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Species composition of endolithic microflora developing in live and dead thalli of crustose coralline algae in the Northern Adriatic Sea

Abstract

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The coralline algae, like all the limestone substrata, can be invaded by endolithic microorganisms. Live and dead thalli of *Hydrolithon farinosum* (Lamouroux) Penrose & Chamberlain, *Lithophyllum incrustans* Philippi, *Sporolithon ptychoides* Heydrich and *Corallina elongata* Ellis & Solander (*Corallinales*, *Rhodophyta*) were therefore examined to discover if, as it happens in the corals, only few endoliths can invade the live coralline algae, while dead thalli can be bored by many organisms. New techniques have been employed.

Introduction

A study on the endolithic microorganisms living in the Northern Adriatic Sea (Italian coast) is currently being carried out in the Department of Biology of the University of Trieste. In particular the microborers, inhabiting into crustose and erect members of *Corallinales* (*Rhodophyta*), are being considered.

Many papers were published about the endolithic microflora developing in corals (Le Campion-Alsumard & al. 1995a; Lukas 1974; Tribollet & al. 2002); the chlorophyte *Ostreobium quekettii* Bornet and Flahault, together with the cyanophyte *Plectonema terebrans* Bornet and Flahault and small fungal hyphae (Kendrick & al. 1982; Le Campion-Alsumard & al. 1995b) was identified to be the major endolithic constituent of live corals.

A more various microflora was described from skeletons of dead corals: *Mastigocoleus testarum* Lagerheim, *Hyella caespitosa* Bornet and Flahault, *H. balani* Lehmann, *Solentia foveolarum* Ercegovic, *S. paulocellulare* (Ercegovic) Le Campion-Alsumard & Golubic, *Phaeophila dendroides* (Crouan & Crouan) Batters, in addition to *Plectonema* and *Ostreobium* are the most common inhabitants.

While the bioerosion of corals by micro- and macroborers has widely been studied by several authors, few studies have been carried out on microendoliths boring crustose coralline algae, despite the coralline algae, like all the limestone substrata, can be invaded by endolithic microorganisms.

Live and dead thalli of *Hydrolithon onkodes* (Heydrich) Penrose & Woelkerling (Tribollet & Payri 2001), *Hydrolithon farinosum* (Lamouroux) Penrose & Chamberlain,

Lithophyllum incrustans Philippi and *Titanoderma pustulatum* (Lamouroux) Nägeli (Ghirardelli 1998, 2002) (*Corallinales*, *Rhodophyta*) have therefore been examined to discover if, as it happens in the corals, only few endoliths have been able to invade the live coralline algae.

Understanding whether the thallus examined is really alive or not has been the main problem of this study. The thallus structure of *Corallinales*, in fact, is multi-axial and in the crustose thalli the filaments grow together so closely that the alga appears to be composed of parenchyma. The crust expands by means of the division of the peripheral cells of the hypothallus (basal filament) and grows in thickness by the division of the uppermost perithallus cells (Van den Hoek & al. 1995). After some time, the basal oldest part of these filaments dies. In many *Rhodophyta* and in particular in the crustose coralline algae, the upper external 20-30 rows of cells of each filament form the live part of the alga.

Bright red thalli can be considered alive at the surface; white thalli are normally dead (Nichetto & Ghirardelli 1994)

In this paper the use of particular techniques, that could help to understand if the algae are alive, are reported.

Materials and methods

Specimens of calcareous red algae (*Lithophyllum incrustans*, *Hydrolithon farinosum*, *Titanoderma pustulatum*, *Sporolithon ptychoides* Heydrich and *Corallina elongata* Ellis & Solander) were collected in the littoral settings of Trieste (Northern Adriatic Sea, Italy) at 2-4 metres depth. They were usually still anchored to little stones representing their substratum. The coralline algae were then removed from the substratum in laboratory.

The live and dead parts of the collected algae were then treated for SEM and light microscope observation, exposed to dyes and examined by an oximeter.

SCANNING ELECTRON MICROSCOPE (SEM). The specimen was fixed with 4% glutaraldehyde in 0,6 M phosphate buffer, rinsed in distilled water, dehydrated in graded alcohol series, critical point dried, mounted on a stub and golden coated. Specimens were then observed with a Leica Stereoscan 430i.

LIGHT MICROSCOPE. Before decalcification, the epilithic algal turf, grown on coralline algae, was removed by sodium hypochlorite and by a vigorous brushing. Carbonate substrate was removed from the cell walls by dissolution in dilute acids (0,6 N Nitric acid) or Perenyi solution (0,5% chromic acid, 10% nitric acid, 70 to 90% alcohol, in relation 3:4:3), after fixation in 3% formaldehyde solution in order to reduce damages to the enclosed endoliths. The decalcified algae were then squashed on a slide. The endoliths, free from the carbonate substrate, were observed using a light microscope.

STEREO LIGHT MICROSCOPE. Specimens, broken perpendicularly to the external surface were observed on a stereo light microscope, without any previous treatment.

USE OF DYES. MTT formazan and alamar blue, able to reveal the viability of the whole coralline algae or the live parts of them, were employed (Mizuta & al. 1997). TREATMENT WITH MTT. Viability of crustose coralline algae was measured using an oxidation-reduction indicator: 3-(4, 5-dimethyl-tiazolyl-2)-2, 5-diphenyl-tetrazolium chloride (MTT). The MTT was reduced to coloured formazan form (bluish-violet) in living *Corallinales*, but

was not reduced in dead *Corallinales*. The coralline algae were incubated for 20 minutes in an MTT buffered solution that was adjusted to 0,5 % (w/v) in 1/15 M phosphate buffer (ph=7). Dead algae remained uncoloured. TREATMENT WITH ALAMAR BLUE. For alamar blue assay, coralline pieces (ca. 8 mm³) were placed in a microcuvette with 1 ml of sea water and 0,05 ml of alamar blue and incubated for about 10-12 hours. After incubation, the medium was supplied to measure absorbance at 570 and 600 nm wavelengths. The reduction rate % of alamar blue was calculated following the method described in the alamar blue assay manual (Biosource). A solution of alamar blue, without algae incubated, was maintained near the experiment to verify the changes that are visible also at first sight. Alamar Blu solution changed colour from blue-violet to red, if it was in contact with live algae. Dead algae did not provoke any changes in the colour of the solution.

CLARK-TYPE ELECTRODE METHOD. The viability of thalli was detected by means of photosynthetic activity, which was closely linked to oxygen production. Oxygen production can be measured by means of the Clark-type electrode method (Delieu & Walcher 1972). The "Clark-type electrode" is able to detect the oxygen which is accumulate during photosynthesis. Red, white and grey thalli of coralline algae were examined by Clark-type electrode and their colour was related to the oxygen production (Nichetto & Ghirardelli 1994). The photosynthetic activity of endoliths living in dead thalli was held in due consideration.

These testing techniques are still at experimental stages.

Results

The live parts of the coralline algae, usually the red ones, were stained bluish violet by MTT, while the white or yellow parts remained uncoloured. However, after culturing algae with alamar blue, the medium used with the live algae changed from blue to red, while in the medium with dead algae, alamar blue was not reduced and did not change colour.

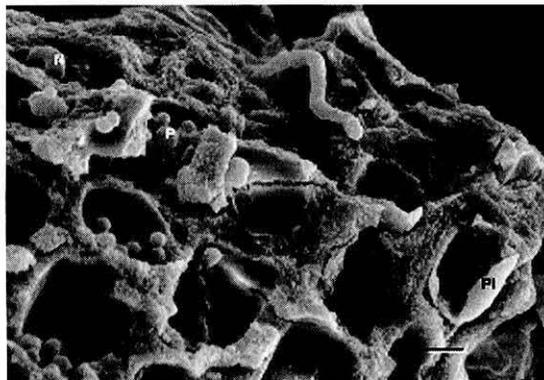


Fig. 1. SEM image of *Plectonema terebrans* in a live thallus of *Lithophyllum incrustans*. Plastids (P), plasma-lemma (Pl) and nuclei (N) are clearly visible in the cells of the alga, testifying their viability. Bar 2 μ m.

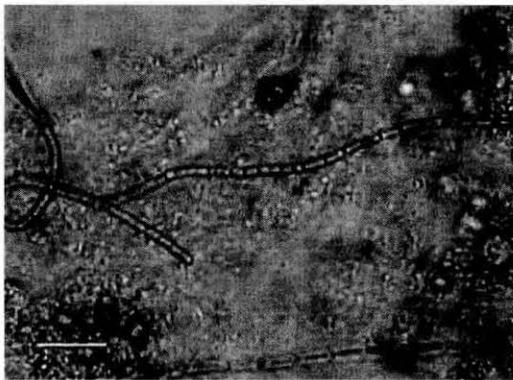


Fig. 2. Light microscope image of *P. terebrans* in a live thallus of *L. incrustans*. Bar 20 μ m.

After having identified live algae or the parts of the thalli that had a good viability, they were isolated, fixed, decalcified and observed with light microscopy. Endolithic microorganisms were identified and classified. The use of dyes allowed to reduce the errors about the presence of endolithic microflora in live algae.

In the specimens observed with SEM the cellular organules, that appeared evident only in the living cells, revealed immediately the live parts of the thalli (Fig. 1).

It was very difficult to find endoliths in the living parts of the coralline algae, as they are usually free from boring organisms. The microflora, if present, consisted almost exclusively of the cyanophyte *Plectonema terebrans* (Figs 1, 2). The chlorophyte *Ostreobium quekettii* (Fig. 3) was rarely extracted from still living thalli of coralline algae.

Dead thalli may be considered as any other calcareous substrate, since only the calcified wall remains after the cells die. The borers penetrate into the thallus from the more exposed surface of the coralline algae, that is usually the upper one, then the microorganisms bore the host, following the calcified cell walls, without a preferential direction. They revealed a numerous number of endolithic microorganisms, belonging to *Cyanophyta* (*Hyella caespitosa*, *H. balani*, *Mastigocoleus testarum* and *Plectonema terebrans*) or *Chlorophyta* (*Ostreobium quekettii* and *Phaeophila dendroides*) and fungi.

On the contrary, the invasion of live coralline algae by endoliths proceeded from the inner surface, that is the dead part of the thallus, and usually stopped when the microborers reached the living cells (Fig. 4).

Only the fungi were able to bore the coralline thallus either in the walls or into the cells.

Conclusions

Contrary to what occurs in the inorganic limestone substrates, that are colonized by a great number of endolithic microorganisms (Golubic 1981), living sea organisms, impregnated with calcium carbonate (corals and coralline algae), react to the invasion of the borings, rejecting the penetration. Only few endoliths *Ostreobium quekettii* (Fig. 3) and *Plectonema terebrans* (Figs 1 and 2) are able to penetrate. The cyanobacterium which is more often

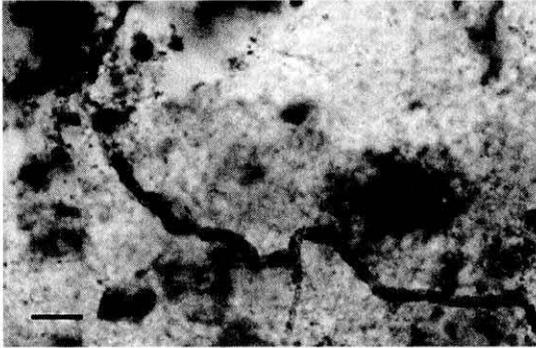


Fig. 3. *Ostreobium quekettii* in a live thallus of *L. incrustans*. Bar 10 μ m.

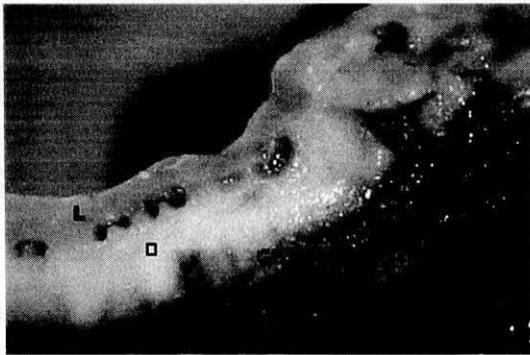


Fig. 4. *L. incrustans* with conceptacles on the upper live (L) surface. The green band (GB) indicates the presence of endoliths in the dead part (D) of the thallus. Bar 1 mm.

found inside live *Corallinales* is *Plectonema terebrans*, synonymous of *Spirocoleus terebrans* (Bornet & Flahault) P. Silva or *Leptolyngbya terebrans* (Bornet & Flahault) Anagnostidis & Komarek, known also as *Schizothrix calcicola* (C. Agardh) Gomont, according to Drouet (1981) that was found either using light (Fig. 2) and scanning electron microscope (Fig. 1) (Giaccone & al. 2003).

As regards crustose coralline algae, a light microscopy examination, preceded by decalcification, not always enabled the evaluation of the viability of the thallus, while SEM observation of fresh specimens certainly solved the problem better, but it was not the simplest method to use.

Specific dyes, such as MTT and alamar blue (Mizuta & al. 1997; Bressan & Babbini 2003) was employed and could solve this problem, as live *Corallinales* thalli reacted in different ways to dyes. This method confirmed that in live thalli the presence of endoliths was limited to two species: *Plectonema terebrans* and *Ostreobium quekettii*.

Erect *Corallinales* (*Corallina elongata*) up until now have been found free from endoliths.

Further investigation is needed to understand if, among the endoliths, fungi are able to cause diseases in the *Corallinales*, as it happens in other algae (Raghukumar 1987) and in corals (Le Campion & al. 1995b).

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