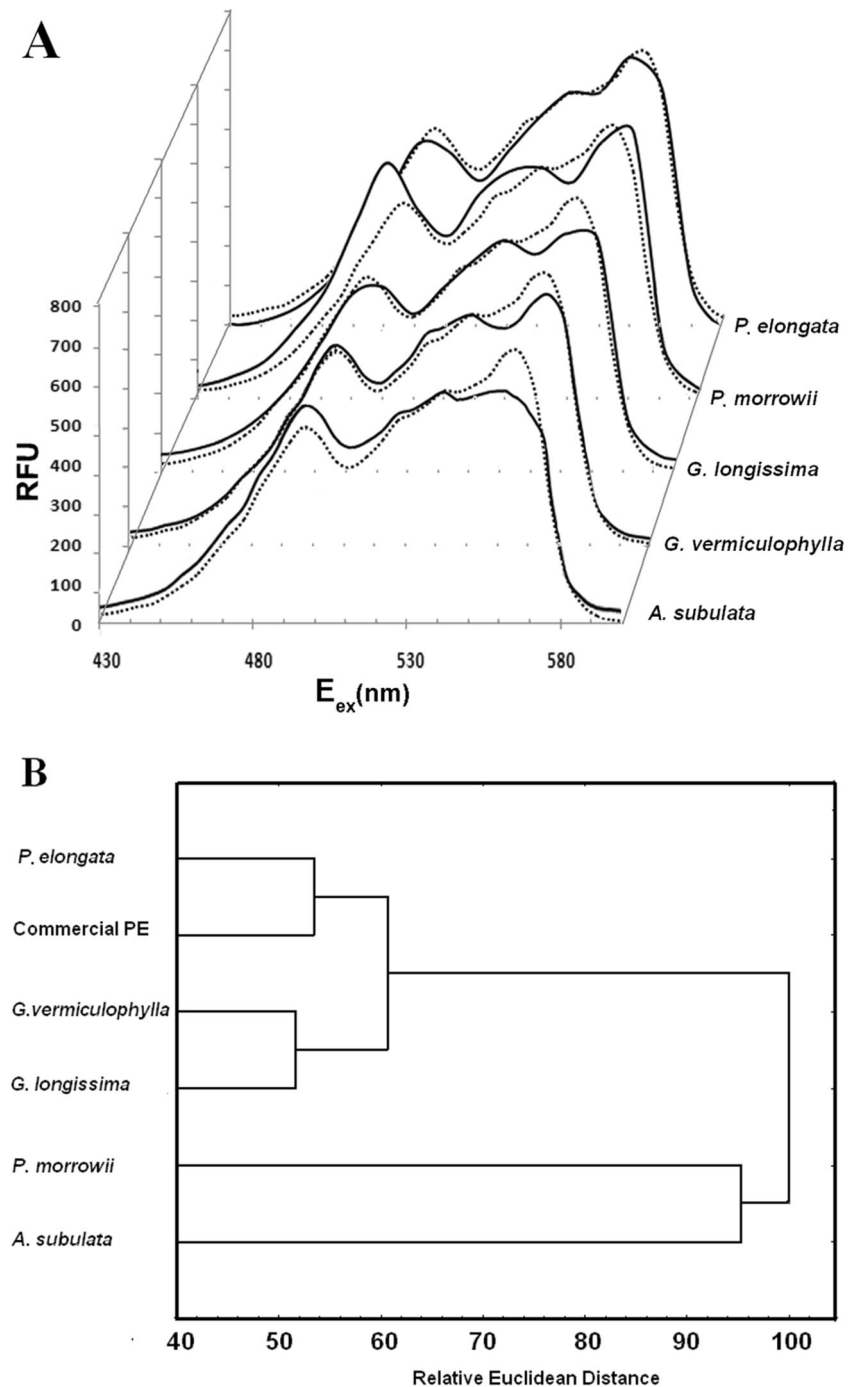
Algal phycobiliprotein content

The scores recorded with the best extracting solutions for each phycobiliprotein are reported in Fig. 5. The highest content of phycobiliprotein was found in *P. elongata*. The comparison with *P. morrowii* shows that the latter did not even reach 20 and 12% of *P. elongata* PE and PC content, respectively. *Agardhiella subulata* displayed the second highest PE content but low PC and APC. Finally, although *G. vermiculophylla* PE and PC values were about 43% lower than those displayed by *P. elongata*, the APC content was similar in both the algae.

Discussion

The coastal transition environments of the Adriatic Sea, such as estuaries and lagoons, are usually characterized by high turbidity and nutrient loads (Sfriso et al. 2017a, b). In these environments, a remarkable algal production of unattached red algae was recorded (Sfriso et al. 1994a; 2017a, b). The attached species, such as *P. morrowii* and *P. elongata*, are present, though they represent a minority in terms of biomass production (Sfriso et al. 1994b). The species involved are

Fig. 2 a Excitation spectra of PE
(4 μg mL^{-1}) extracted from five
red algae (solid line) in
comparison with commercial PE
(dotted line). **b** Single-linkage
clustering dendrogram of the PE
excitation spectra in the range
430–600 nm

mainly Gracilariaceae and unattached red algae, such as
A. subulata (Sfriso et al. 2016a, b). These species could be
not only a valuable raw material for agar extraction, but also a
valuable resource of phycobiliproteins. In this context, a meth-
od for an efficient extraction and quantification of PE, the
main phycobiliprotein in the red algae, was carried out togeth-
er with a spectrofluorimetric characterization.

This is the first report on the effect of pH on the
phycobiliprotein extraction whose effects were only investi-
gated on the stability and fluorescence of pigments
(Zhongzheng et al. 1987; Ogawa et al. 1991; Liu et al. 2009;
Munier et al. 2014). The PE extraction technique we devel-
oped by 1 mM EDTA at pH 9 yields only PE and no other
phycobiliprotein. At the same time, it stops completely PC
and APC from leaking into the solution. Conversely, the best
way to extract PC and APC is by using 1 mM PB at pH 6 for
PC and 1 mM PB at pH 7 for APC, respectively. All authors
who deal with phycobiliprotein extraction describe the use of
extracting solutions at pH between 6 and 7 (Table 1). In ac-
cordance with our data, this pH range ensures the highest total

Fig. 3 Extraction of PE from
A. subulata after 24 h (empty
histograms) and 72 h (gray
histograms) with PB at pH 7 and
(not buffered) EDTA at different
concentrations (0.1, 1, 10, and
100 mM). The error bars show the
coefficient of variation (standard
deviation as a percentage of
mean); the maximum value was
5% (three independent replicates)
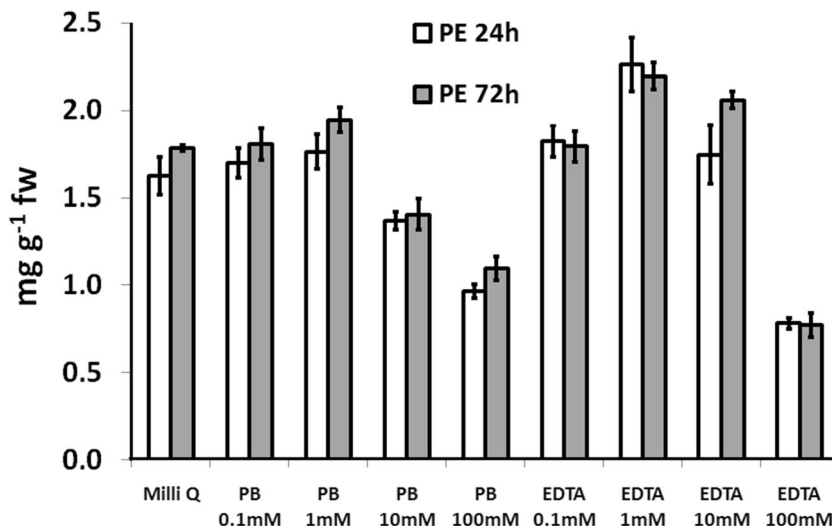
phycobiliprotein yield but not the single maximum yield for
each pigment. This can be obtained tuning the pH of the
extracting solution. The influence of the extractive pH seems
to be crucial for the selective extraction of PE which does not
explain for the disappearance of PC and APC from the
complexant extract. The fluorescence intensity of PE did not
change also in the pH range from 5 to 9 (Ramirez et al. 2000;
Liu et al. 2009); additionally in that range, no fluorescence
quenching was recorded for PC and APC; the same happened
with PB and EDTA solutions.

A one-step ion exchange chromatography purification
method for PE from *P. morrowii* was developed by Liu et al.
(2005). That method allowed to exclude other proteins due to
the different isoelectric points (iPs) and used decreasing pH
gradients. Assuming a similar behavior in the extracting con-
ditions from the sample which behaves as solid phase, a dif-
ference in the iPs of PE, PC, and APC could actually lead to a
differential extraction. The iP of APC from the red alga
*Porphyridium cruentum* (S.F.Gray) Nägeli was reported to

be at pH 5.1 (Ley and Butler 1977), and the iP of PC fell in
the pH range 5.2–5.6 (Bryant 1982). Eventually, the iP of PE
is only at pH 4.7.

The iP distance of PE from pH 9 is the highest in compar-
ison with PC and APC iP values, and that means that PE is the
most soluble pigment at pH 9 (Wang et al. 2014). Moreover,
Algarra et al. (1990) suggested that R-PE should be less
strongly bound to phycobilisome than PC and APC, which
could favor the extraction of the red pigment.

The reliability of the method involving the combined use of
the mortar and pestle with a freeze-thawing cycle and an
EDTA 1 mM solution for the selective extraction of the phy-
coerythrin fluorophore was verified by residual pellet fluores-
cence. This displayed an extraction efficiency of 95 and 98%,
say, a percentage higher than the efficiencies of PE extraction
from cyanobacteria reported in literature: > 73% in Zimba
(2012) and > 85% in Viskari and Colyer (2003).

The investigation on the excitation spectra of the PEs from
different red algae reveals qualitative differences. As expected,

Fig. 4 In all five red-algae spe-
cies, the phycobiliprotein contents
of PE (empty box), PC
(gray box), and APC (solid box)
were extracted in relation to pH
changes. The plot-box diagram
shows quartiles and means (solid
point)
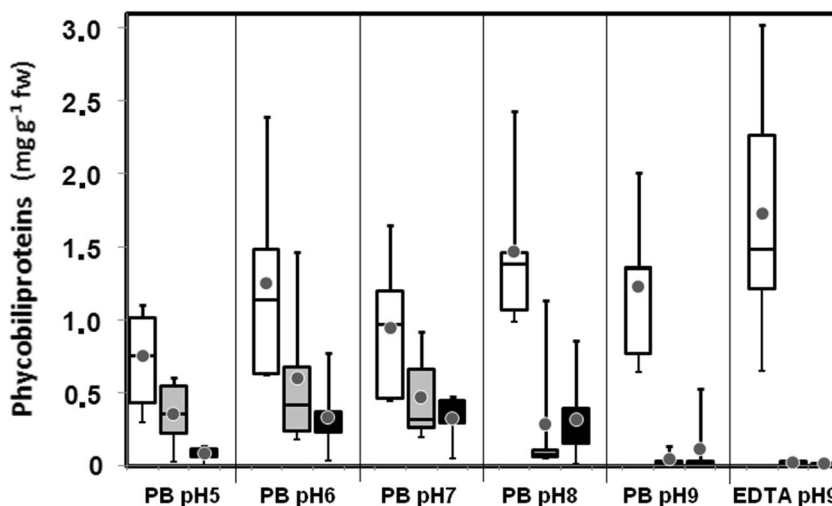
Fig. 5 Phycobiliprotein algal content from five red algae. The error bars show the coefficient of variation (standard deviation as a percentage of mean); the maximum value was 5% (three independent replicates)
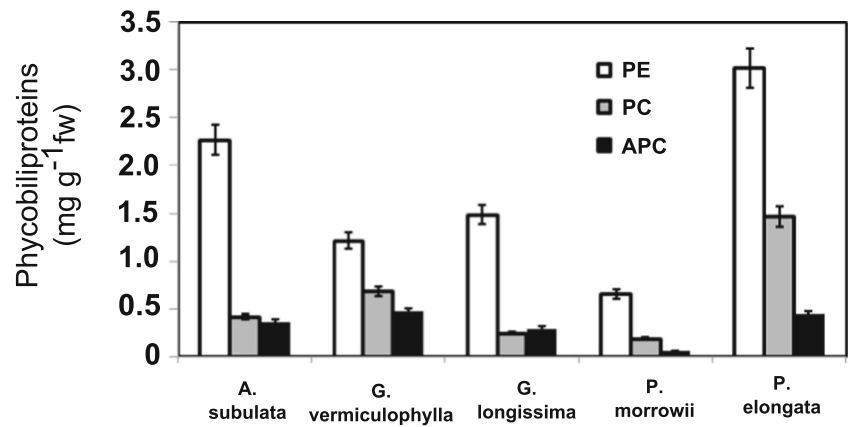
the PE excitation profile from *P. elongata* was the most similar to the commercial standard as it was extracted from the same genus. The same occurs for *G. vermiculophylla* and *G. longissima* which cluster together, but the most different PE profiles were those of *A. subulata* and *P. morrowii*. The differences in the PE excitation profiles reflect those in the biliprotein structure. For the PE of *Polysiphonia urceolata* (Lightfoot *ex* Dillwyn) Greville (which is the same genus as *P. morrowii*), structural differences from other PEs were reported. That is due to an additional γ subunit, which is uncommon because R-PE is usually composed of two trimers of $(\alpha\beta)_3$ subunits linked by only one γ subunit (Chang et al. 1996; Wang et al. 2015). No information instead was found in literature on the reasons why *A. subulata* shows such different characteristics. Consequently, it could be considered in future studies of proteomic characterization.

Although the qualitative differences are important, the production of all these pigments plays a main role in the choice of a cultivable red alga species. The phycobiliprotein global market was estimated at US$ 50 million with prices ranging from US$3 to 50 per milligram (Spolaore et al. 2006; Francavilla et al. 2013), depending on the degree of purity of the proteins.

In Table 2, the algal productions for the investigated species are reported. By multiplying the seaweed production reported by authors for the PE content, we estimated the PE productivity per square meter. Though most of the research

was carried out on *P. morrowii* and *P. elongata*, these sessile species display the lowest biomass production and so the lowest PE production. *Pyropia elongata* showed the highest phycobiliprotein content of all the seaweeds, but in comparison with unattached species, such as Gracilariaceae, the productivity was much lower. *Agardhiella subulata* because of its high PE content and high biomass production seems to be a good candidate for cultivation and PE production. However, we must highlight that this species that grows on the shallow bottoms of the lagoons tends to detach itself from the bottom and start floating in mid-summer leaving not-confined cultivation areas. This event cannot be reported with reference to Gracilariaceae which have a biomass comparable to *A. subulata* but are more stationary. In addition, unattached red algae, such as *Gracilaria* species, are already farmed in many countries (Hugh 2003) and can be cultivated in bioreactors under controlled conditions almost anywhere. Eventually, PE extraction from *Gracilaria* and *Agardhiella* species does not interfere with further treatments of seaweeds and sulfated polysaccharide extraction (agar and carrageenan) can follow (Toffanin et al. 1997; Sfriso et al. 2016a, b). The residual protein enriched material can be used both for fish food production in aquaculture and for metal remediation on soil and water bodies (Vilar et al. 2005, 2006). Further investigations should be carried out to find selective extraction protocols also from other species and for

Table 2 Seaweed production and PE productivity

Species	Seaweed production			Authors	PE productivity
		Unit	kg fw m^{-2} y^{-1}		g m^{-2} y^{-1}
Gracilaria sp.	6.0–62	g fw m^{-2} day^{-1}	2.2–23	Edding et al. (1987)	3.0–31
G. verrucosa	2.0–13	g dw m^{-2} day^{-1}	3.7–24	Mclachlan and Bird (1986)	5.5–36
G. longissima	11–28	kg fw m^{-2}	11–28	Sfriso and Sfriso (2017)	16–41
G. vermiculophylla	14–22	kg fw m^{-2}	14–22	Sfriso and Sfriso (2017)	17–27
P. morrowii	150–240	g fw m^{-2}	0.15–0.24	Sfriso et al. (1994b)	0.10–0.16
P. elongata	360–1700	g fw m^{-2}	0.37–1.7	Sfriso et al. (1994b)	1.10–5.10
A. subulata	0.06	g dw L^{-1} day^{-1}	27	Huang and Rorrer (2002)	61

other phycobiliproteins, especially for APC which resulted to be the pigment with the lowest concentrations.

Conclusions

This study revealed a new improved method for PE selective extraction from red algae by 1 mM EDTA. It also put in evidence the role of pH in PC and APC extraction highlighting that the highest efficiency rate can be obtained at pH 6 and 7, respectively. At pH 9, both PC and APC extraction efficiency was drastically reduced, while PE dissolution increased. The comparison of the fluorescence excitation profiles revealed qualitative differences among PEs from different seaweeds. Such differences were more marked in *A. subulata* and *P. morrowii* in comparison with commercial PE from *Pyropia*. The PE content in unattached red algae (Gracilariaceae and *A. subulata*) was comparable with the one from attached species (*P. morrowii* and *P. elongata*), but as the biomass production from unattached species is from 10 to 100 fold, the production from the attached ones for phycoerythrin extraction could be economically very interesting.

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