Light Gradient and Moonlight Effects on the Ventral Calcified Stripes and the Thallus Aspects of Padina spp. on the Mediterranean Coast of Israel

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Abstract

Light is an important abiotic environmental factor that dictates several dial, monthly and annual life patterns for most of the creatures on earth. Aside from its essential role as energy supporter of life on our planet, light may be dangerous and even deadly when overexposure occurs. Around the Israeli shores, intertidal organisms face high radiation most of the year, which exacerbated at ebb time due to desiccation. The Padina species, i.e., brown macroalgae inhabiting the Israeli abrasion platforms, seem to have means to protect themselves from excess light in such environments. They precipitate CaCO₃ in the form of aragonite needle-shaped crystals arranged in concentric stripes located mainly on the ventral side of the frond. These ventral stripes cover the reproductive organs, which are located behind them on the dorsal side of the frond. The aragonite needle-shaped crystals in the apical stripes change to a flattened amorphous shape in older parts of the frond, probably due to wave erosion. We present here our results regarding the effect of light intensity and moonlight on rate of deposition of aragonite -artificial and natural light, on the aragonite deposition rates and patterns. We found that even a small change in light intensity affects the reflectance from the calcified parts of the thallus. We also describe the deposition patterns of the aragonite in relation to the distribution of Padina’s reproductive organelles.

Materials and Methods

Sampling

Padina samples were collected from abrasion platforms at Tel-Ba-ruch (dp. 1800-07/6706-16, 32°N34°E), from October 2014 until June 2016, a collection area of 100 m² up to 15 cm deep. Samples were immediately placed in glass jars containing seawater. In the lab, medium-to large-size, non-damaged fronds were selected for experimental uses.

Microscopy

ESEM: Samples exposed to different light intensities for 4 days were subsequently examined using an environmental scanning electron microscope 2.2.1 (ESEM). Samples were collected into 50 ml tubes, and fixed in 70%EtOH. From each frond, 3 mm wide and 5-6 cm long vertical stripes, from the apical top to the rhizoids (vertical axis), were cut and glued to carbon paper on 6*3.5 cm aluminum plates. The plates with the samples were covered with carbon for x-ray analysis of the surface elements.

Binocular: Samples for binocular examination were fixed on petri dishes in a drop of seawater, examined, and photographed dorsally and ventrally from the apical top to the rhizoids (vertical axis).

Axio Z1: Samples for Axio Z1 were fixed onto ‘Superfrost+’ slides following histology embedding protocol.

Embedding protocol:
1. 80% ethanol for 1 hour or until start of embedding.
2. 80% ethanol for 1 hour.
3. 96% ethanol for 1 hour.
4. 96% ethanol for 1 hour.
5. 96% ethanol for 1 hour.
6. 100% ethanol for 1 hour.
7. 100% ethanol for 1 hour.
8. 100% ethanol for 1 hour.
9. First clearing agent-chloroform (AR)-for 1 hour.
10. Second clearing agent-chloroform (AR)-for 3 hours.
11. First wax (Paraplast X-tra) at 60°C for 3 hours.
12. Second wax (Paraplast X-tra) at 60°C for 1 hour.

**Histology**

Samples were placed in 70%EtOH at the end of each experiment. In preparation for embedding, they were transferred in the following order: 2 h 80%EtOH, 3 h 96%EtOH, 3 h 100%EtOH, 1 h chloroform, and 4 h chloroform, 60°C Paraplast X-tra wax for 3 h followed by 60°C Paraplast X-tra wax for 1 hour.

The embedded tissue was placed into paraffin blocks (LEICA). The blocks were cut into 5 μm thick slices by a cutting machine, and placed in a 37°C bath in order to straighten them up. The sections were set up on ‘Superfrost+’ slides, dried for one hour on a 40°C plate, and kept in an incubator at 37°C overnight. The slides were stained for 15 min in alizarin red.

**Light effects experiment on Padina pavonica**

In this experiment, we exposed the algae to a range of 9 light intensities. *Padina pavonica* fronds were placed in nine 5 L aquaria, with an average of 11 fronds (+3) in each one. The fronds was separated and cleaned from visible epiphytes and attached to silicon plugs, with their ventral side facing the light source. The aquaria were filled with 35% salinity instant Ocean® water, divided into three light gradient groups by placing them under 3 different volt light bulbs (LEELITE™) as follows: Aquaria #1-3 were placed under a low light gradient (20 W), aquaria #4-6 were placed under a medium light gradient (23 W), and aquaria #7-9 were placed under natural sunlight (high light gradient). In order to generate another gradient within each treatment, aquaria #1, 4, and 7 were covered with a double agronomic net layer, aquaria #2, 5, and 8 were covered with one agronomic net layer, and aquaria #3, 6, and 9 were placed bare, without any net, as seen in figure 1.

Light intensity, as described in table 1, was measured underwater, with the sensor next to the algal fronds, using a dual-wavelength spectrophotometer HK4000 with a 4π quantum sensor, ocean optics. Each is an average of 3 readings.

Algae were collected every day and attached vertically to a white plastic square that served as a background and reflectance control, and its value was subtracted from the stripe readings. The readings took place from the apical’s first calcareous stripe to the eighth stripe. Readings are averages of 14 measurements from different fronds, and its value was subtracted from the stripe readings. The readings took place from the apical’s first calcareous stripe to the eighth stripe. Readings are averages of 14 measurements from different fronds, after which the algae were subsequently fixed in 70%EtOH for histology (for details, see 3.3). The Ca/C ratio was determined by ESEM analysis (for details, see 3.2).

**Moonlight stripe measurement**

Eleven fronds (4.5 cm length average size, without rhizoids) were collected on the 10.4.16 and the 22.5.16, under a newborn moon and full moon, respectively. After patting dry and flattening the algae under a heavy book, the width of the CaCO₃ stripes was measured under a Leica binocular, averaging 5 measurements per stripe in the apical-basal axis where the stripes are wider. The measurements were from the first to the 7th stripe, a total of 35 measurements per alga.

**Statistical methods**

In the Light effects experiment, an ANOVA test, along with Tukey’s post hoc analysis took place. When there was no normal distribution, Kruskal-Wallis post hoc was used through Mann-Whitney multiple comparative correction.

In the Moonlight stripes measurement, two independent samples t-test was applied to compare random samples.
Results

Experiment

Effects of light: Light attenuation is exponential as depth increases, and only 45% of subsurface light remains at a depth of a few meters [9]. In the light experiments, we had 9 light intensities mimicking different depths which the algae inhabit. Under natural light conditions, the algal stripes had a particular reflectance pattern, as the first apical stripe reflected the most while the stripes in the mid-thallus reflected the least (Figure 2). In agreement with the reflectance gradient among the stripes, pigment ratios (460/560 and 680/560) of the calcareous stripes showed less pigment content in the apical stripes than in the mid-thallus (Figure 3).

This gradient light experiment attempted to mimic variant depths. By using equation no. 1, we calculated the approximate depth of each treatment, as seen in figure 4.

\[ I_z = I_o e^{-K_d z} \]

\( I = \)Irradiance, \( Z = \)depth, \( I_o = \)PAR, \( K_d = \)light attenuation coefficient; m\(^{-1}\).

It seems that under a particular light intensity, the reflectance from the calcifying stripes increases as depth reduces (Figure 4, intensities 1.29-2.58 μE).

In contrast to mid-light, algae under high and dim light show the opposite results. The stripe reflectance showed some reduction under 0.9 μE and above 2.58 μE.

In comparing the spectral characters to ESEM, it seems that results are consistent from 19.6 μE upward, while under very low light (8.33 μE downward) the results are the opposite (Figure 5). Under high light, even though the spectral results show some correlation with the ESEM, there is more of a dramatic drop in the reflection rather than in the amount of the CaCO\(_3\).

Figure 2: Light reflectance from the aragonite stripes under variant light conditions: Light reflectance from fresh algae showing the gradient reflectance as the first apical stripe reflects the most and the eighth stripe reflects the least. The pictures on the right show the CaCO\(_3\) morphology in those areas.

Figure 3: Light reflectance from the aragonite stripes under variant light conditions: Pigment ratio of fresh algae indicating the least absorption in the first apical stripe, increasing up to the eighth stripe.

Figure 4: Light reflectance from the aragonite stripes under variant light conditions: Reflectance under variant light intensity mimicking depth gradient. The results indicating that there is less calcification in the depth and more calcification at the shore and under high light conditions (t0=after placing the experiment, t4=after full 4 days).

Figure 5: Light reflectance from the aragonite stripes under variant light conditions: Thallus average Ca/C weight ratio compared with the thallus average reflectance, showing some correlation around mid-light (160-500 μE) while there is some reverse correlation under extreme light conditions. This implies that under some conditions, the morphology overcomes the amount. p-value=2.83e-06.

Pigment quality expression (Figure 6) showed correlation with the spectral results, and it appears to be expressed more under low light (290 μE downward) and, furthermore, tends to be overexpressed over time (t1 to t3). This suggests that under low light, the algae absorb light more than they reflect it, while under high light, reflectance overcomes absorbance.
Spore response: The dorsal side, in which the reproduction organs-gelles grows on, gets lower amount of light, as seen in figure 7. In fresh algae, the meiospores and gametes are pigmented, spherical, and evenly spread along the reproductive stripes (Figure 8). Light stress affected the spores in several ways. Comparing the spores under high light (from 380 μE upwards) to those under low light (290 μE downwards) shows that low light spores are consistently larger (an average of 73±11.43 μm) than high light spores (an average of 59±5.58 μm). In spite of the large size, low light spores (290 μE downwards) did not fill the whole cysts, and some cysts turned empty and transparent (Figure 9), while under mid and high light (380 μE upwards), they were dense, fully diametric, and fully pigmented.

Tissue response: We observed a darkening of the thallus tissue under low light (290 μE downwards) that did not occur under high light. It seems that in the non-calcareous area, in between the aragonite stripes, some group of cells became darker and demonstrated excess pigmentation (Figure 10).

In general, when considering all the parameters, it seems that under 260 μmol quanta m⁻² s⁻¹, the spores had the greatest resemblance to fresh algae in size, density, color, and fullness of the cyst.

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Experiment

Moonlight stripe measurement: Algae that were collected under a newborn moon showed an average stripe width of 1.67 (±0.3) mm.
while algae that were collected under a full moon showed an average stripe width of 1.95 (+0.2) mm, an average of almost 42% wider stripes during the full moon (Figure 11).

Discussion

Along with the life-supporting benefits that sunlight provides, there is also a dangerous side to it when overexposure occurs. Creatures who live along the shoreline need to develop means to protect themselves from excessive radiation, particularly during neap tide. In February 2016, we measured the solar radiation at Tel Baruch, and found an average of 2900 (±5) μmol quanta m⁻² s⁻¹ at sea level and 1825 (±12) μmol quanta m⁻² s⁻¹ at a depth of 10 cm. While microalgae can migrate vertically [10], benthic sublittoral macroalgae can suffer photobleaching when exposed to high radiation; therefore, intertidal species must be able to cope with excessive high radiation [6]. Padina spp. are able to cope with high light and excessive radiation as our light-intensity experiments show by changing the CaCO₃ precipitation according to light intensity the algae exposed to.

Fresh algae: In most cases, the natural reflectance from the aragonite stripes was the highest at the apical stripes, diminishing towards the mid-thallus (Figure 2). This suggests either quantitative approach, i.e., more apical precipitation, and therefore more reflectance, or qualitative approach, i.e., aragonite needles reflect more than when they are partly dissolved (CaCO₃ morphology showed in figure 2, right ESEM pictures). The light reflection is opposite of the trend of pigment visibility, in which there is an increase from the apical stripes toward the mid-thallus (Figure 3). The reflection trend does not necessarily coincide with the amount of aragonite deposited, as found by ESEM (Figure 3, stripes/pas 1-5, n=3). These results strengthen the hypothesis that the crystallography, rather than deposition amount, and the layers of cells placed behind this deposition, affect reflectance.

Both reflectance and the carbonate content of stripes are modified by temporal changes in the deposition/dissolution ratio. This is evident in the age-dependent change in the calcification of the stripes, as it decreases from the growing tip of the frond towards its older parts. We also suggest that the reported change in stripe width according to the lunar cycle may be due to periodic dissolution/deposition of the aragonite cover. As mentioned, Tel-Baruch’s P. pavonica has dorsal reproductive stripes located right behind the ventral aragonite stripes (Figure 7). This particular location strongly implies that the young, delicate reproductive tissue benefits from the protection afforded by the aragonite and that the alga saves energy as the precipitation does not occur all over the thallus. It seems that the spores need enough light to develop to be fully diametric and colored but not accessed light which seems to be a trigger for the cyst to release the spores to the water column [11,12]. The pigment absorption and the aragonite reflectance are inversely correlated since the aragonite hides the pigmented layer underneath it and thus lessens its optical signal.

To conclude the light characters of fresh algae, our results show that the apical stripes reflect more and absorb less, while the mid-thallus stripes are less reflective and more absorbing, regardless of the amount of precipitation.

Light manipulated algae: Under different light conditions that mimic the depth gradient (Figure 4), the algae showed the same reflectance pattern, though reflectance deteriorated dramatically after 4 days under high gradient light (260 μE upward).

In comparing reflectance of CaCO₃ to its amount under extreme light intensities (high and low), it seems that Ca²⁺ amount is opposite, i.e., when there is more Ca²⁺, there is less reflectance, and vice versa. This is not the case under mid-light (160-290 μE), where the spectral and ESEM results seem to be correlated (Figure 5).

These results strengthen the theory that the morphology of CaCO₃ is more effective than its amount. It is plausible that under low light, the needles direct the light to other parts of the thallus (i.e., extra pigmented cells located in-between the ventral CaCO₃ stripes) in order to use it for photosynthesis and signaling, while under high light conditions, the needles reflect and divert light away from the thallus in order to protect the tissue and the spores. This acclimation of extra pigmentation under low light was observed by Foy and Gibson[13] when they exposed the blue cyanobacteria Oscillatoria redekei to different light intensities (13-160 μE) and it seems that these changes in other macro-algae can accrue on a daily bases influenced by the sunlight [2] (Figures 12 and 13).

Comparing young and old calcified stripes, it seems that the needles reflect and spread even the smallest amount of light that reaches the alga in favor of metabolism, while in the old part of the thallus, where the aragonite cover is amorphous, the light is mainly reflected rather than diverted. This idea requires experimental validation. It also seems that the algae can daily acclimate themselves to the light changes that occur during the day. The moonlight experiment strongly supports this theory; showing wider stripes during full moon
(see Section 4.2). The light gradient changed the dorsal organelles properties as well, and under low light, the reproductive stripes were emptier, and the cysts were smaller and transparent (Figures 7 and 8), thus supporting a previous research claiming that light is a spore’s release trigger [14].

Figure 13: Dorsal close up on the spores behind the CaCO₃ stripes. Taking with Leica binocular (Zeiss).

In comparing all the results, it was found that, in vitro, a medium amount of light (160-290 μE) is the most efficient condition allowing precipitation to reflect more light and for the spores to have pigmentation, full cyst diameter, and to develop to a satisfactory size.

The darkening of the thallus, along with the spores’ transparency under extreme low and high light, shows cytology changes –is supporting the idea that a calcification process occurs in order to provide some protection to the tissues, particularly the young ones, i.e., young thalli, new apical cells and reproductive cells. The 40% thicker CaCO₃ stripes under a full moon (Figure 11) gives some reinforcement to the in-vitro-experiment results, and considering all the results together, CaCO₃ deposition is influenced by light.

Conclusion

The algal fauna and the habitat in which Padina spp. flourish in the Mediterranean Sea and at the Israeli shores in particular, cope with more sunny days annually, even at winter time. Our results shows that Padina calcification is motivated and changes according to the environmental amount of light. Under low light stress, the algae probably prefer to divert the light that they get towards the thallus cells (along with darkening some of this cells) for metabolism, while under high light stress, reflection is probably to protect the tissue and the spores and preventing sun radiation damage.

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