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Detecting cells with low RNA content colonizing artworks non-invasively: RNA-FISH

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ABSTRACT: Detection and identification of the microorganisms involved in biodeterioration of cultural heritage materials is vital. Since some artworks have an incalculable value, the use of nondestructive sampling methods is preferred. In this study, the possibility of using RNA-FISH technique for detecting microbial cells with low RNA content, isolated from biodegraded cultural heritage materials, was investigated. So, the possibility of the RNA-FISH protocol implementation as a noninvasive approach was studied. Wood and stone slabs artificially inoculated with yeast and bacteria cells were prepared and different sampling methods were applied to evaluate their performance regarding: i) the number of cells extracted and ii) the signals obtained by the RNA-FISH technique. It was possible to conclude that is possible to apply RNA-FISH for detecting cells with low RNA content and is possible to use noninvasive sampling in wood or stone materials.

1 INTRODUCTION

It is a well-documented phenomenon that microor-, cause deterioration of Cultural Heritage (CH) (Müller *et al*., 2001; Otlewska *et al*., 2014; Rosado *et al*., 2014). For the development of adequate conservation and restoration strategies it is necessary to identify them (Sterflinger and Piñar, 2013). Thus, multiple efforts are focused on the improvement of approaches that allow microbial signalization. An in suspension RNA-FISH (Fluorescence *In Situ* Hybridization) protocol was previously proposed by our group as an alternative for *ex situ* analysis of the metabolically active microorganisms (Vieira *et al.*, 2016). The low RNA content of the target cells has been previously reported as a possible limitation for RNA-FISH application in environmental and CH samples (Hoshino *et al.*, 2008) and the use of noninvasive sampling methods is preferred in CH field. Thus, the aim of this work was to investigate: i) the capacity of the RNA-FISH protocol

cited above to detect yeast (*Rhodotorula sp.*) and bacteria (*Bacillus sp.*) cells with low RNA content; and ii) the possibility of adapting it for its application in samples collected by noninvasive methods.

2. MATERIALS AND METHODS

2.1. *Detection of cells with low RNA content using FISH*

Microbial cells isolated from artworks (Rhodotorula sp. and Bacillus sp.) were cultured and recovered in death phase (with 32 days of growth) from liquid cultures (of Malt Extract for yeast and of Nutrient Broth for bacteria, incubated at 30°C and 120 rpm) and washed with Phosphate-Buffered Saline solution (PBS; 130.0 mM NaCl, 8.0 mM NaH2PO4, 2.7 mM KCl, 1.5 mM KH2PO4, pH 7.2).

The RNA extraction of *Rhodotorula* sp. was performed as described by Schmitt (Schmitt, Brown and Trumpower, 1990) and for *Bacillus* sp. as described by Atshan (Atshan *et al.*, 2012). Total RNA was quantified using a spectrophotometer (Thermo Scientific Multiskan GO μDropTM Plate).

The procedure followed for the application of the RNA-FISH technique was previously described by us (González-Pérez *et al.*, 2017). A blank (without probe) and two assays with addition of individually probes to detect bacteria (EUB338-Cy3) and to detect yeast cells (EUK516-Cy3) were performed. All assays mentioned above were carried out in triplicate.

2.2 *Combination of various types of sampling methods with RNA-FISH detection*

Artificially inoculated slabs were prepared for mimicking cultural heritage biocolonized materials. Wood and stone sterilized slabs (1x1 cm) were inoculated with 1.5 x 10^8 cells of *Rhodotorula* sp. and *Bacillus* sp. cells in exponential phase (resuspended in miliQ water). The slabs were incubated for 2 weeks.

Samples were collected from the slabs using both nondestructive (swabbing with cotton swab for 30 s or pressing 1 cm^2 of filter paper, nylon membrane or nitrocellulose membrane impregnated with sterile distilled water for 30 s) and destructive methods (analyzing the cells after their extraction in suspension from a microsample).After sampling, the collected cells were fixed with absolute ethanol for 1 h. The cellular suspensions obtained were used for determining the cellular recovery capacity using each of the sampling methods selected (by direct counting in *Neubauer* chamber) and for evaluating the performance of the FISH technique. The following steps of the FISH technique were carried out as described previously by our group (González-Pérez *et al.*, 2017). Four different RNA-FISH assays were performed using two individually probes (EUK516-6-FAM and EUB338-Cy3), a probe cocktail containing the two probes in equal concentrations ("Mix-(2)" = EUK516-6-FAM + EUB338-Cy3) and the blank. Each assay was performed in triplicate. The analysis were carried out using the biological microscope BA410E Motic equipped with a 100W Quartz Halogen Koehler illumination with intensity control and with an epi-attachment (EF-UPR-III) and a Power Supply Unit (MOTIC MXH-100).

3 RESULTS

3.1 *Detection of cells with low RNA content using RNA-FISH*

For investigating the possibility of detecting cells from the CH with low RNA content using RNA-FISH technique, cells of *Rhodotorula* sp. and *Bacillus* sp., in death phase, were used. They were analyzed in terms of cellular RNA content and detectability of the signals obtained after the application of the RNA-FISH technique.

The results revealed that the cellular RNA content of the cells in death phase was low (less than $1.25x10^{-3}$ pg/cell for *Rhodotorula* sp. and less than $1.12x10^{-4}$ pg/cell for *Bacillus* sp.), and, as expected, lower than that of the cells in

exponential phase (4.42 x10^{-3} pg/cell for *Rhodotorula* sp. and 4.71x10^{-4} pg/cell for *Bacillus* sp.). After application of the RNA-FISH technique, the results showed that, even with low RNA content the cells were detectable by epifluorescence microscopy (Fig. 1a, b). These results support the possibility of detecting cells with low RNA content by using this technique.

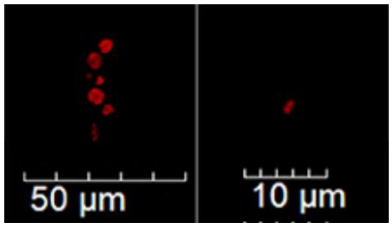

Figure 1. RNA-FISH signals of the *Rhodotorula* sp. (a) and *Bacillus* sp. (b) in death phase.

3.2 *RNA-FISH in combination with various non-invasive sampling methods*

The Number of Cells Recovered (NCR) using a micro-destructive and various noninvasive sampling methods (swabbing and impression methods) as well as their applicability in combination with RNA-FISH technique in wood and stone were evaluated.

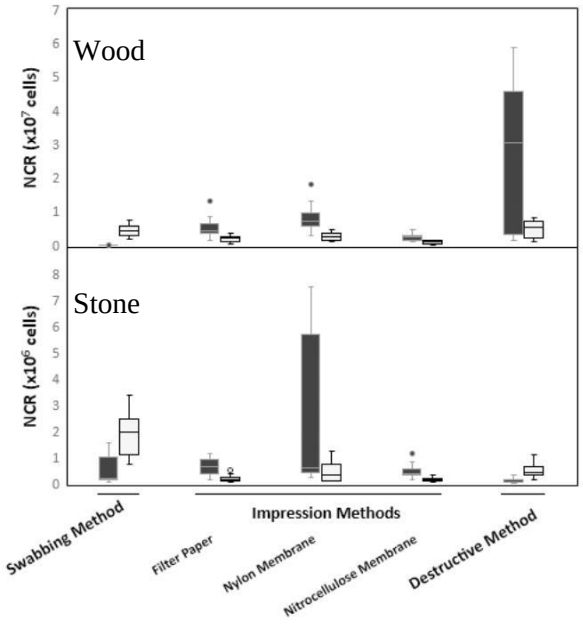

Figure 2. Box plots show the NCR in wood and stone for both microorganisms (■ *Rhodotorula* sp. and ■ *Bacillus* sp.).

The NCR for all sampling methods selected was determined (Fig. 2). The results of this study on wood material showed that the destructive sampling method was the one that allowed to obtain a higher NCR (Fig. 2). However, the fluorescent signals of the microorganisms resulting from combination of the RNA-FISH technique with this sampling method were sometimes masked by the autofluorescence of the support (Fig. 3a). For these reason, even if the NCR obtained for wood are lower using noninvasive sampling methods, they are preferred for further detection and identification of the cells recovered by RNA-FISH. Among the noninvasive methods tested, the impression with nylon membrane and swabbing with cotton swab showed the highest NCR for both yeast and bacteria, respectively (Fig. 2). In the case of stone, as observed for wood, the destructive sampling method showed fluorescent signals due to the support (Fig. 3b), which can

cause false positives. The method that obtained the highest NCR for yeast was the nylon membrane impression method and for bacteria was the swabbing method with cotton swab. (Fig. 2).

It was previously reported by other authors that the efficiency of recovery is influenced by several factors: i) the surface material; ii) the sampling method;
iii) the material used for sampling; iv) the sample processing protocol; v) the properties of the target microorganisms; and vi) the analysis method. Our results evidenced that the sampling method applied affect the efficiency of recovery independently of the support sampled. But it was also possible to determine that the type of microorganism and the type of support are also factors with enormous influence (higher NCRs were obtained for yeast than for bacteria cells recovery independently of the support and sampling method applied and comparing the results obtained for wood and stone applying each of the sampling methods, higher number of cells were recovered from wood).

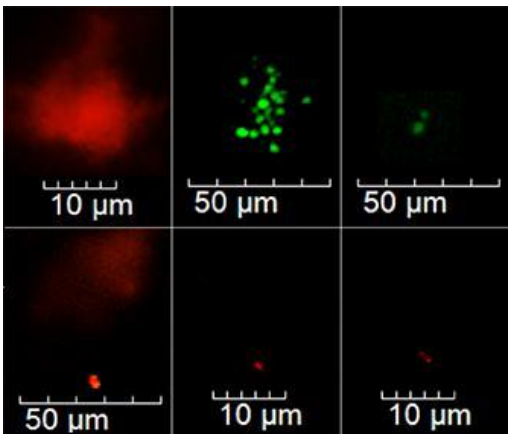

Figure 3. RNA-FISH signals of the microorganisms recovered by the sampling methods selected. Autofluorescence of the support of wood (a) and stone (b) using the destructive sampling method. *Rhodotorula* sp. fluorescent cells (c and e) obtained using nylon membrane impresion on wood (c) and stone (e). *Bacillus* sp. fluorescent cells (d and f) using the cotton swab on wood (d) and using nylon membrane on stone (f).

In spite of the high variability associated to the recovery efficiencies (also reported by Buttner *et al.*, 2007, regardless of the noninvasive sampling method used, good fluorescent signals were detected for both type of microorganisms inoculated (yeast and bacteria) on both supports (wood and stone) when applied FISH technique (Fig. 3 c-f). This evidenced the possibility of combining RNA-FISH and noninvasive sampling for microbial detection/identification in CH samples made of wood or stone.

4 CONCLUSION

This work allowed to conclude that: i) FISH can be applied for detecting cells with low RNA content; and

ii) it is possible to combine noninvasive sampling methods with RNA-FISH for detecting eukaryotic and prokaryotic cells proliferating in wood and stone materials. Furthermore, the proper type of sampling method to be used depends on the support and on the type of microorganism(s) to be analyzed.

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