



Ocean acidification detrimentally affects mineralization and photosynthetic efficiency of the brown alga *Padina pavonica* at a CO₂ vent

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+ This paper is dedicated to the memory of Prof Zvy Dubinsky and Dr Yulia Pinchasov Grinblat, who both played a key role in the research but are no longer with us.

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ABSTRACT.—Increasing atmospheric CO₂ concentrations are causing ocean acidification potentially leading to significant consequences for marine organisms, particularly those that build calcium carbonate structures. In the present study, we examined the effects of decreasing pH on the photosynthetic efficiency and spectral reflectance of the calcifying alga *Padina pavonica* naturally occurring along a natural pH gradient generated by a CO₂ vent near the Island of Panarea (Italy). Based on the photoacoustic determination of photosynthetic light energy utilization efficiency, we found that the photosynthetic efficiency of *P. pavonica* decreased with decreasing pH. In the most acidified sites, calcium carbonate across the algae decreased while the changes in absorption light at 483 nm (carotenoids) in relation to green light (562 nm) ratio indicate an increase in the contribution of carotenoids to algal pigments towards the more acidic sites. Here we show, for the first time, the effects of seawater acidification on the optical properties of a Mediterranean calcifying phototroph under low pH conditions at CO₂ vents.

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The absorption of CO₂ acidifies the oceans, changing seawater chemistry and lowering its pH. Ocean acidification is considered a key threat to the long-term survival of marine calcifying organisms (Doney et al. 2009, Gao et al. 2019). Future forecasts indicate a progressive decrease in ocean pH from current values around 8.1 to ca. pH 7.8 by the year 2100 (Hendriks et al. 2010, Kroeker et al. 2010, Wittmann and Portner 2013, Auzoux-Bordenave et al. 2020). Because the pH scale is logarithmic, the latter indicates an increase of about 100% in ocean acidity (Burger and Frölicher 2023). The increase in seawater CO₂ concentration leads to a decrease in the availability of carbonate ions that reduce the aragonite saturation state, therefore affecting the ability of calcifying marine organisms to build their calcium carbonate exoskeletons and shells (Hoegh-Guldberg et al. 2007, Doney et al. 2009, McCulloch et al. 2012, Auzoux-Bordenave et al. 2020). Marine calcifying organisms include many taxonomic groups: photosynthetic primary producers such as planktonic coccolithophores, encrusting seaweeds, foraminifera, corals, molluscs, echinoderms, and crustaceans (Beaufort et al. 2011). Many of these organisms, which build their calcified structures from calcium carbonate (CaCO₃), show a decrease in the mass of CaCO₃ with seawater acidification (Fine and Tchernov 2007, Fabry et al. 2008, Ries et al. 2009, Goffredo et al. 2014).

Marine photosynthetic organisms carry out about half of the global photosynthetic carbon fixation (Falkowski and Raven 2007, Gao et al. 2019). Although increased CO₂ availability has the potential of stimulating photosynthesis, the stress due to the associated pH decrease may be deleterious (Gao et al. 2019), triggering possible conflicting responses ranging from increased photosynthetic performance (Johnson et al. 2012, Koch et al. 2013, Scherner et al. 2016), to decreased photosynthetic activity (Price et al. 2011, Sinutok et al. 2012), to no response (Israel and Hophy 2002, Hofmann et al. 2012). The photoacoustic method used in the current study is a very reliable and precise method for measuring the quantum yield of photosynthesis. It is a non-invasive method, which means that it does not damage the study organism. Photoacoustics measures the absorption of light by a material and the subsequent conversion of that light energy into heat. This heat can then be detected as an acoustic signal, or sound wave (Dubinsky et al. 1998, Pinchasov-Grinblat et al. 2013). In the case of photosynthesis, photoacoustics can be used to measure the efficiency of the photosynthetic process, which is known as the quantum yield (Φ). The quantum yield is the ratio of the number of light quanta (photons) that are absorbed by the plant and used for photosynthesis to the total number of light quanta that are absorbed by the plant (Dubinsky et al. 1998, Pinchasov-Grinblat et al. 2013).

There is yet limited information regarding the optical properties of photosynthetic organisms. Optical properties can be assessed by spectral analysis which measures the composition of a substance by determining the amount of light reflected from it at different wavelengths. In a spectral analysis of an algal sample, the wavelengths of the algal pigments can be seen. Pigments are chemical substances that give plants their color. The wavelengths of the algal pigments are determined by the type of pigments they have (e.g., chlorophyll, carotenoids). Spectral analysis can be used to distinguish between different types of algae and also differences in the amount of pigments in the algae (Fonseca et al. 2019). Certain types of algae, such as *Padina pavonica*, are known to produce calcite and aragonite surfaces. The amount of these minerals on the algal surface affects the way light is reflected from the alga. As the amount of these minerals decreases, the color of the algal pigments becomes

more apparent, and the amount of light reflected from the algae decreases (Cavaleri et al. 2013). Previous studies have used reflectance data to assess the health and physiological status of photosynthesizing organisms such as corals and calcifying algae (Bürger and Schagerl 2010) in terms of: (1) pigment concentration (Stambler and Shashar 2007, Torres-Pérez et al. 2015); (2) disease state (Anderson et al. 2013); (3) discrimination among healthy, bleached, and dead coral (Nurdin et al. 2012); and (4) effects caused by thermal stress (Russell et al. 2016). Although these studies have used reflectance to assess organismal health conditions, to the best of our knowledge, no work has been done to identify the possible effects of seawater pH reduction on reflectance of calcifying algae at CO₂ vents.

The macroalga *Padina pavonica* (Linnaeus) Thivy, 1960 (Phaeophyceae, Dictyotales, Dictyotaceae) is a brown calcified seaweed commonly found in the Mediterranean Sea, where, due to its fan-shaped thallus, it is also known as Peacock's tail (Cormaci et al. 2012). *Padina pavonica* is quite unique because it is one of the only two species of calcified brown algae known today (the second one being *Newhousia imbricata*; Johnson et al. 2012). The thallus of *P. pavonica* is covered by a layer of extra-cellular aragonite, a calcium carbonate polymorph, deposited as needle-shaped crystals, mainly on the ventral surface of the thallus (Geraldino et al. 2005). *Padina pavonica* is an ideal bioindicator for environmental change as it is sessile and has a long life cycle (Iluz et al. 2017, Segman et al. 2018). Here we investigated the photosynthetic efficiency and color reflectance of *P. pavonica* living along a natural pH gradient generated by a CO₂ vent (Goffredo et al. 2014).

MATERIALS AND METHODS

STUDY SITE AND SAMPLING.—Close to Panarea Island (Mediterranean Sea, Aeolian Archipelago, Italy, 38°38'16"N, 15°06'37"E) there is an area delimited by the islets of Dattilo, Bottaro, Lisca Nera and Lisca Bianca, characterized by a widespread distribution of gas vents (Anzidei et al. 2005). The sampling area is located around the main vent, a volcanic crater 20 × 14 m² wide at depth of 12 m, which emits a sustained column of CO₂ bubbles raising from the seabed towards the sea surface. The hydrothermally stable CO₂ emissions generates a pH gradient around the crater, which extends for about 34 m from the center of the crater (11.6 m deep) to the periphery (9.2 m deep).

Four sampling sites, whose seawater physicochemical parameters have been previously characterized (Goffredo et al. 2014, Fantazzini et al. 2015, Prada et al. 2017), were selected along the gradient: control site (S1), intermediate CO₂ (S2 and S3), and high CO₂ (S4) at the center of the crater. The distances from the center of the crater and the corresponding average pH in Total Scale (T) values for each site were: S1 control, $d = 34$ m, pH_T = 8.07; S2, $d = 13$ m, pH_T = 7.87; S3, $d = 9$ m, pH_T = 7.74; and S4, $d = 3$ m, pH_T = 7.40. *Padina pavonica* samples ($n = 2$ per site) naturally occurring along the gradient were collected in June 2012 from the four sites, placed in containers with seawater from the different sites and transported within 30 min by boat to the Eolo Sub Diving Center, where a temporary wet lab was set up. Samples were maintained at ambient temperature in aerated 2L containers with use of a bubbler and no source of artificial light and immediately measured to avoid alterations of the carbonate chemistry of the seawater due to calcification, respiration and photosynthesis.

PHOTOACOUSTIC EXPERIMENTAL SETUP.—To measure the quantum yield of photosynthesis using photoacoustics, the following steps are taken: the algal sample is placed in a thermostatted cuvette, which is a small chamber that maintains a constant temperature. A microphone or hydrophone is inserted into the water in the cuvette. The signal detectable by an immersed microphone is proportional to the heat generated by a laser pulse. The sample is illuminated with a strong, constant light that is sufficient to saturate the sample. The light absorbed by the photosynthetic pigments in the algal cells is, in part, stored by photochemistry as products of photosynthesis, while the remainder is converted to heat, causing a brief expansion wave sensed by the hydrophone. By exposing the algal samples to continuous saturating background light, no storage of any of the pulse energy can take place, whereas in the absence of such light, a maximal fraction of the pulse energy is stored by photosynthesis. The difference between the acoustic signals that are measured in the light and in the dark is the energy that is stored in photosynthesis. The energy that is stored in photosynthesis is divided by the total energy that is absorbed by the plant by which the plant is illuminated equals the quantum yield of photosynthesis. The measurement is repeated for 128 pulses and the results are averaged. This is done to improve the accuracy and precision of the measurement.

Each sample was placed in a 16 mm square glass cell (PAC = thermostated PhotoAcoustic Cuvette with plant samples), filled with seawater from its collection site. The cell was placed perpendicular to a laser beam that was incident upon the algal thallus and was adjustable to cover a circular area of about 5 mm diameter (Online Fig. S1). A second harmonic laser beam of Opolette 430 at 532 nm was used as a light pulse source. Each pulse caused a thermal expansion wave that was sensed by the submerged hydrophone. After amplification [Amptek A-250 Preamp and Stanford Research 560 amplifier (A)], the photoacoustic signal was recorded on a Tektronix TDS 430A oscilloscope (O) that is triggered by the laser pulse generator. The submersible, stainless steel enclosed homemade detector (D) contained a 10 mm diameter resonating ceramic disc (BM 500, Sensor, Ontario, Canada; Online Fig. S1). When the sample was illuminated by a saturating continuous beam, all absorbed light energy from the concomitant laser pulse was converted to heat. That value was the reference for all the pulse energy. Then, the same series of pulses (32–128 pulses) was aimed at the same samples in the dark. The difference between these signals was proportional to the energy stored by photochemistry. Its fraction of the total energy absorbed by the sample, as measured under the saturating light, is the efficiency of the utilization of light energy in the photosynthetic activity of the sample, Φ , or the quantum yield of photosynthesis (Dubinsky et al. 1998, Pinchasov-Grinblat et al. 2013). The source of the background light E was a quartz-halogen illuminator (Cole-Parmer 4971). We used the Root Mean Square (RMS) values over the time of the recorded signal (about 10 μ s), after each laser pulse.

REFLECTANCE MEASUREMENTS, METHOD, AND INSTRUMENTATION.—Light reflectance was measured using a spectrophotometer equipped with an optical fiber light guide (HR-4000, 600 μ m; Ocean Optics, Florida) of high spectral resolution (1 nm) at the UV-PAR wavelength range (200–1100 nm). Illumination was provided with a custom-made panel of EdiStar 120-W point-source LED (Edison, Karlsbad, Germany), and cooled down by a built-in fan.

The light source was positioned at an angle of approximately 70 degrees relative to the sample being tested, and the optical fiber sensor was positioned at an approximately 70-degree angle on the opposite side, ensuring that no shadows were cast between the light source and the sensor. The light source illuminated the entire algae pad, and the optical fiber was positioned 1 cm above the algae, resulting in a measurement distance of approximately 1 cm from the algae. The algae was placed on a blank white board, which also served as the measurement surface for the background light reflected from the light source. Each measurement was obtained by averaging over 10 measurements per sample. Two sides of the algae were measured.

The reflection of aragonite was measured by calculating the ratio of the light reflected from the algae pad to the light reflected from the white board, expressed as a percentage. The carotenoid content was determined by calculating the absorbance ratio at 483 nm to 562 nm. Changes in the carotenoid content can be inferred from changes in the green light or chlorophyll ratio (Zur et al. 2000, Gitelson et al. 2002, Gitelson et al. 2003, Kira et al. 2015).

MORPHOLOGICAL SEM INVESTIGATIONS.—Morphological investigations were carried out on air-dried samples of *P. pavonica*. Before drying, the samples were rinsed with double distilled water three times, followed by absolute ethanol. Samples were prepared for the Scanning Electron Microscope (SEM) observations on the ventral face of the thalli, the more calcified face. A small sample fragment, about 5 mm × 5 mm, was glued by carbon tape onto an aluminum stub and then coated with a thin layer (about 2 nm) of gold by sputtering to ensure their electrical conductivity. Prior to coating, the samples were dried under vacuum to remove water and volatile substances. The images were collected under high vacuum (10^{-6} Torr) using a Hitachi 6400 FEG scanning electron microscope operating at 20 keV. The signal from secondary electrons was used for the morphological investigation.

STATISTICAL ANALYSES.—Data were checked for normality using a Kolmogorov-Smirnov test ($n > 50$) and Shapiro-Wilk test ($n < 50$) and for homogeneity of variance using Levene's Test. Assumptions for parametric statistics were not fulfilled, thus the nonparametric Kruskal-Wallis and Spearman's rank correlation coefficients were used to test for differences in reflectance and photosynthetic efficiency among sites. All analyses were performed using SPSS v20.

RESULTS

The photoacoustic measurements indicate that the photosynthetic efficiency of *P. pavonica* is significantly positively related to seawater pH ($P < 0.001$; Fig. 1, Online Table S1). In particular, the average photosynthetic efficiency in *P. pavonica* decreased by over 60% from pH 8.1 to 7.4 (Fig. 1, Online Table S1). The reflectance of aragonite (i.e., the reflection of light from the algae surface relative to the reflection of background light from the white panel) showed lower values at the more acidified site (Fig. 2), in agreement with morphological observations of *P. pavonica* samples collected from the four sites showing a marked reduction of mineralized areas at lower pH values (Fig. 3). In particular, the thin aragonite layer covering the thalli in the control was progressively lost towards the more acidic site. At the macroscopic scale, the surface coverage of aragonite crystals significantly decreased with decreasing

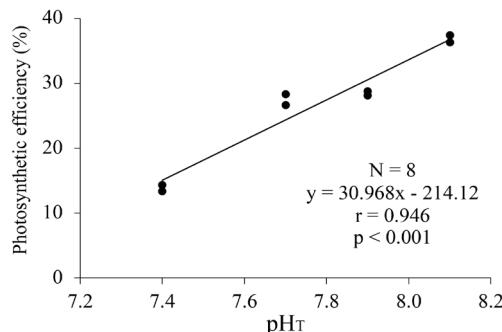


Figure 1. Variations of photosynthetic efficiency with pH in *Padina pavonica*. r = Spearman's correlation coefficient, N = number of individuals.

pH, as shown by the white regions on the thalli which clearly decline from site 1 to site 4 (Fig. 3A, E, I, M). The SEM images showed that crystals in sites 1 and 2 formed a compact mineral layer and were interconnected and fused together. Additionally, the typical crystalline morphology, which is characterized by well-defined faces and angles, was not observed. This makes it difficult to accurately estimate crystal sizes. However, a qualitative observation indicates that the aragonite crystals are smaller at lower pH values. This pattern drastically changed with acidity and in sites 2, 3, and 4, single crystals were visible, even if overlapping. The changes in absorption light at 483 nm (carotenoids) in relation to green light (562 nm) ratio indicate an increase in the amount of carotenoids towards the more acidic sites (Fig. 4).

DISCUSSION

The findings presented here on the photosynthetic efficiency and optical reflectance properties of the calcifying macroalgae *Padina pavonica* assessed along the Panarea pH gradient complement previous findings which showed that this algal species reduced and changed mineralization with increasing pCO₂, from aragonite to the less soluble calcium sulphates (Goffredo et al. 2014, Iluz et al. 2017). The decrease in spectral reflectance of aragonite with increasing acidity in *P. pavonica* is in agreement with morphological SEM observations revealing a decrease in aragonite through the reduction in calcified material, as previously

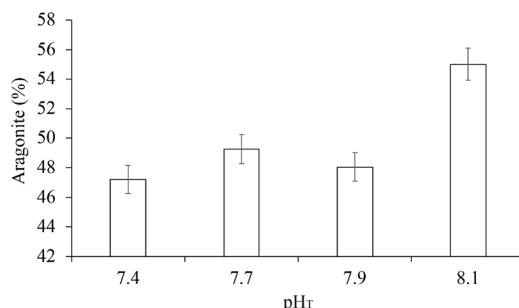


Figure 2. Percent coverage of aragonite in *Padina pavonica* at four sites along the Panarea pH gradient. The percent cover was calculated by comparing the light reflected from the sample to that from a white salt reference. Error bars report the instrumental error for the measurements.

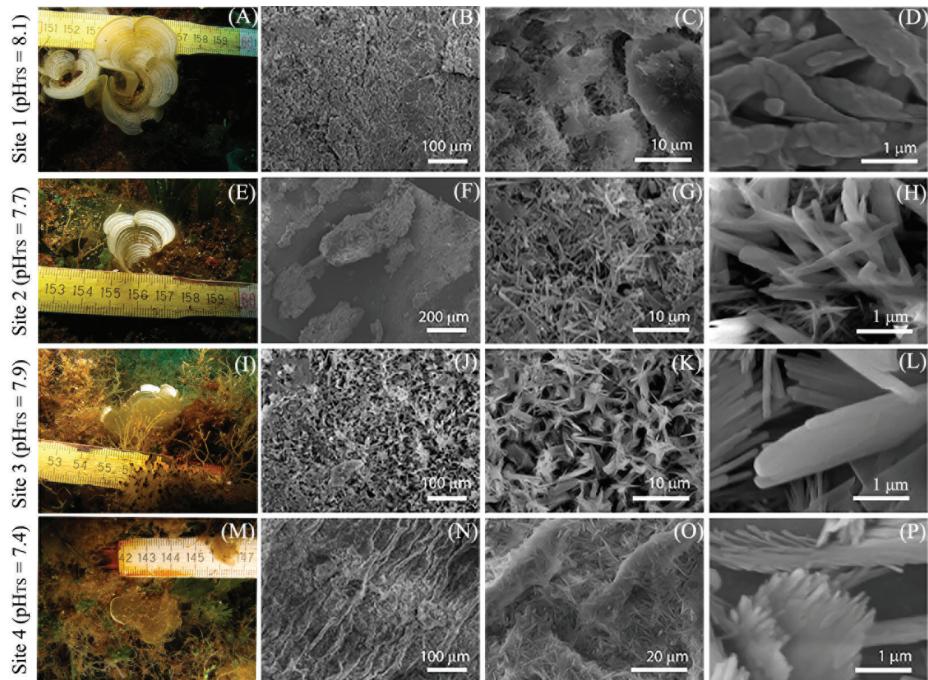


Figure 3. Morphological observations on *Padina pavonica* samples collected in the four sites, with the reduction of calcified material (white areas) with decreasing pH. The SEM images were taken on the more calcified face of the thalli, the ventral side facing the light, in the region close to the external border. The samples from site 1 (A–D), site 2 (E–H), site 3 (I–L), and site 4 (M–P) were photographed underwater (A, E, I, M; a ruler is included in each image to show scale: smallest tick marks = 1 mm) and imaged by scanning electron microscopy (SEM; B–D, F–H, J–L, N–P).

observed (Goffredo et al. 2014, Iluz et al. 2017). A change in aragonite texture along the pH gradient was detected by the SEM observations. Moreover, the observation of only acicular crystals, even if sometimes fused together, suggests the absence of any other crystalline phases besides aragonite. Calcification in macroalgae is thought to: (1) provide mechanical resistance to herbivores and minimizes grazing damage to tissues (Littler and Littler 1980, Padilla 1993), (2) increase the ability for bicarbonate

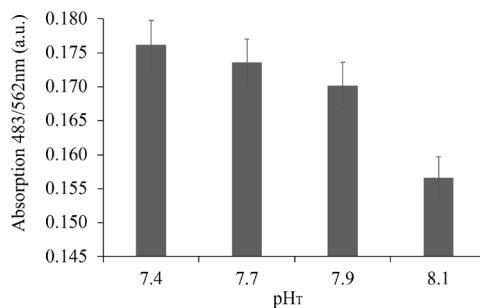


Figure 4. Changes in spectral absorption of *Padina pavonica* (carotenoids: 483/562 nm) at four sites along the Panarea pH gradient. Error bars report the instrumental error for the measurements.

and nutrient assimilation through the generation of protons (McConaughey and Whelan 1997), (3) improve photosynthetic performance (Buapet and Sinutok 2021), and (4) provide protection from photoinhibition (Bürger and Schagerl 2010). Thus, ocean acidification-induced changes in macroalgal calcification could potentially alter physiological and ecological fitness, by affecting thallus rigidity, growth rates, mortality, and photosynthetic efficiency (Nelson 2009). Indeed, our results indicate a decrease in photosynthetic efficiency with decreasing pH in *P. pavonica*, resulting from the reduction in mineralized structures.

The spectral absorption ratio (483 nm / 562 nm) reveals an augmentation in the ratio of total carotenoids to total chlorophyll, rather than a rise in the absolute quantity of carotenoids. Thus, the increase in contribution of carotenoids to algal pigments could be the result of chlorophyll or other pigments showing higher reductions than carotenoids with increased acidification. This finding aligns with a recent study on the green macroalga *Caulerpa cylindracea* at a CO₂ vent showing an increase in carotenoid content accompanied by a decrease in chlorophyll content at lowered pH (Santin et al. 2022). Photosynthetic pigments are not only responsible for harvesting solar energy to carry out photosynthesis, but also for photoprotective functions and antioxidant activity (Kuczynska et al. 2015). This phenotypic response to increased acidification is in agreement with previous studies conducted on the green alga *Chlorococcum* sp. (Zhang et al. 1997, Liu and Lee 2000). For instance, more carotenoids are produced to protect microalgae such as *Chlamydomonas*, *Dunaliella*, *Euglena*, and *Ochromonas* from high light and acidification stress (Faraloni and Torzillo 2017, Gauthier et al. 2020). Here we hypothesize that the increase in carotenoid light absorption in relation to other pigments serve as a protective mechanism as the calcified surface layer is reduced with increasing acidification. Future studies are needed to determine whether the thinning or removal of the aragonite coverage may increase the susceptibility of calcifying algae such as *P. pavonica* to herbivore grazing, UV damage, and high-light photoinhibition. It is of considerable interest trying to predict ecosystem changes brought about by ocean acidification. A growing body of literature suggests that in coming decades less resilient coral reefs will be likely replaced by algal meadows, benefiting from the increasing availability of CO₂, thus forcing a bottom-up change in the assemblages of associated fauna.

DATA AVAILABILITY STATEMENT

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

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