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Correlation tests between relative light unit and colony forming unit for improving adenosine triphosphate bioluminescence analysis in bacterial consolidation treatments on palaeontological heritage

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Abstract

In this article bacterial carbonate mineralization treatments are proposed as a novel strategy for decayed fossils and palaeontological heritage conservation; specifically, by means of inoculation of *Myxococcus xanthus*, a bacterium of proven effectiveness in ornamental stone bioconsolidation.

Bioconsolidation treatments can be very effective, stable, nontoxic, environmentally friendly, and chemically compatible with fossil heritage. The method reproduces what nature has been doing for millennia with fossils that have been permineralized by bacterial calcium carbonate precipitation.

There is, however, some concern that bacterial inoculation could lead to the growth of undesirable microbiota, which could subsequently damage the fossil substrate. Because of this, the use of bacteria on heritage items must be meticulously monitored and analysis strategies should be carried out to detect bacteria viability during and after treatments. For this purpose, adenosine triphosphate assay is proposed in this article as a fast, affordable, portable, and easy-to-use system for conservators. as ATP assay results are relative and difficult to relate to colony forming unit, this study aims to improve their applicability by examining the correlation between ATP analysis and total viable bacteria count in the specific case of *M. xanthus*. This research provides reference and correlatable data to obtain an approximate estimation of *M. xanthus* viable bacterial colonies based on relative light unit data.

KEYWORDS

adenosine triphosphate, bioconsolidation, bioluminescence, correlation tests, palaeontological conservation

1 | INTRODUCTION

1.1 | Palaeontological consolidation: traditional treatments vs. emerging alternatives

Consolidation, in heritage conservation, is the process for restoring surface cohesion of remains to prevent further loss of matrix and to recover mechanical properties. Traditionally, palaeontological consolidation has been carried out by means of hardening products such as adhesives, resins, and waxes. This procedure, documented since the 19th century,[1–3] was developed in parallel with consolidation of monumental stone[4–6] and archaeological assets.[7,8]

Currently, palaeontological consolidation consists in general of impregnation with polymers, mainly acrylic resins.[9–14] These impregnations, common in the so-called palaeontological preparation, were intended to preserve fossil morphology and to improve the hardness to facilitate fossil manipulation for histomorphometric, histopathological, taxonomic, and taphonomic research.[10,15] Therefore, palaeontological consolidation treatments are usually conditioned to the palaeontologists' research needs. In this regard, the international criteria for heritage conservation (UNESCO, ICOM, ICOMOS) are not totally followed.

Resins are usually applied systematically because this is considered an essential process for fossils to acquire a certain consistency,[16] from an initial preconsolidation in the field, with acrylic, nitrocellulose, or vinyl resins accompanied by hydrophilic gauzes to help in the extraction of the fossils and avoid their fragmentation,[10,17–19] up to the progressive acrylic resin application during fossil cleaning in laboratory.[10,11,20] Although polymers applied on porous materials achieve an effective hardening for immediate needs, they also generate changes regarding colour and gloss, can interfere with future analysis,[10,21,22] and can chemically alter the material, increasing the weight and forming surface films with different shrinkage-dilation coefficients. This can cause disruption and delamination, clogging pores,[23] and changing the water–gas behaviour.[24–27]

When polymers age they are submitted to cross-linking reactions and molecular changes which cause solubility loss and which in turn makes the application irreversible.[28,29] Therefore, resin impregnations are irreversible, incompatible, and generally toxic treatments.[26,27,30,31] Furthermore, polymer penetrability is very limited,[26,32] typically from 1 to 3 mm,[25] therefore, its consolidating effect is also limited.

In general, characteristics that conservators usually consider when choosing a consolidant are particle size, viscosity, penetrability, hardness, glass transition temperature, toxicity, and the solvent needed.[6,15] But the decision to consolidate bones and fossils should be based on several factors such as: fossil needs, understanding the properties of the consolidant, and the chemical and physical reactions between bone and consolidant. Also effects of the storage environment on the success of the treatment, the long-term effects of consolidation on remains, the physical stability and the possible future research interests,[10] as well as compatibility and retractability,[23,24] must be considered.

Current conservation trends and the emergence of conservators in institutions that manage palaeontological heritage increasingly rely on the above-mentioned principles.[16,22,33] Furthermore, bearing in mind that fossil bones are mostly composed of a mineral fraction, 60–70 wt% mainly carbonate–hydroxyapatite ($Ca_5(PO_4)_{3-x}(CO_3)_xOH_{x+1}$),[34] inorganic consolidations would be preferable to the application of resins, as they have a better physicochemical affinity with mineral substrates.[35]

When focusing on inorganic products, hydroxyapatite-induced consolidation (HAP) is a type of inorganic consolidation found to be highly compatible with bones. This system has been recently explored in some types of archaeological bone,[26,36,37] after being applied for years in biomedicine[38–41] and in other types of carbonate heritage surfaces.[42–44]

Conversely, some palaeontological consolidation with ethyl silicate, an alkoxysilane compound, has appeared in the published literature.[45,46] Ethyl silicates make colloidal silica that is deposited inside porous structures and, as silica molecules are chemically alike to silicate minerals,[30] they can show good compatibility with fossils that have a silicate-based composition. In contrast, ethyl silicate does not show any affinity with carbonate substrates,[25,30] therefore, it would not be applicable to carbonate fossils, which are most common.[47] In fact, some authors have shown that calcite delays and even inhibits the polymerization of the product.[48–50]

During the time bones are buried, empty spaces are generated when organic matter, such as collagen (20–30 wt%), blood vessels, and cells, decomposes.[45] Inorganic matter such as hydroxyapatite can also leach out and leave spaces or transform, demineralizing the bone.[26] These spaces have been filled over millions of years by precipitated minerals from underground water and the surrounding sediments,[45,51] and by the mineral neoformation from bacterial activity, in the context of a propitious combination of sedimentological, microbial, and geochemical conditions.[52–57] This phenomenon is called fossilization or fossil diagenesis by permineralization and pseudomorphosis, and it depends on the hydrology and the type of burial environment.[51,58] It also depends on relationships established between bones and their environment: collagen loss, microbial action, ion exchange, mineral leaching, and infiltration by organic and mineral compounds found in the soil.[26]

In this research, and in most fossils, bones have been mainly permineralized by calcium carbonate.[47] At the same time, during burial, bones continue to undergo demineralization processes that affect their mechanical properties, therefore, a good way to strengthen bone remains could be the in situ growth of $CaCO_3$ crystals.[59] This system partially imitates the natural fossilization process; moreover, consolidations based on th induction of calcium carbonate have a good physicochemical affinity with carbonate substrates and would therefore be highly compatible.[60] One way to induce calcium carbonate would be through the application of $Ca(OH)_2$ nanoparticles that react with Ambiental CO_2, generating calcium carbonate crystals in the form of aragonite and calcite.[59] Over the last 2 decades nanolimes have been

studied as conservation treatments for the consolidation of wall paintings, limestone, lime mortars, and plasters[60–64] and in recent years in archaeological and palaeontological bone.[59,65]

Another way to induce calcium carbonate in carbonate substrates is by means of bacterial carbonatogenesis. In the 1970s, the research group of Boquet, Boronat and Ramos-Cormenzana at the University of Barcelona demonstrated that most heterotrophic soil bacteria were capable of precipitating calcium carbonate.[66] But it was not until the 1990s when the team, formed by French researchers Adolphe, Castanier, Loubière, and Le Métayer-Levrel, considered the use of microbial-induced calcium carbonate precipitation (MICP) for ornamental stone protection.[67] Since then, this type of bioconsolidation has been tested in applications on monumental stone,[35,68–72] plasters,[25,73] and wall paintings.[74] According to these studies, bioconsolidation promotes greater resistance without plugging the pores, keeping its transpiration to enable gas exchange. Furthermore, it does not alter the colour or the aesthetic stone appearance.

This method replicates what nature has been doing for millennia with carbonate rocks created by bacterial calcium carbonate precipitation.[75,76] In addition, it also imitates some permineralization that occurs during the fossilization process, caused by autogenous microbial activity.[53] It should be noted that the most common autogenous minerals in fossilization are calcium phosphate and calcium carbonate, both of which are very present in bones and burial environment composition.[51,77] In fact, the bulk of fossil records consists of biomineralized remains[53] and, although initial taphonomic changes are induced by bacteria and mineralogy inherent to fossils, most significant diagenetic changes are mainly due to the action of soil microbiota.[58]

All these aspects indicate bacterial carbonate precipitation as a compatible and suitable method for the consolidation of carbonate palaeontological material. The experiments carried out using this methodology have been performed with different bacterial strains and carbonation medium. The basic operation consists of inoculation of a bacterial solution accompanied by a calcium and carbonate-rich medium[35,69–71,78] or the direct activation of the substrates' microbiota by applying a calcium and carbon-rich source medium.[25,72,79–81]

Inoculated bacterial cell walls contain carboxyl and hydroxyl groups that deprotonate in an alkaline environment, generating negative charges. These charges have a strong electrostatic affinity to metal cations in aqueous medium. Because large quantities of calcium ions are not required (Ca^{2+}) in the metabolic bacteria processes, the excess accumulates and saturates outside the cell, which, due to electrostatic attraction forces, remain adhered to the bacterial cell membrane. Once fixed to cell walls, calcium ions bind to carbon also present in medium, and results in the formation of calcium carbonate around the cell.[35] This is an evolutionary mechanism in which organisms can control cellular ionic balance and therefore build support and defence structures both inside and outside the cell.[24] In a normal equilibrium reaction, calcium carbonate in solution precipitation occurs as follows, once a certain degree of saturation has been reached[35,82]: $Ca^{2+} + CO_3^{2-} \leftrightarrow CaCO_3$. In fact, metabolic bacterial processes increase pH through the release of ammonia and other metabolites.[58] In this way, there is a high supersaturation of calcium ions that favours carbonaceous precipitation.[35]

For all these reasons, bacterial carbonate mineralization treatments are here proposed as a novel strategy for the conservation of fossils and palaeontological heritage.

Moreover, this is a nontoxic and environmentally friendly methodology.[83] Certainly, the inoculated bacteria must be safe and nonpathogenic to not constitute a risk to the heritage items or to the restorer.[84] But even with the many advantages of this methodology, use of living bacteria in cultural heritage needs to be thoroughly controlled. Some researchers pay attention to the possible drawbacks, because bacterial inoculation could cause the growth of undesirable microbiota that could subsequently damage treated material.[85] In fact, if biofilm is formed, it can be positive in terms of surface cohesion, but it can also become an undesirable source of organic matter.[86] Therefore, the risks can be minimized by carrying out good rinsing and drying after treatment, followed by monitoring possible microorganism proliferation. In addition, bioconsolidation treatments are required to detect bacteria viability also during treatment in order to obtain good consolidation results. To accomplish this, improved adenosine triphosphate (ATP) assay becomes necessary and essential for bioconsolidation works.

1.2 | ATP bioluminescence analysis in bacterial conservation treatments on cultural heritage

To monitor the viability of bacteria for conservation treatments, classical microbiological methods by plating in culture medium and API biochemical tests have commonly been used.[87,88] Molecular methods such as polymerase chain reaction (PCR)[89,90] or fluorescence in situ hybridization (FISH)[91] have also usually been applied.

For this purpose, the literature also provides rapid, portable, and cost-effective methods based on adenosine triphosphate (ATP) bioluminescence analysis.[88,92–99] This is a semiquantitative technique used since the mid-20th century in hygiene and environmental applications.[100]

ATP is an energy molecule that exists in organisms that can be used as a bioindicator of bacterial viability.[98] Bioluminescence can be used to quantify ATP,[101] being a technology capable of detecting ATP from viable bacteria via the luciferin–luciferase enzyme complex present in fireflies. The enzyme catalyzes the oxidation of ATP to adenylyl-luciferin and produces light when it is combined with ATP and oxygen.[102] The amount of light is proportional to the amount of the existing ATP in the analyzed sample, and it can be quantified by a luminometer.[103,104] Luminometer results are expressed in relative light unit (RLU) that can be indirectly related to the colony forming unit (CFU) of microorganisms.[105]

Unlike other classical and molecular analyses, ATP assay is a portable biochemical technique that provides immediate and useful results for monitoring biorestoration practices.[87] The fast response is an important improvement of this technique because biorestoration

treatments often need to be carried out in situ, or immediate results, that cannot wait for hours or days of incubation, are required.

In bioconsolidation treatments ATP is applied to optimize bacterial consolidation treatment on palaeontological remains and as a post-treatment control technique for microbial growth.

Bacteria consolidation treatment is optimized by checking the viability prior to treatment, i.e. by obtaining quantifiable data in RLU just before application. In addition, for consolidation optimization, ATP makes it possible to verify that bacteria remain active in appropriate quantities, and it is effective during treatment. The luminometer also helps to check that fossils were properly sterilized before treatment, preventing interference when an exogenous bacterium is applied.

As a post-treatment control, the ATP assay is very useful because it allows direct rinsing verification, enabling repetition if necessary (Figure 1). It also enables quick and easy regular controls. If monitoring is done in a traditional or molecular way, it would take several hours or days to obtain results, being incompatible with the required phases of a bioconsolidation treatment.

Some studies on post-treatment controls have confirmed that no undesirable bacterial residues have remained on heritage surfaces and, in these studies, researchers compared bioluminescence results with the total viable bacteria count plates, obtaining consistent and comparable results.[88,101,106,107]

It must be considered that results obtained can only be indirectly related to the CFU.[91] RLU is not equivalent to CFU, but they are proportional[103] and the amount of ATP remained constant with bacterial growth rate[108] in the same environment.[109] However, not all microorganisms contain similar amounts of ATP[109,110] and it is important to verify the results with bacterial count plates or establish correlation for each bacterium.

Therefore, correlation tests can be used as a reference for in situ biorestoration, without a supporting laboratory to plating controls. In this regard, the current research sets out a correlation between RLU and CFU on 20 serial dilutions (10^{-1} to 10^{-20}) of *Myxococcus xanthus*, a bacterium with adequate capacity to generate calcium carbonate when it is applied on monumental limestone for bioconsolidation.[35,78,111–114] *M. xanthus* was selected in this study for carbonate fossils bioconsolidation by using exogenous bacteria.

2 | EXPERIMENTAL

2.1 | Bacterial strain, culture medium, and serial dilutions

The microorganism used in this research was *M. xanthus* from strain 422 of the Spanish Type Culture Collection (CECT). Tryptic Soy Broth (TSB) medium was used (TSB: tryptone, soitone – soya bean peptic digest –, dextrose, NaCl, and K_2HPO_4 buffered at pH 7.3) for its growth. Liquid medium was sterilized by autoclaving at 120°C. The culture was incubated for 48 h at 28°C, the optimal growth duration for *M. xanthus* to achieve a concentration in the order of 10^8 to 10^9/ml concentration. From this initial dilution, 20 decimal serial dilutions in sterile Ringer's solution were prepared ($n = 10$), each one 10 times more dilute than the previous one (10^{-1} to 10^{-20}) at pH 7.3 to avoiding bacterial osmotic shock. Dilutions were homogenized each time using a Vortex®.

A negative control test with sterile Ringer's solution was performed.

2.2 | ATP analysis

To quantify ATP as a bioindicator of microbial activity[95,106] in the different dilutions, a luminometer Hygiena SystemSURE Plus® was used.

FIGURE 1 Example of the ATP assay using a Hygiena SystemSURE Plus® luminometer and UltraSnap® ATP devices (in this case used for bacterial viability control after rinsing and drying on a bioconsolidated miocene *Cheirogaster richardi* fossil)

This equipment indicates the viable microbial presence expressed in RLU. All ATP luminometers measure the light emitted by microorganisms during the enzymatic reaction in fmol per µl (1×10^{-15} mol/µl). Specifically, the Hygiena SystemSURE Plus® luminometer, in conjunction with AquaSnap® dipping ATP devices, was calibrated using a multiplication factor of 5; i.e. 1 fmol is equivalent to 5 RLU. According to the manufacturer[115] and independent studies,[116] the instrument sensitivity is capable of detecting 0.1 fmol. Furthermore, it has a low variability of results in a single sample (coefficient of variation [CV] 9%) and has a linear correlation coefficient (r) between 0.95–0.99 (where 1.0 represents the perfect fit) both in standard solutions and in the diversity of analyzed bacteria.

AquaSnap® ATP devices were used during this experiment (Figure 2) because they are specially formulated to analyze liquid samples. These devices have a calibrated immersion dipper that allows the collection of exactly 100 µl. To discriminate the free ATP belonging to nonviable fragmented cells, two different devices must be used[106,109] in this case AquaSnap Total® and AquaSnap Free®. AquaSnap Total® is coated with an agent that aids in the ATP extraction from microbial cells. In contrast, AquaSnap Free® has not any extraction agent and only detects soluble (free) ATP. Consequently, AquaSnap Total® devices were used to analyze total ATP in a sample; i.e. both intracellular ATP (contained in living cells) and extracellular ATP (released from dead and fragmented cells). Conversely, AquaSnap Free® is only able to compute free ATP, so these devices can discriminate free ATP from the total count. Discrimination of extracellular or free ATP is essential to obtaining a viable bacterial count, therefore both devices had to be used in each sample and results obtained had to be subtracted. During the experiment, 100 µl of each bacterial dilution were analyzed (10^{-1} to 10^{-20}), just after shaking them in a Vortex®. First, a sample of 100 µl was collected with an AquaSnap Total® device, then a second 100 µl sample was collected with an AquaSnap Free® device. Samples were activated by breaking the upper valve of the device, which contains the luciferin–luciferase reagent. Devices were shaken for 5 s and immediately inserted into the luminometer to be analyzed. Results are expressed in RLU. Three ATP assays per sample were carried out, both with AquaSnap Total® and AquaSnap Free®. Viable bacteria RLU data were obtained and processed with a spreadsheet to determine averages, standard deviation, free ATP discrimination, correlation coefficient (r), and determination coefficient (r^2).

FIGURE 2 Here, a 100 µl collection of one serial *Myxococcus xanthus* dilution using the Hygiena SystemSURE Plus® luminometer and AquaSnap Total® ATP devices

2.3 | Total viable bacteria count

In order to establish the correlation between RLU and CFU, 100 µl of each bacterial dilution was collected (10^{-1} to 10^{-20}), just after Vortex® agitation. Subsequently, samples were analyzed by plating the serial 10-fold dilutions onto Tryptic Soy Agar plates and incubated for 48 h at 28°C. Three Tryptic Soy Agar plates per sample dilution were performed. Then, a manual CFU count that had developed on plates was carried out. CFU results were compared with luminometer results in order to determine correlations between the quantification systems. Data were processed statistically with a spreadsheet to obtain averages, standard deviations, correlation coefficient (r), and determination coefficient (r^2).

To discard fungal contamination, one Saboraud Dextrose Agar plate per sample was performed.

2.4 | Statistical analysis

To determine RLU and CFU correlations, RLU and CFU data were analyzed to obtain averages, standard deviation, free ATP discrimination, correlation coefficient (r), and determination coefficient (r^2). Once luminometer results had been collected, all data were entered into a spreadsheet. All repetition data were treated with arithmetic averages and standard deviations. The standard deviation allowed us to know the most common measure of dispersion, indicating the dispersion of the data with respect to the mean. Then, AquaSnap Free® results were subtracted from AquaSnap Total® results, to obtain the viable bacterial ATP-RLU data for each sample and each repetition.

After manual total viable counts, CFU data were also entered into a spreadsheet to calculate means and standard deviations. With final mean data in both ATP analysis and total viable bacteria count a comparison between RLU and CFU was performed. Therefore, correlation coefficient (r) and determination coefficient (r^2) were calculated. Correlation coefficient (r) quantifies the strength of a linear relationship between two variables and determination coefficient (r^2) quantifies the fit of the model to the variable that is trying to be explained. Both

data are very important to assess the correlation between the two test methods.

For data presentation, a table with final mean data and a two-axis graph showing the correlation curves between the two test methods were used.

3 | RESULTS

Data obtained were compiled to examine the correlation between ATP analysis and total viable bacteria count (Figure 3). Results of the replicates were analyzed by averages ± standard deviation, both in total ATP and free ATP measurements. Standard deviation allowed us to know the most common measure of dispersion, pointing out that the measurements only differed by an average of 7.2% in ATP analysis repetitions. This was important in order to verify that the measurement method was reliable in the different repetitions of the ATP analysis.

From the means of the two analyses, average results of viable ATP for each serial dilution were calculated. In statistical values, the difference between total ATP and free ATP is quite linear and correlated, with correlation coefficient results of $r = 0.80$ and determination coefficient of $r^2 = 0.65$. Except for a few cases in the medium values (8–51 RLU), most of the samples had more than 70% intact

Dilution	Total mean ATP (RLU)	Standard deviation	Free mean ATP (RLU)	Standard deviation	Viable mean ATP (RLU)	Total viable bacteria mean count (UFC/ml)
Initial	7879	90	1354	53	6525	>10640
10^{-1}	5947	72	37	4	5911	>10640
10^{-2}	2630	590	308	95	2322	>10640
10^{-3}	212	24	58	11	154	>10640
10^{-4}	51	9	32	4	20	>10640
10^{-5}	51	15	31	8	20	10640
10^{-6}	12	1	9	1	3	4300
10^{-7}	16	3	13	1	2	3020
10^{-8}	14	4	11	6	3	2760
10^{-9}	8	2	6	1	2	1960
10^{-10}	11	5	10	1	1	880
10^{-11}	8	5	6	1	2	1560
10^{-12}	1	1	0	0	1	1010
10^{-13}	1	0	0	0	1	1060
10^{-14}	1	1	0	0	1	1030
10^{-15}	1	0	0	0	1	1240
10^{-16}	0	0	0	0	0	810
10^{-17}	0	0	0	0	0	800
10^{-18}	0	0	0	0	0	260
10^{-19}	0	0	0	0	0	360
10^{-20}	0	0	0	0	0	100
						R=0,968 R^2=0,937

FIGURE 3 Average RLU data for each dilution. Correlation (r) and determination coefficient (r^2) between ATP assay and total viable bacteria mean count in *Myxococcus xanthus* serial dilutions

cells. In samples with higher and lower total ATP concentrations, the percentages ranged from 72% to 100%, with a total average of 62.14%.

For the manual CFU count, three plates of each dilution were counted and statistically treated. The three replicates were analyzed to obtain averages and standard deviation. Standard deviation indicated that the different plating repetitions only differed by an average of 7.8%. These data were in accordance with luminometer results standard deviation, so CFU results can be considered consistent and reliable.

It can be seen that viable ATP mean concentrations ranged from as low as 0 RLU, 0.0 fmol ATP/μl (1×10^{-15} mol/μl) to 6525 RLU, 1305 fmol ATP/μl (1×10^{-15} mol/μl). Once compared with total viable bacteria count, it could be observed that the statistical results were strongly correlated with the linearity results of the correlation coefficient $r = 0.968$ and determination coefficient $r^2 = 0.937$ between the two test methods (Figure 4).

Negative control test with sterile Ringer's solution did not develop viability in any of the analyses performed. Fungal contamination test also did not develop any viability.

4 | DISCUSSION

The main objective of tests carried out in this study has been to examine the correlation between ATP analysis and total viable bacteria count to improve the luminometer's applicability in bioconsolidation treatments, both in laboratory and in situ. From biorestoration needs, the luminometer has been postulated as a quick and portable piece of equipment,[106,107] so it could be useful for improving bioconsolidation processes and in order to achieve fast feedback on-site monitoring. Nevertheless, results obtained were relative and difficult to relate to the CFU, so it was necessary to check the results each time against the bacteria count plates, sacrificing the process immediacy. In this context, the current study has provided correlatable and reference data that can help to obtain an approximate estimation of bacterial colony count for *M. xanthus*.

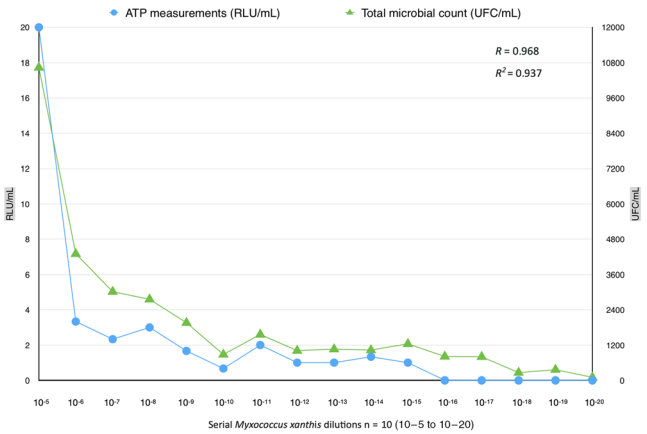

FIGURE 4 Correlation curves between ATP monitoring system and total viable bacteria count in *Myxococcus xanthus* serial dilutions

Checking the viability of bacteria just before treatment and during the 7–10 days that it normally lasts can be a key part of the intervention. Applied bacteria must be viable in high enough concentrations, so obtaining immediate RLU data (in a few seconds) can determine the success of the process. This is presently possible by combining devices that discriminate free ATP and give immediate results in RLU that can be correlated with total viable bacterial count. Results obtained gave a strong correlation $r = 0.968$ and $r^2 = 0.937$ that will now allow the estimation of concentrations in number of colonies if bioconsolidations by *M. xanthus* is used. It was observed that, in a range of three- and four-digit RLU data, bacterial concentrations were very high in *M. xanthus*, i.e. these were concentrations between 1.06×10^9 cells/ml and 4.3×10^9 cells/ml that can be applicable to bioconsolidation treatments.

Checking a correct sterilization of fossils before treatment is very important because it is necessary to avoid unwanted interactions. It is also important to confirm the absence of viable bacteria on fossil surface once the treatment has been completed and during subsequent periodic monitoring. Consequently, it has been verified that when luminometer measurements indicate 1 RLU (0.1 fmol ATP/μl, 1×10^{-15} mol/μl) the approximate range is between 880 and 1240 CFU/ml and when results are 0 RLU (instrument limit) it can be counted from 100 to 810 CFU/ml. These data apply to liquid samples collected with AquaSnap® devices, so it will be necessary to carry out relevant correlations with surface devices in a future study. Moreover, not all devices had the same sensitivity or the same relationship between fmol and RLU. For example, in AquaSnap® devices, 1 fmol of ATP equals 5 RLU and 0.1 fmol ATP/μl equals 1 RLU.[115] Its sensitivity is 0.1 fmol. Among surface devices, UltraSnap® have an equivalence of 1 fmol = 1 RLU and a sensitivity of 1 fmol., whereas SuperSnap® devices have an equivalence of 1 fmol = 5–6 RLU[115,116] and a sensitivity of 0.1 fmol. Therefore, for surface measurements SuperSnap® devices could be used that are equivalent in sensitivity and fmol/RLU ratio to AquaSnap®. Until surface ATP-CFU correlation tests have been carried out, it will be possible to measure fossil ATP with SuperSnap® and use data from this study as a reference while confirming some of the results by plating.

Even so, there are still some questions to be answered that have already been suggested by Shama and Malik[109]: Does the humidity degree affect ATP supply in bacteria? Because then results would not be comparable with analyzed bacterial dilutions with residues of possible bacteria on a surface that has already been bioconsolidated. Furthermore, it has been demonstrated that bacteria isolated from natural environments tend to contain less ATP per cell than those cultivated in laboratories.[109,110] Therefore, all these factors must be taken into account for future studies.

We were able to verify that bacteria viability was very high and correlated in relation to the total RLU, especially in the more concentrated dilutions and in the less concentrated ones. It must be noted that this viability correlation is important because, although devices that allow free ATP discrimination exist for liquid samples, they do not

yet exist for surface samples, at least in portable systems. Consequently, it will be necessary to consider the approximate rates during viability tests on the following days of treatment, in which fossil surface is measured as well as the application dilution. These data should also be taken into account during fossil surface ATP assays after sterilization, after final rinsing and during periodic post-treatment controls. As total ATP:viability ratio may vary in each stock dilution, it may be of interest to establish some correlations on liquid samples with AquaSnap Total® and AquaSnap Free® devices whenever a treatment is initiated.

5 | CONCLUSION

Results indicated that correlation tests between RLU and CFU are a very useful procedure to improve ATP bioluminescence analysis, in particular when immediate results are needed in conservation–restoration treatments or when working in situ.

This is even more important when treatments are applied by a conservator–restorer not trained in microbiology or when there is not immediate access to a supporting laboratory. In addition, it is a cost-effective analysis.

Each bacterium has different quantities of ATP that are proportional to its CFU, as has been proven in several studies in which ATP-CFU correlation tests have been established, usually in industrial and food applications.[117–120] ATP measurements are more correlatable in controlled cultures of the same bacteria and for this reason, ATP assay is not recommended for a general microbial population study of a biological attack on heritage items surfaces. ATP analysis can give approximate and relative data related to the severity of the attack, but it cannot be correlated with CFU in those cases. ATP assay can also be useful as a routine monitoring tool for disinfection in heritage collections or to ensure that cleaning and bacteriological control protocols are carried out satisfactorily. In this case, we go further: thanks to correlations, it is possible to obtain approximate CFU data with immediate results of ATP assay. It should be noted that some of the steps in bioconsolidation process could not be carried out in the time required if the viability analyses were performed in the traditional way by total plate count. Therefore, the main ATP assay disadvantage, that results obtained can only be indirectly related to CFU, becomes minimized. However, it is necessary to carry out many more assay repetitions in order to establish even more accurate correlations. In addition, the range of bacteria studied could also be extended to include other bacteria that can also be used in the field of bioconsolidation.

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COMPETING INTEREST STATEMENT

There are no conflicts to declare.

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