Influence of fungi in the weathering of limestone of Mayan monuments

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Influencia de los hongos en el deterioro de piedra caliza de monumentos mayas

Resumen. La roca caliza deteriorada de monumentos mayas de Yucatán, México, fue analizada por medio de microscopia electrónica de barrido acoplada con análisis por rayos X retrodispersados (SEM-EDAX), difracción de rayos X (XRD) y espectroscopia infrarojo transformada de Fourier (FT-IR). Los cambios en la composición química de la superficie causadas por la biopelícula con la consecuente conversión de calcita a yeso, fueron demostrados. Los géneros más abundantes fueron *Aspergillus*, *Penicillium*, *Fusarium* y *Paecilomyces*. Cepas de *Aspergillus niger* y *Penicillium* sp., fueron seleccionadas por su capacidad de producir metabolitos ácidos. Ambos hongos excretaron ácidos orgánicos cuando fueron incubados (glucónico, succínico, málico y oxálico). *A. niger* fue el productor más activo y también excreto ácido cítrico. El impacto del hongo sobre los cupones liberó calcio de la matriz mineral asociado a la producción de acido oxálico. Sin embargo, el calcio soluble fue considerablemente bajo en los filtrados de cultivos que contenían los cupones de piedra, sugiriendo quelación. El análisis de SEM-EDAX confirmó el papel biodeteriorante de los ácidos micogénicos.

Palabras clave: acidólisis, *Aspergillus*, biopelículas microbianas, piedra caliza, *Penicillium*.

Abstract. Deteriorated limestone from Mayan buildings in Yucatan, Mexico, was analyzed with scanning electron microscopy (SEM) and microprobe with energy dispersive X-ray analysis (EDAX), X-ray diffraction (XRD) and Fourier-Transformed Infrared Spectroscopy (FTIR). Changes in surface chemical composition, caused by the biofilm layer and the conversion of calcite into gypsum, were demonstrated. Representative fungi include *Aspergillus*, *Penicillium*, *Fusarium* and *Paecilomyces*. Strains of *Aspergillus niger* and *Penicillium* sp., were selected for their ability to produce acidic metabolites. Both fungi excreted organic acids when incubated; ion exchange chromatography identified these acids as gluconic, succcinic-malic (coeluted) and oxalic. *A. niger*, the most active acid producer, also excreted citric acid. When grown in the presence of limestone coupons, calcium release from the mineral matrix paralleled the production of oxalic acid. However free calcium was considerably lower in filtrates from limestone coupon-containing culture, suggesting its complexation. SEM and EDAX confirmed that calcium oxalate crystals developed on the surface of the stone coupons. The results show that organic-acid-producing fungi may contribute to the deterioration of limestone monuments.

Kew words: acidelysis, *Aspergillus*, biofilm layers, lime stone, *Penicillium*.

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ORIGINAL

Introduction

Microorganisms participate actively in the weathering of minerals (Banfield and Hamers 1997). Microbial processes leading to the degradation of mineral may include microbial oxidation and reduction, creation and maintenance of appropriate physicochemical conditions, and production of acidic metabolites (Barker *et al*., 1997; Gaylarde and Morton, 2002). These microbially-mediated processes are partially responsible for the chemical and physical weathering of rocks, which lead, eventually, to the formation of soils (Eckhardt, 1985).

Microorganisms may also contribute to the deterioration of stone artifacts such as historical monuments and statues (Warscheid and Braams, 2000). The production of organic and inorganic acids by microflora in the biofilm has been generally recognised as the predominant mechanism of stone deterioration (Eckhardt, 1978; Sand *et al*., 2002). Most authors have tested acid production by isolated microorganisms in laboratory cultures, in the absence of the stone substrate, extrapolating these results to the field situation (Gaylarde *et al*., 2001; Resende *et al*., 1996).

Stone buildings located in tropical and sub-tropical regions throughout the world are particularly vulnerable to microbial deterioration; the prevailing environmental conditions of temperature and humidity are more suitable for microbial growth and development than those in temperate climates. Studies at Mayan archaeological sites in Yucatan, Mexico, have shown that microbial biofilms, dominated by cyanobacterial populations (Gaylarde *et al*., 2001; Ortega-Morales *et al*., 2000, 2005), contributed to the biodegradation of these monuments through active boring by cyanobacteria and probably by supporting growth of organic acid-producing microorganisms (Ortega-Morales *et al*., 2000). Fungi are a group of heterotrophic organisms that have been detected systematically on degraded stone buildings in tropical and temperate regions (Resende *et al*., 1996; Warscheid and Braams, 2000; Gaylarde and Gaylarde, 2005). They may have greater deteriogenic potential than bacteria, as they produce and excrete higher concentrations of organic acids (Palmer *et al*., 1991). In addition, these microorganisms may cause physical biodegradation of stone by the growth of hyphal networks through the pore space system (Urzi *et al*., 2000).

We studied the deterioration of ancient limestone buildings at the archaeological site of Uxmal, Yucatan, Mexico, and investigated the capacity of the fungal populations to produce acid-linked degradation of the stone.

Materials and methods

Stone sampling

Samples were removed from walls of the Anexo Norte building at the archeological site of Uxmal, Yucatan, Mexico. Two stone samples from a severely degraded pillar were collected with an alcohol rinsed chisel and hammer. These surfaces did not show any apparent biofilm coverage to the naked eye. Two samples of sound stone (undegraded, 2 cm below the surface of apparently sound stone) were also removed. Additional samples (n=2) of a powdered whitish material that appeared macroscopically to be salt efflorescences covering indoor walls were also collected. Samples were frozen and conveyed to the laboratory, where they were subdivided for analyses.

Chemical analysis of sound and degraded stone

Degraded stone and whitish powdery material samples were analyzed in duplicate by scanning electron microscopy (SEM) with energy-dispersive spectroscopy (EDS). Briefly, specimens were fixed with 2.5% glutaraldehyde (v/v) for 1 hour, air dried overnight and stored in a vacuum dessicator. The coupons were then fixed to aluminium stubs and examined using a Philips XL 30 instrument operating at 30 kV with EDAX facility.

Fourier Transform Infrared analysis (Spectroscopy) (FTIR) was carried out on lyophilized and powdered (where necessary) stone samples, formed into cylindrical blocks using 3mg stone plus 100 mg KBr. The spectra were obtained in an OPIH345 spectrophotometer between wavelengths 400 and 4000 cm^{-1}. X-ray powder diffraction of samples was performed with a Siemens D 5005 powder diffractometer, CoKα radiation, scanning speed 2Θ=2.0°/min, 30 kV and 20 mA current, and Diffract-EVA software.

Isolation of fungi

Subsamples of weathered stone were chosen for microbiological analysis, as previously described (Ortega-Morales *et al*., 1999). Stone material was ground in a laminar flow hood, using an alcohol-flamed pestle and mortar. Samples were serially diluted in physiological saline, plated on Czapek Dox agar (1% glucose) and incubated in the dark at 25 °C for 14 days. Fungal colonies were counted and most frequent colony types were further purified and identified according to morphological characteristics (Booth, 1971; Domsch *et al*., 1980; Klinch and Pitt, 1988; Pitt, 1991). The fungal strains were maintained on PDA, al 4 °C.

Dissolution experiments

To identify acidogenic strains, fungal isolates were cultured in Czapek Dox broth containing 0.05 % of bromothymol blue as pH indicator. Cultures were incubated at 25 °C for 8 days. The strains capable of decreasing the pH to the lowest values, as indicated by colour change of bromothymol blue to pale yellow, were selected for the dissolution test.

Intact limestone coupons were obtained from a 15 cm core collected from a nearby commercial quarry (Oxkintok, Yucatan). One cm^3 coupons were cut with a diamond saw, sterilized in three cycles in an autoclave and two placed in each of 250 ml conical flasks containing 150 ml of Czapek Dox broth. Flasks were inoculated with 10 ml of a suspension of approx. 10^6 cfu/ml of each selected strain, apart from control flasks. Flasks without limestone coupons were similarly inoculated to check the influence of stone on acid. All flasks were incubated at 25 °C. The rate of limestone dissolution was monitored by taking samples of spent medium from the systems at 0, 4 and 10 days and determining calcium and organic acids in the solution. Growth of the fungi was also monitored by plating on Sabouraud agar.

Organic acids and solubilized calcium determination

Aliquots (20 ml) from the incubated flasks were filtered (0.45 µm Nucleopore filters) and organic acids determined by ion exclusion chromatography, using 0.5 mM HCl eluant at 0.8 ml/min flow through a Dionex 500XL HPICE-AS1 separator column. Identification and quantification of acids were performed by coinjention of standards (Sigma), the free acids or their salts. Succinic and malic acids coeluted in these chromatographic conditions were therefore quantified as a single suc-mal peak. Dissolved calcium was assayed in the same filtered medium by atomic absorption spectrophotometry, using a model GBC 904AA spectrophotometer.

Microscopic and surface analysis of incubated coupons

Coupons were retrieved from the flasks and prepared for SEM analysis, as described above, using gold sputtering to aid the visualization of biological material. Elemental analysis by EDAX was performed on specimens without gold coating in order to determine the chemistry of the coupon surfaces.

Results and discussion

Surface elemental analysis of limestone samples

The chemical composition of the sound and degraded limestone samples, as shown by EDAX, is given in Table 1. The sound sample, as anticipated, consisted mainly of

calcium and oxygen, the components of the limestone rock (calcite, $CaCO_3$), with Mg indicating the presence of dolomite ($CaMgCO_3$). García de Miguel *et al*. (1995) report a similar composition for limestone samples from the Mayan Pyramid of El Jaguar at Tikal, Guatemala.

The deteriorated samples could be divided into solid, but biofilmed, surfaces and badly degraded, powdery surfaces, with little apparent biofilm. The former contained high amounts of carbon and oxygen, as would be expected for an organic layer; this was confirmed by the presence of a considerable amount of nitrogen. The percentage of calcium was low, indicating that the limestone substrate was hidden below the organic biofilm layer. The powdery stone samples gave calcium, carbon and oxygen values in between the other two. Apparently, the limestone substrate was partially covered, either by microorganisms or, perhaps more likely in view of the absence of nitrogen, by their metabolic products. These could be organic acids or extracellular polymeric materials (EPS).

The presence of sulfur in these powdery samples was unexpected, although a similar phenomenon had been noted at other Mayan sites located in unpolluted countryside (Ortega-Morales *et al*., 2005). This indicated that some of the limestone had been converted into gypsum ($CaSO_4 \cdot 2H_2O$) and this was confirmed by XRD (Figure 1). Gypsum is not unusual in urban sites, but the source of the sulfur in this countryside situation is unknown, although cartographic data shows that well water from near by locations contains significant amounts of sulfates.

Table 1. Percentage elemental (EDS) analysis of sound and degraded limestone from Uxmal, Mexico. Values are means of three replicates

Element	Sound stone (%)	Biofilm-covered (%)	Powdery surface (%)
C	8.2	32.2-35.9	15.2-15.6
O	30.7	26.0-34.0	38.2-39.5
N	-	19.9	-
Ca	59.5	1.1-5.1	25.5-26.4
Mg	1.5	0.3-3.5	1.1-1.2
Na	-	1.9-9.6	-
S	-	1.3-1.5	16.9-17.7
Cl	-	0.6-5.7	2.6
K	-	0.4-0.6	-
P	-	0.1	-
Si	-	0.2	-
Al	-	0.1	-

Sodium was present at reasonable levels in the biofilmed samples (Table 1). Other authors have noted this enrichment of sodium in rock samples associated with microbial communities (Johnston and Vestal, 1989; Ferris and Lowson, 1996). The small amounts of potassium, chloride and silicon observed by EDS are probably derived from the original mortar coating, which would contain wood ash from calcining limestone, and the secondary inclusion of other minerals, as we have previously noted (Ortega-Morales *et al*., 2005).

FTIR (Figure 2) confirmed that calcite was present in the undegraded and heavily biofilmed stone (strong bands at 1432cm^{-1}, 872cm^{-1} and 708cm^{-1}, Negrotti *et al*., 1996) and that the reduction in this component in the powdery stone was due to its conversion into gypsum (bands at 1622cm^{-1}, 1140cm^{-1}, 1120cm^{-1}, 671cm^{-1} and 603cm^{-1}). The presence of gypsum was confirmed by EDAX (Figure 3). Gómez-Alarcón *et al*. (1994) also noted a diminution in calcium content, with the formation of gypsum, in a degraded limestone monument in Spain.

Figure 1. Anexo Norte building at Uxmal, Mexico.

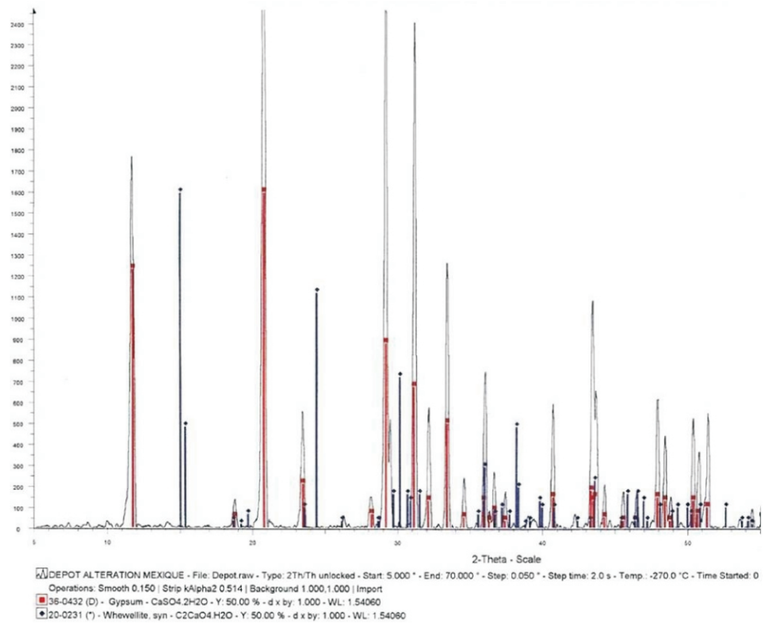


Figure 2. XRD spectrum of powdery degraded stone from the Anexo Norte building, showing the presence of gypsum.

Fungi detected in the biofilms

The fungi identified from degraded stone surfaces at the ancient Mayan site of Uxmal, Yucatan, Mexico, were *Aspergillus fumigatus* Fresen, *Aspergillus niger* Tiegh, *Aspergillus terreus* Thom, *Aureobasidium pullulans* (de Bary) G. Arnaud, *Cunninghamella* sp., *Fusarium* sp., *Paecilomyces* sp. and *Penicillium* sp. These genera are commonly found on stone monuments around the world (Palmer *et al*., 1991; Resende *et al*., 1996), and are typical of the soil mycoflora (Domsch *et al*., 1980). The dominant fungi were *A. niger, A. fumigatus* and *Penicillium* sp. Fungal populations ranged from 10^2 to 10^5cfu/g, similar to results obtained by other investigators on stone buildings in Europe and Latin America (Resende *et al*., 1992; Urzi, 1993; Hirsch *et al*., 1995; Gaylarde *et al*., 2001).

Dissolution experiments and organic acid production

Two fungal strains, *Aspergillus niger* and *Penicillium* sp., were screened for their effect on limestone dissolution. Previous mineralogical analyses have shown that this limestone material is predominantly composed of calcite, with low amounts of aragonite (Ortega-Morales *et al*.,

unpublished results). After four days, *Penicillium* sp. and *A. niger* increased dissolved calcium in the culture medium more than eight- and 23-fold, respectively, relative to the abiotic control (Table 2), even though there was no visible growth in the flasks and colony counts were slightly reduced to around 10^5cfu/ml. At 10 days, fungal numbers had increased to 2 – 4 x 10^5 cfu/ml and the dissolution rate was increased up to 46-fold for *Penicillium* sp. and 112-fold for *A. niger*, compared to the uninoculated control.

Both genera produced gluconic oxalic and suc-mal acids, *A. niger being* the most active producer and also releasing citric acid. Table 2 shows the levels of oxalic acid, which is well known to complex with calcium. *A. niger* reduced the final pH in the medium without limestone coupons from 7.1 to 3.1, while the final pH in non-coupon containing flasks was 6.2, close to that for *Penicillium* sp. (6.8 and 6.4, with and without coupons, respectively).

Braams (1992) found that 41 fungal strains excreted predominantly gluconic, oxalic, citric and fumaric acids, a similar profile to these Uxmal isolates. He did not, however, detect oxalic acid from *A. niger*, probably because the medium utilized was too rich. It has been shown, for basidiomycetes, that oxalic acid production is linked to low nutrient levels (Dutton *et al*., 1993). The carbon source (glucose) in Czapek Dox medium was reduced from 3% to 1%, allowing us to detect the high levels shown here. Nevertheless, we recorded differences in quantitative terms compared with other studies; where up to 4–fold higher concentrations of oxalic acids were observed with similar fungi (Gómez-Alarcón *et al*., 1994).

The flasks containing limestone specimens showed much lower concentrations of oxalic acid than those without coupons, indicating its removal by reaction or chelation with calcium. *A. niger* and species of *Penicillium*, among other fungal genera, have previously been shown to produce calcium oxalate, mainly as the monohydrate form, whewellite, however, small amounts of the dihydrate, weddellite, was also detected (reviewed in Pinna, 1993). The calcium appeared in the form of crystals on the surface of the coupons, in shapes that have been described by Monje and Baran (2002) as typical of calcium oxalate (Figures 4 and 5). EDAX analysis of these crystals showed major peaks of Ca, C and O, confirming their identity (data not shown).

All taxonomic groups of the fungi are able to precipitate calcium oxalate, to a greater or lesser extent (Sterflinger, 2000), although not all species or strains are able to do so (Palmer *et al*., 1991). This activity, when occurring within stone, leads to increasing internal pressure, the final result being catastrophic failure, or spalling, as was seen in our badly degraded limestone samples from Uxmal. These results suggest that organic acid producing fungi contribute to the biodeterioration of Mayan monuments. This

Table 2. Limestone dissolution by organic acid-producing fungal strains isolated from weathered surfaces in buildings at Uxmal, Yucatan, Mexico. N = 2

	Calcium µg/ml			Oxalic acid µg/ml		
Fungus / Day	0	4	10	0	4	10
Penicillium (With stone)	3 (2)	128 (9)	606 (26)	-	5 (1)	8 (2)
Penicillium (No stone)	-	-	-	-	112 (17)	108 (6)
A. niger (With stone)	2 (1)	374 (20)	1,456 (157)	-	-	2 (1)
A. niger (No stone)	-	-	-	-	311 (19)	1,179 (127)
Uninoculated control With stone	8 (1)	16 (6)	13 (2)	-	-	-

Values are means with (SD)

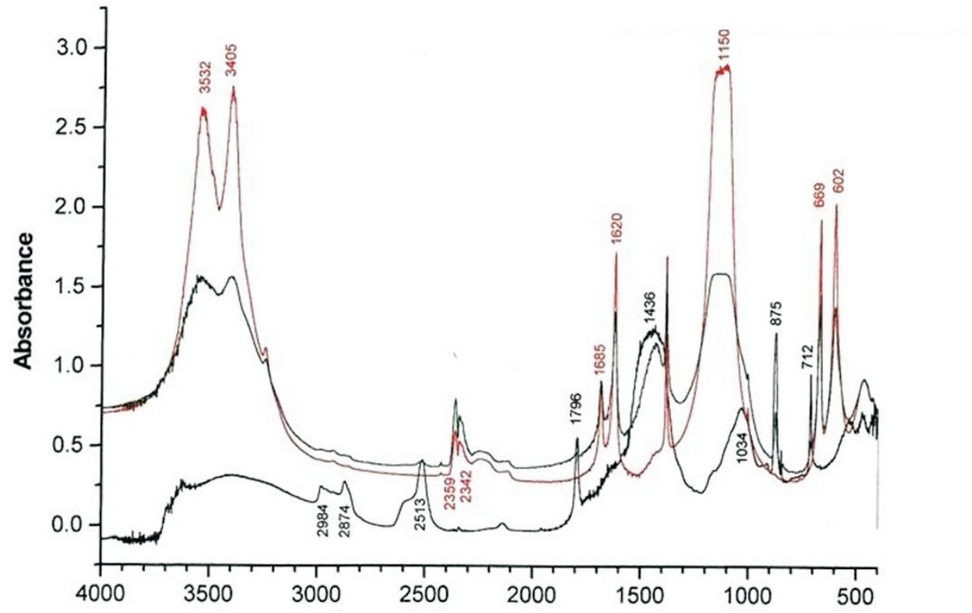


Figure 3. FTIR spectrum of samples from the Anexo Norte, showing peaks indicating the presence of calcite and gypsum.

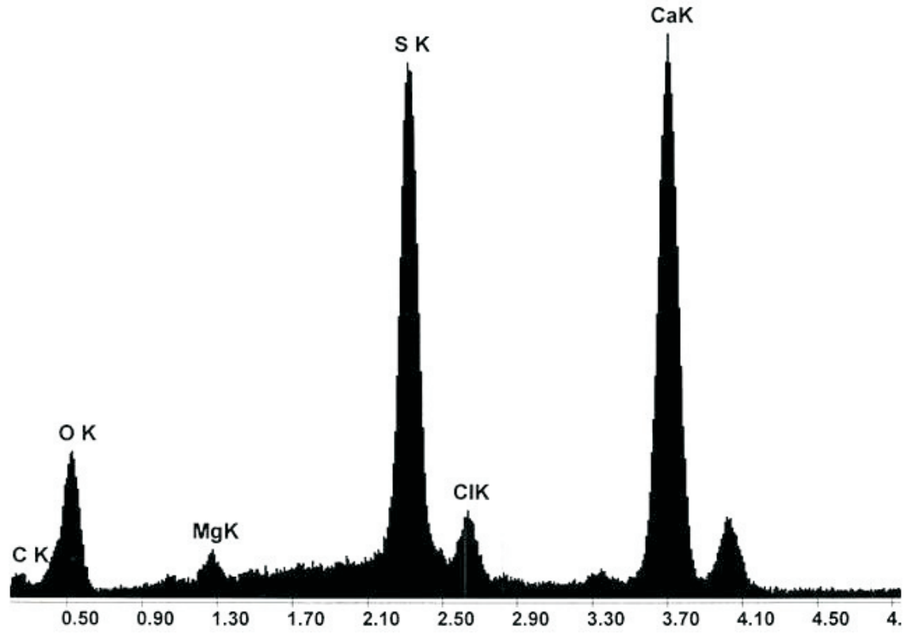
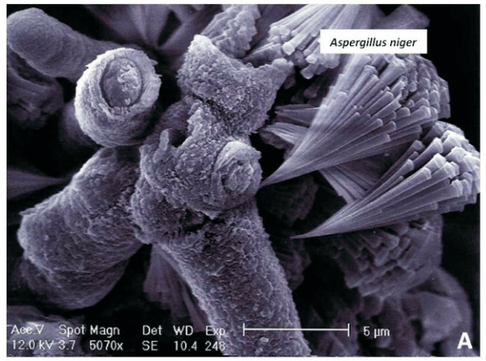


Figure 4. EDS spectrum of heavily biofilmed stone from the Anexo Norte building, showing presence of gypsum.



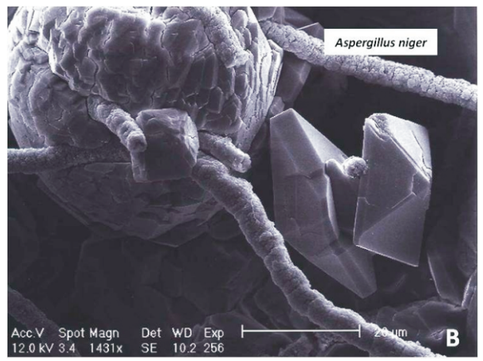


Figure 5. SEM images of crystals formed in coupon-containing cultures incubated with *Aspergillus niger*. A) crystals typical of whewellite, B) crystals typical of weddellite.

heterotrophic mycoflora could be relying for its carbon source on the organic matter provided by the cyanobacterial populations dominating these epilithic biofilms and which, themselves, contribute to limestone biodegradation (Ortega-Morales *et al*., 2000, 2005).

The Yucatan Peninsula along with the rest of the world is experiencing rapid climatic change. Current forecasts include increased carbon dioxide release and precipitation. These are key factors that determine microbial activity. Fungi are proved agents intimately involved in biogeochemical transformations at local and global scales through excretion of organic acids (Gadd, 2007). Other metabolites, not analyzed in this study, such as carbonic anhydrase (CA) could also contribute to the dissolution of carbonate rocks (Li *et al.,* 2005). Climate change is likely to have a major impact on built cultural heritage not only through abiotic exacerbation of stone deterioration, but also by increasing microbial metabolic activity (Bonazza *et al.,* 2009).

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