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An investigation of the presence and variations in abundance of UV-absorbing structures in *Grateloupia turuturu* Yamada (Halymeniaceae, Rhodophyta) from Brittany (France)

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Abstract – Biofouling is a potential problem for any surface immersed in the sea. For algae this can lead to its death; they have therefore developed biofouling defence mechanisms to prevent biofouling. *Grateloupia turuturu* was chosen as a model to isolate some structures with antifouling properties. The aims of our study were then (1) to identify and describe in *G. turuturu* structures similar to gland-cells observed in a variety of species from the order Bonnemaisoniales and (2) to determine their variability in relation to site, stages of development, and the presence of epiphytes. Fluorescent and confocal microscopy was used to highlight structures similar to gland-cells. Using epifluorescence microscopy, the number of "gland cells-like structures" was determined on individuals sampled from three sites in French Brittany (Callot Island, Pointe du Diable, and Fort Bloqué), characterized by different hydrodynamic and fouling exposure conditions, and from three life history phases (gametophyte, tetrasporophyte and carposporophyte). Isolated and grouped fluorescent structures were detected and characterized. Significant variations in the density of those fluorescent structures were found between site and life-history stages. Individuals from the Pointe du Diable had significantly more fluorescent structures than thalli from the two other sites, and they were more numerous on carposporic plants than on either tetrasporophytes or immature gametophytes. More "gland cells-like structures" were also present on plants bearing epiphytes.

***Grateloupia turuturu* / UV-absorbing gland cell / spatial and ontogenic variations / epiphytism / epifluorescence microscopy / confocal microscopy / anti-fouling**

Résumé – Etude de la présence et des variations du nombre de structures absorbant les UV chez *Grateloupia turuturu* Yamada (Halymeniaceae, Rhodophyta) de Bretagne (France). Le biofouling est un problème que rencontre toute surface immergée. Les algues sont particulièrement concernées par ce processus qui peut mener à leur mort ; ce qui les

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conduit à développer des mécanismes de défense pour faire face à cette colonisation. Dans le cadre de notre étude, *Grateloupia turuturu* a été choisie comme modèle afin d'isoler des structures aux propriétés antifouling. Les objectifs de notre étude étaient (1) d'identifier et de décrire chez *G. turuturu* des structures similaires aux cellules glandulaires observées chez certaines espèces de l'ordre Bonnemaisoniales et (2) de déterminer les variations du nombre de structures observées en relation avec le site de prélèvement, le stade de développement de l'algue et la présence d'épiphytes. La microscopie à fluorescence ainsi que la microscopie confocale ont été utilisées pour isoler et décrire des structures similaires aux cellules glandulaires préalablement décrites dans la littérature. La densité de telles structures a été déterminée sur des individus prélevés aléatoirement sur trois sites en Bretagne (Ile Callot, Pointe du Diable, et Fort Bloqué), caractérisés par des conditions hydrodynamiques et une exposition aux épiphytes différentes, et à 3 stades de développement (gamétophyte, tétrasporophyte et carposporophyte). Des structures fluorescentes isolées et groupées ont été mises en évidence et décrites. Les individus provenant de la Pointe du Diable présentent un plus grand nombre de structures fluorescentes que les thalles provenant des deux autres sites. De plus, les structures fluorescentes sont trouvées plus nombreuses sur le stade carposporophyte que sur les stades tétrasporophytes et gamétophytes. Enfin, leur nombre augmente avec la présence d'épiphytes.

***Grateloupia turuturu* / glande cellulaire absorbant les UV / variations spatiales et ontogéniques / épiphytisme / microscopie à épifluorescence / microscopie confocale / anti-fouling**

INTRODUCTION

Any living or non living surface immersed in the sea is exposed to the process of colonisation (Steinberg *et al.*, 1997). The formation of a biofilm may result in an array of problems, including death of the living surface (Kjelleberg & Steinberg, 2001). There is therefore strong evolutionary pressure on marine eukaryotes to develop mechanisms to inhibit or control the settlement and growth of biofoulers on their surface (Steinberg *et al.*, 1997). In order to prevent colonisation, algae have developed many defensive strategies, for example the production of primary and secondary metabolites (Wright *et al.*, 2000).

Like terrestrial plants, secondary metabolites in seaweeds are generally localized in specific structures (Steinberg & de Nys, 2002). In brown algae, phlorotannins are contained within vesicular physodes present at the surface of the thallus (Ragan & Glombitza, 1986). Terpenoids found in the genus *Laurencia* are encapsulated within sub-cellular structures called "corps en cerise" (Young *et al.*, 1980). A variety of species within the red algal order Bonnemaisoniales also present specific structures named gland cells (Wolk, 1968; Dworjanyn *et al.*, 1999). Those structures have been well described in the specie *Delisea pulchra* in which they appear to produce antifouling compounds called furanones (Dworjanyn *et al.*, 1999).

Furanones from the Australian red alga *Delisea pulchra* (Greville) Montagne (Bonnemaisoniaceae, Rhodophyta) are non-polar secondary metabolites of high interest because of their ability to regulate Gram – bacteria density; indeed, this regulation is not based on a simple toxicity, but on interference with the AHL quorum sensing system. Furanones in *Delisea pulchra* are located in gland cells that occur in the outer cortex. The presence of conjugated double bonds in their chemical structure makes them fluoresce when excited by near-UV light.

Fluorescence microscopy therefore allows the detection of gland cells at the surface of algae and to highlight the presence of furanones.

The accidental introduction of *Grateloupia turuturu* Yamada (Halymeniaceae, Rhodophyta) to France, in 1989 (Fort Bloqué) and 1992 (Callot), together with its fast expansion from Normandy to South Brittany (Simon *et al.*, 2001) has raised concerns and interest in this invasive species. *In situ* observations have noted that thalli of this species are free of epiphytes during most of the year, which suggested the possible presence of a chemical defence mechanism.

This preliminary study was carried out i) to determine the possible presence in *G. turuturu* of structures similar to gland cells and ii) to determine the variation in abundance of these structures in relation to life-history stages, geographic location, and the presence of epiphytes.

MATERIAL AND METHODS

Sampling of thalli and field sites

Samples of *Grateloupia turuturu* were collected in February 2003 from three field sites in French Brittany: Callot Island, Pointe du Diable and Fort Bloqué (Fig. 1), chosen because of their different geographical and hydrodynamic features. Wave and water movement at Callot Island is low due to its sheltered location, contrary to Pointe du Diable and Fort Bloqué where it is high. Moreover, *G. turuturu* settled in 1989 and 1992 at Fort Bloqué and Callot Island, respectively, whereas it is newly established at the Pointe du Diable. Moreover, these three sites present thalli of *G. turuturu* with different rates of epiphytism as followed: PD (36.8% of epiphyted thalli) > CI (11.3%) > FB (no epiphyted thallus) (Plouguerné, data not shown).

Three different life-history stages are recognisable in *G. turuturu*: tetrasporophytes, mature gametophytes (bearing carposporophytes) and gametophytes which are distinguished according to Simon *et al.* (2001). Some uncertainties

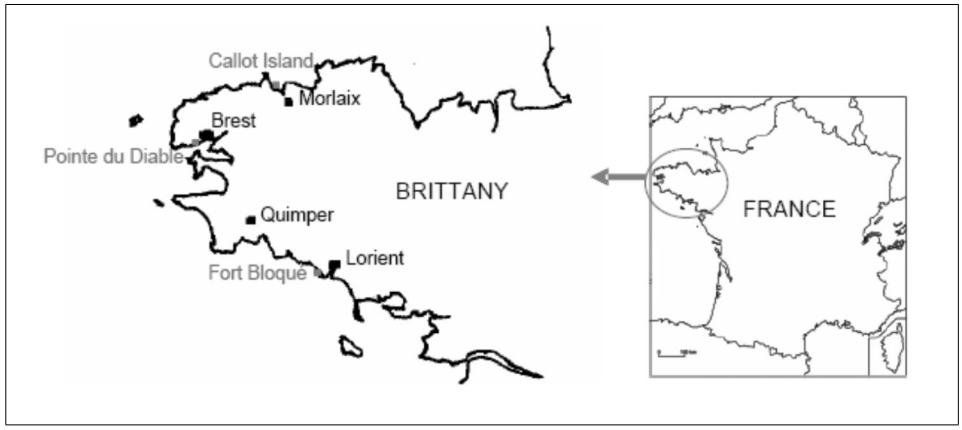

Fig. 1. Location of the three field collection sites of *Grateloupia turuturu* along the coast of Brittany (France).

remained with immature thalli, which can not be classified into one category. Three thalli of each stage were collected at each field site. Once back in the laboratory, the algae were held for a few days in seawater-filled aquaria.

Detection of "gland cell-like structures" using confocal microscopy

The presence of "gland cell-like structures" in tetrasporic and cystocarpic plants collected in November 2003 at Pointe du Diable was determined by confocal microscopy ("Olympus Fluoview"). Preliminary detection by UV fluorescence allowed the isolation of naturally fluorescent structures. These structures were then excited at 390 nm and observed at 440 nm; wavelengths known to be furanone-specific (Dworjanyn *et al.*, 1999).

Determination of the density of "gland cell-like structures" using epifluorescence microscopy

Gland cells were counted on 0.15 cm^2 algal pieces examined under a fluorescence microscope (Leica DMR microscope, Leica DC 100 optic). For each thallus, two pieces were cut from each of three selected regions corresponding to the distal, middle and basal parts of the plant (Fig. 2) and gland cell density calculated as the number of gland cells per cm^2.

Effect of epiphytes on the gland cell density

The influence of epiphytes was tested on 2 epiphyted tetrasporophytes and 3 epiphyted cystocarpic gametophytes of *Grateloupia turuturu* collected at Pointe du Diable, which present the higher rate of epiphyted thalli (see above). The number of the different portions examined on the different thalli was dependent of the length of the individual thallus, with different pieces taken at 5 cm intervals along the length of the thallus. The number of fluorescent structures per 0.15 cm^2 was compared to the presence or absence of epiphytes.

Statistical tests

Relationships between the number of fluorescent structures in *Grateloupia turuturu*, the life-history phase and the field site were statistically assessed. Normality and homoscedasticity were tested, and then experimental data were tested with two-way Anova. A multi-range test was finally used to gather homogenous data. Moreover, a correlation between the number of fluorescent structures and the presence of epiphytes was established. All statistic tests were performed using the Statgraphics software for PC.

RESULTS

Presence of "gland cell-like structures" using epifluorescence and confocal microscopies

Structures illuminated by epifluorescence microscopy were detected across frond surfaces (Fig. 3a), and confocal microscopy confirmed the presence of a specific fluorescence from these structures. Working at two specific

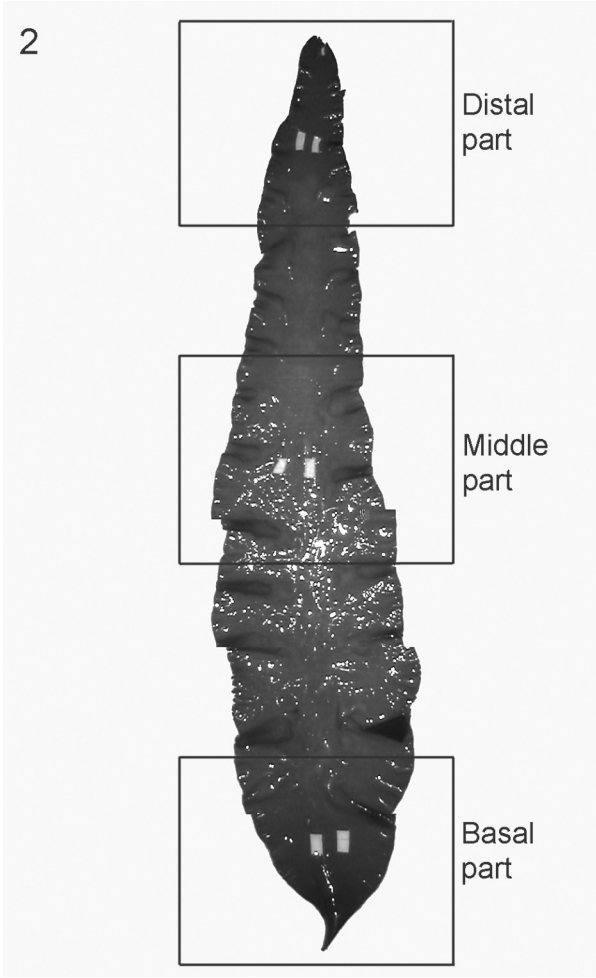

Fig. 2. An example of sample locations for pieces (0.15 cm^2) taken from the thallus of *Grateloupia turuturu* for the counting of fluorescent structures. 2 replicates were collected in each area (basal, middle and distal part).

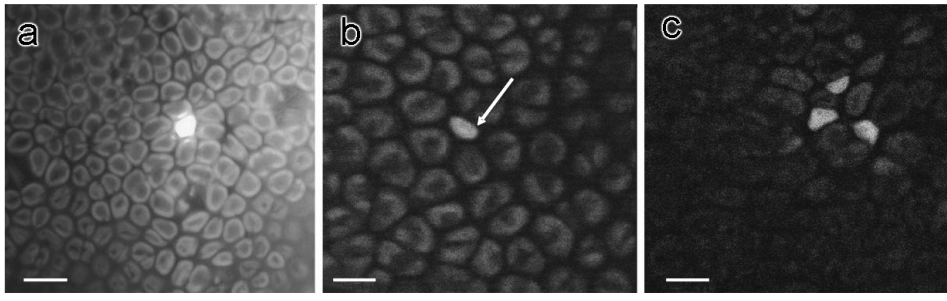

Fig. 3. Fluorescent structure from *Grateloupia turuturu* illuminated by epifluorescence (a) and confocal (b and c) microscopies. Gland cells-like were isolated (a) or grouped (c). The arrow shows an isolated gland cell-like structure. Scale bars = 5.0 µm.

wavelengths for excitation (390 nm) and emission (440 nm) reduced the fluorescence due to compounds other than furanones, and a specific and recurrently-occurring structure was identified. This consisted of a central fluorescent vesicle (5 µm) surrounded by five to six non-fluorescent cells (Fig. 3b). Some grouped cells were also observed (Fig. 3c). These distinctive cells were observed by confocal microscopy to be present at the surface of all thalli and resemble gland cells seen in other red algae from the order Bonnemaisoniales.

Variations in the density of "gland cell-like structures"

The density of fluorescent structures varied significantly with the reproductive phase of *Grateloupia turuturu* ($p < 0.001$, Fig. 4A). The number of fluorescent structures per cm^2 was significantly higher on cystocarpic plants (8523 ± 1132) than on tetrasporophytes (3634 ± 1107) and non-cystocarpic gametophytes (1974 ± 1159). From the multi-range test, densities on these two latter stages were not different.

Significant variation in the density of fluorescent structures also occurred between field sites ($p < 0.001$, Fig. 4B). Higher densities of fluorescent structures occurred in thalli collected at the Pointe du Diable (9654 ± 1175) than those from

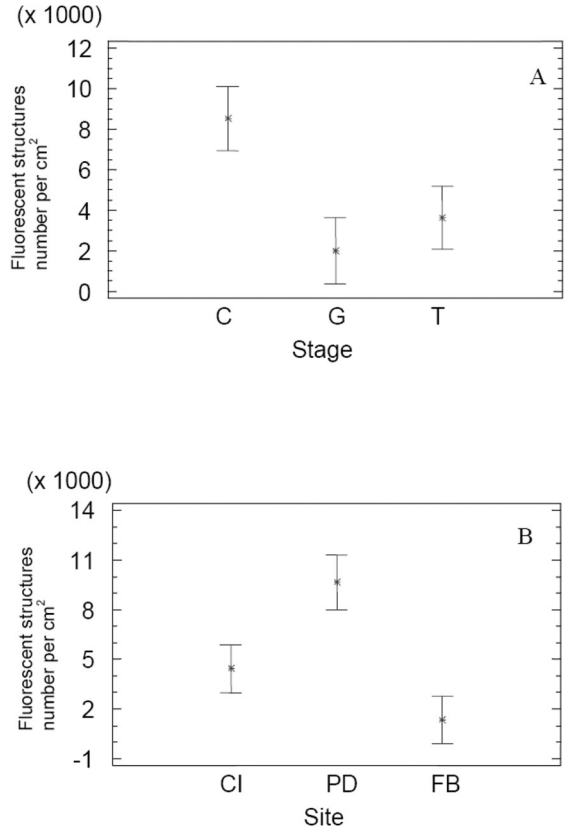

Fig. 4. Variations in fluorescent structures density in *Grateloupia turuturu* thalli from different life phases (A) and field locations (B). C: cystocarpic gametophyte; G: non-cystocarpic gametophyte; T: tetrasporophyte. CI: Callot Island; PD: Pointe du Diable; FB: Fort Bloqué. The data presented are means (± Standard Error).

Callot Island (4433 ± 1018) and Fort Bloqué (1349 ± 1018). The intra-plant variation in the number of fluorescent structures was tested on thalli free of epiphytes and no significant difference was observed ($p > 0.005$, data not shown).

Effect of epiphytes on the density of "gland cell-like structures"

The abundance of fluorescent structures observed on five thalli of *Grateloupia turuturu* collected at Pointe du Diable showed an increase in their density on plants with epiphytes for both tetrasporophytes (Figs 5A and 5C) and cystocarpic gametophytes (Figs 5B, 5D and 5E). Our experimental data set showed a significant correlation between fluorescent structures density and the presence of epiphytes ($R^2 > 0.9$, $p < 0.05$ for the 5 thalli tested). The density of fluorescent structures seemed then correlated with the presence of epiphytes (Fig. 5). Moreover, considering the description of each field site of this study, the density of fluorescent structures seemed to follow the percentage of epiphyted thalli. Indeed, the rate of epiphyted thalli varied with site with more epiphyted thalli observed at PD than at CI and FB (where no epiphyted thallus was noted).

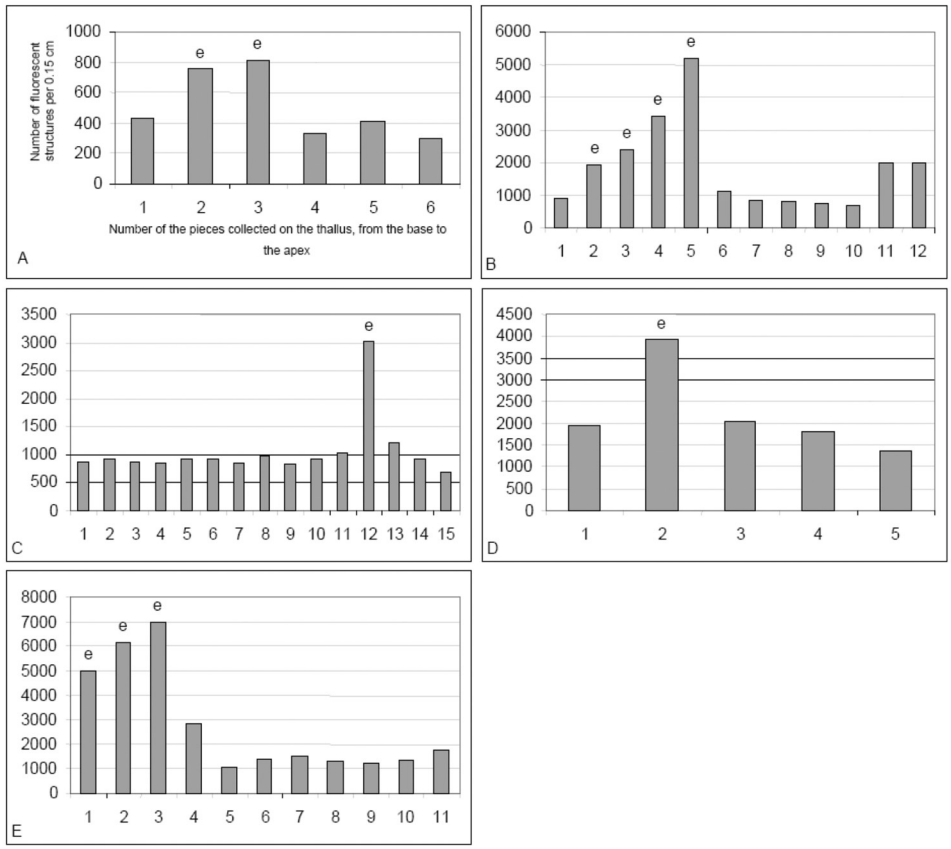

Fig. 5. Fluorescent structures density in *Grateloupia turuturu* in relation to the absence/presence of epiphytes (e) and red algae sporocystes (s). Data were collected on tetrasporophytes (A, C) and on cystocarpic gametophytes (B, D, E).

DISCUSSION

This is the first report of fluorescent structures described in the genus *Grateloupia*. Gland cell densities in *Grateloupia turuturu* were found to vary between sites. In our study, the degree of water movement and substratum differed significantly between the three selected field sites. Fort Bloqué and Pointe du Diable present high hydrodynamism and rocky substratum, whereas Callot Island is more sheltered with sandy substratum. The highest and lowest gland cell densities were from the two high water movement/rocky sites, which makes it difficult to draw any conclusions on the effects of hydrodynamism and substratum on the variations of the density of fluorescent structures.

Intrathallus variations of the density of gland cells were observed in *Delisea pulchra* with a higher density observed at the distal part of the plant (Dworjanyn *et al.*, 1999). This was not the case in *Grateloupia turuturu* plants without epiphytes as no significant intrathallus variation in fluorescent structures density was highlighted.

Epiphytism, as well as changes in nutrients composition and concentration may affect the regulation of secondary metabolites production (Wright *et al.*, 2000), and then induce spatial variations of gland cells as observed in *Grateloupia turuturu*. Our experimental data showed a significant correlation between fluorescent structures density and the presence of epiphytes ($R^2 > 0.9$). This is in accordance with observations made on the field: thalli growing at Pointe du Diable are largely epiphyted.

Studies on spatial variations in furanone content of *Delisea pulchra* in Australia produced contradictory results: Wright *et al.* (2000) showed spatial variations, whereas de Nys *et al.* (1996) found no significant differences between two locations 10 km apart, with only minor variations in levels of individual metabolites observed. However, these studies assessed the level of furanones and not the density of gland cells. Our work looks only at gland cell densities, which may correlate with production of furanones-like compounds, but this can not be quantified.

We also highlighted a significant variation in gland cell density between the three life-history phases investigated with tetrasporophytes > carposporophytes and gametophytes or immature thalli. This result led us to focus our study on only cystocarpic and tetrasporic plants, which can be easily identified *in-situ*. Our results showed that *Grateloupia turuturu* developed more defense to protect tetraspophytes than carpo-, gametophytes or immature thalli from microfouling.

A correlation between the presence of epiphytes and the increase of fluorescent structures density was established in *Grateloupia turuturu*. The production of fluorescent compounds may be ruled not only by the growth mode of the alga, which depends on the type of environmental hydrodynamism, but also on epiphyte and grazer abundance, water-temperature, salinity and light (Wright *et al.*, 2000). In *G. turuturu*, our study demonstrated a high density of fluorescent structures in relation of newly colonized habitat (Pointe du Diable) and also in relation with the rate of epiphyted thalli. To elucidate this point, it will be interesting to find compounds responsible to the fluorescence and to carry out a more thorough ecological and chemical study on *G. turuturu* in the aim to characterize the involved fluorescent compounds.

Epifluorescence and confocal microscopies permitted the observation of "gland cell-like structures" at the surface of *Grateloupia turuturu* thalli which fluoresced at the same wavelength than reported for furanones from *Delisea pulchra*.

Moreover, the fluorescent structures isolated in this study are morphologically closed to gland cells described in *Delisea pulchra*. In a preliminary chemical approach, we then tried to isolate furanones from dichloromethan extracts of *Grateloupia turuturu* and no furanone could be isolated from this alga. We then hypothetized that other kinds of compounds were responsible of the emission of fluorescence at the chosen wavelengths and the chemical approach continues to allow us to characterize the(se) fluorescent compound(s).

The location of those structures at the surface of *Grateloupia turuturu* also suggests a potential role of the produced compound as natural antifoulant (Steinberg & de Nys, 2002). In a recent study, we demonstrated antimicrofouling activity of several extracts of *G. turuturu* (Plouguerné *et al.*, unpublished data).

It is interesting to remark that only one genus of epiphyte, *Polysiphonia* sp. (Le Duff, pers. com.), occurred on the thalli of *Grateloupia turuturu* from Brittany. We can then hypothesize that *G. turuturu* is able to develop defence against microfouling (settlement of a special flora, which is necessary to the settlement of macrofouling) which is not efficient against *Polysiphonia* sp. In such a case, a potential resistance of *Polysiphonia* sp., which allow this specie to colonize *G. turuturu*, must be hypothesized. This last point highlights the potential interest of those fluorescent structures isolated in *G. turuturu*.

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