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Characterisation of Primary and Secondary Stone Bioreceptivity

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Summary

Stone bioreceptivity is its ability to support the colonisation and growth of microorganisms. Primary bioreceptivity is the initial potential for biological colonisation of a sound or freshly cut stone, while secondary bioreceptivity is the potential for biological colonisation of stone weathered by environmental factors and/or colonisers. This research report presents the results from the pilot phase of a research project to address knowledge gaps in stone bioreceptivity.

The pilot phase developed a protocol for determining primary and secondary stone bioreceptivity. It devised a repeatable protocol to measure the characteristics that influence bioreceptivity on a range of different stone types, enabling comparison of colonisation levels. Results from these tests were used to calculate a bioreceptivity index value. Bioreceptivity indices can be used to compare greening rates of different types of stone. They will help specifiers select replacement stones that share similar bioreceptivity properties to pre-existing stones.

The laboratory-based model for measuring bioreceptivity will be used to test biocide alternatives used for treating English limestone stonework in the main phase of the research.

Contributors

This report was prepared by Dr Philip Skipper and Dr Lynda Skipper, University of Lincoln, and Francesca Gherardi and Clara Willett, Historic England.

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Images

Front cover image: Microbial growth on sandstone buttress. © Historic England, 2024.

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Introduction

Stone bioreceptivity is a measure of the physical and chemical characteristics of a material that define its ability to be colonised and maintain a permanent ecosystem of microorganisms, as opposed to a transient presence (Sanmartín *et al* 2021). The various microorganisms - including algae, bacteria and fungi - which colonise the stone surface are collectively known as the microbiome.

There are four types of stone bioreceptivity (Sanmartín *et al* 2021):

- Primary bioreceptivity: the initial potential for biological colonisation of a sound or freshly cut stone after manipulation (extraction from the quarry and cut) for a final function.

- Secondary bioreceptivity: the potential for biological colonisation of stone weathered by environmental factors and/or colonisers. This weathering can be artificially induced through thermal shock and other techniques.

- Tertiary bioreceptivity: the potential of stone to support new growth after conservation treatments that do not leave a surface deposit (for example, laser cleaning, cleaning with deionised water).

- Quaternary bioreceptivity: the potential of stone to support new growth after conservation treatments that leave a surface deposit (for example, biocides, paints).

Information about bioreceptivity of building stone provides specifiers with an understanding of how new stone might respond within a certain environment: that is, whether particular characteristics will encourage biological growth. Within the heritage sector, it enables professionals to select stone for repair that matches the physical characteristics of the original stone and will support biological growth in the same way, allowing the repair to blend in aesthetically over time. Understanding bioreceptivity allows contractors to determine whether the biological growth they are finding on a stone surface is expected, and thus determine whether regrowth after cleaning is also normal. It also offers a basis for specifiers, building owners and custodians to objectively evaluate the effectiveness of biocide alternatives when within the heritage sector there is a particular need to justify new products to statutory and funding bodies and stakeholders.

There is currently no consistent method for measuring bioreceptivity, nor – with the exception of granites (Vázquez-Nion *et al* 2018) – an index for comparing stone types. Prior to this study, no English building stones had been fully characterised

for bioreceptivity, and only primary bioreceptivity measurements are available for stones worldwide (Sanmartín *et al* 2021). The influence of conservation treatments on biopatination and biodeterioration of these stones also needs further study.

The pilot phase of this project aimed to develop a protocol for a standardised laboratory-based bioreceptivity test that could later be adapted for the initial testing of environmentally acceptable biocide alternatives. This included a literature review of previous studies to identify similar bioreceptivity test protocols. Furthermore, this phase involved characterising a selection of English building stones for bioreceptivity (using our protocol) and examining the physical and petrographic properties of these stones to confirm that our protocol aligned with the observed bioreceptivity correlation noted in previous studies. Using a standard protocol will provide a way to compare the effectiveness of different conservation treatments.

This report outlines:

- A review of literature of stone bioreceptivity and weathering studies
- A standardised protocol for calculating primary and secondary bioreceptivity, so that measurements can be compared
- A standardised protocol for artificially weathering stone, based on BS EN 14066:2013, so that it is possible to carry out secondary bioreceptivity testing on weathered stone surfaces
- Primary and secondary bioreceptivity values of the stones tested

1. Review of previous studies of stone bioreceptivity and artificial weathering

To determine a suitable bioreceptivity testing method, we reviewed 450 journal and conference articles on the bioreceptivity of building materials, predominantly stone. These have all been published since Guillitte introduced the concept of different types of bioreceptivity in 1995. With colleagues, Guillitte defined material characteristics to describe stone bioreceptivity that are still used today, such as determining the stone's porosity, surface roughness and mineralogical composition, as well as observing microbiological colonisation rates. The group did not, however, define how to express and quantify bioreceptivity. This has led to a number of different approaches (Sanmartín *et al* 2021).

The majority of the articles reviewed were field trials that used a range of non-comparable techniques to look at:

- Levels of microbiological growth on stone surfaces (Marques *et al* 2014; Gulotta *et al* 2018; Jang and Viles 2021)
- Characterisation of the microbiological species found on the surfaces (Vázquez-Nion *et al* 2016; Antonelli *et al* 2020; Lubelli *et al* 2021)
- Material characterisation papers that then did not continue to test how the characteristics related to bioreceptivity (Camara *et al* 2008)
- Biocide trial case studies that consider how the biocide changed the bioreceptivity (Urzì and De Leo 2007; Sasso *et al* 2016; Sanmartín *et al* 2019; Toreno *et al* 2024)

All of these publications have, undoubtedly, enhanced our understanding of bioreceptivity and provided useful information for the heritage science and conservation communities. However, their results usually cannot be compared to one another, as different measurements have been used. This highlights the need for reliable and robust laboratory-based methods to compare bioreceptivity measurements and the effects of biocides and other conservation treatments on bioreceptivity. Papers that discuss field trial methodologies only were excluded from further review at this stage, because the underlying purpose of this phase of our project is to define a standardised laboratory-based testing process.

From the 450 articles, we identified 31 primary sources that had investigated bioreceptivity in a laboratory-based environment. A further 46 journal articles on the artificial weathering of stone for conservation testing (published between 2012 and 2022) identified 11 studies that had carried out laboratory-based trials on artificial weathering of stone, using various methodologies.

1.1 Stone bioreceptivity properties

The core concept of stone bioreceptivity is to determine which properties are important for the stone's ability to colonise and support ongoing biological growth. Most studies look at the quantity of microorganisms that can grow on surfaces, but many do not measure the stone's properties. Miller *et al*'s 2012 review of bioreceptivity shows that out of 20 studies into primary bioreceptivity (carried out under laboratory conditions), only ten investigated the properties of the stone. Furthermore, the types of tests carried out were inconsistent between the studies (Table 1).

Table 1: A summary of stone property measurements used in ten studies of laboratory-based primary bioreceptivity experiments, adapted from Miller *et al* 2012.

Stone property measurement	**Number of studies**
Texture / petrography	6
Open porosity (%)	8
Surface roughness (µm)	4
Bulk density (g/cm^3)	1
Dry density (g/cm^3)	1
Grain density (g/cm^3)	1
Surface hardness	1
Water content (%)	2
Capillarity coefficient ($g.m^{-2}.s^{-0.5}$)	3
Degree of water saturation (%)	1
Permeability ($kg.m^{-1}.s^{-1}.Pa^{-1}$)	1
pH	3
Chemical composition	7

The work of Miller and colleagues (Miller *et al* 2006, 2009; Sasso *et al* 2016) shows that the main properties that relate to the bioreceptivity of stone are capillarity coefficient and surface roughness. Capillarity coefficient is the rate at which water is transported through the network of pores and microfissures that make up part of the stone matrix. Surface roughness is the small-scale variations in the height of a physical surface. It includes changes to the texture, such as the height and sharpness of peaks, as well as the balance between peaks and troughs (Butler 2008). A surface roughness of 30nm or higher is sufficient for the adherence of microorganisms (Yoda *et al* 2014). Miller *et al* show that these properties directly correlate with the levels of chlorophyll measured: that is, the amount of algae present on a stone.

The geochemistry, or chemical composition, of the stone has been shown to influence the types of species present in the microbiome, (Ennis *et al* 2020; Louati *et al* 2020), but so far it does not appear to influence bioreceptivity.

1.2 Artificial stone weathering methods

To perform secondary bioreceptivity studies, the bioreceptivity must be measured on a weathered surface. We reviewed a range of publications that discuss how to artificially weather stone.

While there are many ways to artificially weather stone (Steiger *et al* 2014), the main one used in bioreceptivity studies is thermal, or heat based. Steiger *et al* note that thermal cycling between 20 and 90 ºC induces deterioration in most types of stone. Table 2 summarises the settings used in a range of studies. The duration of heating for these studies is based on BS EN 14066:2013. However, other studies show that the period of heating makes little difference, providing the sample reaches the maximum temperature throughout (Sassoni *et al* 2011). Thermal shock methodology has also been assessed by petrography and shown to produce structural changes in limestones and bioclastic packstones (stones containing fine fossil fragments and a mud cement) equivalent to natural weathering (Torabi-Kaveh *et al* 2019).

Table 2: Summary of artificial weathering conditions used on stone in artificial weathering studies, some of which also looked at the impact of this on bioreceptivity.

Author	**Material**	**Temperature setting**	**Number of cycles**
Murru *et al* 2018	Carrara marble and Santa Caterina limestone	Ramping up to 600°C for 1 hour, hold at 600°C for 7 hours, rapidly cool to 25°C	2
Ban *et al* 2016	Carrara marble, Obernkirchen and Schlaitdorf sandstones, St Margarethen limestone	Ramping up to 600°C (400°C for marble) for 1 hour, hold at 600°C for 7 hours, rapidly cool to 25°C	5
Abdelhamid *et al* 2022	South Sinai, Suez, El-Minia, Qena, Sohag and Aswan limestones	20°C (6 hours) and 70°C (18 hours)	20
Freire-Lista *et al* 2016	Alpedrete, Cadalso de los Vidrios, Colmenar Viejo and Zarzalejo granites	20°C (12 hours) to 105°C (18 hours)	42
Yu *et al* 2021	Shandong granite	300°C (3 hours) to 25°C (6 hours)	15
Ghobadi and Babazadeh 2015; Freire-Lista *et al* 2016	Upper Red Formation sandstone, Qazvin Province, Iran	20°C (12 hours) to 105°C (18 hours)	20

Although salt crystallisation has been shown to be highly effective for artificially weathering limestones (Abdelhamid *et al* 2022), calcarenites (Raneri *et al* 2018) and granites (Brea *et al* 2008), it is not suitable for bioreceptivity studies. Secondary bioreceptivity measurements with salt-contaminated stone did not work because removing the high levels of salts artificially introduced was challenging. It resulted in an altered bioreceptivity compared to the untreated stone (Vázquez-Nion *et al* 2018).

The alternative to heating the stone is to put it through freeze-thaw cycles. However, this method is rarely used because to be effective for rapid weathering, the stone needs to be frozen to -40ºC or lower (Steiger *et al* 2014).

1.3 Microorganisms studied and culture methods

Chlorophyll is the green pigment found in plants, algae and cyanobacteria which allows them to photosynthesise (convert light energy into chemical energy which they use to grow and live). Chlorophyll levels can be used to measure bioreceptivity. They can be monitored over time on stone that has been exposed to a culture of microorganisms. Choosing a consistent type of microorganism means that the study is repeatable by others. Twenty-two of the 32 studies we reviewed characterised primary bioreceptivity by measuring the chlorophyll *a* levels of algae and cyanobacteria which directly correlate to the number of organisms on the surface. The correlation between chlorophyll *a* levels and the number of organisms means that algae are more easily measurable than bacteria or fungi. Five studies looked at fungal bioreceptivity. The final five studies used a mixture of unknown organisms sampled from the environment.

The culture method (or growing conditions) of the microorganisms on stone is also important, to establish a reproducible growth method. While some studies used complex environmental chambers that simulate rainfall, wind and so on (De Muynck *et al* 2009; Giannantonio *et al* 2009; Sanmartín *et al* 2020), the majority used growth protocols in which a steady temperature was maintained (on average 23°C), with a diurnal light/dark cycle. In some studies, this was achieved simply by placing the samples on a window sill in a temperature controlled room (Miller *et al* 2006; Coutinho *et al* 2016). Typically, researchers used environmental chambers that allowed them to control relative humidity as well as the length of the light period (Escadeillas *et al* 2009; Miller *et al* 2009, 2010; Vázquez-Nion *et al* 2018a; Fuentes and Prieto 2021).

1.3.1 Mixed microorganism species environmental samples

For measuring bioreceptivity in a laboratory environment, it is problematic to use environmental samples of undefined surface growth that has been removed from stone, cultured and then applied to the test surface. The different species within a mixed culture will show significantly different growth rates, ranging from hours to days (Skipper 2018). Although identification is possible, this has significant time and cost implications compared to using organisms that are already identified. Variable combinations of microorganisms also cause issues with reproducibility between different studies.

Mixed cultures comprise bacteria, fungi and algae, which contain a range of different coloured pigments. Environmental samples can, therefore, contain various mixes of pigmentation that will change the colour readings that form part of the bioreceptivity assessment (Urzì *et al* 1992, 1993; Urzì *et al* 2008; Kusumi *et al* 2013; Sakr *et al* 2020; Sanmartín *et al* 2020). In order to repeat the studies, a researcher would need to use the same initial samples to ensure that the starting microbiome is consistent. A sample taken from the same site at a later date could have been exposed to biocides, cleaning fluids or other chemicals that may alter its composition. Results would not be comparable

between different research groups that used different environmental samples. Cultures derived from environmental samples are, therefore, unsuitable for laboratory-based studies because they introduce too many potential variables.

1.3.2 Fungus and lichen samples

The studies that looked at the growth of fungi and lichens used single species samples that were clearly identified (Shirakawa *et al* 2003; Wiktor *et al* 2006; Favero-Longo *et al* 2009; Giannantonio *et al* 2009). In many cases, the fungal culture methodology involved the continuous application of growth media – a mix of minerals, vitamins and other nutrients designed to optimally support fungal growth – to the stone surfaces through the whole growth cycle (Wiktor *et al* 2006; Favero-Longo *et al* 2009; Giannantonio *et al* 2009). This method does not produce an accurate measure of bioreceptivity because growth is artificially enhanced by the additional nutrients.

1.3.3 Algae and cyanobacteria

Bioreceptivity tests that were carried out specifically with algae or cyanobacteria used between three and six different species, which were considered representative of those commonly found in the environment (Tomaselli *et al* 2000; Miller *et al* 2009; Marques *et al* 2014; Coutinho *et al* 2016; Vázquez-Nion *et al* 2017; Sanmartín *et al* 2019). The majority of the studies provided clear methodologies for the growth conditions, with the main variation being the light period and the length of the study. For example, the Vázquez-Nion group protocol requires stone samples to be incubated for three months at 23°C, sitting in sufficient water to allow water absorption by capillarity to occur, at 95% relative humidity with a light/dark cycle of 12/12 hours. However, these conditions for standard growth parameters create practical and cost limitations due to the amount of deionised water required to maintain the relative humidity level. The three-month period is longer than that employed by the majority of research groups (Prieto and Silva 2005; Escadeillas *et al* 2009; Miller *et al* 2010; Sanmartín *et al* 2019, 2020; Fuentes and Prieto 2021) who used an eight-week period with varying light/dark cycle lengths – the most common being 16 hours light/four hours dark.

Reproducibility of Tomaselli *et al* (2000), Coutinho *et al* (2016) and Veeger *et al* (2021) is limited because the methodology is not described in full. Many of the studies used microorganisms isolated locally from the environment. Unless the study was carried out by the same research group, it is likely that the different algae will have different growth rates. Again, this makes it difficult to accurately compare the levels of bioreceptivity identified in the articles.

1.4 Bioreceptivity measurement methods

When Guillitte's group introduced the concept of bioreceptivity in 1995, they did not recommend any particular standard measurement method. Since then, there has been a wide range of approaches taken to measure bioreceptivity in a laboratory setting (Table 3). There are six main measures:

1. Visual observation
2. Percentage of surface area covered by microorganisms
3. Direct count of microorganism cells
4. Colour change by L*a*b* visible colorimetry
5. Spectrofluorometry of the chlorophyll present on the surface
6. Visible light spectrophotometry of extracted chlorophyll

Table 3: Review of methods used to measure bioreceptivity in laboratory-based tests.
'Y' indicates method used in paper.

Paper	Organisms	Surface cover area (%) by macroscopic observation	Surface cover area (%) by image analysis	Surface cover area (%) by measuring chlorophyll fluorescence	Microscopic cell counts	Viable cell count by culture	Colorimetric analysis	Chlorophyll *a* extraction – spectro-fluorometry (µg/cm^2)	Chlorophyll *a* extraction – visible light spectro-photometry (µg/cm^2)	In-vivo chlorophyll *a* fluorescence	Visual inspection by stereomicroscopy of polished cross-sections with periodic acid-Schiff staining
Guillitte and Dreesen (1995)	Cyanobacteria, algae, diatoms, mosses	Y									
Tiano *et al* (1995)	Algae, cyanobacteria			Y	Y						
Saiz-Jimenez *et al* (1995)	Cyanobacteria								Y		
Papida *et al* (2000a)	Environmental microbial population				Y	Y					
Tomaselli *et al* (2000)	Algae, cyanobacteria			Y							
Shirakawa *et al* (2003)	Fungi	Y			Y						
Prieto and Silva (2005)	Cyanobacteria								Y		
Miller *et al* (2006)	Cyanobacteria		Y							Y	
Wiktor *et al* (2006)	Fungi	Y									Y
Urzì and De Leo (2007)	Algae						Y	Y			
Miller *et al* (2008)	Fungal, bacterial or algal suspension mostly of unknown species				Y						
Escadeillas *et al* (2009)	Cyanobacteria		Y								
Miller *et al* (2009)	Fungal, bacterial or algal suspension mostly of unknown species	Y									

Paper	Organisms	Surface cover area (%) by macroscopic observation	Surface cover area (%) by image analysis	Surface cover area (%) by measuring chlorophyll fluorescence	Microscopic cell counts	Viable cell count by culture	Colorimetric analysis	Chlorophyll *a* extraction – spectro-fluorometry (µg/cm^2)	Chlorophyll *a* extraction – visible light spectro-photometry (µg/cm^2)	In-vivo chlorophyll *a* fluorescence	Visual inspection by stereomicroscopy of polished cross-sections with periodic acid-Schiff staining
Favero-Longo *et al* (2009)	Lichens										Y
De Muynck *et al* (2009)	Algae		Y				Y				
Giannantonio *et al* (2009)	Fungi		Y								
Miller *et al* (2010)	Algae		Y						Y		
Wiktor *et al* (2011)	Fungi										Y
Adamson *et al* (2013)	Environmental algae						Y				
Marques *et al* (2014)	Algae, cyanobacteria						Y		Y		
Coutinho *et al* (2016)	Algae, cyanobacteria		Y						Y		
Vázquez-Nion *et al* (2018)	Algae						Y	Y			
Vázquez-Nion, Silva *et al* (2018)	Algae						Y	Y			
Vázquez-Nion, Troiano *et al* (2018)	Algae, cyanobacteria						Y				
Sanmartín *et al* (2019)	Algae						Y	Y			
Sanmartín *et al* (2020)	Algae						Y	Y			
Fuentes and Prieto (2021)	Algae						Y	Y			
Veeger *et al* (2021)	Algae		Y								
Total studies using each method		4	7	2	4	1	10	6	5	1	3

As can be seen in Table 3, many of the studies used multiple methods to determine the level of bioreceptivity. Below, we discuss each of these methods in turn, evaluating how suitable they are for this research project.

1.4.1 Visual observation

The studies using visual observation identify surfaces as having high, medium or low bioreceptivity, based on how they look in comparison with one another. We feel this is the least reliable method. Although it is inexpensive and fast, visual interpretation is subjective. It does not provide a reliable reproducible methodology that can be carried out in different laboratories by different people.

1.4.2 Percentage of surface area covered by microorganisms

Measuring the percentage of surface area covered with growth provides a quantitative measure that – depending on the way the organisms were introduced to the surface – is reproducible. This technique does not, however, reflect the concentration of coverage. For example, 100 cells per mm^2 would be counted as the same percentage area coverage as 1,000 or even 1,000,000 cells per mm^2. As such, this method is best used in conjunction with other measurements (which many of the earlier studies in the field did not do). It is also dependent on there being sufficient contrast between the surface and the microorganisms to allow the area to be accurately measured.

1.4.3 Direct count of microorganism cells

Counting the cells in a sample taken from the surface can provide an accurate measure of the level of colonisation at the time of sampling. While there is an initial high cost in obtaining a microscope, the slides and stains are not expensive. However, this is a time-consuming technique, and it requires trained and experienced microbiologists to carry out the microscopy. Otherwise, the method is highly subjective and prone to error.

1.4.4 Colour change by L*a*b* visible colorimetry

This method provides a quantitative measure of the colour change of a surface and so can provide a good measure of its bioreceptivity. L*a*b* colour space is a method of measuring colour, where L represents a range between white and black, a is a range between red and green, and b is the range between yellow and blue. Differences between colours can be calculated giving a Delta E^*_{ab} (ΔE^*_{ab}) reading (described in section 2.2.4 Colorimetry). However, there is an initial high cost to obtain reliable equipment, and the method is open to bias. For example, one person may instinctively

measure the darkest areas of growth, whereas another may measure the lightest. To avoid microbiological contamination of the surface, the measurement is only a spot measure, rather than a whole sample measure. Additionally, many types of stone, such as Lincolnshire limestone for example, change colour naturally over time and so this must be accounted for.

1.4.5 Spectrofluorometry of chlorophyll

Chlorophyll (the green pigment which plants use to turn light into energy) will fluoresce a red light when exposed to ultraviolet (UV) light. The levels of chlorophyll in a sample or on a surface can be measured using a spectrofluorometer or camera. A camera will provide information similar to the percentage of area covered measurement, but with additional information about the concentration of cells making up the cover, based on the intensity of the red area on the image. A spectrofluorometer gives a relative light unit measure that relates to the amount of chlorophyll *a* present in the sample. While there are several types of chlorophyll (*a*-*e*), chlorophyll *a* directly relates to the number of cells present.

Using a camera or spectrofluorometer are both reproducible methods, and they can produce quantitative results in terms of the area covered and the intensity of the fluorescence. The main limitation for testing samples is the initial cost of the spectrofluorometer (the majority of this type of testing is being carried out by one research group). Both techniques are also highly time dependant, because as soon as the UV exposure stops, the level of fluorescence starts to fall. Even a 1-minute delay in imaging the fluorescence can result in a significant skew in the measurements.

Miller's work on bioreceptivity of Portuguese limestones (Miller *et al* 2009) shows a poor correlation between chlorophyll *a* fluorescence and the stone properties that influence bioreceptivity, especially when compared to spectrophotometry of extracted chlorophyll (*see* 1.4.6). This is primarily due to the fluorescence of components of the stone in the same region. As such, this measure of growth cannot be considered suitable for all stone types.

Unlike absorbance, fluorescence is not an absolute measurement (BMG Labtech 2022). The intensity of the fluorescent signal is usually relative to other measurements, or to a reference measurement taken by the instrument. Consequently, spectrofluorometers measure the light signal emitted by a sample in relative fluorescence units, and readings are only comparable if taken using the same device or specific controls. None of the published studies, other than that by Vázquez-Nion *et al*, used fluorescence controls. The studies, therefore, cannot be compared. Vázquez-Nion's group produced a calibration curve for their work, meaning that all their own laboratory studies are

comparable. They can also be compared to other studies that report results in μg/cm^2 of chlorophyll. However, this curve is not included in their publication, and it is specific to their spectrofluorometer (Vázquez-Nion *et al* 2018b).

1.4.6 Visible light spectrophotometry of extracted chlorophyll

Spectrophotometry measures the level of chlorophyll present in a sample, which is directly related to the number of photosynthetic cells present. It differs from spectrofluorometry because it is a direct colour measurement of the chlorophyll, as opposed to a measurement of the red light emitted from the chlorophyll when excited by UV light.

Readings from a spectrophotometer give a quantitative measure of the amount of growth present on a surface. Unlike spectrofluorometry, this is an absolute reading not a relative one, and it is comparable across studies. The main limitation of the technique is the initial cost of a spectrophotometer. After this, consumables are inexpensive and readily accessible. A number of recent studies have been carried out using adapted smartphone cameras to work as accurate low budget spectrophotometers (Koohkan *et al* 2020). Other limitations relate to the sampling of the surface, as different sampling techniques impact on cross-study comparisons.

1.4.7 Bioreceptivity index

Vázquez-Nion *et al* (2018a) proposed a bioreceptivity index (BI) for granites, with the suggestion that it could be extended to other stones based on readings from chlorophyll extraction and colour change by L*a*b* colorimetry. There are three outputs from the index:

- Bioreceptivity based on growth of algae on the surface (BI_{growth})
- Bioreceptivity based on colour change of the stone due to algal growth (BI_{colour})
- Bioreceptivity index value, the total measurement calculated from BI_{growth} and BI_{colour}

The index runs on a scale of 0 to 10, split into two-increment divisions, and has qualitative descriptions linked to the ranges (Table 4).

Table 4: Bioreceptivity index scoring and a qualitative interpretation of the index results, taken from Vázquez-Nion *et al* 2018a.

Bioreceptivity index	Qualitative description
≤2	Very low bioreceptivity
>2 ≤4	Low bioreceptivity
>4 ≤6	Mild bioreceptivity
>6 ≤8	High bioreceptivity
>8	Very high bioreceptivity

The method for calculating the bioreceptivity index is based on a study of seven different granites, which had previously been noted as supporting different levels of growth. Bioreceptivity is characterised by measuring the chlorophyll *a* levels in the surface growths and the change in colour of the stone surface at the end of the growth period compared to the start. From the results, Vázquez-Nion *et al* demonstrate that the colour change (ΔE^*_{ab}) and the concentration of chlorophyll *a* (measured in μg/cm^2) provide measurements which correlate with the key physical characteristics that control bioreceptivity e.g. open porosity, surface roughness etc., as discussed in section 1.1 of this report.

The following formulae (Equations 1 to 3) were developed to calculate bioreceptivity based on growth and colour. A full explanation of how the equations were conceived can be found in the source paper (Vázquez-Nion *et al* 2018a):

$$BI_{growth} = 10 \cdot \frac{chl\ a\ (\mu g\ cm^2)}{4.14}$$

Equation 1: Bioreceptivity index growth calculation.

$$BI_{colour} = \frac{10 \cdot \Delta E^*_{ab}}{24.25}$$

Equation 2: Bioreceptivity index colour calculation. ΔE^*_{ab} has been calculated from an initial reading of the stone sample compared to a reading of the sample after algal growth.

An overall bioreceptivity index value (total measurement) was calculated from these using the following formula:

$$BI = \frac{2 \cdot BI_{growth} + BI_{colour}}{3}$$

Equation 3: Bioreceptivity index calculation for the total bioreceptivity index.

Vázquez-Nion *et al* acknowledge that the methodology may underestimate bioreceptivity for highly porous rocks, as extracting microorganisms from deep within the pores can be difficult. They do not define a recommended porosity cut-off for the methodology. However, the stone samples tested within the pilot phase of our study did not present this problem. The Vázquez-Nion paper recommends that all three measures should be provided, as end users of the stone may have different requirements. For example, where the aesthetic impact is less important, BI_{growth} or BI would be most suitable. Alternatively, for the integration of a stone into a facade or into the landscape, BI_{colour} or BI may be more appropriate.

1.5 Summary

From our review of the literature, it is clear that there is a high level of variation in the species and the culture methods and conditions used. This means that the results from these studies are not comparable.

1.5.1 Bioreceptivity protocols

The most reproducible methodology for laboratory-based bioreceptivity tests is culturing known algal or cyanobacterial species on sterile stone samples. The species need to come from a quality controlled culture collection, such as the Culture Collection of Algae and Protozoa (CCAP, no date). Our project aims to standardise the culture conditions used and the methods for assessing the level of growth, so that studies are directly comparable.

Research groups with a history of publishing on bioreceptivity have moved away from using direct microbial counts and single measurement techniques. They prefer measuring chlorophyll levels and combining this with measuring the percentage of surface area covered or colour change by L*a*b* colorimetry. The majority of groups used spectrophotometry to measure extracted chlorophyll, as opposed to spectrofluorometry.

A bioreceptivity index has been proposed to allow direct comparison between stone types (Vázquez-Nion *et al* 2018a), which would be a useful tool in this field. However, the work carried out in this project shows that some modifications to the calculation are needed for it to be reliable for all stone types.

In many bioreceptivity articles, we observed that the methods section tends to focus on the analysis of the stone samples and the methods of determining the levels of growth on the stone. It does not cover the earlier stages of the protocol, so information on the types of organisms and how they are initially grown on the stone (culture methodology) are not discussed in enough detail to repeat the work (Guillitte and Dreesen 1995, Papida *et al* 2000; Tomaselli *et al* 2000; Urzì and De Leo 2007; De Muynck *et al* 2009; Sanmartín *et al* 2020; Veeger *et al* 2021).

The discrepancy in techniques means we needed to design a reproducible protocol that can be used to make comparisons between stone types. In particular, it is important to use a defined group of microorganisms that are commercially available, to ensure reproducibility between studies. Our study uses defined algal species, available from the Culture Collection of Algae and Protozoa (CCAP, no date):

- CCAP 211/11B – *Chlorella vulgaris* (type culture)
- CCAP 379/1B – *Stichococcus bacillaris*
- CCAP 219/5A – *Trebouxia decolorans*

All three have been identified as growing in the environment on stone in previous studies (Tomaselli *et al* 2000; Miller *et al* 2008; Macedo *et al* 2009).

1.5.2 Artificial weathering protocols

To artificially weather the stone for measuring secondary bioreceptivity, we have chosen to use thermal shock. This is an effective method, it is relatively straightforward for people to reproduce in different laboratories, and it does not add any chemicals to the stone that could alter the secondary bioreceptivity properties.

The protocols used in this study are based on previous studies of limestone, sandstone and granite (Ghobadi and Babazadeh 2015; Freire-Lista *et al* 2016; Abdelhamid *et al* 2022), which were based on BS EN 14066:2013. Sassoni *et al* (2011) demonstrate that the times – 18 hours at 105 ºC and six hours at 25 ºC – in BS EN 14066:2013 are excessive. In their study, they show that the artificial weathering caused by a one-hour hot / one-hour cold cycle is the same as an 18-hour hot / six-hour cold cycle, and that the level of weathering is temperature dependent, not time dependant. Based on BS EN 14066:2013, 20 cycles should be sufficient to produce a weathered material.

In our study, the material with the lowest thermal diffusivity (the measure of the rate at which temperature changes occur in a material when a difference in temperature is applied) is granite. Using the heat penetration calculation (Thermtest no date; Faghri and Zhang 2006), a sample of the lowest recorded thermal diffusivity granite (0.77) will need 15 minutes for the core to reach the external temperature. As artificial weathering is caused by a rapid change in temperature, a more rapid cycle than that set out in BS EN 14066:2013 is suitable, providing the incubation periods are no less than 30 minutes. While it is not possible to determine an exact period of environmental weathering to this protocol, due to the additional environmental variables, this protocol ensures that all stone types will undergo identical weathering conditions.

2. Methodology

This section covers the methodologies used in this study for testing bioreceptivity and characterising stone properties. The three main stone types selected for the pilot phase were:

- Limestone: Portland limestone (Jordans Basebed, an oolitic limestone from Jordans Quarry, Portland, Dorset, supplied by Albion Stone)
- Sandstone: Howley Park sandstone (a dolomitic sublithic wackestone from Howley Park Quarry 3, Leeds, West Yorkshire, supplied by Hutton Stone)
- Granite: Foggintor granite (a biotite-bearing syenogranite pegmatite from Foggintor Quarry, Dartmoor, Devon, supplied by Blackenstone Quarry, Devon)

In this report, they are referred to as Jordans Basebed limestone, Howley Park sandstone and Foggintor granite, respectively. The three stones are representative of a wide range of English building stones. They are sufficiently different in geochemical and physical properties to ensure that the methodology developed is suitable for the majority of building stones. The limestone and sandstone samples used were a minimum of 10mm thick and 40mm^2, as used by other researchers (Miller *et al* 2012; Ghobadi and Babazadeh 2015; Vázquez-Nion *et al* 2018). Due to cutting constraints, the granite blocks were 50mm^3.

2.1 Artificial weathering of stone

The stone samples were heated to 105 ºC for a minimum of one hour (Genlab Mino/30 oven), immersed in deionised water at 20 ºC for a minimum of one hour, then returned to the higher temperature for the next cycle. This was repeated for a total of 20 hot/cool cycles. Samples were left at temperature overnight where necessary.

The samples were tested for changes to capillarity and open porosity. Unweathered and weathered samples were petrographically analysed.

2.2 Physical and geochemical properties of stones

2.2.1 Capillarity coefficient

Water absorption coefficient by capillarity was carried out on at least three separate samples of unweathered and artificially weathered stone, following the method in BS EN 1925:1999.

2.2.2 Surface roughness

Surface roughness measurements were taken on at least six separate samples of unweathered and artificially weathered stone using a Surtronic S-128 surface roughness tester, as per the manufacturer's guidelines. Scan length was set to 4mm with a PK-02 5μm pick-up giving a 50nm resolution.

2.2.3 Open porosity

Measurements for open porosity were carried out on at least three separate samples of unweathered and artificially weathered stone, following methods in BS EN 1936:2006 and BS EN 13755:2008. Stone samples were oven dried for 24 hours at 70 ºC, or until a consistent dry weight was achieved, and then cooled slowly to room temperature. Each sample was weighed to an accuracy of 0.01g and placed in an evacuation vessel. The samples were covered with deionised water to a set level, and the vessel was covered with a lid. The pressure was then lowered using a vacuum pump for 10 minutes, and the vessel sealed. It was left for two hours under negative pressure to ensure that all the air in the samples was replaced by water. The chamber was then equilibrated to room pressure. Samples were removed, wiped with a damp cloth and weighed to an accuracy of 0.01g. Open porosity as a percentage was calculated as shown in equation 4.

$$Open\ porosity\ (\%) = \frac{wet\ weight - dry\ weight}{wet\ weight} \times 100$$

Equation 4: Open porosity calculation for determining the percentage open porosity.

2.2.4 Colorimetry

Colorimetry was carried out on at least six separate samples of unweathered and artificially weathered stone, across the whole stone surface on both wet and dry samples using a Konica Minolta CM-2600d colorimeter with the medium aperture, as per the manufacturer's guidelines.

Colour change between wet and dry surfaces was calculated as ΔE^*_{ab}, as shown in equation 5 (CIE 2004).

$$\Delta E^*_{ab} = \sqrt{(L_2\text{-}L_1)^2 + (a_2\text{-}a_1)^2 + (b_2\text{-}b_1)^2}$$

Equation 5: Delta E^*_{ab} calculation for determining the change in colour using L*a*b* colour space. Where L represents a range between white and black, a is a range between red and green, and b is the range between yellow and blue.

2.2.5 Geochemical composition

Petrographic analysis was outsourced to Petrolab Ltd, C Edwards Offices, Gweal Pawl, Redruth, Cornwall TR15 3AE. It was completed by Bradley Staniforth, CEng MIMMM, senior geomaterials scientist.

2.3 Bioreceptivity tests

2.3.1 Algal culturing on stone

Three algal species (CCAP211/11B – *Chlorella vulgaris* (type culture), CCAP379/1B – *Stichococcus bacillaris*, CCAP219/5A – *Trebouxia decolorans*) were cultured using standard microbiological technique in BG11 medium (CCAP, UK).

Stone samples were sterilised by autoclave in borosilicate glass Petri dishes or other suitable lidded containers.

Each stone sample was inoculated with 61.6µl/cm^2 of mixed algal culture with an OD750nm (optical density) of 0.2, harvested while the algae are in exponential growth phase. Optical density is the amount of light absorbed by the cells in a sample at a 750nm wavelength, and it provides a measure of the cell mass that is not affected by the pigments in the algae. It gives an inoculum equivalent to 25µg/cm^2 dry weight cells, a similar level to the Vázquez-Nion *et al* (2017, 2018a) and Sanmartín *et al* (2020) studies, which are the most consistent in inoculation levels. The stone sample was inoculated by spreading the culture evenly across the surface using a sterile spreader.

Incubation was carried out at 23 ºC, 80% relative humidity (Fuentes and Prieto 2021), with a diurnal cycle of 16 hours light (16,500 lux, using Luxline Plus 4000K colour temperature strip lights, which are suitable for algal growth), six hours dark (Escadeillas *et al* 2009; Miller *et al* 2010; Sanmartín *et al* 2020) for 56 days in a Climacell 111 chamber. Sterile deionised water was maintained in the containers at a depth of 5mm for the period of the experiment to ensure that water activity (reduction in water availability over time due to evaporation) was not a limiting factor.

Samples were moved weekly in a random order to ensure that no single sample was shaded by the others for too long, as per Vázquez-Nion *et al* (2017).

2.3.2 Colorimetry of algae inoculated stone

Measurements were carried out on at least three separate samples of unweathered and artificially weathered stone. Stone samples were handled aseptically, and colour measurements were taken using a Konica Minolta CM-2600d colorimeter, with the medium aperture. The aperture was wiped down with 70% ethanol pre and post reading, and allowed to air dry in a sterile environment. L*a*b* readings were taken prior to harvesting the cells for chlorophyll extraction. To avoid operator bias in selecting the area measured, each stone was measured at the centre of the sample area.

Colour change was calculated using ΔE^*_{ab} (*see* 2.2.4), using the mean of the L*a*b* readings for each sample.

Vásquez-Nion *et al*'s (2018) methodology for calculating *BI* and BI_{colour} (Equation 2 and Equation 1, respectively, *see* 1.4.7 Bioreceptivity index) compares the colorimetry reading of wet stone before inoculation (L1/a1/b1) with a reading after the stone has been inoculated and incubated (L2/a2/b2). To account for colour changes through alterations in the stone itself during the incubation period, our L1/a1/b1 readings were taken from uninoculated stone after incubation under the same conditions for 56 days.

2.3.3 Algal sampling from stone

Sampling was carried out weekly for the first four weeks to enable the chlorophyll extraction technique to be optimised. This was then changed to fortnightly for the remaining four weeks. Using aseptic technique, a 20mm^2 area of the stone surface was sampled initially. One surface of a sterile cotton swab, wetted with sterile deionized water, was wiped over the surface twice, in a crosshatch pattern, to ensure consistent sampling. Once the surface algae had been removed, the same surface was scraped twice with a scalpel, in a crosshatch pattern, to remove the surface layer of softer stone samples and capture the subsurface algae. The scalpel sample was then applied to

the wetted swab, which contained the surface algae, and the swab tip cut to remove the sample. The swab tip was then placed into a 1.5ml microtube ready for chlorophyll extraction.

2.3.4 Chlorophyll *a* extraction and measurement

Our initial tests showed that the most effective extraction method was using dimethyl sulfoxide (DMSO). Extraction was carried out using a sample in a 1.5ml microtube and adding 200µl DMSO. The sample was vortexed for 30 seconds, to ensure the DMSO fully penetrated the swabs, and then frozen for 4 hours at -20 ºC. The sample was defrosted, and 200µl pure acetone was added to enhance the stability of the chlorophyll. The sample was vortexed again for 30 seconds, to ensure the DMSO and acetone were completely mixed, and then centrifuged for 5 minutes at 15,000rpm to reduce debris in the supernatant (liquid portion of the sample).

The supernatant was measured using a BioDrop Touch spectrophotometer, with a 10mm path length cuvette at 630, 647, 664 and 750nm, and the levels of chlorophyll *a* extracted were calculated as per the method in UNESCO (1966) using Equation 6.

$$\mu g \text{ chl } a \text{ cm}^2 = \frac{S\,[11.85\,(Abs_{664} - Abs_{750}) - 1.54\,(Abs_{647} - Abs_{750}) - 0.08\,(Abs_{630} - Abs_{750})]}{V}$$

Equation 6: Chlorophyll *a* extraction calculation. Where S = volume of DMSO or acetone used for the extraction (0.4 for stone samples) and V = volume/area of culture tested in litres or cm^2 sampled ($2cm^2$ for stone samples).

The mean of the µg chlorophyll *a* cm^2 results for each sample was then applied to the BI_{growth} equation (Equation 1, section 1.4.7 Bioreceptivity index). This result, together with the BI_{colour} result from Section 2.3.2 Colorimetry of algae inoculated stone, was applied to the *BI* equation (Equation 3, *see* 1.4.7 Bioreceptivity index) to give the total bioreceptivity.

2.4 Statistical tests

Unpaired Student's t-tests (a hypothesis test used for comparing the significance of differences between two related sample sets) were carried out using the embedded function in Excel and Equation 7.

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{S^2\left(\frac{1}{n_1} + \frac{1}{n_2}\right)}}$$

$$S^2 = \frac{\sum_{i=1}^{n_1}(x_i - \bar{x}_1)^2 + \sum_{j=1}^{n_2}(x_j - \bar{x}_2)^2}{n_1 + n_2 - 2}$$

Equation 7: Unpaired Student's t-test. Where $\bar{x}_1$ and $\bar{x}_2$ are sample means, S^2 is the pooled sample variance, n_1 and n_2 are the sample sizes, and t is a Student t quartile with n_1+n_2-2 degrees of freedom.

Testing for correlation – statistically significant relationships – between data sets was carried out using the Pearson correlation coefficient via the embedded function in Excel and Equation 8.

$$r = \frac{n(\sum xy) - (\sum x)(\sum y)}{\sqrt{[n(\sum x^2) - (\sum x)^2]\,[n(\sum y^2) - (\sum y)^2]}}$$

Equation 8: Pearson correlation coefficient. Where n is the sample size, x is data set 1 and y is data set 2.

Statistical significance is described through use of a p-value. This is a measure of how likely it is that this result could occur by chance. The p-value ranges between 0 and 1, and the lower the p-value, the more likely it is that the result is significant. In research, a p-value equal to or below 0.05 is considered proof that the results did not occur by chance (Skipper & Skipper, 2024).

3. Stone properties results

The results of the tests carried out in this study are outlined in this section. Bioreceptivity is primarily influenced by surface roughness and other characteristics that affect the availability of accessible water in a stone to microorganisms. A stone's geochemical characteristics will influence the types of microorganisms that grow on it. Measuring the properties of the stones in our study allows us to confirm correlations between the characteristics of each stone type and the results of our bioreceptivity testing.

3.1 Physical properties

We measured physical properties of water absorption by capillarity coefficient, surface roughness and open porosity for the three stones before and after artificial weathering.

Results for the artificially weathered samples were compared to those of the unweathered samples using a Student's t-test, to determine if the weathering had led to a significant physical change. The artificial weathering caused a measurable change in the stones' properties (Table 5). In all three stones, there was a statistically significant difference in water absorption by capillarity and open porosity pre and post artificial weathering. The largest change was predominantly water absorption by capillarity, with Jordans Basebed limestone demonstrating a 27.99% increase and Foggintor granite a 51.66% increase. Howley Park sandstone had a greater increase in water absorption by capillarity (16.99%) than open porosity (6.7%), but unlike the other two stone types showed a significant increase in surface roughness (13.7%).

Table 5: Properties of unweathered and weathered stone samples. Showing average measurements and the p-value for the differences (calculated using a Student's t-test). Where pre and post readings are significantly different ($p<0.05$), the p-value is highlighted green.

Limestone: Portland Jordans Basebed	Unweathered	Weathered	p-value	% change
Water absorption by capillarity ($g.m^{-2}.s^{-0.5}$)	37.379 ±0.5	51.911 ±1	<0.01	27.99
Surface roughness Ra (μm)	8.99	9.12	0.80	1.4
Open porosity (%)	4.98	5.77	0.03	13.6

Sandstone: Howley Park	Unweathered	Weathered	p-value	% change
Water absorption by capillarity ($g.m^{-2}.s^{-0.5}$)	21.648 ±0.3	26.08 ±1.08	0.015	16.99
Surface roughness Ra (µm)	9.81	11.36	0.05	13.7
Open porosity (%)	3.71	3.97	<0.01	6.7

Granite: Foggintor Quarry	Unweathered	Weathered	p-value	% change
Water absorption by capillarity ($g.m^{-2}.s^{-0.5}$)	0.29 ±0.04	0.6 ±0.065	<0.01	51.66
Surface roughness Ra (µm)	3.58	4.35	0.46	17
Open porosity (%)	0.23	0.29	<0.01	22

3.2 Colorimetry

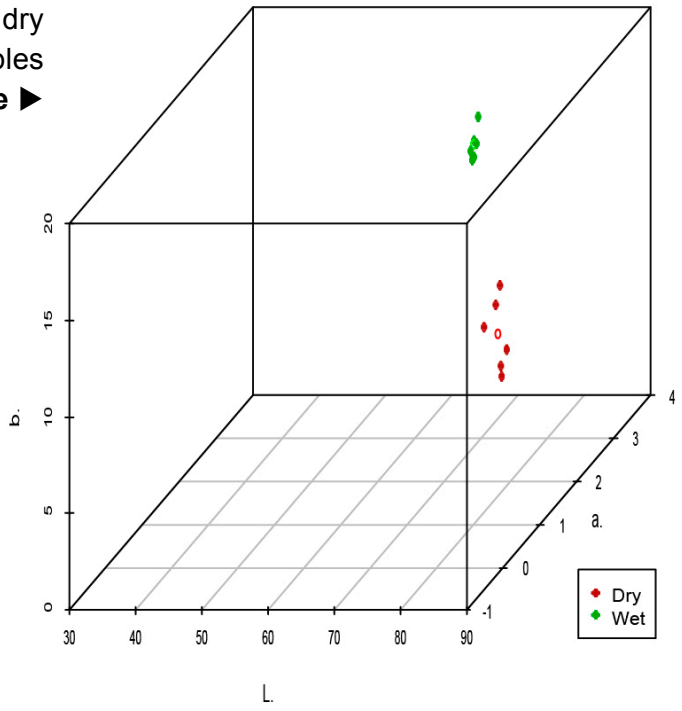
Colorimetry of the three stone types demonstrated that the stones showed a noticeable difference in colour when comparing wet and dry samples (Table 6). When the differences between the wet and dry readings are calculated, this provides a Delta E_{ab} (ΔE^*_{ab}) measure. Delta E ranges from 0 to 100, and it is a measure of whether there is a perceptible change. For example, a colour difference of between 1 and 2 would only be perceptible on close examination, whereas between 11 and 49 would be visibly obvious but still considered similar in colour (Mokrzycki and Tatol 2011).

Foggintor granite demonstrated the highest level of variability in colour due to the variable mineral composition, but it had the greatest overlap of colour readings between wet and dry measurements (Figure 1). Bioreceptivity testing is carried out on wet stone in a humid environment, and these results demonstrate that it is important that the non-inoculated controls for colorimetry and bioreceptivity measurements are carried out under the same conditions.

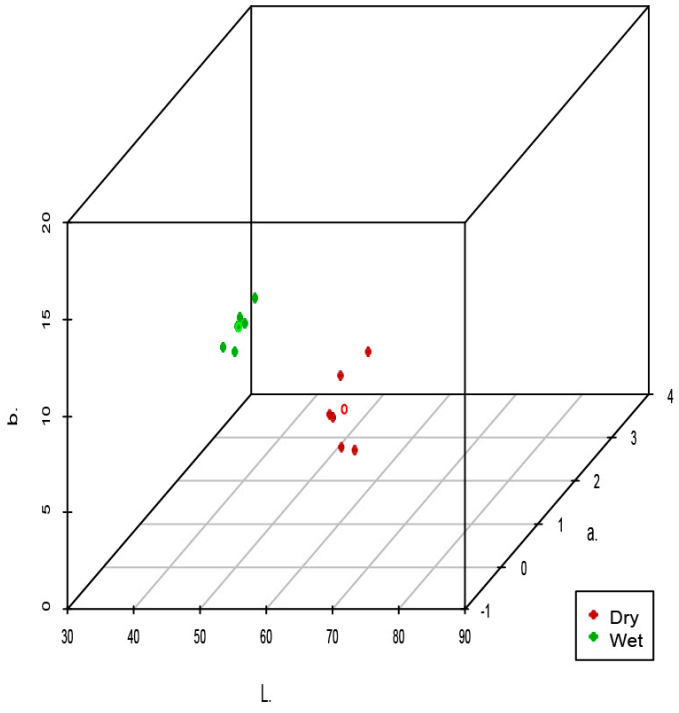
Table 6: L*a* b* readings of the stone samples with a Delta E comparison between wet and dry.

Stone	Wet			Dry			ΔE
	L*	a*	b*	L*	a*	b*	
Jordans Basebed limestone	68.12 ±0.5	3.13 ±0.13	14.88 ±0.54	80.69 ±1.5	1.53 ±0.2	9.04 ±0.89	13.9
Howley Park sandstone	45.45 ±0.53	0.87 ±0.25	10.45 ±0.49	65.17 ±2.4	0.22 ±0.44	7.79 ±1.08	19.9
Foggintor granite	51.17 ±10.25	0.12 ±0.43	6.02 ±1.82	60.56 ±6.29	0.52 ±1.31	6.43 ±4.16	10.4

L*a*b* colourimetry of wet and dry untreated stone samples
Jordans Basebed Limestone ▶



L*a*b* colourimetry of wet and dry untreated stone samples
◀ Howley Park Sandstone



L*a*b* colourimetry of wet and dry untreated stone samples
Foggintor Granite ▶

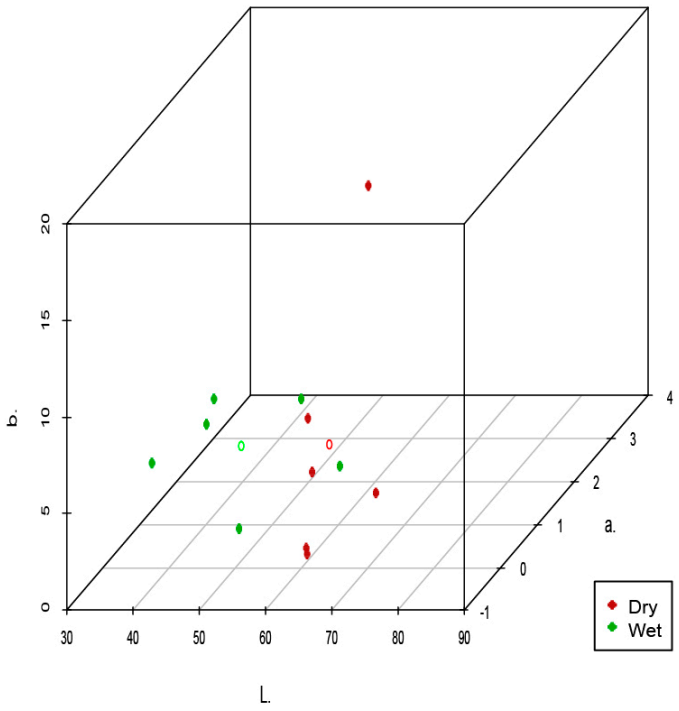


Figure 1: Wet and dry colour measurements of all three stone types plotted in 3D L*a*b* colour space. While the limestone and sandstone show distinct clustering of wet and dry readings, the granite demonstrates an overlap of measurements between wet and dry.

3.3 Petrographic and geochemical analysis

Petrographic analysis demonstrated our chosen weathering protocol had been successful in causing changes to the stone samples. Analysis of the samples clearly showed physical, and in some cases mineralogical, changes in the stones after artificial weathering. A summary of the mineral phases, textural components and measurements of voids/porosity, internal porosity and fractures for each stone pre and post artificial weathering can be found in Appendix A.

Weathered Jordans Basebed limestone showed increased fracturing, combined with a reduction in micritic (microcrystalline calcite) and spartic (crystalline calcite) cements, when compared to the sample that had not undergone thermal shock treatment. There was also an increase in surface variability in the artificially weathered sample, caused by the dissolution of the cementing matrix (Figure 2). In this sample, the change in surface roughness was caused by the dissolution of the mixed sparry and micritic cements, which also opened up voids within the matrix. This correlates with the changes in open porosity and water absorption by capillarity that we observed.

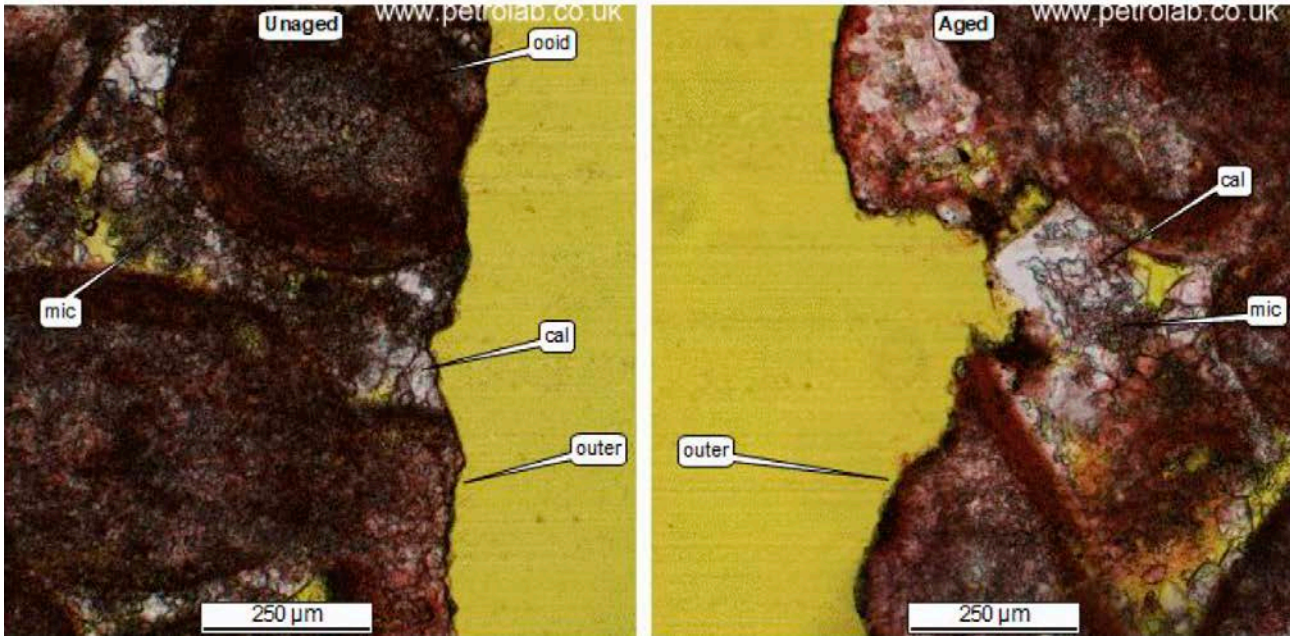


Figure 2: Jordans Basebed limestone sample from the petrography report.
The sample referred to as ‘unaged’ is the unweathered sample; the ‘aged’ sample is artificially weathered. Key: mic = micritic cement, cal = calcite.

For the Howley Park sandstone, the overall porosity showed no major change, but artificial weathering resulted in a larger proportion of connected voids (Figure 3). This supports the results found for the open porosity and water absorption by capillarity (*see* 3.1 Physical properties). There was also a shift in the mineralogical composition of the iron oxide/hydroxide-containing minerals (Figure 4), and decreases in the carbonate and sulphate phases (Figure 5). These were calculated by modal analysis using a Pelcon 64 channel electromechanical point counter, using stepping and traverse intervals of 1mm. The iron oxide/hydroxide-containing minerals that showed an increase in % volume were those that contained manganese and sulphur, which will account for the decrease in the carbonate and sulphate phases. These mineralogical changes are probably associated with the colour change observed in the stone during the testing that is discussed in Section 4.3 Stone bioreceptivity by colorimetry.

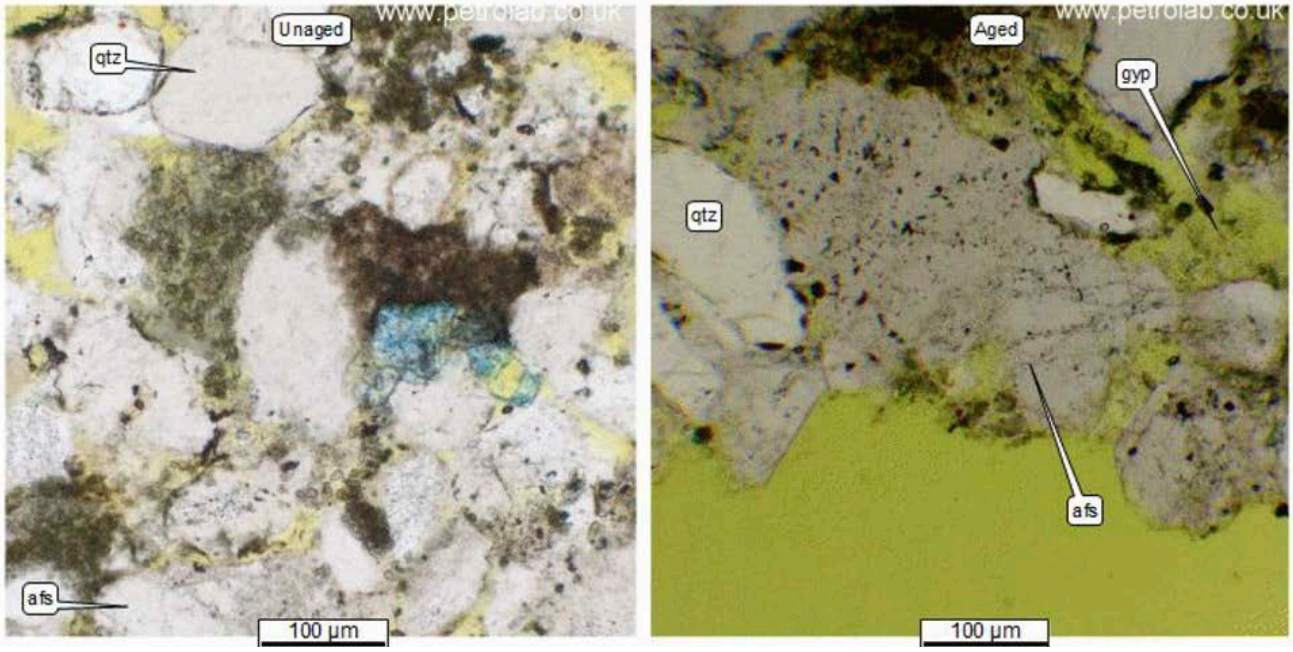


Figure 3: Howley Park sandstone sample from the petrography report. The sample referred to as ‘unaged’ is the unweathered sample; the ‘aged’ sample is artificially weathered. Key: qtz = quartz, afs = alkali feldspar, gyp = gypsum.

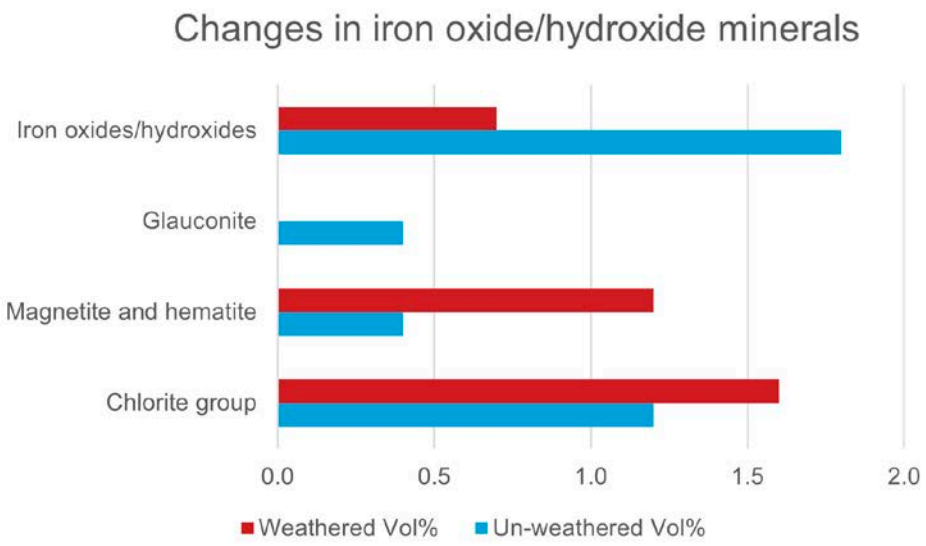


Figure 4: Changes in the iron oxide/hydroxide minerals after artificial weathering in Howley Park sandstone.

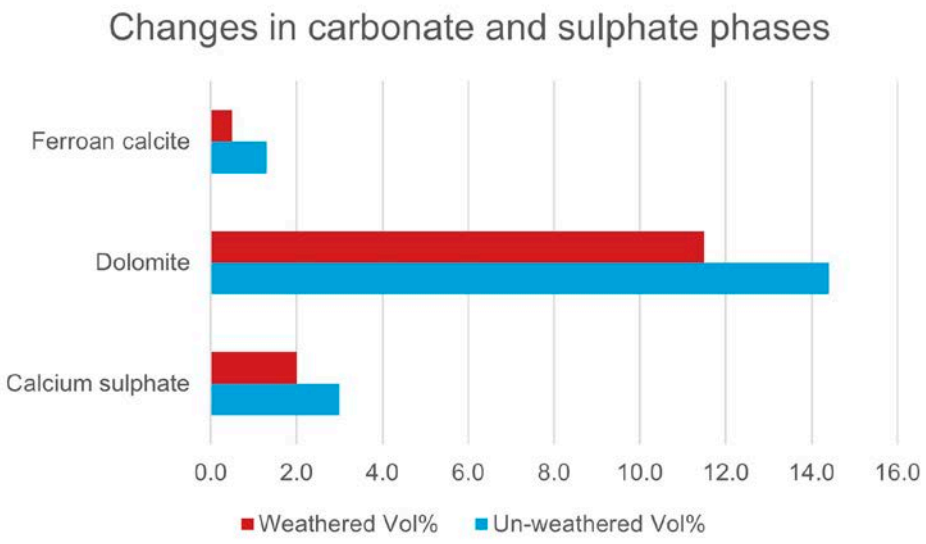


Figure 5: Changes in the carbonate and sulphate phases after artificial weathering in Howley Park sandstone.

The petrographic comparison of unweathered and artificially weathered Foggintor granite demonstrated an increase in fracture occurrence and size (Figure 6), supporting the results in Section 3.1 Physical properties.

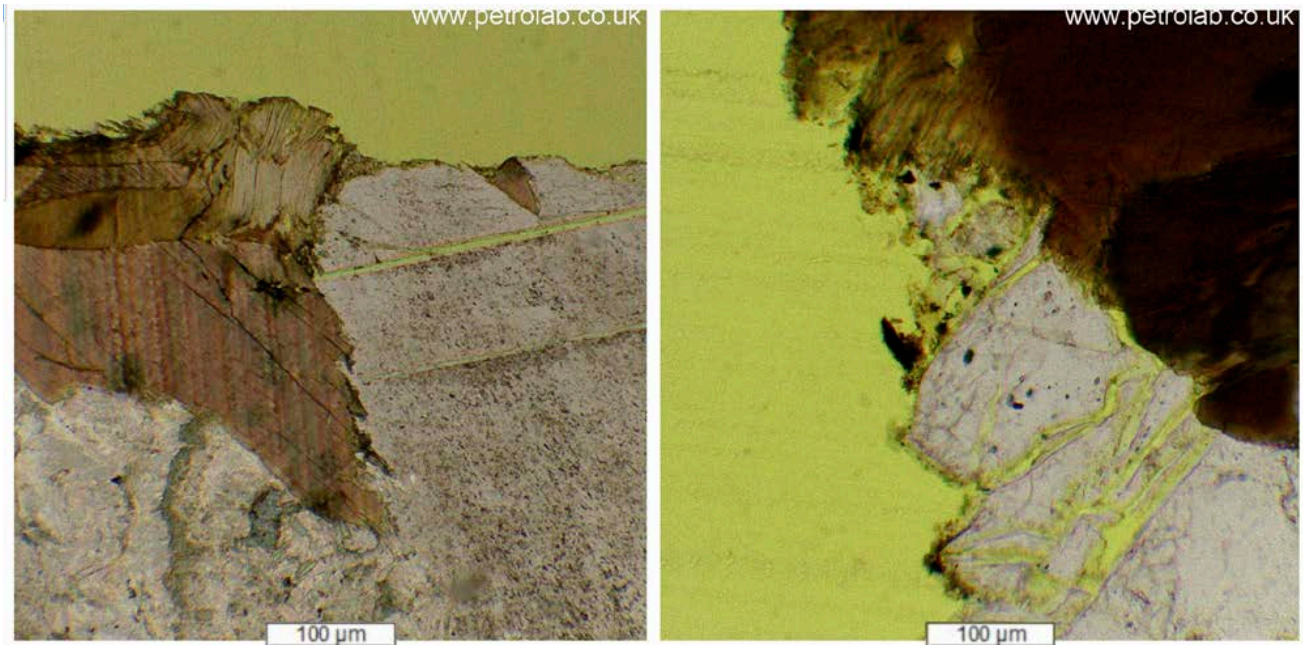


Figure 6: Plane polarised light microscopy of Foggintor granite from the petrography report. Unweathered (left) and artificially weathered (right) samples demonstrate the increase in fracturing, in this case in the biotite mica crystals.

3.4 Summary of stone properties

Our results show a statistically significant change between weathered and unweathered stone, which confirms that the weathering protocol we used was successful. We also characterised differences between the stone types in relation to their geochemistry and their ability to retain and transport water, which we can use to aid our interpretation and understanding of the bioreceptivity results.

4. Bioreceptivity results

Both new and artificially weathered stones were used to produce readings for primary and secondary bioreceptivity.

4.1 Optimisation of chlorophyll extraction protocol

The primary chlorophyll extraction technique in bioreceptivity literature (Ortega-Morales *et al* 2006, 2010; Scheerer 2008; Cappitelli *et al* 2009, 2012; Rossi *et al* 2012) uses acetone as a solvent, with freezing overnight to disrupt the cells. Our trials of this technique showed that the swabs still carried a distinct green colour, indicating the chlorophyll was not being extracted by the acetone. Marine biology literature suggests a range of solvents – predominantly acetone, DMSO, methanol and isopropanol (Shoaf and Lium 1976; Blanke 1992; Simon and Helliwell 1998; Su *et al* 2010) – which is also supported by Prieto and Silva (2005) and Vázquez-Nion *et al* (2016), who used DMSO for extracting terrestrial algae. These solvents were tested with either sonication (breaking cells using high powered sound waves) or freezing for 4 hours at -20 ºC to disrupt the cells. DMSO and methanol were shown to be the most effective solvents for extracting chlorophyll, with DMSO incubated at -20 ºC for 4 hours giving the most complete extraction (Figure 7).

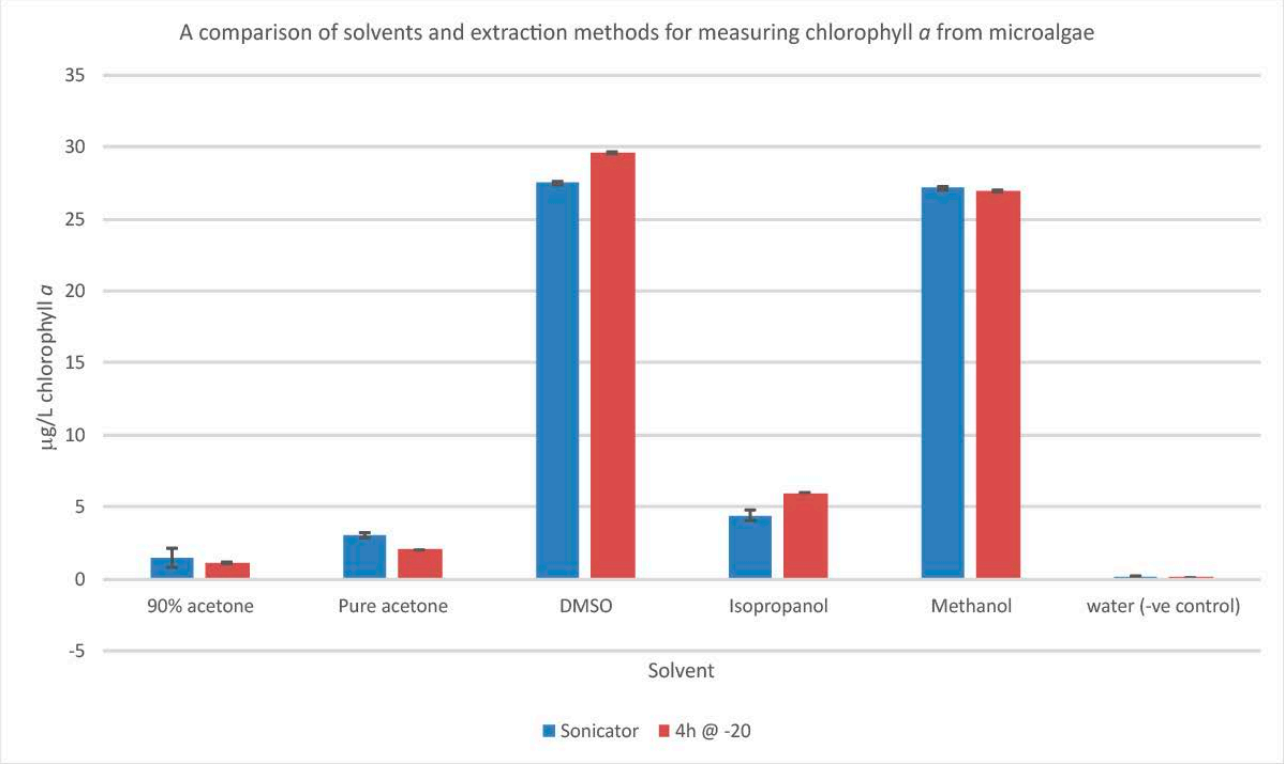


Figure 7: A comparison of solvents and techniques for extracting chlorophyll *a* from terrestrial algae. Using DMSO followed by freezing generated the highest amount.

Literature suggests scraping the surface with a scalpel to remove microalgae. However, our observations showed that this causes sample loss through aerosolisation (dispersal into the air). Combining swab sampling (to capture the surface cells) and scalpel sampling was the most effective collection method. Using this technique (Figures 7 and 8), the majority of algal growth was sampled, with only growth in deep pores remaining.

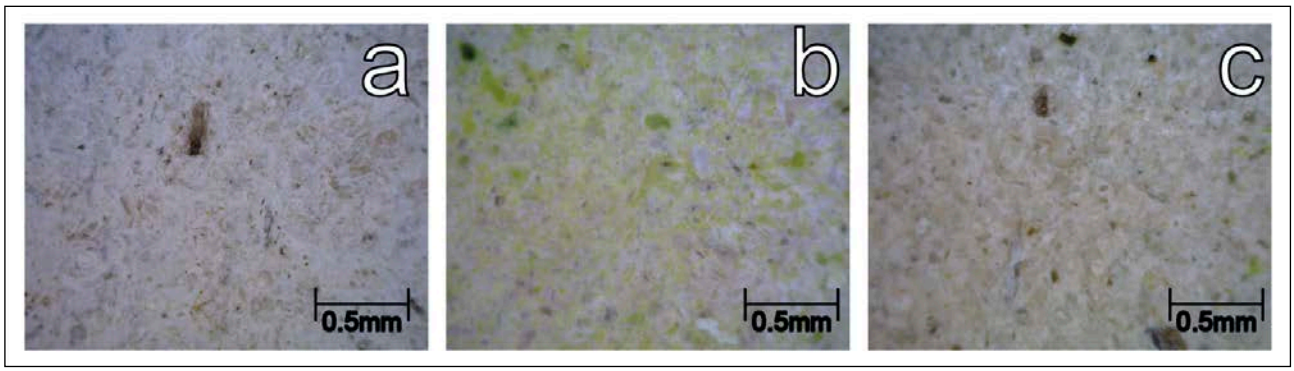


Figure 8: Unweathered Jordans Basebed limestone samples taken at 50x magnification: (a) without algal growth, (b) with algal growth, (c) post sampling.

4.2 Stone bioreceptivity from chlorophyll *a* extraction levels

Measuring chlorophyll *a* extracted from the algae was used to provide a measure of stone bioreceptivity. Each stone type demonstrated a significantly higher level of algae on artificially weathered samples compared to unweathered samples, based on the amount of chlorophyll *a* extracted per cm^2 (p-value <0.05). Jordans Basebed limestone supported the highest level of growth. Foggintor granite and Howley Park sandstone supported similar levels of growth to one another (Figure 9).

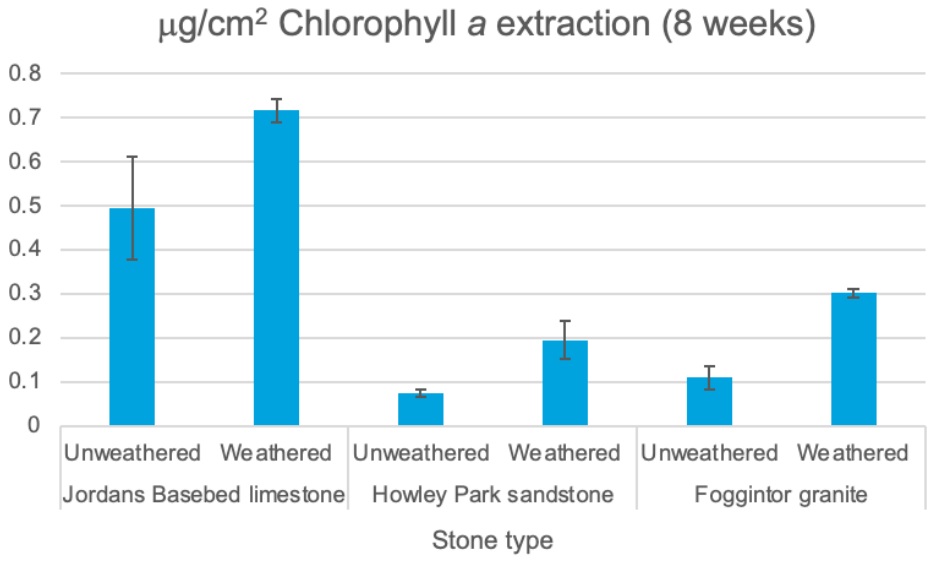


Figure 9: Chlorophyll *a* extraction from sampled algal cells after 8 weeks of growth.

4.3 Stone bioreceptivity by colorimetry

The stone colour was measured at regular intervals for an eight-week period, after adding algae. The first four weeks of data show an increase in green colouration, which demonstrates an initial surge of algal growth, probably caused by addition of the growth media on the stone. This then stabilises over weeks four to eight, as the algae equilibrate with the surface. They achieve a balance between the rates of growth and death in the population due to the bioreceptivity of the stone surface, without the influence of the growth media they were in when added to the surface (Figure 10).

By the eight-week mark, only Foggintor granite demonstrated a significant colour difference (as measured by Student's t-test) between the unweathered and weathered samples. This is in contrast to the readings of chlorophyll *a* levels (*see* 4.2), where there was a significant difference between unweathered and weathered samples for all stone types.

In the case of Howley Park sandstone, any green colouring caused by algae was masked completely by a shift towards red. This may have been caused by the oxidation of the iron minerals in the stone, which were identified in the petrography report.

A control sample was run without the algal inoculum, to determine how much colour change was due to the algae and how much was natural patination of the stone (which occurs in a wet environment). Between weeks two and four, the algal growth on the inoculated stone caused a faster rate of colour change to the stone surface than that on the no algae control. By week six, the green pigments in the algae were counteracting the change in colour of the stone surface, reducing the redness of the stone (compared to the no algae control, (Figure 11). This natural change in colour is problematic for the bioreceptivity index proposed by Vázquez-Nion *et al* (2018a, *see* 4.4).

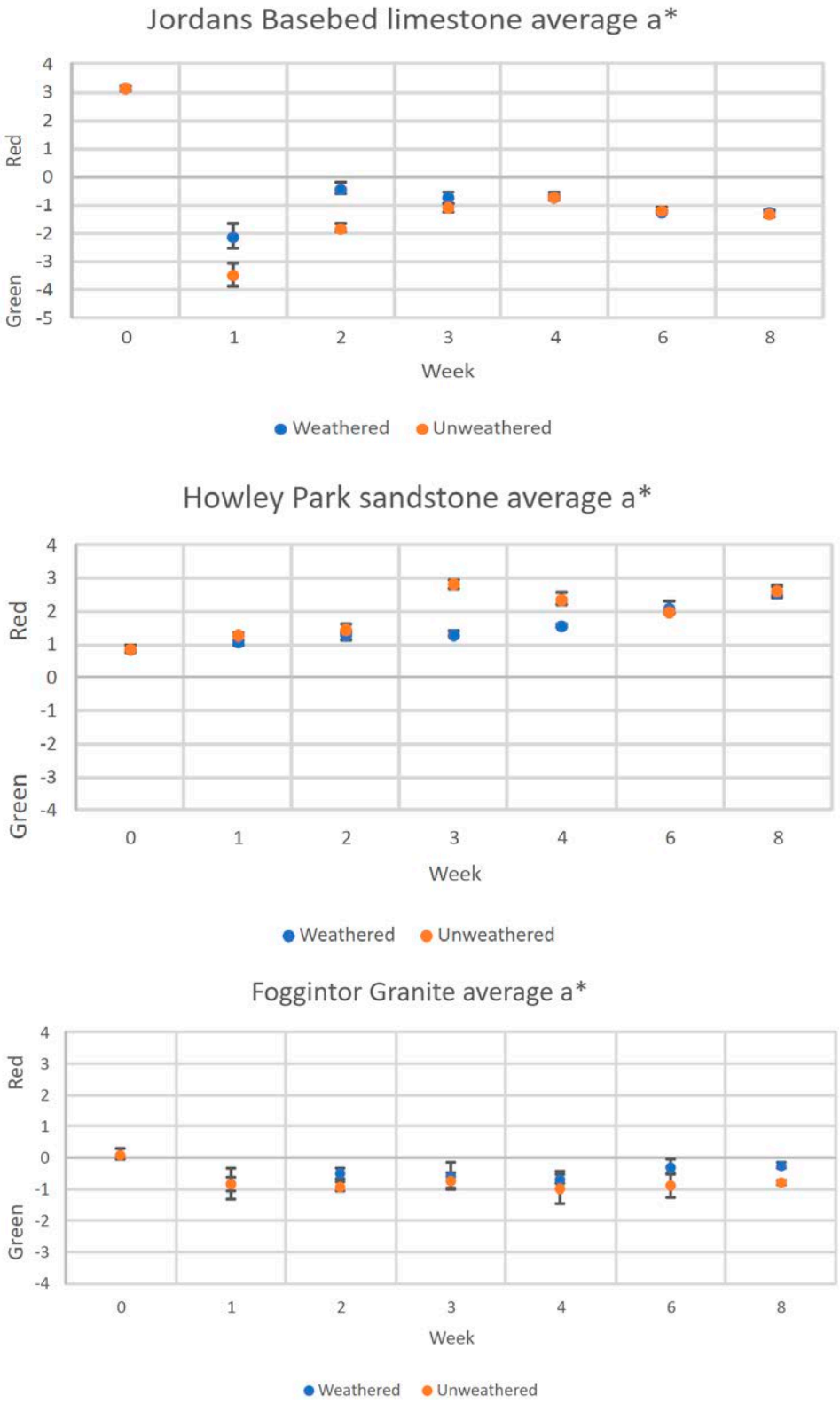


Figure 10: Average a* plotted over 8 weeks for unweathered and weathered samples of Jordans Basebed limestone, Howley Park sandstone and Foggintor granite.

Foggintor and Jordans Basebed both show an initial change towards a greener surface colour, before stabilising over time. Howley Park progressively turns red. The first reading (week 0) on each graph is the wet stone with no algae.

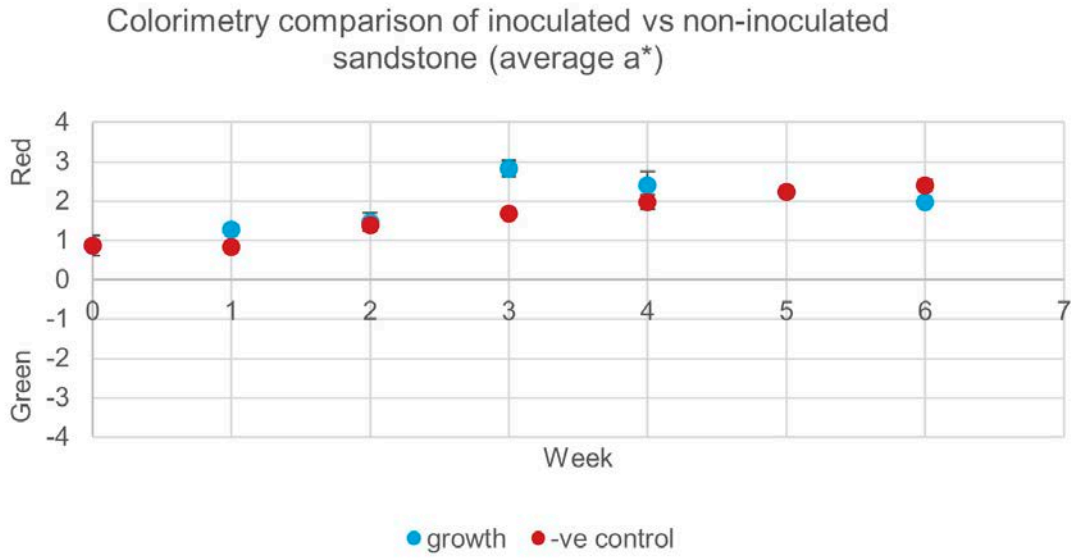


Figure 11: Comparison of inoculated (growth) vs non-inoculated (-ve control) colour change in Howley Park sandstone.

4.4 Vázquez-Nion's bioreceptivity index

Vázquez-Nion *et al* (2018a) proposed a bioreceptivity index for granites based on chlorophyll extraction and colorimetry. They suggested this could be applied to other stones. The index runs from 0 to 10, and it has qualitative descriptions linked to the ranges (see 1.4.7).

We used their equations (see 1.4.7) to evaluate their proposed methodology based on our results. Looking at the overall outcome for both primary (unweathered) and secondary (artificially weathered) bioreceptivity, we can see how these stone types would be categorised (Table 7):

- Jordans Basebed Portland limestone has low primary bioreceptivity (>2 BI ≤4). After weathering, bioreceptivity increases, giving a mild secondary bioreceptivity reading (>4 BI ≤6).
- Howley Park sandstone has very low primary and secondary bioreceptivity (BI ≤2).
- Foggintor granite has very low primary and secondary bioreceptivity (BI ≤2).

Bioreceptivity is consistently higher for weathered samples than it is for unweathered samples.

Table 7: Bioreceptivity index (BI, BI_{colour} and BI_{growth}) results for the three stone types in our study.

Bioreceptivity index	Jordans Basebed limestone		Howley Park sandstone		Foggintor granite	
	Unweathered	Weathered	Unweathered	Weathered	Unweathered	Weathered
BI_{growth}	1.20	1.73	0.18	0.47	0.27	0.73
BI_{colour}	9.06	9.25	4.50	4.76	0.91	1.24
BI	3.82	4.24	1.62	1.90	0.48	0.90

These calculations do not account for stones that undergo colour change in the presence of water, as there can be an artificial increase or reduction in the total *BI* and the BI_{growth} depending on the mineralogical composition of the stone. In this study, only Howley Park sandstone showed this characteristic. However, currently unpublished work by our group has shown this colour change occurs in several different stones, so the impact of this needs to be addressed.

The problem can be overcome by using a non-inoculated control and exposing it to the same conditions as the sample with algae growing on it, before taking the colour reading used for L1/a1/b1 to calculate ΔE^*_{ab} for equation 2 shown in 1.4.7. This produces a different bioreceptivity index result to the one calculated using Vázquez-Nion *et al*'s methodology, as their control was a wetted stone that had not been placed in a climate chamber alongside algal growth samples. This change in how the control sample is prepared means that the natural colour change of the stone, which falsely increases the bioreceptivity index, is accounted for (Figure 12).

Modifying the methodology with the non-inoculated control, using the calculation described in section 2.3.2, gives an overall bioreceptivity index for Howley Park sandstone of 0.56 for the unweathered stone and 0.87 for the artificially weathered stone (Figure 12). This is between a third and a half of that calculated using the Vázquez-Nion *et al* method. Without using a control for colour changes, this could lead to major errors when directly comparing the bioreceptivity of two stones.

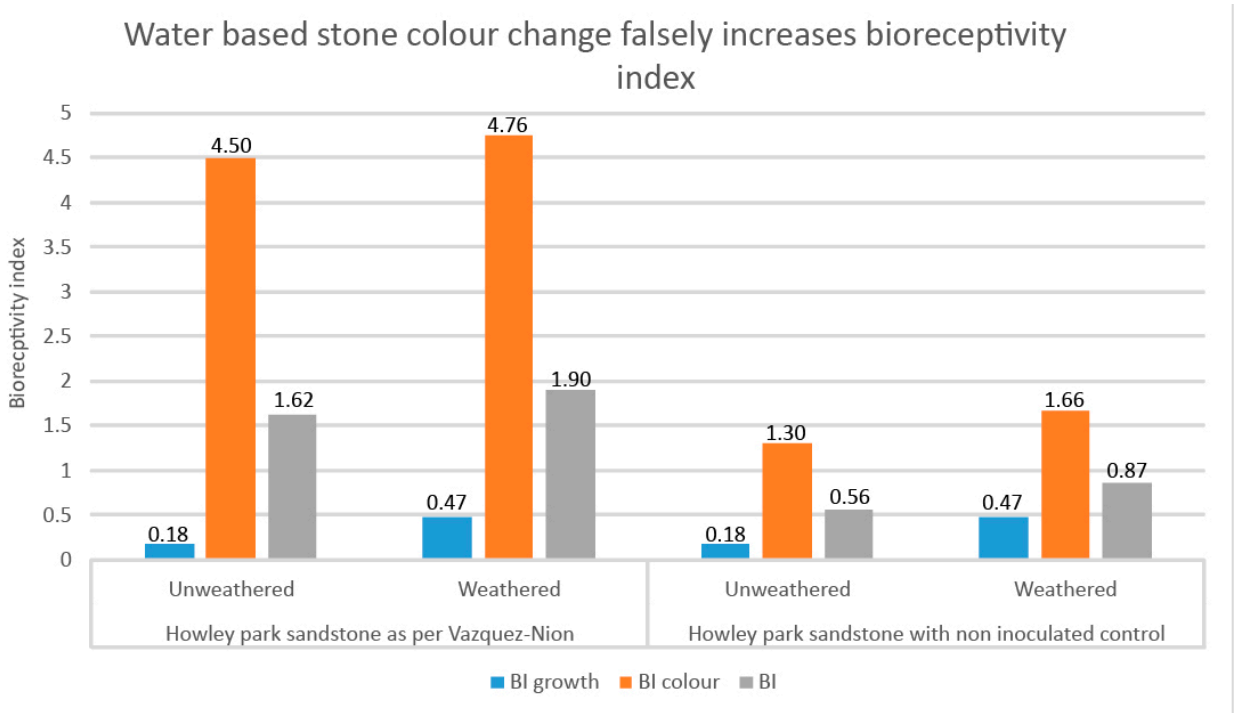


Figure 12: Comparison of bioreceptivity index calculations based on Vázquez-Nion *et al*'s original paper and our modified version. This clearly shows that water-based stone colour change causes an artificial increase in the bioreceptivity measurements for Howley Park sandstone.

4.5 Correlation of stone properties and bioreceptivity

To better understand the relationship between the change in stone properties after artificial weathering and the bioreceptivity measurements, we calculated the Pearson correlation coefficient to compare the data sets. This test can be used to show how two data sets are related. The results range from -1 to 1. If the result is 0, there is no relationship. A positive relationship is where both values go up at the same time; a negative relationship is where one goes up and the other goes down.

The properties evaluated were capillarity coefficient, open porosity and surface roughness, as these have been shown in past studies to directly correlate with the bioreceptivity of the surface. They were compared to the chlorophyll *a* level extracted from the stone, as this is a direct measure of the amount of biological growth on the surface. Correlations were calculated using the chlorophyll *a* level measured at eight weeks, when the growth had equilibrated. There was a positive correlation between capillarity, open porosity and surface roughness for all stone types, whether unweathered or weathered (see Appendix B). This corresponds with the findings in the bioreceptivity literature and supports the validity of this test method.

There was no significant correlation between colour change and chlorophyll *a* levels on any stone. This result is expected and has been found in other papers (Vázquez-Nion *et al* 2018a).

5. Discussion of results

5.1 Primary bioreceptivity

Primary bioreceptivity measurements for all three stone types have been carried out using standardised optimised methodology. Our selection of species, chemicals and equipment is readily available to others carrying out the tests.

We have demonstrated that, although values of capillarity, open porosity and surface roughness can produce suitable results for calculating primary bioreceptivity in a laboratory environment, a combination of chlorophyll *a* levels and colorimetry measurements are the simplest way to produce reliable results. The methodology devised generates results that are comparable between research groups.

5.2 Secondary bioreceptivity

The measurement of surface roughness, open porosity and capillarity coefficient add to our understanding of stone properties and how these parameters correlate with bioreceptivity. Although these measurements are not required for calculating bioreceptivity, they are helpful for describing changes as part of the artificial weathering for secondary bioreceptivity.

The methodology used for artificially weathering samples before measuring secondary bioreceptivity has been shown to create statistically significant changes to the stone surface and matrix. All three stone types demonstrated an increase in capillarity coefficient and open porosity, although not all showed changes to surface roughness.

Petrographic analysis is not required for calculating bioreceptivity. However, it confirmed the mineralogical alterations for artificially weathered sandstone, which informed our proposals for modifying the bioreceptivity index calculation.

We applied the methodology for primary bioreceptivity testing to the weathered stone. Artificial weathering resulted in a statistically significant increase in the bioreceptivity of the stones compared to unweathered samples. Our work demonstrates a repeatable approach to producing a laboratory-based methodology for measuring and calculating secondary bioreceptivity.

5.3 Bioreceptivity index

We have demonstrated that the bioreceptivity index calculation proposed by Vázquez-Nion *et al* (2018a) can be applied to a range of stone types. However, the methodology needs to be modified to take into account stones that undergo a natural colour change in the presence of water.

The index proposed using two-increment divisions to categorise primary bioreceptivity (*see* Table 4). The term ‘mild’ for bioreceptivity in the range of 4 to 6, as suggested by Vázquez-Nion *et al* (2018a), would be more conventionally defined as ‘moderate’. This means the bioreceptivity categories would be very low, low, moderate, high, and very high.

From our data, it appears that this scale is also applicable to secondary bioreceptivity. However, further data from different stone types are needed to determine whether the two-unit increment for categorisation is reasonable. For example, there may be a high proportion of stones clustered in one area, and further definition between stages may be required.

6. Conclusion and next steps

We have successfully achieved the aims of the pilot phase of this project which was to develop and test a protocol for a standardised laboratory-based bioreceptivity test that could be adapted for the initial testing of environmentally acceptable biocide alternatives. We would like to encourage others to carry out their own testing using this protocol. We can then begin to build a shareable database of stone characteristics, for use in maintaining stonework.

The next phase of the project is to test the methodology to measure quaternary bioreceptivity, that is bioreceptivity of a stone surface following treatment with a product that leaves a coating or changes the surface properties. This will be carried out by comparing samples coated with biocidal products with samples which have no coating. Although this is less useful for comparing stone types, the testing will help those working in the building stone sector to understand the impact of conventional and innovative conservation treatments on stone. The methodology could, in future, be extended to other materials and treatments.

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Appendix A

Summary table of the petrographic characteristics of Jordans Basebed limestone, Howley Park sandstone and Foggintor Quarry granite.

Jordans Basebed limestone

Mineral/ phase	Textural component	General formula | specific gravity (s.g.)	Unweathered Vol (%)	Weathered Vol (%)
Ooids	Grain	$CaCO_3$ | sg~2.70	60.8	63.1
Bioclasts	Grain	$CaCO_3$ | sg~2.70	3.3	3.7
Calcite	Grain	$CaCO_3$ | sg~2.70	3.0	3.5
Quartz	Grain	SiO_2 | sg~2.65	1.0	2.1
Opaque minerals	Grain	Typically, pyrite FeS_2 | sg~4.90	<0.5	<0.5
Plagioclase feldspar	Grain	$Na(AlSi_3O_8)$ | sg~2.60	<0.5	<0.5
Accessory phases	Grain	Typically, titanite $CaTi(SiO_4)O$ | sg~3.48	<0.5	<0.5
Iron oxides/ hydroxides	Matrix	Typically, goethite $Fe^{3+}O(OH)$ | sg~3.80	<0.5	<0.5
Phyllosilicate clay minerals	Matrix	Illite and glauconite $K_{0.65}Al_2[Al_{0.65}Si_{3.35}O_{10}](OH)_2$ | sg~2.79	<0.5	traces
Gypsum	Cement	$CaSO_4.2H_2O$ | sg~2.31	<0.5	<0.5
Sparry cement	Cement	$CaCO_3$ | sg~2.70	12.5	9
Micrite cement	Cement	$CaCO_3$ | sg~2.70	7.2	3.1
Voids/porosity	Porosity	-	6.7	9.5
Internal porosity	Porosity	-	5.0	5.1
Fractures	Porosity	-	-	-

Howley Park sandstone

Mineral/ phase	Textural component	General formula | specific gravity (s.g.)	Unweathered Vol (%)	Weathered Vol (%)
Quartz	Grain	SiO_2 | sg~2.65	41.7	41.5
Dolomite	Grain	$CaMg(CO_3)_2$ | sh~2.84	14.4	11.5
Lithic fragments	Grain	Typically, quartzites, claystone and chloritised sediments	7.6	7.4
Alkali feldspar	Grain	Typically, orthoclase $K(AlSi_3O_8)$ | sg~2.55	5.4	6.0
Iron oxides/ hydroxides	Grain	Typically, goethite $Fe^{3+}O(OH)$ | sg~3.80	1.8	0.7
Plagioclase feldspar	Grain	$Na(AlSi_3O_8)$ | sg~2.60	1.4	1.3
Ferroan calcite	Grain	$CaCO_3$ | sg~2.70	1.3	0.5
Chlorite group	Grain	$(Mg,Fe)_5Al(Si_3Al)O_{10}(OH)_8$ | sg~2.90	1.2	1.6
Muscovite mica	Grain	$KAl_2(AlSi_3O_{10})(OH)_2$ | sg~2.77	1.2	0.8
Opaque minerals	Grain	Magnetite and hematite, rarely pyrite FeS_2 | sg~4.90	<0.5	1.2
Accessory phases	Grain	Typically, titanite $CaTi(SiO_4)O$ | sg~3.48	<0.5	0.5
Glauconite	Grain	$K_{0.6}Na_{0.05}Fe^{3+}_{1.3}Mg_{0.4}Fe^{2}+_{0.2}Al_{0.3}Si_{3.8}O_{10}(OH)_2$ | sg~2.64	<0.5	Traces
Phyllosilicate clay minerals	Matrix	Assumed illite $K_{0.65}Al_2[Al_{0.65}Si_{3.35}O_{10}](OH)_2$ | sg~2.79	7.8	12.0
Gypsum	Cement	$CaSO_{4\cdot2}H_2O$ | sg~2.31	3.0	2.0
Microcrystalline silica	Cement	SiO_2 | sg~2.65	0.6	1.3
Voids/porosity	Porosity	-	7.8	9.5
Internal porosity	Porosity	-	4.0	2.0
Fractures	Porosity	-	<0.5	0.0

Foggintor Quarry granite

Mineral/ phase	Textural component	General formula | specific gravity (s.g.)	Unweathered Vol (%)	Weathered Vol (%)
Alkali feldspar	Crystals	$K(AlSi_3O_8)$ | sg~2.55	57.7	37.1
Quartz	Crystals	SiO_2 | sg~2.65	21.3	22.8
Pagioclase feldspar	Crystals	$Na(AlSi_3O_8)$ | sg~2.60	7.8	10.5
Biotite	Crystals	$K(Mg,Fe)_3AlSi_3O_{10}(F,OH)_2$ | sg~3.07	6.1	12.0
Sericite/ Muscovite mica	Crystals	$KAl_2(AlSi_3O_{10})(OH)_2$ | sg~2.77	1.9	2.5
Chlorite	Crystals	$(Mg,Fe)_3(Si,Al)_4O_{10}(OH)_2$. $(Mg,Fe)_3(OH)_6$ | sg~2.90	1.0	1.5
Accessory phases	Crystals	Typically, zircon and apatite	0.7	<0.5
Phyllosilicate clay minerals	Matrix	Typically, kaolinite $K_{0.65}Al_2[Al_{0.65}Si_{3.35}O_{10}](OH)_2$ | sg~2.79	<0.5	2.0
Opaque minerals	Crystals	Typically, pyrite and magnetite FeS_2 | sg~4.90	<0.5	<0.5
Topaz	Crystals	$Al_2SiO_4(F,OH)_2$ | sg~3.55	<0.5	0.7
Voids/porosity	Porosity	-	-	1.0
Internal porosity	Porosity	-	0.4	1.7
Fractures	Porosity	-	2.5	7.7

Appendix B

Correlation between chlorophyll *a* (μg /cm^2) measurements at 8 weeks and the physical characteristics of the stone. Where the p-value is less than 0.05 (shaded green), there is a significant correlation between the chlorophyll levels and the physical characteristics.

Jordans Basebed limestone		**Capillarity**	**Open porosity**	**Surface roughness**
Unweathered	Correlation	0.99994	0.92833	0.97310
Unweathered	p-value	<0.001	0.04	<0.001
Weathered	Correlation	0.99443	0.87250	0.90599
Weathered	p-value	0.003	0.03	0.002

Howley Park sandstone		**Capillarity**	**Open porosity**	**Surface roughness**
Unweathered	Correlation	0.96077	0.98938	0.98273
Unweathered	p-value	0.02	<0.001	0.004
Weathered	Correlation	0.93615	0.884	0.932
Weathered	p-value	0.03	0.009	0.001

Foggintor granite		**Capillarity**	**Open porosity**	**Surface roughness**
Unweathered	Correlation	0.93182	0.99412	0.80681
Unweathered	p-value	0.03	<0.001	0.01
Weathered	Correlation	0.94521	0.99958	0.99807
Weathered	p-value	0.03	<0.001	<0.001

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