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Microbiological and calorimetric investigations on degraded marbles from the Cà d'Oro facade (Venice) [1]

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Abstract

Microbiological, physicochemical and biochemical investigations were carried out on degraded marble samples from the Cà d'Oro facade (Venice) in order to verify the presence of biodeterioration agents. The aerobic heterotrophic bacteria (mean value 10^5 CFU/g marble), which were also observed by scanning electron microscope (SEM), were characterized as belonging to the *Micrococcus* and *Bacillus* genera. Nitrifying and sulphooxidizing bacteria were not found in any samples. The fungi (mean value 10^2 CFU/g marble) were attributed to the *Penicillium* and *Aspergillus* genera. Enrichment cultures revealed the presence of photosynthetic microorganisms belonging to the div. *Cyanophyta* and *Chlorophyta.*

The metabolic activity of the microorganisms derived from the cultural tests, was confirmed by the ATP content extracted directly from the powdered marble samples (average 3170 pg ATP per g marble).

Microbiological alterations of stone are the result of the metabolic activity of the microorganisms, whose heat production is an expression of this activity. Confirmation of the presence of the microorganisms on the degraded marble samples was, therefore, obtained by calorimetric investigations, which revealed that the process is completely exothermic and can be divided into two phases: the first showed the attainment of a plateau, typical of a zero-order reaction; the second was characterized by a peak followed by an abrupt arrest.

The change in ATP concentration, which was followed in parallel, reflected both the DSC trace and the plate counts.

INTRODUCTION

Chemical and microbial atmospheric pollutants are known to contribute to the degradation of artistic stoneworks [1–3]. In Venice, chemical

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pollution from Porto Marghera and gas emissions from boats, microbial pollution from marine aerosols, and humidity, all contribute to incalculable damage.

Among the artistic stoneworks that have been seriously degraded in Venice is Cà d'Oro, a 15th century palace of great interest. The most frequently noticed alterations to its marble facade, identified according to the lexicon defined by the Normal Commission [4], are black dendritic crusts, surface deposits, disintegrations and flaking, found on marbles of oriental origin, red Veronese marble and Istria stone.

The aim of this research is to combine the different investigation techniques in order to observe the presence of biodeterioration microorganisms on these degraded materials.

Firstly, traditional culture techniques were used to verify the presence of aerobic heterotrophic bacteria, nitrifying and sulphooxidizing bacteria, fungi and photosynthetic microorganisms. In addition, the ATP content was determined directly on the deteriorated marble samples in order to evaluate the total metabolic activity of the microflora. The quantity of heat released from the cells was measured by calorimetric investigation.

EXPERIMENTAL

Sampling

Marble samples were taken from the facade using a sterile scalpel and transported to the laboratory in sterile plastic vials. The description of the eleven samples and their relative alterations and materials is reported in Table 1.

TABLE 1

Description of the samples

Sample	Materials	Alterations
1	Oriental marble	Black dendritic crust
2	Oriental marble	Flaking
3	Red Veronese marble	Black dendritic crust
4	Istria stone	Black dendritic crust
5	Oriental marble	Flaking
6	Red Veronese marble	Black flaking
7	Oriental marble	Disintegration
8	Istria stone	Black surface deposit
9	Red Veronese marble	Black surface deposit
10	Red Veronese marble	Black crust
11	Oriental marble	Black crust

Samples

The marble fragment masses ranged from a few milligrams to 1.5 grams. Before performing the analyses, the samples were crushed to a fine powder with a sterile pestle and mortar.

Sample fixation for electron microscopy

The samples were fixed onto aluminium stubs and exposed to osmium vapour overnight. They were then coated with a thin gold–palladium film in a Edwards 306 metallizator (Edwards High Vacuum Ltd., UK). The samples were then examined with a Stereoscan 250 (Cambridge Scientific Instruments Ltd., UK) scanning electron microscopy (SEM). Kodac TRI-X-Pan 120 films were used for the electron micrographs.

Microbiological analyses

Known quantities of powdered degraded marble were suspended in 1 or 2 ml of pH 7 phosphate buffer (0.01 M) and then used for microbiological analyses.

Plate count Agar (Difco) was used for the determination of heterotrophic bacterial counts, the isolation in pure culture and the maintenance of the various microbial strains. Malt agar was used for determination of fungi counts, and the isolation and maintenance of the various fungi strains.

The characterization of the isolated bacterial strains was carried out according to cultural, physiological, enzymatic and biochemical analyses, as reported by Bergey [5] The fungi strains were observed using a stereomicroscope and a phase-contrast microscope and were characterized according to Barnett [6]. The presence of nitrifying and sulphooxidizing bacteria was investigated employing Stanier medium and Pochon and Tardieux medium, respectively [7].

The cultures were incubated at 30°C for 1 month. The presence of nitrite and nitrate was demonstrated with Griess-Llosvay reactive and with Lunge reactive on acidified cultures, respectively. The presence of sulphate was confirmed with $BaCl_2$ on acidified cultures. Chu medium and Detmer medium were employed for the detection of *Cyanophyta* and *Chlorophyta*, respectively [7]. The growth was observed after about 1 month of incubation at 25–30°C under light. Photosynthetic microorganisms were observed using a phase-contrast microscope and were characterized by morphology analysis [8, 9].

ATP determination

The quantitative determination of ATP was carried out on a small amount of powdered marble following the Lumac method [10–12].

Luminescence was given in digital relative luminescence units (RLU) and subsequently converted into ATP contents according to the calibration curve obtained from pure standards. The luminometer used was a Biocounter P 1500 (Lumac) equipped with a set photomultiplicator tube (PMT).

The reliability of this method was confirmed by the ATP determination carried out on sterile marbles to which known quantities of ATP were added.

Two series of tests were carried out. The first, on some samples of deteriorated stone, was to confirm the presence of microorganisms, and the second, on the culture prepared and incubated under the same conditions as those for the calorimetric experiment, was to compare the trend of the calorimetric signal with the ATP concentration curve.

Calorimetric investigations

A Setaram C 80 differential flux calorimeter (DFC) equipped with two cells (10 ml capacity) was used. Each cell was separated into two sections by an aluminium membrane (0.015 mm thick). The upper section was in contact with the exterior through a capillary (0.8 mm inner diameter) containing a mobile shaft with which to pierce the membrane allowing the contents of the two cell sections to mix.

A reference suspension was prepared by adding 400 mg of sterilized marble to 2 ml of pH 7 phosphate buffer (0.1 M); the preparation of the unknown sample suspensions was identical, except that the marble powder was not sterilized. These suspensions were placed in the lower sections of the two calorimetric cells, while the upper sections contained 2 ml of double-concentrated nutrient broth.

Every part of the apparatus in contact with the cell contents had previously been sterilized in an autoclave and every operation was carried out under sterile conditions. Because the two cells were not hermetically seated, the experiment can be considered as aerobic.

The temperature was fixed at 30°C for 48 hours. When thermal equilibrium was reached (after about 90 min) the membranes were pierced. The calorimetric signal was recorded as an ASCII file and elaborated as a Quattro Pro worksheet according to the criteria reported previously [13].

RESULTS AND DISCUSSION

Scanning electronic microscope observations

SEM investigations carried out directly on some degraded marble samples showed a diffuse presence of cocci (Fig. 1). The microorganisms

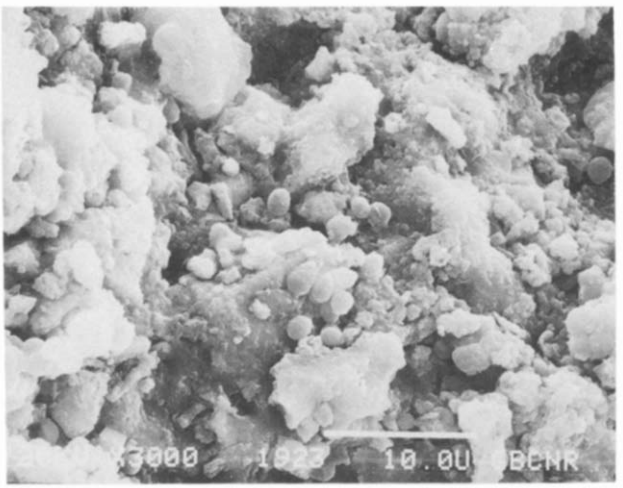

Fig. 1. Cocci on degraded marble (SEM).

are most apparent in the microfissures of the stone, in agreement with the observations of other authors [14].

Schizomycetes

The aerobic heterotrophic bacteria counts are reported in Table 2: the mean value of the counts was 10^5 CFU per g of marble. Some strains were characterized: one of these (coccus, asporigene, with endocellular pigment,

TABLE 2

Schizomycetes and *Eumycetes* counts, the presence of algae, and ATP content

Sample	*Schizomycetes* Calc. from plate count in CFU per g	*Eumycetes* Calc. from plate count in CFU per g	Algae	ATP/pg g ^{1}	*Schizomycetes* Calc. from ATP content in CFU per g
1	5.55×10^4	9.41×10^2	+	8500	8.5×10^6
2	3.38×10^6	2.35×10^2	+	4100	4.1×10^6
3	3.65×10^6	–	–	11000	1.1×10^7
4	8.50×10^3	–	+	ND	ND
5	1.31×10^4	–	+	540	5.4×10^5
6	7.03×10^4	–	+	420	4.2×10^5
7	1.45×10^5	–	+	300	3.1×10^5
8	ND	–	–	ND	ND
9	4.62×10^5	–	–	ND	ND
10	6.90×10^5	–	–	360	3.6×10^5
11	4.01×10^4	–	–	110	1.1×10^5

ND, not determined because of the small quantity of the sample.

Gram+, non-motile and catalase+) was attributed to the *Micrococcus* genus; another strain (rod-shaped, sporigene, Gram+ and catalase+) was attributed to the *Bacillus* genus.

No nitrifying or sulphooxidizing bacteria were found in the samples.

Eumycetes

The fungi counts are reported in Table 2: the mean value of the counts is 10^2 CFU per g of marble.

The fungi strains were isolated and observed with a stereomicroscope and a phase-contrast microscope. The morphology analyses characterized the fungal strains as belonging to the *Penicillium* and *Aspergillus* genera.

Photosynthetic microorganisms

Microorganisms grown in cultural media inoculated with powdered marble samples and observed by phase-contrast microscope were characterized. Some were attributed to the div. *Chlorophyta* and others to the div. *Cyanophyta.*

Calorimetric investigations

The aim of these experiments was to measure the thermal effect of mixing nutrient broth with microbial suspension. The calorimetric signal, $s = \mathrm{d}Q/\mathrm{d}t$, is directly proportional to the speed of the process taking place in the measurement cell [13]

$$S = Q_\infty V = Q_\infty k(1 - \alpha)^n$$

where V is the process rate, k the kinetic constant, n the reaction order, α the advancing degree and Q_∞ the reaction heat, i.e. $Q_\infty = m\Delta_r H$, where m is the total mass of the sample and $\Delta_r H$ the reaction enthalpy. This expression holds for a single event and cannot be directly extended to cases of multi-step processes.

When this event takes place, the signal shows a particular trend [13] which is proportional to the quantity of heat released (or adsorbed) and can, therefore, be referred to as a reliable index of the chemical reactions in the system.

The trace (Fig. 2c) corresponds to an exothermic process that can be qualitatively subdivided in two phases. In the first, the signal reaches a plateau, as expected in a zero-order reaction [13]. The second is characterized by an exothermic peak that starts from the plateau and, thus,

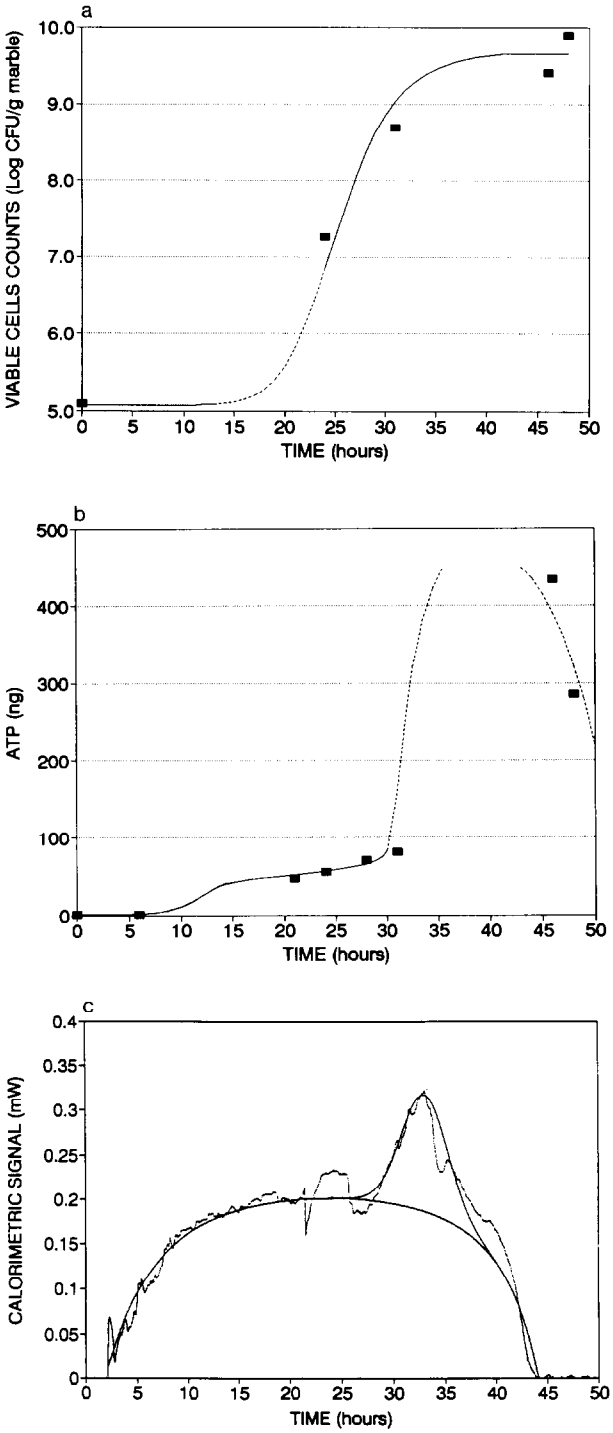

Fig. 2. The monitoring of the metabolic activity: a, viable cells counts; b, ATP content; c, calorimetric signal.

corresponds to a new step in the process or to a change in reaction order.

Because the global process is characterized by an increase in the microbial population, the calorimetric signal corresponds to the thermal effect of the relevant biochemical reactions. A global index of this biochemical activity can be identified by the increase in ATP concentration.

ATP content

The results of the ATP determinations carried out on marble samples are reported in Table 2; the mean value is 3170 pg ATP per g marble. The bacterial cells have around 1 fg ATP (1 fg = 10^{-15} g) per cell [15]. From the ATP values obtained by sample analysis, the corresponding number of *Schizomycetes* was calculated: the results were generally higher than those obtained by cultural analysis.

The differences between the amounts of ATP determined in degraded marble and those derived from bacterial counts were attributed to the presence of eucharistic cells, fungi and *Chlorophyta,* which have ATP contents greater than those of procharistic cells [15]. ATP determinations, together with viable cells counts, were also carried out on parallel cultures prepared and inoculated under the same experimental conditions as those for the calorimetric investigations. Plate counts and ATP contents were determined at successive times (0, 6, 21, 24, 28, 31, 46 and 48 h). The results are reported in Figs. 2a and 2b.

A confirmation of the reliability of ATP as a metabolic bioindicator is possible by comparing the trend of the calorimetric signal with that of the ATP concentration (Fig. 2b). The ATP levels are also characterized by two phases which are chronologically superimposable both on the calorimetric signal and (fairly well) on that of the viable cells counts (Fig. 2a); the small differences among these three curves can be attributed to the three different cultures used for the experiments.

The DSC trace and ATP concentration curve indicate an abrupt arrest of the biochemical activity after 40–45 h. This event which can be explained either by the decrease in the residual substratum or by the accumulation of catabolites that are toxic to the microflora, has also been observed by other authors [16, 17]. The initial lag period of the calorimetric curve ($t < 2.5$ h) can be attributed either to the relatively small number of cells present in the inoculated samples or to the low viability of the cells that live on the stone under unfavourable conditions.

Over longer periods of time, the signal becomes sensitive and indicates that metabolic activity is taking place (with heat release), even when these variations cannot be detected in the ATP determination. The attainment of a plateau was also observed by other authors, either for bacterial cultures [16, 17] or for *Saccharomyces* cultures [18]. In other studies, the curve has also shown two or more peaks, even for pure cultures [16–18].

CONCLUSIONS

This study demonstrates that calorimetry is suitable for the investigation of the microbial growth that takes place under given conditions, because the DSC trace follows the progress of the relevant metabolic activity.

Improvements to the special calorimetric cells used in these experiments are in progress; these will allow investigations under modified atmospheric conditions.

This calorimetric analysis has confirmed the presence of microorganisms on the stone. The plateau phase could correspond to a state of slow microbial activity, which, under favourable conditions, can initiate the process of their multiplication again with accelerated intensity and a consequent deterioration of the marble.

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