

The Effect of Light on Bacterial Activity in a Seaweed Holobiont

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Abstract Holobionts are characterized by the relationship between host and their associated organisms such as the biofilm associated with macroalgae. Considering that light is essential to macroalgae survival, the aim of this study was to verify the effect of light on the heterotrophic activity in biofilms of the brown macroalgae *Sargassum furcatum* during its growth cycle. Measurements of heterotrophic activity were done under natural light levels at different times during a daily cycle and under an artificial extinction of natural light during the afternoon. We also measured *Sargassum* primary production under these light levels in the afternoon. Both measurements were

done with and without photosynthesis inhibitor and antibiotics. Biofilm composition was mainly represented by bacteria but diatoms, cyanobacteria, and other organisms were also common. When a peak of diatom genera was recorded, the heterotrophic activity of the biofilm was higher. Heterotrophic activity was usually highest during the afternoon and the presence of a photosynthesis inhibitor caused an average reduction of 17% but there was no relationship with *Sargassum* primary production. These results indicate that autotrophic production in the biofilm was reduced by the inhibitor with consequences on bacterial activity. Heterotrophic activity was mainly bacterial and the antibiotics chloramphenicol and penicillin were more effective than streptomycin. We suggest primary producers in the biofilm are more important to increase bacterial activity than the macroalgae itself because of coherence of the peaks of heterotrophic and autotrophic activity in biofilm during the afternoon and the effects of autotrophic inhibitors on heterotrophic activity.

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Introduction

Biofilms are ubiquitous features of any immersed surface in aquatic environments including the surfaces of living organisms [1]. Macroalgae or seaweeds provide a highly attractive surface for the development of microbial biofilms; not only do they provide a solid surface for attachment but they also release large amounts of organic carbon which can be utilized by microorganisms [2]. Hence, macroalgae support complex and highly dynamic microbial communities. Understanding of the interaction between these predominantly prokaryotic communities and the

seaweeds on which they grow is relatively poor [3]. This is surprising given that the seaweed/biofilm system potentially provides an ideal model to explore the interface between eukaryotic and prokaryotic ecology and specifically to examine the bidirectional relationship between holobiont partners [4].

The relationship between seaweeds and prokaryotes can operate in a positive and negative sense for both partners [3]. For example, algal products such as dissolved organic matter can stimulate prokaryotic activity [5] but algae can also produce compounds which are toxic to prokaryotes [6]. Products derived from epiphytic prokaryotes such as CO₂, fixed nitrogen, and specific growth factors can benefit macroalgal photoautotrophy and growth [7, 8]. In this holobiont model, biological, chemical, and physical surface properties of seaweeds, which may be determined by factors such as environmental conditions and seaweed age, have an important role in determining the composition and activity of prokaryotic communities [9].

In addressing the nature and direction of the relationship between prokaryote biofilms and eukaryotic seaweeds, one profitable approach can be through measurements of prokaryotic heterotrophic activity by the ³H-leucine incorporation technique [10]. Using *Sargassum* as a biological model, the use of specific inhibitors showed that biofilm heterotrophic activity was mainly bacterial. However, the effect of different antibiotics was tested only in laboratory conditions. In addition, bacterial activity was also inhibited by a eukaryotic inhibitor in dark conditions, suggesting a symbiotic relationship between eukaryotes and prokaryotes in the *Sargassum* model. Finally, bacterial activity was higher when incubations were done in field conditions than in the laboratory, suggesting that natural solar light could be also important to the heterotrophic prokaryotic production. Thus, it is possible that bacterial activity is associated with algal autotrophic production that could change during the daily cycle and by light intensity.

Using *Sargassum furcatum* as a seaweed holobiont model, the aim of this work was to determine if (1) bacterial activity in the biofilm is associated with algal autotrophic production, (2) varies seasonally, (3) varies over the day-night cycle as well as (4) under different light intensities in the afternoon, and (5) different antibiotics have different effects on the bacterial activity. In a series of experiments performed monthly using different ages of *Sargassum*, we also evaluated environmental conditions, observed biofilm by microscopy, and compared biofilm bacterial activity with or without a photosynthesis inhibitor.

Materials and Methods

Sargassum Sampling

Experiments were done in Arraial do Cabo (23° S, 42° W), the main upwelling area on the Brazilian coast located in the Cabo Frio region of Rio de Janeiro state (details in [11]). Sampling

was undertaken monthly in a *Sargassum* bed at 4–6 m depth at the Farol beach station, Cabo Frio Island, between November 2007 and April 2008. Physicochemical water variables (nutrients, chlorophyll, temperature, and salinity) were available over the period of study through the weekly monitoring of the Brazilian navy (Instituto de Estudos do Mar Almirante Paulo Moreira—IEAPM) and methods used are described elsewhere [12]. Solar light radiation was measured every 5 min during all experimental periods using a LICOR LI-1.000 Datalogger/Li-193 SA spherical quantum sensor.

Specimens of *S. furcatum* were collected and selected by size over its growth cycle from November to April [13]. Therefore, individuals up to 5 cm were sampled in November, between 5 and 15 cm in December, between 10 and 20 cm in January, between 15 and 25 cm in February, between 15 and 30 cm in March, and up to 10 cm in April, representing the end of its life cycle. Five individuals in the size classes described above were collected at random within the *Sargassum* bed and transferred in polyethylene pots (500 mL) to the laboratory approximately 200 m away. Biofilm composition was observed by epifluorescence and scanning electron microscopy. Blades of each *Sargassum* individual were used for microscopic observations ($n = 5$) as well as for measurements of bacterial activity ($n = 5$). A pilot study showed no statistical difference in bacterial activity using 5 or 15 replicates.

Experimental Conditions

Bacterial activity was measured at all six sampling dates. To understand the daily pattern of heterotrophic production, measures were made at different times of the day during the afternoon (12:00–15:00), evening (18:00–20:00), night (23:00–01:00), and morning (07:00–09:00) at each sampling event. Light levels vary over this 24-h cycle but covary with other factors including temperature and internal diel rhythms. Thus, to unambiguously determine the effect of light, measures were made at the time of peak light levels during the afternoon (12:00–15:00) over a light extinction sequence using an artificial reduction of natural light (100, 75, 50, 25, 10, 1, and 0%) using different levels of a black mesh.

All measures of activity were made in the sea at a depth of approximately 0.5 m, using a submerged platform supporting an open ended acrylic tube into which algal material was placed. One blade of approximately 1 cm² from each plant ($n = 5$) was incubated in separate 2-mL microcentrifuge tubes (Eppendorf) with local 0.22- μ m filtered water held within a 125-mL Winkler bottle. The dry weight in each square centimeter of *Sargassum* blade was 0.004 ± 0.001 g.

For autotrophic production, five blades were inserted into one 125-mL glass Winkler bottle with local 0.22- μ m filtered water to have an amount of algal biomass that could be detected by the method used. In November, the measurement was determined by the mean of three Winkler bottles since there was no

experimental treatment. In the other months, two treatments were tested (with and without specific inhibitors) and photosynthesis incubation was done in three bottles ($n = 3$).

To directly test the relationship between autotrophic production and bacterial activity, a photosynthesis inhibitor (10 μM diuron) was used at four of the sampling dates at each of the four times of day (Table 1). Bacterial activity was thus compared when photosynthesis was naturally occurring and when inhibited. We also tested different antibiotics in November, January, and March (through comparing bacterial activity with and without the use of antibiotics at the sampling dates): 5 $\mu\text{g L}^{-1}$ streptomycin + 100 units L^{-1} penicillin were used in November, 5 $\mu\text{g L}^{-1}$ streptomycin was used in January, and 0.2 nM chloramphenicol in March samplings (Table 1).

Bacterial Activity

Bacterial activity was determined using an adaptation of the ^3H -leucine incorporation technique [10] following a methodology for periphyton associated with macrophyte roots [14]. Briefly, blades of approximately 1 cm^2 were incubated in 2.0-mL Eppendorf with 35 nM ^3H -leucine in 1.5 mL of 0.22- μm filtered water ($n = 5$). The incubation time was 1.5–3 h, and 80 μL of 100% trichloroacetic acid (TCA) was used to stop the incubation. In addition, all experiments were performed using a killed control where production was terminated at the start by adding 5% TCA just before the input of ^3H -leucine. Following incubation, samples were frozen until protein extraction. Extraction started with a 5-min sonication bath, and then the blade was removed. Samples were centrifuged at 2500 $\times g$ for 15 min, and the supernatant was transferred to a new microcentrifuge tube before being centrifuged again at 13,000 $\times g$ for 10 min, after which time the supernatant was discarded; 1.5 mL of cold TCA (5%) was added, and the centrifugation process was repeated. One milliliter of 80% ethanol was added, and a new centrifugation was done. Finally, 1 mL of scintillation cocktail (CytoScint) was added and, after an overnight period, radioassayed by scintillation

Table 1 The use of antibiotics (penicillin, streptomycin, and chloramphenicol) and photosynthesis inhibitor (diuron) in both autotrophic production and bacterial activity for the experiments realized during the *Sargassum* growth cycle

Month of the experiment	Autotrophic production	Bacterial activity
November	No inhibitor	Penicillin + streptomycin
December	Penicillin + streptomycin	Diuron
January	Streptomycin	Streptomycin
February	Diuron	Diuron
March	Chloramphenicol	Chloramphenicol
April	Diuron	Diuron

counting (TRICARB PACKARD 1600) for 30 min or after the accumulation of 10,000 counts (as determined by [12]). Leucine incorporation rate (in moles per square meter per hour) was calculated considering net disintegrations per minute (DPM), sample area (1 cm^2), leucine concentration, ^3H -leucine specific activity (72 Ci mmol^{-1}), and time of incubation.

Sargassum Autotrophic Production

To measure the *Sargassum* primary production, five blades (1 cm^2) were incubated in 125-mL glass Winkler bottles with local 0.22- μm filtered water. It is important to mention that blades were not axenic since they were sampled from the natural bed. Hence, both microautotrophs and heterotrophs were included in both production and consumption of carbon. Oxygen was fixed to determine its initial concentration. A dark control was used to determine oxygen consumption, and a glass with no blade of *Sargassum* was used to verify phytoplankton production. Then, primary production was determined using the Winkler method after 2–3 h of incubation stopped with oxygen fixation [15]. The results were calculated considering the difference of oxygen between the end and the beginning of the experiment divided by the total dry weight of the five blades and the time of incubation ($\text{ng C g dw}^{-1} \text{h}^{-1}$). The photosynthetic coefficient was between 0.98 and 1.01 [16].

Statistical Analysis

Two-way ANOVA was done considering all factors as fixed and orthogonal and a 0.1 significance level. Four levels were used for the factor time of day (morning, afternoon, evening, and night), seven levels for the factor light incidence (100, 75, 50, 25, 10, 1, 0%), and two levels for the factor inhibitor (with or without). When necessary, data were log transformed and analyses run using WinGmav software (version 5.0). In the experiments at different times of the day, November was omitted owing to lack of measurement in the morning time. Multiple comparisons of levels within significant factors were made using Student Newman Keuls (SNK) tests.

Results

Environmental Conditions During the Experiments

All environmental conditions during sampling can be found in Tables 2 and 3. Light intensity (at approximately 0.5 m depth) was high during the afternoon and morning experiments except in December and January when it was approximately 1000 times lower compared to the other months. During this time, a bloom of phytoplankton was visually observed and the

Table 2 Physical-chemical variables measured in surface (sup) and deep (dp) waters during the sampling and experimental dates

Month of the experiment	Temp. (°C)		OD (mg/L)		P-PO ₄ ³⁻ (μMol L ⁻¹)		N-NO ₂ ⁻ (μMol L ⁻¹)		N-NO ₃ ²⁻ (μMol L ⁻¹)		N-NH ₄ ⁺ (μMol L ⁻¹)		pH	
	sup	dp	sup	dp	sup	dp	sup	dp	sup	dp	sup	dp	sup	dp
November	21.0	20.0	5.6	5.7	0.2	0.3	0.2	0.2	0.8	0.6	1.3	1.8	8.5	8.5
December ^a	23.4	21.5	5.4	5.0	0.1	0.6	0.0	0.4	0.1	1.0	1.1	1.7	8.8	8.7
January	23.4	17.8	5.4	5.1	0.1	0.1	0.0	0.1	0.5	2.2	1.4	1.2	8.4	8.5
February ^b	22.0	21.3	5.2	5.3	0.2	0.3	0.0	0.1	3.1	3.0	0.7	1.1	8.8	8.9
March	23.5	23.2	4.9	4.9	0.3	0.3	0.1	0.0	2.0	1.7	1.3	1.1	8.2	8.3
April	24.3	23.8	5.1	5.1	0.1	0.1	0.0	0.0	0.0	0.0	1.1	1.1	8.4	8.4

^a Two days after experiment^b One day after experiment

Sargassum bed was not visible from the boat (4 m above) during arrival at the experimental area in the morning before the experiment began. In addition, pheophytin concentrations peaked in December and January (2.8 and 1.3 mg/m³, respectively) and chlorophyll *a* in December (1.7 mg/m³). For nutrients, nitrate was highest in February (3.1 μmol/L) and below the detection limit in April. Peak of ammonia was observed in November (1.8 μmol/L) and of phosphate and of nitrite in December (0.6 and 0.4 μmol/L, respectively).

Surface water temperatures over the duration of the experiments varied by a few degrees among months (Table 2). Surface water temperatures over 24 h were around 20.6 °C during the experiments of November and around 23.3 °C in December but ranged from 25.3 to 23.0 °C in February, 27.2 to 24 °C in March, and 25.5 to 24.4 °C in April. In January, upwelling was more intense during sampling since water temperature was below 18 °C in the *Sargassum* bed at 4–6 m depth.

Biofilm composition includes both autotrophic and heterotrophic organisms such as bacteria, phytoflagellates, diatoms, hydrozoans, cyanobacteria, and eukaryotic algae. Diatom abundance peaked in January and the main taxa were *Cocconeis*, *Navicula*, *Nitzschia*, *Licmophora*, and *Striatella* (Baeta-Neves, personal observation). SEM images showed a very dynamic biofilm (supplementary material).

Daily and Seasonal Bacterial Activity

Bacterial activity showed little obvious seasonal variation when measured in the evening, night-time, and morning. When measured in the afternoon, it showed a rapid increase from very low levels in November to a peak of over 300 pgC cm⁻² h⁻¹ in January followed by a gradual decline over the following months (Fig. 1). Two-way ANOVA revealed a significant interaction between the factors month and time of experiment ($p = 0.04$), and SNK confirmed that bacterial activity was significantly highest during the afternoon of January. However, bacterial activity in January was only significantly higher than April ($p = 0.014$), and independently of season, activity was significantly highest during the afternoon ($p \leq 0.001$).

Bacterial Activity in a Simulated Light Extinction During the Afternoon and Its Relationship with *Sargassum* Autotrophic Production

To check if bacterial activity is influenced by light intensity in the afternoon, a series of experiments simulating different degrees of light intensity was done in each month during the *Sargassum* growth cycle. Considering each month, no obvious

Table 3 Concentration of chlorophylls in surface (sup) and deep (dp) waters during the sampling and experimental dates

Month of the experiment	Chlorophyll <i>a</i>		Chlorophyll <i>b</i>		Chlorophyll <i>c</i>		Pheophytin	
	sup	dp	sup	dp	sup	dp	sup	dp
November	0.9	0.9	0.2	0.2	0.3	0.1	0.3	0.9
December ^a	1.8	1.6	0.0	0.0	0.0	0.6	2.8	0.0
January	0.6	0.5	0.0	0.0	0.0	0.0	1.3	0.3
February ^b	1.0	0.7	0.1	0.2	0.5	0.3	0.0	0.0
March	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.1
April	0.6	0.3	0.0	0.0	0.0	0.0	0.6	0.0

^a Two days after the experiment^b One day after the experiment

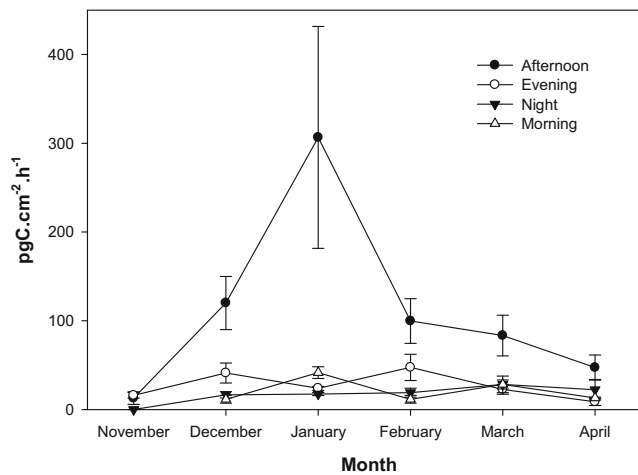


Fig. 1 Seasonal bacterial activity (mean ± SE) at different times of the day during *Sargassum* growth between November 2007 and April 2008 (n = 5)

pattern was observed among treatments (Fig. 2). However, ANOVA and SNK tests revealed that heterotrophic activity was highest under low light conditions in the experiments of

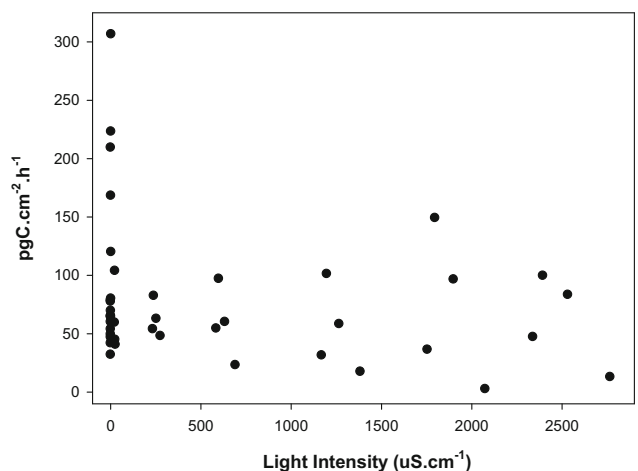
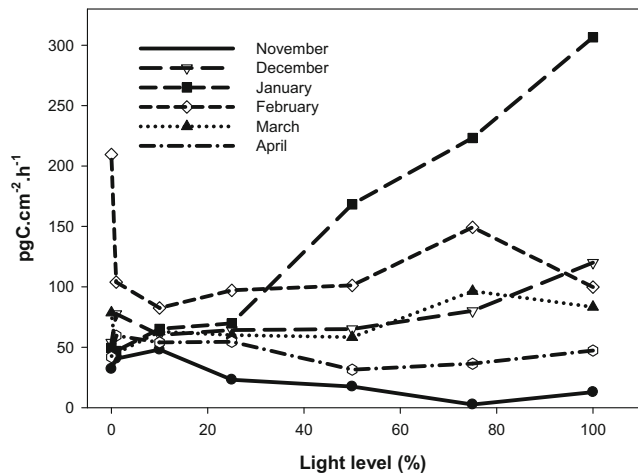


Fig. 2 Mean of bacterial activity at different levels of light during *Sargassum* growth in the experiments done between November 2007 and April 2008 (top, n = 5) and its relationship with light intensity pooling all data (bottom, n = 5)

November ($p \leq 0.001$) and February ($p = 0.01$). Pooling all data in function of light intensity, the highest activity was also observed under low light conditions (Fig. 2). In addition, there was no evident relationship between *Sargassum* primary production and the bacterial activity in the biofilm.

The Effect of Specific Metabolic Inhibitors

The use of 10 μM diuron in February and April inhibited *Sargassum* autotrophic production completely (data not shown) and its effect on bacterial activity was variable (Table 4). In the experiments conducted at different times of day done in November, December, February, and April, the presence of diuron reduced heterotrophic activity between 0 and 66% (in average $17 \pm 11\%$). ANOVA revealed a significant interaction in December ($p = 0.002$) when the presence of diuron reduced bacterial activity significantly during the afternoon and night-time and in April ($p = 0.008$) when heterotrophic activity reduced significantly during the night-time and in the morning (Fig. 3). The effect of diuron on heterotrophic activity in the experiments simulating light extinction during the afternoon was also variable ranging from 0 to 81% (in average $16 \pm 13\%$) and its effect was only significant ($p = 0.02$) under 50% of natural light in November (Table 4).

Antibiotics had different actions on both autotrophic and heterotrophic activities. While penicillin with streptomycin completely inhibited photosynthesis of *Sargassum* and the heterotrophic activity in biofilm (data not shown), the effect of streptomycin alone was light-dependent since the significant reduction of both measurements was observed only under 50% of light in the experiment conducted in January (Fig. 4). Chloramphenicol inhibited bacterial activity significantly ($p \leq 0.001$) and also had a light-dependent effect on *Sargassum* photosynthesis, stopping its activity under high lights conditions (Fig. 4).

Discussion

Studies on the ecology of seaweed holobionts aim to understand the mechanism and function of all microbial members

Table 4 Range in the reduction of bacterial activity (%) under the presence of photosynthesis inhibitor (diuron) in experiments in the daily cycle and simulating light extinction during the afternoon (ns = not significant).

Month	Daily cycle	Afternoon	Significant effect
November	13–66	0–81	50% of light
December	0–59	0–47	afternoon and night
February	0–40	0–44	ns
April	0–39	0–25	morning and night

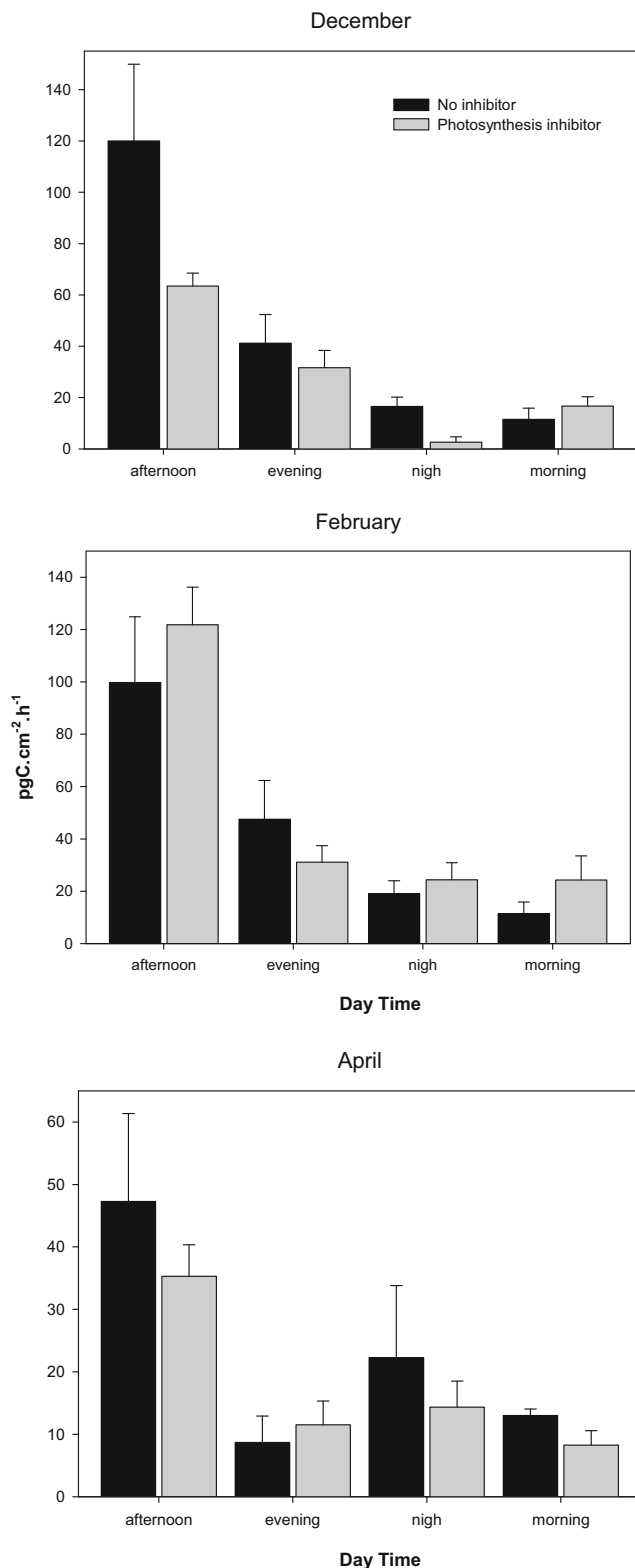


Fig. 3 Mean \pm SE of bacterial activity ($\text{pgC cm}^{-2} \text{h}^{-1}$) at different times of the day under the presence or absence of diuron in the experiments undertaken in December 2007 and February and April 2008 ($n = 5$)

and their ecological role in the alga's life cycle. However, manipulative experiments in field conditions are challenging

[17]. Our observations of the *S. furcatum* holobiont model in its natural environment showed that biofilm composition varied with algal age and with environmental conditions; such variation potentially plays an important role in determining bacterial activity. However, we could not separate the effects of specific environmental conditions from the plant size and age. Observations at different times of the day showed that bacterial activity consistently peaked in the afternoon, when light levels were highest suggesting an association of heterotrophic and autotrophic productions. However, there was no relationship between bacterial activity and light intensity during the afternoon and no obvious correlation between *Sargassum* primary production and the bacterial activity in its biofilm. More studies are needed to check if *Sargassum* also incorporate leucine. Nevertheless, our results showed some evidence of a negative effect of a photosynthesis inhibitor on bacterial activity and the highest activity occurred when diatoms were more abundant suggesting that heterotrophs may be stimulated by the autotrophic production in biofilm. Such prokaryotic-eukaryotic coupling is expected in a healthy holobiont system highlighted herein by the effect of antibiotics on both heterotrophic and autotrophic activities.

In the studied region, upwelling events are seasonal and *Sargassum* growth occurs as a function of its intensity [18]. Thus, upwelling should be considered an important factor causing turnover in community composition of the benthic environment but also of planktonic prokaryotes [19] and eukaryotes [20] which are able to colonize the surface of macroalgal blades [10]. It is expected that *Sargassum* also selects its biofilm composition since *Sargassum* produces compounds with antioxidant, antibacterial, antitumoral, anti-malarial, antiherbivory, and antifouling properties (e.g., [21]), but the concentrations of these compounds may reduce as algae age [22]. We also observed using SEM images the presence of holes indicating that settled organisms detached early increasing spatial heterogeneity and forming new habitats and niches [9] that could enhance productivity [23]. We suggest a higher niche overlap on the youngest blades when higher competition decreased bacterial activity [24] and the peak of activity in January could be associated not only with a more stable habitat but also with the subsequent increase in diatom abundance and diversity. Such increases could enhance bacterial activity by provision of diatom metabolic products. The reduction in bacterial activity after the peak in January could be a function of both diatom detachment and the regressing of *Sargassum* blades (increasing polymer degradation) leading to a reduction in autotrophic enhancement.

Bacterial activity peaked in the afternoon and we suggest its association with bioactive secondary metabolites as a consequence of photosynthesis [17] as well as with microalgal-(nutrient)-leakage after a day of photosynthesis [25] since it peaked during a phytoplankton bloom under an upwelling event. It is known that biofilm conditions vary in

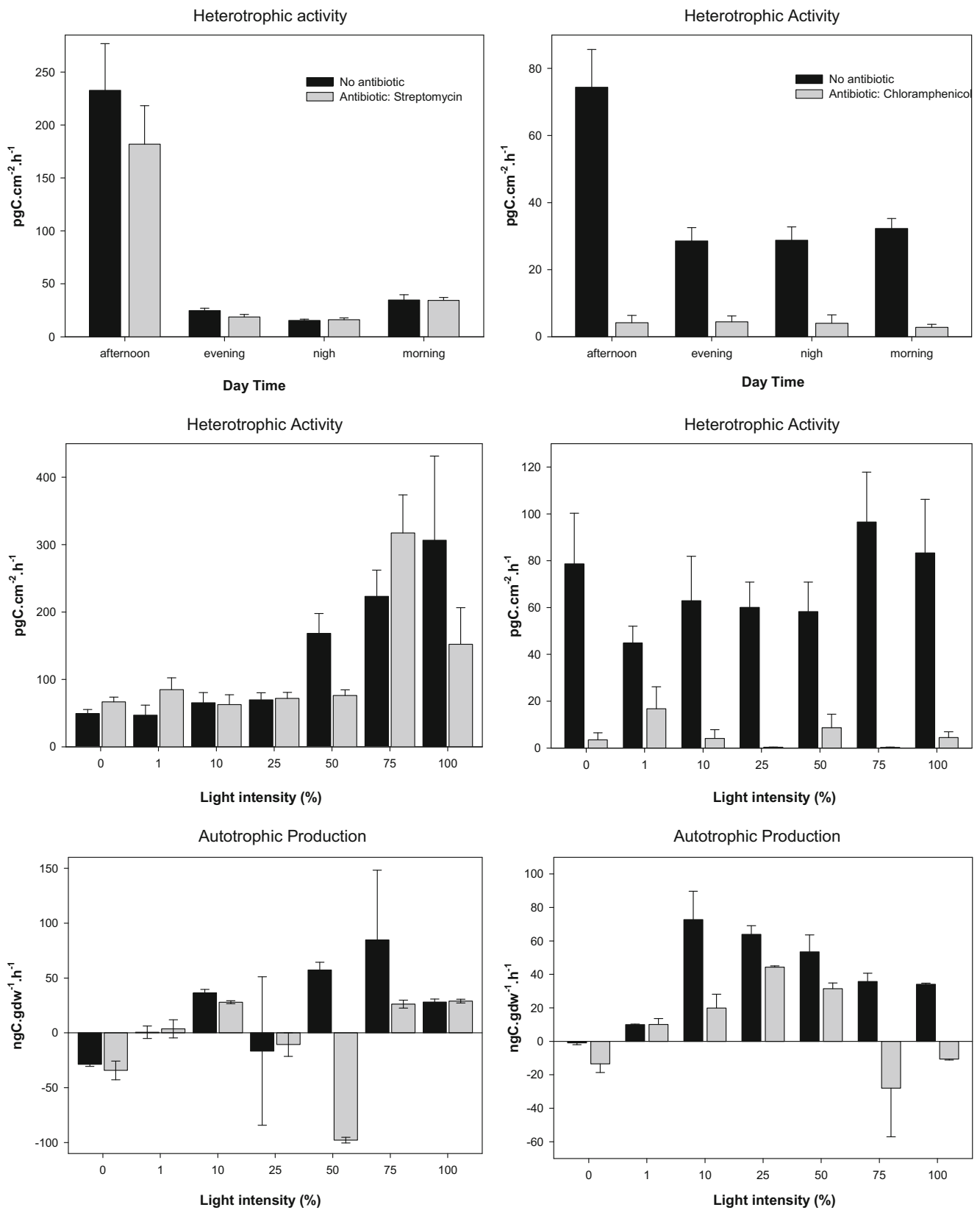


Fig. 4 Mean \pm SEM of biofilm heterotrophic activity in different times of a day and under different levels of light during the afternoon ($n = 5$) as well as in *Sargassum* autotrophic production under different levels of

light during the afternoon ($n = 3$) with and without the antibiotic streptomycin (*left*) and chloramphenicol (*right*) during the experiments conducted in January 2008

the contrasting light circumstances over a day [16] and seasonal variations in the biofilm conditions are strictly associated with light [26]. In addition, bacterial growth is usually higher during daylight hours [27] and the effect of antibiotics showed herein point to Bacteria being the major group incorporating leucine in our measurements, confirming observation in laboratory assays [10]. Similar observations have been made in freshwater epilithic biofilms, where bacterial biomass and activity was highest in the presence of light as a consequence of organic substrates produced by algae [28]. Thus, we suggest that exudation is an important factor for coupling algal primary productivity and bacterial activity in a seaweed holobiont model.

We cannot affirm if diatoms increased bacterial activity because of facilitation or by character displacement, since both enhance productivity over time [24, 29] and we did not measure important biotic factors such as competition, predation, and herbivory [25, 30, 31]. In contrast, the importance of microalgal exudation is partially supported by the effect of photosynthesis inhibitor on bacterial activity. The wide variation in the effect of diuron could be associated with the ability of biofilms to decrease the action of many compounds [32] but also with the direct relationship between grazing pressure on bacteria and the concentration of exudates [25]. However, our results support the hypothesis that on average 17% of the autotrophic production in the biofilm is used by bacteria in the *Sargassum* holobiont model.

Our results, showing different degrees of antibiotic effects, corroborate the work of Nair et al. [33] that showed marine bacteria to be less sensitive to streptomycin than to penicillin and chloramphenicol. Additionally, our results showed a light-dependent effect of streptomycin and chloramphenicol on photosynthesis; such effects may not only be related to their antibiotic properties. Both are directly associated with photoinhibition since they inhibit photosystem II photosynthetic efficiency. Hader et al. [34] showed inhibition of D1 protein biosynthesis in chloroplast of seaweeds, using almost the same concentrations of both substances as in our study (500 and 2 mg/mL, for streptomycin and chloramphenicol, respectively). D1 protein controls electron transport after primary photon absorption and is inhibited by visible and UV lights.

In future studies, we recommend the determination of (1) the importance of periphytic algae to the attached bacterial production and of (2) the specific effect of plant size and environmental conditions in the experimental design. In the first case, it is important to measure exudation rates by both seaweed and periphytic algae to estimate the relationship between bacterial production and primary production of both macroalgae and periphyton. Our photosynthesis measurements included both components and its inhibition resulted in a mean reduction of bacterial activity potentially caused by a reduction in carbon uptake by bacteria from periphyton

or macroalgal exudate [25]. In addition, primary production is also affected by plant age and environmental conditions. Although there are benefits of measuring bacterial activity under natural conditions [10], it is important to check the effect of biotic and abiotic factors in laboratory conditions controlling variables such as secondary compounds and polymer concentrations of seaweed, periphytic algae, and phytoplankton as well as UV intensity, temperature, and substrate concentrations.

In conclusion, solar light is likely important to bacterial activity in the biofilm but not necessarily as a function of the photosynthetic activity of *S. furcatum*. Despite bacterial activity being highest during the afternoon, it was not correlated to light intensity. Bacterial activity peaked during the intermediate age of *Sargassum* when diatoms were abundant in the biofilm, suggesting the importance of periphytic algae.

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